Synthetic optimization of cystobactamids as broad-spectrum antibiotics

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation

von

Moritz Dominik Stappert, M. Sc. RWTH

2024

Referent: Prof. Dr. rer. nat. Mark Brönstrup

Korreferent: Prof. Dr. rer. nat. Andreas Kirschning

Tag der Promotion: 15.04.2024

„Ich weiß Nichts und Sie wissen Nichts… und dennoch reden wir hier, als ob wir schon alles wüssten“

Herbert Wehner

Für Mama und Papa

Kurzzusammenfassung

Bakterielle Resistenz ist allgegenwärtig und wird voraussichtlich ein zukünftiges Problem sein, wie die Berichte und Leitlinien der Weltgesundheitsorganisation (WHO) seit 2014,[1-3] der Centers for Disease Control and Prevention (CDC)[4] und des European Centre of Disease Prevention and Control (ECDC)[5] warnen. Neue molekulare Grundstrukturen für Breitbandantibiotika, die β-Laktame und Chinolone ersetzen können, sind selten und ihre Entwicklung verläuft schleppend,[6] obwohl eine Vielzahl von Verbindungen mit antibakteriellen Eigenschaften aus der Natur bekannt ist.[7] Mit den Cystobactamiden wurde eine vielversprechende Verbindungsklasse von Antibiotika gefunden und intensiv in Richtung eines anwendbaren Medikaments weiterentwickelt.[8-11]

In dieser Arbeit wurde die derzeit bekannte Cystobactamid-Bibliothek synthetisch erweitert und die *in-vitro*-Wirksamkeit der neuen Analoga gegen diverse Bakterien, einschließlich der hochrelevanten ESKAPE-Erreger, bestimmt. Ausgehend von dem aktuellen Cystobactamid CN-CC 861 als Leitstruktur[11] wurden mehrere neue CDE-Fragmente synthetisiert. Verschiedene hochsubstituierte Aromaten wurden synthetisch zugänglich gemacht und als Ring-D-Derivate eingesetzt. Diese Methoden umfassen unter anderem die metallvermittelte aromatische Funktionalisierung und Heterocyclisierungen. Die Cystobactamid-Synthesevorschriften hin zur Zielverbindung wurden verbessert und auf mehrere Derivate angewendet. Eine neue allgemeine SAR von Ring D wurde abgeleitet, indem die Ergebnisse der Aktivitätstests aller neuen Verbindungen ausgewertet wurden. Dabei erwies sich sowohl die Substitution der Hydroxygruppe als auch eine Versteifung zwischen Ring D und E als schwierig. Die Analyse der Aktivität gegen ESKAPE-Erreger führte zu neuen Erkenntnissen über die Auswirkungen dieser Modifikationen, auf deren Grundlage künftige Cystobactamid-Analoga angestrebt wurden. Eine invertierte Amidbindung zwischen Ring C und D erwies sich hier als äußerst vorteilhaft im Hinblick auf die Breitspektrum-Aktivität. Die neuen CDE-Fragmente wurden mit den *Western*-Fragmenten von sowohl der Leitverbindung, als auch von aktuellen Cystobactamid-Favoritstrukturen kombiniert. Letztere enthielten Benzimidazole, Bicyclo[1.1.1]pentan und Pyridin als Strukturelemente, bzw. Isostere für Benzol. Die beiden aktivsten Verbindungen dieser Arbeit waren Teil einer präklinischen *in vivo* ADME-Studie und werden in weiterführende Studien einbezogen. Außerdem wurde Serin als zentrale Aminosäure gut toleriert und stellt eine höher polare Alternative für die zentrale Aminosäure dar, was sich möglicherweise positiv auf die Löslichkeit auswirkt. *Schlagworte: Antibiotika, Cystobactamide, organische Chemie*

Abstract

Bacterial resistance is omnipresent and expected to be a future problem as warned by the World Health Organization (WHO) reports and guidelines since 2014,[1-3] Centers for Disease Control and Prevention (CDC)[4] and European Centre of Disease Prevention and Control (ECDC)[5]. Novel scaffolds for broad-spectrum antibiotics that can displace β-lactams and quinolones are rare and their development is slow,[6] although a variety of compounds with antibacterial properties is known from nature.[7] With the cystobactamids, a highly promising antibiotic compound class was found and comprehensively expanded towards an applicable medication.[8-11]

In this Thesis, the currently known library of cystobactamids was synthetically extended and the *in vitro* efficacy of the novel analogues was determined against various bacteria, including the highly relevant ESKAPE pathogens. Based on the current cystobactamid CN-CC 861 as lead-scaffold,[11] several new CDE-fragments were synthesized. Various highly substituted aromatic systems were synthetically accessed and implemented as ring D derivatives. These methods embrace metal-mediated aromatic functionalization, and heterocyclization among others. The cystobactamid assembly protocols were improved and simplified and applied to several new derivatives. An updated general SAR of ring D was derived by evaluating the activity test results of all new compounds. Thereby, both replacement of the hydroxy group and a rigidification between ring D and E proved difficult. Analysis of the activity against ESKAPE pathogens led to new insights into the effects of these modifications, based on which future cystobactamid analogues were targeted. A reversed amide bond between ring C and D proved to be highly advantageous here in terms of broad-spectrum activity. Novel CDE-fragments were combined with Western-fragments of the lead compound, as well as of current front-running cystobactamids. The latter included benzimidazoles, bicyclo[1.1.1]pentane and pyridin as substructures, resp. isosteres for benzene.

The two most active compounds in this Thesis were part of a preclinical *in vivo* ADME study and will be included in follow-up trials. Besides, serine as central amino acid was found to be well tolerated and represents a more polar alternative for the central alkyl amino acid which probably has a positive effect on solubility.

Keywords: antibiotics, cystobactamids, organic chemistry

Contents

Abbreviations

1,1 ADEQUATE	adequate sensitivity double-quantum transfer experiment
a.u.	arbitrary unit
Ac	Acetyl
AcOH	Acetic acid
acr	Acriflavine resistance protein family
ADME	absorption, distribution, metabolism, and excretion
AIBN	azobis(isobutyronitril)
AlbD	Albicidin resistance factor
All	Allyl
AMR	Antimicrobial resistance
Ar	Aryl
ATP	Adenosine triphosphate
AUC	area under the curve
Bn	Benzyl
Boc	tert-Butyloxycarbonyl
Bz	Benzoyl
Cbz	Carboxybenzyl
CDC	Centers for Disease Control and Prevention
central AA	central α-amino acid
CFU	colony-forming units
COSY	correlation spectroscopy
CRE	Carbapenem-resistant Enterobacteriaceae
Cryo-EM	Cryo-electron microscopy
CuAAC	copper-catalyzed azide-alkyne cycloaddition
Da	Dalton
DAST	Diethylaminosulfur trifluoride
DBDMH	1,3-Dibrom-5,5-dimethylhydantoin
DBP	dibenzoylperoxide
DCM	Dichloromethane
DIAD	Diisopropyl azodicarboxylate
DiPEA	N,N-Diisopropylethylamine
DMA	N,N-Dimethylacetamide
DMAP	4-Dimethylaminopyridine
DMF	N,N-Dimethylformamide
DMPU	N,N′-Dimethylpropyleneurea
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECDC	European Centre of Disease Prevention and Control
EEDQ	N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
Eq	Equivalents
ESI	electrospray-ionization
Et	Ethyl
EtOAc or EA	Ethyl acetate
FCC	flash column chromatography
Fmoc	Fluorenylmethyloxycarbonyl
FQ	Fluoroquinolone
HATU	Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
HBA	hydrogen bond acceptor

HBD	hydrogen bond donor
H-bond	hydrogen bond
HBTU	Hexafluorophosphate Benzotriazole Tetramethyl Uronium
HMBC	heteronuclear multiple bond correlation
HMPA	Hexamethylphosphoramide
HMTA	hexamethylenetetramine
HOAt	1-Hydroxy-7-azabenzotriazole
HPLC	High-performance liquid chromatography
HRMS	high resolution mass spectrometry
HSQC	heteronuclear single quantum coherence
i.v.	intravenous
IC*50*	half maximal inhibitory concentration
*i*Pr	Isopropyl
K_i	affinity constant
LBAT	liver bile acid transporter
LCMS	liquid chromatography coupled with mass spectrometry
logD or logP	Octanol-water partition coefficient
m/z	mass-to-charge-ratio
marR	multiple antibiotic resistance regulator
MDR	multidrug resistant
Me	Methyl
MeCN	Acetonitrile
MHB	Mueller Hinton Broth
MIC	minimum inhibitory concentration
Moc	Methoxycarbonyl
MOM	Methoxymethyl
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRT	mean residence time
MTBE	Methyl-*tert*-butylether
MW	micro wave
NBS	*N*-bromosuccinimide
nBu	*n*-Butyl
NMO	*N*-methylmorpholine *N*-oxide
NMP	*N*-Methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
o xs	over x steps
OATP	organic anion transporter protein
PABA	*para*-amino benzoic acid
PBS	phosphate-buffered saline
Pd/C	palladium on carbon
PDB	Protein Data Bank
PE	petrol ether
PEG	Polyethylene glycol
Ph	Phenyl
pH	pondus hydrogenii
pK_A	acid dissociation constant at logarithmic scale
PKPD	pharmacokinetic pharmacodynamic
PPB	plasma protein binding
ppm	parts per million
Pra	propargylglycine

Py	Pyridine
PyBOP	Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
qnr	Quinolone resistance genes
QSAR	quantitative structure-activity relationship
RP	reverse phase
RT	room temperature
SAR	structure-activity relationship
S_EAr	electrophilic aromatic substitution
S_NAr	nucleophilic aromatic substitution
T3P	Propanephosphonic acid anhydride
*t*Bu	*tert*-butyl
TEA	Triethylamine
Tf	Triflyl
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic acid anhydride
THF	Tetrahydrofurane
THPTA	Tris(3-hydroxypropyltriazolylmethyl)amine
TIPS	Triisopropylsilane
TLC	thin-layer chromatography
TMEDA	Tetramethylethylenediamine
TMS	Trimethylsilyl
Trt	Trityl
u.r.p.	under reduced pressure
UV	ultraviolet
VRE	Vancomycin-resistant Enterococcus
WHO	World Health Organization
δ	chemical shift
λ	wavelength

1 Introduction

The term antibiotic (greek for 'against life') is often exclusively used for microbially produced substances that are active against bacteria.[12-13] However, broader definitions extend this to activity against other microorganisms[14] like archaea, protozoa and some algae and fungi species.[14-15] In this Thesis, the term is used for antibacterial medication, regardless of a natural or synthetic origin.

1.1 Antibiotic Crisis

1.1.1 Recent Antibiotics and resistances

The domain of bacteria is morphologically very heterogeneous, with more than 10^4 known species. Despite this diversity, bacteria are usually divided into Gram-positive and Gram-negative, based on the Gram stain test. Regardless of some exceptions, this is an important classification which specifies the cell wall type.[16] Since many antibiotics such as the first penicillins[17] or glycopeptide antibiotics[18-19] are primarily active against Gram-positive bacteria, this classification is highly relevant. As with every other organism, bacteria are subject to evolutionary selection and adapt to natural as well as synthetic antibiotics.[20] However, the extensive use of broad-spectrum antibiotics is a substantial anthropogenic selection factor and has resulted in increasing resistance since the introduction of the first antibiotics.[21] The resistance-causing genes can occur by random mutation during reproduction and spread by horizontal gene transfer between bacterial cells, i.e. by transformation, transduction and conjugation (see Figure 1 for an overview).[22-23]

Mankind regularly responded to emerging resistances with chemical modifications of the inactive antibiotic, in many cases without deeper understanding of the underlying resistance mechanisms. The first resistances to penicilline could then be traced back to an inactivation by penicillases.[24]

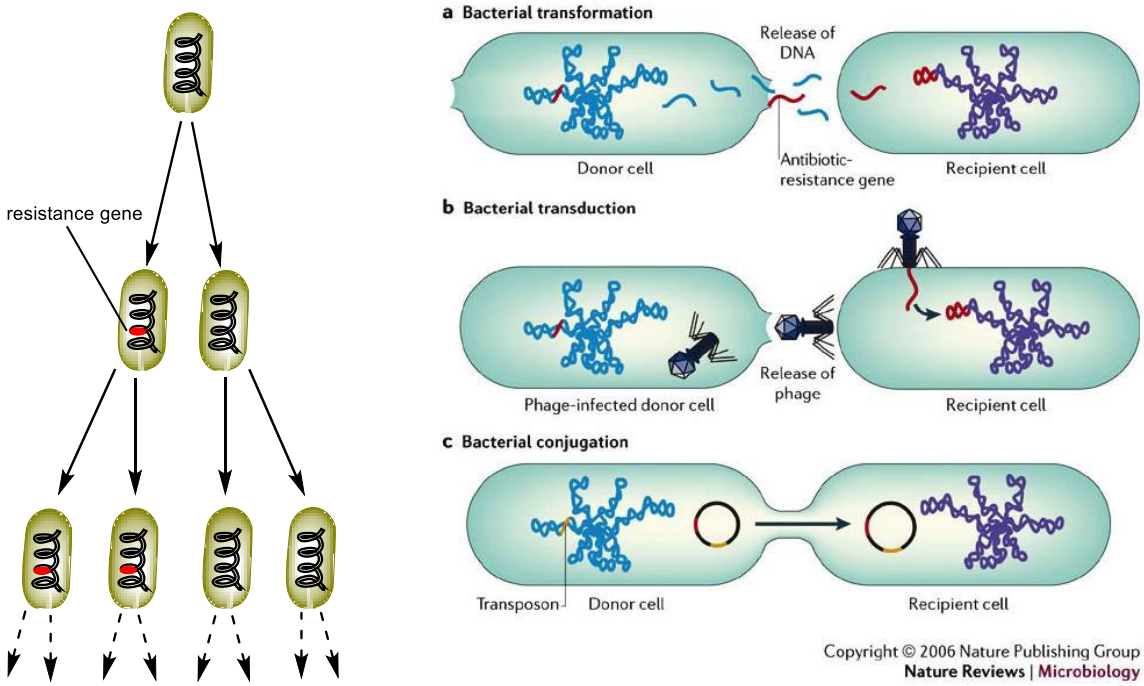


Figure 1: Mechanisms of acquired antimicrobial resistance with vertical transmission by mutant reproduction (left, image adapted from[23]) and horizontal gene transfer (right, image taken from[22]).

These enzymes are able to cleave the β-lactam ring of β-lactam antibiotics such as penicillins (see Figure 2) and are therefore part of the β-lactamases.[25] As a response to this resistance, penicillinase-resistant penicillins such as Methicillin[26] were developed. Aminopenicillins like amoxicillin were found to extend the activity of penicillins to Gram-negative species that were not susceptible to previous penicillins. They are still used as broad-spectrum antibiotics. In parallel, the related cephalosporins were isolated from natural sources and used as another type of β-lactam broad-spectrum antibiotic, being effective against Gram-negative bacteria.[25, 27] Continuous development over five generations adapted the spectrum of activity of cephalosporins to more and more bacterial species. These new developed compounds allowed treatment of a diverse range of severe bacterial infections such as urinary tract- and pseudomonal nosocomial infections.[28]

However, many of the β-lactam antibiotics are nowadays formulated together with a β-lactamase inhibitor such as clavulanic acid to prevent penicillinase-induced degradation.[25, 27] In this context, the carbapenems are also worth mentioning, which show stability against most β-lactamases.[25, 27-28] Representatives of this class are drugs of last resort, only used for treatment of severe multi-drug resistant bacterial infections.[25, 27]

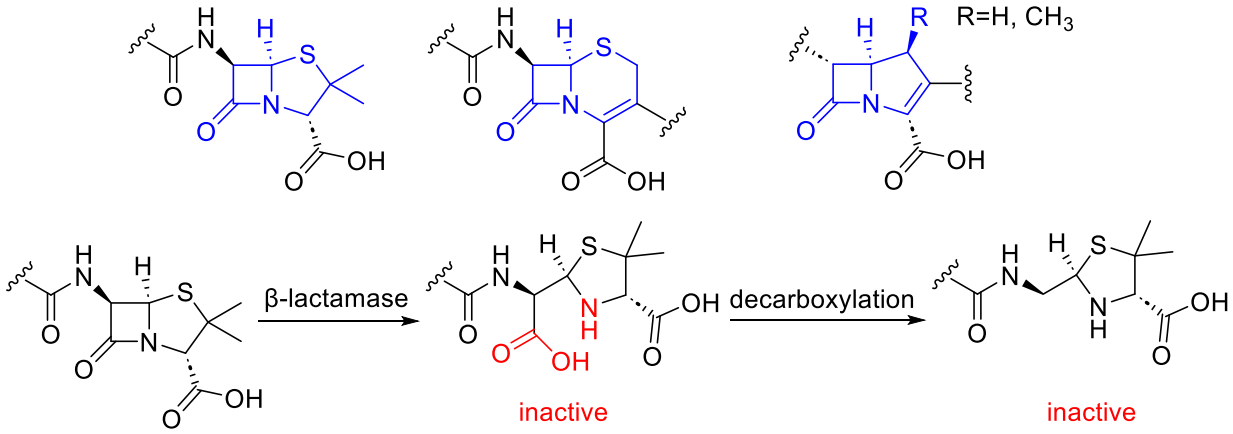


Figure 2: Basic structural elements of penicillines, cephalosporins and carbapenems (f.l.t.r.) with the characteristic bycyclic scaffold marked in blue (top). Inactivation mechanism of β-lactamases, cleaving the β-lactam ring (bottom).

Besides these extensively used β-lactam antibiotics, several other antibiotic compound classes were developed, with both natural and synthetic origin of the underlying lead-structure. Among these most prescribed classes in 2000 and 2010 were macrolide-antibiotics (e.g. Clarithromycin, Azithromycin), tetracyclines (e.g. Tetracycline, Doxycycline) and (fluoro-)quinolones (e.g. Ciprofloxacin, Moxifloxacin). A clear increase in general antibiotic consumption from 2000 to 2010 is noticeable.[29] Resistances are known for all of the mentioned drug classes since at least 1996.[30] (For an overview of sensitivities of bacteria against antibiotics (antibiogram) see also Figure 102 in attachment). In addition, it was repeatedly noted, that more than 30% of all prescribed antibiotic therapies are unnecessary or inappropriate, even in intensive care units.[4, 30-31] A systematic approach to improve this situation is antimicrobial 'stewardship', proposed as early as 1996.[32] This includes guidelines to establish expert teams consisting of physicians, pharmacists, microbiologists, epidemiologists among others, who are regularly advising hospitals, control the use of antimicrobial medication and optimize therapies as well as education.[33-34] Corresponding programs were also implemented in Europe since 1998.[35] Antibiotic consumption in Europe decreased by ~3% at most between 2011 and 2020.[36] While the occurrence of antimicrobial resistances (AMR) is tendencially low in North-european countries, there is a remarkable accumulation in South- and Eastern-european countries.[37]

There is strong evidence, that antimicrobial resistance often spreads between countries in the modern globalized world, as this is demonstrably the case for methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) and

carbapenem-resistant *Enterobacteriaceae* (CRE).[38] Increasing focus is placed on emerging countries such as China and India, since these are two of the biggest producers of antibiotics. High concentrations of antimicrobial agents were measured in the surrounding area of pharmaceutical plants, even in the effluent of water treatment plants in India[39-43] and in water from the strongly agriculturated Haihe River Basin in China[44]. A connection with the increased occurrence of known antibiotic resistance genes, that are also present in Western countries, was established.[42-43, 45-46] Interestingly, it was found that in an antibiotic free environment, a multiresistant *E. coli* strain lost its resistance after ~500 generations. This effect depended on the type of antibiotic and was explained with a fitness recovery of the bacterium.[47]

1.1.2 ESKAPE Pathogens

Especially concerning are the so called ESKAPE bacterial pathogens which comprise at least six highly virulent bacterial pathogens: ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****. aeruginosa*, and ***E****nterobacter spp.* .[48-49] The ESKAPE pathogens were designated "priority status" by WHO in 2017.[50-51] Representatives of these pathogens as the already mentioned MRSA, VRE and CRE show resistance mechanisms against reserve antibiotics that can be classified in mainly four categories: Antimicrobial inactivation (as seen for the β-lactamases), target site modification (e.g. cell wall alteration, reduced receptor affinity), reduced antibiotic accumulation (e.g. efflux pumps, decreased amount of membrane pores) and persistence by formation of inaccessible biofilms (often observed for *P. aeruginosa, S. aureus* and *A. baumannii*).[49, 52]

ESKAPE pathogens are responsible for severe healthcare-associated (nosocomial) infections, especially in surgical wounds, the urinary tract, lower respiratory tract and bloodstream (sepsis).[53-55] Often extended medication with reserve antibiotics is required, connected with the omnipresent danger of a highly life-threatening sepsis in case of insufficient efficacy.

Important reserve antibiotics, defined as 'Watch and Reserve group' by WHO, include cephalosporins of higher generations and carbapenems.[56] In case of a general β-lactam- and fluoroquinolone-resistant infection with Gram-negative bacteria, the last line of defense are often polymyxins (e.g. colistin) and aminoglycosides (e.g. gentamycin, tobramycin). However, these antibiotics often posess serious toxicities.[57] In these cases,

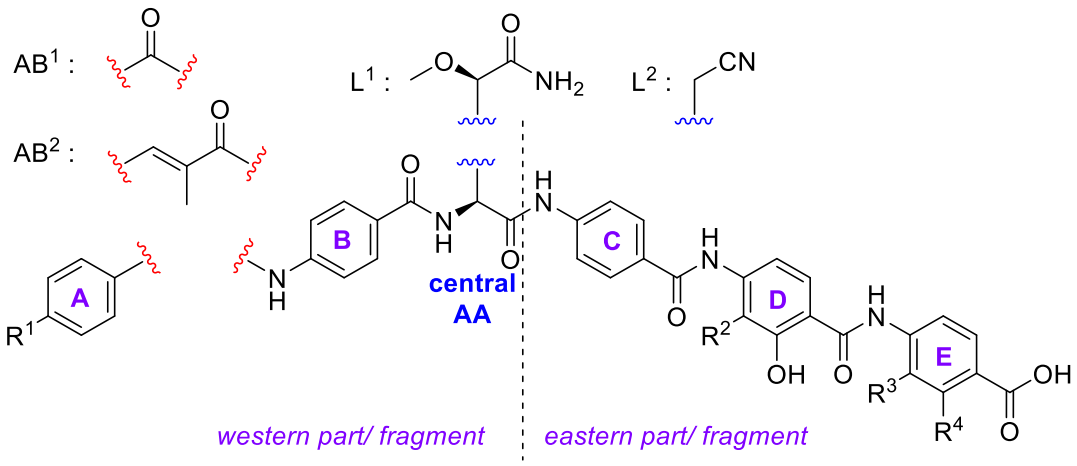
patients are already paying a high price for a lacking development of new agents against multidrug resistant (MDR) infections.[6] Industry as well as academia reduced their engagement during the last decades and correcting this mistake will cost valuable time.[58-59]

1.2 Cystobactamids and related compounds as antibiotics

As already mentioned, many currently used antibiotics were isolated from microorganisms and synthetically optimized to achieve high efficacy. Interestingly, bacteria themselves offer a broad arsenal of such compounds, myxobacteria being just one example of such bacteria. Myxobacteria are soil-based bacteria, producing a diverse range of compounds[60-61] that include, among others, antineoplastic, antifungal and antibiotic agents like epothilon,[62-63] soraphen,[64-65] tubulysin,[66] myxovalargin,[67] and cystobactamids[8].

The cystobactamids were first extracted from myxobacterium *Cystobacter velatus*, characterized and reported in 2014.[8] They are oligomers of *para*-amino benzoic acid (PABA), an important intermediate in the bacterial folate biosynthesis,[68] and show a central α-amino acid (central AA, see Figure 3), being aliphatic and non-proteinogenic. Similar compounds were found in another Myxobacterium *Corallococcus coralloides* (Coralmycins)[69] as well as in plant-disease related proteobacterium *Xanthomonas albilineans* (Albicidins, discovered in 1985[70], structure elucidated in 2015[71]). These compounds show high antibacterial activity, including multiresistant pathogens of the ESKAPE group.[8, 69, 72-73]

In this Thesis, the nomenclature is used for the corresponding partial structures as seen in Figure 3. One important structural difference is found between the rings A and B with cystobactamids and Coralmycin A, each showing an amide bond, and Albicidin featuring a cinnamic acid derivative as ring A instead. A unique substitution pattern consisting of hydroxy- and ether-substituents can be observed in ring D and, except for cystobactamid 861-2, in ring E.



	R¹	AB-linker	central AA	R²	R³	R⁴
Cystobactamid 919-2[8]	O_2N-	AB¹	L¹	*i*PrO-	*i*PrO-	H-
Cystobactamid 861-2[72]	O_2N-	AB¹	L¹	*i*PrO-	H-	H-
Coralmycin A[69]	O_2N-	AB¹	L¹	*i*PrO-	*i*PrO-	HO-
Albicidin[71]	HO-	AB²	L²	MeO-	MeO-	HO-

Figure 3: Structure of cystobactamids and related compounds as found in bacteria. The publication of the first structure elucidation is cited for each compound. In this Thesis, the PABA-units are alphabetically named as ring A-E as shown and the molecule is sectioned in Western and Eastern part or fragment. Other fragments occuring during the synthesis of cystobactamids are named after the PABA-units they contain, e.g. DE-fragment meaning the monopeptide of ring D and E. The connections between two benzene rings are named the same way, e.g. AB-linker meaning the connection between ring A and B.

For the subsequent optimization process, cystobactamid 861-2 was chosen as starting point, as it is the most potent compound of the 13 cystobactamids found. The linear polypeptide structure allows a modular synthesis and therefore a straightforward SAR investigation.

1.2.1 Mode of action

In early investigations cystobactamids were found to be bacterial topoisomerases type IIA inhibitors.[8] topoisomerases participate in the DNA replication process by catalyzing the conversion between topological states often consuming ATP.[74-75] On the one hand this can change the torsional stress that arises, for example, during DNA replication. On the other hand, DNA bundles or coils are formed or dissipated.[76-77] The formation or dissolution of these DNA superstructures by topoisomerases is an essential process in cells during DNA replication, transcription, chromosome restructuring and other cellular transactions.[78-79] An essential mechanistic step here is the passage of one strand of DNA through another strand, both being either single or double strands. Accordingly, one or both DNA strands must be temporarily cleaved for this purpose. Type I and II topoisomerases facilitate single or double strand breaks, respectively.[74] Type II

topoisomerases can be divided in sub-family types A and B, with A comprising bacterial topoisomerase II (also called gyrase[80]) and IV.[81] Bacterial topoisomerase IV (e.g. of *Escherichia coli, E. coli*) is able to dissolve positive and negative supercoiling while gyrase can additionally generate negative supercoils.[74-75, 80, 82] A shortened classification for topoisomerases is seen in Figure 4.

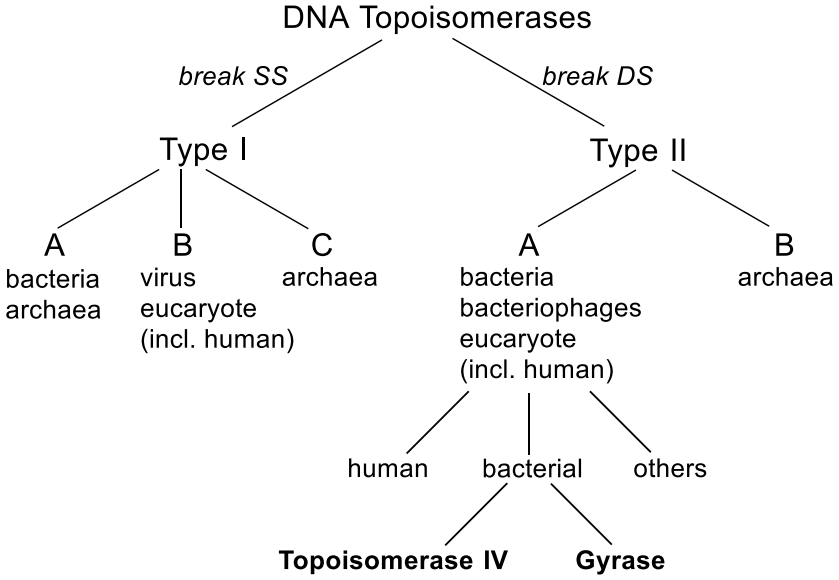


Figure 4: Classification of DNA topoisomerases[81, 83]: The relevant topoisomerases targeted by cystobactamids are shown at the bottom.

Bacterial type IIA topoisomerases are well documented targets for antibiotics, e.g. fluoroquinolones (ciprofloxacin, moxifloxacin, etc.) and aminocoumarines (novobiocin, etc.).[84] Most of the inhibitors act by stabilizing the DNA-enzyme intermediate during cleavage of the DNA, also termed as interfacial inhibition.[85-86] It should be borne in mind that some topoisomerase inhibitors are able to affect human type IIA topoisomerases to some extend, and that therefore an undesirable cytotoxic effect cannot be ruled out in principle with these compounds. Indeed, this cytotoxic effect is used in several anti-cancer drugs as etoposid, doxorubicin and mitoxantron by targeting human type IIA topoisomerases.[87]

Both gyrase and topoisomerase IV are heterotetramers, to be more precise homodimers of two heterodimers. The latter in turn consist of two subunits: GyrA and B for gyrase as well as ParC and E for topoisomerase IV. High homology exists between GyrA and ParC as well as GyrB and ParE.[88] The molecular mechanism of the gyrase inhibition by a cystobactamid-related synthetic analogue of Albicidins was recently identified via Cryo-EM structures.[89] Based on the obtained data, it was assumed that the inhibitor forms a

ternary complex with the cleaved DNA and the topoisomerase after one DNA double strand, the T-segment, was passed through the cleaved DNA double strand, the G-segment. This is a process called strand-passage mechanism[90] and is an essential step for decoiling DNA[91] (see Figure 5).

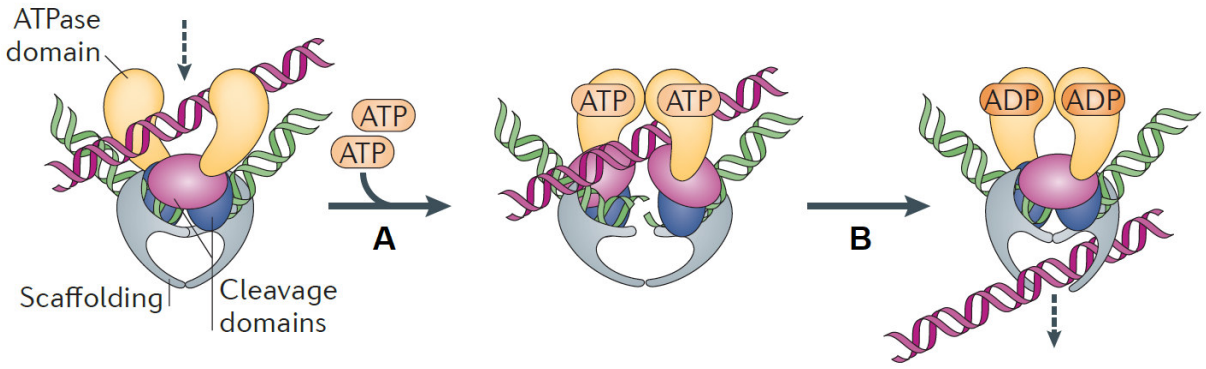


Figure 5: DNA strand-passage mechanism: type IIA topoisomerase (image from[92]) is built of an ATPase domain, a cleavage domain and a scaffolding. During the decoiling an ATP-dependent transient cleavage of DNA occurs (**A**) and one DNA double strand (magenta) is passed through another (green) afterwards (**B**).

Besides this, it should be noted that in addition to this mechanism, a nicking-closing mechanism is known for gyrase variants. This mechanism does not require double- but single-strand cleavage.[93]

In the determined structure the Western part of the Albicidin analogue is found intercalating between the two separated DNA strands while the Eastern part is located as specific binder between two α-helices of the GyrA and GyrA' subunits of *E. coli* (see Figure 6).[89] Due to the *N*-terminal nitrile group, the simple amide between ring A and B, as well as the substitution pattern of ring D, the used inhibitor has high structural similarity to the cystobactamids. Thereby, the observed mechanism is very likely valid for cystobactamid-gyrase interaction as well. In early investigations of cystobactamid mode of action the AB-fragment was confirmed as a key substructure to access low IC_{50} values with gyrase. Moreover, the data suggested that the primary binding site of cystobactamids is located at the GyrA-DNA interface and not at an ATP-binding pocket.[8] The latter would imply competitive inhibition at the ATP-binding site as the mechanism.[94] For the most active compound, the IC_{50}-value for the inhibition of *E. coli* topoisomerase IV was nearly 150-fold higher compared to IC_{50} against gyrase in the same organism. This suggests gyrase as the more relevant target of cystobactamids at least in *E. coli*.[8] A different inhibition mechanism with topoisomerase IV can not be excluded.

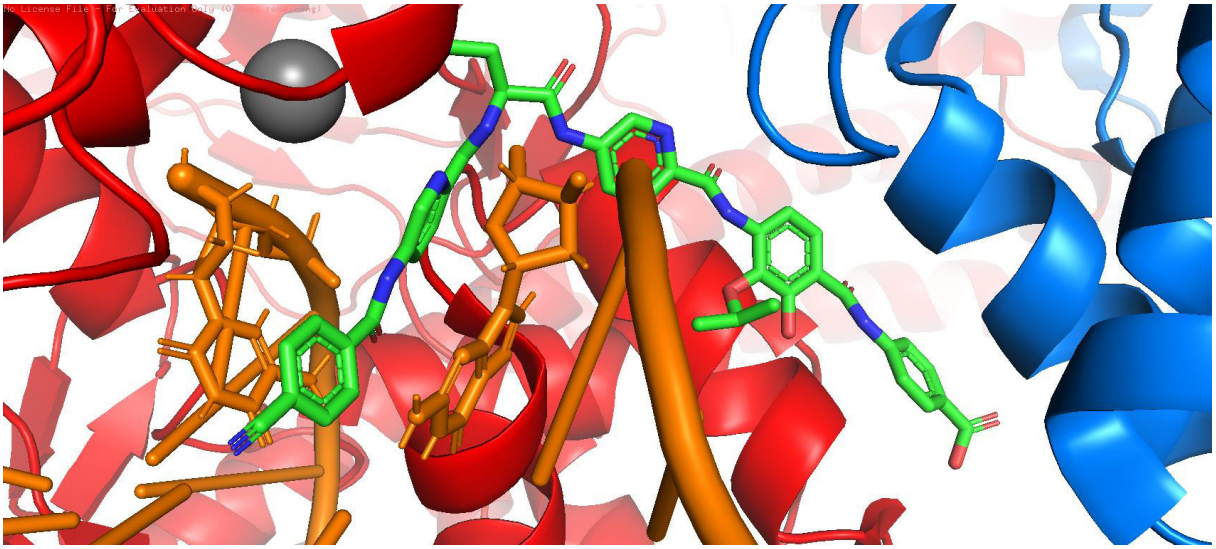
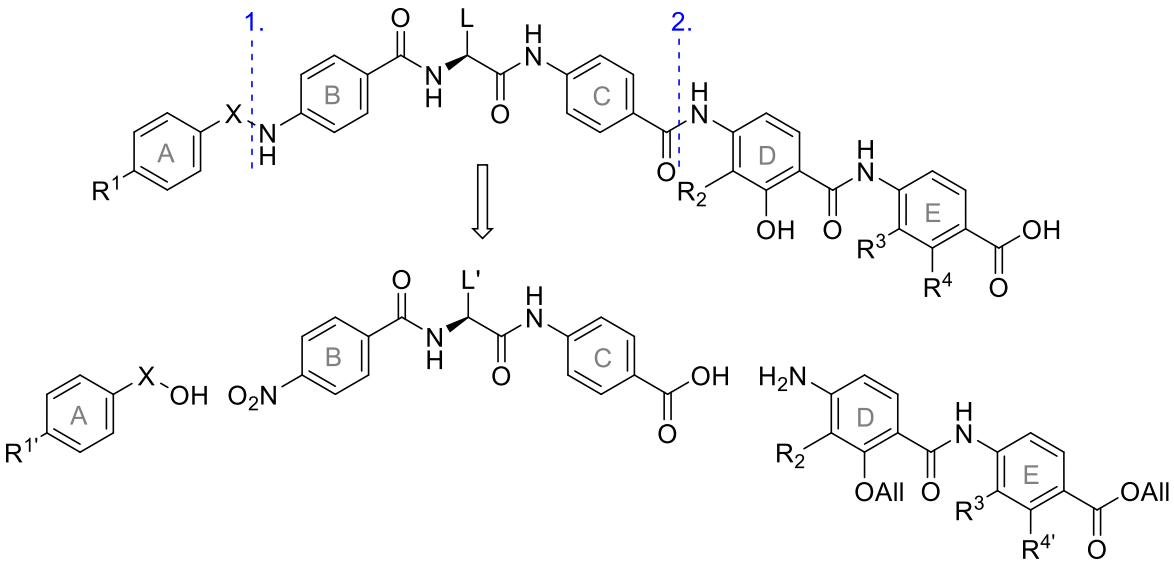


Figure 6: Cryo-EM structure[89] (PDB: 7Z9K) of a cystobactamid-related Albicidin analogue (Albi-1, green) showing interaction of the Western part with the separated DNA double strand (orange, nearest nucleotides are shown as ribbon) to the left and placement of the Eastern part between α-helices of GyrA and GyrA' subunits (red and blue) to the right.

However, the opposite was found for gyrase and topoisomerase IV in *S. aureus*: Almost equal IC_{50} values were found for both enzymes, which argues against a generally higher selectivity of cystobactamids for gyrase (data provided by HIPS). In addition, comparatively low IC_{50} values were measured even for otherwise inactive compounds. This makes it difficult to directly correlate antibacterial activity with gyrase- or topoisomerase IV inhibition.[11]

1.2.2 Totalsynthetic approaches

Due to their peptidic structure, cystobactamids and related compounds are synthesized by coupling pre-assembled building blocks in a divergent synthesis. These building blocks can easily be modified, i.e. by incorporating a substituted PABA monomer. A first total synthesis was published for the Albicidins in 2015 with retrosynthetic cuts between the rings A and B as well as between rings C and D.[73] The same strategy was applied to cystobactamid 861-2 in 2017 which later served as the base for subsequent SAR investigations (see Figure 7).[72]



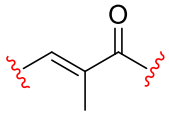
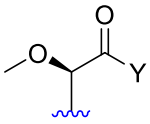
	X :	L :	L' :	R^1:	$R^{1'}$:	R^2:	R^3:	R^4:	$R^{4'}$:
Albicidin:				OH	OAll	OMe	OMe	OH	OAll
Cystobactamid 861-2:				NO_2		O*i*Pr	H	H	

Figure 7: Retrosynthetic strategy for the first total synthesis of Albicidin[73] and cystobactamid 861-2[72].

During early optimizations several cystobactamid derivatives were synthesized by Testolin *et al.* in 2020 using this strategy.[9] An example is shown in Figure 8 and Figure 9 for one of the most potent compounds found in this publication ('cmpd. 22', later named CN-DM 861). Starting from the *N*-Fmoc-protected central AA **1**, ring C **2** was first coupled as the Me-ester of PABA via $POCl_3$ as amide coupling reagent. After deprotection of the N-terminus, carboxylic acid **3** was attached by amide coupling with HBTU, followed by deprotection of the C-terminus to obtain building block **4**.

The DE-fragment **5** was synthesized by reacting the trisubstituted benzoic acid **7** as ring D with allyl 4-aminobenzoate as ring E using $POCl_3$ as amide coupling reagent, followed by nitro reduction.[72] **7** and similar trisubstituted benzoic acids were prepared via multistep syntheses that are described in detail in section 1.2.3. In the next step, the amino function of the DE-fragment was again amide-coupled with the carboxylic acid **4** using $POCl_3$. The remaining nitro group was reduced using Zinc in a mild acidic medium to afford intermediate **6** after acidic cleavage of the trityl group.

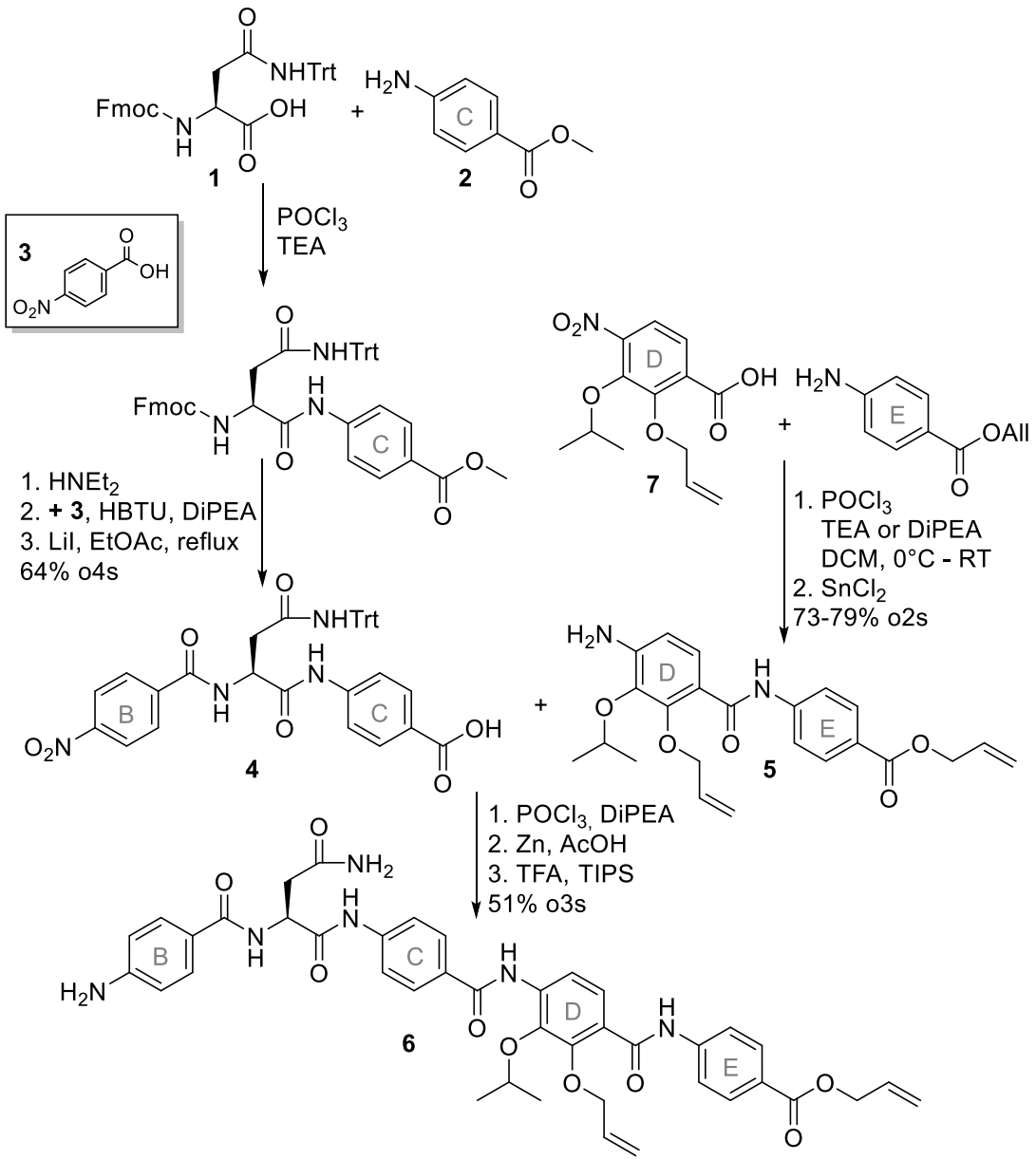


Figure 8: Part I of the synthesis of CN-DM 861, developed by G. Testolin *et al.* [9] and Hüttel *et al.* [72]. The yields for reactions in this work are summarized for consecutive reaction steps where suitable (e.g. over three steps, o3s).

For the final assembly of compound CN-DM 861, 4-cyanobenzoic acid was coupled to the aniline function of intermediate **6** using triphosgene, followed by Pd-catalyzed deprotection of the allyl groups (see Figure 9).[9]

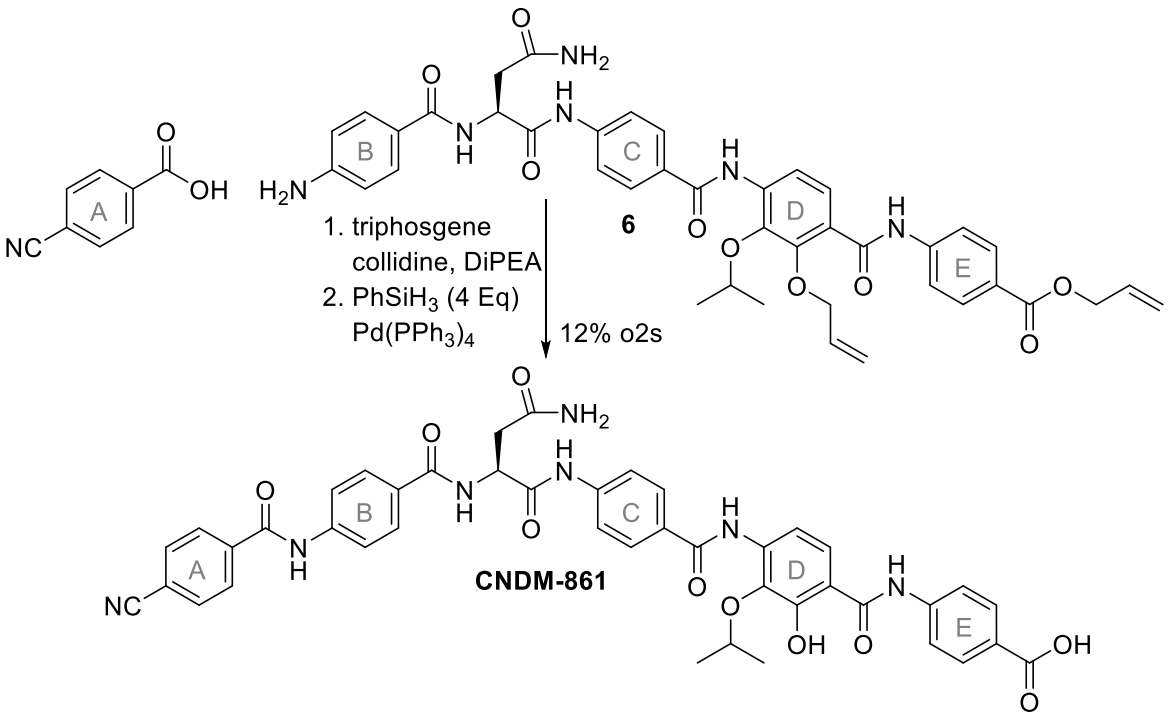


Figure 9: Part II of the synthesis of CN-DM 861 as developed by G. Testolin *et al.* [9].

This route was later improved in terms of more convenient reaction conditions. The retrosynthetic cut was now made between ring B and the central AA, as well as between the latter and ring C (see Figure 10). Here the AB-fragment was coupled last to the central AA.[11] Similar approaches were also published as part of the first cystobactamid total synthesis in 2017,[95] as well as in 2019[96] and will not be discussed here further.

In the modified route in Figure 10, the DE-fragment **8** comprised a *t*Bu-protecting group at the C-terminus and was first coupled to 4-nitrobenzoyl chloride. After subsequent reduction with zinc, the CDE-fragment with the free amine **9** was obtained. For the reduction of nitro functions in cystobactamid intermediates, several methods were published, involving catalytic hydrogenation,[95, 97] $SnCl_2$[72] and zinc[9, 96]. Reductions with zinc in AcOH exhibit some advantages such as low toxicity and good chemoselectivity. Moreover, in such a weak acidic medium the now used *t*Bu protecting group at the C-terminus was not deprotected.[9, 11, 96]

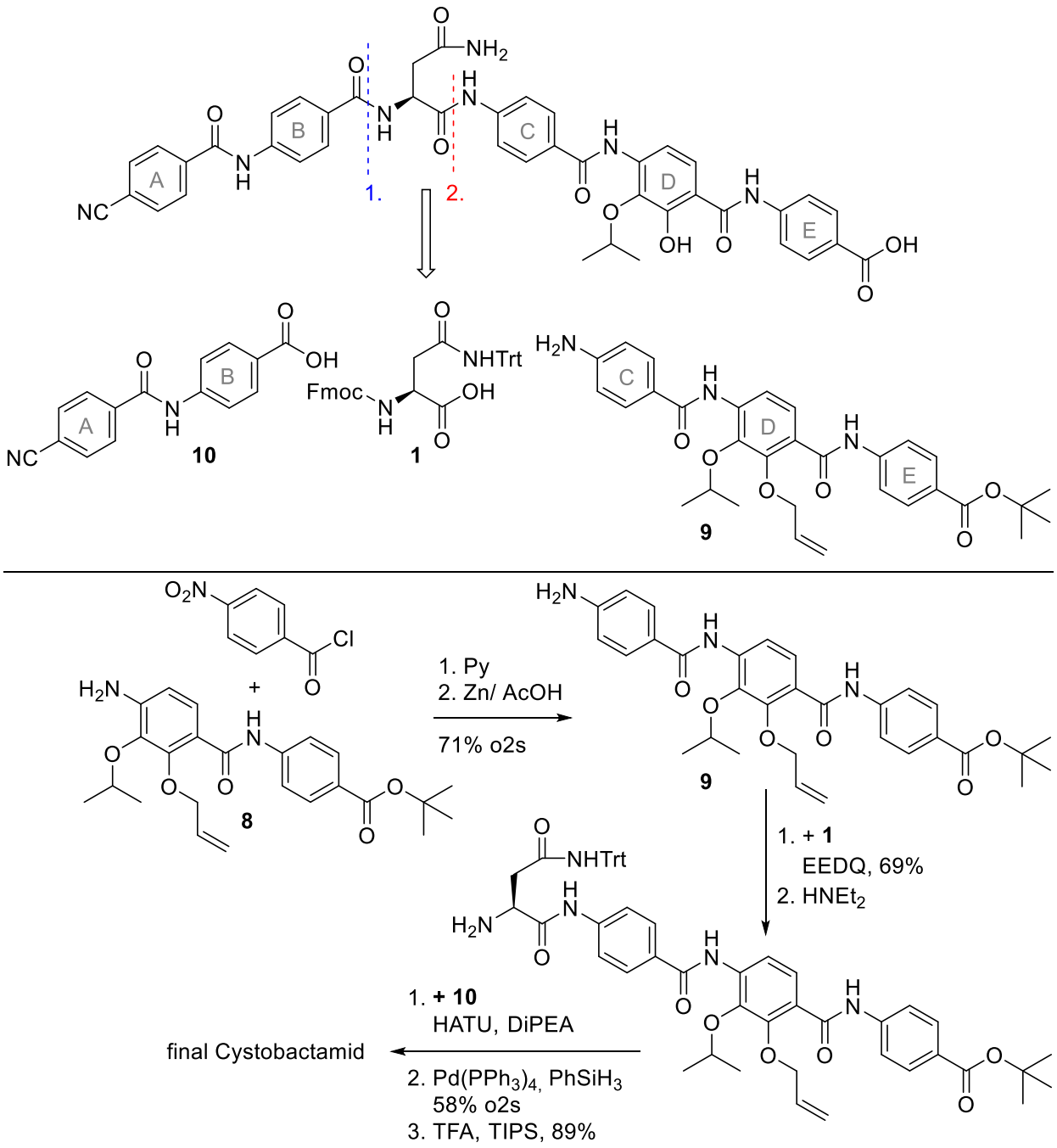


Figure 10: Retrosynthetic strategy for the assembly of cystobactamids with altered retrosynthetic cuts (top); exemplified synthesis with reaction conditions developed by Moeller *et al.* and D. Kohnhäuser (bottom).[11, 96] The reaction sequence is shown as published in the latter source.

The CDE-fragment **9** was subsequently coupled to the double-protected central AA. In this step $POCl_3$ combined with pyridine, EEDQ (*N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline), or T3P[98]/Pyridine were found to be more selective reagents than $POCl_3$/DiPEA, preventing elimination reactions and racemization of the central AA.[11, 96] The Fmoc group was removed using diethylamine before the protected full-length cystobactamid was obtained via HATU amide coupling between the free amine and an AB-

fragment. The phenolic hydroxy group was deprotected by the known catalyzed deallylation with $Pd(PPh_3)_4$ using phenyl silane or aniline as scavenger,[11, 96] followed by final deprotection of the terminal carboxylic acid by acidic hydrolysis with TFA.[11, 95-96] The final compound was isolated from the reaction mixture by preparative reverse phase HPLC (RP-HPLC) using NH_4HCO_3 as additive.[9, 99]

As a last strategy, the retrosynthesis is as shown in Figure 11.[9, 100] In contrast to the previous method, the AB-central AA fragment is assembled first and coupled to the CDE-fragment afterwards. For the latter, a heavily modified analogue was selected, that was proposed as a strong minor-grove binder for DNA during the topoisomerase inhibition process and would therefore increase efficacy.

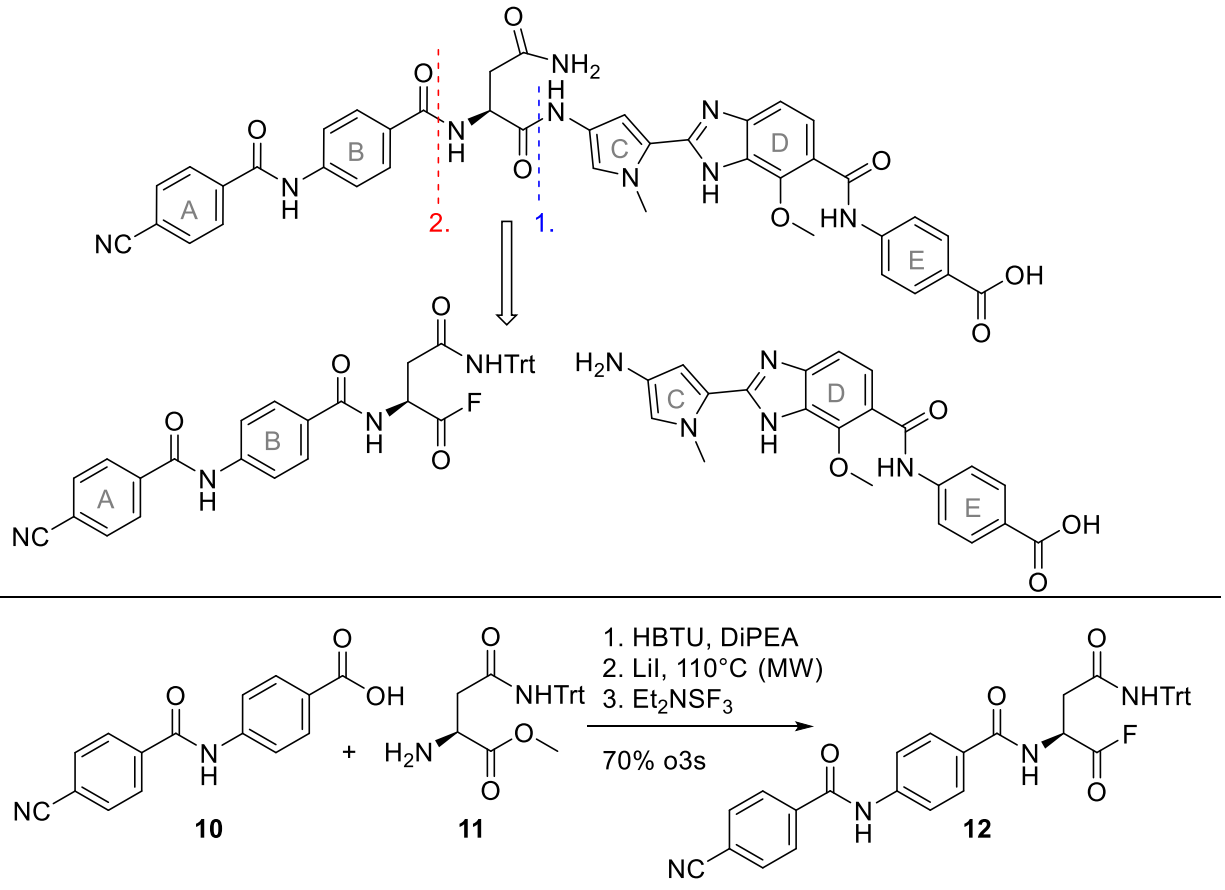


Figure 11: Retrosynthetic strategy with initial assembly of the linker-CDE-fragment and coupling to the AB-fragment (top). Reaction conditions for assembling the AB-linker fragment and activation to acyl fluoride (bottom).[9, 100]

The carboxylic acid of the AB-fragment **10** is first coupled with the amino function of the central AA as methyl ester **11**. After demethylation with LiI at high temperature, the free carboxylic acid can be activated to an acylfluoride **12**. Diethylaminosulfur trifluoride

(DAST) is used as fluorinating reagent. The late-stage coupling of the CDE-fragment is especially suitable for the introduction of multiple new CDE-fragments.

1.2.3 Detailed synthesis of ring D and its incorporation in cystobactamids

The most challenging part of cystobactamid synthesis is the construction of the tetra-substituted ring D, involving the arrangement of four adjacent substituents. In the earliest synthesis of Albicidin[73] a method of Kato *et al.* from 1996[101] was used to introduce the nitrogen substituent by nitration at low temperatures to an earlier described *ortho*-vanilline derivative **13**, followed by reprotection of the phenol as an allyl ether. The aldehyde function was subsequently oxidized to a carboxylic acid **14** using the Pinnick-Lindgren protocol (see Figure 12).[102]

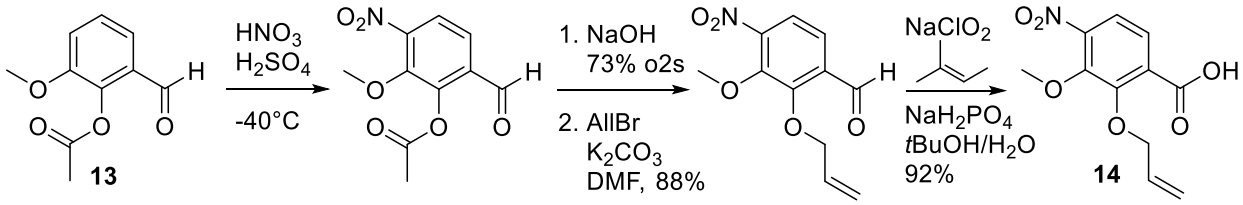


Figure 12: Synthesis of the tetra-substituted ring D as published for the Albicidins in 2015[73] and in earlier work[101-102].

Ring E was then introduced by amide coupling facilitated by triphosgene using collidine as base.[103] It was stated that coupling using T3P was insufficient, however this method was later successfully established in a cystobactamid synthesis[11].

In parallel, another synthesis for ring D was published in 2015[97, 104] using a 12-step procedure starting from 6-bromo *ortho*-vanillin. This was the first synthesis of a ring D building block featuring an isopropoxy instead of a methoxy substituent. Here as well, the nitration was performed using a regioselective method by Anuradha *et al.* involving Nickel(II) nitrate under acidic conditions.[105] The isopropyl group was attached in the next step via Mitsunobu reaction. Several protecting and deprotecting steps followed. Additionally, a shorter synthesis was published for the methoxy derivative, producing carboxylic acid **15** from *ortho*-vanilline in only 4 steps (see Figure 13, method **1**).[97] A similar procedure was published by Kleebauer *et al.* in 2021.[106]

The next contributions were made in 2017 by Cheng *et al.*[95] (Figure 13, method **2**) and Hüttel *et al.*[72] (Figure 13, method **3**) starting from 2,3-dihydroxybenzaldehyde. Here, the 3-isopropyl ether was obtained by a selective Williamson ether synthesis[107] with

isopropyl bromide and NaH. In the next step, an acetyl protecting group was introduced for the remaining hydroxy group to produce **16**. Protecting the hydroxy group with acetyl allowed regioselective nitration in the desired position, as the directing effect of the hydroxy group was now reduced. Nitration was performed at low temperature, using either KNO_3/TFAA or HNO_3 in DCM as the nitronium source.[108] While the acetyl group was maintained as protecting group in method **2**, it was replaced by allyl in method **3**, requiring two more steps. Eventually, the corresponding aldehydes were oxidized to the carboxylic acids **17** and **7** with oxone® or Pinnick conditions.

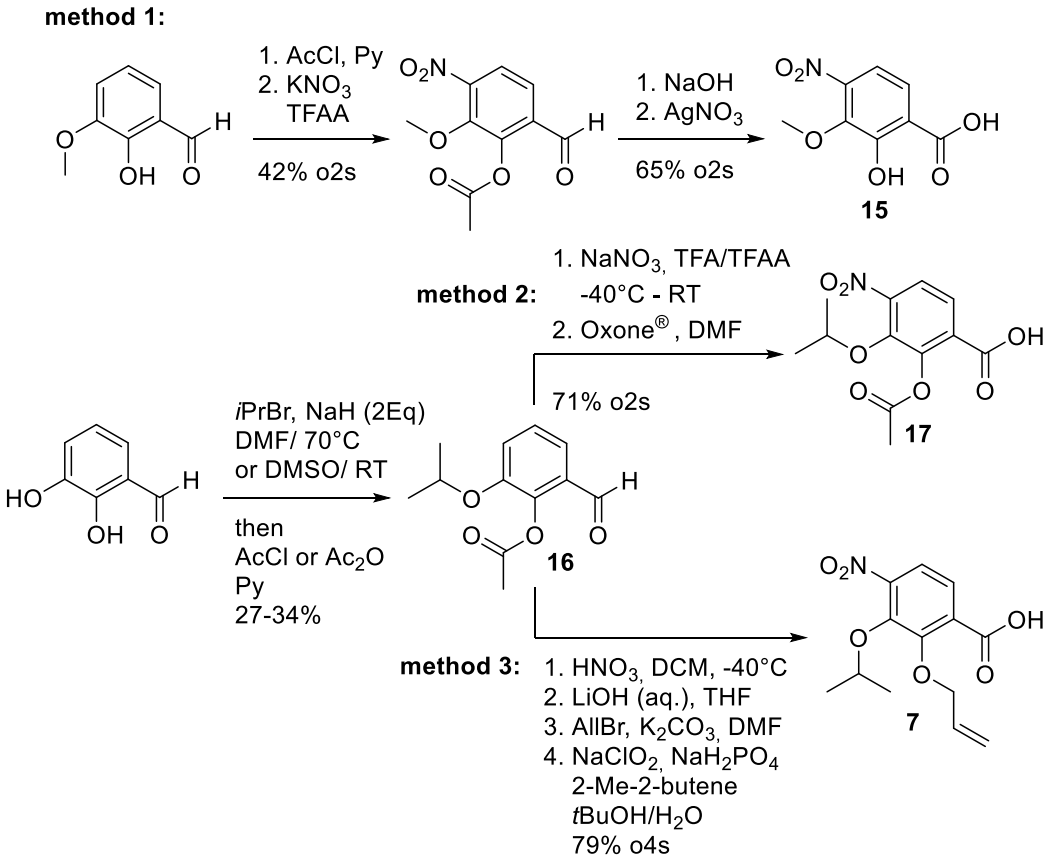


Figure 13: Published syntheses of ring D with method 1)[97, 104] 2)[95] and 3)[72].

An alternative route starting from catechol was implemented by Elgaher *et al.* in 2020 (Figure 14, method **4**).[109] After the nitration of catechol, the isopropylation was performed selectively in *ortho* to the nitro function. This selectivity might be due to the higher acidity of this hydroxy group. The carbonyl function was subsequently introduced by Skattebol-formylation using $MgCl_2$ as *ortho*-directing reagent.[110] The carboxylic acid **18** with free phenol was obtained after oxidation of the aldehyde with $AgNO_3$.

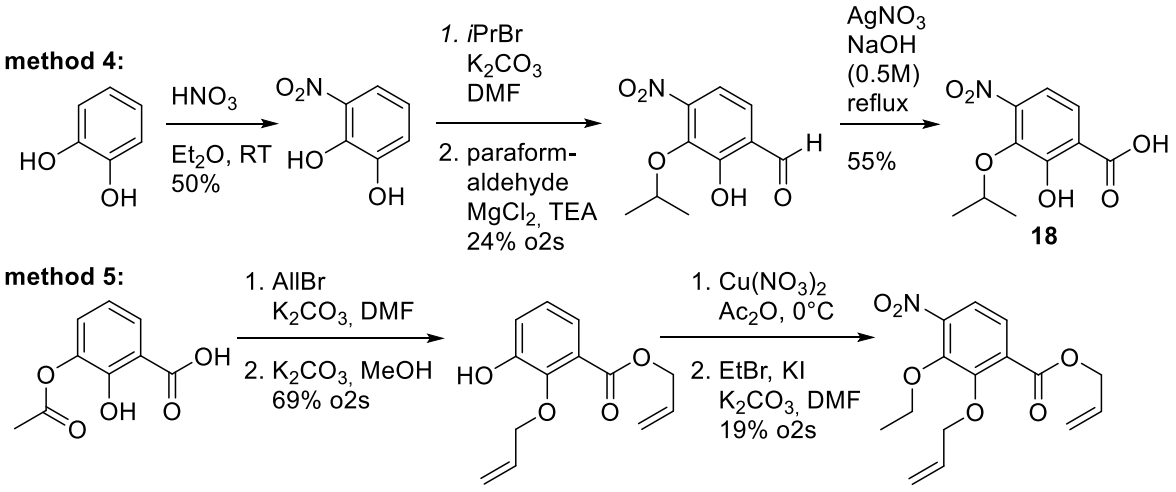


Figure 14: Alternative syntheses for ring D with method 4)[109] introducing methoxy and method 5)[111] ethoxy.

Another protocol that allowed introduction of an ethoxy ether is worth mentioning (Figure 14, method **5**).[111] This synthesis started from 3-acetoxy-2-hydroxybenzoic acid. After allyl protection, the acetyl group was cleaved to form the phenolic substrate for a Menke nitration. The latter involves *ortho* selective substitution using cupric nitrate and Ac_2O.[112-113] However, the allyl group did not seem to withstand those conditions, since the yield is low. Eventually, the ethyl group was introduced by Williamson ether synthesis. Although the selective deprotection of the corresponding carboxylic acid was not posed in the publication, this building block was used as both ring D and E.

Besides this, several derivatives of ring D and similar compounds were synthesized in literature (see Figure 15). For reasons of brevity, a detailed presentation of the synthetic conditions is omitted. One remarkable substitution was implemented for assembly of α-Helix Mimetics showing a hydroquinone structure (Figure 15, bottom right).[114-115]

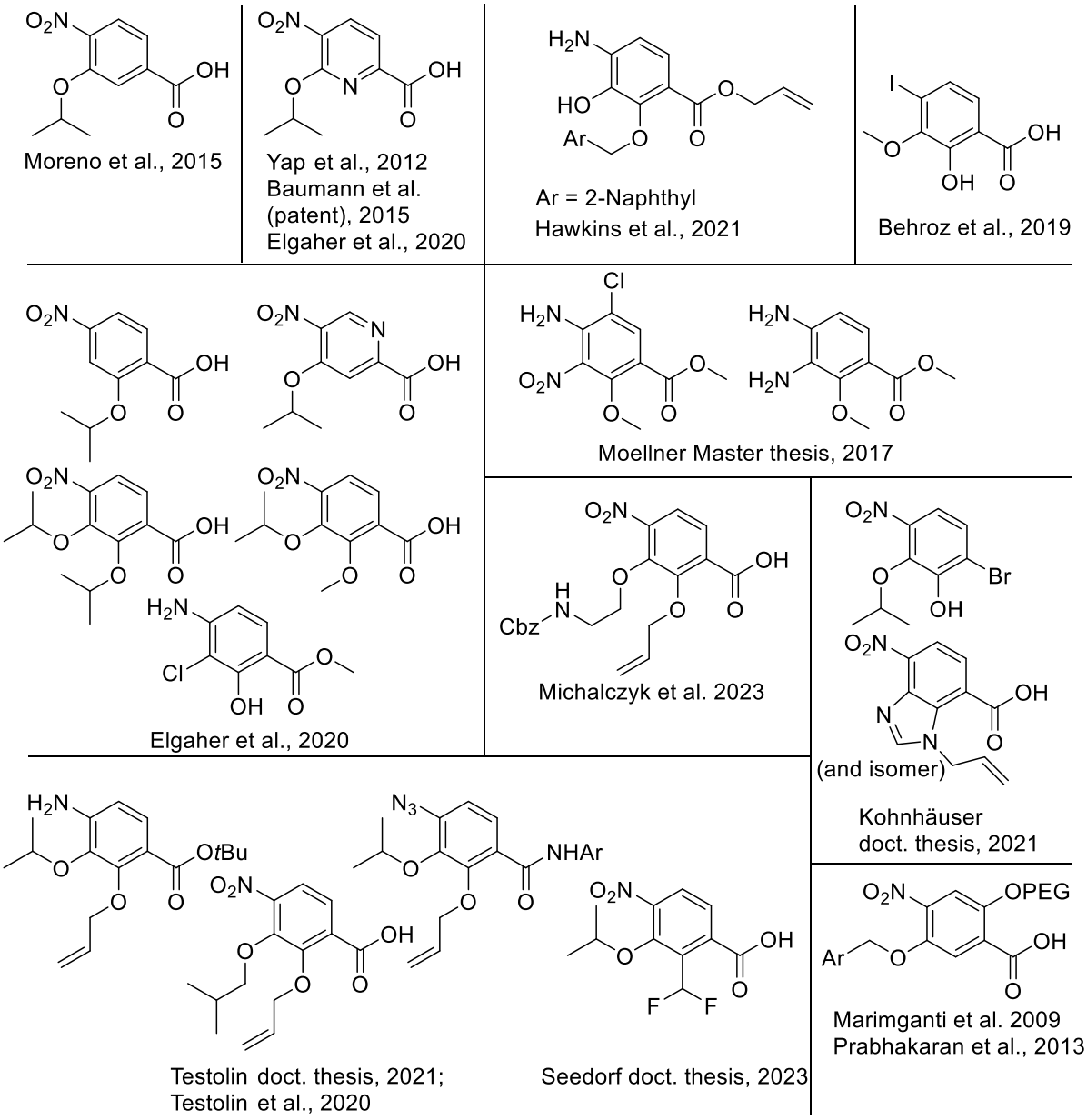


Figure 15: Other modifications of ring D that were published for cystobactamids, Albicidins, Coralmycins and related compounds.[9-11, 89, 97, 99-100, 104, 109, 111, 114-117] First authors are depicted with the year of publication.

Ring D fragments were usually prepared as free carboxylic acid, which was subsequently coupled to ring E. Several coupling conditions were applied, with Figure 16 showing one of the most relevant and convenient methods.[9, 72, 99] $POCl_3$ is used as activation reagent to produce the acylchloride *in situ*, followed by reaction with the aniline function of ring E. After reduction with zinc in AcOH the DE-fragment is obtained.

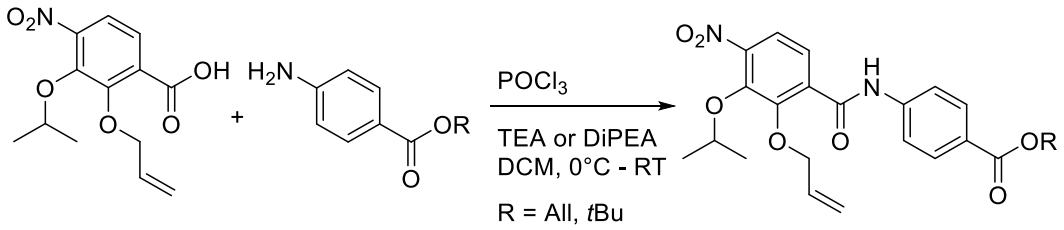


Figure 16: Standard procedure for amide coupling between ring D and E.

1.2.4 Recent SAR-data for the Western fragment

SAR investigations for cystobactamids started immediately with their discovery in 2014,[8] and since then several key compounds have emerged in the optimization process to achieve high broad-spectrum activity. The most important method for the *in vitro* evaluation of the antibiotic efficacy is the minimum inhibitory concentration (MIC)-assay, determining the lowest concentration of a compound preventing visible bacterial growth. In this section, the focus is on the Western part of the cystobactamids. The MIC-values are depicted for key cystobactamids in Table 1 with the structures shown in Figure 17. Where data is available, resistances of a strain against certain types of antibiotics are indicated.

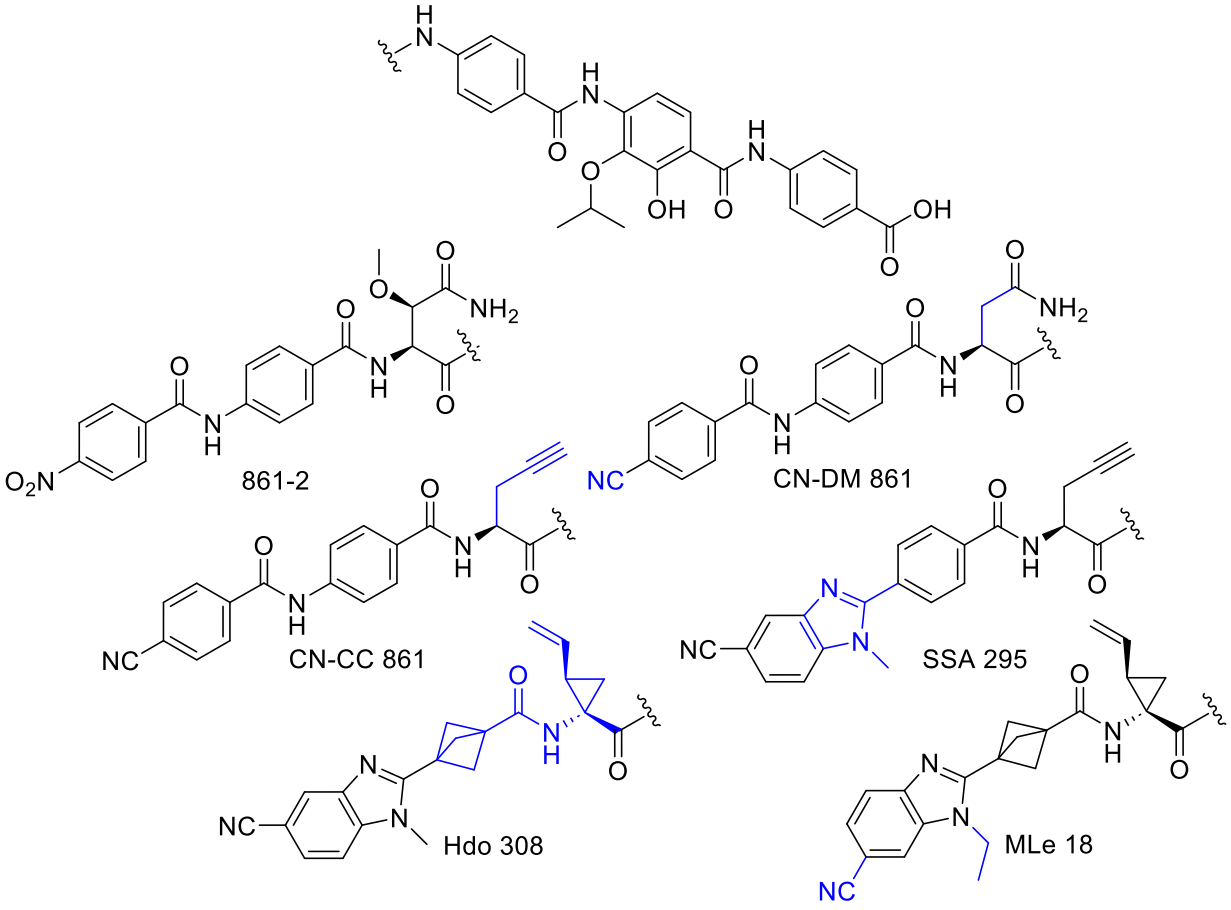


Figure 17: Structures of key compounds with focus on the Western fragment.

Table 1: MIC-values (µg/mL) of cystobactamids from Figure 17. The MIC values for some strains that were missing in the original publications were re-measured by Janetta Coetzee (HIPS).

	861-2 [72]	CN-DM 861 [9]	CN-CC 861 [11]	SSA 295 [118]	HDo 308 [119]	MLe 18 [120]
A. baumannii DSM-30008 (AR)	0.5	0.5-2	0.02-0.03	0.125	0.06	n.d.
A. baumannii ATCC BAA-1710 (FQR)	4-8	64	0.06-0.25	0.25	0.5	≤0.03
A. baumannii CIP-107292 (FQR, CR)	>64	64	0.5-1	1	0.125-0.25	n.d.
A. baumannii R835 (FQR)	n.d.	2->64	1	1	0.5	0.06
A. baumannii ACC00535 (FQR, AR)	n.d.	>32	0.25	0.06-0.125	n.d.	n.d.
A. baumannii NCTC 13301 (CR)	n.d.	>64	2	0.25	0.125	≤0.03
A. baumannii DSM-30007 (AR)	n.d.	n.d.	≤0.03	0.01	n.d.	0.125
A. baumannii 070 NDM-1 (CR)	n.d.	n.d.	2	0.025	n.d.	≤0.03
E. coli ATCC-25922	2*	0.06	0.03-0.06	0.00125-0.016	<0.06	≤0.03
E. coli LM705 (S83L, D87N, S80I, ΔmarR, ΔacrR) (FQR, Gyr, EFL+)	n.d.	0.125-0.25	<0.03-0.2	0.25	1	0.25
S. pneumoniae DSM-11865 (FQR, AR)	n.d.	n.d.	0.125	8	2	1
E. faecium DSM-17050 (VR)	n.d.	n.d.	<0.03	0.5	<0.06	2
S. aureus ATCC-29213	0.125*	1	0.02	4	<0.3	≤0.03
S. aureus MRSA (clin HAP/VAP, pneumo isolate BAL #2524MHH, 2022) (AR)	n.d.	n.d.	≤0.03	2	n.d.	≤0.03
K. pneumoniae CIP-104298 (AR)	64*	0.125-4	8	0.5	1	n.d.
K. pneumoniae R-1525 (FQR)	n.d.	>64	16- >64	>64	<0.3	n.d.
P. aeruginosa PA14	4	4-64	0.5-16	>64	4	n.d.
P. aeruginosa PA14ΔmexAB (EFL-)	1	0.5-1	0.03-0.5	0.5	1	n.d.
E. cloacae ATCC BAA-2468 (CR)	n.d.	1	0.06-0.5	1	2	0.25
E. cloacae DSM-30054	64	1	0.5	n.d.	n.d.	n.d.
E. aerogenes CIP 106754 (AR)	n.d.	(>64)	0.125-4	2	8	4

**E. coli DSM1116,* S. aureus Newman or *K. pneumoniae DSM-30104* used as comparable reference strain. n.d. = not determined

Reference strains are marked in blue. AR = ampicillin- or amoxicillin- resistant, CR = resistant against some carbapenems, EFL+/- = increased or decreased efflux, FQR = fluroquinolone resistant, Gyr = gyrase mutant associated with FQ-resistance, VR = vancomycin resistant. In this table and following MIC-tables this color code is used: green: <1 µg/mL, orange: 1-8 µg/mL, red: >8µg/mL. Ranges are colored for their median value (in exponential series)

Starting from the natural product 861-2 it was shown by Testolin *et al.* that removing the methoxy residue in the central AA retains activity and a cyano group was successfully introduced as a bioisosteric replacement for the *N*-terminal nitro group (compound CN-DM 861).[9, 99] Nitro groups are considered toxicophores.[121] Moreover, the activity against the tested bacterial strains was significantly improved by introducing L-

propargylglycine as central AA in CN-CC 861.[11] In particular, the susceptibility of *A. baumannii* is remarkably high, including strains with fluoroquinolone resistances. In later optimization stages, several new modifications of the AB-system were created,[9-11, 99] with SSA 295 being one of the most remarkable in terms of activity against *A. baumannii*[118]. In this compound, the amide bond between rings A and B was rigidified by implementing a benzimidazole as partial structure. Methylation of one imidazole nitrogen was found to be essential for a high antibacterial activity (Data provided by Evotec). Improved activity could be observed for *A. baumannii* strains, while SSA 295 shows lower activity against *S. aureus* and *P. aeruginosa* reference strains.

As a more exotic descendant, the introduction of a bicyclo[1.1.1]pentane partial structure as ring B as well as a rigidified cyclopropane amino acid led to derivative HDo 308. This compound showed an even higher activity against several *A. baumannii* strains, with MIC values up to three dilution steps lower than those of SSA 295. Shifting the position of the *N*-terminal cyano group from 5- to 6-position results in MIC-values below the lowest dilution tested (0.03 µg/mL) for *A. baumannii*, while substitution of the methyl group with ethyl has a slightly beneficial effect.[120]

The main results of the SAR studies obtained until July 2023, relevant for this Thesis, are summarized in Figure 18 for rings A and B, the central AA and the AB-linker. The results of the SAR studies of the CDE-fragment are explained in section 1.2.5.

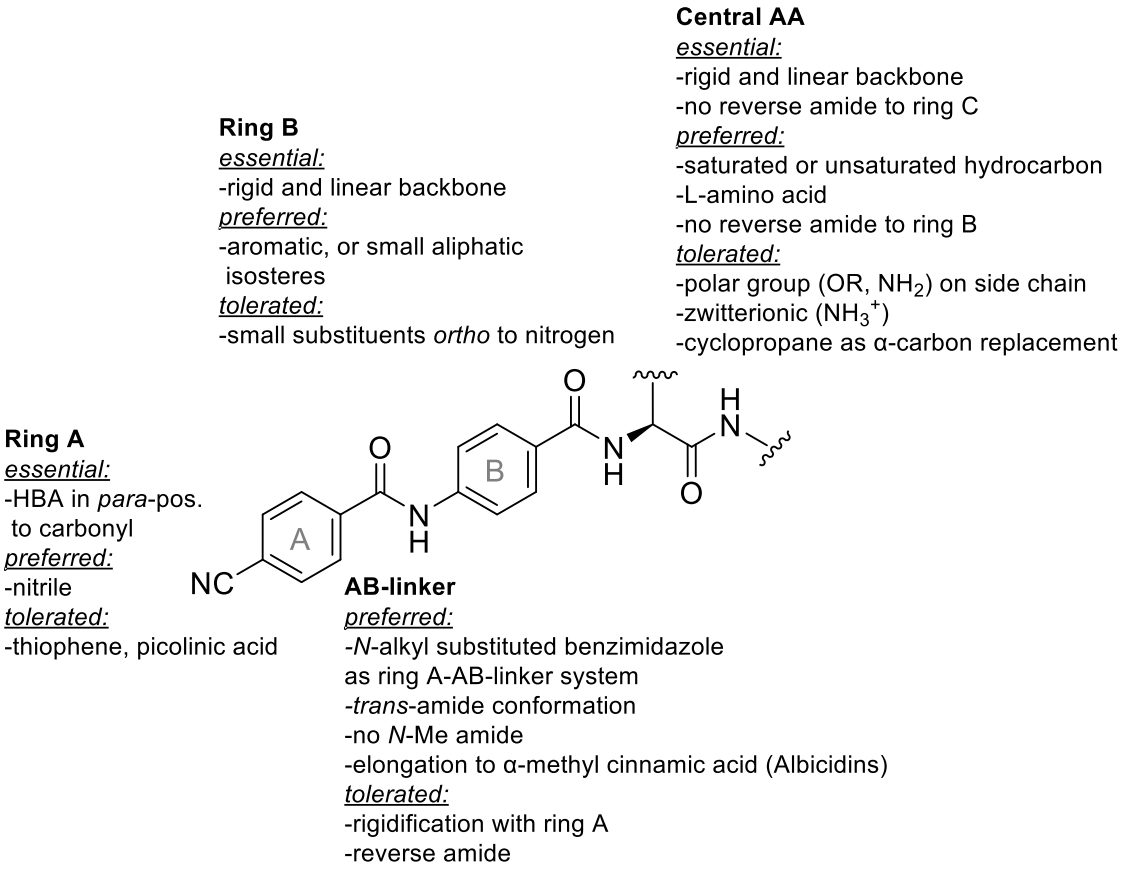
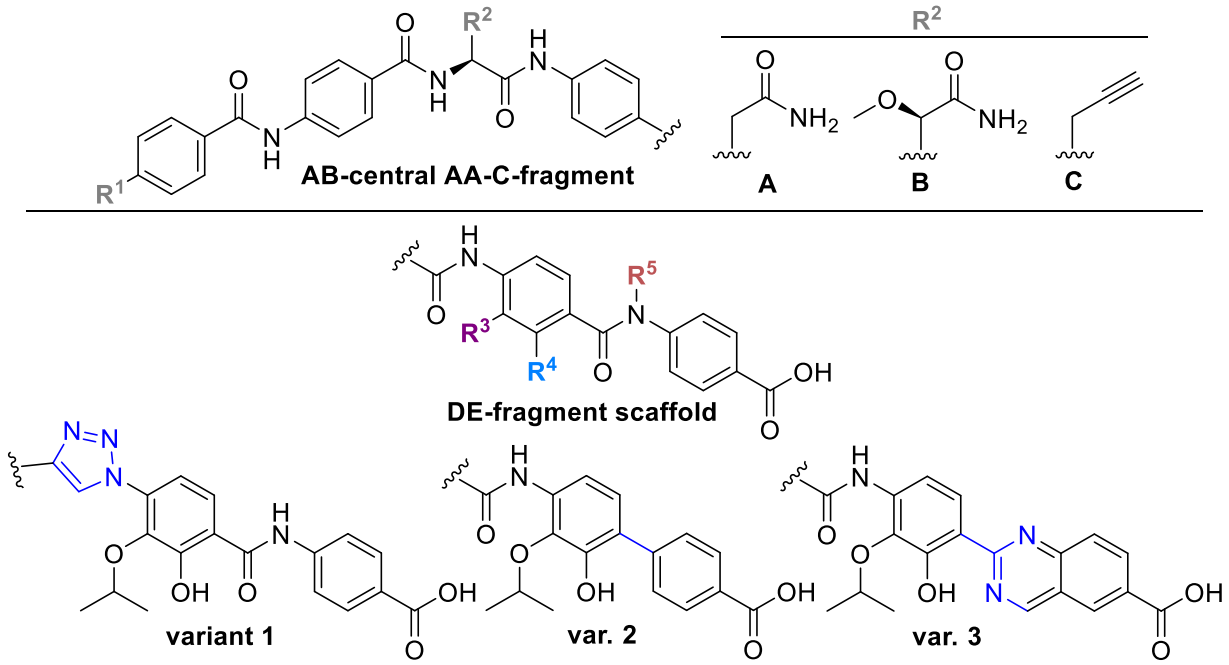


Figure 18: Current SAR results for the Western fragment with focus on structures relevant in this Thesis.[9-11, 99, 119, 122] HBD: hydrogen bond donor, HBA: hydrogen bond acceptor.

1.2.5 Recent SAR-data for the Eastern fragment

The Eastern fragment was focused in later investigations. Ring D occupies a special position here, since the two additional substituents can form H-bonds with the CD- and DE-linkers. Table 2 summarizes the MIC-values of selected compounds with modifications in ring D and the attached amide bonds. Due to different strains tested in these SAR investigations, a general comparability is not given but can be at least assumed for some of the shown strains.

Table 2: MIC-values (in µg/mL) and IC_{50} gyrase values of recent cystobactamids with CD-linker, ring D, and DE-linker modifications. Reference strains are marked in blue.



	CN-DM 861 'Cmpd. 22'[9]	**19** 'Cmpd. 52'[9]	**20** 'Cmpd. 54'[9]	**21** 'Cmpd. 61'[9]	TSD 08 [10]	TSE 40 [10]	DK 477 [11]	DK 674 [11]
R^1, R^2	CN, **A**	NO_2, **A**	CN, **A**	CN, **A**	CN, **B**	CN, **B**	CN, **C**	CN, **C**
R^3	*i*PrO	*i*PrO	*i*BuO	**var. 1**	*i*PrO	*i*PrO	**var. 2**	**var. 3**
R^4	OH	H	OH		CHF_2	OH		
R^5	H	H	H		H	Me		
E. coli wild type [a]	0.5 (WT1)	>64 (WT1)	0.25 (WT1)	0.5 (WT1)	n.d.	>8 (WT4)	>64 (WT3)	>64 (WT2)
E. coli ΔtolC (EFL-)	0.06	0.06	<0.03	<0.03	n.d.	n.d.	<0.03[c]	n.d.
E. coli LM705 (S83L, D87N, S80I, ΔmarR, ΔacrR) (FQR, Gyr, EFL+)	n.d.	n.d.	n.d.	n.d.	1	>64	n.d.	>64[d]
S. aureus ATCC-29213 (WT)	0.25	>64	0.25	<0.03	8	>64	>64[b]	>64
P. aeruginosa PA14	2	>64	>64	8	>64	>64	>64	>64
P. aeruginosa PA14ΔmexAB (EFL-)	0.25	1	>64	2	16	>64	>64	>64
IC_{50} *E. coli* gyrase [µM]	0.08	n.d.	0.9[e]	1.5[99]	n.d.	n.d.	n.d.	n.d.

[a] Different wild types were used: WT1: DSM1116, WT2: MG1655, WT3: BW25113, WT4: ATCC-25922; [b] S. aureus Newman as reference strain; [c] E. coli ΔacrB mutant; [d] E. coli CH448 (S83L. QnrS) (FQR, Gyr) mutant; [e] for R^1 = NO_2, EFL+/- = increased or decreased efflux, FQR = Fluroquinolone resistant, Gyr = gyrase mutant associated with FQ-resistance.

Regardless of the structural differences in the *N*-terminal group and the central AA, it can be seen, that removal of the hydroxy substituent as HBD in **19** highly decreased activity against *E. coli* and *S. aureus* wildtypes as well as *P. aeruginosa* ref. strain. However, *E. coli* and *P. aeruginosa* with downregulated efflux system were still susceptible.[9, 99]

Substitution of hydroxy with a difluoromethyl group in TSD 08 reduced activity.[10] Besides, elongation of the ether residue to isobutoxy in **20** was partly beneficial.[9, 99]

With closer view to the CD linker, isosteric replacement of the amide with a triazole in **21** was tolerated.[9, 99] On the other side, the annihilation of all activity by introducing a *N*-methylated DE-linker in TSE 40 indicated the importance of an unsubstituted amide.[10] Similar results were obtained for DK 477 with a biphenyl system consisting of rings D and E and thus without a DE-linker. However, residual activity is again observed for the *E. coli* mutant with decreased efflux. Annulation of ring E with pyrimidin as isostere for the DE-amide in DK 674 formed a quinazoline and led to a complete activity loss.[11]

With regard to gyrase inhibition, the very different IC_{50} values of CN-DM 861 and **21** are striking, which should actually be in the same order of magnitude according to the identical activity in the MIC-assay. This is an indication of the difficult correlation of IC_{50} and MIC mentioned in section 1.2.1.

Knowledge of the SAR at ring C is still limited, as only a few modifications have been synthesized at this site. However, the introduction of 5-aminopicolinic acid as a pyridine anologue of ring C was tolerated and activity was well maintained in combinations of this analogue with various Western fragments[10-11, 123].

When evaluating the SAR of ring E, some interesting results were obtained in relation to the carboxylic acid. The substitution of the carboxylic acid with a $CONH_2$-group can retain activity in both *E. coli* wildtype and mutant with reduced efflux, while activity against *P. aeruginosa* and *S. aureus* is not present.[9, 99] Introduction of a sulfonic acid led to complete loss of activity.[123] However, the introduction of a tetrazole largely preserved activity.[124] Further examples are given in the Results section and are discussed there together with the corresponding compounds synthesized in this Thesis. Modifications of the benzene core of ring E were not investigated in this Thesis and are therefore not described in detail here.

From these observations, the SAR shown in Figure 19 was derived for the Eastern fragment:

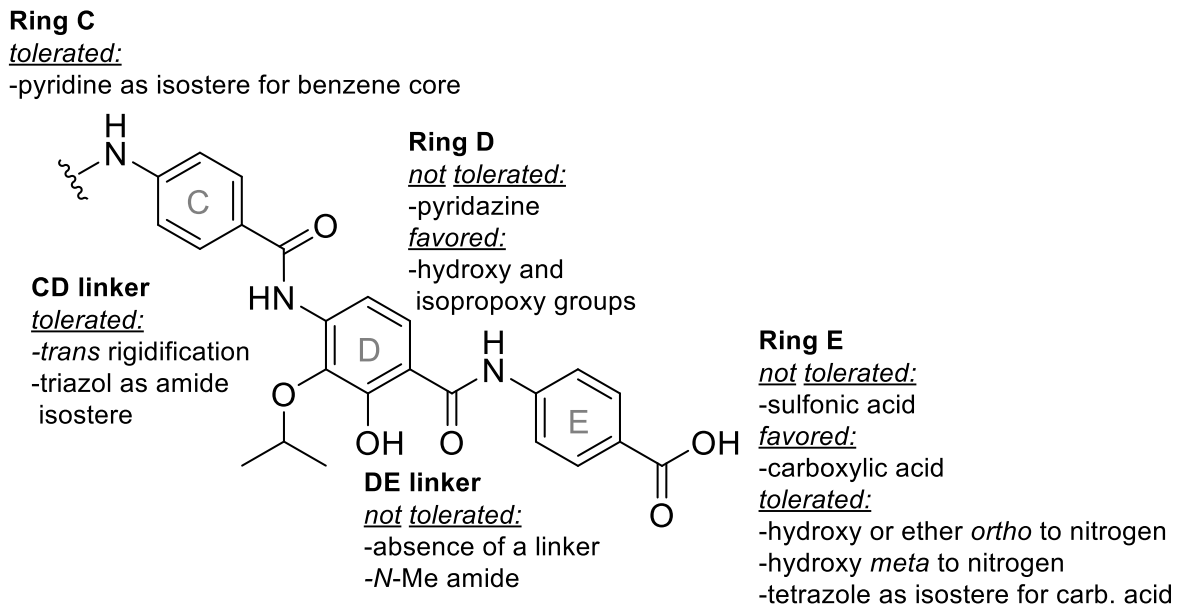


Figure 19: Current SAR results for the CDE-system.[9-11, 99, 118-119, 123]

1.2.6 Physicochemical properties of recent cystobactamids

In later physicochemical investigations, aequeos solubility and $logD_{7.4}$ values were determined along with the degree of plasma protein binding (PPB) (expounded in section 1.3) for cystobactamids. Data for the most important cystobactamids is listed in Table 3.

Table 3: Physicochemical properties and PPB data of selected cystobactamids.

	861-2	CN-DM 861	CN-CC 861	SSA 295	DK 444
aq. solubility pH 7.4 [µg/mL] thermodyn.[a)]	<1	<1	n.d.	<1	901
aq. solubility pH 7.4 [µg/mL] kinetic[b)]	n.d.	<10	n.d.	n.d.	800-1000
$logD_{7.4}$ [a),c)]	1.76	1.49	2.26	n.d.	1.72
PPB (human) [b)]	n.d.	99.71±0.18	100±0.0	99.96±0.1	n.d.

[a)] data provided by Evotec,[b)] data provided by K. Rox (HZI),[c)] determined via HPLC-retention time

Solubility of solids can be separated in thermodynamic solubility, which is the concentration of the compound in a saturated solution in thermodynamic equilibrium, and kinetic solubility. The latter may be higher than the thermodynamic solubility, as it is possible that the solid to be dissolved may constitute a well soluble form but not the thermodynamically most stable. This is especially the case if the compound forms a readily soluble non-crystalline solid and precipitates from solution in a less soluble, but thermodynamically more stable crystalline form.[125] Both thermodynamic and kinetic aequeos solubility of recent cystobactamids are low at physiological pH, except for DK 444. This compound constitutes the structure of CN-CC 861 with bicyclo[1.1.1]pentane as

aliphatic isosteric group for ring B. Therefore, this structural motif was highly promising for further developments. The log D values need to be considered carefully, since the measurement was conducted chromatographically by comparison of the retention time of the compound with those of standards. This enables a high-throughput determination of log D, but is less precise than a direct determination via a shaken-flask extraction experiment. However, the values are in an acceptable range. The amounts of PPB however are near 100%, indicating a drastically low physiological availability of the compounds (further explanations in section 1.3).

1.3 Medicinal Chemistry and Pharmacology of cystobactamids

1.3.1 Rational design of SAR studies

A main difficulty within the process of optimizing a lead compound is to find feasible analogues that fulfill all physicochemical, pharmacokinetic and pharmacodynamic demands. Even before existence of computers, drugs were designed and optimized by simple empirical methods. First approaches can be traced back to 1860s, when the idea of connecting "chemical constitution and physiological action" was first raised and applied to natural bioactive compounds.[126-127] However, implementing practicable methods was found to be difficult. A main point of discussion was the question whether pharmacological activity of a drug was more related to their chemical structure or physical properties.[128] Almost one century later these different perspectives were generalized and quantified by Hansch *et al.* .[129-130] This resulted in quantitative structure-activity relationship (QSAR) models that also allowed computer-aided optimization as well as modelling of physicochemical properties and biological activity.

For the evaluation of biological activity, a huge library of compounds is often synthesized, which is unfeasible for non-commercial research. Therefore, there is a demand for a method, that allows deriving a beneficial chemical structure based of few compounds. The operational scheme of Topliss[131] is one vivid example, based on the work of Hansch *et al.*[129-130] and Craig[132] (see Figure 20).

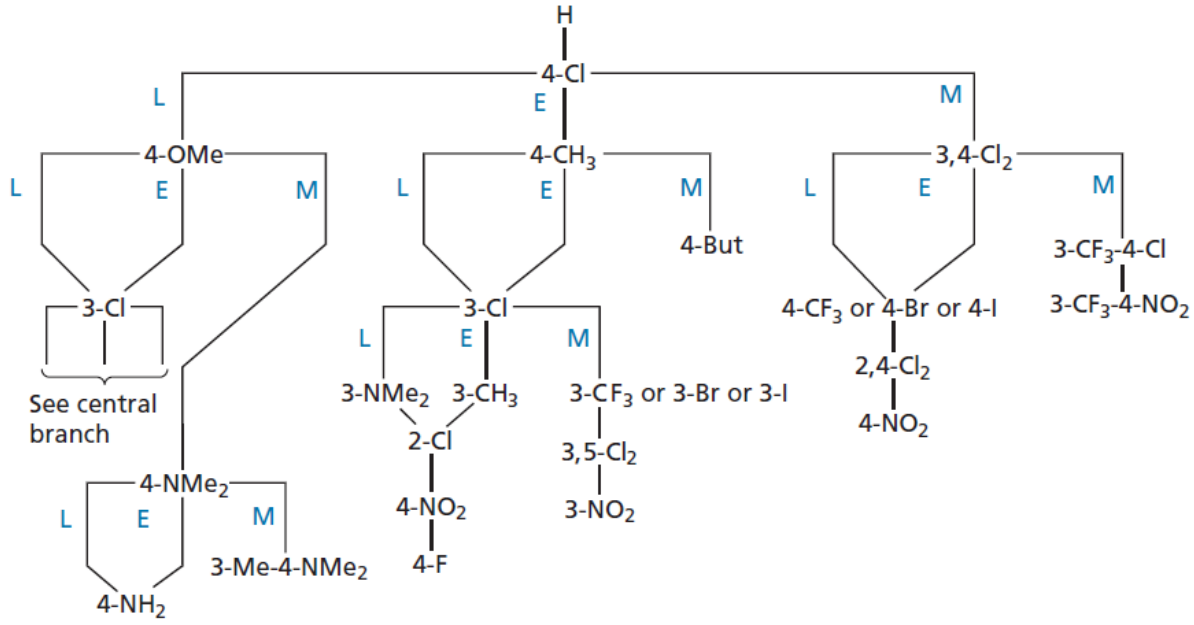


Figure 20: Topliss-scheme of aromatic substitution (image from[133]) for a bioactive compound with a non-substituted phenyl residue. The starting point is a replacement of the phenyl group with a 4-chlorophenyl moiety. Depending on how the activity of the compound changes with this modification (lower (L), equal (E) or more (M) activity), this moiety is replaced successively with differently substituted phenyl residues. Thereby, a systematic search for the best substitution pattern is enabled.

This scheme provides a connection between compound activity and the kind of aromatic substituents or aliphatic side chains that can be introduced to improve the activity.[133] In an extended sense, this approach can also be applied to PABA oligomers such as cystobactamids. Thus, the SAR of ring B was investigated by successively exchanging the substituents and accompanying evaluation of the MIC-activity.[11]

Another simple drug optimization strategy is to replace distinct functional groups with a new structurally similar moiety which is then called isostere in this context. If this change results in comparable physical properties or *in vivo* activity of the tested compound, this isostere is called a bioisostere.[134-135] In this Thesis the term bioisostere is used in the context of *in vivo* activity. In the case of cystobactamids the successful substitution of the *N*-terminal nitro group by a nitrile function is a good example. For amides as well as benzene rings, several isosteric replacements are known, with Figure 21 showing relevant examples.

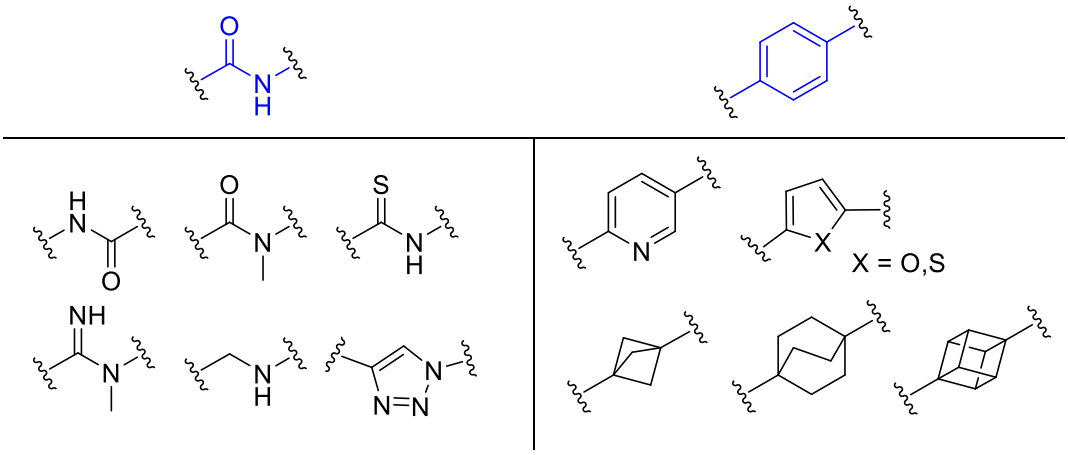


Figure 21: Selected well-established isosteric groups for amide[136-137] and *p*-phenylene[138].

With focus on ring D substituents, isosteric replacements for the phenolic hydroxy function include secondary amines with one electron withdrawing substituent to increase acidity as most popular example.[137, 139-140] For ethers, the substitution of the oxygen atom with methylene or omission of oxygen are feasible alternatives. However, this ether substitution often shows anomalous results.[137]

Besides this, the rigidification of a specific flexible bond within a drug is an established way to modulate activity. A compound molecule with flexible bonds can adopt many different conformations. But after it binds to the target, the number of possible conformations is drastically reduced to mainly one conformation, the active conformation. This restriction results in an unfavorable entropy decrease which increases the free energy ΔG for the binding process. However, when conformational flexibility is reduced by fixing (pre-organizing) the assumed active conformation, the entropy decrease will be limited and therefore the free energy will be lower. Because a lower free energy is directly related to a higher binding constant and a lower dissociation constant (according to $K = \exp[-\Delta G/(RT)]$), higher affinity and/or selectivity of the ligand to the target are usually the result.[141-142] Such an example is found when one compares the two opioid-analgetics morphine and pethidin (both μ-opioid receptor agonists), with morphine having additional bridging elements and a higher binding affinity to the μ-opioid receptor as well as a higher potency (Figure 22 a).[143-144] The principle of rigidification is especially relevant for modifications in the cystobactamid DE-fragment. Here, an intramolecular hydrogen bond (H-bond) between the phenolic hydroxy group and the adjacent amide can be rigidified, thereby affecting activity. Salicylamides are rarely found in commercial drugs. Simpler but close examples are found in literature

(Figure 22 b and c).[145-146] The rigidified compounds show comparable affinity constants or IC_{50} values with their non-rigidified counterpart. However, these are only examples and a general tendency is not derivable.

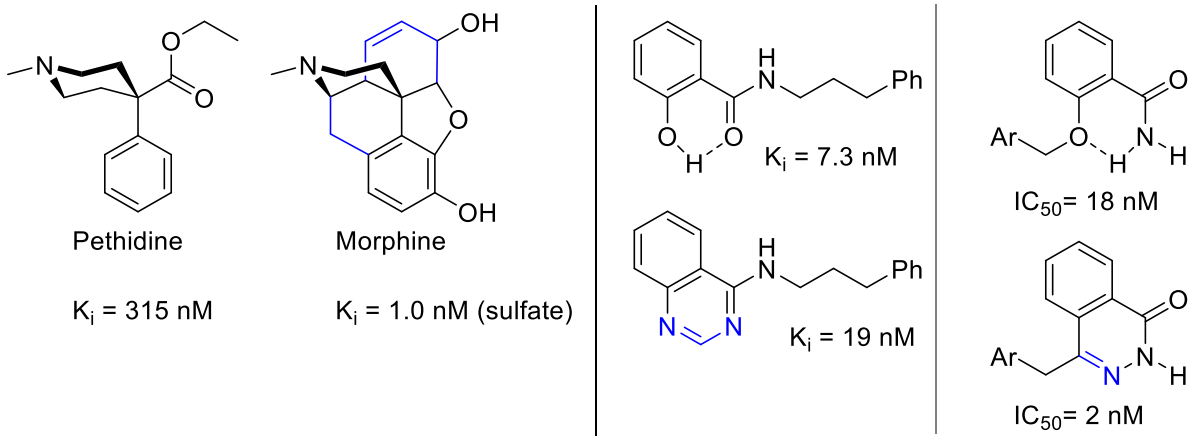


Figure 22: Examples for improved efficacy of drugs by rigidification: a) pethidine and morphine as opioid agonists, b) rigidification of intramolecular H-bond in a competitive inhibitor of scylatone dehydratase[145] and c) rigidification of the opposite H-bond binding mode in a poly(ADP-ribose) polymerase inhibitor[146].

Rigidifications were already successfully implemented in cystobactamids, e.g. with a benzimidazole between ring A and B as well as a cyclopropane derivative as central AA (see section 2.2.4).

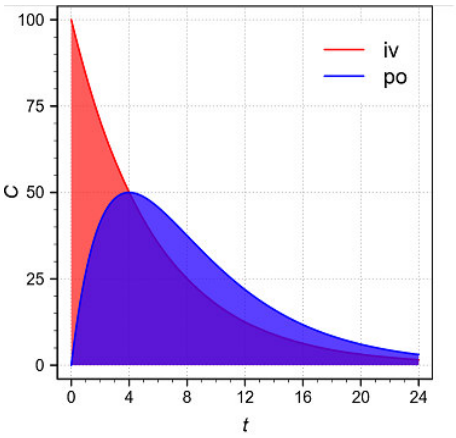
1.3.2 Pharmacokinetic parameters

SAR investigations of cystobactamids use the MIC as metric for *in vitro* activity determined for several bacterial strains. Subsequent steps follow including the determination of physicochemical properties such as solubility and log P as well as pharmacokinetic parameters. For promising substances, further pharmacokinetic and pharmacodynamic investigations in animal models ensue. This section goes into greater detail about the pharmacokinetic parameters.

Pharmacokinetics is often summarized in the acronym ADME standing for absorption, distribution, metabolism and elimination/excretion of a drug in an organism. The letter L, representing the liberation of a drug from its formulation, is often prepended to consider its effect to the bioavailability of the drug.[147] Bioavailability ('F') can be measured for different application routes, e.g. oral bioavailability, and is described by a quotient (see Figure 23). Here, the determination of the plasma concentration of the substance over time is of key importance. A time-concentration curve is then interpolated from the measuring points for an intravenous (i.v.) and oral application. Classically, a

decay curve (i.v.) and an accumulation-decay-curve (oral) are observed, respectively (see Figure 23). Integration of the curves delivers the area under the curve (AUC) which is used in the calculation of the bioavailability. The bioavailability of an i.v. application is assumed to be 100% and it is therefore used as reference.[25]

It should be noted that besides the classical solution of the drug in infusion medium there are several types of i.v. formulations (e.g. liposomes, micellar solutions) that may show different release rates.[148]



$$F = \frac{AUC_x}{AUC_{i.v.}}$$

Figure 23: Exemplified plasma concentration curves for i.v. (red) or oral (blue) application (schematic) with the formula for calculating bioavailability (F), AUC_x, $AUC_{i.v.}$: area under curve for a specific application route x and for i.v. application. The higher F the more efficient the application route.

After oral application and passage of the highly acidic stomach, absorption of the drug into the hepatic portal vein takes place mainly in the small bowel, by passing through the intestinal wall.[149-150] Important factors here are e.g. the solubility of the compound in aqueous solution as well as its lipophilicity, expressed by the log P value:[147, 151]

$$\log P = -\log(K_{OW}) = -\log\left(\frac{c_{octanol}}{c_{water}}\right)$$

K_{OW} : partition coefficient (equilibrium constant)

Since the log P value is only related to one specific species of a compound, an additional value is needed for ion-forming compounds like amines and carboxylic acids, that includes all present species at a certain pH. This value, called log D, is usually determined in a buffered aqueous solution at pH 7.4, since this is the physiologically most relevant pH (e.g. pH~7.4 for human plasma) and easy to adjust with PBS-buffer.[147]

The substance enters the liver via the hepatic portal vein, where it is metabolized in varying amounts. A combination of passive diffusion, facilitated diffusion by carriers or

transporters and active transport is relevant for the passage of a substance through membranes.[25, 152] This also applies to the uptake from the blood into the liver cells, more precisely into hepatocytes, however specialized transporters play a role that needs to be emphasized. The liver is adorned with such transporters, that enable metabolization of a plethora of chemically very diverse substances. For example, organic anion transporter proteins (OATPs) are able to transport mainly anionic compounds (e.g. carboxylates from carboxylic acids such as bile acids or cystobactamids) into liver cells.[25] Another important influx transporter is the liver bile acid transporter (LBAT), which participates in the enterohepatic circulation of a drug. This circulation leads to repeated metabolization which in turn can produce higher levels of inactive or toxic metabolites.[153] Despite of this enterohepatic circulation, a versatile machinery of liver enzymes can produce such metabolites that typically facilitate excretion of the parent compound.

The metabolism can be divided in two phases: Phase I are functionalization reactions, with include oxidations e.g. by cytochrome P450 enzymes, reductions and hydrolyses. Phase II involves conjugation of the compound with polar molecules like glucoronic acid or sulfate. This usually results in higher polarity and thus aqueous solubility of the substance and extends elimination routes from the body. While metabolization in many cases reduces the desired biological effect of the administered compound, metabolites sometimes may contribute to the activity or even resemble the active form of a drug (active metabolite). Toxic metabolites can be produced as well, causing undesirable side effects.[25]

The higher solubility can lead to faster elimination which can also mean that the compound is excreted without fulfilling its intended task. A certain percentage of the compound passes through liver and distributes via body fluids like blood in the body, reaching all organs. However, the transfer of a substance from the blood, more precisely the inner space of blood vessels, to its intended site of action can be hindered by binding to plasma proteins (plasma protein binding, PPB).[25] Lipophilic compounds tend to have high PPB.[147] Besides, an important value to be determined is the virtual volume of distribution V_D. The higher the volume the less compound is found in plasma after administration of a certain dose.

$$V_D = \frac{d}{c_{plasma}}$$

d : administered dose, c_{plasma} : plasma mass concentration

Depending on the intended site of action, a different V_D value is targeted. For example, if the site of action is in tissue, the value should not be too small. A drug that is readily uptaken into tissue may reach first well-perfused organs (e.g. heart and lung) and later in other organs. The brain is reached only by few substances because of the blood-brain barrier. In addition, high lipophilicity, as indicated by a high log P or log D value, may facilitate the exit of a drug from blood vessels through lipid bilayer membranes. However, a substance can also accumulate in the intercellular (interstitial) space, not reaching intracellular space. In addition, lipophilic compounds can accumulate in fat tissue.[25]

The effect of a drug or an active metabolite on the organism is terminated by the elimination and excretion. Depending on physicochemical properties such as solubility and volatility, several elimination routes exist, with elimination via the liver or kidney being the most important. The ability of an organ to eliminate a substance can be described by its clearance. The clearance is the elimination rate of a drug divided by its plasma concentration. This leads to an expression in volume per time, describing e.g. the volume of blood being purged from the compound by the organ in a certain period of time. The higher this value the faster the compound is eliminated by this organ.[25, 147]

2 Objective of this Thesis

The objective was to extend the current repertoire of cystobactamids synthetically with two intentions: Obtain knowledge about the nebulous role of the Eastern part and improve the current most rewarding structures.[9-11, 118-119, 122] The final goal of this optimization process is to generate superior broad-spectrum antibacterial activity, in particular against the ESKAPE pathogens (*E. coli, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter spp.*). The improvement of activity against *A. baumannii* is an interim goal.

CN-CC 861 was determined as being highly active[11] and was selected as scaffold for modifications of the Eastern part (see Figure 24). On that note, the first general aim was to build new variants of the CDE-fragment, assembly of the full cystobactamids and evaluation of their antibacterial activity in MIC assays in the end.

With particular focus on ring D modifications, the intention was to synthesize highly substituted aromatic systems for the first time. Concomitantly, new derivatives of ring C and E ought to be introduced to complement SAR-information. Full assembly of the cystobactamids will require adaption and enhancement of existing methods. A deepened SAR of the CDE-fragment is then made possible by evaluating final activity data. Substitution of the hydroxy function at ring D is assumed to deliver more detailed information about the mode of action, as this function was assumed to have a decisive influence on efficacy. New findings here are then transferable to future modifications.

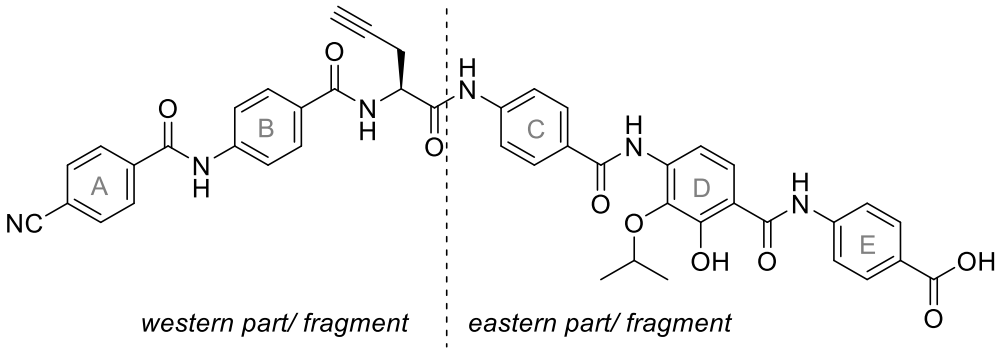


Figure 24: Structure of lead scaffold cystobactamid CN-CC 861.

In addition, the new modifications in the CDE-fragment are expected to be combined with simultaneously developed and promising Western part substructures. A combination of the most active ring D derivative with these substructures is a reasonable next step. To

improve pharmacological parameters of the cystobactamids, the most active compounds can be further tested in *in vitro* and *in vivo* ADME-studies. In addition, a characterization and optimization of physicochemical properties as solubility and plasma protein binding is desired.

The high antibacterial activity of already known cystobactamids will therefore be optimized towards critical ESKAPE pathogens. Together with the *in vitro* and *in vivo* ADME-studies this is of central importance for the preclinical phase. With the syntheses of new tetrasubstituted ring D derivatives, advanced synthetic methods of aromatic chemistry will be applied and developed.

3 Results

3.1 New strategies for assembling full cystobactamids

General assembly strategy

As a starting point for SAR investigations, CN-CC 861[11] was selected as the main scaffold showing (S)-2-aminopent-4-ynoic acid as central AA. Starting from this compound, three retrosynthetic paths were pursued as shown in Figure 25. Path 1 represents one of the published assembly strategy for cystobactamids with the retrosynthetic cut between ring B and the amine of the AA-CDE-fragment.[11, 96, 154] Path 2 features the disconnection between the AB-central AA-fragment and the CDE-fragment. Reaction conditions suitable for this bond formation were developed in recent publications[9, 100] and were improved in this Thesis to allow introduction of several new ring D analogues.

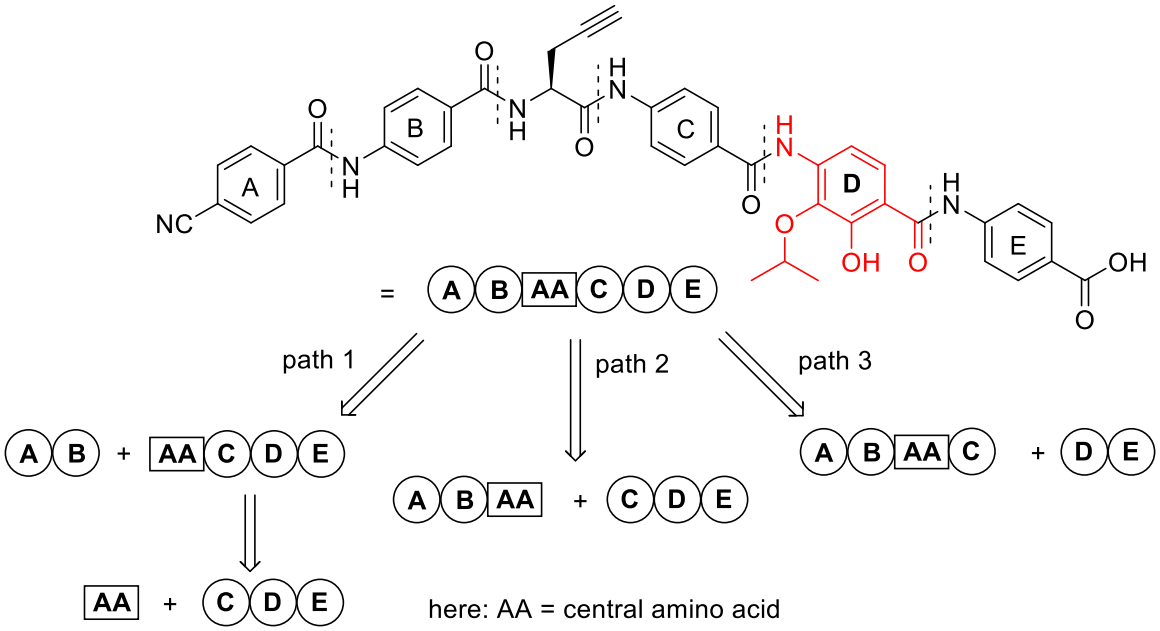


Figure 25: Structure of cystobactamid CN-CC 861[11] with retrosynthetic paths used in the following synthesis. The rings are abbreviated with the corresponding letter with ring D highlighted in red. Coupling of the rings takes place by forming the intermediate amide bond from the carboxylic acid or equivalent moieties of one ring with the amino function of the other ring. Reaction conditions were optimized for paths 2 and 3 in this Thesis.

Paths 1 and 2 involve the synthesis of the full CDE-fragment before the full assembly. The synthesis of the CDE-fragments followed a published assembly strategy for cystobactamids[11] and is exemplified for one D ring derivative in Figure 26. The first step is the amide coupling between the carboxylic acid moiety of **22** with *tert*-butyl 4-aminobenzoate. For this coupling either $(COCl)_2$, $POCl_3$ or T3P were used as activation reagent[9, 11, 72, 96] in combination with either DiPEA, TEA, or Pyridine as base. These strong

activating conditions were considered sufficient to facilitate the attack of the weak aniline-nucleophile. Indeed, the amide coupling of the shown substrate proceeded with sufficiently high yield, but left room for optimization, as described later. For the following nitro reduction of **23, 24** and all other DE- and CDE-fragments with nitro functions, zinc in diluted AcOH[155] was selected as standard method in this Thesis.

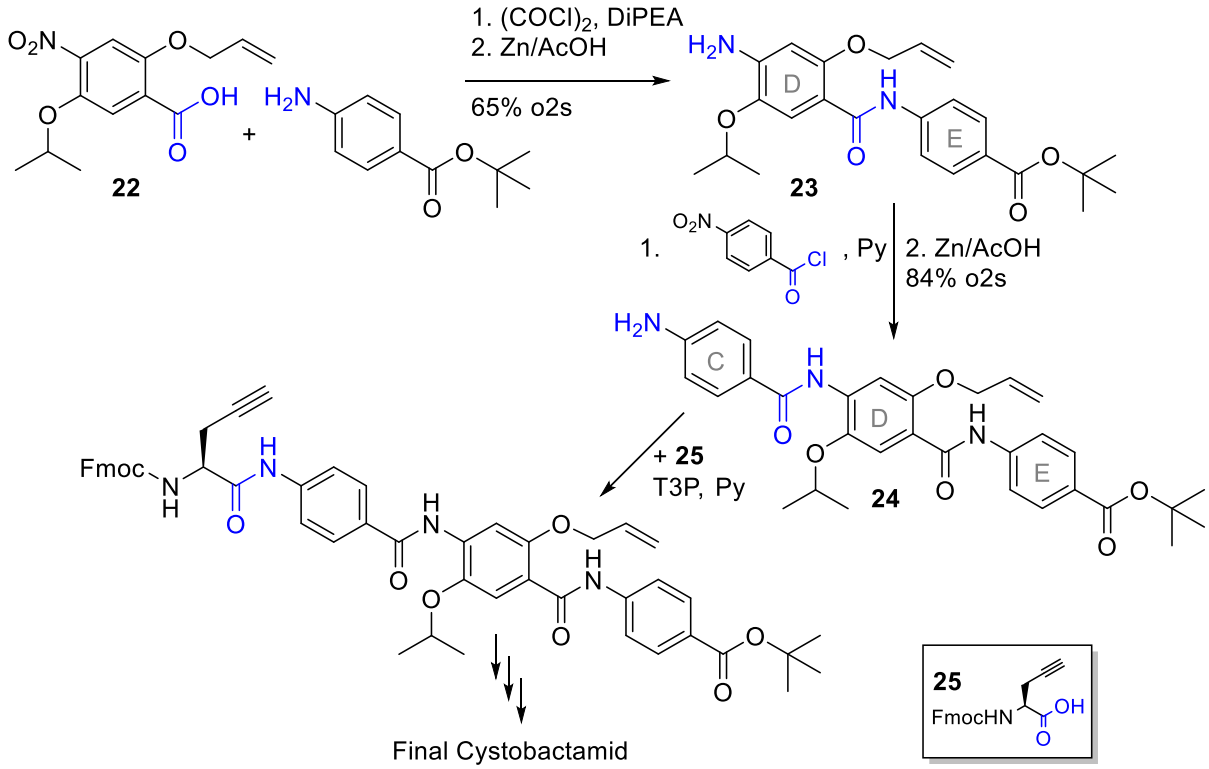


Figure 26: Example for assembling the AA-CDE-fragment starting with a D ring derivative, here a hydroquinone.

Attachment of ring C followed by known reaction of the amino function of the DE-fragment with 4-nitrobenzoyl chloride and subsequent nitro reduction. Afterwards, the amide coupling between *N*-Fmoc-propargylglycine (Fmoc-L-Pra-OH) **25** and the amino function of the CDE-fragment was conducted with T3P as activation reagent according to the literature. The deprotection of the central AA was performed afterwards.[11] Interestingly, imine formation of the free amine with acetone impurities was found as a minor side reaction and proves a high nucleophilicity of such aliphatic amines compared to aromatic anilines. In contrast, the latter were in fact found to be inert towards acetone as eluent component.

By switching the synthetic strategy from path 1 to path 2, the synthetically most valuable building block (ring D) could be introduced at a later stage of synthesis. Two more

reaction steps, starting from the ring D-carboxylic acid, could be saved. An example for this alternative coupling is shown in Figure 27. The synthesis of the required AB-central AA-fragment **26** as carboxylic acid is described in section 3.3.1. As mentioned in the introduction, similar fragments were previously synthesized and activated as acid fluoride, requiring isolation of this compound.[9, 100] Therefore, a direct amide coupling starting from the carboxylic acid was considered to save an additional purification step. Since T3P has been shown to be a highly efficient activating reagent for the carboxyl function of the central AA[11], it was assumed that this reagent would also facilitate amide coupling with the new AB-central AA-fragment. In this way, the latter was coupled with the CDE-fragment **27** to produce the full cystobactamid **003** after final deprotection of the *C*-terminal *t*Bu group.

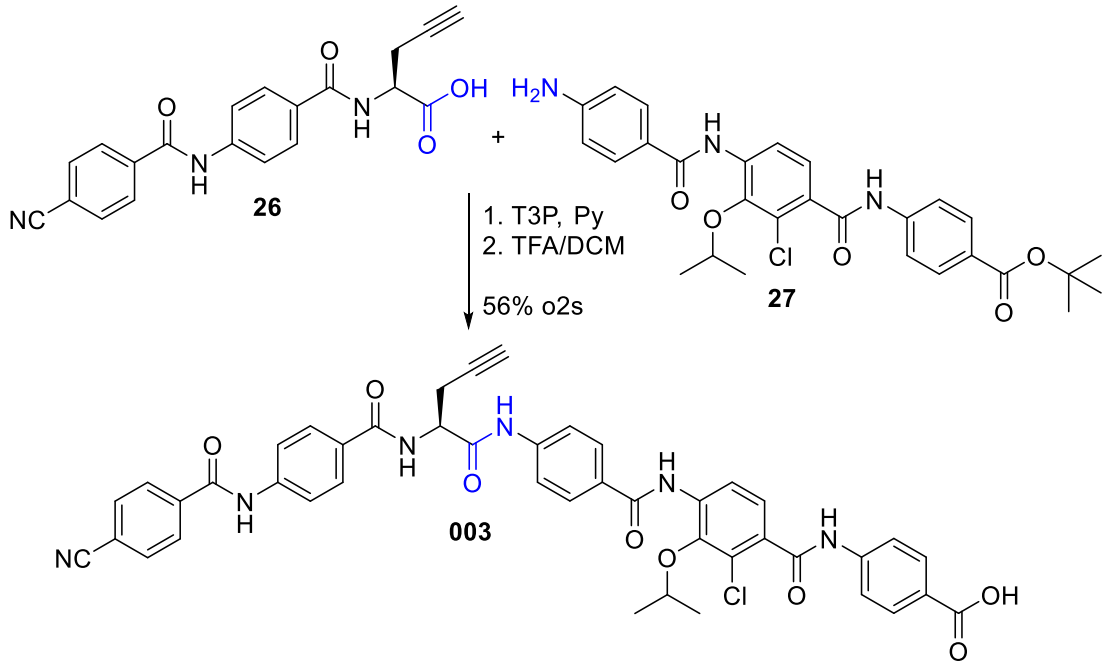


Figure 27: Final assembly and deprotection according to retrosynthetic path II, exemplified for a chloro derivative **003** developed in this Thesis. The synthesis of the AB-central AA-fragment **26** is described in section 3.3.1.

To further reduce the number of reaction steps after preparing the ring D carboxylic acid, reaction conditions for path 3 were further investigated. By following this strategy, the final assembly would solely require the synthesis of the DE- instead of the CDE-fragment. As a benchmark for this kind of reaction, the coupling of the AB-central AA-C fragment **28** with DE-fragment **29** was first chosen, using $POCl_3$ as activating reagent (Figure 28 a). The synthesis of **29** is described later in section 3.2.2, while **28** was provided by D. Kohnhäuser. Unfortunately, the reaction delivered an undefinable product mixture,

indicating $POCl_3$ as unselective reagent for this reaction. Low yields for this kind of amide coupling were already reported for similar substrates.[11]

It was assumed that the amide coupling of an aliphatic and thus more electrophilic carboxylic acid such as **30** (synthesis described in experimental part) with a DE-fragment like **31** would be much faster. The reaction was performed using a large excess of T3P with DiPEA at 60°C (Figure 28 b).

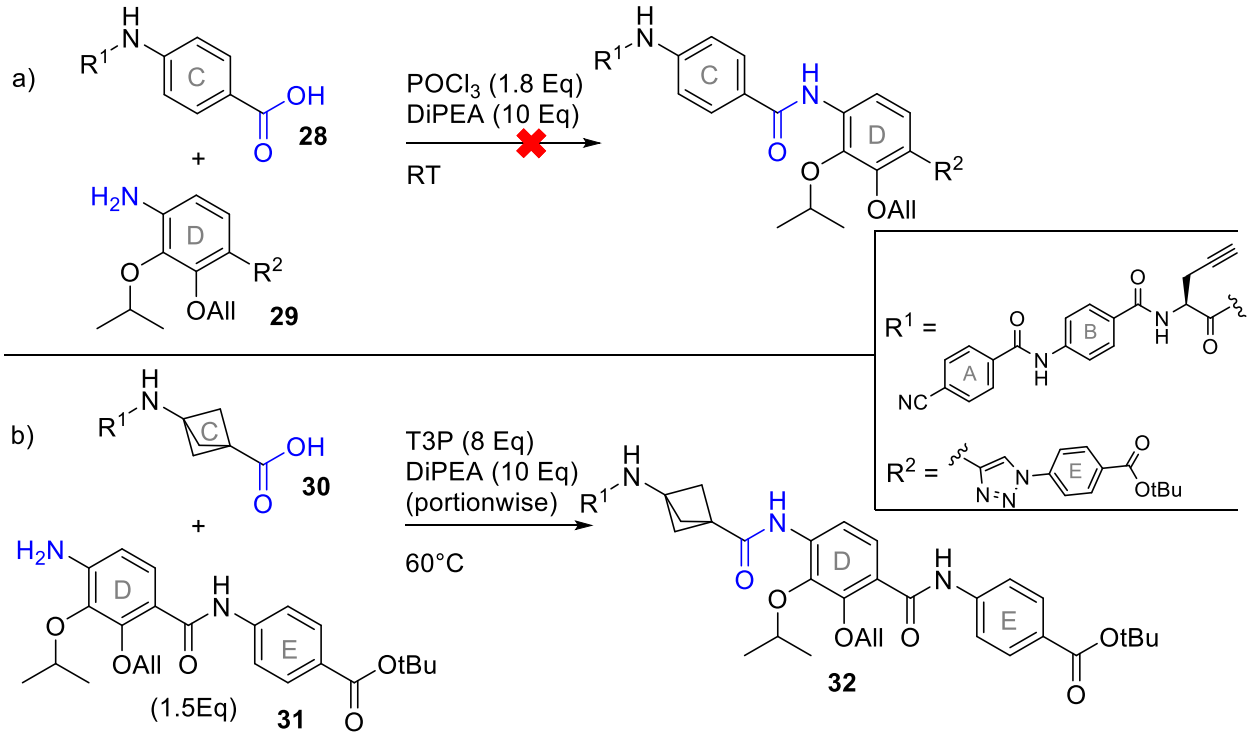


Figure 28: Reaction conditions tried for the direct amide coupling of the AB-central AA-C fragments **28** and **30** with **29** and **31**, respectively (path 3).

Indeed, the reaction proceeded slowly, but sufficiently selective formation of **32** occured. Although this reaction was successful, it was feared that the amide coupling between an aromatic carboxylic acid on ring C and the DE-fragment might not take place, or only under such harsh conditions that the central amino acid might undergo racemization. Therefore, the retrosynthetic strategy of path 3 was not pursued further.

An overview of the different assembly methods used for the cystobactamids in this Thesis is listed in the experimental part (section 5.3).

3.2 Optimization of the Eastern part

The extension of the SAR of the Eastern-part, or CDE-fragment, was the next focal point. At this point, the following nomenclature is proposed for describing the main conformations of the amide backbone of the CDE-fragment.

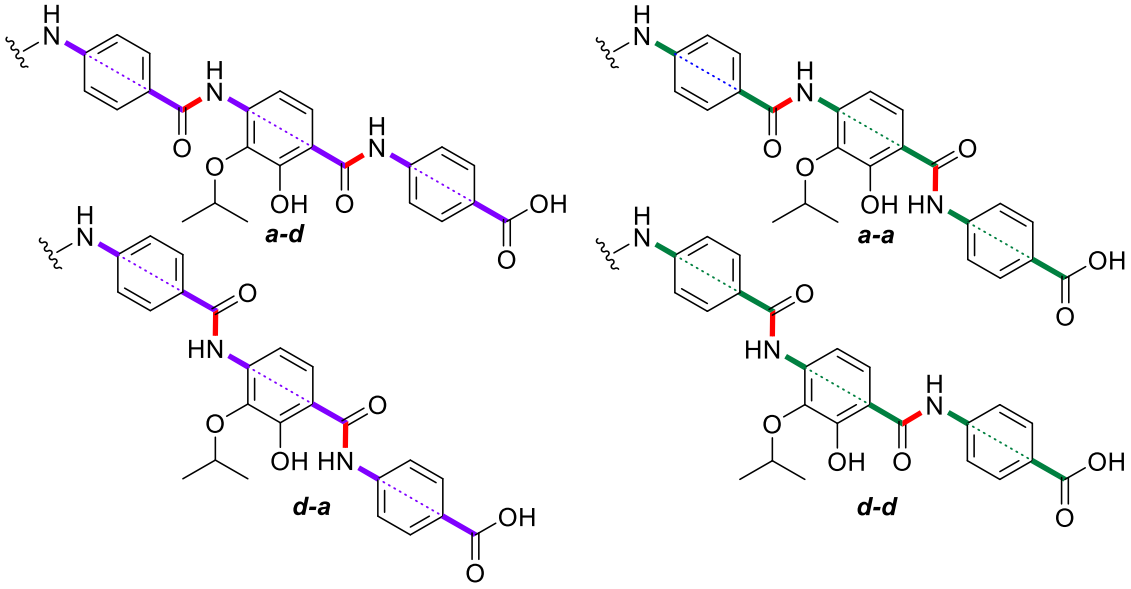


Figure 29: Proposed nomenclature for conformations of the CDE-fragment used in this Thesis. The amide 'backbone' is highlighted in purple for antiperiplanar and green for synperiplanar conformations. The conformers are named after the orientation of the carbon-nitrogen bond (red) of CE- and DE-amide linkers relative to the isopropoxy or hydroxy group. Here, *a* and *d* indicate approximate and distant orientation.

Here, *a-d* and *d-a* are antiperiplanar conformations, while *a-a* and *d-d* are synperiplanar conformations.

3.2.1 Synthesis of ring D derivatives

A strong focus of recent cystobactamid research was set to rings A, B, C and the central amino acid. Comparatively little attention was devoted to ring D, therefore the first target was to synthesize new ring D analogues and investigate the SAR for this essential[9, 11, 99] cystobactamid substructure (see also section 1.2.5). For instance the absence of the hydroxy group led to almost full loss of activity.

The synthesis of ring D analogues usually require arrangement of four adjacent substituents to the benzene ring. Therefore regioselectivity problems are likely to occur during the synthesis.

Hydroquinone derivative

As a formally simple change, the position of the hydroxy group was moved from *ortho* to *para* relative to the isopropoxy group. Thereby, the type of substituents and the electron density of ring D are largely preserved, but the topology of HBDs is rearranged. One result is the absence of a possible H-bond interaction between the hydroxy- and the isopropoxy group. Moreover the resulting compound will show a different steric fit in a possible binding pocket.

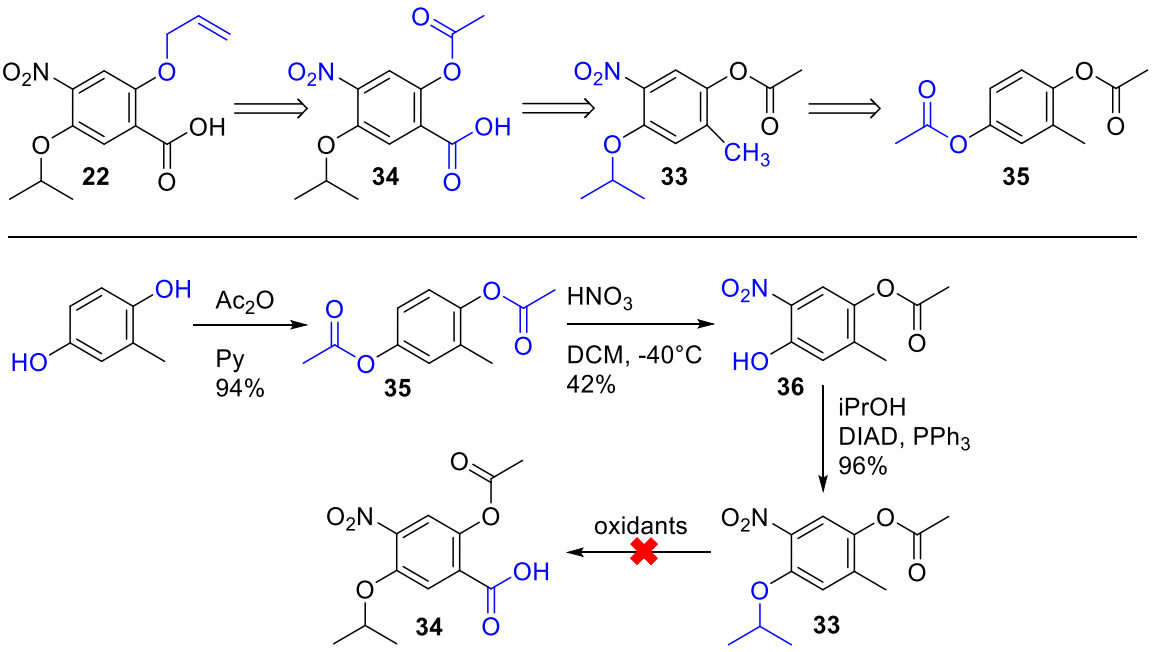


Figure 30: Retrosynthetic strategy 1 for hydroquinone derivative (top) and attempted synthesis (bottom).

As a first approach the synthesis was planned and synthetic conditions were tried according to Figure 30. The desired ring D carboxylic acid **22** was intended to be prepared from **33** via **34** by full oxidation of the benzylic methyl group. This key substitution was chosen to circumvent the *meta*-directing effect of a carboxylic acid in a nitration, and to implement methyl as an *ortho, para* directing group. Compound **33** was considered as accessible from diacetylester **35** which in turn would be prepared from 2-methyl-2-hydroxyphenol. This acetylation would prevent side reactions, since the direct nitration of 2-methyl-2-hydroxyphenol was expected to produce undesired benzoquinones as oxidation products, as this was reported for similar compounds.[156] Because the hydroquinone system is highly electron-rich, this is a reasonable reaction behaviour. Therefore the educt was first diacetylated with acetic anhydride in pyridine to reduce the electron density in the benzene ring. The nitration was attempted with **35**, assuming that the directing effect of the acetoxy substituent in *m*-Me position would add to the *para* directing effect of the methyl group and thereby the electrophile would be directed to the desired position. Surprisingly, a mono-deacetylation was observed simultaneously to the nitration. After isolation of the regioisomer **36**, its structure was elucidated by HSQC, HMBC and 1,1-ADEQUATE NMR experiments. From the ^{1}H NMR spectrum it was concluded, that the aromatic protons must be in *para* position to each other (Figure 31 a). The revealed structure is shown in Figure 31 b) with key interactions in NMR.

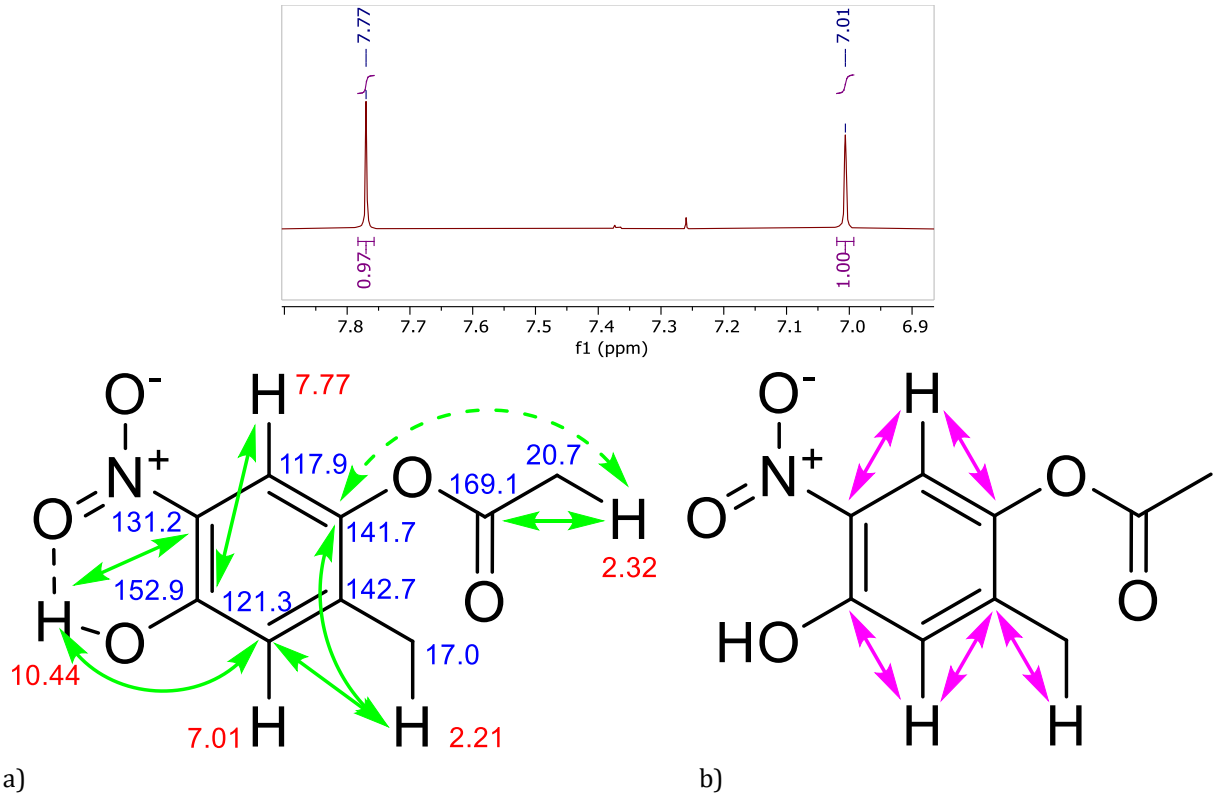


Figure 31: Excerpt from ^{1}H NMR spectrum (top) of **36**, showing singlet signals for the aromatic protons. a) HSQC/HMBC NMR data for **36**. Explicit HMBC interactions shown in green, b) 1,1 ADEQUATE-NMR data for **36** with interactions in pink.

It is plausible that the ester cleavage may have occured due to acidic hydrolysis facilitated by nitric acid. Moreover, the exposure of the free hydroxy group may be favoured in *ortho* position to the nitro group as this may lead to a higher thermodynamic stabilization of especially this regioisomer by the resulting intramolecular H-bond.

Next, the isopropoxy group was introduced through a Mitsunobu reaction which is suitable especially for acidic phenols such as in compound **36**. Accordingly, a high yield of **33** was obtained for this step. For this and the following Mitsunobu reactions, the intermediate betain reagent was formed in advance from diisopropylazodicarboxylate (DIAD) and triphenylphosphine (PPh_3) before the substrates were added. This way, possible side reactions of DIAD with the phenol or nitro functions were prevented. Encouraged by this outcome, the oxidation towards benzoic acid **34** was studied next (see Figure 32).

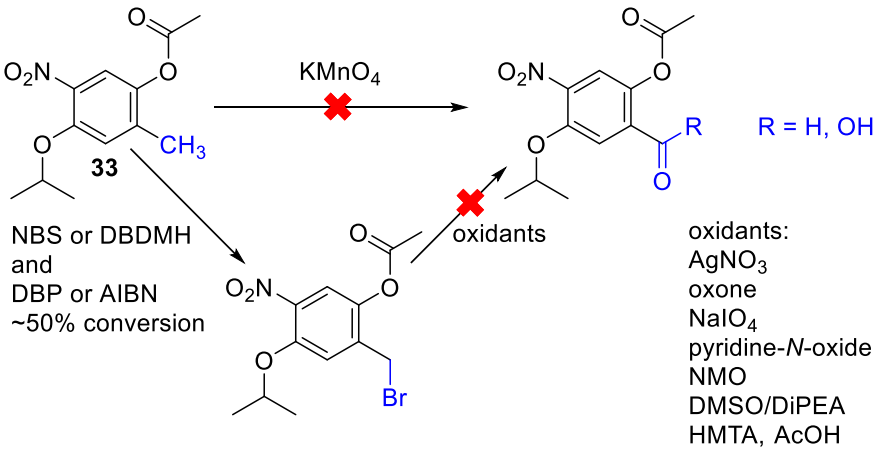


Figure 32: Attempted oxidation methods for conversion of the benzylic methyl group of compound **33** to an aldehyde or a carboxylic acid.

Unfortunately, the classical oxidation with $KMnO_4$ was not successful, therefore an oxidation with brominating reagents such as *N*-bromosuccinimide (NBS) and 1,3-dibromo-5,5-dimethylhydantoin (DBDMH) in combination with initiators such as dibenzoylperoxide (DBP) and azobis(isobutyronitril) (AIBN) was tried next. The monobromination occurred only partly, regardless of reaction conditions. Nevertheless, the further oxidation to the benzoic acid was tried unsuccessfully using several oxidants as silver nitrate,[157] oxone,[158] sodium periodate,[159] pyridine *N*-oxide,[160] *N*-methylmorpholine *N*-oxide (NMO, Ganem oxidation),[161] DMSO/DiPEA (Kornblum oxidation) and hexamethylenetetramine (HMTA)/AcOH (Sommelet reaction). A considerable turnover was observed only with HMTA/AcOH, but the product was identified as the benzyl amine which is the product of a known competing reaction[162] (Delépine reaction).

Due to consistantly low conversion, continuing with the oxidation step was found to be unfeasible and another retrosynthetic strategy was tried (see Figure 33). Here the carboxylic function and the oxygen substituents are already implemented and after functionalization of the latter, the nitro substituent is introduced. Starting with commercially available methyl 2,5-dihydroxybenzoate, the selective isopropoxylation following literature conditions[95, 163] gave a 33% yield of the isopropylether, followed by acetylation with acetic anhydride in pyridine to obtain compound **37**. As the isopropoxy group is the strongest directing group in this aromatic system, a nitration was expected to be directed in *ortho* position to this group.

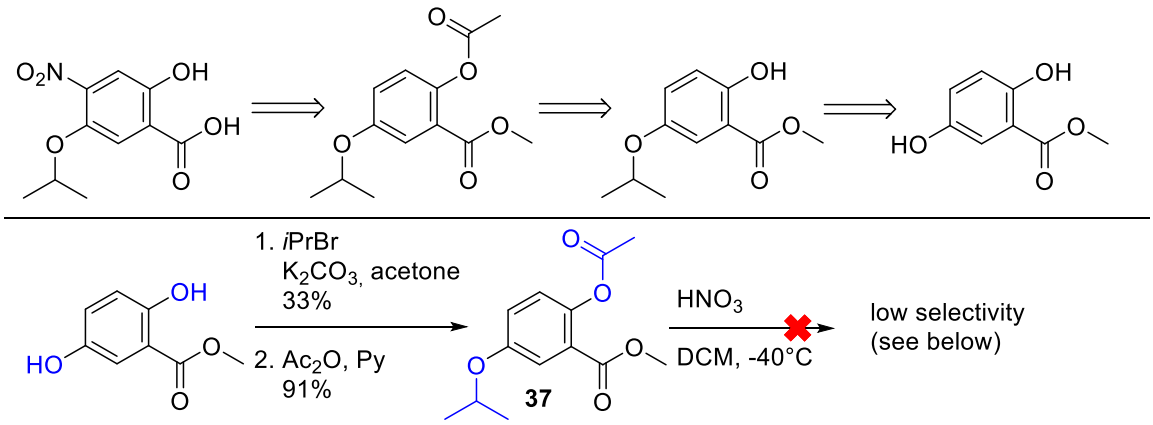


Figure 33: Retrosynthetic strategy 2 for hydroquinone derivative and attempted synthesis.

The nitration led to a ~1:3 regioisomeric mixture. After chromatographic separation of the mixture, the NMR spectrum of the minor component beared two signals from aromatic protons with coupling constants indicating ^{4}J–or ^{5}J–coupling. From this it was assumed that either the desired regioisomer **38a** or the regioisomer **38b** had beed formed. Meanwhile, the major component showed an overlay of two aromatic proton signals with characteristic ^{3}J–coupling constants (see Figure 34) thus indicating regioisomer **39**.

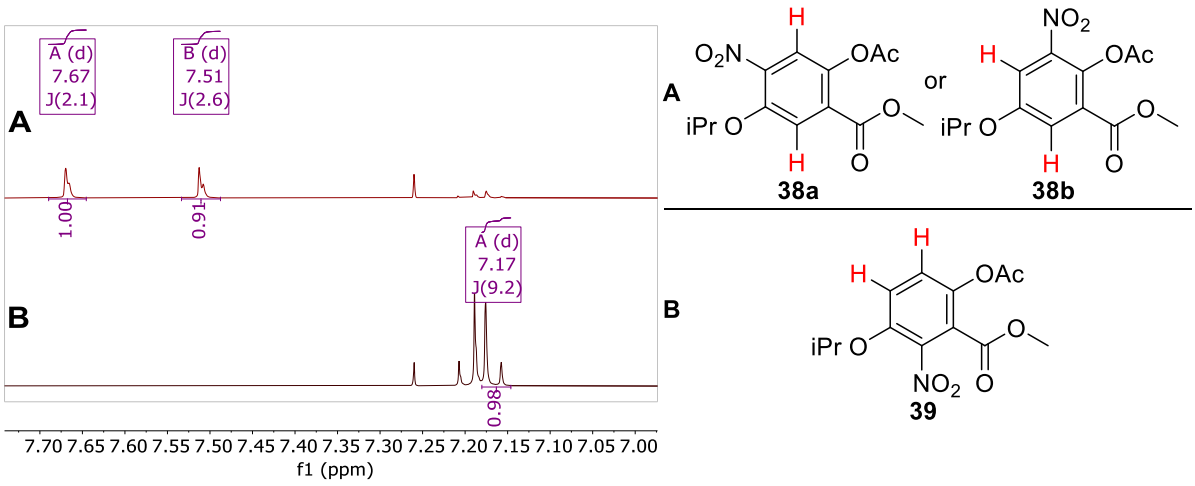


Figure 34: Left: Excerpt from the compared ^{1}H NMR spectra showing doublet signals for both the aromatic protons of the first (top) and second compound (bottom). The observed coupling constants indicate either a ^{5}J or ^{4}J coupling for **A** and show a ^{3}J coupling for **B**. Right: the proposed matching structures.

Admittedly, the structure of the minor component was not fully confirmed but even assuming it was the correct regioisomer, then the conversion of 19% (by NMR) was too low to deem this method as useful. Moreover, since another strategy was pursued in parallel (see below) and was ultimately more successful, this synthesis route was eventually abandoned.

An alternative retrosynthesis by adapting a known synthesis protocol was designed and tested (Figure 35).[115] This protocol involves a low-yielding Elbs oxidation as first step and achieves the direct introduction of an oxygen electrophile in *para* position to a phenol in strongly alkaline water with peroxodisulfates. It often requires several days reaction time and bears low educt conversion.[164-165] The isolation of the product **40** from residual educt by precipitation was more complicated than reported, since both substances precipitated in similar amounts after acidification of the reaction mixture. A more precise adjustment of the pH during the workup and subsequent filtration led to a total yield of 18%. The next step included the formation of a cyclic acetonide in a TFA/TFAA mixture.[114-115] Here the reaction was also very slow, requiring refluxing for 6 days.

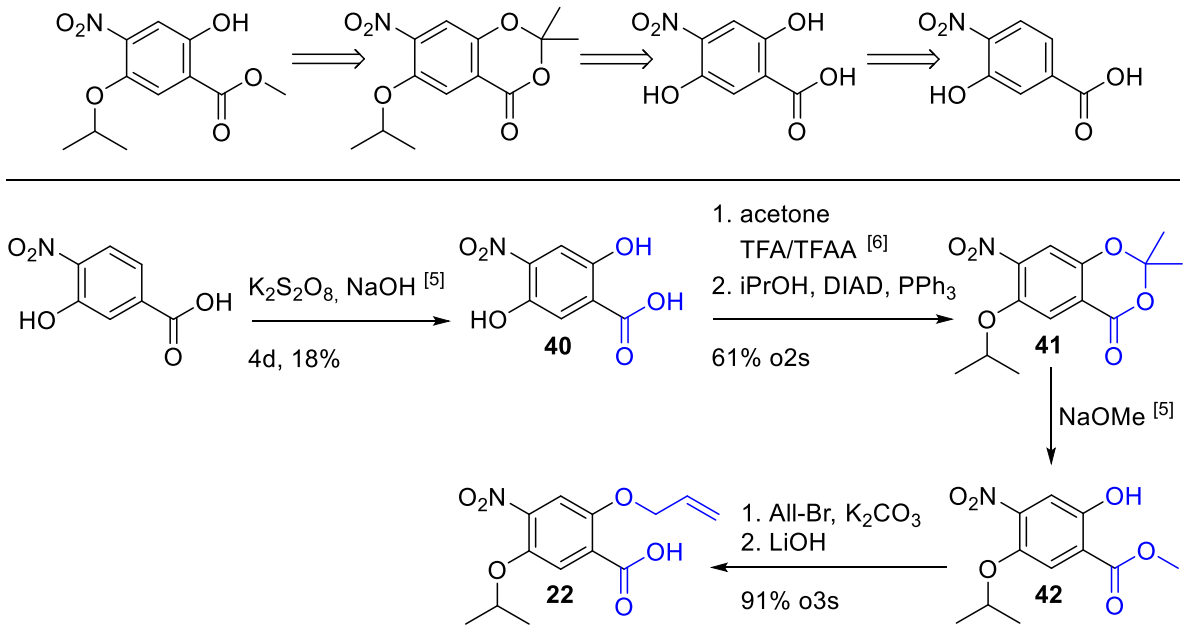


Figure 35: Retrosynthetic strategy 3 adapted from[115] for the hydroquinone derivative (top) and successfully established synthesis (bottom).

The acetonide function was found to be very sensitive to any medium alkaline aequeos solution, requiring careful product control during the workup. After this step, the hydroxy group in *ortho* position to the nitro group was isopropylated via a Mitsunobu reaction, using the protocol as applied in retrosynthetic strategy 1. Compound **41** was then isolated in acceptable yield of 61% over two reaction steps. The cyclic acetonide moiety was hydrolysed with sodium methoxide to obtain the fully functionalized methyl ester **42**. After protection of the phenolic hydroxy group with allyl bromide, the methyl ester was cleaved by saponification with LiOH to obtain the free carboxylic acid **22** in good yield. Thereby, the desired hydroquinone substitution pattern was successfully implemented.

Subsequent coupling with *tert*-butyl 4-aminobenzoate was initially tried with $POCl_3$/DiPEA and more successfully with $(COCl)_2$ as the activation reagent. The directly following reduction of the nitro group delivered the hydroquinone DE-fragment in 65% yield over 2 steps. The full cystobactamid **001** was then synthesized according to path 1 (see Figure 36 and section 3.1), as reported in[11].

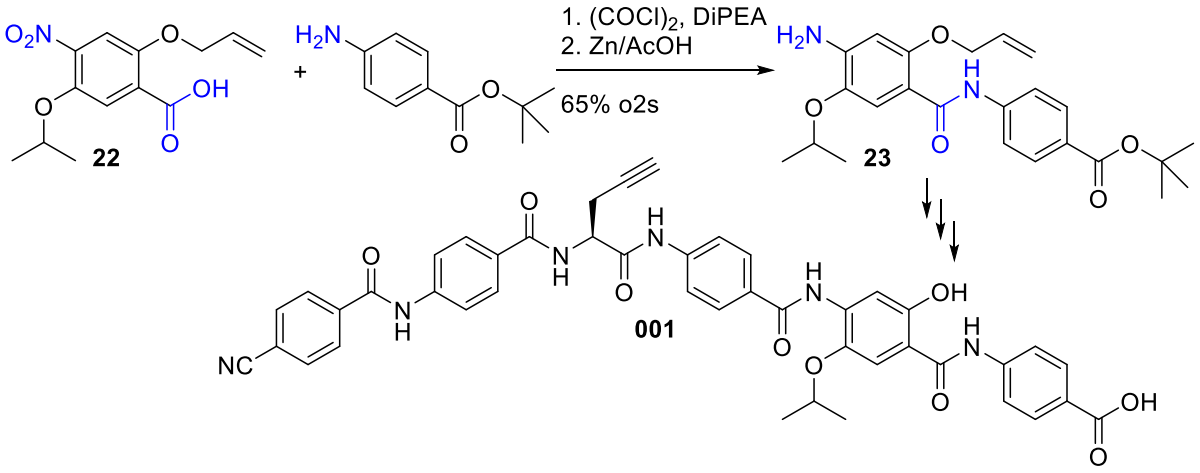


Figure 36: Assembly of the hydroquinone DE-fragment and structure of the final cystobactamid **001**.

Halogen derivatives

The focus was now centered on isosteres of the hydroxy group with halogens being a starting point. Aryl halides differ from hydroxy as benzene substituent, as halogen substituents are exclusively poor HBAs and are more lipophilic than hydroxy.[142] As a hypothesis, it was expected that an aryl fluoride, as the least lipophilic aryl halide, would show similar activity behaviour to the original hydroxy derivative. In addition, aryl halides would offer a possibility for further modifications of ring D, e.g. cross-coupling reactions.

Three derivatives with fluoro, chloro and bromo substituents were synthesized. An iodo derivative was excluded due to its similarity to bromine and an expected high reactivity in metal-mediated reactions as they are used in the cystobactamid assembly. The synthesis was initially optimized for the chloro derivative and later transferred to the other halogen derivatives. Details for the retrosynthetic strategy are shown in Figure 37 together with the later established synthesis. The starting point for the synthesis were commercially available aldehydes **43,** in which three substituents were already present. After the introduction of the isopropoxy moiety, the nitration should be conducted to obtain the complete substitution pattern **44** (strategy I). In the case of an interference of the isopropoxy function with the nitration reaction, it was intended to proceed with

strategy II, in which the starting material was first nitrated, followed by isopropoxylation of the hydroxy group.

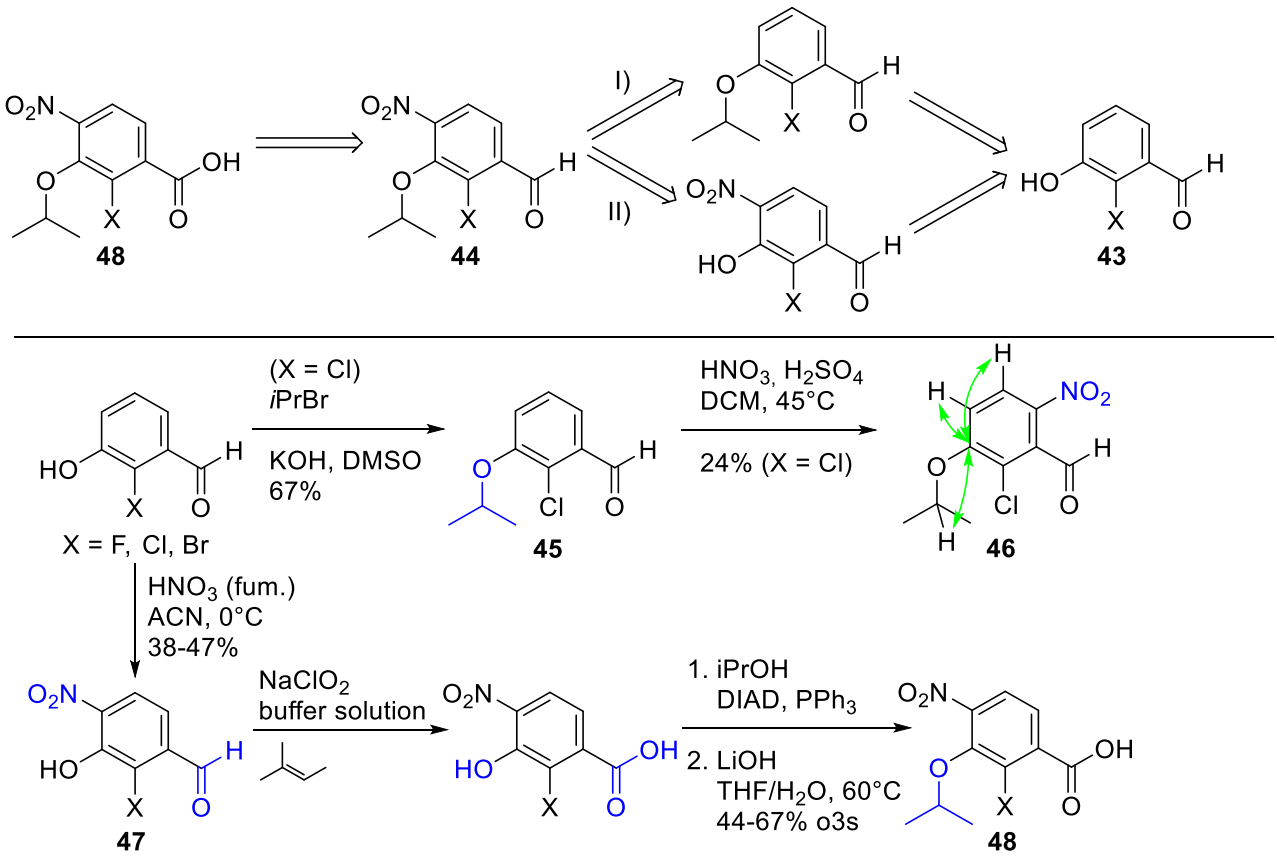


Figure 37: Retrosynthetic strategies for halogen ring D derivatives (top) with later developed reaction conditions (bottom). The structure of the undesired nitro regioisomer was elucidated by HMBC (key interactions shown in green). Similar intensities of the interaction peaks of both aromatic protons indicated ^{2}J or ^{3}J-coupling with carbon, confirming the structure shown.

Starting with commercially available 2-halo-3-hydroxybenzaldehyde, the isopropoxy group was first introduced by Williamson ether synthesis (strategy I). The subsequent nitration of **45** was slow, thus requiring a 2:1-mixture of HNO_3 and H_2SO_4 as a 10 vol% solution in DCM and a temperature of 45°C, indicating a lower reactivity of the aromatic system. This can be rationalized by the inactivating effect of halogen substituents towards S_EAr. The reaction selectively yielded one regioisomer, which, however, was identified as the undesired regioisomer **46** by 2D NMR. This is in contrast to the regioselectivity observed in the nitration, which is part of the standard ring D synthesis and where the desired regioisomer is formed instead. However, it is known that benzoic acids with a +M substituent in the *meta*-position are subject to the so-called *ortho*-effect in S_EAr reactions.[166-168] This effect would lead here to a preferential substitution in the *ortho*-position to the carbonyl function. In the standard ring D synthesis, the acetoxy-substituent might reduce this effect.

The synthesis was rearranged so that the starting material was first nitrated (strategy II) after optimization of the original nitration protocol[72, 95]. A higher reaction temperature of 0°C compared to -40°C was required here as well, confirming the low reactivity of these aromatic compounds. A regioisomeric mixture was obtained and 2D NMR spectroscopy was conducted with both of the isolated regioisomers to identify the desired compound **47a**. Key interactions are shown in Figure 38. The ratio of the regioisomers was nearly 1:1.

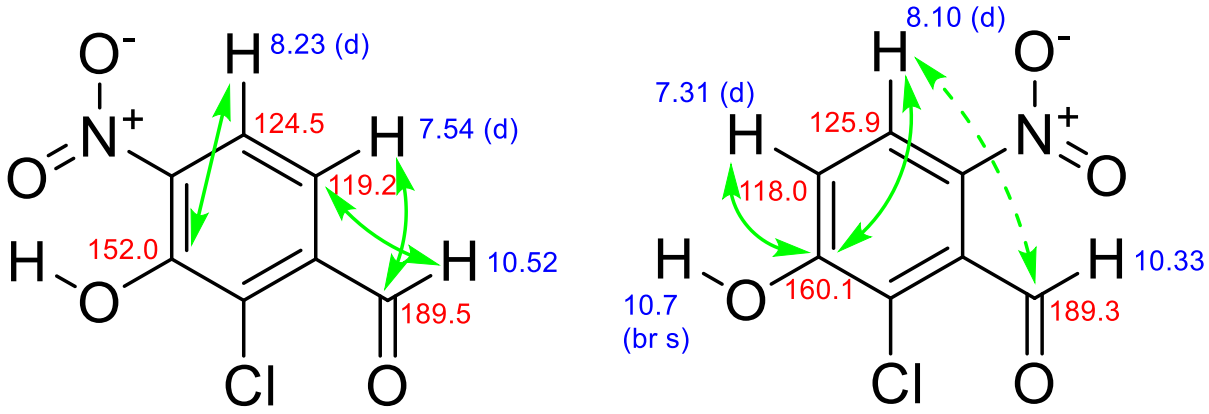


Figure 38: 2D NMR data with key interactions for the 4-nitro (**47a**, a) and 6-nitro (b) products for the nitration of the chloro- derivative. Explicit HMBC interactions are shown in green with a weak interaction indicated as dashed arrow.

The next step would have been the isopropoxylation via Mitsunobu reaction according to the own protocol. However, the aldehyde function of **47a** proved to be partially reactive, as several unidentified byproducts were observed. Instead, the Pinnick oxidation was conducted next for all halogen analogues, following the known protocol[72]. Mitsunobu-isopropylation and saponification of the intermediate isopropyl esters with LiOH yielded the desired ring D carboxylic acids **48** which were finally isolated by acid-base extraction without the need of further chromatographic purification.

The subsequent synthesis of the CDE-fragments followed the usual protocol. The fluoro- and chloro- substituted fragments were obtained without synthetic problems and successfully assembled to give the complete cystobactamids **002** and **003**, following retrosynthetic path 2 (Figure 39 and section 3.1).

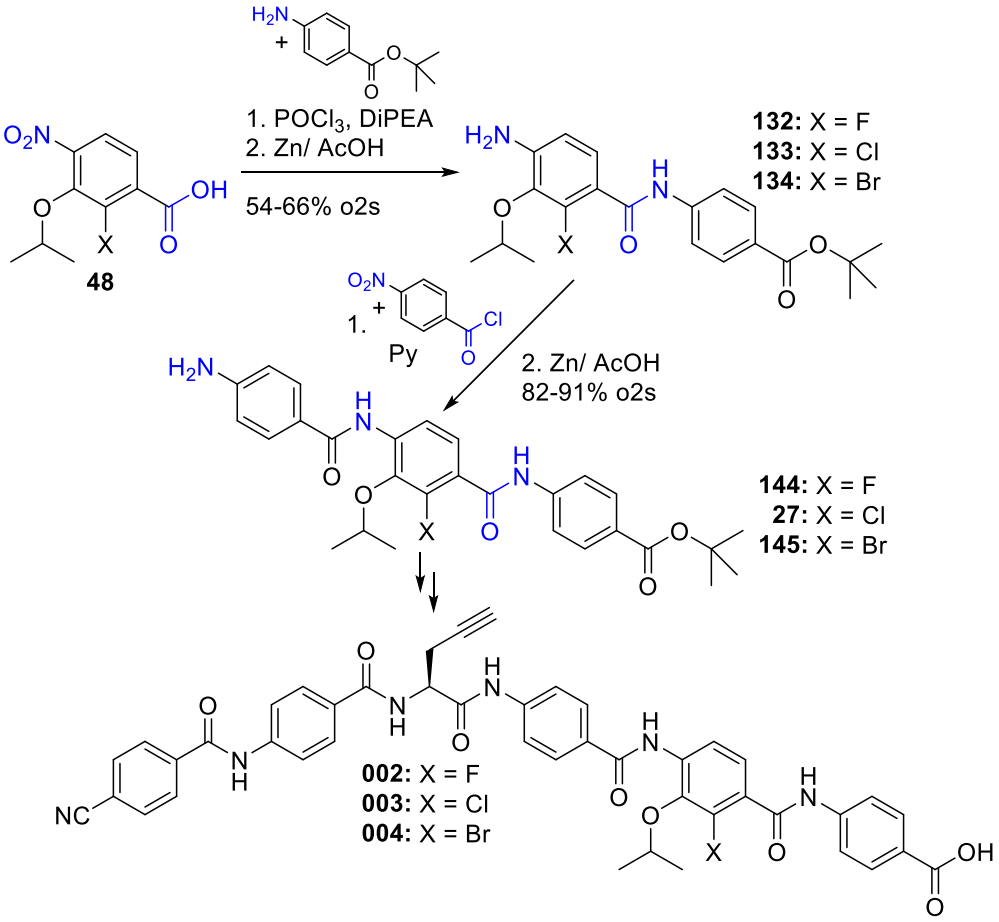
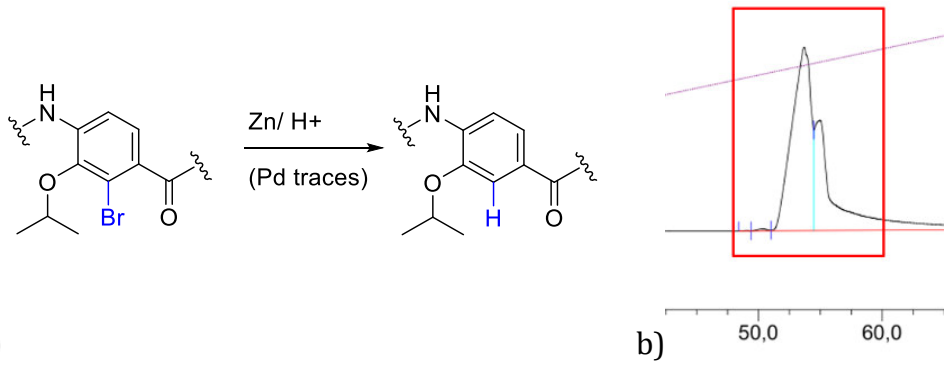


Figure 39: Assembly of the halogen CDE-fragment and structure of the final cystobactamids **002**, **003** and **004**.

The bromo-CDE-fragment was contaminated with a substance identified as product of a reductive side-reaction (see Figure 40 a). Here zinc/AcOH proved to be disadvantageous because it probably facilitated the substitution of bromine by hydrogen in the acidic medium present. The reduction of carbon-bromide bonds by zinc/AcOH was reported for heteroaromatics,[169] but traces of Pd catalyst residues in the reaction flask may also have catalyzed this reaction in a similar manner to a Negishi coupling. This reduction occured simultaneously with each nitro reduction and the product was not identified until the CDE-fragment was fully synthesized. Since only two steps were missing to finalize the full cystobactamid **004**, the contamination was tolerated. Separation of the bromo derivative from the hydrogen derivative was not possible with basic HPLC, but the desired compound was isolated with acidic HPLC after careful adjustment of the gradient program (see Figure 40 b).



a) b)

Figure 40: a) observed carbon-halide reduction during reduction with zinc. b) Excerpt from HPLC-chromatogram bearing the sufficient separation of cystobactamids with bromo and hydrogen substituent by HPLC, details are listed in experimental part.

Despite the still poor separation, sufficient quantities of the pure desired compound were obtained for further experiments.

Amino derivatives

An amino group is both an HBA and HBD and was therefore considered as a possible bioisosteric group for hydroxy. Moreover, secondary arylamines are known isosteric groups for phenol (as mentioned in section 2.3.1) and may be readily prepared from amines. Two retrosynthetic strategies were designed to access compound **49**, as seen in Figure 41. The amine function would require an appropriate protecting group for the final assembly of the cystobactamid. It was assumed that Boc or Fmoc protecting groups would decompose during subsequent nitrations and, in addition, that their high bulkiness would impede amide coupling between ring D and ring E. Therefore either acetyl or trifluoroacetyl were considered as more stable and less bulkier alternatives.

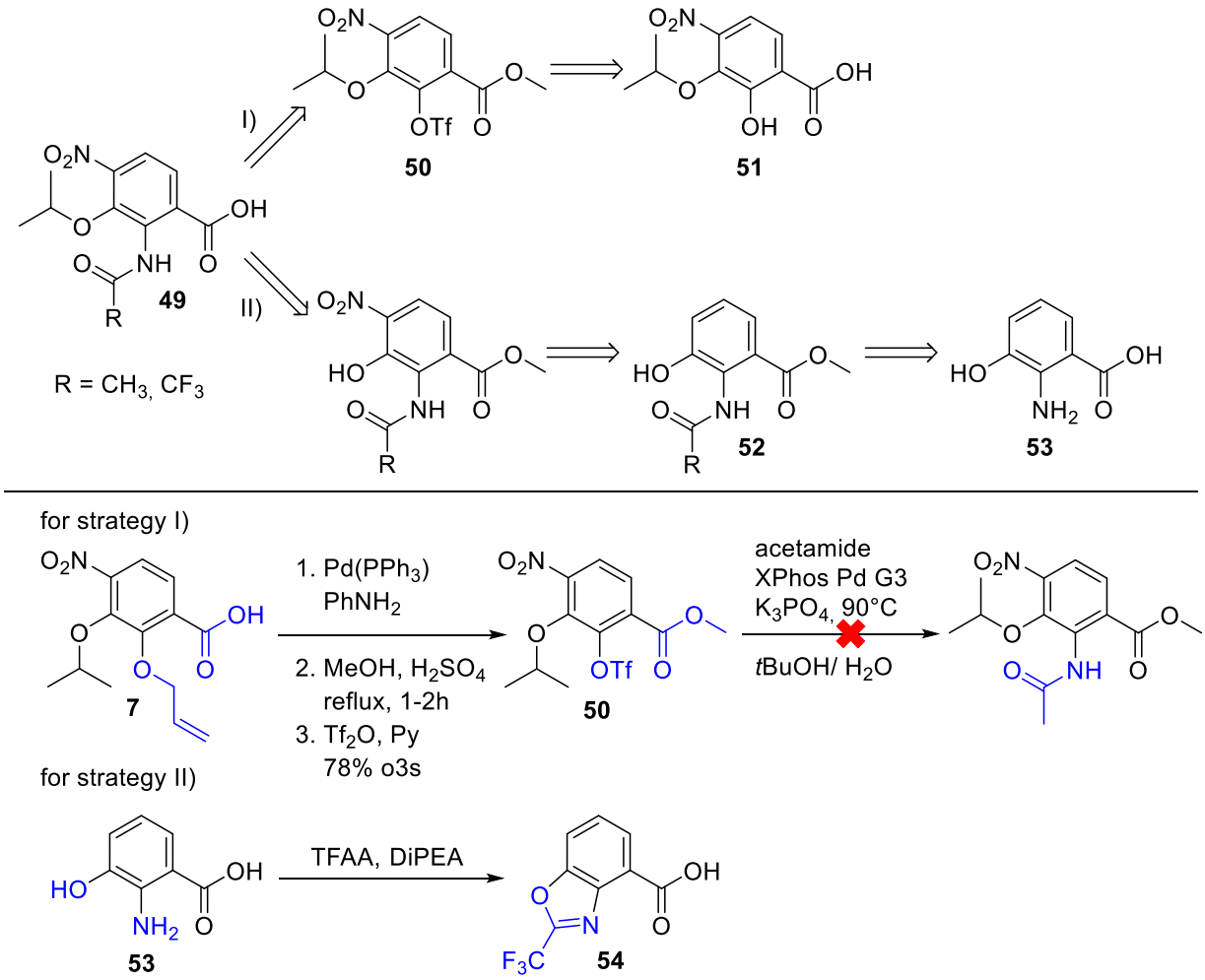


Figure 41: Retrosynthetic strategies for amino derivative (top) and failed syntheses (bottom).

Strategy I included a Buchwald Hartwig cross coupling to introduce a nitrogen substituent in *ortho* position to the carboxyl function from **50**. This required the conversion of the hydroxy function of compound **51** to a good leaving group such as triflate. To prevent side

reactions with the carboxylic acid, the methyl ester would be formed before the Buchwald Hartwig coupling. Therefore the allyl protecting group of the original ring D carboxylic acid **7** was first cleaved using a Pd-catalyzed deallylation protocol (see section 1.2.2). Esterification of the carboxylic acid with methanol followed, using classical conditions. A triflyl ester with the phenolic hydroxy group was then formed with triflic anhydride in pyridine to introduce a good leaving group, producing compound **50**. The Buchwald Hartwig reaction conditions were adapted from a literature method[170] using acetamide as substrate, but were not successful. A high steric hindrance by two groups each in *ortho* position to the triflyl group seemed like a reasonable explanation for the negative outcome of this reaction. The use of the less bulky ammonia may circumvent this problem, but would require a high-pressure resistant reaction vessel, which was not available at the time. An alternative strategy was assumed as most reasonable option.

Strategy II involved the regioselective nitration of compound **52** for the introduction of the nitrogen substituent in *para* position to the carboxyl function. A direct nitration of the starting material **53** would likely introduce the nitro function in *para* position to the amino function, as it is the strongest directing group. Therefore the strong directing effect of the amino substituent needed to be reduced, similar as it was known from former ring D syntheses[72, 99] by adding an acyl group that simultaneously functions as protecting group. The hydroxy group of compound **52** would now be the strongest directing group. Compound **52** was accessible via *N*-acylation of the commercially available starting material **53**. Trifluoro acetate was first tried as a substituent because the deprotecting conditions[171-172] are usually milder compared to those for acetyl cleavage. Unfortunately, the formation of a benzoxazole **54** was observed during the synthesis that excluded this protecting group. This heterocyclization is attributed to the high electrophilicity of the carboxyl function, which therefore reacts rapidly in an intramolecular reaction with the adjacent phenolic hydroxy group.

Acetyl was used as a less electrophilic alternative and was introduced after Fischer esterification of **53** with methanol (see Figure 43). The ester formation proceeded quite slowly, presumably because the amino group was protonated under such strong acidic conditions, thereby preventing the initial protonation of the carboxylic acid by electrostatic repulsion. The acetyl group was then introduced by acylation with acetic anhydride in pyridine to obtain **55**. Similarly as reported in literature[173], The acylation

firstly produced a mixture of *O*-, *N*- and diacylated material. The *O*-acetylated product was converted to the thermodynamically more stable *N*-acetylated compound after 1-2 days in solution.

Reaction conditions were optimized for the subsequent nitration, by varying the solvent, the reaction temperature and the reaction times. The obtained regioisomeric mixtures were quantified by UV absorption via LCMS as seen in Table 4.

Table 4: Screening conditions for the nitration of methyl 2-acetamido-3-hydroxy-4-nitrobenzoate with outcome (composition of the product mixture). Samples were taken after the listed reaction time t. The composition was determined by LCMS by means of the areas of the corresponding UV signals (λ=255 nm) of educt, 4-nitro (desired regioisomer), 6-nitro and 4,6-dinitro regioisomers.

Entry	Solvent	T [°C]	t [min]	educt	6-NO_2	4-NO_2	4,6-$(NO_2)_2$
1	DCM	-40	10	41%		50%	9%
2	DCM	0	20	2%	19%	25%	54%
3	Ac_2O	RT	10	-	-	-	~100%
4	Ac_2O/DCM*	-40	10	19%	14%	41%	26%
5	Ac_2O/DCM*	-40	30	41%		30%	29%
6	acetone	RT	10	28%		53%	19%
7	acetone	-40 - 0	55	30%		60%	10%
8	THF	RT	15	-	13%	-	87%
9	MeCN	RT	15	-	3%	-	97%

*solution of 1.1 Eq Ac_2O in DCM

The main undesired side reaction was the double nitration that was observed in nearly every entry. This may be due to the fact that the aromatic system is strongly activated by nitrogen and oxygen substituents. As expected, mono- and double-nitration proceeded faster at RT in several solvents (entries 3, 6, 8 and 9). The known reaction protocol in DCM at -40°C (entry 1) was found to be acceptable, bearing the lowest amount of dinitration, but the nitration in acetone (entry 6 and 7) showed a slightly better product fraction of maximal 60%. Nevertheless, both DCM and acetone were later used for the reaction, as acetone is known to be not completely inert to nitric oxide, which can form in nitric acid.[174] The nitration in acetone at lower temperature (entry 7) was scaled up first and a final yield of 27-33% was obtained after isolation of the correct regioisomer. The structure was verified by 2D NMR spectroscopy and by comparison with data from the other isolated regioisomer. Key interactions are shown in Figure 42.

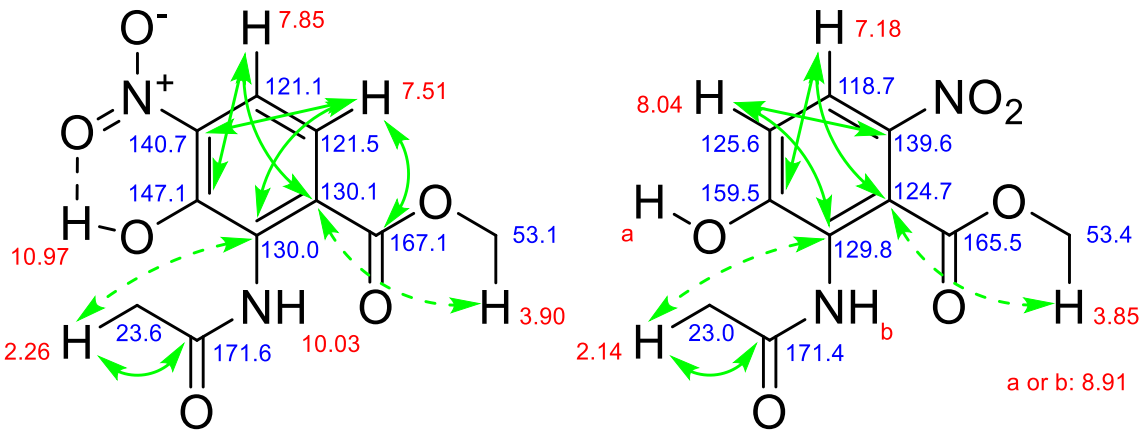


Figure 42: 2D NMR data for the 4-nitro (**56a**, a) and 6-nitro (b) products for the nitration of the acetamide. Explicit HMBC interactions shown in green, with weak interactions indicated as dashed arrows.

A Mitsunobu reaction was again chosen for the isopropoxylation of the hydroxy function of **56a**, followed by hydrolysis of the Me-ester function. The free carboxylic acid **49a** was obtained with an acceptable yield of 72% over 2 steps. The entire reaction sequence is shown in Figure 43.

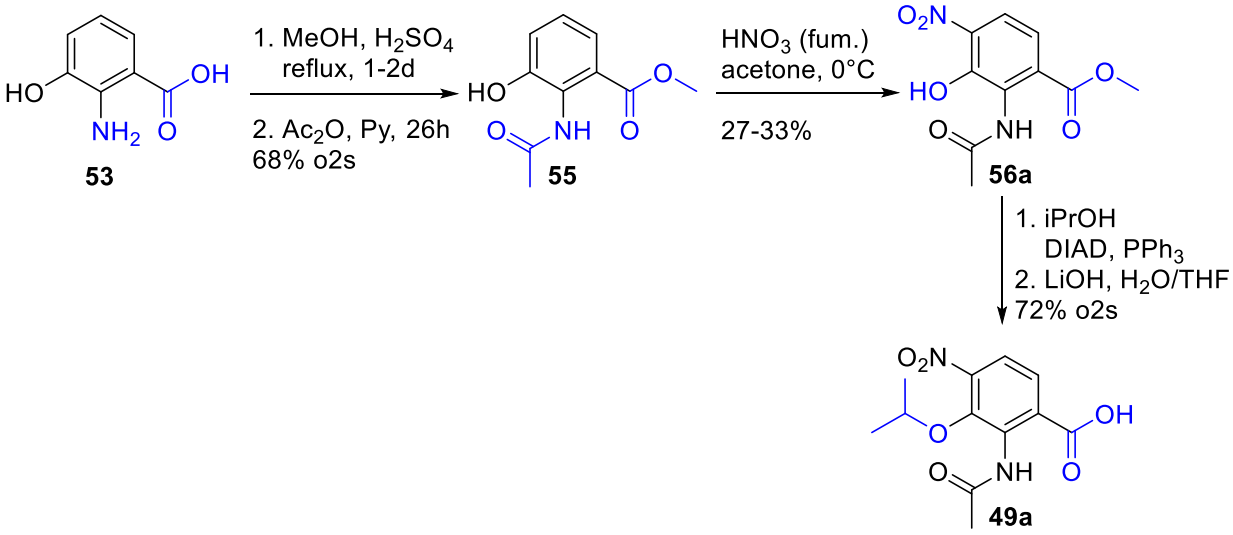


Figure 43: Synthesis of the acetamido ring D derivative starting from 2-amino-3-hydroxybenzoic acid.

The cleavage of the acetamido group was intended to take place next. First, however, the amide coupling with ring E was performed in order to investigate possible side reactions. Since the nitrogen function in the form of an amide in *ortho* position to the carboxylic acid still represented a nucleophile, it was feared that side reactions such as homocoupling of ring D could accur during activation. However, the standard amide coupling using oxalyl chloride and pyridine resulted in cyclization, forming an oxoquinazoline (**57**, see Figure 44) that was identified by LCMS and NMR.

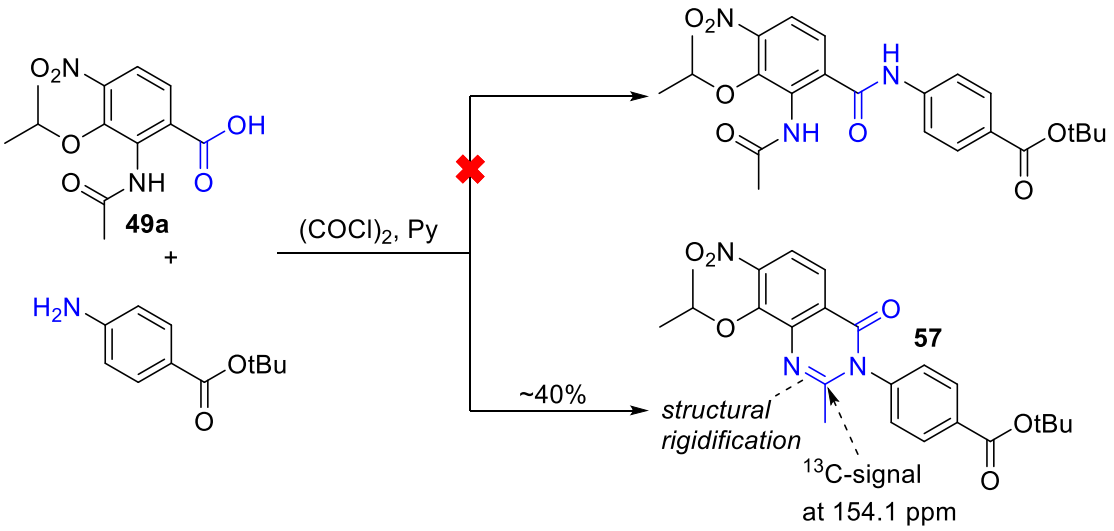


Figure 44: Amide coupling between ring D and E with expected product (top) and observed cyclization product (**57**, bottom).

This compound provided a beneficial structure for the SAR investigation for two reasons: Firstly, a rigidification of the DE-fragment was achieved while maintaining the amide bond as key feature of the DE-linker. A similar example is found in literature.[145] Secondly, this structure would verify the need for an HBD in *ortho* position to the carboxylic acid. Therefore the assembly to the full cystobactamid **012** was continued via path 2 (see Figure 45 and section 3.1) involving CDE-fragment **58**.

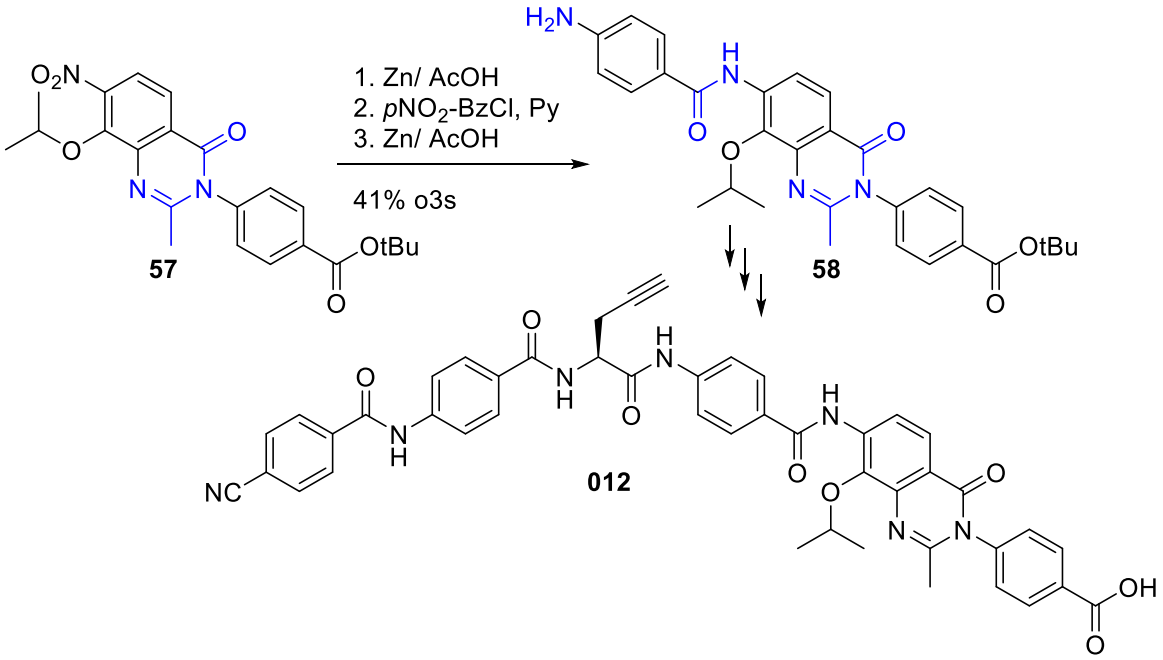


Figure 45: Assembly of the CDE-fragment **58** and structure of the final cystobactamid **012**.

To determine the effect of the methyl group attached to the oxoquinazoline, the synthesis of the demethylated analogue was planned by resubstituting compound **59** with

formyl[175] and continuing the reaction sequence with it (see Figure 46). Formyl amide was also later established as a protecting group for the amine, requiring only mild deprotecting conditions. After cleavage of the Me-ester and acetamido group by refluxing in strongly alkaline aequeos solution, the formyl amide was introduced by using acetic anhydride in formic acid[176]. The amide coupling with ring E formed the corresponding oxoquinazoline **60**, as it had been observed before. $(COCl)_2$ was assumed to be a less selective coupling reagent, as the yield for the oxoquinazoline **57** was only 40%, so $POCl_3$ was used here instead. Interestingly, the coupling did not take place when DiPEA was used as a base. Only addition of pyridine enabled the heterocyclization. This indicates that only the formation of a charged acylpyridinium salt may be sufficient to facilitate activation and heterocyclization. The synthesis towards the full cystobactamid **013** was continued with the method mentioned above.

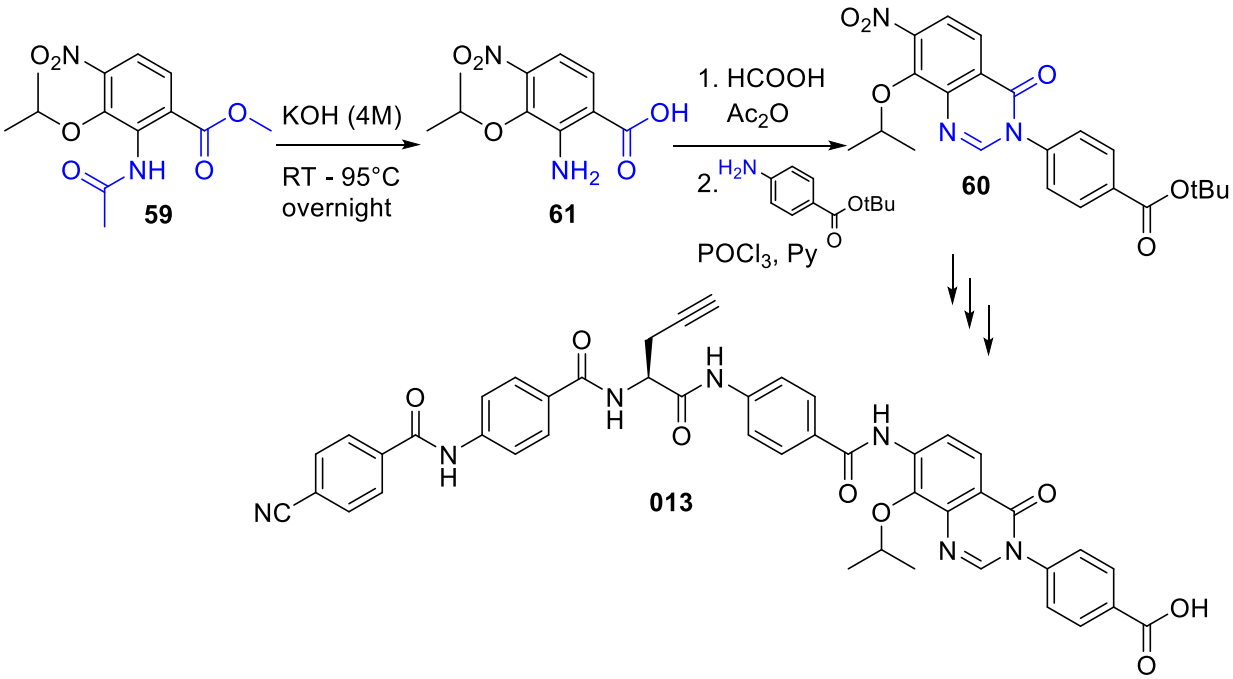


Figure 46: Deprotection of **59**, formylation, subsequent coupling with ring E and incorporation of the DE-fragment to the final cystobactamid **013**.

The originally intended introduction of the free amino group as replacement for the hydroxy group was now pursued further. Because the overall yield of the tetrasubstituted benzene **49a** was quite low (14% o5s), it was intended to simplify its synthesis. A new method was developed, allowing preparation of carboxylic acid **61** in 4 instead of 5 steps with an overall yield of 20% o4s, including only one chromatographic purification (see Figure 47). Starting from compound **53**, a formyl group was introduced first with the known method. The nitration was performed at -40°C in DCM as solvent.

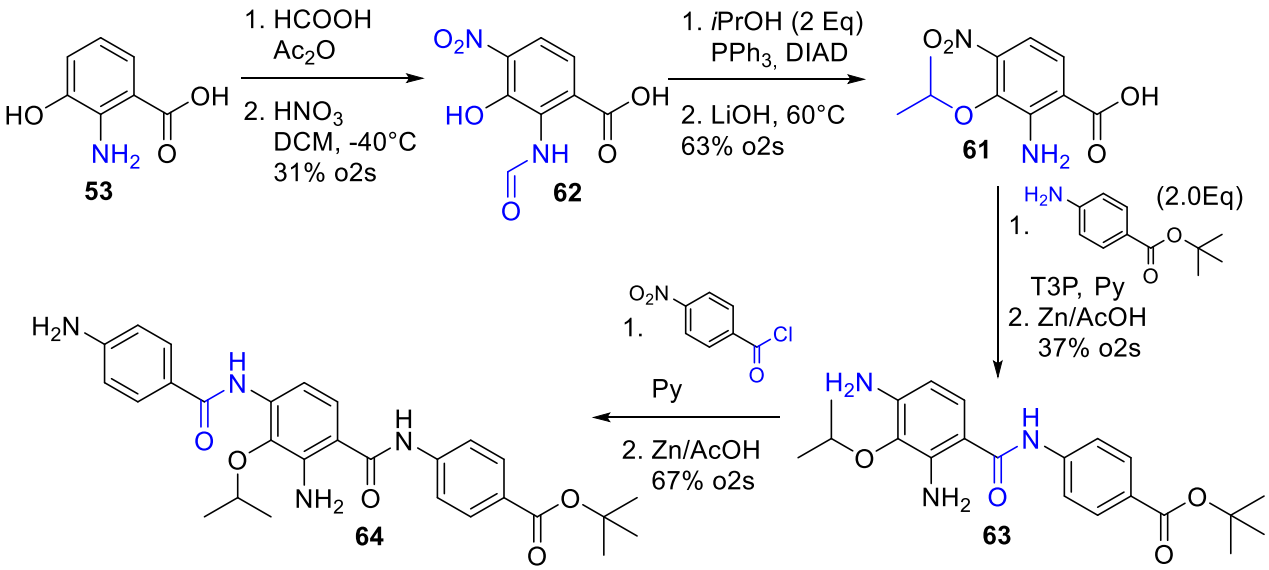


Figure 47: Shortened synthesis to obtain the free amino group at ring D and subsequent assembly to the CDE-fragment.

The correct regioisomer **62** was obtained by crystallization and filtration from toluene, since it was less soluble than the other regioisomer. Toluene was also found to be an effective quenching reagent to prevent multiple nitration. The carboxylic acid and hydroxy groups were subsequently isopropylated by Mitsunobu reaction. Saponification with LiOH removed both the methyl and formyl group to give **61** within only four steps. The Boc-protection of the free amino group was tried with Boc_2O and Py, but was unsuccessful, possibly due to sterical hindrance by the adjacent isopropoxy and carboxylic acid moieties combined with low nucleophilicity of the aromatic amino group. Based on this observation, it was assumed that this hindrance was high enough to prevent self-oligomerization of ring D during amide coupling with ring E. Therefore amide coupling was performed with an excess of ring E and without protecting the amino group. Subsequently, an attempt was made to protect the product with a Boc group using a stronger base (KO*t*Bu), which was also unsuccessful. Further attempts to protect the amino function were omitted. After the reduction of the nitro function at ring D, the DE-fragment **63** was obtained in a suitable yield of 37% over 2 steps. The subsequent amide coupling with ring C proceeded successfully, showing a sufficient selectivity of 88% (LCMS) with respect to the product of double substitution and a yield of 74% o2s for **64** after reduction of the nitro group. After final assembly and deprotection via path 2 (see section 3.1), the desired amino substituted cystobactamid **005** (see Figure 48) was obtained in 52% yield over 2 steps. A detailed analysis of the 2D NMR spectroscopy data of the full cystobactamid can be found in section 5.2. From this data the correct regioisomer was confirmed.

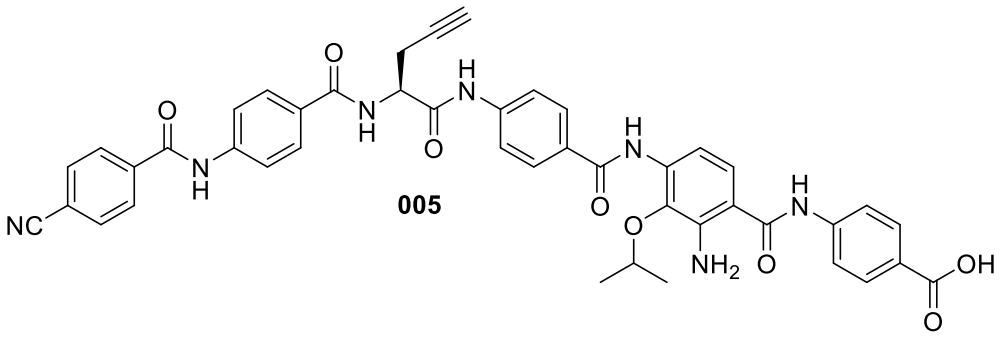


Figure 48: Structure of cystobactamid **005** with a free amino group at ring D.

The next target involved a rigidification strategy complementary to that of the rigidified oxoquinazoline structure mentioned before. In the former structure the amide bond between rings D and E was fixed to the 'distant' (*a*-*d*- or *d*-*d*-) conformation (Figure 49, left) and would now be fixed in the 'adjacent' (*a*-*a*- or *d*-*a*-) conformation (Figure 49, right).

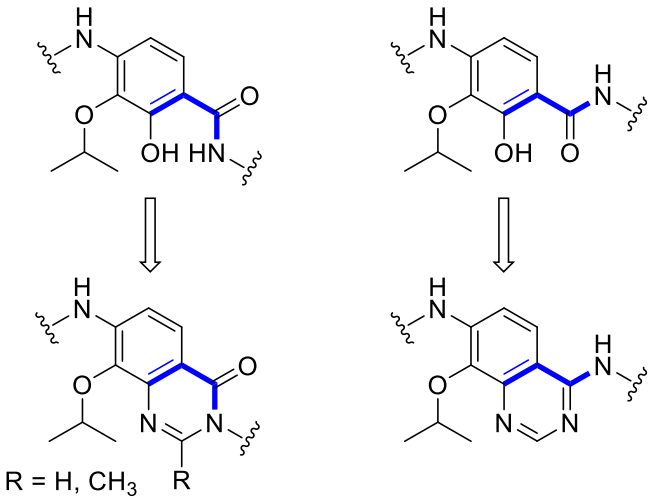


Figure 49: Ring D section of cystobactamids showing the two most likely rotations of the amide bond between ring D and E (top) and a possible rigidification by heterocyclization (bottom).

This aminoquinazoline was thought to be accessible via the same strategies used for the previously synthesized amino derivatives (see Figure 50). Strategy I started from the methyl ester **65**, following the earlier developed approach for accessing a free amino group as substitute for the hydroxy function (compare Figure 43) but using a formylamide instead of an acetamide function. This way **66** was intended to be synthesized to allow subsequent heterocyclization, forming benzopyrimidone **67**.

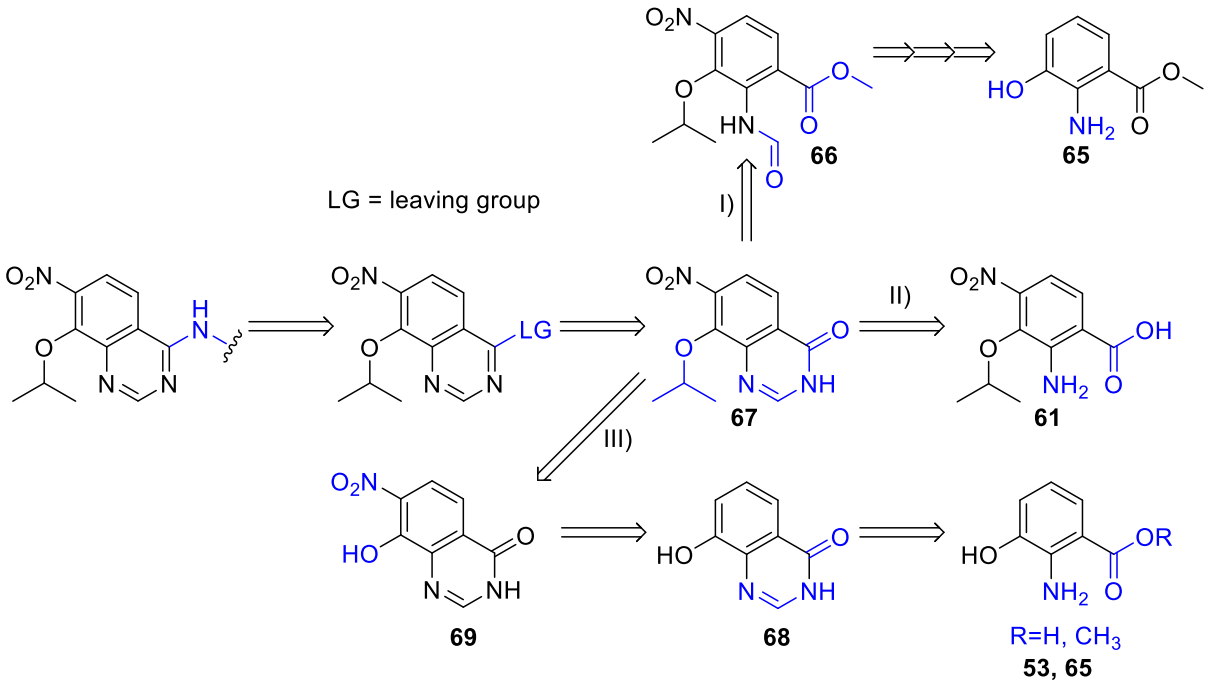


Figure 50: Retrosynthetic approaches for the synthesis of the aminoquinazoline with three strategies to approach the benzopyrimidone **67** with all strategies initiating from already accessible structures.

This strategy was realized by an initial formylation by using the known method followed by nitration with HNO_3 in DCM at -40°C. Isomer separation was again followed by Mitsunobu-isopropylation, giving compound **66** (see Figure 51). Inspired by methods of Nishino *et al.*[177] and Tanaka *et al.*[178], the cyclization to benzopyrimidone **67** was carried out in concentrated methanolic solution of ammonia at 60°C in a closed flask. Deformylation was observed as a side reaction. The benzopyrimidone **67** was obtained in a yield of 60% and its structure was verified by 2D NMR. With this compound prepared, coupling with ring E would continue after the oxo function of **67** was converted to a good leaving group.

An alternative synthesis of the key compound **67** was also planned using strategy II that involved the conversion of already accessible compound **61** with formamidine acetate, as the latter is a known condensing reagent towards the formation of pyrimidines.[179-181] This conversion was successfully carried out in a typical reaction setup with EtOH/ dioxane as solvents at higher temperature, but gave a low yield of 38% for **67**.

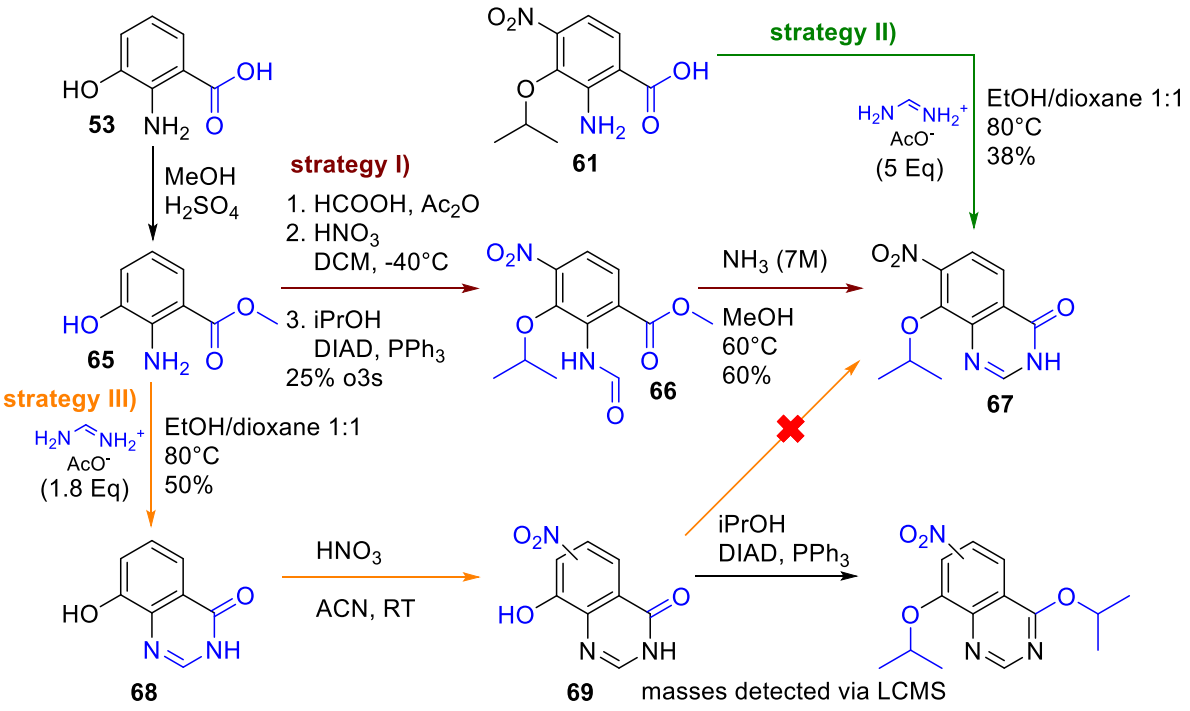


Figure 51: Summary of synthetic strategies to obtain benzopyrimidone **67**.

Since both strategies I and II included five to six synthetic steps with a maximum overall yield of below 15%, it was considered worthwhile to investigate strategies which would reduce the synthetic effort. Using strategy III, preparation of **67** would be accessible in only four steps from the commercially available educt **53** and initial protection of the amino group would not be required. In this way, benzopyrimidone **68** would be synthesized first, followed by nitration with the hydroxy group as the strongest directing group, allowing the desired regioisomer **69** to be formed. The Mitsunobu-isopropylation would subsequently give compound **67.** Similar to strategy I, this strategy was started with Me ester **65**, and the benzopyrimidone **68** was formed by condensation with formamidine acetate with a yield of 50%. The following nitration was observed to convert **68** to a mononitrated compound whose structure was not identified. However, it was found that the Mitsunobu reaction of the crude product led to double isopropoxylation. This indicates the high acidity of the enolizable pyrimidone, given that a pK_A of 9.81[182] for the hydroxy function of 4-hydroxyquinazoline was reported. This allows the participation as an acidic group in the Mitsunobu reaction, analogous to a phenolic hydroxy group.

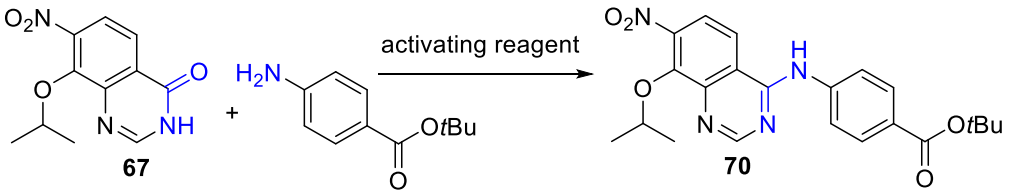


Figure 52: Intended coupling reaction between the benzopyrimidone **67** and the ring E building block.

With the benzopyrimidone **67** in hand, it was assumed that the coupling with ring E would take place between the oxo function of **67** and the amino group of *tert*-butyl 4-aminobenzoate (see Figure 52) to produce the desired DE-fragment **70**. Similar to the activation of carboxylic acids, amide coupling reagents such as PyBOP, T3P/pyridine or $POCl_3$/pyridine were now tested as activation reagents in dilute solution. All methods showed no significant product formation. It was assumed that the reaction in dilute solution was too slow, therefore the reaction was repeated in pure $POCl_3$ as solvent at 90-100°C. In fact, the formation of the chloropyrimidine was observed after reaction overnight. The chloropyrimidine was found to be quite sensitive to hydrolysis. To allow easier removal of the reagent, $POCl_3$ was replaced with $SOCl_2$ to exploit a lower boiling point of 76°C versus 106°C for $POCl_3$. The conversion was low, but enough material was obtained in this way to test the amination with ring E. The amination was performed in DMF at 100°C, and conversion to the product of ~20% was observed via LCMS. However, the isolation and characterization of the product was not successful. Indeed, it is known from literature, that the low reactivity of pyrimidones towards chlorinating reagents as $POCl_3$ may result from a reversible self-dimerization of the chloro pyrimidone with educt.[183] This may explain the low conversion observed for this step. As an alternative reaction condition, a direct conversion of the benzopyrimidone **67** with ring E was tried with triphenyl phosphite in refluxed toluene, delivering only traces of the desired product.

The synthesis was rigorously replanned to obtain the desired pyrimidine ring **70** only after formation of the DE-fragment **71** (see Figure 53). This DE-fragment had already been prepared before, thus circumventing the difficult coupling of ring E to a pyrimidone such as **67**.

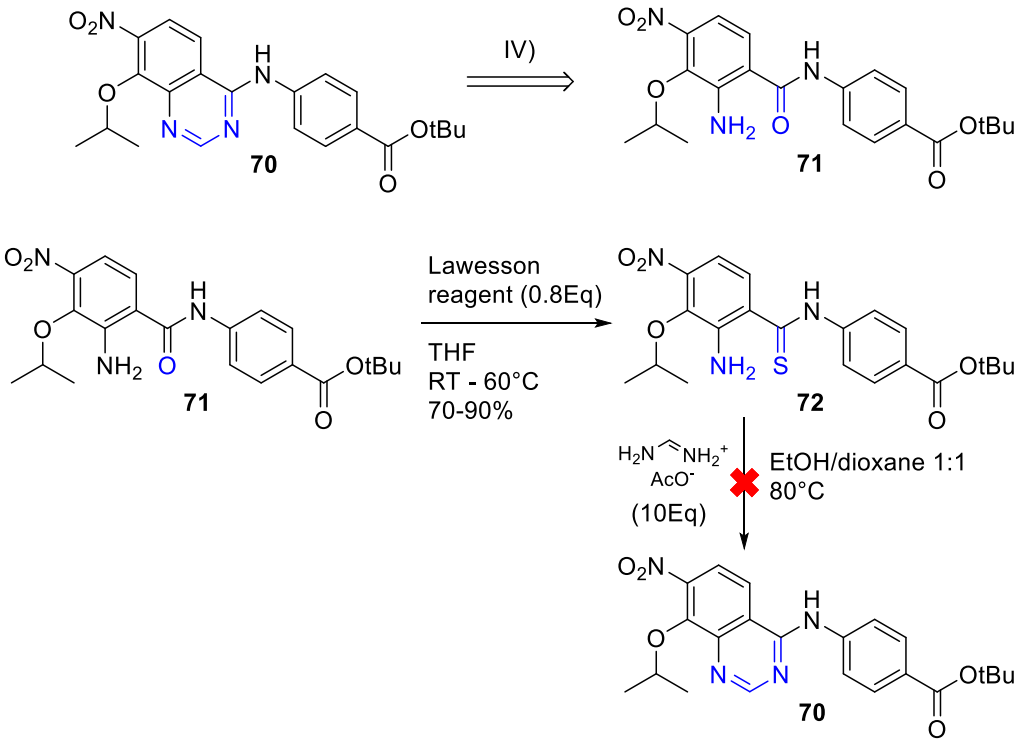


Figure 53: Retrosynthetic strategy IV for synthesis of the aminoquinazoline **70** (top) and reaction conditions tried (bottom).

To implement this strategy, free amine **71** was used as starting point and the amide was first converted to an activated thioamide **72** using the Lawesson reagent[184]. This reagent represents a dimer of the actual sulfur-transferring species. The use of a stoichiometric amount of this reagent led to incomplete turnover, while an excess of one equivalent of Lawesson reagent gave condensation products of the reagent itself with **72**. An optimum betwenn conversion and side reaction was determined with an excess of 0.8 equivalents of the Lawesson reagent, and the thioamide **72** was isolated in >70% yield. Subsequent heterocyclization with formamidine actetate exhibited low and nonselective formation of the desired product, even with a large excess of the reagent. The separation and characterization of the product was found to be unfeasible, hence the synthesis of the aminoquinazoline was not further pursued.

Pyridine derivative

The focus was now set on a pyridine as a substitute for the hydroxy function at ring D. This substitution was found to result in high activity against strains of *E. coli* and *Salmonella* in the cystobactamid-related Albicidins[185] and was also implemented in a truncated version of cystobactamids[109]. For the latter compound bearing such a pyridine modification, a higher resistance to AlbD was found compared to the standard analogue.[109] This enzyme catalyzes the amide cleavage between ring C and D and

therefore inactivates the Albicidins and cystobactamids.[186] In addition, the substitution of the benzene ring with an electron-deficient pyridine may allow the introduction of new interesting moieties via S_NAr.

The synthesis of the required carboxylic acid **73** was mainly adapted as reported by Elgaher *et al.*[109], starting from 2,6-dichloro-3-nitropyridine and is shown in Figure 54.

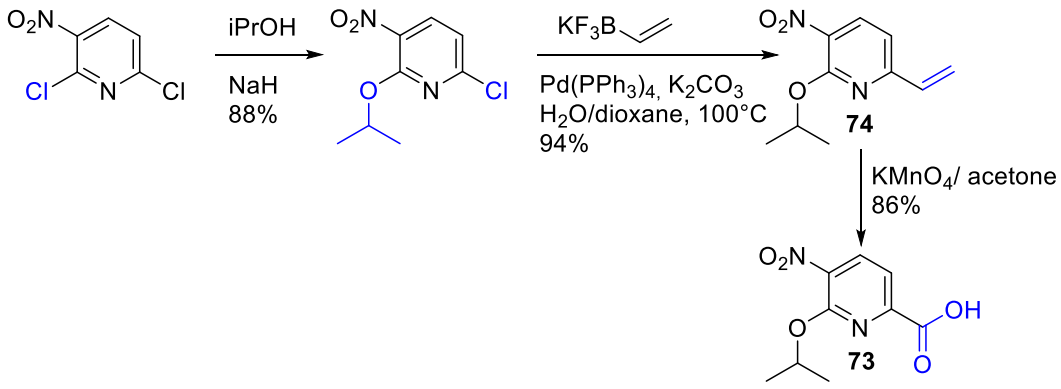


Figure 54: Synthesis of a pyrimidine analogue of ring D similar as reported[109]. The reaction conditions for the Suzuki coupling were introduced in this Thesis.

As a modification, the Stille coupling introducing a vinyl group to give **74** was replaced by a Suzuki coupling which offered less toxic reagents and delivered a high yield. The final assembly was completed following the method described in section 3.1 (retrosynthetic path 2, see Figure 55 left side). As an alternation, a new scaffold was introduced as well (see Figure 55 right side), following retrosynthetic path 1. Cystobactamids with this strong variation of the AB-central AA fragment showed superior results in MIC-testings[119] (see section 1.2.4, Figure 18, compound HDo 308). Similar results were expected with the pyridine at ring D, as this modification apparently retained activity. Furthermore, the new scaffold was chosen as an additional Western fragment for combinations with other derivatives later synthesized in this Thesis.

The derivative showing the standard scaffold **006** was obtained in a yield of 66% over 2 steps via the previously discussed retrosynthetic strategy (see Figure 55). The new scaffold was combined with the CDE-fragment **75** as well by using a modificated AB-fragment **76**, yielding the final cystobactamid **006b** in 10% over 4 steps.

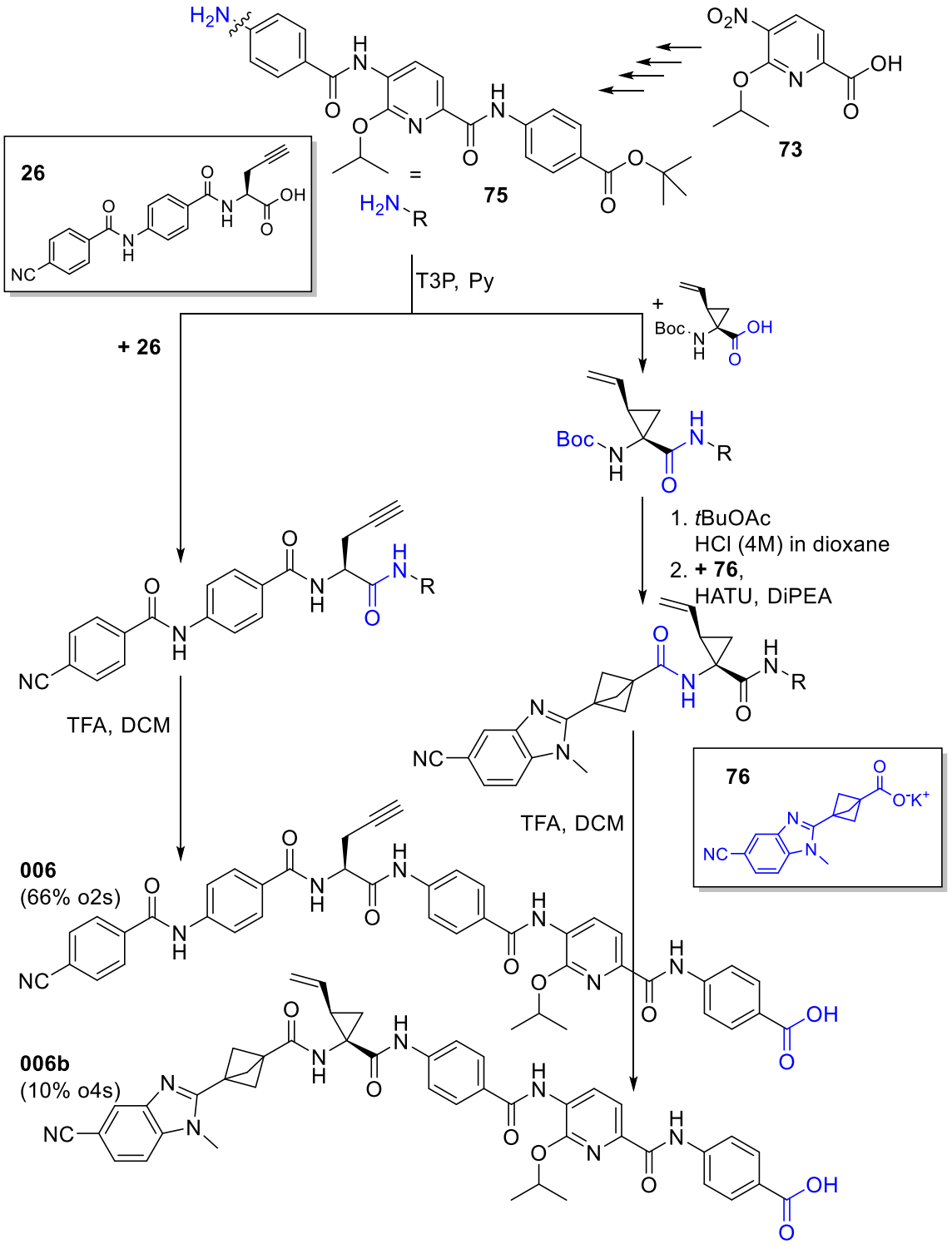


Figure 55: Assembly of the CDE-fragment **75** with the standard Western part (left path) and with a modified Western part with new scaffold (right path) using AB-fragment **76**. The resulting cystobactamids **006** and **006b** are shown.

The synthesis of the new AB-central AA scaffold is described in detail in section 3.3.2. The pyridine analogue **73** was later used for developing a non-acidic cystobactamid (see section 3.4.1).

Methoxy derivative

Emphasis was now placed to substitutions of the isopropoxy group. Investigations of G. Testolin *et al.*[9, 99] revealed a high decrease of activity when isobutoxy was used instead, indicating that an elongation of the ether residue might not be beneficial (see also section 1.2.5). On the other side a methoxy group was found in the cystobactamid-related antibacterial Albicidins[71, 73] and was introduced in a shortened version of the cystobactamids[109], however it was not included in recently optimized cystobactamid structures. The corresponding ring D fragment **77** was synthesized and provided by C. Leitner with the free amino function and with the *t*Bu protected carboxylic acid.

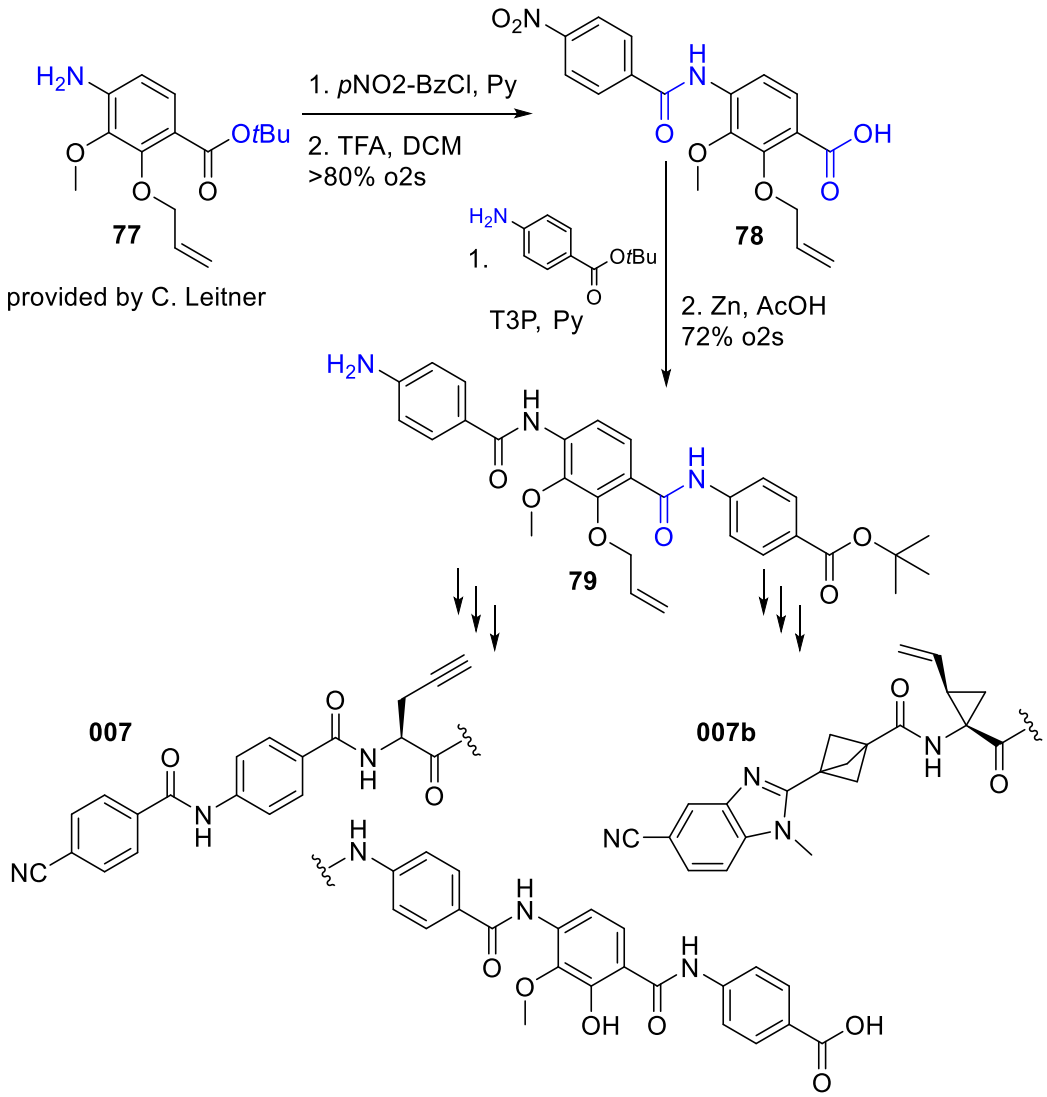


Figure 56: Assembly of the CDE-fragment **79** with the standard Western part (left path) and with the new scaffold (right path). The resulting cystobactamids **007** and **007b** were obtained analogously as seen for the pyridine derivative.

This pattern of protecting groups required initial coupling with ring C, followed by *t*Bu-ester hydrolysis to form **78**. This CD-fragment was subsequently reacted with ring E, and reduction of the nitro function gave the CDE-fragment **79** (see Figure 56).

The full assembly was achieved by following retrosynthetic path 2, forming the final cystobactamid **007** in a yield of 33% over three steps from the CDE-fragment **79**. Moreover, fragment **79** was also combined with the additional Western fragment in analogy with the pyridine ring D analogue (retrosynthetic path 1) to give cystobactamid **007b**.

Isobutyl derivative

A more ambitious modification was masterminded, namely the introduction of isobutyl as substitute for isopropoxy. In the resulting ring D structure, there is no longer an HBA between the DE-linker and the phenolic hydroxy group. Several retrosynthetic strategies were suggested: With strategy I) (see Figure 57), the introduction of the C_4-group was envisaged by an intramolecular Fries rearrangement of ester **80** and subsequent reduction of the ketone **81**, e.g. by the Wolff-Kishner reaction to produce **82**.

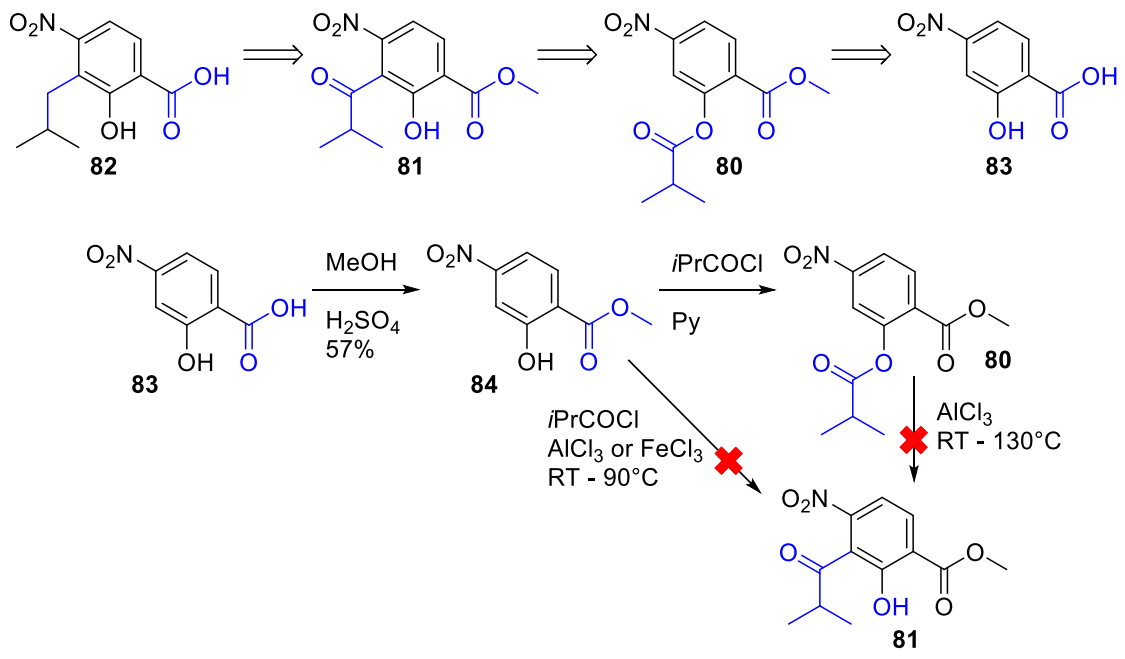


Figure 57: Retrosynthetic strategy I) and attempted synthesis for an isobutyl ring D derivative.

The required arylester **80** was obtained in two steps from commercially available benzoic acid **83**. Unfortunately, the rearrangement to **81** was not observed with $AlCl_3$ as Lewis acid at high temperature. Instead, only esterhydrolysis was observed. The direct aromatic

acylation of intermediate **84** using $AlCl_3$ or $FeCl_3$ and isobutyryl chloride did not occur as well. It was assumed that the aromatic ring is not sufficiently activated to allow electrophilic substitution, as it bears both a nitro and a carboxy group.

In a similar approach, a Claisen rearrangement was tried with aryl ether **85** (provided by D. Kohnhäuser) at high temperature in a polar aprotic solvent to form **86**. Exclusively decomposition products were observed under the conditions tested (see Figure 58).

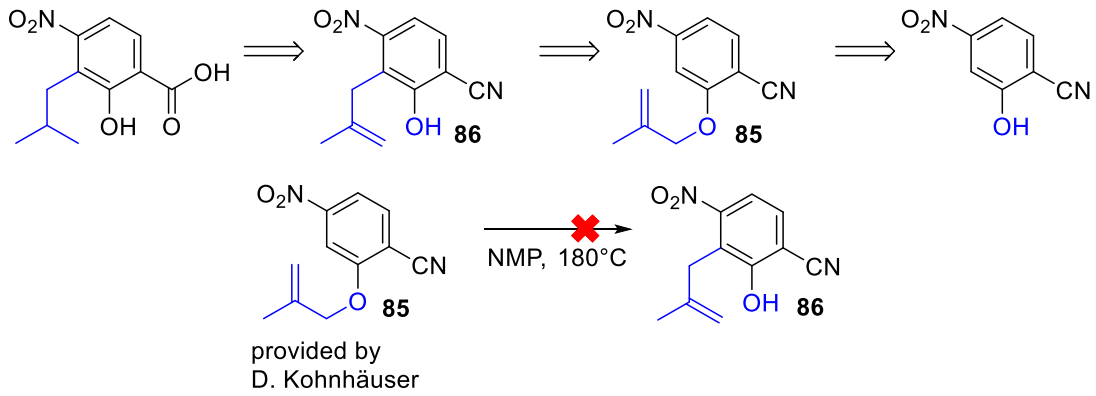


Figure 58: Retrosynthetic strategy II) and attempted synthesis for an isobutyl ring D derivative.

An approach analogous to the known ring D synthesis (see section 1.2.3) was viewed as not practical, since the alkyl substituent replacing isopropoxy would no longer be the strongest directing group. It was expected that the adjacent acetoxy group would direct the electrophilic attack in *para* to itself instead (see Figure 59).

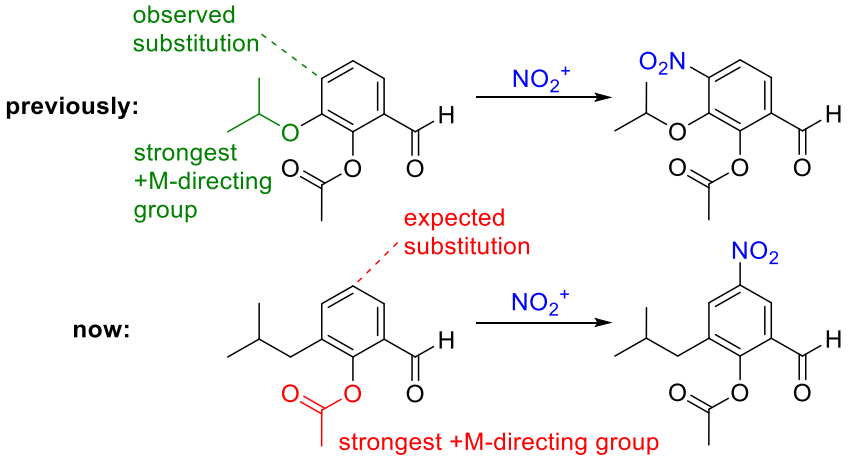


Figure 59: Comparison of the regioselectivity for the nitration as published for the cystobactamid ring D synthesis[72] (top) and for the intended nitration of the isobutyl ring D analogue (bottom).

It was assumed that the aromatic ring, which bears a nitro and a carboxyl group and thus two –M substituents, was too electron deficient to allow introduction of a substituent by S_EAr. Therefore, the focus was set to electron rich and activated systems as a starting

point. A promising approach was thought to be the introduction of the alkyl chain by directed ortho lithiation[187-188]. This method allows the organolithium-mediated selective aromatic substitution of hydrogen by an electrophile in *ortho* position to a directing group (reviewed in[189-190]). Such directing groups include moieties capable of complexing the lithium atom (as seen in Figure 60, compound **87**) and therefore stabilizing the Li-aryl bond. Fortunately, the benzene core of ring D is adorned with oxygen and nitrogen substituents *meta* to each other, that were considered to be easily functionalized, so that both provide metal-directing groups and thus would force the substitution to their middle position. A MOM-group and an alkoxycarbonyl-group were therefore selected as substituents for the hydroxy and the amino function, as in compound **88**.

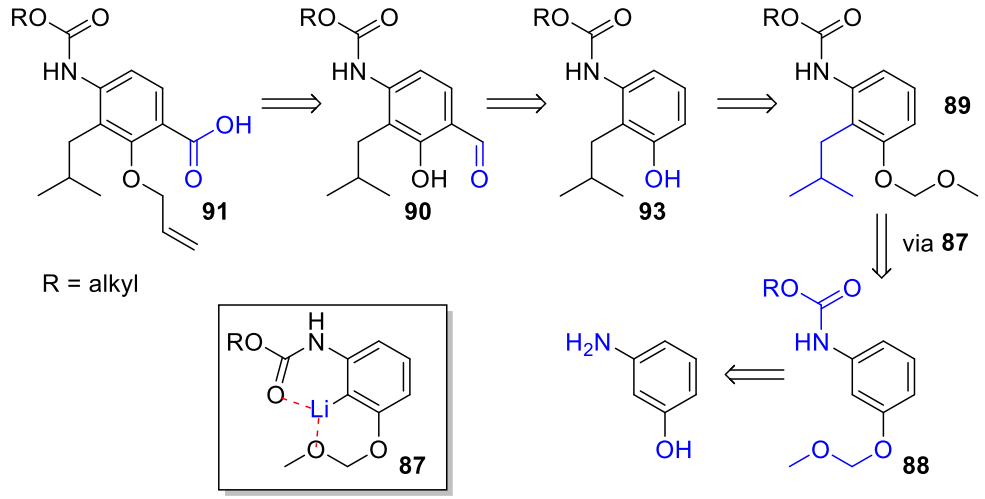


Figure 60: Retrosynthetic strategy III) for an isobutyl ring D derivative using the ortho lithiation method and stabilizing effect of alkoxycarbonyl and MOM-groups on lithium in the lithiation intermediate **87** (boxed).

After the successful introduction of the alkyl chain forming **89**, the MOM group would be cleaved to reveal the phenolic hydroxy moiety. This moiety was expected to direct formyl as electrophile selectively in *ortho* position by a magnesium-mediated Skattebol formylation[110], delivering **90**. After completion of the substitution pattern, allyl protection of the hydroxy group and oxidation to the carboxylic acid **91** would follow.

Coincidentally, the targeted lithiation method with the exact substitution pattern and a methoxycarbonyl- (Moc-) group as directing moiety for nitrogen was found in the literature[191]. Herein, the Moc-group was found to provide better regioselectivity than a Boc-group. Accordingly, the synthesis was started with functionalization of 3-aminophenol with MOMCl and NaH as base, followed by reaction with methyl chloroformate.[191-192] Compound **88a** was obtained with a yield of 56% over two steps.

Having the two necessary directing groups in place, the conditions for a directed *ortho* lithiation were investigated next as shown in Figure 61.

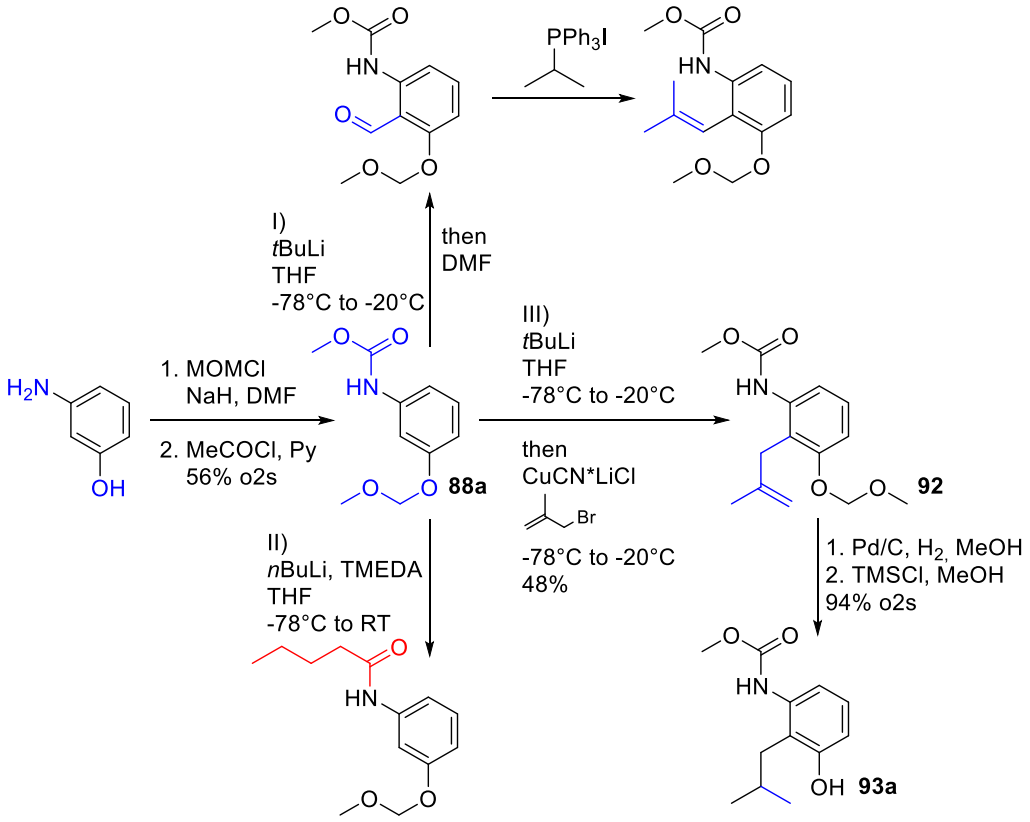


Figure 61: Selection of investigated conditions for introducing isobutyl to ring D by ortho lithiation with strategy III) being the successful approach.

In literature, *t*BuLi was selected as lithiation reagent since weaker reagents such as *n*BuLi or *s*BuLi were found to be insufficiently reactive, even when tetramethylethylenediamine (TMEDA) was used as complexing reagent.[191, 193] Initially, DMF was chosen as the electrophile (conditions I) to explore the best reaction conditions, including temperature, solvent and the effect of adding TMEDA. A detailed table is found in the attachment (Table 18). The main observations were firstly that *t*BuLi was adequate and the use of *n*BuLi may lead to substitution reactions at the Moc group (conditions II), secondly that mixtures of *t*BuLi and TMEDA were not stable and thirdly that best results could be achieved in a temperature range of -78 to -20°C (conditions I and III). The so obtained benzaldehyde was first intended to react with isopropyl triphenylphosphonium iodide to obtain a methallyl substituent. Hydrogenation of the methallyl group was expected to provide the isobutyl group. To enable a more direct introduction of the isobutyl chain, the electrophile was later changed to methallyl bromide. It was assumed that the direct reaction with isobutyl bromide in the presence of a strong base such as *t*BuLi would lead to the

elimination of HBr and thus to a low conversion. The use of methallyl bromide would circumvent such elimination, and this reagent is also a stronger electrophile. The direct reaction of the lithiated educt with methallyl bromide gave the desired product, but also showed notable side reactions. To further optimize this reaction, an attempt was made to perform a transmetalation of the lithiated aromatic ring with a cuprate. Such cuprates are known to facilitate the conversion of alkyl and alkenyl halides in lithiation reactions.[194-197] The cuprate reagent was prepared in solution by solving CuCN and dry LiCl in THF and then added to the lithiated educt. This was followed by the addition of methallyl bromide. The use of the cuprate indeed led to an increased product formation while diminishing the amount of by-products. After scaling up the reaction, compound **92** was isolated in 48% yield. 2D NMR spectroscopy revealed the correct regioisomer (see Figure 62). One aromatic proton (7.51 ppm) showed unusual broadening of the signal, but assignment to a carbon atom was possible (115.3 ppm).

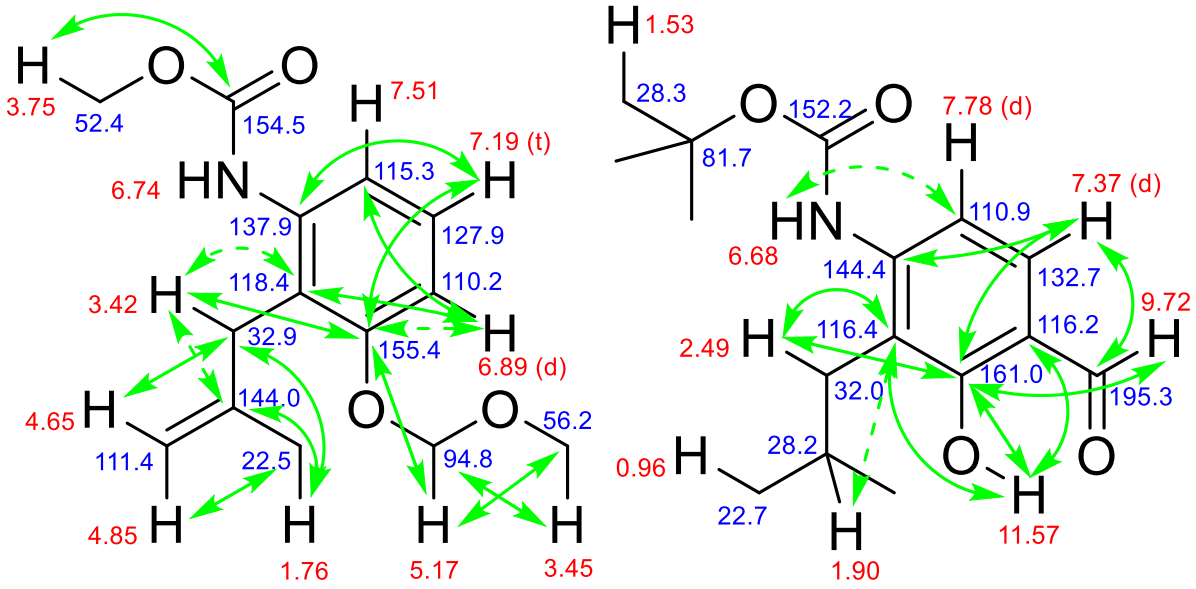


Figure 62: 2D NMR key interactions of the alkylation product (**92**, left) and the aldehyde intermediate (**90a**, right). Explicit HMBC interactions are shown in green with weak interactions indicated as dashed arrows.

Hydrogenation with H_2 on palladium/carbon and deprotection of the hydroxy group by acidic cleavage of the MOM group gave **93a**, which in turn was intended to be formylated in the subsequent Skattebol formylation. By applying this method, similar compounds were selectively formylated in *ortho* to the hydroxy group in a previous cystobactamid synthesis[109]. However, in this case the desired product was isolated in a low yield of 18%. The cause was thought to be slow conversion, as the reaction stagnated at an unidentified intermediate, for which a neutral mass of 281 was determined by LCMS. This indicated a formylation of the intermediate benzyl alcohol that formed during the Skattebol reaction.

The specific site of this formylation was not elucidated in detail. However, in the MS-fragmentation pattern of this compound a mass of 264 was found as well, thus indicating cleavage of hydroxy as it is typical for a benzylic alcohol function. The observed by-product was slowly consumed while the amount of desired aldehyde increased. Therefore, a reversible side reaction was assumed and thus the formylation of the benzene core as irreversible side reaction was excluded. Therefore it was assumed that cyclic acetal **94** had formed (see Figure 64). A kinetic measurement was performed by measuring the composition of the reaction mixture over time. The plot is shown in Figure 63.

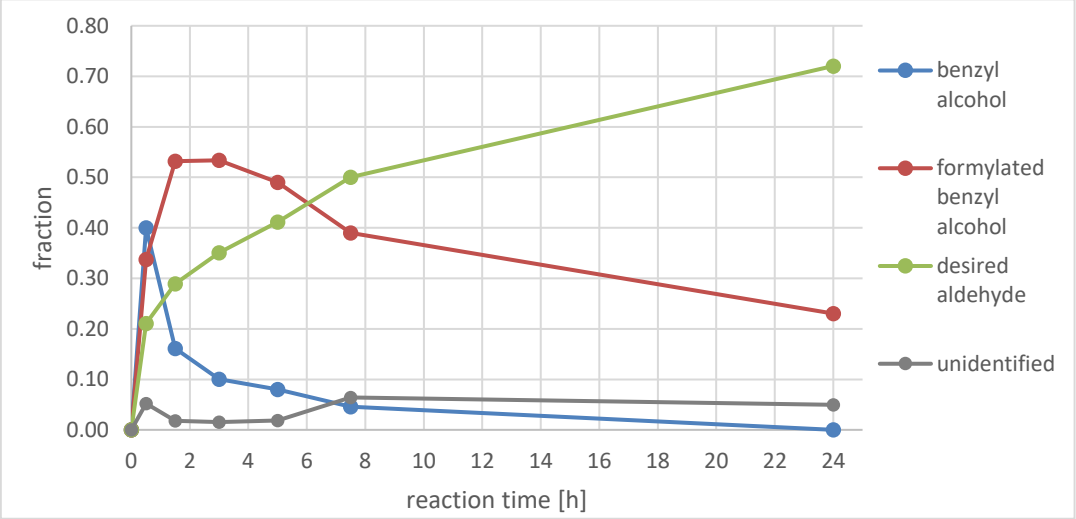


Figure 63: Kinetic plot for the investigated Skattebol-formylation. The fractions of the desired product and the intermediate compounds were determined by LCMS based on the peak areas of UV-absorption at λ = 254nm.

A possible mechanism for the Skattebol reaction is depicted in Figure 64. Starting from **93a** the phenol function is first deprotonated by TEA and a magnesium alkoxide is formed (**a**). Formaldehyde is complexed by magnesium, which directs the following electrophilic attack in the *ortho* position. After rearomatization of **b**, the benzyl alkoxide **95** is formed. The renewed coordination of formaldehyde enables a hydride shift of one methylene hydrogen to formaldehyde and thereby the oxidation of the benzyl alcohol to the aldehyde by rearrangement (**c**). This magnesium complex can either be hydrolyzed to form compound **90a**, or the magnesium complex partially dissociates to **d**, leading to a new reaction with formaldehyde to form a cyclic acetal **94a**. This cyclic acetal would be observed in LCMS as compound **94** after hydrolysis. However, the formation of **94a** is assumed to be rapid but reversible, so that the desired aldehyde **90a** will form only after

a longer reaction time. Taking this into account, a yield of 35% was obtained after the reaction time was increased from 2 h to 24 h.

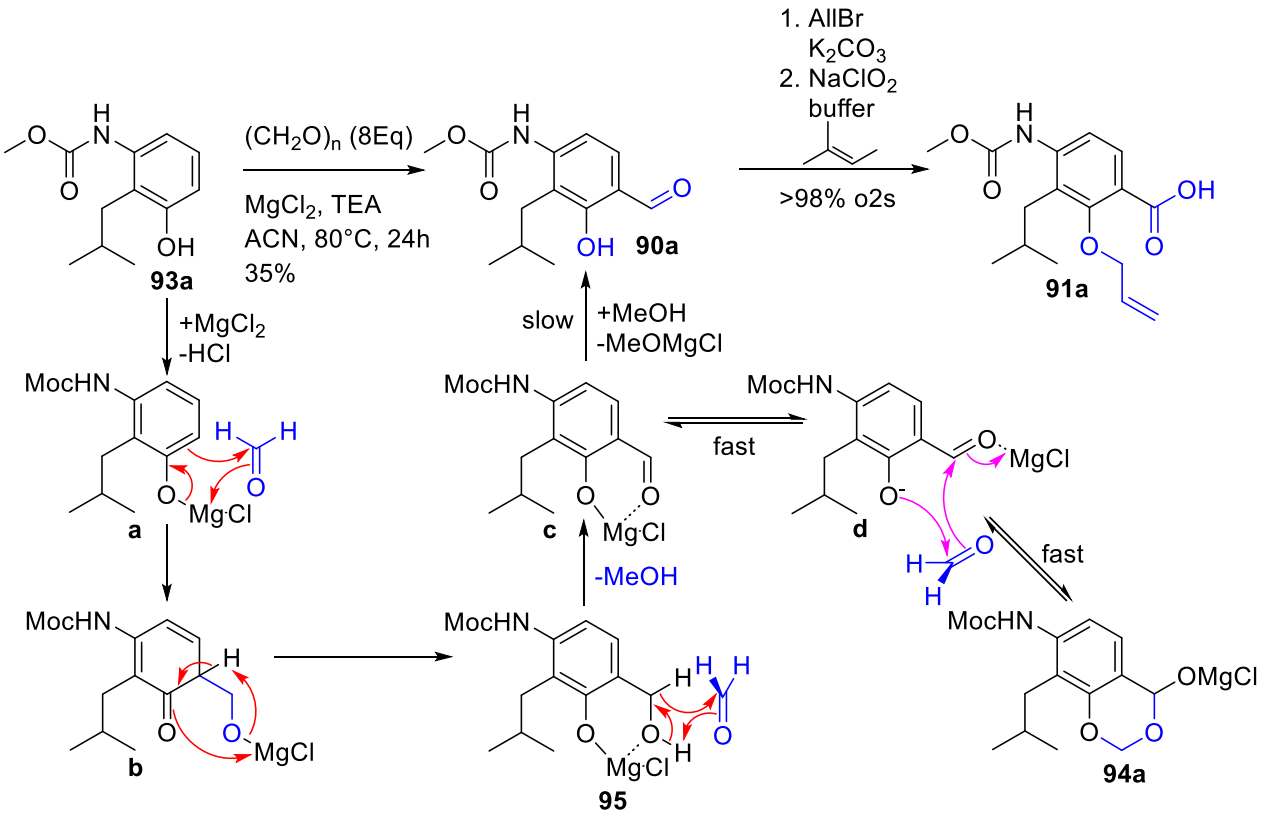


Figure 64: Skattebol-formylation of compound **93a** with proposed mechanism below and subsequent conversion of **90a** to the targeted isobutyl ring D carboxylic acid **91a**.

The hydroxy group of **90a** was subsequently protected with allyl and the aldehyde function was oxidized via a known method[9, 99] to give the carboxylic acid **91a**. This compound was then amide-coupled with *tert*-butyl 4-aminobenzoate to obtain the DE-fragment. The deprotection to the *N*-terminal amino function was scheduled next, but turned out to be difficult, since the Moc protecting group was unremovable under mild aqueous acidic or basic conditions. Other known methods such as deprotection by trimethylsilyl iodide[198] and lithium *n*-propanethiolate[199] led to simultaneous deprotection of the allyl group. A reprotection of the hydroxy group with allyl bromide after synthesis of the CDE-fragment also failed to generate useful material. The stability of the Moc group in acidic medium can be rationalized as being higher compared to a Boc group due to the less favoured formation of the weak electrofuge Me^+, compared to Me_3C^+. Moreover, the nucleophilic attack of the allyl group with strong nucleophiles such as iodide or thiolates might be generally faster than the S_N2-reaction with the Moc-methyl group. However, these fundamentally different mechanisms cannot be directly compared.

In an alternative attempt (see Figure 65), the Moc group of **93a** was cleaved by hydrolysis in strong alkaline aqueous medium similar to amide hydrolysis. The amino group was then reprotected with Boc_2O[200] followed by Skattebol formylation. This time both reaction time and yield could be improved by using *N,N'*-dimethylpropyleneurea (DMPU) as cosolvent. The similar metal ion complexing reagent hexamethylphosphoramide (HMPA) was reported in the literature to accelerate the oxidation from benzyl alcohol to benzaldehyde by reducing the acidity of magnesium and therefore increasing the electron density in the aromatic system.[201] However HMPA is highly carcinogenic, and DMPU is a less toxic alternative for metal mediated reactions.[202] Indeed, the desired benzaldehyde **90b** was obtained after 2 h reaction time in 43% yield.

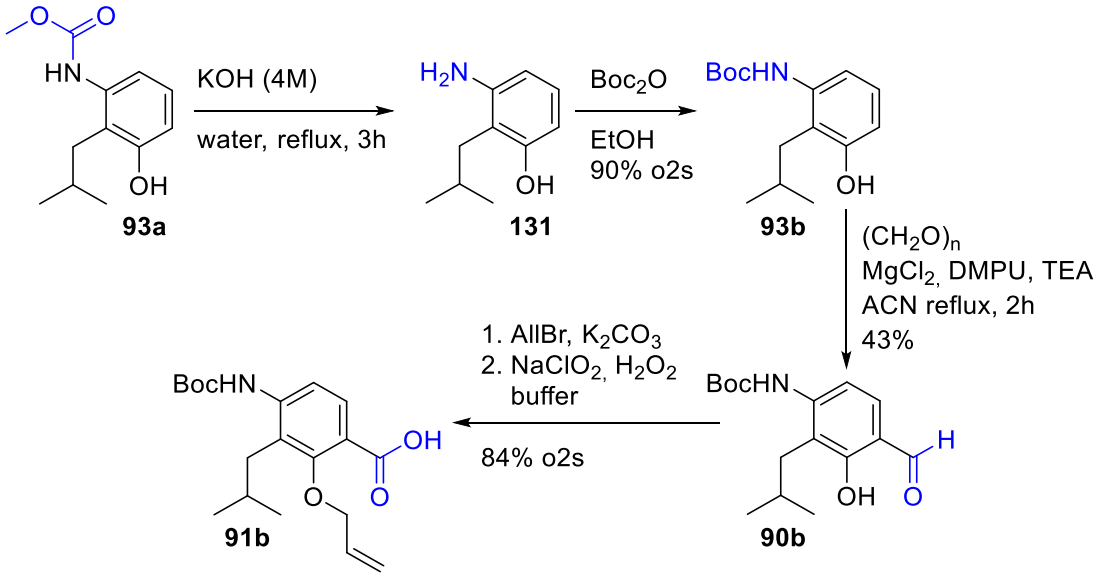


Figure 65: Final steps towards the isobutyl-analogue for ring D.

The hydroxy function was again protected with allyl bromide, followed by aldehyde oxidation. The usual scavenger 2-Me-2-butene for the Pinnick oxidation produced unseparable by-products during the oxidation, that interfered with the subsequent amide coupling. As an improvement, this scavenger was replaced by hydrogen peroxide, which produced no organic by-products. The carboxylic acid obtained **91b** was coupled to ring E. To allow orthogonal deprotection of the amino group on ring D, the terminal carboxylic acid was allyl protected this time. A convenient side-effect was that the final deprotecting only involved one step. The assembly of the CDE-fragment was performed via the previously discussed method (retrosynthetic path 2, see Figure 66). The final cystobactamid **008** was obtained from this CDE-fragment in a yield of 54% over two steps.

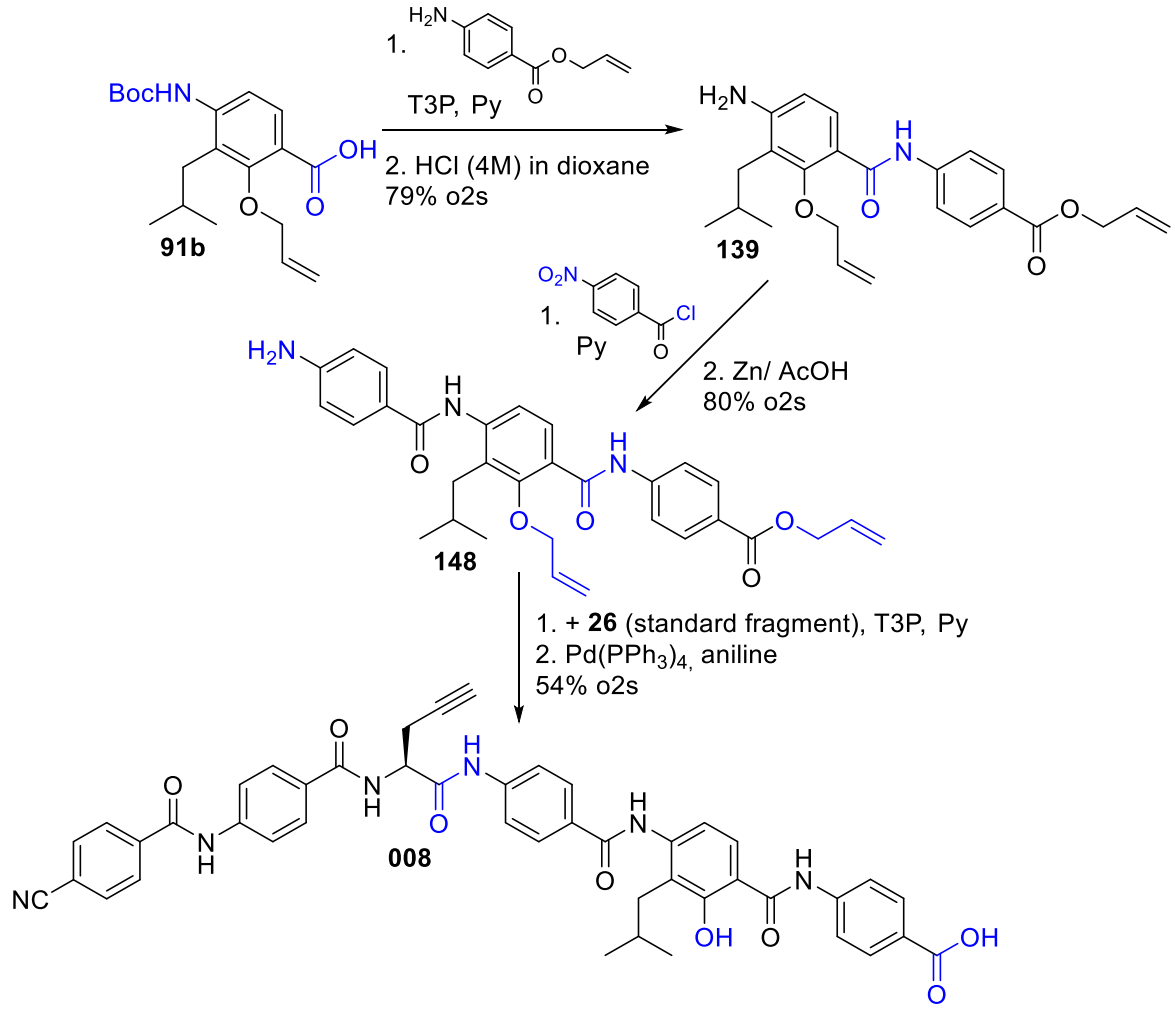


Figure 66: Assembly of the isobutyl cystobactamid **008**.

3.2.2 Synthesis of amide isosteres attached to ring D

In addition to the substitution of the ring D core, the SAR-investigation was extended to variations in the adjacent amide bonds. The following target molecules were proposed (Figure 67).

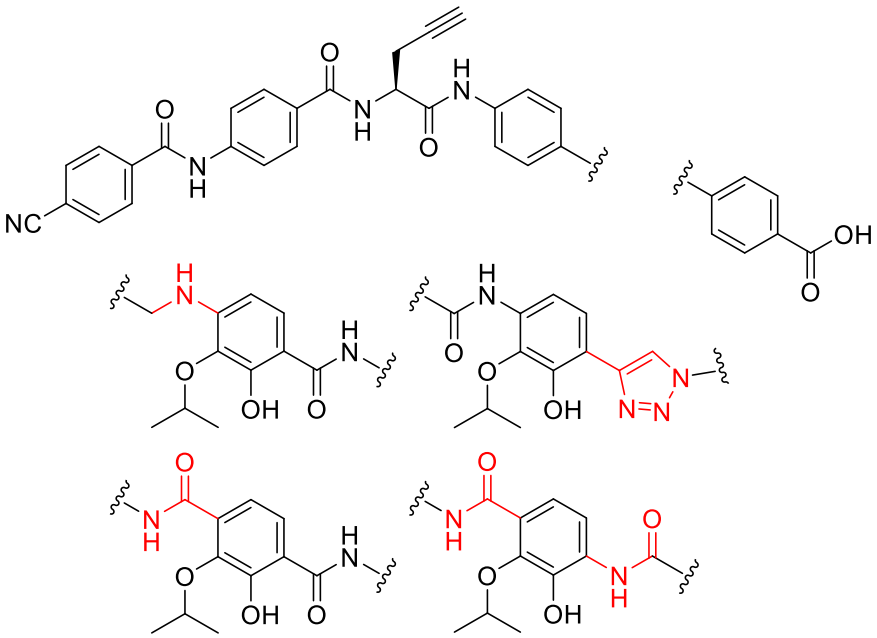


Figure 67: Suggested cystobactamid derivatives containing amide isosters at ring D.

Benzylamine derivative

A benzylamine function was chosen as isosteric replacement for the CD-linker (see also section 1.3.1). This substitution enlarges the conformational space of the CD-linker. This function would be introduced in a reductive amination between 4-nitrobenzaldehyde and the amino group of the DE-fragment **8,** using sodium triacetoxyborohydride as the reductant. The latter is a mild reduction reagent and was prepared *in situ* by adding 3 equivalents of acetic acid to sodium borohydride. The coupling proceeded slowly, even after repeated addition of the reagent. A major problem seemed to be that the premature inactivation of 4-nitrobenzaldehyde by reduction of the aldehyde function was still faster than the intended reductive amination. Attempts to form the intermediate imine first, removing the generated water through molecular sieves, also failed. It was assumed that steric hindrance by the isopropoxy group decreased the reaction rate. The conversion was finally increased to an acceptable amount by multiple workup of the reaction solution, isolation of the educt/product-mixture and renewed addition of reagents. The purification was attempted by column chromatography, but the product was obtained as a mixture. Next, a Boc-protection of this mixture was tried using Boc_2O and DMAP as

catalyst, followed by nitro reduction by the method discribed previously. The CDE-fragment **96** was finally obtained in 9.2% yield over 3 steps (see Figure 68).

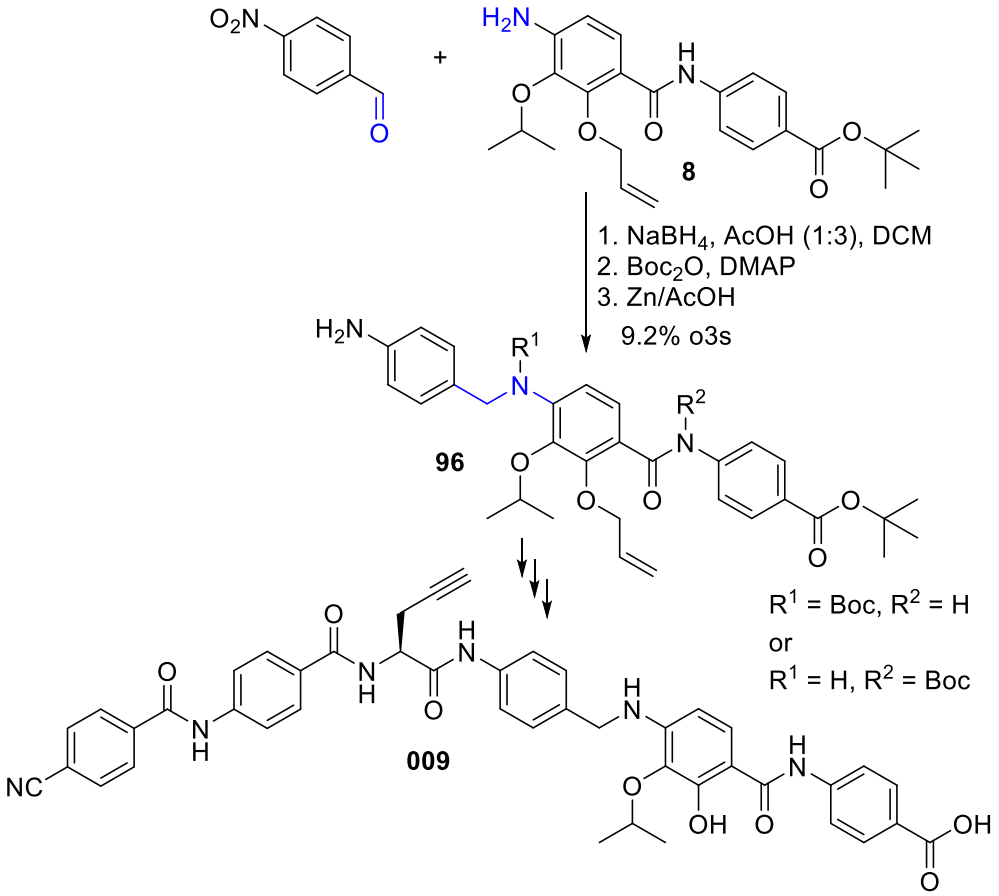


Figure 68: Synthesis of the benzylamine CDE-fragment **96** and structure of the final cystobactamid **009**. The exact position of the Boc protection was not verified.

However, it is assumed that the Boc protection was not necessary, because the secondary amine itself is blocked sterically by benzyl and phenyl substituents as well as the isopropoxy residue of ring D. Therefore, substitution should be in favour to the free amino group. The full cystobactamid **009** was obtained from the CDE-fragment **96** via retrosynthetic path 2 in 9.5% yield over 3 steps.

Terephthalic acid derivative

As another CD-linker modification, the amide bond was inverted (so called reverse amide) by the incorporation of a terephthalic acid analogue of ring D **97**. This analogue was provided by D. Kohnhäuser and synthesized from 2,3-dihydroxyterephthalic acid via formation of the dimethylester, Mitsunobu-reaction with isopropanol and selective hydrolysis according to Figure 69.[122]

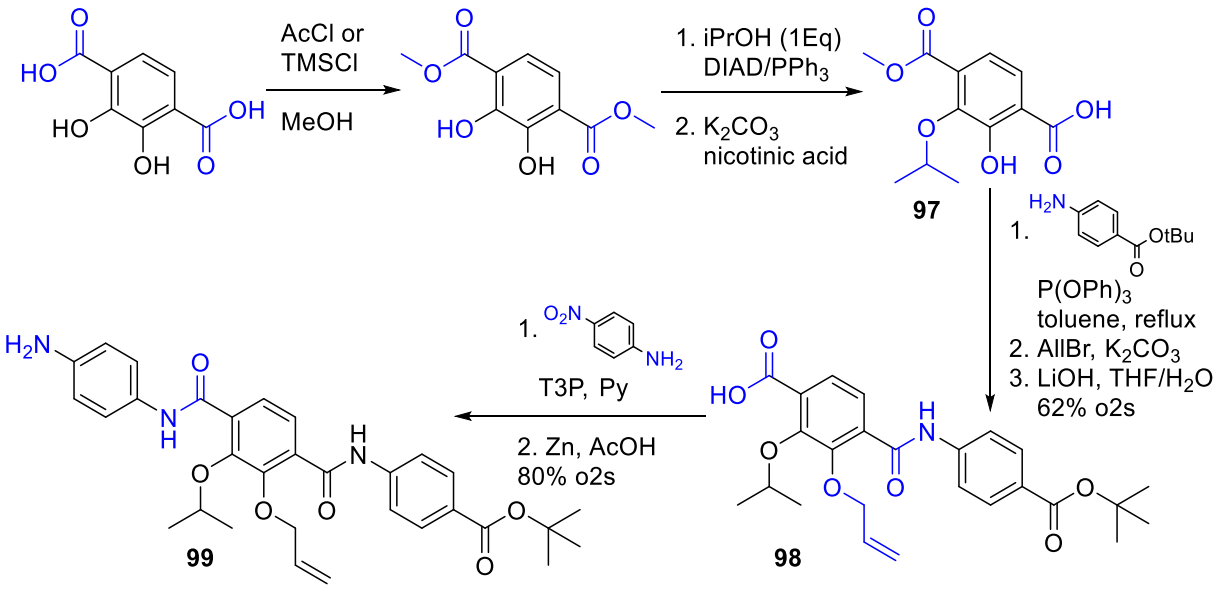


Figure 69: Synthesis of the CDE-fragment **99** showing a terephthalic acid structure for ring D. Monocarboxylic acid **97** was provided by D. Kohnhäuser[122]. The synthesis of **99** from **97** was performed in this Thesis.

Therefore a monoprotected dicarboxylic acid **97** was obtained which allowed amide coupling with *tert*-butyl 4-aminobenzoate. This coupling was achieved with selectivity for the amine over the hydroxy group by using triphenylphosphite as activation reagent in refluxed toluene to afford **98**. The hydroxy group was then protected as an allyl ether, followed by hydrolysis of the methyl ester. Amide coupling with 4-nitroaniline and the subsequent reduction of the nitro group progressed with high conversion, however the CDE-fragment **99** turned out to be a sensitive compound. This sensitivity was evident from a change in the color of the material and the observation of by-products on the TLC. Because the storage of the material under argon and in a light protected flask could prevent this, an oxidation reaction was considered to be the most likely reason for the by-product formation. Highly electron rich diaminobenzenes are known to be easily oxidizable to diimines, which react further to form oligomeric structures.[203]

The so obtained CDE-fragment **99** was first assembled with the standard Western fragment to give compound **010**. After a high potency of this compound was measured in MIC-assays, this fragment was also combined with the new scaffold as seen for the pyridine and methoxy derivative. A regioisomer of this new scaffold was included as well, carrying the nitrile function at the 6-position of the benzimidazole (see Figure 70).

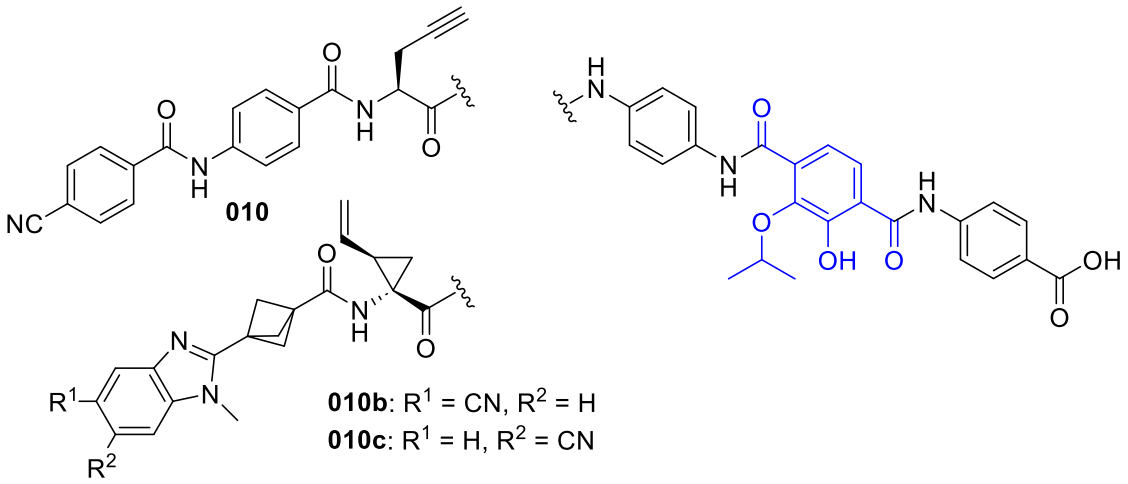


Figure 70: Synthesized cystobactamids **010**, **010b** and **010c**, showing the terepthalic acid modification at ring D.

Triazole derivative

As a next point, isosteres for the amide function between ring D and E were investigated. A 1,2,3-triazol as isostere for this DE-linker was considered as useful for two reasons: First, the angle between the major axes of ring D and E would be fixed at ~144°, mimicking a *trans*-conformation of the amide bond. Second, an H-bond would still be possible between the phenolic group and one triazole nitrogen (see Figure 71).

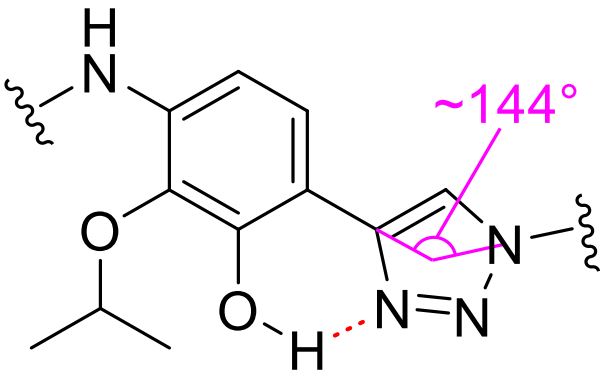


Figure 71: Features of a 1,2,3-triazol as DE-linker. The hydroxy function can still act as an HBD for one triazole nitrogen.

1,2,3-Triazoles are usually accessible from the copper-catalyzed azide-alkyne cycloaddition (CuAAC). This reaction was intended to take place between the azide function of 4-azidobenzoic acid and an alkyne analogue of ring D **100**. This ring D analogue was considered accessible by Sonogashira coupling between a corresponding aryl bromide **101** with ethynyltrimethylsilane (see Figure 72). Therefore **101** was synthesized via a known procedure[11] to investigate this strategy. The following Sonogashira reaction was adapted from literature[204], producing the protected alkyne **102** in good yield. The TMS and acetyl groups were cleaved under alkaline conditions.

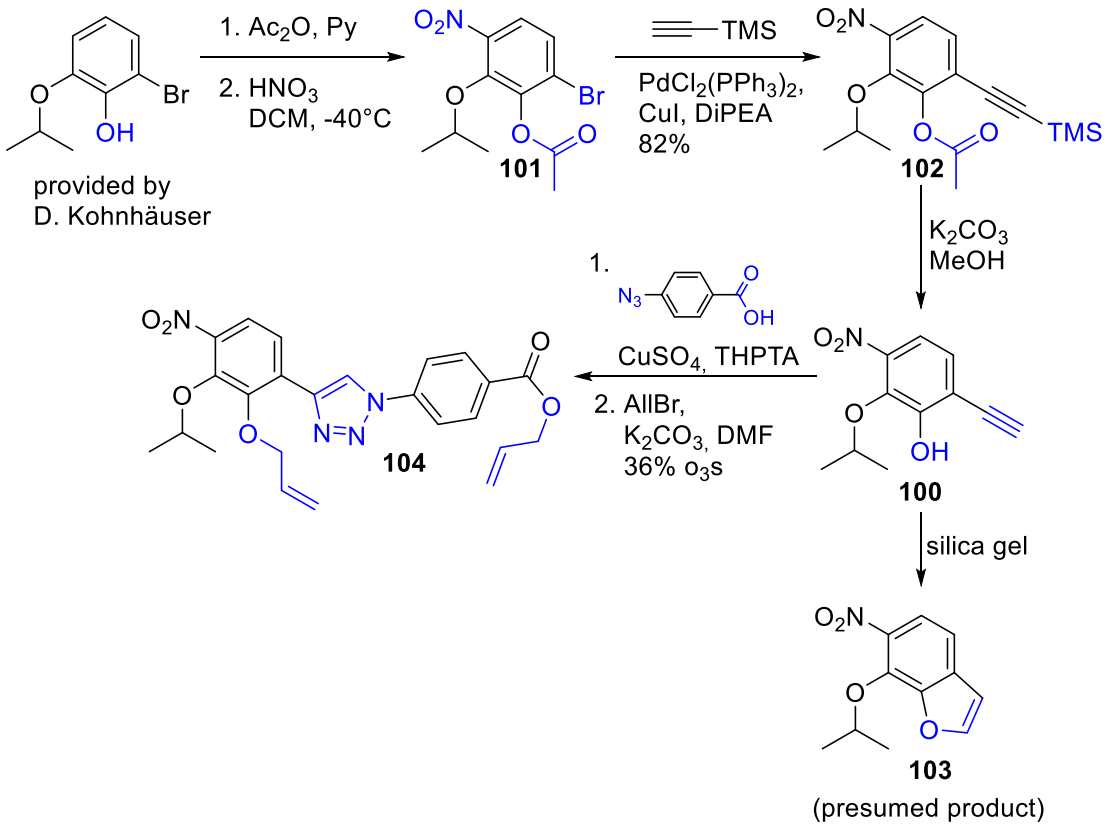


Figure 72: Synthesis of a DE-fagment **104** carrying the triazole moiety.

The isolation of the resulting alkyne **100** was first attempted, but led to cyclization, presumably forming a benzofurane **103**. Therefore the following CuAAC between **100** and 4-azidobenzoic acid was performed without further purification of **100**. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)[205] was used as a complexing reagent for copper, as the reaction did not take place without THPTA. After allyl protection of both the phenolic hydroxy group and the carboxylic acid, compound **104** was obtained in 3 steps in an acceptable yield of 36% over three steps. The full cystobactamid **011** (structure see Figure 73) was then assembled via the previously discussed method (retrosynthetic path 2).

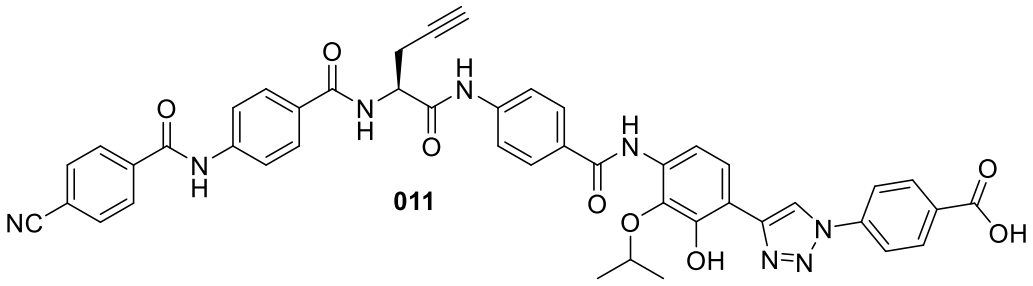


Figure 73: Structure of the triazol cystobactamid (**011**).

Derivative with switched amide bonds at ring D

The inversion of an amide bond (reverse amide) is a common method for introducing an isosteric group for amides.[137] In the case of the terephthalic acid derivative mentioned before, the amide between ring C and D had already been inverted. Since the inversion of only the DE-linker would lead to an aromatic system with four +M-substituents, a high oxidation sensitivity was assumed for such a compound. This would firstly hinder its synthesis due to undesired side reactions and secondly provoke its metabolic oxidation to a Michael-acceptor system, which are known toxicophores[11]. Thereby, it was proposed that both amide bonds at ring D should be inverted to obtain structure **105** (see Figure 74).

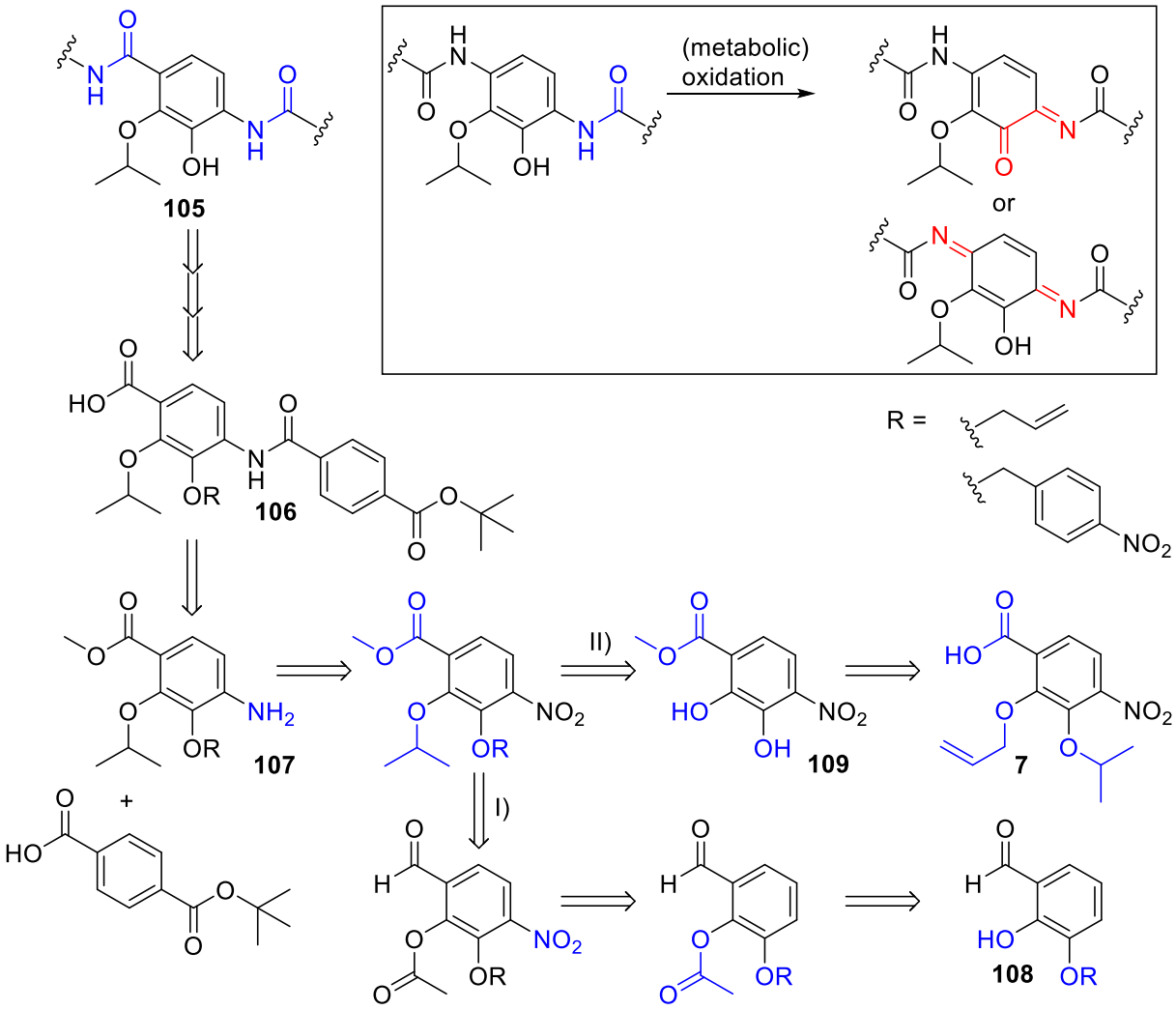


Figure 74: Possible oxidation of a ring D analogue with inverted DE-linker (box, top right). Retrosynthetic strategies towards a ring D derivative **105** showing double amide bond inversion.

Similar to the terephthalic acid derivative, the DE-fragment **106** would be synthesized first, bearing a free carboxylic acid function. Therefore an inverted ring D analogue **107**

would be required. For the synthesis of this analogue two strategies were proposed: Strategy I was similar to the known standard ring D synthesis, starting with 2,3-dihydroxybenzaldehyde with a protected 3-hydroxy group **108**. Therefore, a protecting group is required that is stable against nitration reagents while maintaining the +M effect of the 3-hydroxy function. The 2-hydroxy function would be acetylated to reduce its directing effect. After selective nitration, aldehyde oxidation and methyl ester formation, the nitro group would be reduced to obtain **107**. Strategy II started from the standard ring D (compound **7**) and after complete removal of the ether functions and methyl-protection of the carboxylic acid function, **109** would be obtained. For the next step, a higher acidity of the hydroxy function in *ortho*-position to the nitro group was assumed and would be exploited to selectively protect this hydroxy function with a Mitsunobu reaction. The isopropyl group would then be introduced in a second Mitsunobu-reaction. The nitro reduction of the resulting compound would then provide **107**.

Pursuing strategy I, the allyl group was introduced by a Williamson ether synthesis protocol[107], followed by acetylation with acetic anhydride in pyridine to form **110**. The nitration of this compound at -40°C in DCM delivered a complex reaction mixture that may have occured from an oxidation of the allylic group. As already mentioned in the introduction, the nitration of a similar allyloxy substituted benzene was published for the synthesis of Albicidins as well. However, the yield was also low here, indicating similar side reactions.[111] Therefore 4-nitrobenzyl was investigated next as protecting group. This group was considered a more stable alternative to a benzyl protecting group with regard to nitration, as it bears an electron deficient benzene ring which should not be nitrated as a result. However, introduction of this group by Williamson ether synthesis was not possible and showed several side reactions. Alternatively, the 2-hydroxy group was first selectively allyl-protected using only one equivalent of NaH, and then the 4-nitrobenzyl group was introduced in the 3-position with K_2CO_3 as a base in MeCN. The allyl group was subsequently cleaved by using a Riley oxidation procedure[206] and compound **111** was obtained after acetylation. However, nitration also failed here, showing no reaction at -40°C in DCM and several side reactions at higher temperatures. Changing the solvent to the more polar MeCN was also not successful here. The attempted syntheses are shown in Figure 75 (top).

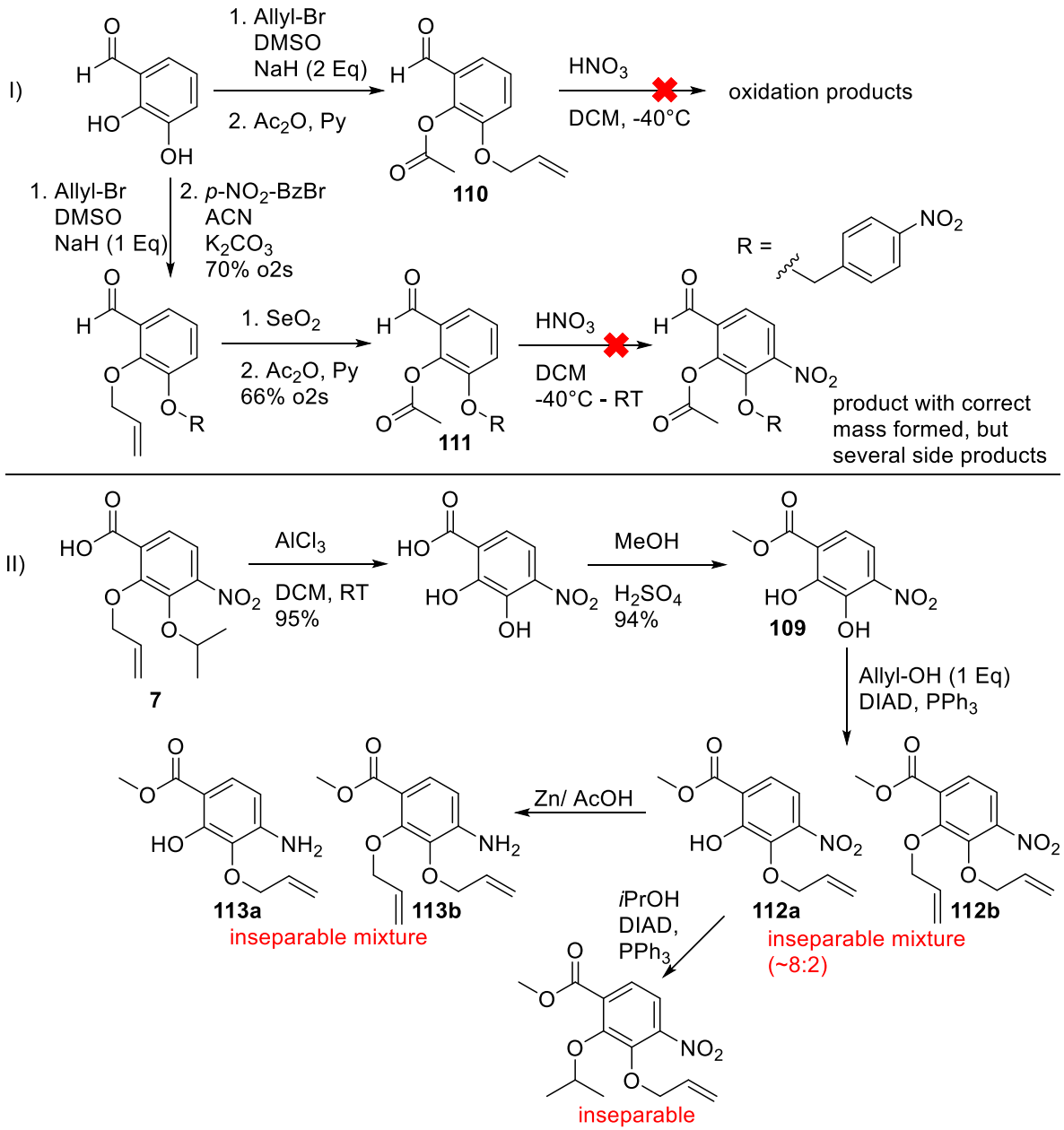


Figure 75: Attempts for the synthesis of a ring D derivative with doubly inverted amide bonds.

As larger amounts of the ring D standard fragment were available, strategy II was tried instead (Figure 75, bottom), which starts with the cleavage of the two ether substituents using $AlCl_3$ as a strong Lewis acid. After successful cleavage, the carboxylic acid was again methylated by Fischer esterification to form **109** in 94% yield. The Mitsunobu reaction was conducted by first adding allyl alcohol and then isopropanol, assuming selectivity for the hydroxy group in *ortho*-position to the nitro group. Clear regioselectivity for one monoallyl ether was observed after chromatographic separation but this monoallyl ether was in mixture with the diallylether. The separation of the regioisomers, which were supposed to be **112a** and **112b**, was not possible by normal phase chromatography. After proceeding with the nitro reduction to the coresponding amines **113a** and **113b**, the

separation could not be achieved either. One possible solution would be here, to isolate the monoallyl ether **112a** first by acid-base extraction, as the phenolate should be water soluble. After isolation of **112a** the isopropoxylation would selectively give the desired compound. The initial allylation of **109** with one equivalent of allyl bromide is considered as an alternative to the Mitsunobu reaction. However, due to the high electrophilicity and therefore low selectivity of allyl bromide, low regioselectivity towards the two hydroxy groups is expected here. In conclusion, further investigations into strategy II were not carried out in this case, as the use of the synthetically demanding standard ring D fragment as a starting point was considered uneconomical. The synthesis of the switched amide derivative was not pursued further.

3.2.3 SAR study of ring D

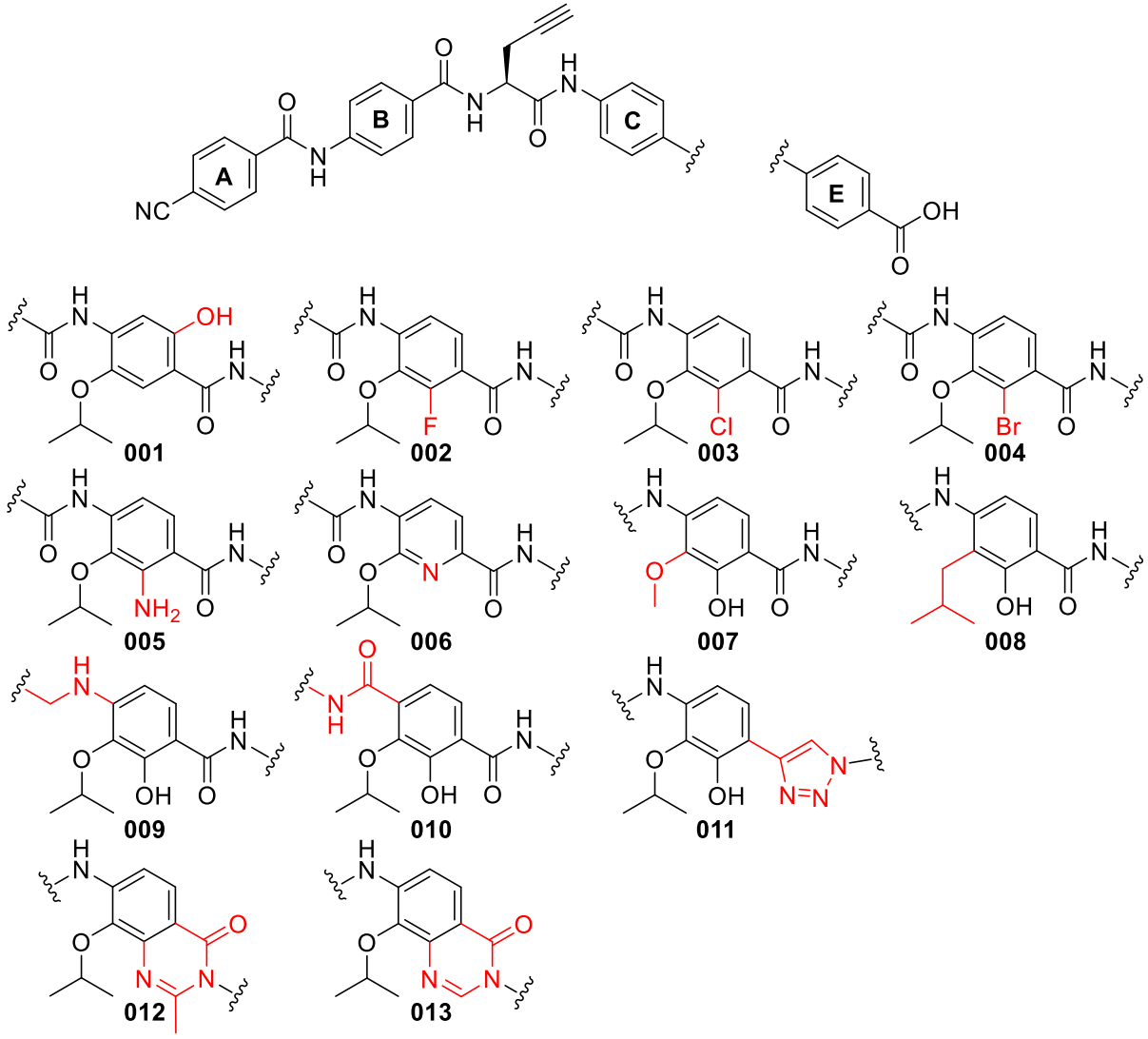


Figure 76: Overview of ring D analogues synthesized in this Thesis.

MIC evaluation

For the following SAR investigations on ring D, all substituents, including the two attached amide linkers, had to be considered, as the latter can act as intramolecular HBA and HBD with other ring D substituents. As a metric for activity, MICs were measured and are divided in two parts: The MICs for compounds with a substitute for the hydroxy-group are listed in Table 5 and the MICs for compounds with substitutes for the isopropoxy- and amide substituents later in Table 6. Measurements were conducted by Janetta Coetzee (Helmholtz-Institut für Pharmazeutische Forschung Saarland, HIPS) and technical employees at HIPS. The full tables are found in the supporting material.

Table 5: MIC-values (in µg/mL) for ring D analogues with modifications of the hydroxy group.

	Ref CN-CC 861	Hydro-quinone derivative **001**	Fluoro der. **002**	Chloro der. **003**	Bromo der. **004**	Amino der. **005**	Pyridine der. **006**
A. baumannii DSM-30008 (AR)	0.02-0.03	1	0.06	>8	>8	n.d	n.d.
A. baumannii ATCC BAA-1710 (FQR)	0.06-0.25	>64	>8	>8	>8	>64	>64
A. baumannii CIP-105742 (AR)	0.02	2	0.25-0.5	>8	>8	n.d	n.d.
A. baumannii CIP-107292 (FQR, CR)	0.5-1	>64	>8	>8	>8	>64	8
A. baumannii R835 (FQR)	1	>64	>8	>8	>8	>64	>64
E. coli ATCC-25922	0.03-0.06	1	4	>64	>64	0.005	≤0.03
E. coli LM705 (S83L, D87N, S80I, ΔmarR, ΔacrR) (FQR, Gyr, EFL+)	<0.03-0.2	>64	>64	>64	>64	>64	>64
S. pneumoniae DSM-11865 (FQR, AR)	0.125	>64	>64	>64	>64	0.2	n.d.
E. faecium DSM-17050 (VR)	<0.03	>64	4	>64	>64	>64	0.25
S. aureus ATCC-29213	0.02	>64	>64	>64	>64	>64	>64
K. pneumoniae CIP-104298 (AR)	8	>64	>64	>64	>64	n.d.	n.d.
K. pneumoniae KP10581 (waaC::Tn30) (FQR)	0.06-0.25	>64	>64	>64	>64	>64	>64
K. pneumoniae R1525 (QnrA1) (FQR)	16- >64	>64	>64	>64	>64	>64	>64
P. aeruginosa PA14	0.5-16	>64	>64	>64	>64	n.d.	n.d.
P. aeruginosa PA14ΔmexAB (EFL-)	0.03-0.5	>64	>64	>64	>64	n.d.	n.d.
E. cloacae ATCC BAA-2468 (CR)	0.06-0.5	>64	>64	>64	>64	>64	>64
E. aerogenes CIP 106754 (AR)	0.125-4	>64	>64	>64	>64	>64	>64

Reference strains are marked in blue. FQR = fluroquinolone resistant, AR = ampicillin- or amoxicillin-resistant, CR = resistant against some carbapenems, Gyr = gyrase mutant associated with FQ-resistance, EFL+/- = increased or decreased efflux, VR = vancomycin resistant.

At first sight it can be seen that modifying the hydroxy group is a very risky business. Switching the position of hydroxy in *para* to the isopropoxy group led to a near complete loss of activity. Residual activity was observed here for *E. coli* reference strain and non-fluoroquinolone-resistant *A. baumannii* strains. The substitution of the hydroxy function with a fluoro substituent showed nearly the same pattern of activity, while the substitutions with chloro and bromo substituents removed activity across all tested strains. In general, such a complete loss of activity through all different bacterial species seemed to be common for those compounds, which beared no HBD in *ortho* to the DE-amide linker. However, substituting an amino group for the hydroxyl group also led to a loss of activity. From this it can be concluded that the presence of an HBD alone is not sufficient, but that the specific H-bond pattern of a phenolic hydroxy group is decisive

here. There is also no improvement with the pyridine derivative, which underlines this theory. It is remarkable that the single substitution of the hydroxy group affects so many different bacterial species simultaneously. This may be a hint to a highly conserved binding site. However, if the assumed mechanism of gyrase inhibition[89] as determined from the cryo-EM structures is accurate, there are no H-bond interactions of the hydroxy group at least with the gyrase binding site. To be more precise, the distance between the phenolic hydroxy group and the nearby α-helices of the gyrase subunits is too high.

However, the comparison of IC_{50}-values for one cystobactamid on different organisms (*E. coli* and *A. baumannii*) showed varying inhibition for gyrase and topoisomerase IV. This shows that inhibition of these enzymes may not correlate with antibacterial activity.[122] Taken this into account, one can assume that there may be other essential interactions of the cystobactamids with unknown targets (bacterial membrane, membrane pores, transporters, etc.) in the bacterial cell. Nevertheless, the main question is, which conformation of the cystobactamid is the one relevant for activity. The type of intramolecular H-bonding has a great influence on the stabilization of a conformation, and especially in ring D several such H-bonds are possible.

Figure 77 shows the possible intramolecular H-bond interactions for the tested derivatives with replacements for the hydroxy function. These interactions can stabilize different conformations of the amide bonds to each other, namely antiperiplanar (*a-d* and *d-a*) and synperiplanar (*a-a* and *d-d*) conformations (see section 3.2 for the nomenclature).

In all these cases the CD-linker can be oriented in such a way that H-bonding with the isopropoxy function is possible. This results in the stabilization of the *d-d* and *d-a* conformations for the standard ring D derivative (Figure 77, first two structures). An H-bond interaction of the hydroxy group with the isopropoxy function is only possible here if the isopropoxy function is oriented in such a way that its oxygen electron pairs point in the direction of the hydroxy proton (see 3D structure). Due to the assumed tetrahedral geometry of the isopropoxy oxygen, this results in a rotation of the alkyl chain out of the aromatic plane. This allows the adjacent amide group to adopt such a conformation that its proton is able to form an H-bond with the isopropoxy oxygen.

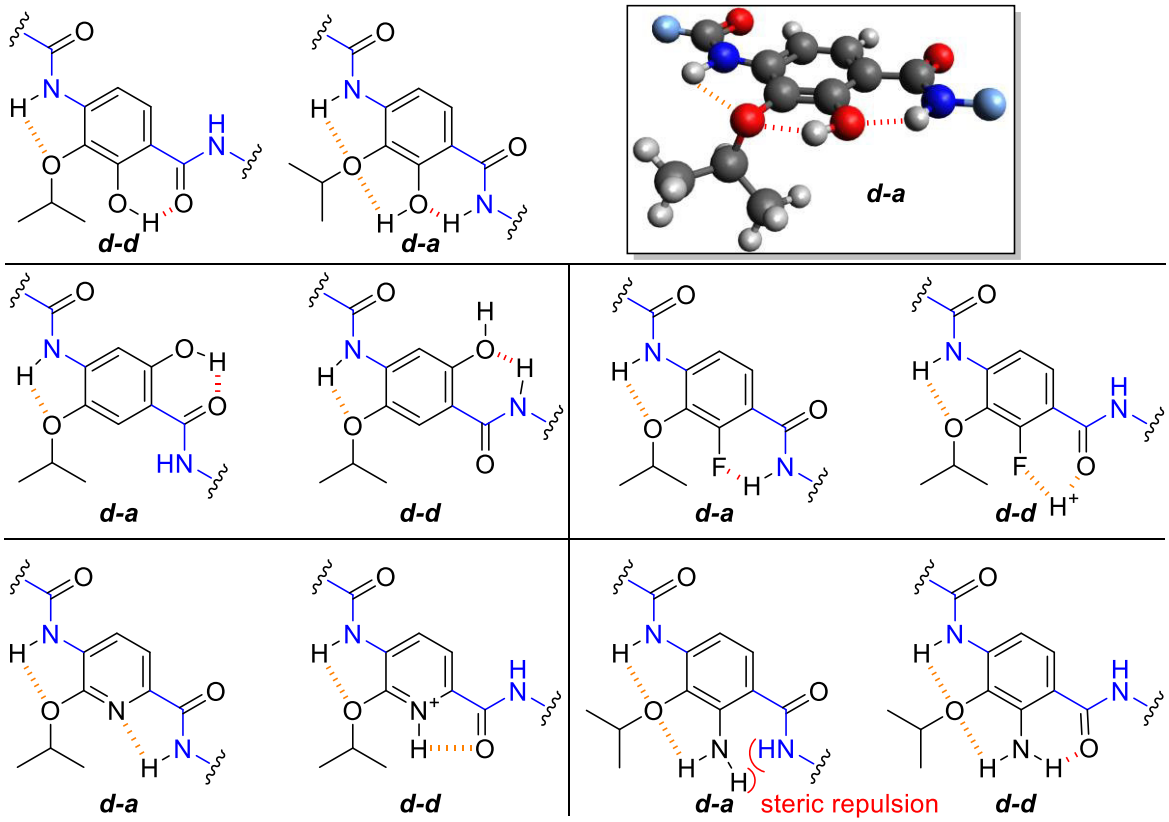


Figure 77: Possible H-bonds (dashed) and resulting possible conformations (with namings) for standard ring D (top, with the most likely 3D-structure boxed, made with *Avogadro*), for the hydroquinone derivative (middle left), the fluoro derivative (middle right), the pyridine derivative (bottom left) and the amino derivative (bottom right). Presumably weak H-bonds are highlighted in orange in contrast to red. The amide 'backbone' is highlighted in blue.

The mentioned Cryo-EM structures of the related Albicidins in complex with gyrase display this antiperiplanar *d-a* conformation, however here the CD- and DE-amide linkers are not strictly in the aromatic plane and are twisted up to ~30°.[89]

In the hydroquinone derivative, the isopropoxy and hydroxy groups show the same H-bonding with the adjacent amide groups, but the resulting conformations are reversed to what was seen for the standard ring D. The *d-a* conformation from before now corresponds to the d-d conformation in the hydroquinone derivative. Assuming that the *d-a* conformation is indeed the activity-relevant conformation of the standard cystobactamid, the low activity of the hydroquinone derivative may therefore indicate that the likewise preferred *d-d* conformation here is precisely not the required activity-relevant conformation. Furthermore, there is no H-bond between the hydroxy group and the isopropoxy function in the hydroquinone derivative, which can additionally result in a lower conformer stabilization. However, the different molecular shape of the hydroquinone derivative may also be partly responsible for the lower activity.

It must be taken into account that the hydroxy group can also be present as a phenolate, which restricts the conformations according to Figure 78.

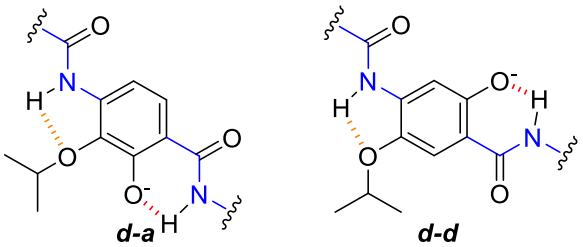


Figure 78: Possible H-bonds of the deprotonated standard ring D for comparison (left) and the deprotonated hydroquinone ring D analogue (right) for the most likely conformations.

In this state, the *d-d* conformation in the hydroquinone derivative would indeed be the most stabilized.

By replacing the hydroxy group with fluorine, the HBD is removed and with it a possible H-bond with the ethereal oxygen. An interaction of fluorine with the adjacent amide proton of the DE-linker is still possible (resulting in the *d-a* conformation) but is assumed to be weak[207]. Consequently, only this *d-a* conformation is possible, which is less stabilized here compared to the standard ring D. However, the carbon-fluorine bond is the most polarized bond compared to all other carbon-halogen bonds, and fluorine may attract protons or cations by ion-dipol interaction (usual energy range of ion-dipol interaction: 12-20 kJ/mol[208], of H-bond: 16-60 kJ/mol[207]). This would allow a proton bridge between fluorine and the adjacent amide-oxygen and therefore stabilization of this conformation to a lesser extend. Certainly, such an interaction is possible for the hydroxy group as well.

In the case of the pyridine derivative, the low activity is probably due to a greater distance between the aromatic nitrogen and the adjacent amide-proton, weakening the H-bond between them and leading to less stabilization of the *d-a* conformation. The five-membered ring formed in this way would also occur with a protonated pyridine, leading to the *d-d* conformation. However, none of these conformations may have such a strong stabilization as present in the standard ring D. The amino substituted ring D system, in turn, may adopt only the *d-d* conformation, since the *d-a* conformation will result in sterical clash between the amino function and the close amide-proton of the DE-linker. The geometry of the amino function in aniline is slightly pyramidalized and not planar.[209] The results for the amino derivative suggest that stabilizing the *d-d* conformation is not beneficial for activity.

For all shown replacements of the hydroxy group, the anion-forming moiety is no longer present. This means that at this position, ion-ion interactions with other molecules are no longer possible. Instead, the lipophilicity might be increased here.

The MIC values for the residual modifications at ring D are seen in Table 6 starting with modifications of the isopropoxy group. Here the substitution with a methoxy function led to a light decrease in activity for some *A. baumannii* and *S. aureus* strains as well as a significant activity loss against the Gram-negative *E. cloacae*.

Table 6: MIC-values (in µg/mL) for substitutions of the isopropoxy group at ring D as well as isosteres for the amide bonds attached to ring D. For the structures of the cystobactamids see Figure 76.

	Ref CN-CC 861	methoxy derivative **007**	isobutyl der. **008**	benzyl-amine der. **009**	tere-phthalic acid der. **010**	triazol der. **011**	methyl-oxoquina-zolin der. **012**	oxoquina-zolin der. **013**
A. baumannii DSM-30008 (AR)	0.02-0.03	n.d.	1	0.5	0.125	>8	8	1-2
A. baumannii ATCC BAA-1710 (FQR)	0.06-0.25	0.25	0.25	0.5	<0.03	>8	0.5-1	>8
A. baumannii CIP-105742 (AR)	0.02	n.d.	<0.03	0.25	<0.03	>8	4	2
A. baumannii CIP-107292 (FQR, CR)	0.5-1	4	>64	>8	0.06	>8	>8	>8
A. baumannii R835 (FQR)	1	4	>64	>8	0.5	>8	>8	>8
E. coli ATCC-25922	<0.03-0.2	<0.03	<0.03	1	<0.03	>64	4	1
E. coli LM705 (S83L, D87N, S80I, ΔmarR, ΔacrR) (FQR, Gyr, EFL+)	0.03-0.06	<0.03	>64	>64	0.06	>64	>64	>64
S. pneumoniae DSM-11865 (FQR, AR)	0.125	<0.03	>64	>64	<0.03	>64	>64	>64
E. faecium DSM-17050 *(VR)*	<0.03	n.d.	<0.03	0.25	<0.03	>64	4	1
S. aureus ATCC-29213	0.02	0.5	16-32	0.5-1	0.06	>64	4	8
K. pneumoniae CIP-104298 (AR)	8	n.d.	>64	>64	<0.03	>64	>64	>64
K. pneumoniae R1525 *(FQR)*	16- >64	32	>64	>64	<0.03	>64	>64	>64
E. cloacae ATCC BAA-2468 (CR)	0.06-0.5	16	>64	>64	0.25	>64	>64	>64
E. aerogenes CIP 106754 (AR)	0.125-4	4	>64	>64	1	>64	>64	>64

Reference strains are marked in blue. FQR = fluroquinolone resistant, AR = ampicillin- or amoxicillin-resistant, CR = resistant against some carbapenems, Gyr = gyrase mutant associated with FQ-resistance, EFL+/- = increased or decreased efflux, VR = vancomycin resistant.

The activity of the methoxy derivative against other strains was almost equal to that of the reference compound. In contrast to this, the substitution of the ethereal oxygen with a methylene group (isobutyl derivative) strongly decreased activity against several species, with residual activity against some *A. baumannii* strains, the *E. coli* reference

strain and a VRE strain. The benzylamine derivative exhibited nearly the same pattern of activity with better activity against the *S. aureus* reference strain.

In contrast to this, the terephthalic acid derivative showed exceptional activity against every tested strain that even exceeded the referenced cystobactamid. The triazol derivative, however, was completely inactive on the tested strains. The two oxaquinazoline derivatives do not differ much in their activity, which was however low overall. Again, residual activities can be observed for some non-fluoroquinolone resistant *A.baumannii* strains, the *E. coli* reference strain, as well as the gram-positive species *E. faecium* (VRE) and the *S. aureus* reference. Possible H-bond interactions are exemplified in Figure 79.

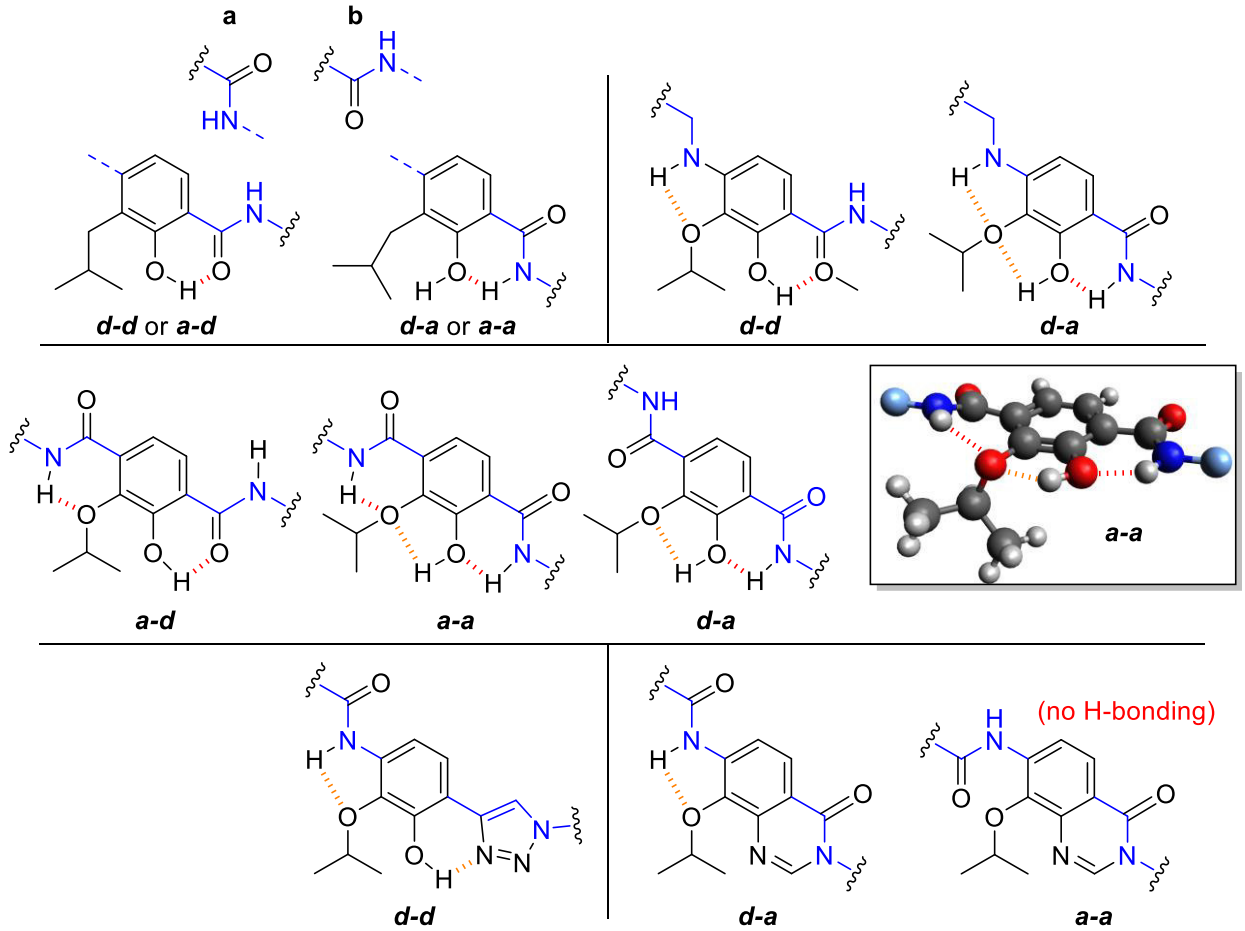


Figure 79: Relevant conformers and possible H-bonds (red dashed) in the isobutyl derivative (top left), the benzylamine derivative (top right), the terephthalic acid derivative (middle) with proposed 3D structure (middle right, made with *Avogadro),* the triazol derivative (bottom left) and the oxoquinazoline derivative (bottom right). Presumably weak H-bonds are highlighted in orange in contrast to red.

Apart from a lower bulkiness of the ether function compared to the standard ring D, the methoxy derivative has an identical H-bond pattern and is therefore not shown here. However, the isobutyl residue shows no interactions with the adjacent CD-linker or with

the hydroxy group and is solely a sterical factor. In principle, all periplanar conformers are possible here, although the *d*-*d* and *d*-*a*-conformers (**a**) should be preferred over the *a*-*a* and *a*-*d* conformers (**b**) to minimize sterical hindrance between the isobutyl residue and the oxygen of the CD-linker. Simultaneously, the H-bonding patterns of the hydroxy group with the adjacent DE-linker are the same as in standard ring D. It can be concluded that replacing the isopropoxy oxygen as HBA with a methylene group considerably enlarges the conformational space and possibly reduces target affinity, thus explaining the significant loss of activity.

The same argument can be applied to the benzylamine derivative. Because this derivative lacks conjugation of the CD-linker with the adjacent aromatic system of ring C, the rotational barrier is much lower compared to an amide. The substitution of the amide-carbonyl function with a less bulky methylene group enhances this effect. Therefore the conformational space is enlarged which eventually may result in lower target affinity. In addition, the basicity of the nitrogen in the benzylamine is higher than in the standard amide bond which can modulate the strength of H-bonding.

In the previous cystobactamids, the H-bond between the proton of the CD-linker and the isopropoxy oxygen is considered to be moderately strong between these moieties in the five-membered ring geometry (see Figure 80). This H-bond is expected to be stronger in the terephthalic acid derivative due to a closer proximity of these moieties. In this derivative the intramolecular H-bond is formed within a six-membered ring, while the standard ring D shows a five-membered ring. This H-bond stabilizes either the *a*-*d*- or the *a*-*a* conformation. A *d*-*a* conformation is also possible if there is no interaction between the CD-linker and the isopropoxy function. Here the *a*-*a* conformation and its proposed 3D-structure (see Figure 79) must be emphasized, since it features the highest number (3) of H-bonds of all conformers. This *a*-*a* conformation shows the same H-bonding pattern as the *d*-*a* conformation of the standard cystobactamid. However, both conformations lead to differing orientations of ring C relative to ring D. Assuming that the *a*-*a* conformation is indeed the one relevant for the higher activity of the terephthalic acid derivative, the high activity may even be enhanced by appropriate further rigidification of the CD-linker with ring D. Admittedly, the *a*-*a* conformation was not observed in cryo-EM data of the Albicidin-gyrase-DNA complex[89] or in a conformational analysis of the

cystobactamid CDE-fragment[109]. The next step would therefore be to determine the cryo-EM structure of the terephthalic acid derivative in the complex.

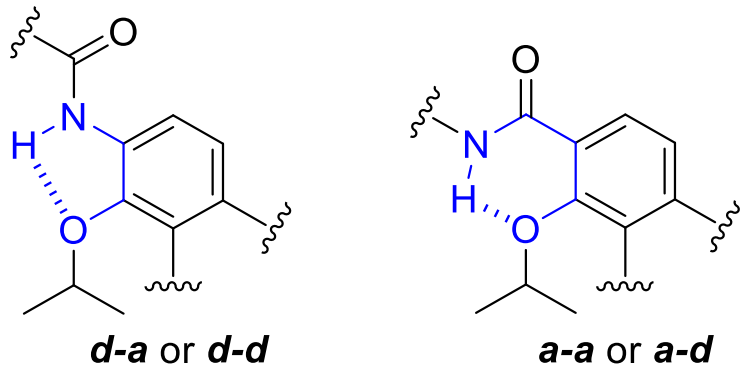


Figure 80: H-bonding between the proton of the CD-linker and the adjacent isopropoxy oxygen with a five-membered cycle for the standard ring D (left) and a six-membered cycle for the terephthalic acid derivative (right).

Another aspect is that the aromatic system on ring D is more electron-deficient than in the standard cystobactamid. This in turn may substantially lower the pK_A of the phenol group and thus increase the degree of deprotonation at physiological pH.

For the triazol derivative, there is only one relevant H-bond interaction between the hydroxy group as HBD and the triazole ring as HBA. This H-bond is assumed to be relatively weak because the distance between the hydroxy function and the triazol nitrogen is relatively large. Additionally, the central axis of ring E is not parallel to the central axis of ring D with an intersection angle of ~36°, in deviation from the standard cystobactamid. This results in a different orientation of the terminal carboxylic acid moiety and thus possibly to an insufficient interaction with the target.

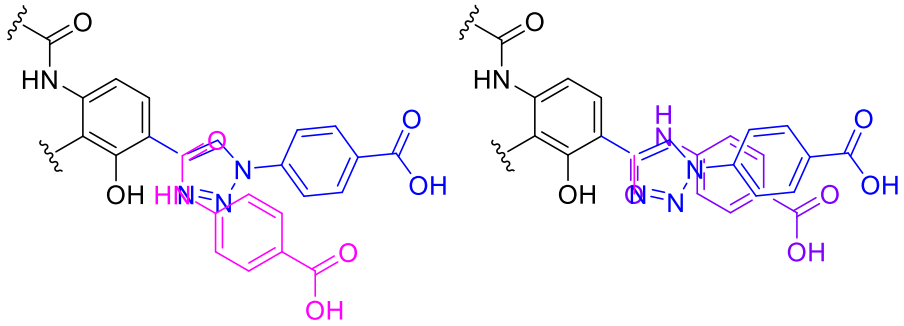


Figure 81: Overlay of the assumed conformation of the triazol substituted DE-fragment (blue) with the *d-a*-(pink, left), and the *d-d* conformer (purple, right) of the standard DE-fragment. Both standard DE-conformers show a crucially different orientation of the terminal carboxylic acid than the triazol DE-fragment.

The rigidification between rings D and E by introducing the oxoquinazoline replaced the hydroxy group (HBD and HBA) with a nitrogen substituent (only HBA). There is only one

H-bond possible between the proton of the CD-linker and the oxygen of the isopropoxy function. Together with the rigidification, only the *d*-*a*-conformation will be considerably stabilized. However, with the previous argumentation, one should expect an active compound with this modification. The absence of the hydroxy group itself could be the reason why this is not the case here. Analogous to the amino derivative from before, the nitrogen substituent as replacement for the hydroxy group bears only one free electron pair. The orientation of this electron pair will differ from that seen in standard ring D and might lead to an unfavourable rotation of the isopropoxy residue.

One other assumption is that besides the gyrase inhibition there might exist other essential interactions of the cystobactamids within the bacterial cell, e.g. with transporter proteins. Since salicylamides have been found to complex metal ions and act as iron accumulators (Siderophores) in bacteria[210-211], cystobactamids with this moiety could act as such a metal complex and therefore be more easily taken up by bacteria. Another explanation for the low activity is that due to the coplanarity of the DE-linker with ring D, the conformational space might be so restricted that there is no room left for intermediate conformations, which might be necessary for the successful cystobactamid-gyrase binding.

From the analysis of all intramolecular H-bond interactions in relation to the observed MIC activity, some trends can be identified. For the discussed compounds except for the terephthalic acid derivative, the *d*-*a*-conformation may represent the activity-relevant conformation. Only in this conformation, a sterical hindrance between the CD-amide oxygen and the isopropoxy-oxygen is avoided and an H-bond of the hydroxy group with both the isopropoxy residue and the adjacent amide-proton is possible. In the terephthalic acid derivative, the *a*-*a* conformation features the equivalent H-bond pattern. Taking into account the results seen for substitutions of the hydroxy group, it seems reasonable that the latter can not be exchanged by other moieties with similar H-bonds. In the recent structural study of the Albicidins[89], it is also assumed that the hydroxy group is mainly important for intramolecular H-bonding when only gyrase inhibition is considered. However, it is still possible that the hydroxy group interacts with unidentified biomolecules on the pathway of the cystobactamid from outside the bacterium to the bacterial gyrase.

With respect to the benzyl amine and isobutyl derivatives, free rotation of the CD-amide linker is not beneficial and should be avoided in future compounds. The oxygen in the ether function is an important HBA and stabilizes the coplanarity of ring C and D. This stabilization seems to be increased by switching the CD-amide and thereby allowing stronger H-bonding.

In order to be able to make a final statement about the prevailing activity-relevant conformation in the CDE-fragment, further investigations should include the determination of IC_{50} values on the individual target enzymes of each pathogen, as they are structurally different. Alternatively, energies of the entire ligand-target-DNA complex can be calculated, which can be difficult due to the numerous effects that must be taken into account for such a large complex. Finally, a synthetically very demanding complete rigidification of all H-bonds involved at ring D could also confirm that the specified conformations are the activity-relevant conformations.

pK_A determination

In all these models, it was assumed that the phenolic hydroxy group remains protonated. However, when a phenolate is formed under physiological conditions, the electron density in the aromatic system increases, and ionic and various H-bond interactions occur intra- and intermolecularly. To predict the ionic state under physiological conditions, the pK_A for the phenolic hydroxy group was determined. For this purpose, a titration curve was recorded using a reference cystobactamid (DK 444, provided by D. Kohnhäuser) with increased solubility in water. A pK_A of 7.1 was determined, details are further explained in section 3.4.3. From this data, it can be assumed that at physiological pH of ~7.4, the phenol function can adopt both protonated and deprotonated forms. However, unexpected protonation states can also be present at the cystobactamid binding site in any enzymes.

3.2.4 Synthesis of ring E derivatives

Isosteres for the terminal carboxylic acid have been studied to a lesser extend in previous SAR investigations (see section 1.2.5).[9, 99, 122-124] It seemed reasonable to extend the knowledge about the SAR of this functional group in order to clarify the question which electronic properties are tolerated at all. In order to specify these properties, the effect of acidity and the type of H-bond pattern of the terminal group were investigated. Thus, a benzyl alcohol function and a nitro group were selected as isosteric groups for the carboxylic acid.

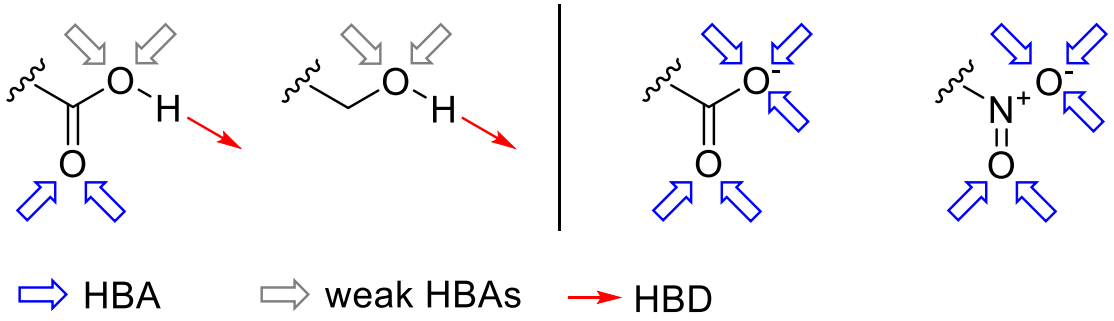


Figure 82: H-bonding patterns of carboxylic acid and carboxylate compared with benzyl alcohol and nitro groups, respectively.

By comparing activities of the cystobactamids with these isosteric groups, it was expected to obtain informations on the H-bond pattern responsible for activity. The *C*-terminal carboxylate may also enter into ionic interactions with a target molecule. The activity of a benzyl alcohol derivative lacking a carboxylic oxo function as a strong HBA would allow statements about the importance of such a strong HBA.[142] In turn, the nitro group as an isoster for the carboxylic acid is isoelectronic to the carboxylate (see Figure 82), so it is an HBA but not an HBD[212], and it is always neutral. Therefore, the importance of a charged group here was investigated. Such a charged group may also enable ion-ion interactions with other molecules. The nitro group is rarely used as an isosteric group for a carboxylic acid owing to its bad reputation as a toxicophore[121] so this derivative would primarily be used in activity tests to obtain SAR insights.

Benzyl alcohol derivative

As a first point, a benzyl alcohol function was tested as a substitute for the carboxylic acid. This drastically increased the pK_A, with the result, that the *C*-terminal group cannot form an anion at a physiological pH of 7.4. The corresponding ring E analogue **114** was

synthesized starting from commercially available *p*-nitrobenzyl alcohol, that was first protected with a trityl group and then the nitro group was reduced (see Figure 83).

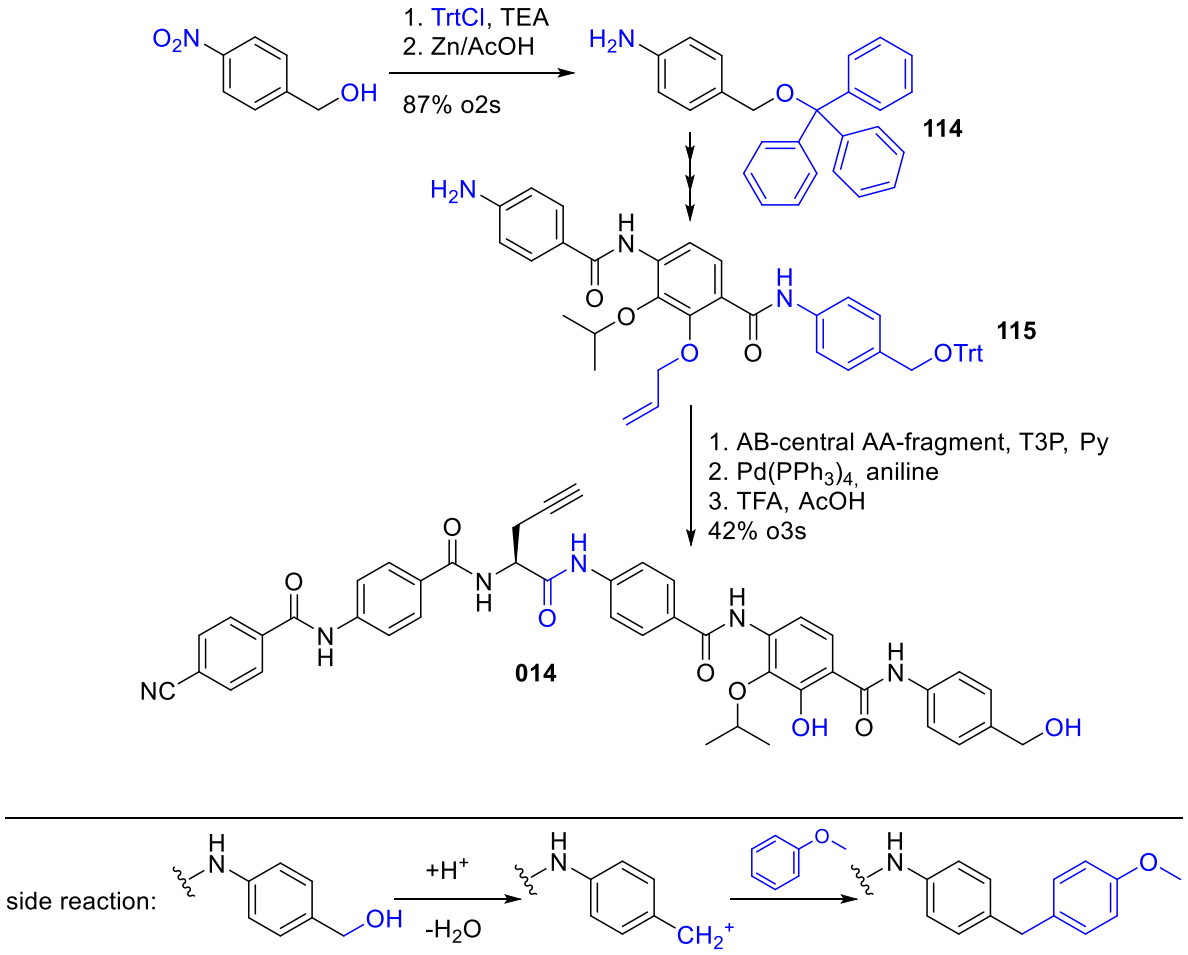


Figure 83: Protecting of *p*-nitrobenzyl alcohol and incorporation as ring E analogue in the CDE-fragment **115**. The final assembly steps are shown, with the last deprotecting step showing an irreversible side reaction when anisol was used as scavenger (bottom).

The full cystobactamid was assembled as described above in 42% yield over 3 steps from the CDE-fragment **115**. The trityl protecting group was cleaved off in the last step using TFA according to a previous protocol[9]. As the generated trityl cation can react back with the benzyl alcohol function to the educt, triisopropylsilane (TIPS) is used as scavenger here. Since it was expected that TIPS would undergo side reactions with the alkyne function, anisol was first tried as such, but it reacted as a nucleophile with the intermediate benzyl cation of the cystobactamid (see Figure 83, bottom). Acetic acid was now used as an experimental scavenger, as a weaker nucleophile. In addition, the reaction was later quenched by dilution in alkaline aqueous medium to suppress further side reactions.

Nitro derivative

The synthesis of a derivative with a nitro group as substitute for the *C*-terminal carboxylic acid was started by first exchanging the nitro group of standard ring D (provided by Evotec) **7** with a Boc protected amino group (see Figure 84). This way the reduction of the nitro group at ring E would be circumvented. The respective *N*-Boc-carboxylic acid **116** was synthesized by nitro reduction and subsequent *N*-Boc protection with Boc_2O[213] in an acceptable yield of 60% over two steps. T3P-facilitated amide coupling with *p*-nitroaniline followed and the DE-fragment **117** with a free amino function at ring D was obtained after acidic Boc deprotection in a mediocre yield of 41% over two steps.

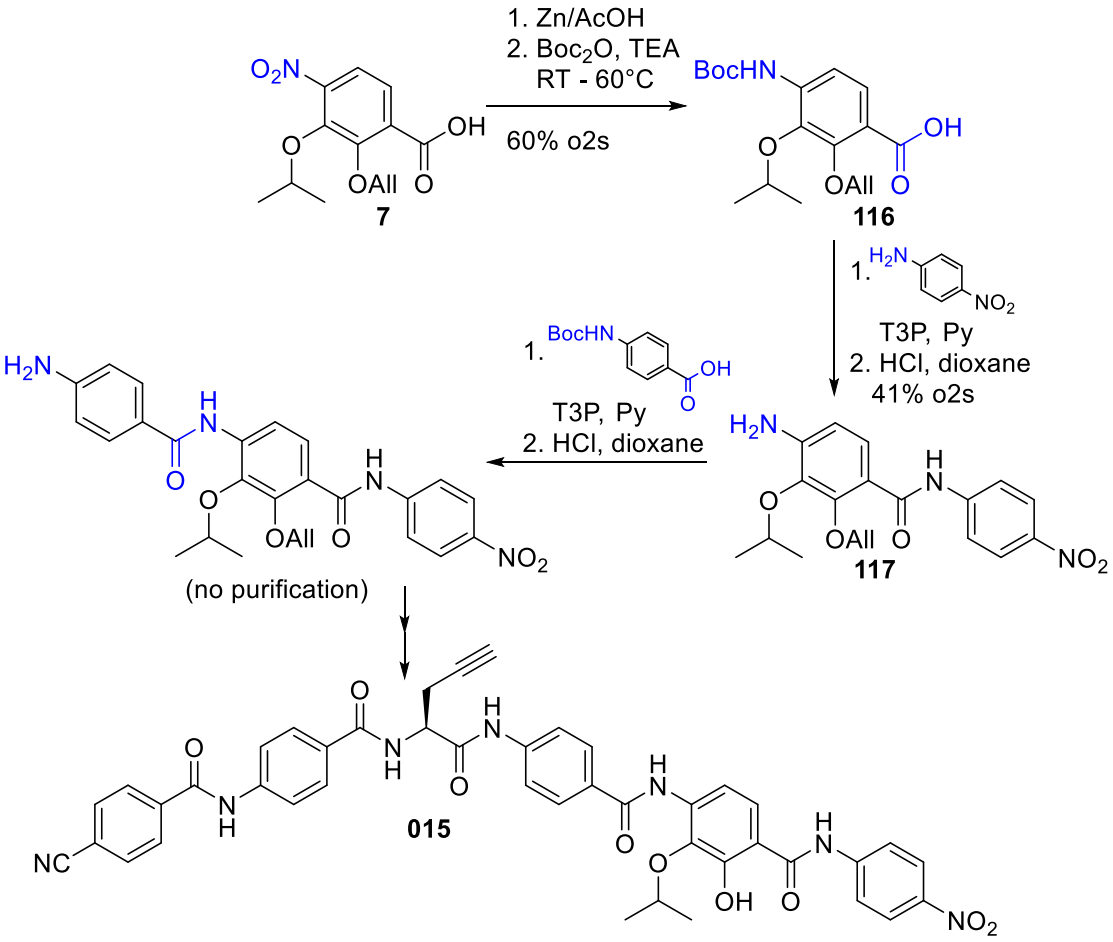


Figure 84: Synthesis of a CDE-fragment, featuring a nitro group as substitute for the terminal carboxylic acid, and structure of the final cystobactamid **015**.

Here, separation of the product from the remaining *p*-nitroaniline proved difficult due to similar R_f values. The DE-fragment **117** was now coupled to 4-((*tert*-butoxycarbonyl)amino)benzoic acid, again to avoid subsequent nitro reduction. This reaction gave a by-product that was later identified as the anhydride of the benzoic acid and could not be removed. Therefore, the final cystobactamid **015** was assembled without

intermediate purification in 4 steps with a yield of 41%. The product still had a purity of ~90 mol% as determined by NMR after purification by basic HPLC, but this purity level was finally accepted for the first MIC-tests.

3.2.5 SAR study of ring E

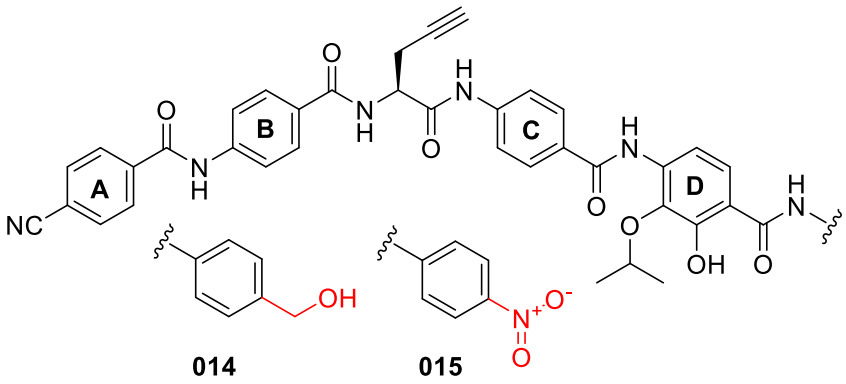


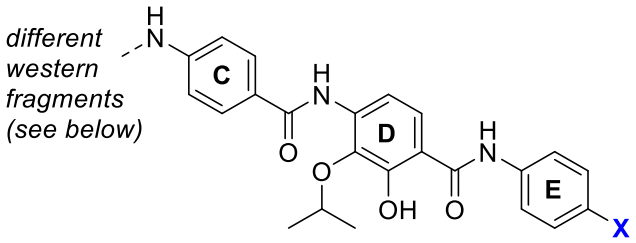
Figure 85: Modifications for ring E synthesized in this Thesis.

MIC evaluation

The MICs of the two derivatives shown in Figure 85 were determined as seen in Table 7. The previously reported[9, 99, 122-124] MICs of cystobactamids in which the *C*-terminal carboxylic acid was replaced are included to allow a concluding discussion. These feature an amide group (-$CONH_2$), a sulfonic acid, a sulfonamide and a tetrazole function. These compounds were synthesized at very different times in the cystobactamid project and tested on different bacterial strains, which makes a direct comparison difficult. In addition, they feature different Western fragments. On the basis of existing activity data and available informations on the tested strains, comparability is therefore only suggested.

Both derivatives synthesized in this Thesis were poorly active in the tested ESKAPE spectrum, sharing residual activity against the *E. coli* reference strain and a clinically isolated *S. aureus* strain. An activity against one carbapenem-resistant strain of *A. baumannii* and the *S. aureus* reference strain was seen for the benzyl alcohol derivative. In turn, the nitro derivative was active against a fluoroquinolone resistant mutant of *S. pneumoniae* and a strain of VRE. The benzyl alcohol derivative exhibited a similar activity pattern as the sulfonamide, but was inactive against almost every tested *A. baumannii* strain in comparison. A sulfonamide derivative featuring the standard cystobactamid structure of CN-CC 861 was not synthesized at this point and therefore can not be taken as reference.

Table 7: MIC-values (in µg/mL) for two isosteric replacements of the *C*-terminal carboxylic acid on ring E in this Thesis (indicated in black) with the reference cystobactamid CN-CC 861 and recent derivatives for comparison (indicated in grey).



X =	-COOH (CN-CC 861)	-$CONH_2$ a) [9, 99]	-SO_3H b) [123]	-SO_2NH_2 c) [122]	Tetrazole b) [124]	-CH_2OH **014** (this Thesis)	-NO_2 **015** (this Thesis)
A. baumannii ATCC BAA-1710 (FQR)	0.06-0.25	n.d.	>64	4	n.d.	>64	>64
A. baumannii CIP-107292 (FQR, CR)	0.5-1	n.d.	>64	2	n.d.	n.d.	>64
A. baumannii R835 (FQR)	1	n.d.	>64	4	n.d.	>64	n.d.
A. baumannii DSM-30007 (AR)	≤0.03	n.d.	>64[f]	2	>64[f]	>64	>64
A. baumannii NCTC 13301 (CR)	2	n.d.	n.d.	8	n.d.	1	>64
E. coli ATCC-25922	0.03-0.06	~1[d]	>64	<0.03	<0.03[d]	≤0.03	0.1
E. coli LM705 (S83L, D87N, S80I, ΔmarR, ΔacrR) (FQR, Gyr, EFL+)	<0.03-0.2	≤0.03[e]	>64	4	<0.03[e]	>64	>64
S. pneumoniae DSM-11865 (FQR, AR)	0.125	n.d.	>64	>64	n.d.	>64	0.05-0.08
S. pneumoniae PRSP clin. HAP/VAP isolate (MHH. tbd)	0.25	n.d.	n.d.	<0.03	n.d.	0.25	1.6
E. faecium DSM-17050 (VR)	<0.03	n.d.	>64	n.d.	n.d.	>64	0.125-0.2
S. aureus ATCC-29213	0.02	64	>64	0.25	0.25	0.25	>64
S. aureus MRSA (clinical pneumo isolate BAL #2524MHH, 2022) (AR)	≤0.03	n.d.	n.d.	0.125	n.d.	1	0.25
P. aeruginosa PA14	0.5-16	>64	>64	>64[g]	>64	n.d.	>64[g]
P. aeruginosa PA14ΔmexAB (EFL-)	0.03-0.5	>64	>64	n.d.	0.06	n.d.	n.d.
E. cloacae ATCC BAA-2468 (CR)	0.06-0.5	n.d.	>64	n.d.	n.d.	>64	>64

Reference strains are marked in blue. FQR = fluoroquinolone resistant, AR = ampicillin- or amoxicillin-resistant, CR = resistant against some carbapenems, Gyr = gyrase mutant associated with FQ-resistance, EFL+/- = increased or decreased efflux, VR = vancomycin resistant. [a]NO_2 as *N*-terminal group and asparagine as central AA. [b]asparagine or methoxyasparagine as central AA. [c][1.1.1]-bicyclopentane as isostere for ring B and threonine as central AA.[d]E. coli DSM-1116 used. [e]E. coli ΔTolC used. [f]A. baumannii DSM-30008 used. [g]P. aeruginosa PAO1 used.

In addition to the example shown, combinations of the sulfonamide group with other structural modifications are known, that showed a similar trend in activity.[122]

Even though the tetrazol derivative showed a comparably high activity, it appears that the best broad-spectrum activity is given with a *C*-terminal carboxylic acid. An HBD with only a weak HBA (benzyl alcohol) or an exclusive HBA (nitro) as isosteres are not sufficient for this purpose. However, the sulfonamide, benzyl alcohol and nitro derivatives showed shared activity against single strains of *E. coli, S. aureus and S. pneumoniae*. Neither strong

HBAs nor acidic groups may be therefore required for activity against these strains. In contrast, high activity against *A. baumannii* appears to be optimal for the derivative with a terminal carboxylic acid and decreases with stronger acidic groups such as a sulfonic acid (pK_A = 0.7 for benzene sulfonic acid[214]) or less acidic groups such as a sulfonamide (pK_A = 10.1 for benzene sulfonamide in water[215]) and a benzyl alcohol (pK_A~15) as isosteric replacements. The tetrazol is also inactive against the tested *A. baumannii* strain.

Furthermore, at least HBDs and acidity at the *C*-terminal group seem to be superfluous for those bacterial strains that are sensitive to the nitro derivative. However, to achieve activity against *A. baumannii,* this is not sufficient, and slightly acidic functions may additionally be required. The tetrazole function features a similar pK_A value to a carboxylic acid (pK_A = 4.4 for 5-phenyl tetrazole[216]) and showed indeed activity. However, the lack of activity against *A. baumannii* suggests that a carboxylic acid or isoelectronic groups are mandatory here. At the other end, the sulfonic acid derivative is completely inactive, which could be due to its high acidity.

As a conclusion, one can assume that the *C*-terminal function may contain at least one stronger HBA and be moderately acidic (pK_A of ~4-10) to show notable activity against the tested species. Slightly acidic functions as sulfonamide seem to be tolerated to a lesser extent. Such an anionic function might then also form a salt bridge as well. However, residual activity of cystobactamids with *C*-terminal groups, that do not have the characteristics mentioned, cannot be ruled out. With a molecular shape similar to that of a carboxylic acid, broader activity can be obtained. A pure HBA function may be sufficient for isolated activity, but only the combination with an HBD seems to extend activity to a broader spectrum, including *A. baumannii*.

In order to verify this hypothesis in detail, a further SAR investigation of the terminal carboxylic acid is required.

3.3 Optimization of the Western part

The following part of this Thesis contributed to the objective to further improve cystobactamids for high broad-spectrum efficacy *in vitro* and *in vivo*, with a focus on achieving superior activity against *A. baumannii*. At the same time, new synthetic methods for the assembly of the Western part were introduced.

3.3.1 Synthesis of the standard AB-central amino acid-fragment

Standard AB-fragment

The standard AB-fragment **10** was synthesized and obtained in a similar amide coupling reaction as reported (see Figure 86).[217] However, after precipitation of the product from the reaction mixture with brine and subsequent filtration analogously as reported, an unexpectedly high product mass was obtained. A detection reaction with $AgNO_3$ revealed that the product indeed contained NaCl. After careful washing with water, a yield of 69% was obtained. Amide coupling with unprotected L-propargylglycine as the central AA using HATU as activating reagent followed, but double coupling of the amino acid was observed as side reaction. Since this by-product was difficult to remove, it was thought that a protection of the carboxylic acid function would circumvent the problem. Therefore, the carboxylic function of the amino acid was methyl-esterificated first with TMSCl in MeOH. This method was chosen as it featured mild acidic catalysis and the by-products were volatile. The so obtained hydrochloride **118** was coupled with the standard AB-fragment **10**. The chromatographic purification of the product was found to be problematic because of the low solubility of the product (formation of insoluble liquid-crystal-like aggregates). Therefore the purification was omitted at this step and the material was directly deprotected with LiOH to obtain the carboxylic acid **26** (see Figure 86).

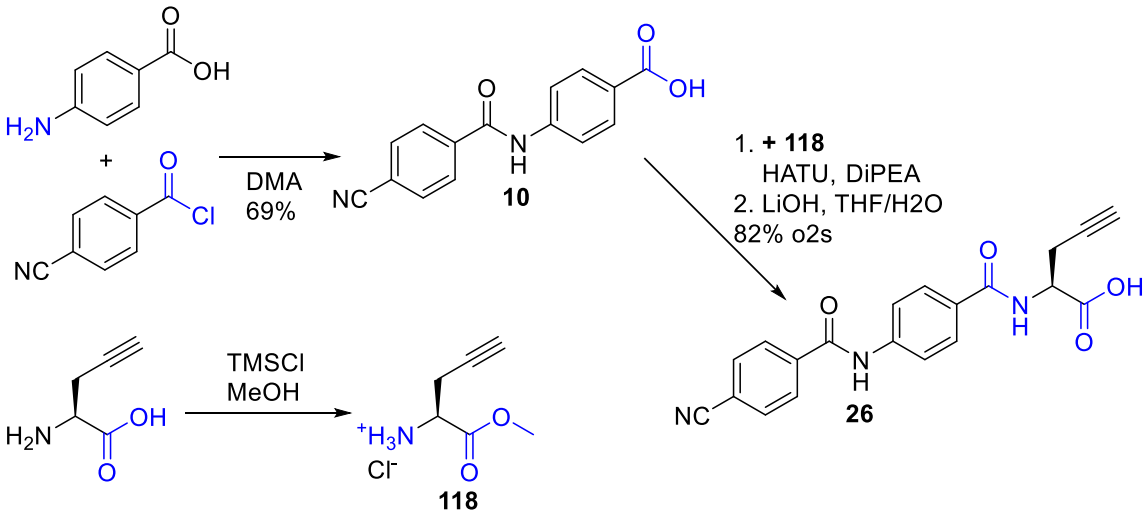


Figure 86: Assembly of the standard AB-central amino acid-fragment.

The crude product mixture contained residual amounts of the AB-fragment **10**. Interestingly, after dilution of the alkaline reaction mixture between water and an organic solvent, the desired product was detected almost exclusively in the aqueous phase, while

10 remained in the organic phase. It was assumed that the product **26** had a better solubility in alkaline solution than the aromatic carboxylic acid **10** due to its aliphatic carboxylic acid function. Therefore the purification of **26** was successfully performed by acid-base extraction and the AB-central AA-fragment was obtained in a yield of 82% over two steps.

3.3.2 Synthesis of new AB-amino acid scaffolds

Inspired by the high efficacy of the heavily rescaffolded cystobactamid HDo 308 described in section 1.2.4 against ESKAPE pathogens, further derivatives were synthesized that included the corresponding modifications in rings A, B and the central AA. This specific combination of three alterations, a benzimidazole as ring A, a [1.1.1] bicyclopentane as ring B and a rigidifying cyclopropane structure in the central AA (see Figure 87), showed exceptional activity against *A. baumannii*. To further investigate the capabilities of this highly divergent structural motif, it was combined with some of the new modifications in the Eastern part.

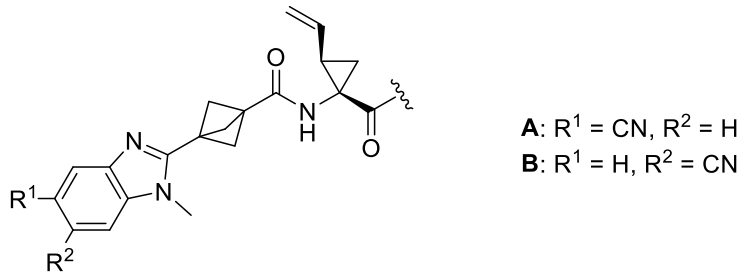


Figure 87: Structure of the modified AB-linker fragment. Variant **A** was primarily used as alternative fragment and variant **B** was introduced later.

Synthesis of a benzimidazole-bicyclo[1.1.1]pentane fragment

For the synthesis of the respective AB-fragment, a known method was adapted, starting from 4-fluoro-3-nitrobenzonitrile that reacted in a S_NAr with methylamine. Subsequent nitro reduction and heterocyclization of the resulting diamine **119** with the aldehyde function of **120**, subsequent oxidation with Cu(II) and oxygen, and cleavage of the *t*Bu-group gave a similar AB-fragment **121** (see Figure 88).[122]

The heterocyclization was now attempted with **119** as diamine and **122** as carboxylic acid to form the imidazol ring directly. The reaction was performed in a similar way to the amide coupling method used in the synthesis of the terephthalic acid (see section 3.2.2). The intermediate was purified chromatographically and then its methyl ester function was saponificated. The free carboxylic acid thus obtained formed a poorly soluble

precipitate that could not be extracted from the aqueous phase. The isolation of the product by filtration was not successful as well, as the solid contained impurities. It was now assumed that a stoichiometric amount of KOH would also accomplish the hydrolysis and give the potassium salt of the desired AB-fragment **76** directly after simply removing the reaction solvents.

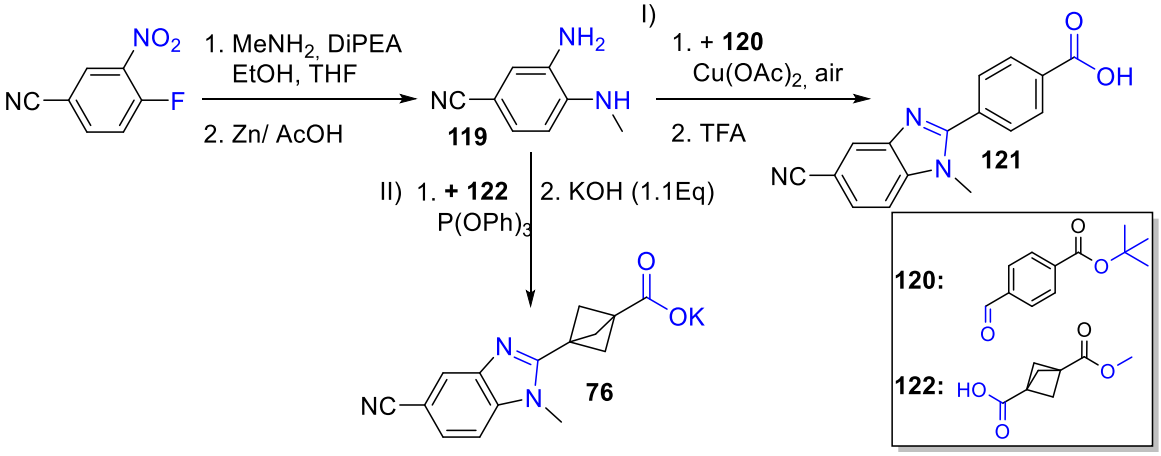


Figure 88: Synthesis of AB-fragments with benzimidazole as ring A modification, including the former synthesis strategy I)[122] and new substitution method, developed in this Thesis II).

Indeed, **76** was obtained after the reaction with a slight excess of KOH in aqueous solution and after lyophillization of the reaction solution. The corresponding 6-cyano-regioisomer **76b** was prepared[119] analogously to **76**.

Coupling with the central amino acid

The carboxylate thus obtained was intended to be coupled to the central AA. This would have resulted in an AB-central AA-fragment, in analogy to the standard AB-central AA-fragment mentioned earlier in this section. To this end, (1*S*,2*R*)-1-amino-2-vinylcyclopropane-1-carboxylic acid was first methyl-protected to give **123**. However, this reaction proceeded insufficiently, and a mixture of protected and unprotected amino acid was formed. Purification of this mixture by acid-base extraction was dispensed with for the the time being, as this would have meant that a large amount of material would not have been converted in the next reaction step. It was also assumed that if the carboxylic acid was so inert, its initial protection would not be necessary. This mixture was now added to the AB-fragment, followed by the known coupling by HATU and DiPEA as base. Again, insufficient turnover was observed. Additional HATU reagent led to an intramolecular reaction, forming a heterocycle **124** (see Figure 89), whose mass was identified by LCMS.

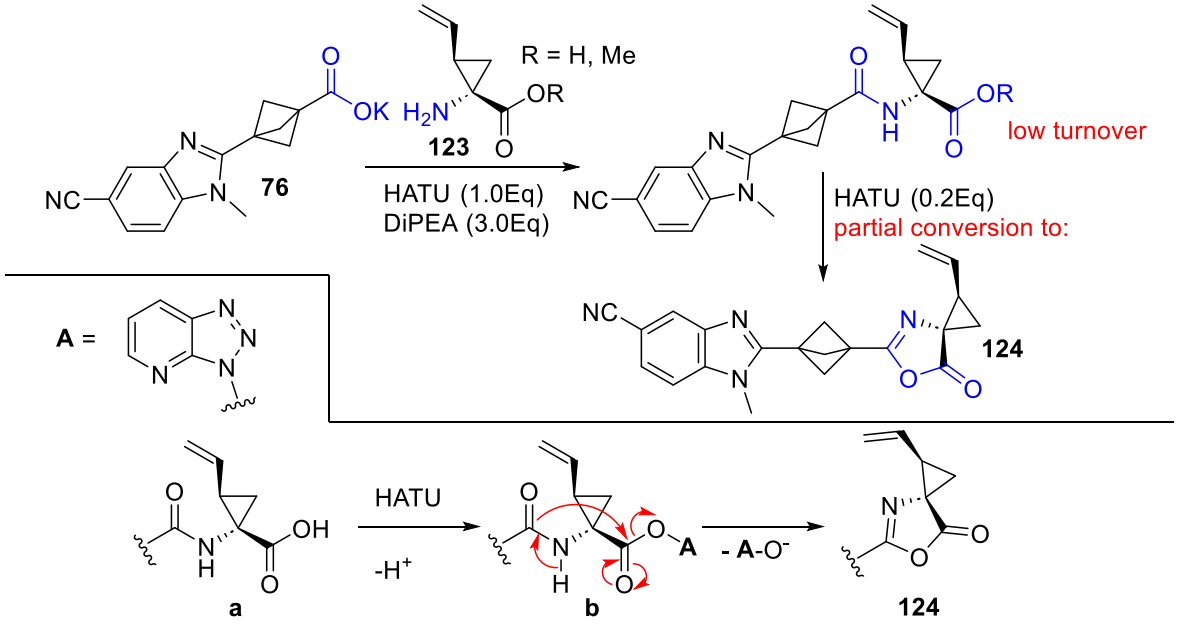


Figure 89: Amide coupling of modified AB-fragment with *C*-terminal protected cyclopropane amino acid and observed heterocyclization product. A possible mechanism is suggested below.

This heterocyclization was assumed to start from the free carboxylic acid **a**. After deprotonation it is activated by HATU to form an HOAt-ester **b**. Subsequently, this ester is attacked intramolecularly by the nearby amide oxygen forming the five-membered heterocyclus in **124**. It can be assumed that this intramolecular attack and the formation of a spiro compound is driven by the cyclopropane ring, that preorganizes the reacting groups into close proximity (Thorpe-Ingold effect[218]), comparable to the accelerated lactonisation in trimethyl-lock systems[219-220]. However, the cyclopropane residue has a widening effect on the N-C-COOR bond angle, and compared to a system with two geminal methyl substituents, only a small contribution to the acceleration of the reaction is to be expected according to the literature.[221-222]

From this observation it was derived that such an intramolecular side reaction would also take place during the T3P-mediated amide coupling between the AB-central AA-fragment and a CDE-fragment. Therefore, the synthesis of the AB-central AA-fragment was not developed further and it was proceeded with the established assembly method[11, 96, 154] (see section 3.1, retrosynthetic path 1).

The *N*-Boc protected cyclopropane amino acid **125** was commercially available. As the first CDE-fragment for coupling with **125**, a modified variant **126** of the standard CDE-fragment was used as a kind of benchmark. This fragment showed a pyridine at ring C, which reduced the nucleophilicity of the amino group. It was now interesting to see

whether the sterically hindered carboxylic acid function of **125** would be reactive enough for activation by T3P and subsequent amide coupling (see Figure 90). Indeed, the reaction was found to proceed slowly, reaching a maximum turnover of ~55% (LCMS). Addition of additional T3P and **125** could not improve this outcome.

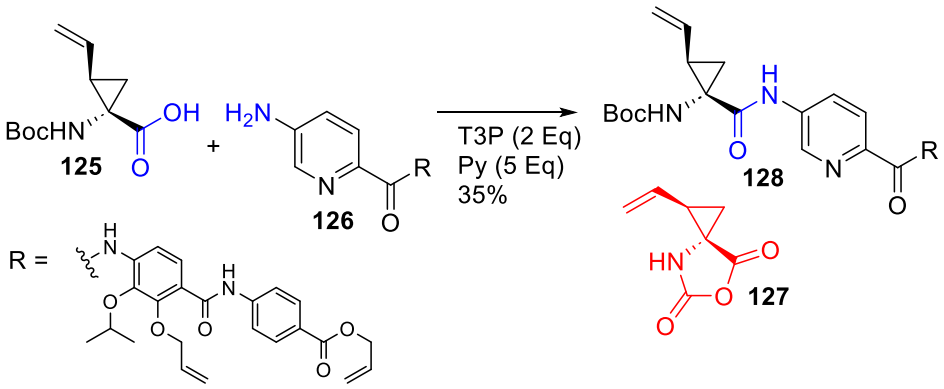


Figure 90: Amide coupling between the *N*-Boc-protected cyclopropane amino acid **125** and a weakly nucleophilic CDE-fragment **126** with observed side-product (**127**, red).

Strangely enough, also in this case a cyclization of the amino acid was observed, forming an unknown by-product that was detectable by LCMS with a mass of 176 Da. The by-product showed remarkable UV-absorption and was found to be stable in aequeos acidic solution. This by-product was finally found to be the cyclic anhydride **127** as elucidated by 2D NMR experiments. The chromatographic separation of the reaction mixture gave the desired AA-CDE-fragment **128** in 35% yield.

Further experiments revealed that an initial excess of carboxylic acid was beneficial and subsequent alkaline work-up was required to remove this excess. The Boc protecting group was cleaved under acidic conditions with mediocre selectivity towards the terminal *t*Bu group. The final assembly of the cystobactamid ended in a yield of 36% over 3 steps (see Figure 91).

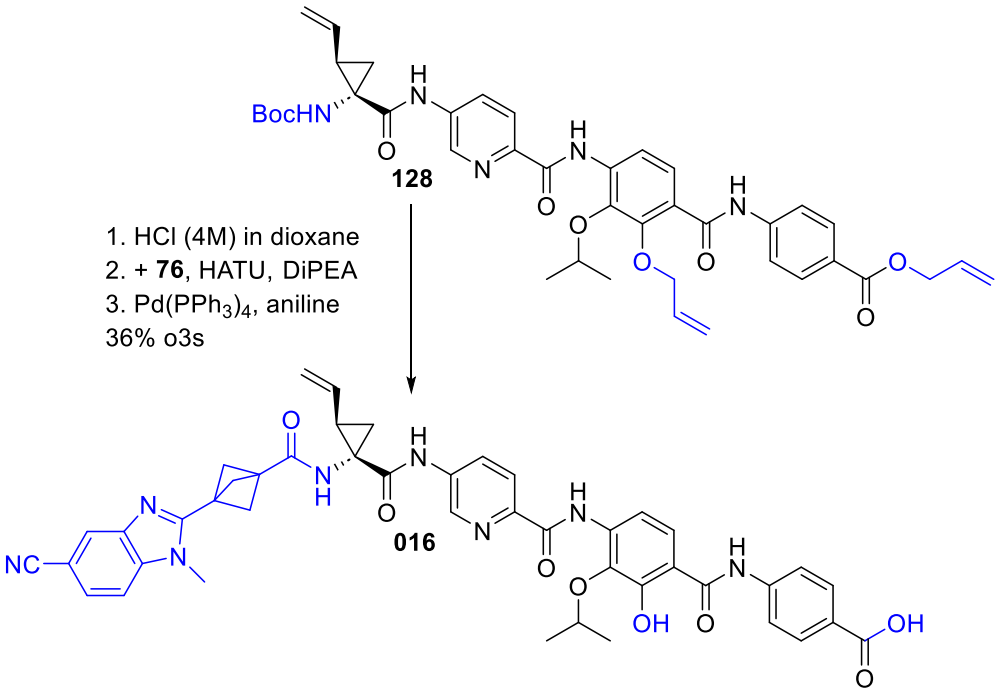


Figure 91: Final assembly of cystobactamid **016**.

The only partial orthogonality of the *N*-Boc protecting group prompted further synthetic optimization. Using *t*BuOAc as a competing reactant in large excess did not prove beneficial. As an alternative approach, the commercially available cyclopropane amino acid was intended to be reprotected with an Fmoc group in analogy with the standard AB-central AA-fragment. For this purpose, the Boc protecting group was first cleaved from **125** and then the free amino function was newly protected with an Fmoc protecting group by adapting a known method[223] (see Figure 92).

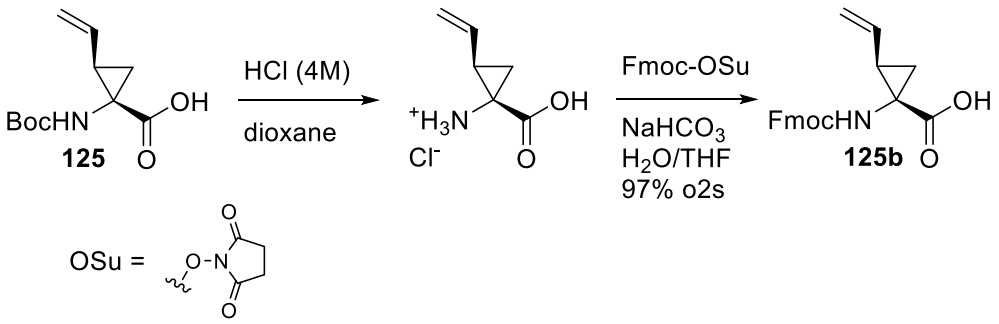


Figure 92: Reprotection of the cyclopropane amino acid.

The product **125b** was obtained in a very good yield and successfully coupled to several CDE-fragments (see chapter 5 for details), which now allowed a fully orthogonal deprotection.

3.3.3 SAR study for combinations of ring D analogues with the optimized Western fragment

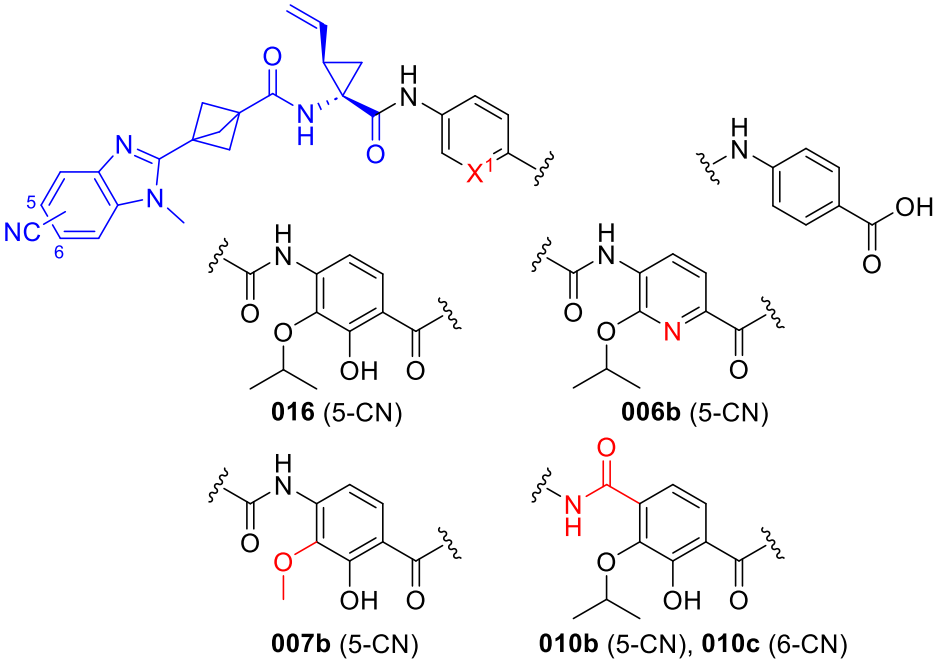


Figure 93: Synthesized combinations of the optimized Western fragments with modified rings C and D. X^1 = N for Cytobactamid **016**, X^1= CH for cystobactamids **006b**, **007b**, **010b** and **010c**. A combination with X^1 = CH and standard ring D was already synthesized (HDo 308)[119] and is used as reference in the following analysis.

MIC evaluation

In the following analysis, the synthesized derivatives are compared with HDo 308 as cystobactamid reference[119]. For reasons of space, the corresponding derivatives with standard Western fragment are not listed here. The bacterial strains listed in the MIC table (Table 8) were changed to achieve a stronger representation of clinically relevant strains and especially *A. baumannii*.

First of all, the substitution of ring C with a pyridine was tolerated and even increased the activity for most of the *A. baumannii* strains, which makes this derivative one of the most active compounds in this Thesis. As already shown in section 3.2.3, substitution of the phenolic hydroxy group at ring D by a pyridine led to extensive inactivity with a residual activity for the *E. coli* reference strain, one VRE-strain and, with an MIC of 8 µg/mL, one non-fluoroquinolone-resistant *A. baumannii* strain. A similar result is observed here. It is interesting that even in combination with the improved Western part, activity is not restored.

Table 8: MIC-values (in µg/mL) for combinations of optimized Western fragments with ring C and D modifications and two cystobactamid references.

	Ref. HDo 308	Ring C pyridine derivative **016**	Ring D pyridine der. **006b**	Methoxy der. **007b**	Tere-phthalic acid der. **010b**	Tere-phthalic acid der. **010c**	std. Tere-phthalic acid der. **010**
A. baumannii ATCC BAA-1710 (FQR)	0.5	0.025	16	4	2	1	<0.03-0.05
A. baumannii CIP-107292 (FQR, CR)	0.125-0.25	0.025	16	0.5	8-16	8-16	0.06
A. baumannii R835 (FQR)	0.5	0.025	16	4	n.d.	n.d.	0.5
A. baumannii DSM-30007 (AR)	n.d.	≤0.01	2	1	4	n.d.	0.05
A. baumannii 038 OXA-23 (CR)	n.d.	≤0.01	4	1	1	0.5	0.025
A. baumannii 070 NDM-1 (CR)	n.d.	≤0.01	32	2	2	1	0.25
A. baumannii NCTC 13301 (CR)	0.125	0.06	>64	16	8	4	2
E. coli ATCC-25922	<0.06	≤0.03	≤0.03	≤0.03	n.d.	n.d.	<0.03
E. coli LM705 (S83L, D87N, S80I, ΔmarR, ΔacrR) (FQR, Gyr, EFL+)	1	0.125	>64	1	0.5	0.125-0.25	0.06
S. pneumoniae DSM-11865 (FQR, AR)	2	2	>64	n.d.	n.d.	n.d.	<0.03
S. pneumoniae clin. HAP/VAP isolate (MHH. tbd) (AR)	n.d.	≤0.5	>64	2	n.d.	n.d.	16
E. faecium DSM-17050 (VR)	<0.06	≤0.03	>64	0.25	0.005	0.0025	<0.03
S. aureus ATCC-29213	<0.3	≤0.03	>64	2	0.2	0.05	0.06
S. aureus MRSA (clinical pneumo isolate BAL #2524MHH, 2022) (AR)	n.d.	≤0.5	>64	4	≤0.03125	≤0.03125	<0.5
K. pneumoniae KP10581 (waaC::Tn30) (FQR)	<0.06	≤0.03	>64	0.06	0.04	n.d.	<0.03
K. pneumoniae R1525 (FQR)	<0.3	0.5	>64	2	1	0.5	<0.03
P. aeruginosa PAO1 (ref. strain)	n.d.	8	16	16	16-32	8-16	<0.03
P. aeruginosa clinical HAP/VAP isolate 4MRGN, BAL #2182MHH,2021 (CR)	n.d.	16	>64	>64	64	64	16
E. cloacae ATCC BAA-2468 (CR)	2	1	>64	4	0.25-0.5	0.125	0.25
E. aerogenes CIP 106754 (AR)	8	2	>64	32	2	1	1

Reference strains are marked in blue. FQR = fluroquinolone resistant, AR = ampicillin- or amoxicillin-resistant, CR = resistant against some carbapenems, Gyr = gyrase mutant associated with FQ-resistance, EFL+/- = increased or decreased efflux, VR = vancomycin resistant.

Substitution of the isopropoxy substituent with a methoxy function also followed the trend observed in section 3.2.3, meaning a significant decrease in activity for all tested *A. baumannii* strains compared to the cystobactamid reference. This activity decrease for *A. baumannii* was even higher than the one observed for the combination of the methoxy-CDE-fragment with the standard Western fragment. This also applied to some *S. aureus*, *S. pneumoniae* and *E. coli* strains while the new combination in turn showed better activity against *K. pneumoniae*.

This also suggests that the beneficial effect of the new Western scaffold on cystobactamid activity varies depending on the bacterial species. It is worth noting that the combination of the new Western fragment with L-propargylglycine as central AA actually proved to be detrimental to antibacterial activity.[119] A general positive effect of the new Western fragment can therefore not be assumed.

The high activity of the terephthalic acid analogue of ring D against *A. baumannii* was also not maintained by switching to the improved Western fragment. The efficacy here was reduced by 2-7 dilution steps compared to the combination with the standard Western fragment. These findings are somehow surprising, given that the combination of the terephthalic acid ring D analogue with the standard Western fragment was highly active against *A. baumannii*. One possible rational is that the rigidification of the central AA impairs the enhanced H-bond between the isopropoxy function in ring D and its adjacent amide function (see section 3.2.3). If this changes the conformation, the affinity to the target may be decreased. Another, simplier explanation for this inconsistency would be a measurement error. Apart from this, the activity of the new terephthalic acid analogues was still high on all other strains tested. Switching the *N*-terminal cyano group from 5- to 6-position did not have a remarkable influence, although this was recently found to result in improved activity for some derivatives[119-120, 122].

In summary, although the activity against *A. baumannii* was improved by combining the pyridine at ring C with the new Western fragment, the combination with the standard Western fragment seems to be generally more reliable here. However, further combinations of the terephthalic acid ring D with other promising Western parts (as mentioned in the introduction) should be considered in the future.

3.4 Pharmacological optimization

As outlined in section 1.2.4, the low solubility of the cystobactamids as well as their high amount of PPB were problematic. In addition, experiments with hepatocytes pointed to a possibly high susceptibility of cystobactamids to transporters such as OATPs.[224] The structure of the standard cystobactamid CN-CC 861 was now modified to target these issues, as explained in the following section. Physicochemical properties were determined for selected cystobactamids.

3.4.1 Tackling problems resulting from cystobactamid acidity

One possible reason for low physiological activity of a drug may be the OATP mechanism (see section 1.3.2), which can facilitate the transport of substances into metabolizing tissue (e.g. liver). As a result, the plasma concentration of an active substance may decrease rapidly. To inhibit this process, acidic moieties should be replaced. In addition, such a replacement may also lower PPB, since cystobactamids as acidic compounds were expected to have a high affinity to plasma proteins as well.

Pyridine-sulfonamide derivatives

The pyridine analogue of ring D now served to investigate the absence of an acidic group at ring D, as it lacks the acidic phenolic hydroxy group. Unfortunately, activity data for the cystobactamid featuring this analogue are not available yet. As already mentioned in section 3.2.5, a sulfonamide group was recently introduced as an isosteric replacement for the *C*-terminal carboxylic acid, resulting in moderately active compounds.[122] This isosteric group was now intended to be combined with the pyridine analogue to obtain a non-acidic cystobactamid.

For that purpose, the pyridine-D-carboxylic acid was coupled with ring E, which had a protected sulfonamide group (material provided by D. Kohnhäuser). The protecting group chosen here was *N,N*-bis(2,4-dimethoxybenzyl),[172, 225] which can be cleaved under mildly acidic conditions. The corresponding CDE-fragment was poorly soluble in organic solvents, presumably due to the protecting group containing two more aromatic moieties. Because this poor solubility was observed for the final cystobactamids as well, the usual purification by basic HPLC, as well as a chromatographic purification on the normal phase failed. The low solubility was now exploited to isolate the final compounds by precipitation and subsequent filtration. Similar to the pyridine derivative, cystobactamids with both standard- and new scaffold were synthesized (structures see Figure 94).

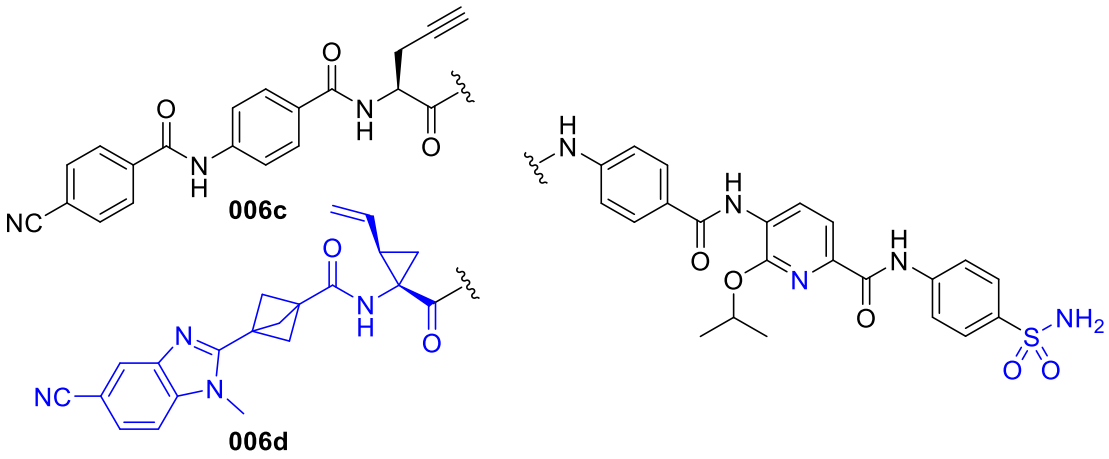


Figure 94: Synthesized cystobactamids **006c** and **006d** with standard- (top left) and new scaffolded Western fragment (bottom left), featuring the pyridine analogue of ring D and a sulfonamide as isosteric replacement for the *C*-terminal carboxylic acid.

3.4.2 Adressing solubility

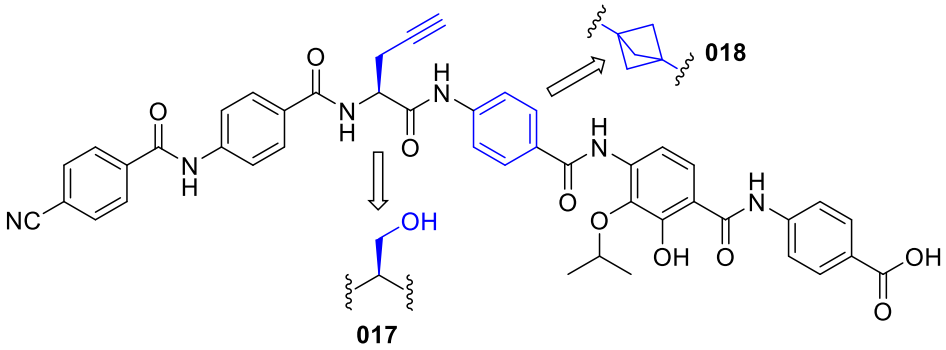


Figure 95: Proposed modifications of cystobactamid CN-DM 861 in this Thesis to improve its solubility.

Introduction of bicyclo [1.1.1] pentane at ring C

As shown in section 1.2.4, the substitution of ring B by an aliphatic bicyclo [1.1.1] pentane group resulted in good solubility of the final cystobactamid. The substitution of aromatic systems by aliphatic isosteric groups is a well-known method to improve solubility and log P.[138] This isosteric group was also introduced as a substitute for all aromatic rings in cystobactamids except ring C and D.[118, 122] Since the synthesis of a corresponding ring D derivative was not considered feasible, the respective ring C derivative was synthesized according to the final assembly path 3 as already outlined in section 3.1 (Figure 28 b).

Introduction of serine as central amino acid

Recently, it was found that replacement of the standard central AA with threonine was tolerated in terms of activity and both improved solubility and lowered PPB (values of ~86% were determined here in mice).[122] To expand knowledge about the effect of a hydroxy group in the central AA, serine was introduced as the central AA. To enable

orthogonal deprotection, *N*-Fmoc-*O*-*t*Bu-serine was chosen as a building block for the central AA and coupled with the standard CDE-fragment (assembly path 1). After cleavage of the Fmoc protecting group, final assembly with the standard AB-fragment followed. Anisol was then used as a scavenger for the final deprotection of the two *t*Bu-groups with TFA. The final compound **017** was obtained in 45% o4s from the AA-CDE fragment (see Figure 96).

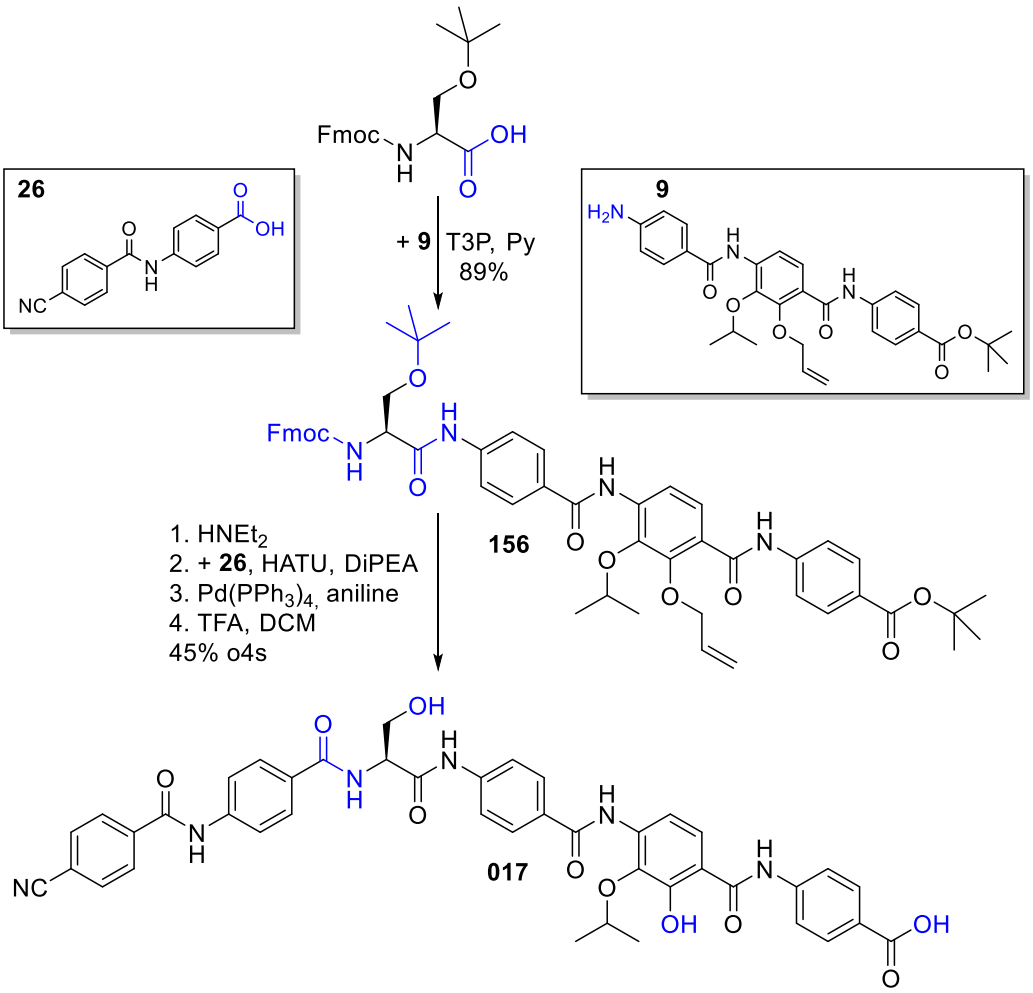


Figure 96: Assembly of the serine-cystobactamid **017**.

3.4.3 SAR study and determination of physicochemical properties

MIC evaluation

In a first step, the compounds were tested for their efficacy against bacteria. The results of the MIC tests are found in Table 9. Bacterial strains were selected in such a way that clinically relevant strains are better represented.

Table 9: MIC-values (in µg/mL) for derivatives in pharmacological optimization.

	CN-CC 861	pyridine-sulfonamide derivative I **006c**	pyridine-sulfonamide der. II **006d**	serine der. **017**	ring C-aliph. isostere der. **018**
A. baumannii ATCC BAA-1710 (FQR)	0.06-0.25	>64	>64	0.25	>8
A. baumannii CIP-107292 (FQR,CR)	0.5-1	>64	>64	8	>8
A. baumannii R835 (FQR)	1	>64	>64	1	>8
A. baumannii DSM-30007 (AR)	≤0.03	>64	>64	0.125	n.d.
A. baumannii 038 OXA-23 (CR)	0.5	>64	>64	0.5	n.d.
A. baumannii 070 NDM-1 (CR)	2	>64	>64	>64	n.d.
E. coli ATCC-25922	0.03-0.06	4	4	<0.03	0.04
E. coli LM705 (S83L, D87N, S80I, ΔmarR, ΔacrR) (FQR, Gyr, EFL+)	<0.03-0.2	>64	>64	0.06	64
S. pneumoniae DSM-11865 (FQR, AR)	0.125	>64	>64	<0.03	16
E. faecium DSM-17050 (VR)	<0.03	>64	>64	0.04	4
S. aureus ATCC-29213	0.02	>64	>64	0.125	8
S. aureus MRSA clin HAP/VAP isolate (tbd. MHH) (clin pneumo isolate BAL #2524MHH, 2022) (AR)	≤0.03	>64	>64	0.05	n.d.
K. pneumoniae KP10581 (waaC::Tn30) (FQR)	0.06-0.25	>64	>64	0.5	8
K. pneumoniae R1525 (FQR)	16- >64	>64	>64	0.5	64
P. aeruginosa PA14	0.5-16	n.d.	n.d.	n.d.	64
P. aeruginosa PA14ΔmexAB (EFL-)	0.03-0.5	n.d.	n.d.	n.d.	2
P. aeruginosa PAO1	>64	>64	>64	>64	n.d.
P. aeruginosa clin. HAP/VAP isolate (tbd. MHH) 4MRGN (clin pneumo isolate BAL #2182MHH, 2021) (CR)	>64	>64	>64	>64	n.d.
E. cloacae ATCC BAA-2468 (CR)	0.06-0.5	>64	>64	<0.03	32
E. aerogenes CIP 106754 (AR)	0.125-4	>64	>64	0.125	>64

Reference strains are marked in blue. FQR = Fluroquinolone resistant, AR = ampicillin- or amoxicillin-resistant, CR = resistant against some carbapenems, Gyr = gyrase mutant associated with FQ-resistance, EFL+/- = increased or decreased efflux, VR = Vancomycin resistant.

As one might expect, both sulfonamides showed almost complete inactivity with the pyridine function on ring D, with the *E. coli* reference strain being the only exception. The same argumentation can be applied here as for the pyridine derivative, with a much weaker H-bond between the pyridine nitrogen and the adjacent amide bond being held responsible compared to the standard DE-fragment. As already mentioned in section 3.2.5, the substitution of the terminal carboxylic acid by a sulfonamide group led to a lower activity, so that an additional attenuating effect can be assumed here.

However, the serine derivative showed high activity against Gram-positive strains with MICs of ≤0.5 µg/mL and high efficacy against all *E. coli* and *Enterobacter* strains tested. The activity against *A. baumannii* strains is comparable with the one of CN-CC 861 with one exception (070 NDM-1). Furthermore, in contrast to CN-CC 861, activity is achieved

against one additional fluoroquinolone-resistant *K. pneumoniae* strain. In general, this compound is highly promising and may also offer a solution to the problem of solubility.

On the other hand, the introduction of a bicyclo [1.1.1] pentane isostere to ring C showed a negative effect on activity. For example **018** was completely inactive against the tested *A. baumannii* strains. The *E. coli* reference strain was susceptible to this compound and slight residual activity was observed for some Gram-positive species, requiring drug concentrations of 2-8 µg/mL. One reason for this could be the missing aromaticity of ring C and thus the lack of conjugation between ring C and the CD-linker. This, in turn, should allow free rotation of the DE-fragment along the major axis of ring C. A lower binding affinity to relevant bacterial targets and thus a lower activity could be the consequence. A similar effect was already observed for the benzylamine and isobutyl derivatives (see section 3.2.3). Another possible reason is that bicyclo [1.1.1] pentane deviates from the planar shape of a benzene ring and is also more contracted. Both may negatively influence relevant interactions of the cystobactamid within the bacterial cell, especially with gyrase. Considering structural data of the interactions in the albicidin-gyrase-DNA complex,[89] slight deviations in the three-dimensional shape of the CDE-system might already show large effects in the highly narrow space between the two gyrase subunits.

Table 10: Comparing MIC-values (in µg/mL) for bicyclo [1.1.1] pentane substituted derivatives with the corresponding ring. The selection of bacterial strains was adapted to attain comparability.

	CN-CC 861	ring A replacement[118] SSA 196	ring B repl.[122] DK 444	ring C repl. **018**
A. baumannii DSM-30008 (AR)	0.02-0.03	2	0.125-1	8
A. baumannii ATCC BAA-1710 (FQR)	0.06-0.25	4	0.25-4	>8
E. coli LM705 (S83L, D87N, S80I, ΔmarR, ΔacrR) (FQR, Gyr, EFL+)	<0.03-0.2	2	0.5-1	>64
E. coli CH448 (FQR, Gyr)	0.04	0.5	0.06	n.d.
E. coli MG1655	n.d.	0.125	n.d.	n.d.
K. pneumoniae CIP-104298 (AR)	8	32	0.25-0.5	32
K. pneumoniae KP10581 (waaC::Tn30) (FQR)	0.06-0.25	4	0.25-1	8
K. pneumoniae DSM-30104	0.5	32	4	n.d.

FQR = fluoroquinolone resistant, AR = ampicillin- or amoxicillin-resistant, Gyr = gyrase mutant associated with FQ-resistance, EFL+/- = increased or decreased efflux.

The MICs of all synthesized cystobactamids with bicyclo[1.1.1]pentane replacements were now compared. While activity was preserved when ring B was substituted with this isosteric group, this was less the case when ring A was substituted[118, 122] (see Table 10)

and even less so for ring C. The particularly low activity of the substitution at ring C is in agreement with the observed general trend that structural changes in the Eastern fragment have a more severe impact on activity than in the Western fragment.

Evaluation of solubility

Despite of the low activity of the cystobactamid with the bicyclo [1.1.1] pentane substitute for ring C, it was still interesting to investigate the effects of this replacement on solubility and log D. The solubility of the bicyclo [1.1.1] pentane derivative at ring C was determined as being >1mg/mL at pH 7.4 and 9.0 (measured by Evotec). This is one of the highest solubilities of all cystobactamids and the highest solubility of all derivatives with an isosteric replacement by bicyclo [1.1.1] pentane (see Table 11). One possible explanation is that the intermolecular attraction of cystobactamids by aromatic π-π interactions is weakened by the substitution of one aromatic ring with an aliphatic element. In this way, the interaction with the solvent can be higher in comparison, which in turn may lead to a higher solubility.[226]

Table 11: Aqueous solubilities of cystobactamids with bicyclo [1.1.1] pentane as isoster for the corresponding ring at different pH. This data was provided by Evotec.

	Ring A[118] SSA 196	Ring B[122] DK 444	Ring C **018**
pH 7.4 [µg/mL]	18	901	>1000
pH 9.0 [µg/mL]	922	889	>1000

The determination of solubility for the serine derivative was not carried out because the required instrumental equipment was no longer available at this time. However, since this derivative carried an additional polar hydroxy group, being both an HBD and HBA, it was expected to have a higher solubility compared to CN-CC 861. A higher polarity might also allow lower values for PPB.

Evaluation of log P

For some key derivatives, the log D values at pH 7.4 were measured using a shake-flask method as reviewed and evaluated by Valko[227] and Andres *et al.*[228] .

In summary, all measured cystobactamids had log $D_{7.4}$ values between 1 and 4 which is within the usual range of commercially available drugs (see Table 12). The standard cystobactamid CN-CC 861 showed a log $D_{7.4}$ value of 2.26 in previous measurements (performed by Evotec, see section 1.2.4), which is in accordance with the value found here (2.4±0.2).

Table 12: Measured log D values for some cystobactamids at pH 7.4. For further details see experimental part.

derivative	log $D_{7.4}$	standard deviation error
CN-CC 861	2.4	±0.2
Fluoro (**002**)	3.0	±0.2
Oxoquinazoline (**013**)	2.4	±0.1
Methoxy (**007**)	1.1	±0.2
Isobutyl (**008**)	2.4	±0.2
Terephthalic acid (**010**)	1.5	±0.5
Terephthalic acid-combination (**010b**)	1.6	±0.0
Pyridine C-ring-combination (**016**)	3.0	±0.0
Benzyl alcohol (**014**)	n.d.*	-
Nitro (**015**)	n.d.*	-

*values could not be determined due to solubility issues and very low peak intensities measured for the aqueous phase. The estimated log D values are therefore estimated to be much higher compared to the other values.

Evaluation of pK_A

The determination of pK_A values was considered an essential point, as the cystobactamids feature two acidic functions and can thus pass through three protolysis stages. Knowledge of the predominant species at a physiological pH of 7.4 was an important piece of information to obtain here, e.g. to predict ionic interactions of the cystobactamid with other molecules. The pK_A values of a representative cystobactamid were measured by titration in aqueous solution. It was feared that precipitation of the cystobactamid could falsify the results, as the solubility of the cystobactamids is generally low. Therefore, a compound was chosen that beared high water solubility combined with high activity in MIC-tests, as it was the case for DK 444, the cystobactamid with the bicyclo [1.1.1] pentane moiety as ring B isostere (see Table 11, material provided by D. Kohnhäuser). A titration curve was recorded (see Figure 97), in which acidification was started from the dianionic salt. Latter was prepared by adding 2.5 equivalents of KOH. In this way, the less soluble neutral form was kept kinetically dissolved for a sufficient time during the titration.

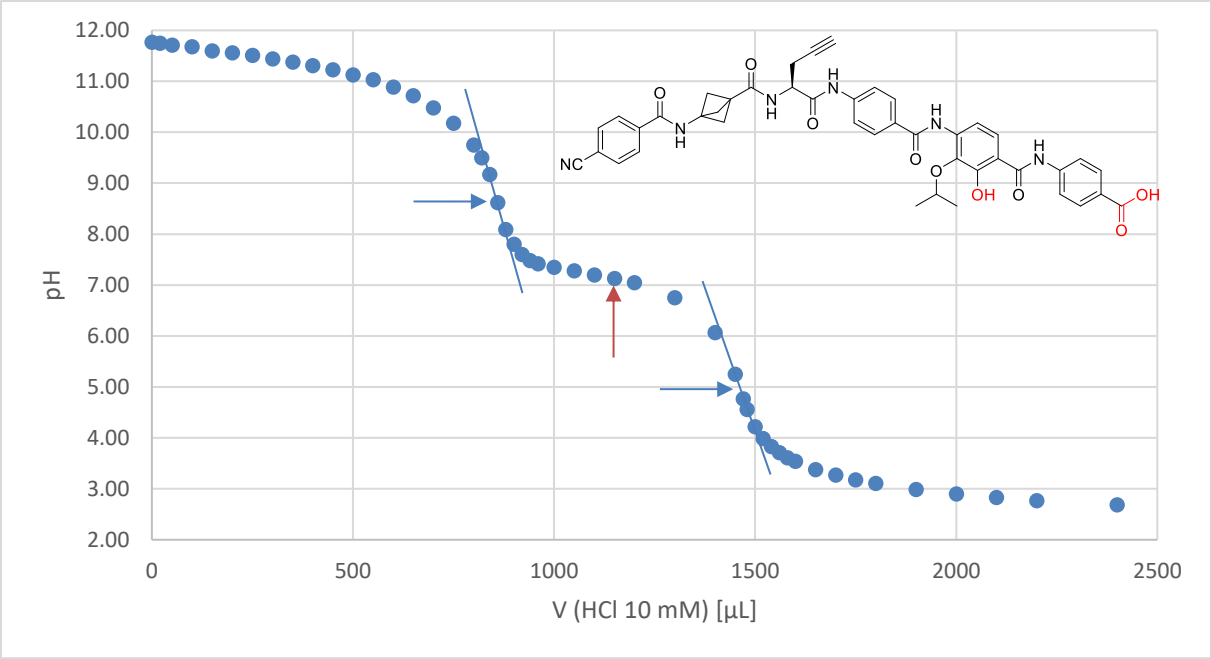


Figure 97: Titration curve for DK 444 (structure shown). For sample preparation, 2.5 Eq of KOH was initially added to a suspension of the cystobactamid (5 mg) in deionized water (3 mL). The clear solution was then titrated with HCl (10 mM). The half equivalence point (red arrow) was determined from the two tangents of the equivalence points (blue arrows). Partial precipitation was observed underneath a pH of ~7.

From this titration curve, at least one pK_A value could be derived by evaluating the half equivalence point at a volume of ~1200 µL HCl-solution. A pK_A of 7.1 was determined which probably corresponds to the phenolic hydroxy function. This value is smaller than the values found in literature for similar molecules (e.g. $pK_A = 8.27$ for 2-hydroxybenzamide[229]). The physiologically relevant pH of 7.4 is reached between the first equivalence point and the half equivalence point. At this pH the *C*-terminal carboxylic acid should therefore be deprotonated (for comparison: $pK_A = 4.88$ for *p*-aminobenzoic acid[230]), but the phenolic hydroxy group can be present in both protonated or deprotonated states.

3.4.4 Pharmacokinetic evaluation of selected compounds

The two most promising cystobactamids of this Thesis (see Figure 98) were chosen for the investigation of pharmacokinetic properties. As a model organism CD-1 mice were chosen. The compounds were tested in a cassette study by K. Rox, in which other test compounds were tested in the same animal (not shown here). The concentrations were kept as low as possible here to avoid mutual interference of the tested compounds as far as possible. Plasma concentrations were determined as shown in Figure 99.

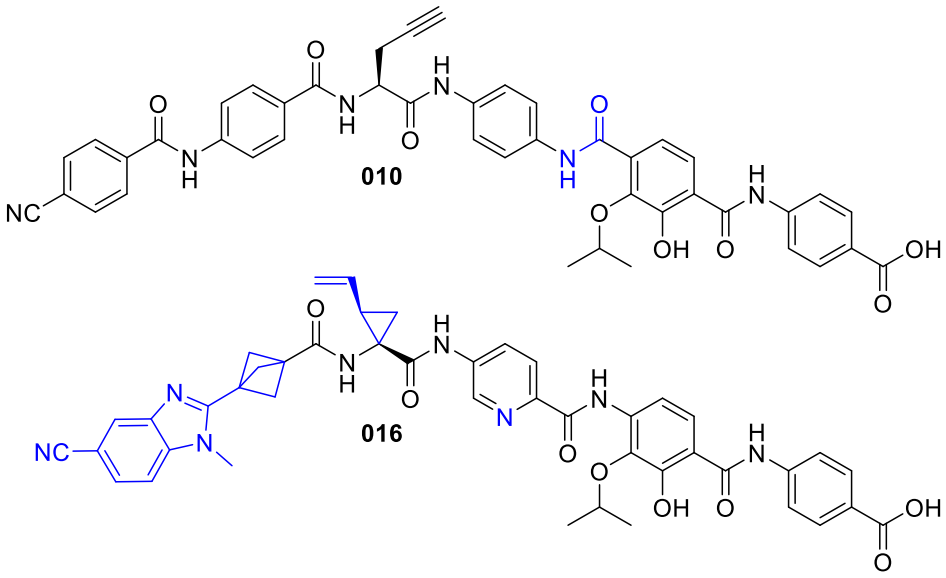


Figure 98: Cystobactamids **010** and **016,** selected for the pharmacokinetic evaluation in cassette PK study.

Table 13: Determined parameters for the two selected compounds determined in cassette PK study (measurements and analysis conducted by K. Rox).

	meaning	010	016
t1/2 [h]	plasma half-life	0.88 ± 0.1	0.6 ± 0.2
C0 [µg/mL]	extrapolated initial mass conc.	2.2 ± 1.5	0.70 ± 0.56
AUC 0-t [ng/mL*h]	area under the curve (extrapolated)	1207 ± 832	152 ± 45
MRT [h]	mean residence time	0.70 ± 0.2	0.30 ± 0.2
Vz [L/kg]	volume of distribution in elimination phase	1.39 ± 1.0	5.72 ± 0.0
Cl [mL/min/kg]	clearance of plasma (extrapolated)	17.90 ± 12.3	113.39 ± 33.2

Compounds were measured in separated cassettes with 4 other compounds, respectively. N=2 mice per cassette, initial dosage: 1 mg/kg *i.v.*, The underlying plasma concentration curve is shown below.

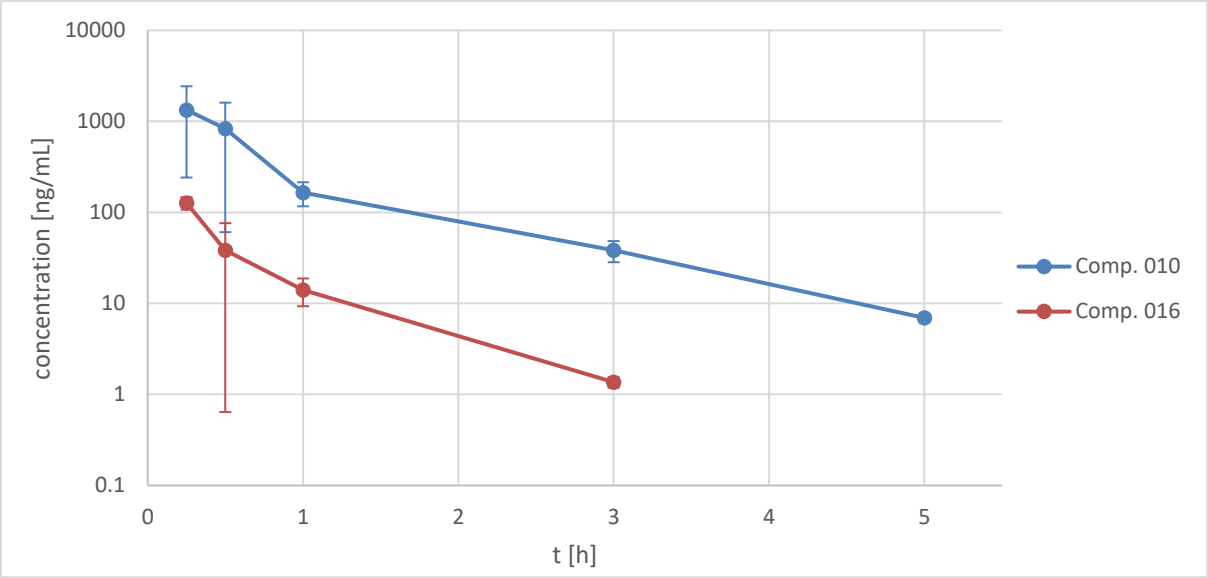


Figure 99: Plasma concentration curves for data shown in Table 13 for cystobactamids **010** and **016**. Error bars were determined via standard deviation of two cassette experiments per compound (n=2).

The plasma concentration curve for **010** shows a higher concentration of this compound in mice plasma then **016**. This is reflected in a higher AUC for this compound, implying higher exposure. Ideally, one would expect a linear decay in the logarithmic plot. The non-linearity observed here indicates that the underlying model is only partially accurate. The very high error bar observed for **016** at t = 0.5 h could be due to a missed vein injection. As mentioned above, a mutual influence of the tested compounds cannot be excluded, as they were each tested together with four other compounds in one animal. Inaccuracies in the determined measured values can also occur due to the low concentrations used.

The plasma half life seems to be quite low with a value of less than 1 h for both compounds. This would usually require relatively high dosage and more frequent administration of the drug. For comparison, the half life of Morphine in human is 1.5-4.5 h,[231] of Methotrexate 3-15 h,[232] and of Fluoxetine 4-6 d.[233] However, the comparison of half-lives in mice and humans can at best show a tendency.

The extrapolated initial plasma concentration is ~2 µg/mL for **010** and ~0.7 µg/mL for **016**. With a dose of 1 mg/kg given per mice and a common weight of 20-40 g/mice[234] one can calculate an administered mass of 20-40 µg compound per mice. Given an average blood volume of 76-80 mL/kg or 1.5-3.2 mL per mice[234], the extrapolated initial concentration corresponds to an extrapolated mass of 3-6 µg for **010** and 1-2 µg compound per mice for **016**. This means that after the injection and initial distribution, a large amount of the administered compound is not detectable in the blood and may

already have been absorbed to the tissue or bound to plasma proteins or blood cells. However, due to the non-linearity of the logarithmic plot, the extrapolated initial concentrations have a high uncertainty.

The mean residence time is higher for **010** than for **016** (0.7 vs 0.3 h), with >2 h being a desirable value. The volume of distribution in the elimination phase Vz is lower for **010** than that for the other compound tested (1.4 vs. 5.7 L/kg), implying that this compound was less absorbed to tissues in the terminal phase. A lower amount of PPB could also be a reason. Matching to this, the measured log $D_{7.4}$ value of 1.5 indicates lower lipophilicity for **010** compared to **016** ($logD_{7.4}$ = 3.0). This also shows that the prediction of some ADME-properties is also possible using the measured log $D_{7.4}$ values. However, plasma protein binding can not be deduced from this value and might still be high. The clearance from plasma is higher for the best-of compound so this compound might be eliminated faster from organism.

In summary these parameters suggest that **010** shows slightly better exposure and higher plasma concentrations than **016**. This might be a main result of higher polarity as seen from its lower log $D_{7.4}$ value. The lower exposure of **016** might additionally be due to a higher degree of degradation. Thus, the standard Western fragment remains a valuable structural feature for improving pharmacokinetics.

4 Conclusion and outlook

25 novel cystobactamids were synthesized in this Thesis. 19 of these compounds feature modifications of the synthetically challenging ring D. New cystobactamid Eastern fragments were combined with the recently optimized and proven Western fragment of CN-CC 861[11] as well as with the current best-of Western fragments. By evaluating activity in MIC-tests, many new findings were added to the previous SAR.

The library of ring D analogues was expanded by introducing new functional groups as substitute for the so important hydroxy- and isopropoxy groups. Various new synthetic protocols were developed and well-known methods were adapted and further developed. New methods were introduced for the full assembly of cystobactamids and previous protocols were further optimized. An extensive MIC-activity testing followed, including highly critical ESKAPE pathogens.

Based on the evaluation of these completely new compounds, the SAR for the Eastern fragment was updated. Key findings are illustrated in Figure 100.

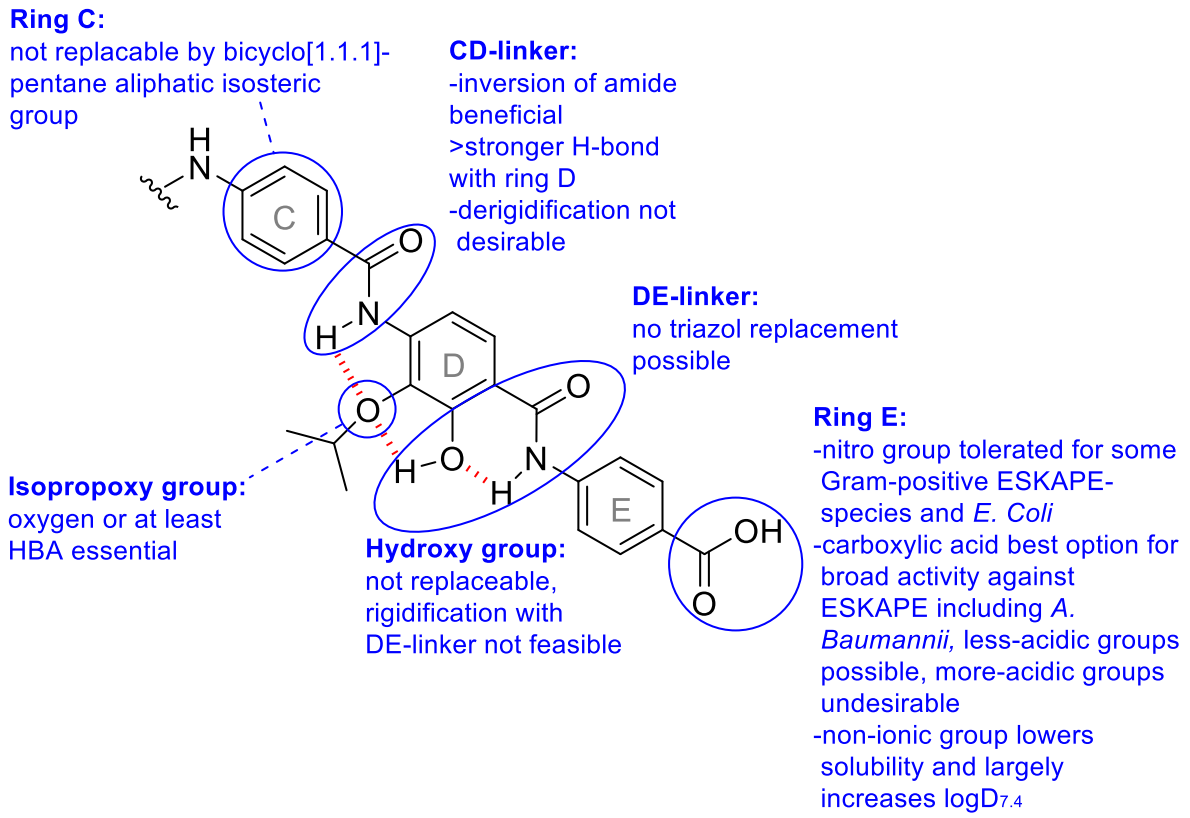


Figure 100: Main outcome of the new SAR for the CDE-fragment. For details consult section 3.2. The shown conformation (*d-a* conformation) is assumed to be the most relevant one for activity.

As the most important modification, the CD-linker was switched to form the reverse amide, resulting in increased activity. In addition to the instrumental analysis by NMR, LCMS and HRMS, the cystobactamids were characterized with respect to pharmacologically relevant properties such as solubility, log P and pK_A. *In vivo* pharmacokinetic parameters were determined for the two most promising compounds, a terephthalic acid derivative and a derivative containing both a new Western fragment and a pyridine analogue of ring C.

The mentioned terephthalic acid derivative was combined with other promising Western fragments in this work, more combinations should be prepared in the future. Some proposed structures are shown in Figure 101. Indeed, the *in vivo* studies in mice have revealed that the standard Western fragment is still a valuable combination, but the benzimidazole for ring A, as mentioned in the introduction, should be considered as an alternative. It should be possible to improve solubility with the introduction of more hydrophilic central AAs. It should still be kept in mind that some best-of combinations may not be advantageous in terms of activity.

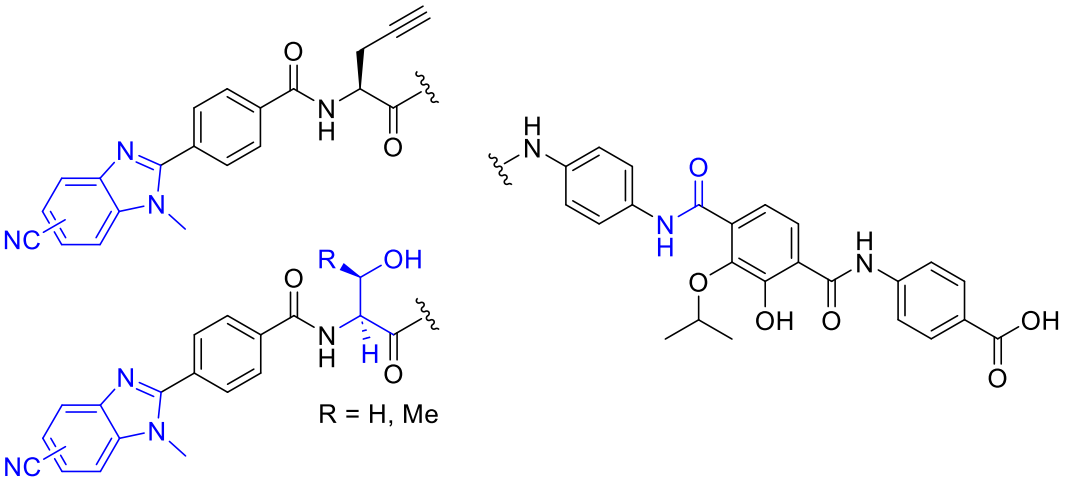


Figure 101: Proposed next structural combinations including the terephthalic acid analogue of ring D.

As noted by D. Kohnhäuser,[11] there is sometimes a low correlation between IC_{50} inhibition levels for *E. coli* gyrase or topoisomerase IV and MIC activity. Similar experiments[122] with other ESKAPE pathogens such as *S. aureus* and *A. baumannii* confirmed this observation, suggesting that other unknown mechanisms might be partly responsible for cystobactamid activity. Therefore, further studies should target this question, e.g. by renewed target-fishing experiments and determination of IC_{50}-values for the target enzymes in all ESKAPE pathogens. The salicylamide structure in the DE-system

is probably a key feature here, as it might play a role other than just bearing extensive intramolecular H-bonds.

The results presented in this work demonstrate the great versatility of cystobactamids as antibiotics and lead structures for rational drug design. Rationalization of SAR in the critical DE fragment region provided further information and improvements for cystobactamids. The development of new syntheses for CDE-fragment analogues provided detailed insights into the aromatic chemistry of oligoarylamides. In combination of these new CDE-fragments with the best-of Western fragments of current frontrunners, the next steps towards a clinical trial have already been initiated.

5 Experimental Procedures

5.1 Materials and general methods for synthesis and testing

5.1.1 Solvents and eluents

Solvents for synthesis and purification were used as delivered, if not stated else. Usage of dry solvents is indicated where their use was assumed to be necessary, involving the following dry solvents (manufacturer):

acetonitrile, acetone, dimethylformamide, dimethylsulfoxide, 1,4-dioxane, isopropanol, methanol, methylene chloride, pyridine, tetrahydrofurane, toluene (Acros Organics)

Organic solvents for HPLC were used as HPLC grade and degassed before use. Water was either used as HPLC grade or purified with Milli-Q water filter device (Elga Veolia Purelab flex) before use.

5.1.2 Educt/product handling and general reaction setup

All non-volatile solid reactants and reagents were dried under high vacuum before use. Reactions were conducted in glass ware (flasks, tubes or vials) using magnetic stirring. Glass ware was cleaned by hand and using washing machines and was checked optically for contamination before use. The use of inert gas is indicated where it was employed. Products were isolated from their solutions at a rotary evaporator (Heidolph Hei-Vap Advant) using a membrane pump (Vacuubrand PC3001 VARIO select). For storage, products were transferred to small flasks as solution, followed by renewed removal of the solvent. Further drying was conducted under high vacuum with an oil rotary vane pump (Vacuubrand RZ 2.5).

5.1.3 Reaction control

The reaction mixtures were monitored by using thin-layer chromatography (TLC) and/or LCMS. For sample extraction, a truncated metal canula was used and the sample was diluted before analysis. TLC was conducted with silica-coated aluminum plates and fluorescence-indicator (TLC Silica gel 60 F_{254}, Merck (Darmstadt)). After development, the TLC was radiated with UV light (λ=254 nm or λ=365 nm) for evaluation. For a closer assignment of the spots to expected products the TLC-plates were dyed using Ninhydrin-solution in EtOH (1.0 g Ninhydrin in 100 mL EtOH) for amines, a solution of $FeCl_3$ in EtOH (50 mL) and water (50 mL) or hydrochloric acid (0.1 M, 50 mL) for phenols, a bromo cresol green solution (40 mg in aqueous NaOH (0.1 M, 100 mL)) for carboxylic acids, or a

$KMnO_4$-solution (1.0 g in a solution of K_2CO_3 (2.0 g) in H_2O (100 mL)) for oxidizable compounds. Subsequently, the plates were treated with a heat gun.

Crude products and purified products were analyzed on an Agilent LCMS device (Agilent Technologies 1260 Infinity II) with MeCN/H_2O as eluent system using LCMS method 6 (see attachment). Mass spectra were generated in positive mode with ESI as ionization method with a Quadrupole mass spectrometer (Agilent Technologies 6130 ES superior). Solvents for LCMS were used with Formic Acid (0.1%) added to both MeCN and H_2O before measurement.

5.1.4 Column chromatography

Reaction products were purified and isolated using manual or automated flash-column chromatography (FCC). For this purpose, silica 60 gel (particle size: 40-63 µm, otherwise noted) was used as stationary phase. The dimensions of the silica column was adapted to the reaction scale and the estimated separation efficiency: Silica was used in 10-300 fold mass, compared to either the mass of the reactant/ reactants (without reagents) or the theoretically accessible mass of product (at 100% yield). The crude product was loaded as solid on column, being absorbed on either silica or Celite beforehand. The mass of this absorbant was throughout adjusted to 1/10 mass of the column package-silica. The composition of eluents was adjusted to the separation using analysis of product mixtures by TLC. Fractions were analyzed by TLC, spot detection and staining was analogous to TLC reaction control.

5.1.5 HPLC/LCMS

Preparative HPLC was implemented on a Thermo Scientific Ultimate 3000 HPLC-system using either a basic or an acidic eluent system. It was applied to purify the final cystobactamids. Further details for sample preparation is mentioned in section 5.2.

Analytical LCMS was conducted on an Agilent LCMS device (Agilent Technologies 1260 Infinity II) as mentioned in section 5.1.3, using method 6 (see attachment).

The eluent programs used are listed in the attachment.

5.1.6 NMR spectroscopy

NMR spectra were measured in deuterated solvents either with a 500 MHz-NMR-spectrometer (Bruker Advance-III HD 500 MHz, Frequencies: ^{1}H NMR: 500 MHz, ^{13}C NMR: 126 MHz) or a 700 MHz-NMR-spectrometer (Bruker Advance-III HD 700 MHz, Frequencies: ^{1}H NMR: 700 MHz, ^{13}C NMR: 176 MHz). All spectra were measured at room temperature and ^{13}C NMR-spectras were measured ^{1}H-decoupled. Chemical shifts (δ) of the compounds are given in ppm, the coupling constants J are given in Hz. The spectra were always referenced to the respective residual solvent peak given in table Table 14.

Table 14: Reference peaks for NMR data depending on solvent.

NMR-solvent	^{1}H [ppm]	^{13}C [ppm]
$CDCl_3$	7.26	77.16
DMSO-*d6*	2.50	39.52
acetone-*d6*	2.05	29.84
THF-*d8*	3.58	65.57
MeOH-*d4*	4.87	49.00

Multiplicities are given according to Table 15:

Table 15: Abbreviations of the multiplicities for NMR data used in this Thesis.

multiplicity	abbreviation
singlet	s
doublet	d
doublet of doublet	dd
doublet of triplet	dt
triplet	t
quartet	q
heptet	hept
multiplet	m
broad	br

Yields were determined by weighting and also corrected by NMR when solvent impurities would substantially falsify yield. The correction was done using following equation:

$$Y_{corrected} = Y_{measured} \frac{M_P J_P N_I}{M_P J_P N_I + M_I J_I N_P}$$

M_P : product molar mass, J_P : integral of observed product peak, N_P : number of protons assigned to product peak, M_I : molar mass of main impurity, J_I : integral of observed impurity peak, N_I : number of protons assigned to impurity peak

For spectra of key substances in the synthesis, assignment of signals to the corresponding atom was verified by 2D NMR validation.

In cases of common solvent impurities in the NMR sample, the observed number of signals in NMR may not correspond with the expected number. Signals assignable to known impurities (solvent, educt etc.)[235] are not listed.

5.1.7 HRMS

HRMS spectra were recorded on a Bruker maXis HD spectrometer. Mass signals are given in their *m/z* values with the relative intensities in brackets together with the calculated mass.

5.1.8 MIC testing

Strains were cultivated according to standard procedures. Bacterial cryo-cultures were plated on fresh CASO agar and incubated at appropriate conditions for 24 hours. The following day, 1-2 colonies were picked and suspended into 0.9% NaCl (Merck KGaA, Darmstadt, Germany) to reach a McFarland value of 0.2 - 0.5. The suspension was resuspended into fresh MHBII (cation adjusted) medium. The resuspension corresponding to approximately 5 x 10^6 colony-forming units (CFU)/mL. The test culture (75 µL) was added to 75 µL of a serial dilution of the test compounds in 96 well assay plate (Corning, #3788). All compound were used from a 5 mg/ml stock solution and were tested at final starting concentrations of 64 µg/ml. Ciprofloxacin was tested in parallel as a positive control. The highest DMSO concentration in the assay was 1%, which had no growth effect.

In the case of obviously ineffective cystobactamids the MIC-panel was not tested completely.

5.1.9 Cassette PK study

The study was conducted in CD-1 outbred mice, male. The applied intravenous dose was 1 mg/kg. The mentioned compounds were measured in two separated cassettes, together with 4 other compounds.

5.1.10 Determination of log D

Methods for determination of log D values were adapted from[227-228]. PBS buffer (pH=7.4, pH meter checked) and octanol were mutually saturated by shaking in separating funnel. Phases separated at least 24 hours. Every sample was measured in glass vials (0.5 mL,

2 mL, 4 mL, 8 mL). The cystobactamids were diluted as DMSO solution with PBS buffer to 0.10 mM (standard solution). From this, phase mixtures of PBS/octanol were made in at least two of these ratios: 1:1, 5:1, 10:1, 50:1. The phase mixtures were shaken strongly for at least 10 min per hand, then left to separate for at least 8 h. The lower phase was extracted with an Eppendorf pipet tip after careful removal of octanol residues. The aequeos fraction was measured by UV-absorption in HPLC device (Shimadzu Prominence). All samples were measured in such a way that at most half of the maximum UV intensity was reached. Log D values were determined by using following formula:

$$\log D = \log\left(\left(\frac{A_{\mathrm{st}}}{A_w} \cdot \frac{v_{\mathrm{inj}(w)}}{v_{\mathrm{inj}(\mathrm{st})}} r - 1\right)\frac{V_w}{V_o}\right)$$

log D: pH dependent log P, Ast: Peak area for standard solution, Aw: Peak area for sample solution, $v_{inj(w)}$: injection volume for aequos phase, $v_{inj(st)}$: inj. vol. for standard solution, r: dilution factor of standard solution, V_w/V_o: volume fraction of aequos phase and organic phase.

For every measurement, triple determination was conducted with varying injection volumina and arithmetic mean was calculated.

5.1.11 pKa determination

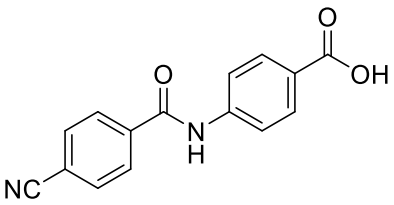
For sample preparation, 2.5 Eq of KOH (1.0 M) was initially added slowly to a suspension of the cystobactamid DK 444 (5 mg) in deionized, non-degassed water (3.0 mL). The clear solution was then titrated with HCl (10 mM, 10-200 µL increments). The half equivalence point was determined from the two equivalence points. The pH was determined using a Schott CG840 pH meter that was calibrated with buffer solutions at pH 4.00 and 7.00. Partial precipitation was observed underneath a pH of ~7.

5.2 Synthetic procedures

The following syntheses are sorted by the corresponding fragments, using the nomenclature used for cystobactamids.

AB-fragments

*Synthesis of 4-(4-cyanobenzamido)benzoic acid **(10)**[217]*

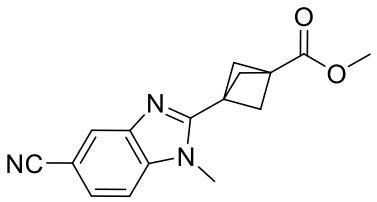


4-amino benzoic acid (2.00 g, 14.6 mmol, 1.00 Eq) was solved in DMA (44 mL) and DMAP (71 mg, 0.58 mmol, 0.040 Eq) was added. 4-cyano benzoyl chloride (2.41 g, 14.6 mmol, 1.00 Eq) was added and the reaction mixture stirred 18 h at RT. The mixture was added to a heavily stirred brine solution (150 mL) which was then filtrated (Note: Precipitate forms a very fine powder, small pore size required). The colorless precipitate was dried u.r.p. overnight to give a fine colorless powder. Because the precipitate contained greater amounts of NaCl, the solid was resuspended, filtered and washed with water several times and dried u.r.p. overnight to give a fatty colorless solid, 2.66 g, 69%.

^{1}H NMR (500 MHz, DMSO): δ = 12.79 (s, 1H), 10.74 (s, 1H), 8.11 (d, *J* = 8.4, 2H), 8.04 (d, *J* = 8.2, 2H), 7.96 (d, *J* = 8.9, 2H), 7.91 (d, *J* = 8.9, 2H).

^{13}C NMR (126 MHz, DMSO): δ = 166.9, 164.6, 142.9, 138.6, 132.5, 130.3, 128.7, 125.9, 119.7, 118.3, 114.1.

*Synthesis of methyl 3-(5-cyano-1-methyl-1H-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate **(129)***



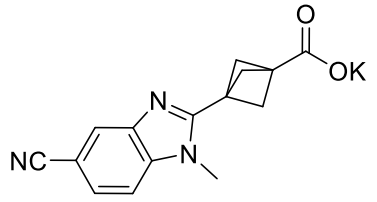
3-(methoxycarbonyl)bicyclo[1.1.1]pentane-1-carboxylic acid **122** (100 mg, 0.588 mmol, 1.00 Eq) and 3-amino-4-(methylamino)benzonitrile **119** (provided by D. Kohnhäuser, 91 mg, 1.05 Eq) were solved in dry toluene (1.5 mL) under Argon atmosphere and $P(OPh)_3$ (0.20 mL, 1.3 Eq) was added. The light purple solution was refluxed for 17 h while being screened with LCMS. The mixture was diluted between EA and NaH_2PO_4-sol. (1 M) (20 mL each). The aqueous phase was extracted with EA (2x15 mL) and the combined organic phases were dried over Na_2SO_4 before removing solvents u.r.p.. The crude product was purified by FCC (solid loading, 80x reactant mass, PE/EA, 40%EA->50%) to give a light purple crystalline solid (140 mg, 85%).

^{1}H NMR (700 MHz, $CDCl_3$): δ = 8.04 (s, 1H), 7.53 (dd, *J* = 8.4, 1.5, 1H), 7.36 (d, *J* = 8.4, 1H), 3.86 (s, 3H), 3.74 (s, 3H), 2.67 (s, 6H, 3x CH_2).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 169.2, 153.6, 141.4, 138.6, 126.2, 124.4, 119.8, 110.3, 105.4, 54.0, 52.0, 39.6, 35.9, 31.0.

HRMS (ESI): calculated for $[M+H]^+$: 282.1237, found: 282.1241.

*Synthesis of potassium 3-(5-cyano-1-methyl-1H-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate **(76)***



methyl 3-(5-cyano-1-methyl-1*H*-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate **129** (51.0 mg, 0.181 mmol, 1.00 Eq) was suspended in THF (0.5 mL) and KOH (0.50 M, 381 µL, 1.05 Eq) was added. The clear reaction solution was stirred at RT for 3 h while being monitored by LCMS. Solvents were removed u.r.p. and by coevaporation with EA and MeCN to give an off-colorless solid, 58.4 mg, >98%, (Note: the product contained

~5% residual educt which might be more if the reaction is conducted at a higher scale. Therefore the reaction temperature can be increased).

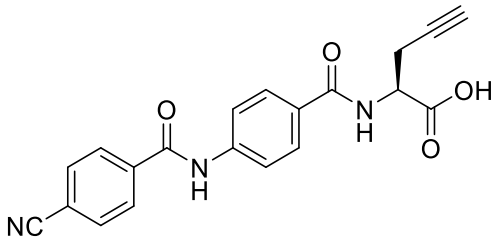
^{1}H NMR (700 MHz, DMSO): δ = 8.06 (dd, *J* = 1.6, 0.6, 1H), 7.70 (dd, *J* = 8.4, 0.7, 1H), 7.59 (dd, *J* = 8.3, 1.6, 1H), 3.85 (s, 3H), 2.21 (d, *J* = 0.9, 6H).

^{13}C NMR (176 MHz, DMSO): δ = 172.1, 155.9, 141.5, 139.0, 125.2, 123.4, 120.1, 111.4, 103.4, 53.1, 43.1, 34.4, 30.8.

HRMS (ESI): calculated for $[M+H]^+$ (carboxylic acid): 268.1081, found: 268.1083.

Central AAs and AB-central AA-fragments

Synthesis of (S)-2-(4-(4-cyanobenzamido)benzamido)pent-4-ynoic acid ***(26)***



a) Under Argon atmosphere: (*S*)-Propargyl glycine (340 mg, 3.01 mmol, 1.00 Eq) was suspended in MeOH (10 mL) and TMS-Cl (1.9 mL, 15 mmol, 5.0 Eq) was added. The clear solution was stirred overnight before being concentrated u.r.p. and coevaporated several times with MeCN. The intransparent viscous liquid was directly coupled in the next step.

b) Adapted from[11, 96]: 4-(4-cyanobenzamido)benzoic acid **10** (762 mg, 2.86 mmol, 1.00 Eq) and HATU (1.09 mg, 1.00 Eq) were solved in dry DMF (20 mL) and DiPEA (1.0 mL, 2.0 Eq) was added. The reaction was stirred for 2 h while reaction was monitored by LCMS. Then a solution of the material from part a) in dry DMF (7 mL) and DiPEA (1.0 mL, 2.0 Eq) were added and the reaction stirred 1.5 h. The solution was neutralized with HCl (5.7 mL, 2 M) and concentrated to a volume of ~5 mL. (Note: smaller amounts of acid may increase yield). DCM (~5 mL) and water were added to the concentrate until great amounts of precipitate were formed. The precipitate was filtered through pore size 4 and washed several times with water before it was dried u.r.p. to give ~0.94 g crude product.

The filtrate solution was extracted with DCM (3x) and EA (3x), organic phases were combined and dried over Na_2SO_4. The solution was concentrated u.r.p. before water was

added to the residual solution (containing DMF) to result in precipitation. The precipitate was filtered off and washed with water. This precipitate and the precipitate from before were combined.

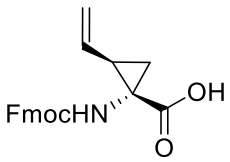
The collected precipitates were suspended in a THF/water mixture (20/20 mL) and LiOH*H_2O (0.96 g, 8.0 Eq) was added. The reaction stirred for 1.5 h before solving the reaction solution between H_2O and EA (80 mL each). (Note: Here the product stays in the alkaline aqueous phase and educt residuals stay in the organic phase). The aqueous phase was then acidified with HCl (2 M, 15 mL) and extracted with EA (1x80 mL, 2x50 mL). The organic phases were combined afterwards, dried over Na_2SO_4 and solvents were removed u.r.p. and by coevaporation (3-4x) with chloroform and heptane. (Note: acetone or MeOH should be avoided here). The product was obtained as a colorless fatty solid (849 mg, 82% o2s).

^{1}H NMR (500 MHz, DMSO): δ = 12.88 (br s, 1H), 10.70 (s, 1H), 8.70 (d, *J* = 7.9, 1H), 8.13 (d, *J* = 8.7, 2H), 8.04 (d, *J* = 8.5, 2H), 7.93 – 7.87 (m, 4H), 4.54 (ddd, *J* = 8.9, 7.8, 5.4, 1H), 2.87 (t, *J* = 2.6, 1H), 2.80 – 2.67 (m, 2H).

^{13}C NMR (126 MHz, DMSO): δ = 172.0, 165.7, 164.5, 141.6, 138.7, 132.5, 129.0, 128.6, 128.2, 119.6, 118.3, 114.0, 80.9, 72.8, 51.7, 20.8.

HRMS (ESI): calculated for $[M+H]^+$: 362.1135, found: 362.1135.

Synthesis of (1S,2R)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-vinylcyclopropane-1-carboxylic acid ***(125b)***



a) (1S,2R)-1-((*tert*-butoxycarbonyl)amino)-2-vinylcyclopropane-1-carboxylic acid **125** (200 mg, 1.00 Eq) was solved in an HCl/dioxane solution (4 M, 4.4 mL, 20 Eq) and the reaction was stirred at RT for 3 h while being screened with LCMS. The mixture was diluted with DCM and the solvents were removed u.r.p. and under high vacuum. The material was directly used in next step.

b) adapted from[223]: The material was solved in THF (1.8 mL) and water (1.8 mL) and $NaHCO_3$ (148 mg, 2.0 Eq) was added. After 10 min the gas generation was finished and Fmoc N-hydroxysuccinimide ester (297 mg, 1.00 Eq) was added portionwise. The mixture was stirred at RT overnight. After the end of reaction was checked by LCMS the reaction mixture was diluted between EA and HCl (0.1 M) (25 mL each) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were washed with brine (5 mL), dried with Na_2SO_4 and the solvents were removed u.r.p. and by coevaporation with heptane. Yield: 331 mg, >98%, NMR corrected yield: 298 mg, 97%.

The material contained NHS (6 mol%) but was used without further purification.

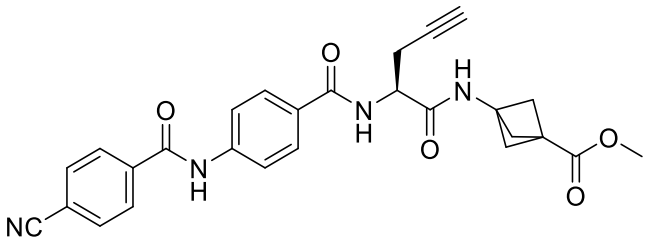
^{1}H NMR (500 MHz, DMSO): δ = 12.61 (s, 1H), 8.12 (s, 1H), 7.91 – 7.86 (m, 2H), 7.74 – 7.64 (m, 2H), 7.45 – 7.38 (m, 2H), 7.36 – 7.28 (m, 2H), 5.70 (ddd, *J*=17.2, 10.3, 9.2, 1H), 5.26 (dd, *J*=17.2, 2.1, 1H), 5.07 (dd, *J*=10.2, 2.1, 1H), 4.31 – 4.14 (m, 3H), 2.16 – 2.09 (m, 1H), 1.56 (dd, *J*=7.8, 4.9, 1H), 1.31 (dd, *J*=9.4, 5.0, 1H).

^{13}C NMR (126 MHz, DMSO): δ = 172.3, 156.1, 143.8, 140.7, 134.8, 127.6, 127.1, 125.3, 120.1, 117.1, 65.5, 46.6, 32.7, 25.2, 22.5.

HRMS (ESI): calculated for $[M+H]^+$: 350.1387, found: 350.1388.

AB-central AA-C-fragments

Synthesis of methyl (S)-3-(2-(4-(4-cyanobenzamido)benzamido)pent-4-ynamido)bicyclo[1.1.1]pentane-1-carboxylate ***(30b)***



Methyl 3-((*tert*-butoxycarbonyl)amino)bicyclo[1.1.1]pentane-1-carboxylate (200 mg, 0.83 mmol, 1.0 Eq) was solved in an HCl/MeOH solution (1.25 M, 7 mL) and the reaction was stirred overnight. The solvent was removed u.r.p. and MeOH residues were removed by coevaporation with MeCN (3x) to give a colorless solid.

Adapted from[11]: A part of this solid (HCl-salt, 71 mg, 0.40 mmol, 1.0 Eq) and the standard AB-central AA-fragment **26** (130 mg, 0.36 mmol, 0.90 Eq) were solved in dry EA (5 mL) and pyridine (0.16 mL, 5.0 Eq) and T3P (50%w in EA, 0.48 mL, 2.0 Eq) were added. The reaction was stirred for 1-2 h showing a precipitate, then DiPEA (0.14 mL, 2.0 Eq) was added to increase turnover resulting in clearing of the suspension. After another 1 h reaction and screening by TLC and LCMS the reaction mixture was diluted between DCM (20 mL) and HCl (1 M, 20 mL) and the aqueous phase was extracted with DCM (2x20 mL). The organic phases were washed with brine and water (20 mL each), dried over Na_2SO_4 and the solvent was removed u.r.p.. The material was purified by FCC (silica mass=100x reactant mass, solid loading, PE/EA, up to 60% EA) leading to exclusive elution of impurities and precipitation of the product on top of the column. The purified product was then eluted with acetone and the solvent was removed u.r.p. to get an off-colorless solid. Yield: 94 mg, 49%.

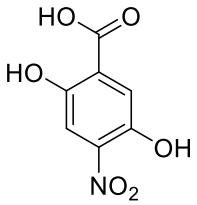
^{1}H NMR (500 MHz, DMSO): δ = 10.69 (s, 1H), 8.76 (s, 1H), 8.50 (d, *J*=8.4, 1H), 8.13 (d, *J*=8.7, 2H), 8.05 (d, *J*=8.7, 2H), 7.93 (d, *J*=9.0, 2H), 7.88 (d, *J*=9.0, 2H), 4.55 (d, *J*=22.8, 1H), 3.60 (s, 3H), 2.82 (t, *J*=2.6, 1H), 2.72 – 2.55 (m, 2H), 2.23 (s, 6H).

^{13}C NMR (126 MHz, DMSO): δ = 170.6, 169.3, 165.6, 164.4, 141.6, 138.7, 132.5, 129.1, 128.6, 128.4, 119.5, 118.3, 114.0, 81.0, 72.7, 53.9, 52.1, 51.5, 45.4, 35.6, 21.5.

HRMS (ESI): calculated for $[M+H]^+$: 485.1819, found: 485.1815.

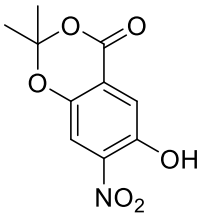
Ring D analogues

*Synthesis of 2,5-dihydroxy-4-nitrobenzoic acid **(40)***



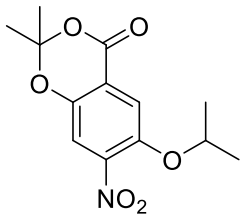
Adapted from[115]: 3-Hydroxy-4-nitrobenzoic acid (5.00 g, 27.4 mmol, 1.00 Eq) was dissolved in aqueous NaOH (2 M, 100 mL) and a solution of $K_2S_2O_8$ (7.76 g, 1.05 Eq) in H_2O (150 mL) was added. The reaction mixture was stirred at room temperature for 5 d. The reaction mixture was then cooled in an ice bath and strongly acidified by adding conc. H_2SO_4 (~15 mL). The resulting precipitate was removed by filtration and the product was extracted with small amounts of water. The aqueous solution was then refluxed for 1 h. The resulting precipitate was washed with small amounts of water and transferred with acetone in a flask, solvents were removed u.r.p. and the solid was dried in vacuum. A red-brown solid was obtained, yield: 0.961 g, 18%. Analytical data was in agreement with literature.

*Synthesis of 6-hydroxy-2,2-dimethyl-7-nitro-4H-benzo[d][1,3]dioxin-4-one **(130)***



Adapted from[114]: 2,5-dihydroxy-4-nitrobenzoic acid **40** (400 mg, 2.01 mmol, 1.00 Eq) was solved in TFA (2.6 mL) in a 2-neck-flask and TFAA (1.96 mL, 7.00 Eq) and acetone (1.0 mL, 7.0 Eq) were added. The mixture was refluxed for 6 d. The reaction mixture was concentrated carefully u.r.p., solved in EtOAc (20 mL) and washed with water (3x10 mL) before washing with saturated $NaHCO_3$-solution (1x15 mL). EtOAc (15 mL) was added and the organic phase was washed again with brine (1x15 mL) before drying over Na_2SO_4 and concentrating u.r.p. The black mush was withdrawn on Celite and purified by FCC (50x reactant mass, PE/EA, 80/20, silica particle size: 63-200 µm). After solvent removal u.r.p. a dark brown solid was obtained, yield: 328 mg, 68%. The analytical data was in agreement with literature.

Synthesis of 6-isopropoxy-2,2-dimethyl-7-nitro-4H-benzo[d][1,3]dioxin-4-one ***(41)***



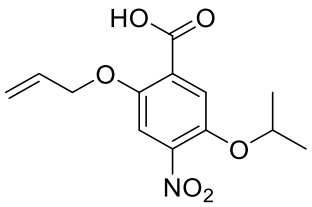
Adapted from[236]: In a nitrogen flushed flask PPh_3 (362 mg, 1.10 Eq) was solved in dried THF (2 mL) at 0°C and DIAD (0.27 mL, 1.1 Eq) was added. To the resulting yellowish suspension, **130** (300 mg, 1.25 mmol, 1.00 Eq) was added as a solution in dry THF (1.5 mL), its container was washed with 0.5 mL, that was also added. *i*PrOH (145 µL, 1.50 Eq) dried over molecular sieve (4 Å) was added and the reaction was then stirred 2 h at RT. To the mixture PE was added (30 mL) and the precipitate was filtered through celite. The solution was freed from solvent over silica u.r.p. and purified by FCC (PE/EA, 85/15, silica particle size: 63-200 µm) to give 319 mg, 90% yield of a yellowbrown liquid that slowly formed crystals.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.64 (s, 1H), 7.29 (s, 1H), 4.70 – 4.61 (m, 1H), 1.38 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 159.8, 148.9, 146.2, 145.8, 116.9, 116.4, 114.0, 107.6, 73.9, 25.9, 21.9.

HRMS (ESI): calculated for $[M+H]^+$: 282.0973, found: 282.0978.

Synthesis of 2-(allyloxy)-5-isopropoxy-4-nitrobenzoic acid ***(22)***



Adapted from[115]:

a) **41** (300 mg, 1.07 mmol, 1.00 Eq) was solved in MeOH (25 mL), sodium methoxide (173 mg, 3.00 Eq) was added and the reaction mixture stirred for 15 min at RT. HCl (1 M, 3 mL) was added and the solution was concentrated u.r.p.. The residue was solved in MTBE (25 mL) and washed with H_2O (2x15 mL). The organic layer was dried with Na_2SO_4 and solvents were removed u.r.p. to afford a dark-yellow solid. Yield: 262 mg, 96%.

b) The crude product **42** (250 mg, 980 µmol, 1.00 Eq) was solved in DMF (8 mL) and allyl bromide (169 µL, 2.00 Eq) and K_2CO_3 (203 mg, 1.50 Eq) were added. (Note: Educt and product have very similar Rf-values with PE/EA) Reaction was finished after 3.5 h (reaction shows kinetic behaviour of 0. order) and the suspension was diluted between MTBE (100 mL) and water (100 mL). The aqueous phase was extracted with MTBE (1x50 mL) before adding NaCl until saturation. The aqueous phase was further extracted with MTBE (2x50 mL). Solvent was removed u.r.p. from combined organic phases to obtain product: 299 mg, >98%, yellow solid. The product was directly used in next step.

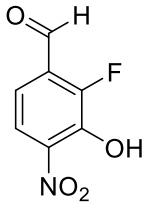
c) The crude product was solved in THF/water (2.5 mL/2.5 mL) and LiOH monohydrate (178 mg, 4.20 Eq) was added. The reaction was stirred for 3 h, before HCl (1 M, 10 mL) and 20 mL water were added and the aequos phase was extracted with MTBE (3x20 mL). Solvents were removed from organic phase u.r.p. to give a yellow-brownish solid (276 mg, 96% o3s).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.87 (s, 1H), 7.44 (s, 1H), 6.13 – 6.01 (m, 1H), 5.55 – 5.43 (m, 2H), 4.78 (dt, *J*=5.7, 1.3, 2H), 4.73 – 4.64 (m, 1H), 1.37 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 164.3, 150.1, 145.7, 143.8, 130.5, 122.5, 121.4, 121.3, 110.7, 73.9, 72.0, 21.9.

HRMS (ESI): calculated for $[M+H]^+$: 282.0973, found: 282.0978.

*Synthesis of 2-fluoro-3-hydroxy-4-nitrobenzaldehyde **(47c)***



2-fluoro-3-hydroxy-benzaldehyde (150 mg, 1.07 mmol, 1.00 Eq) was solved in MeCN (11 mL) and HNO_3 (fum., 0.45 mL, 10 Eq) was added dropwise at 0°C. The reaction was stirred for 30 min at 0°C while being screened continuously by LCMS and then quenched by adding sat. $NaHCO_3$ sol. (20 mL). The pH was adjusted to ~1-3 (color change!) with HCl (1 M). The aqueous phase was extracted with EA (2x20 mL), the combined organic phases were washed with brine (1x10 mL), dried over Na_2SO_4 and the solvent was removed u.r.p.. The correct regioisomer was isolated by FCC (solid-loading, 85x theoretical product mass, PE/EA/AcOH, 85/10/5) to give yellow crystals (yield: 76 mg, 38%).

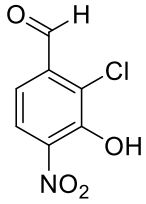
^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.51 (s, 1H), 10.45 (d, *J*=0.8, 1H), 8.01 (ddd, *J*=9.0, 2.1, 0.9, 1H), 7.45 (dd, *J*=9.1, 6.0, 1H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 185.6 (d, *J*=7.0, COH), 156.3, 154.2, 145.6, 129.5, 128.8, 120.1 (d, *J*=4.0, C_{Ar}-H), 116.7 (C_{Ar}-H).

^{19}F NMR (471 MHz, $CDCl_3$): δ = -139.4.

HRMS (ESI): calculated for $[M+H]^+$: 186.0197, found: 186.0199.

*Synthesis of 2-chloro-3-hydroxy-4-nitrobenzaldehyde **(47a)***



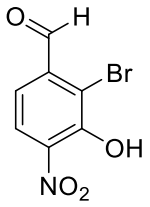
2-chloro-3-hydroxy-benzaldehyde (400 mg, 2.55 mmol, 1.00 Eq) was solved in MeCN (25 mL) and HNO_3 (fum., 1.1 mL, 10 Eq) was added dropwise at 0°C. The reaction was stirred for 30 min at 0°C while being screened continuously by LCMS and then quenched by adding sat. $NaHCO_3$ sol. (40 mL) and diluted between water/EA (75 mL each phase). The pH of the aqueous phase was adjusted to ~1 (color-change from orange to yellow!) with HCl(aq., 2 M). The aqueous phase was extracted with EA (2x30 mL), the combined organic phases were washed with brine (1x25 mL), dried over Na_2SO_4 and the solvent was removed u.r.p.. The correct regioisomer was isolated by FCC (solid-loading, 75x theoretical product mass, PE/EA/AcOH, 85/10/5->50/50/0) to give yellow crystals (yield: 225 mg, 44%).

^{1}H NMR (500 MHz, Acetone): δ = 10.52 (d, *J*=0.9, 1H), 8.23 (dd, *J*=8.9, 0.9, 1H), 7.54 (d, *J*=8.9, 1H).

^{13}C NMR (126 MHz, Acetone): δ = 189.5 (COH), 152.0, 138.7, 138.6, 128.7, 124.5 (C_{Ar}-H), 119.2.

HRMS (ESI): calculated for $[M+H]^+$: 201.9902, found: 201.9901.

*Synthesis of 2-bromo-3-hydroxy-4-nitrobenzaldehyde **(47b)***



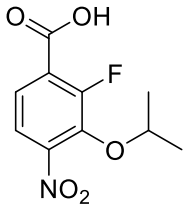
2-bromo-3-hydroxy-benzaldehyde (100 mg, 0.497 mmol, 1.00 Eq) was solved in MeCN (5.0 mL) and HNO_3 (fum., 0.21 mL, 10 Eq) was added dropwise at 0°C. The reaction was stirred for 30 min at 0°C while being screened continuously by LCMS and then quenched by adding sat. $NaHCO_3$ sol. (10 mL) and diluted between water/EA (50 mL each). The organic phase was collected and the pH of the aqueous phase was adjusted to ~4-5 (color-change from orange to yellow!) with HCl (aq.). The aqueous phase was then extracted with EA (2x30 mL), the combined organic phases were dried over Na_2SO_4 and solvent was removed u.r.p.. The correct regioisomer was isolated by FCC (solid-loading, 100x theoretical product mass, PE/EA/AcOH 90/5/5->85/10/5). Yield: 54 mg, 44%.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 11.28 (s, 1H), 10.46 (d, *J*=0.9, 1H), 8.21 (dd, *J*=8.9, 0.9, 1H), 7.54 (d, *J*=8.9, 1H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 190.6 (COH), 152.9, 139.4, 136.6, 124.2 (C_{Ar}-H), 119.6 (C_{Ar}-H), 119.0.

HRMS (ESI): calculated for $[M+H]^+$: 245.9397/ 247.9376, found: 245.9395/ 247.9375.

*Synthesis of 2-fluoro-3-isopropoxy-4-nitrobenzoic acid **(48c)***



a) Adapted from[72]: 2-Fluoro-3-hydroxy-4-nitro-benzaldehyde **47c** (72 mg, 0.39 mmol, 1.0 Eq) was suspended in a *t*BuOH/THF-mixture (1.6 mL each) and 2-Me-2-butene (0.41 mL, 10 Eq) was added. $NaClO_2$ (80%, 88 mg, 2.0 Eq) solved in aqueous NaH_2PO_4-sol. (1 M, 0.74 mL) at 0°C was added dropwise in 3 portions after 0 min, 15 min and 1 h. 2-Me-2-butene (0.21 mL, 5 Eq) was added after 3 h and $NaClO_2$ (80%, 3x22 mg, 3x0.5 Eq) was added again in aqueous NaH_2PO_4-sol. (1 M, 3x0.18 mL) after 3 h, 3.5 h and 4 h. After 5 h and screening by LCMS the reaction mixture was concentrated u.r.p. to ~half volume,

diluted between EA/HCl (0.5 M) (30 mL each), the pH was controlled to be ~1 and the aqueous phase was extracted with EA (2x20 mL). The organic phases were collected, dried over Na_2SO_4 and solvents were removed u.r.p. to get a yellow solid directly used in next reaction.

b) PPh_3 (224 mg, 2.2 Eq) was solved in dry THF (1.5 mL) and DIAD (0.17 mL, 2.2 Eq) was added at 0°C. After precipitation *i*PrOH (75µL, 2.5 Eq) was added and then the product from a) (78 mg, 1.0 Eq) was added as solution in dry THF (2.5 mL) at 0°C. The reaction was stirred at RT overnight and then $LiOH*H_2O$ (163 mg, 10 Eq) and H_2O (4.0 mL) were added to the reaction solution. The mixture was then stirred vigourously at RT for 3 h while being screened with TLC. After 2.5 h the mixture was diluted between EA and NaOH (0.5 M) (30 mL each). The organic phase was extracted with NaOH (0.5 M, 2x10 mL) and organic phase was analysed for product residuals by TLC. The combined aqueous phases were washed with DCM (2x10 mL) and then acidified with HCl (2 M, 20 mL). The product was now extracted with EA (3x20 mL), the organic phases were dried over Na_2SO_4 and a beige solid was obtained (52 mg, 44% o2s) after solvent removal u.r.p..

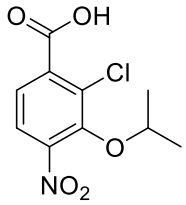
^{1}H NMR (500 MHz, Acetone): δ = 7.80 (dd, *J*=8.7, 6.4, 1H), 7.73 (dd, *J*=8.7, 1.7, 1H), 4.69 (heptd, *J*=6.2, 1.3, 1H), 1.34 (dd, *J*=6.1, 0.9, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 163.9, 157.6, 155.5, 148.8, 140.8, 130.5, 126.5 (C_{Ar}-H), 124.8 (d, *J*=10.1), 119.8 (d, *J*=4.9, C_{Ar}-H), 79.9, 22.6.

^{19}F NMR (471 MHz, Acetone): δ = -122.7.

HRMS (ESI): calculated for $[M+H]^+$: 244.0616, found: 244.0616.

*Synthesis of 2-chloro-3-isopropoxy-4-nitrobenzoic acid **(48a)***



a) Adapted from[72]: 2-Chloro-3-hydroxy-4-nitro-benzaldehyde **47a** (220 mg, 1.10 mmol, 1.0 Eq) was suspended in a *t*BuOH/THF mixture (4.5 mL each) and 2-Me-2-butene (1.2 mL, 10 Eq) was added. $NaClO_2$ (80%, 0.25 g, 2.0 Eq) solved in aqueous NaH_2PO_4-sol. (1 M, 2.0 mL) at 0°C was added dropwise in 3 portions after 0 min, 15 min and 1 h. 2-Me-

2-butene (0.58 mL, 5 Eq) was added after 3 h and $NaClO_2$ (80%, 3x62 mg, 3x0.5 Eq) was added again in aqueous NaH_2PO_4-sol. (1 M, 3x0.50 mL) after 3 h, 3.5 h and 4 h. After 4 h 45 min and screening by LCMS the reaction mixture was concentrated u.r.p. to ~half volume, diluted between EA/HCl (0.5 M) (50 mL each), the pH was controlled to be ~1 and the aqueous phase was extracted with EA (2x30 mL). The organic phases were collected, dried over Na_2SO_4 and solvents were removed u.r.p. to get a yellow solid directly used in next reaction.

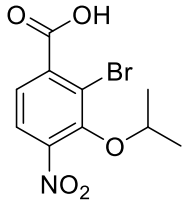
b) PPh_3 (0.63 g, 2.2 Eq) was solved in dry THF (3.5 mL) and DIAD (0.47 mL, 2.2 Eq) was added at 0°C. After precipitation *i*PrOH (0.21 mL, 2.5 Eq) was added and then the product from a) (238 mg, 1 Eq) was added dropwise as solution in dry THF (6.5 mL) at 0°C. The reaction was stirred at RT overnight and then $LiOH*H_2O$ (0.46 g, 10 Eq) and water (10 mL) were added to the reaction solution. The mixture was heated to 60°C for 3.5 h under vigourous stirring. After 2.5 h the mixture was diluted between EA and NaOH (0.5 M) (50 mL each). The organic phase was extracted with NaOH (0.5 M, 2x20 mL) and organic phase was analysed for product residuals by TLC. The combined aqueous phases were washed with DCM (2x15 mL) and then acidified with HCl (2 M, 45 mL). The product was now extracted with EA (4x30 mL), the organic phases were dried over Na_2SO_4 and a colorless-orange solid was obtained (138 mg, 49% o2s) after solvent removal u.r.p..

^{1}H NMR (500 MHz, Acetone): δ = 7.88 (d, *J*=8.5, 1H), 7.71 (d, *J*=8.4, 1H), 4.62 (hept, *J*=6.1, 1H), 1.31 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 166.0, 148.4, 138.2, 133.1-129.5 (m), 125.9 (C_{Ar}-H), 123.7 (C_{Ar}-H), 79.9, 22.4.

HRMS (ESI): calculated for $[M+H]^+$: 260.0321, found: 260.0320.

*Synthesis of 2-bromo-3-isopropoxy-4-nitrobenzoic acid **(48b)***



a) Adapted from[72]: 2-Bromo-3-hydroxy-4-nitro-benzaldehyde **47b** (48.4 mg, 1.0 Eq) was solved in a THF/*t*BuOH-mixture (0.8 mL each) and 2-Me-2-butene (0.21 mL, 10 Eq) was added at 0°C to avoid evaporation. $NaClO_2$ (80%, 27 mg, 1.2 Eq) solved in aqueous

NaH_2PO_4-solution (1 M, 0.20 mL) at 0°C was added and the reaction stirred for 2 h 45 min at RT. The reaction mixture was diluted between EA/HCl (0.1 M) (30 mL each), the pH was controlled to be ~1-2 and the aqueous phase was extracted with EA (2x10 mL). The organic phases were collected, dried over Na_2SO_4 and solvents were removed u.r.p. to get a yellow solid directly used in next reaction.

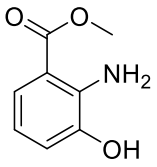
b) PPh_3 (119 mg, 2.3 Eq) was solved in dry THF (0.5 mL) and DIAD (89 µL, 2.3 Eq) was added at 0°C. After precipitation *i*PrOH (40µL, 2.6 Eq) was added and then the product from a) (51.5 mg, 1.0 Eq) was added as solution in dry THF (1.5 mL) at 0°C. The reaction was stirred at RT and *i*PrOH (8 µL, 0.5 Eq) was added again after 1.5 h. After 2 h $LiOH*H_2O$ (87 mg, 10 Eq) and water (2.0 mL) were added to the reaction solution. The mixture was stirred vigourously for 30 min at RT, then at 60°C while being screened by TLC. After 4 h the reaction mixture was diluted between EA (30 mL) and brine/NaOH (1 M) (25 mL/ 5 mL). The organic phase was extracted with H_2O (1x15 mL), the combined aqueous phases were washed with EA (20 mL) and then acidified with HCl (1 M). The product was now extracted with EA (2x10 mL, 1x15 mL), the organic phases were dried over Na_2SO_4 and an off-colorless-solid was obtained (42.1 mg, 67% o2s) after solvent removal u.r.p. and by coevaporation with *n*-heptane.

^{1}H NMR (500 MHz, Acetone): δ = 7.92 (d, *J*=8.4, 1H), 7.64 (d, *J*=8.4, 1H), 4.59 (hept, *J*=6.3, 1H), 1.31 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 166.9, 149.2, 147.9, 141.2, 125.6, 124.6, 118.9, 80.0, 22.3.

HRMS (ESI): calculated for $[M+H]^+$: 303.9815/ 305.9795, found: 303.9818/ 305.9798.

*Synthesis of methyl 2-amino-3-hydroxybenzoate **(65)***



2-Hydroxy-3-amino benzoic acid **53** (1.12 g, 7.28 mmol, 1.00 Eq) was suspended in MeOH (50 mL) and H_2SO_4 (98%, 2.5 mL, 6.5 Eq) was added dropwise forming a clear dark brown solution. The mixture was refluxed overnight and after 24 h solvent volume was reduced to ~half volume by removing the refluxing device. Then NaOH (2 M) was added dropwise

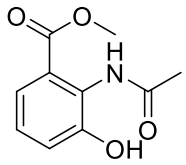
until neutralization occured. The solution was diluted between DCM (100 mL) and H_2O (100 mL) and the aqueous phase was extracted with DCM (3x50 mL). Combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p. to give 909 mg, 75% yield of a dark brown solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.48 (dd, *J* = 8.2, 1.4, 1H), 6.82 (dd, *J* = 7.6, 1.4, 1H), 6.50 (t, *J* = 7.9, 1H), 5.4 (br s, 3H), 3.87 (s, 3H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.9, 143.2, 140.7, 123.5, 118.1, 115.2, 111.6, 51.8.

HRMS (ESI): calculated for $[M+H]^+$: 168.0656, found: 168.0657.

*Synthesis of methyl 2-acetamido-3-hydroxybenzoate **(55)***

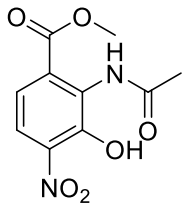


Crude methyl 2-amino-3-hydroxybenzoate **65** (0.91 g, 5.4 mmol, 1.0 Eq) and acetic anhydride (0.54 mL, 1.05 Eq) were stirred in dry pyridine (15 mL) at RT for 26 h. The reaction was screened by LCMS (Note: first a mixture of *O*- or *N*-acetylated product is formed which slowly converts to the *N*-acetylated product). The reaction solution was diluted between DCM (50 mL) and HCl (2 M, 100 mL). The aqueous phase was extracted with DCM (3x30 mL) and organic phases were washed with H_2O (1x30 mL). Solvents were removed u.r.p. and a brown solid was obtained (1.12 g, 99%, NMR: 90%) that was used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 11.21 (s, 1H), 9.88 (s, 1H), 7.61 (dd, *J* = 7.8, 1.5, 1H), 7.24 (dd, *J* = 8.1, 1.5, 1H), 7.14 (t, *J* = 8.0, 1H), 3.93 (s, 3H), 2.34 (s, 4H).

HRMS (ESI): calculated for $[M+H]^+$: 210.0761, found: 210.0759.

*Synthesis of methyl 2-acetamido-3-hydroxy-4-nitrobenzoate **(56a)***



Methyl 2-acetamido-3-hydroxybenzoate **55** (300 mg, 1.43 mmol, 1.00 Eq) was solved in dry acetone (14.3 mL) under Argon atmosphere. After stirring at 0°C for 10 min, HNO_3

(fuming, 0.48 mL, 8.0 Eq) was added dropwise. The reaction progress was then monitored carefully by LCMS. After 75 min HNO_3 (0.18 mL, 3.0 Eq) was added again and the reaction was stirred further for 45 min while being screened by LCMS.

The reaction was quenched by pouring the solution into sat. $NaHCO_3$ sol. (50 mL). The aqueous phase was extracted with DCM (3x40 mL or 1x50 mL) (organic phase 1). Then the pH of the aequos solution was adjusted to pH~5, changing its color from red to yellow and the solution was further extracted with DCM (3x40 mL) (organic phase 2). Organic phase 2 was dried over Na_2SO_4 and solvent was removed u.r.p.. To improve the yield organic phase 1 was reextracted with water and sat. $NaHCO_3$ sol. (40 mL each), followed by neutralization and extraction with DCM (2x30 mL). Dried over Na_2SO_4, this solution was combined with organic phase 2.

The reaction was repeated with same amounts of material but the second portion of HNO_3 (3.0 Eq) was already added after 15 min and the reaction was already quenched after 1 h. The workup was the same as shown above. The crude products from both reactions were combined and purified by FCC (solid loading, 80x reactant mass, PE/EA/AcOH, 65/30/5). After solvent removal u.r.p. and by coevaporation with heptane, the desired regioisomer was obtained as a yellow solid: 209 mg, 29%, NMR: 27%.

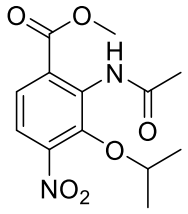
A mixed fraction was also collected, containing both regioisomers (238 mg).

^{1}H NMR (500 MHz, Acetone): δ = 10.97 (s, 1H), 10.03 (s, 1H), 7.85 (d, *J*=8.7, 1H), 7.51 (d, *J*=8.9, 1H), 3.90 (s, 3H), 2.26 (s, 3H).

^{13}C NMR (126 MHz, Acetone): δ = 171.6, 167.1, 147.1, 140.7, 130.1, 130.0, 121.5, 121.1, 53.1, 23.6.

HRMS (ESI): calculated for $[M+H]^+$: 255.0612, found: 255.0611.

Synthesis of Methyl 2-acetamido-3-isopropoxy-4-nitrobenzoate ***(59)***



PPh_3 (56 mg, 2.0 Eq) was solved in dry THF (0.2 mL) under Ar-atmosphere and DIAD (42 µL, 2.0 Eq) was added at 0°C. After precipitation occured, *i*PrOH (16.4 µL, 2.0 Eq) was

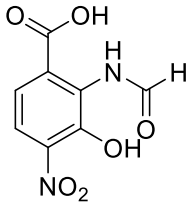
added and then a solution of methyl 2-acetamido-3-hydroxy-4-nitrobenzoate **56a** (27 mg, 0.11 mmol, 1.0 Eq) in dry THF (0.6-0.8 mL) was added dropwise to the reaction. The reaction was stirred for 1.5 h and monitored by TLC, before solving the solution between brine and EA (15 mL each). The aqueous phase was extracted with EA (1x20 mL or 2x15 mL) and the combined organic phases were dried over Na_2SO_4. Solvents were removed u.r.p. to get a brown oil. This crude material was either used directly in next reaction or was purified by FCC (solid loading, 300x reactant mass, PE/acetone, 75/25) to give a brownish solid, yield 17.7 mg, 56%.

^{1}H NMR (700 MHz, $CDCl_3$): δ = 8.15 (s, 1H), 7.63 (d, *J* = 8.6, 1H), 7.59 (d, *J* = 8.6, 1H), 4.23 (hept, *J* = 6.2, 1H), 3.91 (s, 3H), 2.20 (s, 3H), 1.26 (d, *J* = 6.2, 6H).

^{13}C NMR (176 MHz, $CDCl_3$): δ = 168.6, 166.2, 146.1, 144.8, 132.7, 130.9, 124.9, 121.1, 79.6, 53.0, 23.8, 22.5.

HRMS (ESI): calculated for $[M+H]^+$: 297.1081, found: 297.1081.

*Synthesis of 2-formamido-3-hydroxy-4-nitrobenzoic acid **(62)***



a) 2-amino-3-hydroxy benzoic acid **53** (1.00 g, 6.53 mmol, 1.00 Eq) was solved in HCOOH (pure, 20 mL) and Ac_2O (0.65 mL, 1.05 Eq) was added dropwise. After 1 h the precipitate was isolated, using pore size filter 4. Water (3x10 mL) was used for washing and the precipitate was collected mechanically and by solvation in EA (fraction A).

The washing solution was diluted between water (100 mL) and EA (50 mL) and the aqueous phase was extracted with EA (2x50 mL). The organic phases were combined, washed with brine (25 mL) and dried over Na_2SO_4 (fraction B). After LCMS analysis both fractions were combined and solvents were removed u.r.p. and by coevaporation with heptane to give a grey-reddish powder (1.13 g). The material was used without further purifications in the next step.

b) A part of the intermediate (0.80 g, 4.4 mmol, 1.0 Eq) was suspended in dry DCM (45 mL) under Argon atmosphere and cooled down to -25°C. Then HNO_3 (fum., 1.85 mL,

10.0 Eq) was added over 10 min resulting in a dark brown lumpy suspension. After 3 h the reaction mixture was quantitatively poured into water (50 mL, then 150 mL added portionwise) forming two phases. After extraction, the organic phase was collected and the aqueous phase (together with a precipitate between the two phases) was extracted with EA (4x40 mL). The combined organic phases were washed with brine, dried over Na_2SO_4 before adding toluene (100 mL) as a scavenger for nitronium. The orange brown-solution was concentrated u.r.p. to the toluene fraction (down to ~80 mbar, 40°C).

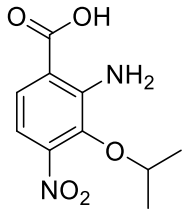
The residual suspension (~50 mL) was stored over 4 d in fridge forming a dark brown precipitate. The precipitate was isolated using pore size filter 4. Toluene (2x10 mL) was used for washing and the product was collected mechanically and by solvation in EA. The solvent was removed u.r.p. to give a yellow-brown fine solid (327 mg, 31% o2s). Note: A purity of 96% of this regioisomer was confirmed by LCMS while the filtrate contained almost exclusively a different regioisomer.

^{1}H NMR (500 MHz, DMSO): δ = 13.36 (br s, 1H), 10.85 (br s, 1H), 10.07 (s, 1H), 8.25 (s, 1H), 7.84 (d, *J*=8.7, 1H), 7.41 – 7.23 (m, 1H).

^{13}C NMR (126 MHz, DMSO): δ = 166.6, 160.7, 146.6, 138.9, 133.9, 126.1, 121.5, 119.6.

HRMS (ESI): calculated for $[M+H]^+$: 227.0299, found: 227.0299.

*Synthesis of 2-amino-3-isopropoxy-4-nitrobenzoic acid **(61)***



Method 1:

a) PPh_3 (230 mg, 1.1 Eq) was solved in dry THF (3 mL) under Argon atmosphere and DIAD (0.17 mL, 1.1 Eq) was added at 0°C. The reagent solution was stirred for 15 min at 0°C before adding methyl 2-acetamido-3-isopropoxy-4-nitrobenzoate **59** (203 mg, 0.799 mmol, 1.00 Eq) as a solution in dry THF (5 mL) dropwise. After this, *i*PrOH (92 µL, 1.5 Eq) was added and the reaction was stirred for 3 h while being monitored with TLC.

b) The reaction mixture was concentrated u.r.p. to ~half volume, then KOH (4 M, 15 mL) was added and the mixture was stirred at 80°C before raising the temperature to 95°C

after 1 h. After 2 h the heat source was switched off and the reaction mixture was stirred overnight. The next day the temperature was increased again to 95°C to ensure full conversion. After 2.5 h and reaction control by LCMS the reaction solution was extracted with EA (1x30 mL). The organic phase was extracted with NaOH (1 M, 2x20 mL) and the combined aqueous phases were washed with DCM (2x15 mL). After the pH was adjusted to ~7 using conc. HCl. the neutralized aqueous phase was extracted with EA (3x20 mL). Combining the organic phases was followed by drying over Na_2SO_4. Solvent removal u.r.p. and coevaporation with heptane (5x) resulted in a dark orange-brown solid (139 mg, 72% o2s).

Method 2:

a) PPh_3 (0.66 g, 2.1 Eq) was solved in dry THF (5.0 mL) under Argon atmosphere and DIAD (0.49 mL, 2.1 Eq) was added dropwise at 0°C forming a precipitate after few minutes. The mixture was stirred for 10 min and then *i*PrOH (0.23 mL, 2.5 Eq) was added. After 10 min 2-formamido-3-hydroxy-4-nitrobenzoic acid **62** (270 mg, 1.19 mmol, 1.00 Eq) was added dropwise as suspension in dry THF (7.0 mL) over 10 min. Then the mixture was stirred at RT for 1 h before it was diluted with EA. After adsorbtion on silica the material was purified by FCC (solid loading, 130x theoretical product mass, cyclohexane/EA 85/15->80/20->70/30) to give an off colorless solid that was dried under HV overnight and directly used in next step:

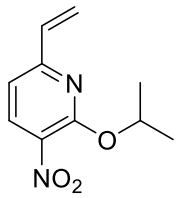
b) The crude material was suspended in a THF/water mixture (6 mL each) and $LiOH*H_2O$ (0.50 g, 10 Eq) was added. The reaction was then stirred at 60°C while being screened with LCMS. After 4 h water (6 mL) was added again and 2 h later the mixture was concentrated u.r.p. and the residue was diluted between DCM and H_2O (70 mL each). The aqueous phase was washed with DCM (1x30 mL), brine (5 mL) was added and washing was continued with EA (3x30 mL). The aqueous phase was then acidified to pH~5 by adding HCl (1 M) before it was extracted with EA (4x30 mL). The combined organic phases were dried over Na_2SO_4 before solvents were removed u.r.p. and by multiple coevaporation with heptane to give yellow-orange crystals (181 mg, 63% o2s). (Note: Reextraction of the former organic washing solution could not raise the yield significantly)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.74 (d, *J* = 8.9, 1H), 6.96 (d, *J* = 9.0, 1H), 4.28 (hept, *J* = 6.1, 1H), 1.34 (d, *J* = 6.1, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0, 148.2, 146.7, 138.4, 127.0, 112.0, 110.2, 78.9, 22.7.

HRMS (ESI): calculated for $[M+H]^+$: 241.0819, found: 241.0820.

*Synthesis of 2-isopropoxy-3-nitro-6-vinylpyridine **(74)***



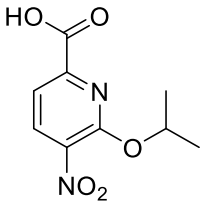
Potassium vinyltrifluoroborate (618 mg, 2.00 Eq), K_2CO_3 (415 mg, 1.30 Eq) and 6-chloro-2-isopropoxy-3-nitropyridine (synthesized as described in[109], 500 mg, 2.31 mmol, 1.00 Eq) were subsequently added to a Schlenk flask. Dioxane (11 mL) and H_2O (3.0 mL) were added under Argon atmosphere and after a solution was formed, the mixture was freezed in liquid nitrogen. The flask was evacuated before refilling with Ar and warming up to RT. This procedure was repeated once again before $Pd(PPh_3)_4$ (67 mg, 0.025 Eq) was added. After one more freeze-degas cycle the mixture was stirred under Ar at 100°C for 17 h. The reaction mixture was diluted between H_2O/EA (50 mL each) and the aqueous phase was extracted with EA (2x30 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was purified by FCC (solid loading, 50x reactant mass, cyclohexane/EA, 95/5) to give a yellow oil (450 mg, 94%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.21 (d, *J* = 8.1, 1H), 6.89 (d, *J* = 8.1, 1H), 6.72 (dd, *J* = 17.1, 10.5, 1H), 6.37 (dd, *J* = 17.2, 1.4, 1H), 5.62 (dd, *J* = 10.5, 1.5, 1H), 5.60 – 5.52 (m, 1H), 1.44 (d, *J* = 6.3, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 157.6, 155.7, 136.1, 135.1, 122.6, 114.0, 70.8, 22.0.

HRMS (ESI): calculated for $[M+H]^+$: 209.0921, found: 209.0921.

*Synthesis of 6-isopropoxy-5-nitropicolinic acid **(73)***



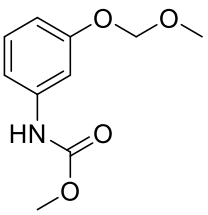
Adapted from[109]: 2-isopropoxy-3-nitro-6-vinylpyridine **74** (442 mg, 2.12 mmol, 1.00 Eq) was solved in acetone (7 mL) and a solution of $KMnO_4$ (1.34 g, 4.00 Eq) in acetone/water (18 mL each) was added within 1 min. The reaction was stirred at RT for 20 h. The mixture was basified by mixing with NaOH (1 M, 2.1 mL) and the dark brown suspension was filtered through a pad of celite. The precipitate was washed with water and acetone and the pH of the yellow filtrate was adjusted to pH~4 by adding $NaHSO_4$-sol. (1 M). The solution was concentrated u.r.p. and the remaining aqueous solution was extracted with EA (4x20 mL). The combined organic phase was washed with brine (1x10 mL) before drying over Na_2SO_4 and removing solvents u.r.p.. An orange crystalline solid (413 mg, 86%) was obtained.

^{1}H NMR (500 MHz, DMSO): δ = 13.73 (s, 1H), 8.50 (d, *J* = 8.0, 1H), 7.75 (d, *J* = 8.0, 1H), 5.51 (hept, *J* = 6.2, 1H), 1.35 (d, *J* = 6.1, 6H).

^{13}C NMR (126 MHz, DMSO): δ = 164.4, 154.2, 149.2, 136.3, 136.1, 117.8, 71.0, 21.6.

HRMS (ESI): calculated for $[M+H]^+$: 227.0663, found: 227.0660.

*Synthesis of methyl (3-(methoxymethoxy)phenyl)carbamate **(88a)***



Adapted from[191-192]:

In an Ar flushed flask 3-aminophenol (10.0 g, 91.6 mmol, 1.00 Eq) was solved in dry DMF (50 mL) and NaH (60% in mineral oil, 3.66 g, 1.00 Eq) was added in portions. After gas evolution calmed down, the mixture was stirred at 50°C for 30 min. The brownish suspension was stirred for 30 min before adding MOMCl (technical, 7.0 mL, 1.0 Eq) dropwise. The reaction was stirred further at 50°C. After 2 h, NaH (60% in mineral oil,

1.32 g, 0.60 Eq) was added again in portions before adding MOMCl (technical, 2.8 mL, 0.40 Eq) after 15 min dropwise.

The reaction mixture was diluted with water (200 mL). The aqueous reaction solution was extracted with EA (1x200 mL, 2x150 mL). The organic phase was dried over Na_2SO_4 and the solution was concentrated until ~100 mL.

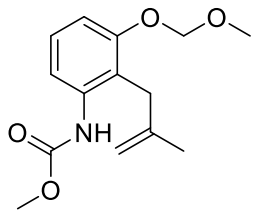
Dry pyridine (15 mL, 2.0 Eq) was added to the product solution and then Methyl chloroformiate (7.8 mL, 1.1 Eq) was added dropwise over 15 min. The reaction was stirred for 15 min at RT before solving between EA (100 mL) and H_2O (100 mL) and extracting the aqueous phase with EA (1x50 mL). The organic phase was washed with HCl (1 M, 200 mL). The combined aqueous phases were extracted again with EA (2x50 mL) after pH check. The combined organic phases were washed with brine (1x30 mL) before removing solvent u.r.p. over silica. The product was purified by filtration over silica (PE/acetone, 100/0->90/10->85/15) to obtain a clear oil (10.8 g, 56% o2s).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.20 (t, J = 8.2, 1H, H-Ar), 7.17 (s, 1H), 6.99 (d, J = 7.8, 1H, H-Ar), 6.74 (ddd, J = 8.2, 2.2, 0.9, 1H, H-Ar), 6.64 (s, 1H), 5.17 (s, 2H, O-CH_2-O), 3.77 (s, 3H, CH_3), 3.47 (s, 3H, CH_3).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 158.0 (NH-COO), 154.0 (C_{Ar}), 139.2 (C_{Ar}), 123.0 (C_{Ar}), 112.3 (C_{Ar}), 111.4 (C_{Ar}), 106.9 (C_{Ar}), 94.6 (O-CH_2-O), 56.2 (CH_3), 52.5 (CH_3).

HRMS (ESI): calculated for $[M+H]^+$: 212.0918, found: 212.0918.

*Synthesis of methyl (3-(methoxymethoxy)-2-(2-methylallyl)phenyl)carbamate **(92)***



Preparation of cuprate solution: LiCl (4.16 g, 98.1 mmol) was heated extensively u.r.p. while gassing and degassing with Ar. CuCN (4.40 g, 49.1 mmol) and dry THF (48 mL) were added under inert conditions and the green mixture was stirred until salts were solved.

Adapted from[191, 195]: Under strict Ar-atmosphere: **88a** (4.00 g, 18.9 mmol, 1.00 Eq) and a stirring bar were added to a Schlenk flask and dried u.r.p. before gassing with Ar. Then

dry THF (100 mL) was added and the mixture was cooled to -78°C by cryostate. *t*BuLi (1.6 M in pentane, 24.9 mL, 2.10 Eq) was added dropwise over 20 min, resulting in a yellow solution. The reaction was stirred for 15 min before raising temperature to -20°C over 70 min. A suspension formed which turned to a solution again above -30°C. The mixture was stirred for additional 1 h at -20°C, then cooled down again to -78°C. The mentioned solution of CuCN*2 LiCl in THF (21 mL, 1.1 Eq) was added dropwise over 20 min. After 40 min further stirring, 3-bromo-2-methylpropene (0.48 mL, 1.0 Eq) was added dropwise and the mixture was slowly warmed to -20°C over 80 min.

The reaction was quenched by adding ammonia (13% in H_2O, 130 mL) and stirring the solution vigourously for 20 min. The blueish suspension was concentrated u.r.p. and was then extracted with EA (3x70 mL). The combined organic phases were washed with brine (1x20 mL) and dried over Na_2SO_4 before removing solvent u.r.p.. The purification by FCC (solid loading, 30x reactant mass, PE/EA, 93/7->90/10->85/15) led to a slightly yellowish transparent oil after solvent removal (2.43 g, 48%).

R_f(PE/EA, 70/30)=0.55

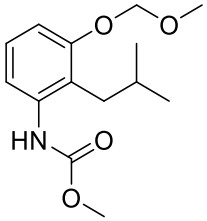
^{1}H NMR (700 MHz, $CDCl_3$): δ = 7.52 (s, 1H), 7.19 (t, *J* = 8.3, 1H, H-Ar), 6.88 (d, *J* = 8.3, 1H, H-Ar), 6.75 (s, 1H), 5.17 (s, 2H, O-CH_2-O), 4.85 (s, 1H, CH_2=C), 4.65 (s, 1H, CH_2=C), 3.75 (s, 3H, CH_3), 3.45 (s, 3H, CH_3), 3.42 (s, 2H, CH_2-Ar), 1.76 (s, 3H, C-CH_3).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 155.4 (C_{Ar}), 154.5 (NH-COO), 144.0 ($C_2\underline{C}$=C), 137.9 (C_{Ar}), 127.7 (HC_{Ar}-$\underline{C}_{Ar}$-$C_{Ar}H$), 118.3* (C_{Ar}), 115.3* (C_{Ar}), 111.4 (CH_2=C), 110.1 (C_{Ar}), 94.8 (O-CH_2-O), 56.2 (CH_2-O-$\underline{C}H_3$), 52.4 (COO-$\underline{C}H_3$), 32.9 (CH_2-Ar), 22.4 (C-$\underline{C}H_3$).

*assignment proved by HSQC

HRMS (ESI): calculated for $[M+H]^+$: 266.1387, found: 266.1387.

*Synthesis of methyl (3-(methoxymethoxy)-2-isobutylphenyl)carbamate **(89a)***



Compound **92** (2.42 g, 9.12 mmol, 1.00 Eq) was solved in MeOH (90 mL) and Pd/C (0.49 g, 0.05 Eq) was added. The flask was filled with H_2 by multiple degassing and gassing with a

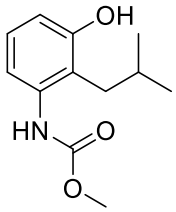
balloon and the reaction was stirred vigourously. After 3.5 h the black suspension was filtrated over celite and a pore size 4 filter. The solvent was removed u.r.p. and by coevaporation with heptane to give a transparent oil (2.37 g, 97%).

^{1}H NMR (700 MHz, $CDCl_3$): δ = 7.46 (s, 1H), 7.14 (t, J = 8.2, 1H, H-Ar), 6.88 (d, J = 8.3, 1H), 6.43 (s, 1H), 5.17 (s, 2H, O-CH_2-O), 3.77 (s, 3H, O-CH_3), 3.47 (s, 3H, O-CH_3), 2.51 (d, J = 7.4, 2H, CH_2-Ar), 1.91 – 1.80 (m, 1H, C<u>H</u>$(CH_3)_2$), 0.94 (d, J = 6.7, 6H, CH(C<u>H</u>$_3)_2$).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 155.9 (C_{Ar}-O), 154.6 (NH-COO), 136.7 (C_{Ar}), 127.1 (C_{Ar}), 115.6 (C_{Ar}), 110.0 (C_{Ar}), 94.7 (O-CH_2-O), 56.2 (CH_2-O-<u>C</u>H_3), 52.5 (COO-<u>C</u>H_3), 33.5 (CH_2-Ar), 28.9 (<u>C</u>$(CH_3)_2$), 22.8 (C(<u>C</u>$H_3)_2$).

HRMS (ESI): calculated for $[M+H]^+$: 268.1544, found: 268.1543.

*Synthesis of methyl (3-hydroxy-2-isobutylphenyl)carbamate **(93a)***



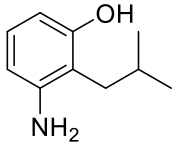
Compound **89a** (0.70 g, 2.6 mmol, 1.0 Eq) was solved in MeOH (13 mL) and TMSCl (1.7 mL, 5.0 Eq) was added dropwise. The reaction was stirred at RT for 2 h before removing the reaction solvent u.r.p. and coevaporate with heptane (3x) to give a brownish gum becoming slowly a fluffy solid (0.57 g, 97%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.37 (s, 1H), 7.05 (t, *J* = 8.1, 1H, H-Ar), 6.55 (d, *J* = 8.0, 1H, H-Ar), 6.43 (s, 1H), 5.01 (br s, 1H, OH), 3.77 (s, 3H, CH_3), 2.47 (d, *J* = 7.5, 2H, CH_2-Ar), 1.93 – 1.82 (m, 1H, C<u>H</u>$(CH_3)_2$), 0.95 (d, *J* = 6.6, 6H, CH(C<u>H</u>$_3)_2$).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 154.7 (NH-COO), 154.2 (C_{Ar}-O), 136.8 (C_{Ar}), 127.1 (C_{Ar}), 114.70 (C_{Ar}), 111.60 (C_{Ar}), 52.6 (COO-<u>C</u>H_3), 33.4 (CH_2-Ar), 28.8 (<u>C</u>$(CH_3)_2$), 22.7 (C(<u>C</u>$H_3)_2$).

HRMS (ESI): calculated for $[M+H]^+$: 224.1281, found: 224.1282.

*Synthesis of 3-amino-2-isobutylphenol **(131)***



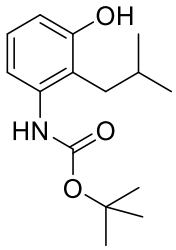
Compound **93a** (224 mg, 1.00 mmol, 1.00 Eq) was solved in KOH (4 M, 10 mL) and refluxed for 3 h. The reaction solution was poured into NaH_2PO_4-solution (1 M, 50 mL) and the aqueous phase was extracted with EA (2x30 mL). Solvents were removed u.r.p. to give a brown crystalline solid (158 mg, 95%).

^{1}H NMR (700 MHz, DMSO-d_6): δ = 8.64 (s, 1H, OH), 6.63 (t, *J* = 7.9, 1H, H-Ar), 6.08 (dd, *J* = 7.9, 1.2, 1H, H-Ar), 6.03 (dd, *J* = 7.9, 1.2, 1H, H-Ar), 4.56 (s, 2H, NH_2), 2.29 (d, *J* = 7.4, 2H, CH_2-Ar), 1.88 – 1.82 (m, 1H, C<u>H</u>$(CH_3)_2$), 0.85 (d, *J* = 6.6, 6H, CH(C<u>H</u>$_3)_2$).

^{13}C NMR (176 MHz, DMSO-d_6) δ = 155.9 (C_{Ar}-O), 147.5 (C_{Ar}-N), 126.1 (C_{Ar}), 111.45 (C_{Ar}), 106.3 (C_{Ar}), 103.5 (C_{Ar}), 32.3 (CH_2-Ar), 27.1 (<u>C</u>$(CH_3)_2$), 22.6 (C(<u>C</u>$H_3)_2$).

HRMS (ESI): calculated for $[M+H]^+$: 166.1227, found: 166.1229.

*Synthesis of tert-butyl (3-hydroxy-2-isobutylphenyl)carbamate **(93b)***



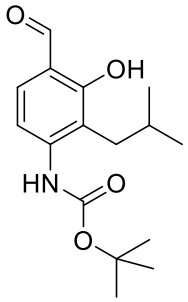
The previous product **131** (154 mg, 0.932 mmol, 1.00 Eq) was solved in dry EtOH (3.5 mL) and Boc_2O (0.32 mL, 1.5 Eq) was added dropwise over 5 min before the mixture was refluxed. The reaction solution was concentrated u.r.p. after 2 h 15 min until crystals were formed. Then the rest of Boc_2O was removed by coevaporation with EA/heptane to give a brownish solid (252 mg, >98%, NMR corrected yield: 95%).

^{1}H NMR (700 MHz, DMSO-d_6): δ = 9.17 (s, 1H, NH-COO), 8.27 (s, 1H, OH), 6.90 (t, *J* = 8.0, 1H, H-Ar), 6.68 (dd, *J* = 8.0, 1.1, 1H, H-Ar), 6.60 (dd, *J* = 8.0, 1.2, 1H, H-Ar), 2.43 (d, *J* = 7.2, 2H, CH_2-Ar), 1.82 – 1.74 (m, 1H, C<u>H</u>$(CH_3)_2$), 1.42 (s, 9H, C$(CH_3)_3$), 0.81 (d, *J* = 6.7, 6H, CH(C<u>H</u>$_3)_2$).

^{13}C NMR (176 MHz, DMSO-d_6) δ = 155.8 (C_{Ar}-O), 154.0 (NH-COO), 137.5 (C_{Ar}-N), 125.7 (C_{Ar}), 123.3 (C_{Ar}), 117.4 (C_{Ar}), 111.8 (C_{Ar}), 78.2 (<u>C</u>$(CH_3)_3$), 32.9 (CH_2-Ar), 28.2 (C(<u>C</u>$H_3)_2$), 26.9 ($(CH_3)_2$<u>C</u>H), 22.5 ((<u>C</u>$H_3)_2$CH).

HRMS (ESI): calculated for $[M+H]^+$: 266.1751, found: 266.1751.

Synthesis of tert-butyl (4-formyl-3-hydroxy-2-isobutylphenyl)carbamate ***(90b)***



A flask equipped with **93b** (179 mg, 0.675 mmol, 1.00 Eq) and heat gun dried $MgCl_2$ (129 mg, 2.00 Eq) was flushed with Ar and the content was suspended in dry MeCN (3.4 mL). Then dry TEA (0.37 mL, 4.0 Eq), DMPU (0.16 mL, 2.0 Eq) and paraformaldehyde (162 mg, 8.0 Eq) were added subsequentially. The mixture was stirred at 80°C getting yellow after few minutes.

After 2 h the mixture was diluted between HCl (0.1 M, 30 mL) and EA (30 mL) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were washed with brine (1x10 mL) and dried over Na_2SO_4 before the product was isolated by FCC (solid loading, 85x reactant mass, PE/EA, 95/5) to obtain colorless crystals (86 mg, 43%).

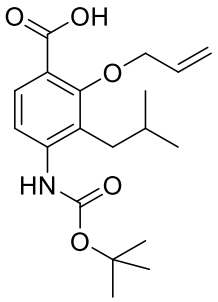
^{1}H NMR (700 MHz, $CDCl_3$): δ = 11.57* (s, 1H, OH), 9.72* (s, 1H, CHO), 7.78* (d, *J* = 8.7, 1H, H-C_{Ar}-C_{Ar}-N), 7.37* (d, *J* = 8.7, 1H, H-C_{Ar}-C_{Ar}-CHO), 6.68 (s, 1H, NH-COO), 2.49 (d, *J* = 7.5, 2H, CH_2-Ar), 1.93 – 1.87* (m, 1H, C**H**$(CH_3)_2$), 1.53* (s, 9H, $C(CH_3)_2$), 0.96* (d, *J* = 6.8, 6H, $(CH_3)_2$CH).

^{13}C NMR (176 MHz, $CDCl_3$): δ = 195.3* (COH), 161.0* (C_{Ar}-O), 152.2* (NH-COO), 144.4* (C_{Ar}-N), 132.7* (C_{Ar}-C_{Ar}-COH), 116.4 (C_{Ar}), 116.2 (C_{Ar}), 110.9* (C_{Ar}-C_{Ar}-N), 81.7 ($C(CH_3)_3$), 32.0* (CH_2-Ar), 28.3* ($C(CH_3)_3$), 28.2* ($(CH_3)_2CH$), 22.7 ($(CH_3)_2$CH).

*assignment proved by HSQC/HMBC

HRMS (ESI): calculated for $[M+H]^+$: 294.1700, found: 294.1701.

Synthesis of 2-(allyloxy)-4-((tert-butoxycarbonyl)amino)-3-isobutylbenzoic acid ***(91b)***



Adapted from[72]:

a) **90b** (85 mg, 0.29 mmol, 1.0 Eq) and K_2CO_3 (0.16 g, 4.0 Eq) were secured under Ar and dry DMF (1.5 mL) was added. Then allyl bromide (30 µL, 1.2 Eq) was added and the reaction was stirred at RT for 2 h. The mixture was diluted between EA/H_2O (25 mL each) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were dried over Na_2SO_4 before removing solvent u.r.p. to give a yellow oil (yield: 96 mg, 99%) directly used in the next step.

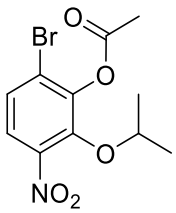
b) A part of the material from a) (86 mg, 0.26 mmol, 1.0 Eq) was solved in MeCN (1.3 mL) and aqueous NaH_2PO_4-sol. (0.5 M, 0.52 mL) and H_2O_2 (30%, 79 µL) were added. $NaClO_2$ (80%, as 1 M solution in NaH_2PO_4-buffer (1 M), 0.36 mL, 1.4 Eq) was added and the reaction was stirred at RT. After 45 min $NaClO_2$-solution (0.16 mL, 0.60 Eq) was added again and after 1 h Na_2SO_3-sol. (1 M, 5 mL) and NaH_2PO_4-sol. (1 M, 15 mL) were added. The mixture was extracted with EA (3x20 mL) and after drying the combined organic phases over Na_2SO_4 solvents were removed u.r.p.. A colorless crystalline solid (yield: 76 mg, 84% o2s) was obtained.

^{1}H NMR (700 MHz, $CDCl_3$): δ = 7.97 (d, *J*=8.8, 1H, C_{Ar}-H), 7.91 (d, *J*=8.9, 1H, C_{Ar}-H), 6.54 (s, 1H, N-H), 6.14 – 6.04 (m, 1H), 5.48 (dq, *J*=17.2, 1.6, 1H), 5.37 (dq, *J*=10.4, 1.2, 1H), 4.42 (d, *J*=5.6, 2H), 2.48 (d, *J*=7.4, 2H), 1.92 – 1.83 (m, 1H, -C<u>H</u>$(CH_3)_2$), 1.53 (s, 9H), 0.95 (d, *J*=6.6, 6H, -CH(C<u>H</u>$_3)_2$).

^{13}C NMR (176 MHz, $CDCl_3$): δ = 166.5, 157.4, 152.5, 142.8, 132.1, 131.6, 123.9, 119.6, 117.2, 117.1, 81.7, 77.1, 33.7, 28.9, 28.4, 22.8.

HRMS (ESI): calculated for $[M+H]^+$: 350.1962, found: 350.1962.

Synthesis of 6-bromo-2-isopropoxy-3-nitrophenyl acetate ***(101)***[11]



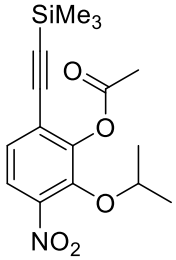
a) 2-bromo-6-isopropoxyphenol (synthesized as reported[11], 2.20 g, 9.5 mmol, 1.00 Eq) was added to a nitrogen-flushed flask and dry pyridine (4.5 mL, 6.0 Eq) and Ac_2O (1.8 mL, 2.0 Eq) were added at 0°C. The reaction was stirred for 2 h at RT. HCl(aq) (1 M, 80 mL) and brine (120 mL) were added, the aequeous phase was extracted with Et_2O (3x50 mL) then the organic phase was washed with brine (2x8 mL). Solvent was removed under reduced pressure to give a colorless oil, 2.55 g (97%). The product was directly used in the next step.

b) The intermediate (747 mg, 2.73 mmol, 1.00 Eq) was solved in DCM (4.5 mL), the solution was added dropwise to fuming HNO_3 (1.5 mL, 13 Eq) at -40°C (cooled with MeCN/dry ice mixture). After full educt conversion the reaction mixture was quenched with 25 mL water and the aqueous phase was extracted with DCM (3x20 mL). The product was adsorbed on celite and purified by FCC (PE/EA, 90/10, silica particle size: 63-200 µm). Product was obtained as yellow crystals, yield: 693 mg, 80%.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.63 (d, 1H, *J*=9.0, Ar-H), 7.43 (d, 1H, *J*=8.9, Ar-H), 4.44 (hept, 1H, *J*=6.1, C-H), 2.39 (s, 3H, CO-CH_3), 1.31 (d, 6H, *J*=6.3, CH($C\underline{H}_3$)$_2$).

^{13}C NMR (126 MHz, $CDCl_3$): 166.9, 145.7, 144.5, 144.4, 127.1, 123.4, 123.1, 79.7, 22.6, 20.6.

Synthesis of 2-isopropoxy-3-nitro-6-((trimethylsilyl)ethynyl)phenyl acetate ***(102)***



Adapted from[204]: 6-bromo-2-isopropoxy-3-nitrophenyl acetate **101** (516 mg, 1.62 mmol, 1.00 Eq), CuI (31 mg, 0.10 Eq) and $PdCl_2(PPh)_3$ (57 mg, 0.05 Eq) were added to a flask which subsequently was flushed with nitrogen (3x). Dry DMF (2 mL) and DiPEA (1.4 mL, 5.0 Eq) were added and Ethynyl-TMS (0.48 g, 3.0 Eq) was introduced as a

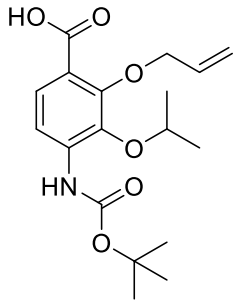
solution in dry DMF (2 mL). The mixture turned its color to brownish and was stirred overnight at 35°C. The reaction mixture was diluted with DCM (30 mL) and organic phase was washed with HCl (1 M, 2x30 mL). The aqueous phase was reextracted with DCM (2x 25 mL). The solvent was removed u.r.p. from the combined organic phases. The crude product was absorbed on Celite and purified by FCC (PE/EA, 90/10, silica particle size: 63-200 µm) (note: purification is necessary for the next reaction step) to give 445 mg, 82% of a brown solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.65 (d, *J*=8.5, 1H), 7.27 (d, *J*=8.5, 1H), 4.42 (hept, *J*=6.2, 1H), 2.36 (s, 3H), 1.30 (d, *J*=6.1, 6H), 0.25 (s, 9H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 167.2, 147.3, 144.9, 144.8, 126.8, 124.1, 122.3, 104.7, 97.8, 79.3, 22.6, 20.7, -0.2.

HRMS (ESI): calculated for $[M+H]^+$: 336.1262, found: 336.1259.

Synthesis of 2-(allyloxy)-4-((tert-butoxycarbonyl)amino)-3-isopropoxybenzoic acid ***(116)***



a) Standard ring D **7** (provided by Evotec, 300 mg, 1.07 mmol, 1.00 Eq) was solved in THF (5.0 mL) and EtOH (5.0 mL) and AcOH (1.2 mL, 20 Eq) were added. Then Zn powder (0.70 g, 10 Eq) was added and the mixture was vigourously stirred for 22 h. The reaction solution was diluted between brine and EA (30 mL each) and the aqueous phase was extracted with EA (1x20 mL). The organic phases were combined, dried over Na_2SO_4 and solvents were removed u.r.p. and by coevaporation with heptane/EA and MTBE to remove AcOH residues. The yellow highly viscous oil was freezed in liquid nitrogen and the solid was grinded with a spatula. The material was then further dried under HV and used directly in the next step.

b) Adapted from[213]: The material was solved in dioxane (2.7 mL) and H_2O (1.4 mL) and TEA (0.22 mL, 1.5 Eq) were added. Then Boc_2O (0.37 mL, 1.5 Eq) was added dropwise and the reaction was stirred at RT overnight. After LCMS analysis TEA (74 µL, 0.50 Eq) and

Boc_2O (0.12 mL, 0.50 Eq) were added again. The temperature was increased up to 60°C after ~1 h. 2.5 h after the addition the temperature was decreased to RT, TEA (74 µL, 0.50 Eq) and Boc_2O (0.12 mL, 0.50 Eq) were added again and the mixture was stirred again overnight. The mixture was then diluted between EA (30 mL) and HCl (0.1 M, 50 mL) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. LCMS showed a turnover of 84% (UV). The crude material was purified by FCC (solid loading, cyclohexane/EA/AcOH, 90/5/5->85/10/5->40/55/5) to give 227 mg, 60% of a yellowish solid.

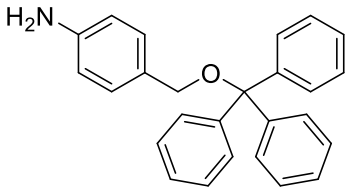
^{1}H NMR (700 MHz, THF): δ = 11.05 (s, 1H, COOH), 7.90 (d, *J*=8.8, 1H, C_{Ar}-H), 7.58 (d, *J*=8.8, 1H, C_{Ar}-H), 7.57 (s, 1H, H-N), 6.13 (ddt, *J*=17.3, 10.4, 5.6, 1H), 5.34 (dq, *J*=17.2, 1.7, 1H), 5.17 – 5.14 (m, 1H), 4.63 (hept, *J*=6.1, 1H), 4.54 (dt, *J*=5.7, 1.5, 2H), 1.51 (s, 9H), 1.27 (d, *J*=6.1, 6H).

^{13}C NMR (176 MHz, THF): δ = 166.3, 153.1, 152.9, 140.7, 138.9, 135.7, 127.7, 120.7, 117.2, 114.0, 80.9, 76.9, 75.4, 28.5, 22.7.

HRMS (ESI): calculated for $[M+H]^+$: 352.1755, found: 352.1755.

Ring E analogues

Synthesis of 4-((trityloxy)methyl)aniline ***(114)***



a) 4-Nitrobenzyl alcohol (1.00 g, 6.53 mmol, 1.00 Eq) and Trityl chloride (1.82 g, 1.00 Eq) were solved in dry DCM (16 mL) under Argon atmosphere. Dry TEA (1.09 mL, 1.20 Eq) was added dropwise and the reaction (turning from yellow to purple within minutes) was stirred at RT while being screened by LCMS. After ~1.5 h the color disappeared and a precipitate was formed and after 3 h the solvents were removed from the mixture u.r.p..

b) The residue was solved again in THF/EtOH mixture (16 mL each). Then AcOH (3.7 mL, 10 Eq) and Zn powder (2.13 g, 5.00 Eq) were added before stirring the reaction vigourously at RT. The reaction was screened by LCMS and after 1 h the reaction mixture was diluted between EA (70 mL) and HCl (0.4 M, 150 mL). The aqueous phase was extracted with EA (3x30 mL), the combined organic phases were washed with sat. $NaHCO_3$ sol. (50 mL) and the basic aqueous phase was extracted again with EA (2x30 mL). All organic phases were combined, dried over Na_2SO_4 and solvents were removed u.r.p.. The crude material was purified by automated FCC (solid loading, 30x reactants mass, cyclohexane/EA, 100/0->85/15) and solvents were removed u.r.p. to give a nearly colorless very sticky foam. Yield: 2.08 g, 87%.

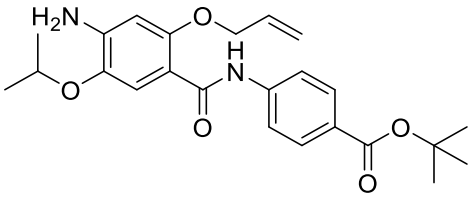
^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.57 – 7.54 (m, 6H), 7.36 – 7.32 (m, 6H), 7.30 – 7.24 (m, 3H), 7.22 – 7.18 (m, 2H), 6.69 (d, *J*=8.5, 2H), 4.08 (s, 2H, H_2C-O), 3.64 (s, 2H, H_2N).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 145.7, 144.4, 129.1, 128.9, 128.7, 127.9, 127.0, 115.1, 86.9, 65.8.

HRMS (ESI): calculated for $[M+H]^+$: 366.1853, found: 366.1856.

DE-fragments

Synthesis of tert-butyl 4-(2-(allyloxy)-4-amino-5-isopropoxybenzamido)benzoate ***(23)***



a) Under Argon atmosphere: 2-(allyloxy)-5-isopropoxy-4-nitrobenzoic acid **22** (36.9 mg, 131 µmol, 1.00 Eq) was solved in dry THF (1.2 mL) and $(COCl)_2$ (12 µL, 1.1 Eq) was added. DiPEA (45 µL, 2.0 Eq) was added at 0°C and the solution was stirred at 0°C for 30 min leading to gas generation. Then a solution of *tert*-butyl 4-aminobenzoate (27.9 mg, 1.10 Eq) in dry THF (1.2 mL) and DiPEA (33 µL, 1.5 Eq) were added dropwise at 0°C. The solution was stirred at RT until reaction control showed the end of reaction. The reaction solution was diluted between MTBE (20 mL) and brine (20 mL) and the aqueous phase was extracted with MTBE (20 mL). Combined organic phases were washed with NaOH (0.1 M, 20 mL) and the aqueous phase was extracted with MTBE (20 mL). Combined organic phase were washed with brine (20 mL). Because the aqueous phase still contained UV-absorbing substances it was acidified and reextracted again with EA. Organic phases were combined and solvents removed u.r.p.. The crude product was directly used in next step.

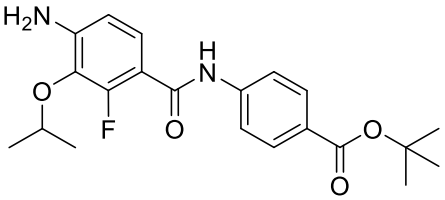
b) Adapted from[9, 96]: Zn dust (86 mg, 10 Eq) was added in two portions to a solution of the crude product in THF/EtOH (1 mL each) and AcOH (75 µL, 10 Eq). After 15 min vigourously stirring AcOH (75 µL, 10 Eq) was added and the reaction was stirred further while being monitored by LCMS. After 1 h the reaction solution was diluted between MTBE (20 mL) and sat. $NaHCO_3$ sol. (20 mL) and the aqueous phase was extracted with MTBE (2x20 mL). Combined organic phases were washed with water (20 mL), and dried over Na_2SO_4 before removing solvents u.r.p. to get the crude product which was purified by FCC (solid loading, 100x reactants mass, PE/EA, 90/10->85/15->80/20). The product was obtained as yellow solid (36.2 mg, 65% o2s).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.21 (s, 1H, H-N), 7.95 (d, *J*=8.9, 2H, H-$C_{Ar,E}$), 7.70 (s, 1H, H-$C_{Ar,D}$), 7.68 (d, *J*=9.0, 2H, H-$C_{Ar,E}$), 6.34 (s, 1H, H-$C_{Ar,D}$), 6.17 (ddt, *J*=17.2, 10.4, 5.8, 1H), 5.52 (dq, *J*=17.2, 1.4, 1H), 5.45 (dq, *J*=10.4, 1.1, 1H), 4.65 – 4.56 (m, 3H), 4.43 (s, 2H, H_2N), 1.59 (s, 9H), 1.34 (d, *J*=6.0, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 165.7, 164.0, 152.4, 142.9, 142.6, 139.8, 132.1, 130.7, 126.8, 120.3, 118.9, 116.4, 110.8, 99.7, 80.8, 71.6, 71.1, 28.4, 22.3.

HRMS (ESI): calculated for $[M+H]^+$: 427.2228, found: 427.2231.

Synthesis of tert-butyl 4-(4-amino-2-fluoro-3-isopropoxybenzamido)benzoate ***(132)***



a) 2-fluoro-3-isopropoxy-4-nitro-benzoic acid **48c** (51 mg, 0.21 mmol, 1.0 Eq) and DiPEA (0.18 mL, 5.0 Eq) were solved in dry DCM (1.7 mL) under Argon atmosphere and $POCl_3$ (23 µL, 1.2 Eq) was added at 0°C. The reaction was stirred 5 min at 0°C, then at RT. After 1.5 h, *tert*-butyl 4-aminobenzoate (51 mg, 1.3 Eq, solution in dry DCM (1.7 mL)) was added dropwise. After 2 h DiPEA (0.18 mL, 5.0 Eq) was added again and the reaction was stirred overnight at RT. After LCMS reaction control the reaction mixture was diluted between EA/HCl (0.5 M) (25 mL each) and the aqueous phase was extracted with EA (2x15 mL). The organic phase was washed with brine (10 mL), NaOH (0.5 M, 2x15 mL) and brine (2x10 mL). (The alkaline aqueous solution was later acidified with HCl (2 M), then extracted with EA (2x15 mL) to recover educt). The combined organic phases containing product were dried over Na_2SO_4 and solvents were removed u.r.p. to give 105 mg crude.

b) Adapted from[9, 96]: The crude material was solved in dry THF (1.5 mL) under Ar and EtOH (1.5 mL) was added. AcOH (0.12 mL, 10 Eq) and Zn powder (69 mg, 5.0 Eq) were added and the reaction was stirred vigourously. After 30 min, 2 h and 3.5 h AcOH (3x0.12 mL, 3x10 Eq) and Zn powder (3x69 mg, 3x5.0 Eq) were added again. After 4.5 h reaction and screening with LCMS the mixture was diluted between EA/sat. $NaHCO_3$ sol. (20 mL each) and the aqueous phase was extracted with EA (2x15 mL). The combined organic phases were washed with brine (10 mL), dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was purified by FCC (solid loading, 80x theoretical product mass, PE/acetone, 85/15->80/20) to give a pale yellow gum, yield: 52.8 mg, 65% o2s.

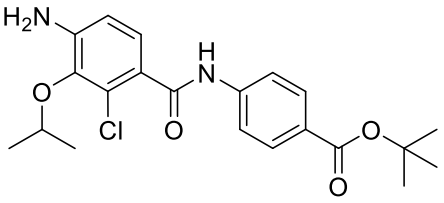
^{1}H NMR (500 MHz, Acetone): δ = 9.22 (d, *J*=7.6, 1H, H-NCO), 7.94 (d, *J*=9.0, 2H), 7.89 (d, *J*=9.0, 2H), 7.42 (t, *J*=8.4, 1H), 6.65 (dd, *J*=8.7, 1.4, 1H), 5.39 – 5.29 (m, 2H, H_2N), 4.49 – 4.38 (m, 1H), 1.58 (s, 9H), 1.33 (dd, *J*=6.2, 0.8, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 165.7, 163.3, 155.8 (d, *J*=246), 148.6 (d, *J*=6.3), 144.2, 131.8 (d, *J*=15.3), 131.0 (C_{Ar}-H), 127.6, 126.8 (d, *J*=3.8), 119.8 (C_{Ar}-H), 112.1 (d, *J*=11.3), 110.7, 80.9, 76.6, 28.4, 22.7.

^{19}F NMR (471 MHz, Acetone): δ = -131.0.

HRMS (ESI): calculated for $[M+H]^+$: 389.1871, found: 389.1871.

Synthesis of tert-butyl 4-(4-amino-2-chloro-3-isopropoxybenzamido)benzoate ***(133)***



a) Note: The first step was splitted to two equal-sized batches: 2-chloro-3-isopropoxy-4-nitrobenzoic acid **48a** (98 mg in total, 0.38 mmol, 1.0 Eq) and DiPEA (0.32 mL, 5.0 Eq) were solved in dry DCM (3.0 mL) under Argon atmosphere and $POCl_3$ (41 µL, 1.2 Eq) was added at 0°C. The reaction was stirred 5 min at 0°C, then at RT. After ~1 h DiPEA (0.32 µL, 5.0 Eq) and *tert*-butyl 4-aminobenzoate (91 mg, 1.25 Eq, solution in dry DCM (3.0 mL)) were added. The reaction was continued overnight either at 5°C or RT. Both batches were now combined.

After LCMS reaction control the reaction mixture was diluted between EA and HCl (0.5 M) (40 mL each) and the aqueous phase was extracted with EA (2x20 mL). The organic phase was washed with brine (10 mL), sat. $NaHCO_3$ sol. (20 mL), NaOH (0.5 M, 2x20 mL) and brine (1x20 mL). (The alkaline aqueous solution was later acidified with HCl (2 M), then extracted with DCM (2x20 mL) to recover educt). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was directly used in the next step:

b) Adapted from[9, 96]: The crude material was solved in dry THF (2.5 mL) under Ar and EtOH (2.5 mL) was added. AcOH (0.22 mL, 10 Eq) and Zn powder (123 mg, 5.00 Eq) were added and the reaction was stirred vigourously. After 30 min and 2 h AcOH (2x0.22 mL,

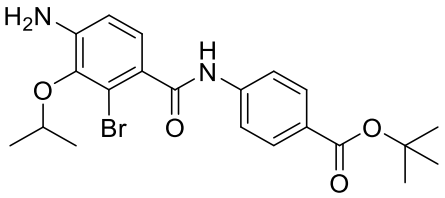
2x10 Eq) and Zn powder (2x123 mg, 2x5.00 Eq) were added again. After 3 h reaction and screening with LCMS the mixture was diluted between DCM/sat. $NaHCO_3$ sol. (30 mL each) and the aqueous phase was extracted with DCM (2x20 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was purified by FCC (solid loading, 100x theoretical product mass, PE/acetone, 85/15->80/20) to give an off-colorless foam, yield: 82.2 mg, 54% o2s.

^{1}H NMR (500 MHz, Acetone): δ = 9.52 (s, 1H, H-NCO), 7.95 (d, *J*=8.9, 2H), 7.89 (d, *J*=9.0, 2H), 7.16 (d, *J*=8.2, 1H), 6.76 (d, *J*=8.4, 1H), 5.16 – 5.05 (m, 2H), 4.53 (hept, *J*=6.1, 1H), 1.58 (s, 9H), 1.34 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 166.2, 165.7, 146.7, 144.4, 141.2, 131.1, 127.6, 126.4, 126.1, 125.9, 119.4, 113.5, 80.9, 76.2, 28.4, 22.7.

HRMS (ESI): calculated for $[M+H]^+$: 405.1576, found: 405.1575.

Synthesis of tert-butyl 4-(4-amino-2-bromo-3-isopropoxybenzamido)benzoate ***(134)***



a) 2-bromo-3-isopropoxy-4-nitrobenzoic acid **48b** (43.8 mg, 0.144 mmol, 1.00 Eq) and DiPEA (0.12 mL, 5.0 Eq) were solved in dry DCM (1.2 mL) and $POCl_3$ (16 µL, 1.2 Eq) was added at 0°C. The reaction was stirred 5 min at 0°C, then at RT. After 1.5 h *tert*-butyl 4-aminobenzoate (35 mg, 1.25 Eq, solution in dry DCM (1.2 mL)) was added dropwise. After 2 h, DiPEA (0.12 mL, 5.0 Eq) was added again and the reaction was stirred overnight at RT. After LCMS reaction control the reaction mixture was diluted between EA/HCl (0.5 M) (25 mL each) and the aqueous phase was extracted with EA (2x15 mL). The organic phase was washed with brine (10 mL), NaOH (0.5 M, 2x15 mL) and brine (2x10 mL) (=organic phase 1). The washing solution was acidified with HCl (2 M), extracted with EA (2x15 mL) and this organic phase was again washed with NaOH (0.2 M, 20 mL) (=organic phase 2). (The alkaline aqueous solution was later acidified with HCl, then extracted with EA (2x15 mL) to recover educt). Organic phases 1 and 2 were combined, dried over Na_2SO_4 and solvents were removed u.r.p. to give 87 mg crude material.

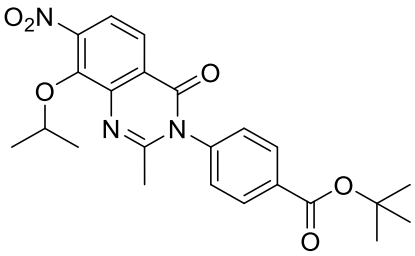
b) Adapted from[9, 96]: The crude material was solved in dry THF (1.0 mL) under Ar and EtOH (1.0 mL) was added. AcOH (82 µL, 10 Eq) and Zn powder (47 mg, 5.0 Eq) were added and the reaction was stirred vigourously. After 30 min, 2 h and 3.5 h AcOH (3x82 µL, 3x10 Eq) and Zn powder (3x47 mg, 3x5.0 Eq) were added again. After 4 h reaction and screening with LCMS, the mixture was diluted between EA/sat. $NaHCO_3$ sol. (20 mL each) and the aqueous phase was extracted with EA (2x15 mL). The combined organic phases were washed with brine (10 mL), dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was purified by FCC (solid loading, 100x theoretical product mass, PE/acetone, 85/15->80/20) to give a pale yellow gum, yield: 46.5 mg, 72% o2s, NMR: 66%.

^{1}H NMR (500 MHz, Acetone): δ = 9.51 (s, 1H, H-NCO), 7.95 (d, *J*=8.9, 2H), 7.89 (d, *J*=9.0, 2H), 7.10 (d, *J*=8.2, 1H), 6.79 (d, *J*=8.2, 1H), 5.07 (s, 2H, H_2N), 4.57 (hept, *J*=6.1, 1H), 1.58 (s, 9H), 1.35 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 167.2, 165.7, 146.4, 144.4, 142.3, 131.1, 128.7, 127.6, 125.8, 119.4, 116.5, 114.2, 80.9, 76.3, 28.4, 22.6.

HRMS (ESI): calculated for $[M+H]^+$: 449.1071/ 451.1050, found: 449.1069/ 451.1051.

*Synthesis of tert-butyl 4-(8-isopropoxy-2-methyl-7-nitro-4-oxoquinazolin-3(4H)-yl)benzoate **(57)***



a) Methyl 2-acetamido-3-isopropoxy-4-nitrobenzoate **59** (27 mg, 0.11 mmol, 1.0 Eq, non-purified product) was solved in THF/water (0.5 mL each) and LiOH*H_2O (22 mg, 5.0 Eq) was added. After stirring for 45 min the reaction solution was diluted between EA and H_2O (10 mL each). The organic phase was extracted again with NaOH (0.1 M, 10 mL) and the combined aqueous phases were washed with EA (2x10 mL) (organic phase 1) before acidification with HCl (conc., ~3 mL) and extraction with EA (3-4x 15 mL) (organic phase 2). To ensure full product recovery organic phase 1 was extracted again with NaOH (2x20 mL). The aqueous phase was acidified with HCl (conc.) and extracted with EA (2x20 mL). This fraction and organic phase 2 were combined, dried over Na_2SO_4 and

solvents were removed u.r.p. and by coevaporation with heptane to give a colorless-yellowish solid (22.2 mg) used without further purification.

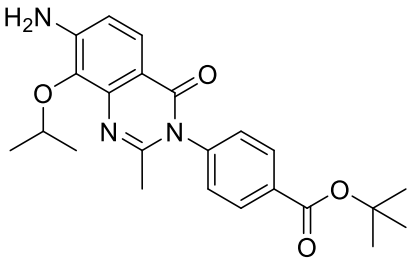
b) The vacuum-dried solid (22.2 mg crude, 79 µmol, 1.0 Eq) was suspended in dry DCM (0.8 mL) under Ar and $(COCl)_2$ was added (8.4 µL, 1.25 Eq). Then a catalytic amount of DMF (0.4 µL, 0.07 Eq) was added and the reaction stirred 1 h while being screened by LCMS. Additional $(COCl)_2$ (5.0 µL, 0.75 Eq) and DMF (0.4 µL, 0.07 Eq) were added and the reaction was stirred 30 min before removing solvents and reagent u.r.p. and high vacuum. The residue was solved in dry DCM (0.8 mL) and *tert*-butyl 4-aminobenzoate (21 mg, 1.4 Eq) and pyridine (19 µL, 3.0 Eq) were added. The reaction was stirred for 1 h while being screened with TLC. The reaction mixture was then diluted between DCM and water (15 mL each) and the aqueous phase was extracted with DCM (1x15 mL). The aqueous phase was acidified with HCl (1 M) and extracted again with EA (2x20 mL). The combined organic phases were dried over Na_2SO_4 and solvent was removed u.r.p.. The crude product was purified by FCC (solid loading, 100x reactants mass, PE/EA, 85/15) to give a light green oil. A mixed fraction was washed again with $NaHSO_4$ (1 M, 2x10 mL) and HCl (0.1 M, 6x) and combined with the other product fractions to give 14.5 mg (42% o2s, 31% o3s) material.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.20 (d, *J* = 8.5, 2H), 8.01 (d, *J* = 8.7, 1H), 7.63 (d, *J* = 8.5, 1H), 7.34 (d, *J* = 8.7, 2H), 5.30 (hept, *J* = 6.2, 1H), 2.28 (s, 3H), 1.63 (s, 9H), 1.40 (d, *J* = 6.1, 6H).

^{13}C NMR (126 MHz, CDCl3): δ = 164.6, 161.2, 154.1, 148.4, 146.6, 143.1, 140.7, 133.6, 131.5, 128.0, 124.3, 121.5, 121.1, 82.1, 79.4, 28.3, 24.8, 22.6.

HRMS (ESI): calculated for $[M+H]^+$: 440.1816, found: 440.1818.

*Synthesis of tert-butyl 4-(7-amino-8-isopropoxy-2-methyl-4-oxoquinazolin-3(4H)-yl)benzoate **(135)***



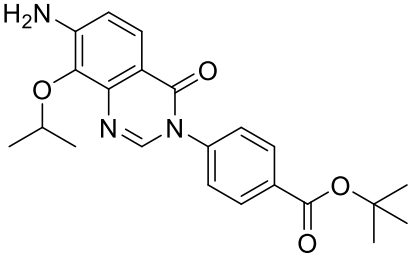
Adapted from[9, 96]: *tert*-butyl 4-(8-isopropoxy-2-methyl-7-nitro-4-oxoquinazolin-3(4H)-yl)benzoate **57** (12.9 mg, 29.4 µmol, 1.00 Eq) was solved in EtOH (0.33 mL) and dry THF (0.13 mL). Then AcOH (44 µL, 27 Eq) and Zn dust (15 mg, 8.0 Eq) were added and the reaction was stirred at RT and monitored with LCMS. Another portion Zn dust was added (15 mg, 8.0 Eq) after 1.5 h and after 2 h the suspension was diluted between sat. $NaHCO_3$ sol. (15 mL) and DCM (15 mL). The aqueous suspension was extracted with DCM (2x10 mL) and the organic phase was dried over Na_2SO_4 and solvent removed u.r.p. to get 10.9 mg, 91% of a light yellow solid used without further purification.

^{1}H NMR (500 MHz, CDCl3): δ = 8.16 (d, J = 8.7, 2H), 7.82 (d, J = 8.5, 1H), 7.31 (d, J = 8.7, 2H), 6.84 (d, J = 8.5, 1H), 4.93 (hept, J = 6.2, 1H), 2.27 (s, 3H), 1.62 (s, 9H), 1.40 (d, J = 6.3, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 164.8, 161.7, 146.7, 141.5, 136.5, 133.0, 131.5, 131.2, 128.4, 128.2, 123.3, 115.3, 112.1, 81.8, 76.6, 28.3, 24.5, 22.8.

HRMS (ESI): calculated for $[M+H]^+$: 410.2074, found: 410.2075.

*Synthesis of tert-butyl 4-(7-amino-8-isopropoxy-4-oxoquinazolin-3(4H)-yl)benzoate **(136)***



a) 2-amino-3-isopropoxy-4-nitrobenzoic acid **61** (60 mg, 0.25 mmol, 1.0 Eq) was solved in HCOOH (98%, 2.5 mL) and Ac_2O (24.8 µL, 1.05 Eq) was added. The reaction mixture was stirred for 1 h 15 min while being monitored by LCMS. The reaction was quenched by solving the reaction mixture between DCM (20 mL) and H_2O (15 mL). The aqueous

phase was extracted with DCM (3x20 mL) and the organic phases were combined and dried over Na_2SO_4 before removing solvent u.r.p. to give a brown-yellow solid (50.8 mg, 76%) directly used in next reaction.

b) Note: The following reaction step was first conducted with DiPEA as base leading to very small turnover and the reactants and products were therefore recovered by aequeos workup.

The recovered material containing the crude formyl-intermediate (45.5 mg, 0.17 mmol, 1.0 Eq) and *tert*-butyl 4-aminobenzoate (49 mg, 1.5 Eq) was solved in dry DCM (1.7 mL) together with DMF (2.0 µL, 15 mol%). Then $POCl_3$ (31 µL, 2.0 Eq) was added and the reaction was stirred for 1 h before adding *tert*-butyl 4-aminobenzoate again (49 mg, 1.5 Eq) and dry pyridine (55 µL, 4.0 Eq). After 2 h stirring at RT the reaction mixture was diluted between DCM/HCl (0.5 M) (20 mL each) and the aqueous phase was extracted with DCM (2x15 mL). Combined organic phases were washed with brine (10 mL), the washing solution was reextracted with DCM (1x10 mL) and all organic phases were combined and dried over Na_2SO_4. The solvent was removed u.r.p. to give a brown crude product directly used in the next step.

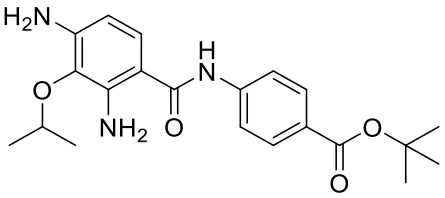
c) Adapted from[9, 96]: The crude product was suspended in THF/EtOH mixture (1.2 mL each) and AcOH (0.29 mL, 30 Eq) was added. Then Zn dust (166 mg, 15 Eq) was added and the reaction was stirred vigourously while being monitored by LCMS. After 3 h the mixture was diluted between EA/sat. $NaHCO_3$ sol. (20 mL each). The aqueous phase was extracted with EA (2x15 mL) and the combined organic phases were washed with brine (1x5 mL). The organic phase was dried over Na_2SO_4, filtered through pore size 4 and solvents were removed u.r.p.. The residue was purified by FCC (solid loading, 100x theoretical product mass, PE/EA, 70/30) to give 30.2 mg (34% o3s) of an off colorless gum.

^{1}H NMR (500 MHz, Acetone): δ = 8.14 (s, 1H, N_2C-H), 8.12 (d, *J* = 8.7, 2H), 7.77 (d, *J* = 8.5, 1H), 7.66 (d, *J* = 8.9, 1H), 6.99 (d, *J* = 8.5, 1H), 5.42 (s, 2H, H_2N), 5.04 (hept, *J* = 6.1, 1H), 1.62 (s, 9H), 1.33 (d, *J* = 6.3, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 165.3, 160.5, 148.7, 145.6, 142.9, 142.9, 137.4, 132.7, 130.8, 128.2, 123.3, 115.9, 113.4, 81.8, 76.4, 28.3, 22.8.

HRMS (ESI): calculated for $[M+H]^+$: 396.1918, found: 396.1918.

Synthesis of tert-butyl 4-(2,4-diamino-3-isopropoxybenzamido)benzoate ***(63)***



a) 2-amino-3-isopropoxy-4-nitrobenzoic acid **61** (80.5 mg, 0.335 mmol, 1.00 Eq) and *tert*-butyl 4-aminobenzoate (130 mg, 2.00 Eq) were suspended in dry DCM (2.7 mL) under Argon atmosphere and dry pyridine (68 µL, 2.5 Eq) was added. After a clear solution was formed T3P (50%-sol. in EA, 0.30 mL, 1.5 Eq) was added dropwise. The reaction was carefully screened by LCMS and after 75 min the mixture was diluted between HCl (0.1 M) and EA (30 mL each) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were dried over Na_2SO_4 before removing solvents u.r.p.. The material was directly used in the next step.

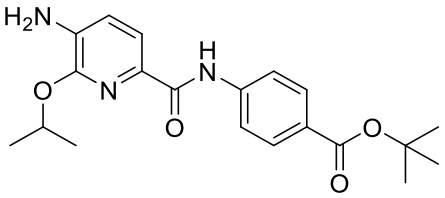
b) Adapted from[9, 96]: The material was solved in a THF/EtOH-mixture (2.5 mL each) and Zn dust (219 mg, 10.0 Eq) was added. Then AcOH (2x0.19 mL, 2x10 Eq) was added dropwise directly and after 30 min while the reaction was screened by LCMS. After 1 h 20 min AcOH (0.19 mL, 10.0 Eq) was added again. After 1 h 45 min the mixture was diluted between sat. $NaHCO_3$ sol./EA (30 mL each) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were washed with brine (5 mL) and dried over Na_2SO_4 before removing solvents u.r.p.. The material was then purified by FCC (solid loading, 100x reactants mass (21 g-scale column), cyclohexane/EA, 85/15->80/20->70/30) to give an almost colorless solid after solvent removal u.r.p. and coevaporation with Cy (3x). Yield: 48.4 mg, 37% o2s.

^{1}H NMR (500 MHz, THF): δ = 9.04 (s, 1H, H-NCO), 7.88 (d, *J*=8.9, 2H), 7.77 (d, *J*=9.0, 2H), 7.20 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 6.11 (s, 2H, H_2N), 5.98 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 4.69 (s, 2H, H_2N), 4.34 (hept, *J*=6.3, 1H), 1.57 (d, *J*=1.1, 9H), 1.30 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, THF): δ = 168.8, 165.7, 146.8, 145.9, 145.2, 131.0, 130.9, 126.8, 125.0, 119.5, 106.8, 104.1, 80.4, 73.2, 28.5, 23.0.

HRMS (ESI): calculated for $[M+H]^+$: 386.2075, found: 386.2075.

Synthesis of tert-butyl 4-(5-amino-6-isopropoxypicolinamido)benzoate ***(137)***



a) 6-isopropoxy-5-nitropicolinic acid **73** (207 mg, 0.915 mmol, 1.00 Eq) and *tert*-butyl 4-aminobenzoate (230 mg, 1.30 Eq) were solved in dry EA (9 mL) under Argon atmosphere. Then dry pyridine (0.30 mL, 4.0 Eq) was added, T3P (50% sol. in EA, 1.09 mL, 2.00 Eq) was added dropwise and the yellow solution was stirred at RT. After ~5 min a colorless precipitate was formed. The reaction was screened with LCMS and after 1 h the reaction mixture was diluted between EA/HCl (0.5 M) (40 mL each). The aqueous phase was extracted with EA (3x20 mL) and the combined organic phases were dried over Na_2SO_4. Solvents were removed u.r.p. to give a light yellow amorphous solid that was taken directly for next reaction.

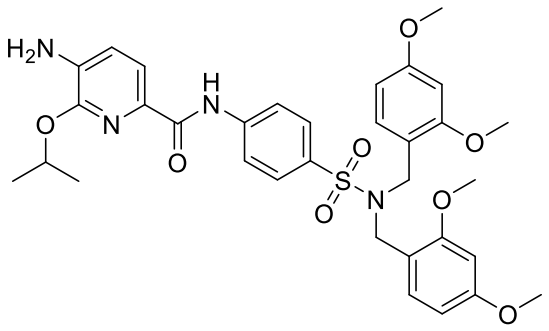
b) Adapted from[9, 96]: The crude product was secured under Ar and solved in THF/EtOH (4.5 mL each). Then AcOH (1.05 mL, 20.0 Eq) and Zn powder (598 mg, 10.0 Eq) were added and the mixture was stirred vigourously at RT while being monitored with LCMS. After 1.5 h the mixture was suspended between EA/sat. $NaHCO_3$ sol. (50 mL each) and the aqueous phase was extracted with EA (2x25 mL). The combined organic phases were washed with brine (1x10 mL), dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was purified by FCC (solid loading, 80x theoretical product mass, PE/EA, 80/20) and solvents were removed u.r.p. and by coevaporation with MTBE and cyclohexane to give 371 mg, >98%, NMR: 81% (cyclohexane residue), of a light brown highly viscous oil.

^{1}H NMR (700 MHz, CD_3CN): δ = 9.73 (s, 1H, H-NCO), 7.95 (d, *J*=8.8, 2H), 7.83 (d, *J*=8.8, 2H), 7.63 (d, *J*=7.9, 1H), 7.00 (d, *J*=7.9, 1H), 5.54 (hept, *J*=6.2, 1H), 4.77 (s, 2H, H_2N), 1.57 (s, 9H), 1.42 (d, *J*=6.1, 6H).

^{13}C NMR (176 MHz, CD_3CN): δ = 166.1, 164.0, 150.6, 143.6, 137.6, 134.5, 131.2, 127.7, 119.7, 119.2, 118.6, 81.4, 69.8, 28.4, 22.3.

HRMS (ESI): calculated for $[M+H]^+$: 372.1918, found: 372.1920.

Synthesis of 5-amino-N-(4-(N,N-bis(2,4-dimethoxybenzyl)sulfamoyl)phenyl)-6-isopropoxypicolinamide ***(138)***



a) 6-isopropoxy-5-nitropicolinic acid **73** (50 mg, 0.22 mmol, 1.0 Eq) and 4-amino-N,N-bis(2,4-dimethoxybenzyl)benzenesulfonamide (provided by D. Kohnhäuser, 110 mg, 1.05 Eq) were solved in dry DCM (2.2 mL) under Argon atmosphere before adding dry pyridine (54 µL, 3.0 Eq). Then $POCl_3$ (21.2 mg, 1.05 Eq) was added dropwise and the reaction was stirred at RT while being screened by LCMS. After 45 min the mixture was suspended between EA/HCl (0.1 M) (30 mL each), then brine (10 mL) was added and the aqueous phase was extracted with EA (2x20 mL). Combined organic phases were dried over Na_2SO_4 before removing solvents u.r.p. to give a yellow foam. The crude material was directly used in the next step.

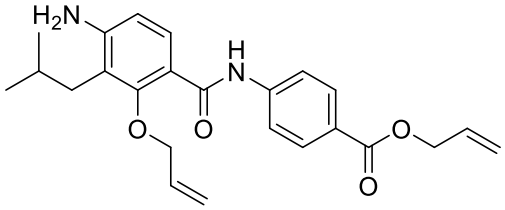
b) Adapted from[9, 96]: The crude material was suspended in a THF/EtOH mixture (1.1 mL each) and AcOH (0.25 mL, 20 Eq) and Zn powder (145 mg, 10.0 Eq) were added. THF (1.1 mL) was added again to enhance solubility and the mixture was stirred vigourously at RT while being screened by LCMS. After 2 h and 3.5 h AcOH (2x0.13 mL, 2x10 Eq) and Zn powder (2x72 mg, 2x5.0 Eq) were added again. After 4 h the mixture was diluted between EA/sat. $NaHCO_3$ sol. (25 mL each) and the aqueous phase was extracted with EA (2x30 mL). Combined organic phases were dried over Na_2SO_4 before removing solvents u.r.p.. The crude material was purified by FCC (solid loading, 80x theoretical product mass, PE/acetone, 70/30) to give an off colorless solid after removing solvents u.r.p. and by coevaporation with heptane/EA. Yield: 119 mg, 83% o2s.

^{1}H NMR (500 MHz, THF): δ = 9.77 (s, 1H, H-NCO), 7.88 (d, *J*=8.9, 2H), 7.69 (d, *J*=8.9, 2H), 7.66 (d, *J*=7.8, 1H, H-$C_{Ar,D}$), 7.09 (d, *J*=8.4, 2H), 6.92 (d, *J*=7.8, 1H, H-$C_{Ar,D}$), 6.35 (dd, *J*=8.4, 2.4, 2H), 6.31 (d, *J*=2.3, 2H), 5.54 (hept, *J*=6.1, 1H), 5.25 (d, *J*=9.3, 2H, H_2N), 4.30 (s, 4H, $N(CH_2)_2$), 3.71 (s, 6H, 2xCH_3O), 3.59 (s, 6H, 2xCH_3O), 1.44 (d, *J*=6.3, 6H).

^{13}C NMR (126 MHz, THF): δ = 163.5, 161.4, 159.2, 150.3, 143.3, 138.0, 136.4, 134.1, 131.2, 129.0, 119.3, 118.7, 118.6, 118.1, 104.8, 98.4, 69.3, 55.4, 55.3, 46.5, 22.5.

HRMS (ESI): calculated for $[M+H]^+$: 651.2483, found: 651.2487.

*Synthesis of allyl 4-(2-(allyloxy)-4-amino-3-isobutylbenzamido)benzoate **(139)***



a) 2-(allyloxy)-4-((*tert*-butoxycarbonyl)amino)-3-isobutylbenzoic acid **91b** (37.6 mg, 0.108 mmol, 1.00 Eq) and 4-amino allylbenzoate (synthesized as reported in[72], 31 mg, 1.6 Eq) were solved in dry EA (1.1 mL) and pyridine (43 µL, 5.0 Eq) was added. After stirring for 5 min, T3P (50% in EA, 0.13 mL, 2.0 Eq) was added dropwise. The mixture was stirred at RT and screened by LCMS. After 1 h the mixture was diluted between EA/HCl (0.1 M) (20 mL each), the aqueous phase was extracted with EA (2x20 mL), combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p..

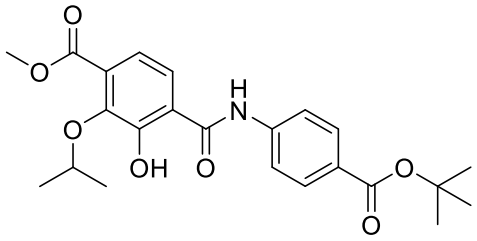
b) The crude material was solved in HCl (sol. in dioxane, 4 M, 1.0 mL) and the reaction was stirred at RT. After 1.5 h and screening by TLC the reaction mixture was diluted between EA/sat. $NaHCO_3$ sol. (20 mL each) and the aqueous phase was extracted with EA (2x20 mL) before drying the combined organic phases over Na_2SO_4 and removing solvents u.r.p.. The crude product was purified by FCC (solid loading, 100x theoretical product mass, PE/EA, 85/15->70/30) to give a light red gum, 34.6 mg, 79% o2s.

^{1}H NMR (700 MHz, $CDCl_3$): δ = 10.00 (s, 1H), 8.03 (d, *J*=8.8, 2H), 7.89 (d, *J*=8.5, 1H), 7.74 (d, *J*=8.8, 2H), 6.63 (d, *J*=8.5, 1H), 6.12 – 6.00 (m, 2H), 5.51 (dd, *J*=17.2, 1.6, 1H), 5.41 (dd, *J*=17.2, 0.9, 1H), 5.36 (dd, *J*=10.5, 1.4, 1H), 5.28 (dd, *J*=10.4, 1.3, 1H), 4.81 (d, *J*=5.7, 2H), 4.4 (br s, 1H, H_2N), 4.35 (d, *J*=5.1, 2H), 2.43 (d, *J*=7.3, 2H), 1.98 (hept, *J*=6.8, 1H), 0.99 (d, *J*=6.7, 6H).

^{13}C NMR (176 MHz, $CDCl_3$): δ = 166.0, 164.1, 156.6, 149.4, 143.2, 132.6, 132.5, 131.0, 130.9, 125.0, 119.2, 119.1, 118.4, 118.2, 116.7, 112.7, 75.8, 65.5, 33.8, 28.2, 22.9.

HRMS (ESI): calculated for $[M+H]^+$: 409.2122, found: 409.2123.

Synthesis of methyl 4-((4-(tert-butoxycarbonyl)phenyl)carbamoyl)-3-hydroxy-2-isopropoxybenzoate ***(140)***



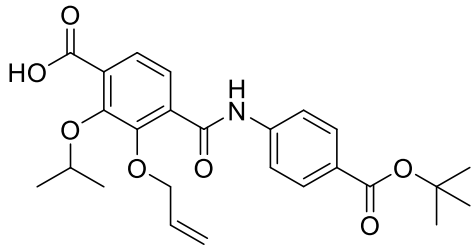
Adapted from[122]: 2-hydroxy-3-isopropoxy-4-(methoxycarbonyl)benzoic acid **97** (provided by D. Kohnhäuser, 131 mg, 515 µmol, 1.00 Eq) and *tert*-butyl 4-aminobenzoate (149 mg, 1.50 Eq) were suspended in dry toluene (1.3 mL) under Argon atmosphere and $P(OPh)_3$ (0.18 mL, 1.3 Eq) was added. The reaction was refluxed for 24 h and the product was directly purified by FCC (solid loading, 75x theoretical product mass, PE/EA, 90/10->85/15->80/20). (Note: TLC was not sufficient to identify product fractions, instead LCMS was used). The product was isolated with a yield of 168 mg, 76% containing an unquantified residual fraction of phenolic compounds.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.27 (s, 1H), 9.22 (s, 1H), 8.00 (d, *J* = 9.0, 2H), 7.72 (d, *J* = 9.0, 2H), 7.71 (d, *J* = 8.5, 2H), 7.40 (d, *J* = 8.5, 1H), 4.54 (hept, *J* = 6.1, 1H), 3.94 (s, 3H), 1.60 (s, 9H), 1.36 (d, *J* = 6.1, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 166.0, 165.4, 164.6, 151.7, 145.8, 141.3, 130.8, 130.2, 128.2, 123.3, 121.4, 120.3, 119.8, 81.2, 78.7, 52.6, 28.4, 22.5.

HRMS (ESI): calculated for $[M+H]^+$: 430.1861, found: 430.1862.

Synthesis of 3-(allyloxy)-4-((4-(tert-butoxycarbonyl)phenyl)carbamoyl)-2-isopropoxybenzoic acid ***(98)***



Adapted from[122]:

a) Methyl 4-((4-(*tert*-butoxycarbonyl)phenyl)carbamoyl)-3-hydroxy-2-isopropoxybenzoate purified material **140** (162 mg, 0.377 mmol, 1.00 Eq) and K_2CO_3 (104 mg, 2.00 Eq) were suspended in dry DMF (1.9 mL) under Argon atmosphere. The suspension was stirred for 10 min before adding AllBr (36 µL, 1.1 Eq) and stirring further at RT while being screened with LCMS. After 5 h reaction the mixture was diluted between EA/HCl (0.1 M) (30 mL each) and the aqueous phase was extracted with EA (2x20 mL). Organic phases were combined, dried over Na_2SO_4 and solvents were removed u.r.p. to give a yellowish oil directly used in saponification.

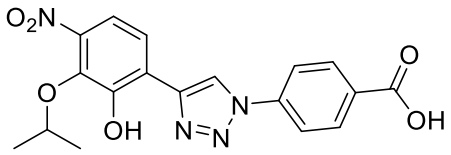
b) The crude material and LiOH*H_2O (63 mg, 4.0 Eq) were solved in a THF/H_2O mixture (1.3 mL each) forming a 2-phase system. The reaction was stirred at RT while being screened with LCMS. After 3 h the mixture was diluted between EA/HCl (0.1 M) (20 mL each) and the aqueous phase was extracted with EA (2x20 mL). Organic phases were combined and dried over Na_2SO_4 before removing solvents u.r.p.. The crude product was purified by FCC (solid loading, 85x reactant mass, PE/EA/AcOH, 80/15/5). Pure product fractions were identified with NMR and the product was isolated as a colorless fluffy solid, 115 mg, 67% o2s, 51% o3s.

^{1}H NMR (500 MHz, Acetone): δ = 11.50 (br s, 1H), 10.23 (s, 1H), 7.97 (d, *J* = 8.7, 2H), 7.87 (d, *J* = 9.0, 2H), 7.75 (d, *J* = 8.2, 1H), 7.69 (d, *J* = 8.2, 1H), 6.17 (ddt, *J* = 17.2, 10.4, 5.8, 1H), 5.51 (dq, *J* = 17.2, 1.6, 1H), 5.31 (dq, *J* = 10.4, 1.2, 1H), 4.76 (dt, *J* = 5.8, 1.4, 2H), 4.75 – 4.68 (m, 1H), 1.59 (s, 9H), 1.33 (d, *J* = 6.1, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 166.4, 165.6, 163.8, 151.5, 151.4, 143.5, 134.1, 133.4, 131.2, 128.2, 126.8, 125.5, 119.9, 119.8, 119.3, 81.1, 78.4, 75.8, 28.3, 22.6.

HRMS (ESI): calculated for $[M+H]^+$: 456.2017, found: 456.2018.

*Synthesis of 4-(4-(2-hydroxy-3-isopropoxy-4-nitrophenyl)-1H-1,2,3-triazol-1-yl)benzoic acid **(141)***



a) 2-isopropoxy-3-nitro-6-((trimethylsilyl)ethynyl)phenyl acetate **102** (100 mg, 0.298 mmol, 1.00 Eq) was solved in MeOH (7 mL) and K_2CO_3 (82 mg, 2.0 Eq) was added. The mixture stirred 15 min, before it was diluted between DCM (30 mL), water (30 mL) and HCl (1 M, 2 mL). The aqueous phase was extracted with DCM (2x20 mL). Organic phases were combined and solvents were removed u.r.p. to use this material in the next step.

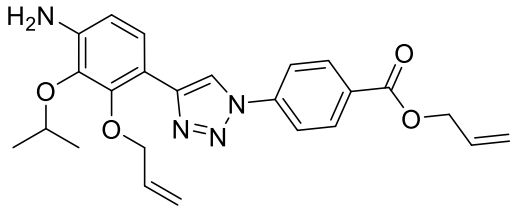
b) The crude product **100** was solved in *t*BuOH (3 mL) and 4-Azidobenzoic acid (73 mg, 0.45 mmol, 1.5 Eq) was added. Then a solution of $CuSO_4$ pentahydrate (7.4 mg, 0.10 Eq), Na-ascorbate (30 mg, 0.50 Eq) and THPTA (8 mg, 0.06 Eq) in H_2O (2 mL) was added. The reaction produced a suspension after few minutes and was stirred overnight. The reaction mixture was concentrated u.r.p. to remove *t*BuOH and was then diluted between EA (30 mL) and water (30 mL). The aqueous phase was extracted with EA (2x30 mL). The combined organic phases were washed with brine (1x30 mL) and dried over Na_2SO_4. To purify the product, the organic phase was concentrated slowly u.r.p. at 40°C. Once precipitation occured, the solution was put in fridge for 90 min and was filtrated over pore size 4. After washing with small portions of cold EA, the precipitate was washed out with acetone and the solution was concentrated u.r.p.. The filtrate solution was concentrated again u.r.p. at 60°C until precipitation and was stored at RT over weekend. The filtration was repeated and the solid was washed with MeCN and extracted with acetone to give a yield of 74 mg, 65% o2s product as a pale yellow solid. NMR showed 6 mol% educt residue that was tolerated.

^{1}H NMR (500 MHz, THF): δ = 11.70 (br s, 1H, COOH), 10.24 (s, 1H, OH), 9.06 (s, 1H, $H_{triazol}$), 8.24 (d, *J*=8.7, 2H), 8.08 (d, *J*=8.7, 2H), 7.88 (d, *J*=8.7, 1H), 7.44 (d, *J*=8.7, 1H), 4.61 (hept, *J*=6.1, 1H), 1.34 (d, *J*=6.2, 6H).

^{13}C NMR (126 MHz, THF): δ = 172.0, 166.8, 150.7, 145.6, 145.2, 141.3, 141.0, 132.4, 122.1, 122.0, 121.6, 120.9, 116.5, 78.8, 22.7, 20.5.

HRMS (ESI): calculated for $[M+H]^+$: 385.1143, found: 385.1140.

*Synthesis of allyl 4-(4-(2-(allyloxy)-4-amino-3-isopropoxyphenyl)-1H-1,2,3-triazol-1-yl)benzoate **(142)***



a) 4-(4-(2-hydroxy-3-isopropoxy-4-nitrophenyl)-1*H*-1,2,3-triazol-1-yl)benzoic acid **141** (70 mg, 0.18 mmol, 1.0 Eq) and K_2CO_3 (101 mg, 4.00 Eq) were suspended in DMF (1.8 mL) and allyl bromide (63 µL, 4.0 Eq) was added. The reaction was stirred overnight, then the reaction mixture was diluted between MTBE (25 mL) and water (25 mL) and aqueous phase extracted with MTBE (1x25 mL). Solvent was removed u.r.p. and DMF was removed by coevaporation with heptane (3x). Crude product (**104**) yield: 80 mg, 95%.

b) Adapted from[9, 96]: The crude product **104** (80 mg, 1.0 Eq) was suspended in EtOH (2.5 mL) and AcOH (0.35 µL, 35 Eq) in a glass vial flushed with Ar and the solution cooled in ice bath. Zn powder (112 mg, 10.0 Eq) was added in two portions over 15 min and the mixture was stirred at RT while vigourously stirred. The solution was diluted after 1 h between MTBE (25 mL) and sat. $NaHCO_3$ sol. (25 mL). The aqueous phase was extracted again with MTBE (1x25 mL) and the combined organic phases were washed with brine (25 mL) and dried over Na_2SO_4.

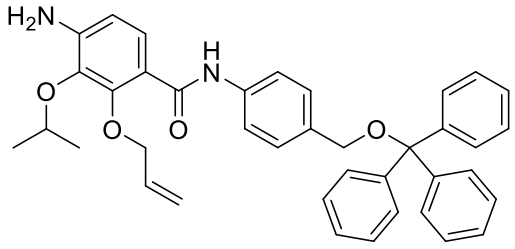
The reaction and workup was repeated with following amounts: protected Triazole (110 mg, 237 µmol, 1.00 Eq), EtOH (3.5 mL), AcOH (0.45 mL, 33 Eq), Zn powder (154 mg, 10.0 Eq). Crude products were combined and purified by FCC (solid loading, 75x silica mass, PE/EA, 80/20->70/30). Solvent was removed u.r.p. overnight to give a yield of 143 mg, 76% o2s.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.51 (s, 1H), 8.23 (d, *J*=8.9, 2H), 7.92 (d, *J*=8.4, 1H), 7.87 (d, *J*=9.0, 2H), 6.75 (d, *J*=8.5, 1H), 6.17 – 6.01 (m, 2H), 5.51 – 5.40 (m, 2H), 5.36 – 5.28 (m, 2H), 4.86 (dt, *J*=5.8, 1.4, 2H), 4.64 (hept, *J*=6.1, 1H), 4.56 (dt, *J*=5.5, 1.5, 2H), 1.36 (d, *J*=6.3, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 165.3, 149.2, 144.8, 140.9, 140.6, 138.7, 134.5, 132.1, 131.6, 129.9, 123.0, 119.6, 118.9, 118.8, 117.4, 115.7, 112.7, 75.5, 72.7, 66.1, 22.9.

HRMS (ESI): calculated for $[M+H]^+$: 435.2027, found: 435.2025.

Synthesis of 2-(allyloxy)-4-amino-3-isopropoxy-N-(4-((trityloxy)methyl)phenyl)benzamide ***(143)***



a) 4-((trityloxy)methyl)aniline **114** (200 mg, 0.547 mmol, 1.00 Eq) and standard ring D (provided by Evotec, 169 mg, 1.10 Eq) were solved in dry EA (2.7 mL) under Argon atmosphere and dry pyridine (0.13 mL, 3.0 Eq) was added. Then T3P (50% sol. in EA, 0.65 mL, 2.0 Eq) was added dropwise and the reaction was stirred at RT. After 1 h the mixture was diluted between EA/HCl (0.1 M) (30 mL each) and the aqueous phase was extracted with EA (2x20 mL) before drying over Na_2SO_4 and removing solvents u.r.p.. The crude material was directly used in the next step.

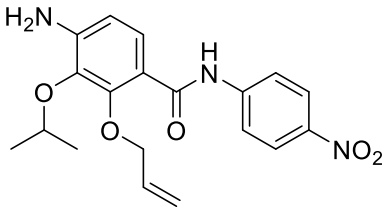
b) Adapted from[9, 96]: The crude material was solved in THF/EtOH mixture (2.7 mL each) under Argon atmosphere. Then AcOH (0.63 mL, 20 Eq) and Zn powder (0.36 g, 10 Eq) were added and the reaction was vigourously stirred at RT while being screened by LCMS. After 3 h the reaction mixture was diluted between EA/sat. $NaHCO_3$ sol. (40 mL each) and the aqueous phase was extracted with EA (2x20 mL). After collecting the organic phases and drying over Na_2SO_4 solvents were removed u.r.p.. The crude material was purified by FCC (solid loading, 50x theoretical product mass, cyclohexane/EA, 80/20->75/25) to give a light yellow foam (solidification was supported by freezing the sample in liquid N_2). Yield: 292 mg, 89%.

^{1}H NMR (500 MHz, THF): δ = 9.98 (s, 1H, H-NCO), 7.72 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 7.70 (d, *J*=8.5, 2H), 7.52 (d, *J*=7.0, 6H), 7.32 – 7.26 (m, 8H), 7.21 (t, *J*=7.3, 3H), 6.53 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 6.26 – 6.15 (m, 1H), 5.53 (dq, *J*=17.1, 1.7, 1H), 5.35 (dq, *J*=10.4, 1.4, 1H), 5.07 – 4.98 (m, 2H, H_2N), 4.70 (dt, *J*=5.6, 1.5, 2H), 4.57 (hept, *J*=6.2, 1H), 4.10 (s, 2H), 1.32 (d, *J*=6.3, 6H).

^{13}C NMR (126 MHz, THF): δ = 163.4, 151.8, 148.5, 145.4, 140.0, 137.0, 134.7, 134.3, 129.7, 128.6, 128.4, 128.1, 127.7, 120.0, 118.6, 116.1, 111.2, 87.9, 75.7, 75.0, 66.5, 22.9.

HRMS (ESI): calculated for $[M+H]^+$: 599.2905, found: 599.2908.

Synthesis of 2-(allyloxy)-4-amino-3-isopropoxy-N-(4-nitrophenyl)benzamide ***(117)***



a) 2-(allyloxy)-4-((*tert*-butoxycarbonyl)amino)-3-isopropoxybenzoic acid **116** (50 mg, 0.14 mmol, 1.0 Eq) and 4-nitroaniline (31 mg, 1.6 Eq) were solved in dry EA (1.0 mL) under Argon atmosphere and dry pyridine (48 µL, 4.2 Eq) was added. Then T3P (50% sol. in EA, 0.25 mL, 3.0 Eq) was added and the reaction was stirred 24 h at RT. After reaction control by TLC the reaction solution was diluted between EA/HCl (0.1 M) (25 mL each) and the aqueous phase was extracted with EA (2x20 mL) before collecting organic phases and drying them over Na_2SO_4. The solvent was removed u.r.p. to give a yellow oil directly used in the next reaction.

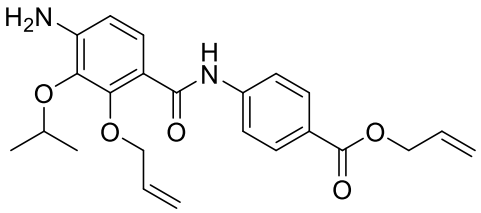
b) The crude material was suspended in HCl (sol. in dioxane, 4 M, 1.4 mL) under Argon atmosphere and the suspension was stirred at RT. The reaction was screened by TLC and after 1 h 40 min the suspension was concentrated u.r.p.. The crude material was purified by FCC (solid loading, 110x theoretical product mass, cyclohexane/EA, 75/25) to give 21.7 mg, 41% of a yellow gum.

^{1}H NMR (500 MHz, THF): δ = 10.42 (s, 1H, H-NCO), 8.18 (d, *J*=9.3, 2H), 7.92 (d, *J*=9.3, 2H), 7.72 (d, *J*=8.7, 1H), 6.56 (d, *J*=8.7, 1H), 6.21 (ddt, *J*=17.2, 10.4, 5.7, 1H), 5.53 (dq, *J*=17.2, 1.7, 1H), 5.36 (dq, *J*=10.5, 1.2, 1H), 5.28 – 5.12 (m, 2H, H_2N), 4.73 (dt, *J*=5.6, 1.4, 2H), 4.57 (hept, *J*=6.1, 1H), 1.32 (d, *J*=6.3, 6H).

^{13}C NMR (126 MHz, THF): δ = 164.3, 152.2, 149.5, 146.7, 143.8, 136.9, 134.6, 128.5, 125.7, 119.8, 119.2, 115.0, 111.4, 75.9, 75.3, 23.0.

HRMS (ESI): calculated for $[M+H]^+$: 372.1554, found: 372.1557.

Synthesis of allyl 4-(2-(allyloxy)-4-amino-3-isopropoxybenzamido)benzoate ***(5)***



a) Allyl 4-aminobenzoate (520 mg, 2.93 mmol, 1.00 Eq) and standard ring D-fragment (provided by Evotec, 915 mg, 1.11 Eq) were solved in dry DCM (10 mL) under Argon atmosphere before adding pyridine (0.71 mL, 3.0 Eq). Then $POCl_3$ (0.28 mL, 1.05 Eq) was added dropwise at 0°C and the reaction was stirred at RT for 1 h. The mixture was diluted between EA/HCl (1 M) (30 mL each) and the aqueous phase was extracted with EA (2x20 mL) before washing the combined organic phases with brine (1x10 mL), drying over Na_2SO_4 and removing solvents u.r.p.. The crude product was directly used in the next step.

b) Adapted from[9, 96]: The crude product was solved in THF/EtOH (15 mL each) and AcOH (3.4 mL, 20 Eq) and Zn dust (1.92 g, 10.0 Eq) were added. The reaction was stirred vigourously for 1 h and monitored by LCMS before filtrating the solution and concentrating the filtrate u.r.p.. The residue was diluted between EA/sat. $NaHCO_3$ sol. (30 mL each) and the aqueous phase was extracted with EA (3x20 mL) before drying the combined organic phases over Na_2SO_4 and removing solvents u.r.p.. The crude material was purified by FCC (solid loading, 40x theoretical product mass, PE/acetone, 80/20). A yellow oil was obtained (725 mg, 60% o2s).

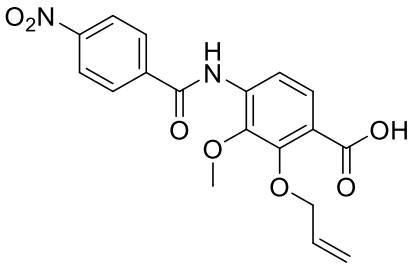
^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.29 (s, 1H), 8.04 (d, *J*=8.7, 2H), 7.86 (d, *J*=8.5, 1H), 7.74 (d, *J*=8.9, 2H), 6.71 (d, *J*=8.7, 1H), 6.19 – 6.00 (m, 2H), 5.49 (dq, *J*=17.1, 1.5, 1H), 5.44 – 5.36 (m, 2H), 5.28 (dq, *J*=10.4, 1.4, 1H), 5.11 (br s, 2H, H_2N), 4.81 (dt, *J*=5.6, 1.4, 2H), 4.69 (dt, *J*=5.8, 1.4, 2H), 4.61 (hept, *J*=6.3, 1H), 1.35 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 166.0, 163.5, 150.8, 144.9, 143.2, 137.4, 132.7, 132.5, 131.0, 127.6, 125.0, 119.7, 119.1, 118.2, 116.9, 112.1, 75.8, 74.7, 65.5, 22.8.

HRMS (ESI): calculated for $[M+H]^+$: 411.1915, found: 411.1917.

CD-fragments

Synthesis of 2-(allyloxy)-3-methoxy-4-(4-nitrobenzamido)benzoic acid ***(78)***



tert-butyl 2-(allyloxy)-4-amino-3-methoxybenzoate **77** (provided by C. Leitner, 400 mg, 1.43 mmol, 1.00 Eq) and 4-nitrobenzoyl chloride (279 mg, 1.05 Eq) were solved in dry DCM (7.0 mL) under Argon atmosphere and dry pyridine (0.23 mL, 2.0 Eq) was added. After 1 h, solvents were removed from the reaction mixture u.r.p. and by coevaporation with EA/heptane to give a yellowish crude product. The crude product was secured under Ar, solved in HCl/dioxane (4 M, 10 mL) and the reaction was stirred for 40 min. The reaction mixture was quenched by diluting in water (70 mL) and extracting with EA (1x50 mL, 2x30 mL). The combined organic phases were washed with brine (10 mL) and water (10 mL), dried over Na_2SO_4 and solvents were removed u.r.p.. The residue was solved again in HCl/dioxane (4 M, 6.0 mL) and the reaction was stirred for 1 h. Solvents were removed u.r.p. to obtain a pale yellow amorphous solid (553 mg, NMR: >98%) containing residual amounts of 4-nitrobenzoic acid (~8 mol%).

^{1}H NMR (700 MHz, DMSO): δ = 12.86 (s, 1H), 10.11 (s, 1H, H-NCO), 8.37 (d, *J*=8.9, 2H), 8.17 (d, *J*=8.9, 2H), 7.74 (d, *J*=8.6, 1H), 7.50 (d, *J*=8.5, 1H), 6.09 (ddt, *J*=17.3, 10.5, 5.6, 1H), 5.39 (dq, *J*=17.2, 1.7, 1H), 5.23 (dq, *J*=10.4, 1.3, 1H), 4.53 (dt, *J*=5.6, 1.5, 2H), 3.85 (s, 3H).

^{13}C NMR (176 MHz, DMSO): δ = 166.5, 164.3, 151.4, 149.3, 145.8, 140.0, 135.1, 134.2, 129.4, 125.3, 123.8, 123.6, 118.8, 117.5, 74.5, 60.9.

HRMS (ESI): calculated for [M+H]+: 373.1031, found: 373.1033.

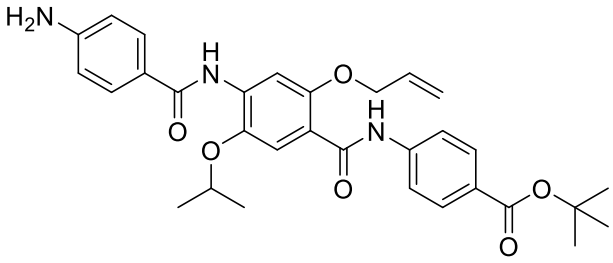
CDE-fragments

NMR spectra of CDE-fragments in $CDCl_3$ or acetone often show unexpected multiplet signals which may be explained by either salt formation with acidic impurities or from aggregates formed in solution. Deuterated DMSO, MeCN or THF were therefore used as NMR-solvent, if necessary.

General procedure **A** (coupling of 4-nitrobenzoyl chloride with the DE-fragments):

Adapted from[96]: The DE-fragment and 4-nitrobenzoyl chloride were solved in dry DCM under Argon atmosphere. Then dry pyridine was added and the solution was stirred at RT for the given amount of time while being screened via TLC or LCMS. The mixture was diluted between HCl (0.1-0.5 M, 15-30 mL) and DCM (1x15-30 mL) and the aqueous phase was extracted with DCM (1-2x15-30 mL). The combined organic phases were washed with H_2O (1x5-10 mL) when required and dried with Na_2SO_4. The solvent was removed u.r.p. to give the crude intermediate that was directly used in the subsequent nitro reduction. If a workup was omitted, only the solvent was removed u.r.p..

Synthesis of tert-butyl 4-(2-(allyloxy)-4-(4-aminobenzamido)-5-isopropoxybenzamido) benzoate ***(24)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **23** (23 mg, 54 µmol, 1.0 Eq), 4-nitrobenzoyl chloride (10.6 mg, 1.05 Eq) and dry pyridine (5.3 µL, 65 µmol, 1.2 Eq) in dry DCM (1.0 mL) for 2 h.

Deviating from the general aqueous workup, this workup was performed: The mixture was diluted between HCl (0.1 M, 15 mL) and DCM (1x15 mL), the aqueous phase was extracted with DCM (1x15 mL) and washed with sat. $NaHCO_3$ solution (1x20 mL). Combined organic phases were dried with Na_2SO_4 and the solvent was removed u.r.p. to give a yellow solid that was directly used in the next step.

b) Adapted from[9, 96]: The crude product was solved in THF/EtOH (1.0 mL+0.5 mL) and AcOH (47 µL, 15 Eq) and Zn powder (53 mg, 15 Eq) were added in two portions directly

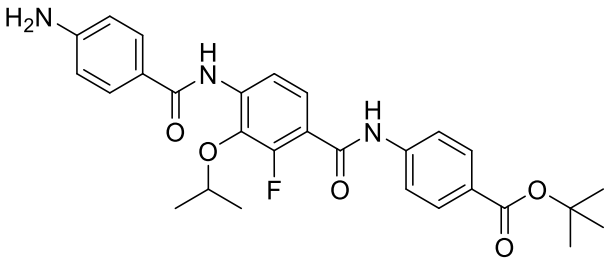
and after 10 min. The reaction was stirred 2 h before the suspension was diluted between MTBE (15 mL) and sat. $NaHCO_3$ sol. (15 mL). The aqueous phase was extracted with MTBE (1x15 mL) and combined organic phases were washed with brine (1x20 mL), dried over Na_2SO_4 and solvents were removed u.r.p.. The pale yellow colored solid was purified by FCC (solid loading, 100x reactants mass, PE/EA, 75/25) and solvents were removed u.r.p. to give a yellowish oil, yield: 25 mg, 84% o2s, purity: ~90 wt% (impurity: low boiling residues from petrol ether). The product was dried further at high vacuum overnight.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 10.36 (s, 1H, H-NCO), 8.76 (s, 1H, H-NCO), 8.51 (s, 1H, H-$C_{Ar,D}$), 7.97 (d, *J*=8.9, 2H), 7.84 (s, 1H, H-$C_{Ar,D}$), 7.72 (d, *J*=8.9, 2H), 7.70 (d, *J*=9.0, 2H), 6.81 (d, *J*=8.5, 2H), 6.21 (ddt, *J*=17.2, 10.5, 6.0, 1H), 5.57 (dq, *J*=17.2, 1.4, 1H), 5.48 (dq, *J*=10.2, 1.0, 1H), 4.79 (d, *J*=6.0, 2H), 4.75 – 4.68 (m, 1H), 1.59 (s, 9H), 1.41 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 165.6, 165.2, 163.3, 151.5, 149.3, 142.6, 140.8, 133.9, 131.9, 130.8, 129.0, 127.2, 124.7, 120.7, 119.1, 115.5, 115.4, 115.2, 104.7, 80.9, 72.4, 71.2, 28.4, 22.4.

HRMS (ESI): calculated for $[M+H]^+$: 546.2599, found: 546.2598.

*Synthesis of tert-butyl 4-(4-(4-aminobenzamido)-2-fluoro-3-isopropoxybenzamido) benzoate **(144)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **132** (47.6 mg, 0.123 mmol, 1.00 Eq), 4-nitrobenzoyl chloride (24.2 mg, 1.07 Eq) and dry pyridine (20 µL, 2.0 Eq) in dry DCM (2.5 mL) for 3 h. An aqueous workup was omitted.

b) Adapted from[9, 96]: The crude product was suspended in a THF/EtOH-mixture (1.4 mL each) under Argon atmosphere and Zn powder (80 mg, 10 Eq) and AcOH (0.14 mL, 20 Eq) were added. The reaction was stirred vigourously and Zn (2x40 mg, 2x5.0 Eq) and AcOH (2x70 µL, 2x10 Eq) were added again after 1 h and 2.5 h. After 3.5 h and LCMS control the mixture was diluted between EA/sat. $NaHCO_3$ sol. (30 mL each) and the aqueous phase

was extracted with EA (2x15 mL). The organic phase was washed with brine (10 mL) and dried over Na_2SO_4. The solvents were removed u.r.p. and the material was purified by FCC (solid loading, 75x theoretical product mass, PE/EA, 60/40). The product was obtained as a green-colorless gum (60.5 mg, 97% o2s, NMR: 88% o2s). For the next reaction the product was coevaporated with DCM/heptane to give an off-colorless solid.

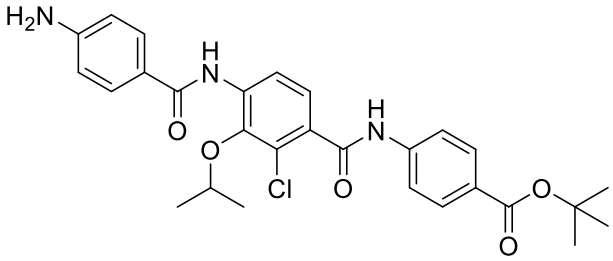
^{1}H NMR (500 MHz, Acetone): δ = 9.61 (d, *J*=4.3, 1H, H-NCO), 8.77 (s, 1H, H-NCO), 8.39 (dd, *J*=8.8, 1.4, 1H), 7.98 (d, *J*=8.9, 2H), 7.91 (d, *J*=9.0, 2H), 7.75 (d, *J*=8.9, 2H), 7.56 (dd, *J*=8.7, 7.6, 1H), 6.79 (d, *J*=8.7, 2H), 5.44 (s, 2H, H_2N), 4.65 – 4.56 (m, 1H), 1.59 (s, 9H), 1.41 (dd, *J*=6.3, 0.8, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 165.6, 165.3, 163.0, 153.8 (d, *J*=248), 153.6, 143.8, 138.4 (d, *J*=5.0), 135.4 (d, *J*=14.1), 131.1 (C_{Ar}-H), 129.8 (C_{Ar}-H), 128.1, 125.5 (d, *J*=2.9), 122.2, 119.9 (C_{Ar}-H), 119.7 (d, *J*=12.0), 115.8 (d, *J*=4.0), 114.3, 81.0, 78.4 (d, *J*=5.2), 28.3, 22.9.

^{19}F NMR (471 MHz, Acetone): δ = -130.7.

HRMS (ESI): calculated for $[M+H]^+$: 508.2242, found: 508.2242.

*Synthesis of tert-butyl 4-(4-(4-aminobenzamido)-2-chloro-3-isopropoxybenzamido) benzoate **(27)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **133** (76.8 mg, 0.190 mmol, 1.00 Eq), 4-nitrobenzoyl chloride (37.0 mg, 1.05 Eq) and dry pyridine (30.6 µL, 2.0 Eq) in dry DCM (3.0 mL) for 1-2 h. An aqueous workup was omitted and residual pyridine was coevaporated with acetone and toluene.

b) Adapted from[9, 96]: The crude product was suspended in a THF/EtOH-mixture (2.0 mL each) and Zn powder (124 mg, 10.0 Eq) and AcOH (0.22 mL, 20 Eq) were added. The reaction was stirred vigourously and Zn (2x62 mg, 2x5.0 Eq) and AcOH (2x0.11 mL, 2x10 Eq) were added again after 1 h and 2.5 h. After 3.5 h and LCMS control the mixture

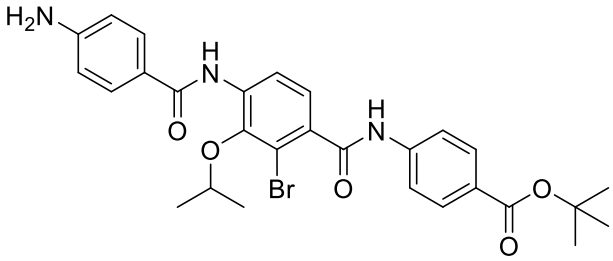
was diluted between EA/sat. $NaHCO_3$ sol. (40 mL each) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were washed with brine (10 mL) and dried over Na_2SO_4. Solvents were removed u.r.p. and the material was purified by FCC (solid loading, 75x theoretical product mass, PE/EA, 60/40->50/50). The product was obtained as green-colorless gum (99.2 mg, 100% o2s, NMR-corrected yield: 90.5 mg, 91%). For the next reaction the product was coevaporated with DCM/heptane to give an off-colorless solid.

^{1}H NMR (500 MHz, Acetone): δ = 9.84 (s, 1H, H-NCO), 8.75 (s, 1H, H-NCO), 8.42 (d, *J*=8.4, 1H), 7.98 (d, *J*=8.9, 2H), 7.92 (d, *J*=8.9, 2H), 7.79 (d, *J*=8.9, 2H), 7.39 (d, *J*=8.4, 1H), 6.79 (d, *J*=8.7, 2H), 5.43 (s, 2H, H_2N), 4.60 (hept, *J*=6.2, 1H), 1.59 (s, 9H), 1.37 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 165.8, 165.6, 165.3, 153.5, 144.6, 143.9, 137.1, 133.1, 131.1, 129.9, 128.0, 125.4, 124.8, 122.2, 119.7, 119.6, 114.3, 81.0, 78.0, 28.3, 22.7.

HRMS (ESI): calculated for $[M+H]^+$: 524.1947, found: 524.1947.

*Synthesis of tert-butyl 4-(4-(4-aminobenzamido)-2-bromo-3-isopropoxybenzamido) benzoate **(145)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **134** (43 mg, 95 µmol, 1.0 Eq), 4-nitrobenzoyl chloride (19.3 mg, 1.09 Eq) and dry pyridine (15.4 µL, 2.00 Eq) in dry DCM (2.0 mL) for 3.0 h. An aqueous workup was omitted.

b) Adapted from[9, 96]: The crude product was suspended in a THF/EtOH-mixture (1.0 mL each) under Argon atmosphere and Zn powder (62 mg, 10 Eq) and AcOH (0.11 mL, 20 Eq) were added. The reaction was stirred vigourously and Zn (2x31 mg, 2x5.0 Eq) and AcOH (2x55 µL, 2x10 Eq) were added again after 1 h and 2.5 h. After 3.5 h and LCMS control the mixture was diluted between EA/sat. $NaHCO_3$ sol. (30 mL each) and the aqueous phase was extracted with EA (2x15 mL). The combined organic phases were washed with brine (10 mL) and dried over Na_2SO_4. The material was purified by FCC (solid loading, 75x

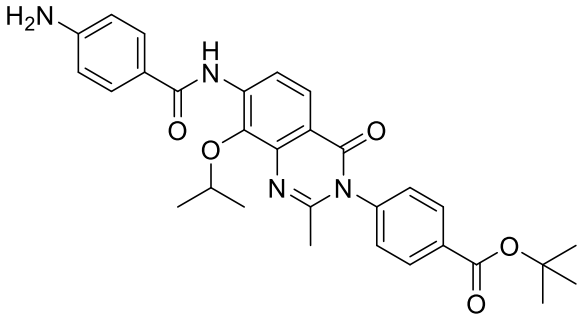
theoretical product mass, PE/EA, 60/40) to give a green-colorless gum (46.3 mg, 85% o2s, purity: 82 mol% (NMR)) that was found by LCMS to be contaminated with a debrominated byproduct (NMR-signals not listed here).

^{1}H NMR (700 MHz, CD_3CN): δ = 8.87 (s, 1H, H-NCO), 8.59 (s, 1H, H-NCO), 8.40 (d, *J*=8.4, 1H), 7.99 – 7.94 (m, 2H), 7.75 (d, *J*=8.6, 2H), 7.72 (d, *J*=8.6, 2H), 7.31 (d, *J*=8.4, 1H), 6.73 (d, *J*=8.6, 2H), 4.76 (s, 2H), 4.68 – 4.61 (m, 1H), 1.57 (d, *J*=1.6, 9H), 1.36 (d, *J*=6.2, 6H).

^{13}C NMR (176 MHz, CD_3CN): δ = 167.0, 166.0, 165.7, 153.2, 146.0, 143.5, 137.0, 131.3, 130.0, 128.5, 125.0, 122.7, 120.8, 120.4, 119.8, 115.3, 114.5, 81.6, 78.5, 28.4, 22.6.

HRMS (ESI): calculated for $[M+H]^+$: 568.1469/ 570.1421, found: 554.1442/ 570.1426.

*Synthesis of tert-butyl 4-(7-(4-aminobenzamido)-8-isopropoxy-2-methyl-4-oxoquinazolin-3(4H)-yl)benzoate **(58)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **135** (10 mg, 23 µmol, 1.0 Eq), 4-nitrobenzoyl chloride (4.5 mg, 1.05 Eq) and dry pyridine (2.9 µL, 1.6 Eq) in dry DCM (0.5 mL) for 2.0 h.

b) Adapted from[9, 96]: The crude material was solved in a EtOH/THF mixture (0.3 mL each) and AcOH (33 µL, 25 Eq) and Zn dust (11 mg, 7.5 Eq) were added. The reaction was screened by TLC and LCMS and after 1 h Zn dust (11 mg, 7.5 Eq) was added again and the reaction was stirred overnight. The next day THF (0.5 mL) and Zn dust (11 mg, 7.5 Eq) were added again and the reaction was stirred for 1 h before adding AcOH (33 µL, 25 Eq). To ensure full turnover Zn dust (11 mg, 7.5 Eq) and AcOH (13 µL, 10 Eq) were added again 1.5 h after the last addition and the reaction was stirred for additional 15 min. The reaction solution was diluted between DCM/sat. $NaHCO_3$ sol. (15 mL each) and the aqueous phase was extracted with DCM (3x10 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p. . The crude material was purified by

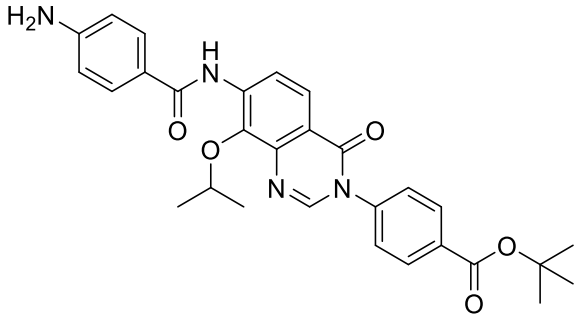
FCC (solid loading, 100x theoretical product mass, PE/EA, 60/40) to get a pale yellow gum, yield: 6.2 mg, 51% o2s, purity: ~90% (NMR).

^{1}H NMR (700 MHz, CD_3CN): δ = 8.95 (s, 1H, H-NCO), 8.62 (d, *J*=8.8, 1H, H-$C_{Ar,D}$), 8.14 (d, *J*=8.1, 2H, H-C_{Ar}), 7.89 (d, *J*=8.8, 1H, H-$C_{Ar,D}$), 7.73 (d, *J*=8.6, 2H, H-C_{Ar}), 7.46 (d, *J*=8.1, 2H, H-C_{Ar}), 6.75 (d, *J*=8.7, 2H, H-C_{Ar}), 5.24 – 5.18 (m, 1H, H-$C_{iPr}O$), 4.76 (s, 2H, H_2N), 2.16 (s, 3H, H_3C), 1.61 (s, 9H, $(H_3C)_3$), 1.41 (d, *J*=6.1, 6H, $(H_3C)_2C$).

^{13}C NMR (HSQC, HMBC, 176 MHz, CD_3CN): δ = 165.7 (C=O), 165.6 (C=O), 162.5 ($C_{Ar,D}$-<u>C</u>=O), 154.2 (<u>C</u>(=N)(N)CH_3), 153.1 (C_{Ar}-NH_2), 143.1 (C_{Ar}), 141.7 ($C_{Ar,D}$-N), 141.0 ($C_{Ar,D}$-OiPr), 138.9 ($C_{Ar,D}$-N), 133.7 (C_{Ar}), 131.5 (C_{Ar}-H), 129.8 (C_{Ar}-H), 129.6 (C_{Ar}-H), 123.1 (C_{Ar}), 122.3 ($C_{Ar,D}$-H), 118.5 ($C_{Ar,D}$-H), 117.9 ($C_{Ar,D}$-CO), 114.5 (C_{Ar}-H), 82.4 (<u>C</u>$(CH_3)_3$), 78.2 (<u>C</u>$(CH_3)_2$), 28.2 (C(<u>C</u>$H_3)_3$), 24.8 (CH_3), 22.8 ($(\underline{C}H_3)_2C$).

HRMS (ESI): calculated for $[M+H]^+$: 529.2445, found: 529.2442.

*Synthesis of tert-butyl 4-(7-(4-aminobenzamido)-8-isopropoxy-4-oxoquinazolin-3(4H)-yl) benzoate **(146)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **136** (28.3 mg, 71.6 µmol, 1.00 Eq), 4-nitrobenzoyl chloride (17.8 mg, 1.35 Eq) and dry pyridine (12 µL, 2.0 Eq) in dry DCM (1.5 mL) overnight.

b) Adapted from[9, 96]: The crude material was suspended in dry THF/EtOH (1.0 mL each) and AcOH (82 µL, 20 Eq) was added. Then Zn dust (47 mg, 10 Eq) was added and the reaction was stirred vigourously while being screened by LCMS. AcOH (82 µL, 20 Eq) and Zn dust (47 mg, 10 Eq) were added again after 75 min. After 2 h reaction the mixture was diluted between DCM/sat. $NaHCO_3$ sol. (20 mL each) and the organic phase was extracted with DCM (2x15 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was removed u.r.p.. The material was purified by FCC (solid loading, 100x

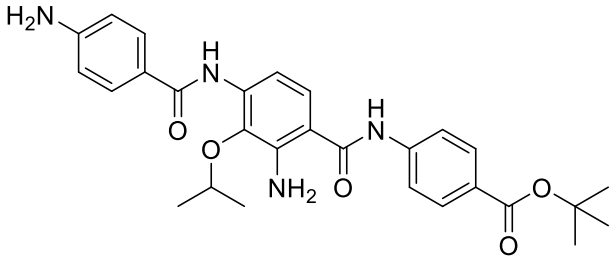
theoretical product mass, PE/Acetone, 70/30) to give a colorless solid after removing solvents u.r.p. and by coevaporation with heptane. Yield: 29.1 mg, 79%.

^{1}H NMR (700 MHz, CD_3CN): δ = 8.96 (s, 1H, H-NCO), 8.70 (d, *J*=8.8, 1H, H-$C_{Ar,D}$), 8.13 (d, *J*=8.4, 2H, H-C_{Ar}), 8.12 (s, 1H, H-C(=N)(N)), 8.00 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 7.74 (d, *J*=8.6, 2H, H-C_{Ar}), 7.58 (d, *J*=8.4, 2H, H-C_{Ar}), 6.75 (d, *J*=8.7, 2H, H-C_{Ar}), 5.19 (hept, *J*=6.0, 1H, H-C_{iPr}O), 4.77 (s, 2H, H_2N), 1.60 (d, *J*=1.4, 9H, $(H_3C)_3$), 1.40 (d, *J*=6.1, 6H, $(H_3C)_2C$).

^{13}C NMR (HSQC, HMBC, 176 MHz, CD_3CN): δ = 165.8 (C=O), 165.6 (C=O), 161.1 ($C_{Ar,D}$-C=O), 153.2 (C_{Ar}-NH_2), 146.1 (H-C(=N)(N)), 142.5 (C_{Ar}), 142.2 ($C_{Ar,D}$-N), 141.5 ($C_{Ar,D}$-OiPr), 139.3 ($C_{Ar,D}$-N), 133.3 (C_{Ar}), 131.1 (C_{Ar}-H), 130.0 (C_{Ar}-H), 128.3 (C_{Ar}-H), 123.0 (C_{Ar}), 122.6 ($C_{Ar,D}$-H), 119.6 ($C_{Ar,D}$-H), 119.3 ($C_{Ar,D}$), 114.5 (C_{Ar}-H), 82.4 ($\underline{C}(CH_3)_3$), 78.4 ($\underline{C}(CH_3)_2$), 28.3 ($C(\underline{C}H_3)_3$), 22.9 ($(\underline{C}H_3)_2C$).

HRMS (ESI): calculated for $[M+H]^+$: 515.2289, found: 515.2288.

*Synthesis of tert-butyl 4-(2-amino-4-(4-aminobenzamido)-3-isopropoxybenzamido) benzoate **(64)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **63** (30 mg, 78 µmol, 1.0 Eq), 4-nitrobenzoyl chloride (7.2+7.9 mg, 0.50+0.55 Eq, directly and after 30 min) and dry pyridine (19 µL, 3.0 Eq) in dry DCM (0.80 mL) for 1 h 15 min. EA was used as extraction solvent.

b) Adapted from[9, 96]: The crude material was suspended in a THF/EtOH mixture (0.60 mL each) and Zn dust (51 mg, 10 Eq) was added while stirring the reaction vigourously. Then AcOH (2x45 µL, 2x10 Eq) was added directly and after 30 min while the reaction was screened by LCMS. After 2 h Zn dust (25 mg, 5.0 Eq) and AcOH (45 µL, 10 Eq) were added again. After 3 h the reaction mixture was put in -70°C freezer overnight before the reaction was warmed to RT, AcOH (45 µL, 10 Eq) was added again and the reaction was stirred vigourously for 45 min. The mixture was then diluted between EA/sat. $NaHCO_3$

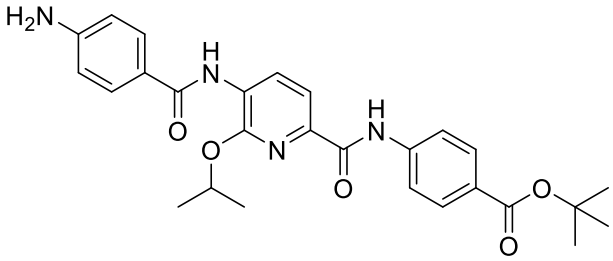
sol. (25 mL each) and the aqueous phase was extracted with EA (2x20 mL) before washing the combined organic phases with brine (5 mL) and drying over Na_2SO_4. The crude material was purified by FCC (solid loading, 100x theoretical product mass, cyclohexane/EA, 70/30->50/50) to give an almost colorless solid after solvent removal and coevaporation with cyclohexane (yield: 29.2 mg, 74% o2s, NMR: 67% o2s).

^{1}H NMR (700 MHz, THF): δ = 9.37 (s, 1H, H-NCO), 8.55 (s, 1H, H-NCO), 7.91 (d, *J*=8.7, 2H), 7.83 – 7.79 (m, 3H), 7.70 (d, *J*=8.6, 2H), 7.42 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 6.63 (d, *J*=8.6, 2H), 6.11 (s, 2H, H_2N), 5.18 (s, 2H, H_2N), 4.43 (hept, *J*=6.1, 1H), 1.58 (s, 9H), 1.35 (d, *J*=6.1, 6H).

^{13}C NMR (176 MHz, THF): δ = 168.7, 165.6, 164.8, 153.3, 145.3, 144.7, 137.5, 134.4, 130.9, 129.6, 127.4, 124.5, 123.1, 119.9, 114.0, 112.4, 108.5, 80.6, 74.8, 28.5, 22.9.

HRMS (ESI): calculated for $[M+H]^+$: 505.2446, found: 505.2448.

*Synthesis of tert-butyl 4-(5-(4-aminobenzamido)-6-isopropoxypicolinamido)benzoate **(75)***



a) Note: *tert*-butyl 4-(5-amino-6-isopropoxypicolinamido)benzoate **137** (354 mg crude DE-fragment) contained cyclohexane and was freeze-dried under vacuum to remove cyclohexane residues for this purpose, ending in a mass amount of 308 mg.

The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **137** (218 mg, assumed as 1.00 Eq), 4-nitrobenzoyl chloride (123 mg, 1.13 Eq) and dry pyridine (0.14 mL, 3.0 Eq) in dry DCM (6.0 mL) for 45 min. EA was used as extraction solvent.

b) Adapted from[9, 96]: The crude product was solved in THF/EtOH (3.0 mL each) under Argon atmosphere and AcOH (0.67 mL, 20 Eq) and Zn powder (384 mg, 10.0 Eq) were added. The mixture was stirred vigourously at RT while being monitored with LCMS. After 2.5 h the mixture was suspended between EA/sat. $NaHCO_3$ sol. (40 mL each) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was purified by

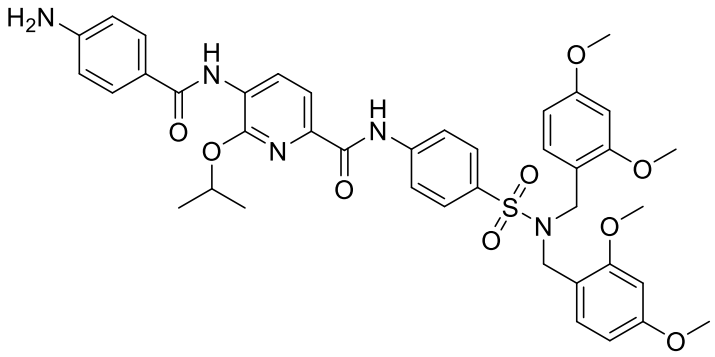
FCC (solid loading, 80x theoretical product mass, cyclohexane/EA, 70/30->60/40->PE/acetone, 60/40) and solvents were removed u.r.p. to give two fractions, a pure fraction showing a beige fine powdered solid, 180 mg, 63% o2s.

^{1}H NMR (700 MHz, DMSO): δ = 10.24 (s, 1H, H-NCO), 8.95 (s, 1H, H-NCO), 8.60 (d, *J*=8.1, 1H), 7.97 (d, *J*=8.7, 2H), 7.92 (d, *J*=8.7, 2H), 7.77 (d, *J*=8.0, 1H), 7.67 (d, *J*=8.7, 2H), 6.64 (d, *J*=8.6, 2H), 5.93 (s, 2H, H_2N), 5.78 (hept, *J*=6.2, 1H), 1.55 (s, 9H), 1.43 (d, *J*=6.2, 6H).

^{13}C NMR (176 MHz, DMSO): δ = 165.0, 164.6, 162.5, 152.9, 152.0, 142.3, 139.9, 129.9, 129.3, 128.1, 126.7, 126.3, 119.7, 119.7, 116.4, 112.8, 80.4, 69.5, 27.9, 21.8.

HRMS (ESI): calculated for $[M+H]^+$: 491.2289, found: 491.2292.

*Synthesis of 5-(4-aminobenzamido)-N-(4-(N,N-bis(2,4-dimethoxybenzyl)sulfamoyl)phenyl)-6-isopropoxypicolinamide **(147)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **138** (119 mg, 183 µmol, 1.00 Eq), 4-nitrobenzoyl chloride (36 mg, 1.05 Eq) and dry pyridine (44 µL, 3.0 Eq) in dry DCM (1.8 mL).

b) Adapted from[9, 96]: The crude product was suspended in a THF/EtOH mixture (1.8 mL/0.90 mL) and AcOH (0.21 mL, 20 Eq) was added. Then Zn powder (119 mg, 10.0 Eq) was added and the mixture was stirred vigourously while being monitored by LCMS. After 1.5 h and 3.2 h AcOH (2x0.10 mL, 2x10 Eq) was added and after 4 h 15 min Zn (60 mg, 5.0 Eq) and AcOH (0.10 mL, 10 Eq) were added again. After 5.5 h the mixture was suspended between EA/sat. $NaHCO_3$ sol. (25 mL each) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The material was purified by FCC (solid loading, 85x theoretical product mass, cyclohexane/acetone, 70/30->60/40), solvents were removed

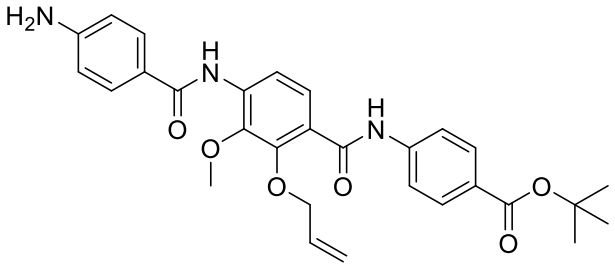
u.r.p. and by coevaporation with EA/heptane. A beige solid (122 mg, 87% o2s) was obtained.

^{1}H NMR (500 MHz, THF): δ = 9.86 (s, 1H, H-NCO), 8.98 (d, *J*=8.1, 1H, H-$C_{Ar,D}$), 8.62 (s, 1H, H-NCO), 7.91 (d, *J*=8.9, 2H), 7.88 (d, *J*=8.1, 1H, H-$C_{Ar,D}$), 7.71 (d, *J*=8.9, 2H), 7.67 (d, *J*=8.5, 2H), 7.10 (d, *J*=8.4, 2H), 6.64 (d, *J*=8.7, 2H), 6.36 (dd, *J*=8.3, 2.4, 2H), 6.31 (d, *J*=2.4, 2H), 5.73 – 5.65 (m, 1H), 5.35 – 5.27 (m, 2H, H_2N), 4.31 (s, 4H, $N(CH_2)_2$), 3.71 (d, *J*=1.7, 6H, 2xCH_3-O), 3.60 (d, *J*=1.5, 6H, 2xCH_3-O), 1.53 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, THF): δ = 165.6, 162.8, 161.5, 159.2, 153.8, 151.9, 142.8, 140.1, 137.1, 131.3, 129.8, 129.0, 128.7, 126.8, 122.3, 119.8, 118.1, 117.8, 114.0, 104.8, 98.5, 70.9, 55.4, 55.3, 46.5, 22.3.

HRMS (ESI): calculated for $[M+H]^+$: 770.2854, found: 770.2859.

*Synthesis of tert-butyl 4-(2-(allyloxy)-4-(4-aminobenzamido)-3-methoxybenzamido) benzoate **(79)***



a) 2-(allyloxy)-3-methoxy-4-(4-nitrobenzamido)benzoic acid **78** (300 mg, 0.806 mmol, 1.00 Eq) and *tert*-butyl 4-aminobenzoate (164 mg, 1.05 Eq) were suspended in dry EA (4.0 mL) under Argon atmosphere. Then dry pyridine (0.33 mL) and T3P (sol. in EA, 50%, 0.48 mL) were added and the reaction was stirred at RT. After 2.0 h EA (4.0 mL) was added to the mixture to enhance solubility of the reactants. After 3 h reaction and monitoring by TLC/LCMS the suspension was diluted between EA/HCl (0.5 M) (30 mL each) and the aqueous phase was extracted with EA (1x20 mL) (Note: The product may form a precipitate on top of the organic phase). Combined organic phases were washed with brine (1x10 mL) before removing solvent u.r.p.. The yellow crude product (498 mg) was directly used in the next step.

b) Adapted from[9, 96]: The crude product was suspended in THF/EtOH (8 mL/4 mL) and AcOH (0.92 mL, 20 Eq) was added. Then Zn dust (0.53 mg, 10 Eq) was added and the

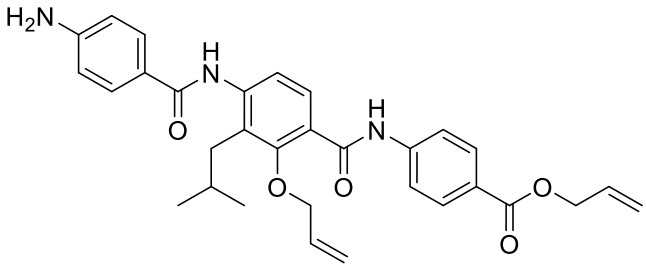
reaction was stirred vigourously at RT. After 1 h 45 min AcOH (0.46 mL, 10 Eq) and Zn dust (0.26 mg, 5.0 Eq) were added again. The reaction was screened by LCMS and after 2.0 h the suspension was filtrated and the filtrate was diluted between EA/sat. $NaHCO_3$ sol. (40 mL each). The aqueous phase was extracted with EA (2x20 mL), combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The material was purified by FCC (solid loading, 75x theoretical product mass, PE/acetone, 80/20->70/30->0/100) to give a brownish crystalline solid (301 mg, 72% o2s, NMR: 68% o2s). The product contained ~10 mol% of a byproduct (LCMS: 313 m/z, C-E coupling product).

^{1}H NMR (700 MHz, DMSO): δ = 10.49 (s, 1H, H-NCO), 9.16 (s, 1H, H-NCO), 7.89 (d, *J*=8.8, 2H), 7.86 (d, *J*=8.5, 1H), 7.83 (d, *J*=8.8, 2H), 7.71 (d, *J*=8.7, 2H), 7.39 (d, *J*=8.5, 1H), 6.62 (d, *J*=8.7, 2H), 6.03 (ddt, *J*=17.3, 10.5, 5.6, 1H), 5.85 (s, 2H), 5.39 (dq, *J*=17.2, 1.7, 1H), 5.20 (dq, *J*=10.4, 1.3, 1H), 4.60 (dt, *J*=5.6, 1.5, 2H), 3.90 (s, 3H), 1.55 (s, 9H).

^{13}C NMR (176 MHz, DMSO): δ = 165.0, 164.6, 164.4, 152.6, 149.1, 143.9, 143.0, 135.4, 133.6, 130.1, 129.4, 126.0, 125.9, 123.8, 120.2, 118.8, 118.1, 117.9, 112.7, 80.3, 74.5, 60.8, 27.8.

HRMS (ESI): calculated for $[M+H]^+$: 518.2304, found: 518.2289.

Synthesis of allyl 4-(2-(allyloxy)-4-(4-aminobenzamido)-3-isobutylbenzamido)benzoate ***(148)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **139** (57.9 mg, 142 µmol, 1.00 Eq), 4-nitrobenzoyl chloride (32 mg, 1.2 Eq) and dry pyridine (23 µL, 2.0 Eq) in dry DCM (2.8 mL) for 2 h. EA was used as extraction solvent.

b) Adapted from[9, 96]: The crude product was solved in THF/EtOH (1.5 mL each) and AcOH (0.16 mL, 20 Eq) and Zn powder (93 mg, 10 Eq) were added. After 30 min AcOH (0.10 mL, 12 Eq) and Zn powder (56 mg, 6.0 Eq) were added and after 130 min AcOH

(50 µL, 6.0 Eq) was added again. After 2.5 h the mixture was diluted between EA/sat. $NaHCO_3$ sol. (30 mL each) and the aqueous phase was extracted with EA (2x20 mL) before drying the combined organic phases over Na_2SO_4 and removing solvent u.r.p.. The material was purified by FCC (solid loading, 100x theoretical product mass, PE/EA, 50/50) to obtain an off colorless powder after coevaporation with heptane (59.9 mg, 80% o2s). NMR contains ~10 mol% impurity.

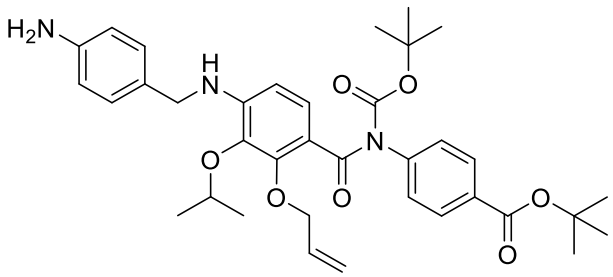
Note: When the substance was solved in $CDCl_3$ the solution turned into a solid gel-like mass (formation of salt?).

^{1}H NMR (700 MHz, CD_3CN): δ = 9.76 (s, 1H, H-NCO), 8.19 (s, 1H, H-NCO), 8.02 (d, *J*=8.7, 2H), 7.84 – 7.79 (m, 3H), 7.72 – 7.67 (m, 3H), 6.71 (d, *J*=8.7, 2H), 6.13 – 6.04 (m, 2H), 5.48 (dq, *J*=17.2, 1.7, 1H), 5.42 (dq, *J*=17.2, 1.6, 1H), 5.28 (ddq, *J*=10.4, 5.9, 1.5, 2H), 4.80 (dt, *J*=5.5, 1.6, 2H), 4.70 (s, 2H, H_2N), 4.42 (dt, *J*=5.2, 1.6, 2H), 2.64 (d, *J*=7.2, 2H), 0.92 (d, *J*=6.6, 6H).

^{13}C NMR (176 MHz, CD_3CN): δ = 166.4, 166.4, 165.2, 156.7, 152.9, 144.2, 142.3, 134.1, 133.8, 133.7, 131.5, 130.3, 130.1, 129.5, 126.2, 125.4, 123.2, 122.4, 120.2, 114.4, 114.3, 76.7, 66.1, 34.9, 29.9, 23.0.

HRMS (ESI): calculated for $[M+H]^+$: 528.2493, found: 528.2493.

*Synthesis of tert-butyl 4-(2-(allyloxy)-4-((4-aminobenzyl)amino)-N-(tert-butoxycarbonyl)-3-isopropoxybenzamido)benzoate **(96)***



(proposed structure)

a) *tert*-butyl 4-(2-(allyloxy)-4-amino-3-isopropoxybenzamido)benzoate **8** (standard DE-fragment, provided by Evotec, 300 mg, 0.703 mmol, 1.00 Eq) and 4-nitrobenzaldehyde (106 mg, 1.00 Eq) were solved in dry DCM (2.0 mL) under Argon atmosphere. The mixture was stirred 4 d and monitored by LCMS (reaction showed no significant progress). Then AcOH (0.36 mL, 9.0 Eq) was added and $NaBH_4$ (80 mg, 3.0 Eq) was added

portionwise at 0°C. The reaction mixture was stirred 1.5 h, before it was diluted between DCM/sat. $NaHCO_3$ sol. (15 mL each) and the aqueous phase was extracted with DCM (15 mL). The combined organic phases were washed with H_2O (15 mL) and the washing solution was reextracted with DCM (5 mL). All organic phases were combined and dried over Na_2SO_4 before the solvent was removed u.r.p..

To the crude material, 4-nitrobenzaldehyde (106 mg, 1.00 Eq) was added and the mixture was solved in DCM (2.0 mL). AcOH (0.36 mL, 9.0 Eq) was added and the reaction was stirred for 1.5 h at RT, then at 40°C overnight. The next day, DiPEA (1.62 mL, 13.5 Eq) was added in two portions directly and after 2 h and the mixture was stirred further under reflux, still showing no significant process (analysis by LCMS). The workup of the reaction mixture was performed as known, but with DCM/ HCl (1 M) and the solvent was removed from the combined organic phases u.r.p.. Molecular sieve (4Å), AcOH (0.36 mL, 9.0 Eq) and DCM (4 mL) were added to the concentrated solution and then $NaBH_4$ (80 mg, 3.0 Eq) was added at 0°C. The reaction solution was again stirred overnight at RT, subsequently diluted between DCM/sat. $NaHCO_3$ sol. (30 mL each) and the aqueous phase was extracted with DCM (2x15 mL). The combined organic phases were washed with H_2O (15 mL) and dried over Na_2SO_4. The crude product was purified by FCC (solid loading, 40x reactants mass, PE/EA, 80/20->70/30) and used as a mixed fraction with 4-benzylalcohol directly in the next step.

b) The mixed fraction and DMAP (spatula tip) were solved in dry THF (1.0 mL) under Argon atmosphere. Boc_2O (0.16 mL, 3.0 Eq) and dry TEA (0.11 mL, 3.5 Eq) were added dropwise and the reaction was stirred for 5.5 h at RT while being screened by LCMS. MeOH (5 mL) was added to the reaction mixture and solvents were removed u.r.p.. The crude material was directly used in next step.

c) Adapted from[9, 96]: The crude material was suspended in THF/EtOH (1 mL each) and AcOH (0.26 mL, 20 Eq) and Zn (149 mg, 10.0 Eq) were added. After 30 min AcOH (0.13 mL, 10 Eq) and Zn (75 mg, 5.0 Eq) were added again and the reaction was stirred overnight. The reaction solution was diluted between DCM/water (20 mL each). The aqueous phase was extracted with DCM (2x15 mL) and the collected organic phases were dried over Na_2SO_4 before removing solvents u.r.p.. The product was isolated by FCC (solid loading, 80x reactant mass, PE/acetone, 90/10->85/15). The product fraction was

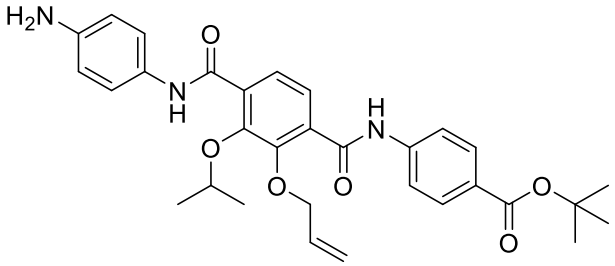
identified by LCMS and solvents were removed u.r.p. to give a light yellow gum, yield: 41.1 mg, 9.2% o3s (air sensitive!).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.98 (d, *J*=8.7, 2H), 7.28 – 7.21 (m, 3H), 7.11 (d, *J*=8.5, 2H), 6.66 (d, *J*=8.4, 2H), 6.44 – 6.35 (m, 1H, H-$C_{Ar,D}$), 6.12 – 6.03 (m, 1H), 5.40 (dq, *J*=17.2, 1.6, 1H), 5.23 (dq, *J*=10.4, 1.3, 1H), 4.89 (br s, 1H, H-N-CH_2), 4.59 – 4.49 (m, 3H), 3.84 (br s, 2H, H_2N), 1.59 (s, 9H), 1.32 (s, 9H), 1.23 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ= 169.7, 165.2, 152.7, 149.6, 146.9, 145.5, 143.5, 136.3, 133.9, 130.5, 129.9, 128.5, 127.7, 126.5, 118.9, 117.4, 115.3 (2x), 105.8, 82.6, 81.0, 75.0, 74.3, 47.1, 28.1, 27.6, 22.6.

HRMS (ESI): calculated for $[M+H]^+$: 632.3330, found: 632.3324.

*Synthesis of tert-butyl 4-(2-(allyloxy)-4-((4-aminophenyl)carbamoyl)-3-isopropoxybenzamido)benzoate **(99)***



a) 3-(allyloxy)-4-((4-(*tert*-butoxycarbonyl)phenyl)carbamoyl)-2-isopropoxybenzoic acid **98** (402 mg, 0.883 mmol, 1.00 Eq) and 4-nitroaniline (146 mg, 1.20 Eq) were solved in dry EA (6.0 mL) under Argon atmosphere and dry pyridine (0.21 mL, 3.0 Eq) was added. The solution was stirred for few minutes before adding T3P (sol. in EA, 50%, 1.05 mL, 2.00 Eq) dropwise. The reaction was stirred at RT while being screened with LCMS. After 7 h the reaction mixture was diluted between EA/HCl (0.1 M) (30 mL each) and the aqueous phase was extracted with EA (2x20 mL). Organic phases were combined and dried over Na_2SO_4 before removing solvents u.r.p.. The crude product was directly used in the next step.

b) Adapted from[9, 96]: The crude material was solved in THF/EtOH (7.0 mL each) and AcOH (1.01 mL, 20.0 Eq) and Zn powder (0.58 g, 10 Eq) were added. After 1.5 h stirring at RT the mixture was diluted between EA/sat. $NaHCO_3$ sol. (40 mL each) and the aqueous phase was extracted with EA (2x30 mL) before drying over Na_2SO_4 and removing solvent

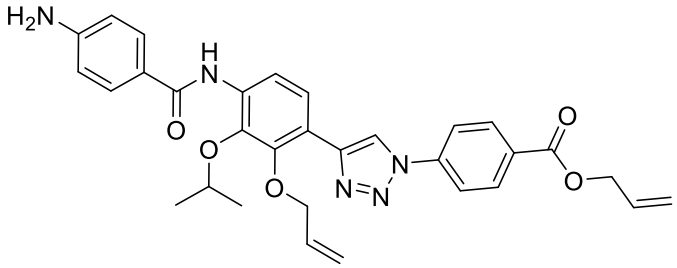
u.r.p.. The product was purified by FCC (solid loading, 50x reactant mass, cyclohexane/EA, 60/40->50/50) to obtain a yellow solid after coevaporation with MTBE/heptane (1x) and freeze pumping with liquid N_2 (2x). The yellow solid was stored under Argon atmosphere and its container was wrapped in Aluminium foil in the fridge (450 mg, 93% o2s, air/light sensitive!).

^{1}H NMR (500 MHz, CDCl3): δ = 10.18 (s, 1H, H-NCO), 9.68 (s, 1H, H-NCO), 8.09 (s, 2H), 8.00 (d, J = 8.9, 2H), 7.74 (d, J = 8.9, 2H), 7.50 (d, J = 8.9, 2H), 6.72 (d, J = 8.9, 2H), 6.16 (ddt, J = 17.1, 10.4, 6.0, 1H), 5.51 (dq, J = 17.1, 1.4, 1H), 5.43 (dq, J = 10.4, 1.1, 1H), 4.83 (hept, J = 6.3, 1H), 4.73 (dt, J = 6.0, 1.3, 2H), 3.66 (s, 2H, H_2N), 1.60 (s, 9H), 1.40 (d, J = 6.3, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 165.5, 162.2, 161.8, 150.2, 148.6, 143.6, 141.9, 132.9, 132.2, 130.8, 129.9, 129.5, 127.8, 127.2, 126.9, 121.7, 120.4, 119.3, 115.7, 81.1, 78.3, 75.6, 28.4, 22.5.

HRMS (ESI): calculated for $[M+H]^+$: 546.2599, found: 546.2599.

*Synthesis of allyl 4-(4-(2-(allyloxy)-4-(4-aminobenzamido)-3-isopropoxyphenyl)-1H-1,2,3-triazol-1-yl)benzoate **(149)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **142** (130 mg, 0.299 mmol, 1.00 Eq), 4-nitrobenzoyl chloride (58 mg, 0.31 mmol, 1.05 Eq) and dry pyridine (45 µL, 2.0 Eq) in dry DCM (6.0 mL) for 16 h.

b) Adapted from[9, 96]: For the following step the material was splitted in two equally sized batches: The crude material was suspended in EtOH (5.1 mL) and AcOH (0.63 µL, 37 Eq) under Argon atmosphere and the solution was cooled in an ice bath. Zn powder (0.21 g, 11 Eq) was added in two portions over 15 min and the mixture was vigourously stirred at RT. The reaction was screened by LCMS and after 2 h or 3.5 h both approaches were combined. The reaction solution was diluted between MTBE (50 mL) and sat. $NaHCO_3$ sol.

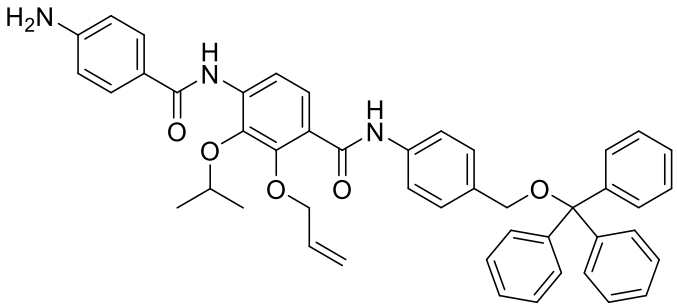
(50 mL). The aqueous phase was extracted again with MTBE (1x50 mL) and the combined organic phases were washed with brine (50 mL). After drying over Na_2SO_4 and rinsing of the Na_2SO_4-solid with EA the solvents were removed u.r.p.. Because the reaction was not completed, the solid was again suspended in a EtOH/THF (5 mL/2 mL) under Argon atmosphere. AcOH (0.65 mL, 35 Eq) and Zn powder (0.21 g, 10 Eq) were added in 2 portions over 15 min and the reaction was stirred for 1 h 45 min. After reaction control by TLC and LCMS the mixture was diluted between EA and sat. $NaHCO_3$ sol.(50 mL each) and the aqueous phase was extracted with EA (2x50 mL). The combined organic phases were washed with brine (1x70 mL) and dried over Na_2SO_4. The crude product was purified by FCC (solid loading, 75x reactants mass, PE/EA, 65/35->60/40) to give a yield of 130 mg, 79% o2s.

^{1}H NMR (700 MHz, Acetone): δ = 8.87 (s, 1H), 8.73 (s, 1H), 8.43 (d, *J*=8.7, 1H), 8.28 (d, *J*=8.8, 2H), 8.12 (d, *J*=8.8, 2H), 8.04 (d, *J*=8.7, 1H), 7.76 (d, *J*=8.6, 2H), 6.79 (d, *J*=8.6, 2H), 6.24 – 6.16 (m, 1H), 6.16 – 6.09 (m, 1H), 5.51 (dq, *J*=17.2, 1.7, 1H), 5.47 (dq, *J*=17.2, 1.7, 1H), 5.38 (s, 2H), 5.32 – 5.29 (m, 2H), 4.88 (dt, *J*=5.6, 1.5, 2H), 4.79 (hept, *J*=6.2, 1H), 4.66 (dt, *J*=5.6, 1.5, 2H), 1.39 (d, *J*=6.1, 6H).

^{13}C NMR (176 MHz, Acetone): δ = 165.6, 165.2, 153.4, 149.1, 144.9, 141.5, 141.1, 135.8, 135.4, 133.6, 132.2, 131.0, 129.8, 123.3, 123.1, 121.2, 120.9, 120.6, 118.5, 118.2, 116.8, 114.5, 76.8, 73.8, 66.5, 23.0.

HRMS (ESI): calculated for $[M+H]^+$: 554.2398, found: 554.2388.

Synthesis of 2-(allyloxy)-4-(4-aminobenzamido)-3-isopropoxy-N-(4-((trityloxy)methyl) phenyl)benzamide ***(115)***



a) The nitro-CDE-fragment was obtained according to general procedure A, involving the reaction of the DE-fragment **143** (150 mg, 0.251 mmol, 1.00 Eq), 4-nitrobenzoyl chloride

(56 mg, 1.2 Eq) and dry pyridine (60 µL, 3.0 Eq) in dry DCM (2.5 mL) for 1 h. EA was used as extraction solvent.

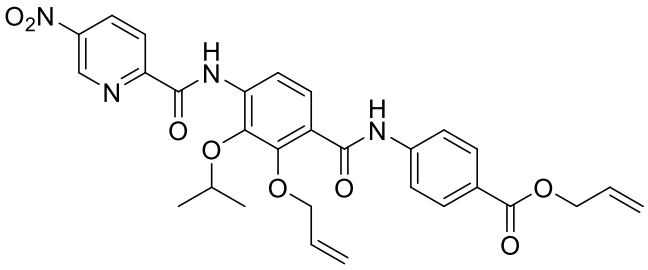
b) Adapted from[9, 96]: The crude material was suspended in a THF/EtOH mixture (1.2 mL each) and AcOH (0.29 mL, 20 Eq) and Zn powder (164 mg, 10.0 Eq) were added. The reaction was screened with LCMS and after 3 h 15 min the mixture was diluted between EA/sat. $NaHCO_3$ sol. (25 mL each). The aqueous phase was extracted with EA (2x25 mL) and after collecting the organic phases and drying over Na_2SO_4 solvents were removed u.r.p.. The material was purified by FCC (solid loading, 70x theoretical product mass, cyclohexane/EA, 70/30->60/40) to give a yellowish solid after solvent removal by coevaporation with DCM/heptane (2x). Yield: 163 mg, 91% o2s, NMR: 84% o2s.

^{1}H NMR (500 MHz, THF): δ = 9.92 (s, 1H, H-NCO), 8.72 (s, 1H, H-NCO), 8.46 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 7.85 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 7.73 (d, *J*=8.5, 2H), 7.70 (d, *J*=8.7, 2H), 7.52 (d, *J*=7.2, 6H), 7.35 – 7.26 (m, 8H), 7.22 (t, *J*=7.3, 3H), 6.64 (d, *J*=8.7, 2H), 6.26 – 6.15 (m, 1H), 5.52 (dq, *J*=17.1, 1.7, 1H), 5.34 (dq, *J*=10.5, 1.4, 1H), 5.23 (s, 2H), 4.79 – 4.68 (m, 3H), 4.12 (s, 2H, H_2C-O), 1.38 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, THF): δ = 164.8, 163.0, 153.4, 150.4, 145.4, 140.1, 139.5, 139.0, 135.0, 134.4, 129.7, 128.6, 128.5, 127.8, 127.2, 123.3, 122.8, 120.2, 119.0, 116.3, 114.0, 87.9, 77.2, 75.5, 66.5, 23.0.

HRMS (ESI): calculated for $[M+H]^+$: 718.3276, found: 718.3280.

Synthesis of allyl 4-(2-(allyloxy)-3-isopropoxy-4-(5-nitropicolinamido)benzamido)benzoate ***(150)***



5-Nitropicolinic acid (110 mg, 0.654 mmol, 1.00 Eq) was filled in a Schlenk flask under Argon atmosphere and a solution of DMF in DCM (10 mM, 3.3 mL) was added under Schlenk conditions. To the suspension oxalyl chloride (59 µL, 1.05 Eq) was added. The mixture was stirred for 1.5 h at RT while being screened with LCMS (A MeOH quench of

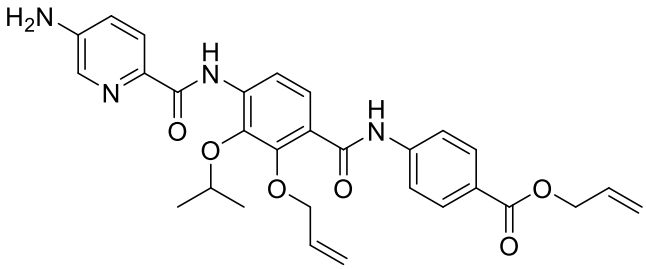
the sample was applied to identify the acid chloride). After 1.5 h a solution of allyl 4-(2-(allyloxy)-4-amino-3-isopropoxybenzamido)benzoate **5** (256 mg, 0.95 Eq) and dry pyridine (126 µL, 2.5 Eq) in dry DCM (3.1 mL) was added dropwise and the mixture was stirred further at RT while being monitored with LCMS. The mixture was quenched after 1 h by adding brine (30 mL). The aqueous phase was extracted with EA (3x20 mL) before drying the combined organic phases over Na_2SO_4 and removing solvent u.r.p. and by coevaporation with acetone/heptane. A yellow solid (318 mg, 91%) was obtained.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.78 (s, 1H, H-NCO), 10.27 (s, 1H, H-NCO), 9.51 (d, *J* = 2.6, 1H, H-C_{Ar}-N_{Ar}), 8.73 (dd, *J* = 8.5, 2.4, 1H, H-C_{Ar}-C_{Ar}-C_{Ar}-N_{Ar}), 8.54 (*J* = 8.9, 2H, 2x H-$C_{Ar,D}$), 8.10 (d, *J* = 8.9, 1H, H-C_{Ar}-C_{Ar}-N_{Ar}), 8.07 (d, *J* = 8.7, 2H), 7.77 (d, *J* = 8.7, 2H), 6.16 (ddt, *J* = 16.3, 10.4, 5.8, 1H), 6.05 (ddt, *J* = 17.4, 10.7, 5.6, 1H), 5.52 (dq, *J* = 17.2, 1.6, 1H), 5.46 – 5.38 (m, 2H), 5.30 (dq, *J* = 10.4, 1.4, 1H), 4.82 (dt, *J* = 5.6, 1.4, 2H), 4.78 – 4.70 (m, 3H), 1.44 (d, *J* = 6.3, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 166.0, 162.7, 159.9, 154.0, 149.8, 145.9, 144.1, 142.8, 139.9, 136.8, 133.3, 132.5, 132.3, 131.1, 127.7, 125.5, 123.4, 122.4, 120.3, 119.3, 118.3, 115.6, 75.1 (2x), 65.6, 22.8.

HRMS (ESI): calculated for $[M+H]^+$: 561.1980, found: 561.1984.

*Synthesis of allyl 4-(2-(allyloxy)-4-(5-aminopicolinamido)-3-isopropoxybenzamido) benzoate **(126)***



Adapted from[9, 96]:

Allyl 4-(2-(allyloxy)-3-isopropoxy-4-(5-nitropicolinamido)benzamido)benzoate **150** (223 mg, 0.398 mmol, 1.00 Eq) was solved in THF/EtOH (2.0 mL/1.0 mL) and AcOH (0.46 mL, 20 Eq) and Zn dust (260 mg, 10.0 Eq) were added. The mixture was stirred vigourously for 1 h while being screened with LCMS. Then the mixture was quenched by solving between sat. $NaHCO_3$ sol. (100 mL) and EA (50 mL), the phase mixture was

filtered through pore size 4 and the aqueous phase was extracted with EA (3x30 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was purified by FCC (solid loading, 90x reactant mass, PE/EA, 60/40->50/50) to give a pale yellow amorphous solid. Yield: 121 mg, 57%.

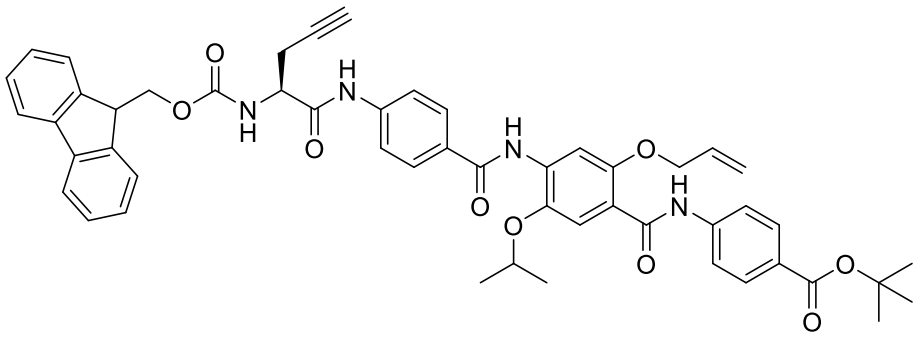
^{1}H NMR (700 MHz, CD_3CN): δ = 10.64 (s, 1H, H-NCO), 10.11 (s, 1H, H-NCO), 8.46 (d, *J*=8.8, 1H), 8.07 (d, *J*=2.8, 1H), 8.02 (d, *J*=8.2, 2H), 7.94 (d, *J*=8.5, 1H), 7.86 (d, *J*=8.8, 1H), 7.81 (d, *J*=8.5, 2H), 7.12 (ddd, *J*=8.4, 2.7, 0.9, 1H), 6.20 – 6.13 (m, 1H), 6.11 – 6.04 (m, 1H), 5.52 – 5.48 (m, 1H), 5.44 – 5.39 (m, 1H), 5.34 (dt, *J*=10.5, 1.2, 1H), 5.28 (dt, *J*=10.5, 1.2, 1H), 4.91 (s, 2H, H_2N), 4.79 (dq, *J*=5.5, 1.5, 2H), 4.74 (dq, *J*=5.8, 1.2, 2H), 4.73 – 4.67 (m, 1H), 1.40 (d, *J*=6.2, 6H).

^{13}C NMR (176 MHz, CD_3CN): δ = 166.4, 164.1, 163.6, 151.0, 148.6, 144.2, 140.4, 139.3, 139.0, 136.0, 134.1, 133.8, 131.5, 127.4, 126.1, 124.4, 122.5, 121.2, 120.2, 119.8, 118.2, 115.2, 77.7, 75.6, 66.0, 22.8.

HRMS (ESI): calculated for $[M+H]^+$: 531.2238, found: 531.2241.

AA-CDE-fragments

Synthesis of tert-butyl (S)-4-(4-(4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)pent-4-ynamido)benzamido)-2-(allyloxy)-5-isopropoxybenzamido)benzoate ***(151)***



Adapted from[11]:

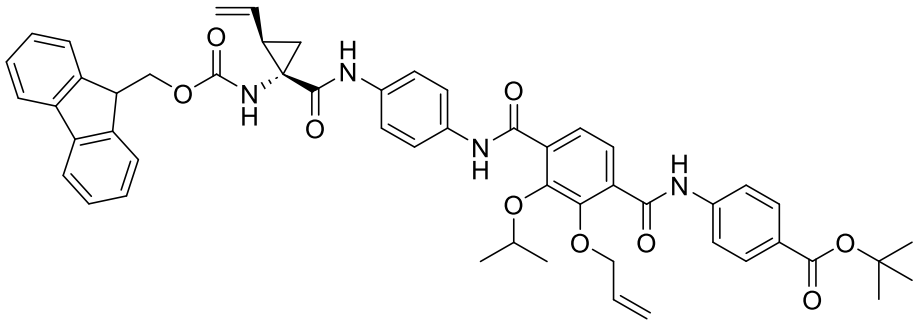
CDE-fragment **24** (36 mg, 66 µmol, 1.0 Eq) was solved in dry EA (1.4 mL) under N_2 atmosphere. This solution was added to an ice cold solution of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)pent-4-ynoic acid **25** (Fmoc-L-Pra-OH, 33 mg, 1.5 Eq) and pyridine (16 µL, 3.0 Eq) in dry EA (0.16 mL). The reaction mixture was cooled to 0°C and T3P (50% sol. in EA, 82 µL, 2.0 Eq) was added dropwise. The yellow solution was stirred for 2 h at 0°C, and was screened by TLC. After 2.2 h the reaction was stopped by solving

the reaction solution between H_2O (2 mL), HCl (1 M, 1.5 mL) and EA (2 mL). The aqueous phase was extracted with EA (2x) and the combined organic phases were washed with brine (2 mL). The solvent was removed u.r.p. and the residue was purified by FCC (solid loading, 50x reactants mass, PE/EA, 65/35). To remove residual amounts of carboxylic acid, solvents were removed u.r.p. from the solution, MTBE (15 mL) was added and the resulting solution was washed with sat. $NaHCO_3$ sol. (3x10 mL). After solvent removal a colorless solid was obtained, yield: 45 mg, 79%.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.36 (s, 1H, H-NCO), 8.84 (s, 1H, H-NCO), 8.52 (s, 1H), 8.47 (br s, 1H, H-NCO), 7.98 (d, *J*=8.8, 2H), 7.87 (d, *J*=8.8, 3H), 7.77 (d, *J*=7.6, 2H), 7.71 (d, *J*=8.8, 2H), 7.69 (d, *J*=8.8, 2H), 7.59 (d, *J*=7.4, 2H), 7.40 (t, *J*=7.5, 2H, H-$C_{fluoren}$), 7.30 (t, *J*=7.2, 2H, H-$C_{fluoren}$), 6.22 (ddt, *J*=17.2, 10.4, 5.9, 1H, H-C(=CH_2)), 5.63 – 5.54 (m, 2H, H-NCOO, H_2C=C), 5.49 (dq, *J*=10.4, 1.0, 1H, H_2C=C), 4.81 (dt, *J*=5.9, 1.1, 2H, H_2C-O), 4.75 (hept, *J*=6.0, 1H, H-C_{iPr}O), 4.58 – 4.44 (m, 3H, H-C(Ar)$_2$, H_2C-O), 4.25 (t, *J*=6.7, 1H, H-C), 2.97 – 2.64 (m, 2H, H_2C(CCH)), 2.17 (t, *J*=2.6, 1H, H-CC), 1.60 (s, 9H, $(H_3C)_3C$), 1.42 (d, *J*=6.1, 6H, $(H_3C)_2CH$).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.4, 165.6, 164.6, 163.2, 156.7, 151.4, 143.6, 143.5, 142.6, 141.5, 140.9, 133.4, 131.9, 130.8, 130.5, 128.3, 128.1, 128.0, 127.3, 125.0, 120.7, 120.3, 120.0, 119.1, 116.0, 115.6, 104.9, 80.9, 79.1, 77.4, 72.5, 71.3, 67.7, 54.3, 47.3, 28.4, 27.1, 22.5.

Synthesis of tert-butyl 4-(4-((4-((1S,2R)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-vinylcyclopropane-1-carboxamido)phenyl)carbamoyl)-2-(allyloxy)-3-isopropoxybenzamido)benzoate ***(152)***



Chemical Formula: $C_{52}H_{52}N_4O_9$
Exact Mass: 876,3734

Adapted from[11]:

CDE-fragment **99** (150 mg, 0.275 mmol, 1.00 Eq) and (1S,2R)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-vinylcyclopropane-1-carboxylic acid **125b** (‘*N*-Fmoc-

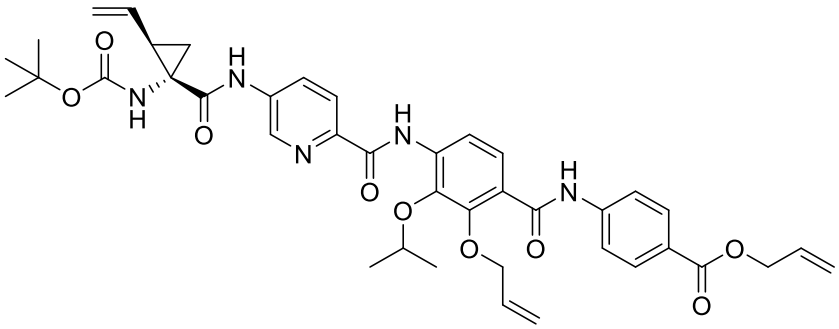
cyclopropane AA', 106 mg, 1.10 Eq) were solved in dry EA (2.0 mL) under Argon atmosphere. Then dry pyridine (67 µL, 3.0 Eq) was added and T3P (50% sol. in EA, 0.33 mL, 2.0 Eq) was added dropwise before stirring the reaction for 2 h 45 min. The solution was diluted between EA/HCl (0.1 M) (40 mL each) and the aqueous phase was extracted with EA (2x20 mL). Combined organic phases were dried over Na_2SO_4 before removing solvents u.r.p.. The material was purified by FCC (solid loading, 70x theoretical product mass, cyclohexane/EA, 70/30->60/40) to give 203 mg, 84% (NMR: 80%) of a yellowish foam.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.17 (s, 1H, H-NCO), 9.87 (s, 1H, H-NCO), 8.74 (d, *J* = 9.5, 1H, H-NCO), 8.13 – 8.08 (m, 2H, H-$C_{Ar,D}$), 8.00 (d, *J* = 8.9, 2H), 7.81 – 7.71 (m, 4H), 7.67 (d, *J* = 9.0, 2H), 7.61 – 7.54 (m, 2H), 7.48 (d, *J* = 8.9, 2H), 7.44 – 7.35 (m, 2H), 7.34 – 7.27 (m, 2H), 6.16 (ddt, *J* = 16.3, 10.4, 6.0, 1H, H-C(=CH_2)(CH_2O)), 5.70 – 5.58 (m, 1H, H-C(CH_2)(CH)), 5.52 (dq, *J* = 17.2, 1.5, 1H), 5.48 – 5.39 (m, 2H, H_2C=C, H-NCO), 5.28 (d, *J* = 17.1, 1H, H_2C=C), 5.13 – 5.07 (m, 1H, H_2C=C), 4.84 (hept, *J* = 6.0, 1H, H-C_{iPr}O), 4.74 (dd, *J* = 5.9, 1.1, 2H, H_2C-O), 4.64 – 4.54 (m, 2H, H_2C-O), 4.22 (t, *J* = 6.0, 1H, H-C(Ar)$_2$), 2.09 (q, *J* = 8.4, 1H, H-$C_{cycloprop.}$(C)$_3$), 1.93 – 1.87 (m, 1H, $H_aH_bC_{cycloprop.}$), 1.60 (s, 9H, $(H_3C)_3C$), 1.42 – 1.36 (m, 6H, $(H_3C)_2C$), 1.30 – 1.23 (m, 1H, $H_aH_bC_{cycloprop.}$).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.3, 167.2, 165.4, 162.1 (2x), 150.3, 148.7, 143.5, 143.5, 141.8, 141.5, 133.6, 132.5, 132.1, 130.9, 130.3, 128.0, 127.9, 127.3, 127.2, 127.0, 124.9, 124.9, 121.0, 120.5, 120.4, 120.3, 119.3, 117.9, 81.1, 78.5, 75.7, 67.2, 47.3, 42.8, 28.4, 25.6, 22.5-22.4 (m), 20.5.

HRMS (ESI): calculated for $[M+H]^+$: 877.3807, found: 877.3812.

Synthesis of allyl 4-(2-(allyloxy)-4-(5-((1S,2R)-1-((tert-butoxycarbonyl)amino)-2-vinylcyclopropane-1-carboxamido)picolinamido)-3-isopropoxybenzamido)benzoate ***(128)***



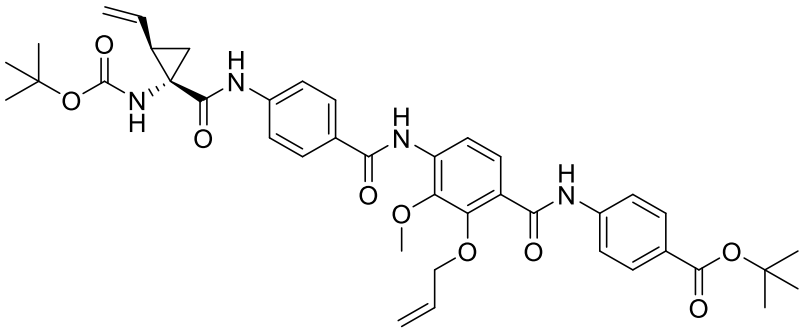
CDE-fragment **128** (50.0 mg, 94.2 µmol, 1.00 Eq), (1S,2R)-1-((*tert*-butoxycarbonyl)amino)-2-vinylcyclopropane-1-carboxylic acid **125** ('*N*-Boc-cyclopropane AA', 22.5 mg, 1.05 Eq) and DMAP (1.3 mg, 0.11 Eq) were suspended in EA (1.0 mL). Pyridine (23 µL, 3.0 Eq), and T3P (84.1 µL, 1.50 Eq) were added and the solution was stirred at RT while being monitored with TLC. After 3 d, NaOH (0.5 M, 15 mL) was added to the mixture to destroy anhydride byproducts and the aqueous phase was extracted with EA (2x15 mL). The pH of the aqueous phase was adjusted to ~2-3 before combining all phases again. After mixing of the two phases the aqueous phase was extracted again with EA (2x15 mL). The combined organic phases were dried over Na_2SO_4 and solvent was removed u.r.p.. The crude product was purified by FCC (solid loading, 100x reactants mass, PE/EA, 60/40) to give 25.5 mg, 37% (NMR: 35%) of an off colorless solid.

^{1}H NMR (700 MHz, $CDCl_3$): δ = 10.70 (s, 1H, H-NCO), 10.29 (s, 1H, H-NCO), 9.66 (br s, 1H, H-NCO), 8.83 (s, 1H, H-$C_{Ar,C}$), 8.51 (d, *J* = 8.8, 1H, H-C_{Ar}), 8.23 (d, *J* = 8.5, 1H, H-C_{Ar}), 8.08 – 8.03 (m, 4H, H-C_{Ar}), 7.76 (d, *J* = 8.8, 2H, H-$C_{Ar,E}$), 6.16 (ddt, *J* = 17.2, 10.3, 5.9, 1H), 6.05 (ddt, *J* = 17.2, 10.4, 5.6, 1H), 5.67 – 5.53 (m, 2H, H-C(CH_2)(CH), H-NCO), 5.51 (dq, *J* = 17.1, 1.5, 1H, HC=C), 5.44 – 5.38 (m, 2H, H_2C=C), 5.33 – 5.27 (m, 2H, H_2C=C), 5.12 (dd, *J* = 10.3, 1.6, 1H, HC=C), 4.82 (dt, *J* = 5.6, 1.5, 2H, $H_2C_{Allyl}O$), 4.75 (d, *J* = 5.8, 2H, $H_2C_{Allyl}O$), 4.66 (hept, *J* = 6.1, 1H, H-$C_{iPr}O$), 2.19 (q, *J* = 8.3, 1H, H-$C_{cycloprop.}(C)_3$), 1.90 (br s, 1H, $H_aH_bC_{cycloprop.}$), 1.52 (s, 9H, $(H_3C)_3C$), 1.42 (dd, *J* = 6.1, 2.0, 6H, $(H_3C)_2C$), 1.35 – 1.19 (m, 1H, $H_aH_bC_{cycloprop.}$).

^{13}C NMR (176 MHz, $CDCl_3$): δ = 168.4, 166.0, 163.0, 161.9, 157.7, 149.9, 144.7, 142.9, 139.6, 139.4, 138.1, 137.8, 133.2, 132.5 (2x), 131.1, 127.5, 127.2, 125.4, 123.3, 121.3, 120.0, 119.3, 118.3 (2x), 115.5, 82.1, 77.0, 75.0, 65.6, 43.1, 32.0, 29.1, 28.4, 22.7.

HRMS (ESI): calculated for $[M+H]^+$: 740.3290, found: 740.3295.

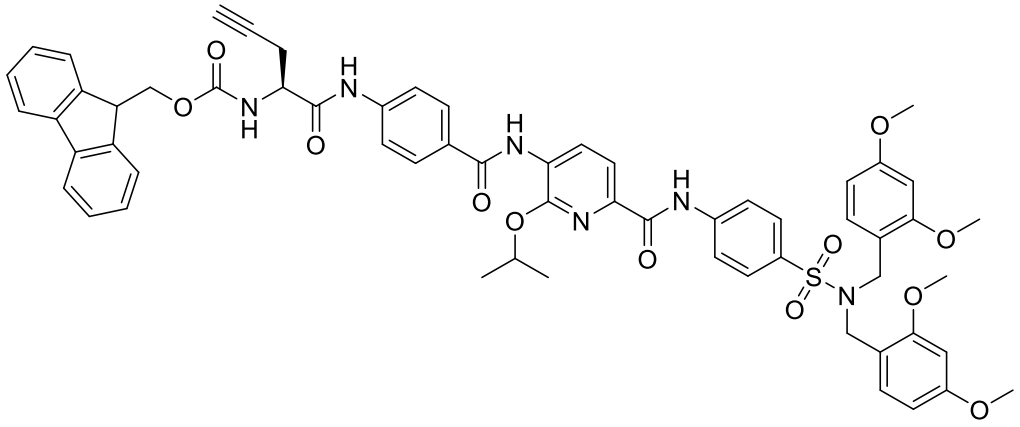
Synthesis of tert-butyl 4-(2-(allyloxy)-4-(4-((1S,2R)-1-((tert-butoxycarbonyl)amino)-2-vinylcyclopropane-1-carboxamido)benzamido)-3-methoxybenzamido)benzoate ***(153)***



The *N*-Boc-cyclopropane AA **125** (18 mg, 79 µmol, 1.0 Eq) and CDE-fragment **79** (43 mg, 1.05 Eq) were solved in dry EA (0.8 mL) under Ar at 0°C. Dry pyridine (22 µL, 3.4 Eq) was added and then T3P (50% sol. in EA, 94 µL, 2.0 Eq) was added portionwise over 10 min. The suspension was stirred further in ice bath while the reaction was screened by LCMS. After 45 min dry DMF (0.10 mL) was added to enhance solubility. After 3 h the reaction was stored overnight in fridge. Then the mixture was diluted between EA/HCl (0.1 M) (15 mL each) and the aqueous phase was extracted with EA (2x15 mL). The combined organic phases were washed with brine (1x5 mL) dried over Na_2SO_4 and solvents were removed u.r.p.. The material was purified using FCC (solid loading, 100x theoretical product mass, cyclohexane/EA, 60/40) obtaining a brownish foam (55.8 mg, 97%, NMR: 85%) after removing solvent u.r.p. and by coevaporation with MTBE. The product contained ~15 mol% impurities and was used without further purification and NMR-characterization in the next reaction.

HRMS (ESI): calculated for $[M+H]^+$: 727.3338, found: 727.3341.

Synthesis of (9H-fluoren-9-yl)methyl (S)-(1-((4-((6-((4-(N,N-bis(2,4-dimethoxybenzyl)sulfamoyl)phenyl)carbamoyl)-2-isopropoxypyridin-3-yl)carbamoyl)phenyl)amino)-1-oxopent-4-yn-2-yl)carbamate ***(154)***



Adapted from[11]:

CDE-fragment **147** (59 mg, 76 µmol, 1.0 Eq) and Fmoc-L-Pra-OH **25** (26.8 mg, 1.05 Eq) were suspended in dry EA (0.80 mL) under Argon atmosphere. Then dry pyridine (18 µL, 3.0 Eq) and T3P (50% sol. in EA, 91 µL, 2.0 Eq) were added and the reaction was stirred vigourously at RT showing a clearification after ~30 min. After 1 h reaction and screening by LCMS the mixture was diluted between EA/HCl (0.1 M) (25 mL each) and the aqueous phase was extracted with EA (2x20 mL) before drying combined organic phases over Na_2SO_4 and removing solvents u.r.p.. The material was purified by FCC (solid loading, 100x theoretical product mass, cyclohexane/EA, 60/40->50/50), solvents were removed u.r.p. and by coevaporation with *n*-heptane to give a beige solid. Yield: 75.6 mg, 91% (NMR: ~85%).

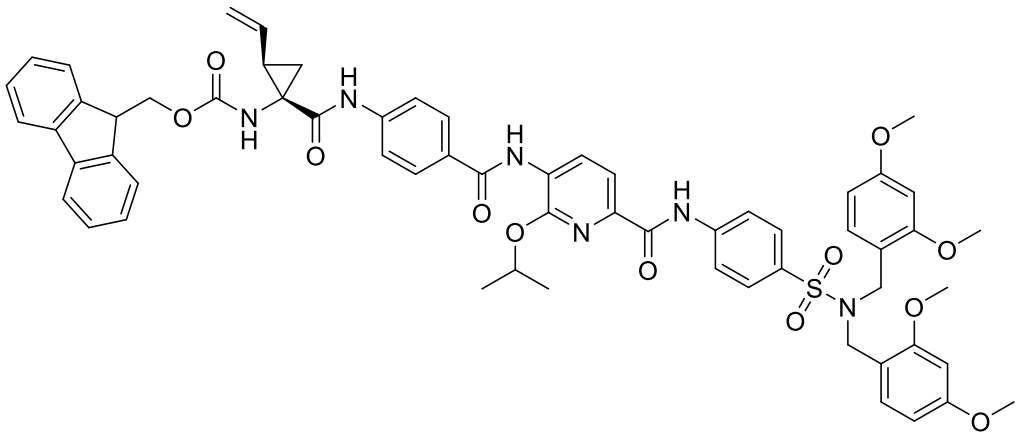
^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.65 (s, 1H, H-NCO), 8.96 (d, *J*=8.1, 1H, H-$C_{Ar,D}$), 8.56 (s, 1H, H-NCO), 8.55 (br s, 1H, H-NCO), 7.97 (d, *J*=8.1, 1H, H-$C_{Ar,D}$), 7.86 (d, *J*=8.9, 2H), 7.78 (d, *J*=7.3, 2H), 7.75 (d, *J*=9.0, 2H), 7.73 – 7.66 (m, 4H), 7.60 (d, *J*=7.8, 2H), 7.41 (t, *J*=7.3, 2H, H-$C_{fluoren}$), 7.31 (t, *J*=7.2, 2H, H-$C_{fluoren}$), 7.16 (d, *J*=8.4, 2H, 2x H-C_{Ar}(C_{Ar}-CH_2-N)), 6.37 (dd, *J*=8.4, 2.4, 2H, 2x H-C_{Ar}(C_{Ar}-OMe)(C_{Ar}-H)), 6.26 (d, *J*=2.4, 2H, 2x H-C_{Ar}(C_{Ar}-OMe)$_2$), 5.70 – 5.60 (m, 1H), 5.49 (hept, *J*=6.3, 1H, H-C_{iPr}O), 4.58 – 4.46 (m, 3H), 4.37 (s, 4H, N(CH_2)$_2$), 4.25 (t, *J*=6.8, 1H), 3.76 (s, 6H, 2x CH_3-O), 3.61 (s, 6H, 2xCH_3-O), 2.96 – 2.67 (m, 2H), 2.17 (t, *J*=2.7, 1H), 1.55 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 168.4, 164.9, 162.2, 160.3, 158.3, 156.7, 150.9, 143.6, 143.5, 141.5, 141.0, 139.5, 136.0, 130.7, 129.9, 128.5, 128.4, 128.0, 127.3, 126.9, 126.7,

125.0, 120.3, 120.0, 119.1, 117.5, 117.1, 104.0, 98.0, 79.0, 72.5, 70.7, 67.7, 55.5, 55.1, 54.2, 47.2, 46.3, 22.3, 22.0.

HRMS (ESI): calculated for $[M+H]^+$: 1087.3906, found: 1087.3905.

*Synthesis of (9H-fluoren-9-yl)methyl ((1S,2R)-1-((4-((6-((4-(N,N-bis(2,4-dimethoxybenzyl)sulfamoyl)phenyl)carbamoyl)-2-isopropoxypyridin-3-yl)carbamoyl)phenyl)carbamoyl)-2-vinylcyclopropyl)carbamate **(155)***



Adapted from[11]:

CDE-fragment **147** (65 mg, 84 µmol, 1.0 Eq) and the *N*-Fmoc-cyclopropane AA **125b** (35 mg, 1.2 Eq) were suspended in dry EA (1.25 mL) under Argon atmosphere and dry pyridine (20 µL, 3.0 Eq) was added. T3P (0.10 mL, 2.0 Eq) was added and the reaction was stirred overnight at RT. After reaction control by LCMS the mixture was diluted between EA/HCl (0.1 M) (30 mL each) and the aqueous phase was extracted with EA (2x20 mL). The combined organic phases were washed with sat. $NaHCO_3$ sol. (20 mL) and dried over Na_2SO_4 before solvents were removed u.r.p.. The material was purified by FCC (solid loading, 100x reactant mass, cyclohexane/EA, 60/40->50/50) to give a beige clumpy solid after solvent removal u.r.p. and coevaporation with Cy and DCM. Yield: 81.1 mg, 87%, (NMR: 75% (contains Cy)).

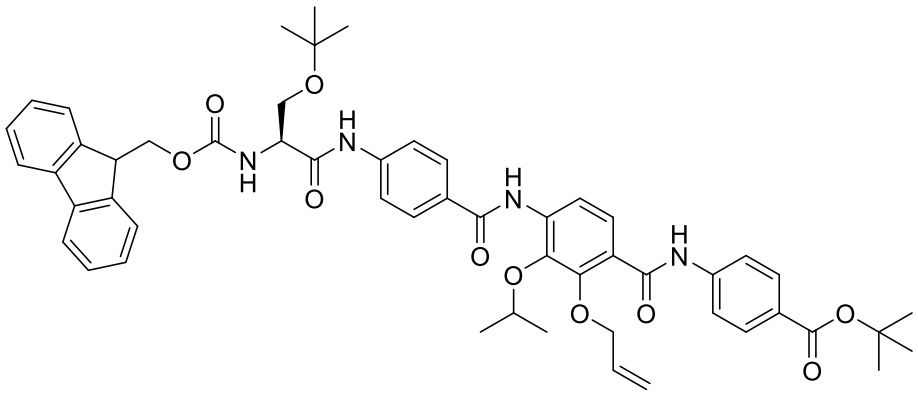
^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.61 (s, 1H, H-NCO), 9.06 (s, 1H, H-NCO), 8.92 (d, *J*=8.1, 1H, H-$C_{Ar,D}$), 8.51 (s, 1H, H-NCO), 7.94 (d, *J*=8.1, 1H, H-$C_{Ar,D}$), 7.81 – 7.66 (m, 8H), 7.64 – 7.52 (m, 4H), 7.42 – 7.33 (m, 2H), 7.32 – 7.20 (m, 2H), 7.15 (d, *J*=8.4, 2H, 2x H-C_{Ar}(C_{Ar}-CH_2-N)), 6.35 (dd, *J*=8.4, 2.4, 2H, 2x H-C_{Ar}(C_{Ar}-OMe), 6.26 (d, *J*=2.4, 2H, 2x H-C_{Ar}(C_{Ar}-OMe)$_2$), 5.96 (s, 1H, H-NCOO), 5.72 – 5.58 (m, 1H, H-C(=CH_2)(C)), 5.46 (hept, *J*=6.3, 1H, H-C_{iPr}O), 5.28 (d, *J*=17.4, 1H, H_2C=C), 5.11 (dd, *J*=10.3, 1.8, 1H H_2C=C), 4.54 (s, 2H, H_2C-O), 4.37 (s, 4H,

N(CH_2)$_2$), 4.20 (t, *J*=6.5, 1H, H-C(Ar)$_2$), 3.74 (s, 6H, 2x CH_3-O), 3.60 (s, 6H, 2x CH_3-O), 2.13 (q, *J*=8.5, 1H, H-$C_{cycloprop.}$(C)$_3$), 1.92 (dd, *J*=7.9, 5.7, 1H, $\underline{H_a}H_bC_{cycloprop.}$), 1.55 (dd, *J*=6.3, 1.7, 6H, (H_3C)$_2$C), 1.35 – 1.29 (m, 1H, $H_a\underline{H_b}C_{cycloprop.}$).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 167.8, 165.0, 162.1, 160.3, 158.2, 157.6, 150.8, 143.7 – 143.3 (m), 142.0, 141.4, 141.0, 139.3, 135.8, 133.4, 130.7, 129.0, 128.4, 128.1, 128.0, 127.2, 126.9, 126.5, 125.0 – 124.9 (m), 120.2, 119.7, 119.0, 118.1, 117.4, 117.0, 104.0, 97.9, 70.7, 67.3, 55.4, 55.0, 47.2, 46.3, 42.9, 34.3, 22.2, 20.7.

HRMS (ESI): calculated for $[M+H]^+$: 1101.4063, found: 1101.4051.

Synthesis of tert-butyl (S)-4-(4-(4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(tert-butoxy)propanamido)benzamido)-2-(allyloxy)-3-isopropoxybenzamido)benzoate ***(156)***



Adapted from[11]:

Standard CDE-fragment **9** (provided by EVOTEC France, 100 mg, 183 µmol, 1.00 Eq) and *N*-Fmoc-Ser(Trt)-OH (77 mg, 1.1 Eq) were solved in dry EA (1.6 mL) under Argon atmosphere. Then dry pyridine (77 µL, 5.2 Eq) and T3P (50% sol. in EA, 0.22 mL, 2.0 Eq) were added and the reaction was stirred at RT while being screened by LCMS. After 2 h T3P (50% sol. in EA, 0.11 mL, 1.0 Eq) was added again and after 3 h the mixture was diluted between EA (30 mL) and HCl (0.1 M, 30 mL). The aqueous phase was then extracted with EA (2x15 mL). The combined organic phases were dried over Na_2SO_4 and the material was purified by FCC (solid loading, 80x theoretical product mass, cyclohexane/EA, 70/30). After solvent removal u.r.p. and by coevaporation with DCM (3x) the material was dried under HV forming a beige sticky foam, yield: 148 mg, 89%.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.19 (s, 1H, H-NCO), 9.01 (s, 1H, H-NCO), 8.75 (s, 1H, H-NCO), 8.50 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 8.07 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 7.98 (d, *J*=8.9, 2H), 7.91 (d, *J*=8.7, 2H), 7.78 (d, *J*=7.9, 2H), 7.74 (d, *J*=8.9, 2H), 7.69 (d, *J*=8.9, 2H), 7.62 (d, *J*=7.3, 2H), 7.41 (t, *J*=7.5, 2H, H-$C_{fluoren}$), 7.37 – 7.27 (m, 2H, H-$C_{fluoren}$), 6.14 (ddt, *J*=17.1, 10.4, 5.8, 1H), 5.85 – 5.77 (m, 1H, H-NCOO), 5.50 (dq, *J*=17.1, 1.5, 1H, H_2C=C), 5.41 (dq, *J*=10.4, 1.1, 1H, H_2C=C), 4.80 – 4.72 (m, 1H, H-C_{iPr}O), 4.70 (dt, *J*=6.0, 1.4, 2H, H_2C-O), 4.47 (d, *J*=7.2, 2H, H_2C-O), 4.40 (s, 1H, $\underline{H_a}H_bC(CH)(O\mathit{t}Bu)$), 4.26 (t, *J*=6.9, 1H, H-C), 3.99 – 3.88 (m, 1H, $H_a\underline{H_b}C(CH)(O\mathit{t}Bu)$), 3.48 (t, *J*=8.8, 1H, H-C), 1.60 (s, 9H, $(H_3C)_3$-OOC), 1.39 (d, *J*=6.3, 6H, $(H_3C)_2CH$), 1.28 (d, *J*=17.7, 9H, $(H_3C)_3$-OC).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 169.0, 165.6, 164.3, 162.7, 149.4, 143.8, 143.8, 142.3, 141.5, 141.2, 139.1, 137.7, 132.3, 130.8, 130.1, 128.4, 128.0, 127.6, 127.4, 127.2, 125.2, 121.7, 120.2, 120.2, 119.6, 119.1, 115.8, 80.9, 75.3, 75.1, 67.4, 61.8, 55.0, 47.3, 28.4, 27.7, 23.0.

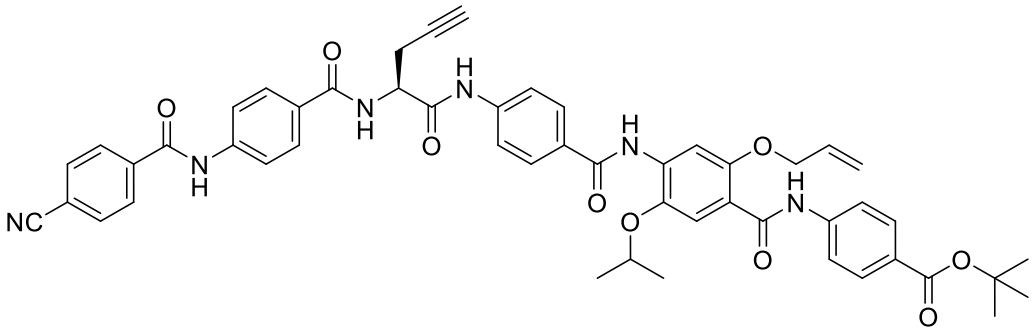
HRMS (ESI): calculated for $[M+H]^+$: 911.4226, found: 911.4226.

Cystobactamid precursors

General procedure **B** (coupling between the standard AB-central AA-fragment and CDE-fragments):

The CDE-fragment and the standard AB-central AA-fragment **26** were suspended under Argon atmosphere in dry EA and pyridine and T3P (50% solution in EA) were added. The reaction was stirred at RT for the given amount of time while being screened by TLC or LCMS. Clarification of the original solution was often observed. The reaction solution was diluted between EA (20-30 mL) and an adequate amount of HCl (0.1-1 M, 15-30 mL) and the aqueous phase was extracted with EA (2x15-20 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was removed u.r.p. to give the crude product or intermediate. If a workup was omitted, only the solvent was removed u.r.p..

*Synthesis of Allyl/tBu-protected p-hydroquinone cystobactamid **(157)***



Adapted from[11, 96]:

a) Note: following reactions were conducted in one small (6 µmol) and one large (41 µmol educt) batch that were combined before column purification.

The Fmoc-protected AA-CDE-fragment **151** (40 mg, 41 +5.6 µmol, 1.0 Eq) was suspended in MeCN (0.8 mL) under nitrogen atmosphere and $HNEt_2$ (81 µL, 17 Eq) was added at 0°C. Reaction stirred 1 h at 0°C and 1 h at RT. Educt was fully converted as shown with LCMS. Solvents were removed from the mixture u.r.p. and by multiple coevaporation with MeCN. The crude product was further dried u.r.p. and used directly in next step.

b) The crude material, 4-(4-cyanobenzamido)benzoic acid **10** (16 mg, 60 µmol, 1.3 Eq) and HATU (23 mg, 60 µmol, 1.3 Eq) were solved in DMF (0.6 mL) under N_2 atmosphere at 0°C. After 5 min, DiPEA was added (24 µL, 3.0 Eq) and the reaction mixture stirred further at 0°C until LCMS showed full educt conversion. The reaction was quenched with HCl

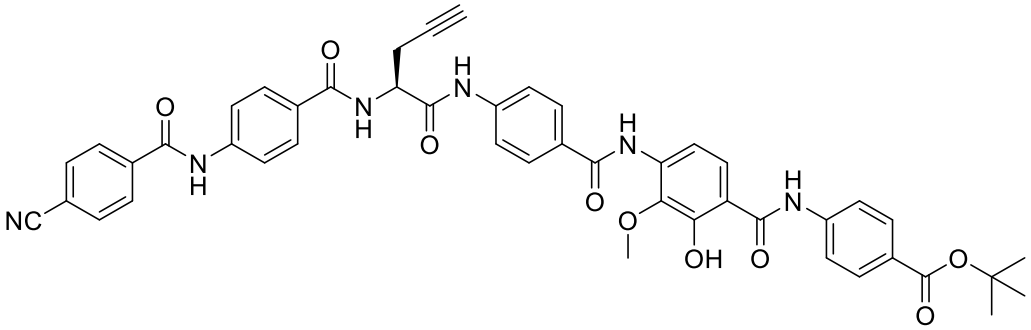
(0.1 M, 3 mL) and brine (12 mL) was added before the aqueous phase was extracted with EA and the organic phase was washed with brine. Solvents were removed u.r.p. to get a yellowish solid that was purified by FCC (solid loading, PE/EA, 40/60->50/50). A colorless solid was obtained, yield: 33 mg, 80% o2s, NMR: <70% o2s. The product contained ~50 mol% tetramethylurea and minor impurities and was used without further purification.

^{1}H NMR (500 MHz, Acetone): δ = 10.34 (s, 1H, H-NCO), 10.01 (s, 1H, H-NCO), 9.90 (s, 1H, H-NCO), 9.01 (s, 1H, H-NCO), 8.51 (s, 1H, H-$C_{Ar,D}$), 8.18 (d, *J*=8.7, 2H), 8.13 (d, *J*=7.6, 1H, H-NCO), 8.02 (d, *J*=9.0, 2H), 7.98 – 7.92 (m, 8H), 7.91 – 7.83 (m, 4H), 7.77 (s, 1H), 6.34 (ddt, *J*=17.2, 10.4, 5.8, 1H), 5.64 (dq, *J*=17.2, 1.4, 1H), 5.47 (dq, *J*=10.4, 1.0, 1H), 4.97 (q, *J*=7.5, 1H), 4.86 (d, *J*=5.8, 2H), 4.81 – 4.72 (m, 1H), 3.00 – 2.86 (m, 2H), 2.52 (t, *J*=2.6, 1H), 1.59 (s, 9H), 1.43 (d, *J*=6.1, 6H).

^{13}C NMR (126 MHz, Acetone): δ = 170.1, 167.3, 165.6, 165.2, 165.0, 163.6, 152.1, 143.9, 143.3, 143.0, 141.8, 139.8, 134.6, 133.5, 133.3, 131.2, 129.3 (3x), 129.0, 127.7, 120.4, 120.3 (2x), 120.2, 119.7, 118.7, 116.9, 116.3, 115.9, 105.9, 81.0, 80.7, 73.2, 72.4, 71.6, 54.6, 28.4, 22.3, 22.2.

HRMS (ESI): calculated for $[M+H]^+$: 889.3556, found: 889.3540.

Synthesis of Methoxy cystobactamid (tBu protected) ***(158)***



a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **79** (80 mg, 0.15 mmol, 1.0 Eq), the standard AB-central AA-fragment **26** (61 mg, 1.1 Eq), dry pyridine (62 µL, 5.0 Eq) and T3P-solution (0.18 mL, 2.0 Eq) in dry EA (1.5 mL) for 2 h 15 min.

b) Adapted from[11]: The crude product, meldrum acid (71 mg, 3.2 Eq) and $Pd(PPh_3)_4$ (8.9 mg, 0.05 Eq) were solved in dry THF (2.3 mL) under Argon atmosphere. The reaction was stirred for 1 h at RT, monitored by LCMS and solvents were then removed u.r.p.. The

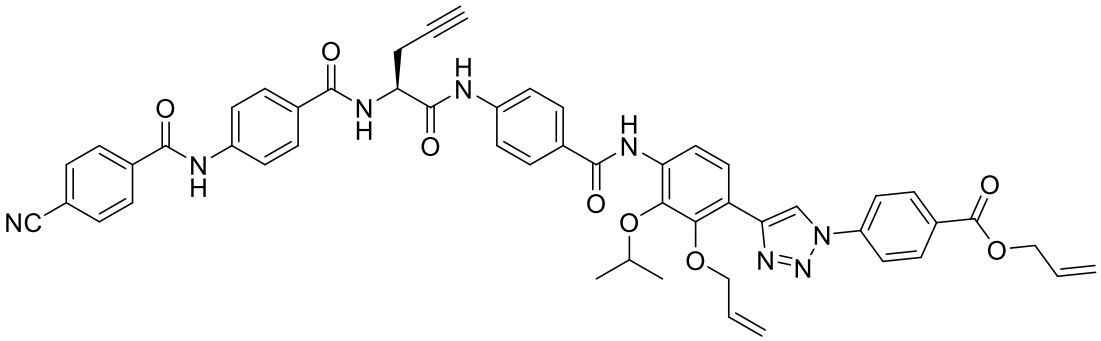
material was purified by FCC (solid loading, 150x reactant mass, PE/EA/AcOH, 60/35/5->55/40/5->50/45/5->35/60/5). Product fractions were identified by LCMS. Solvents were removed u.r.p. and by coevaporation with heptane from the purified product to give a yellow solid, yield: 96 mg, 76% o2s, NMR:68% o2s.

^{1}H NMR (500 MHz, Acetone): δ = 10.99 (br s, 1H), 10.06 (s, 1H), 9.90 (s, 1H), 9.03 (s, 1H), 8.19 (d, *J*=8.7, 2H), 8.15 (d, *J*=7.3, 1H), 8.04 – 7.94 (m, 11H), 7.92 – 7.86 (m, 4H), 7.81 (d, *J*=8.9, 1H), 5.00 – 4.93 (m, 1H), 4.01 (s, 3H), 2.99 – 2.85 (m, 2H), 2.51 (t, *J*=2.7, 1H), 1.58 (s, 9H).

^{13}C NMR (126 MHz, Acetone): δ = 172.2, 170.2 – 170.0 (m), 169.6, 167.3 – 167.1 (m), 165.6, 165.3 – 165.1 (m), 143.1, 143.0, 142.9, 139.9, 139.8, 133.3, 131.0, 130.6, 130.2, 129.4, 129.3, 129.3, 129.2, 128.0, 123.9, 120.9, 120.4, 120.3, 120.1, 120.0, 118.7, 115.9, 109.4, 81.0, 80.8, 72.4, 60.7, 54.5, 28.3, 22.2.

HRMS (ESI): calculated for $[M+H]^+$: 821.2930, found: 821.2927.

*Synthesis of diallyl protected Triazol cystobactamid **(159)***



The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **149** (51 mg, 92 µmol, 0.95 Eq), the standard AB-central AA-fragment **26** (35 mg, 97 µmol, 1.0 Eq), dry pyridine (after 30 min, 39 µL, 5.0 Eq) and T3P-solution (0.12 mL, 2.0 Eq) in dry EA (1.5 mL) for 3 h.

The material was purified with FCC (solid loading, 100x reactants mass, PE/EA, 50/50) and a mixed fraction was purified again with FCC (solid loading, PE/EA, 60/40). Total yield: 47 mg, 54%.

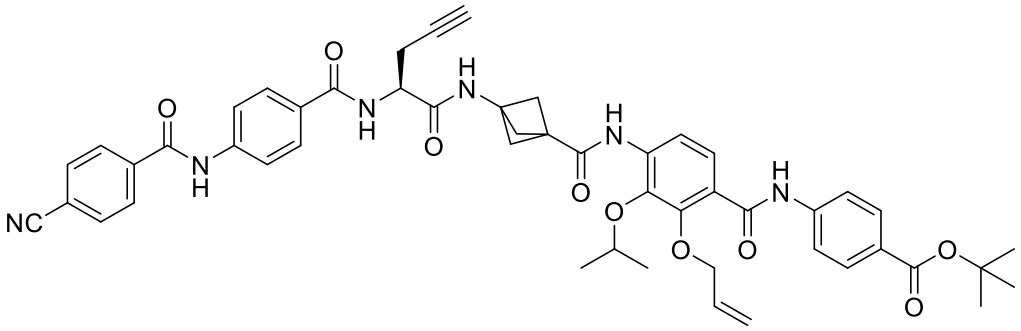
^{1}H NMR (700 MHz, Acetone): δ = 9.99 (s, 1H, H-NCO), 9.85 (s, 1H, H-NCO), 8.93 (s, 1H, H-NCO), 8.88 (s, 1H), 8.38 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 8.28 (d, *J*=8.9, 2H), 8.19 (d, *J*=8.8, 2H), 8.11 (d, *J*=8.9, 2H), 8.09 (d, *J*=7.9, 1H, H-NCO), 8.06 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 8.02 (d, *J*=8.9, 2H),

8.00 (d, *J*=8.9, 2H), 7.98 – 7.96 (m, 2H), 7.95 (d, *J*=6.6, 2H), 7.90 (d, *J*=8.9, 2H), 6.20 (ddt, *J*=17.3, 10.5, 5.6, 1H), 6.12 (ddt, *J*=17.3, 10.5, 5.6, 1H), 5.51 (dq, *J*=17.2, 1.7, 1H), 5.47 (dq, *J*=17.2, 1.6, 1H), 5.32 – 5.29 (m, 2H), 4.97 (td, *J*=7.6, 6.3, 1H), 4.88 (dt, *J*=5.6, 1.5, 2H), 4.79 (hept, *J*=6.2, 1H), 4.66 (dt, *J*=5.6, 1.5, 2H), 2.96 (ddd, *J*=17.0, 6.3, 2.7, 1H), 2.89 (ddd, *J*=17.0, 7.6, 2.7, 1H), 2.51 (t, *J*=2.7, 1H), 1.39 (d, *J*=6.1, 6H).

^{13}C NMR (176 MHz, Acetone): δ = 170.1, 167.3, 165.5, 165.2, 164.7, 149.0, 144.6, 143.1, 143.0, 141.5, 141.4, 139.9, 135.3, 135.1, 133.5, 133.3, 132.1, 130.9, 130.6, 130.3, 129.3 (2x), 128.9, 123.1, 121.3 (2x), 120.8, 120.4, 120.2, 118.7, 118.4, 118.1, 117.1, 115.9, 80.7, 76.9, 73.7, 72.4, 66.3, 54.5, 22.9, 22.2.

HRMS (ESI): calculated for $[M+H]^+$: 897.3355, found: 897.3339.

*Synthesis of Allyl/tBu-protected bicyclopentane cystobactamid **(32)***



a) methyl (*S*)-3-(2-(4-(4-cyanobenzamido)benzamido)pent-4-ynamido)bicyclo[1.1.1]pentane-1-carboxylate **30b** (80 mg, 0.17 mmol, 1.0 Eq) and LiOH*H_2O (55 mg, 8.0 Eq) were solved in a THF/water mixture (2 mL each) and the reaction was stirred for 1 h. The reaction mixture was suspended between HCl (0.5 M) and DCM (20 mL each). Because of insolubility of the product, the resulting suspension was filtered through pore size 3. The organic phase of the filtrate was washed again with HCl (0.5 M) and the aqueous phase of the filtrate was extracted with DCM and MTBE. The precipitate of the filtration was washed with HCl (0.5 M) and DCM before combining it with the other organic phases (Note: product is better soluble in acetone). Solvents were then removed u.r.p., the residue was resolved in acetone (~15 mL) and the suspension was filtered again through a syringe filter to remove salt impurities. The filter was extracted with acetone (~2x5 mL) again and solvent was removed u.r.p. and by coevaporation with MeCN/MeOH. The crude material was dried overnight at HV and directly used in the next step.

Note: The following step was performed in two batches with same scale and is described as one batch here:

b) The crude material and the standard DE-fragment (95 mg, 1.4 Eq) were suspended in dry EA (4.0 mL). DiPEA (0.13 mL, 7.5 Eq) and T3P (50% sol. in EA, 0.40 mL, 4.1 Eq) were incrementally added at 60°C, while the reaction process was screened by LCMS. The reaction was stirred overnight at 60°C. T3P (50% sol. in EA, 0.40 mL, 4.1 Eq) and DiPEA (34 µL, 2.0 Eq) were added again incrementally over 2 h. After reaction control by TLC, the two reaction batches were combined, diluted between EA and HCl (0.5 M) (50 mL each) and the aqueous phase was extracted with EA (1x50 mL). The combined organic phases were washed with sat. $NaHCO_3$ sol. (2x30 mL) and brine (1x30 mL) and dried over Na_2SO_4 before removing solvent u.r.p.. The material was purified by FCC (solid loading, 100x reactants mass, PE/acetone, 70/30->60/40->50/50) and solvents were removed u.r.p. to obtain off-colorless crystals, 90 mg, 62% o2s.

^{1}H NMR (700 MHz, Acetone): δ = 10.17 (s, 1H), 9.99 (s, 1H), 8.31 (s, 1H), 8.24 (d, *J*=8.8, 1H), 8.20 – 8.18 (m, 2H), 8.17 (s, 1H), 7.99 – 7.93 (m, 9H), 7.84 (d, *J*=8.9, 2H), 7.79 (d, *J*=8.7, 1H), 6.19 (ddt, *J*=17.2, 10.4, 5.7, 1H), 5.54 (dq, *J*=17.2, 1.5, 1H), 5.35 (dq, *J*=10.4, 1.2, 1H), 4.77 – 4.69 (m, 4H), 2.87 (ddd, *J*=17.0, 5.6, 2.7, 1H), 2.76 (ddd, *J*=17.1, 7.9, 2.7, 1H), 2.46 – 2.44 (m, 7H), 1.58 (s, 9H), 1.37 (d, *J*=6.2, 6H).

^{13}C NMR (176 MHz, Acetone): δ = 171.4, 167.8, 166.8, 165.6, 165.2, 163.6, 150.6, 143.8, 142.9, 140.2, 139.9, 137.9, 134.0, 133.3, 131.2, 130.5, 129.4, 129.2, 127.9, 126.9, 123.8, 120.4, 119.8, 119.5, 118.7, 116.0, 115.9, 81.0 (2x), 77.5, 75.5, 72.1, 54.6, 53.4, 46.0, 39.4, 28.3, 22.9, 22.4.

HRMS (ESI): calculated for $[M+H]^+$: 879.3712, found: 879.3703.

Final cystobactamids

Preparative HPLC and NMR analysis:

the crude product was solved in THF and a NH_4HCO_3-solution (10 mM) (<2 mL each). If a precipitate was observed DMSO or NaOH (1 M) was added dropwise until it was solved. The solution was then injected manually. After purification and LCMS control of the product fractions, the fractions were combined. The solvent was removed after freezing in liquid nitrogen and lyophillization over at least 2 days. Afterwards, a sample of the product was solved in d6-DMSO for NMR measurement. In addition to the final yield, the purity is also given, if it was significantly lower than 95%. For the final cystobactamids, NMR assignments were verified by 2D NMR spectroscopy.

Preparation for MIC-testing: After NMR analysis, the concentration of the NMR-solution was adjusted to 5.0 mg/mL with d6-DMSO. Details for MIC-testing are listed in section 5.1.

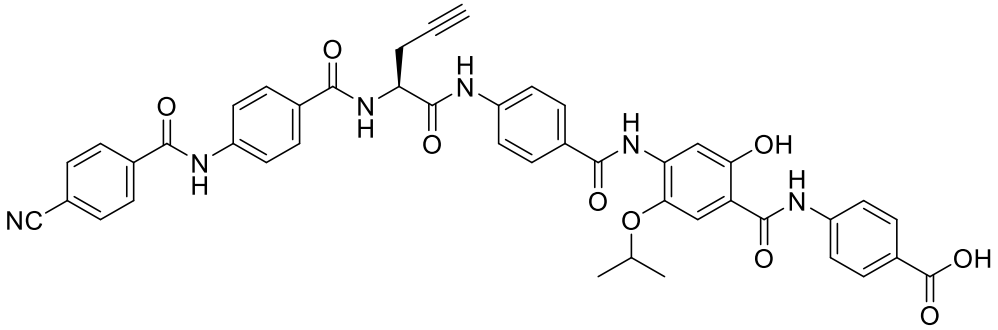
General procedure **B** (coupling between the standard AB-central AA-fragment and CDE-fragments):

The CDE-fragment and the standard AB-central AA-fragment **26** were suspended under Argon atmosphere in dry EA and pyridine and T3P (50% solution in EA) were added. The reaction was stirred at RT for the given amount of time while being screened by TLC or LCMS. Clarification of the original solution was often observed. The reaction solution was diluted between EA (20-30 mL) and an adequate amount of HCl (0.1-1 M, 15-30 mL) and the aqueous phase was extracted with EA (2x15-20 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was removed u.r.p. to give the crude product or intermediate. If a workup was omitted, only the solvent was removed u.r.p..

General procedure **C** (final deprotection of the *t*Bu-protecting group):

Adapted from[11]: The crude material was suspended or solved in dry DCM under Argon atmosphere and TFA was added dropwise at 0°C or RT. The reaction was stirred at RT for the given amount of time while being screened by LCMS. The reaction solution was diluted with DCM or MeCN and solvents were then removed u.r.p. and by multiple coevaporation with DCM, MeCN or THF. The material was then purified by preparative HPLC by using the given HPLC method. Usually, fluffy colorless solids with low density were obtained.

Synthesis of hydroquinone (ring D) cystobactamid ***(001)***



a) Adapted from[11]: The cystobactamid-precursor **157** (20 mg, 22 µmol, 1.0 Eq) and aniline (6.0µL, 3.0 Eq) were solved subsequently in dry THF (0.7 mL). $Pd(PPh_3)_4$ (2.6 mg, 0.1 Eq) was added and the solution was stirred for 1.5 h changing its color from orange to red. TLC showed nearly full educt conversion after 30 min. The reaction mixture was diluted with acetone and was concentrated on silica followed by FCC (100x reactant mass, PE/EA/AcOH, 50/50/1->60/40/1). Solvent was removed u.r.p. to give a yellowish solid directly used in next step.

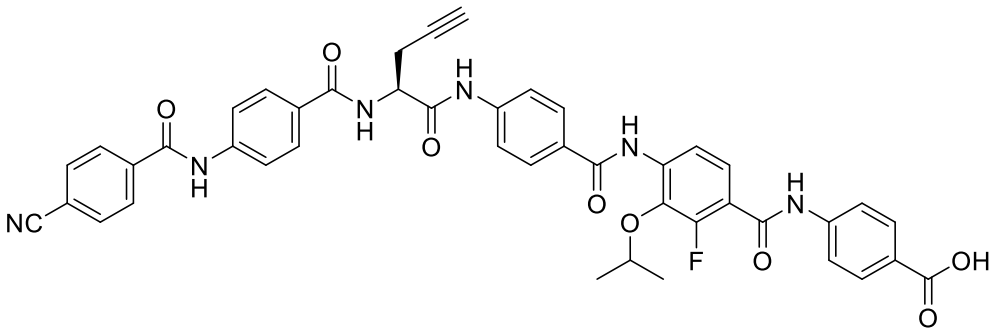
b) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (0.85 mL) and TFA (160 µL, 100 Eq). The reaction was conducted at RT for 1 h and HPLC method 1 was used. Yield: 7.2 mg, 43% o2s.

^{1}H NMR (500 MHz, DMSO): δ = 12.76 (br s, 1H), 11.63 (br s, 1H), 10.71 (s, 1H, H-NCO), 10.66 – 10.53 (m, 2H), 9.16 (s, 1H), 8.78 (d, *J*=7.6, 1H, H-NCO), 8.13 (d, *J*=8.9, 2H, H-C_{Ar}), 8.05 (d, *J*=8.5, 2H, H-C_{Ar}), 7.99 – 7.94 (m, 5H, H-C_{Ar}), 7.93 – 7.88 (m, 4H, H-C_{Ar}), 7.85 – 7.81 (m, 4H, H-C_{Ar}), 7.65 (s, 1H, , H-$C_{Ar,D}$), 4.84 – 4.77 (m, 1H, <u>H</u>-C(NH)$(C)_2$), 4.61 (hept, *J*=6.3, 1H, H-C_{iPr}O), 2.94 (t, *J*=2.6, 1H, H-CC), 2.85 – 2.69 (m, 2H, <u>H</u>$_2$C(CCH)), 1.35 (s, 6H, $(H_3C)_2C$).

^{13}C NMR (176 MHz, DMSO): δ = 169.7 (C=O), 166.9 (C=O), 165.9 (C=O), 165.5 (C=O), 164.5 (C=O), 164.1 (C=O), 152.8 (C-OH), 142.4 (C_{Ar}), 142.3 (C_{Ar}), 141.7 (C_{Ar}), 140.1 (C_{Ar}), 138.7 (C_{Ar}), 134.1 (C_{Ar}), 132.5 (C_{Ar}-H), 130.3 (C_{Ar}), 128.9 (C_{Ar}), 128.6 (C_{Ar}-H), 128.4 (C_{Ar}-H), 128.3 (C_{Ar}-H), 125.8 (C_{Ar}), 119.9 (C_{Ar}), 119.5 (2x, C_{Ar}-H), 119.0 (C_{Ar}), 118.3 (CN), 114.7 (C_{Ar}-H), 114.0 (C_{Ar}), 112.1 (C_{Ar}), 109.0 (C_{Ar}-H), 80.6 (<u>C</u>CH), 73.2 (C_{iPr}-H), 72.6 (C<u>C</u>H), 53.5 (<u>C</u>H-$(C)_2$(N)), 21.8 ($(\underline{C}H_3)_2C$), 21.4 ($\underline{C}H_2$-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 793.2617, found: 793.2606.

Synthesis of fluoro (ring D) cystobactamid ***(002)***



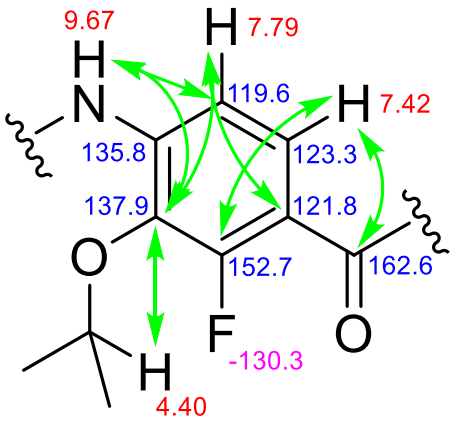
a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **144** (47.2 mg, 93.0 µmol, 1.00 Eq), the standard AB-central AA-fragment **26** (37.0 mg, 1.10 Eq), dry pyridine (38 µL, 5.0 Eq) and T3P-solution (0.11 mL, 2.0 Eq) in dry EA (1.4 mL) for 2.5 h.

b) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (1.4 mL) and TFA (0.36 mL, 50 Eq). The reaction was conducted at RT for 2 h and HPLC method 1 was used. Yield: 51.3 mg, 69% o2s.

^{1}H NMR (700 MHz, DMSO): δ = 12.90 (br s, 1H), 10.73 (s, 1H, H-NCO), 10.66 (s, 1H, H-NCO), 10.64 (s, 1H, H-NCO), 9.67 (s, 1H, H-NCO), 8.85 (d, *J*=7.5, 1H, H-NCO), 8.13 (d, *J*=8.6, 2H, H-C_{Ar}), 8.05 (d, *J*=8.6, 2H, H-C_{Ar}), 7.99 (d, *J*=9.0, 2H, H-C_{Ar}), 7.97 (d, *J*=8.8, 2H, H-C_{Ar}), 7.93 (d, *J*=8.8, 2H, H-C_{Ar}), 7.90 (d, *J*=8.8, 2H, H-C_{Ar}), 7.84 – 7.78 (m, 5H, H-C_{Ar}, H-$C_{Ar,D}$), 7.42 (dd, *J*=8.4, 7.1, 1H, H-$C_{Ar,D}$), 4.80 (td, *J*=8.1, 6.2, 1H, H-C(NH)$(C)_2$), 4.40 (hept, *J*=5.9, 1H, H-C_{iPr}O), 2.93 (t, *J*=2.6, 1H, HCC), 2.84 – 2.74 (m, 2H, H_2C(CCH)), 1.29 (d, *J*=6.0, 6H, $(H_3C)_2$C).

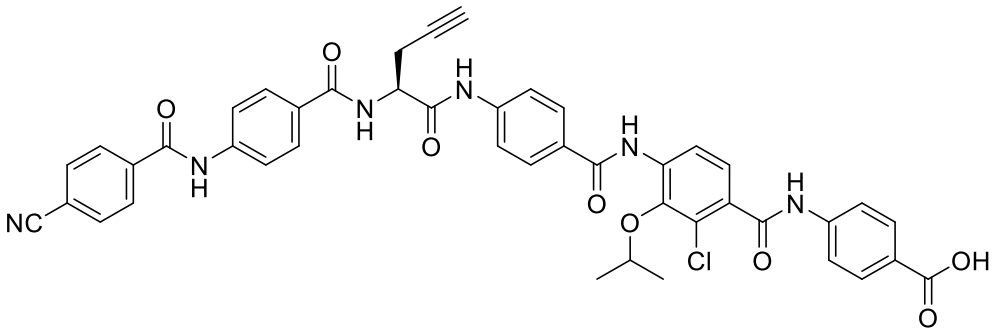
^{13}C NMR (176 MHz, DMSO): δ = 169.7 (C=O), 167.2 (C=O), 165.9 (C=O), 164.5 (C=O), 164.5 (C=O), 162.6 ($C_{Ar,D}$-C=O), 152.7 (d, *J*=249.0, $C_{Ar,D}$-F), 142.3 (C_{Ar}), 142.2 (C_{Ar}), 141.7 (C_{Ar}), 138.7 (C_{Ar}), 137.9 (d, *J*=13.2, $C_{Ar,D}$-OiPr), 135.8 ($C_{Ar,D}$), 132.5 (C_{Ar}-H), 130.3 (C_{Ar}-H), 128.9 (C_{Ar}), 128.6 (C_{Ar}-H), 128.6 (C_{Ar}-H), 128.4 (C_{Ar}-H), 128.4 (C_{Ar}), 123.4 ($C_{Ar,D}$-H), 121.8 (d, *J*=13.5, $C_{Ar,D}$), 119.6 ($C_{Ar,D}$-H), 119.5 (C_{Ar}-H), 118.9 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.3 (CN), 114.0 (C_{Ar}), 80.7 (CCH), 77.3 (C_{iPr}-H), 73.2 (C_{iPr}-H, CCH), 53.6 (CH-$(C)_2$(N)), 22.3 ((CH_3)$_2$C), 21.4 (CH_2-CCH).

^{19}F NMR (471 MHz, DMSO): δ = -130.3.



HRMS (ESI): calculated for $[M+H]^+$: 795.2573, found: 795.2574.

Synthesis of chloro (ring D) cystobactamid ***(003)***



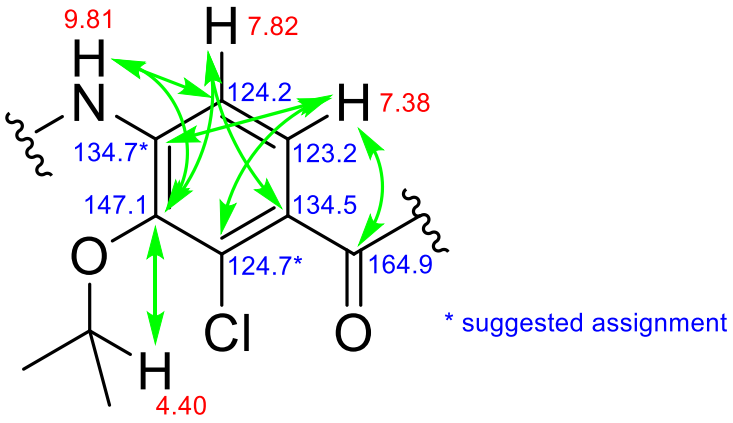
a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **27** (63.8 mg, 0.122 mmol, 1.00 Eq), the standard AB-central AA-fragment **26** (48.4 mg, 1.10 Eq), dry pyridine (49 µL, 5.0 Eq) and T3P-solution (0.14 mL, 2.0 Eq) in dry EA (1.8 mL) for 2 h.

b) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (1.8 mL) and TFA (0.47 mL, 50 Eq). The reaction was conducted at RT for 2 h and HPLC method 1 was used. Yield: 54.9 mg, 56% o2s.

^{1}H NMR (700 MHz, DMSO): δ = 12.78 (br s, 1H), 10.82 (s, 1H, H-NCO), 10.72 (s, 1H, H-NCO), 10.60 (s, 1H, H-NCO), 9.81 (s, 1H, H-$N_{D\text{-ring}}$CO), 8.80 (d, *J*=7.5, 1H, H-NCO), 8.13 (d, *J*=8.6, 2H, H-C_{Ar}), 8.05 (d, *J*=8.4, 2H, H-C_{Ar}), 8.02 (d, *J*=8.8, 2H, H-C_{Ar}), 7.97 (d, *J*=8.8, 2H, H-C_{Ar}), 7.94 (d, *J*=8.6, 2H, H-C_{Ar}), 7.90 (d, *J*=8.8, 2H, H-C_{Ar}), 7.85 – 7.79 (m, 5H, H-C_{Ar}, H-$C_{Ar,D}$), 7.38 (d, *J*=8.2, 1H, H-$C_{Ar,D}$), 4.80 (td, *J*=8.3, 6.2, 1H, H-C(NH)$(C)_2$), 4.40 (hept, *J*=6.1, 1H, H-C_{iPr}O), 2.94 (t, *J*=2.7, 1H, HCC), 2.85 – 2.73 (m, 2H, H_2C(CCH)), 1.26 (d, *J*=6.0, 6H, $(H_3C)_2$C).

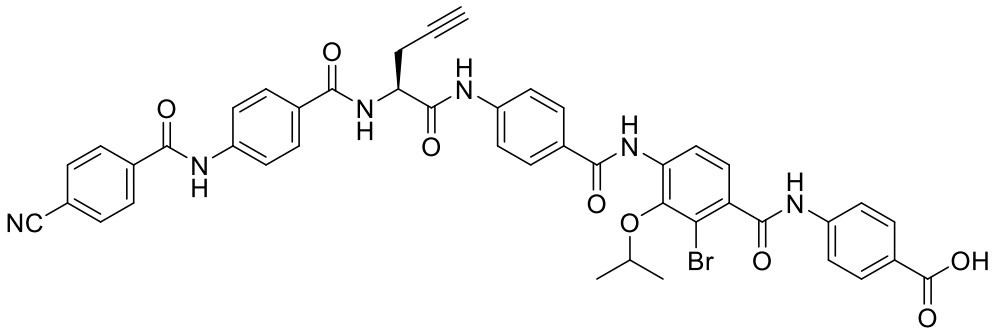
^{13}C NMR (176 MHz, DMSO): δ = 169.7 (C=O), 167.0 (C=O), 165.9 (C=O), 164.9 ($C_{Ar,D}$-C=O), 164.5 (C=O), 164.5 (C=O), 147.1 (C_{Ar}-OiPr), 142.7 (C_{Ar}), 142.1 (C_{Ar}), 141.7 (C_{Ar}), 138.7

(C_{Ar}), 134.7 ($C_{Ar,D}$), 134.5 ($C_{Ar,D}$), 132.5 (C_{Ar}-H), 130.4 (C_{Ar}-H), 128.9 (C_{Ar}), 128.6 (C_{Ar}-H), 128.6 (C_{Ar}-H), 128.4 (C_{Ar}-H), 128.3 (C_{Ar}), 126.3 (C_{Ar}), 124.7 ($C_{Ar,D}$), 124.2 ($C_{Ar,D}$-H), 123.2 ($C_{Ar,D}$-H), 119.5 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.3 (CN), 114.0 (C_{Ar}), 80.7 (CCH), 76.9 (C_{iPr}-H), 73.2 (C_{iPr}-H, CCH), 53.5 (CH-$(C)_2$(N)), 22.2 ($(CH_3)_2$C), 21.4 (CH_2-CCH).



HRMS (ESI): calculated for $[M+H]^+$: 811.2278, found: 811.2278.

*Synthesis of bromo (ring D) cystobactamid **(004)***



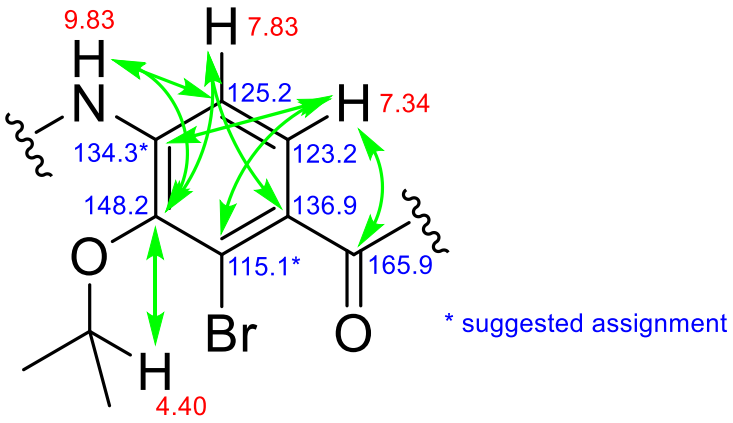
a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **145** (38.3 mg, 67.4 µmol, 1.00 Eq), the standard AB-central AA-fragment **26** (26.8 mg, 1.10 Eq), dry pyridine (27 µL, 5.0 Eq) and T3P-solution (80 µL, 2.0 Eq) in dry EA (1.0 mL) for 2 h.

b) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (1.0 mL) and TFA (0.26 mL, 50 Eq). The reaction was conducted at RT for 2 h and HPLC method 2 was used. Because the isolation of the targeted compound from byproduct was not successful, the product mixture was separated again with the half amount of the material by using acidic preparative HPLC (method 5, Note: The behavior of the product mixture was modelled before with reverse phase TLC). The product

fractions were again collected and lyophillized. Yield (for half of the product): 6.5 mg, 22%.

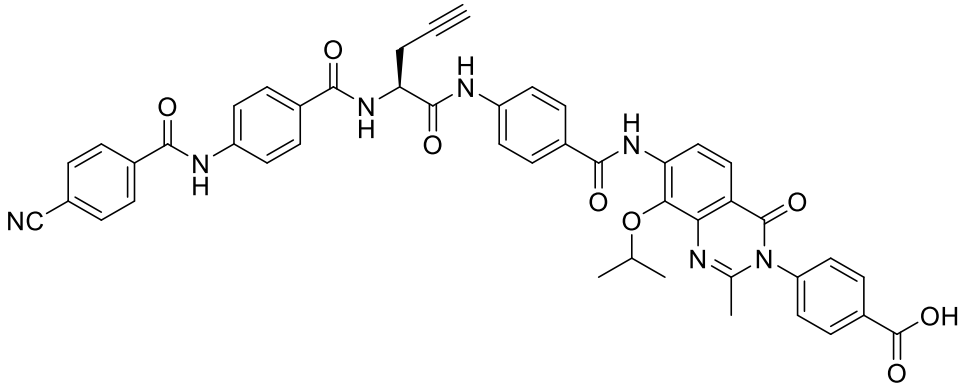
^{1}H NMR (700 MHz, DMSO): δ = 12.78 (br s, 1H), 10.82 (s, 1H, H-NCO), 10.71 (s, 1H, H-NCO), 10.57 (s, 1H, H-NCO), 9.83 (s, 1H H-$N_{D\text{-}ring}$CO), 8.78 (d, *J*=7.5, 1H, H-NCO), 8.13 (d, *J*=8.6, 2H, H-C_{Ar}), 8.05 (d, *J*=8.6, 2H, H-C_{Ar}), 8.02 (d, *J*=8.8, 2H, H-C_{Ar}), 7.96 (d, *J*=9.0, 2H, H-C_{Ar}), 7.94 (d, *J*=8.6, 2H, H-C_{Ar}), 7.90 (d, *J*=8.8, 2H, H-C_{Ar}), 7.85 – 7.82 (m, 3H, H-C_{Ar}, H-$C_{Ar,D}$), 7.81 (d, *J*=8.8, 2H, H-C_{Ar}), 7.34 (d, *J*=8.2, 1H, H-$C_{Ar,D}$), 4.83 – 4.77 (m, 1H, H-C(NH)$(C)_2$), 4.42 (hept, *J*=6.2, 1H, H-C_{iPr}O), 2.94 (t, *J*=2.6, 1H, HCC), 2.85 – 2.72 (m, 2H, H_2C(CCH)), 1.26 (d, *J*=6.0, 6H, $(H_3C)_2$C)).

^{13}C NMR (176 MHz, DMSO): δ = 169.7 (C=O), 166.9 (C=O), 165.9 ($C_{Ar,D}$-C=O), 165.9 (C=O), 164.5 (C=O), 164.5 (C=O), 148.2 (C_{Ar}-OiPr), 142.9 (C_{Ar}), 142.1 (C_{Ar}), 141.7 (C_{Ar}), 138.7 (C_{Ar}), 136.9 ($C_{Ar,D}$), 134.3 ($C_{Ar,D}$), 132.5 (C_{Ar}-H), 130.4 (C_{Ar}-H), 128.9 (C_{Ar}), 128.6 (C_{Ar}-H), 128.6 (C_{Ar}-H), 128.4 (C_{Ar}-H), 128.3 (C_{Ar}), 126.0 (C_{Ar}) 125.2 ($C_{Ar,D}$-H), 123.2 ($C_{Ar,D}$-H), 119.5 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.3 (CN), 115.1 ($C_{Ar,D}$), 114.0 (C_{Ar}), 80.7 (CCH), 76.9 (C_{iPr}-H), 73.2 (C_{iPr}-H, CCH), 53.5 (CH-$(C)_2$(N)), 22.2 (($CH_3)_2$C), 21.4 (CH_2-CCH).



HRMS (ESI): calculated for $[M+H]^+$: 855.1773/ 857.1752, found: 855.1771/ 857.1760.

Synthesis of Me-oxoquinazoline (ring D) cystobactamid ***(012)***



a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **58** (6.2 mg, 12 µmol, 1.0 Eq), the standard AB-central AA-fragment **26** (5.7 mg, 1.35 Eq), dry pyridine (4.7 µL, 5.0 Eq) and T3P-solution (14 µL, 2.0 Eq) in dry EA (0.18 mL) for 2 h. An aqueous workup was omitted.

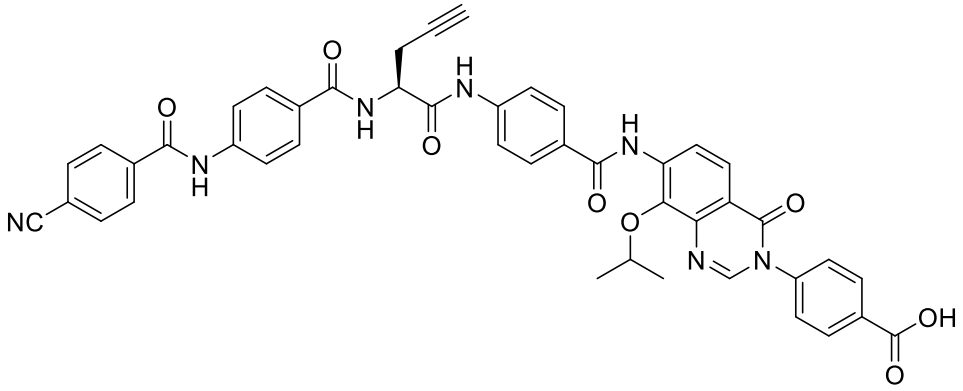
b) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (0.18 mL) and TFA (45 µL, 50 Eq). The reaction was conducted at 0°C for 2 h and HPLC method 1 was used, including lyophillization over 7 days. Yield: 4.9 mg, 51% o2s.

^{1}H NMR (700 MHz, DMSO): δ = 13.21 (br s, 1H, HOOC), 10.71 (s, 1H, H-NCO), 10.61 (s, 1H, H-NCO), 9.55 (s, 1H, H-NCO), 8.78 (d, *J*=7.5, 1H, H-NCO), 8.25 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 8.13 (d, *J*=8.7, 2H), 8.11 (d, *J*=8.6, 2H), 8.05 (d, *J*=8.6, 2H), 7.99 (d, *J*=8.9, 2H), 7.97 (d, *J*=8.9, 2H), 7.90 (d, *J*=9.0, 2H), 7.86 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 7.84 (d, *J*=8.9, 2H), 7.60 (d, *J*=8.4, 2H), 5.00 (hept, *J*=6.1, 1H, H-C_{iPr}O), 4.81 (q, *J*=7.7, 1H, H-C(NH)$(C)_2$), 2.94 (t, *J*=2.6, 1H, H-CC), 2.85 – 2.73 (m, 2H, H_2C(CCH)), 2.16 (s, 3H, H_3C-C), 1.34 (d, *J*=6.2, 6H, $(H_3C)_2$C).

^{13}C NMR (176 MHz, DMSO): δ = 169.7 (C=O), 166.7 (C=O), 166.0 (C=O), 164.5 (C=O), 164.3 (C=O), 160.9 (C=O), 153.1 ($C_{pyrim.}$-CH_3), 142.2 (C_{Ar}), 142.2 (C_{Ar}), 141.7 (C_{Ar}), 141.6 (C_{Ar}), 141.1 (C_{Ar}), 138.7 (C_{Ar}), 136.9 (C_{Ar}), 132.5 (C_{Ar}-H), 130.5 (C_{Ar}-H), 128.9 (C_{Ar}), 128.9 (C_{Ar}-H), 128.6 (C_{Ar}-H), 128.5 (C_{Ar}), 128.4 (C_{Ar}-H), 128.4 (C_{Ar}-H), 121.0 ($C_{Ar,D}$-H), 120.3 ($C_{Ar,D}$-H), 119.5 (C_{Ar}-H), 119.0 (C_{Ar}-H), 118.3 (CN), 117.6 (C_{Ar}), 114.0 (C_{Ar}), 80.6 (CCH), 77.1 (C_{iPr}-H), 73.2 (CCH), 53.5 (CH-$(C)_2$(N)), 24.6 (CH_3-$C_{pyrim.}$), 22.5 ((CH_3)$_2$C), 21.4 (CH_2-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 816.2776, found: 816.2776.

*Synthesis of oxoquinazoline (ring D) cystobactamid **(013)***



a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **146** (28 mg, 50 µmol, 1.0 Eq), the standard AB-central AA-fragment **26** (19.7 mg, 1.10 Eq), dry pyridine (20 µL, 5.0 Eq) and T3P-solution (59 µL, 2.0 Eq) in dry EA (0.75 mL) for 2 h.

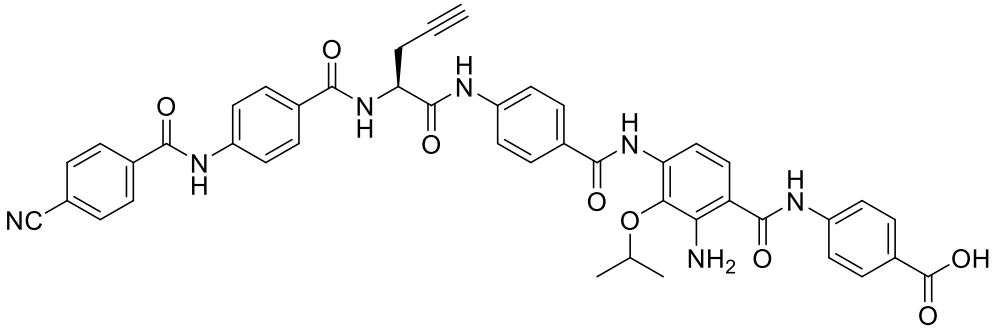
b) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (0.75 mL) and TFA (0.19 mL, 50 Eq). The reaction was conducted at RT for 2 h and HPLC method 1 was used. Yield: 29.5 mg, 74%.

^{1}H NMR (500 MHz, DMSO): δ = 13.28 (s, 1H, COOH), 10.72 (s, 1H, H-NCO), 10.63 (s, 1H, H-NCO), 9.62 (s, 1H, H-NCO), 8.81 (d, *J*=7.5, 1H, H-NCO), 8.40 (s, 1H, H-$C_{pyrim.}$), 8.32 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 8.13 (d, *J*=8.7, 2H, H-C_{Ar}), 8.10 (d, *J*=8.5, 2H, H-C_{Ar}), 8.05 (d, *J*=8.5, 2H, H-C_{Ar}), 8.03 – 7.94 (m, 5H, H-C_{Ar}), 7.90 (d, *J*=9.0, 2H, H-C_{Ar}), 7.85 (d, *J*=8.9, 2H, H-C_{Ar}), 7.68 (d, *J*=8.5, 2H, H-C_{Ar}), 4.99 (hept, *J*=6.3, 1H, H-C_{iPr}O), 4.85 – 4.77 (m, 1H, <u>H</u>-C(NH)$(C)_2$), 2.94 (t, *J*=2.6, 1H, H-CC), 2.79 (qdd, *J*=16.6, 7.5, 2.7, 2H, <u>H</u>$_2$C(CCH)), 1.32 (d, *J*=6.1, 6H, $(H_3C)_2$C).

^{13}C NMR (126 MHz, DMSO): δ = 169.7 (C=O), 166.8 (C=O), 166.0 (C=O), 164.5 (C=O), 164.4 (C=O), 159.5 (C=O), 146.1 ($C_{pyrim.}$-H), 142.6 (C_{Ar}), 142.3 (C_{Ar}), 141.7 (C_{Ar}), 141.6 (C_{Ar}), 140.8 (C_{Ar}), 138.7 (C_{Ar}), 137.3 (C_{Ar}), 132.5 (C_{Ar}-H), 130.1 (C_{Ar}-H), 128.9 (C_{Ar}), 128.6 (C_{Ar}-H), 128.5 (C_{Ar}-H), 128.4 (C_{Ar}-H), 127.6 (C_{Ar}-H), 121.5 ($C_{Ar,D}$-H), 121.3 (C_{Ar}-H), 119.5 (C_{Ar}-H), 119.0 (C_{Ar}, C_{Ar}-H), 118.3 (CN), 114.0 (C_{Ar}), 80.7 (<u>C</u>CH), 77.1 (C_{iPr}-H), 73.2 (C<u>C</u>H), 53.5 (<u>C</u>H-$(C)_2$(N)), 22.3 ($(\underline{C}H_3)_2$C), 21.4 (<u>C</u>H_2-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 802.2620, found: 802.2620.

*Synthesis of free amine (ring D) cystobactamid **(005)***

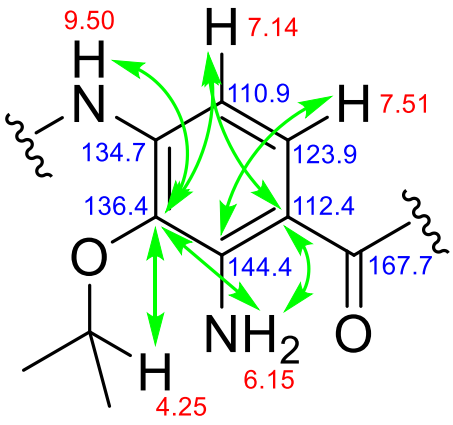


a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **64** (26.5 mg, 52.5 µmol, 1.00 Eq), the standard AB-central AA-fragment **26** (19 mg, 1.0 Eq), dry pyridine (13 µL, 3.0 Eq) and T3P-solution (47 µL, 1.5 Eq) in dry EA (0.80 mL) for 1 h.

b) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (0.80 mL) and TFA (0.20 mL, 50 Eq). Anisol (11.5 µL, 2.0 Eq) was added as scavenger. The reaction was conducted at RT for 1 h and HPLC method 1 was used. Yield: 21.8 mg, 52% o2s.

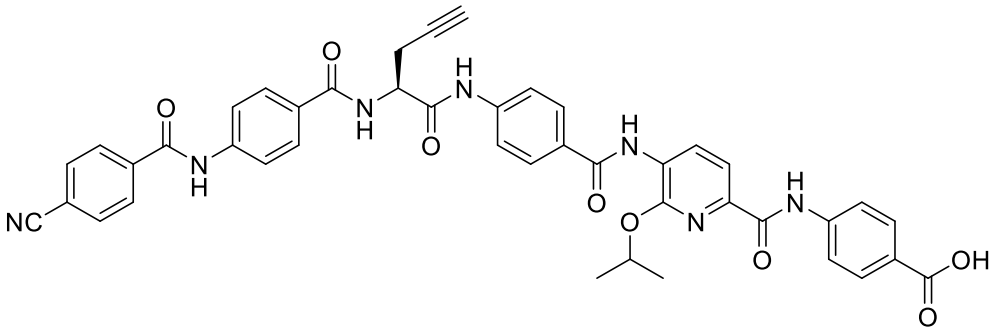
^{1}H NMR (700 MHz, DMSO): δ = 12.71 (s, 1H, COOH), 10.71 (s, 1H, H-NCO), 10.56 (s, 1H, H-NCO), 10.28 (s, 1H, H-NCO), 9.50 (s, 1H, H-$N_{D\text{-}ring}$CO), 8.78 (d, *J*=7.5, 1H, H-NCO), 8.13 (d, *J*=8.4, 2H, H-C_{Ar}), 8.05 (d, *J*=8.4, 2H, H-C_{Ar}), 8.00 (d, *J*=8.8, 2H, H-C_{Ar}), 7.96 (d, *J*=8.8, 2H, H-C_{Ar}), 7.94 – 7.88 (m, 4H, H-C_{Ar}), 7.85 (d, *J*=8.8, 2H, H-C_{Ar}), 7.80 (d, *J*=8.7, 2H, H-C_{Ar}), 7.51 (d, *J*=8.9, 1H, H-$C_{Ar,D}$-$C_{Ar,D}$-CO), 7.14 (d, *J*=8.6, 1H, H-$C_{Ar,D}$-$C_{Ar,D}$-NCO), 6.15 (s, 2H, H_2N), 4.80 (q, *J*=7.9, 1H, H-C(NH)$(C)_2$), 4.25 (hept, *J*=6.3, 1H, H-C_{iPr}O), 2.94 (t, *J*=2.6, 1H, H-CC), 2.84 – 2.73 (m, 2H, H_2C(CCH)), 1.24 (d, *J*=6.1, 6H, $(H_3C)_2$C).

^{13}C NMR (176 MHz, DMSO): δ = 169.7 (C=O), 167.7 ($C_{Ar,D}$-C=O), 167.0 (C=O), 165.9 (C=O), 164.5 (C=O), 164.2 (C=O), 144.4 (C_{Ar}-NH_2), 143.3 (C_{Ar}), 142.0 (C_{Ar}), 141.7 (C_{Ar}), 138.7 (C_{Ar}), 136.4 ($C_{Ar,D}$-OiPr), 134.7 ($C_{Ar,D}$-NCO), 132.5 (C_{Ar}-H), 130.1 (C_{Ar}-H), 128.9 (C_{Ar}), 128.7 (C_{Ar}), 128.6 (C_{Ar}-H), 128.4 (C_{Ar}-H), 128.4 (C_{Ar}-H), 125.4 (C_{Ar}), 123.9 (H-$C_{Ar,D}$-$C_{Ar,D}$-C=O), 119.6 (C_{Ar}-H), 119.5 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.3 (CN), 114.0 (C_{Ar}), 112.4 ($C_{Ar,D}$-C=O), 110.9 (H-$C_{Ar,D}$-$C_{Ar,D}$-NCO), 80.7 (CCH), 74.2 (C_{iPr}-H), 73.2 (CCH), 53.5 (CH-$(C)_2$(N)), 22.2 ($(CH_3)_2$C), 21.4 (CH_2-CCH).



HRMS (ESI): calculated for $[M+H]^+$: 792.2777, found: 792.2775.

*Synthesis of pyridine (ring D) cystobactamid **(006)***

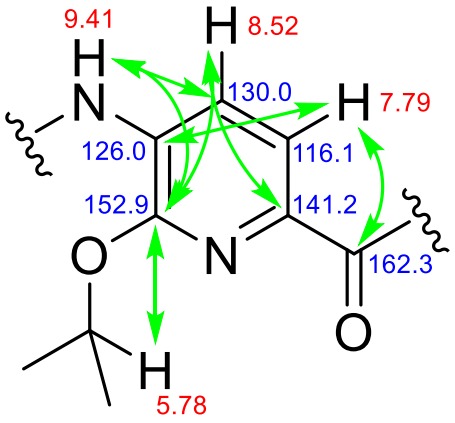


a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **75** (27.1 mg, 55.2 µmol, 1.00 Eq), the standard AB-central AA-fragment **26** (21.4 mg, 1.07 Eq), dry pyridine (13 µL, 3.0 Eq) and T3P-solution (66 µL, 2.0 Eq) in dry EA (0.60 mL) for 1.5 h.

b) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (0.83 mL) and TFA (0.21 mL, 50 Eq). The reaction was conducted at RT for 1 h and HPLC method 1 was used, including lyophillization over 4 d. Yield: 28.4 mg, 66% o2s.

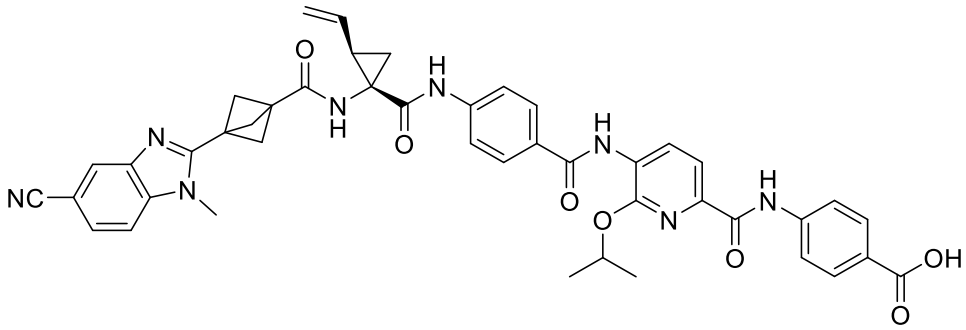
^{1}H NMR (700 MHz, DMSO): δ = 10.74 (s, 1H, H-NCO), 10.69 (s, 1H, H-NCO), 10.21 (s, 1H, H-NCO), 9.41 (s, 1H, H-N-$C_{Ar,D}$), 8.88 (d, *J* = 7.6, 1H, H-NCO), 8.52 (d, *J* = 8.0, 1H, H-$C_{Ar,D}$), 8.13 (d, *J* = 8.6, 2H, H-C_{Ar}), 8.04 (d, *J* = 8.7, 2H, H-C_{Ar}), 7.99 – 7.94 (m, 6H, H-C_{Ar}), 7.92 (d, *J* = 8.7, 2H, H-C_{Ar}), 7.90 (d, *J* = 8.9, 2H, H-C_{Ar}), 7.84 (d, *J* = 8.8, 2H, H-C_{Ar}), 7.79 (d, *J* = 7.9, 1H, H-$C_{Ar,D}$), 5.78 (hept, *J* = 6.1, 1H, H-C_{iPr}O), 4.83 – 4.78 (m, 1H, H-C(NH)$(C)_2$), 2.93 (t, *J* = 2.6, 1H, HCC), 2.85 – 2.74 (m, 2H, H_2C(CCH)), 1.42 (d, *J* = 6.1, 6H, $(H_3C)_2$C).

^{13}C NMR (176 MHz, DMSO): δ = 169.7 (C=O), 167.4 (C=O), 165.9 (C=O), 164.9 (C=O), 164.5 (C=O), 162.3 ($C_{Ar,D}$-C=O), 152.9 ($C_{Ar,D}$-OiPr), 142.3 (C_{Ar}), 141.7 (C_{Ar}), 141.4 (C_{Ar}), 141.2 ($C_{Ar,D}$-CO), 138.7 (C_{Ar}), 132.5 (H-C_{Ar}), 130.1 (H-C_{Ar}), 130.0 (H-$C_{Py,4}$), 128.9 (C_{Ar}), 128.6 (H-C_{Ar}), 128.4 (H-C_{Ar}), 128.6 (H-C_{Ar}), 128.4 (C_{Ar}), 126.0 ($C_{Ar,D}$-NH), 119.5 (H-C_{Ar}), 119.5 (H-C_{Ar}), 118.9 (H-C_{Ar}), 118.3 (CN), 116.1 (H-$C_{Py,3}$), 114.0 (C_{Ar}), 80.7 (CCH), 73.2 (CCH), 69.6 (C_{iPr}-H), 53.6 (CH-$(C)_2$(N)), 21.8 ($(CH_3)_2$C), 21.4 (CH_2-CCH).



HRMS (ESI): calculated for $[M+H]^+$: 778.2620, found: 778.2619.

Synthesis of new scaffolded pyridine (ring D) cystobactamid ***(006b)***



a) The CDE-fragment **75** (32.5 mg, 66.3 µmol, 1.00 Eq) and the *N*-Boc-cyclopropane amino acid **125** (15.8 mg, 1.05 Eq) were suspended in dry EA (0.66 mL) and dry pyridine (16 µL, 3.0 Eq) was added. Then T3P (50% sol. in EA, 2x39 µL, 2x1.0 Eq) was added in two portions, one portion directly and one after 30 min. The mixture was stirred at RT while being screened by LCMS. Because product formation was very slow, after 2 h 45 min pyridine (11 µL, 2.0 Eq) and T3P (39 µL, 1.0 Eq) were added again. After 4 h the reaction suspension was diluted between EA/HCl (0.1 M) (20 mL each) and the aqueous phase was extracted with EA (2x10 mL). The combined organic phases were dried over Na_2SO_4 before removing solvents u.r.p.. The crude product was purified by FCC (solid loading,

100x theoretical product mass, cyclohexane/acetone, 75/25). The product fractions were identified by LCMS, solvent was removed u.r.p. and by coevaporation with MTBE/Cy. The product (12.9 mg crude) was used without further purification and characterization.

b) Adapted from[119]: The crude material (12.9 mg, 18.4 µmol, 1.00 Eq) was solved in *t*Bu-acetate (0.10 mL) and dioxane (0.10 mL). Then a solution of HCl in dioxane (4 M, 46 µL, 10 Eq) was added and the reaction was stirred at RT while being screened with LCMS. After 2 h 15 min and 3.5 h HCl in dioxane (4 M, 2x46 µL, 2x10 Eq) was added again. After 5 h reaction time the solvents were removed u.r.p. at 40°C and by coevaporation with toluene and MTBE (3x). The intermediate was dried overnight at HV and was directly used in the next reaction.

c) Adapted from[11, 96]: The crude material, potassium 3-(5-cyano-1-methyl-1*H*-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate (6.8 mg, 1.2 Eq) and HATU (8.4 mg, 1.2 Eq) were solved in dry DMF (0.15 mL) under Argon atmosphere. DiPEA (9.4 µL, 3.0 Eq) was added and the mixture was stirred at RT while being monitored by LCMS. After 1 h the solution was diluted between EA (15 mL), HCl (0.1 M) (5 mL) and brine (5 mL) and the aqueous phase was extracted with EA (2x10 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was directly used in the next reaction.

d) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (0.28 mL) and TFA (71 µL, 50 Eq). The reaction was conducted at RT for 1.5 h and HPLC method 1 was used, including lyophillization over 5 d. Yield: 5.2 mg, 9.9% o4s.

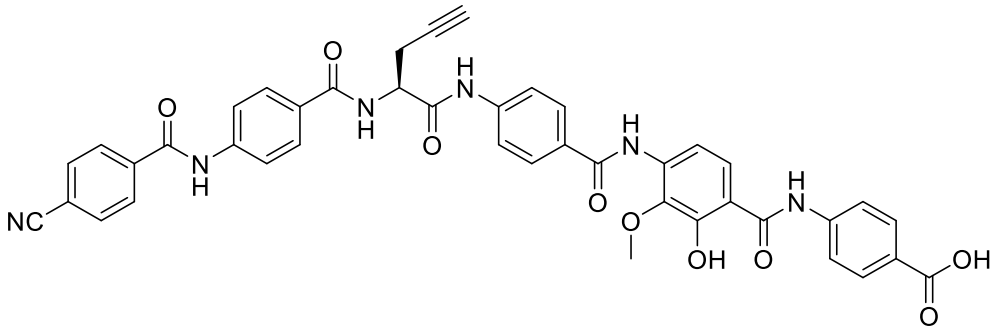
^{1}H NMR (700 MHz, DMSO): δ = 10.88 (br s, 1H, H-NCO), 10.09 – 9.99 (m, 2H, H-NCO), 9.38 (s, 1H, H-NCO), 8.52 (d, *J*=8.0, 1H, H-$C_{Ar,D}$), 8.10 (d, *J*=1.6, 1H, H-$C_{Ar,A}$), 7.97 – 7.92 (m, 4H, H-C_{Ar}), 7.92 – 7.89 (m, 2H, H-C_{Ar}), 7.78 (d, *J*=7.9, 1H, H-$C_{Ar,D}$), 7.77 – 7.73 (m, 2H, H-C_{Ar}), 7.72 (d, *J*=8.5, 1H, H-$C_{Ar,A}$), 7.62 (dd, *J*=8.4, 1.6, 1H, H-$C_{Ar,A}$), 5.75 (hept, *J*=6.3, 1H, H-C_{iPr}O), 5.53 (ddd, *J*=17.1, 10.5, 8.9, 1H, H-C=CH_2), 5.22 (dd, *J*=17.0, 1.9, 1H, H_aH_bC=C), 5.01 (dd, *J*=10.3, 2.2, 1H, H_aH_bC=C), 3.83 (s, 3H, H_3C-N), 2.55 (s, 6H, 3x $H_2C_{bicyclo}$), 2.26 (q, *J*=8.8, 1H, H-$C_{cycloprop.}(C)_3$), 1.86 – 1.81 (m, 1H, $H_aH_bC_{cycloprop.}$), 1.43 (dd, *J*=6.1, 2.4, 6H, $(H_3C)_2$C), 1.25 – 1.19 (m, 1H, $H_aH_bC_{cycloprop.}$).

^{13}C NMR (176 MHz, DMSO): δ = 169.9 (C=O), 169.5 (C=O), 168.4 (C=O), 164.9 (C=O), 161.9 (C=O), 154.3 (C(-N)(=N)), 152.9 ($C_{Ar,D}$), 142.9 (C_{Ar}), 141.4 ($C_{Ar,D}$), 141.3 ($C_{Ar,A}$), 139.0

($C_{Ar,A}$), 138.7 (C_{Ar}), 135.9 (C_{Ar}), 134.6 (H<u>C</u>=CH_2), 129.9 ($C_{Ar,D}$-H), 129.6 (C_{Ar}-H), 128.5 (C_{Ar}-H), 128.0 (C_{Ar}), 125.8 ($C_{Ar,D}$-NH), 125.5 ($C_{Ar,A}$-H), 123.6 ($C_{Ar,A}$-H), 120.0 (CN), 119.1 (C_{Ar}-H), 119.0 (C_{Ar}-H), 116.9 (<u>C</u>H_2=CH), 115.8 ($C_{Ar,D}$-H), 111.6 ($C_{Ar,A}$-H), 103.7 (<u>C</u>$_{Ar,A}$-CN), 69.6 (C_{iPr}-H), 53.1 (3x $C_{bicyclo}H_2$), 42.0 (<u>C</u>$(C)_3(N)$), 41.0 (C_{bicylo}), 34.8 (C_{bicylo}), 31.2 ($C_{cycloprop.}$-H), 30.8 (CH_3-N), 21.8 ($(\underline{C}H_3)_2C$), 20.2 ($C_{cycloprop.}H_2$).

HRMS (ESI): calculated for $[M+H]^+$: 793.3093, found: 793.3093.

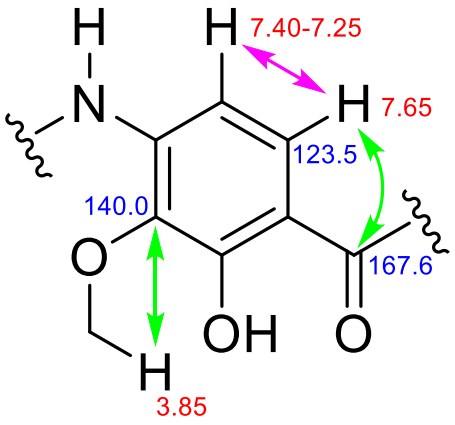
*Synthesis of methoxy (ring D) cystobactamid **(007)***



The final product was obtained according to the general *t*Bu-deprotection procedure using the cystobactamid-precursor **158** (84.4 mg, 103 µmol, 1.00 Eq), DCM (1.5 mL) and TFA (0.40 mL, 50 Eq). The reaction was conducted at RT for 1 h 40 min and HPLC method 1 was used, including lyophillization over 3 d. Yield: 37.9 mg, 48%.

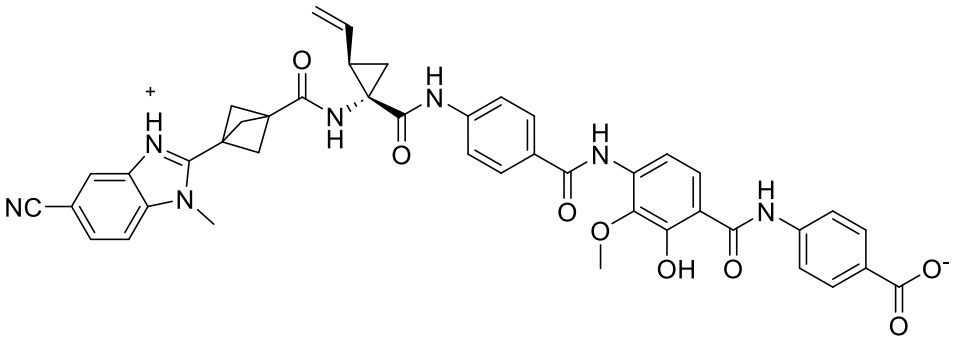
^{1}H NMR (500 MHz, DMSO): δ = 12.59 (br s, 2H), 10.73 (s, 1H, H-NCO), 10.58 (s, 1H, H-NCO), 9.28 (br s, 1H, H-NCO), 8.79 (d, *J*=7.6, 1H, H-NCO), 8.13 (d, *J*=8.9, 2H, H-C_{Ar}), 8.05 (d, *J*=8.7, 2H, H-C_{Ar}), 7.98 – 7.95 (m, 2H, H-C_{Ar}), 7.95 – 7.89 (m, 6H, H-C_{Ar}), 7.84 – 7.77 (m, 4H, H-C_{Ar}), 7.65 (d, *J*=9.0, 1H, H-$C_{Ar,D}$), 7.40 – 7.25 (m, 1H, H-$C_{Ar,D}$), 4.81 (td, *J*=8.2, 6.3, 1H, <u>H</u>-C(NH)$(C)_2$), 3.85 (s, 3H, H_3CO), 2.94 (t, *J*=2.7, 1H, HCC), 2.85 – 2.71 (m, 2H, <u>H</u>$_2$C(CCH)).

^{13}C NMR (126 MHz, DMSO): δ = 169.7 (C=O), 167.6 ($C_{Ar,D}$-C=O), 167.1 (C=O), 166.0 (C=O), 164.5 (C=O), 164.3 (C=O), 143.7 (C_{Ar}), 141.9 (C_{Ar}), 141.7 (C_{Ar}), 140.0 ($C_{Ar,D}$-OMe), 138.7 (C_{Ar}), 134.9 (C_{Ar}), 132.6 (C_{Ar}-H), 130.4 (C_{Ar}-H), 129.2 (C_{Ar}), 129.0 (C_{Ar}), 128.7 (C_{Ar}-H), 128.5 (C_{Ar}-H), 128.5 (C_{Ar}-H), 124.8 (C_{Ar}), 123.5 ($C_{Ar,D}$-H), 119.5 (C_{Ar}-H), 119.5 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.7 ($C_{Ar,D}$), 118.3 (CN), 114.5 (C_{Ar}), 114.1 (C_{Ar}), 80.7 (<u>C</u>CH), 73.2 (C<u>C</u>H), 59.4 (OCH_3), 53.5 (<u>C</u>H-$(C)_2(N)$), 21.5 (<u>C</u>H_2-CCH).



HRMS (ESI): calculated for $[M+H]^+$: 765.2304, found: 765.2303.

Synthesis of new scaffolded methoxy (ring D) cystobactamid ***(007b)***



a) Adapted from[119]: The *N*-Boc protected amino acid-CDE-fragment **153** (47.5 mg, 65.4 µmol, 1.00 Eq) was solved in *t*Bu-acetate (0.33 mL) and dioxane (0.33 mL). Then a solution of HCl in dioxane (4 M, 49 µL, 3.0 Eq) was added dropwise and the reaction was screened by LCMS. HCl solution (4 M, 2x98 µL, 2x6.0 Eq) was added again after 30 min and 1 h. After 5 h reaction time the solvents were removed u.r.p. at 40°C and by coevaporation with toluene and MTBE. The product was directly used in the next step.

b) Adapted from[11, 96]: The crude product, potassium 3-(5-cyano-1-methyl-1*H*-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate **76** (23.9 mg, 1.20 Eq) and HATU (28.6 mg, 1.15 Eq) were solved in dry DMF (0.50 mL) under Argon atmosphere and DiPEA (33 µL, 3.0 Eq) was added. The mixture was stirred at RT while being screened with LCMS. After 1 h the solution was diluted between EA/HCl (0.1 M) (20 mL each) and brine (10 mL) and the aqueous phase was extracted with EA (2x15 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was directly used in the next step.

c) Adapted from[11]: The crude product and $Pd(PPh_3)_4$ (spatula tip) were solved in dry THF (0.65 mL) under Argon atmosphere. Then aniline (18 µL, 3.0 Eq) was added and the reaction was stirred at RT while being screened with LCMS. After 1 h the reaction mixture was diluted with EA and solvents were removed to purify the material by FCC (solid loading, PE (or Cy)/EA/AcOH, 50/45/5->40/55/5->25/72/3->0/100/0) to obtain a colorless fluffy solid that was used without further characterization in the next step.

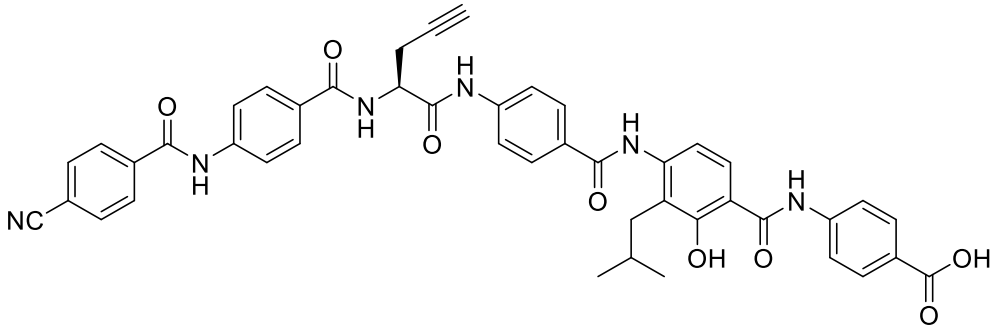
d) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (1.0 mL) and TFA (0.18 mL, 35 Eq). The reaction was conducted at RT for 1 h and HPLC method 1 was used, including lyophillization over 5 d. Yield: 12.7 mg, 25% o4s.

^{1}H NMR (700 MHz, DMSO): δ = 12.52 (br s, 1H), 9.83 (s, 1H, H-NCO), 9.14 (s, 1H, H-NCO), 8.64 (s, 1H, H-NCO), 8.14 – 8.12 (m, 1H, H-$C_{Ar,A}$), 7.91 (d, *J*=8.8, 2H, H-C_{Ar}), 7.89 (d, *J*=8.7, 2H, H-C_{Ar}), 7.79 (d, *J*=8.7, 2H, H-C_{Ar}), 7.75 (d, *J*=8.5, 1H, H-$C_{Ar,A}$), 7.73 (d, *J*=8.8, 2H, H-C_{Ar}), 7.64 (dd, *J*=8.4, 1.6, 1H, H-$C_{Ar,A}$), 7.57 (d, *J*=9.1, 1H, H-$C_{Ar,D}$), 7.18 (br s, 1H, H-$C_{Ar,D}$), 5.57 (ddd, *J*=17.2, 10.4, 9.0, 1H, H-C=CH_2), 5.28 (dd, *J*=17.0, 1.9, 1H, H_aH_bC=C), 5.08 (dd, *J*=10.3, 2.0, 1H, H_aH_bC=C), 3.89 (s, 3H, H_3C-N), 3.85 (s, 3H, H_3C-O), 2.56 (s, 6H, 3x $H_2C_{bicyclo}$), 2.33 – 2.26 (m, 1H, H-$C_{cycloprop.}(C)_3$), 1.89 – 1.85 (m, 1H, $H_aH_bC_{cycloprop.}$), 1.28 – 1.22 (m, 1H, $H_aH_bC_{cycloprop.}$).

^{13}C NMR (176 MHz, DMSO): δ = 170.0 (C=O), 167.8 (C=O), 167.5 (C=O), 167.1 (C=O), 164.1 (C=O), 154.2 (C(-N)(=N)), 144.3 (C_{Ar}), 141.7 (C_{Ar}), 141.4 ($C_{Ar,A}$), 140.5 ($C_{Ar,D}$-OMe), 139.0 ($C_{Ar,A}$), 134.4 (HC=CH_2), 130.4 (C_{Ar}-H), 129.5 (C_{Ar}), 128.2 (C_{Ar}-H), 125.5 ($C_{Ar,A}$-H), 124.1 (C_{Ar}), 123.7 ($C_{Ar,A}$-H), 123.6 ($C_{Ar,D}$-H), 120.0 (CN), 119.2 (C_{Ar}-H), 119.0 (C_{Ar}-H), 117.2 ($C$$H_2$=CH), 115.0 ($C_{Ar}$), 111.6 ($C_{Ar,A}$-H), 103.7 ($C_{Ar,A}$-CN), 59.0 ($CH_3$-O), 53.1 (3x $C_{bicyclo}H_2$), 41.9 ($C(C)_3(N)$), 40.8 (C_{bicylo}), 34.9 (C_{bicylo}), 31.5 ($C_{cycloprop.}$-H), 30.9 (CH_3-N), 20.5 ($C_{cycloprop.}H_2$).

HRMS (ESI): calculated for $[M+H]^+$: 780.2777, found: 780.2776.

Synthesis of isobutyl (ring D) cystobactamid ***(008)***

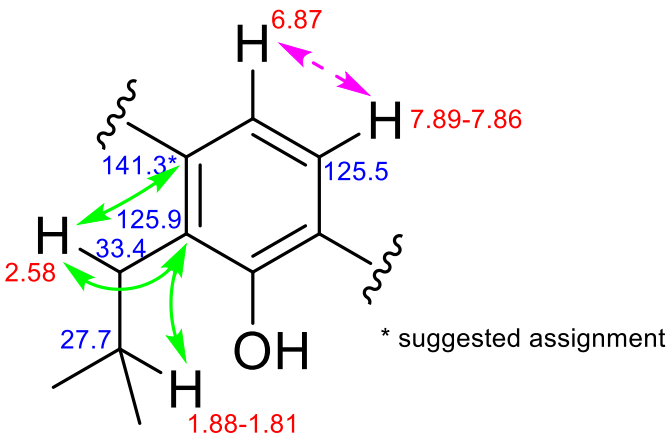


a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **148** (30.7 mg, 58.2 µmol, 1.00 Eq), the standard AB-central AA-fragment **26** (23.1 mg, 1.10 Eq), dry pyridine (23 µL, 5.0 Eq) and T3P-solution (69 µL, 2.0 Eq) in dry EA (1.0 mL) for 1 h. Precipitation of the product was observed during the workup, thus drying over Na_2SO_4 was omitted.

b) Adapted from[11]: The crude product and $Pd(PPh_3)_4$ (3.4 mg, 0.05 Eq) were solved in dry THF (1.0 mL) under Argon atmosphere before adding aniline (32 µL, 6.0 Eq). The reaction was stirred at RT while being monitored with LCMS. Solvents were removed from the reaction mixture after 1.5 h and the crude material was purified by preparative HPLC using HPLC method 1. The combined product fractions were lyophillized to give a nearly colorless solid: 24.9 mg, 54% o2s, (purity: 95 mol%).

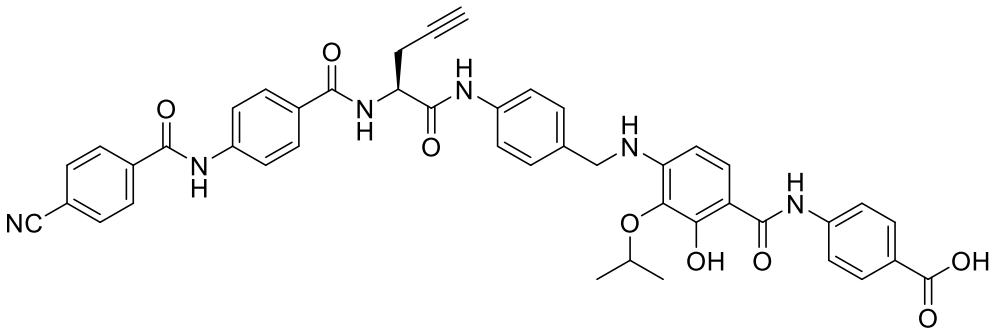
^{1}H NMR (700 MHz, DMSO): δ = 12.74 (br s, 2H), 10.73 (s, 1H, H-NCO), 10.56 (s, 1H, H-NCO), 9.71 (br s, 1H), 8.80 (d, *J*=7.5, 1H, H-NCO), 8.13 (d, *J*=8.6, 2H, H-C_{Ar}), 8.04 (d, *J*=8.6, 2H, H-C_{Ar}), 7.99 – 7.95 (m, 4H, H-C_{Ar}), 7.94 (d, *J*=8.6, 2H, H-C_{Ar}), 7.90 (d, *J*=8.8, 2H, H-C_{Ar}), 7.89 – 7.86 (m, 1H, H-$C_{Ar,D}$), 7.83 (d, *J*=8.7, 2H, H-C_{Ar}), 7.79 (d, *J*=8.9, 2H, H-C_{Ar}), 6.87 (br s, 1H, H-$C_{Ar,D}$), 4.81 (td, *J*=8.1, 6.4, 1H, $\underline{H}$-C(NH)$(C)_2$), 2.93 (t, *J*=2.6, 1H, HCC), 2.86 – 2.72 (m, 2H, $\underline{H}_2$C(CCH)), 2.58 (d, *J*=7.1, 2H, $\underline{H}_2$C-$C_{Ar,D}$), 1.89 – 1.81 (m, 1H, $\underline{H}$-C$(CH_3)_2$), 0.81 (d, *J*=6.7, 6H, $(\underline{H}_3C)_2CH$).

^{13}C NMR (176 MHz, DMSO): δ = 169.6 (C=O), 168.9 (C=O), 167.1 (C=O), 165.9 (C=O), 164.6 (C=O), 164.5 (C=O), 141.7 (C_{Ar}), 141.6 (C_{Ar}), 141.3 (C_{Ar}), 138.7 (C_{Ar}), 132.5 (C_{Ar}-H), 130.2 (C_{Ar}-H), 129.3 (C_{Ar}), 129.0 (C_{Ar}), 128.6 (C_{Ar}-H), 128.5 (C_{Ar}-H), 128.4 (C_{Ar}-H), 126.3 (C_{Ar}), 125.9 ($C_{Ar,D}$-iBu), 125.5 (C_{Ar}-H), 120.2 (C_{Ar}), 119.5 (C_{Ar}-H), 119.4 (C_{Ar}), 118.7 (C_{Ar}-H), 118.6 (C_{Ar}), 118.3 (CN), 114.0 (C_{Ar}), 113.8 (C_{Ar}), 80.7 ($\underline{C}$CH), 73.2 (C$\underline{C}$H), 53.4 ($\underline{C}$H-$(C)_2$(N)), 33.4 ($\underline{C}H_2$-$C_{Ar,D}$), 27.7 ($\underline{C}(CH_3)_2$), 22.7 (($\underline{C}H_3)_2C$), 21.4 ($\underline{C}H_2$-CCH).



HRMS (ESI): calculated for $[M+H]^+$: 791.2824, found: 791.2824.

*Synthesis of benzylamine (ring D) cystobactamid **(009)***



a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **96** (40 mg, 63 µmol, 1.0 Eq), the standard AB-central AA-fragment **26** (24.0 mg, 1.05 Eq), dry pyridine (26µL, 5.0 Eq) and T3P-solution (75 µL, 2.0 Eq) in dry EA (1.0 mL) for 1 h 45 min.

b) Adapted from[11]: The crude material and aniline (17 µL, 3.0 Eq) were solved in dry THF (0.63 mL). $Pd(PPh_3)_4$ (7.3 mg, 0.10 Eq) was added under Argon atmosphere and the solution was stirred while being screened with LCMS and TLC. After 2.5 h the temperature was increased to 40°C. Aniline (17 µL, 3.0 Eq) was added after 30 min and the reaction was stirred overnight at 40°C. Subsequently, solvents were removed from the reaction solution and the material was purified by FCC (solid loading, PE/acetone, 80/20->70/30). A brownish gum was obtained (15 mg) that was directly used in the next step. Note: An unsuccessful attempt was made to remove impurities by extraction with DCM/HCl (0.1 M) and sat. $NaHCO_3$ sol.

c) The final product was obtained according to the general *t*Bu-deprotection procedure using the crude precursor material (15 mg, 16 µmol, 1.0 Eq), DCM (0.18 mL) and TFA

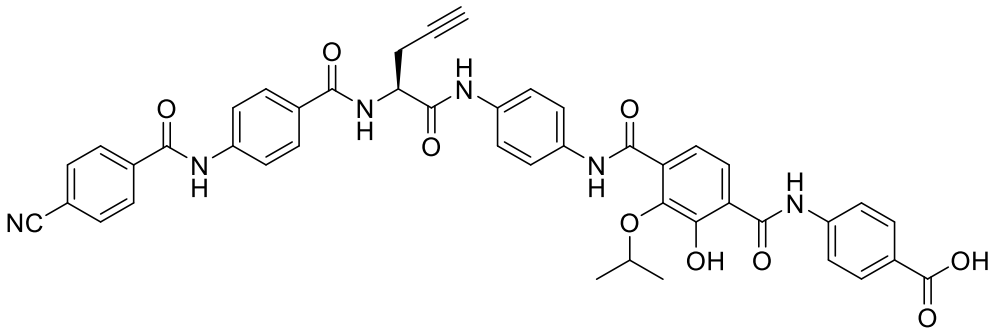
(45 µL, 50 Eq). The reaction was conducted at RT for 1 h 45 min and HPLC method 1 was used, including lyophillization over 5 d. Yield: 4.7 mg, 9.5% o3s.

^{1}H NMR (700 MHz, DMSO): δ = 12.70 (br s, 1H, HOOC), 12.42 (s, 1H, HO), 10.69 (s, 1H, H-NCO), 10.20 (s, 1H, H-NCO), 10.14 (s, 1H, H-NCO), 8.68 (d, *J*=7.6, 1H, H-NCO), 8.12 (d, *J*=8.1, 2H, H-C_{Ar}), 8.04 (d, *J*=7.7, 2H, H-C_{Ar}), 7.94 (d, *J*=8.5, 2H, H-C_{Ar}), 7.90 (d, *J*=8.5, 2H, H-C_{Ar}), 7.88 (d, *J*=8.6, 2H, H-C_{Ar}), 7.77 (d, *J*=8.7, 2H, H-C_{Ar}), 7.55 (m, 3H, H-C_{Ar}, H-$C_{Ar,D}$), 7.27 (d, *J*=8.7, 2H, H-C_{Ar}), 6.42 (s, 1H, H$\underline{\text{H}}$N-CH_2), 6.10 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 4.77 – 4.72 (m, 1H, H-$\underline{\text{C}}$(NH)$(C)_2$), 4.57 (hept, *J*=6.1, 1H, H-C_{iPr}O), 4.39 (d, *J*=6.2, 2H, $\underline{\text{H}}_2$C-NH), 2.90 (dt, *J*=2.7, 1.3, 1H, HCC), 2.73 (qdd, *J*=16.7, 7.4, 2.7, 2H, $\underline{\text{H}}_2$C(CCH)), 1.26 (d, *J*=6.2, 6H, $(H_3C)_2$C).

^{13}C NMR (176 MHz, DMSO): δ = 169.4 (C=O), 169.0 (C=O), 167.0 (C=O), 165.8 (C=O), 164.4 (C=O), 154.4 (C_{Ar}-OH), 147.3 (C_{Ar}), 142.5 (C_{Ar}), 141.6 (C_{Ar}), 138.7 (C_{Ar}), 137.4 (C_{Ar}), 135.0 (C_{Ar}), 132.5 (C_{Ar}-H), 130.1 (C_{Ar}-H), 129.8 ($C_{Ar,D}$-OiPr), 129.0 (C_{Ar}), 128.6 (C_{Ar}-H), 128.4 (C_{Ar}-H), 127.2 (C_{Ar}-H), 125.6 (C_{Ar}), 123.9 ($C_{Ar,D}$-H), 120.2 (C_{Ar}-H), 119.5 (C_{Ar}-H), 119.4 (C_{Ar}-H), 118.3 (CN), 114.0 (C_{Ar}), 103.8 (C_{Ar}), 101.6 ($C_{Ar,D}$-H), 80.7 ($\underline{\text{C}}$CH), 73.1 (C$\underline{\text{C}}$H), 72.9 (C_{iPr}-H), 53.3 ($\underline{\text{C}}$H-$(C)_2$(N)), 45.1 (CH_2-NH), 22.2 (($\underline{\text{C}}H_3)_2$C), 21.5 ($\underline{\text{C}}H_2$-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 779.2824, found: 779.2829.

*Synthesis of terephthalic acid (ring D) cystobactamid **(010)***



a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **99** (68.8 mg, 126 µmol, 1.00 Eq), the standard AB-central AA-fragment **26** (52.4 mg, 1.15 Eq), dry pyridine (51 µL, 5.0 Eq) and T3P-solution (0.15 mL, 2.0 Eq) in dry EA (2.0 mL) for 2 h.

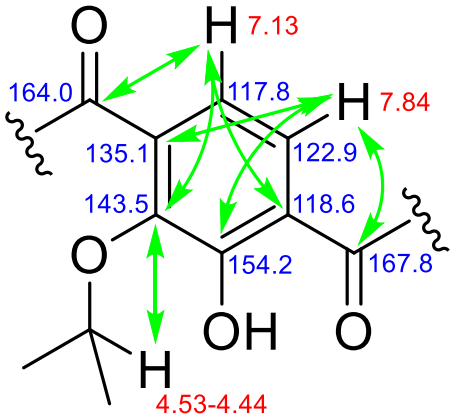
b) Adapted from[11]: The crude product, fresh $Pd(PPh_3)_4$ (7.3 mg, 0.05 Eq) and aniline (35 µL, 3.0 Eq) were solved in dry THF (1.9 mL) under Argon atmosphere and the reaction was stirred at RT while being monitored with LCMS. After 1.5 h the solvent was removed u.r.p. and the product was isolated by FCC (solid loading, 100x theoretical product mass,

PE/EA/AcOH, 50/50/2->40/60/2->60/40/10) to give a brownish solid that was used directly in the next step.

c) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (1.5 mL) and TFA (0.39 mL, 40 Eq). The reaction was conducted at RT for 1.5 h and HPLC method 1 was used. Yield: 45.7 mg, 46% o3s.

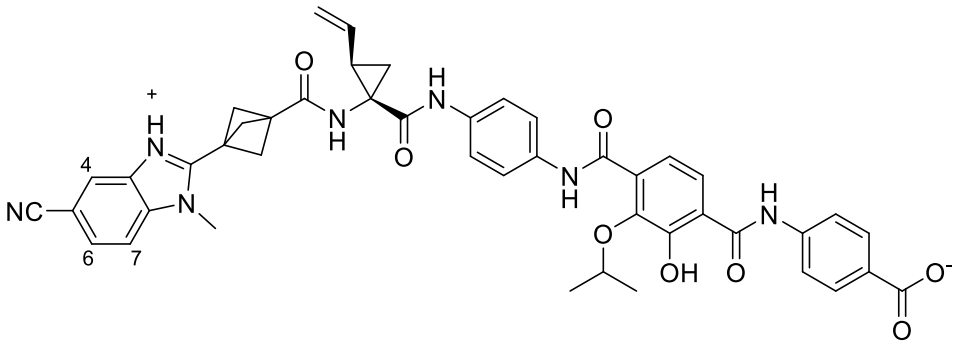
^{1}H NMR (500 MHz, DMSO): δ = 12.87 (br s, 1H, HOOC), 12.01 (br s, 1H, HO-$C_{Ar,D}$), 10.89 (br s, 1H, H-NCO), 10.72 (s, 1H, H-NCO), 10.30 (s, 1H, H-NCO), 10.26 (s, 1H, H-NCO), 8.73 (d, *J* = 7.8, 1H, H-NCO), 8.13 (d, *J* = 8.7, 2H, H-C_{Ar}), 8.05 (d, *J* = 8.7, 2H, H-C_{Ar}), 8.01 – 7.94 (m, 4H, H-C_{Ar}), 7.92 – 7.85 (m, 4H, H-C_{Ar}), 7.84 (d, *J*=8.5, 1H, H-$C_{Ar,D}$), 7.68 (d, *J* = 9.2, 2H, H-C_{Ar}), 7.62 (d, *J* = 9.2, 2H, H-C_{Ar}), 7.13 (d, *J* = 8.2, 1H, H-$C_{Ar,D}$), 4.81 – 4.75 (m, 1H, H-C(NH)$(C)_2$), 4.53 – 4.44 (m, 1H, H-C_{iPr}O), 2.93 (t, *J* = 2.6, 1H, HCC), 2.83 – 2.68 (m, 2H, H_2C(CCH)), 1.21 (d, *J* = 6.1, 6H, $(H_3C)_2$C).

^{13}C NMR (126 MHz, DMSO): δ = 168.9 (C=O), 167.8 (HO-$C_{Ar,D}$-$C_{Ar,D}$-C=O), 166.9 (C=O), 165.9 (C=O), 164.5 (C=O), 164.0 (iPrO-$C_{Ar,D}$-$C_{Ar,D}$-C=O), 154.2 ($C_{Ar,D}$-OH), 143.5 ($C_{Ar,D}$-OiPr), 142.0 (C_{Ar}), 141.7 (C_{Ar}), 138.7 (C_{Ar}), 135.1 ($C_{Ar,D}$), 134.7 (C_{Ar}), 134.6 (C_{Ar}), 132.6 (C_{Ar}-H), 130.3 (C_{Ar}-H), 129.1 (C_{Ar}), 128.7 (C_{Ar}-H), 128.5 (C_{Ar}-H), 126.3 (C_{Ar}), 122.9 ($C_{Ar,D}$-H), 120.6 (C_{Ar}-H), 119.9 (C_{Ar}-H), 119.9 (C_{Ar}-H), 119.5 (C_{Ar}-H), 118.6 ($C_{Ar,D}$), 118.4 (CN), 117.8 ($C_{Ar,D}$-H), 114.1 (C_{Ar}), 80.9 (CCH), 75.9 (C_{iPr}-H), 73.1 (CCH), 53.3 (CH-$(C)_2$(N)), 22.2 ((CH_3)$_2$C), 21.6 (CH_2-CCH).



HRMS (ESI): calculated for $[M+H]^+$: 793.2617, found: 793.2616.

Synthesis of new scaffolded terephthalic acid (ring D) cystobactamid ***(010b)***



a) Adapted from[11]: The *N*-Fmoc-protected AA-CDE-fragment **152** (61 mg, 70 µmol, 1.0 Eq) was solved in dry MeCN (1.0 mL) under Argon atmosphere and $HNEt_2$ (0.11 mL, 15 Eq) was added. The reaction was screened by LCMS, after 2 h the mixture was diluted with MeCN and solvents were removed u.r.p. and by coevaporation with MeCN (4x). The material was further dried under HV overnight.

b) Adapted from[11, 96]: The crude material was solved in dry DMF (0.70 mL) under Argon atmosphere together with potassium 3-(5-cyano-1-methyl-1*H*-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate **76** (23.3 mg, 1.10 Eq) and HATU (29.1 mg, 1.10 Eq). DiPEA (35 µL, 3.0 Eq) was added and the mixture was stirred at RT while being screened with LCMS. After 2 h the solution was diluted between EA/HCl (0.1 M) (20 mL each) and brine (10 mL) and the aqueous phase was extracted with EA (2x15 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The crude product was directly used in the next step.

c) Adapted from[11]: The material was suspended in dry THF (1.0 mL) and anilin (19 µL, 3.0 Eq) under Argon atmosphere. Then $Pd(PPh_3)_4$ (spatula tip) was added and the reaction was stirred overnight at RT. Solvents were removed from the reaction mixture and the material was purified by FCC (solid loading, 100x theoretical product mass, cyclohexane/acetone, 60/40) to give a colorless solid that was used without further purification in the final deprotection.

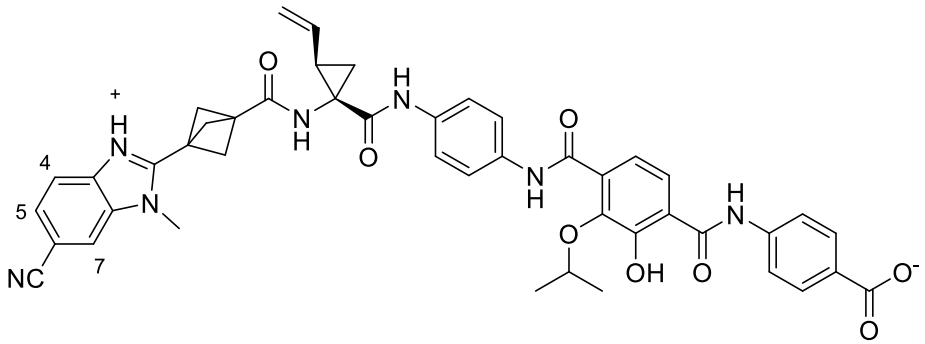
d) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (1.0 mL) and TFA (0.27 mL, 15 Eq). The reaction was conducted at RT for 1 h 50 min and HPLC method 1 was used, including lyophillization over 3 d. Yield: 38.5 mg, 69% o4s.

^{1}H NMR (500 MHz, DMSO): δ = 12.54 (br s, 1H), 10.45 (s, 1H, H-NCO), 9.50 (s, 1H, H-NCO), 8.61 (s, 1H, H-NCO), 8.18 – 8.08 (m, 1H, H-$C_{Ar,A,\mathbf{4}}$), 7.88 (d, *J*=8.9, 2H, H-C_{Ar}), 7.81 (d, *J*=8.9, 2H, H-C_{Ar}), 7.75 (dd, *J*=8.5, 0.8, 1H, H-$C_{Ar,A,\mathbf{7}}$), 7.67 (d, *J*=9.0, 2H, H-C_{Ar}), 7.64 (dd, *J*=8.4, 1.5, 1H, H-$C_{Ar,A,\mathbf{6}}$), 7.58 (d, *J*=8.4, 1H, H-$C_{Ar,D}$), 7.50 (d, *J*=9.2, 2H, H-C_{Ar}), 6.66 (d, *J*=7.8, 1H, H-$C_{Ar,D}$), 5.59 (ddd, *J*=17.1, 10.5, 9.2, 1H, $\underline{H}$-C=CH_2), 5.27 (dd, *J*=17.1, 1.8, 1H, $H_a\underline{H_b}$C=C), 5.11 – 5.00 (m, 2H, $\underline{H_a}H_b$C=C, H-C_{iPr}O), 3.89 (s, 3H, H_3C-N), 2.55 (s, 6H, 3x $H_2C_{bicyclo}$), 2.25 – 2.19 (m, 1H, H-$C_{cycloprop.}(C)_3$), 1.85 – 1.80 (m, 1H, $\underline{H_a}H_bC_{cycloprop.}$), 1.25 – 1.18 (m, 7H, $(H_3C)_2$C, $H_a\underline{H_b}C_{cycloprop.}$).

^{13}C NMR (126 MHz, DMSO): δ = 169.9 (C=O), 167.2 ($C_{cycloprop.}$-$\underline{C}$=O), 167.2 ($C_{Ar,D}$-C=O), 167.2 (C=O), 164.9 (C=O), 154.2 (C(-N)(=N)), 144.6 (C_{Ar}), 141.4 ($C_{Ar,A}$-N=C), 139.0 ($C_{Ar,A}$-NMe), 134.9 (C_{Ar}), 134.8 (H$\underline{C}$=CH_2), 134.1 (C_{Ar}), 130.5 (C_{Ar}-H), 125.6 ($C_{Ar,A,\mathbf{6}}$-H), 123.9 (C_{Ar}), 123.7 ($C_{Ar,A,\mathbf{4}}$-H), 123.6 ($C_{Ar,D}$-H), 123.6 (C_{Ar}), 120.7 (C_{Ar}-H), 120.1 (C_{Ar}), 120.1 (CN), 119.5 (C_{Ar}-H), 118.9 (C_{Ar}-H), 116.9 ($\underline{C}H_2$=CH), 111.6 ($C_{Ar,A,\mathbf{7}}$-H), 103.7 ($\underline{C}_{Ar,A}$-CN), 72.0 (C_{iPr}-H), 53.1 (3x $C_{bicyclo}H_2$), 41.6 ($\underline{C}(C)_3(N)$), 40.9 (C_{bicylo}), 34.9 (C_{bicylo}), 31.5 ($C_{cycloprop.}$-H), 30.9 (CH_3-N), 22.4 – 22.2 (m, $(\underline{C}H_3)_2$C), 20.5 ($C_{cycloprop.}H_2$).

HRMS (ESI): calculated for $[M+H]^+$: 808.3090, found: 808.3091.

*Synthesis of alternatively scaffolded terephthalic acid (ring D) cystobactamid **(010c)***



a) Adapted from[11]: The *N*-Fmoc-protected AA-CDE-fragment **152** (69 mg, 79 µmol, 1.0 Eq) was solved in MeCN (1.2 mL) and $HNEt_2$ (0.12 mL, 15 Eq) was added. The reaction was screened by LCMS and after 1 h 20 min the reaction mixture was diluted with MeCN. Solvents were removed u.r.p. and by coevaporation with MeCN (3x) followed by drying under HV overnight. The raw material was directly used in the next reaction.

b) Adapted from[11, 96]: The crude material, potassium 3-(6-cyano-1-methyl-1*H*-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate **76b** (26.4 mg, 1.10 Eq) and

HATU (32.9 mg, 1.10 Eq) were solved in dry DMF (0.80 mL) under Argon atmosphere. DiPEA (40 µL, 3.0 Eq) was added and the mixture was stirred at RT. After 2 h and reaction control by LCMS the solution was diluted between EA/HCl (0.1 M) (20 mL each) and brine (10 mL) and the aqueous phase was extracted with EA (2x15 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The material was directly used in the next step.

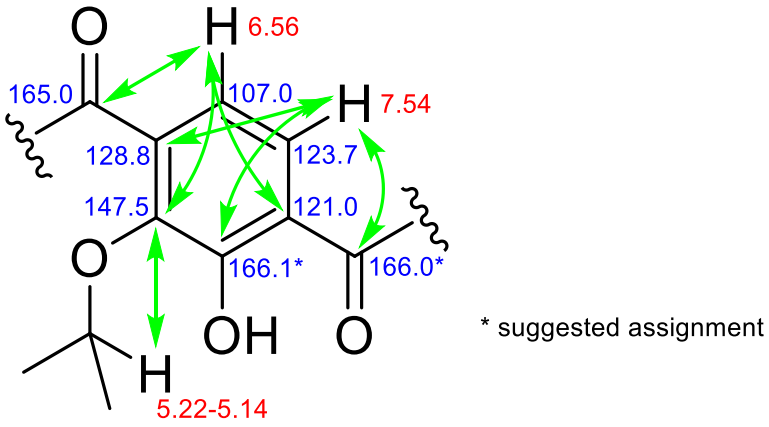
c) Adapted from[11]: The crude material and $Pd(PPh_3)_4$ (spatula tip) were solved in dry THF (0.8 mL) and aniline (22 µl, 3.0 Eq) was added. The reaction was stirred at RT while being screened with LCMS. After 2 h 45 min the solvents were removed u.r.p. from the mixture and the material was purified by FCC (solid loading, 100x theoretical product mass, cyclohexane/acetone, 70/30->60/40->50/50). After solvent removal u.r.p. and coevaporation with cyclohexane (2x) a brownish sticky solid was obtained that was used without further characterization.

d) The final product was obtained according to the general *t*Bu-deprotection procedure using DCM (1.2 mL) and TFA (0.30 mL, 50 Eq). The reaction was conducted at RT for 1.5 h and HPLC method 1 was used, including lyophillization over 6 d. Yield: 47.3 mg, 74% o4s.

^{1}H NMR (500 MHz, DMSO-d6): δ = 15.42 (s, 1H, HO), 10.51 (s, 1H, H-$N_{C\text{-}ring}$(CO)$C_{Ar,D}$), 9.92 – 9.84 (m, 1H, H-$N_{E\text{-}ring}$), 9.23 – 9.13 (m, 1H, H-NCO), 8.22 – 8.17 (m, 1H, H-$C_{Ar,A,\mathbf{7}}$), 7.83 (d, J=7.5, 2H, H-C_{Ar}), 7.74 (d, J=8.8, 1H, H-$C_{Ar,A,\mathbf{4}}$), 7.66 (d, J=9.0, 2H, H-C_{Ar}), 7.61 (d, J=8.5, 2H, H-C_{Ar}), 7.59 – 7.55 (m, 3H, H-$C_{Ar,A,\mathbf{5}}$, H-C_{Ar}), 7.54 (d, *J*=8.4, 1H, H-$C_{Ar,D}$), 6.57 (d, J=8.4, 1H, H-$C_{Ar,D}$), 5.63 – 5.51 (m, 1H, $\underline{H}$-C=CH_2), 5.25 (dd, J=17.1, 2.0, 1H, $H_a\underline{H_b}$C=C), 5.22 – 5.14 (m, 1H, H-C_{iPr}O), 5.04 (dd, J=10.1, 2.2, 1H, $\underline{H_a}H_b$C=C), 3.87 (s, 3H, H_3C-N), 2.55 (s, 6H, 3x $H_2C_{bicyclo}$), 2.21 (q, J=8.9, 1H, H-$C_{cycloprop.}$(C)$_3$), 1.85 – 1.78 (m, 1H, $\underline{H_a}H_b$C$_{cycloprop.}$), 1.26 – 1.18 (m, 7H, $(H_3C)_2$C, $H_a\underline{H_b}$C$_{cycloprop.}$).

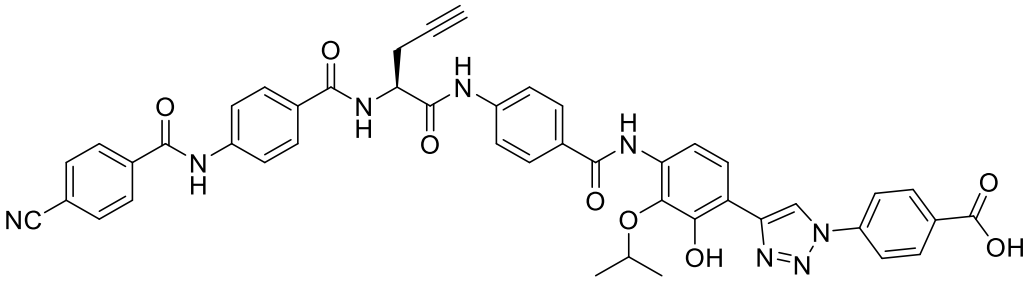
^{13}C NMR (126 MHz, DMSO): δ = 170.5 (C=O), 169.9 (C=O), 167.4 (C=O), 166.6 ($C_{Ar,D}$-OH), 166.0 ($N_{E\text{-}ring}$-C=O), 165.0 ($N_{C\text{-}ring}$-$\underline{C}$(=O)-$C_{Ar,D}$), 155.0 (C(-N)(=N)), 147.5 ($C_{Ar,D}$-OiPr), 144.8 ($C_{Ar,A}$-N=C), 141.5 (C_{Ar}), 135.8 ($C_{Ar,A}$-NMe), 134.9 (H$\underline{C}$=CH_2), 134.8 (C_{Ar}), 134.3 (C_{Ar}), 133.6 (C_{Ar}), 129.8 (C_{Ar}-H), 128.8 ($C_{Ar,D}$), 125.0 ($C_{Ar,A,\mathbf{5}}$-H), 123.7 ($C_{Ar,D}$-H), 121.0 ($C_{Ar,D}$), 120.6 (C_{Ar}-H), 120.1 (CN), 119.8 ($C_{Ar,A,\mathbf{4}}$-H), 119.4 (C_{Ar}-H), 117.8 (C_{Ar}-H), 116.8 ($\underline{C}H_2$=CH), 115.5 ($C_{Ar,A,\mathbf{7}}$-H), 107.0 ($C_{Ar,D}$-H), 103.7 ($\underline{C}_{Ar,A}$-CN), 71.2 (C_{iPr}-H), 53.1 (3x $C_{bicyclo}H_2$), 41.7

(C(C)$_3$(N)), 41.0 (C_{bicylo}), 34.9 (C_{bicylo}), 31.4 ($C_{cycloprop.}$-H), 30.9 (CH_3-N), 22.3 ((CH_3)$_2$C), 20.3 ($C_{cycloprop.}H_2$).



HRMS (ESI): calculated for $[M+H]^+$: 808.3090, found: 808.3089.

*Synthesis of triazole (ring D) cystobactamid **(011)***



Note: The following reaction was divided in two equal-sized batches and is treated here as one batch:

Adapted from[11]: The cystobactamid-precursor **159** (36 mg, 40 µmol, 1.0 Eq) and aniline (24 µL, 6.5 Eq) were solved in dry THF (1.1 mL). Fresh $Pd(PPh_3)_4$ (5.1 mg, 0.11 Eq) was added under Argon atmosphere and the reaction solution was stirred for 1.5-2 h. After reaction control by LCMS the reaction mixture was concentrated u.r.p. and the residue was purified by preparative HPLC (method 1). After removing solvents u.r.p. at the rotary evaporator and by lyophillization the product was obtained as an almost colorless solid. Yield: 19.6 mg, 60%.

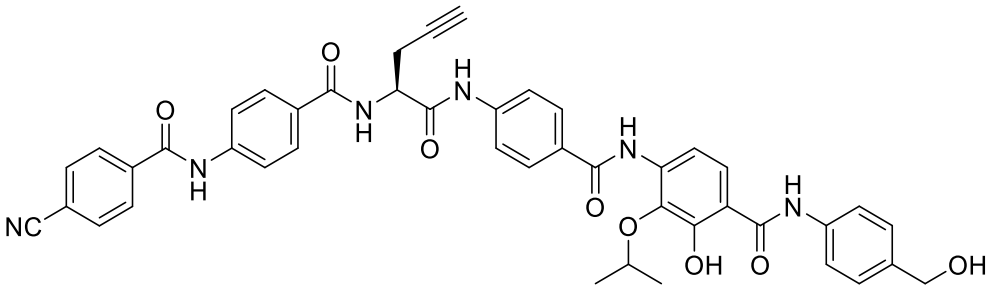
^{1}H NMR (500 MHz, DMSO): δ = 10.72 (s, 1H), 10.59 (s, 1H), 9.52 (s, 1H), 9.13 (s, 1H, H-CNN), 8.81 (d, *J*=7.6, 1H, HNCO), 8.16 (d, *J*=8.9, 2H), 8.13 (d, *J*=8.7, 2H), 8.10 (d, *J*=8.9, 2H), 8.05 (d, *J*=8.9, 2H), 8.01 – 7.95 (m, 4H), 7.90 (d, *J*=9.0, 2H), 7.81 (d, *J*=9.0, 2H), 7.77 (d, *J*=8.5, 1H, H-$C_{Ar,D}$), 7.46 (d, *J*=8.5, 1H, H-$C_{Ar,D}$), 4.80 (q, *J*=7.9, 1H, H-C(NH)(C)$_2$), 4.39 (hept, *J*=6.0,

1H, H-C_{iPr}O), 2.94 (t, *J*=2.6, 1H, H-CC), 2.86 – 2.72 (m, 2H, $\underline{H_2}$C(CCH)), 1.26 (d, *J*=6.3, 6H, $(H_3C)_2C$).

^{13}C NMR (126 MHz, DMSO): δ = 169.7 (C=O), 166.7 (C=O), 166.0 (C=O), 164.5 (C=O), 164.2 (C=O), 147.9 (C_{Ar}), 144.8 (C_{Ar}), 141.9 (C_{Ar}), 141.7 (C_{Ar}), 139.0 (C_{Ar}), 138.7 (C_{Ar}), 138.6 (C_{Ar}), 132.5 (C_{Ar}-H), 132.4 (C_{Ar}), 131.0 (C_{Ar}-H), 129.0 (C_{Ar}), 128.9 (C_{Ar}), 128.6 (C_{Ar}-H), 128.4 (C_{Ar}-H), 128.4 (C_{Ar}-H), 121.1 ($C_{Ar,D}$-H), 120.6 ($C_{triazol}$-H), 119.9 (C_{Ar}-H), 119.5 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.3 (CN), 115.9 ($C_{Ar,D}$-H), 114.2 (C_{Ar}), 114.0 (C_{Ar}), 80.7 ($\underline{C}$CH), 75.1 (C_{iPr}-H), 73.2 (C$\underline{C}$H), 53.5 ($\underline{C}$H-$(C)_2$(N)), 22.1 (($\underline{C}H_3)_2C$), 21.4 ($\underline{C}H_2$-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 817.2729, found: 817.2737.

*Synthesis of benzyl alcohol (ring E) cystobactamid **(014)***



a) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the CDE-fragment **115** (40.0 mg, 55.7 µmol, 1.00 Eq), the standard AB-central AA-fragment **26** (22.1 mg, 1.10 Eq), dry pyridine (13 µL, 3.0 Eq) and T3P-solution (66 µL, 2.0 Eq) in dry EA (0.80 mL) for 1 h 45 min.

b) Adapted from[11]: The material was solved in dry THF (0.55 mL) under Argon atmosphere and aniline (15 mg, 3.0 Eq) was added. $Pd(PPh_3)_4$ (spatula tip) was added and the reaction was stirred at RT. After 1.5 h the mixture was diluted with DCM and solvents were removed u.r.p. and by coevaporation with EA/heptane (3x) before purifying the material by FCC (solid loading, 100x theoretical product mass, cyclohexane/EA/AcOH, 50/50/1). The product was obtained as an off white solid and directly used in the final deprotection.

Note: a small amount of the material was used to examine the best deprotecting conditions. Anisol was insuccessfully tried as a scavenger.

c) The crude material (40.7 mg, 39.9 µmol, 1.00 Eq) was solved in dry DCM (0.80 mL) under Argon atmosphere before adding AcOH (0.14 mL, 60 Eq). The mixture was stirred

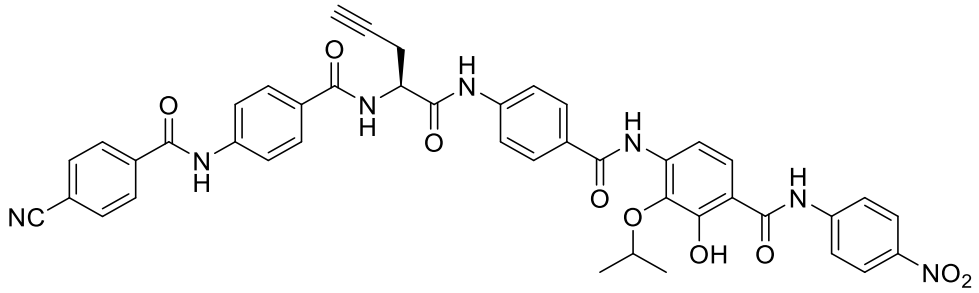
at 0°C while adding TFA (92 µL, 30 Eq) slowly dropwise. The reaction was further stirred at 0°C while being screened by LCMS. After 70 min and after 1 h 45 min TFA (46+31 µL, 15+10 Eq) was added again and after 1 h 50 min the reaction was quenched by adding H_2O (0.5 mL) and NaOH (5 M) to the mixture to adjust the pH to ~12. (Note: this is necessary to avoid the back reaction of trityl cation with the alcohol). THF (1.5 mL) and DMSO (0.3 mL) were added to the suspension (volume ~1.5 mL) followed by stirring for ~15 min. After sonicating the suspension the material was purified by HPLC (method 3) and the product fractions were combined and lyophillized over 5 d. A colorless fluffy solid was obtained, yield: 18.1 mg, 58%, 42% o3s, purity: 95 w% (trityl dimer as impurity).

^{1}H NMR (700 MHz, DMSO): δ = 14.78 (br s, 1H), 10.73 (br s, 1H), 10.57 (s, 1H, H-NCO), 8.93 (s, 1H, H-$N_{D\text{-}ring}$CO), 8.78 (d, *J*=7.7, 1H, H-NCO), 8.13 (d, *J*=8.6, 2H, H-C_{Ar}), 8.04 (d, *J*=8.6, 2H, H-C_{Ar}), 7.96 (d, *J*=8.8, 2H, H-C_{Ar}), 7.90 (d, *J*=8.9, 2H, H-C_{Ar}), 7.87 (d, *J*=8.8, 2H, H-C_{Ar}), 7.81 (d, *J*=8.8, 2H, H-C_{Ar}), 7.64 (d, *J*=8.4, 2H, H-C_{Ar}), 7.49 (d, *J*=8.7, 1H, H-$C_{Ar,D}$), 7.21 (d, *J*=8.6, 2H, H-C_{Ar}), 7.15 (d, *J*=8.8, 1H, H-$C_{Ar,D}$), 5.03 (br s, 1H, $\underline{H}$O-CH_2), 4.96 (hept, *J*=6.7, 1H, H-C_{iPr}O), 4.81 (td, *J*=8.2, 6.3, 1H, $\underline{H}$-C(NH)$(C)_2$), 4.43 (s, 2H, $\underline{H_2}$COH), 2.93 (t, *J*=2.6, 1H, HCC), 2.85 – 2.72 (m, 2H, $\underline{H_2}$C(CCH)), 1.20 (d, *J*=6.3, 6H, $(H_3C)_2$C).

^{13}C NMR (176 MHz, DMSO): δ = 169.6 (C=O), 167.0 ($C_{Ar,D}$-$\underline{C}$=O), 165.9 (C=O), 164.5 (C=O), 163.2 (C=O), 141.7 (C_{Ar}), 141.7 (C_{Ar}), 139.5 (C_{Ar}), 138.7 (C_{Ar}), 137.4 ($C_{Ar,D}$-OiPr), 135.6 (C_{Ar}), 134.3 ($C_{Ar,D}$), 132.5 (C_{Ar}-H), 130.4 (C_{Ar}), 129.6 (C_{Ar}), 129.0 (C_{Ar}), 128.6 (C_{Ar}-H), 128.4 (C_{Ar}-H), 127.7 (C_{Ar}-H), 127.1 (C_{Ar}-H), 123.5 ($C_{Ar,D}$-H), 119.5 (C_{Ar}-H), 119.1 (C_{Ar}-H), 119.1 (C_{Ar}-H), 118.3 (CN), 115.6 ($C_{Ar,D}$), 114.0 (C_{Ar}), 102.0 ($C_{Ar,D}$-H), 80.7 ($\underline{C}$CH), 73.2 (C$\underline{C}$H), 70.8 (C_{iPr}-H), 62.9 (CH_2OH), 53.5 ($\underline{C}$H-$(C)_2$(N)), 22.6 (($\underline{C}H_3)_2$C), 21.4 ($\underline{C}H_2$-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 779.2824, found: 779.2819.

Synthesis of Nitro (ring E) cystobactamid ***(015)***



a) 2-(allyloxy)-4-amino-3-isopropoxy-N-(4-nitrophenyl)benzamide **117** (21.7 mg, 1.0 Eq) and 4-((*tert*-butoxycarbonyl)amino)benzoic acid (15.2 mg, 1.10 Eq) were solved in dry EA (0.60 mL) under Argon atmosphere before adding dry pyridine (14 µL, 3.0 Eq). Then T3P (50% sol. in EA, 70 µL, 2.0 Eq) was added and the reaction was stirred at RT while being screened by LCMS. After 1.5 h the temperature was raised to 60°C and the reaction was stirred further overnight. The mixture was then diluted between EA/HCl (0.1 M) (20 mL each) and the aqueous phase was extracted with EA (2x15 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The material was purified by FCC (solid loading, 100x theoretical product mass, cyclohexane/EA, 80/20). After the solvents were removed u.r.p. the raw product was resolved again in MTBE (20 mL) and subsequently washed with NaOH (1 M, 20 mL). The aqueous phase was extracted with MTBE (1x15 mL) followed by drying the combined organic phases over Na_2SO_4 and removing solvents u.r.p.. To support drying of the material it was freezed in liquid nitrogen under high vacuum. A light yellow foam was obtained that was used directly in the next step (crude mass: 26 mg).

b) The crude material (26 mg, 44 µmol, 1.0 Eq) was solved in HCl (4 M sol. in dioxane, 0.55 mL, 50 Eq) under Argon atmosphere and the reaction was stirred at RT. After few hours a precipitate was formed and after 3 h HCl (4 M sol. in dioxane, 0.55 mL, 50 Eq) was added again. After 4 h the mixture was diluted between MTBE/sat. $NaHCO_3$ sol. (20 mL each) and the aqueous solution was extracted with MTBE (2x15 mL). The combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. Solvents were removed from the material u.r.p. and by coevaporation with acetone, then MTBE (3x). The crude material was directly used in the next step.

c) The protected cystobactamid precursor was obtained according to general procedure B, involving reaction of the crude CDE-fragment, the standard AB-central AA-fragment **26**

(16 mg, 1.0 Eq), dry pyridine (11 µL, 3.0 Eq) and T3P-solution (52 µL, 2.0 Eq) in dry EA (0.50 mL) for 1.5 h.

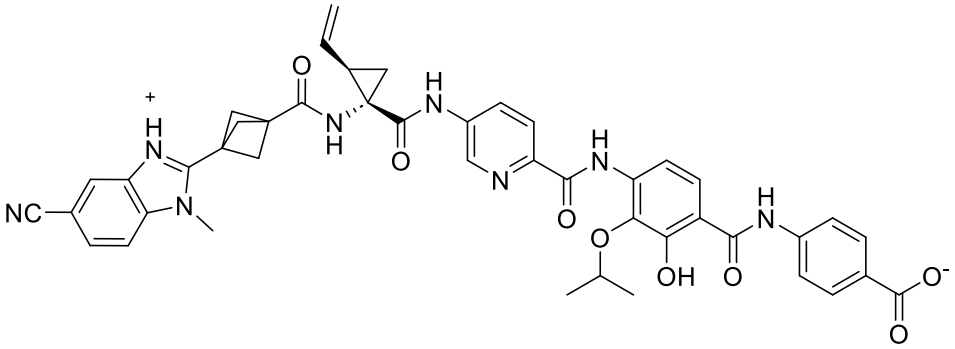
d) Adapted from[11]: The crude material was solved in dry THF (0.50 mL) under Argon atmosphere before adding aniline (12 µL. 3.0 Eq) and a spatula tip of $Pd(PPh_3)_4$. The reaction was screened by LCMS and after 2 h solvents were evaporated u.r.p.. The material was purified by preparative HPLC (method 4) and product fractions were collected. After lyophillization a yellowish fluffy solid was obtained. Yield: 19.1 mg, 41% o4s, purity ~90 mol% (UV/ LCMS).

^{1}H NMR (700 MHz, DMSO): δ = 11.98 (s, 1H, HO), 10.88 (br s, 1H, H-NCO), 10.71 (s, 1H, H-NCO), 10.58 (s, 1H, H-NCO), 9.43 (s, 1H, H-$N_{D\text{-}ring}$CO), 8.77 (d, *J*=7.5, 1H, H-NCO), 8.29 (d, *J*=9.2, 2H, H-C_{Ar}), 8.13 (d, *J*=8.4, 2H, H-C_{Ar}), 8.05 (d, *J*=8.5, 2H, H-C_{Ar}), 8.02 (d, *J*=9.3, 2H, H-C_{Ar}), 7.98 – 7.95 (m, 4H, H-C_{Ar}), 7.90 (d, *J*=8.8, 2H, H-C_{Ar}), 7.85 – 7.80 (m, 3H, H-$C_{Ar,D}$, H-C_{Ar}), 7.70 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 4.84 – 4.77 (m, 1H, <u>H</u>-C(NH)$(C)_2$), 4.54 (hept, *J*=5.9, 1H, H-C_{iPr}O), 2.94 (t, *J*=2.6, 1H, HCC), 2.84 – 2.73 (m, 2H, <u>H</u>$_2$C(CCH)), 1.27 (d, *J*=6.1, 6H, $(H_3C)_2$C).

^{13}C NMR (176 MHz, DMSO): δ = 169.7 (C=O), 168.5 ($C_{Ar,D}$-<u>C</u>=O), 166.0 (C=O), 164.5 (C=O), 164.2 (C=O), 154.0 ($C_{Ar,D}$-OH), 144.4 (C_{Ar}), 142.9 (C_{Ar}), 142.2 (C_{Ar}), 141.7 (C_{Ar}), 138.7 (C_{Ar}), 137.2 (C_{Ar}), 136.5 ($C_{Ar,D}$-OiPr), 132.5 (C_{Ar}-H), 128.9 (C_{Ar}), 128.6 (C_{Ar}-H), 128.5 (C_{Ar}), 128.4 (C_{Ar}-H), 128.4 (C_{Ar}-H), 124.8 (C_{Ar}-H), 123.1 ($C_{Ar,D}$-H), 120.9 (C_{Ar}-H), 119.5 (C_{Ar}-H), 119.0 (C_{Ar}-H), 118.3 (CN), 114.0 (C_{Ar}), 112.8 ($C_{Ar,D}$), 112.3 ($C_{Ar,D}$-H), 80.6 (<u>C</u>CH), 74.9 (C_{iPr}-H), 73.2 (C<u>C</u>H), 53.5 (<u>C</u>H-$(C)_2$(N)), 22.3 ($(\underline{C}H_3)_2$C), 21.4 (<u>C</u>H_2-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 794.2569, found: 794.2572.

Synthesis of new scaffolded pyridine (ring C) cystobactamid ***(016)***



a) Adapted from[96]: The *N*-Boc protected AA-CDE-fragment **128** (25.5 mg, 34.5 µmol, 1.00 Eq) was solved in a solution of HCl in dioxane (4 M, 0.80 mL) under Argon atmosphere. The reaction mixture was stirred at RT while being continously screened with TLC. After 30 min solvents and HCl were removed u.r.p. and the material was directly used in next step.

b) Adapted from[11, 96]: potassium 3-(5-cyano-1-methyl-1*H*-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate **76** (10.5 mg, 1.0 Eq) and HATU (14.4 mg, 1.10 Eq) were added to the crude material and the reactants were secured solved in dry DMF (0.80 mL) under Argon atmosphere. Then DiPEA (18 µL, 3.0 Eq) was added and the reaction mixture was stirred at RT while being screened with TLC. After 1 h the mixture was diluted between EA/HCl (0.1 M) (15 mL each) and the aqueous phase was extracted with EA (2x15 mL) (the phase separation was accelerated by addition of solid NaCl). The organic phases were combined and dried over Na_2SO_4 before solvents were removed u.r.p.. The crude material was directly used in final deprotection.

c) Adapted from[11]: The crude product and $Pd(PPh_3)_4$ (3.2 mg, 0.08 Eq) were added to a flask under Argon atmosphere and solved in dry THF (0.70 mL). Aniline (19 µL, 6.0 Eq) was added and the reaction was stirred at RT for 3 h while being screened with TLC. Solvents were removed u.r.p. and the crude product was purified by basic HPLC (method 1). A pure and a mixed product fraction was obtained, the latter containing aniline. Solvents were removed from the pure fraction by lyophillization over 5 days, yield: 10.1 mg, 36% o3s. This fraction was used for MIC- and ADME-testing.

The mixed fraction (~30 mL solution) was carefully acidified with HCl (1 M, 1.0 mL) to adjust the pH to ~3-4. A precipitate was formed that was extractable with EA (10 mL). The aqueous phase was extracted again with EA (2x10 mL) and the combined organic

phases were washed with brine before removing solvents u.r.p.. The residue was extracted from the solid using DMSO-*d6*. The DMSO was then removed u.r.p. at 60°C u.r.p. . The material was purified again by preparative HPLC (method 1) and lyophillized to give 6.9 mg additional compound. The identity of both fractions was checked by LCMS.

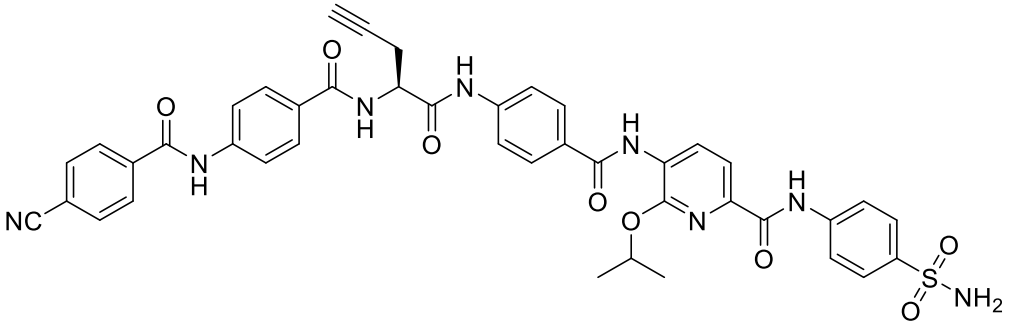
NMR data of first fraction:

^{1}H NMR (500 MHz, DMSO): δ = 12.79 (br s, 1H), 12.49 (br s, 1H), 10.82 (br, 1H, H-NCO), 10.74 (s, 1H, H-NCO), 10.14 (s, 1H, H-NCO), 8.97 (d, *J*=2.7, 1H, H-$C_{Ar,C}$-$N_{Ar,C}$), 8.63 (s, 1H, H-NCO), 8.32 (dd, *J*=8.5, 2.4, 1H, H-$C_{Ar,C}$-$C_{Ar,C}$-NH), 8.18 (d, *J*=8.5, 1H, H-$C_{Ar,C}$-$C_{Ar,C}$-$N_{Ar,C}$), 8.14 (d, *J*=1.8, 1H, H-$C_{Ar,A}$), 8.07 – 7.99 (m, 1H, H-$C_{Ar,D}$), 7.95 (d, *J*=8.9, 2H, H-C_{Ar}), 7.90 – 7.81 (m, 3H, H-$C_{Ar,D}$, H-C_{Ar}), 7.76 (d, *J*=8.5, 1H, H-$C_{Ar,A}$), 7.65 (dd, *J*=8.4, 1.5, 1H, H-$C_{Ar,A}$), 5.61 (ddd, *J*=17.1, 10.5, 9.0, 1H, H-C=CH_2), 5.30 (dd, *J*=17.1, 1.8, 1H, H_aH_bC=C), 5.09 (dd, *J*=10.2, 2.1, 1H, H_aH_bC=C), 4.79 – 4.65 (m, 1H, H-C_{iPr}O), 3.89 (s, 3H, H_3C-N), 2.56 (s, 6H, 3x $H_2C_{bicyclo}$), 2.39 (q, *J*=8.9, 1H, H-$C_{cycloprop.}(C)_3$), 1.94 – 1.87 (m, 1H, $H_aH_bC_{cycloprop.}$), 1.33 (dd, *J*=6.2, 1.9, 6H, $(H_3C)_2$C), 1.30 – 1.21 (m, 1H, $H_aH_bC_{cycloprop.}$).

^{13}C NMR (126 MHz, DMSO): δ = 170.1 (C=O), 168.7 (C=O), 168.5 (C=O), 167.0 (C=O), 161.2 (C=O), 154.2 (C(-N)(=N)), 143.6 ($C_{Ar,C}$), 142.4 (C_{Ar}), 141.4 ($C_{Ar,A}$), 140.0 (H-$C_{Ar,C}$-$N_{Ar,C}$), 139.0 ($C_{Ar,A}$), 138.5 ($C_{Ar,C}$), 136.5 ($C_{Ar,D}$), 134.2 (HC=CH_2), 130.3 (C_{Ar}-H), 127.9 (H-$C_{Ar,C}$-$C_{Ar,C}$-N), 126.0 (C_{Ar}), 125.6 ($C_{Ar,A}$-H), 123.7 ($C_{Ar,A}$-H), 123.6 ($C_{Ar,D}$-H), 122.8 (H-$C_{Ar,C}$-$C_{Ar,C}$-$N_{Ar,C}$), 120.5 (C_{Ar}-H), 120.1 (CN), 117.5 ($C$$H_2$=CH), 112.1 ($C_{Ar}$), 111.6 ($C_{Ar,A}$-H), 107.5 (br, C_{Ar}), 103.7 ($C_{Ar,A}$-CN), 74.3 (C_{iPr}-H), 53.2 (3x $C_{bicyclo}H_2$), 41.8 ($C(C)_3$(N)), 40.8 (C_{bicylo}), 35.0 (C_{bicylo}), 31.7 ($C_{cycloprop.}$-H), 30.9 (CH_3-N), 22.4 ((CH_3)$_2$C), 21.2 ($C_{cycloprop.}H_2$).

HRMS (ESI): calculated for $[M+H]^+$: 809.3042, found: 809.3041.

Synthesis of pyridine (ring D) sulfonamide (ring E) cystobactamid ***(006c)***



a) Adapted from[11]: The *N*-Fmoc-protected amino acid-CDE-fragment **154** (59.6 mg, 54.8 µmol, 1.00 Eq) was solved in dry MeCN (0.82 mL) under Argon atmosphere and $HNEt_2$ (85 µL, 15 Eq) was added. The mixture was stirred at RT for 2 h while being screened with LCMS. After 2 h the solvent was removed from the mixture u.r.p. and by coevaporation with MeCN (5x) and the crude material was dried 1 h u.r.p..

b) Adapted from[11, 96]: 4-(4-cyanobenzamido)benzoic acid **10** (16.1 mg, 1.10 Eq) and HATU (22.9 mg, 1.10 Eq) were added to the crude material and all reactants were solved in dry DMF (0.55 mL) under Argon atmosphere. DiPEA (28 µL, 3.0 Eq) was added and the reaction was stirred at RT while being screened with LCMS. After 45 min the mixture was diluted between EA/HCl (0.1 M), (20 mL each) and brine (10 mL). The aqueous phase was extracted with EA (3x15 mL) before drying the combined organic phases over Na_2SO_4 and removing solvents u.r.p.. Further drying was tried using coevapoartion with several solvents but was unsuccessful. The crude material was directly used in the next step.

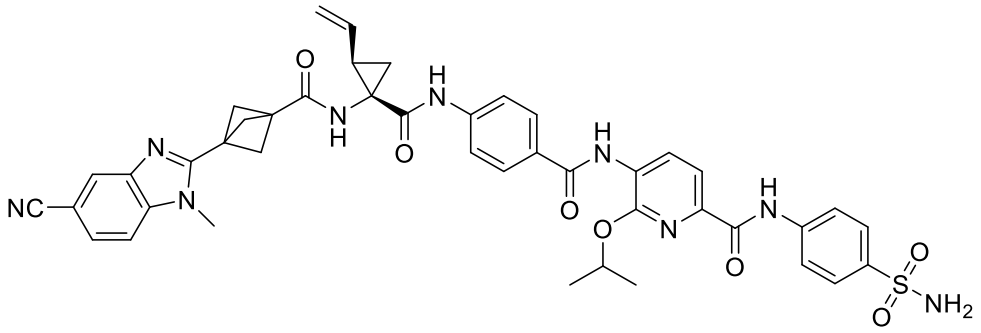
c) The material was suspended in dry DCM (0.82 mL) under Argon atmosphere and TFA (0.21 mL, 50 Eq) was added dropwise. The reaction mixture was stirred at RT for 2 h showing a color change to dark purple after ~1 h. After monitoring by LCMS the mixture was diluted with DCM and solvents were removed u.r.p. and by coevaporation with DCM (3x). Since purification using acidic preparative HPLC (method 1) was unsuccessful, the impure fractions were combined again and purified on normal phase column by FCC (silica mass: 4 g, solid loading, cyclohexane/EA, 70/30->50/50->30/70->0/100->acetone, 100%->MeOH, 100%). (Note: Elution was not observed until eluting with acetone). This material and pure fractions from the first purification were combined, solvents were removed u.r.p. and the solid residue was suspended in H_2O, supported by ultrasound. After lyophillization of the solution (over 5 d) a beige solid was obtained, yield: 17.4 mg, 39%, purity: 93 mol% (UV/LCMS).

^{1}H NMR (500 MHz, DMSO): δ = 10.76 (s, 1H, H-NCO), 10.67 (s, 1H, H-NCO), 10.30 (s, 1H, H-NCO), 9.43 (s, 1H, H-$N_{D\text{-}ring}$CO), 8.83 (d, *J*=7.6, 1H, H-NCO), 8.53 (d, *J*=8.1, 1H, H-$C_{Ar,D}$), 8.14 (d, *J*=8.5, 2H, H-C_{Ar}), 8.05 (d, *J*=8.4, 2H, H-C_{Ar}), 8.02 (d, *J*=8.9, 2H, H-C_{Ar}), 7.98 – 7.93 (m, 4H, H-C_{Ar}), 7.91 (d, *J*=8.9, 2H, H-C_{Ar}), 7.86 – 7.82 (m, 4H, H-C_{Ar}), 7.80 (d, *J*=8.1, 1H, H-$C_{Ar,D}$), 7.31 (s, 2H, H_2N), 5.80 (hept, *J*=6.0, 1H, H-C_{iPr}O), 4.85 – 4.77 (m, 1H, H-C(NH)$(C)_2$), 2.94 (t, *J*=2.7, 1H, HCC), 2.87 – 2.72 (m, 2H, H_2C(CCH)), 1.42 (d, *J*=6.1, 6H, $(H_3C)_2$C).

^{13}C NMR (126 MHz, DMSO): δ = 169.8 (C=O), 165.9 (C=O), 164.9 (C=O), 164.5 (C=O), 162.5 ($C_{Ar,D}$-C=O), 153.0 ($C_{Ar,D}$-OiPr), 142.4 (C_{Ar}), 141.7 (C_{Ar}), 141.1 (C_{Ar}), 141.0 ($C_{Ar,D}$), 139.1 (C_{Ar}), 138.7 (C_{Ar}), 132.5 (C_{Ar}-H), 130.0 ($C_{Ar,D}$-H), 128.9 (C_{Ar}), 128.7 (C_{Ar}-H), 128.7 (C_{Ar}-H), 128.5 (C_{Ar}-H), 128.4 (C_{Ar}), 126.6 (C_{Ar}-H), 126.1 ($C_{Ar,D}$), 120.1 (C_{Ar}-H), 119.5 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.3 (CN), 116.3 ($C_{Ar,D}$-H), 114.0 (C_{Ar}), 80.7 (CCH), 73.2 (CCH), 69.5 (C_{iPr}-H), 53.5 (CH-$(C)_2$(N)), 21.8 ((CH_3)$_2$C), 21.5 (CH_2-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 813.2450, found: 813.2450.

Synthesis of new scaffolded pyridine (ring D) sulfonamide (ring E) cystobactamid ***(006d)***



a) Adapted from[11]: The *N*-Fmoc-protected amino acid-CDE-fragment **155** (70.0 mg, 63.6 µmol, 1.00 Eq) was solved in dry MeCN (0.95 mL) under Argon atmosphere and $HNEt_2$ (0.10 mL, 15 Eq) was added. The mixture was stirred at RT while reaction screening by LCMS. After 1.5 h solvents were removed from the mixture u.r.p. and by coevaporation with MeCN (4x). The crude product was dried overnight under high vacuum and was directly used in the next step.

b) Adapted from[11, 96]: potassium 3-(5-cyano-1-methyl-1*H*-benzo[d]imidazol-2-yl)bicyclo[1.1.1]pentane-1-carboxylate **76** (21.4 mg, 1.10 Eq) and HATU (26.6 mg, 1.10 Eq) were added to the crude product. Dry DMF (0.65 mL) was added under Argon atmosphere, followed by DiPEA (32 µL, 3.0 Eq). The reaction was stirred at RT while being screened with LCMS. After 1 h 20 min the mixture was diluted between EA/HCl (0.1 M),

(20 mL each) and brine (20 mL). The aqueous phase was extracted with EA (2x15 mL) before drying the combined organic phases over Na_2SO_4 and removing solvents u.r.p.. The material was purified by FCC (solid loading, 120x theoretical product mass, DCM/EtOH, 97/3->95/5) to give an off-white solid after solvent removal u.r.p. and coevaporation with $CHCl_3$ (1x), MeCN (2x) and DCM (3x) (66 mg crude).

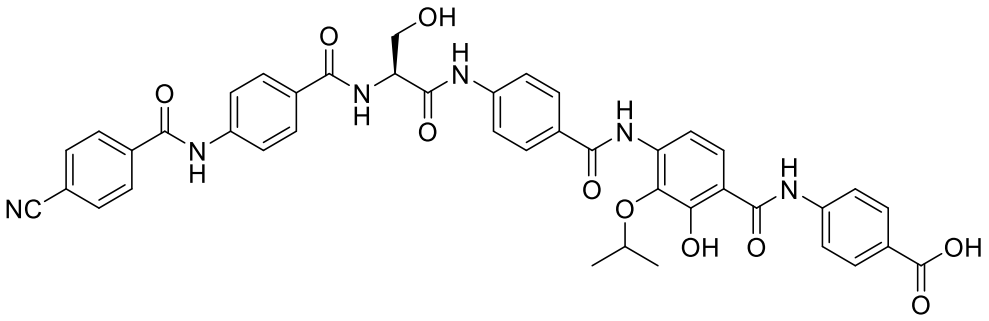
c) Adapted from[11]: The crude product (66 mg, 58 µmol, 1.0 Eq) was solved in dry DCM (0.88 mL) under Argon atmosphere and Anisole (13 µL, 2.0 Eq) was added. Then TFA (0.22 mL, 50 Eq) was added dropwise resulting in a color change from orange to dark purple over ~1 h. After 1 h 45 min and reaction control by LCMS the purple product solution was transferred dropwise into stirred Et_2O (12 mL). The resulting precipitate was filtered off by using pore size 4 filter and the residue was rinsed again with portions of Et_2O (3x3 mL). The off-colorless precipitate was quantitatively transferred from the filter to a flask. Then the product was resuspended in MeCN/H_2O (ca. 2 mL each) and lyophillized over 4 d to give 48.4 mg, 92% o3s.

^{1}H NMR (700 MHz, DMSO): δ = 10.29 (s, 1H, H-NCO), 9.86 (s, 1H, H-N-$C_{Ar,D}$), 9.41 (s, 1H, H-NCO), 8.59 (s, 1H, H-NCO), 8.52 (d, *J*=8.0, 1H, H-$C_{Ar,D}$), 8.14 (dd, *J*=1.4, 0.6, 1H, H-$C_{Ar,A}$), 8.02 (d, *J*=8.9, 2H, H-C_{Ar}), 7.95 (d, *J*=8.9, 2H, H-C_{Ar}), 7.84 (d, *J*=8.8, 2H, H-C_{Ar}), 7.80 (d, *J*=8.0, 1H, H-$C_{Ar,D}$), 7.78 – 7.75 (m, 3H, H-$C_{Ar,A}$, H-C_{Ar}), 7.66 (dd, *J*=8.4, 1.5, 1H, H-$C_{Ar,A}$), 7.30 (s, 2H, H_2N), 5.79 (hept, *J*=6.1, 1H, H-$C_{iPr}O$), 5.57 (ddd, *J*=17.2, 10.4, 9.0, 1H, <u>H</u>-C=CH_2), 5.29 (dd, *J*=17.0, 1.8, 1H, H_a<u>H_b</u>C=C), 5.08 (dd, *J*=10.3, 2.1, 1H, <u>H_a</u>H_bC=C), 3.90 (s, 3H, H_3C-N), 2.59 – 2.55 (m, 6H, 3x $H_2C_{bicyclo}$), 2.34 – 2.28 (m, 1H, H-$C_{cycloprop.}(C)_3$), 1.90 – 1.85 (m, 1H, <u>H_a</u>$H_b$$C_{cycloprop.}$), 1.42 (dd, *J*=6.2, 1.7, 6H, $(H_3C)_2C$), 1.28 – 1.24 (m, 1H, H_a<u>H_b</u>$C_{cycloprop.}$).

^{13}C NMR (176 MHz, DMSO): δ = 170.0 (C=O), 167.9 (C=O), 164.9 (C=O), 162.5 (C=O), 154.1 (C(-N)(=N)), 153.0 ($C_{Ar,D}$-OiPr), 142.1 (C_{Ar}), 141.1 (C_{Ar}), 141.0 ($C_{Ar,D}$), 140.9 ($C_{Ar,A}$), 139.1 (C_{Ar}), 138.9 ($C_{Ar,A}$), 134.4 (H<u>C</u>=CH_2), 129.9 ($C_{Ar,D}$-H), 128.6 (C_{Ar}-H), 128.5 (C_{Ar}), 126.5 (C_{Ar}-H), 126.1 ($C_{Ar,D}$-), 125.7 ($C_{Ar,A}$-H), 123.5 ($C_{Ar,A}$-H), 120.1 (C_{Ar}-H), 119.9 (CN), 119.2 (C_{Ar}-H), 117.2 (<u>C</u>H_2=CH), 116.2 ($C_{Ar,D}$-H), 111.7 ($C_{Ar,A}$-H), 103.9 (<u>C</u>$_{Ar,A}$-CN), 69.6 (C_{iPr}-H), 53.1 (3x $C_{bicyclo}H_2$), 41.9 (<u>C</u>$(C)_3(N)$), 40.8 (C_{bicylo}), 34.8 (C_{bicylo}), 31.5 ($C_{cycloprop.}$-H), 30.9 (CH_3-N), 21.8 ($(CH_3)_2C$), 20.5 ($C_{cycloprop.}H_2$).

HRMS (ESI): calculated for $[M+H]^+$: 828.2923, found: 828.2924.

Synthesis of serine (central AA) cystobactamid ***(017)***



a) Adapted from[11]: The *N*-Fmoc-protected serine(Trt)-CDE-fragment **156** (57.0 mg, 62.6 µmol, 1.00 Eq) was solved in MeCN (1.0 mL) and $HNEt_2$ (0.10 mL, 15 Eq) was added. The reaction was stirred at RT while being screened by LCMS. After 1 h the solvent was removed u.r.p. and by coevaporation with MeCN (2x). The beige foam was further dried under HV overnight and directly used in the next step.

b) Adapted from[11, 96]: To the crude product, the standard AB-central AA-fragment **26** (18.3 mg, 1.10 Eq) and HATU (26 mg, 1.1 Eq) were added and the mixture was solved in dry DMF (0.65 mL) under Argon atmosphere. DiPEA (32 µL, 3.0 Eq) was added and the reaction was stirred at RT. The reaction solution was directly used in the next step.

c) Adapted from[11]: After 2 h 10 min dry THF (0.65 mL), aniline (17 µL, 3.0 Eq) and $Pd(PPh_3)_4$ (spatula tip) were added to the reaction solution. The reaction was then stirred under Argon atmosphere at RT while being screened by LCMS. After 2 h reaction the mixture was diluted between EA/HCl (0.1 M) (20 mL each) and the organic phase was extracted with EA (2x15 mL). Combined organic phases were dried over Na_2SO_4 and solvents were removed u.r.p.. The material was purified by FCC (solid loading, 100x theoretical product mass, cyclohexane/acetone, 75/25->70/30->60/40) to give a colorless fine solid after solvent removal u.r.p. and coevaporation with cyclohexane/acetone. The purified material (crude mass: 51 mg) was directly used in the next step.

d) Adapted from[11]: The material (51 mg, 57 µmol, 1.0 Eq) was solved in dry DCM (0.85 mL) under Argon atmosphere and anisole (12.4 µL, 2.00 Eq) and TFA (0.22 mL, 50 Eq) were added. The reaction was screened by LCMS and after 2 h the flask was flushed with Ar to concentrate the solution by evaporating DCM. After 3 h 15 min TFA (0.22 mL, 50 Eq) was added again and after 4.5 h the reaction mixture was stirred at 40°C in the opened flask. 15 min later solvents were removed u.r.p., by coevaporation with MeCN and

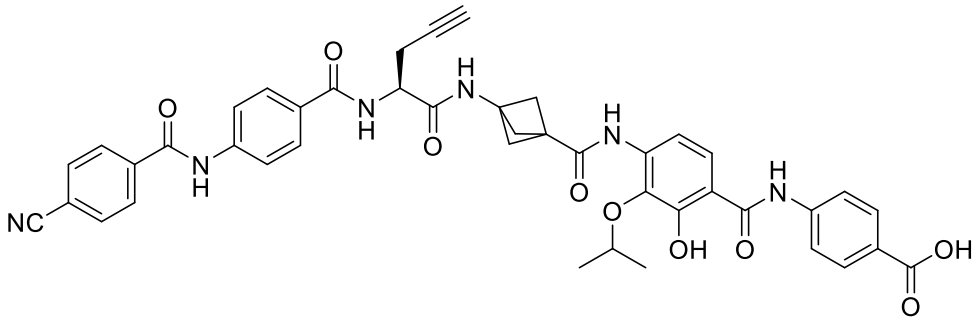
by lyophillization overnight. The material was purified by HPLC (method 1) and the product fractions were lyophillized over 5 d to obtain a colorless fluffy solid, yield: 21.9 mg, 49%, 45% o4s.

^{1}H NMR (500 MHz, DMSO): δ = 12.84 (s, 1H), 12.30 (s, 1H), 10.72 (s, 1H, H-NCO), 10.66 (br s, 1H), 10.46 (s, 1H, H-NCO), 9.41 (s, 1H, H-$N_{D\text{-}ring}$CO), 8.43 (d, *J*=7.3, 1H, H-NCO), 8.13 (d, *J*=8.5, 2H, H-C_{Ar}), 8.05 (d, *J*=8.5, 2H, H-C_{Ar}), 7.99 – 7.94 (m, 6H, H-C_{Ar}), 7.90 (d, *J*=9.0, 2H, H-C_{Ar}), 7.88 – 7.81 (m, 5H, H-C_{Ar}, H-$C_{Ar,D}$), 7.69 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 5.14 (t, *J*=5.7, 1H, HO-CH_2), 4.69 (q, *J*=6.0, 1H, H-C(NH)$(C)_2$), 4.54 (hept, *J*=6.3, 1H, H-C_{iPr}O), 3.89 – 3.79 (m, 2H, H_2C-OH), 1.26 (d, *J*=6.1, 6H, $(H_3C)_2$C).

^{13}C NMR (126 MHz, DMSO): δ = 169.9 (C=O), 168.5 (C=O), 166.9 (C=O), 166.0 (C=O), 164.5 (C=O), 164.2 (C=O), 154.2 ($C_{Ar,D}$-OH), 142.4 (C_{Ar}), 142.1, 141.6 (C_{Ar}), 138.7 (C_{Ar}), 137.0 (C_{Ar}), 136.4 ($C_{Ar,D}$-OiPr), 132.6 (C_{Ar}-H), 130.2 (C_{Ar}-H), 129.2 (C_{Ar}), 128.7 (C_{Ar}-H), 128.4 (C_{Ar}-H), 128.4 (C_{Ar}-H), 128.3 (C_{Ar}), 126.2 (C_{Ar}), 122.9 ($C_{Ar,D}$-H), 120.7 (C_{Ar}-H), 119.5 (C_{Ar}-H), 118.8 (C_{Ar}-H), 118.3 (CN), 114.1 (C_{Ar}), 112.5 ($C_{Ar,D}$), 112.1 ($C_{Ar,D}$-H), 74.8 (C_{iPr}-H), 61.5 (CH_2-OH), 57.1 ($\underline{C}$H-$(C)_2$(N)), 22.3 (($\underline{C}H_3)_2$C).

HRMS (ESI): calculated for $[M+H]^+$: 785.2566, found: 785.2564.

*Synthesis of bicyclopentane (ring C) cystobactamid **(018)***



a) Adapted from[11]: The cystobactamid-precursor **32** (80 mg, 91 µmol, 1.0 Eq) and aniline (25 µL, 3.0 Eq) were solved in dry THF (1.0 mL) and $Pd(PPh_3)_4$ (11 mg, 0.10 Eq) was added under Argon atmosphere. The solution was stirred for 1.5 h at 40°C. After reaction control by LCMS, the reaction mixture was concentrated u.r.p.. The crude material was purified by FCC (solid loading, 100x reactant mass, PE/Acetone, 80/20->75/25->70/30->60/40) to give yellowish crystals (50 mg crude) that were used directly in the next step.

b) The final product was obtained according to the general *t*Bu-deprotection procedure using the crude material (50 mg, 60 µmol, 1.0 Eq), DCM (0.50 mL) and TFA (0.23 mL,

50 Eq). The reaction was conducted at 0°C for 2 h and at RT for 10 min and HPLC method 1 was used, including lyophillization over 5 d. (Note: Precipitation of the product was first tried insuccessfully by pouring a solution of the crude product in DMF into water) Yield: 16.2 mg, 23% o2s.

^{1}H NMR (500 MHz, DMSO): δ = 12.26 (br s, 1H), 10.71 (s, 1H, H-NCO), 10.64 (br s, 1H, H-NCO), 8.79 (s, 1H, H-NCO), 8.75 (s, 1H, H-NCO), 8.52 (d, *J*=8.2, 1H, H-NCO), 8.12 (d, *J*=8.4, 2H, H-C_{Ar}), 8.04 (d, *J*=8.3, 2H, H-C_{Ar}), 7.98 – 7.91 (m, 4H, H-C_{Ar}), 7.88 (d, *J*=8.8, 2H, H-C_{Ar}), 7.82 (d, *J*=8.7, 2H, H-C_{Ar}), 7.78 (d, *J*=8.9, 1H, H-$C_{Ar,D}$), 7.57 (d, *J*=8.8, 1H, H-$C_{Ar,D}$), 4.59 – 4.45 (m, 2H, H-C(NH)$(C)_2$, H-C_{iPr}O), 2.83 (t, *J*=2.6, 1H, H-CC), 2.66 (d, *J*=73.3, 2H, H_2C(CCH)), 2.33 (s, 6H, 3x $H_2C_{bicyclo}$), 1.25 (d, *J*=6.1, 6H, $(H_3C)_2C$).

^{13}C NMR (126 MHz, DMSO): δ = 170.7 (C=O), 168.5 (C=O), 167.3 (C=O), 167.0 (C=O), 165.8 (C=O), 164.6 (C=O), 154.5 (C_{Ar}), 142.1 (C_{Ar}), 141.6 (C_{Ar}), 138.7 (C_{Ar}), 136.4 (C_{Ar}), 135.5 (C_{Ar}), 132.6 (C_{Ar}-H), 130.3 (C_{Ar}-H), 129.2 (C_{Ar}), 128.7 (C_{Ar}-H), 128.5 (C_{Ar}-H), 126.2 (C_{Ar}), 123.1 ($C_{Ar,D}$-H), 120.7 (C_{Ar}-H), 119.6 (C_{Ar}-H), 118.4 (CN), 114.1 (C_{Ar}), 112.5 (C_{Ar}), 111.1 ($C_{Ar,D}$-H), 81.1 (CCH), 74.8 (C_{iPr}-H), 72.8 (CCH), 53.6 (3x $C_{bicyclo}$), 52.3 (CH-$(C)_2$(N)), 44.9 ($C_{bicyclo}$), 38.3 ($C_{bicyclo}$), 22.4 ($(CH_3)_2$C), 21.6 (CH_2-CCH).

HRMS (ESI): calculated for $[M+H]^+$: 783.2774, found: 783.2791.

5.3 Overview of synthetic routes

Table 16: Part I, products of the corresponding reaction are given with their number, x = intermediate, not isolated; - = not performed.

Nick-name (inspired by James Bond)	Dr.No	Stromberg	Kananga	Scara-manga	Largo	Drax	Renard	Khan	Zorin	Koskov	Sanchez	Q	Honey
Generic name	*Pahyno-bactid*	*Suflu-bactid*	*Suklo-bactid*	*Subro-bactid*	*Demepydo-bactid*	*Depydo-bactid*	*Damino-bactid*	*Supy-bactid*	*Combo-bactid P*	*Combo-bactid PS1*	*Combo-bactid PS2*	*Methoxy-bactid*	*Combo-bactid M2*
Type of coupling *Cmpd. Nr.*	**001**	**002**	**003**	**004**	**012**	**013**	**005**	**006**	**006b**	**006c**	**006d**	**007**	**007b**
ring D with E													
$POCl_3$/DiPEA and Zn reduction	-	**132**	**133**	**134**	-	-	-	-	-	-	-	-	-
$(COCl)_2$/DiPEA and Zn reduction	**23**	-	-	-	-	-	-	-	-	-	-	-	-
$(COCl)_2$/Py/DMF (cat.) and Zn reduction	-	-	-	-	**57 and 135**	-	-	-	-	-	-	-	-
$POCl_3$/Py/DMF (cat.) and Zn reduction	-	-	-	-	-	**136**	-	-	-	-	-	-	-
$POCl_3$/Py and Zn reduction	-	-	-	-	-	-	-	-	-	**138**	**138**	-	-
T3P/Py and Zn reduction	-	-	-	-	-	-	**63**	**137**	**137**	-	-	-	-
ring C with D													
with acid chloride and Zn reduction	-	-	-	-	-	-	-	-	-	-	-	**78**	**78**
ring C with DE													
with acid chloride and Zn reduction	**24**	**144**	**27**	**145**	**58**	**146**	**64**	**75**	**75**	**147**	**147**	-	-
others													
CD with ring E, T3P/Py and deprotection	-	-	-	-	-	-	-	-	-	-	-	**79**	**79**
CDE with central AA, T3P/Py	**151**	-	-	-	-	-	-	-	**x**	**154**	**155**	-	**153**
CDE with AB-central AA, T3P/Py	-	**x**	**x**	**x**	**x**	**x**	**x**	**x**	-	-	-	**x**	-
AB with AA-CDE, HATU/DiPEA	**157**	-	-	-	-	-	-	-	**x**	**x**	**x**	-	**x**
Deprotections													
allyl with Pd-Tetrakis	**x**	-	-	-	-	-	-	-	-	-	-	**158**	**x**
*t*Bu or other group sensitive to acid/ TFA	**001**	**002**	**003**	**004**	**012**	**013**	**005**	**006**	**006b**	**006c**	**006d**	**007**	**007b**

Table 17: Part II, products of the corresponding reaction are given with their number, x = intermediate, not isolated; - = not performed.

Nick-name (inspired by James Bond)	Kristatos	Blofeld	Bond	Tanja	Pussy	Klebb	Trevelyan	Carver	Leiter	Domino	Goldfinger
Generic name	*Sibu-bactid*	*Cebylam-bactid*	*Cedeswi-bactid*	*Combo-bactid T1*	*Combo-bactid T2*	*Detrazo-bactid*	*Emethol-bactid*	*Enitro-bactid*	*Combo-bactid I*	*Serino-bactid*	*Cebiclo-bactid*
Type of coupling *Cmpd. Nr.*	**008**	**009**	**010**	**010b**	**010c**	**011**	**014**	**015**	**016**	**017**	**018**
Coupling ring D with E											
$POCl_3$/Py and Zn reduction	-	-	-	-	-	-	-	-	**5**	-	-
T3P/Py and Zn reduction	**139**	-	-	-	-	-	**143**	**117**	-	-	-
$P(OPh)_3$ or $CuSO_4$/ Na-ascorbate (+Zn reduction)	-	-	**140 and 98**	**x**	**x**	**141 and 142**	-	-	-	-	-
Coupling ring C with DE											
with acid chloride and Zn reduction	**148**	-	-	-	-	**149**	**115**	-	**150 and 126**	-	-
via imine formation and Zn reduction	-	**96**	-	-	-	-	-	-	-	-	-
T3P/Py and Zn reduction	-	-	**99**	**99**	**99**	-	-	**x**	-	-	-
others											
CD with ring E, T3P/Py	-	-	-	-	-	-	-	-	-	-	-
CDE with central AA, T3P/Py	-	-	-	**152**	**152**	-	-	-	**128**	**156**	-
CDE with AB-central AA, T3P/Py	**x**	**x**	**x**	-	-	**159**	**x**	**x**	-	-	-
AB with AA-CDE, HATU/DiPEA	-	-	-	**x**	**x**	-	-	-	**x**	**x**	-
AB-central AA with ring C, T3P/DiPEA and deprotection	-	-	-	-	-	-	-	-	-	-	**30b**
AB-central AA-C with DE, T3P/DiPEA	-	-	-	-	-	-	-	-	-	-	**30 and 32**
Deprotections											
allyl with Pd-Tetrakis	**008**	**x**	**x**	**x**	**x**	**011**	**x**	**015**	**016**	**x**	**x**
*t*Bu or other group sensitive to acid/ TFA	-	**009**	**010**	**010b**	**010c**	-	**014**	-	-	**017**	**018**

6 Closing comment

This Thesis was written as part of a collaboration between academia (Helmholtz centers HZI and HIPS, and Leibniz University of Hanover) and an industrial partner (Evotec in France). It can not be overemphasized, how important this type of collaboration is in the field of antiinfectives. Many pharmaceutical companies have narrowed their activities to more lucrative fields as antineoplastic agents, immunosupressants and drugs for cardiovascular diseases. Medicinal academia has adapted their research respectively, to get funding support from industry. To overcome this lack of development, large efforts and support will be required on all levels: federal and state governments, ministries of education, research and health as well as pharmaceutical industry and academia themself.

To further allow a sustainable transfer of knowledge, the current academial system has to be rolled up in terms of allowing scientists to have permanent employment. Nowadays, the latter is almost exclusively reserved for full professors and the so called academic mid-level positions are discontinued. The most important currency or metric of success turned out to be the amount of published papers. But precisely with regard to translational sciences, the exclusive focus of gaining large numbers of publication and high impact will have no future and will destroy people's trust in research.

In times of increasing denial of proven facts and anti-democratic propaganda, communication between science and the public will play a deciding role. The task of scientists is to give people knowledge, not to show each other who is better. Although this may be a naive view, this basic idea should always be taken into account when allocating research funds or setting up a research project.

Acknowledgements

Firstly, I would like to thank Prof. Dr. Mark Brönstrup for providing the great opportunity for generating this doctoral thesis on such an important and interdisciplinary scientific topic. Our discussions were always purposeful, respectful and rational within a reasonable framework. From my own experience I can say that this open-minded scientific working is absolutely not a matter of course. Project progress was always ensured by adequate laboratory equipment. Furthermore, I thank the referees for assessing my thesis.

Particular thanks are owed to Dr. Daniel Kohnhäuser for our daily scientific and dry-humorous discussions in the cystobactamid-project, as well as Dr. Dominik Heimann and Dr. Stefan Saretz as project contributors. It can not be denied that both scientific and social exchange is essential for valuable scientific work but is often underestimated in the scientific community.

Valuable discussion partners of the cystobactamid-project were also Prof. Dr. Andreas Kirschning, Tim, Ruben and Danny at the Leibniz University in Hanover, along with Evotec cooperators from Lyon and Toulouse. For the latter I would like to thank especially Dr. Jean-François Sabuco for his support during my doctoral project as part of the thesis-committee, together with Prof. Dr. Marc Stadler from HZI.

Several more people were so important for an enjoyable and successful daily work and scientific progress:

Dr. Kirsten Harmrolfs, Christel Kakoschke and Esther Surges from the NMR-department. Janetta Coetzee (HIPS) for the MIC-testing. Dr. Katharina Rox, Dr. Jennifer Herrmann (HIPS) for biological assays and PK-studies. Ulrike Beutling for HRMS measurement. Dr. Matthias Göhl for organochemical-synthetic advice. Bettina Mehner and Dr. Werner Tegge for office and scientific organization. Susanne Daenicke and Antje Ritter for laboratory support. I am grateful to the entire CBIO research family for a pleasant and scientific working atmosphere with a great helpfulness. It was also fun and enlightening to supervise my former Bachelor student Mila Le.

I of course also thank my family and friends for their support and my colleagues and friends for their proofreading.

Additionally, a few points and personal details are worth being grateful for. This is an incomplete itemization, a priorization by order is not intended: Thanks to Eugenio for his lessons in Italian culture, to Julia, Carsten and Patrick in South-German culture, to Ravi and Adi in Indian culture, food and humour, to our 'Golf' in British culture. Karo and Bettina Mehner are faithful and charming companions and good souls. Simon and Heike always had open ears and mouths for diverse scientific topics, Hannah and Tamara a vivid and charming way to discuss science. Thomas has enviable photo-shop skills. Travis has top English wording skills and a huge stamina to swim all the way from Australia to Europe. Anna at least as much to walk from Flensburg or swim from Ireland to Braunschweig. Raimo is and Werner was an experienced calm anchor in the CBIO. Matthias is a master of synthetic simplification and Daniel a master of slothification.

Attachment

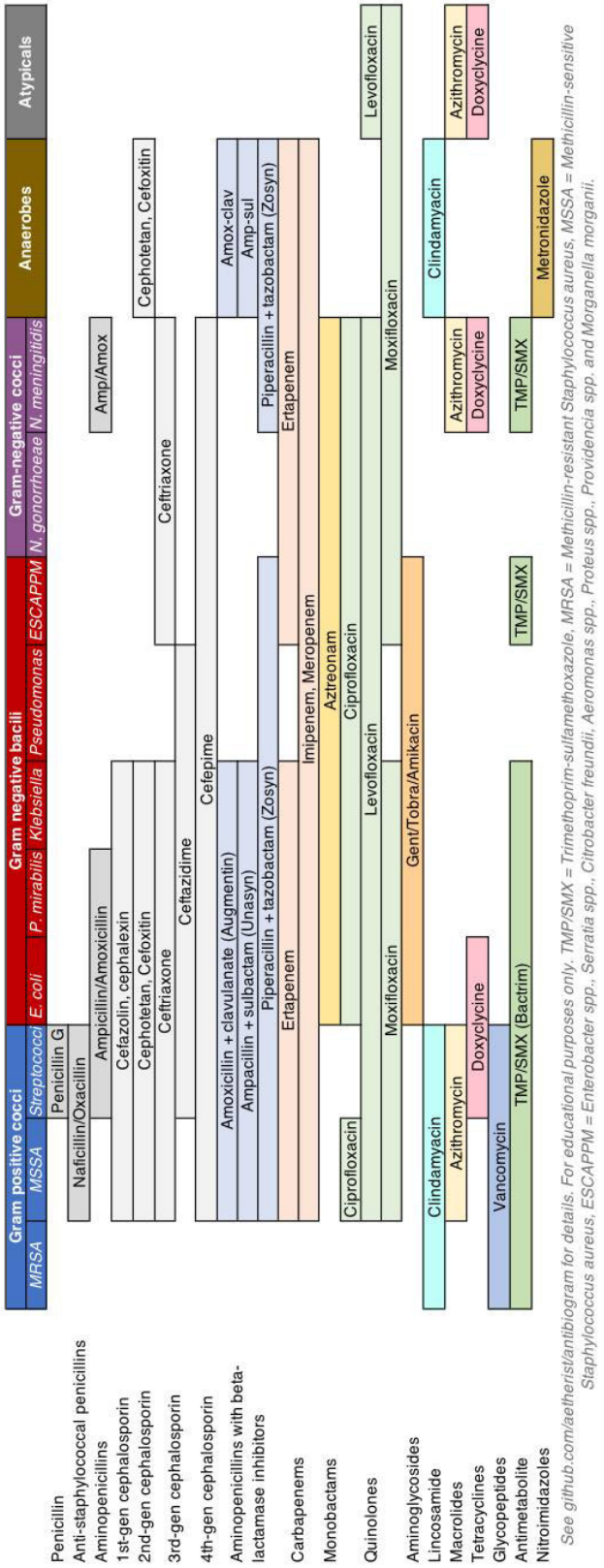


Figure 102: Antibiogram for common bacterial pathogens (https://github.com/aetherist/antibiogram, accessed: 18.08.2023) There may be already isolated resistances that are not included here.

Table 18: Screened reaction condition for the isobutenylation by lithiation.

entry	Li-reagent (2.1-2.2 Eq)	TMEDA [Eq]	solvent	T [°C] for lithiation	T [°C] for substitution	electro-phile	outcome
1	*t*BuLi	-	hexane/ MTBE	-78	RT	DMF (1.1 Eq)	low product formation
2	*t*BuLi	-	THF	-78 - -20	-78 - -20	DMF (5.5 Eq)	product formation
3	*t*BuLi	-	THF	-78 - -20	-78 - -20	DMF (5.5 Eq)	product formation
4	*t*BuLi	2.2	THF	-78	RT	DMF (5.5 Eq)	no reaction
5	*n*BuLi	2.2	THF	-78 - -20	-20	DMF (5.5 Eq)	~40% product formation
6	*n*BuLi	2.2	THF	-40 - 0	0	DMF (5.5 Eq)	mainly decomposition
7	*s*BuLi	2.2	THF	-78	-78 - RT	DMF (5.0 Eq)	no product formation
8	*t*BuLi	-	THF	-78 - -20	-78 - -20	MAB (1.0 Eq)	product formation
9	*t*BuLi	-	THF	-78 - -20	-78 - -20	MAB +C (1.0 Eq)	~50% higher product formation, cleaner reaction
10	*t*BuLi	-	THF	-78 - -20	-78 - -20	MAB +C (1.0 Eq)	upscaled batch, 48% yield

MAB = methallyl bromide; MAB+C = prior to addition of MAB a solution of CuCN (1.1 Eq) and LiCl (2.2 Eq) in THF was added.

Table 19: Conditions for HPLC method 1. A binary eluent system of water with 10 mM NH_4HCO_3 additive (A) and ACN without additive (B) was used. Column: Phenomenex Luna 5µm C18(2) 100A, 250x21.2 mm (00G-4252-P0-AX)

t [min]	Flow [mL/min]	B [%]
0.0	8.0	10.0
5.0	10.0	17.5
40.0	10.0	70.0
45.0	10.0	95.0
50.0	10.0	95.0
55.0	10.0	10.0
60.2	0.1	10.0

Table 20: Conditions for HPLC method 2. A binary eluent system of water with 10 mM NH_4HCO_3 additive (A) and ACN without additive (B) was used. Column: Phenomenex Luna 5µm C18(2) 100A, 250x21.2 mm (00G-4252-P0-AX)

t [min]	Flow [mL/min]	B [%]
0.0	8.0	10.0
5.0	10.0	17.5
50.0	10.0	70.0
52.0	10.0	95.0
60.0	10.0	95.0
62.0	10.0	10.0
68.0	0.1	10.0

Table 21: Conditions for HPLC method 3. A binary eluent system of water with 10 mM NH_4HCO_3 additive (A) and ACN without additive (B) was used. Column: Phenomenex Luna 5µm C18(2) 100A, 250x21.2 mm (00G-4252-P0-AX)

t [min]	Flow [mL/min]	B [%]
0.0	7.0	10.0
5.0	7.0	17.5
60.0	7.0	70.0
70.0	7.0	95.0
79.0	7.0	95.0
80.0	7.0	10.0
90.0	0.1	10.0

Table 22: Conditions for HPLC method 4. A binary eluent system of water with 10 mM NH_4HCO_3 additive (A) and ACN without additive (B) was used. Column: Phenomenex Luna 5µm C18(2) 100A, 250x21.2 mm (00G-4252-P0-AX)

t [min]	Flow [mL/min]	B [%]
0.0	8.0	10.0
5.0	10.0	17.5
40.0	10.0	70.0
50.0	10.0	95.0
55.0	10.0	95.0
60.0	10.0	10.0
65.2	0.1	10.0

Table 23: Conditions for HPLC method 5. A binary eluent system of water with 0.1% HCOOH additive (A) and ACN with 0.06% HCOOH additive (B) was used. Column: Phenomenex Luna 5 µm C18(2) 100A, 250x21.2 mm (00G-4252-P0-AX)

t [min]	Flow [mL/min]	B [%]
0.0	10.0	30.0
5.0	10.0	30.0
10.0	10.0	40.0
65.0	10.0	60.0
75.0	10.0	60.0
80.0	10.0	100.0
90.0	10.0	100.0
95.0	10.0	10.0
105.0	0.5	10.0

Table 24: Conditions for LCMS method 6. A binary eluent system of water with 0.1% HCOOH additive (A) and ACN with 0.06% HCOOH additive (B) was used. Column: Phenomenex Gemini 3 µm NX-C18 110A, 50x2 mm (00B-4453-B0)

t [min]	Flow [mL/min]	B [%]
0.0	1.5	5.0
3.0	1.5	95.0
4.5	1.5	95.0
4.5	1.5	5.0
5.0	1.5	5.0

Further supplementary information can be found in the DVDs attached to this thesis.

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Curriculum vitae

Personal data

Name	Moritz Dominik Stappert
Born	20.06.1994 in Aachen
Nationality	DE

Work experience and education

Since 04/2020	**LUH and Helmholtz Centre for Infection Research** doctoral researcher (Prof. Dr. Mark Brönstrup)
10/2016-06/2019	**Master of Science RWTH Aachen University (Chemistry)** Specialization in synthesis/ bioactive compounds and catalysis Master thesis at DWI Leibniz-Institute for Interactive Materials in Aachen and at RWTH (Prof. Dr. Herrmann). Research internship at Uniklinikum Aachen (Institute of Physiology)
10/2013-09/2016	**Bachelor of Science RWTH Aachen University (Chemistry)** Bachelor thesis at the Institute for Organic Chemistry at RWTH (Prof. Dr. Weinhold).
2004-2013	**Einhard Gymnasium Aachen** Abitur

Other skills

Foreign languages	Fluent in written and spoken English
IT experience	ChemOffice, Origin, MNova, Search tools (SciFinder, Reaxys, etc.), Python, MSOffice, Bruker Compass, and further
Others	Member of the German Chemical Society (GDCh) and VAA–Chemistry Managers (VAA). Speaker of the HZI-doctoral initiative/representation (Doit) 2023

Publications

Patents:	‘Novel cystobactamid derivatives’, patent application filed 2023.
Conference contributions:	Poster presentations at
	‘Frontiers in Medicinal Chemistry’, 2023, Vienna
	‘Helmholtz Drug Discovery Conference’, 2023, Braunschweig.