Structural optimization and photopharmacological studies of the cystobactamids class of natural products

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Zusammenfassung

Es besteht aktuell ein dringender Bedarf neue Antibiotika zu entdecken, die speziell gegen Gramnegative Bakterien aktiv sind, da diese die schlimmste Bedrohung für die öffentliche Gesundheit darstellen. In der Vergangenheit war die Natur die Hauptquelle für antibiotische Strukturen und sie kann auch immer noch neue chemische Leitstrukturen liefern.

Cystobactamide sind neue Naturstoffprodukte, die aus Myxobakterien isoliert wurden. Sie verfügen über ein oligomeres Gerüst, das sich aus Resten der para-Aminobenzoesäure zusammensetzt und verfügen über eine bemerkenswerte Aktivität gegen klinisch relevante Gramnegative Bakterien.

In dieser Dissertation wird eine medizinisch-chemische Untersuchung dieser neuen Klasse von Naturstoffen präsentiert, wobei das Cystobactamid 861-2 als Leitstruktur diente.

Um die verschiedenen Einheiten des Moleküls zu adressieren und um eine weite Bandbreite an Diversifikation einzufügen, wurden multiple synthetische Zugänge zu den Cystobactamiden etabliert. Insgesamt wurden mehr als 50 Derivate synthetisiert, die es erlaubten, extensive Struktur-Aktivitätsbeziehungen zu etablieren.

Im Rahmen dieser Arbeit wurden Cystobactamide mit deutlich verbesserten antibakteriellen Eigenschaften identifiziert. Bemerkenswerterweise konnte auch das Aktivitätsspektrum auf weitere hoch relevante Gramnegative Bakterien wie *K. pneumoniae* und *E. cloacae* erweitert werden. Weiterhin wurden verschiedene Analoga mit einer Aktivität gegen *P. aeruginosa* synthetisiert und die antimikrobielle Potenz gegen ausgewählte Pathogene wie *A. baumannii* wurde ebenfalls verbessert.

Weiterhin wurden zwei mögliche bakterielle Resistenzmechanismen untersucht. Es konnte gezeigt werden, dass das Protein Tsx die Aufnahme der Cystobactamide nicht beeinflusst und dass die hydrolytische Instabilität gegenüber der Endopeptidase AlbD durch eine strukturelle Innovation potentiell umgangen werden könnte.

Zuletzt wurden photoschaltbare Cystobactamide mit jeweils einer N- oder C-terminalen Modifikation synthetisiert. Diese photoresponsiven Antibiotika erbrachten interessante Informationen über den Bindungsmodus der Cystobactamide an ihr Zielenzym.

Zusammenfassend wurde das chemische Gerüst des Cystobactamid-Naturstoffs optimiert und dessen Aktivität gegen Gramnegative Bakterien verbessert. Es konnten essentielle medizinisch-chemische Struktur-Aktivitätsbeziehungen definiert werden. Dies bestätigt und unterstreicht das große Potential dieser Naturstoffklasse, um neue Antibiotika für die Zukunft hervorzubringen.

Schlagworte: Cystobactamiden, Antibiotika, medizinische Chemie, organische Chemie.

Abstract

There is an urgent need to discover novel antibiotics, particularly with activity against Gram-negative bacteria that are the most threatening for public health. In the past, nature has been the main source of antibiotic scaffolds and can still provide novel chemical architectures. Cystobactamids are novel natural products isolated from *Myxobacteria* that possess an intriguing oligomeric scaffold composed of para-aminobenzoic acid derived moieties and show remarkable activity against clinically relevant Gram-negative bacteria.

This thesis details a medicinal chemistry investigation of this novel natural product class based around cystobactamid 861-2. Multiple synthetic routes to the cystobactamids were established in order to prepare analogues with variations at different sites of the molecular scaffold. In sum more than 50 derivatives were synthesized and extensive structure-activity relationships were established. Cystobactamids with clearly improved antibacterial properties were identified from this study. Notably, the spectrum coverage of the class was extended to highly relevant Gram-negative bacteria such as *K. pneumoniae* and *E. cloacae*. Furthermore, several analogs active against *P. aeruginosa* were synthesized and the antimicrobial potency was enhanced against selected pathogens such as *A. baumannii.*

Additionally, two possible bacterial resistance mechanisms were investigated. It was demonstrated that the protein Tsx does not affect the uptake of the cystobactamids and that the hydrolytic instability determined by the endopeptidase AlbD can potentially be overcome with a structural innovation, replacing the critical amide bond with a triazole.

Finally, photoswitchable cystobactamids were synthesized with either the N- or C-terminal side of the molecule modified. These photoresponsive antibiotics suggested intriguing information regarding the binding mode of the cystobactamids with their target enzyme.

To sum up, the chemical scaffold of the cystobactamids natural product was optimized and the activity against Gram-negative bacteria was enhanced. Essential structure activity relationships have been defined. This confirmed and highlighted the great potential of this class of natural products to yield novel antibiotics for the future.

Keywords: Cystobactamids, antibiotics, medicinal chemistry, organic chemistry.

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1 INTRODUCTION

1.1 CHEMICAL INNOVATION IN ANTIBACTERIAL DRUG DISCOVERY

The so called "antibiotics era" started in the 30s with the discovery of Penicillin G[1] and the sulfonamides[2]. In both cases, serendipity played a pivotal role. Different other classes followed shortly afterwards such as tetracylines, macrolides and cephalosporins. The molecular targets of these chemical entities are bacterial biochemical pathways which span from the cellular peripheral cell wall biosynthesis to the deeper protein synthesis and DNA replication. An overview of the selected established antibiotics classes with general information regarding their discovery and molecular target is given in the image below.

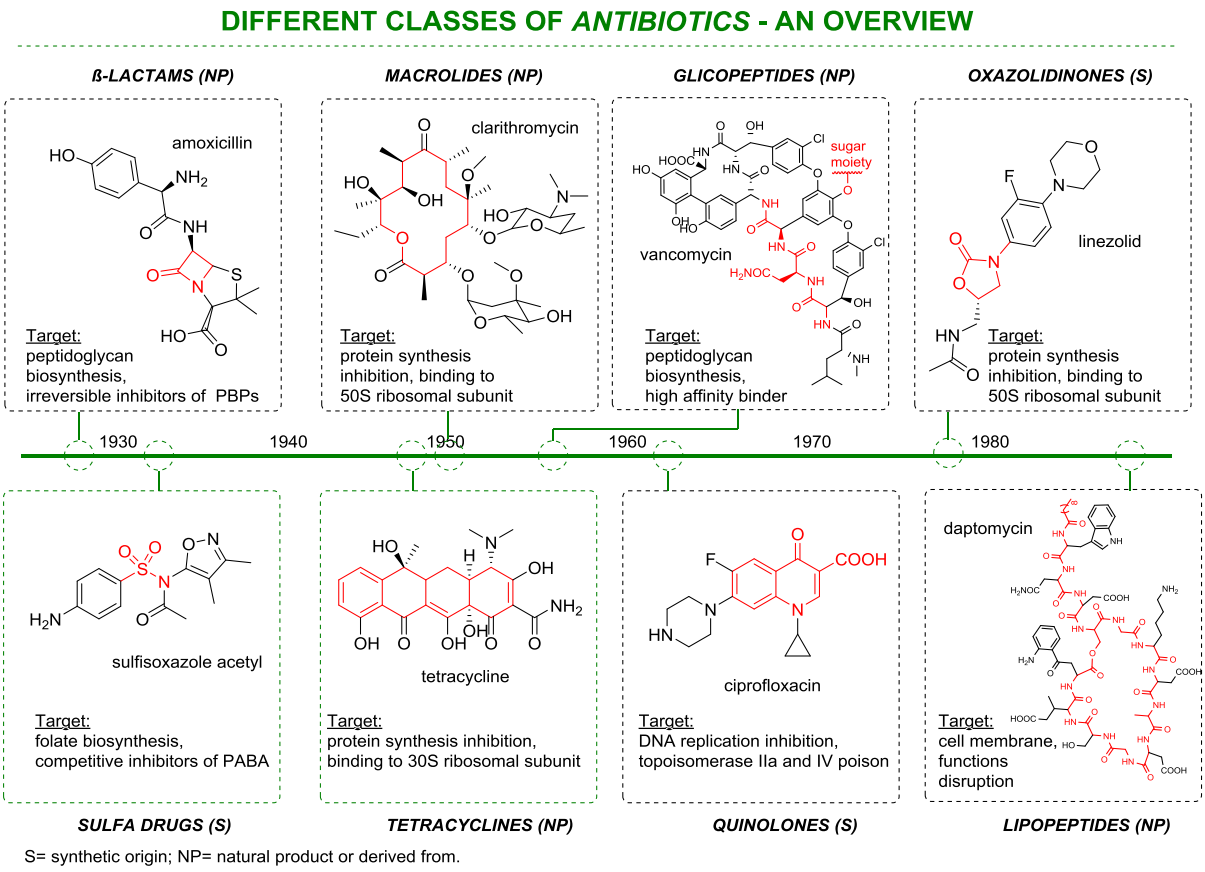


Figure 1: Representation of the selected antibiotics classes with general information, image adapted from[3].

The armamentarium of antibiotics can be classified based on their origin in two major sub-groups. On one side, we can find molecules which are or derive from natural products and on the other side compounds of synthetic origin.

In all cases, after the discovery of the first-in-class molecule, medicinal chemistry efforts led to further generations within the class, yielding antibiotics with improved properties such as broadened spectrum, better pharmacokinetic properties and avoidance of bacterial resistance.

Thanks to their reactive moiety the ß-lactam antibiotics are covalent inhibitors that target the penicillin binding proteins (PBPs). These enzymes are DD-transpeptidases which have the function to cross-link the peptide side chains of peptidoglycan[4].

Carbapenems are the most active compounds within the ß-lactam class against Gram-negative bacteria. Their discovery goes back to the late 60s, when the search for ß-lactamase inhibitors led to the isolation of the natural product thienamycin[5].

Noteworthy, they possess two important structural differences compared to the other ß-lactams, which have a significant influence on their antimicrobial properties. These are the alkyl side chain and the stereochemistry of the four membered ring. The lead scaffold of thienamycin was optimized in order to decrease the chemical instability given by the primary amino group, increase the potency and reduce the instability in vivo[6] (Fig. 2).

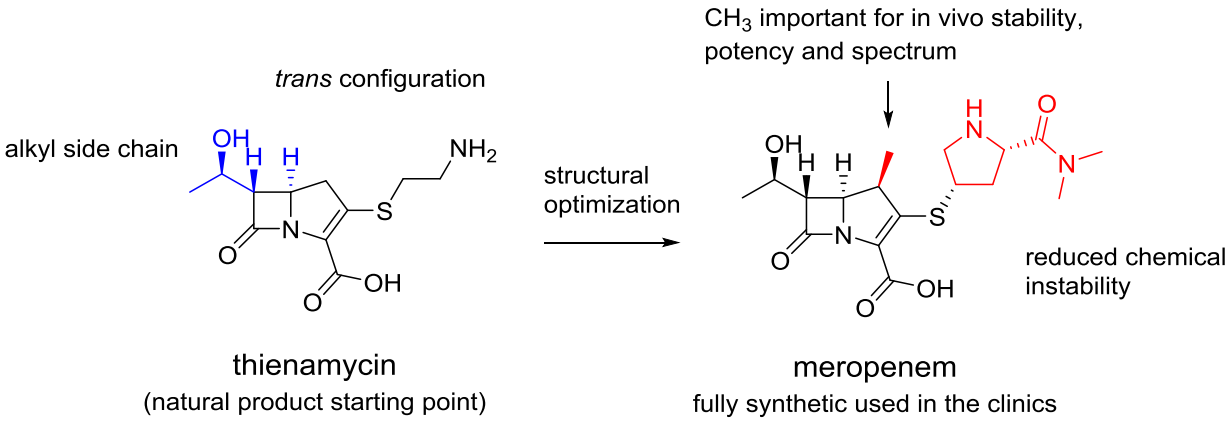


Figure 2: Chemical evolution of carbapenems.

After the discovery of a natural product, its structural optimization can follow two ways[7]. On one hand this can be achieved by semisynthesis and on the other hand by total synthesis. There are two important factors that affect the choice of the strategy to pursue. One is the availability of the natural product by fermentation and the other one the feasibility of the synthesis, which strongly depends on the structural complexity. Via semisynthesis the possible number of modifications is limited, since it depends on the tolerance of the functional groups present on the molecule. While total synthesis enables potential access to derivatives that cannot be obtained with the former approach. The ideal synthesis should be practical and scalable.

A great example, where both approaches have been used, comes from the tetracycline (TC) antibiotics[8,9]. They are protein synthesis inhibitors, targeting the 30S subunit of the ribosome, thus preventing the binding of aminoacyl-tRNA.

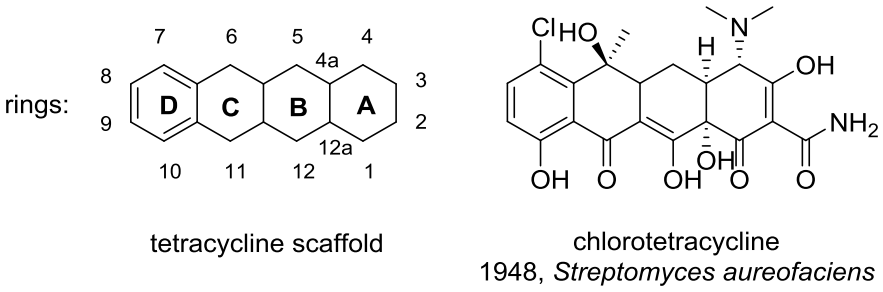


Figure 3: “Naked” tetracycline scaffold and first, naturally occurring, isolated TC.

In 1952 L. Conover discovered that the Cl-C bond of chlorotetracycline could be selectively cleaved under hydrogenolytic conditions to obtain tetracycline (natural product itself)[10]. At first glance this finding might not appear as significant as it actually was. In fact, tetracycline represents the first antibiotic obtained via semisynthesis. At the time, this had a seismic effect on the way of thinking of the scientists. It showed that natural products do not only represent a source of new antibiotics by themselves, but they can also be considered the starting point for the development of molecules with improved drug-like and antibacterial properties.

To highlight the importance and the potential of the semisynthetic approach, a major improvement within the TC class was obtained with the synthesis of 6-deoxytetracyclin[11 12]. It was found that the C-O bond at the C6 could be also cleaved under hydrogenolytic

conditions. Derivatives lacking the –OH are more stable under both acidic and basic conditions, therefore this finding opened new chances for further semisynthetic modifications. This culminated in the synthesis of minocycline (1967)[13 14], a derivative with even broader spectrum and restored activity against some TC-resistant staphylococci.

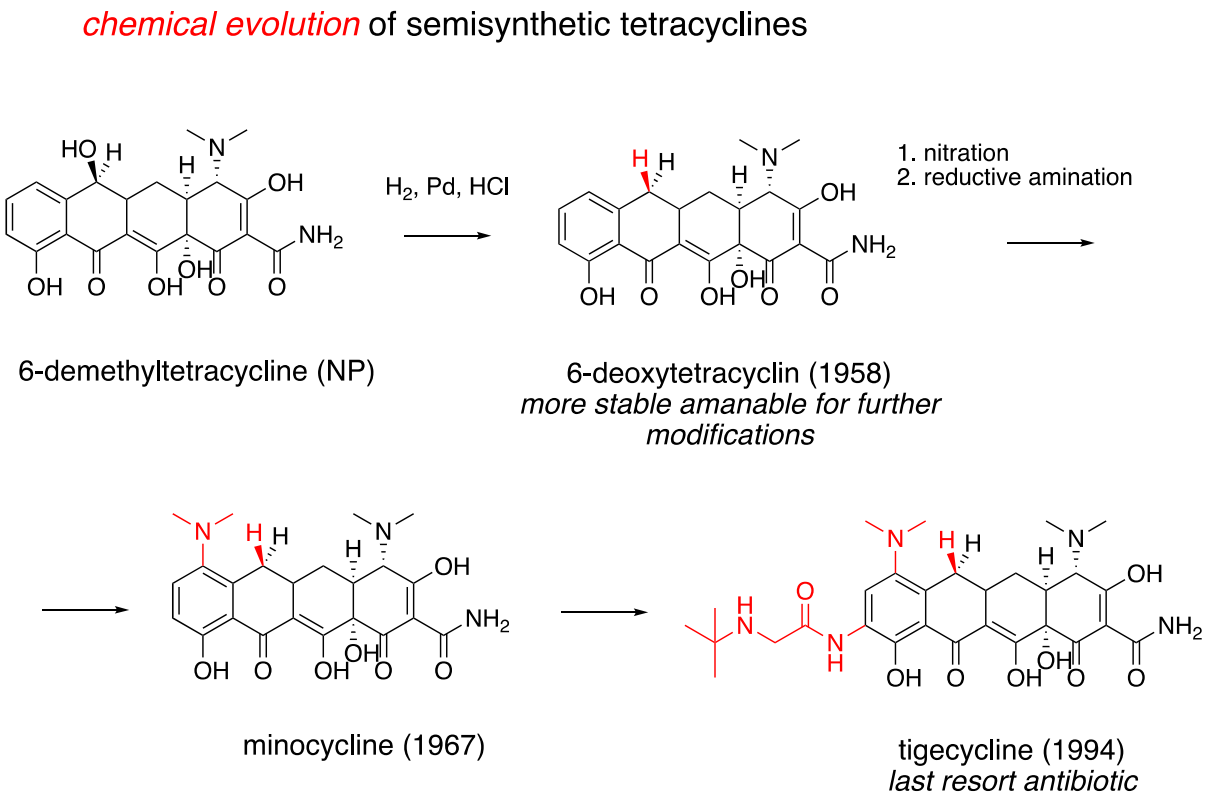


Figure 4: Relevant steps of the semisynthetic modifications that led to the glycylcyclines.

More than 20 years later, the glycylcyclines were discovered (Fig. 4). Among these tigecycline was obtained[15,16], an antibiotic with greatly extended spectrum especially against TC-resistant bacteria. Together with colistin[17,18], it is a last line antibiotic, the last therapeutic option when all other classes fail.

The first total syntheses of tetracycline antibiotics were reported in the 60S, but weren´t practical synthetic routes. In this regard, a great advancement came from the work of A. Myers. He and coworkers reported a synthesis that relies on a modular strategic assembly of two major building blocks[9].

The older syntheses of tetracyclines[19 20 21] employed strategies to build the molecule from the left to right side (D→A approach), therefore they did not offer much space for the synthesis of new derivatives. The new synthesis based instead on a "D+AB approach" and

opened a much broader chemical space for diversification (Fig. 5). Over the past decade, more than 3000 novel, fully synthetic tetracyclines were synthesized. Out of this pool, the most advanced molecule in clinical trials is eravacycline[22].

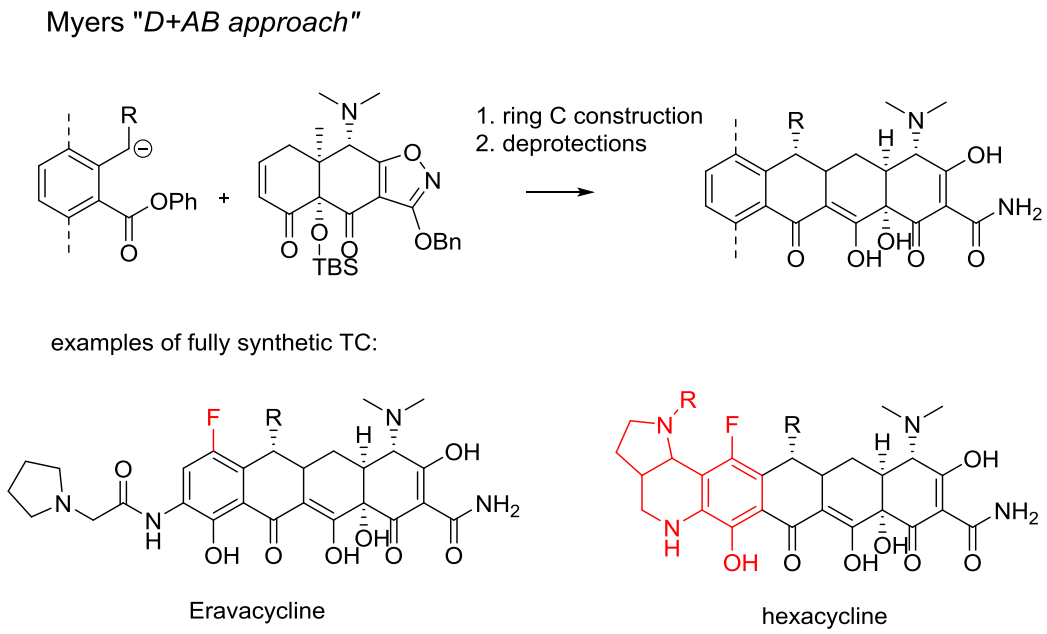


Figure 5: Myers approach used for the synthesis of the TC and selected examples for giving the idea of the broad range of possibilities for modifications of the D ring.

A key core structure of more than 25 antibiotics approved by the FDA is the one of the quinolones[23]. The first approved drug of the class is nalidixic acid, which has been developed at Sterling Drug. As for other antibacterial classes, serendipity played a pivotal role in the discovery of the hit structure. A byproduct of the production process of chloroquine was found to possess moderate antibacterial properties (Fig. 6). This finding triggered the medicinal chemistry program that led to the discovery of naldixic acid.

The Sterling patent that covers nalidixic acid was filed in 1961 (published 1962), interestingly similar findings had also been protected by an earlier patent application (1957), published in 1960 from the Imperial Chemical Industries (ICI)[24 25 26].

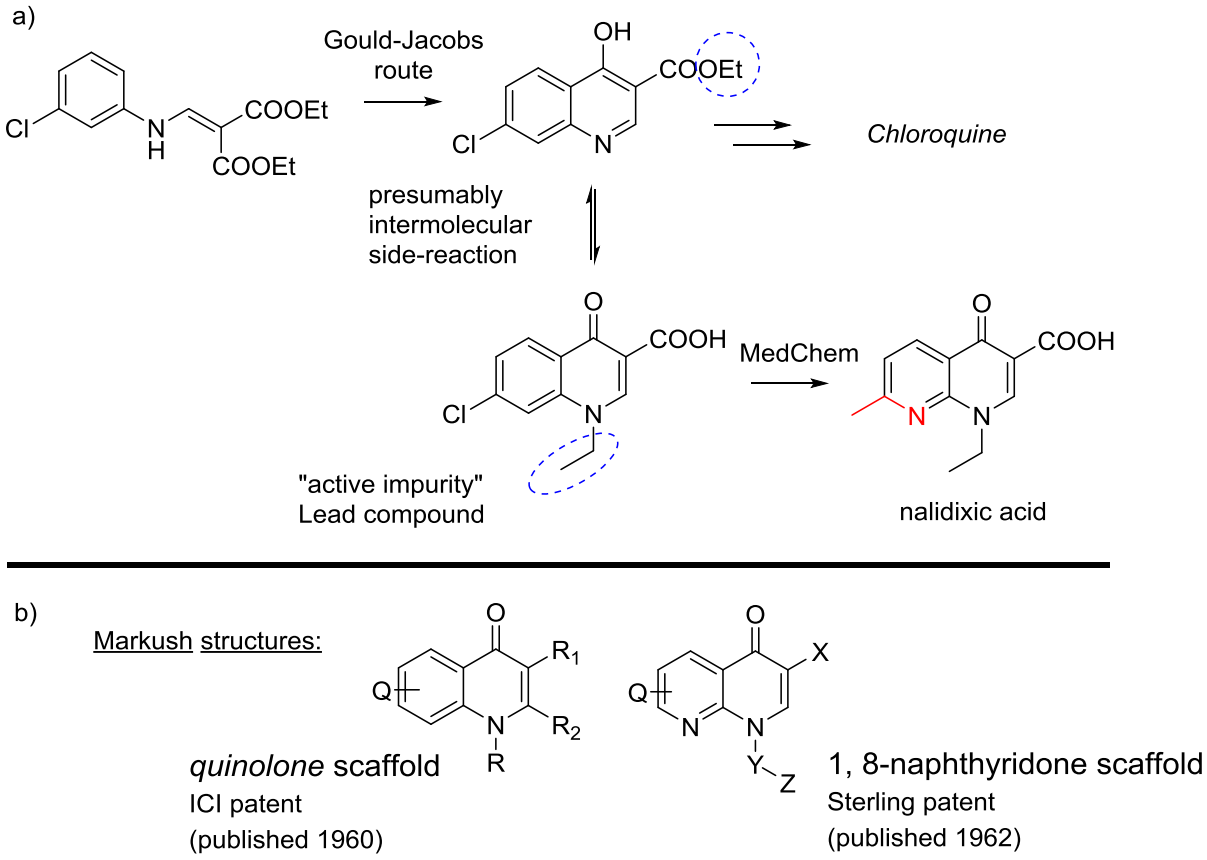


Figure 6: a) Reactions sequence used for the production of chloroquine at Sterling Drug in the 60s and that gave origin to the active impurity that led to nalidixic acid. b) Markush structures covered from ICI and Sterling Drug patents from the 60s.

It appears clear that the discovery of the antibacterial properties of the quinolone was actually disclosed by ICI. We could speculate that this could have been the reason for moving from a quinolone to a 1, 8-naphthyridone scaffold at Sterling drug. Nevertheless, nalidixic acid was approved[27], while no quinolone was brought into the market from ICI. The selection of a wrong clinical candidate could possibly be the reason for ICI's lack of success.

The first quinolones had a narrow spectrum, limited against certain Gram-negative bacteria. A first advancement in the class came with the discovery of norfloxacin, followed by ciprofloxacin[28,29]. This 2nd generation of quinolones has key substituents at the C6 and C7 positions. Particularly, the introduction of a fluorine-atom at the former and a basic moiety at the latter extended the spectrum against Gram-negative bacteria such as *P. aeruginosa* and improved the activity. Further generations of quinolones such as the 3rd and 4th present additional variants at the C7 basic moiety and C8 position (Fig. 7). Modifications of these two

have led to derivatives with increased potency against Gram-positive and improved pharmacokinetic properties[30].

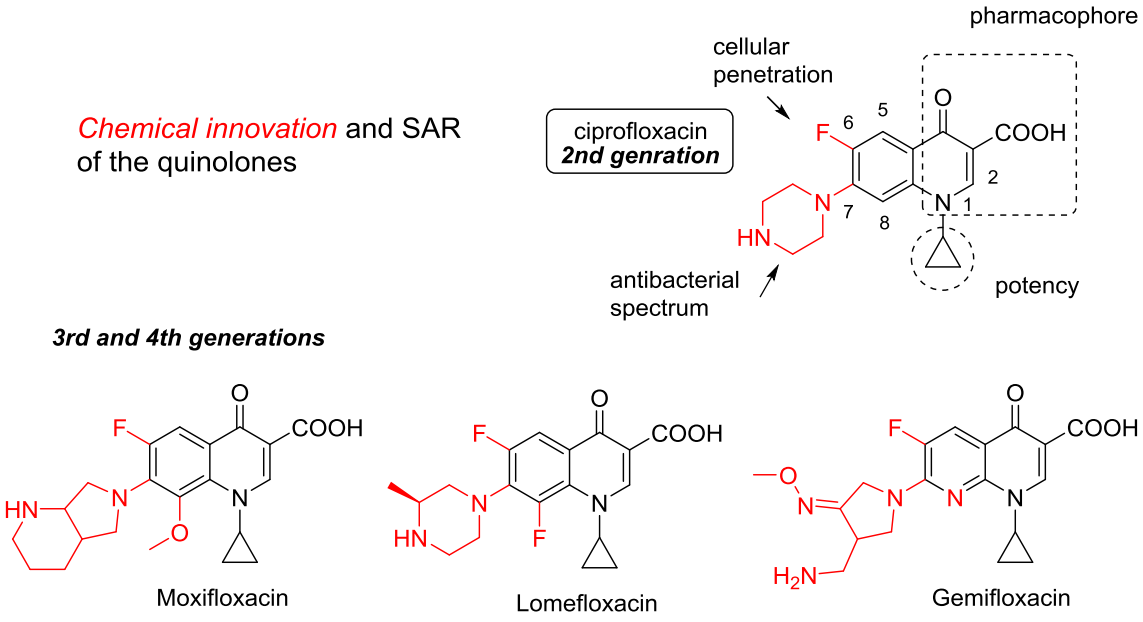


Figure 7: Sum up of the quinolones SAR and examples of compounds belonging to the latest generations.

The target enzymes of this successful class of antibiotics are the bacterial topoisomerases IIa (gyrase) and IV. They are implied in the modification of the DNA topology, with the former being the only enzyme in the prokaryotic kingdom that possesses the ability to introduce negative supercoils. The quinolones inhibit both enzymes by stabilizing the DNA-topoisomerase cleavage complex.

To conclude, structural optimization played an essential role in antibacterial drug discovery. This can be done with the semisynthetic or fully synthetic approach, with the former being more challenging but also the one that gives wider possibilities for modifications.

Most antibiotics are derived from natural products produced by microorganisms that use them to compete for survival in their environment. This also means that these compounds are not ideally designed or ready for being used as medicines.

The antibiotic history tells us that this gap can be filled and the development of optimized drugs can be achieved. Modifications of promising lead scaffolds led to compounds with

extended antibacterial spectrum, improved pharmacokinetic properties and reduced bacterial resistance.

In this scenario the discovery of novel chemical architectures with promising activities, nowadays especially against Gram-negative bacteria, is essential in order to give the possibility to medicinal chemists to develop novel antibiotics.

1.2 ANTIBIOTICS IN CLINICAL DEVELOPMENT

The need of novel solutions to treat bacterial infections, particularly those caused by Gram-negative bacteria that are members of the so called *ESKAPE* group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) is an important medical need[31]. The increasing level of resistance to the current antibiotics, the decreasing number of big pharma companies over the past four decades involved in antibacterial drug discovery [32] and the lack of structural innovation are among the causes of the current situation.

Most of the antibiotics currently used derive from scaffolds which had been discovered during the so called "golden age of antibacterials discovery" that took place only between the 40s and 60s. The following four decades instead could be seen as "golden age of MedChem"[33,34] (Fig. 8), big efforts have been spent on optimizing privileged structures such as the β-lactams and quinolones. This strategy brought several antibiotics to the market but without any profound diversification from the structural point of view.

It is reasonable to think that these classes of antibiotics are almost exhausted sources. Therefore the finding of novel antibacterial scaffolds is highly desirable and could potentially open new rich sources for the development of antibiotics of the future.

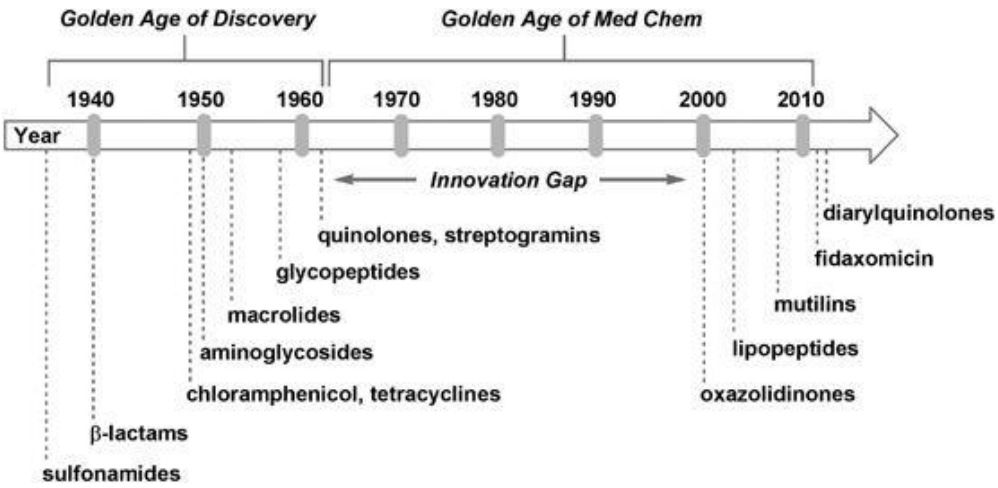


Figure 8: Years of discovery of the main classes of antibiotics currently available on the market. Image taken from[33].

1.2.1 **An overview of the pipeline**

Over the last decade the awareness of the *antibiotic crisis* has significantly increased, as a consequence more and more organizations went back to antibacterial drug discovery. In order to see which are the current situation and future perspectives for novel therapeutic options, the list of antibiotics currently in clinical development for the US market has been analyzed. Particular attention was given to compounds presenting a new chemical architecture, either possessing novel mechanism of action or acting on established targets[35].
In March 2017, 39 chemical entities were undergoing clinical trials at different stages ranging from phase I to III. Of these, ***18*** possess a ***novel chemical scaffold***, 12 acting with an established and ***six*** with a ***new mechanism of action***. All the other ones belong to established antibiotic classes such as ß-lactams, quinolones, macrolides, oxazolidinones, tetracyclines, aminoglycosides and pleuromutilins.

1.2.1.1 Selected novel chemical scaffolds

The clinical canditates possessing a new chemical scaffold and mechanism of action have been selected and will be presented in this section.
MGB-BP-3 is a minor grove binder, interfering with transcription factors, it leads to deregulation of the bacterial cell homeostasis[36]. The rational design of such compound class has been inspired by *nature*, particularly mimicking minor groove binder natural products such a distamycin A and netropsin [37] (Fig. 9).

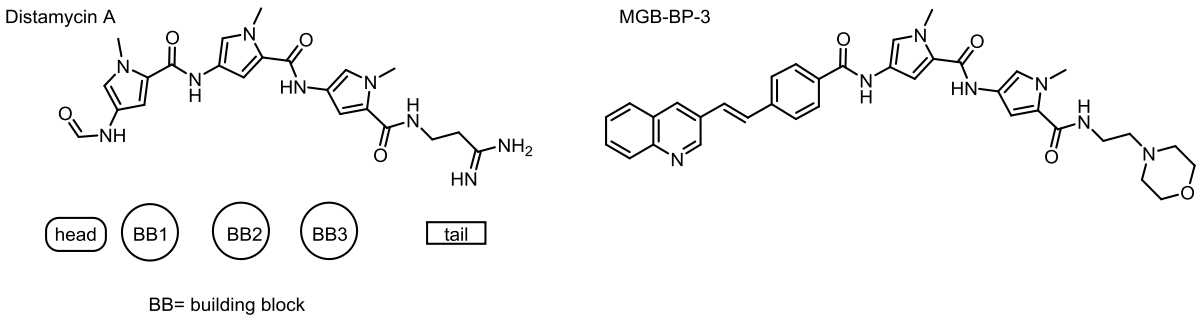


Figure 9: Structure of Distamycin A with schematic disconnection in five components and the clinical candidate deriving from it.

POL7080, also called murepavadin, is a very narrow spectrum antibiotic, because it is active only against *P. aeruginosa*. It originated from a library of peptidomimetics of the host-defense peptide protegrin I. The very narrow spectrum is not the sole novelty of the molecule, in fact this compound does not act unspecifically disrupting the bacterial cell membrane but it has a specific protein target, which is usually not the case for antimicrobial peptides. The target LptD is an outer membrane protein involved in the biogenesis of the lipopolysaccharide (LPS) and is widely distributed in Gram-negative bacteria [38]. The fact that inhibitors of this protein would not need to cross the challenging barrier of the outer membrane, makes of it a very interesting novel target.

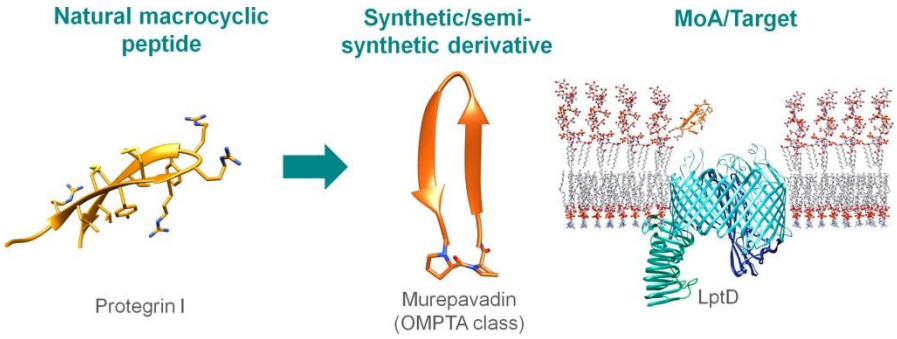


Figure 10: The pathogen-specific murepavadin derives from protegrin I but it acts with a different MoA, the protein target is the outer membrane protein LptD. Image taken from[39].

Debio 1450, also named afabicin (phase II), targets FabI, and enzyme that catalyzes the reduction step in the fatty acid elongation cycle (essential for bacteria) (Fig. 11). Two isoforms of this enzyme are present in the prokaryotic kingdom. Bacteria possess either one of them such as *S. aureus* and *E. coli* or both like for instance enterococci and *P. aeruginosa*[40]. For this reason, this new interesting target would not allow the development of broad spectrum antibiotics.

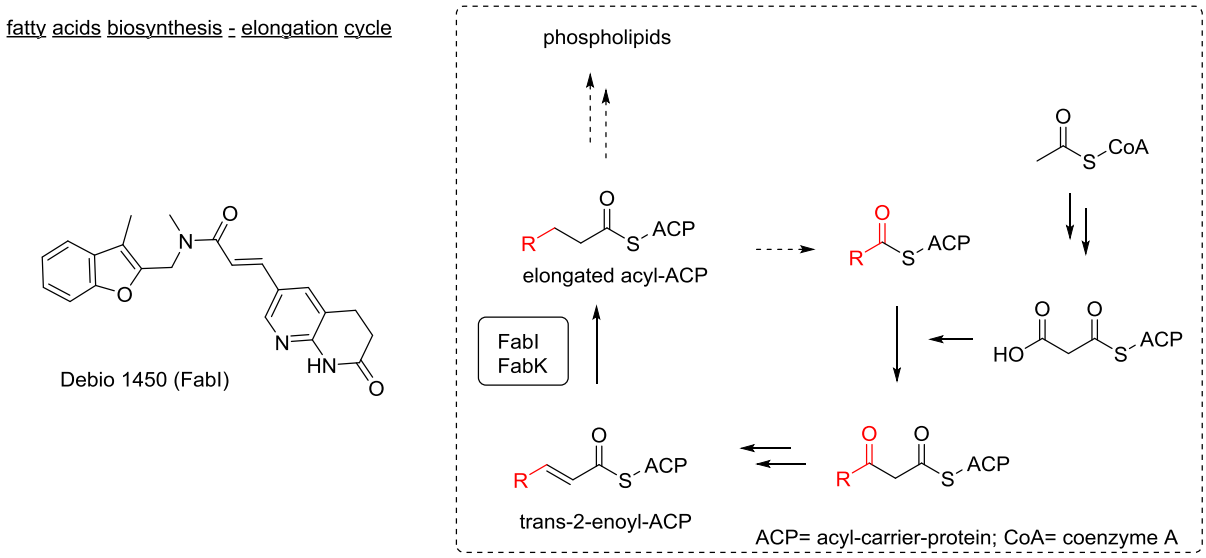


Figure 11: Structure of the clinical candidate and the biochemical pathway that it targets.

Aminoacyl tRNA synthetases (aatRS) are enzymes that catalyze the attachment of the appropriate amino acid to its cognate tRNA carrier to form aminoacyl-tRNA. Inhibition of these enzymes has direct consequences on the correct functionality of protein synthesis. CRS3123, currently in phase I clinical trials, has the scaffold of a diaryldiamine. It is a fully synthetic molecule derived from the structural optimization of a hit discovered during a high throughput screening campaign. It inhibits the methionyl-tRNA synthetase (MetRS), showing potent antibacterial activity against Gram-positive bacteria (Fig. 12). The aatRNAs are very interesting drug targets, but the development of efficient inhibitors is hindered by different factors such as limited homology of bacterial enzymes, resistance development together with the common challenge of the low permeability of Gram-negative bacteria.

A 5th novel chemical scaffold in the pipeline is represented by brilacidin, an arylamide foldamer that mimics host-defense peptides (HDP-mimetics). It interferes with the correct functionality of the cell membrane but has the advantage of improved pharmacokinetic properties [41]. It is selective for bacterial over eukaryotic cells, remarkably it shows activity against both Gram-positive and negative bacteria (among them *P. aeruginosa*) [42].

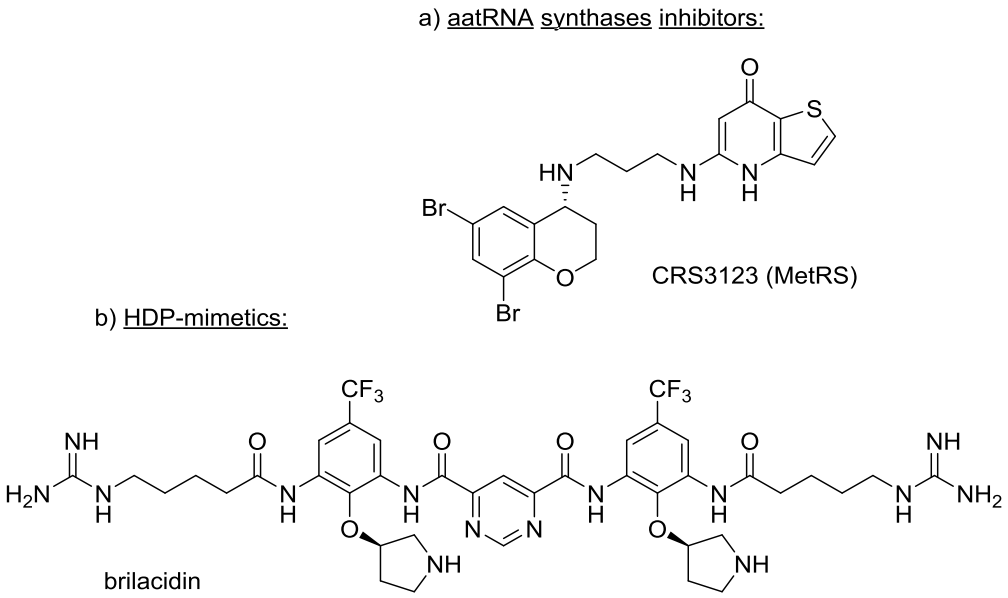


Figure 12: a) Structure of CRS3123 and b) of brilacidin, synthetic compound design to mimic host defense peptides.

A new interesting strategy being pursued at Spero therapeutics is the so called potentiator approach. SPR741, the representative compound, derives from polymyxin B. Interestingly, this molecule does not possess antimicrobial activity itself, but has the capability to expand the spectrum and enhance the potency of several "Gram-positive antibiotics" to MDR Gram-negative bacteria. It interferes with the integrity of the outer and inner membrane, thus facilitating the entering of chemical entities into the bacterial cells[43].

The other 12 compounds that have a novel chemical scaffolds, target well-established enzymatic targets such β-lactamases, DNA gyrase or ribosomes.

1.2.1.2 ***Outlook***

At first glance, the antibiotic pipeline seems to be quite robust considering the total number (39) of compounds present, which span from the early stage to the advanced clinical trials. Considering the statistic, which says that one out of five molecules will reach the market, the current perspective foresees the approval of about eight new antibiotics in a close feature.
As recently stated by the WHO there is an urgent need to find therapeutic solutions against resistant Gram-negative bacteria[44]. From this point of view, a closer look at the data indicates that out of the 39 candidates, those possessing clear activity against resistant Gram-negative (ESKAPE pathogens) are eleven. Of these, ten belong to well-established

antibiotic scaffolds, such as cyclines (2), β-lactam alone or combination of them with a β–lactamase inhibitor (6, of which 5 are combinations), aminoglycoside (1) and fluoroquinoilone (1).

In sum, among the clinical candidates only two molecules targets Gram-negative bacteria and possesses a new chemical scaffold. This highlights that there is a lack of innovation and the need for new therapeutic solutions remains to be still high.

Table 1: NP= natural product; S= synthetic; NP/S= drug is composed of two molecules, one of synthetic origin and one derived from nature, the numbers is represented by the combination of beta lactam and BLs inhibitors; N=nature.

Potential new antibiotics		
	total	*Active against MDR Gram-neg.*
NP derived	*14*	*4*
S origins	*17*	*1*
NP/S	*6*	*5*
N inspired	*2*	*1*

Nature still plays a main inspirational source for antibacterial drug discovery campaigns (table 1). It could be noted that finding substances active against MDR Gram-negative is very difficult and only around 25% of the compounds currently in clinical development have these specifications. Noteworthy, substances of synthetic origins possess generally poor activities against Gram-negative bacteria.

To conclude, increasing efforts in the field of antibiotic drug discovery are fueling the pipeline with an important number of clinical candidates. However, novel chemical scaffolds with broad spectrum activity against resistant Gram-negative bacteria are not present. Therefore the finding of new lead structures with such specifications is still of very high importance in order to develop innovative antibiotics.

1.3 PABA-DERIVED OLIGOMERIC NEW ANTIBIOTIC SCAFFOLDS

1.3.1 **Structural features, mechanism of action and biosyntheses.**

The isolation of natural products from microorganisms is still a valuable strategy to pursue in antibacterial drug discovery. In fact, nature can still provide novel structural templates with promising antimicrobial activity.

In this context, albicidin, cystobactamids and coralmycins represent a good example. These natural products were recently identified [45-50] and remarkably possessed a new molecular scaffold and activity against clinically relevant Gram-negative bacteria.

These intriguing classes of natural products can be seen as oligomers of p-aminobenzoic acid (PABA). Particularly, they can be disconnected into six subunits, a central aliphatic amino acid and five aromatic moieties. The central amino acid holds a fragment composed of two subunits at the amino functional group and one composed of three at the carboxylic acid, the PABA-derived building blocks are connected together via amidic bonds (Fig. 13).

A unique structural feature shared by the three classes is an aromatic ring possessing an uncommon tetrasubstitution pattern, with alkoxy and hydroxy moieties present respectively at the *meta* and *ortho* position of the PABA unit. For albicidin a methoxy (MeO) is present while for the other two classes an isopropoxy (*i*-PrO).

Two other characteristic moieties that differ among these three classes are the central building block and the last aromatic subunit at the N-terminus [46,47,49,51].

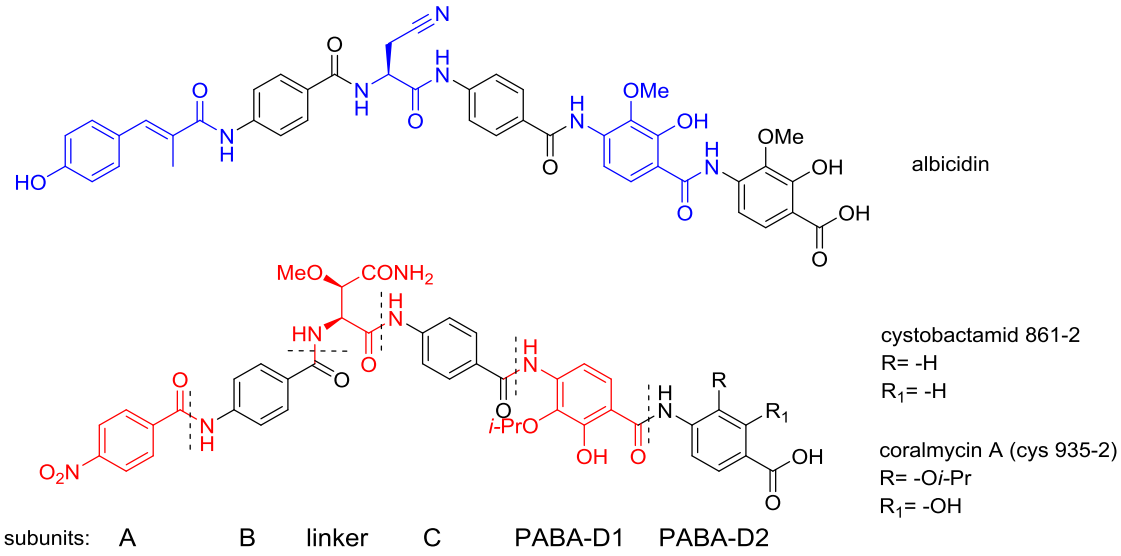


Figure 13: Chemical structure of the representatives of these three new classes of Gram-negative active natural products. The moieties representing the structural differences are highlighted.

These natural products inhibit bacterial growth by targeting DNA gyrase with activities in the nanomolar range, notably even higher than the one of ciprofloxacin[47,52]. They also inhibit bacterial topoisomerase IV but with inferior activity. It was shown that both natural products do not possess cytotoxic effects at concentrations of 10 µM for albicidin and 50 µg/mL for cystobactamids.

DNA gyrase is the only bacterial enzyme that is able to introduce negative supercoils into the DNA at the expense of two molecules of ATP. It is a tetramer constituted of two homologous subunits (A_2B_2). A classification of gyrase inhibitors is done based on the target subunit, molecules acting on GyrA in general interfere with the catalytic function while those targeting GyrB are competitive inhibitors of the binding sites of ATP. Two famous classes of antibiotics targeting subunits A and B are the quinolones and the aminocumarines, respectively.

The first step of the catalytic cycle of DNA gyrase is the recognition of a DNA strand from the C-terminal domains (CTD). This brings the DNA into a wrapped conformation around the enzyme. Binding of two molecules of ATP triggers conformational changes that bring the two subunits B closer together and arrange the DNA into the correct position for being cleaved. Here, the two strands of DNA will be opened by the activity of a catalytic triade composed by tyrosin, histidine and arginine, in which the phenol moiety is responsible for the nucleophilic attack at the phosphate group. Once the double strand is open, ATP hydrolysis furnishes the

energy necessary for translocating the other segment through the DNA gate, hydrolysis of the second ATP will liberate the DNA strand and restore the enzyme in the initial conformation ready to start a new cycle (Fig. 14) [53].

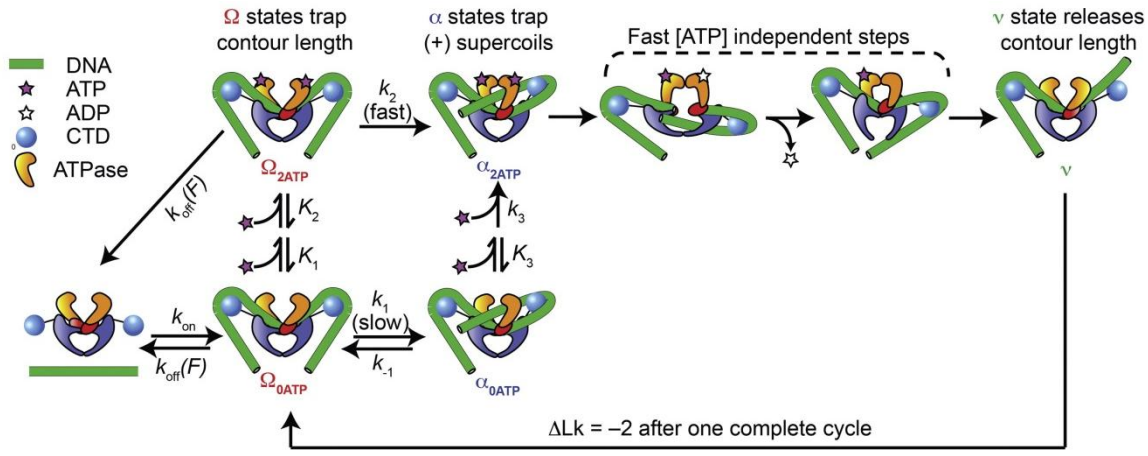


Figure 14: Schematic representation of gyrase subunits and the catalytic cycle that introduces negative supercoils into the DNA. The starting point is at the bottom left side of the figure, adopted from [54].

The co-crystal structure with the quinolone (Fig. 15) [55,56] revealed that once the double strand of DNA is covalently attached to the catalytic Tyr, two molecules intercalate freezing the complex enzyme-DNA in this status. One of the key interactions is between the ketone and the carboxylic acid with a non-catalytic Mg^{2+} ion that functions as bridge to a Ser and Glu/Asp. Mutation of these two residues (S83 and an acidic residue four amino acids downstream such as D87) leads to decrease inhibitory activity (30-60 folds).This is one of the most clinically relevant resistance mechanisms for the quinolones.

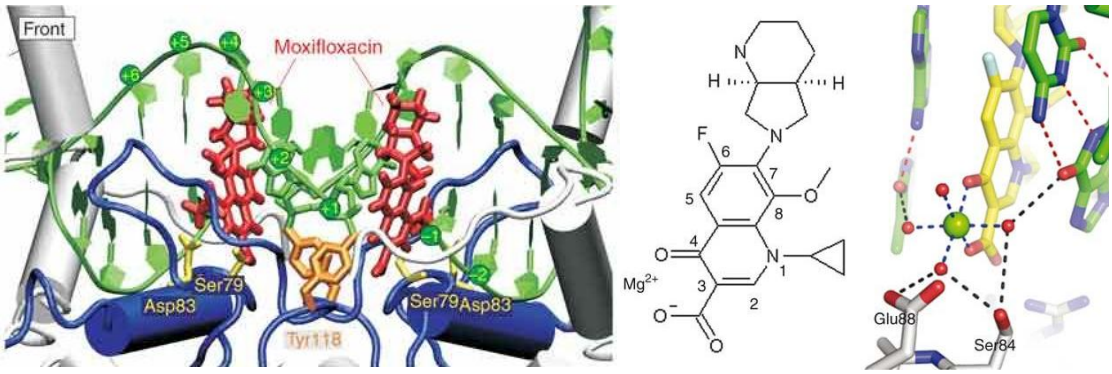


Figure 15: Co-crystal structures of moxifloxacin and truncated version of topoisomerase IIa (left image) and IV (right image) from *Streptococcus pneumoniae and A. baumannii* respectively; image take from[55,56].

Both cystobactamids and albicidin stabilize the DNA gyrase cleavage complex in a similar way to the one of the quinolone. The activity of cystobactamids and albicidin against bacterial strains carrying these gyrase mutations was found to be lower in comparison to the corresponding wild types. On the other hand, it was affected to a smaller extent (two-six folds), then thus indicating that their binding site possibly partially overlaps. Nevertheless, this data should be considered in a critical way, gyrase is a complex large enzyme and during the catalytic cycle many conformational changes are required for correct functionality. It could be also speculated that the target mutations entailing resistance to quinolones lead to changes in the 3D arrangements of certain parts of the enzyme, thereby decreasing the binding affinity of cystobactamids and albicidin.

These natural products are nonribosomal peptide synthetase (NRPS) derived. In both cases the proposed biosynthesis involves a series of modules composed by adenylation (A), thiolation (T) and condensation (C) domains that assemble them from the N- to the C-terminus. Additionally, at the beginning of the “assembly line” during albicidin biosynthesis we can also find a polyketide synthase (PKS) module which builds up the para-coumaric acid derivative starting from *p*-hydroxybenzoic acid. In case of the cystobactamids, a module for the oxidation of the anilino to nitro group is instead present (Fig. 16).

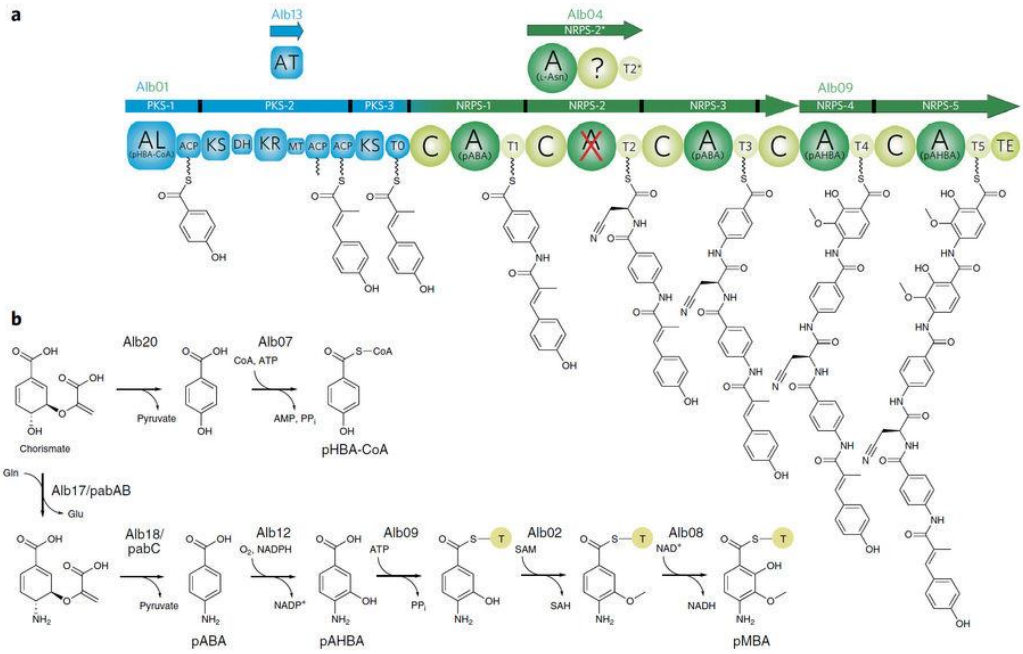


Figure 16: Proposed albicidin biosynthesis. a) Assembly line composed of PKS (blue) and NRPS (green) modules; b) enzymatic steps for the synthesis of the tetrasubstituted aromatic ring. The biosynthesis of cystobactamids follows the same assembly sequence with the difference that the PKS module is replaced by an additional NRPS (image from[46]).

The synthesis of the characteristic tetrasubstituted aromatic ring starts from PABA, that is first hydroxylated, presumably at the position 3 as shown for albicidin[46], loaded on a T domain and only then it undergoes methylation followed by oxidation of the position 2, thus affording the building block which is incorporated in the oligopeptide scaffold.

The cystobactamids show heterogeneity in their structures. One of the features that distinguishes different derivatives is the alkyl substituent of the phenol in position 3 of PABA-D1 and D2 rings. The alkyl substituent can be a methyl, an ethyl, most commonly an isopropyl or even a *sec*-butyl [51]. The different level of alkylation is achieved in a unique way[57]. CysS is the cobalamin-dependent radical *S*-adenosylmethionine (SAM) methyltransferase responsible for this. In brief, upon reductive cleavage of methionine from SAM, 5´-deoxyadenosyl radical (5´dA) is generated. This will react with the methoxy moiety, in this way a radical is generated that will then be quenched by methyl cobalamin (in so doing acting as a methyl donor). This process can be seen as a natural "C-H" activation. Starting from the methoxy moiety, different alkyl branches can be biosynthesized by successive radical methylation following the same mechanism (Fig 17).

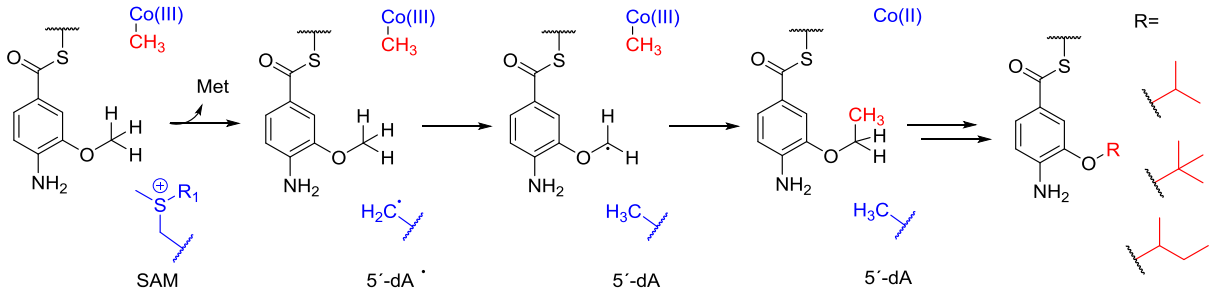


Figure 17: Biosynthesis of branched alkoxy group of cystobactamids. For simplification the redox system $[4Fe\text{-}4S]^{+1/+2}$ is not depicted, this has an essential role in the recycling process of cobalamine. Methylcobalamine is also generated in a SAM dependent manner.

1.3.2 **Albicidins**

Albicidin was first discovered in 1985 by Birch and coworkers, it is a secondary metabolite produced by the sugarcane pathogen *Xanthomonas albilineans*[45]. These scientists spent significant efforts in understanding the biological properties of this molecule [58-64]. Despite the promising activity, investigation of this antibiotic scaffold has been hindered for decades because the structure couldn´t be elucidated due to the extremely low production yield.

In 2014, Cociancich *et al.* optimized a protocol for the heterologous expression[46], the enigmatic structure of this natural product could be elucidated and its total synthesis was established [6566]. Retrosynthetically, the molecule was disconnected into three main fragments (Fig. 18) which have then been assembled starting from the right to the left part. In this synthesis, BTC had been extensively used for the coupling steps. Final deprotection of the allyl groups afforded the desired final product. Both enantiomers were obtained and this definitely confirmed that the configuration of the β-cyanoalanine of natural albicidin is *S*. The biological activities of them were assessed against clinically relevant pathogens and in the DNA supercoiling assay. Interestingly, although both enantiomers inhibit gyrase at the same level (IC_{50} around 40 nM), the *S* isomer had a broader antibiotic spectrum.

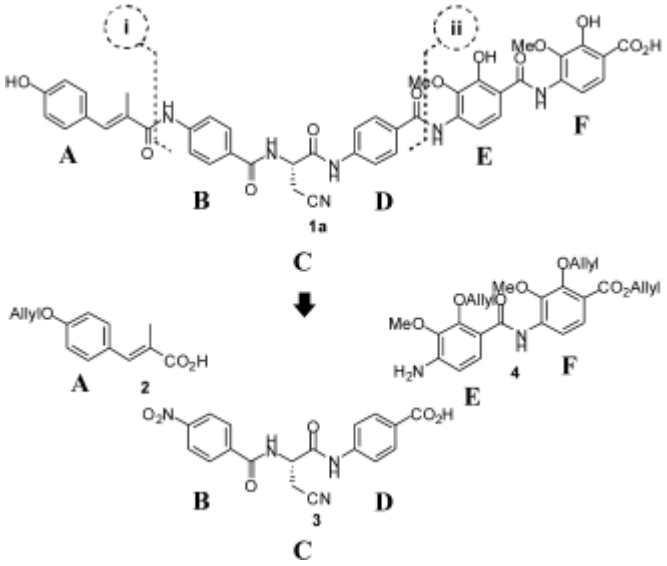


Figure 18: Retrosynthetic analysis of albicidin. Final assembly of the natural product starts with the coupling of fragments 4 and 3; reduction of the nitro group followed by coupling of fragment 2 gives fully protected albicidin (Figure taken from[65]).

Albicidin is not the sole natural occurring derivative of this class. Trace amounts of carbamoyl albicidin (C-Alb) were also found during the purification process. Biochemical assays and chemical synthesis demonstrated that the carbamoyl residue was added at the N-terminus [67]. The antibacterial properties of these two natural derivatives were very similar. The IC_{50} value for gyrase inhibition for C-Alb was found to be five-fold higher than for Alb. This indicated that the N-terminal side of the molecule is an important point of interaction with the target enzyme. Therefore it represents an interesting point for medicinal chemistry investigations.

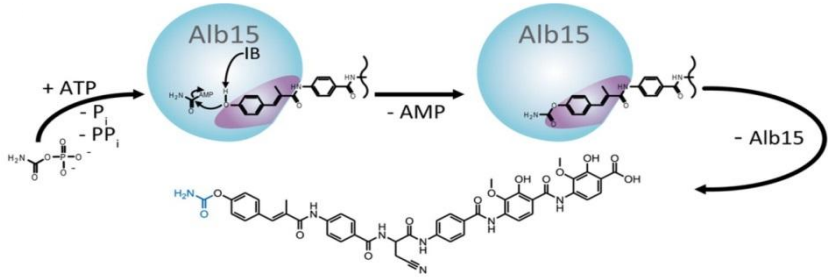


Figure 19: Schematic representation of post-NRPS carbamoylation of albicidin catalyzed by Alb15 (Figure take from[67]).

In 2016, two reports on SAR studies of albicidin were published by the Süssmuth group. Considering the disconnection of the molecule into six subunits, two of them have been assessed, particularly the central cyanoalanine and the N-terminal methyl coumaric acid [68,69].

For the investigation of the central part, the original β-cyanoalanine has been replaced by amino acids with different characteristics in term of polarity and electronic properties (Fig. 20).

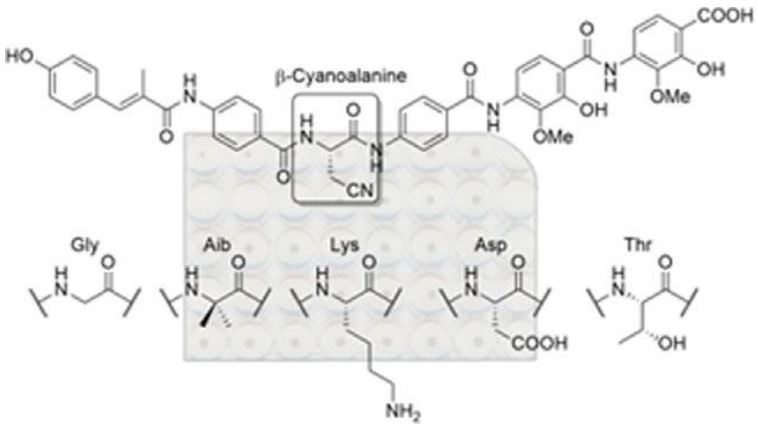


Figure 20: Albicidin derivatives bearing modifications of the central part. Imagine take from[68].

Gyrase inhibitory activities of these derivatives were all in the same range as albicidin, thus indicating that chemical modifications of the central part are well tolerated.

On the other hand, MIC values showed important differences, compounds bearing charged residues had drastically lower antibacterial activities, the three other instead showed inhibition properties similar to albicidin. This suggested that cell penetration might be the issue in case of the former compounds.

During the investigation of the N-terminal acyl residue, the position of the phenol on the aromatic ring, the relevance of the Michael acceptor and the lipophilicity were investigated (Fig. 21). Also in this case, it turned out that cell penetration is a limiting factor for the antibacterial activity, in fact some derivatives had comparable gyrase IC_{50} but worse MIC values.

Compounds with increased hydrophilicity had both worse enzyme inhibitory and antibacterial properties, moving the hydroxyl group to the *meta* and *ortho* positions had the same effect. The presence of the methyl group in the Michael acceptor moiety is important.

Finally, they showed that substitution of the -OH with a lipophilic substituent such as F atom or a CF_3 moiety was in general well tolerated.

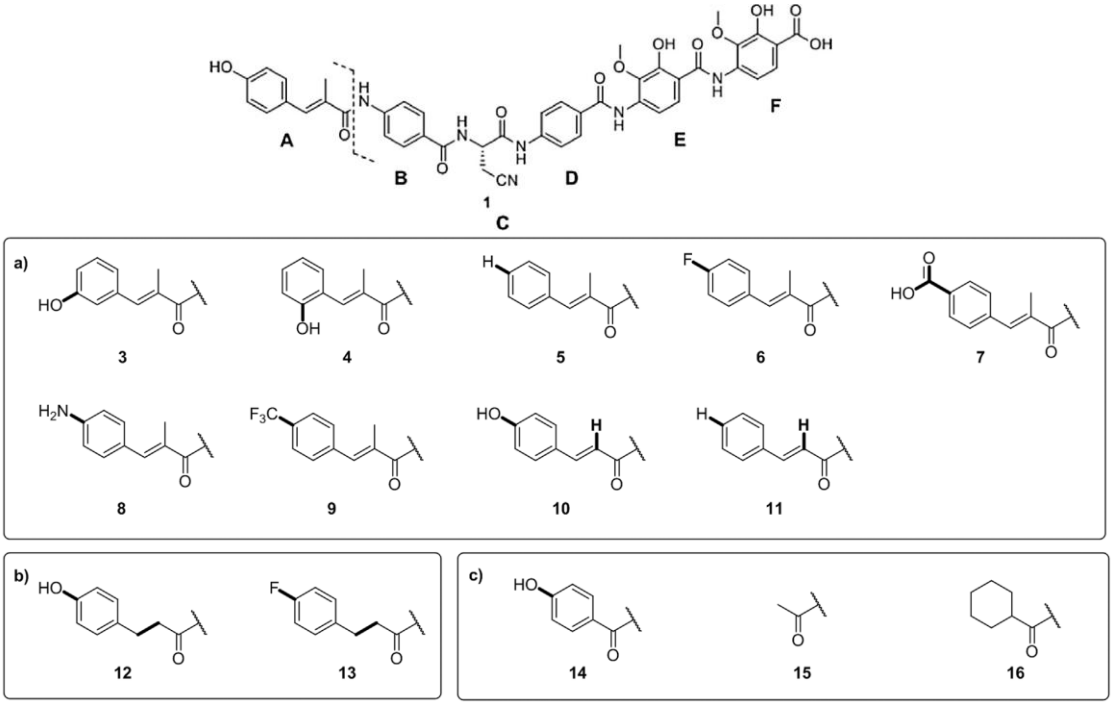


Figure 21: Series of modifications of the N-terminal building block (compound numbering according to the original publication). Image take from[69].

For natural products, the development of resistance occurs in parallel with their biosynthesis. As soon as a new compound is produced, one or multiple mechanisms will also be developed for self-protection. In this regard, several works on albicidin were published [58-64,70-74].

X. albilineans defends itself from albicidin in three ways, these are mediated by the proteins AlbF, AlbG and a characteristic gyrase that carries multiple mutations.

AlbF is part of the DHA14 drug efflux pumps family that presents 14 transmembrane helices. It is very specific for albicidin and has a certain homology with other known efflux pumps for natural products [75-77]. Heterologous expression of this pump confers high resistance level in *E. coli* (ca. 3000-fold).

AlbG is part of the pentapeptide-repeat proteins (PRP) family, the protein folds forming an eight coil β-helix structure and is active as a homodimer. In general, the binding of such proteins to topoisomerase obstacles access to the binding site of the drug [78].

The third element that confers resistance at the microorganism producer is a gyrase enzyme that presents several mutations in comparison with the one of *E. coli*.

There are three other resistance mechanisms known for albicidin which are not related to the self-protection of the producer microorganism. The first is the alteration of an outer membrane protein related to albicidin uptake. Tsx is found in *E. coli* and facilitates the uptake of nucleosides. Mutations at this protein channel entailed resistance to albicidin up to 100-fold[59].

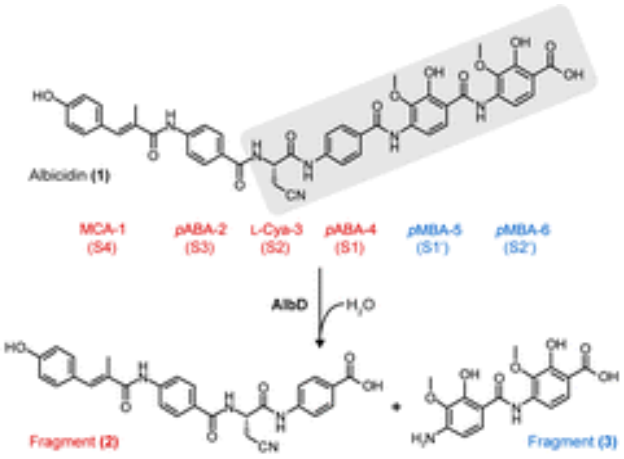
A second type of resistance factor is represented by proteins with high-affinity binding properties for albicidin. They were named AlbA and AlbB, the first one was found in *K. oxytoca* and the latter in *Alcaligenes denitrificans*[58,61].

AlbA is the one that has been better characterized, it is composed of 221 amino acids and its natural role in *K. oxytoca* is not known. It binds albicidin with a K_d of 6.4×10^{-8} M. This high affinity binding entails quick conformational changes in the protein, the antibiotic is captured in a stable complex and cannot interact with the target enzyme.

The last resistance mechanism known is the hydrolytic inactivation catalyzed by AlbD. This endopeptidase cleaves albicidin site-specifically into two inactive fragments. The cleavage site is the amide bond between the building blocks S1 and S1´ (Fig. 22). A set of fragments of albicidin and modified versions of it have been synthesized and tested against AlbD in order to gain an understanding of the minimal recognition and cleavage motif[71].

It was found that hydrolytic activity was retained or even enhanced for most of the synthesized fragments. A fragment where the methoxy moiety of S1´ was replaced with the more sterically demanding isopropoxy found in the cystobactamids was also synthesized. The hydrolytic activity of AlbD was retained, demonstrating that the cystobactamids can potentially be substrate of this endopeptidase. Interestingly, the R-isomer of albicidin was cleaved with a significantly lowered efficiency.

a)



b)

Substrate	Substrate residues						RCR[1]	SD[2]
	S4	S3	S2	S1	S1'	S2'		
albicidin (**1**)	MCA	*p*ABA	L-Cya	*p*ABA	*p*MBA	*p*MBA	1.00	0.00
ent-albicidin (ent-**1**)	MCA	*p*ABA	D-Cya	*p*ABA	*p*MBA	*p*MBA	0.35	0.05
tetrapeptide (**4**)			L-Cya	*p*ABA	*p*MBA	*p*MBA	1.77	0.03
tripeptide (**5**)				*p*ABA	*p*MBA	*p*MBA	1.44	0.01
modified tripeptide (**6**)				*p*ABA	*p*IBA	*p*IBA	1.21	0.15
tripeptide (**7**)			L-Cya	*p*ABA	*p*MBA		-0.25	0.34
acetylated tripeptide (**8**)		Ac-	L-Cya	*p*ABA	*p*MBA		1.77	0.03
amidated tripeptide (**9**)			L-Cya	*p*ABA	*p*MBA	$-NH_2$	0.35	0.07
dipeptide (**10**)				*p*ABA	*p*MBA		-0.13	0.22

Figure 22: a) Representation of albicidin cleavage site. b) Table containing selected set of fragments synthesized and results obtained in the enzymatic assay with recombinant AlbD; RCR= relative cleavage ratios determined by HPLC-MS; SD= standard deviation; figure and table taken from [71].

It is yet to be understood how likely the horizontal transmission of all these resistance factors to other microorganisms is. This is an issue that definitely could undermine and affect the potential of albicidin as new antibiotic, at the same time having already understood so much about the potential resistance mechanisms could aid the design of strategies to overcome such problems in advance.

Considering the structural similarities and the same MoA, it is understandable to think that also the activity of the cystobactamids could be affected. On the other hand, this likelihood should be properly verified by means of biological experiments to guide the early-stage drug development process.

1.3.3 **Cystobactamids**

The first report on the cystobactamids has been published in 2014, approximately at the same time as the structure elucidation and total synthesis of albicidin[46,47,65]. In this work, the analogs cys 919-2, 919-1 and 507 were presented. They were isolated as main components from the fermentation of *cystobacter* sp. Cbv34 with a very low production titer. Obtaining sufficient amounts of these molecules for pre-clinical investigations remains still very tedious.

The first two compounds have very similar structures, with the only difference in the central aliphatic building block. Where as for cys 919-2 a α-amino acid connects the two large aromatic fragments, beta connection is present in cys 919-1 (Fig. 23). Cys 507 is a truncated version of the first two molecules, possibly isolated as a hydrolytic byproduct. This suggestion finds partial confirmation with its total synthesis[79]. Antibacterial activity tests of the synthetic material revealed that the molecule is virtually inactive, therefore the originally assessed activity could derive from impurity traces of highly potent cystobactamids. Cys 919-2 possessed clearly higher antibacterial properties than 919-1.

The configuration of the two chiral carbons present in central MeO-Asn moieties was firstly misassigned as "*2S, 3S"*. Due to the absence of a racemase in the biosynthetic gene cluster, the first carbon is defined as *S*.

The mistake was identified with the work of the Kirschning group (data unpublished) and reported by Kim et al. [49]. The assigned stereochemistry was revised to be "*2S, 3R"*.

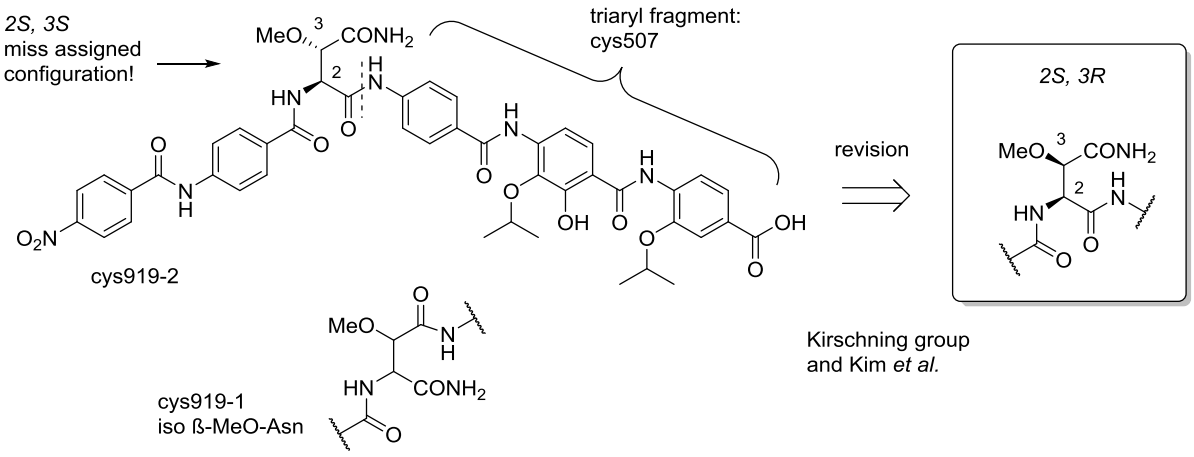


Figure 23: Structures of the first three reported cystobactamids; depiction of the originally assigned stereochemistry and the correct one.

The correct stereochemistry was confirmed to be the revised one with the total synthesis of cys919-2 [48]. The synthesis relies on the disconnection of the molecule in three fragments, the central linker and the two aromatic portions (Fig. 24). One of the interesting points of the synthetic strategy used is the "self-activation" of the ß-methoxy aspartate as cyclic anhydride. Thanks to this, the known side reactions of Asn and Asp that can take place in the activation of the carboxylic acid moiety could be avoided.

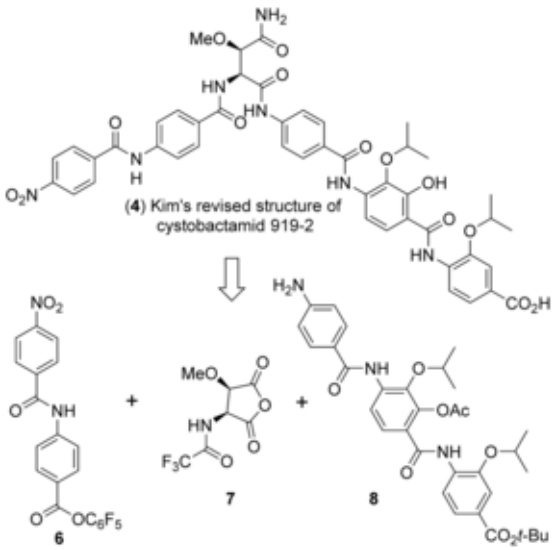
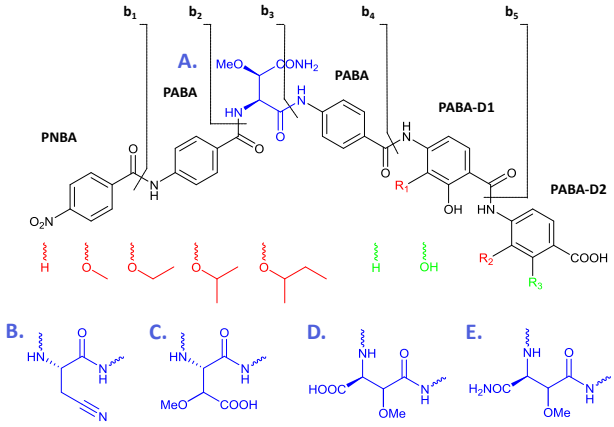


Figure 24: Structure of the three building blocks used for the total synthesis of cys919-2. Figure taken from [48].

As already mentioned, the cystobactamids are a heterogeneous family of natural products. After the first publication and patent of 2014[80], new derivatives with superior activities against Gram-negative bacteria (table 2), remarkably active also against *P. aeruginosa,* were isolated and characterized from *Myxococcus sp.* SBCy9270 [5181]. The new derivatives showed structural differences at the linker region, PABA-D1 and D2 (Fig. 25). It was confirmed that the optimal linker variant is the ß-MeO-Asn and that a simple modification such as replacement of the primary amide with a carboxylic acid have a drastic effect on the activity (Table 2). The best substitution pattern for PABA-D1 and D2 was found to be the one of cystobactamid 861-2 (cys 861-2) that together with cys 935-2 had the best antibacterial profile being active against all the tested clinically relevant pathogens with the exception of *K. pneumoniae*.



Cystobactamid	Linker	R^1	R^2	R^3
919-1	E	Isopropoxy	Isopropoxy	H
919-2	A	Isopropoxy	Isopropoxy	H
449	-	Isopropoxy	H	H
507	-	Isopropoxy	Isopropoxy	H
861-2	A	Isopropoxy	H	H
871	B	Isopropoxy	Isopropoxy	H
877-2	A	Ethoxy	Methoxy	H
891-2	A	Isopropoxy	Methoxy	H
905-2	A	Isopropoxy	Ethoxy	H
920-1	C	Isopropoxy	Isopropoxy	H
920-2	D	Isopropoxy	Isopropoxy	H
934-2	D	Isopropoxy	1-Methylpropoxy	H
935-2	A	Isopropoxy	Isopropoxy	OH

Figure 25: Schematic representation of the second series of natural cystobactamids discovered from *Myxococcus* sp. SBCy9270. Figure taken from[51].

These molecules were published only very recently and practically at the same time with the total synthesis of cys 919-2. In this report, together with the discovery of these new natural cystobactamids, the total synthesis of the most active congener (cys 861-2) has been published. The author of the thesis is also a coauthor of the mentioned manuscript and was involved in the synthesis of cys 861-2.

Table 2: Antimicrobial activities of second series of cystobactamids isolated, table adapted from[51].

cystobactamid	919-2	861-2	871	877-2	905-2	920-1	920-2	934-2	935-2
Acinetobacter baumannii DSM-30008	8	0.5	16	> 64	64	> 64	> 64	> 64	2
Escherichia coli (WT)	0.5	0.13	16	2	4	> 64	> 64	> 64	0.5
Klebsiella pneumoniae DSM-30104	> 64	> 64	nd	nd	nd	nd	nd	nd	nd
P. aeruginosa DSM-24600 (CRE)[b]	64	1	64	64	> 64	> 64	> 64	> 64	8
Proteus vulgaris DSM-2140	4	0.25	2	4	32	> 64	> 64	> 64	1

[a] Ciprofloxacin. [b] Carbapenem-resistant *Enterobacteriaceae*.

1.4 PHOTOPHARMACOLOGY

Photopharmacology is an emerging field of research. It is based on molecules that upon irradiation with light of specific wavelength undergo reversible structural changes. Thus the bioactivity of a molecule became controllable by light. Such "smart" molecules can be identified as *photoswitchable drugs* [82-87]. This type of drugs would offer the advantage of site- and temporal-specific activation, thus for instance offering the possibility of limiting peripheral side effects. To date, this field is in a growing phase, with the research around it being still at its early stage development needing for ground breaking works.

A known drug can be transformed into a photoswitchable one by the insertion of a proper photoswitchable moiety in the original scaffold. The design of such drug is preferably guided by structure-activity relationships, since it is essential that the biological properties of the parent compound are at least in part retained.

Different photoswitchable moieties have been reported for their suitability for being used in this field, these are azobenzenes[88], diarylethenes[89], spiropyrans[90] and others such as acylhydrazones[91] (Fig. 26).

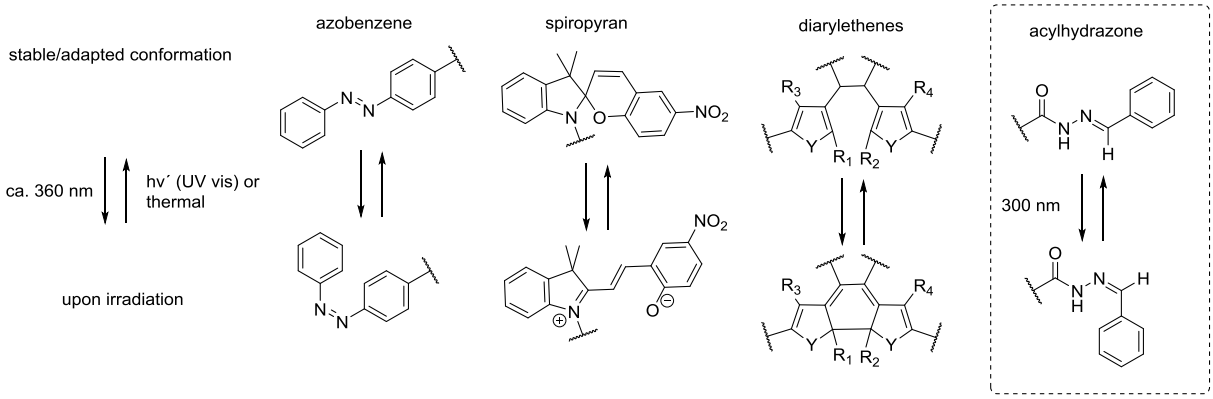


Figure 26: Overview of reported photoswichable moieties that have found or can potentially find application in photopharmacology. For the first three examples on the left, photoswitchable drugs that incorporate them are known, for the acylhydrazone in the square not yet.

A molecular photoswictch undergoes isomerization upon absorption of photons, the moiety can then go back to the most stable state by different irradiation or thermal relaxation.

In order to have photoconversion, a molecule has to be irradiated with light of the wavelength that is in the proximity of an absorbance maximum. Quantitative isomerization

can be achieved if the two absorption spectra of the isomers don´t overlap in the region of irradiation.

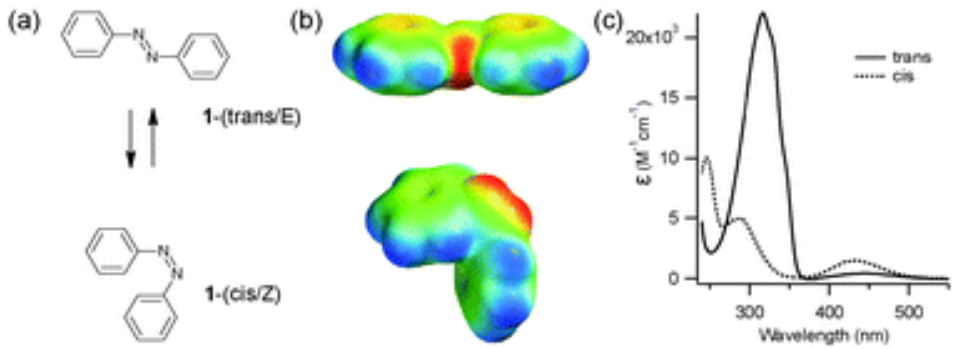


Figure 27: a) Chemical structure of azobenzene in its two isomeric states; b) electrostatic potential of the structures (red: negative; blue: positive); c) UV spectra of *trans* and *cis* azobenzene. Image taken from[92].

There are some important requirements that the ideal photoswitch should fulfill for photopharmacological applications[85]:

- The change in concentration of the most active isomer should be enough in order to reach differences in biological activity. It is desirable that this difference is as pronounced as possible, with the optimal case in which one of the two isomers is inactive ;
- The isomerization process should be achieved by using light in the near infrared region, this is for two reasons, to ensure tissue penetration and to avoid tissue damage;
- The thermal relaxation process that brings the irradiated isomer to the more stable conformation should occur within a time frame compatible with the application of the drug (range of minutes or hours);
- The molecule should not undergo side reactions in photoswitching cycles (photobleaching);
- The molecule should possess proper pharmacokinetic parameters such as solubility in water of both isomers, metabolic stability and no general cytotoxic effects.

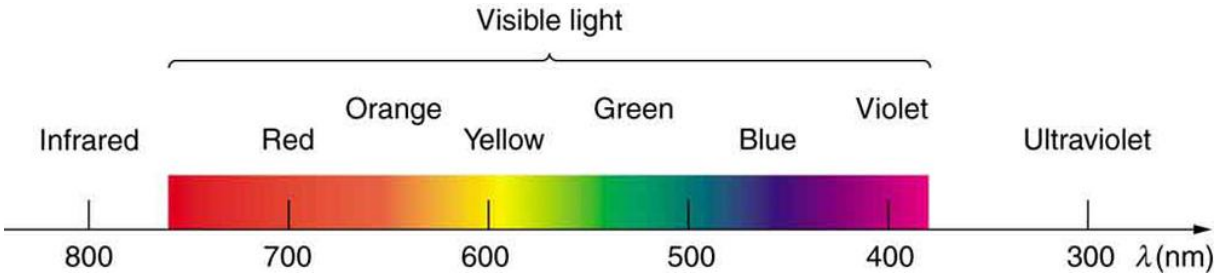


Figure 28: Electromagnetic spectrum. Image taken from[93].

Azobenzene is the moiety that has been best understood and found the broadest application in photopharmacology so far[83,90,94,95].

The simplest azobenzene moiety can be switched from *trans* to *cis* with light of 365 nm (Fig. 27 c). The absorption properties can be modulated modifying either the aromatic rings or the substituents on them[96-100101]. Particularly, in the successful strategies pursued, the UV spectrum of the photoswitchable moiety could be red shifted introducing substituents at the *ortho* positions or replacing one of them with a heterocycle (Fig. 29).

A drawback of photoswitchable drugs incorporating azobenzene is the potential metabolic instability [102,103]. Nevertheless, if the use of azobenzene will encounter such issues, photopharmaclogy could still rely on the others photoswitchable moieties.

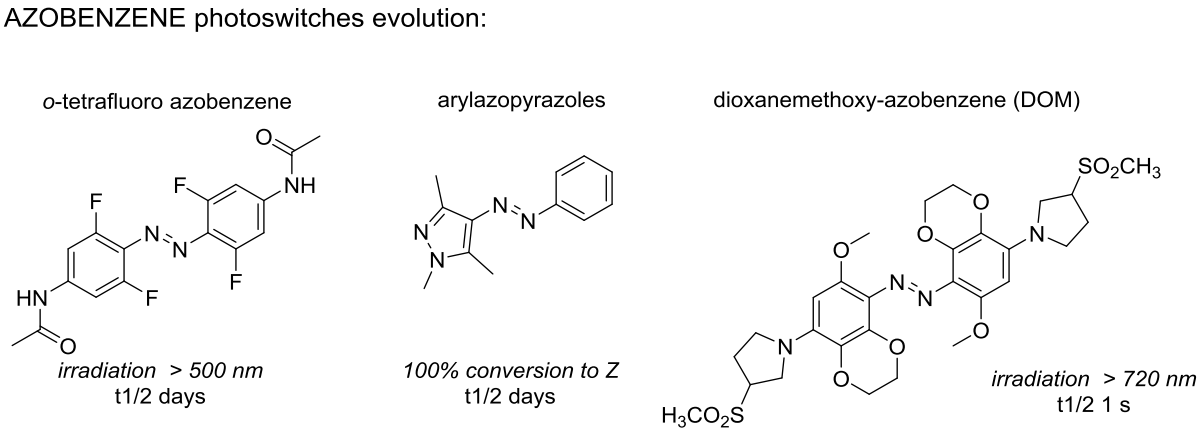


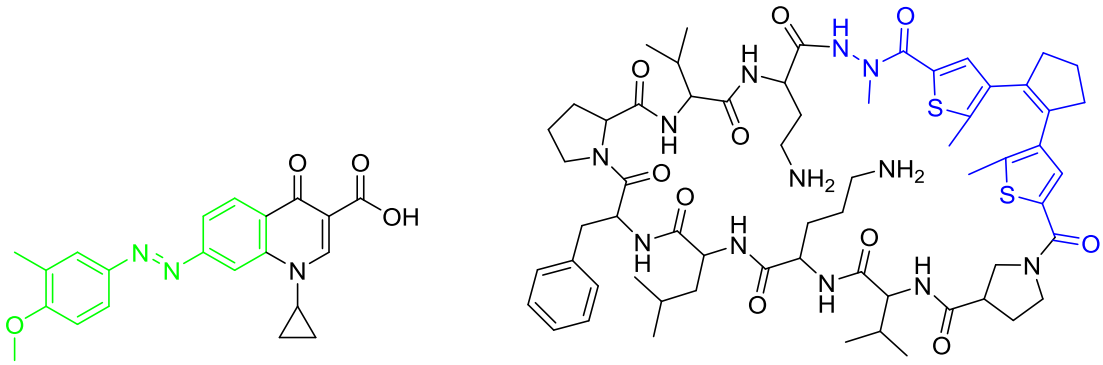
Figure 29: Chemical structures of latest azobenzene unit reported with tuned properties in the direction to reach long relaxation time, switching in the near IR region, total conversion in both isomeric states.

In the literature we can find different examples of these innovative types of medicine, among them there are photoswitchable antibiotics. Possible advantages that such smart

antibiotics could offer are limited level of active antibiotics in the environment thus limiting the onset of bacterial resistance, protection of human microbiota and possibly decreased side effects.

In a pioneering work, azobenzenes were merged into the antibacterially active quinolone scaffold [88]. The light induced *cis* isomer was more active than the corresponding *trans* with a four-fold difference in MICs for *E. coli*. A high conversion rate (ca 90% at the PSS) and it had a half-life in solution of several hours could be reached (Fig. 30).

Another example of photoswitchable antimicrobials was derived from the cyclic peptide gramicidin. Also in this case, up to ten fold differences in antimicrobial activities for the two isomers could be seen.



Feringa´s photoswitchable quinolone *Photoswitchable Gramicidin derivative*

Figure 30: Chemical structure examples of selected molecules possessing the best profile as photoswitchable antibiotics in the first work on the quinolone (2013) and gramicidin (2014).

To conclude, pioneering works[83,84,88,90,102,104] have demonstrated the basic functional principle of a photoswitchable drug. Such proofs of concept rised considerably the interest in this field, which still needs of major advancements in order to reach potential application in the human body.

2 AIM OF THE THESIS

There is a critical need for novel antibiotics, and the discovery of innovative molecular scaffolds active against Gram-negative bacteria is of particular importance.
Nature has been the richest source of antibiotics, and it still provides useful bioactive molecules that serve as starting points for drug development campaigns.
In this context the cystobactamids represent a promising lead scaffold for the development of a novel antibiotic due to their broad antibacterial activity and novel chemical architecture. The most active congener of this natural product class is cystobactamid 861-2 (Fig. 31), which showed remarkably good activities against clinically relevant Gram-negative bacteria such as *E. coli* and *P. aeruginosa*, but lacked inhibitory activity against other relevant pathogens such as *K. pneumoniae* and *E. aerogenes.* For these reasons, it was selected as the starting point of my doctoral studies.

The aim of this thesis is to invent cystobactamid analogs with enhanced antibiotic properties. Extending the spectrum coverage of this natural product class and improving the antimicrobial potency against critical bacteria, such as *P. aeruginosa* and *A. baumannii*, are of most importance. Furthermore, the potential to break bacterial resistance mechanisms against cystobactamids by chemical modifications should be investigated.

To reach these objectives, a synthetic route to the cystobactamids has to be established. After that, the optimization of the N-terminal part of the molecule will be pursued, followed by the assessment of the central and C-terminal parts, in order to establish structure-activity relationships that would aid the design of the next generations of cystobactamid analogs.
Two known resistance mechanisms for the related antibiotic albicidin will be considered within this thesis, i.e. the outer membrane protein Tsx and the endopeptidase AlbD. Regarding the latter, a solution to solve the potential enzymatic instability of the cystobactamids should be found.

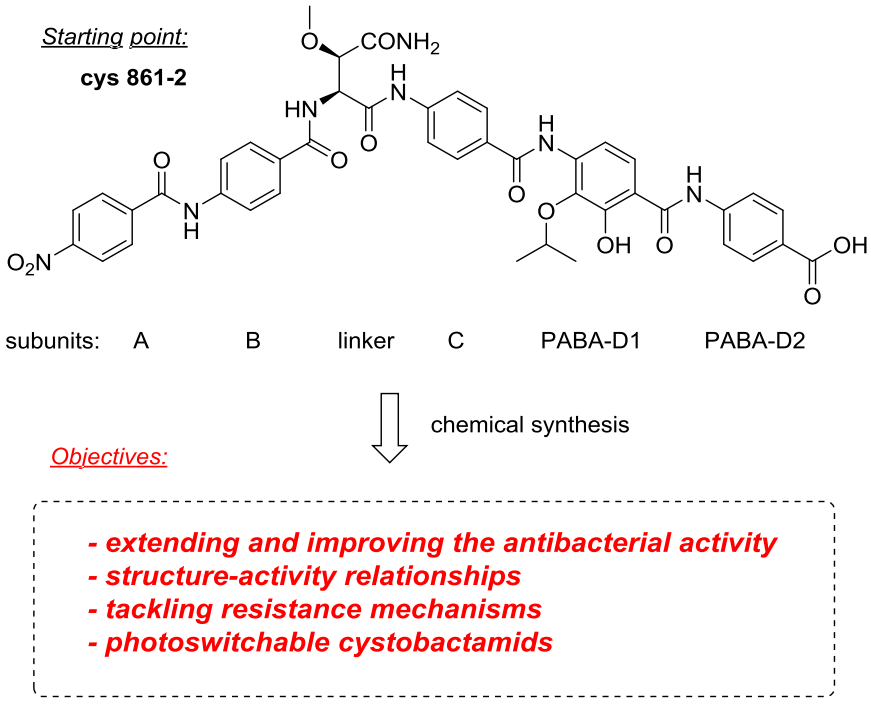


Figure 31: Lead structure of the natural derivative possessing the best antibacterial profile and the objectives of the thesis.

Additionally, my doctoral studies aim at a broader chemical and biological investigation of this natural product class. The potential of the cystobactamids scaffold to generate innovative, photoswitchable antibiotics will be investigated. These are “smart” molecules that offer spatial and temporal control of the antibacterial activity with light.

The specific objectives are to design and synthesize photoswitchable cystobactamids with a photoresponsive moiety inserted either at the N- or C- terminus, and to assess the application of a new type of photoswitch in photopharmacology.

3 RESULTS and DISCUSSION

3.1 STRUCTURAL SIMPLIFICATION

First, the opportunity of using a simplified structural template for medicinal chemistry investigations was assessed. Two compounds were designed in order to gain an understanding which the essential structural requirements for antibacterial activity are. For this reason, compounds bearing structural simplifications at two of the most complex moieties of the cystobactamids were targeted. With these analogs the relevance of the MeO- group in the Asn linker and the one of the characteristic phenol at the tetrasubstituted ring were assessed (Fig. 32).

In order to do this, the first two key steps of the project were the establishment of:

- a more concise synthesis of PABA-D1
- a synthetic access to the cystobactamid scaffold.

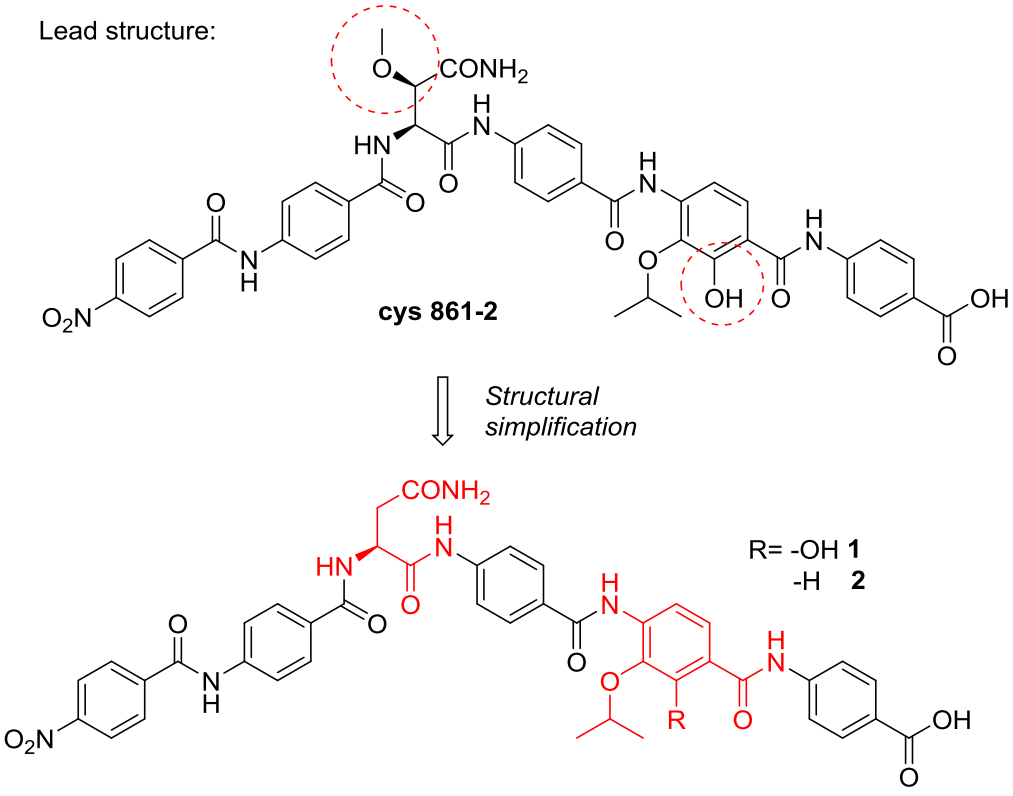


Figure 32: Structure of cys 861-2 and the first two targeted derivatives.

3.1.1 **PABA-D1 synthesis**

Ring PABA-D1 shows an unprecedented substitution pattern, with hydroxyl and isopropoxy moieties on the same side of the aromatic PABA unit. Three different synthetic strategies starting from commercially available compounds have been pursued in order to access the desired building block.

All of them relied on the introduction of a fourth substituent on an aromatic compound where at least three other functionalities were already in place (Fig. 33).

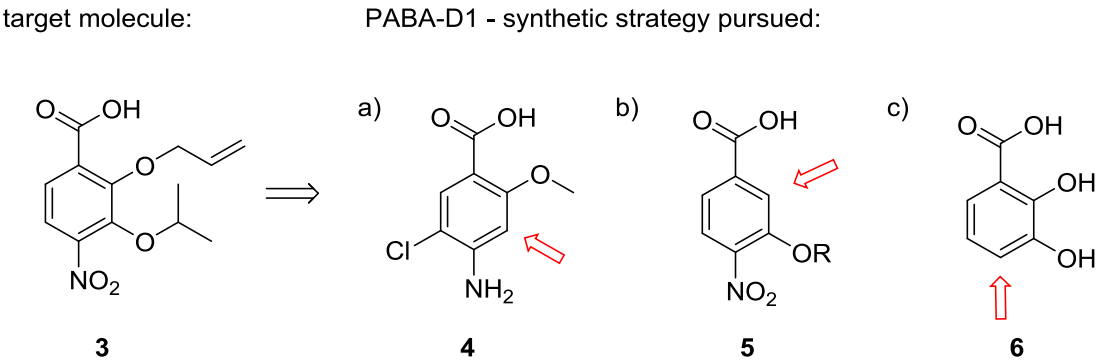
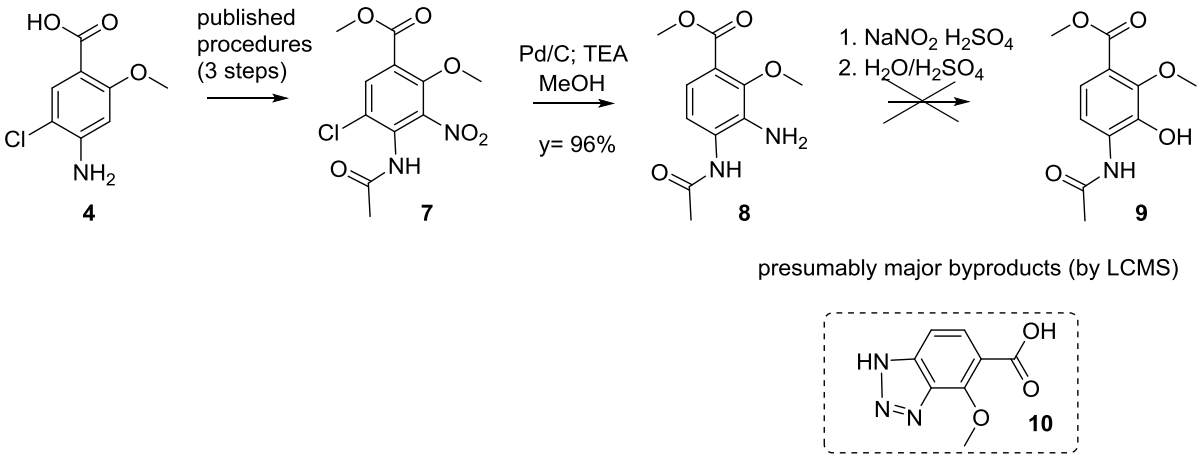


Figure 33: Starting materials employed in the three different routes pursued to access PABA-D1, the red arrows indicate the position where the 4th substituent should be introduced.

3.1.1.1 Strategy a)

Starting from **4** (Fig. 33), upon initial functional group interconversions, the introduction of the oxygen at the *meta* position to the carboxylic acid was planned. This should have been achieved via nitration followed by reduction and diazotation of the corresponding aromatic amine. Finally, the phenol was planned to be generated by treatment in water of the diazonium species (Scheme 1).



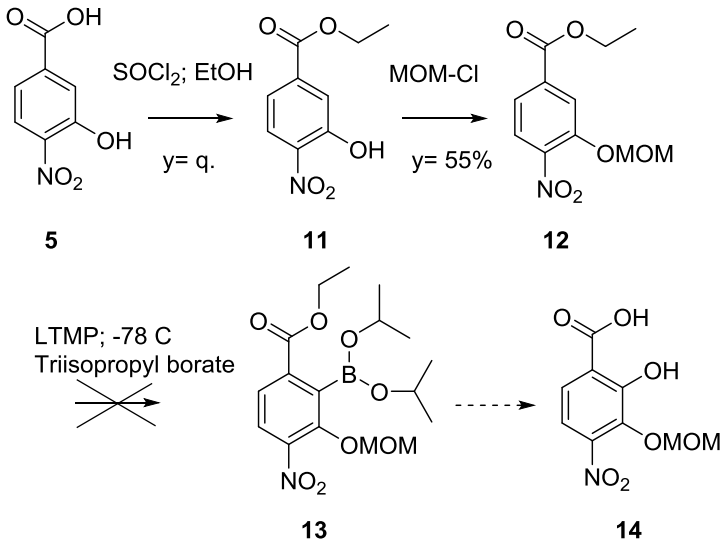
Scheme 1: First strategy attempted to access PABA-D1.

The first three steps were carried out following procedures previously reported[105]. An esterification was conducted, followed by acetylation of the aniline moiety and then nitration of the *meta* position to give compound **7**. Reductive cleavage of the C-Cl bond was carried out with Pd/C in methanol and TEA to afford the desired arene **8** for the critical step (H_2 pressure higher than 1 atm was important for a positive reaction outcome). At this point, the planned introduction of the phenol moiety via diazotation and treatment in water turned out to be unsuccessful due to undesired side reactions (Scheme 1). Possibly, the formation of the reactive diazonium salt was followed by intramolecular cyclization to afford the benzotriazole scaffold **10**. Under different reaction conditions hydrolysis of the ester and acetyl amide were observed. Due to the undesired side reactions this synthetic strategy was not further pursued.

3.1.1.2 Strategy b)

In the second synthetic route used, the starting material was arene **5** (Fig. 33). The first two steps saw esterification followed by protection of the phenol with MOM (Scheme 2) using adapted versions of previously reported experimental procedures[106]. The planned key step in this route was the introduction of the OH moiety ortho to the carboxylic acid. Desirably, it would have been achieved with directed ortho lithiation, followed by trapping of the carbanion with triisopropyl borate and final oxydation of the C-B bond to a phenol. Selectivity towards the desired position should have been obtained due to additive directing effects of both ester and MOM that functions as both protecting and directing group[107]. The

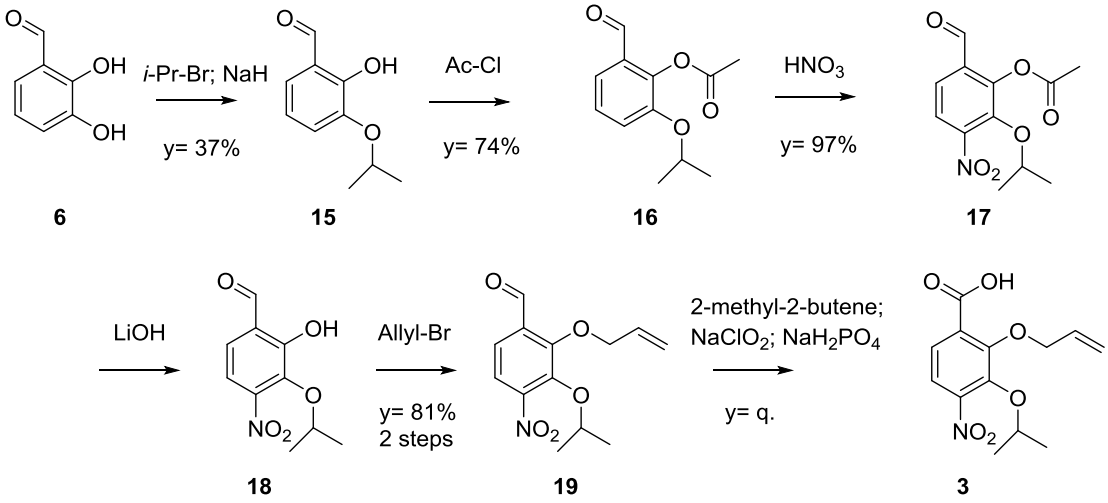
strategy turned out to be unsuccessful, probably due to the instability of the nitro group under such strong basic and nucleophilic conditions.



Scheme 2: Second strategy attempted to access PABA-D1.

3.1.1.3 Strategy c)

Synthetic access to PABA-D1 was established starting from arene **6** (Fig. 33). Differently from the two previous strategies attempted, the tetrasubstitution pattern was achieved introducing the nitrogen at the para position instead of an oxygen either at the *meta* or *ortho* position.



Scheme 3: Successful strategy used to access PABA-D1.

In the first step 2,3-dihydroxybenzaldehyde (**6**) was alkylated selectively with isopropyl bromide using sodium hydride as base[108], followed by acetylation of the phenol in position 2 (Scheme 3). Arene **16** was nitrated at the *para*-position with fuming nitric acid, selectivity for the para position could be achieved by lowering the temperature to -40°C. Cleavage of the acetyl group followed by alkylation with allyl bromide provided aldehyde **19**, which was finally oxidize to the corresponding carboxylic acid (**3**) using Pinnick reaction. The building block obtained was used without further purification in the coupling step that will be described in a coming section.

In doing so, a new synthetic access to PABA-D1 was established. The first and only synthesis previously published required 12 steps with an overall yield of 6%, while the one presented herein had six steps with overall yield of 22%. These results have already been published [51].

3.1.2 **Synthetic access to cystobactamid analogs**

The first cystobactamid derivative targeted (**2**) bears two major modifications with respect to cystobactamid 861-2, it lacks both the methoxy group in the central Asn moiety and the phenol on PABA-D1. Retrosynthetically, it was disconnected in three fragments (Fig. 34). During the synthesis, trityl was chosen as protecting group of the primary amide to avoid side reactions in the coupling steps, and the aniline moieties were masked as nitro groups.

A different approach that relied on the disconnection in two fragments (fragments A and B of the figure below already coupled) was also attempted but it turned out to be unsuccessful due to the very poor solubility and reactivity of the larger intermediate.

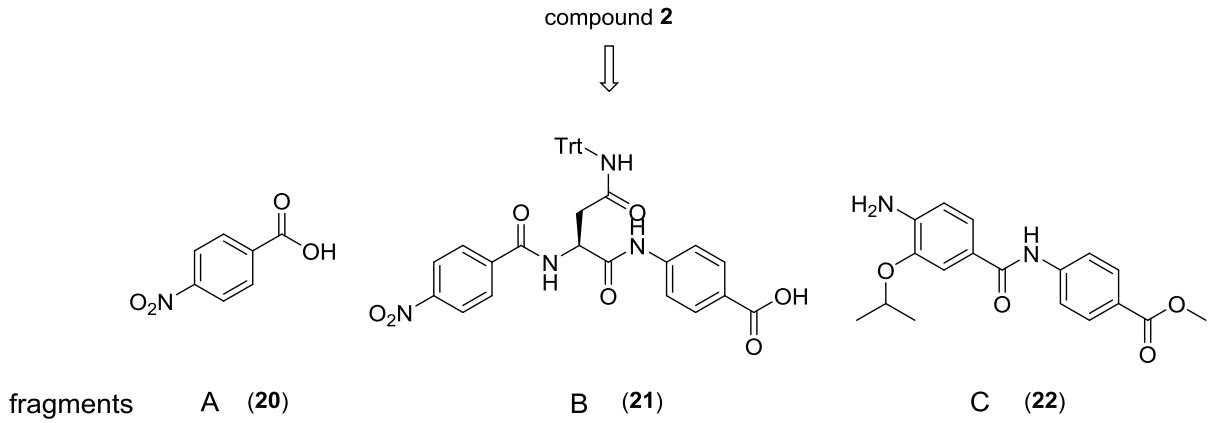


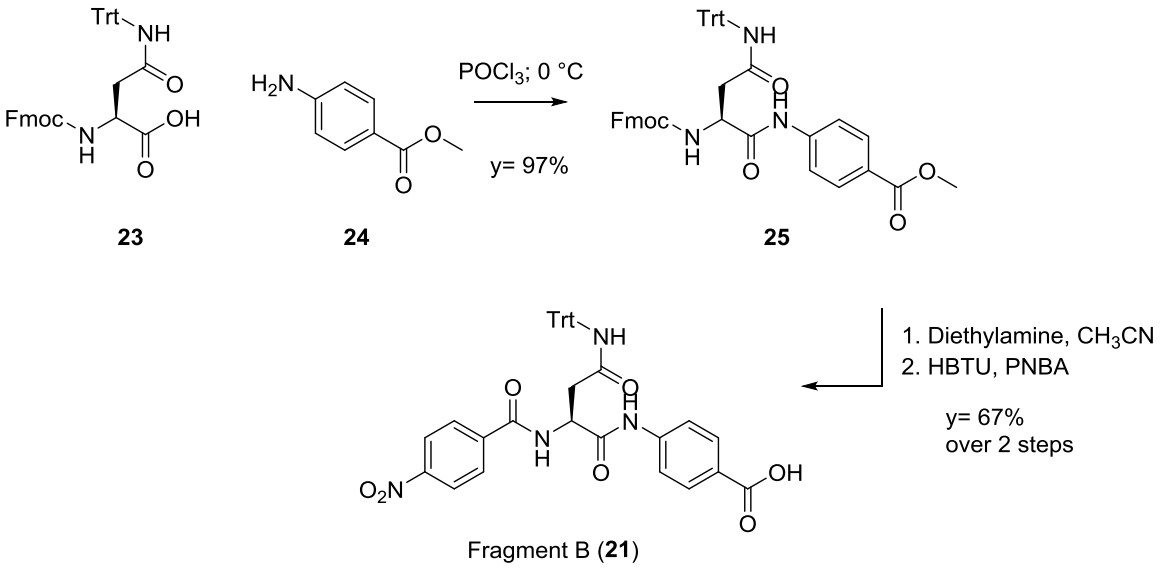
Figure 34: Retrosynthetic disconnection of derivative **2**.

3.1.2.1 Fragments synthesis:

3.1.2.1.1 Fragment B

For the synthesis of central fragment B (Scheme 4), Fmoc-Asn(Trt)-OH (**23**) and methyl *p*-aminobenzoate (**24**) were coupled efficiently by means of $POCl_3$ at 0 °C. Keeping the temperature low was important in order to avoid racemization[109]. Using this chlorinating reagent aspartimide formation was avoided. This common side reaction of asparagine, although trityl protected, was observed with other coupling reagents, such as HATU.

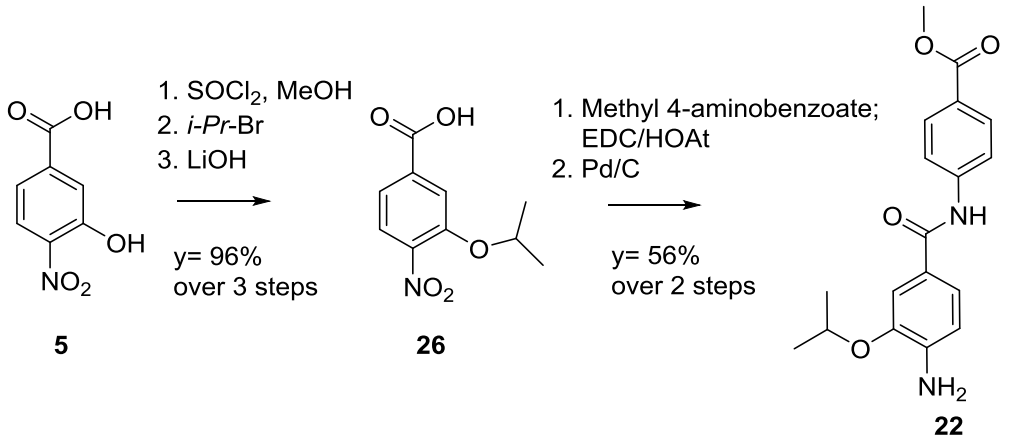
Installation of the second PABA unit was carried out using HBTU and para nitro benzoic acid (PNBA), upon deprotection of Fmoc protecting group. Hydrolysis of methyl ester using standard conditions led to complete racemization. This could be avoided treating it with excess of LiI in ethyl acetate at refluxing conditions to afford carboxylic acid **21**[110].



Scheme 4: Synthesis of central fragment B.

3.1.2.1.2 Fragment C

The synthesis of the diaryl building block was carried out with a five step sequence (Scheme 5). In brief, esterification of **5** using $SOCl_2$ in MeOH followed by alkylation and hydrolysis afforded carboxylic acid **26** in nearly quantitative yield. This was coupled with commercially available methyl ester of PABA by means of EDC and HOAt in a moderate yield, finally the nitro group was reduced with $SnCl_2$ in ethanol to afford amine **22**.



Scheme 5: Synthesis of fragment C.

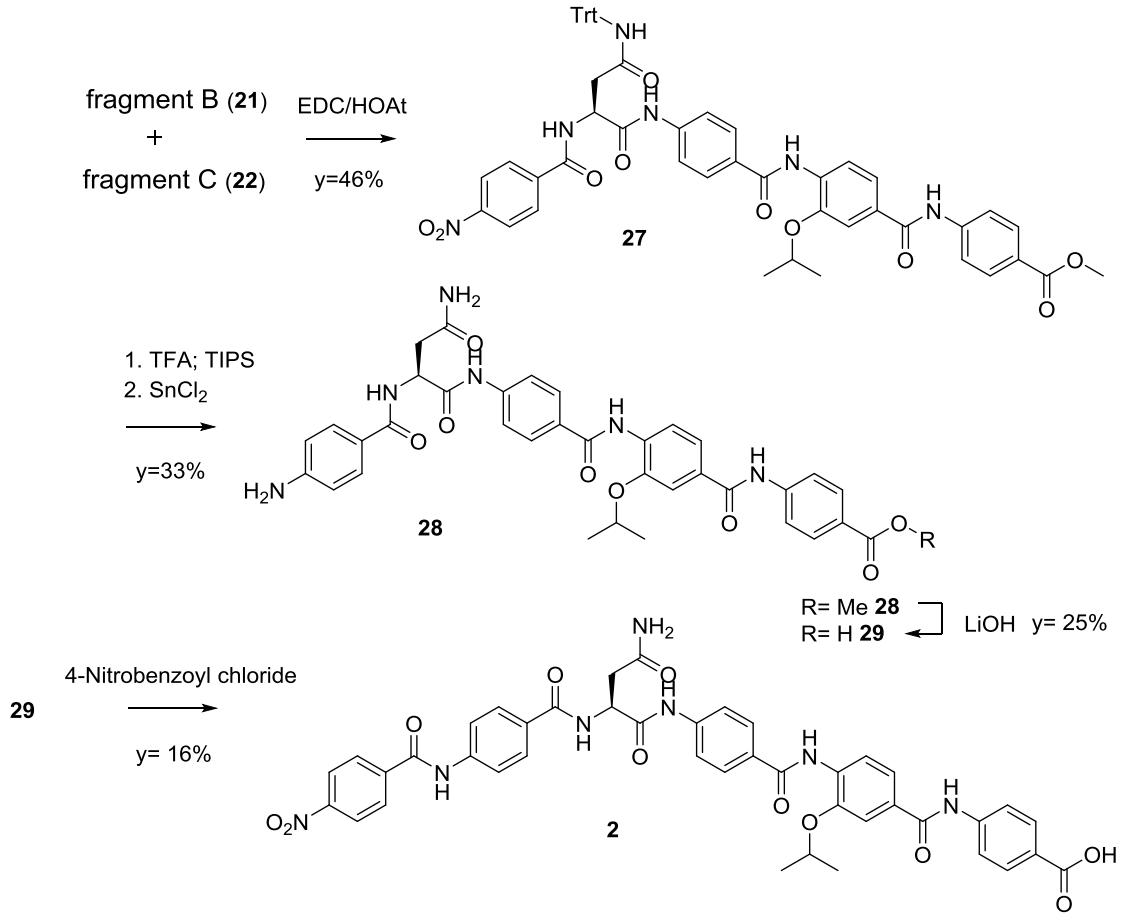
3.1.2.2 Final assembly of the molecule:

The final steps of the route started with the assembly of fragments B and C (**21** and **22**). This turned out to be the most difficult coupling step of the whole synthesis due to the low reactivity of the hindered aniline moiety and the carboxylic acid fragment. The coupling reagents employed were ECD/HOAt using excess of collidine as base, the reaction proceeded well but required 6 days, after which compound **27** was isolated with around 50% yield (Scheme 6). These reaction conditions most probably altered the enantiomeric excess of the molecule and therefore alternative ones were needed.

The trityl (Trt) protecting group was then removed with TFA and TIPS (carbocation scavenger) in DCM to liberate the primary amide. The intermediate was directly used in the reduction step of the nitro moiety to give the corresponding aniline **28** (Scheme 6).

With the growing oligomer also the sensitivity of the peptide bonds to basic conditions increased. Therefore the methyl ester of **28** was cleaved before the introduction of the last aromatic ring. The hydrolysis was carried out in THF/water using 2 eq. of base (LiOH) with a reaction time of 2.5 days. Carrying it out with a higher amount of base led to increased amounts of hydrolytic by-products.

Aromatic ring A, activated as an acyl chloride, was coupled to amino acid **29** using Schotten Baumann conditions, and the desired product **2** was isolated by preparative RP-HPLC.



Scheme 6: Final steps of the synthesis

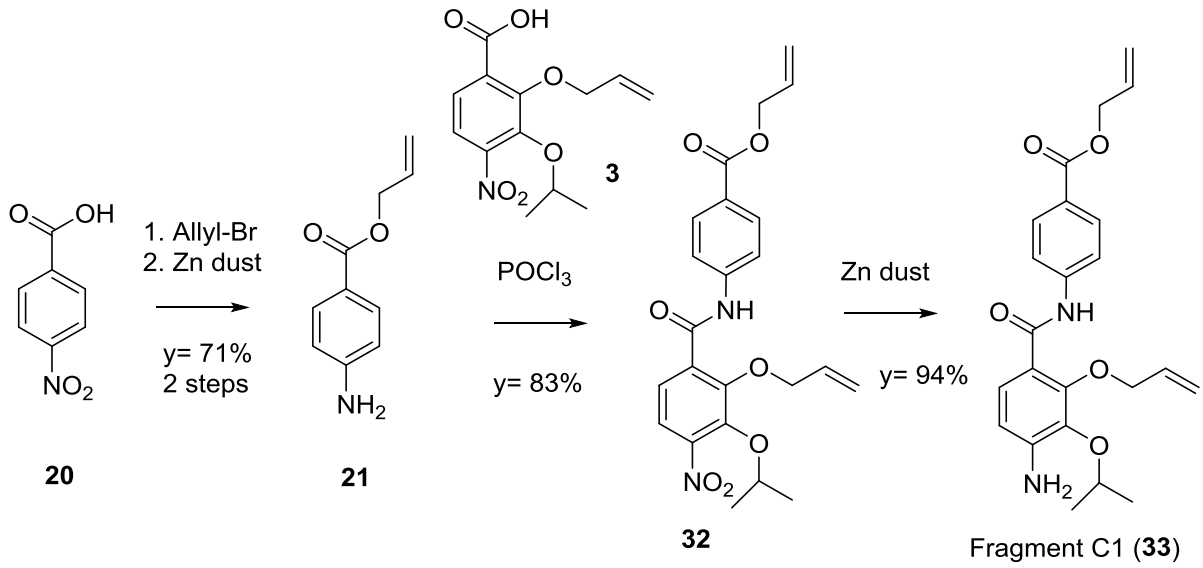
3.1.2.3 Cystobactamid des methoxy 861-2

With the established synthesis of PABA-D1 at hand, also the structurally simplified analog lacking only the methoxy moiety (compound **1**) was targeted. For its synthesis, an adapted route of the one just described was employed. Major differences concerned fragment C and the experimental procedures of the critical coupling step discussed before, in order to avoid potential racemization.

In particular, for the coupling step of the two larger fragments stronger conditions were needed, in order to shorten the reaction time and thus to avoid racemization. The degree of racemization was assessed in each critical step of the synthesis using the Marfey assay [111] (see section 3.1.4).

A different protecting group strategy was adopted for fragment C1, in order to avoid the basic conditions needed for methyl ester hydrolysis. Additionally, the phenol present on PABA-D1 needed to be protected for a positive outcome of critical coupling steps. For these

reasons, both phenol and carboxylic acid were protected with an allyl group that can be removed under neutral conditions. Compound **32** was obtained by coupling the allyl ester of PABA (**21**) with PABA-D1 building block (**3**), with $POCl_3$ as the coupling reagent (Scheme 7). Reduction of the nitro group with Zn^0 and 10% acetic acid in EtOH afforded aniline **33**. In most of the reactions where a NO_2 group had to be reduced, either Zn^0 in acidic conditions or $SnCl_2$ were the methods employed. The former method should be favored over the second one due to the more practical work-up of the reaction and the lower toxicity of the waste materials generated.



Scheme 7: Assembly of fragment C bearing PABA-D1 moiety in which the phenol is masked as an allyl ether.

Aniline **33** was then coupled to central fragment B (**21**). The most efficient reagent found was once again $POCl_3$. Standard coupling reagents, such as HATU or EDC, were found to be inefficient. Whereas pre-activation of the carboxylic acid with other chlorinating agents such as oxalyl or thionyl chloride mainly led to decomposition, presumably due to side reactions of the amide of the Asn side chain (although Trt protected).

With the conditions employed, these side reactions could be avoided presumably because the carboxylic acid was activated in situ. The order of addition of the reagents turned out to be important. When adding first DiPEA and then $POCl_3$, formation of the mixed anhydride of **21** was mainly observed, with traces amount of product generated (despite the reactive nature of the byproduct, no reaction could be observed with the aniline even under enforced conditions such as heating for prolonged time). A different outcome was observed when $POCl_3$ was carefully added first, followed by the base. In this case, formation of the

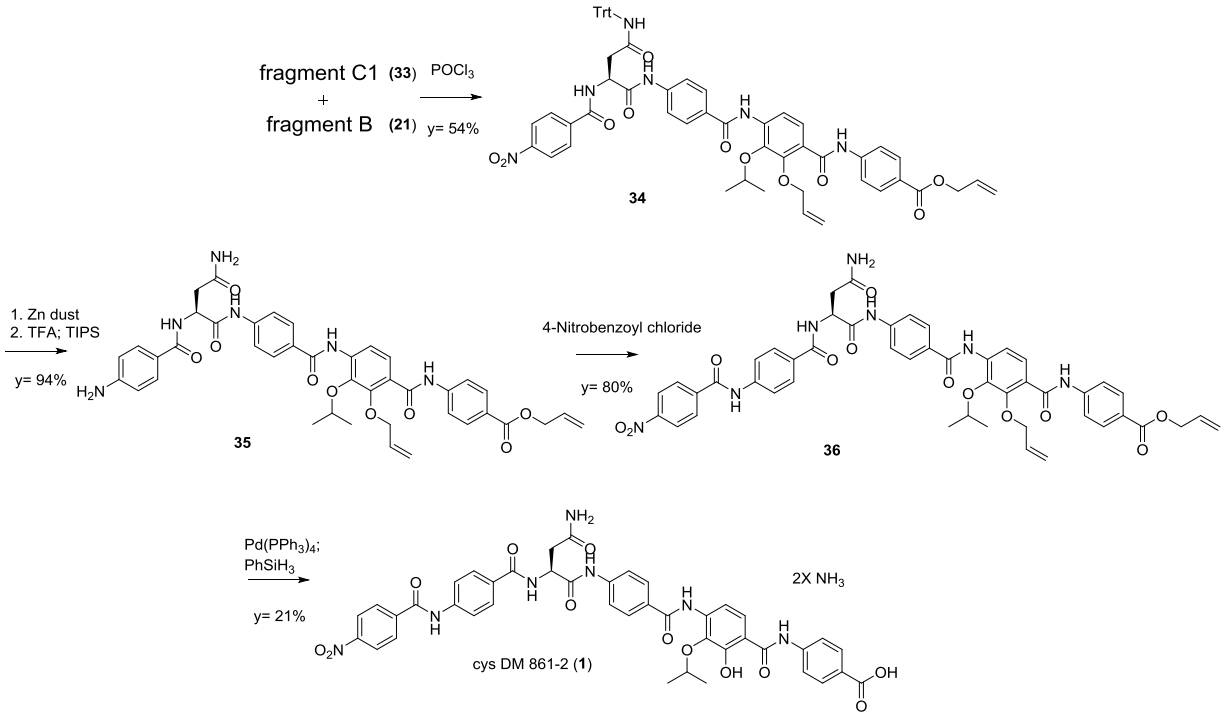
desired coupling product was obtained, and only traces of the byproduct were present (Scheme 8).

In sum, the reagent of choice also for this critical coupling step was $POCl_3$, the desired intermediate could be obtained in around 60% yield with a shortened reaction time and partial racemization could be avoided.

As described before, removal of the Trt group and reduction of the nitro group afforded advanced amine **35**. The last aromatic ring PNBA was activated to acyl chloride and installed in good yield. Allyl protected cystobactamid **36** was then treated with tetrakis(triphenylphosphine)palladium^0 and phenyl silane as scavenger to give the desired derivative, which was purified with preparative RP-HPLC using a gradient of 10 mM aqueous NH_4HCO_3 and acetonitrile.

The establishment of a proper purification method was not trivial due to the low solubility of the molecule in either water or organic solvents. Using an acidic system for the reversed phase purification, only small amounts of product with unsatisfactory purity could be isolated. A convenient method was found using the condition described above, shifting the pH to slightly basic (ca. 8). In this manner, the solubility of the cystobactamid was clearly enhanced and its decomposition could be avoided.

In sum, the synthesis of the derivative cys DM861-2 (**1**) could be achieved in 12 steps (longest linear sequence) with an overall yield of 1.9%.



Scheme 8: End game of the synthesis of cystobactamid des methoxy 861 (cys DM861).

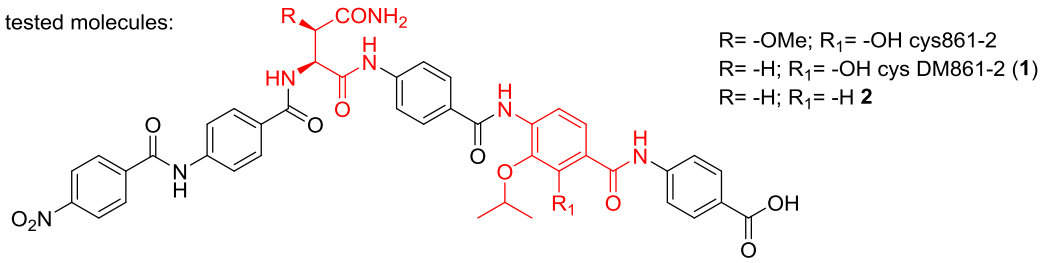
3.1.3 **Biological activities**

In order to assess the tolerability of the introduced structural changes, the two synthesized cystobactamid analogs were tested against *E. coli, P. aeruginosa, S. aureus* and the respective mutants of the two Gram-negative bacteria lacking a major efflux pump (TolC for *E. coli* and MexAB for *P. aeruginosa*) (Table 3).

The compound lacking both –OMe and –OH (**2**) was active only against the mutant bacterial strains, thus suggesting that this molecule is subject to efflux processes.

Cystobactamid desmethoxy 861-2 (**1**) retained inhibitory activity in the low µg/mL range comparable to the parent compound against the tested strains with the exception of *P. aeruginosa* wild type (PA WT). A comparison of the activity between mutant and WT strains suggested that this molecule is substrate for the multidrug efflux system MexAB, whereas efflux mediated by TolC seems to play a minor role.

Table 3: Antimicrobial and *E. coli* (Ec) gyrase inhibitory activities of structurally simplified analogs in comparison with the ones of cys 861-2.



MIC [µg/mL]	*E. coli* ΔtolC	*E. coli* WT	*P. aeruginosa* ΔmexAB	*P. aeruginosa* WT	*S. aureus*	EC gyrase IC_{50} [µM]
Reference Cys 861-2	0.125	0.125	0.5	2	0.5	0.22[a]
2	0.06	> 64	1	> 64	> 64	nd
1	0.06	0.13	1	> 64	0.5	0.11[a]

MIC and DNA supercoiling assays performed at the Helmholtz institute for pharmaceutical research Saarland, department of microbial natural products (MINS) by Dr. Jennifer Hermann.

Considering the comparable ability to inhibit gyrase, which was even higher for the simplified analog DM861-2 (**1**), it seems that the central MeO- and the -OH on PABA-D1 moieties are important structural features of the cystobactamid scaffold for evading the efflux pump systems of the pathogens.

Although cystobactamid DM861-2 lacked activity against *P. aeruginosa* WT, it represents a simplified structural template for establishing structure-activity relationships of this new class of natural products.

As the established synthetic route allowed accessing a larger number of analogs compared to routes based on methoxy-asparagine, it was decided to design further derivatives with an asparagine unit. This decision was also supported by the fact that albicidin (lacking of the methoxy in the central β-cyanoalanine) was reported to be active on *P. aeruginosa*. This thus suggested that activity against this pathogen could be mediated not only by the central linker unit, but also by other structural modifications of the scaffold.

3.1.4 **Marfey analysis**

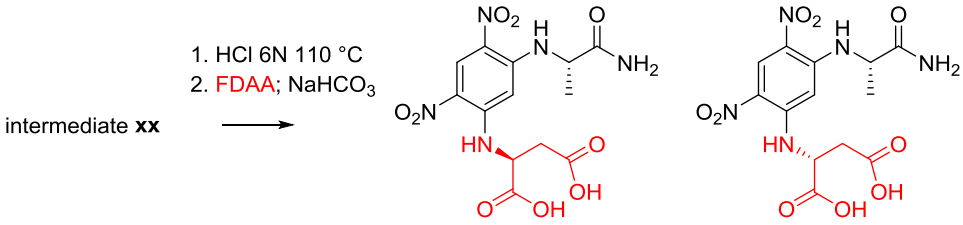
The steps in which partial racemization could have occurred, due to the reaction conditions, were investigated with Marfey´s analysis[112] [113] [114] (Table 4) (for experimental details see section 4.2.1.1.).

The derivatization procedure in brief, the desired intermediate was hydrolyzed with HCl to generate aspartate. After lyophilization, the sample was reacted with FDAA reagent 1% in acetone and subjected to LCMS analysis. The amount of each diastereoisomer is determined by area integration of the extracted ion chromatogram (EIC 384.10±0.3 -All MS).

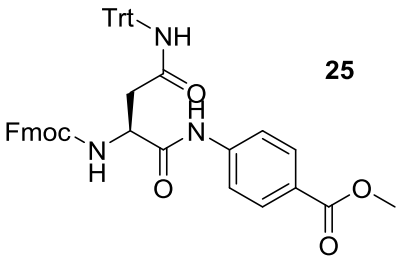
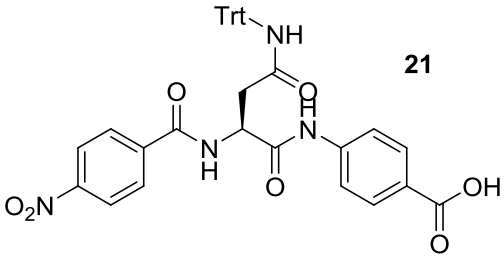
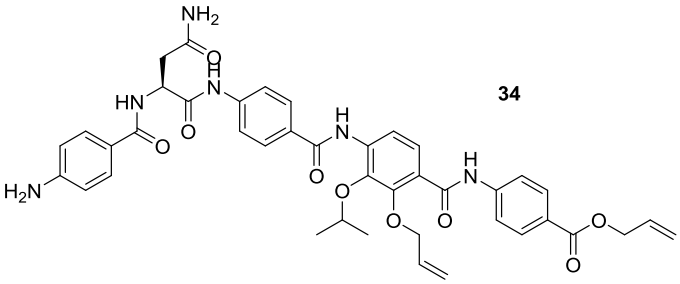
During the derivatization process, partial racemization occurs as assessed with commercially available **23.**

Taken together, the information presented in Table 4 proved that the synthetic route established for the synthesis of **1** did not entail significant racemization of the chiral Asn.

Table 4: General scheme describing Marfey´s method and selection of intermediates analyzed.



Chemical Formula: $C_{13}H_{15}N_5O_9$
Exact Mass: 385,0870

Molecule	% of (*S, S*) diastereoisomer
Fmoc-Asn(Trt)-OH (**23**)	95.8
 **25**	96.5
 **21**	94.0
 **34**	94.0
DM 861-2 (**1**)	91.2

3.2 N-terminal OPTIMIZATION

After assessing possible structural simplifications, the medicinal chemistry investigation was continued using DM861-2 as structural template. It was decided to first investigate structural variations of the N-terminus, this was for different reasons.

First, the established synthetic access sees the late-stage introduction of aromatic ring A. Furthermore, the structural difference to albicidin and the published albicidins activity data [67,69] suggested that this part of the molecule can tolerate different structural motifs and that modifications of it can be exploited to enhance the enzymatic inhibitory activity.

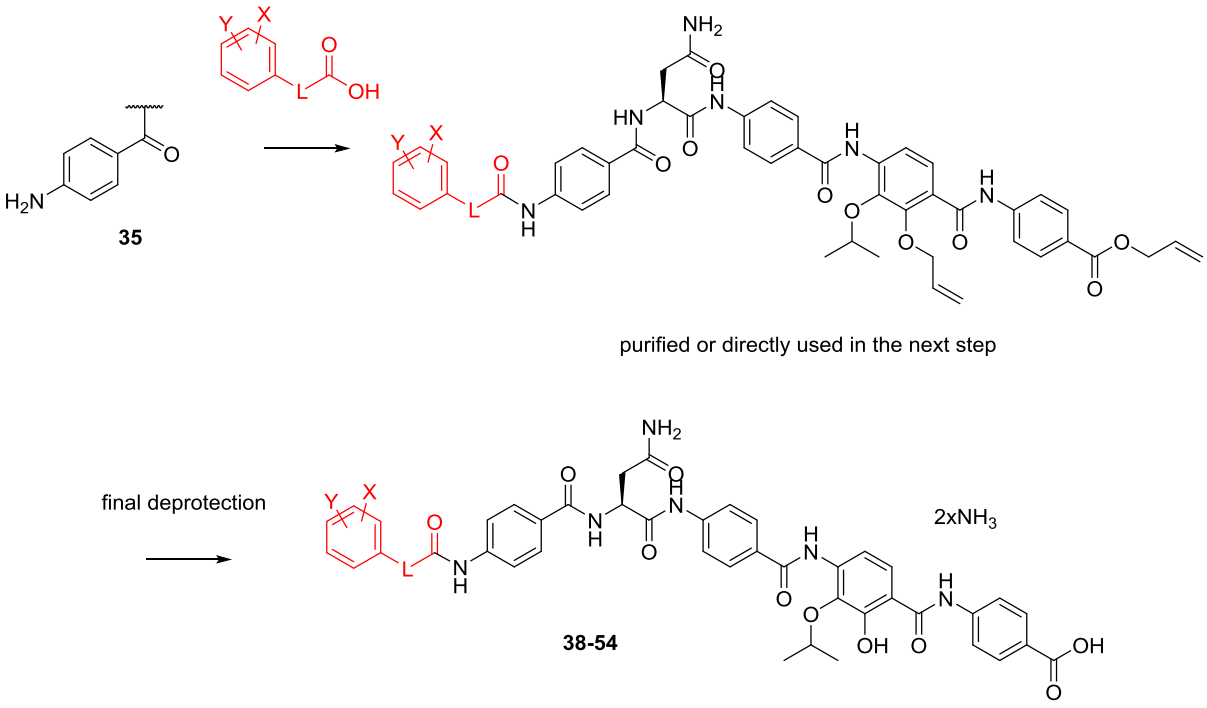
Additionally, the presence of a NO_2 group confers very low solubility properties and potentially could be the cause of metabolic instability and toxicity.

Therefore, the aims of this first round of analogs synthesis were to find a replacement for this undesirable functional group, to gain knowledge of the structure-activity relationships of the N-terminus and to enhance the antibacterial activity of the cystobactamids.

3.2.1 **General synthetic scheme**

The general synthetic Scheme 9 has been followed for the synthesis of derivatives bearing modifications at ring A. Amine **35** was synthesized on a gram scale and coupled to different aromatic acyl moieties that were either purchased or synthesized. Coupling reactions were carried out upon activation of the acid to acyl chloride by means of BTC and collidine[65] or oxalyl chloride. Alternatively, when the acid was bearing a moiety which could give side reactions with such reagents, a milder activation strategy was adopted. A combination of EDC and HOAt was chosen, which could still ensure strong enough coupling conditions even if the reaction time was longer in these instances.

Allyl protected cystobactamid analogs could be either purified or directly employed in the final deprotection step. Most of the cystobactamids were purified by preparative RP-HPLC with a water/acetonitrile gradient that contained NH_4HCO_3 (10 mM) as modifier, thus they were obtained as ammonium salt. Depending on the analog, the overall yield over these two steps could vary from around 10% to 50%.



Scheme 9: General scheme of the last coupling and final deprotection steps.

3.2.1.1 Synthesis of variants of building block A

Variants of building block A (Fig. 35) were prepared for being used in the coupling step of Scheme 9. They represent variations of para nitrobenzoic acid that is present in the original scaffold of the cytobactamids.

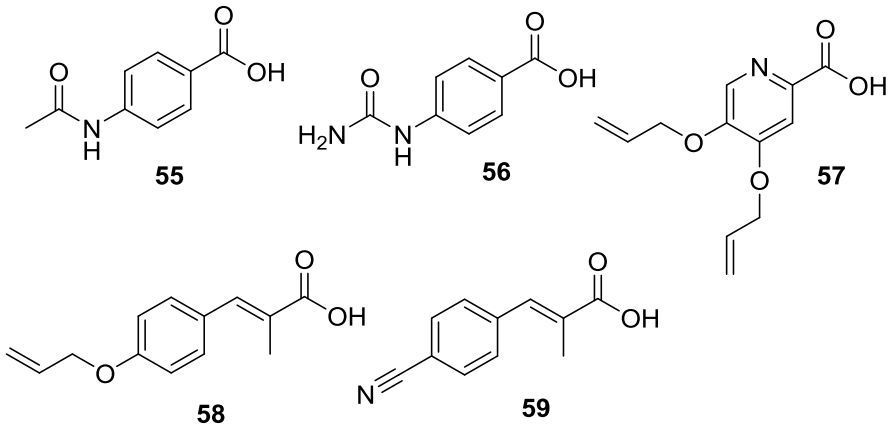
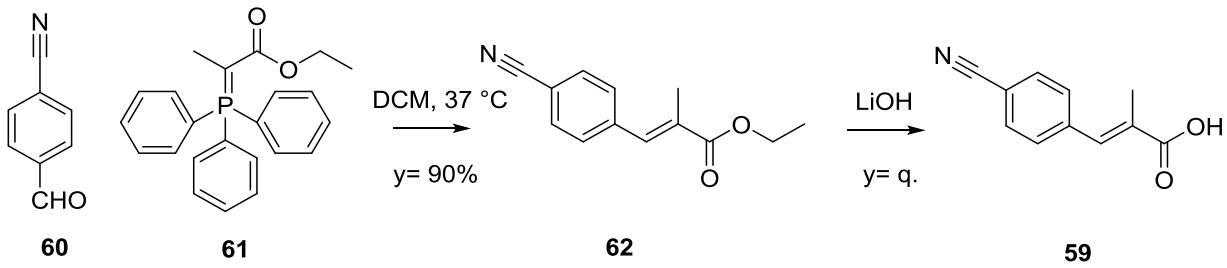


Figure 35: Different building blocks A prepared.

4-Acetamidobenzoic acid[115] (**55**) and (E)-3-(4-(allyloxy)phenyl)-2-methylacrylic acid[65] (**58**) were prepared according to reported experimental procedures while (E)-3-(4-cyanophenyl)-2-methylacrylic acid[65] (**59**) and 4,5-bis(allyloxy)picolinic acid [116117] (**57**) with only minor adaptations.

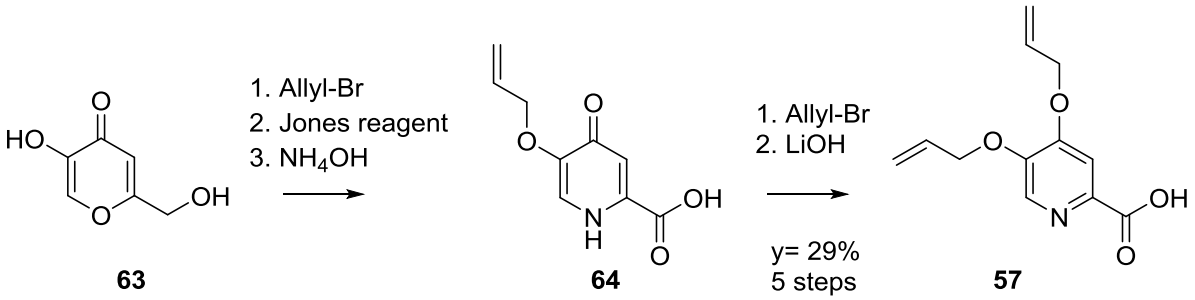
For **59**, a Wittig reaction between 4-cyanobenzaldehyde and 2-(triphenylphosphoranylidene) propionate afforded mainly the desired E isomer, which was isolated with good yield, finally hydrolysis of ester **62** with LiOH afforded the desired building block (Scheme 10).



Scheme 10: Synthesis of modified N-terminal acyl residue of albicidin.

The synthesis of the siderophore protected moiety (**57**) started from Kojic acid (**63**). Mono alkylation followed by oxidation of the primary alcohol to the corresponding carboxylic acid and finally exchange of the oxygen part of the ring with nitrogen, simply upon treatment in ammonium hydroxide, afforded the siderophore scaffold 4-pyridon-2-carboxylic acid (**64**) (Scheme 11).

Any attempts to couple this molecule with the amine **35** failed, thus it was decided to protect also the ketone. Treating **64** with slightly excess of allyl bromide afforded the tris allyl protected pyridine derivative, which underwent hydrolysis under standard condition to afford the desired carboxylic acid **57**.



Scheme 11: Synthesis of siderophore protected moiety.

The benzoic acid derivative **56** was synthesized starting from the methyl ester of PABA. Formation of the urea was carried out by treating the aniline with BTC, the resulting carbamoyl chloride was subsequently quenched with NH_4OH. Hydrolysis of the ester gave the desired carboxylic acid.

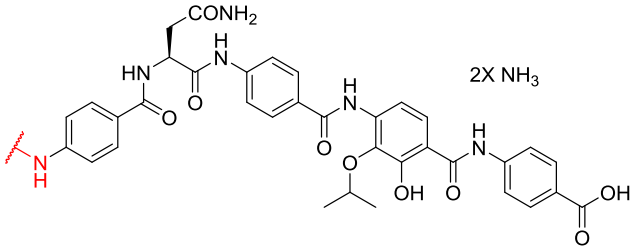
3.2.2 **1th series of ring A analogs**

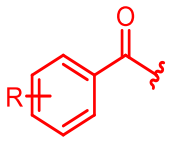
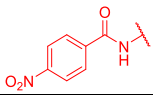
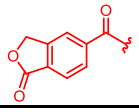
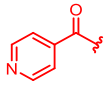
The aim of the first series of analogs (Table 5) was to understand which features the substituent on the aromatic ring A should possess. Particularly, its electronic properties, position and polarity were assessed. The apolar nitro was reduced to an amino group or replaced by a substituent with different characteristics, for instance the acetamido moiety. The rationale behind this compound was to insert an additional amide group that ideally could have extended the possibility of amidic H-bond interactions. In other molecules, the original substituent was moved to the *meta* and *ortho* positions. Bioisosteric substitutions have been pursued with the analogs bearing a cyano, trifluoromethyl, methanesulfonyl moieties or with compounds **52** and **53**.

The design of **56** bearing the urea was inspired by carbamoyl albicidin and the hope to increase the solubility of the molecule. An extended cystobactamid was also obtained, where a third aromatic ring had been added at the N-terminal. A compound bearing the same substitution pattern of PABA-D1 at ring A was also synthesized.

The biological activities and structures of these first N-terminal analogs are presented in Table 5.

Table 5: Chemical structure of derivatives synthesized in the first series of ring A modifications and their biological characterization (antibacterial activities and *E. coli* gyrase inhibitory properties for selected analogs).



				MIC [µg/mL]					EC gyrase IC_{50} [µM]
Compound				*E. coli* ΔtolC	*E. coli* WT	*P. ae.* ΔmexAB	*P. ae.* WT	*S. au.*	
	R								
	o	*m*	*p*						
Reference DM861-2	*-H*	*-H*	*-NO_2*	0.125	0.5	1	> 64	1	0.11[a]
38	*-H*	*-H*	*-NHAc*	0.06	16	> 64	> 64	64	nd
39	*-H*	*-H*	*-H*	0.5	> 64	2	> 64	> 64	4.0[b]
40	*-H*	*-H*	*-F*	0.5	0.5	2	> 64	2	nd
41	*-H*	*-H*	*-NH_2*	32	2	4	> 64	> 64	26.8[b]
42	*-H*	*-NO_2*	*-H*	0.25	> 64	0.5	> 64	> 64	2.2[b]
43	*-H*	*-NH_2*	*-H*	2	32	4	> 64	> 64	nd
44	*-NO_2*	*-H*	*-H*	2	8*	16*	> 64	> 64	nd
45	*-H*	*-H*	*-$NHCONH_2$*	0.03	0.5	4	> 64	64	8.7[b]
46	*-H*	*-H*	*-CN*	0.06	0.5	0.25	2	0.25	0.08[a]
47	*-H*	*-H*	*-CF_3*	≤ 0.03	0.5	64*	> 64	16-32	nd
48	*-H*	*-H*		> 64	0.5*	> 64	> 64	> 64	2.5[b]
49	*-OH*	*i-PrO*	*-NO_2*	> 64	> 64	> 64	> 64	> 64	>50[b]
50	*-OH*	*i-PrO*	*-NH_2*	0.01	2	2-4	32*	> 64	50[b]
51	*-H*	*-H*	*-SO_2CH_3*	<0.03	2	4*	>64	>64	nd
52				<0.03	<0.03	1	>64	1	1.4[b]
53				<0.03	1	2	> 64	> 64	
54				<0.03	<0.03	>64	>64	>64	5.5[b]

P. ae.= *P. aeruginosa*, *S. au.*= *S. aureus*; [a] determined at the department of microbial natural products

(MINS), [b] determined at the department of chemical biology (CBIO), further details are given in the experimental part; nd= not determined; * the value could not be determined unambiguously and might be higher than assigned. Dr. Jennifer Hermann and Katarina Cirnski performed the MIC assays. [a] Dr. Jennifer Hermann and [b] Dr. Peter-Hans Prochnow performed the DNA supercoiling assay.

For the brief discussion the reference compound will be DM861-2 (first line of Table 5).

Moving the substituent to different positions of the aromatic ring as well as introducing polar EDG, was in general deleterious for the antibacterial activity and also for the efficiency to inhibit gyrase. For instance, the compound with the NO_2 at the *meta* position (**42**) had a worse antibacterial profile compared to DM861-2 and a ten-fold weaker gyrase IC_{50} value. Even more pronounced was the effect of the nitro reduction on gyrase inhibitory activity, as the compound bearing an aniline moiety (**41**) showed a 100-fold decreased IC_{50}. The *ortho* position seemed to be the worst, compound **44** was active only against mutant *E. coli*.

On the other side, molecules bearing EWG, apolar substituents such F, CF_3, CN (**40**, **47**, **46**) had in general good antibacterial profiles comparable to the one of the reference structure, and also the gyrase inhibitory activity was in the same sub-µM range.

Remarkably, the derivative **46** with a cyano group as a NO_2 bioisoster was active also against *P. aeruginosa* WT and had the highest gyrase inhibitory activity among all the cystobactamids tested so far (both natural and synthetic ones).

Considering the success of the CN moiety, other compounds with NO_2 bioisosters were synthesized, these are the one with the pyridyl moiety, a γ-lacton fused to the aromatic ring, trifluoromethyl and the methanesulfonyl group (**53**, **52**, **47** and **51**). All of them possessed good to very good activity against both *E. coli* strains tested but were lacking activity against *P. aeruginosa* WT.

Compounds **49** and **50** that bear the same substitution pattern of PABA-D1 had activities that are not in line with the previous information on SAR. For these, the analog bearing a NH_2 showed a better antibacterial profile compared to the one with the NO_2, that was inactive against all the pathogens tested. Interestingly, although the IC_{50} of the former was around 50 µM, far from the range of the best cystobactamids, it showed very good activity against mutant *E. coli*.

Gyrase A inhibitors can target also topoisomerase IV, as it was shown for albicidin and cystobactamids. The high IC_{50} of analog **50**, but still good MIC values might be rationalized by an enhanced topoisomerase IV affinity or additional capability to inhibit other

topoisomerases. This is only a speculation but if so a possible explanation could be that certain structural variations entail changes at the overall shape of the molecule, resulting in increased affinity for alternative target enzymes.

The extended, heptameric cystobactamid **48** showed good gyrase activity (2.5 µM), but it was lacking of antibacterial properties with the exception of only *E. coli*. Interestingly, this molecule was active against the wild type but not the ΔtolC strain.

The last analog of the Table is a confirmation of the information already gained on aromatic ring A: the four fluorine substituents decreased the overall electron density of the ring, as a consequence improved IC_{50} and MICs values were obtained in comparison to the *para* NH_2 monosubstituted derivative (**41**).

In conclusion, these results represent the first structure-activity relationships of the N-terminus established with synthetic cystobactamids. Particularly, they clearly indicate that aromatic ring A is very important for the interaction with the target enzyme and can mediate cellular penetration. Optimal binding is reached with an electron poor aromatic ring, and the preferable position of the substituent is the *para*. Notably, a substitution pattern conferring *P. aeruginosa* WT activity was found, this indicated that evasion of the multi-efflux pump system (MexAB) could be modulated also via proper N-terminal modification, and not only with variations of the central linker region.

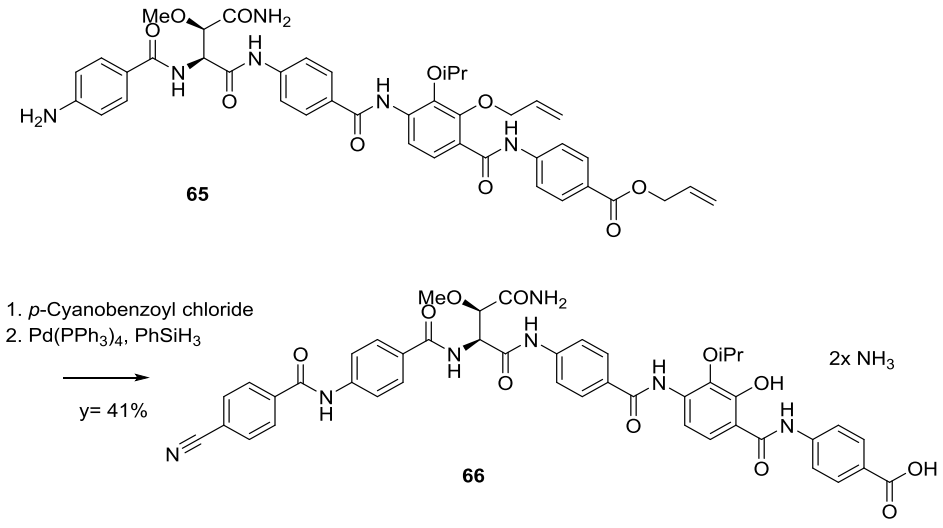
3.2.3 **2nd series of ring A analogs**

3.2.3.1 Aromatic ring and connection with the carbonyl

Building on the promising findings of the first series of analogs, in which the position and the electronic properties of the substituent on the aromatic ring were mainly investigated, a second series of derivatives was designed that aimed at assessing the introduction of an additional substituent on ring A and the aromatic ring-carbonyl group connectivity. Additionally, the methoxy moiety at the central Asn linker was also reintroduced in the scaffold of the best analog found in the first series.

For the synthesis of these new derivatives, the same procedures described in section 3.2.1 were used. Also in this case, the carboxylic acids building blocks were either synthesized (section 3.2.1.1) or purchased.

The synthesis of the molecule bearing the methoxy at the central Asn started from advanced intermediate **65**[51]. For the last two steps (Scheme 12), similar procedures of the ones already described were followed. In brief, *para*-cyanobenzoyl chloride was coupled to **65** in THF, and the obtained product was directly employed in the allyl cleavage step. The desired product (**66**) was isolated by RP-HPLC using a gradient of water and acetonitrile buffered with 10 mM NH_4HCO_3.



Scheme 12: Final two steps of the synthesis of compound **66**.

As mentioned before, albicidin possesses a coumaric acid derivative rather than benzoic acid moiety at the N-terminus. In order to investigate the influence of this difference, **67** was synthesized (Table 6). Additionally, the OH- group was also replaced with a cyano moiety (**68**), because of the favourable properties of the cyano group found in the first series of analogs.

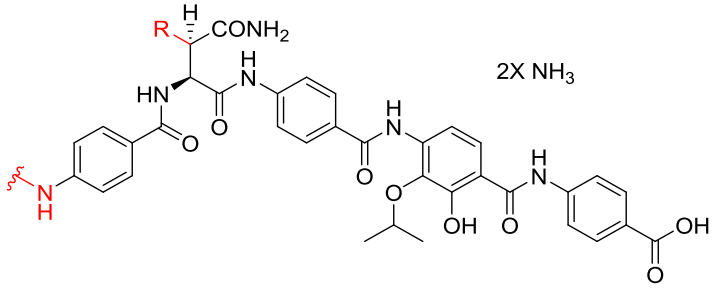
After these two molecules, derivatives that aimed at the investigation of the aromatic ring-carbonyl connectivity were synthesized. Particularly, the electron density given by double bond of the Michael acceptor in albicidin was mimicked by the introduction of heteroatoms such as an oxygen or a sulfur (**71**, **72**) atom and simplified analogs with one and two methylene spacer (**69**, **70**) were synthesized.

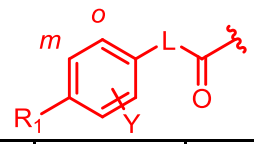
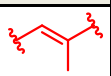
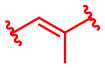
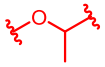
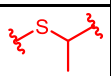
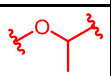
It was found that the two analogs with the double bond had the best antimicrobial profile, and any attempts to replace it led to complete loss of activity against *P. aeruginosa* and *S. aureus*. Considering that nearly all the analogs possessed comparably potent gyrase IC_{50}´s (low µM range), the reason for absence of bacterial inhibition was presumably again lack of penetration due to efflux.

The structurally simplified molecules bearing a CH_2 or a $(CH_2)_2$ spacer also showed efflux issues. Additionally the shorter linker yielded a ten-fold reduced gyrase activity in comparison to all the other compounds. Since this reduction cannot be attributed to the electronic properties of the aromatic ring, the reason could be that the overall conformation taken by the molecule becomes unfavorable.

In another set of molecules, second substituents with different electronic properties were introduced on the 4-cyanobenzoic acid moiety. First, a methyl group was placed either at the *ortho* or *meta* position (**74** and **75**) and since the former compound was less active, it was decided to focus only on the latter position. Hence, EDG and EWG groups were introduced.

Table 6: Depiction and biological characterization of cystobactamid analogs bearing a second substituent or a spacer unit between carbonyl and aromatic ring A.



Compound	R				MIC [µg/mL]					EC gyrase IC_{50} [µM]
		R_1	Y	L	*E. coli* ΔtolC	*E. coli* WT	*P. ae.* ΔmexAB	*P. ae.* WT	*S. aureus*	
Reference CNDM 861-2	*-H*	*-CN*	*/*	*/*	0.06	0.5	0.25	2	0.25	0.08[a]
67	*-H*	*-OH*	*/*		0.06	0.25	0.25	1	0.25	nd
68	*-H*	*-CN*	*/*		<0.03	0.125	0.25	1*	0.06	nd
69	*-H*	*-CN*	*/*	*$-CH_2-$*	<0.03	1	>64	>64	>64	16.3[b]
70	*-H*	*-CN*	*/*	*$-(CH_2)_2-$*	<0.03	0.125	0.5*	>64	>64	1.0[b]
71	*-H*	*-CN*	*/*		<0.03	0.06	>64	>64	>64	1.8[b]
72	*-H*	*-CN*	*/*		<0.03	0.25	>64	>64	>64	0.8[b]
73	*-H*	*-H*	*/*		4	> 64	> 64	> 64	> 64	nd
74	*-H*	*-CN*	*$-CH_3$ (o)*	*/*	0.03	0.5	2	8*	64	1.7[b]
75	*-H*	*-CN*	*$-CH_3$ (m)*	*/*	0.03	0.5	0.5	4	2	1.6[b]
76	*-H*	*-CN*	*-F (m)*	*/*	≤ 0.03	0.25	0.5	8	2	0.4[b]
77	*-H*	*-CN*	*-Cl (m)*	*/*	<0.03	<0.03	0.25	>64	<0.03	0.1[b]
78	*-H*	*-CN*	*$-OCH_3$ (m)*	*/*	<0.03	<0.03	0.25	>64	>64	1.8[b]
66	*-OMe*	*-CN*	*/*	*/*	<0.03	<0.03	<0.03	0.5	<0.03	0.4[b]

P. ae.= *P. aeruginosa*, *S. au.*= *S. aureus*; [a] determined at the department of microbial natural products (MINS), [b] determined at the department of chemical biology (CBIO), further details are given in the experimental part; nd= not determined; * the value could not be determined unambiguously and might be higher than assigned.

All these derivatives retained potent activities against both *E. coli* strains and the mutant of *P. aeruginosa*, while they showed differences among them against *P. aeruginosa* WT and

S. aureus. The only two that retained moderate activity against the former pathogen (4-8 µg/mL) were **76** and **75**, meta-substituted with F and CH_3.

It is striking to see how small modifications such as the replacement of a methyl with a chlorine group, which are functional groups that have the same size, had a drastic effect on the activity against *P. aeruginosa* and on the other side a positive one against *S. aureus*, with a more than ten-fold improvement of the MIC value.

All these derivatives had a gyrase inhibitory activity between 0.1 and 2 µM, with the chloro analog **77** being the most potent; steric effects or electrostatic repulsion could possibly determine these differences in IC_{50}´s.

A comparison between antibacterial profiles and enzymatic activity for this set of analogs, once again highlighted that the optimization of the potency on the enzyme does not go in parallel with the antibacterial activity, especially against *P. aeruginosa.*

Since fundamental understanding of the structural requirements needed for highly potent gyrase inhibition is already reached, it could be more important in the future to focus on establishing the structure-activity relationships that determine cellular uptake.

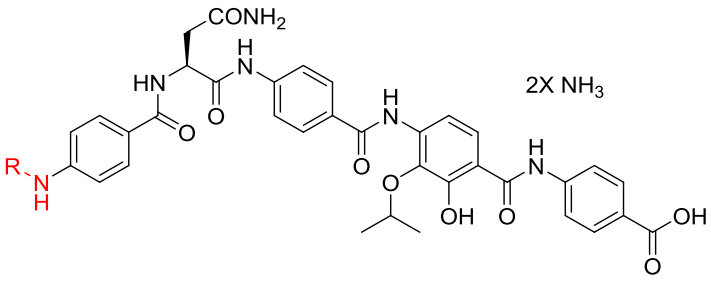
In this context, to confirm the relevance of the MeO- at the central Asn linker, compound **66** was designed. The biological properties of this molecule revealed that the optimized ring A and the central β-MeO-Asn had a positive additive effect. Noteworthy, this compound possessed a broader antibacterial spectrum and clearly improved activities against all the tested strains in comparison to both cys 861-2 and **46** (for instance four-fold improvement against *P. aeruginosa* WT), although its gyrase IC_{50} was not the most potent among all the analogs synthesized.

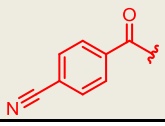
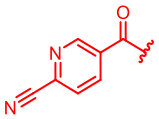
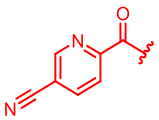
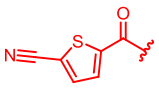
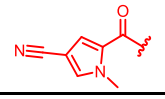
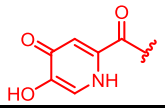
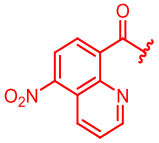
3.2.3.2 Heterocyclic moieties

Within the second round of investigation of unit A, it was decided to assess also the possibility to replace the phenyl ring, while maintaining the optimized substitution pattern (Table 7). This was for gaining further understanding in the SAR of this part of the molecules and for increasing the solubility. Heterocyclic moieties with different electronic properties and ring sizes were selected, for instance two cyano-pyridines. This choice was partially driven by the knowledge that an electron poor ring confers higher inhibitory properties.

A bioisosteric substitution was also attempted, and thiophen was chosen for this purpose. Pyrrole was selected due to its reported ability to interacts with DNA bases[105]. A compound with a bicyclic system (**85**) was designed to assess the effect of a bulky moiety that is potentially able to boost the pi stacking interactions. A compound with a pyridinone moiety (**84**) was chosen to exploit whether iron-chelating properties would be beneficial for activity. The compound represents a strategy that has been especially used in β-lactams research, because incorporation of a siderophore in the antibiotic scaffold should enhance permeation into the bacterial cell through the iron uptake pathways [116,118].

Table 7: Depiction and biological characterization of the derivatives where the N-terminal phenyl ring was replaced by heterocycles.



	MIC [µg/mL]						EC gyrase IC_{50} [µM]
Compound	R	*E. coli* ΔtolC	*E. coli* WT	*P. ae.* ΔmexAB	*P. ae.* WT	*S. aureus*	
Reference CNDM 861-2		0.06	0.5	0.25	2	0.25	0.08[a]
80		<0.03	<0.03	1	>64	64	0.5[b]
81		<0.03	<0.03	0.25	0.5	<0.03	0.9[b]
82		<0.03	<0.03	0.25	0.5	0.25	0.5[b]
83		<0.03	<0.03	0.5	>64	64	0.7[b]
84		>64	>64	>64	>64	>64	3.6[b]
85		0.5*	0.25	>64	>64	>64	0.3[b]

P. ae.= *P. aeruginosa*, *S. au.*= *S. aureus*; [a] determined at the department of microbial natural products (MINS), [b] determined at the department of chemical biology (CBIO), further details are given in the experimental part; nd= not determined; * the value could not be determined unambiguously and might be higher than assigned.

All the derivatives possess IC_{50} values in the sub-µM range with the exception of the siderophore-cystobactamid (**84**), that had an activity in the low µM range. Unfortunately, this molecule turned out to be totally inactive against all the tested strains, even against the mutants lacking the efflux pump. Considering the significantly higher polarity of this residue

in comparison with all active compounds, it could be speculated that this prevents cellular penetration.

The nitro-quinoline analog (**85**) possessed the best IC_{50} value (0.3 µM) among the compounds in this subsection but it wasn´t active against neither *P. aeruginosa* nor *S. aureus*.

Among the remaining four, the derivative bearing the 5-cyanothiophene-2-carboxylic acid and 5-cyanopicolinic acid moieties (**82** and **81**) showed the most interesting antibacterial profile. They possessed excellent MICs against all the pathogens tested.

To sum up, within the second round of investigation of ring A five new highly potent derivatives had been discovered, with three of them possessing clearly improved activity with respect to the lead structure cys 861-2. The structural motif of ring A could be further optimized to reach *P. aeruginosa* inhibition in the sub-µg/mL range.

In term of SAR, it was shown that the introduction of a second substituent can be tolerated but it is not beneficial for the activity. The presence of different spacers between ring and carbonyl group led to different results, with the Michael acceptor system as present in albicidin being the best moiety for the activity. Whether the positive effect is due to the chemical reactivity or to the evasion of efflux pumps has to be demonstrated yet. Nevertheless, considering the activity against gyrase, a 1,4-addition is presumably not critical in the inhibition mechanism. It could thus be speculated that the electronic properties and influence on the molecular conformation/shape of the methacryl moiety are the reasons for the positive effect on the activity. This would also match with the results of the first series of ring A analogs, where it was seen that a higher activity was observed with lipophilic moieties.

It is very interesting to see that nearly equally potent activities can be achieved with different types of N-termini, such as the simpler benzoic acid of the cystobactamids as well as the more sterically demanding tail of albicidin.

An additional point of reflection is given by the fact that the aromatic ring at the N-terminal residue of albicidin bears an EDG group. Nonetheless this substitution pattern yields optimal binding and antibacterial properties, thus standing partially in contrast with the cystobactamid SAR established herein. At the same time, it should be kept in consideration that in one of the reports on the SAR of albicidin[69] it was shown that a derivative with a

simpler 4-hydroxybenzoyl residue (Michael acceptor deleted) had significantly lower antibacterial properties compared to the parent natural product, which is in line with my findings.

For all these reasons, it appears that this small lipophilic linker has a particular influence on the final activity, but the knowledge developed so far does not permit to have a clear understanding of its effect. Trying to gain such knowledge would probably partially overlap with the work already done on albicidin.

Two new heterocyclic moieties that further boost the antimicrobial activities, remarkably against *P. aeruginosa* WT, have been identified. Notably, this proved that the PABA moieties of the natural product scaffold can be substituted by different aromatic systems, a strategy that could be adopted to increase the solubility of the molecule.

Finally, I have confirmed that the β-MeO-Asn linker allows reaching better antibacterial properties compared to the simpler Asn, and that the information obtained from the optimization of ring A can be translated to the original scaffold, yielding a positive additive effect on the antimicrobial activity.

These two series of derivatives modified at ring A proved that **1** had been a valid alternative scaffold for the medicinal chemistry investigation of the natural cystobactamid 861-2. It could be envisaged that novel analogs bearing the new optimal heterocycles and a β-MeO-Asn could potentially possess even better activities.

The evaluation of the antimicrobial properties against a broader panel of pathogens of clinical relevance for the most promising analogs will be presented in another section of this thesis.

3.2.4 **Second synthetic access, modifications of rings A-B**

The structural optimization of the cystobactamid proceeded involving aromatic unit B. In order to have the possibility to introduce a higher level of diversification of the scaffold at the N-terminus efficiently, a second synthetic access had been established (Fig. 36). The new synthesis allowed late-stage modifications of both aromatic units A and B at the same time. This modular synthesis relied on the disconnection of the molecule in the three fragments readily accessible on gram scale.

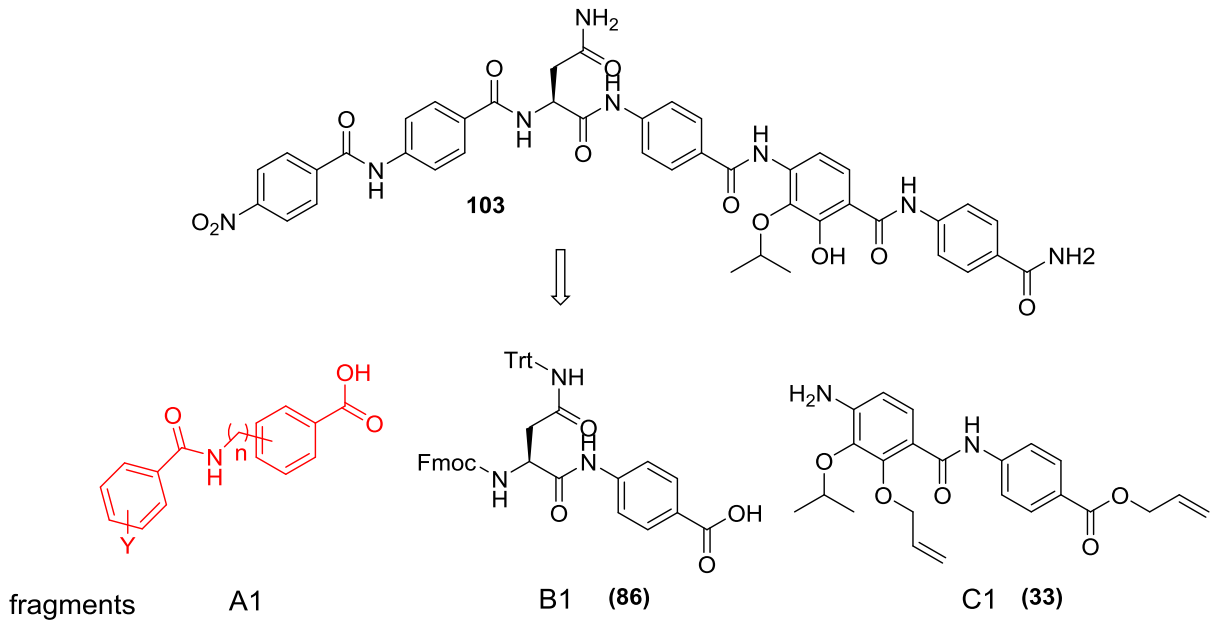


Figure 36: Retrosynthetic disconnection of the second synthetic access to cystobactamid analogs. Fragment A1 is given as a general formula to highlight the possibilities of variation of this part.

With the new synthesis, the higher reactivity of the primary amine of central building block B1 (**86**) was exploited in the last step, this was to ensure mild reaction conditions and avoid the need of protecting groups for the phenol and carboxylic acid.

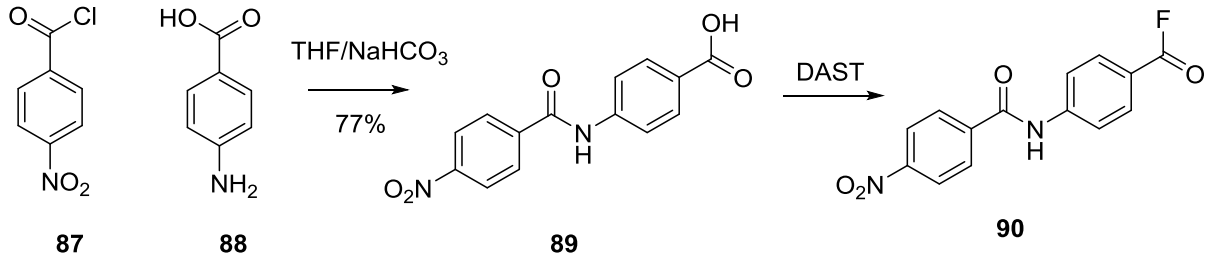
Other reasons for the design of the new synthesis were to shorten the longest linear sequence and to increase the overall yield.

Compound **103** bears an amide at the C-terminus instead of a carboxylic acid. The synthesis of **103** with the new approach proved the applicability of this synthesis for investigation of the carboxylic acid function of the PABA-D2 ring.

In the next sections, the synthesis of the fragments will be described, followed by the final assembly of the molecule.

3.2.4.1 Fragments synthesis:

3.2.4.1.1 Fragments A1



87 **88** **89** **90**

Scheme 13: Synthesis of fragment A1.

Fragment A1 (**89**) was readily synthesized coupling PABA and 4-nitrobenzoyl chloride via Schotten Baumann conditions (Scheme 13). The compound was obtained in good yield upon filtration and trituration of the reaction mixture. The carboxylic acid was then activated as an acyl fluoride by means of DAST and directly used in the coupling step.

This activation strategy has the advantages that (i) acyl fluorides react selectively with amines rather than oxygen-based nucleophiles, (ii) they are produced with an easy and quick procedure, and (iii) the activated species is usually a solid that can be isolated and stored for several days. For these reasons it was initially preferred over standard coupling reagents and acyl chlorides.

It should be mentioned that **89** possessed very bad solubility in any organic solvent, and also the acyl fluoride product had a poor solubility in both DCM and THF. This rendered the work up of the reaction tedious with concomitant loss of material. Therefore alternative activation strategies were investigated for the synthesis of other analogs.

3.2.4.1.2 Variants of fragments A1

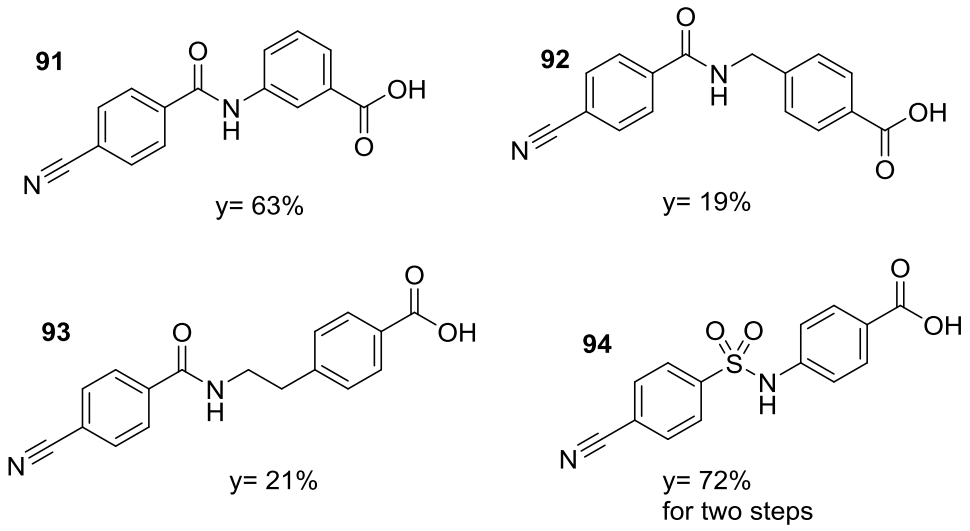
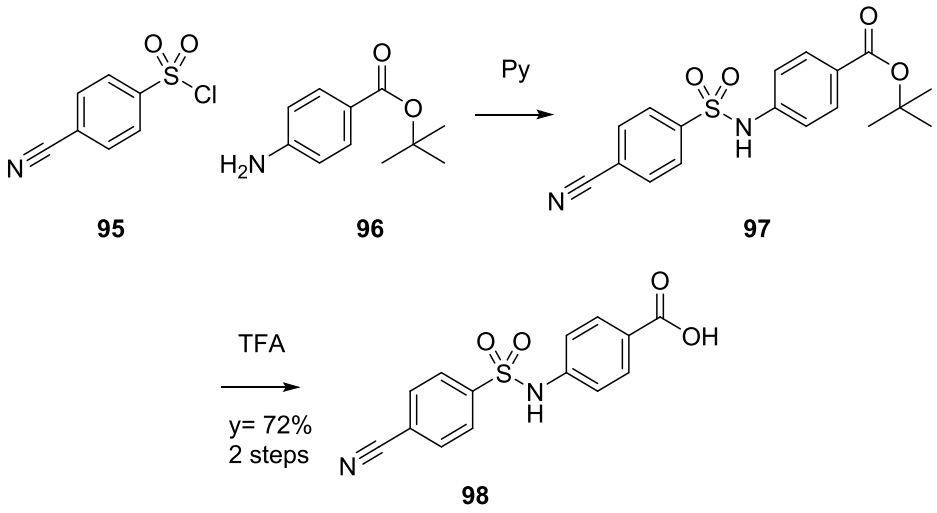


Figure 37: Different fragments A1 synthesized.

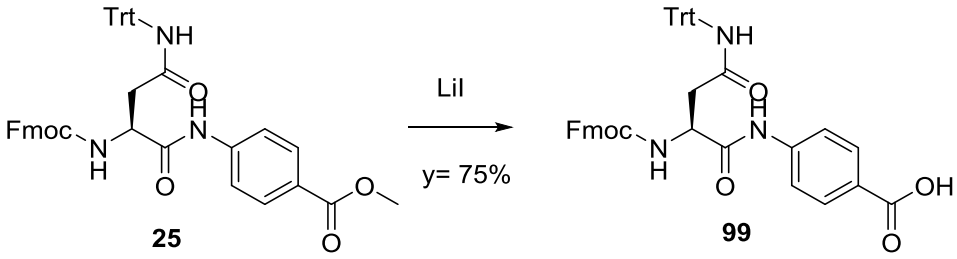
The fragments depicted in Figure 37 were also synthesized starting from the corresponding amines and 4-cyanobenzoyl chloride.

For the sulfonamide building block **98**, the synthesis started from *tert*-butyl ester of PABA and the commercially available sulfonyl chloride **95**, which were coupled in THF and pyridine. The crude compound (**97**) was treated with TFA to deprotect the carboxylic acid moiety, and an aqueous work up afforded pure **98** (Scheme 14).



Scheme 14: Synthesis of sulfonamide variant of fragment A1.

3.2.4.1.3 Fragments B1



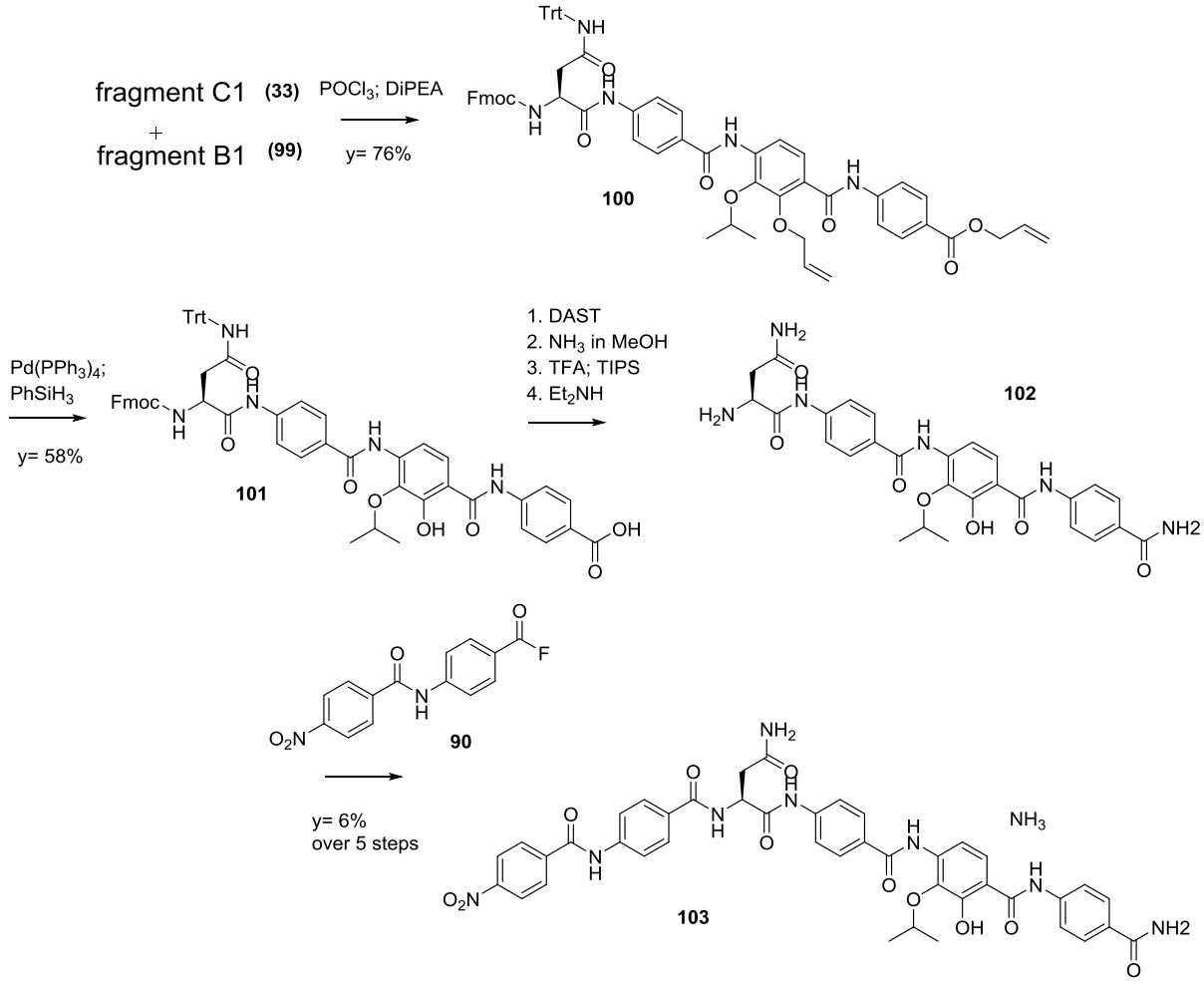
Scheme 15: Synthesis of building block B1.

Fragment B1 was synthesized treating ester **25** with lithium iodide to obtain the corresponding carboxylic acid (Scheme 15).

3.2.4.2 Final assembly of the molecule:

The assembly strategy pursued was the same as in the first synthetic access, starting coupling fragments B1 and C1 by means of $POCl_3$ (Scheme 16). Alternative coupling methods such as HATU and activation of the carboxylic acid as an acyl fluoride did not afford the desired product even under enforced conditions such as MW assisted reaction. Also in such instances, only starting materials were present, highlighting once again the low reactivity of these intermediates.

Nevertheless, the established chlorinating reagent afforded the desired product in around 75% yield. It should be mentioned that purification of the desired intermediate by flash chromatography was not easy due to the tailing behavior on the column. In fact, the compound isolated partially contained carboxylic acid starting material. It turned out to be more convenient to complete the purification in the next step rather than repeating additional flash chromatographies. In fact, after removal of the allyl groups, which was carried out using tetrakis(triphenylphosphine)palladium(0) and phenylsilane in THF, the product had a better behavior on silica gel, thus the separation from the impurities was easier.



Scheme 16: Last steps of the second synthetic access established.

Introduction of the primary amide was planned only at this point to avoid side reactions in the coupling step using $POCl_3$. Carboxylic acid **101** could be activated without any side reactions of the free phenol by means of DAST. After work up, the corresponding acyl fluoride was directly treated with a solution 7 N of NH_3 in MeOH to afford the desired primary amide. Both reactions proceeded smoothly with a clean profile. Because the solubilities of the intermediates were low, it was decided to skip the purification step and directly use the crude product in the deprotection steps of the trityl and Fmoc. After trituration of the solid with petrolether, the primary amine **102** was coupled to pre-activated fragment A1 (**90**) in THF. The reaction was stirred for a few hours and then purified by RP-HPLC with a gradient of water and acetonitrile buffered with 10 mM NH_4HCO_3.

The biological properties of **103** will be discussed in the section on the C-terminal optimization.

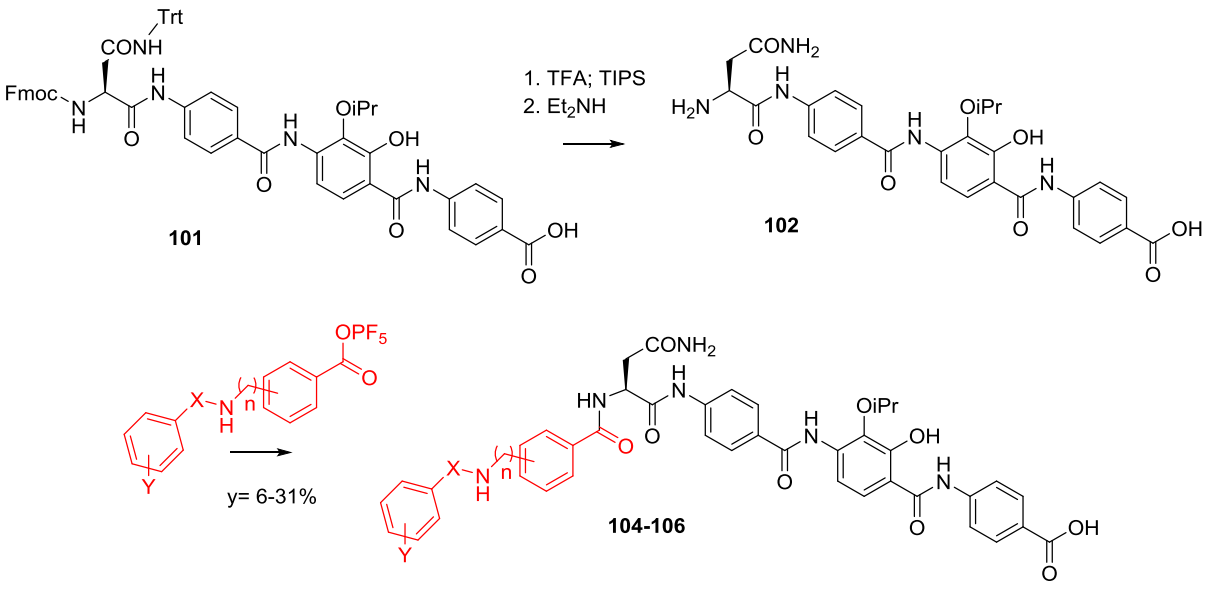
Using this new sequence, three new analogs, that were not accessible with the first synthetic route, were obtained. Omitting the steps for the introduction of the primary amide, the procedures adopted were the same as described above, with the exception that fragments A1 were activated as pentafluorophenyl esters as reported by Trauner [48]. It was decided to use this activation to overcome the solubility issues previously encountered.

Activation and coupling reactions were carried out in DMF with a reaction time of three hours. After extraction of the product with EtOAc, the crude materials were purified by RP-HPLC. The final yields over these three steps varied from 6 to 31%. It should be mentioned that all the reactions had a very clean profile, thus presumably the low yield is due to loss of material during work up and chromatography.

To sum up, a new shortened synthetic access could be established that offers the possibility to access derivatives with modifications at either the N- or C-terminus.

In terms of overall yield, it is difficult to make a comparison with the first synthesis, since different compounds have been synthesized.

Considering the analog where the highest yield was obtained, the overall yield of the longest linear sequence (11 steps) was 3%.

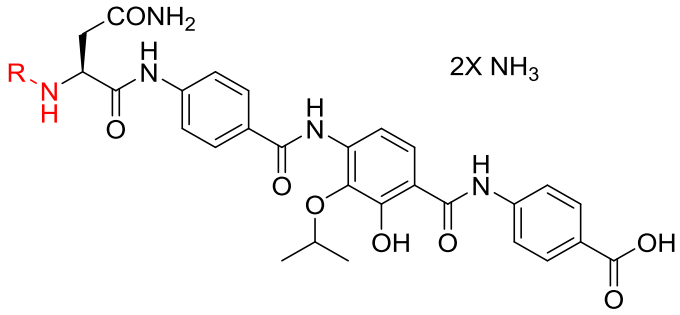


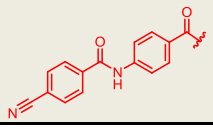
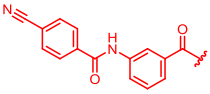
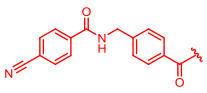
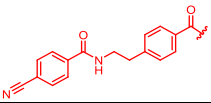
Scheme 17: General scheme depicting the last steps of the synthesis of analogs bearing wider modifications of the N-terminus (rings A and B).

The three analogs **104**-**106** (Table 8) possess modifications at the connectivity amino group-aromatic ring of unit B. Particularly, in two of them a methylene and an ethylene spacers

were introduced to extend the molecule length. In other words, a small spacer was introduced between the amide and the B ring. The other compound presents a more profound variation, in fact the *para* connectivity of the aromatic building blocks was broken with a *meta* connection, a modification that presumably entails significant effects on the final conformation and shape of the molecule.

Table 8: Analogs that present the optimized ring A and variations at unit B.



		MIC [µg/mL]					EC gyrase IC_{50} [µM]
Compound	R	*E. coli* ΔtolC	*E. coli* WT	*P. ae.* ΔmexAB	*P. ae.* WT	*S. aureus*	
Reference (CNDM 861-2)		0.06	0.5	0.25	2	0.25	0.08[a]
104		>64	>64	>64	>64	>64	nd
105		1	>64	>64	>64	>64	nd
106		0.125	0.25	4	>64	>64	nd

P. ae.= *P. aeruginosa*; [a] determined at the department of microbial natural products (MINS); nd= not determined.

Gyrase IC_{50} values are not available for this small set of compounds. Nevertheless from the antibacterial activity we can see that the drastic modification of the *meta* connection as in **104** was not tolerated, as it led to total loss of antibacterial properties (Table 7).

Introduction of CH_2 spacers gave comparable results to the analogs previously presented investigating the ring A-carbonyl group connection. Similarly, the shorter benzyl amine derivative (**105**) was active only against mutant *E. coli*, while the extended ethyleneamine derivative (**106**) showed inhibition of both *E. coli* strains and of *P. aeruginosa* ΔmexAB. Also

in this case, it could be assumed that the reasons for such differences in activity are not the electronic properties of the aromatic rings but rather the permitted conformational arrangement, which in one instance is not ideal for proper binding with the target enzyme.
The information given is just the starting point for a proper investigation of aromatic unit B, that could potentially go on with the replacement of the phenyl ring with a thiophene, a pyridine or other heterocycles.

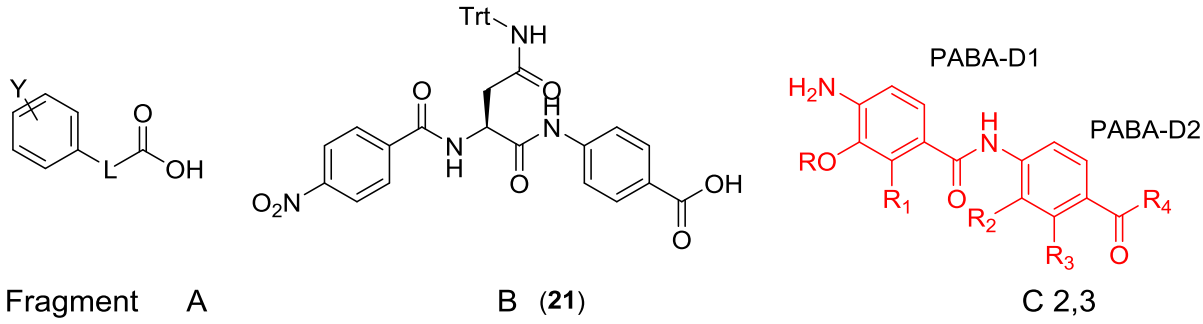
3.3 C-terminal OPTIMIZATION

3.3.1 C-terminal modifications

In this section the synthesis and biological evaluation of analogs with modifications at rings PABA-D1 and D2 will be discussed.

For the synthesis of such analogs, the previously established synthetic access has been employed with variations of fragment C (Fig. 38). This synthesis allowed modifications of the N-terminus at the same time, thus giving the possibility to assess the effect of variations of both sides.

One of the two analogs bears an isobutyl group as substituent on PABA-D1 and the other has the substitution pattern at PABA-D2 of natural cys 935-2[51], which together with cys 861-2 was the best naturally occurring cystobactamid. The former modification was designed in order to assess the relevance of the isopropyl present on the natural scaffold and to investigate an alkyl moiety that presumably cannot be biosynthesized in the natural derivatives[57].

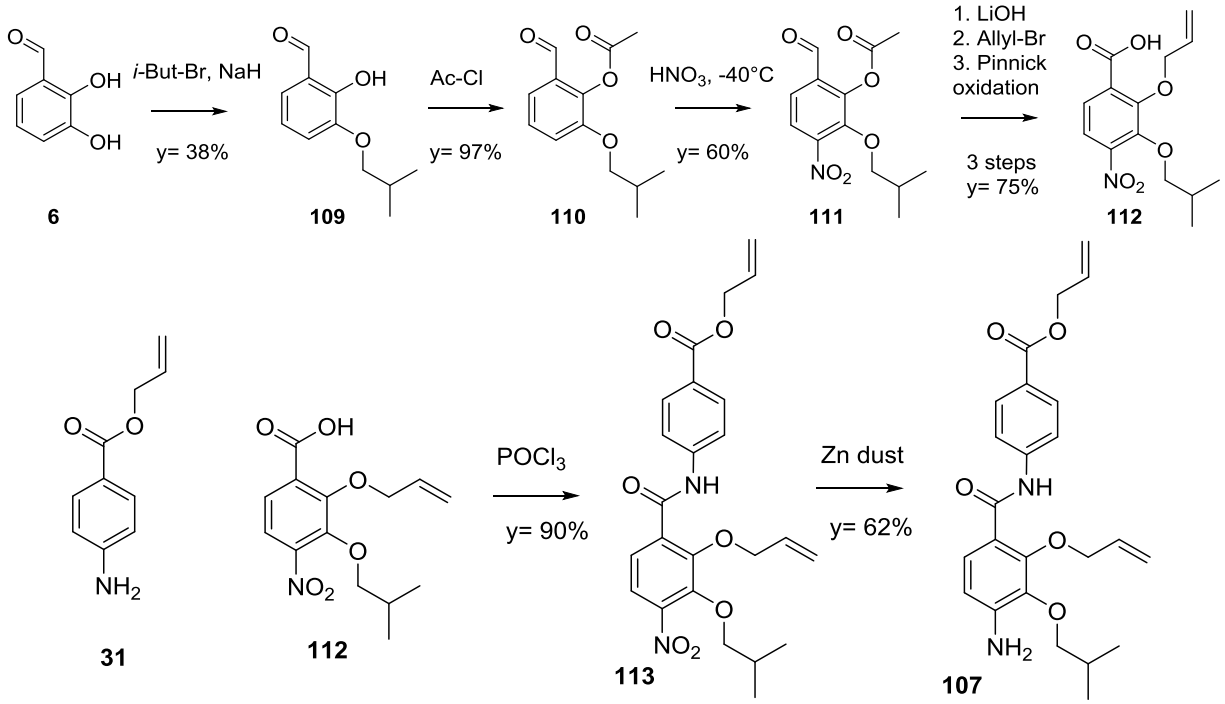


Fragment	R	R1	R2	R3	R4
C2 (**107**)	*i*-But	-OAllyl	-H	-H	-OAllyl
C3 (**108**)	*i*-Pr	-OAllyl	-O*i*-Pr	-OAllyl	-OAllyl

Figure 38: Schematic representation of building blocks used for the synthesis of analogs bearing C-terminal modifications. Fragment C is highlighted in red and in the table the fragments used in the synthesis are depicted.

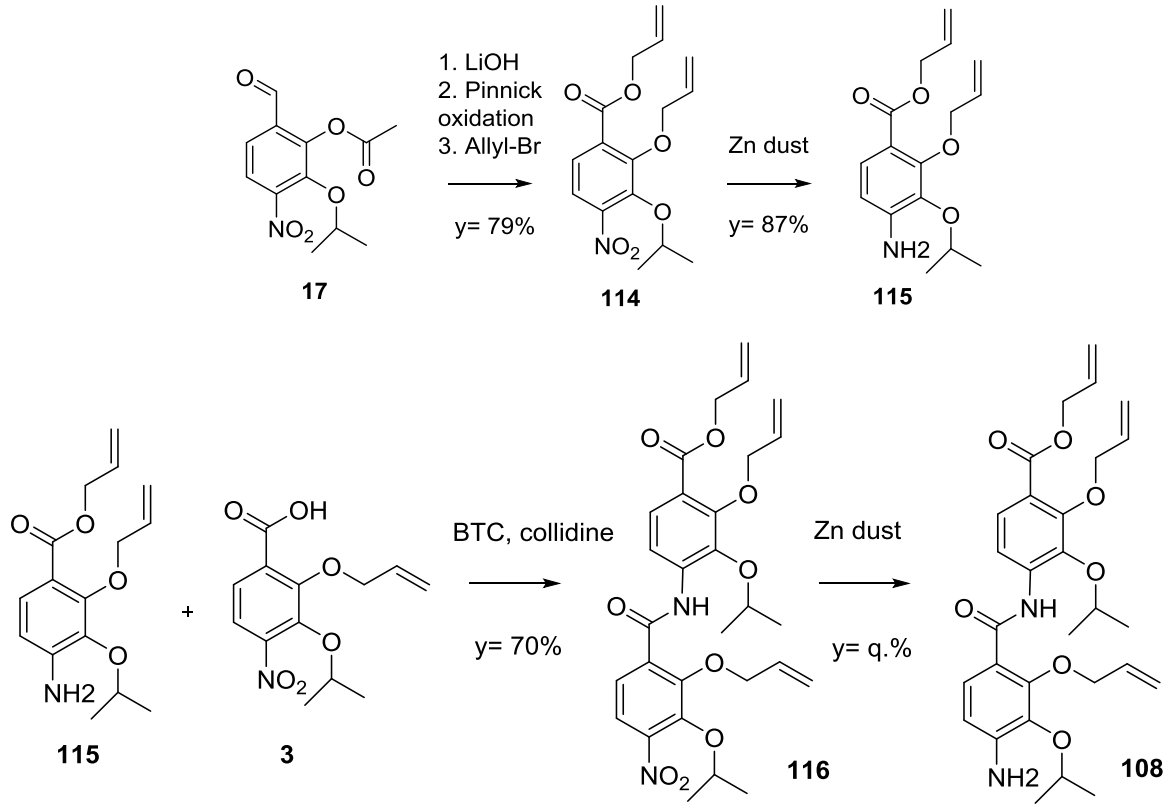
3.3.1.1 Fragment C2 and C3 synthesis

The synthesis of fragment C2 (**107**) followed the same approach employed for the construction of PABA-D1, with the difference of using isobutyl bromide as alkylating reagent in the first step. In all the steps the yields were comparable to the previously described synthesis of fragment C1 (Scheme 18).



Scheme 18: Synthesis of *i*-But PABA-D1 and assembly of the diaryl fragment C2.

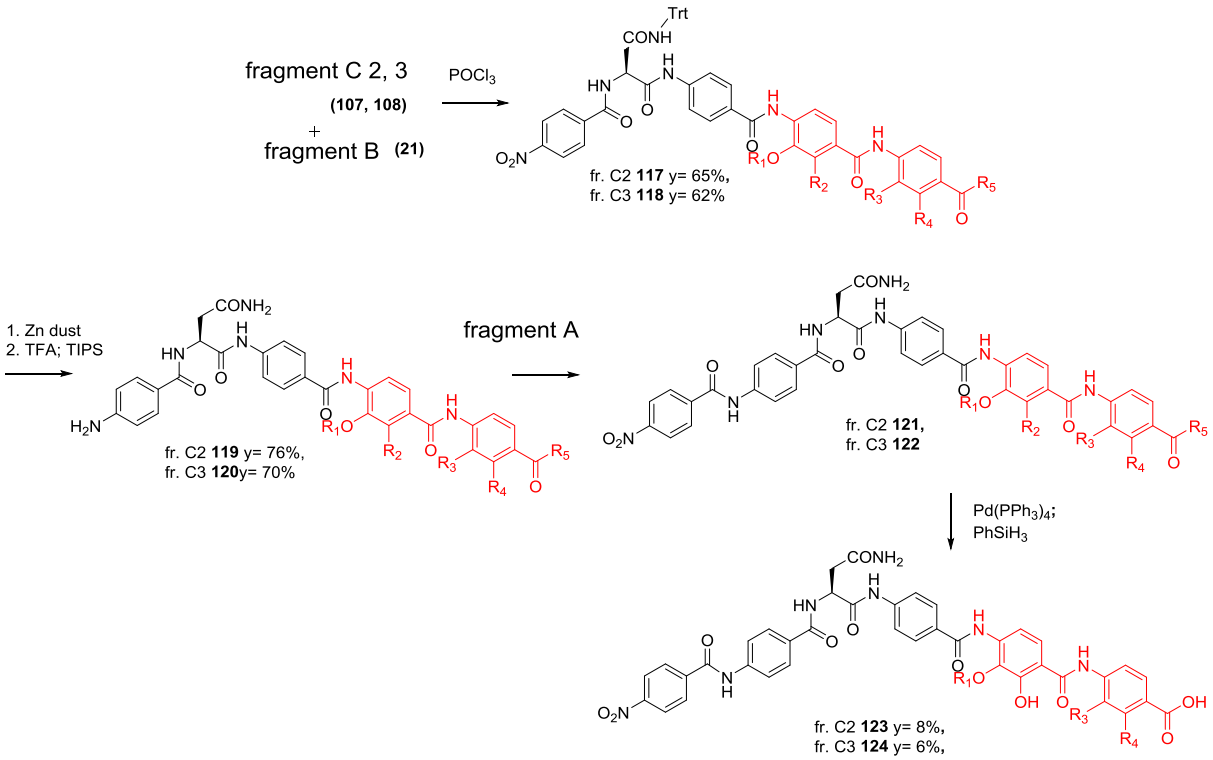
The synthesis of fragment C3 commenced with chemical modifications of nitro aldehyde **17** to afford amine **115**. It was carried out with a four step linear sequence that mainly consisted of functional group interconversions with a good overall yield and the need of only 2 purifications (Scheme 19). Coupling of this amine with PABA-D1 was done using BTC and collidine to activate the carboxylic acid, and finally the NO_2 group of **116** was reduced with Zn dust in EtOH and 10% acetic acid.



Scheme 19: Synthesis of fragment C3, construction of modified PABA-D2 and coupling to PABA-D1.

3.3.1.2 Final assembly of the molecules

The final assembly strategy adopted is the one described for the synthesis of cys DM861-2 and depicted in Scheme 20 with no major differences found in all the steps (for detailed information see experimental part).

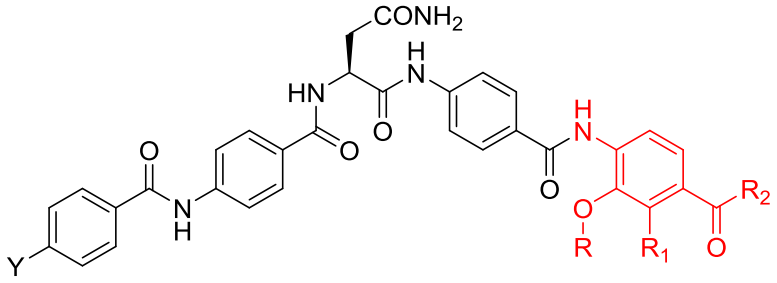


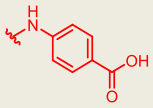
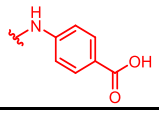
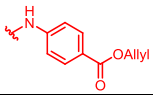
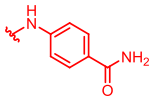
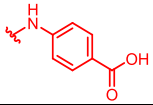
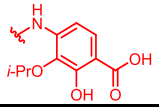
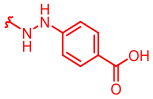
Scheme 20: General synthetic route employed for the assembly of the two analogs described in this section and also used for accessing molecules with modification at both C- and N- terminus.

3.3.1.3 *Biological properties*

The table below provides a sum up of all the molecules with modifications at PABA-D1 and 2. Together with the analogs described in the previous section others are given such as the one bearing a primary amide at the C-terminal (**103**) and the structurally simplified analogs missing the phenol at PABA-D1 (**2**). Allyl protected cys DM861-2 (**36**) was also tested and has been included in this set of compounds.

Table 9: Structures and activities of analogs bearing modifications at the PABA-D1 and D2 rings.



					MIC [µg/ml]					EC gyrase IC_{50} [µM]
Compound	Y	R	R_1	R_2	*E. coli* ΔtolC	*E. coli* WT	*P. ae.* ΔmexAB	*P. ae.* WT	*S. aureus*	
Reference Molecule (DM861-2)	*-NO_2*	*i-Pr*	*-OH*		0.06	0.13	1	> 64	0.5	0.11[a]
2	*-NO_2*	*i-Pr*	*-H*		0.06	> 64	1	> 64	> 64	nd
36	*-NO_2*	*i-Pr*	*OAllyl*		> 64	> 64	> 64	> 64	> 64	nd
103	*-NO_2*	*i-Pr*	*-OH*		≤ 0.03	1*	> 64	> 64	64	0.2[b]
123	*-NO_2*	*i-But*	*-OH*		2*	> 64	> 64	> 64	1	0.9[b]
124	*-NO_2*	*i-Pr*	*-OH*		0.125	0.5	1-2	> 64	0.05	0.2[b]
125	*-CN*	*i-Pr*	*-OH*		0.25	>64	>64	>64	>64	nd

P. ae.= *P. aeruginosa*; [a] determined at the department of microbial natural products (MINS); [b] determined at the department of chemical biology (CBIO); nd= not determined; * the value could not be determined unambiguously and might be higher than assigned.

Despite a remarkable gyrase inhibition, the derivative with the primary amide **103** was inactive against both *P. aeruginosa* strains and *S. aureus*. Taken together this information suggested that permeability issues might be the reason for the lowered antibacterial properties.

The same could probably be stated also for "*i*-Bu- cystobactamid" (**123**), where the insertion of a CH_2 in the isopropoxy moiety had a seismic, negative effect on the antibacterial activity.

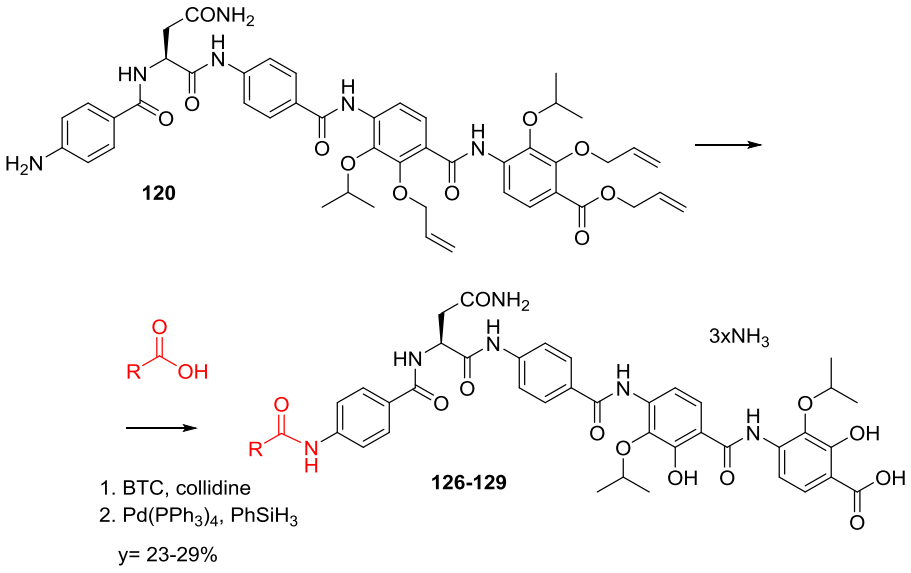
As expected, the addition of isopropoxy and hydroxyl moieties on PABA-D2 (**124**) was well tolerated, this analog was comparably potent against all tested strains as the reference compound (Table 9). Therefore, to better evaluate these two structural variants, the evaluation of the antimicrobial activity on a larger panel of pathogens was required. Additionally, it was also decided to investigate analogs bearing at the same time the optimized N-terminal motifs (section 3.3.2).

The last analog of Table 9 bears a hydrazine at the *para* position of the benzoic acid moiety. It has been synthesized with a synthetic strategy that will be discussed in the next section of the thesis. The introduction of this additional nitrogen was very deleterious for the antimicrobial properties, as **125** was active only against *E. coli* ΔtolC.

3.3.2 **N-terminal modifications of the cys 935-2 scaffold**

Due to the potent antibacterial properties of the desmethoxy (DM) analog of cys935-2 (**124**), compounds bearing its substitution pattern of PABA-D2 and the most promising moieties found in the structural optimization of the N-terminus were designed.

Amine **120** was synthesized on a gram scale and used in the coupling steps with activated rings A as described for other analogs in the previous sections, followed by cleavage of the allyl groups (Scheme 21).



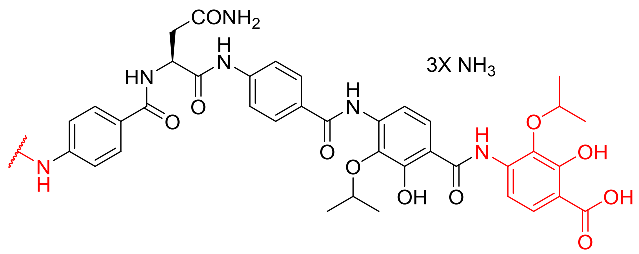
Scheme 21: Final coupling and deprotection steps for the synthesis of cys DM-935-2 analogs.

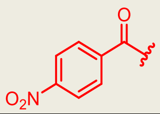
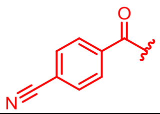
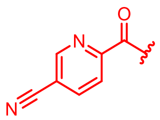
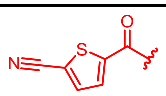
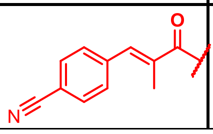
All selected fragments A bear a nitrile substituent, additionally they include variations of the phenyl ring and its connectivity with the carbonyl moiety (Table 10).

All analogs were highly potent against the tested strains with the sole exception of *P. aeruginosa* WT. This partially suggested that the simpler PABA as last aromatic unit at the C-terminus represents a superior structural motif.

In order to establish more detailed SAR, the antibacterial properties on a larger panel of bacteria were determined. They will be presented in another section of the thesis.

Table 10: Structures and biological characterization of molecules bearing the cys 935-2 substitution pattern at PADA-2 and optimized N-terminal residues.



		MIC [µg/mL]					
Compound	R	*E. coli* ΔtolC	*E. coli* WT	*P. ae.* ΔmexAB	*P. ae.* WT	*S. aureus*	EC gyrase IC_{50} [µM]
Reference ***124***		0.125	0.5	1	> 64	0.05	0.2^{b}
126		≤ 0.03	0.25	1	2*	≤ 0.03	0.6^{b}
127		<0.03	0.06	0.5	>64	<0.03	nd
128		0.06	0.125	2	>64	0.06	nd
129		<0.03	0.125	0.25	1*	0.06	0.2^{b}

P. ae.= *P. aeruginosa*; [b] determined at the department of chemical biology (CBIO); nd= not determined; * the value could not be determined unambiguously and might be higher than assigned

3.4 TACKLING POSSIBLE RESISTANCE MECHANISMS

Albicidin was discovered in 1985 and during the following two decades, several studies on its biological characterization were reported. Some of these aimed at the identification of resistance mechanisms [58-60,62-64,70,72,73,119]. To date six proteins conferring resistance to albicidin are known (Table 11).

Table 11: Resistance mechanisms known for albicidin (Alb); dm= defending microorganism, pm= producer microorganism.

Name	Description and function	Origin
Tsx	Protein channel/ nucleosides uptake	*E. coli* (dm)
AlbD	Endopeptidase (*P. dispersa*)/ hydrolytic cleavage of Alb	*P. dispersa* (dm)
AlbA and AlbB	high-affinity binding protein/ sequestration of Alb	*K. oxytoca and* *A. denitrificans* (dm)
AlbF	DHA14 drug efflux pumps	*X. albilineans* (pm)
AlbG	pentapeptide-repeat protein/ prevent binding to gyrase	*X. albilineans* (pm)
Mutated gyrase	gyrase carrying multiple mutations/ reduced susceptibility to albicidin	*X. albilineans* (pm)

The last three were found in the producer microorganism used for self-protection, whereas the former ones are used by the defending microorganisms.

Considering that antibiotic resistance is one of the major causes that led to the current antibiotics crisis, the understanding of the potential mechanisms for a new antibiotic candidate is considered an important step in the advancement of early stage drug candidates.

Due to the structural similarities between albicidin and the cystobactamids, the resistance mechanisms of the former could probably also affect the activity of the latter. Therefore, the influence of two of the six resistance mechanisms was investigated for cystobactamids, namely Tsx and AlbD.

3.4.1 **The outer membrane protein Tsx**

Deletion of the protein channel Tsx that is present on the outer membrane of *E. coli* resulted in 100 fold loss of inhibitory properties for albicidin, as demonstrated by Birch and coworkers[59].

In order to assess the effect of Tsx deletion on cystobactamids activity, MICs for selected analogs (Table 12) against two Δtsx strains and the corresponding WTs were measured.

Table 12: Minimal inhibitory concentrations of selected cystobactamid analogs; details regarding the bacterial strains can be found in section 4.1.1.

Compound	MIC [µg/mL] *E. coli*			
	WT1	Δtsx1	WT2	Δtsx2
Cys 861-2	0.2	0.4	0.2-0.4	0.8
*DM861-2 (**1**)*	0.1	1.6	0.1	0.2
*CNDM861-2 (**46**)*	0.05	0.1	0.05	0.1

The activity data of both mutants and WTs were all in the same range, with the sole exception of compound **1** against the bacterial strains denoted with the number one (16-fold reduction in activity). These results indicate that the activity of cystobactamids is not significantly affected by deletion of Tsx. Therefore, it cannot be considered a resistance mechanism for them.

This finding highlights that despite the structural similarities there are important differences at the molecular level for the two lead structures albicidin and cystobactamid.

3.4.2 **The endopeptidase AlbD**

The enzyme AlbD was found in *Pantoea dispersa*, a Gram-negative bacterium usually harmless for human beings that has therefore no clinical relevance[120]. Nonetheless, a putative horizontal gene transfer to clinically relevant bacterial strains could create serious problems for the efficacy of albicidin.

This enzyme cleaves the molecule in two inactive fragments, specifically at the amide bond that connects ring C and D.

Süssmuth and coworkers showed that also the cystobactamids could potentially be substrate of the endopeptidase[71]. In their work, they found that a catalytic triad, composed of Ser, His and Asp, is probably present in the active site, suggesting that the amide bond is hydrolyzed by the primary alcohol of Ser.

In order to overcome this resistance mechanism, the hydrolytically stable cystobactamid **130** was designed, in which the critical amide bond is bioisosterically substituted by a triazole (Fig. 39).

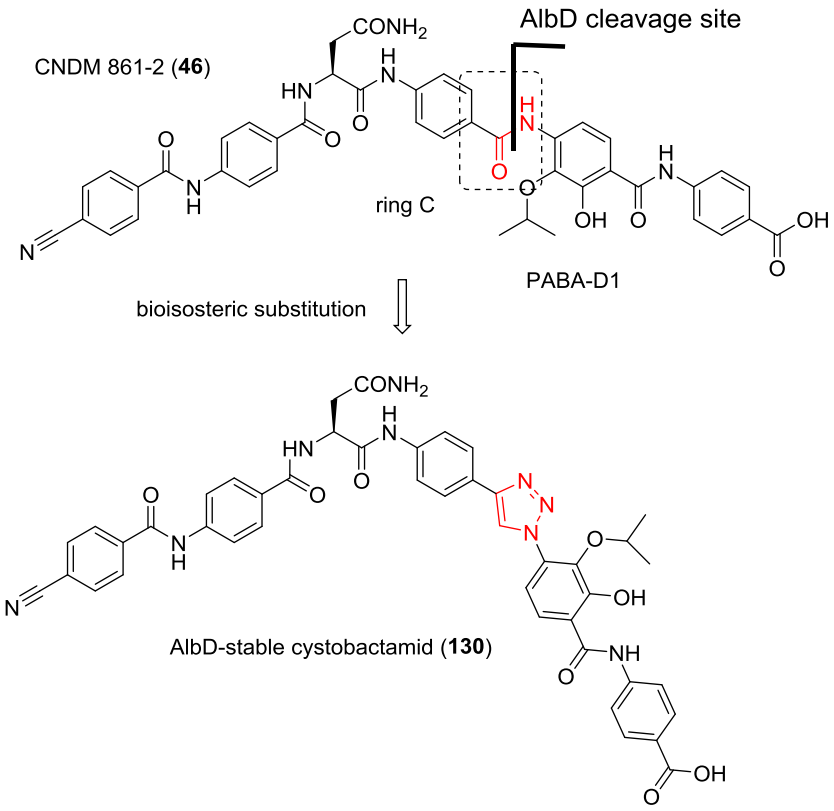


Figure 39: Structures of potentially AlbD-sensitive cystobactamid (top) and the stable one (bottom). In red are highlighted the critical amide bond and the bioisoster moiety.

Retrosynthetically, the molecule was disconnected into the two fragments **131** and **132**, with the formation of the triazole moiety planned at the end of the synthesis (Fig. 40). Compared to the previously established synthetic routes, aromatic rings A and B are already connected to the central Asn moiety (**131**). The modified fragment C-N_3 (**132**) bears an azide instead of the aniline. The protecting group strategy had been revised. Protection of the phenol was needed in the coupling of fragment C and B of the previous syntheses, whereas this was not necessary in the click chemistry approach.

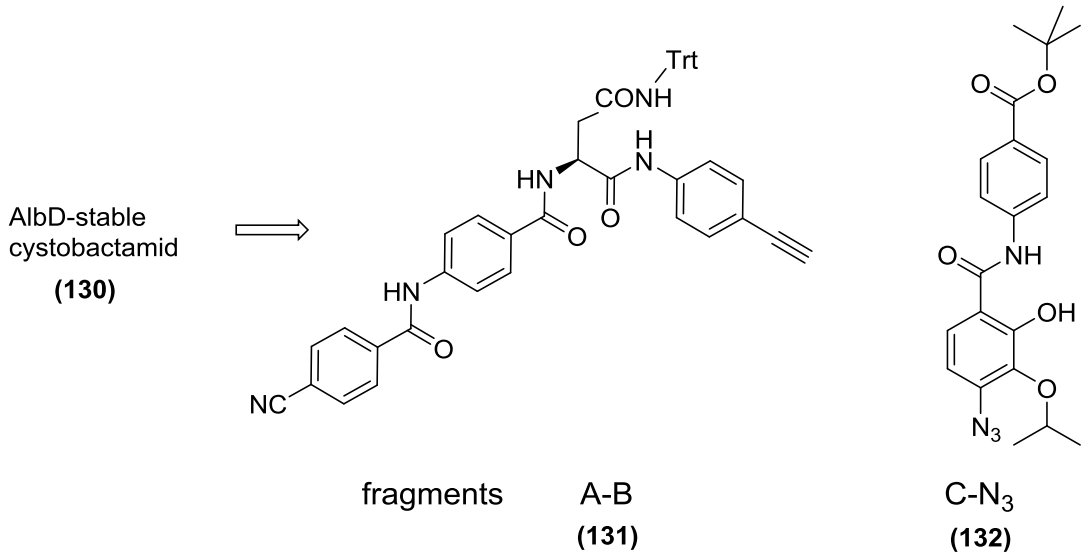
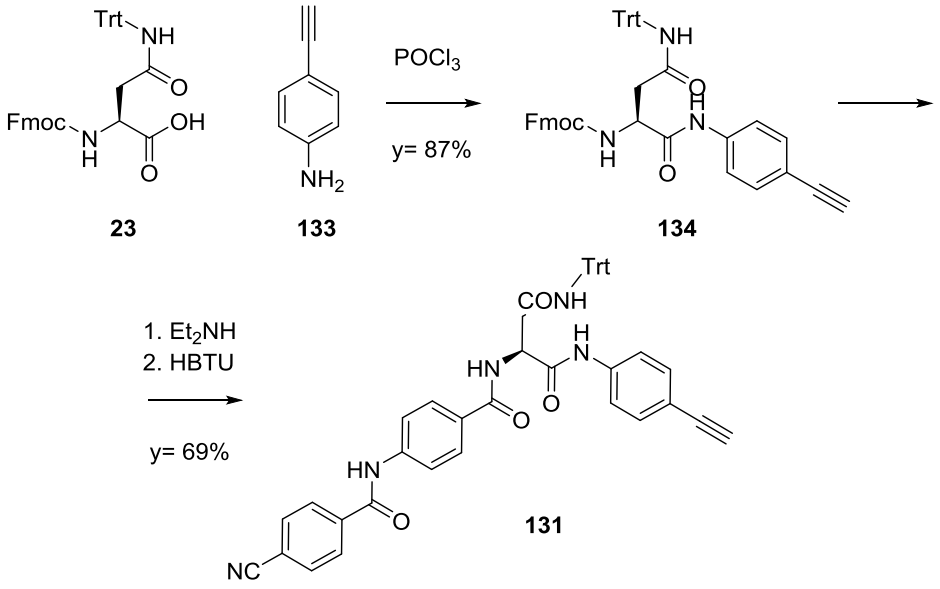


Figure 40: Retrosynthetic disconnection of **130**.

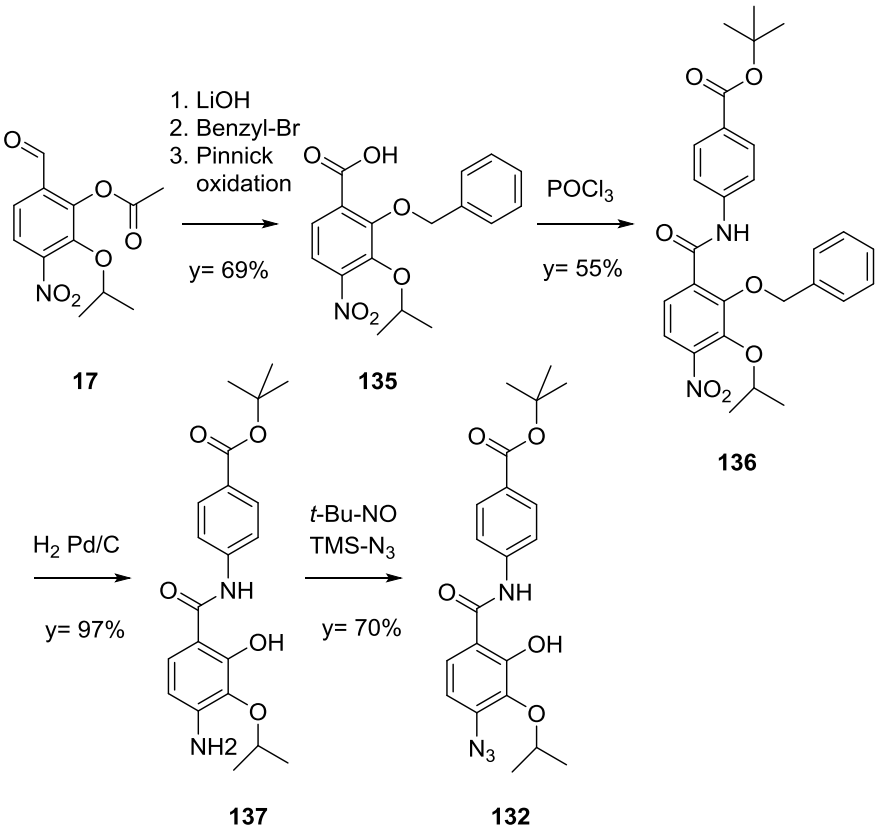
3.4.2.1 Fragments synthesis

The synthesis of fragment A-B commenced with the formation of the amide bond between Fmoc-Asn-(Trt)-OH and 4-ethynylaniline with the established experimental procedure ($POCl_3$, 0°C, few hours reaction time) to afford the desired product **134** in good yield (Scheme 22). The two aromatic rings were installed using HBTU as coupling reagent following deprotection of the Fmoc group. The diaryl unit was synthesized as described for the synthesis of fragments A1 (section 3.2.4.1.1).



Scheme 22: Synthetic steps for the synthesis of fragment A-B.

Fragment C-N_3 was built up starting from nitro aldehyde **17** with a sequence of functional group interconversions to give benzyl-protected PABA-D1 (**135**), which was then coupled with $POCl_3$ to *t*Bu-4-aminobenzoate to obtain the diaryl intermediate **136**. Finally reduction of the nitro group using H_2 on Pd/C entailed concomitant deprotection of the phenol to afford amine **137** (Scheme 23). This was converted to the azide **132** via Sandmeyer reaction: Diazotaion with tert-butyl nitrite was followed by treatment of the diazonium salt with trimethylsilyl azide that furnished the nucleophile. This critical step proceeded smoothly in a good yield.



Scheme 23: Synthesis of fragment C-N_3.

The two fragments **131** and **132** were coupled efficiently via 1,3 dipolar cycloaddition using $CuSO_4$, Na-ascorbate and TBTA as a catalyst[121,122]. After extraction from the reaction mixture, the crude product was treated with TFA in DCM using TIPS as a scavenger to cleave both acid labile protecting groups. The desired product **130** was purified by preparative RP-HPLC with a gradient of water and acetonitrile buffered with 10 mM NH_4HCO_3 in a 55% yield for these two steps. To sum up, the synthesis of this new derivative was accomplished with a longest linear sequence of 11 steps and an overall yield of 7%.

The biological properties of **130** were assessed against the small panel of pathogens and in the DNA supercoiling assay (Table 13). For the following discussion the analog bearing the amide bond instead of the triazole (CNDM 861-2) was selected as the most appropriate reference compound.

"Triazole cystobactamid" was active against all tested strains, notably also against *P. aeruginosa* WT. In comparison to the parent compound, it had higher MIC values against *P. aeruginosa*. On the other hand, the activity against *S. aureus* and both *E. coli* strains was

enhanced. This suggested that presumably the reduced activity against *P. aeruginosa* might not be due to an impaired inhibition efficiency of the target enzyme but rather once again cellular uptake. To prove this, a triazole cystobactamid bearing also the MeO- moiety at the central Asn linker could give interesting information, since it appears that this unusual amino acid enhances the permeation of the antibiotic into the bacterial cells.

The hydrolytic stability towards AlbD of the novel analog still remains to be proven with a proper biochemical assay. Nevertheless, it is reasonable to assume that the molecule would not be cleaved between ring C and PABA-D1.

Table 13: Biological characterization of clickable cystobactamid.

	MIC [µg/ml]					EC gyrase IC_{50} [µM]
compound	*E. coli* ΔtolC	*E. coli* WT	*P. ae.* ΔmexAB	*P. ae.* WT	*S. aureus*	
*CNDM 861-2 (**46**)*	0.06	0.5	0.25	2	0.25	0.08[a]
130	<0.03	0.06	2	8	<0.03	1.5[b]

P. ae.= *P. aeruginosa*; [a] determined at the department of microbial natural products (MINS); [b] determined at the department of chemical biology (CBIO).

To conclude, one of the potential resistance mechanisms of the cystobactamids could be overcome by chemical modification of the sensitive amide moiety. Notably, the designed compound retained antibacterial activities against all tested strains including *P. aeruginosa* WT. The new analog was obtained with a shorter sequence compared to the previously established ones and in clearly higher overall yield (7% versus 1.9% of the first synthetic access).

Additionally, click chemistry could offer a convenient alternative to the tedious coupling reaction of fragments C and B, thus facilitating the synthesis of novel analogs. For these reasons, triazole cystobactamid could represent a new structural template for medicinal chemistry investigation of parts of the molecule unexplored so far.

3.5 ANTIMICROBIAL ACTIVITIES ON A LARGER PANEL OF PATHOGENS

Together with the establishment of SAR, the aim of this thesis was to broaden the spectrum coverage of the cystobactamids and to enhance their activity.

The compounds having the best antimicrobial profile on the small panel of pathogens were selected and tested against clinically relevant Gram-positive and Gram-negative bacteria (Fig. 41 and Table 14 a) and b)). A selection criterion was the presence of activity against *P. aeruginosa* WT.

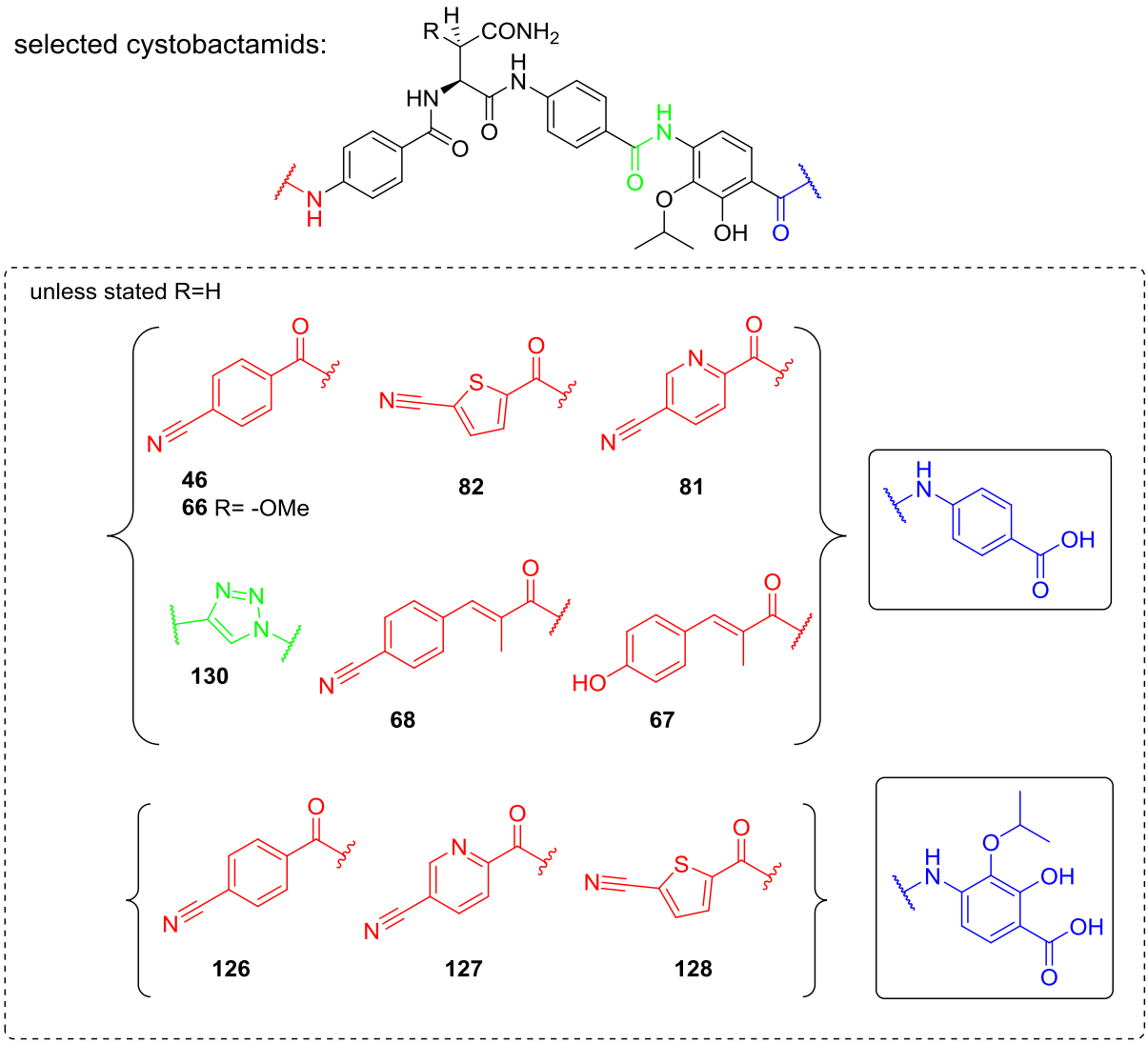


Figure 41: Selected cytobactamid analogs possessing the best antimicrobial profiles. The parts of the scaffold modified with respect to cystobactamid 861-2 are highlighted with colors.

In the extended panel of microorganism, we can find for instance *A. baumannii, E. cloacae, K. pneumoniae*, two resistant strains of *P. aeruginosa,* that express extended spectrum β-lactamases (ESBL), fluoroquinolone resistant *E. coli* (gyrase mutated) and others.

The reference compounds used were the two lead natural products albicidin and cys 861-2 as well as ciprofloxacin. Albicidin was synthesized following the reported experimental procedure [65].

The aim of these antimicrobial tests was to assess the potential of selected new synthetic analogs as broad spectrum antibiotics and to understand whether they had improved antibacterial properties compared to the reference compounds.

Remarkably, some derivatives displayed potent activity against *E. aerogenes, E. cloacae* and *K. pneumoniae*, whereas both albicidin and cys 861-2 were inactive (Table 14).

The MICs against some critical pathogens could also be enhanced in comparison to the reference natural products. An example is *A. baumannii,* against which activity in the sub-μg/mL range could be reached (0.06 μg/mL for **68**), that is clearly improved in comparison to cys 861-2 (1 μg/mL) and albicidin (>64 μg/mL). Noteworthy, the activity of **68** against *A. baumannii* exceeded even the one of ciprofloxacin by five-fold.

This is not the sole example where the novel analogs did better than ciprofloxacin. Also *E. faecalis, S. epidermidis* and one of the ESBL strain of *P. aeruginosa* are inhibited at lower concentrations by the synthetic analogs.

The activity against a fluoroquinolone resistant *E. coli* was also assessed. The resistance is determined by the mutations at the active site of gyrase, as discussed in the introduction. The activity of ciprofloxacin against such bacteria is drastically reduced compared to the corresponding WT (ca. 100 fold), and the ones of albicidin and cystobactamids were reported to be only partially affected (two-eight fold) [47,52]. This led to the hypothesis that the binding site of the natural products partially overlaps with the one of the quinolones.

New synthetic analogs having the same activity against both *E. coli* WT and fluoroquinolone resistant strains were found, and notably also compounds possessing even higher inhibitory properties against *E. coli* carrying the resistance mechanism. Explanations of these findings could be that this potential resistance mechanism can be overcome by proper chemical modifications, or that actually the binding site of the cystobactamid does not overlap with the one of the quinolone.

Table 14 a) and b): Antimicrobial properties of selected cystobactamid analogs on a large panel of Gram-positive and Gram-negative bacteria of clinical interest in comparison with three reference compounds.

12 a)

MIC [µg/mL]	**Cys 861-2**	**Alb**	**CIP**	**46**	**130**	**126**	**67**	**68**
E. faecalis	1	4	0.64	0.5	16	nd	0.5	0.5
S. epidermidis	0.5	0.5	0.3	<0.03	0.125	nd	0.25	<0.03
A. baumannii	1	>64	0.32	1	8	0.5	0.25	0.06
***E. coli* WT**	0.06	0.06	0.005	0.25*	16	0.125	0.13	0.125
***E. coli* WT-3 [*gyrA*(S83L,D87G)]**	0.5	0.06	0.64	0.25	2	nd	0.06	0.125
E. aerogenes	>64	>64	0.08	0.06*	1	> 64	1	0.25
E. cloacae	>64	>64	0.16	0.25*	>64	nd	>64	>64
***P. aeruginosa* ESBL 1**	4	16	6.4	4*	16	nd	>64	>64
***P. aeruginosa* ESBL 2**	2	16	0.16	4	16	nd	2	32
K. pneumoniae	>64	>64	0.02	0.5*	>64	> 64	>64	>64
C. freundii	0.125	<0.03	0.003	<0.03	0.5	0.125	0.13	0.06
S. marcescens	>64	>64	0.32	>64	>64	> 64	64	>64
P. vulgaris	0.25	<0.03	0.005	0.25	2	0.25	0.13	<0.03
P. mirabilis	32	>64	0.04	>64	16	nd	2	>64

Alb= albicidin; CIP= ciprofloxacin; ESBL= extended spectrum β-lactamases; *E. coli* WT-3 carries the characteristic mutations conferring resistance to fluoroquinolone; detailed bacterial strains information is present in the experimental part. * value could not be determined unambiguously and might be higher than assigned.

12 b)

MIC [µg/mL]	**Cys 861-2**	**Alb**	**CIP**	**81**	**82**	**66**	**127**	**128**
E. faecalis	1	4	0.64	0.125	0.25	0.125	<0.03	<0.03
S. epidermidis	0.5	0.5	0.3	1	0.5	<0.03	<0.03	<0.03
A. baumannii	1	>64	0.32	0.125	0.125	1	0.25	1
***E. coli* WT**	0.06	0.06	0.005	2	1	2	0.5	0.5
***E. coli* WT-3 [*gyrA*(S83L,D87G)]**	0.5	0,06	0.64	0.25	0.125	0.25	2	0.5
E. aerogenes	>64	>64	0.08	0.25	0.25	2	1	1
E. cloacae	>64	>64	0.16	>64	>64	>64	>64	>64
***P. aeruginosa* ESBL 1**	4	16	6.4	>64	8	2	>64	>64
***P. aeruginosa* ESBL 2**	2	16	0.16	4	8	1	>64	>64
K. pneumoniae	>64	>64	0.02	>64	>64	8	4	1
C. freundii	0.125	<0.03	0.003	<0.03	<0.03	<0.03	0.5	0.5
S. marcescens	>64	>64	0.32	>64	>64	>64	>64	>64
P. vulgaris	0.25	<0.03	0.005	0.06	0.06	0.125	1	1
P. mirabilis	32	>64	0.04	4	>64	4	8	>64

Alb= albicidin; CIP= ciprofloxacin; ESBL= extended spectrum β-lactamases; *E. coli* WT-3 carries the characteristic mutations conferring resistance to fluoroquinolone; detailed bacterial strains information is present in the experimental part. * value could not be determined unambiguously and might be higher than assigned.

The analogs discussed in this section bear modifications at the N-terminus (ring A) and/or at the C-terminus (ring PABA-D2). Regarding ring A nearly all of them possess a cyano group on a phenyl ring that in some instances is replaced by a heterocycle or connected to the carbonyl group with the methacryl moiety found in albicidin. Others possess an additional isopropoxy and hydroxyl groups at PABA-D2 together with the optimized N-terminal acyl residues (Fig. 41).

Two compounds that go out of this Scheme are **130** and **66**. The former present a triazol bioisoster of an amide bond, and the latter has the methoxy at the central asparagine unit (3*R* configuration).

Interestingly, “triazole cystobactamid” **130** has practically the same spectrum coverage as the corresponding analog bearing the original amide **46**. However, it shows in general lower inhibitory properties.

The overall good spectrum coverage of this molecule indicates that a bioisosteric substitution is tolerated. Therefore, this molecule represents an interesting scaffold for further medicinal chemistry investigations, since a critical coupling step of the synthesis can be avoided using a more efficient reaction (1,3 dipolar cycloaddition). Possibly, modifications of other units of the molecule could lead to enhanced MIC values.

Derivative **66** has the broadest antibacterial spectrum coverage. Remarkably, it possesses activity in the low μg/mL range against all the tested bacteria with the sole exceptions of *E. cloacae* and *S. marcescens*. This cystobactamid shows potent MIC values against critical bacterial strains such as *P. aeruginosa* ESBL, against which the inhibition was clearly higher even compared to ciprofloxacin.

These results indicate that the presence of the methoxy in the central Asn is beneficial for the spectrum coverage. On the other hand, it is noteworthy that **66** does not present the lowest MIC values against all the tested strains. This thus suggests that a certain strain-dependent effect influences the activity of the cystobactamids derivatives.

To sum up, I have synthesized several novel cystobactamids with broadened spectrum and clearly improved antibacterial activity in comparison to both cys 861-2 and albicidin. Against some bacteria, even the activity of ciprofloxacin could be exceeded. These results indicate that the antibacterial properties of the cystobactamids can really be improved by structural optimization. This further confirms the great potential of these novel natural products as lead scaffold for the development of a novel antibiotic.

3.6 STRUCTURE-ACTIVITY RELATIONSHIPS

The understanding of the structural requirements that give optimal biological properties to a molecule is an essential step in early-stage drug development. In the case of the cystobactamids, optimal biological properties include a broad and potent antibacterial spectrum. The analogs presented in this thesis are of relevance for their potent antibacterial activity and, because they allow the establishment of structure-activity relationships (SAR).

In this work, a deep understanding of the N-terminus of the cystobactamid scaffold was obtained, together with relevant information regarding the central aliphatic linker and two aromatic units at the C-terminus (Fig. 42).

The full length of the molecule is essential for the activity, shortening it of a single aromatic unit either at the N- or C- terminus led to total loss of activity.

For optimal inhibition of gyrase and also for good antibacterial properties, aromatic ring A should be electron poor, a cyano group confers the best antibacterial properties. The optimal position of the substituent on ring A is *para*. Replacement of the phenyl ring with heterocycles is possible and has a beneficial effect on the activity. Finally, the presence of a short rigid spacer, such as a substituted double bond, between aromatic ring and carbonyl was well tolerated.

The "*para*-connectivity" of the aromatic building blocks seemed to be essential. For instance, moving the aniline group of ring B to the *meta* position had a drastic negative effect on the activity.

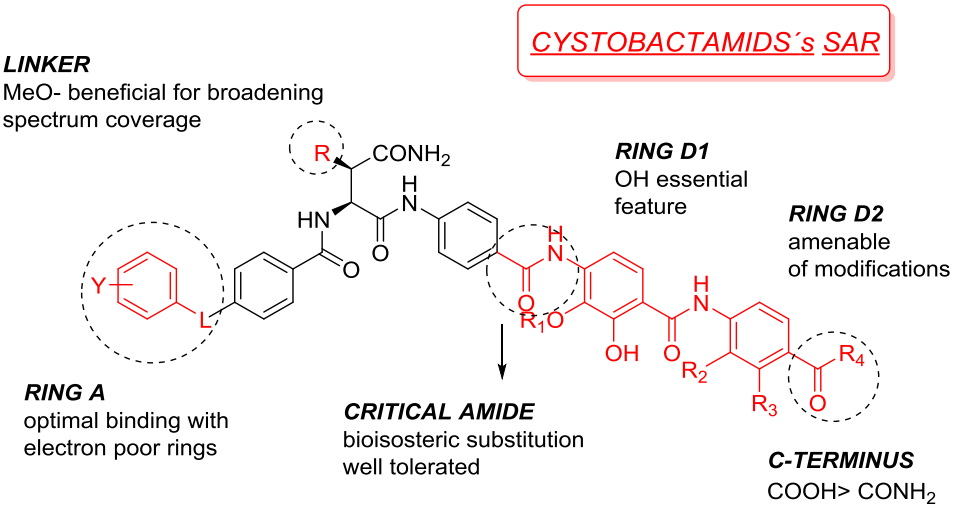


Figure 42: Structure activity relationships of cystobactamids. The parts of the molecule investigated in this thesis are highlighted in red.

The structural simplification in the aliphatic linker region was well tolerated, however, the presence of the original methoxy group at the central asparagine moiety, found in the natural cystobactamids, seemed to be beneficial for a broader spectrum coverage.

Moving to the C-terminus, PABA-D1 represents the most critical part of the molecule, the changes done here led to drastic losses of activity. The amidic bond between ring D1 and D2 is unusually acidic (pK_a lower than 8), this could be partially explained by the presence of the phenol that allows stabilization of the anion generated (Fig. 43).

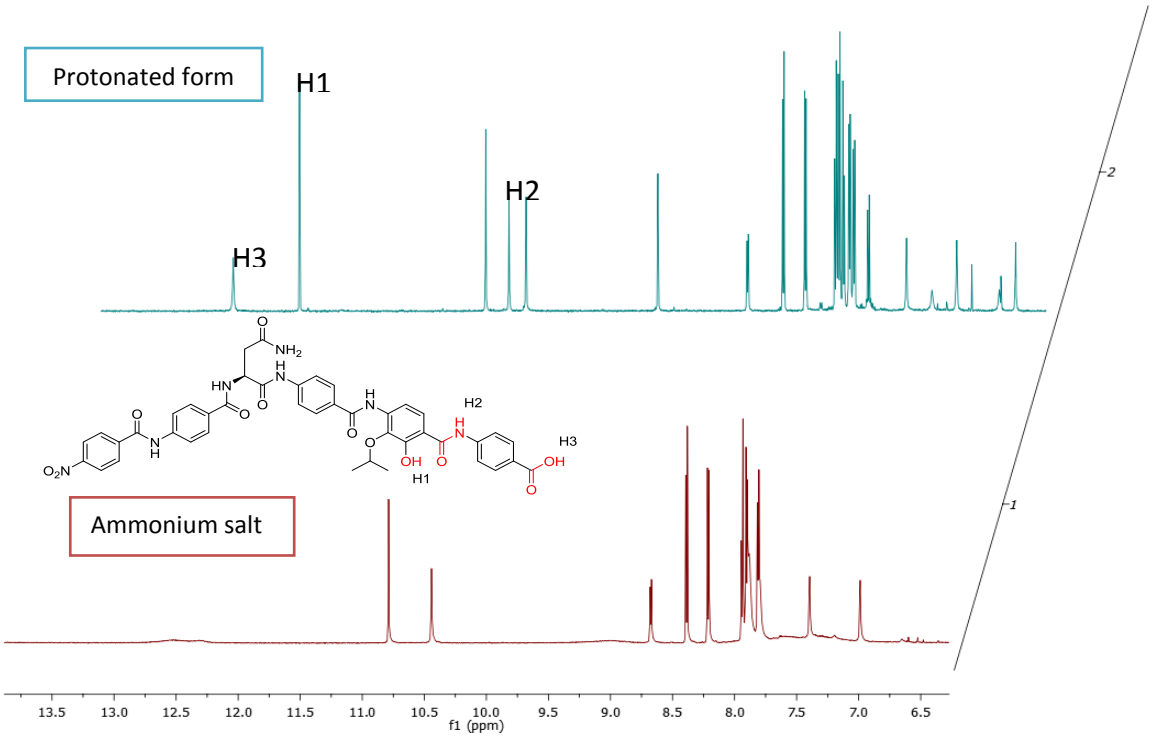


Figure 43: Enlargement of H-NMR spectra of two different forms of cys DM 861-2 (**1**). This highlights the acidity of the amide connecting PABA-D1 and D2.

Understanding of the binding mode of the cystobactamids would be required in order to better clarify the role of this critical part of the molecule, that could be either mediating optimal gyrase inhibition or enabling cellular uptake (the gyrase IC_{50} value for analog **2** was not determined).

The introduction of substituents on PABA-D2 was well tolerated. This thus suggested that aromatic unit D2 is more amenable of modifications than PABA-D1. The carboxylic acid at the *para* position was by far the best option among those attempted.

The replacement of the amide bonds between the aromatic units with triazole bioisosters was showed to be in principle possible.

Compounds that bear simultaneously the optimal features identified for the N- and/or C-terminus and central part had very potent antimicrobial profile. This demonstrates that multiple rational changes at the molecular architecture of the cystobactamids can have positive additive effects on the activity.

It should be kept in mind that potent gyrase inhibitions not always correlated to good antibacterial properties. For this reason, the SARs presented here not only reflect good enzymatic inhibition, but also sufficient entry of the antibiotic into the bacterial cells.

A rational systematic investigation of the other building blocks of the scaffold will possibly reveal additional favorable structural features. That, merged together with the information generated in my work, will allow the design of fully optimized cystobactamids with potentially even better antibacterial profile and improved drug-like properties.

3.7 PHOTOSWITCHABLE CYSTOBACTAMIDS

In the introduction of the thesis, we have seen the photoswitchable moieties reported in the literature and some application examples of them in photopharmacology, with azobenzene as the most widely investigated an d best understood congener so far.

For their characteristic oligomeric PABA-derived scaffold, the cystobactamids represent a perfect template for the design of photoswitchable antibiotics. Ideally, the activity of the two isomeric forms should consistently differ, and preferably the thermodynamically less stable isomer should be the one with the higher antibacterial properties. This is to limit the exposure to the active drug to specific sites and time ranges.

In this section of the thesis, the attempts to create cystobactamids with antibacterial properties that can be modulated with light will be presented. In particular, azobenzene and acylhydrazone were used as photoswitchable moieties for addressing either the N- or C-terminal side of the molecule.

3.7.1 **Azobactamids**

3.7.1.1 Chemistry

It is desirable that a photoswitchable drug has activity in the same range of the parent compound, therefore the design of it should be guided by SAR and/or by the rule of caution to vary the structural features of the molecule as little as possible.

For this reason, considering the “available” functional moieties presented in section 1.5, azobenzene represented the most suitable one for the transformation of the cystobactamid architecture into a photoconvertable antibiotic. In order to be as little invasive as possible, the amide group that connects rings A and B was replaced with an azo (-N=N-) moiety (Fig. 44). Additionally, I investigated also the effect of substituents with different electronic features at the *para* position of ring A on the activity and photochromic properties.

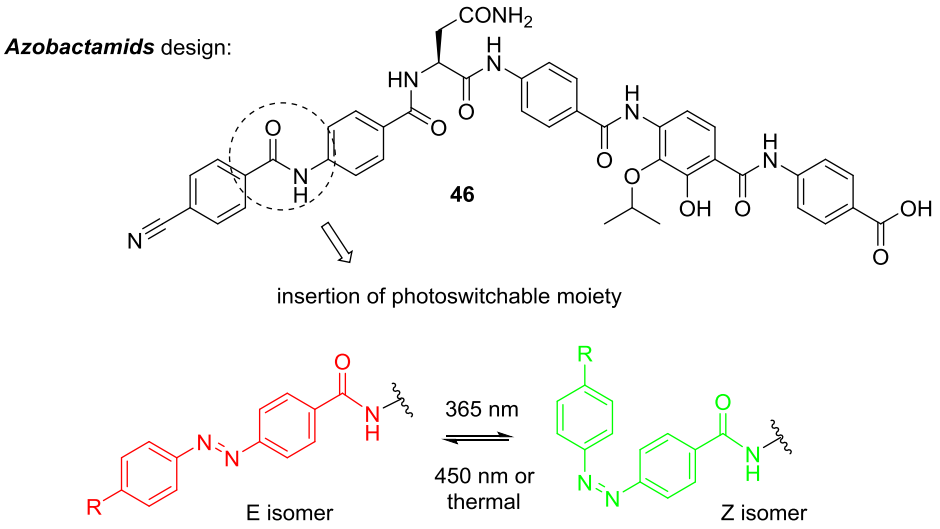
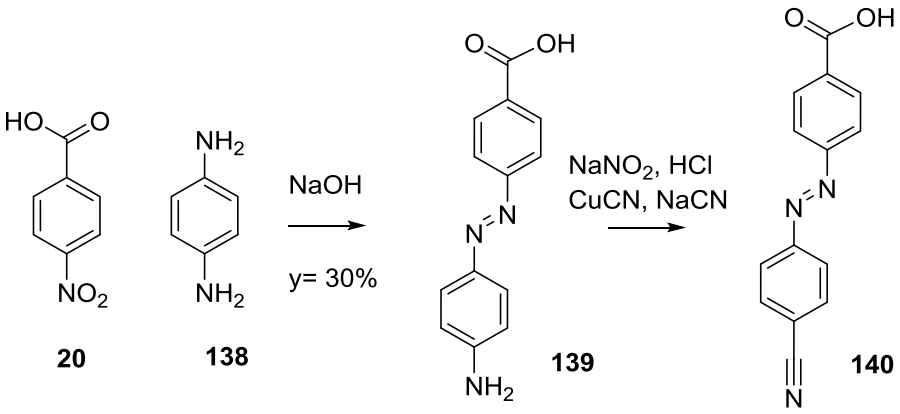


Figure 44: Design of azobactamids, highlighted in red and green the two isomeric states of the molecule.

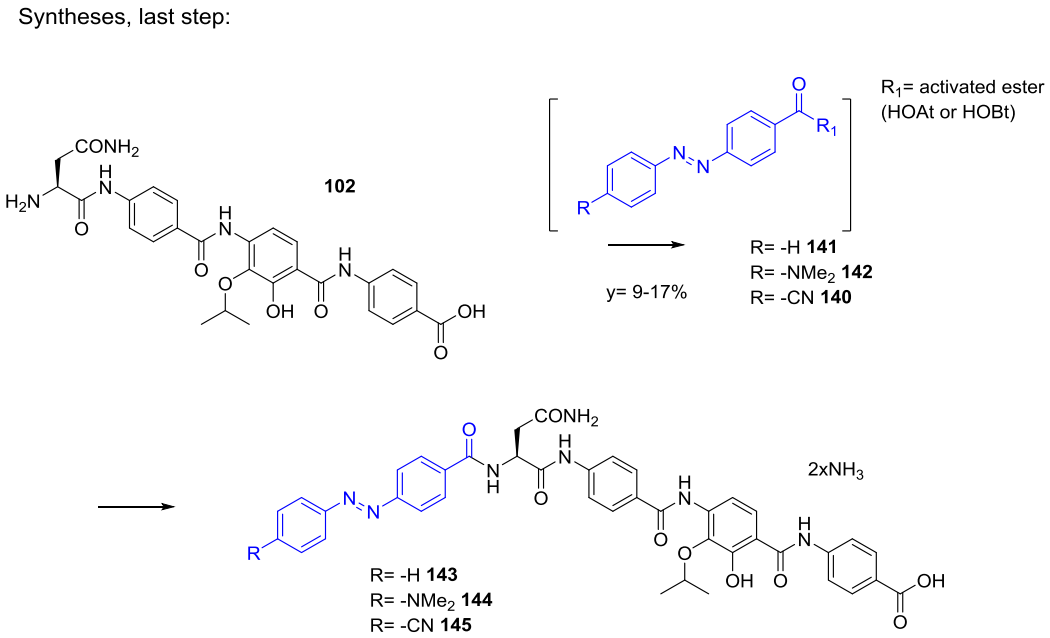
Three molecules were designed bearing an EDG, EWG and no substituent on aromatic ring A. For their synthesis, the disconnection strategy that allows introduction of aromatic units A-B in the last step was used. Two of the three azobenzene building blocks needed were commercially available, whereas the one bearing a nitrile had to be synthesized. The choice was based on the SAR developed (see Section 3.2).

The synthesis of this building block commenced with the formation of the asymmetric azo benzene unit **139**, which had to offer on one side a coupling anchor and on the other side a functional group amenable to modifications. For this purpose, *para*-nitrobenzoic acid and benzene-1,4-diamine were condensed together in a solution of NaOH at refluxing conditions[95]. In the next step, diazotation followed by Sandmeyer reaction afforded the desired *para*-cyano derivative **140** (Scheme 24).



Scheme 24: Synthesis of azobenzene building block.

The different fragments A-B bearing the azo moiety were coupled to the amine **102** as pre-activated esters (Scheme 25). Activation was carried out by means of either EDC/HOAt or HBTU in DMF. After extraction of the product from the reaction solution (DMF), the crude materials were purified by RP-HPLC to afford the desired compounds with yields between 9 and 17%.



Scheme 25: Last step of the synthesis of azobactamids.

3.7.2 **Hydrazobactamid**

The SAR discussed earlier showed that modifications at the C-terminus are in general less tolerated than those at the N-terminus. For this reason, the insertion of a photoswitchable moiety on this side of the molecule would potentially offer a higher probability to generate derivatives where the two isomers have a large difference in antibacterial properties, generating ideally a real ON/OFF antibiotic (active/inactive).

Acylhydrazones, reported as photoswitches for the first time in 2015[91], represented an interesting opportunity for addressing this side of the molecule (Fig. 45). This was because they introduced only small structural alterations, their synthetic access was straightfoward, and they represented a novelty in photopharmacology.

The key step for the formation of this moiety relies on the high efficiency of the reaction between aldehydes and hydrazides. For this reason, the formation of the hydrazine moiety was envisaged at the end of the sequence.

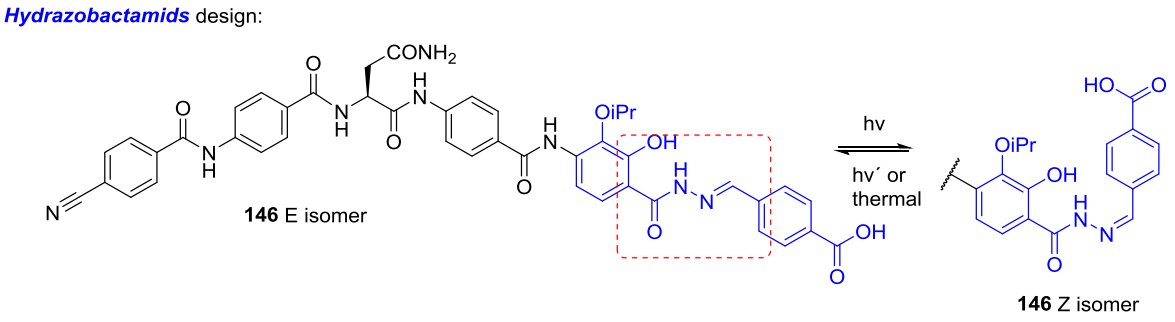
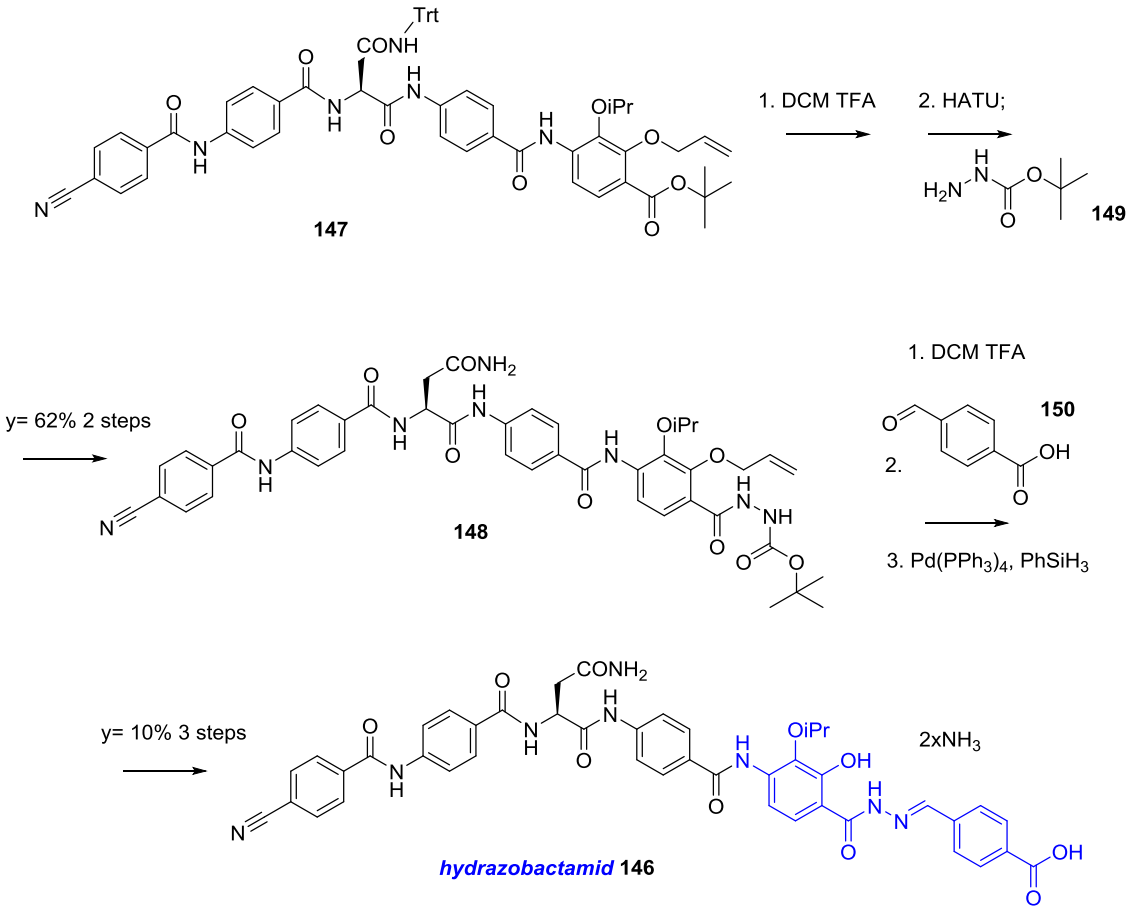


Figure 45: Addressing the C-terminus using a novel photoswitchable moiety, acylhydrazone. The two isomeric states of hydrazobactamid analog are depicted, in blue the photoswitchable moiety involving PABA-D1 and D2.

The synthesis of this photoswitchable cystobactamid started from advanced fragment **147** (Scheme 26). Its synthesis was the subject of the master thesis of Tim Mollner, who has been working under my supervision. It is an additional, novel synthetic approach established to access cystobactamid analogs and it was designed for a deeper investigation of ring PABA-D2[123].

The acid labile protecting groups were cleaved with TFA in DCM to afford the free carboxylic acid at PABA-D1, which was preactivated with HATU and then treated with an excess of *tert*-butyl hydrazinecarboxylate to afford the product **148** in more than 60% yield over 3 steps.

This was treated with TFA to liberate the amino group, condensation of the 5th aromatic ring occurred readily after addition of a solution of aldehyde in THF. Final deprotection of the phenolic allyl group afforded the desired product **146** after RP-HPLC purification.



Scheme 26: Finals steps of the synthesis of hydrazobactamid derivative.

3.7.3 **Photocromic and biological evaluation**

3.7.3.1 UV spectra

In this section the UV spectra of the three azobactamids **143**-**145** are given, in red the spectrum of the pure *trans*-isomer, while in green is depicted the one at the photostationary state (PSS) after irradiation with UV light of 365 nm.

At the PSS, **143** and **145** possess an initial, small absorption maximum at around 260 nm. Following, a strong maximum at around 330 nm for both forms is present, that is more intens for the *trans* isomer (characteristic spectral behavior for unsubstituted azobenzenes). In the UV-vis range, the *cis* isomer should possess a slightly higher maximum than the *trans*, but this is practically absent for azobactamids. This atypical feature is difficult to explain, it might be determined by the numerous aromatic rings of the molecules, that potentially can establish extensive conjugation and/or intramolecular pi stacking interactions.

For **144**, the electron donating dimethyl amino moiety creates a so-called push-pull system with the acyl moiety on the opposite site. This entails a decrease of the absorption band at 330 nm with concomitant appearance of another strong maximum in the UV-vis range. Irradiation of the *trans* isomer with light of 365 nm did not afford any change of the UV-vis spectrum. Since an absorption minimum is present in this point of the spectrum, it could be assumed that this is the reason for the lack of photoconversion. On the other hand, the *cis* isomer of push-pull systems are described in the literature for possessing a short half-life[124], therefore also this explanation cannot be excluded. Irradiation experiments with light of wavelength in the visible range (between 400-500 nm) would be required to better assess the photoswitchable properties of **144**, that potentially represents a photoswitchable antibiotic that can be switched in the UV-vis range.

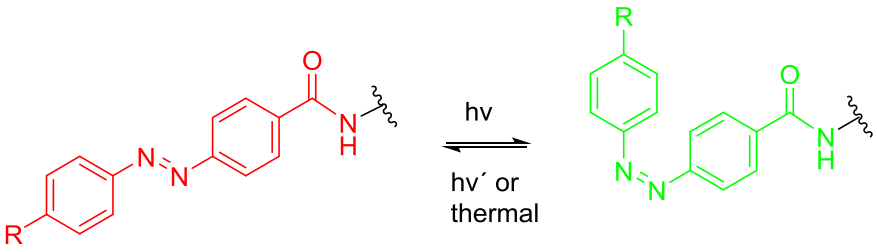
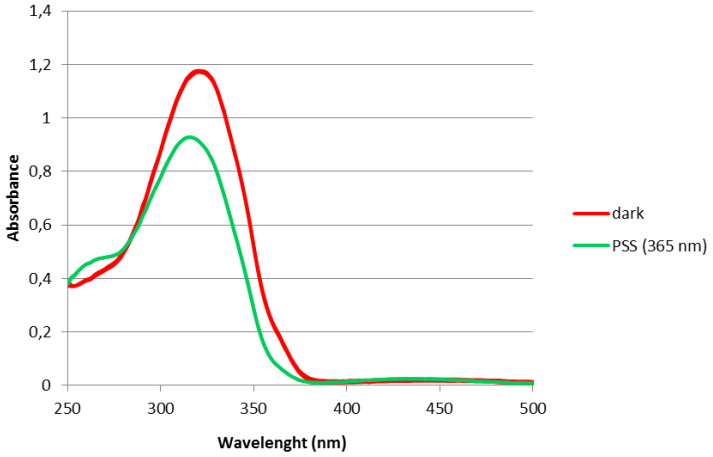
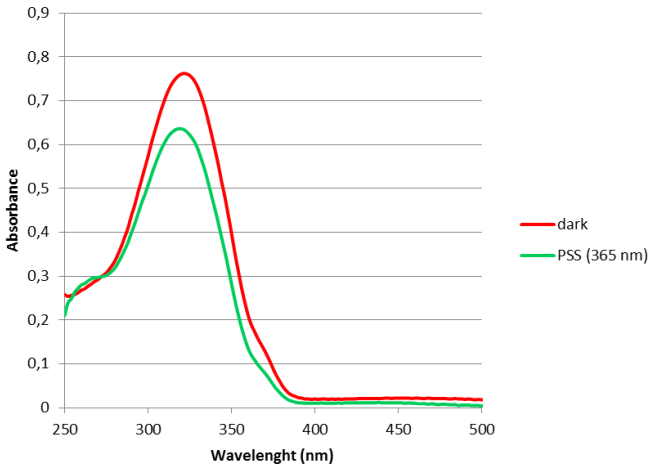


Figure 46: General principle of azobenzene photoconversion, molecular switching is possible upon irradiation with a certain wavelength (hv) or by thermal relaxation.

R= H (**143**)photostationary state(PSS) 80% *cis*.



R= -CN (**145**) PSS 50% *cis*.



R= -N(Me)$_2$ (**144**) no photoconversion with UV light of 365 nm.

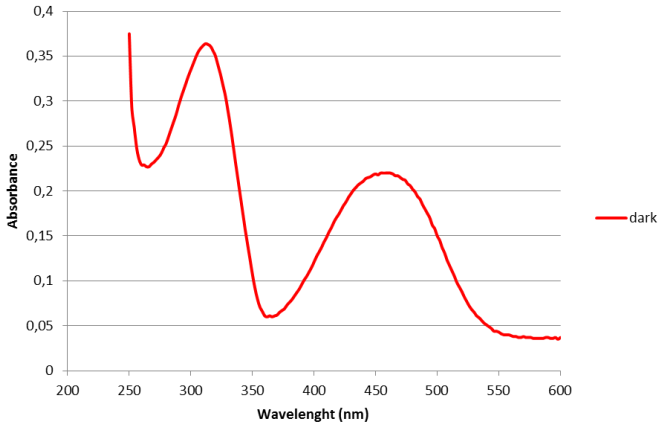
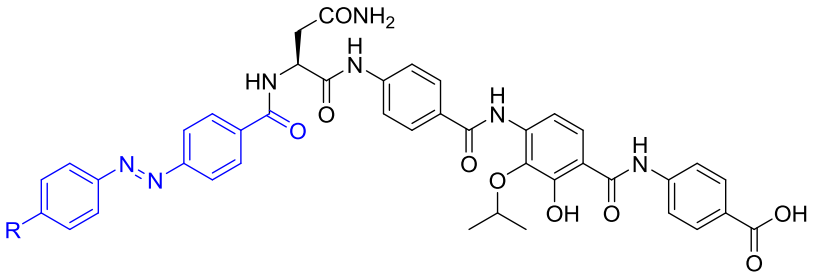


Figure 47: UV spectra of azobactamids synthesized, the PSS was determined by LCMS analysis immediately after irradiation of the probe (0.18 mM solution in DMSO +0.1% HCOOH) with light of 365 nm.

3.7.3.2 Modulation of biological properties with light

The biological properties of the azobenzene analogs were assessed by the gyrase supercoiling assay and by determining the minimal inhibitory concentration against *E. coli* BW25113 (Table 15). To the best of my knowledge, this represents the first example in which a photoswitchable antibiotic is characterized by means of both enzymatic and cell-based assays.

Table 15: Assessment of the difference of biological activities between *trans* and *cis* isomers of the photoswitchable cystobactamids.



R		MIC E. Coli [µg/ml]	*EC gyrase IC_{50} [µM]* [b]
-H (**143**)	*trans*	0.35	6.1
	cis	0.18	3.4
-CN (**145**)	*trans*	0.64	0.5
	cis	0.45	0.1
-N(Me)$_2$ (**144**)	*trans*	0.13	0.9
	cis	/	/

Compound **143** and **145** were tested either after they were left in the dark or after irradiation with light of 365 nm. Whereas only the *trans* isomer of **144** could be tested due to the lack of photoswitching after irradiation (365 nm).

All the molecules showed potent MIC values in the low µg/mL range as well as good IC_{50}s in the DNA supercoiling assay.

The *cis* isomer of both **143** and **145** possessed higher inhibitory properties compared to their corresponding *trans* form in both assays. The widest difference in inhibitory activity between the pair of isomers was found with the CN derivative **145** (five-fold in the enzymatic assay).

These results demonstrated that the molecular design was successful. Furthermore, they also confirmed the previously established SAR, reporting that an electron poor aromatic ring A confers better gyrase activity.

Because the *trans* geometry is more thermodynamically favorable for an amide bond, the finding that the *cis* isomer of azobactamids were more active than the *trans* isomer was surprising. At the same time, this placed azobactamids in the ideal case for a photoswitchable drug, since they offer the possibility to specifically "*turn ON*" the most active antibiotic form. Additionally, this provided meaningful information regarding the binding mode of cystobactamids with their target enzyme, suggesting that the inhibitors may adopt a *cis*-conformation upon binding to gyrase.

The characterization of the photoswitchable and biological properties of hydrazobactamid **146** has not been completed yet. Attempts to switch the *trans* to the *cis* isomer with 365 nm light irradiation did not afford any appreciable conversion. Nevertheless, the effect of this modification on the biological activity was assessed. Promisingly, the molecule was still very active against *E. coli* with an MIC of 0.03 µg/mL.

Irradiation experiments with a proper source of light will allow the understanding of the photocromic (isomer ratio at the PSS state, half-life of *cis* isomer, robustness) and biological properties of this novel photoswitchable antibiotic.

It is also desirable that the conformational change on this side of the molecule will bring even higher difference between the biological activities of the two isomers.

In conclusion, I synthesized four photoswitchable cystobactamids. Three of them have modification at the N-terminus and one at the C-terminus. Either azobenzene or acylhydrazone was used as photoswitchable moiety.

The biological evaluation of the analogs was done with both DNA supercoiling and antibacterial assays, domonstrating that the cystobactamids are suitable scaffolds for photoswitchable drugs. Interestingly, the study revealed that the *cis* isomer of azobactamids was more active than the *trans*.

To the best of my knowledge, this work has two important novelties:

- It is the first time that the activity of a photoswitchable antibiotic is described by means of both cell based and enzymatic assays
- Hydrazobactamid **146** represents the first application of acylhydrazones in photopharmacology.

3.8 OUTLOOK

Based on the results presented here, the structural optimization of the cystobactamids should be further explored targeting the central linker region and the aromatic units B and C (Fig. 48).

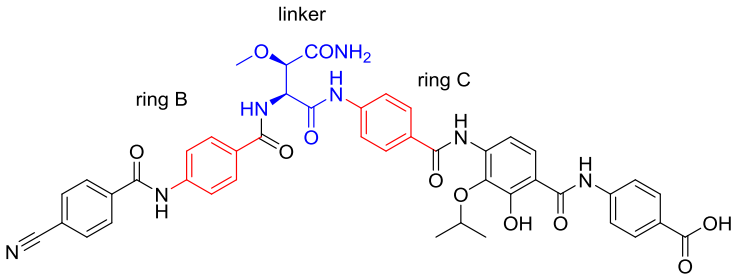


Figure 48: Structure of analog **66** that could be used as lead structure.

Additionally, quantitative LCMS-based studies, for assessing the concentration of the inhibitor inside the bacterial cells, could be performed with selected cystobactamids. These would be very useful in order to confirm that the reasons for the observed discrepancies between gyrase IC_{50} and MIC values for some analogs are due to different penetration and/or accumulation properties of the molecules.

The photopharmacological studies on the cystobactamids should also go on. The synthesis of cystobactamids that incorporate two photoswitchable moieties at the same time could be of particular interest. This would bring to a photoswitchable antibiotic with four isomeric states that would represent an absolute novelty in the field and potentially offer molecules with very large differences in biological properties (Fig. 49).

Finally, the photoswitchable antibiotics revealed that the *cis* isomer is the more active, to confirm this finding, analogs with the N-terminus rigidified in this conformation could be synthesized.

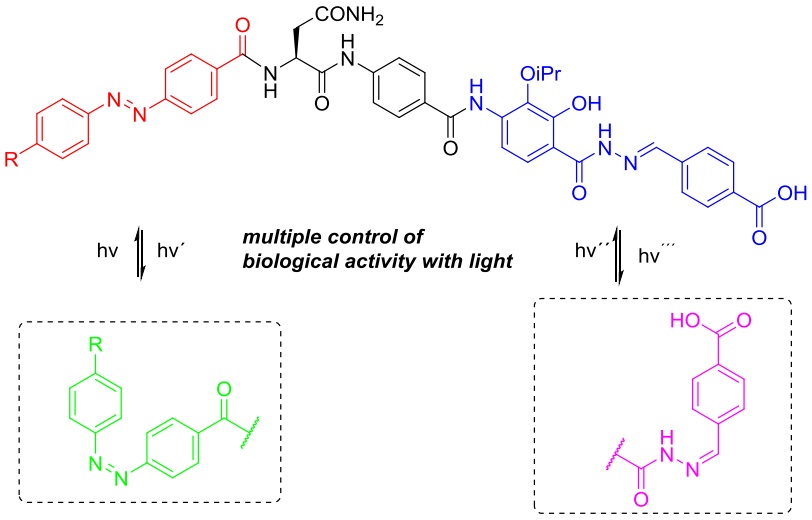


Figure 49: Design of double photoswitchable cystobactamids.

3.9 CONCLUSION

The two main objectives of my research project were the synthesis of analogs of cystobactamid 861-2 with improved antibacterial properties, particularly against Gram-negative bacteria, and the establishment of structure-activity relationships of this novel natural product scaffold.

In the course of my doctoral studies, I have established two synthetic routes to cystobactamid analogs. One of them gave the possibility to introduce late-stage modifications of ring A, and this was used for a deep investigation of the N-terminus as well as for variations at the C-terminal side. The second route allowed the synthesis of molecules with wider modifications of the N-terminus (both ring A and B). The former synthesis has a longest linear sequence of 12 steps with overall yield ranging from 0.6 to 3.6%, while the latter has 11 steps and overall yield between 0.5 and 2.8%.

In sum, more than 50 cystobactamid derivatives were synthesized. Their biological properties were evaluated by means of MIC and DNA supercoiling assays. The information generated allowed the establishment of comprehensive SAR of the N-terminal part of the molecule and also basic understandings of the C-terminus and the central linker.

Analogs with clearly improved antibiotic properties against clinically relevant pathogens were synthesized. Remarkably, the activity of the cystobactamids was extended to Gram-negative bacteria such as *E. aerogenes, E. cloacae* and *K. pneumoniae.* Additionally, the potency of the compounds was also enhanced and in some cases it even exceeded the one of ciprofloxacin (eg against *A. baumanii* and *P. aeruginosa* ESBL).

Furthermore two potential resistance mechanisms were assessed. The nucleosides importer Tsx turned out to have only a minor effect on the activity of the cystobactamids. This demonstrated that despite the structural similarities with albicidin, important differences at the molecular level might be present between these two natural product classes.

The potential hydrolytic instability towards AlbD could be presumably overcome by substituting the critical amide bond with a triazol bioisoster. The derivative obtained retained the antibacterial properties and might offer a new structural template for further medicinal chemistry studies.

I have also investigated the applicability of the cystobactamids in photopharmacology. Four photoswitchable antibiotics were synthesized with either the N- or the C-terminus modified.

These molecules carry either an azobenzene or an acylhydrazone as photoswitchable moiety. Notably, the latter derivative represents the first application of acylhydrazone in the field of photopharmacology.

The difference of biological properties of the two isomeric states was assessed by means of both enzymatic and cellular assays. Interestingly, the information obtained suggested that the *cis* conformation of the amide bond at the N-terminus enables optimal binding with the target enzyme.

To conclude, my doctoral studies dealt with medicinal chemistry investigations on the cystobactamids. Molecules with extended antibacterial spectrum and enhanced antimicrobial potency against critical Gram-negative bacteria have been synthesized. These discoveries demonstrate the great potential of this innovative antibacterial scaffold to generate new broad spectrum antibiotics in the future.

4 EXPERIMENTAL PART

4.1 BIOLOGY

4.1.1 Minimal inhibitory concentrations (MIC) determination

All microorganisms were handled according to standard procedures and were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) or the American Type Culture Collection (ATCC). Tsx-deficient *Escherichia* coli and corresponding parental strains were purchased from the Coli Genetic Stock Center (CGSC). Fluoroquinolone-resistant strains *Escherichia coli* WT-3 and *E. coli* wild-type were kindly provided by Prof. Dr. P. Heisig (University of Hamburg, Germany). *Pseudomonas aeruginosa* PA14 and PA14Δ*mexAB* were kindly provided by Prof. Dr. S. Häußler (Twincore, Hannover). cystobactamids were prepared as DMSO stocks (5 $mgmL^{-1}$). Minimum inhibitory concentrations were determined according to standard procedures as described elsewhere[47]. Single colonies of the bacterial strains were suspended in cation-adjusted Müller-Hinton broth to achieve a final inoculum of approximately 10^4 $CFUmL^{-1}$. Serial dilutions of cystobactamids (0.03 to 64 μgmL^{-1}) were prepared in sterile 96-well plates and the bacterial suspension was added. Growth inhibition was assessed after overnight incubation (16-18 h) at 30-37°C. MIC values are defined as the lowest concentration of antibiotic that inhibited visible growth.

Bacterial strains used for the biological characterization of most of the compounds:

- *Escherichia coli* DSM-1116 (WT)
- *Escherichia coli* TolC-deficient
- *Pseudomonas aeruginosa* PA14
- *Pseudomonas aeruginosa* PA14Δ*mexAB*
- *Staphylococcus aureus* ATCC29213
- *Escherichia coli* BW25113 (CSGC# 7636) (WT1)
- *Escherichia coli* JW0401-1 (CGSC# 8574) Tsx-deficient (Δtsx1; parental strain: BW25113)
- *Escherichia coli* MG1655 (CGSC# 6300) (WT2)
- *Escherichia coli* χ2844 (CGSC# 6683) Tsx-deficient (Δtsx2; parental strain: MG1655)

4.1.2 **DNA supercoiling assay**

The half-inhibitory concentrations (IC_{50}) against gyrase are denoted in the text with the letter a) and b). These indicate two different laboratories where the assay had been carried out.

a) Stays for "department of microbial natural products- Helmholtz institute for pharmaceutical research Saarland" (MINS), the assay was done by Dr. Jennifer Hermann.
b) Stays for "department of chemical biology- Helmholtz centre for infection research" (CBIO), Dr. Peter-Hans Prochnow performed the assay.

The assays were carried out as follow:

4.1.2.1 Method a)

Commercial *E. coli* gyrase supercoiling assay kits (Inspiralis, Norwich, UK) were used to determine half-inhibitory concentrations (IC_{50}). Cystobactamids were prepared as DMSO stocks [assay concentration range: 0.05 to 25 µM, DMSO concentration: 0.1 % (*v/v*)] and the reference drug ciprofloxacin was prepared as acidified (HCl) aqueous solution. Assays were performed according to the manufacturer's protocol. In brief, *E. coli* gyrase (1 U) was mixed with the inhibitors and 0.5 µg relaxed plasmid was added after 10 min equilibration at room temperature. Reactions were quenched after 30 min at 37 °C by the addition of DNA gel loading buffer containing 10 % (*w/v*) SDS. Relaxed (REL) and supercoiled (SC) plasmids were separated on 0.8 % (w/v) agarose gels and visualized by ethidium bromide staining. Each gel was additionally loaded with two control reaction samples (1: without enzyme; 2: solvent control). Image analysis (intensity of REL and SC plasmid bands) was performed in ImageJ[125]. The respective values were normalized to the controls and the ratio SC/REL was used to determine IC_{50} values by sigmoidal curve fitting in Origin (OriginLab, Northampton, MA).

4.1.2.2 Method b)

Relaxed plasmid DNA was prepared by mixing 25 µg of circular pUC19 with 6.5 U of Topoisomerase I [Thermo] in a total volume of 50 µl Topoisomerase buffer (250 mM Tris, [pH 7.5], 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM DTT, 0.5 mM EDTA, 150 µg/ml BSA). The

reaction was run for 90 min at 37°C and eventually purified via DNA spin-columns according to the vendor's manual. DNA-concentration was adjusted to 25 ng/µl according to OD_{260nm}.
Supercoiling assays were performed in 0.2 ml reaction tubes. For a single reaction, 7.8 µl of H_2O were mixed with 3 µl of 5× gyrase buffer [NEB] and 0.2 µl of DNA-gyrase [NEB]. Then 1 µl of pre-diluted compound of interest or 1 µl of solvent (negative control) was added followed by 3 µl of relaxed plasmid DNA (= 75 ng). The reaction was carried out for 30 min at 37°C and subsequently stopped by setting temperature to 60°C for 10 min. The concentrations of compounds tested were either 50 µM, 25 µM or 10 µM with three-fold serial dilutions down to 0.07 µM, 0.03 µM or 0.01 µM, respectively.
All reactions were separated on an Ethidium bromide-free agarose gel. After electrophoresis, gels were stained for 5 min in Ethidium bromide solution (10 mg/ml) and UV-fluorescence was recorded.

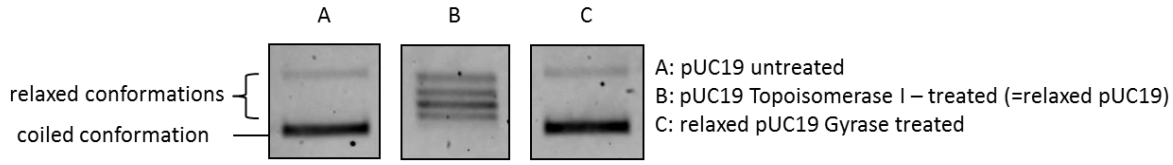


Figure 50: Exemplary result of a DNA supercoiling assay assay.

For evaluation, result images were loaded to the software Image Lab 5.0 [BioRad]. Digitally, each gel was divided into lanes and bands corresponding to coiled pUC19 were detected in each lane by densitometric analysis. Hereafter, intensities of each band were expressed relatively to the intensity of the untreated control. These values were then plotted on an x/y graph and IC_{50} was calculated by non-linear regression using graph pad prism.

4.1.3 **Experiments with photoswitchable cystobactamids**

A stock solution in DMSO+ 1% HCOOH of cystobactamids (0.375 mM) was irradiated for 1 h with light of 365 nm (40 W, 220 V). After this, DNA supercoiling assay and MIC determination were carried out with the same procedure described above. The E. coli strain used is BW25113. After irradiation the percentage of the *cis* isomer at the PSS was assessed by means of LCMS, using the UV trace (254 nm). The half-life of the molecules was also assessed in the same way and it showed to be compatible with the assays time.

4.2 CHEMISTRY

4.2.1 **Materials and methods**

Commercially available reagents and solvents were used as supplied. All reactions were performed in oven-dried glassware under an atmosphere of nitrogen gas unless otherwise stated.

NMR spectra were recorded using a Bruker Advance-III HD 500 MHz or Bruker Advance-III HD 700 MHz spectrometer. Multiplicities are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, septet (hept), m = multiplet, br = broad signal. Chemical shift values of ^{1}H and ^{13}C NMR spectra are commonly reported as values in ppm relative to residual solvent signal as internal standard.

High resolution mass spectra were recorded on a Bruker maXis HD spectrometer using positive or negative electrospray ionization (ESI).

LCMS measurements were performed using a Agilent technologies 1200 series (LC) coupled to Bruker amaZon SL (ion trap MS) using a Gemini-NX 3u C18 110A 50x2.0 mm column (for Marfey's method), or on Agilent technologies 1260 Infinity II (LC) coupled to Agilent technologies 6130 a (quadrupol MS) using an Agilent poroshell 120 SB-C18 2.7 µm 2.1x30 mm column (for reaction monitoring).

Analytical thin-layer chromatography was performed using pre-coated silica gel 60 F_{254} plates (Merck, Darmstadt), and the spots were visualized with UV light at 254 nm or alternatively by staining with potassium permanganate or cerium sulfate.

Chromatographic separations were performed by automated flash chromatography using Grace Reveleris® X2 flash chromatography system or via flash chromatography using silica gel 60M MACHEREY-NAGEL (0.040-0.063 mm; 230–400 mesh).

Preparative reversed phase high performance liquid chromatography was carried out with a Thermo Scientific Dionex (UltiMate 3000 HPLC system) with a Phenomenex 006-4252-P0 Luna C18 (250 mm × 21.2 mm, 5 µm) column.

For microwave assisted reaction, Biotage® Initiator+ was used.

Freezedrying was done using LYO Christ alpha 1-4 coupled to high vacuum oil pump. UV spectra were recorded using a microplate spectrophotometer PowerWave™ XS/XS2.

4.2.1.1 Marfey assay

The sample (5 µmol) is treated with HCl 6N at 110 °C for 6 hours. The resulting mixture/solution is dried via Freeze-drying, the residue thus obtained is treated with $NaHCO_3$ saturated solution (100 µL) and a solution 1% of Marfey reagent (FDAA) in acetone (200 µL). Reaction stirred at 40 °C for 1 h and quenched with HCl 1 N (100 µL). Sample analyzed by LCMS. (column, Gemini-NX 3u C18 110 A 50.0x2.0 mm)
Results are given in % of the two diasteroisomers formed upon derivatization. The method itself, presumably during the hydrolysis step, entails partial racemization, which was quantified in around 5%.

4.2.1.2 Preparative RP-HPLC purifications

Purifications via preparative RP-HPLC were carried out using two possible conditions:

- Condition A: 10-95% CH_3CN + 0.1% HCOOH in water + 0.1% HCOOH in 40 min.
  The sample was dissolved in DMSO and loaded on the HPLC system
- Condition B: gradient 10-70% CH_3CN in water 10 mM NH_4HCO_3 in 40 min.
  The sample was dissolved in THF (1 mL) and cooled to 0 °C. Then a few drops of DMSO and 1M aqueous NH_4HCO_3 solution (1 mL) were added. The solution pH was adjusted to 9 ca. by dropwise addition of 1M NaOH. The mixture was filtered through a syringe filter (CHROMAFIL® PET-45/15, 45 µm pore size, 15 mm diameter) and directly injected into the HPLC system.

The collected fractions were lyophilized after their identity and purity was verified by LCMS.

4.2.2 **Experimental procedures**

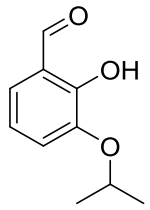
The experimental procedures that I report in this section have been also used for the filing of a European patent application[126].

The experimental procedures used for the synthesis of PABA-D1 have been already published in the manuscript[51].

A citation in the name of the molecule indicates that the compound has already been synthesized in the cited document or that the experimental procedure used for the reaction has been adapted from the cited document.

4.2.2.1 Structural simplification

2-Hydroxy-3-isopropoxybenzaldehyde (15)[108]



Chemical Formula: $C_{10}H_{12}O_3$
Exact Mass: 180,0786

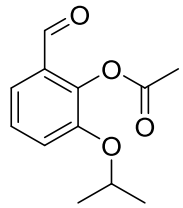
A solution of 2,3-dihydroxybenzaldehyde (20 g; 145 mmol) in DMSO (100 mL) was added dropwise, keeping low the temperature with an ice bath, to a previously prepared suspension of NaH (7.0 g; 292 mmol) in DMSO (250 mL). The mixture stirred at r.t. for two hours, then 2-Bromopropane (13.6 mL; 145 mmol) was added slowly keeping low the temperature. The reaction mixture was stirred for 36 hours, quenched with HCl followed by NH_4Cl until pH 5 reached. Work up done in several portions as follows: 300 mL of the mixture were further diluted with H_2O (1200 mL) and extracted with Et_2O (3x 200 mL). Organic phases dried over Na_2SO_4 and reduced under pressure to give 30 g of dark oil. Crude residue chromatographed on silica gel, isocratic condition (Pet. Et. DCM 7:3) to give 9.78 g of a yellow oil (54 mmol, y= 37%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.96 (s, 1H), 9.92 (s, 1H), 7.19 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.15 (dd, *J* = 8.0, 1.2 Hz, 1H), 6.94 (t, *J* = 7.9 Hz, 1H), 4.59 (m, *J* = 6.1 Hz, 1H), 1.39 (d, *J* = 6.1 Hz, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 196.4, 153.0, 146.4, 125.3, 122.7, 121.3, 119.5, 72.1, 22.0.

HRMS (ESI) calculated for C10H13O3 ($M+H^+$) 181.0859, found 181.0858.

2-Formyl-6-isopropoxyphenyl acetate (16)



Chemical Formula: $C_{12}H_{14}O_4$
Exact Mass: 222,0892

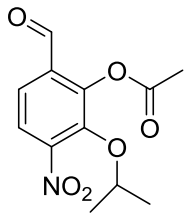
Acetyl chloride (4.25 mL; 59.76 mmol) was added dropwise to a stirred solution of 2-hydroxy-3-isopropoxybenzaldehyde (9.78 g; 54.33 mmol) and pyridine (9.65 mL; 119.53 mmol) in DCM (540 mL) at 0° C. Reaction stirred for 5 min. at 0 °C then temperature raised to r.t. and stirring prolonged for 1 h. Reaction quenched with HCl 1 N, organic phase partially reduced under vacuum, washed with HCl 1 N (200 mL), brine (200 mL), dried over sodium sulphate and reduced under vacuum to give 12.2 g of a yellow oil, which was chromatographed on silica gel with a gradient 2-10% EtOAc in Pet. Et, to give 8.89 g of a pale yellow oil (40.04 mmol; y= 74%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 10.14 (s, 1H), 7.44 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.30 (t, *J* = 8.0 Hz, 1H), 7.21 (dd, *J* = 8.3, 1.3 Hz, 1H), 4.59 – 4.53 (m, 1H), 2.39 (s, 3H), 1.34 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, $CDCl_3$) δ 188.8, 168.7, 150.2, 142.9, 129.5, 126.6, 121.0, 120.6, 71.9, 22.0, 20.5.

HRMS (ESI) calculated for C12H15O4 ($M+H^+$) 223.0965, found 223.0968.

6-Formyl-2-isopropoxy-3-nitrophenyl acetate (17)



Chemical Formula: $C_{12}H_{13}NO_6$
Exact Mass: 267,0743

Fuming nitric acid (17.5 mL, 420 mmol) was cooled to −40 °C under a nitrogen atmosphere. A solution of 2-formyl-6-isopropoxyphenyl acetate (6.50 g, 29.3 mmol) in 40 mL of dry DCM was added dropwise while the mixture was vigorously stirred and kept at −40 °C. The solution was stirred for an additional 1.5 hours before being poured into 150 mL of ice water. The mixture was then extracted with DCM (4 × 50 mL) and the combined organic

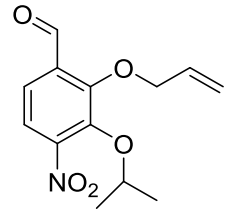
extracts were dried over sodium sulphate. The solvent was removed under vacuum to afford the desired compound as an orange oil (7.59 g, 28.4 mmol, y= 97%), containing around 17% of deactylated product.

^{1}H NMR (500 MHz, DMSO) δ 10.10 (d, *J* = 0.5 Hz, 1H), 8.00 (dd, *J* = 8.5, 0.4 Hz, 1H), 7.81 (d, *J* = 8.5 Hz, 1H), 4.46 (hept, *J* = 6.1 Hz, 1H), 2.44 (s, 3H), 1.22 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 189.4, 168.3, 148.4, 145.5, 143.1, 131.8, 125.4, 122.0, 78.9, 22.1, 20.4.

HRMS (ESI) calculated for C12H14NO6 ($M+H^+$) 268.0816, found 268.0811.

2-(Allyloxy)-3-isopropoxy-4-nitrobenzaldehyde (19)



Chemical Formula:
$C_{13}H_{15}NO_5$
Exact Mass: 265,0950

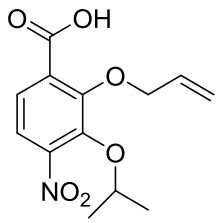
6-Formyl-2-isopropoxy-3-nitrophenyl acetate (2.10 g; 7.9 mmol) was dissolved in THF (40 mL) and water (20 mL), then LiOH (1.89 g; 79.0 mmol) dissolved in water (20 mL) was added at 0 °C, reaction stirred overnight. In the morning, pH adjusted to 1, solvent partially reduced under vacuum and watery phase extracted with $CHCl_3$ (150 mL) three times, combined organic phases dried over sodium sulphate and reduced under vacuum to give a yellow oil, which was used in the next step without further purification. Residue was dissolved in DMF (20 mL), K_2CO_3 (2.18 g; 15.8 mmol) followed by allyl bromide (1.026 mL; 11.85 mmol) were added, reaction stirred 24 h at r.t.. Reaction diluted with water (200 mL) and EtOAc (200 mL), aqueous phase extracted with EtOAc (150 mL). Combined organic phases washed with brine (300 mL), dried over sodium sulphate and reduced under vacuum to give 4 g of a crude material, which was chromatographed on silica gel with a gradient 0-10% EtOAc in Pet. Et. to give 1.69 g a yellow oil (6.37 mmol; y= 81%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.39 (d, *J* = 0.9 Hz, 1H), 7.64 (d, *J* = 8.5 Hz, 1H), 7.51 (dd, *J* = 8.5, 0.9 Hz, 1H), 6.05 (m, *J* = 17.1, 10.3, 6.1 Hz, 1H), 5.40 (pseudo dq, *J* = 17.1, 1.4 Hz, 1H), 5.33 (pseudo dq, *J* = 10.3, 2.2, 1.0 Hz, 1H), 4.74 – 4.72 (m, 1H), 4.68 (m, 1H), 1.32 (d, *J* = 6.2 Hz, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 188.7, 156.3, 150.0, 145.1, 133.0, 132.0, 122.3, 120.3, 119.5, 78.3, 75.7, 22.4.

HRMS (ESI) calculated for C13H15NO5Na (M+Na^+) 288.0842, found 288.0839.

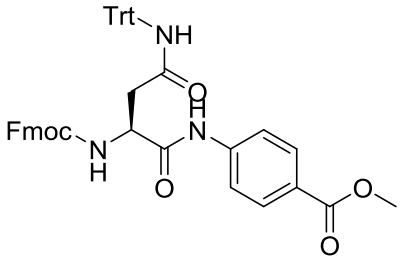
2-(Allyloxy)-3-isopropoxy-4-nitrobenzoic acid (3)[127]



Chemical Formula: $C_{13}H_{15}NO_6$
Exact Mass: 281,0899

2-(Allyloxy)-3-isopropoxy-4-nitrobenzaldehyde (1.69 g; 6.38 mmol) and 2-Methyl-2-butene (7.2 mL; 70 mmol) were dissolved in *t*-BuOH (48 mL). Then a solution of $NaClO_2$ 80% (0.87 g; 7.65 mmol) in Monosodium phosphate monohydrate solution 1 N (7.2 mL) was added dropwise to the solution. Reaction stirred for 1 h, t hen quenched by adding a solution of Na_2SO_3 (14.0 mmol in 10 mL). Mixture partially reduced under vacuum, diluted with EtOAc (100 mL) and HCl 1 N (100 mL), aqueous phase extracted again with EtOAc (50 mL), organic phases reunited washed with brine (150 mL) and dried over sodium sulphate. Solvent reduced under vacuum to give 1.9 g (6.38 mmol; y= q.) of a dark residue, which was used in the next step without further purification.

Methyl (S)-4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzoate (25)[109]



Chemical Formula: $C_{46}H_{39}N_3O_6$
Exact Mass: 729,2839

$POCl_3$ (2.22 mL; 23.84 mmol) was added at 0 °C under N_2 atmosphere to a stirred solution of Fmoc-Asn(Trt)OH (14.22 g; 23.84 mmol), TEA (5.51 mL; 39.74 mmol) and methyl 4-aminobenzoate (3.00 g; 19.87 mmol) in DCM (330 mL). Reaction stirred at 0 °C for 2 hours,

quenched with HCl 1N and ice. Organic phase washed with HCL 1 N (300 mL), brine (330 mL) and dried over sodium sulphate. The solvent was removed under reduced pressure, the residue thus obtained was chromatographed on silica gel with a gradient 0-10% EtOAc in DCM to give 13.97 g of a white solid(19.16 mmol; y= 96%).

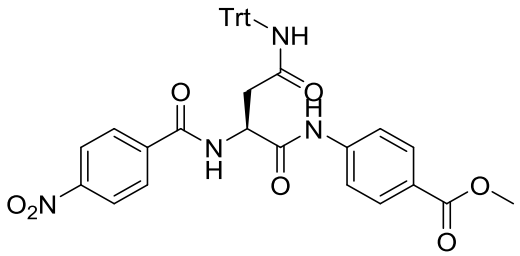
^{1}H NMR (700 MHz, DMSO) δ 10.44 (s, 1H), 8.62 (s, 1H), 7.91 (dd, *J* = 15.9, 8.2 Hz, 4H), 7.80 (d, *J* = 7.9 Hz, 1H), 7.78 – 7.72 (m, 4H), 7.41 (q, *J* = 7.6 Hz, 2H), 7.30 (dt, *J* = 22.8, 7.3 Hz, 2H), 7.23 – 7.15 (m, 15H), 4.48 – 4.43 (m, 1H), 4.36 (dd, *J* = 10.6, 7.1 Hz, 1H), 4.30 (dd, *J* = 10.5, 7.1 Hz, 1H), 4.23 (t, *J* = 7.0 Hz, 1H), 2.75 (dd, *J* = 14.6, 9.8 Hz, 1H), 2.62 (dd, *J* = 14.6, 5.0 Hz, 1H).

^{13}C NMR (176 MHz, DMSO) δ 170.9, 168.4, 165.8, 155.8, 144.7, 143.8, 143.8, 143.4, 140.7, 130.2, 128.6, 127.6, 127.4, 127.1, 126.3, 125.3, 125.2, 124.0, 120.1, 118.7, 69.4, 65.8, 52.9, 51.9, 46.7, 38.3, 20.8.

HRMS (ESI) calculated for C46H40N3O6 ($M+H^+$) 730.2912, found 730.2925.

Marfey: 96.5% S enantiomer, 3.5% R enantiomer

Methyl (S)-4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzoate



Chemical Formula: $C_{38}H_{32}N_4O_7$
Exact Mass: 656,2271

Methyl (S)-4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzoate (12.26 g; 16.82 mmol) was dissolved in a 20% solution of diethylamine in acetonitrile (195 mL), solution stirred for 30 min. The solvent was removed under vacuum, the residue was dissolved in CH_3CN and evaporated twice. The pale yellow gum and 4-Nitrobenzoic acid (3.09 g; 18.5 mmol) were suspended in CH_3CN (140 mL), HBTU (7.02 g; 18.5 mmol) followed by DiPEA (6.754 mL, 38.85 mmol) were added at 0 °C. The reaction mixture was stirred for 3 hours and quenched with $NaHCO_3$ saturated solution. The solvent was partially evaporated under reduced pressure, the residue was dissolved in EtOAc (300 mL). Organic phase washed with $NaHCO_3$ saturated solution (300 mL), HCl 1 N (300 mL), brine (300 mL) dried over sodium sulphate and evaporated under vacuum. The residue thus

obtained was triturated with Pet. Et. and chromatographed on silica gel with a gradient 0-10% EtOAc in DCM to give 8.64 g of a yellow solid (13.17 mmol; y= 78%).

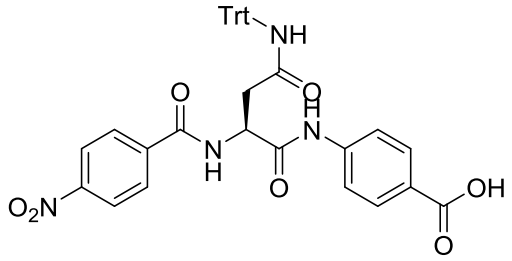
^{1}H NMR (500 MHz, DMSO) δ 10.57 (s, 1H), 9.20 (d, *J* = 7.6 Hz, 1H), 8.68 (s, 1H), 8.41 – 8.35 (m, 2H), 8.18 – 8.13 (m, 2H), 7.95 – 7.90 (m, 2H), 7.80 – 7.75 (m, 2H), 7.23 – 7.13 (m, 15H), 4.93 (m, 1H), 3.82 (s, 3H), 2.98 (dd, *J* = 14.9, 10.5 Hz, 1H), 2.75 (dd, *J* = 14.8, 4.6 Hz, 1H).

^{13}C NMR (126 MHz, DMSO) δ 170.5, 168.3, 165.8, 164.6, 149.2, 144.7, 143.3, 139.3, 130.2, 129.0, 128.5, 127.4, 126.4, 124.1, 123.6, 118.8, 69.4, 52.1, 51.9, 37.9.

HRMS (ESI) calculated for C38H33N4O7 ($M+H^+$) 657.2344, found 657.2348.

Marfey: 94.2% S enantiomer, 5.8% R enantiomer

(S)-4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzoic acid (21)[110]



Chemical Formula: $C_{37}H_{30}N_4O_7$
Exact Mass: 642,2114

Methyl (S)-4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzoate (5.7 g; 8.69 mmol) and lithium iodide (9.32 g; 69.52 mmol) were mixed in EtOAc (80 mL) and heated to 90 °C for 5 days. After cooling, mixture diluted with EtOAc (200 mL) and HCl 1 N (200 mL), organic phase washed with water (2x 200 mL), brine (200 mL), dried over sodium sulphate and reduced under vacuum. The residue was chromatographed on silica gel with a gradient 0-20% MeOH in DCM to give 5.39 g of yellow solid (7.44 mmol; y= 86%).

^{1}H NMR (700 MHz, DMSO) δ 12.72 (s, 1H), 10.52 (s, 1H), 9.19 (d, *J* = 7.6 Hz, 1H), 8.67 (s, 1H), 8.40 – 8.36 (m, 2H), 8.17 – 8.13 (m, 2H), 7.91 – 7.88 (m, 2H), 7.76 – 7.72 (m, 2H), 7.22 – 7.14 (m, 15H), 4.93 (m, 1H), 2.98 (dd, *J* = 14.9, 10.6 Hz, 1H), 2.75 (dd, *J* = 14.9, 4.5 Hz, 1H).

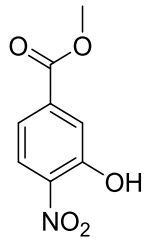
^{13}C NMR (176 MHz, DMSO) δ 170.4, 168.3, 166.9, 164.6, 149.2, 144.7, 142.9, 139.3, 130.3, 129.0, 128.5, 127.4, 126.4, 123.6, 118.6, 69.4, 66.3, 52.0, 38.0.

^{13}C NMR (176 MHz, DMSO) δ 170.4, 168.3, 166.9, 164.6, 149.2, 144.7, 142.9, 139.3, 130.3, 129.0, 128.5, 127.4, 126.4, 123.6, 118.6, 69.4, 52.0, 38.0.

HRMS (ESI) calculated for C37H29N4O7 ($M-H^+$) 641.2041, found 641.2045.

Marfey: 94.0 % S enantiomer, 6.0% R enantiomer

Methyl 3-hydroxy-4-nitrobenzoate



Chemical Formula: $C_8H_7NO_5$
Exact Mass: 197,0324

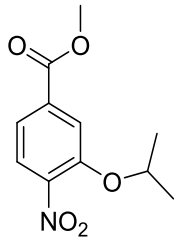
3-Hydroxy-4-nitrobenzoic acid (2.5 g; 13.66 mmol) was dissolved in a MeOH (35.0 mL), thionyl chloride (2.5 mL; 34.15mmol) was added dropwise at 0 °C, the solution was warmed to r.t. and then heated to 70 °C for 1 hour. The solvent was evaporated under reduced pressure, the residue was diluted with water (90 mL) and EtOAc (90 mL), water again extracted twice with EtOAc (2x90 mL). Combined organic phases washed with brine (150 mL), dried over sodium sulphate and evaporated under reduced pressure to give 2.64 g of a yellow solid (13.40 mmol; y= 98%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 10.50 (s, 1H), 8.18 (d, *J* = 8.8 Hz, 1H), 7.83 (d, *J* = 1.8 Hz, 1H), 7.62 (dd, *J* = 8.8, 1.8 Hz, 1H), 3.97 (s, 3H).

^{13}C NMR (176 MHz, $CDCl_3$) δ 164.9, 154.7, 138.0, 135.8, 125.3, 121.7, 120.6, 53.0.

HRMS (ESI) calculated for C8H6NO5 (M-H$^+$) 196.0251, found 196.0249.

Methyl 3-isopropoxy-4-nitrobenzoate



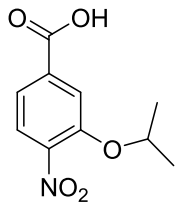
Chemical Formula: $C_{11}H_{13}NO_5$
Exact Mass: 239,0794

Methyl 3-hydroxy-4-nitrobenzoate (1.94 g; 9.85 mmol) and K_2CO_3 (1.72 g; 11.82 mmol) were mixed in DMF (32 mL). 2-Bromopropane (1.55 mL; 15.76 mmol) was added and the mixture heated to 70 °C overnight. Reaction diluted with Et_2O (320 mL) and water (320 mL). Organic phase washed with brine (300 mL), dried over sodium sulphate and reduce under vacuum to give 2.25 g of a yellow oil (9.41 mmol; y= 96%).

^{1}H NMR (700 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.3 Hz, 1H), 7.74 (d, *J* = 1.6 Hz, 1H), 7.64 (dd, *J* = 8.3, 1.6 Hz, 1H), 4.77 (hept, *J* = 6.1 Hz, 1H), 3.96 (s, 3H), 1.41 (dd, *J* = 6.0, 2.5 Hz, 6H).

^{13}C NMR (176 MHz, $CDCl_3$) δ 165.4, 150.8, 143.8, 134.5, 125.1, 121.1, 117.0, 73.0, 52.8, 21.8.

3-Isopropoxy-4-nitrobenzoic acid (26)



Chemical Formula: $C_{10}H_{11}NO_5$
Exact Mass: 225,0637

Methyl 3-hydroxy-4-nitrobenzoate (1.94 g; 9.85 mmol) and K_2CO_3 (1.72 g; 11.82 mmol) were mixed in DMF (32 mL). 2-Bromopropane (1.55 mL; 15.76 mmol) was added and the mixture heated to 70 °C overnight. The reaction was diluted with Et_2O (320 mL) and water (320 mL). The organic phase was washed with brine (300 mL), dried over sodium sulphate and reduce under vacuum to give 2.25 g of a yellow oil (9.41 mmol; y= 96%).

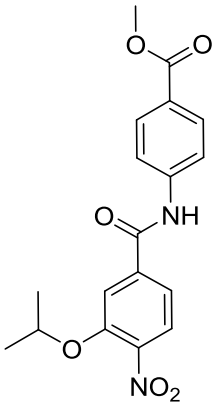
Part of this material (1.45 g; 6.07 mmol) was dissolved in THF (33 mL) and water (15 mL). To this mixture, a solution of LiOH (1.45 g; 60.70 mmol) in water (18 mL) was added. Reaction stirred at r.t. for 3 h., pH adjusted to 1 and solvent partially reduced under vacuum. The precipitate thus formed was collected by filtration and washed with Pet. Et. twice to give 1.36 g of a pale yellow solid (6.04 mmol; y= q.).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 – 7.77 (m, 2H), 7.73 (dd, *J* = 8.3, 1.5 Hz, 1H), 4.79 (hept, *J* = 6.1 Hz, 1H), 1.43 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 169.4, 150.8, 133.3, 125.2, 121.8, 117.4, 73.2, 21.8.

HRMS (ESI) calculated for C10H10NO5 ($M-H^+$) 224.0564, found 224.0565.

Methyl 4-(3-isopropoxy-4-nitrobenzamido)benzoate



Chemical Formula: $C_{18}H_{18}N_2O_6$
Exact Mass: 358,1165

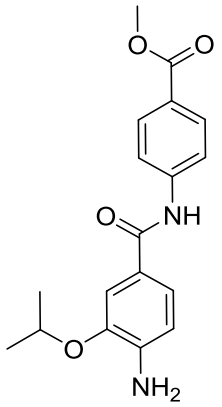
Methyl 4-aminobenzoate (79 mg; 0.52 mmol), 3-isopropoxy-4-nitrobenzoic acid (100 mg; 0.44) and HOAt (91; 0.67 mmol) were mixed together in DMF (1.2 mL). To this yellow solution, EDC (102 mg; 0.53 mmol) followed by lutidine (0.258 mL; 2.22 mmol) were added. Reaction stirred overnight, diluted with EtOAc (20 mL) and HCl 1 N (20 mL), watery phase extracted again with EtOAc (20 mL). Organic phases reunited dried over sodium sulphate and reduced under vacuum, the residue thus obtained chromatographed on silica gel with a gradient 3-40% EtOAc in Pet. Et. to give 112 mg (0.31 mmol; y= 60%) of a pale yellow solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.11 – 8.06 (m, 2H), 7.98 (s, 1H), 7.83 (d, *J* = 8.3 Hz, 1H), 7.76 – 7.72 (m, 2H), 7.67 (d, *J* = 1.6 Hz, 1H), 7.36 (dd, *J* = 8.3, 1.7 Hz, 1H), 4.85 – 4.74 (m, 1H), 3.92 (s, 3H), 1.42 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 166.5, 163.9, 151.7, 143.1, 141.4, 139.2, 131.0, 126.6, 125.7, 119.5, 117.2, 115.8, 73.3, 52.2, 21.8.

HRMS (ESI) calculated for C18H19N2O6 ($M+H^+$) 359.1238, found 359.1239.

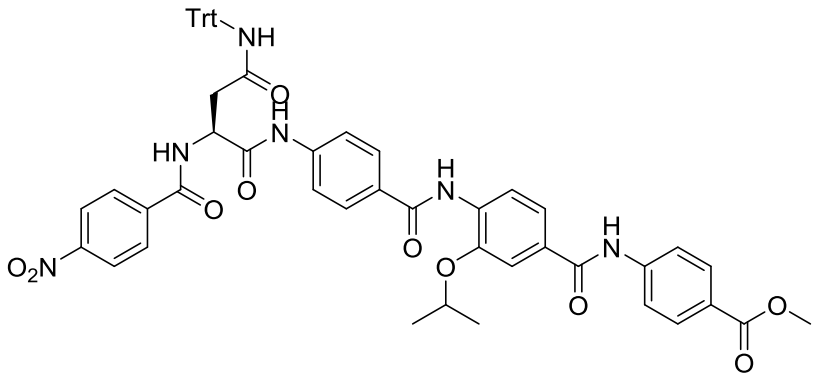
Methyl 4-(4-amino-3-isopropoxybenzamido)benzoate (22)



Chemical Formula: $C_{18}H_{20}N_2O_4$
Exact Mass: 328,1423

Methyl 4-(3-isopropoxy-4-nitrobenzamido)benzoate (800 mg; 2.23 mmol) was dissolved in MeOH (24 mL). The solution was degassed with Argon for 10 min. Pd (80 mg) was added, reaction stirred under an H_2 atmosphere overnight. Pd filtered out over a pad of celite and solvent evaporated under vacuum. The oil thus obtained was chromatographed on silica gel with a gradient 10-40% EtOAc in Pet. Et. to give 790 mg (2.40 mmol; y= 93%) of a white solid.
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 – 8.03 (m, 1H), 8.03 – 7.98 (m, 2H), 7.78 – 7.71 (m, 2H), 7.46 (d, *J* = 1.8 Hz, 1H), 7.28 (dd, *J* = 8.2, 1.8 Hz, 1H), 6.80 (d, *J* = 8.1 Hz, 1H), 4.71 – 4.62 (m, 1H), 3.91 (s, 3H), 1.37 (d, *J* = 6.0 Hz, 6H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 166.8, 165.5, 145.7, 142.7, 130.9, 125.4, 119.7, 119.0, 114.6, 112.9, 71.2, 52.1, 22.2.
HRMS (ESI) calculated for C18H21N2O4 ($M+H^+$) 329.1496, found 329.1495.

Methyl (S)-4-(3-isopropoxy-4-(4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzamido)benzamido)benzoate (27)



Chemical Formula: $C_{55}H_{48}N_6O_{10}$
Exact Mass: 952,3432

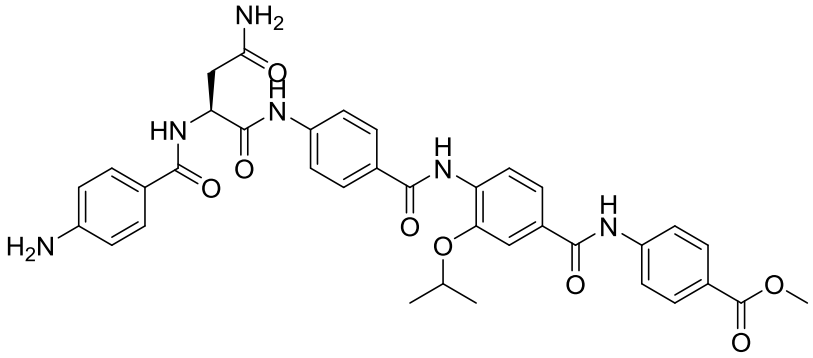
(S)-4-(2-(4-Nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzoic acid (325 mg; 0.506 mmol), methyl 4-(4-amino-3-isopropoxybenzamido)benzoate (194 mg; 0.592 mmol), HOAt (103 mg; 0.759 mmol) were mixed in DMF (1.25 mL). To this solution, EDC (116 mg; 0.607 mmol) followed by lutidine (0.295 mL; 2.530 mmol) were added. Reaction stirred at r.t. for 6 days, reaction diluted with EtOAc (75 mL) and HCl 1 N (75 mL), the organic phase washed with $NaHCO_3$ saturated solution (50 mL) and brine (50 mL), dried over sodium sulphate and reduced under vacuum to give an orange material, which was chromatographed on silica gel with a gradient 0-3% MeOH in DCM to give 220 mg of desired product (0.23 mmol; y= 46%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.61 (s, 1H), 8.80 (s, 1H), 8.69 (d, *J* = 8.4 Hz, 1H), 8.48 (d, *J* = 6.3 Hz, 1H), 8.31 – 8.26 (m, 2H), 8.09 – 8.05 (m, 2H), 7.99 (s, 1H), 7.98 – 7.94 (m, 2H), 7.88 – 7.84 (m, 2H), 7.78 – 7.74 (m, 2H), 7.61 (d, *J* = 1.9 Hz, 1H), 7.60 – 7.55 (m, 2H), 7.41 (dd, *J* = 8.5, 1.9 Hz, 1H), 7.32 – 7.24 (m, 15H), 7.14 (s, 1H), 5.09 – 5.04 (m, 1H), 4.88 – 4.76 (m, 1H), 3.92 (s, 3H), 3.31 (dd, *J* = 15.7, 2.7 Hz, 1H), 2.75 (dd, *J* = 15.6, 7.3 Hz, 1H), 1.47 (dd, *J* = 6.0, 2.9 Hz, 6H).

^{13}C NMR (176 MHz, $CDCl_3$) δ 171.2, 168.8, 166.6, 165.7, 165.1, 164.5, 150.1, 146.6, 143.9, 142.2, 141.0, 138.3, 132.4, 131.0, 130.4, 129.4, 128.6, 128.6, 128.2, 128.2, 127.4, 125.8, 124.0, 119.8, 119.2, 118.9, 118.8, 112.1, 72.0, 71.4, 52.1, 51.2, 37.5, 22.3.

HRMS (ESI) calculated for C55H49N6O10 ($M+H^+$) 953.3505, found 953.3483.

Methyl (S)-4-(4-(4-(4-amino-2-(4-aminobenzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzamido)benzoate (28)



Chemical Formula: $C_{36}H_{36}N_6O_8$
Exact Mass: 680,2595

Methyl (S)-4-(3-isopropoxy-4-(4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)-benzamido)benzamido)benzoate (238 mg; 0.25 mmol) was dissolved in DCM (7.5 mL), Tips (0.154 mL; 0.75 mmol) followed by TFA (2.5 mL) were added at 0 °C. Reaction stirred 2 h at

r.t. then solved removed under vacuum, residue take up and evaporated twice with DCM (10 mL) then triturated 3x with ice cold Pet. Et..

Tin(II) chloride dehydrate (338 mg; 1.50 mmol) was mixed with the crude residue coming from the former step (0.25 mmol) in EtOH (5 mL), the solution was stirred at r.t. overnight.

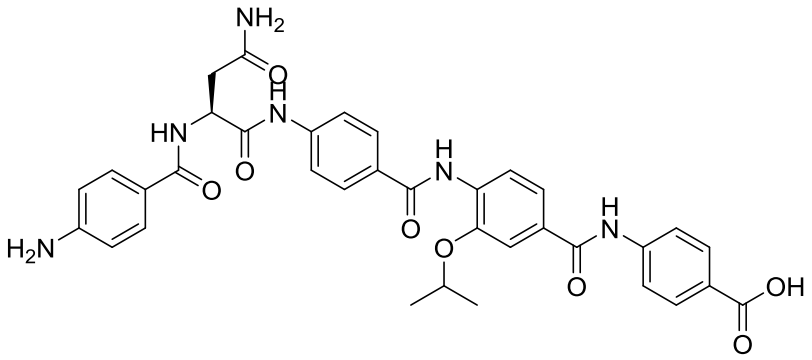
Solvent was evaporated under vacuum, the residue dissolved in EtOAc (50 mL), washed with $NaHCO_3$ (50 mL) saturated solution, which was extracted twice again with EtOAc (2x30 mL). Organic phases reunited washed with brine (100 mL), dried over sodium sulphate and reduced under vacuum. The residue was chromatographed on silica gel with a gradient 0-10% MeOH in DCM to give 50 mg of desired product (0.082 mmol; y= 33%).

^{1}H NMR (700 MHz, DMSO) δ 10.47 (s, 1H), 10.38 (s, 1H), 9.25 (s, 1H), 8.22 (d, *J* = 7.4 Hz, 1H), 8.20 (d, *J* = 8.9 Hz, 1H), 7.99 – 7.90 (m, 6H), 7.82 – 7.78 (m, 2H), 7.64 (dd, *J* = 6.9, 1.8 Hz, 2H), 7.62 – 7.59 (m, 2H), 7.37 (s, 1H), 6.97 (s, 1H), 6.57 – 6.54 (m, 2H), 5.67 (s, 2H), 4.85 (q, *J* = 7.1 Hz, 1H), 4.79 (hept, *J* = 6.0 Hz, 1H), 3.84 (s, 3H), 2.64 (d, *J* = 7.1 Hz, 2H), 1.37 (d, *J* = 6.0 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.4, 171.1, 166.4, 165.8, 165.3, 164.2, 151.9, 147.8, 143.7, 142.5, 131.7, 130.4, 130.1, 129.1, 128.4, 128.3, 124.2, 121.3, 120.5, 120.4, 119.7, 118.8, 113.1, 112.4, 71.5, 51.9, 51.5, 36.9, 21.8.

HRMS (ESI) calculated for C36H37N6O8 ($M+H^+$) 681.2667, found 681.2673.

(S)-4-(4-(4-(4-Amino-2-(4-aminobenzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzamido)benzoic acid (29)



Chemical Formula: $C_{35}H_{34}N_6O_8$
Exact Mass: 666,2438

Methyl (S)-4-(4-(4-(4-amino-2-(4-aminobenzamido)-4-oxobutanamido)benzamido)-3-iso-propoxybenzamido)benzoate (20 mg, 0.029 mmol) was mixed into THF (1.4 mL) and water (1.4 mL). To this mixture, a solution 0.1 M of LiOH (0.59 mL; 0.059 mmol) was added at 0 °C.

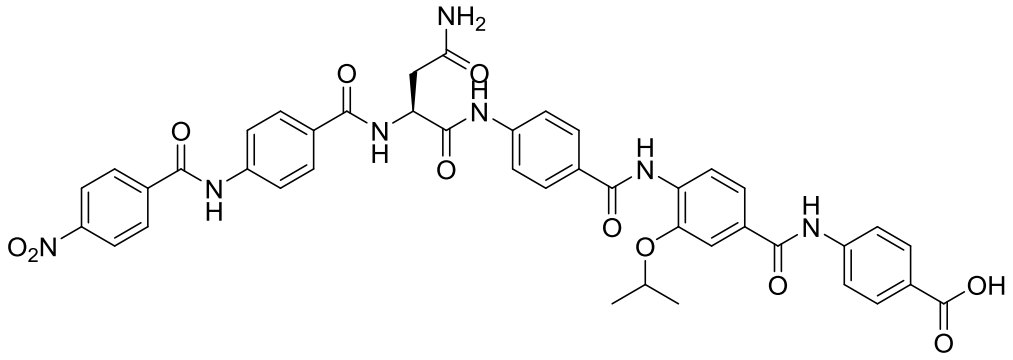
Reaction stirred for 2.5 days. Reaction quenched with acetic acid, solvent reduced under vacuum and residue purified by preparative HPLC with a gradient 20-90% CH_3CN in water both phases +0.1 % TFA to obtain 4.8 mg of desired product (0.0073 mmol; y= 25%).

^{1}H NMR (500 MHz, DMSO) δ 12.78 (br, 1H), 10.45 (s, 1H), 10.40 (s, 1H), 9.26 (s, 1H), 8.24 (d, *J* = 7.2 Hz, 1H), 8.19 (d, *J* = 8.9 Hz, 1H), 7.92 (m, 6H), 7.80 (d, *J* = 8.8 Hz, 2H), 7.66 – 7.59 (m, 4H), 7.38 (s, 1H), 6.98 (s, 1H), 6.57 (d, *J* = 8.6 Hz, 2H), 5.72 (br, 1H), 4.85 (q, *J* = 7.0 Hz, 1H), 4.79 (dt, *J* = 12.1, 6.0 Hz, 1H), 2.67 – 2.60 (m, 2H), 1.37 (d, *J* = 6.0 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.4, 171.0, 166.9, 166.4, 165.2, 164.2, 147.8, 143.3, 142.5, 131.7, 130.4, 130.2, 129.1, 128.5, 128.3, 125.4, 121.3, 120.5, 119.6, 118.8, 113.1, 112.6, 71.6, 51.5, 36.9, 21.8.

HRMS (ESI) calculated for C35H35N6O8 ($M+H^+$) 667.2511, found 667.2512.

(S)-4-(4-(4-(4-Amino-2-(4-(4-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzamido)benzoic acid (30)



Chemical Formula: $C_{42}H_{37}N_7O_{11}$
Exact Mass: 815,2551

To a suspension of (S)-4-(4-(4-(4-amino-2-(4-aminobenzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzamido)benzoic acid (4.8 mg; 0.0072 mmol) in THF (0.25 mL) and $NaHCO_3$ saturated solution (0.25 mL), 4-nitrobenzoyl chloride (2.6 mg; 0.014 mmol) was added at 0 °C. Reaction stirred for 2.5 hours then quenched with AcOH, solvent partially removed under vacuumn, residue dissolved in DMSO, salts filtered out, and purified by preparative HPLC with a gradien 20-90% CH_3CN in water both phases +0.1% TFA to obtain 1 mg of desired compound (0.0012 mmol; y= 16%).

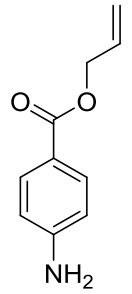
^{1}H NMR (700 MHz, DMSO) δ 12.75 (br, 1H), 10.79 (s, 1H), 10.46 (s, 1H), 10.44 (s, 1H), 9.26 (s, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.40 – 8.37 (m, 2H), 8.21 (dt, *J* = 17.0, 6.2 Hz, 3H), 7.96 – 7.89 (m,

10H), 7.82 (d, *J* = 8.8 Hz, 2H), 7.65 – 7.63 (m, 2H), 7.40 (s, 1H), 6.99 (s, 1H), 4.93 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.79 (hept, *J* = 6.1 Hz, 1H), 2.69 (d, *J* = 7.8 Hz, 2H), 1.37 (d, *J* = 6.0 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 166.9, 165.8, 165.2, 164.2, 149.3, 147.8, 143.3, 141.5, 140.3, 131.7, 130.5, 130.2, 129.3, 129.2, 128.6, 128.3, 128.3, 125.4, 123.6, 121.4, 120.5, 119.6, 118.9, 113.1, 71.6, 51.6, 36.8, 21.8.

HRMS (ESI) calculated for C42H38N7O11 ($M+H^+$) 816.2624, found 816.2633.

Allyl 4-aminobenzoate (31)



Chemical Formula: $C_{10}H_{11}NO_2$
Exact Mass: 177,0790

K_2CO_3 (2.5 g, 17.95 mmol) was added to a stirred mixture of 4-nitrobenzoic acid (5.0 g; 30.0 mmol) and Allyl bromide (2.9 mL; 33.0 mmol) in DMF (50 mL). Reaction was stirred for 24 hours. Et_2O (500 mL) and water (500 mL) were added, the organic phase washed then with $NaHCO_3$ satured solution twice (400 mL) and once with brine (400 mL), dried over sodium sulphate, evaporated under reduced pressure to obtain 6.1 g (30.0 mmol; y= q.) of a yellow oil. Product was used in the next step without further purification.

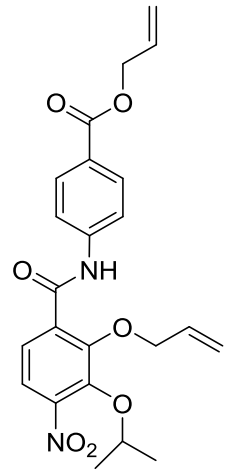
Zinc dust (27 g; 541 mmol) was added over 30 min to a solution of allyl 4-nitrobenzoate(5.6 g; 27 mmol) in acetic acid (100 mL). Reaction stirred overnight at r.t.. It was quenched with $NaHCO_3$, watery phase extracted twice with EtOAc, washed again with $NaHCO_3$ and brine, dried over sodium sulphate and reduced under vacuum to give around 4.5 g of a crude residue, which was chromatographed on silica gel with a gradient 5-30% EtOAc in Pet. Et. to afford 3.42 g (19 mmol; y= 71%) of a white solid.

^{1}H NMR (500 MHz, DMSO) δ 7.74 – 7.55 (m, 2H), 6.62 – 6.51 (m, 2H), 6.00 (m, 3H), 5.34 (pseudo dq, *J* = 17.2, 1.7 Hz, 1H), 5.22 (pseudo dq, *J* = 10.5, 1.4 Hz, 1H), 4.68 (dt, *J* = 5.3, 1.5 Hz, 2H).

^{13}C NMR (126 MHz, DMSO) δ 165.5, 153.6, 133.3, 131.1, 117.2, 115.6, 112.6, 64.0.

HRMS (ESI) calculated for C10H12NO2 ($M+H^+$) 178.0863, found 178.0867.

Allyl 4-(2-(allyloxy)-3-isopropoxy-4-nitrobenzamido)benzoate (32)



Chemical Formula: $C_{23}H_{24}N_2O_7$
Exact Mass: 440,1584

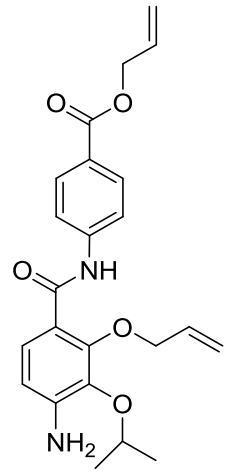
$POCl_3$ (0.68 mL; 7.32 mmol) was added at 0 °C to a stirred solution of 2-(allyloxy)-3-isopropoxy-4-nitrobenzoic acid (7.32 mmol, crude), TEA (1.7 mL; 12.20 mmol) and allyl 4-aminobenzoate (1.08 g; 6.10 mmol) in DCM (100 mL) under nitrogen. Reaction stirred for 3 h, then quenched with $NaHCO_3$ saturated solution, solvent partially reduced under vacuum, then diluted with EtOAc (150 mL) and water (150 mL), aqueous phase extracted again twice with EA (2x 100 mL), organic phases reun ited washed with HCl 1 N (300 mL) and brine (300 mL), dried over sodium sulphate and reduced under vacuum to give a crude material, which was chromatographed on silica gel with a gradient 5-30% to give 2.22 g (5.04 mmol; y= 83%) of a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.17 (s, 1H), 8.08 (d, *J* = 8.7 Hz, 3H), 7.74 (d, *J* = 8.6 Hz, 2H), 7.63 (d, *J* = 8.8 Hz, 1H), 6.24 – 5.87 (m, 2H), 5.55 – 5.22 (m, 4H), 4.80 (dd, *J* = 20.6, 5.9 Hz, 4H), 4.64 (hept, *J* = 12.4, 6.3 Hz, 1H), 1.34 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (176 MHz, $CDCl_3$) δ 165.7, 161.1, 151.5, 148.2, 144.7, 141.9, 132.3, 131.5, 131.0, 130.5, 126.2, 126.1, 121.1, 120.0, 119.4, 118.3, 78.7, 75.7, 65.5, 22.4.

HRMS (ESI) calculated for C23H25N2O7 ($M+H^+$) 441.1656, found 441.1654

Allyl 4-(2-(allyloxy)-4-amino-3-isopropoxybenzamido)benzoate (31)



Chemical Formula: $C_{23}H_{26}N_2O_5$
Exact Mass: 410,1842

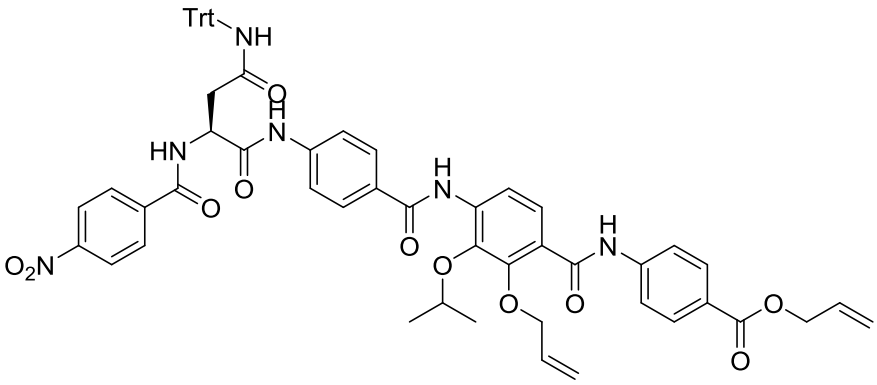
Tin(II) chloride dehydrate (4.3 g; 19.09 mmol), allyl 4-(2-(allyloxy)-3-isopropoxy-4-nitrobenzamido)benzoate (1.4 g; 3.18 mmol) were dissolved in EtOH (32 mL), the solution was stirred at r.t. overnight. The solvent was removed under vacuum, $NaHCO_3$ saturated solution (300 mL) and EtOAc (300 mL) were added to the residue. The aqueous phase extracted again with EtOAc (200 mL). The organic phases reunited were washed with brine (400 mL), dried over sodium sulphate and reduced under vacuum. The crude residue thus obtained was chromatographed on silica gel with a gradient EtOAc 10-40% in Pet. Et. to give 1.22 g (2.98 mmol; 95%)of a yellow oil.

^{1}H NMR (500 MHz, DMSO) δ 10.26 (s, 1H), 8.01 – 7.86 (m, 2H), 7.86 – 7.74 (m, 2H), 7.39 (d, *J* = 8.6 Hz, 1H), 6.56 (d, *J* = 8.6 Hz, 1H), 6.26 – 5.93 (m, 2H), 5.59 (s, 2H), 5.48- 5.35 (m, 2H), 5.30- 5.20 (m, 2H) 4.78 (dt, *J* = 5.4, 1.5 Hz, 2H), 4.61 (dt, *J* = 5.6, 1.4 Hz, 2H), 4.46 (hept, *J* = 6.1 Hz, 1H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 165.0, 163.9, 150.6, 147.8, 143.7, 135.3, 133.6, 132.8, 130.3, 126.1, 123.6, 118.8, 118.1, 117.7, 114.6, 109.9, 74.4, 73.7, 64.8, 40.1, 40.0, 39.9, 39.8, 39.8, 39.7, 39.6, 39.5, 39.3, 39.2, 39.0, 22.2.

HRMS (ESI) calculated for C23H27N2O5 ($M+H^+$) 411.1914, found 411.1900

Allyl (S)-4-(2-(allyloxy)-3-isopropoxy-4-(4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzamido)benzamido)benzoate (34)



Chemical Formula: $C_{60}H_{54}N_6O_{11}$
Exact Mass: 1034,3851

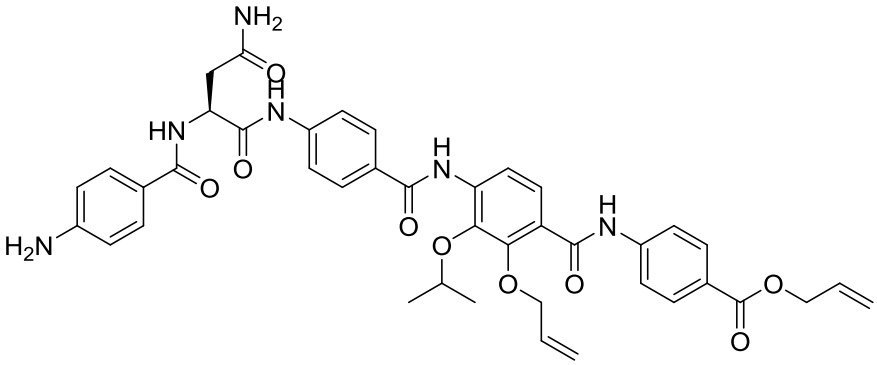
$POCl_3$ (3.20 mmol) as a solution in DCM (1:9) was added dropwise to a solution of allyl 4-(2-(allyloxy)-4-amino-3-isopropoxybenzamido)benzoate (0.53 g; 1.28 mmol) and (S)-4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzoic acid (2.05 g, 3.20 mmol) in THF (13 mL) at 0 °C, followed by DiPEA (1.78 mL; 10.24 mmol) as a solution in DCM (1:1). Reaction stirred at r.t. for 6 h, quenched with HCl 1 N and ice, solvent partially reduced under vacuum and residue diluted with EtOAc (250 mL) and HCl 1N (250 mL), organic phase washed with brine (250 mL) and dried over sodium sulphate. Solvent removed under vacuum, the crude residue was chromatographed on silica gel with a gradient EtOAc 20-75% in Pet. Et to give 940 mg of a orange residue (0.95 mmol; 54%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.20 (s, 1H), 9.63 (s, 1H), 8.74 (s, 1H), 8.50 (d, *J* = 8.9 Hz, 1H), 8.47 (d, *J* = 6.2 Hz, 1H), 8.29 (d, *J* = 8.8 Hz, 2H), 8.07 (dd, *J* = 8.8, 4.9 Hz, 3H), 7.96 (d, *J* = 8.8 Hz, 2H), 7.88 (d, *J* = 8.7 Hz, 2H), 7.77 (d, *J* = 8.7 Hz, 2H), 7.59 (d, *J* = 8.7 Hz, 2H), 7.33 – 7.24 (m, 15H), 7.15 (s, 1H), 6.15 (m, 1H), 6.05 (m, 1H), 5.50 (dd, *J* = 17.1, 1.2 Hz, 1H), 5.43 (m, 2H), 5.30 (dd, *J* = 10.4, 1.1 Hz, 1H), 5.07 (m, 1H), 4.82 (d, *J* = 5.6 Hz, 2H), 4.76 (dt, *J* = 12.3, 6.2 Hz, 1H), 4.71 (d, *J* = 5.9 Hz, 2H), 3.31 (dd, *J* = 15.6, 2.7 Hz, 1H), 2.76 (dd, *J* = 15.6, 7.3 Hz, 1H), 1.39 (dd, *J* = 6.1, 3.9 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 168.3, 166.9, 165.0, 164.6, 164.3, 149.5, 149.2, 144.7, 143.5, 142.6, 142.2, 139.3, 135.6, 133.7, 132.8, 130.6, 130.3, 129.0, 128.5, 128.4, 128.0, 127.4, 127.2, 126.4, 124.2, 123.6, 119.0, 118.9, 118.7, 117.8, 117.8, 76.3, 74.3, 69.4, 64.8, 52.1, 38.0, 22.3.

HRMS (ESI) calculated for C60H55N6O11 ($M+H^+$) 1035.3923, found 1035.3943.

Allyl (S)-4-(2-(allyloxy)-4-(4-(4-amino-2-(4-aminobenzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzamido)benzoate (35)



Chemical Formula: $C_{41}H_{42}N_6O_9$
Exact Mass: 762,3013

Zn dust (0.95 g; 15.5 mmol) was added portionwise over few minutes to a stirred solution of allyl (S)-4-(2-(allyloxy)-3-isopropoxy-4-(4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)-butanamido)benzamido)benzamido)benzoate (1.30 g; 1.26 mmol) in THF (6.0 mL), EtOH (4.8 mL) and AcOH (1.2 mL). Reaction stirred for 5 h, the mixture was filtered through celite, the solvent was reduced under vacuum. The crude was used in the next step without further purification.

The residue was dissolved in DCM (18.7 mL), Tips (0.775 mL; 3.78 mmol) followed by TFA (6.3 mL) were added at 0 °C. Reaction stirred 2 h at r.t. then solved removed under vacuum, residue take up and evaporated twice with DCM (15 mL) then triturated 3x with ice cold Pet. Et.. The crude thus obtained was purified on silica gel with a gradient 0-10% MeOH in DCM to give 1.02 g of a yellow solid (1.18 mmol; y= 94%).

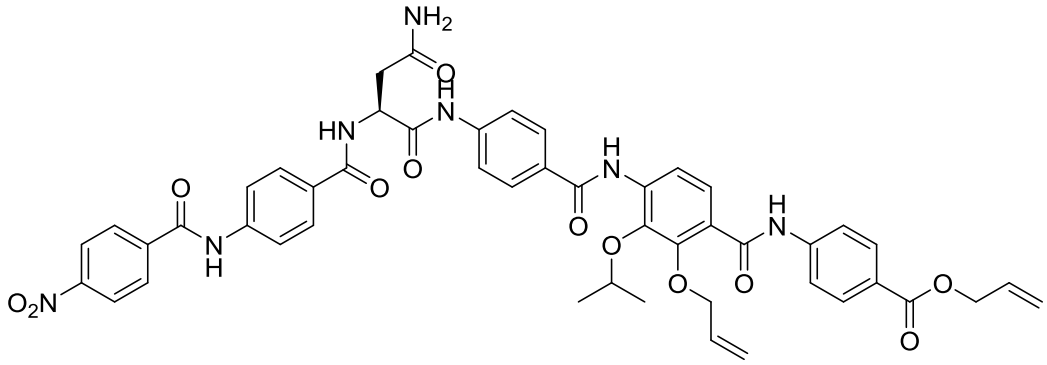
^{1}H NMR (500 MHz, DMSO) δ 10.59 (s, 1H), 10.38 (s, 1H), 9.52 (s, 1H), 8.23 (d, *J* = 7.3 Hz, 1H), 7.98 (t, *J* = 9.4 Hz, 4H), 7.88 (d, *J* = 8.8 Hz, 2H), 7.80 (dd, *J* = 11.0, 8.7 Hz, 3H), 7.62 (d, *J* = 8.7 Hz, 2H), 7.40 (d, *J* = 8.5 Hz, 2H), 6.97 (s, 1H), 6.56 (d, *J* = 8.7 Hz, 2H), 6.11 – 5.97 (m, 2H), 5.68 (s, 2H), 5.44 – 5.35 (m, 2H), 5.31 - 5-18 (m, 2H), 4.85 (dd, *J* = 14.1, 7.1 Hz, 1H), 4.80 (d, *J* = 5.3 Hz, 2H), 4.61 (d, *J* = 5.5 Hz, 2H), 4.53 – 4.44 (m, 1H), 2.65 (d, *J* = 7.0 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 171.5, 171.1, 166.4, 165.0, 164.7, 164.3, 151.9, 149.5, 143.5, 142.6, 142.5, 135.7, 133.7, 132.8, 130.4, 129.1, 128.4, 128.2, 127.1, 124.2, 123.6, 120.4, 119.0, 118.8, 117.8, 117.8, 116.0, 112.5, 76.3, 74.3, 64.9, 51.5, 40.1, 40.0, 39.9, 39.8, 39.8, 39.7, 39.6, 39.5, 39.3, 39.2, 39.0, 36.9, 22.3, 20.8.

HRMS (ESI) calculated for C41H43N6O9 ($M+H^+$) 763.3086, found 763.3085.

Marfey: 94.0% S enantiomer, 6.0% R enantiomer

Allyl (S)-4-(2-(allyloxy)-4-(4-(4-amino-2-(4-(4-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzamido)benzoate (36)[65]



Chemical Formula: $C_{48}H_{45}N_7O_{12}$
Exact Mass: 911,3126

Collidine (0.166 mL; 1.26 mmol) was added dropwise at 0 °C to a solution of 4-nitro benzoic acid (92 mg; 0.551 mmol) and bis(trichloromethyl) carbonate (49 mg; 0.165 mmol) in THF (10 mL). Reaction stirred at r.t. for 20 min then added to a solution of allyl (S)-2-(allyloxy)-4-(2-(allyloxy)-4-(4-(2-(4-aminobenzamido)-3-cyanopropanamido)benzamido)-3-methoxybenzamido)-3-methoxybenzoate (120 mg; 0.157 mmol) and DiPEA (0.273 mL; 1.57 mmol) in THF (10 mL). Reaction stirred for 4 h then quenched with HCl 1 N and ice. Solvent partially reduced under vacuum, EtOAc (40 mL) and HCl 1N (40 mL) were added, organic phase washed with $NaHCO_3$ saturated solution (30 mL), brine (30 mL) and dried over sodium sulphate. The solvent was removed under reduced pressure, the residue was purified on silica gel with a gradient 0-10% MeOH in DCM to give 114 mg of a yellow residue (0.125 mmol; y= 80%).

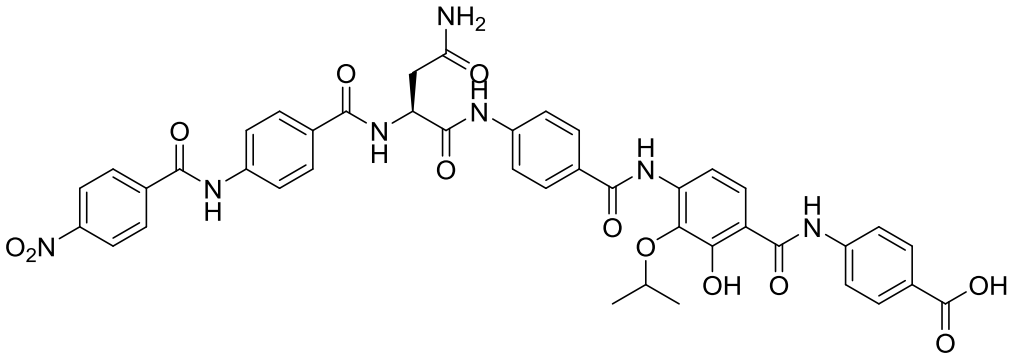
^{1}H NMR (700 MHz, DMSO) δ 10.79 (s, 1H), 10.58 (s, 1H), 10.45 (s, 1H), 9.52 (s, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.41 – 8.36 (m, 2H), 8.23 – 8.19 (m, 2H), 8.00 – 7.95 (m, 4H), 7.94 (m, 2H), 7.91 (m, 2H), 7.87 (d, *J* = 8.6 Hz, 2H), 7.81 (m, 3H), 7.40 (d, *J* = 8.4 Hz, 2H), 6.99 (s, 1H), 6.04 (m, 2H), 5.39 (m, 2H), 5.28 (ddd, *J* = 10.5, 2.9, 1.4 Hz, 1H), 5.20 (ddd, *J* = 10.5, 2.9, 1.3 Hz, 1H), 4.92 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.79 (d, *J* = 5.3 Hz, 2H), 4.61 (d, *J* = 5.5 Hz, 2H), 4.49 (tt, *J* = 12.2, 6.1 Hz, 1H), 2.69 (d, *J* = 7.9 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 165.8, 164.9, 164.7, 164.3, 164.2, 149.5, 149.3, 143.5, 142.6, 142.4, 141.5, 140.3, 135.7, 133.7, 132.8, 130.3, 129.3, 129.2, 128.4, 128.3,

128.3, 127.1, 124.2, 123.6, 119.5, 119.0, 119.0, 118.8, 117.8, 117.8, 76.2, 74.3, 64.8, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C48H46N7O12 ($M+H^+$) 912.3199, found 912.3196.

(S)-4-(4-(4-(4-Amino-2-(4-(4-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (1)



Chemical Formula: $C_{42}H_{37}N_7O_{12}$
Exact Mass: 831,2500

Phenyl silane (0.012 mL; 0.099 mmol) followed by palladium-tetrakis(triphenylphosphine (6.3 mg; 0.0055 mmol) was added to a solution of allyl (S)-4-(2-(allyloxy)-4-(4-(4-amino-2-(4-(4-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzamido)benzoate (20 mg; 0.022 mmol) in THF (2.75 mL). Reaction stirred overnight and purified by preparative RP-HPLC to afford to obtain 3.8 mg of a white material (0.0046 mmol; y= 21%).

According to the purification method used, the desired structure could be obtained either in its protonated form or as ammonium salt.

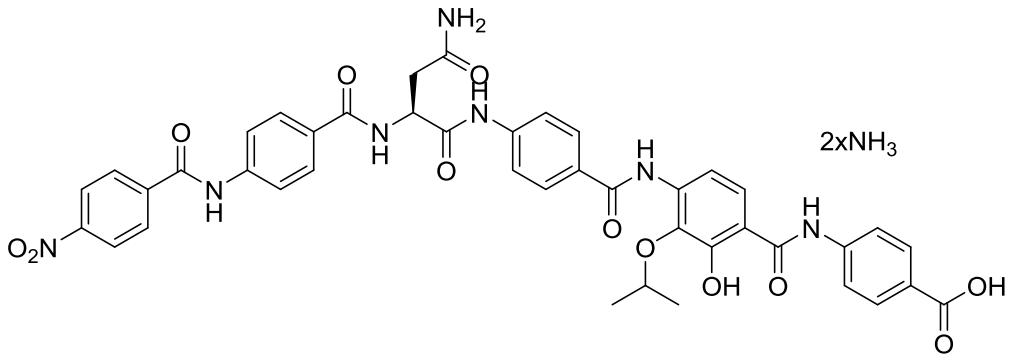
Condition A:

protonated form

^{1}H NMR (700 MHz, DMSO) δ 12.82 (s, 1H), 12.29 (s, 1H), 10.79 (s, 1H), 10.60 (s, 1H), 10.46 (s, 1H), 9.40 (s, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.41 – 8.35 (m, 2H), 8.24 – 8.18 (m, 2H), 7.99 – 7.92 (m, 6H), 7.90 (d, *J* = 8.8 Hz, 2H), 7.85 (m, 3H), 7.82 (d, *J* = 8.8 Hz, 2H), 7.70 (d, *J* = 8.8 Hz, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.57 – 4.51 (m, 1H), 2.69 (d, *J* = 7.6 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

Condition B:

ammonium salts



Chemical Formula: $C_{42}H_{37}N_7O_{12}$
Exact Mass: 831,2500

^{1}H NMR (700 MHz, DMSO) δ 15.41 (br, 1H), 10.80 (s, 1H), 10.46 (s, 1H), 8.87 (s, 1H), 8.72 (d, *J* = 7.3 Hz, 1H), 8.41 – 8.35 (m, 2H), 8.23 – 8.18 (m, 2H), 7.96 – 7.92 (m, 2H), 7.90 (d, *J* = 8.8 Hz, 2H), 7.84 (d, *J* = 8.9 Hz, 2H), 7.81 (d, *J* = 8.9 Hz, 2H), 7.76 (d, *J* = 7.8 Hz, 2H), 7.59 (br, 2H), 7.44 (d, *J* = 8.7 Hz, 1H), 7.42 (s, 1H), 7.09 (d, *J* = 8.7 Hz, 1H), 6.98 (s, 1H), 5.06 – 4.99 (m, 1H), 4.93 (q, *J* = 7.1 Hz, 1H), 2.69 (d, *J* = 7.4 Hz, 2H), 1.19 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.6, 166.9, 165.7, 165.3, 164.2, 163.1, 149.3, 142.0, 141.5, 140.3, 137.5, 129.5, 129.3, 129.3, 128.3, 127.6, 124.2, 123.7, 123.6, 119.5, 119.0, 117.7, 70.3, 51.6, 36.8, 22.7.

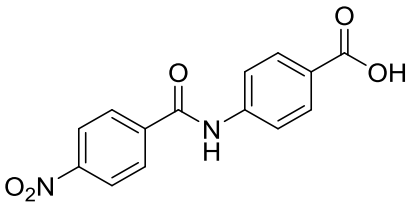
HRMS (ESI) calculated for C42H38N7O12 ($M+H^+$) 832.2573, found 832.2580.

Marfey: 91.2% S enantiomer, 8.8% R enantiomer

4.2.2.2 N-terminal OPTIMIZATION

4.2.2.2.1 Synthesis of variants of building block A

4-(4-Nitrobenzamido)benzoic acid (89)



Chemical Formula: $C_{14}H_{10}N_2O_5$
Exact Mass: 286,0590

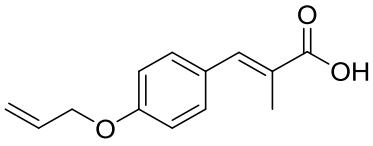
4-Aminobenzoic acid (800 mg; 5.84 mmol) was dissolved in THF (4.0 mL) and $NaHCO_3$ saturated solution (4.0 mL), to it 4-nitrobenzoyl chloride (1.08 g; 5.84 mmol) was added at 0 °C. Reaction stirred for 2 hours, precipitate collected by filtration, washed with water and THF, dried under high vacuum to give 1.30 g of a solid (4.55 mmol; y= 77%).

^{1}H NMR (500 MHz, DMSO) δ 12.80 (br, 1H), 10.83 (s, 1H), 8.43 – 8.35 (m, 2H), 8.25 – 8.14 (m, 2H), 7.99 – 7.94 (m, 2H), 7.94 – 7.90 (m, 2H).

^{13}C NMR (126 MHz, DMSO) δ 166.9, 164.3, 149.3, 142.8, 140.3, 130.3, 129.4, 126.0, 123.6, 119.7.

HRMS (ESI) calculated for C14H9N2O5 ($M-H^+$) 285.0517, found 285.0536.

(E)-3-(4-(Allyloxy)phenyl)-2-methylacrylic acid (58)[65]



Chemical Formula: $C_{13}H_{14}O_3$
Exact Mass: 218,0943

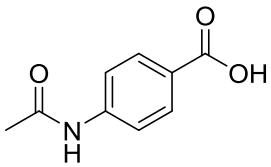
Synthesized according to exp. procedure reported.

^{1}H NMR (700 MHz, DMSO) δ 12.35 (br, 1H), 7.54 (d, *J* = 1.1 Hz, 1H), 7.46 – 7.42 (m, 2H), 7.03 – 6.99 (m, 2H), 6.05 (ddt, *J* = 17.3, 10.5, 5.2 Hz, 1H), 5.40 (dq, *J* = 17.3, 1.7 Hz, 1H), 5.27 (dq, *J* = 10.5, 1.5 Hz, 1H), 4.61 (dt, *J* = 5.3, 1.5 Hz, 2H), 2.03 (d, *J* = 1.5 Hz, 3H).

^{13}C NMR (176 MHz, DMSO) δ 169.6, 158.3, 137.4, 133.5, 131.4, 128.1, 126.2, 117.6, 114.7, 68.2, 13.9.

HRMS (ESI) calculated for C13H13O3 ($M-H^+$) 217.0870, found 217.0873.

4-Acetamidobenzoic acid (55)[115]



Chemical Formula: $C_9H_9NO_3$
Exact Mass: 179,0582

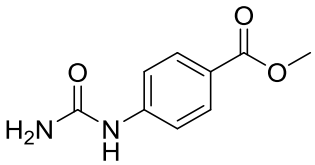
Synthesized according to exp. procedure reported.

^{1}H NMR (500 MHz, DMSO) δ 12.67 (br, 1H), 10.23 (s, 1H), 7.90 – 7.84 (m, 2H), 7.68 (d, *J* = 8.7 Hz, 2H), 2.08 (s, 3H).

^{13}C NMR (126 MHz, DMSO) δ 168.8, 166.9, 143.3, 130.4, 124.9, 118.1, 24.1.

HRMS (ESI) calculated for C9H8NO3 (M-H$^+$) 178.0510, found 178.0510.

Methyl 4-ureidobenzoate



Chemical Formula: $C_9H_{10}N_2O_3$
Exact Mass: 194,0691

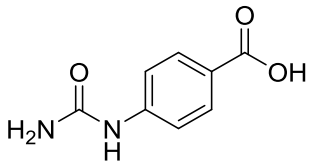
A solution of methyl 4-aminobenzoate (500 mg; 3.31 mmol) and DiPEA (0.86 mL; 4.97 mmol) in DCM (10 mL) was added dropwise at 0 °C to a solution of BTC (328 mg; 1.10 mmol) in DCM (20 mL). Reaction stirred at 0 °C for 2 h. Solvent reduced under vacuum, residue dissolved in 5 mL of DCM and added to a cooled solution of NH_4OH conc.. The mixture was stirred for 2 hours, the solid was collected by filtration, washed with water and twice with Et_2O, dried under high vacuum to give 410 mg of a white powder (2.11 mmol; y= 64%).

^{1}H NMR (500 MHz, DMSO) δ 8.98 (s, 1H), 7.87 – 7.79 (m, 2H), 7.57 – 7.48 (m, 2H), 6.05 (s, 2H), 3.79 (s, 3H).

^{13}C NMR (126 MHz, DMSO) δ 166.0, 155.6, 145.3, 130.3, 121.6, 116.8, 51.7.

HRMS (ESI) calculated for C9H11N2O3 (M+H$^+$) 195.0764, found 195.0766.

4-Ureidobenzoic acid (56)



Chemical Formula: $C_8H_8N_2O_3$
Exact Mass: 180,0535

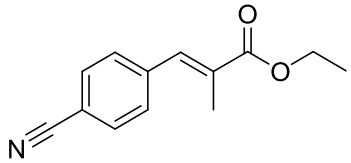
Methyl 4-ureidobenzoate (150 mg; 0.77 mmol) was suspended in THF (4 mL) and water (2 mL). A solution of LiOH (185 mg; 7.7 mmol) in water (2 mL) was added to the suspension at 0 °C. Reaction stirred overnight, pH adjusted to 1, the precipitate was collected by filtration washed with water and three times with Et_2O, dried at high vacuum to give 80 mg of a white powder (0.44 mmol; y= 58%).

^{1}H NMR (500 MHz, DMSO) δ 12.50 (br, 1H), 8.90 (s, 1H), 7.84 – 7.77 (m, 2H), 7.54 – 7.45 (m, 2H), 6.02 (s, 1H).

^{13}C NMR (126 MHz, DMSO) δ 167.1, 155.6, 144.9, 130.4, 122.9, 116.7.

HRMS (ESI) calculated for C8H7N2O3 ($M-H^+$) 179.0462, found 179.0470.

Ethyl (E)-3-(4-cyanophenyl)-2-methylacrylate (62)



Chemical Formula: $C_{13}H_{13}NO_2$
Exact Mass: 215,0946

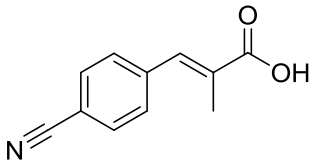
4-Hydroxybenzaldehyd (500 mg; 3.82 mmol) was dissolved in DCM (3.0 mL) at rt, ethyl-2-(triphenylphosphoranylidene)propionate (1.06 g; 2.94 mmol) was added. The mixture was stirred overnight at 37°C then concentrated under reduced pressure. The crude was purified on silica gel with a gradient 1-50% EtOAc in Pet. Et. to give 570 mg of white solid (2.65 mmol; y= 90%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70 – 7.66 (m, 2H), 7.64 (s, 1H), 7.47 (d, *J* = 8.2 Hz, 2H), 4.29 (q, *J* = 7.1 Hz, 2H), 2.10 (d, *J* = 1.5 Hz, 3H), 1.35 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 167.9, 140.6, 136.4, 132.1, 131.8, 130.0, 118.6, 111.7, 61.3, 14.3, 14.2.

HRMS (ESI) calculated for C13H14NO2 ($M+H^+$) 216.1019, found 216.1014.

(E)-3-(4-Cyanophenyl)-2-methylacrylic acid (59)



Chemical Formula: $C_{11}H_9NO_2$
Exact Mass: 187,0633

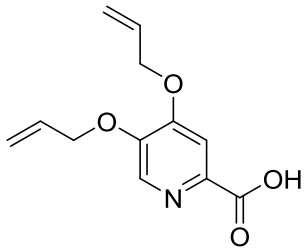
Ethyl (E)-3-(4-cyanophenyl)-2-methylacrylate (150 mg; 0.67 mmol) was dissolved in THF (3.35 mL) and water (1.68 mL), solution cooled to 0 °C and a mixture of LiOH (167 mg; 6.98 mmol) in water (1.68 mL) added to it. Reaction stirred at 0 C for 10 min. then to r.t. overnight. Reaction quenched adjusting pH to 1, then diluted with EtOAc (20 mL) and HCl 1 N (20 mL), organic solvent washed with brine and dried over sodium sulphate. Solvent reduce under vacuum to afford 125 mg of a white solid (0.67 mmol; y= q.).

^{1}H NMR (500 MHz, DMSO) δ 7.91 – 7.87 (m, 2H), 7.65 (d, *J* = 8.2 Hz, 2H), 7.61 (s, 1H), 2.02 (d, *J* = 1.5 Hz, 3H).

^{13}C NMR (126 MHz, DMSO) δ 168.9, 140.4, 135.8, 132.3, 130.3, 127.6, 118.7, 110.6, 14.0.

HRMS (ESI) calculated for C11H10NO2 ($M+H^+$) 188.0706, found 188.0709.

4,5-Bis(allyloxy)picolinic acid (57)[116]



Chemical Formula: $C_{12}H_{13}NO_4$
Exact Mass: 235,0845

Synthesized according experimental procedures reported using allyl bromide instead of benzyl bromide.

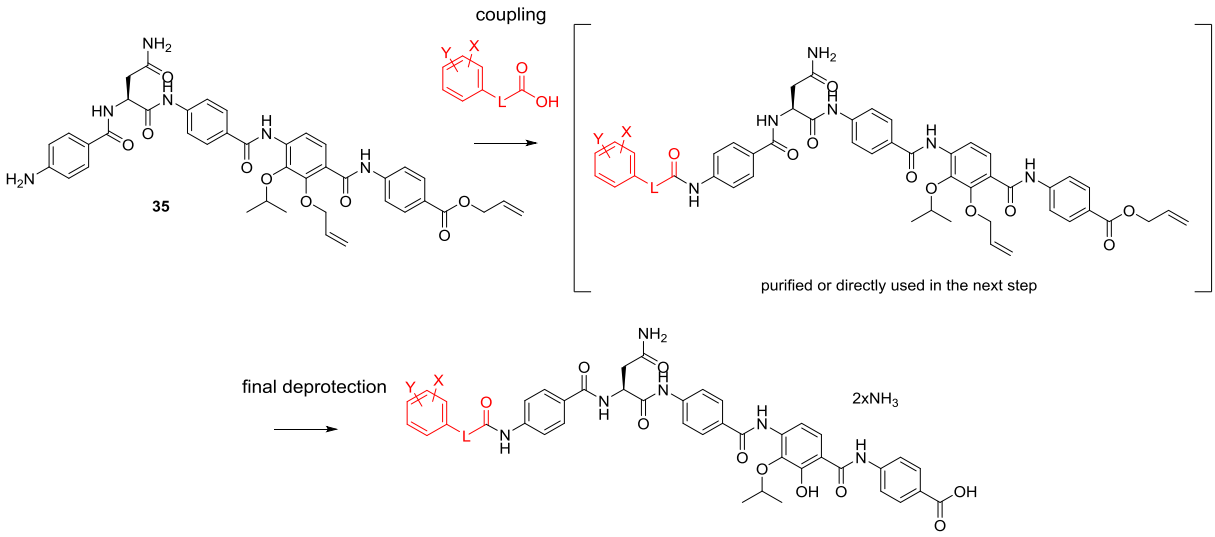
^{1}H NMR (500 MHz, MeOD) δ 8.16 (s, 1H), 7.82 (s, 1H), 6.11 (m, 2H), 5.54 – 5.44 (m, 2H), 5.36 (m, 2H), 4.86 (dt, *J* = 5.3, 1.5 Hz, 2H), 4.77 (dt, *J* = 5.3, 1.5 Hz, 2H).

^{13}C NMR (126 MHz, MeOD) δ 165.4, 159.9, 149.2, 144.4, 133.6, 132.9, 131.0, 119.5, 119.2, 110.5, 72.0, 71.5.

HRMS (ESI) calculated for C12H14NO4 ($M+H^+$) 236.0917, found 236.0922.

4.2.2.2.2 General scheme

Synthetic scheme used for the synthesis of analogs bearing modifications of ring A. In all cases, amine **35** was coupled to a carboxylic acid and then deprotection of allyl groups followed.



4.2.2.2.3 General procedures

Coupling:

The last aromatic ring was installed activating the carboxylic acid to acyl chloride or activated ester by means of:

- A. BTC collidine
- B. oxalyl chloride
- C. EDC, HOAt

A. Collidine (8 eq.) was added dropwise at 0 °C to a solution of desired carboxylic acid (3.5 eq.) and bis(trichloromethyl) carbonate (1.05 eq.) in THF (M= 0.02). Reaction stirred at r.t. for 20 min then added to a solution of amine **35** (1 eq.) and DiPEA (10 eq.) in THF (M= 0.02). Reaction stirred for few hours then quenched with HCl 1 N and ice. Solvent partially reduced under vacuum, EtOAc and HCl 1N were added, organic phase washed with $NaHCO_3$ saturated solution, brine and dried over sodium sulphate. The solvent was removed under reduced pressure, the residue was purified on silica gel or directly used in the next step without further purification.

B. The desired carboxylic acid (1.0 eq.) is suspended in DCM (M= 0.5), oxalyl chloride (1.5 eq.) followed by catalytic DMF were added at 0 °C. Reaction stirred until all the carboxylic acid has reacted, reaction monitored by TLC. Solvent reduced under vacuum, residue dissolved again in DCM and evaporated twice.

The desired acyl chloride (1.5 eq.) as a solution in THF (M= 0.08) was added at 0 °C to a stirred solution of amine **35** (1.0 eq.) and DiPEA (5 eq.) in THF (M= 0.08), reaction stirred at 0 °C for 10 min then warmed to r.t., stirring prolonged until completion of the reaction (monitored by TLC and/or LCMS). Reaction quenched with HCl 1N/ice, solvent partially reduced under vacuum, mixture diluted with EtOAc and HCl 1 N, organic phase washed with brine and dried over sodium sulphate. The residue thus obtained could be purified on silica gel or directly used in the next step without further purification.

C. Carboxylic acid (3.0 eq.), EDC (3.0 eq), HOAt (3.5 eq.) were mixed together in DMF (M= 0.2), to this solution collidine was added (8.0 eq.), reaction stirred for 20 min then added to a solution of amine **35** (1.0 eq.) in DMF (M= 0.2). Reaction heated to 40 °C for

several hours until reaction completed. Reaction diluted with EtOAc and HCl 1 N, organic phase washed with brine and dried over sodium sulphate, solvent reduced under vacuum, the residue was used in the next step without further purification.

Final deprotection

Phenyl silane (2 eq. x n of allyl group) followed by tetrakis(triphenylphosphine)palladium^0 (0.25 eq.) were added to a solution of allyl protected cystobactamid derivative (1.0 eq.) in THF (M= 0.01). Reaction stirred overnight and purified by preparative RP-HPLC using condition A or B (described in section 4.2.1.2).

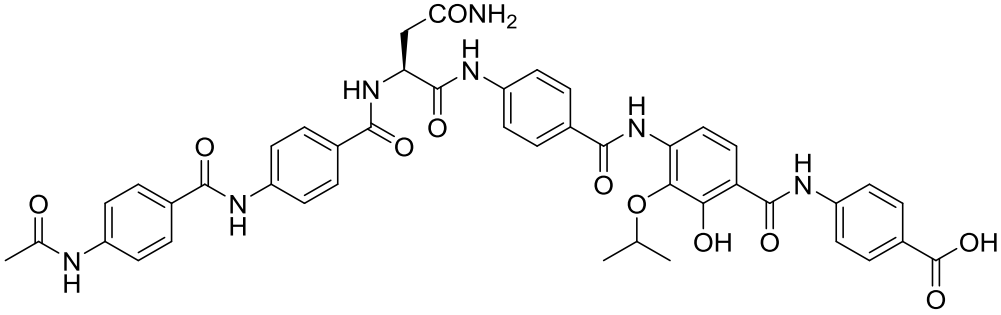
4.2.2.2.4 1th and 2nd series of analogs ring A

For coupling step, final deprotection and purification, the general proceduers described in section 4.2.2.2.3 were used.

The carbocylic acids employed in the coupling step with amine **35** were purchased or synthesized according to procedures described herein.

All compounds purified according to condition B were obtained as ammonium salts. The number of counteranions varies and depends on the functional groups present on the molecule. The given numbers with the structures are estimations according to H-NMR data.

(S)-4-(4-(4-(2-(4-(4-Acetamidobenzamido)benzamido)-4-amino-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (38)



Chemical Formula: $C_{44}H_{41}N_7O_{11}$
Exact Mass: 843,2864

Amine **35** (15 mg, 0.016 mmol) was coupled with carboxylic acid **55** using coupling conditions C followed by final deprotection.

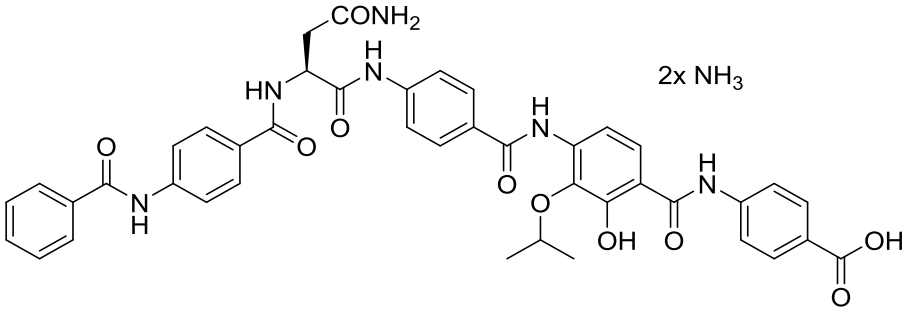
Desired compound purified by preparative RP-HPLC condition A, to obtain 1.1 mg of desired product as a white solid (0.0013 mmol, y= 8%).

^{1}H NMR (700 MHz, DMSO) δ 12.78 (br, 1H), 12.29 (s, 1H), 10.60 (s, 1H), 10.45 (s, 1H), 10.33 (s, 1H), 10.23 (s, 1H), 9.40 (s, 1H), 8.63 (d, *J* = 7.3 Hz, 1H), 7.95 (m, 6H), 7.92 – 7.87 (m, 3H), 7.85 (m, 3H), 7.82 (d, *J* = 8.7 Hz, 2H), 7.71 (m, 3H), 7.39 (s, 1H), 7.35 (dd, *J* = 8.0, 1.4 Hz, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.54 (dt, *J* = 12.3, 6.1 Hz, 1H), 2.69 (d, *J* = 8.1 Hz, 2H), 2.09 (s, 3H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.8, 168.8, 168.5, 166.9, 165.8, 165.2, 164.2, 154.1, 142.5, 142.5, 142.2, 142.0, 137.0, 136.3, 133.9, 133.7, 130.2, 129.7, 128.7, 128.7, 128.5, 128.3, 128.2, 127.3, 126.3, 122.8, 120.7, 119.3, 119.0, 118.1, 112.4, 112.2, 74.9, 51.6, 36.8, 24.1, 22.3.

HRMS (ESI) calculated for C44H42N7O11 (M-H^{+}) 844.2937, found 844.2930.

(S)-4-(4-(4-(4-Amino-2-(4-benzamidobenzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (39)



Chemical Formula: $C_{42}H_{38}N_6O_{10}$
Exact Mass: 786,2649

Amine **35** (40 mg, 0.052 mmol) was coupled with benzoic acid using coupling conditions A followed by final deprotection.

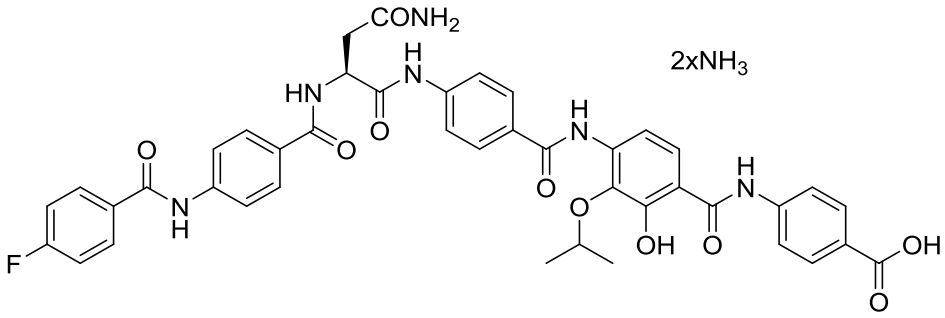
Desired compound purified by preparative RP-HPLC condition B, to obtain 13.8 mg of desired product as a white solid (0.018 mmol, y= 34%).

^{1}H NMR (700 MHz, DMSO) δ 12.74 (br, 1H), 12.30 (br, 1H), 10.48 (s, 1H), 10.45 (s, 1H), 9.33 (br, 1H), 8.65 (d, *J* = 7.3 Hz, 1H), 7.99 – 7.96 (m, 2H), 7.94 (dd, *J* = 15.6, 6.5 Hz, 4H), 7.92 – 7.89 (m, 4H), 7.83 (dd, *J* = 17.1, 8.7 Hz, 4H), 7.79 (s, 1H), 7.61 (dd, *J* = 11.6, 4.2 Hz, 2H), 7.55 (t, *J* = 7.6 Hz, 2H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.61 (s, 1H), 2.69 (d, *J* = 7.9 Hz, 2H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.3, 168.3, 166.9, 165.8, 164.0, 142.4, 142.1, 136.5, 134.7, 131.8, 130.2, 128.7, 128.4, 128.3, 128.2, 127.7, 127.4, 123.0, 120.4, 119.3, 118.9, 51.6, 36.8, 22.4.

HRMS (ESI) calculated for C42H37N6O10 (M-H$^+$) 785.2577, found 785.2577.

(S)-4-(4-(4-(4-Amino-2-(4-(4-fluorobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (40)



Chemical Formula: $C_{42}H_{37}FN_6O_{10}$
Exact Mass: 804,2555

Amine **35** (40 mg, 0.052 mmol) was coupled with 4-fluorobenzoic acid using coupling conditions A followed by final deprotection.

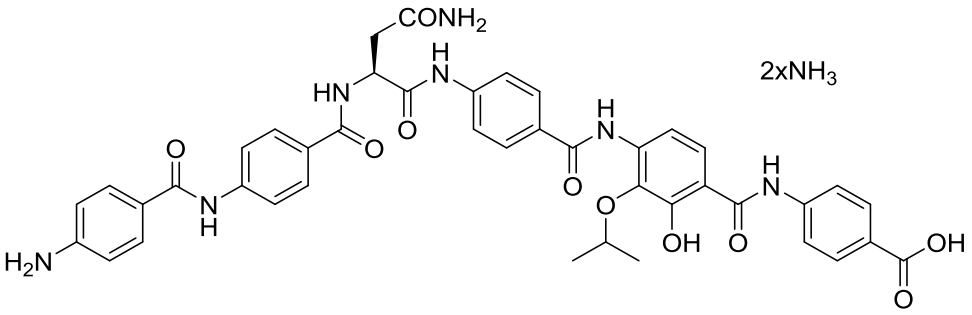
Desired compound purified by preparative RP-HPLC condition B, to obtain 3.2 mg of desired product as a white solid (0.004 mmol, y= 8%).

^{1}H NMR (500 MHz, DMSO) δ 12.80 (br, 1H), 12.29 (br, 1H), 10.62 (br, 1H), 10.49 (s, 1H), 10.46 (s, 1H), 9.39 (s, 1H), 8.65 (d, *J* = 7.3 Hz, 1H), 8.09 – 8.03 (m, 2H), 7.96 (t, *J* = 8.4 Hz, 4H), 7.90 (q, *J* = 9.0 Hz, 4H), 7.84 (dd, *J* = 17.4, 8.8 Hz, 5H), 7.68 (d, *J* = 8.6 Hz, 1H), 7.43 – 7.35 (m, 3H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.1, 7.1 Hz, 1H), 4.59 – 4.51 (m, 1H), 2.69 (d, *J* = 7.2 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 171.3, 170.8, 168.4, 166.9, 165.8, 164.7, 164.2, 163.2, 142.5, 142.0, 136.4, 131.1, 130.6, 130.5, 130.2, 128.8, 128.3, 122.8, 120.6, 119.4, 118.9, 115.5, 115.3, 112.5, 74.8, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C42H36FN6O10 (M-H$^+$) 803.2482, found 803.2498.

(S)-4-(4-(4-(4-Amino-2-(4-(4-aminobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (41)



Chemical Formula: $C_{42}H_{39}N_7O_{10}$
Exact Mass: 801,2758

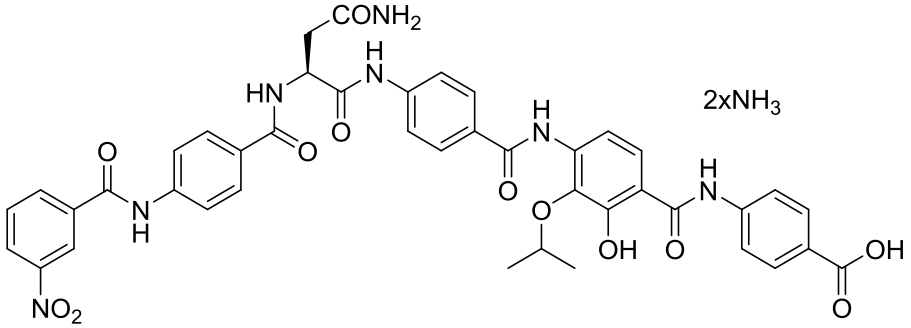
Compound **40** was isolated as a by-product of the synthesis of **1**, obtained 0.7 mg.

^{1}H NMR (700 MHz, DMSO) δ 15.31 (s, 1H), 10.46 (s, 1H), 9.98 (s, 1H), 8.87 (s, 1H), 8.68 (d, *J* = 7.0 Hz, 1H), 7.86 (d, *J* = 9.9 Hz, 4H), 7.82 (dd, *J* = 19.7, 8.8 Hz, 4H), 7.75 (dd, *J* = 11.3, 8.6 Hz, 4H), 7.55 (d, *J* = 8.5 Hz, 2H), 7.44 (d, *J* = 8.7 Hz, 2H), 7.08 (d, *J* = 8.7 Hz, 1H), 6.98 (s, 1H), 6.60 (d, *J* = 8.6 Hz, 2H), 5.80 (s, 2H), 5.02 (dt, *J* = 12.4, 6.1 Hz, 1H), 4.91 (dd, *J* = 14.2, 7.2 Hz, 1H), 2.69 (d, *J* = 7.0 Hz, 2H), 1.19 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 169.8, 166.8, 165.9, 165.5, 165.3, 163.1, 152.4, 142.7, 142.0, 141.5, 137.5, 134.0, 129.6, 129.5, 128.2, 127.9, 127.5, 123.7, 120.7, 119.0, 119.0, 117.5, 116.1, 112.5, 100.6, 70.3, 51.7, 36.9, 22.7.

HRMS (ESI) calculated for C42H38N7O10 (M-H$^+$) 800.2686, found 800.2733.

(S)-4-(4-(4-(4-Amino-2-(4-(3-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (42)



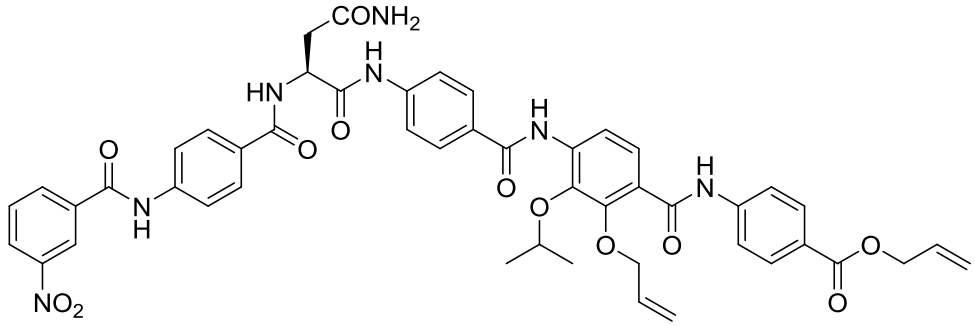
Chemical Formula: $C_{42}H_{37}N_7O_{12}$
Exact Mass: 831,2500

Amine **35** (40 mg, 0.052 mmol) was coupled with 3-nitrobenzoic acid using coupling conditions A. Allyl protected derivative was purified on silica gel with a gradient 0-10% MeOH in DCM to give 45 mg of white slid (0.049 mmol, y= 95%).

The final deprotection was done on 20 mg of intermediate (0.022 mmol). The desired compound was purified by preparative RP-HPLC using condition B, to obtain 6.8 mg of

desired product (0.008 mmol, y= 31%) and 2.2 mg of compound **43**, which was formed as a by-product of the reaction.

Allyl protected intermediate:



Chemical Formula: $C_{48}H_{45}N_7O_{12}$
Exact Mass: 911,3126

^{1}H NMR (700 MHz, DMSO) δ 10.80 (s, 1H), 10.58 (s, 1H), 10.45 (s, 1H), 9.52 (s, 1H), 8.82 (t, *J* = 2.0 Hz, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.46 (ddd, *J* = 8.2, 2.3, 1.0 Hz, 1H), 8.44 – 8.41 (m, 1H), 8.00 – 7.96 (m, 4H), 7.96 – 7.93 (m, 2H), 7.93 – 7.89 (m, 2H), 7.86 (t, *J* = 8.0 Hz, 3H), 7.83 – 7.79 (m, 3H), 7.40 (d, *J* = 8.4 Hz, 2H), 6.99 (s, 1H), 6.08 – 5.99 (m, 2H), 5.39 (m, 2H), 5.24 (m, 2H), 4.93 (dd, *J* = 14.0, 7.3 Hz, 1H), 4.79 (dd, *J* = 3.9, 1.4 Hz, 2H), 4.61 (d, *J* = 5.5 Hz, 2H), 4.52 – 4.46 (m, 1H), 2.71 – 2.68 (m, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 165.8, 164.9, 164.7, 164.3, 163.6, 149.5, 147.8, 143.5, 142.6, 142.4, 141.5, 136.0, 135.7, 134.3, 133.7, 132.8, 130.3, 130.3, 129.2, 128.4, 128.3, 127.1, 126.4, 124.2, 123.6, 122.5, 119.6, 119.0, 118.8, 117.8, 117.8, 76.2, 74.3, 64.8, 51.6, 36.8, 22.3.

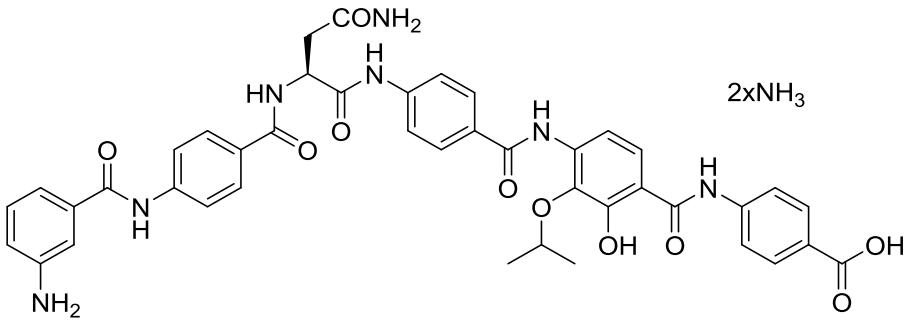
Final compound 42:

^{1}H NMR (700 MHz, DMSO) δ 12.79 (br, 1H), 12.29 (br, 1H), 10.80 (s, 1H), 10.62 (br, 1H), 10.46 (s, 1H), 9.36 (s, 1H), 8.82 (t, *J* = 1.8 Hz, 1H), 8.68 (d, *J* = 7.2 Hz, 1H), 8.46 (dd, *J* = 8.2, 1.5 Hz, 1H), 8.43 (d, *J* = 7.9 Hz, 1H), 7.98 – 7.89 (m, 8H), 7.86 (dd, *J* = 15.4, 7.9 Hz, 3H), 7.82 (d, *J* = 8.7 Hz, 3H), 7.66 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.93 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.58 (s, 1H), 2.70 (d, *J* = 7.5 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.4, 166.9, 165.8, 164.1, 163.6, 147.8, 142.5, 141.5, 136.4, 136.0, 134.3, 130.3, 130.2, 129.2, 128.3, 128.3, 126.4, 122.9, 122.5, 120.6, 119.6, 118.9, 74.5, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C42H38N7O12 ($M+H^+$) 832.2573, found 832.2565.

(S)-4-(4-(4-(4-Amino-2-(4-(3-aminobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (43)



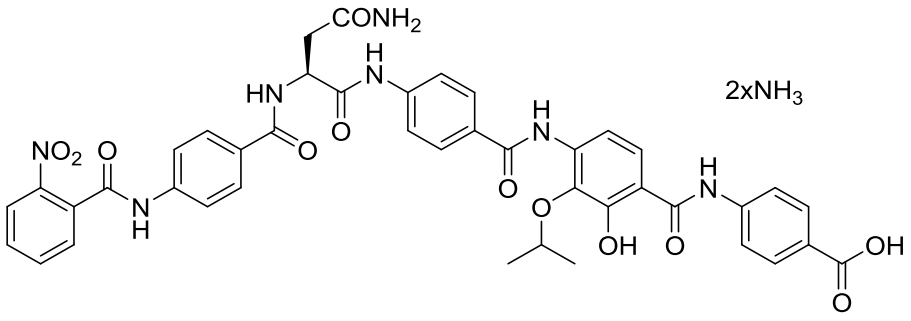
Chemical Formula: $C_{42}H_{39}N_7O_{10}$
Exact Mass: 801,2758

^{1}H NMR (700 MHz, DMSO) δ 15.86 (br, 1H), 12.45 (br, 1H), 10.43 (s, 1H), 10.30 (s, 1H), 8.94 (br, 1H), 8.62 (d, *J* = 7.3 Hz, 1H), 7.91 – 7.83 (m, 9H), 7.79 (dd, *J* = 20.6, 8.7 Hz, 4H), 7.49 (br, 1H), 7.40 (s, 1H), 7.16 (t, *J* = 7.8 Hz, 1H), 7.10 (d, *J* = 1.8 Hz, 1H), 7.08 (d, *J* = 7.8 Hz, 1H), 6.98 (s, 1H), 6.76 (dd, *J* = 7.9, 1.5 Hz, 1H), 5.33 (s, 2H), 4.95 (br, 1H), 4.92 (dd, *J* = 14.0, 7.3 Hz, 1H), 2.71 – 2.66 (m, 2H), 1.20 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 167.5, 167.2, 166.6, 165.9, 163.3, 148.8, 142.3, 142.1, 135.6, 130.4, 128.8, 128.5, 128.2, 127.7, 123.6, 119.2, 119.0, 117.0, 114.8, 113.0, 51.6, 36.8, 22.6.

HRMS (ESI) calculated for C42H38N7O10 ($M-H^+$) 800.2686, found 800.2691.

(S)-4-(4-(4-(4-Amino-2-(4-(2-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (44)



Chemical Formula: $C_{42}H_{37}N_7O_{12}$
Exact Mass: 831,2500

Amine **35** (20 mg, 0.026 mmol) was coupled with 2-nitrobenzoic acid using coupling conditions B followed by final deprotection.

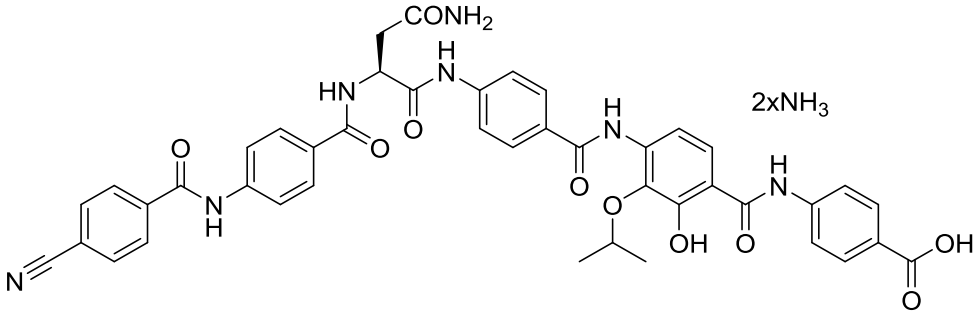
Desired compound was purified by preparative RP-HPLC condition B, to obtain 2.4 mg of desired product as a white solid (0.003 mmol, y= 11%).

^{1}H NMR (700 MHz, DMSO) δ 12.72 (br, 1H), 12.30 (br, 1H), 10.91 (s, 1H), 10.45 (s, 1H), 9.29 (br, 1H), 8.67 (d, *J* = 7.3 Hz, 1H), 8.17 (dd, *J* = 8.2, 0.8 Hz, 1H), 7.93 (t, *J* = 8.8 Hz, 6H), 7.89 (td, *J* = 7.5, 0.9 Hz, 1H), 7.85 – 7.80 (m, 6H), 7.80 – 7.75 (m, 4H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 13.9, 7.3 Hz, 1H), 4.64 (br, 1H), 2.69 (dd, *J* = 6.8, 3.4 Hz, 2H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.2, 166.9, 165.7, 164.4, 164.0, 146.4, 142.4, 141.6, 136.6, 134.2, 132.4, 131.1, 130.3, 129.3, 129.0, 128.5, 128.1, 124.3, 123.0, 118.9, 118.7, 51.6, 36.8, 22.4.

HRMS (ESI) calculated for C42H36N7O12 (M-H^{+}) 830.2427, found 830.2439.

(S)-4-(4-(4-(4-Amino-2-(4-(4-cyanobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (46)

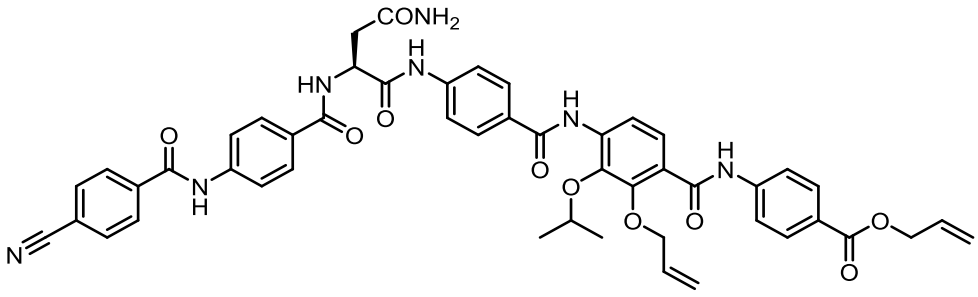


Chemical Formula: $C_{43}H_{37}N_7O_{10}$
Exact Mass: 811,2602

Amine **35** (25 mg, 0.033 mmol) was coupled with 4-cyanobenzoic acid using coupling conditions A. Allyl protected intermediate was purified on silica gel with a gradient 0-10% MeOH in DCM to give 18 mg (0.02 mmol, y= 61%) of allyl protected cystobactamid derivative.

Final deprotection afforded the desired compound, which was purified by preparative RP-HPLC using condition B to obtain 3.3 mg of desired product (0.004 mmol, y= 20%).

Allyl protected intermediate:



Chemical Formula: $C_{49}H_{45}N_7O_{10}$
Exact Mass: 891,3228

^{1}H NMR (700 MHz, DMSO) δ 10.70 (s, 1H), 10.57 (s, 1H), 10.45 (s, 1H), 9.52 (s, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.13 (d, *J* = 8.4 Hz, 2H), 8.05 (d, *J* = 8.4 Hz, 2H), 7.98 (dd, *J* = 8.7, 6.9 Hz, 4H), 7.93 (d, *J* = 8.8 Hz, 2H), 7.88 (dd, *J* = 15.2, 8.7 Hz, 4H), 7.81 (dd, *J* = 8.6, 5.6 Hz, 3H), 7.40 (d, *J* = 8.4 Hz, 2H), 6.99 (s, 1H), 6.09 – 5.98 (m, 2H), 5.43 – 5.34 (m, 2H), 5.30 – 5.18 (m, 2H), 4.92 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.79 (d, *J* = 5.3 Hz, 2H), 4.61 (d, *J* = 5.4 Hz, 2H), 4.49 (dt, *J* = 12.3, 6.1 Hz, 1H), 2.69 (d, *J* = 7.5 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 165.8, 165.0, 164.7, 164.5, 164.3, 149.5, 143.5, 142.6, 142.4, 141.6, 138.7, 135.7, 133.7, 132.8, 132.5, 130.3, 129.1, 128.6, 128.4, 128.3, 127.1, 124.2, 123.6, 119.5, 119.0, 118.8, 117.8, 117.8, 114.0, 76.3, 74.3, 64.8, 51.6, 36.8, 22.3.

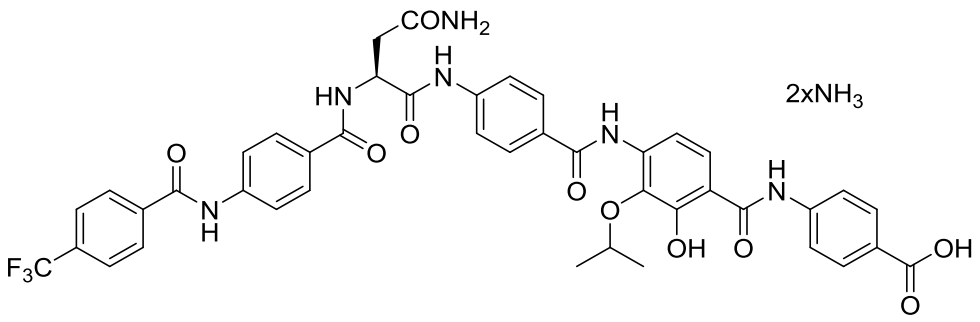
Final compound 46:

^{1}H NMR (500 MHz, DMSO) δ 12.81 (br, 1H), 12.29 (br, 1H), 10.70 (s, 1H), 10.61 (br, 1H), 10.46 (s, 1H), 9.37 (br, 1H), 8.67 (d, *J* = 7.3 Hz, 1H), 8.15 – 8.10 (m, 2H), 8.07 – 8.03 (m, 2H), 7.98 - 7.93 (m, 4H), 7.93 – 7.87 (m, 4H), 7.83 (dd, *J* = 16.0, 8.8 Hz, 5H), 7.67 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (q, *J* = 7.1 Hz, 1H), 4.56 (br, 1H), 2.69 (d, *J* = 7.2 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.4, 166.9, 165.8, 164.5, 164.1, 142.5, 141.6, 138.7, 136.4, 132.5, 130.2, 129.1, 128.6, 128.3, 128.3, 122.9, 120.6, 119.5, 118.9, 118.3, 114.0, 74.8, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C43H36N7O10 (M-H^{+}) 810.2529, found 810.2538.

(S)-4-(4-(4-(4-Amino-4-oxo-2-(4-(4-(trifluoromethyl)benzamido)benzamido)butanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (47)



Chemical Formula: $C_{43}H_{37}F_3N_6O_{10}$
Exact Mass: 854,2523

Amine **35** (15 mg, 0.020 mmol) was coupled with 4-trifluoromethyl benzoic acid using coupling conditions A followed by final deprotection.

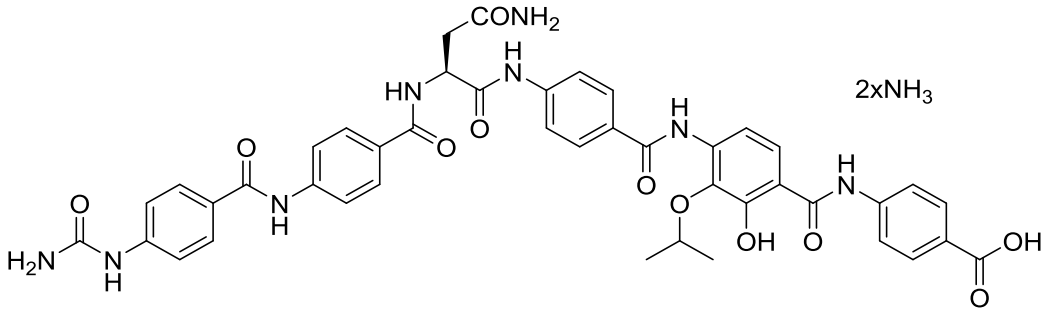
Desired compound purified by preparative RP-HPLC using condition B, to obtain 5.3 mg of desired product as a white solid (0.0062 mmol, y= 31%).

^{1}H NMR (700 MHz, DMSO) δ 12.80 (br, 1H), 12.29 (br, 1H), 10.69 (s, 1H), 10.62 (br, 1H), 10.46 (s, 1H), 9.37 (br, 1H), 8.67 (d, *J* = 7.3 Hz, 1H), 8.17 (d, *J* = 8.1 Hz, 2H), 7.98 – 7.92 (m, 8H), 7.90 (d, *J* = 8.9 Hz, 2H), 7.85 (d, *J* = 8.7 Hz, 2H), 7.82 (d, *J* = 8.8 Hz, 3H), 7.67 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (q, *J* = 7.2 Hz, 1H), 4.57 (br, 1H), 2.69 (d, *J* = 7.6 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.4, 166.9, 165.8, 164.7, 164.1, 142.5, 141.7, 138.5, 136.9, 136.4, 131.6, 131.4, 130.2, 129.1, 128.7, 128.3, 128.3, 125.4, 125.4, 124.7, 123.1, 122.9, 120.6, 119.5, 118.9, 74.7, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C43H36F3N6O10 (M-H$^+$) 853.2450, found 853.2438.

(S)-4-(4-(4-(4-Amino-4-oxo-2-(4-(4-ureidobenzamido)benzamido)butanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (45)



Chemical Formula: $C_{43}H_{40}N_8O_{11}$
Exact Mass: 844,2817

Amine **35** (20 mg, 0.026 mmol) was coupled with carboxylic acid **56** using coupling conditions C followed by final deprotection.

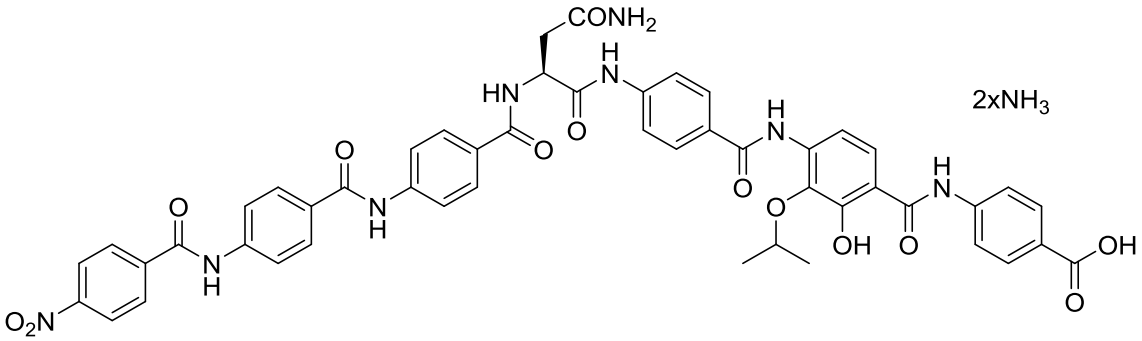
Desired compound purified by preparative RP-HPLC condition B to obtain 6.2 mg of desired product as a white solid (0.0073 mmol, y= 28%).

^{1}H NMR (700 MHz, DMSO) δ 12.55 (br, 1H), 10.44 (s, 1H), 10.26 (s, 1H), 9.15 (br, 1H), 8.89 (s, 1H), 8.63 (d, *J* = 7.3 Hz, 1H), 7.93 – 7.87 (m, 10H), 7.81 (d, *J* = 8.8 Hz, 4H), 7.66 (br, 1H), 7.56 – 7.52 (m, 2H), 7.39 (s, 1H), 6.99 (s, 1H), 6.01 (s, 2H), 4.92 (dd, *J* = 14.0, 7.3 Hz, 1H), 4.77 (br, 1H), 2.69 (d, *J* = 8.2 Hz, 2H), 1.23 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 167.9, 167.0, 165.9, 165.3, 163.8, 155.7, 144.0, 142.3, 142.3, 130.3, 128.8, 128.4, 128.2, 128.0, 126.6, 123.2, 119.2, 119.0, 116.6, 51.6, 36.8, 22.5.

HRMS (ESI) calculated for C43H39N8O11 (M-H$^+$) 843.2744, found 843.2759.

(S)-4-(4-(4-(4-Amino-2-(4-(4-(4-nitrobenzamido)benzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (48)



Chemical Formula: $C_{49}H_{42}N_8O_{13}$
Exact Mass: 950,2871

Amine **35** (15 mg, 0.020 mmol) coupled with carboxylic acid **89** using coupling conditions C followed by final deprotection.

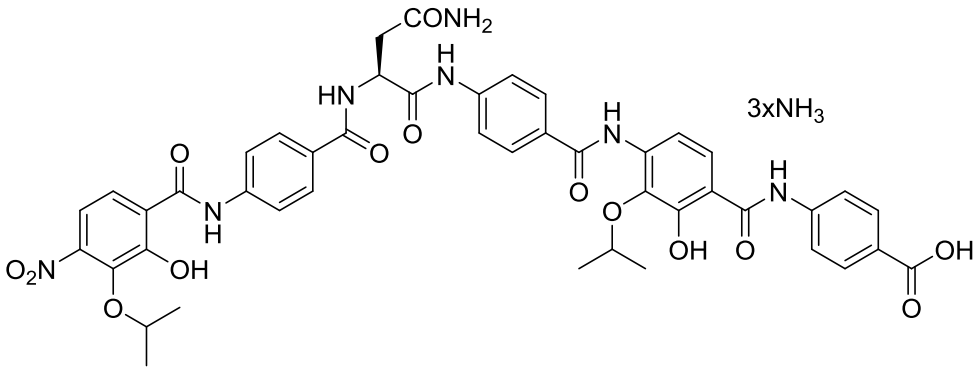
Desired compound was purified by preparative RP-HPLC condition B, to obtain 3.9 mg of desired product as a white solid (0.0041 mmol, y= 20%).

^{1}H NMR (700 MHz, DMSO) δ 12.61 (br, 1H), 12.32 (br, 1H), 10.84 (s, 1H), 10.44 (s, 1H), 10.41 (s, 1H), 9.16 (br, 1H), 8.65 (d, *J* = 7.2 Hz, 1H), 8.42 – 8.37 (m, 2H), 8.25 – 8.19 (m, 2H), 8.04 (d, *J* = 8.7 Hz, 2H), 7.96 (d, *J* = 8.8 Hz, 2H), 7.94 – 7.86 (m, 9H), 7.82 (d, *J* = 8.7 Hz, 4H), 7.67 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.93 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.76 (br, 1H), 2.69 (d, *J* = 7.7 Hz, 2H), 1.23 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 167.0, 165.8, 165.1, 164.3, 163.7, 149.3, 142.3, 142.1, 141.9, 140.3, 130.3, 129.8, 129.3, 128.7, 128.6, 128.3, 128.0, 123.6, 123.2, 119.6, 119.3, 119.0, 51.6, 36.8, 22.5.

HRMS (ESI) calculated for C49H41N8O13 (M-H$^+$) 949.2799, found 949.2848.

(S)-4-(4-(4-(4-Amino-2-(4-(2-hydroxy-3-isopropoxy-4-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (49)

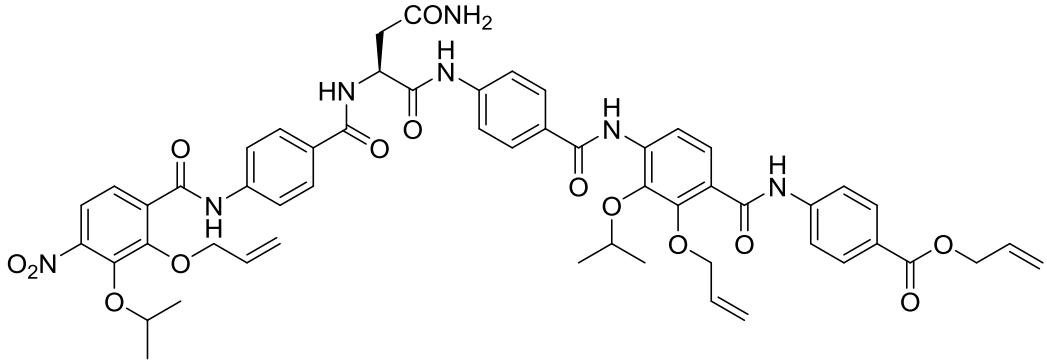


Chemical Formula: $C_{45}H_{43}N_7O_{14}$
Exact Mass: 905,2868

Amine **35** (40 mg, 0.052 mmol) was coupled with carboxylic acid **3** using coupling conditions A, the intermediate was purified on silica gel with a gradient 0-10% MeOH in DCM to give 40 mg (0.095 mmol, y= 87%) of allyl protected cystobactamid derivative.

Followed final deprotection on 15 mg of intermediate (0.015 mmol), the desired compound was purified by preparative RP-HPLC using condition B to obtain 2.5 mg of desired product (0.0028 mmol, y= 18%) and 1.2 mg of compound 50, which was formed as a by-product of the reaction.

Allyl protected intermediate:



Chemical Formula: $C_{54}H_{55}N_7O_{14}$
Exact Mass: 1025,3807

^{1}H NMR (700 MHz, DMSO) δ 10.66 (s, 1H), 10.58 (s, 1H), 10.44 (s, 1H), 9.52 (s, 1H), 8.66 (d, *J* = 7.3 Hz, 1H), 7.98 (dd, *J* = 8.7, 6.6 Hz, 4H), 7.92 (d, *J* = 8.8 Hz, 2H), 7.87 (d, *J* = 8.6 Hz, 2H), 7.83 – 7.76 (m, 5H), 7.72 (d, *J* = 8.4 Hz, 1H), 7.46 (d, *J* = 8.4 Hz, 1H), 7.40 (d, *J* = 8.4 Hz, 2H), 6.99 (s, 1H), 6.09 – 5.93 (m, 3H), 5.43 - 5.36 (m, 2H), 5.36 - 5.26 (m, 2H), 5.22 – 5.16 (m, 2H), 4.92 (dd, *J* = 13.9, 7.4 Hz, 1H), 4.81 – 4.78 (m, 2H), 4.70 – 4.65 (m, 1H), 4.62 – 4.59 (m, 4H), 4.52 – 4.46 (m, 1H), 2.71 – 2.67 (m, 1H), 1.26 (d, *J* = 6.1 Hz, 6H), 1.24 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 165.7, 164.9, 164.7, 164.3, 163.7, 149.9, 149.5, 146.8, 143.5, 143.4, 142.6, 142.4, 141.4, 136.1, 135.7, 133.7, 133.1, 132.8, 130.3, 129.1,

128.5, 128.4, 128.3, 127.1, 124.2, 123.6, 123.2, 119.2, 119.0, 118.8, 118.8, 118.3, 117.8, 117.8, 77.2, 76.2, 74.6, 74.3, 64.8, 51.6, 36.8, 22.3, 22.1.

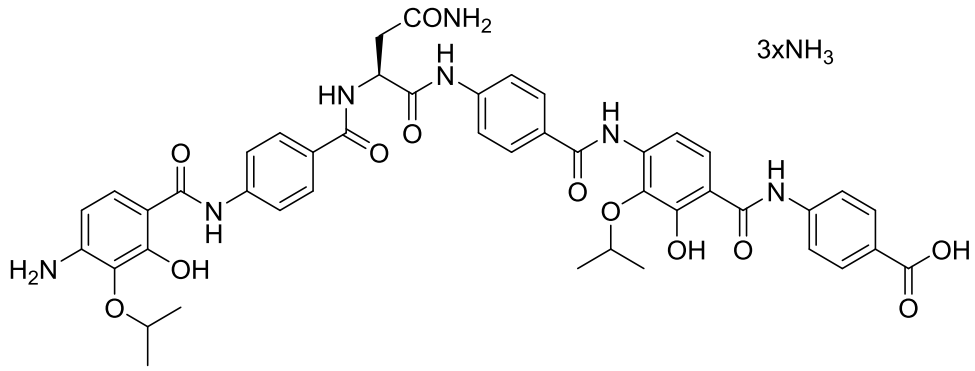
Final compound 49:

^{1}H NMR (700 MHz, DMSO) δ 12.82 (br, 1H), 12.29 (br, 1H), 10.61 (br, 1H), 10.43 (s, 1H), 9.40 (s, 1H), 8.60 (d, *J* = 7.2 Hz, 1H), 7.96 (dd, *J* = 14.7, 8.7 Hz, 4H), 7.91-7.75 (m, 9H), 7.69 (d, *J* = 8.7 Hz, 1H), 7.59 (d, *J* = 8.3 Hz, 1H), 7.40 (s, 1H), 6.98 (s, 1H), 6.48 (br, 1H), 5.10 (br, 1H), 4.91 (dd, *J* = 13.9, 7.2 Hz, 1H), 4.54 (dt, *J* = 11.9, 5.9 Hz, 1H), 2.69 (dd, *J* = 6.7, 3.2 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H), 1.14 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.8, 168.5, 166.3, 166.0, 164.2, 154.1, 147.2, 142.5, 137.0, 136.4, 130.2, 128.5, 128.3, 123.4, 122.8, 120.9, 120.7, 118.9, 118.8, 112.5, 112.2, 74.8, 51.6, 36.8, 22.3, 22.2.

HRMS (ESI) calculated for C45H44N7O14 ($M+H^{+}$) 906.2941, found 906.2940.

(S)-4-(4-(4-(4-Amino-2-(4-(4-amino-2-hydroxy-3-isopropoxybenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (50)



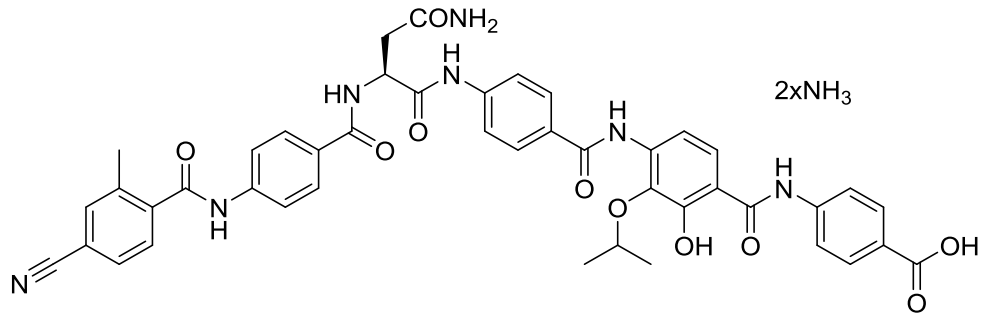
Chemical Formula: $C_{45}H_{45}N_7O_{12}$
Exact Mass: 875,3126

^{1}H NMR (700 MHz, DMSO) δ 12.82 (br, 1H), 12.63 (s, 1H), 12.28 (br, 1H), 10.61 (br, 1H), 10.45 (s, 1H), 10.11 (s, 1H), 9.39 (s, 1H), 8.65 (d, *J* = 7.3 Hz, 1H), 7.95 (dd, *J* = 12.0, 8.7 Hz, 4H), 7.89 (d, *J* = 8.7 Hz, 2H), 7.83 (dd, *J* = 24.7, 8.7 Hz, 4H), 7.78 (d, *J* = 8.8 Hz, 2H), 7.68 (br, 2H), 7.59 (d, *J* = 9.0 Hz, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 6.26 (d, *J* = 8.8 Hz, 1H), 5.63 (s, 2H), 4.92 (dd, *J* = 14.0, 7.3 Hz, 1H), 4.55 (br, 1H), 4.46 (dt, *J* = 12.3, 6.1 Hz, 1H), 2.70 – 2.67 (m, 2H), 1.26 (d, *J* = 6.1 Hz, 6H), 1.22 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 169.4, 168.4, 166.9, 165.9, 164.2, 155.7, 148.1, 142.5, 142.0, 141.3, 137.0, 136.4, 130.2, 129.6, 128.8, 128.3, 128.3, 128.2, 123.6, 122.9, 120.6, 120.3, 118.9, 105.3, 103.4, 72.8, 51.6, 36.8, 22.3, 22.2.

HRMS (ESI) calculated for C45H46N7O12 ($M+H^+$) 876.3199, found 876.3200.

(S)-4-(4-(4-(4-Amino-2-(4-(4-cyano-2-methylbenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (74)



Chemical Formula: $C_{44}H_{39}N_7O_{10}$
Exact Mass: 825,2758

Amine **35** (20 mg, 0.026 mmol) was coupled with 4-Cyano-2-methylbenzoic acid using coupling conditions A followed by final deprotection.

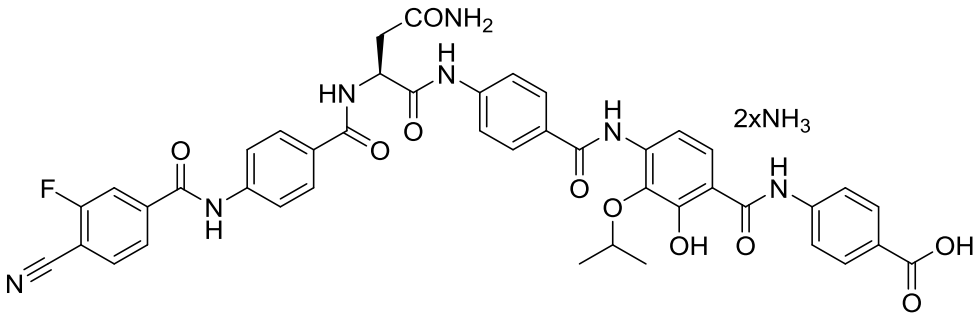
The desired compound was purified by preparative RP-HPLC using condition B to obtain 4.5 mg of desired product as a white solid (0.005 mmol, y= 21%).

^{1}H NMR (700 MHz, DMSO) δ 12.75 (br, 1H), 12.29 (br, 1H), 10.73 (s, 1H), 10.46 (s, 1H), 9.31 (br, 1H), 8.66 (d, *J* = 7.3 Hz, 1H), 7.93 (m, 6H), 7.82 (m, 7H), 7.78 (br, 1H), 7.69 (d, *J* = 7.9 Hz, 1H), 7.65 – 7.55 (br, 2H), 7.39 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.63 (br, 1H), 2.69 (d, *J* = 8.1 Hz, 2H), 2.42 (s, 3H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.3, 166.9, 166.6, 165.7, 164.0, 142.4, 141.6, 141.1, 136.8, 136.5, 134.0, 130.2, 129.7, 129.0, 128.4, 128.2, 123.0, 120.3, 118.9, 118.9, 118.4, 112.3, 51.6, 36.8, 22.4, 18.8.

HRMS (ESI) calculated for C44H38N7O10 ($M-H^+$) 824.2686, found 824.2705.

(S)-4-(4-(4-(4-Amino-2-(4-(4-cyano-3-fluorobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (76)



Chemical Formula: $C_{43}H_{36}FN_7O_{10}$
Exact Mass: 829,2508

Amine **35** (20 mg, 0.026 mmol) was coupled with 4-Cyano-3-fluorobenzoic acid using coupling conditions A followed by final deprotection.

Desired compound was purified by preparative RP-HPLC condition B, to obtain 5.7 mg of desired product as a white solid (0.007 mmol, y= 27%).

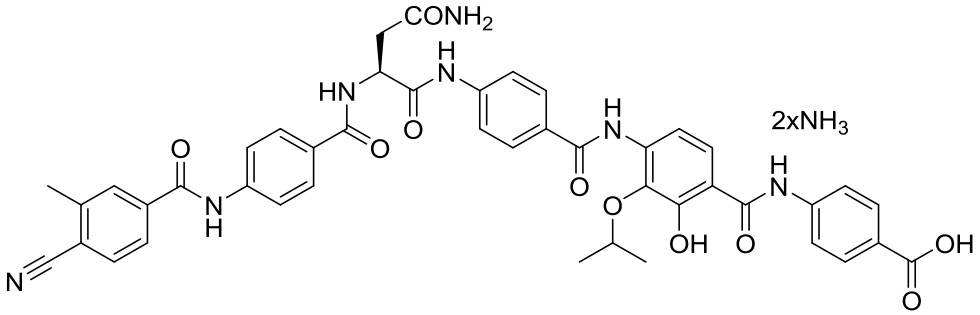
^{1}H NMR (500 MHz, DMSO) δ 12.78 (br, 1H), 12.29 (br, 1H), 10.74 (s, 1H), 10.46 (s, 1H), 9.35 (br, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.15 (dd, *J* = 8.0, 6.7 Hz, 1H), 8.08 (dd, *J* = 10.1, 1.5 Hz, 1H), 7.97 (m, 2H), 7.94 (d, *J* = 8.7 Hz, 5H), 7.88 (d, *J* = 8.9 Hz, 2H), 7.83 (dd, *J* = 15.2, 8.8 Hz, 5H), 7.64 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.1, 7.1 Hz, 1H), 4.59 (br, 1H), 2.69 (d, *J* = 7.2 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.2, 170.7, 168.4, 166.9, 165.7, 164.1, 161.5, 142.5, 141.5, 141.5, 141.3, 136.4, 134.3, 129.3, 128.4, 128.2, 124.7, 124.7, 122.9, 120.5, 119.6, 118.9, 115.8, 115.7, 113.6, 102.9, 102.8, 51.6, 36.8, 22.3.

^{19}F NMR (471 MHz, DMSO) δ -107.68 (dd, *J* = 9.8, 6.8 Hz).

HRMS (ESI) calculated for C43H35FN7O10 (M-H^{+}) 828.2435, found 28.2447.

(S)-4-(4-(4-(4-Amino-2-(4-(4-cyano-3-methylbenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (75)



Chemical Formula: $C_{44}H_{39}N_7O_{10}$
Exact Mass: 825,2758

Amine **35** (20 mg, 0.026 mmol) was coupled with 4-Cyano-3-methylbenzoic acid using coupling conditions A followed by final deprotection.

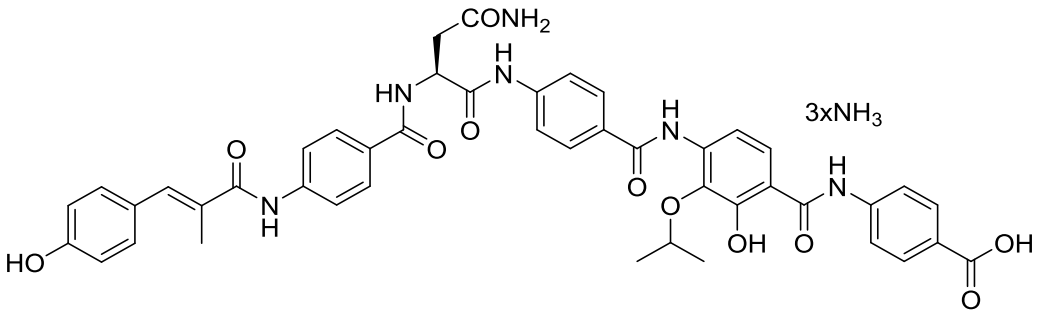
The desired compound was purified by preparative RP-HPLC using condition B to obtain 5.2 mg of desired product as a white solid (0.006 mmol, y= 24%).

^{1}H NMR (700 MHz, DMSO) δ 12.77 (br, 1H), 12.29 (br, 1H), 10.66 (s, 1H), 10.46 (s, 1H), 9.34 (br, 1H), 8.67 (d, *J* = 7.3 Hz, 1H), 8.02 (s, 1H), 7.98 – 7.90 (m, 8H), 7.90 – 7.87 (m, 2H), 7.87 – 7.76 (m, 5H), 7.67 – 7.58 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.60 (br, 1H), 2.69 (d, *J* = 7.5 Hz, 2H), 2.59 (s, 3H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.3, 166.9, 165.8, 164.6, 164.1, 142.4, 141.9, 141.6, 138.6, 136.5, 132.8, 132.6, 130.2, 129.4, 129.2, 129.1, 128.3, 128.2, 125.8, 125.4, 122.9, 120.4, 119.5, 118.9, 117.5, 114.4, 51.6, 36.8, 22.3, 20.0.

HRMS (ESI) calculated for C44H38N7O10 (M-H$^+$) 824.2686, found 824.2694.

(S,E)-4-(4-(4-(4-Amino-2-(4-(3-(4-hydroxyphenyl)-2-methylacrylamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (67)

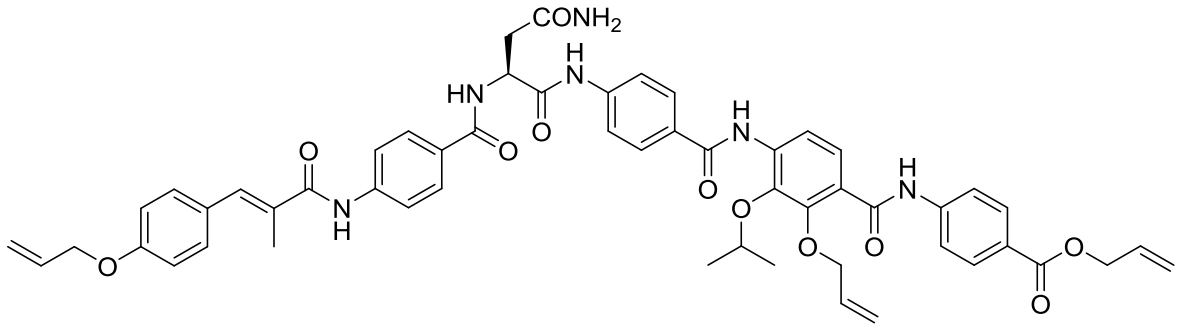


Chemical Formula: $C_{45}H_{42}N_6O_{11}$
Exact Mass: 842,2912

Amine **35** (40 mg, 0.052 mmol) was coupled with carboxylic acid **58** coupling conditions A, the intermediate was purified on silica gel with a gradient 0-10% MeOH in DCM to give 50 mg (0.052 mmol, y= q.) of allyl protected cystobactamid derivative.

Followed final deprotection on 15 mg of intermediate (0.016 mmol), the desired compound was purified by preparative RP-HPLC using condition B to obtain 1.0 mg of desired product (0.0012 mmol, y= 7%).

Allyl protected intermediate:



Chemical Formula: $C_{54}H_{54}N_6O_{11}$
Exact Mass: 962,3851

^{1}H NMR (700 MHz, DMSO) δ 10.58 (s, 1H), 10.44 (s, 1H), 10.12 (s, 1H), 9.52 (s, 1H), 8.62 (d, *J* = 7.3 Hz, 1H), 7.98 (dd, *J* = 8.8, 7.0 Hz, 4H), 7.90 – 7.85 (m, 4H), 7.85 – 7.79 (m, 5H), 7.45 (d, *J* = 8.8 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 2H), 7.30 (s, 1H), 7.06 – 7.01 (m, 2H), 6.99 (s, 1H), 6.09 - 5.98 (m, 3H), 5.44 – 5.35 (m, 3H), 5.28 (dq, *J* = 10.5, 1.4 Hz, 2H), 5.20 (ddd, *J* = 10.5, 2.9, 1.3 Hz, 1H), 4.91 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.79 (dd, *J* = 4.0, 1.4 Hz, 2H), 4.63 - 4.60 (m, 4H), 4.49 (dt, *J* = 12.3, 6.2 Hz, 1H), 3.85 – 3.73 (m, 1H), 2.69 (d, *J* = 7.8 Hz, 2H), 2.12 (d, *J* = 1.3 Hz, 3H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.8, 168.7, 165.9, 165.0, 164.7, 164.3, 158.0, 149.5, 143.5, 142.6, 142.4, 142.3, 135.7, 133.7, 133.6, 133.3, 13 2.8, 131.1, 130.7, 130.3, 128.4, 128.3, 128.2, 127.1, 124.2, 123.6, 119.1, 119.0, 118.8, 117.8, 117.8, 117.6, 114.7, 107.0, 97.2, 76.3, 74.3, 68.2, 64.8, 51.6, 36.8, 22.3, 14.5.

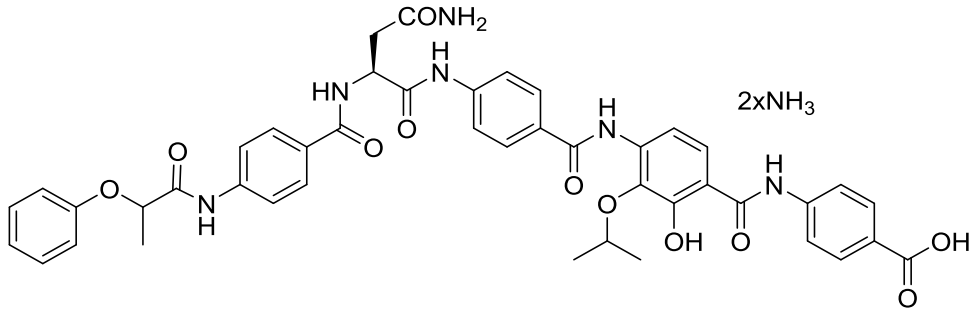
Final compound 67:

^{1}H NMR (700 MHz, DMSO) δ 12.79 (br, 1H), 12.29 (br, 1H), 10.60 (br, 1H), 10.45 (s, 1H), 10.08 (s, 1H), 9.76 (s, 1H), 9.38 (br, 1H), 8.61 (d, *J* = 7.2 Hz, 1H), 7.98 – 7.90 (m, 4H), 7.90 – 7.76 (m, 10H), 7.39 (s, 1H), 7.35 (d, *J* = 8.7 Hz, 2H), 7.26 (s, 1H), 6.99 (s, 1H), 6.84 (d, *J* = 8.6 Hz, 2H), 4.91 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.56 (br, 1H), 2.68 (d, *J* = 7.5 Hz, 2H), 2.11 (d, *J* = 1.2 Hz, 3H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.8, 168.8, 166.9, 165.9, 164.1, 162.3, 162.2, 157.5, 142.4, 133.8, 131.3, 130.2, 129.5, 128.2, 127.3, 126.6, 122.8, 120.6, 119.0, 118.9, 115.4, 51.6, 36.8, 22.3, 14.5.

HRMS (ESI) calculated for C45H41N6O11 (M-H$^+$) 841,2839, found 841,2844.

4-(4-(4-((2S)-4-Amino-4-oxo-2-(4-(2-phenoxypropanamido)benzamido)butanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (73)

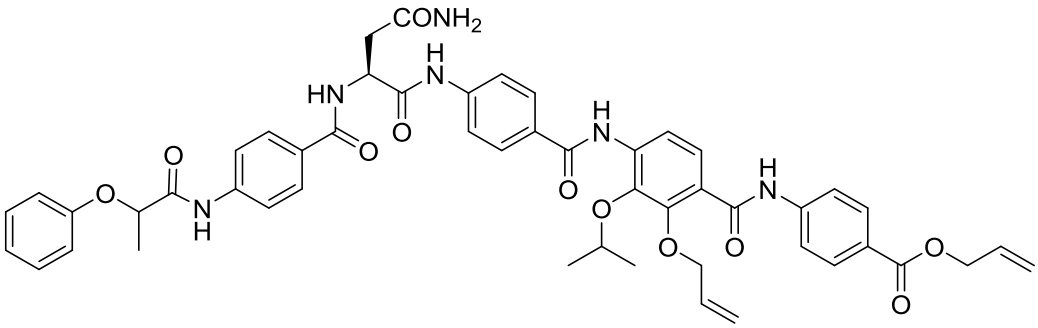


Chemical Formula: $C_{44}H_{42}N_6O_{11}$
Exact Mass: 830,2912

Amine **35** (40 mg, 0.052 mmol) was coupled with 2-phenoxypropanoic acid using coupling conditions A, the intermediate was purified on silica gel with a gradient 0-10% MeOH in DCM to give 30 mg (0.033 mmol, y= 63%) of allyl protected cystobactamid derivative.

Followed final deprotection on 15 mg of intermediate (0.016 mmol), the desired compound was purified by preparative RP-HPLC using condition B to obtain 7.5 mg of desired product (0.009 mmol, y= 56%).

Allyl protected intermediate:



Chemical Formula: $C_{50}H_{50}N_6O_{11}$
Exact Mass: 910,3538

^{1}H NMR (500 MHz, Acetone) δ 10.25 (s, 1H), 10.02 (s, 1H), 9.52 (s, 1H), 8.99 (s, 1H), 8.40 (dd, *J* = 8.8, 2.3 Hz, 1H), 8.37 (d, *J* = 7.5 Hz, 1H), 8.08 – 8.03 (m, 2H), 8.00 – 7.96 (m, 2H), 7.94 – 7.88 (m, 4H), 7.88 – 7.82 (m, 5H), 7.34 – 7.29 (m, 2H), 7.15 (s, 1H), 7.05 – 7.01 (m, 2H), 6.99

(tt, *J* = 7.4, 1.0 Hz, 1H), 6.54 (s, 1H), 6.27 - 6.17 (m, 1H), 6.14 – 6.04 (m, 1H), 5.50 (m, 2H), 5.32 (m, 2H), 5.10 – 5.05 (m, 1H), 4.89 (q, *J* = 6.7 Hz, 1H), 4.83 – 4.76 (m, 5H), 2.93 (qd, *J* = 15.9, 6.0 Hz, 2H), 1.61 (d, *J* = 6.7 Hz, 3H), 1.39 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (176 MHz, Acetone) δ 173.7, 171.5, 171.0, 167.2, 166.0, 165.0, 163.9, 158.3, 150.7, 144.4, 143.6, 142.6, 140.8, 138.6, 138.3, 134.1, 133.9, 131.5, 130.6, 130.4, 130.2, 130.1, 129.2, 126.9, 126.2, 123.8, 122.7, 122.0, 120.2, 120.1, 120.0, 119.6, 118.1, 116.7, 116.6, 116.6, 115.9, 77.5, 75.9, 75.6, 65.9, 52.5, 37.1, 22.9, 18.8.

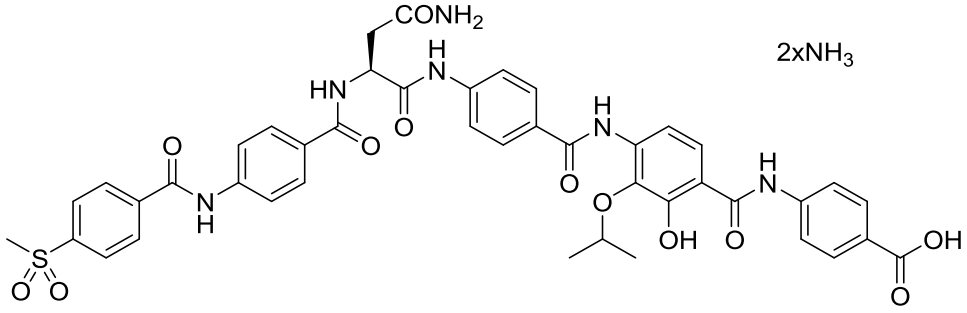
Final compound 73:

^{1}H NMR (700 MHz, DMSO) δ 12.76 (br, 1H), 12.29 (br, 1H), 10.71 (br, 1H), 10.44 (s, 1H), 10.36 (s, 1H), 9.33 (br, 1H), 8.62 (d, *J* = 7.3 Hz, 1H), 7.94 (dd, *J* = 15.5, 8.6 Hz, 4H), 7.85 (dd, *J* = 16.2, 8.7 Hz, 4H), 7.80 (d, *J* = 8.7 Hz, 3H), 7.74 (d, *J* = 8.8 Hz, 2H), 7.62 (br, 1H), 7.38 (s, 1H), 7.30 (dd, *J* = 8.6, 7.4 Hz, 2H), 6.96 (dd, *J* = 13.2, 7.6 Hz, 4H), 4.93 – 4.87 (m, 2H), 4.60 (br, 1H), 2.69 – 2.65 (m, 2H), 1.56 (d, *J* = 6.6 Hz, 3H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 170.9, 170.3, 170.1, 168.0, 166.6, 165.3, 163.7, 156.8, 142.1, 140.9, 136.1, 129.9, 129.2, 128.4, 128.0, 127.8, 122.6, 120.9, 120.0, 118.5, 118.4, 114.7, 73.3, 51.2, 36.4, 22.0, 18.2.

HRMS (ESI) calculated for C44H41N6O11 (M-H^{+}) 829.2839, found 829.2830.

(S)-4-(4-(4-(4-Amino-2-(4-(4-(methylsulfonyl)benzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (51)



Chemical Formula: $C_{43}H_{40}N_6O_{12}S$
Exact Mass: 864,2425

Amine **35** (25 mg, 0.032 mmol) was coupled with 4-(methylsulfonyl)benzoic acid using coupling conditions A followed by final deprotection.

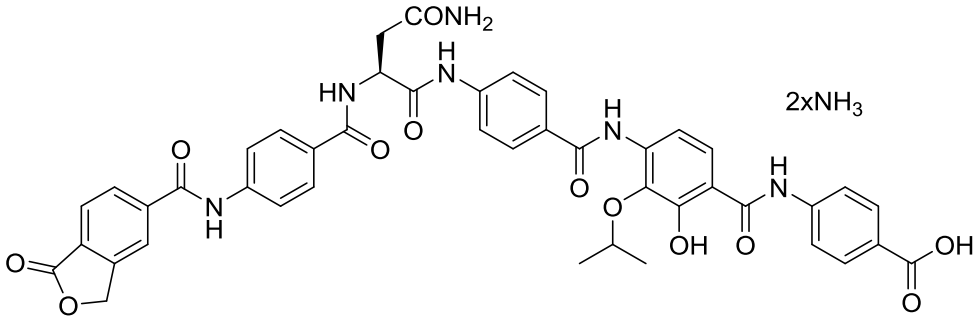
Desired compound was purified by preparative RP-HPLC using condition B to obtain 8.6 mg of desired product as a white solid (0.010 mmol, y= 31%).

^{1}H NMR (700 MHz, DMSO) δ 15.35 (s, 1H), 10.76 (s, 1H), 10.57 (s, 1H), 8.90 (s, 1H), 8.87 (s, 1H), 8.21 (d, *J* = 8.1 Hz, 2H), 8.09 (d, *J* = 8.1 Hz, 2H), 7.96 (d, *J* = 8.5 Hz, 2H), 7.90 (d, *J* = 8.4 Hz, 2H), 7.83 (s, 4H), 7.79 (d, *J* = 8.0 Hz, 2H), 7.58 (d, *J* = 8.0 Hz, 2H), 7.51 (s, 1H), 7.45 (d, *J* = 8.7 Hz, 1H), 7.08 (d, *J* = 8.7 Hz, 1H), 6.98 (s, 1H), 5.02 (dt, *J* = 12.0, 5.9 Hz, 1H), 4.92 (dd, *J* = 13.4, 7.2 Hz, 1H), 3.30 (s, 3H), 2.73 (dd, *J* = 14.8, 8.7 Hz, 1H), 2.68 (dd, *J* = 14.8, 5.1 Hz, 1H), 1.20 (d, *J* = 5.7 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 166.9, 165.7, 165.3, 164.6, 163.1, 143.3, 142.0, 141.8, 141.6, 139.2, 137.5, 134.0, 133.4, 129.7, 129.5, 129.2, 128.8, 128.4, 128.3, 127.6, 127.1, 127.0, 126.8, 123.7, 119.5, 119.0, 117.6, 116.0, 100.6, 70.3, 51.9, 43.3, 36.9, 22.7.

HRMS (ESI) calculated for C43H39N6O12S (M-H^{+}) 863.2352, found 863.2364.

(S)-4-(4-(4-(4-Amino-4-oxo-2-(4-(1-oxo-1,3-dihydroisobenzofuran-5-carboxamido)benzamido)butanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (52)



Chemical Formula: $C_{44}H_{38}N_6O_{12}$
Exact Mass: 842,2548

Amine **35** (25 mg, 0.032 mmol) was coupled with 1-oxo-1,3-dihydroisobenzofuran-5-carboxylic acid using coupling conditions A followed by final deprotection.

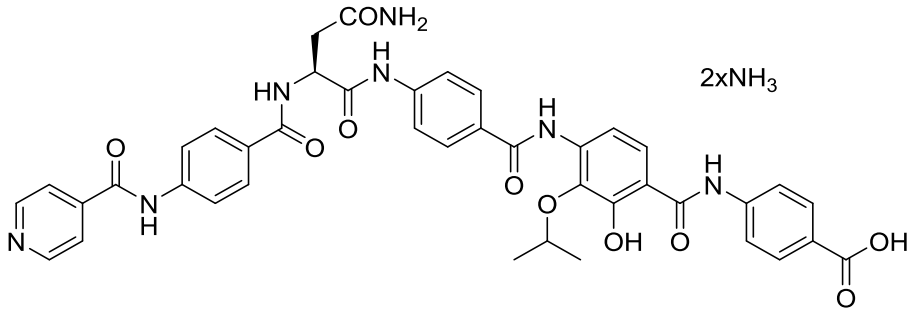
The desired compound was purified by preparative RP-HPLC using condition B to obtain 4.9 mg of desired product as a white solid (0.0058 mmol, y= 18%).

^{1}H NMR (700 MHz, DMSO) δ 15.33 (s, 1H), 10.81 (s, 1H), 10.56 (s, 1H), 8.87 (s, 2H), 8.23 (s, 1H), 8.14 (d, *J* = 8.1 Hz, 1H), 8.01 (d, *J* = 8.0 Hz, 1H), 7.95 (d, *J* = 8.8 Hz, 2H), 7.91 (d, *J* = 8.7 Hz, 2H), 7.83 (q, *J* = 9.1 Hz, 4H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.56 (d, *J* = 8.5 Hz, 2H), 7.51 (s, 1H), 7.44 (d, *J* = 8.7 Hz, 1H), 7.08 (d, *J* = 8.7 Hz, 1H), 6.98 (s, 1H), 5.51 (s, 2H), 5.05 – 5.00 (m, 1H), 4.92 (dd, *J* = 13.9, 7.6 Hz, 1H), 2.73 (dd, *J* = 15.1, 8.6 Hz, 1H), 2.68 (dd, *J* = 15.1, 5.5 Hz, 1H), 1.19 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 170.0, 166.8, 165.7, 165.3, 165.0, 163.1, 147.4, 142.0, 141.6, 140.0, 137.5, 134.0, 133.4, 131.2, 129.7, 129.5, 129.1, 128.5, 128.3, 128.1, 127.5, 127.4, 127.1, 125.0, 124.2, 123.7, 122.5, 120.0, 119.5, 119.0, 117.5, 116.0, 100.6, 70.3, 70.1, 51.9, 36.9, 22.7.

HRMS (ESI) calculated for C44H37N6O12 (M-H$^+$) 841.2475, found 841.2478.

(S)-4-(4-(4-(4-Amino-2-(4-(isonicotinamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (53)



Chemical Formula: $C_{41}H_{37}N_7O_{10}$
Exact Mass: 787,2602

Amine **35** (25 mg, 0.032 mmol) was coupled with isonicotinic acid using coupling conditions A followed by final deprotection.

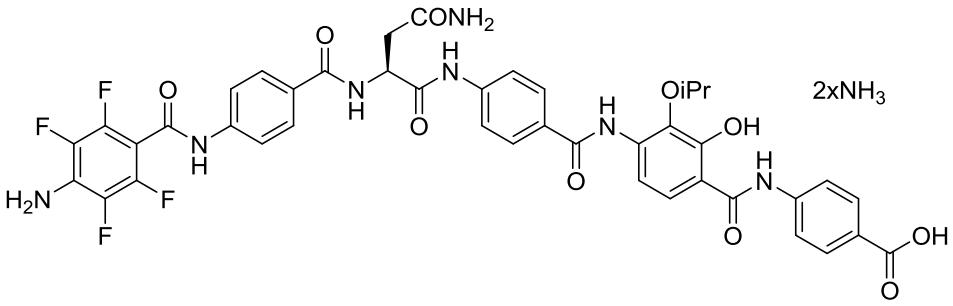
The desired compound was purified by preparative RP-HPLC using condition B to obtain 5.5 mg of desired product as a white solid (0.007 mmol, y= 22%).

^{1}H NMR (700 MHz, DMSO) δ 15.70 (br, 1H), 12.48 (br, 1H), 10.73 (s, 1H), 10.46 (s, 1H), 8.88 (s, 1H), 8.80 (d, *J* = 5.8 Hz, 2H), 8.73 (d, *J* = 7.0 Hz, 1H), 7.94 (d, *J* = 8.7 Hz, 2H), 7.91 – 7.86 (m, 4H), 7.86 – 7.77 (m, 6H), 7.70 (br, 2H), 7.45 (d, *J* = 8.7 Hz, 1H), 7.42 (s, 1H), 7.10 (d, *J* = 8.7 Hz, 1H), 6.98 (s, 1H), 5.01 (dt, *J* = 12.2, 6.0 Hz, 1H), 4.92 (q, *J* = 7.1 Hz, 1H), 2.69 (d, *J* = 7.0 Hz, 2H), 1.20 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 167.1, 165.7, 165.3, 164.3, 163.2, 150.3, 142.0, 141.7, 141.4, 137.5, 134.2, 130.0, 129.5, 129.3, 128.3, 127.6, 124.2, 123.7, 121.6, 119.5, 119.0, 117.9, 115.7, 100.9, 70.4, 51.7, 36.8, 22.7.

HRMS (ESI) calculated for C41H38N7O10 (M-H$^+$) 788.2675, found 788.2659.

(S)-4-(4-(4-(4-Amino-2-(4-(4-amino-2,3,5,6-tetrafluorobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (54)



Chemical Formula: $C_{42}H_{35}F_4N_7O_{10}$
Exact Mass: 873,2382

Amine **35** (25 mg, 0.033 mmol) was coupled with 4-azido-2,3,5,6-tetrafluorobenzoic acid using coupling conditions A followed by final deprotection.

Purification by preparative RP-HPLC following condition B was done to obtain 4.3 mg of amine 54 as a degradation byproduct of the desired azide (0.0069 mmol, y= 15%).

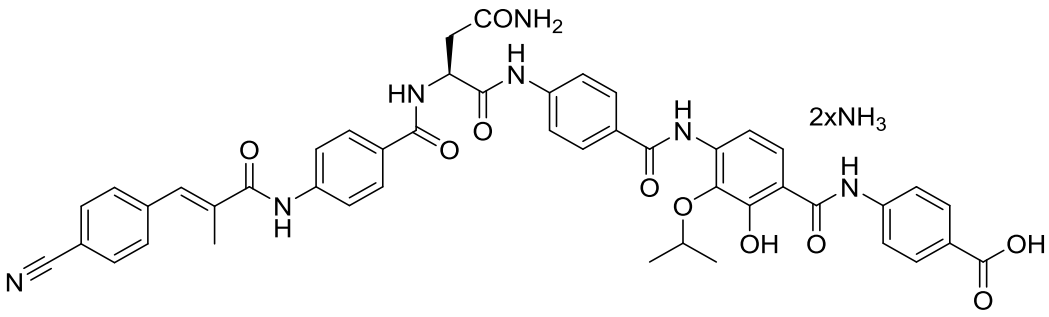
^{1}H NMR (500 MHz, DMSO) δ 12.64 (br, 2H), 10.83 (s, 1H), 10.45 (s, 1H), 9.23 (br, 1H), 8.67 (d, *J* = 7.3 Hz, 1H), 7.96 – 7.88 (m, 6H), 7.82 (dd, *J* = 8.7, 7.1 Hz, 4H), 7.76 (d, *J* = 8.7 Hz, 2H), 7.72 (br, 1H), 7.51 (br, 1H), 7.39 (s, 1H), 6.99 (s, 1H), 6.49 (s, 2H), 4.92 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.70 (br, 1H), 2.70 – 2.66 (m, 2H), 1.24 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.1, 167.0, 165.7, 163.9, 157.2, 144.4, 143.0, 142.3, 141.2, 136.7, 135.8, 134.3, 130.3, 129.2, 128.6, 128.1, 123.1, 119.9, 118.9, 118.6, 100.95 (t, *J* = 19.5 Hz, C), 51.6, 36.8, 22.4.

^{19}F NMR (471 MHz, DMSO) δ -145.12 (d, *J* = 16.0 Hz), -161.56 (d, *J* = 16.1 Hz).

HRMS (ESI) calculated for C42H36F4N7O10 ($M+H^+$) 874.2454, found 874.2458.

(S,E)-4-(4-(4-(4-Amino-2-(4-(3-(4-cyanophenyl)-2-methylacrylamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (68)



Chemical Formula: $C_{46}H_{41}N_7O_{10}$
Exact Mass: 851,2915

Amine **35** (25 mg, 0.032 mmol) was coupled with carboxylic acid **59** using coupling conditions A followed by final deprotection.

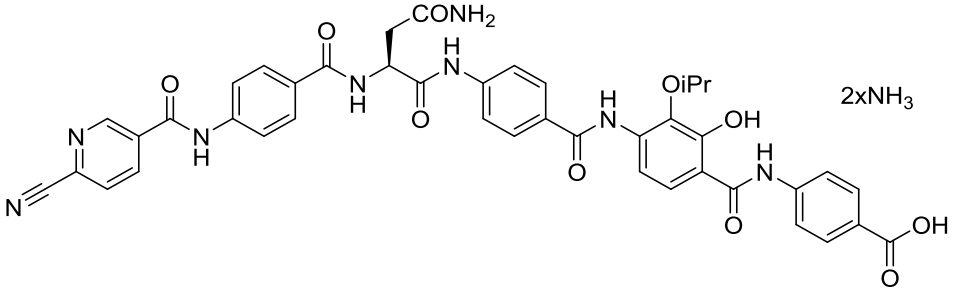
The desired compound was purified by preparative RP-HPLC using condition B to obtain 6.4 mg of desired product as a white solid (0.0075 mmol, y= 24%).

^{1}H NMR (700 MHz, DMSO) δ 12.80 (br, 1H), 12.29 (br, 1H), 10.65 (br, 1H), 10.47 (s, 1H), 10.26 (s, 1H), 9.37 (s, 1H), 8.64 (d, *J* = 7.2 Hz, 1H), 7.95 (dd, *J* = 11.5, 8.8 Hz, 4H), 7.91 (dd, *J* = 10.4, 8.6 Hz, 4H), 7.88 – 7.80 (m, 7H), 7.67 (d, *J* = 8.3 Hz, 3H), 7.40 (s, 1H), 7.37 (s, 1H), 6.99 (s, 1H), 4.92 (q, *J* = 7.1 Hz, 1H), 4.59 – 4.53 (m, 1H), 2.69 (d, *J* = 7.0 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.8, 168.4, 168.1, 166.9, 165.8, 164.1, 142.5, 142.1, 142.0, 140.6, 136.9, 136.4, 135.8, 132.4, 132.0, 131.7, 130.2, 130.1, 128.6, 128.3, 126.9, 126.1, 122.9, 120.6, 119.2, 118.9, 118.7, 112.5, 110.3, 74.7, 51.6, 36.8, 22.3, 14.6.

HRMS (ESI) calculated for C46H41N7NaO10 ($M+Na^{+}$) 874.2807, found 874.2814.

(S)-4-(4-(4-(4-Amino-2-(4-(6-cyanonicotinamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (80)



Chemical Formula: $C_{42}H_{36}N_8O_{10}$
Exact Mass: 812,2554

Amine **35** (25 mg, 0.033 mmol) was coupled with 6-cyanonicotinic acid using coupling conditions A followed by final deprotection.

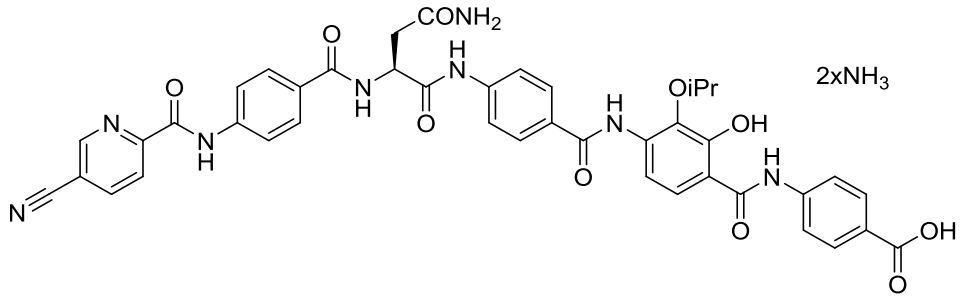
The desired compound was purified by preparative RP-HPLC using condition B to obtain 4.6 mg of desired product (0.0057 mmol, y= 17%).

^{1}H NMR (500 MHz, DMSO) δ 12.76 (br, 1H), 12.31 (br, 1H), 10.87 (s, 1H), 10.47 (s, 1H), 9.34 (br, 1H), 9.26 – 9.22 (m, 1H), 8.69 (d, *J* = 7.2 Hz, 1H), 8.54 (dd, *J* = 8.1, 2.2 Hz, 1H), 8.26 (d, *J* = 8.1 Hz, 1H), 7.95 (dt, *J* = 6.7, 3.2 Hz, 6H), 7.91 – 7.74 (m, 7H), 7.63 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.1, 7.1 Hz, 1H), 4.60 (s, 1H), 2.69 (d, *J* = 7.0 Hz, 2H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.2, 170.7, 168.3, 166.9, 165.7, 164.1, 163.0, 150.2, 142.4, 141.3, 137.3, 136.5, 134.5, 133.4, 130.2, 129.4, 128.8, 128.4, 128.2, 122.9, 120.4, 119.5, 118.9, 117.1, 74.4, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C42H35N8O10 (M-H$^+$) 811.2482, found 811.2480.

(S)-4-(4-(4-(4-Amino-2-(4-(5-cyanopicolinamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (81)



Chemical Formula: $C_{42}H_{36}N_8O_{10}$
Exact Mass: 812,2554

Amine **35** (25 mg, 0.033 mmol) was coupled with 5-cyanopicolinic acid using coupling conditions A followed by final deprotection.

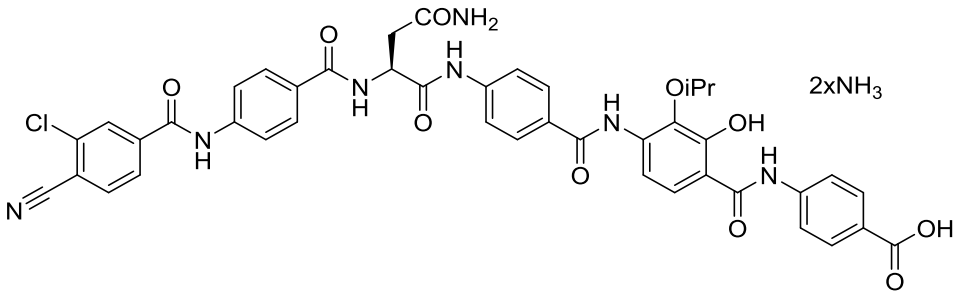
The desired compound was purified by preparative RP-HPLC using condition B to obtain 2.5 mg of desired product (0.0031 mmol, y= 9%).

^{1}H NMR (500 MHz, DMSO) δ 12.76 (br, 1H), 12.30 (br, 1H), 11.03 (s, 1H), 10.46 (s, 1H), 9.33 (br, 1H), 9.22 (d, *J* = 1.3 Hz, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.60 (dd, *J* = 8.2, 2.0 Hz, 1H), 8.31 (d, *J* = 8.1 Hz, 1H), 8.05 (d, *J* = 8.8 Hz, 2H), 8.00 – 7.89 (m, 6H), 7.83 (dd, *J* = 13.2, 8.8 Hz, 5H), 7.62 (s, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.60 (br, 1H), 2.69 (d, *J* = 7.3 Hz, 2H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.3, 166.9, 165.8, 164.0, 161.6, 152.3, 151.5, 142.4, 142.3, 140.9, 136.5, 130.2, 129.4, 128.3, 128.2, 122.9, 122.5, 120.3, 119.7, 118.9, 116.6, 111.7, 51.6, 36.8, 22.4.

HRMS (ESI) calculated for C42H35N8O10 (M-H$^+$) 811.2482, found 811.2461.

(S)-4-(4-(4-(4-Amino-2-(4-(3-chloro-4-cyanobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (77)



Chemical Formula: $C_{43}H_{36}ClN_7O_{10}$
Exact Mass: 845,2212

Amine **35** (25 mg, 0.033 mmol) was coupled with 3-chloro-4-cyanobenzoic acid using coupling conditions A followed by final deprotection.

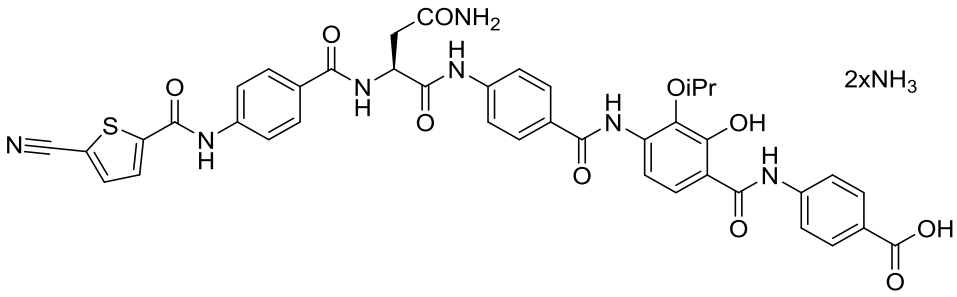
The desired compound was purified by preparative RP-HPLC using condition B to obtain 4.5 mg of desired product (0.0053 mmol, y= 16%).

^{1}H NMR (500 MHz, DMSO) δ 12.78 (br, 1H), 12.30 (br, 1H), 10.76 (s, 1H), 10.46 (s, 1H), 9.36 (br, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.29 (d, *J* = 1.6 Hz, 1H), 8.19 (d, *J* = 8.1 Hz, 1H), 8.07 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.98 – 7.91 (m, 5H), 7.91 – 7.78 (m, 6H), 7.65 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (q, *J* = 7.1 Hz, 1H), 4.57 (br, 1H), 2.69 (d, *J* = 7.1 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.2, 170.7, 168.4, 166.9, 165.7, 164.1, 163.1, 142.5, 141.3, 140.3, 136.4, 135.6, 134.9, 130.2, 129.3, 128.9, 128.4, 128.2, 127.3, 122.9, 120.5, 119.5, 118.9, 115.6, 114.5, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C43H37ClN7O10 ($M+H^+$) 846.2285, found 846.2297.

(S)-4-(4-(4-(4-Amino-2-(4-(5-cyanothiophene-2-carboxamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (82)



Chemical Formula: $C_{41}H_{35}N_7O_{10}S$
Exact Mass: 817,2166

Amine **35** (25 mg, 0.033 mmol) was coupled with 5-cyanothiophene-2-carboxylic acid using coupling conditions A followed by final deprotection.

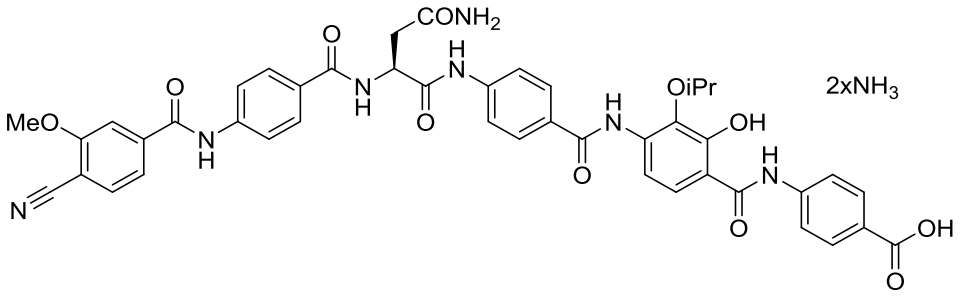
The desired compound was purified by preparative RP-HPLC using condition B to obtain 6.6 mg of desired product (0.0053 mmol, y= 24%).

^{1}H NMR (500 MHz, DMSO) δ 12.71 (br, 1H), 12.35 (br, 1H), 10.79 (s, 1H), 10.46 (s, 1H), 9.34 (br, 1H), 8.70 (d, *J* = 7.3 Hz, 1H), 8.15 (d, *J* = 4.1 Hz, 1H), 8.08 (d, *J* = 4.1 Hz, 1H), 7.98 - 7.92 (m, 6H), 7.87 - 7.77 (m, 7H), 7.64 (br, 1H), 7.40 (s, 1H), 7.00 (s, 1H), 4.93 (dd, *J* = 14.1, 7.1 Hz, 1H), 4.61 (br, 1H), 2.70 (d, *J* = 7.3 Hz, 2H), 1.26 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.2, 170.7, 168.3, 166.9, 165.7, 164.1, 158.5, 146.8, 142.4, 140.8, 139.7, 136.7, 136.5, 130.2, 129.5, 129.2, 128.4, 128.2, 125.8, 122.9, 120.4, 119.7, 118.9, 113.8, 112.8, 112.5, 74.3, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C41H36N7O10S (M+H^{+}) 818.2239, found 818.2237.

(S)-4-(4-(4-(4-Amino-2-(4-(4-cyano-3-methoxybenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (78)



Chemical Formula: $C_{44}H_{39}N_7O_{11}$
Exact Mass: 841,2708

Amine **35** (25 mg, 0.033 mmol) was coupled with 4-cyano-3-methoxybenzoic acid using coupling conditions A followed by final deprotection.

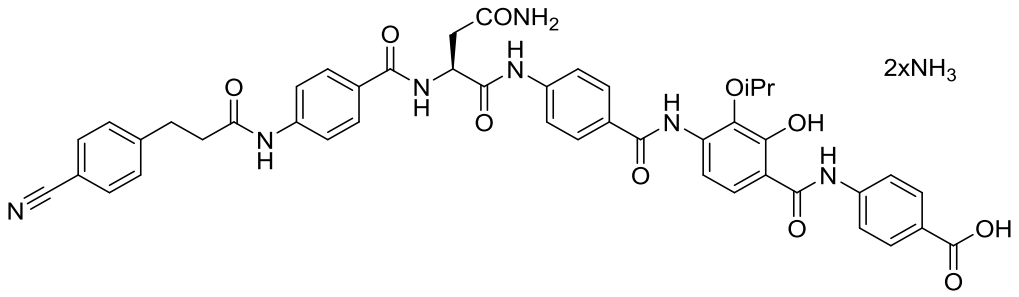
The desired compound was purified by preparative RP-HPLC using condition B to obtain 4.8 mg of desired product (0.0057 mmol, y= 17%).

^{1}H NMR (500 MHz, DMSO) δ 12.80 (br, 1H), 12.29 (br, 1H), 10.66 (s, 1H), 10.47 (s, 1H), 9.38 (s, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 7.98 – 7.92 (m, 7H), 7.91 – 7.79 (m, 7H), 7.72 (d, *J* = 1.3 Hz, 1H), 7.69 – 7.63 (m, 2H), 7.40 (s, 1H), 7.00 (s, 1H), 4.92 (q, *J* = 7.1 Hz, 1H), 4.61 – 4.53 (m, 1H), 4.03 (s, 3H), 2.69 (d, *J* = 7.1 Hz, 2H), 1.25 (t, *J* = 8.4 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.2, 170.7, 168.4, 166.9, 165.7, 164.4, 164.1, 160.8, 142.5, 142.1, 141.5, 140.7, 136.9, 136.4, 133.9, 130.2, 129.2, 128.3, 128.3, 126.1, 122.9, 120.6, 120.1, 119.6, 118.9, 115.9, 112.6, 111.4, 103.0, 74.6, 56.6, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C44H40N7O11 (M+H^{+}) 842.2780, found 842.2784.

(S)-4-(4-(4-(4-Amino-2-(4-(3-(4-cyanophenyl)propanamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (70)



Chemical Formula: $C_{45}H_{41}N_7O_{10}$
Exact Mass: 839,2915

Amine **35** (25 mg, 0.033 mmol) was coupled with 3-(4-cyanophenyl)propanoic acid using coupling conditions A followed by final deprotection.

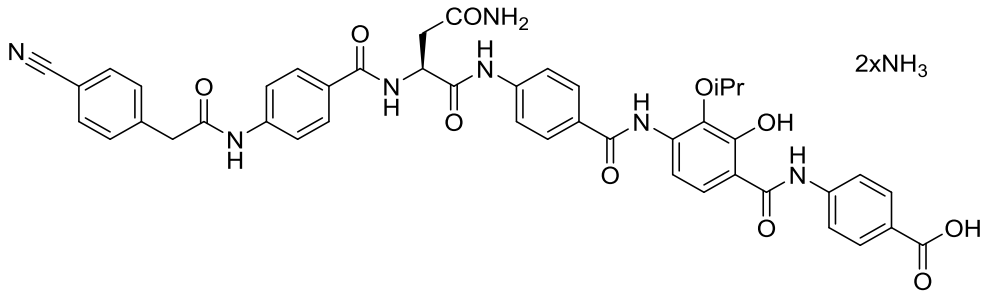
The desired compound was purified by preparative RP-HPLC condition B to obtain 4.7 mg of desired product (0.0057 mmol, y= 17%).

^{1}H NMR (500 MHz, DMSO) δ 12.80 (br, 1H), 12.29 (br, 1H), 10.63 (br, 1H), 10.44 (s, 1H), 10.19 (s, 1H), 9.39 (s, 1H), 8.60 (d, *J* = 7.3 Hz, 1H), 8.00 – 7.91 (m, 4H), 7.89 – 7.78 (m, 7H), 7.78 – 7.74 (m, 2H), 7.72 – 7.64 (m, 3H), 7.48 (d, *J* = 8.4 Hz, 2H), 7.38 (s, 1H), 6.98 (s, 1H), 4.90 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.55 (dt, *J* = 12.1, 6.0 Hz, 1H), 3.01 (t, *J* = 7.6 Hz, 2H), 2.71 (t, *J* = 7.6 Hz, 2H), 2.67 (d, *J* = 7.6 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 170.4, 168.4, 166.9, 165.8, 164.2, 147.3, 142.5, 142.0, 141.9, 137.0, 136.3, 132.2, 130.2, 129.4, 128.4, 128.3, 128.2, 126.2, 122.8, 120.6, 119.0, 118.9, 118.1, 112.5, 112.2, 108.9, 74.8, 51.6, 37.1, 36.8, 30.6, 22.3.

HRMS (ESI) calculated for C45H42N7O10 ($M+H^+$) 840.2988, found 840.2971.

(S)-4-(4-(4-(4-Amino-2-(4-(2-(4-cyanophenyl)acetamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (69)



Chemical Formula: $C_{44}H_{39}N_7O_{10}$
Exact Mass: 825,2758

Amine **35** (25 mg, 0.033 mmol) was coupled with 2-(4-cyanophenyl)acetic acid using coupling conditions A followed by final deprotection.

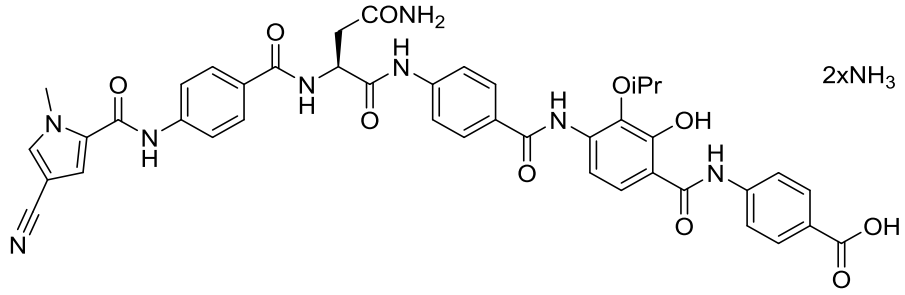
The desired compound was purified by preparative RP-HPLC condition B to obtain 6.4 mg of desired product (0.0078 mmol, y= 24%).

^{1}H NMR (500 MHz, DMSO) δ 12.74 (br, 1H), 12.30 (br, 1H), 10.49 (s, 1H), 10.44 (s, 1H), 9.35 (br, 1H), 8.62 (d, *J* = 7.3 Hz, 1H), 7.94 (dd, *J* = 11.1, 8.9 Hz, 4H), 7.90 – 7.74 (m, 9H), 7.68 (d, *J* = 8.8 Hz, 2H), 7.65 (br, 1H), 7.54 (d, *J* = 8.2 Hz, 2H), 7.39 (s, 1H), 6.98 (s, 1H), 4.90 (q, *J* = 7.1 Hz, 1H), 4.58 (s, 1H), 3.82 (s, 2H), 2.67 (d, *J* = 7.0 Hz, 2H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.5, 168.4, 166.9, 165.7, 164.1, 142.4, 141.8, 141.5, 136.4, 132.2, 132.1, 131.9, 130.5, 130.2, 128.4, 128.2, 122.9, 122.5, 120.5, 118.9, 118.2, 109.5, 74.4, 51.6, 43.0, 36.8, 22.3.

HRMS (ESI) calculated for C44H38N7O10 (M-H^{+}) 824.2686, found 824.2689.

(S)-4-(4-(4-(4-Amino-2-(4-(4-cyano-1-methyl-1H-pyrrole-2-carboxamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (83)



Chemical Formula: $C_{42}H_{38}N_8O_{10}$
Exact Mass: 814,2711

Amine **35** (20 mg, 0.033 mmol) was coupled with 4-cyano-1-methyl-1H-pyrrole-2-carboxylic acid using coupling conditions C. Allyl protected intermediate was purified on silica gel with a gradient 0-10% MeOH in DCM.

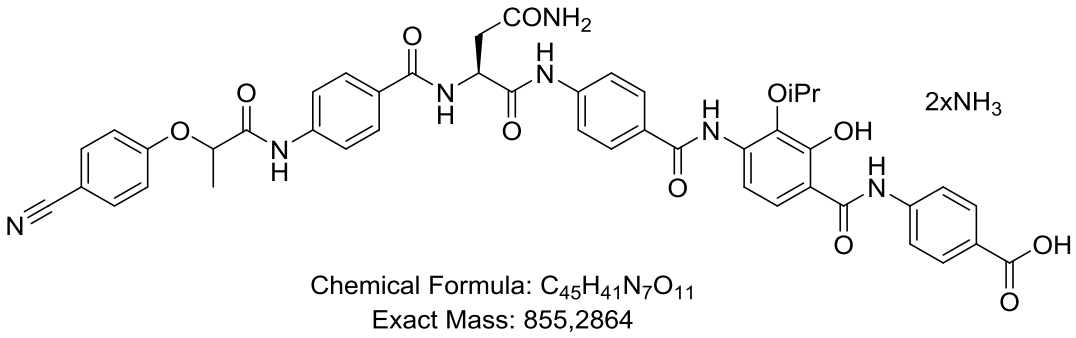
Final deprotection afforded the desired compound, which was purified by preparative RP-RP-HPLC using condition B to obtain 1.4 mg of desired product (0.002 mmol, y= 8%).

^{1}H NMR (500 MHz, DMSO) δ 12.51 (br, 1H), 10.43 (s, 1H), 10.24 (s, 1H), 8.99 (br, 1H), 8.64 (d, *J* = 7.3 Hz, 1H), 7.92 – 7.88 (m, 2H), 7.88 – 7.83 (m, 5H), 7.83 – 7.75 (m, 6H), 7.53 (br, 1H), 7.45 (d, *J* = 1.8 Hz, 1H), 7.39 (s, 1H), 7.23 (br, 1H), 6.98 (s, 1H), 4.92 (dd, *J* = 14.1, 7.1 Hz, 2H), 3.92 (s, 3H), 2.68 (d, *J* = 7.2 Hz, 2H), 1.21 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 167.6, 167.1, 165.7, 163.4, 158.6, 142.1, 141.6, 135.0, 130.4, 129.2, 128.7, 128.3, 127.8, 126.9, 123.5, 119.1, 119.0, 116.4, 115.9, 90.0, 51.6, 37.1, 36.8, 22.6.

HRMS (ESI) calculated for C42H39N8O10 (M+H$^+$) 815.2784, found 815.2777.

4-(4-(4-((2S)-4-Amino-2-(4-(2-(4-cyanophenoxy)propanamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (71)



Amine **35** (25 mg, 0.033 mmol) was coupled with 2-(4-cyanophenoxy)propanoic acid using coupling conditions A followed by final deprotection.

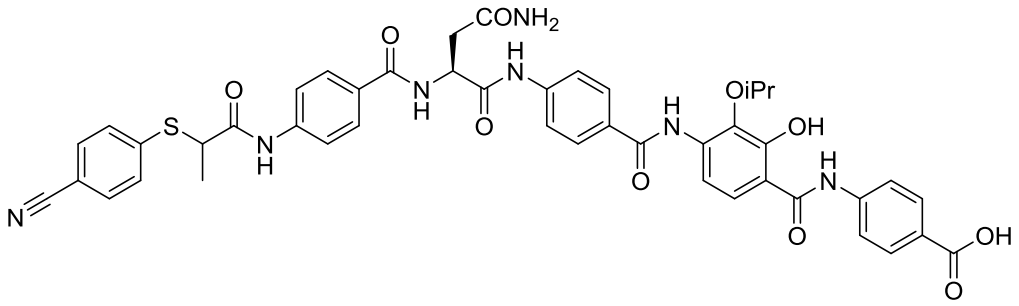
The desired compound was purified by preparative RP-HPLC condition B to obtain 6.9 mg of desired product (0.0081 mmol, y= 24%).

^{1}H NMR (500 MHz, DMSO) δ 12.61 (br, 2H), 10.45 (s, 2H), 9.27 (br, 1H), 8.64 (d, *J* = 7.3 Hz, 1H), 7.97 – 7.85 (m, 6H), 7.85 – 7.77 (m, 6H), 7.75 (br, 1H), 7.71 (d, *J* = 8.8 Hz, 2H), 7.55 (br, 1H), 7.39 (s, 1H), 7.15 – 7.08 (m, 2H), 6.98 (s, 1H), 5.07 (q, *J* = 6.6 Hz, 1H), 4.90 (q, *J* = 7.1 Hz, 1H), 4.66 (br, 1H), 2.70 – 2.65 (m, 2H), 1.59 (d, *J* = 6.6 Hz, 3H), 1.24 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 171.0, 169.4, 168.2, 167.0, 165.7, 163.9, 160.7, 142.4, 141.1, 136.6, 134.3, 130.3, 128.9, 128.6, 128.4, 128.1, 123.0, 120.1, 119.0, 118.9, 118.9, 116.0, 103.5, 73.7, 51.6, 36.8, 22.4, 18.3.

HRMS (ESI) calculated for C45H42N7O11 (M+H$^+$) 856.2937, found 856.2938.

4-(4-(4-((2S)-4-Amino-2-(4-(2-((4-cyanophenyl)thio)propanamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (72)



Chemical Formula: $C_{45}H_{41}N_7O_{10}S$
Exact Mass: 871,2636

Amine **35** (25 mg, 0.033 mmol) was coupled with 2-((4-cyanophenyl)thio)propanoic acid using coupling conditions A followed by final deprotection.

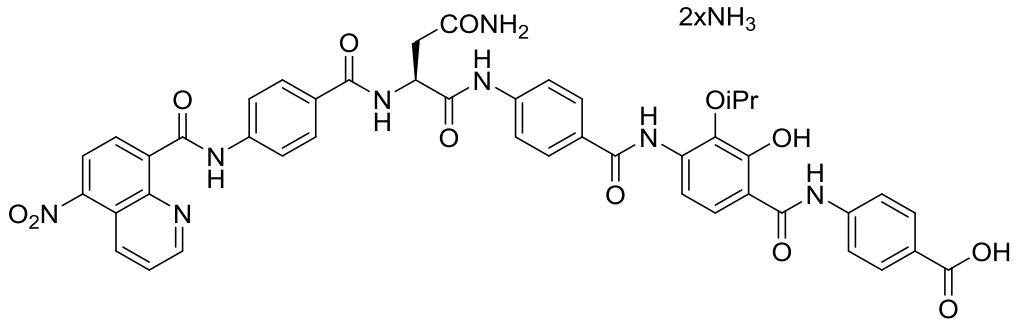
The desired compound was purified by preparative RP-HPLC using condition B to obtain 6.0 mg of desired product (0.0069 mmol, y= 21%).

^{1}H NMR (500 MHz, DMSO) δ 12.78 (br, 1H), 12.30 (br, 1H), 10.66 (br, 1H), 10.50 (s, 1H), 10.45 (s, 1H), 9.35 (br, 1H), 8.63 (d, *J* = 7.3 Hz, 1H), 7.95 (t, *J* = 9.5 Hz, 5H), 7.91 – 7.83 (m, 5H), 7.80 (ddd, *J* = 8.7, 4.6, 2.6 Hz, 6H), 7.66 (d, *J* = 8.8 Hz, 4H), 7.59 – 7.49 (m, 3H), 7.39 (s, 1H), 6.98 (s, 1H), 4.90 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.58 (s, 1H), 4.34 (q, *J* = 6.9 Hz, 1H), 2.69 – 2.66 (m, 2H), 1.53 (d, *J* = 6.9 Hz, 3H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 171.3, 170.7, 169.6, 168.4, 166.9, 165.7, 164.1, 142.5, 142.0, 141.4, 136.5, 132.7, 130.2, 128.9, 128.8, 128.5, 128.2, 122.9, 120.5, 118.9, 118.6, 118.5, 108.5, 51.6, 45.1, 36.8, 22.3, 17.8.

HRMS (ESI) calculated for C45H42N7O10S ($M+H^+$) 872.2708, found 872.2715.

(S)-4-(4-(4-(4-Amino-2-(4-(5-nitroquinoline-8-carboxamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (85)



Chemical Formula: $C_{45}H_{38}N_8O_{12}$
Exact Mass: 882,2609

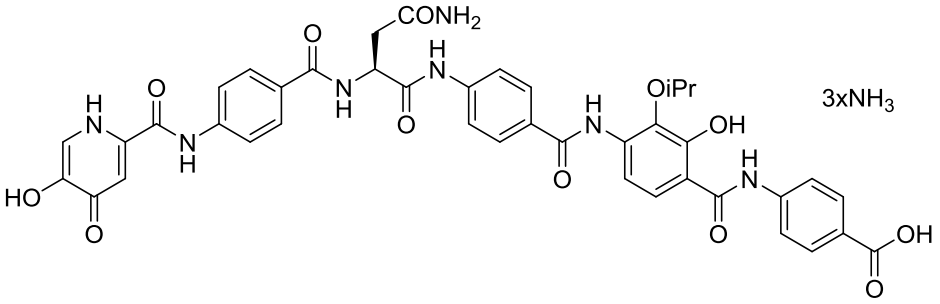
Amine **35** (25 mg, 0.033 mmol) was coupled with 5-nitroquinoline-8-carboxylic acid using coupling conditions A followed by final deprotection.

The desired compound was purified by preparative RP-HPLC using condition B to obtain 2.1 mg of desired product (0.0024 mmol, y= 7%).

^{1}H NMR (500 MHz, DMSO) δ 12.83 (br, 1H), 12.29 (br, 1H), 12.08 (s, 1H), 10.62 (br, 1H), 10.48 (s, 1H), 9.40 (s, 1H), 9.25 (dd, *J* = 4.2, 1.5 Hz, 1H), 8.93 (dd, *J* = 8.9, 1.6 Hz, 1H), 8.71 (d, *J* = 7.3 Hz, 1H), 8.56 (d, *J* = 8.0 Hz, 1H), 8.45 (d, *J* = 8.0 Hz, 1H), 8.02 – 7.90 (m, 8H), 7.84 (dd, *J* = 13.5, 8.7 Hz, 5H), 7.69 (d, *J* = 8.7 Hz, 1H), 7.41 (s, 1H), 7.01 (s, 1H), 4.94 (dd, *J* = 14.1, 7.0 Hz, 1H), 4.59 – 4.51 (m, 1H), 2.74 – 2.67 (m, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

HRMS (ESI) calculated for C45H39N8O12 (M+H$^+$) 883.2682, found 883.2682.

(S)-4-(4-(4-(4-Amino-2-(4-(5-hydroxy-4-oxo-1,4-dihydropyridine-2-carboxamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (84)



Chemical Formula: $C_{41}H_{37}N_7O_{12}$
Exact Mass: 819,2500

Amine **35** (25 mg, 0.033 mmol) was coupled with 4,5-bis(allyloxy)picolinic acid (**57**) using coupling conditions A followed by final deprotection.

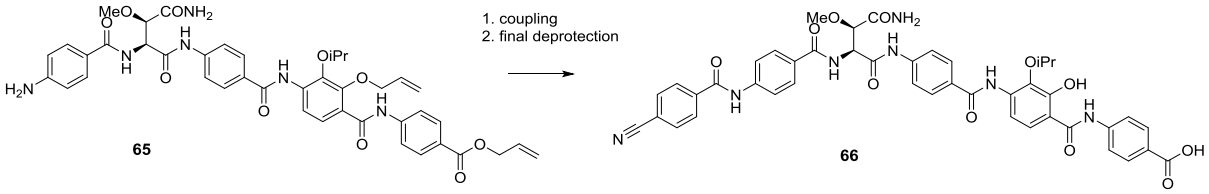
Purification by preparative RP-HPLC following condition B was done to obtain 11.5 mg of desired compound (0.0136 mmol, y= 41%).

^{1}H NMR (700 MHz, DMSO) δ 15.83 (br, 1H), 10.51 (s, 1H), 10.41 (s, 1H), 8.88 (s, 1H), 8.62 (d, *J* = 7.4 Hz, 1H), 8.49 (s, 1H), 7.96 (d, *J* = 8.8 Hz, 2H), 7.88 (d, *J* = 8.8 Hz, 2H), 7.83 (t, *J* = 8.0 Hz, 5H), 7.80 (d, *J* = 8.8 Hz, 2H), 7.74 (d, *J* = 8.2 Hz, 2H), 7.45 (d, *J* = 8.7 Hz, 1H), 7.39 (s, 1H), 7.32 (br, 1H), 7.11 (d, *J* = 8.7 Hz, 1H), 6.97 (s, 1H), 5.03 – 4.98 (m, 1H), 4.91 (dd, *J* = 14.1, 7.3 Hz, 1H), 2.68 (d, *J* = 8.1 Hz, 2H), 1.19 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.4, 170.7, 170.3, 166.9, 165.9, 165.3, 164.6, 163.1, 142.0, 141.7, 137.5, 134.0, 131.1, 129.7, 129.5, 128.3, 128.1, 127.6, 127.5, 127.0, 124.2, 123.7, 119.1, 118.0, 117.6, 116.0, 107.7, 100.7, 70.4, 51.7, 36.9, 22.7.

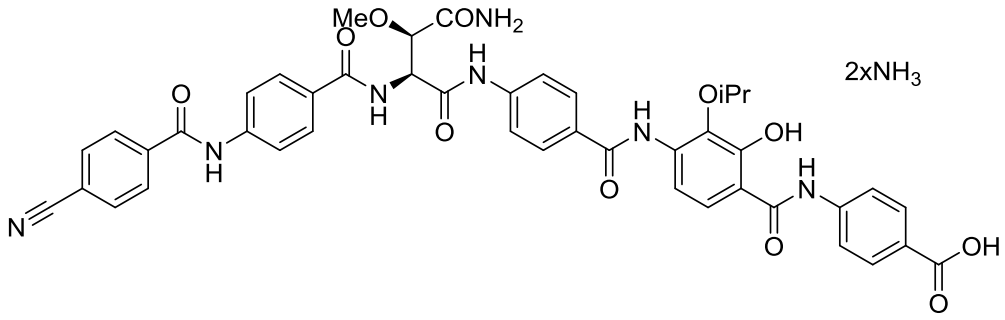
HRMS (ESI) calculated for C41H38N7O12 ($M+H^+$) 820.2573, found 820.2571.

Synthetic scheme depicting the final steps of the synthesis of compound **66:**



The synthesis of amine **65** is described in details in the published manuscript[51].

4-(4-(4-((2S,3R)-4-Amino-2-(4-(4-cyanobenzamido)benzamido)-3-methoxy-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (66)



Chemical Formula: $C_{44}H_{39}N_7O_{11}$
Exact Mass: 841,2708

Amine **65** (12 mg, 0.015 mmol) was coupled to 4-cyanobenzoic acid using coupling conditions A followed by final deprotection.

Purification by preparative RP-HPLC using condition B afforded 5.2 mg of desired compound (0.0062 mmol, y= 41%).

^{1}H NMR (700 MHz, DMSO) δ 12.70 (br, 1H), 12.28 (br, 1H), 10.72 (s, 1H), 10.57 (s, 1H), 9.34 (br, 1H), 8.46 (d, *J* = 8.1 Hz, 1H), 8.14 – 8.11 (m, 2H), 8.04 (d, *J* = 8.4 Hz, 2H), 7.95 (dd, *J* = 8.2, 5.4 Hz, 4H), 7.92 – 7.86 (m, 4H), 7.84 (d, *J* = 8.6 Hz, 4H), 7.80 (br, 1H), 7.62 (br, 1H), 7.54 (s, 1H), 7.47 (s, 1H), 4.92 (t, *J* = 8.1 Hz, 1H), 4.60 (br, 1H), 4.09 (d, *J* = 8.1 Hz, 1H), 3.31 (s, 3H), 1.26 (d, *J* = 6.1 Hz, 6H).

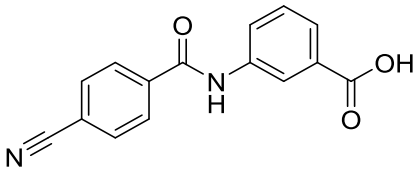
^{13}C NMR (176 MHz, DMSO) δ 170.9, 168.7, 168.3, 166.9, 165.5, 164.5, 164.1, 142.2, 141.8, 138.7, 136.5, 132.5, 130.2, 128.9, 128.6, 128.3, 122.9, 120.4, 119.6, 119.0, 118.3, 114.0, 107.0, 80.0, 57.7, 55.8, 45.8, 22.4.

HRMS (ESI) calculated for C44H40N7O11 ($M+H^+$) 842.2780, found 842.2771.

4.2.2.2.5 Second synthetic access, modification rings A-B

4.2.2.2.5.1 Fragments synthesis

3-(4-Cyanobenzamido)benzoic acid (91)



Chemical Formula: $C_{15}H_{10}N_2O_3$
Exact Mass: 266,0691

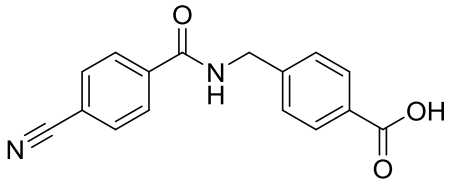
3-Aminobenzoic acid (230 mg; 1.68 mmol) was dissolved in THF (1.2 mL) and $NaHCO_3$ saturated solution (1.2 mL), to it 4-cyanobenzoyl chloride (277 mg; 1.68 mmol) was added at 0 °C. The reaction was stirred for 2 hours, pH adjusted to 1, the precipitate was collected by filtration, washed with HCl 1 N, triturated with Et_2O and dried at high vacuum to give 280 mg of a white solid (1.05 mmol; y= 63%).

^{1}H NMR (500 MHz, DMSO) δ 13.02 (br, 1H), 10.67 (s, 1H), 8.42 (t, *J* = 1.8 Hz, 1H), 8.14 (d, *J* = 8.4 Hz, 2H), 8.04 (d, *J* = 8.4 Hz, 3H), 7.71 (d, *J* = 7.8 Hz, 1H), 7.50 (t, *J* = 7.9 Hz, 1H).

^{13}C NMR (126 MHz, DMSO) δ 167.1, 164.3, 139.0, 138.6, 132.5, 131.3, 129.0, 128.6, 124.8, 124.5, 121.2, 118.3, 114.0.

HRMS (ESI) calculated for C15H9N2O3 ($M-H^+$) 265.0619, found 265.0606.

4-((4-Cyanobenzamido)methyl)benzoic acid (92)



Chemical Formula: $C_{16}H_{12}N_2O_3$
Exact Mass: 280,0848

4-(Aminomethyl)benzoic acid (287 mg; 1.9 mmol) was dissolved in THF (2.0 mL) and NaOH 1 N (5.7 mL), to it 4-cyanobenzoyl chloride (314 mg; 1.9 mmol) was added at 0 °C. Reaction

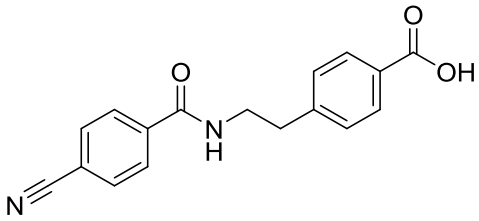
stirred for 2 hours, pH adjusted to 1, compound extracted with EtOAc (50 mL), organic phase washed with brine (50 mL) dried over sodium sulphate and reduced under vacuum. The crude was chromatographed on silica gel with a gradient 0-10% MeOH in DCM to give 100 mg of a white solid (0.36 mmol; y= 19%).

^{1}H NMR (500 MHz, DMSO) δ 12.87 (br, 1H), 9.37 (t, *J* = 5.9 Hz, 1H), 8.08 – 8.02 (m, 2H), 8.01 – 7.96 (m, 2H), 7.94 – 7.88 (m, 2H), 7.43 (d, *J* = 8.5 Hz, 2H), 4.56 (d, *J* = 5.9 Hz, 2H).

^{13}C NMR (126 MHz, DMSO) δ 167.2, 165.0, 144.3, 138.1, 132.5, 129.4, 128.1, 127.2, 118.3, 113.7, 42.6.

HRMS (ESI) calculated for C16H11N2O3 (M-H^{+}) 279.0775, found 279.0794.

4-(2-(4-Cyanobenzamido)ethyl)benzoic acid (93)



Chemical Formula: $C_{17}H_{14}N_2O_3$
Exact Mass: 294,1004

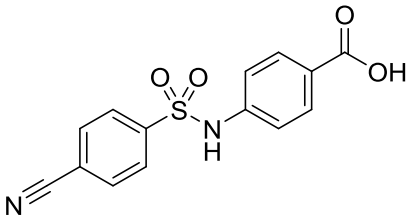
4-(2-Aminoethyl)benzoic acid (165 mg; 0.82 mmol) was dissolved in THF (3.0 mL) and NaOH 1 N (3.5 mL), to it 4-cyanobenzoyl chloride (108 mg; 0.66 mmol) was added at 0 °C. Reaction stirred for 1.5 hours, pH adjusted to 1, compound extracted with EtOAc (30 mL), organic phase washed with brine (30 mL) dried over sodium sulphate and reduced under vacuum. The crude was chromatographed on silica gel with a gradient 0-10% MeOH in DCM to give 45 mg of a white solid (0.14 mmol; y= 21%).

^{1}H NMR (500 MHz, DMSO) δ 12.82 (br, 1H), 8.83 (t, *J* = 5.6 Hz, 1H), 7.98 – 7.92 (m, 4H), 7.88 – 7.84 (m, 2H), 7.36 (d, *J* = 8.3 Hz, 2H), 3.53 (dd, *J* = 13.0, 7.1 Hz, 2H), 2.93 (t, *J* = 7.2 Hz, 2H).

^{13}C NMR (126 MHz, DMSO) δ 167.2, 164.8, 144.7, 138.5, 132.4, 129.4, 128.9, 128.0, 118.3, 113.5, 40.5, 34.8.

HRMS (ESI) calculated for C17H13N2O3 (M-H^{+}) 293.0932, found 293.0951.

4-((4-Cyanophenyl)sulfonamido)benzoic acid (98)



Chemical Formula: $C_{14}H_{10}N_2O_4S$
Exact Mass: 302,0361

Tert-butyl 4-aminobenzoate (140 mg; 0.725 mmol) and pyridine (0.58 mL; 7.25 mmol) were dissolved in THF (10 mL), to this solution a solution of 4-cyanobenzenesulfonyl chloride (146 mg; 0.725 mmol) in THF (2 mL) was added at r.t.. Reaction stirred overnight, quenched with HCl 1 N and ice, solvent partially reduced under vacuum, residue diluted with EtOAc (30 mL) and water (30 mL), organic phase washed with $NaHCO_3$ saturated solution (30 mL), brine (30 mL) and dried over sodium sulphate. Solvent evaporated under reduced pressure and crude used in the next step without further purification.

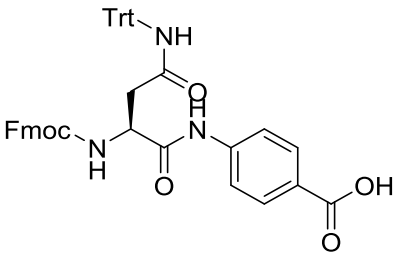
The crude residue was dissolved in DCM (6.0 mL) and TFA (1.2 mL) was added at 0 °C. Reaction stirred for 1.5 h, solvent reduced under vacuum, residue dissolved in EtOAc (40 mL), the organic phase was washed with HCl 1 N (40 mL), brine (40 mL) and dried over sodium sulphate. Solvent removed under vacuum to afford 160 mg of pure product (0.52; y= 72%).

^{1}H NMR (500 MHz, DMSO) δ 12.81 (br, 1H), 11.05 (s, 1H), 8.10 – 8.04 (m, 2H), 8.00 – 7.95 (m, 2H), 7.86 – 7.80 (m, 2H), 7.23 – 7.18 (m, 2H).

^{13}C NMR (126 MHz, DMSO) δ 167.1, 143.7, 141.7, 134.1, 131.3, 127.9, 126.7, 119.2, 118.0, 116.2.

HRMS (ESI) calculated for C14H9N2O4S ($M-H^+$) 301.0289, found 301.0301.

(S)-4-(2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzoic acid (99)



Chemical Formula: $C_{45}H_{37}N_3O_6$
Exact Mass: 715,2682

Methyl (S)-4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzoate (1.0 g; 1.37 mmol), lithium iodide (1.8 g; 13.72 mmol) were mixed in EtOAc (14 mL) and heated at 110 °C for 12 h in a microwave synthesizer. Mixture was diluted with EtOAc (100 mL) and HCL 1N (100 mL), organic phase washed with brine (100 mL), dried over sodium sulphate and reduced under vacuum. The residue was chromatographed on silica gel with a gradient 0-5% MeOH in DCM to give 0.74 g of a white solid (1.03 mmol; y= 75%).

^{1}H NMR (500 MHz, DMSO) δ 12.70 (s, 1H), 10.41 (s, 1H), 8.62 (s, 1H), 7.90 (dd, *J* = 8.1, 3.9 Hz, 4H), 7.80 (d, *J* = 7.9 Hz, 1H), 7.77 – 7.70 (m, 4H), 7.42 (dd, *J* = 13.7, 7.0 Hz, 2H), 7.34 – 7.26 (m, 2H), 7.25 – 7.14 (m, 15H), 4.49 – 4.41 (m, 1H), 4.36 (dd, *J* = 10.4, 7.0 Hz, 1H), 4.30 (dd, *J* = 10.4, 7.0 Hz, 1H), 4.23 (t, *J* = 6.9 Hz, 1H), 2.75 (dd, *J* = 14.6, 9.8 Hz, 1H), 2.66 – 2.58 (dd, *J* = 14.0, 4.4 Hz, 1H).

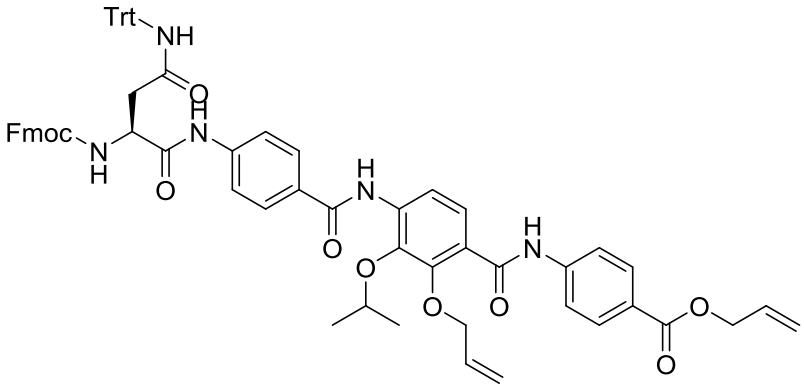
^{13}C NMR (126 MHz, DMSO) δ 170.8, 168.4, 166.9, 155.8, 144.7, 143.8, 143.0, 140.7, 130.3, 128.6, 127.6, 127.4, 127.1, 126.3, 125.3, 125.2, 120.1, 118.6, 69.4, 65.8, 52.8, 46.7, 38.4.

HRMS (ESI) calculated for C45H38N3O6 ($M+H^+$) 716.2755, found 716.2745.

Marfey: 93.9% S enantiomer, 6.1% R enantiomer

4.2.2.2.5.2 Final assembly

Allyl (S)-4-(4-(4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzamido)-2-(allyloxy)-3-isopropoxybenzamido)benzoate (100)



Chemical Formula: $C_{68}H_{61}N_5O_{10}$
Exact Mass: 1107,4418

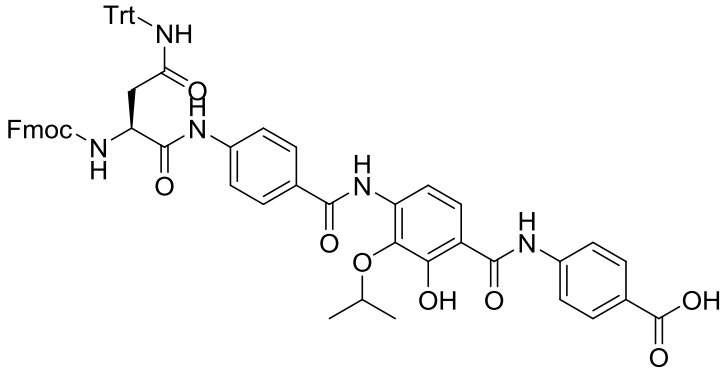
$POCl_3$ (1.92 mmol) as a solution in DCM (1:9) was added dropwise to a solution of allyl 4-(2-(allyloxy)-4-amino-3-isopropoxybenzamido)benzoate (0.315 g; 0.77 mmol) and (S)-4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzoic acid (1.37 g, 1.92 mmol) in THF (8 mL) and DCM (4 mL) at 0 °C, followed by DiPEA (1.78 mL; 10.24 mmol) as a solution in DCM (1:1). Reaction stirred at r.t. for 6 h, quenched with HCL 1 N and ice, solvent partially reduced under vacuum and residue diluted with EtOAc (200 mL) and HCl 1N (200 mL), organic phase washed with brine (200 mL) and dried over sodium sulphate. Solvent removed under vacuum, the crude residue was chromatographed on silica gel with a gradient EtOAc 20-75% in Pet. Et to give 750 mg of a orange residue (0.68 mmol; 76%).

^{1}H NMR (700 MHz, DMSO) δ 10.58 (s, 1H), 10.42 (s, 1H), 9.53 (s, 1H), 8.64 (s, 1H), 7.99 (d, *J* = 8.6 Hz, 2H), 7.93 – 7.85 (m, 4H), 7.81 (m, 4H), 7.78 – 7.72 (m, 3H), 7.42 (m, 3H), 7.35 – 7.26 (m, 3H), 7.26 – 7.14 (m, 15H), 6.04 (m, 2H), 5.39 (m, 2H), 5.24 (m, 2H), 4.80 (d, *J* = 5.3 Hz, 2H), 4.62 (d, *J* = 5.5 Hz, 2H), 4.52 – 4.44 (m, 2H), 4.37 (m, 1H), 4.31 (m, 1H), 4.24 (dd, *J* = 14.3, 7.1 Hz, 1H), 2.77 (td, *J* = 14.1, 9.9 Hz, 1H), 2.64 (td, *J* = 15.1, 5.0 Hz, 1H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 170.8, 168.5, 166.9, 165.0, 164.7, 164.3, 155.8, 149.5, 144.7, 143.8, 143.5, 143.0, 142.6, 142.3, 140.7, 135.7, 133.7, 132.8, 130.3, 128.6, 127.4, 127.1, 126.3, 125.3, 125.2, 124.2, 123.6, 120.1, 119.0, 118.8, 118.6, 117.8, 117.8, 76.3, 74.3, 69.4, 65.8, 64.8, 52.9, 46.7, 30.4, 22.3.

HRMS (ESI) calculated for C68H62N5O10 ($M+H^+$) 1108.4491, found 1108.4514.

(S)-4-(4-(4-(2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (101)



Chemical Formula: $C_{62}H_{53}N_5O_{10}$
Exact Mass: 1027,3792

Phenyl silane (0.25 mL; 2.02 mmol) followed by palladium-tetrakis(triphenylphosphine (138 mg; 0.12 mmol) was added to a solution of allyl (S)-4-(4-(4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzamido)-2-(allyloxy)-3-isopropoxybenzamido)benzoate (530 mg; 0.48 mmol) in THF (19.0 mL). Reaction stirred for 3 hours, quenched by addition of few drops of acetic acid and filtered through celite. Solvent removed under reduced pressure, the crude residue was purified on silica gel with a gradient 0-10% MeOH in DCM +1% acetic acid to give 285 mg of a yellow solid (0.28 mmol; y= 58%).

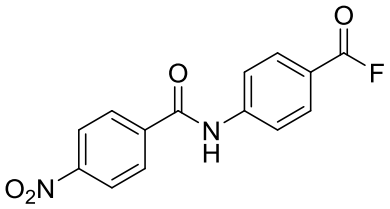
^{1}H NMR (500 MHz, DMSO) δ 12.36 (br, 1H), 12.30 (s, 1H), 10.61 (s, 1H), 10.43 (s, 1H), 9.41 (s, 1H), 8.64 (s, 1H), 8.00 – 7.93 (m, 3H), 7.90 (d, *J* = 7.6 Hz, 2H), 7.86 (m, 3H), 7.81 (dd, *J* = 8.0, 4.8 Hz, 3H), 7.75 (dd, *J* = 7.4, 3.8 Hz, 2H), 7.71 (d, *J* = 8.9 Hz, 1H), 7.42 (dd, *J* = 12.6, 7.4 Hz, 2H), 7.35 – 7.27 (m, 3H), 7.27 – 7.15 (m, 15H), 4.58 – 4.51 (m, 1H), 4.48 (dd, *J* = 14.2, 8.4 Hz, 1H), 4.38 (dd, *J* = 10.4, 7.1 Hz, 1H), 4.31 (dd, *J* = 10.5, 7.0 Hz, 1H), 4.24 (t, *J* = 6.8 Hz, 1H), 2.78 (dd, *J* = 14.5, 9.7 Hz, 1H), 2.64 (dd, *J* = 14.2, 5.2 Hz, 1H), 1.27 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 172.0, 170.8, 168.5, 168.5, 166.9, 164.2, 155.8, 154.1, 144.7, 143.8, 142.4, 142.0, 140.7, 137.0, 136.3, 130.2, 128.6, 128.3, 127.7, 127.4, 127.1, 126.3, 126.3, 125.3, 125.2, 124.9, 122.8, 120.7, 120.1, 118.9, 112.4, 112.2, 74.9, 69.4, 65.8, 52.9, 46.7, 30.4, 22.3.

HRMS (ESI) calculated for C62H52N5O10 (M-H$^+$) 1026.3720, found 1026.3716.

Marfey: 96.2% S enantiomer, 3.8% R enantiomer

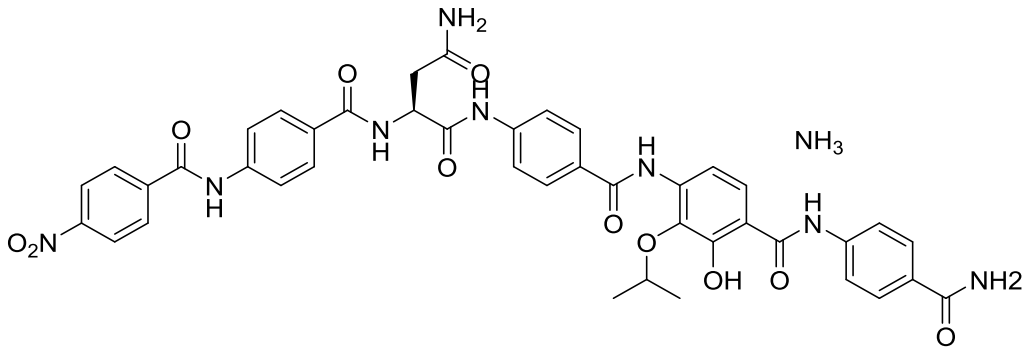
4-(4-Nitrobenzamido)benzoyl fluoride (90)



Chemical Formula: $C_{14}H_9FN_2O_4$
Exact Mass: 288,0546

Diethylaminosulfur trifluoride (13 µL; 0.095 mmol) was added was added at 0 °C to a mixture of 4-(4-nitrobenzamido)benzoic acid (**89**) (50 mg; 0.17 mmol) in DCM (1.5 mL). The reaction was stirred for 20 minutes, then diluted with DCM and washed with ice water, organic phase dried over sodium sulphate and reduced under vacuum to give 4-(4-nitrobenzamido)benzoyl fluoride.

(S)-N1-(4-((4-((4-Carbamoylphenyl)carbamoyl)-3-hydroxy-2-isopropoxyphenyl)carbamoyl)phenyl)-2-(4-(4-nitrobenzamido)benzamido)succinamide (103)



Chemical Formula: $C_{42}H_{38}N_8O_{11}$
Exact Mass: 830,2660

Diethylaminosulfur trifluoride (13 µL; 0.095 mmol) was added at 0 °C to a mixture of (S)-4-(4-(4-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylamino)butanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (65 mg; 0.063 mmol) in DCM (0.7 mL). The reaction was stirred for 20 minutes, then diluted with DCM (20 mL) and washed with ice water (20 mL), organic phase dried over sodium sulphate and reduced under vacuum.

The residue was dissolved in THF (0.5 mL), the solution added to a NH_3 in MeOH 7N at 0 °C. Reaction stirred 15 min then solvent removed under vacuum. Compound dissolved in DCM (2 mL), Tips (0.1 mL) followed by TFA (0.5 mL) were added at 0 °C. The reaction was stirred

for 2 hours, the solvent was reduced under vacuum, the residue dissolved again in DCM and evaporated twice. The crude thus obtained was dissolved in a 20% solution of diethylamine in acetonitrile (2 mL), the solution was stirred for 30 min. The solvent was removed under reduced pressure, the residue was dissolved in CH_3CN and evaporated twice. Residue triturated three times with Pet. Et. and used in the coupling step.

A solution of 4-(4-nitrobenzamido)benzoyl fluoride (0.069 mmol) in THF (1 mL) was added to a solution of the crude from the previous step (0.048 mmol) and DiPEA (42 µL; 0.24 mmol) in DCM/THF 1:1 (1 mL). The reaction was stirred for 3 hours and then purified by RP-HPLC using method B to obtain 3.2 mg of desired compound (0.0039 mmol; y= 6%).

^{1}H NMR (700 MHz, DMSO) δ 12.40 (br, 1H), 10.79 (s, 1H), 10.58 (br, 1H), 10.46 (s, 1H), 9.35 (br, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.41 – 8.37 (m, 2H), 8.23 – 8.19 (m, 2H),7.94 (dd, *J* = 8.9, 2.2 Hz, 4H), 7.90 (dd, *J* = 8.6, 5.7 Hz, 5H), 7.82 (d, *J* = 8.8 Hz, 3H), 7.78 (d, *J* = 8.7 Hz, 2H), 7.66 (br, 1H), 7.40 (s, 1H), 7.28 (br, 1H), 6.99 (s, 1H), 4.93 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.58 (br, 1H), 2.69 (d, *J* = 7.7 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.4, 167.3, 165.8, 164.2, 164.1, 149.3, 142.5, 141.5, 140.3, 136.4, 129.3, 129.2, 128.6, 128.2, 123.6, 122.8, 120.5, 119.6, 118.9, 51.6, 36.8, 22.3.

HRMS (ESI) calculated for C42H37N8O11 ($M-H^+$) 829.2587, found 829.2588.

4.2.2.2.5.3 derivatives synthesized

General procedures:

Trityl deprotection

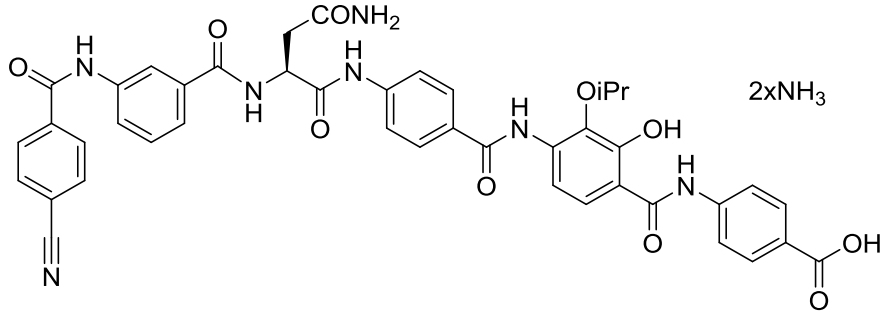
The compound was dissolved in DCM (M= 0.1), Tips (3 eq.) followed by TFA (20%) were added at 0 °C. The reaction was stirred for 2 hours at r.t. then the solvent was removed under vacuum, the residue was suspended and evaporated twice with DCM, finally it was triturated 3x with ice cold Pet. Et..

Fmoc deprotection

The compound was dissolved in a 20% solution of diethylamine in acetonitrile (M= 0.05- 0.1) and the reaction was stirred until completion as monitored by TLC or LCMS (in general reaction time 30 min-1 hour). The solvent was removed under reduced pressure and the residue was dissolved in CH_3CN and evaporated twice. Finally it was left at high vacuum overnight.

Starting from intermediate **101**, after Trityl and Fmoc deprotection (according to general procedures described above), the crude amine **102** was coupled to a carboxylic acid (fragments A1) to obtain the following compounds:

(S)-4-(4-(4-(4-Amino-2-(3-(4-cyanobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (104)



Chemical Formula: $C_{43}H_{37}N_7O_{10}$
Exact Mass: 811,2602

Carboxylic acid **91** was activated to the corresponding pentafluorophenyl ester following reported procedure [48].

Activated ester (17.3 mg; 0.04 mmol) was dissolved in DMF (0.1 mL) and added at 0°C to a stirred solution of amine **102** (0.02 mmol) and DiPEA (18 µL; 0.10 mmol). The reaction was

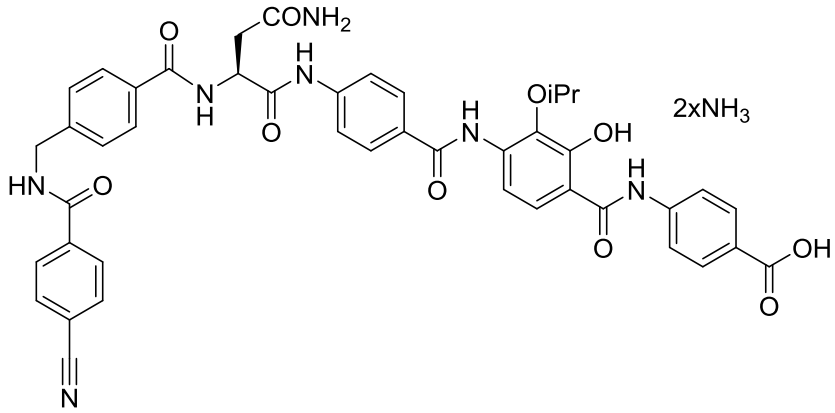
stirred for three hours at r.t. then diluted with EtOAc and a solution of ice cold HCl, organic phase washed with brine and evaporated under vacuum. The residue was purified by preparative RP-HPLC with a gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min. to give 0.9 mg of desired product (0.0011 mmol; y= 5%).

^{1}H NMR (700 MHz, DMSO) δ 15.36 (br, 1H), 10.70 (s, 1H), 10.48 (s, 1H), 8.87 (s, 1H), 8.83 (d, *J* = 6.6 Hz, 1H), 8.54 (s, 1H), 8.25 (s, 1H), 8.15 (d, *J* = 8.3 Hz, 2H), 8.04 (d, *J* = 8.3 Hz, 2H), 8.01 (d, *J* = 7.9 Hz, 1H), 7.82 (dd, *J* = 22.5, 8.7 Hz, 4H), 7.76 (d, *J* = 8.3 Hz, 2H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.57 (d, *J* = 7.7 Hz, 2H), 7.49 (t, *J* = 7.9 Hz, 1H), 7.44 (d, *J* = 8.7 Hz, 2H), 7.08 (d, *J* = 8.7 Hz, 1H), 6.97 (s, 1H), 5.02 (dt, *J* = 12.3, 6.1 Hz, 1H), 4.93 (dd, *J* = 13.9, 7.3 Hz, 1H), 2.72 – 2.68 (m, 2H), 1.19 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.5, 166.2, 165.4, 165.2, 164.2, 163.1, 142.0, 138.8, 137.5, 134.6, 134.0, 132.5, 129.7, 129.5, 128.6, 127.6, 123.7, 123.3, 122.9, 120.1, 119.0, 118.3, 117.6, 116.0, 113.9, 100.7, 70.3, 51.7, 36.8, 22.7.

HRMS (ESI) calculated for C43H38N7O10 ($M+H^+$) 812.2675, found 812.2671.

(S)-4-(4-(4-(4-Amino-2-(4-((4-cyanobenzamido)methyl)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (105)



Chemical Formula: $C_{44}H_{39}N_7O_{10}$
Exact Mass: 825,2758

Carboxylic acid coupling partner (**92**) was activated to the corresponding pentafluorophenyl ester following reported procedure [48].

Activated ester (18.0 mg; 0.04 mmol) was dissolved in DMF (0.1 mL) and added at 0°C to a stirred solution of amine **102** (0.02 mmol) and DiPEA (18 µL; 0.10 mmol). The reaction was stirred for three hours at r.t. then diluted with EtOAc and a solution of ice cold HCl, organic phase washed with brine and evaporated under vacuum. The residue was purified by

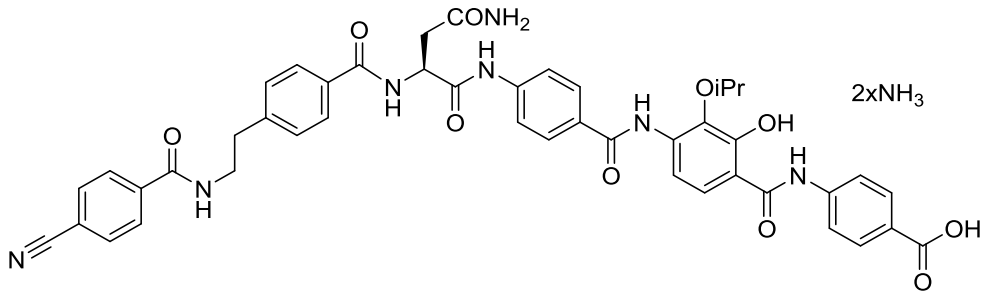
preparative RP-HPLC with a gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min. to give 3.2 mg of desired product (0.0039 mmol; y= 19%).

^{1}H NMR (700 MHz, DMSO) δ 12.49 (br, 1H), 10.44 (s, 1H), 9.36 (t, *J* = 5.9 Hz, 1H), 9.26 (s, 1H), 8.69 (d, *J* = 7.3 Hz, 1H), 8.05 (d, *J* = 8.4 Hz, 2H), 7.98 (d, *J* = 8.4 Hz, 2H), 7.93 (dd, *J* = 13.9, 8.6 Hz, 4H), 7.88 – 7.78 (m, 6H), 7.75 (d, *J* = 7.8 Hz, 1H), 7.55 (br, 1H), 7.43 (d, *J* = 8.2 Hz, 2H), 7.38 (s, 1H), 6.98 (s, 1H), 4.91 (dd, *J* = 13.9, 7.3 Hz, 1H), 4.65 (br, 1H), 4.56 (d, *J* = 5.9 Hz, 2H), 2.68 (dd, *J* = 6.6, 4.4 Hz, 2H), 1.24 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.6, 168.2, 167.0, 166.1, 165.0, 163.9, 142.8, 142.4, 138.2, 136.6, 132.5, 130.3, 128.6, 128.1, 127.6, 127.0, 123.0, 120.1, 118.9, 118.3, 113.7, 51.6, 42.6, 36.8, 22.4.

HRMS (ESI) calculated for C44H40N7O10 ($M+H^+$) 826.2831, found 826.2822.

(S)-4-(4-(4-(4-Amino-2-(4-(2-(4-cyanobenzamido)ethyl)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (106)



Chemical Formula: $C_{45}H_{41}N_7O_{10}$
Exact Mass: 839,2915

The carboxylic acid coupling partner (**93**) was activated to the corresponding pentafluorophenyl ester following reported procedure [48].

Activated ester (18.4 mg; 0.04 mmol) was dissolved in DMF (0.1 mL) and added at 0°C to a stirred solution of amine **102** (0.02 mmol) and DiPEA (18 µL; 0.10 mmol). The reaction was stirred for three hours at r.t. then diluted with EtOAc and a solution of ice cold HCl, organic phase washed with brine and evaporated under vacuum. The residue was purified by preparative RP-HPLC with a gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min. to give 5.3 mg of desired product (0.0063 mmol; y= 32%).

^{1}H NMR (700 MHz, DMSO) δ 15.34 (s, 1H), 10.53 (s, 1H), 8.85 (m, 3H), 7.95 (s, 4H), 7.82 (ddd, *J* = 25.7, 15.6, 8.3 Hz, 8H), 7.57 (d, *J* = 8.4 Hz, 2H), 7.47 (s, 1H), 7.44 (d, *J* = 8.7 Hz, 1H), 7.35 (d, *J* = 8.2 Hz, 2H), 7.08 (d, *J* = 8.7 Hz, 1H), 6.96 (s, 1H), 5.02 (dt, *J* = 12.3, 6.1 Hz, 1H), 4.90 (dd, *J* =

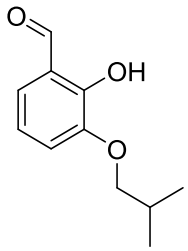
13.8, 7.6 Hz, 1 H), 3.54 (dd, *J* = 13.2, 6.9 Hz, 2H), 2.92 (t, *J* = 7.1 Hz, 2H), 2.68 (ddd, *J* = 20.6, 15.1, 7.1 Hz, 2H), 1.19 (d, *J* = 6.0 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 166.9, 166.1, 165.3, 164.8, 163.1, 143.1, 142.0, 141.7, 138.5, 137.5, 134.0, 132.4, 131.9, 129.7, 129.5, 128.6, 128.0, 127.6, 127.5, 124.2, 123.7, 119.0, 118.3, 117.6, 116.0, 113.5, 100.6, 70.3, 51.8, 40.7, 36.9, 34.7, 22.7.

HRMS (ESI) calculated for C45H42N7O10 ($M+H^+$) 840.2988, found 840.2994.

4.2.2.3 C-terminal OPTIMIZATION

2-Hydroxy-3-isobutoxybenzaldehyde (109)



Chemical Formula: $C_{11}H_{14}O_3$
Exact Mass: 194,0943

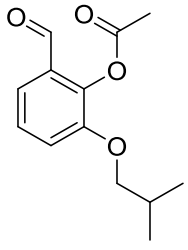
A solution of 2,3-dihydroxybenzaldehyde (15 g; 109 mmol) in DMSO (75 mL) was added drop wise, keeping low the temperature with an ice bath, to a previously prepared suspension of NaH (8.72 g; 218 mmol) in DMSO (180 mL). The mixture stirred at r.t. for two hours, then isobutyl bromide (11.9 mL; 109 mmol) was added slowly keeping the temperature low. The reaction mixture was stirred for 42 hours, quenched with HCl followed by NH_4Cl until pH 5 was reached. Work up done in several portions as follows: 300 mL of the mixture were further diluted with H_2O (1200 mL) and extracted with Et_2O (3x 200 mL). Organic phases dried over Na_2SO_4 and reduced under pressure to give 30 g of dark oil. The crude residue was chromatographed on silica gel using isocratic condition (Pet. Et. DCM 7:3) to give 8.04 g of a yellow oil (41 mmol, y= 38%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.98 (s, 1H), 9.92 (s, 1H), 7.17 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.11 (dd, *J* = 8.0, 1.2 Hz, 1H), 6.93 (t, *J* = 7.9 Hz, 1H), 3.81 (d, *J* = 6.7 Hz, 2H), 2.17 (dp, *J* = 13.3, 6.7 Hz, 1H), 1.05 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 196.5, 152.1, 147.9, 124.5, 121.0, 119.7, 119.5, 75.9, 28.2, 19.2.

HRMS (ESI) calculated for C11H15O3 ($M+H^+$) 195.1016, found 195.1014.

2-Formyl-6-isobutoxyphenyl acetate (110)



Chemical Formula: $C_{13}H_{16}O_4$
Exact Mass: 236,1049

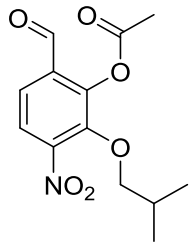
Acetyl chloride (5.93 mL; 55.2 mmol) was added dropwise to a stirred solution of 2-hydroxy-3-isobutoxybenzaldehyde (**109**) (7.15 g; 36.8 mmol) and pyridine (8.90 mL; 110.0 mmol) in DCM (340 mL) at 0 °C. The reaction was stirred for 5 min. at 0 °C then temperature raised to r.t., stirring was prolonged for 1 h. The reaction was quenched with HCl 1 N, organic phase partially reduced under vacuum, washed with HCl 1 N (200 mL), brine (200 mL), dried over sodium sulphate and reduced under vacuum. The crude was chromatographed on silica gel with a gradient 2-10% EtOAc in Pet. Et to give 8.46 g of a pale yellow oil (35.85 mmol; y= 97%).

^{1}H NMR (700 MHz, DMSO) δ 10.12 (s, 1H), 7.46 (dd, *J* = 7.2, 2.5 Hz, 1H), 7.42 – 7.37 (m, 2H), 3.82 (d, *J* = 6.3 Hz, 2H), 2.34 (s, 3H), 2.00 (dp, *J* = 13.2, 6.6 Hz, 1H), 0.96 (d, *J* = 6.8 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 190.0, 168.5, 150.9, 141.1, 128.9, 127.0, 120.4, 119.4, 74.6, 27.7, 20.2, 18.8.

HRMS (ESI) calculated for C13H16NaO4 ($M+Na^{+}$) 259.0941, found 259.0943.

6-Formyl-2-isobutoxy-3-nitrophenyl acetate (111)



Chemical Formula: $C_{13}H_{15}NO_6$
Exact Mass: 281,0899

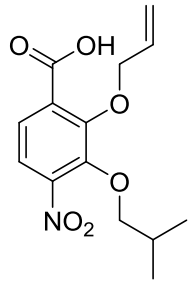
Fuming nitric acid (5.0 mL, 118.4 mmol) was cooled to −40 °C under a nitrogen atmosphere. A solution of 2-formyl-6-isobutoxyphenyl acetate (**110**) (3.50 g, 14.8 mmol) in 8.0 mL of dry DCM was added dropwise while the mixture was vigorously stirred and kept at −40 °C. The solution was stirred for an additional 1.5 hours before being poured into 100 mL of ice water. The mixture was then extracted with DCM (4 × 50 mL) and the combined organic extracts were dried over sodium sulphate. The solvent was removed under vacuum, the crude thus obtained was chromatographed on silica gel with a gradient 5-40% EtOAc in Pet. Et. to afford 2.45 g of the desired compound (8.73 mmol, y= 59%).

^{1}H NMR (500 MHz, DMSO) δ 10.13 – 10.09 (s 1H), 8.03 – 8.00 (m, 1H), 7.83 (d, *J* = 8.5 Hz, 1H), 3.84 (d, *J* = 6.2 Hz, 2H), 2.44 (s, 3H), 2.01 – 1.93 (m, 1H), 0.94 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 189.4, 168.5, 147.5, 145.2, 144.7, 132.0, 125.5, 122.3, 81.5, 28.5, 20.4, 18.5.

HRMS (ESI) calculated for C13H15NNaO6 ($M+Na^+$) 304.0792, found 304.0792.

2-(Allyloxy)-3-isobutoxy-4-nitrobenzoic acid (112)



Chemical Formula: $C_{14}H_{17}NO_6$
Exact Mass: 295,1056

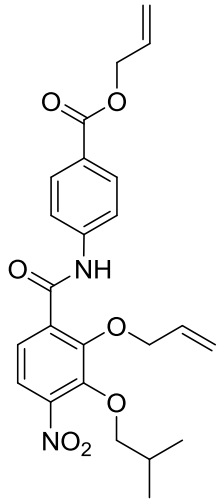
6-Formyl-2-isobutoxy-3-nitrophenyl acetate (**111**) (4.10 g; 14.6 mmol) was dissolved in THF (70 mL) and water (35 mL), then LiOH (3.50 g; 146.0 mmol) dissolved in water (35 mL) was added at 0 °C, the reaction was stirred overnight. In the morning, pH adjusted to 1, solvent partially reduced under vacuum and watery phase extracted with $CHCl_3$ (150 mL) three times, combined organic phases dried over sodium sulphate and reduced under vacuum to give a yellow oil, which was used in the next step without further purification. Residue was dissolved in DMF (30 mL), K_2CO_3 (4.03 g; 29.2 mmol) followed by allyl bromide (1.89 mL; 21.9 mmol) were added, the reaction was stirred for 24 h at r.t.. Reaction mixture diluted with water (200 mL) and EtOAc (200 mL), aqueous phase extracted with EtOAc (150 mL). Combined organic phases washed with brine (300 mL), dried over sodium sulphate and reduced under vacuum to give a crude material, which was dissolved with 2-Methyl-2-butene (15.5 mL; 146 mmol) in *t*-BuOH (100 mL). Then a solution of $NaClO_2$ 80% (1.98 g; 17.52 mmol) in monosodium phosphate monohydrate solution 1 N (16.2 mL) was added dropwise to the solution. Reaction stirred for 1 h, t hen quenched by adding a solution of Na_2SO_3 (34.0 mmol in 10 mL). Mixture partially reduced under vacuum, diluted with EtOAc (200 mL) and HCl 1 N (200 mL), aqueous phase extracted again with EtOAc (100 mL), organic phases reunited washed with brine (250 mL) and dried over sodium sulphate. Solvent reduced under vacuum, crude chromatographed on silica gel with a gradient 0-10% MeOH in DCM to afford 3.01 g of the desired compound (10.22 mmol, y= 70%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.90 (d, *J* = 8.7 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 1H), 6.08 (m, 1H), 5.44 (dq, *J* = 17.1, 1.3 Hz, 1H), 5.39 (ddd, *J* = 10.3, 1.9, 0.9 Hz, 1H), 4.81 – 4.77 (m, 2H), 3.93 (d, *J* = 6.5 Hz, 2H), 2.12 (dp, *J* = 13.3, 6.7 Hz, 1H), 1.03 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 165.0, 152.7, 148.2, 146.4, 131.1, 127.5, 126.8, 121.7, 119.8, 82.0, 76.5, 29.0, 19.0.

HRMS (ESI) calculated for C14H16NO6 ($M-H^+$) 294.0983, found 294.0995.

Allyl 4-(2-(allyloxy)-3-isobutoxy-4-nitrobenzamido)benzoate (113)



Chemical Formula: $C_{24}H_{26}N_2O_7$
Exact Mass: 454,1740

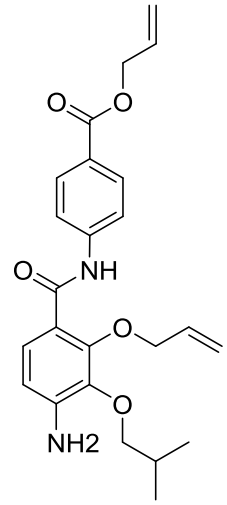
To a stirred solution of 2-(allyloxy)-3-isobutoxy-4-nitrobenzoic acid (500 mg; 1.69 mmol), allyl 4-aminobenzoate (250 mg; 1.41 mmol) and TEA (0.38 mL; 2.82 mmol) in dry DCM (28 mL), $POCl_3$ (0.16 mL; 1.69 mmol) was added drop wise at 0 °C. Reaction stirred for 4 hours then quenched by addition of $NaHCO_3$ saturated solution, solvent partially reduced under vacuum and residue dissolved in EtOAc (50 mL). The organic phase was washed with $NaHCO_3$ saturated solution (50 mL), HCl 1 N (50 mL), brine (50 mL), dried over sodium sulphate and reduced under vacuum. The crude thus obtained was purified on silica gel with a gradient 2-20% EtOAc in Pet. Et. to give 400 mg of desired product (0.88 mmol; y= 62%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.09 (m, 3H), 7.79 – 7.73 (m, 2H), 7.64 (d, *J* = 8.8 Hz, 1H), 6.08 (m, 2H), 5.48 (dq, *J* = 17.1, 1.3 Hz, 1H), 5.45 – 5.39 (m, 2H), 5.30 (dq, *J* = 10.5, 1.3 Hz, 1H), 4.83 (dt, *J* = 5.7, 1.4 Hz, 2H), 4.76 (dt, *J* = 6.1, 1.0 Hz, 2H), 3.97 (d, *J* = 6.5 Hz, 2H), 2.20 – 2.11 (m, 1H), 1.06 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 165.6, 161.1, 151.3, 147.3, 146.5, 141.8, 132.3, 131.5, 131.0, 130.8, 126.3, 126.1, 121.2, 120.1, 119.4, 118.3, 82.0, 76.2, 65.5, 29.1, 19.0.

HRMS (ESI) calculated for C24H27N2O7 ($M+H^+$) 455.1813, found 455.1806.

Allyl 4-(2-(allyloxy)-4-amino-3-isobutoxybenzamido)benzoate (107)



Chemical Formula:
$C_{24}H_{28}N_2O_5$
Exact Mass: 424,1998

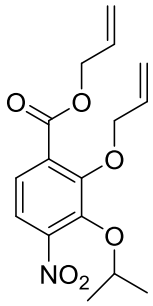
Allyl 4-(2-(allyloxy)-3-isobutoxy-4-nitrobenzamido)benzoate (350 mg; 0.77 mmol) was dissolved in EtOH (13.5 mL) and AcOH (1.5 mL), the solution was cooled to 0 °C and to it Zn dust (500 mg; 7.70 mmol) was added portion wise over few minutes. Reaction stirred at r.t. for 4 hours then mixture filtered over a pad of celite and solvent reduced under vacuum to afford 295 mg of desired compound (0.70 mmol; y= 90%).

^{1}H NMR (500 MHz, DMSO) δ 10.24 (s, 1H), 7.98 – 7.90 (m, 2H), 7.84 – 7.78 (m, 2H), 7.41 – 7.36 (m, 1H), 6.58 (d, *J* = 8.6 Hz, 1H), 6.05 (m, 2H), 5.54 (s, 2H), 5.41 (ddq, *J* = 17.1, 15.3, 1.6 Hz, 2H), 5.27 (m, 2H), 4.78 (dt, *J* = 5.4, 1.5 Hz, 2H), 4.61 (dt, *J* = 5.6, 1.3 Hz, 2H), 3.67 (d, *J* = 6.5 Hz, 2H), 2.11 (tt, *J* = 13.6, 6.8 Hz, 1H), 1.01 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 165.0, 163.9, 150.4, 146.6, 143.6, 137.2, 133.5, 132.8, 130.3, 126.2, 123.7, 118.8, 118.3, 117.8, 114.8, 110.2, 78.5, 74.4, 64.8, 28.5, 19.2.

HRMS (ESI) calculated for C24H27N2O5 ($M-H^+$) 423.1925, found 423.1933.

Allyl 2-(allyloxy)-3-isopropoxy-4-nitrobenzoate (114)



Chemical Formula: $C_{16}H_{19}NO_6$
Exact Mass: 321,1212

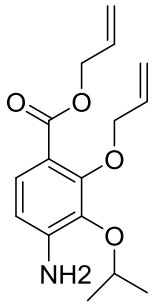
6-Formyl-2-isopropoxy-3-nitrophenyl acetate (0.9 g; 3.37 mmol) was dissolved in THF (17 mL) and water (8.5 mL), then LiOH (0.81 g; 33.70 mmol) dissolved in water (8.5 mL) was added at 0 °C, reaction stirred overnight. In the morning, pH adjusted to 1, solvent partially reduced under vacuum and watery phase extracted with $CHCl_3$ (100 mL) three times, combined organic phases dried over sodium sulphate and reduced under vacuum to give a yellow oil. This was dissolved with 2-Methyl-2-butene (3.75 mL; 35.39 mmol) in *t*-BuOH (25 mL), then a solution of $NaClO_2$ 80% (0.46 g; 4.04 mmol) in monosodium phosphate monohydrate solution 1 N (3.75 mL) was added dropwise to the solution. The reaction was stirred for 1 h, it was then quenched by adding a solution of Na_2SO_3 (8.0 mmol in 5 mL). Mixture partially reduced under vacuum, diluted with EtOAc (100 mL) and HCl 1 N (100 mL), aqueous phase extracted again with EtOAc (50 mL), organic phases reunited washed with brine (150 mL) and dried over sodium sulphate. Solvent removed under vacuum, the residue was dissolved in DMF (9.0 mL), K_2CO_3 (1.40 g; 10.11 mmol) followed by allyl bromide (0.73 mL; 8.43 mmol) were added, reaction stirred 24 h at r.t.. The reaction was diluted with water (100 mL) and EtOAc (100 mL), aqueous phase extracted with EtOAc (50 mL). Combined organic phases washed with brine (100 mL), dried over sodium sulphate and reduced under vacuum, the crude material was chromatographed on silica gel with a gradient 0-10% EtOAc in Pet. Et. to give 0.84 g a yellow oil (2.63 mmol; y= 79%).

^{1}H NMR (500 MHz, DMSO) δ 7.70 (d, *J* = 8.5 Hz, 1H), 7.57 (d, *J* = 8.5 Hz, 1H), 6.08 – 5.97 (m, 2H), 5.40 (m, 2H), 5.28 (m, 2H), 4.81 (dt, *J* = 5.6, 1.4 Hz, 2H), 4.64 (dt, *J* = 12.3, 6.1 Hz, 1H), 4.56 (dt, *J* = 5.8, 1.4 Hz, 2H), 1.20 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 164.1, 151.7, 147.8, 143.8, 133.2, 132.1, 130.4, 124.8, 119.1, 118.7, 118.5, 77.2, 74.8, 66.0, 22.0.

HRMS (ESI) calculated for C16H19NNaO6 ($M+Na^+$) 344.1105, found 344.1105.

Allyl 2-(allyloxy)-4-amino-3-isopropoxybenzoate (115)



Chemical Formula: $C_{16}H_{21}NO_4$
Exact Mass: 291,1471

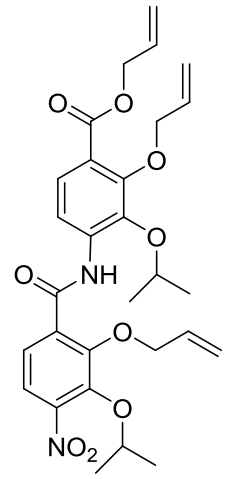
Allyl 2-(allyloxy)-3-isopropoxy-4-nitrobenzoate 700 mg; 2.17 mmol) was dissolved in EtOH (19.8 mL) and AcOH (2.2 mL), the solution was cooled to 0 °C and to it Zn dust (1.42 g; 21.7 mmol) was added portion wise. The reaction was stirred at r.t. for 4 hours then mixture filtered over a pad of celite and solvent reduced under vacuum, the residue was chromatographed on silica gel with a gradient 1-20% EtOAc in Pet. Et. to afford 550 mg of desired compound (1.89 mmol; y= 87%).

^{1}H NMR (500 MHz, DMSO) δ 7.33 (d, *J* = 8.6 Hz, 1H), 6.46 (d, *J* = 8.7 Hz, 1H), 6.10 – 5.94 (m, 2H), 5.62 (s, 2H), 5.34 (m, 2H), 5.20 (m, 2H), 4.66 (dt, *J* = 5.4, 1.5 Hz, 2H), 4.45 – 4.38 (m, 3H), 1.21 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 164.6, 153.1, 148.5, 136.2, 134.6, 133.2, 127.2, 117.5, 116.8, 111.0, 109.2, 74.1, 73.6, 64.2, 40.0, 39.9, 39.8, 39.8, 39.7, 39.5, 39.3, 39.2, 39.0, 22.1.

HRMS (ESI) calculated for C16H22NO4 ($M+H^+$) 292.1543, found 292.1541.

Allyl 2-(allyloxy)-4-(2-(allyloxy)-3-isopropoxy-4-nitrobenzamido)-3-isopropoxybenzoate (116)



Chemical Formula: $C_{29}H_{34}N_2O_9$
Exact Mass: 554,2264

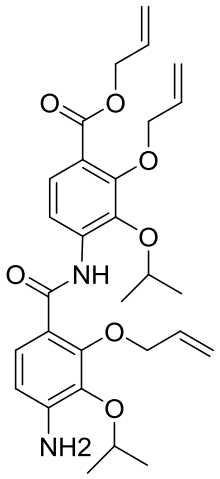
Collidine (1.44 mL; 11.00 mmol) was added dropwise at 0 °C to a solution of 2-(allyloxy)-3-isopropoxy-4-nitrobenzoic acid (579 mg; 2.06 mmol) and bis(trichloromethyl) carbonate (204 mg; 0.69 mmol) in THF (13 mL). The reaction was stirred at r.t. for 20 min then added to a cooled solution of allyl 2-(allyloxy)-4-amino-3-isopropoxybenzoate (400 mg; 1.37 mmol) and DiPEA (2.38 mL; 13.7 mmol) in THF (13 mL). The reaction was stirred for 20 hours at r.t., then quenched with water and solvent partially reduced under vacuum, the mixture was diluted with Et_2O (50 mL) and HCl 1N (50 mL), watery phase extracted again with Et_2O (50 mL). The combined organic phases were washed with brine (100 mL), dried over sodium sulphate and reduced under vacuum. The crude was chromatographed on silica gel with a gradient 1-20% EtOAc in Pet. Et. to give 535 mg of desired product (0.97 mmol; y= 70%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.73 (s, 1H), 8.42 (d, *J* = 8.8 Hz, 1H), 8.02 (d, *J* = 8.8 Hz, 1H), 7.68 (d, *J* = 8.8 Hz, 1H), 7.62 (d, *J* = 8.8 Hz, 1H), 6.18 – 6.00 (m, 3H), 5.45 – 5.35 (m, 2H), 5.34 – 5.27 (m, 2H), 5.26 (m, 2H), 4.83 – 4.76 (m, 5H), 4.65 (dt, *J* = 12.3, 6.2 Hz, 1H), 4.58 (dt, *J* = 5.9, 1.3 Hz, 2H), 1.37 (d, *J* = 6.2 Hz, 6H), 1.28 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 165.0, 161.3, 151.8, 151.2, 147.9, 145.0, 140.4, 137.4, 133.8, 132.2, 131.7, 131.6, 127.0, 125.9, 121.8, 121.1, 119.9, 118.5, 118.0, 115.1, 78.7, 76.3, 76.2, 74.9, 65.6, 22.5, 22.4.

HRMS (ESI) calculated for C29H35N2O9 ($M+H^+$) 555.2337, found 555.2335.

Allyl 2-(allyloxy)-4-(2-(allyloxy)-4-amino-3-isopropoxybenzamido)-3-isopropoxybenzoate (108)



Chemical Formula: $C_{29}H_{36}N_2O_7$
Exact Mass: 524,2523

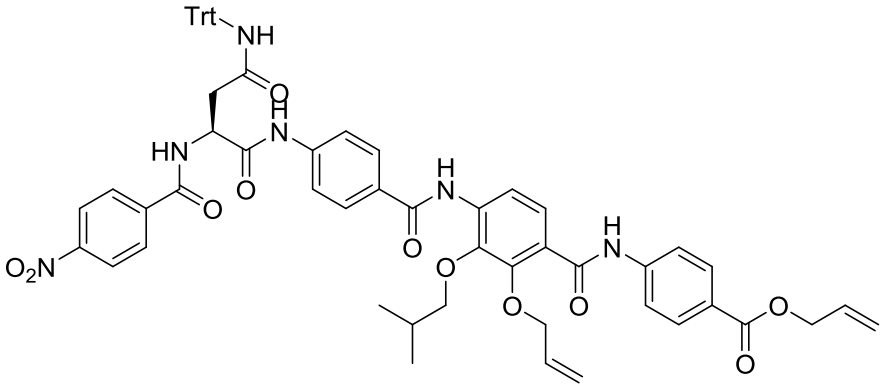
Allyl 2-(allyloxy)-4-(2-(allyloxy)-3-isopropoxy-4-nitrobenzamido)-3-isopropoxybenzoate (250 mg; 0.45 mmol) was dissolved in EtOH (9.0 mL) and AcOH (1.0 mL), the solution was cooled to 0 °C and to it Zn dust (295 mg; 4.50 mmol) was added portion wise. The reaction was stirred at r.t. for 5 hours then the mixture was filtered over a pad of celite and solvent reduced under vacuum, residue dissolved in DCM (30 mL), organic phase washed with $NaHCO_3$ saturated solution and dried over sodium sulphate. The solvent was removed under reduced pressure to give 220 mg of desired product (0.42 mmol; y= 93%).

^{1}H NMR (500 MHz, DMSO) δ 10.75 (s, 1H), 8.34 (d, *J* = 8.8 Hz, 1H), 7.55 (d, *J* = 8.7 Hz, 1H), 7.53 (d, *J* = 8.8 Hz, 1H), 6.58 (d, *J* = 8.7 Hz, 1H), 6.12 – 5.98 (m, 3H), 5.70 (s, 2H), 5.43 – 5.36 (m, 2H), 5.31 (m, 2H), 5.27 – 5.19 (m, 2H), 4.75 (dt, *J* = 5.5, 1.5 Hz, 2H), 4.67 (d, *J* = 6.5 Hz, 2H), 4.61 – 4.55 (m, 1H), 4.53 (dt, *J* = 5.7, 1.4 Hz, 2H), 4.45 – 4.39 (m, 1H), 1.29 (d, *J* = 6.2 Hz, 6H), 1.23 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 164.5, 162.9, 151.2, 150.6, 148.3, 139.5, 138.5, 135.1, 134.0, 133.0, 132.7, 126.7, 126.2, 119.8, 119.3, 118.1, 117.5, 114.2, 113.1, 110.3, 75.7, 74.8, 74.2, 74.1, 65.0, 22.2, 22.0.

HRMS (ESI) calculated for C29H37N2O7 ($M+H^+$) 525.2595, found 525.2591.

Allyl (S)-4-(2-(allyloxy)-3-isobutoxy-4-(4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzamido)benzamido)benzoate (117)



Chemical Formula: $C_{61}H_{56}N_6O_{11}$
Exact Mass: 1048,4007

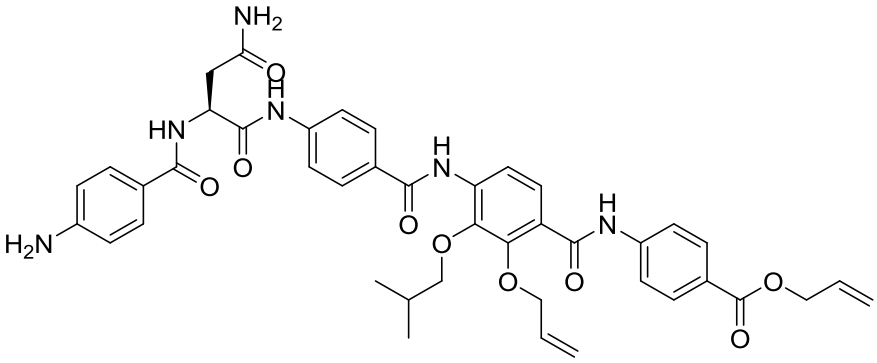
$POCl_3$ (2.3 mmol) as a solution in DCM (1:9) was added dropwise to a solution allyl 4-(2-(allyloxy)-4-amino-3-isobutoxybenzamido)benzoate (0.390 g; 0.92 mmol) and (S)-4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzoic acid (1.48g, 2.3 mmol) in THF (9.2 mL) and DCM (7.0 mL) at 0 °C, followed by DiPEA (1.28 mL; 7.36 mmol) as a solution in DCM (1:1). The reaction was stirred at r.t. for 4 hours, then it was quenched with HCL 1 N and ice, the solvent was partially reduced under vacuum and residue diluted with EtOAc (200 mL) and HCl 1N (200 mL), organic phase washed with brine (200 mL) and dried over sodium sulphate. The solvent was removed under vacuum, the crude residue was chromatographed on silica gel with a gradient EtOAc 20-90% in Pet. Et to give 626 mg of an orange residue (0.60 mmol; 65%).

^{1}H NMR (500 MHz, DMSO) δ 10.58 (s, 1H), 10.52 (s, 1H), 9.58 (s, 1H), 9.21 (d, *J* = 7.6 Hz, 1H), 8.70 (s, 1H), 8.41 – 8.37 (m, 2H), 8.19 – 8.15 (m, 2H), 7.99 (dd, *J* = 8.8, 2.0 Hz, 4H), 7.87 (d, *J* = 8.7 Hz, 2H), 7.78 (d, *J* = 8.8 Hz, 2H), 7.69 (d, *J* = 8.5 Hz, 1H), 7.40 (d, *J* = 8.4 Hz, 1H), 7.23 – 7.14 (m, 15H), 6.10 – 5.96 (m, 2H), 5.44 – 5.38 (m, 1H), 5.36 (ddd, *J* = 17.2, 3.1, 1.6 Hz, 1H), 5.28 (ddd, *J* = 10.5, 2.6, 1.3 Hz, 1H), 5.19 (dd, *J* = 10.5, 1.3 Hz, 1H), 4.97 – 4.91 (m, 1H), 4.79 (d, *J* = 5.3 Hz, 2H), 4.60 (d, *J* = 5.5 Hz, 2H), 3.82 (d, *J* = 6.2 Hz, 2H), 3.00 (dd, *J* = 14.8, 10.3 Hz, 1H), 2.77 (dd, *J* = 14.8, 4.5 Hz, 1H), 1.99 (tt, *J* = 13.4, 6.7 Hz, 1H), 0.94 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 168.3, 164.9, 164.6, 164.5, 149.4, 149.2, 144.8, 144.7, 143.5, 142.2, 139.3, 134.8, 133.6, 132.8, 130.3, 129.0, 128.5, 128.5, 127.6, 127.4, 126.4, 124.2, 123.6, 119.9, 119.0, 118.7, 117.8, 79.4, 74.6, 69.4, 64.8, 52.1, 38.0, 28.6, 19.0.

HRMS (ESI) calculated for C61H57N6O11 ($M+H^+$) 1049.4080, found 1049.4096.

Allyl (S)-4-(2-(allyloxy)-4-(4-(4-amino-2-(4-aminobenzamido)-4-oxobutanamido)benzamido)-3-isobutoxybenzamido)benzoate (119)



Chemical Formula: $C_{42}H_{44}N_6O_9$
Exact Mass: 776,3170

Zn dust (0.314 g; 4.8 mmol) was added portion wise over few minutes to a stirred solution of intermediate **117** (0.25 g; 0.24 mmol) in THF (1.5 mL), EtOH (1.4 mL) and AcOH (0.15 mL). The reaction was stirred for 5 hours, the mixture was filtered through celite and the solvent was reduced under vacuum. The crude was used in the next step without further purification.

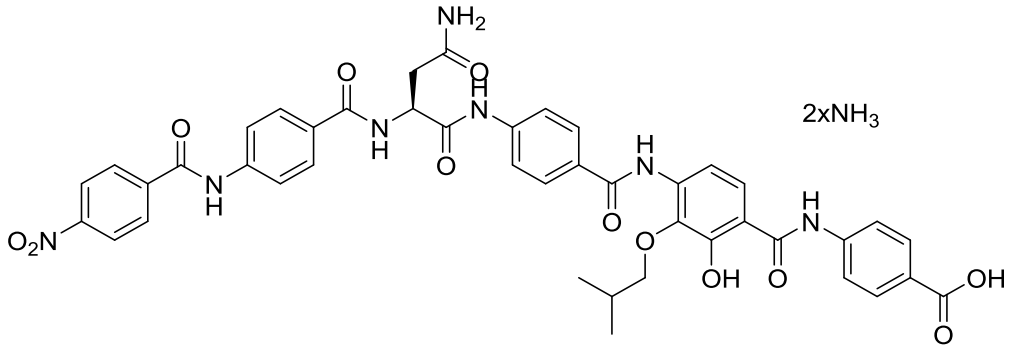
The residue was dissolved in DCM (9.0 mL), Tips (0.148 mL; 0.72 mmol) followed by TFA (3.0 mL) were added at 0 °C. The reaction was stirred for 2 hours at r.t. then the solvent was removed under vacuum, the residue take up and evaporated twice with DCM (5 mL) then triturated 3x with ice cold Pet. Et.. The crude thus obtained was purified on silica gel with a gradient 0-10% MeOH in DCM to give 140 mg of a yellow solid (0.18 mmol; y= 76%).

^{1}H NMR (500 MHz, DMSO) δ 10.58 (s, 1H), 10.36 (s, 1H), 9.56 (s, 1H), 8.22 (d, *J* = 7.4 Hz, 1H), 7.98 (dd, *J* = 11.9, 5.0 Hz, 4H), 7.87 (d, *J* = 8.8 Hz, 2H), 7.77 (d, *J* = 8.9 Hz, 2H), 7.69 (d, *J* = 8.5 Hz, 1H), 7.63 – 7.59 (m, 2H), 7.39 (d, *J* = 8.4 Hz, 2H), 6.97 (s, 1H), 6.58 – 6.54 (m, 2H), 6.10 – 5.96 (m, 2H), 5.70 (br, 2H), 5.41 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.36 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.28 (ddd, *J* = 10.5, 3.0, 1.4 Hz, 1H), 5.19 (ddd, *J* = 10.5, 3.0, 1.3 Hz, 1H), 4.84 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.79 (dt, *J* = 5.3, 1.4 Hz, 2H), 4.60 (d, *J* = 5.5 Hz, 2H), 3.81 (d, *J* = 6.2 Hz, 2H), 2.66 – 2.62 (m, 2H), 1.99 (dp, *J* = 13.2, 6.6 Hz, 1H), 0.94 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 171.5, 171.0, 166.4, 165.0, 164.6, 164.5, 151.8, 149.4, 144.7, 143.5, 142.4, 134.8, 133.6, 132.8, 130.4, 129.1, 128.4, 128.3, 127.6, 124.2, 123.5, 120.4, 119.9, 119.0, 118.6, 117.8, 112.5, 79.4, 74.6, 64.9, 51.5, 36.8, 28.6, 19.0.

HRMS (ESI) calculated for C42H45N6O9 ($M+H^+$) 777.3243, found 777.3225.

(S)-4-(4-(4-(4-Amino-2-(4-(4-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isobutoxybenzamido)benzoic acid (123)



Chemical Formula: $C_{43}H_{39}N_7O_{12}$
Exact Mass: 845,2657

Collidine (0.048 mL; 0.36 mmol) was added dropwise at 0 °C to a solution of 4-nitro benzoic acid (26 mg; 0.16 mmol) and bis(trichloromethyl) carbonate (14.0 mg; 0.047 mmol) in THF (2.3 mL). Reaction stirred at r.t. for 20 min then added to a solution of amine **119** (35 mg; 0.045 mmol) and DiPEA (0.078 mL; 0.45 mmol) in THF (2.3 mL). The reaction was stirred for 3 hours then quenched with HCl 1 N and ice. The solvent was partially reduced under vacuum, EtOAc (25 mL) and HCl 1N (25 mL) were added, organic phase washed with $NaHCO_3$ saturated solution (20 mL), brine (20 mL) and dried over sodium sulphate. The solvent was removed under reduced pressure, the residue thus obtained was used in the next step without further purification.

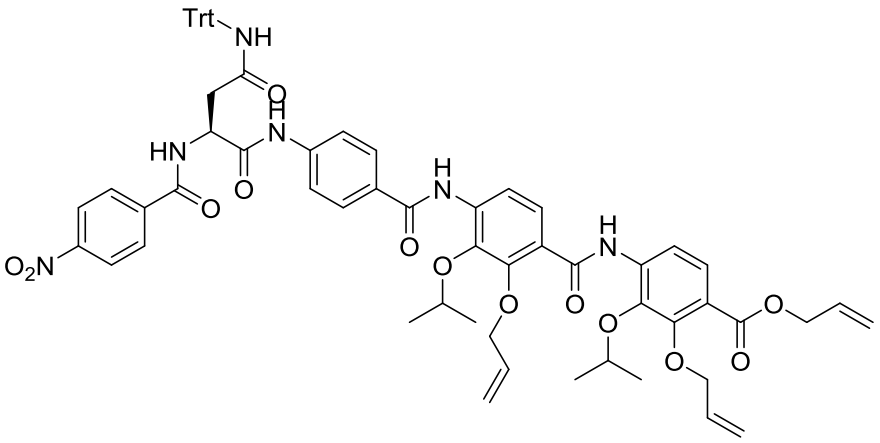
Phenyl silane (0.022 mL; 0.18 mmol) followed by palladium-tetrakis(triphenylphosphine (13.0 mg; 0.011 mmol) was added to a solution of the crude residue (0.045 mmol) in THF (4.5 mL). Reaction stirred for 3 hours and purified by preparative RP-HPLC with a gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min to afford 3.0 mg of desired product (0.0036 mmol; y= 8%).

^{1}H NMR (700 MHz, DMSO) δ 12.78 (br, 1H), 12.27 (br, 1H), 10.79 (s, 1H), 10.61 (br, 1H), 10.44 (s, 1H), 9.36 (br, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.41 – 8.36 (m, 2H), 8.24 – 8.19 (m, 2H), 7.99 – 7.92 (m, 6H), 7.90 (d, *J* = 8.8 Hz, 2H), 7.85 (d, *J* = 8.6 Hz, 2H), 7.79 (d, *J* = 8.8 Hz, 3H), 7.53 (br, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 13.9, 7.3 Hz, 1H), 3.83 (d, *J* = 6.4 Hz, 2H), 2.69 (dd, *J* = 6.9, 2.9 Hz, 2H), 2.01 (dp, *J* = 13.3, 6.6 Hz, 1H), 0.95 (d, *J* = 6.7 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 168.3, 166.9, 165.7, 164.3, 164.2, 149.3, 142.4, 141.5, 140.3, 130.2, 129.3, 129.2, 128.3, 128.3, 123.6, 122.9, 120.4, 119.5, 118.7, 78.5, 51.6, 36.8, 28.6, 19.1.

HRMS (ESI) calculated for C43H38N7O12 ($M-H^+$) 844.2584, found 844.2593.

Allyl (S)-2-(allyloxy)-4-(2-(allyloxy)-3-isopropoxy-4-(4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzamido)benzamido)-3-isopropoxybenzoate (118)



Chemical Formula: $C_{66}H_{64}N_6O_{13}$
Exact Mass: 1148,4531

$POCl_3$ (1.42 mmol) as a solution in DCM (1:9) was added dropwise to a solution of allyl 2-(allyloxy)-4-(2-(allyloxy)-4-amino-3-isopropoxybenzamido)-3-isopropoxybenzoate (0.300 g; 0.57 mmol) and (S)-4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)butanamido)benzoic acid (0.914 g, 1.42 mmol) in THF (7.2 mL) and DCM (5.0 mL) at 0 °C, followed by DiPEA (0.79 mL; 4.56 mmol) as a solution in DCM (1:1). Reaction stirred at r.t. for 5 h, quenched with HCl 1 N and ice, solvent partially reduced under vacuum and residue diluted with EtOAc (200 mL) and HCl 1N (200 mL), organic phase washed with brine (200 mL) and dried over sodium sulphate. Solvent removed under vacuum, the crude residue was chromatographed on silica gel with a gradient EtOAc 20-90% in Pet. Et to give 407 mg of an orange residue (0.35 mmol; 62%).

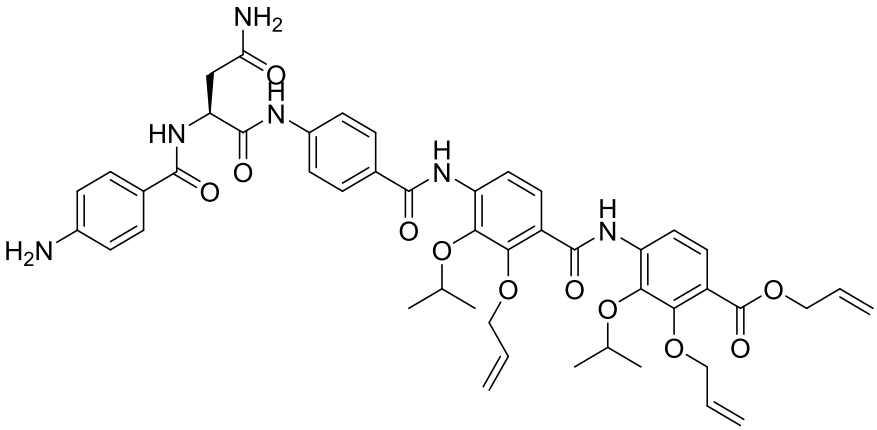
^{1}H NMR (500 MHz, $CDCl_3$) δ 10.73 (s, 1H), 9.63 (s, 1H), 8.74 (s, 1H), 8.47 (d, *J* = 2.3 Hz, 1H), 8.45 (d, *J* = 2.4 Hz, 1H), 8.44 (s, 1H), 8.27 – 8.24 (m, 2H), 8.00 (d, *J* = 8.9 Hz, 1H), 7.97 – 7.93 (m, 3H), 7.86 (d, *J* = 8.8 Hz, 2H), 7.67 (d, *J* = 8.8 Hz, 1H), 7.60 – 7.57 (m, 2H), 7.30 – 7.22 (m, 15H), 6.20 – 6.00 (m, 3H), 5.40 (tq, *J* = 17.2, 1.5 Hz, 2H), 5.33 – 5.25 (m, 2H), 5.25 – 5.21 (m, 2H), 5.12 – 5.06 (m, 1H), 4.80 (dt, *J* = 5.7, 1.3 Hz, 2H), 4.79 – 4.72 (m, 2H), 4.70 (d, *J* = 6.7 Hz, 2H), 4.59 (dt, *J* = 5.9, 1.3 Hz, 2H), 3.34 – 3.22 (m, 1H), 2.78 (dd, *J* = 15.6, 7.2 Hz, 1H), 1.41 (dd, *J* = 6.1, 3.3 Hz, 6H), 1.29 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.1, 168.8, 165.6, 165.1, 164.1, 162.8, 151.9, 149.9, 149.0, 143.9, 141.1, 140.2, 139.2, 138.3, 138.2, 137.2, 133.9, 132.9, 132.3, 128.6, 128.1, 128.0,

127.3, 127.2, 127.0, 123.8, 122.6, 120.7, 120.3, 119.8, 119.4, 119.2, 118.4, 117.8, 115.5, 115.0, 76.6, 76.0, 75.7, 74.8, 71.2, 65.5, 51.2, 37.6, 22.8, 22.5.

HRMS (ESI) calculated for C66H65N6O13 ($M+H^+$) 1149.4604, found 1149.4593.

Allyl (S)-2-(allyloxy)-4-(2-(allyloxy)-4-(4-(4-amino-2-(4-aminobenzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzamido)-3-isopropoxybenzoate (120)



Chemical Formula: $C_{47}H_{52}N_6O_{11}$
Exact Mass: 876,3694

Zn dust (0.56 g; 8.6 mmol) was added portionwise over few minutes to a stirred solution of allyl (S)-4-(2-(allyloxy)-3-isopropoxy-4-(4-(2-(4-nitrobenzamido)-4-oxo-4-(tritylamino)-butanamido)benzamido)benzamido)benzoate (0.49 g; 0.43 mmol) in THF (2.7 mL), EtOH (2.4 mL) and AcOH (0.27 mL). Reaction stirred for 5 hours, the mixture was filtered through celite, the solvent was reduced under vacuum. The crude was used in the next step without further purification.

The residue was dissolved in DCM (16.5 mL), Tips (0.264 mL; 1.29 mmol) followed by TFA (5.5 mL) were added at 0 C. Reaction stirred 2 h at r.t. then solved removed under vacuum, residue take up and evaporated twice with DCM (10 mL) then triturated 3x with ice cold Pet. Et.. The crude thus obtained was purified on silica gel with a gradient 0-10% MeOH in DCM to give 263 mg of a yellow solid (0.30 mmol; y= 70%).

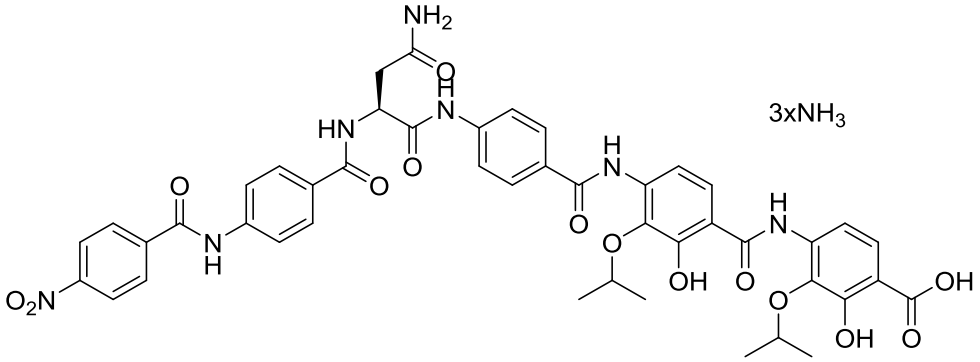
^{1}H NMR (500 MHz, DMSO) δ 10.89 – 10.54 (m, 1H), 10.39 (t, *J* = 7.9 Hz, 1H), 9.58 – 9.53 (m, 1H), 8.35 (d, *J* = 8.8 Hz, 1H), 8.22 (d, *J* = 7.4 Hz, 1H), 7.97 (d, *J* = 8.7 Hz, 2H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.84 – 7.77 (m, 3H), 7.63 – 7.59 (m, 2H), 7.56 (dd, *J* = 11.9, 8.7 Hz, 1H), 7.38 (s, 1H), 6.97 (s, 1H), 6.58 – 6.53 (m, 2H), 6.13 – 5.86 (m, 3H), 5.67 (br, 2H), 5.46 – 5.22 (m, 5H), 5.10 – 5.01 (m, 1H), 4.86 (m, 2H), 4.81 – 4.71 (m, 2H), 4.68 (d, *J* = 5.4 Hz, 1H), 4.62 (dt, *J* = 12.3, 6.1 Hz,

1H), 4.54 (d, *J* = 5.7 Hz, 1H), 4.46 (m, 2H), 2.64 (d, *J* = 7.1 Hz, 2H), 1.29 (dd, *J* = 12.4, 7.4 Hz, 3H), 1.24 (m, 6H), 1.19 (d, *J* = 6.1 Hz, 3H).

^{13}C NMR (126 MHz, DMSO) δ 171.5, 171.1, 168.7, 166.4, 164.5, 164.4, 152.9, 151.9, 149.8, 142.4, 141.3, 139.9, 137.7, 137.4, 136.6, 136.3, 134.0, 132.7, 132.2, 129.1, 128.5, 126.2, 125.5, 123.5, 120.4, 118.8, 118.5, 118.1, 117.7, 116.5, 114.6, 112.5, 76.6, 75.4, 74.2, 65.7, 65.1, 51.5, 36.9, 22.3, 22.1.

HRMS (ESI) calculated for C47H53N6O11 ($M+H^+$) 877.3767, found 877.3780.

(S)-4-(4-(4-(4-Amino-2-(4-(4-nitrobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)-2-hydroxy-3-isopropoxybenzoic acid (124)



Chemical Formula: $C_{45}H_{43}N_7O_{14}$
Exact Mass: 905,2868

Collidine (0.036 mL; 0.272 mmol) was added dropwise at 0 °C to a solution of 4-nitro benzoic acid (20 mg; 0.12 mmol) and bis(trichloromethyl) carbonate (11.6 mg; 0.039 mmol) in THF (1.75 mL). Reaction stirred at r.t. for 20 min then added to a solution of amine **120** (30 mg; 0.034 mmol) and DiPEA (0.060 mL; 0.34 mmol) in THF (1.75 mL). Reaction stirred for 3 h then quenched with HCl 1 N and ice. Solvent partially reduced under vacuum, EtOAc (25 mL) and HCl 1N (25 mL) were added, organic phase washed with $NaHCO_3$ saturated solution (20 mL), brine (20 mL) and dried over sodium sulphate. The solvent was removed under reduced pressure, the residue thus obtained was used in the next step without further purification.

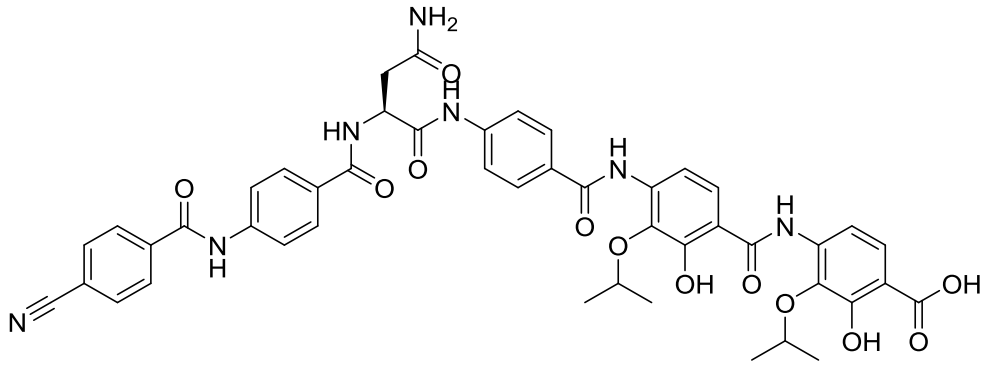
Phenyl silane (0.026 mL; 0.21 mmol) followed by palladium-tetrakis(triphenylphosphine (9.8 mg; 0.0085 mmol) was added to a solution of the crude residue (0.034 mmol) in THF (3.5 mL). The reaction was stirred overnight and purified by preparative RP-HPLC with a gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min to afford 1.7 mg of desired product (0.0046 mmol; y= 6%).

^{1}H NMR (700 MHz, DMSO) δ 11.26 (s, 1H), 10.90 (s, 1H), 10.79 (s, 1H), 10.45 (s, 1H), 9.60 (s, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.42 – 8.35 (m, 2H), 8.24 – 8.18 (m, 2H), 7.99 – 7.88 (m, 8H), 7.80 (dd, *J* = 8.7, 5.3 Hz, 4H), 7.54 – 7.48 (m, 2H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.69 (dt, *J* = 12.3, 6.1 Hz, 1H), 4.31 (dt, *J* = 12.2, 6.1 Hz, 1H), 2.71 – 2.65 (m, 2H), 1.31 – 1.23 (m, 12H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 165.8, 164.3, 164.2, 163.6, 162.3, 150.4, 149.3, 142.4, 141.5, 140.3, 138.4, 136.3, 134.0, 129.3, 129.2, 128.4, 128.3, 125.0, 124.8, 123.6, 119.5, 118.8, 116.4, 115.3, 112.5, 109.9, 75.7, 74.0, 51.6, 36.8, 22.0, 21.9.

HRMS (ESI) calculated for C45H42N7O14 (M-H^{+}) 904.2795, found 904.2786.

(S)-4-(4-(4-(4-Amino-2-(4-(4-cyanobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)-2-hydroxy-3-isopropoxybenzoic acid (126)



Chemical Formula: $C_{46}H_{43}N_7O_{12}$
Exact Mass: 885,2970

Compound **126** was synthesized starting from amine **120** (30 mg; 0.034 mmol) and 4-Cyanobenzoic acid (18 mg; 0.12 mmol) using the same experimental procedure employed for the synthesis of compound **124**.

The compound was purified by preparative RP-HPLC (gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min) to afford 8.7 mg of desired product (0.010 mmol; y=29%).

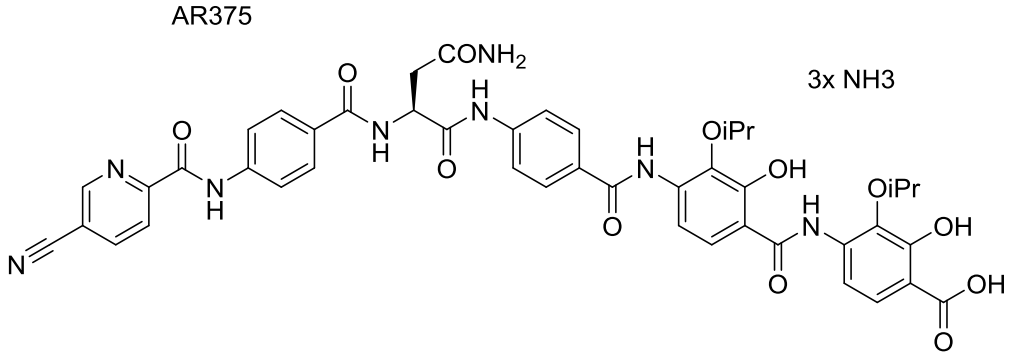
^{1}H NMR (700 MHz, DMSO) δ 11.25 (s, 1H), 10.93 (s, 1H), 10.70 (s, 1H), 10.44 (s, 1H), 9.60 (s, 1H), 8.67 (d, *J* = 7.3 Hz, 1H), 8.13 (d, *J* = 8.4 Hz, 2H), 8.05 (d, *J* = 8.4 Hz, 2H), 7.96 (t, *J* = 8.9 Hz, 3H), 7.91 (dd, *J* = 26.2, 8.8 Hz, 4H), 7.80 (m, 3H), 7.54 (d, *J* = 8.8 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 1H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.0, 7.3 Hz, 1H), 4.69 (dp, *J* = 12.3, 6.1 Hz, 1H), 4.30 (td, *J* = 12.1, 6.0 Hz, 1H), 2.71 – 2.67 (m, 2H), 1.27 (dd, *J* = 7.5, 6.3 Hz, 12H).

^{13}C NMR (176 MHz, DMSO) δ 172.0, 171.3, 170.7, 165.8, 164.5, 164.3, 163.6, 150.4, 142.4, 141.6, 138.7, 138.4, 136.3, 133.9, 132.5, 129.1, 128.6, 128.4, 128.3, 125.0, 124.9, 119.5,

118.8, 118.3, 116.4, 115.4, 114.0, 110.1, 75.7, 74.1, 51.6, 40.0, 39.9, 39.8, 39.6, 39.5, 39.4, 39.3, 39.2, 36.8, 22.0, 21.9.

HRMS (ESI) calculated for C46H42N7O12 ($M-H^+$) 884.2897, found 884.2917.

(S)-4-(4-(4-(4-Amino-2-(4-(5-cyanopicolinamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)-2-hydroxy-3-isopropoxybenzoic acid (127)



Chemical Formula: $C_{45}H_{42}N_8O_{12}$
Exact Mass: 886,2922

Compound **127** was synthesized starting from amine **120** (20 mg; 0.023 mmol) and 5-cyanopicolinic acid (12 mg; 0.08 mmol) using the same experimental procedure employed for the synthesis of compound **124**.

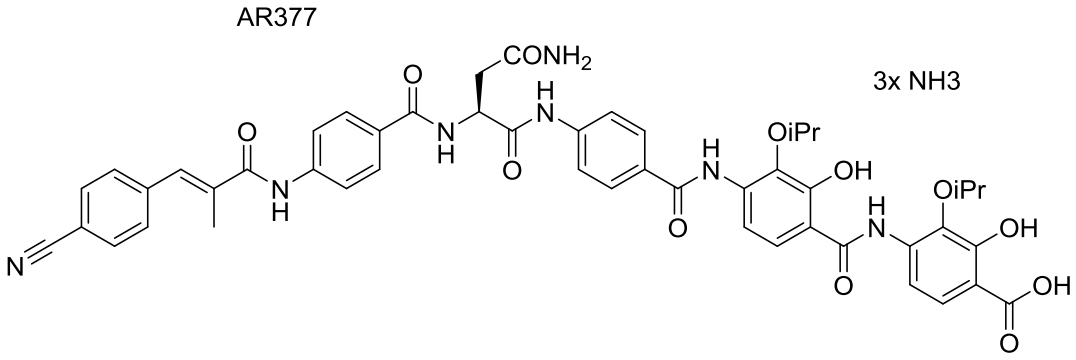
Preparative RP-HPLC (gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min) afforded 5.6 mg of desired product (0.0063 mmol; y=27%).

^{1}H NMR (700 MHz, DMSO) δ 11.26 (s, 1H), 11.03 (s, 1H), 10.89 (s, 1H), 10.43 (s, 1H), 9.59 (s, 1H), 9.22 (dd, *J* = 1.9, 0.7 Hz, 1H), 8.67 (d, *J* = 7.3 Hz, 1H), 8.60 (dd, *J* = 8.2, 2.0 Hz, 1H), 8.35 – 8.27 (m, 1H), 8.04 (d, *J* = 8.8 Hz, 2H), 7.97 (d, *J* = 8.8 Hz, 2H), 7.93 (d, *J* = 8.7 Hz, 3H), 7.80 (dd, *J* = 8.7, 4.8 Hz, 3H), 7.51 (t, *J* = 9.5 Hz, 2H), 7.40 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 13.9, 7.4 Hz, 1H), 4.72 – 4.66 (m, 1H), 4.31 (dt, *J* = 12.2, 6.1 Hz, 1H), 2.73 – 2.65 (m, 2H), 1.27 (dd, *J* = 5.8, 5.1 Hz, 12H).

^{13}C NMR (176 MHz, DMSO) δ 171.9, 171.3, 170.7, 165.8, 164.3, 163.6, 161.6, 152.3, 151.5, 150.4, 142.4, 142.3, 140.9, 138.4, 136.3, 134.0, 129.4, 128.4, 128.3, 124.9, 124.8, 122.5, 119.7, 118.8, 116.6, 116.4, 115.3, 111.7, 109.9, 75.7, 74.0, 51.6, 36.8, 22.0, 21.9.

HRMS (ESI) calculated for C45H43N8O12 ($M+H^+$) 887.2995, found 887.2992.

(S,E)-4-(4-(4-(4-Amino-2-(4-(3-(4-cyanophenyl)-2-methylacrylamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)-2-hydroxy-3-isopropoxybenzoic acid (129)



Chemical Formula: $C_{49}H_{47}N_7O_{12}$
Exact Mass: 925,3283

Amine **120** (25 mg, 0.033 mmol) was coupled with 5-cyanothiophene-2-carboxylic acid using coupling conditions A (general procedures section 4.2.2.2.3) followed by final deprotection. Desired compound purified by preparative RP-HPLC using condition B to obtain 6.6 mg of desired product (0.0071 mmol, y= 22%).

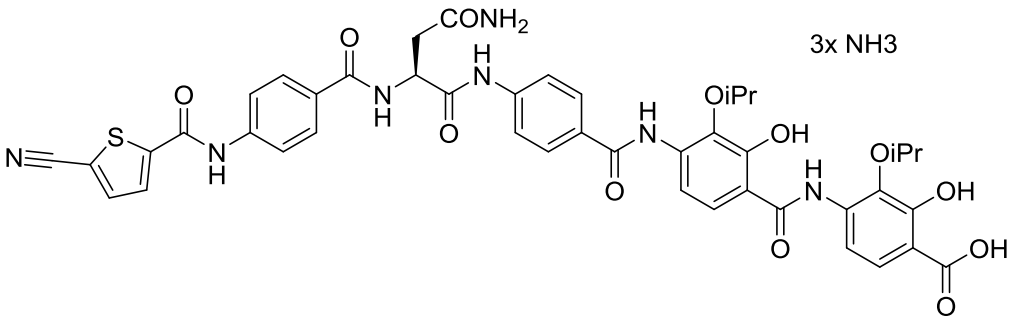
^{1}H NMR (700 MHz, DMSO) δ 11.26 (s, 1H), 10.91 (s, 1H), 10.44 (s, 1H), 10.26 (s, 1H), 9.60 (s, 1H), 8.64 (d, *J* = 7.2 Hz, 1H), 7.97 (d, *J* = 8.7 Hz, 2H), 7.95 (d, *J* = 6.3 Hz, 1H), 7.91 (dd, *J* = 11.4, 8.6 Hz, 4H), 7.83 (d, *J* = 8.7 Hz, 2H), 7.80 (dd, *J* = 8.8, 1.9 Hz, 3H), 7.67 (d, *J* = 8.3 Hz, 2H), 7.53 (d, *J* = 8.8 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 1H), 7.40 (s, 1H), 7.37 (s, 1H), 6.99 (s, 1H), 4.92 (dd, *J* = 14.0, 7.1 Hz, 1H), 4.69 (dt, *J* = 12.3, 6.1 Hz, 1H), 4.31 (dt, *J* = 12.2, 6.1 Hz, 1H), 2.69 (d, *J* = 7.5 Hz, 2H), 2.13 (d, *J* = 0.8 Hz, 3H), 1.27 (t, *J* = 6.2 Hz, 12H).

^{13}C NMR (176 MHz, DMSO) δ 172.0, 171.3, 170.7, 168.1, 165.8, 164.3, 163.6, 162.3, 155.0, 150.4, 142.4, 142.0, 140.6, 138.4, 136.3, 135.8, 134.0, 132.4, 131.7, 130.1, 128.6, 128.4, 128.3, 125.0, 124.8, 119.2, 118.8, 116.4, 115.4, 110.3, 110.1, 75.7, 74.1, 51.6, 36.8, 22.0, 21.9, 14.6.

HRMS (ESI) calculated for C49H48N7O12 ($M+H^+$) 926.3355, found 926.3337.

(S)-4-(4-(4-(4-Amino-2-(4-(5-cyanothiophene-2-carboxamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)-2-hydroxy-3-isopropoxybenzoic acid (128)

AR376



Chemical Formula: $C_{44}H_{41}N_7O_{12}S$
Exact Mass: 891,2534

Compound **128** was synthesized starting from amine **120** (20 mg; 0.023 mmol) and 5-cyanothiophene-2-carboxylic acid (12 mg; 0.08 mmol) using the same experimental procedure employed for the synthesis of compound **124**.

Preparative RP-HPLC (gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min) afforded 4.7 mg of desired product (0.0053 mmol; y=23%).

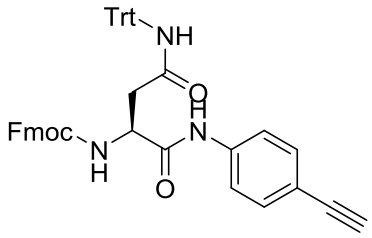
^{1}H NMR (700 MHz, DMSO) δ 11.27 (br, 1H), 10.88 (br, 1H), 10.78 (s, 1H), 10.44 (s, 1H), 9.59 (s, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.14 (d, *J* = 4.1 Hz, 1H), 8.08 (d, *J* = 4.0 Hz, 1H), 7.97 (d, *J* = 8.7 Hz, 2H), 7.94 (d, *J* = 8.7 Hz, 2H), 7.92 (br, 1H), 7.84 (d, *J* = 8.7 Hz, 2H), 7.80 (m, 3H), 7.51 (t, *J* = 7.8 Hz, 2H), 7.39 (s, 1H), 6.98 (s, 1H), 4.92 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.69 (dt, *J* = 12.3, 6.1 Hz, 1H), 4.31 (dt, *J* = 12.2, 6.1 Hz, 1H), 2.73 – 2.65 (m, 2H), 1.27 (m, 12H).

^{13}C NMR (176 MHz, DMSO) δ 171.9, 171.3, 170.7, 165.7, 164.3, 163.6, 158.5, 150.4, 146.8, 142.4, 140.8, 139.7, 138.4, 136.2, 134.0, 129.5, 129.2, 128.4, 124.9, 124.7, 119.7, 118.8, 116.4, 115.3, 113.8, 112.5, 109.9, 75.7, 73.9, 51.6, 36.8, 22.0, 21.9.

HRMS (ESI) calculated for C44H42N7O12S ($M+H^+$) 892.2607, found 892.2615.

4.2.2.4 AlbD-stable cystobactamid

(9H-Fluoren-9-yl)methyl (1-((4-ethynylphenyl)amino)-1,4-dioxo-4-(tritylamino)butan-2-yl)carbamate (134)



Chemical Formula:
$C_{46}H_{37}N_3O_4$
Exact Mass: 695,2784

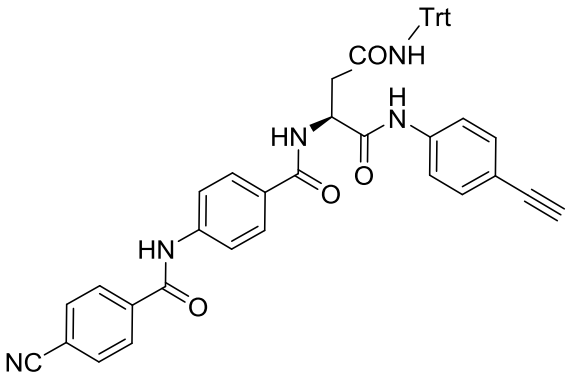
$POCl_3$ (40 µL, 0.43 mmol) was added at 0 °C to a stirred solution of Fmoc-Asn(Trt)-OH (254 mg, 0.43 mmol), triethylamine (60 µL, 0.43 mmol) and 4-ethynylaniline (25 mg, 0.215 mmol) in DCM (4 mL) under nitrogen. The reaction was stirred at °0 C for 2 hours. $NaHCO_3$ (5 mL) saturated solution and EtOAc (20 mL) were added, the organic phase washed again with $NaHCO_3$ (5 mL) and brine (20 mL), dried over sodium sulphate and reduced under vacuum to give a yellow oil which was chromatographed on silica gel with a solution Hexane/EtOAc 7:3 to give 131 mg of a white solid (0.19 mmol; y= 87 %).

^{1}H NMR (500 MHz, DMSO) δ 10.27 (s, 1H), 8.61 (s, 1H), 7.90 (d, J = 7.5 Hz, 2H), 7.78 (d, J = 8.0 Hz, 1H), 7.74 (dd, J = 7.3, 4.3 Hz, 2H), 7.65 (d, J = 8.7 Hz, 2H), 7.46 – 7.38 (m, 3H), 7.37 – 7.25 (m, 2H), 7.25 – 7.12 (m, 15H), 4.44 (td, J = 9.0, 5.3 Hz, 1H), 4.36 (dd, J = 10.4, 7.0 Hz, 1H), 4.29 (dd, J = 10.4, 7.0 Hz, 1H), 4.23 (t, J = 6.9 Hz, 1H), 4.09 (s, 1H), 2.75 (dd, J = 14.5, 9.8 Hz, 1H), 2.61 (dd, J = 14.5, 5.0 Hz, 1H).

^{13}C NMR (126 MHz, DMSO) δ 170.5, 168.5, 155.8, 144.7, 143.8, 140.7, 139.5, 132.3, 128.6, 127.7, 127.4, 127.1, 126.3, 125.3, 125.2, 120.1, 119.1, 116.2, 83.6, 79.9, 69.4, 65.8, 52.8, 46.7, 38.4.

HRMS (ESI) calculated for $C_{46}H_{36}N_3O_4$ (M-H$^+$) 694.2711, found 694.2690.

(S)-2-(4-(4-Cyanobenzamido)benzamido)-N1-(4-ethynylphenyl)-N4-tritylsuccinamide (131)



Chemical Formula: $C_{46}H_{35}N_5O_4$
Exact Mass: 721,2689

To a solution of 4-(4-cyanobenzamido) benzoic acid (15.5 mg, 0.058 mmol) and HBTU (27 mg, 0.77 mmol) in dry DMF (0.4 mL) DIPEA (30 µL, 0.174 mmol) was added. The mixture was stirred for 15 minutes before being added to a solution of 2-amino-*N*1-(4-ethynylphenyl)-*N*4-tritylsuccinamide (34 mg, 0.07 mmol) in dry DMF (0.6 mL), which was obtained cleaving the Fmoc protecting group from (9H-fluoren-9-yl)methyl (1-((4-ethynylphenyl)amino)-1,4-dioxo-4-(tritylamino)butan-2-yl)carbamate using standard conditions as already described herein (a 20% solution of diethylamine in CH_3CN).

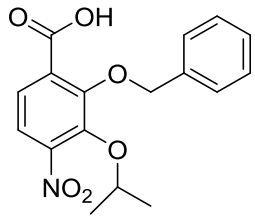
The solution was stirred for 3 hours, diluted with brine (5 mL) and water (5 mL) and extracted with ethyl acetate (3 × 5 mL). The combined organic extracts were washed with 1N HCl (5 mL), saturated aqueous $NaHCO_3$ solution (5 mL) and brine (5 mL). After drying over anhydrous Na_2SO_4, the solvent was removed by distillation at reduced pressure and the residue was subjected to flash chromatography with a gradient MeOH 0-5% in DCM to afford 29 mg of desired product as a pale yellow solid (0.04 mmol, y= 69%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.41 (s, 1H), 8.75 (s, 1H), 8.07 (s, 1H), 7.92 (d, *J* = 8.0 Hz, 2H), 7.65 (d, *J* = 8.1 Hz, 4H), 7.59 (d, *J* = 7.9 Hz, 2H), 7.42 (s, 1H), 7.37 (s, 4H), 7.25 – 7.13 (m, 15H), 5.05 – 4.94 (m, 1H), 3.19 (d, *J* = 12.3 Hz, 1H), 3.05 (s, 1H), 2.70 (dd, *J* = 15.3, 6.7 Hz, 1H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.9, 169.1, 167.0, 164.3, 143.9, 141.1, 138.2, 138.0, 132.8, 132.4, 128.7, 128.6, 128.3, 128.0, 127.2, 120.0, 119.6, 117.9, 117.8, 115.3, 83.3, 77.0, 71.0, 51.1, 37.9.

HRMS (ESI) calculated for $C_{46}H_{34}N_5O_4$ ($M\text{-}H^+$) 720.2616, found 720.2618.

2-(Benzyloxy)-3-isopropoxy-4-nitrobenzoic acid (135)



Chemical Formula:
$C_{17}H_{17}NO_6$
Exact Mass: 331,1056

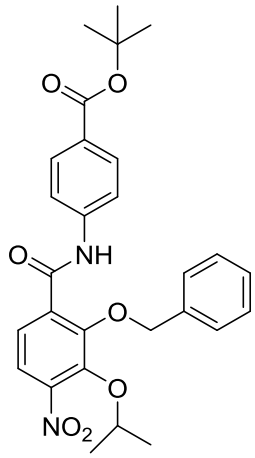
6-Formyl-2-isopropoxy-3-nitrophenyl acetate (2.0 g; 7.49 mmol) was dissolved in THF (38 mL) and water (19 mL), then LiOH (1.42 g; 74.9 mmol) dissolved in water (19 mL) was added at 0 C, reaction stirred overnight. In the morning, the pH was adjusted to 1, solvent partially reduced under vacuum and aqueous phase extracted with $CHCl_3$ (150 mL) three times, combined organic phases dried over sodium sulphate and reduced under vacuum to give a yellow oil, which was used in the next step without further purification. Residue was dissolved in DMF (18 mL), K_2CO_3 (2.07 g; 14.98 mmol) followed by benzyl bromide (1.34 mL; 11.24 mmol) were added, reaction stirred 24 h at r.t.. Reaction diluted with water (200 mL) and EA (200 mL), aqueous phase extracted with EtOAc (150 mL). Combined organic phases washed with brine (300 mL), dried over sodium sulphate and reduced under vacuum to give a crude material, which was dissolved with 2-Methyl-2-butene (8.35 mL; 78.65 mmol) in *t*-BuOH (45 mL). Then a solution of $NaClO_2$ 80% (1.02 g; 8.99 mmol) in Monosodium phosphate monohydrate solution 1 N (8.4 mL) was added dropwise to the solution. Reaction stirred for 1 h, t hen quenched by adding a solution of Na_2SO_3. Mixture partially reduced under vacuum, diluted with EtOAc (200 mL) and HCl 1 N (200 mL), aqueous phase extracted again with EtOAc (100 mL), organic phases reunited washed with brine (250 mL) and dried over sodium sulphate. Solvent reduced under vacuum, crude chromatographed on silica gel with a gradient 0-10% MeOH in DCM to afford 1.7 g of the desired compound (5.14 mmol, y= 69%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (d, *J* = 8.7 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 1H), 7.46 – 7.37 (m, 5H), 5.31 (s, 2H), 4.74 – 4.67 (m, 1H), 1.34 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 165.7, 153.0, 149.0, 144.8, 134.4, 129.4, 129.1, 128.9, 127.6, 126.6, 119.7, 78.7, 77.3, 22.3.

HRMS (ESI) calculated for C17H17NNaO6 ($M+Na^+$) 354.0948, found 354.0950.

Tert-butyl 4-(2-(benzyloxy)-3-isopropoxy-4-nitrobenzamido)benzoate (136)



Chemical Formula:
$C_{28}H_{30}N_2O_7$
Exact Mass: 506,2053

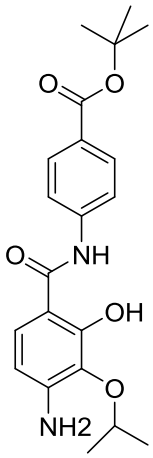
$POCl_3$ (0.031 mL; 0.33 mmol) was added at 0 °C to a stirred solution of tert-butyl 4-(2-(benzyloxy)-3-isopropoxy-4-nitrobenzamido)benzoate (110 mg; 0.33 mmol), TEA (0.077mL; 0.55 mmol) and tert-butyl 4-aminobenzoate (53 mg; 0.27 mmol) in DCM (4.5 mL) under nitrogen. Reaction stirred 2.5 h, then quenched with $NaHCO_3$ saturated solution, solvent partially reduced under vacuum , then diluted with EtOAc (20 mL) and $NaHCO_3$ saturated solution (20 mL), organic phase then washed with HCl 1 N and brine, dried over sodium sulphate and reduced under vacuum to give around 200 mg of crude material which was chromatographed on silica gel with a gradient 5-30% EtOAc in PetEt to give 75 mg of a yellow oil (0.15 mmol; y= 55%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.94 (s, 1H), 8.09 (d, *J* = 8.8 Hz, 1H), 7.89 – 7.85 (m, 2H), 7.66 (d, *J* = 8.8 Hz, 1H), 7.47 – 7.36 (m, 5H), 7.28 – 7.23 (m, 2H), 5.31 (s, 2H), 4.73 (hept, *J* = 6.2 Hz, 1H), 1.60 (s, 9H), 1.41 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 165.3, 160.9, 151.8, 148.1, 144.5, 141.1, 134.5, 130.6, 130.4, 129.6, 129.2, 129.2, 127.8, 126.3, 119.9, 119.1, 81.0, 78.7, 77.7, 28.2, 22.4.

HRMS (ESI) calculated for C28H31N2O7 ($M+H^+$) 507.2126, found 507.2120.

Tert-butyl 4-(4-amino-2-hydroxy-3-isopropoxybenzamido)benzoate (137)



Chemical Formula:
$C_{21}H_{26}N_2O_5$
Exact Mass: 386,1842

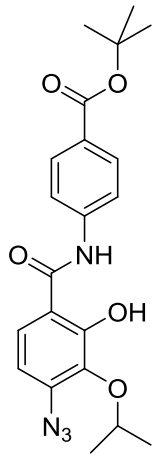
Tert-butyl 4-(2-(benzyloxy)-3-isopropoxy-4-nitrobenzamido)benzoate (1.22 g; 2.41 mmol) was dissolved in MeOH (35 mL). The solution was purged with N_2, then Pd/C (240 mg) was added and solution purged with H_2. The reaction was stirred under an H_2 atmosphere for 2 h, afterwards the mixture was filtered over a pad of celite and solvent removed under reduced pressure. The crude thus obtained was chromatographed on silica gel with gradient 0-20% MeOH in DCM to give 906 mg of desired product (2.37 mmol; y= 97%).

^{1}H NMR (500 MHz, DMSO) δ 12.57 (s, 1H), 10.15 (s, 1H), 7.92 – 7.84 (m, 2H), 7.83 – 7.78 (m, 2H), 7.59 (d, *J* = 8.9 Hz, 1H), 6.26 (d, *J* = 8.8 Hz, 1H), 5.66 (s, 2H), 4.46 (dt, *J* = 12.3, 6.1 Hz, 1H), 1.55 (s, 9H), 1.22 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (126 MHz, DMSO) δ 169.9, 165.1, 156.2, 148.7, 143.1, 130.3, 130.0, 126.6, 124.2, 120.7, 105.8, 103.9, 80.8, 73.2, 28.3, 22.7.

HRMS (ESI) calculated for C21H27N2O5 ($M+H^+$) 387.1914, found 387.1902.

Tert-butyl 4-(4-azido-2-hydroxy-3-isopropoxybenzamido)benzoate (132)



Chemical Formula:
$C_{21}H_{24}N_4O_5$
Exact Mass: 412,1747

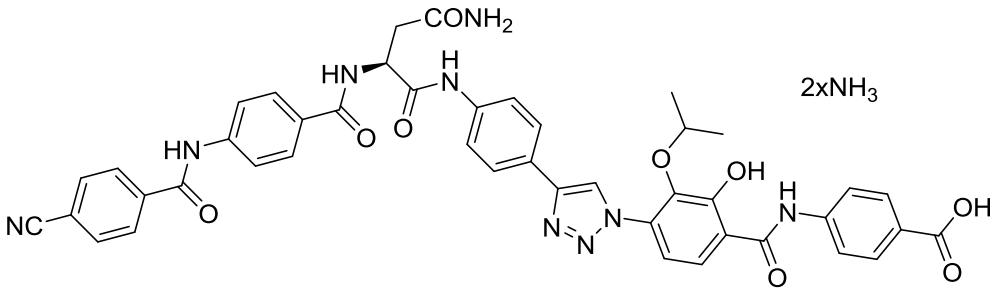
Tert-butyl 4-(4-amino-2-hydroxy-3-isopropoxybenzamido)benzoate (40 mg, 0.10 mmol) was dissolved in acetonitrile (2 mL) and the reaction mixture was cooled to 0 °C, then terbutylnitrite (18.5 µL, 0.15 mmol) and trimethylsilylazide (20.4 µL, 0.15 mmol) were subsequently added dropwise and the reaction mixture was allowed to stir at room temperature for 2 hours. After evaporation of the volatiles, the crude mixture was subjected to purification by flash column chromatography using petroleum ether / ethyl acetate 8:2 as an eluent to obtain 30 mg of a pale yellow orange solid (0.072 mmol, y= 70 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.49 (s, 1H), 8.56 (s, 1H), 8.02 (d, *J* = 8.7 Hz, 2H), 7.68 (d, *J* = 8.8 Hz, 2H), 7.49 (d, *J* = 8.8 Hz, 1H), 6.65 (d, *J* = 8.8 Hz, 1H), 4.76 (hept, 6.1 Hz, 1H), 1.61 (s, 9H), 1.37 (d, *J* = 6.2 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 166.1, 165.2, 154.0, 140.9, 138.0, 137.9, 130.7, 128.2, 122.6, 119.6, 113.1, 110.9, 81.0, 76.0, 28.2, 22.2

HRMS (ESI) calculated for $C_{21}H_{23}N_4O_5$ (M-H$^+$) 411.1674, found 411.1662.

4-(4-(4-(4-(4-Amino-2-(4-(4-cyanobenzamido)benzamido)-4-oxobutanamido)phenyl)-1H-1,2,3-triazol-1-yl)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (130)



Chemical Formula: $C_{44}H_{37}N_9O_9$
Exact Mass: 835,2714

(S)-2-(4-(4-Cyanobenzamido)benzamido)-N1-(4-ethynylphenyl)-N4-tritylsuccinamide (9 mg, 0.012 mmol) and tert-butyl 4-(4-azido-2-hydroxy-3-isopropoxybenzamido)benzoate (5 mg, 0.012 mmol) were dissolved in 300 µL DMSO/THF mixture (2:1) and then sodium ascorbate (1.4 mg, 0.0072 mmol) previously dissolved in 10 µL of water was added followed by TBTA (2.5 mg, 0.0048 mmol) previously dissolved in DMSO (10 µL). Finally; copper sulfate (0.2 mg, 0.0012 mmol) was added as a solid and the reaction mixture was allowed to stir at room temperature for 2 hours. After extraction with ethyl acetate (3x1 mL), the organic layer was washed with NH_4Cl saturated solution, water and brine, dried over sodium sulfate and evaporated under reduced pressure to obtain 14 mg (0.012 mmol, y= q.) of a yellow oil. The residue was used in the next step without further purification. Part of the residue (10 mg, 0.0088 mmol) was dissolved in DCM (250 µL), then TFA (50µL) and Tips (10 µL) were subsequently added and the reaction mixture was allowed to stir at room temperature for 3 hours. After evaporation of the volatiles, the crude residue was purified by preparative RP-HPLC using water (10 mM NH_4HCO_3) / acetonitrile to afford the pure compound as a white solid (4 mg, 0.0048 mmol, 55%).

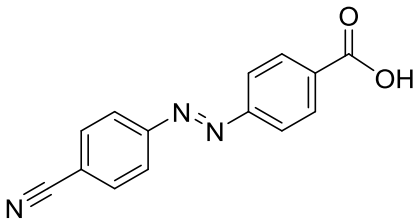
^{1}H NMR (700 MHz, DMSO) δ 15.18 (s, 1H), 10.74 (s, 1H), 10.31 (s, 1H), 8.80 (d, *J* = 5.7 Hz, 1H), 8.69 (s, 1H), 8.14 (d, *J* = 8.4 Hz, 2H), 8.04 (d, *J* = 8.3 Hz, 2H), 7.95 (d, *J* = 8.7 Hz, 2H), 7.89 (d, *J* = 8.7 Hz, 2H), 7.87 (d, *J* = 8.6 Hz, 2H), 7.79 (d, *J* = 8.1 Hz, 2H), 7.74 (d, *J* = 8.6 Hz, 2H), 7.61 (d, *J* = 8.5 Hz, 1H), 7.59 (d, *J* = 8.3 Hz, 2H), 7.47 (s, 1H), 6.97 (s, 1H), 6.34 (d, *J* = 8.5 Hz, 1H), 4.92 (dd, *J* = 13.8, 7.7 Hz, 1H), 4.83 (hept, *J* = 6.1 Hz, 1H), 2.74 – 2.65 (m, 2H), 0.93 (d, *J* = 6.0 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.4, 170.2, 169.8, 166.7, 166.3, 165.7, 164.4, 145.1, 141.5, 141.1, 138.7, 132.5, 129.7, 129.2, 128.6, 128.3, 126.4, 125.9, 125.6, 123.9, 122.5, 119.7, 119.5, 119.4, 118.3, 117.7, 103.1, 70.8, 51.7, 40.0, 37.0, 22.2.

HRMS (ESI) calculated for $C_{44}H_{36}N_9O_9$ ($M\text{-}H^+$) 834.2641, found 834.2661.

4.2.2.5 Photoswitchable cystobactamids

(E)-4-((4-Cyanophenyl)diazenyl)benzoic acid (140)



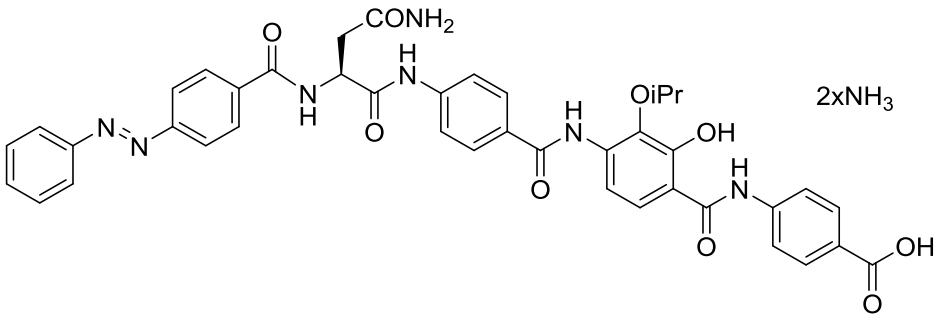
Chemical Formula: $C_{14}H_9N_3O_2$
Exact Mass: 251,0695

A solution of NaCN in water (345 mg, 7.05 mmol in 4 mL) was added at 0°C to a stirred mixture of CuCN (241 mg, 2.69 mmol) in water (2 mL), the mixture was stirred at 0° for 30 min..

(E)-4-((4-aminophenyl)diazenyl)benzoic acid[95] was suspended in 2 N HCl (4 mL) and cooled to 0°C, then a solution of $NaNO_2$ (150 mg, 2.18 mmol) in water (0.45 mL) was added dropwise keeping the temperature low. The mixture was stirred for 20 min at 0°C and then Na_2CO_3 was added portion wise to quench the excess of HCl. The resulting mixture was then added dropwise at 0°C to the solution of Cu/Na cyanide. The reaction was stirred for 1 h hour at 0°C, then 200 mL of HCl 1N and EtOAc were added, watery phase extracted again 3 times with EtOAc (80 mL). The combined organic phases were washed with brine (200 mL) and dried over sodium sulphate. The solvent was removed under vacuum to obtain 450 mg of a crude product that was used in the next step without further purification.

Starting from intermediate **101**, after Trityl and Fmoc deprotection (see general procedures section 4.2.2.2.5.3), amine **102** was coupled to a desired carboxylic acid (fragment A1) to obtain the following photoswitchable cystobactamids:

(S,E)-4-(4-(4-(4-Amino-4-oxo-2-(4-(phenyldiazenyl)benzamido)butanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (143)



Chemical Formula: $C_{41}H_{37}N_7O_9$
Exact Mass: 771,2653

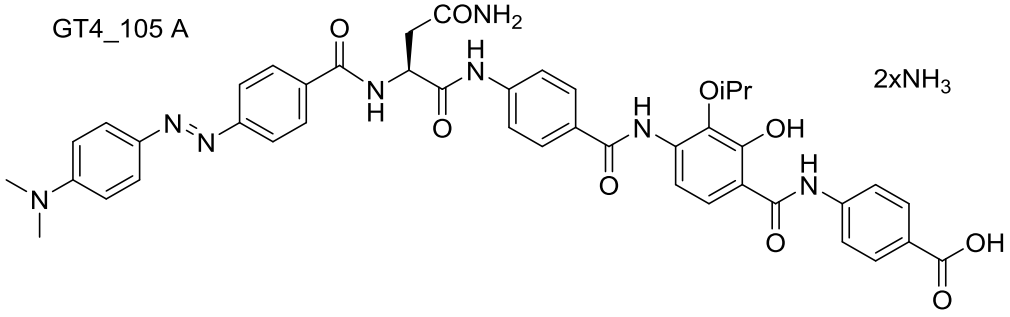
(E)-4-(Phenyldiazenyl)benzoic acid (0.039 mmol) was mixed with HBTU (0.039 mmol) in DMF (0.1 mL), to this solution DiPEA (0.117 mmol) was added. The reaction was stirred 15 min. then added to the crude amine **102** (0.019 mmol). The reaction was stirred overnight and purified by preparative RP-HPLC with a gradient 10-95% CH_3CN in water 10 mM NH_4HCO_3 in 40 min. to give 2.5 mg of desired product (0.0032 mmol; y= 17%).

^{1}H NMR (700 MHz, DMSO) δ 12.79 (br, 1H), 12.29 (br, 1H), 10.62 (br, 1H), 10.49 (s, 1H), 9.36 (br, 1H), 8.94 (d, *J* = 7.3 Hz, 1H), 8.14 – 8.10 (m, 2H), 8.01 – 7.98 (m, 2H), 7.98 – 7.92 (m, 6H), 7.87 – 7.81 (m, 5H), 7.66 (br, 1H), 7.64 – 7.60 (m, 3H), 7.41 (s, 1H), 7.01 (s, 1H), 4.97 (dd, *J* = 13.9, 7.3 Hz, 1H), 4.58 (br, 1H), 2.74 – 2.70 (m, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.2, 170.5, 168.4, 166.9, 165.6, 164.1, 153.4, 151.9, 142.4, 136.4, 136.0, 132.0, 130.2, 129.6, 128.9, 128.2, 122.9, 122.8, 122.3, 120.5, 118.9, 51.7, 36.7, 22.3.

HRMS (ESI+): *m/z* for C41H38N7O9 ($M+H^+$): calculated: 772.2726, found: 772.2724.

(S,E)-4-(4-(4-(4-Amino-2-(4-((4-(dimethylamino)phenyl)diazenyl)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (144)



Chemical Formula: $C_{43}H_{42}N_8O_9$
Exact Mass: 814,3075

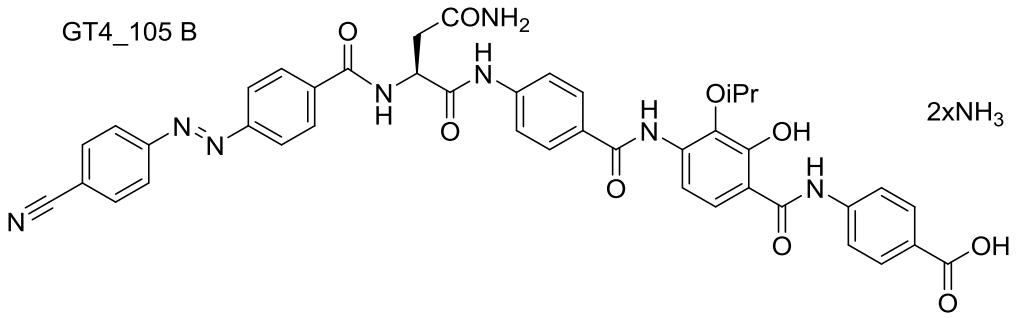
(E)-4-((4-(Dimethylamino)phenyl)diazenyl)benzoic acid (11.7 mg, 0.044 mmol) was mixed in DMF (0.2 mL) with HOAt (5.9 mg, 0.041 mmol) and EDC (7.8 mg, 0.041 mmol), then collidine (0.038 mL, 0.29 mmol) was added. Reaction stirred for 30 min. then crude amine **102** as a solution in DMF (0.2 mL) was added to the activated ester. Reaction stirred at r.t. overnight, then diluted with EtOAc (15 mL), THF (5 mL) and HCl (20 mL), organic phase evaporated. The crude residue was purified by preparative RP-HPLC using condition B as described in section 4.2.1.2 to obtain 2.1 mg of desired product (0.0026 mmol, y= 9%).

^{1}H NMR (500 MHz, DMSO) δ 12.79 (br, 1H), 12.30 (br, 1H), 10.63 (br, 1H), 10.49 (s, 1H), 9.38 (s, 1H), 8.85 (d, *J* = 7.3 Hz, 1H), 8.28 (br, 1H), 8.07 – 8.01 (m, 2H), 7.95 (dd, *J* = 8.6, 6.1 Hz, 4H), 7.87 – 7.80 (m, 9H), 7.67 (d, *J* = 8.4 Hz, 1H), 7.40 (s, 1H), 7.00 (s, 1H), 6.86 (d, *J* = 9.3 Hz, 2H), 4.95 (q, *J* = 7.1 Hz, 1H), 4.61 – 4.52 (m, 1H), 3.08 (s, 6H), 2.71 (d, *J* = 7.1 Hz, 2H), 1.26 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.2, 170.6, 168.4, 166.9, 165.8, 164.1, 154.1, 152.9, 142.6, 142.5, 136.9, 136.4, 134.1, 130.2, 128.7, 128.4, 128.3, 125.1, 122.9, 121.5, 120.6, 118.9, 111.6, 74.6, 51.7, 36.7, 34.3, 22.3.

HRMS (ESI) calculated for C43H41N8O9 (M-H^{+}) 813.3002, found 813.2995.

(S,E)-4-(4-(4-(4-Amino-2-(4-((4-cyanophenyl)diazenyl)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzamido)benzoic acid (145)



Chemical Formula: $C_{42}H_{36}N_8O_9$
Exact Mass: 796,2605

(E)-4-((4-Cyanophenyl)diazenyl)benzoic acid (10.9 mg, 0.044 mmol) was mixed in DMF (0.2 mL) with HOAt (5.9 mg, 0.041 mmol) and EDC (7.8 mg, 0.041 mmol), then collidine (0.038 mL, 0.29 mmol) was added. Reaction stirred for 30 min. then crude amine **102** (0.029 mmol) as a solution in DMF (0.2 mL) was added to the activated ester. Reaction stirred at r.t. overnight, then diluted with EtOAc (15 mL), THF (5 mL) and HCl (20 mL), organic phase

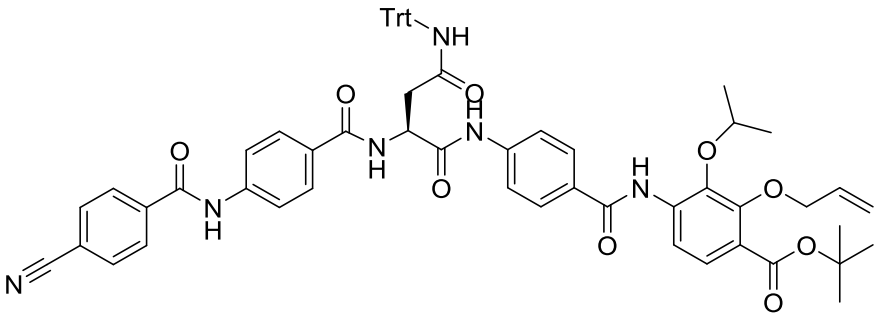
evaporated. The crude residue was purified by preparative RP-HPLC using condition B as described in section 4.2.1.2 to obtain 2.1 mg of desired product (0.0038 mmol, y= 13%).

^{1}H NMR (700 MHz, DMSO) δ 12.58 (br, 1H), 12.35 (br, 1H), 10.49 (s, 1H), 9.15 (br, 1H), 8.98 (d, *J* = 7.3 Hz, 1H), 8.15 – 8.13 (m, 2H), 8.13 – 8.10 (m, 2H), 8.08 – 8.06 (m, 2H), 8.06 – 8.03 (m, 2H), 7.91 (d, *J* = 7.6 Hz, 4H), 7.84 – 7.78 (m, 5H), 7.66 (br, 1H), 7.41 (s, 1H), 7.00 (s, 1H), 4.97 (dd, *J* = 13.9, 7.4 Hz, 1H), 4.75 (br, 1H), 2.75 – 2.69 (m, 2H), 1.23 (d, *J* = 6.0 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.2, 170.5, 167.0, 165.5, 153.9, 153.2, 136.9, 133.9, 130.3, 129.0, 128.0, 123.4, 122.8, 119.0, 118.3, 113.7, 51.7, 36.7, 22.5.

HRMS (ESI) calculated for C42H37N8O9 ($M+H^{+}$) 797.2678, found 797.2678.

Tert-butyl (S)-2-(allyloxy)-4-(4-(2-(4-(4-cyanobenzamido)benzamido)-4-oxo-4-(trityl-amino)butanamido)benzamido)-3-isopropoxybenzoate (147)

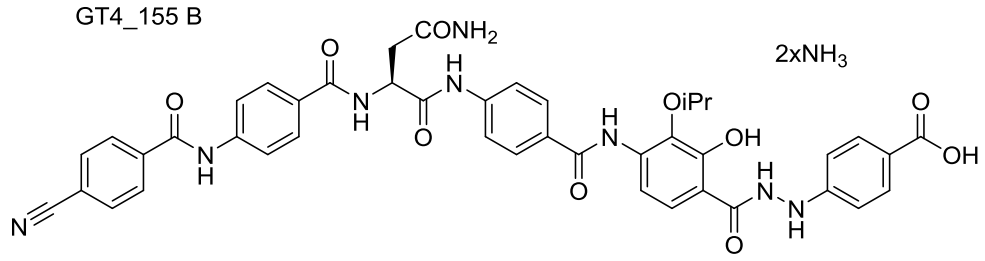


Chemical Formula: $C_{62}H_{58}N_6O_9$
Exact Mass: 1030,4265

The synthesis of this intermediate is described in Tim Mollner master thesis and in the European patent application cited in section 4.2.2.

This molecule is one of the starting materials used in the experimental procedures described below.

(S)-4-(2-(4-(4-(4-Amino-2-(4-(4-cyanobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzoyl)hydrazineyl)benzoic acid (125)



Chemical Formula: $C_{43}H_{38}N_8O_{10}$
Exact Mass: 826,2711

Tert-butyl (S)-2-(allyloxy)-4-(4-(2-(4-(4-cyanobenzamido)benzamido)-4-oxo-4-(trityl-amino)butanamido)benzamido)-3-isopropoxybenzoate (**147**) (21 mg, 0.021 mmol) was suspended in DCM (2 mL), the mixture was cooled to 0°C, then Tips (13 µL, 0.06 mmol) followed by TFA (0.5 mL) were added. Reaction stirred 2 hours at r.t., then solvent removed under vacuum, the residue was dissolved in DCM and evaporated twice. The crude product was left at the high vacuum povernight. The residue (0.021 mmol) was mixed with HATU (8.0 mg, 0.021 mmol) in DMF (0.5 mL) and then DiPEA (18.3 µL, 0.105 mmol) was added. Reaction stirred 30 min at r.t. then 4-hydrazineylbenzoic acid (8.6 mg, 0.063 mmol) as a solution in DMF (0.1 mL) was added. Reaction stirred for 3 hours at r.t. then EtOAc (20 mL) and HCl 1N/ice (20 mL) were added. The organic phase was washed with brine, dried over sodium sulphate and removed under vacuum.

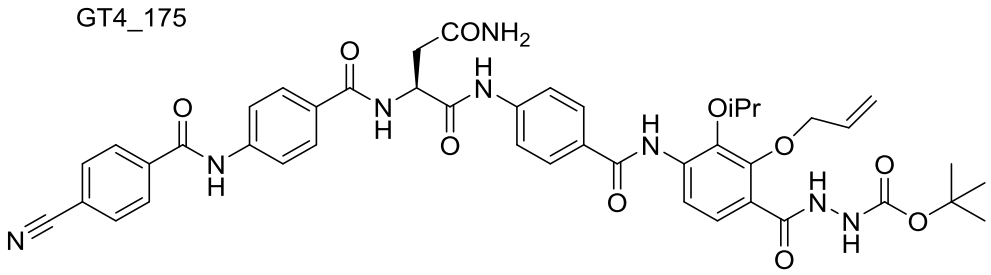
Part of the residue (0.005 mmol) was suspended in THF (0.5 mL), $PhSiH_3$ (1.23 µL, 0.01 mmol) followed by Pd-tetrakis (1.3 mg, 0.0012 mmol) were added. The reaction was stirred for two hours and then purified on preparative RP-HPLC using condition B as described in section 4.2.1.2 to obtain1.2 mg of desired product (0.0015 mmol, y= 29%).

^{1}H NMR (700 MHz, DMSO) δ 12.00 (br, 1H), 10.71 (s, 1H), 10.45 (s, 1H), 9.00 (s, *J* = 15.8 Hz, 1H), 8.97 (br, 1H), 8.70 (d, *J* = 7.1 Hz, 2H), 8.57 (br, 1H), 8.13 (d, *J* = 8.2 Hz, 2H), 8.04 (d, *J* = 8.3 Hz, 2H), 7.93 (d, *J* = 8.8 Hz, 2H), 7.91 – 7.84 (m, 4H), 7.82 – 7.78 (m, 2H), 7.68 (d, *J* = 8.4 Hz, 2H), 7.41 (s, 2H), 7.23 (br, 1H), 6.98 (s, 1H), 6.73 (d, *J* = 8.1 Hz, 2H), 4.92 (q, *J* = 7.1 Hz, 1H), 4.83 (br, 1H), 2.69 (d, *J* = 6.9 Hz, 2H), 1.21 (d, *J* = 6.0, 3.8 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 167.5, 165.7, 164.4, 163.4, 157.1, 142.1, 141.6, 138.7, 135.3, 133.9, 132.5, 130.9, 129.2, 128.6, 128.3, 127.8, 123.8, 119.5, 119.0, 118.3, 114.0, 110.3, 107.7, 72.9, 51.6, 36.8, 22.6, 22.5.

HRMS (ESI) calculated for C43H39N8O10 ($M+H^+$) 827.2784, found 827.2798.

Tert-butyl (S)-2-(2-(allyloxy)-4-(4-(4-amino-2-(4-(4-cyanobenzamido)benzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzoyl)hydrazine-1-carboxylate (148)



Chemical Formula: $C_{44}H_{46}N_8O_{10}$
Exact Mass: 846,3337

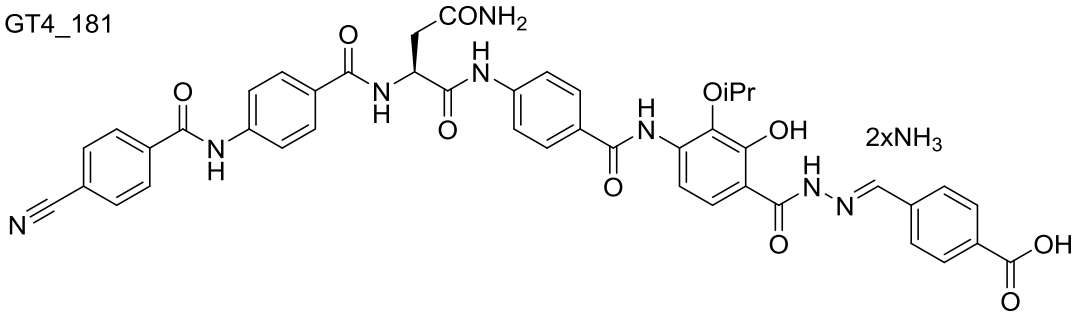
Tert-butyl (S)-2-(allyloxy)-4-(4-(2-(4-(4-cyanobenzamido)benzamido)-4-oxo-4-(trityl-amino)butanamido)benzamido)-3-isopropoxybenzoate (**147**) (75 mg, 0.073 mmol) was suspended in DCM (3 mL), the mixture was cooled to 0°C, then Tips (42 µL, 0.204 mmol) followed by TFA (0.75 mL) were added. Reaction stirred 2 hours at r.t., then solvent removed under vacuum, the residue was dissolved in DCM and evaporated twice. The crude product was left at the high vacuum povernight. The residue (0.073 mmol) was mixed with HATU (29.1 mg, 0.077 mmol) in DMF (1.7 mL) DiPEA (63.5 µL, 0.365 mmol) was added. The reaction was stirred for 30 min, then a solution of *tert*-Butyl carbazate (30 mg, 0.219 mmol) in DMF (0.5 mL) was added at 0°C.The reaction was stirred for 3 hours at r.t., then EtOAc (30 mL) and HCl 1N/ice 1:1 (30 mL) were added. The organic phase was washed with $NaHCO_3$ (30 mL), brine (20 mL) and dried over sodium sulphate. The crude resiued was chromatographed on silica gel with a gradient 0-10% MeOH in DCM to give 38 mg of desired product (0.045 mmol, y= 62%).

^{1}H NMR (700 MHz, DMSO) δ 10.70 (s, 1H), 10.44 (s, 1H), 9.73 (s, 1H), 9.44 (s, 1H), 8.96 (s, 1H), 8.66 (d, *J* = 7.3 Hz, 1H), 8.14 – 8.11 (m, 2H), 8.06 – 8.03 (m, 2H), 7.97 – 7.91 (m, 4H), 7.91 – 7.87 (m, 2H), 7.80 (d, *J* = 8.8 Hz, 2H), 7.78 (d, *J* = 9.3 Hz, 1H), 7.39 (s, 1H), 7.29 (d, *J* = 8.3 Hz, 1H), 6.99 (s, 1H), 6.15 – 6.07 (m, 1H), 5.36 (dd, *J* = 17.2, 1.6 Hz, 1H), 5.23 (d, *J* = 10.4 Hz, 1H), 4.92 (dd, *J* = 14.0, 7.2 Hz, 1H), 4.58 (d, *J* = 5.3 Hz, 2H), 4.48 – 4.41 (m, 1H), 2.69 (d, *J* = 7.9 Hz, 2H), 1.44 (s, 9H), 1.23 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 165.7, 165.4, 164.4, 164.2, 155.3, 149.8, 142.4, 141.6, 138.7, 135.5, 134.0, 132.5, 129.1, 128.6, 128.3, 125.0, 123.8, 119.5, 118.8, 118.5, 118.3, 118.1, 114.0, 79.2, 76.0, 74.5, 51.6, 36.8, 28.1, 22.3.

HRMS (ESI) calculated for C43H41N8O9 ($M-H^+$) 813.3002, found 813.2995.

(S,E)-4-((2-(4-(4-(4-Amino-2-(4-(4-cyanobenzamido)benzamido)-4-oxobutanamido)benzamido)-2-hydroxy-3-isopropoxybenzoyl)hydrazineylidene)methyl)benzoic acid (146)



Chemical Formula: $C_{44}H_{38}N_8O_{10}$
Exact Mass: 838,2711

Tert-butyl (S)-2-(2-(allyloxy)-4-(4-(4-amino-2-(4-(4-cyanobenzamido)benzamido)-4-oxobutanamido)benzamido)-3-isopropoxybenzoyl)hydrazine-1-carboxylate (**148**) (18 mg, 0.021 mmol) was suspended in DCM (0.8 mL) and to the mixture TFA (0.2 mL) was added. The mixture turned into a solution and reaction stirred for 1.5 hours, the solvent was removed under vacuum and the residue dissolved in DCM (1 mL) and evaporated again twice.

A solution of 4-formylbenzoic acid (3.9 mg, 0.026 mmol) in THF (0.2 mL) was added to the residue from the previous reaction. Suddenly a dense white precipitate formed. The solvent was removed under vacuum and the residue left at high vacuum overnight. The crude was suspended in THF (2.1 mL), $PhSiH_3$ (10.3 µL, 0.084 mmol) followed by Pd-tetrakis (6.1 mg, 0.0053 mmol) were added. The reaction was stirred for 3 hours and then the purified by preparative RP-HPLC using condition B as described in section 4.2.1.2 to obtain 1.8 mg of a white material (0.0021 mmol, y= 10%).

^{1}H NMR (700 MHz, DMSO) δ 12.86 (br, 1H), 10.70 (s, 1H), 10.47 (s, 1H), 9.27 (s, 1H), 8.68 (d, *J* = 7.3 Hz, 1H), 8.51 (s, 1H), 8.15 – 8.11 (m, 1H), 8.04 (d, *J* = 8.4 Hz, 1H), 8.01 (d, *J* = 8.1 Hz, 1H), 7.93 (dd, *J* = 7.8, 5.8 Hz, 1H), 7.89 (d, *J* = 8.8 Hz, 1H), 7.86 (d, *J* = 8.1 Hz, 1H), 7.82 (d, *J* = 8.7 Hz, 1H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.58 (br, 1H), 7.41 (s, 1H), 6.99 (s, 1H), 4.92 (q, *J* = 7.1 Hz, 1H), 4.65 (br, 1H), 2.69 (d, *J* = 7.0 Hz, 2H), 1.25 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (176 MHz, DMSO) δ 171.3, 170.7, 167.0, 166.3, 165.8, 164.5, 164.0, 142.4, 141.6, 138.7, 138.3, 136.5, 134.1, 132.5, 132.0, 129.8, 129.2, 128.6, 128.3, 128.1, 127.1, 122.4, 119.5, 118.9, 118.3, 114.0, 73.9, 55.2, 51.6, 36.8, 22.4.

HRMS (ESI) calculated for C44H39N8O10 ($M+H^+$) 839.2784, found 839.2781.

5 ABBREVIATIONS

AcOH= acetic acid

Alb= albicidin

Allyl-Br= allyl bromide

Asn= L-asparagine

ATP= adenosine triphosphate

BTC= bis(trichloromethyl) carbonate

CIP= ciprofloxacin

cys= cystobactamid

Collidine= 2,4,6-collidine

COSY= correlation spectroscopy

DAST= diethylaminosulfur trifluoride

DiPEA= *N*,*N*-diisopropylethylamine

DCM= dichloromethane

DMF= *N*,*N*-dimethylformamide

DMSO= dimethyl sulfoxide

DNA= deoxyribonucleic acid

ECD= 1-ethyl-3-(3-dimethylaminopropyl)carbodiimid

EDG= electron donating group

EIC= extracted ion chromatogram

ESI= electrospray ionization

Et= ethyl

Et_2O= diethylether

EtOAc= ethyl acetate

EtOH= ethanol

EWG= electron withdrawing group

FDA= United States food and drug administration

FDAA=1-fluoro-2-4-dinitrophenyl-5-L-alanine amide

Fmoc= fluorenylmethoxycarbonyl

h= hours

HATU= 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-

b]pyridinium 3-oxid hexafluorophosphate

HBTU= 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HOAt= 1-hydroxy-7-azabenzotriazol

HRMS= high resolution mass spectrometry

i-But-Br= isobutyl bromide

IC_{50}= half maximal inhibitory concentration

i-Pr= iso propoxy

i-Pr-Br= isopropyl bromide

LTMP= lithium tetramethylpiperidin

LCMS= liquid chromatography–mass spectrometry

$M+H^+$= molecular ion plus one hydrogen atom

$M-H^+$= molecular ion minus one hydrogen atom

$M+Na^+$= molecular ion plus one sodium atom

m-= meta-

Me= methyl

MIC= minimum inhibitory concentration

MoA= mechanism of action

MOM= methoxymethyl ether

MOM-Cl= (chlormethyl)methylether

MS= mass

MW= microwave reactor synthetizer

n= number

NaH= sodium hydride

nd= not determined

NMR= nuclear magnetic resonance

p-= para-

PABA= para aminobenzoic acid

PRP= pentapeptide-repeat

Pet. Et= petrolether

Pd/C= palladium on activated charcoal

Ph= phenyl

$PhSiH_3$= phenylsilane

Py= pyridine

PNBA= *p*-nitrobenzoic acid

Ppm= parts per million

PSS= photostationary state

RP-HPLC= reversed phase high-performance liquid chromatography

SAR= structure-activity relationships

Sec But= sec butyl

rt= room temperature

TBTA= tris(benzyltriazolylmethyl)amine

tBu-= *tert*-butyl-

t-BuOH= tert butanol

TEA= trimethylamine

TFA= trifluoroacetic acid

THF= tetrahydrofuran

TIPS= triisopropylsilane

TLC= thin-layer chromatography

TMS-N_3= trimethylsilyl azide

Trt= trityl

UV= ultraviolet

WT= wild type

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7 SUPPLEMENTARY INFORMATION

The NMR spectra are reported in the CD-ROM attached to the thesis.

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