Synthesis and Characterisation of wALAD Inhibitors and Novel ABAL Probes

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A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.

Ein Gelehrter in seinem Laboratorium ist nicht nur ein Techniker; er steht auch vor den Naturgesetzen wie ein Kind vor der Märchenwelt.

Marie Curie

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Contents

1. Abstract

The here presented work comprises two projects with highly interdisciplinary topics in the chemical biology field. The development of novel small molecule ALAD inhibitors which might show their potential as novel antifilarial, antiplasmodial and antimicrobial agents, herbicides or chemical biology tools was the main task of the first project (**Section A**).[1]

wALADin1 is a benzimidazole-based drug-like small molecule that was found by high throughput screening and was identified as a potent species-specific inhibitor for the endobacterial enzyme wALAD with effectiveness in *in vitro* and *ex vivo* studies.[1b] Several wALADin1 derivatives were synthesised in this work to further characterise the influence on the inhibitory activity of the 3-trifluoromethylbenzyl and 2-[(2-thienylcarbonyl)amino]ethyl group present in the benzimidazole scaffold of wALADin1.[1b, 2] These studies revealed the importance of the unaltered 3-trifluoromethylbenzyl-moiety (R^2) for the inhibitory activity of the wALADin compounds and the necessity of the 2-[(2-thienylcarbonyl)amino]ethyl-moiety (R^1) for species-specificity, although irrelevant for wALAD inhibition.[1b] Additionally, the obtained wALADin compounds were tested in cross species structure activity relationship studies and showed contrasting biological results with various ALAD orthologues from plants, bacteria, metazoa and protozoa.[1c] Moreover, an antiplasmodial effect on blood stage *Plasmodium falciparum* was investigated for wALADin1, wALADin2 and wALADin3 that demonstrably does not result from inhibitory activity on the PfALAD orthologue.[1d]

The second project comprised the development of novel probes for aptamer-based affinity labelling (ABAL) of proteins, an approach for the rational, proteome-wide identification of proteins that bind to a particular aptamer (**Section B**).[3]

This is achieved by labelling aptamers with photoreactive probes and cross-linking them to their target protein in a UV light-dependent and highly specific manner. Successful identification requires isolation of pure samples for mass spectrometry analysis, which was not obtained using the initial ABAL probe. To improve the ABAL procedure novel ABAL probes were developed. These probes carry biotin in combination with a chemically cleavable linker or desthiobiotin as purification tags to enable mild elution of the captured complex and besides phenyl azide (PA),

1,2,4,5-tetrafluorophenyl-3-azide (TPA) and 3-phenyl-3-(trifluoromethyl)-3H-diazirine (TPD) were used as potentially more potent photoreactive moieties.

These novel ABAL probes might help to tap the full potential of the ABAL procedure and develop it into a standard procedure for identifying unknown target proteins.

2. Zusammenfassung

Die hier vorgestellte Arbeit behandelt zwei höchst interdisziplinäre Projekte im Bereich der Chemischen Biologie. Das erste Projekt befasst sich hauptsächlich mit der Entwicklung von neuen Small-Molecule-ALAD-Inhibitoren, die als potentielle antifilarische, antiplasmodische und antimikrobische Mittel, Herbizide oder Werkzeuge der chemischen Biologie Verwendung finden könnten (**Abschnitt A**).[1]

wALADin1 ist ein auf Benzimidazol basierendes Small-Molecule, das in einem Screeningverfahren mit hohem Durchsatz ermittelt und als potenter, speziesspezifischer Inhibitor gegen das endobakterische Enzym wALAD mit Wirkung in *in-vitro*- und *ex-vivo*-Studien, identifiziert wurde.[1b, 2] In der hier vorgestellten Arbeit wurde eine Vielzahl von wALADin1-Derivaten synthetisiert um den Einfluss der am Benzimidazolring vorhandenen 3-Trifluormethylbenzyl- und 2-[(2-thienylcarbonyl)amino]ethylgruppe auf die inhibierende Wirkung zu untersuchen.[1b, 2] Diese Untersuchungen belegen, dass die modifizierte 3-Trifluormethylbenzylgruppe (R^2) unverzichtbar für die inhibierende Wirkung der wALAD-Inhibitoren ist und dass die 2-[(2-thienylcarbonyl)amino]ethylgruppe (R^1) wiederum eine entscheidende Rolle für die speziesspezifische Inhibition spielt, auch wenn sie keinerlei Einfluss auf die wALAD-Inhibition hat.[1b, 2] Ebenfalls wurden die erhaltenen wALADin-Verbindungen in artübergreifenden Struktur-Wirkung-Beziehungsstudien getestet und zeigten gegensätzliche biologische Resultate bei verschiedenen ALAD-Orthologen von Pflanzen, Bakterien, Metazoen und Protozoen.[1c] Darüber hinaus wurde ein antiplasmodischer Effekt von wALADin1, wALADin2 und wALADin3 auf das Merozoitenstadium von *Plasmodium falciparum* festgestellt, der nachweislich nicht auf der Inhibition des PfALAD-Orthologs beruht.[1d]

Das zweite Projekt befasst sich mit der Entwicklung von neuen Sonden zur aptamer-basierten Affinitätsmarkierung (ABAL) von Proteinen, einem Ansatz zur rationalen, proteomweiten Identifizierung von Proteinen die an ein bestimmtes Aptamer binden (**Abschnitt B**).[3] Dies wird durch das Markieren der Aptamere mit photoreaktiven Sonden erreicht, die durch UV-Licht-Bestrahlung hochspezifisch mit ihrem Zielprotein vernetzt werden. Die erfolgreiche Identifizierung erfordert, dass die aus der Vernetzung und anschließenden Isolierung erhaltenen Proben für die massenspektrometrische Analyse rein sind, was bisher nicht erreicht werden konnte. Um die Ergebnisse der ABAL-Strategie zu verbessern, wurden neue ABAL-Sonden

entwickelt. Diese Sonden enthalten zur Aufreinigung entweder Biotin in Verbindung mit einem chemisch spaltbaren Linker oder Desthiobiotin, um die milde Elution des eingefangenen Komplexes zu ermöglichen sowie Phenylazid (PA), 1,2,4,5-tetrafluorophenyl-3-azid (TPA) und 3-phenyl-3-(trifluoromethyl)-3H-diazirin (TPD) als photoreaktive Gruppen.

Diese neuen ABAL-Sonden werden möglicherweise helfen das volle Potential des ABAL-Verfahrens zu entfalten und es zu einer Standardanwendung zur Identifizierung von unbekannten Zielproteinen weiterzuentwickeln.

A Synthesis and characterisation of wALAD inhibitors

1. Abstract

Lymphatic filariasis and Onchocerciasis are vector-borne diseases which mainly occur in third world countries and are caused by human filarial nematodes.[4] These diseases manifest in destroyed and distorted tissue or blindness. To eliminate these massive public health problems, consecutive mass drug administration programs are carried out using single-dose combination therapies with classical anthelmintics.[5] These therapies merely focus on preventing transmission and have developed increasing suboptimal responses of patients and drug resistance of the filarial nematodes which points to a need for the development of novel antifilarial drugs or treatment options.[6]

Antibiotic therapies have revealed the endosymbiotic α-proteobacteria *Wolbachia*, as a promising target for novel antifilarial treatments, but are not suitable for mass drug distribution.[7] Genome sequence analysis of *Wolbachia*[8] and its filarial hosts[9] identified the enzyme δ-aminolevulinic acid dehydratase (ALAD) within the heme biosynthetic pathway as a promising target in antifilarial drug development. wALADin1 was identified as a potent species-specific inhibitor for the endobacterial enzyme wALAD with effectiveness in *in vitro* and *ex vivo* studies.[1b]

In a previous study, chemical modification of wALADin1 revealed the general importance of the carboxylic acid-moiety (R^3) and the necessity of its presence at the C5-carbon of the benzimidazole scaffold.[1a] Based on this study, several wALADin1 derivatives were synthesised in the work presented here to further characterise the influence on the inhibitory activity of the other substituent groups present in the benzimidazole scaffold.[1b, 2] These studies revealed that the 3-trifluoromethylbenzyl-moiety (R^2) is also essential for the inhibitory activity of the wALADin compounds and any alteration on this group results in a decrease of inhibitory activity. The 2-[(2-thienylcarbonyl)amino]ethyl-moiety (R^1) has no influence on the inhibitory activity against wALAD, but plays an important role for species-specific inhibition.[1c]

Additionally, the obtained wALADin compounds showed contrasting biological results varying between inhibition, no effect and stimulation in cross species structure activity relationship studies testing several ALAD-orthologues from plants, bacteria,

metazoa and protozoa.[1c] Remarkable, was the antiplasmodial effect of wALADin1, wALADin2 and wALADin3 on blood stage *Plasmodium falciparum.*[1d] These compounds were able to reduce parasitemia to almost 0 % and wALADin2 was identified as a potent inhibitor of Plasmodium motility and invasion. Additional experiments with the PfALAD orthologue further revealed that the antiplasmodial effect of the wALADin compounds does not result from inhibitory activity on this orthologue and that the antiplasmodial activity is a result of wALADin-interaction with an alternative target.

These results lead to wALADin compounds as potential lead structures in the development of novel antifilarial, antiplasmodial and antimicrobial agents, herbicides or chemical biology tools.

2. Zusammenfassung

Lymphatische Filariose und Onchozerkose sind vektorübertragene Krankheiten, die vermehrt in Ländern der Dritten Welt auftreten und durch Filarien ausgelöst werden. [4] Diese Krankheiten führen zu schweren Gewebeschäden oder Blindheit. Um diese massiven Gesundheitsprobleme zu eliminieren, werden wiederholt Einzeldosis-Kombinationstherapien klassischer Anthelmintika in großangelegten Massenbehandlungen verabreicht.[5] Diese Therapien bewirken hauptsächlich die Eindämmung der Übertragung und führen vermehrt zu Nebenwirkungen bei Patienten und Resistenz der Filarien gegen die Medikamente, was die Entwicklung neuer Medikamente oder Behandlungsoptionen nötig macht.[6]

Durch antibiotische Therapien wurden die endosymbiotischen α-Proteobacteria *Wolbachia* als vielversprechendes Ziel für neue Behandlungsansätze entdeckt, jedoch sind sie nicht für großangelegte Massenbehandlungen geeignet.[7] Bei der Genomsequenzanalyse von *Wolbachia*[8] und den Wirtsfilarien[9] wurde das Enzym δ-Aminolävulinsäure-Dehydratase (ALAD), welches ein Teil des Häm-Biosynthesewegs darstellt, als vielversprechende Quelle für die Entdeckung neuer Medikamente gegen Filarien identifiziert. wALADin1 wurde als potenter, speziesspezifischer Inhibitor gegen das endobakterische Enzym wALAD identifiziert, das seine Wirkung in *in-vitro*- und *ex-vivo*-Studien unter Beweis gestellt hat.[1b, 2]

In einer vorherigen Studie wurde durch chemische Modifikation von wALADin1 die allgemeine Relevanz der Karbonsäuregruppe (R^3) und die Wichtigkeit ihrer Positionierung am C5-Kohlenstoff des Benzimidazolrings aufgedeckt.[1a] Basierend auf dieser Studie wurde in der hier vorgestellten Arbeit eine Vielzahl von wALADin1-Derivaten synthetisiert um den Einfluss der verbleibenden Benzimidazolsubstituenten auf die inhibierende Wirkung zu untersuchen.[1b, 2] Diese Untersuchungen belegen, dass die 3-Trifluormethylbenzylgruppe (R^2) ebenfalls unverzichtbar für die inhibierende Wirkung der wALAD-Inhibitoren ist und selbst kleinste Veränderungen an dieser Gruppe zur Verminderung dieser Wirkung führen. Die 2-[(2-thienylcarbonyl)amino]ethylgruppe (R^1) hingegen hat keinerlei Einfluss auf die inhibierende Wirkung der Moleküle gegen wALAD, allerdings spielt sie eine entscheidende Rolle für die speziesspezifische Inhibition.[1c]

Darüber hinaus zeigten die erhaltenen wALADin-Verbindungen gegensätzliche biologische Resultate wie Inhibition, kein Effekt und Stimulation in artübergreifenden

Struktur-Wirkung-Beziehungsstudien, in denen mehrere ALAD-Orthologe von Pflanzen, Bakterien, Metazoen und Protozoen getestet wurden.[1c] Hervorstechend war der antiplasmodische Effekt von wALADin1, wALADin2 und wALADin3 auf das Merozoitenstadium von *Plasmodium falciparum.*[1d] Diese Verbindungen reduzierten die Parasitenbelastung auf annähernd 0 % und wALADin2 stellte sich als potenter Inhibitor der Plasmodien-Beweglichkeit und des Eindringens von Plasmodium-Sporozoite in Leberzellen des Wirtes heraus. Zusätzliche Experimente mit dem PfALAD-Ortholog zeigten jedoch, dass der antiplasmodische Effekt der wALADin-Verbindungen nicht auf der Inhibition dieses Orthologs beruht sondern auf der wALADin-Interaktion mit einem alternativen Zielmolekül beruhen muss.

Diese Ergebnisse liefern die wALADin-Verbindungen als potenzielle Leitstruktur für die Entwicklung von neuen antifilarischen, antiplasmodischen und antimikrobischen Mitteln, Herbiziden oder Werkzeugen der chemischen Biologie.

3. Introduction

3.1. Parasitic diseases

Lymphatic filariasis and Onchocerciasis are parasitic diseases in humans caused by an infection with human filarial nematodes which are common in the tropical regions of America, Asia, and Africa.[4] The host's immune response and inflammatory reactions to these infections lead to chronic diseases, manifesting in destroyed and distorted tissue or blindness. These diseases are rated as major public health problems in endemic regions by the World Health Organization (WHO). The WHO announced that more than 120 million people suffer from lymphatic filariasis[5a] and approximately 18 million from Onchocerciasis[5b] with more than 1.4 billion inhabitants of endemic countries at risk of developing these disabling and stigmatising chronic diseases.

The pathologies in lymphatic filariasis are hydrocele and lymphedema that can develop into elephantiasis.[10] Elephantiasis is an oedema with thickening of the skin and underlying tissue and affects mainly the lower extremities, and in less frequent cases, the ears and mucus membrane.

In Onchocerciasis pathologies are depigmentation of the skin and dermatitis, while it can also manifest in ocular lesions leading to keratitis, severe visual impairment and blindness.[4, 11]

In both diseases the endosymbiotic α-proteobacteria *Wolbachia* is a provocative driver of inflammation. *Wolbachia* lipoproteins promote chronic inflammation and are associated with side-effects in antifilarial chemotherapy. Common therapies that target filarial nematodes release huge amounts of bacteria upon death of the filarial host, which subsequently induces severe inflammatory reactions.[12]

One third of the infected people show clinical disease while the remaining develop no overt symptoms, although infected with millions of vigorously motile worms. Presumably, a high rate of inflammatory responses of the human hosts to dead or dying adult worms brings forth the development of clinical disease.[4]

Lymphatic filariasis and Onchocerciasis can be diagnosed by identifying microfilariae in the blood of the patient for the former and in skin snips for the latter.[13] Although these methods are widely used, they are time-consuming and cumbersome. For this reasons rapid and sensitive techniques have been developed for the diagnosis of these diseases.[14]

Immunochromatographic card tests (ICT) have simplified the diagnosis of *bancroftian* filariasis by detecting antigens. This technique is advantageous not only due to its simplicity but also since it can detect latent infections.[15] An equivalent method for Onchocerciasis has not been established yet due to a lack of suitable antigen detection assays, but rapid card tests based on antibody detection have been produced and assessed with promising results.[16]

3.2. Human filarial nematodes

Causative agents of lymphatic filariasis and Onchocerciasis are the filarial nematodes *Wuchereria bancrofti, Brugia malayi,* and *Onchocerca volvulus.* The former two are known to induce lymphatic filariasis, while the latter is associated with Onchocerciasis. The site of parasitism of the adult worms of *Wuchereria bancrofti* and *Brugia malayi* is in dilated nests within the lymphatic vessel which block the lymphatic system and cause the pathologies described above. *Wuchereria bancrofti and Brugia malayi* mainly affect the legs and arms. Cases of affected genitals are also known, but more commonly reported for infections with *Wuchereria bancrofti.*

The adult worms of *Onchocerca volvulus* are found in nodules within the subcutaneous and deep tissues. Microfilariae migrate into the skin and eyes and cause severe inflammation that leads to the impairment of skin and eyes previously described.

In their desired tissue-sites, adult worms survive for more than a decade reproducing and releasing millions of microfilariae (L1 larvae) (**Figure 1**). Microfilariae of *Wuchereria bancrofti* and *Brugia malayi* migrate to the blood and microfilariae of *Onchocerca volvulus* within the dermis where they are ingested during the next blood meal of their required arthropod vector. Mosquitos and the black fly serve as biological vectors and intermediate hosts. Within the arthropod, the microfilariae develop into infective larvae (L3 larvae) which migrate to the head of the arthropod, enter the proboscis, and infect the human host upon the arthropods' next blood meal.

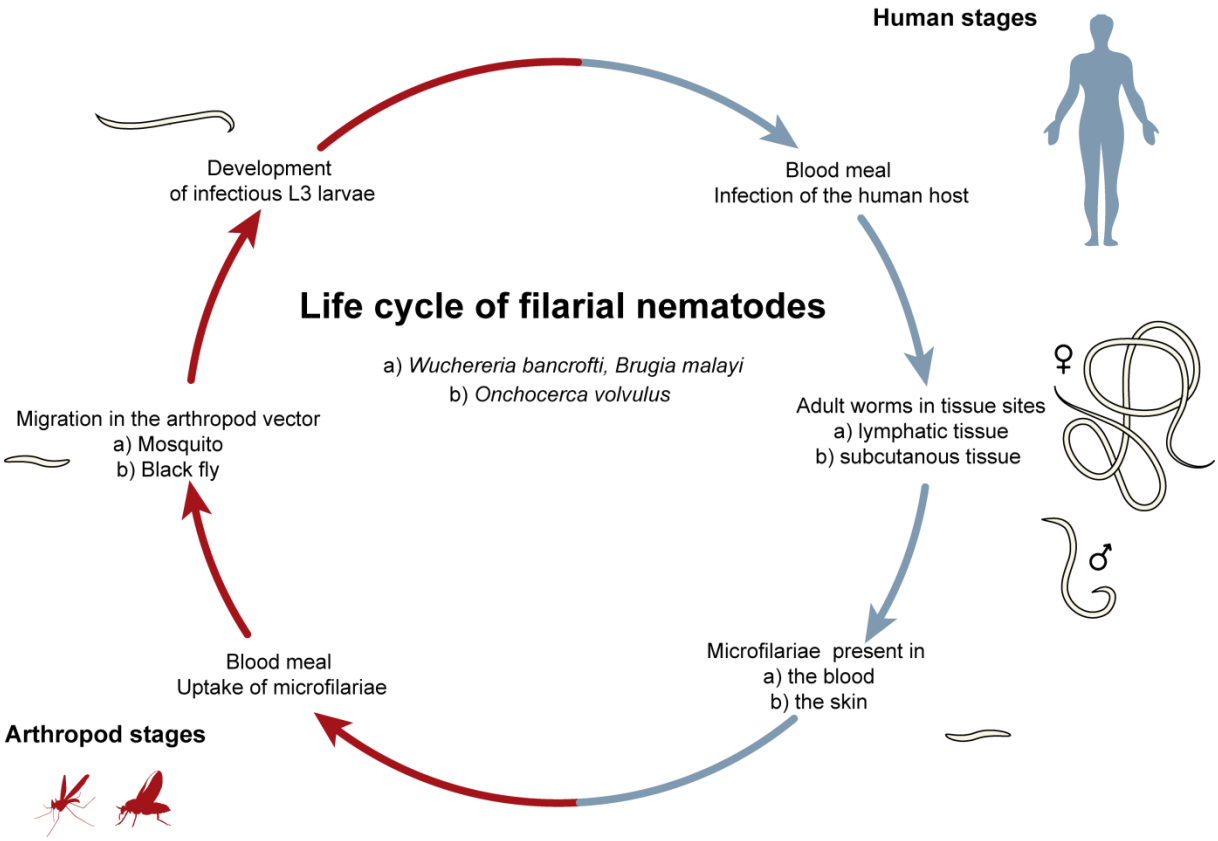


Figure 1: Life cycle of filarial nematodes. a) *Wuchereria bancrofti and Brugia malayi.* b) *Onchocerca volvulus.* The figure shows an adapted version of the figure found in reference[2].

3.3. *Wolbachia* endobacteria

The bacteria found in filarial nematodes responsible for lymphatic filariasis and Onchocerciasis are a group of endosymbiotic α-proteobacteria termed *Wolbachia*. Currently, there is only one valid species within the genus *Wolbachia* called *Wolbachia pipientis,*[17] which comprises nine phylogenetic lineages that are still continuously updated. Only two of these lineages are found in filarial nematodes. These are exclusively present in the subfamilies of the filarial nematodes *Onchocerca spp.*, *Dirofilaria spp.*, *Brugia spp*. and *Wuchereria bancrofti* and are generally found in vacuoles in the cytoplasm of the nematodes' cells.[18]

The relationship between *Wolbachia* and these filarial nematodes is mutual and has been stable and species-specific for long evolutionary periods. The numbers of *Wolbachia* are generally lower in male than in female worms. Within female nematodes, *Wolbachia* are mainly found in the reproductive tract which enables the extranuclear transmission of the endobacteria from the female worm to its offspring. The numbers of *Wolbachia* in microfilariae are low but multiply during their development into adult worms. Knowledge of genome sequences of *Wolbachia*[8] as well as *B. malayi*[9] revealed

a division of functions between the nematode and the endosymbiont. Several biochemical pathways for the synthesis of essential molecules were found to be either present in *Wolbachia* or its filarial host. The endosymbiont, for example, lacks the genes for *de novo* synthesis of several essential amino acids, vitamins and cofactors and therefore, these have to be provided by its filarial host.

On the other hand, filarial nematodes are incapable of *de novo* synthesis of heme, purine and flavins and depend on *Wolbachia* to deliver these essential molecules.

Heme is an essential cofactor for many proteins such as cytochromes, haemoglobins, peroxidises, and catalases. These proteins are involved in critical biological processes, including oxidative metabolism and electron transport. Antibiotics were used to reduce numbers of Wolbachia, confirming the filarial nematode's dependence on Wolbachia to maintain fertility and embryogenesis.[7] These experiments resulted in a sex-ratio shift and defects in molting[19] in the subsequent generation. These findings were not observed in experiments using *Wolbachia*-free filarial nematodes.[11] The involvement of a heme-dependent cytochrome in molting and reproductive processes of filarial nematodes[20] is a possible explanation for the defects in these processes after depletion of *Wolbachia*.

Wolbachia play a crucial role in pathological processes of filarial diseases. Their lipoproteins are inflammatory ligands that induce innate inflammation by binding and activating toll-like receptors.[21] Additionally, components of *Wolbachia* promote recruitment and activation of neutrophils[22] in the cornea which is the causative agent of stromal haze that results in blindness.[23]

3.4. Antifilarial drugs

Classical anthelmintics

To eliminate Onchocerciasis and lymphatic filariasis as massive public health problems, mass drug administration programs (MDA) are carried out in endemic countries. Single-dose combination therapies with classical anthelmintics such as diethylcarbamazine or ivermectin each combined with albendazole are administered in these programs.

Diethylcarbamazine (**Figure 2**) is a synthetic organic compound which is used as an anthelmintic drug for the treatment of lymphatic filariasis.[24] It is effective against all parasite-induced lymphatic filariasis by efficiently depleting microfilariae. Its effect on

adult worms is only weak.[25] The mode of action of diethylcarbamazine is not yet understood,[26] but its dependence on host components for its *in vivo* activity such as the arachidonic acid pathway, 5-lipooxygenase pathway, cyclooxygenase and inducible nitric oxide pathways has been confirmed in studies by McGarry et al.[27]

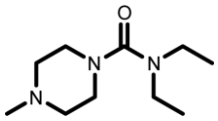


Figure 2: Structure of diethylcarbamazine[1a].

Diethylcarbamazine-induced rapid killing of high amounts of microfilariae in infected individuals is accompanied by the release of large amounts of *Wolbachia.* This causes adverse reactions and depending on the site of parasitemia manifest in systemic inflammation.[28] Administration of diethylcarbamazine to Onchocerciasis patients led to blindness since death of ocular residing microfilariae resulted in strong inflammation causing ocular lesions.[4, 29]As a response, diethylcarbamazine administration was abolished in areas where Onchocerciasis is co-endemic.[29]

In Onchocerciasis patients and in co-endemic regions ivermectin is administered.[30] Ivermectin is a macrocyclic lactone derivative that belongs to the group of avermectins which are metabolism products of the actinobacteria *Streptomyces avermitilis*. Ivermectin is a mixture of two semisynthetic avermectins B_{1a} and B_{1b} in a 9:1 ratio (**Figure 3**).

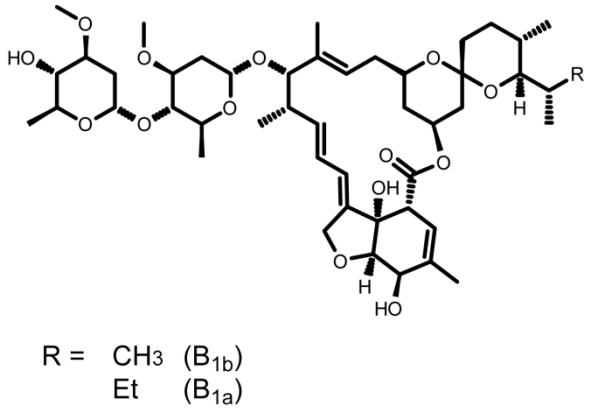


Figure 3: Structure of ivermectin.[30]

The mode of action is the hyperpolarisation of glutamate-sensitive ion channels which results in immobilisation of microfilariae.[31] Ivermectin mainly eliminates microfilariae, leaving most adult worms unaffected.[31] These will resume production of microfilariae if not treated periodically for the entire adult worms' lifespan.

In treatments using Ivermectin, severe inflammatory reactions are observed upon massive elimination of microfilariae. These reactions are due to the release of *Wolbachia* and are similar to those for diethylcarbamazine. Especially in patients infected with the loiasis causing eye worm Loa loa[32], severe encephalopathy was observed when treated with ivermectin.[33]

Albendazole is given in combination with diethylcarbamazine or ivermectin as a lymphatic filariasis treatment to enhance the period of reduced microfilariae in the periphery. Albendazole (**Figure 4**) is a benzimidazole derivative that is effective in depleting microfilariae by destabilisation of microtubules in the nematode.[34] Recently it was reported that the *in vivo* metabolite of albendazole is capable of reducing *Wolbachia* and that the antifilarial effect of albendazole is most likely a combination of destabilisation of microtubules and the depletion of *Wolbachia*.[35] These effects are only observed when treatment is administered over a prolonged time period with high doses of albendazole.[36] The dose used in combination with diethylcarbamazine or ivermectin probably has no added effect on adult worms, but merely enhance the existing effects.[37]

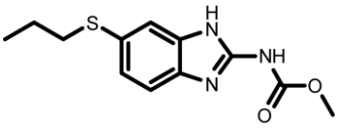


Figure 4: Structure of albendazole[1a].

Antibiotics depleting *Wolbachia*

As previously stated, human filarial nematodes that carry *Wolbachia* depend on their endobacteria to maintain essential functions. The depletion of *Wolbachia* as an attempt to develop novel antifilarial treatments is therefore an obvious consideration. Previous studies showed that antibiotic treatments using tetracycline are capable of depleting *Wolbachia* in filarial nematodes *in vivo,* resulting in long-term sterility of female filarial hosts and premature death of adult worms after 12 months.[11, 38] The observed effect was limited to tetracycline antibiotics, for example doxycycline, and could not be observed for several other antibiotics tested.[39] Doxycycline (**Figure 5**) was used as *Wolbachia*-depleting treatment in clinical trials. It was the first drug to show a prominent depletion of macrofilarial activity especially in onchocerciasis.[40] The mode of action of this antibiotic is the inhibition of protein translation by preventing the attachment of the aminoacyl-tRNAs to the ribosomal acceptor site. Additionally, doxycycline shows a bacteriostatic effect.[2, 41] Treated individuals showed substantial improvement in lymphatic pathological features and decreased severity of lymphedema

and hydrocele.[42] Similar to animal tests, doxycycline causes long-term sterility and death of adult worms. The slow drug action of doxycycline and the delayed death of filarial populations prevent *Wolbachia*-mediated inflammatory adverse reactions and severe and often fatal adverse events of common therapies. Furthermore, no adverse side-effects should be observed in loiasis co-infected patients since the filarial nematode Loa loa lacks *Wolbachia*. This should prevent severe side-effects as seen in ivermectin-treated co-infected patients.

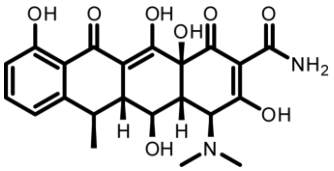


Figure 5: Structure of doxycycline.[39]

Therapies using doxycycline are good therapeutic options, yet they require long term treatment up to six weeks and show contraindications for children, pregnant and breast-feeding women.[2] At this point, doxycycline therapies are especially valuable for individuals under close observation by medical personnel, rather than in mass drug distribution.[17, 43] Nevertheless, first trials with combination therapies using rifampicin, an antibiotic used for tuberculosis treatment, were successful to make treatment regimens more suitable for mass drug distribution.[44] These therapies are not yet fully developed, but represent a giant step toward novel therapies for mass drug distribution programs.

3.5. A novel antifilarial drug target

The effect of present antifilarial drugs used in mass drug distribution programs is indisputable. Nevertheless, the effect is limited to reduction of transmission by merely depleting microfilariae and temporarily paralyzing adult worms. This results in the need of consecutive treatment of infected individuals for the entire lifespan of the adult worm entailing treatment for more than a decade. Additionally, observations of drug resistance of filarial nematodes against diethylcarbamazine and ivermectin have been reported which leaves the future effectiveness of these drugs in mass drug distribution uncertain.[43, 45]

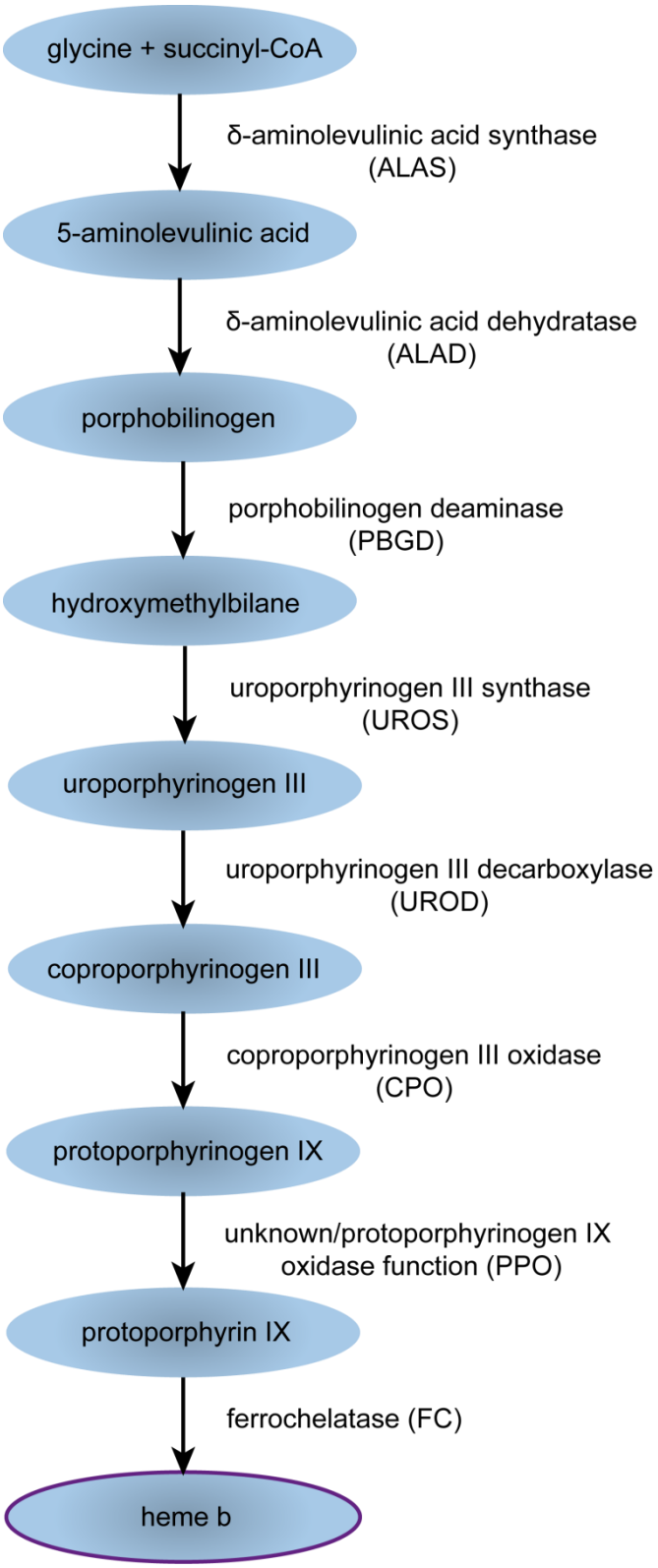
With tetracycline antibiotics, like doxycycline, the first steps were taken towards strong macrofilariae-depleting treatments and biosynthetic pathways in *Wolbachia* were identified as suitable targets for antifilarial drug discovery.

Genome sequence analysis revealed several essential biosynthetic pathways which exclusively occur in *Wolbachia* and not in its filarial host. One of these pathways, the heme biosynthetic pathway, was selected as a potential target. This choice is advantageous, since the products of this pathway are also indispensable for the filarial host.[8-9] Heme is essential in many vital processes like oxygen transport (haemoglobin/myoglobin)[46] and oxidative phosphorylation in bacteria (cytochrome b and c oxidase),[47] to cite just two examples.

Heme is a heterocyclic organic molecule consisting of four pyrrolic groups that are methine bridged at their α-positions. At its centre, heme complexes an iron ion, which can either be present as a ferrous (Fe^{2+}) or a ferric (Fe^{3+}) ion.

In eukaryotic cells and α-proteobacteria the initial step of the heme biosynthetic pathway is the synthesis of 5-aminolevulinic acid by aminolevulinic acid synthase-catalysed reaction of glycine and succinyl-CoA (**Scheme 1**). In the next step the pyrrole porphobilinogen is formed by δ-aminolevulinic acid dehydratase-catalysed asymmetric condensation of two 5-aminolevulinic acid molecules. The linear hydroxymethylbilane is then produced by porphobilinogen deaminase mediated tetramerisation and subsequently cyclised to uroporphyrinogen III by uroporphyrinogen III synthase. In the next three steps, the different side chains are introduced by using uroporphyrinogen III decarboxylase to form coproporphyrinogen III, coproporphyrinogen III oxidase to form protoporphyrinogen IX and protoporphyrinogen IX oxidase to form protoporphyrin IX. The incorporation of the ferrous ion is the final step in the synthesis of the most common heme form, heme b.[48]

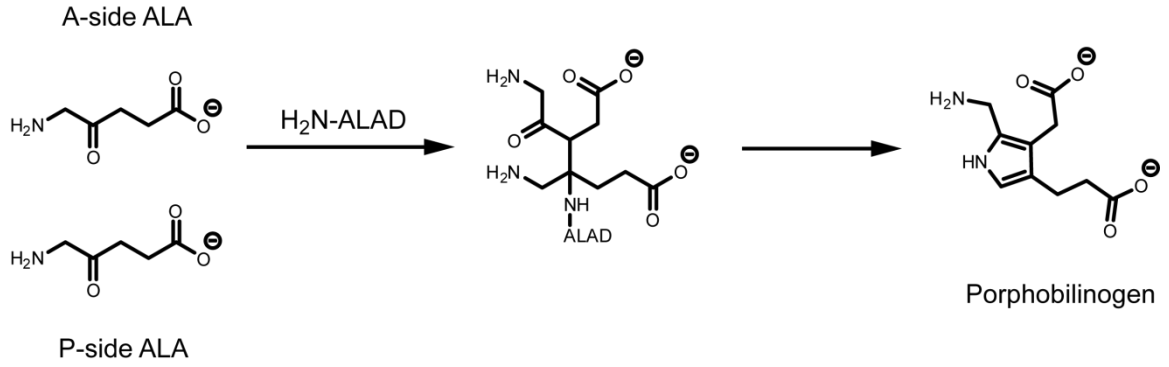
Humans and *Wolbachia* share this type of heme biosynthesis, but show a deep evolutionary distance between homologues in this pathway. The comparison between *Wolbachia* and human aminolevulinic acid dehydratase (wALAD and hALAD) indicates significant structural und functional variations between these two enzymes.[1a, 49] hALAD contains a zinc ion(Zn^{2+})-binding cysteine rich sequence,[50] while wALAD requires magnesium ions (Mg^{2+}) and is Zn^{2+}-independent.[51] Binding of the respective metal ion is essential for enzymatic activity. The differences in metal binding sites and the structural and functional variations make wALAD an ideal species-specific inhibition target.



Scheme 1: Heme biosynthesis pathway. The figure shows an adapted version of the figure found in reference[51].

Porphobilinogen is obtained via the asymmetric condensation of two 5-aminolaevulinic acid (ALA) molecules which is catalysed by aminolevulinic acid dehydratase.[1a, 49, 52] One ALA forms a Schiff base at the propanoic acid side (P-side ALA) with a conserved lysine residue of ALAD and binds to the second ALA which contributes

the acetic acid side (A-side ALA). The pyrrole ring of porphobilinogen is then formed via aldol condensation in a subsequent step (**Scheme 2**).



Scheme 2: Synthesis of porphobilinogen.[52]

The formation of porphobilinogen can be monitored by addition of modified Ehrlich reagent, containing *p*-dimethylaminobenzaldehyde (DMAB) in glacial acetic acid, perchloric acid, and trichloroacetic acid.[1b, 2] Added to the sample, DMAB forms a red coloured complex with the pyrroles of porphobilinogen. This colour shift can be spectrometrically detected at 555 nm.[53]

3.6. Small molecule inhibitors

With wALAD identified as a suitable species-specific target for new antifilarial drug approaches, molecules showing acceptable inhibitory activity were identified by high throughput screening. A chemical library of almost 18,000 drug-like small molecules[54] was screened for inhibitory activity on the wALAD orthologue. In this screening three benzimidazole-based hit structures were identified that specifically inhibited wALAD in a species-selective and dose-dependent manner and showed structural similarity. Besides the benzimidazole based core structure they contained a substituted benzyl group (R^1, green), a substituted aryl or aromatic heterocycle attached to a carbonylaminoethyl chain (R^2, red) and a carboxylic acid (R^3, blue) (**Figure 6**). The most promising of these three inhibitory compounds was termed wALADin1, while the remaining two were discarded due to their low inhibitory activity. wALADin1 inhibited wALAD with a half maximum concentration (IC_{50}) of approximately 11 µM, while experiments with the human ALAD orthologue revealed a marginally inhibitory activity with an IC_{50} of approximately 740 µM. This species-specificity was proven to be valid for the optimum pH of both orthologues, pH 7.5 for hALAD and pH 8 for wALAD.[1b].

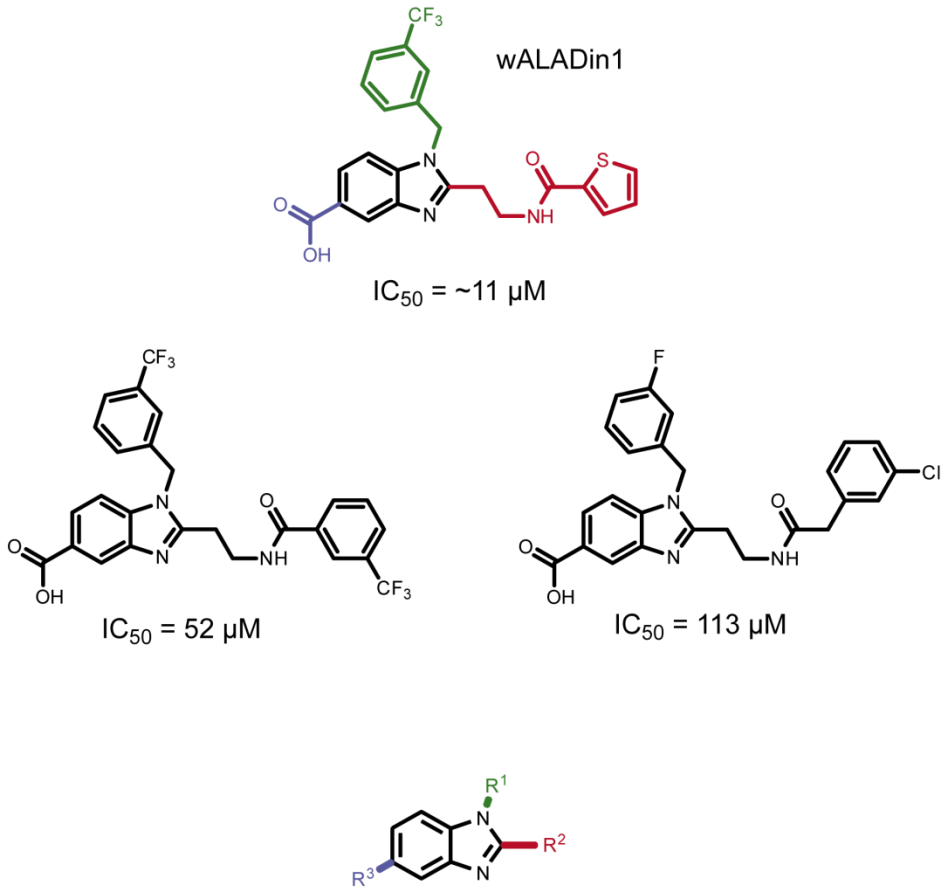


Figure 6: Benzimidazole hit structures from the high throughput screening of ~18,000 drug-like small molecules.[1a]

Further investigation of 50 benzimidazole-based structures with overall similarity to the hit structures but without the carboxylic acid (R^3, blue) and the examination of a wALADin1 derivative lacking the carboxylic acid as well revealed the contribution and the necessity of the carboxylic acid moiety to the inhibitory activity of the compounds.[2] Further determination of the relationship between the side chains and the inhibitory activity of wALADin1 were required and investigations were started during my diploma thesis[1a] and continued in this work.

A structure activity relationship study (SAR) was initiated during my diploma thesis starting with investigating the influence of the carboxylic acid's position on the inhibitory activity. Therefore, wALADin1 derivatives were synthesised, carrying the carboxylic acid at different positions of the benzimidazole core (**Figure 7**).

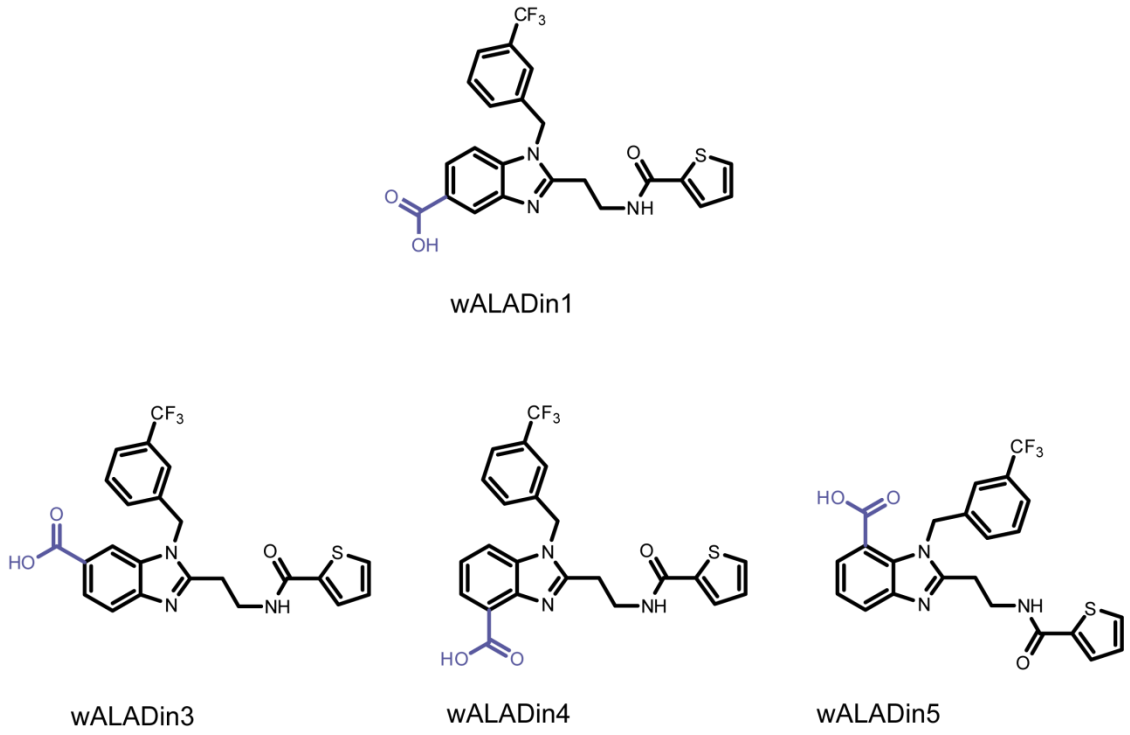
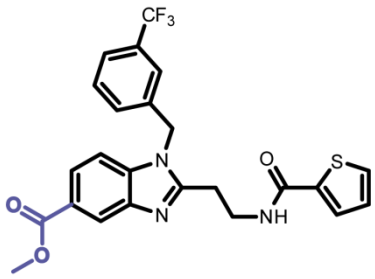


Figure 7: wALADin1 and its R^3-repositioned derivatives.[1a]

In collaboration with the Institute of Medical Microbiology, Immunology and Parasitology (IMMIP) the obtained compounds **3**, **4** and **5** were tested for their inhibitory activity against wALAD mostly by Dr. Christian Lentz. wALADin3 and wALADin5 showed inhibitory activity, but were considerably weaker inhibitors than wALADin1(**Section 5.3 Table 2**). wALADin4 was inactive in the wALAD assay.

To investigate how modifying the carboxylic acid affected the inhibitory activity, the methyl ester **59**, which is a precursor in the synthesis of wALADin1,[1b] was tested for its inhibitory activity against wALAD and showed none at all. This led to the conclusion that the carboxylic acid has to be present and unaltered for inhibitory effects to occur and that the position of the carboxylic acid has a great impact on the inhibitory activity of the wALADin compounds. In comparison to the initial compound wALADin1, all the synthesised regioisomeric derivatives proved to be considerably less active or even inactive.



59

Figure 8: Methyl ester 59, a precursor in the synthesis of wALADin1.[1a, 1b]

The specific binding of wALADin1 to wALAD was then further demonstrated by the IMMIP via thermal shift assays.[2] In these assays the stabilisation of the protein by ligand binding results in a rightward shift of the protein's melting curve. This curve is displayed as an increase in fluorescence intensity of the environmentally sensitive fluorescent dye Sypro Orange.[55]

At the beginning of this study, the mode of action of wALADin1 was not fully discovered but was assumed to be based on a competitive/non-competitive mechanism that involved functional competition of wALADin1 with Mg^{2+}-binding or binding to a site that interferes with the allosteric activation process. In the meantime the inhibitory mechanism of wALADin1 has been identified. wALAD is known to exist in different oligomeric states which are either di-, hexa- or octameric. The former two are inactive assemblies since they lack subunit interactions required to stabilise a closed conformation of the active site lid, while the latter usually act as the active assemblies. wALADin1 disturbs the oligomeric equilibrium of wALAD and the interconversion between hexameric and octameric states by stabilising the low activity hexamers.[1c]
wALADin1 showed antifilarial activity with a half maximal effective concentration (EC_{50}) of approximately 100 µM in *ex vivo* experiments using the *Wolbachia*-containing rodent filarial nematode *Litomosoides sigmodontis* as a model system. Treated worms showed a significant reduction in motility and viability and the effect was proven to be *Wolbachia*-dependant. Due to the inactivity of wALADin4 in the enzymatic assays, it was chosen as a potential negative control compound for *in vitro* testing on filarial worms with wALADin1. In these *in vitro* tests wALADin4 proved to be much more toxic than wALADin1 by killing the filarial nematodes without the reduction of *Wolbachia* and was discarded as a negative control.

Nevertheless, *in vivo* experiments with L. sigmodontis infected mice delivered no antifilarial effect. The lack of an *in vivo* effect of wALADin1 is most likely a result of pharmacokinetic deficiencies.[2]

4. Aim of project

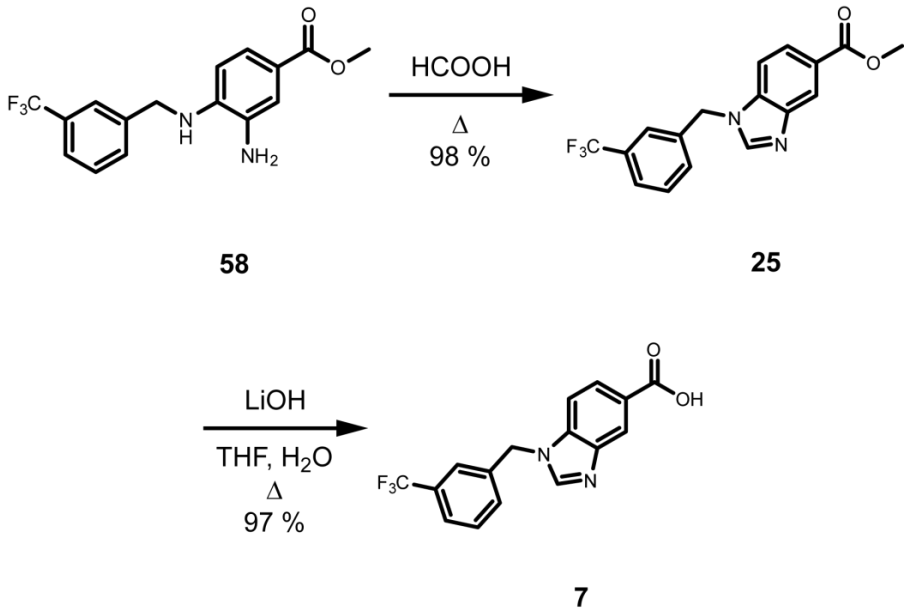
For the future treatment and eradication of filarial diseases, novel chemotherapeutic treatments need to be developed. In this case wALAD represents a promising target for novel antifilarial drug approaches. The identification of the species-specific and dose dependant wALAD inhibitor wALADin1 provides a good basis for the development of novel chemotherapeutics. However, desirable half maximal inhibitory concentrations of potential drug candidates lie in the nanomolar range, while the IC_{50} of wALADin1 is merely located in the low micromolar range. Additionally, the inhibitory activity of wALADin1 could not be confirmed in *in vivo* experiments, likely due to pharmacokinetic deficiencies. To be able to improve the inhibitory activity and modify functional groups for the improvement of pharmacokinetic properties or the use as chemical biology tools, the influence of all side chains (R^1-R^3) on the inhibitory activity has to be well understood. For this reason a variety of different wALADin1 derivatives with altered side chains were synthesised in this work and compared in a structure activity relationship study.

5. Results and discussion

The previously mentioned SAR was continued in this work. Since the investigations of the influence of the R^3-moiety revealed the importance of the presence of the carboxylic acid and its position on the benzimidazole core structure, the study was continued by determining the influence of the R^1- and R^2-moiety on the inhibitory activity. For this reason derivatives were synthesised either lacking a side chain or carrying moieties with altered positions or different electron demands. These derivatives were then tested for their inhibitory activity to gain full knowledge of the possibilities of modification and optimisation. The synthesis and inhibitory activity of R^1- and R^2- modified wALADin1 derivatives are reported below. All enzymatic assay, except the once described in **section 7.14,** were carried out by Dr. Christian Lentz at the IMMIP.

5.1. Investigation of the R^2-moiety

The influence of the R^2-moiety to the inhibitory activity was determined by examining a wALADin1 derivative lacking the R^2-moiety. The wALADin7 termed molecule was synthesised by cyclisation of the precursor **58**[1b] without prior attachment of the carboxylic acid **57 (Scheme 3)**. This reaction succeeded in a single step solvent and catalyst free reaction by formulating compound **58** using formic acid, which resulted in subsequent cyclisation.[56] The resulting compound **25** was obtained in excellent yields and was hydrolysed using lithium hydroxide monohydrate in a final step to obtain the R^2-free wALADin7 in excellent overall yields of 78 %.



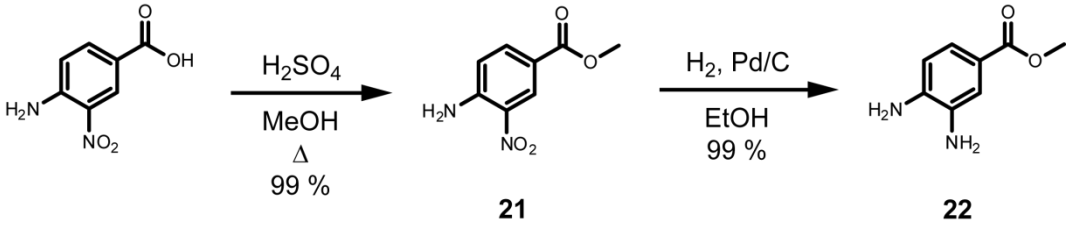
Scheme 3: Synthesis of wALADin7.

wALADin7 was tested for its inhibitory activity against wALAD and proved to be similarly potent to wALADin1 (~11 µM) with an IC_{50} of 13 µM. Based on this results further investigations on the R^2-moiety were deferred.

5.2. Investigation of the R^1-moiety

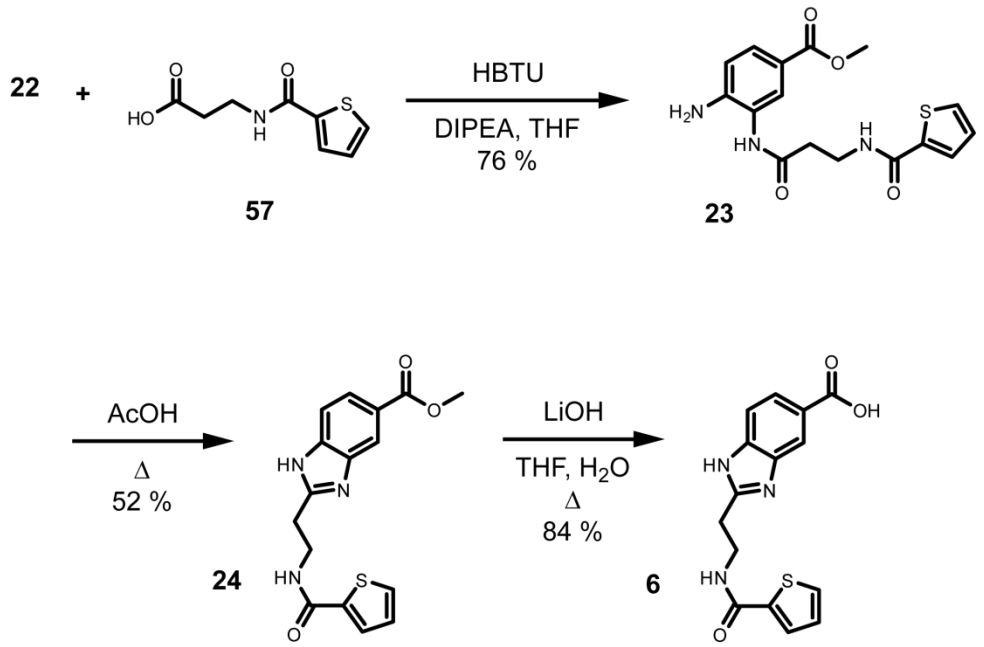
wALADin6

The influence of the R^1-moiety was investigated by synthesising a derivative lacking the R^1-moiety (wALADin6) as well as several derivatives with a variety of modified benzyl groups. A suitable starting material for the synthesis of wALADin6 was the 4-amino-3-nitrobenzoic acid. After esterification of the carboxylic acid the resulting methyl ester **21** was reduced to the dianiline **22** using hydrogen promoted by palladium on activated carbon (**Scheme 4**).



Scheme 4: Esterification of the starting material and reduction of 21.

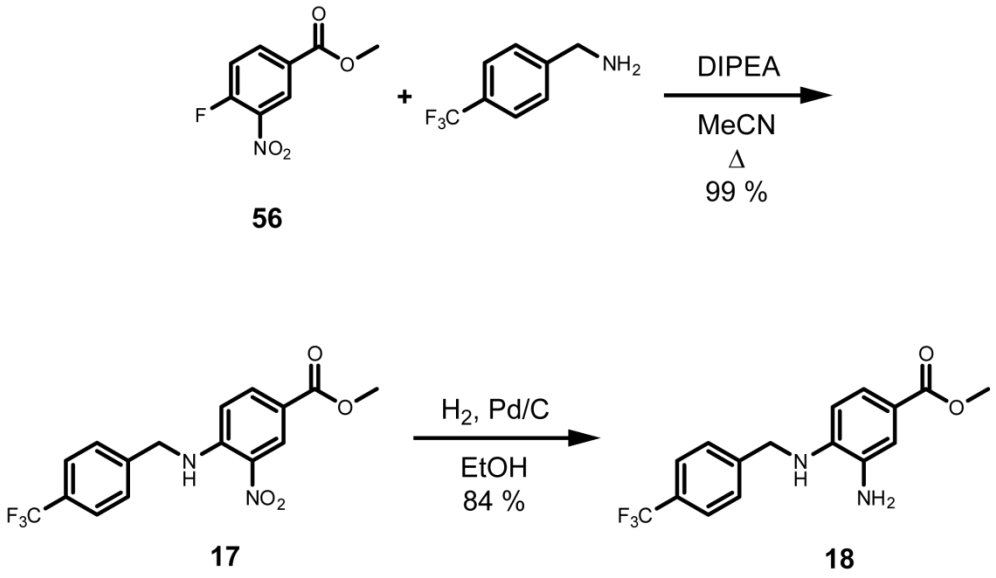
The HBTU-mediated coupling reaction of the dianiline **22** and the carboxylic acid **57** resulted exclusively in the formation of a single amide bond and furnished the aniline **23** in good yields (**Scheme 5**). Acid promoted cyclisation was carried out in a similar fashion as previously described using acetic acid and resulted in the tautomeric compound **24.** NMR measurements showed that compound **24** exists as constitutional isomer which readily interconverts between the two forms with the proton bound to either of the two benzimidazole nitrogens which results in a shift of the double bond. Hydrolysis of compound **24** using lithium hydroxide monohydrate furnished the final compound wALADin6 in overall yields of 32 %.



Scheme 5: Synthesis of wALADin6.

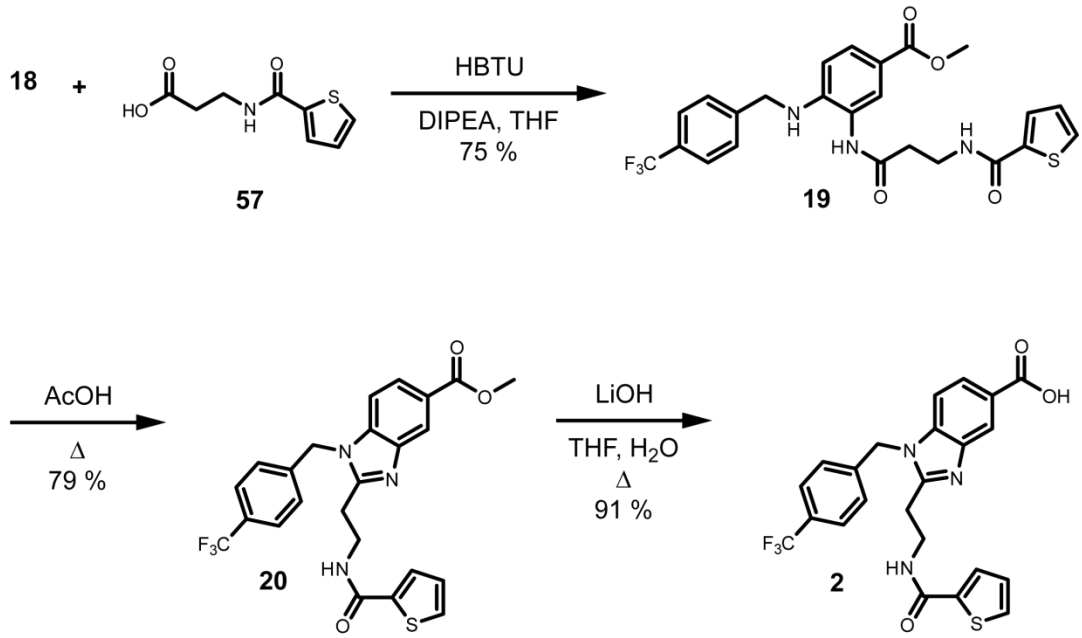
wALADin2

wALADin2 is a wALADin1 derivative with the trifluoromethyl group of the R^1-moiety in para position instead of ortho. The Synthesis of wALADin2 followed a similar synthetic pathway as for wALADin1. The methyl ester **56** enabled installation of 4-trifluormethylbenzylamine via ipso-nucleophilic substitution of the fluoro-group (**Scheme 6**). Subsequently, the furnished nitro aniline **17** was reduced to its corresponding aniline **18** using hydrogen promoted by palladium on activated carbon.[57]



Scheme 6: Nucleophilic aromatic displacement of 56 and reduction of 17.

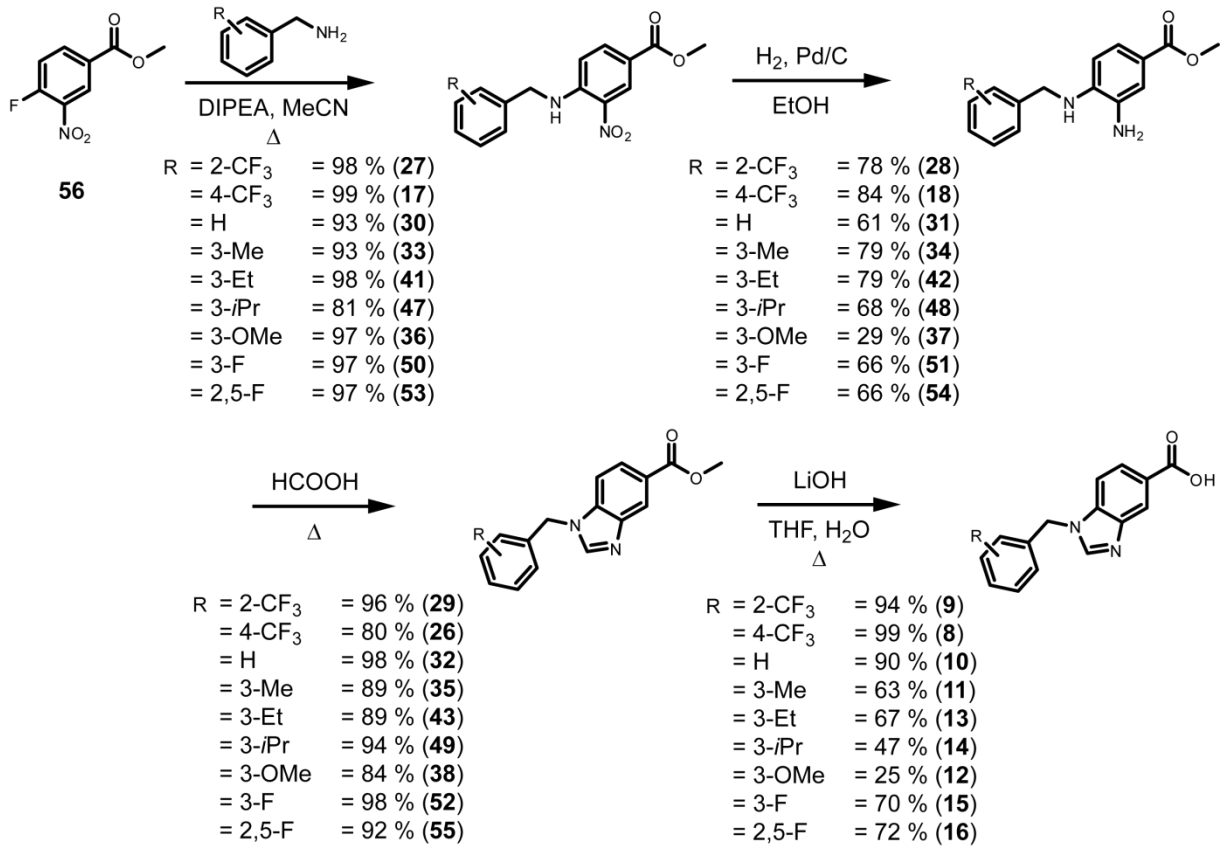
Carboxylic acid **57** was coupled to aniline **18** in an HBTU mediated coupling reaction (**Scheme 7**). HBTU is a benzotriazole based coupling reagent that activates the carboxylic acid by forming an active ester to promote the amide bond formation. After recrystallisation of the crude product the cyclisation precursor **19** was obtained in a moderate yield. The following step furnished the benzimidazole core structure **20** of wALADin2 by acid promoted cyclisation of **19** using acetic acid.[58] In a final step **20** was hydrolysed using lithium hydroxide monohydrate to give **2** in excellent yields.[59] wALADin2 was furnished in overall yields of 44 %.



Scheme 7: Synthesis of wALADin2 (2).

wALADin8-16

Since the R^2-moiety showed no significant influence on the inhibitory potency of wALADin7 the decision was made to proceed with compounds lacking the R^2-moiety. Nine derivatives were synthesised all following a similar synthetic pathway. The methyl ester **56** was coupled to the respective benzylamine in an ipso-nucleophilic substitution reaction of the fluoro-group (**Scheme 8**). The resulting compounds **17**, **27**, **30**, **33**, **36**, **41**, **47**, **50**, **53** were reduced to their corresponding anilines **18**, **28**, **31**, **34**, **37**, **42**, **48**, **51**, **54** using hydrogen promoted by palladium on activated carbon. The following step furnished the benzimidazole core structures **26**, **29**, **32**, **35**, **38**, **43**, **49**, **52**, **55** by acid promoted cyclisation of the respective precursors using formic acid. In a final step the respective precursors were hydrolysed using lithium hydroxide monohydrate to give **8-16** in excellent yields.



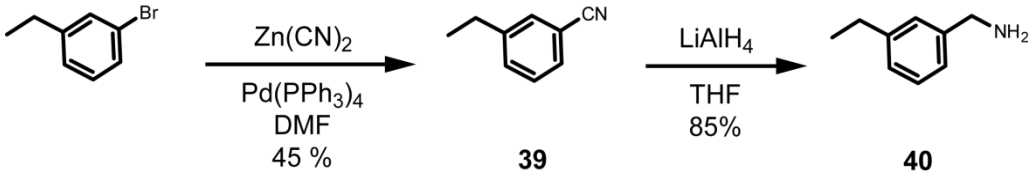
Scheme 8: Synthesis of wALADin8-16.

Compound	Overall yield [%]
8	65
9	69
10	49
11	43
12	6
13	41
14	34
15	43
16	42

Table 1: Overall yields of wALADin 8-16.

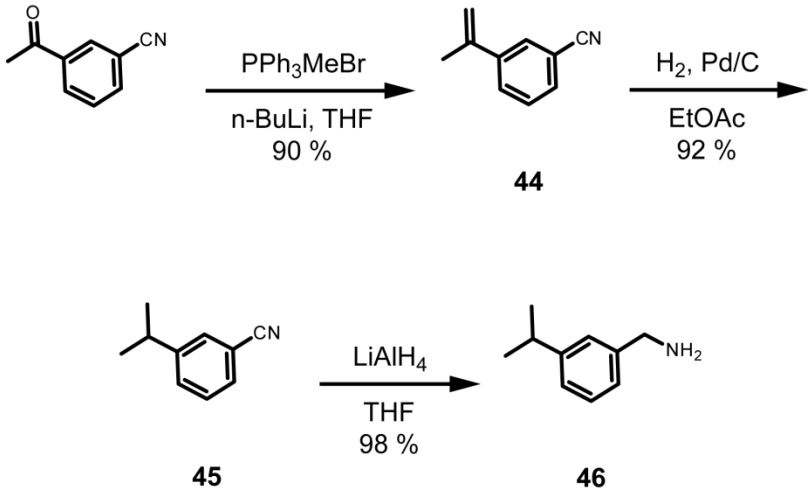
All benzylamines used were commercially available except for 3-ethylbenzylamine (**40**) and 3-isopropylbenzylamine (**46**) used for the synthesis of wALADin13 and wALADin14. Benzylamine **40** was obtained in a 2-step synthesis starting by converting the 1-bromo-3-ethylbenzene into the corresponding benzonitrile **39** using zinc cyanide

promoted by tetrakis(triphenylphosphine)palladium (**Scheme 9)**.[60] Compound **39** was then reduced to the corresponding benzylamine **40** using lithium aluminium hydride and furnished the desired compound in overall yields of 38 %.



Scheme 9: Synthesis of 3-ethylbenzylamine (40).

Benzylamine **46** was obtained in a 3-step synthesis using 3-acetylbenzonitril as starting material (**Scheme 10**). 3-Acetylbenzonitril was converted to the isopropenyl **44** by Wittig reaction.[61] In this reaction the phosphorus ylide methylenetriphenylphosphorane was generated by deprotonation of the methyltriphenylphosphonium bromide with n-butyl lithium. The generated ylide then reacted with the carbonyl group of the 3-acetylbenzonitril to form an intermediate species that converts into a four membered heterocyclic structure termed oxaphosphatane. Cleavage of the oxaphosphatane results in triphenylphosphine oxide and the desired isopropenyl **44**. Reduction of compound **44** using hydrogen promoted by palladium on activated carbon led to the isopropyl **45** which was reduced by lithium aluminium hydride to furnish the desired amine **46** in good overall yields of 81 %.



Scheme 10: Synthesis of 3-isopropylbenzylamine (46).

Comparison of R[1]-modified wALADin1 derivatives

Enzymatic assays using the synthesised R[1]-modified derivatives revealed the importance of this moiety. Replacement of the R[1]-moiety with hydrogen in wALADin6 resulted in a total depletion of inhibitory activity. Modifying the R[1]-moiety generally

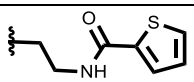
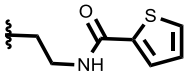
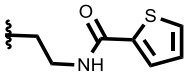
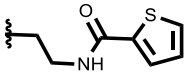
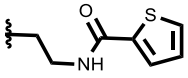
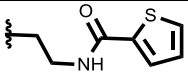
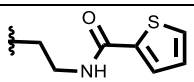
caused a decrease in inhibitory activity. Attachment of an unsubstituted benzyl moiety resulted in an 18-fold less active compound. Even minor changes like the repositioning of the trifluoromethyl group to either the 2-position as in wALADin9 or to the 4-position as in wALADin2 and wALADin8 resulted in a weakening of the inhibitory potency of these compounds. The 4-position was more tolerated, resulting in only 4-fold and 8-fold weaker inhibitors compared to 27-fold for wALADin9. Replacement of the electron-withdrawing CF_3-group with a fluoro-group either in a 3-mono or 2,5-disubtituted fashion as in wALADin15 and wALADin16 did not furnish an increase in inhibitory activity and resulted in 34- and 64-fold less active compounds. Attempts using benzyl moieties with electron-donating groups such as methyl-, ethyl-, isopropyl- or methoxy-moieties as in wALADin11, wALADin13, wALADin14 or wALADin12 did not furnish more potent inhibitors as well. This leads to the conclusion that the R^1-moiety in general and the 3-trifluoromethyl benzyl-moiety in particular is optimal among the series of compounds investigated.

5.3. Overall comparison of the wALADin1 and its derivatives

The structure activity relationship study revealed the influence of the different moieties of the wALAD inhibitors. The importance of the carboxylic acid-moiety was underlined by the results of thermal shift assays carried out by Dr. Christian Lentz at the IMMIP. These assays revealed that the less potent compounds wALADin3 and wALADin5 also bind to and stabilise the structure of wALAD but with smaller shifts in the melting curve as seen with wALADin1. The inactivity of wALADin4 was also displayed in its failure to induce a shift of the melting curve, which indicates no binding to the protein. These results indicate a functional role of the carboxylic acid-moiety in inhibition most likely through direct interactions with the wALAD protein by, for example, salt bridge formation.

The 3-trifluoromethyl substituent on the R^1-benzyl-moiety is preferred over all other substituents investigated. The substitution of the CF_3-group (wALADin7) with a CH_3-group as in wALADin11 results in a 10-fold lower inhibitory activity. This decrease in inhibitory activity is not only a result of reduced size of the benzyl-substituents since wALADin14, which carries an equally bulky isopropyl-substituent,[62] shows a 17-fold reduced inhibitory activity. The higher inhibitory activity of the CF_3-substituted compounds is possibly a result of the combined effects induced by the fluorines. These

effects can be, for example, solvation sphere bridged direct interaction with the protein and increased lipophilicity of the compound.

				wALAD		hALAD	
Compound	R^1-moiety	R^2-moiety	R^3-Moiety	IC_{50} [µM]	R^2	IC_{50} [µM]	R^2
wALADin1	3-CF_3-benzyl		C_5-CO_2H	11.1 ± 1.0	0.9517	739 ± 103	0.8582
wALADin2	4-CF_3-benzyl		C_5-CO_2H	38.6 ± 6.2	0.9670	618 ± 105	0.9186
wALADin3	3-CF_3-benzyl		C_6-CO_2H	317 ± 53	0.9022	*	-
wALADin4	3-CF_3-benzyl		C_4-CO_2H	*	-	*	-
wALADin5	3-CF_3-benzyl		C_7-CO_2H	164 ± 14	0.9551	*	-
wALADin6	-H		C_5-CO_2H	*	-	*	-
wALADin7	3-CF_3-benzyl	-H	C_5-CO_2H	13.0 ± 1.2	0.9638	197 ± 20	0.9457
wALADin8	4-CF_3-benzyl	-H	C_5-CO_2H	87.7 ± 11	0.9487	173 ± 12	0.9730
wALADin9	2-CF_3-benzyl	-H	C_5-CO_2H	293 ± 67	0.8503	145 ± 7.2	0.9854
wALADin10	benzyl	-H	C_5-CO_2H	197 ± 33	0.9042	213 ± 6.3	0.9934
wALADin11	3-Me-benzyl	-H	C_5-CO_2H	134 ± 17	0.9394	222 ± 11	0.9864
wALADin12	3-OMe-benzyl	-H	C_5-CO_2H	205 ± 12	0.9922	156 ± 7.8	0.9881
wALADin13	3-Et-benzyl	-H	C_5-CO_2H	168 ± 24	0.8573	n/a	n/a
wALADin14	3-*i*Pr-benzyl	-H	C_5-CO_2H	217 ± 29	0.8664	n/a	n/a
wALADin15	3-F-benzyl	-H	C_5-CO_2H	373 ± 61	0.8700	n/a	n/a
wALADin16	2,5-F-benzyl	-H	C_5-CO_2H	703 ± 192	0.8940	n/a	n/a
Compound59	3-CF_3-benzyl		C_5-CO_2Me	*	-	*	-

*absence of inhibitory activity, n/a = not determined

Table 2: Inhibitory activity of wALADin1 and its derivatives.

The R^2-moiety has no influence on the inhibitory activity against wALAD, but investigating the inhibitory potency of all R^2-H compounds on the human ALAD revealed a significantly higher inhibitory potential for the inhibition of the hALAD than the compounds containing the R^2-moiety. IC_{50}-values of the R^2-H compounds against hALAD ranged between 145 µM and 222 µM, while the most potent R^2-moiety containing compound, wALADin2, had an IC_{50}-Value of approximately 618 µM. This indicates a direct influence of the R^2-moiety on the species-selectivity of the wALADin compounds which is possibly a result of steric hindrance of the bulky R^2-moiety at the hALAD binding site.

Summarising the results, the structure activity relationship studies revealed that the carboxylic acid moiety on the C5-carbon and the 3-trifluoromethyl benzyl-moiety are essential for the inhibitory activity of the wALADin compounds, while the 2-[(2-thienylcarbonyl)amino]ethyl-moiety did not have any influence on the inhibitory activity against wALAD. Still it plays an important role for species-specific inhibition. This results in wALADin1 and wALADin7 as the most potent wALAD inhibitors in this study.

5.4. Cross species structure activity relationship studies

As previously described, wALADin1 and its derivatives specifically inhibit wALAD in a mixed competitive/non-competitive manner by disrupting the Mg^{2+}-dependant activation of the enzyme while leaving the Mg^{2+}-independent activation of the human orthologue mostly unaffected. This led to the question if this specificity also applies to other organisms for which the products of the heme biosynthetic pathway are equally essential. For this reason the wALADin compounds described in this work were tested in a cross species structure activity relationship study (SAR) on different ALAD orthologues by Dr. Christian Lentz and Silke Strassel at the IMMIP.[1c]

ALAD orthologues of the bacteria *Escherichia coli* (Ec), *Vibrio cholera* (Vc), *Yersinia enterocolitica* (Ye), *Pseudomonas aeruginosa* (Pa), the parasite *Toxoplasma gondii* (Tg) and the chloroplast protein of *Pisum sativum* (Ps) were chosen to determine the effect of wALADin1 and its derivatives on different ALAD orthologues.

Enzymatic activity of EcALAD is Zn^{2+}-dependant[63] while the enzymatic activity of PsALAD[64] and TgALAD[65] requires catalytic Mg^{2+}. VcALAD[50], YeALAD[50] and PaALAD[66] do not require divalent metal ions for catalytic activity, but all six

orthologues are allosterically activated by Mg^{2+}. Additionally, the ALAD orthologue of the fruit fly *Drosophila melanogaster* was also investigated. Like hALAD, DmALAD is Zn^{2+}-dependant and does not require allosteric activation by Mg^{2+} for enzymatic activity. The investigations revealed that the wALADin compounds are not merely inhibitors but also have the ability to stimulate the enzyme.[1c]

PsALAD was inhibited in a competitive/non-competitive manner and all wALADin compounds showed comparable inhibitory activity as seen for wALAD. Repositioning the carboxylic acid to C4, C6 or C7 position and alterations of the benzyl-moiety resulted in a decrease in inhibitory activity while the removal of the R^2-side chain had no significant effect on the inhibitory activity. Only minor differences were observed, for example, that wALADin5 was inactive on PsALAD while it showed weak inhibitory activity on wALAD and vice versa for wALADin6.

EcALAD, YeALAD, TgALAD and VcALAD were stimulated by wALADin1 and some derivatives. Unlike the SAR for inhibition with wALADin compounds stimulation SAR showed different tendencies. In contrast to the decrease of inhibitory activity in inhibition assays, repositioning of the carboxylic acid group was well accepted, with wALADin3 being one of the two most potent compounds in stimulation assays. Removal of the R^2-side chain or any modification on the benzyl moiety, except the repositioning of the CF_3-moiety from 3- to 4-position, were not tolerated and led to abrogation of the stimulating effect. These findings resulted in a set of stimulators for the ALAD-orthologues stated with wALADin2 and wALADin3 being the most potent compounds.

The enzymatic assays carried out by Dr. Christian Lentz and Silke Strassel at the IMMIP indicated that the stimulating effect of the wALADin compounds is protein concentration dependant since high protein concentration abrogated stimulation.[1c] This led to the conclusion that the stimulating mode of action of the wALADin compounds is based on promoting and stabilising the enzymatically active assemblies by modifying the oligomeric equilibrium.

PaALAD represents a special case: Under the respective conditions used for the inhibition and stimulation assays the PaALAD was either inhibited or stimulated by the wALADin compounds. This phenomenon was exclusively observed for PaALAD.

In general, DmALAD was similarly insensitive to wALADin1 and all derivatives carrying the R^2-moiety as seen for the human orthologue. One exception is seen in wALADin3 with the carboxylic acid in C6 position which shows a week inhibitory effect

on the fruit fly orthologue. In contrast, removal of the R^2-moiety does not generally result in a rise of inhibitory activity as seen for hALAD. It appears that some modifications on the benzyl moiety have an abrogating effect on the inhibitory activity that exceeds the activating effect of the R^2-moiety removal. This is displayed in the insensitivity of DmALAD towards wALADin8, wALADin9, wALADin10.

All tested ALAD-orthologues could be assigned to one of the three categories inhibition, stimulation or insensitivity. For each category a different set of wALADin compounds with specific chemical features is most effective. This results in a specific SAR profile for each category which is shared exclusively by the members of the same category. PaALAD which was inhibited and stimulated under the respective conditions showed a SAR profile corresponding to that of the other members present in the according category and indicated that the decision between inhibition and stimulation is not only functional.[1c] Furthermore, hALAD and DmALAD, members of the insensitivity category, both lack an allosteric Mg^{2+}-binding site. This leads to the assumption of a correlation between the presence of an allosteric Mg^{2+}-binding site and inhibition/stimulation. Nevertheless, further predictions of wALADin responsiveness based on the investigations of the predicated oligomeric architectures and the dependence of catalytic metal ions made by the IMMIP could not be made due to the lack of unambiguous patterns in the response of the ALAD orthologues to the wALADin compounds.[1c]

These experiments revealed the diversity of the wALADin compounds and added further properties to their effector spectrum. The insensitivity of the human orthologue towards some wALADin compounds and therefore the enabled species specific ALAD-inhibition of human pathogens poses an up to now unique characteristic. Revealing the inhibitory effect on plant ALAD additionally shows a potential for herbicidal use of the wALADin compounds and even the stimulating properties of some wALADin compounds are not necessarily a disadvantage for the development of therapeutic applications. The stimulatory activity might proof effective against organisms in which overproduction of porphyrins and precursors lead to phototoxic effects in the targeted organism.[67] All in all the wALADin compounds and future derivatives might deliver new herbicidal and antibiotic therapies based on ALAD inhibition or stimulation.

5.5. Investigation of antiplasmodial activity.

Another parasite for which the products of the heme biosynthetic pathway are essential and which is known to synthesise heme *de novo* is the malaria parasite *Plasmodium falciparum*.[68] The ALAD orthologue of *Plasmodium falciparum* (PfALAD) is also allosterically activated by Mg^{2+}[69] and therefore poses a potential target for the wALADin compounds. The parasite has the ability to not only use the endogenously occurring ALAD but also the human orthologue which is imported from infected erythrocytes.[70] Although only 10 % of the ALAD-activity in the malaria parasite is attributed to PfALAD, it is presumed that PfALAD is essential during certain life stages or apicoplast homeostasis.[71] To determine the antiplasmodial activity, the wALADin compounds described in this work were investigated for their activity on different life cycle stages of *Plasmodium*.[1d]

Activity assays for the blood stage type were carried out by incubating *Plasmodium falciparum* erythrocyte cultures[72] with wALADin compounds for 72 h by Dr. Christian Lentz and Martina Fendler.[1d] An antiplasmodial effect was observed for wALADin1, wALADin2 and wALADin3 which were able to reduce parasitemia to almost 0 %.

The SAR profile of *Plasmodium falciparum* did not show the characteristics of the inhibition SAR profile of the ALAD orthologues previously described for the enzymatic assays but had more resemblance with the stimulation SAR profile. Repositioning of the carboxylic acid group from C5- to C6-position, as in wALADin3, was well accepted and resulted in a greater inhibitory activity of the compound. Removal of the R^2-side chain or any modification on the benzyl moiety, except the repositioning of the CF_3-moiety from 3- to 4-position, were not tolerated and led to abrogation of the antiplasmodial effect.

The activity of the wALADin compounds against sporozoites and liver stages was investigated by Dr. Julia Sattler at the Parasitology Unit of the University Hospital Heidelberg by using the rodent parasite *Plasmodium berghei ANKA*.[1d] The investigation of the gliding motility of infective sporozoites incubated with the effective wALADin compounds revealed that wALADin1 and wALADin3 prevented sporozoites' adhesion to the glass slides at concentrations of 40 µM. wALADin2 on the other hand was much more potent and reduced the gliding motility by 75 % at a concentration of 200 nM. Since gliding motility is essential for infection, additional experiments were carried out by the Parasitology Unit of the University Hospital Heidelberg to investigate if reduction of the gliding motility of wALADin2 is sufficient enough to also prevent infection.[1d]

Immortalised human hepatoma cells were treated with wALADin2-preincubated sporozoites and revealed that invasion of sporozoites into hepatoma cells was blocked. Besides the greater motility and invasion inhibiting properties of wALAdin2 over wALADin1, wALADin2 also showed no cytotoxicity against human hepatoma cells while wALADin1 was determined to be moderately antiproliferative.

Additional enzymatic assays using the PfALAD were carried out by Dr. Christian Lentz and Silke Strassel to determine if the antiplasmodial effect is a result of PfALAD inhibition.[1d] These assays revealed that the wALADin compounds have almost no effect on the enzyme itself. These findings lead to the conclusion that the antiplasmodial activity is a result of wALADin-interaction with an alternative target and explains the different SAR profile in culture experiments.

These studies identified wALADin2 as a potent inhibitor of Plasmodium motility and invasion. Unlike wALADin1 it has no cytotoxic effects on human hepatoma cells and affects motility and invasion of *Plasmodium* with concentrations in a nanomolar range. Besides wALADin2's potential as a candidate for novel antiplasmodial treatments, it might also deliver useful new possibilities as a tool for identifying the elements of the gliding machinery of *Plasmodium* parasites that are presently unknown.[73] Before these new advantageous properties of wALADin2 can be used in their full potential, identification of the actual target is necessary.

6. Outlook

6.1. The future of wALADins as drug candidates

ALAD poses a suitable target for species-specific inhibition of the heme biosynthetic pathway since the different orthologues seem to have evolved in a way that enables exclusive human pathogen ALAD targeting. The potential of the combined findings described in this work is indisputable but the compounds investigated so far still have disadvantages that prevent the development into novel therapies. The inhibitory activities of potential drug candidates are desired to lie at least in the nanomolar range to enable low-dose administration that will still lead to bioactive concentrations in the organism. The wALADin compounds' inhibitory activities only lie in the low micromolar range and are not effective in *in vivo* studies which is partially contributed to the low effectivity of these compounds. The SAR studies carried out in this work did not result in more potent ALAD inhibitors but gave a greater insight into the influence of the different functional groups on the inhibitory activity of wALADin compounds. Additionally, this data can be used to perform virtual screening to identify wALADin-like compounds that show improved activity.[74]

An alternative approach is to identify the binding site of the wALADin compounds and use the obtained structural information to specifically tailor a more potent wALADin-based inhibitor. Co-crystallisation is the most commonly used strategy for identifying the binding site of effectors to their target, but can be inconvenient when the desired crystals are difficult to obtain as for wALAD and PsALAD. For the other ALAD-orthologues used in the cross species SAR crystallisation has been described and co-crystallisation studies with the wALADin compounds are currently carried out.

Another method for identifying the binding site of the wALADin compounds on the ALAD-orthologues could be an affinity-based proteomic profiling (ABPP) approach[75] where the wALADin compounds are chemically modified (see **section 6.2**) to be covalently bound to the target by UV irradiation. Digestion and purification of the complex then enables identification of the binding site via LC-MS analysis. The same modified compounds could also be used to investigate possible secondary targets of the wALADin compounds.

Overcoming these obstacles will clear the way for the development of wALADin and wALADin-based compounds into novel herbicidal and antibiotic therapies.

6.2. Target identification of wALADin2 in *Plasmodium falciparum*

As previously described the antiplasmodial effect of wALADin2 does not result from inhibitory activity on the *Plasmodium falciparum* ALAD-orthologue. To investigate the actual target of wALADin2, a cross-linkable wALADin2-based clickable probe is the preferable tool for these investigations. Modifications on wALADin2 need to be small to not affect the interaction with the target and maintain antiplasmodial activity of the modified wALADin2. A good approach might be a modified version of the published all-in-one 3-(1,1-difluoroprop-2-ynyl)-3H-diazirin-3-yl functional moiety,[76] which enables introduction of a cross-linkable and clickable moiety on just one modification site (**Figure 9**).

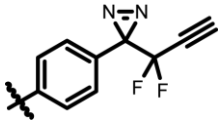
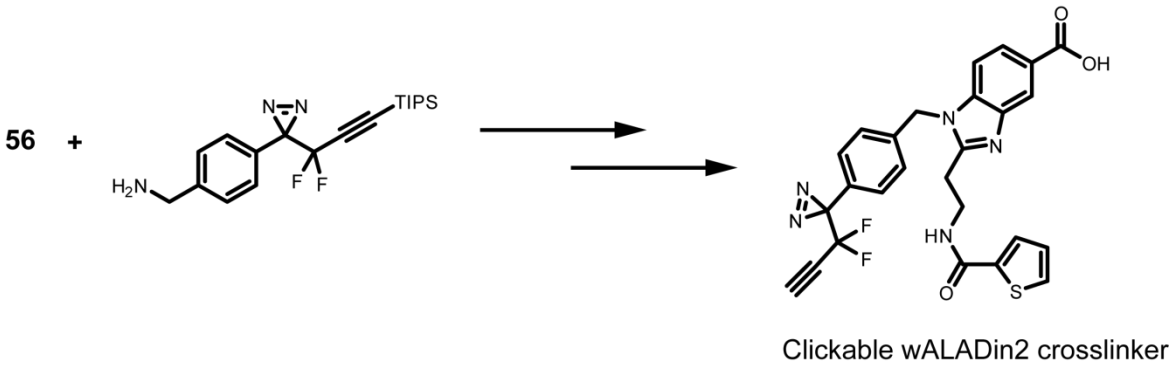


Figure 9: All-in-one 3-(1,1-difluoroprop-2-ynyl)-3Hdiazirin-3-yl functional moiety.[76]

Synthesis of the 3-(1,1-difluoroprop-2-ynyl)-3Hdiazirin-3-yl benzylamine can be carried out by coupling of a suitably protected 4-Iodobenzylamine with methyl 2,2-difluoro-4-(triisopropylsilyl)-3-butynoate[77] and conversion of the carbonyl group to diazirine.[76] The resulting precursor, 3-(1,1-difluoroprop-2-ynyl)-3Hdiazirin-3-yl benzylamine, can then be used as reagent in the ipso-nucleophilic substitution reaction to prepare the clickable wALADin2 crosslinker (**Scheme 11**).



Scheme 11: Synthesis of the cross-linkable wALADin2-based clickable probe.

Implied that the functionalisation of wALADin2 has only a neglectable effect on target binding and antiplasmodial activity, target identification is then accomplished by incubating plasmodial lysate with the wALADin2-based probe and covalent crosslinking via UV irradiation. Coupling of the cross-linked complexes with desthiobiotin linkers via

click reaction enables purification and subsequent identification of the target molecule via LC-MS analysis.

7. Experimental section

7.1. General methods

NMR-spectroscopy

^{1}H- and ^{13}C-NMR-spectra were measured with a nuclear magnetic resonance spectrometer AM300 (^{1}H = 300 MHz; ^{13}C = 75.5 MHz), AM400 (^{1}H = 400 MHz; ^{13}C = 100.6 MHz) und AM500 (^{1}H = 500 MHz; ^{13}C = 126.0 MHz) from *BRUKER,* Karlsruhe. D_6-DMSO and $CDCl_3$ were used as solvents. The chemical shifts were plotted as δ-values in ppm. The ^{1}H-spectra were calibrated on the d_6-DMSO residual content at δ = 2.50 ppm or on the $CDCl_3$ residual content at δ = 7.26 ppm. The ^{13}C-spectra were calibrated on the d_6-DMSO residual content at δ = 39.5 ppm or on the $CDCl_3$ residual content at δ = 77.0 ppm. The analysis of the spectra was carried out with *MestRec 4.7.0.0* from *Mestrelab Research S.L.*

The following abbreviations were used for the multiplicities:

s singlet
d doublet
dd doublet of doublets
t Triplet
app. q apparent quartet
sept septet
m multiplet

The coupling constant *J* were quoted in Hertz and with the letters indicating the atom. The letters used are identical to the letters shown on the structures.

Mass spectrometry

EI-Mass spectra were measured on a MAT-95XL from *Finnigan*, Bremen. ESI-Mass spectra were measured with a micrOTOF-Q flight time spectrometer from *Bruker Daltonik,* Bremen using an *Agilent* 1200 Series HPLC-facility.

Flash chromatography

Flash chromatography was carried out using pre-packed silica gel columns on a PuriFlash 430 from Interchim, France.

TLC

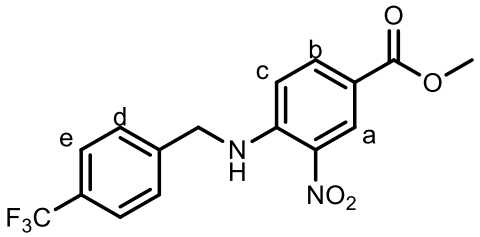
Analytical TLC was performed on a 0.25 mm thickness plates pre-coated with Merck Kieselgel 60 F_{254} silica gel. TLC were visualised under UV (254) nm.

Compounds

wALADin1, wALADin3, wALADin4, wALADin5 and the precursors methyl 4-fluoro-3-nitrobenzoate (**56**), 3-(thiophene-2-carboxamido) propanoic acid (**57**), methyl 3-amino-4-(3-(trifluoromethyl)benzylamino)benzoate (**58**) and methyl 2-(2-(thiophene-2-carboxamido)ethyl)-1-(3-(trifluoromethyl)benzyl)-1H-benzo[d]imidazole-5-carboxylate (**59**) were prepared as described in my diploma thesis[1a] and in reference[1b].

7.2. wALADin2

Methyl 3-nitro-4-(4-(trifluoromethyl)benzylamino)benzoate (17)



4-trifluormethylbenzylamine (7.53 mmol, 1.08 mL) was added in one portion to a stirred solution of **56** (5.02 mmol, 1.00 g) and diisopropylethylamine (10.0 mmol, 1.73 mL) in acetonitrile (51.0 mL) at room temperature. The reaction mixture was refluxed for one hour.

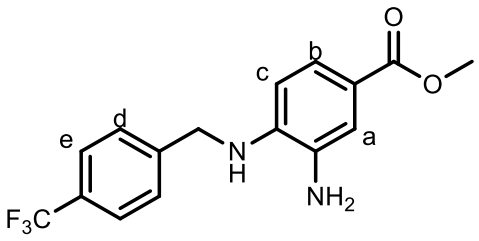
The reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate (30.0 mL) and water (10.0 mL). The reaction mixture was washed with water (2x 10.0 mL) and saturated sodium chloride solution (1x 10.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **17** as a yellow solid. Yield 1.77 g (99 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 9.16 (t, 1H, $^3J_{NH,CH2}$ = 6.3 Hz, ArN<u>H</u>), 8.62 (d, 1H, $^4J_{a,b}$ = 2.1 Hz, H_a), 7.87 (dd, 1H, $^4J_{b,a}$ = 2.0 Hz, $^3J_{b,c}$ = 9.0 Hz, H_b), 7.70 (d, 2H, $^3J_{e,d}$ = 8.1 Hz, H_e), 7.58 (d, 2H, $^3J_{d,e}$ = 8.1 Hz, H_d), 6.93 (d, 1H, $^3J_{c,b}$ = 9.1 Hz, H_c), 4.81 (d, 2H, $^3J_{CH2,NH}$ = 6.3 Hz, ArC<u>H</u>$_2$), 3.80 (s, 3H, OC<u>H</u>$_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.6 ($\underline{C}O_2R$), 147.0 ($\underline{C}_{Ar}NH$), 142.9 ($\underline{C}_{Ar}CH_2$), 135.5 (C_b), 131.0 ($\underline{C}_{Ar}NO_2$), 128.2 (C_a), 127.9 ($\underline{C}_{Ar}CF_3$), 127.5 (C_d), 125.3 (C_e), 122.8 ($\underline{C}F_3$), 116.2 ($\underline{C}_{Ar}CO_2R$), 114.9 (C_c), 51.9 ($O\underline{C}H_3$), 45.3 ($Ar\underline{C}H_2$).

HRMS calculated for $C_{16}H_{13}F_3N_2O_4$ 354.0827. Found 354.0831.

Methyl 3-amino-4-(4-(trifluoromethyl)benzylamino)benzoate (18)



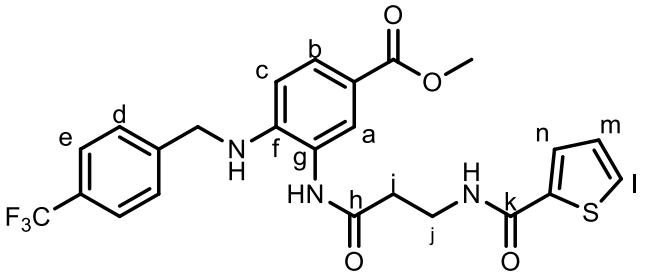
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.17 g) was added to a stirred suspension of **17** (5.70 mmol, 1.67 g) in ethanol (47.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 5 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The remaining solid was suspended in ether and filtered to give **18** as a white solid. Yield 1.28 g (84 %)

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 7.69 (d, 2H, $^3J_{e,d}$ = 8.1 Hz, H_d), 7.56 (d, 2H, $^3J_{d,e}$ = 8.0 Hz, H_d), 7.23 (d, 1H, $^4J_{a,b}$ = 2.0 Hz, H_a), 7.11 (dd, 1H, $^4J_{b,a}$ = 2.0 Hz, $^3J_{b,c}$ = 8.3 Hz, H_b), 6.31 (d, 1H, $^3J_{c,b}$ = 8.4 Hz, H_c), 6.06 (t, 1H, $^3J_{NH,CH2}$ = 5.9 Hz, ArNH), 4.85 (s, 2H, $ArN\underline{H}_2$), 4.50 (d, 2H, $^3J_{CH2,NH}$ = 5.8 Hz, $ArC\underline{H}_2$), 3.71 (s, 3H, $OC\underline{H}_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.6 ($\underline{C}O_2R$), 144.6 ($\underline{C}_{Ar}CH_2$), 139.6 ($\underline{C}_{Ar}NH$), 134.3 ($\underline{C}_{Ar}NH_2$), 127.6 (C_d), 127.5 ($\underline{C}_{Ar}CF_3$), 125.1 (C_e), 122.9 (CF_3), 120.2 (C_b), 117.2 ($\underline{C}_{Ar}CO_2R$), 114.2 (C_a), 108.4 (C_c), 51.0 ($O\underline{C}H_3$), 45.6 ($Ar\underline{C}H_2$).

HRMS calculated for $C_{16}H_{15}F_3N_2O_2$ 324.1086. Found 324.1083.

Methyl 3-(3-(thiophene-2-carboxamido)propanamido)-4-((4-(trifluoromethyl)benzyl) amino)benzoate (19)



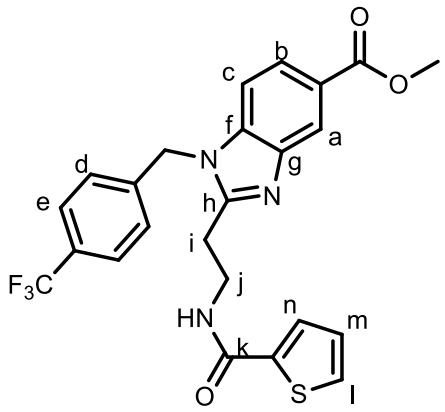
Diisopropylethylamine (10.1 mmol, 1.80 mL) was added to a stirred suspension of **57** (6.06 mmol, 1.21 g) in THF (41.0 mL) followed by HBTU (6.06 mmol, 2.30 g) at room temperature. After 5 min **18** (4.04 mmol, 1.31 g) was added. The reaction mixture was stirred for 15 hours at room temperature. The reaction mixture was filtered and concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (45.0 mL) and washed with hydrochloric acid (1M, 3 x 25.0 mL) and saturated sodium hydrogen carbonate (3 x 25.0 mL). The resulting precipitate was removed and the organic layer was washed with saturated sodium chloride (1 x 25.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was recrystallized from chloroform to give **19** as a white solid. Yield 1.54 g (75 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 9.36 (s, 1H, $C_gN\underline{H}$), 8.70 (t, 1H, $^3J_{NH,CH2}$ = 5.5 Hz, $C_fN\underline{H}$), 7.76 (d, 1H, $^3J_{n,l}$ = 3.6 Hz, H_n), 7.73 (s, 1H, H_a), 7.72 (d, 1H, $^3J_{l,n}$ = 4.7 Hz, H_l), 7.66 (d, 2H, $^3J_{e,d}$ = 8.2 Hz, H_e), 7.57 (d, 2H, $^3J_{d,e}$ = 8.4 Hz, H_d), 7.55 – 7.53 (m, 1H, H_b), 7.12 (dd, 1H, $^3J_{m,n}$ = 3.9 Hz, $^3J_{m,l}$ = 4.7 Hz, H_m), 6.59 (t, 1H, $^3J_{NH,CH2}$ = 6.1 Hz, $C_kON\underline{H}$), 6.46 (d, 1H, $^3J_{c,b}$ = 8.7 Hz, Hc), 4.54 (d, 2H, $^3J_{CH2,NH}$ = 5.8 Hz, $ArC\underline{H}_2$), 3.73 (s, 3H, $OC\underline{H}_3$), 3.60 (app. q, 2H, $^3J_{j,I}$ = 6.5 Hz, H_j), 2.67 (t, 2H, $^3J_{i,j}$ = 6.7 Hz, H_i).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 170.1 (C_h), 165.9 ($\underline{C}O_2R$), 161.2 (C_k), 146.6 (C_f), 144.3 ($\underline{C}_{Ar}CH_2$), 139.8 ($\underline{C}C_hO$), 130.5 (C_a), 128.5 (C_b), 127.9, 127.9 (C_l, C_n), 127.7 (C_m), 127.4 (C_d), 125.6 ($\underline{C}_{Ar}CF_3$), 125.0 (C_e), 122.8 (CF_3), 122.4 (C_g), 115.9 ($\underline{C}_{Ar}CO_2R$), 109.7 (C_c), 51.2 ($O\underline{C}H_3$), 45.1 ($Ar\underline{C}H_2$), 36.0 (C_i), 35.9 (C_j).

HRMS calculated for $C_{24}H_{22}F_3N_3O_4S$ 528.1181. Found 528.1175.

Methyl 2-(2-(thiophene-2-carboxamido)ethyl)-1-(4-(trifluoromethyl)benzyl)-1*H*-benzo [*d*]imidazole-5-carboxylate (20)



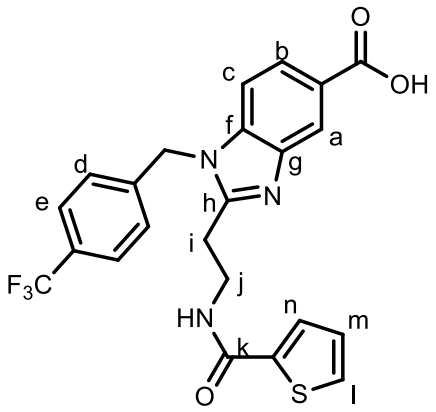
A stirred suspension of **19** (2.93 mmol, 1.48 g) in acetic acid (15.0 mL) was refluxed for 48 hours at 100 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in dichloromethane (25.0 mL). The reaction mixture was washed with water (2x 20.0 mL) and saturated sodium chloride (1x 20.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was recrystallized from chloroform to give **20** as a white solid. Yield 1.13 g (79 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.71 (t, 1H, $^3J_{NH,CH2}$ = 5.7 Hz, C_kONH), 8.25 (d, 1H, $^4J_{a,b}$ = 1.5 Hz, H_a), 7.84 (dd, 1H, $^4J_{b,a}$ = 1.6 Hz, $^3J_{b,c}$ = 8.5 Hz, H_b), 7.73 (dd, 1H, $^4J_{l,n}$ = 1.1 Hz, $^3J_{l,m}$ = 5.0 Hz, H_l), 7.70 (dd, 1H, $^4J_{n,l}$ = 1.1 Hz, $^3J_{n,m}$ = 3.7 Hz, H_n), 7.65 (d, 2H, $^3J_{e,d}$ = 8.2 Hz, H_e), 7.57 (d, 1H, $^3J_{c,b}$ = 8.6 Hz, H_c), 7.28 (d, 2H, $^3J_{d,e}$ = 8.1 Hz, H_d), 7.12 (dd, 1H, $^3J_{m,n}$ = 3.7 Hz, $^3J_{m,l}$ =5.0 Hz, H_m), 5.70 (s, 2H, $ArCH_2$), 3.86 (s, 3H, OCH_3), 3.75 (app. q, 2H, $^3J_{j,I}$ = 7.1 Hz, H_j), 3.15 (t, 2H, $^3J_{i,j}$ = 7.3 Hz, H_i).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.6 (CO_2R), 161.2 (C_k), 155.3 (C_h), 141.9 ($C_{Ar}CH_2$), 141.1 (C_g), 139.6 (C_f), 138.4 (CC_kO), 130.6 (C_l), 127.9 (C_n), 127.7 (C_m), 126.9 (C_d), 125.5 (C_e), 125.3 ($C_{Ar}CF_3$), 123.3 (C_b), 123.2 ($C_{Ar}CO_2R$), 122.6 (CF_3), 120.2 (C_a), 110.2 (C_c), 51.8 (OCH_3), 45.7 ($ArCH_2$), 37.1 (C_j), 26.9 (C_i).

HRMS calculated for $C_{24}H_{20}F_3N_3O_3S$ + Na 510.1075. Found 510.1070.

2-(2-(Thiophene-2-carboxamido)ethyl)-1-(4-(trifluoromethyl)benzyl)-1*H*-benzo [*d*]imidazole-5-carboxylic acid (2)



Lithium hydroxide (11.0 mmol, 0.46 g) was added to a suspension of **20** (2.19 mmol, 1.07 g) in tetrahydrofuran and water (2:1, 24.0 mL) at room temperature. The reaction mixture was refluxed for 15 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (35.0 mL). The reaction mixture was washed with ethyl acetate (1x 15.0 mL). Hydrochloric acid (2M, 5.00 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **2** as a white solid. Yield 0.94 g (91 %).

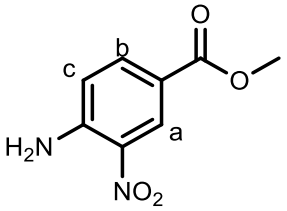
^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.72 (s, 1H, $CO_2\underline{H}$), 8.73 (t, 1H, $^{3}J_{NH,CH2}$ = 5.7 Hz, $C_kON\underline{H}$), 8.23 (d, 1H, $^{4}J_{a,b}$ = 1.5 Hz, H_a), 7.83 (dd, 1H, $^{4}J_{b,a}$ = 1.5 Hz, $^{3}J_{b,c}$ = 8.5 Hz, H_b), 7.73 (dd, 1H, $^{4}J_{l,n}$ = 1.1 Hz, $^{3}J_{l,m}$ = 5.0 Hz, H_l), 7.70 (dd, 1H, $^{4}J_{n,l}$ = 1.1 Hz, $^{3}J_{n,m}$ = 3.8 Hz, H_n), 7.65 (d, 2H, $^{3}J_{e,d}$ = 8.2 Hz, H_e), 7.53 (d, 1H, $^{3}J_{c,b}$ = 8.6 Hz, H_c), 7.28 (d, 2H, $^{3}J_{d,e}$ = 8.1 Hz, H_d), 7.12 (dd, 1H, $^{3}J_{m,n}$ = 3.7 Hz, $^{3}J_{m,l}$ = 5.0 Hz, H_m), 5.69 (s, 2H, $ArC\underline{H}_2$), 3.75 (app. q, 2H, $^{3}J_{j,i}$ = 7.1Hz, H_j), 3.14 (t, 2H, $^{3}J_{i,j}$ = 7.3 Hz, H_i).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 167.7 ($\underline{C}O_2R$), 161.2 (C_k), 155.0 (C_h), 141.9 ($\underline{C}_{Ar}CH_2$), 141.2 (C_g), 139.6 ($\underline{C}C_kO$), 138.2 (C_f), 130.6 (C_l), 127.9, 127.7 (C_m, C_n), 127.0 (C_d), 125.5 (C_e), 125.3 ($\underline{C}_{Ar}CF_3$), 124.6 ($\underline{C}_{Ar}CO_2R$), 123.4 (C_b), 122.6 (CF_3), 120.3 (C_a), 109.9 (C_c), 45.7 ($Ar\underline{C}H_2$), 37.2 (C_j), 26.9 (C_i).

HRMS calculated for $C_{23}H_{18}F_3N_3O_3S$ + Na 496.0919. Found 496.0913.

7.3. wALADin6

Methyl 4-amino-3-nitrobenzoate (21)



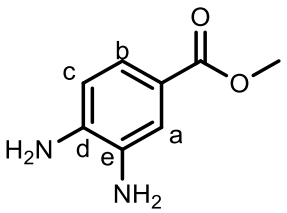
Sulphuric acid (0.40 mL) was added to a stirred solution of 4-amino-3-nitrobenzoic acid (11.0 mmol, 2.00 g) in ethanol (110 mL) and refluxed for 15 hours. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in ethyl acetate (40.0 mL). The mixture was washed with saturated sodium hydrogen carbonate (3x 20.0 mL), water (3x 20.0 mL) and saturated sodium chloride (1x 20.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **21** as a yellow solid. Yield 2.13 g (99 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.53 (d, 1H, $^4J_{a,b}$ = 2.0 Hz, H_a), 7.97 (s, 2H, ArN$\underline{H}_2$), 7.83 (dd, 1H, $^4J_{b,a}$ = 2.1 Hz, $^3J_{b,c}$ = 8.9 Hz, H_b), 7.05 (d, 1H, $^3J_{c,b}$ = 9.0 Hz, H_c), 3.80 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.7 ($\underline{C}O_2R$), 148.7 ($\underline{C}_{Ar}NH_2$), 134.6 (C_b), 129.4 ($\underline{C}_{Ar}NO_2$), 127.9 (C_a), 119.2 (C_c), 116.0 ($\underline{C}_{Ar}CO_2R$), 51.8 (O$\underline{C}H_3$).

HRMS calculated for $C_8H_8N_2O_4$ 196.0484. Found 196.0488.

Methyl 3,4-diaminobenzoate (22)



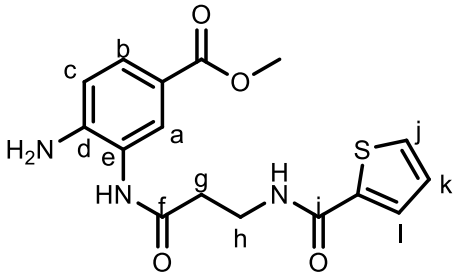
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.2g) was added to a stirred suspension of **21** (10.4 mmol, 2.04 g) in ethanol (104 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 5 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The remaining solid was suspended in ether and filtered to give **22** as a pale orange solid. Yield 1.55 g (99 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 7.16 (d, 1H, $^{4}J_{a,b}$ = 2.0 Hz, H_a), 7.09 (dd, 1H, $^{4}J_{b,a}$ = 2.0 Hz, $^{3}J_{b,c}$ = 8.1 Hz, H_b), 6.51 (d, 1H, $^{3}J_{c,b}$ = 8.1 Hz, H_c), 5.27 (s, 2H, H_d or H_e), 4.65 (s, 2H, H_d or H_e), 3.71 (s, 3H, O$\underline{C}H_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.7 ($\underline{C}O_2R$), 140.4, 133.7 (C_d, C_e), 120.0 (C_b), 117.0 ($\underline{C}_{Ar}CO_2R$), 114.7 (C_a), 112.5 (C_c), 50.9 (O$\underline{C}H_3$).

HRMS calculated for $C_8H_{10}N_2O_2$ 166.0742. Found 166.0746.

Methyl 4-amino-3-(3-(thiophene-2-carboxamido)propanamido)benzoate (23)



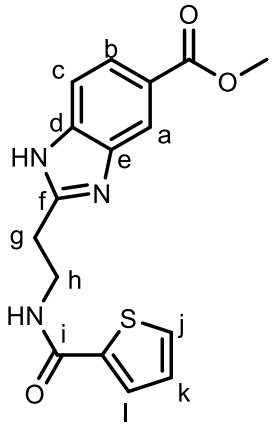
Diisopropylethylamine (26.0 mmol, 4.50 mL) was added to a stirred suspension of **57** (15.6 mmol, 3.11 g) in THF (104 mL) followed by HBTU (15.6 mmol, 5.92 g) at room temperature. After 5 min **22** (10,4 mmol, 1.73 g) was added. The reaction mixture was stirred for 15 hours at room temperature. The reaction mixture was filtered and concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (60 mL) and washed with hydrochloric acid (1M, 2 x 15.0 mL) and saturated sodium hydrogen carbonate (2 x 15.0 mL). The resulting precipitate was removed and the organic layer was washed with saturated sodium chloride (1 x 15.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was recrystallized from chloroform to give **23** as a pink solid. Yield 2.74 g (76 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 9.16 (s, 1H, $C_eN\underline{H}$), 8.64 (t, 1H, $^{3}J_{NH,CH2}$ = 5.6 Hz, $C_iON\underline{H}$), 7.84 (d, 1H, $^{4}J_{a,b}$ = 2.0 Hz, H_a), 7.75 (dd, 1H, $^{4}J_{l,j}$ = 1.1 Hz, $^{3}J_{l,k}$ = 3.7 Hz, H_l), 7.73 (dd, 1H, $^{4}J_{j,l}$ = 1.1 Hz, $^{3}J_{j,k}$ = 5.0 Hz, H_j), 7.52 (dd, 1H, $^{4}J_{b,a}$ = 2.0 Hz, $^{3}J_{b,c}$ = 8.5 Hz, H_b), 7.14 (dd, 1H, $^{3}J_{k,l}$ = 3.7 Hz, $^{3}J_{k,j}$ = 5.0 Hz, H_k), 6.71 (d, 1H, $^{3}J_{c,b}$ = 8.5 Hz, H_c), 5.76 (s, 2H, C_dNH_2), 3.74 (s, 3H, OCH_3), 3.54 (app. q, 2H, $^{3}J_{h,g}$ = 6.7 Hz, H_h), 2.63 (t, 2H, $^{3}J_{g,h}$ = 6.9 Hz, H_g).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 169.7 ($\underline{C}_f$), 166.0 ($\underline{C}O_2R$), 161.1 (C_i), 147.0 (C_d), 139.9 ($\underline{C}C_iO$), 130.5 (C_j), 127.9 (C_a), 127.7 (C_b), 127.7 (C_k), 127.4 (C_l), 121.7 (C_e), 115.9 ($\underline{C}_{Ar}CO_2R$), 114.1 (C_c), 51.2 (O$\underline{C}H_3$), 35.8 (C_g, C_h).

HRMS calculated for $C_{16}H_{17}N_3O_4S$ + Na 370.0837. Found 370.0832.

Methyl 2-(2-(thiophene-2-carboxamido)ethyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (24)



A stirred suspension of **23** (7.17 mmol, 2.49 g) in acetic acid (72.0 mL) was refluxed for 5 hours at 100 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in dichloromethane (50 mL). The reaction mixture was washed with water (2x 30.0 mL) and saturated sodium chloride (1x 30.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was recrystallized from chloroform to give **24** as a white solid. Yield 1.23 g (52 %).

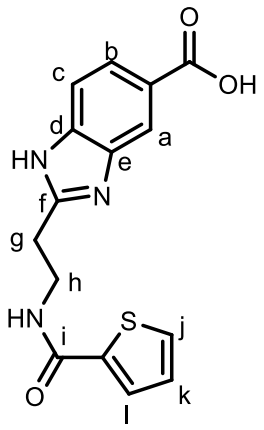
Exists as tautomer:

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.68 (s, 2H, $C_{Ar}N\underline{H}$), 8.70 (t, 2H, $^3J_{NH,h}$ = 5.3 Hz, $C_iON\underline{H}$), 8.16, 8.04 (s, 1H, H_a, $H_{a'}$), 7.81 (d, 1H, $^3J_{b,c}$ = 8.7 Hz, H_b), 7.78 (d, 1H, $^3J_{b',c'}$ = 8.1 Hz, $H_{b'}$), 7.74 (dd, 2H, $^4J_{j,l}$ = 1.1 Hz, $^3J_{j,k}$ = 5.0 Hz, H_j), 7.71 (dd, 2H, $^4J_{l,j}$ = 1.1 Hz, $^3J_{l,k}$ = 3.7 Hz, H_l), 7.63 (d, 1H, $^3J_{c,b}$ = 8.3 Hz, H_c), 7.52 (d, 1H, ^3J = 8.3 Hz, $H_{c'}$), 7.13 (dd, 2H, $^3J_{k,l}$ = 3.7 Hz, $^3J_{k,j}$ = 5.0 Hz, H_k), 3.85 (s, 6H, $OC\underline{H}_3$), 3.71 (app. q, 4H, $^3J_{h,g}$ = 7.1 Hz, H_h), 3.12 (app. bs, 4H, H_g).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.8 ($\underline{C}O_2R$), 166.6 ($\underline{C}O_2R'$), 161.1 (C_i), 156.2 (C_f), 155.1 ($C_{f'}$), 146.8 (C_d), 142.8 ($C_{d'}$), 139.7 ($\underline{C}C_iO$), 137.8 (C_e), 133.8 ($C_{e'}$), 130.5 (C_k), 127.9 (C_j), 127.7 (C_l), 122.8 (C_b), 122.6 ($\underline{C}_{Ar}CO_2R$), 122.4 ($\underline{C}_{Ar}CO_2R'$), 122.1 ($C_{b'}$), 119.7 (C_a), 117.9 (C_c), 112.4 ($C_{a'}$), 110.8 ($C_{c'}$), 51.8 ($O\underline{C}H_3$), 37.6 (C_h), 28.9 (C_g).

HRMS calculated for $C_{16}H_{15}N_3O_3S$ + Na 352.0732. Found 352.0726.

2-(2-(Thiophene-2-carboxamido)ethyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (6)



Lithium hydroxide (9.11 mmol, 0.38 g) was added to a suspension of **24** (1.82 mmol, 0.60 g) in tetrahydrofuran and water (2:1, 18.0 mL) at room temperature. The reaction mixture was refluxed for 4 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (10.0 mL). The reaction mixture was washed with ethyl acetate (2x 5.0 mL). Hydrochloric acid (2M, 10.0 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **6** as a white solid. Yield 0.48 g (84 %).

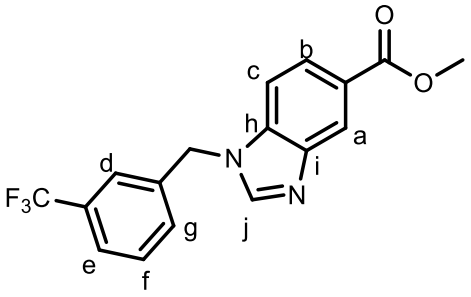
^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.50 (s, 2H, $CO_2\underline{H}$, N$\underline{H}$), 8.72 (t, 1H, $^{3}J_{NH,h}$ = 5.6 Hz, $C_iON\underline{H}$), 8.09 (s, 1H, H_a), 7.78 (dd, 1H, $^{4}J_{b,a}$ = 1.5 Hz, $^{3}J_{b,c}$ = 8.4 Hz, H_b), 7.73 (dd, 1H, $^{4}J_{j,l}$ = 1.1 Hz, $^{3}J_{j,k}$ = 5.0 Hz, H_j), 7.72 (dd, 1H, $^{4}J_{l,j}$ = 1.1 Hz, $^{3}J_{l,k}$ = 3.8 Hz, H_l), 7.54 (d, 1H, $^{3}J_{c,b}$=8.4Hz, H_c), 7.12 (dd, 1H, $^{3}J_{k,l}$ = 3.8 Hz, $^{3}J_{k,j}$ = 4.9 Hz, H_k), 3.71 (app. q, 2H, $^{3}J_{h,g}$ = 7.1 Hz, H_h), 3.12 (t, 2H, $^{3}J_{g,h}$ = 7.2 Hz, H_g).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 167.9 ($\underline{C}O_2R$), 161.2 (C_i), 155.3 (C_f), 141.9 (C_d), 139.8 ($\underline{C}C_iO$), 138.7 (C_e), 130.5 (C_l), 128.0 (C_j), 127.7 (C_k), 123.9 ($\underline{C}_{Ar}CO_2R$), 122.7 (C_b), 116.7 (C_a), 114.0 (C_c), 37.7 (C_h), 28.9 (C_g).

HRMS calculated for $C_{15}H_{13}N_3O_3S$ + Na 338.0576. Found 338.0570.

7.4. wALADin7

Methyl 1-(3-(trifluoromethyl)benzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (25)



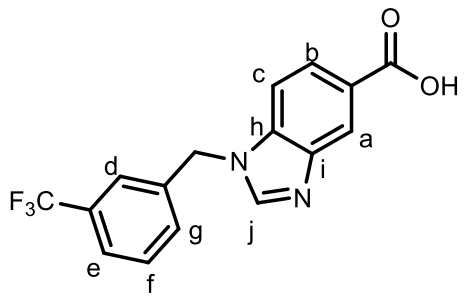
A stirred suspension of **58** (8.05 mmol, 2.61 g) in formic acid (21.0 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralised with sodium hydrogen carbonate (700 mL). The reaction mixture was extracted with ethyl acetate (3 x 100 mL) and the organic layer was washed with water (3 x 50.0 mL) and sodium chloride (1 x 50.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **25** as an off-white solid. Yield 2.63 g (98 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.63 (s, 1H, H_j), 8.28 (dd, 1H, $^5J_{a,c}$ = 0.5 Hz, $^4J_{a,b}$ = 1.5 Hz, H_a), 7.87 (dd, 1H, $^4J_{b,a}$ = 1.6 Hz, $^3J_{b,c}$ = 8.5 Hz, $H_{b,}$) 7.77 (s, 1H, H_d), 7.69 (dd, 1H, $^5J_{c,a}$ = 0.6 Hz, $^3J_{c,b}$ = 8.6 Hz, H_c), 7.65 – 7.68 (m, 1H, H_e), 7.55 – 7.60 (m, 2H, H_f, H_g), 5.68 (s, 2H, ArC$\underline{H}_2$), 3.85 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.5 ($\underline{C}O_2R$), 146.3 (C_j), 143.0, 137.9, 136.8 (C_h, C_i, $\underline{C}_{Ar}CH_2$), 131.4 (C_f or C_g), 129.8 (C_f or C_g), 125.2 ($\underline{C}_{Ar}CF_3$), 124.5 ($\underline{C}_{Ar}CO_2R$), 124.0 (C_e), 123.5 (C_d), 123.4 (C_b), 122.5 (CF_3), 121.2 (C_a), 110.6 (C_c), 51.9 (O$\underline{C}H_3$), 47.0 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{17}H_{13}F_3N_2O_2$ 334.0929. Found 334.0929.

1-(3-(Trifluoromethyl)benzyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (7)



Lithium hydroxide (37.5 mmol, 1.58 g) was added to a suspension of **25** (7.51 mmol, 2.51 g) in tetrahydrofuran and water (2:1, 75.0 mL) at room temperature. The reaction

mixture was refluxed for 15 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (50.0 mL). The reaction mixture was washed with ethyl acetate (2x 50.0 mL). Hydrochloric acid (2M, 50.0 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **7** as a white solid. Yield 2.34 g (97 %).

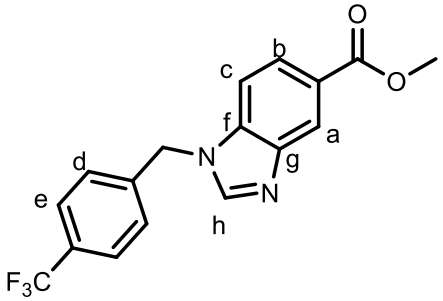
^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.80 (s, 1H, $CO_2\underline{H}$), 8.66 (s, 1H, H_j), 8.27 (d, 1H, $^{4}J_{a,b}$ = 1.0 Hz, H_a), 7.87 (dd, 1H, $^{4}J_{b,a}$ = 1.5 Hz, $^{3}J_{b,c}$ = 8.5 Hz, H_b), 7.78 (s, 1H, H_d), 7.65 – 7.68 (m, 2H, H_c,H_e), 7.57 – 7.58 (m, 2H, H_f, H_g), 5.68 (s, 2H, Ar$C\underline{H}_2$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 167.6 ($\underline{C}O_2R$), 146.1 (C_j), 142.7, 137.9, 136.5 (C_h, C_i, $\underline{C}_{Ar}CH_2$), 131.4 (C_f or C_g), 129.8 (C_f or C_g), 125.2 ($\underline{C}_{Ar}CF_3$), 124.7 ($\underline{C}_{Ar}CO_2R$), 124.5 (C_e), 124.0 (C_d), 123.9 (C_b), 122.5 (CF_3), 121.2 (C_a), 110.5 (C_c), 47.1 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{16}H_{11}F_3N_2O_2$ 320.0773. Found 320.0775.

7.5. wALADin8

Methyl 1-(4-(trifluoromethyl)benzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (26)



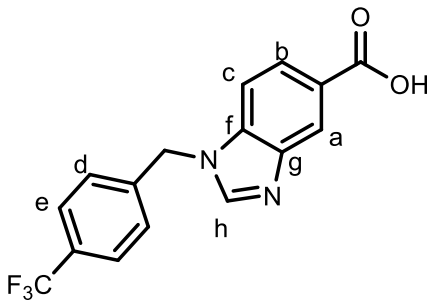
A stirred suspension of **17** (4.63 mmol, 1.50 g) in formic acid (8.50 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralised with sodium hydrogen carbonate (300 mL). The reaction mixture was extracted with ethyl acetate (3 x 100 mL) and the organic layer was washed with water (3 x 100 mL) and sodium chloride (1 x 100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **26** as a white solid. Yield 1.05 g (80 %).

^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.61 (s, 1H, H_h), 8.28 (d, 1H, $^{4}J_{a,b}$ = 1.0 Hz, H_a), 7.86 (dd, 1H, $^{4}J_{b,a}$ = 1.6 Hz, $^{3}J_{b,c}$ = 8.5 Hz, H_b), 7.72 (d, 2H, $^{3}J_{e,d}$ = 8.1 Hz, H_e), 7.64 (dd, 1H, $^{5}J_{c,a}$ = 0.5 Hz, $^{3}J_{c,b}$ = 8.5 Hz, H_c), 7.49 (d, 2H, $^{3}J_{d,e}$ = 8.0 Hz, H_d), 5.69 (s, 2H, Ar$C\underline{H}_2$) 3.86 (s, 1H, O$C\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.5 ($\underline{C}O_2R$), 146.4 (C_h), 143.0 (C_f), 141.2 ($\underline{C}_{Ar}CH_2$), 141.2 (C_g), 136.8 ($\underline{C}_{Ar}CF_3$), 128.5 (C_d), 125.6 (C_e), 123.5 (C_b), 123.4 ($\underline{C}_{Ar}CO_2R$), 122.1 (CF_3), 121.2 (C_a), 110.7 (C_c), 51.9 ($O\underline{C}H_3$), 47.1 ($Ar\underline{C}H_2$).

HRMS calculated for $C_{17}H_{13}F_3N_2O_2$ 334.0929. Found 334.0931.

1-(4-(Trifluoromethyl)benzyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (8)



Lithium hydroxide (12.6 mmol, 0.53 g) was added to a suspension of **26** (2.51 mmol, 0.84 g) in tetrahydrofuran and water (2:1, 27.0 mL) at room temperature. The reaction mixture was refluxed for 6 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (20.0 mL). The reaction mixture was washed with ethyl acetate (1x 10.0 mL). Hydrochloric acid (2M, 5.00 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **8** as a white solid. Yield 0.80 g (99 %).

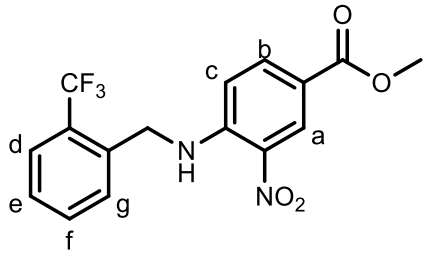
^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.59 (s, 1H, H_h), 8.28 (d, 1H, $^4J_{a,b}$ = 1.0 Hz, H_a), 7.86 (dd, 1H, $^4J_{b,a}$ = 1.5 Hz, $^3J_{b,c}$ = 8.5 Hz, H_b), 7.71 (d, 2H, $^3J_{e,d}$ = 8.1 Hz, H_e), 7.59 (d, 1H, $^3J_{c,b}$ = 8.5 Hz, H_c), 7.49 (d, 2H, $^3J_{d,e}$ = 8.1 Hz, H_d), 5.68 (s, 2H, $ArC\underline{H}_2$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 167.7 ($\underline{C}O_2R$), 146.1 (C_h), 143.0 (C_g), 141.3 (C_f), 136.5 ($\underline{C}_{Ar}CH_2$), 128.5 ($\underline{C}_{Ar}CF_3$), 127.9 (C_d), 125.5 (C_e), 125.0 ($\underline{C}_{Ar}CO_2R$), 123.8 ($\underline{C}_b$), 122.6 (CF_3), 121.3 (C_a), 110.3 (C_c), 47.1 ($Ar\underline{C}H_2$).

HRMS calculated for $C_{16}H_{11}F_3N_2O_2$ 320.0773. Found 320.0771.

7.6. wALADin9

Methyl 3-nitro-4-(2-(trifluoromethyl)benzylamino)benzoate (27)



2-trifluormethylbenzylamine (7.53 mmol, 1.10 mL) was added in one portion to a stirred solution of **56** (5.02 mmol, 1.00 g) and diisopropylethylamine (10.0 mmol, 1.80 mL) in acetonitrile (50.0 mL) at room temperature. The reaction mixture was refluxed for one hour.

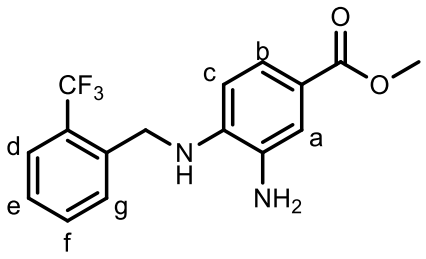
The reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate (30.0 mL) and water (10.0 mL). The reaction mixture was washed with water (2x 10.0 mL) and saturated sodium chloride solution (1x 10.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **27** as a yellow solid. Yield 1.74 g (98 %).

^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 9.07 (t, 1H, $^3J_{NH,CH2}$ = 6.1 Hz, ArN<u>H</u>), 8.64 (d, 1H, $^4J_{a,b}$ = 2.1 Hz, H_a), 7.90 (dd, 1H, $^4J_{b,a}$ = 2.0 Hz, $^3J_{b,c}$ = 9.0 Hz, H_b), 7.78 (d, 1H, $^3J_{g,f}$ = 7.3 Hz, H_g), 7.62 (dd, 1H, $^3J_{f,g}$ = 7.6 Hz, H_f), 7.50 (dd, 2H, $^3J_{d,e}$ = 6.7 Hz, H_d,H_e), 6.76 (d, 1H, $^3J_{c,b}$ = 9.1 Hz, H_c), 4.86 (d, 2H, $^3J_{CH2,NH}$ = 6.0 Hz, ArC<u>H</u>$_2$) 3.80 (s, 3H, OC<u>H</u>$_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.5 (<u>C</u>O_2R), 146.9 (<u>C</u>$_{Ar}$NH), 135.7 (C_b), 132.8 (C_f), 131.1 (<u>C</u>$_{Ar}CH_2$), 128.2 (C_a), 127.7 (C_d, C_e), 126.1 (C_g), 125.9 (<u>C</u>$_{Ar}NO_2$), 125.5 (<u>C</u>$_{Ar}CF_3$), 122.5 (<u>C</u>F_3), 116.4 (<u>C</u>$_{Ar}CO_2$R), 114.4 (C_c), 51.9 (O<u>C</u>H_3), 42.7 (Ar<u>C</u>H_2).

HRMS calculated for $C_{16}H_{13}F_3N_2O_4$ + Na 377.0725. Found 377.0720.

Methyl 3-amino-4-(2-(trifluoromethyl)benzylamino)benzoate (28)



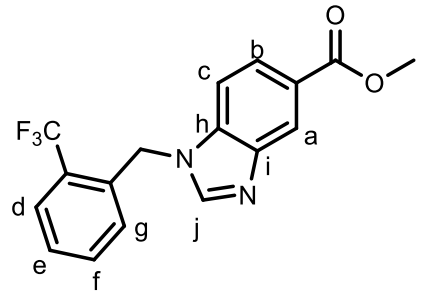
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.17 g) was added to a stirred suspension of **27** (4.69 mmol, 1.66 g) in ethanol (47.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 5 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The remaining solid was suspended in ether and filtered to give **28** as a white solid. Yield 1.18 g (78 %)

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 7.75 (d, 1H, $^{3}J_{d,e}$ = 7.7 Hz, H_d), 7.61 (dd, 1H, $^{3}J_{f,e/g}$ = 7.5 Hz, H_f), 7.52 (d, 1H, $^{3}J_{g,f}$ = 7.7 Hz, H_g), 7.46 (dd, 1H, $^{3}J_{e,d/f}$ = 7.5 Hz, H_e), 7.26 (d, 1H, $^{4}J_{a,b}$ = 2.0 Hz, H_a), 7.11 (dd, 1H, $^{4}J_{b,a}$ = 1.9 Hz, $^{3}J_{b,c}$ = 8.3 Hz, H_b), 6.18 (d, 1H, $^{3}J_{c,b}$ = 8.3 Hz, H_c), 6.06 (t, 1H, $^{3}J_{NH,CH2}$ = 5.7 Hz, ArN<u>H</u>), 4.89 (s, 2H, ArN<u>H</u>$_2$), 4.56 (d, 2H, $^{3}J_{CH2,NH}$ = 5.4 Hz, ArC<u>H</u>$_2$), 3.71 (s, 3H, OC<u>H</u>$_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.6 (<u>C</u>O$_2$R), 139.5 (<u>C</u>$_{Ar}$CH$_2$), 137.7 (<u>C</u>$_{Ar}$NH), 134.4 (<u>C</u>$_{Ar}$NH$_2$), 132.5 (C_f), 128.1 (C_g), 127.2 (C_e), 126.1 (<u>C</u>$_{Ar}$CF$_3$), 125.7 (C_d), 123.1 (CF$_3$), 120.2 (C_b), 117.4 (<u>C</u>$_{Ar}$CO$_2$R), 114.4 (C_a), 108.0 (C_c), 51.0 (O<u>C</u>H$_3$), 42.7 (Ar<u>C</u>H$_2$).

HRMS calculated for $C_{16}H_{15}F_3N_2O_2$ + H 325.1164. Found 325.1158.

Methyl 1-(2-(trifluoromethyl)benzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (29)



A stirred suspension of **28** (3.52 mmol, 1.14 g) in formic acid (8.80 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralised with sodium hydrogen carbonate (200 mL). The reaction mixture was extracted with ethyl

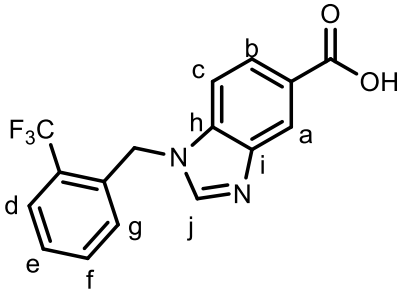
acetate (3 x 50 mL) and the organic layer was washed with water (3 x 40 mL) and sodium chloride (1 x 40 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **29** as a white solid. Yield 1.13 g (96 %).

^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.49 (s, 1H, H_j), 8.32 (d, 1H, $^4J_{a,b}$ = 1.0 Hz, H_a), 7.81 – 7.87 (m, 2H, H_b, H_d), 7.52 – 7.59 (m, 2H, H_f, H_g), 7.42 (dd, 1H, $^5J_{c,a}$ = 0.4 Hz, $^3J_{c,b}$ = 8.5 Hz, H_c), 6.80 (d, 1H, $^3J_{e,d/f}$ = 6.6 Hz, H_e), 5.77 (s, 2H, ArC$\underline{H}_2$), 3.86 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.4 ($\underline{C}O_2R$), 146.7 (H_j), 143.0 (H_h), 137.0 (H_i), 134.4 ($\underline{C}_{Ar}CH_2$), 133.1, 128.3 (H_f, H_g), 127.8 (H_e), 126.3 (H_d), 125.8 ($\underline{C}_{Ar}CF_3$), 123.7 (H_b), 123.5 ($\underline{C}_{Ar}CO_2R$), 122.3 (CF_3), 121.3 (H_a), 110.4 (H_c), 51.9 (O$\underline{C}H_3$), 44.6 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{17}H_{13}F_3N_2O_2$ + Na 357.0827. Found 357.0821.

1-(2-(Trifluoromethyl)benzyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (9)



Lithium hydroxide (16.5 mmol, 0.69 g) was added to a suspension of **29** (3.29 mmol, 1.10 g) in tetrahydrofuran and water (2:1, 33.0 mL) at room temperature. The reaction mixture was refluxed for 5 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (30.0 mL). The reaction mixture was washed with ethyl acetate (1x 10.0 mL). Hydrochloric acid (2M, 10.0 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **9** as a white solid. Yield 0.99 g (94 %).

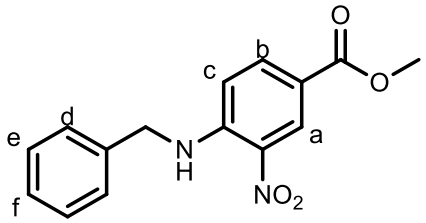
^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.80 (s, 1H, $CO_2\underline{H}$), 8.47 (s, 1H, H_j), 8.31 (d, 1H, $^4J_{a,b}$ = 1.0 Hz, H_a), 7.84 (ddd, 2H, $^4J_{b,a}$ = 1.5 Hz, $^3J_{b,c}$ = 8.4 Hz, $^3J_{d,e}$ = 8.1 Hz, H_b, H_d), 7.50 – 7.58 (m, 2H, H_f, H_g), 7.39 (d, 1H, $^3J_{c,b}$ = 8.5 Hz, H_c), 6.80 (d, 1H, $^3J_{e,f}$ = 7.3 Hz, H_e), 5.77 (s, 2H, ArC$\underline{H}_2$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 167.6 ($\underline{C}O_2R$), 146.5 (C_j), 143.0 (C_h), 136.7 (C_i), 134.5 ($\underline{C}_{Ar}CH_2$), 133.1, 128.3 (C_f or C_g), 127.8 (C_e), 126.2 (C_d), 125.5 ($\underline{C}_{Ar}CF_3$), 124.7 ($\underline{C}_{Ar}CO_2R$), 124.0 (C_b), 122.8 (CF_3), 121.4 (C_a), 110.1 (C_c), 44.6 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{16}H_{11}F_3N_2O_2$ 320.0773. Found 320.0771.

7.7. wALADin10

Methyl 4-(benzylamino)-3-nitrobenzoate (30)



Benzylamine (22.6 mmol, 2.46 mL) was added in one portion to a stirred solution of **56** (15.0 mmol, 3.00 g) and diisopropylethylamine (30.0 mmol, 5.21 mL) in acetonitrile (151.0 mL) at room temperature. The reaction mixture was refluxed for one hour.

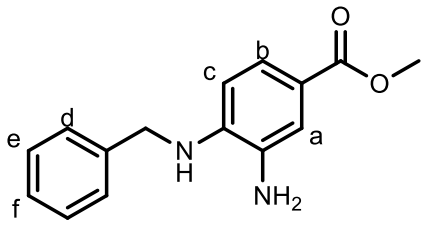
The reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate (90.0 mL) and water (30.0 mL). The reaction mixture was washed with water (2x 30.0 mL) and saturated sodium chloride solution (1x 30.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **30** as a yellow solid. Yield 4.03 g (93 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 9.07 (t, 1H, $^3J_{NH,CH2}$= 6.1 Hz, ArN$\underline{H}$), 8.62 (d, 1H, $^4J_{a,b}$ = 2.1 Hz, H_a), 7.88 (dd, 1H, $^4J_{b,a}$ = 1.7 Hz, $^3J_{b,c}$ = 9.1 Hz, H_b), 7.32 – 7.39 (m, 4H, H_d, H_e), 7.23 – 7.28 (m, 1H, H_f), 6.99 (d, 1H, $^3J_{c,b}$ = 9.1 Hz, H_c), 4.69 (d, 2H, $^3J_{CH2,NH}$ = 6.2 Hz, ArC$\underline{H}_2$), 3.80 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.6 ($\underline{C}O_2R$), 147.1 ($\underline{C}_{Ar}NH$), 137.7 ($\underline{C}_{Ar}CH_2$), 135.3 (C_b), 130.7 ($\underline{C}_{Ar}NO_2$), 128.5 (C_d or C_e), 128.2 (C_a), 127.0 (C_f), 126.8 (C_d or C_e), 115.9 ($\underline{C}_{Ar}CO_2R$), 115.0 (C_c), 51.8 (O$\underline{C}H_3$), 45.7 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{15}H_{14}N_2O_4$ + Na 309.0852. Found 309.0846.

Methyl 3-amino-4-(benzylamino)benzoate (31)



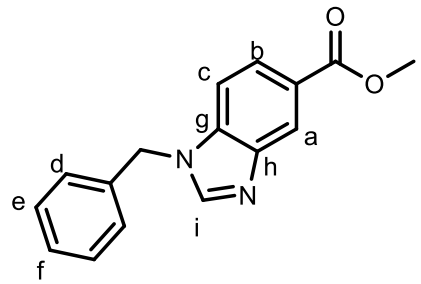
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.20 g) was added to a stirred suspension of **30** (6.99 mmol, 2.00 g) in ethanol (70.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 5 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The remaining solid was suspended in ether and filtered to give **31** as an off-white solid. Yield 1.10 g (61 %)

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 7.30 -7.37 (m, 4H, H_d, H_e), 7.21 – 7.25 (m, 1H, H_f), 7.21 (d, 1H, $^{4}J_{a,b}$ = 2.0 Hz, H_a), 7.12 (dd, 1H, $^{4}J_{b,a}$ = 2.0 Hz, $^{3}J_{b,c}$ = 8.3 Hz, H_b), 6.36 (d, 1H, $^{3}J_{c,b}$ = 8.4 Hz, H_c), 5.91 (t, 1H, $^{3}J_{NH,CH2}$ = 5.8 Hz, ArN<u>H</u>), 4.83 (s, 2H, ArN<u>H</u>$_2$), 4.39 (d, 2H, $^{3}J_{CH2,NH}$ = 5.8 Hz, ArC<u>H</u>$_2$), 3.71 (s, 3H, OC<u>H</u>$_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.6 (<u>C</u>O$_2$R), 140.0 (<u>C</u>$_{Ar}$CH$_2$), 139.4 (<u>C</u>$_{Ar}$NH), 134.2 (<u>C</u>$_{Ar}$NH$_2$), 128.2 (C_e), 127.0 (C_d), 126.6 (C_f), 120.2 (C_b), 116.8 (<u>C</u>$_{Ar}$CO$_2$R), 114.1 (C_a), 108.4 (C_c), 50.9 (O<u>C</u>H$_3$), 46.1 (Ar<u>C</u>H$_2$).

HRMS calculated for $C_{15}H_{16}N_2O_2$ 256.1212. Found 256.1210.

Methyl 1-benzyl-1*H*-benzo[*d*]imidazole-5-carboxylate (32)



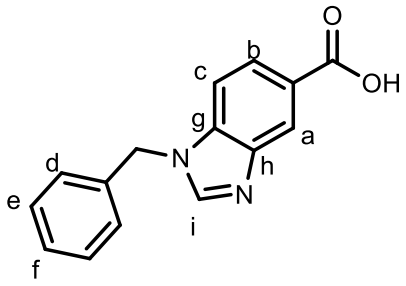
A stirred suspension of **31** (4.06 mmol, 1.04 g) in formic acid (10.1 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralised with sodium hydrogen carbonate (330 mL). The reaction mixture was extracted with ethyl acetate (3 x 50 mL) and the organic layer was washed with water (3 x 50 mL) and sodium chloride (1 x 50 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **32** as an off-white solid. Yield 1.06 g (98 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.58 (s, 1H, H_i), 8.27 (dd, 1H, $^{5}J_{a,c}$ = 0.5 Hz, $^{4}J_{a,b}$ = 1.5 Hz, H_a), 7.85 (dd, 1H, $^{4}J_{b,a}$ = 1.6 Hz, $^{3}J_{b,c}$ = 8.5 Hz, H_b), 7.65 (dd, 1H, $^{5}J_{c,a}$ = 0.6 Hz, $^{3}J_{c,b}$ = 8.5 Hz, H_c), 7.26 - 7.36 (m, 5H, H_d, H_e, H_f), 5.55 (s, 2H, ArC$\underline{H}_2$), 3.85 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.5 ($\underline{C}O_2R$), 146.3 (C_i), 143.0 (C_g), 136.9 (C_h), 136.4 ($\underline{C}_{Ar}CH_2$), 128.6 ($\underline{C}_{Ar}CO_2R$), 127.7 (C_d or C_e), 127.2 (C_f), 123.3 (C_d or C_e), 123.2 (C_b), 121.1 (C_a), 110.7 (C_c), 51.8 (O$\underline{C}H_3$), 47.7 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{16}H_{14}N_2O_2$ 266.1055. Found 266.1055.

1-Benzyl-1*H*-benzo[*d*]imidazole-5-carboxylic acid (10)



Lithium hydroxide (19.2 mmol, 0.80 g) was added to a suspension of **32** (3.83 mmol, 1.02 g) in tetrahydrofuran and water (2:1, 39.0 mL) at room temperature. The reaction mixture was refluxed for 3 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (30.0 mL). The reaction mixture was washed with ethyl acetate (1x 10.0 mL). Hydrochloric acid (2M, 10.0 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **10** as an off-white solid. Yield 0.87 g (90 %).

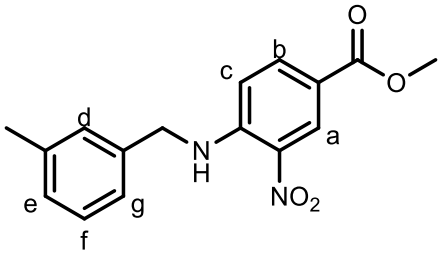
^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.73 (s, 1H, $CO_2\underline{H}$), 8.57 (s, 1H, H_i), 8.26 (d, 1H, $^{4}J_{a,b}$ = 1.0 Hz, H_a), 7.85 (dd, 1H, $^{4}J_{b,a}$ = 1.5 Hz, $^{3}J_{b,c}$ = 8.5 Hz, H_b), 7.61 (d, 1H, $^{3}J_{c,b}$ = 8.5 Hz, H_c), 7.31 (m, 5H, H_d, H_e, H_f), 5.55 (s, 2H, Ar$\underline{C}H_2$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 167.6 ($\underline{C}O_2R$), 146.1 (C_i), 143.0 (C_g), 136.7 (C_h), 136.5 ($\underline{C}_{Ar}CH_2$), 128.6 (C_d or C_e), 127.7 (C_f), 127.3 (C_d or C_e), 124.4 ($\underline{C}_{Ar}CO_2R$), 123.6 (C_b), 121.2 (C_a), 110.5 (C_c), 47.7 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{15}H_{12}N_2O_2$ 252.0899. Found 252.0902.

7.8. wALADin11

Methyl 4-((3-methylbenzyl)amino)-3-nitrobenzoate (33)



3-methylbenzylamine (7.53 mmol, 0.94 mL) was added in one portion to a stirred solution of **56** (5.02 mmol, 1.00 g) and diisopropylethylamine (10.0 mmol, 1.71 mL) in acetonitrile (50.0 mL) at room temperature. The reaction mixture was refluxed for one hour.

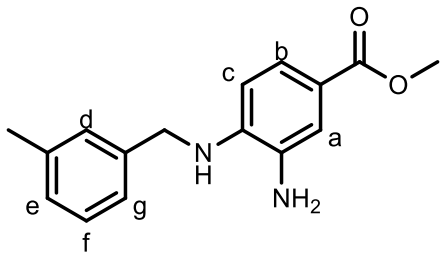
The reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate (70.0 mL) and water (30.0 mL). The reaction mixture was washed with water (2x 30.0 mL) and saturated sodium chloride solution (1x 30.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **33** as a yellow solid. Yield 1.47 g (98 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 9.03 (t, 1H, $^3J_{NH,CH2}$ = 6.1 Hz, ArN<u>H</u>), 8.61 (d, 1H, $^4J_{a,b}$ = 2.1 Hz, H_a), 7.87 (dd, 1H, $^4J_{b,a}$ = 1.9 Hz, $^3J_{b,c}$ = 9.0 Hz, H_b), 7.22 (dd, 1H, $^3J_{f,e/g}$ = 7.5 Hz, H_f), 7.18 (s, 1H, H_d), 7.15 (d, 1H, $^3J_{g,f}$ = 7.6 Hz, H_g), 7.07 (d, 1H, $^3J_{e,f}$ = 7.4 Hz, H_e), 6.98 (d, 1H, $^3J_{c,b}$ = 9.1 Hz, H_c), 4.64 (d, 2H, $^3J_{CH2,NH}$ = 6.1 Hz, ArC<u>H</u>$_2$), 3.80 (s, 3H, OC<u>H</u>$_3$), 2.27 (s, 3H, ArC<u>H</u>$_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.6 (<u>C</u>O_2R), 147.1 (<u>C</u>$_{Ar}$NH), 137.6 (<u>C</u>$_{Ar}$CH$_2$), 137.6 (<u>C</u>$_{Ar}$CH$_3$), 135.3 (C_b), 130.6 (<u>C</u>$_{Ar}$NO$_2$), 128.4 (C_f), 128.2 (C_a), 127.7 (C_e), 127.3 (C_d), 123.8 (C_g), 115.9 (<u>C</u>$_{Ar}$CO$_2$R), 115.0 (C_c), 51.8 (O<u>C</u>H$_3$), 45.7 (Ar<u>C</u>H$_2$), 20.9 (Ar<u>C</u>H$_3$).

HRMS calculated for $C_{16}H_{16}N_2O_4$ + Na 323.1008. Found 323.1002.

Methyl 3-amino-4-((3-methylbenzyl)amino)benzoate (34)



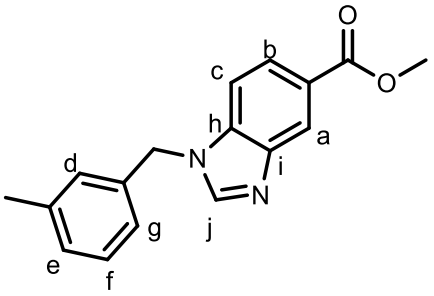
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.16 g) was added to a stirred suspension of **33** (5.19 mmol, 1.56 g) in ethanol (52.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 5 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The remaining solid was suspended in ether and filtered to give **34** as a white solid. Yield 1.11 g (79 %).

^{1}H NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 7.23 - 7.11 (m, 5H, H_a, H_b, H_d, H_f, H_g), 7.04 (d, 1H, $^{3}J_{e,f}$ = 7.2 Hz, H_e), 6.36 (d, 1H, $^{3}J_{c,b}$= 8.3 Hz, H_c), 5.88 (t, 1H, $^{3}J_{NH,CH2}$ = 5.7 Hz, ArN<u>H</u>), 4.83 (s, 2H, ArN<u>H</u>$_2$), 4.34 (d, 2H, $^{3}J_{CH2,NH}$ = 5.7 Hz, ArC<u>H</u>$_2$), 3.71 (s, 3H, OC<u>H</u>$_3$), 2.28 (s, 3H, ArC<u>H</u>$_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.6 (<u>C</u>O$_2$R), 140.1 (<u>C</u>$_{Ar}$CH$_2$), 139.4 (<u>C</u>$_{Ar}$NH), 137.2 (<u>C</u>$_{Ar}$CH$_3$), 134.2 (<u>C</u>$_{Ar}$NH$_2$), 128.1 (C_d), 127.6 (C_f), 127.3 (C_e), 124.1 (C_g), 120.2 (C_b), 116.8 (<u>C</u>$_{Ar}$CO$_2$R), 114.1 (C_a), 108.3 (C_c), 50.9 (O<u>C</u>H$_3$), 46.2 (Ar<u>C</u>H$_2$), 20.9 (Ar<u>C</u>H$_3$).

HRMS calculated for $C_{16}H_{18}N_2O_2$ + H 271.1446. Found 271.1441.

Methyl 1-(3-methylbenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (35)



A stirred suspension of **34** (4.11 mmol, 1.11 g) in formic acid (50.1 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralised with sodium hydrogen carbonate (1400 mL). The reaction mixture was extracted with ethyl acetate (3 x 200 mL) and the organic layer was washed with water (3 x 100 mL) and

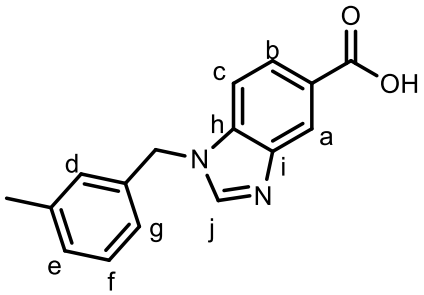
sodium chloride (1 x 100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **35** as an off-white solid. Yield 1.03 g (89 %).

^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.57 (s, 1H, H_j), 8.27 (d, 1H, $^5J_{a,c}$ = 0.5 Hz, $^4J_{a,b}$ = 1.5 Hz, H_a), 7.86 (dd, 1H, $^4J_{b,a}$ = 1.6 Hz, $^3J_{b,c}$ = 8.5 Hz, H_b), 7.64 (dd, 1H, $^5J_{c,a}$ = 0.5 Hz, $^3J_{c,b}$ = 8.5 Hz, H_c), 7.22 (dd, 1H, $^3J_{f,e/g}$ = 7.5 Hz, H_f), 7.15 (s, 1H, H_d), 7.09 (m, 2H, H_e, H_g), 5.50 (s, 2H, Ar$\underline{C}H_2$), 3.85 (s, 3H, OC$\underline{H}_3$), 2.24 (s, 3H, Ar$\underline{C}H_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.5 ($\underline{C}O_2R$), 146.3 (C_j), 143.0 (C_i), 137.8 (C_h), 136.9 ($\underline{C}_{Ar}CH_3$), 136.3 ($\underline{C}_{Ar}CH_2$), 128.5 (C_f), 128.4 (C_g), 127.8 (C_d), 124.4 (C_e), 123.3 ($\underline{C}_{Ar}CO_2R$), 123.2 (C_b), 121.1 (C_a), 110.7 (C_c), 51.8 (O$\underline{C}H_3$), 47.6 (Ar$\underline{C}H_2$), 20.8 (Ar$\underline{C}H_3$).

HRMS calculated for $C_{17}H_{16}N_2O_2$ + Na 303.1110. Found 303.1104.

1-(3-Methylbenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (11)



Lithium hydroxide (18.4 mmol, 0.76 g) was added to a suspension of **35** (3.67 mmol, 1.03 g) in tetrahydrofuran and water (2:1, 37.0 mL) at room temperature. The reaction mixture was refluxed for 4 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (25.0 mL). The reaction mixture was washed with ethyl acetate (2x 10.0 mL). Hydrochloric acid (2M, 10.0 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **11** as a white solid. Yield 0.62 g (63 %).

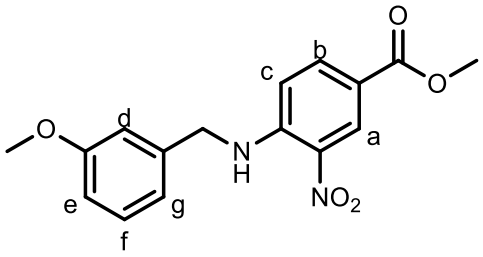
^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.74 (s, 1H, $CO_2\underline{H}$), 9.29 (s, 1H, H_j), 8.33 (d, 1H, $^5J_{a,c}$ = 0.5 Hz, $^4J_{a,b}$ = 1.4 Hz, H_a), 7.97 (dd, 1H, $^4J_{b,a}$ = 1.5 Hz, $^3J_{b,c}$ = 8.6 Hz, H_b), 7.79 (dd, 1H, $^5J_{c,a}$ = 0.4 Hz, $^3J_{c,b}$ = 8.6 Hz, H_c), 7.26 – 7.17 (m, 3H, H_d, H_e, H_f), 7.11 (d, 1H, $^3J_{g,f}$ = 7.7 Hz, H_g), 5.63 (s, 2H, ArC$\underline{H}_2$), 2.25 (s, 3H, ArC$\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.9 ($\underline{C}O_2R$), 144.9 (C_j), 138.0 (C_h), 137.2 (C_i), 135.2 ($\underline{C}_{Ar}CH_3$), 135.1 ($\underline{C}_{Ar}CH_2$), 128.7 (C_d), 128.6 (C_f), 128.1 (C_e), 126.5 ($\underline{C}_{Ar}CO_2R$), 125.0 (C_b), 124.7 (C_g), 118.9 (C_a), 112.0 (C_c), 48.6 (Ar$\underline{C}H_2$), 20.8 (Ar$\underline{C}H_3$).

HRMS calculated for $C_{16}H_{14}N_2O_2$ + H 267.1133. Found 267.1128.

7.9. wALADin12

Methyl 4-((3-methoxybenzyl)amino)-3-nitrobenzoate (36)



3-methoxybenzylamine (7.53 mmol, 0.97 mL) was added in one portion to a stirred solution of **56** (5.02 mmol, 1.00 g) and diisopropylethylamine (10.0 mmol, 1.71 mL) in acetonitrile (50.0 mL) at room temperature. The reaction mixture was refluxed for one hour.

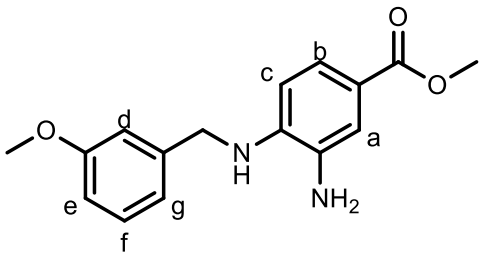
The reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate (70.0 mL) and water (30.0 mL). The reaction mixture was washed with water (2x 30.0 mL) and saturated sodium chloride solution (1x 30.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **36** as a yellow solid. Yield 1.54 g (97 %).

^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] =9.05 (t, 1H, $^3J_{NH,CH2}$ = 6.0 Hz, ArN$\underline{H}$), 8.62 (d, 1H, $^4J_{a,b}$ = 2.1 Hz, H_a), 7.89 (dd, 1H, $^4J_{b,a}$ = 2.0 Hz, $^3J_{b,c}$ = 9.1 Hz, H_b), 7.25 (dd, 1H, $^3J_{f,e/g}$ = 7.9 Hz, H_f), 6.99 (d, 1H, $^3J_{c,b}$ = 9.1 Hz, H_c), 6.94 (s, 1H, H_d), 6.93 (d, 1H, $^3J_{e,f}$ = 7.3 Hz, H_e), 6.83 (d, 1H, $^3J_{g,f}$ = 7.1 Hz, H_g), 4.65 (d, 2H, $^3J_{CH2,NH}$ = 6.1 Hz, ArC$\underline{H}_2$), 3.80 (s, 1H, $CO_2C\underline{H}_3$), 3.72 (s, 1H, $OC\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.6 ($\underline{C}O_2R$), 159.3 ($\underline{C}_{Ar}OCH_3$), 147.1 ($\underline{C}_{Ar}NH$), 139.3 ($\underline{C}_{Ar}CH_2$), 135.3 (C_b), 130.7 ($\underline{C}_{Ar}NO_2$), 129.6 (C_f), 128.2 (C_a), 118.8 (C_e), 115.9 ($\underline{C}_{Ar}CO_2R$), 115.0 (C_c), 112.6 (C_d), 112.3 (C_g), 54.8 ($O\underline{C}H_3$), 51.8 ($CO_2\underline{C}H_3$), 45.6 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{16}H_{16}N_2O_5$ + Na 339.0957. Found 339.0951.

Methyl 3-amino-4-((3-methoxybenzyl)amino)benzoate (37)



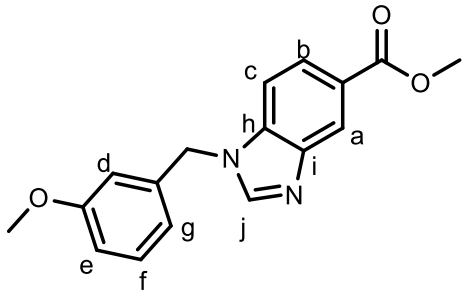
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.15 g) was added to a stirred suspension of **36** (4.68 mmol, 1.48 g) in ethanol (49.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 1 hour using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The remaining solid was suspended in ether and filtered to give **37** as an off-white solid. Yield 0.39 g (29 %).

^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 7.26 – 7.21 (m, 2H, H_d,H_f), 7.12 (dd, 1H, $^{4}J_{b,a}$ = 2.2 Hz, $^{3}J_{b,c}$ = 8.2 Hz, H_b), 6.92 (d, 2H, $^{4}J_{a,b}$ = 2.2 Hz, H_a, H_g), 6.80 (d, 1H, $^{3}J_{e,f}$ = 7.9 Hz, H_e), 6.36 (d, 1H, $^{3}J_{c,b}$ = 8.3 Hz, H_c), 5.91 (t, 1H, $^{3}J_{NH,CH2}$ = 5.0Hz, ArN<u>H</u>), 4.83 (s, 2H, ArN<u>H</u>$_2$), 4.36 (d, 2H, $^{3}J_{CH2,NH}$ = 5.4 Hz, ArC<u>H</u>$_2$), 3.72 (s, 3H, $CO_2C\underline{H}_3$), 3.71 (s, 3H, $OC\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.6 ($\underline{C}O_2R$), 159.2 ($\underline{C}_{Ar}OCH_3$), 141.2 ($\underline{C}_{Ar}CH_2$), 140.0 ($\underline{C}_{Ar}NH$), 134.2 ($\underline{C}_{Ar}NH_2$), 129.2 (C_f), 120.2 (C_b), 119.1 (C_g), 116.9 ($\underline{C}_{Ar}CO_2R$), 114.1 (C_d), 112.7 (C_a), 111.8 (C_e), 108.4 (C_c), 54.8 ($CO_2\underline{C}H_3$), 50.9 ($O\underline{C}H_3$), 46.1 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{16}H_{18}N_2O_3$ + H 287.1395. Found 287.1390.

Methyl 1-(3-methoxybenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (38)



A stirred suspension of **37** (1.20 mmol, 0.34 g) in formic acid (3.30 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralised with sodium hydrogen carbonate (90.0 mL). The reaction mixture was extracted with ethyl acetate (3 x 40.0 mL) and the organic layer was washed with water (3 x 20.0 mL) and

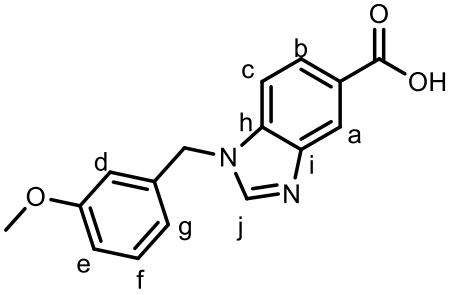
sodium chloride (1 x 30.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **38** as a beige solid. Yield 0.30 g (84 %).

^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.58 (s, 1H, H_j), 8.27 (d, 1H, $^4J_{a,b}$ = 1.3 Hz, H_a), 7.86 (dd, 1H, $^4J_{b,a}$ = 1.5 Hz, $^3J_{b,c}$ = 8.5 Hz, H_b), 7.67 (d, 1H, $^3J_{c,b}$ = 8.5 Hz, H_c), 7.24 (dd, 1H, $^3J_{f,e/g}$ = 7.9 Hz, H_f), 6.93 (d, 1H, $^4J_{d,e/g}$ = 1.8 Hz, H_d), 6.85 (dd, 2H, $^4J_{e/g,d}$ = 2.4 Hz, $^3J_{e/g,f}$ = 8.5 Hz, H_e,H_g), 5.51 (s, 2H, ArC$\underline{H}_2$), 3.85 (s, 3H, $CO_2C\underline{H}_3$), 3.71 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.5 ($\underline{C}O_2R$), 159.3 ($\underline{C}_{Ar}OCH_3$), 146.3 (C_j), 143.0 (C_h), 137.9 (C_i), 136.9 ($\underline{C}_{Ar}CH_2$), 129.8 (C_f), 123.3 (C_b), 123.2 ($\underline{C}_{Ar}CO_2R$), 121.1 (C_a), 119.3 (C_g), 113.2 (C_d), 112.9 (C_e), 110.8 (C_c), 54.9 (O$\underline{C}H_3$), 51.8 ($CO_2\underline{C}H_3$), 47.6 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{17}H_{16}N_2O_3$ + H 297.1239. Found 297.1234.

1-(3-Methoxybenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (12)



Lithium hydroxide (4.61 mmol, 0.19 g) was added to a suspension of **38** (0.92 mmol, 0.27 g) in tetrahydrofuran and water (2:1, 9.80 mL) at room temperature. The reaction mixture was refluxed for 2.5 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (2.00 mL). The reaction mixture was washed with ethyl acetate (2x 2.00 mL). Hydrochloric acid (2M, 1.50 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **12** as beige solid. Yield 0.06 g (25 %).

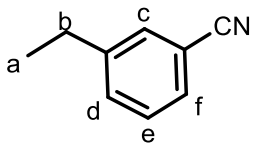
^{1}H NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.77 (s, 1H, $CO_2\underline{H}$), 8.60 (s, 1H, H_j), 8.26 (d, 1H, $^4J_{a,b}$ = 1.0 Hz, H_a), 7.86 (dd, 1H, $^4J_{b,a}$ = 1.4 Hz, $^3J_{b,c}$ = 8.5 Hz, H_b), 7.64 (d, 1H, $^3J_{c,b}$ = 8.5 Hz, H_c), 7.25 (dd, 1H, $^3J_{f,e/g}$ = 7.9 Hz, H_f), 6.93 (d, 1H, $^4J_{d,e/g}$ = 1.6 Hz, H_d), 6.85 (dd, 2H, $^4J_{e/g,d}$ = 2.0 Hz, $^3J_{e/g,f}$ = 8.1 Hz, H_e, H_g), 5.51 (s, 2H, ArC$\underline{H}_2$), 3.71 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 167.6 ($\underline{C}O_2R$), 159.3 ($\underline{C}_{Ar}OCH_3$), 146.0 (C_j), 142.7 (C_h), 137.9 (C_i), 136.6 ($\underline{C}_{Ar}CH_2$), 129.8 (C_f), 124.5 ($\underline{C}_{Ar}CO_2R$), 123.7 (C_b), 121.1 (C_a), 119.3 (C_g), 113.3 (C_d), 112.9 (C_e), 110.6 (C_c), 54.9 ($O\underline{C}H_3$), 47.7 ($Ar\underline{C}H_2$).

HRMS calculated for $C_{16}H_{14}N_2O_3$ + H 283.1082. Found 283.1077.

7.10. wALADin13

3-Ethylbenzonitrile (39)

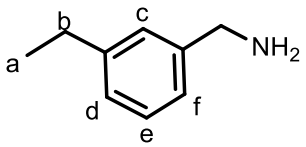


A stirred suspension of 1-bromo-3-ethylbenzene (27.0 mmol, 5.00 g), zinc cyanide (14.9 mmol, 1.75 g) and tetrakis(triphenylphosphine)palladium (1.35 mmol, 1.56 g) in dimethylformamide (50.0 mL) was refluxed for 4 hours at 80 °C under an argon atmosphere. The reaction mixture was concentrated under reduced pressure and the residue was suspended in ethanol. The suspension was filtered and the filtrate was concentrated under reduced pressure. The crude product was distilled (4.10^{-5} bar; 40 °C) to give **39** as colourless oil. Yield 1.70 g (45 %).

^{1}H-NMR (300 MHz, $CDCl_3$, 298 K) [ppm] = 7.39-7.27 (m, 2H, H_c, H_f), 7.19-7.09 (m, 2H, H_d, H_e), 2.63 (q, 2H, $^3J_{b,a}$ = 7.6 Hz, H_b), 1.24 (t, 3H, $^3J_{a,b}$ = 7.6 Hz, H_a).

^{13}C-NMR (75 MHz, $CDCl_3$, 298 K) [ppm] = 146.68 ($\underline{C}_{Ar}C_b$), 131.09 (C_f), 130.00 (C_d), 129.96 (C_c), 128.85 (C_e), 126.68 ($C_{ar}\underline{C}N$), 122.52 ($\underline{C}N$), 28.72 (C_b), 15.50 (C_a).

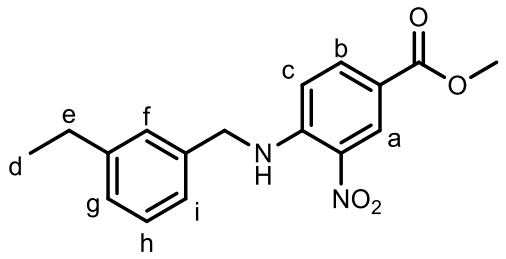
3-Ethylbenzylamine (40)



Lithium aluminium hydride (71.3 mmol, 2.71 g) was slowly added to a stirred solution of **39** (13.0 mmol, 1.70 g) in tetrahydrofuran (140 mL) at 0 °C and stirred at room temperature for 48 hours. The reaction mixture was cooled to 0 °C and diluted with ether (75.0 mL). The reaction mixture was quenched with saturated sodium sulphate (80.0 mL) and filtered. The filtrate was concentrated under reduced pressure to give **40** as yellow solid. Yield 1.49 g (85 %).

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 7.31-7.25 (m, 1H, H_e), 7.19-7.10 (m, 3H, H_c, H_d, H_f), 3.87 (s, 2H, ArC$\underline{H}_2$), 2.67 (q, 2H, $^{3}J_{b,a}$ = 7.6 Hz, H_b), 1.27 (t, 3H, $^{3}J_{a,b}$ = 7.6 Hz, 3H, H_a).

Methyl 4-((3-ethylbenzyl)amino)-3-nitrobenzoate (41)



40 (7.55 mmol, 1.02 g) was added in one portion to a stirred solution of **56** (5.03 mmol, 1.00 g) and diisopropylethylamine (10.1 mmol, 1.71 mL) in acetonitrile (50.0 mL) at room temperature. The reaction mixture was refluxed for two hours.

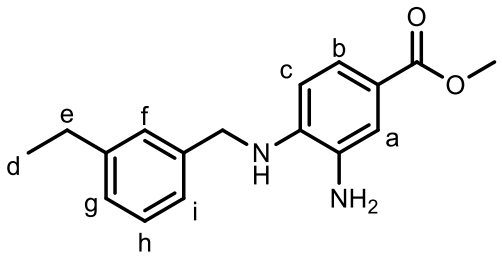
The reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate (100 mL) and water (40.0 mL). The reaction mixture was washed with water (2x 40.0 mL) and saturated sodium chloride solution (1x 40.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **41** as a yellow solid. Yield 1.39 g (88 %).

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 9.03 (t, 1H, $^{3}J_{NH,CH2}$ = 6.2 Hz, ArN$\underline{H}$), 8.62 (d, 1H, $^{4}J_{a,b}$ = 2.1 Hz, H_a), 7.88 (dd, 1H $^{3}J_{b,c}$ = 9.1, $^{4}J_{b,a}$ = 2.1 Hz, H_b), 7.30-7.04 (m, 4H, H_f, H_h, H_g, H_i), 7.00 (d, 1H, $^{3}J_{c,b}$ = 9.1 Hz, H_c), 4.65 (d, 1H $^{3}J_{CH2,NH}$ = 6.1 Hz, ArC$\underline{H}_2$), 3.80 (s, 3H, OC$\underline{H}_3$), 2.57 (q, 2H, $^{3}J_{e,d}$ = 7.6 Hz, H_e), 1.15 (t, 3H, $^{3}J_{d,e}$ = 7.6 Hz, H_d).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 164.8 ($\underline{C}O_2R$), 147.3 ($\underline{C}_{Ar}NH$), 144.1 ($\underline{C}_{Ar}C_e$), 137.7 ($\underline{C}_{Ar}CH_2$), 135.5 (C_b), 130.8 (C_a), 128.6 ($\underline{C}_{Ar}NO_2$), 128.3 (C_h), 126.6 (C_f), 126.4 (C_g), 124.2 (C_i), 116.0 ($\underline{C}_{Ar}CO_2R$), 115.2 (C_c), 52.0 (O$\underline{C}H_3$), 45.9 (Ar$\underline{C}H_2$), 28.1 (C_e), 15.5 (C_d).

HRMS calculated for $C_{17}H_{18}N_2O_4$ 314.1267. Found 314.1262.

Methyl 3-amino-4-((3-ethylbenzyl)amino)benzoate (42)



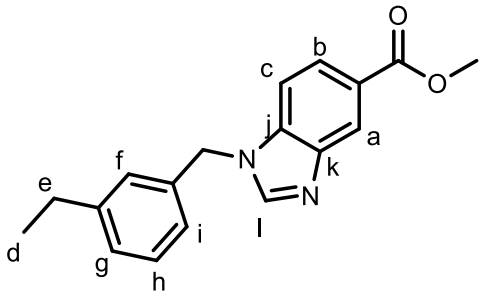
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.16 g) was added to a stirred suspension of **41** (5.06 mmol, 1.60 g) in ethanol (50.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 3 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The remaining solid was suspended in ether and filtered to give **42** as an off-white solid. Yield 1.14 g (79 %)

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 7.29-7.04 (m, 6H, H_h, H_f, H_g, H_i, H_b, H_a), 6.38 (d, 1H, $^{3}J_{c,b}$ = 8.3 Hz, H_c), 5.87 (t, 1H, $^{3}J_{NH,CH2}$ = 5.8 Hz, ArN<u>H</u>), 4.83 (s, 2H, ArN<u>H</u>$_2$), 3.71 (s, 3H, OC<u>H</u>$_3$), 2.58 (q, 2H, $^{3}J_{e,d}$ = 7.6 Hz, H_e), 1.16 (t, 6H, $^{3}J_{d,e}$ = 7.6 Hz, H_d).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 166.78 (CO_2R), 143.73 (<u>C</u>$_{Ar}C_e$), 140.24 (<u>C</u>$_{Ar}CH_2$), 139.51 (<u>C</u>$_{Ar}$NH), 134.32 (<u>C</u>$_{Ar}NH_2$), 128.28 (C_h), 126.6, (C_f), 126.22 (C_g) 124.50 (C_i), 120.37 (C_b), 116.94 (<u>C</u>$_{Ar}CO_2R$), 114.23 (C_a), 108.47 (C_c), 51.08 (O<u>C</u>H$_3$), 46.42 (Ar<u>C</u>H$_2$), 28.16 (C_e), 15.58 (C_d).

HRMS calculated for $C_{17}H_{20}N_2O_2$ 284.1525. Found 284.1518.

Methyl 1-(3-ethylbenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (43)



A stirred suspension of **42** (3.97 mmol, 1.13 g) in formic acid (9,90 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralised with sodium hydrogen carbonate (30.0 mL). The reaction mixture was extracted with ethyl acetate (3 x 50.0 mL) and the organic layer was washed with water (3 x 50.0 mL) and

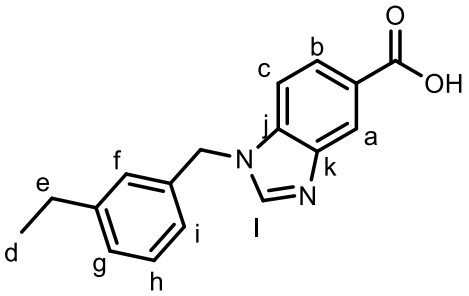
sodium chloride (1 x 50.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **43** as a light red solid. Yield 1.04 g (89 %).

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 8.57 (s, 1H, H_l), 8.26 (d, 1H, $^4J_{a,b}$ = 1.5 Hz, H_a), 7.85 (dd, $^3J_{b,c}$ = 8.6 Hz, $^4J_{b,a}$ = 1.6 Hz, H_b), 7.65 (d, 1H, $^3J_{c,b}$ = 8.5 Hz, H_c), 7.26-7.17 (m, 2H, H_h, H_f), 7.16-7.04 (m, 2H, H_g, H_i), 5.51 (s, 2H, ArC$\underline{H}_2$), 3.84 (s, 3H, OC$\underline{H}_3$), 2.55 (q, 1H, $^3J_{e,d}$ = 7.6 Hz, H_e), 1.14 (t, 3H, $^3J_{d,e}$ = 7.6 Hz, H_d).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 166.66 (CO2R), 146.42 ($C_{Ar}C_e$), 144.30 (C_l), 143.14 (C_k), 137.05 (C_j), 136.51 ($C_{Ar}CH_2$), 128.72 (C_f), 127.29 (C_h), 126.88 (C_b), 124.72 ($\underline{C}_{Ar}CO_2R$), 123.45 (C_i), 123.31 (C_g), 121.22 (C_a), 110.87 (C_c), 51.97 (O$\underline{C}$H3), 47.85 (Ar$\underline{C}H_2$), 28.00 (C_e), 15.44 (C_d).

HRMS calculated for $C_{18}H_{18}N_2O_2$ 294.1368. Found 294.1361.

1-(3-Ethylbenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (13)



Lithium hydroxide (16.9 mmol, 0.71 g) was added to a suspension of **43** (3.40 mmol, 1.00 g) in tetrahydrofuran and water (2:1, 35.0 mL) at room temperature. The reaction mixture was refluxed for 3.5 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (25.0 mL). The reaction mixture was washed with ethyl acetate (1x 20.0 mL). Hydrochloric acid (2M, 15.0 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **13** as an off-white solid. Yield 0.64 g (67 %).

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 12.88 (s, 1H, $CO_2\underline{H}$), 9.49-9.28 (m, 2H, H_l, H_b), 8.34 (s, 1H, H_a), 7.99 (d, 1H $^3J_{c,b}$ = 8.5 Hz, H_c), 7.83 (d, 1H, $^3J_{h,i/g}$ = 8.6 Hz, H_h), 7.36-7.11 (m, 3H, H_g, H_i, H_f), 5.66 (s, 2H, ArC$\underline{H}_2$), 2.56 (q, 2H, $^3J_{e,d}$ = 7.6 Hz, H_e), 1.13 (t, 3H, $^3J_{d,e}$ = 7.6 Hz, H_d).

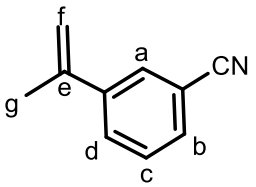
^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 166.95 ($\underline{C}O_2H$), 144.88 ($\underline{C}_{Ar}C_e$), 144.47 (C_l), 136.62 (C_j), 135.24 (C_k), 135.02 ($\underline{C}_{Ar}CH_2$), 128.84 (C_f), 127.71 (C_h), 127.29 (C_b),

126.92 ($\underline{C}_{Ar}CO_2H$), 125.34 (C_i), 125.13 (C_g), 118.77 (C_a), 112.29 (C_c), 48.97 ($Ar\underline{C}H_2$), 28.00 (C_e), 15.46 (C_d).

HRMS calculated for $C_{17}H_{16}N_2O_2$ 280.1212. Found 280.1205.

7.11. wALADin14

3-(Prop-1-en-2-yl)benzonitrile (44)



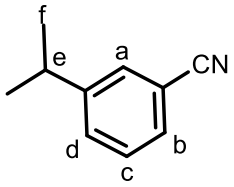
n-Butyl lithium (40.0 mmol, 25.0 mL) in tetrahydrofurane (40.0 mL) was added dropwise at 0 °C to a stirred solution of methyltriphenylphosphonium bromide (40.0 mmol, 14.g) in tetrahydrofurane (90.0 mL). After half an hour 3-Acetylbenzonitril (27.0 mmol, 4.00 g) in tetrahydrofurane (180 mL) was added and stirred for 3 hours. The reaction mixture was partitioned between ethyl acetate (150 mL) and saturated ammonium chloride solution (250 mL) and the organic phase was washed with water (2x 80.0 mL) and saturated sodium chloride solution (1x 100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using cyclohexane: ethyl acetate (19:1) as eluent to give **44** as colourless oil. Yield 3.49 g (90 %).

^{1}H-NMR (300 MHz, $CDCl_3$, 298 K) [ppm] = 7.72-7.66 (m, 2H, H_b,H_a), 7.54 (dt, 1H, $^3J_{c,b}$ = 7.7 Hz, $^3J_{c,d}$ = 7.7 Hz, $^5J_{c,a}$ = 1.4 Hz, H_c), 7.42 (td, 1H, $^3J_{d,c}$ = 7.7 Hz, $^4J_{d,b}$ = 0.7 Hz, $^4J_{d.a}$ = 0.7 Hz, H_a), 5.41 (p, 1H, $^2J_{f,f'}$ = 0.9 Hz, $H_{f/f'}$), 5.20 (p, 1H, $^2J_{f,f'}$ = 1.4 Hz, $H_{f/f'}$), 2.15 (d, 3H, $^4J_{g,f}$ = 1.4 Hz, H_g).

^{13}C-NMR (75 MHz, $CDCl_3$, 298 K) [ppm] = 142.47 (C_e), 141.41 ($\underline{C}_{Ar}C_a$), 130.85 (C_b), 129.89 (C_d), 129.28 (C_a), 129.16 (C_c), 119.03 ($\underline{C}N$), 114.79 (C_f), 112.54 ($\underline{C}_{Ar}CN$), 21.59 (C_g).

HRMS calculated for $C_{10}H_9N$ 143.0735. Found 143.0759.

3-Isopropylbenzonitrile (45)



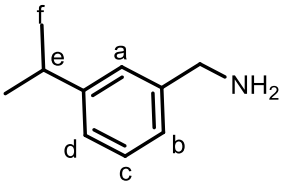
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.35 g) was added to a stirred solution of **44** (24.4 mmol, 3.49 g) in ethyl acetate (90.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 16 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure to give **45** as colourless oil. Yield 3.26 g (92 %).

^{1}H-NMR (300 MHz, $CDCl_3$, 298 K) [ppm] = 7.48-7.32 (m, 2H, H_b, H_a), 7.26-7.09 (m, 2H, H_c, H_d), 2.96-2.83 (m, 1H, H_e), 1.23 (d, 6H, $^{3}J_{f,a}$ = 6.9 Hz, H_f).

^{13}C-NMR (75 MHz, $CDCl_3$, 298 K) [ppm] = 149.16 ($\underline{C}_{Ar}C_e$), 130.25 (C_d), 129.20 (C_a), 128.48 (C_b), 125.81 (C_c), 119.30 ($\underline{C}N$), 112.41 ($\underline{C}_{Ar}CN$), 34.01 (C_e), 23.77 (C_f).

HRMS calculated for $C_{10}H_9N$ 145.0891. Found 145.0891.

3-Isopropylbenzylamine (46)



Lithium aluminium hydride (123.5 mmol, 4.67 g) was slowly added to a stirred solution of **45** (22.5 mmol, 3.26 g) in tetrahydrofuran (250 mL) at 0 °C and stirred at room temperature for 48 hours. The reaction mixture was cooled to 0 °C and diluted with ether (125 mL). The reaction mixture was quenched with saturated sodium sulphate (130 mL) and filtered. The filtrate was concentrated under reduced pressure and the residue was purified by column chromatography on silica gel using cyclohexane: ethyl acetate (19:1) as eluent to give **46** as yellow solid. Yield 3.14 g (98 %).

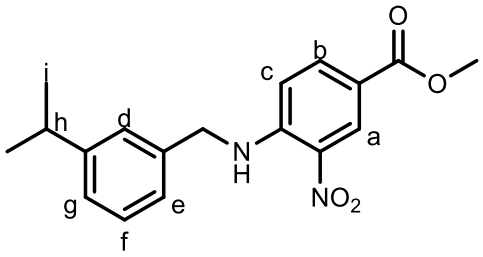
^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 7.24-7.16 (m, 2H, H_a, H_c), 7.12 (dt, 1H, $^{3}J_{d,c}$ = 7.8 Hz, $^{5}J_{d,a}$ = 1.5 Hz, $^{5}J_{d,b}$ = 1.5 Hz, H_d), 7.06 (dt, 1H, $^{3}J_{b,c}$ = 7.5 Hz , $^{5}J_{b,a}$ = 1.6 Hz,

$^{5}J_{b,d}$ = 1.6 Hz, H_b), 3.69 (d, 2H, $^{3}J_{CH2,NH}$ = 0.8 Hz, ArC$\underline{H}_2$), 2.85 (sept, 1H, $^{3}J_{e,f}$ = 6.9 Hz, H_e), 1.20 (d, 6H, $^{3}J_{f,e}$ = 6.9 Hz, H_f).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 148.08 ($\underline{C}_{Ar}C_e$), 144.19 ($\underline{C}_{Ar}CH_2$), 127.96 (C_a), 124.96 (C_c), 124.48 (C_b), 124.01 (C_d), 45.79 (Ar$\underline{C}H_2$), 33.48 (C_e), 23.95 (C_f).

HRMS calculated for $C_{10}H_{15}N$ 149.1204. Found 149.1195.

Methyl 4-((3-isopropylbenzyl)amino)-3-nitrobenzoate (47)



46 (7.55 mmol, 1.12 mL) was added in one portion to a stirred solution of **56** (5.03 mmol, 1.00 g) and diisopropylethylamine (10.0 mmol, 1.73 mL) in acetonitrile (51.0 mL) at room temperature. The reaction mixture was refluxed for 2 hours.

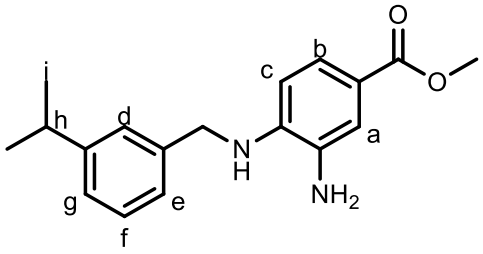
The reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate (100 mL) and water (50.0 mL). The reaction mixture was washed with water (2x 50.0 mL) and saturated sodium chloride solution (1x 50.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using cyclohexane: ethyl acetate (19:1) as eluent to give **47** as yellow solid. Yield 1.01 g (61 %).

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 9.02 (t, 1H, ^{3}JNH,3 = 6.1 Hz, ArN$\underline{H}$), 8.62 (d, 1H, $^{4}J_{a,b}$ = 2.1 Hz, H_a), 7.88 (dd, 1H $^{3}J_{b,a}$ = 9.0, $^{4}J_{b,c}$ = 2.1 Hz, H_b), 7.31-7.21 (m, 2H, H_d, H_f), 7.20-7.10 (m, 2H, H_g, H_e), 7.01 (d, 1H, $^{3}J_{b,c}$ = 9.1 Hz, H_c), 4.66 (d, $^{3}J_{CH2,NH}$ = 6.1 Hz, ArC$\underline{H}_2$), 3.80 (s, 3H, OC$\underline{H}_3$), 2.85 (sept, 1H, $^{3}J_{h,i}$ = 6.9 Hz, H_h), 1.17 (d, 6H, $^{3}J_{i,h}$ = 6.9 Hz, H_i)

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 164.75 ($\underline{C}O_2R$), 148.75 ($\underline{C}_{Ar}NH$), 147.31 ($\underline{C}_{Ar}C_h$), 137.67 ($\underline{C}_{Ar}CH_2$), 135.48 (C_b), 130.75 (C_a), 128.59 ($C_{Ar}NO_2$), 128.30 (C_f), 125.11 (C_d), 124.31 (C_e, C_g), 116.02 (C_c), 115.15 ($\underline{C}_{Ar}CO_2R$), 51.97 (O$\underline{C}H_3$), 45.99 (Ar$\underline{C}H_2$), 33.35 (C_h), 23.82 (C_i).

HRMS calculated for $C_{18}H_{20}N_2O_4$ 328.1423. Found 328.1418.

Methyl 3-amino-4-((3-isopropylbenzyl)amino)benzoate (48)



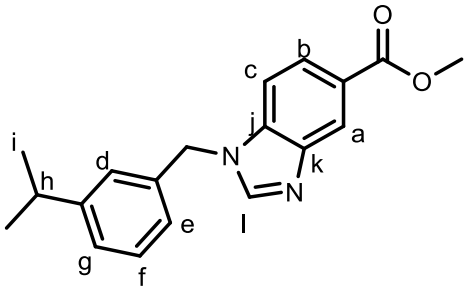
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.23 g) was added to a stirred suspension of **47** (7.00 mmol, 2.30 g) in ethanol (75.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 3 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The remaining solid was suspended in ether (80.0 mL) and filtered to give **48** as off-white solid. Yield 1.35 g (65 %)

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 7.24-7.26 (m, 2H, H_f, H_g), 7.21 (d, 1H, $^{3}J_{e,f}$ = 7.21 Hz, H_e), 7.05-7.20 (m, 3H, H_a, H_b, H_d), 6.39 (d, 1H, $^{3}J_{c,b}$ = 8.3 Hz, H_c), 5.87 (t, 1H, $^{3}J_{NH,CH2}$ = 5.8 Hz, ArN<u>H</u>), 4.35 (d, 2H, $^{3}J_{CH2;NH}$ = 5.6 Hz, ArC<u>H</u>$_2$), 3.71 (s, 3H, OC<u>H</u>$_3$), 2.85 (sept, 1H, $^{3}J_{h,1}$ = 6.9 Hz, H_h), 1.18 (d, 6H, $^{3}J_{i,h}$ = 6.9 Hz, H_i).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 166.79 (<u>C</u>O$_2$R), 148.39 (<u>C</u>$_{Ar}$C$_h$), 140.29 (<u>C</u>$_{Ar}$CH$_2$), 139.46 (<u>C</u>$_{Ar}$NH), 134.33 (<u>C</u>$_{Ar}$NH$_2$), 128.27 (C$_f$), 125.36 (C$_d$), 124.67 (C$_e$), 120.37 (C$_g$), 116.94 (C$_b$), 114.23 (<u>C</u>$_{Ar}$CO$_2$R), 108.48 (C$_a$), 51.08 (O<u>C</u>H3), 46.53 (Ar<u>C</u>H$_2$), 33.41 (C$_h$), 23.90 (C$_i$).

HRMS calculated for $C_{18}H_{22}N_2O_2$ 298.1681. Found 298.1674.

Methyl 1-(3-isopropylbenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (49)



A stirred suspension of **48** (4.86 mmol, 1.31 g) in formic acid (11.0 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralised with sodium hydrogen carbonate (45.0 mL). The reaction mixture was extracted with ethyl acetate (3 x 50.0 mL) and the organic layer was washed with water (3 x 50.0 mL) and

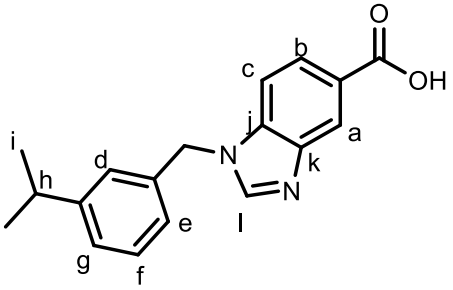
sodium chloride (1 x 50.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **49** as light-red solid. Yield 1.41 g (94 %).

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 8.58 (s, 1H, H_l), 8.27 (dd, 1H, $^4J_{a,b}$ = 1.6 Hz, $^5J_{a,c}$ = 0.7 Hz, H_a), 7.86 (dd, $^3J_{b,c}$ = 8.5 Hz, $^4J_{b,a}$ = 1.6 Hz, H_b), 7.68 (dd, 1H, $^3J_{c,b}$ = 8.5 Hz, $^5J_{c,a}$ = 0.7 Hz, H_c), 7.19-7.30 (m, 2H, H_f, H_d), 7.15 (dt, $^3J_{g,f}$ = 7.8 Hz, $^4J_{g,d}$ = 1.6 Hz, H_g), 7.07 (dt, $^3J_{e,f}$ = 7.5 Hz, ^4Je,d = 1.5 Hz, H_e), 5.52 (d, 2H, $^3J_{CH2,NH}$ = 5.6 Hz, ArC$\underline{H}_2$), 3.85 (s, 3H, OC$\underline{H}$3), 2.82 (sept, 1H, $^3J_{h,i}$ = 6.9 Hz, H_h), 1.14 (d, 6H, $^3J_{i,h}$ = 6.9 Hz, H_i).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 166.65 (CO_2R), 148.94 ($\underline{C}_{Ar}C_h$), 146.40 (C_l), 143.13 (C_k), 137.06 (C_j), 136.49 ($\underline{C}_{Ar}CH_2$), 128.72 (C_f), 125.75 (C_d), 125.59 (C_b), 124.81 ($C_{Ar}CO_2$R), 123.43 (C_e), 123.30 (C_g), 121.23 (C_a), 110.87 (C_c), 51.96 (O$\underline{C}H_3$), 47.91 (Ar$\underline{C}H_2$), 33.28 (C_h), 23.74 (C_i).

HRMS calculated for $C_{19}H_{20}N_2O_2$ 308.1525. Found 308.1518.

1-(3-Isopropylbenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (14)



Lithium hydroxide (20.4 mmol, 0.85 g) was added to a suspension of **49** (4.09 mmol, 1.26 g) in tetrahydrofuran and water (2:1, 40.0 mL) at room temperature. The reaction mixture was refluxed for 3.5 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (30.0 mL). The reaction mixture was washed with ethyl acetate (1x 40.0 mL). Hydrochloric acid (2M, 20.0 mL) was added to the aqueous phase and the resulting precipitate was filtered to give **14** as a white solid. Yield 1.13 g (94 %).

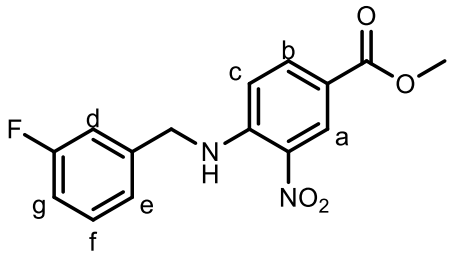
^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) [ppm] = 12.82 (s, 1H, $CO_2\underline{H}$), 8.71 (s, 1H, H_a), 8.27 (s, 1H, H_l), 7.88 (dd, 1H $^3J_{b,a}$ = 8.6 Hz, $^4J_{b,c}$ = 1.6 Hz, H_b), 7.68 (d, 1H, $^3J_{c,b}$ = 8.6 Hz, H_c), 7.08-7.28 (m, 4H, H_d, H_g, H_f, H_e), 5.54 (s, 2H, ArC$\underline{H}_2$), 2.83 (sept, 1H, $^3J_{h,i}$ = 6.9 Hz, H_h), 1.14 (d, 6H, $^3J_{i,h}$ = 6.9 Hz, H_i)

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) [ppm] = 166.65 ($\underline{C}O_2H$), 148.94 ($\underline{C}_{Ar}C_h$), 146.40 (C_l), 143.13 (C_j), 137.06 (C_k), 136.49 ($\underline{C}_{Ar}CH_2$), 128.72 (C_f), 125.75 (C_d), 125.59 (C_b), 124.81 ($\underline{C}_{Ar}CO_2R$), 123.43 (C_e), 123.30 (C_g), 121.23 (C_a), 110.87 (C_c), 51.96 ($O\underline{C}H_3$), 47.91 ($Ar\underline{C}H_2$), 33.28 (C_h), 23.74 (C_1).

HRMS calculated for $C_{19}H_{20}N_2O_2$ 294.1368. Found 294.1376.

7.12. wALADin15

Methyl 4-((3-fluorobenzyl)amino)-3-nitrobenzoate (50)



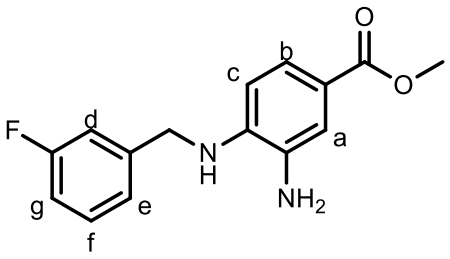
3-Fluorobenzylamine (7.53 mmol, 0.86 mL) was added in one portion to a stirred solution of **56** (5.00 mmol, 1.00 g) and diisopropylethylamine (10.00 mmol, 1.74 mL) in acetonitrile (50.0 mL). The reaction mixture was refluxed for 2 hours; cooled down to room temperature and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate (40 mL) and water (15 mL). The organic layer was washed with water (3x15 mL) and saturated sodium chloride solution (15 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **50** as a yellow solid. Yield 1.57 g (97 %).

^{1}H-NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 9.09 (t, 1H, $^3J_{NH,CH2}$ = 6.2 Hz, ArN$\underline{H}$), 8.60 (d, 1H, $^4J_{a,b}$ = 2.0 Hz, H_a), 7.86 (dd, 1H, $^3J_{b,c}$ = 8.8 Hz, $^4J_{b,a}$ = 1.6 Hz, H_b), 7.40 – 7.34 (m, 1H, H_f), 7.22 – 7.18 (m, 2H, H_d, H_e), 7.09 – 7.04 (m, 1H, H_g), 6.94 (d, 1H, $^3J_{c,b}$ = 9.1 Hz, H_c), 4.70 (d, 2 H, $^3J_{CH2,NH}$ = 6.4 Hz, Ar$C\underline{H}_2$), 3.79 (s, 3H, O$C\underline{H}_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.7 ($\underline{C}O_2R$), 162.3 (d, 1C, $^1J_{CF}$ = 242 Hz, $\underline{C}_{Ar}F$), 147.1 ($\underline{C}_{Ar}NH$), 141.0 (d, 1C, $^3J_{CF}$ = 7.1 Hz, $\underline{C}_{Ar}CH_2$), 135.5 (C_b), 130.9 ($\underline{C}_{Ar}NO_2$), 130.5 (d, 1C, $^3J_{CF}$ = 8.3 Hz, C_f), 128.3 (C_a), 122.8 (d, 1C, $^4J_{CF}$ = 2.6 Hz, C_e), 116.1 ($\underline{C}_{Ar}CO_2R$), 115.0 (C_c), 113.8 (d, 1C, $^2J_{CF}$ = 20.8 Hz, C_g), 113.6 (d, 1C, $^2J_{CF}$ = 21.7 Hz, C_d), 51.9 ($O\underline{C}H_3$), 45.2 (d, 1C, $^4J_{CF}$ = 1.5 Hz, Ar$\underline{C}H_2$).

HRMS calculated for $C_{15}H_{13}FN_2O_4$ 304.0859. Found 304.0854.

Methyl 3-amino-4-((3-fluorobenzyl)amino)benzoate (51)



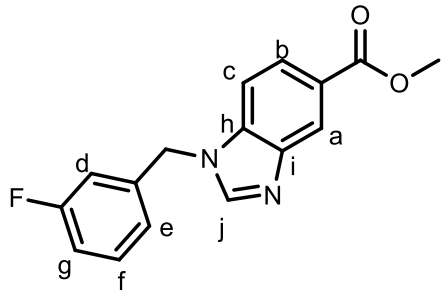
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.16 g) was added to a stirred suspension of **50** (5.16 mmol, 1.57 g) in ethanol (50 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 2.5 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The solid residue was suspended in ether and filtered to give **51** as off-white solid. Yield 0.93 g (66 %).

^{1}H-NMR (400 MHz, d_6-DMSO, 298 K) δ [ppm] = 7.39 – 7.33 (m, 1H, H_f) 7.22 (d, 1H, $^{3}J_{a,b}$ = 2.0 Hz, H_a), 7.21 – 7.14 (m, 2H, H_g, H_e), 7.12 (dd, 1H, $^{3}J_{b,c}$ = 8.4 Hz, $^{4}J_{b,a}$ = 2.0 Hz, H_b), 7.07 – 7.02 (m, 1H, H_d), 6.34 (d, 1H, $^{3}J_{c,b}$ = 8.4 Hz, H_c), 5.98 (t, 1H, $^{3}J_{NH,CH2}$ = 6.0 Hz, ArN<u>H</u>), 4.84 (s, 2H, ArN<u>H</u>$_2$), 4.41 (d, 2H, $^{3}J_{CH2,NH}$ = 6.0 Hz, ArC<u>H</u>$_2$), 3.71 (s, 3H, OC<u>H</u>$_3$).

^{13}C-NMR (100 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.5 (<u>C</u>O$_2$R), 162.3 (d, 1C, $^{1}J_{CF}$ = 242 Hz, <u>C</u>$_{Ar}$F), 142.9 (d, 1C, $^{3}J_{CF}$ = 6.7 Hz, <u>C</u>$_{Ar}$CH$_2$), 139.8 (<u>C</u>$_{Ar}$NH), 134.4 (<u>C</u>$_{Ar}$NH$_2$), 130.2 (d, 1C, $^{3}J_{CF}$ = 8.2 Hz, C_f), 123.0 (d, 1C, $^{4}J_{CF}$ = 2.7 Hz, C_e), 120.3 (C_b), 117.2 (<u>C</u>$_{Ar}$CO$_2$R), 114.3 (C_a), 113.6 (d, 1C, $^{2}J_{CF}$ = 21.4 Hz, C_g), 113.4 (d, 1C, $^{2}J_{CF}$ = 20.8 Hz, C_d), 108.5 (C_c), 51.0 (O<u>C</u>H$_3$), 45.6 (d, 1C, $^{4}J_{CF}$ = 1.6 Hz, Ar<u>C</u>H$_2$).

HRMS calculated for $C_{15}H_{15}FN_2O_2$ 274.1117. Found 274.1112.

Methyl 1-(3-fluorobenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylate (52)



A stirred suspension of **51** (3.10 mmol, 0.85 g) in formic acid (8.0 mL) was refluxed for 1.5 hours at 110°C. The reaction mixture was cooled to 0°C and neutralised with saturated sodium hydrogen carbonate solution (265 mL). The reaction mixture was

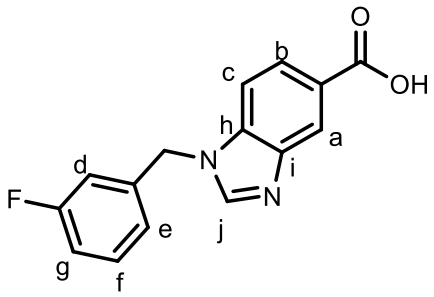
extracted with ethyl acetate (3x40 mL) and the organic layer was washed with water (3x40 mL) and saturated sodium chloride solution (40 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **52** as off-white solid. Yield 0.86 g (98 %).

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.60 (s, 1H, H_j), 8.27 (d, 1H, $^4J_{a,b}$ = 1.2 Hz, H_a), 7.86 (dd, 1H, $^3J_{b,c}$ = 8.5 Hz, $^4J_{b,a}$ = 1.5 Hz, H_b), 7.66 (d, 1H, $^3J_{c,b}$ = 8.5 Hz, H_c), 7.42 – 7.34 (m, 1 H, H_f), 7.23 – 7.08 (m, 3 H, H_d, H_g, H_e), 5.58 (s, 2 H, ArC$\underline{H}_2$), 3.85 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 166.5 (CO_2R), 162.1 (d, 1C, $^1J_{CF}$ = 243 Hz, $\underline{C}_{Ar}F$), 146.1 (C_j), 143.1 (C_h), 139.3 (d, 1C, $^3J_{CF}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2$), 136.8 (C_i), 130.8 (d, 1C, $^3J_{CF}$ = 8.3 Hz, C_f), 123.5 (C_b), 123.4 (d, 1C, $^4J_{CF}$ = 2.7 Hz, C_e), 123.4 ($\underline{C}_{Ar}CO_2R$), 121.2 (C_a), 114.7 (d, 1C, $^2J_{CF}$ = 20.8 Hz, C_g), 114.3 (d, 1C, $^2J_{CF}$ = 21.8 Hz, C_d), 110.7 (C_c), 51.9 (O$\underline{C}H_3$), 47.1 (d, 1C, $^4J_{CF}$ = 1.8 Hz, Ar$\underline{C}H_2$).

HRMS calculated for $C_{16}H_{13}FN_2O_2$ 284.0956. Found 284.0961.

1-(3-Fluorobenzyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid (15)



Lithium hydroxide (16.2 mmol, 0.68 g) was added to a stirred suspension of **52** (3.24 mmol, 0.92 g) in tetrahydrofuran (22 mL) and water (11 mL) at room temperature. The reaction mixture was refluxed for 3 hours at 60°C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (14 mL). The reaction mixture was washed with ethyl acetate (10 mL). Hydrochloric acid (8.4 mL, 2M) was added to the aqueous phase and the resulting precipitate was filtered to give **15** as off-white solid. Yield 0.61 g (70 %).

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 12.8 (s, 1H, $CO_2\underline{H}$), 8.60 (s, 1H, H_j), 8.27 (d, 1H, $^4J_{a,b}$ = 1.2 Hz, H_a), 7.86 (dd, 1H, $^3J_{b,c}$ = 8.4 Hz, $^4J_{b,a}$ = 1.5 Hz, H_b), 7.64 (d, 1H,

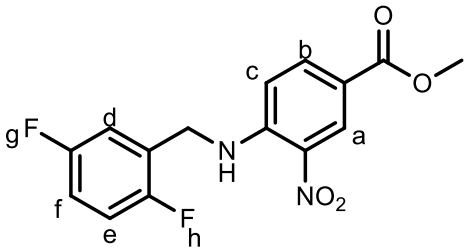
$^{3}J_{c,b}$ = 8.4 Hz, H_c), 7.42 – 7.34 (m, 1 H, H_f), 7.23 – 7.08 (m, 3 H, H_d, H_g, H_e), 5.58 (s, 2 H, ArC$\underline{H}_2$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 167.6 ($\underline{C}O_2H$), 162.1 (d, 1C, $^{1}J_{CF}$ = 243 Hz, $C_{Ar}F$), 146.1 (C_j), 142.9 (C_h), 139.3 (d, 1C, $^{3}J_{CF}$ = 7.2 Hz, $\underline{C}_{Ar}CH_2$), 136.6 (C_i), 130.8 (d, 1C, $^{3}J_{CF}$ = 8.3 Hz, C_f), 124.6 ($\underline{C}_{Ar}CO_2H$), 123.8 (C_b), 123.4 (d, 1C, $^{4}J_{CF}$ = 2.8 Hz, C_e), 121.2 (C_a), 114.7 (d, 1C, $^{2}J_{CF}$ = 20.8 Hz, C_g), 114.3 (d, 1C, $^{2}J_{CF}$ = 21.8 Hz, C_d), 110.6 (C_c), 47.2 (d, 1C, $^{4}J_{CF}$ = 1.8 Hz, Ar$\underline{C}H_2$).

HRMS calculated for $C_{15}H_{11}FN_2O_2$ 270.0805. Found 270.0805.

7.13. wALADin16

Methyl 4-((2,5-difluorobenzyl)amino)-3-nitrobenzoate (53)



2,5-Difluorobenzylamine (7.53 mmol, 0.88 ml) was added in one portion to a stirred solution of **56** (5.00 mmol, 1.00 g) and diisopropylethylamine (10.00 mmol, 1.74 ml) in acetonitrile (50.0 ml). The reaction mixture was refluxed for 2 hours; cooled down to room temperature and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate (40 ml) and water (15 ml). The organic layer was washed with water (3 x 15 ml) and saturated sodium chloride solution (15 ml). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give **53** as yellow solid. Yield 1.66 g (97 %).

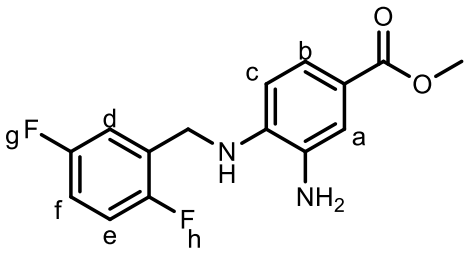
^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 8.97 (t, 1H, $^{3}J_{NH,CH2}$ = 6.3 Hz, ArN$\underline{H}$), 8.62 (d, 1H, $^{3}J_{a,b}$ = 2.1 Hz, H_a), 7.91 (dd, 1H, $^{3}J_{b,c}$ = 9.0 Hz, $^{4}J_{b,a}$ = 1.8 Hz, H_b), 7.33 - 7.25 (m, 1H, H_e,), 7.22 – 7.11 (m, 2 H, H_f, H_d), 6.96 (d, 1H, $^{3}J_{c,b}$ = 9.0 Hz, H_c), 4.73 (d, 2 H, $^{3}J_{CH2,NH}$ = 6.3 Hz, ArC$\underline{H}_2$), 3.81 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.7 ($\underline{C}O_2R$), 158.2 (dd, 1C, $^{1}J_{CF}$ = 238 Hz, $^{4}J_{CF}$ = 2.0 Hz, $\underline{C}_{Ar}F_g$), 156.1 (dd, 1C, $^{1}J_{CF}$ = 239 Hz, $^{4}J_{CF}$ = 2.0 Hz, $\underline{C}_{Ar}F_h$), 146.9 ($\underline{C}_{Ar}NH$), 131.2 ($C_{Ar}NO_2$), 135.6 (C_b), 128.2 (C_a), 126.8 (dd, 1C, $^{2}J_{CF}$ = 17 Hz, $^{3}J_{CF}$ = 7.5 Hz,

$\underline{C}_{Ar}CH_2$), 116.9 (dd, 1C, $^2J_{CF}$ = 24 Hz, $^3J_{CF}$ = 8.8 Hz, C_e), 116.4 ($\underline{C}_{Ar}CO_2R$), 114.8 – 115.6 (m, 2C, C_f, C_d), 114.5 (C_c), 51.9 ($O\underline{C}H_3$), 39.9 – 39.6 (m, 1C, $Ar\underline{C}H_2$).

HRMS calculated for $C_{15}H_{12}F_2N_2O_4$ 322.0765. Found 322.0759

Methyl 3-amino-4-((2,5-difluorobenzyl)amino)benzoate (54)



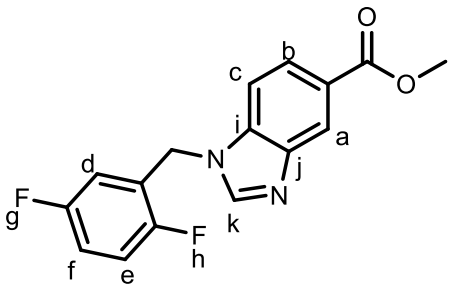
Palladium on activated carbon (5 % Pd) (wt 10 %, 0.17 g) was added to a stirred suspension of **53** (5.15 mmol, 1.66 g) in ethanol (50 ml) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the suspension for 2.5 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure. The solid residue was suspended in ether and filtered to give **54** as off-white solid. Yield 1.00 g (66 %)

^{1}H-NMR (300 MHz, d_6-DMSO, 298 K) δ [ppm] = 7.30 – 7.22 (m, 1H, H_e), 7.23 (d, 1H, $^3J_{a,b}$ = 2.1 Hz, H_a), 7.17 – 7.10 (m, 2H, H_f, H_d), 7.15 (dd, 1H, $^3J_{b,c}$ = 8.1 Hz, $^4J_{b,a}$ = 2.1 Hz, H_b), 6.37 (d, 1H, $^3J_{c,b}$ = 8.1 Hz, H_c), 5.87 (t, 1H, $^3J_{NH,CH2}$ = 6.0 Hz, $ArN\underline{H}$), 4.86 (s, 2H, $ArN\underline{H}_2$), 4.42 (d, 2H, $^3J_{CH2,NH}$ = 6.0 Hz, $ArC\underline{H}_2$), 3.72 (s, 3H, $OC\underline{H}_3$).

^{13}C-NMR (75 MHz, d_6-DMSO, 298 K) δ [ppm] = 164.7 ($\underline{C}O_2R$), 158.2 (dd, 1C, $^1J_{CF}$ = 238 Hz, $^4J_{CF}$ = 2.0 Hz, $\underline{C}_{Ar}F_g$), 156.3 (dd, 1C, $^1J_{CF}$ = 238 Hz, $^4J_{CF}$ = 2.0 Hz, $\underline{C}_{Ar}F_h$), 139.5 ($\underline{C}_{Ar}NH$), 134.5 ($\underline{C}_{Ar}NH_2$), 128.4 (dd, 1C, $^2J_{CF}$ = 17 Hz, $^3J_{CF}$ = 7.3 Hz, $\underline{C}_{Ar}CH_2$), 120.3 (C_b), 117.5 ($\underline{C}_{Ar}CO_2R$), 116.6 (dd, 1C, $^2J_{CF}$ = 24 Hz, $^3J_{CF}$ = 8.7 Hz, C_e), 114.7 – 115.2 (m, 2C, C_f, C_d), 114.4 (C_a), 108.3 (C_c), 51.1 ($O\underline{C}H_3$), 39.8 ($Ar\underline{C}H_2$).

HRMS calculated for $C_{15}H_{14}F_2N_2O_2$ 292.1023. Found 292.1018

Methyl 1-(2,5-difluorobenzyl)-1H-benzo[d]imidazole-5-carboxylate (55)

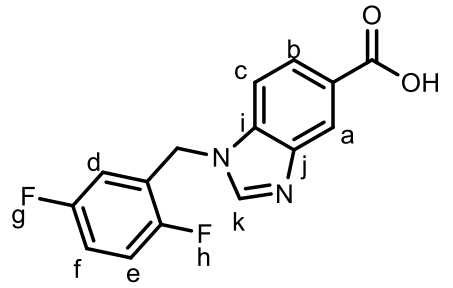


A stirred suspension of **54** (2.98 mmol, 0.87 g) in formic acid (7.45 mL) was refluxed for 30 minutes at 110 °C. The reaction mixture was cooled to 0 °C and neutralized with sodium hydrogen carbonate (150 mL). The reaction mixture was extracted with ethyl acetate (3 x 50 mL) and the organic layer was washed with water (3 x 50 mL) and sodium chloride (1 x 50 mL). The organic layer was dried (magnesium sulfate) and concentrated under reduced pressure to give **55** as off-white solid. Yield 0.83 g (92 %).

^{1}H-NMR (400 MHz, DMSO-d6, 298 K) δ [ppm] = 8.51 (s, 1H, H_k); 8.26 (dd, 1H, $^{5}J_{a,c}$ = 0.5 Hz, $^{4}J_{a,b}$ = 1.5 Hz, H_a); 7.87 (dd, 1H, $^{4}J_{b,a}$ = 1.6 Hz, $^{3}J_{b,c}$ = 8.5 Hz, H_b), 7.67 (d, 1H, $^{3}J_{c,b}$ = 8 Hz, H_c), 7.18 - 7.32 (m, 3H, H_d, H_f, H_e), 5.60 (s, 2H, ArC$\underline{H}_2$), 3.84 (s, 3H, OC$\underline{H}_3$).

^{13}C-NMR (100 MHz, DMSO-d6, 298 K) δ [ppm] = 166.6 ($\underline{C}O_2R$), 159.3 ($^{1}J_{C,F}$ = 241 Hz, $^{4}J_{C,F}$ = 3.5 Hz, $\underline{C}_{Ar}F_g$), 157.6 ($^{1}J_{C,F}$ = 243 Hz, $^{4}J_{C,F}$ = 2.9 Hz, $\underline{C}_{Ar}F_h$), 146.5 (C_k), 143.0 (C_i), 136.8 (C_j), 125.3 ($\underline{C}_{Ar}CH_2$), 123.7 (C_b), 123.5 ($\underline{C}_{Ar}CO_2R$), 121.3 (C_a), 117.5 (C_f), 117.2 (C_e), 116.9 (C_d), 110.6 (C_c), 52.0 (O$\underline{C}H_3$), 42.3 (Ar$\underline{C}H_2$).

1-(2,5-Difluorobenzyl)-1H-benzo[d]imidazole-5-carboxylic acid (16)



Lithium hydroxide (15.55 mmol, 0.65 g) was added to a suspension of **55** (2.75 mmol, 0.83 g) in tetrahydrofuran and water (2:1, 39 mL) at room temperature. The reaction mixture was refluxed for 3 hours at 60 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (15 mL). The reaction mixture was washed with ethyl acetate (1 x 15 mL). Hydrochloric acid (2 M, 10 mL) was added to

the aqueous phase and the resulting precipitate was filtered to give **16** as off-white solid. Yield 0.65 g (72 %).

^{1}H-NMR (400 MHz, DMSO-d6, 298 K) δ [ppm] = 9.78 (s, 1H, H_k); 8.37 (s, H_a); 8.1 (dd, 1H, $^{4}J_{b,a}$ = 1.6 Hz, $^{3}J_{b,c}$ = 8.4 Hz, H_b), 7.93 (d, 1H, $^{3}J_{c,b}$ = 11.5 Hz, H_c), 7.22 - 7.55 (m, 3H, H_d, H_f, H_e), 5.84 (s, 2H, ArC$\underline{H}_2$).

^{13}C-NMR (100 MHz, DMSO-d6, 298 K) δ [ppm] = 166.5 ($\underline{C}O_2H$), 159.7 ($^{1}J_{C,F}$ = 241.3 Hz, $^{4}J_{C,F}$ = 2.2 Hz, $\underline{C}_{Ar}F_g$), 156.5 ($^{1}J_{C,F}$ = 242.9 Hz, $^{4}J_{C,F}$ = 3.3 Hz, $C_{Ar}F_h$), 144.6 (C_k), 134.1 (C_i), 133.2 ($\underline{C}_{Ar}CO_2H$),128.3 (C_j), 126.4 (C_b), 121.3 ($\underline{C}_{Ar}CH_2$), 117.8 (C_a), 117.7 (C_f), 117.5 (C_e), 117.1 (C_d), 112.8 (C_c), 47.7 (Ar$\underline{C}H_2$).

HRMS calculated for $C_{15}H_{10}F_2N_2O_2$ - H 287.0632. Found 287.0638.

7.14. Enzymatic Assays

All enzymatic assays, except for the ones listed below, were carried out by Dr. Christian Lentz at the Institute of Medical Microbiology, Immunology and Parasitology (IMMIP).[2]

Protein-buffer mix

	Final concentration
Tris, pH 8	100 mM
MgCl2	1 mM
DTT	5 mM
wALAD	500 µM

Modified Ehrlich's Reagent

DMAB	0,4 g
Acetic acid	17,1 mL
Perchloric acid	4,9 mL
Trichloroacetic acid (12 %)	3 mL

wALAD inhibition assay

2 µL compound dissolved in DMSO (final concentration row 0.26 µM – 533 µM) was pipetted to 23 µL protein-buffer mix and incubated for 30 minutes. 5 µL 5-aminolevulinic acid (200 µM final concentration) dissolved in water was added and the assay plates were sealed, spun down and incubated at 37 °C for 20 minutes. The

reaction was stopped by addition of 200 µL modified Ehrlich's Reagent and incubated for 10 minutes at room temperature. The absorption was read at 555 nm.

Compound	IC_{50} [µM]
wALADin13	168
wALADin14	217
wALADin15	373
wALADin16	703

B Synthesis and characterisation of novel ABAL probes

1. Abstract

Aptamers are single stranded oligonucleotides, which have gained increasing importance in biotechnological and therapeutic applications. They offer molecular recognition properties that rival those of the commonly used antibodies.[78]

In aptamer-based affinity labelling (ABAL) aptamers are labelled with photoreactive probes and can then be cross-linked to their target protein in a UV light-dependent and highly specific manner.[3] This method enables the identification of unknown target proteins of aptamers which emerge from selection processes carried out against complex target structures. For the successful use of this method it is crucial that cross-linking and isolation of the protein results in a pure sample for mass spectrometry analysis. The originally used ABAL probe, sulfo-SBED consists of a lysine core structure with biotin as a purification tag and phenyl azide (PA) as a moiety for UV induced crosslinking. The sulfo-SBED-modified aptamer failed to deliver a pure sample of the target protein from cell lysate.[3] Contaminations are most likely a result of co-eluted endogenously biotinylated proteins and unspecifically cross-linked proteins.

To improve the ABAL procedure novel ABAL probes were developed. These probes either carry desthiobiotin or biotin in combination with a chemically cleavable linker as purification tags to enable mild elution of the captured complex. The resulting new elution conditions should significantly reduce the amount of co-eluted endogenously biotinylated proteins.

Besides PA which is used as a reference, 1,2,4,5-tetrafluorophenyl-3-azide (TPA) and 3-phenyl-3-(trifluoromethyl)-3H-diazirine (TPD) are used as photoreactive moieties in the novel ABAL probes. TPA and TPD are more stable than PA and have a lower tendency to undergo side reactions.[79] This reduces the possibility of unspecifically cross-linked protein. These improvements combined in the novel ABAL probes, have a high potential to eliminate contamination of the resulting samples.

This part of the thesis describes the development and synthesis of the novel ABAL probes which might help to tap the full potential of the ABAL procedure and develop it into a standard procedure for identifying unknown target proteins.

2. Zusammenfassung

Aptamere sind einzelsträngige Oligonukleotide, die vermehrt in biotechnologischen und therapeutischen Anwendungen Verwendung finden. Sie zeigen dabei molekulare Erkennungseigenschaften, die denen von Antikörpern in nichts nachstehen.[78]

Bei der aptamer-basierten Affinitätsmarkierung werden Aptamere mit photoreaktiven Sonden markiert und dann gezielt durch UV-Bestrahlung mit ihren Zielproteinen vernetzt.[3] Diese Methode macht eine Identifizierung von unbekannten Zielproteinen von Aptameren, die gegen komplexe Zielstrukturen selektiert wurden, möglich. Für die erfolgreiche Anwendung dieser Methode ist es wichtig, dass die aus der Vernetzung und anschließenden Isolierung erhaltenen Proben rein sind, damit eine massenspektrometrische Analyse erfolgen kann. Die ursprünglich verwendete ABAL-Sonde, sulfo-SBED, baut auf einer Lysin-Struktur auf, die Biotin zur Aufreinigung und Phenylazid (PA) zur Vernetzung enthält. Mit den sulfo-SBED-modifizierten Aptameren konnten bislang keine reinen Proben aus Zelllysaten erhalten werden, da Verunreinigungen, höchstwahrscheinlich verursacht durch endogen biotinylierte und unspezifisch vernetzte Proteine, vorliegen.[3]

Um die Ergebnisse der ABAL-Strategie zu verbessern, wurden neue ABAL-Sonden entwickelt. Diese Sonden enthalten zur Aufreinigung entweder Desthiobiotin oder Biotin in Verbindung mit einem chemisch spaltbaren Linker, um die milde Elution des eingefangenen Komplexes zu ermöglichen. Die daraus resultierenden neuen Elutionsbedingungen sollten die Menge an co-eluierten endogen biotinylierten Proteinen deutlich verringern.

Neben PA, das als Referenz verwendet wird, werden auch 1,2,4,5-tetrafluorophenyl-3-azide (TPA) und 3-phenyl-3-(trifluoromethyl)-3H-diazirine (TPD)als photoreaktive Gruppen in den neuen ABAL-Sonden verwendet. TPA und TPD sind stabiler als PA und haben eine geringere Tendenz Nebenreaktionen einzugehen, was die Wahrscheinlichkeit von unspezifisch vernetzten Proteinen reduziert.[79] Die Kombination dieser Verbesserungen in den neuen ABAL-Sonden hat das Potenzial Verunreinigungen in den resultierenden Proben zu eliminieren.

Dieser Teil der Arbeit beschreibt die Entwicklung und Synthese dieser neuen ABAL-Sonden, die möglicherweise helfen können das volle Potenzial des ABAL-Verfahrens zu entfalten und es zu einer Standardanwendung zur Identifizierung von unbekannten Zielproteinen weiterzuentwickeln.

3. Introduction

3.1. Aptamers

Aptamers are single stranded oligonucleotides, which are known for their ability to fold into complex secondary structures. These structures form binding pockets for specific recognition and tight binding to a variety of targets. A resulting effect can be the blockage of single protein domains affecting protein functions. Aptamers have the ability to bind ligand molecules with high affinity and specificity, which is a result of non-covalent interactions such as van der Waals forces, hydrogen bonds, aromatic ring stacking and electrostatic forces.[80] For this reason, dissociation constants of aptamer-ligand complexes can reach the picomolar range.[81]

Aptamers are obtained by systematic evolution of ligands by exponential enrichment (SELEX).[82] In this process aptamers are derived from combinatorial libraries of about 10^{13} - 10^{15} different sequences according to their specific binding affinity. Sequences can be entirely random, depending on the complexity of the library, except for their constant flanking regions at the 3'- and 5'-end. These flanking regions serve as primers for subsequent amplification. DNA and RNA libraries can be chemically synthesised using standard oligonucleotide synthesisers. Commonly, RNA libraries are obtained by *in vitro* transcription of the corresponding DNA sequences.

At the beginning of the SELEX procedure (**Figure 10**) the library is exposed to the desired target immobilised on a solid support usually in the form of activated agarose or coated magnetic beads. Unbound sequences are then removed by increasingly stringent washing steps. The bound sequences are isolated and amplified to prepare for the next round of selection. DNA sequences are amplified by polymerase chain reaction (PCR) and RNA sequences by reverse transcription PCR with subsequent *in vitro* transcription of the obtained complementary DNA. Multiple rounds of selection with increasing selection pressure and amplification lead to a decrease of library diversity and enrichment of the best binding ligands. The cycle of repeating rounds is stopped when no further change of affinity can be observed. This indicates the completion of the SELEX procedure. Subsequent to the last round the enriched library is cloned and sequenced to obtain the individual sequence of each member. These sequences are then analysed by grouping them into families according to homologies in their random region and finally,

testing the presumed aptamers for binding affinity and specificity.[83] Due to this procedure aptamers of high binding affinity to a variety of targets have been developed.

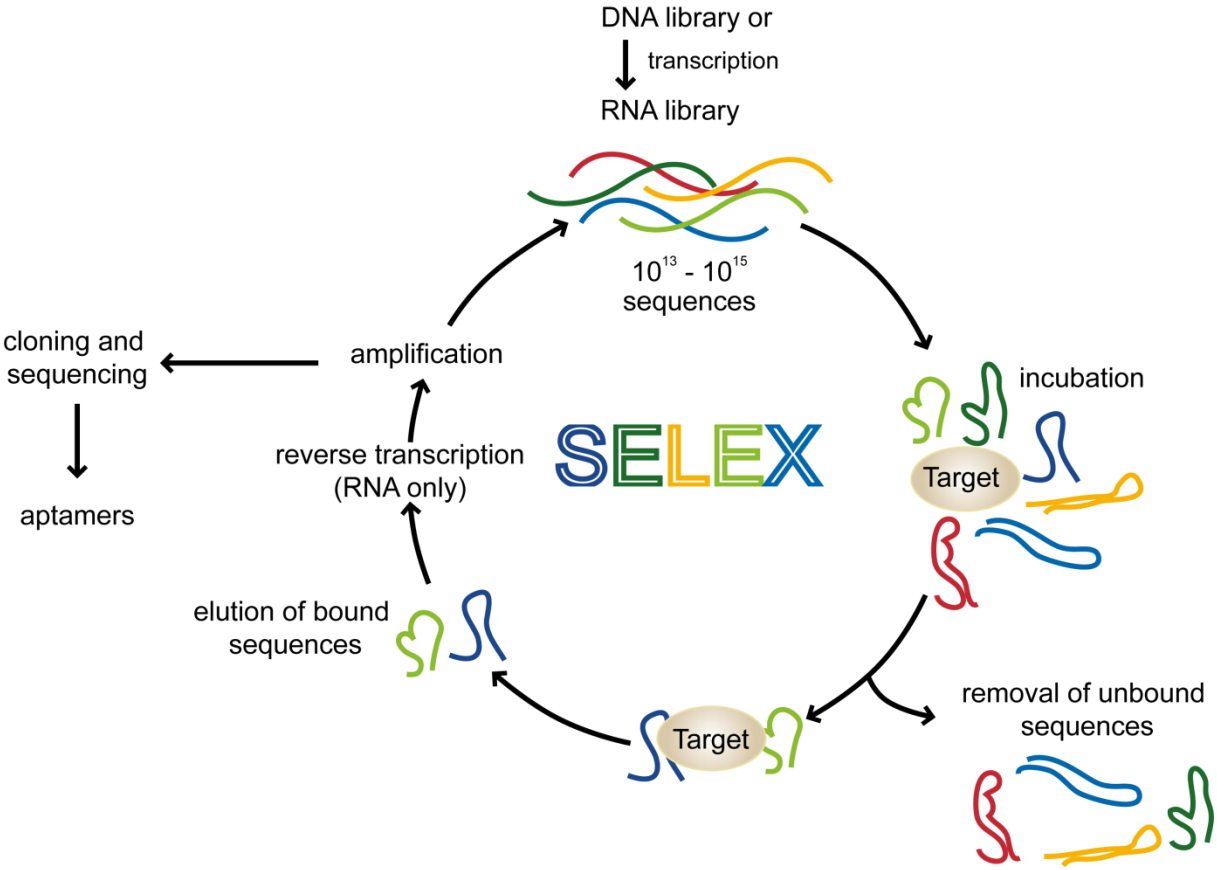


Figure 10: Schematic diagram of systematic evolution of ligands by exponential enrichment (SELEX).[82]

These targets are metal ions, small molecules, peptides, proteins, viruses, bacteria, and even whole cells,[84] tissue samples[85] and living organisms.[86] Automated platforms are new technologies like that enable simultaneous selection of aptamers to multiple proteins in a high throughput manner and therefore make aptamers easily obtained and valuable tools.[87]

Aptamers were first described in literature in the in the early 1990s. Since then they have gained more and more importance as alternatives to long established antibody-based diagnostics as well as biotechnological products for research, diagnostics, and therapy. This is amongst others a result of their comparable molecular recognition properties to those of antibodies. Moreover, antibodies have disadvantages compared to aptamers.[88] Antibodies are initially generated within animals, therefore generating antibodies against molecules, which negatively affect the living organism, is difficult. Subsequently, in cell culture, identifying and producing rare antibodies for a specific target requires screening of a large number of colonies, which can make the process

expensive and time consuming. When cell culture is applied common problems like accidental loss or death of cell lines is an issue. Antibodies have the tendency of varying performance from batch to batch, which makes optimisation of immunoassays necessary for each batch. The identification of antibodies is restricted by *in vivo* parameters and therefore recognition of targets can only be carried out under physiological conditions and identified antibodies cannot be modified on demand. Antibodies have a limited shelf-life and denature irreversibly due to their temperature sensitivity.

Aptamers on the other hand are obtained by SELEX, which is an *in vitro* process. Therefore, SELEX conditions can be altered to obtain aptamers with fitting properties and under suitable conditions, for example, for *in vitro* diagnostics. The aptamer sequences are chemically synthesised and can be purified to a very high degree. That is why they hardly show any batch-to-batch variation. Additionally, aptamers can be chemically modified by attaching reporter molecules or functional groups at precise locations during the synthesis. Aptamers are suitable for long-term storage and are temperature stable. When denatured they can be readily regenerated.[89]

Aptamers can be easily chemically modified by introduction of altered nucleotides (**Figure 11**). This can either be accomplished through enzymatic incorporation or chemical synthesis. Modifications on the ribose ring of RNA aptamers can drastically improve their properties. For example, the modifying of the 2'-position of the ribose ring with a fluoro, amino or methoxy group can enhance the serum stability from less than one second to more than 81 hours.[90]

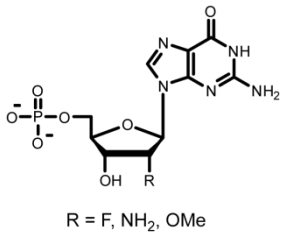
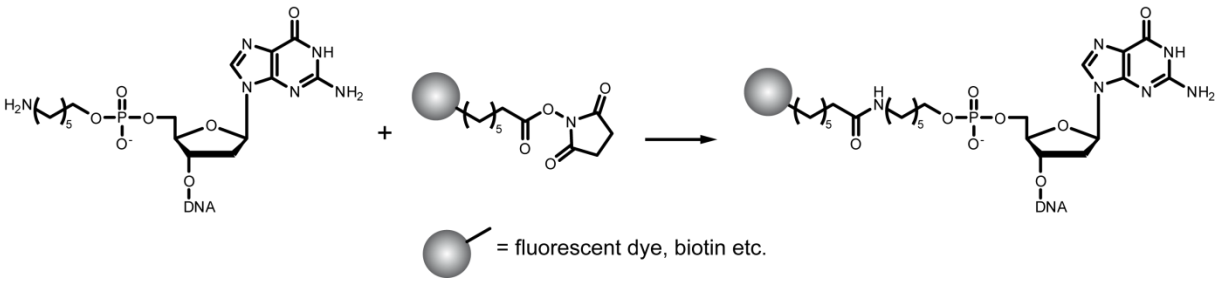


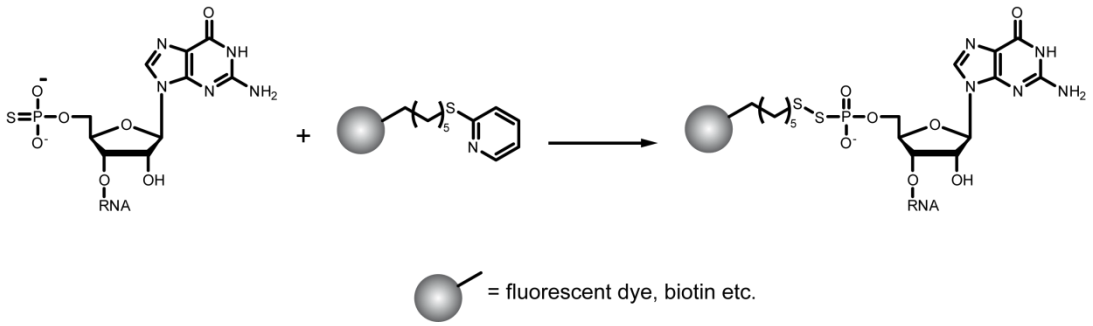
Figure 11: 2'-modification of the ribose ring.[90]

Apart from stabilising modifications, aptamers can also be altered to covalently bind to functional molecules such as fluorescent dyes or biotin. Therefore aptamers are modified with aminohexyl or thiohexyl phosphoramidites at their 3'- or 5'-end during synthesis.[91] Molecules carrying the corresponding functionalities can then be attached post-synthetically (**Scheme 12**).



Scheme 12: Functionalisation of 5'-aminohexyl modified DNA.[91]

Another method to modify RNA aptamers is the incorporation of guanosine-monophosphorothioate (GMPS) at the 5'-end. GMPS is incorporated by T7-transcription of the complementary double stranded DNA. The obtained GMPS-primed RNA can then be functionalised with the desired molecule by disulphide-bond formation (**Scheme 13**).[92]



Scheme 13: Functionalisation of GMPS-primed RNA.[92]

Besides the use for *in vitro* applications, aptamers can also be applied in extra- and intracellular applications. In extracellular applications, cell-surface proteins, which are the main route of communication between cells and their external environment, are targeted. Alterations in cell-surface proteins can be directly linked to a large number of human diseases and therefore these proteins deliver an excellent target for disease diagnoses, therapeutics and prognosis.[93] Aptamers selected for purified, soluble cell-surface proteins often show little or no binding under physiological conditions. Reasons for this can be that the binding domain of the protein is masked or the protein might only be functional when co-presented with other cell-surface components. One way to overcome these problems is a SELEX procedure using cell membrane preparations containing the desired protein as a target.[94] This complex target SELEX often requires significantly more cycles to obtain enrichment of the best binding sequences, but is generally conducted similarly as for *in vitro* selection.

Another way is to use whole living cells as targets in SELEX.[95] Most commonly for this cell-SELEX procedure, the desired protein receptor is over-expressed on unrelated cells and these cells are then incubated with the RNA or DNA library. Unbound targets can be easily washed off and the surface-bound sequences are eluted by using heat, EDTA or, in the case of RNA, Triazol. With these sequences the SELEX procedure is continued as described for the *in vitro* SELEX. A major advantage of cell-SELEX is the capability to obtain aptamers against diseased or differentiated cells without prior knowledge of the responsible target protein. Aptamers obtained this way are valuable tools for specific recognition and sorting of diseased cells.

However, when targeting a specific protein, co-selection of aptamers targeting other proteins present on the cell surface has to be prevented. Hence, SELEX procedures targeting membrane preparations or whole cells need to contain additional counter-selection steps. During these steps the binding sequences are incubated with membrane preparations or cells that are similar to the initial ones, but do not contain the desired target. Binding sequences are removed and the original SELEX procedure is continued.

A problem of cell-SELEX is that dead cells, which occur during the selection procedure, non-specifically bind to nucleic acids. This leads to the enrichment of false-positive aptamers. To overcome this problem a digital high-speed fluorescence-activated cell sorter (FACS) can be used to separate viable and dead cells, after incubation with the library, based on their differential light-scattering characteristics.[84] The obtained viable cells are then processed as described.

In intracellular applications genes, proteins, metabolites and all the other molecules making up a cell are analysed to precisely understand how they interconnect and function in their diseased and normal states. This knowledge can help to develop new strategies in drug target discovery and novel therapeutic concepts.[96] Here too, aptamers have proved to be able to interrupt, modulate, or define the functions of a wide range of target proteins within cells. Although aptamers cannot readily cross cell membranes, it has been shown that they can be introduced either by intracellular expression,[97] lipofection,[98] microinjection[99] or by employing nanoparticles.[100] Within the cell these so called intramers maintain their ability to modulate the functions of their target protein and can be utilised for proteins in the cytoplasm as well as in the nucleus.[96]

Nevertheless, some applications afford molecules that can readily cross cell membranes, be applied in a spatio-temporally controlled fashion, act transiently and employ effects that are reversible. Chemical compounds or more precise drug-like small molecules often combine these properties and hence, have an advantage compared to aptamers.[54b] On the other hand, identification of small molecule inhibitors can be laborious since their screening requires methods that are specifically designed for the desired target and often screens deliver no positive result. Since aptamers can be obtained for a great variety of targets and conditions, aptamer-displacement assays were developed to identify small molecules with similar inhibitory properties as the corresponding aptamer. In these assays small molecule libraries are screened for compounds that are able to replace the bound aptamer and therefore identify small molecules that are effective and specific inhibitors.

3.2. Aptamer-based affinity labelling (ABAL)

As previously mentioned, Cell-SELEX can be used to obtain aptamers against diseased or differentiated cells without prior knowledge of the responsible target protein. In some cases this may be sufficient, for example, when identifying and sorting diseased cells, but if the selected aptamers are supposed to be developed into therapeutics, biomarkers or analytical devices, knowledge of the target protein is crucial.

An attempt to provide a method to identify unknown protein targets of aptamers was the 2012 developed aptamer-based affinity labelling (ABAL) which is based on the concept of affinity-based proteomic profiling (ABPP). ABPP uses active site-directed chemical probes that carry photoreactive groups for covalent binding after UV irradiation, to identify their target proteins within the proteome by mass spectrometry techniques. Other than in classical proteomic approaches, this method does not merely measure protein abundance but delivers information of protein functional sites.[101]

The principle of ABAL involves the covalent crosslinking of an aptamer, modified with a photoreactive probe, to a target protein by UV irradiation and subsequent purification and identification of the target protein. As depicted in **Figure 12**, the aptamer is modified with the ABAL probe containing an aryl azide group for covalent binding and biotin for purification. The modified aptamer is incubated with the target protein, either present in cell lysates, membrane preparations or on living cells and then cross-linked to the target protein by UV irradiation with a wavelength of 360 nm. Covalently bound

aptamer-protein complexes are then isolated by incubation with magnetic streptavidin coated beads, that bind to the biotin linker included in the photoreactive probe and subsequent elution of the purified complex.[3] Commonly, purified complexes are then digested with trypsin and analysed via LC-MS analysis.[102]

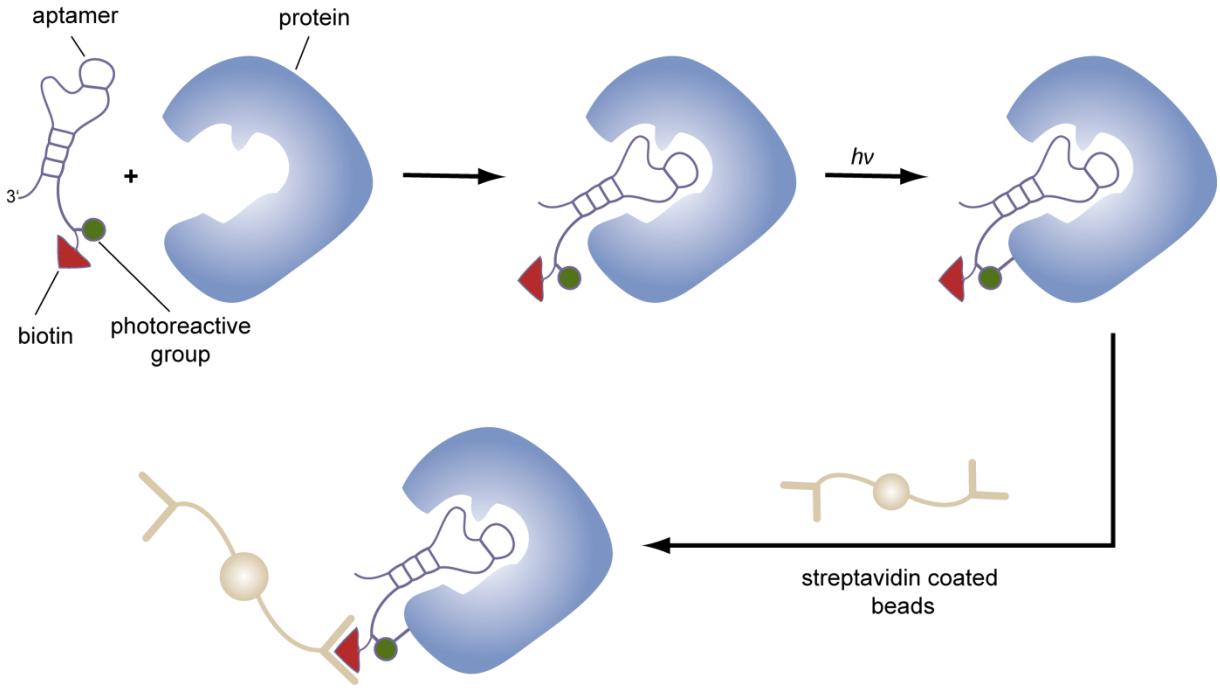


Figure 12: Schematic diagram of the aptamer-based affinity labelling (ABAL). The figure shows an adapted version of the figure found in reference[3].

To enable a wide applicability of the ABAL-procedure the aptamer is modified at its 5'-end with the photoreactive probe sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azidobenzamido)-hexanoamido) ethyl-1,3'-dithioproprionate (sulfo-SBED). This makes it possible to modify the aptamers in a post-SELEX procedure. The sulfo-SBED-moiety is a commercially available, heterobifunctional chemical cross-linker which was originally employed for label-transfer reactions to identify protein-protein interactions.[103] As shown in **Figure 13** sulfo-SBED molecules carry an aryl azide group on the one end and a sulfo-NHS-ester group on the other. The former is capable to covalently crosslink under UV irradiation to nearly any protein functional group while the latter binds to primary amines. The included disulphide bond provides a cleaving-site for bond-cleavage by reducing agents (**chapter B3.4**). Furthermore, sulfo-SBED also includes a biotin moiety, which has a remarkably high affinity to avidin and streptavidin

and therefore provides a good basis for enrichment of the aptamer-protein complexes with avidin or streptavidin matrices.[104]

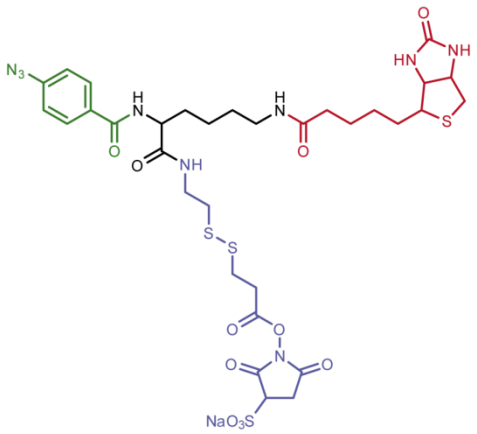


Figure 13: Structure of sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3'-dithioproprionate (Sulfo-SBED).

ABAL has been applied to different aptamer-protein complexes, consisting of aptamers with diverse secondary structures including hairpin and G-quadruplex structures, to reinforce the assumption of universal applicability. This combined with the applicability in highly complex biological samples such as cell lysates and membranes of living cells makes it also a potential method for identifying possible, additional targets (off-target effects) of aptamers selected to modulate protein functions within cells. These so called intramers modulate the biological function of the target protein and make it possible to assign biological answer to the modulated function of the addressed protein. The unambiguity of this assignment could then be verified by using ABAL to rule out off-target effects.

3.3. Photocrosslinking

In order to be able to identify the unknown binding proteins of aptamers, the aptamer-target complex needs to be isolated. Although the binding of the aptamer to the target protein is highly affine and tight, it is not covalent and consequently aptamer-protein complexes often dissociate under denaturing conditions.

Photoaffinity labelling (PAL) is a method to covalently crosslink proteins to their corresponding ligand for example small molecules, enzymes or other proteins[105] and forms the basis for both ABPP and ABAL. A key element for all these methods is the

photoreactive group which develops a reactive species upon irradiation at a certain wavelength that covalently binds the ligand to the target.

A suitable photoreactive group has to meet certain criteria: [106]
First of all the photoreactive group has to be stable in biological media and should not show light independent background reactivity. This prevents cross-reactivity or decomposition of the photoreactive group which would cause an increase in off-target labelling and a decrease in the desired complex concentration. Compared to the ligand it modifies, the photoreactive group should be less sterically demanding and the modification should have low to no influence on the biological activity of the ligand. Furthermore, the reactive species formed by irradiation with specific wavelengths has to be highly reactive and short-lived to be able to crosslink the target structure before dissociation of the complex. Otherwise this would as well result in off-target labelling.
Based on these criteria three types of photoreactive groups have emerged that are commonly used in PAL and ABPP: Benzophenones, aryl azide and aryl diazirines.

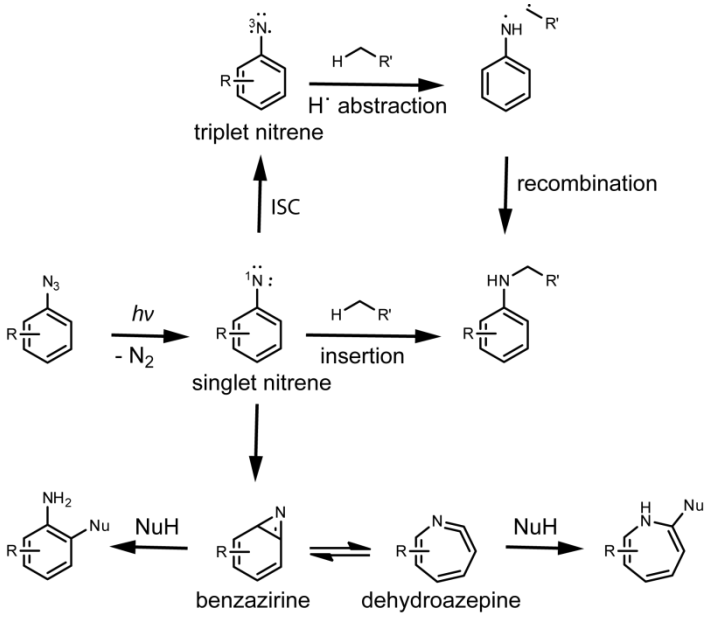
Aryl azides[79]

Irradiation of aryl azides with UV light below 300 nm results in elimination of molecular nitrogen and the formation of a highly energetic and reactive singlet aryl nitrene. Within 10 - 100 ps the singlet aryl nitrene undergoes rearrangement to form the benzazirine which can then form a dehydroazepine via ring expansion. At low temperatures or in methanol as a solvent the singlet nitrene converts into triplet nitrene by intersystem crossing. All four species can subsequently react with suitable functions by different mechanisms. Singlet nitrene reacts like an electrophile and therefore readily inserts into C-H (**Scheme 14**), N-H and O-H bonds. Triplet nitrene reacts in a diradical manner by abstracting hydrogen from an adjacent C-H followed by crosslinking to the produced carbon radical. Benzazirine and dehydroazepine are long-lived electrophiles and can only react with adjacent nucleophiles.

The major advantages of aryl azides are their comparatively small size and their easy preparation. Aryl azides are commonly prepared in a two-step synthesis starting from the corresponding aniline.

A drawback of aryl azides are the side-reactions that can occur when used in biological buffers. Both reactions, the aerobic oxidation of the triplet nitrene to the corresponding nitro species and the reduction of the initial azide to the amine by

dithiothreitol (DTT), reduce the concentration of the available crosslinking reagent and therefore the crosslinking yields.



Scheme 14: Chemistry of aryl azides after photolysis. The scheme shows an adapted version of the scheme found in reference[79].

Other disadvantages are the low absorption wavelengths, which can damage the biological system and the formed benzazirine and dehydroazepine, which cause off-target labelling due to their slow reaction time. These disadvantages can be easily overcome by using perfluorinated aryl azides. Introduction of electron-withdrawing groups causes a shift to higher maximum absorption wavelengths ranging from 300 nm to 460 nm which reduces damaging effects on biological systems. Electron-withdrawing groups on the aryl moiety also cause singlet nitrene to rearrange much slower which prevents off-target effects.[107]

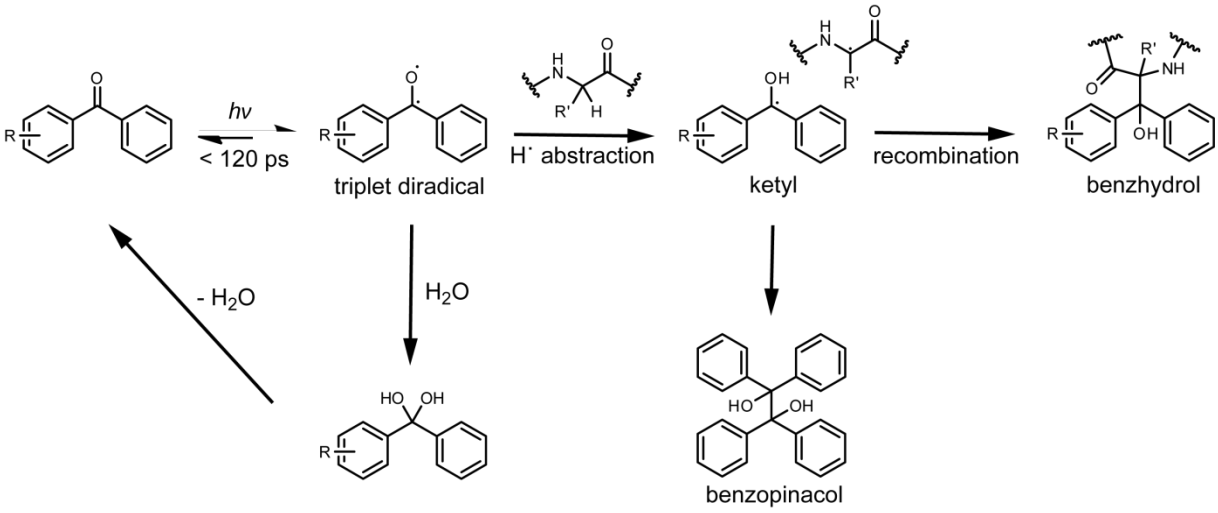
Benzophenones[79]

Excitation with UV light ranging from 350 - 360 nm causes an electron of the oxygen to promote from its non-binding sp^2-like n-orbital to an antibinding π*-orbital of the carbonyl carbon. Interaction of this so formed triplet diradical with weak C-H or O-H bonds is then possible due to the electrophilicity of the oxygen. This interaction results in the abstraction of the hydrogen and the subsequent crosslinking of the formed ketyl and alkyl radicals. The dimerisation of ketyl radicals to benzopinacol occurs only in small amounts due to the difference in reaction rates (**Scheme 15**). Hydrogen abstraction preferably takes place at benzylic positions, amino acid α-positions, tertiary

carbon centres, and heteroatom-stabilised positions due to stabilisation of the generated carbon radical.

Advantages of benzophenones are their inertness to most of the commonly used organic solvents, which enables chemical modification of the chosen compound under standard conditions without prior inactivation of the photoreactive group. Moreover, they show a higher chemical stability compared to azides and diazirines, since benzophenones can undergo several cycles of excitation and relaxation in the absence of abstractable hydrogens. The reactive triplet state can last up to 120 µs and after that readily relaxes into the ground state, maintaining its binding and photoactivatable properties. Therefore, reactions take place even in the presence of water and bulk nucleophiles. The excitation wavelength of benzophenones lies between 350 and 360 nm, which is suitable for biological systems.

Besides these advantages, that make benzophenones seem like ideal photocrosslinking reagents, they suffer from some disadvantages. Irradiation for up to 30 min is necessary to obtain reasonable crosslinking efficiency and this prolonged irradiation time and lifetime can cause non-specific labelling.[108] Additionally, the introduction of the benzophenones increases the bulkiness and hydrophobicity of the modified molecule. This is a potential negative influence on the ligand-target interaction and makes benzophenones less suitable for complexes with low affinity binding.[109]



Scheme 15: Chemistry of benzophenones after photolysis. The scheme shows an adapted version of the scheme found in reference[79].

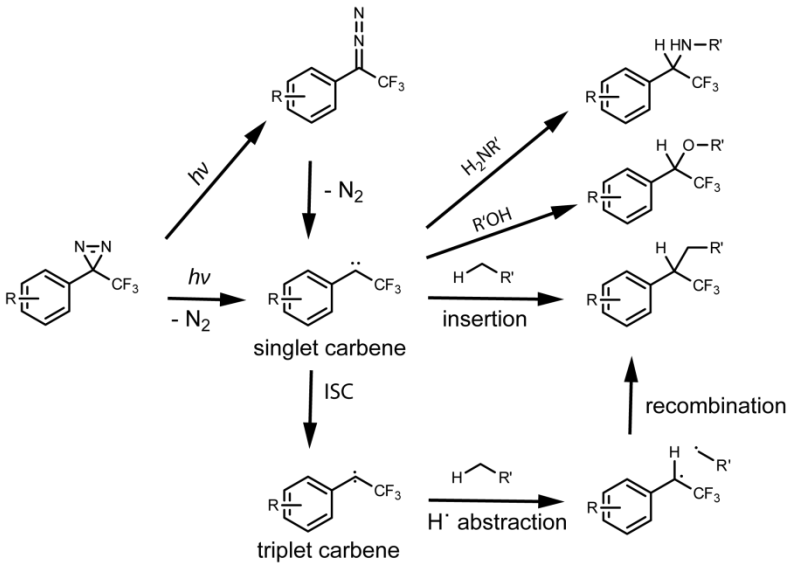
Aryl diazirines[79]

Upon irradiation with UV light ranging from 350 - 380 nm aryl diazirines can undergo two different reactions. Competitively, either a singlet carbene or a diazoisomer is

formed. The diazoisomer of unsubstituted 3-alkyl-3H-diazirines is long-lived and can be converted into the singlet carbene in a relatively slow process by continued irradiation. Unfortunately this elevates the occurrence of aspecific binding and hydrolysis. Introduction of an electron-withdrawing group stabilises the diazoisomer and therefore prevents hydrolysis. The Singlet carbene is a highly reactive short-lived species which can convert into triplet carbene via intersystem crossing. Singlet carbene is readily inserted into C-H, O-H or N-H bonds without discrimination of the reaction site. Triplet carbene reacts in an analogous manner to triplet nitrene by hydrogen abstraction with subsequent coupling to the formed carbon radical (**Scheme 16**).

Due to their enhanced stability compared to unsubstituted 3-alkyl-3H-diazirines, 3-aryl-3-(trifluoromethyl)-3H-diazirine have come to be the commonly used diazirine photocrosslinkers. Nevertheless, they still show undesirable side reactions such as the oxidation of the triplet carbene by molecular oxygen to the corresponding ketone. Another disadvantage is the elimination of HF after insertion into N-H bonds. This results in an enamine-imine equilibrium that hydrolyses to the corresponding ketone upon loss of the cross-linked molecule in aqueous environments.[110]

A major advantage of the aryl diazirines is their absorption wavelength ranging in areas that causes no significant damage to biological systems. Beyond that aryl diazirines are stable against an impressive variety of different conditions. Their stability against strongly acidic, strongly basic, oxidising and reducing conditions can be of interest for the synthesis of modified compounds as well as for the crosslinking application itself. The relatively small size of aryl diazirines similar to that of aryl azides minimises structural changes of the modified compound and potentially reduces a loss of activity.



Scheme 16: Chemistry of aryl diazirines after photolysis. The scheme shows an adapted version of the scheme found in reference[79].

Summary

Perfluorinated aryl azide (TPA) and aryl diazirine (TPD) show superior photoreactive properties compared to the other available moieties. TPA and TPD are less bulky compared to benzophenones and therefore have potentially less negative influence on the ligand-target interactions. Both photoreactive groups are activated using wavelengths above 300 nm which reduces the damaging effects on biological systems and are able to insert into N-H, C-H and O-H bonds after irradiation.

Despite the similar advantages, except for the bulkiness, and the higher stability of benzophenones in aqueous solutions they seem less suitable due to their prolonged irradiation time required to obtain reasonable crosslinking efficiency. This additionally causes rearrangement into a variety of different crosslinking products which is highly undesirable for the unambiguous identification of cross-linked targets.

A final evaluation, if TPA or TPD is the ideal photoreactive group, requires the synthesis of the TPA- and TPD-ABAL probe and testing of each probe in an actual ABAL experiment.

3.4. Cleavable linker

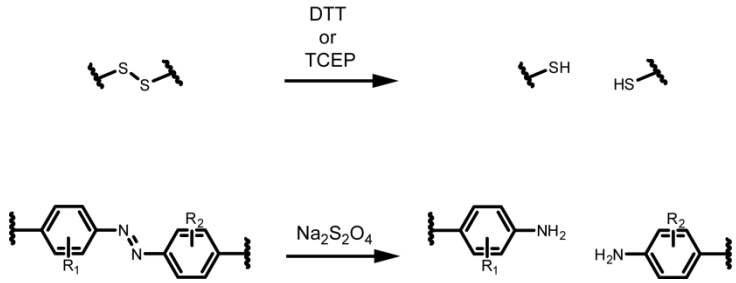
An essential step in ABPP and ABAL is the identification of the target protein. This is usually accomplished by purification and subsequent mass-spectrometry-based identification. Affinity purification on streptavidin beads using biotin as affinity tags has become the preferred strategy. This method takes full advantage of the high affinity of

biotin for streptavidin. Nevertheless, it is limited by its harsh elution conditions which include either denaturation of streptavidin by heating the resin in a denaturing buffer,[111] trypsin digestion of resin-bound proteins,[112] or elution of proteins with excess free biotin.[113] These elution strategies lead to contamination of the sample by coeluting non-specifically bound and/or naturally biotinylated proteins as well as resin-based peptides.[114]

Introducing a molecule chain which contains a chemical bond that can be selectively broken allows the selective release of target proteins under mild conditions. This so called cleavable linker strategy[115] is a valuable addition for biotin-streptavidin-based affinity purification.

There are a variety of cleavable moieties investigated to cleave at various conditions. Disulphide and azobenzene, for example, are the only cleavable moieties that cleave under reducing conditions (**Scheme 17**). Disulphide bonds are readily cleaved with mild reducing agents like dithiothreitol (DTT), β-mercaptoethanol or tris(2-carboxyethyl)phosphine (TCEP).[116] Because of that and its straightforward synthesis disulphide bonds are often used in chemical biology applications. Nonetheless, disulphide linkers have many disadvantages. Electrophilic and nucleophilic polar reagents cause a thiol exchange which can lead to non-specific cleavage under intracellular conditions. Even the mere cellular environment with its reducing properties can cleave the disulphide bond.

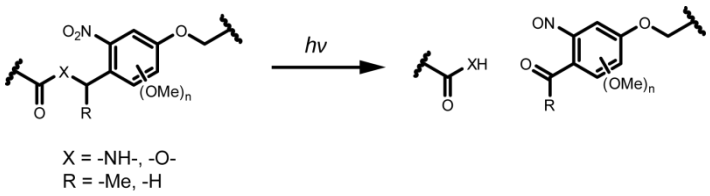
Azobenzene on the other hand is stable under mild reducing conditions such as DTT or TCEP used in biological applications. It is cleaved into two aniline moieties by using sodium dithionite, a mild and bio-orthogonal reducing agent.[117] Azobenzene cleavage requires several washing steps with high concentration of the reducing agent which can result in denaturation of the protein.



Scheme 17: Cleavage of disulphide (top) and azobenzene probes.[116-117]

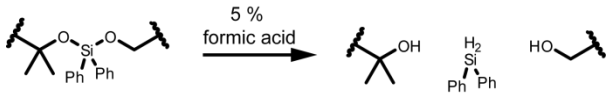
Alternatively, photosensitive groups can be used in cleavable linkers. They are stable under a variety of chemical conditions and can be triggered highly selective by

irradiation with UV light. The photocleavable moieties such as *ortho*-nitrobenzyl derivatives are applied in a variety of biological studies (**Scheme 18**).[115] However, since ABAL already requires UV irradiation for photo-crosslinking, photosensitive groups are not suitable for the use in ABAL approaches.



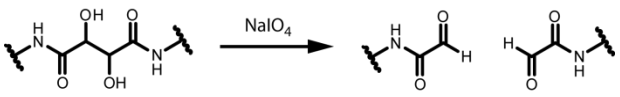
Scheme 18: Cleavage of linkers with *ortho*-nitrobenzyl derivatives.[115]

Another type of cleavable moieties can be cleaved under acidic conditions. To maintain biocompatibility acid cleavable moieties must respond to minor changes close to the physiological pH since strong acidic conditions can lead to denaturing of proteins and DNA. An acid cleavable linker based on a diphenyldialkoxysilane cleavable bond has been recently developed.[118] It is cleaved by incubation with 5 % formic acid at room temperature (**Scheme 19**). Although, these cleaving conditions are the mildest acidic reported so far, possible denaturation of proteins cannot be entirely ruled out.



Scheme 19: Cleavage of linkers with diphenyldialkoxysilane.[118]

Vicinal diols are one example of oxidation sensitive moieties and can be cleaved by sodium periodate to form two aldehydes (**Scheme 20**). Due to this ability sodium periodate is the most frequently used biocompatible oxidising agent. In general, proteins are stable under the mild, neutral cleaving conditions of sodium periodate.[119] Side reactions are of minor concern since they only occur by cleavage of linked carbohydrates or by oxidation of N-terminal serine and threonine residues which are rare protein N-termini.



Scheme 20: Cleavage of vicinal diols.[119]

The variety of cleavable linkers with different cleaving conditions makes it possible to find suitable cleavable linkers for nearly every application.[115] For the synthesis of

cleavable ABAL probes a moiety is necessary that is stable under biological conditions such as the extra- and intracellular environment. At the same time the cleaving conditions should not influence the aptamer-protein interaction, for example, by denaturing the protein, the DNA or both. Oxidation sensitive moieties or more precisely vicinal diols meet these requirements and therefore are a suitable moiety for a cleavable ABAL probe.

4. Aim of project

The ABAL procedure was developed to identify unknown target proteins of aptamers which were selected from complex media such as cell lysates, membrane preparations or whole cells. Vinkenborg et al. showed that the ABAL procedure is applicable to different aptamer-protein complexes with diverse secondary structures and that these known proteins can be cross-linked to their targets in complex media, followed by subsequent purification and identification via western blotting.[3] They also reported that western blot analysis of the crosslinking of the aptamer-protein complex showed several additional bands to the band matching the molecular mass of the targeted protein. Vinkenborg et al. presumed that these bands are a result of endogenously biotinylated proteins which were also isolated during the streptavidin based purification and unspecifically cross-linked protein. For the identification of unknown proteins, the protein has to be isolated in high purity and separated from endogenously biotinylated counterparts to ensure the successful analysis by using mass spectrometry strategies.

The aim of this project was to develop novel ABAL probes that have the potential to yield pure cross-linked products suitable for mass spectrometry analysis (**Figure 14**). So far Sulfo-SBED was used as ABAL probe and is a commercially available photocrosslinking reagent which was originally designed for identifying protein-protein interactions.[3] The main cause of endogenously biotinylated protein impurities present in the samples of the isolated protein is the harsh denaturing conditions necessary to elute the bound samples. Using the structurally related desthiobiotin instead of biotin as a moiety for enrichment has the potential to significantly reduce the amount of eluted impurities. The reason for that is the looser bond of the desthiobiotin-streptavidin system that makes it possible to easily displace the desthiobiotin-containing probes by using biotin-containing buffers under mild conditions.[120]

Another method to reduce the elution of impurities is the introduction of a cleavable linker into the ABAL probe. The cleaving conditions of the linker as well as its position in the ABAL probe are crucial for the effectiveness of the cleavable linker strategy. The originally used sulfo-SBED includes a disulphide linker, but due to its position in the ABAL probe merely the initially modified aptamer is removed from the target protein. The protein itself remains attached to the biotin and therefore to the streptavidin coated carrier which makes elution under harsh denaturing conditions necessary. A crucial

disadvantage of the disulphide bond is that it is cleaved in reducing environments.[116] Protein stabilising additives such as DTT are often necessary to assure proper folding and retain activity of the protein. DTT is also a reducing agent and would accordingly cause the disulphide bond to cleave prematurely when both are present simultaneously. In an ABAL procedure this would result in early release of the aptamer and nonspecific labelling of the free probe after irradiation.

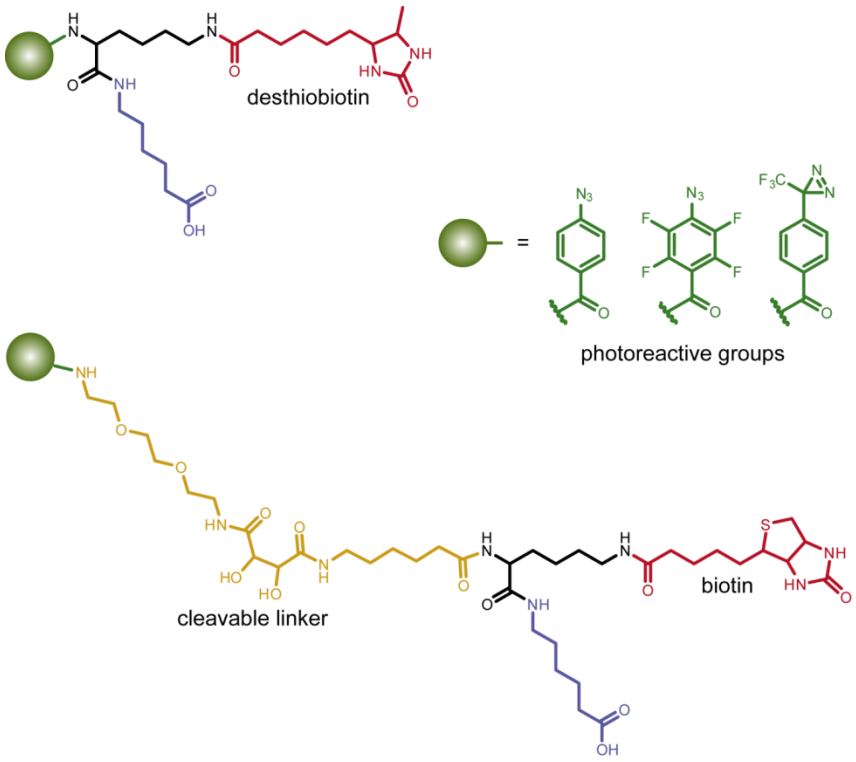


Figure 14: Structures of novel ABAL probes. Each ABAL probe includes either a phenyl azide (PA) 1,2,4,5-tetrafluorophenyl-3-azide (TPA) and 3-phenyl-3-(trifluoromethyl)-3H-diazirine (TPD) moiety. Top: Desthiobiotin ABAL probe. Bottom: Cleavable ABAL probe.

A more suitable moiety for an effective cleavable linker strategy is a vicinal diol, which is cleaved by addition of sodium periodate.[119] These conditions do not naturally occur at any time of the ABAL procedure. Just as important as the cleaving conditions is the position of the cleavable linker in the ABAL probe. To prevent the necessity to elute the protein from the streptavidin carrier it is essential to position the cleavable linker in direct proximity of the crosslinking moiety. At this position cleavage of the linker elutes and isolates the protein from the streptavidin carrier and the rest of the probe.

In sulfo-SBED phenyl azide (PA) is the photoreactive moiety. PA is known to show a significant amount of side reactions that can notably reduce the crosslinking efficiency and can lead to unspecific crosslinking which can be the cause of impurities in isolated

protein samples. Good alternatives are 1,2,4,5-tetrafluorophenyl-3- azide (TPA) and 3-phenyl-3-(trifluoromethyl)-3*H*-diazirine (TPD), which are similar in size but much more stable towards unwanted side reactions.[79]

Based on these considerations two sets of compounds containing desthiobiotin or a cleavable linker were designed. Each set of compounds included PA, TPA or TPD as photoreactive group. The synthesis of these compounds is shown in this work.

5. Results and discussion

To develop a suitable synthesis route for the novel ABAL probes the structure of the probes was retrosynthetically analysed (**Figure 15**). Similar to sulfo-SBED the design of the novel ABAL probes is based on a lysine core structure (black) to which the different functional moieties are attached. These moieties are either biotin variants(red), one of the photoreactive groups PA, TPD or TPA (green), the cleavable linker (orange) and a linker for attachment of the aptamer (blue). These compounds are termed dependent on the elution strategy they enable as desthiobiotin (D) or cleavable (C) ABAL probes. Additionally, the abbreviation of the photoreactive group present is preceded.

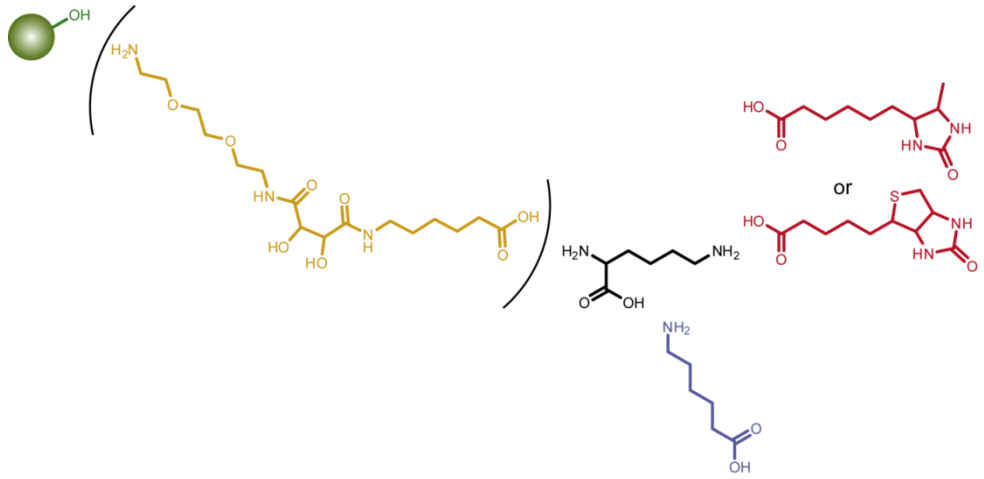
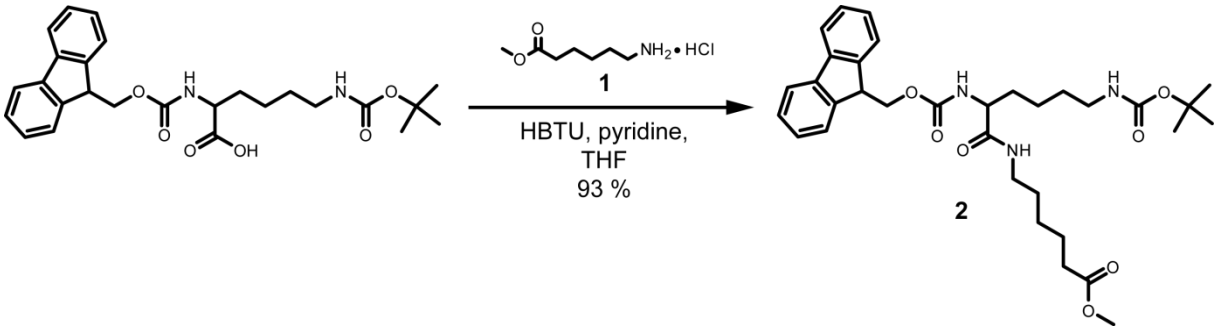


Figure 15: Retrosynthetically determined fragments for novel ABAL probe synthesis.

5.1. Desthiobiotin ABAL probes

To be able to attach the functional moieties to the lysine core structure, considerations were made concerning the order of moiety attachment. The only differences between each of the desthiobiotin compounds are the photoreactive groups. Introducing the photoreactive group as one of the last steps made it possible to follow a single synthesis route until this step. It was necessary to position differently-cleaved protecting groups on the two amines of the lysine molecule. This so called orthogonal protection strategy made it possible to address the reaction sites independently as needed. A fluorenylmethyloxycarbonyl (fmoc)[121]- and *tert*-butyloxycarbonyl (boc)[122]-protected lysine was chosen since it meets the requirements and is commercially available. The fmoc-protection group can be removed under mild basic conditions using mainly amine bases while the boc-protection group on the other hand can be removed under acidic conditions. Both protection groups are stable under the deprotection

conditions of the other. The introduction of the linker for the attachment of the aptamer was the first step in the synthesis of desthiobiotin ABAL probes. The carboxylic acid of the lysine and the amine of the methyl 6-aminocaproate hydrochloride (**1**) were coupled to form an amide bond using HBTU as a coupling reagent (**Scheme 21**). HBTU is one of the most common coupling reagents and is used to activate the carboxylic acid by forming an active ester to accelerate the reaction.

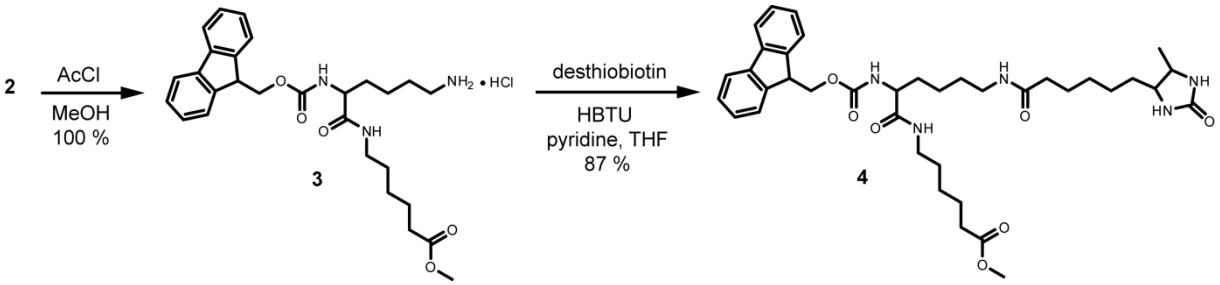


Scheme 21: Coupling of 6-aminocaproate hydrochloride to diprotected lysine.

In compound **1** the amine is present as a hydrochloric salt which needs to be converted into the free amine in order to enable the reaction. Commonly, diisopropylethylamine (DIPEA) is added as a base in coupling reactions to obtain the free amine, but in this particular case the amine base DIPEA caused cleavage of the fmoc-protection group. Pyridine did not deprotect the amine and was added instead to ensure the availability of free methyl 6-aminocaproate.[123] After completion of the reaction, excess pyridine was removed by washing with one molar hydrochloric acid. This mild acidic solution did not affect the boc-protection group and the pure coupling product **2** was obtained after recrystallisation from methanol in excellent yields.

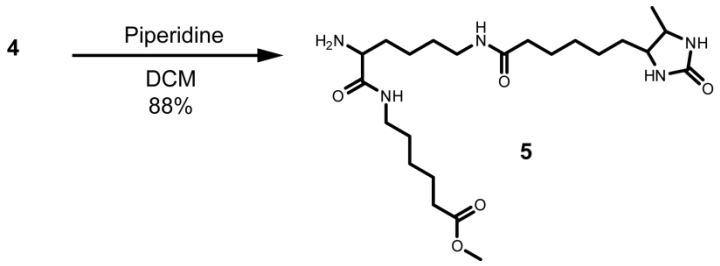
The following coupling step afforded prior acidic deprotection of the boc-protected amine. Hydrochloric acid was generated *in situ* by reaction of acetyl chloride with methanol[122] to provide the acidic deprotection conditions (**Scheme 22**). Protonation of the boc group results in the loss of the tert-butyl cation and subsequent decarboxylation. In this case, precipitation of the reaction mixture in a less polar solvent proved to be an efficient purification method and resulted in the pure amine hydrochloride salt **3** in quantitative yields.

Desthiobiotinolation of the obtained amine **3** by HBTU mediated coupling using pyridine as a base and purification by column chromatography furnished compound **4** in good yields.



Scheme 22: Deprotection of the boc-protected amine and desthiobiotinolation.

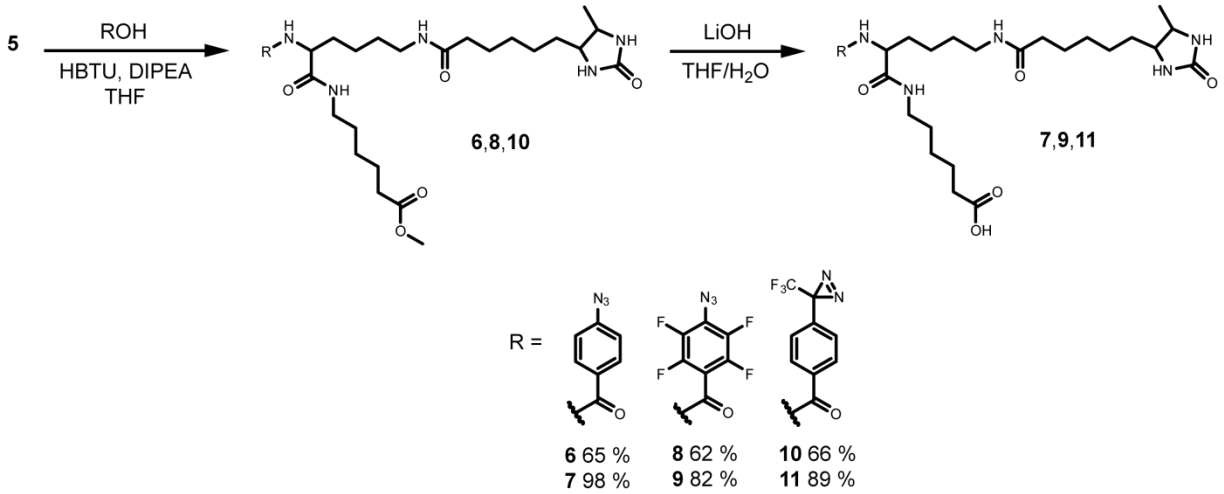
The deprotection of the fmoc-protected amine of compound **4** was then carried out using 20 % piperidine in dichloromethane (**Scheme 23**).[121] The mild base piperidine abstracts the acidic proton of the fmoc group and rearrangement leads to decarboxylation and the formation of 9-methylene-9*H*-fluorene. The free amine **5** was furnished in good yields after purification via column chromatography.



Scheme 23: Deprotection of the fmoc-protected amine.

Compound **5** forms the basis for all three desthiobiotin ABAL probes. Either 4-Azidobenzoic acid, 4-azido-2,3,5,6-tetrafluorobenzoic acid and 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid were then introduced into compound **5** in a HBTU mediated coupling reaction. Since the fmoc group was no longer present in compound **5** common coupling reaction conditions using DIPEA as a base could be applied (**Scheme 24**).[124] The presence of the photoreactive groups in all compounds from this point on afforded handling and storage in the absence of light. This was necessary to prevent premature activation of the photoreactive groups and therefore unspecific crosslinking. The methyl ester of each resulting compound **6**, **8** and **10** was treated with lithium hydroxide monohydrate to furnish the free carboxylic acids **7**, **9** and **11** in excellent yields.[59]

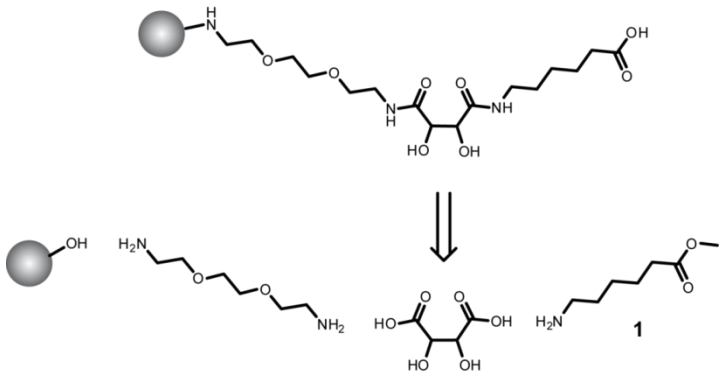
These routes result in a convenient 6-step synthesis for each of the desthiobiotin ABAL probe with overall yields of 36 % for the TPAD, 42 % for the TPDD and 45 % for the PAD ABAL probe.



Scheme 24: Coupling of the photoreactive moieties and final hydrolysis.

5.2. Cleavable ABAL probe‡

In preparation for the synthesis of the cleavable ABAL probe the linker containing the cleavable vicinal diol function had to be synthesised. To ensure accessibility of the diol function and the moieties attached to either side it was necessary to position the diol function in between two chains acting as spacers.[118] 2,2'-(Ethane-1,2-diylbis(oxy))diethanamine and the previously used compound **1** were chosen as spacer and L-tartaric acid served as the vicinal diol function (**Scheme 25**).

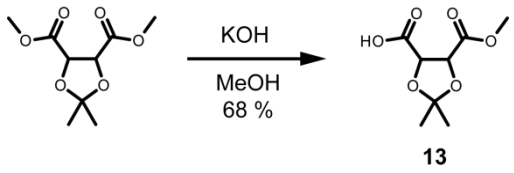


Scheme 25: Retrosynthetically determined fragments for the cleavable linker synthesis.

For sufficient synthesis, an orthogonal protection strategy needed to be applied to L-tartaric acid, since the synthesis required independent addressing of the two carboxylic acid functions and protection of the vicinal diol function. The commercially

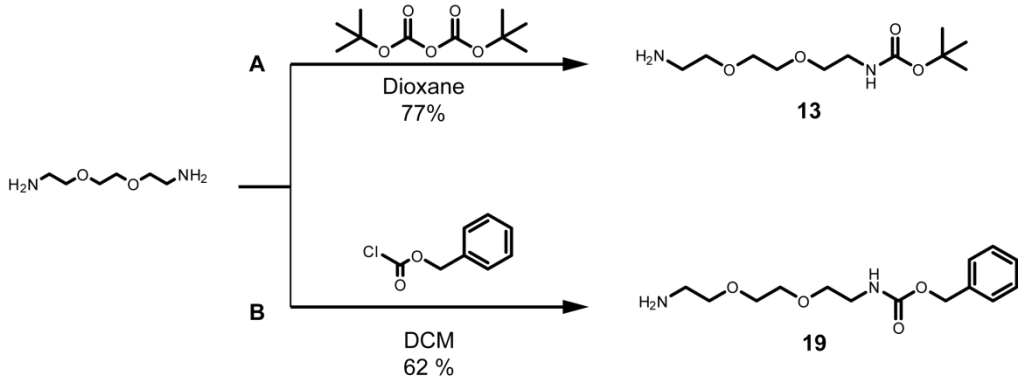
‡ The synthesis of the phenyl azide cleavable ABAL probe was carried out with assistance of Christian Deutsch, who I supervised during his Bachelor thesis. The synthesis of this compound is also described in his Bachelor thesis.

available (−)-dimethyl 2,3-*O*-isopropylidene-L-tartrate proved to be a suitable starting material[119] even though this compound carries two identical protection groups on the carboxylic acids. It was possible to selectively deprotect only one of the carboxylic acids under basic conditions by using a methanolic solution with one equivalent of potassium hydroxide in respect to (−)-dimethyl 2,3-*O*-isopropylidene-L-tartrate (**Scheme 26**).



Scheme 26: Single-sided carboxylic acid deprotection of (−)-dimethyl 2,3-O-isopropylidene-L-tartrate.

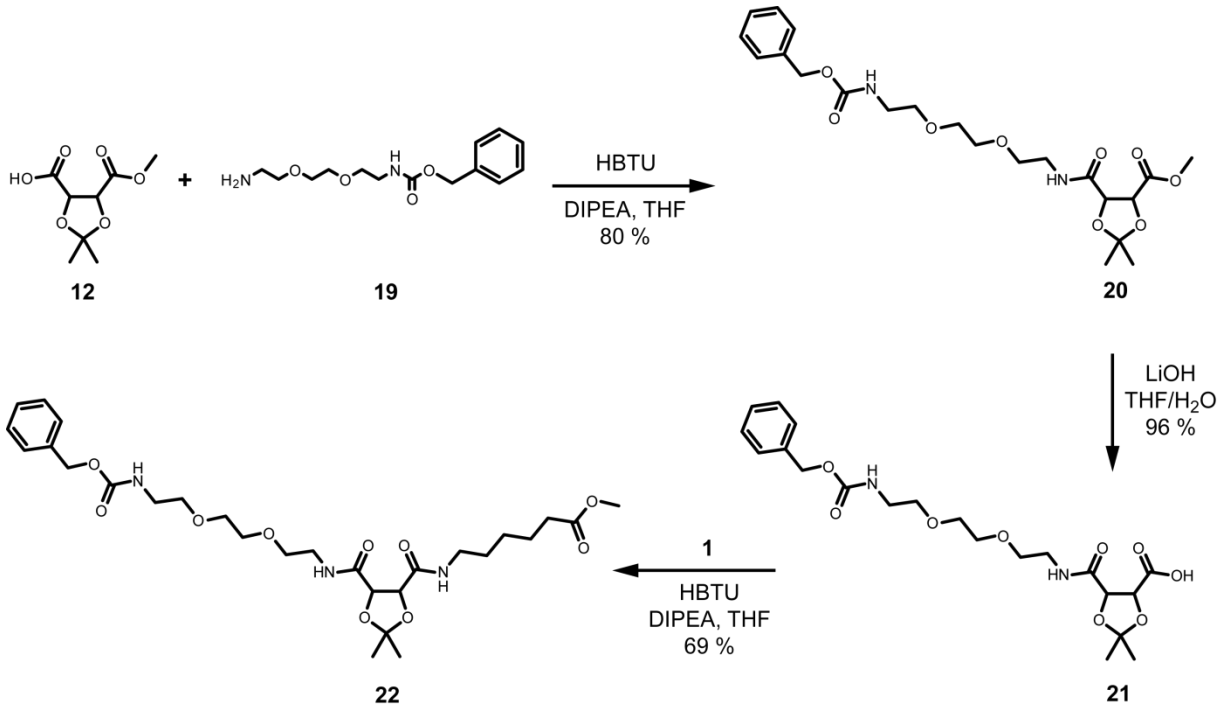
2,2'-(Ethane-1,2-diylbis(oxy))diethanamine was boc-protected using excess 2,2'-(ethane-1,2-diylbis(oxy))diethanamine to ensure monoprotection of one of the two amines (**Scheme 27A**).[125] The boc-protection group was chosen since it enabled simultaneous deprotection of the protected amine and the vicinal diol at a later stage of synthesis. This strategy proved to be a wrong approach particularly as the gained polarity of the resulting molecule **17** increased its solubility in water tremendously. This caused the resulting compound to be not-extractable from the aqueous reaction mixture of the last cleavable linker synthesis step. For this reason a carboxybenzyl (Z)-protection group was chosen to protect one amine of the 2,2'-(ethane-1,2-diylbis(oxy))diethanamine (**Scheme 27B**). The ability to cleave the Z-protection group by promoted reduction with hydrogen[126] enables independent amine and vicinal diol deprotection.



Scheme 27: Single-sided protection of 2,2'-(ethane-1,2-diylbis(oxy))diethanamine. A Boc-protection. B carboxybenzyl (Z)-protection.

The carboxylic acid **12** and the Z-protected compound **19** were then coupled in a HBTU mediated reaction to furnish the coupling product **20** in good yields (**Scheme 28**).

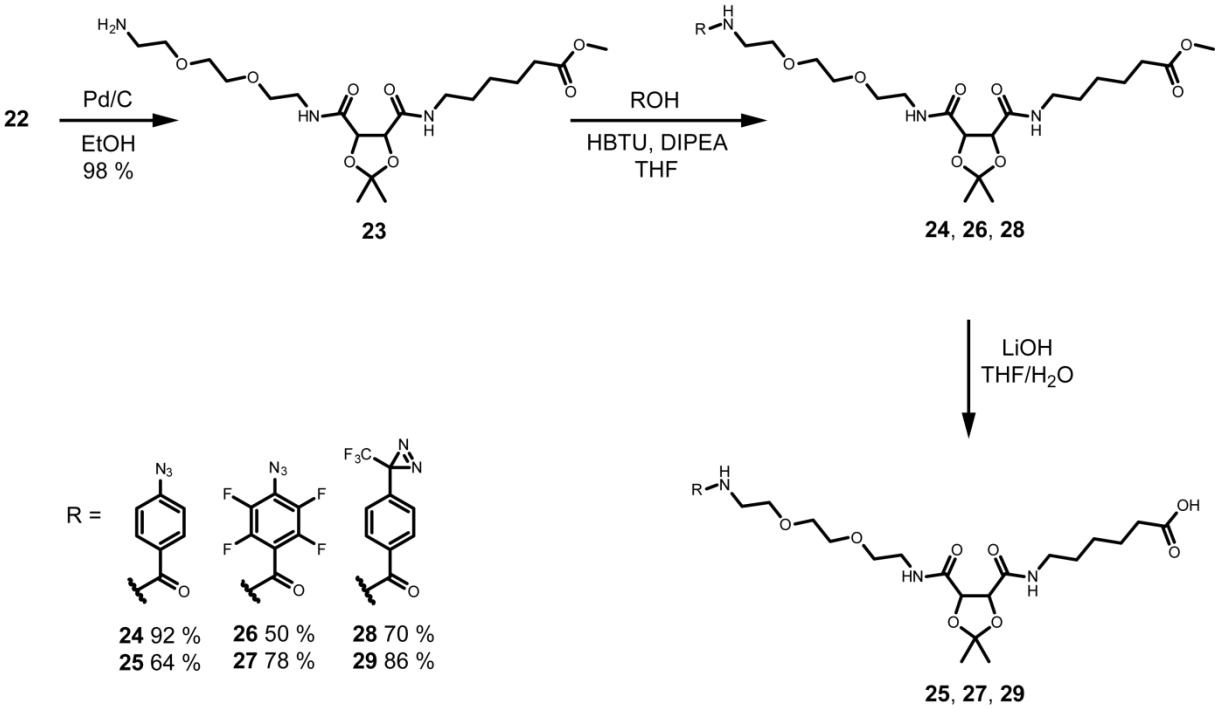
To be able to introduce the second spacer the remaining methyl ester of compound **20** was hydrolysed by using lithium hydroxide monohydrate. The carboxylic acid **21** was obtained in excellent yields and compound **1** was attached via HBTU mediated coupling to furnish the coupling product **22** in acceptable yields.



Scheme 28: Synthesis of the cleavable linker precursor 22.

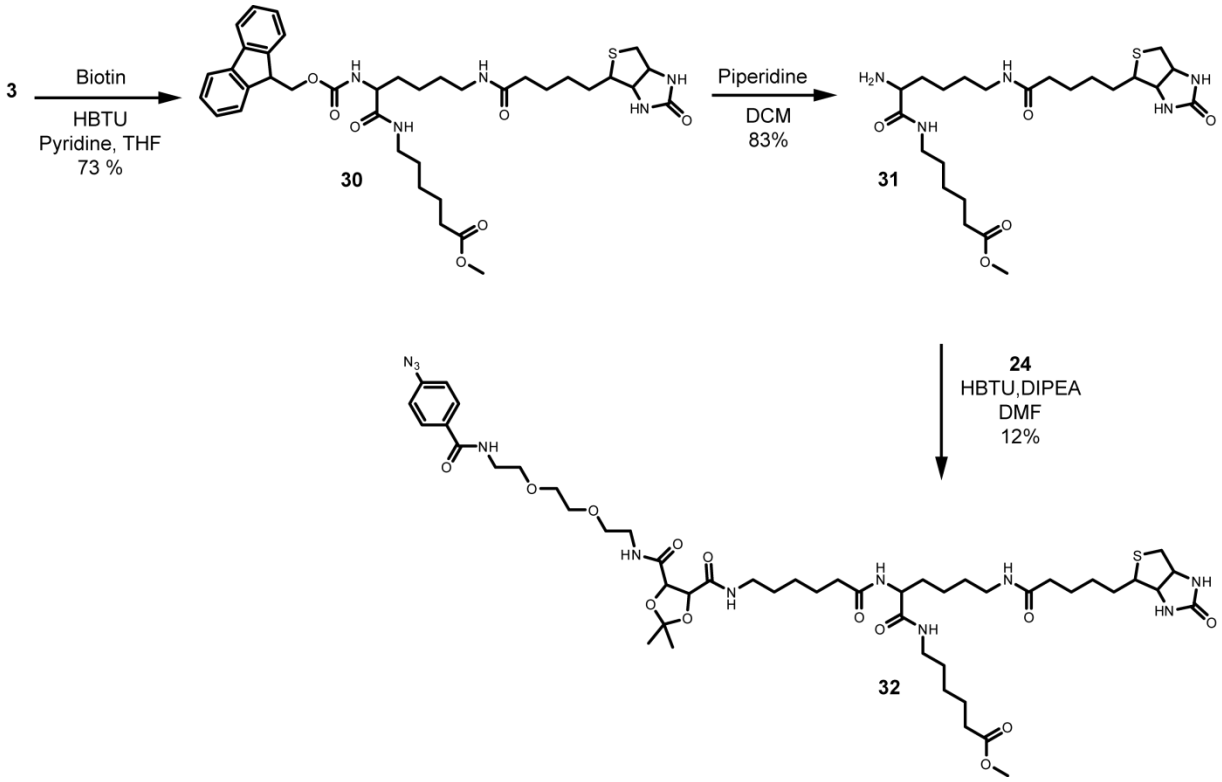
The carboxybenzyl protected compound **22** was cleaved using hydrogen promoted by palladium on activated carbon (**Scheme 29**).[127] This deprotection strategy left the acetal-protection group intact and therefore enabled smooth handling of the resulting compound **23** which was obtained in excellent yields. Compound **23** builds the basis for all photoreactive cleavable linkers.

The introduction of the three photoreactive groups again afforded handling and storage of the resulting compounds in absence of light. Each of the three photoreactive groups was attached to compound **23** using HBTU as a coupling reagent. As a final step carboxylic acid was obtained from the methyl ester via lithium hydroxide monohydrate mediated hydrolysis. This route yielded the three cleavable photoreactive building blocks in an 8-step synthesis. The cleavable PA and the TPD linker show similar overall yields of 13 % and the cleavable TPA linker an overall yield of 9 %. The generally low overall yields are mainly accorded to the low yields of the selective Z-protection and the selective carboxylic acid deprotection. Nevertheless, the synthesis is acceptable due to the inexpensive starting materials.



Scheme 29: Synthesis of the cleavable linkers. PAC linker **25**, TPAC linker **27** and TPDC linker **29**.

With the photoreactive cleavable linkers in hand the synthesis of the cleavable ABAL probes followed a similar strategy as the synthesis of the desthiobiotin ABAL probe, sharing identical starting materials and initial synthesis steps. After the synthesis of compound **3** the synthesis route of the cleavable ABAL probes differed to the extent that compound **3** was coupled to biotin (**Scheme 30**). The coupling reaction was carried out under similar conditions as described for the desthiobiotin ABAL probe using HBTU as a coupling reagent and pyridine as base instead of diisopropylethylamine.[123] The biotinylated compound **30** showed reduced solubility in high extent intensifying handling. Hence, solvents for the aqueous work up were tested, proving a mixture of dichloromethane/methanol in a ratio 3:1 to be the best choice. Purification only succeeded using column chromatography. This required dry loading of the sample and addition of 1 % triethylamine to the solvent mixture of dichloromethane and methanol to deprotonate excess biotin and thereby to prevent its migration on the column.

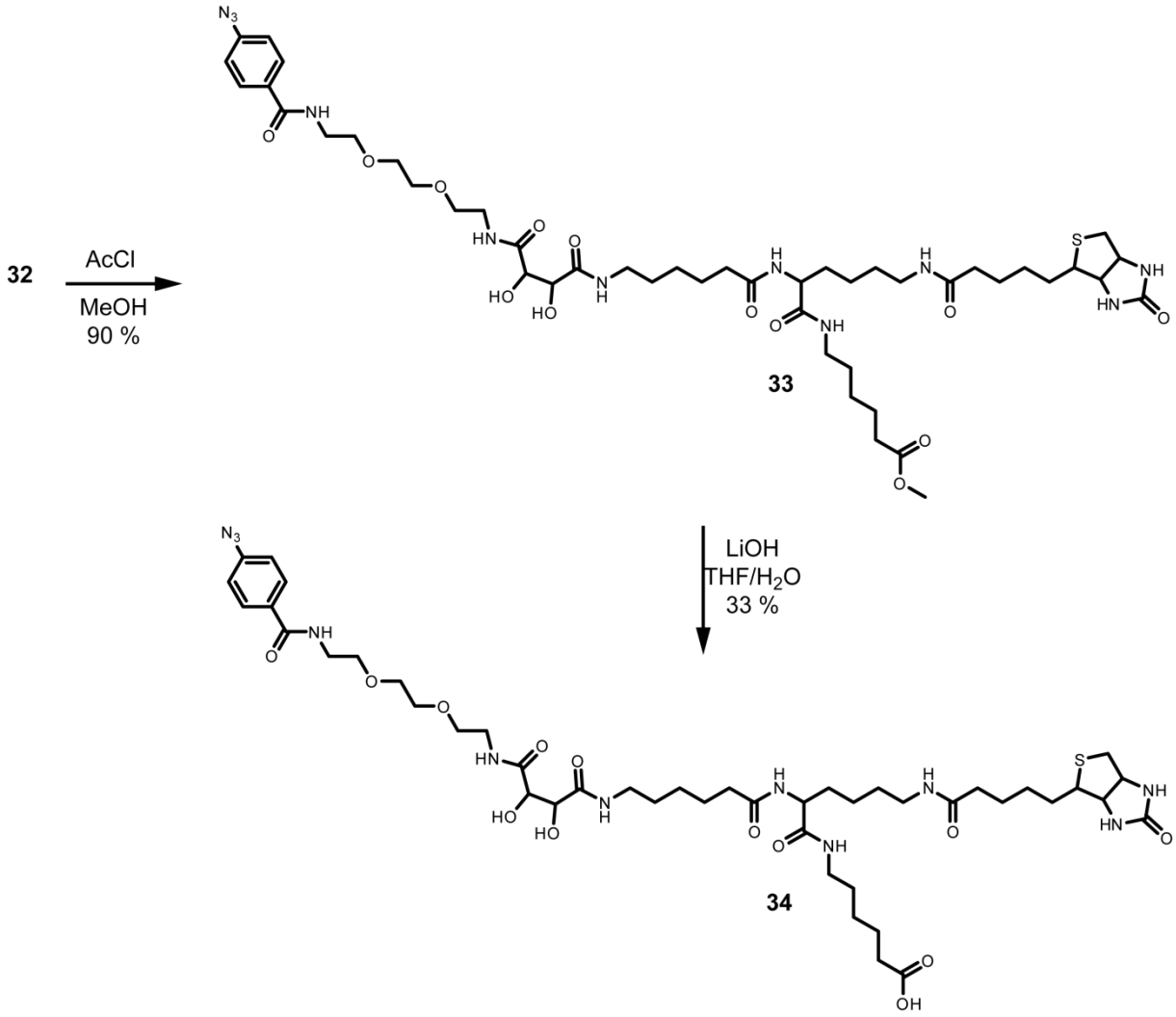


Scheme 30: Synthesis of the cleavable ABAL probe precursor 32.

The fmoc-protection group of the pure compound **30** was then removed by using 20 % piperidine in dichloromethane.[121] Purification was carried out by column chromatography using similar conditions as before, whereas in this case the triethylamine was used to prevent protonation of the free amine **31** on the slightly acidic silica gel and thereby enhance migration on the column.

To assure that the further synthesis conditions were also suitable for the introduction of the cleavable photoreactive linker the coupling reaction was carried out using only the PA cleavable linker **24**. The linker **24** was coupled to the amine **31** using HBTU as coupling reagent and dimethylformamide (DMF) as solvent.[124] DMF is a polar organic solvent which is commonly used for reactions with polar reagents to ensure a homogenous reaction mixture. In this case even while using DMF precipitation occurred during the reaction and reoccurred after adding several portions of dimethylformamide. This situation resulted in the coupling product yields of only 12 % after 96 hours reaction time most likely due to the fact that the amine **31** was removed from the reaction mixture by precipitation before completion of the reaction. Alternating the reaction conditions and exchanging the coupling reagent by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide[128] (EDC) to increase the amount of coupled product **32** did not deliver an increase in yield.

To be able to show the general validity of the synthetic pathway, synthesis was continued with the amounts of compound **32** obtained from the previous reaction. As a following step the acetal-protected diol was deprotected by using acetyl chloride and methanol (**Scheme 31**). With this reaction mixture for *in situ* hydrochloric acid generation the free diol **33** was successfully obtained in excellent yields. The rise of polarity due to the free diols increased the solubility of the compound to an extent that made it possible to carry out the hydrolysis of the methyl ester with lithium hydroxide monohydrate in aqueous tetrahydrofuran.[59] The work up of the hydrolysis reaction requires precipitation of the carboxylate ion from an aqueous solution by protonation with an aqueous hydrochloric acid solution and filtration of the formed compound **34**. The final product **34** was obtained in rather poor yields of 33 %, most likely due to the loss of material during precipitation and filtration of the small amounts of sample. Nevertheless it was shown that the pathway generally succeeds.



Scheme 31: Synthesis of the PA cleavable ABAL probe.

The coupling reaction of the cleavable linker **24** and the amine **31** is clearly the bottle neck of this synthetic pathway. The major problem of the coupling reaction seems to be the poor solubility of the biotinylated compound **31**. For the large scale synthesis of the PAC ABAL probe and the synthesis of the TPAC and TPDC ABAL probes an alternative synthetic pathway, introducing biotin as one of the last steps should be taken into consideration (**Chapter B6**).

5.3. Aptamer ABAL probe

The PAD ABAL probe and the aptamer C10.35 were chosen to investigate the most suitable reaction conditions to generate the ABAL aptamer probes. C10.35 is a DNA-aptamer which targets the Sec7 domain of the guanine nucleotide exchange factor cytohesin-2 (**Figure 16**) and was one of the aptamer-protein complexes used to prove the validity of the ABAL procedure for different aptamer-protein complexes.

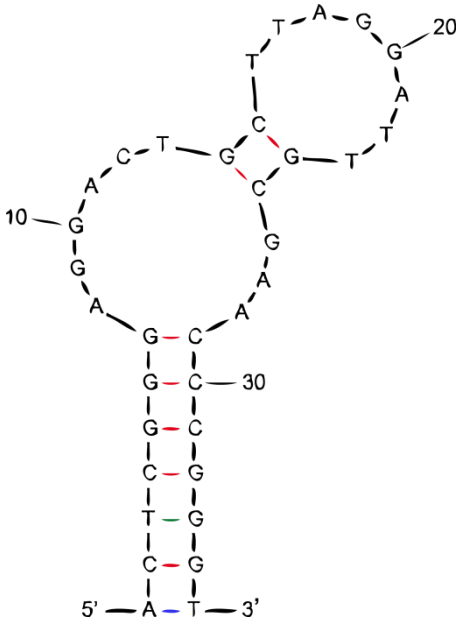


Figure 16: Predicted structure of the cytohesin-2 Sec 7 aptamer C10.35. The figure shows an adapted version of the figure found in reference[3].

The generation of the ABAL aptamer probe is an amide bond formation carried out in aqueous buffers. Suitable buffers must lack carboxylic acids and amines to prevent unwanted by-products. In this case the zwitterionic buffering agent 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was used to prepare a buffer at pH 8.3 which also included sodium chloride. This buffer was chosen due to its good coupling performance in the initial ABAL experiments.[3]

As previously described, coupling reagents are used to accelerate the amide bond formation by activating the carboxylic acid. EDC is a commonly used coupling reagent in aqueous buffers due to its good solubility in water. It activates the carboxylic acid by forming an *O*-acylisourea intermediate which is then displaced by nucleophilic attack of the amine.[129]

Coupling between the 5'-hexylamino-modified aptamer C10.35 and the PAD ABAL probe was carried out with 100-fold excess of the PAD ABAL probe and EDC. The reaction mixture was incubated for 19 hours in a Thermomixer at 300 rpm and LC-MS analysis showed a yield of 45 % for the desired C10.35 PAD ABAL probe (**Table 3**). In an attempt to increase the coupling yield the reaction was repeated at 65 °C. The higher temperature increased the reaction yield only marginally to 49 %.

Coupling reagent	Reaction temperature [°C]	Reaction time [h]	Reaction yield [%]
EDC	RT	19	45
EDC	65	17	49
EDC + OxymaPure	RT	19	4
EDC + Sulfo-NHS	4	2	13
EDC + Sulfo-NHS	4	3	17
EDC + Sulfo-NHS	4	4	20
EDC + Sulfo-NHS	4	19	42
EDC + Sulfo-NHS	RT	2	29
EDC + Sulfo-NHS	RT	3	0
EDC + Sulfo-NHS	RT	4	31
EDC + Sulfo-NHS	RT	19	64

Table 3: Conditions and results of the coupling reaction.

The formed *O*-acylisourea intermediate is prone to hydrolysis in aqueous buffers, which is most likely the reason for the moderate yields in this coupling reaction. Approaches to prevent hydrolysis of the active ester comprise either reduction of the reaction time or stabilisation of the active ester. Hence, the reaction was carried out using the additive ethyl 2-cyano-2-(hydroxyimino)acetate (OxymaPure) which is combined with carbodiimide coupling reagents to improve their performance.[130] OxymaPure is an alternative to the commonly used benzotriazol-based additives which were reported to show explosive properties. In a coupling reaction using both EDC and OxymaPure the *O*-acylisourea intermediate is primarily formed. OxymaPure then reacts with the intermediate to form the oxime active ester, which stabilises the approach of

the amine.[131] The reaction furnished the desired coupling product in very poor yields of only 4 % which proved this approach as unsuccessful.

In a different approach the reaction was carried out using an analog of *N*-hydroxysuccinimide (NHS), *N*-hydroxysulfosuccinimide (sulfo-NHS). Both NHS and sulfo-NHS are water soluble, but sulfo-NHS has the advantage over NHS that the formed active ester remains water-soluble due to the charged sulfonate group. Similar to the coupling reaction with the additive OxymaPure primarily the *O*-acylisourea intermediate is formed which then reacts with sulfo-NHS to form the sulfo-NHS ester. The hydrophilic sulfo-NHS ester hydrolyses very slowly compared to its reaction rate with amino groups.[132] Nevertheless, the half-life of the sulfo-NHS ester decreases depending on the water-content, temperature and pH of the reaction mixture. For this reason the coupling reaction was carried out at room temperature and at 4 °C and reaction yields were analysed for different reaction times. For both temperatures yields increased over time, but the reactions at room temperature furnished better yields and the higher maximum yield of 64 % after 19 hours. The differing value at room temperature after 3 hours is most likely due to handling or measurement errors and can be ignored.

These results show that the C10.35 PAD ABAL probe can be generated in good yields using EDC and sulfo-NHS as coupling reagents at room temperature.

6. Outlook

6.1. Verification of the improved properties of the novel ABAL probes

The work described in this part of the thesis displays the successful synthesis of novel ABAL probes which, in future, might have the potential to make ABAL a standard procedure for identifying unknown target proteins of aptamers selected from highly complex systems.

To achieve this, the improved properties of the novel ABAL probes have to be verified by analysing aptamer-protein complexes with structurally diverse aptamers to underline universal applicability of the novel probes in the ABAL procedure. Therefore, these aptamers and suitable negative controls have to be modified with the novel ABAL probes using the method determined in this work. Reverse-phase HPLC could then be used to separate the modified aptamers from the unreacted novel ABAL probes and the unfunctionalised aptamers. A potential negative influence of the ABAL probe on the binding properties of the modified aptamer needs to be ruled out by competitive filter binding experiments. In these experiments the 5'-ABAL-modified aptamers compete with the corresponding unfunctionalised aptamers for the binding on the target proteins. Comparison of the obtained IC_{50} values of unfunctionalised aptamers, the ABAL-modified aptamers, and the negative-control aptamer will make it possible to draw conclusions about the influence of the novel ABAL probes on the binding properties of the ABAL-modified aptamer.

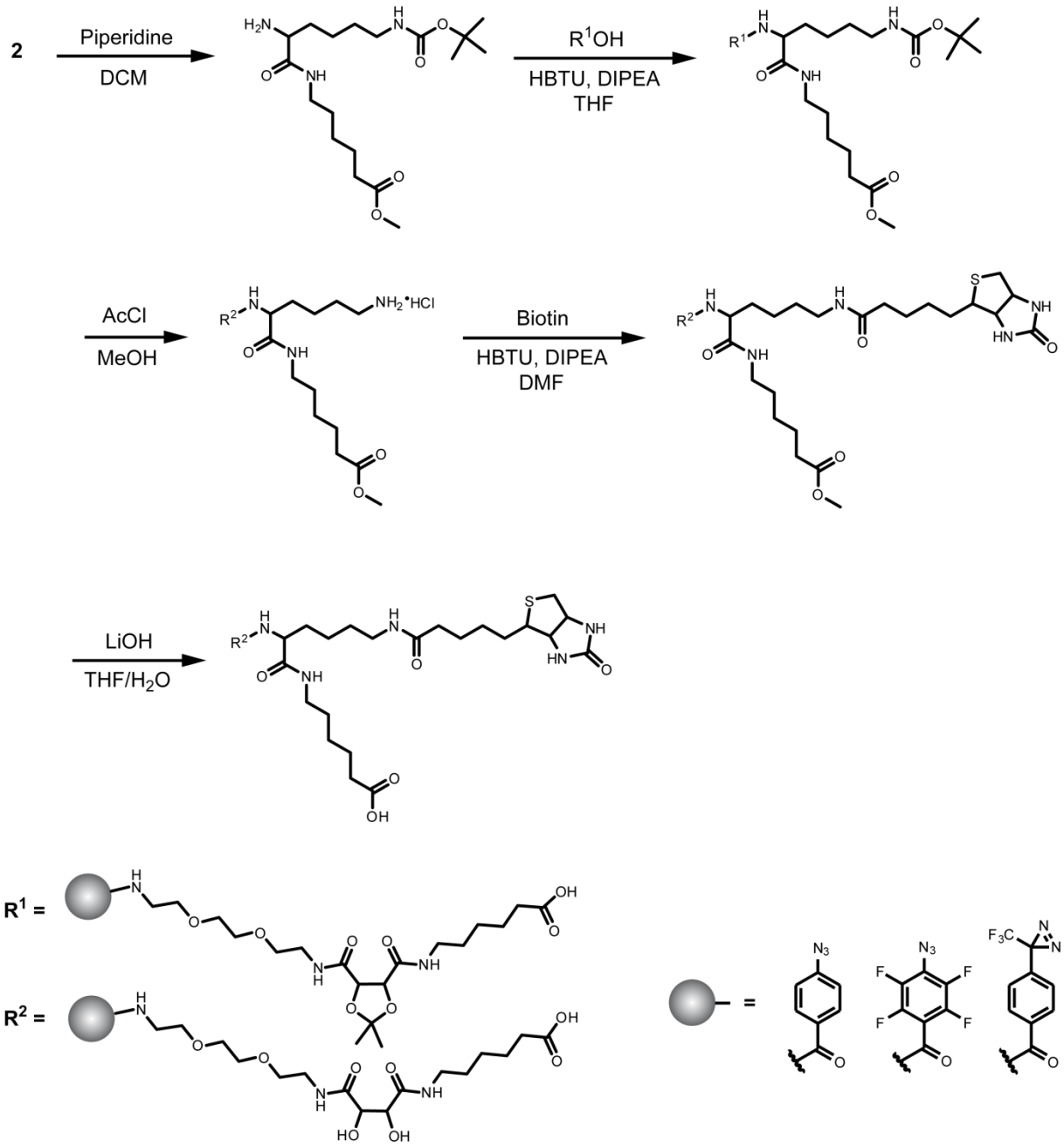
The crosslinking efficiency of the ABAL-modified aptamers needs to be determined as well. For this the ABAL procedure has to be carried out for the ABAL-modified aptamers using purified protein as well as protein in cell lysate. Elution of the proteins from streptavidin-coated beads can then be carried out using either biotin-containing buffers in the case of the desthiobiotin probes or sodium periodate-containing buffers in the case of the cleavable variant. The western blot analysis of the experiments using the purified protein will provide information about the most efficient cross-linkers. In the case of the experiments using protein in cell lysate, the western blot analysis will at best show just a single band corresponding to the target protein. If so, this will prove the success of the novel ABAL probes.

For the cleavable ABAL-modified aptamers, additional experiments need to be carried out to determine optimal cleaving conditions and efficiency. For this, streptavidin beads

loaded with the cross-linked aptamer-protein complex should be eluted using sodium periodate-containing buffer. The beads should then be treated with common elution strategies such as boiling in sodium dodecyl sulphate to elute all remaining proteins. Both fractions can then be analysed via western blotting to determine optimal cleavage conditions.

6.2. Alternative synthetic pathway for cleavable ABAL probes

The synthesis of the PAC ABAL probe only furnished poor yields most likely due to the low solubility of the biotinylated compound **31** and the resulting poor handleability. Introduction of biotin as one of the last steps might solve the problem and increase overall yields (**Scheme 32**).



Scheme 32: Alternative synthetic pathway for the synthesis of cleavable ABAL probes.

The only disadvantage of this strategy is that the synthetic pathway splits into three individual pathways at an early stage of the synthesis one for each cleavable linker. This makes the synthesis of the three cleavable ABAL probes in parallel more complex. **Scheme 32** shows the alternative synthetic pathway in which at first compound **2** is fmoc-deprotected and the cleavable linker attached. After the deprotection of the amine and the vicinal diol in a single step, the biotinylation is carried out, leaving only the hydrolysis as a last synthesis step. This strategy should most likely result in a synthetic pathway with better overall yields.

7. Experimental section

7.1. General methods

NMR-spectroscopy

^{1}H- and ^{13}C-NMR-spectra were measured with a nuclear magnetic resonance spectrometer AM300 (^{1}H = 300 MHz; ^{13}C = 75.5 MHz), AM400 (^{1}H = 400 MHz; ^{13}C = 100.6 MHz) und AM500 (^{1}H = 500 MHz; ^{13}C = 126.0 MHz) from *BRUKER,* Karlsruhe. D_6-DMSO was used as solvent. The chemical shifts were ploted as δ-values in ppm. The ^{1}H-spectra were calibrated on the d_6-DMSO residual content at δ = 2.50 ppm and the ^{13}C-spectra were calibrated on the d_6-DMSO residual content at δ = 39.5 ppm. Increment calculations of the Program *ChemBioDraw Ultra 12.0* from *Cambridge Soft* were used to assign some signals. The analysis of the spectra was carried out with *MestReNova 8* from *Mestrelab Research S.L.*

The following abbreviations were used for the multiplicities:

s Singlet
d Doublet
dd Doublet of Doublets
t Triplet
q quartet
m multiplet

The coupling constant *J* were quoted in Hertz and with the letters indicating the atom. The letters used are identical to the letters shown on the structures.

Mass spectroscopy

The mass spectra were measured in the chemical institute. EI-Mass spectra were measured on a MAT-95XL from *Finnigan*, Bremen. ESI-Mass spectra were measured with a micrOTOF-Q flight time spectrometer from *Bruker Daltonik,* Bremen using an *Agilent* 1200 Series HPLC-facility.

Flash chromatography

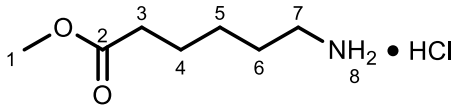
Flash chromatography was carried out using pre-packed silica gel columns on a PuriFlash 430 from Interchim, France.

TLC

Analytical TLC was performed on a 0.25 mm tickness plates pre-coated with Merck Kieselgel 60 F_{254} silica gel. TLC were visualised under UV (254) nm or by using Phosphorus molybdic acid and Ninhydrin dyes.

7.2. Desthiobiotin ABAL probes

Methyl 6-aminohexanoate hydrochloride (1)



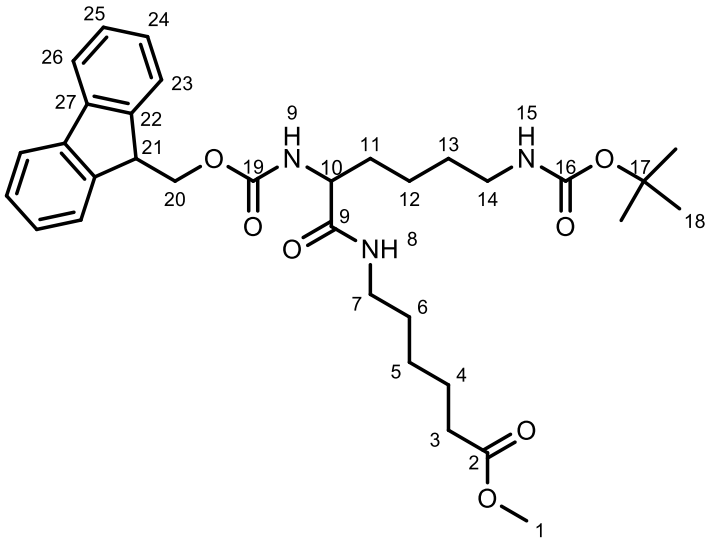
Acetyl chloride (228 mmol, 16.3 mL) was added to 6-Aminocaproic acid (76.2 mmol, 10.0 g) in methanol (135 mL) at 0 °C and stirred for 18 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in methanol (40.0 mL) and poured into diethyl ether (320 mL). The resulting precipitate was filtered to obtain **1** as white solid. Yield 12.7 g (92 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 8.12 (s, 3H,H_8), 3.58 (s, 3H, H_1), 2.76 – 2.65 (m, 2H, H_7), 2.29 (t, $^{3}J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.60 – 1.48 (m, 4H, H_4, H_6), 1.36 – 1.25 (m, 2H, H_5).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 173.2 (C_2), 51.2 (C_1), 38.5 (C_7), 33.1(C_3), 26.6 (C_4), 25.3 (C_5), 23.9 (C_6).

HRMS calculated for $C_7H_{16}NO_2Cl$ + H 146.1181. Found 146.1176

Methyl 6-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-((tert-butoxycarbonyl)amino)hexanamido)hexanoate (2)



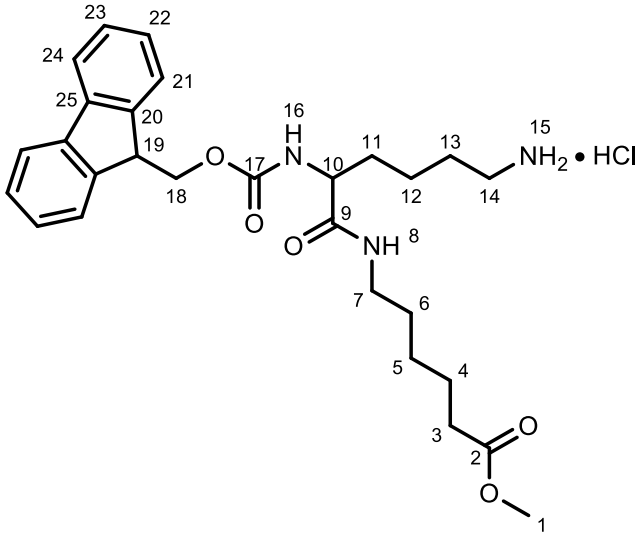
Pyridine (142 mL) was added to a stirred solution of Fmoc-Lys(Boc)-OH (28.4 mmol, 13.2 g) in tetrahydrofuran (142 mL) followed by HBTU (31.2 mmol, 11.8 g) at room temperature. After 15 minutes **1** (31.2 mmol, 5.67 g) was added. The reaction mixture was stirred for 72 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in dichloromethane (150 mL) and washed with hydrochloric acid (1M, 3 x 100 mL), saturated sodium hydrogen carbonate solution (3 x 100 mL) and saturated sodium chloride solution (100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was recrystallized from methanol to give **2** as a white solid. Yield 15.7 g (93 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 7.88 (d, $^3J_{26,25}$ = 7.7 Hz, 2H, H_{26}), 7.81 (t, $^3J_{8,7}$ = 5.2 Hz, 1H, H_8), 7.76 – 7.68 (m, 2H, H_{23}), 7.45 – 7.27 (m, 5H, H_9, H_{24}, H_{25}), 6.74 (t, $^3J_{15,14}$ = 5.5 Hz, 1H, H_{15}), 4.34 – 4.13 (m, 3H, H_{10},H_{20}), 3.92 – 3.85 (m, 1H, H_{21}), 3.55 (s, 3H, H_1), 3.09 – 2.98 (m, 2H, H_7), 2.91 - 2.84 (m, 2H, H_{14}), 2.25 (t, $^3J_{3,4}$ = 7.3 Hz, 2H, H_3), 1.59 - 1.44 (m, 4H, H_4, H_{11}), 1.40 - 1.33 (m, 13H, H_6, H_{13}, H_{18}), 1.30 – 1.11 (m, 4H, H_5, H_{12}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_2), 171.7 (C_9), 155.9 (C_{16}), 155.6 (C_{19}), 143.9 (C_{22}), 140.7 (C_{27}), 127.6 (C_{25}), 127.0 (C_{24}), 125.3 (C_{23}), 120.1 (C_{26}), 77.3 (C_{17}), 65.6 (C_{10}), 54.7 (C_{21}), 51.2 (C_1), 46.7 (C_{20}), 39.7 (C_{14}), 38.2 (C_7), 33.2 (C_3), 31.8 (C_{13}), 29.2 (C_6), 28.7 (C_{11}), 28.3 (C_{18}), 25.8 (C_5), 24.1 (C_4), 22.9 (C_{12}).

HRMS calculated for $C_{33}H_{45}N_3O_7$ + Na 618.3156. Found 618.3150.

Methyl 6-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-aminohexanamido) hexanoate (3)



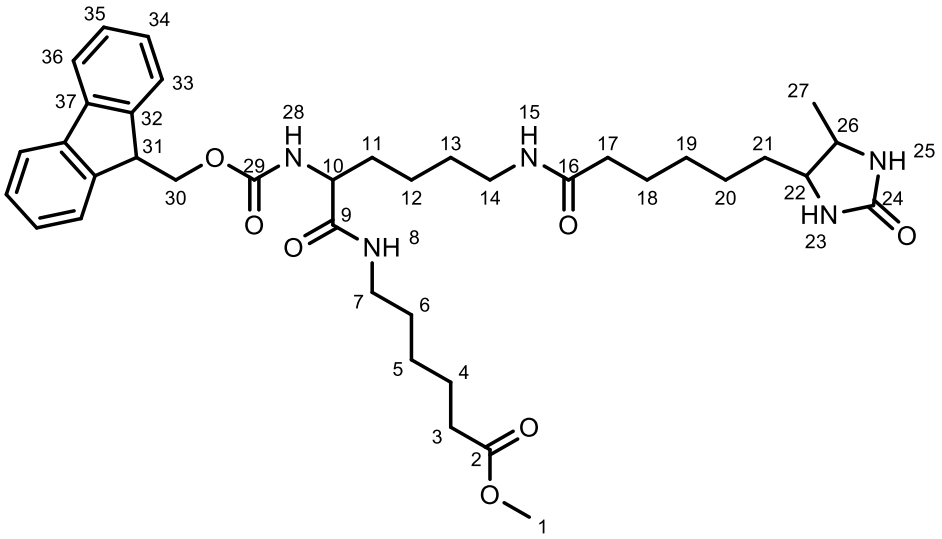
Acetyl chloride (79.1 mmol, 5.60 mL) was added to **2** (26.4 mmol, 15.7 g) in methanol (264 mL) at 0 °C and refluxed for 1 hour. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in methanol (80.0 mL) and poured into diethyl ether (640 mL). The resulting precipitate was filtered to obtain **3** as white solid. Yield 14.0 g (100 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 8.01 (s, 3H, H_{15}), 7.95 – 7.86 (m, 3H, H_8, H_{24}), 7.75 – 7.72 (m, 2H, H_{21}), 7.49 – 7.37 (m, 3H, H_{16}, H_{23}), 7.32 (ddd, $^{3}J_{22,21/23}$ = 7.4 Hz, $^{4}J_{22,24}$ = 1.2 Hz, 2H, H_{22}), 4.33 – 4.17 (m, 3H, H_{10}, H_{18}), 3.94 – 3.89 (m, 1H, H_{19}), 3.56 (s, 3H, H_1), 3.12 – 2.95 (m, 2H, H_7), 2.78 – 2.71 (m, 2H, H_{14}), 2.26 (t, $^{3}J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.65 - 1.45 (m, 6H, H_{11}, H_4, H_{13}), 1.44 – 1.16 (m, 6H, H_6, H_5, H_{12}).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 173.2 (C_2), 171.6 (C_9), 155.9 (C_{17}), 143.7 (C_{20}), 140.7 (C_{25}), 127.6 (C_{23}), 127.0 (C_{22}), 125.3 (C_{21}), 120.1 (C_{24}), 65.5 (C_{10}), 54.5 (C_{19}), 51.1 (C_1), 46.6 (C_{18}), 38.4 (C_{14}), 38.2 (C_7), 33.2 (C_3), 31.3 (C_{13}), 28.6 (C_6), 26.5 (C_{11}), 25.7 (C_5), 24.1 (C_4), 22.4 (C_{12}).

HRMS calculated for $C_{28}H_{37}N_3O_5$ +Na 518.2631. Found 518.2625.

Methyl 6-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-(6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamido)hexanamido)hexanoate (4)



Pyridine (24.0 mL) was added to a stirred solution of desthiobiotin (4.67 mmol, 1.00 g) in tetrahydrofuran (24.0 mL) followed by HBTU (5.13 mmol, 1.95 g) at room temperature. After 15 minutes **3** (5.13 mmol, 2.73 g) was added. The reaction mixture was stirred for 72 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in dichloromethane (150 mL) and washed with hydrochloric acid (1M, 3 x 100 mL), saturated sodium hydrogen carbonate solution (3 x 100 mL) and saturated sodium chloride solution (100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was dissolved in methanol (80.0 mL) and poured into diethyl ether (640 mL). The resulting precipitate was filtered to obtain **4** as yellow solid. Yield 2.82 g (87 %).

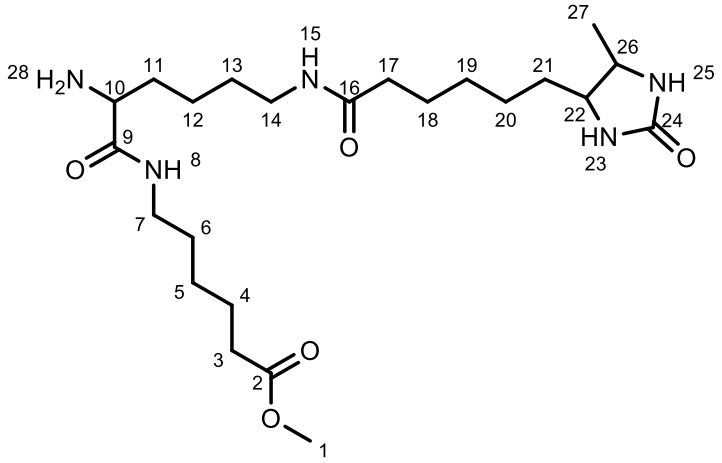
^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 7.89 (d, $^3J_{36,35}$ = 7.5 Hz, 2H, H_{36}), 7.86 - 7.79 (m, 1H, H_8), 7.74 – 7.70 (m, 3H, H_{15}, H_{33}), 7.46 – 7.37 (m, 3H, H_{28}, H_{35}), 7.32 (ddd, $^3J_{34,33/35}$ = 7.4 Hz, $^4J_{33,36}$ = 1.2 Hz, 2H, H_{34}), 6.28 (s, 1H, $H_{23/25}$), 6.11 (s, 1H, $H_{23/25}$), 4.30 - 4.16 (m, 3H, H_{10}, H_{30}), 3.93 - 3.87 (m, 1H, H_{31}), 3.64 – 3.53 (m, 4H, H_1, H_{26}), 3.51 - 3.42 (m, 1H, H_{22}), 3.10 – 2.93 (m, 4H, H_7, H_{14}), 2.26 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 2.02 (t, $^3J_{17,18}$ = 7.4 Hz, 2H, H_{17}), 1.62 – 1.43 (m, 6H, H_4, H_{11}, H_{13}), 1.42 – 1.11 (m, 14H, H_5, H_6, H_{12}, H_{18}, H_{19}, H_{20}, H_{21}), 0.94 (d, $^3J_{27,26}$ = 6.3 Hz, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 173.2 (C_2), 171.8 (C_{16}), 171.6 (C_9), 162.7 (C_{24}), 155.8 (C_{29}), 143.8 (C_{32}), 140.7 (C_{37}), 127.6 (C_{35}), 127.0 (C_{34}), 125.3 (C_{33}), 120.0 (C_{36}), 65.5 (C_{10}), 54.9 (C_{22}), 54.6 (C_{31}), 51.1 (C_1), 50.2 (C_{26}), 46.6 (C_{30}), 38.2 (C_7, C_{14}), 35.3

(C_{17}), 33.2 (C_3), 31.7 (C_{21}), 29.5 (C_6), 28.8 (C_{19}), 28.7 (C_5), 28.6 (C_{20}), 25.7 (C_{18}), 25.5 (C_{12}), 25.2 (C_{13}), 24.1 (C_{11}), 22.9 (C_4), 15.4 (C_{27}).

HRMS calculated for $C_{38}H_{53}N_5O_7$ + Na 714.3843. Found 714.3837.

Methyl 6-(2-amino-6-(6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamido) hexanamido)hexanoate (5)



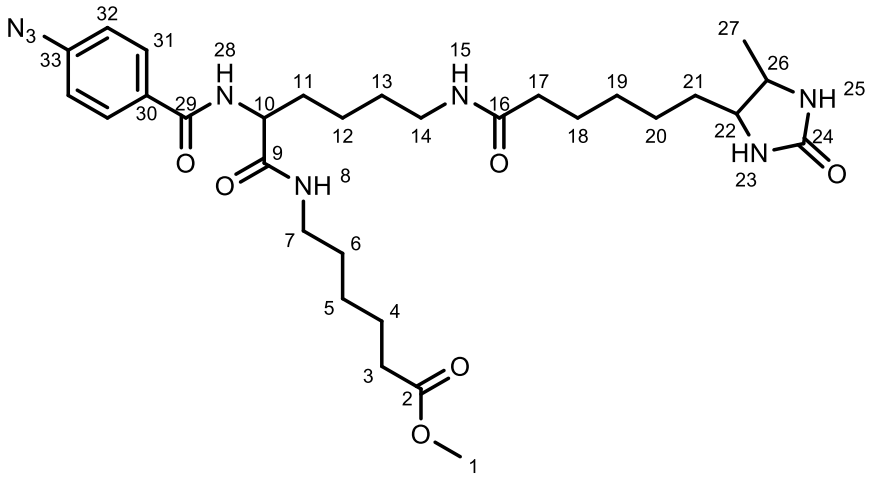
Piperidine (8.00 mL) was added to **4** (4.08 mmol, 2.82 g) in dichloromethane (32.0 mL) at 0 °C and stirred for 1 hours at room temperature. Toluene (40.0 mL) was added and the reaction mixture was concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using dichloromethane, methanol and triethylamine (90:9:1) as eluent to give **5** as a pale yellow solid. Yield 1.68 g (88 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.15 (t, $^3J_{8,7}$ = 5.7 Hz, 1H, H_8), 7.77 (t, $^3J_{15,14}$ = 5.6 Hz, 1H, H_{15}), 6.30 (s, 1H, $H_{23/25}$), 6.12 (s, 1H, $H_{23/25}$), 3.65 - 3.56 (s, 4H, H_1, H_{26}), 3.50 - 3.42 (m, 1H, H_{22}), 3.39 – 3.30 (m, 1H, H_{10}), 3.16 – 2.92 (m, 4H, H_7, H_{14}), 2.28 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 2.03 (t, $^3J_{17,18}$ = 7.4 Hz, 2H, H_{18}), 1.69 – 1.09 (m, 20H, H_4, H_5, H_6, H_{11}, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{21}), 0.95 (d, $^3J_{27,26}$ = 6.3 Hz, 3H, H_{27}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_9), 171.8 (C_2), 171.8 (C_{16}), 162.8 (C_{24}), 55.0 (C_{22}), 53.5 (C_{10}), 51.2 (C_1), 50.2 (C_{26}), 38.2 (C_7), 38.2 (C_{14}), 35.3 (C_{17}), 33.2 (C_{11}), 32.9 (C_3), 29.5 (C_{21}), 28.9 (C_6), 28.7 (C_{13}), 28.6 (C_{19}), 25.8 (C_5), 25.6 (C_{18}), 25.2 (C_{20}), 24.1 (C_4), 22.2 (C_{12}), 15.5 (C_{27}).

HRMS calculated for $C_{23}H_{43}N_5O_5$ + Na 492.3162. Found 492.3156.

Methyl 6-(2-(4-azidobenzamido)-6-(6-(5-methyl-2-oxoimidazolidin-4-yl) hexanamido)hexanamido)hexanoate (6)



Diisopropylethylamine (4.80 mmol, 0.83 mL) was added to a stirred solution of 4-Azidobenzoic acid (1.74 mmol, 284 mg) in tetrahydrofuran (17.4 mL) followed by HBTU (1.92 mmol, 728 mg) at room temperature. After 15 minutes **5** (1.92 mmol, 900 mg) was added. The reaction mixture was stirred for 18 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in dichloromethane (70.0 mL) and washed with hydrochloric acid (1M, 3 x 40.0 mL), saturated sodium hydrogen carbonate (3 x 40.0 mL) and saturated sodium chloride (40.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was dissolved in methanol (40.0 mL) and poured into diethyl ether (320 mL). The resulting precipitate was filtered to obtain **6** as yellow solid. Yield 695 mg (65 %).

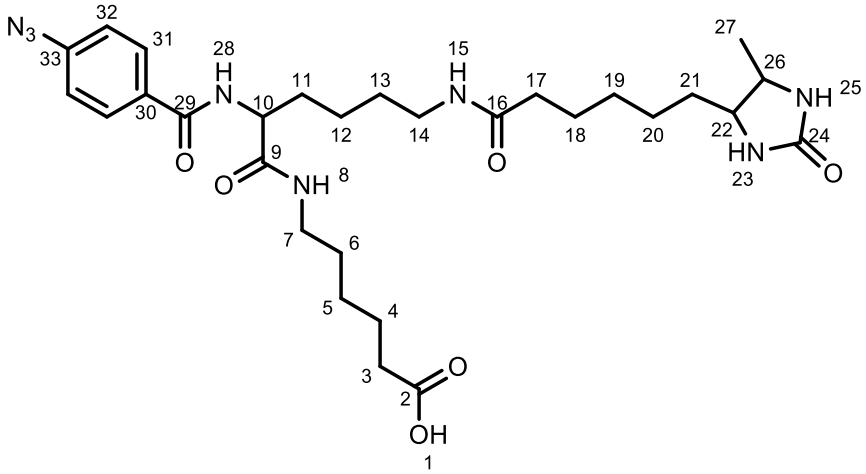
^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] =8.36 (d, $^3J_{28,10}$ = 7.9 Hz, 1H. H_{28}), 7.97 - 7.90 (m, 3H, H_8, H_{31}), 7.72 (t, $^3J_{15,14}$ = 5.5 Hz, 1H, H_{15}), 7.19 (d, $^3J_{32,31}$ = 8.6 Hz, 2H, H_{32}), 6.29 (s, 1H, $H_{23/25}$), 6.12 (s, 1H, $H_{23/25}$), 4.34 (q, $^3J_{10,11/28}$ = 7.6 Hz, 1H, H_{10}), 3.71 - 3.53 (m, 4H, H_1, H_{26}), 3.51 – 3.45 (m, 1H, H_{22}), 3.12 – 2.97 (m, 4H, H_7, H_{14}), 2.27 (t, $^3J_{3,4}$ = 7.3 Hz, 2H, H_3), 2.01 (t, $^3J_{17,18}$ = 7.3 Hz, 2H, H_{17}), 1.70 (q, $^3J_{11,12}$ = 8.1 Hz, $^3J_{11,10}$ = 7.6 Hz, 2H, H_{11}), 1.60 - 1.09 (m, 18H, H_4, H_5, H_6, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{21}), 0.95 (d, $^3J_{27,26}$ = 6.2 Hz, 3H, H_{27}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] =173.2 (C_9), 171.9 (C_2), 171.6 (C_{16}), 165.3 (C_{29}), 162.8 (C_{24}), 142.3 (C_{33}), 130.7 (C_{30}), 129.4 (C_{31}), 118.7 (C_{32}), 54.9 (C_{22}), 53.5 (C_{10}), 51.1

(C_1), 50.2 (C_{26}), 38.2 (C_7), 38.1 (C_{14}), 35.3 (C_{17}), 33.2 (C_3), 31.4 (C_{11}), 29.5 (C_{21}, C_{13}), 28.9 (C_6), 28.7 (C_{19}), 25.7 (C_5), 25.5 (C_{20}), 25.2 (C_{18}), 24.1 (C_4), 23.2 (C_{12}), 15.5 (C_{27}).

HRMS calculated for $C_{30}H_{46}N_8O_6$ + Na 637.3438. Found 637.3433.

6-(2-(4-azidobenzamido)-6-(6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamido) hexanamido)hexanoic acid (7)



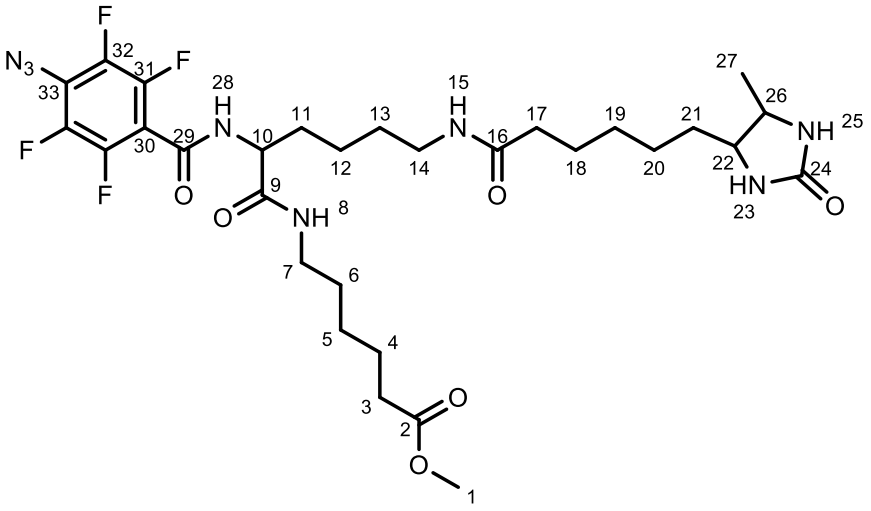
A solution of lithium hydroxide (0.59 mmol, 25.0 mg) in water (1.00 mL) was added to a stirred solution of **6** (0.12 mmol, 72.0 mg) in tetrahydrofuran (2.00 mL) at room temperature. The reaction mixture was refluxed for 1 hour at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (3.00 mL). The reaction mixture was washed with ethyl acetate (3.00 mL). Hydrochloric acid (2M, 3.00 mL) was added to the aqueous phase and the resulting precipitate was filtered to obtain **7** as a pale yellow solid. Yield 69.0 mg (98 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 12.03 (bs, 1H, H_1), 8.36 (d, $^3J_{28,10}$ = 7.8 Hz, 1H, H_{28}), 8.04 – 7.87 (m, 3H, H_8, H_{31}), 7.72 (t, $^3J_{15,14}$ = 5.7 Hz, 1H, H_{15}), 7.19 (d, $^3J_{32,31}$ = 8.8 Hz, 1H, H_{32}), 6.49 – 5.92 (m, 2H, H_{23}, H_{25}), 4.34 (q, $^3J_{10,11/28}$ = 7.5 Hz, 1H, H_{10}), 3.59 (p, $^3J_{26,25/27}$ = 6.6 Hz, 1H, H_{26}), 3.51 - 3.42 (m, 1H, H_{22}), 3.10 – 2.97 (m, 4H, H_7, H_{14}), 2.17 (t, $^3J_{3,4}$ = 7.3 Hz, 2H, H_3), 2.01 (t, $^3J_{17,18}$ = 7.4 Hz, 2H, H_{17}), 1.69 (q, $^3J_{11,10/12}$ = 7.8 Hz, 2H, H_{11}), 1.56 – 1.02 (m, 18H, H_4, H_5, H_6, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{21}), 0.95 (d, $^3J_{27,26}$ = 6.3 Hz, 3H, H_{27}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 174.4 (C_2), 171.9 (C_9), 171.6 (C_{16}), 165.3 (C_{29}), 162.8 (C_{24}), 142.3 (C_{33}), 130.8 (C_{30}), 129.5 (C_{31}), 118.7 (C_{32}), 55.0 (C_{22}), 53.5 (C_{10}), 50.2 (C_{26}), 38.4 (C_7), 38.2 (C_{14}), 35.4 (C_{17}), 33.6 (C_3), 31.4 (C_{21}), 29.5 (C_{11}), 28.9 (C_6, C_{13}), 28.7 (C_{19}), 25.9 (C_5), 25.6 (C_{18}), 25.2 (C_{20}), 24.2 (C_4), 23.2 (C_{12}), 15.5 (C_{27}).

HRMS calculated for $C_{29}H_{44}N_8O_6$ +Na 623.3282. Found 623.3276.

Methyl 6-(2-(4-azido-2,3,5,6-tetrafluorobenzamido)-6-(6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamido)hexanamido)hexanoate (8)



Diisopropylethylamine (2.40 mmol, 0.41 mL) was added to a stirred solution of 4-azido-2,3,5,6-tetrafluorobenzoic acid (0.96 mmol, 226 mg) in tetrahydrofuran (10.0 mL) followed by HBTU (1.06 mmol, 402 mg) at room temperature. After 15 minutes **5** (1.06 mmol, 498 mg) was added. The reaction mixture was stirred for 18 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in dichloromethane (50.0 mL) and washed with hydrochloric acid (1M, 3 x 30.0 mL), saturated sodium hydrogen carbonate solution (3 x 30.0 mL) and saturated sodium chloride solution (30.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was dissolved in methanol (30.0 mL) and poured into diethyl ether (240 mL). The resulting precipitate was filtered to obtain **8** as yellow solid. Yield 406 mg (62 %).

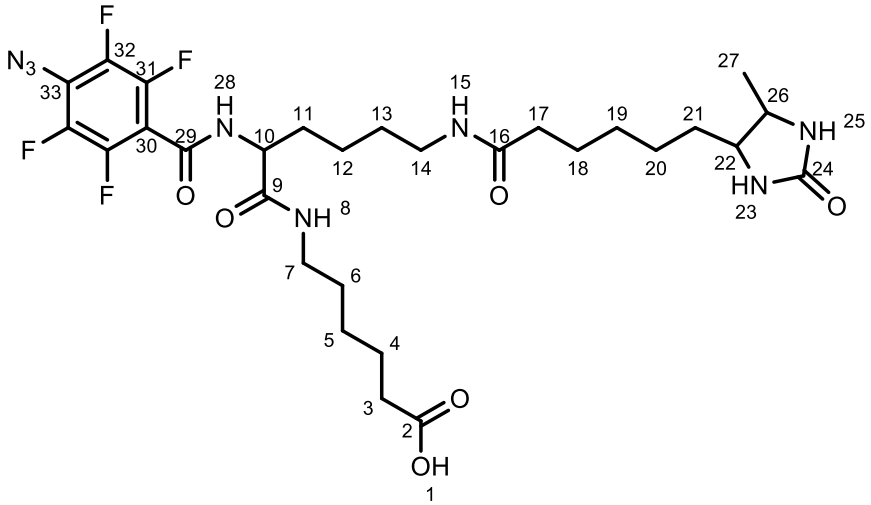
^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 9.04 (d, $^3J_{28,10}$ = 8.1 Hz, 1H, H_{10}), 8.05 (t, $^3J_{8,7}$ = 5.7 Hz, 1H, H_8), 7.72 (t, $^3J_{15,14}$ = 5.7 Hz, 1H, H_{15}), 6.29 (s, 1H, $H_{23/25}$), 6.12 (s, 1H, $H_{23/25}$), 4.39 (td, $^3J_{10,28}$ = 8.4 Hz, $^3J_{10,11}$ = 5.3 Hz, 1H, H_{10}), 3.62 - 3.56 (m, 4H, H_1, H_{26}), 3.50 – 3.42 (m, 1H, H_{22}), 3.18 – 2.91 (m, 4H, H_7, H_{14}), 2.28 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 2.02 (t, $^3J_{17,18}$ = 7.4 Hz, 2H, H_{17}), 1.76 – 1.05 (m, 20H, H_4, H_5, H_6, H_{11}, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{21}), 0.95 (d, $^3J_{27,26}$ = 6.2 Hz, 3H, H_{27}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_9), 171.9(C_2), 170.5 (C_{16}), 162.8 (C_{29}), 156.7 (C_{24}), 144.6 (C_{32}), 141.4 (C_{31}), 120.7 (C_{30}), 112.3 (C_{33}), 55.0 (C_{22}), 53.2 (C_{10}), 51.2

(C_1), 50.2 (C_{26}), 38.3 (C_7), 38.2 (C_{14}), 35.4 (C_{17}), 33.3 (C_3), 31.9 (C_{11}), 29.5 (C_{21}), 28.8 (C_6, C_{13}), 28.6 (C_{19}), 25.7 (C_5), 25.6 (C_{18}), 25.2 (C_{20}), 24.1 (C_4), 22.7 (C_{12}), 15.5 (C_{27}).

HRMS calculated for $C_{30}H_{42}F_4N_8O_6$ + Na 709.3061. Found 709.3056.

6-(2-(4-azido-2,3,5,6-tetrafluorobenzamido)-6-(6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamido)hexanamido)hexanoic acid (9)



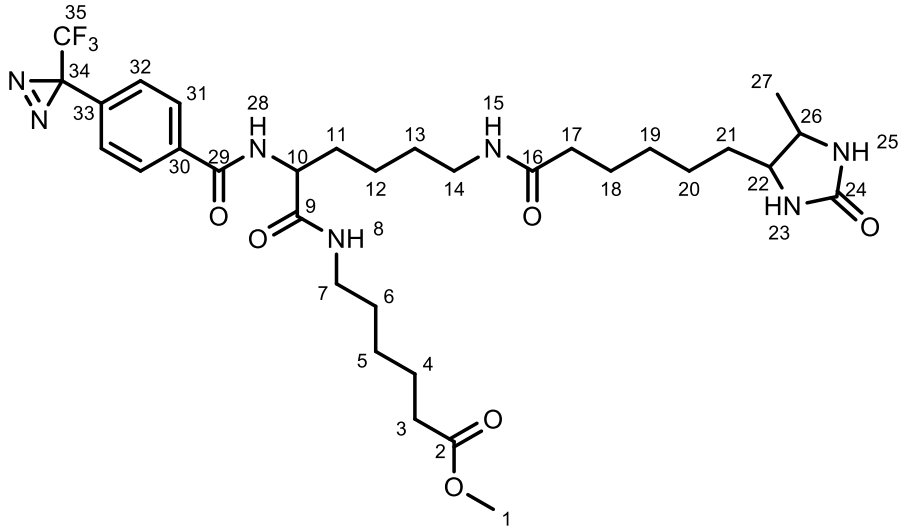
A solution of lithium hydroxide (2.70 mmol, 113 mg) in water (2.00 mL) was added to a stirred solution of **8** (0.54 mmol, 371 mg) in tetrahydrofuran (4.00 mL) at room temperature. The reaction mixture was refluxed for 1 hour at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (8.00 mL). The reaction mixture was washed with ethyl acetate (4.00 mL). Hydrochloric acid (2M, 4.00 mL) was added to the aqueous phase and the resulting precipitate was filtered to obtain **9** as a pale yellow solid. Yield 299 mg (82 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 12.00 (s, 1H, H_1), 9.04 (d, $^3J_{28,10}$ = 8.2 Hz, 1H, H_{28}), 8.05 (t, $^3J_{8,7}$ = 5.7 Hz, 1H, H_8), 7.72 (t, $^3J_{15,14}$ = 5.6 Hz, 1H, H_{15}), 6.30 (s, 1H, $H_{23/25}$), 6.12 (s, 1H, $H_{23/25}$), 4.39 (td, $^3J_{10,28}$ = 8.4 Hz, $^3J_{10,11}$ = 5.3 Hz, 1H, H_{10}), 3.63 - 3.57 (m, 1H, H_{26}), 3.52 – 3.40 (m, 1H, H_{22}), 3.17 – 2.90 (m, 4H, H_7, H_{14}), 2.18 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 2.02 (t, $^3J_{17,18}$ = 7.4 Hz, 2H, H_{17}), 1.72 – 1.09 (m, 20H, H_4, H_5, H_6, H_{11}, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{21}), 0.95 (d, $^3J_{27,26}$ = 6.4 Hz, 3H, H_{27}).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 174.4 (C_2), 171.9(C_9), 170.4 (C_{16}), 162.8 (C_{29}), 156.7 (C_{24}), 144.1 (C_{32}), 141.7 (C_{31}), 120.7 (C_{30}), 112.3 (C_{33}), 55.0 (C_{22}), 53.2 (C_{10}), 50.2 (C_{26}), 38.3 (C_7), 38.2 (C_{14}), 35.3 (C_{17}), 33.6 (C_3), 31.9 (C_{11}), 29.5 (C_{21}), 28.7 (C_6, C_{13}), 28.7 (C_{19}), 25.8 (C_5), 25.6 (C_{18}), 25.2 (C_{20}), 24.2 (C_4), 22.7 (C_{12}), 15.5 (C_{27}).

HRMS calculated for $C_{29}H_{40}F_4N_8O_6$ - H 671.2929. Found 671.2934.

Methyl 6-(6-(6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamido)-2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzamido)hexanamido)hexanoate (10)



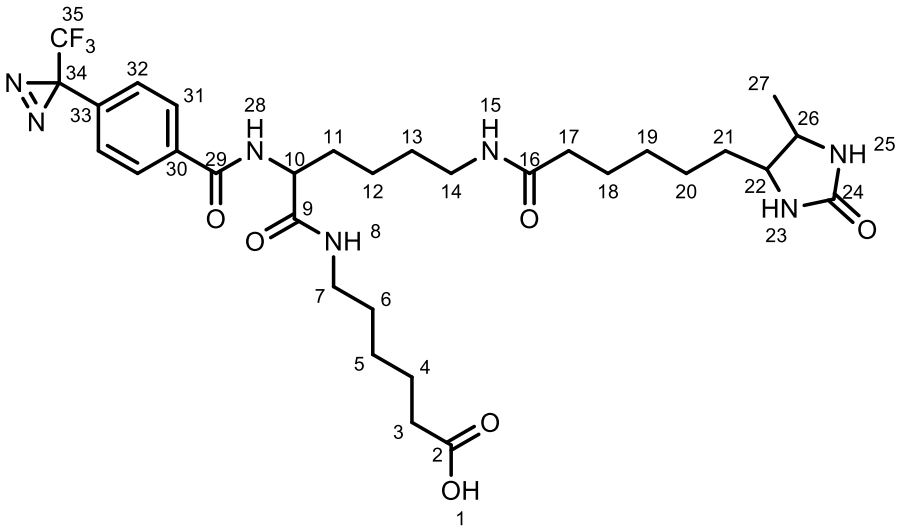
Diisopropylethylamine (2.40 mmol, 0.41 mL) was added to a stirred solution of 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid (0.96 mmol, 221 mg) in tetrahydrofuran (10.0 mL) followed by HBTU (1.06 mmol, 402 mg) at room temperature. After 15 minutes **5** (1.06 mmol, 498 mg) was added. The reaction mixture was stirred for 18 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in dichloromethane (50.0 mL) and washed with hydrochloric acid (1M, 3 x 30.0 mL), saturated sodium hydrogen carbonate solution (3 x 30.0 mL) and saturated sodium chloride solution (30.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The residue was dissolved in methanol (30.0 mL) and poured into diethyl ether (240 mL). The resulting precipitate was filtered to obtain **10** as yellow solid. Yield 435 mg (66 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.53 (d, $^3J_{28,10}$ = 7.9 Hz, 1H, H_{28}), 8.01 (d, $^3J_{32.31}$ = 8.6 Hz, 2H, H_{32}), 7.93 (t, $^3J_{8,7}$ = 5.7 Hz, 1H, H_8), 7.71 (t, $^3J_{15,14}$ = 5.7 Hz, 1H, H_{15}), 7.37 (d, $^3J_{31,32}$ = 8.1 Hz, 2H, H_{31}), 6.30 (s, 1H, $H_{23/25}$), 6.12 (s, 1H, $H_{23/25}$), 4.35 (td, $^3J_{10,28}$ = 8.2 Hz, $^3J_{10,11}$ = 5.9 Hz, 1H, H_{10}), 3.64 - 3.55 (m, 4H, H_1, H_{26}), 3.53 – 3.38 (m, 1H, H_{22}), 3.08 – 2.97 (m, 4H, H_7, H_{14}), 2.26 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 2.00 (t, $^3J_{17,18}$ = 7.4 Hz, 2H, H_{17}), 1.78 – 1.63 (m, 2H, H_{11}), 1.58 – 1.06 (m, 18H, H_4, H_5, H_6, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{21}), 0.94 (d, $^3J_{27,26}$ = 6.3 Hz, 3H, H_{27}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 173.2 (C_9), 171.9 (C_2), 171.4 (C_{16}), 165.2 (C_{29}), 162.8 (C_{24}), 135.7 (C_{33}), 130.2 (C_{30}), 128.5 (C_{32}), 126.2 (C_{31}), 123.6 (C_{35}), 119.9 (C_{34}), 55.0 (C_{22}), 53.5 (C_{10}), 51.1 (C_1), 50.2 (C_{26}), 38.3 (C_7), 38.1 (C_{14}), 35.3 (C_{17}), 33.2 (C_3), 31.3 (C_{11}), 29.5 (C_{21}), 28.9 (C_6), 28.7 (C_{13}), 28.7 (C_{19}), 25.7 (C_5), 25.5 (C_{18}), 25.2 (C_{20}), 24.1(C_4), 23.1 (C_{12}), 15.4 (C_{27}).

HRMS calculated for $C_{32}H_{46}F_3N_7O_6$ + Na 704.3360. Found 704.3354.

6-(6-(6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamido)-2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzamido)hexanamido)hexanoic acid (11)



A solution of lithium hydroxide (2.94 mmol, 123 mg) in water (2.00 mL) was added to a stirred solution of **10** (0.59 mmol, 401 mg) in tetrahydrofuran (4.00 mL) at room temperature. The reaction mixture was refluxed for 1 hour at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (10.0 mL). The reaction mixture was washed with ethyl acetate (5.00 mL). Hydrochloric acid (2M, 5.00 mL) was added to the aqueous phase and the resulting precipitate was filtered to obtain **11** as off-white solid. Yield 351 mg (89 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 11.98 (s, 1H, H_1), 8.53 (d, $^3J_{28,10}$ = 7.8 Hz, 1H, H_{28}), 8.01 (d, $^3J_{32,31}$ = 8.6 Hz, 2H, H_{32}), 7.94 (t, $^3J_{8,7}$ = 5.7 Hz, 1H, H_8), 7.72 (t, $^3J_{15,14}$ = 5.6 Hz, 1H, H_{15}), 7.37 (d, $^3J_{31,32}$ = 8.1 Hz, 2H, H_{31}), 6.30 (s, 1H, $H_{23/25}$), 6.12 (s, 1H, $H_{23/25}$), 4.35 (td, $^3J_{10,28}$ = 8.2 Hz, $^3J_{10,11}$ = 5.7 Hz, 1H, H_{10}), 3.64 – 3.55 (m, 1H, H_{26}), 3.49 – 3.44 (m, 1H, H_{22}), 3.10 – 2.96 (m, 4H, H_7, H_{14}), 2.17 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_{17}), 2.00 (t, $^3J_{17,18}$ = 7.4 Hz, 2H, H_{17}), 1.77 – 1.62 (m, 2H, H_{11}), 1.54 – 1.08 (m, 18H, H_4, H_5, H_6, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{21}), 0.95 (d, $^3J_{27,26}$ = 6.3 Hz, 3H, H_{27}).

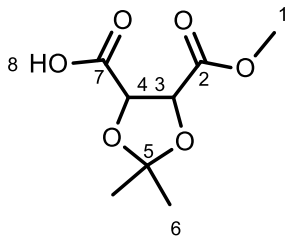
^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 174.4 (C_2), 171.9 (C_9), 171.4 (C_{16}), 165.2 (C_{29}), 162.8 (C_{24}), 135.8 (C_{33}), 130.2 (C_{30}), 128.5 (C_{32}), 126.2 (C_{31}), 123.6 (C_{35}), 119.9 (C_{34}), 55.0 (C_{22}), 53.5 (C_{10}), 50.2 (C_{26}), 38.4 (C_7), 38.1 (C_{14}), 35.3 (C_{17}), 33.6 (C_3), 31.3 (C_{11}), 29.5 (C_{21}), 28.9 (C_6), 28.8 (C_{13}), 28.7 (C_{19}), 25.9 (C_5), 25.5 (C_{18}), 25.2 (C_{20}), 24.2 (C_4), 23.1 (C_{12}), 15.4 (C_{27}).

HRMS calculated for $C_{31}H_{44}F_3N_7O_6$ - 2H + Na 688.3047. Found 688.3052.

7.3. Cleavable linkers

The synthesis of the phenyl azide cleavable ABAL probe described in **section 7.3** and **7.4** was carried out with assistance of Christian Deutsch, who I supervised during his Bachelor thesis. The synthesis of this compound is also described in his Bachelor thesis.[133]

5-(methoxycarbonyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid (12)



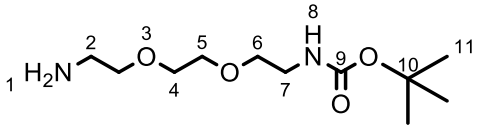
To a solution of (−)-Dimethyl 2,3-*O*-isopropylidene-L-tartrate (54.2 mmol, 10.0 mL) in methanol (90.0 mL) potassium hydroxide (54.2 mmol, 3.58 g) in methanol (90.0 mL) was added drop wise during a time period of two hours. The reaction mixture was stirred for three hours and concentrated under reduced pressure. The residue was dissolved in diethyl ether (100 mL) and extracted with saturated sodium hydrogen carbonate solution (3 x 50.0 mL). The aqueous phase was acidified with hydrochloric acid (1M, pH 2) and extracted with diethyl ether (3 x 100 mL). The organic layer was washed with saturated sodium chloride solution (100 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give **12** as colourless oil. Yield 7.53 g (68 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 13.26 (s, 1H, H_8), 4.79 (d, $^3J_{4,3}$ = 5.0 Hz, 1H, H_4), 4.73 (d, $^3J_{3,4}$ = 5.0 Hz, 1H, H_3), 3.71 (s, 3H, H_1), 1.38 (s, 6H, H_6).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 170.9 (C_7), 170.1 (C_2), 112.6 (C_5), 76.6 (C_3), 76.5 (C_4), 52.4 (C_1), 26.4 (C_6).

HRMS calculated for $C_8H_{12}O_6$ - H 203.0556. Found 203.0561.

***Tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (13)**



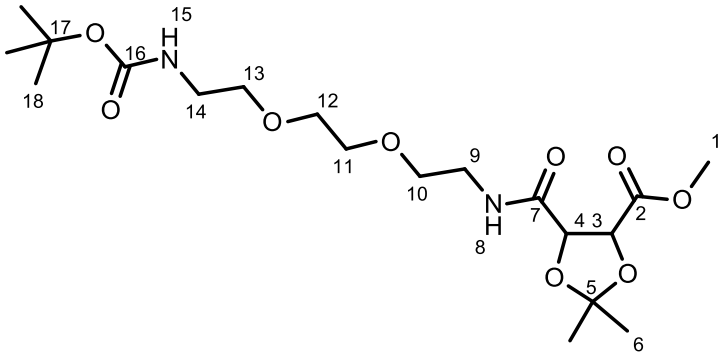
To a solution of 2,2'-(ethylendioxy)bis(ethylamine) (376 mmol, 55.8 mL) in 1,4-dioxan (400 mL) di-*tert*-butyl dicarbonate (53.6 mmol, 11.7 g) in 1,4-dioxan (240 mL) was added drop wise during a time period of two hours. The reaction mixture was stirred for 15 hours and concentrated under reduced pressure. The residue was dissolved in dichloromethane (300 mL) and washed with water (3 x 200 mL) and saturated sodium chloride solution (200 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **13** as pale yellow oil. Yield 10.3 g (77 %).

^{1}H-NMR (300 MHz, DMSO-d_6) δ [ppm] = 6.76 (t, $^3J_{8,7}$ = 6.0 Hz , 1H, H_8), 3.49 (s, 4H, H_4, H_5), 3.40 – 3.33 (m, 4H, H_3, H_6), 3.05 (q, $^3J_{7,6/8}$ = 6.0 Hz, 2H, H_7), 2.63 (t, $^3J_{2,3}$ = 5.8 Hz, 2H, H_2), 1.37 (s, 9H, H_{11}).

^{13}C-NMR (75 MHz, DMSO-d_6) δ [ppm] = 155.5 (C_9), 77.5 (C_{10}), 73.1 (C_3), 69.5, 69.5 (C_4, C_5), 69.1 (C_6), 41.3 (C_2), 39.7 (C_7), 28.2 (C_{11}).

HRMS calculated for $C_{11}H_{24}N_2O_4$ + H 249.1814. Found 249.1809.

Methyl 5-((2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylate (14)



Diisopropylethylamine (39.3 mmol, 6.80 mL) was added to a stirred solution of **1** (15.7 mmol, 3.21 g) in tetrahydrofuran (157 mL) followed by HBTU (17.3 mmol, 6.56 g) at room temperature. After 15 minutes **13** (17.3 mmol, 4.30 g) was added. The reaction mixture was stirred for 48 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (150 mL) and washed with hydrochloric acid (1M, 3 x 100 mL), saturated sodium

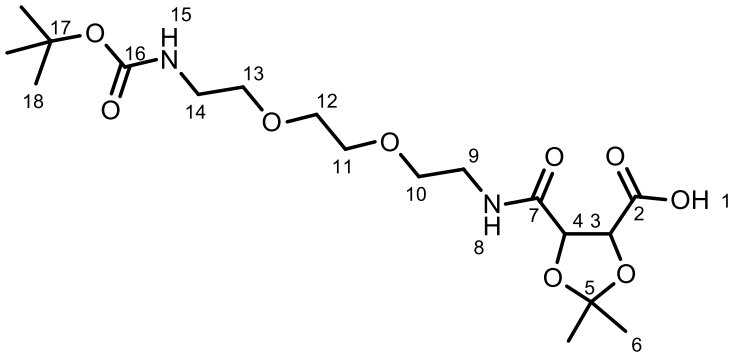
hydrogen carbonate solution (3 x 100 mL) and saturated sodium chloride solution (100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using cyclohexane and ethyl acetate (1:3) as eluent to give **14** as a colourless oil. Yield 5.30 g (78 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.05 (t, $^3J_{8,9}$ = 5.7 Hz, 1H, H_8), 6.73 (t, $^3J_{15,14}$ = 5.8 Hz, 1H, H_{15}), 4.71 (d, $^3J_{4,3}$ = 5.4 Hz, 1H, H_4), 4.61 (d, $^3J_{3,4}$ = 5.4 Hz, 1H, H_3), 3.70 (s, 3H, H_1), 3.49 (s, 4H, H_{11}, H_{12}), 3.44 (t, $^3J_{10,9}$ = 5.8 Hz, 2H, H_{10}), 3.37 (t, $^3J_{13,14}$ = 6.2 Hz, 2H, H_{13}), 3.33 – 3.17 (m, 2H, H_9), 3.06 (q, $^3J_{14,13/15}$ = 6.0 Hz, 2H, H_{14}), 1.39 (s, 6H, H_6), 1.37 (s, 9H, H_{18}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 170.3 (C_7), 168.6 (C_2), 155.6 (C_{16}), 112.5 (C_5), 77.7, 77.6, 76.4 (C_3, C_4, C_{17}), 69.5, 69.2, 68.6 (C_{10}, C_{11}, C_{12}, C_{13}), 52.3 (C_7), 39.7 (C_9), 38.7 (C_{14}), 28.2 (C_{18}), 26.3 (C_6).

HRMS calculated for $C_{19}H_{34}N_2O_9$ + Na 457.2161. Found 457.2157.

5-((2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid (15)



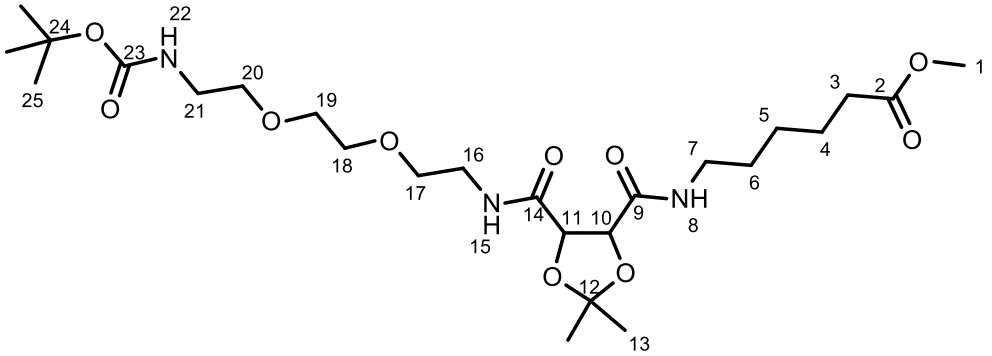
Lithium hydroxide (81.1 mmol, 3.40 g) in water (65.0 mL) was added to a solution of **14** (16.2 mmol, 7.04 g) in tetrahydrofuran (110 mL) at room temperature. The reaction mixture was refluxed for 1 hour at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (140 mL). The reaction mixture was washed with ethyl acetate (2x 70.0 mL). The aqueous phase was acidified with hydrochloric acid (1M, pH 2) and extracted with dichloromethane (3x 140 mL). The organic phase was washed with a saturated sodium chloride solution (140 mL), dried ($MgSo_4$) and concentrated under reduced pressure to give **15** as pale orange oil. Yield 4.83 g (71 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 13.02 (s, 1H, H_1), 8.04 (t, $^{3}J_{8,9}$ = 5.7 Hz, 1H, H_8), 6.73 (t, $^{3}J_{15,14}$ = 5.8 Hz, 1H, H_{15}), 4.60 – 4.54 (m, 2H, H_3, H_4), 3.49 (s, 4H, H_{11}, H_{12}), 3.44 (t, $^{3}J_{10,9}$ = 5.9 Hz, 2H, H_{10}), 3.37 (t, $^{3}J_{13,14}$ = 6.1 Hz, 2H, H_{13}), 3.33 – 3.16 (m, 2H, H_9), 3.06 (q, $^{3}J_{14,13/15}$ = 6.0 Hz, 2H, H_{14}), 1.40 – 1.36 (m, 15H, H_6, H_{18}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 171.3 (C_7), 168.8 (C_2), 155.5 (C_{16}), 112.1 (C_5), 77.7, 77.6, 76.6 (C_3, C_4, C_{17}), 69.4, 69.4, 69.1, 68.6 (C_{10}, C_{11}, C_{12}, C_{13}), 39.6 (C_9), 38.4 (C_{14}), 28.2 (C_{18}), 26.3, 26.3 (C_6).

HRMS calculated for $C_{18}H_{32}N_2O_9$ + Na 443.2005. Found 443.2000.

Methyl 6-(5-((2,2-dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-yl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxamido)hexanoate (16)



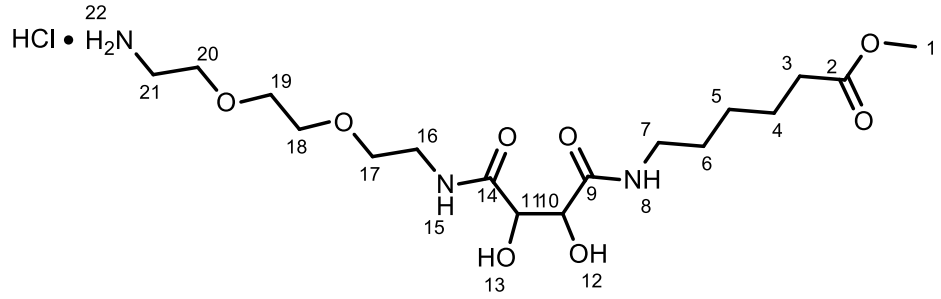
Diisopropylethylamine (46.1 mmol, 7.98 mL) was added to a stirred solution of **15** (46.1 mmol, 7.98 mL) in tetrahydrofuran (185 mL) followed by HBTU (20.3 mmol, 7.69 g) at room temperature. After 15 minutes **1** (20.3 mmol, 3.68 g) was added. The reaction mixture was stirred for 96 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (220 mL) and washed with hydrochloric acid (1M, 3x 140 mL), saturated sodium hydrogen carbonate solution (3x 140 mL) and saturated sodium chloride solution (100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure to give **16** as pale orange oil. Yield 8.59 g (85 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.09 – 7.99 (m, 2H, H_8, H_{15}), 6.74 (t, $^{3}J_{22,21}$ = 5.7 Hz, 1H, H_{22}), 4.50 (d, $^{3}J_{11,10}$ = 6.0 Hz, 1H, H_{11}), 4.46 (d, $^{3}J_{10,11}$ = 6.0 Hz, 1H, H_{10}), 3.58 (s, 3H, H_1), 3.49 (s, 4H, H_{18}, H_{19}), 3.44 (t, $^{3}J_{17,16}$ = 5.9 Hz, 2H, H_{17}), 3.37 (t, $^{3}J_{20,21}$ = 6.1 Hz, 2H, H_{20}), 3.30 – 3.14 (m, 2H, H_{16}), 3.14 – 2.95 (m, 4H, H_7, H_{21}), 2.28 (t, $^{3}J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.60 – 1.20 (m, 21H, H_4, H_5, H_6, H_{13}, H_{18}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_2), 169.1(C_9), 168.7 (C_{14}), 155.6 (C_{23}), 111.7 (C_{12}), 77.7, 77.6, 77.5 (C_{10}, C_{11}, C_{24}), 69.5, 69.5, 69.2, 68.7 (C_{17}, C_{18}, C_{19}, C_{20}), 51.2 (C_1), 39.7 (C_{16}), 38.4, 38.3 (C_7, C_{21}), 33.2 (C_3), 28.6 (C_6), 28.2 (C_{25}), 26.2, 26.2 (C_{13}), 25.7 (C_5), 24.1 (C_4).

HRMS calculated for $C_{25}H_{45}N_3O_{10}$ + H 548.3183. Found 548.3178.

Methyl 1-amino-11,12-dihydroxy-10,13-dioxo-3,6-dioxa-9,14-diazaicosan-20-oate hydrochloric salt (17)



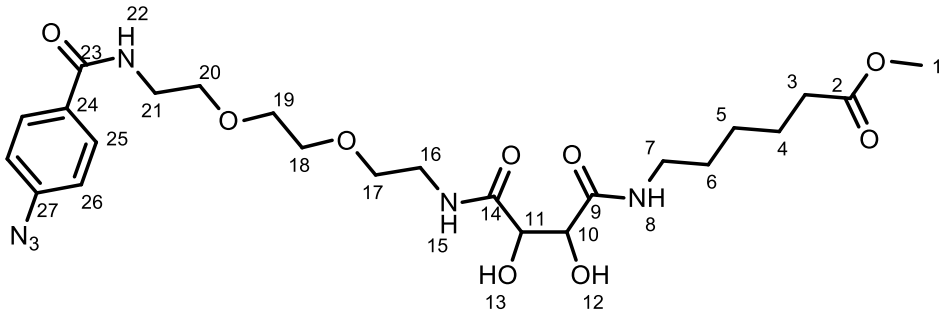
Acetyl chloride (40.4 mmol, 2.90 g) was added to **16** (13.5 mmol, 7.37 g) in methanol (135 mL) at 0 °C and stirred for 15 hours at room temperature. The reaction mixture was concentrated under reduced pressure to give **17** as pale orange oil. Yield 5.95 g (99 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.05 (s, 2H, H_{22}), 7.71 – 7.63 (m, 2H, H_8, H_{15}), 5.26 (s, 2H, H_{12}, H_{13}), 4.24 – 4.19 (m, 2H, H_{11}, H_{10}), 3.66 – 3.60 (m, 2H, H_{16}), 3.59 – 3.54 (m, 7H, H_1, H_{18}, H_{19}), 3.45 (t, $^3J_{17,16}$ = 5.9 Hz, 2H, H_{17}), 3.35 - 3.21 (m, 2H, H_{21}), 3.08 (q, $^3J_{7,6/8}$ = 6.7 Hz, 2H, H_7), 2.99 – 2.90 (m, 2H, H_{22}), 2.28 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.56 – 1.18 (m, 6H, H_4, H_5, H_6).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_2), 172.1 (C_{14}), 171.7 (C_9), 72.5, 72.5 (C_{10}, C_{11}), 69.6, 69.4, 68.9, 66.6 (C_{17}, C_{18}, C_{19}, C_{20}), 51.2 (C_1), 38.5, 38.3, 38.2 (C_7, C_{16}, C_{21}), 33.2 (C_3), 28.8 (C_6), 25.7 (C_5), 24.2 (C_4).

HRMS calculated for $C_{17}H_{33}N_3O_8$ + H 408.2346. Found 408.2340.

Methyl 1-(4-azidophenyl)-13,14-dihydroxy-1,12,15-trioxo-5,8-dioxa-2,11,16-triazadocosan-22-oate (18)



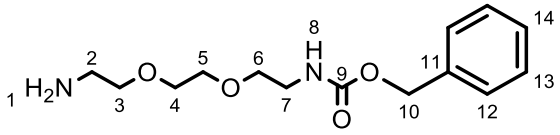
Diisopropylethylamine (4.00 mmol, 0.70 mL) was added to a stirred solution of 4-azidobenzoic acid (1.76 mmol, 0.78 g) in tetrahydrofuran (16.0 mL) followed by HBTU (1.76 mmol, 0.67 g) at room temperature. After 15 minutes **17** (1.60 mmol, 0.26 g) was added. The reaction mixture was stirred for 18 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (25.0 mL) and washed with hydrochloric acid (1M, 2 x 10.0 mL), saturated sodium hydrogen carbonate solution (3 x 10.0 mL) and saturated sodium chloride solution (10.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using dichloromethane and methanol (9:1) as eluent to give **18** as a yellow oil. Yield 397 mg (45 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 8.52 (t, $^3J_{22,21}$ = 5.5 Hz, 1H, H_{22}), 7.92 - 7.88 (m, 2H, H_{25}), 7.63 (q, $^3J_{8/15,7/16}$ = 6.0 Hz, 2H, H_8, H_{15}), 7.21 – 7.17 (m, 2H, H_{26}), 5.58 (d, $^3J_{12/13,10/11}$ = 7.3 Hz, 1H, $H_{12/13}$), 5.47 (d, $^3J_{12/13,10/11}$ = 7.3 Hz, 1H, $H_{12/13}$), 4.23 - 4.20 (m, 2H, H_{10}, H_{11}), 3.58 (s, 3H, H_1), 3.56 – 3.50 (m, 6H, H_{18}, H_{19}, H_{20}), 3.45 – 3.38 (m, 4H, H_{17}, H_{21}), 3.31 – 3.16 (m, 2H, H_{16}), 3.08 (q, $^3J_{7,8/6}$ = 6.7 Hz, 2H, H_7), 2.28 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.51 (p, $^3J_{4,3/6}$ = 7.4 Hz, 2H, H_4), 1.45 – 1.37 (m, 2H, H_6), 1.29 – 1.20 (m, 2H, H_5).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_2), 172.1(C_9), 171.7 (C_{14}), 165.3 (C_{23}), 142.2 (C_{27}), 130.9 (C_{24}), 129.1 (C_{25}), 118.9 (C_{26}), 72.5, 72.5 (C_{10}, C_{11}), 69.5, 69.5, 68.9, 68.9 (C_{17}, C_{18}, C_{19}, C_{20}), 51.1 (C_1), 39.2, 38.2, 38.1 (C_7, C_{16}, C_{21}), 33.2 (C_3), 28.8 (C_6), 25.7 (C_5), 24.1 (C_4).

HRMS calculated for $C_{24}H_{36}N_6O_9$ + Na 575.2442. Found 575.2436.

Benzyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate **(19)**



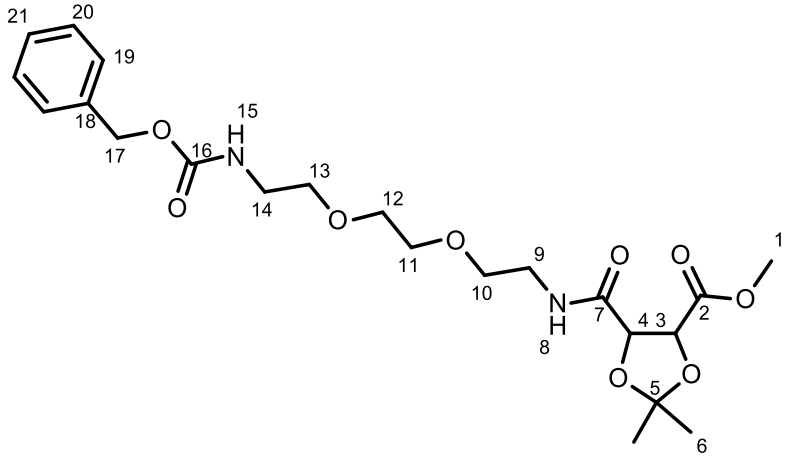
To a solution of 2,2'-(ethylendioxy)bis(ethylamine) (100 mmol, 14.6 mL) in dichloromethane (72.0 mL) benzyl chloroformate (10.0 mmol, 1.42 mL) in dichloromethane (50.0 mL) was added drop wise and the reaction mixture was stirred for 21 hours at 0 °C. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in dichloromethane (50.0 mL) and washed with water (3 x 25.0 mL) and saturated sodium chloride solution (25.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using ethyl acetate and methanol (9:1) as eluent to give **19** as yellow oil. Yield 1.74 g (62 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 7.40 – 7.25 (m, 5H, H_{12}, H_{13}, H_{14}), 5.01 (s, 2H, H_{10}), 3.52 – 3,47 (m, 4H, H_4, H_5), 3.43 (t, $^3J_{3,2}$ = 6.0 Hz, 2H, H_3), 3.34 (t, $^3J_{6,7}$ = 5.8 Hz, 2H, H_6), 3.21 – 3.10 (m, 2H, H_2), 2.63 (t, $^3J_{7,6}$ = 5.8 Hz, 2H, H_7).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 156.1 (C_9), 137.2 (C_{11}), 128.3, 127.7 (C_{12}, C_{13}, C_{14}), 73.0 (C_6), 69.5 (C_4, C_5), 69.1 (C_3), 65.2 (C_{10}), 41.3 (C_7), 40.2 (C_2).

HRMS calculated for $C_{14}H_{22}N_2O_4$ + H 283.1659. Found 283.1652.

Methyl 2,2-dimethyl-5-((3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-yl)carbamoyl)-1,3-dioxolane-4-carboxylate (20)



Diisopropylethylamine (35.5 mmol, 6.10 mL) was added to a stirred solution of **12** (14.2 mmol, 2.90 g) in tetrahydrofuran (142 mL) followed by HBTU (15.6 mmol, 5.92 g)

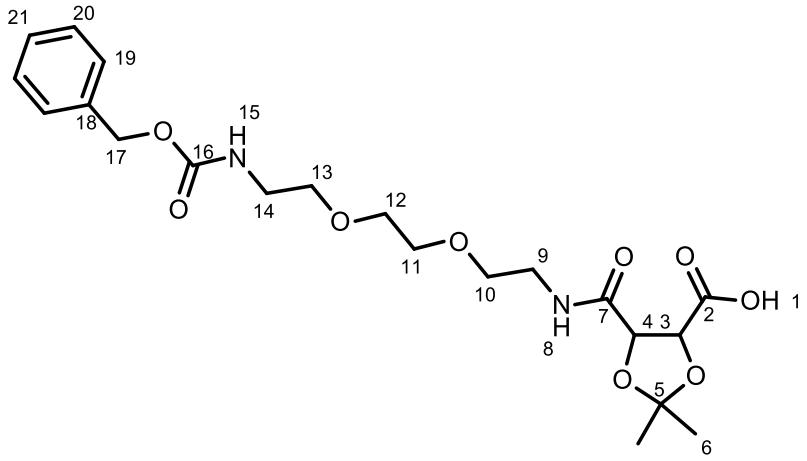
at room temperature. After 15 minutes **19** (15.6 mmol, 4.41 g) was added. The reaction mixture was stirred for 96 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (150 mL) and washed with hydrochloric acid (1M, 2 x 100 mL), saturated sodium hydrogen carbonate solution (2 x 100 mL) and saturated sodium chloride solution (100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using cyclohexane and ethyl acetate (1:3) as eluent to give **20** as a yellow oil. Yield 5.31 g (80 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.05 (t, $^3J_{8,9}$ = 5.7 Hz, 1H, H_8), 7.41 – 7.20 (m, 6H, H_{15}, H_{19}, H_{20}, H_{21}), 5.01 (s, 2H, H_{17}), 4.71 (d, $^3J_{4,3}$ = 5.4 Hz, 1H H_4), 4.61 (d, $^3J_{3,4}$ = 5.4 Hz, 1H, H_3), 3.69 (s, 3H, H_1), 3.52 – 3.10 (m, 12H, H_9, H_{10}, H_{11}, H_{12}, H_{13}, H_{14}), 1.39 (s, 6H, H_6).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 170.3 (C_7), 168.6 (C_2), 156.1 (C_{16}), 137.2 (C_{18}), 128.3, 127.7 (C_{19}, C_{20}, C_{21}), 112.5 (C_5), 77.7 (C_4), 76.4 (C_3), 69.5, 69.1, 68.6 (C_{10}, C_{11}, C_{12}, C_{13}), 65.2 (C_{17}), 52.3 (C_1), 40.1 (C_9), 38.4 (C_{14}), 26.2 (C_6).

HRMS calculated for $C_{22}H_{32}N_2O_9$ + H 469.2186. Found 469.2181.

2,2-dimethyl-5-((3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-yl)carbamoyl)-1,3-dioxolane-4-carboxylic acid (21)



Lithium hydroxide (56.7 mmol, 2.38 g) in water (38.0 mL) was added to a solution of **20** (11.3 mmol, 5.31 g) in tetrahydrofuran (76.0 mL) at room temperature. The reaction mixture was refluxed for 2 hours at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (100 mL). The reaction

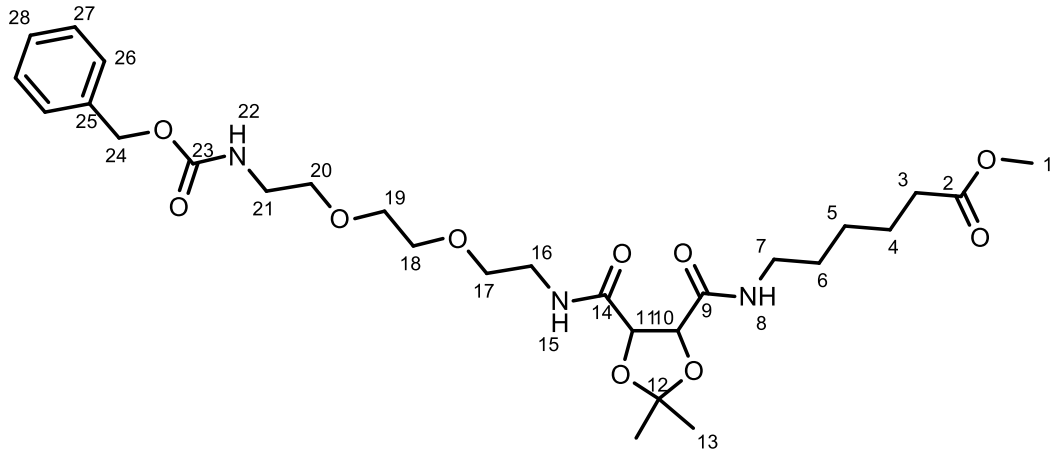
mixture was washed with ethyl acetate (50 mL). The aqueous phase was acidified with hydrochloric acid (1M, pH 2) and extracted with ethyl acetate (3 x 100 mL). The organic phase was washed with a saturated sodium chloride solution (100 mL), dried ($MgSo_4$) and concentrated under reduced pressure to give **21** as yellow oil. Yield 4.92 g (96 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.03 (t, $^3J_{8,9}$ = 5.7 Hz, 1H, H_8), 7.40 – 7.19 (m, 6H, H_{15}, H_{19}, H_{20}, H_{21}), 5.00 (s, 2H, H_{17}), 4.61 – 4.53 (m, 2H, H_3, H_4), 3.52 – 3.09 (m, 12H, H_9, H_{10}, H_{11}, H_{12}, H_{13}, H_{14}), 1.38 - 1.37 (m, 6H, H_6).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 171.3 (C_2), 168.9 (C_7), 156.2 (C_{16}), 137.2 (C_{18}), 128.3, 127.7 (C_{19}, C_{20}, C_{21}), 112.1 (C_5), 77.7 (C_4), 76.6 (C_3), 69.5, 69.5, 69.1, 68.6 (C_{10}, C_{11}, C_{12}, C_{13}), 65.2 (C_{17}), 40.2 (C_9), 38.5 (C_{14}), 26.3, 26.3 (C_6).

HRMS calculated for $C_{21}H_{30}N_2O_9$ - H 453.1873. Found 453.1879.

Methyl 6-(2,2-dimethyl-5-((3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-yl)carbamoyl)-1,3-dioxolane-4-carboxamido)hexanoate (22)



Diisopropylethylamine (5.28 mmol, 0.91 mL) was added to a stirred solution of **21** (2.11 mmol, 0.96 mL) in tetrahydrofuran (22.0 mL) followed by HBTU (2.32 mmol, 880 mg) at room temperature. After 15 minutes **1** (2.32 mmol, 340 mg) was added. The reaction mixture was stirred for 96 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (50.0 mL) and washed with hydrochloric acid (1M, 2 x 25.0 mL), saturated sodium hydrogen carbonate solution (2 x 25.0 mL) and saturated sodium chloride solution (25.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on

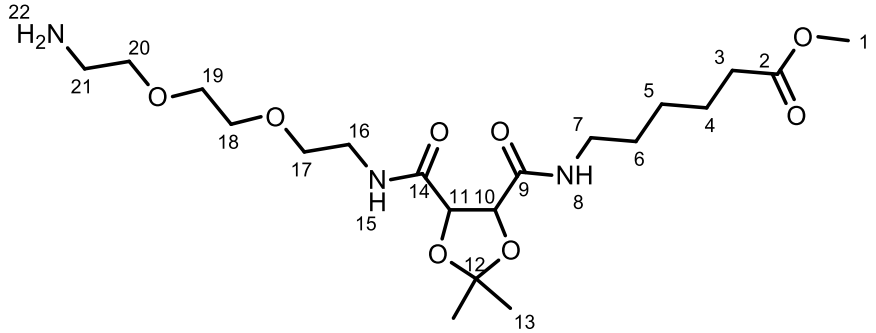
silica gel using dichloromethane, ethyl acetate and methanol (9:1:0 → 9:0:1) as eluent to give **22** as a yellow oil. Yield 850 mg (69 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 8.06 (t, $^3J_{8,7}$ = 5.8 Hz, 1H, H_8), 8.01 (t, $^3J_{15,16}$ = 5.8 Hz, 1H, H_{15}), 7.39 – 7.27 (m, 5H, H_{26}, H_{27}, H_{28}), 7.24 (t, $^3J_{22,21}$ = 5.9 Hz, 1H, H_{22}), 5.00 (s, 2H, H_{24}), 4.49 (d, $^3J_{11,10}$ = 6.1 Hz, 1H, H_{11}), 4.46 (d, $^3J_{10,11}$ = 6.0 Hz, 1H, H_{10}), 3.57 (s, 3H, H_1), 3.52 – 3.46 (m, 4H, H_{18}, H_{19}), 3.44 – 3.39 (m, 4H, H_{17}, H_{20}), 3.34 – 2.94 (m, 6H, H_7, H_{16}, H_{21}), 2.27 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.57 – 1.19 (m, 12H, H_4, H_5, H_6, H_{13}).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_2), 169.1 (C_9), 168.7 (C_{14}), 156.1 (C_{23}), 137.2 (C_{25}), 128.3, 127.7 (C_{26}, C_{27}, C_{28}), 111.7 (C_{12}), 77.6, 77.5 (C_{10}, C_{11}), 69.5, 69.4, 69.1, 68.6 (C_{17}, C_{18}, C_{19}, C_{20}), 65.2 (C_{24}), 51.1 (C_1), 40.1 (C_{16}), 38.4 (C_{21}), 38.3 (C_7), 33.2 (C_3), 28.6 (C_6), 26.1 (C_5), 25.7 (C_{13}), 24.1 (C_4).

HRMS calculated for $C_{28}H_{43}N_3O_{10}$ + H 582.3026. Found 582.3021.

Methyl 6-(5-((2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxamido)hexanoate (23)



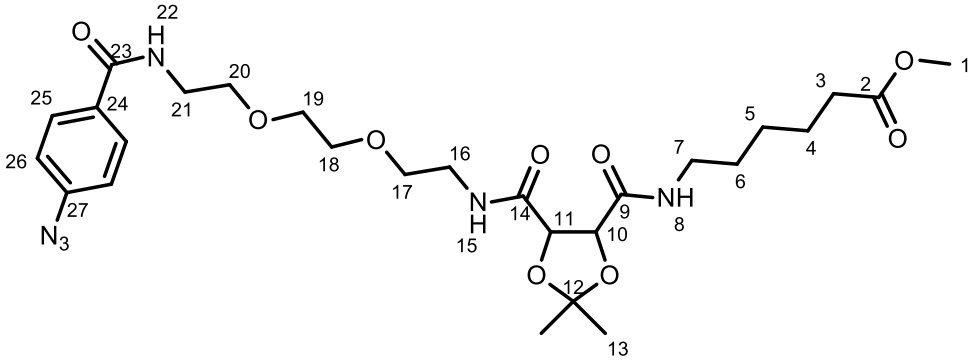
Palladium on activated carbon (5 % Pd) (wt 10 %, 85.0 mg) was added to a solution of **22** (1.46 mmol, 850 mg) in ethanol (15.0 mL) at room temperature. The reaction mixture was purged with argon and then hydrogen was bubbled through the solution for 2 hours using a balloon. The reaction mixture was filtered over Celite and concentrated under reduced pressure to give **23** as a yellow oil. Yield 640 mg (98 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 8.08 (t, $^3J_{8,7}$ = 5.9 Hz, 1H, H_8), 8.03 (t, $^3J_{15,16}$ = 5.9 Hz, 1H, H_{15}), 4.50 (d, $^3J_{11,10}$ = 6.1 Hz, 1H, H_{11}), 4.46 (d, $^3J_{10,11}$ = 6.0 Hz, 1H, H_{10}), 3.58 (s, 3H, H_1), 3.54 – 3.47 (m, 4H, H_{18}, H_{19}), 3.44 (t, $^3J_{17,16}$ = 5.9 Hz, 2H, H_{17}), 3.41 – 2.94 (m, 6H, H_7, H_{16}, H_{20}), 2.66 (t, $^3J_{21,20}$ = 5.8 Hz, 2H, H_{21}), 2.28 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.57 - 1.19 (m, 12H, H_4, H_5, H_6, H_{13}).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 173.2 (C_2), 169.1(C_9), 168.7 (C_{14}), 111.7 (C_{12}), 77.6, 77.5 (C_{10}, C_{11}), 72.6 (C_{20}), 69.5 (C_{18}, C_{19}), 68.6 (C_{17}), 51.1 (C_1), 41.1 (C_{21}), 38.4 (C_{16}), 38.3 (C_7), 33.2 (C_3), 28.8 (C_6), 26.2 (C_5), 25.7 (C_{13}), 24.1(C_4).

HRMS calculated for $C_{20}H_{37}N_3O_8$ + Na 470.2479. Found 470.2473.

Methyl 6-(5-((2-(2-(2-(4-azidobenzamido)ethoxy)ethoxy)ethyl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxamido)hexanoate (24)



Diisopropylethylamine (3.25 mmol, 0.60 mL) was added to a stirred solution of 4-Azidobenzoic acid (1.30 mmol, 212 mg) in tetrahydrofuran (13.0 mL) followed by HBTU (1.43 mmol, 542 mg) at room temperature. After 15 minutes **23** (1.43 mmol, 638 mg) was added. The reaction mixture was stirred for 96 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (20.0 mL) and washed with hydrochloric acid (1M, 2 x 10.0 mL), saturated sodium hydrogen carbonate solution (2 x 10.0 mL) and saturated sodium chloride solution (10.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using dichloromethane and methanol (98:2 → 9:1) as eluent to give **24** as a yellow oil. Yield 707 mg (92 %).

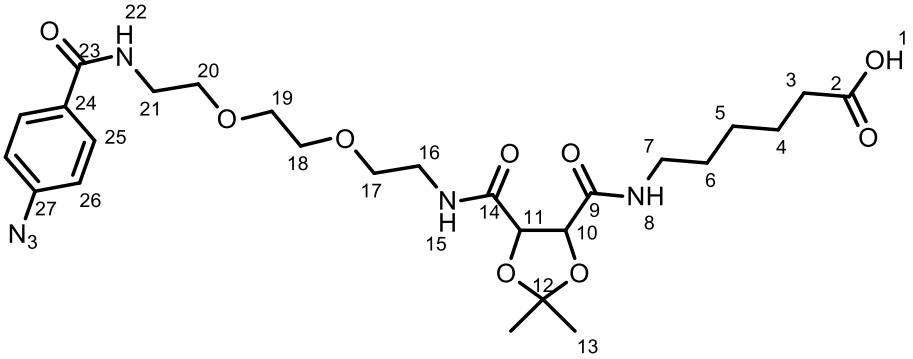
^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.51 (t, $^3J_{22,21}$ = 5.6 Hz, 1H, H_{22}), 8.06 (t, $^3J_{8,7}$ = 5.9 Hz, 1H, H_8), 8.01 (t, $^3J_{15,16}$ = 5.8 Hz, 1H, H_{15}), 7.92 – 7.87 (m, 2H, H_{25}), 7.21 – 7.17 (m, 2H, H_{26}), 4.50 (d, $^3J_{11,10}$ = 6.0 Hz, 1H, H_{11}), 4.46 (d, $^3J_{10,11}$ = 6.0 Hz, 1H, H_{11}), 3.57 (s, 3H, H_1), 3.55 - 3.49 (m, 6H, H_{18}, H_{19}, H_{20}), 3.46 – 3.37 (m, 4H, H_{17}, H_{21}), 3.30 – 2.99 (m, 4H, H_7, H_{16}), 2.28 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.57 – 1.17 (m, 12H, H_4, H_5, H_6, H_{13}).

^{13}C NMR (101 MHz, DMSO-d_6) δ = 173.2 (C_2), 169.1(C_9), 168.7(C_{14}), 165.3 (C_{23}), 142.2 (C_{27}), 130.9 (C_{24}), 129.0 (C_{25}), 118.8 (C_{26}), 111.7 ($_{C12}$), 77.6, 77.5 (C_{10}, C_{11}), 69.5, 69.5,

68.9, 68.6 (C_{17}, C_{18}, C_{19}, C_{20}), 51.1 (C_1), 39.2 (C_{21}), 38.4 (C_{16}), 38.3 (C_7), 33.2 (C_3), 28.5 (C_6), 26.2 (C_5), 25.7 (C_{13}), 24.1 (C_4).

HRMS calculated for $C_{27}H_{40}N_6O_9$ + Na 615.2755. Found 615.2749.

6-(5-((2-(2-(2-(4-azidobenzamido)ethoxy)ethoxy)ethyl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxamido)hexanoic acid (25)



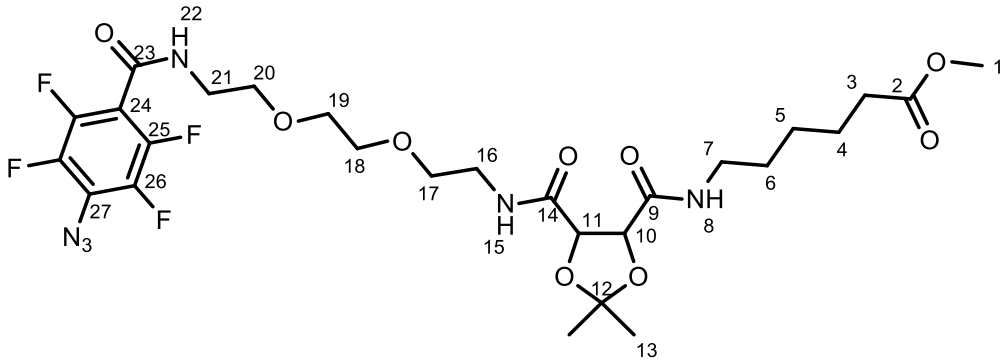
Lithium hydroxide (5.50 mmol, 231 mg) in water (4.00 mL) was added to a solution of **24** (1.10 mmol, 652 mg) in tetrahydrofuran (8.00 mL) at room temperature. The reaction mixture was refluxed for 2 hours at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (40.0 mL). The reaction mixture was washed with ethyl acetate (20.0 mL). The aqueous phase was acidified with hydrochloric acid (1M, pH 2) and extracted with ethyl acetate (3 x 20.0 mL). The organic phase was washed with a saturated sodium chloride solution (20.0 mL), dried ($MgSo_4$) and concentrated under reduced pressure to give **25** as an orange oil. Yield 408 mg (64 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 12.39 (s, 1H, H_1), 8.51 (t, $^3J_{22,21}$ = 5.5 Hz, 1H, H_{22}), 8.12 – 8.00 (m, 2H, H_8, H_{15}), 7.92 – 7.87 (m, 2H, H_{25}), 7.22 – 7.16 (m, 2H, H_{26}), 4.55 - 4.43 (m, 2H, H_{10}, H_{11}), 3.56 – 3.49 (m, 6H, H_{18}, H_{19}, H_{20}), 3.46 – 2.98 (m, 8H, H_7, H_{16}, H_{17}, H_{21}), 2.18 (t, $^3J_{3,4}$ = 7.3 Hz, 2H, H_3), 1.53 – 1.20 (m, 12H, H_4, H_5, H_6, H_{13}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 174.4 (C_2), 169.1 (C_9), 168.7 (C_{14}), 165.3 (C_{23}), 142.2 (C_{27}), 130.9 (C_{24}), 129.1 (C_{25}), 118.8 (C_{26}), 111.7 (C_{12}), 77.6, 76.7 (C_{10}, C_{11}), 69.5, 69.5, 68.9, 68.6 (C_{17}, C_{18}, C_{19}, C_{20}), 38.4 , 38.4, 38.3 (C_7, C_{16}, C_{21}), 33.5 (C_3), 28.6 (C_6), 26.3 (C_5), 25.8 (C_{13}), 24.1 (C_4).

HRMS calculated for $C_{26}H_{38}N_6O_9$ - H 577.2622. Found 577.2628.

Methyl 6-(5-((2-(2-(2-(4-azido-2,3,5,6-tetrafluorobenzamido)ethoxy)ethoxy) ethyl) carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxamido)hexanoate (26)



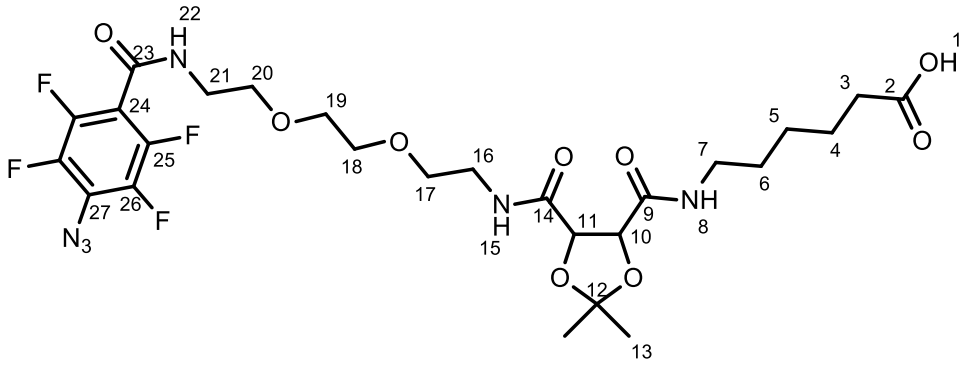
Diisopropylethylamine (12.0 mmol, 2.10 mL) was added to a stirred solution of 4-azido-2,3,5,6-tetrafluorobenzoic acid (4.81 mmol, 1.13 g) in tetrahydrofuran (53.0 mL) followed by HBTU (5.29 mmol, 2.01 g) at room temperature. After 15 minutes **23** (5.29 mmol, 2.37 g) was added. The reaction mixture was stirred for 72 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in dichloromethane (100 mL) and washed with hydrochloric acid (1M, 3 x 50.0 mL), saturated sodium hydrogen carbonate solution (3 x 50.0 mL) and saturated sodium chloride solution (50.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using dichloromethane, ethyl acetate and methanol (9:1:0 → 95:0:5) as eluent to give **26** as an orange oil. Yield 1.59 g (50 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.96 (t, $^3J_{22,21}$ = 5.5 Hz, 1H, H_{22}), 8.09 - 8.00 (m, 2H, H_8, H_{15}), 4.49 (d, $^3J_{11,10}$ = 6.0 Hz, 1H, H_{11}), 4.45 (d, $^3J_{10,11}$ = 6.0 Hz, 1H, H_{10}), 3.58 (s, 3H, H_1), 3.56 – 3.50 (m, 6H, H_{18}, H_{19}, H_{20}), 3.46 – 3.38 (m, 4H, H_{16}, H_{17}), 3.34 – 2.98 (m, 4H, H_7, H_{21}), 2.28 (t, $^3J_{3,4}$ = 7.4 Hz, 2H, H_3), 1.56 – 1.21 (m, 12H, H_4, H_5, H_6, H_{13}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_2), 169.1 (C_9), 168.7 (C_{14}), 157.0 (C_{23}), 111.7 (C_{12}), 77.6, 77.5 (C_{10}, C_{11}), 69.6, 69.5, 68.6, 68.6 (C_{17}, C_{18}, C_{19}, C_{20}), 51.1 (C_1), 39.4 (C_{16}), 38.4, 38.3 (C_7, C_{21}), 33.2 (C_3), 28.6 (C_6), 26.2 (C_5), 25.7 (C_{13}), 24.1 (C_4).

HRMS calculated for $C_{27}H_{36}F_4N_6O_9$ + Na 687.2378. Found 687.2381.

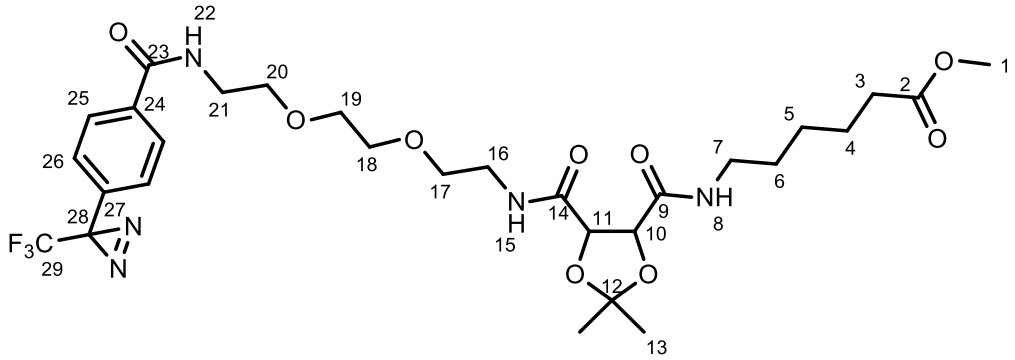
6-(5-((2-(2-(2-(4-azido-2,3,5,6-tetrafluorobenzamido)ethoxy)ethoxy)ethyl) carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxamido)hexanoic acid (27)



Lithium hydroxide (12.0 mmol, 500 mg) in water (8.00 mL) was added to a solution of **26** (2.39 mmol, 1.59 g) in tetrahydrofuran (16.0 mL) at room temperature. The reaction mixture was refluxed for 2 hours at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (15.0 mL). The reaction mixture was washed with ethyl acetate (10.0 mL). The aqueous phase was acidified with hydrochloric acid (1M, pH 2) and extracted with ethyl acetate (3 x 40.0 mL). The organic phase was washed with a saturated sodium chloride solution (40.0 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give **27** as an orange oil. Yield 1.21 g (78 %).

HRMS calculated for $C_{26}H_{33}F_4N_6O_9$ - H 649.2245. Found 649.2251.

Methyl 6-(2,2-dimethyl-5-((2-(2-(2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzamido)ethoxy)ethoxy)ethyl)carbamoyl)-1,3-dioxolane-4-carboxamido) hexanoate (28)



Diisopropylethylamine (12.7 mmol, 2.20 mL) was added to a stirred solution of 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid (4.62 mmol, 1.06 g) in tetrahydrofuran (47.0 mL) followed by HBTU (5.08 mmol, 1.93 g) at room temperature. After 30 minutes **23** (5.08 mmol, 2.27 g) was added. The reaction mixture was stirred

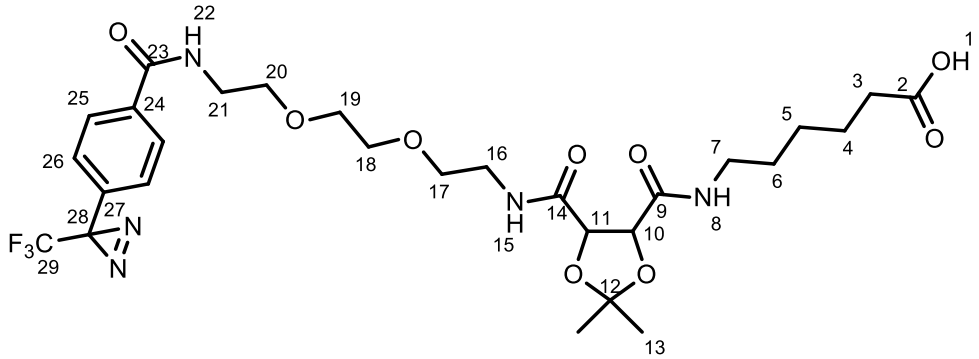
for 72 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in ethyl acetate (100 mL) and washed with hydrochloric acid (1M, 3 x 50.0 mL), saturated sodium hydrogen carbonate solution (3 x 50.0 mL) and saturated sodium chloride solution (50.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using dichloromethane, ethyl acetate and methanol (9:1:0 → 9:0:1) as eluent to give **28** as a red oil. Yield 2.12 g (70 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.66 (t, $^3J_{22,21}$ = 5.5 Hz, 1H, H_{22}), 8.09 - 7.99 (m, 2H, H_8, H_{15}), 7.98 – 7.93 (m, 2H, H_{25}), 7.39 – 7.35 (m, 2H, H_{26}), 4.50 (d, $^3J_{11,10}$ = 6.0 Hz, 1H, H_{11}), 4.46 (d, $^3J_{10,11}$ = 6.1 Hz, 1H, H_{10}), 3.57 (d, $^5J_{1,3}$ = 1.6 Hz, 3H, H_1), 3.55 – 3.50 (m, 6H, H_{18}, H_{19}, H_{20}), 3.46 – 3.38 (m, 4H, H_{16}, H_{17}), 3.34 – 2.96 (m, 4H, H_7, H_{21}), 2.28 (td, $^3J_{3,4}$ = 7.4 Hz, $^5J_{3,1}$ = 2.4 Hz, 2H, H_3), 1.56 – 1.20 (m, 12H, H_4, H_5, H_6, H_{13}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 173.2 (C_2), 169.1 (C_9), 168.7 (C_{14}), 165.2 (C_{23}), 135.9 (C_{27}), 130.1 (C_{24}), 128.1 (C_{25}), 126.4 (C_{26}), 123.5 (C_{29}), 119.9 (C_{28}), 111.7 (C_{12}), 77.6, 77.5 (C_{10}, C_{11}), 69.5, 69.5, 68.8, 68.6 (C_{17}, C_{18}, C_{19}, C_{20}), 51.1 (C_1), 39.5 (C_{16}), 38.4, 38.3 (C_7, C_{21}), 33.2 (C_3), 28.6 (C_6), 26.2 (C_5), 25.7 (C_{13}), 24.1 (C_4).

HRMS calculated for $C_{29}H_{40}F_3N_5O_9$ + Na 682.2676. Found 682.2670.

6-(2,2-dimethyl-5-((2-(2-(2-(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzamido) ethoxy)ethoxy)ethyl)carbamoyl)-1,3-dioxolane-4-carboxamido)hexanoic acid (29)



Lithium hydroxide (12.7 mmol, 530 mg) in water (9.00 mL) was added to a solution of **29** (2.55 mmol, 1.68 g) in tetrahydrofuran (18.0 mL) at room temperature. The reaction mixture was refluxed for 2 hours at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (15.0 mL).

The reaction mixture was washed with ethyl acetate (10.0 mL). The aqueous phase was acidified with hydrochloric acid (1M, pH 2) and extracted with ethyl acetate (3 x 40.0 mL). The organic phase was washed with a saturated sodium chloride solution (40.0 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give **29** as an red oil. Yield 1.41 g (86 %).

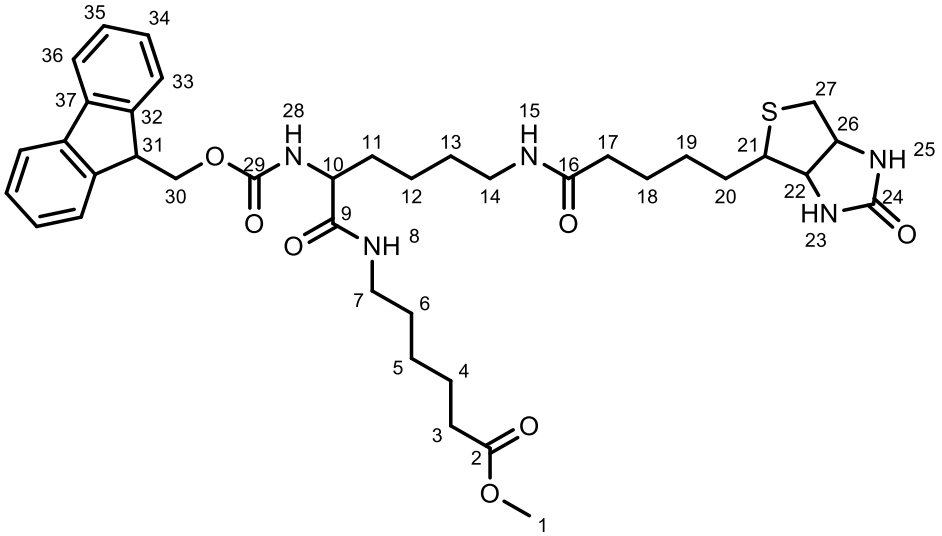
^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 12.17 (s, 1H, H_1), 8.66 (t, $^3J_{22,21}$ = 5.5 Hz, 1H, H_{22}), 8.09 – 7.99 (m, 2H, H_8, H_{15}), 7.97 – 7.93 (m, 2H, H_{25}), 7.40 – 7.35 (m, 2H, H_{26}), 4.50 (d, $^3J_{11,10}$ = 5.9 Hz, 1H, H_{11}), 4.46 (d, $^3J_{10,11}$ = 6.0 Hz, 1H, H_{10}), 3.55 – 3.50 (m, 6H, H_{18}, H_{19}, H_{20}), 3.45 – 2.96 (m, 8H, H_7, H_{16}, H_{17}, H_{21}), 2.18 (td, $^3J_{3,4}$ = 7.2 Hz, $^5J_{3,1}$ = 1.6 Hz, 2H, H_3), 1.56 – 1.21 (m, 12H, H_4, H_5, H_6, H_{13}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 174.4 (C_2), 169.1 (C_9), 168.7 (C_{14}), 165.2 (C_{23}), 135.9 (C_{27}), 130.2 (C_{24}), 128.1 (C_{25}), 126.4 (C_{26}), 123.6 (C_{29}), 119.9 (C_{28}), 111.7 (C_{12}), 77.6, 77.5 (C_{10}, C_{11}), 69.5, 69.5, 68.8, 68.6 (C_{17}, C_{18}, C_{19}, C_{20}), 39.5 (C_{16}), 38.4, 38.3 (C_7, C_{21}), 33.5 (C_3), 28.6 (C_6), 26.2 (C_5), 25.8 (C_{13}), 24.1 (C_4).

HRMS calculated for $C_{28}H_{38}F_3N_5O_9$ + Na 668.2520. Found 668.2514.

7.4. Cleavable ABAL probes

Methyl 6-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanamido) hexanoate (30)



Pyridine (27.0 mL) was added to a stirred solution of biotin (5.13 mmol, 1.25 g) in dimethylformamide (27.0 mL) followed by HBTU (5.64 mmol, 2.14 g) at room

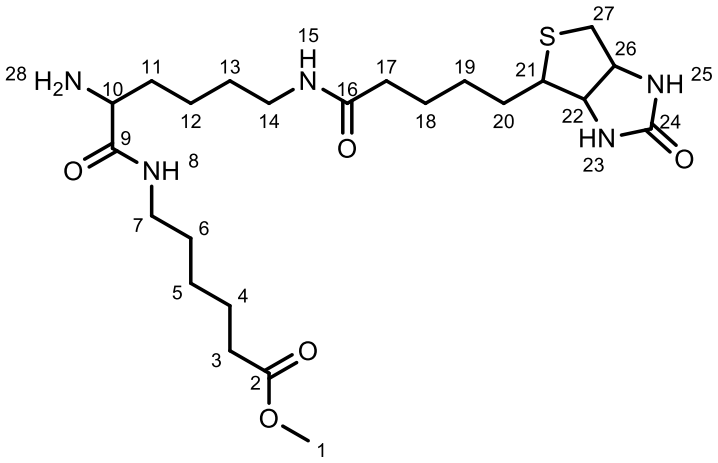
temperature. After 15 minutes **3** (5.64 mmol, 3.00 g) was added. The reaction mixture was stirred for 96 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in dichloromethane and methanol (3:1, 400 mL) and washed with hydrochloric acid (1M, 3 x 200 mL) and saturated sodium chloride solution (50.0 mL). The organic layer was filtered and the precipitate was purified by column chromatography on silica gel using dichloromethane, methanol and triethylamine (90:9:1) as eluent to give **30** as an off-white solid. Yield 2.72 g (73 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ [ppm] = 7.89 (d, $^{3}J_{36,35}$ = 7.5 Hz, 2H, H_{36}), 7.82 (t, $^{3}J_{8,7}$ = 5.6 Hz, 1H, H_8), 7.74 – 7.72 (m, 3H, H_{15}, H_{33}), 7.44 – 7.38 (m, 3H, H_{28}, H_{35}), 7.32 (dd, $^{3}J_{34,33/35}$ = 7.4 Hz, 2H, H_{34}), 6.41 (s, 1H, $H_{23/25}$), 6.35 (s, 1H, $H_{23/25}$), 4.31 - 4.19 (m, 4H, H_{26}, H_{30}, H_{31}), 4.13 – 4.08 (m, 1H, H_{22}), 3.90 (td, $^{3}J_{10,11}$ = 8.5 Hz, $^{3}J_{10,28}$ = 5.3 Hz, 1H, H_{10}), 3,56 (s, 3H, H_1), 3.10 – 2.96 (m, 5H, H_7, H_{14}, H_{21}), 2.80 (dd, $^{2}J_{27,27}$ = 12.4 Hz, $^{3}J_{27,26}$ = 5.1 Hz, 1H, H_{27}), 2.57 (d, $^{2}J_{27,27}$ = 12.4 Hz, 1H, H_{27}), 2.26 (t, $^{3}J_{3,4}$ = 7.4 Hz, 2H, H_3), 2.04 (t, $^{3}J_{17,18}$ = 7.4 Hz, 2H, H_{17}), 1.65 – 1.20 (m, 18H, H_4, H_5, H_6, H_{11}, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}).

^{13}C NMR (101 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_2), 171.8 (C_{16}), 171.7 (C_9), 162.7 (C_{24}), 155.9 (C_{29}), 143.9 (C_{32}), 140.7 (C_{37}), 127.6 (C_{35}), 127.1 (C_{34}), 125.3 (C_{33}), 120.1(C_{36}), 65.6 (C_{30}), 61.1 (C_{22}), 59.2 (C_{26}), 55.4 (C_{21}), 54.7 (C_{10}), 51.2 (C_1), 46.7 (C_{31}), 39.9 (C_{27}), 38.3, 38.3 (C_7, C_{14}), 35.3 (C_{17}), 33.2 (C_3), 31.7 (C_6), 28.9 (C_{13}), 28.7 (C_{19}), 28.2 (C_{11}), 28.1 (C_5), 25.8 (C_{18}), 25.3 (C_4), 24.1 (C_{20}), 23.0 (C_{12}).

HRMS calculated for $C_{38}H_{51}N_5O_7S$ + Na 744.3407. Found 744.3401.

Methyl 6-(2-amino-6-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido) hexanamido)hexanoate (31)

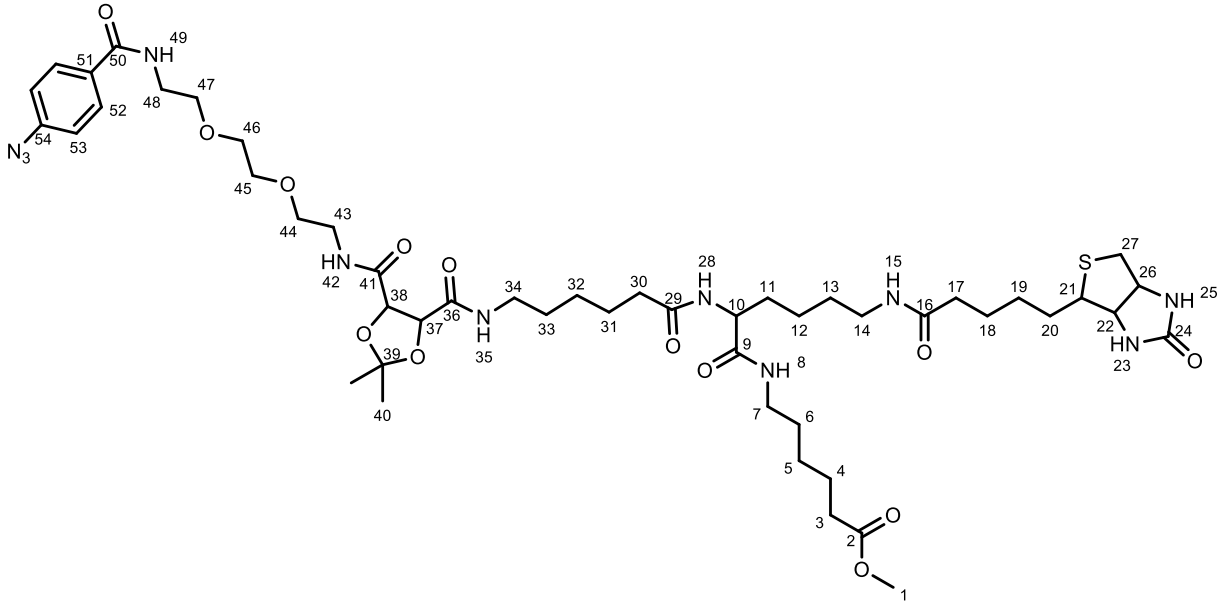


Piperidine (11.0 mL) was added to **30** (3.74 mmol, 2.70 g) in dichloromethane (44.0 mL) at 0 °C and stirred for 1 hour at room temperature. Toluene (44.0 mL) was added and the reaction mixture was concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using dichloromethane, methanol, triethylamine (90:9:1) as eluent to give **31** as a white solid. Yield 1.56 g (83 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 7.79 (t, $^{3}J_{8,7}$ = 5.8 Hz, 1H, H_8), 7.72 (t, $^{3}J_{15,14}$ = 5.6 Hz, 1H, H_{15}), 6.41 (s, 1H, $H_{23/25}$), 6.35 (s, 1H, $H_{23/25}$), 4.33 – 4.27 (m, 1H, H_{26}), 4.14 – 4.10 (m, 1H, H_{22}), 3.57 (s, 3H, H_1), 3.12 - 2.95 (m, 6H, H_7, H_{10}, H_{14}, H_{21}), 2.81 (dd, $^{2}J_{27,27}$ = 12.4, $^{3}J_{27,26}$ = 5.1 Hz, 1H, H_{27}), 2.57 (d, $^{2}J_{27,27}$ = 12.4 Hz, 1H, H_{27}), 2.28 (t, $^{3}J_{3,4}$ = 7.4 Hz, 2H, H_3), 2.03 (t, $^{3}J_{17,18}$ = 7.3 Hz, 2H, H_{17}), 1.67 – 1.18 (m, 18H, H_4, H_5, H_6, H_{11}, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 174.8 (C_2), 173.3 (C_{16}), 171.8 (C_9), 162.7 (C_{24}), 61.1 (C_{22}), 59.2 (C_{26}), 55.4 (C_{21}), 54.6 (C_{10}), 51.2 (C_1), 39.8 (C_{27}), 38.4, 38.1 (C_7, C_{14}), 35.2 (C_{17}), 34.9 (C_6), 33.2 (C_3), 29.2 (C_{13}), 28.8 (C_{19}), 28.2 (C_{11}), 28.0 (C_5), 25.8 (C_{18}), 25.3 (C_4), 24.2 (C_{20}), 22.8 (C_{12}).

Methyl 6-(2-(6-(5-((2-(2-(2-(4-azidobenzamido)ethoxy)ethoxy)ethyl)carbamoyl)-2,2-dimethyl-1,3-dioxolane-4-carboxamido)hexanamido)-6-(5-(2-oxohexahydro-1H-thieno [3,4-d]imidazol-4-yl)pentanamido)hexanamido)hexanoate (32)



Diisopropylethylamine (4.03 mmol, 0.70 mL) was added to a stirred solution of **25** (1.61 mmol, 930 mg) in dimethylformamide (34.0 mL) followed by HBTU (1.77 mmol, 670 mg) at room temperature. After 15 minutes **31** (1.77 mmol, 880 mg) was added. The reaction mixture was stirred for 96 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The resulting solid was dissolved in dichloromethane and methanol (9:1, 45.0 mL) and washed with hydrochloric acid (1M, 3 x 22.0 mL), saturated sodium hydrogen carbonate solution (3 x 22.0 mL) and saturated sodium chloride solution (22.0 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel using dichloromethane and methanol (9:1) as eluent to give **32** as a yellow solid. Yield 204 mg (12 %).

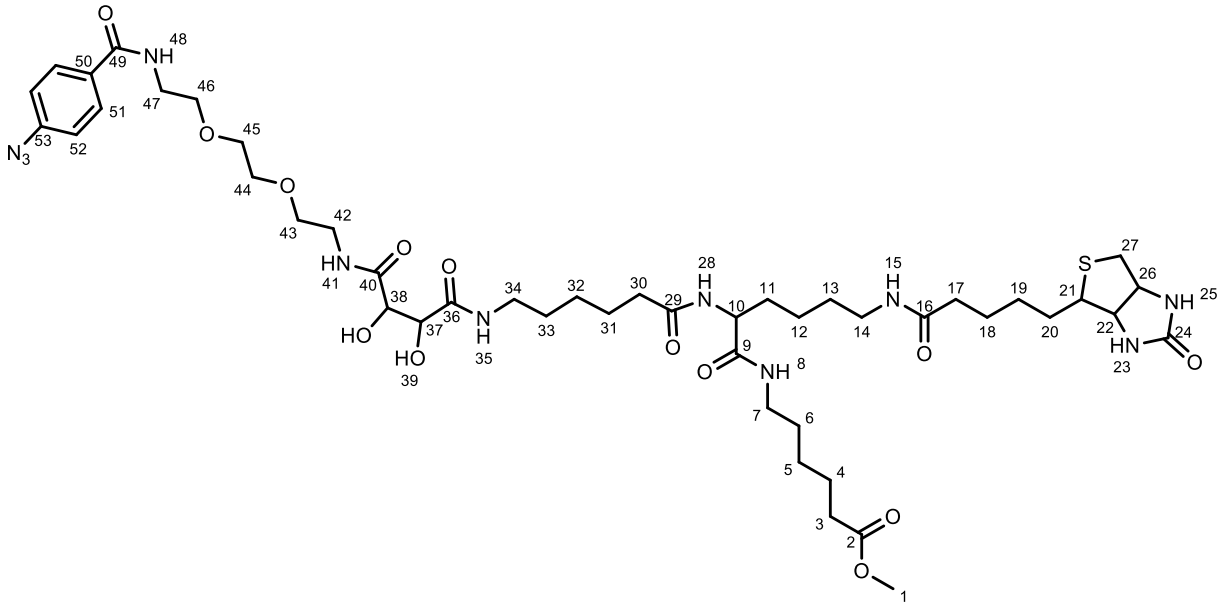
^{1}H NMR (500 MHz, DMSO-d_6) δ [ppm] = 8.52 (t, $^3J_{49,48}$ = 5.6 Hz, 1H, H_{49}), 8.06 (t, $^3J_{35,34}$ = 5.9 Hz, 1H, H_{35}), 8.02 (t, $^3J_{42,43}$ = 5.8 Hz, 1H, H_{42}), 7.91 – 7.88 (m, 2H, H_{52}), 7.83 - 7.81 (m, 2H, H_8, H_{28}), 7.72 (t, $^3J_{15,14}$ = 5.6 Hz, 1H, H_{15}), 7.21 – 7.18 (m, 2H, H_{53}), 6.41 (s, 1H, $H_{23/25}$), 6.35 (s, 1H, $H_{23/25}$), 4.50 (d, $^3J_{38,37}$ = 6.0 Hz, 1H, H_{38}), 4.46 (d, $^3J_{37,38}$ = 6.0 Hz, 1H, H_{37}), 4.31 - 4.29 (m, 1H, H_{26}), 4.17 – 4.10 (m, 2H, H_{10}, H_{22}), 3.57 (d, $^5J_{1,3}$ = 1.6 Hz, 3H, H_1), 3.54 – 3.49 (m, 6H, H_{45}, H_{46}, H_{47}), 3.44 – 3.39 (m, 4H, H_{44}, H_{48}), 3.31 – 3.16 (m, 2H, H_{43}), 3.12 – 2.94 (m, 7H, H_7, H_{14}, H_{21}, H_{34}), 2.82 (dd, $^2J_{27,27}$ = 12.5 Hz, $^3J_{27,26}$ = 5.1 Hz, 1H,

H_{27}), 2.57 (d, $^{2}J_{27,27}$ = 12.4 Hz, 1H, H_{27}), 2.27 (td, $^{3}J_{3,4}$ = 7.3 Hz, $^{5}J_{3,1}$ = 2.5 Hz, 2H, H_3), 2.12 - 2.07 (m, 2H, H_{30}), 2.03 (t, $^{3}J_{17,18}$ = 7.4 Hz, 2H, H_{17}), 1.64 – 1.14 (m, 30H, H_4, H_5, H_6, H_{11}, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{31}, H_{32}, H_{33}, H_{40}).

^{13}C NMR (126 MHz, DMSO-d_6) δ [ppm] = 173.3 (C_{29}), 172.0 (C_2), 171.8 (C_{16}), 171.6 (C_9), 169.1 (C_{36}), 168.7 (C_{41}), 165.3 (C_{50}), 162.7 (C_{24}), 142.2 (C_{54}), 130.9 (C_{51}), 129.1 (C_{52}), 118.8 (C_{53}), 111.7 (C_{39}), 77.6, 77.5 (C_{37}, C_{38}), 69.5, 69.5, 68.9, 68.6 (C_{44}, C_{45}, C_{46}, C_{47}), 61.0 (C_{22}), 59.2 (C_{26}), 55.4 (C_{21}), 52.3 (C_{10}), 51.2 (C_1), 39.9, 39.8 (C_{27}, C_{43}), 38.4, 38.4, 38.2, 38.2 (C_7, C_{14}, C_{34}, C_{48}), 35.2 (C_{17}), 35.1 (C_{30}), 33.2 (C_3), 31.9 (C_6), 28.9 (C_{13}), 28.7 (C_{33}), 28.7 (C_{19}), 28.2 (C_{11}), 28.0 (C_5), 26.2 (C_{40}), 26.0 (C_{32}), 25.7 (C_{18}), 25.3 (C_{31}), 25.0 (C_4), 24.1(C_{20}), 22.8 (C_{12}).

HRMS calculated for $C_{38}H_{53}N_5O_7$ + Na 714.3843. Found 714.3837.

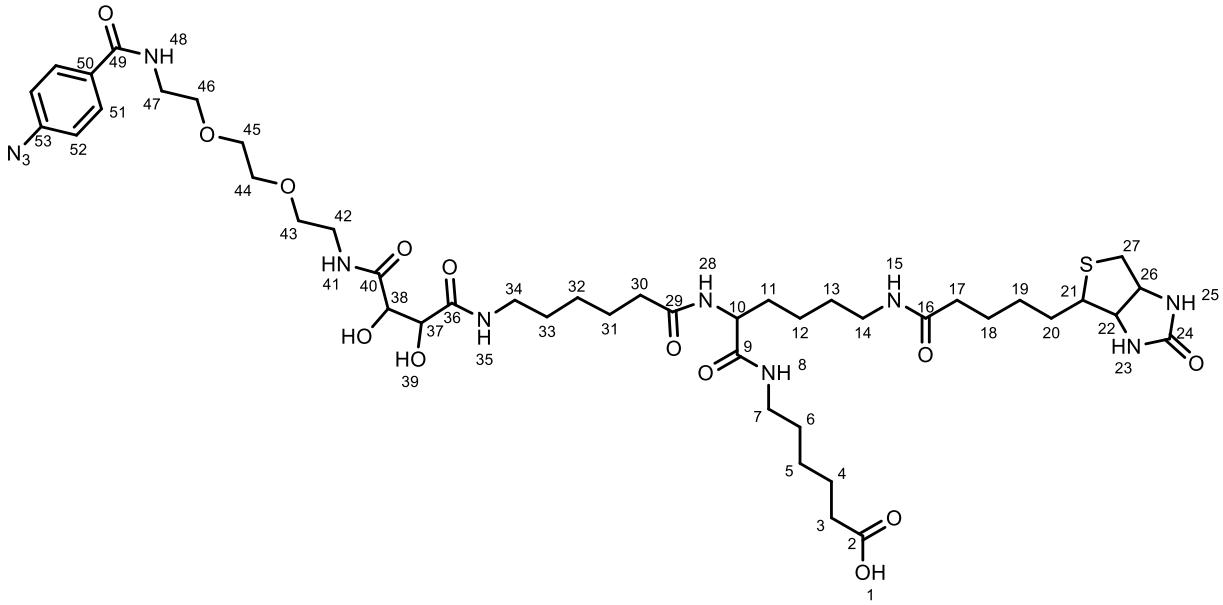
Methyl 1-(4-azidophenyl)-13,14-dihydroxy-1,12,15,22,25-pentaoxo-24-(4-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)butyl)-5,8-dioxa-2,11,16,23,26-pentaazadotriacontan-32-oate (33)



Acetyl chloride (0.58 mmol, 0.05 mL) was added to **32** (0.19 mmol, 204 mg) in methanol (2.00 mL) at 0 °C and stirred for 48 hours at room temperature. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in methanol (2.00 mL) and poured into diethyl ether (10.0 mL). The resulting precipitate was filtered to obtain **33** as pale yellow solid. Yield 175 mg (90 %).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 – 8.52 (m, 1H, H_{49}), 8.24 – 8.03 (m, 1H, H_{35}), 7.96 – 7.81 (m, 5H, H_8, H_{28}, H_{42}, H_{52}), 7.64 (t, $^3J_{15,14}$ = 5.6 Hz, 1H, H_{15}), 7.20 – 7.16 (m, 2H, H_{53}), 5.41 (s, 2H, H23/25), 4.33 – 4.28 (m, 1H, H_{26}), 4.21 (d, $^3J_{37/38,39/40}$ = 6.1 Hz, 2H, H_{37}, H_{38}), 4.16 – 4.10 (m, 2H, H_{10}, H_{22}), 3.57 (d, $^5J_{1,3}$ = 1.7 Hz, 3H, H_1), 3.55 – 3.48 (m, 6H, H_{45}, H_{46}, H_{47}), 3.45 – 3.36 (m, 4H, H_{44}, H_{48}), 3.34 – 3.17 (m, 2H, H_{43}), 3.13 – 2.88 (m, 9H, H_7, H_{14}, H_{21}, H_{34}, H_{39}, H_{40}), 2.82 (dd, $^2J_{27,27}$ = 12.5 Hz, $^3J_{27,26}$ = 5.0 Hz, 1H, H_{27}), 2.58 (d, $^2J_{27,27}$ = 12.4 Hz, 1H, H_{27}), 2.31 – 2.23 (m, 2H, H_3), 2.14 – 2.00 (m, 4H, H_{17}, H_{30}), 1.73 – 1.12 (m, 24H, H_4, H_5, H_6, H_{11}, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{31}, H_{32}, H_{33}).

1-(4-azidophenyl)-13,14-dihydroxy-1,12,15,22,25-pentaoxo-24-(4-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)butyl)-5,8-dioxa-2,11,16,23,26-pentaazadotriacontan-32-oic acid (34)



A solution of lithium hydroxide (0.72 mmol, 30.0 mg) in water (0.50 mL) was added to a stirred solution of **33** (0.14 mmol, 147 mg) in tetrahydrofuran (1.00 mL) at room temperature. The reaction mixture was refluxed for 3 hours at 70 °C. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in water (6.00 mL). The reaction mixture was washed with ethyl acetate (3.00 mL). Hydrochloric acid (2M, 3.00 mL) was added to the aqueous phase and the resulting precipitate was filtered to obtain **34** as an off- white solid. Yield 49.0 mg (33 %).

^{1}H NMR (300 MHz, DMSO-d_6) δ [ppm] = 8.56 (t, $^3J_{49,48}$ = 5.6 Hz, 1H, H_{49}), 7.93 - 7.77 (m, 5H, H_{28}, H_{35}, H_{42}, H_{52}), 7.63 (t, $^3J_{8/15,7/14}$ = 5.9 Hz, 2H, H_8, H_{15}), 7.21 – 7.16 (m, 2H, H_{53}),

4.34 – 4.28 (m, 1H, H_{26}), 4.23 – 4.20 (m, 2H, H_{37}, H_{38}), 4.18 – 4.11 (m, 2H, H_{10}, H_{22}), 3.57 - 3.50 (m, 6H, H_{45}, H_{46}, H_{47}), 3.45 – 3.37 (m, 4H, H_{44}, H_{48}), 3.34 - 3.15 (m, 2H, H_{43}), 3.14 – 2.92 (m, 7H, H_7, H_{14}, H_{21}, H_{34}), 2.82 (dd, $^2J_{27,27}$ = 12.5 Hz, $^3J_{27,26}$ = 5.0 Hz, 1H, H_{27}), 2.58 (d, $^2J_{27,27}$ = 12.4 Hz, 1H, H_{27}), 2.20 – 2.00 (m, 6H, H_3, H_{17}, H_{30}), 1.65 – 1.14 (m, 24H, H_4, H_5, H_6, H_{11}, H_{12}, H_{13}, H_{18}, H_{19}, H_{20}, H_{31}, H_{32}, H_{33}).

^{13}C NMR (75 MHz, DMSO-d_6) δ [ppm] = 174.4 (C_{29}), 172.2 (C_2), 172.1 (C_{16}), 172.0 (C_9), 171.8(C_{36}), 171.7 (C_{41}), 165.4 (C_{50}), 162.8 (C_{24}), 142.2 (C_{54}), 130.9 (C_{51}), 129.1 (C_{52}), 118.9 (C_{53}), 72.5 (C_{37}, C_{38}), 69.6, 68.9 (C_{44}, C_{45}, C_{46}, C_{47}), 61.1 (C_{22}), 59.3 (C_{26}), 55.4 (C_{21}), 52.5 (C_{10}), 40.1(C_{27}, C_{43}), 38.3, 38.3 (C_7, C_{14}, C_{34}, C_{48}), 35.2 (C_{17}), 35.2 (C_{30}), 33.7 (C_3), 31.9 (C_6), 29.0 (C_{33}), 28.9 (C_{13}), 28.8 (C_{19}), 28.2 (C_{11}), 28.0 (C_5), 26.1 (C_{32}), 25.9 (C_{18}), 25.3 (C_{31}), 25.1 (C_4), 24.2 (C_{20}), 22.9 (C_{12}).

HRMS calculated for $C_{45}H_{71}N_{11}O_{13}S$ + 2Na 525.7375. Found 525.7369.

7.5. C10.35 PAD ABAL probe

EDC mediated coupling

PAD ABAL probe was freshly dissolved in dry DMSO to a concentration of 20 mM. Coupling of the ABAL probe to the 5'-hexylamino-modified aptamer C10.35 was performed with 4 mM PAD ABAL probe, in a buffer consisting of 25 mM Hepes, 100 mM NaCl, 20 % dry DMSO, 4mM EDC and 40 µM aptamer. The reaction mixture was incubated in a Thermomixer at 300 rpm and was then desalted using Amicon Ultra 3K centrifugal filters and analysed via LC-MS.

Reaction Temperatur	Reaction Time	Reaction Yield [%]
RT	19 h	45
65 °C	17 h	49

EDC + Oxyma Pure mediated coupling

PAD ABAL probe was freshly dissolved in dry DMSO to a concentration of 20 mM. Coupling of the ABAL probe to the 5'-hexylamino-modified aptamer C10.35 was performed with 4 mM PAD ABAL probe, in a buffer consisting of 25 mM Hepes, 100 mM NaCl, 20 % dry DMSO, 4mM EDC and 4mM Oxyma Pure and 40 µM aptamer. The reaction mixture was incubated in a Thermomixer at 300 rpm and was then desalted using Amicon Ultra 3K centrifugal filters and analysed via LC-MS.

Reaction Temperatur	Reaction Time	Reaction Yield
RT	19 h	4

EDC + sulfo-NHS mediated coupling

A mixture of 20 mM PAD ABAL probe, 20 mM EDC and 20 mM sulfo-NHS in dry DMSO was freshly prepared and incubated for 30 minutes in a Thermomixer at 600 rpm. Coupling of the ABAL probe to the 5'-hexylamino-modified aptamer C10.35 was performed with 4 mM ABAL mixture, in a buffer consisting of 25 mM Hepes, 100 mM NaCl, 20 % dry DMSO and 40 µM aptamer. The reaction mixture was incubated in a Thermomixer at 600 rpm and was then desalted using Amicon Ultra 3K centrifugal filters and analysed via LC-MS.

Reaction Temperatur	Reaction Time	Reaction Yield
RT	2 h	29
RT	3 h	0
RT	4 h	31
RT	19 h	64
4 °C	2 h	13
4 °C	3 h	17
4 °C	4 h	20
4 °C	19 h	42

C Appendix

List of abbreviations

ABAL	Aptamer-based affinity labelling
ABPP	Affinity-based proteomic profiling
ALA	5-Aminolevulinic acid
ALAD	δ-Aminolevulinic acid dehydratase
Boc	*tert*-Butyloxycarbonyl
DIPEA	Diisopropylethylamine
DMAB	p-Dimethylaminobenzaldehyde
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC_{50}	Half maximal effective concentration
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EI	Electron ionization
ESI	Electrospray-ionisation
FACS	fluorescence-activated cell sorter
Fmoc	fluorenylmethyloxycarbonyl
GMPS	guanosine-monophosphorothioate
HBTU	O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOBt	1-hydroxy-1H-benzotriazole
HPLC	High-performance liquid chromatography
IC_{50}	half maximum concentration
ICT	Immunochromatographic card tests
LC-MS	Liquid chromatography–mass spectrometry
MDA	mass drug administration programs
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance spectroscopy
NOE	Nuclear Overhauser effect

PA	Phenyl azide
PAL	Photoaffinity labelling
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SELEX	Systematic evolution of ligands by exponential enrichment
Sulfo-NHS	N-hydroxysulfosuccinimide
Sulfo-SBED	Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azidobenzamido)-hexanoamido) ethyl-1,3'-dithioproprionate
TBAF	Tetra-n-butylammonium fluoride trihydrate
TCEP	Tris(2-carboxyethyl)phosphine
TLC	Thin layer chromatography
TPA	1,2,4,5-tetrafluorophenyl-3-azide
TPD	3-phenyl-3-(trifluoromethyl)-3H-diazirine
Tris	Tris(hydroxymethyl)aminomethane
WHO	World Health Organization

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Analytics

The NMR and HRMS spectra for all synthesised compounds presented in this work are compiled on a multimedia CD enclosed with this work.

Curriculum Vitae

Victoria Simone Halls

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