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Antibacterial sulfonimidamide-based oligopeptides as type I signal peptidase inhibitors: Synthesis and biological evaluation

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ABSTRACT

Oligopeptide boronates with a lipophilic tail are known to inhibit the type I signal peptidase in *E. coli*, which is a promising drug target for developing novel antibiotics. Antibacterial activity depends on these oligopeptides having a cationic modification to increase their permeation. Unfortunately, this modification is associated with cytotoxicity, motivating the need for novel approaches. The sulfonimidamide functionality has recently gained much interest in drug design and discovery, as a means of introducing chirality and an imine-handle, thus allowing for the incorporation of additional substituents. This in turn can tune the chemical and biological properties, which are here explored. We show that introducing the sulfonimidamide between the lipophilic tail and the peptide in a series of signal peptidase inhibitors resulted in antibacterial activity, while the sulfonamide isostere and previously known non-cationic analogs were inactive. Additionally, we show that replacing the sulfonamide with a sulfonimidamide resulted in decreased cytotoxicity, and similar results were seen by adding a cationic sidechain to the sulfonimidamide motif. This is the first report of incorporation of the sulfonimidamide functional group into bioactive peptides, more specifically into antibacterial oligopeptides, and evaluation of its biological effects.

1. Introduction

The lack of new antibiotics, alongside the increase in resistance to existing antibiotics, is a current alarming problem that researchers all over the world are fighting to address. To discover new antibiotics, novel lead structures that are potent and specific, and possess minimal toxic effects, are needed. In medicinal chemistry, optimization of validated hits is a fundamental task involving strategies such as bioisosteric replacements of atoms or groups [1]. The sulfonamide (SA) functional group, one of the first examples of a carboxamide isostere in drug design [2], has played a major role in various important biologically-active compounds such as diuretics, antivirals, and antibiotics [3]. Replacing one of the SA oxygens with a nitrogen gives the isostere sulfonimidamide (SIA), which has gained much momentum over the past ten years due to its unique chemical properties and new possibilities in drug design. The extra nitrogen in the SIA scaffold acts as a weakly basic imine, providing a reactive handle for incorporating chemical modifications. This substitution furthermore introduces chirality into the molecule, in contrast to the achiral SA or carboxylic acids [4]. Previous reports have shown that properties such as metabolic stability and solubility are often improved with the SIA compared to the corresponding SAs in small molecules [4,5]. Until now, the major studies regarding SIAs have focused on the development and optimization of novel synthetic routes to small molecule SIAs, whereas the evaluation of SIA-based compounds in medicinal chemistry has been less explored. Examples of small molecule SIA-based analogs of known bioactive compounds are seen in Fig. 1, including oncolytic sulfonylureas [6], an SIA analog of the Alzheimer's drug Begacestat [7], antimicrobial trifluoromethylated SIAs [8], and an analog of the cancer drug Tasisulam [9].

A review by Arvidsson's group in 2016 [4] focusing on SIAs in medicinal chemistry also highlighted the fact that most emphasis

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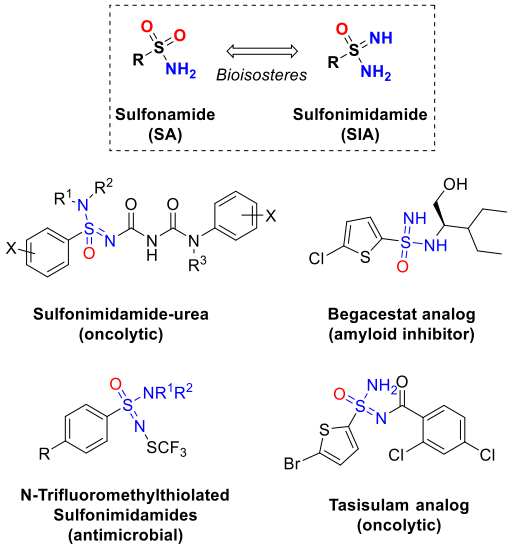


Fig. 1. Examples of sulfonimidamide-based analogs of small molecule bioactive compounds.

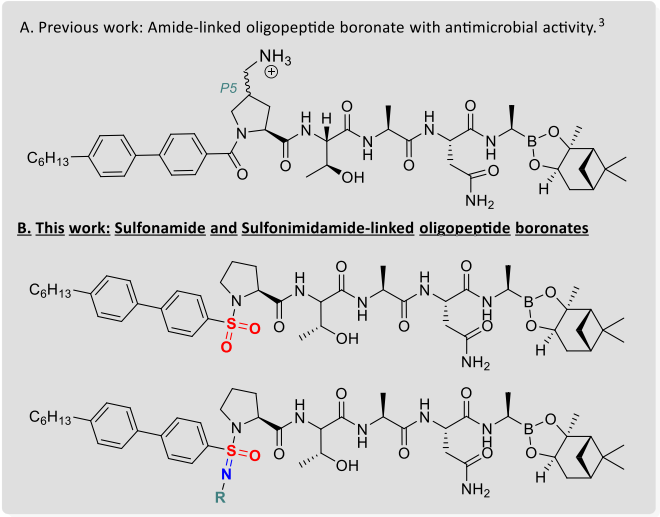


Fig. 2. Outline of this work, in which replacing the amide linker in the previous oligopeptide boronate hit **(A)** with the sulfonamide and sulfonimidamide moiety **(B)** is explored.

has been placed on studies of the drug-related and physicochemical properties of SIAs, in contrast to the paucity of studies on biological evaluation. To the best of our knowledge, no evaluation of SIA in bioactive peptides has yet been presented.

Multidrug-resistant Gram-negative bacteria have become one of the primary concerns in the fight against antibiotic resistance, as there are currently few effective drugs available to treat such infections. The double cell membrane and special efflux mechanisms make it very challenging to develop new effective Gram-negative antibiotics [10–12]. Type I signal peptidase (SPase I), commonly referred to as LepB in *Escherichia coli* [13], is a promising drug target as it is limited to bacteria and vital for their survival and virulence. SPase is a highly specific enzyme designed to cleave pre-proteins to release a mature protein from the bacterial cytoplasmic membrane. The active site is located at the outer surface of the cytoplasmic membrane, making it accessible to any inhibitor capable of crossing the outer membrane of Gram-negative bacteria. The peptidase has a unique Ser-Lys catalytic dyad that differs from typical eukaryotic serine proteases, which have a Ser-His-Asp catalytic triad architecture [14–16]. Targeting the Ser-Lys dyad and thus blocking the SPase activity leads to the accumulation of the pre-proteins in the membrane, eventually killing the bacteria. Despite the five classes of molecules, including arylomycins [17–19], krisynomycin [20], 5S-penems [21,22], beta-aminoketone [23], and substrate-based oligopeptides [24–27], that have been identified as inhibitors of SPase I, there is to our knowledge still no candidate drug in advanced stages of development. In our laboratory, De Rosa et al., previously optimized substrate-based oligopeptides by replacing an aldehyde warhead with a boronic ester (Fig. 2A), which improved the *Ec*LepB IC_{50} from low micromolar to low nanomolar, and at the same time imparted whole-cell antibacterial activity on wild-type strains [26]. Although promising starting points, these potent oligopeptide boronates were shown to be toxic to human liver cells (HepG2) and to have hemolytic effects. Therefore, improvements are necessary to reduce the toxic effects while maintaining the antibacterial activity of these compounds. Further optimization of the linear boronic ester inhibitors was recently attempted in our lab, resulting in novel boronic ester-linked macrocycles [27]. These compounds were also potent inhibitors of the enzyme, but unfortunately did not solve the existing problems; when the toxic effect was avoided, the antibacterial activity was reduced. Investigation of the structure-activity relationships of the substrate-based oligopeptides has identified some key structural features important for antibacterial activity. A bulky lipophilic tail at the N-terminus, an electrophilic and highly-reactive warhead at the C-terminus, and a positive charge around the P5 position are all important for antimicrobial activity (Fig. 2). In particular, the lipophilic group is crucial for enzymatic inhibition. This tail seems to be needed for anchoring to the membrane where the LepB target is situated; it thus helps the peptide part of the inhibitor to reach the binding site. However, previous studies indicated that the lipophilic tail might also be correlated with cytotoxicity and hemolytic side effects [26,27]. To develop sufficiently potent lead molecules with an acceptable toxicity profile, further exploration of the lipophilic tails is of interest. Additionally, designs for improved antibacterial activity of the oligopeptides in previous studies relied on either the addition of a cationic side chain as seen in Fig. 2A [25] or replacement of the proline with the cationic amino acid ornithine [27]. The aim of the present work was to investigate the effect of the SIA functionality, introduced between the lipophilic tail and P5, on antibacterial potency via modulating permeability or efflux properties. Effects on toxicity and hemolysis of SIA-containing oligopeptides were also evaluated. Furthermore, the SIA offers an additional hydrogen bond donor or acceptor, allows for an additional substituent, introduces chirality, and alters the geometry between the peptide and the lipophilic chain compared to the amide-linked oligopeptide. We wanted to take advantage of these features and explore the differences of the SA versus SIA functionalities in the oligopeptides. We furthermore saw an opportunity to apply and challenge our previously reported synthetic protocols [28,29] for introducing the SIA group into peptides in a medicinal chemistry context.

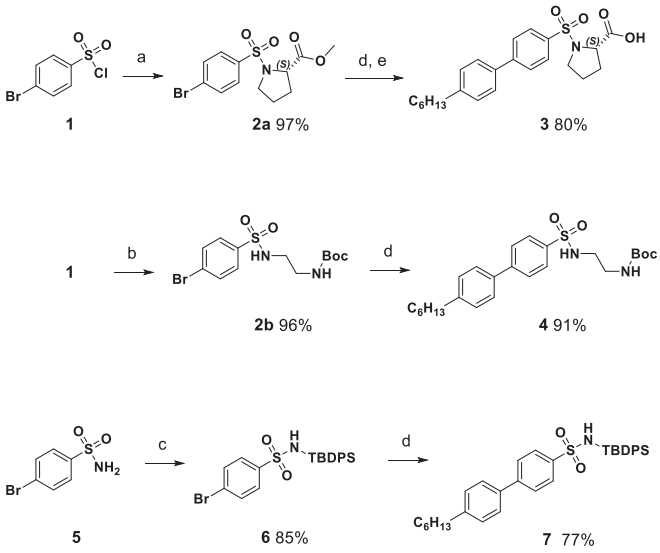
We report herein the design, synthesis, and biological evaluation of novel SA- and SIA-linked oligopeptides targeting the *E. coli* type I signal peptidase.

2. Results and discussion

2.1. Design and chemistry

The oligopeptide boronates with lipophilic tails designed and synthesized in this study were based on a Pro-Thr-Ala-Asn peptide sequence with an amino boronic ester as warhead at the N-terminus, a rigid biphenyl tail, and with SA or SIA as a linker functionality. Furthermore, for comparison, a SA-linked analog was designed with a flexible dodecane tail to investigate if there were differences from the more rigid tail analog. The SIA-linked oligopeptides were designed with the same sequence as the SA-linked analog for direct comparison of the linkers. As previously mentioned, the SIA group introduces chirality, therefore it was important to be able to separate the two diastereomers to investigate them for possible differences. To facilitate simple separation of the diastereomers, the SIA building blocks were designed with the tert-butyldiphenylsilyl (TBDPS)-protected SA. This bulky group has been shown to enhance differences between isomers; the fact that it is highly UV-active also makes the separation much easier [28]. We have previously shown that the TBDPS group is compatible with solid-phase peptide chemistry (SPPS), and is stable when cleaving the peptide with 10–20% hexafluoroisopropanol (HFIP) in DCM from a 2-chlorotritylchloride (2-CTC) polymer resin, whereas it was removed when using standard cleavage conditions such as TFA [29]. As highlighted in the initial study on the oligo-boronates [26], introducing a primary amine as a substituent on the proline in P5 was important for antibacterial activity. With this in mind, we designed an analog with an additional cationic sidechain (aminoethyl), utilizing the SIA moiety. The overall synthetic approach is described below where the SA building blocks were first prepared in solution (Scheme 1), the desired peptide sequences on solid phase (Scheme 2), then the building blocks were attached to the peptide sequence (Scheme 3), and finally the boronic ester warhead was coupled to the free C-terminus (Scheme 4).

The syntheses of the SA-based building blocks 3, 4 and 7 are outlined in Scheme 1. Compound 3 was prepared by a substitution of a bromobenzenesulfonyl chloride 1 with a proline methyl ester, followed by a Suzuki-Miyaura cross-coupling of a (4-hexylphenyl) boronic acid, then basic hydrolysis of the methyl ester to yield the free carboxylic acid at the proline. Likewise, compound 4 was prepared by substitution of an N-Boc-etylene diamine with bromobenzenesulfonyl-chloride, followed by Suzuki-Miyaura cross-coupling of the (4-hexylphenyl) boronic acid. The preparation of compound 7 started by protecting a bromobenzenesulfonamide 5 with a TBDPS group, which was followed with Suzuki-Miyaura cross-coupling of the (4-hexylphenyl) boronic acid to obtain the hexyl-biphenyl tail. The tripeptide 10 and tetrapeptide 11 were synthesized by manual SPPS on 2-CTC resin using standard protocols for N-terminal 9-fluorenylmethyloxycarbonyl-protected amino acids (Fmoc-AA-OH), using N,*N*-diisopropyl-ethylamine (DIPEA) and 3-[Bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate (HBTU) and 20% piperidine in DMF for Fmoc removal (Scheme 2). First, the Fmoc-Asn-(Trt)-OH 8 was attached to a 2-CTC resin to yield 9, which was further reacted with the remaining Fmoc-AAs, DIPEA, and HBTU and finally the Fmoc group was removed by treating the resin with 20% piperidine solution in DMF to yield 10 and 11. To further react the peptides with the SA-based building blocks, the commercially-available sulfonyl chloride 1a was coupled to the tetramer 11 (Scheme 3-c) and the building block 3 was attached directly to the trimer 10 (Scheme 3-a). Both peptides were then cleaved off the solid phase, resulting in the SA-linked oligopeptides 12–13 (Scheme 3-d).

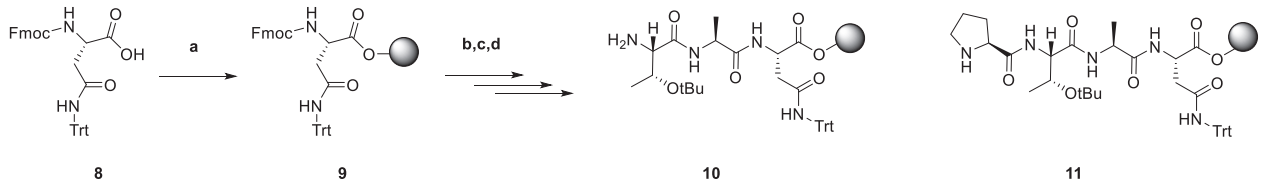


Scheme 1. Syntheses of the sulfonamide-based building blocks **3, 4** and **7**. Reagents and conditions: a) (S)-methyl pyrrolidin-1-ium-2-carboxylate chloride, Et_3N, DCM, overnight b) *tert*-butyl N-(2-aminoethyl) carbamate, Et_3N, DCM, 16h c) tert-butyl-chlorodiphenylsilane, Et_3N, anhydrous THF, overnight, 50 C d) (4-hexylphenyl) boronic acid, $Pd(PPh_3)_4$, K_2CO_3, Tol/EtOH (1:1), 4h e) LiOH, THF/MeOH/H_2O (3:2:1), 12h.

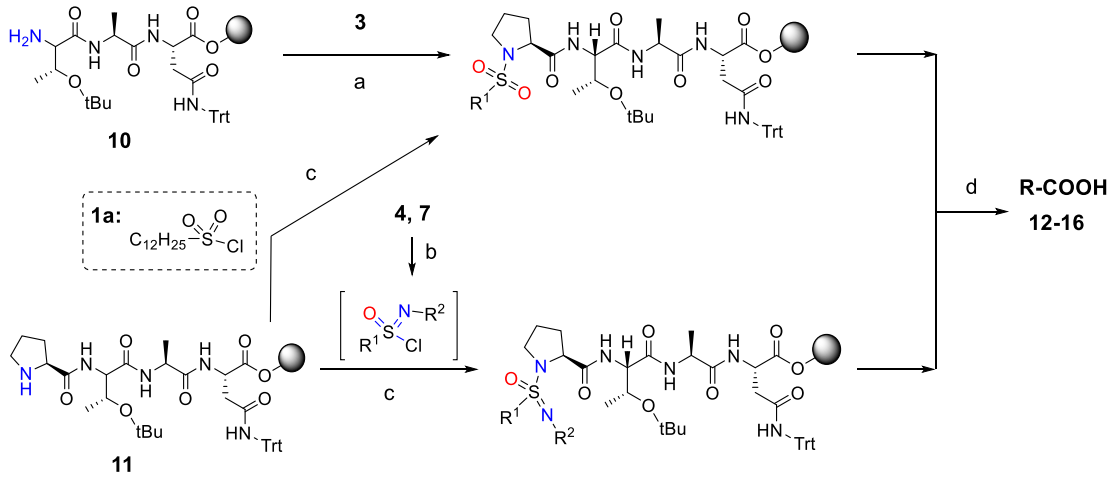
The syntheses of the SIA-linked oligopeptides started with converting the SA building blocks **4** and **7** to a sulfonimidoyl-chlorides (SIC) in solution, with the chlorinating agent triphenyl-phosphine dichloride (Scheme 3-b). The SIC solution was then added directly to the tetramer **11** on solid-phase as seen in Scheme 3-c. Any excess of unreacted building blocks was then simply washed away from the oligopeptide-bound resins, after which the resins were treated with 20% HFIP in DCM to selectively release the oligopeptides as free C-terminal acids **14**–**16** (Scheme 3-d), with the sidechain protecting groups still intact. Thanks to the TBDPS group, diastereomers **14** and **15** were easily separated by preparative HPLC, in contrast to the diastereomers of the Boc-protected aminoethyl compound **16** which were inseparable under these conditions. After purification of the acids by preparative reversed-phase HPLC, the final step included the attachment of the reactive boronic ester warhead via peptide coupling of the commercially-available (*R*)-boroAla-(+)-pinanediol, in solution, resulting in the final compounds **17**–**21** (Scheme 4). It is however well known that activation of C-terminal carboxylic acids of peptides can cause racemization via oxazolone formation [30]. To avoid this as much as possible, the activation was carried out at low temperature for 3 min before the addition of the amino boronate. However, as previously observed by De Rosa et al., racemization was unavoidable, resulting in partial loss of chiral integrity at the asparagine chiral side chain, and yielding mixtures of epimers in ratios ranging from 90:10 to 50:50. The epimers could not be separated in the preparative RP-HPLC, and thus the final compounds were isolated and tested as mixtures of epimers in analogy with the corresponding inhibitors evaluated by De Rosa et al. [26].

2.2. In vitro biology

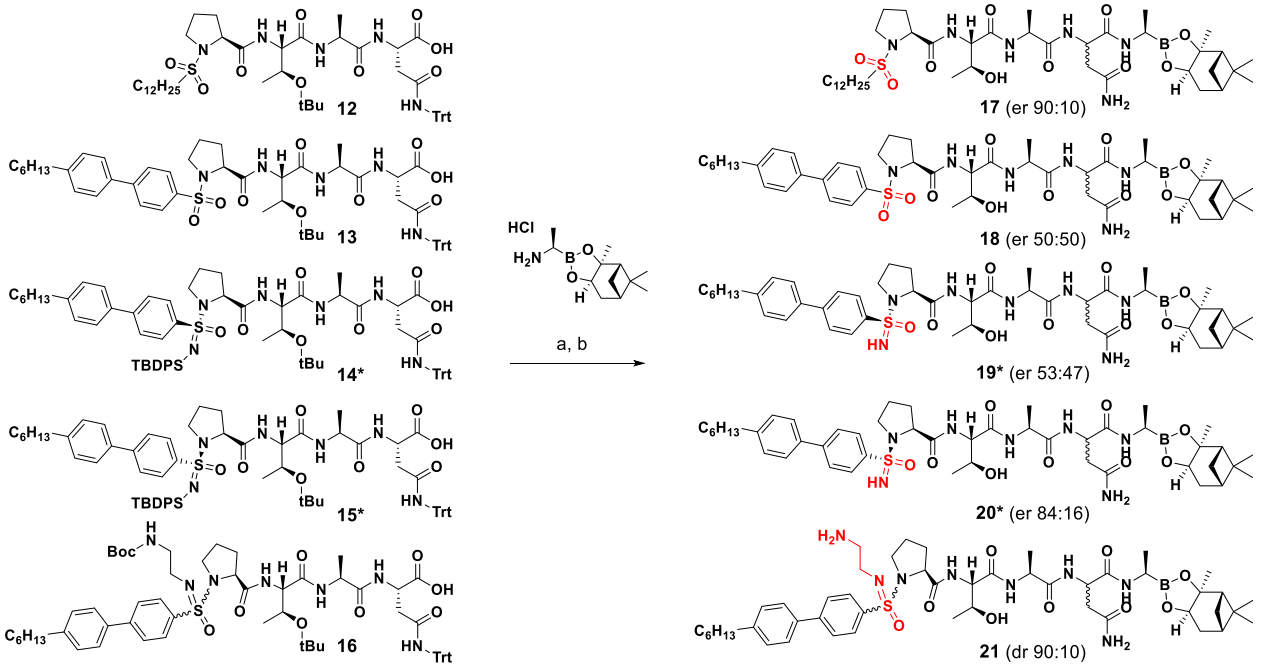
The final compounds **17**–**21** were evaluated for *in vitro* inhibitory potency against the *E. coli* SPase in a FRET-based functional assay, reported in Table 1 as IC_{50} values (μM) [26]. The compounds were also evaluated for their minimal inhibitory concentration (MIC), against a panel of seven Gram-negative bacterial strains and one Gram-positive organism, *S. aureus* [26]. The *S. aureus* spsB gene encodes a catalytically active and essential Type I signal peptidase



Scheme 2. Solid-phase synthesis of peptides **10** and **11.** *Reagents and conditions*: a) 2-CTC, DIPEA, DCM, r. t., 2 h, then MeOH, r. t., 0.5 h; b) 20% piperidine in DMF, twice, 5 min + 20 min, r. t.; c) Fmoc-AA-OH, HBTU, DIPEA, DMF, r. t., 5 h; d) 20% piperidine in DMF (5 + 20 min), DMF wash.



Scheme 3. Synthetic route of the SA- and SIA linked oligopeptides **12**–**16.** Reagents and conditions: a) HBTU, DIPEA, DMF, 4h b) PPh_3Cl_2, DIPEA, anhydrous DCM c) DIPEA, N_2, 0 d) HFIP in DCM (1:4), 1h at rt.

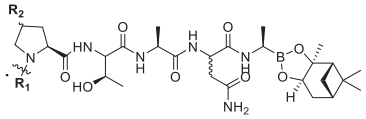


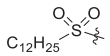
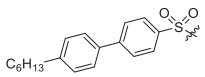
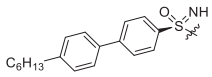
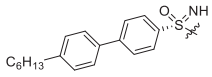
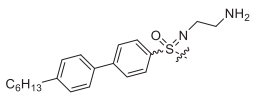
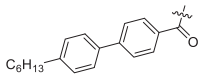
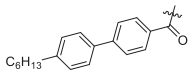
Scheme 4. Final step in the synthesis of SA- and SIA-linked oligopeptide boronic acids **17**–**21** by peptide coupling of the boronic ester warhead to the linear oligopeptide carboxylic acids **12**–**16.** Reagents and conditions: a) (*R*)-boroAla-(+)-pinanediol hydrochloride, HATU, DIPEA, DCM, 0 °C, 1h; b) TFA/DCM 1:1, 2h. *Relative stereochemistry. The epimeric ratio (er) and the diastereomeric ratio (dr) of the final compounds are seen in parentheses.

enzyme with similar conserved sequence motifs to LepB in *E. coli* [31]. Further, the oligopeptides were evaluated on the human liver cell line HepG2 for cytotoxicity, and in a hemolysis assay where the compounds (100 μM) were tested, using red blood cells from heparinized human blood, values greater than 1% in the hemolysis assay are regarded as a red flag [26].

All of the new oligopeptides (**17**–**21**) were able to inhibit the LepB protease, with IC_{50}s ranging from 0.056 to 1.11 μM.

Table 1
Structures and *in vitro* testing results for compounds **17**–**23**.



Cmpd. nr.	R_1	R_2	*Ec*LepB IC_{50} (μM)	MIC (μg/mL)								Cytotoxicity IC_{50} (μM)[i]	Hemolysis (%)
				E. c.[a]	E. c.[b]	E. c.[c]	P. a.[d]	P. a.[e]	K. p.[f]	A. b.[f]	S. a.[h]		
17		H	0.056	>64	8	16	>64	32	>64	>64	≤1	1.20	0.10 ± 0.01
18		H	0.071	>64	>64	>64	>64	>64	>64	>64	>64	0.58	14.5 ± 8.8
19		H	1.07	>64	4	8	>64	>64	>64	>64	4	2.95	2.15 ± 0.24
20		H	1.11	>64	4	32	>64	>64	>64	>64	4	2.99	0.35 ± 0.31
21		H	0.133	64	16	8	>64	>64	>64	64	≤1	5.20	5.32 ± 0.8
22[j]		H	0.012	>64	64	16	>64	>64	>64	>64	4	4.7	1.1 ± 0.2
23[j]		$CH_2NH_3^+$	0.018	32	4	1	64	8	16	32	≤1	18.6	5.7 ± 0.3

[a]E. coli ATCC 25922 WT; [b]E. coli tolC (CH3130, efflux-defective mutant isogenic to ATCC 25922); [c]E. coli D22 (CGSG 5163, lps mutant, drug-hypersensitive); [d]P. aeruginosa PAO1 WT; [e]P. aeruginosa efflux defective PAO750 (isogenic to PAO1); [f]K. pneumoniae ATCC 13883 WT; [g]A. baumanii ATCC 19606 WT; [h]S. aureus ATCC 29213 (Gram-positive WT reference); [i]Cytotoxicity of HepG2 cells; [j]Reference compounds from De Rosa et al. [25].

As seen in Table 1, the SA-linked peptides **17** and **18** were more potent inhibitors of the enzyme (0.056–0.071 μM) compared to the SIA-linked peptides **19**–**21** (0.133–1.11 μM), but not as potent as the previously reported amide-linked peptides **22** and **23** (0.012–0.018 μM) [26]. The difference between **22** and **23** is that the latter has a cationic sidechain in P5, which makes the compound active on bacteria, able to inhibit growth at 4 μg/mL on the efflux-defective *E. coli* strain and at 32 μg/mL on wild-type. Compound **22** and its new SA analog **18** do not have a cationic side chain and lack antibacterial activity even on the efflux-defective strain (>64 μg/mL). However, the SIA analogs **19**–**20** both inhibit bacterial growth on the efflux-defective strain at 4 μg/mL, equally potent to compound **23,** which relies on the cationic side chain for this activity. The drawback of the cationic sidechain in **23** was strong (5.7%) hemolysis, whereas the hemolytic effects of the SIA-analogs **19**–**20** were lower, at 0.35–2.2%. The SIA analogs **19**–**21** were also active on the hypersensitive *E. coli* strain at 8–32 μg/mL, however, no activity was observed on wild-type strains, possibly because they are substrates for efflux pump activity. The cytotoxic effects of the SIA-linked peptides were 5-fold less than the SA analogs (~2.97 μM vs. 0.58 μM), however without reaching safe levels, defined as at least ten times lower than enzyme potency. Adding the cationic aminoethyl group directly on the SIA handle (diastereomers **21**) resulted in enhanced inhibition of the LepB protease as compared to the unsubstituted SIA peptides (**19** and **20**) and 9-fold suppression of cytotoxicity compared to the SA (0.58 μM vs. 5.20 μM). However, it should be noted that the values are rough and should be analyzed with caution since the compounds are a mixture of epimers/diastereomers. Thus, it might be that the inhibition potency, as well as cytotoxicity, for individual stereoisomers could be higher. Since all compounds in Table 1 (**17**–**23**) are mixed with certain amounts of D-Asn epimer, which could not be separated, the exact influence of this epimer remains unknown.

The initial set of compounds were all designed with the biphenyl lipophilic tail however, to evaluate whether the flexibility of the tail in these types of compounds would have any impact on activity or toxicity, we prepared **17**, a direct analog of **18** but with a flexible tail. Notably, the more flexible tail had an impact on the antibacterial activity, resulting in activity on the efflux-defective and hypersensitive *E. coli* mutants, and on *S. aureus*, unlike epimers **18**, which were inactive on all strains. The compounds showing MIC activity against *S. aureus* (Table 1) are believed to do so by targeting the SpsB enzyme but at present we have no direct evidence to support this. The alternative possibility is that the activity on *S. aureus* reflects general cytotoxicity, however, there is no correlation between MIC on *S. aureus* and cytotoxicity measured by hemolysis or IC_{50} on HepG2 cells (Table 1). The flexibility of the tail did not result in any difference in cytotoxicity, however, the more flexible analog had negligible hemolytic effects (0.1%) compared to the more rigid one, where 14.5% hemolysis was observed. The same effect was seen by De Rosa et al. [26], i.e. the more flexible tail was moderately less hemolytic.

3. Conclusions

In exploring the effects of novel bioisosteric replacements, a number of sulfonamide and sulfonimidamide-based oligopeptides were designed and synthesized. The goal was to study whether such small changes in large bioactive peptides would result in marked differences in their properties. The biological evaluation of the sulfonimidamide-based oligopeptides indicated that the bioisosteric replacement was tolerated, but did not result in optimized inhibition compared to the previously-reported amide-based oligopeptides. However, in the case of the sulfonimidamide **20**, hemolysis was decreased to basal levels, yet the compound was active on an efflux-defective *E. coli* strain. Further, replacing the sulfonamide with sulfonimidamide decreased the cytotoxicity and led to improved antibacterial properties. Additionally, utilizing the sulfonimidamide motif for adding a basic aminoethyl side chain to the "N" handle resulted in reduced cytotoxicity compared to the sulfonamide. We foresee that the synthetic approaches herein demonstrated and the awareness of SIA's ability to affect various properties of bioactive peptides will inspire and facilitate exploration of this functionality in peptide-based drug discovery.

4. Experimental section

4.1. Chemical synthesis

All chemicals and solvents were purchased from Sigma Aldrich, Fisher Scientific, FluoroChem, Enamine and Ark Pharm chemicals and were used without further purification. Analytical thin-layer chromatography (TLC) was performed using Merck aluminum sheets precoated with silica gel 60 F_{254}. Column chromatography was performed on Merck silica gel 60 (40–63 μm) ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury Plus instruments; ^{1}H at 399.9 MHz and ^{13}C at 100.6 MHz at 25 °C. Chemical shifts (δ) are reported in ppm and coupling constants (J) are reported in hertz (Hz). Exact molecular masses were determined on Micromass Q-Tof 2 mass spectrometer equipped with an electrospray ion source. Analytical RP-HPLC-MS was performed on a Gilson RP-HPLC system with a Finnigan AQA quadrupole low-resolution mass spectrometer in positive or negative ESI mode using an Onyx Monolithic C_{18} 3 × 50 mm, 2.6 μm particle size, 100 Å pore size (Phenomenex) with gradients of MeCN in 0.05% aqueous HCOOH as mobile phase at a flow rate of 2 mL/min. Preparative RP-HPLC was performed on a system equipped with a Nucleodur C18 HTec 5 μm column (150 × 21.2 mm) or a Phenomenex C8 5 μm column (150 × 21.2 mm), using a H_2O/MeCN gradient with 0.1% TFA or H_2O/MeCN gradient, flow rate 14 mL/min with 20 mM triethylammonium acetate (TEAA), in both cases using UV detection at 220 nm and 254 nm. High-resolution molecular masses (HRMS) were determined on a Waters acuity UPLC mass spectrometer with an ESI source, 7-T hybrid linear ion trap (LTQ), flow rate 0.25 mL/min (MeCN/H_2O 1:1). Compounds **1**, **1a, 5,** and **8** were purchased, and compounds **2a** and **2b** are commercially available but synthesized in this work. The rest of the compounds are new entities and have been characterized by ^{1}H NMR and ^{13}C NMR and ESI (MS). All final compounds were ≥95% pure as determined by analytical HPLC and NMR.

4.1.1. Synthesis of methyl ((4-bromophenyl)sulfonyl)-L-prolinate (2a)

To a solution of (S)-2-(methoxycarbonyl)pyrrolidin-1-ium chloride (12.7 mmol, 2.10 g) in dry DCM (35 mL) in a round bottom flask, commercially available 4-bromo-benzenesulfonyl chloride (19.0 mmol, 4.86 g) and triethylamine (38.0 mmol, 5.30 mL) was carefully added at 0 C and left stirring for 30 min and then at rt overnight. The mixture was diluted with 1 M aq. HCl (35 mL) and DCM (5 mL) and the two phases were separated in a funnel. The organic phase was washed twice with 1 M HCl (30 mL). The combined organic layers were washed twice with brine (25 mL), dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The title compound **2a** was obtained as white solid (4.30 g, 97% yield) and used in the next step without further purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.75 (AA′ of an AA'XX', 2H), 7.66 (XX′ of an AA'XX', 2H), 4.35 (dd, J = 8.2, 3.6 Hz, 1H), 3.71 (s, 3H), 3.45 (m, 1H), 3.35 (m, 1H), 2.09 (m, 1H), 2.06–1.91 (m, 2H), 1.83 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.2, 137.3, 132.1, 128.8, 127.6, 60.2, 52.3, 48.1, 30.7, 24.5. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{12}H_{15}NO_4SBr$ 347.9905; found, 347.9901.

4.1.2. Synthesis of tert-butyl (2-((4-bromophenyl)sulfonamido) ethyl) carbamate (2b)

Commercially available 4-bromo-benzenesulfonate chloride (2.16 mmol, 553 mg) was dissolved in 6 mL DCM, N-Boc-ethylenediamine (1.95 mmol, 308 μL) and TEA (6.49 mmol, 905 μL) was added dropwise and the mixture stirred overnight. After the completion of the reaction, excess TEA was boiled away and then the reaction mixture was diluted with 50 mL of water and extracted two times with DCM, the organic phases collected and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude product then purified by silica gel chromatography (isohexane/ethyl acetate 1:1) and a white solid **2b** (797 mg, 96% yield) was obtained. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.81 (AA′ of an AA'XX', 2H), 7.71 (XX′ of an AA'XX', 2H), 7.33 (bs, 1H), 6.76 (t, J = 5.8 Hz, 1H), 2.94 (m, 2H), 2.76 (m, 2H), 1.34 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.9, 140.2, 132.8, 129.0, 126.7, 78.3, 42.7, 40.6, 28.6. $C_{13}H_{19}BrN_2O_4S$, MS (ESI): *m/z* 758.9 [2 M + H]$^+$. HRMS-ESI (*m/z*): $[M + CH_3CN + Na]^+$ calcd for $C_{15}H_{22}BrN_3O_4SNa$ 442.0412; found, 442.0405.

4.1.3. Synthesis of ((4′-hexyl-[1,1′-biphenyl]-4-yl)sulfonyl)-L-proline (3)

A 30 mL vial was charged with sulfonamide 2a (1.44 mmol, 500 mg) and hexylphenyl-boronic acid (2.87 mmol, 592 mg) followed by addition of toluene/EtOH (1:1, 11 mL), 4.0 M potassium carbonate (5.74 mmol, 1.44 mL) and at last $Pd(PPh_3)_4$ (0.0717 mmol, 82.9 mg). The vial was sealed and flushed with N_2 gas and heated to 90 °C and left stirring for 3 h when it was stopped after TLC and LC-MS results confirmed that the starting materials were consumed. The reaction mixture was washed with 1 M HCl (2 × 15 mL) and DCM (2 × 15 mL) the organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. To the reaction mixture, LiOH (4.18 mmol, 90.6 mg) was added along with 6 mL of THF/MeOH/H_2O (3:2:1) and left stirring overnight. The reaction mixture was washed again with 1 M HCl (2 × 10 mL) and DCM (2 × 10 mL) the organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The crude mixture was then purified by flash column chromatography (EtOAc/iso-hexane, 4:6). The pure fractions with the product were collected and concentrated affording 3 as a white solid (432 mg, 80% yield). $C_{23}H_{29}NO_4S$, MS (ESI): *m/z* 416.1 $[M+H]^+$. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.93 (AA' of an AA'XX', 2H), 7.74 (XX' of an AA'XX', 2H), 7.54 (AA' of an AA'XX', 2H), 7.29 (XX' of an AA'XX', 2H), 4.33 (m, 1H), 3.56 (m, 1H), 3.33 (m, 1H), 2.66 (t, J = 7.8, 7.4 Hz, 2H), 2.15 (m, 1H), 2.07–1.92 (m, 2H), 1.69–1.62 (m, 2H), 1.34–1.31 (m, 2H), 1.27–1.25 (m, 6H), 0.89 (distorted triplet, w = 13.9 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-d) δ 176.6, 146.5, 144.2, 136.8, 135.9, 129.6, 128.5, 128.0, 127.6, 60.9, 49.3, 36.1, 32.2, 31.8, 31.1, 29.4, 25.1, 23.1, 14.6. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{23}H_{30}NO_4S$, 416.1817; found, 416.1882.

4.1.4. Synthesis of tert-butyl (2-((4′-hexyl-[1,1′-biphenyl])-4-sulfonamido)ethyl)carbamate (**4**)

A 30 mL vial was loaded with sulfonamide **2b** (1.23 mmol, 466 mg) and hexylphenyl-boronic acid (2.46 mmol, 506 mg) followed by addition of 4.0 M K_2CO_3 (4.91 mmol, 1.23 mL), 10 mL Toluene/EtOH (1:1) and triphenyl-phosphine)palladium (0) (0.0614 mmol, 71.0 mg) was added. The vial was sealed, and the reaction mixture was stirred at 100 °C for 4h. Upon completion, the reaction mixture was cooled down to rt and diluted with DCM (30 mL) and brine solution (30 mL) were added, and the two phases separated. The organic phases were collected and concentrated *in vacuo* and purified by column chromatography (1.5:1 iso-hexane/ethyl acetate) yielding 515 mg of **4** as white solid (91% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91 (m, 1H), 7.85 (AA'XX' of an AA'XX', 4H), 7.67 (XX' of an AA'XX', 2H), 7.32 (AA' of an AA'XX', 2H), 6.79 (t, J = 5.8 Hz, 1H), 2.97 (m, 2H), 2.78 (m, 2H), 2.62 (t, J = 8.05, 2H), 1.59 (m, 2H), 1.34 (s, 9H), 1.31–1.24 (m, 6H), 0.86 (distorted triplet, w = 14.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.9, 144.3, 143.4, 139.3, 136.3, 134.6, 129.5, 127.60, 127.58, 127.4, 78.3, 42.8, 35.2, 31.6, 31.3, 28.8, 28.6, 22.5, 14.4. HRMS-ESI (m/z): [M + MeCN + Na] for $C_{27}H_{39}N_3O_4NaS$ Calcd: 524.2559, Found: 524.2557 (M + MeCN + Na).

4.1.5. Synthesis of 4-bromo-N-(tert-butyldiphenylsilyl)benzenesulfonamide (**6**)

In a solution of commercially available 4-bromobenzenesulfonamide (4.24 mmol, 1.00 g) in dry THF (25 mL), tert-butyl-chloro-diphenylsilane (8.46 mmol, 2.20 mL) and triethylamine (16.9 mmol, 2.36 mL) was added and left stirring at 50 °C overnight. After the completion of the reaction the solvent was removed *in vacuo* and the product recrystallized from pentane/MeOH resulting in 1.70 g of compound **6** as grey crystal (85% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.33 (s, 1H), 7.61 (AA′ of an AA'XX', 2H), 7.57 (BB′ of an BB'CC'D, 4H), 7.47 (D of an BB'CC'D, 2H), 7.37 (CC′ of an BB'CC'D, 4H), 7.31 (XX′ of an AA'XX', 2H), 0.96 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 143.5, 136.0, 132.1, 132.0, 130.3, 128.01, 127.95, 125.8, 27.2, 18.6. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{22}H_{23}BrNO_2SSi$, 472.0402; found, 472.0410.

4.1.6. Synthesis of N-(tert-butyldiphenylsilyl)-4′-hexyl-[1,1′-biphenyl]-4-sulfonamide (**7**)

A 30 mL vial was loaded with TBDPS protected sulfonamide **6** (0.887 mmol, 421 mg) and hexylphenyl-boronic acid (1.77 mmol, 366 mg) followed by addition of 4.0 M K_2CO_3 (3.55 mmol, 0.887 mL), 10 mL Toluene/EtOH (1:1) and lastly triphenyl-phosphine)palladium (0) (0.0444 mmol, 51.3 mg) was added. The vial was sealed, and the reaction mixture stirred at 90 °C for 3h. Upon completion, the reaction mixture was cooled down to rt and diluted with DCM (30 mL) and brine solution (30 mL) added, and the two phases separated. The organic phases were collected and concentrated *in vacuo* and purified by column chromatography (0.5:3; ethyl acetate/iso-hexane) yielding 380 mg of **7** as white solid (77% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.23 (s, 1H), 7.66 (AA′ of an AA'XX', 2H), 7.63 (AA′ of an AA'XX', 2H), 7.60 (BB′ of an BB'CC'D, 4H), 7.48 (XX of an AA'XX', 2H), 7.45 (D of an BB'CC'D, 2H), 7.37 (CC′ of an BB'CC'D, 4H),7.33 (XX of an AA'XX', 2H), 2.63 (t, J = 7.6 Hz, 2H), 1.60 (m, 2H), 1.34–1.27 (m, 6H), 0.96 (s, 9H), 0.87 (distorted triplet, w = 13.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 143.6, 143.2, 136.5, 136.1, 132.4, 130.3, 129.5, 127.9, 127.3, 126.9, 126.7, 115.4, 35.2, 31.6, 31.3, 28.8, 27.2, 22.6, 18.7, 14.4. (ESI): m/z 556.8 $[M+H]^+$. HRMS-ESI (m/z): $[M - H]^+$ calcd for $C_{34}H_{40}NO_2SSi$, 554.2549; found, 554.2531.

4.1.7. General synthesis of the peptides **12**–**16**

The peptides **10** and **11** were synthesized on a 1.0 mmol scale by manual solid-phase peptide synthesis (SPPS) from 2-Chlorotritylchloride (2-CTC) polymer resin (1.63 mmol/g) in a 12 mL disposable syringe fitted with porous polyethylene filter. For the Fmoc-amino acids the following sidechain protecting groups were used: Thr-(*t*-Bu), Asn-(Trt). Standard Fmoc conditions were used and the Fmoc protecting group was removed by treatment with 20% piperidine in DMF (2 × 3 mL, 5 + 20 min) and the polymer was washed with DMF (5 × 3 mL, 5 × 2 min). Coupling of the first amino acid Fmoc-Asn-(Trt)-OH (0.8 equiv.) to the 2-CTC resin (1 equiv.) was performed in anhydrous DCM (7.5 mL) in the presence of DIPEA (3 equiv.) under agitation for 2 h at rt, after which MeOH (1.2 mL) was added to cap the unreacted sites of the resin (15 min agitation). The MeOH was removed by filtration and the resin washed with DCM, DMF and again DCM and dried under vacuum overnight. 1.46 g of Fmoc-Asn-(Trt)-CTC resin were obtained, with a new loading of 0.68 mmol/g. Next, coupling of the appropriate amino acid, Fmoc-AA-OH (4 equiv.) was performed in DMF (3 mL) using *N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 4 equiv.), in the presence of *N,N*-diisopropylethylamine (DIPEA, 8 equiv.), shaking at rt for 4 h. In the case of α-branched amino acids (e.g. Fmoc-Thr (*t*-Bu)-OH) or secondary amine (Fmoc-Pro-OH), the coupling was carried out overnight. After each coupling, the resin was washed with DMF (5 × 3 mL, 5 × 2 min) and subsequently de-protected and washed as described above. After completion of the coupling cycle the resin was washed with several portions of DMF, MeOH and DCM before it was dried under high vacuum overnight. The final peptides were cleaved from the resin by treatment with a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/DCM 1:4 (5 mL), followed by agitation for 1h at rt. The resin was filtered off and washed several times with DCM. The filtrate was collected in a centrifuge tube and concentrated in a stream of nitrogen and the residue was directly purified. The crude peptides were dissolved in H_2O/MeCN and filtered through a 0.45 μm nylon membrane and purified in 6–8 runs by RP-HPLC. Selected fractions were analyzed by RP-HPLC-MS, and those containing pure product were combined and lyophilized.

4.1.8. Synthesis of N^2–*O*-(tert-butyl)-N-((dodecylsulfonyl)-L-prolyl)-L-allothreonyl-L-alanyl-N^4-trityl-L-asparagine (**12**)

In a 12 mL disposable syringe, fitted with porous polyethylene filter, the resin bound tetramer **11** was added (0.135 mmol, 300 mg, loading 0.45 mmol/g) and swelled in DCM for 5 min. Then DCM was filtered away and solution of 20% piperidine in DMF added and reacted on rotating wheel. Into a falcon tube, commercially-available dodecane sulfonyl chloride **1a** was added (0.552 mmol, 149 mg) with TEA (1.11 mmol, 0.154 mL) dissolved in 12 mL anhydrous DCM. This solution was then added to the syringe containing the resin-bound peptide and left rotating on wheel for 5h. The leftover reaction mixture was then washed away and the resin washed with DCM (3 × 4 mL), DMF (3 × 4 mL), THF (2 × 4 mL) and DCM (2 × 4 mL). The peptide was then cleaved from the resin and the filtrate and two DCM washes were collected and the solvent removed *in vacuo*. The crude peptide was purified by preparative RP-HPLC (0.05% HCOOH in MeCN/H_2O, 90–100% gradient, 20 min, product eluted after 10 min) and the fractions containing the desired compound were combined and lyophilized to get 88.1 mg (70% yield) of desired protected peptide as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.65 (s, 1H), 8.76 (s, 1H), 8.20 (d, J = 8.1 Hz, 1H), 7.77 (d, J = 8.1 Hz, 1H), 7.73 (d, J = 7.2 Hz, 1H), 7.27–7.23 (m, 6H), 7.19–7.15 (m, 9H), 4.46 (m, 1H), 4.39 (m, 1H), 4.36 (m, 1H), 4.21 (dd, J = 8.1, 3.2 Hz, 1H), 4.02 (m, 1H), 3.46–3.33 (m, 2H), 3.19–3.06 (m, 2H), 2.74 (dd, J = 15.1, 7.2 Hz, 1H), 2.57 (m, 1H), 2.14 (m, 1H), 1.93 (m, 1H), 1.89–1.79 (m, 2H), 1.73–1.61 (m, 2H), 1.34 (m, 2H), 1.30–1.27 (m, 3H), 1.25–1.22 (m, 16H), 1.14 (s, 9H), 1.00 (d, J = 6.2 Hz, 3H), 0.84 (distorted triplet, w = 13.6 Hz, 3H). ^{13}C NMR

(101 MHz, DMSO-d_6) δ 173.2, 171.95, 171.89, 169.2, 168.9, 145.2, 129.0, 127.9, 126.8, 74.3, 69.9, 67.0, 61.4, 57.9, 49.1, 48.9, 48.3, 31.8, 31.4, 29.4, 29.2 (2C), 29.0, 28.5, 28.2, 25.0, 23.0, 22.6, 19.5, 14.4. MS (ESI): *m/z* 932.3 $[M+H]^+$. HRMS-ESI (*m/z):* $[M+H]^+$ calcd for $C_{51}H_{73}N_5O_9NaS$, 954.5027; found, 954.5022.

4.1.9. Synthesis of N^2—O-(tert-butyl)-N-(((4′-hexyl-[1,1′-biphenyl]-4-yl)sulfonyl)-L-prolyl)-L-allothreonyl-L-alanyl-N^4-trityl-L-asparagine (**13**)

The analog was prepared according to the general peptide synthesis using the trimer **10** (the Fmoc group was removed prior coupling by the general method of deprotection) (0.754 mmol, 877 mg, loading: 0.53 mmol/g), the sulfonamide building block **3** (1.04 mmol, 433 mg) in anhydrous DMF (7 mL), and adding HBTU (1.04 mmol, 394 mg) and DIPEA (2.26 mmol, 0.394 mL). The peptide was then cleaved from the resin and was directly purified by preparative RP-HPLC (0.05% HCOOH in MeCN/H_2O, 90—100% gradient, 20 min, product eluted after 8 min) and the fractions containing the desired compound were combined and lyophilized to get 528 mg (70% yield) of desired protected peptide as white soft solid. MS (ESI): *m/z* 1000.4.^{1}H NMR (400 MHz, DMSO-d_6) δ 12.65 (s, 1H), 8.70 (s, 1H), 8.25 (d, J = 8.0 Hz, 1H), 7.97 (m, 1H) 7.95 (AA′ of an AA'XX', 2H), 7.89 (XX of an AA'XX', 2H), 7.77 (d, J = 7.2 Hz, 1H) 7.67 (AA′ of an AA'XX', 2H), 7.33 (XX of an AA'XX', 2H), 7.27—7.22 (m, 6H), 7.20—7.15 (m, 9H) 5.16 (m, 1H), 4.49 (m, 1H) 4.40 (m, 1H), 4.36 (m, 1H), 4.25 (m, 1H), 4.05 (m, 1H), 3.42 (m, 1H), 3.22 (m, 1H) 2.77 (m, 1H) 2.62 (m, 3H), 1.86 (m, 1H), 1.76 (m, 1H) 1.73 (m, 1H), 1.61 (m, 2H) 1.55 (m, 1H), 1.28 (m, 10H), 1.16 (s, 9H), 1.05 (d, J = 6.3 Hz, 3H) 0.86 (distorted triplet, w = 14.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.2, 172.0, 171.5, 169.2, 168.9, 162.8, 145.2, 145.0, 143.6, 136.1, 135.7, 129.6, 129.0, 128.7, 127.9 127.6, 127.4, 126.8, 74.4, 69.9, 67.0, 61.7, 58.0, 49.7, 48.3, 38.4, 36.2, 35.2, 31.6, 31.3, 31.1, 28.8, 28.5, 24.7, 22.5, 19.6, 19.5, 14.4. $[M+H]^+$. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{57}H_{70}N_5O_9S$, 1000.4894; found, 1000.4902.

4.1.10. Synthesis of N^2—O-(tert-butyl)-N-(((S)—N-(tert-butyldiphenyl-silyl)-4′-hexyl-[1,1′-biphenyl]-4-sulfonimidoyl)-L-prolyl)-L allothreonyl-L-alanyl-N^4-trityl-L-asparagine (**14**)

To a stirred suspension of PPh_3Cl_2 (0.147 mmol, 49.2 mg) in dry DCM (1.1 mL) under a N_2 atmosphere was added DIPEA (0.306 mmol, 53.4 μL). The reaction mixture was stirred for 20 min at rt and thereafter cooled to 0 °C. A solution of the TBDPS-protected sulfonamide building block **7** (0.123 mmol, 80.0 mg) in dry DCM (4 mL) was added, formation of a light brown-yellow solution was observed immediately. The reaction mixture was stirred for 30 min at 0 °C then added to the DMF swelled tetramer-bound resin **11** (0.341 mmol, 832 mg, loading: 0.41 mmol/g), in a syringe for 2h on rotating wheel. The peptide was then washed with 3 × 5 mL of DCM and DMF and then cleaved from the resin and directly purified by preparative RP-HPLC (0.05% HCOOH in MeCN, 30 min, product eluted after 16 min) and the fractions containing the desired compound were combined and lyophilized to get 59.5 mg (65.7% yield) of desired protected peptide as white soft solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.55 (broad s, 1H), 8.78 (broad s, 1H), 8.31 (d, J = 7.9 Hz, 1H), 8.21 (d, J = 7.3 Hz, 1H) 8.18 (d, J = 7.7 Hz, 1H) 7.83 (AA′ of an AA'XX, 2H), 7.83 (d, J = 8.0 Hz, 1H), 7.81 (d, J = 7.2 Hz, 1H) 7.78 (AA′ of an AA'XX, 2H) 7.72 (XX′ of an AA'XX, 2H) 7.70 (BB′ of an BB'CC'D, 4H) 7.63 (D of an BB'CC'D, 2H), 7.34 (CC′ of an BB'CC'D, 4H), 7.30 (XX of an AA'XX', 2H), 7.24 (m, 6H), 7.17 (m, 9H), 4.46 (m, 1H), 4.40 (m, 2H), 4.18 (dd, J = 8.0, 3.0 Hz, 1H), 3.91 (m, 1H), 3.20 (m, 1H), 3.25 (m, 1H), 2.69 (dd, J = 15.2, 6.0 Hz, 1H), 2.61 (m, 2H), 2.56 (m, 1H), 1.75 (m, 1H), 1.67 (m, 1H), 1.58 (m, 2H), 1.50 (m, 1H), 1.27—1.24 (m, 6H), 1.11—1.09 (m, 10H), 0.99 (m, 9H), 0.90 (m, 3H) 0.85 (distorted t, w = 13.7 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.2, 172.0, 171.8, 169.3, 168.6, 145.3, 145.3, 143.7, 143.2, 140.4, 136.3, 136.0, 135.6, 135.5, 129.5, 129.5, 129.0, 128.2, 127.9, 127.8, 127.8, 127.3, 127.0, 126.8, 74.5, 69.8, 67.8, 66.9, 62.5, 57.4, 49.9, 49.0, 48.4, 48.2, 40.5, 35.2, 31.6, 31.5, 31.3, 28.8, 28.5, 28.3, 27.5, 24.6, 22.5, 20.8, 19.62, 19.57, 18.7, 14.4. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{73}H_{87}N_6O_8SSi$, 1235.6075; found, 1235.6100.

4.1.11. Synthesis of N^2—O-(tert-butyl)-N-(((R)-N-(tert-butyldiphenylsilyl)-4′-hexyl-[1,1′-biphenyl]-4-sulfonimidoyl)-L-prolyl)-L-allothreonyl-L-alanyl-N^4-trityl-L-asparagine (**15**)

To a stirred suspension of PPh_3Cl_2 in dry DCM (1 mL) under a N_2 gas DIPEA (0.306 mmol, 53.4 μL) was added and the reaction mixture stirred for 30 min at rt then cooled to 0 °C. A solution of the TBDPS-protected sulfonamide building block **7** (0.123 mmol, 80.0 mg) in dry DCM (4 mL) was added. The reaction mixture was stirred for 30 min then added to the DMF swelled tetramer bound resin **11** (0.341 mmol, 832 mg, loading: 0.41 mmol/g), in a filter containing syringe and left rotating for 2h. The peptide was then washed with 3 × 5 mL of DCM and DMF and then cleaved from the resin. The crude was directly purified by preparative RP-HPLC (0.05% HCOOH in MeCN, 30 min, product eluted after 14 min) and the fractions containing the desired compound were combined and lyophilized to get 54.9 mg (63.7% yield) of desired protected peptide as white soft solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.56 (broad s, 1H), 9.13 (broad s, 1H), 8.20 (d, J = 7.9 Hz, 1H), 7.94 (AA′ of an AA'XX, 2H), 7.83 (d, J = 8.0 Hz, 1H), 7.81 (d, J = 7.2 Hz, 1H) 7.78 (AA′ of an AA'XX, 2H) 7.72 (XX′ of an AA'XX, 2H) 7.70 (BB′ of an BB'CC'D, 4H) 7.63 (D of an BB'CC'D, 2H), 7.34 (CC′ of an BB'CC'D, 4H), 7.29 (XX′ of an AA'XX', 2H), 7.24 (m, 6H), 7.17 (m, 9H), 4.47 (m, 1H), 4.42 (m, 1H), 4.25 (dd, J = 8.0, 3.0 Hz, 1H), 4.15 (dd, J = 8.5, 3.5 Hz, 1H), 4.06 (m, 1H), 3.29 (m, 1H), 3.25 (m, 1H), 2.76 (dd, J = 15.2, 6.0 Hz, 1H), 2.61 (m, 2H), 2.56 (m, 1H), 1.83 (m, 1H), 1.67 (m, 1H), 1.58 (m, 3H), 1.50 (m, 1H), 1.27—1.24 (m, 6H), 1.11 (m, 12H), 1.02 (m, 12H), 0.85 (distorted t, w = 14.1 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 173.2, 172.0, 171.9, 169.2, 169.0, 145.2, 143.9, 143.3, 139.0, 136.3, 136.1, 135.5, 129.5, 129.0, 128.4, 127.9, 127.3, 127.2, 126.8, 74.3, 69.8, 66.8, 61.8, 58.0, 50.6, 49.7, 48.3, 40.5, 38.5, 35.2, 31.6, 31.3, 31.1, 28.8, 28.7, 28.4, 27.5, 24.7, 22.5, 19.7, 19.52, 19.46, 14.4. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{73}H_{87}N_6O_8SSi$, 1235.6075; found, 1235.6102.

4.1.12. Synthesis of N^2—N-((N-(2-((tert-butoxycarbonyl)amino)ethyl)-4′-hexyl-[1,1′-biphenyl]-4-sulfonimidoyl)-L-prolyl)-O-(tert-butyl)-L-allothreonyl-L-alanyl-N^4-trityl-L-asparagine (**16**)

To a stirred suspension of PPh_3Cl_2 in dry DCM (1 mL) under a N_2 atmosphere was added DIPEA (1.32 mmol, 230 μL). The reaction mixture was stirred for 30 min at rt then cooled to 0 °C. A solution of the Boc-protected sulfonamide building block **4** (0.430 mmol, 198 mg) in dry DCM (4 mL) was added, formation of a clear light brown yellow solution was observed immediately. The reaction mixture was stirred for 30 min at 0 °C then added to the DMF swelled tetramer bound resin **11** (0.341 mmol, 832 mg, loading: 0.41 mmol/g), in a filter containing syringe and left on a rotating wheel for 2h. The peptide was then washed with 3 × 5 mL of DCM and DMF and then cleaved from the resin and directly purified by preparative RP-HPLC (0.05% HCOOH in MeCN for 30 min. The product eluted after 16 min) and the fractions containing the desired compound were combined and lyophilized to get 54.9 mg (63.7% yield) of desired protected peptide as white fluffy solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.07 (broad s, 1H), 8.32 (d, J = 8.1 Hz, 1H), 8.16 (d, J = 7.7 Hz, 1H), 7.98 (d, J = 8.3 Hz, 1H), 7.88 (d, J = 7.2 Hz, 1H), 7.84 (m, 1H), 7.65 (m, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.27 (m, 3H), 7.24 (m, 6H), 7.17 (m, 12H), 4.45—4.38 (m, 2H), 4.36 (m, 1H), 4.21 (m, 1H), 3.99 (m, 1H), 3.80 (m, 1H), 2.99 (m, 1H), 2.87 (m, 1H), 2.70 (m, 2H), 2.63 (m, 1H), 2.54 (s, 9H), 2.06 (m, 2H), 1.75 (m, 1H), 1.67 (m, 2H), 1.60 (m, 1H), 1.37 (m, 3H), 1.26 (m, 7H), 1.16 (m, 2H), 1.13 (s, 10H), 1.05 (m, 1H), 0.99 (d, J = 6.3 Hz, 3H), 0.86 (distorted triplet,

w = 13.6 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.8, 171.3, 168.9, 168.7, 168.6, 144.8, 129.1, 128.6, 127.4, 126.3, 77.4, 73.8, 69.3, 66.8, 61.7, 59.8, 57.6, 57.3, 49.4, 47.9, 46.4, 42.4, 42.2, 42.0, 40.4, 34.8, 31.1, 30.8, 30.3, 28.4, 28.0, 25.2, 22.1, 19.1, 18.9, 14.0. $C_{64}H_{84}N_7O_{10}S$ MS (ESI): *m/z* 1142.4 $[M+H]^+$. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{64}H_{84}N_7O_{10}S$, 1142.5995; found, 1142.6000.

4.1.13. General method for coupling the amino-boronic ester warhead to peptides **12**–**16** and deprotection of sidechain protecting groups

To a solution of the peptide (1 equiv.) in anhydrous DCM (2–4 mL), HATU (2 equiv.) and DIPEA (3–10 equiv.) were added under N_2 flow and the mixture was stirred at 0 °C for 5 min, after which the warhead (2 equiv.) was added and the reaction mixture was stirred for 30 min and then removed from the ice bath and stirred for another 30 min at rt till completion (monitoring via LC-MS). After completion, the solvent was removed under N_2 stream and the crude dissolved in minimum amount of DMSO for purification by RP-HPLC, the pure product was then used for the final step, deprotection of the sidechain protecting groups. The crude or pure protected peptides (1 equiv.) were dissolved in a solution of 95% TFA in H_2O or in a solution of TFA/DCM/TES (10:9:1) and stirred at rt till completion (1 h). The solvents were removed under reduced pressure and the residues were purified by preparative RP-HPLC with TEAA/H_2O/MeCN mobile system.

4.1.14. Synthesis of (2S)-2-((2S)-2-((3R)-2-((S)-1-(dodecylsulfonyl) pyrrolidine-2-carboxamido)-3-hydroxybutan-amido) propanamido)-N^1-((R)-1-((3aS,4S,6S,7aR)-3a,5,5-trimethyl-hexahydro-4,6-methanobenzo[d] [1–3]dioxaborol-2-yl)ethyl) succinamide (**17**)

The final compound **17** was prepared according to the general procedure using peptide **12** (0.0429 mmol, 40.0 mg), and the commercially available warhead (*R*)-BoroAla-(+)-Pinanediol hydrochloride (0.0858 mmol, 22.3 mg) in anhydrous DCM (1.2 mL) and in the presence of HATU (0.0858 mmol, 32.6 mg) and DIPEA (0.172 mmol, 29.9 μL). The solvent was removed, and the crude mixture dissolved in 900 μL DMSO and purified by multiple runs into the preparative RP-HPLC (20 mM TEAA MeCN/H_2O, 90–100% gradient for 20 min, product eluted at 9 min). The fractions with the desired product were collected at 214 nm and lyophilized yielding 40.1 mg of pure white compound (84% yield) (RP-HPLC purity: C8 column 100%, MS (ESI): *m/z* 1137.9) with sidechains still protected. Next, the deprotection of the side chains was performed according to the general method (TFA/DCM/TES, 10:9:1, 3 mL) for 1h at rt. The solvent was removed and the oily crude purified by preparative RP-HPLC (20 mM TEAA MeCN/H2O, 55–100% 28 min gradient; product eluted after 8 min) affording 11.5 mg (39% yield) of desired oligopeptide boronate **17** (epimeric ratio 90:10). Major epimer: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (broad s, 1H), 8.08 (d, J = 7.7 Hz, 1H), 7.95 (d, J = 6.8 Hz, 1H), 7.71 (d, J = 8.5 Hz, 1H), 7.35 (s, 1H), 6.96 (s, 1H), 5.02 (d, J = 5.2 Hz, 1H), 4.59 (q, J = 6.9 Hz, 1H), 4.36 (dd, J = 8.6, 3.6 Hz, 1H), 4.25 (m, 1H), 4.21 (m, 1H), 4.09 (m, 1H), 4.07 (m, 1H), 3.46–3.35 (m, 2H), 3.20–3.07 (m, 2H), 2.52 (m, 2H), 2.49–2.45 (m, 2H), 2.21 (m, 1H), 2.13 (m, 1H), 2.01 (m, 2H), 1.93 (m, 1H), 1.88–1.82 (m, 2H), 1.78 (m, 1H), 1.70–1.63 (m, 2H), 1.62 (m, 1H), 1.40–1.33 (m, 4H), 1.28–1.17 (m, 21H), 1.03 (d, J = 6.3 Hz, 3H), 0.99 (m, 2H), 0.84 (distorted triplet, w = 13.5 Hz, 3H), 0.80 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.1, 172.2, 171.2, 170.2, 83.3, 76.1, 67.0, 61.5, 57.9, 52.2, 49.1, 49.0, 48.5, 47.1, 38.1, 31.8, 29.5, 29.42, 29.38, 29.3, 29.2, 29.0, 28.2, 27.6, 25.0, 24.4, 23.0, 22.6, 20.1, 18.2, 16.9, 14.4. RP-HPLC purity: C8 column >95%. $C_{40}H_{71}BN_6O_{10}S$ MS (ESI): *m/z* 839.5 $[M+H]^+$. HRMS-ESI (*m/z*): $[M + Na + H]^+$ calcd for $C_{40}H_{72}BN_6O_{10}S$ + Na, 861.4938; found, 861.4949.

4.1.15. Synthesis of (2S)-2-((2S)-2-((3R)-2-((S)-1-((4′-hexyl-[1,1′-biphenyl]-4-yl)sulfonyl)pyrrolidine-2-carboxamido)-3-hydroxy butanamido)propanamido)-N1-((R)-1-((3aS,4S,6S,7aR)-3a,5,5-trimethylhexahydro-4,6-methanobenzo[d] [1–3]dioxaborol-2-yl) ethyl)succinamide (**18**)

Final compound **18** was prepared according to the general procedure using the peptide **13** (0.114 mmol, 114 mg), and the warhead (*R*)-BoroAla-(+)-Pinanediol hydrochloride (0.181 mmol, 46.9 mg) in anhydrous DCM and in the presence of HATU (0.228 mmol, 86.6 mg) and DIPEA (1.14 mmol, 198 μL). DCM was evaporated and de-protection of the side chains was performed according to the general method (TFA/DCM, 10:9, 3 mL). The crude yellow oil was purified by preparative RP-HPLC (using TEAA as the buffer, to avoid hydrolysis of the boronate to boronic acid), affording 8.1 mg (9.2% yield) of the desired oligopeptide **18** as white soft solid and as a 50:50 mixture of epimers. Characterization for the major epimer: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.37 (d, J = 8.0 Hz, 1H), 8.26 (m, 2H), 8.00 (m, 1H), 7.93 (AA′ of an AA'XX', 4H), 7.68 (XX′ of an AA'XX', 2H), 7.50 (m, 1H), 7.34 (XX′ of an AA'XX', 2H), 7.11 (m, 2H), 5.02 (m, 1H), 4.81 (m, 1H), 4.31 (m, 1H), 4.27 (m, 1H), 4.09 (m, 2H), 3.92 (m, 1H), 3.65 (m, 2H), 3.62 (m, 1H), 3.45 (m, 1H), 3.21 (m, 1H), 3.14 (m, 3H), 2.64 (m, 3H), 1.84 (m, 1H), 1.76 (m, 1H), 1.70 (m, 1H), 1.60 (m, 2H), 1.55 (m, 1H), 1.29 (m, 2H), 1.26 (m, 6H), 1.24 (m, 8H), 1.09 (m, 1H), 0.96 (m, 2H), 0.86 (distorted triplet, w = 11.9 Hz, 3H), 0.82 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 177.3, 172.4, 171.3, 170.0, 169.8, 144.6, 143.1, 135.6, 135.0, 129.1, 128.3, 127.1, 126.3, 118.1, 97.2, 76.4, 66.7, 65.7, 61.4, 59.9, 57.7, 54.9, 49.2, 45.8, 35.9, 34.8, 31.1, 30.8, 30.7, 29.0, 28.3, 26.2, 24.2, 22.1, 19.7, 18.1, 17.6, 16.7, 14.9, 14.0, 12.5, 8.6. $C_{46}H_{67}BN_6O_{10}S$ MS (ESI): *m/z* 907.47 $[M+H]^+$. RP-HPLC purity: C8 column >95%. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{46}H_{68}N_6O_{10}SB$, 907.4811; found, 907.4818.

4.1.16. Synthesis of (S)-2-((S)-2-((2S,3S)-2-((S)-1-((R)-4′-hexyl-[1,1′-biphenyl]-4-sulfonimidoyl)pyrrolidine-2-carboxamido)-3-hydroxy butanamido)propanamido)-N^1-((R)-1-((3aS,4S,6S,7aR)-3a,5,5-trimethylhexahydro-4,6-methanobenzo[d] [1–3]dioxaborol-2-yl)ethyl)succinamide (**19**)

The final compound **19** was prepared according to the general procedure using the peptide **14** (0.049 mmol, 51.8 mg), and the warhead (*R*)-BoroAla-(+)-Pinanediol hydrochloride (0.0837 mmol, 31.8 mg) in anhydrous DCM (1 mL) and in the presence of HATU (0.0837 mmol, 31.8 mg) and DIPEA (0.209 mmol, 36.5 μL). DCM was evaporated and de-protection of the side chains was performed according to the general method (TFA/DCM, 10:9, 3 mL). The crude yellow oil was purified by preparative RP-HPLC (using TEAA as the buffer, to avoid hydrolysis of the boronate to boronic acid), affording the total yield of the epimers 15 mg (25% yield) of the desired oligopeptide **19** as white solid and as a 53:47 mixture of epimers. Characterization for the major epimer: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.63 (m, 1H), 8.39 (d, J = 6.9 Hz, 1H), 8.27 (d, J = 8.2 Hz, 1H), 8.06 (d, J = 7.9 Hz, 1H), 7.97 (AA′ of an AA'XX', 2H), 7.90 (AA′ of an AA'XX', 2H), 7.80 (d, J = 8.4 Hz, 1H), 7.68 (XX′ of an AA'XX', 2H), 7.34 (XX′ of an AA'XX', 2H), 6.97 (m, 1H), 5.12 (m, 1H), 4.66 (s, 1H), 4.62 (d, J = 7.0 Hz, 1H), 4.32 (m, 1H), 4.27 (m, 1H), 4.17 (m, 1H), 4.07 (m, 1H), 3.92 (m, 1H), 3.51–3.43 (m, 1H), 3.13 (m, 1H), 2.62 (m, 3H), 2.45 (m, 1H), 2.38 (m, 1H), 2.19 (m, 1H), 2.01 (m, 1H), 1.84 (m, 1H), 1.79 (m, 2H), 1.72–1.69 (m, 3H), 1.65 (m, 1H), 1.62–1.57 (m, 2H), 1.51 (m, 1H), 1.27 (m, 6H), 1.27–1.22 (m, 8H), 1.09–1.06 (m, 3H), 1.00 (m, 2H), 0.86 (distorted triplet, w = 13.8 Hz, 3H), 0.80 (broad s, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.6, 172.3, 172.2, 170.8, 170.7, 143.9, 142.9, 135.7, 135.4, 129.1, 128.6, 127.9, 126.9, 82.9, 75.7, 66.7, 62.5, 57.4, 51.7, 49.9, 48.9, 48.7, 47.6, 45.7, 37.6, 36.6, 36.2, 34.7, 31.1, 30.8, 28.9, 28.3, 27.1, 26.0, 24.3, 23.9, 22.1, 20.1, 17.7, 17.5, 16.4, 14.0. $C_{46}H_{68}BN_7O_9S$, MS (ESI): *m/z* 906.47 $[M+H]^+$. RP-HPLC purity: C8 column >95%. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{46}H_{69}N_7O_9SB$,

906.4971; found, 906.5002.

*4.1.17. Synthesis of (2S)-2-((2S)-2-((3R)-2-((S)-1-((S)-4′-hexyl-[1,1′-biphenyl]-4-sulfonimidoyl)pyrrolidine-2-carbox-amido)-3-hydroxy butan-amido)propanamido)-N[1]-((R)-1-((3aS,4S,6S,7aR)-3a,5,5-trimethyl-hexa-hydro-4,6-methanobenzo[d] [1–3] dioxaborol-2-yl) ethyl)succinamide (**20**)*

The final compound **20** was prepared according to the general procedure using the peptide **15** (0.0404 mmol, 50.0 mg), and the warhead (*R*)-BoroAla-(+)-Pinanediol hydrochloride (0.0808 mmol, 21.0 mg) in anhydrous DCM (1.5 mL) in the presence of HATU (0.0808 mmol, 30.7 mg) and DIPEA (0.202 mmol, 35.2 μL). DCM was evaporated and de-protection of the side chains was performed according to the general method (TFA/DCM, 10:9, 2 mL). The crude yellow oil was purified by preparative RP-HPLC (using TEAA as the buffer, to avoid hydrolysis of the boronate to boronic acid), affording the total yield of the epimers 7.0 mg (12% yield) of the desired oligopeptide **20** as white solid and as a 84:16 mixture of epimers. Characterization for the major epimer: ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.63 (m, 1H), 8.06 (d, J = 7.9 Hz, 1H), 7.99 (m, 1H), 7.97 (AA′ of an AA'XX', 2H), 7.90 (AA′ of an AA'XX', 2H), 7.80 (d, J = 8.4 Hz, 1H), 7.68 (XX′ ozf an AA'XX', 2H), 7.36 (s, 1H), 7.34 (XX′ of an AA'XX', 2H), 6.97 (m, 1H), 5.12 (m, 1H), 4.76 (s, 1H), 4.62 (q, J = 7.0 Hz, 1H), 4.27 (m, 1H), 4.23 (m, 1H), 4.17 (m, 1H), 4.09 (m, 1H), 4.06 (m, 1H), 3.41 (m, 1H), 3.14 (m, 1H), 2.62 (m, 3H), 2.38 (m, 1H), 2.19 (m, 1H), 2.01 (m, 1H), 1.84 (m, 1H), 1.79 (m, 1H), 1.72–1.69 (m, 3H), 1.65 (m, 1H), 1.62–1.57 (m, 2H), 1.51 (m, 1H), 1.27 (m, 6H), 1.24 (m, 6H), 1.21 (m, 3H), 1.08 (m, 3H), 1.00 (m, 2H), 0.86 (distorted triplet, w = 13.8 Hz, 3H), 0.80 (s, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.6, 172.3, 172.2, 170.8, 170.7, 143.9, 142.9, 135.7, 135.4, 129.1, 128.6, 127.9, 126.9, 82.9, 75.7, 66.7, 62.5, 57.4, 51.7, 49.9, 48.9, 48.7, 47.6, 45.7, 37.6, 36.6, 36.2, 34.7, 31.1, 30.8, 28.9, 28.3, 27.1, 26.0, 24.3, 23.9, 22.1, 20.1, 17.7, 17.5, 16.4, 14.0. $C_{46}H_{68}BN_7O_9S$, MS (ESI): m/z 906.47 $[M+H]^+$. RP-HPLC purity: C8 column >95%. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{46}H_{69}N_7O_9SB$, 906.4971; found, 906.5002.

*4.1.18. Synthesis of 2-((((S)-2-(((2S,3S)-1-(((S)-1-(((S)-4-amino-1,4-dioxo-1-(((R)-1-((3aS,4S,6S,7aR)-3a,5,5-trimethylhexahydro-4,6-methanobenzo[d] [1–3]dioxaborol-2-yl)ethyl)amino)butan-2-yl) amino)-1-oxopropan-2-yl)amino)-3-hydroxy-1-oxobutan-2-yl) carbamoyl)pyrrolidin-1-yl) (4′-hexyl-[1,1′-biphenyl]-4-yl) (oxo)-l6-sulfanylidene)amino)ethan-1-aminium (**21**)*

The final compound **21** was prepared according to the general procedure using the peptide **16** (0.0446 mmol, 50.9 mg), and the warhead (*R*)-BoroAla-(+)-Pinanediol hydrochloride (0.0891 mmol, 23.1 mg) in anhydrous DCM (900 μL) in the presence of HATU (0.0891 mmol, 33.9 mg) and DIPEA (0.178 mmol, 31.0 μL). DCM was evaporated and de-protection of the side chains was performed according to the general method (TFA/DCM, 10:9, 2 mL). The crude yellow oil was purified by preparative RP-HPLC (using TEAA as the buffer, to avoid hydrolysis of the boronate to boronic acid), affording the total yield of the epimers 8.1 mg (14% yield) of the desired oligopeptide **21** as white solid and as a 90:10 mixture of epimers. Characterization for the major epimer: ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.06 (broad s, 2H), 8.71 (m, 2H), 8.31 (d, J = 8.1 Hz, 1H), 8.23 (d, J = 7.0 Hz, 1H), 8.17 (d, J = 8.2 Hz, 1H), 8.09 (d, J = 8.3 Hz, 1H) 8.05 (AA′ of an AA'XX, 2H), 7.89 (XX′ of an AA'XX, 2H) 7.78 (m, 1H), 7.69 (AA′ of an AA'XX, 2H), 7.34 (XX of an AA'XX', 2H), 4.61 (m, 1H), 4.37 (m, 2H), 4.28 (m, 2H), 4.23 (m, 1H), 4.06 (m, 1H), 3.64 (m, 1H), 3.38 (m, 1H), 3.27 (m, 1H), 2.90 (m, 1H), 2.63 (m, 2H), 2.19 (m, 2H), 2.02 (m, 2H), 1.88 (m, 2H), 1.76 (m, 1H), 1.59 (m, 1H), 1.31–1.27 (m, 8H), 1.24 (m, 3H), 1.22 (m, 2H), 1.20 (m, 1H), 1.09 (m, 3H), 1.04 (m, 3H), 1.00 (m, 3H), 0.94 (d, J = 6.5 Hz, 3H), 0.86 (distorted t, w = 13.9 Hz, 3H), 0.80 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 173.7, 172.1, 171.9, 170.6, 170.1, 158.0, 157.7, 143.1, 135.7, 129.1, 128.5, 127.5, 127.0, 75.7, 51.7, 47.5, 45.7, 41.1, 40.0, 39.9, 39.7, 39.5, 39.4, 39.2, 39.0, 37.7, 36.2, 34.8, 31.1, 30.8, 28.9, 28.3, 28.1, 27.1, 26.0, 24.0, 23.9, 22.1, 22.0 (2C), 20.0, 16.4, 14.0, 8.6. RP-HPLC purity: C8 column >95%. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{48}H_{73}BN_8O_9S$, 949.5393; found, 949.5410.

4.2. Bioassays

The *E. coli* signal peptidase activity and inhibition experiments were conducted in a FRET assay with the two fluorophore labeled substrates; dabcyl-VEVGGTATA↓GAFSRPGLE-(EDANS)) and dabcyl-KLTFGTVKPVQAIA↓GYEILE-(EDANS)-OH (arrows indicate the expected cleavage sites). The linear increase in fluorescence, corresponding to substrate cleavage, was monitored at 22 °C for 2 h. For the inhibition tests, EcLepB was pre-incubated with the compound for 10 min at 22 °C, then the reaction was followed at a substrate (dabcyl-VGGTATAGAFSRPGLE (EDANS)-OH) concentration of 8 μM; final EcLepB concentration was estimated to be 50 nM. Reaction rates were plotted as a function of inhibitor concentration, and half maximal inhibitory concentration (IC_{50}) values were determined by a non-linear regression analysis of the sigmoidal dose–response curves in GraphPad Prism (GraphPad software Inc., CA, USA). The cloning, expression and purification of the *E. coli* LepB protein is fully described in the supporting information by De Rosa et al. [26].

For assessing the minimal inhibitory concentration (MIC), the compounds were prepared in Mueller-Hinton II medium and dispensed into a 96-well round-bottomed plate to give final assay concentrations from 64 μg/mL down to 0.25 μg/mL (two-fold dilution series in 10 wells, with two control wells: medium control with no bacteria or compound, and growth control with bacteria added but no compound). Plates were covered and incubated without shaking for 16–20 h at 35 °C ± 2 °C. MIC was read visually, as complete inhibition of growth by the unaided eye, using the medium-only wells as the control [26].

The compounds were evaluated for hemolytic activity using red blood cells from heparinized human blood. Red blood cells (RBC) were washed three times in Tyrode buffer (130 mM NaCl, 4 mM KCl, 2.8 mM Na acetate, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, 1 mM CaCl2, adjusted to pH 7.4) and resuspended in the same buffer. Final concentrations in the hemolysis assay were 100 μM compound, 1% DMSO, and 50% RBC, assayed in a 200 μL vol. in a microtiter plate. The mixture was incubated at 37 °C for 45 min with shaking (250 rpm). After incubation, RBCs were removed by centrifugation and clear plasma was transferred to a fresh plate, and the amount of hemoglobin measured using a spectrophotometer at 540 nm. The complete lysis control contained 2% Triton X-100 (in Tyrode buffer) instead of compound; the negative control contained Tyrode buffer but no compound. Percent hemolysis was calculated as: [Abs compound] − [Abs negative control]/[Abs complete lysis control] − [Abs negative control] x 100. Values greater than 1% hemolysis at 100 μM were regarded as a red flag [26].

In vitro cytotoxicity was determined by a fluorometric microculture cytotoxicity assay using the HepG2 cell line from ATCC, and cultured in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/100 μg/mL) and L-glutamine 2 mM. HepG2 cells were passaged 2 times/week and used maximally for 20 passages. Cells were finally seeded in Nunc 384-well assay plates at a density of 1000 cells/well. Cytotoxicity was assessed after 72 h with cell survival presented as survival index (SI, %) defined as fluorescence in test wells in percent of control cultures with blank values subtracted. Criteria for a successful assay included a signal-to-noise ratio in control cultures >10, CV < 30% and a positive control (Bortezomib) SI of <5%. The half maximal inhibitory concentration (IC_{50}) was determined from

log concentration-effect curves in GraphPad Prism using a non-linear regression analysis. Detailed procedure is reported by De Rosa et al. [26].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships which have or could be perceived to have influenced the work reported in this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113699.

Author contributions

AB carried out the synthesis, data analysis, and wrote the manuscript. AS, AK, and EZ were involved in the project planning, supervision, data analysis, and in manuscript editing. The enzymatic studies and data analysis were performed by LL. The microbiology and hemolysis assays were performed by SC. SLM and DH were involved in the supervision, data analysis, and in manuscript editing. All authors listed contributed to the manuscript.

References

[1] G. Klebe, in: Drug Design: Methodology, Concepts, and Mode-Of-Action, Springer Berlin Heidelberg, Berlin, Heidelberg, 2013, https://doi.org/10.1007/978-3-642-17907-5_8.
[2] B. Carlo, M.H. Donna, L. Amos, B. Smith, Carboxylic acid (Bio)lsosteres in drug design, ChemMedChem 8 (2013) 385–395, https://doi.org/10.1002/cmdc.201200585.Carboxylic.
[3] F. Carta, A. Scozzafava, C.T. Supuran, Sulfonamides: a patent review, Expert Opin. Ther. Pat. 22 (2012) 747–758, https://doi.org/10.1517/13543776.2012.698264.
[4] P.K. Chinthakindi, T. Naicker, N. Thota, T. Govender, H.G. Kruger, P.I. Arvidsson, Sulfonimidamides in medicinal and agricultural chemistry, Angew. Chem. Int. Ed. 56 (2017) 4100–4109, https://doi.org/10.1002/anie.201610456.
[5] M. Frings, C. Bolm, A. Blum, C. Gnamm, Sulfoximines from a medicinal chemist's perspective: physicochemical and in vitro parameters relevant for drug discovery, Eur. J. Med. Chem. 126 (2017) 225–245, https://doi.org/10.1016/j.ejmech.2016.09.091.
[6] J.E. Toth, G.B. Grindey, W.J. Ehlhardt, J.E. Ray, G.B. Boder, J.R. Bewley, K.K. Klingerman, S.B. Gates, S.M. Rinzel, R.M. Schultz, L.C. Weir, J.F. Worzalla, Sulfonimidamide analogs of oncolytic sulfonylureas, J. Med. Chem. 40 (1997) 1018–1025, https://doi.org/10.1021/jm960673l.
[7] F. Sehgelmeble, J. Janson, C. Ray, S. Rosqvist, S. Gustavsson, L.I. Nilsson, A. Minidis, J. Holenz, D. Rotticci, J. Lundkvist, P.I. Arvidsson, Sulfonimidamides as sulfonamides bioisosteres: rational evaluation through synthetic, in vitro, and in vivo studies with γ-secretase inhibitors, ChemMedChem 7 (2012) 396–399, https://doi.org/10.1002/cmdc.201200014.
[8] N. Thota, P. Makam, K.K. Rajbongshi, S. Nagiah, N.S. Abdul, A.A. Chuturgoon, A. Kaushik, G. Lamichhane, A.M. Somboro, H.G. Kruger, T. Govender, T. Naicker, P.I. Arvidsson, N-Trifluoromethylthiolated Sulfonimidamides and Sulfoximines: Anti-microbial, anti-mycobacterial, and cytotoxic activity, ACS Med. Chem. Lett. 10 (2019) 1457–1461, https://doi.org/10.1021/acsmedchemlett.9b00285.
[9] A.D. Steinkamp, L. Schmitt, X. Chen, K. Fietkau, R. Heise, J.M. Baron, C. Bolm, Synthesis of a sulfonimidamide-based analog of Tasisulam and its biological evaluation in the melanoma cell lines SKMel23 and A375, skin pharmacol, Physiol. 29 (2017) 281–290, https://doi.org/10.1159/000453042.
[10] F. Prestinaci, P. Pezzotti, A. Pantosti, Pathogens and Global Health Antimicrobial resistance: a global multifaceted phenomenon Antimicrobial resistance: a global multifaceted phenomenon, Glob. Health 109 (2015) 309–318, https://doi.org/10.1179/2047773215Y.0000000030.
[11] U. Theuretzbacher, K. Outterson, A. Engel, A. Karlén, The global preclinical antibacterial pipeline, Nat. Rev. Microbiol. 18 (2020) 275–285, https://doi.org/10.1038/s41579-019-0288-0.
[12] L.L. Silver, Challenges of antibacterial discovery, Clin. Microbiol. Rev. 24 (2011) 71–109, https://doi.org/10.1128/CMR.00030-10.
[13] M. Paetzel, Bacterial signal peptidases. https://doi.org/10.1007/978-3-030-18768-2_7, 2019.
[14] M.T. Black, J.G.R. Munn, A.E. Allsop, On the catalytic mechanism of prokaryotic leader peptidase 1, Biochem. J. 282 (1992) 539–543, https://doi.org/10.1042/bj2820539.
[15] M.T. Black, Evidence that the catalytic activity of prokaryote leader peptidase depends upon the operation of a serine-lysine catalytic dyad, J. Bacteriol. 175 (1993) 4957–4961, https://doi.org/10.1128/jb.175.16.4957-4961.1993.
[16] M. Paetzel, R.E. Dalbey, N.C.J. Strynadka, Crystal structure of a bacterial signal peptidase apoenzyme. Implications for signal peptide binding and the Ser-Lys dyad mechanism, J. Biol. Chem. 277 (2002) 9512–9519, https://doi.org/10.1074/jbc.M110983200.
[17] T.C. Roberts, M.A. Schallenberger, J. Liu, P.A. Smith, F.E. Romesberg, Initial efforts toward the optimization of arylomycins for antibiotic activity, J. Med. Chem. 54 (2011) 4954–4963, https://doi.org/10.1021/jm1016126.
[18] P.A. Smith, M.F.T.T. Koehler, H.S. Girgis, D. Yan, Y.Y. Chen, Y.Y. Chen, J.J. Crawford, M.R. Durk, R.I. Higuchi, J. Kang, J. Murray, P. Paraselli, S. Park, W. Phung, J.G. Quinn, T.C. Roberts, L. Rougé, J.B. Schwarz, E. Skippington, J. Wai, M. Xu, Z. Yu, H. Zhang, M.-W.W. Tan, C.E. Heise, Optimized arylomycins are a new class of Gram-negative antibiotics, Nature 561 (2018) 189–194, https://doi.org/10.1038/s41586-018-0483-6.
[19] A. Höltzel, D.G. Schmid, G.J. Nicholson, S. Stevanovic, J. Schimana, K. Gebhardt, H.-P. Fiedler, G. Jung, Arylomycins A and B, new biaryl-bridged lipopeptide antibiotics produced by Streptomyces sp. Tü 6075. II. Structure elucidation, J. Antibiot. (Tokyo) 55 (2002) 571–577, https://doi.org/10.7164/antibiotics.55.571.
[20] A.G. Therien, J.L. Huber, K.E. Wilson, P. Beaulieu, A. Caron, D. Claveau, K. Deschamps, R.G.K. Donald, A.M. Galgoci, M. Gallant, X. Gu, N.J. Kevin, J. Lafleur, P.S. Leavitt, C. Lebeau-Jacob, S.S. Lee, M.M. Lin, A.A. Michels, A.M. Ogawa, R.E. Painter, C.A. Parish, Y.-W. Park, L. Benton-Perdomo, M. Petcu, J.W. Phillips, M.A. Powles, K.I. Skorey, J. Tam, C.M. Tan, K. Young, S. Wong, S.T. Waddell, L. Miesel, Broadening the spectrum of β-lactam antibiotics through inhibition of signal peptidase type I, antimicrob, Agents Chemother 56 (2012) 4662–4670, https://doi.org/10.1128/AAC.00726-12.
[21] A.E. Allsop, G. Brooks, G. Bruton, S. Coulton, P.D. Edwards, I.K. Hatton, A.C. Kaura, S.D. McLean, N.D. Pearson, T.C. Smale, R. Southgate, Penem inhibitors of bacterial signal peptidase, Bioorg. Med. Chem. Lett 5 (1995) 443–448, https://doi.org/10.1016/0960-894X(95)00052-U.
[22] X.E. Hu, N.K. Kim, L. Grinius, C.M. Morris, C.D. Wallace, G.E. Mieling, T.P. Demuth, Synthesis of (5S)-Tricyclic penems as novel and potent inhibitors of bacterial signal peptidases, Synthesis (Stuttg) 11 (2003) 1732–1738.
[23] J. Ollinger, T. O'Malley, J. Ahn, J. Odingo, T. Parish, Inhibition of the sole type I signal peptidase of Mycobacterium tuberculosis is bactericidal under replicating and nonreplicating conditions, J. Bacteriol. 194 (2012) 2614–2619, https://doi.org/10.1128/JB.00224-12.
[24] G. Bruton, A. Huxley, P. O'Hanlon, B. Orlek, D. Eggleston, J. Humphries, S. Readshaw, A. West, S. Ashman, M. Brown, K. Moore, A. Pope, K. O'Dwyer, L. Wang, Lipopeptide substrates for SpsB, the Staphylococcus aureus type I signal peptidase: design, conformation and conversion to α-ketoamide inhibitors, Eur. J. Med. Chem. 38 (2003) 351–356, https://doi.org/10.1016/S0223-5234(03)00040-0.
[25] P. Buzder-Lantos, K. Bockstael, J. Anné, P. Herdewijn, Substrate based peptide aldehyde inhibits bacterial type I signal peptidase, Bioorg. Med. Chem. Lett 19 (2009) 2880–2883, https://doi.org/10.1016/J.BMCL.2009.03.064.
[26] M. De Rosa, L. Lu, E. Zamaratski, N. Szałaj, S. Cao, H. Wadensten, L. Lenhammar, J. Gising, A.K. Roos, D.L. Huseby, R. Larsson, P.E. Andrén, D. Hughes, P. Brandt, S.L. Mowbray, A. Karlén, Design, synthesis and in vitro biological evaluation of oligopeptides targeting E. coli type I signal peptidase (LepB), Bioorg. Med. Chem. 25 (2017) 897–911, https://doi.org/10.1016/J.BMC.2016.12.003.
[27] N. Szałaj, L. Lu, A. Benediktsdottir, E. Zamaratski, S. Cao, G. Olanders, C. Hedgecock, A. Karlén, M. Erdélyi, D. Hughes, S.L. Mowbray, P. Brandt, Boronic ester-linked macrocyclic lipopeptides as serine protease inhibitors targeting Escherichia coli type I signal peptidase, Eur. J. Med. Chem. 157 (2018) 1346–1360, https://doi.org/10.1016/j.ejmech.2018.08.086.
[28] P.K. Chinthakindi, A. Benediktsdottir, A. Ibrahim, A. Wared, C.-J.J. Aurell, A. Pettersen, E. Zamaratski, P.I. Arvidsson, Y. Chen, A. Sandström, Synthesis of sulfonimidamide-based amino acid building blocks with orthogonal protecting groups, Eur. J. Org Chem. (2019) 1045–1057, https://doi.org/10.1002/ejoc.201801541.
[29] P.K. Chinthakindi, A. Benediktsdottir, P.I. Arvidsson, Y. Chen, A. Sandström, Solid phase synthesis of sulfonimidamide pseudopeptides and library generation, Eur. J. Org Chem. (2020) 3796–3807, https://doi.org/10.1002/ejoc.202000108.
[30] C.A.G.N. Montalbetti, V. Falque, Amide bond formation and peptide coupling, Tetrahedron 61 (2005) 10827–10852, https://doi.org/10.1016/j.tet.2005.08.031.
[31] K.M. Cregg, E.I. Wilding, M.T. Black, Molecular cloning and expression of the spsB gene encoding an essential type I signal peptidase from Staphylococcus aureus, J. Bacteriol. 178 (1996) 5712–5718, https://doi.org/10.1128/jb.178.19.5712-5718.1996.