Microcin C and albomycin analogues with aryl-tetrazole substituents as nucleobase isosters are selective inhibitors of bacterial aminoacyl tRNA synthetases but lack efficient uptake

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Abstract

In 1998 Cubist Pharmaceuticals patented a series of aminoacyl tRNA synthetase (aaRS) inhibitors based on aminoacyl sulfamoyladenines (aaSAs) where the adenine was substituted by aryl-tetrazole moieties linking to the ribose fragment via a two-carbon spacer. Although being strong specific inhibitors of bacterial IleRS, these compounds proved not successful in vivo, due to low cell permeability and strong binding to serum albumin. In this work we attempted to improve these compounds by combining them with microcin C (McC) or albomycin (*i.e.* Siderophore Drug Conjugate (SDC)) transport modules. We found that aryl-tetrazole variants of McC and albomycin still lacked antibacterial activity. However, these compounds were readily processed by the *E. coli* aminopeptidases with the release of toxic aaRS inhibitors. Hence, the lack of activity in whole-cell assays was due to inability of the new compounds to be taken up by the cells, indicating that the nucleotide moieties of McC and albomycin strongly contribute to facilitated transport of these compounds inside the cell.

Introduction

Increasing resistance to antibiotics is a major problem worldwide and provides the stimulus for development of new bacterial inhibitors. The process of bacterial protein synthesis is a validated target of antibiotic action. Aminoacyl-tRNA synthetases (aaRSs) play an indispensable role in protein synthesis. These proteins aminoacylate tRNAs with cognate amino acids in a two-stage process whereby the amino acid is first activated with adenosine monophosphate (AMP) and in a second step esterified to the 2'- or 3'-hydroxy group of the 3'-end of respective tRNA. The best known aaRS inhibitors to date are aminoacyl sulfamoyl adenosines that are poorly hydrolysable isosters of aminoacyladenylates, which are used by the aaRSs as reactive intermediates to esterify the amino acids to their cognate tRNA.¹ To date, a wealth of compounds was synthesized that target various aaRSs.¹⁻⁵ Unfortunately these compounds show low selectivity towards microbial aaRSs or suffer from other limitations like poor cell permeability.

A US patent by Cubist Pharmaceuticals[®]⁶ reported on the synthesis and evaluation of a new type of aaRS inhibitors. Although still based upon aminoacyl sulfamoyladenine, these analogues contained an aryl-tetrazole moiety, which substituted the adenine part, and was connected through a two-carbon linker to ribose. The tetrazole moiety is linked to either one or two five- or six-membered heterocycles. The advantage of these compounds compared to aaSAs is their high selectivity for bacterial aaRSs relative to the human homologues. The two most important compounds, along with IleSA (**1**) are depicted in Scheme 1 and their inhibitory properties are listed in Table 1. As can be seen, aryl-tetrazole-containing compounds **2** and **3** exhibit good activity against *E. coli* IleRS and poor activity against the human homologue (an especially profound difference is seen with CB168 (**2**)). Of numerous compounds of this class synthesized and tested, only CB432 (**3**) showed moderate activity against a broad range of bacteria including *Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus*, *Streptococcus pneumoniae*, *S. pyogenes*, *Enterococcus faecium*, *E. faecalis*, *Bacillus subtilis*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, and *Moraxella catarrhalis* with MIC values ranging between 2 and 100 µg/mL.⁷ In addition, CB432 (**3**) showed activity during treatment of mice infected with *S. pyogenes*.⁷ However, this compound could not be further pursued as a potential antibiotic due to its high affinity to serum albumin.⁷

Microcin C (**4a**, McC) is a natural compound that is produced by Enterobacteriaceae. McC consist of an adenosine, to which a heptapeptide is attached via a C-terminal phosphoamide bond. The biosynthesis and mode of action of McC have been reviewed comprehensively elsewhere.⁸ In *E. coli*, a single locus, *yejABEF*, encoding an inner membrane ABC-transporter, is responsible for the uptake of McC.⁶ Transport through the outer membrane is facilitated by OmpF. Once inside the cell, McC is metabolized by a peptide deformylase and one of three aminopeptidases, PepA, PepB, or PepN, to liberate a non-hydrolyzable analogue of aspartyl adenylate (**4b**). This compound is able to selectively

inhibit AspRS.⁹ Because of its facilitated transport, with subsequent processing and release of a toxic part, McC belongs to the Trojan horse class of antibacterial agents.

Albomycin (**5a**) is another natural aaRS inhibitor that relies on a Trojan-horse strategy to increase the uptake of a toxic part that is poorly permeable on its own. Albomycin is a so-called siderophore-drug-conjugate (SDC), with the siderophore part consisting of a tripeptide of *N*⁵-acetyl-*N*⁵-hydroxy-L-ornithines, mimicking siderophore ferrichrome. The siderophore part is *C*-terminally attached to a 4'-thio-6'-amino heptonic acid via a special linker. A modified cytosine, resembling a nucleobase is attached at the 1'-position. Albomycin is taken up by FhuA-TonB complex, a ferrichrome transport mechanism present in many bacteria and capable of transporting siderophores and, in some cases, also sideromycins across the bacterial membranes.¹⁰ Once inside the cell, it is released from its iron by ferric reductase and processed by PepN with release of active moiety **5b**, which resembles seryl adenylate and inhibits SerRS.¹¹

We were interested to determine, whether compounds designed by Cubist Pharmaceuticals® when attached to transport modules of McC and albomycin, could function as aryl-tetrazole containing Trojan-horse inhibitors. We concentrated on two different lead compounds for this analysis, CB168, which was chosen for its great potential as a selective inhibitor of bacterial IleRS, and CB432 - because of its broad-range antibacterial activity, low-level inhibition of human IleRS, and moderate efficacy in an animal model. Our expectation was that Trojan-horse inhibitors based on aryl tetrazole containing aaRS inhibitors would be superior to natural compounds for further development.

Results

Design and synthesis of McC and albomycin analogues carrying a 2-carbon linked aryl-tetrazole as adenine substitution

As mentioned above, two of the compounds developed by Cubist Pharmaceuticals® (**2** and **3**) were used as lead compounds for our study. Compound **2** was further modified in several directions, by converting it into an McC analogue (yielding compound **32**), by replacing the isoleucine part of compound **32** with leucine (**33**) or aspartic acid as found in McC (**34**). In addition, compound **1** was also converted into a SDC by coupling it to a hydroxamate-based siderophore (**37**), affording compound **38**. Lead compound **3** was likewise converted into a McC analogue, by coupling it to the McC peptide, affording compound **35**. In a related compound **36**, the *N*-terminal formyl group of McC peptide was omitted and the alanine at position six was substituted by a serine. The expectation was that this compound may be processed more efficiently once inside the cell due to expected lack of requirement for peptide deformylase caused by a presence of a possible endopeptidase cleavage site. Finally, compound **3** was also coupled with the hydroxamate-based siderophore (**37**), affording compound **39**.

The 5-phenyltetrazole (**7**) was synthesized following a method described by Herbst and Wilson.¹² The 5-(4-phenoxy)phenyltetrazole (**9**) was synthesized as described by Hill *et al.*⁶ (Scheme 2). Following the example of Cubist Pharmaceuticals® the design of the starting materials **2** and **3** was kept identical. The synthetic procedure however was modified, and was largely based on the work by Lee *et al.*,¹³ Mandal *et al.*¹⁴ and most importantly Ohruí *et al.*¹⁵ (Scheme 3). Starting from ribose (**10**), the 2'- and 3'-hydroxyl groups were protected with an isopropylidene moiety and the 5'-hydroxyl with a *p*-anisyl-diphenylmethyl (MMTr) protecting group to afford **12**. Following the method described by Ohruí *et al.*¹⁵ a Wittig-Moffat reaction was carried out, providing compound **13**. Reduction of the ester, followed by a Mitsunobu reaction with one of the tetrazole derivatives **7** or **9**, yielded respectively compounds **15** and **16**. These subsequently were selectively deprotected to liberate the 5'-hydroxyl group affording **17** and **18**. Sulfamoylation of the latter compounds according to previous protocols¹⁶ gave **19** and **20**. Previously, we already described a convenient method for the coupling of peptides to sulfamoylated adenosine (Scheme 4).^{17, 18} Analogous procedures were followed here to obtain compounds **32-36** as described in Scheme 3. The SDCs (**38** and **39**) were synthesized by coupling of compound **2** or **3** to [*N*²-(benzyloxycarbonyl)-*N*⁵-acetyl-*N*⁵-*O*-acetyl-L-ornithinyl]-[*N*⁵-acetyl-*N*⁵-*O*-acetyl-L-ornithinyl]-*N*⁵-acetyl-*N*⁵-*O*-acetyl-L-ornithine (**37**) using HBTU. The synthesis of this hydroxamate-based siderophore **37** was described earlier by Miller *et al.*¹⁹ Subsequent removal of the three *O*-acetyl groups with DIPEA, and removal by hydrogenation of the Cbz-group yielded compounds **38** and **39** (Scheme 5).

Antibacterial activity of McC and albomycin analogues

The ability of new McC-like compounds to inhibit growth of *E. coli* was determined. In addition to the wild-type *E. coli* K-12 BW28357, a derivative strain Ara-Yej (BW39758) with genomic *yejABEF* operon under control of the arabinose-inducible *araBAD* promoter was tested. In the absence of arabinose, the Ara-Yej strain is virtually resistant to McC. However, when induced, the Ara-Yej strain is more sensitive to McC and its analogues than the wild-type due to higher levels of YejABEF transporter. Both strains were grown in LB medium with or without 5 mM (L)-arabinose in the presence of various concentrations of compounds **32-36**. Unfortunately, with the exception of McC control, none of the compounds showed activity.

The antibacterial activities of all compounds against *S. aureus* ATCC 6538, *S. epidermidis* RP62A (ATCC 35984), *Pseudomonas aeruginosa* PAO1, *Sarcina lutea* ATCC 9341, and *Candida albicans* CO11 were also tested. The positive control, compound **2**, was active against *S. aureus*, in agreement with original findings of Cubist Pharmaceuticals® and also showed activity against *C. albicans* at 50 µM. Among the newly synthesized compounds, only **39** showed statistically significant but very weak activity against *S. aureus* and *C. albicans* after 11 hours of incubation as shown in a Student T-test ($p=0.01$) (Figure 1). To a lesser extent also *C. albicans* was inhibited after 11 h ($p=0.1$).

To understand the reason(s) for the disappointing lack of antibacterial activity of new compounds, in vitro analyses were performed and the ability of all compounds targeting IleRS (compounds **2, 3, 32, 35, 36, 38, 39**) to inhibit tRNA^{Ile} aminoacylation reaction in *E. coli* extracts was determined (Figure 2). All compounds showed good activity against IleRS in wild-type cell extracts and, with the exception of compounds **2** and **3**, were devoid of activity in extracts prepared from McC and albomycin-resistant cells lacking peptidases PepA, PepB, and PepN. The result thus indicates that new compounds are processed in an expected way and the released active moiety is able to inhibit the IleRS target (compounds **2** and **3** do not require processing for target inhibition).

A similar result was obtained for compounds **27** and **33** (Figure 3): these LeuRS inhibitors showed activity against LeuRS in wild-type cell extracts. In extracts prepared from cells lacking aminopeptidase activity, compound **33** was inactive, while compound **27** showed activity. Finally, compound **34**, expected to target AspRS, was devoid of inhibitory activity even in wild-type cell extracts.

Discussion

In search of new antibacterial compounds targeting aaRSs, researchers from Cubist Pharmaceuticals (Lexington, USA) identified a number of new entities that are based on aminoacyl sulfamoyladenoses, which are well-known non-hydrolyzable isosters of aminoacyl adenylates. It was found that selectivity of aminoacyl sulfamoyl adenosines can be increased by replacing adenine by a two-carbon linked aryl-tetrazole. Unfortunately these compounds were shown to exhibit low whole-cell activity and to bind avidly to serum albumin. Previously, we have shown that McC and its synthetic analogues show great potential as antibacterial compounds and that the McC concept could be further exploited by endowing different antibacterial compounds with the McC peptide to enhance their uptake.^{17, 18} A related Trojan horse approach was investigated by several groups using siderophore-conjugated antibiotics.²⁰ In order to improve the antibacterial potency of Cubist compounds, we here successfully converted them into McC or SDCs analogues. Next, the whole-cell activity as well as the inhibitory action of these compounds against their respective aaRSs in in vitro aminoacylation experiments was determined.

Three important conclusions can be drawn from these results. First, synthetic SDC-prodrugs have been often found unable to release the active moiety, which therefore could not reach its target,²¹ making the approach ineffective. Our results clearly show that, although no antibacterial activity was observed with new McC or SDCs analogues, these compounds did show strong activity in cell extracts and the activity was dependent on processing by cellular peptidases. Hence, it must be concluded that all compounds were efficiently metabolized, liberating the active moiety which inhibited the aminoacylation reaction of IleRS and LeuRS. This shows that the approach presented in this study could surpass issues related to the release of the active principle observed in previous work. Secondly, it must be concluded that, in case of the McC analogues, the absence of antibacterial activity against *E. coli* is attributable to the failure of uptake. In our previous work,^{17, 18} it was clearly shown that the YejABEF transporter, responsible for McC uptake, tolerates modifications in McC peptide moiety relatively well. In this paper, the only modification compared to natural McC or its active chemically synthesized analogues targeting LeuRS or IleRS is the replacement of the adenine base by two-carbon-linked aryl-tetrazoles. Therefore, the YejABEF transporter is able to recognize the nucleotide moiety of McC and may be a selective transporter of peptidyl adenylates or very closely resembling derivatives. The same may be true for the SDCs that would rely on FhuA and the TonB complex for efficient uptake. Further research is required to resolve this issue. However, it is clearly shown that substitution of the base results in absence of ability to pass the bacterial membrane of the tested *E. coli* strains. Therefore, it would be highly desirable to investigate whether efficient uptake by one or both transport systems can be obtained by modifying the two-carbon-linked aryl-tetrazole moiety. Attempts to synthesize SDCs, by conjugation of the here presented siderophore with simple aminoacyl-sulfamoyl-adenosines proved ineffective for reasons of instability of these compounds.

In spite of all our attempts to promote uptake of well-known inhibitors, none of the newly synthesized congeners showed antibacterial activity, while in vitro, all compounds (compound **34** excluded) proved potent inhibitors of either LeuRS or IleRS. However, since compound **34** did not inhibit AspRS, it can be concluded that not all modifications in the amino acid constituency are allowed. Since we have shown before that all McC analogues comprising L-amino acids can be efficiently metabolized,¹⁷ it is unlikely that this observation results from problems during the metabolism. This suggests that LeuRS and IleRS can accommodate and effectively bind the aryl-tetrazole moiety substituting for adenine, while AspRS does not allow for such modification. An explanation for this observation may be given by the fact that class II aaRSs, in contrast to class I aaRSs, have a well conserved ATP binding site, whereby the ATP is stacked between a phenylalanine of motive 2 and an arginine in motive 3. In addition, Brick *et al.* and Nakama *et al.* showed that there is no initial hydrogen bonding of the adenine N⁶ group with the KMSKS loop for isoleucyl and tyrosyl-tRNA synthetases.^{22, 23} Therefore, adenine mimics such as the aryl-tetrazoles as presented in this manuscript may be accepted as good isosters of adenine.

Materials and Methods

Reagents and solvents were purchased from commercial suppliers (Acros, Sigma-Aldrich, Bachem, Novabiochem) and used as provided, unless indicated otherwise. DMF and THF were of analytical grade and were stored over 4Å molecular sieves. For reactions involving Fmoc-protected amino acids and peptides, DMF for peptide synthesis (low amine content) was used. All other solvents used for reactions were analytical grade and used as provided. Reactions were carried out in oven-dried glassware under a nitrogen atmosphere with stirring at room temperature, unless indicated otherwise.

¹H and ¹³C NMR spectra of the compounds dissolved in DMSO-*d*₆ or D₂O were recorded on a Bruker UltraShield Avance 300 MHz or 500 MHz spectrometer. The chemical shifts are expressed as δ values in parts per million (ppm), using the residual solvent peaks (DMSO: ¹H, 2.50 ppm; ¹³C, 39.52 ppm; HOD: ¹H, 4.79 ppm) as a reference. Coupling constants are given in Hertz (Hz). The peak patterns are indicated by the following abbreviations: bs = broad singlet, d = doublet, m = multiplet, q = quadruplet, s = singlet and t = triplet. High resolution mass spectra were recorded on a quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard ESI interface; samples were infused in 2-propanol/H₂O (1:1) at 3 μL.min⁻¹.

For TLC, precoated aluminium sheets were used (Merck, Silica gel 60 F₂₅₄). The spots were visualized by UV light at 254 nm. Column chromatography was performed on ICN silica gel 60A 60–200. Preparative HPLC of peptides was done using a Waters Xbridge prep. C18 (19 × 150 mm) column connected to a Waters 1525 binary HPLC pump and a Waters 2487 dual absorbance detector. Final products were purified using a PLRP-S 100Å column connected to a Merck-Hitachi L6200A Intelligent pump. Eluent compositions are expressed as v/v. Purity was checked by analytical HPLC

on a Inertsil ODS-3 (C-18) (4.6 x 100 mm) column, connected to a Shimadzu LC-20AT pump using a Shimadzu SPD-20A UV-detector. Recordings were performed at 254 nm and 214 nm.

Synthesis of 5-phenyltetrazole (7)

Benzonitrile (**6**) (15.5 mL), sodium azide (13 g) and acetic acid (11.4 mL) were dissolved in *n*-butanol (60 mL) and refluxed. After three days another portion of sodium azide (2.60 g), acetic acid (3.8 mL) and *n*-butanol (20 mL) were added and the mixture was stirred for another 24 h. The mixture was cooled down till 0 °C and a white precipitate was formed. Water (250 mL) was added and the mixture was concentrated to 100 mL. The resulting solution was neutralized with 1 N NaOH and was next washed with toluene (2 x 100 mL). The water layer was acidified with 1N HCl until a white precipitate was formed. This precipitate was washed with cold water (0 °C) and recrystallized from 20% isopropanol in water. The white crystals were filtered yielding 11.31 g (77.4 mmol, 39%).

¹³C-NMR (75 MHz, DMSO-*d*₆): 124.23 (*i*-aryl), 127.08 (*o*-aryl), 129.52 (*m*-aryl), 131.36 (*p*-aryl), 155.38 (C-tetrazole).

ESI-MS calcd. for C₇H₇N₄ ([M+H]⁺): 147.07; found: 146.90.

Synthesis of 5-(4-phenoxy)phenyltetrazole (9)

4-Phenoxybenzonitrile (**8**) (200 mg, 1.02 mmol, 1.0 eq.), dibutyltin oxide (79 mg, 3.16 mmol, 3.0 eq), and trimethylsilylazide (679 μL, 5.17 mmol, 5.0 eq) were dissolved in toluene (5 mL) and refluxed for 5 h, after which the reaction mixture was cooled down to rt. To this mixture another 100 mL of toluene was added and the organic layer was extracted twice with 50 mL of 1.6 N NaOH. The aqueous layer was subsequently washed twice with 50 mL diethyl ether, after which the aqueous layer was acidified to pH 6 with concentrated HCl. The aqueous layer was then extracted with EtOAc. The organic layer was dried over Na₂SO₄, filtered and evaporated yielding 215 mg (87%) of the desired compound.

¹³C-NMR (75 MHz, DMSO-*d*₆): 119.49, 120.24, 120.52, 125.36, 129.90, 131.23, 156.41, 160.16.

HR-MS: calcd. for C₁₃H₁₀N₄O₁ ([M+H]⁺): 239.0933; found: 239.0918.

Synthesis of 5-*O*-*p*-anisyl diphenylmethyl-2,3-*O*-isopropylidene-β-D-ribofuranose (**12**)

A solution of D(-)-ribose (**10**) (6.50 g, 43.16 mmol, 1 eq.), acetone (80 mL) and H₂SO₄ (0.2 mL) was stirred for 30 min. at rt under Ar. NaHCO₃ (0.43 g, 5.14 mmol, 0.12 eq.) was added and the mixture was stirred for 30 min, filtered and the volatiles were evaporated, yielding 2',3'-*O*-isopropylidene-β-D-ribofuranose (**11**). The crude product was dissolved in pyridine (30 mL). Next, a solution of monomethoxytrityl-chloride (MMTr-Cl) (10 g, 32 mmol, 0.67 eq.) in pyridine was added. This mixture was stirred overnight at rt under Ar. The volatiles were evaporated and the residue was purified by flash-chromatography (20% EtOAc in hexane with 0.1% Et₃N). The fractions containing the desired product, as determined by TLC analysis, were evaporated yielding product **12** (2.8 g, 6.054 mmol, 43% calculated over two steps).

¹³C-NMR (75 MHz, CDCl₃): 24.98, 25.31, 26.36 and 26.75 (2x isopropylidene CH₃); 55.39 (O-CH₃); 65.19 and 65.56 (C5'); 79.68 and 82.27(C1'); 80.32 and 86.23 (C3'); 82.41 and 87.20 (C2'); 87.44 and 88.04 (C4'); 98.22, 103.66, 112.41, 113.33, 113.48 (trityl); 113.56 (C-isopropylidene); 127.51, 128.26, 128.63, 130.63, 134.79, 135.33, 143.73, 144.40, 159.04 (aromatic signals).
ESI-MS calcd. for C₂₈H₃₀O₆Na ([M+Na]⁺): 485.19; found: 484.80.

Synthesis of ethyl-3,6-anhydro-7-O-p-anisylidiphenylmethyl-2-deoxy-4,5-O-isopropylidene-D-allo-heptanoate (13)

Product **12** (11.0 g, 23.8 mmol, 1 eq.) and carbethoxymethylene-triphenylphosphorane (12.45 g, 35.7 mmol, 1.5 eq.) were dissolved in CH₃CN (100 mL). The solution was refluxed for 8 h. The solution was evaporated yielding a pale yellow oil.
The product was purified by flash-chromatography (22% EtOAc in hexane). The fractions containing the desired product were evaporated affording product **13** (12.50 g, 23.485 mmol, 99%).
¹³C-NMR (75 MHz, CDCl₃): 14.44 (CH₃ ethyl); 25.89 and 27.77 (2x CH₃ isopropylidene); 39.00 (C2); 55.38 (C1); 60.85 (CH₂ ethyl); 64.44 (C7); 81.17 (C3); 82.57 (C5); 83.77 (C4); 84.54 (C6); 86.61 (MMTr-C(Ph₃)); 113.34 (trityl); 114.39 (C-isopropylidene); 127.10, 128.03, 128.70, 130.65, 135.71, 144.57, 144.62, 158.81 (aromatic signals), 170.75 (C=O).
ESI-MS calcd. for C₃₂H₃₆O₇Na ([M+Na]⁺): 555.24; found: 554.83.

Synthesis of 3,6-anhydro-7-O-p-anisylidiphenylmethyl-2-deoxy-4,5-O-isopropylidene-D-allo-heptitol (14)

Compound **13** (12.50 g, 23.48 mmol, 1 eq.) was dried overnight *in vacuo*. THF (10 mL) was added and to the solution, LiAlH₄ (1.34 g, 35.23 mmol, 1.5 eq.) was carefully added. After 30 min, the reaction was quenched by adding 2-propanol (50 mL). The volatiles were evaporated and the product was purified by flash-chromatography (40% EtOAc in hexane). The fractions containing the desired product **14** were collected and evaporated. Yield: 9.5 g (19.38 mmol, 83%).
¹³C-NMR (125 MHz, DMSO-*d*₆): 25.95 (CH₃ isopropylidene), 27.88 (CH₃ isopropylidene), 36.09 (C2), 55.55 (CH₃ para-anisyl), 61.21 (C1), 64.40 (C7), 82.48 (C3), 83.65 (C5), 84.35 (C4), 85.10 (C6), 86.71 (C-trityl), 113.48 (C-isopropylidene), 114.82, 127.25, 128.17, 128.76, 130.72, 144.60, 144.66, 158.94 (aromatic signals).
ESI-MS calcd. for C₃₀H₃₄O₆ ([M+H]⁺): 513.22; found: 513.00.

Synthesis of 3,6-anhydro-7-O-p-anisylidiphenylmethyl-1,2-dideoxy-4,5-O-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol (15)

Product **14** (9.35 g, 19.1 mmol, 1.0 eq.) and triphenylphosphine (Ph₃P, 10.0 g, 38.2 mmol, 1.0 eq.) were combined and dried *in vacuo*. THF (140 mL) was added and the mixture was cooled to 0 °C. Diethyl 1,2-diazenedicarboxylate (DEAD) was slowly added to the cooled mixture over 1 h, after which **7** (3.6 g, 24.8 mmol, 0.33 eq.) was added. The mixture was stirred for 3 h at rt under Ar, after which the volatiles were evaporated and the product was purified using flash-chromatography (10%

MeOH in chloroform). All fractions containing the desired product were collected and evaporated yielding **15** (9.0 g, 14.6 mmol, 76%).

¹³C-NMR (125 MHz, CDCl₃): 25.91 and 27.81 (2x CH₃ isopropylidene); 31.91 (C2); 50.46 (C1); 55.54 (O-CH₃); 64.56 (C7); 81.66 (C3); 82.71 (C5); 83.82 (C4); 85.01 (C6); 114.78 (C-isopropylidene); 113.49, 127.17, 127.29, 127.81, 128.19, 128.76, 129.18, 130.56, 130.70, 135.75, 144.59, 158.95, 165.45 (aromatic signals).

ESI-MS calcd. for C₃₇H₃₉N₄O₅ ([M+H]⁺): 641.27; found: 640.79.

Synthesis of 3,6-anhydro-2-deoxy-7-hydroxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol (**17**)

Product **15** (5.6 g, 9.1 mmol, 1.0 eq.) was dissolved in diethylether/HOAc (20 mL, 1 v/v) and refluxed for 10 h. The mixture was evaporated and neutralized with NaHCO₃(sat.). The mixture was extracted with ether (2 x 20 mL) and washed with brine (20 mL). The organic layer was dried (Na₂SO₄), filtered and evaporated. The product could easily be purified by flash-chromatography (60% EtOAc in hexane). Yield: 1.2 g (3.4 mmol, 38%).

¹H-NMR (500 MHz, DMSO-*d*₆): 1.25 (s, 3H, CH₃), 1.40 (s, 3H, CH₃), 2.17-2.33 (m, 2H, C1) 3.44-3.46 (*t*AB, 2H, C7, *J*_{AB}=11.60 Hz /*J*_{A,OH}≈*J*_{A,6}≈5.4 Hz/*J*_{B,OH}≈*J*_{B,6}≈5.3 Hz), 3.83-3.87 (m, 2H, C6, C3), 4.45-4.47 (dd, 1H, C4, *J*=4.2/6.5), 4.59-4.61 (dd, 1H, C5, *J*=3.3/6.5), 4.79-4.84 (m, 2H, C2), 4.87-4.88 (*t*, 1H, OH, *J*=5.5 Hz), 7.54-7.58 (m, 1H, *m/p*-aryl), 8.05-8.05 (d, 1H, *o*-aryl, *J*=1.5 Hz), 8.07-8.07 (d, 1H, *o*-aryl, *J*=1.8 Hz).

¹³C-NMR (125 MHz, DMSO-*d*₆): 25.34 and 27.23 (2x CH₃ isopropylidene), 32.88 (C2), 49.95 (C1), 61.88 (C7), 80.94 (C3), 81.90 (C5), 83.99 (C4), 84.84 (C6), 113.00 (C-isopropylidene), 126.39 (C-aryl), 127.05 (*i*-aryl), 129.33 (C-aryl), 130.59 (*p*-aryl), 164.11 (tetrazole).

ESI-MS calcd. for C₁₇H₂₃N₄O₄ ([M+H]⁺): 347.17; found: 346.74.

Synthesis of 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-sulfamate (**19**)

Chlorosulfonyl isocyanate (2.3 mmol, 198 μL) and formic acid (2.2 mmol, 85.2 μL) were combined at 0 °C. A white foam was formed and the mixture was allowed to come to rt. After 15 min., the mixture was cooled to 0 °C again and acetonitrile (1 mL) was added. This mixture was stirred for 15 min. The obtained product was used as such for the next step.

An amount of **17** (450 mg, 1.3 mmol) was dissolved in dimethylacetamide (DMAc, 2.3 mL) at rt. This solution was cooled to 0 °C and the cooled chlorosulfonamide was added. This mixture was stirred for 50 min at 0 °C to rt. After 50 min the mixture was cooled to 0 °C again and Et₃N (0.46 mL) was added. The mixture was stirred for 15 min. Then MeOH (2.3 mL) was added and the mixture was stirred for another 15 min. The solvents were evaporated. The desired product is taken up in EtOAc and washed twice with NaHCO₃ (50 mL) and once with brine (50 mL). The organic layer was dried (Na₂SO₄), filtered and evaporated. Yield of **19**: 410 mg (0.96 mmol, 74%).

¹H-NMR (500 MHz, DMSO-*d*₆): 1.32 (s, 3H, CH₃), 1.48 (s, 3H, CH₃), 2.31-2.48 (m, 2H, C1), 3.21-3.33 (m, 2H, 7C), 3.99-4.02 (m, 2H, C6, C3), 4.48-4.52 (dd, 1H, C4, *J*=4.6/6.5 Hz), 4.71-4.74 (dd, 1H, C5, *J*=2.8/6.5 Hz), 4.79-4.84 (m, 2H, C2), 7.51-7.53 (m, 3H, *m/p*-aryl), 8.09-8.13 (m, 2H, *o*-aryl, *J*=1.5 Hz)
¹³C-NMR (125 MHz, DMSO-*d*₆): 26.44 and 28.46 (2x CH₃ isopropylidene), 34.99 (C2), 52.03 (C1), 71.08 (C7), 83.89 (C3), 84.03 (C5), 84.29 (C4), 86.68 (C6), 116.52 (C-isopropylidene), 128.59 (C-aryl), 129.43 (*i*-aryl), 130.93 (C-aryl), 132.41 (*p*-C-aryl), 167.05 (tetrazole).
ESI-MS calcd. for C₁₇H₂₆N₅O₆S₁ ([M+H]⁺): 426.14; found: 426.15.

Synthesis of 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[N-(Boc-Ile)-sulfamate] (21)

Product **19** (95 mg, 0.22 mmol, 1.0 eq.) was dried together with *N*-*tert*-butoxycarbonyl-isoleucine-*O*-succinimide (110 mg, 0.33 mmol, 1.5 eq.) *in vacuo*. Diazobicyclo[5,4,0]undecene, (DBU, 50 μL, 1.5 eq.) was added. This mixture was stirred for 8 h. The volatiles were evaporated and the product was purified using flash-chromatography (40% hexane in EtOAc). The fractions containing the product were collected and evaporated yielding product **21** (20 mg, 0.03 mmol, 14%).
¹³C-NMR (75 MHz, CDCl₃): 11.58 (Ile-δ-CH₃), 16.04 (Ile-γ-CH₃), 25.21 (Ile-γ'-CH₃), 25.73 (isopropylidene CH₃), 27.59 (isopropylidene CH₃), 28.71 (*t*Bu-Boc), 33.33 (C2), 37.45 (Ile-β-CH₂), 50.17 (C1), 63.16 (Ile-α-CH), 69.11 (C7), 80.32 (C3), 81.80 (C5), 82.33 (C4), 84.82 (C6), 115.17 (C-isopropylidene), 127.18 (C-aryl), 127.67 (*i*-C-aryl), 129.19 (C-aryl), 130.63 (*p*-C-aryl), 157.23 (C=O Boc), 165.44 (tetrazole), 182.3 (C=O Ile).
ESI-MS calcd. for [M-H]⁻: C₂₈H₄₃N₆O₉S; 637.27; found: 637.11.

Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[N-(Ile)-sulfamate] (2)

To **21** (20 mg, 0.03 mmol, 1 eq.) was added a mixture of TFA/water (5:2). This mixture was reacted for 2 h. The volatiles were evaporated and co-evaporated with toluene. The desired product was purified using HPLC (10% MeOH in water). Yield **1**: 4.5 mg (0.01 mmol, 30%).
¹H-NMR (500 MHz, MeOD): 0.95-0.98 (t, 3H, Ile-δ-CH₃, *J*=7.4 Hz), 1.05-1.06 (d, 3H, Ile-γ'-CH₃, *J*=7.0 Hz), 1.23-1.31 (m, 1H, Ile-γ_B-CH₂), 1.58-1.66 (m, 1H, Ile-γ_A-CH₂), 1.99 (m, 1H, Ile-β-CH), 2.19-2.26 and 2.41-2.47 (m, 2H, C2), 3.57-3.57 (d, 1H, Ile-α-CH, *J*=4.0 Hz), 3.78-3.81 (m, 1H, C4), 3.83-3.85 (t, 1H, C5, *J*=5.5 Hz), 4.00-4.03 (dd, 1H, C6, *J*=3.7/ 7.7 Hz), 4.11-4.13 (dd, 1H, C3, *J*=4.5/9.6 Hz), 4.17-4.24 (dAB, 1H, C7, *J*_{A,4'}=3.63/*J*_{A,B}=10.81 Hz), 4.90 (m, 2H, C1), 7.50-7.51 (m, 3H, *m/p*-phenyl), 8.09-8.11 (m, 2H, *o*-phenyl).
¹³C-NMR (125 MHz, MeOD): 12.18 (Ile-δ-CH₃), 15.56 (Ile-γ-CH₃), 25.60 (Ile-γ'-CH₂), 34.19 (C2), 38.15 (Ile-β-CH), 51.40 (C1), 61.22 (Ile-α-CH₂), 70.25 (C7), 73.00 (C3), 76.06 (C5), 80.70 (C6), 83.43 (C4), 127.77 (*i*-C-aryl), 128.72 (*o*-C-aryl), 130.13 (*m*-C-aryl), 131.58 (*p*-C-aryl), 166.22 (tetrazole), 173.99 (C=O Ile).

374 HR-MS calcd. for $C_{20}H_{29}N_6O_7S$ ($[M-H]^+$): 497.1819; found: 497.1822.

375 **Synthesis of peptide coupled 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[N-(Ile)-**
376 **sulfamate] (32)**

377 f-MR(Pbf)T(*t*Bu)GN(Trt)A-OH (7 mg, 0.006 mmol, 2eq.) was combined with HOBt (1.5 mg, 0.01
378 mmol, 3 eq.) and dried *in vacuo*. DMF (0.5 mL) and DIC (1.6 μ L, 0.01 mmol, 3 eq.) were added and
379 the solution was stirred for 1 h. The previously obtained product **1** (1.5 mg, 0.003 mmol, 1 eq.) and
380 DIPEA (1 μ L, 0.006 mmol, 2 eq.) were added. This solution was stirred overnight. The evaporated
381 desired product was purified on a Porapak®-column (25% to 100% CH_3CN in water).

382 The protecting groups were removed by addition of a mixture of TFA/water/thioanisol (92.5/7.5/2.5,
383 v/v/v). This solution was stirred for 2 h. The mixture was coevaporated with toluene. Next, the mixture
384 was purified using HPLC (2% CH_3CN in water).

385 HR-MS calcd. for $C_{45}H_{71}N_{16}O_{16}S_2$ ($[M-H]^+$): 1155.4676; found: 1155.4664.

386 **Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[N-[[N^5 -acetyl- N^5 -O-hydroxyl-L-**
387 **ornithinyl]-[N^5 -acetyl- N^5 -O-hydroxyl-L-ornithinyl]-[N^5 -acetyl- N^5 -O-hydroxyl-L-ornithinyl]-L-Ile)-**
388 **sulfamate] (38)**

389 Compound **1** (25 mg, 0.05 mmol) was dissolved in DMF (0.5 mL), to which Et_3N (13 μ L, 0.1 mmol, 2
390 eq.) was added. This mixture was next combined with a mixture **37** (32.4 mg, 0.05 mmol, 1 eq.) and
391 HBTU (23 mg, 0.06 mmol, 1.2 eq.), which were dissolved in DMF (0.5 mL). The reaction mixture
392 was stirred overnight at rt under Ar. The following day, the DMF was evaporated and the residue was
393 taken up in EtOAc. The organic layer was washed two times with water. Subsequently, the EtOAc was
394 evaporated and the product was dissolved in H_2O/CH_3CN , whereby the volume of CH_3CN was kept
395 to a minimum. This mixture was then purified over a Porapak™ column (0 to 100 % CH_3CN in H_2O).
396 The fractions containing the product were dried, affording the desired product, still containing side
397 products. Yield: 21 mg (34%).

398 ESI-MS calcd. for $C_{55}H_{77}N_{12}O_{21}S$ ($[M-H]^+$): 1273.5; found: 1273.1.

399 The crude product (10.5 mg, 8.2 μ mol) was dissolved in MeOH, containing 6% DIPEA, and stirred for
400 24 h. The product was dried and dissolved in H_2O/CH_3CN . The product was next purified by RP-
401 HPLC (5 to 80 % CH_3CN in H_2O), yielding 1.8 mg (5%) of the desired compound.

402 ^{13}C -NMR (125 MHz, MeOD): 12.08 (CH_3 DIPEA), 13.20 (Ile- δ - CH_3), 16.37 (Ile- γ - CH_3), 17.38
403 (DIPEA), 19.41 (Orn- γ - CH_2), 20.27 (*N*-acetyl- CH_3), 25.79 (Ile- γ' - CH_2), 30.29 (Orn- β - CH_2), 34.15
404 (C_2), 38.59 (Ile- β -CH), 43.79 (DIPEA- CH_2), 51.43 (C_1), 54.21 (Orn- α -CH), 55.83 (DIPEA-CH),
405 61.62 (Ile- α - CH_2), 67.76 (Cbz- CH_2), 69.81 (C_7), 73.08 (C_3), 76.10 (C_5), 80.45 (C_6), 83.73 (C_4),
406 127.77-138.46 (aromatic signals), 158.77 ($C=O$ Cbz), 166.15 (tetrazole), 173.84 ($C=O$ Ile).

407 HR-MS calcd. for $C_{49}H_{71}N_{12}O_{18}S$ ($[M-H]^+$): 1147.4730; found: 1147.4774.

Next, the compound was dissolved in 5% aqueous DMF (100 μ L), to which a solution of FeCl₃ (10 μ L of a 0.5 M solution) was added. Hereto, 10 mg of 10% Pd-C was added and the reaction mixture was stirred for 5 h under hydrogen atmosphere. Subsequently, the reaction mixture was filtered and evaporated, yielding 1.2 mg of the desired compound.

HR-MS calcd. for C₄₁H₆₅N₁₂O₁₆S ([M-H]⁻): 1013.4360; found: 1013.4360.

Synthesis of 3,6-anhydro-2-deoxy-4,5-isopropylidene-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol-7-[N-(Boc-L-Ile)-sulfamate] (22)

Further synthesis was performed analogous to the preparation of **21**, affording 260 mg (88% total yield) of the title compound.

¹³C-NMR (125 MHz, CDCl₃): 11.85 (Ile- δ -CH₃), 15.90 (Ile- γ -CH₃), 25.22 (Ile- γ' -CH₃), 25.69 (isopropylidene CH₃), 27.56 (isopropylidene CH₃), 28.67 (t-Bu-Boc), 33.33 (C2), 37.47 (Ile- β -CH₂), 50.13 (C1), 69.00 (C7), 79.95 (C3), 81.78 (C5), 82.38 (C4), 84.78 (C6), 115.01 (C-isopropylidene), 118.85, 119.84, 122.41, 124.27, 128.81, 130.21, 156.61, 156.88, 159.64 (aromatic signals), 156.51 (C=O Boc), 164.98 (tetrazole).

ESI-MS calcd. for C₃₄H₄₅N₆O₁₀S ([M-H]⁻): 729.29; found: 729.18.

Synthesis of 3,6-anhydro-2-deoxy-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol-7-[N-(L-Ile)-sulfamate] (3)

Further synthesis was performed analogous to the preparation of **1**, affording 260 mg (88% total yield) of the title compound.

¹H-NMR (500 MHz, MeOD): 0.88-0.93 (t, 3H, Ile- δ -CH₃, *J*=7.4 Hz), 0.98-0.99 (d, 3H, Ile- γ' -CH₃, *J*=7.0 Hz), 1.23-1.27 (m, 1H, Ile- γ_B -CH₂), 1.52-1.57 (m, 1H, Ile- γ_A -CH₂), 1.93 (m, 1H, Ile- β -CH), 2.15-2.36 (m, 2H, C2), 3.48-3.49 (d, 1H, Ile- α -CH, *J*=4.0 Hz), 3.73-3.81 (m, 1H, C4), 3.95-3.96 (m, 1H, C5), 4.05-4.11 (m, 4H, C3, C1, C6, C7), 6.99-7.37 (m, aromatic signals).

¹³C-NMR (125 MHz, MeOD): 13.03 (Ile- δ -CH₃), 16.39 (Ile- γ -CH₃), 26.56 (Ile- γ' -CH₃), 34.99 (C2), 30.06 (Ile- β -CH₂), 52.18 (C1), 62.27 (Ile- α -CH₂), 70.65 (C7), 73.86 (C3), 76.86 (C5), 81.59 (C4), 84.34 (C6), 107.38-161.84 (aromatic signals).

HR-MS calcd. for C₂₆H₃₃N₆O₈S ([M-H]⁻): 589.2081; found: 589.2090.

Synthesis of peptide coupled 3,6-anhydro-2-deoxy-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol-7-[N-(L-Ile)-sulfamate] (35)

Further synthesis was performed analogous to the preparation of **32**, affording 1.0 mg (18% total yield) of the title compound.

HR-MS calcd. for [M-H]⁻: C₅₁H₇₅N₁₆O₁₇S₂ 1247.4938; found: 1247.4900.

Synthesis of peptide coupled 3,6-anhydro-2-deoxy-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol-7-[N-(L-Ile)-sulfamate] (36)

The required hexapeptide Boc-MR(Pbf)T(*t*Bu)GN(Trt)S(*t*Bu)-OH was prepared in analogy to the formylated derivative f-MR(Pbf)T(*t*Bu)GN(Trt)A-OH [ref. 18] (HR-MS calcd. for [M-H]⁺: C₆₉H₉₇N₁₀O₁₅S₂ 1369.6581; found: 1369.6570). Further synthesis was performed analogous to the preparation of **32**, affording 1.0 mg (18% total yield) of the title compound. HR-MS calcd. for C₅₁H₇₆N₁₆O₁₈S₂ ([M-H]⁺): 1263.4887; found: 1263.4774.

Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenoxyphephenyltetrazole]-D-allo-heptitol-7-[N-[[N⁵-acetyl-N⁵-O-hydroxyl-L-ornithinyl]-[N⁵-acetyl-N⁵-O-hydroxyl-L-ornithinyl]-[N⁵-acetyl-N⁵-O-hydroxyl-L-ornithinyl]-L-Ile)-sulfamate] (39)

Further synthesis was performed analogous to the preparation of **38**, affording 1.3 mg (20% total yield) of the title compound. HR-MS calcd. for C₅₅H₇₅N₁₂O₁₉S ([M-H]⁺): 1239.4992; found: 1239.4967.

Synthesis of 3,6-anhydro-2-deoxy-4,5-O-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[N-(Boc-Leu)]-sulfamate (23)

Further synthesis was performed analogously to the preparation of **21**, affording 550 mg (82% total yield) of the title compound.

¹³C-NMR (75 MHz, CDCl₃): 22.18 (Leu-δ_B-CH₃), 23.41 (Leu-δ_A-CH₃), 25.13 (Leu-γ-CH), 25.74 (CH₃ isopropylidene), 27.60 (CH₃ isopropylidene), 28.68 (*t*Bu Boc), 33.35 (C2), 41.27 (Leu-β-CH₃), 50.18 (C1), 56.51 (Leu-α), 69.71 (C7), 80.74 (C3), 81.88 (C5), 82.28 (C4), 84.83 (C6), 115.26 (C-isopropylidene), 127.21 (C-aryl), 127.66 (*i*-C-aryl), 129.22 (C-aryl), 130.67 (*p*-C-aryl), 156.69 (Boc C=O), 165.48 (tetrazole).

ESI-MS calcd. for C₂₈H₄₃N₆O₉S ([M+H]⁺): 639.28; found: 639.20.

Synthesis of 2-deoxy-4,5-dihydroxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-N-(Leu)-sulfamate (27)

Further synthesis was performed analogously to the preparation of **1**, affording 460 mg (89% total yield) of the title compound.

¹H-NMR (300 MHz, MeOD): 0.96-0.97 (d, 3H, Leu-δ_B-CH₃, *J*=5.9 Hz), 0.98-0.99 (d, 3H, Leu-δ_A-CH₃, *J*=6.1 Hz), 1.56-1.62 and 1.76-1.80 (m, 2H, C1), 1.76-1.80 (m, 1H, Leu-γ-CH), 2.20-2.26 (m, 1H, Leu-β_A-CH₂), 2.41-2.43 (m, 1H, Leu-β_B-CH₂), 3.57-3.60 (dd, 1H, Leu-α, *J*=5.2/8.5 Hz), 3.77-3.80 (m, 1H, C4), 3.83-3.85 (t, 1H, C5, *J*=4.8 Hz), 4.00-4.02 (dd, 1H, C6, *J*=3.7/7.5 Hz), 4.11-4.19 (m, 1H, C3 and C7), 4.90 (m, 2H, C2), 7.50-7.51 (m, 1H, *m/p*-aryl), 8.10-8.11 (dd, 1H, *o*-aryl, *J*=1.5/7.26 Hz).

¹³C-NMR (75 MHz, MeOD): 22.13 (Leu-δ_B-CH₃), 23.23 (Leu-δ_A-CH₃), 25.86 (Leu-γ-CH), 34.14 (C2), 42.33 (Leu-β-CH₂), 51.36 (C1), 55.55 (Leu-α), 69.75 (C7), 73.01 (C3), 76.02 (C5), 80.59 (C4), 83.53 (C6), 127.74 (C-aryl), 128.71 (*i*-C-aryl), 130.09 (C-aryl), 131.54 (*p*-C-aryl), 166.20 (tetrazole), 176.47 (C=O Leu).

475 HR-MS calcd. for C₂₀H₂₉N₆O₇S ([M-H]⁻): 497.1819; found: 497.1822.

476 **Synthesis of peptide coupled 2-deoxy-4,5-dihydroxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-N-(Leu)-**
477 **sulfamate (33)**

478 Further synthesis was performed analogously to the preparation of **32**, affording 1.8 mg (4% total
479 yield) of the title compound.

480 HRMS calcd for C₄₅H₇₁N₁₆O₁₆S₂ ([M-H]⁻): 1155.4676; found: 1155.4683.

481 **Synthesis of 3,6-anhydro-2-deoxy-4,5-O-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[N-[N-**
482 **benzyloxycarbonyl-L-aspartyl(O-*t*Bu)]-sulfamate] (24)**

483 A solution of Z-aspartyl(O-*tert*-butyl)-O-Su (460 mg, 0.15 mmol, 2.0 eq.), 3,6-anhydro-2-deoxy-4,5-
484 O-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-sulfamate (**19**) (320 mg, 0.75 mmol, 1 eq.)
485 and DBU (171 μ l, 0.9 mmol, 1.2 eq.) in DMF (5 mL) was stirred at rt for 8 h. DMF was evaporated
486 under reduced pressure. Next, the residue was purified by flash chromatography (10% MeOH in
487 CH₂Cl₂). Fractions containing the desired product were evaporated affording **24** as colourless oil.
488 Yield: 420 mg (81%).

489 ¹³C-NMR (75 MHz, CDCl₃): 25.57 (isopropylidene CH₃), 27.47 (isopropylidene CH₃), 28.35 (*t*-Bu),
490 29.84 (C2), 38.45 (Asp-CH₂) 50.04 (C1), 53.59 (C α), 67.46 (CH₂-Cbz), 69.83 (C7), 81.78 (C3), 81.96
491 (C-*t*Bu), 82.09 (C5), 82.46 (C4), 84.76 (C6), 115.47 (C-isopropylidene), 127.18 (C-aryl), 127.67 (*i* C-
492 aryl), 128.51 (C-aryl), 129.21 (*p* C-aryl), 130.64 (C-aryl), 136.44 (*i* C-aryl), 157.44 (C=O Asp),
493 165.49 (tetrazole), 172.42 (C=O Asp).

494 ESI-MS calcd. for C₃₈H₆₂N₇O₁₁SSi₂ ([M+H]⁺): 880.4; found: 880.1.

495 **Synthesis of 3,6-anhydro-2-deoxy-4,5-O-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[N-[L-**
496 **aspartyl(O-*tert*-butyl)]-sulfamate] (25)**

497 Compound **24** (420 mg) was dissolved in a mixture of MeOH with 4.4% HCOOH (15 mL) while Pd/C
498 (100 mg) was added. The mixture was stirred for 0.5 h, after which it was filtered and evaporated
499 yielding a white foam (**25**). Yield: 250 mg (76%)

500 ¹³C-NMR (75 MHz, CDCl₃): 25.79 (isopropylidene CH₃), 27.63 (isopropylidene CH₃), 28.39 (*t*-Bu-
501 Boc), 33.42 (C2), 35.63 (Asp-CH₂) 50.27 (C1), 53.05 (Asp-C α), 69.53 (C7), 81.91 (3C), 82.02 (C-
502 *t*Bu), 82.44 (C5), 83.11 (C4), 84.84 (C6), 115.02 (C-isopropylidene), 127.18 (C-aryl), 127.75 (*i* C-
503 aryl), 129.22 (C-aryl), 130.61 (*p* C-aryl), 165.39 (tetrazole), 171.49 and 174.10 (C=O Asp).

504 ESI-MS calcd. for C₃₂H₃₁N₆O₉S ([M-H]⁻): 555.2; found: 554.9.

505 **Synthesis of peptide coupled 3,6-anhydro-2-deoxy-4,5-O-isopropylidene-1-[5-phenyltetrazole]-D-allo-**
506 **heptitol-7-[N-[L-aspartyl(O-*tert*-butyl)]-sulfamate] (34)**

507 Further synthesis was performed analogously to the preparation of **32**, affording 1.7 mg (15% total
508 yield) of the title compound.

509 HRMS calcd. for $C_{42}H_{67}N_{16}O_{17}S_2$ ($[M+H]^+$): 1131.4311; found: 1131.4850.

510 **Synthesis of 3,6-anhydro-7-*O*-*p*-anisylidiphenylmethyl-1,2-dideoxy-4,5-*O*-isopropylidene-1-[5-(4-**
511 **phenoxy)phenyltetrazole]-D-allo-heptitol (16)**

512 Further synthesis was performed analogously to the preparation of **15**, affording 410 mg (71% total
513 yield) of the title compound.

514 ^{13}C -NMR (75 MHz, $CDCl_3$): 25.93 and 27.83 (2x CH_3 isopropylidene), 33.96 (C2), 50.45 (C1), 55.56
515 (O- CH_3), 64.57 (C7), 81.68 (C3), 82.72 (C5), 83.83 (C4), 85.02 (C6), 86.79 (C-trityl), 114.79 (C-
516 isopropylidene), 113.49, 118.93, 119.89, 122.63, 124.28, 127.29, 128.19, 128.77, 128.85, 130.26,
517 130.72, 135.76, 144.61, 156.72, 158.96 (aromatic signals); 159.64 (tetrazole).

518 ESI-MS calcd. for $C_{43}H_{42}N_4O_6Na$ ($[M+Na]^+$): 733.30; found: 733.24.

519 **Synthesis of 3,6-anhydro-2-deoxy-7-hydroxy-4,5-*O*-isopropylidene-1-[5-(4-phenoxy)phenyltetrazole]-D-**
520 **allo-heptitol (18)**

521 Further synthesis was performed analogous to the preparation of **17**, affording 248 mg (98% total
522 yield) of the title compound.

523 1H -NMR (500 MHz, $CDCl_3$): 1.34 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 2.26-2.56 (m, 2H, C1) 3.67-3.46
524 (ABX, 2H, 7C, $J_{AB}=12.60$ Hz/ $J_{AX}\approx 3.0$ / $J_{BX}\approx 3.75$ Hz), 3.95-4.05 (m, 2H, 6C, 3C), 4.34-4.38 (m, 1H,
525 4C), 4.64-4.89 (m, 3H, 5C, 2C), 7.07-7.19 and 7.36-7.41 and 8.09-8.12 (aromatic signals).

526 ^{13}C -NMR (125 MHz, $CDCl_3$): 25.71 and 27.65 (2x CH_3 isopropylidene), 33.18 (C2), 50.23 (C1),
527 62.90 (C7), 81.75 (C3), 81.86 (C5), 84.79 (C4), 84.83 (C6), 115.17 (C-isopropylidene), 118.87,
528 119.91, 122.35, 124.30, 128.79, 130.23, 156.59, 159.72 (aromatic signals) 165.03 (tetrazole).

529 ESI-MS calcd. for $C_{23}H_{26}N_4O_5Na$ ($[M+Na]^+$): 461.18; found: 461.25.

530 **Synthesis of 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol-**
531 **7-sulfamate (20)**

532 Further synthesis was performed analogous to the preparation of **19**, affording 260 mg (88% total
533 yield) of the title compound.

534 ^{13}C -NMR (75 MHz, $CDCl_3$): 25.69 and 27.59 (2x CH_3 isopropylidene), 33.74 (C2), 50.28 (C1), 70.57
535 (C7), 81.59 (C3), 82.35 (C5), 82.74 (C4), 85.05 (C6), 118.93, 120.01, 124.43, 128.89, 130.31
536 (aromatic signals).

537 HR-MS calcd. for $C_{23}H_{28}N_5O_7S$ ($[M+H]^+$): 518.1709; found: 518.1708.

538

Biological activity

Whole cell activity determinations

The respective bacteria were grown overnight in LB medium and cultured again the following day in fresh LB medium or LB-medium containing 5 mM (L)-arabinose. Compounds were titrated in a 96-well plate using either LB-medium +/- 5 mM (L)-arabinose to dilute the compounds. To each well, 85 µL LB-medium +/- 5 mM (L)-arabinose was added to a total volume of 90 µL. Next, 10 µL of bacterial cell culture grown to a OD600 of 0.1 was added. The cultures were next placed into a Tecan Infinite M200® incubator and shaken at 37°C, subsequently the OD600 was determined after 8 h. All experiments have been performed in triplicate.

Bacterial strains used for the evaluations: *E. coli* Ara-Yej (BW39758), expressing the *yejABEF* transporter upon L-arabinose induction; *E. coli* K-12 (BW28357), used as the wild type control. The antibacterial activities of all compounds were determined by monitoring the optical density of suspensions of cell-cultures of the following strains: *Staphylococcus aureus* ATCC 65388, *Staphylococcus epidermidis* RP62A (ATCC 35984), *Pseudomonas aeruginosa* PAO1, *Sarcina lutea* ATCC 9341, *Candida albicans* CO11.

Aminoacylation experiments

To assess the degree of inhibition of the aminoacylation reaction, in vitro tests were performed using the relevant S30 cell extracts.

Preparation of S30 cell extracts. Cells were grown in 50 mL LB-medium. After centrifugation at 3000 × g for 10 min the supernatant was discarded and the pellet was resuspended in 40 mL buffer containing: Tris.HCl or Hepes.KOH (pH = 8.0) (20 mM), MgCl₂ (10 mM), KCl (100 mM). The cell suspension was centrifuged again at 3000 × g. This procedure was repeated twice. The pellet was resuspended in 1 mL of the following buffer Tris.HCl or Hepes.KOH (pH = 8.0) (20 mM), MgCl₂ (10 mM), KCl (100 mM), DTT (1 mM) and kept at 0 °C. Subsequently, the cells were sonicated for 10 s and left at 0 °C for 10 min. This procedure was repeated 5-8 times. The lysate was centrifuged at 15000 g for 30 min at +4 °C.

tRNA aminoacylation reaction. To 1 µL of solution containing inhibitor, 3 µL of *E. coli* S30 extracts was added. Next, 16 µL of the following aminoacylation mixture was added: Tris.HCl (30 mM, pH 8.0), DTT (1 mM), bulk of *E. coli* tRNA (5 g/l), ATP (3 mM), KCl (30 mM), MgCl₂ (8 mM), and the specified, ¹⁴C-radiolabeled amino acid (40 µM, 200 mCi/mmol). The reaction products were precipitated in cold 10% TCA on Whatman 3MM papers, 5 min. after the aminoacylation mixture was added. The aminoacylation reaction was carried out at room temperature. Depending on whether or not processing was needed, variable time intervals were included between the addition of the cell extract and the addition of the aminoacylation mixture. After thorough washing with cold 10% TCA,

the papers were washed twice with acetone and dried on a heating plate. Following the addition of scintillation liquid (12 mL), the amount of radioactivity was determined in a Tri-card 2300 TR liquid scintillation counter. Inhibitions reported are based on relative amounts of the observed radioactivity (expressed in cpm) normalized versus the respective control value. ¹⁴C-Radiolabeled amino acids and scintillation liquid were purchased from Perkin Elmer.

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FIGURE LEGENDS

Figure 1. Inhibitory activity of compounds **3** and **39** against *S. aureus* and *C. albicans* at 50 µM concentrations. OD600 refers to the optical density of the cell culture measured at 600 nm. Marginal but statistically significant inhibition of *S. aureus* by compound **39** was found.

Figure 2. Inhibition of the aminoacylation reaction.

A) IleRS inhibition in S30 extracts of McC-sensitive *E. coli* cells.

B) IleRS in S30 extracts of McC-resistant *E. coli* cells lacking peptidases A, B and N.

In all tests, the different extracts were incubated with the respective IleRS inhibitors (25 µM), taking samples after 15 min for evaluation of the aminoacylation reaction. The amounts of aminoacylated tRNA^{Ile} were measured *via* liquid scintillation counting and normalized versus the respective control.

Figure 3. Inhibition of LeuRS by compounds **27** and **33**. In all tests, the different extracts were incubated with the respective IleRS inhibitors (25 µM), taking samples after 15 min for evaluation of the aminoacylation reaction. The amounts of aminoacylated tRNA^{Ile} were measured *via* liquid scintillation counting and normalized versus the respective control.

Table 1. Respective inhibition constants of compounds **1-3** on IleRS isolated from different organisms (µg/mL).

Scheme 1. IleRS inhibitors with aryl-tetrazole substituents for adenine and chemical structures for microcin C and albomycin.

Scheme 2. Synthesis of 5-aryl tetrazoles.

A) Synthesis of 5-phenyl tetrazole: a) benzonitrile, NaN₃, HOAc in *n*-butanol, 72 h, 125 °C.

B) Synthesis of 5-(4-phenoxy)phenyltetrazole: a) 4-phenoxybenzonitrile, dibutyltin oxide, trimethylsilylazide in toluene, 5 h, 95 °C. b) work-up with NaOH (1.6 N).

Scheme 3. Synthesis of the aryl tetrazole derivatives. Reagents and conditions: a) D (-) ribose, H₂SO₄, acetone, 30 min, rt. b) **11**, monomethoxytrityl-Cl, pyridine, 16 h, rt. c) **12**, carbethoxymethylenetriphenylphosphorane, CH₃CN, 8 h, 95 °C. d) **13**, LiAlH₄, THF, 4 h, rt. e) **14**, Ph₃P, DEAD, 5-phenyltetrazole or 5-(4-phenoxy)phenyltetrazole, THF, 0 °C. f) **15** or **16**, ether/HOAc, 10 h, 55 °C. g) **17** or **18**, sulfamoylchloride, DMAc, 0 °C. h) **19** or **20**, *N*-α-Cbz or Boc-L-aminoacyl- (*t*Bu)-succinimide, DBU in DMF, 6 h, rt.

Scheme 4. General procedure for the preparation of McC analogues **32-36**: i) for R³ = Z-group, H₂, Pd/C in MeOH, 3 h, rt. j) for R³ = Boc-group TFA/H₂O (5:2), 4 h, 0 °C to rt. k) protected peptide (respectively f-MRTGNA for **28-30** or Boc-MRTGNS for **31**), HOBt, DIC, DIPEA, DMF, 16 h, rt. l) TFA/thioanisole/H₂O (90/2.5/7.5), 2 h, rt.

Scheme 5. General procedure for the preparation of hydroxamate based siderophore - IleRS conjugated inhibitors, carrying either a phenyl tetrazole (**2**) or a phenoxyphenyl tetrazole (**3**) moiety. a) protected peptide, HBTU, Et₃N in DMF, 16 h, rt. b) DIPEA (6%) in MeOH, 5h, rt. c) H₂, Pd/C in aqueous DMF with FeCl₃, 3 h, rt.

Table 1.

Entry	<i>S. aureus</i>	<i>E. faecalis</i>	<i>E. coli</i>	Human
IleSA (1)	2.9	2	2	20
CB 168 (2)	5	20	1.3	3000
CB 432 (3)	0.5	9	3.2	450

Table of contents

Selective inhibition of bacterial aminoacyl tRNA synthetases was accomplished in the past using aminoacyl sulfamoyladenines and substituting aryltetrazole moieties for the adenine heterocycle. While these compounds proved not successful in vivo, conjugation to peptidic Trojan horses or siderophore drug conjugate (SDC) transport modules envisaged improved uptake.

Microcin C and albomycin analogues with aryl-tetrazole substituents as nucleobase isosters are selective inhibitors of bacterial aminoacyl tRNA synthetases but lack efficient uptake

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Proposed outlook of figures and schemes

Figures

Figure 1. (A and B)

20 A)

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22 B)

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24
25 **Figure 2.** (A and B)
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30

31 **Figure 3.**

32

CB168: R¹ = -C₆H₅, R² = -CH(CH₃)CH₂CH₃

CB432: R¹ = -C₆H₄OC₆H₅, R² = -CH(CH₃)CH₂CH₃

32-35: R³ = fMRTGNA

32: R¹ = -C₆H₅, R² = -CH(CH₃)CH₂CH₃

33: R¹ = -C₆H₅, R² = -CH₂CH(CH₃)₂

34: R¹ = -C₆H₅, R² = -CH₂COOH

35: R¹ = -C₆H₄OC₆H₅, R² = -CH(CH₃)CH₂CH₃

36: R¹ = -C₆H₄OC₆H₅, R² = -CH(CH₃)CH₂CH₃, R³ = MRTGNS⁻

38: R¹ = -C₆H₅

39: R¹ = -C₆H₄OC₆H₅

Microcin C (4a)

Processed Microcin C (4b)

Albomycin (5a)

Y = N CO NH₂ delta2

Y = NH epsilon

Y = O delta 1

Processed albomycin (5b)

34

35 **Scheme 1.**

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A)

B)

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39 **Scheme 2.**

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43 **Scheme 3.**

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51 **Scheme 5.**

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55 **Table of contents**

56 Selective inhibition of bacterial aminoacyl tRNA synthetases was accomplished in the past using
 57 aminoacyl sulfamoyladenines and substituting aryltetrazole moieties for the adenine heterocycle.
 58 While these compounds proved not successful in vivo, conjugation to peptidic Trojan horses or
 59 siderophore drug conjugate (SDC) transport modules envisaged improved uptake.

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