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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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In 1998, Cubist Pharmaceuticals patented a series of aminoacyl tRNA synthetase (aaRS) inhibitors based on aminoacyl sulfamoyladenosines (aaSAs), in which the adenine was substituted by aryl-tetrazole moieties linked to the ribose fragment by a two-carbon spacer. Although potent and specific inhibitors of bacterial IleRS, these compounds did not prove 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 *E. coli* aminopeptidases with the release of toxic aaRS inhibitors. Hence, the lack of activity in whole-cell assays was due to an inability of the new compounds to be taken up by the cells, thus 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 is esterified in a second step 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 hydrolyzable isosteres of aminoacyladenylates, which are used by 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 such as poor cell permeability.

A U.S. patent by Cubist Pharmaceuticals (Lexington, USA)[6] reported the synthesis and evaluation of a new type of aaRS inhibitors. Although still based upon aminoacyl sulfamoyladenosine, these analogues contain an aryl-tetrazole in place of the adenine moiety, 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 the numerous compounds of this class that have been synthesized and tested, only CB432 (**3**) showed moderate activity against a broad range of bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus pneumoniae Streptococcus pyogenes, Enterococcus faecium, Enterococcus faecalis, Bacillus subtilis, Haemophilus influenzae, Klebsiella pneumoniae*, and *Moraxella catarrhalis*, with MIC values ranging between 2 and 100 $\mu g\,mL^{-1}$.[7] In addition, **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 produced by *Enterobacteriaceae*. McC consist of an adenosine to which a heptapeptide is attached through a C-terminal phosphoamide bond. The biosynthesis and mode of action of McC have

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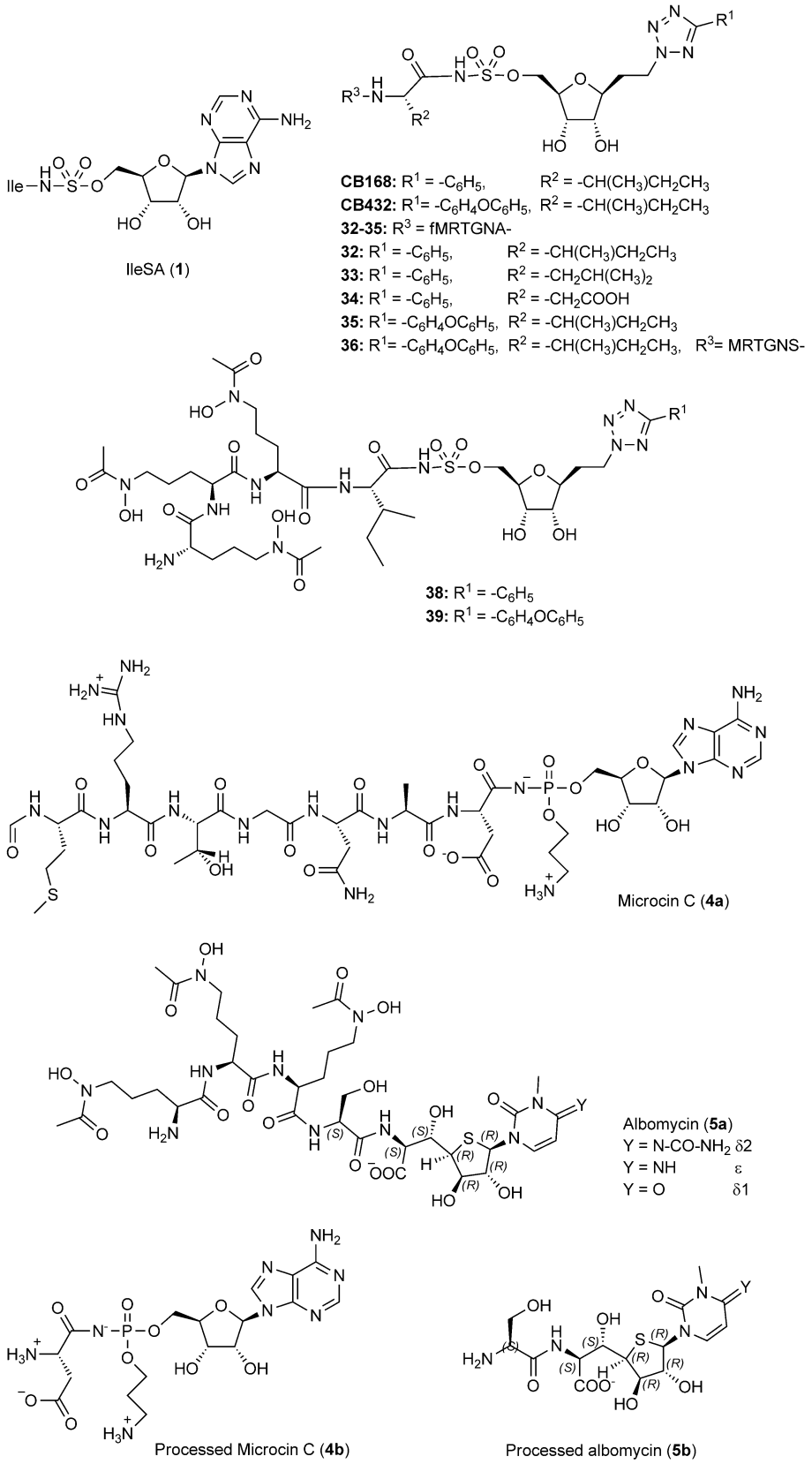


Scheme 1. IleRS inhibitors with aryl-tetrazole substitutes for adenine, and chemical structures for microcin C and albomycin.

been reviewed comprehensively elsewhere.[8] In *E. coli*, the 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 nonhydrolyzable 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 moiety, 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 moiety that is poorly permeable on its own. Albomycin is a so-called siderophore–drug conjugate (SDC), with the siderophore consisting of a tripeptide of *N*-5-acetyl-*N*-5-hydroxy-L-ornithines, mimicking the siderophore ferrichrome. The siderophore is C-terminally attached to a 4′-thio-6′-amino heptonic acid through a special linker. A modified cytosine resembling a nucleobase is attached at the 1′-position. Albomycin is taken up by the FhuA–TonB complex, a ferrichrome transport mechanism present in many bacteria and capable of transporting siderophores and, in some cases, sideromycins, across bacterial membranes.[10] Once inside the cell, it is released from iron by ferric reductase and processed by PepN with release of the 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 inhibi-

Table 1. Respective inhibition constants of compounds **1**–**3** on IleRS isolated from different organisms [μg mL^{-1}].

	S. aureus	*E. faecalis*	*E. coli*	Human
IleSA (**1**)	2.9	2	2	20
CB168 (**2**)	5	20	1.3	3000
CB432 (**3**)	0.5	9	3.2	450

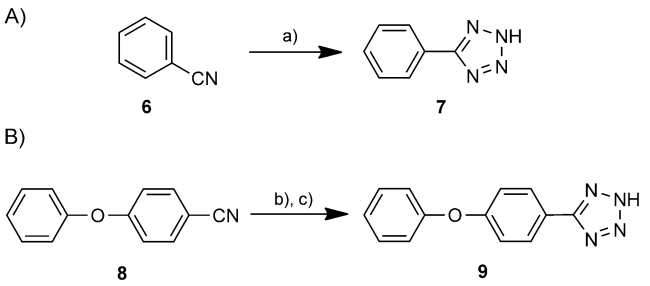
tors. We concentrated on two different lead compounds for this analysis, CB168, which was chosen for its great potential as a selective inhibitor of bacterial IleRS, and CB432, because of its broad-range antibacterial activity, low level inhibition of human IleRS, and moderate efficacy in an animal model. 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 an adenine substitute

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 by several strategies: by converting it into an McC analogue (yielding compound **32**), and by replacing the isoleucine moiety 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 related compound **36**, the N-terminal formyl group of the McC peptide was omitted, and the alanine at position 6 was replaced by a serine. The expectation was that this compound would be processed more efficiently once inside the cell, due to the expected lack of requirement for peptide deformylase caused by the presence of a possible endopeptidase cleavage site. Finally, compound **3** was coupled with the hydroxamate-based siderophore (**37**), affording compound **39**.

The 5-phenyltetrazole (**7**) was synthesized by 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 starting materials **2** and **3** was retained. However, the synthetic procedure was modified, largely based on work by Lee et al.,[13] Mandal et al.[14] and most importantly, Ohrui et al.[15] (Scheme 3). Starting from ribose (**10**), the 2′- and 3′-hydroxy groups were protected with an isopropylidene moiety and the 5′-hydroxy with a *p*-anisyl-diphenylmethyl (MMTr) protecting group to afford **12**. Following the method described by Ohrui et al.,[15] a Wittig–Moffat reaction was carried out, providing compound **13**. Reduction of the ester, followed by a Mitsunobu reaction with tetrazole derivative **7** or **9**, yielded compounds **15** and **16**, respectively. Subsequently, these compounds were selectively deprotected to liberate the 5′-hydroxy group, affording **17** and **18**. Sulfamoylation of the latter compounds according to previous protocols[16] gave **19** and **20**. Previously, we 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. 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**) by 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 of the Cbz group by hydrogenation yielded compounds **38** and **39** (Scheme 5).

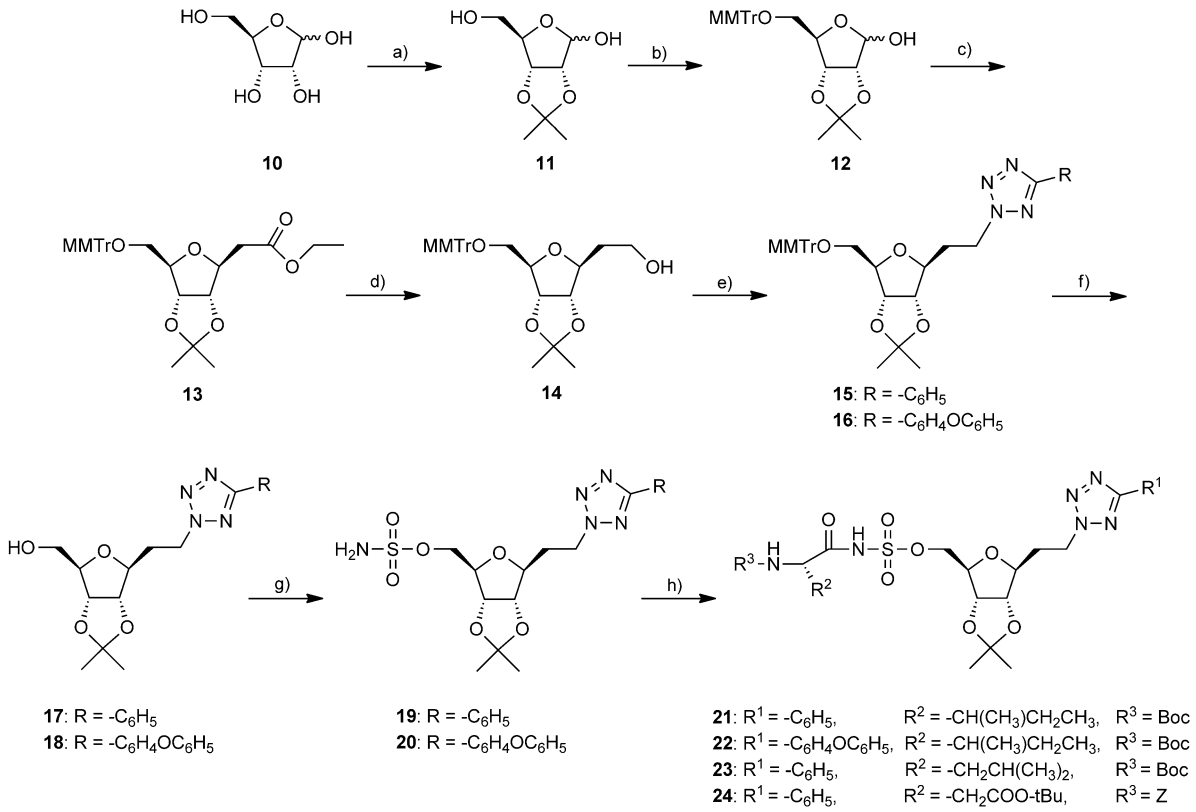


Scheme 2. Synthesis of 5-aryl tetrazoles. A) Synthesis of 5-phenyl tetrazole: a) benzonitrile, NaN_3, HOAc in *n*BuOH, 96 h, 125 °C. B) Synthesis of 5-(4-phenoxy)phenyltetrazole tetrazole: b) 4-phenoxybenzonitrile, dibutyltinoxide, trimethylsilylazide in toluene, 5 h, 95 °C; c) work-up with NaOH (1.6 N).

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 wild-type *E. coli* K-12 BW28357, a derivative strain Ara-Yej (BW39758) with the 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 strain due to higher levels of the 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 the 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 the 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, although very weak, activity against *S. aureus* and *C. albicans* after 11 h incubation as shown in a Student's T-test ($p = 0.01$) (Figure 1). To a lesser extent, *C. albicans* was also inhibited after 11 h ($p = 0.1$).



Scheme 3. Synthesis of aryl tetrazole derivatives. a) D(−)-ribose, H_2SO_4, acetone, 30 min, RT; b) **11**, monomethoxytrityl-Cl, pyridine, 16 h, RT; c) **12**, carbethoxymethylenetriphenylphosphorane, CH_3CN, 8 h, 95 °C; d) **13**, $LiAlH_4$, THF, 4 h, RT; e) **14**, Ph_3P, 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.

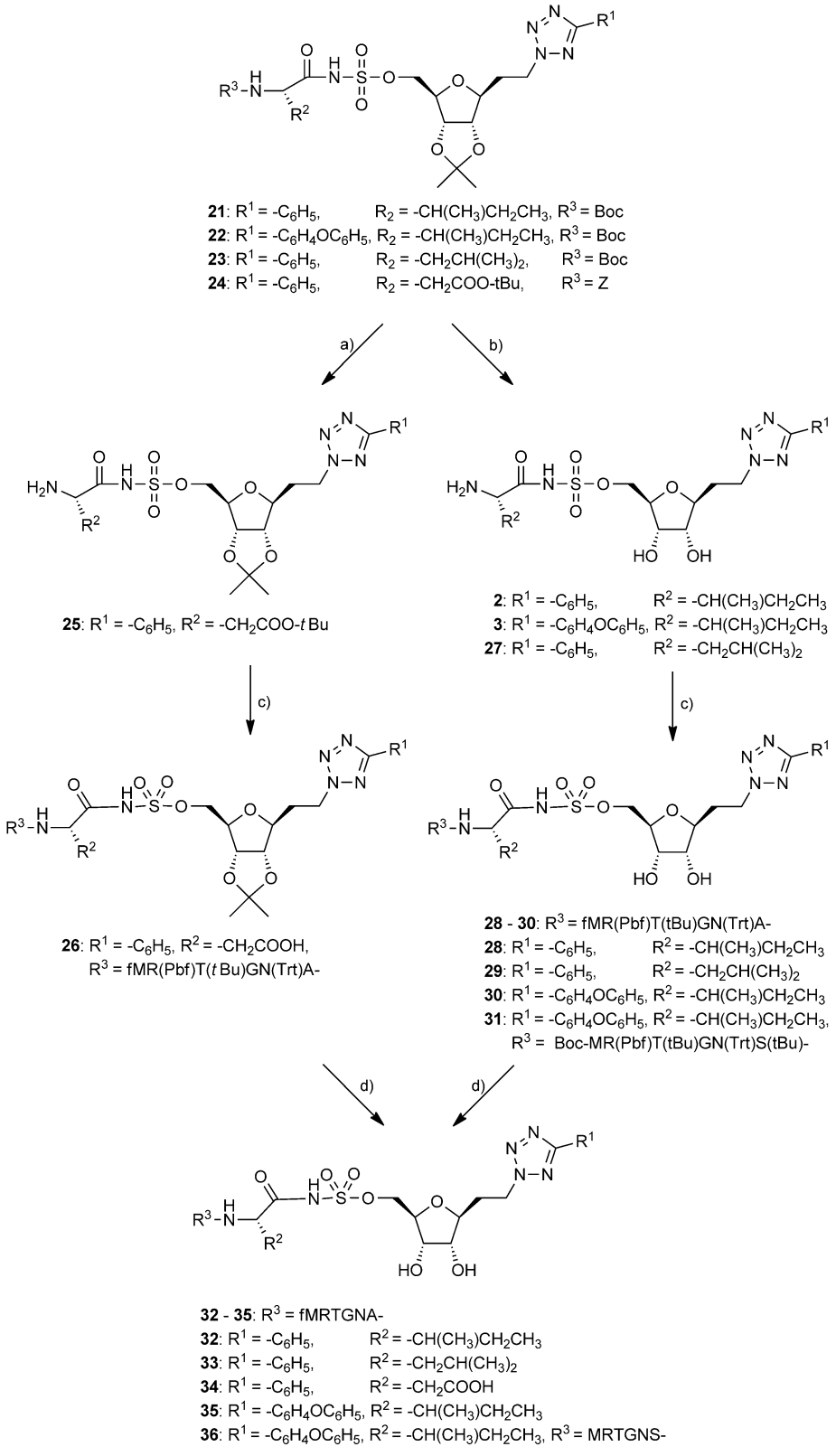
To understand the reason(s) for the disappointing lack of antibacterial activity of new compounds, in vitro analyses were performed, and the ability of all compounds targeting IleRS (compounds **2**, **3**, **32**, **35**, **36**, **38**, and **39**) to inhibit the $tRNA^{Ile}$ aminoacylation reaction 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 results thus indicate 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 identified a number of new entities that are based on aminoacyl sulfamoyladenosines, which are well-known nonhydrolyzable isosteres of aminoacyl adenylates. It was found that selectivity of aminoacyl sulfamoyl adenosines can be increased by replacing adenine with 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 this 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 the Cubist compounds, we successfully converted them into McC or SDC analogues. The whole-cell activity as well as the inhibitory action of these compounds against their respective aaRSs in in vitro aminoacylation experiments was determined.

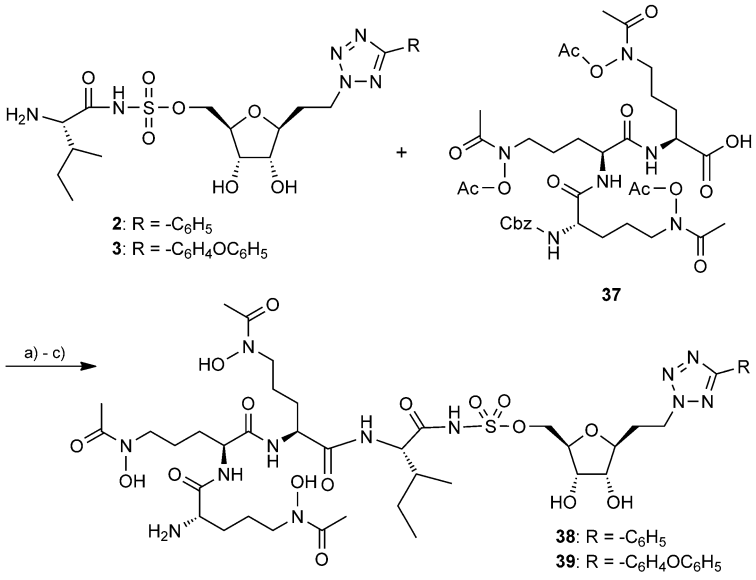
Three important conclusions can be drawn from these results. First, synthetic SDC-prodrugs have often been found unable to release the active moiety, which therefore cannot reach its target,[21] making this approach ineffective. Our results clearly show that, although no antibacterial activity was observed with new McC or SDC 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,



Scheme 4. General procedure for the preparation of McC analogues **32**–**36**: a) for R^3 = Z-group, H_2, Pd/C in MeOH, 3 h, RT; b) for R^3 = Boc group, TFA/H_2O (5:2), 4 h, 0 °C to RT; c) protected peptide (f-MRTGNA for **28**–**30** or Boc-MRTGNS for **31**), HOBt, DIC, DIPEA, DMF, 16 h, RT; d) TFA/thioanisole/H_2O (90:2.5:7.5), 2 h, RT.

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 moiety observed in previous work. Secondly, it must be concluded that, in the case of McC analogues, the absence of antibacterial activity against *E. coli* is attributable to failure of uptake. In our previous work,[17,18] it was clearly shown that the YejABEF transporter, responsible for McC uptake, tolerates modifications to the 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 derivatives with very close resemblance. The same may be true for SDCs that 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 the 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 siderophore presented here with simple aminoacyl-sulfamoyl-adenosines proved ineffective due to the 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 (excluding compound **34**) proved potent inhibitors of either LeuRS or IleRS. However, since compound **34** did not inhibit AspRS, it



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, Et_3N in DMF, 16 h, RT; b) DIPEA (6%) in MeOH, 5 h, RT; c) H_2, Pd/C in aqueous DMF with $FeCl_3$, 3 h, RT.

can be concluded that not all modifications of 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 metabolism. This suggests that LeuRS and IleRS can accommodate and effectively bind the aryl-tetrazole moiety substitution 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 in which ATP is stacked between a phenylalanine of motif 2 and an arginine in motif 3. In addition, Brick et al. and Nakama et al. showed that there is no initial hydrogen bonding of the adenine N-6 group with the KMSKS loop for isoleucyl and tyrosyl-tRNA synthetases.[22,23] Therefore, adenine mimics such as the aryl-tetrazoles presented in this manuscript may be accepted as good isosteres of adenine.

Experimental Section

Reagents and solvents were purchased from commercial suppliers (Acros, Sigma–Aldrich, Bachem, Novabiochem) and used as provided, unless otherwise indicated. DMF and THF were of analytical grade and were stored over 4 Å 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 otherwise indicated.

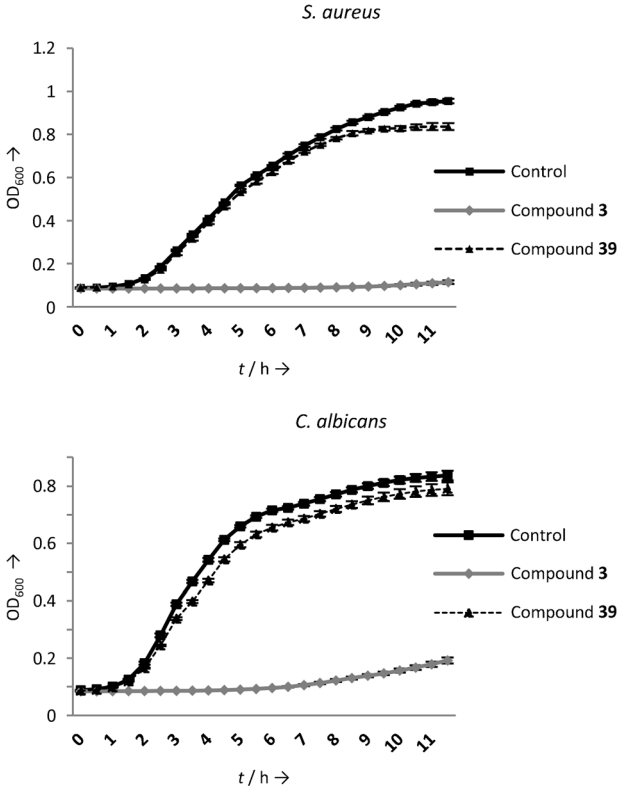


Figure 1. Inhibitory activity of compounds **3** and **39** against *S. aureus* and *C. albicans* at 50 μM concentrations. OD_{600} refers to the optical density of the cell culture measured at 600 nm. Marginal but statistically significant inhibition of *S. aureus* by compound **39** was found.

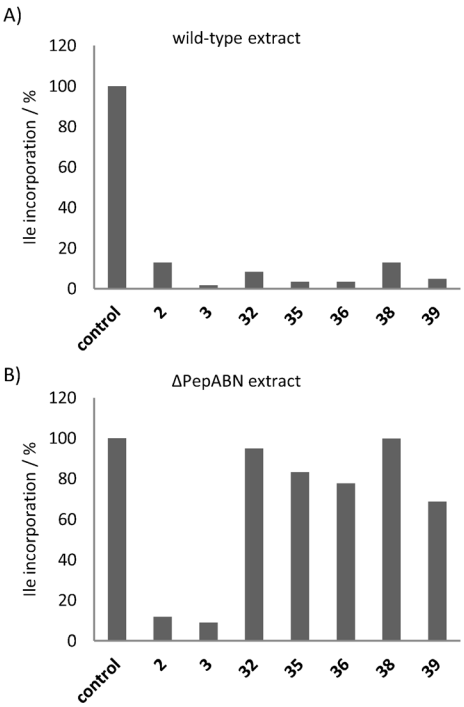


Figure 2. Inhibition of the aminoacylation reaction. A) IleRS inhibition in S30 extracts of McC-sensitive *E. coli* cells. B) IleRS in S30 extracts of McC-resistant *E. coli* cells lacking peptidases A, B, and N. In all tests, the different extracts were incubated with the respective IleRS inhibitors (25 μM), with samples taken after 15 min for evaluation of the aminoacylation reaction. The amounts of aminoacylated $tRNA^{Ile}$ were measured through liquid scintillation counting and normalized versus the respective control.

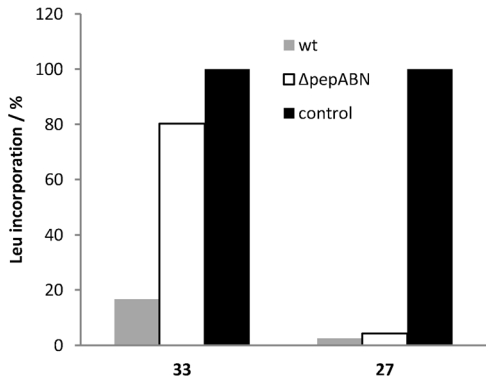


Figure 3. Inhibition of LeuRS by compounds **27** and **33**. In all tests, the different extracts were incubated with the respective IleRS inhibitors (25 μM), with samples taken after 15 min for evaluation of the aminoacylation reaction. The amounts of aminoacylated $tRNA^{Ile}$ were measured through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

^1H and ^{13}C NMR spectra of the compounds dissolved in $[D_6]DMSO$ or D_2O were recorded on a Bruker UltraShield Avance 300 MHz or 500 MHz spectrometer. Chemical shifts are expressed as δ values in parts per million (ppm) using residual solvent peaks (DMSO: ^1H, $\delta = 2.50$ ppm; ^{13}C, $\delta = 39.52$ ppm; HOD: ^1H, $\delta = 4.79$ ppm) as a reference. Coupling constants are given in Hertz (Hz). The peak patterns are indicated by the following abbreviations: br s = 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 propan-2-ol/H_2O (1:1) at 3 μL min^{-1}.

Precoated aluminium sheets were used for TLC (Merck, Silica gel 60 F_{254}). 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 preparative 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; 4.6×100 mm) column, connected to a Shimadzu LC-20AT pump using a Shimadzu SPD-20A UV detector. Recordings were performed at 254 and 214 nm.

Synthesis of 5-phenyltetrazole (7): Benzonitrile (**6**) (15.5 mL), sodium azide (13 g, 15.5 mL) and acetic acid (11.4 mL) were dissolved in *n*-BuOH (60 mL) and stirred at reflux. After 3 d, another portion of sodium azide (2.60 g), acetic acid (3.8 mL), and *n*BuOH (20 mL) were added, and the mixture was stirred for another 24 h. The mixture was cooled to 0 °C, and a white precipitate 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 then washed with toluene (2×100 mL). The water layer was acidified with 1 N HCl until a white precipitate 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%): ^{13}C NMR (75 MHz, $[D_6]DMSO$): $\delta = 124.23$ (*i*-aryl), 127.08 (*o*-aryl), 129.52 (*m*-aryl), 131.36 (*p*-aryl), 155.38 ppm (C-tetrazole); ESI-MS calcd for $C_7H_7N_4$: 147.07 $[M+H]^+$; found: 146.90.

Synthesis of 5-(4-phenoxy)phenyltetrazole (9): 4-Phenoxybenzonitrile (**8**) (200 mg, 1.02 mmol, 1.0 equiv), dibutyltinoxide (79 mg, 3.16 mmol, 3.0 equiv), and trimethylsilylazide (679 μL, 5.17 mmol, 5.0 equiv) were dissolved in toluene (5 mL) and stirred at reflux for 5 h, after which the reaction mixture was cooled to room temperature. Additional toluene (100 mL) was added to this mixture, and the organic layer was extracted twice with 50 mL NaOH (1.6 N). 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 Na_2SO_4, filtered, and evaporated, yielding 215 mg (87%) of the desired compound: ^{13}C NMR (75 MHz, $[D_6]DMSO$): $\delta = 119.49$, 120.24, 120.52, 125.36, 129.90, 131.23, 156.41, 160.16 ppm; HR-MS: calcd for $C_{13}H_{10}N_4O_1$: 239.0933 $[M+H]^+$; found: 239.0918.

Synthesis of 5-*O*-*p*-anisyldiphenylmethyl-2,3-*O*-isopropylidene-β-D-ribofuranose (12): A solution of D(−)-ribose (**10**) (6.50 g, 43.16 mmol, 1 equiv), acetone (80 mL), and H_2SO_4 (0.2 mL) was stirred for 30 min at room temperature under argon. $NaHCO_3$ (0.43 g, 5.14 mmol, 0.12 equiv) was added, and the mixture was stirred for 30 min, filtered, and the volatiles 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 equiv) in pyridine was added. This mixture was stirred overnight at room temperature under argon. The volatiles were evaporated, and the residue was purified by flash chromatography (20% EtOAc in hexane with 0.1% Et_3N). Fractions containing the desired product, as determined by TLC analysis, were evaporated to yield product **12** (2.8 g, 6.054 mmol, 43% calculated over two steps): ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 24.98$, 25.31, 26.36, and 26.75 (2×isopropylidene CH_3), 55.39 (O-CH_3), 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 ppm (aromatic signals); ESI-MS calcd for $C_{28}H_{30}O_6Na$: 485.19 $[M+Na]^+$; found: 484.80.

Synthesis of ethyl-3,6-anhydro-7-*O*-*p*-anisyldiphenylmethyl-2-deoxy-4,5-*O*-isopropylidene-D-allo-heptanoate (13): Product **12** (11.0 g, 23.8 mmol, 1 equiv) and carbethoxymethylene-triphenylphosphorane (12.45 g, 35.7 mmol, 1.5 equiv) were dissolved in CH_3CN (100 mL). The solution was stirred at reflux for 8 h, then was evaporated to yield a pale yellow oil. The product was purified by flash chromatography (22% EtOAc in hexane). Fractions containing the desired product were evaporated, affording product **13** (12.50 g, 23.485 mmol, 99%): ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.44$ (CH_3 ethyl), 25.89 and 27.77 (2×CH_3 isopropylidene), 39.00 (C2), 55.38 (C1), 60.85 (CH_2 ethyl), 64.44 (C7), 81.17 (C3), 82.57 (C5), 83.77 (C4), 84.54 (C6), 86.61 (MMTr-C(Ph_3)), 113.34 (trityl), 114.39 (C-isopropylidene), 127.10, 128.03, 128.70, 130.65, 135.71, 144.57, 144.62, and 158.81 (aromatic signals), 170.75 ppm (C=O); ESI-MS calcd for $C_{32}H_{36}O_7Na$: 555.24 $[M+Na]^+$; found: 554.83.

Synthesis of 3,6-anhydro-7-*O*-*p*-anisyldiphenylmethyl-2-deoxy-4,5-*O*-isopropylidene-D-allo-heptitol (14): Compound **13** (12.50 g, 23.48 mmol, 1 equiv) was dried overnight in vacuo. THF (10 mL) was added, then $LiAlH_4$ (1.34 g, 35.23 mmol, 1.5 equiv) was carefully added to the solution. After 30 min, the reaction was quenched by adding propan-2-ol (50 mL). The volatiles were evaporated, and the product was purified by flash chromatography (40% EtOAc in hexane). Fractions containing the desired product **14** were collected and evaporated to yield 9.5 g (19.38 mmol, 83%): ^{13}C NMR (125 MHz, $[D_6]DMSO$): $\delta = 25.95$ (CH_3 isopropylidene), 27.88 (CH_3 isopropylidene), 36.09 (C2), 55.55 (CH_3 *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, and 158.94 ppm (aromatic signals); ESI-MS calcd for $C_{30}H_{34}O_6$: 513.22 $[M+H]^+$; found: 513.00.

Synthesis of 3,6-anhydro-7-*O*-*p*-anisyldiphenylmethyl-1,2-dideoxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol (15): Product **14** (9.35 g, 19.1 mmol, 1.0 equiv) and triphenylphosphine (Ph_3P, 10.0 g, 38.2 mmol, 1.0 equiv) 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 equiv) was added. The mixture was stirred for 3 h at room temperature under argon, 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%): ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 25.91$ and 27.81 ($2CH_3$ isopropylidene), 31.91 (C2), 50.46 (C1), 55.54 (O-CH_3), 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, and 165.45 ppm (aromatic signals); ESI-MS calcd for $C_{37}H_{39}N_4O_5$: 641.27 $[M+H]^+$; 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 equiv) was dissolved in diethyl ether/HOAc (20 mL, 1 *v/v*) and refluxed for 10 h. The mixture was evaporated and neutralized with $NaHCO_{3(sat.)}$. The mixture was then extracted with ether (2×20 mL) and washed with brine (20 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated. The product was easily purified by flash chromatography (60% EtOAc in hexane) to yield 1.2 g (3.4 mmol, 38%): ^{1}H NMR (500 MHz, [D_6]DMSO): $\delta = 1.25$ (s, 3H; CH_3), 1.40 (s, 3H; CH_3), 2.17–2.33 (m, 2H; C1), 3.44–3.46 (*t*AB, $J_{AB} = 11.60$ Hz, $J_{A,OH} \approx J_{A,6} \approx 5.4$ Hz, $J_{B,OH} \approx J_{B,6} \approx 5.3$ Hz, 2H; C7), 3.83–3.87 (m, 2H; C6, C3), 4.45–4.47 (dd, $J = 4.2/6.5$, 1H; C4), 4.59–4.61 (dd, $J = 3.3$, 6.5 Hz, 1H; C5), 4.79–4.84 (m, 2H; C2), 4.87–4.88 (*t*, $J = 5.5$ Hz, 1H; OH), 7.54–7.58 (m, 1H; *m/p*-aryl), 8.05 (d, $J = 1.5$ Hz, 1H; *o*-aryl), 8.07 ppm (d, $J = 1.8$ Hz, 1H; *o*-aryl); ^{13}C NMR (125 MHz, [D_6]DMSO): $\delta = 25.34$ and 27.23 ($2CH_3$ 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 ppm (tetrazole); ESI-MS calcd for $C_{17}H_{23}N_4O_4$: 347.17 $[M+H]^+$; 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 formed, and the mixture was allowed to come to room temperature. After 15 min, the mixture was cooled to 0 °C again, and CH_3CN (1 mL) was added. This mixture was stirred for 15 min. The resulting product was used as such for the next step.

Compound **17** (450 mg, 1.3 mmol) was dissolved in dimethylacetamide (DMAc, 2.3 mL) at room temperature. This solution was cooled to 0 °C, and cooled chlorosulfonamide was added. This mixture was stirred for 50 min from 0 °C to room temperature. After 50 min, the mixture was cooled to 0 °C again, and Et_3N (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, and the desired product was redissolved in EtOAc and washed twice with $NaHCO_3$ (50 mL) and once with brine (50 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to yield **19** (410 mg, 0.96 mmol, 74%): ^{1}H NMR (500 MHz, [D_6]DMSO): $\delta = 1.32$ (s, 3H; CH_3), 1.48 (s, 3H; CH_3), 2.31–2.48 (m, 2H; C1), 3.21–3.33 (m, 2H; C7), 3.99–4.02 (m, 2H; C6, C3), 4.48–4.52 (dd, $J = 4.6$, 6.5 Hz, 1H; C4), 4.71–4.74 (dd, $J = 2.8/6.5$ Hz, 1H; C5), 4.79–4.84 (m, 2H; C2), 7.51–7.53 (m, 3H; *m/p*-aryl), 8.09–8.13 ppm (m, 2H; *o*-aryl, $J = 1.5$ Hz); ^{13}C NMR (125 MHz, [D_6]DMSO): 26.44 and 28.46 ($2CH_3$ 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 $C_{17}H_{26}N_5O_6S_1$: 426.14 $[M+H]^+$; 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 equiv) was dried with *N*-*tert*-butoxycarbonyl-isoleucine-*O*-succinimide (110 mg, 0.33 mmol, 1.5 equiv) in vacuo. Diazobicyclo[5,4,0]undecene, (DBU, 50 μL, 1.5 equiv) was added and this mixture was stirred for 8 h. The volatiles were evaporated, and the product was purified using flash chromatography (40% hexane in EtOAc). Fractions containing the product were collected and evaporated, yielding product **21** (20 mg, 0.03 mmol, 14%): ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 11.58$ (Ile-δ-CH_3), 16.04 (Ile-γ-CH_3), 25.21 (Ile-γ′-CH_3), 25.73 (isopropylidene CH_3), 27.59 (isopropylidene CH_3), 28.71 (*t*Bu-Boc), 33.33 (C2), 37.45 (Ile-β-CH_2), 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 ppm (C=O Ile); ESI-MS calcd for $C_{28}H_{43}N_6O_9S$: 637.27 $[M-H]^-$; found: 637.11.

Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-(Ile)-sulfamate] (2): A mixture of TFA/water (5:2) was added to **21** (20 mg, 0.03 mmol, 1 equiv), and the mixture was allowed to react for 2 h. The volatiles were evaporated, then the residue was coevaporated with toluene. The desired product was purified using HPLC (10% MeOH in water) to yield **1** (4.5 mg, 0.01 mmol, 30%): ^{1}H NMR (500 MHz, MeOD): $\delta = 0.95$–0.98 (t, $J = 7.4$ Hz, 3H; Ile-δ-CH_3), 1.05–1.06 (d, $J = 7.0$ Hz, 3H; Ile-γ′-CH_3), 1.23–1.31 (m, 1H; Ile-γ_B-CH_2), 1.58–1.66 (m, 1H; Ile-γ_A-CH_2), 1.99 (m, 1H; Ile-β-CH), 2.19–2.26 and 2.41–2.47 (m, 2H; C2), 3.57–3.57 (d, $J = 4.0$ Hz, 1H; Ile-α-CH), 3.78–3.81 (m, 1H; C4), 3.83–3.85 (t, $J = 5.5$ Hz, 1H; C5), 4.00–4.03 (dd, $J = 3.7/7.7$ Hz, 1H; C6), 4.11–4.13 (dd, $J = 4.5$, 9.6 Hz, 1H; C3), 4.17–4.24 (dAB, $J_{A,4'} = 3.63$, $J_{A,B} = 10.81$ Hz, 1H; C7), 4.90 (m, 2H; C1), 7.50–7.51 (m, 3H; *m/p*-phenyl), 8.09–8.11 ppm (m, 2H; *o*-phenyl); ^{13}C NMR (125 MHz, MeOD): $\delta = 12.18$ (Ile-δ-CH_3), 15.56 (Ile-γ-CH_3), 25.60 (Ile-γ′-CH_2), 34.19 (C2), 38.15 (Ile-β-CH), 51.40 (C1), 61.22 (Ile-α-CH_2), 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 ppm (C=O Ile); HR-MS calcd for $C_{20}H_{29}N_6O_7S$: 497.1819 $[M-H]^-$; found: 497.1822.

Synthesis of peptide coupled 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-(Ile)-sulfamate] (32): f-MR(Pbf)T-(*t*Bu)GN(Trt)A-OH (7 mg, 0.006 mmol, 2 equiv) was combined with HOBt (1.5 mg, 0.01 mmol, 3 equiv) and dried in vacuo. DMF (0.5 mL) and DIC (1.6 μL, 0.01 mmol, 3 equiv) were added, and the solution was stirred for 1 h. Previously obtained product **1** (1.5 mg, 0.003 mmol, 1 equiv) and DIPEA (1 μL, 0.006 mmol, 2 equiv) were added. This solution was stirred overnight, then the evaporated desired product was purified on a Porapak-column (25% to 100% CH_3CN in water). The protecting groups were removed by addition of TFA/water/thioanisol (92.5:7.5:2.5, *v/v/v*). This solution was stirred for 2 h, then the mixture was coevaporated with toluene. Next, the mixture was purified using HPLC (2% CH_3CN in water).

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

Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[[*N*-5-acetyl-*N*-5-*O*-hydroxyl-L-ornithinyl]-[*N*-5-acetyl-*N*-5-*O*-hydroxyl-L-ornithinyl]-[*N*-5-acetyl-*N*-5-*O*-hydroxyl-L-ornithinyl]-L-Ile)-sulfamate] (38): Et_3N (13 μL, 0.1 mmol, 2 equiv) was added to compound **1** (25 mg, 0.05 mmol) dissolved in DMF (0.5 mL). This mixture was next combined with a mixture of **37** (32.4 mg, 0.05 mmol, 1 equiv) and HBTU (23 mg, 0.06 mmol, 1.2 equiv), which were dissolved in DMF (0.5 mL). The reaction mixture was stirred overnight at room temperature under argon. The following day, the DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed twice with water. Subsequently, the EtOAc was evaporated, and the product was dissolved in water/CH_3CN, during which the volume of CH_3CN was kept to a minimum. This mixture was then purified over a Porapak column (0 to 100% CH_3CN in water). Fractions containing the product were dried, affording the desired product, still containing side products (yield: 21 mg, 34%): ESI-MS calcd for $C_{55}H_{77}N_{12}O_{21}S$: 1273.5 $[M-H]^-$; found: 1273.1.

The crude product (10.5 mg, 8.2 μmol) was dissolved in MeOH containing 6% DIPEA and was stirred for 24 h. The product was dried and dissolved in H_2O/CH_3CN. The product was next purified by RP-HPLC (5 to 80% CH_3CN in H_2O), yielding 1.8 mg (5%) of the desired compound: ^{13}C NMR (125 MHz, MeOD): $\delta = 12.08$ (CH_3 DIPEA), 13.20 (Ile-δ-CH_3), 16.37 (Ile-γ-CH_3), 17.38 (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 (C2), 38.59 (Ile-β-CH), 43.79 (DIPEA-CH_2), 51.43 (C1), 54.21 (Orn-α-CH), 55.83 (DIPEA-CH), 61.62 (Ile-α-CH_2), 67.76 (Cbz-CH_2), 69.81 (C7), 73.08 (C3), 76.10 (C5), 80.45 (C6), 83.73 (C4), 127.77–138.46 (aromatic signals), 158.77 (C=O Cbz), 166.15 (tetrazole), 173.84 ppm (C=O Ile); HR-MS calcd for $C_{49}H_{71}N_{12}O_{18}S$: 1147.4730 $[M-H]^-$; found: 1147.4774.

Next, the compound was dissolved in 5% aqueous DMF (100 μL), and a solution of $FeCl_3$ (10 μL of a 0.5 M solution) was added. Next, 10 mg of 10% Pd-C were 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 $C_{41}H_{65}N_{12}O_{16}S$: 1013.4360 $[M-H]^-$; 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): Synthesis analogous to the preparation of **21** was performed to affording 260 mg (88% total yield) of compound **22**: ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 11.85$ (Ile-δ-CH_3), 15.90 (Ile-γ-CH_3), 25.22 (Ile-γ′-CH_3), 25.69 (isopropylidene CH_3), 27.56 (isopropylidene CH_3), 28.67 (*t*Bu-Boc), 33.33 (C2), 37.47 (Ile-β-CH_2), 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 ppm (tetrazole); ESI-MS calcd for $C_{34}H_{45}N_6O_{10}S$: 729.29 $[M-H]^-$; found: 729.18.

Synthesis of 3,6-anhydro-2-deoxy-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol-7-[*N*-(L-Ile)-sulfamate] (3): Synthesis was performed, analogous to the preparation of **1**, to afford 260 mg (88% total yield) of the compound **3**: ^1H NMR (500 MHz, MeOD): $\delta =$ 0.88–0.93 (t, $J = 7.4$ Hz, 3H; Ile-δ-CH_3), 0.98–0.99 (d, $J = 7.0$ Hz, 3H; Ile-γ′-CH_3), 1.23–1.27 (m, 1H; Ile-γ_B-CH_2), 1.52–1.57 (m, 1H; Ile-γ_A-CH_2), 1.93 (m, 1H; Ile-β-CH), 2.15–2.36 (m, 2H; C2), 3.48–3.49 (d, $J = 4.0$ Hz, 1H; Ile-α-CH), 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); ^{13}C NMR (125 MHz, MeOD): $\delta = 13.03$ (Ile-δ-CH_3), 16.39 (Ile-γ-CH_3), 26.56 (Ile-γ′-CH_3), 34.99 (C2), 30.06 (Ile-β-CH_2), 52.18 (C1), 62.27 (Ile-α-CH_2), 70.65 (C7), 73.86 (C3), 76.86 (C5), 81.59 (C4), 84.34 (C6), 107.38–161.84 ppm (aromatic signals); HR-MS calcd for $C_{26}H_{33}N_6O_8S$: 589.2081 $[M-H]^-$; 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): Synthesis was performed, analogous to the preparation of **32**, to afford 1.0 mg (18% total yield) of the compound **35**: HR-MS calcd for $C_{51}H_{75}N_{16}O_{17}S_2$: 1247.4938 $[M-H]^-$; 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 an analogous manner to the formylated derivative f-MR(Pbf)T(*t*Bu)GN(Trt)A-OH:[18] HR-MS calcd for $[M-H]^-$: $C_{69}H_{97}N_{10}O_{15}S_2$ 1369.6581; found: 1369.6570. Synthesis analogous to the preparation of **32** was performed to afford 1.0 mg (18% total yield) of compound **36**: HR-MS calcd for $C_{51}H_{76}N_{16}O_{18}S_2$: 1263.4887 $[M-H]^-$; found: 1263.4774.

Synthesis of 3,6-anhydro-2-deoxy-1-[5-phenoxyphenyltetrazole]-D-allo-heptitol-7-[*N*-[[*N*-5-acetyl-*N*-5-*O*-hydroxyl-L-ornithinyl]-[*N*-5-acetyl-*N*-5-*O*-hydroxyl-L-ornithinyl]-[*N*-5-acetyl-*N*-5-*O*-hydroxyl-L-ornithinyl]-L-Ile)-sulfamate] (39): Synthesis analogous to the preparation of **38** was performed to afford 1.3 mg (20% total yield) of compound **39**: HR-MS calcd for $C_{55}H_{75}N_{12}O_{19}S$: 1239.4992 $[M-H]^-$; 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): Synthesis was performed, analogous to the preparation of **21**, to afford 550 mg (82% total yield) of compound **23**: ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 22.18$ (Leu-δ_B-CH_3), 23.41 (Leu-δ_A-CH_3), 25.13 (Leu-γ-CH) 25.74 (CH_3 isopropylidene), 27.60 (CH_3 isopropylidene), 28.68 (*t*Bu Boc), 33.35 (C2), 41.27 (Leu-β-CH_3), 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 ppm (tetrazole); ESI-MS calcd for $C_{28}H_{43}N_6O_9S$: 639.28 $[M+H]^+$; found: 639.20.

Synthesis of 2-deoxy-4,5-dihydroxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-*N*-(Leu)-sulfamate (27): Synthesis analogous to the preparation of **1** was performed to afford 460 mg (89% total yield) of compound **27**: ^1H NMR (300 MHz, MeOD): $\delta = 0.96$–0.97 (d, $J =$ 5.9 Hz, 3H; Leu-δ_B-CH_3), 0.98–0.99 (d, $J = 6.1$ Hz, 3H; Leu-δ_A-CH_3) 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-CH_2), 2.41–2.43 (m, 1H; Leu-β_B-CH_2), 3.57–3.60 (dd, $J = 5.2$, 8.5 Hz, 1H; Leu-α), 3.77–3.80 (m, 1H; C4), 3.83–3.85 (t, $J = 4.8$ Hz, 1H; C5), 4.00–4.02 (dd, $J = 3.7/7.5$ Hz, 1H; C6), 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 ppm (dd, $J = 1.5$, 7.26 Hz, 1H; *o*-aryl); ^{13}C NMR (75 MHz, MeOD): $\delta = 22.13$ (Leu-δ_B-CH_3), 23.23 (Leu-δ_A-CH_3), 25.86 (Leu-γ-CH), 34.14 (C2), 42.33 (Leu-β-CH_2), 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 ppm (C=O Leu); HR-MS calcd for $C_{20}H_{29}N_6O_7S$: 497.1819 $[M-H]^-$; found: 497.1822.

Synthesis of peptide-coupled 2-deoxy-4,5-dihydroxy-1-[5-phenyltetrazole]-D-allo-heptitol-7-*N*-(Leu)-sulfamate (33): Synthesis was performed, analogous to the preparation of **32**, to afford 1.8 mg (4% total yield) of the compound **33**: HR-MS calcd for $C_{45}H_{71}N_{16}O_{16}S_2$: 1155.4676 $[M-H]^-$; found: 1155.4683.

Synthesis of 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[*N*-benzyloxycarbonyl-L-aspartyl-(*O*-*t*Bu)]-sulfamate] (24): A solution of (*Z*)-aspartyl-(*O*-*tert*-butyl)-*O*-Su (460 mg, 0.15 mmol, 2.0 equiv), 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-sulfamate (**19**) (320 mg, 0.75 mmol, 1 equiv), and DBU (171 μL, 0.9 mmol, 1.2 equiv) in DMF (5 mL) was stirred at room temperature for 8 h. DMF was evaporated under reduced pressure, then the residue was purified by flash chromatography (10% MeOH in CH_2Cl_2). Fractions containing the desired product were evaporated to afford **24** as colorless oil (420 mg, 81%). ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 25.57$ (isopropylidene CH_3), 27.47 (isopropylidene CH_3), 28.35 (*t*Bu), 29.84 (C2), 38.45 (Asp-CH_2) 50.04 (C1), 53.59 (Cα), 67.46 (CH_2-Cbz), 69.83 (C7), 81.78 (C3), 81.96 (C-*t*Bu), 82.09 (C5), 82.46 (C4), 84.76 (C6), 115.47 (C-isopropylidene), 127.18 (C-aryl), 127.67 (*i* C-aryl), 128.51 (C-aryl), 129.21 (*p* C-aryl), 130.64 (C-aryl), 136.44 (*i* C-aryl), 157.44 (C=O Asp), 165.49 (tetrazole), 172.42 ppm (C=O Asp); ESI-MS calcd for $C_{38}H_{62}N_7O_{11}SSi_2$: 880.4 $[M+H]^+$; found: 880.1.

Synthesis of 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[L-aspartyl-(*O*-*tert*-butyl)]-sulfamate] (25): Compound **24** (420 mg) was dissolved in a mixture of MeOH with 4.4% HCOOH (15 mL), and Pd/C (100 mg) was added. The mixture was stirred for 0.5 h, after which it was filtered and evaporated to yield **25** as a white foam: (250 mg, 76%): ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 25.79$ (isopropylidene CH_3), 27.63 (isopropylidene CH_3), 28.39 (*t*Bu-Boc), 33.42 (C2), 35.63 (Asp-CH_2) 50.27 (C1), 53.05 (Asp-Cα), 69.53 (C7), 81.91 (C3), 82.02 (C-*t*Bu), 82.44 (C5), 83.11 (C4), 84.84 (C6), 115.02 (C-isopropylidene), 127.18 (C-aryl), 127.75 (*i* C-aryl), 129.22 (C-aryl), 130.61 (*p* C-aryl), 165.39 (tetrazole), 171.49 and 174.10 ppm (C=O Asp); ESI-MS calcd for $C_{32}H_{31}N_6O_9S$: 555.2 $[M-H]^-$; found: 554.9.

Synthesis of peptide-coupled 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-phenyltetrazole]-D-allo-heptitol-7-[*N*-[L-aspartyl-(*O*-*tert*-butyl)]-sulfamate] (34): Synthesis was performed, analogous to the preparation of **32**, to afford 1.7 mg (15% total yield) of compound **34**: HR-MS calcd for $C_{42}H_{67}N_{16}O_{17}S_2$: 1131.4311 $[M+H]^+$; found: 1131.4850.

Synthesis of 3,6-anhydro-7-*O*-*p*-anisyldiphenylmethyl-1,2-dideoxy-4,5-*O*-isopropylidene-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol (16): Synthesis was performed, analogously to the preparation of **15**, to afford 410 mg (71% total yield) of compound **16**: ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 25.93$ and 27.83 ($2\times CH_3$ isopropylidene), 33.96 (C2), 50.45 (C1), 55.56 (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-isopropylidene), 113.49, 118.93, 119.89, 122.63, 124.28, 127.29, 128.19, 128.77, 128.85, 130.26, 130.72, 135.76, 144.61, 156.72, 158.96 (aromatic signals), 159.64 ppm (tetrazole); ESI-MS calcd for $C_{43}H_{42}N_4O_6Na$: 733.30 $[M+Na]^+$; found: 733.24.

Synthesis of 3,6-anhydro-2-deoxy-7-hydroxy-4,5-*O*-isopropylidene-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol (18): Synthesis was performed, analogous to the preparation of **17**, to afford 248 mg (98% total yield) of compound **18**: ^1H NMR (500 MHz, $CDCl_3$): $\delta = 1.34$ (s, 3H; CH_3), 1.53 (s, 3H; CH_3), 2.26–2.56 (m, 2H; C1) 3.67–3.46 (ABX, $J_{AB} = 12.60$ Hz/$J_{AX} \approx 3.0$/$J_{BX} \approx 3.75$ Hz, 2H; C7), 3.95–4.05 (m, 2H; C6, C3), 4.34–4.38 (m, 1H; C4), 4.64–4.89 (m, 3H; C5, C2), 7.07–7.19 and 7.36–7.41 and 8.09–8.12 ppm (aromatic signals); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 25.71$ and 27.65 ($2\,CH_3$ isopropylidene), 33.18 (C2), 50.23 (C1), 62.90 (C7), 81.75 (C3), 81.86 (C5), 84.79 (C4), 84.83 (C6), 115.17 (C-isopropylidene), 118.87, 119.91, 122.35, 124.30, 128.79, 130.23, 156.59, 159.72 (aromatic signals), 165.03 ppm (tetrazole); ESI-MS calcd for $C_{23}H_{26}N_4O_5Na$: 461.18 $[M+Na]^+$; found: 461.25.

Synthesis of 3,6-anhydro-2-deoxy-4,5-*O*-isopropylidene-1-[5-(4-phenoxy)phenyltetrazole]-D-allo-heptitol-7-sulfamate (20): Synthesis was performed, analogous to the preparation of **19**, to afford 260 mg (88% total yield) of compound **20**: ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 25.69$ and 27.59 ($2\times CH_3$ isopropylidene), 33.74 (C2), 50.28 (C1), 70.57 (C7), 81.59 (C3), 82.35 (C5), 82.74 (C4), 85.05 (C6), 118.93, 120.01, 124.43, 128.89, 130.31 ppm (aromatic signals); HR-MS calcd for $C_{23}H_{28}N_5O_7S$ ($[M+H]^+$): 518.1709; found: 518.1708.

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 L-arabinose (5 mM). Compounds were titrated in a 96-well plate using either LB medium ± L-arabinose (5 mM) to dilute the compounds. To each well, 85 μL LB medium ± L-arabinose (5 mM) was added to a total volume of 90 μL. Next, 10 μL of bacterial cell culture, grown to a OD_{600} of 0.1, was added. The cultures were next placed into a Tecan Infinite M200 incubator and shaken at 37 °C; subsequently the OD_{600} 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) was 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 centrifution 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), $MgCl_2$ (10 mM), KCl (100 mM). The cell suspension was centrifuged again at 3000 *g*. This procedure was repeated twice, then the pellet was resuspended in 1 mL of the following buffer: Tris·HCl or HEPES·KOH (pH 8.0, 20 mM), $MgCl_2$ (10 mM), KCl (100 mM),and DTT (1 mM) and was 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, then the lysate was centrifuged at 15 000 *g* for 30 min at 4 °C.

tRNA aminoacylation reaction: E. coli S30 extracts (3 μL) were added to 1 μL of solution containing inhibitor. Next, 16 μL of the following aminoacylation mixture were added: Tris·HCl (30 mM, pH 8.0), DTT (1 mM), bulk of *E. coli* tRNA (5 $g\,L^{-1}$), ATP (3 mM), KCl (30 mM), $MgCl_2$ (8 mM), and the specified ^{14}C-radiolabeled amino acid (40 μM, 200 $mCi\,mmol^{-1}$). The reaction products were precipitated in cold 10% TCA on Whatman 3 MM 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. Reported inhibitory values are based on

relative amounts of the observed radioactivity (expressed in cpm), normalized versus the respective control value. ^{14}C-Radiolabeled amino acids and scintillation liquid were purchased from Perkin Elmer.

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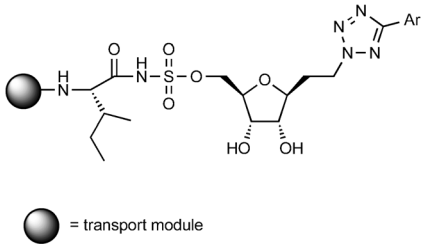
Microcin C and Albomycin Analogues with Aryl-tetrazole Substituents as Nucleobase Isosters Are Selective Inhibitors of Bacterial Aminoacyl tRNA Synthetases but Lack Efficient Uptake



Synthetases' Achilles' heel? Selective inhibition of bacterial aminoacyl tRNA synthetases was previously accomplished by using aminoacyl sulfamoyladenosines and substituting aryltetrazole moieties for the adenine heterocycle. While these compounds did not prove successful in vivo, conjugation to peptidic Trojan horses or siderophore drug conjugate (SDC) transport modules envisaged improved uptake.

