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Extended targeting potential and improved synthesis of Microcin C analogs as antibacterials

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ABSTRACT

Microcin C (McC) (**1**) is a potent antibacterial compound produced by some *Escherichia coli* strains. McC functions through a Trojan-Horse mechanism: it is actively taken up inside a sensitive cell through the function of the YejABEF-transporter and then processed by cellular aminopeptidases. Processed McC (**2**) is a non-hydrolysable aspartyl-adenylate analog that inhibits aspartyl-tRNA synthetase (AspRS). A new synthesis is described that allows for the production of a wide variety of McC analogs in acceptable amounts. Using this synthesis a number of diverse compounds was synthesized with altered target specificity. Further characteristics of the YejABEF transporters were determined using these compounds.

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1. Introduction

Bacterial antibiotic resistance is a global problem and high resistance profiles against antibiotics currently in use are ever increasing. Hereto, different resistance mechanisms have been developed by bacteria of which many are based on either enzymatic detoxification or excretion of the drug via an efflux process.[1] To overcome these deficiencies in our armamentarium against pathogens, new classes of antibiotics need to be developed.[2–4] Since the discovery of antibiotics, nature has been the predominant source of anti-microbial compounds or at least served as a source of initial lead compounds. Even today, nature provides new antimicrobial compounds that can be used to tackle the shortcomings in our antibiotics library. However, many valuable and intrinsically interesting natural compounds often lack the necessary properties such as bioavailability and drug uptake. Alternatively, some natural examples use a Trojan Horse strategy to smuggle active cargo into the cell using either siderophore conjugates as iron carrying species[5] or peptidic sequences recognized by a specific reporter.[6] We herein consider natural peptide-nucleotide inhibitor of enteric bacteria Microcin C (McC, **1**, Fig. 1) as a lead compound in the development of a new class of antibiotics.

McC functions through a Trojan-Horse mode of action, whereby the peptide moiety is recognized by a YejABEF-peptide transporter which transports the prodrug across the bacterial membrane.[7] Once inside the sensitive cell, McC is metabolized by peptide-deformylase (PDF) and next by several broad specificity aminopeptidases.[8] This causes the active compound (i.e., aspartyl-phosphoramidate-adenosine, (**2**)) to be released. This product resembles Asp-AMP (**3**), the activated aspartic-acid that normally would be esterified to the 3′-hydroxyl-group of $tRNA^{Asp}$. The processed McC, however, contains a more stable N–P bond which is not hydrolyzed inside the active pocket of AspRS and thus functions as an inhibitor of this enzyme.[9]

The production of McC is catalyzed by several enzymes encoded in the same operon that codes for the McC heptapeptide.[10] The attachment of the heptapeptide chain to adenosine via the phosphoramidate linker can only be achieved if the C-terminal amino acid is asparagine, which is indeed encoded by the last codon of the *mccA* gene coding for the heptapetide. In view of the mechanism of formation of the phosphoramidate bond, no other amino acid at the seventh position of the McC-peptide would allow for further maturation into a functional McC.[11] Upon activation and coupling of the peptide chain, the C-terminal asparagine is hereby modified into aspartic acid.[12] In analogy with the strong inhibitory properties of **2** on AspRS, it is well known that aminoacyl-sulfamoyl-adenosine compounds can also target different aaRSs, but these compounds suffer from poor bioavailability. Therefore, the attachment of an uptake enhancer, such as the hexapeptide unit of McC, could improve their potency and would allow targeting virtually any aaRS.

In earlier work three McC-analogs were synthesized which contained different amino acids at the seventh position of the

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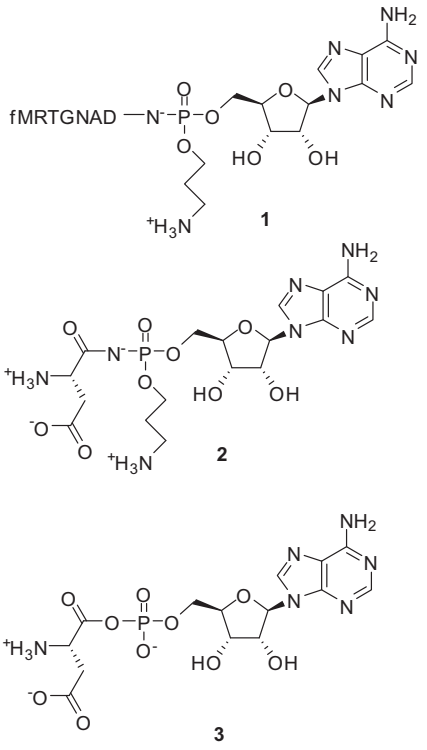


Figure 1. Chemical structures related to the natural product: McC (**1**) in unprocessed form; metabolized McC (**2**) in its active form as bioisoster of the natural AspRS intermediate aspartyl-adenylate (**3**).

McC-analog (i.e., XD-SA, XE-SA, XL-SA, whereby X stands for the N-terminal hexapeptide part of McC; D, E, and L for aspartic acid, glutamic acid and leucine respectively; and SA stands for 5′-*O*-sulfamoyl-adenosine).[13] These compounds lacked a N-terminal formyl group. All three proved to be active and work via the same mechanism as natural McC, which is through removal of the hexapeptide part following transporter mediated uptake and concomitant inhibition of the respective tRNA synthetase by the metabolite. Following these results, we now report on the development of a new synthetic approach to improve the synthetic yield of the synthesis and to extend the range of different McC-like compounds that could be synthesized. This improved procedure is used to generate and characterize a new series of McC analogs targeting various aaRSs and to further characterize the requirements for efficient transport by the YejABEF transporter.

2. Results

2.1. Design and synthesis of McC analogs

aaSAs were created in analogy to previously published methodology[13] with the modification that orthogonal protecting groups were used for the side chains with respect to the alpha-amine of the respective amino acids (see Scheme 1). Amino acylated SAs were prepared by condensation of either L-*N*-benzyloxycarbonyl-aminoacyl(*O*-*tert*-butyl or *tert*-butylox ycarbonyl)]-*O*-succinimide analogs with the adenosine derivative **4** using DBU in DMF. The Z-group of the resulting products (**5a** and **5e**) was next removed by hydrogenation. The aaSAs containing an amino acid not requiring protection of its side chain (**5b**–**d** and **5f**–**g**) were prepared by condensation of the respective L-*N*-*tert*-butyloxycarbonyl-aminoacyl]-*O*-succinimide precursors with **4**. The Boc-group was removed by acidolysis and the TBDMS-protecting groups of all compounds were removed using $Et_3N{\cdot}3HF$. This strategy allowed for the synthesis of aaSAs 7**a**–**g** only protected at the amino acid side chain.

The hexapeptide was assembled on a super-acid-sensitive resin, allowing the use of moderately acid-sensitive protecting groups. The fully protected, formylated hexapeptide (fM-R(Pbf)-T(tBu)-G-N(Trt)-A) was then coupled to the partially protected aaSAs using standard peptide-synthesis reagents such as DIC, HOBt, and DIPEA. The obtained products **8a**–**g** were subsequently deprotected yielding the respective McC-analogs (**9a–e**).

Following the here described methodology, a new set of analogs was created, in order to further extend the synthesis and evaluation of McC-analogs carrying different amino acids at the seventh position. These compounds carried either an aspartic acid (**9a**), alanine (**9b**), leucine (**9c**), glycine (**9d**), lysine (**9e**), isoleucine (**9f**) or valine (**9g**) as the target-determining amino acid. With these analogs also an N-terminal formyl group was included as it was found to improve the recognition by the YejABEF-transporter.

Identity and purity of the final heptapeptidic compounds **9a–g** was established by 1D and 2D NMR and MS/MS analysis. A combination of 2D HSQC–TOCSY and HMBC experiments allowed unambiguous assignment of all signals and confirmed the amino acid sequence of the peptide chain, which was further corroborated by MS/MS analysis.

2.2. Antibacterial activity of McC analogs

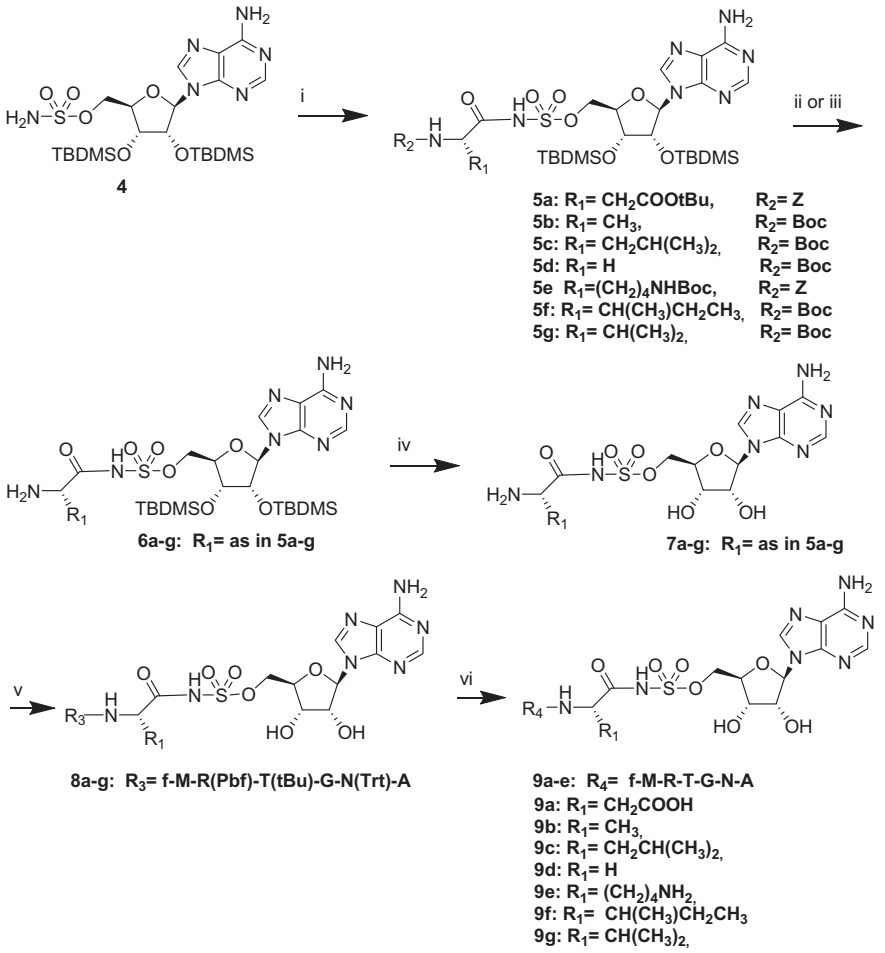
The growth inhibitory properties on McC-sensitive *Escherichia coli* were determined for all new compounds by measuring the optical density reached by identical cell cultures in wells of microtiter plates in the presence of various concentrations of the respective inhibitors.

As shown earlier, the intracellular target is determined by the *C*-terminal amino acid, which remains attached onto the sulfamoyl-adenosine following intracellular metabolization.[13] To facilitate the activity evaluation and the mechanism of action studies of newly synthesized compounds, an *E.coli* tester strain Ara-Yej (BW39758) was used, where the genomic yejABEF operon is under control of the arabinose-inducible araBAD promoter. In the presence of L-arabinose higher amounts of Yej-transporters will be displayed at the inner-membrane. The wild-type *E. coli* K-12 BW28357 and the Ara-Yej strain were either grown in LB-medium with or without 5 mM (L)-arabinose. The antibacterial activities of the various McC-analogs (**9a**–**g**) were determined by monitoring the optical density of suspensions of cell-cultures. In parallel, *E. coli* with disrupted *yej*-, or *pepA,B,N*-genes, grown on M63 agar-plates were used to confirm the McC mode of action.

As can be seen in Figure 2, compounds **1** (reference), **9a**, **9b**, and **9e** displayed activity at concentrations $\leq$0.63 μM when challenged with Ara-Yej cells cultured in arabinose containing medium. In contrast to **9b**, the more hydrophobic compounds **9c, 9d, 9f**, and **9g** showed reduced activity. *E. coli* cells with a disrupted *yej* gene (with concomitant loss of the active transport modality) and cells without functional aminopeptidases A, B and N (with loss of formation of the active principle) proved resistant to these compounds, indicating the same mode of action as McC.

3. Discussion

We previously suggested that deviations from the native McC-structure could be quite extensive.[13] This is further exemplified here with McC analogs affording different intracellular active compounds upon metabolization. Even if more extensive alterations were incorporated, such as a lysine as the target determining amino acid, activities were in the same range as observed for native McC. However, the potency of the compounds **9c, 9d, 9f**, and **9g** was lower in comparison to **9a**, **9b**, and **9e**. The reduced



Scheme 1. Reagents and conditions: General scheme affording the various McC analogs with R_4 being the formylated hexapeptide. (i) *N*-α-CBZ-L-aminoacyl-(*t*Bu or Boc)-succinimide, DBU in DMF, 6 h, rt. (ii) For R_2 = Z-group, H_2, Pd/C in MeOH, 3 h, rt. (iii) For R_2 = Boc-group of TFA/H_2O (5:2), 4 h, 0 °C to rt. (iv) $Et_3N{\cdot}3HF$ in THF, 16 h, rt. (v) Protected peptide (1 equiv), HOBt (4 equiv), DIC(4 equiv) and DIEA(2 equiv) in DMF, 16 h, rt. R_3 may be either Boc-, or formyl-group. (vi) TFA/thioanisole/H_2O (90/2.5/7.5), 2 h, rt.

activity for compounds with a more hydrophobic amino acid at the C-terminal end is remarkable, as M Brown et al. found a K_i of 0.01 nM of I-SA for IleRS.[14] Therefore the relatively low activity of the compound fXI-SA (**9f**) must be attributed to either uptake or metabolization issues. As shown in Figure 2 (graph **9f**), the strain with upregulated Yej-transporters is more sensitive to this compound. Therefore it may be concluded that the Yej-transporter discriminates between the constituencies of the peptide chains. The polarity may be a decisive factor since compounds containing amino acids at the seventh position with more aliphatic side chains display lower activities in comparison to compounds containing aspartic acid (**9a**) or lysine (**9e**), with the alanine derivative **9b** as a notable exception. However, compound **9c** containing leucine also showed a five-fold higher potency compared to **9f**. Therefore it may be concluded that not only the polarity, but also more specific recognition by the transporter is of importance for uptake of these compounds, as processing of the different analogs to the respective active metabolites is straightforward. This was shown before for the glycine analog **9d** as well as for several shortened compounds which all proved active in an in vitro assay, where in presence of cellular extracts tRNA aminoacylation was inhibited. Hence, metabolization is a prerequisite, but not an issue for the activity of the different congeners.[15] Neither is amino acid usage per se a decisive factor in the activity profile, as many McC variants obtained by mutagenesis proved active.[11] The latter methodology however, did not allow for analogs with a different amino acid at the seventh position, targeting different tRNA synthetases.

The synthetic scheme as presented here is a significant improvement upon the earlier reported synthesis with up to 15-fold improved overall yields. The new methodology allows for the preparation of sufficient amounts of different McC analogs, allowing further studies on the biology of this fascinating class of compounds and enabling the design of further improved analogs. Following this method, new compounds could be created that may not only inhibit other aaRSs, but even other targets. Furthermore, compounds that suffer from low bioavailability could be improved following the proposed synthetic scheme in order to turn these potential drugs in Trojan Horse-like compounds.

4. Materials and methods

4.1. Chemistry

General: Reagents and solvents were purchased from commercial suppliers (Acros, Sigma–Aldrich, Bachem, Novabiochem) and used as provided, unless indicated otherwise. DMF and THF were 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

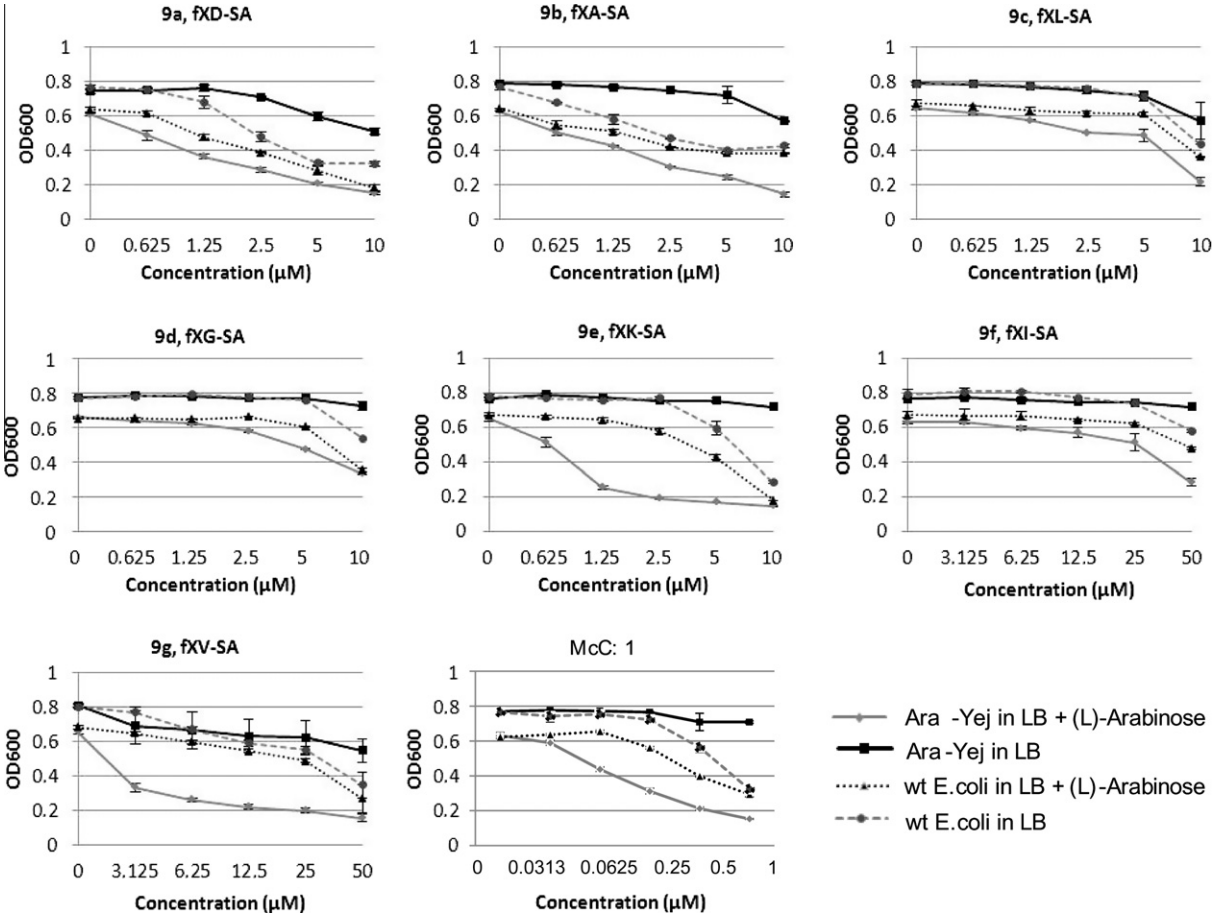


Figure 2. In vitro screening of McC analogs. Cell growth of the wild-type *E. coli* and of the Ara-Yej strain is shown in presence of different concentrations of **9a–g** and the reference compound **1** with or without arabinose induction.

provided. Reactions were carried out in oven-dried glassware under a nitrogen atmosphere and stirred at room temperature, unless indicated otherwise.

Standard ^{1}H and ^{13}C NMR spectra of the compounds were recorded on a Bruker UltraShield Avance 300 MHz or 500 MHz spectrometer. Spectra were recorded in DMSO-d_6 or D_2O. The chemical shifts are expressed as δ values in parts per million (ppm), using the residual solvent peaks (DMSO: ^{1}H, 2.50 ppm; ^{13}C, 39.60 ppm; HOD: ^{1}H, 4.79 ppm with dioxane added for ^{13}C, 67.19 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 = multiple, 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/H_2O (1:1) at 3 μL min^{-1}. High resolution NMR spectra of the final compounds **9a–g** were recorded on a Bruker Avance II 600 with a gradient cryoprobe. Methodology and 1D and 2D NMR spectra for analysis of the different analogs **9a–g** are included in the Supplementary data.

For TLC, precoated aluminium sheets were used (Merck, Silica gel 60 F_{254}). The spots were visualized by UV light. Column chromatography was performed on ICN silica gel 60A 60–200. For size exclusion chromatography, a 2 × 30 cm column of Sephadex LH-20 was used as the solid phase and MeOH/H_2O (7:3 v/v) as the eluent. 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 100 Å column connected to a Merck-Hitachi L6200A Intelligent pump. Eluent compositions are expressed as v/v.

4.1.1. Synthesis of 5′-*O*-[*N*-[L-Aspartyl(*O*-*tert*-butyl)]-sulfamoyl] adenosine (7a)

A solution of Z-Aspartyl(*O*-*tert*-butyl)-*O*-Su (1 g, 2.38 mmol, 1.0 equiv), 5′-*O*-sulfamoyl-2′,3′-di-*O*-(*tert*-butyldimethylsilyl)-adenosine (**4**) (1.23 g, 2.14 mmol, 0.9 equiv) and DBU (356 μL, 2.38 mmol, 1.0 equiv) in DMF (7 mL) was stirred at rt for 8 h under nitrogen atmosphere. DMF was evaporated under reduced pressure. Next, the reaction mixture was purified by flash chromatography (CH_2Cl_2, 1% Et_3N, 2.5–10% MeOH). Fractions containing the desired product were evaporated giving a yellow oil (**5a**). Yield: 1.7 g (90%). ESI-MS [M+H^+]: $C_{38}H_{62}N_7O_{11}SSi_2$ Calculated: 880.4 Found: 880.1

The obtained product **5a** (1.7 g) was dissolved in MeOH at 0 °C and Pd/C (0.17 g) was added. The solution was stirred under hydrogen atmosphere for 3 h. The mixture was filtered and evaporated yielding a white–yellow foam (**6a**). Yield: 1.1 g (76%). The product **6a** (1.1 g) was carefully dried and dissolved in THF (15 mL) and $Et_3N{\cdot}3HF$ (1 mL). After 3 h, another 0.8 mL of $Et_3N{\cdot}3HF$ was added and the reaction mixture was stirred for another 22 h. The reaction mixture was evaporated and the reaction mixture was purified by flash chromatography (CH_2Cl_2, 5–20% MeOH). Fractions containing the desired product were evaporated, yielding **7a**: 670 mg (88%) ^{1}H NMR (D_2O): 1.04–1.09 (t, 9H, Et_3N, J = 7.16), 1.44 (s, 9H, *t*Bu), 2.67–2.75 (q, 6H,Et_3N CH_2, J = 7.12), 3.68–3.72 (t, 2H, $Asp_{\alpha}H$,

J = 5.70), 4.12–4.19 (m, 4H, 3′, 4′, 5′), 4.63–4.67 (t, 1H, 2′H, *J* = 5.36), 5.94–5.96 (d, 1H, 1′, *J* = 5.82), 7.31 (s, 1H, NH_2), 8.18 (s, 1H, 2H), 8.41 (s, 1H, 8H).

^{13}C NMR (D_2O): 27.02 (*t*Bu-CH_3), 35.71 (Asp-β-C), 51.67 (Asp-α-C), 68.14 (5′-C), 70.08 (3′-C), 74.14 (2′-C), 82.05 (4′-C), 83.88 (*t*Bu-C), 87.37 (1′-C), 118.06 (5-C), 139.49 (8-C), 148.31(4-C), 152.19 (2-C), 154.74 (6-C), 170.50 and 173.81 (Asp- C═O).

ESI-MS calcd for $C_{18}H_{26}N_7O_9S$ $[M-H]^-$: 516.2; found: 515.8.

4.1.2. Synthesis of 5′-*O*-[*N*-[L-alanyl]-sulfamoyl] adenosine (7b)

A solution of Boc-Alanyl-O-Su (1 g, 3.5 mmol, 1.0 equiv), compound **4** (1.8 g, 3.2 mmol, 0.9 equiv) and DBU (450 μL, 3 mmol, 0.9 equiv) in DMF (7 mL) was stirred at rt for 8 h under nitrogen atmosphere. DMF was evaporated under reduced pressure. Next, the reaction mixture was purified by flash chromatography (CH_2Cl_2, 1% Et_3N, 2.5–10% MeOH). Fractions containing the desired product were evaporated giving a yellow oil (5b). Yield: 2.09 g (90%). Compound **5b** was next treated with TFA/H_2O (5/2 v/v) for 5 h at rt, after which the volatiles were evaporated to yield the product **6b**. Compound **6b** was carefully dried and dissolved in THF (15 mL) and $Et_3N{\cdot}3HF$ (1 mL). After 3 h, another 0.8 mL of $Et_3N{\cdot}3HF$ was added and the reaction mixture was stirred further for 22 h. The reaction mixture was evaporated and the residue was purified by flash chromatography (CH_2Cl_2, 5–20% MeOH). Fractions containing the desired product **7b** were evaporated. Yield: 710 mg (63%)

^{1}H NMR (D_2O): 1.22–1.27 (t, 9H, Et_3N-CH_3, *J* = 7.34), 1.43–1.45 (d, 3H, Ala-β-H, *J* = 7.2), 2.61–2.68 (q, 6H, Et_3N–CH_2, *J* = 7.35), 3.45–3.52 (q, 1H, Ala-α-H, *J* = 7.19), 4.35–4.41 (m, 4H, 3′, 4′, 5′,), 4.45–4.48 (t, 1H, 2′-H, *J* = 4.7), 6.08–6.10 (d, 1H, 1′-H, *J* = 6.0), 7.28 (bs, 2H, NH_2, exchangable with D_2O), 8.22 (s, 1H, 2H), 8.36 (s, 1H, 8H).

^{13}C NMR (D_2O): 17.07 (Ala-β-C), 52.01 (Ala-α-C), 68.89 (5′-C), 70.75 (3′-C), 74.71 (2′-C), 82.88 (4′-C), 88.03 (1′-C), 119.17 (5-C), 140.36 (8-C), 149.45 (4-C), 153.11 (2-C), 156.00(6-C), 177.12 (Asp- C═O).

ESI-MS calcd for $C_{13}H_{18}N_7O_7S$ $[M-H]^-$: 416.4; found: 416.2.

4.1.3. Synthesis 5′-*O*-[*N*-[L-leucyl]-sulfamoyl] adenosine (7c)

This compound was synthesized analogously to compound **7b**.

^{1}H NMR (D_2O): 0.87–0.89 (d, 3H, Leu-δ, *J* = 6), 0.89–0.90 (d, 3H, Leu-δ, *J* = 6), 1.64–1.73 (m, 3H, Leu-γ, β-H), 3.75–3.76(m, 1H, Leu-α-H), 4.42–4.45 (m, 3H, 3′„5′), 4.50–4.52 (t. 1H, 4′-H, *J* = 4.8) 4.74-4.76 (t, 1H, 2′-H, *J* = 4.8), 6.15–6.16 (d, 1H, 1′-H, *J* = 5.31), 8.42 (s, 1H, 2H), 8.51 (s, 1H, 8H).

^{13}C NMR (D_2O): 20.3 (Leu-δB-CH_3), 21.45 (Leu-δA-CH_3), 23.51 (Leu-γ-CH), 39.46 (Leu-β-CH2), 53.25 (Leu-α-CH), 68.67 (5′-C), 69.49 (3′-C), 73.76 (2′-C), 81.88 (4′-C), 88.02 (1′-C), 118.29 (5-C), 142.06 (8-C), 144.17 (4-C), 147.91 (2-C), 149.50 (6-C), 174.14 (Leu- C═O).

ESI-MS calcd for $C_{16}H_{26}N_7O_7S$ $[M+H]^+$: 460.48; found: 460.16.

4.1.4. Synthesis of 5′-*O*-[*N*-[L-glycyl]-sulfamoyl] adenosine (7d)

This compound was synthesized analogously to compound **7b**.

^{1}H NMR (D_2O): 3.70 (s, 2H, α-H), 4.41–4.50 (m, 4H, 3′,4′,5′), 4.69–4.73 (t, 1H, 2′-H, *J* = 4.6), 6.12–6.14 (d, 1H, 1′-H, *J* = 5.31), 8.15 (s, 1H, 2H), 8.34 (s, 1H, 8H).

^{13}C NMR (D_2O): 42.72 (Gly- α-C), 68.11 (5′-C), 70.06 (3′-C), 74.06 (2′-C), 82.15 (4′-C), 87.35 (1′-C), 118.43 (5-C), 139.54 (8-C), 148.73 (4-C), 152.37 (2-C), 156.15 (6-C), 172.69 (Gly –C═O).

ESI-MS calcd for $C_{12}H_{16}N_7O_7S$ $[M-H]^-$: 402.37; found: 402.31.

4.1.5. Synthesis of 5′-*O*-[*N*-[L-lysyl(*N*-*tert*-butyloxycarbonyl)]-sulfamoyl]adenosine (7e)

This compound was synthesized analogously to compound **7a**.

^{1}H NMR (D_2O): 1.26–1.44 (m, Lys-γ and δ), 1.38 (s, 9H, *t*Bu), 1.77–1.84 (m, Lys-β), 2.87 (m, 2H, Lys-ε), 3.75–3.79 (t, 2H, Lys-α, *J* = 5.77), 4.41–4.46 (m, 4H, 3′,4′,5′), 4.50–4.53 (t, 1H, 2′, *J* = 4.26), 6.12–6.14 (d, 1H, *J* = 5.7), 8.26 (s, 1H, 2H), 8.43 (s, 1H, 8H)

^{13}C NMR (D_2O): 21.89 (Lys-γ), 28.26 (*t*Bu-CH_3), 29.12 (Lys-δ), 31.14 (Lys-β), 40.15 (Lys-ε), 56.03 (Lys-α), 68.97 (5′-C), 70.76 (3′-C), 74.73 (2′-C), 82.87 (4′-C), 87.69 (1′-C), 119.57 (5-C), 140.39 (8-C), 149.68 (4-C), 153.57 (2-C), 156.23 (6-C), 158.77 (Boc –C–O), 176.31 (Lys –C═O).

ESI-MS calcd for $C_{21}H_{35}N_8O_9S$ $[M+H]^+$: 575.22; found: 575.00.

4.1.6. Synthesis of 5′-*O*-[*N*-[L-isoleucyl]-sulfamoyl]adenosine (7f)

This compound was synthesized analogously to compound **7b**.

^{1}H NMR (D_2O): 0.84–0.89 (t, 3H, Ile-δ, *J* = 7.38), 0.97–0.99 (d, 3H, Ile-γ′-H, *J* = 7.02), 1.1–1.6 (m, 2H, Ile-$\gamma_{A/B}$-H), 1.9–2,1 (m, 1H, Ile-β-H), 3.75–3.76(d, 1H, Ile-α-H), 4.41–4.50 (m, 4H, 3′,4′,5′), 4.54–4.57 (t, 1H, 2′-H, *J* = 4.6), 6.12–6.14 (d, 1H, 1′-H, *J* = 5.31), 8.24 (s, 1H, 2H), 8.41 (s, 1H, 8H).

^{13}C NMR (D_2O): 11.5 (, Ile-δ-CH_3), 15.1 (Ile-γ′-CH_3), 24.7 (Ile-γ-CH_2), 36.9 (Ile-β-CH), 60.7 (Ile-α-CH), 68.9 (5′-C), 70.79 (3′-C), 74.69 (2′-C), 82.94 (4′-C), 87.93 (1′-C), 119.13 (5-C), 140.43 (8-C), 149.47 (4-C), 153.05 (2-C), 155.79 (6-C), 175.61 (Ile- C═O).

HR-MS calcd for $C_{16}H_{24}N_7O_9S_1$ $[M-H]^-$: 458.1548; found: 458.1450.

4.1.7. Synthesis of 5′-*O*-[*N*-[L-valyl]-sulfamoyl] adenosine (7g)

This compound was synthesized analogously to compound **7b**.

^{1}H NMR (D_2O):0.96–0.98 (0.84-0.89 (t, 3H, Ile-δ, *J* = 7.38), 0.97–0.99 (d, 3H, Ile-γ′-H, *J* = 7.02), 1.1–1.6 (m, 2H, Ile-$\gamma_{A/B}$-H), 1.9–2,1 (m, 1H, Ile-β-H), 3.75–3.76(d, 1H, Ile-α-H), 4.41–4.50 (m, 4H, 3′,4′,5′), 4.54–4.57 (t, 1H, 2′-H, *J* = 4.6), 6.12–6.14 (d, 1H, 1′-H, *J* = 5.31), 8.24 (s, 1H, 2H), 8.41 (s, 1H, 8H).

^{13}C NMR (D_2O): 16.94(Val- γ_B-CH_3), 18.56 (Ile-γ_A-CH_3), 30.45 (Val-β-CH), 61.45 (Val-α-CH), 68.91 (5′-C), 70.78 (3′-C), 74.65 (2′-C), 82.95 (4′-C), 87.90 (1′-C), 119.20 (5-C), 140.31 (8-C), 149.54 (4-C), 153.47 (2-C), 156.15 (6-C), 176.38 (Val- C═O).

ESI-MS calcd for $C_{15}H_{22}N_7O_7S$ $[M-H]^-$: 444.45; found: 444.30.

4.1.8. Synthesis of fMRTGNAD-SA (9a)

The peptide Formyl-methionyl-arginyl(2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulfonyl)-threonyl(*t*Bu)-glycyl-asparaginyl (trityl)-alanyl-OH was synthesized on a 2-chlorotrityl chloride resin using standard Fmoc-based solid phase peptide chemistry. The protected peptide was cleaved from the resin using a mixture of HOAc/Trifluoroethanol/DCM (1/1/8, v/v) in 30 min. Following RP-HPLC purification, the peptide (20 mg, 16.13 μmol, 1.0 equiv) and HOBt (9 mg, 64.52 μmol, 4.0 equiv) were dissolved in DMF (500 μL) and DIC (10 μL, 64.52 μmol, 4.0 equiv) was added. This mixture was stirred for 1 h at rt under argon atmosphere. DIPEA (7.5 μL, 40.33 μmol, 2.5 equiv) was added and the mixture was added to the adenosine analog **7a** (16.68 mg, 32.26 μmol, 2.0 equiv) and stirred for 16 h at rt under argon. Next, the volatiles were evaporated and the residue was taken up in a mixture of CH_3CN/water. This was purified on a PoraPak Rxn® column (CH_3CN 25–100% in water). The fractions containing the product were evaporated yielding **8a**. The product **8a** was subsequently deprotected using a mixture of 90% TFA, 7.5% H_2O and 2.5% thioanisole. The volatiles were evaporated and co-evaporated 3 times with toluene (10 mL). The remaining title product (**9a**, fMRTGNAD-SA) was redissolved in H_2O, filtered and purified by RP-HPLC (solvent A: 25 mM TEAB in H_2O; solvent B: 25 mM TEAB in CH_3CN; see Supplementary data for HPLC analysis of all final compounds).

Yield (as calculated over coupling and deprotection): 1.398 mg (13.9%). HR-MS calcd for $C_{39}H_{60}N_{17}O_{18}S_2$ $[M-H]^-$: 1118.3822; found: 1118.3789.

4.1.9. Synthesis of fMRTGNAA-SA (9b)

Further synthesis to obtain **9b** (yield: 2.3 mg (11%)) was performed analoguously to the synthesis of **9a**. HR-MS calcd for $C_{38}H_{62}N_{17}O_{16}S_2$ $[M+H]^+$: 1076.4002; found: 1076.3849.

4.1.10. Synthesis of fMRTGNAL-SA (9c)

Further synthesis to obtain **9c** (yield: 0.7 mg (7%)) was performed analoguously to the synthesis of **9a**. HR-MS calcd for $C_{41}H_{68}N_{17}O_{16}S_2$ $[M+H]^+$: 1118.4471; found: 1118.4482.

4.1.11. Synthesis of fMRTGNAG-SA (9d)

Further synthesis to obtain **9d** (yield: 1.8 mg (8%)) was performed analoguously to the synthesis of **9a**. HR-MS calcd for $C_{37}H_{58}N_{17}O_{16}S_2$ $[M-H]^-$: 1060.3689; found: 1060.3702.

4.1.12. Synthesis of fMRTGNAK-SA (9e)

Further synthesis to obtain **9e** (yield: 1.2 mg (2%)) was performed analoguously to the synthesis of **9a**. HR-MS calcd for $C_{41}H_{67}N_{18}O_{16}S_2$ $[M-H]^-$: 1131.4400; found: 1132.3250.

4.1.13. Synthesis of fMRTGNAI-SA (9f)

Further synthesis to obtain **9f** (yield: 1.2 mg (5.6%)) was performed analoguously to the synthesis of **9a**. HR-MS calcd for $C_{41}H_{66}N_{17}O_{16}S_2$ $[M-H]^-$: 1116.4314; found: 1116.4326.

4.1.14. Synthesis of fMRTGNAV-SA (9g)

Further synthesis to obtain **9g** (yield: 3.8 mg (19%)) was performed analoguously to the synthesis of **9a**. HR-MS calcd for $C_{40}H_{64}N_{17}O_{16}S_2$ $[M-H]^-$: 1102.4158; found: 1102.4165.

4.1.15. Biology

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 to an OD600 of 0.1. Compounds were titrated in a 96 well-plate using either LB-medium with or without 5 mM L-arabinose. To each well 85 μL LB-medium with or without 5 mM L-arabinose was added to a total volume of 90 μL followed by 10 μL of bacterial cell culture. The cultures were next incubated at 37 °C and the OD600 was determined after 8 h.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.052.

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