Simplified Novel Muraymycin Analogues; using a Serine Template Strategy for Linking Key Pharmacophores

Bhautikkumar Patel,[a,b,c] Rachel V Kerr,[d] Dr Alpeshkumar K Malde,[e,f] Dr Matthew Zunk,[a,b,c] Prof Timothy DH Bugg,[d] A/Prof Gary Grant,[a,b,c] and Dr Santosh Rudrawar*[a,b,c]

Abstract: The present status of antibiotic research requires an urgent invention of novel agents that act on multidrug resistant bacteria. The World Health Organization (WHO) has classified antibiotic-resistant priority bacterium into critical, high and medium priority according to the urgency of need for new antibiotics. Naturally occurring uridine-derived 'nucleoside antibiotics' have shown promising activity against numerous priority resistant organisms *via* inhibition of the transmembrane protein MraY (translocase I), which is yet to be explored in a clinical context. The catalytic activity of MraY is an essential process for bacterial cell viability and growth including that of priority organisms. Muraymycins are one subclass of naturally occurring MraY inhibitors. Despite having potent antibiotic properties, the structural complexity of muraymycins advocates for simplified analogues as potential lead structures. Herein we report the systematic structure-activity relationship (SAR) study of serine template linked simplified muraymycin-type analogues. This preliminary SAR lead study of serine template analogues has successfully revealed that the complex structure of naturally occurring muraymycins could be easily simplified to afford bioactive scaffolds against resistant priority organisms. This study will pave the way for the development of novel antibacterial lead compounds based on a simplified serine template.

Introduction

Bacterial resistance against clinically used antibiotics is an emerging health concern in contemporary healthcare.[1,2] To address growing global resistance, the World Health Organization (WHO) has published a list of antibiotic-resistant priority pathogens.[3] The major objective of publishing this list was to guide and promote research and development (R&D) of new unexplored classes of antibiotics aimed at treating multidrug resistant pathogens.[4] Ideally, these novel antibiotics should target clinically unexploited modes of action to avoid cross resistance with existing drugs. Bacterial cell-wall peptidoglycan layer biosynthesis has been a promising antibacterial target for decades led by the discovery of β-lactam (penicillin) and glycopeptide (vancomycin) classes of antibiotics.[5] However, these cell wall inhibitors target the late extracellular steps of peptidoglycan synthesis. The early intracellular steps of peptidoglycan synthesis are not well explored in clinical context. Therefore development of antibacterial agents targeting enzymes involved in these initial stages could provide an exciting opportunity to meet the current need for novel targets.[6] The transmembrane enzyme MraY (phospho-*N*-acetylmuramoyl-pentapeptide-transferase; translocase I) is one such enzyme with its active site located on the cytosolic side of the membrane, which fulfils the requirement of a novel target.[7] MraY catalyses the first membrane-associated step of peptidoglycan formation which involves transfer of an UDP-*N*-acetylmuramoyl (UDP-MurNAc)-pentapeptide (-L-Ala-γ-D-Glu-diaminopimelic acid/L-Lys-D-Ala-D-Ala) (Park's nucleotide) to the lipid carrier [C_{55} isoprenoid phosphate (C_{55}-P)/C_{50} decaprenyl phosphate (C_{50}-P)], resulting in the formation of lipid I (undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide) (Figure **1**).[8]

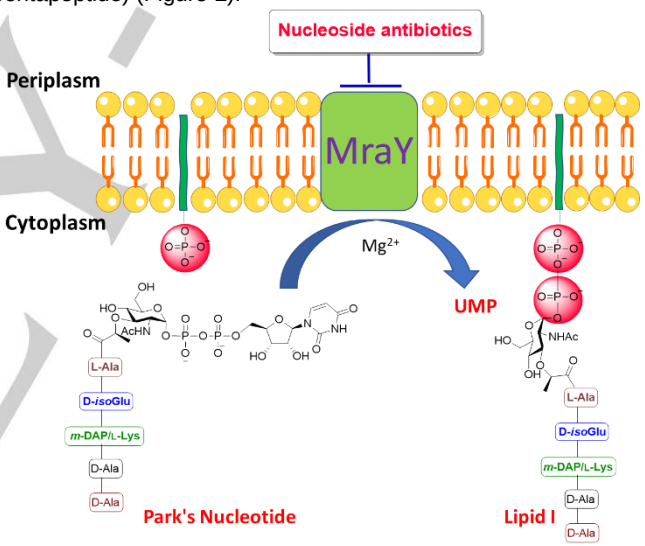


Figure 1. Reaction catalysed by the bacterial transmembrane enzyme MraY.[8]

The MraY enzyme (translocase I) has been identified as the target of bacterial nucleoside antibiotics, a class of bacterial derived natural products containing a nucleoside core structure.[9,10] Due to their promising antibacterial activity against a broad spectrum of Gram-positive bacteria, nucleoside antibiotics have been considered as promising lead structures for the development of novel antibacterial agents. Based on their structural characteristics, nucleoside antibiotics have been classified into four classes: *i*) ribosamino-uridines, *ii*) uridylpeptides, *iii*) tunicamycins, and *iv*) capuramycin.[11,12] Muraymycins belong to the family of ribosamino-uridine type nucleoside antibiotics, which were first discovered as a collection of 19 structurally related secondary metabolites from a broth of a *Streptomyces* sp. in 2002.[13] More recently novel members of the muraymycins class of natural products have been isolated from the same bacterial strains.[14] Muraymycin A1 has been reported active against Gram-positive *Staphylococcus* (MIC: 2-16 μg/mL), *Enterococcus* (MIC: 16-64 μg/mL), as well as Gram-negative *E. coli* (MIC: <0.03 μg/mL).[15]

[a] Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD 4222, Australia.
[b] School of Pharmacy and Pharmacology, Griffith University, Gold Coast, QLD 4222, Australia. E-mail: s.rudrawar@griffith.edu.au
[c] Quality Use of Medicines Network, Griffith University, Gold Coast, QLD 4222, Australia.
[d] Department of Chemistry, University of Warwick Coventry CV4 7AL, United Kingdom
[e] Institute for Glycomics, Griffith University, Gold Coast, QLD 4222, Australia.
[f] MaldE Scientific, https://maldescientific.com, Australia

In general, the muraymycin scaffold contains a (5′*S*, 6′*S*)-glycyluridine (GlyU) core structure linked to a urea peptide moiety by an alkyl linker.[16] The urea peptide contains the non-proteinogenic amino acid epicapreomycidine derived from a cyclic arginine derivative. Based on the structural diversity of the central L-leucine (Leu) unit of the peptide moiety, muraymycins have been classified into four sub-classes, being series A to D (Figure **2**).[17] Subclasses A, B and C contain a (3*S*)-3-hydroxyl-L-leucine, which are further distinguished between each other by varying substitutions at the 3-hydroxyl position. Series A muraymycins (e.g., A4 **1**) are 3-*O*-acylated with ω-functionalised fatty acids terminating with a guanidinium moiety. While series B muraymycins (e.g. B5 **2**) are 3-*O*-acylated with unfunctionalised terminally branched fatty acids. The C-type muraymycins (e.g. C2 **3**) are not *O*-acylated at the 3-hydroxy-L-leucine, and the D-series of muraymycin D2 **4**) contain proteinogenic non-hydroxylated L-leucine at this position (Figure **2**).

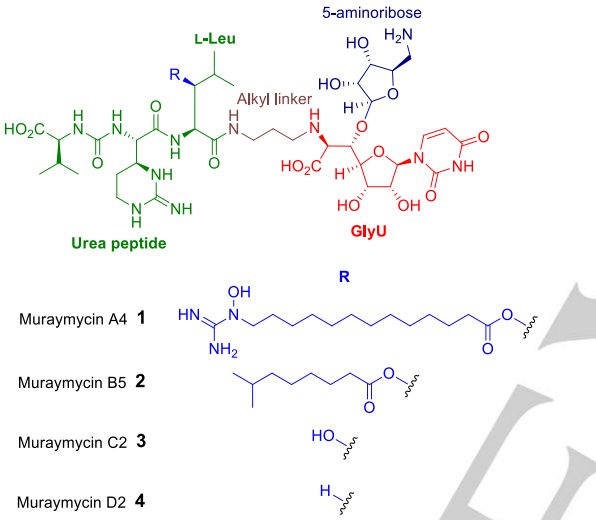


Figure 2. Chemical diversity of naturally occurring muraymycin sub-classes A-D

A significant challenge in order to create drug-like compounds is muracycin's complex and synthetically challenging structure.[18a-f,19,20a-b] To date, two approaches are reported in the literature towards the total synthesis of naturally occurring muraymycins.[18a,b] Tanino et al. reported the first total synthesis of muraymycin D2 in total 18 steps with a 4.3% overall yield.[18b] While the second report for stereo-controlled total synthesis of muraymycin D1 was published by Kurosu et al. in 19 steps with overall yield of only 15.9%.[18a] Moreover, Kurosu et al. evaluated muraymycin D1 and its amide derivatives for antibacterial activity.[18a] These muraymycin analogues exhibited growth inhibitory activity against *Mycobacterium tuberculosis* (MIC_{50} 1.56-6.25 μg/mL) and strong enzyme inhibitory activities against the bacterial phosphotransferases (MurX and WecA) (IC_{50} = 0.096-0.69 μM).[18a] Furthermore, Kurosu et al. created an anticancer DPAGT1 inhibitor from a muraymycin biosynthetic intermediate.[18c] Another group of scientists from Wyeth also worked on the semisynthetic access and have synthesised a total of 16 derivatives of muraymycin C1.[18d] Structure-activity relationship (SAR) studies of these synthesised analogues suggested that the mono-substituted derivatives demonstrated good inhibitory activity against MraY or MurG (6.25 μg/mL), which importantly, correlated with the lipophilicity of the substituents introduced.[18d] Kristin et al. reported solid phase-based synthetic strategy for accessing muraymycin analogues.[18e] Recently, Anna et al. explored variations in the nucleoside unit of muraymycin antibiotics.[18f]

To address this synthetic complexity of muraymycin-based natural products, the prime goal of our research is to investigate bioactive, structurally simplified muraymycin analogues, which are active against a range of bacterial strains. Herein, we describe the full details of our efforts to simplify the complex structure of muraymycins based on a serine template strategy for linking key pharmacophores. This study includes a preliminary SAR for our serine template linked simplified muraymycin analogues toward discovery of novel structures effective against a range of drug-resistant bacterial strains.

Results and Discussion

Design

To achieve the primary aim of synthesising simplified analogues of muraymycins, it was essential to understand the structural orientation of the key pharmacophores present in the muraymycins. To start, key interactions reported for the co-crystal structure of MraY (*Aquifex aeolicus*) in complex with the muraymycin D2 **4** (Figure **3**), were investigated.[21] This crystallographic study revealed that the 5″-aminoribosyl and uracil moieties of muraymycin D2 (MD2) bind to the nucleoside-binding pocket of MraY like a two-pronged electrical plug inserted into a socket.[21] Moreover, these interactions were found to be the most critical for binding of muraymycin D2 with the MraY enzyme.[21] Specifically, the 5″-amino group of the aminoribose moiety interacts with Asp-193 through a hydrogen bond and an electrostatic interaction.[21] The importance of the aminoribose moiety has been substantiated. A 5'-defunctionalised (5'-deoxy) muraymycin analogue was essentially inactive.[22] Another very specific key interaction found was for the uracil nucleobase, which binds in a neighboring pocket interacting with Asn-255, Asp-196, Lys-70 through hydrogen bonding as well as with Phe-262 *via* π-stacking.[21] These crystallographic findings were found to be in line with reported literature while evaluating the core aminoribosylated glycyluridine (GlyU) against MraY (from *Escherichia coli*).[23] It was found that the core aminoribosylated glycyluridine unit exhibit significant anti-MraY activity (IC_{50} = 50 μM), reflecting the minimal structural requirement responsible for antibacterial activity against MraY (from *Escherichia coli*).[23] After having sufficient literature reports supporting the aminoribose and uridine moieties as an essential structural requirement for interaction in the active site of MraY,[21-23] it was decided to retain both the pharmacophores within our intended simplified muraymycin scaffold.

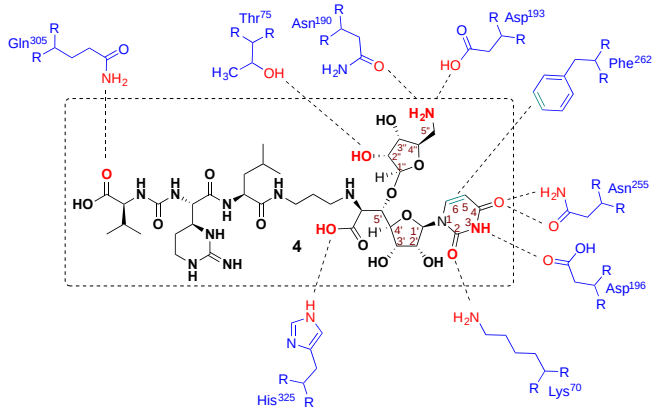


Figure 3. Key interactions of muraymycin D2 with the MraY enzyme (PDB: 5CKR).[21]

Next, regarding substitutions on the core pharmacophore, the literature reports that there is strong hydrophobic interaction between muraymycin D1 and the MraY hydrophobic tunnel.[24] The significant antibacterial activity of lipophilic side chain containing molecules suggests that the side chain itself might be contributing to the membrane permeability of the molecules as MraY is an integral membrane protein and its active site exists on the cytoplasmic side of the membrane.[24] As the peptidoglycan lipid bilayer is a common barrier for bacteria, incorporating a lipophilic group appears necessary in simplified analogues to afford any antibacterial activity.[24] Following the trend reported for inclusion of a lipophilic side chain,[24] it was decided to retain this characteristic while designing our simplified muraymycin scaffold.

In this work, the principle objective was to link key pharmacophores with a simplified linker to afford rapid access to biologically active muraymycins analogues. Having encountered the significant contribution of the 5″-aminoribose,[21-23] uridine[21,23] and lipophilic side chain,[24] we turned our attention to link these pharmacophores together to achieve bioactive muraymycins. Naturally occurring muraymycins contain a 5′-glycyluridine core unit with specific stereochemistry to hold key pharmacophores in a specific orientation. Synthetic access to this 5′-glycyluridine (GlyU) core unit is not trivial and involves multi-step challenging synthetic routes.[25] Henceforth, to achieve the prime objective of chemically simplified muraymycins analogues, we have selected a serine template as a linker to which the desired key pharmacophores can be linked *via* conventional amide-coupling methodologies (**5**, Figure **4**). Additionally, we also aimed to vary the lipophilic side chain by investigating the linear carbon chain length to achieve optimised antibacterial activity. Moreover, as revealed in the X-ray co-crystal structure,[21] orientation of both 5″-aminoribosyl and uracil moieties of muraymycin is crucial for binding with the MraY enzyme. So, in this proof-of-principle study, we aimed to investigate the stereochemical outcome of the proposed serine template by using both enantiomers L and D-serine amino acids as starting points, while performing total synthesis of serine template analogues.

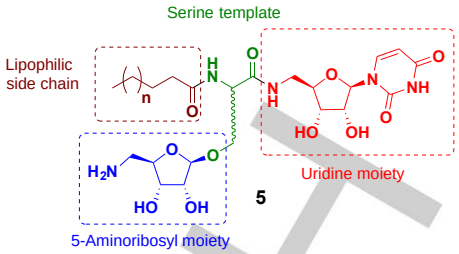


Figure 4. The proposed design of our simplified serine template-based muraymycin analogous.

Chemistry

Investigation of the serine template basic scaffold

The SAR study of the serine template linked truncated muraymycins analogues was initiated by synthesis and biological evaluation of the core serine template (L-serine) linked structure **6**, comprising uridine, 5′-aminoribose and octanoic acid as key pharmacophores. This core serine template linked molecule **6** could be synthesised from their synthetic equivalents, including the isopropylidene protected 5′-amino uridine **7**, the ribose building block **8** (glycosyl donor) and the serine template (glycosyl acceptor) **13** as described in retrosynthetic analysis (Figure **5**).

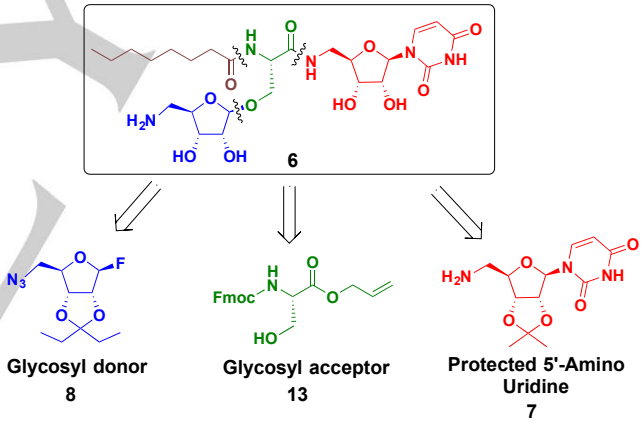


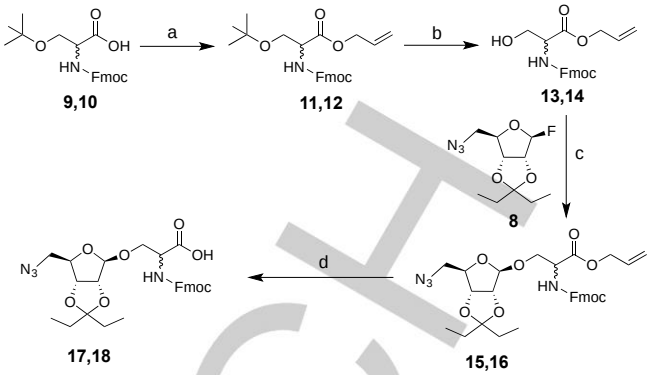
Figure 5. The retrosynthetic analysis of core serine template (L-serine) linked structure **6**.

Synthesis of the serine template based muraymycin analogue **6** was started by independently synthesising each of building blocks *via* modified literature reported methods. The isopropylidene protected 5′-amino uridine **7** was synthesised from commercially available uridine in four synthetic steps in quantitative yields (58%).[26] After protection of the secondary hydroxyl groups (2′,3′-diol) of uridine with isopropylidene unit, the primary hydroxyl group (5′-hydroxyl) was activated through addition of a *p*-toluenesulfonyl ester to produce a tosylate product. Subsequent nucleophilic substitution of the tosyl group with NaN_3 gave the azido derivative in high yield (95%). The building block **7** was prepared by reduction of the corresponding isopropylidene protected 5'-azido uridine readily obtained from commercially available uridine in four steps, according to known routes.[26] To avoid the undesired Michael addition already described in Staudinger conditions for this reaction,[27] a catalytic hydrogenation

was preferred and its duration was optimised to limit the formation of over reduced side product. In these optimised conditions (Pd/C, MeOH, 1 hour) the expected amine **7** was obtained in 90% yield, it was used without further purification in the next reaction due to its inherent instability

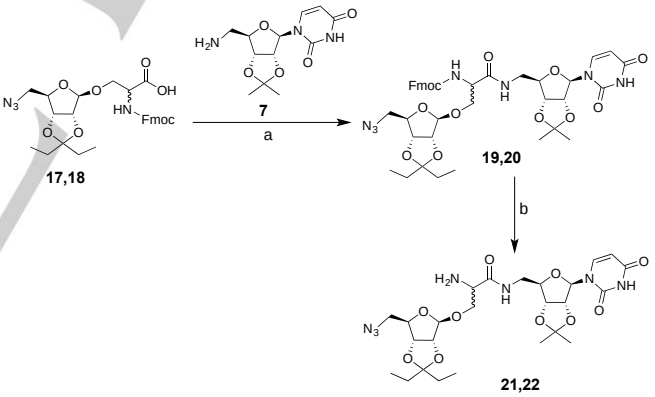
Synthesis of the ribose building block **8** as a glycosyl donor was carried out concurrently. Prior to synthesis, a thorough literature search related to key structural features that aminoribose linkage should contain to have antibacterial active was undertaken.[28] Importantly, ribosyl building blocks must be β-glycosides to retain antibacterial activity, in naturally occurring muraymycins.[28a] The introduction of a 5′-aminoribose moiety in a β-selective manner was a major challenge towards the total synthesis of our simplified serine template analogues.[28a] Hirano et al. have reported an optimised protocol towards this desired outcome.[28a] They established that to achieve β-selectivity for the glycosylated product, the ribofuranoside must have a 3-pentylidene protecting group across the 2,3-diol, a fluoride leaving group at *C*-1 and an azide group at the *C*-5 position.[28a] Therefore, based on this report we decided to use the optimised ribose building block **8** as our glycosyl donor,[28a] starting the synthesis from commercially available D-ribose.

In order to assemble the various synthetic fragments together, we also required synthesis of the serine template (glycosyl acceptor) (Scheme **1**). For the preparation of this compound, commercially available Fmoc-Ser(*t*Bu)-OH **9** (L-isomer) or **10** (D-isomer) was first converted into the allyl ester L-**11** or D-**12**. The *tert*-butyl ether was then deprotected with TFA to afford the desired serine intermediate (glycosyl acceptor) L-**13** or D-**14** ready for the glycosylation reaction with **8**. Having all the building blocks in hand, we first planned to couple the uridine **7** and ribose **8** together with the serine template L-**13** or D-**14** followed by linking of the lipophilic side chain. The Hirano et al.[28] had evaluated different reaction conditions for this transformation such as using various activators like $BF_3{\cdot}OEt_2$, $AgOTf/Cp_2HfCl_2$, $AgOTf/SnCl_2$ (or) $AgClO_4/SnCl_2$ under varying temperature conditions. They observed the use of $BF_3{\cdot}OEt_2$ as an activating agent at 0°C was the best outcome in comparison with other conditions.[28] The ribosylation reaction was followed as reported by the Hirano et al.[28] and reproduced successfully yielding the glycosylated product with only β-anomer L-**15** or D-**16**. After purification, the glycosylated product L-**15** or D-**16** were reacted for deallylation to afford the corresponding acid compounds. However, initial deallylation attempts by using $Pd(PPh_3)_2Cl_2$ and phenylsilane resulted in undesired side product with reduction of the azide functionality. This was assumed due to the inherent reduction properties of organosilane (phenylsilane) to act as a hydride donor. The issue was resolved by replacing phenylsilane with *N*-methylaniline for deallylation of the glycosylated products L-**15** or D-**16**, which afforded the desired corresponding acid intermediates L-**17** or D-**18** efficiently (Scheme **1**).



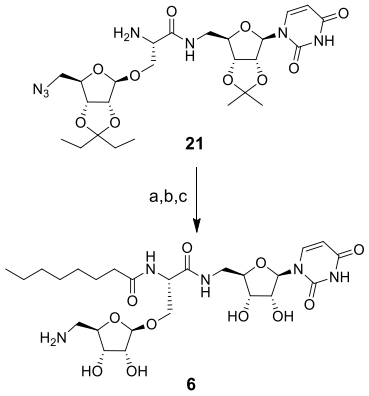
Scheme 1. Reagents and conditions: a) allyl bromide, DIPEA, acetonitrile, 40°C, 4 h, 90% (for comd. **11**), 87% (for comd. **12**) b) TFA:DCM (1:1), rt, 1 h, 85% (for comd. **13**), 84% (for comd. **14**), c) $BF_3{\cdot}OEt_2$, CH_2Cl_2, MS 4Å, 0°C, 1 h, 62% (for comd. **15**), 61% (for comd. **16**), d) $Pd(PPh_3)_4$, CH_2Cl_2, *N*-methylaniline, rt, 1 h, 84% (for comd. L-**17**), 82% (for comd. D-**18**).

After the successful linking of the ribose intermediate **8** with the serine template (either in **15** or **16**), we planned to introduce the uridine pharmacophore **7** on to the serine template. The plan was executed by reacting either acid intermediates L-**17** or D-**18** with the previously synthesised 5′-amino uridine **7** *via* a conventional amide coupling reaction (Scheme **2**). The resulting amide product L-**19** or D-**20** was further reacted for Fmoc deprotection to yield the corresponding amino compound L-**21** or D-**22** (Scheme **2**). This amine product (**21** or **22** Scheme **2**) is an advanced precursor for the synthesis of our target serine template analogues.

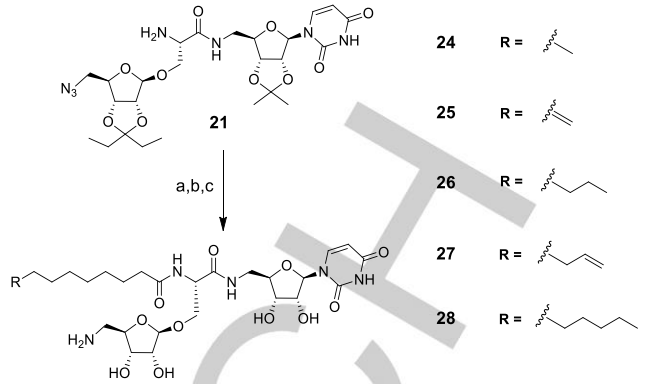


Scheme 2. Reagents and conditions: a) EDC·HCl, HOBt, DMF, rt, overnight, 84% (for comd. **19**), 82% (for comd. **20**), b) piperidine (20%), DMF, rt, 1 h.

After optimisation of the conditions toward the advanced stage amine intermediate **21** and **22**, we focused on preparing our target structures. This work began by optimisation towards the western zone of muraymycins, preparing the synthetically accessible core structure **6**, having three building blocks including the uridine, the aminoribose and lipophilic side chain (octanoic functional group) linked together onto the serine template. The designed target molecule **6** was synthesised starting from **21** in three reaction steps (Scheme **3**). Firstly, the free amino group of the amine product **21** was reacted for amide coupling with octanoic acid. Next the azide group of ribose was subsequently reduced to the amine under Staudinger reaction conditions. Finally, the target compound **6** was produced by a global acidic deprotection of the amino intermediate with 80% TFA in water (Scheme **3**), followed by its isolation through preparative HPLC.



Scheme 3. Reagents and conditions: a) EDC·HCl, HOBt, DMF, rt, overnight, b) PPh_3, H_2O, THF/toluene (1:1), 50°C, 18 h, c) aqueous TFA (80%), rt, 6 h, 26% in 4 steps.



Scheme 4. Reagents and conditions: a) EDC·HCl, HOBt, DMF, rt, overnight, b) PPh_3, H_2O, THF/toluene (1:1), 50°C, 18 h, c) aqueous TFA (80%), rt, 5-6 h, 37% (for comd. **24**), 31% (for comd. **25**), 36% (for comd. **26**), 27% (for comd. **27**), 29% (for comd. **28**).

Investigation of the lipophilic side chain

Owing to the lipophilic side chain contribution toward the membrane permeability of muraymycins, further work was carried out to investigate the importance of this moiety. We wanted to evaluate the effect of increasing lipophilicity of the synthetically accessible core structure **6**, and how that impacted on antibacterial properties. Therefore, different target molecules were designed with varied lipophilicity in the side chain (**23,** Figure **6**).

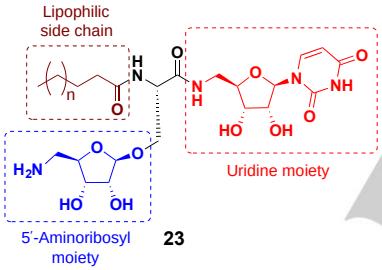


Figure 6. The proposed serine template (L-serine) linked scaffold with varied lipophilic side chains **23**

Previous SAR studies have reported that addition of a lipophilic side chain beyond octanoic acid to nonanoic **24**, undecanoic **26** and tridecanoic **28** functional groups helped increase antimicrobial activity. Therefore, we began work on inducing increased chain length intermediates to mimic the most active natural product muraymycin A1 (C13 carbon length). Furthermore, introduction of unsaturation increases hydrophobicity of the molecule, which should enhance membrane permeability. With this idea in mind, we also synthesised extended target molecules having linear lipophilic side chain with unsaturation, such as 8-nonenoic **25** and undecylenic **27** functional groups (Scheme **4**). At this stage, all of the synthesised target molecules (**24**-**28**) were evaluated for antibacterial activity (see biological evaluation section).

Investigation of the amino acid spacer

After the successful creation of the lipophilic side chain intermediates **24**-**28**, we next focused on investigating the simplified peptide unit in place of synthetically challenging urea-peptide moiety of the western zone. Previously, Tanino et al. performed a systematic SAR study of the accessory urea-peptide moiety.[24] In this study, diverse muraymycin analogues were synthesised by replacing the native L-*epi*-capreomycidine with simple amino acids such as L-arginine, L-ornithine, L-methionine and L-valine.[24] The results from biological evaluation suggested that the synthetically challenging western zone could be simplified by using amino acids with a lipophilic side chain.[24] Inspired by this SAR study[24] which was carried out towards simplification of the western zone of the muraymycin structure, we planned to introduce a simplified amino acid spacer between the serine template and the lipophilic side chain. The reasoning behind the introduction of a spacer in between the serine template and lipophilic side chain was to mimic the alkyl linker which is present between the core 5′-glycyluridine and lipophilic side chain of naturally occurring muraymycins.[13,15] It was envisaged that introduction of the spacer molecule would orientate the introduced pharmacophores in order to best retain antibacterial activity. The approach employed uses different amino acids as a spacer, selecting them based on their varied polarity (**29**, Figure **7**). We selected non-polar aromatic amino acids such as phenylalanine and tryptophan, a polar aromatic amino acid such as tyrosine and a polar aliphatic amino acid such as lysine.

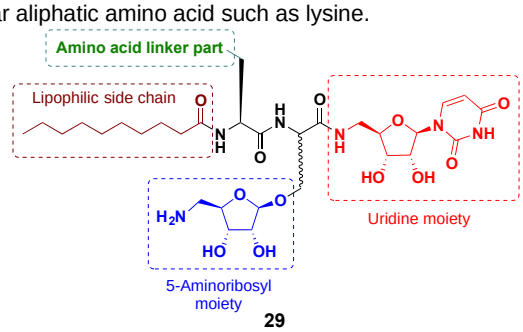
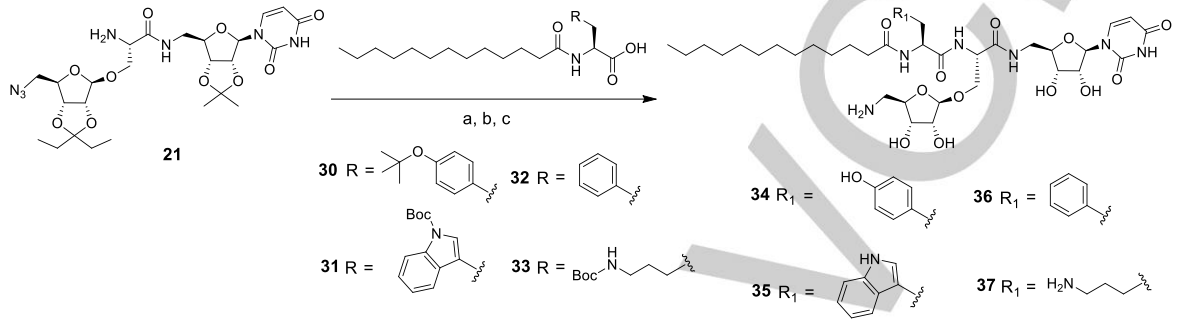


Figure 7. The proposed serine template (L/D-serine) linked scaffold having different amino acids as spacer unit **29**.

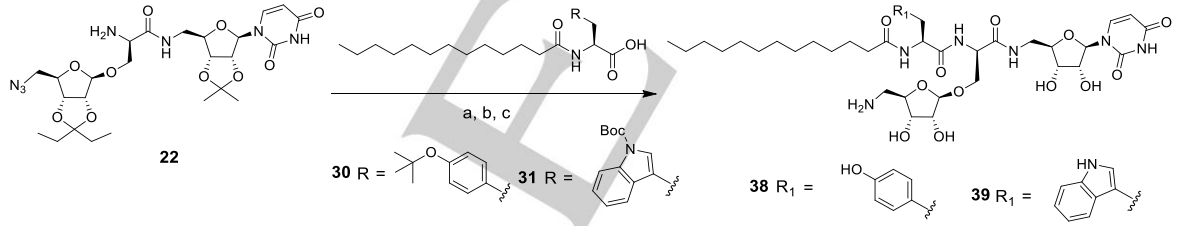
Synthesis of target molecules with the amino acid spacer was initiated by independent synthesis of acid intermediates **30**-

33 followed by linking them on to core structure of the serine template **21** (Scheme **5**). The acid intermediates, which all contain different amino acids (tyrosine **30**, tryptophan **31,** phenylalanine **32** and lysine **33**) and a tridecanoic lipophilic side chain (mimicking the natural product muraymycin A1), were prepared by a conventional four step synthetic protocol as summarised in SI. With the successful synthesis of the acid intermediates **30**-**33**, each was efficiently transferred to the core serine template structure **21** in a similar manner to the creation of compound **6**. Following on from the successful amide coupling reaction of **21** with the acid intermediates **30**-**33**, the resulting amide coupled products were further reacted under Staudinger reaction conditions to produce the amino intermediates, which were all globally deprotected under acidic conditions to afford our final target molecules **34**-**37** (Scheme **5**).



Scheme 5. Reagents and conditions: a) EDC·HCl, HOBt, DMF, rt, overnight, b) PPh_3, H_2O, THF/toluene (1:1), 50°C, 18 h, c) aqueous TFA (80%), rt, 5-6 h, 39% (for compd. **34**), 44% (for compd. **35**), 44% (for compd. **36**), 42% (for compd. **37**).

At this stage of our SAR study of simplified serine template analogues of muraymycins, we were interested in investigating stereochemical constraints related to the serine template itself and how this may contribute toward antibacterial activity. Therefore, we designed target molecules by using tyrosine and tryptophan as spacer molecule and tridecanoic lipophilic side chain, except having opposite stereochemistry of serine template by using D-serine as a linker. Synthesis of the target molecules **38** and **39** was performed exactly in the same fashion except starting from the advanced stage precursor **22** (*R*, by using D-serine as linker) (Scheme **6**).



Scheme 6. Reagents and conditions: a) EDC·HCl, HOBt, DMF, rt, overnight, b) PPh_3, H_2O, THF/toluene (1:1), 50°C, 18 h, c) aqueous TFA (80%), rt, 5-6 h, 48% (for compd. **38**), 31% (for compd. **39**).

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Biological Evaluation

All newly synthesised serine template linked muraymycins analogues **6,24**-**28,34**-**37,38**-**39** were evaluated for antibacterial activity against a range of clinically relevant bacterial pathogens, including *S. aureus* ATCC 43300 (methicillin-resistant), *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27853, *A. baumannii* ATCC 19606. Colistin and vancomycin were used as positive bacterial inhibitor standards for Gram-negative and Gram-positive bacteria, respectively (MIC values are depicted in Table **1**). Interestingly, these microorganisms are all priority 1 (critical) or 2 (high) listed pathogens according to WHO classification.[3] The results obtained from a dose response assay to determine the minimum inhibitory concentration (MIC) are summarised in Table **1**. Each sample concentration was prepared in 384-well, non-binding surface plates (NBS; Corning 3640) for each bacterial strain. We first screened the core serine template analogue **6** linked with three pharmacophores, including uridine, aminoribose and the lipophilic side chain (octanoic acid). However, contrary to our expectation, the core serine template compound **6** did not show any antibacterial activity against all the screened bacterial pathogens (MIC > 32 μg/mL). As the most active analogue of naturally occurring muraymycins A1 contains a lipophilic side chain of *n* = 13, we were anxious to investigate the effect of increasing lipophilicity on antibacterial activity, comparable to that of the natural product. Therefore, we have synthesised and screened compounds **24**-**28** having a longer linear lipophilic side chain such as nonanoic **24**, undecanoic **26**, and tridecanoic **28** functional groups. We also explored molecules with higher lipophilicity, which contained unsaturated lipophilic side chains, such as undecylenic **27** and 8-nonenoic **25** functional groups. However, none of the lipophilic side chain analogues **24**-**28** showed any antibacterial activity against resistant bacterial pathogens (MIC > 32 μg/mL). This set of biological results suggested that increasing lipophilicity equivalent to that of the naturally occurring muraymycin A1 was also not enough to generate a significant antibacterial agent. Therefore, it was clear that linking only three key units was not sufficient to generate the bioactive scaffold. We

had envisaged that further optimisation of the western zone was therefore necessary.

To optimise western zone of the serine template analogues, we planned to introduce an amino acid spacer in between the serine template and lipophilic side chain. As we wanted to mimic the lipophilicity of the natural product, we planned to retain linearity of *n = 13* carbon chain (tridecanoic functional group) as present in muraymycin A1.[13,15] We then selected representative amino acids with varying polarity such as non-polar aromatic (phenylalanine **36**, tryptophan **35**), polar aromatic (tyrosine **34**), and polar aliphatic (lysine **37**) amino acids. The antibacterial activity of this series of compounds was then evaluated, and the results are summarised in Table **1**. Interestingly, compounds possessing tryptophan **35** and phenylalanine **36** amino acid linker were found to be moderately active against multiple WHO priority resistant strains. The phenylalanine linker containing molecule **36** was active against *S. aureus* ATCC 43300 (methicillin-resistant) (MIC = 32 μg/mL), *E. coli* ATCC 25922 (MIC = 32 μg/mL) and *P. aeruginosa* ATCC 27853 (MIC = 32 μg/mL). The tryptophan linker containing molecule **35** was also active against multiple bacterial strains, including *S. aureus* ATCC 43300 (methicillin-resistant) (MIC = 32 μg/mL) and *A. baumannii* ATCC 19606 (MIC = 32 μg/mL). However, the tyrosine linker analogue **34**, exhibited no antibacterial activity (MIC > 32 μg/mL). Additionally, we investigated a polar aliphatic amino acid (lysine amino acid linker **37**) which was also found inactive. Based on the results we had obtained the serine template with a non-polar aromatic amino acid (phenylalanine and tryptophan) linker appeared to be essential for antibacterial activity. Conversely, the presence of a polar aromatic amino acid (tyrosine) or polar aliphatic amino acid (lysine) linker resulted in loss of antibacterial properties. Therefore, the antibacterial activity of our simplified serine template analogues was well-correlated with the presence of lipophilicity in the linker part of serine template scaffold. This is consistent with the observation by Mansour and co-workers, where lipophilicity of semi-synthetic muraymycin C1 analogues directly related to the biological activity.[18d]

After optimising the minimum structural requirement of serine template scaffold, we further investigated its stereochemical requirement towards antibacterial activity. This was achieved by screening two more compounds, which contained the D-serine template with a tryptophan **39** and tyrosine **38** as spacers, along with the lipophilic side chain mimicking the natural product muraymycin A1 (*n* = 13 carbon length, tridecanoic functional group). Though having similar structural characteristics, these analogues **38** and **39** were designed by replacing the natural L-serine template with the unnatural amino acid D-serine. Interestingly, antimicrobial screening results for molecules **38** and **39** were similar to that observed for the L-serine template analogues **34** and **35**. The tryptophan linker containing molecule **39** showed activity against multiple bacterial strains, including *S. aureus* ATCC 43300 (methicillin-resistant) (MIC = 16 μg/mL) and *A. baumannii* ATCC 19606 (MIC = 32 μg/mL). However, analogue **38**, the tyrosine linker, molecule had lost antibacterial activity (MIC > 32 μg/mL). These results were in line with the previous observation that non-polar aromatic amino acid linkers were active antimicrobials, whilst polar aromatic amino acid linkers reduced this observed activity (MIC > 32 μg/mL). These results also allowed us to summarise that the presence of an unnatural amino acid template containing D-serine, as seen for compound **39** lead to an increase in antibacterial activity against *S. aureus* ATCC 43300 (methicillin-resistant) in comparison with the natural amino acid template (L-serine) **35**. However, equivalent results were observed against *A. baumannii* ATCC 19606 in both cases (D-serine **39** and L-serine **35**). These results clearly show that a presence of unnatural amino acid template (D-serine) replacing natural amino acid template (L-serine) could form either equipotent or more potent antibacterial scaffold.

Next we turned to the screening of the serine template analogues for MraY inhibitory activity (Table 2). This radiochemical MraY assay was performed at 100 μM final concentration against membranes containing overexpressed *E. coli* MraY by a previously described method.[29] Interpretation of these results reveal that the inhibition of the MraY enzymatic activity does not correlate with the antibacterial activity. The most active hit compounds **35, 36** and **39** showed MraY inhibition of only 3.0%, 0.0% and 5.7%, respectively. However, in contrast the compounds **34** and **37** have shown significant MraY inhibition of 25.3% and 19.7%, respectively. We believe that this contradiction between *in vitro* antibacterial activity and MraY enzyme inhibition could be possible due partly to uptake issues, and the possibility of other targets. Also, there are minor differences in the sequences of the MraY enzymes across different bacterial species,[10b] so it is possible that compounds **35**,**36** and **39** are more active against MraY enzymes from different bacteria.

Table 2. The inhibitory activity of structurally simplified serine template analogues of muraymycins against MraY enzyme[a]

Compound	% MraY Inhibition
6	9.3
24	11.3
25	9.0
26	3.3
27	6.4
28	7.1
34	25.3
35	3.0
36	0.0
37	19.7
38	4.4
39	5.7

[a]Assay was performed at 100 μM final concentration against membranes containing overexpressed *E. coli* MraY. This MraY enzyme inhibitory assay was performed with controls which included a no inhibitor negative control, and a positive inhibitor control tunicamycin (100 μM). IC_{50} was measured from a plot of enzyme activity *vs*. inhibitor concentration where the negative control was defined as 100% activity.

Selective toxicity towards bacterial strains is a key concern in the development of chemotherapeutics for infectious disease. Subsequently, determining the specificity of our synthesised compounds towards bacterial cells and determination of their toxicity towards mammalian cell is an important aspect of potential clinical drug development. Accordingly, the lead serine template analogues **35**, **36** and **39** were evaluated *via in-vitro* cytotoxicity assay against *human embryonic kidney cells* (*HEK-293*, ATCC

CRL-1573) as well as for haemolytic assay against human red blood cells (*RBC*). The three screened serine template analogues **35**, **36** and **39** were found to be free from any significant cytotoxicity. However, in contrast all of them **35**, **36** and **39** were found to be haematotoxic against human red blood cells (*RBC*).

Table 1. Antibacterial and cytotoxicity values (μg/mL) of structurally simplified serine template analogues of muraymycins **35,36,39**

Compound	MIC (μg/mL)					Cytotoxicity	
	Gram-positive bacteria[a]	Gram-negative bacteria[b]				*HEK-293*[c] (CC50)	*RBC*[d] (HC50)
	Sa	Ec	Kp	Pa	Ab		
36	32	32	>32	32	>32	>32	11.26
35	32	>32	>32	>32	32	>32	11.54
39	16	>32	>32	>32	32	>32	<=0.25
Vancomycin	1	-	-	-	-	-	-
Colistin	-	0.125	0.25	0.25	0.25	-	-

[a] Sa (*Staphylococcus aureus* MRSA ATCC 43300).

[b] Ec (*Escherichia coli* ATCC 25922); Kp (*Klebsiella pneumoniae* ATCC 700603); Pa (*Pseudomonas aeruginosa* ATCC 27853); Ab (*Acinetobacter baumannii* ATCC 19606).

[c] *HEK-293*, *Human embryonic kidney cells* (ATCC CRL-1573, CC50).

[d] *RBC*, *Human red blood cells* (HC50).

Proposed binding mode of serine template analogues

With exact MraY inhibition data of serine template compounds in hand, we were interested if the relative inhibition potencies of our serine template analogues could be correlated to their structural characteristics by *in silico* modelling. To predict the putative binding mode of the most active molecules **34** and **37** against a MraY enzyme, a combination of semi-automated docking and molecular dynamics (MD) simulations was employed. The initial structures of muraymycin D2 **4** (from the crystal structure), molecules **34** and **37** (derived from the guided docking) in complex with the MraY enzyme are given in Figure **S1**. To mimic the cell membrane environment, each protein-ligand complex was inserted into a model lipid bilayer. The stability of these complexes was evaluated using the root mean square deviation (RMSD) of protein backbone and ligand atoms from two independent 50 ns MD simulations for each of the complexes. The RMSD plots (Figure S2) indicates that the protein is stable in all three complexes. The crystal structure-derived bound conformation of Muramycin D2 **4** shows the lowest RMSD amongst all ligands, and validates the MD simulation protocol and parameters. Molecules **34** and **37** show slightly higher RMSD values indicating the re-arrangement of the docked conformations to reach a more stable state. The representative bound conformations of ligands obtained after 50 ns of MD simulations are shown in Figure **8**. The key pharmacophores (GlyU and 5-aminoribose) of **34** and **37** bind to the negatively charged cavity as observed for Muramycin D2 **4**. The rest of the pharmacophores (including the aliphatic chain) can hop around and bind to the protein surface around the cavity. The aliphatic chain inserts into a hydrophobic cavity at the junction of TM5 and TM9 helices. The aliphatic chain plays a crucial role of being an anchor and we believe this proposed binding of the serine template analogues could be a guide to the rational design of selective inhibitors for MraY.

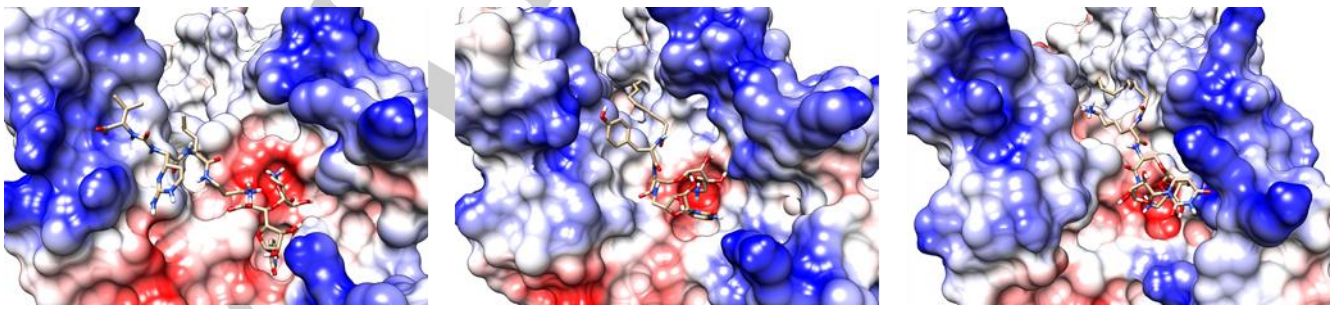

Figure 8. Representative structure of muraymycin D2 **4** (left) and serine template analogue **34** and **37** (middle and right, respectively) in complex with MraY from *Aquifex aeolicus*, after 50 ns of MD simulations with the position of **4** derived from the previously reported X-ray co-crystal structure[21] and the position of **34** and **37** derived from the guided docking. The electrostatic surface of the protein is shown with the colour spectrum red (negative) to white (neutral) to blue (positive) regions.

Conclusion

In conclusion, a systematic SAR study of simplified serine template analogues was investigated. The simplified analogues of muraymycins, having their key pharmacophores linked on a serine template were synthesised through a multistep protocol. Interestingly, these compounds have shown their antibacterial potential against WHO priority organisms classified as priority 1 (critical) and priority 2 (high). Initial antimicrobial screening results revealed that three pharmacophores, including uridine, 5-aminoribose and the lipophilic side chain, were not enough to fulfil minimum structural requirements for activity. Moreover, an increment of linearity or unsaturation in the lipophilic side chain was also not efficient for generating an active scaffold. However, the introduction of a non-polar aromatic amino acid (phenylalanine and tryptophan) spacer was found to be crucial for affording antimicrobial activity against resistant bacterial strains. Furthermore, evaluation of the effect of changed stereochemistry within the core serine templet indicated that serine holding opposite stereochemistry (D-serine) either retained or increased antibacterial potential in the case of a non-polar aromatic amino acid (tryptophan) spacer. The lipophilic side chain was found to act as a crucial anchor for the binding and inhibition of MraY. Subsequently, the screening of the serine template analogues for enzymatic activity revealed two active hit compounds, having significant inhibition against overexpressed *E. coli* MraY enzyme.

Moving forward, active molecules were screened for mammalian cell toxicity. The hit compounds were found to be non-toxic towards *human embryonic kidney cells* and toxic against human red blood cells. Overall, the SAR study of our novel serine template linked molecules suggests that the complexity present in the naturally occurring muraymycins could be simplified to a large extent. On the basis of our results, the binding mode of the active serine template compounds with an MraY enzyme was proposed. The knowledge obtained from our SAR study will provide further direction towards the design of potent muraymycin analogues with simplified structural characteristics. This preliminary study has set the stage for a new generation of novel antibacterial lead compounds acting against priority microorganisms, specifically against the ones classified by WHO. Work along this line is ongoing in our laboratories, including determining the mode of action as well as further lead optimisation to achieve more potent antibacterial agents.

Experimental Section

Full experimental details are disclosed in the Supporting Information.

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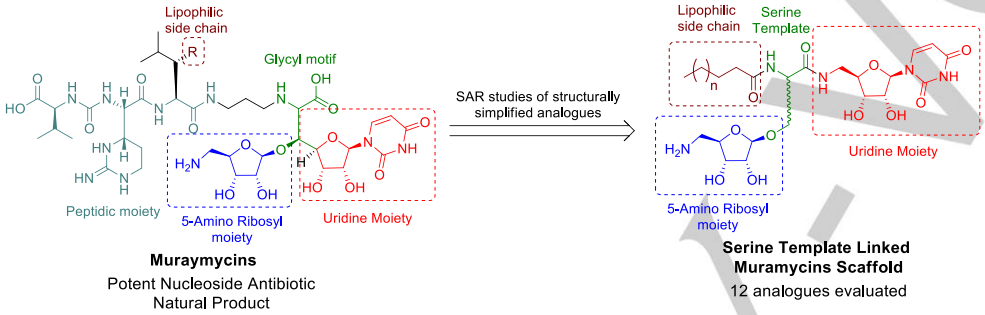
Keywords: Antibiotics • Nucleoside natural product • MraY • Muraymycins • Structure-activity relationship

References

[1] C.L. Ventola, *Pharm. Ther.* **2015**, *40*, 277.
[2] C.L. Ventola, *Pharm. Ther.* **2015**, *40*, 344.
[3] E. Tacconelli, E. Carrara, A. Savoldi, S. Harbarth, M. Mendelson, D.L. Monnet, C. Pulcini, G. Kahlmeter, J. Kluytmans, Y. Carmeli, *Lancet Infect. Dis.* **2018**, *18*, 318.
[4] C.T. Walsh, T.A. Wencewicz, *J. Antibiot.* **2014**, *67*, 7.
[5] K.A. Abrahams, G.S. Besra, *Parasitology* **2018**, *145*, 116.
[6] B. Patel, P. Ryan, V. Makwana, M. Zunk, S. Rudrawar, G. Grant, *Eur. J. Med. Chem.* **2019**, *171*, 462.
[7] T.D. Bugg, A.J. Lloyd, D.I. Roper, *Infect. Disord. Drug Targets* **2006**, *6*, 85.
[8] B.C. Chung, J. Zhao, R.A. Gillespie, D.-Y. Kwon, Z. Guan, J. Hong, P. Zhou, S.-Y. Lee, *Science* **2013**, *341*, 1012.
[9] K.-i. Kimura, T.D. Bugg, *Nat. Prod. Rep.* **2003**, *20*, 252.
[10] a) M. Winn, R.J. Goss, K.-i. Kimura, T.D. Bugg, *Nat. Prod. Rep.* **2010**, *27*, 279; b) T.D.H. Bugg, R.V. Kerr, *J. Antibiot.* **2019**, *72*, 865.
[11] C. Dini, *Curr. Top. Med. Chem.* **2005**, *5*, 1221.
[12] M. Kotnik, P.S. Anderluh, A. Prezelj, *Curr. Pharm. Des.* **2007**, *13*, 2283.
[13] L.A. McDonald, L.R. Barbieri, G.T. Carter, E. Lenoy, J. Lotvin, P.J. Petersen, M.M. Siegel, G. Singh, R.T. Williamson, *J. Am. Chem. Soc.* **2002**, *124*, 10260.
[14] Z. Cui, X. Wang, S. Koppermann, J.S. Thorson, C. Ducho, S.G. Van Lanen, *J. Nat. Prod.* **2018**, *81*, 942.
[15] a) D. Wiegmann, S. Koppermann, M. Wirth, G. Niro, K. Leyerer, C. Ducho, *Beilstein J. Org. Chem.* **2016**, *12*, 769; b) Y. Takeoka, T. Tanino, M. Sekiguchi, S. Yonezawa, M. Sakagami, F. Takahashi, H. Togame, Y. Tanaka, H. Takemoto, S. Ichikawa, *ACS Med. Chem. Lett.* **2014**, *5*, 556.
[16] D. Wiegmann, S. Koppermann, C. Ducho, *Molecules* **2018**, *23*, 3085.
[17] A. Spork, S. Koppermann, R. Linder, C. Ducho, *Molecules* **2018**, *23*, 2868.
[18] a) K. Mitachi, B.A. Aleiwi, C.M. Schneider, S. Siricilla, M. Kurosu, *J. Am. Chem. Soc.* **2016**, *138*, 12975; b) T. Tanino, S. Ichikawa, M. Shiro, A. Matsuda, *J. Org. Chem.* **2010**, *75*, 1366; c) K. Mitachi, S. M. Kurosu, S. Eslamimehr, M. R. Lemieux, Y. Ishizaki, W. M. Clemons, M. Kurosu, *Org. Lett.* **2019**, *21*, 876; d) Y. -I. Lin, Z. Li, G. D. Francisco, L. A. McDonald, R. A. Davis, G. Singh, Y. Yang, T. S. Mansour, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2341; e) K. Leyerer, S. Koppermann, C. Ducho, *Eur. J. Org. Chem.* **2019**, *2019*, 7420; f) A. Heib, G. Niro, S. C. Weck, S. Koppermann, C. Ducho, *Molecules* **2020**, *25,* 22.
[19] A. Katsuyama, S. Ichikawa, *Chem. Pharm. Bull.* **2018**, *66*, 123.
[20] a) T. Tanino, S. Ichikawa, B. Al-Dabbagh, A. Bouhss, H. Oyama, A. Matsuda, *Med. Chem. Lett.* **2010**, *1*, 258; b) K. Mitachi, S.M. Kurosu, S. Eslamimehr, M.R. Lemieux, Y. Ishizaki, W.M. Clemons Jr, M. Kurosu, *Org. Lett.* **2019**, *21*, 876.
[21] a) B.C. Chung, E.H. Mashalidis, T. Tanino, M. Kim, A. Matsuda, J. Hong, S. Ichikawa, S.-Y. Lee, *Nature* **2016**, *533*, 557; b) E. H. Mashalidis, B. Kaeser, Y. Terasawa, A. Katsuyama, D.-Y. Kwon, K. Lee, J. Hong, S. Ichikawa, S.-Y. Lee, *Nature Commun.* **2019**, *10*, 2917.
[22] S. Koppermann, Z. Cui, P.D. Fischer, X. Wang, J. Ludwig, J.S. Thorson, S.G. Van Lanen, C. Ducho, *ChemMedChem* **2018**, *13*, 779.
[23] a) C. Dini, P. Collette, N. Drochon, J. Guillot, G. Lemoine, P. Mauvais, J. Aszodi, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1839; b) M. J. Fer, A. Bouhss, M. Patrão, L. Le Corre, N. Pietrancosta, A. Amoroso, B. Joris, D. Mengin-Lecreulx, S. Calvet-Vitale, C. Gravier-Pelletier, *Org. Biomol. Chem.* **2015**, 13, 7193.
[24] a) T. Tanino, B. Al-Dabbagh, D. Mengin-Lecreulx, A. Bouhss, H. Oyama, S. Ichikawa, A. Matsuda, *J. Med. Chem.* **2011**, *54*, 8421; b) O. Ries, C. Carnarius, C. Steinem, C. Ducho, *MedChemComm* **2015**, *6*, 879.
[25] B. Patel, G. Grant, M. Zunk, S. Rudrawar, *Eur. J. Org. Chem.* **2019**, *2019*, 6005.
[26] A. Babič, S. Gobec, C. Gravier-Pelletier, Y. Le Merrer, S. Pečar, *Tetrahedron* **2008**, *64*, 9093.
[27] A. Bozzoli, W. Kazmierski, G. Kennedy, A. Pasquarello, A. Pecunioso, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2759.
[28] a) S. Hirano, S. Ichikawa, A. Matsuda, *J. Org. Chem.* **200**7, 72, 9936; b) L. Le Corre, C. Gravier-Pelletier, Y. Le Merrer, *Eur. J. Org. Chem*. **2007**, 2007, 5386.
[29] A. Mihalyi, S. Jamshidi, J. Slikas, *Bioorg. Med. Chem.* **2014**, *22*, 4566.

Entry for the Table of Contents



Systematic SAR study of 12 structurally simplified serine template linked muraymycin analogues were conducted. Our lead compounds are active against WHO priority organisms classified as priority 1 (critical) and priority 2 (high), including one Gram-positive and four Gram-negative bacterial strains. This preliminary investigation of serine template analogues has successfully revealed that the complex structure of naturally occurring muraymycins could be easily simplified to afford bioactive scaffolds active against resistant priority organisms and has set the stage for development of novel class of antibacterial agents.