Synthesis and Medicinal Chemistry of Muraymucins, Nucleoside Antibiotics

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Received August 29, 2017

Muraymucins, isolated from a culture broth of \textit{Streptomyces} sp., are members of a class of naturally occurring nucleoside antibiotics. They are strong inhibitors of the phospho-MurNAc-pentapeptide translocase (MraY), which is responsible for the peptidoglycan biosynthesis. Since MraY is an essential enzyme among bacteria, muraymucins are expected to be a novel antibacterial agent. In this review, our efforts to synthesize muraymucin D2, simplify the chemical structure, improve antibacterial spectrum, and solve the X-ray crystal structure of the muraymucin D2/MraY complex are described.

Key words muraymucin; nucleoside antibiotics; antibacterial agent; total synthesis

Introduction

Multidrug resistant pathogens, such as methicillin-resistant \textit{Staphylococcus aureus} (MRSA) and vancomycin-resistant \textit{Staphylococcus aureus} (VRSA), pose an ongoing public health concern. Keeping pace with their mutations continues to be a priority and challenge for the medical community.\textsuperscript{1} In choosing novel antibacterial agents to address this problem, several factors should be considered: the target must be essential for growth, the mechanism different from existing drugs, and the initial “hit” scaffold amenable to structural changes that allow for potency optimization and efficacy to generate “lead” compounds.\textsuperscript{2–6} Antibacterial drugs tend to have higher molecular weight and increased polarity, and they are known to occupy a unique physicochemical property space and are remarkably different from drugs of other therapeutic areas.\textsuperscript{7–11} Since the antibacterial drugs currently in use originated largely from natural products, natural products are still a rich source for the discovery of novel antibacterial agents.\textsuperscript{12,13}

Muraymucins (MRYs) (Fig. 1), isolated from a culture broth of \textit{Streptomyces} sp.,\textsuperscript{14} are members of a class of naturally occurring nucleoside antibiotics. The MRYs are strong inhibitors of the phospho-MurNAc-pentapeptide translocase (MraY), which is responsible for the formation of the lipid I in the peptidoglycan biosynthesis. Since MraY is an essential enzyme among bacteria, it has a potential to be a novel target for the development of general antibacterial agents.\textsuperscript{15,16} The MRYs possessing a lipophilic side chain that is required to exhibit excellent antimicrobial activity against Gram-positive bacteria.\textsuperscript{17} In particular, the efficacy of the MRYs in \textit{S. aureus} infected mice represents a promising lead for the development of new antibacterial agents.\textsuperscript{14}

In this review, our efforts to synthesize MRY D2 (1), simplify the chemical structure, improve the antibacterial spectrum, and solve an X-ray crystal structure of the MRY D2/MraY complex are described.

1. Total Synthesis of Muraymucin D2

Common features of MRYs include a 5'-O-aminoribosyl-5'-C-glycyluridine moiety and an amino acid-urea-amino acid motif involving \textit{\textalpha}-epi-capreomycidine (\textit{l-epi-Cpm}), which is a cyclic guanidine amino acid. The promising biological properties in conjunction with their interesting chemical structures

**Fig. 1. Structures of Muraymucins**

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render the MRYs intriguing and challenging synthetic targets. Since chemical modifications of the natural product are limited, totally chemical supply would be necessary for pursuing a structure–activity relationship (SAR) to develop novel antibacterial agents, and the strategic synthetic route is preferred for accessibility to a range of analogs.

The Ugi four-component reaction (U4CR) has long been considered a useful method for the preparation of N-acyl-α-aminocarboxyamides from an aldehyde, an amine, an isonitrile and a carboxylic acid. Because of the inherent high convergent potential of the multi-component assembly, the U4CR has been applied not only to medicinal chemistry for the preparation of libraries but also to the total synthesis of complex molecules. We retrosynthetically divided MRY D2 into the urea dipeptide for U4CR (Chart 1). Among the four components, the urea dipeptide moiety contains rare L-epi-Cpm. The structure of the L-epi-Cpm can be regarded as an arginine analog, where the terminal nitrogen atom of the guanidine is inserted into the C–H bond at the β-position constructing a cyclic guanidine moiety. Therefore, we embarked on the synthesis of an L-epi-Cpm unit by using the C–H insertion of the sulfamate.

Synthesis of the urea dipeptide containing the L-epi-Cpm residue is described in Chart 2. The sulfamate, which was prepared from the commercially available δ-N-tert-butoxycarbonyl (Boc)-α-N-benzoxycarbonyl (Cbz)-l-ornithine (7) in 66% yield over 3 steps, was treated with 10 mol% bis[rhodium(α,α,α’,α’-tetramethyl-1,3-benzenedi-propionic acid)] (Rh(esp)2) catalyst in the presence of PhI(OAc)2 and MgO in refluxing CH2Cl2 for 1.5 h, and the desired oxathiazinane derivatives 11a and 11b were obtained in 47% yield (11a:11b=1:2). After 11a was converted to the S-methylothiourea derivative 12, construction of the cyclic guanidine skeleton was performed through cyclization promoted by HgBr2 and the following desulfonylation upon acetolysis of the oxathiazinane ring gave 13 in 82% yield over 2 steps. After removal of the acetyl group of 13, the amine obtained by selective removal of the Cbz group of 14 was reacted with 15 to provide 16. Finally, the alcohol 16 was oxidized to the carboxylic acid 17.

The isonitrile component linked to the 6′-N-alkyl-5′-O-amoribosyl-C-glycyluridine was prepared from uridine (Chart 3). Oxidation of 2′,3′-O-isopropylideneuridine (18) with 2-iodoxybenzoic acid (IBX) followed by the two-carbon elongation with Ph3P=CHCO2Me provided 19 (trans/cis=37/1). When the Sharpless aminohydroxylation of 19 was carried out with (DHQD)2AQN as a chiral ligand (15 mol% of K2[Os2(OH)4], 15 mol% of (DHQD)2AQN, 3 eq of benzyl carbamate, 2.6 eq of NaOH, iPrOH–H2O, 15°C) afforded 20 as the major diastereomer in 72% overall yield. The ribosylation with the ribosyl fluoride possessing a sterically hindered 3-pentylidene group using BF3·OEt2 as an activator at 0°C afforded the desired 23 with excellent β-selectivity (β/α=24/1). The azide group in 23 was reduced under Staudinger’s conditions to the corresponding amine, which was sequentially protected with a Boc group to give 24. Then, methyl ester in 24 was converted to a desired carboxylic acid 25. In order to employ the final global deprotection at the last stage under acidic conditions to obtain the highly polar and base-sensitive target molecule, the carboxylic acid 25 was protected as a tert-butyl ester to give 26 in 66% yield. Hydrogenolysis of the Cbz protecting group cleanly afforded the amine, reductive alkylation of which was conducted with N-2,2,2-trichloroethoxycarbonyl (Troc)-3-aminopropenal to
give the secondary amine 28 in 94% yield over 2 steps. Upon removal of the Troc group of 28 with Zn, the corresponding amine derivative was converted to the formamide 29. Treatment of 29 with triphosgene and Et$_3$N in CH$_2$Cl$_2$ at −78°C gave the isonitrile 30 in the clean and quantitative conversion.

With the carboxylic acid 17 and the isonitrile 30 in hand, we undertook the final assemblage by the U4CR (Chart 4). The U4CR in our synthetic strategy to the MRYs required the use of ammonia as an amine. Ammonium acetate or ammonium chloride is generally used; however, the low reactivity and side-reactions limited the yield. In our study, 2,4-dimethoxybenzylamine was chosen as a “masked” ammonia, and the resulting 2,4-dimethoxybenzyl (DMB) group at the tertiary amide site after the U4CR can be removed by acid-catalyzed hydrolysis. Compounds 17, 30, isovaleraldehyde, and 2,4-dimethoxybenzylamine were mixed without any solvent at room temperature for 72 h provided the desired Ugi products 31 in 54% yield as a mixture of diastereomers (1:1) at the α-position of the Leu residue. The final deprotection of 31 successfully afforded (−)-MRY D2 (1) and epi-MRY D2 (32) after HPLC separation of the diastereomers and we have accomplished the first total synthesis of 1.

2. Medicinal Chemistry of Muraymycins

Synthetic (−)-MRY D2 exhibited potent inhibitory activity against its target enzyme MraY (Table 1, IC$_{50}$ 0.01 µM, $K_i$ 7.6 nM). Although inhibitory activity of epi-MRY D2 (32) was weakened, it still showed potent inhibitory activity (IC$_{50}$ 0.09 µM, $K_i$ 49 nM). As for the mode of inhibition, these compounds present a common behaviour with respect to the substrates of MraY: they were all competitive and noncompetitive inhibitors toward the nucleotide and the lipid substrates, respectively. However, they showed no antibacterial activity when they were subjected to a range of Gram-positive bacteria up to 64 µg/mL (Table 1). Previous studies by us and others associated with antibacterial nucleoside natural products indicated that the aminoribosyluridine moiety is the essential structural feature that interacts with the active site of the MraY enzyme. That the lipophilic side chain is not essential for MraY inhibition but rather for antibacterial activity clearly indicates that the side chain contributes to the membrane-permeability of the molecule because MraY is an integral membrane protein and its active site exists on the cytoplasmic side of the membrane. The urea-peptide moiety is regarded as an accessory motif linking the aminoribosyluridine moiety to the lipophilic moiety. Its role and structural requirement for the biological activity are totally unknown. We next planned a two-directional optimization on both the lipophilic side chain and the urea-peptide moieties by a common synthetic strategy using the U4CR (Fig. 2).

We designed and synthesized analogues such as 35a–d and 36a–d, which were linked to a hydrophobic substituent on the
MRY core structure via a C–C bond, as chemically and biologically stable isosteres of the MRYs (Fig. 3). The lipophilic analogues 35a and 36a were found to be weaker inhibitors of MraY than MRY D2, but still potent (Table 1, IC₅₀ = 0.33, 0.74 µM, respectively). The types of inhibition of 35a and 36a are competitive with the nucleotide and non-competitive with the lipid substrate and these properties are similar to those of 1. The substitution of the leucine side chain by a long carbon chain decreased the \( K_i \) by factors of 30 and 90 for compounds 35a and 36a, respectively. In each case, the compound with the amino acid residue having the \( \text{L} \)-configuration displayed a higher affinity for the enzyme target compared to that of the \( \text{D} \)-configuration.

The antibacterial activity of this series of compounds was then evaluated (Table 2). The impact of the lipophilic substituent on antibacterial activity was very good and both 35a and 36a exhibited good antibacterial activity against a range of Gram-positive bacterial pathogens including \( \text{S. aureus} \) SR3637 (MRSA) and \( \text{Enterococcus faecium} \) SR7917 (VRE) with minimum inhibitory concentration (MIC) values of 0.25–4 \( \mu \)g/mL. Thus, membrane-permeability plays an important role in terms of the antibacterial activity among this class of natural products.

We next investigated the impact of the accessory urea-pep-
tide moiety on antibacterial activity with a pentadecyl group as the lipophilic side chain. Several mutated analogues, where the L-epi-Cpm was replaced by the L-Cpm, L-Arg, and L-ornithine (Orn), respectively, were prepared to investigate the role of the cyclic guanidine functionality (Fig. 4). Truncated analogues at the L-Val residue were also prepared.

The antibacterial activity of this series of compounds was evaluated, and the results are summarized in Table 3. Overall, the stereochemistry at the newly formed stereogenic center in the U4CR did not influence the antibacterial activity. All analogues were also active against MRSA or VRE. Both of the L-Cpm and L-Arg mutated analogues exhibited antibacterial activity to a range of Gram-positive bacteria with MICs of 1–4 µg/mL. The results indicate that the stereochemistry or cyclic structure of the L-epi-Cpm residue found in MRYs is not important for antibacterial activity. Although the guanidine functionality is preferred, a less basic and simple amino functional group is tolerated enough to exhibit moderate antibacterial activity because the activity of the L-Orn mutated analogues was still active with the MIC values of 2–8 µg/mL. Interestingly, the analogue where the accessory motif was completely removed from 37 or 38, was found to be a much weaker MraY inhibitor with an IC₅₀ value of 5 µM, which was a 6–12 fold reduction of the inhibitory activity compared to 35a and 36a (Table 2). These results indicate that the partial structure of the urea-peptide

Table 1. Inhibitory Activities of the Synthesized Compounds Against MraY

<table>
<thead>
<tr>
<th>IC₅₀ (µM)</th>
<th>Mode of inhibition</th>
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<tbody>
<tr>
<td>MraY</td>
<td>WecA</td>
</tr>
<tr>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>32</td>
<td>0.09</td>
</tr>
<tr>
<td>35a</td>
<td>0.33</td>
</tr>
<tr>
<td>36a</td>
<td>0.74</td>
</tr>
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</table>

a) The activities of the compounds were tested against purified MraY from B. subtilis. The assay was performed in a reaction mixture of 10 mL containing, in final concentrations, 100 mM Tris-HCl, pH 7.5, 40 mM MgCl₂, 1.1 mM C₅₅-P, 250 mM NaCl, 0.25 mM UDP-MurNAc-[¹⁴C]pentapeptide (337Bq), and 8.4 mM N-lauroyl sarcosine. The mixture was incubated for 30 min at 37°C. The radiolabeled substrate UDP-MurNAc-pentapeptide and reaction product (lipid I, product of MraY) were separated by TLC on silica gel plates. The radioactive spots were located and quantified with a radioactivity scanner. IC₅₀ values were calculated with respect to a control assay without the inhibitor. Data represent the mean of independent triplicate determinations. Lineweaver–Burk plots were used to determination of the Kᵢ values and the type of inhibition assuming a ping-pong Bi Bi kinetic mechanism as proposed previously by Heydanek et al.

Fig. 2. Two-Directional Optimization via U4CR
accessory motif is a contributing factor in the interaction with the MraY enzyme to result in strong antibacterial activity. Two-directional optimization was effectively achieved by the SAR of several analogues resulting from the U4CR.

_Pseudomonas aeruginosa_ is a common nosocomial Gram-negative bacterial pathogen that is intrinsically resistant to a variety of drugs currently used in the clinic, and immunocompromised patients are at greater risk of the infection. Consequently, it is urgent to develop novel anti-

_Pseudomonas_ agents that should also be active against multi-drug resistant _P. aeruginosa_.60–62) The MraY enzyme is also conserved among Gram-negative bacteria, however the natural MRYs are not effective to these Gram-negative bacteria, presumably as a consequence of outer membrane impermeability. The fact that 37e exhibited weak antibacterial activity against _P. aeruginosa_ ATCC 25619 (MIC 64 µg/mL) prompted us to expand the antibacterial spectrum of MRYs to _P. aeruginosa_. It is expected that chemical modification of our MRY analogues 37 modulates outer membrane permeability of _P. aeruginosa_ and makes it possible to expand antibacterial activity toward this pathogen. From the SAR study, necessity of a lipophilic side chain and a guanidino group at the accessory moiety was implied to be important for the anti-

_Pseudomonas_ activity.60) Therefore, we diversified the relative positions of these two functional groups to the core aminoribosyluridine moiety in order to optimize the orientation of the three moieties as shown in Fig. 5. For example, the lipophilic side chain and the guanidino group were linked in a linear manner to give analogue 40. Interconversion of the branching functional groups of 46 gave analogue 47. Analogue 48 is a hybrid-type of 45–47.

The anti-

_Pseudomonas_ activity of this series of the compounds was then evaluated (Table 4). The analogues 40 and 41 did not show any activity. The analogues 42 and 43, which have the shorter lipophilic side chain, exhibited no antibacterial activity, whereas 44 and 45 with the longer side chain showed moderate activity and the antibacterial activity was also well-correlated to the length of the lipophilic side chain. The branching analogues 46 and 47 have a similar anti-

_Pseudomonas_ activity to 45. The hybrid-type analogue 48...
was the strongest activity among tested in this study. Selective toxicity against bacterial cell is an important issue on development of antibacterial agents. Cytotoxic activity of 48 against human hepatocellular liver carcinoma (HepG2) cells was then evaluated and 48 exhibited less cytotoxicity (IC$_{50}$ 34 µg/mL). Furthermore, the metabolic stability of 48 was briefly evaluated by treatment of these analogues with human or rat liver microsome and around 90% of 48 was unaffected by human liver microsomes, and 48 was revealed to be metabolically stable. As described here, simplification and optimization through the extensive SAR of MRYs led us to discover a potential antibacterial lead compound active against P. aeruginosa.

### 3. X-Ray Crystal Structure of the MRY D2/MraY Complex

Structure-based drug design is a rational approach in drug discovery process. In structure-based drug design, the three-dimensional structure of a target protein complexed with its interacting small molecules is used to guide drug discovery. The X-ray crystal structure of MraY, which is a target enzyme of MRYs, has long been difficult to solve because it is an integral membrane protein. Recently we present the crystal structure of MraY from *Aquifex aeolicus* (MraY$_{AA}$) in complex with MRY D2$^{24}$ (Fig. 6). Overall interaction found in the complex structure are in good accordance with our SAR studies of MRYs. Of note is a dynamics of MraY$_{AA}$. Namely,
upon binding MRY D2, MraY AA undergoes remarkably large conformational rearrangements near the active site, which lead to the formation of a nucleoside-binding pocket and a peptidic-binding site. MRY D2 binds the nucleoside-binding pocket like a two-pronged plug inserting into a socket. The peptidic moiety also contributes to the binding by anchoring MRY D2 to MraY, presumably contributing to its specificity for MraY. This could be a guide to the rational design of selective inhibitors for MraY and these efforts are in due course.

4. Conclusion

The first total synthesis of MRY D2 has been accomplished and the details are described. Our approach was quite effective and has provided ready access to a range of analogues simply by altering the aldehyde or carboxylic acid component. The systematic SAR study of the MRYs was then investigated. The impact of the lipophilic substituent on antibacterial activity was very large and analogues exhibited good activity against a range of Gram-positive bacterial pathogens, including MRSA and VRE. The SAR of the accessory urea-peptide moiety suggested that it could be simplified to a large extent. Moreover, some analogues exhibit antibacterial activity against *P. aeruginosa*. Compared to synthetic drugs, many biologically relevant natural products possess large, complex, or labile chemical structures, which may restrict chemical modifications in a SAR study. Therefore, it is important to design less structurally complex targets with comparable or superior activity that could be made in a practical manner.

The X-ray co-crystal structure of MRY D2 and MraY AA was also solved. The three-dimensional structure enables us to pursue structure-based drug design of potent inhibitors for MraY in near the future and these efforts are in due course.

We hope this review is useful for the researchers in the field of synthetic organic chemistry and medicinal chemistry of...
nucleosides and natural products.

Conflict of Interest The authors declare no conflict of interest.

References