

Expansion of Antibacterial Spectrum of Muraymycins toward *Pseudomonas aeruginosa*

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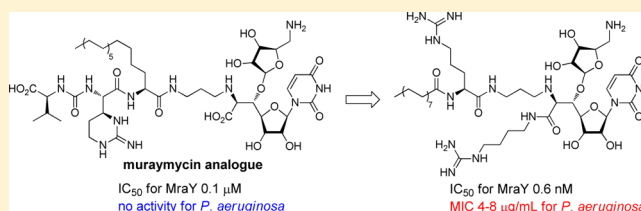
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Supporting Information

ABSTRACT: It is urgent to develop novel anti-*Pseudomonas* agents that should also be active against multidrug resistant *P. aeruginosa*. Expanding the antibacterial spectrum of muraymycins toward *P. aeruginosa* was investigated by the systematic structure–activity relationship study. It was revealed that two functional groups, a lipophilic side chain and a guanidino group, at the accessory moiety of muraymycins were important for the anti-*Pseudomonas* activity, and analogue **29** exhibited antibacterial activity against a range of *P. aeruginosa* strains with the minimum inhibitory concentration values of 4–8 $\mu\text{g/mL}$.

KEYWORDS: Antibiotics, drug-resistance, *MraY*, muraymycin, peptidoglycan, anti-*Pseudomonas*



Generally, Gram-negative bacterial pathogens are less sensitive to antibacterial drugs in comparison with Gram-positive bacteria because they possess an outer membrane, which acts as a permeability barrier. Among them, *Pseudomonas aeruginosa* is a common nosocomial pathogen that is intrinsically resistant to a variety of drugs currently used in the clinic, and immuno-compromised patients are at greater risk of infection. The increased rate of acquired multidrug resistance in *P. aeruginosa* also complicates anti-*Pseudomonas* chemotherapy. Consequently, it is urgent to develop novel anti-*Pseudomonas* agents that should also be active against multidrug resistant *P. aeruginosa*.^{1–3} In choosing novel antibacterial agents, the target must be essential for their growth, a mechanism of action of the agent should be different from existing drugs. The initial lead compounds should also be amenable to structural changes that allow for optimization of the potency and efficacy.^{4–8} Muraymycins (MRYs) (Figure 1, **1**), isolated from a culture broth of *Streptomyces* sp.,^{9,10} are members of a class of naturally occurring 6'-*N*-alkyl-5'- β -*O*-aminoribosyl-*C*-glycyluridine antibiotics.¹¹ The MRYs having a lipophilic side chain have been shown to exhibit excellent antimicrobial activity against Gram-positive bacteria. The MRYs inhibit the formation of lipid II and peptidoglycan and are inhibitors of phospho-MurNac-pentapeptide transferase (*MraY*), which is responsible for the formation of lipid I in the peptidoglycan biosynthesis pathway.^{12–16} The enzymes involved in the biosynthesis of peptidoglycan are essential for bacterial life and represent important targets for antibacterial chemotherapy. Recently, we have investigated the systematic structure–activity relationship (SAR) study of the MRYs.^{17–19}

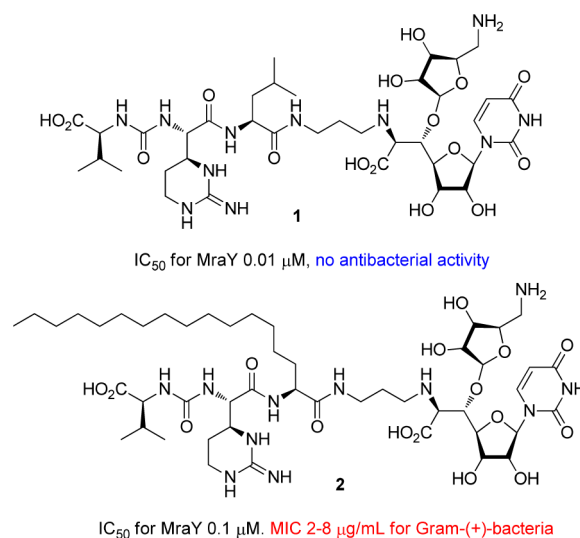


Figure 1. Structures and biological activity of muraymycin D2 and its lipophilic analogue.

These studies revealed that the impact of the lipophilic substituent on antibacterial activity was very large, and the lipophilic analogue **2** showed a potent antibacterial activity against a range of Gram-positive bacteria including methicillin-

Received: January 9, 2014

Accepted: March 6, 2014

Published: March 6, 2014

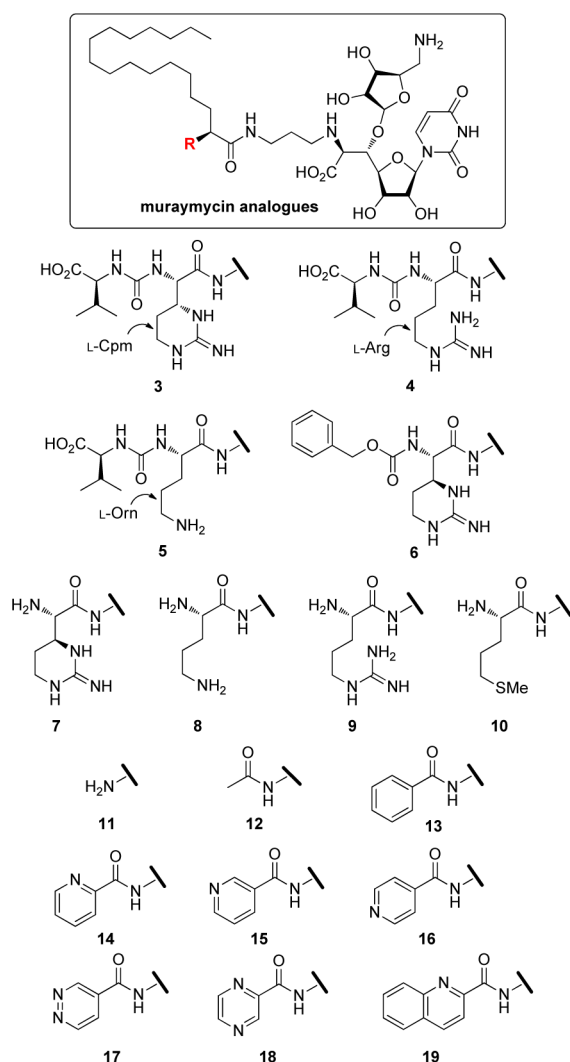
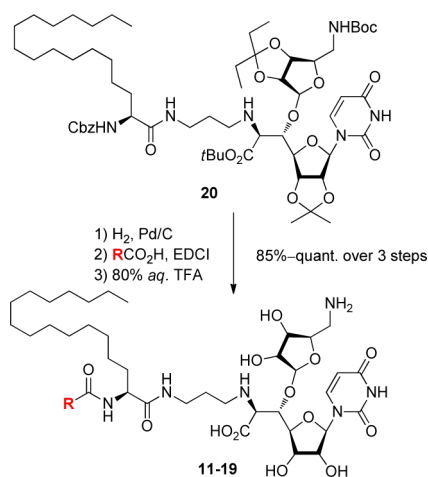


Figure 2. Structures of muraymycin analogues.

Scheme 1. Synthesis of Acyl Analogues 11–19



resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE).¹⁸ An accessory urea-dipeptide moiety could also be simplified to a large extent, and the truncated analogues 3–10 (Figure 2) retained the antibacterial activity.¹⁹ Other biological properties of these analogues were also investigated, and it was found that these analogues are

selective inhibitors of *MraY* and are nontoxic to human liver hepatocellular (HepG2) cells. Consequently, the *MraY* inhibitors are promising leads for antibacterial agents active against drug-resistant Gram-positive bacterial pathogens. 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. Here we describe our efforts to expand antibacterial spectrum of the MRYs toward *P. aeruginosa*.

It is expected that chemical modification of our MRY analogues 2–10 modulates outer membrane permeability of *P. aeruginosa* and makes it possible to expand antibacterial activity toward this pathogen. However, antibacterial evaluation of 2–10 for *P. aeruginosa* has not been investigated yet. We initiated this program by revisiting anti-*Pseudomonas* activity of 2–10. In addition, simple acyl derivatives 11–19 were also planned to evaluate (Figure 2). As shown in Scheme 1, these analogues were easily obtained by the Cbz deprotection of 6'-*N*-aminopropyl-5'- β -*O*-aminoribosyl-*C*-glycyluridine 20,¹⁵ acylation of the liberated amine, and global deprotection by aqueous TFA over 3 steps.

First, the inhibitory activity of the analogues on the purified *MraY* enzyme (*Staphylococcus aureus*) was examined by fluorescence-based *MraY* assay^{20,21} using UDP-MurNAc-dansylpentapeptide, where the formation of dansylated lipid I was monitored by fluorescence enhancement (excitation at 355 nm and emission at 535 nm), and the results are summarized in Table 1. As a result, most of the analogues exhibited strong inhibitory activity with a nanomolar range of the IC_{50} s. Anti-*Pseudomonas* activity of 1–19 was next evaluated against a range of *P. aeruginosa* strains.²² As expected, MRY D2 (1) showed a very weak activity, and its lipophilic analogue 2 did not show any activity. Analogues 7–12 exhibited a moderate activity with the MIC values of 8–64 μ g/mL. Analogue 9, which possesses the guanidino and the amino functional groups, was the most active among those tested (MIC values of 8–32 μ g/mL). It is a great contrast that the analogues 3 and 4 also have guanidino groups but show no anti-*Pseudomonas* activity. Since these analogues have the terminal carboxylic acid, it would partially neutralize the positive charge of the guanidino group. Newly synthesized analogues 13–19, which have an aromatic ring at the accessory moiety, were inactive to a range of *P. aeruginosa* strains. As a result, analogues possessing net positive charges at the accessory moiety tend to have the expanded antibacterial activity toward *P. aeruginosa* although there are some exceptions.

A revisit of the antibacterial evaluation of muraymycin analogues gave us a direction for a further molecular design for expanding the antibacterial spectrum to *P. aeruginosa*. Thus, necessity of a lipophilic side chain and a guanidino group at the accessory moiety was implied to be important for the anti-*Pseudomonas* activity from the SAR in Table 1. Therefore, we planned to diversify 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 Figure 3. For example, the lipophilic side chain and the guanidino group were linked in a linear manner to give analogue 21. Interconversion of the lipophilic side chain and the guanidino group gave *N*-acyl-*L*-arginine analogues 23–26. Analogues 3–19 contains *L*-pentadecylglycine residue, the preparation of which required a number of steps. The molecular design of 23–26 enables us to use a commercially

Table 1. Screening of Muraymycin Analogues 1–19 as Anti-Pseudomonas Agents

compound	MraY IC ₅₀ (nM)	MIC (μg/mL) ^a				
		<i>P. aeruginosa</i> PAO1	<i>P. aeruginosa</i> YY165 (ΔmexB)	<i>P. aeruginosa</i> ATCC 25619	<i>P. aeruginosa</i> SR 27156	<i>P. aeruginosa</i> ATCC 27853
1	2.8	>64	64	64	32	>64
2	1.1	>64	>64	>64	>64	>64
3	0.8	>64	>64	>64	>64	>64
4	0.7	>64	>64	>64	>64	>64
5	0.8	>64	>64	>64	>64	>64
6	4.2	>64	>64	>64	>64	>64
7	2.4	>64	32	64	64	>64
8	3.8	64	32	32	32	64
9	2.2	16	8	8	16	32
10	8.5	>64	16	16	64	64
11	2.6	>64	32	32	>64	>64
12	2.7	64	32	32	64	>64
13	29.6	>64	>64	>64	>64	>64
14	6.4	>64	>64	64	>64	>64
15	9.6	>64	>64	64	>64	>64
16	8.1	>64	>64	64	>64	>64
17	13.5	>64	64	64	>64	>64
18	33.1	>64	>64	64	>64	>64
19	105	>64	>64	>64	>64	>64
doripenem	n.d.	0.5	0.25	0.063	0.031	0.5

^aMICs were determined by a microdilution broth method as recommended by the CLSI with cation-adjusted Mueller–Hinton broth (CA-MHB). Serial 2-fold dilutions of each compound were made in appropriate broth, and the plates were inoculated with 5×10^4 CFU of each strain in a volume of 0.1 mL. Plates were incubated at 35 °C for 20 h, and then MICs were scored.

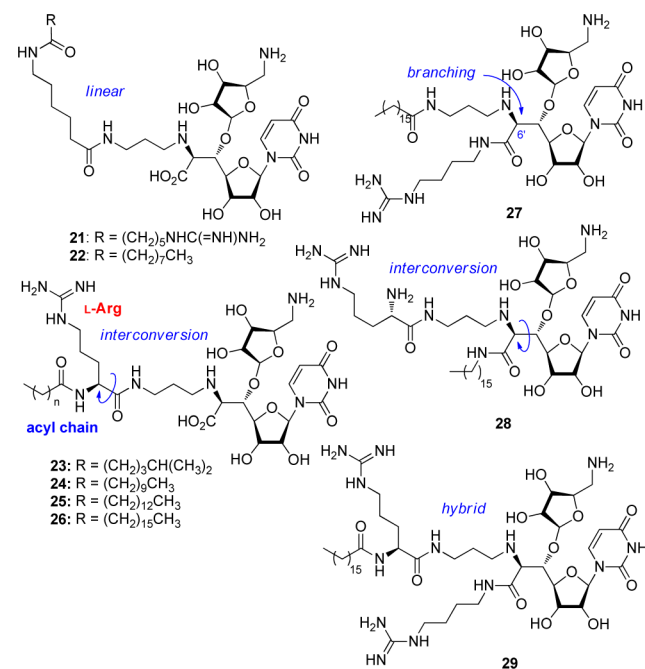
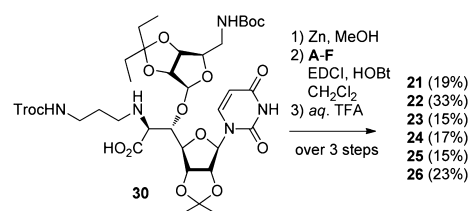
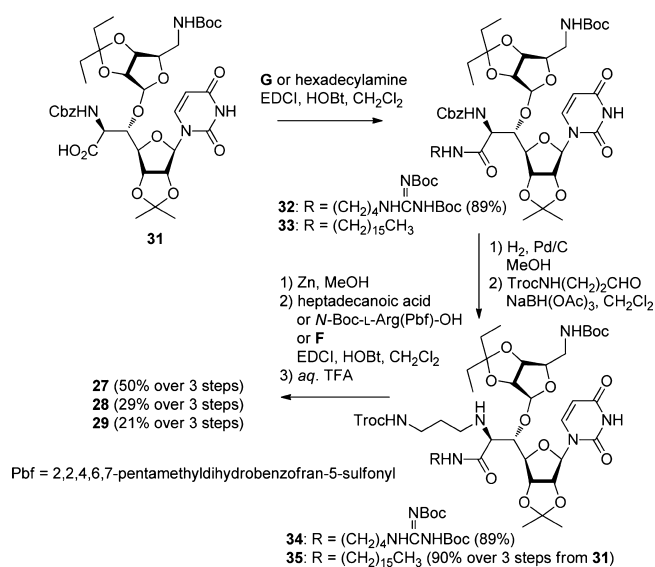


Figure 3. Molecular design of analogues 21–29.

Scheme 2. Synthesis of Analogues 21–26



Scheme 3. Synthesis of Branched Analogues 27–29



available L-Arg derivative and simple fatty acids. Analogue 27 is a branched version of 26, where the guanidino group located in the accessory moiety is moved to the carboxylic acid at the 6'-position of the uridine core structure. An interconversion of the branching functional groups of 27 gave 28. Analogue 29 is a hybrid-type of 26–28. Preparation of 21–26 is summarized in Scheme 2. The key intermediate 30^{13–15} for the synthesis of muraymycin D2 and its derivatives was used as a starting material. The trichloroethoxycarbonyl (Troc) group was removed by Zn in MeOH, and the liberated amine was acylated with the corresponding carboxylic acids A–F (see Supporting Information) by EDCI and HOBT in CH₂Cl₂. Finally, the global deprotection by aqueous TFA successfully

Table 2. MraY Inhibitory and Anti-Pseudomonas Activity of Analogues 21–29

compound	MraY IC ₅₀ (nM)	MIC (μg/mL) ^a				
		<i>P. aeruginosa</i> PAOI	<i>P. aeruginosa</i> YY165 (ΔmexB)	<i>P. aeruginosa</i> ATCC 25619	<i>P. aeruginosa</i> SR 27156	<i>P. aeruginosa</i> ATCC 27853
21	1491	>64	>64	>64	>64	>64
22	97.3	>64	>64	>64	>64	>64
23	225	>64	>64	>64	>64	>64
24	9.8	>64	>64	32	>64	>64
25	2.1	32	16	16	16	32
26	1.6	16	8	16	16	32
27	0.14	16	8	8	8	16
28	12.2	32	16	16	16	16
29	0.60	4	4	8	8	8
doripenem	n.d.	0.5	0.25	0.063	0.031	0.5

^aMICs were determined by a microdilution broth method as recommended by the CLSI with cation-adjusted Mueller–Hinton broth (CA-MHB). Serial 2-fold dilutions of each compound were made in appropriate broth, and the plates were inoculated with 5×10^4 CFU of each strain in a volume of 0.1 mL. Plates were incubated at 35 °C for 20 h and then MICs were scored.

Table 3. Metabolic Stability of Muraymycin Analogues 26, 27, and 29^a

	26	27	29
human	89.5% ^b	90.0%	86.9%
rat	81.1%	80.7%	69.8%

^aRat and human liver microsomes were incubated at 37 °C in Tris-HCl buffer (pH 7.4) containing 50 mM Tris-HCl, 150 mM KCl, 10 mM MgCl₂, 1 mM β-NADPH, and 2 μM compounds. The protein concentration was 0.5 mg/mL, and the final volume was 0.2 mL. Incubations were terminated by the addition of 2-fold volume of ice-cold acetonitrile/methanol 1:1, v/v after 0 and 30 min of incubation. Samples were centrifuged at 3000 rpm for 10 min prior to LC–MS/MS analysis of the supernatants. Experiments were conducted in duplicate. ^bPercentage of the remaining material after treatment with liver microsomes.

gave 21–26, respectively. Scheme 3 illustrates the preparation of analogues 27–29. Another key intermediate carboxylic acid 31^{23,24} was coupled with Boc-protected 4-aminobutyl guanidine G (see Supporting Information) by EDCI and HOBt to give 32 in 89% yield. After the Cbz protecting group was removed by hydrogenolysis catalyzed by Pd/C, reductive alkylation of the resulting amine with TrocHNCH₂CH₂CHO afforded 34 in 89% yield. Removal of the Troc group (Zn and MeOH), acylation with palmitic acid (EDCI and HOBt), and deprotection (80% aqueous TFA) provided 27 in 50% yield over three steps. In a manner similar to the synthesis of 27, the analogue 28 and the hybrid-type analogue 29 were prepared.

The MraY inhibitory activity of 21–29 was evaluated (Table 2). The linear analogues 21 and 22 reduced the inhibitory activity. As for interconverted analogues 23–26, the length of the lipophilic side chain is important for the MraY inhibitory activity. Thus, the analogue 23 with the short side chain largely reduced the activity with the IC₅₀ value of 225 nM. Increasing the length of the side chain resulted in an increase of the inhibitory activity, and the length and the activity were well-correlated. Analogue 26 with C16 side chain exhibited the IC₅₀ value of 1.6 nM. Analogues 27 and 29 exhibited strong MraY inhibitory activity (IC₅₀ = 0.14 nM for 27 and 0.60 nM for 29, respectively). The anti-Pseudomonas activity of this series of the compounds was then evaluated (Table 2). The analogues 21 and 22 did not show any activity. The analogues 23 and 24, which have the shorter lipophilic side chain, exhibited no antibacterial activity, whereas 25 and 26 with the longer side

chain showed moderate activity. As was indicated by the first screening shown in Table 1, two functional groups, a lipophilic side chain and a guanidino group, at the accessory moiety were important for the anti-Pseudomonas activity. The antibacterial activity was also well-correlated to the length of the lipophilic side chain. The branching analogues 27 and 28 have a similar anti-Pseudomonas activity to 26. Finally, the hybrid-type analogue 29 was the strongest activity among those tested in this study. It is worthy to mention that *P. aeruginosa* YY165 is slightly more sensitive to these analogues than other strains tested in this study. *P. aeruginosa* YY165 is a deletion mutant of MexB, which is an inner membrane transporter component of the MexAB-OprM multidrug efflux system that confers multidrug resistance. The sensitivity of analogues to *P. aeruginosa* YY165 indicates that they could be a substrate of the MexAB-OprM multidrug efflux system and excluded outside the cell. The analogues 27 and 29 are almost equipotent in terms of MraY inhibitory and anti-Pseudomonas activities. Selective toxicity is a key concern in the chemotherapy for infectious diseases. Since the chemical structure of 27 and 29 contains the long lipophilic side chain and positively charged guanidine functionality, it is suspected that the analogues 27 and 29 act as detergents, which are sometimes cytotoxic. Accordingly, their cytotoxic activity against human hepatocellular liver carcinoma (HepG2) cells was then evaluated. Analogue 27 showed cytotoxicity with an IC₅₀ value of 4.5 μg/mL. Under these conditions, 29 exhibited less cytotoxicity (IC₅₀ 34 μg/mL). With the observed higher therapeutic index than 27, analogue 29 could be a lead for further development as anti-Pseudomonas agent although much improvement to reduce the toxicity is necessary. Finally, the metabolic stability of 26, 27, and 29 was briefly evaluated by treatment of these analogues with human or rat liver microsome at 37 °C for 30 min and LC–MS/MS analysis of remaining analogues (Table 3). Around 90% of each analogue was unaffected by human liver microsomes, and these analogues were revealed to be metabolically stable especially to human liver microsomes.

In conclusion, antibacterial spectrum of the MRYs toward *P. aeruginosa* was investigated by the systematic SAR study of the MRYs. It was revealed that two functional groups, a lipophilic side chain and a guanidino group, at the accessory moiety of MRYs were important for the anti-Pseudomonas activity. The knowledge obtained from our SAR study of the MRYs would

provide future direction toward the expansion of the antibacterial spectrum of the MRYS to find more potent and selective agents active against *P. aeruginosa*.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis and characterization of synthesized compounds, fluorescence-based *MraY* assay, antibacterial activity evaluation, and metabolic stability testing. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Funding

This research was supported by JSPS Grant-in-Aid for Challenging Exploratory Research (Grant Number 22659020 to S.I.) and Scientific Research (B) (Grant Number 25293026 to S.I.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Ms. S. Oka and Ms. A. Tokumitsu (Center for Instrumental Analysis, Hokkaido University) for measurement of the mass spectra. We are also thankful for Ms. M. Okane and Mr. K. Uotani (Shionogi Techno Advance Research Co., Ltd.) for MIC measurement and Mr. K. Sekiguchi (Shionogi Co., Ltd.) for evaluating metabolic stability.

■ ABBREVIATIONS

Cbz, benzyloxycarbonyl; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxy-1*H*-benzotriazol; HepG2, human hepatocellular liver carcinoma; IC₅₀, 50% inhibitory concentration; MIC, minimum inhibitory concentration; MRY, muraymycin; *MraY*, phospho-MurNAc-pentapeptide transferase; MRSA, methicillin-resistant *Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; SAR, structure–activity relationship; TFA, trifluoroacetic acid; Troc, trichloroethoxycarbonyl; C₅₅-P, undecaprenyl–phosphate; VRE, vancomycin-resistant *Enterococci*

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