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Expansion of Antibacterial Spectrum of Muraymycins toward *Pseudomonas aeruginosa*

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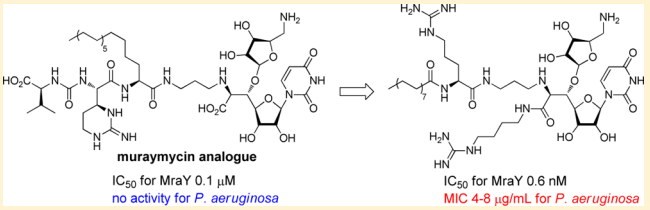
Supporting Information

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 μg/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]

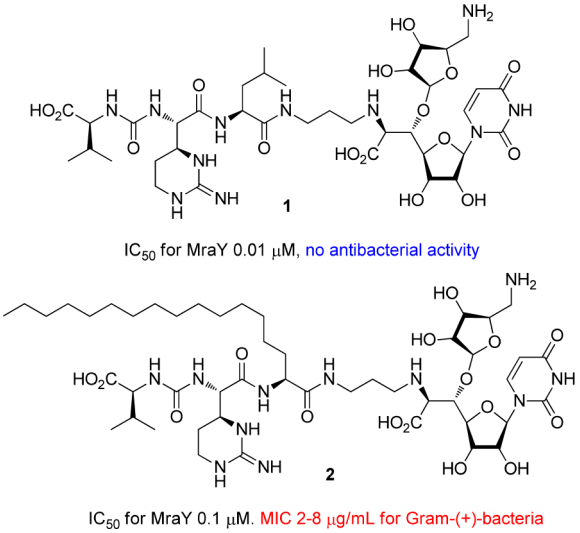


Figure 1. Structures and biological activity of muraymycin D2 and its lipophilic analogue.

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-

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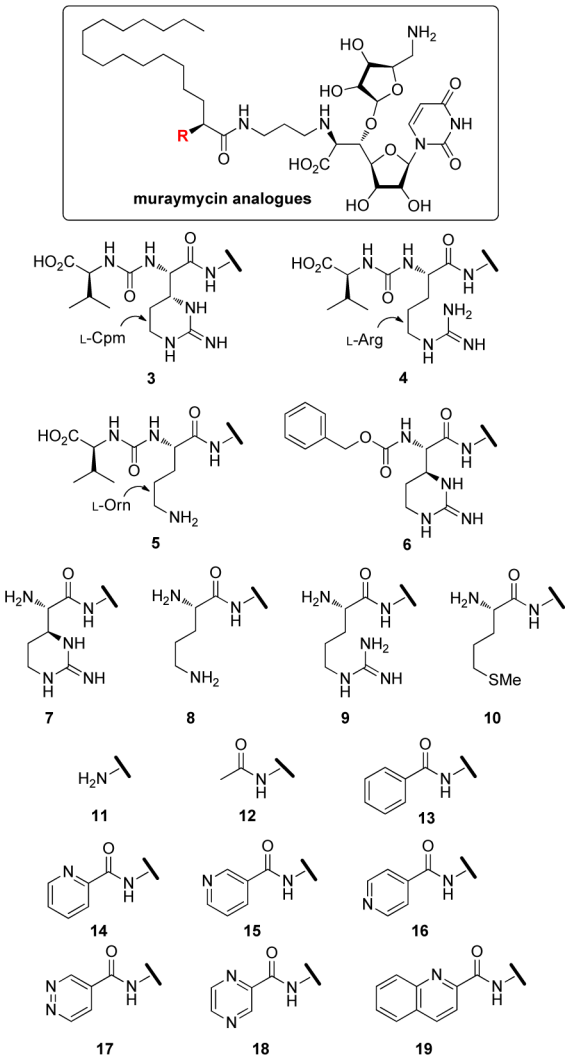
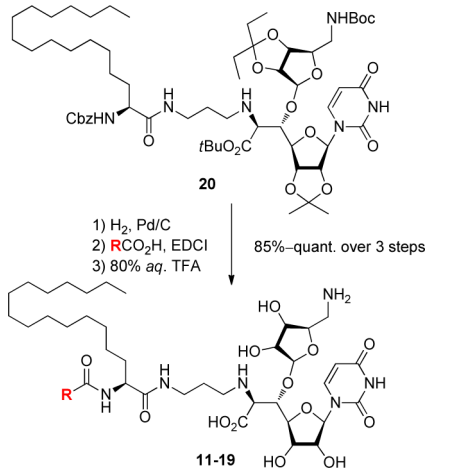


Figure 2. Structures of muraymycin analogues.

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 μg/mL. Analogue **9**, which possesses the guanidino and the amino functional groups, was the most active among those tested (MIC values of 8−32 μg/mL). It is a great contrast that the analogues **3** and **4** also have guanidine 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-pentadecylglycin residue, the preparation of which required a number of steps. The molecular design of **23**−**26** enables us to use a commercially

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

		MIC (μg/mL)[a]				
compound	MraY IC_{50} (nM)	*P. aeruginosa* PAO1	*P. aeruginosa* YY165 (ΔmexB)	*P. aeruginosa* ATCC 25619	*P. aeruginosa* SR 27156	*P. aeruginosa* 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

[a]MICs 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.

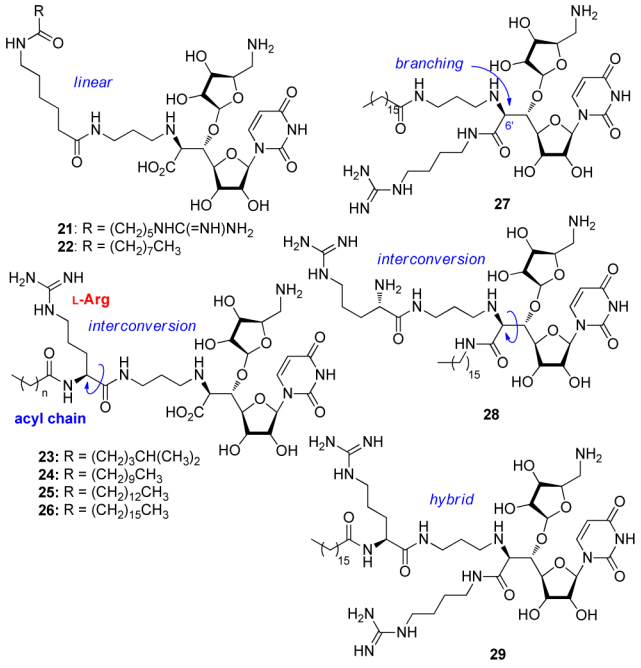
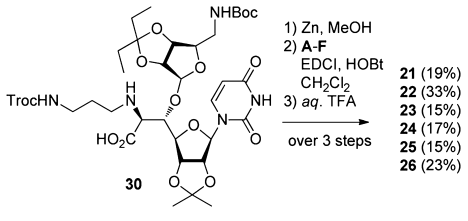
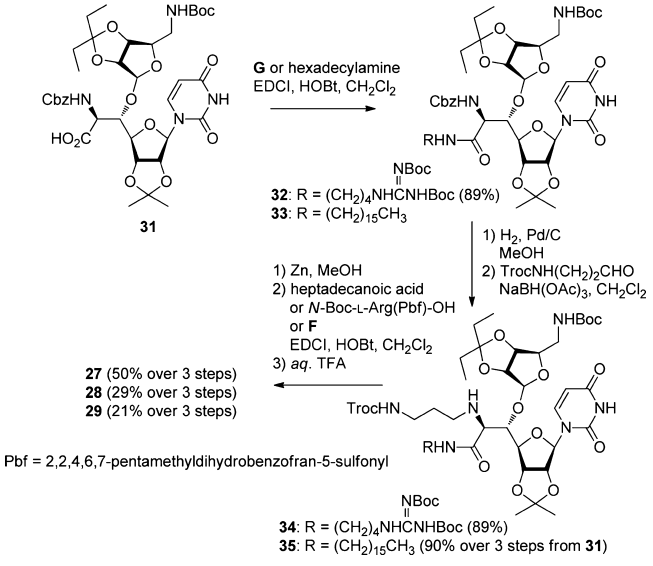


Figure 3. Molecular design of analogues **21**−**29**.

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 CH_2Cl_2. Finally, the global deprotection by aqueous TFA successfully

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

		MIC (μg/mL)[a]				
compound	MraY IC_{50} (nM)	*P. aeruginosa* PAOl	*P. aeruginosa* YY165 (ΔmexB)	*P. aeruginosa* ATCC 25619	*P. aeruginosa* SR 27156	*P. aeruginosa* 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

[a]MICs 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%

[a]Rat 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 $MgCl_2$, 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. [b]Percentage 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 $TrocHNCH_2CH_2CHO$ 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 IC_{50} 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 IC_{50} value of 1.6 nM. Analogues **27** and **29** exhibited strong MraY inhibitory activity (IC_{50} = 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 IC_{50} value of 4.5 μg/mL. Under these conditions, **29** exhibited less cytotoxicity (IC_{50} 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

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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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Notes

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■ ABBREVIATIONS

Cbz, benzyloxycarbonyl; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HOBt, 1-hydroxy-1*H*-benzotriazol; HepG2, human hepatocellular liver carcinoma; IC_{50}, 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; C_{55}−P, undecaprenyl−phosphate; VRE, vancomycin-resistant *Enterococci*

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