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Antibacterial Muraymycins from Mutant Strains of *Streptomyces* sp. NRRL 30471

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Abstract

Muraymycins are nucleoside antibiotics isolated from *Streptomyces* sp. NRRL 30471 and several mutant strains thereof that were generated by random, chemical mutagenesis. Reinvestigation of two mutant strains using new media conditions led to the isolation of three new muraymycin congeners named B8, B9 and C6 (**1–3**), as well as a known muraymycin C1. Structures of the compounds were elucidated by HRMS and 1D- and 2D-NMR spectroscopic analyses. Complete 2D NMR assignments for the known muraymycin C1 are also provided for the first time.

Compounds **1** and **2**, which differ from other muraymycins by having an elongated, terminally branched fatty acid side chain, had picomolar IC₅₀ values against *Staphylococcus aureus* and *Aquifex aeolicus* MraY and showed good antibacterial activity against *S. aureus* (MIC = 2 and 6 µg/mL, respectively) and *Escherichia coli* *tolC* (MIC = 4 and 2 µg/mL, respectively). Compound **3**, which is characterized by an N-acetyl modification of the primary amine of the dissaccharide core that is shared among nearly all of the reported muraymycin congeners, greatly reduced its inhibitory and antibacterial activity compared to nonacylated muraymycin C1, which possibly indicates this modification is used for self-resistance.

Graphical Abstract

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#Zheng Cui and Xiachang Wang contributed equally to this work.

Supporting Information Available: ¹H NMR, ¹³C NMR, ¹H-¹H COSY, HSQC, HMBC, TOCSY, NOESY, and HR-ESI-MS of compounds **1–3** and muraymycin C1. The Supporting Information is available free of charge via the Internet at <http://pubs.acs.org>.

Muraymycins, initially reported as a mixture of nineteen congeners from the fermentation broth of *Streptomyces* sp. NRRL 30471,¹ were discovered using biological assays to identify inhibitors of Lipid II and peptidoglycan cell wall biosynthesis.^{1–4} Muraymycin A1, one of the more potent of the congeners, was shown to have good activity against *Staphylococcal* (MIC 2 to 16 $\mu\text{g/mL}$) and *E. coli imp* mutant (MIC $<0.03 \mu\text{g/mL}$), and was further shown to be effective in a murine model of *Staphylococcus aureus* infection (ED_{50} 1.1 mg/kg). Follow-up efforts have revealed that muraymycins likely exhibit antibacterial activity by directly inhibiting translocase I (MraY). MraY initiates the lipid cycle of cell wall biosynthesis by catalyzing the transfer and attachment of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to a lipid carrier, undecaprenylphosphate, to form UMP and undecaprenyl-diphospho-MurNAc-pentapeptide, also referred to as Lipid I.^{5–9} Notably, several muraymycin synthetic variants have been screened against MraYs from different species, achieving IC_{50} values as low as 0.7 nM.⁸ However, only two naturally occurring muraymycins have been directly tested against MraY to date: muraymycin D1 and D2, both prepared by total synthesis and yielding IC_{50} values of 10 nM and 2.8 nM, respectively.^{6,9} Our understanding of muraymycin structure-activity relationship from these studies has recently been reviewed.¹⁰

Muraymycins have several unique structural features when compared with clinically used antibiotics. They are classified as lipopeptidyl nucleoside antibiotics, consisting of a (5'S, 6'S)-glycylyluridine (GlyU) core that is modified with two distinct components: an aminoribose attached by a traditional O-glycosidic bond and an unusual peptide attached by a propylamine linker (Figure 1).^{1,4} The different congeners have been categorized into four types (series A-D) based on structural variation of a Leu residue of the peptide moiety. The D series, which includes the aforementioned muraymycins D1 and D2, contain an unmodified L-Leu and are structurally the simplest. The C series of muraymycins contain a (3S)-3-hydroxy-L-Leu, and the A and B series are O-acylated variants of the hydroxylated L-Leu. The B series are characterized by a branched, saturated fatty acid ranging from seven to ten linear carbons (represented by muraymycin B2 in Figure 1) and the A series characterized by a terminal guanidinium-substituted fatty acid ranging from fourteen to sixteen linear atoms in length (represented by muraymycin A1 in Figure 1).¹

As part of our on-going efforts to characterize the inhibitory properties and establish structure-activity relationships of naturally occurring muraymycins toward the target MraY, we report the discovery, structural elucidation, and activity of three new muraymycins (**1–3**) from *Streptomyces* sp. NRRL30473 and NRRL 30477, two strains previously derived from *Streptomyces* sp. NRRL 30471 by random, chemical mutagenesis. Importantly, a complete suite of 1D and 2D NMR spectroscopic analysis was performed, and the entirety of the

NMR spectral data is provided in the public domain for the first time for any muraymycin congener. Compounds **1** and **2**, named muraymycin B8 and B9, respectively, differ from other muraymycins by having an elongated, terminally branched fatty acid side chain. Both are shown to be potent inhibitors of *Staphylococcus aureus* and *Aquifex aeolicus* *MraY* *in vitro*. Compound **3**, named muraymycin C6, is characterized by an N-acetyl modification of the primary amine of the aminoribose. The inhibitory activity of **3** against *MraY* is significantly reduced, which is consistent with an important role of this amine in target binding and suggests acetylation as a possible means of self-resistance by the producing strain.

RESULTS AND DISCUSSION

Initial attempts to produce muraymycins using the published fermentation conditions^{1,3} were unsuccessful. Hence, a few different media were examined. Using analytical scale fermentation with LC-MS analysis, production of muraymycins was observed using PM-1 media, which was previously employed for the production of capuramycin, a structurally distinct nucleoside antibiotic from a different *Streptomyces* sp.¹¹ As a consequence, a 100 mL seed culture of *Streptomyces* sp. strain NRRL 30473 was grown for three days and used to inoculate 10 L of PM-1 media. After fermentation for seven days, followed by extraction, fractionation, and resolution of the components within the crude extract, five known muraymycins were identified by LC-MS analysis (A1, B1, B2, B3, and B6). Two new congeners, muraymycins B8 (**1**, yield: 0.6 mg/L) and B9 (**2**, yield: 0.2 mg/L), with masses that did not match prior reports, were purified by reverse-phase HPLC. Using identical fermentation conditions with *Streptomyces* sp. strain NRRL 30477, one new congener, muraymycin C6 (**3**, yield: 0.4 mg/L), and one known muraymycin C1 (yield: 0.5 mg/L) were obtained and likewise isolated for structural elucidation and activity testing.

The molecular formula of **1** (C₆₄H₉₃N₁₁O₁₈) was determined by HR-ESI-MS (Figure S1). An initial analysis of the ¹H and ¹³C NMR data (Tables 1 and 2) in conjunction with the HSQC spectra in DMSO-*d*₆ showed the presence of 54 carbon resonances, which were ascribed to 7 methyls (including 1 methoxyl), 18 methylenes, 20 methines (including 2 sp² methines), and 9 amide/acid carbonyls (Figures S2–S4). The NMR and UV data for **1** displayed typical features of a muraymycin compound, including the GlyU nucleoside, ribofuranoside, aminopropane, epicapreomycinidine, urea, valine and hydroxy-leucine. Further interpretation of the 1D and 2D NMR data (including ¹H-¹H COSY, HMBC and TOCSY; Figures S5–S7) revealed the structure of **1**, which was similar to muraymycin B1¹ except for the fatty acid chain, which was identified as 14-methyl-pentadecanoic acid through 2D NMR (Figure 2). A strong HMBC correlation from H-22 (δ_{H} 4.92) to C-1' (δ_{C} 172.0) unambiguously determined the position of the pentadecanoic acyl group on hydroxy-leucine. Based on the NOESY spectrum (Figure S8), the relative configuration of **1** was assigned to be identical with that reported for muraymycin B1.¹ Thus, the structure of compound **1** was established as a new member of the muraymycin B family and subsequently named muraymycin B8.

Compound **2** was assigned the molecular formula C₆₂H₈₉N₁₁O₁₈ on the basis of HR-ESI-MS (Figure S9). A difference of C₂H₄ with **1** and their similar NMR spectroscopic data was

suggestive of **2** as a muraymycin B congener (Figures S10-S14). The fatty acid chain for **2** was identified as 12-methyl-tridecanoic acid from a thorough analysis of the 2D NMR data (Figure 2). The relative configuration of **2** was also established by NOESY (Figure S15). Compound **2** was designated as muraymycin B9.

The UV and NMR spectroscopic data for **3** (C₄₀H₆₅N₁₁O₁₈) revealed characteristic features of muraymycins (Tables 1 and 2, and Figures S16-S22) and were similar to that reported for muraymycin C1,¹ which was likewise isolated and structurally elucidated from this strain (Figures S23-S28).³ Specifically, **3** displayed an extra acetyl substitution of a methyl (δ_C 21.9 and δ_H 1.85) and a carboxylic acid (δ_C 174.1). The key HMBC correlations from H₂-16 (δ_H 3.05 and 3.41) to δ_C 174.1 established the *N*-acetyl at C-16. Based upon this analysis, the structure of **3** was established as 16-*N*-acetyl-muraymycin C1, a new muraymycin C congener and subsequently named muraymycin C6. Of note, 16-*N*-acetyl-muraymycin C1 was previously prepared from muraymycin C1 via chemical semisynthesis,² and the ¹H NMR spectrum of the naturally occurring congener matched the semisynthetic variant.

The newly isolated muraymycins were evaluated for inhibition of *MraY* activity. An *in vitro* fluorescence-based assay for *MraY* was previously developed and refined by multiple groups,^{12–16} which we have adapted and optimized for screening.¹⁷ In short, this assay required the synthesis of a dansylated derivative of the *MraY* substrate UDP-MurNAc-pentapeptide to generate a fluorescent readout, and overproduction of recombinant *S. aureus* and *A. aeolicus* *MraY*. The *MraY* homologue from *S. aureus* (*SaMraY*) was used as a crude membrane preparation as attempts to solubilize and purify the protein resulted in loss of activity. In contrast, the solubilization and purification of the *MraY* homologue from *Aquifex aeolicus* (*AaMraY*) was feasible,^{18,19} and the protein was used accordingly in the *in vitro* assays for *MraY* inhibition. The results for the inhibitory activities (IC₅₀ values) of the new muraymycins **1–3** along with the known compound muraymycin C1 are listed in Table 3. To summarize, **1**, **2**, and muraymycin C1 had potent IC₅₀ values ranging from 4–22 pM against *SaMraY* and 68–350 pM against *AaMraY*, which is significantly lower than IC₅₀ values reported for synthetic analogues and naturally occurring muraymycins D1 and D2 against *MraY* from different sources (D1, 11 nM against crude *MraY* from *Mycobacterium tuberculosis*;⁹ D2, 10 nM against purified *MraY* from *Bacillus subtilis*,⁶ and 2.8 nM against *SaMraY*⁸). The comparable IC₅₀ values with or without the acid chain is consistent with prior conclusions that a long chain, aliphatic component might not significantly contribute to *MraY* inhibition.^{5,6,10} Contrastingly, when compared with muraymycin C1, compound **3** showed reduced activity against both enzymes. This result is consistent with a significant role of the intact aminoribosyl group for *MraY* binding, which was postulated based upon analysis of the X-ray structure of muraymycin D2 in complex with *AaMraY*.¹⁸ It has been found that a conformational rearrangement occurs upon muraymycin D2 binding that generates a pocket for opportune interactions with the aminoribose.^{18,19}

We also examined the bacterial growth inhibition of **1–3** and muraymycin C1 against *S. aureus* and two strains of *E. coli*. Interestingly, the longer fatty acid side chains of **1** and **2** converted the B series from inactive against *S. aureus* (MIC > 32 µg/mL for muraymycin B2 and B6)^{1,3} to active against *S. aureus* (MIC = 2–6 µg/mL for muraymycin B8 and B9). Furthermore, this activity is comparable to or better than muraymycin A1,²⁰ which was

previously noted as the most potent muraymycin,^{1,3} hence warranting a continued evaluation of these new muraymycins as antistaphylococcal antibiotics. Contrastingly, muraymycin C1 and **3**, both of which lack a fatty acid side chain, were inactive against *S. aureus*. Taken together with the IC₅₀ values for MraY inhibition, this result suggests that the fatty acid unit indeed plays a role in cellular uptake as suggested.⁶ In contrast to the MIC data against *S. aureus*, the addition of the fatty acid side chain and increase in its length slightly reduced the activity against both efflux-deficient *E. coli tolC* (MIC for the B series range from 1–4 µg/mL) and efflux-competent *E. coli* BL21(DE3) (MIC from 8–64 µg/mL). Similarly to the trends observed with the IC₅₀ values, however, acetylation of muraymycin C1 at the aminoribose significantly reduced the MIC against all strains.

In conclusion, we examined the metabolic profile of *Streptomyces* sp. strain NRRL 30473 and *Streptomyces* sp. strain NRRL 30477 with the goal of developing a muraymycin production platform and expanding upon the known family of muraymycins. Four muraymycins were characterized by a suite of spectroscopic methods, including three new members of the muraymycin family: B8, B9 and C6 (**1–3**). Muraymycin B8 and B9 showed promising antibacterial activity and pM inhibition against *Sa*MraY and *Aa*MraY. Contrastingly, muraymycin C6, the first N-acetylated version of muraymycin to be isolated, showed reduced activity in all screens suggesting acetylation might be a mechanism of self-resistance.

EXPERIMENTAL SECTION

General Experimental Procedures.

UV spectra were recorded on a GE Ultraspec 8000 spectrometer. All NMR data was recorded at 400 MHz for ¹H and 100 MHz for ¹³C with Varian Inova NMR spectrometers or 600 MHz for ¹H and 150 MHz for ¹³C with an Agilent DD2 NMR spectrometer. LC-MS was conducted with an Agilent 6120 Quadrupole MSD mass spectrometer equipped with an Agilent 1200 Series Quaternary LC system and an Eclipse XDB-C₁₈ column (150 × 4.6 mm, 5 µm). HR-ESI-MS spectra were recorded on an AB SCIEX Triple TOF 5600 System. Analytic HPLC was performed with Waters Alliance 2695 separation module equipped with a Waters 2998 diode array detector and an analytical Apollo C₁₈ column (250 mm × 4.6 mm, 5 µm). Semipreparative HPLC was performed with a Waters 600 controller and pump equipped with a 996 diode array detector, 717 plus autosampler, and an Apollo C₁₈ column (250 × 10 mm, 5 µm) purchased from Grace. All solvents used were of ACS grade and purchased from Pharmco-AAPER. Sephadex LH-20 (25–100 µm) was purchased from GE Healthcare. A solution of resazurin sodium salt (Alfa Aesar) was prepared to 1 mg/mL in sterile distilled water.

Fermentation, Extraction, Isolation and Purification.

Streptomyces sp. NRRL 30473 and NRRL 30477 were cultivated on MS agar plates at 28 °C for 7 days. Chunks of the corresponding agar with bacterial growth were added to five 250 mL Erlenmeyer flasks, each containing 50 mL TSBG [Tryptic soy broth (BD) supplemented with 20 g/L glucose], at 30 °C on a rotary shaker (250 rpm) for 72 h. The cultures were used to inoculate 200 flasks (250 mL), each containing 50 mL PM-1 medium.

¹¹ PM-1 was composed of glucose 2%, soluble starch 1%, pressed yeast 0.9%, peptone (Bacto) 0.5%, meat extract (Fluka) 0.5%, NaCl 0.5%, CaCO₃ 0.3% and CB-442 (NOF Co., Ltd. Japan) 0.01% (pH 7.4, before sterilization). The fermentation was continued for 7 days at 23 °C on a rotary shaker (210 rpm).

All culture flasks were combined and centrifuged at 6000 × g for 15 min to separate the mycelium and water phase. The mycelial-cake portion was extracted with methanol (3 × 1 L) by sonication, and the organic phase was evaporated to afford 15 g of brown crude extract. Amberlite XAD-16 (4%, Sigma) resin was added to the water phase and stirred for 12 h. The resin was washed with water until the effluent became colorless, and then eluted with 3 L of methanol. The MeOH extract was concentrated under reduced pressure to obtain a crude extract (12 g). Components of the mycelium crude extract and water phase crude extract were subjected to Sephadex LH-20 column, and methanol was used to elute compounds at a flow rate at 2 mL/min to yield twelve fractions. Fraction I was further purified by using a semipreparative HPLC (CH₃CN–H₂O, 0.01 % TFA; flow rate: 3.0 mL/min) to yield **1** (6 mg) and **2** (2 mg). Compound **3** (4 mg) and muraymycin C1 (5 mg) were isolated from fraction II by semipreparative HPLC (CH₃CN–H₂O, 0.01 % TFA; flow rate: 3.0 mL/min).

muraymycin B8 (**1**): white amorphous powder; UV (MeOH) $\lambda_{\max}$ (log *e*) 260 (3.83) nm; ¹³C and ¹H NMR data, see Tables 1 and 2; (+)-HR-ESI-MS: *m/z* 1184.6757 [M + H]⁺ (calcd for C₆₄H₉₄N₁₁O₁₈, 1184.6778).

muraymycin B9 (**2**): white amorphous powder; UV (MeOH) $\lambda_{\max}$ (log *e*) 258 (1.93) nm; ¹³C and ¹H NMR data, see Tables 1 and 2; (+)-HR-ESI-MS: *m/z* 1156.6458 [M + H]⁺ (calcd for C₆₂H₉₀N₁₁O₁₈, 1156.6465).

muraymycin C6 (**3**): white amorphous powder; UV (MeOH) $\lambda_{\max}$ (log *e*) 261 (3.95) nm; ¹³C and ¹H NMR data, see Tables 1 and 2; (+)-HR-ESI-MS: *m/z* 988.4598 [M + H]⁺ (calcd for C₄₀H₆₆N₁₁O₁₈, 988.4587).

Antimicrobial activity.

The protocol used for the determination of the minimum inhibitory concentration (MIC) was as described previously with minor modifications.²¹ The bacterial strains *S. aureus* subsp. *aureus* (Newman strain), *E. coli* *tolC* mutant JW5503 (*E. coli* genetic stock center), and *E. coli* BL21(DE3) were used as model strains for antimicrobial susceptibility assays. All strains were grown in appropriate liquid or on agar plates using LB media (BD244620) for *S. aureus* and LB media supplemented with 50 µg/mL kanamycin for *E. coli* *tolC* mutant. Individual strains were grown in 5 mL of medium for 16 h at 37 °C with shaking (250 rpm), and then were diluted 1000-fold into 4.5 mL of medium and incubated until OD₆₀₀ reaching 0.4. An aliquot of the suspensions was again diluted 1000-fold. Aliquots (90 µL) of each diluted culture were transferred into the individual well of a 96-well plate supplied with 5 µL of the tested compound. Maximum final concentration of 256 µg/mL or 32 µg/mL with serial dilutions was maintained to obtain the antimicrobial activities and compared to the negative control 1% DMSO or water alone. The culture plates were incubated at 37 °C for 16 h with shaking (160 rpm) for *E. coli* strains and (50 rpm) for *S. aureus*. The OD₆₀₀ of

each well was measured using BioTek™ Synergy™ 2 Multi-Mode Microplate Readers. The acquired OD₆₀₀ values were normalized to the negative control wells (100% viability). Resazurin solution (5 µL) was also added to each well, and the plates were shaken for 10 s and incubated at 37 °C for another 3 h to allow resazurin to convert to resorufin by viable bacteria. The minimal concentration of the tested compound that caused growth inhibition was recorded as the MIC.

Overexpression of MraY from *S. aureus*.

The overexpression of *SaMraY* in *E. coli* was performed as described, and a crude membrane preparation was employed for *in vitro* MraY assays.¹⁷

Overexpression of MraY from *Aquifex aeolicus*.

For the overexpression of *AaMraY* in *E. coli*, the plasmid previously reported by Lee and co-workers^{18,19} was used. This plasmid contained the codon-optimized *mraY* gene from *Aquifex aeolicus* encoding a fusion protein with decahistidine maltose-binding protein (His-MBP) and a PreScission protease cleavage site for removal of the tag. The plasmid was transformed into *E. coli* C41(DE3) cells and MraY was expressed in terrific broth (TB) medium containing 35 µg/mL kanamycin. Protein expression was induced at OD₆₀₀ ~1.0 at 37 °C for 3 h using 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation at 5750 × g. All further steps were carried out at 4 °C. The cells were resuspended in 10–20 mL resuspension buffer per liter culture [resuspension buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM β-mercaptoethanol (βME), lysozyme, DNase I and one cOmplete™ EDTA-free Protease Inhibitor Cocktail tablet], the cells were lysed by sonication (30% amplitude, 3/10 s pulse, 7/10 s pause for 15 min). *n*-Dodecyl-β-D-maltoside (DDM, 30 mM) was added and the resultant lysate was stirred for 2 h. After centrifugation at 30000 × g for 45 min, the cleared lysate was loaded onto pre-equilibrated TALON Superflow™ resin (~0.5 mL/L culture), imidazole was added to a final concentration of 7 mM and the mixture was rotated at 4 °C overnight. Subsequently, the resin was washed with 20 column volumes of washing buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM βME, 10 mM imidazole, 1 mM DDM] and eluted with 10–20 mL elution buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM βME, 250 mM imidazole, 1 mM DDM]. Dithiothreitol (DTT, final concentration of 1 mM), ethylenediaminetetraacetic acid (EDTA, final concentration of 1 mM) and PreScission protease (ratio to His-MBP-MraY 1:100) were added and the solution was incubated at 4 °C overnight. The cleaved protein was separated on a HiLoad 16/600 Superdex 200 pg in a buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM DTT and 5 mM *n*-decyl-β-D-maltopyranoside (DM). The resultant solution was employed as a source of solubilized and purified *AaMraY* for *in vitro* MraY assays.

Fluorescence-based MraY assay.

In vitro MraY assays were performed using a previously reported adapted version¹⁷ of Bugg's fluorescence-based method.^{12–16} Fluorescence intensity over time was measured at λ_{ex} = 355 nm and λ_{em} = 520 nm (BMG Labtech POLARstar Omega, 384-well plate format). Each well contained a total volume of 20 µL with 100 mM Tris-HCl buffer (pH 7.5), 200 mM KCl, 10 mM MgCl₂, 0.1% Triton X-100, 0–5% DMSO, 50 mM undecaprenyl

phosphate, 7.5 mM dansylated Park's nucleotide (synthetic or semi-synthetic),¹⁷ a protein preparation (see below) and the potential inhibitor at various concentrations.

For *Sa*MraY, 1 μ L of a crude membrane preparation with a total protein concentration of 1.0 mg/mL was used, and the reaction was initiated by the addition of the protein preparation. For *Aa*MraY, 1 μ L of a solution of purified protein was used and preincubated with the assay mixture (including the inhibitor but excluding the dansylated Park's nucleotide) at room temperature for 30 min. The reaction was then initiated by the addition of Park's nucleotide. Concentration of the purified *Aa*MraY was 0.1 mg/mL. The protein preparations of *Sa*MraY and *Aa*MraY showed comparable activity in the absence of inhibitors. MraY activity at a certain inhibitor concentration was determined by a linear fit of the fluorescence intensity curve from 0 to 2 min. This measure of enzymatic activity was plotted against the logarithmic inhibitor concentration and fitted with a sigmoidal fit using the formula shown below, thus furnishing the reported IC₅₀ values. Data were collected in triplicate.

$$y = A1 + \frac{(A2 - A1)}{1 + 10^{\log(x_0 - x) \cdot p}}$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of representative muraymycins.

Figure 2.
 ^1H - ^1H COSY (—) and selected HMBC (→) correlations of **1–3**.

Table 1.

¹H NMR data (400 MHz, *J* in Hz) of compounds **1–3** and muraymycin Cl.

no.	δ^a	$2^{a,b}$	δ^c	no.	δ^a	$2^{a,b}$	δ^c
1	7.75, d (8.0)	7.75, d (9.5)	7.62, d (6.4)	25	0.79 ^e	0.79 ^e	0.76, d (5.3)
2	5.70 ^d	5.68 ^d	5.73, d (6.4)	27	4.39, m	4.38, m	4.19, m
5	5.66 ^d	5.66 ^d	5.62, d (2.3)	28	3.60, m	3.61, m	3.71, m
6	4.13, m	4.14, m	4.25, m	29	1.70, m	1.71, m	1.72, m
7	3.98, m	3.99, m	4.12, m	30	3.19, 3.29, m	3.19, 3.29, m	3.12, 3.19, m
8	4.10, m	4.11, m	4.18, m	34	4.03, m	4.03, m	3.93, m
9	4.42, m	4.42, m	4.43, dd (0.8, 3.1)	35	2.02, m	2.03, m	1.96, m
10	3.58, m	3.57, m	3.92, m	36	0.86 ^e	0.86 ^e	0.75, d (5.2)
12	5.11, br s	5.11, br s	5.11, d (1.9)	37	0.82 ^e	0.82 ^e	0.70, d (5.8)
13	3.54, m	3.54, m	3.68, m	3-NH	11.42, s	11.39, s	
14	4.03, m	4.05, m	4.08, m	13-OCH ₃	3.27, s	3.28, s	3.25, s
15	3.95, m	3.94, m	3.95, m	16-NH ₂	8.02, br s	8.02, br s	
16	3.03, m	3.03, m	3.05, 3.41, m	16-NHC(=O)CH ₃			1.85, s
17	2.87, 2.94, m	2.88, 2.95, m	2.95, 3.13, m	19-NH	8.23, br s	8.21, br s	
18	1.73, m	1.73, m	1.74, m	21-NH	8.50, d (8.2)	8.47, d (9.6)	
19	2.93, 3.16, m	2.92, 3.17, m	3.05, 3.18, m	27-NH	6.65, d (8.2)	6.66, d (9.3)	
21	4.44, m	4.44, m	4.14, m	30-NH	8.02, br s	8.02, br s	
22	4.92, dd (2.7, 6.4)	4.92, dd (1.1, 5.8)	3.47, dd (3.5, 6.6)	31-NH	7.84, br s	7.83, br s	
23	1.89, m	1.90, m	1.57, m	32-NH	6.50, d (9.5)	6.50, d (10.1)	
24	0.82 ^e	0.82 ^e	0.65, d (5.3)				

no.	1 ^a	2 ^{a,b}	3 ^c	CI ^c	no.	1 ^a	2 ^{a,b}	3 ^c	CI ^c
Fatty acid part for 1			1.48 2.24 1.23	1.22 1.11 0.83	Fatty acid part for 2		1.49 2.26 1.23	1.23 1.13 0.83	CI ^c

^a measured in DMSO-*d*₆;^b measured at 600 MHz;^c measured in D₂O;^{d,e} overlapped signals.

Table 2.

¹³C NMR data (100 MHz) of compounds **1–3** and muraymycin Cl.

no.	1 ^a	2 ^{a,b}	3 ^c	Cl ^c	no.	1 ^a	2 ^{a,b}	3 ^c	Cl ^c
1	141.6, CH	141.5, CH	141.9, CH	142.3, CH	21	53.4, CH	53.4, CH	56.1, CH	56.0, CH
2	101.7, CH	101.7, CH	101.9, CH	102.2, CH	22	75.0, CH	75.0, CH	74.4, CH	74.6, CH
3	163.1, C	163.1, C	165.9, C	165.9, C	23	27.8, CH	27.8, CH	29.1, CH	29.2, CH
4	150.3, C	150.3, C	151.2, C	151.2, C	24	15.7, CH ₃	15.7, CH ₃	14.6, CH ₃	14.7, CH ₃
5	90.6, CH	90.6, CH	91.1, CH	91.8, CH	25	19.6, CH ₃	19.6, CH ₃	18.7, CH ₃	18.7, CH ₃
6	72.6, CH	72.5, CH	72.8, CH	72.5, CH	26	169.5, C	169.5, C	171.2, C	171.1, C
7	70.0, CH	69.9, CH	69.0, CH	69.1, CH	27	54.8, CH	54.8, CH	55.3, CH	55.4, CH
8	82.8, CH	82.8, CH	83.4, CH	83.7, CH	28	50.0, CH	50.0, CH	49.1, CH	49.1, CH
9	76.3, CH	76.3, CH	76.4, CH	76.3, CH	29	20.4, CH ₂	20.4, CH ₂	20.3, CH ₂	20.3, CH ₂
10	62.5, CH	62.5, CH	63.6, CH	62.8, CH	30	35.6, CH ₂	35.6, CH ₂	35.6, CH ₂	35.7, CH ₂
11	168.8, C	168.7, C	169.9, C	170.3, C	31	153.7, C	153.7, C	153.6, C	153.6, C
12	105.2, CH	105.1, CH	106.5, CH	106.7, CH	32	157.5, C	157.5, C	158.7, C	158.7, C
13	84.1, CH	84.1, CH	83.9, CH	83.6, CH	33	173.7, C	173.6, C	177.3, C	176.4, C
14	71.8, CH	71.7, CH	70.1, CH	70.5, CH	34	57.5, CH	57.5, CH	59.4, CH	58.6, CH
15	79.3, CH	79.3, CH	82.9, CH	79.6, CH	35	30.3, CH	30.3, CH	30.1, CH	29.9, CH
16	42.1, CH	42.0, CH	41.7, CH	42.2, CH	36	19.1, CH ₃	19.1, CH ₃	18.5, CH ₃	18.3, CH ₃
17	45.5, CH ₂	45.5, CH ₂	45.4, CH ₂	45.6, CH ₂	37	17.4, CH ₃	17.4, CH ₃	16.6, CH ₃	16.6, CH ₃
18	25.5, CH ₂	25.4, CH ₂	25.2, CH ₂	25.0, CH ₂	14-OCH ₃	57.5, CH ₃	57.4, CH ₃	57.8, CH ₃	57.9, CH ₃
19	36.1, CH ₂	36.0, CH ₂	35.8, CH ₂	35.8, CH ₂	16-NHCOCH ₃			174.1, C	
20	169.2, C	169.1, C	172.5, C	172.5, C	16-NHCOCH ₃			21.9, CH ₃

^a measured in DMSO-*d*₆

η measured at 150 MHz;

ϵ measured in D₂O.

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Table 3.*In vitro* MraY assays and antimicrobial activities.

compounds	<i>S. aureus</i> MraY IC ₅₀ (pM)	<i>A. aeolicus</i> MraY IC ₅₀ (pM)	<i>S. aureus</i> growth inhibition MIC (μg/mL)	<i>E. coli</i> <i>tolC</i> growth inhibition MIC (μg/mL)	<i>E. coli</i> BL21(DE3) growth inhibition MIC (μg/mL)
1	4.0±0.7	(6.8±0.5)×10 ¹	2	4	64
2	22.1±3.2	(3.2±0.2)×10 ²	6	2	32
3	93±8	(1.8±0.4)×10 ³	>32	16	256
muraymycin C1	15.7±1.2	(3.5±0.4)×10 ²	>32	1	8