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Synthesis and evaluation of cyclopentane-based muraymycin analogs targeting MraY

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ABSTRACT

Antibiotic resistance is one of the most challenging global health issues and presents an urgent need for the development of new antibiotics. In this regard, phospho-MurNAc-pentapeptide translocase (MraY), an essential enzyme in the early stages of peptidoglycan biosynthesis, has emerged as a promising new antibiotic target. We recently reported the crystal structures of MraY in complex with representative members of naturally occurring nucleoside antibiotics, including muraymycin D2. However, these nucleoside antibiotics are synthetically challenging targets, which limits the scope of medicinal chemistry efforts on this class of compounds. To gain access to active muraymycin analogs with reduced structural complexity and improved synthetic tractability, we prepared and evaluated cyclopentane-based muraymycin analogs for targeting MraY. For the installation of the 1,2-*syn*-amino alcohol group of analogs, the diastereoselective isocyanoacetate aldol reaction was explored. The structure–activity relationship analysis of the synthesized analogs suggested that a lipophilic side chain is essential for MraY inhibition. Importantly, the analog **20** (JH-MR-23) showed antibacterial efficacy against *Staphylococcus aureus*. These findings provide insights into designing new muraymycin-based MraY inhibitors with improved chemical tractability.

1. Introduction

The global burden of multidrug-resistant infections is a major threat to global public health [1]. A decline in chemical and pharmaceutical research to develop new antibiotics has resulted in few truly new antibiotics in the pipeline. At the same time, widespread antibiotic resistance is steadily eroding the effectiveness of existing treatment options. Therefore, an urgent need exists for new antibiotics with novel modes of action.

Peptidoglycan is a cross-linked polymer of carbohydrates and amino acids that comprises the cell wall of both Gram-negative and Gram-positive bacteria [2]. It is essential for bacterial survival as it is responsible for maintaining cell shape by stabilizing the membrane against osmotic pressure. Hence, biosynthesis of peptidoglycan is a well-established target for antibiotic development. The late stages of peptidoglycan biosynthesis (i.e., cross-linking) have been extensively explored, which has resulted in the development of the penicillin and vancomycin classes of antibiotics. However, the early stages have been underexplored despite the fact that there are many natural product inhibitors targeting these stages. Therefore, these early steps offer excellent opportunities for new antibiotic development.

Among the enzymes involved in the early stages of peptidoglycan biosynthesis, phospho-MurNAc-pentapeptide translocase (MraY) is a member of the polyprenyl-phosphate *N*-acetylhexosamine 1-phosphate-transferase (PNPT) superfamily. MraY is an integral membrane protein that catalyzes the first membrane step of bacterial cell wall biosynthesis, the transfer of the peptidoglycan precursor phospho-MurNAc-pentapeptide to the lipid carrier undecaprenyl phosphate (C_{55}–P) [3]. This is an essential membrane step of peptidoglycan biosynthesis, and as a result, the inhibition of MraY leads to cell lysis. Therefore, MraY has long been considered a

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promising target for the development of new antibiotics [4–6]. However, despite many years of effort, the development of antibiotics targeting MraY has been stagnant largely due to insufficient understanding of MraY structure, function, and inhibition. Recently, we reported the crystal structures of *Aquifex aeolicus* MraY ($MraY_{AA}$) alone and in complex with representative members of the liposidomycin/caprazamycin, capuramycin, and mureidomycin classes of nucleoside inhibitors [7–9]. The analysis of these crystal structures enhanced our understanding of the mechanisms of MraY catalysis and inhibition by natural products and paved the way to the development of new MraY-targeting antibiotics.

Among the naturally occurring nucleoside antibiotics, the muraymycins (Fig. 1a) were isolated from a broth of a *Streptomyces* sp. and represent a promising class of new nucleoside antibiotics targeting MraY [10]. They have a glycyl-uridine motif connected via an aminopropyl linker to a urea peptide moiety consisting of L-leucine or L-hydroxyleucine, L-epicapreomycidine, and L-valine. The promising MraY inhibitory activity of muraymycins makes them attractive candidates for future antibacterial agent development.

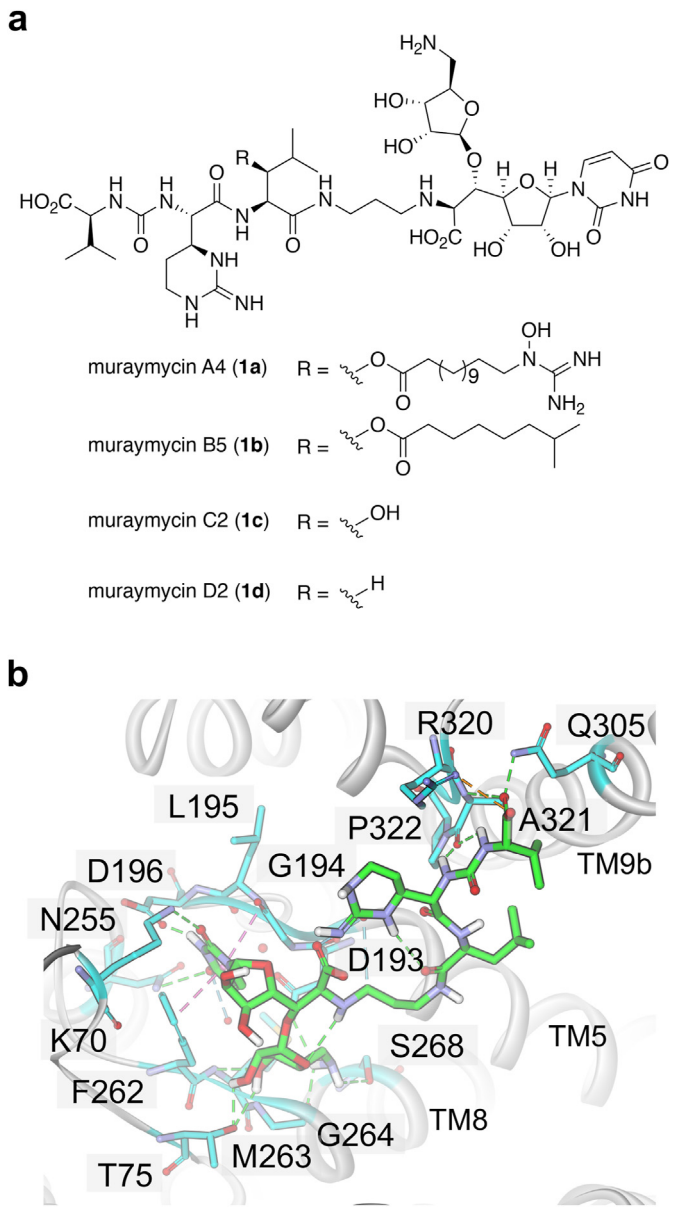


Fig. 1. (a) The structure of muraymycins. (b) Conformation of muraymycin D2 (**1d**, green color) in the **1d**-bound MraY structure (PDB: 5CKR). The hydrogen bond, water–hydrogen bond, π–π stacking interaction, and salt bridge interaction between **1d** and MraY are shown in green, cyan, magenta, and orange dashed lines, respectively. Water molecules are presented as red spheres.

Since the first total synthesis of muraymycin D2 reported by Ichikawa, Matsuda, and co-workers [11], there have been a number of reports on the synthesis and biological evaluation of muraymycins and muraymycin analogs [12–27]. However, despite great efforts in the synthesis of muraymycins, they are still challenging synthetic targets. Moreover, the structure–activity relationship (SAR) of muraymycins reported to date has primarily focused on the peptide motif and the 5′-position with the amino ribose [16–18]; little is known about the role of the ribose core of muraymycins in MraY inhibition [28]. Therefore, development of muraymycin analogs with modifications on the ribose moiety would help to elucidate the role of the ribose unit of muraymycins in MraY inhibition.

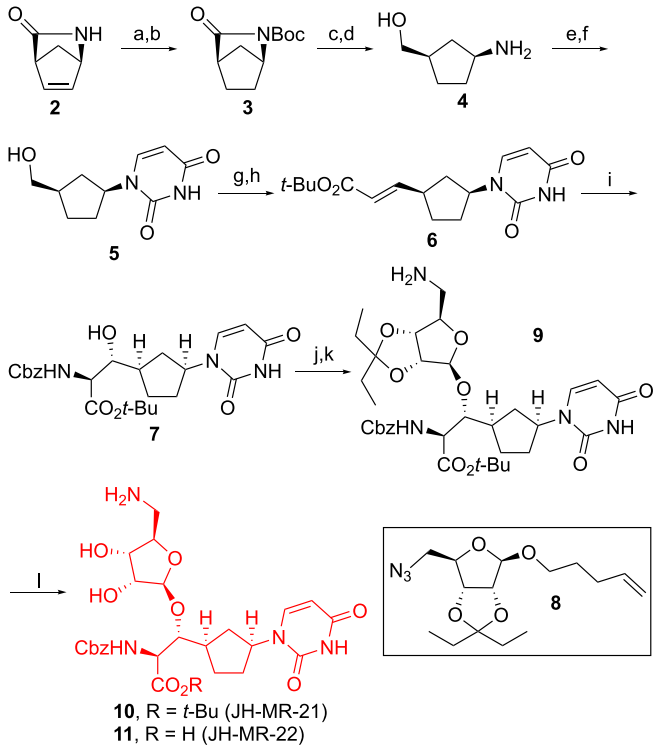
Towards this goal, we embarked on the synthesis of cyclopentane core analogs of muraymycins. As the ribose of muraymycins has no specific interaction with the amino acid residues within the active site of MraY (Fig. 1b) [8], we reasoned that a cyclopentane with a similar ring conformation as the tetrahydrofuran ring of the ribose would be an excellent substitute for the ribose moiety of muryamycins. More importantly, it is more amenable to modifications with various substituents than the ribose ring of muraymycins. Indeed, it has been reported that the replacement of a ribose with a cyclopentane ring improved the biological activity of the cyclopentane analog over the original compound [29]. By substituting the ribose of muraymycins with a cyclopentane ring, we anticipated to gain access to active muraymycin analogs with reduced structural complexity and/or improved synthetic tractability. Here we report the synthesis and evaluation of cyclopentane core-based muraymycin analogs.

2. Results and discussion

2.1. Chemistry

The synthesis of the key intermediate **5** started with the commercially available (1*R*,4*S*)-2-azabicyclo[2.2.1]hept-5-en-3-one (**2**) (Scheme 1). Catalytic hydrogenation of **2** followed by *N*-Boc protection gave the lactam **3** [30] (81% over two steps). Next, the reductive ring cleavage of **3** with $NaBH_4$ and boiling water catalyzed neutral *N*-Boc deprotection [31] gave the known amine **4** [30] in good yield (64% over two steps). To introduce a uracil group, we coupled **4** with 3-ethoxyacryloyl isocyanate following the previously reported procedure [32]. The final acid-mediated cyclization of the urea intermediate completed the synthesis of the key intermediate **5**.

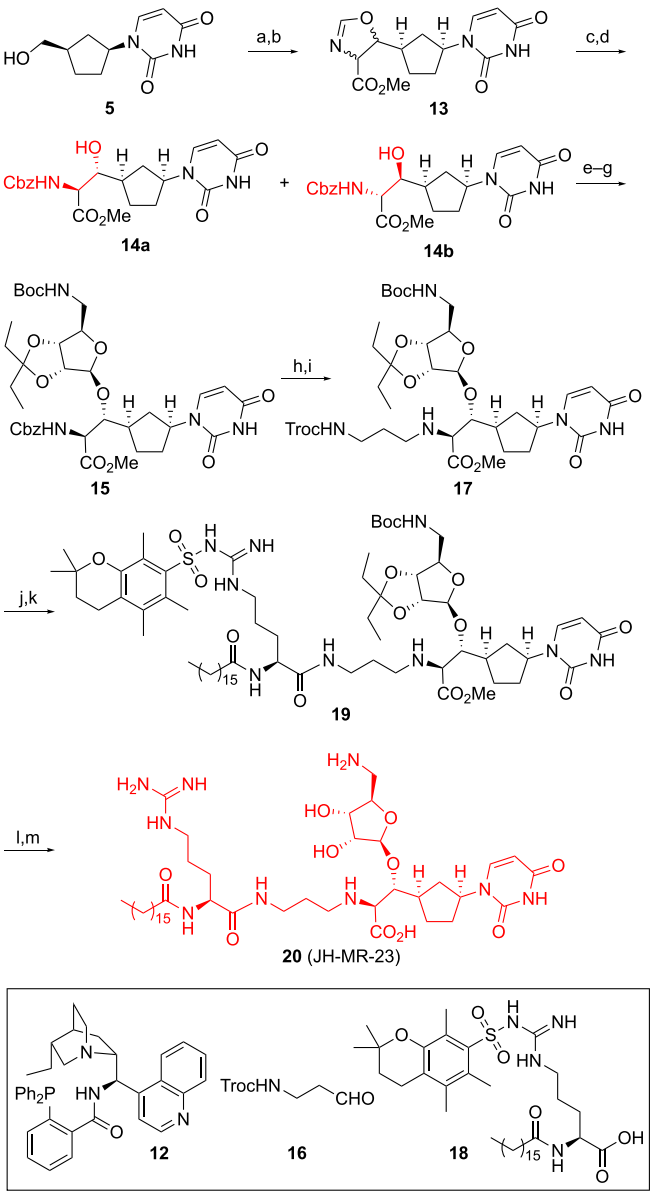
After the preparation of **5**, the installation of the 1,2-*syn*-amino alcohol moiety of muraymycins began with the oxidation of **5** to the corresponding aldehyde. Initial attempts for the oxidation of **5** such as Swern or PCC oxidation did not provide the desired aldehyde, which led us to use of the IBX oxidation conditions reported by Matsuda and co-workers [33]. The IBX oxidation of **5** smoothly proceeded to provide the corresponding aldehyde, which was converted to the α,β-unsaturated ester **6** by treating with (*tert*-butoxycarbonylmethylene)triphenylphosphorane. The Sharpless aminohydroxylation reaction [33] of **6** was carried out with $(DHQD)_2AQN$ as a chiral ligand to afford the 1,2-*syn*-amino alcohol **7** (46%) as a single diastereomer. Interestingly, the aminohydroxylation reaction of **6** did not give other diastereomeric 2,3-amino alcohols as previously reported by others [34]. However, the stereoselective aminohydroxylation reaction of **6** suffered from the reproducibility and low-yield issues, which prompted us to



Scheme 1. Synthesis of cyclopentane-based MraY inhibitors **10** (JH-MR-21) and **11** (JH-MR-22).[a] [a]Reagents and conditions: (a) Pd/C, H_2, MeOH, 25 °C, 5 h; (b) Boc_2O, DMAP, MeCN, 25 °C, 1 h, 81% for two steps; (c) $NaBH_4$, MeOH, 25 °C, 3 h, 80%; (d) H_2O, reflux, 20 h, 80%; (e) 3-ethoxyacryloyl isocyanate, 4 Å MS, DMF, −20 to 25 °C, 15 h, 49%; (f) 1 N H_2SO_4, reflux, 30 min, 68%; (g) IBX, MeCN, 80 °C, 1.5 h; (h) $Ph_3P{=}CHCO_2t$-Bu, THF, 0 °C, 12 h, 45% for two steps; (i) $CbzNH_2$, $K_2OsO_2(OH)_4$, $(DHQD)_2AQN$, *t*-BuOCl, *n*-PrOH, 0.6 N NaOH, 5 to 25 °C, 2 h, 46% (dr > 20:1); (j) **8**, NIS, TESOTf, CH_2Cl_2, 4 Å MS, 25 °C, 30 min, 53%; (k) PPh_3, THF/toluene (1/1), H_2O, 25 °C, 12 h, 63%; (l) 80% TFA in THF/H_2O (1/1), 25 °C, 14 h; For **10**: 41%; For **11**: 91%.

explore other methods for the installation of the 1,2-*syn*-amino alcohol moiety (*vide infra*). Next, the glycosylation reaction of **7** with various glycosyl donors and activators was explored. After an extensive search for reaction conditions (see the Supplementary Information for details), treatment of **7** with the *n*-pentenyl glycoside **8** [35], TESOTf, and NIS provided the desired glycosylation product in 53% as a single diastereomer. The azide group of the glycosylation product was reduced under Staudinger's conditions to give the amine **9** (63%). The final global deprotection under acidic conditions gave either the partially deprotected *t*-Bu ester **10** (JH-MR-21) or the fully deprotected carboxylic acid **11** (JH-MR-22) as the final product depending on purity of **9** and reaction time.

Since the lipophilic peptide chains of muraymycins play an important role in MraY inhibition [11,16,18], we embarked on the synthesis of a cyclopentane analog with a lipophilic side chain (analog **20**) starting from the common intermediate **5**. As mentioned above, when we prepared the cyclopentane-based analogs **10** and **11**, the Sharpless aminohydroxylation gave low yield and inconsistent diastereoselectivity. To address these issues, we turned our attention to the diastereoselective isocyanoacetate aldol reaction reported by Dixon and co-workers [36] (Scheme 2). The IBX oxidation of **5** followed by the coupling of the resulting aldehyde with methyl isocyanoacetate in the presence of chiral aminophosphine ligand **12** and Ag_2O proceeded to give a 2:1 mixture of the aldol products **14a** and **14b**. The yield and stereoselectivity of the isocyanoacetate aldol reaction were sensitive to catalyst activation time and temperature (see the Supplementary Information



Scheme 2. Synthesis of a cyclopentane-based MraY inhibitor with a lipophilic side chain **20** (JH-MR-23).[a]
[a]Reagents and conditions: (a) IBX, MeCN, 80 °C, 1 h, 70%; (b) methyl isocyanoacetate, Ag_2O, **12**, EtOAc, 4 Å MS, −78 to 0 °C, 1.5 days; (c) 4 N HCl, THF, 25 °C, 1 h; (d) CbzCl, $NaHCO_3$, THF/H_2O (2/1), 0 °C, 15 h, 20% for three steps; (e) **8**, NIS, TESOTf, CH_2Cl_2, 4 Å MS, 25 °C, 30 min, 60%; (f) Zn powder, NH_4Cl, EtOH/H_2O (3/1), 25 °C, 1.5 h; (g) Boc_2O, $NaHCO_3$, CH_2Cl_2, 25 °C, 2 h, 52% for two steps; (h) Pd/C, H_2, MeOH, 25 °C, 3 h; (i) **16**, $NaBH_3CN$, HOAc, MeOH, 25 °C, 15 h, 59% for two steps; (j) Zn powder, NH_4Cl, MeOH, 25 °C, 25 h; (k) **18**, EDCI, HOBt, CH_2Cl_2, 25 °C, 20 h, 12% for two steps; (l) $Ba(OH)_2{\cdot}8H_2O$, THF/H_2O (4/1), 0 to 25 °C, 20 h, 40%; (m) 80% TFA in H_2O, 25 °C, 18 h, quantitative.

for details). When we adopted the procedure reported by Shibasaki and co-workers (CuCl, PPh_3 and DIPEA) [37], the isocyanoacetate aldol reaction gave a 1:5 (**14a**:**14b**) mixture. To unambiguously establish the configuration of the major aldol reaction product to be (2*S*,3*R*), we subjected **13** to acid-catalyzed hydrolysis and subsequent Cbz protection. The NMR spectral data of the Cbz-protected major aldol product **14a** was identical with the major diastereomer of the Sharpless aminohydroxylation reaction (Scheme 1), confirming that the major isocyanoacetate aldol product was the desired 1,2-*syn*-amino alcohol (see the Supplementary Information for details).

Following the isocyanoacetate aldol reaction, the 1,2-*syn*-amino alcohol **14** was treated with the glycosyl donor **8** [35], TESOTf, and NIS to afford the glycosylation product in 60%. The azide reduction of the glycosylation product by Zn powder and NH_4Cl followed by a subsequent Boc protection of the resulting amine gave the Boc-protected amine **15** in 52% for two steps. The Cbz deprotection of **15** by Pd/C followed by reductive alkylation of the resulting amine with **16** [38] and $NaBH_3CN$/HOAc gave the carbamate **17** in 59% for two steps. The Troc group of **17** was removed by treatment with Zn powder in MeOH, and the resulting amine was acylated with the carboxylic acid **18** [16] in the presence of EDCI and HOBt to afford the amide **19**. Treatment of **19** with $Ba(OH)_2{\cdot}8H_2O$ provided the carboxylic acid (40%). Finally, the global deprotection by aqueous TFA successfully completed the synthesis of the cyclopentane-based analog **20** (JH-MR-23) with a lipophilic side chain.

2.2. Biological characterization

After the completion of analog synthesis, we performed the UMP-Glo™ glycosyltransferase assay [39] to assess the effect of the cyclopentane core on MraY inhibition. The analogs (**10**, **11** and **20**) we prepared inhibited MraY activity in a dose-dependent manner (Fig. 2a–c). Among the analogs tested, the analog **20** (JH-MR-23) showed the most potent inhibitory activity (IC_{50}: 75 ± 9 μM) against MraY from *Aquifex aeolicus* ($MraY_{AA}$). The analogs **10** (JH-MR-21) and **11** (JH-MR-22) without a lipophilic side chain exhibited significantly lower inhibitory activity (IC_{50}: 340 ± 42 μM for **10** and 500 ± 69 μM for **11**) than **20**. This data indicated that a lipophilic side chain is crucial for MraY inhibition. However, none of them was as potent as muraymycin D2 [8]. We then tested the biological activity of our most potent analog **20** (JH-MR-23) against *Staphylococcus aureus* (strain *SA*113) and determined the minimal inhibitory concentration (MIC) is 54 ± 6.8 μg/mL (Fig. 2d).

3. Conclusion

MraY is an essential enzyme in the peptidoglycan biosynthesis and a promising target for new antibiotic development. We recently reported the crystal structures of MraY in complex with representative members of the natural nucleoside inhibitors, including muraymycin D2. To harness our recent findings and to improve the chemical tractability of muraymycin analogs, we prepared cyclopentane-based muraymycin analogs by replacing the ribose and lipophilic peptide chain groups of muraymycins with a cyclopentane ring and a modified lipophilic side chain. We also explored the diastereoselective isocyanoacetate aldol reaction for the installation of the 1,2-*syn*-amino alcohol group of muraymycins. We found that our cyclopentane analogs are less potent than muraymycin D2 (**1d**) in inhibiting MraY activity. Future structural and functional studies are necessary to elucidate the reason for the low efficacy of cyclopentane analogs in MraY function. However, among the cyclopentane analogs, analog **20** (JH-MR-23) has the most potent MraY inhibition and exhibits antibacterial activity against the Gram-positive bacteria *S. aureus* to levels comparable to some of the reported muramycin analogs [18]. Our SAR analysis of the analogs suggested that a lipophilic side chain is important for MraY inhibition and antibacterial efficacy. Our recent structural and functional analysis revealed that MraY contains six druggable hot spots, all of which can be exploited in a combinatorial manner to improve existing MraY inhibitors or develop new types of MraY inhibitors [6]. Because our cyclopentane-based muraymycin analogs are more amenable to modification with

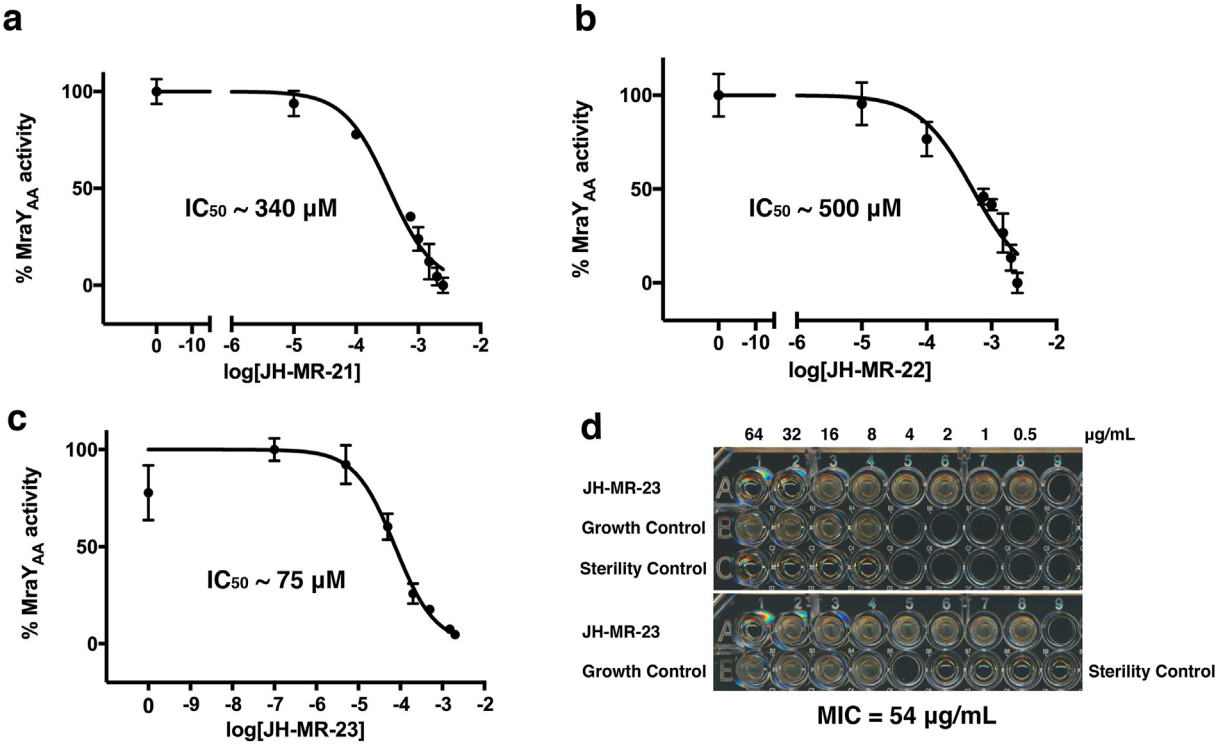


Fig. 2. Dose-response curves and IC_{50} values of (a) **10** (JH-MR-21), (b) **11** (JH-MR-22), and (c) **20** (JH-MR-23) with $MraY_{AA}$ solubilized in the detergent CHAPS. Each IC_{50} measurement was made by using the UMP-Glo™ assay. Data are shown as the mean ± standard deviation of three technical replicates. (d) MIC of **20** (JH-MR-23) against *S. aureus* (strain *SA*113) is 54 ± 6.8 μg/mL. Representative images of the MIC determination for **20** (JH-MR-23) with growth and sterility controls (4 wells each) as indicated in row B and C (n = 8, MIC value is reported as mean ± standard error).

various substituents, our synthetic route will be a valuable foundation for the future development of new muraymycin-derived antibiotics targeting MraY.

4. Materials and methods

4.1. Synthesis of cyclopentane-based muraymycin analogs

General chemistry procedures. All reactions were conducted in oven-dried glassware under nitrogen or argon. Unless otherwise stated all reagents were purchased from commercial suppliers and used without further purification. All solvents were American Chemical Society (ACS) grade or better and used without further purification except tetrahydrofuran (THF), which was freshly distilled from sodium/benzophenone each time before use. Analytical thin layer chromatography (TLC) was performed with glass backed silica gel (60 Å) plates with fluorescent indication (Whatman). Visualization was accomplished by UV irradiation at 254 nm and/or by staining with *p*-anisaldehyde solution. Flash column chromatography was performed by using silica gel (particle size 230–400 mesh, 60 Å). All ^{1}H spectra were recorded with a Varian 400 (400 MHz) and a Bruker 500 (500 MHz) spectrometer. All NMR δ values are given in parts per million (ppm) and are referenced to the residual solvent signals ($CDCl_3$: δ = 7.26 ppm, CD_3OD: δ = 3.31 ppm, $(CD_3)_2SO$: δ = 2.50 ppm) for ^{1}H NMR spectra, or the solvent signals for ^{13}C spectra. Coupling constants (*J*) are given in Hertz (Hz) and multiplicities are indicated using the conventional abbreviation (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or overlap of non-equivalent resonances, br = broad). Electrospray ionization (ESI) mass spectrometry (MS) was recorded with an Agilent 1100 series (LC/MSD trap) spectrometer in order to obtain the molecular masses of compounds.

tert-Butyl (1*S*,4*R*)-3-oxo-2-azabicyclo[2.2.1]heptane-2-carboxylate (**3**). [Reduction] To a solution of the commercially available (1*R*,4*S*)-2-azabicyclo[2.2.1]hept-5-en-3-one (**2**) (10 g, 91.70 mmol) in anhydrous MeOH (180 mL) was added 10% palladium on activated carbon (3 g). After stirring at 25 °C for 5 h under H_2 atmosphere, the reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated *in vacuo* to give the corresponding lactam [30] (9.17 g). The crude lactam was used in the following step without further purification: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.75 (br s, 1H), 3.89 (s, 1H), 2.74 (s, 1H), 1.94–1.91 (m, 1H), 1.88–1.80 (m, 2H), 1.65–1.57 (m, 2H), 1.42–1.36 (m, 1H); [Boc Protection] A mixture of the lactam (9.17 g, 82.51 mmol), Boc_2O (28.60 g, 131.04 mmol), and DMAP (5.04 g, 41.25 mmol) in anhydrous MeCN (300 mL) was stirred at 25 °C for 1 h. The solvents were removed *in vacuo* and the residue was purified by column chromatography (silica gel, hexanes/EtOAc, 5/2) to afford the known Boc-protected lactam **3** [30] (15.68 g, 81% for two steps) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.53 (s, 1H), 2.85 (s, 1H), 1.95–1.90 (m, 2H), 1.80–1.73 (m, 2H), 1.51 (s, 9H), 1.43–1.39 (m, 2H).

((*1R,3S*)-3-Aminocyclopentyl)methanol (**4**). A mixture of **3** (15.68 g, 74.25 mmol) and $NaBH_4$ (5.62 g, 14.85 mmol) in MeOH (300 mL) was stirred at 25 °C for 3 h. The solvents were removed *in vacuo*, and the residue was partitioned between EtOAc and H_2O. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/EtOAc, 2/1) to afford the Boc-protected amino alcohol (12.79 g, 80%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.90 (br s, 1H), 3.56 (d, *J* = 5.0 Hz, 2H), 2.19–2.10 (m, 2H), 1.92–1.83 (m, 1H), 1.74–1.71 (m, 1H), 1.47–1.42 (m, 2H), 1.40 (s, 9H), 1.14–1.10 (m, 1H); [Boc Deprotection] A solution of the Boc-protected amino alcohol (12.79 g, 59.44 mmol) in H_2O (700 mL) was stirred at 100 °C for 20 h. The solvents were removed to give the amino alcohol **4** [30] (5.47 g, 80%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.58–3.46 (m, 3H), 2.75 (br s, 2H), 2.40–2.30 (m, 1H), 1.99–1.92 (m, 1H), 1.80–1.69 (m, 3H), 1.50–1.45 (m, 1H), 1.38–1.28 (m, 1H); HRMS (ESI) *m/z* 116.1073 [$(M+H)^+$ calcd for $C_6H_{13}NO$ 116.1069].

1-((1*S*,3*R*)-3-(Hydroxymethyl)cyclopentyl)pyrimidine-2,4(1*H*,3*H*)-dione (**5**). [Coupling] To a solution of **4** (4.50 g, 39.1 mmol) in anhydrous DMF (135 mL) were slowly added 4 Å molecular sieves and 3-ethoxyacryloyl isocyanate [40] (84.5 mL, 50.8 mmol) in anhydrous benzene at −20 °C. After stirring at 25 °C for 15 h, the molecular sieves were filtered off and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 20/1) to afford the urea intermediate (4.89 g, 49%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (d, *J* = 7.3 Hz, 1H), 8.41 (s, 1H), 7.62 (d, *J* = 12.1 Hz, 1H), 5.24 (d, *J* = 12.2 Hz, 1H), 4.21–4.14 (m, 1H), 3.96 (q, *J* = 7.1 Hz, 2H), 3.58 (d, *J* = 5.8 Hz, 2H), 2.28–2.12 (m, 2H), 2.09–1.90 (m, 1H), 1.85–1.75 (m, 1H), 1.65–1.55 (m, 2H), 1.52–1.41 (m, 1H), 1.35 (t, *J* = 7.0 Hz, 3H); [Uracil Formation] The urea (4.50 g, 17.56 mmol) was dissolved in 1 N H_2SO_4 (150 mL) and the resulting reaction mixture was refluxed under N_2 atmosphere. After stirring for 30 min, the reaction was quenched by an addition of saturated aqueous 2 N NaOH, and the resulting mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 40/1) to afford the uracil alcohol **5** (2.50 g, 68%) as a colorless oil: ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 11.20 (br s, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 5.56 (d, *J* = 8.0 Hz, 1H), 4.77–4.64 (m, 1H), 4.56 (br s, 1H), 3.36 (d, *J* = 6.2 Hz, 2H), 2.08–1.92 (m, 2H), 1.90–1.78 (m, 1H), 1.70–1.60 (m, 2H), 1.55–1.43 (m, 1H) 1.38–1.27 (m, 1H); HRMS (ESI) *m/z* 211.1082 [$(M+H)^+$ calcd for $C_{10}H_{14}N_2O_3$ 211.1077].

tert-Butyl (*E*)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)cyclopentyl)acrylate (**6**). [Oxidation] To a solution of **5** (2.50 g, 11.89 mmol) in anhydrous MeCN (400 mL) was added IBX (8.32 g, 29.72 mmol). After stirring at 80 °C for 1.5 h, the insoluble was filtered off and the filtrate was concentrated *in vacuo* to afford the corresponding aldehyde (2.50 g). The crude aldehyde was used in the following step without further purification: ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 9.98 (br s, 1H), 9.71 (s, 1H), 7.63 (d, *J* = 8.0 Hz, 1H), 5.60 (d, *J* = 8.0 Hz, 1H), 5.08–4.90 (m, 1H), 3.05–2.95 (m, 1H), 2.29–2.21 (m, 2H), 2.18–2.08 (m, 2H), 1.95–1.89 (m, 1H), 1.79–1.69 (m, 1H); [Wittig Reaction] To a cooled (0 °C) solution of the aldehyde (300 mg, 1.44 mmol) in anhydrous THF (15 mL) was added (*tert*-butoxycarbonylmethylene)triphenylphosphorane (2.10 g, 5.76 mmol). After stirring at 0 °C for 12 h, the solvents were removed *in vacuo* and the residue was purified by column chromatography (silica gel, hexanes/*i*-PrOH, 6/1) to afford the α,β-unsaturated ester **6** (198.33 mg, 45% for two steps) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.83 (s, 1H), 7.23 (d, *J* = 8.1 Hz, 1H), 6.84 (dd, *J* = 7.3, 1.4 Hz, 1H), 5.76 (s, 1H), 5.74 (d, *J* = 6.6 Hz, 1H), 5.04–4.90 (m, 1H), 2.82–2.62 (m, 1H), 2.28–2.23 (m, 1H), 2.20–2.13 (m, 1H), 2.04–1.91 (m, 1H), 1.80–1.64 (m, 2H), 1.55–1.50 (m, 1H), 1.46 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.79, 163.09, 151.00, 148.78, 140.65, 122.65, 102.89, 80.50, 56.06, 40.38, 37.74, 30.11, 28.13.

tert-Butyl (2*S*,3*R*)-2-(((benzyloxy)carbonyl)amino)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)cyclopentyl)-3-hydroxy propanoate (**7**). *tert*-Butyl hypochlorite (446 μL, 3.95 mmol) was added to a solution of benzyl carbamate (92.40 mg, 3.91 mmol) in 0.6 N NaOH/*n*-PrOH (1/1, 15 mL) at 15 °C. After stirring at 15 °C for 15 min, the reaction mixture was warmed to 25 °C and sequentially treated with $(DHQD)_2AQN$ (167.10 mg, 0.19 mmol) in *n*-PrOH (2.50 mL), **6** (200 mg, 0.65 mmol) in *n*-PrOH (2.5 mL), and

$K_2OsO_2(OH)_4$ (71.80 mg, 0.19 mmol) in *n*-PrOH (2.50 mL). After stirring at 25 °C for 2 h, the reaction was quenched by an addition of H_2O and the resulting mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 50/1) to afford the amino alcohol **7** (140 mg, 46%) as a yellow solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (s, 1H), 7.38–7.26 (m, 6H), 5.72 (d, J = 8.4 Hz, 1H), 5.52–5.44 (m, 1H), 5.13 (s, 2H), 4.87–4.78 (m, 1H), 4.34–4.27 (m, 1H), 3.94–3.87 (m, 1H), 2.32–2.19 (m, 2H), 2.18–2.08 (m, 2H), 1.75–1.68 (m, 1H), 1.47 (s, 10H), 1.28–1.19 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.39, 163.83, 157.16, 156.76, 151.16, 141.51, 136.26, 136.18, 102.40, 82.72, 67.11, 66.96, 60.45, 57.90, 56.83, 40.88, 34.61, 29.90, 26.95, 21.06, 14.19; HRMS (ESI) *m/z* 474.2236 [(M+H)$^+$ calcd for $C_{24}H_{31}N_3O_7$ 474.2235].

tert-Butyl (2*S*,3*R*)-3-(((3a*R*,4*R*,6*R*,6a*R*)-6-(aminomethyl)-2,2-diethyltetrahydrofuro[3,4-*d*] [1,3]dioxol-4-yl)oxy)-2-(((benzyloxy) carbonyl)amino)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1 (2*H*)-yl)cyclopentyl)propanoate (**9**). [Glycosylation] To a solution of **7** (90 mg, 0.19 mmol) in CH_2Cl_2 (8 mL) were added **8** [35] (78.70 mg, 0.25 mmol), 4 Å molecular sieves, and NIS (74.80 mg, 0.33 mmol). After stirring at 25 °C for 10 min, TESOTf (20 μL, 0.09 mmol) was added and the resulting mixture was stirred for 30 min. The reaction was quenched by an addition of saturated aqueous $NaHCO_3$, and the resulting mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 40/1) to afford the corresponding azide (60 mg, 53%) as a yellow solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.20 (br s, 1H), 7.41–7.26 (m, 5H), 7.22 (d, J = 7.7 Hz, 1H), 6.06 (d, J = 9.6 Hz, 1H), 5.72 (d, J = 8.2 Hz, 1H), 5.11 (s, 1H), 5.09 (d, J = 13.9 Hz, 2H), 4.91–4.77 (m, 1H), 4.58–4.52 (m, 2H), 4.30 (d, J = 9.8 Hz, 1H), 4.27–4.21 (m, 1H), 4.09 (q, J = 7.2 Hz, 2H), 4.00 (d, J = 7.2 Hz, 1H), 3.50–3.32 (m, 2H), 2.30–2.17 (m, 5H), 2.00–1.89 (m, 2H), 1.70–1.60 (m, 2H), 1.58–1.48 (m, 2H), 1.45 (s, 9H), 0.90–0.78 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.07, 171.12, 169.29, 156.88, 151.07, 141.02, 136.50, 117.42, 111.13, 102.54, 86.28, 84.66, 84.22, 82.54, 81.94, 66.97, 60.37, 56.52, 56.21, 53.19, 40.94, 35.55, 29.58, 29.45, 28.87, 27.94, 21.01, 14.17, 8.35, 7.35, 6.77, 6.39; HRMS (ESI) *m/z* 721.3161 [(M+Na)$^+$ calcd for $C_{34}H_{46}N_6O_{10}$ 721.3168]; [Azide Reduction] To a solution of the azide (5 mg, 0.007 mmol) in H_2O (0.05 mL) and THF/toluene (1/1, 1 mL) was added PPh_3 (3.70 mg, 0.01 mmol). After stirring at 25 °C for 12 h, the solvents were removed *in vacuo* and the residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 10/1) to afford the amine **9** (3 mg, 63%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38–7.24 (m, 6H), 5.71 (d, J = 8.2 Hz, 1H), 5.14–5.09 (m, 3H), 4.84–4.78 (m, 1H), 4.62–4.49 (m 3H), 4.35 (d, J = 9.4 Hz, 1H), 4.24–4.17 (m, 1H), 4.03 (d, J = 8.4 Hz, 1H), 2.90–2.84 (m, 1H), 2.72–2.68 (m, 1H), 2.58–2.50 (m, 1H), 1.69–1.62 (m, 6H), 1.57–1.51 (m, 5H), 1.45 (s, 9H), 0.90–0.79 (m, 6H); HRMS (ESI) *m/z* 673.3444 [(M+H)$^+$ calcd for $C_{34}H_{48}N_4O_{10}$ 673.3443].

tert-Butyl (2*S*,3*R*)-3-(((2*R*,3*R*,4*S*,5*R*)-5-(aminomethyl)-3,4-dihydroxytetrahydrofuran-2-yl)oxy)-2-(((benzyloxy)carbonyl)amino)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)cyclopentyl) propanoate (**10**). Compound **9** (3 mg, 0.004 mmol) was treated with 80% TFA in THF/H_2O (1/1, 1 mL) and stirred at 25 °C for 14 h. The reaction mixture was concentrated *in vacuo* and triturated from CH_2Cl_2 to afford the partially deprotected *t*-Bu ester **10** (JH-MR-21) and the fully deprotected carboxylic acid **11** (JH-MR-22). The global deprotection reaction **10** or **11** as the final product depending on purity of **9** and reaction time. Compounds **10** and **11** were purified by HPLC (YMC J'sphere ODS M80, 10 mm × 150 mm, 0.1% formic acid, a linear gradient from 60 to 99% of MeCN–H_2O for 30 min, flow injection: 1 mL/min) to afford **10** (1 mg, 41%) and **11** (2 mg, 91%): For **10**, t_R 3.263 min; HRMS (ESI) *m/z* 605.2816 [(M+H)$^+$ calcd for $C_{29}H_{40}N_4O_{10}$ 605.2711]; For **11**, t_R 3.329 min; ^{1}H NMR (500 MHz, CD_3OD) δ 7.70 (d, J = 7.9 Hz, 1H), 7.40–7.25 (m, 5H), 5.69 (d, J = 7.9 Hz, 1H), 5.50 (s, 2H), 5.12 (d, J = 9.8 Hz, 1H), 5.03 (d, J = 9.8 Hz, 1H), 4.96 (s, 1H), 4.18 (s, 1H), 4.11–4.03 (m, 2H), 4.02–3.95 (m, 2H), 3.96–3.90 (m, 1H), 3.14–3.08 (m, 2H), 2.18–2.11 (m, 2H), 2.10–2.00 (m, 1H), 1.90–1.80 (m, 1H), 1.78–1.70 (m, 1H), 1.68–1.58 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 164.85, 157.75, 157.52, 151.56, 151.40, 142.87, 128.11, 127.80, 127.72, 127.68, 110.36, 101.25, 85.71, 80.86, 78.42, 75.13, 72.05, 66.61, 56.91, 56.74, 56.40, 42.71, 41.07, 34.87, 28.73, 26.22, 22.80; HRMS (ESI) *m/z* 549.2188 [(M+H)$^+$ calcd for $C_{25}H_{32}N_4O_{10}$ 549.2191].

Methyl 5-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl) cyclopentyl)-4,5-dihydrooxazole-4-carboxylate (**13**). [Oxidation] To a solution of **5** (2.50 g, 11.89 mmol) in anhydrous MeCN (400 mL) was added IBX (8.32 g, 29.72 mmol). After stirring at 80 °C for 1 h, the insoluble was filtered off and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 30/1) to afford the corresponding aldehyde (1.72 g, 70%); [Aldol Reaction] The pre-catalyst **12** [36] (28.10 mg, 0.04 mmol) was dissolved in EtOAc (0.60 mL) and Ag_2O (5.57 mg, 0.02 mmol) was added at −78 °C. The resulting mixture was stirred for approximately 1 min before sequentially treated with methyl isocyanoacetate (51.50 μL, 0.56 mmol) and powdered 4 Å molecular sieves at −78 °C. The aldehyde (200 mg, 0.96 mmol) in EtOAc (1 mL) was added to the reaction mixture. The resulting mixture was slowly warmed to 0 °C and stirred at 0 °C for 1.5 d Ag_2O was removed by filtering through a pad of Celite with MeOH to afford the *trans*-oxazoline **13**. The crude **13** was used in next step without further purification.

Methyl (2*S*,3*R*)-2-(((benzyloxy)carbonyl)amino)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)cyclopentyl)-3-hydroxy propanoate (**14a**). [Hydrolysis] To a solution of **13** (200 mg, 0.65 mmol) in THF (1.40 mL) was added 4 N HCl in dioxane (0.70 mL) at 0 °C. After stirring under N_2 at 25 °C for 1 h, the reaction mixture was concentrated *in vacuo* to afford the crude amino alcohol, which was used in the following step without further purification; [Cbz Protection] To a solution of the crude amino alcohol (200 mg, 0.67 mmol) in THF/H_2O (2/1, 6 mL) were added $NaHCO_3$ (113 mg, 1.35 mmol) and CbzCl (0.14 mL, 1.01 mmol). After stirring at 0 °C for 15 h, the reaction was quenched by an addition of H_2O, and the resulting mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 30/1) to afford the *syn*-amino alcohol **14a** (84.9 mg, 20.5% for three steps) and the diastereomeric *syn*-amino alcohol **14b** (42.7 mg, 10.3% for three steps) each as a white solid: Data for the (2*S*,3*R*)-*syn*-amino alcohol: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43–7.28 (m, 5H), 7.23 (d, J = 7.8 Hz, 1H), 5.76 (d, J = 9.5 Hz, 1H, –NH), 5.70 (d, J = 8.0 Hz, 1H), 5.12 (s, 2H), 4.82–4.74 (m, 1H), 4.47 (d, J = 9.7 Hz, 1H), 4.01 (d, J = 8.4 Hz, 1H), 3.76 (s, 3H), 2.29–2.20 (m, 1H), 2.18–2.05 (m, 2H), 1.94–1.85 (m, 1H), 1.78–1.70 (m, 1H), 1.68–1.57 (m, 2H); HRMS (ESI) *m/z* 432.1771 [(M+H)$^+$ calcd for $C_{21}H_{25}N_3O_7$ 432.1765]; Data for the diastereomeric (2*R*,3*S*)-*syn*-amino alcohol: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (br s, 1H), 7.42–7.28 (m, 5H), 7.20 (d, J = 8.0 Hz, 1H), 5.74 (d, J = 7.5 Hz, 1H), 5.46 (dd, J = 11.8, 9.8 Hz, 2H), 5.14 (s, 2H), 4.89–4.86 (m, 1H), 4.66 (d, J = 9.8 Hz, 1H), 3.74 (s, 3H), 3.67–3.60 (m, 1H), 2.41–2.30 (m, 1H), 2.21–2.07 (m, 2H), 1.74–1.70 (m, 1H), 1.68–1.64 (m, 1H), 1.50–1.46 (m, 2H).

Methyl (2*S*,3*R*)-2-(((benzyloxy)carbonyl)amino)-3-(((3a*R*,4*R*,6-*R*,6a*R*)-6-(((*tert*-butoxycarbonyl)amino)methyl)-2,2-diethyltetrahydrofuro[3,4-*d*] [1,3]dioxol-4-yl)oxy)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)cyclopentyl)propanoate (**15**).

[Glycosylation] To a solution of **14** (20 mg, 0.04 mmol) in CH_2Cl_2 (1.50 mL) were added **8** [35] (20 mg, 0.06 mmol), 4 Å molecular sieves, and NIS (18 mg, 0.08 mmol). After stirring at 25 °C for 10 min, TESOTf (5 μL, 0.02 mmol) was added and the resulting solution was stirred for 30 min. The reaction was quenched by an addition of saturated aqueous $NaHCO_3$, and the resulting mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 30/1) to afford the corresponding azide (15.70 mg, 60%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (br s, 1H), 7.42–7.29 (m, 5H), 7.22 (d, J = 8.1 Hz, 1H), 6.05 (d, J = 9.9 Hz, 1H), 5.74 (dd, J = 8.0, 2.4 Hz, 1H), 5.14 (d, J = 3.4 Hz, 2H), 5.08 (s, 1H), 4.88–4.85 (m, 1H), 4.60 (dd, J = 5.9, 1.6 Hz, 1H), 4.54 (d, J = 6.1 Hz, 1H), 4.47 (d, J = 9.7 Hz, 1H), 4.28–4.23 (m, 1H), 4.07 (d, J = 8.3 Hz, 1H), 3.75 (s, 3H), 3.48–3.40 (m, 2H), 2.33–2.25 (m, 2H), 2.20–2.14 (m, 1H), 2.01–1.91 (m, 2H), 1.80–1.70 (m, 2H), 1.69–1.63 (m, 2H), 1.60–1.50 (m, 2H), 0.98–0.78 (m, 6H); HRMS (ESI) *m/z* 657.2879 [$(M+H)^+$ calcd for $C_{31}H_{40}N_6O_{10}$ 657.2883]; [Azide Reduction] To a solution of the azide (19 mg, 0.02 mmol) in EtOH/H_2O (3/1, 0.80 mL) were added activated Zn powder (2.60 mg, 0.03 mmol) and NH_4Cl (3.70 mg, 0.07 mmol). After stirring at 25 °C for 1.5 h, the reaction was quenched by an addition of saturated aqueous $NaHCO_3$, and the resulting mixture was diluted with CH_2Cl_2. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to afford the corresponding amine. The crude amine was used in the following step without further purification: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (br s, 1H), 7.40–7.29 (m, 5H), 7.25–7.21 (d, J = 8.2 Hz, 1H), 5.75 (d, J = 8.0 Hz, 1H), 5.12 (s, 2H), 5.09 (s, 1H), 4.91–4.82 (m, 1H), 4.60–4.50 (m, 3H), 4.25 (br s, 1H), 4.08 (d, J = 7.6 Hz, 1H), 3.77 (s, 3H), 2.97–2.92 (m, 1H), 2.87–2.82 (m, 1H), 2.28–2.21 (m, 3H), 2.15–2.10 (m, 1H), 2.05–1.90 (m, 1H), 1.75–1.69 (m, 2H), 1.68–1.64 (m, 2H), 1.60–1.50 (m, 2H), 0.92–0.82 (m, 6H); [Boc Protection] To a solution of the crude amine (8 mg, 0.01 mmol) in anhydrous CH_2Cl_2 (1 mL) were added $NaHCO_3$ (2 mg, 0.02 mmol) and Boc_2O (11 mg, 0.04 mmol). After stirring at 25 °C for 2 h, the reaction was quenched by an addition of H_2O, and the resulting mixture was diluted with CH_2Cl_2. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 20/1) to afford the Boc-protected glycosylation product **15** (9.20 mg, 52% for two steps) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (s, 1H), 7.40–7.29 (m, 5H), 7.23 (d, J = 8.9 Hz, 1H), 5.75 (dd, J = 5.8, 2.2 Hz, 1H), 5.58–5.45 (m, 1H), 5.40 (br s, 1H), 5.12 (d, J = 7.5 Hz, 2H), 5.07 (s, 1H), 4.91–4.82 (m, 1H), 4.60 (d, J = 6.2 Hz, 1H), 4.54 (d, J = 9.8 Hz, 1H), 4.49 (d, J = 6.1 Hz, 1H), 4.25 (t, J = 5.1 Hz, 1H), 4.04 (d, J = 8.0 Hz, 1H), 3.79 (s, 3H), 3.25–3.18 (m, 2H), 2.28–2.21 (m, 2H), 2.00–1.88 (m, 1H), 1.75–1.62 (m, 6H), 1.60–1.50 (m, 11H), 0.92–0.82 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.43, 163.96, 156.52, 156.01, 150.82, 141.03, 136.00, 128.31, 128.25, 117.17, 112.51, 106.68, 87.00, 86.31, 85.24, 82.21, 79.52, 67.41, 56.69, 56.39, 52.94, 43.06, 41.00, 35.28, 29.64, 29.29, 29.00, 26.87, 8.40, 7.55; HRMS (ESI) *m/z* 753.3324 [$(M+Na)^+$ calcd for $C_{36}H_{50}N_4O_{12}$ 753.3317].

Methyl (2*S*,3*R*)-3-(((3a*R*,4*R*,6*R*,6a*R*)-6-(((*tert*-butoxycarbonyl)amino)methyl)-2,2-diethyltetrahydrofuro[3,4-*d*] [1,3]dioxol-4-yl)oxy)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)cyclopentyl)-2-((3-(((2,2,2-trichloroethoxy)carbonyl)amino)propyl)amino)propanoate (**17**). [Cbz Deprotection] To a solution of **15** (45 mg, 0.06 mmol) in anhydrous MeOH (2.25 mL) was added 10% palladium on activated carbon (27 mg). After stirring under H_2 atmosphere at 25 °C for 3 h, the reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated *in vacuo* to give the corresponding amine. The crude amine was used in the following step without further purification; HRMS (ESI) *m/z* 597.3138 [$(M+H)^+$ calcd for $C_{28}H_{44}N_4O_{10}$ 597.3130]; [Reductive amination] To a solution of the crude amine (45 mg, 0.05 mmol) in anhydrous MeOH (3 mL) were treated with **16** [38] (22.60 mg, 0.09 mmol) in HOAc (30 μL, 0.54 mmol) and $NaBH_3CN$ (15 mg, 0.24 mmol). After stirring at 25 °C for 15 h, the reaction was quenched by an addition of saturated aqueous $NaHCO_3$, and the resulting mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 30/1) to afford the carbamate **17** (27 mg, 59% for two steps) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (br s, 1H), 7.25–7.22 (m, 1H), 5.87–5.76 (m, 1H), 5.73 (d, J = 8.1 Hz, 1H), 5.06 (s, 1H), 4.88–4.79 (m, 1H), 4.72–4.70 (m, 2H), 4.60 (d, J = 6.0 Hz, 1H), 4.50–4.46 (m, 1H), 4.29–4.24 (m, 1H), 3.75 (s, 3H), 3.36–3.29 (m, 3H), 3.26–3.16 (m, 4H), 2.93–2.82 (m, 1H), 2.49–2.32 (m, 2H), 2.31–2.23 (m, 1H), 2.21–2.12 (m, 1H), 1.84–1.80 (m, 2H), 1.66–1.49 (m, 4H), 1.48–1.43 (m, 3H), 1.42 (s, 12H), 0.86–0.84 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.02, 173.79, 163.00, 154.58, 150.81, 141.21, 116.90, 112.71, 103.69, 102.56, 86.96, 85.51, 82.32, 79.22, 74.46, 74.39, 64.08, 56.73, 53.43, 52.36, 46.54, 43.17, 41.04, 39.80, 37.22, 35.01, 32.21, 29.90, 29.82, 29.42, 29.04, 28.99, 27.08, 8.41, 7.52; HRMS (ESI) *m/z* 828.2740 [$(M+H)^+$ calcd for $C_{34}H_{52}Cl_3N_5O_{12}$ 828.2751].

Methyl (2*S*,3*R*)-3-(((3a*R*,4*R*,6*R*,6a*R*)-6-(((*tert*-butoxycarbonyl)amino)methyl)-2,2-diethyltetrahydrofuro[3,4-*d*] [1,3]dioxol-4-yl)oxy)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)cyclopentyl)-2-((3-((*S*)-2-heptadecanamido-5-(3-((2,2,5,6,8-pentamethylchroman-7-yl)sulfonyl)guanidino)pentanamido)propyl)amino)propanoate (**19**). [Troc Deprotection] A solution of **17** (27 mg, 0.03 mmol) in anhydrous MeOH (1.50 mL) were treated with NH_4Cl (49 mg, 0.92 mmol) and Zn powder (97% purity, 31.80 mg, 0.48 mmol). After stirring at 25 °C for 25 h, the insoluble was filtered through a pad of Celite, and the filtrate was concentrated *in vacuo* to give the corresponding amine. The crude amine was used in the following step without further purification; HRMS (ESI) *m/z* 654.3713 [$(M+H)^+$ calcd for $C_{31}H_{51}N_5O_{10}$ 654.3709]; [Coupling] To a solution of the crude amine (27 mg, 0.04) in CH_2Cl_2 (1 mL) were added EDCI (3.20 mg, 0.01 mmol), HOBt (1.9 mg, 0.01 mmol) and **18** [16] (8.40 mg, 0.01 mmol). After stirring at 25 °C for 20 h, the reaction was quenched by an addition of 1 N HCl solution, and the reaction mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The combined organic layers were purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 30/1) to afford the amide **19** (5 mg, 12% for two steps) as a white solid: ^{1}H NMR (400 MHz, CD_3OD) δ 7.67 (d, J = 8.0 Hz, 1H), 5.67 (d, J = 7.9 Hz, 1H), 5.08 (s, 1H), 4.64–4.54 (m, 2H), 4.33–4.22 (m, 1H), 4.18–4.10 (m, 1H), 3.92–3.87 (m, 1H), 3.73 (s, 3H), 3.40–3.20 (m, 3H), 3.18–3.09 (m, 2H), 2.75–2.72 (m, 1H), 2.68–2.62 (m, 2H), 2.55–2.54 (m, 6H), 2.49–2.39 (m, 2H), 2.23–2.18 (m, 3H), 2.11–2.06 (m, 4H), 1.99 (s, 1H), 1.90–1.47 (m, 25H), 1.42 (s, 3H), 1.29 (s, 3H), 1.27 (s, 9H), 1.24–1.12 (m, 21H), 0.88–0.86 (m, 9H); HRMS (ESI) *m/z* 1328.8127 [$(M+H)^+$ calcd for $C_{68}H_{113}N_9O_{15}S$ 1328.8150].

Methyl (2*S*,3*R*)-3-(((2*R*,3*R*,4*S*,5*R*)-5-(aminomethyl)-3,4-dihydroxytetrahydrofuran-2-yl)oxy)-3-((1*R*,3*S*)-3-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)cyclopentyl)-2-((3-((*S*)-5-guanidino-2-heptadecanamidopentanamido)propyl)amino)propanoate formate (**20**). [Hydrolysis] $Ba(OH)_2{\cdot}8H_2O$ (3.20 mg, 0.01 mmol) was added to a solution of **19** (5 mg, 0.003 mmol) in THF/H_2O (4/1, 0.10 mL) at 0 °C and the resulting mixture was stirred at 0 °C for 10 min. The

mixture was warmed to 25 °C and stirred for 20 h. The reaction was quenched by an addition of 1 N HCl and diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 5/1) to afford the corresponding carboxylic acid (2 mg, 40%) as a white solid: ^{1}H NMR (500 MHz, CD_3OD) δ 7.70 (d, J = 8.0 Hz, 1H), 5.69 (d, J = 7.9 Hz, 1H), 5.18 (s, 1H), 4.70–4.58 (m, 2H), 4.30–4.25 (m, 1H), 4.22–4.18 (m, 1H), 3.82 (br s, 1H), 3.22–3.18 (m, 3H), 3.13–3.08 (m, 2H), 2.71–2.68 (m, 3H), 2.60–2.57 (m, 6H), 2.42–2.37 (m, 2H), 2.23–2.18 (m, 3H), 2.10 (s, 2H), 2.01 (s, 1H), 1.88–1.53 (m, 25H), 1.42 (s, 3H), 1.31 (s, 3H), 1.28 (s, 9H), 1.26–1.20 (m, 21H), 0.88–0.86 (m, 9H); HRMS (ESI) *m/z* 1314.7990 [$(M+H)^+$ calcd for $C_{67}H_{111}N_9O_{15}S$ 1314.7993]; [Global Deprotection] After the carboxylic acid (2 mg, 0.001 mmol) was treated with 80% TFA in H_2O (0.30 mL), the resulting mixture was stirred at 25 °C for 18 h. The reaction mixture was concentrated *in vacuo* and triturated from CH_2Cl_2 to afford **20** (2 mg, quantitative) as a white solid: HRMS (ESI) *m/z* 880.5862 [$(M+H)^+$ calcd for $C_{43}H_{77}N_9O_{10}$ 880.5866].

4.2. UMP-Glo assay

The UMP-Glo™ glycosyltransferase assay [39] was performed according to the manufacturer's specifications (Promega Corporation). Reaction mixtures containing 250 μM undecaprenyl phosphate (C_{55}–P) and 150 μM UDP-MurNAc-pentapeptide (UM5A) were initiated with the addition of 50 nM $MraY_{AA}$. The reaction buffer consisted of 100 mM Tris-HCl, 500 mM NaCl, 10 mM $MgCl_2$, and 20 mM (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS, Anatrace). To determine the IC_{50} for **10** (JH-MR-21), the following concentrations were used: 0, 0.5, 5, 25, 100, 250, 750, 1000 μM. Reactions conducted in the presence of **10** had a final concentration of 2% DMSO. To determine the IC_{50} for **11** (JH-MR-22), the following concentrations were used: 0, 10, 100, 750, 1000, 1500, 2000, and 2500 μM. Reactions conducted in the presence of **11** had a final concentration of 5% DMSO. To determine the IC_{50} for **20** (JH-MR-23), the following concentrations were used: 0, 0.1, 5, 50, 200, 500, 1500, 2000 μM. Reactions conducted in the presence of **20** had a final concentration of 4% DMSO. All reactions were carried out for 5 min at 45 °C. A SpectraMax M3 multi-mode microplate reader in luminescence mode was used to make measurements, which were normalized to a negative control reaction without enzyme. Data were fit by using GraphPad Prism 7 software and IC_{50} values were calculated using the log(inhibitor) versus response–variable slope model. Hill slope used for **10** (JH-MR-21), **11** (JH-MR-22), and **20** (JH-MR-23) is −1.2, −1, −0.9 respectively.

4.3. MIC

MIC was determined using the established protocol [41]. Briefly, *S. aureus SA*113 (ATCC) were grown overnight on TB agar plate at 37 °C. Direct colony resuspension method was used to resuspend *S. aureus* colonies in 2 × YT media to match with 0.5 MacFarland standard and then diluted 100-fold. The inoculum (50 μL) was diluted with 50 μL 2 × YT media supplemented with different concentrations of **20** (JH-MR-23). The MIC was performed in 96-well plate (Corning 3596); the plate was incubated overnight at 37 °C. Eight biological replicates (n = 8) were performed, and the average was reported as the MIC value.

Author contributions

S.-H.K., W.Y.L., A.H., and E.H.M. contributed equally; S.-Y.L. and J.H. held overall responsibility for the study; All authors have given approval to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113272.

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