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Synthesis and antibacterial activity against *Clostridium difficile* of novel demethylvancomycin derivatives

Si-Ji Zhang [a], Qing Yang [a], Liang Xu [b], Jun Chang [a,*], Xun Sun [a,c,*]

[a] Department of Natural Products Chemistry, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, China
[b] Department of Chemistry of Medicinal Natural Products, West China School of Pharmacy, Sichuan University, Chengdu 610041, China
[c] Key Lab of Smart Drug Delivery, Ministry of Education & PLA, Fudan University, 826 Zhangheng Road, Shanghai 201203, China

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ABSTRACT

To explore the structure–activity relationships (SAR) of demethylvancomycin (**2**) and find more effective new chemical entities than known glycopeptides for the treatment of *Clostridium difficile* (*C. difficile*), 17 novel N-substituted (*N*-arylmethylene or -aliphatic substituents) demethylvancomycin derivatives were prepared. These analogues have been evaluated in vitro for their antibacterial activities against *C. difficile* and *Enterococcus faecium* (*E. faecium*). Compounds **5d**, **5h**, and **5i** with *N*-arylmethylene substituents, structurally similar to Oritavancin, showed more potent antibacterial activity against *C. difficile* than vancomycin (**1**) or demethylvancomycin (**2**). Meanwhile, compound **5k** with an undecyl side chain showed the most potent antibacterial activity against *E. faecium* (vancomycin-resistant strain).

Clostridium difficile (*C. difficile*) is a species of Gram-positive bacteria of the genus Clostridium that causes severe diarrhea, colitis and pseudomembranous colitis, collectively known as *C. difficile* infection (CDI), when competing bacteria in the gut flora have been wiped out by antibiotics.[1] CDI has been under investigation since 1974, and now is a significant and growing problem with at least 250,000 hospitalized cases per year with a mortality rate of 1–2.5%, and an estimated cost of several hundred million dollars annually in the United States.[2] Pathogenic strains of *C. difficile* produce exotoxins called toxin A and toxin B, which cause mucosal damage and inflammation of the colon.[3] The three main risk factors for CDI are hospitalization, age, and an increasing use of broad spectrum antibiotics, that is, fluoroquinolones, clindamycin, ampicillin, amoxicillin, and cephalosporins.[4]

Vancomycin (**1**) (Fig. 1) and metronidazole have proven to be the most effective treatments for CDI since the 1980s.[5] Due to its low cost, oral metronidazole is preferred in most cases for the treatment. Meanwhile, oral vancomycin is considered to be the last resort, because of the potential for the selection of vancomycin-resistant *Enterococcus* (VRE) and its high cost.[6] However, metronidazole showed reduced efficacy against CDI caused by hypervirulent strains,[7] and metronidazole treatment may also result in significant side effects, including neuropathy, seizures and abdominal pain.[8] Recent studies with fidaxomicin, a narrow-spectrum antibiotic with high selectivity for *C. difficile* over other bacteria, showed cure rates similar to that of vancomycin but a reduced rate of relapse.[9] Fidaxomicin was approved by the FDA in May 2011 and could replace vancomycin as a first-line treatment for CDI.[10] Several other approaches, such as probiotic therapy, toxin-binding polymers, and monoclonal antibodies against *C. difficile* toxins have been assessed in the management of CDI; however, their clinical efficacy is still under evaluation.[11] Therefore, it is still urgent to find drug candidate with better efficacy and avoiding of drug resistance for a better treatment of CDI.

Demethylvancomycin (**2**) was first isolated from Van-23, a strain of *Amycolatopsis orientalis* from soil sample collected in Guizhou Province, China, in 1959 and has been clinically used in China since 1967. It differs from vancomycin only in that methyl group on the amino group of the N-terminal residue of vancomycin has been replaced by a hydrogen atom. It shows similar activity and mode of action to vancomycin against Gram-positive bacteria.[12] Recent studies have shown that introducing a hydrophobic side chain on the nitrogen of the amino sugar moiety in glycopeptides was found to be effective for potent activity against VRE while maintaining potency against methicillin-resistant *Staphylococcus aureus* (MRSA).[13] Telavancin, Oritavancin, and Dalbavancin are demonstrated as the successful examples (Fig. 1). Among them, Televancin was approved by FDA in 2009 for the treatment of complicated skin and skin structure infections (cSSSIs) caused by Gram-positive bacteria, including MRSA. Both Dalbavancin and Oritavancin are in the late stages of clinical trials for the treatment

* Corresponding authors. Tel./fax: +86 21 51980003.
E-mail addresses: jchang@fudan.edu.cn (J. Chang), sunxunf@shmu.edu.cn (X. Sun).

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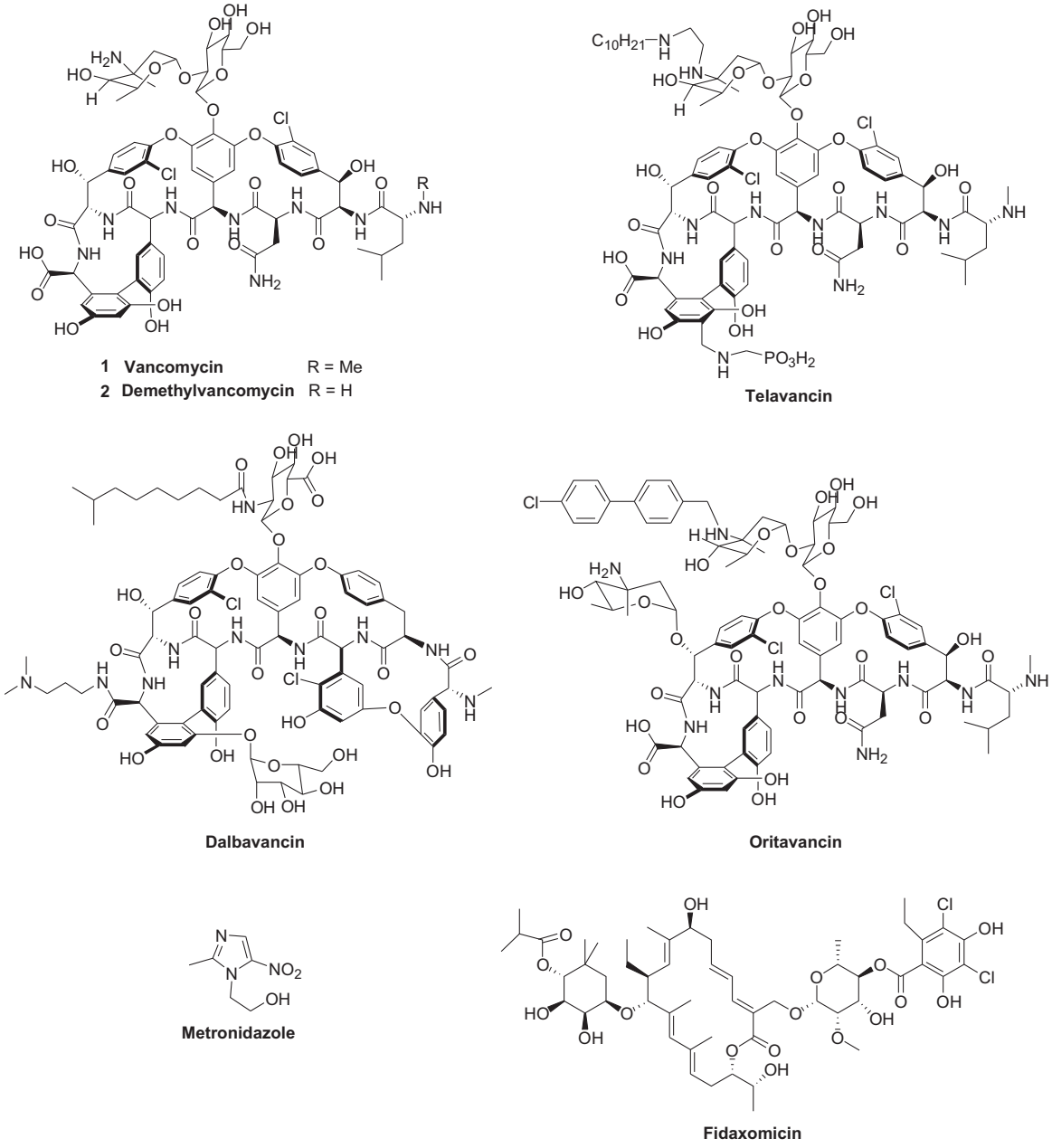


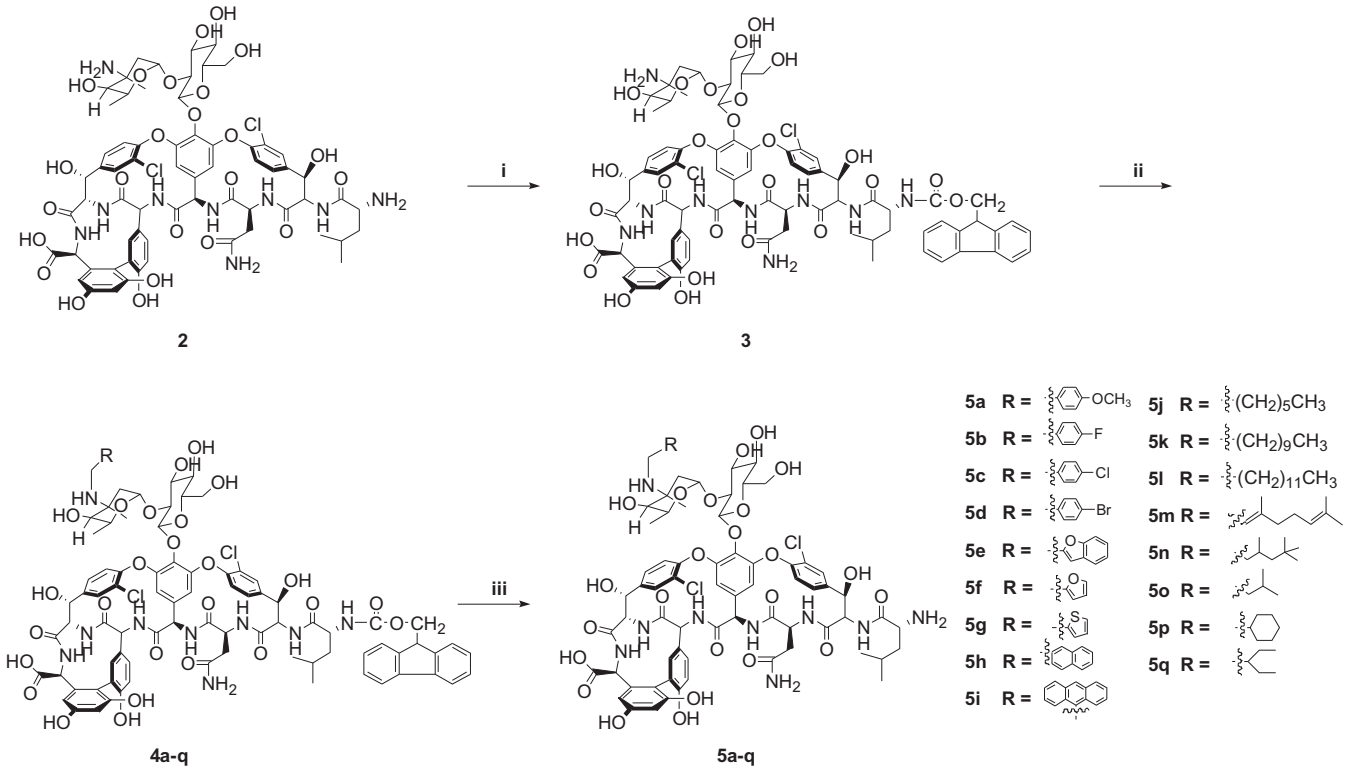
Figure 1. Structures of glycopeptides, metronidazole, and Fidaxomicin.

of cSSSIs.[14] Structurally, Televancin, a novel derivative of *N*-decylaminoethylvancomycin, contains a hydrophilic group of phosphonomethylaminomethyl group,[15] and Dalbavancin is the *N*-(8-methyl) nonylic acyl derivative of the glycopeptide A40926, produced by *Actinomadura strain* ATCC397271.[16] After appended a hydrophobic group on the nitrogen of the amino sugar moiety, the in vitro antibacterial activities have been improved for both Televancin and Dalbavancin. Oritavancin is the *N*-4′-chlorobiphenylmethyl derivative of A82846B, structurally similar to vancomycin,[17] in which the 4′-chlorobiphenylmethyl group can disrupt the cell membrane of Gram-positive bacteria.[18] Although Telavancin, Oritavancin, and Dalbavancin were reported to show greater activity against *C. difficile* than vancomycin,[19] there has been few report on antibacterial activity against *C. difficile* related to demethylvancomycin derivatives. Interestingly, demethylvancomycin is cheaper and easily available as a starting material in China.

Based on the above mentioned successful examples of structural modifications applied on vancomycin, our strategy is to introduce a variety of hydrophobic substituents onto nitrogen of the amino sugar moiety of demethylvancomycin, such as arylmethylene side chains (e.g. substituted benzyl, heteroarylmethylene, and fused arylmethylene group), or aliphatic side chains (e.g. straight-chain alkyl, branched-chain alkyl, alkenyl and cycloalkyl group). It is anticipated that some promising new chemical entities could be obtained from these series of compounds for the treatment of *C. difficile*.

Herein we designed and synthesized 17 demethylvancomycin derivatives, and their antibacterial activity in vitro against *C. difficile* and *Enterococcus faecium* (vancomycin-resistant strain) have been evaluated.

Demethylvancomycin (**2**) was chosen as a starting material for the synthesis of **5a–q** (Scheme 1). The N-terminal free amino group of (**2**) was first protected with *N*-fluorenylmethoxycarbonyl (Fmoc) in dioxane/H_2O (1:1) to obtain **3** in 85% yield.[20] Worth to mention, the chemoselective protection of amino groups by Fmoc at the N-terminus turned out to be problematic here. After numerous



Scheme 1. Reagents and conditions: (i) DIEA (2 equiv), FmocCl (1.1 equiv)/dixane:H_2O (v:v = 1:1), rt, 2.5 h; (ii) (a) aldehyde (5 equiv), DIEA (2 equiv)/DMF, rt, 1 h;(b) $NaCNBH_3$ (3 equiv), TFA (3 equiv)/MeOH, rt, 8–48 h; (iii) piperidine (15 equiv) /DMF, rt, 15 min.

tries, it was found that the N-terminal free amino group was selectively protected by Fmoc (1.1 equiv) with DIEA (2 equiv) at rt for 2 h because of little steric hindrance at N-terminus. Compound **3** was subjected to reductive alkylation with desired aldehydes in DIEA, followed by adding $NaBH_3CN$ as a reducing agent, to afford *N*-Fmoc-demethylvancomycin derivatives (**4a–q**) in 80–90% yields.[20,21] After removing the Fmoc protecting group on **4a–q** with piperidine in DMF at room temperature for 15 min, compounds (**5a–q**) were obtained after further purification via preparative HPLC with CH_3CN aq as the eluant which was another difficult problem leading to the lower yield.[22] The overall yields for each target compound are illustrated in Table 1 and their structures were unambiguously confirmed by spectroscopic analysis, including NMR and mass spectra (ESI and HRESI).

The in vitro antibacterial activity of the compounds (**5a–q**) against Gram-positive bacteria including *C. difficile* (ATCC 43255, ATCC 700057, ATCC700792, IQCC23903), and *E. faecium* (vancomycin-resistant strain, ATCC700802) was evaluated compare to vancomycin (**1**) and demethylvancomycin (**2**). Minimum inhibitory concentration (MIC) values were determined using agar two-fold dilution method according to CLSI (Table 1).[23]

The preliminary results showed that both arylmethylene and aliphatic derivatives of demethylvancomycin maintained good antibacterial activities, complying with the design ideas. Compounds **5c–d**, **5h–j**, **5n** had noticeable antibacterial activity

Table 1
Overall yield, HRESI-MS data and antibacterial activity of the compounds **5a–5q**

	Overall yield (%)	HRESI-MS (*m*/*z*)		*C. difficile* (MIC, μg/mL)				*E. faecium* (MIC, μg/mL)
		Found	Calculated	ATCC 43255	ATCC 700057	ATCC 700792	IQCC 23903	ATCC 700802
1				1	0.5	1	0.5	32
2				0.5	0.5	0.5	0.5	>32
5a	5.8	777.74139 $[M+2H]^{2+}$	777.74331 $[M+2H]^{2+}$	0.5	0.5	1	0.5	16
5b	23.4	771.73029 $[M+2H]^{2+}$	771.73332 $[M+2H]^{2+}$	0.5	0.5	0.5	0.5	16
5c	22.3	779.71967 $[M+2H]^{2+}$	779.71854 $[M+2H]^{2+}$	0.25	0.5	0.25	0.25	16
5d	19.6	1602.38135 $[M+H]^{+}$	1602.37929 $[M+H]^{+}$	0.25	0.25	0.25	0.25	8
5e	22.5	782.73303 $[M+2H]^{2+}$	782.73582 $[M+2H]^{2+}$	1	0.5	1	0.5	8
5f	10.4	757.72870 $[M+2H]^{2+}$	757.72766 $[M+2H]^{2+}$	0.5	0.5	0.5	0.5	32
5g	7.6	765.71771 $[M+2H]^{2+}$	765.71624 $[M+2H]^{2+}$	0.5	0.5	0.5	0.5	32
5h	19.4	787.74725 $[M+2H]^{2+}$	787.74585 $[M+2H]^{2+}$	0.25	0.25	0.125	0.25	16
5i	17.2	812.75543 $[M+2H]^{2+}$	812.75368 $[M+2H]^{2+}$	0.25	0.5	0.25	0.25	2
5j	16.5	766.76782 $[M+2H]^{2+}$	766.76933 $[M+2H]^{2+}$	0.5	0.25	0.5	0.25	8
5k	18.8	794.80133 $[M+2H]^{2+}$	794.80063 $[M+2H]^{2+}$	0.5	0.5	0.5	0.5	0.5
5l	17.1	808.81726 $[M+2H]^{2+}$	808.81628 $[M+2H]^{2+}$	1	2	2	1	4
5m	3.2	785.77832 $[M+2H]^{2+}$	785.77715 $[M+2H]^{2+}$	0.5	0.5	0.5	0.5	16
5n	15.6	780.78090 $[M+2H]^{2+}$	780.78498 $[M+2H]^{2+}$	0.5	0.25	0.5	0.25	8
5o	23.9	752.75293 $[M+2H]^{2+}$	752.75368 $[M+2H]^{2+}$	1	1	1	0.5	32
5p	22.6	1530.51587 $[M+H]^{+}$	1530.51573 $[M+H]^{+}$	1	0.5	1	0.5	16
5q	15.8	759.76123 $[M+2H]^{2+}$	759.75423 $[M+2H]^{2+}$	2	2	2	2	32

against *C. difficile* (2–8 times more active than **1** or **2**). Among them, compound **5h** showed more promising results in terms of antibacterial activity to both *C. difficile* and *E. faecium*. Moreover, compounds **5d–e**, **5i–l, 5n** showed significant activity against *E. faecium* (vancomycin-resistant strain), especially compound **5k,** the most active one (MIC = 0.5 μg/mL), was 64 times more potent than that **1** or **2**.

The antibacterial activities of compounds **5b–5d** against four *C. difficile* strains and *E. faecium* strain were improved as the inductive effect of halogen atom reduced (**5d** > **5c** > **5b**), while the antibacterial activity of *p*-methoxybenzyl derivative (**5a**) was the weakest. Replacement of the aromatic ring with heterocyclic rings such as the furan ring (**5f**) or the thiophene group (**5g**) provided no advantage over the phenyl analogs (**5a–d**), while the fused aromatic analogs (**5e**, **5h**, and **5i**) exhibited an improvement in activity against four *C. difficile* strains and *E. faecium*.

As shown in Table 1, except **5j** and **5n**, aliphatic derivatives of demethylvancomycin (**5j–q**) did not show remarkably antibacterial activity against four *C. difficile* strains compare to **1** or **2**. Interestingly, for analogs **5j–l**, the antibacterial activity against four *C. difficile* strains decreased (**5j** > **5k** > **5l**) when the length of the side chain was extended.

In summary, 17 N-substituted demethylvancomycin derivatives (**5a–q**) with arylmethylene and the aliphatic substituents on the nitrogen of the amino sugar moiety were synthesized in three steps from demethylvancomycin (2) and their in vitro antibacterial activities against *C. difficile* and *E. faecium* were evaluated. Compounds **5d**, **5h,** and **5i** showed more potent activity against *C. difficile* and *E. faecium* (vancomycin-resistant strain) than vancomycin or demethylvancomycin. To our surprise, it was found that demethylvancomycin derivatives appended with arylmethylene side chains, similar to Oritavancin, showed more antibacterial activities against *C. difficile* than those with aliphatic side chains, similar that in Telavancin and Dalbavancin. Compound **5k**, with an undecyl side chain, showed noticeable antibacterial activity against *E. faecium* (vancomycin-resistant strain), 64 times more potent than that of **1** or **2**. Further SAR and mechanism study will be reported in due course.

Acknowledgments

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22. *Representative experiment for the preparation of compound* ***5c***: Compound **2** (2.0 g, 1.35 mmol) and *N*-fluorenylmethoxycarbonyl (384 mg, 1.49 mmol) was dissolved in dioxane/H_2O (1:1, 20 mL). The reaction mixture was added DIEA (0.45 mL, 2.70 mmol), and stirred for 2 h at room temperature. Ethyl acetate was added, and the precipitate was filtered, washed by ethyl acetate and dried in vacuo to give compound **3** in 85% yield. Compound **3** (99 mg, 0.06 mmol) was dissolved in DMF (2 mL), and DIEA (0.019 mL, 0.114 mmol), 4-chlorobenzaldehyde (39 mg, 0.28 mmol) was added. After stirred for 1 h at room temperature, $NaBH_3CN$ (10 mg, 0.17 mmol) and TFA (0.012 mL, 0.17 mmol) was added. The reaction mixture was stirred for additional 4 days and then 5 ml of anhydrous ether was added. The precipitate was filtered, washed by ethyl acetate and dried in vacuo to give compound **4c** in 90% yield. **4c** (96 mg, 0.05 mmol) was dissolved in DMF (2 mL), and piperidine (0.079 mL, 0.81 mmol) was added. After stirred for 15 min at room temperature, 5 ml of anhydrous ether was added. The precipitate was filtered, washed by ethyl acetate and dried in vacuo to give crude product. Further HPLC purification [gradient eluant: CH_3CN-water 5–70% (0.1% TFA)] provided the desired fractions. The eluent was concentrated to a volume of 20 mL and neutralized with saturated sodium bicarbonate to remove the TFA, and then was extracted with n-butanol (20 mL × 3). The organic layer was separated and washed with water, then evaporated in vacuo to dryness. The solid was collected by filtration and washed with acetone and dried in vacuo to give pure compound **5c** (24 mg) as an off white solid. ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 7.83 (s, 1H), 7.55 (m, 1H), 7.49 (m, 5H), 7.44 (m, 1H), 7.30 (d, J = 8.4 Hz, 1H), 7.18 (d, J = 8.4 Hz, 1H), 7.12 (s, 1H), 6.75 (d, J = 8.4 Hz, 1H), 6.69 (d, J = 8.4 Hz, 1H), 6.37 (d, J = 2.2 Hz, 1H), 6.23 (d, J = 2.2 Hz, 1H), 5.74 (s, 1H), 5.64 (s, 1H), 5.32 (m, 1H), 5.27 (m, 1H), 5.12 (s, 1H), 5.08 (s, 2H), 4.79 (m, 1H), 4.63 (d, J = 6.3 Hz, 1H), 4.43 (m, 1H), 4.40 (m, 1H), 4.16 (m, 1H), 4.08 (m, 1H), 4.03 (m, 1H), 3.98 (s, 2H), 3.66 (d, J = 9.8 Hz, 1H), 3.54 (d, J = 9.8 Hz, 1H), 3.49 (m, 1H), 3.30 (overlapped by D_2O, 4H), 2.67 (m, 2H), 2.04 (d, J = 13.2 Hz, 1H), 1.78 (d, J = 13.2 Hz, 1H), 1.66 (m, 1H), 1.58 (m, 1H), 1.53 (m, 1H), 1.44 (s, 3H), 1.09 (d, J = 6.3 Hz, 3H), 0.88 (m, 6H); ^{13}C NMR (DMSO-d_6, 150 MHz) δ: 172.98, 170.71, 169.61, 168.28, 158.67, 158.46, 157.65, 157.02, 155.54, 152.91, 151.61, 150.26, 148.71, 142.95, 136.49, 136.17, 134.08, 132.78, 132.22, 131.71, 128.98, 128.91, 127.86, 127.56, 126.91, 126.55, 125.94, 124.69, 123.81, 121.98, 118.54, 116.58, 108.17, 106.10, 105.16, 102.81, 101.28, 97.00, 78.40, 77.45, 77.31, 72.02, 70.63, 68.80, 63.58, 62.26, 61.67, 60.07, 59.50, 57.13, 55.33, 54.06, 51.76, 51.31, 42.25, 33.29, 23.87, 23.23, 22.26, 19.62, 17.35; ESI-MS m/z: 779.72 $[M+2H]^{2+}$; ESI-FTMS m/z: calcd. for $C_{72}H_{78}Cl_3N_9O_{24}$ $[M+2H]^{2+}$: 779.71854, found 779.71967.
23. The minimum inhibitory concentration (MIC) values of the novel compounds against *E. faecalis* (ATCC 700802) and *C. difficile* (ATCC 43255, ATCC 700057, ATCC 700792, IQCC 23903) were tested using vancomycin and demethylvancomycin as a positive control. MIC values were determined using an agar dilution method according to the methods of CLSI. Compounds were dissolved in 50% water in DMSO to prepare a stock solution that had a concentration of 320 μg/mL. Serial twofold dilutions were prepared from the stock solution with sterile water and then 10-fold diluted with Mueller-Hinton (MH) agar medium to provide concentration ranges of 16–0.03125 μg/mL. The tested organisms were grown in MH broth medium at 35 °C for 8 h and were adjusted to the turbidity of the 0.5 McFarland standard. The bacterial suspensions were inoculated onto the drug-supplemented MH agar plates with a multipoint inoculator and incubated at 35 °C for 16 h.