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FULL PAPER

Bis-Cyclic-Guanidine as a Novel Class of Compounds Potent Against *Clostridium Difficile*

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Abstract: *Clostridium difficile* infection (CDI) symptoms range from diarrhea to severe toxic megacolon and even death. Due to its rapid acquisition of resistance, *C. difficile* is listed as an urgent antibiotic-resistant threat and has surpassed methicillin-resistant Staphylococcus aureus (MRSA) as the most common hospital-acquired infections in the USA. To combat the pathogen, the new structural class of pseudo peptides that exhibit antimicrobial activities could play an important role. Herein, we report that bis-cyclic guanidine compounds that exhibit potent antibacterial activity against *C. difficile* with decent selectivity. Eight compounds showed high in vitro potency against *C. difficile* UK6 with MIC of 1.0 μg/mL, and cytotoxic selectivity index (SI) up to 37. Moreover, the most selective compound **13** is also effective upon the treatment of *C. difficile*-induced diseases in the mouse model of CDI and appears to be a very promising new candidate for the treatment of CDI.

Introduction

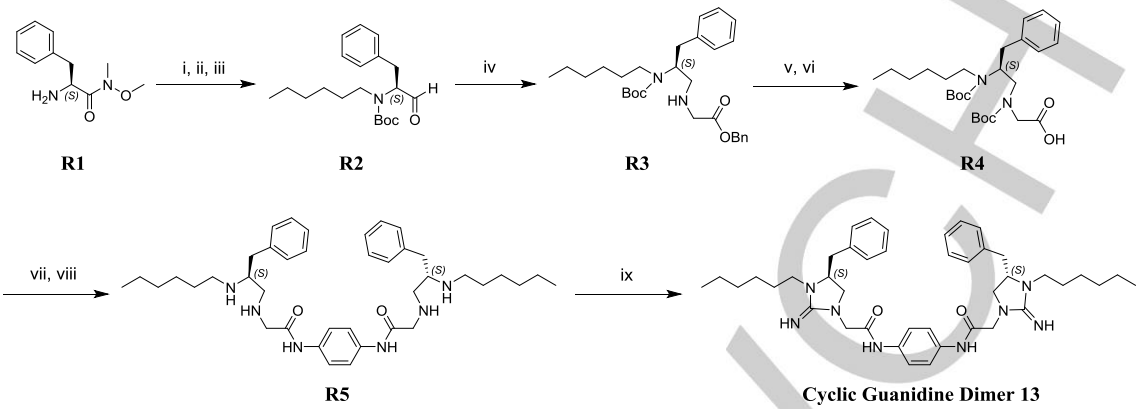
Clostridium difficile (*C. difficile*) is a Gram-positive, spore-forming, anaerobic and toxigenic microbe. Symptoms of *C. difficile* infection (CDI) range from uncomplicated diarrhea to pseudomembranous colitis and even toxic megacolon.[1] *C. difficile* is recognized as the most common cause of hospital-associated diarrhea,[2] and may lead to more related complications,[3] resulting increasingly infectious morbidity and mortality. More alarmingly, the emergence of hypervirulent strains NAP1/BI/027 has been associating with higher mortality rates in North America and several countries in Europe.[4] Antimicrobial therapeutic options with oral vancomycin and metronidazole are effective for severe and mild-to-moderate CDI respectively.[4b, 5] Treatment options for severe CDI include the use of the newly developed antimicrobial agents such as fidaxomicin and fecal microbiota transplantation (FMT), which was identified as an effective treatment for CDI recurrence.[6] However, initial therapy with metronidazole and vancomycin has been associated with increased the rate of failure and recurrence.[7] Fidaxomicin is more reliable but more expensive than metronidazole/vancomycin. The Centers for Disease Control and Prevention has listed *C. difficile* as an urgent antibiotic-resistant threat.[8] Although *C. difficile* has not yet developed significant resistance to the antibiotics most used for CDI treatment, it is highly likely that these resistance phenotypes will emerge, as has occurred through the use of clindamycin and the fluoroquinolones.[9]

Novel antibiotics are in urgent need to more effectively treat CDI. Bis-guanidine related compounds have been reported to bear antiseptic and antibacterial activities, such as hexamidine,[10] norspermidine analogues,[11] teixobactin,[12] Brilacidin,[13] and amphipathic xanthone derivatives[14] etc. This type of compounds display antimicrobial activity against Gram-positive organisms including Methicillin-resistant *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus epidermidis*, and Vancomycin-Resistant *Enterococci faecalis*, and relatively weaker activity against Gram-negative organisms such as *K. pneumoniae* and *P. aeruginosa*.[15] Recently, we have discovered a new type of symmetric bis-cyclic guanidine compounds[15] bearing amphipathic structures that could mimic mechanism of action of host-defense peptides (HDPs).[16] These membrane active, amphipathic compounds showed potent and broad-spectrum activity against both Gram-positive and Gram-negative bacteria. However, to the best of our knowledge, compounds bearing with guanidine groups [17] have been rarely explored for bactericidal activities against *C. difficile*.[18] Herein, we report the antibacterial activity of these dimeric cyclic guanidines against *C. difficile* in vitro and in vivo.

Results and Discussion

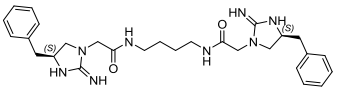
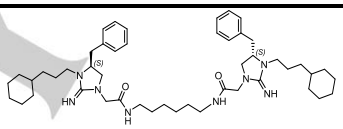
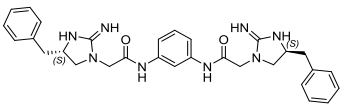
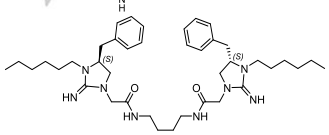
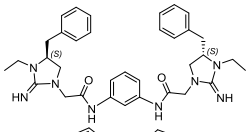
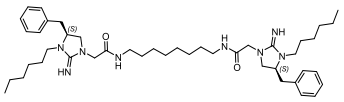
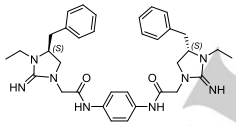
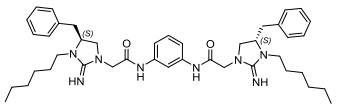
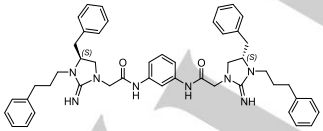
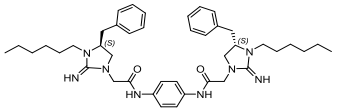
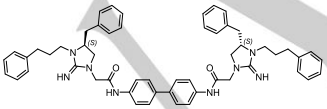
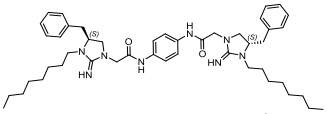
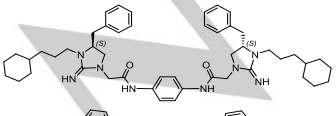
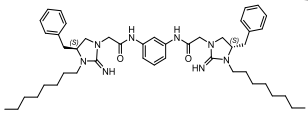
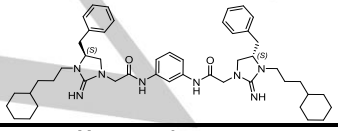
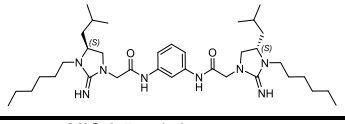
The bis-cyclic guanidine library was synthesized following the same procedure reported previously.[15] Synthesis of compound **13** was shown as an example of typical synthesis process (Scheme 1). Intermediate R4 was obtained from easily accessible reagent R1 through straightforward way with decent yield. The linear intermediate R5 was obtained by coupling between R4 and diamine (*p*-phenylenediamine) in the presence

of hydroxybenzotriazole (HOBt) and *N,N'*-dicyclohexylcarbodiimide (DCC) followed by removing Boc protecting group. R5 could be easily cyclized in the presence of cyanogen bromide to furnish the final bis-cyclic guanidine compound **13**.



Scheme 1 Typical synthesis protocol for bis-cyclic guanidine derivative **13**. (i) Hexanal, $NaBH_3CN$, MeOH, AcOH, room temperature (rt), 3 h; (ii) Boc_2O, $NaHCO_3$, THF, H_2O, rt, 5 h; (iii) $LiAlH_4$, THF, –20 °C, 30 min; (iv) Glycine benzyl ester, $NaBH_3CN$, MeOH, AcOH, room temperature, 3 h; (v) Boc_2O, $NaHCO_3$, THF, H_2O, rt, 5 h; (vi) H_2, Pd-C, MeOH, 2 h; (vii) Benzene-1,4-diamine, HOBt, DIPEA, DCC, DMF, rt, 24 h; (viii) TFA/DCM (50:50, v/v), rt, 2 h; (ix) CNBr, MeCN, rt, 12 h.

Table 1 The structure of compounds **1**–**16** and their antibacterial activity against *C. difficile*.

Cpd #	Structure	Log P[a]	MIC (μg/mL)	Cpd #	Structure	Log P	MIC (μg/mL)
1		1.21	128	**9**		6.12	2.0
2		2.28	32	**10**		4.31	8.0
3		3.17	32	**11**		5.58	2.0
4		3.23	8.0	**12**		5.31	1.0
5		5.51	1.0	**13**		5.38	1.0
6		6.56	1.0	**14**		5.78	1.0
7		5.57	4.0	**15**		5.72	1.0
8		5.55	1.0	**16**		5.01	1.0
Vancomycin				MIC 0.5 μg/mL			

[a]Log P, calculated by Alogps 2.1 program.

The antibacterial potency of these cyclic guanidine dimers on the hypervirulent *C. difficile* UK6 was assessed and described as minimal inhibitory concentration (MIC). As shown in Table 1, the majority of these quinoline compounds displayed potent *in vitro* activity, with MICs in the range of 1.0–4.0 μg/mL. Compounds **1** and **2** that do not bear hydrophobic groups on the guanidine residues displayed weak activity, especially compound **1** showed a MIC of 128 μg/mL. When ethyl group was installed on the guanidine to furnish compounds, i.e. **3** and **4**, they exhibited activity against *C. difficile*, with MIC of 8.0 μg/mL for compound **4**. Compound **5** and **6** have 3-phenylpropyl group attached on the nitrogen atoms of guanidine groups and showed potent antimicrobial activity (MIC=1.0 μg/mL), due to the enhanced ability of interaction with the bacterial membrane. When hydrophobic cyclohexane propyl group was conjugated on the guanidine position to give compounds **7**–**9**, the activities increased as the change of the linker between two cyclic guanidine rings from *p*-phenylenediamine to *m*-phenylenediamine, and then 1,6-hexamethylene, with MICs of 4.0, 1.0, and 2.0 μg/mL respectively. Compound **10** that has aliphatic chain C_6H_{13} on the guanidine rings only showed MIC of 8 μg/mL, where the linker was kept as 1,4-butylene group. However, the activity bounced back when the linker was replaced with 1,8-octamethylene (**11**, MIC=2.0 μg/mL), *m*-phenylenediamine (**12**, MIC=1.0 μg/mL), *p*-phenylenediamine (**13**, MIC=1.0 μg/mL).

Interestingly, replacement of the aliphatic chain on the guanidine rings with increased chains (C_8H_{17}) did not compromise the activity, with the same MIC values of 1.0 μg/mL in the afforded compounds **14** and **15**. Replacing the benzyl group (initially starting from α-Phenylalanine) with isobutyl group (initially starting from α-Leucine) produced the compound **16**, which was also potent, with MIC vale of 1.0 μg/mL, very close to that of the positive control Vancomycin under the same assay condition.

Table 2 The cytotoxicity assessment of active compounds (MIC < 4 μg/mL).

Cpd	MIC (μg/mL)	[a]CC_{50} (μg/mL)		*SI (CC_{50}/MIC)	
		HEK293T	HepG2	HEK293T	HepG2
5	1	20.6	36.8	20.6	36.8
6	1	26.5	31.7	26.5	31.7
8	1	33.9	30.1	33.9	30.1
9	2	42.7	40.3	21.35	20.15
11	2	25.1	24.0	12.55	12
12	1	32.3	33.1	32.3	33.1
13	1	31.8	37.3	31.8	37.3
14	1	29.3	26.8	29.3	26.8
15	1	25.7	25.9	25.7	25.9
16	1	24.3	18.1	24.3	18.1

[a]CC_{50}, the 50% cytotoxic centration; SI: selective index, SI = CC_{50}/MIC.

To determinate the cytotoxicity of ten active compounds (MIC < 4 μg/mL) out of sixteen compounds, MTT assays were performed on human liver cancer cell line HepG2 and human embryonic kidney cell line HEK293T. As shown in Table 2, 3-phenylpropyl modified compounds **5** and **6**, and cyclohexane propyl modified compounds **8** and **9** showed about 20–30-fold selectivity index (SI) (CC_{50} on human cells/MIC on *C. difficile* cells). Aliphatic side chain bearing compound **11** only displayed ten-fold SI, however, compounds **12**–**15** all had low cytotoxicity against both cell lines, especially compound **13** had 37-fold SI against HEK293T cells. Compound **12** and **13** were also not hemolytic even at the concentration of 250 μg/mL.[15] The selectivity decreased slightly when the benzyl group was replaced with isobutyl group. Overall, the compound **13** has the best SI among all the compounds tested.

To further investigate the impact of hydrophobicity on the activity profile, we measured HPLC retention time (RT) (Table S1) and determined log *P* values (Table 1) of all compounds. Generally, the antibacterial activity of compounds increases with longer RT value when RT is shorter than 27.25 min. When RT is longer than 27.25 min, the activity of compounds did not decrease, however, the cytotoxicity of compounds increased (Table 2). Similar trend can be obtained from the correlation of activity with log *P* value. Based on these results, we could conclude that the antibacterial activity and the selectivity of this type of compounds could be improved by carefully tuning the balance between hydrophobicity and hydrophilicity, which is of great help on design HDPs mimics in the future.

Efficacy of compound **13** was further evaluated in mouse model of CDI. As shown in Figure 1a, the *C. difficile* UK6 challenged control group lead to 90% of diarrhea, while the administration of compound **13** displayed significant improvement for overcoming CDI over the entire experimental period (five days). After 3 days, 90% of survival was observed with the administration of compound **13** compared to the control group where only 40% of mouse survived (Figure 1b). Five days later, 60% of the mice were still alive with the treatment of compound **13**, while only 40% of survival was exhibited in the *C. difficile* UK6 challenged control group. These data indicated that compound **13** could improve the diarrhea and survival of mice challenged with *C. difficile* UK6, a hypervirulent strain.

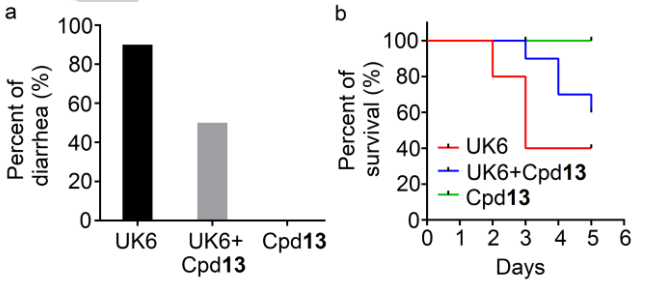


Figure 1. In vivo efficacy of the compound **13** in mouse model of *C. difficile* infection. Two groups of mice (UK6 and UK6+cpd **13**, n = 10 per group) were challenged with *C. difficile* spores at 10^6 /mouse in the absence or presence of compound **13**, after pretreatment of antibiotics. The third group mice (n = 5) were administered compound **13** only as controls. (a) Percent of diarrhea with or without treatment of compound **13**. (b) Survival rates of mice with or without treatment of compound **13**. Results were analyzed by the two-way ANOVA method. The differences between the UK6 group and the treatment group (UK6 + compound **13**) are statistically significant, $p < 0.05$.

The amount of *C. difficile* in fecal samples after treatment were also determined. As shown in Figure 2, one day after the infection, the amount of *C. difficile* in feces from mice treated with compound **13** was 50% less than that of *C. difficile* UK6 challenged group. After 5 days, the amount of *C. difficile* in fecal samples from control group continued to increase, while mice treated with compound **13** were observed with significant decrease of *C. difficile* in fecal samples, down by 80% compared to control levels. This result demonstrated that compound **13** had good efficacy on the inhibition of *C. difficile* in mice.

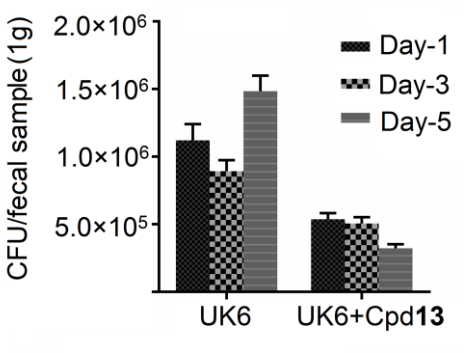


Figure 2. *C. difficile* in fecal samples from compound **13**-treated (UK6+cpd 13) or control (UK6) mice. Two groups of mice (UK6 and UK6+cpd **13**, n = 10 per group) were challenged with C. difficile spores at 10^6 /mouse in the absence or presence of compound **13**, after pretreatment of antibiotics. Fecal samples were collected, and C. difficile spores were determined as described in methods. *C. difficile* isolated in fecal from mouse treated with compound **13** showed significantly decrease compared with that of UK6 challenged group (Two-way ANOVA, P<0.001)

Conclusions

We have reported a series of membrane active bis-cyclic guanidines (molecular mass 600–900) which displayed potency against *C. difficile* UK6, an emerging hypervirulent bacteria. In vitro studies demonstrated that eight out of sixteen cyclic guanidine dimeric compounds showed MIC value of 1.0 μg/mL against *C. difficile*, very close to that of Vancomycin (MIC=0.5 μg/mL). Moreover, the cyclic guanidine dimers also revealed significant efficacy in mouse model of CDI. Further modifications of these compounds may lead to novel potent antibiotics against *C. difficile*.

Experimental Section

General information.

The starting material to synthesize R1 was purchased from Chem-Impex International, Inc. Solvents and other reagents were purchased from either Sigma-Aldrich or Fisher Scientific and were used without further purification. The final products were purified on a Waters Breeze 2 HPLC system and lyophilized on a Labconco lyophilizer. The purity of the compounds was determined to be >95% by analytical HPLC (1 mL/min flow, 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 50 min was used) and the data is shown in the Supporting Information. The NMR spectra were obtained on a Varian Inova 500 instrument.

Synthesis of the intermediate build block R4.

Compound **R1** (TFA salt, 13.6 g, 42.3 mmol) was dissolved in MeOH and treated with TEA (5.8 mL, 42.3 mmol) before adding to a solution of hexanal (5.2 mL, 42.3 mmol) in MeOH and acetic acid (5.1 mL, 82.6 mmol). After stirring for 10 min under ice/H_2O bath, $NaBH_3CN$ (5.6 g, 82.6 mmol) was added portion wise. The reaction was stirred for 3 h at room temperature before solvent was removed. The crude mixture was treated with $NaHCO_3$ (aq.) and extracted with EtOAc, and the organic layer was separated and evaporated to give an oil crude, which was purified by silica gel column chromatography to give 8.9 g of the desired secondary amine. Boc_2O (8 g, 36.6 mmol) was added in the THF/H_2O (1:1, v/v) solution of this intermediate containing $NaHCO_3$ (5.1 g, 61 mmol) and allowed to react for 5 h, after which EtOAc was added and the organic layer was collected. The solvent was removed in reduced pressure to give the colorless crude, which was purified by flash column chromatography to give 9.1 g of compound **R2**. Next, compounds **R2** was taken in THF and reduced by $LiAlH_4$ (926 mg, 23.2 mol) for 30 min at -20 °C, then water was added to quench the reaction. The mixture was extracted with EtOAc, and the organic layer was separated, the solvent was removed in vacuo to give the crude **R3** (7.2 g), which was used in the next reaction without any further purification. BOC protecting group was attached as the same procedure for attaching BOC onto compound **R2**, followed by hydrogenation to remove benzyl protecting group in MeOH to give the building block **R4** (7.5 g) as a white solid after filtration and concentration.

Building block **R4** (400 mg, 0.81 mmol), HOBt (249 mg, 1.6 mmol), DIPEA (284 μL, 1.6 mmol), and *p*-Phenylenediamine (53 mg, 0.49 mmol) was dissolved in DMF (3 mL) and then DCC (335 mg, 1.6 mmol) was added. The reaction mixture was stirred at room temperature for 24 h. The afforded byproduct DCU was filtered off and the filtration was added into water and extracted with EtOAc (×3). The organic phase was combined and washed with 1M HCl (×2), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude oil compound was treated with TFA in DCM (1:1, v/v) for 2 h to completely remove BOC protecting groups to yield crude compound **R5**. Subsequently, **R5** was dissolved in acetonitrile (3 mL), to which CNBr (4 eq.) was added carefully (caution: very toxic). The reaction was stirred for 12 h at room temperature. 1M NaOH solution was added carefully, followed by proper amount of bleach to deactivate excessive CNBr. The mixture was filtered through a millpore filter and purified by HPLC purification on Waters HPLC system, and the desired fraction was lyophilized to give the pure product **13**.

The other compounds were synthesized according to the same procedure as compound **13**. Different aldehydes were used at the first step to give different compounds with various side chains.

The NMR data of compounds **1**–**5**, **12**, **13**, and **16** are consist with the data in the previous paper.[15] The NMR and HRMS of other compounds are shown below:

Compound **6**: ^{1}H NMR (500 MHz, CD_3OD) δ 7.64 (d, J = 7.5 Hz, 4H), 7.57 (d, J = 7.5 Hz, 4H), 7.18–7.33 (m, 20H), 4.22–4.27 (m, 2H), 4.14 (s, 4H), 3.63 (t, J = 9.5 Hz, 2H), 3.51–3.57 (m, 2H), 3.42 (dd, J = 9.5, 5.0 Hz, 2H), 3.34 (dd, J = 9.0, 5.5 Hz, 2H), 3.09 (dd, J = 13.5, 4.5 Hz, 2H), 2.85 (dd, J = 14.0, 8.5 Hz, 2H), 2.62–2.74 (m, 4H), 1.92–2.06 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD) δ 165.1, 158.0, 140.9, 137.2, 136.2, 135.7, 134.3, 129.0 (2C), 128.5 (2C), 128.2 (2C), 128.0 (2C), 126.8, 126.6 (2C), 125.8, 119.8, 58.0, 51.7, 42.3, 37.4, 32.1 (2C), 28.3. HRMS (ESI) $C_{54}H_{59}N_8O_2$ $[M+H]^+$ calcd = 851.4755; found = 851.4742.

Compound **7**: ^{1}H NMR (500 MHz, CD_3OD) δ 7.53 (s, 4H), 7.31–7.34 (m, 4H), 7.24–7.28 (m, 6H), 4.27–4.32 (m, 2H), 4.13, 4.10 (ABq, J = 18.0 Hz, 4H), 3.68 (t, J = 9.0 Hz, 2H), 3.41–3.48 (m, 4H), 3.25 (ddd, J = 15.0, 9.5, 5.5 Hz, 2H), 3.15 (dd, J = 13.5, 5.0 Hz, 2H), 2.89 (dd, J = 13.5, 8.0 Hz, 2H), 1.58–1.76 (m, 14H), 1.15–1.32 (m, 12H), 0.90–0.97 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD) δ 165.1, 158.0, 135.8, 134.3, 128.9 (2C), 128.5 (2C), 126.8, 120.1 (2C), 57.9, 51.8, 47.0, 43.1, 37.6, 37.5, 37.3, 33.6, 33.0, 32.9, 26.3, 26.0, 24.0. HRMS (ESI) $C_{48}H_{67}N_8O_2$ $[M+H]^+$ calcd = 787.5381; found = 787.5374.

Compound **8**: ^{1}H NMR (500 MHz, CD_3OD) δ 8.04 (s, 1H), 7.31–7.34 (m, 4H), 7.24–7.28 (m, 9H), 4.26–4.32 (m, 2H), 4.14, 4.10 (ABq, J = 18.0 Hz, 4H), 3.68 (t, J = 9.5 Hz, 2H), 3.41–3.47 (m, 4H), 3.25 (ddd, J = 14.5, 9.0, 5.5 Hz, 2H), 3.15 (dd, J = 13.5, 5.0 Hz, 2H), 2.89 (dd, J = 13.5, 8.0 Hz, 2H), 1.60–1.75 (m, 14H), 1.14–1.32 (m, 12H), 0.90–0.96 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD) δ 165.2, 158.0, 138.6, 135.8, 128.9 (2C), 128.5 (2C), 126.8, 120.1 115.3, 58.0, 51.7, 47.0, 43.1, 37.6, 37.3, 33.7, 33.0, 32.9, 26.3, 26.0 (2C), 24.0. HRMS (ESI) $C_{48}H_{67}N_8O_2$ $[M+H]^+$ calcd = 787.5381; found = 787.5357.

Compound **9**: ^{1}H NMR (500 MHz, CD_3OD) δ 7.31–7.34 (m, 4H), 7.25–7.27 (m, 6H), 4.24–4.30 (m, 2H), 3.93, 3.89 (ABq, J = 17.5 Hz, 4H), 3.60 (t, J = 9.5 Hz, 2H), 3.42 (ddd, J = 14.5, 9.0, 6.0 Hz, 2H), 3.33 (dd, J = 9.5, 6.0 Hz, 2H), 3.21–3.26 (m, 2H), 3.19 (t, J = 7.0 Hz, 4H), 3.13 (dd, J = 13.5, 5.0 Hz, 2H), 2.85 (dd, J = 13.5, 8.0 Hz, 2H), 1.65–1.74 (m, 12H), 1.58–1.62 (m, 2H), 1.50 (p, J = 6.5 Hz, 4H), 1.32–1.35 (m, 4H), 1.14–1.31 (m, 12H), 0.85–0.95 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD) δ 166.9, 158.0, 135.8, 128.9 (2C), 128.5 (2C), 126.8, 57.9, 51.6, 46.6, 43.1, 39.1, 37.5, 37.3, 33.7, 33.0, 32.9, 28.8, 26.3, 26.1, 26.0 (2C), 24.0. HRMS (ESI) $C_{48}H_{74}N_8O_2$ $[M+H]^+$ calcd = 795.6006; found = 795.5998.

Compound **10**: ^{1}H NMR (500 MHz, CD_3OD) δ 7.30–7.34 (m, 4H), 7.24–7.26 (m, 6H), 4.24–4.30 (m, 2H), 3.94, 3.90 (ABq, J = 18.0 Hz, 4H), 3.59 (t, J = 9.5 Hz, 2H), 3.45 (ddd, J = 15.0, 9.0, 6.5 Hz, 2H), 3.34 (dd, J = 9.5, 6.0 Hz, 2H), 3.23–3.29 (m, 2H), 3.19–3.23 (m, 4H), 3.13 (dd, J = 13.5, 5.0 Hz, 2H), 2.87 (dd, J = 14.0, 8.0 Hz, 2H), 1.51–1.54 (m, 4H), 1.28–1.36 (m, 12H), 0.92 (t, J = 6.5 Hz, 6H). ^{13}C NMR (125 MHz, CD_3OD) δ 167.0, 157.9, 135.8, 129.0 (2C), 128.5 (2C), 126.8, 57.9, 51.6, 46.7, 42.9, 38.7, 37.5, 31.1, 26.6, 26.2, 25.8, 22.1, 12.9. HRMS (ESI) $C_{40}H_{63}N_8O_2$ $[M+H]^+$ calcd = 687.5068; found = 687.5056.

Compound **11**: ^{1}H NMR (500 MHz, CD_3OD) δ 7.31–7.34 (m, 4H), 7.24–7.27 (m, 6H), 4.24–4.30 (m, 2H), 3.94, 3.90 (ABq, J = 18.0 Hz, 4H), 3.59 (t, J = 9.0 Hz, 2H), 3.45 (ddd, J = 15.0, 9.0, 6.5 Hz, 2H), 3.34 (dd, J = 9.5, 5.5 Hz, 2H), 3.25 (ddd, J = 15.0, 9.0, 5.5 Hz, 2H), 3.18 (t, J = 7.0 Hz, 4H), 3.14 (dd, J = 13.5, 5.0 Hz, 2H), 2.86 (dd, J = 13.5, 8.0 Hz, 2H), 1.54–1.69 (m, 4H),1.50 (t, J = 6.0 Hz, 4H), 1.30–1.36 (m, 20H), 0.92 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, CD_3OD) δ 166.9, 157.9, 135.8, 128.9 (2C), 128.5 (2C), 126.8, 57.9, 51.5, 46.7, 42.8, 39.2, 37.5, 31.2, 28.9, 26.6, 26.5, 25.8, 22.2, 12.9. HRMS (ESI) $C_{44}H_{71}N_8O_2$ $[M+H]^+$ calcd = 743.5694; found = 743.5675.

Compound **14**: ^{1}H NMR (500 MHz, CD_3OD) δ 8.02 (d, J = 1.0 Hz, 1H), 7.31–7.34 (m, 4H), 7.24–7.28 (m, 9H), 4.27–4.32 (m, 2H), 4.14, 4.11 (ABq, J = 18.0 Hz, 4H), 3.67 (t, J = 9.5 Hz, 2H), 3.47 (ddd, J = 15.5, 9.5, 7.0 Hz, 2H), 3.42 (dd, J = 9.5, 5.5 Hz, 2H), 3.27 (ddd, J = 15.0, 9.5, 5.5 Hz, 2H), 3.16 (dd, J = 13.5, 5.0 Hz, 2H), 2.89 (dd, J = 14.9, 8.5 Hz, 2H), 1.59–1.70 (m, 4H), 1.30–1.34 (m, 20H), 0.91 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, CD_3OD) δ 165.2, 158.0, 138.6, 135.8, 129.0 (2C), 128.5 (2C), 126.8, 115.3, 111.2, 58.0, 51.7, 42.8, 37.5, 31.5, 28.9 (2C), 26.7, 26.1, 22.3, 13.0. HRMS (ESI) $C_{46}H_{67}N_8O_2$ $[M+H]^+$ calcd = 763.5381; found = 763.5359.

Compound **15**: ^{1}H NMR (500 MHz, CD_3OD) δ 7.52 (s, 4H), 7.31–7.34 (m, 4H), 7.24–7.28 (m, 6H), 4.27–4.33 (m, 2H), 4.12, 4.09 (ABq, J = 18.5 Hz, 4H), 3.67 (t, J = 9.5 Hz, 2H), 3.48 (ddd, J = 15.5, 9.0, 7.0 Hz, 2H), 3.42 (dd, J = 9.5, 5.5 Hz, 2H), 3.25–3.29 (m, 2H), 3.16 (dd, J = 14.0, 5.0 Hz, 2H), 2.89 (dd, J = 13.5, 8.5 Hz, 2H), 1.58–1.71 (m, 4H), 1.27–1.35 (m, 20H), 0.91 (t, J = 6.5 Hz, 6H). ^{13}C NMR (125 MHz, CD_3OD) δ 165.0, 158.0, 135.8, 134.3, 129.0 (2C), 128.5 (2C), 126.7, 120.0 (2C), 57.9, 51.7, 47.0, 42.8, 37.5, 31.5, 28.9 (2C), 26.6, 26.0, 22.3, 13.0. HRMS (ESI) $C_{46}H_{67}N_8O_2$ $[M+H]^+$ calcd = 763.5381; found = 763.5358.

Minimum inhibitory concentrations (MICs) against bacteria.

The antimicrobial activities of the cyclic guanidine dimers against *C. difficile* UK6 were tested using media and methods recommended by the Clinical and Laboratory Standards Institute for susceptibility testing of anaerobes.[19] Compounds at 5 mg/ml were added to wells of 96-well microplates containing UK6 culture at a density of 0.5 McFarland (100 µL/well) in BHIS medium to make final concentrations of extracts ranging from 128 µg/ml to 0.5 µg/ml at a two-fold reduction. The plates were incubated at 37 °C for 24 h. The MICs were determined as the lowest concentration that completely inhibits the bacteria growth in the wells. Vancomycin was included as positive controls.

MTT assay.

MTT (3-(4,5-dimethylthiazol-2yl)-2,5-dipheynyltetrazolium bromide; Sigma–Aldrich, St. Louis, MO) cell viability assay was performed to evaluate the cytotoxicity of the compounds on human HepG2 and HEK293T cell lines. HepG2 is an immortalized cell line consisting of human liver carcinoma cells. HEK293T is a specific cell line originally derived from human embryonic kidney cells grown in tissue culture. Both cells were maintained in a Dulbecco's Modified Eagle Medium (DMEM with, 4.5 g/L Glucose, L-Glutamine and Sodium Pyruvate, Corning; Corning, Manassas, VA) containing 10% FBS (Thermo Scientific) and 1% penicillin/streptomycin at 37°C in 5% CO_2. Cells (10^4 cells/ well) were plated in 96-well plates. After incubation overnight, cells were treated with the compounds at concentrations from 128 µg/ml to 0.125 µg/ml or 1% DMSO (as a control reagent) for 24 h at 37°C. Then 10 µl of MTT stock solution (5 mg/ml) were added to cells in each well, and further incubated for 4 h at 37°C. After careful removal of media from each well without disturbing cells, 100 µl of DMSO was added to each well, and incubated for 15 min at 37°C. Absorbance at 540 nm was read in a Synergy HTX multi-mode reader (Bio Tek Instruments, Inc. Winooski VT). Data were analyzed using Graphpad PRISM 6 (GraphPad Software, Inc., La Jolla, CA), and the 50% cytotoxic concentration (IC_{50}) was reported as a concentration of compound that reduced the cell viability by 50% when compared to untreated controls. Then CC_{50} was determined to establish a selectivity index (SI) (SI = CC_{50}/MIC).

Evaluation of compounds in mouse model of *C. difficile* infection (CDI).

C57BL/6 female mice (6-week old) were purchased from Charles River Laboratories, MA. During the experiment, the mice were housed in groups of 5 animals per cage under the same conditions. All animal experiments were approved by the institutional committee for animal care and use at the University of South Florida. The experimental design is illustrated in Figure S1. Twenty-five mice were divided into three groups (group 1–3). Group 1 (n = 10) were challenged with spores of *C. difficile* UK6. Groups 2 (n = 10) were challenged with spores of *C. difficile* UK6, and treated by compound **13**. Group 3 (n = 5) were only treated with compound **13** without infection. The mice were given drinking water containing a mixture of six antibiotics including ampicillin (200 mg/kg), kanamycin (40 mg/kg), gentamycin (3.5 mg/kg), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg) and vancomycin (4.5 mg/kg) for 5 days, and then received autoclaved water for 2 days, followed by a single dose of clindamycin (10 mg/kg) intraperitoneally 1 day before (day-1) challenge day. In the challenge day (day 0), mice in groups 1–2 were challenged with *C. difficile* UK6 spores at 10^6 colony-forming unit (CFU) by gavage. At 4 hours post challenge, the mice in groups 2 were given one dose of compound **13** (50 mg/kg) via oral routine. From the first day post challenge (day 1), mice in group 2 received one dose of compound **13** twice a day (50 mg/kg/day) for five days. Meanwhile, the mice in group 3 were also given compound **13** at the same time with the same dose to determine the toxicity of the compound to the mice. After *C. difficile* challenge and/or compound treatment, mice were monitored twice a day during the experiment for weight changes, diarrhea (defined as soft or watery feces) and other symptoms of the disease.

Fecal samples were collected at the 1st, 3rd, and 5th day post challenge for *C. difficile* spore enumeration. Fecal samples were weight and shocked in 95% ethanol (0.1 g/ml) for 1 hour followed by serial dilution in PBS, spreading on BHI plates supplemented with 10% taurocholic acid, and incubation in an anaerobic chamber. After incubation for 48 hours, the colonies on plates in three duplicates for the selected dilutions were counted.

Statistical analysis.

Data were analyzed using GraphPad Prism 6.0 (GraphPad Software Inc.). Statistical analyses were performed using the Kaplan-Meier survival analysis (survival rate) and the two-way ANOVA method (results of diarrhea rate and the amount of *C. difficile* in fecal samples after treatment

were expressed as means ± standard errors). *P* values less than or equal to 0.05 were considered significant.

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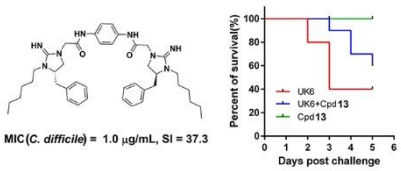
Entry for the Table of Contents



Clostridium difficile is listed as an urgent antibiotic-resistant threat and has surpassed MRSA as the most common hospital-acquired infections. Here we reported a series of small molecular antibacterial agents based on the dimeric cyclic guanidine scaffold, which display remarkable antibacterial activity toward *C. difficile*. The antibacterial efficacy was further evaluated in mouse model of *C. difficile* infection.