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Brief Article

Structure-Activity Relationship Studies of a Series of Semi-synthetic Lipopeptides Leading to the Discovery of Surotomycin, a Novel Cyclic Lipopeptide Being Developed for the Treatment of *Clostridium difficile*-Associated Diarrhea

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Structure-Activity Relationship Studies of a Series of Semi-synthetic Lipopeptides Leading to the Discovery of Surotomycin, a Novel Cyclic Lipopeptide Being Developed for the Treatment of *Clostridium difficile*-Associated Diarrhea

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Supporting Information for publication

ABSTRACT: Novel cyclic lipopeptides with different acyl tails were synthesized via a semi-synthetic approach. Structure-activity relationship studies revealed that lipophilicity, chain length, and the location of key aromatic functionalities of the tail modulated activity. The lead compound surotomycin exhibited significantly improved in vitro activity compared with daptomycin (MIC_{90} 0.5 μg/mL vs 2 μg/mL) against *C. difficile* including NAP1 epidemic strains. In hamster efficacy studies, surotomycin protected animals at a dose of 0.5 mg/kg, PO.

INTRODUCTION

Clostridium difficile-associated diarrhea (CDAD) is one of the leading causes of healthcare-associated infections in the US.[1,2] CDAD is caused by the overgrowth of the anaerobic bacillus *C. difficile* in the lower gastrointestinal tract.[3] This overgrowth is often triggered by the use of broad spectrum antibiotics, which alters the protective microbiota in the intestinal tract allowing opportunistic pathogens such as *C. difficile* to flourish.[3,4] *C. difficile* toxin production can cause epithelial damage and mucosal inflammation resulting in the clinical symptoms associated with CDAD which ranges in severity from mild diarrhea to severe and life-threatening pseudomembranous colitis.[5] CDAD represents an increasing clinical and economic burden and the incidence of hospitalizations and mortality remain high.[5,6] Reported increases in CDAD incidence may be due to more frequent and sensitive testing and to the emergence of particular epidemic strains, for example the BI/NAP1/027 strain.[7,8] While CDAD is traditionally considered a healthcare-associated infection, it is increasing in incidence in the community setting, and community-associated CDAD may account for approximately 20% - 27% of cases.[1]

Optimal patient treatment strategies involve eradication of the pathogen without additional release of toxin, while allowing for minimal disruption of the intestinal microbiota, and minimization of spore production.[7] Depending on the severity of CDAD, current treatment recommendations include metronidazole, vancomycin and fidaxomicin.[5] While initial treatment response is observed in approximately 90% of patients, recurrence of CDAD represents a major problem with rates of approximately 30% - 50% following treatment with vancomycin and metronidazole.[8-11] Fidaxomicin has been shown to lower recurrence compared with vancomycin in clinical trials (15.4% vs 25.3%, respectively).[11]

Even with the current arsenal of antibiotics, the need for novel and effective therapies that can treat virulent strains with low recurrence rates remains. In this study we report the preparation of a series of daptomycin (DAP) (Figure 1) tail analogs via a semi-synthetic approach, and structure activity relationship (SAR) studies with the aim of identifying a compound with potent activity against *C. difficile* strains.[12,13] In addition to improving the potency against *C. difficile*, we also wanted to try to minimize the potential for resistance selection in a manner similar to that observed with DAP, therefore, we also tested the activity of analogs against DAP nonsusceptible isolates, including *Staphylococcus aureus*. This approach led to the discovery of surotomycin, which is currently in phase 3 development for the treatment of CDAD.

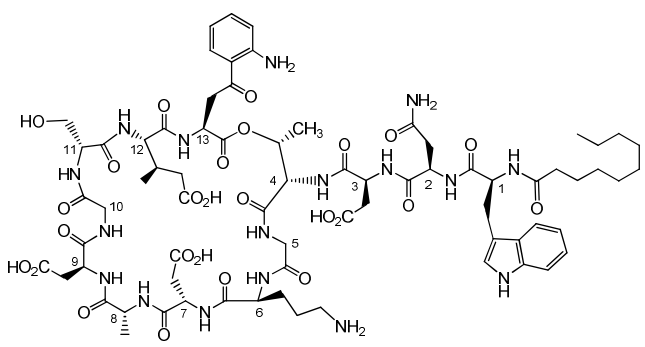
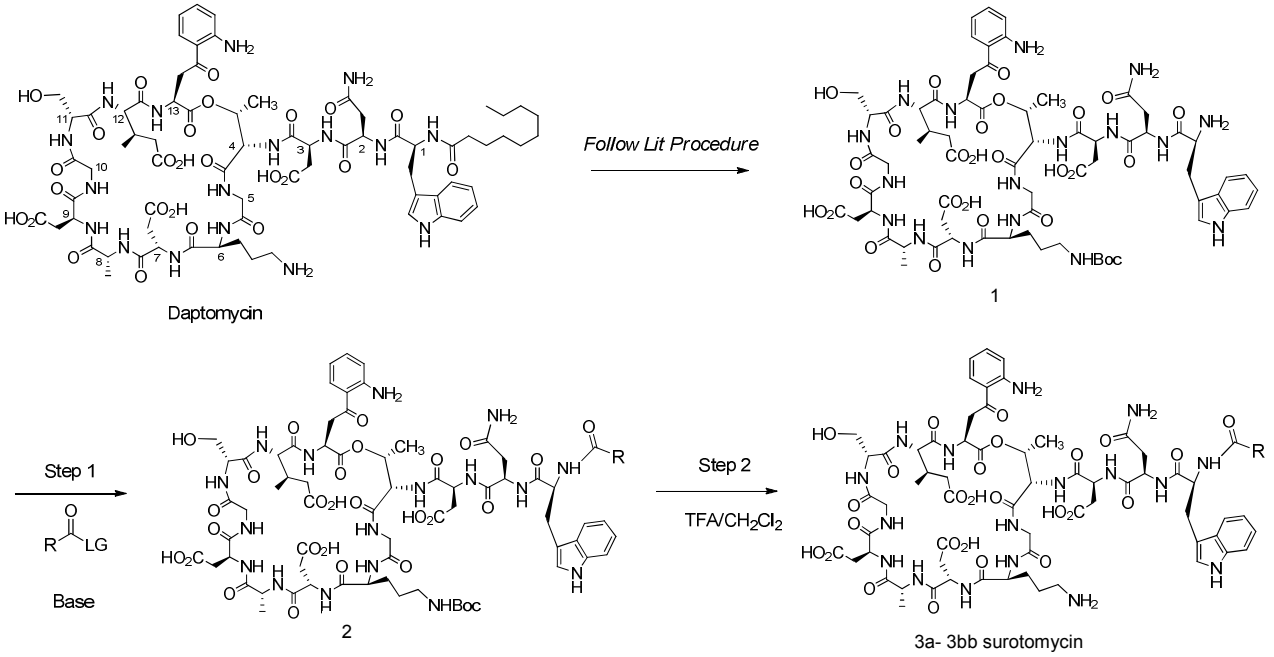


Figure 1. Structure of Daptomycin.[14]

RESULTS AND DISCUSSION

General synthetic route for the preparation of cyclic lipopeptide tail analogs is illustrated in Scheme 1. The C10 tail of DAP was replaced with a variety of commercially available or readily-accessible aliphatic/aromatic moieties through a semi-synthetic approach. The key Boc-Deacyl-Dap intermediate (**1**) was prepared through chemical and enzymatic transformations following literature procedures.[15] Amide-coupling with acyl chloride/PFP ester was employed to introduce different hydrophobic tails. Finally, the Boc group was removed by TFA, and the resulting compounds were purified by reverse phase HPLC to afford the final products (**3a – 3bb** and surotomycin). The in vitro antibacterial activity of the synthesized lipopeptides was assessed by determining the minimal inhibitory concentrations (MICs) against a panel of bacteria according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[16]

Scheme 1. Representative Synthetic Schemes of Lipopeptides.



The screening strategy was designed to identify compounds with potent activity against *C. difficile*, improved activity against isolates with decreased susceptibility to DAP and excellent in vivo efficacy. To support this goal, the strains tested included 30 *C. difficile* clinical isolates (11 NAP1 strains), DAP susceptible *S. aureus* (ATCC#29213), lab derived DAP resistant mutants of *Enterococcus faecium* ATCC #6569 (DAP^R *E. faecium*) and *Enterococcus faecalis* ATCC #49452 (DAP^R *E. faecalis*), and 28 DAP nonsusceptible *S. aureus* strains. Antimicrobial activities of the tested compounds against *C. difficile* are represented as a MIC_{90} where 90% of tested strains had this MIC (μg/mL) or lower (**Table 1 and 2**).

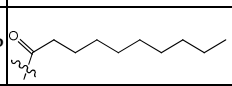
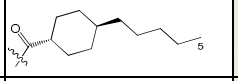
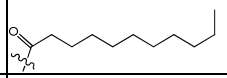
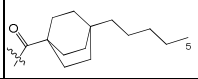
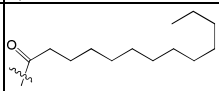
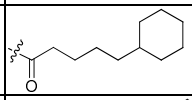
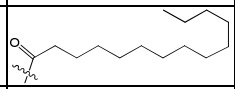
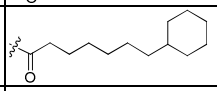
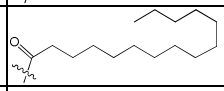
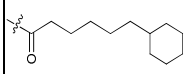
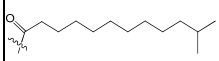
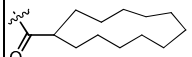
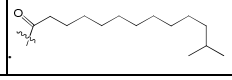
Initially, a series of aliphatic tail containing lipopeptides were prepared (**Table 1**). Among all the straight chain analogs (**DAP,** and **3a–3d**), the 10 carbon tail (**DAP**) gave the optimal *C. difficile* MIC_{90}. As the chain length is extended, there is a trend of reduced potency against *C. difficile*, and an improved activity against DAP resistant *E faecium* and *E. faecalis*. It was also found that analogs with a terminal branching methyl chain (**3e** and **3f**) demonstrated improved *C. difficile* MIC_{90} potency relative to their straight-chain homologs (**3b** and **3c**). Cyclohexyl ring containing analog **3g** displayed a 4-fold improved MIC_{90} against *C. difficile* vs **DAP**. In comparison to **3g**, analogs with cyclohexyl at the terminal position (**3i-3k**) were less potent. Compound **3h** with a bicyclo-octane ring is 2-fold more potent than **DAP** against *C. difficile*, while cyclododecane analog (**3l**) showed weak anti-*C. difficile* activities.

To further explore SAR of the tail structures, analogs were prepared with aromatic ring containing acyl tails. As indicated in **Table 2**, lipophilicity, chain length, and the location of key aromatic functionalities of the tail all impacted activity. Among all the analogs synthesized, surotomycin displayed the best overall in vitro biological profile. It exhibited a 4-fold improvement of *C. difficile* MIC_{90} over DAP, and it was also >8 fold more potent against DAP resistant *E. faecium* and *E. faecalis*.

In comparison to surotomycin, shortening the tail by 1 carbon (**3t**) reduced potency against *C. difficile* and the other tested strains. For the phenyl alkyl series, the C7 alkyl substitution (**3p**) provided an optimum *C. difficile* MIC_{90} (0.5 μg/mL). When the phenyl ring of **3p** was replaced by a thiophene, the resulting analog **3r** displayed reduced *C. difficile* activities (MIC_{90} 2 vs 0.5 μg/mL). Analogs with a terminal phenyl ring (**3m** and **3n**) had significantly weaker antibacterial activities (MIC_{90} 8-16 μg/mL). For the biphenyl ether alkyl series, the C7 ether analog (**D27**) was shown to be the most potent (MIC_{90} = 1 μg/mL); while the biaryl tail analog without alkyl substitution (**D28**) had reduced activities against *C. difficile* (MIC_{90} = 16 μg/mL) and the other tested strains. This study also aimed to identify compounds with superior activity against DAP nonsusceptible isolates. In comparison with DAP, surotomycin showed a 2- to 16-fold improvement in activity against 28 DAP nonsusceptible *S. aureus* strains (**Table 3**).

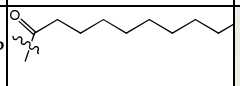
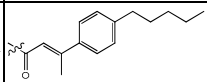
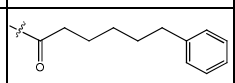
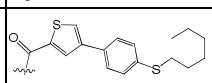
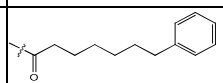
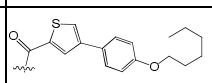
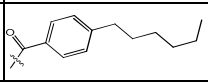
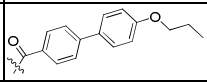
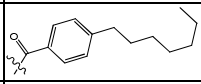
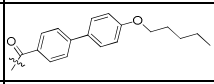
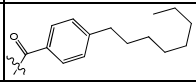
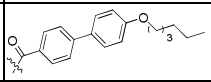
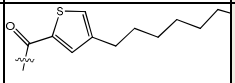
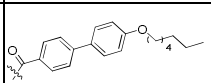
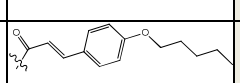
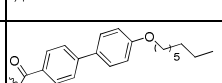
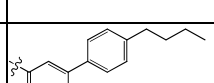
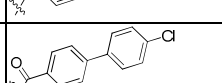
Compounds that passed the screening criteria for in vitro potency were further evaluated in a hamster *C. difficile* infection model using a very low dose, 0.5 mg/kg once a day, to identify the most potent in vivo compounds.[17] The survival rates from day 1 to day 25 after oral dosing once daily for 5 consecutive days of prepared cyclic lipopeptides or vancomycin are presented in **Figure 2**. Surotomycin demonstrated superior in vivo efficacy compared with other analogs when tested in the CDAD hamster model. It fully protected infected animals at 0.5 mg/kg. This result is comparable to or better than vancomycin. Other lipopeptide analogs (**3g, 3h**, **3p**, **3q** and **3r**) demonstrated partial protection at 0.5 mg/kg.

Table 1. In Vitro Antibacterial Activity of Aliphatic Tail-containing Lipopeptides (MICs, μg/mL). MIC90 Values are Provided for 30 *C. difficile* Isolates Tested and MIC Values are Provided for *S. aureus*, *E. faecium* and *E. faecalis* Isolates Tested

Cpd	Structure	*C. difficile*[a] MIC_{90}	*S. aureus* ATCC #29213	DAP^R *E. faecium*[b]	DAP^R *E. faecalis*[c]	Cpd	Structure	*C. difficile*[a] MIC_{90}	*S. aureus* ATCC #29213	DAP^R *E. faecium*[b]	DAP^R *E. faecalis*[c]
DAP		2	0.5	>32	>32	3g		0.5	0.5	16	>32
3a		4	2	>32	32	3h		1	0.25	16	32
3b		8	0.5	8	8	3i		4	1	32	>32
3c		16	1	4	4	3j		1	0.5	8	16
3d		16	1	4	4	3k		2	1	16	>32
3e		1	0.25	4	>32	3l		4	1	32	>32
3f		1	0.125	4	16						

[a]30 *C. difficile* clinical isolates were tested for MIC_{90}.
[b]Lab derived DAP resistant mutant of *E. faecium* ATCC# 6569.
[c]Lab derived DAP resistant mutant of *E. faecalis* ATCC# 49452.

Table 2. In Vitro Antibacterial Activity of Aromatic Tail-containing Lipopeptides (MICs, μg/mL). MIC90 Values are Provided for 30 *C. difficile* Isolates Tested and MIC Values are Provided for *S. aureus* (Sa42), *E. faecium* (Efm384) and *E. faecalis* (Efs312) Isolates Tested

Cpd	Structure	*C. difficile*[a] MIC_{90}	*S. aureus* ATCC #29213	DAP^R *E. faecium*[b]	DAP^R *E. faecalis*[c]	Cpd	Structure	*C. difficile*[a] MIC_{90}	*S. aureus* ATCC #29213	DAP^R *E. faecium*[b]	DAP^R *E. faecalis*[c]
DAP		2	0.5	>32	>32	SUR		0.5	0.25	4	4
3m		16	16	>32	>32	3u		8	8	16	>32
3n		8	16	>32	>32	3v		4	0.5	2	8
3o		4	1	32	>32	3w		4	0.5	32	>32
3p		0.5	0.25	4	8	3x		2	0.25	2	4
3q		1	0.5	4	8	3y		2	0.5	1	2
3r		2	0.125	16	32	3z		2	0.25	1	4
3s		4	4	>32	>32	3aa		1	0.125	4	16
3t		2	1	32	>32	3bb		16	4	>32	>32

[a]30 *C. difficile* clinical isolates were tested for MIC_{90}.
[b]Lab derived DAP resistant mutant of *E. faecium* ATCC# 6569.
[c]Lab derived DAP resistant mutant of *E. faecalis* ATCC# 49452.
SUR, surotomycin.

Table 3. In Vitro Activity of DAP and Surotomycin Against DAP Nonsusceptible *S. aureus* strains (MICs, μg/mL).

Strain ID#	Sa42	Sa399	Sa278	Sa1145	Sa1146	Sa1179	Sa1326	Sa1616	Sa5003	Sa5004	Sa5005	Sa5006	Sa5008	Sa5010
DAP	0.5	ND[a]	16	1	1	2	4	16	2	1	4	2	2	0.5
Surotomycin	0.125	0.125	1	0.25	0.25	0.25	0.5	2	0.25	0.25	1	0.5	0.5	0.25
Strain ID#	Sa5012	Sa5014	Sa5016	Sa5017	Sa5030	Sa5033	Sa5034	Sa5059	Sa5060	Sa5063	Sa5076	Sa5080	Sa5081	Sa5091
DAP	2	0.5	4	4	8	1	2	4	4	8	2	4	4	2
Surotomycin	0.25	0.25	0.5	0.5	1	0.25	0.5	1	1	1	0.5	1	1	0.5

[a]Multiple end points.

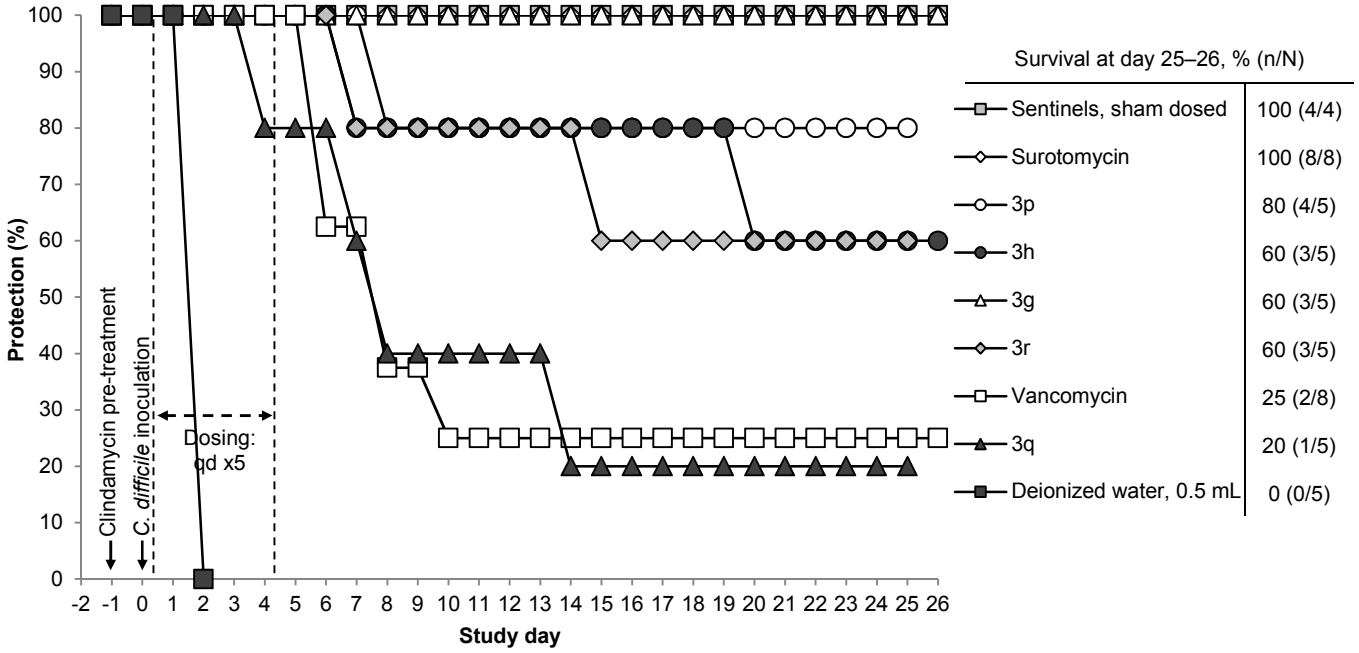


Figure 2. In Vivo Efficacy Study of Lipopeptide Analogs in Hamster CDAD Model (lipopeptide analog and vancomycin administrations were 0.5 mg/kg, PO qd x5 days, deionized water administration was 0.5 mL, PO qd x5)

CONCLUSIONS

A series of DAP tail analogs were prepared, and the relationship between the tail structures and their anti-*C. difficile* activities is discussed. Surotomycin was identified as an antibacterial development candidate. Surotomycin is a new member of the cyclic lipopeptide family of antibiotics and has been shown to cause cell membrane depolarization of the *S. aureus* cell membrane without increasing membrane permeability, resulting in loss of cell viability and negligible cell lysis.[18-20] It exhibited significantly improved in vitro activities compared with DAP against *C. difficile* including NAP1 strains. In addition, surotomycin showed an 8-fold increase in potency against lab derived DAP resistant *E. faecium* and *E. faecalis* and enhanced activity against DAP nonsusceptible *S. aureus* isolates. Surotomycin has also been shown to have good activity, MIC_{90} = 0.125 and 0.5, against *C. difficile* strains with reduced susceptibility to vancomycin (MIC of ≥4 μg/mL) and metronidazole (MIC of ≥8 μg/mL).[21] Further in vitro data also indicate that surotomycin is less disruptive to the gastrointestinal microbiota than vancomycin with MIC_{90} ≥8,192μg/mL for the *Bacteroides fragilis* group and *Prevotella* spp.[22] In the hamster CDAD model efficacy studies, surotomycin protected animals at a dose of 0.5 mg/kg. Surotomycin is a new agent with potent and selective antimicrobial activity and is currently in phase 3 clinical development for the treatment of CDAD.

EXPERIMENTAL SECTION

Synthesis of surotomycin. Deacylated BOC-protected DAP (3.50 g, 2.23 mmol) and sodium bicarbonate (1.13 g, 61.0 mmol) were dissolved in THF (130 mL) and water (50 mL). The resulting solution was cooled to 0°C and a solution of (E)-3-(4-pentylphenyl) but-2-enoyl chloride (1.96 g, 7.82 mmol) in THF (20 mL) was then introduced. The reaction mixture was warmed to room temperature and stirred for 4 h. The mixture was concentrated in vacuo to remove THF. The remaining aqueous solution was loaded onto a C18 flash chromatography column (35 mm× 300 mm, Bondesil HF C18 resin purchased from Varian). The column was first washed with water to remove salt and then with methanol to wash out product. Crude product of compound **2** (3.46 g) was afforded as a white solid after removal of methanol. MS m/z 1780.8 $(M + H)^+$. TFA (10 mL) was added to a solution of crude compound **2** (3.46 g,) in DCM (50 mL) at room temperature. The reaction mixture was stirred vigorously for 45 min and added slowly to vigorously stirring diethyl ether (100 mL). The resulting yellow precipitate was collected by filtration. The crude product was purified by preparative HPLC to afford the TFA

salt of surotomycin (0.75 g). MP carbonate resin (purchased from Biotage) was added to the solution of surotomycin TFA salt (0.70 g, 0.39 mmol) in anhydrous methanol (30 mL). The reaction mixture was stirred at room temperature for 4 h. The resin was removed by filtration and rinsed with methanol. The methanol solution was concentrated under vacuum to give surotomycin as an off-white solid (408 mg). MS m/z 1680.7185 (M + H)+, calcd 1680.7176. Purity of the final product was ≥95% as assessed by HPLC and NMR.

MIC assays. The in vitro antibacterial activity of the synthesized lipopeptides was assessed by determining the MICs against a panel of bacteria according to CLSI guidelines.[16] The strains tested included 30 *C. difficile* clinical isolates (11 NAP1 strains), DAP susceptible *S. aureus* (ATCC#29213), and lab derived DAP resistant *E. faecium* and *E. faecalis*. Briefly, *C. difficile* MICs were determined by the agar dilution method where serial two-fold dilutions of the test compounds were prepared, and added to molten agar (Brucella agar supplemented with Vitamin K_1, hemin, 5% lysed sheep blood and adjusted to contain 50 mg/L Ca^{2+}), mixed, and poured into Petri plates. *C. difficile*, equal to the turbidity of the 0.5 McFarland, was prepared in Brucella broth and used to inoculate the agar plates. Plates were incubated anaerobically at 37°C for 48 h. The MIC was the concentration of drug that inhibited growth or markedly reduced growth as compared with the drug-free control plate. Broth microdilution MICs were determined for *S. aureus* ATCC #29213 and *E. faecalis* and *E. faecium* in Mueller-Hinton broth adjusted to contain 50 mg/L Ca^{2+} (MHBc) per CLSI methodologies except that cultures were incubated at 37°C with rotation (200 rpm).

Hamster efficacy studies. All experiments utilized *C. difficile* ATCC #43596, a clinical isolate from the feces of a patient with pseudomembranous colitis. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Merck and Co., Inc., before the studies were initiated. Male Syrian golden hamsters (*Mesocricetus auratus*, Charles River Laboratories, Wilmington, MA) were pretreated with 10 mg/kg clindamycin subcutaneously 24 h before bacterial challenge. An inoculum of 20 *C. difficile* spores in sterile saline was administered orally. Inoculated hamsters (n=5-8 hamsters/group) were treated beginning 4 h after spore inoculation with vancomycin or various lipopeptide analogs (0.5 mg/kg oral once daily for 5 days). Lipopeptide analogs and vancomycin dosing solutions were prepared and diluted to desired concentration in sterile, deionized water. CDAD was confirmed as the cause of death by observations at necropsy, including wet tail and/or macroscopic cecal alterations.

ASSOCIATED CONTENT

Supporting Information.
Detailed experimental procedures and analytical data for surotomycin and representative compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

CDAD, *Clostridium difficile*-associated diarrhea; DAP, daptomycin; MIC, minimum inhibitory concentration; *S. aureus, Staphylococcus aureus*; *E. faecalis, Enterococcus faecalis*; *E. faecium, Enterococcus faecium*.

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