Journal of Medicinal Chemistry

Article

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Discovery of LFF571: An Investigational Agent for *Clostridium difficile* Infection

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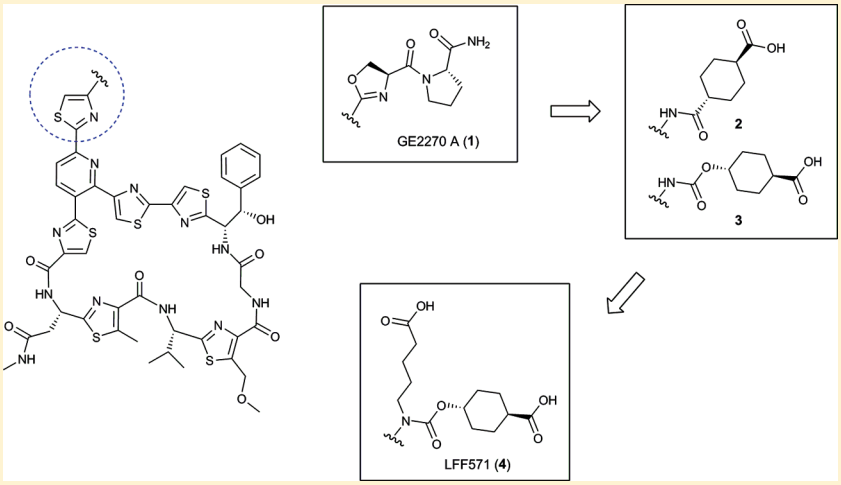
Supporting Information



ABSTRACT: *Clostridium difficile* (*C. difficile*) is a Gram positive, anaerobic bacterium that infects the lumen of the large intestine and produces toxins. This results in a range of syndromes from mild diarrhea to severe toxic megacolon and death. Alarmingly, the prevalence and severity of *C. difficile* infection are increasing; thus, associated morbidity and mortality rates are rising. 4-Aminothiazolyl analogues of the antibiotic natural product GE2270 A (**1**) were designed, synthesized, and optimized for the treatment of *C. difficile* infection. The medicinal chemistry effort focused on enhancing aqueous solubility relative to that of the natural product and previous development candidates (**2**, **3**) and improving antibacterial activity. Structure−activity relationships, cocrystallographic interactions, pharmacokinetics, and efficacy in animal models of infection were characterized. These studies identified a series of dicarboxylic acid derivatives, which enhanced solubility/efficacy profile by several orders of magnitude compared to previously studied compounds and led to the selection of LFF571 (**4**) as an investigational new drug for treating *C. difficile* infection.

INTRODUCTION

C. difficile is a spore forming, anaerobic, Gram positive bacterium. *C. difficile* infection results from spore germination in the gut of infected persons and subsequent toxin production, resulting in severe inflammation and diarrhea in infected patients. *C. difficile* infections often follow broad spectrum antibiotic use that disturbs normal and beneficial gut flora.[1] The prevalence and severity of *C. difficile* infections appear to be rising, in part due to changes in hospital environments, rising numbers of patients with risk factors for disease (e.g., elderly patients), an increasing use of broad spectrum antibiotics, and a higher proportion of bacterial isolates with increased toxin production.[2,3] Annually in the U.S., there are at least 50 000 admissions, >250 000 cases, and more than 15 000 deaths due to *C. difficile* infection.[4] The estimated annual U.S. cost for managing *C. difficile* infections is conservatively estimated at $3.2 billion.[5]

Therapies for *C. difficile* infection exist, but they are inadequate (e.g., efficacy, tolerability). Vancomycin (**5**, Figure 1), metronidazole (not shown), and fidaxomicin[6] (**6**) are used for

Received: December 14, 2011
Published: February 8, 2012

dx.doi.org/10.1021/jm201685h | *J. Med. Chem.* 2012, 55, 2376−2387

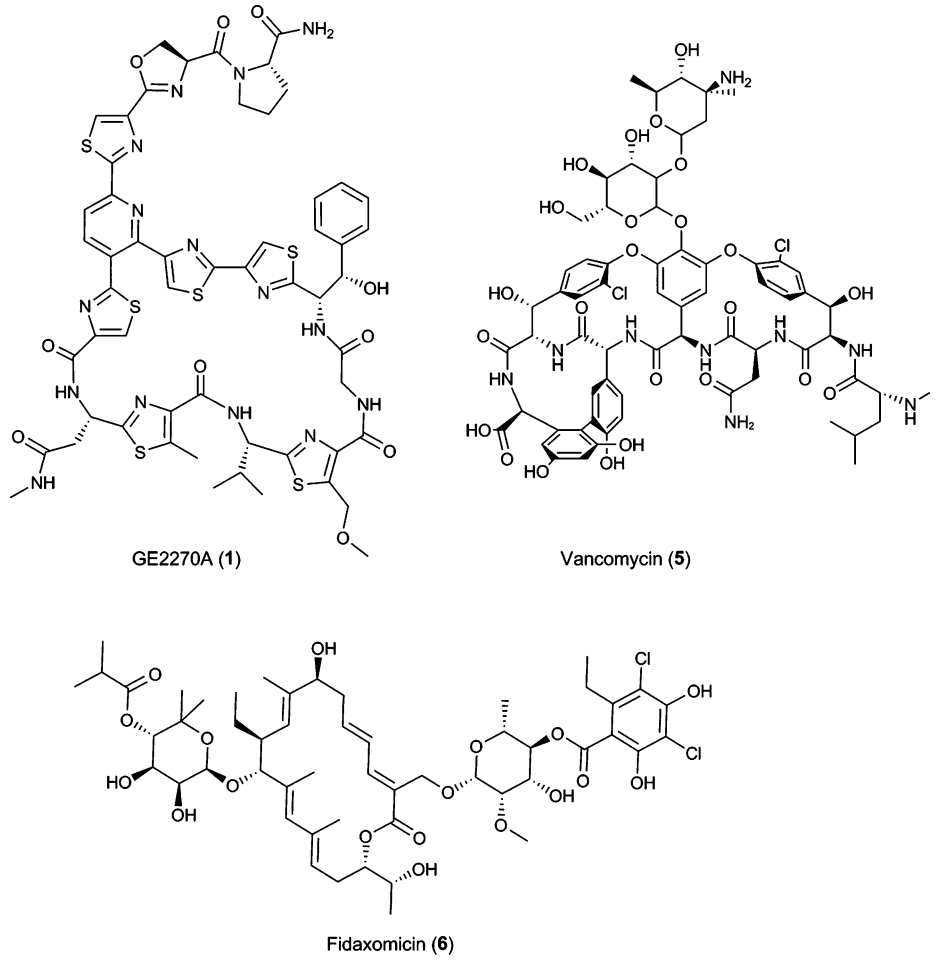


Figure 1. Structures of **1** and natural product-based marketed *C. difficile* antibiotics **5** and **6**.

treating *C. difficile* infection. While they have comparable initial response rates, **5** and metronidazole treatments encounter significant disease relapse.[7]

The discovery of new anti-infective chemical entities is a high priority in research and development.[8] There exists a pressing need for antibacterial chemical templates that act via novel mechanisms of action and are potent against clinically resistant organisms and recalcitrant infections. Historically, natural products have been excellent sources for antibacterial therapies and medicinal chemistry discovery starting points. Indeed many marketed antibiotics are natural products themselves or derivatives thereof (e.g., **5**, **6**).[9]

In 1991, Selva and co-workers from Lepetit Research Institute reported the structure and activity of the natural product antibiotic **1**. This thiopeptide-based secondary metabolite was isolated from a fermentation broth of *Planobispora rosea* and found to inhibit the prokaryotic chaperone elongation factor Tu (EF-Tu).[10] Like **5** and **6**, **1** is a macrocyclic natural product of high molecular weight (1291 Da). However, key structural differences relative to **5** and **6** include the presence of aromatic thiazole rings, the dearth of solubilizing functional groups (e.g., amines, carboxylic acids, sugars), and the mechanism of action (EF-Tu). The in vitro antibiotic activity of **1** against *C. difficile*, as well as a range of Gram positive pathogens including methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE), and group A streptococci, is excellent, with minimum inhibitory concentrations (MIC) in the sub μg/mL range.

Although the in vitro antimicrobial activities of **1** are excellent, the aqueous solubility was unmeasurable in our assays (<0.005 nM).[11] Also, EF-Tu is located in the cytoplasm, which presented a particularly formidable drug discovery hurdle, as soluble derivatives would also need to cross the cell membrane in order to exert their antibacterial effect, unlike **5** which targets the cell wall itself. Thus, an increase in aqueous solubility with the retention of cellular antimicrobial activity represented an arduous challenge for the optimization effort.

Previously, we described lead finding and optimization activities associated with 4-aminothiazolyl analogues of **1**.[12] On the basis of in vitro and in vivo potency, two distinct carboxylic acid based antibacterials (**2** and **3**) were further investigated as preclinical development candidates for the treatment of infections caused by *C. difficile* and other Gram positive organisms.[13] A compelling feature of these molecules included the combination of potent antibacterial activity coupled with improved aqueous solubility through the addition of a single carboxylic acid functional group. Cocrystallography with EF-Tu guided our optimization efforts, which focused on amide and urethane-based aminothiazolyl linkers to orient carboxylic acid containing side chains toward Arg223 of domain II of EF-Tu (Figure 2). Since monocarboxylate-containing compounds (**2**, **3**) retained antibacterial activity and significantly increased aqueous solubility, we investigated additional solubilizing functional groups and general structure−activity relationship expansion of this novel class of antibacterials. Given the proximity of Arg223 and Arg262 within domain II of EF-Tu to the

dx.doi.org/10.1021/jm201685h | *J. Med. Chem.* 2012, 55, 2376−2387

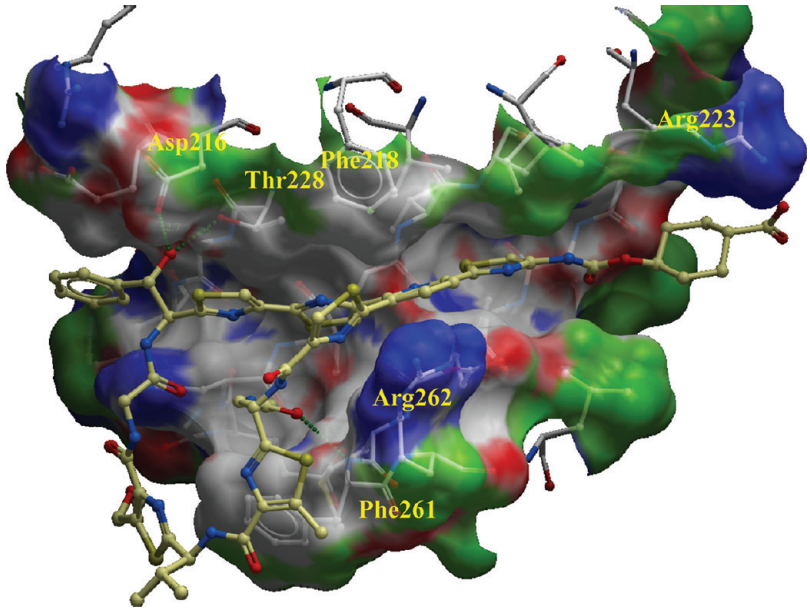


Figure 2. Cocrystal of *E. coli* EF-Tu and carboxylic acid **3**.

monocarboxylate-based ligands, we envisioned additional interactions using additional carboxylates. Thus, we designed, synthesized, and evaluated di- and tricarboxylic acid bearing analogues of **1**. Herein, we report the structure-guided design, synthesis, structure−activity relationships, and biological evaluation of di- and tricarboxylic acid analogues of **1**. These efforts led to the initiation of clinical investigations of the safety and efficacy of LFF571 (**4**) for the treatment of *C. difficile* infections in humans.

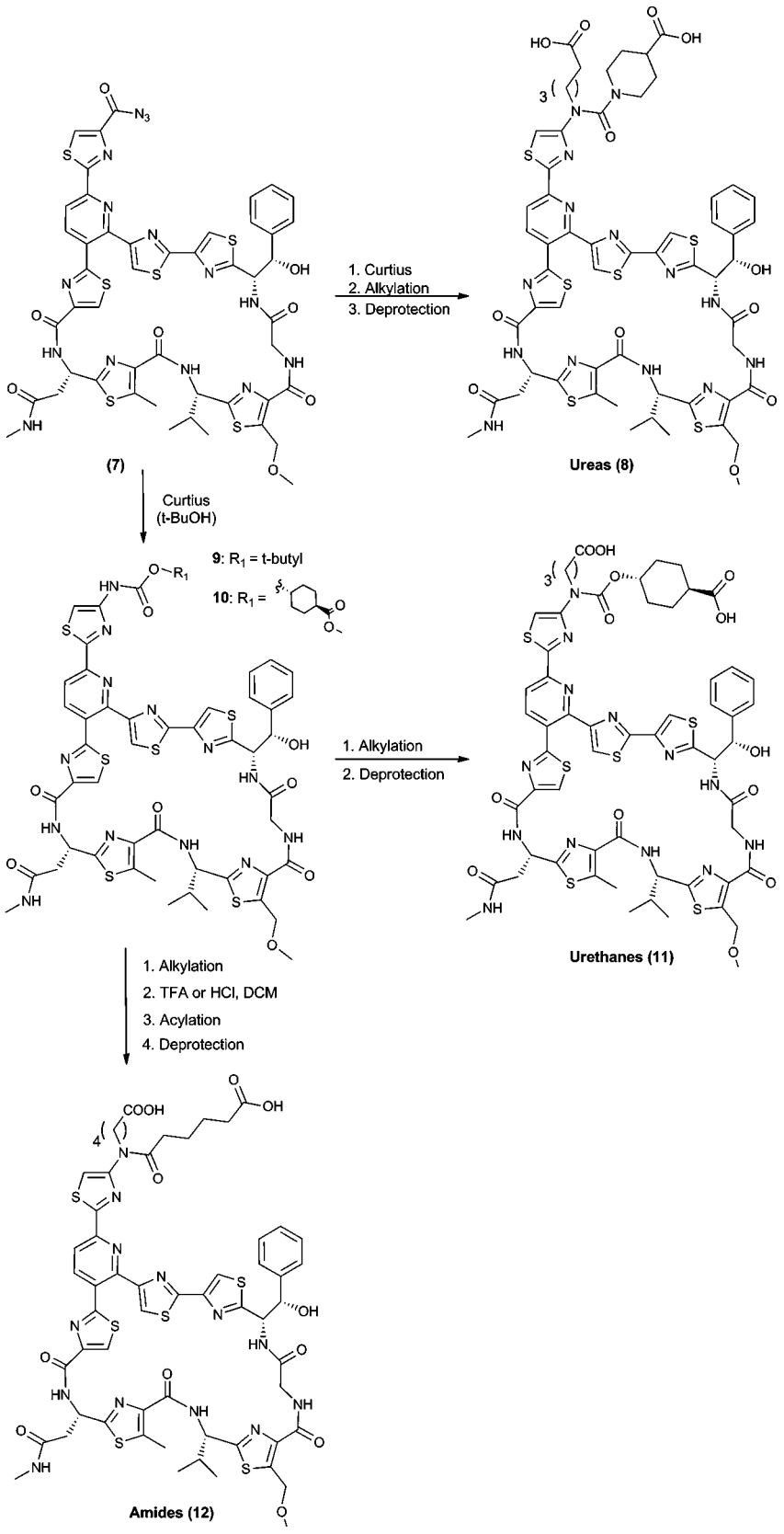
RESULTS

1 was first subjected to a previously reported acid-catalyzed rearrangement, basic hydrolysis,[14] and acyl derivatization which afforded acylazide **7** in high overall yield (not shown).[3] In order to evaluate amide, urethane, and urea-linked diacid containing analogues, three synthetic routes were employed (Scheme 1). For urea derivatives (e.g., **8**), the azide was heated in the presence of secondary amines and the resulting tertiary urea esters were selectively alkylated on the internal nitrogen. The ester protecting groups were then hydrolyzed, which afforded the ureas (**8**). For urethane derivatives (e.g., **11**), various alcohols were utilized in the Curtius reaction (e.g., **9**, **10**), followed by selective alkylation of the urethane nitrogen and ester hydrolysis (e.g., **11**). We speculate that the pseudobenzylic nature of the urethane and urea nitrogens allowed for selective manipulation over other functional groups (amides) within the structurally complex macrocycle. For amide-based diacid derivatives (e.g., **12**), direct alkylation of the amide nitrogen failed, so the acylazide was heated in *tert*-butyl alcohol (80 °C), which furnished the boc-protected aminothiazole (**9**). Selective alkylation proceeded at the urethane nitrogen, followed by boc removal, acylation of the secondary amine, and final ester hydrolysis (e.g., **12**). Alternatively, multiple carboxylic acid functional groups could be installed in a single side chain during the Curtius reaction (vide infra).

In order to further improve the aqueous solubility while retaining antibacterial activity, di- and tricarboxylic acid analogues derived from amides, ureas, and urethanes were evaluated for minimum inhibitor concentrations (MIC, Tables 1−3). Five organisms comprised our antibacterial panel: *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *C. difficile*.[15] *Streptococcus pneumonia* was also evaluated (not shown) but did not differ significantly from *S. pyogenes*. Select compounds were also evaluated in surface plasmon resonance experiments to determine the K_d and also in an in vitro transcription/translation assay measuring protein synthesis inhibition in cell extracts.[16] These assays, in conjunction with the MICs, assisted in the evaluation of cellular uptake of compounds that lost antibacterial activity. Methyl derivatization of the amide linker retained moderate antibacterial activity in three organisms (MIC of **13**: 2 to >64 μg/mL) and also first demonstrated a method for selective alkylation of the boc protected urethane nitrogen, which revealed a new area for medicinal chemistry exploration. Moderate antibacterial activity was also realized with amides terminating in succinic acid (MIC of **14**, **15**: 4 to >32 μg/mL), with a slight stereochemical preference. Thus, to our surprise, retention of moderate antibacterial activity was permissible while incorporating multiple ionizable functional groups. Unfortunately, all efforts to improve antibacterial activity by separating the carboxylic acid functional groups failed (**16**−**26**). These results included varying the tether lengths of the second amide side chain (**16**, **17**) and adding carbocycles, which had previously improved antibacterial activity (**18**−**20**).[4] Urea-based diacids also initially failed to retain even moderate antibacterial activity, especially when the internal nitrogen was substituted (**21**−**23**). However, when carboxylates were positioned on a piperidine-derived urea, moderate activity was retained (MIC of **24**: 0.25−4 μg/mL). Heterocyclic derivatives also failed to retain moderate antibacterial activity (**25**, **26**). Of the five target organisms, *C. difficile* was the most sensitive to the compounds. In all cases, the compounds inhibited protein synthesis in bacterial cell extracts (30−90% at 2 μM, not shown) which compares equally to **1** (IC_{50} = 2.0 μM). This indicated that the observed SAR results from variable cell penetration and not reduced pathway inhibition. Furthermore, the dicarboxylate analogues that retained moderate activity (**14**, **15**) were profiled in aqueous solubility assays and displayed a dramatic increase in solubility

dx.doi.org/10.1021/jm201685h | *J. Med. Chem.* 2012, 55, 2376−2387

Scheme 1. Synthesis of 4-Aminothiazolyl Diacid Analogues

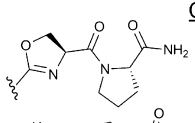
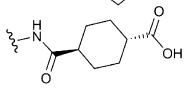
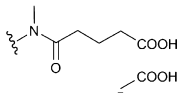
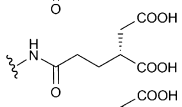
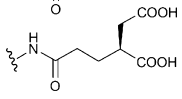
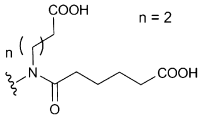
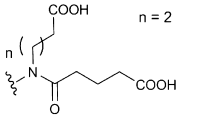
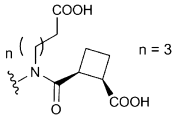
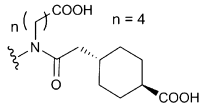
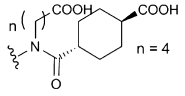
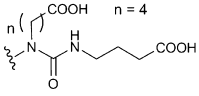
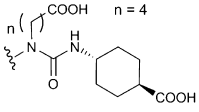
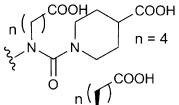
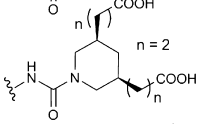
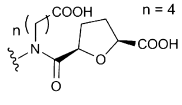
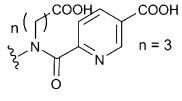


in neutral buffer (>15 mg/mL). Thus, at the outset of the diacid investigation, compounds containing a second carboxylic acid displayed improved aqueous solubility by over an order of magnitude compared to monoacid containing congeners (**2**: ~0.3 mg/mL); however, the antibacterial activity of the diacids required further improvement.

In the urethane subseries (Table 2), derivatizing the urethane nitrogen with carboxylic acid containing functional groups

dx.doi.org/10.1021/jm201685h | J. Med. Chem. 2012, 55, 2376–2387

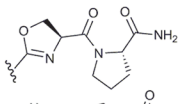
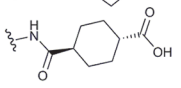
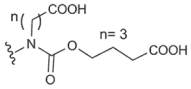
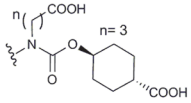
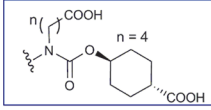
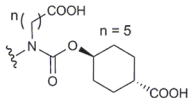
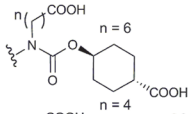
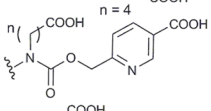
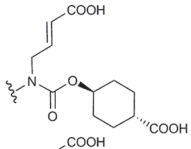
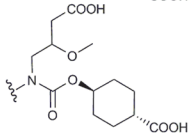
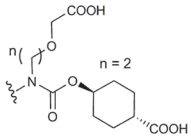
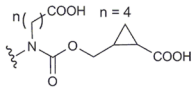
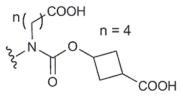
Table 1. SAR of Amide and Urea Diacids

		MIC assay: μg/mL					
	Compound	*E. faecalis*	*E. faecium*	*S. aureus*	*S. pyogenes*	*C. difficile*	aq solubility pH 7.4 (mg/mL)
	**1**	0.5	0.25	0.25	1	0.015	<0.001
	**2**	0.06	0.06	0.125	4	0.06	0.3
	**13**	2	4	2	>64	n/a	
	**14**	4	8	8	>32	4	15.1
	**15**	16	32	32	>32	4	17.6
	**16**	32	32	16	>32	2	
	**17**	>32	>32	>32	>32	>4	
	**18**	16	32	32	>32	4	
	**19**	8	>32	4	32	0.25	
	**20**	32	>32	16	>32	2	
	**21**	>32	>32	32	>32	4	
	**22**	16	>32	16	32	1	
	**23**	>32	>32	>32	>32	>4	
	**24**	2	4	4	4	0.25	
	**25**	>32	>32	>32	>32	>4	
	**26**	16	32	32	>32	4	

retained very potent antibacterial activity (MIC of **27**−**30**, **4**: 0.03−2 μg/mL). In addition, carbocyclic derivatives showed improved potency by an order of magnitude in three out of five target organisms (MIC of **28**−**30**, **4**: 0.03−2 μg/mL), which is consistent with previous optimization studies of the monoacid subseries.[4] This finding contrasts with the above amide-based diacid analogues, which failed to retain potent antibacterial activity (Table 1). Unfortunately, pyridine containing derivatives

dx.doi.org/10.1021/jm201685h | *J. Med. Chem.* 2012, 55, 2376−2387

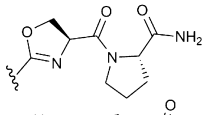
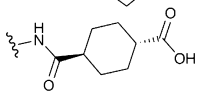
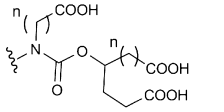
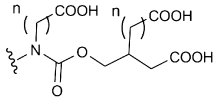
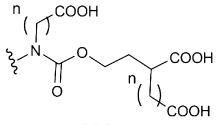
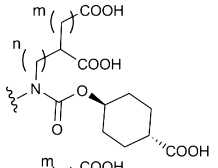
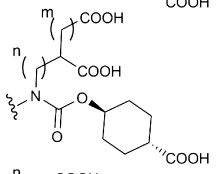
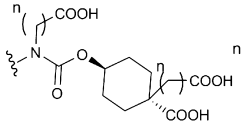
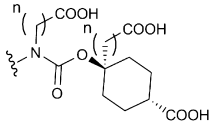
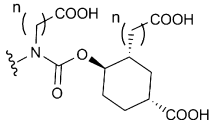
Table 2. SAR of Urethane Diacids

		MIC assay: μg/mL						
	Compound	*E. faecalis*	*E. faecium*	*S. aureus*	*S. pyogenes*	*C. difficile*	ED_{50} (mpk) *Staph., Ent.*	aq solubility pH 7.4 (mg/mL)
	**1**	0.5	0.25	0.25	1	0.015		<0.001
	**2**	0.06	0.06	0.125	4	0.06	5.2, 0.6	0.3
	**27**	0.5	0.5	1	2	0.03	11.0	12.5
	**28**	<0.06	0.06	0.25	1	0.06	2.6, 2.2	
	**4**	<0.06	<0.06	0.125	1	0.03	6.9, 2.2	12
	**29**	<0.06	<0.06	0.125	1	0.06	9.6, 0.8	
	**30**	<0.06	0.125	0.25	2	0.06	6.6, 1.0	
	**31**	1	1	2	4	0.125		
	**32**	0.25	0.5	0.5	2	0.03		
	**33**	1	1	1	4	0.06		
	**34**	2	2	4	4	0.125		
	**35**	0.125	0.5	0.25	2	0.03		
	**36**	0.25	0.25	0.5	2	0.03		

(**31**), unsaturated congeners (**32**), heteroatom incorporation (**33**, **34**), and other carbocyclics (**35**, **36**) failed to improve the antibacterial activity further, although all retained good to excellent potency (MIC of 0.03−4 μg/mL). As before, *C. difficile* was the most sensitive target organism. In all cases, the compounds inhibited protein synthesis in bacterial cell extracts (67−86% at 2 μM, not shown) which compares equally to **1** (IC_{50} = 2.0 μM). This indicated that the SAR results from variable cell penetration and not reduced pathway inhibition. The K_d values of select compounds were determined with EF-Tu·GDPNP. Compound **1** had a K_d of 3 nM, while compound **2** had a higher K_d (43 nM). Compounds **27**, **29**, and **4** were equivalent (8 nM, 6 nM, 6 nM, respectively), indicating that differences in MIC (e.g., in staphylococcus and enterococci) are most likely the result of variable cell penetration. The most potent urethane diacid analogues (**4**, **27**−**30**) were subsequently profiled in a mouse sepsis model of infection utilizing *S. aureus* and *E. faecalis*.[17] The effective doses (ED_{50}) for all five compounds evaluated in both infection models ranged from 0.8 to 11.0 mg/kg. Importantly, the in vivo results correlated with the in vitro MIC findings (*E. faecalis* ED_{50} less than *S. aureus* ED_{50}). In addition, two of the most potent diacid congeners (**4**, **27**) were

dx.doi.org/10.1021/jm201685h | *J. Med. Chem.* 2012, 55, 2376−2387

Table 3. SAR of Urethane Triacids

		MIC assay: μg/mL				
Compound	*E. faecalis*	*E. faecium*	*S. aureus*	*S. pyogenes*	*C. difficile*
	**1**	0.5	0.25	0.25	1	0.015
	**2**	0.06	0.06	0.125	4	0.06
 n = 4	**37**	16	32	32	>32	2
 n = 4	**38**	8	8	8	32	1
 n = 4	**39**	8	16	16	32	0.5
 n = 4 m = 2	**40**	8	8	16	8	0.25
 n = 3 m = 6	**41**	0.25	0.5	1	1	0.06
 n = 4	**42**	0.5	1	1	4	0.03
 n = 4	**43**	4	8	8	16	n/a
 n = 4	**44**	4	4	8	4	0.25

profiled in solubility assays and retained very high aqueous solubility at neutral pH (>10 mg/mL). On the basis of the potent in vitro and in vivo antibacterial results as well as the substantial aqueous solubility, urethane diacids **4** and **27** were subsequently selected for further characterization as development candidates.

Since the addition of a second carboxylic acid functional group retained antibacterial activity and improved aqueous solubility over that of the monoacid class and the natural product itself, we next evaluated triacid analogues of **1** in order to determine whether a third anionic group would retain antibacterial activity/cell penetration. Accordingly, the third carboxylic acid functional group was explored at various points within the most potent diacid structural templates (e.g., **4**, **27**). Similar to the mono- and diacid subseries, in the triacid subseries, the antibacterial activity was dependent on the location of the third carboxylate functional group. Indeed, moderate antibacterial activity was achieved utilizing acyclic scaffolds containing three carboxylates (Table 3, MIC of **37**−**39**: 0.5 to >32 μg/mL). Again, improved antibacterial activities were observed in the carbocyclic subseries (e.g., MIC of **40**−**44**: 0.25−8 μg/mL), albeit reduced from the most potent diacid and monoacid-containing analogues. In particular, maximizing the distance between the carboxylates improved antibacterial activity (e.g., **40**, **41**), and placement within the cyclohexyl ring also affected activity because 4,4-disubstituted cyclohexanes (**42**) proved to be superior to 1,1 disubstituted (**43**) and 1,2 disubstituted (**44**) analogues. In all cases, the compounds inhibited protein synthesis in bacterial cell extracts (50−79% at 2 μM, not shown), which compares equally to **1** (IC_{50} = 2.0 μM).

dx.doi.org/10.1021/jm201685h | *J. Med. Chem.* 2012, 55, 2376−2387

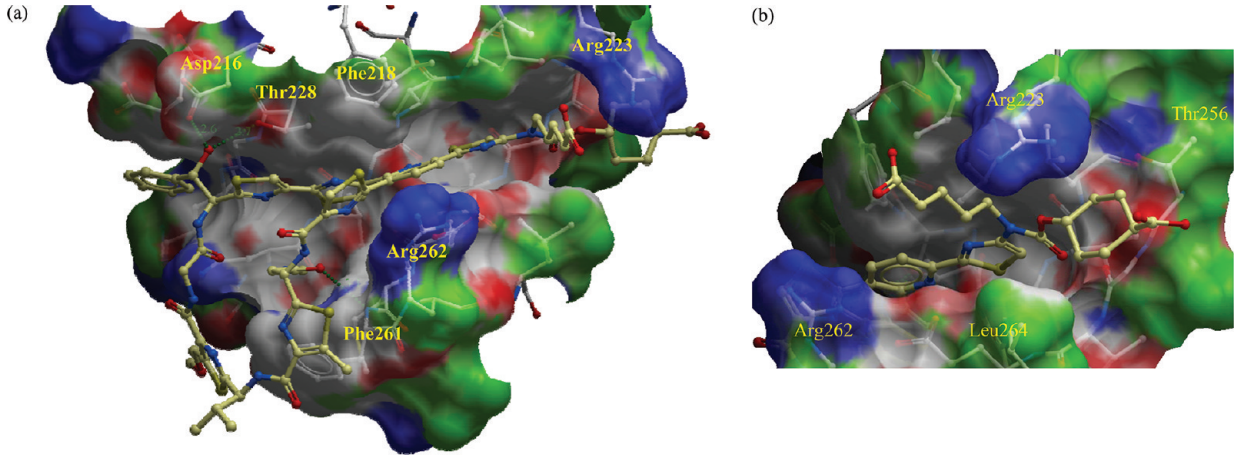


Figure 3. (a) Cocrystal of *E. coli* EF-Tu (domain 2 shown) and **4**. (b) Protein environment surrounding the diacid side chain of **4**.

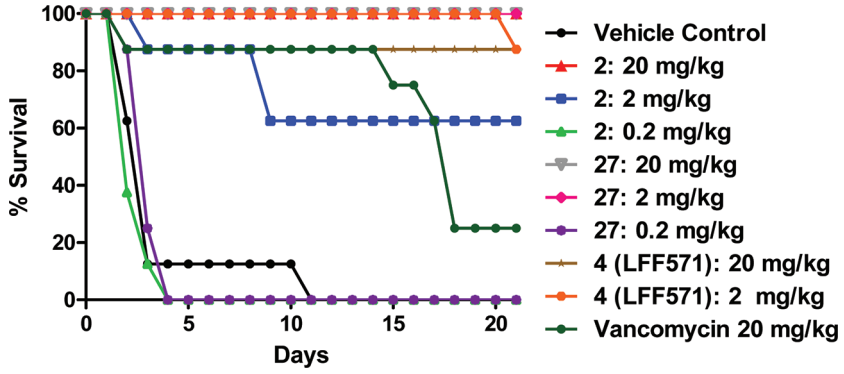


Figure 4. Characterization of **4**, **27**, **2**, and **5** in the golden Syrian hamster model of *C. difficile* infection (eight animals per dose group).

This indicated that the SAR results from variable cell penetration and not reduced pathway inhibition. Although some compounds displayed potent activities across the panel of organisms tested (e.g., **41**, **42**), no additional benefit was realized, especially given the increased structural and synthetic complexity compared to the diacid series.

Cocrystal studies of several diacid congeners and *E. coli* EF-Tu were conducted in order to better understand the observed structure–activity relationships.[18] The homology between *E. coli* EF-Tu and *C. difficile* EF-Tu is high (86% within 5 Å of the binding site). The macrocyclic conformation of **4** was quite similar compared to previous studies (e.g., **2**, **3**), with intermolecular H-bond interactions noted between the hydroxyphenylalanine moiety of with two residues, Thr228 and Asp216. Further interactions included an additional intermolecular hydrogen bond between the amide side chain of **4** and Phe261. Phe218 also formed a T-stack interaction with the pyridine ring of **4**. The urethane aminothiazolyl linker favorably positions the two acid-containing side chains out to solvent accessible areas and in proximity to Arg 223 and Arg226 (Figure 3b).

4 was evaluated in rat pharmacokinetic studies and displayed consistent results with previous experiments.[10] Importantly, very low oral bioavailability was observed ($F < 0.1\%$) resulting from low absorption, which was ideal for the dosing requirements for treating *C. difficile* infection (i.e., oral nonabsorbed route), a pathogen that infects the lumen of the large intestine. Therefore, compounds **4** and **27** and a previous development candidate **2** were evaluated in a golden Syrian hamster model of *C. difficile* infection (Figure 4), which is used to study treatments for the initial infection and relapse stages of *C. difficile* infection.[19] In the experiment, clindamycin was administered on day −1, which cleared the gut flora of the animals, increasing their susceptibility to *C. difficile* infection. On day 0, approximately 1×10^7 CFU of *C. difficile* were administered via oral gavage. **5**, **27**, **2**, **4**, or vehicle control (saline) was administered orally once a day from days 1 to 5, and the animals were observed from days 6 to 21. For compound **2**, a dose dependent effect was observed with 0%, 62.5%, and 100% survival in the 0.2, 2, and 20 mg/kg groups, respectively. For compound **27**, there was 0% survival for the 0.2 mg/kg group and 100% for the 2 and 20 mg/kg groups. For compound **4**, a similar effect was observed for the 2 and 20 mg/kg groups. Importantly, all three thiopeptides proved to be superior at lower doses (2 mg/kg) to **5** (20 mg/kg, 25% survival). Also importantly, animals that were successfully treated with **27**, **2**, **4**, or **5** had no detectable levels of *C. difficile* toxins A and B in their cecal contents when measured at the end of the study (via ELISA, day 21). From these experiments and others, we consider a difference of one animal between dose groups and vehicle controls as statistically insignificant. All three thiopeptides (**2**, **27**, **4**) had very limited systemic exposure in the infected hamsters using sparse PK sampling (Table 4), compared to very high exposure in the gut as measured by cecum sampling. Thus, in both uninfected rats and infected hamsters, the thiopeptides had very limited systemic exposure while maintaining high exposure in the intestinal tract, which is an ideal profile (i.e., oral nonabsorbed) for treating *C. difficile*

dx.doi.org/10.1021/jm201685h | *J. Med. Chem.* 2012, 55, 2376–2387

Table 4. Summary of Pharmacokinetics in *C. difficile* Infected Hamsters after 5 Days of Oral Dosing with 20 mg/kg Test Compounds

	cecum (μM)		plasma (nM)	
compd	1 h	3 h	1 h	3 h
2	18	39	BLQ	BLQ
27	129[a]	72	63.9	BLQ
4	61	208[a]	44.8	5.2

[a] $^2/_3$ of animals were above the limit of quantitation.

infection. To our knowledge, this study represents the first evaluation for the thiopeptide class in the golden Syrian hamster model of *C. difficile* infection.

DISCUSSION AND CONCLUSIONS

In summary, diacid- and triacid-containing analogues of the antibiotic natural product **1** were designed, synthesized, and evaluated for Gram positive bacterial growth inhibition. Several cycloalkylurethane-based diacid and triacids retained antibacterial activity in vitro across a panel of Gram positive organisms. **4** was selected for further characterization as a development candidate on the basis of excellent in vitro and in vivo potency and very high aqueous solubility. Cocrystallographic studies of **4** demonstrated a similar macrocyclic conformation compared to previous analogues, and intermolecular protein interactions within domain 2 of EF-Tu rationalized the observed structure−activity relationships. **4** was further characterized in oral PK studies, which demonstrated very low absorption, an ideal characteristic for its intended use as an oral nonabsorbed treatment for *C. difficile* infection. In the golden Syrian hamster model of *C. difficile* infection, **4** and other thiopeptides proved to be superior to **5** at lower doses. Taken together, these studies culminated in the selection of **4** for clinical development. Currently, a clinical study evaluating **4** for treating of *C. difficile* infections is ongoing.

EXPERIMENTAL SECTION

Compound Synthesis and Characterization. Synthetic procedures and compound characterization data are found in the Supporting Information. Compound purity was assessed by two distinct 20 min HPLC runs to confirm >95% purity.

MIC Assays. MIC assays were conducted according to broth microdilution methods described by the Clinical and Laboratory Standards Institute (CLSI, M07-A8, 2009).[15a] Bacterial strains included *E. faecalis* (ATCC 29212), *E. faecium* (Prof. Chopra, University of Leeds, U.K., strain 7130724), *S. aureus* (MRSA from Prof. Willinger, isolated from a pharyngeal smear, AKH Vienna A4.018), *S. pyogenes* (ATCC BAA-595), *C. difficile* (ATCC 43255).[15b]

Mouse Systemic Infection Model. The studies conducted were approved by the Institutional Animal Care and Use Committee of the Novartis Institutes for BioMedical Research Inc., Cambridge, MA. Animals were maintained under controlled conditions with free access to food and water. Female CD1 mice (21−25 g, Charles River Laboratories, Wilmington, MA) were used for infections with *S. aureus* (ATCC 49951) and *E. faecalis* (NB04025, a clinical isolate from the Novartis bacterial collection that is resistant to erythromycin, tetracycline, and gentamicin, courtesy of Dr. B. Willinger, Vienna General Hospital, Austria). Lethal infections were induced by intraperitoneal injection of a freshly prepared bacterial suspension of 1×10^8 CFU/mouse in either 50% sterile rat fecal extract (*E. faecalis*) or 0.9% NaCl (*S. aureus*). The injected bacterial dose corresponded to 10−100 times the minimal lethal dose as determined from previous lethal dose titration studies. Therapy for *E. faecalis* infections was initiated immediately following the bacterial inoculation, while for *S. aureus* infections, therapy was initiated 1 and 5 h after inoculation. Compounds were administered via tail vein bolus injection at several dose levels to groups of six mice each. Following inoculation the mice were observed for 5 days. In addition, body temperature was monitored by electronic microtransponders (Bio Medic Data Systems, Inc. Seaford, DE) that were implanted in mice subcutaneously prior to infection. The 50% effective dose (ED_{50}), the dose providing protection to 50% of mice, and the 95% confidence limits (95% CI) were calculated from the survival data at day 5 by probit analysis using the program Systat (SPSS Inc.).

Solubility Measurements. An amount of 1−2 mg of compound was weighed into 1 mL glass tubes, and a fixed volume of each vehicle was added to yield approximately 20 mg/mL compound. After initial mixing using brief vortexing and sonication (5−10 min), samples were equilibrated by shaking for 24 h at room temperature. After equilibration, the vials were visually examined. If a clear solution was obtained, solubility was reported as >*X* mg/mL (where *X* is the starting concentration in that sample) and the pH of the sample was recorded. Suspensions or solutions with visible particles were filtered through 0.22 μm PVDF membrane filters. Their pH was recorded, and dissolved drug concentration was analyzed using a RP-HPLC assay.

In Vitro Transcription/Translation Assays. The *E. coli* S30 extract extract system for circular DNA (Promega catalog no. L1020) was used per recommendation of the manufacturer with slight modifications. Briefly, 3.5 μL of 286 ng/μLtemplate DNA (pBESTluc) was mixed with 1.0 μL each of 1 mM "methioninemius" and "cysteine minus" amino acid mixes, 8 μL of S30 premix, 6 μL of S30 extract, and 0.5 μL of the test agents at 40× final concentration in a total volume of 20 μL. The reaction mixtures were incubated for 2 h at 37 °C in a 384-well flat-bottom white plate (Corning, catalog no. 3704). The formation of luciferase was measured by adding equal volume (20 μL) of Steady-Glo luciferase reagent (Promega catalog no. 27104), and emitted light was detected with a luminometer (Molecular Devices, LMaxll plate reader). Kirromycin and puromycin, which are known to be protein synthesis inhibitors, were used as positive controls. Ampicillin was used as a negative control. The rabbit reticulocyteTnT Quick coupled transcription/translation system (Promega catalog no. L1170) was used as recommended by the manufacturer except that the assay volumes were scaled down from 50 to 20 μL. Briefly the assay components consisted of 16 μL of TnT Quick mastermix, 3 μL of pT7luc at 167 ng/μL, 0.5 μL of methionine, and 0.5 μL of the test compound at 40× final concentration in a final volume of 20 μL. The reaction mixtures were incubated at 37 °C for 75 min. Luminescence was detected as described above.

In Vitro Biotinylation of *E. coli* EF-Tu. EF-Tu proteins were first buffer exchanged to 1× PBS by dialysis and then treated with EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce). The amount of the cross-linking reagent, the reaction time, and the temperature were controlled to allow only one molecule of biotin to cross-link with one molecule of EF-Tu. Under these conditions, about 25−30% of protein was 1:1 biotinylated. The rest of EF-Tu was not biotinylated (Figure S1) and was separated from the biotinylated EF-Tu immobilized on streptavidin (SA) sensor chip by flowing buffer over the chip surface.

Determination of Equilibrium Binding Constant (K_D) and Kinetic Binding Parameters of Thiopeptides by SPR. Biotinylated EF-Tu was immobilized onto a streptavidin (SA) sensor chip preconditioned with 40 mM NaOH and 1 M NaCl. Immobilization was controlled to achieve maximum response unit (R_{max}) of 50−100. For standard direct binding studies of thiopeptide binding to the GTP-bound form of EF-Tu, a running buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 μM GDPNP, 1 mM $MgCl_2$, 1 mM TCEP, 0.05% P20 (GE Healthcare), and 3% DMSO was used. SPR experiments were performed on a Biacore T-100 instrument (GE Healthcare). Instrument parameters used for EF-Tu/TP interactions were as follows: flow rate 40 μL/min; contact (association) time = 240 s; dissociation time = 600−900 s depending on TP's dissociation rate. All binding studies were conducted at room temperature. SPR data were analyzed using BiaEvaluation software (GE Healthcare) to obtain K_D.

Rat Pharmacokinetic Studies. *N* = 2 or 3, iv PK studies were performed with male Sprague−Dawley rats weighing 220−270 g that

dx.doi.org/10.1021/jm201685h | J. Med. Chem. 2012, 55, 2376−2387

are approximately 6−10 weeks old, obtained from Harlan Research Laboratories (South Easton, MA), each bearing dual implanted jugular vein cannula. The rats were fasted overnight before use and for 8 h after dosing. Blood samples were taken into K_2-EDTA coated tubes and then centrifuged to yield plasma sample for analysis by LC−MSMS. Bioanalysis of rat plasma, from both in-life and protein binding experiments, were performed by LC−MSMS using a system with the following configuration: Agilent liquid chromatograph (Santa Clara, CA), LEAP Technologies CTC-PAL autosampler (Carrboro, NC), and Applied Biosystems API 4000 mass spectrometer (Framingham, MA). LC was performed in gradient mode with reversed phase C18 columns (2.1 mm × 30−50 mm × 3.5−5 μm particle size). The mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Gradients were run from 5% B to 95% B in ∼3.5 min. Plasma samples were protein precipitated with acetonitrile containing glyburide as the internal standard (Sigma-Aldrich, St. Louis, MO).

Oral Dosing Solution Formulations. *Compound 2.* The concentration used was 10 mg/mL. Ingredients for the amount for 1 mL (percentage) were as follows: 0.1 N NaOH [250 μL (25% v/v)], PEG-400 [200 μL (20% v/v)], 10% Cremophor EL in purified water [250 μL (25% v/v)], purified water [297 μL (29.7%v /v)], 1 N HCl [3 μL (0.3% v/v)]. The compound was weighed in a vial, and to it was added 0.1 N NaOH to completely wet the compound. The mixture was stirred for 60 min at 37 °C, and to it was added PEG-400. The mixture was stirred for 5 min to obtain a yellow solution. To it was added 10% Cremophor EL solution followed by purified water, and the mixture was stirred for 5 min (a clear solution results). The pH of the solution was adjusted to 7.5−8.0 using 1 N HCl. If the pH is above 8, it was adjusted with an additional amount of 1 N HCl. Final formulation is a light-yellow clear solution with pH of 7.5−8.0. Solution is physically stable for at least 24 h at room temperature. Final concentration of Cremophor EL in formulation is 2.5%. To prepare lower concentrations, 10 mg/mL solution was diluted with pH 7.4 phosphate buffered saline (0.15 M PBS). The diluted solutions are stable for 24 h at room temperature.

Compound 27. The concentration was 10 mg/mL. Ingredients (amount for 1 mL (percentage) were as follows: 0.1 N NaOH [150 μL (15% v/v)], PEG-400 [200 μL (20% v/v)], 10% Cremophor EL in purified water [250 μL (25% v/v)], purified water [400 μL (40% v/v)]. The compound was weighed in a vial, and to it was added 0.1 N NaOH. The mixture was sonicated/stirred for 20−30 min to completely dissolve the compound. To it was added PEG-400, and the mixture was stirred for 5 min. The solution remained clear. To it was added 10% Cremophor EL solution followed by purified water, and the mixture was stirred for 5 min. The solution remained clear. The final pH of the solution was measured and recorded. Final formulation is a light-yellow clear solution with pH of 7.0−8.0. Solution is physically stable for at least 24 h at room temperature. Final concentration of Cremophor EL in formulation is 2.5%. To prepare lower concentrations, 10 mg/mL solution was diluted with pH 7.4 phosphate buffered saline (0.15 M PBS). The diluted solutions are stable for 24 h at room temperature.

Compound 4. The concentration was 10 mg/mL. Ingredients (amount for 1 mL) were as follows: 0.1 N NaOH [100 μL (10% v/v)], PEG-400 [200 μL (20% v/v)], 10% Cremophor EL in purified water [250 μL (25% v/v)], purified water [450 μL (45% v/v)]. The compound was weighed in a vial. To it was added 0.1 N NaOH, and the mixture was sonicated/stirred for 20−30 min to completely dissolve the compound. To it was added PEG-400, and the mixture was stirred for 5 min. The solution remained clear. To it was added 10% Cremophor EL solution followed by purified water, and the mixture was stirred for 5 min. The solution remained clear. The final pH of the solution was measured and recorded. Final formulation is a light-yellow clear solution with pH of 7.0−8.0. Solution is physically stable for at least 24 h at room temperature. Final concentration of Cremophor EL in formulation is 2.5%. To prepare lower concentrations, the 10 mg/mL solution was diluted with pH 7.4 phosphate buffered saline (0.15 M PBS). The diluted solutions are stable for 24 h at room temperature.

Hamster Pharmacokinetics. The pharmacokinetics of compounds **2**, **27**, and **4** were determined following 20 mg/kg oral dosing in infected hamsters using sparse plasma and cecal sampling. Six hamsters were administered 0.6 mL of each compound at 3.3 mg/mL in appropriate vehicle once a day for 5 days. At 1 and 3 h after the fifth dose, three hamsters at each time point were euthanized by CO_2 inhalation and both plasma and cecal samples taken. Blood samples were obtained by cardiac puncture and centrifuged to obtain plasma (K_2-EDTA). The plasma was harvested within 30 min after drawing the blood and then stored at −80 °C. Cecal contents were weighed and then frozen at −80 °C after collection. For quantification of thiopeptides in cecal samples, cecal tissue weight was recorded by subtracting an average tube weight. To each sample, phosphate buffered saline (PBS) with 25% acetonitrile was added at 1.5× volume of the tissue weight (1:2.5 dilution). Samples were sonicated for 10 min and homogenized with an Omni-TH tissue homogenizer for 30−60 s. Next the homogenized samples were centrifuged for 10 min at 4000 rpm. An amount of 5 μL of the resulting cecal supernatant was further diluted with 45 μL of control cecal homogenate supernatant (1:10 dilution) resulting in a final dilution of 1:25. In addition, a 10 μL aliquot of control hamster plasma was added to each cecal sample and standard. The samples were then extracted by precipitation of proteins with acetonitrile (200 μL) containing 100 ng/mL internal standard (glyburide). Samples were subsequently vortexed for 5 min followed by centrifugation at approximately 4000 rpm for 5 min. The resulting supernatant (∼150 μL) was transferred into a clean 96-well plate, and 50 μL of water was added and vortexed for 2 min.

Hamster Model of *C. difficile* Infection. Hamsters were kept under controlled conditions with 12 h dark and 12 h light cycles, 68−72 °F constant temperature, 50% relative humidity, and 10−15 exchanges of fresh HEPA filter air per hour. Animals were kept in 7.5 in. wide by 6 in. deep cages (Alternative Design Manufacturing, Silom Springs, AR) with sterilized Bed O-Cob bedding (corn cob) and free access to water and standard rodent chow (Harlan). Animal were hamster-golden Syrian (*Mesocricetus auratus*), Michigan-206 Golden Syrian wild-type from Harlan, male, 90−110 g, 5−6 weeks old. Studies described were approved by the Institutional Animal Care and Use Committee (IACUC) of the Novartis Institutes for BioMedical Research Inc., Cambridge under protocol number OS 20,061.

C. difficile ATCC 43255 was received from American Type Culture Collection and stored at −80 °C in Brucella broth supplemented with 20% glycerol. On day −1, all animals received a single subcutaneous injection of clindamycin (10 mg/kg). At 24 h after clindamycin pretreatment, hamsters were infected by oral gavage with approximately 10^6 CFU of the *C. difficile* culture. All culture work was completed within 30 min, and all cultures were back in anaerobic conditions by this time. Briefly, strains were resuspended from overnight plates (in reinforced clostridial medium + 1% oxyrase (Oxyrase, Inc., Mansfield, OH) and diluted to 1×10^7 CFU/mL (OD_{600} = 1.1−1.3 nm, dilute 1:100). Hamsters were immediately infected with 0.75 mL inoculum (∼7.5×10^6 CFU/hamster final). An aliquot of inoculum was diluted 1:1000 and spiral plated (100 μL, slow deposition) on TSAB or RCMA (×2) and incubated at 37 °C anaerobically for determination of infection titer. Hamsters were administered the test compound (8 animals per dose level) starting 24 h after infection. Single antibiotic doses were administered orally and formulated as described above. Antibiotics were administered 1 time per day and continued for up 5 days. The control group was administered the solution vehicle alone. Animals were observed two times a day for the duration of the experiment. General observations included signs for mortality and morbidity and for the presence of diarrhea ("wet tail"), overall appearance (activity, general response to handling, touch, ruffled fur) and recorded. Animals judged to be in a moribund state will be euthanized. Criteria used to assign a moribund state are extended periods (5 days) of weight loss, progression to an emaciated state, prolonged lethargy (more than 3 days), signs of paralysis, skin erosions or trauma, hunched posture, and a distended abdomen. Observations continued, with any deaths or euthanasia recorded for up to 21 days postinfection (for relapse).

Any animal that died during the observation period was necropsied, and the contents of their cecums were removed, diluted with an equal volume of PBS, and frozen at −80 °C until processing (efficacy study). All surviving animals were euthanized by CO_2 inhalation and sampled in a similar manner as above.

Cecum homogenates are assayed for the presence of *C. difficile* yoxins A and B using the Wampole *C. difficile* Tox A/B II ELISA assay kit (VWR catalog no. 89013-262) in accordance with the manufacturer's directions.

Crystallization and X-ray of Compound 4·EF-Tu Complex. To form the EF-Tu·NVP·**4** complex, 10 mg/mL protein (227 μM), *E. coli* EF-Tu protein in a buffer containing 50 mM Tris, pH 8, and 50 mM NaCl were incubated with 1 mM compound **4** for 1 h at 4 °C. The sample was centrifuged at 20000*g* to remove any resulting precipitant. Crystallization was carried out using 300 nL of the protein sample plus 300 nL of crystallization solution containing 0.1 mM Tris, pH 8.23, 18% PEG3350, 0.2 M $MgSO_4$, using a sitting drop format and equilibrated against a reservoir of the crystallization solution. The crystal was flash frozen with liquid nitrogen after being stabilized in a cryo-buffer containing 0.1 mM Tris, pH 8.23, 18% PEG3350, 0.2 M $MgSO_4$, 15% ethylene glycol.

X-ray Data Collection. Initial data from a single crystal of the EF-Tu·**4** complex were collected on a ADSC Quantum 210 CCD detector using synchrotron radiation (λ = 1 Å) at the IMCA-CAT beamline 17-ID of the Argonne Photon Source. Data were collected using φ rotations of 0.5°, and 180° of total data were collected.

X-ray Data Processing, Structure Determination, and Refinement. Data from the EF-Tu·**4** complex were processed using the HKL2000 Suite, version 0.95.[20] Data processing statistics are shown in Table 5. Crystals were diffracted to 2.7 Å resolution in the space group *P*2(1)2(1)2 with a unit cell of a = 83.303 Å, b = 132.473 Å, c = 37.433 Å, $\alpha = \beta = \gamma = 90°$. Data collection statistics are shown in Table 5.

Table 5

	4
Data Collection Parameters (Highest Resolution Shell)	
resolution range (Å)	50−2.7 (2.87−2.7)
total observations	70876
unique reflections	11365
completeness (%)	93.5 (77.2)
I/σ	20.0 (2.8)
R_{sym}	0.099 (0.416)
Refinement Parameters	
R_{work}/R_{free}	0.233/0.315
protein atoms	2995
heterogen atoms (Mg/GDP/compd)	92
solvent atoms	89
average *B*-factor (Å^2)	47.4
Root Mean Squared Deviations from Ideal Value	
bond lengths (Å)	0.007
bond angles (deg)	1.4

The structure of the EF-Tu·GDP·**4** complex was determined by the molecular replacement as implemented in PHASER,[21] using *E. coli* EF-Tu protein as a search model (1D8T). The resulting molecular replacement solution contained one EF-Tu·GDP·**4** protein complex in the asymmetric unit. Refinement was carried out with CNX[22] using a single round of rigid-body refinement, following several cycles of simulated annealing refinement, *B*-factor refinement, and model building with the COOT software package.[23] Water molecules, GDP, and the Mg^{2+} ion were added prior to addition of the ligand **4**. Refinement produced a final model with excellent geometry (rmsd bond lengths of 0.007 Å, rmsd bond angles of 1.4°), and *R*-factors of R_{work} and R_{free} of 23.3% and 31.5%, respectively.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank all of the contributors to the EFT project team.

■ ABBREVIATIONS USED

MIC, minimum inhibitory concentration; EC_{50}, 50% effective dose; G+, Gram positive; MRSA, methicillin resistant *Staphylococcus aureus*; VRE, vancomycin resistant enterococci; *S. aureus*, *Staphylococcus aureus*; *E. faecalis*, *Enterococcus faecalis*; *E. faecium*, *Enterococcus faecium*; *S. pyogenes*, *Streptococcus pyogenes*; EF-Tu, elongation factor Tu

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