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Chemical Genomics, Structure Elucidation, and *in Vivo* Studies of the Marine-Derived Anticlostridial Ecteinamycin

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Abstract

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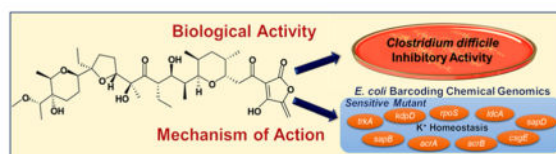
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.7b00388.

1D and 2D NMR spectra, RDC procedures along with considerations of DP4 probability and Pearson's correlations, experimental data for all membrane depolarization assays, potassium release assays, chemical genomic analyses, and additional experimental data (PDF)

A polyether antibiotic, ecteinamycin (**1**), was isolated from a marine *Actinomadura* sp., cultivated from the ascidian *Ecteinascidia turbinata*. ^{13}C enrichment, high resolution NMR spectroscopy, and molecular modeling enabled elucidation of the structure of **1**, which was validated on the basis of comparisons with its recently reported crystal structure. Importantly, ecteinamycin demonstrated potent activity against the toxigenic strain of *Clostridium difficile* NAP1/B1/027 (MIC = 59 ng/ μL), as well as other toxigenic and nontoxigenic *C. difficile* isolates both *in vitro* and *in vivo*. Additionally, chemical genomics studies using *Escherichia coli* barcoded deletion mutants led to the identification of sensitive mutants such as *trkA* and *kdpD* involved in potassium cation transport and homeostasis supporting a mechanistic proposal that ecteinamycin acts as an ionophore antibiotic. This is the first antibacterial agent whose mechanism of action has been studied using *E. coli* chemical genomics. On the basis of these data, we propose ecteinamycin as an ionophore antibiotic that causes *C. difficile* detoxification and cell death *via* potassium transport dysregulation.

Graphical Abstract



Clostridium difficile infections (CDI) represent a tremendous health threat in both the United States and other industrialized nations, necessitating the clear need for new therapeutic approaches and drug entities. Although precise numbers are not yet available for 2016, recent statistics tell a very grim story, especially in the U.S. In 2015, the Centers for Disease Control and prevention reported that about half a million Americans were afflicted with this deadly bacterial infection and that of these, ~30 000 died within 30 days of initial diagnosis.¹ Indeed, the CDC recently listed *C. difficile* as one of the three most highly ranked dangerous infectious pathogens receiving the designation of “urgent.”¹ *C. difficile* is now widely recognized also as the most common microbial cause of healthcare-associated infections in the U.S., and costs to the national healthcare system exceed \$4.8 billion annually. Notably, the U.S. is not alone in facing this health threat. New and hypervirulent strains known as NAP1/B1/027 have emerged and, partly by virtue of their resistance to previously employed therapeutics, have driven epidemics in the U.S., Canada, Britain, and several European nations.^{2–4}

C. difficile is a spore-forming Gram-positive anaerobic bacterium associated with pseudomembranous colitis and antibiotic-associated diarrhea in patients previously administered antimicrobial drugs.^{2–4} CDI arises from disruption of normal intestinal microflora, thus creating an opportunity for ingested spores to germinate, colonize the GI tract, and produce toxins able to inflict profound damage. The production of Rho-inactivating toxins A (TcdA) and B (TcdB) in the GI tracts of those afflicted lies at the heart of the bacterium’s toxicity in human hosts; both are proinflammatory and cytotoxic, causing disruption of the actin cytoskeleton and impairment of tight junctions in human intestinal epithelial cells.^{2–7} The actions of TcdA and TcdB lead to fluid accumulation and extensive

damage to the large intestine. More recent studies have shown that, in addition to serving as Rho-glucosylases, TcdA and TcdB trigger release of inflammatory cytokines from mast cells and macrophages as well as from epithelial cells, leading to further fluid secretion and intestinal inflammation.⁸ Notably, particularly virulent strains, such as NAP1/B1/027, have been noted to generate a third toxin referred to as *C. difficile* binary toxin (CDT), an actin-specific ADP-ribosyltransferase that disrupts the actin cytoskeleton.^{9–13} CDT expression and activity is associated with increased *C. difficile* cell adherence and subsequent pathogenicity.^{9–13} Taken together, it has become clear that TcdA, TcdB, and CDT are major determinants driving both the lethality and virulence of *C. difficile* in humans.

Although the advent of fecal transplants^{14,15} and recent approval of the antidifficile drug fidaxomicin^{16,17} have helped to stem the tide of CDIs, the emergence of resistance to a wide range of antibiotics, such as clindamycin,¹⁸ fluoroquinolones,¹⁹ vancomycin, and metronidazole,^{20–25} and ever increasingly virulent strains such as NAP1/B1/027 underscore the urgent need for new therapeutics. Central to new approaches for combatting *C. difficile* has been the recognition that toxins A and B enter GI cells *via* receptor-mediated endocytosis following target cell surface associations with combined repetitive oligopeptides (CROPs) embedded within the toxin C-termini.^{26–29} Endosomal acidification triggers conformational changes in TcdA and TcdB; this triggers vesicle membrane insertion and translocation of the catalytically active N-terminus into the cytosol. This activation/delivery mechanism is not unlike those of other bacterial toxins, has been hypothesized to represent a unique opportunity for drug targeting, and appears to highlight membrane function as an exploitable point of intervention.³⁰ Importantly, examples of ionophore-driven disruption of assorted ion pumping mechanisms are well established and, in fact, form the basis of a number of commercially crucial anticoccidial feed additives. Monensin (Rumensin), lasalocid (Bovatec), and laidlomycin propionate (Cattlyst) are all polyether antibiotics that disrupt ion concentration gradients (Ca^{2+} , K^+ , H^+ , Na^+) within microorganisms leading to futile ion cycles.³¹ Additionally, several reports have shown that monensin can reduce the cytopathogenic effect of *C. difficile* toxins, and TcdB induced apoptosis relies on ATP-sensitive potassium channels.^{32–34} Taken together, these data indicate that ionophore antibiotics could play a dual role as CDI antibiotics, by helping clear bacteria and reducing the effect of toxins on epithelial cells in the gut.

In our search for novel antibiotics, we isolated a novel polyether antibiotic, ecteinamycin (**1**; see Figure 1), from a marine *Actinomadura* sp. cultivated from the ascidian *Ecteinascidia turbinata* (Herdman, 1880).^{35,36} The generation of ¹³C-enriched samples and acquisition of ¹³C–¹³C COSY data along with other spectroscopic methods expedited the determination of the new carbon skeleton. The relative configuration was assigned, in part, using residual dipolar coupling (RDC)-based methods and was validated on the basis of the recently described crystal structure for **1**.³⁷ The ability of **1** to serve, as suggested by its structure, as a biologically active ionophore was also validated (Supporting Information).

Bioassays with **1** revealed its potent activity against Gram-positive bacteria. Especially notable, **1** displayed excellent selectivity for *C. difficile* (MIC 59 ng/mL). The results of chemical genomics studies, combined with its structure and activity in potassium release and membrane depolarization assays, highlight **1** as a new polyether ionophore antibiotic.³⁵ This

is, to our knowledge, the first instance of an *E. coli* chemical genomics study being used to delineate a drug or drug candidate's mechanism of action (MOA). Notably, alterations in the transport of H⁺, K⁺, Na⁺, Ca⁺, and/or Mg²⁺ ions by **1** may lie at the heart of this agent's toxicity toward *C. difficile*; indeed, the results of chemical genomics studies support this early hypothesis and identify selectivity for H⁺/K⁺. With respect to disrupting endosomal and vesicular trafficking, which we hypothesized was the mechanism by which monensin reduced effects of both *C. difficile* toxins, we examined the effect of **1** on yeast *via* chemical genomics. The data clearly indicated that **1** disrupted vesicular trafficking *via* pH/ion effects providing mechanistic support for how ionophore antibiotics such as monensin abrogate the effects of *C. difficile* toxins.

Mechanistic studies of pathogenic and virulent processes often unveil new therapeutic opportunities and are therefore crucial to drug discovery. This appears to be the case with TcdA and TcdB as overlaid with our discovery of **1**. Several structural features of **1**, such as the acyltetramic acid moiety, are found also in other polyether ionophores, such as tetronomycin (**2**)³⁸ and tetronasin (**3**).³⁹ However, as a whole, ecteinamycin contains a new carbon skeleton, and its potent selectivity against *C. difficile* renders **1** a potential antibiotic. Moreover, an *in vivo* mouse model indicated that **1** has low oral availability. This, combined with its excellent MIC against *C. difficile*, makes **1** very exciting since antibiotics intended to work in the lower GI would ideally have low oral bioavailability yet potent activity when presented with relevant target organisms.

RESULTS AND DISCUSSION

LCMS-PCA Assisted in Microbial Strain Selection

LCMS profiling of 34 marine-derived bacterial extracts and subsequent multivariate analysis by principal component analysis (PCA)⁴⁰ led us to pursue the strain WMMB499, which demonstrated unique chemistry compared to other strains by PCA (Supporting Information, Figure S1). WMMB499 was found to produce three classes of novel natural products: (i) halogenated electrophilic polyketides halomaduronones A–D,⁴¹ (ii) antifungal polyketide forazolone A,⁴² which have previously been reported, and (iii) the new polyether antibiotic **1**.

Structure Elucidation of Ecteinamycin

HRMS data revealed the molecular formula of **1** to be C₃₈H₆₀O₁₂ (Supporting Information, Figure S2), and extensive 1D and 2D NMR data (Supporting Information, Table S1) enabled us to determine the majority of the 2D structure for **1**. To improve the overall efficiency of structure elucidation, we used our previously published approach of ¹³C isotopic enrichment followed by rapid acquisition of ¹³C–¹³C COSY data.^{43,44} In the absence of crystallographically generated structure information at the time, we employed a combination of ROESY correlations (Supporting Information, Figure S11), coupling constants, ¹³C chemical shifts, molecular modeling, and other spectroscopic methods to partially determine the relative configuration of **1** (see Supporting Information). In addition to these efforts, we also employed residual dipolar couplings (RDC) studies to investigate the stereochemistry of **1**. A new method of employing RDCs was developed in parallel and recently published.⁴⁵ Although the RDC approach was successful, we evaluated extensive DFT calculations and

included aspects of those in the Supporting Information. Importantly, our stereochemical assignments have recently been validated by recently reported crystallographic and biological studies of **1** and its chlorinated congener non-thmicin.³⁷

Ecteinaamycin Inhibits Growth of Toxigenic *C. difficile* Strains at Nanogram Levels

Screening of **1** against a panel of Gram-positive and Gram-negative bacteria revealed potent antimicrobial activities for ecteinaamycin (Table 1). Ecteinaamycin was particularly potent against Gram-positive bacteria, consistent with the established activity of ionophore antibiotics and especially those used as feed additives.³³ Given the growing importance of CDIs, we subjected a number of *C. difficile* strains to **1**, including the highly virulent NAP1/B1/027, and MICs were determined. Notably, **1** displayed excellent potency (MIC 59 ng/mL) against NAP1/B1/027, although significant antimicrobial actions were clearly exerted against other *C. difficile* strains as well. Also noteworthy is that **1**, in our hands, was ~20-fold more active against *C. difficile* than against other bacteria (Table 1). For example, **1** displayed MICs of 16, 0.125, and 8 $\mu\text{g/mL}$ against *E. coli*, *S. aureus* (both methicillin sensitive and resistant), and *P. aeruginosa*, respectively. That **1** is so much more active against *C. difficile* than other microbes suggests that **1**, and possibly other ionophores, may represent extremely important new therapeutics for CDIs. This notion is further strengthened by the fact that **1** was much more effective against *C. difficile* than the currently employed first-line clinical antibiotics metronidazole (MIC = 2.0 $\mu\text{g/mL}$)^{46–48} and vancomycin (MIC = 1.0–2.0 $\mu\text{g/mL}$).^{46–48} Notably, nigericin, a well-established ionophore antibiotic, has also been reported to be potent against *C. difficile* (MIC of 2.5 ng/mL), and a number of other tetramic-acid-containing compounds have shown activity against *C. difficile in vitro*.^{49,50} Monensin, despite its established antibiotic activities and ionophore activity, is nowhere near as active (MIC = 0.5 $\mu\text{g/mL}$) against *C. difficile* as **1**.⁴⁸

Ecteinaamycin Depolarizes Cell Membranes and Interferes with Potassium Cation Homeostasis

The potent and selective activity of **1** against *C. difficile* inspired us to probe this new natural product's mechanism of action. A membrane depolarization assay^{50,51} and *E. coli* chemical genomic profiling *via* barcode sequencing were employed to shed light on this issue.⁵² First, flow cytometry was used to evaluate the impact of **1** on bacterial membrane potentials. Methicillin-sensitive *S. aureus* (MSSA) cells were subjected to either 6 or 32 $\mu\text{g/mL}$ of **1** (Figure 2). Additionally, all cells, except the DMSO negative control, were treated with DiOC₂(3) dye and analyzed by flow cytometry.

Compound **1** very rapidly induced membrane depolarization at 6 $\mu\text{g/mL}$ (Figure 2) although the magnitude of depolarization was greater with CCCP, an established depolarizing agent. In parallel, studies with the established anticoccidial agent salinomycin revealed that ecteinaamycin's MSSA depolarization activity far surpassed that of salinomycin sodium salt (Supporting Information).^{53–55} Subsequent potassium-release assays also employing MSSA confirmed salinomycin's excellent ionophoric activity^{53,54} and established, for the first time, ecteinaamycin's ionophore activity (Supporting Information). These data highlight a clear and consistent system in which membrane depolarization by **1** aligns with structure-informed expectation and demonstrable potassium release/ionophoric capacity. In total,

these data strongly affirm the hypothesis that ionophore function provides at least one basis for the antibacterial activity of **1**, albeit at concentrations well above the MIC for **1** against *C. difficile*. It must also be noted however that the results of these assays do not exclude the potential relevance of other *C. difficile* specific targets/pathways exploited by **1**, particularly at low concentrations.

Alongside depolarization assays, chemical genomic profiling with barcoded *E. coli* deletion mutants was used to understand the effects of **1** on bacterial cells. Chemical genomics has been used to determine MOAs and molecular targets for many bioactive compounds, including natural products using barcoded *S. cerevisiae*.^{56–58} However, *E. coli* barcoded libraries have not been used. Given the structural resemblance to other ionophore antibiotics as well as the depolarization data, ecteinamycin was a good candidate for a proof of concept study using *E. coli* chemical genomics. A library of 6000 *E. coli* deletion mutants was combined and treated with ecteinamycin. The genomic DNAs were extracted, and mutant-specific DNA barcodes were amplified and sequenced by Illumina sequencing. Ecteinamycin-sensitive and resistant mutants were determined by quantification of DNA barcodes. The resulting chemical genomic profile (Figure 3) provided significant insight into ecteinamycin's MOA, albeit at concentrations of **1** far higher than necessary to elicit *C. difficile* activity.⁵² In general, however, chemical genomic studies require significantly higher concentrations due to the large number of cells required to represent all knockout strains.

E. coli deficient in potassium processing, and presumably, membrane function, proved particularly sensitive to the effects of **1**. Deletion of *trkA*, *kdpD*, and *sapD* genes drove enrichment of the sensitive mutants following exposure to **1**. The loss of TrkA, the NAD binding domain of the Trk potassium transporter; *sapD*, which is required for ATP-dependence of Trk K⁺ uptake; and KdpD, a sensor kinase for the Kdp (K⁺ dependence) operon, characterized particularly susceptible strains, thus indicating the K⁺ ion as a likely target for **1**.^{59–62} Transport systems and channels, such as the TRK and KDP pathways, are central to regulating potassium accumulation and movement across membranes. Microbial cell membrane integrity is key for homeostasis maintenance, survival, electron transport, and ATP biosynthesis. Taken together, these data firmly support the preceding membrane depolarization assays supporting the idea that, like other ionophores, **1** interferes with bacterial potassium homeostasis and membrane integrity and that this leads to cell killing.^{59–62} This result is consistent with previous work by Hurdle *et al.* wherein the membrane-depolarizing reutericyclins were found to kill *C. difficile* in stationary or nongrowth phases.⁶³ Subsequently, Wu *et al.* suggested that ion/proton gradients across the *C. difficile* membrane play key roles in cell viability and concluded that *C. difficile* is susceptible to antibiotics targeting cell membrane function.⁴⁸ While we could not rule out a specific target in *C. difficile* that differs from *E. coli*, our data were consistent with ecteinamycin acting as an ionophore antibiotic.

Strains resistant to **1** were characterized by alterations in stress response genes (GO: 0033554) and lipid binding (GO: 0008289; Figure 3). Specifically, *sspA* and *rseB* deletion mutants displayed resistance to **1**. The stringent starvation protein A (SspA), a polymerase-associated protein, is involved in pathogenicity and cellular stress-response under conditions

of nutrient deficiency and acidic pH,^{64–67} whereas RseB antagonizes the activity of the sigma E stress-response (RpoE) transcription factor and binds periplasmic lipopolysaccharides.^{68–71} Upon exposure to **1**, deletion of the *cusC* gene was also enriched in *E. coli* relative to the ~6000 strains. This gene encodes for lipoprotein component factor CusC in the outer membrane, which is part of the Cu⁺/Ag⁺ detoxification efflux pump system.^{72–74} In contrast to chemical genomics data for cells sensitive to **1**, the corresponding data related to **1** resistance do not underscore any one particular mechanism or set of mechanisms driving cellular response. Instead, these data suggest that cellular enrichments may have more to do with delayed autolysis mechanisms than with *bona fide*, long-term resistance to **1**.

In parallel, chemical genomic profiling with yeast, *Saccharomyces cerevisiae*, was used to understand the effects of **1** on eukaryotic cells and to shed further insight into how ionophore antibiotics like monensin attenuate the effects of *C. difficile* toxins.^{35,36} Ecteinamycin was screened against over 4000 deletion mutant yeast strains. Genomic DNA was extracted, and mutant-specific DNA barcodes were amplified and sequenced by Illumina as with the *E. coli* studies.

This resulted in a distinct chemical genomic profile of strains responsive to **1** at 250 µg/mL (Supporting Information Table S3). Relative to our existing data set of known compounds (Piotrowski *et al. in review*), we found the top two activity correlations of **1** to be with the polyether antibiotics duamycin and nigericin ($P < 0.0001$).^{56–58} Both duamycin and nigericin act by generating ion channels in cellular membranes and inhibiting Golgi function in eukaryotic cells. The mutant *S. cerevisiae* strains most sensitive to **1** ($P < 0.0001$) had significant gene ontology (GO) enrichment for the biological process “post-Golgi-mediated transport” ($P = 1.19 \times 10^{-6}$), driven by sensitive mutants of genes *KES1*, *APL4*, *MON2*, *DRS2*, *APM2*, and *VPS9*. Further implicating impaired Golgi functioning as an ecteinamycin sensitivity determinant, we identified a hypomorph of *SEC14* as the only significantly sensitive ($P = 0.03$) essential gene mutant in a screen using the DAMP collection.⁷⁵ *SEC14* is a phosphatidylinositol/phosphatidylcholine transfer protein required for correct trans-Golgi network dynamics.⁷⁶

We then correlated the chemical genomic profile to the genetic interaction network for *S. cerevisiae*.⁷⁷ Among the top 20 correlated genetic mutant profiles, we saw significant enrichments for mutants in genes involved in the GO processes “intracellular pH reduction” [$P = 7.31 \times 10^{-9}$, GO: 0051452]. This enrichment was driven by *VMA1*, *VMA8*, *VMA9*, *VMA10*, and *VMA11*: all are subunits of a yeast vacuolar ATPase and crucial to maintaining intracellular pH regulation. On the basis of structural and functional similarities to other polyether antibiotics, these data suggest that ecteinamycin works, in eukaryotic systems, by forming ion channels, disrupting Golgi dynamics, and impairing other vesicle-mediated transport systems. It is perhaps noteworthy that significant differences in MOA are apparent for **1** when considering prokaryotic cells versus eukaryotic cells, but that, in all cases, biological activities clearly stem from the ionophoric nature of **1**.

Cytotoxicity, Bioavailability, and *in Vivo* Efficacy

In vitro cytotoxicity of **1** was evaluated in mammalian erythrocytes (sheep red blood cells), in a metabolism-independent manner. The hemolysis assay is based on the survival of erythrocytes upon treatment with **1** for 1 h. The lysis agent, Triton, and amphotericin B were used as positive controls. Hemolysis of the red blood cells was measured by the release of hemoglobin at a wavelength of 570 nm. Notably, **1** proved incapable of inducing hemolysis at any of the concentrations tested (Supporting Information, Figure S18); this contrasts sharply with the positive amphotericin control which induced hemolysis in a dose-dependent manner. Ecteinamycin treatment, even at 20 μM , enabled 99.9% erythrocyte survival. By contrast, exposure to 20 μM amphotericin (positive control) correlated to only 30.1% survival. These data strongly support the notion that **1**, although toxic to bacteria, does not display overt toxicity toward mammalian cells.

Bioavailability

A pharmacokinetic study in mice was carried out to determine the oral bioavailability of **1**. Mice were dosed orally with **1** at 5 mg/kg, or by retro-orbital intravenous (IV) injection at 2.5 mg/kg. Accounting for this difference in dosage, the oral bioavailability was determined to be 10.9% after 30 min of treatment and 29.3% after 1 h of treatment, indicative of generally poor oral bioavailability (Supporting Information, Figures S19–S23). Such a trait of most agents might be considered disadvantageous. However, given the localization of the *C. difficile* target organism to the lower GI tract in humans, the intestinal lining in particular, these data are very exciting.⁷⁸ Notably, neither vancomycin nor fidaxomicin is well absorbed orally, and this trait of both agents has been attributed to their past success in countering CDIs.¹⁶ It is perhaps very important to recognize that this report represents the first demonstration of oral bioavailability levels for an ionophoric *C. difficile* selective antibiotic drug lead. Indeed, the low oral bioavailability of **1** supports earlier hypotheses in the field that ionophores may represent excellent anti-*C. difficile* therapeutic agents.

In Vivo Efficacy

A preliminary *in vivo* study in mice was performed using **1** as a therapeutic against *C. difficile* in a modified murine model for *C. difficile* infection.⁷⁹ Three experimental mouse groups were used: a healthy control ($n = 5$), *C. difficile* control ($n = 5$), and a group treated with 30 ng of ecteinamycin ($n = 5$). The baseline weight loss and the health scores (Figure 4) were collected for all groups. Mice in the *C. difficile* control group began to decline in health by 30 h postinfection based on the collected health parameters. At 24 h postinfection, each mouse in the ecteinamycin group was dosed with 30 ng of **1**. The preliminary data indicate that the average health score values are significantly different at the 0.05 level and the 0.000125 level, as evaluated by one-way ANOVA (overall ANOVA) and the Bonferroni test, respectively. *C. difficile* control mice lost 16% of baseline weight at 33 h postinfection, and 20% at 48 h, whereas mice treated with 30 ng of **1** lost 9% and 12% at the same time points. The weight loss due to initial diarrhea, which is a characteristic symptom of *C. difficile* pathogenesis, affected the health scores.

In addition, *C. difficile* clearance was measured in collected feces of experimental mice at seven time points (Figure 5). At 48 h after infection, a 1- \log_{10} reduction in *C. difficile*

burden (CFU/g feces) was observed in the experimental group treated with 30 ng of ecteinamycin compared to that of the *C. difficile* control group. Nevertheless, there is a need for more *in vivo* studies in different animal models in order to establish ecteinamycin efficacy.

Conclusion

CDIs constitute a global health crisis that continues to grow in both scope and scale. Very few agents are effective at controlling *C. difficile*, although there exists ample evidence in the literature to suggest that ionophoric feed additives may represent useful therapeutics. Ecteinamycin (**1**), a polyether antibiotic, was isolated from a marine-derived *Actinomadura* sp. (Strain WMMB499) and its structure elucidated by ^{13}C - ^{13}C COSY NMR of ^{13}C -labeled **1** and other spectroscopic methods including a newly developed and highly useful approach exploiting RDC data. Most importantly, **1** demonstrated potent antibacterial activity, especially against a wide array of *C. difficile* strains; this was the case both *in vitro* and *in vivo*. MOA studies with *E. coli* and *S. cerevisiae* barcoded deletion mutants revealed that, indeed, the ability of **1** to serve as an ionophore able to interfere with bacterial ion processing likely explains the compound's potent activity against *C. difficile*. Despite its low MICs against *C. difficile* strains, cell depolarization assays with **1** called for high concentrations due to short exposure times; *E. coli* chemical genomics also called for high concentrations of **1** due to the large number of mutant strains treated and commensurately high cell density of the assay. Ecteinamycin (**1**) appears to show no discernible toxicity to noncancerous mammalian cells as reflected by its lack of hemolytic activity. We have also shown that **1** has limited oral bioavailability, a trait highly desirable when attempting to kill/clear *C. difficile* within the human intestinal lining. Additionally, previous data on monensin as well as the yeast chemical genomics results shown here also establish the hypothesis that ionophore antibiotics might reduce effects of *C. difficile* toxins by disrupting vesicle-mediated trafficking. Taken together, these findings highlight the newly discovered marine natural product **1** as an extremely promising therapeutic lead agent against *C. difficile*. These data also underscore the potential use of other ionophores as effective therapeutics for CDIs, an assertion that has been previously suggested by others but perhaps not examined with any one agent in as rigorous a fashion as in the case here for ecteinamycin.

METHODS

See the Supporting Information for details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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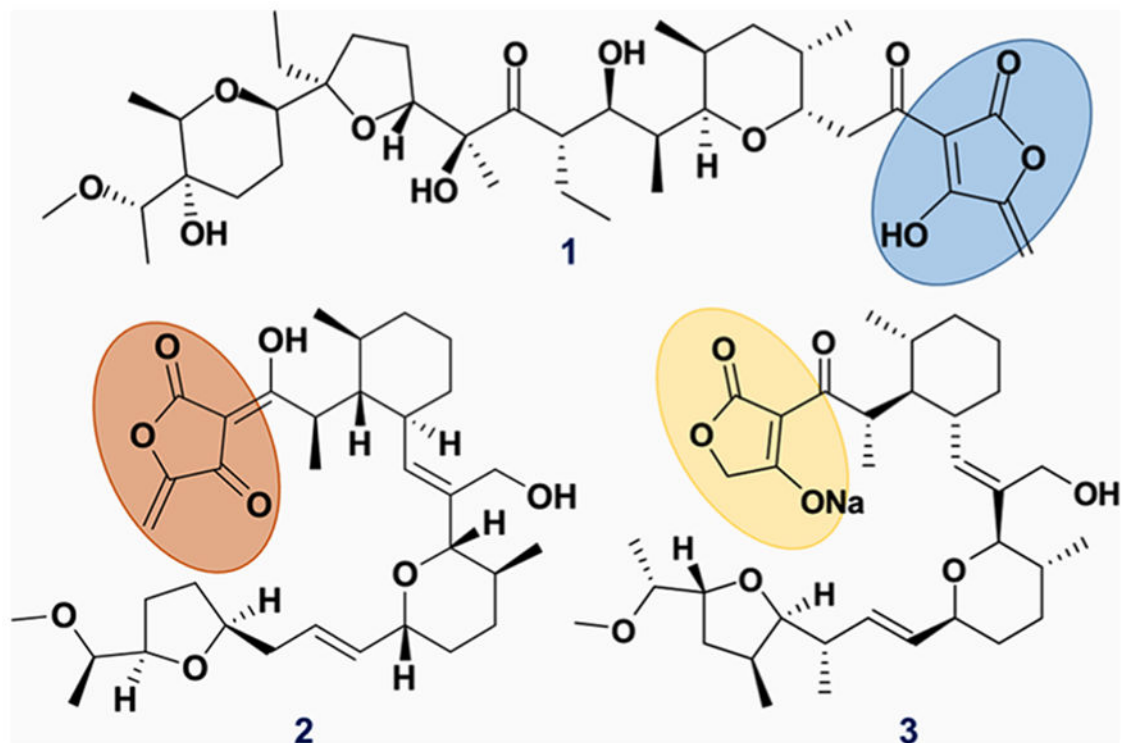


Figure 1. Polyether ionophores sharing the characteristic acyltetramic acid moiety. Ecteinamycin (1), tetronomycin (2), and tetronasin (3).

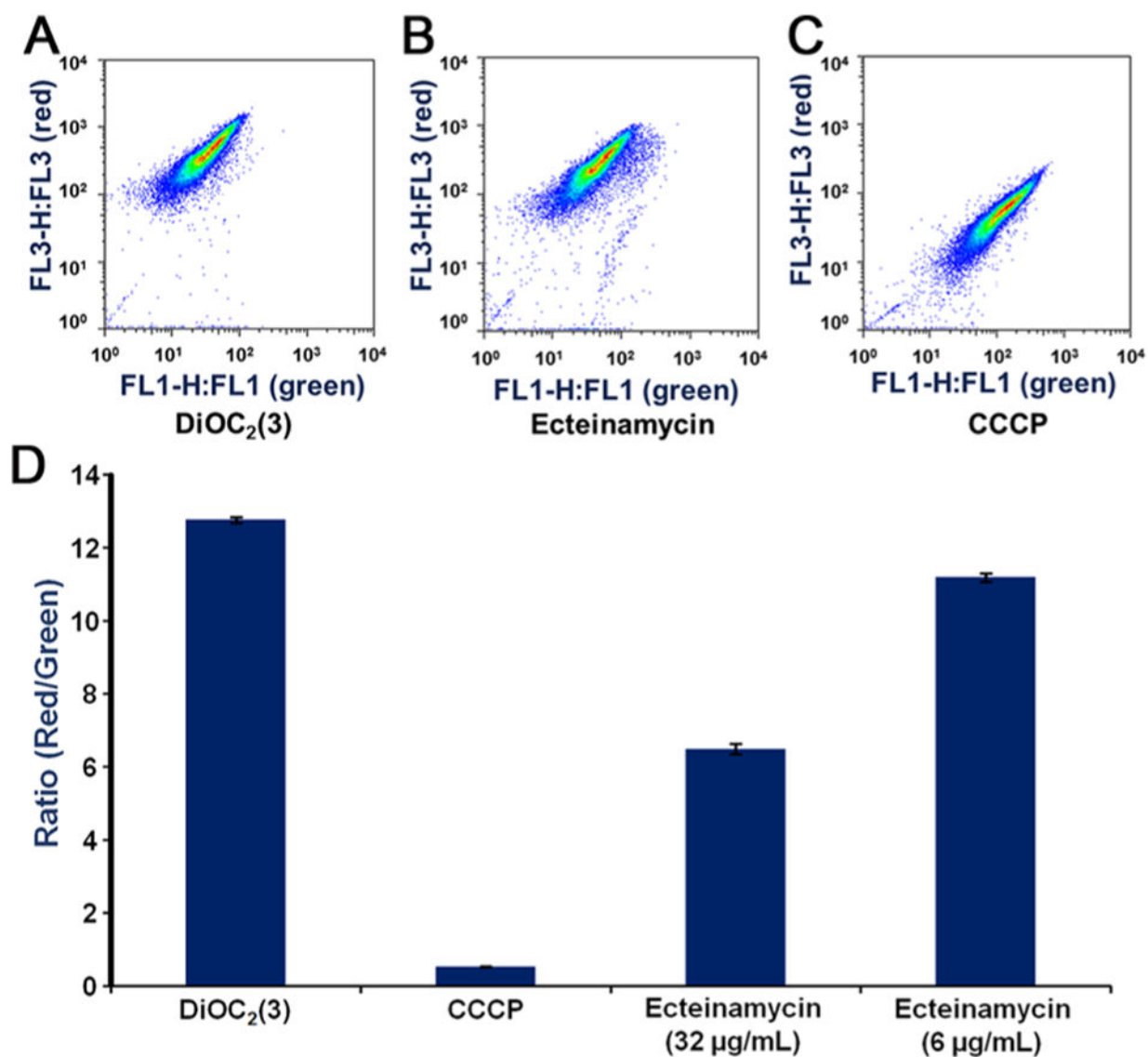


Figure 2. Membrane depolarization assay. Flow cytometry-based analysis of MSSA cells treated with DiOC₂(3) dye control (A), ecteina mycin (B), and CCCP (C). (D) The ratio of red to green fluorescence for MSSA cells treated with DiOC₂(3) dye and the corresponding compound. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used as a positive control. CCCP is a protonophore that disrupts membrane potential.^{50,51}

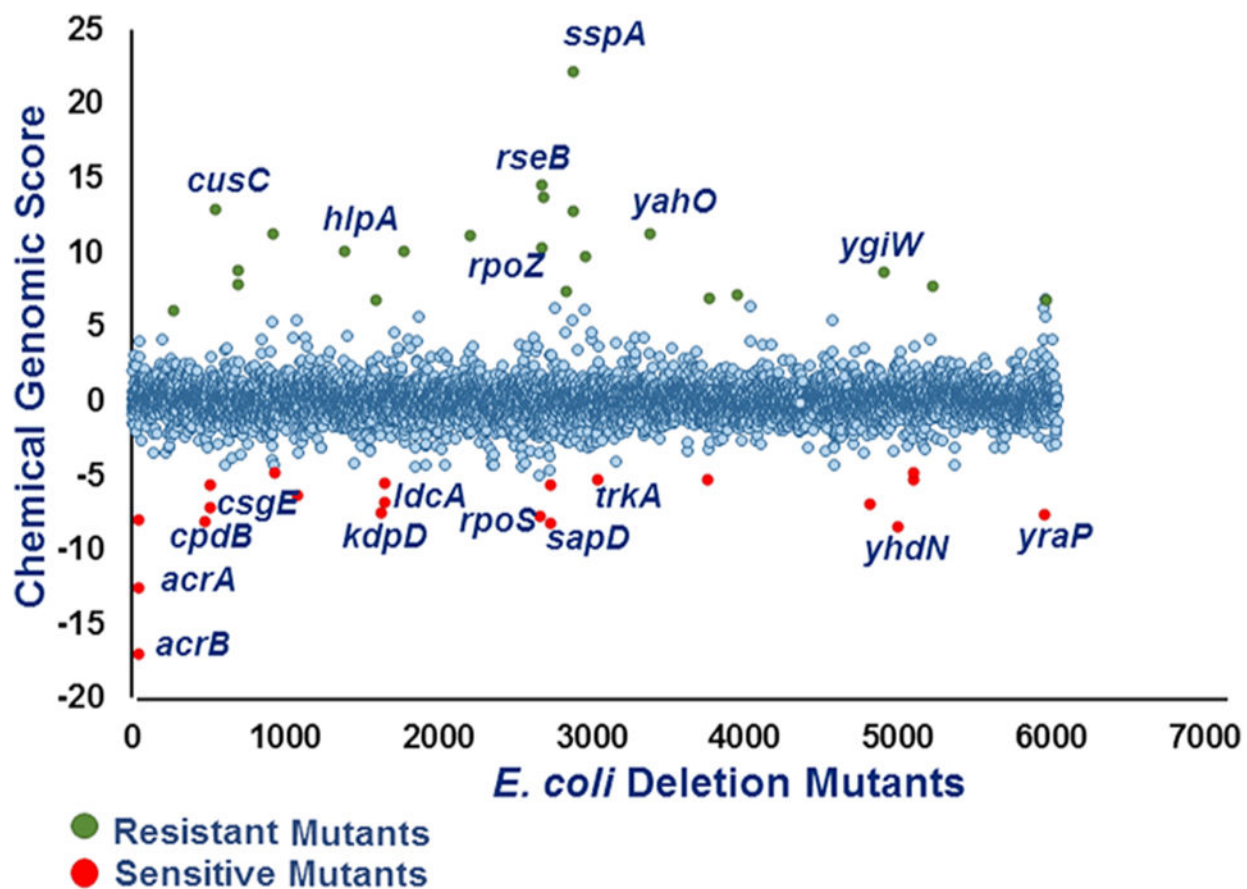


Figure 3. Chemical genomic profile of ecteinamycin. The chemical genomic analysis was performed in triplicate at [1] = 62.5 $\mu\text{g}/\text{mL}$. In green, *E. coli* mutants deficient in *sspA*, *rseB*, *cusC*, and other genes proved resistant to the actions of **1**. In red, *E. coli* sensitive to **1** are deficient in *trkA*, *sapD*, *kdpD*, and other indicated genes (Table 2).

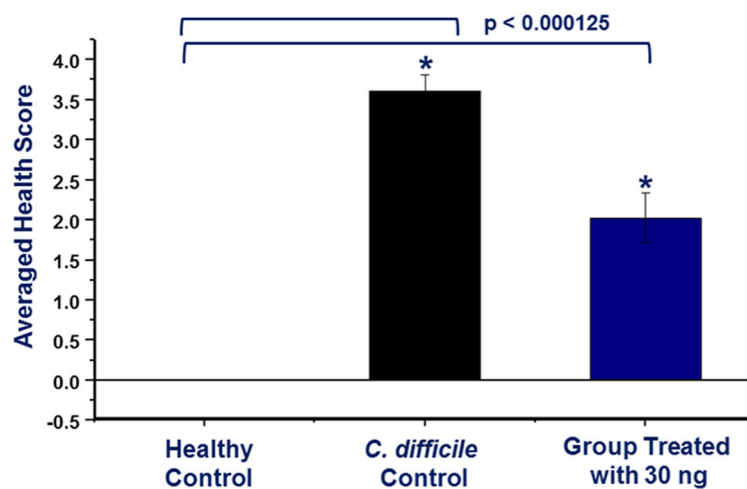


Figure 4. Effect of **1** on *C. difficile* challenged mice. Experimental groups: healthy control (uninfected), *C. difficile* control, and group treated with 30 ng of ecteinamycin. Health scores from 27–48 h were averaged for each experimental group.

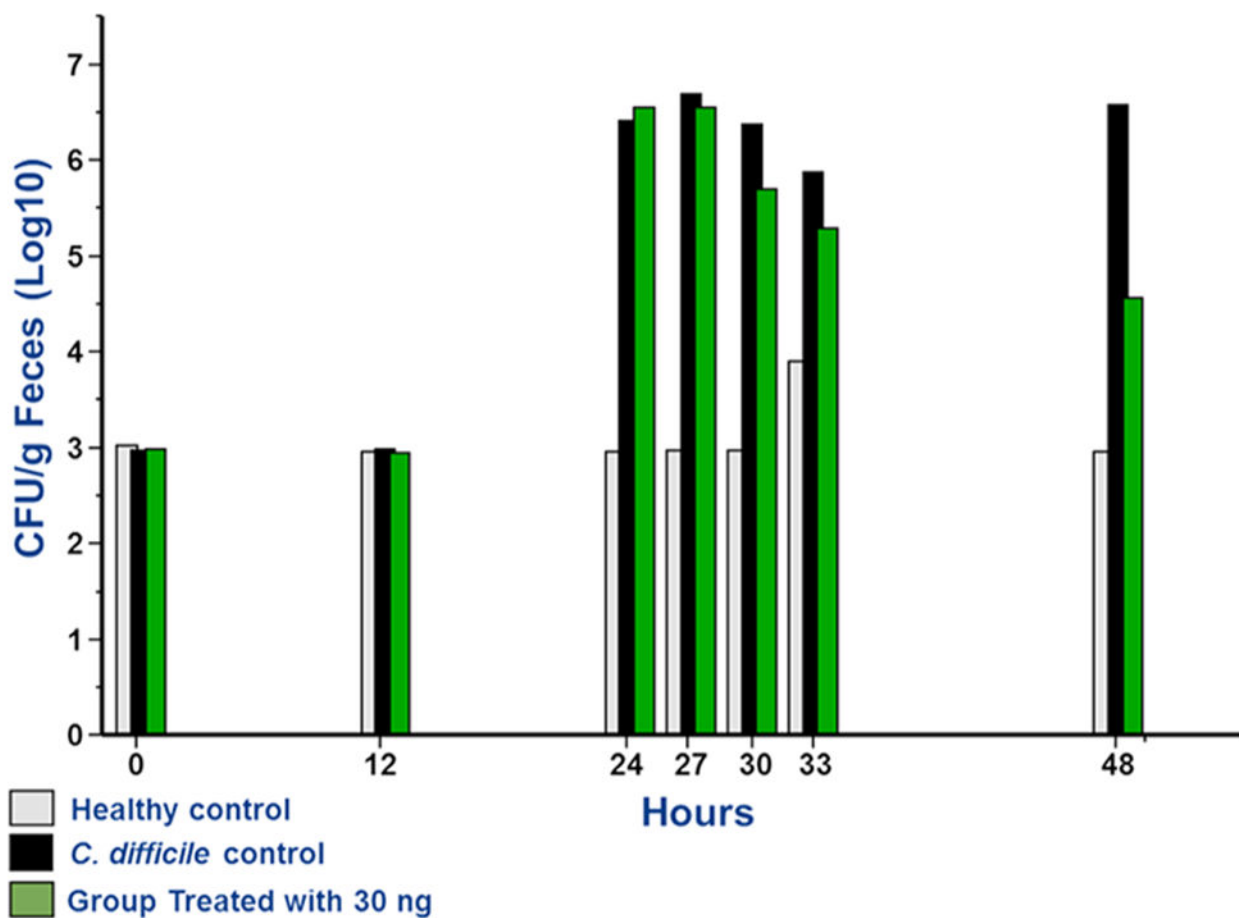


Figure 5. *C. difficile* clearance. Feces samples collected at seven different time points were diluted in PBS and plated onto selective *C. difficile*-Brucella agar, incubated anaerobically for 48 h, and subsequently enumerated. For healthy controls at all time points, as well as the others at time points 0 and 12 h, the CFU/g was zero, given that the limit of detection of the method is $<3.00 \times 10^4$ ($\log_{10} = 4.4771$).

Table 1Minimum Inhibitory Concentration (MIC) of 1³⁵

microorganism	MIC ($\mu\text{g/mL}$)
<i>E. coli</i>	16.0
<i>S. aureus</i> (methicillin-sensitive)	0.125
<i>S. aureus</i> (methicillin-resistant)	0.125
Vancomycin-resistant <i>Enterococcus</i>	0.25
<i>P. aeruginosa</i>	8.0
<i>C. difficile</i> ATCC 43255 ^a	0.059
<i>C. difficile</i> BAA-1870 ^a (strain NAP1/B1/027)	0.059
<i>C. difficile</i> BAA-1382 ^a (strain 630)	0.059
<i>C. difficile</i> ATCC 700057 ^b	0.117
<i>C. difficile</i> ATCC 9689 ^{a,c}	0.059
<i>C. difficile</i> B6/6 005 ^{a,c}	0.117
<i>C. difficile</i> B6/6 006 ^{a,c}	0.117
<i>C. difficile</i> ICCD 001 ^{a,c}	0.059
<i>C. difficile</i> ICCD 006 ^{a,c}	0.059

^aToxigenic.^bNontoxigenic.^cClinical isolate.

Table 2Ecteinamycin Sensitive *E. coli* Deletion Mutants

disrupted gene	fold change	adj. <i>P</i> value	gene function
<i>acrB</i>	0.086930074	1.49e ⁻⁶⁵	component of AcrAB-ToIC multidrug efflux system
<i>acrA</i>	0.08687022	2.59e ⁻⁶⁰	component of AcrAB-ToIC multidrug efflux system
<i>trkA</i>	0.145154921	4.19e ⁻²⁷	NAD-binding component of TrK potassium transporter
<i>sapD</i>	0.196735935	4.74e ⁻²¹	component of peptide uptake ABC trans-porter, required for ATP-dependence of TrK K ⁺ uptake systems
<i>csgE</i>	0.368679357	9.8e ⁻²⁰	component of curli secretion and assembly complex
<i>ldcA</i>	0.341380178	3.45e ⁻¹⁸	L,D-carboxypeptidase A; cytoplasmic protease that cleaves the terminal D-alanine from cytoplasmic muropeptides
<i>sapB</i>	0.241016831	1.28e ⁻¹⁶	component of peptide uptake ABC transporter
<i>kdpD</i>	0.373882707	2.10e ⁻¹⁵	sensor kinase for the kdp (K ⁺ -dependence) operon
<i>fis</i>	0.416051589	9.27e ⁻¹⁵	transcriptional activator for rRNA operons, bends DNA
<i>rpoS</i>	0.35004657	1.15e ⁻¹³	component of RNA polymerase σ 38