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Evaluation of Derivatives of (+)-Puupehenone against *Clostridioides difficile* and Other Gram-Positive Bacteria

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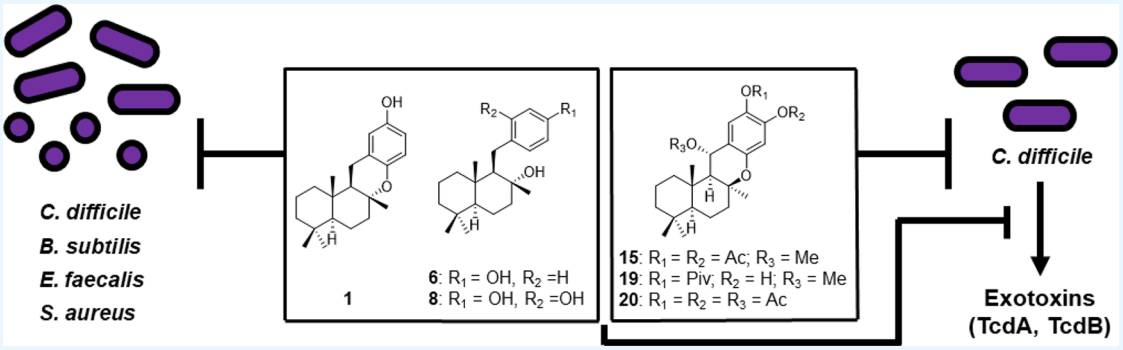

ABSTRACT: Patients receiving healthcare are at higher risk of acquiring healthcare-associated infections, which cause a significant number of illnesses and deaths. Most pathogens responsible for these infections are highly resistant to multiple antibiotics, prompting the need for discovery of new therapeutics to combat these evolved threats. We synthesized structural derivatives of (+)-puupehenone, a marine natural product, and observed growth inhibition of several clinically relevant Gram-positive bacteria, particularly *Clostridioides difficile*. The most potent compounds—(+)-puupehenone, **1**, **15**, **19**, and **20**—all inhibited *C. difficile* in the range of 2.0−4.0 μg/mL. Additionally, when present in the range of 1−8 μg/mL, a subset of active compounds—(+)-puupehenone, **1**, **6**, **15**, and **20**—greatly reduced the ability of *C. difficile* to produce exotoxins, which are required for disease in infected hosts. Our findings showcase a promising class of compounds for potential drug development against Gram-positive pathogens, such as *C. difficile*.

INTRODUCTION

Healthcare-associated infections (HAIs) pose a significant risk to patients undergoing treatment in hospitals and other healthcare facilities.[1] These infections lead to thousands of deaths and cost the U.S. healthcare system several billions of dollars each year.[2,3] There is undoubtedly a high prevalence in the United States as approximately 1 in 31 hospital patients carried an HAI in 2015.[4] The most common HAIs reported from that survey were pneumonia, gastrointestinal infections, and surgical-site infections; moreover, the most common pathogens responsible for these HAIs were found to be *Clostridioides* (formerly *Clostridium*) *difficile*, *Staphylococcus aureus*, and *Escherichia coli*.[4] Several other pathogens were also reported, such as *Pseudomonas aeruginosa* and species belonging to the *Klebsiella*, *Enterobacter*, and *Enterococcus* genera.[4] The challenge in treating HAIs stems from the fact that several of these clinical isolates are resistant to multiple antibiotics; indeed, 45% of *S. aureus* isolates from the aforementioned survey were methicillin-resistant (MRSA) while 3% of *E. coli*, *Klebsiella*, and *Enterobacter* isolates were resistant to at least one carbapenem.[4] According to the Centers for Disease Control and Prevention (CDC), over 2.8 million infections and 35,000 deaths are caused by antibiotic-resistant pathogens each year.[5] Therefore, new therapeutics must be developed to successfully treat these HAIs.

C. difficile is a Gram-positive, spore-forming anaerobe that causes antibiotic-associated diarrhea, generally representing 15−25% of all known cases.[6−9] *C. difficile* infection (CDI) follows the clearance or disturbance of the normal gut flora, usually after antibiotic treatment.[9] The disease manifestation of CDI is caused by two virulence factors, toxins A (TcdA) and B (TcdB), which target intestinal epithelial cells and inactivate host Rho proteins via glucosylation, ultimately resulting in the disruption of the actin cytoskeleton and tight junctions; additionally, both exotoxins cause severe inflammation and are known to induce programmed cell death pathways, such as apoptosis and necrosis.[10−15] The severity of CDI underscores

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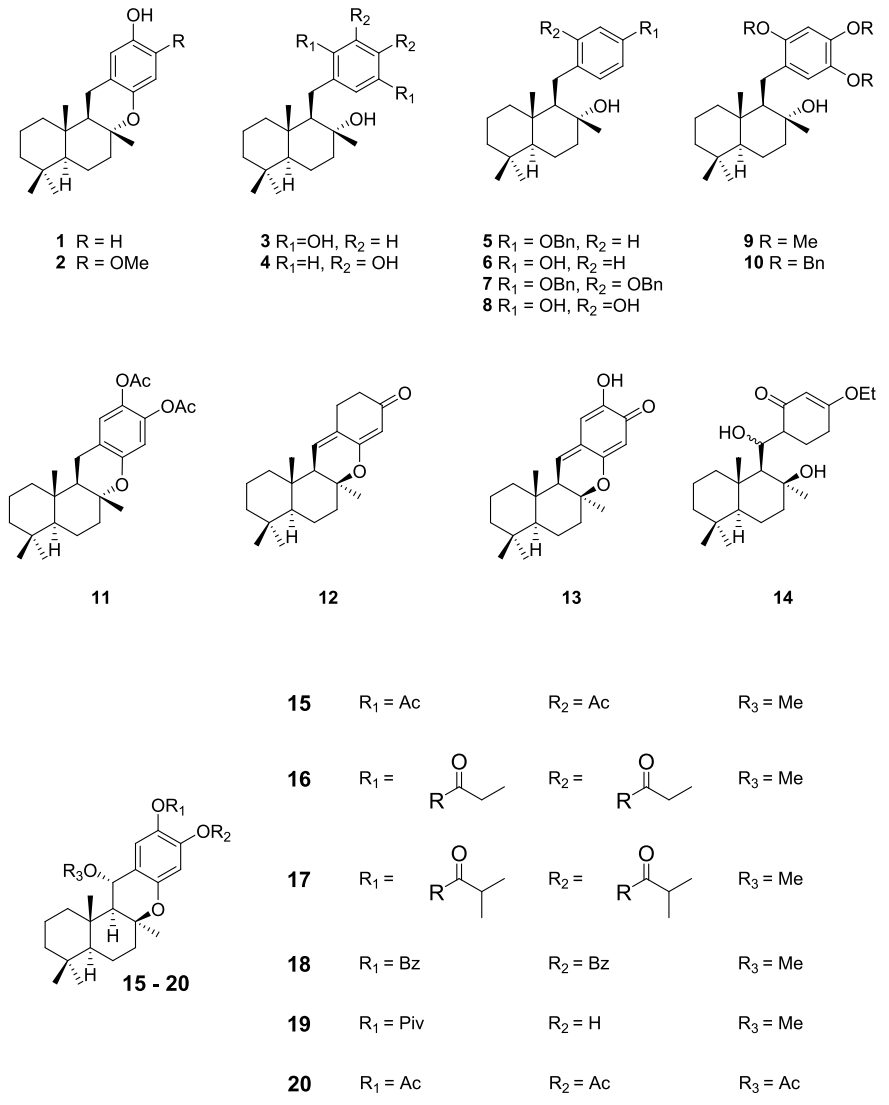

Figure 1. Synthetic meroterpenoid library.

its significance as an HAI; for example, the CDC reported that *C. difficile* was responsible for approximately 223,900 infections in hospitalized patients and 12,800 deaths in the United States in 2017.[5] In addition to its severity, CDI is difficult to treat since the disease recurrence rate (15−35%) increases after each subsequent treatment.[16,17] The primary recommended antibiotics for CDI are fidaxomicin and vancomycin.[18−21] Though metronidazole was previously recommended as the first-line treatment for CDI, it has since become obsolete due to the high frequency of treatment failures and recurrence; similar data has been reported from studies of vancomycin as well.[22,23] Compared to metronidazole and vancomycin, fidaxomicin is typically associated with a lower rate of recurrence.[24,25] Furthermore, while the frequency of antibiotic resistance in *C. difficile* is relatively low for all three drugs,[26,27] resistant isolates have been reported in the literature;[23,28] for example, a fidaxomicin-resistant clinical isolate with a minimum inhibitory concentration (MIC) of >64 μg/mL has recently been characterized.[29] These issues are further complicated with the emergence of a hypervirulent strain of *C. difficile*, designated as North American pulsed-field gel electrophoresis type 1 (NAP1). Isolated from several outbreaks in the early 2000s, NAP1 produces more exotoxins due to a mutation in *tcdC*, which encodes a negative regulator of toxin production; exhibits higher resistance to several antibiotics with a particular emphasis on fluoroquinolones; forms spores at a higher frequency; and produces an additional binary toxin called CDT.[12] Thus, there is an urgent need to identify new antimicrobials that not only possess activity against *C. difficile* but also lower the rates of CDI recurrence and antibiotic resistance.

Historically, the ocean has been recognized as a potential resource for chemically diverse compounds as many novel marine natural products exhibiting antimicrobial activity against drug-resistant microbes have been identified.[30,31] We recently discovered that (+)-puupehenone, a meroterpenoid isolated from deep water marine sponges,[32,33] exhibited antimicrobial activity against multiple strains of *C. difficile*.[34] Based on that report, we created a chemical library of (+)-puupehenone derivatives with the intent of finding a more potent compound that could serve as a foundation for further investigation and structure−activity relationship studies.[35] We then challenged *C. difficile* and other bacterial species with this library to evaluate their effects on organisms implicated in HAIs. Additionally, knowing that fidaxomicin inhibits *C. difficile* toxin production,[36] we wanted to investigate if these

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compounds could modulate the expression of these toxins in a similar fashion. In this present study, we report the activity of (+)-puupehenone derivatives against *C. difficile* NAP1 and other Gram-positive bacteria. We also report that several derivatives with activity against NAP1 were able to substantially decrease toxin production in a concentration-dependent manner.

RESULTS AND DISCUSSION

We previously synthesized a library of 20 compounds to be screened for activity against several Gram-positive and Gram-negative bacteria.[35] The structures of these compounds are shown in Figure 1. Several of these compounds had promising activity against *C. difficile* and other Gram-positive bacteria, as seen in Table 1. Our (+)-puupehenone (compound **13**) exhibited an MIC of 2.0 μg/mL against *C. difficile* NAP1, which was more potent than a previous report where (+)-puupehenone obtained from a commercial supplier exhibited an MIC of 8.0 μg/mL against the same strain.[34] Against *Bacillus subtilis*, *Enterococcus faecalis*, and *S. aureus*, (+)-puupehenone curiously had no observable activity. By comparison, compound **1** was less potent against *C. difficile* at 4.0 μg/mL but inhibited *B. subtilis*, *E. faecalis*, and *S. aureus* at 3.9, 1.9, and 7.8 μg/mL, respectively. Interestingly, the addition of the methoxy group to compound **1** (subsequently generating compound **2**) rendered it inactive against all species except *B. subtilis*. Moreover, we observed that compounds **6** and **8** also possessed activity against all Gram-positive bacteria at varying degrees. Compound **6** inhibited *C. difficile* at 16.0 μg/mL and also inhibited *B. subtilis*, *E. faecalis*, and *S. aureus* at 7.9, 15.8, and 16.9 μg/mL, respectively; however, compound **8** was more potent against *C. difficile* at 8.0 μg/mL but exhibited similar MICs against *B. subtilis* (8.2 μg/mL), *E. faecalis* (16.2 μg/mL), and *S. aureus* (16.6 μg/mL). Compound **3** did not inhibit *C. difficile* or *E. faecalis* but did inhibit the growth of *B. subtilis* and *S. aureus* at an MIC of 33.2 μg/mL for both organisms. Oddly enough, while compound **4** is a structural isomer of compounds **3** and **8**, it did not exhibit any activity against our panel of organisms, demonstrating that the placement of groups on the aryl ring is critical for the activity of these compounds. The ester compounds **15** and **19** both inhibited *C. difficile* at 4.0 μg/mL, while only compound **19** exhibited an MIC of 22.23 μg/mL against *B. subtilis* and *E. faecalis*. The other ester compounds did not inhibit the growth of each organism and may be hindered by the steric bulk of the ester group. Finally, we observed that compound **20** had an MIC of 2.0 μg/mL against *C. difficile*, similar to (+)-puupehenone. None of these compounds were active against *E. coli* or *P. aeruginosa* when tested at concentrations up to 100 μM, which we found to be consistent with published *in vitro* activity against these two organisms.[32] These data support the idea that this library harbors a strict specificity for Gram-positive bacteria.

Table 1. MICs of Compounds against Gram-Positive Bacteria[a]

	MIC (μg/mL) for (+)-puupehenone derivatives against Gram-positive bacteria			
compounds	*C. difficile*	*B. subtilis*	*E. faecalis*	*S. aureus*
fidaxomicin	0.125	N/A	N/A	N/A
vancomycin	1	N/A	N/A	N/A
1	4.0	3.9	1.9	7.8
2	-	8.6	-	-
3	-	33.2	-	33.2
6	16.0	7.9	15.8	16.9
8	8.0	8.3	16.2	16.6
(+)-puupehenone (**13**)	2.0	-	-	-
15	4.0	-	-	-
19	4.0	22.2	22.2	-
20	2.0	-	-	-

[a]MICs for *B. subtilis*, *E. faecalis*, and *S. aureus* were determined visually by the REMA assay. The MICs for *C. difficile* were determined by a modified broth microdilution assay. *C. difficile* NAP1, *B. subtilis* ATCC 23857, *E. faecalis* ATCC 29212, and *S. aureus* ATCC 25923. (-) = no activity seen. N/A = not applicable.

Because the disease state of CDI is primarily due to the pathogen's toxins, we focused only on the subset of meroterpenoids that exhibited activity against *C. difficile* NAP1 and assessed each compound's ability to reduce toxin production in the organism. To do so, we sampled the spent medium of each NAP1 culture challenged with 0.5×, 0.25×, and 0.125× the MIC of each compound (sub-MICs) and immunoblotted for TcdA. To account for changes in biomass, we performed a semi-quantitative analysis of TcdA production by normalizing the target bands from western blots to the amount of total protein in each sample. We observed no significant difference in the amount of TcdA produced by NAP1 in the supplemented brain heart infusion (BHIS) medium and BHIS augmented with the compound vehicle, 5% dimethyl sulfoxide (DMSO) (Figure 2a).

For this experiment, we employed fidaxomicin (MIC = 0.125 μg/mL; Table 1) as a positive control and subsequently noted a substantial reduction in TcdA levels at all sub-MICs (Figure 2b), which is consistent with its reported toxin-suppressing activity.[36,37] Additionally, we were curious to see if vancomycin (MIC = 1 μg/mL; Table 1)—a CDI therapeutic that is not expected to modulate toxin production—would have any effect on TcdA levels in our assay. Treating vancomycin as a type of negative control, we repeated the experiment and were surprised to see a partial reduction in toxin levels at 1 μg/mL (Figure 2c). Despite this unexpected result, it must be noted that the effects of vancomycin on toxin levels are wildly different in several *C. difficile* strains according to previous reports.[36,38−41] Interestingly, we found that 1 μg/mL (+)-puupehenone, while inferior to fidaxomicin, was superior to vancomycin in reducing toxin production to the point where TcdA was undetectable (Figure 2d). Additionally, compounds **1** and **6** decreased the amount of toxin in a concentration-dependent manner (Figure 2e,f) while compounds **8**, **15**, and **19** did not significantly change toxin levels in comparison (Figure 2g−i). At low sub-MICs, however, compounds **1** (0.5 and 1 μg/mL) and **8** (1 and 2 μg/mL) elicited an increase in toxin production (Figure 2e,g), suggesting a complex dose-dependent regulatory mechanism. Finally, while also inferior to fidaxomicin, compound **20** was observed to substantially reduce toxin levels in a similar manner, but this effect did not significantly change at increased doses (Figure 2j). These results strongly indicate that certain chemical modifications are important for these compounds to specifically target this virulence mechanism.

The data generated from this compound library suggest that several of these meroterpenoids, such as compound **1**, show promise as potential antimicrobials for HAIs caused by certain Gram-positive pathogens, like *E. faecalis* and MRSA. Addition-

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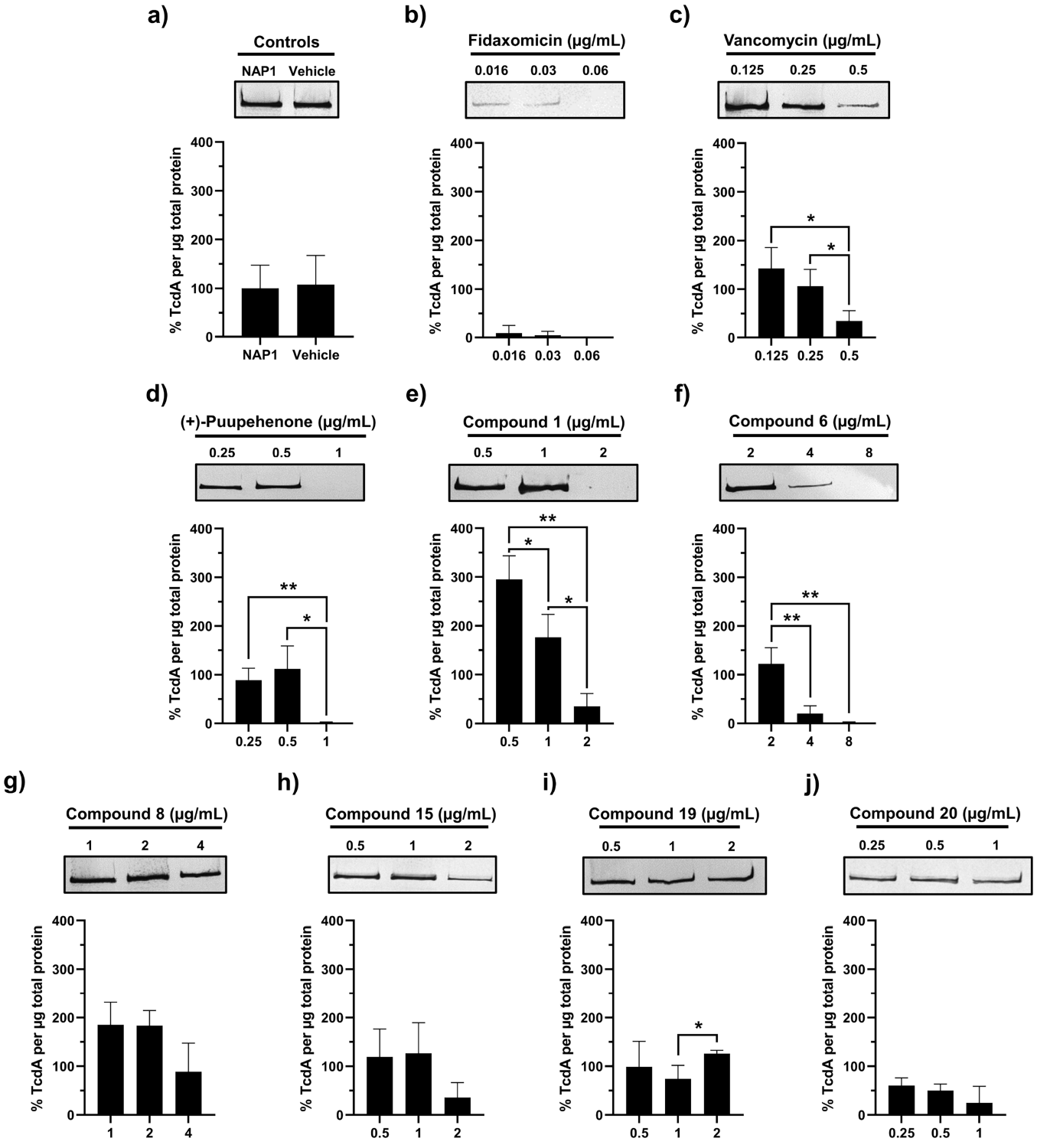

Figure 2. (+)-Puupehenone and several derivatives reduce toxin production in *C. difficile* NAP1. After a 48 h incubation of NAP1 challenged with several dilutions of each compound, extracellular toxin in spent media derived from triplicate cultures was assessed with western blots using a monoclonal antibody against TcdA. Semi-quantitative analysis of toxin levels was performed via densitometry of TcdA band intensity with respect to total protein as measured by the Bradford assay. Semi-quantitative plots are shown below each representative blot. (a) Toxin production in BHIS (NAP1) and BHIS with 5% DMSO (vehicle). (b) Toxin production from cultures challenged with sub-MICs of fidaxomicin, (c) vancomycin, and (d–j) (+)-puupehenone and selected derivatives. Data points represent the means of adjusted toxin levels derived from triplicate cultures, while error bars represent standard deviations. Statistical analysis was performed in GraphPad Prism 8 using unpaired *t*-tests. $*P \leq 0.05$; $**P \leq 0.01$.

ally, since (+)-puupehenone and some of its derivatives, such as compound **20**, were successful in reducing NAP1 toxin production *in vitro*, these meroterpenoids may also have the potential for further development as therapeutics for CDI. Development will surely benefit from future experiments centered on uncovering the mechanism of action of these compounds. Specifically, assessment of gene expression at sub-MICs via transcriptomics and sequencing of mutants resistant

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to certain derivatives will help achieve this goal. Additionally, since spore formation is a core process in *C. difficile* transmission and pathogenesis, it would be beneficial to determine if these compounds possess any sporicidal activity or if they at least modulate processes such as sporulation and germination.

METHODS

Bacterial Growth Conditions. *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 29212), *B. subtilis* (ATCC 23857), and *E. coli* (ATCC 25922) were obtained from American Type Culture Collection (ATCC). Frozen stocks of *P. aeruginosa*, *S. aureus*, *E. faecalis*, and *B. subtilis* were grown in tryptic soy broth (TSB) at 37 °C for 12−24 h until the mid-exponential phase, which corresponded to an optical density at 600 nm (OD_{600}) of 0.6. Frozen stocks of *E. coli* were cultured in Super Optimal broth with catabolite repression (SOC) medium at 37 °C for 12 h until the mid-exponential phase (OD_{600} of 0.6).[42] All OD_{600} readings were recorded with a Laxco MicroSpek DSM Cell Density Meter. All cultures were then diluted 1000-fold into their respective media to prepare inocula.

For all studies regarding *C. difficile*, we used a NAP1 strain isolated from several outbreaks.[43] Anaerobic conditions were defined by maintaining an atmosphere of 1.0% H_2, 5% CO_2, and >90% N_2 in a Coy anaerobic chamber. NAP1 was routinely grown in BHIS broth: 37 g/L brain heart infusion, 5 g/L yeast extract, and 0.1% (w/v) L-cysteine.[44]

MIC Determination. The MICs of test compounds and ampicillin (Acros Organics) were determined by broth microdilution and the resazurin microtiter assay (REMA). Freshly grown cultures of *P. aeruginosa*, *S. aureus*, *B. subtilis*, *E. faecalis*, and *E. coli* were used as inocula at 1000-fold dilutions in TSB and SOC media, respectively. After a 24 h incubation of each organism challenged with two-fold serial dilutions of each compound, the MIC was scored as the lowest concentration where no growth was observed. The plates were then stained with resazurin stock solution added to each well, incubated for 4−5 h, and observed for color change from blue to pink. The MIC was scored at the lowest concentration that retained its blue color. The assay was repeated in triplicate.

Antimicrobial susceptibility tests (ASTs) of NAP1 were performed with a modified broth microdilution procedure as per the Clinical and Laboratory Standards Institute (CLSI) standard M11.[45] Briefly, 96-well assay plates were pre-loaded with 95 μL of BHIS and 5 μL of 20× test compounds to achieve a final concentration range of 0.0625−16 μg/mL in 2-fold increments. Test compounds were serially diluted in 100% DMSO with the exception of vancomycin hydrochloride (Gold Biotechnology) which was dissolved and diluted in deionized water. Fidaxomicin (APExBIO Technology) was similarly diluted in 100% DMSO to achieve a final concentration range of 0.008−1 μg/mL. Assay plates were stored in the anaerobic chamber at room temperature to reduce overnight. A single colony of NAP1 was grown overnight in BHIS at 37 °C. The overnight culture was initially diluted the next day with pre-reduced saline (0.85% NaCl) to match the turbidity of a 0.5 McFarland standard. The inoculum was finally prepared with a subsequent 15-fold dilution in saline. Pre-reduced assay plates were inoculated with 10 μL of diluted cell suspension, stored in a half-sealed plastic bag to prevent evaporation, and incubated at 37 °C for 48 h. After incubation, growth in each well was measured by reading the OD_{600} using a BioTek Epoch 2 plate reader. The assay was performed in triplicate.

Toxin Analysis. Toxin production was determined by analyzing the amount of extracellular toxin in triplicate cultures from the ASTs described above. At 48 h, the total protein of each culture was determined with the Bradford assay using bovine serum albumin (BSA) as a standard.[46] Cultures were then centrifuged at 5000*g* for 5 min to clear the supernatants, which were separately collected and frozen at −20 °C. After thawing at room temperature, cell-free supernatants were mixed 1:1 with 2× Laemmli buffer and incubated in a sand bath at 100 °C for 5 min. Twenty microliters of denatured samples was loaded onto 7.5% Tris-glycine gels and electrophoresed at 200 V for 1 h. After electrophoresis, samples were transferred to polyvinylidene difluoride membranes at 4 °C overnight at 30 V. Membranes were incubated in a blocking buffer (20 mM Tris−HCl, 150 mM NaCl, 0.01 mM ethylenediaminetetraacetic acid, 0.1% Tween 20, 1% BSA, [pH 7.5]) for 1 h at room temperature. TcdA was detected with a monoclonal mouse anti-TcdA antibody (PCG4.1, Novus Biologicals) and a rabbit anti-mouse IgG antibody conjugated with alkaline phosphatase. Blots were visualized with a ChemiDoc XRS+ imaging system (Bio-Rad).

Semi-quantitative analysis of TcdA was performed using Image Lab 6.0 software (Bio-Rad). Briefly, after subtracting background noise in each blot, the peak corresponding to the intensity of the TcdA band (arbitrary units) was selected with the software's Lane Profile tool. The peak density was divided by the total protein amount of each respective sample, resulting in an adjusted toxin level with respect to apparent biomass. Adjusted values of triplicate cultures were averaged together and converted to a percentage using the Normalize tool in GraphPad Prism 8. To define 100%, the TcdA value from NAP1 in BHIS (5% DMSO) was chosen for fidaxomicin and all test compounds. For vancomycin, the TcdA value from NAP1 in BHIS was defined as 100%. In both cases, 0 was defined as 0%. During our investigation, we observed that the MICs of these compounds against *C. difficile* occasionally increased by 2-fold on different days. This variable activity prevented us from performing statistical analysis from multiple experiments as the sub-MICs were not always the same. Since there were no changes to the methodology described, several other reasons could have accounted for decreased activity, such as compound stability, freeze−thaw, and adherence to plastic. Nevertheless, the toxin results were reproducible, irrespective of the exact sub-MICs on different days.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04471.

Table of available CAS registry numbers for compounds **1**−**20** (PDF)

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Author Contributions

M.A.J., A.D.L., W.T.S., and S.J.S. wrote or edited the manuscript. M.A.J. performed the ASTs with *C. difficile* and immunoblots of TcdA. A.D.L. and A.S. synthesized the compounds. A.D.L. performed the MIC determinations for *P. aeruginosa*, *S. aureus*, *B. subtilis*, *E. faecalis*, and *E. coli*.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Haque, M.; Sartelli, M.; McKimm, J.; Abu Bakar, M. Health care-associated infections - an overview. *Infect Drug Resist.* **2018**, *11*, 2321−2333.

(2) Stone, P. W. Economic burden of healthcare-associated infections: an American perspective. *Expert Rev. Pharmacoecon. Outcomes Res.* **2009**, *9*, 417−422.

(3) Forrester, J. D.; Maggio, P. M.; Tennakoon, L. Cost of health care-associated infections in the United States. *J. Patient Saf.* **2021**, *18*, e477−e479.

(4) Magill, S. S.; O'Leary, E.; Janelle, S. J.; Thompson, D. L.; Dumyati, G.; Nadle, J.; Wilson, L. E.; Kainer, M. A.; Lynfield, R.; Greissman, S.; et al. Changes in prevalence of health care-associated infections in U.S. Hospitals. *N. Engl. J. Med.* **2018**, *379*, 1732−1744.

(5) CDC. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.

(6) Hall, I. C.; O'Toole, E. Intestinal flora in new-born infants: with a description of a new pathogenic anaerobe, *Bacillus difficilis*. *Am. J. Dis. Child.* **1935**, *49*, 390−402.

(7) Barbut, F.; Petit, J. C. Epidemiology of *Clostridium difficile*-associated infections. *Clin. Microbiol. Infect.* **2001**, *7*, 405−410.

(8) Bartlett, J. G.; Gerding, D. N. Clinical recognition and diagnosis of *Clostridium difficile* infection. *Clin. Infect. Dis.* **2008**, *46*, S12−S18.

(9) Rupnik, M.; Wilcox, M. H.; Gerding, D. N. *Clostridium difficile* infection: New developments in epidemiology and pathogenesis. *Nat. Rev. Microbiol.* **2009**, *7*, 526−536.

(10) Voth, D. E.; Ballard, J. D. *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin. Microbiol. Rev.* **2005**, *18*, 247−263.

(11) Di Bella, S.; Ascenzi, P.; Siarakas, S.; Petrosillo, N.; di Masi, A. *Clostridium difficile* Toxins A and B: Insights into pathogenic properties and extraintestinal effects. *Toxins* **2016**, *8*, 134.

(12) Fatima, R.; Aziz, M. The hypervirulent dtrain of *Clostridium difficile*: NAP1/B1/027 - A brief overview. *Cureus* **2019**, *11*, No. e3977.

(13) Majumdar, A.; Govind, R. Regulation of *Clostridioides difficile* toxin production. *Curr. Opin. Microbiol.* **2022**, *65*, 95−100.

(14) Kordus, S. L.; Thomas, A. K.; Lacy, D. B. *Clostridioides difficile* toxins: mechanisms of action and antitoxin therapeutics. *Nat. Rev. Microbiol.* **2022**, *20*, 285−298.

(15) Aktories, K. Another surprise in receptor binding of *C. difficile* toxins. *Innovation* **2022**, *3*, No. e100261.

(16) Kelly, C. P.; LaMont, J. T. *Clostridium difficile*–more difficult than ever. *N. Engl. J. Med.* **2008**, *359*, 1932−1940.

(17) Marsh, J. W.; Arora, R.; Schlackman, J. L.; Shutt, K. A.; Curry, S. R.; Harrison, L. H. Association of relapse of *Clostridium difficile* disease with BI/NAP1/027. *J. Clin. Microbiol.* **2012**, *50*, 4078−4082.

(18) McDonald, L. C.; Gerding, D. N.; Johnson, S.; Bakken, J. S.; Carroll, K. C.; Coffin, S. E.; Dubberke, E. R.; Garey, K. W.; Gould, C. V.; Kelly, C.; et al. Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin. Infect. Dis.* **2018**, *66*, 987−994.

(19) Gupta, A.; Cifu, A. S.; Khanna, S. Diagnosis and treatment of *Clostridium difficile* infection. *JAMA* **2018**, *320*, 1031−1032.

(20) Guery, B.; Galperine, T.; Barbut, F. *Clostridioides difficile*: diagnosis and treatments. *BMJ* **2019**, *366*, No. el4609.

(21) van Prehn, J.; Reigadas, E.; Vogelzang, E. H.; Bouza, E.; Hristea, A.; Guery, B.; Krutova, M.; Noren, T.; Allerberger, F.; Coia, J. E.; et al. European Society of Clinical Microbiology and Infectious Diseases: 2021 update on the treatment guidance document for *Clostridioides difficile* infection in adults. *Clin. Microbiol. Infect.* **2021**, *27*, S1−S21.

(22) Vardakas, K. Z.; Polyzos, K. A.; Patouni, K.; Rafailidis, P. I.; Samonis, G.; Falagas, M. E. Treatment failure and recurrence of *Clostridium difficile* infection following treatment with vancomycin or metronidazole: a systematic review of the evidence. *Int. J. Antimicrob. Agents* **2012**, *40*, 1−8.

(23) Dureja, C.; Olaitan, A. O.; Hurdle, J. G. Mechanisms and impact of antimicrobial resistance in *Clostridioides difficile*. *Curr. Opin. Microbiol.* **2022**, *66*, 63−72.

(24) Louie, T. J.; Miller, M. A.; Mullane, K. M.; Weiss, K.; Lentnek, A.; Golan, Y.; Gorbach, S.; Sears, P.; Shue, Y. K.; Group, O. P. T. C. S. Fidaxomicin versus vancomycin for *Clostridium difficile* infection. *N. Engl. J. Med.* **2011**, *364*, 422−431.

(25) Cornely, O. A.; Miller, M. A.; Louie, T. J.; Crook, D. W.; Gorbach, S. L. Treatment of first recurrence of *Clostridium difficile* infection: Fidaxomicin versus vancomycin. *Clin. Infect. Dis.* **2012**, *55 Suppl 2*, S154−S161.

(26) Swanson, R. N.; Hardy, D. J.; Shipkowitz, N. L.; Hanson, C. W.; Ramer, N. C.; Fernandes, P. B.; Clement, J. J. In vitro and in vivo evaluation of tiacumicins B and C against *Clostridium difficile*. *Antimicrob. Agents Chemother.* **1991**, *35*, 1108−1111.

(27) Golan, Y.; Epstein, L. Safety and efficacy of fidaxomicin in the treatment of *Clostridium difficile*-associated diarrhea. *Ther. Adv. Gastroenterol.* **2012**, *5*, 395−402.

(28) Sholeh, M.; Krutova, M.; Forouzesh, M.; Mironov, S.; Sadeghifard, N.; Molaeipour, L.; Maleki, A.; Kouhsari, E. Antimicrobial resistance in *Clostridioides (Clostridium) difficile* derived from humans: A systematic review and meta-analysis. *Antimicrob. Resist. Infect. Control* **2020**, *9*, 158.

(29) Schwanbeck, J.; Riedel, T.; Laukien, F.; Schober, I.; Oehmig, I.; Zimmermann, O.; Overmann, J.; Gross, U.; Zautner, A. E.; Bohne, W. Characterization of a clinical *Clostridioides difficile* isolate with markedly reduced fidaxomicin susceptibility and a V1143D mutation in *rpoB*. *J. Antimicrob. Chemother.* **2019**, *74*, 6−10.
(30) de Vries, D. J.; Beart, P. M. Fishing for drugs from the sea: status and strategies. *Trends Pharmacol. Sci.* **1995**, *16*, 275−279.
(31) Barbosa, F.; Pinto, E.; Kijjoa, A.; Pinto, M.; Sousa, E. Targeting antimicrobial drug resistance with marine natural products. *Int. J. Antimicrob. Agents* **2020**, *56*, No. 106005.
(32) Ravi, B. N.; Perzanowski, H. P.; Ross, R. A.; Erdman, T. R.; Scheuer, P. J.; Finer, J.; Clardy, J. Recent research in marine natural products: The puupehenones. *Pure Appl. Chem.* **1979**, *51*, 1893−1900.
(33) Kohmoto, S.; McConnell, O. J.; Wright, A.; Koehn, F.; Thompson, W.; Lui, M.; Snader, K. M. Puupehenone, a cytotoxic metabolite from a deep water marine sponge, Stronglyophora hartmani. *J. Nat. Prod.* **1987**, *50*, 336.
(34) Self, W. T. Antimicrobial compositions comprising puupehenone for the treatment of *Clostridium difficile* infection. US 20,180,325,863 A1, 2018.
(35) Si, A.; Landgraf, A. D.; Geden, S.; Sucheck, S. J.; Rohde, K. H. Synthesis and evaluation of marine natural product-inspired meroterpenoids with selective activity toward dormant *Mycobacterium tuberculosis*. *ACS Omega* **2022**, *7*, 23487−23496.
(36) Babakhani, F.; Bouillaut, L.; Sears, P.; Sims, C.; Gomez, A.; Sonenshein, A. L. Fidaxomicin inhibits toxin production in *Clostridium difficile*. *J. Antimicrob. Chemother.* **2013**, *68*, 515−522.
(37) Aldape, M. J.; Packham, A. E.; Heeney, D. D.; Rice, S. N.; Bryant, A. E.; Stevens, D. L. Fidaxomicin reduces early toxin A and B production and sporulation in *Clostridium difficile* in vitro. *J. Med. Microbiol.* **2017**, *66*, 1393−1399.
(38) Onderdonk, A. B.; Lowe, B. R.; Bartlett, J. G. Effect of environmental stress on *Clostridium difficile* toxin levels during continuous cultivation. *Appl. Environ. Microbiol.* **1979**, *38*, 637−641.
(39) Drummond, L. J.; Smith, D. G. E.; Poxton, I. R. Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by *Clostridium difficile*. *J. Med. Microbiol.* **2003**, *52*, 1033−1038.
(40) Gerber, M.; Walch, C.; Loffler, B.; Tischendorf, K.; Reischl, U.; Ackermann, G. Effect of sub-MIC concentrations of metronidazole, vancomycin, clindamycin and linezolid on toxin gene transcription and production in *Clostridium difficile*. *J. Med. Microbiol.* **2008**, *57*, 776−783.
(41) Zarandi, E. R.; Mansouri, S.; Nakhaee, N.; Sarafzadeh, F.; Moradi, M. Toxin production of *Clostridium difficile* in sub-MIC of vancomycin and clindamycin alone and in combination with ceftazidime. *Microb. Pathog.* **2017**, *107*, 249−253.
(42) Hanahan, D. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **1983**, *166*, 557−580.
(43) Warny, M.; Pepin, J.; Fang, A.; Killgore, G.; Thompson, A.; Brazier, J.; Frost, E.; McDonald, L. C. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* **2005**, *366*, 1079−1084.
(44) Sorg, J. A.; Dineen, S. S. Laboratory maintenance of *Clostridium difficile*. *Curr. Protoc. Microbiol.* **2009**, 9A 1.
(45) CLSI. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria. 9th ed. CLSI standard M11*. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.
(46) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

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