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Structure–Activity Studies of Divin: An Inhibitor of Bacterial Cell Division

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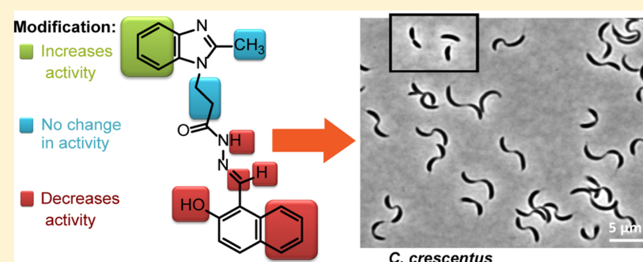
Supporting Information

ABSTRACT: We describe the synthesis and structure–activity relationship (SAR) studies of divin, a small molecule that blocks bacterial division by perturbing the assembly of proteins at the site of cell septation. The bacteriostatic mechanism of action of divin is distinct from other reported inhibitors of bacterial cell division and provides an opportunity for assessing the therapeutic value of a new class of antimicrobial agents. We demonstrate a convenient synthetic route to divin and its analogues, and describe compounds with a 10-fold increase in solubility and a 4-fold improvement in potency. Divin analogues produce a phenotype that is identical to divin, suggesting that their biological activity comes from a similar mechanism of action. Our studies indicate that the 2-hydroxynaphthalenyl hydrazide portion of divin is essential for its activity and that alterations and substitution to the benzimidazole ring can increase its potency. The SAR study provides a critical opportunity to isolate drug resistant mutants and synthesize photoaffinity probes to determine the cellular target and biomolecular mechanism of divin.



KEYWORDS: *Divin, antimicrobial, synthesis, SAR*

The development of new antimicrobial agents to combat the emergence of multidrug resistance among clinical pathogens continues to be a challenge.[1] One approach to antimicrobial agent discovery centers upon developing compounds that bind specifically to a cellular target (e.g., DNA gyrase).[2,3] Another approach that has been gaining traction recently is the identification of compounds that have potent bactericidal or bacteriostatic activity through phenotypic assays/screens, without considering specific cellular targets *per se*.[4,5] Membrane-targeting antibiotics represent one class of compounds that have emerged from this approach.[6−8]

We recently introduced a new compound, divin (**1**), that was discovered using the second track of antibiotic development mentioned above.[9] Divin inhibits late stages of bacterial cell division and produces mother and daughter cells that remain physically fused together and share a common cytoplasm.[9] Peptidoglycan insertion at the septum is blocked in divin-treated cells, and the result is a bacteriostatic effect in Gram-negative and Gram-positive bacteria. In our initial studies, the low solubility of **1** in culture media prevented us from isolating, characterizing, and deep sequencing spontaneous mutants that displayed reduced susceptibility to **1**. Rather than focusing on the identification of the cellular target, we studied how divin altered the spatial and temporal localization of division proteins at the divisome and found that four late-stage division proteins (i.e., FtsQ, FtsL, FtsW, and FtsB) were mislocalized from the division plane. Unlike the classes of molecules that have been reported to bind FtsZ and inhibit division,[10,11] divin inhibits assembly of the functional divisome without directly affecting FtsZ.[9] The unique interaction between **1** and the bacterial divisome makes it a useful chemical probe for studying the dynamics of bacterial division;[11] however, its low solubility and modest activity limits its application and prevents the identification of its target.

In this letter, we lay the groundwork for identifying the target of **1** by performing a structure–activity relationship (SAR) study. We demonstrate a convenient synthetic route to divin analogues and describe compounds with improved solubility and potency. Divin analogues with enhanced solubility will enable us to isolate drug resistant mutants and use genomics to identify the drug target. Alternatively, synthesizing a focused library of analogues has guided us in the design of a photoaffinity probe to identify the molecular target of divin using a pull-down approach.[9] These studies lay the groundwork for identifying the molecular target of divin and the development of therapeutic agents for treating clinical infections.

Performing SAR studies required that we develop a flexible synthetic strategy to manipulate elements of **1** while enabling maximum diversification of the structure. We developed a synthetic approach centered upon a sequential, three component coupling of the benzimidazole, the linker, and the

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2-hydroxy-1-naphthaldehyde components of divin (Figure 1).[12,13] Each of the three components could be modified independently before they were coupled to produce a range of analogues for characterization and biological testing.

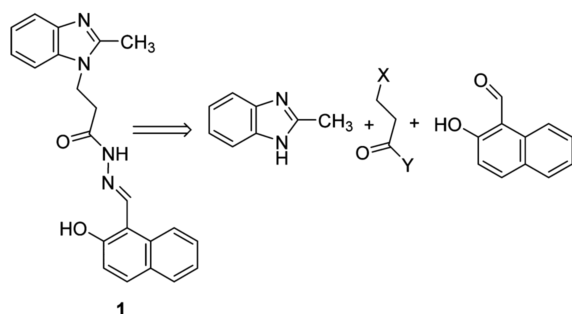
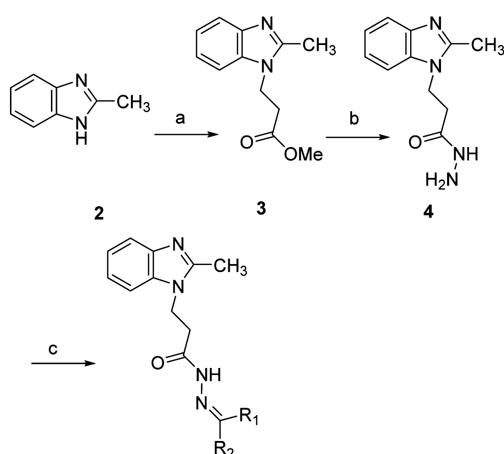


Figure 1. Retrosynthetic strategy for the synthesis of divin and its analogues.

To prepare analogues with variations in the bottom region of the compound, we reacted 2-methyl-1*H*-benzimidazole **2** with methyl 3-bromopropionate in the presence of anhydrous K_2CO_3 to prepare ester **3** in 96% yield (Scheme 1). We synthesized the key intermediate 3-(2-methyl-1*H*-benzimidazol-1-yl) propanohydrazide **4** by hydrazinolysis of **3**. Compounds **1** and **5a−j** were prepared by the condensation of **4** and corresponding aldehydes with yields ranging from 32% to 93% at a scale of 0.1−0.5 mmol.

Scheme 1. Synthesis of Divin Analogues by Varying the Structure of the Naphthalenyl Moiety[a]

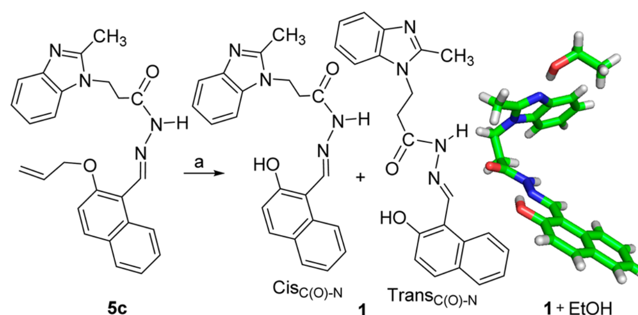


5a: R1 = H, R_2 = 2-hydroxy-1-phenyl (75%)
5b: R1 = H, R_2 = 2-methoxy-1-naphthalenyl (82%)
5c: R1 = H, R_2 = 2-allyloxy-1-naphthalenyl(90%)
5d: R1 = H, R_2 = 1-naphthalenyl (58%)
5e: R1 = H, R_2 = 4-hydroxy-1-naphthalenyl (90%)
5f: R1 = H, R_2 = 4-dimethylamino-1-naphthalenyl (81%)
5g: R1 = H, R_2 = 4-fluoro-1-naphthalenyl (32%)
5h: R1 = H, R_2 = 2-fluoro-1-phenyl (36%)
5I: R1 = H, R_2 = 2, 6-dihydroxy-1-naphthalenyl (93%)
5J: R1 = CH_3, R_2 = 2-hydroxy-1-naphthalenyl (79%)
1: R1 = H, R_2 = 2-hydroxy-1-naphthalenyl (79%)

[a]Reagents and conditions: (a) methyl 3-bromopropionate, K_2CO_3, DMF, 85 °C, 96%; (b) hydrazine, MeOH, 65 °C, 85%; (c) aldehyde, AcOH, EtOH, reflux, 79%.

It is well-known that *N*-acylhydrazones may exist as geometrical isomers (*E*/*Z*) with respect to the imine bonds and as conformational isomers (cis/trans) around the amide N−C(O) bond.[14,15] In our study, we obtained a crystal structure of **1**·EtOH (Scheme 2), which demonstrated that in the solid state the imine of **1** exists in the *E* configuration and the N−C(O) existed as trans rotamers (C═O anti to the NH). In solution, the N−C(O) group of **1** interconverts between cis and trans rotamers (Scheme 2). In the ^{1}H NMR spectra of **1**, we observed two sets of nuclei resonances for N═CH, $-CH_2-$C(O), and some of the protons in the benzylidene moiety. The signals belonging to N═CH and $-CH_2-$C(O) protons were observed at 8.89 (trans), 8.80 (cis) ppm and 2.72 (trans), 3.15 (cis) ppm, respectively. The ^{13}C NMR spectra of **1** indicated two sets of resonances for most of the carbon nuclei. We assigned the two rotamers based on the ^{1}H downfield shift (0.43 ppm) of $-CH_2C(O)-$ in the cis-isomer (C═O syn to the NH) as compared with trans-isomer and ^{13}C downfield shift of carbonyl carbon of the cis-isomer (170.71 ppm) relative to the trans-isomer (C═O anti to the NH, 165.95 ppm), which arises due to the reduced contribution of the conjugative form N^+═C−O^- present in the cis-isomer.

Scheme 2. Alternative Synthesis of 1 and Conformational Isomerization[a]



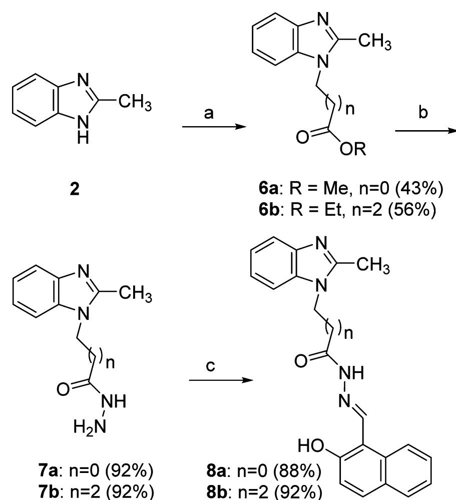
[a]Reagents and conditions: (a) $Pd(PPh_3)_4/PhSiH_3$, 98%.

The two rotamers equilibrated thermally in solution and the relative percentage varied depending on the polarity of the solvent. We determined the ratio of the two rotamers by ^{1}H NMR, as they interconvert too fast to be differentiated by reverse phase HPLC analysis (Figure S3, Supporting Information). As the polarity of the solvent increased, we observed that the percentage of the $trans_{C(O)\text{-}N}$ isomer increased. On the basis of the integrated ratio of the relevant peaks of **1** in the ^{1}H NMR spectrum, 60% of the compound was in the trans-isomer at 25 °C in $CDCl_3/CD_3OD$ (95/5%), 74% in DMSO-d_6 (neat), and 83% in D_2O/DMSO-*d6* (60/40%).

The isomerization of the other analogues in solution depended on their structures. In general, analogues containing a C-2-naphthalenyl or phenyl hydroxyl group (i.e., **5a** and **5i**) had an isomerization pattern that was similar to **1** and consisted of a mixture of $trans_{C(O)\text{-}N}$ and $cis_{C(O)\text{-}N}$ isomers in which the trans-isomer was the major product. Analogues lacking a C-2 naphthalenyl or a free hydroxyl group (i.e., **5b**-**5i**) primarily existed as the $cis_{C(O)\text{-}N}$ isomer in solution. Analogue **5c** (Scheme 2) contained an allyl-protected hydroxyl group. In $CDCl_3/CD_3OD$ (95/5%), **5c** primarily existed in the cis-isomer (84%) according to ^{1}H NMR measurements. We removed the allyl protecting group in **5c** using $Pd(PPh_3)_4/PhSiH_3$ and produced **1** in which the trans-isomer was the major product.

We selected two linkers, one lacking a methylene group and the other containing an additional methylene group, to explore the relationship between the length of the linker and the activity of analogues (Scheme 3). Specifically, we reacted 2-methyl-1*H*-benzimidazole (**2**) with methyl 3-bromoacetate or ethyl 4-bromobutyrate in the presence of K_2CO_3 to yield the corresponding ester **6a** and **6b** in 43% and 56% yield, respectively. After hydrazinolysis of **6a** and **6b** using hydrazine hydrate, we reacted the resulting hydrazides **7a** and **7b** with 2-hydroxy-1-naphthaldehyde to form the analogues **8a** and **8b** in 88% and 92% yields, respectively.

Scheme 3. Synthesis of Divin Analogues Containing Different Linker Lengths[a]



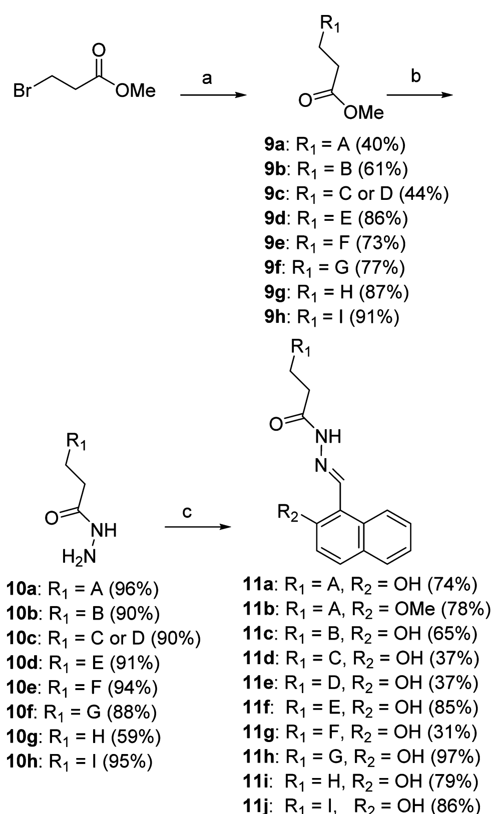
[a]Reagents and conditions: (a) methyl bromoacetate or ethyl 4-bromobutyrate, K_2CO_3, DMF, 85 °C; (b) hydrazine, MeOH, 65 °C; (c) aldehyde, AcOH, EtOH, reflux.

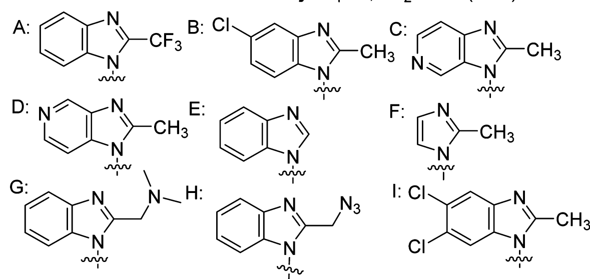
To efficiently prepare divin analogues containing variations in the top region of the molecule, the benzimidazole moiety, we initially attempted to couple the linker to 2-methoxy-1-naphthaldehyde, followed by alkylation of the product with 2-methyl-1*H*-benzimidazole. This strategy was unsuccessful, and we used the above 3-step sequence to prepare analogues containing variations to the benzimidazole region of **1**. We alkylated imidazoles (A-I) with methyl 3-bromopropionate to afford the corresponding esters **9a–h** in 40–91% yields (Scheme 4). Hydrazinolysis of **9a–h** using hydrazine hydrate provided **10a–h** in 59–96% yield. We prepared analogues **11a–j** by condensing **10a–h** with 2-hydroxy-1-naphthaldehyde.

Compounds **9b**, **9c**, **10b**, and **10c** were inseparable mixtures of C-5 and C-6 substituted regioisomers arising from N^1/N^3 competitive alkylation.[16] From these intermediates, we synthesized analogue **11c** as a mixture of regioisomers. Four sets of signals in the ^1H and ^{13}C NMR spectra indicated that **11c** existed as both C-5/C-6 regio- and C(O)–N trans/cis isomers. As expected, the C-5/C-6 dichloro-substituted analogue **11j** produced a single regioisomer. The two regiosiomers **11d** and **11e** that we produced from **10c** were separable by flash chromatography. All of the other analogues existed as a mixture of $trans_{C(O)-N}$ and $cis_{C(O)-N}$ rotamers in solution that were similar to **1**.

Condensing **4** with 2-acetyloxy-1-naphthaldehyde yielded **1**, presumably due to the instability of C-2 phenol ester. To acylate the phenol group of **1**, we treated **1** with Ac_2O in the presence of pyridine at 25 °C, which yielded diester **12** in 45% yield (Scheme 5). We also reduced **1** to the corresponding amine **13** in 43% yield.

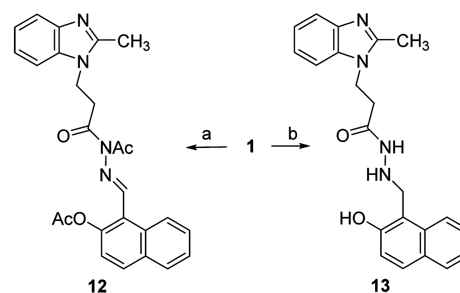
Scheme 4. Synthesis of Divin Analogues by Varying the Benzimidazole Moiety[a]





[a]Reagents and conditions: (a) benzimidazoles, K_2CO_3, DMF, 85 °C; (b) hydrazine, MeOH, 65 °C; (c) aldehyde, AcOH, EtOH, reflux.

Scheme 5. Other Modifications to the Structure of Divin[a]



[a]Reagents and conditions: (a) Ac_2O/Pyr., 45%; (b) $NaCNBH_3$, MeOH, 43%.

Treating Gram-negative infections can be difficult as the outer membrane reduces the bioavailability of antibiotics. We hence decided to screen analogues of **1** against Gram-negative bacterial strains. We determined the minimum inhibitory concentration (MIC) of each analogue against the model Gram-negative bacteria *Caulobacter crescentus* CB15N (wild type) and *Escherichia coli* BW25113 Δ*tolC* using a CLSI protocol based on serial dilution;[17,18] *E. coli* BW25113 Δ*tolC* lacks a central component of one family of efflux drug pumps, and the genetic modification increases the susceptibility of cells to antibiotics. The results are listed in Table 1.

Table 1. MIC of Divin Analogues against *C. crescentus* CB15N (Wild Type) and *E. coli* BW25113 Δ*tolC* Cells; MIC Values are Reported in μM

	MIC			MIC	
compd	CR[a]	EC[b]	compd	CR[a]	EC[b]
1	5	12.5	**11a**	7.5	>50
5a	15	50	**11b**	>20	>50
5b	>20	>50	**11c**	5	7.5
5c	>20	>50	**11d**	10	25
5d	>20	50	**11e**	5	12.5
5e	>20	>50	**11f**	7.5	12.5
5f	>20	>50	**11g**	7.5	12.5
5g	>20	>50	**11h**	5	12.5
5h	>20	>50	**11i**	5	12.5
5i	5	12.5	**11j**	3.8	6.3
5j	>20	>50	**12**	>40	50
8a	5	25	**13**	40	50
8b	5	10			

[a] *C. crescentus* CB15N cells. [b] *E. coli* BW25113 Δ*tolC* cells.

We found that removing, alkylating, or acylating the phenyl hydroxyl group of divin reduced the activity of analogues (i.e., **5d**, **5b**, **5c**, and **12**), thereby indicating the importance of this moiety on the activity of the compound. Changing the position of the phenyl hydroxyl to the C-4 position (i.e., **5e**) or replacing the C-4 hydroxyl group with a dimethylamino or fluoro group (i.e., **5f** and **5g**) dramatically reduced the biological activity. The second aromatic ring of the naphthalenyl moiety was essential for the activity of compounds. Removing the second aromatic ring resulted in inactive analogues (i.e., **5a** and **5h**). Adding a hydroxyl group to the second ring (i.e., **5i**) did not affect the activity.

We found that the imine bond was essential for the activity of **1**. Reducing the imine group (i.e., **13**) increased the MIC. The hydrogen on the imine group was required for activity, as replacing it with a methyl group (i.e., **5j**) produced inactive analogues. We found that the length of the alkyl linker is not important for the activity of **1**; analogues with a shorter (**8a**) or longer linker (**8b**) had the same MIC as **1** against *C. crescentus* CB15N. Analogue **8b** was slightly more active against *E. coli* BW25113 Δ*tolC* than **1**, while **8a** was ~2-fold less active than **1**.

The benzamidazole ring was the region of divin that was most tolerant to modification; a range of alterations to the benzamidazole group led to small or no changes in the MIC of the corresponding analogues. The methyl group on the benzimidazole ring was not critical to the activity of **1**. We found that removing or substituting the methyl group with CF_3, $CH_2N(Me)_2$, or CH_2N_3 (i.e., **11f**, **11a**, **11h**, and **11i**) did not change the MIC significantly; the only exception was the 3-fold reduction in MIC of the CF_3 analogue **11a** against *E. coli* BW25113 Δ*tolC*. Analogue **11a** had an MIC that was nearly identical to **1** against *C. crescentus* CB15N. The benzene ring in the benzimidazole group was not essential, as removing it (i.e., **11g**) did not change the MIC significantly. Introducing new substituents on the benzene ring improved the MIC of analogue (i.e., **11c**, **11e**, and **11j**) against *E. coli* BW25113 Δ*tolC*; the alterations did not change the activity against *C. crescentus* CB15N. The dichloro analogue **11j** had a MIC against *E. coli* BW25113 Δ*tolC* that was 2-fold lower than **1**.

A key goal of this study was to introduce structural modifications that improved the solubility of **1** without affecting its activity. We determined the solubility of a set of divin analogues in M8 media (with 2% DMSO) using a light scattering assay.[19,20] M8 is the media we used to study the activity of divin and analogues against *E. coli BW25113* Δ*tolC* cells and a serial of clinic pathogenic strains of bacteria. The results indicated that some modifications led to large improvements in solubility while maintaining the biological activity of the analogues (Table 2). Compound **8b** contained a linker that was longer than **1**, and the analogue was 6 times more soluble than **1** in M8 media. Introducing a chloride into the benzimidazole ring (**11c**) increased the solubility 10-fold compared to **1**.

Table 2. Solubility of Active Divin Analogues in M8 Media (with 2% DMSO); Solubility is Expressed as μM

compd	solubility	compd	solubility
1	20	**11f**	30
8a	20	**11g**	10
8b	120	**11h**	90
11a	30	**11i**	20
11c	210	**11j**	90
11e	90		

We previously demonstrated that **1** is a bacteriostatic agent by measuring the time-dependent viability of bacterial cells treated with **1**.[9] Treating *C. crescentus* CB15N cells with **1** at its MIC (5 μM) arrested cell division after the initiation of the midcell constriction (Figure 2). To evaluate if the most active analogues had the same phenotypic effect as **1** on cells, we imaged *C. crescentus* CB15N cells treated with divin analogues (at a concentration equivalent to the MIC) after 17 h of incubation (Figure 2). All of the compounds produced the same phenotype, which suggests that they share the same mechanism of action as **1**. We measured the time-dependent viability of *C. crescentus* CB15N cells treated with **11c** and **11j** (Figure S1, Supporting Information). The results indicated that analogues **11c** and **11j** are bacteriostatic, which is similar in mechanism to **1**.[9]

We also explored the efficacy of **1** and **11j** against a panel of clinically relevant strains of bacteria (Table 3). For these experiments, our choice of **11j** versus **8b** or **11c** was primarily due to its improved activity. Compounds **1** and **11j** inhibit the growth of *Shigella boydii*, *Enterobacter aerogenes*, *Vibrio cholera*, and other clinical pathogens. Analogue **11j** has a particularly low MIC (3 μM) against *V. cholera* and a 4-fold increase in potency against *E. aerogenes* compared to **1**.

In this letter, we describe the synthesis of a set of divin analogues and an SAR analysis of these compounds against cells of *C. crescentus* CB15N and *E. coli* BW25113 Δ*tolC*. These

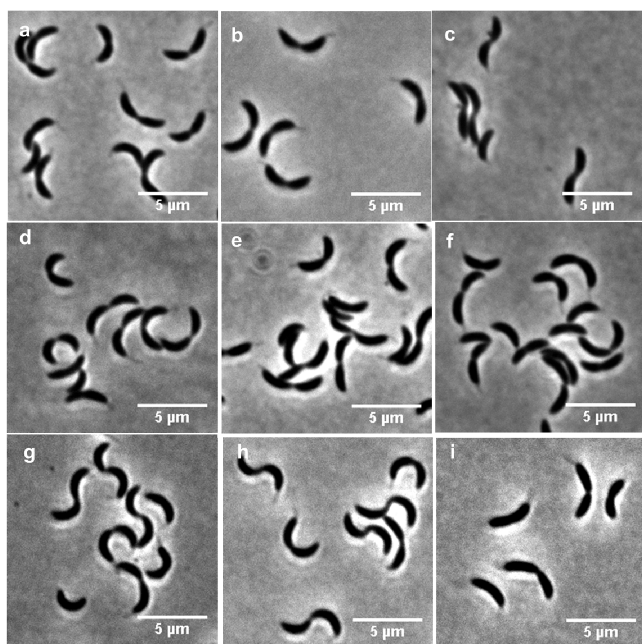


Figure 2. Divin analogues produced the same cellular phenotype with Gram-negative bacteria as did **1**. This figure shows panels of *C. crescentus* CB15N cells treated with different analogues and DMSO control: (a) **1**; (b) **8b**; (c) **11c**; (d) **11e**; (e) **11f**; (f) **11g**; (g) **11i**; (h) **11j**; and (i) DMSO.

Table 3. MIC of 1 and 11j against Various Pathogenic Strains of Bacteria; MIC Is Expressed As μM[a]

pathogenic bacteria	MIC	
	1	11J
Morganella morganii	>50	>50
Klebsiella pneumonia	>50	50
Acinetobacter baumannii	50	50
Salmonella typhimurium	50	25
Shigella boydii	50	25
Enterobacter aerogenes	50	12
Vibrio cholera	6	3

[a]All strains are clinical isolates.

studies have enabled us to identify three analogues (**8b**, **11c**, and **11j**) with improved solubility and potency. Importantly, these compounds produce the same phenotype as **1**, which suggests that they share a similar mechanism of action.

Our studies indicate that the 2-hydroxy-1-naphthalenyl portion of **1** is primarily responsible for its activity and that alterations and substitution to the benzimidazole ring can increase its potency. Compounds **11c** and **11j** were more active than **1** against *E. coli* BW25113 Δ*tolC*, and their solubility was 10- and 4-fold higher than **1** in M8 media, respectively. The solubility improvement of these analogues will enable us to pursue the molecular target by isolation and characterization of spontaneous mutants that display reduced susceptibility to the compound. More importantly, the SAR results enabled us to design and synthesize additional analogues that include a reactive azide group and/or a photoactivated diazirine probe, which exhibit virtually identical in vivo activity to **1**.[9,21] We anticipate that these probes will enable us to identify the molecular target of **1**, and we plan to report the results of these studies at a later date.

■ ASSOCIATED CONTENT

Supporting Information

All experimental details, ^{1}H and ^{13}C spectroscopic data, and other related material. 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.

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