Identification of Borinic Esters as Inhibitors of Bacterial Cell Growth and Bacterial Methyltransferases, CcrM and MenH

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As bacteria continue to develop resistance toward current antibiotics, we find ourselves in a continual battle to identify new antibacterial agents and targets. We report herein a class of boron-containing compounds termed borinic esters that have broad spectrum antibacterial activity with minimum inhibitory concentrations (MIC) in the low microgram/mL range. These compounds were identified by screening for inhibitors against *Caulobacter crescentus* CcrM, an essential DNA methyltransferase from Gram negative α-proteobacteria. In addition, we demonstrate that borinic esters inhibit menaquinone methyltransferase in Gram positive bacteria using a new biochemical assay for MenH from *Bacillus subtilis*. Our data demonstrate the potential for further development of borinic esters as antibacterial agents as well as leads to explore more specific inhibitors against two essential bacterial enzymes.

Introduction

The need to discover novel small molecules that kill bacterial cells or prevent their growth, without affecting the human host has been an ongoing challenge that has reached critical dimensions as increasing numbers of pathogens develop antibiotic resistance. Most existing antibiotics have been derived from natural products and are thus already associated with naturally occurring resistance genes in the antibiotic producing microbe. As resistance has spread, antibiotic potency and effectiveness has been maintained by continual chemical modification.

We have used a mechanism-based approach to develop inhibitors against the *Caulobacter crescentus* cell cycle regulated methyltransferase, CcrM, an essential DNA methyltransferase enzyme found in most α-proteobacteria, including the pathogens *Brucella abortus* and *Agrobacterium tumefaciens*.[1,2] CcrM plays a central role in cell cycle progression[3,4] and virulence.[1] Deletion of CcrM results in cell death, and overexpression leads to aberrant cell division.[1,2,4−6] CcrM catalyzes the transfer of a methyl group from *S*-adenosylmethionine (AdoMet) to the 6-N-position of adenine in GANTC sequences in DNA (Figure 1A).[7] Structural[8,9] studies on other methyltransferases indicate that a dramatic conformational change occurs within the ternary complex that results in the extrahelical localization of the substrate adenine base in an enzyme active site proximal to the AdoMet cofactor. Two independent studies[10,11] suggest that after this base-flipping process, the transfer of the methyl group proceeds by a direct S_N2 mechanism from the cofactor AdoMet to the 6-amino group of the target adenine base. After transfer of the methyl group, the methylated adenine base flips back into the DNA helix, followed by the sequential release of *S*-adenosylhomocysteine (SAH) and methylated DNA.[7] Known inhibitors of CcrM include the natural product sinefungin, which is an analogue of the cofactor AdoMet; however, it inhibits all AdoMet requiring enzymes, including eukaryotic cytosine methyltransferases, thus making it toxic to mammalian cells and unsuitable as a therapeutic agent.[12]

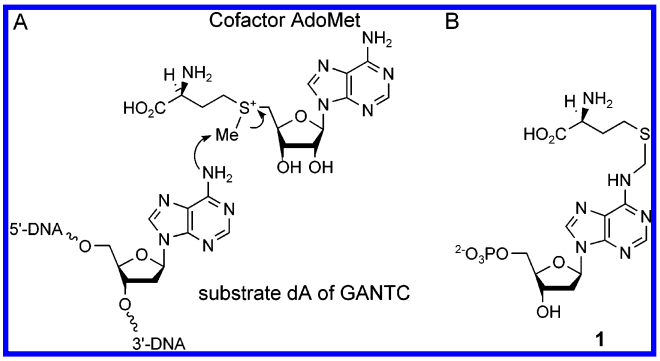

Figure 1. A. Reaction mechanism of the methyl transfer from AdoMet to adenine catalyzed by CcrM. B. The multisubstrate adduct inhibitor designed to test the extrahelical adenine binding site hypothesis and that demonstrated selective inhibition of CcrM over HhaI.[13]

In the absence of a crystal structure of CcrM we hypothesized the existence of an extrahelical adenine-binding site located between the DNA and the AdoMet binding sites. To test this hypothesis, we previously reported a multisubstrate adduct inhibitor of CcrM,

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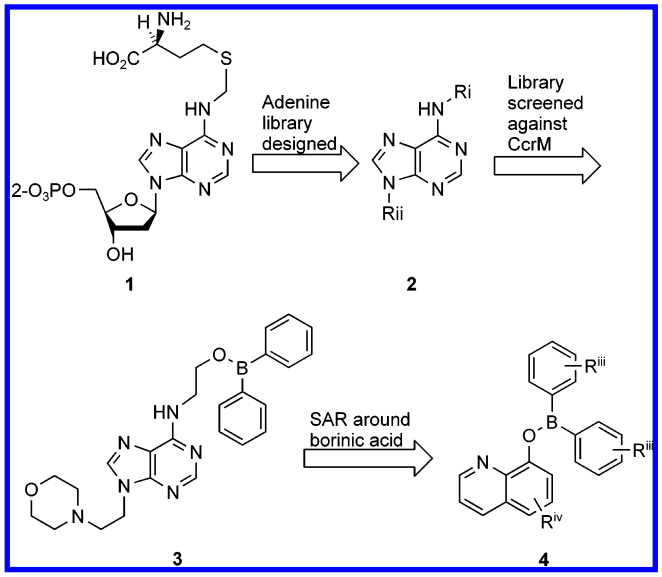


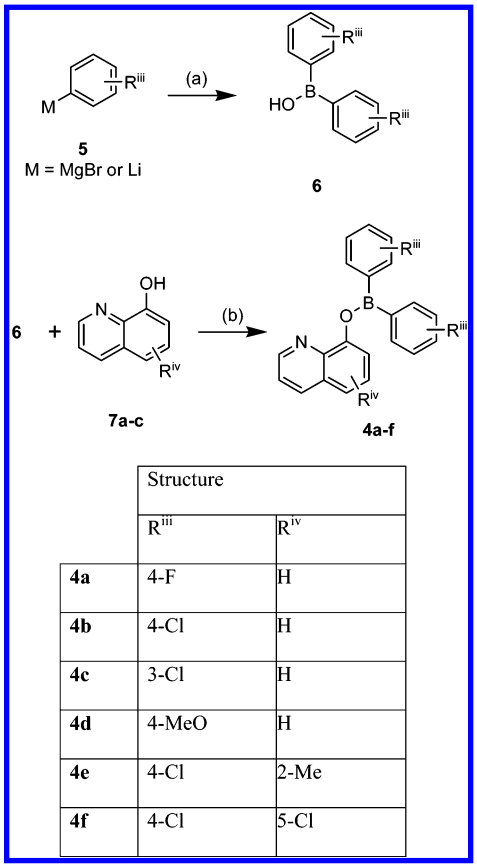
Figure 2. Evolution of diphenylborinic acid quinoline ester library (**4**). Using the multisubstrate adduct inhibitor (**1**) as a lead, a combinatorial library was designed based upon an adenine scaffold (**2**). The best CcrM inhibitor from the library (**3**) contained a borinic ester group, which was the focus of the final iteration to give the borinic acid quinoline esters (**4**).

such as compound **1** (Figure 1B), which was developed by fusing the methionine moiety of AdoMet to the 6-N-position of adenosine monophosphate via a methylene spacer to correctly position the functional groups.[13] These compounds were found to selectively inhibit CcrM over the C-5 cytosine methyltransferase HhaI, suggesting that CcrM contains an extrahelical adenine-binding site.

In this report we describe the design of a series of borinic esters as inhibitors against CcrM. Furthermore, these borinic esters showed both Gram positive and Gram negative antibacterial activity with minimum inhibitory concentrations (MICs) in the low microgram/mL range. As CcrM is not found in Gram positive bacteria, we investigated the potential target in these bacteria and identified MenH, an essential menaquinone methyltransferase. A newly developed biochemical assay for MenH from *Bacillus subtilis* and preliminary structure−activity relationship (SAR) results against this enzyme demonstrate that the borinic esters are inhibitors of *B. subtilis* both in vivo and in vitro.

Chemistry. The success of the multisubstrate inhibitor (**1**, Figure 1B) against CcrM[13] led us to choose adenine as a scaffold to construct an informed library of adenine derivatives substituted at the 6-N- and the 9-positions in order to extend the chemical diversity. Using solution phase combinatorial chemistry, we synthesized a small mixed library (ca. 1000 members, relative to 1 000 000 members, typical of random libraries) of adenine derivatives substituted at the 6-N-,9- or 6-N-,7-positions (the 6-N-,9-substitution pattern (**2**) is shown in Figure 2). The choice of R^i and R^{ii} deliberately included substituents that would provide a variety of side chain volumes and geometries. This library was screened for activity against *C. crescentus* cell growth[13] and in parallel, for direct inhibition of CcrM in vitro. We identified six leads that were bactericidal toward *C. crescentus* at a concentration of 100 μM and also inhibited CcrM activity completely at this level. All converged to a diphenylborinic acid substituent linked via an ethylene spacer to the 6-N-position, with various substituents at the 7- or 9-position. Upon deconvolution of this set we found the best of these six inhibitors contained the 2-morpholinoethyl group at the 9-position (**3**, Figure 2). In an attempt to improve the physicochemical properties of these borinic esters (**3**) yet retain the activity of our initial leads, we studied the SAR of the borinic ester group. Keeping the diphenyl borinic acid group constant, we replaced the ester group, containing the adenine nucleus, with other heteroaromatic groups. We found that only the 8-hydroxyquinoline ester series (**4**)[14] provided these improvements and also enhanced activity against CcrM. Therefore, we focused our efforts on this quinoline series.

Scheme 1. Syntheses of Diphenyl Borinic Acid Quinoline Esters (**4**)[a]



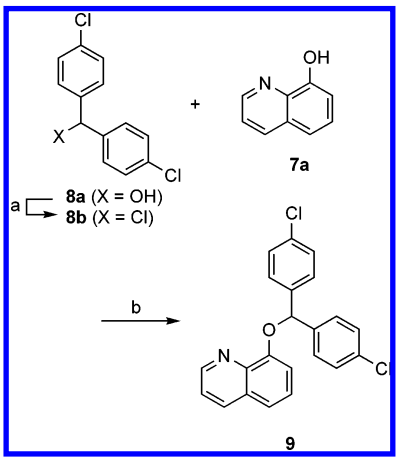
	Structure	
	R^{iii}	R^{iv}
4a	4-F	H
4b	4-Cl	H
4c	3-Cl	H
4d	4-MeO	H
4e	4-Cl	2-Me
4f	4-Cl	5-Cl

[a] Conditions: (a) BCl_3, THF, −78 °C to r.t; (b) EtOH, reflux.

Diphenylborinic acid quinoline esters (**4a**−**f**) were synthesized as shown in Scheme 1. Arylmetal reagents (**5**) were treated with 0.5 mol equiv of boron trichloride at −78 °C in THF. This was stirred overnight at room temperature and after workup gave the diphenylborinic acid (**6**). Without further purification, the diphenylborinic acid (**6**) was treated with 8-hydroxyquinoline derivatives (**7a**−**c**) in ethanol at reflux. Upon cooling, the diphenylborinic acid quinoline ester (**4**) usually crystallized from the solution in high purity.

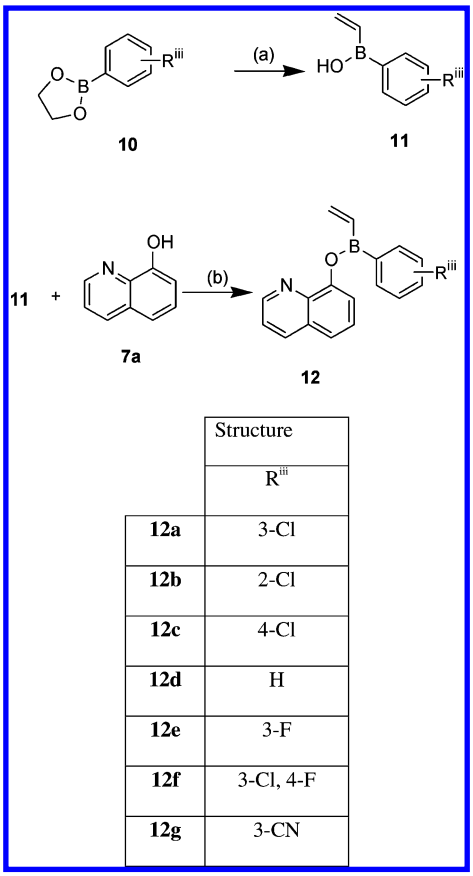
To understand the importance of the boron atom in these compounds, we synthesized an analogue of compound **4b** whereby the boron was replaced with carbon to give compound **9** (Scheme 2). We also surmised that the size of substituents attached to the central boron atom might influence the bioactivities of these compounds. To test this hypothesis, compounds **12a**−**g**

Scheme 2. Synthesis of Carbon Analogue (**9**) of **4b**[a]



[a] Conditions: (a) $SOCl_2$, CH_2Cl_2. (b) K_2CO_3, DMF, 60 °C.

Scheme 3. Syntheses of Vinyl-phenyl Borinic Acid Quinonline Esters (**12**)[a]



	Structure
	R^{iii}
12a	3-Cl
12b	2-Cl
12c	4-Cl
12d	H
12e	3-F
12f	3-Cl, 4-F
12g	3-CN

[a] Conditions: (a) vinylMgBr, THF, −78 °C to r.t.; (b) EtOH, reflux.

(Scheme 3) in which one of the two aryl groups is replaced with a less sterically hindered vinyl group were designed. As shown in Scheme 3, aryl boronic acid ethylene glycol esters (**10**) were reacted with vinylmagnesium bromide at −78 °C under anhydrous conditions and allowed to warm to room temperature to yield the asymmetrical vinyl-aryl borinic acids (**11**) after hydrolysis with HCl solution. As a final step, the borinic acid (**11**) was treated with 8-hydroxyquinoline (**7a**) in ethanol at reflux to furnish the final product (**12**), usually as a crystalline solid upon cooling. If the product did not crystallize, the addition of diethyl ether or diisopropyl ether to the ethanol would cause precipitation of the product.

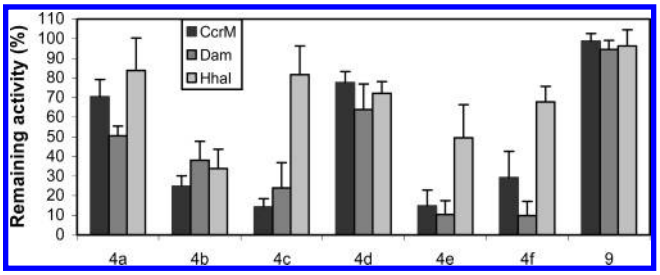


Figure 3. Inhibition of DNA methyltransferases, CcrM, Dam, and HhaI, by diphenylborinic acid quinoline esters (**4a**−**f**) and carbon analogue (**9**). Concentration of inhibitor was 100 μM. Assay measured the transfer of [^{3}H-Me] from Adomet to DNA substrate as a percent, relative to the absence of inhibitor (100% activity).

Biological Results and Discussion

In Vitro Activity against DNA Methyltransferases. Compounds **4a**−**f** and **9** were screened in vitro for activity against CcrM and two other DNA methyltransferases, Dam, a bacterial adenine DNA methyltransferase and HhaI, a bacterial cytosine methyltransferase. The results are shown in Figure 3, which shows the remaining enzyme activity when screened at 100 μM.[15] Compounds bearing a chloro group on the borinic acid moiety (**4b**, **4c**, **4e**, and **4f**) showed potent inhibitory activity against CcrM, whereas compounds **4a** and **4d** did not show significant inhibition. Furthermore, compounds **4c**, **4e**, and **4f** showed some selectivity for the adenine methyltransferases, CcrM and Dam, over the cytosine methyltransferase, HhaI. The carbon analogue (**9**) showed no activity against these enzymes, demonstrating that boron is important for the enzyme inhibition.

Inhibitor Kinetics of CcrM. Steady-state kinetic studies utilizing CcrM and a hemimethylated DNA substrate were conducted to assess the nature and degree of inhibition by **4e** in the presence of variable DNA concentrations and at saturating AdoMet levels.[15] Standard reciprocal plots of rate versus DNA concentration showed an uncompetitive inhibition pattern. In the presence of variable AdoMet concentrations and at saturating DNA levels, the effect of **4e** on CcrM activity was more complex. From the standard reciprocal plots, the observed inhibition pattern was uncompetitive at low concentration of inhibitor and noncompetitive at higher levels. A global fit to the data was achieved with a model in which the inhibitor initially binds to the binary CcrM•DNA complex followed by a second molecule of inhibitor competing with AdoMet binding at the active site. The calculated inhibition constants K_i's were 9 μM (uncompetitive inhibition) and 30 μM (mixed noncompetitive inhibition).[15] The inhibitors did not appear to bind to the free enzyme.

Microbiological Activity of the Borinic Esters. We tested the borinic esters (**4** and **12**) for microbiological activity against both Gram negative and Gram positive bacteria. The results of this study are shown in Table 1. We found that most of the compounds inhibited the growth of Gram negative bacteria in the low μg/mL range except for *Yersinia pseudotuberculosis*. *Francisella tularensis* was most sensitive to diaryl

Table 1. Minimum Inhibitory Concentration MIC (μG/mL) of Borinic Acid Quinoline Esters (**4a**−**c**,**e**,**f** and **12a**−**g**), and the Carbon Analogue **9** against Gram Positive and Gram Negative Bacteria

	structure		Gram positive							Gram negative				
	R[iii]	R[vi]	*S. aureus* ATCC 29213	*S. epidermidis* ATCC 12228	*E. faecalis* ATCC 29212	*E. faecium* CT-26	*B. subtilis* ATCC 23857	*B. anthracis*	*M. tuberculosis* H37Rv	*C. crescentus*	*M. catarrhalis*	*F. tularensis*	*Y. pseudotuberculosis* ATCC 29833	*H. influenzae* ATCC 4976
4a	4-F	H	4	2	32	32			0.62	12.5	2			4
4b	4-Cl	H	2	2	4	4	2	6.25	0.31	4	2	0.03	64	8
4c	3-Cl	H	1	2	32	4	2	6.25	0.62		2	0.01	32	8
4e	4-Cl	2-Me	16	8	32	16				2		6		
4f	4-Cl	5-Cl	0.5	0.25	32	2	32	5.3	0.62	2.6	≤0.125	0.01	8	0.25
9	carbon analogue		>64	>64	>64	>64	>64		>5				>64	>64
12a	3-Cl	H	2	1	64	4	4	8	0.62		4		16	4
12b	2-Cl	H	2	1	64	2	4		0.31		1	16	32	2
12c	4-Cl	H	2	1	>64	4	4		0.62		1		32	2
12d	H	H	2	1	>64	4					2		32	16
12e	3-F	H	2	1	>64	2	2	8	0.31		1	16	32	2
12f	3-Cl, 4-F	H	2	1	32	2	4		0.62		2		32	4
12g	3-CN	H	1	1	8	2	4	8	0.62		2	8	32	2

borinic esters with sub μg/mL activity. As neither CcrM nor Dam is present in Gram positive bacteria, we did not expect this series of compounds to provide broad spectrum antibacterial activity. Yet these compounds had equivalent activity against these pathogens, particularly compound **4f**, which showed MIC values of below 1 μg/mL against three out of seven Gram positive bacteria (Table 1). The two compounds that were most selective for CcrM in vitro **4c** and **4e** showed different specificities: compound **4c** exhibited activity against both Gram positive and Gram negative bacteria, but compound **4e** was active only against Gram negative bacteria. Interestingly, only compounds **4b** and **12g** showed activity against *Enterococcus faecalis*. Activity against *F. tularensis* was greatly reduced when a vinyl group replaced one aromatic ring. The carbon analogue (**9**) was also tested but, as expected, showed no activity. The activity of the borinic esters is comparable to clinically used antibiotics such as erythromycin (MIC_{90} *B. anthracis* 1.5 μg/mL),[16] gentamycin (MIC_{90} *B. anthracis* 0.38 μg/mL[16] MIC_{90} *F. tularensis* 1 μg/mL),[17] and streptomycin (MIC_{90} *F. tularensis* 4 μg/mL).[17] We have a full ADME-Tox profile study (plasma exposure, tissue distribution, serum binding, etc.) for these compounds underway. The data will be subject for a separate manuscript.

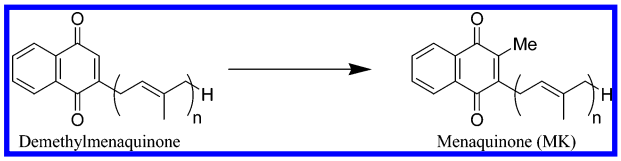
Mechanism of Action Study in Gram Positive Bacteria. As a subset of the borinic esters showed activity against Gram positive bacteria, which do not contain CcrM, we set out to investigate their mechanism of action. To identify putative target enzymes, we attempted to isolate mutants of *B. subtilis* that were resistant to compounds **4b** and **4c**. However, even though chemical mutagenesis was used, we were unable to obtain resistant mutants. Therefore, as the initial concept of the research targeted a methyltransferase, we tested the genes encoding the essential methyltransferases in *B. subtilis*, which are *menH*, *trmD*, *trmU*, *cspR*, *ydiO*, and *ydiP*. These genes were identified by the *B. subtilis* functional genomics project.[18] Strains were constructed that placed each of the essential methyltransferase genes under the control of a chromosomally integrated copy of the isopropyl β-D-thiogalactoside (IPTG) inducible promoter P_{spac}.[19] As the concentration of IPTG was reduced, less protein was expressed. We reasoned that if the borinic esters targeted any of these methyltransferases, the minimal inhibitory concentrations should decrease as IPTG concentrations were reduced. The MICs were determined for compounds **4b** and **4c** at different concentrations of IPTG that were empirically determined to give equivalent growth rates in the absence of inhibitor for each of the strains. The *trmD*, *trmU*, *cspR*, *ydiO*, and *ydiP* strains showed no significant changes of sensitivities for **4b** and **4c** (data not shown). The only strain that showed significant change of sensitivity with altered methyltransferase gene transcription was the *menH* strain. In *B. subtilis*, the *menH* gene, which encodes the essential menaquinone methyltransferase, is upstream of *hepT* (a gene required for the synthesis of the isoprenoid tail prior to its attachment to the menaquinone ring); therefore, the P_{spac} promoter will regulate both *menH* and *hepT* genes. Thus, either *menH* or genes upstream in the menaquinone biosynthetic pathway might be the target for these compounds.

Table 2. Relative Increase in Sensitivity of the *B. subtilis* menH Strain to Borinic Acid Quinoline Esters (**4b**,**c** and **12a**−**g**)

	R[v]	increase in sensitivity
4b	4-Cl	none
4c	3-Cl	16×
12a	3-Cl	64×
12b	2-Cl	10×
12c	4-Cl	none
12d	H	none
12e	3-F	16×
12f	3-Cl, 4-F	64×
12g	3-CN	24×

Additional compounds **12a**−**g** were selected to test their SAR against the *menH* strain (Table 2). The strain was most sensitive to the 3-chlorophenyl vinyl borinic acid quinoline ester (**12a**). When the chloro group was removed, giving **12d**, the sensitivity was lost entirely, indicating an important role for this chloro group. Moving the chloro group from the 3-position to the 2-position, giving **12b**, also reduced sensitivity from 64-fold to 10-fold. However, all sensitivity was lost again when the chloro group was moved to the 4-position, giving **12c**. These data suggested the optimum position for the chloro group was meta to the boron. Replacing the chloro group with fluoro, giving **12e**, or with a cyano group, giving **12g**, also resulted in reduced sensitivity again confirming the importance of the chloro group.

Scheme 4. Methyl Transfer Reaction by MenH in Menaquinone Biosynthesis[a]



[a] Conditions: MenH, AdoMet; $n = 3-9$ or larger.

Finally, addition of a fluoro group para to boron and ortho to the 3-chloro group, giving **12f**, retained equal sensitivity to that of compound **12a**. Since the *menH* strain may affect transcription of both *menH* and *hepT*, either gene products could be the target for these borinic esters. To determine if these compounds acted on MenH directly, we set up a biochemical assay for menaquinone methyltransferase.

In Vitro Activity against MenH. MenH is a methyltransferase that catalyzes the final step in the biosynthesis of menaquinone (MK). It transfers a methyl group from AdoMet onto the aromatic ring of the substrate demethylmenaquinone (DMMK) (Scheme 4). MK is involved in respiratory and photosynthetic electron-transport employing membrane-bound protein complexes.[20] In *B. subtilis*, MK is essential for successful endospore formation and germination[21] and is involved in regulation of cytochrome formation.[22,23] Most Gram positive bacteria contain menaquinone as the sole quinone whereas most aerobically grown Gram negative bacteria have ubiquinone (Q) as the sole quinone. Those Gram negative bacteria, such as *Klebsiella aerogenes* and *E. coli*, that can be grown anaerobically, contain menaquinone.

MenH is an attractive target for a novel class of antibiotics because it has no direct or functional homologues in mammalian cells, unlike the ubiquinone methyltransferases. Most in vitro assays of menaquinone methyltransferase reported to date use whole cell extracts[22,24] with a few exceptions.[25−27]

Following the method of Ogura et al.[22] we used whole cell lysates of *E. coli* JM109 containing the overexpressed *B. subtilis* MenH protein (described in Experimental Methods) and 1,4-dihydroxy-2-naphthoic acid as the substrate. After the reaction, MK was extracted from the cell lysate using a mixture of pentane and 2-propanol. The organic extract from each reaction was collected, the products were separated by HPLC and identified by MS. Using 1,4-dihydroxy-2-naphthoic acid as the substrate, wild-type *E. coli* (containing no *B. subtilis menH* insert) predominantly yielded Q_8 (ubiquonone with eight prenyl subunits). In the *E coli* strain containing the *B. subtilis menH* insert, both Q_8 and MK_8 (MK with eight prenyl subunits) were produced (Figure 4B). Production of MK_8 instead of MK_7, which is found in *B. subtilis*, suggests that the prenyl units were added according to the *E. coli* system, as MK_8 is the predominate quinone in anaerobically grown *E. coli*. When the inhibitor *S*-adenosylhomocysteine (SAH) was added, the amount of MK_8 was dramatically reduced (Figure 4C). This demonstrated that the MenH was expressed and active in *E. coli* whole cell lysates. Thus, this strain would allow us to monitor the inhibition of the MenH menaquinone methyltransferase.

To screen the inhibitory activity of our borinic esters in *E. coli* JM109 with *menH* insert as described above,

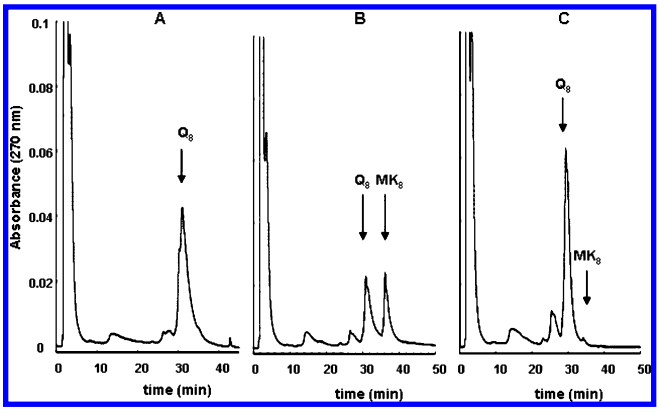


Figure 4. HPLC traces of lipid extracts from (A) *E. coli* JM109 without *B. subtilis menH* insert (B) *E. coli* JM109 with *B. subtilis* MenH overexpressed (C) *E. coli* JM109 with *B. subtilis* MenH overexpressed and inhibitor (SAH) showing the levels of Q_8 (ubiquinone with eight prenyl units) and MK_8 (menaquinone with eight prenyl units) as a result of ubiquinone methyltransferase activity or menaquinone methyltransferase activity.

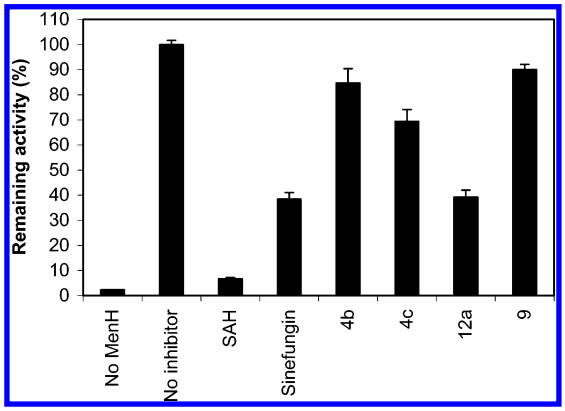


Figure 5. Inhibition of MenH by natural inhibitors SAH and sinefungin, borinic esters **4b**,**c** and **12a**, and carbon analogue **9**. Concentration of inhibitor was 100 μM. Assay measured the transfer of [^{3}H]-Me from AdoMet to menaquinone as a percent, relative to the absence of inhibitor (100% activity).

we used [^{3}H-Me]-AdoMet to monitor the transfer of the methyl group to menaquinone by liquid scintillation counting of the organic extraction. To determine the effect of activity due to the co-extracted *E. coli* ubiquinone, *E. coli* lysates without the *menH* insert were used as a control. The methyltransferase inhibitors sinefungin and SAH were also tested in this assay revealing that SAH had greater inhibitory activity than sinefungin (Figure 5). Although sinefungin is usually found to be a better methyltransferase inhibitor than SAH, this was found not the case for methyltransferases from *Streptomyces* spp.[28]

Compounds **4b**, **4c**, **9**, and **12a** were tested for in vitro inhibition of MenH (Figure 5). Although compound **4b** showed good inhibition activity against the CcrM DNA methyltransferase in vitro, it showed no significant inhibition of the *B. subtilis* MenH methyltransferase. Compound **12a** showed more than 50% inhibition, which was comparable to the activity of sinefungin. Not surprisingly, the carbon analogue (**9**) was inactive in this in vitro assay, which we attribute to the unique structural, conformational, and physical organic character that only the boron atom can bring to this class of compounds.

Thus, MenH appears to be a target of the borinic ester **12a**, which showed the greatest sensitivity against the *B. subtilis menH* strain (Table 2). However, this does not exclude the possibility that **12a** may also target the *hepT* gene product or another enzyme in the pathway. Indeed, the differences between the MIC data (Table 1) and in vitro sensitivity data (Table 2) and our inability to obtain resistant mutant suggest that MenH may not be the sole target of these borinic esters.

Conclusion

In this report, we showed the evolution of borinic acid quinoline esters designed to target the essential enzyme, CcrM, a DNA methyltransferase from Gram negative α-proteobacteria, to its application as a broad spectrum antibiotic inhibiting Gram positive bacteria containing the essential enzyme menaquinone methyltransferase, MenH. We showed these boron-containing compounds have broad-spectrum activity against several pathogenic strains of both Gram positive and Gram negative bacteria and that the boron is essential for its antibacterial activity. In an attempt to identify a target enzyme in Gram positive bacteria, we cloned, overexpressed, and developed an assay for *B. subtilis* MenH. These borinic esters could be used as leads for the development of inhibitors against bacteria cell growth or more specifically against the essential methyltransferase enzymes, such as CcrM and MenH.

Experimental Methods

^{1}H NMR spectra were recorded on a Bruker Avance 400 (400 MHz), Bruker AMX360 (360 MHz), and Varian Oxford 300 (300 MHz). MALDI mass spectra were obtained using a Perspective Biosystems Voyager-DE STR, FAB mass spectra were obtained using a Kratos Analytical MS-50 TC. APCI mass spectra were recorded on a Perspective Biosystems Mariner. Microanalyses were recorded by Atlantic Microlab Inc., P.O. Box 2288, Norcross, GA 30091. Analytical thin-layer chromatography (TLC) were performed with Whatman silica gel aluminum backed plates of thickness 250 μm and fluorescent at 254 nm, and by using the solvent systems indicated. Flash column chromatography was performed with Selecto Scientific silica gel, 32−64 μm particle size. Melting points were obtained using a Mel-Temp II melting point apparatus with a Fluke K1 K/J type thermocouple digital thermometer and are uncorrected. Purity by HPLC was determined using a betabasic-18 4.6 mm × 15 cm column from Keystone Scientific Inc. with a linear gradient of 0 to 40% acetonitrile in 10 mM triethylammonium acetate over 20 min. Chemicals were purchased from Acros Organics and Aldrich Chemical Co. and were used without further purification. Unmethylated lambda phage DNA and litmus 29 isolated from the *E. coli* strain DH5α were purchased from New England Biolabs (Beverly, MA). Reverse phase HPLC (Waters Corporation, pump 600, PDA detector 996) was performed with a C18 column (Nova-pak, 3.9 × 150 mm) for product identification. *B. subtilis* strain 168 for cloning *menH* was acquired from American Type Culture Collection. Pfu DNA polymerase was purchased from Promega (Madison, WI) and restriction enzymes were purchased from New England Biolabs and used as recommended by manufacturers. *E. coli* JM109 strain was acquired free of charge from New England Biolabs. Incorporation of [^{3}H-Me]-AdoMet was monitored by Beckman LS 6800 for liquid scintillation counting.

General Procedure for the 6-N-,9-Substituted Adenine Combinatorial Library. A combinatorial library was prepared in a two-step, one-pot procedure by solution phase using a 96-well glass microtiter plate. Three plates were prepared using the following methodology. Potassium carbonate (7.5 mg) was dispensed into each well of a 96-well microtiter plate. DMF (140 μL) was added to each well followed by 6-chloropurine (30 μL, 0.5 M solution in DMF). To each row, one different alkyl halide (15 μL, 1 M solution in DMF) was added and a lid was placed on top of the microtiter plate. The plate was heated at 45 °C for 24 h to give a regioisomeric mixture of 7- and 9-substituted chloropurines. After this time, one to three different alkylamines (5 μL, 1 M solution in DMF) were added to each column and a lid was placed on top of the plate. This plate was heated at 90 °C for 24 h to furnish a mixed library of 6-N-,9- and 6-N-,7-substituted adenine products. The plate was cooled to room temperature, and the solution in each well was removed from the solid potassium salts. The final concentration of each reaction was adjusted to approximately 17 mM in DMF, assuming quantitative yield, and these solutions were tested for activity without further purification. We used HPLC and LC-MS to analyze a cross-section of this library. We determined that the first reactions proceeded with a 7-:9-substitution ratio of around 2:3, and the overall reaction proceeded with yields varying between 50 and 95%.

General Method for the Synthesis of Diarylborinic Acids (4). Trichloroborane or tribromoborane (1 mol equiv) was added to either tetrahydrofuran (0.2 M) or diethyl ether (0.2 M) under argon and cooled to −78 °C. The arylmagnesium bromide or aryllithium (2 mol equiv), in tetrahydrofuran, diethyl ether, cyclohexane, toluene, or mixtures of these solvents, was added dropwise to the cold reaction. The reaction was allowed to warm to room temperature and stirred overnight. The solvents were removed in vacuo, and the residue was dissolved in diethyl ether. The reaction was stirred rapidly and hydrolyzed by the slow addition of 1 N hydrochloric acid. Stirring was discontinued, the layers were separated, and the organic layer was washed with saturated aqueous NaCl. The organic layer was dried ($MgSO_4$) and filtered, and the solvent was removed in vacuo to give the crude product as an oil. This oil was dissolved in ethanol to an estimated concentration of 1 M and divided into portions to be used in the subsequent precipitation with the various complexing agents.

Bis(4-fluorophenyl)borinic Acid 8-Hydroxyquinoline Ester (4a). Bis(4-fluorophenyl)borinic acid was prepared using the method above and was treated with 8-hydroxyquinoline (0.5 M solution in ethanol). A yellow precipitate formed immediately and was collected by filtration and washed with ethanol; mp 166−167 °C; ^{1}H NMR (360 MHz, $CDCl_3$): δ 8.53 (d, J = 5.1 Hz, 1H), 8.45 (d, J = 8.2 Hz, 1H), 7.70 (dd, J = 8.2, 7.7 Hz, 1H), 7.66 (dd, J = 8.2, 5.1 Hz, 1H), 7.39 (dd, J = 8.8, 6.7 Hz, 4H), 7.30 (d, J = 8.2 Hz, 1H), 7.20 (d, J = 7.7 Hz, 1H), 6.97 (t, J = 8.8 Hz, 4H); MS (+ve APCI) *m/z* 345 ([M + H]$^+$, ^{10}B), 346 ([M + H]$^+$, ^{11}B), 368 ([M + Na]$^+$, ^{11}B); Anal. ($C_{21}H_{14}NOBF_2$) C, H, N.

Bis(4-chlorophenyl)borinic Acid 8-Hydroxyquinoline Ester (4b). Bis(4-chlorophenylborinic acid was prepared using the method above and was treated with 8-hydroxyquinoline (0.5 M solution in ethanol). A yellow precipitate formed immediately and was collected by filtration and washed with ethanol; mp 192−194 °C; ^{1}H NMR (360 MHz, $CDCl_3$): δ 8.49 (dd, J = 4.6, 1.0 Hz, 1H), 8.43 (dd, J = 8.2, 1.0 Hz, 1H), 7.70 (dd, J = 8.5, 7.7 Hz, 1H), 7.66 (dd, J = 8.2, 4.6 Hz, 1H), 7.31 (d, J = 8.2 Hz, 4H), 7.26 (d, J = 8.5 Hz, 1H), 7.21 (d, J = 8.2 Hz, 4H), 7.17 (d, J = 7.7 Hz, 1H); MS (+ve APCI) *m/z* 377 ([M + H]$^+$, ^{10}B, ^{35}Cl, ^{35}Cl), 378 ([M + H]$^+$, ^{11}B, ^{35}Cl, ^{35}Cl), 379 ([M + H]$^+$, ^{10}B, ^{35}Cl, ^{37}Cl), 380 ([M + H]$^+$, ^{11}B, ^{35}Cl, ^{37}Cl), 381 ([M + H]$^+$, ^{10}B, ^{37}Cl, ^{37}Cl), 382 ([M + H]$^+$, ^{11}B, ^{37}Cl, ^{37}Cl); Anal. ($C_{21}H_{14}NOBCl_2$) C, H, N.

Bis(3-chlorophenyl)borinic Acid 8-Hydroxyquinoline Ester (4c). Bis(3-chlorophenyl)borinic acid was formed using the method above and was treated with 8-hydroxyquinoline (1.0 M in ethanol). The solvent was removed in vacuo, and the residue was purified by flash chromatography on silica gel using CH_2Cl_2/hexane (1:1) to elute. The solvent was evaporated and the product crystallized upon the addition of ethanol. The solid was collected by filtration and washed with cold ethanol to yield the title product as a yellow solid; mp 144−145 °C; ^{1}H NMR (360 MHz, CD_3OD): δ 8.83 (d, J = 5.0 Hz, 1H), 8.63 (d, J = 8.2 Hz, 1H), 7.78 (dd, J = 8.6, 5.0 Hz, 1H), 7.67 (t, J = 8.2, 1H), 7.36 (d, J = 8.2, 1H), 7.26−7.09 (m, 9H); MS (+ve

ESI) *m/z* 377 ([M + H]$^+$, ^{10}B, ^{35}Cl, ^{35}Cl), 378 ([M + H]$^+$, ^{11}B, ^{35}Cl, ^{35}Cl), 379 ([M + H]$^+$, ^{10}B, ^{35}Cl, ^{37}Cl), 380 ([M + H]$^+$, ^{11}B, ^{35}Cl, ^{37}Cl), 381 ([M + H]$^+$, ^{10}B, ^{37}Cl, ^{37}Cl), 382 ([M + H]$^+$, ^{11}B, ^{37}Cl, ^{37}Cl); Anal. ($C_{21}H_{14}NOBCl_2$) C, H, N.

Bis(4-methoxyphenyl)borinic Acid 8-Hydroxyquinoline Ester (4d). Bis(4-methoxyphenyl)borinic acid was formed using the method above and was treated with 8-hydroxyquinoline (0.5 M in ethanol) causing the title product to precipitate from the solution. The solid was collected by filtration and washed with cold ethanol to yield the product as a yellow solid; mp 222−224 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.53 (d, J = 5.0 Hz, 1H), 8.38 (d, J = 8.5 Hz, 1H), 7.67 (t, J = 8.0 Hz, 1H), 7.61 (dd, J = 8.3, 5.0 Hz, 1H), 7.38 (d, J = 8.5 Hz, 4H), 7.24 (d, J = 8.2 Hz, 1H), 7.17 (d, J = 7.7 Hz, 1H), 6.85 (d, J = 8.6 Hz, 4H), 3.78 (s, 6H); MS (+ve MALDI, CHCA) *m/z* 369 ([M + H]$^+$, ^{10}B), 370 ([M + H]$^+$, ^{11}B); Anal. ($C_{23}H_{20}BNO_3$) C, H, N.

Bis(4-chlorophenyl)borinic Acid 8-Hydroxyquinaldine Ester (4e). Bis(4-chlorophenyl)borinic acid was formed using the method above and was treated with 8-hydroxyquinaldine (0.5 M solution in ethanol). The product was collected by filtration and washed with ethanol; mp 155−156 °C; ^{1}H NMR (360 MHz, $CDCl_3$): δ 8.21 (d, J = 8.6 Hz, 1H), 7.46 (t, J = 8.4 Hz, 1H), 7.27 (d, J = 8.2 Hz, 1H), 7.18−7.11 (m, 9H), 6.97 (d, J = 7.8 Hz, 1H) 2.38 (s, 3H); MS (+ve, APCI) *m/z* 392 (M$^+$, ^{11}B, ^{35}Cl, ^{35}Cl), 394 (M$^+$, ^{11}B, ^{35}Cl, ^{37}Cl), 396 (M$^+$, ^{11}B, ^{37}Cl, ^{37}Cl); Anal. ($C_{22}H_{16}NBOCl_2$) C, H, N.

Bis(4-chlorophenyl)borinic Acid 5-Chloro-8-hydroxyquinoline Ester (4f). Bis(4-chlorophenyl)borinic acid was formed using the method above and was treated with 5-chloro-8-hydroxyquinoline (0.5 M solution in ethanol). The product was collected by filtration and washed with ethanol; mp 154−156 °C; ^{1}H NMR (360 MHz, $CDCl_3$): δ 8.56 (d, J = Hz, 1H), 8.44 (d, J = Hz, 1H), 7.64 (m, 1H), 7.57 (d, J = Hz, 1H), 7.14 (m, 9 H), 6.98 (d, 1H); MS (+ve, ESI) *m/z* 412 ([M + H]$^+$, ^{10}B, ^{35}Cl, ^{35}Cl, ^{35}Cl), 413 ([M + H]$^+$, ^{11}B, ^{35}Cl, ^{35}Cl, ^{35}Cl); Anal. ($C_{21}H_{13}NBOCl_2$) C, H, N.

4,4′-Dichlorobenzhydryl Chloride (8b). To a solution of 4,4-dichlorobenzhydrol (2.00 g, 7.94 mmol) in dichloromethane (20 mL) was added thionyl chloride (2.0 mL) dropwise at 0 °C, and the mixture was stirred at room temperature for overnight. The solvent was removed under reduced pressure, and the residue was extracted with ethyl acetate. The organic layer was washed with water and dried on anhydrous $MgSO_4$. The solvent was removed under reduced pressure to afford the desired product (2.24 g, quant), which was used for the next step without further purification.

8-[Bis(4-chlorophenyl)methoxy]quinoline (9). To a solution of compound **8b** (1.04 g, 3.83 mmol) and 8-hydroxyquinoline (500 mg, 3.44 mmol) in DMF (5 mL) was added K_2CO_3 (529 mg, 3.83 mmol), and the mixture was stirred 60 °C for overnight. The solvent was removed under reduced pressure, and the residue was extracted with ethyl acetate. The organic layer was washed with water (three times) and dried on anhydrous $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (5:1 hexane/ethyl acetate) to give the desired product (800 mg, 61%) as a white powder; mp 106−107 °C; ^{1}H NMR (300 MHz, DMSO-d_6,): δ 6.89 (s, 1H), 7.17 (d, J = 6.7 Hz, 1H), 7.37 (d, J = 8.2 Hz, 1H), 7.42 (d, J = 8.5 Hz, 4H), 7.47 (t, J = 7.3 Hz, 1H), 7.56 (dd, J = 8.2, 4.1 Hz, 1H), 7.59 (d, J = 8.5 Hz, 4H), 8.30 (dd, J = 8.2, 1.8 Hz, 1H), 8.94 (dd, J = 4.1, 1.8 Hz, 1H); MS (+ve APCI) *m/z* 380 ([M + H]$^+$, ^{35}Cl, ^{35}Cl), 382 ([M + H]$^+$, ^{35}Cl, ^{37}Cl), 384 ([M + H]$^+$, ^{37}Cl, ^{37}Cl). Anal. ($C_{22}H_{15}NOCl_2$) C, H, N.

(3-Chlorophenyl) Vinyl Borinic Acid 8-Hydroxyquinoline Ester (12a). To a THF solution (50 mL) of 3-chlorophenylboronic acid ethylene glycol ester (**10**) (2.89 g, 15.8 mmol), which was prepared by refluxing the mixture of 3-chlorophenylboronic acid and ethylene glycol (1:1 molar ratio) in toluene under nitrogen for 3 h, was added a vinylmagnesium bromide THF solution (1.0 M, 17.5 mL, 17.5 mmol) at −78 °C under nitrogen. The reaction mixture was stirred for 2 h from − 78 °C to room temperature and for 1 h with a water bath. Hydrochloric acid (6 M, 6 mL) was added, and the mixture was rotary evaporated. The residue was dissolved in ethyl acetate (150 mL), washed with brine (2 × 100 mL), dried, and evaporated. The crude solid (2.98 g) of (3-chlorophenyl)-vinylborinic acid was mixed with 8-hydroxyquinoline (2.0 g, 13.8 mmol) in ethanol (75 mL). The mixture was stirred for 15 min and ethanol was removed by rotary evaporation. The residue was purified by flash column chromatography over silica gel eluted with a mixed solvent of hexane and ethyl acetate (4:1, v/v) giving the title compound as yellow crystals (1.15 g, 25%): mp 145−147 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.21 (dd, 1H), 5.44 (dd, 1H), 6.40 (dd, 1H), 7.12−7.26 (m, 3H), 7.30−7.42 (m, 3H), 7.68 (t, 1H), 7.88 (dd, 1H), 8.74 (dd, 1H), 8.80 (d, 1H); MS (ESI+) *m/z* 294 (M + H)$^+$; Anal. ($C_{17}H_{13}BClNO$) C, H, N.

(2-Chlorophenyl) Vinyl Borinic Acid 8-Hydroxyquinoline Ester (12b). This was prepared from 2-chlorophenylboronic acid using a method similar to that described for the synthesis of **12a** to give the product in 49% yield as yellow crystals: mp 95−97 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.12 (dd, 1H), 5.40 (dd, 1H), 6.54 (dd, 1H), 7.08 (d, 1H), 7.16−7.20 (m, 3H), 7.38 (d, 1H), 7.50−7.55 (m, 1H), 7.65 (t, 1H), 7.84 (dd, 1H), 8.75 (d, 1H), 8.80 (d, 1H); MS (ESI+) *m/z* 294 (M + H)$^+$; Anal. ($C_{17}H_{13}BClNO$) C, H, N.

(4-Chlorophenyl) Vinyl Borinic Acid 8-Hydroxyquinoline Ester (12c). This was prepared from 4-chlorophenylboronic acid using a method similar to that described for the synthesis of **12a** giving the product in 14% yield as yellow crystals: mp 119−121 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.20 (dd, 1H), 5.44 (dd, 1H), 6.42 (dd, 1H), 7.13 (d, 1H), 7.22−7.28 (m, 2H), 7.36−7.44 (m, 3H), 7.68 (t, 1H), 7.88 (dd, 1H), 8.75 (d, 1H), 8.92 (d, 1H); MS (ESI+) *m/z* 294 (M + H)$^+$; Anal. ($C_{17}H_{13}BClNO\cdot0.25H_2O$) C, H, N.

Phenyl Vinyl Borinic Acid 8-Hydroxyquinoline Ester (12d). This was prepared from phenylboronic acid using a method similar to that described for the synthesis of **12a** giving the product in 21% yield as yellow crystals: ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.20 (dd, 1H), 5.44 (dd, 1H), 6.42 (dd, 1H), 7.0−7.5 (m, 7H), 7.68 (t, 1H), 7.86 (dd, 1H), 8.73 (d, 1H), 8.92 (d, 1H); MS (ESI+) *m/z* 260 (M + H)$^+$; Anal. ($C_{17}H_{14}BNO$) C, H, N.

(3-Fluorophenyl) Vinyl Borinic Acid 8-Hydroxyquinoline Ester (12e). This was prepared from 3-fluorophenylboronic acid using a method similar to that described for the synthesis of **12a** giving the product in 40% yield as yellow crystals: mp 111−114 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.21 (dd, 1H), 5.44 (dd, 1H), 6.42 (dd, 1H), 6.94 (tt, 1H), 7.10−7.28 (m, 4H), 7.40 (d, 1H), 7.67 (t, 1H), 7.87 (dd, 1H), 8.74 (d, 1H), 8.97 (d, 1H); MS (ESI+) *m/z* 278 (M + H)$^+$; Anal. ($C_{17}H_{13}BFNO$) C, H, N.

(3-Chloro-4-fluorophenyl) Vinyl Borinic Acid 8-Hydroxyquinoline Ester (12f). This was prepared from 3-chloro-4-fluorophenylboronic acid using a method similar to that described for the synthesis of **12a** giving the product in 48% yield as yellow crystals: mp 86−88 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.20 (dd, 1H), 5.46 (dd, 1H), 6.40 (dd, 1H), 7.14 (d, 1H), 7.20 (t, 1H), 7.33−7.49 (m, 3H), 7.68 (t, 1H), 7.88 (dd, 1H), 8.74 (d, 1H), 8.98 (d, 1H); MS (ESI+) *m/z* 312 (M + H)$^+$; Anal. ($C_{17}H_{12}BClFNO$) C, H, N.

(3-Cyanophenyl) Vinyl Borinic Acid 8-Hydroxyquinoline Ester (12g). This was prepared from 3-cyanophenylboronic acid using a method similar to that described for the synthesis of **12a** giving the product in 53% yield as yellow crystals: mp 115−117 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.23 (dd, 1H), 5.47 (dd, 1H), 6.42 (dd, 1H), 7.17 (d, 1H), 7.38−7.45 (m, 2H), 7.60−7.80 (m, 4H), 7.89 (dd, 1H), 8.76 (d, 1H), 9.60 (d, 1H); MS (ESI+) *m/z* 285 (M + H)$^+$; Anal. ($C_{18}H_{13}BN_2O$) C, H, N.

CcrM Inhibition Assay. Methyltransferase activity was measured by monitoring the incorporation of [^{3}H]-Me from [^{3}H]-AdoMet into DNA. A stock solution containing 250 nM CcrM, 5 μM N645/50 mer, 150 mM potassium acetate, 5 mM 2-mercaptoethanol in pH 7.5 HEPES buffer was prepared. Aliquots were placed in eppendorf tubes and inhibitors were added from concentrated stock solutions (16.7 mM in DMF or

Chart 1

DMSO) to reach the appropriate final concentrations (500 or 100 μM) in a total of 15 μL. Reactions were initiated by the addition of [^{3}H]AdoMet at a final concentration of 50 μM. After 40 min at 30 °C, 5 μL aliquots were removed from the reaction and spotted onto DE81 anion exchange filter circles. The filters were allowed to dry and then washed with 3 × 200 mL of 0.3 M ammonium formate to remove unreacted [^{3}H]AdoMet followed by 2 × 200 mL 95% ethanol wash and finally a 200 mL ether wash. The filters were allowed to air-dry and counted by standard liquid scintillation techniques. Control reactions in the absence of inhibitors were used to determine the extent of inhibition. High throughput screening was carried out similarly in Tris-HCl buffer (50 μM, pH 7.5) with plasmid DNA as substrate Litmus 29 (New England Biolabs): 250 μM DNA, 3 μM sites) 100 μM inhibitor candidate and enzyme, potassium acetate, and 2-mercaptoethanol concentrations as described above. Assays were initiated with [$^{14}CH_3$]AdoMet (50 μM, 34 Ci/mol) in a volume of 10 μL in a PCR plate and incubated for 30 min at 30 °C. Four microliter aliquots were then spotted on DE81 paper with a multichannel pipet and washed and dried as described above. Data were collected with a Molecular Dynamics model 425S phosphorimager and analyzed with the spotfinder utility in ImageQuant 3.3.

For the in-depth steady-state kinetic inhibition study the DNA concentration was varied from 0.5 to 5 times K_M of 1 μM, **4e** levels ranged 0 to 75 μM, and AdoMet was at 50 μM. From reciprocal plots of the rates vs DNA levels. $K_i \approx 11.2 \pm 0.2$ μM for **4e**.

The AdoMet concentration was varied from 1 to 10 times K_M of 6 μM, **4e** levels ranged from 0 to 75 μM and DNA was at 5 μM. From reciprocal plots $K_i \approx 9.7 \pm 2.3$ μM (uncompetitive) and $K_i \approx 30.2 \pm 6.7$ μM (noncompetitive) for **4e**.

DNA Substrate [N645/50 mer] (Chart 1). Synthesis of DNA was acheived on an Expedite BioSystems DNA synthesizer and purified as previously described.[29]

Determination of Minimum Inhibitory Concentration (MIC) against *Caulobacter cresentus* and Other Bacteria. Liquid cultures of *C. crescentus* were grown to mid-log phase in PYE media[30] at 30 °C and diluted for use in broth macrodilution assays with the different compounds.[31] The standard inoculum used was 10^5 cfu/mL, and visible growth was assayed after 18 h. In all other bacteria the MICs were determined according to the NCCLS guidelines.

Construction of the *menH* Strain. The spectinomycin resistance gene was amplified from pPE30[32] with the primers GAGGAATTCTAAAAAATTTAGAAGCCAATGAAATCT GAGGAATTCTAAAATCTGATTACCAATTAGAATGAA and the resulting PCR amplification product was digested with *E*co RI and cloned into the *Mun* I digested plasmid pMUTIN4.[33] The spectinomycin derivative pMUTIN4 was designated pMUTINS1. The ribosomal binding site, start codon, and 331 base pairs of *menH* coding sequence were amplified from the *B. subtili*s genome using the following primers TCAAAGCTTGGAAGAAGGGTAAACCATTATG and CATGAATTCAAGGAAGCTCCATCGCATTTCC and cloned into the *Hin*d III and *Eco* RI sites in pMUTINS1. The plasmid bearing this insert was confirmed by DNA sequence and designated pMENS1. The *Eco* RI primer was designed so it would introduce an in-frame stop codon into the *menH* coding region once integrated into the *B. subtilis* genome and therefore placing the only functional copy of *menH* under P_{spac} control. The plasmid pMENS1 was introduced into *B. subtilis*,[34] and transformants were selected on low salt LB agar containing 150 μg/mL spectinomycin and 1 mM IPTG. Transformants were tested for IPTG growth dependence, and the pMENS1 insertion was checked using the following primers AAAGGGGGATGTGCTGCAAGGCGATT and TTTGGGGACAAGGGTGATATTTTTGC.

Determination of Minimum Inhibitory Concentration (MIC) of IPTG Conditional *B. subtilis*. MIC assays were performed as described by the NCCLS protocol M7-A5 for microdilution antibacterial susceptibility tests, with the addition of 40 μg/mL spectinomycin and either 1 mM IPTG or 30 μM IPTG. The sensitivity data was determined by dividing the MIC for the *menH* strain in MHB media containing 40 μg/mL spectinomycin and 1 mM IPTG by the MIC in MHB media containing 40 μg/mL spectinomycin and 30 μM IPTG. 30 μM IPTG was determined empirically as the concentration that allowed cells to grow where their A_{600} end points were not significantly different from cells grown in 1 mM IPTG. Furthermore the control compounds gentamycin and 8-hydroxyquinoline showed no change in MIC.

Cloning *B. subtilis menH*. Boiled cells of *B. subtilis* strain 168 were used as a template for PCR. The PCR conditions were 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 15 min using Pfu DNA polymerase. The primers used were *menH* forward (5′-GCATGAATTCCATATGCAGGACTCAAAAGAACAGCGCG-3′) and *menH* reverse (5′-GCATGTGGATTCTTTCCATCCGATATGCGTGGCAGCG-3′). The PCR product was cut with NdeI and BamHI restriction enzymes and cloned into pMAL-c2x (New England Biolabs) vector before transformed into *E. coli* JM109.

Expression and Isolation of MenH. *E. coli* JM109 cells with *menH* were grown in LB media at 37 °C to $OD_{600} = 0.6-0.8$ and induced with IPTG (final concentration 1 mM). The cells were collected by centrifugation after 3 h, resuspended in a solution of 20 mM Tris-HCl, pH 7.5 with 1 mM EDTA and 10 mM BME, disrupted with a sonicator, and stored at −80 °C until they were used in an assay. The level of MenH overexpression was monitored by 12% SDS−PAGE electrophoresis.

MenH Assay. The inhibitor candidates (final concentration of 100 μM) were added to 500 μL of solution containing 200 μL of disrupted *E. coli* cells from previous procedure, 0.1 M Tris-HCl buffer pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, 1 μM FPP, 1 μM 1,4-dihydroxy-2-naphthoic acid (in EtOH:ether 2:1, v/v), 1 μM IPP, and 20 μM unlabeled AdoMet mixed with [^{3}H]-labeled AdoMet (3.01×10^3 dpm/reaction). The samples were incubated at 37 °C for 1 h with occasional rocking. The reactions were quenched by adding 500 μL of 0.1 M acetic acid in methanol. The expected menaquinone product was extracted with hexane/2-propanol (3:2, v/v, 3 × 3 mL), and the level of [^{3}H]-Me incorporation was monitored by a liquid scintillation counter. The assays were performed in triplicate.[22]

MenH Product Identification. The extract from each reaction above was concentrated to dryness and dissolved into 100 μL of acetonitrile (AN). The samples were injected into HPLC reverse phase column (C18) and eluted with a gradient (70% AN/H_2O to 100% AN) over 30 min at 1 mL/min flow rate. The eluants were monitored by following absorbance at 220 and 270 nm followed by MS analysis: Q_8 (APCI(−), calculated for $C_{49}H_{74}O_4$ 726.56 observed M^+ [726.42], M + 2 [728.47]);MK_8 (APCI(−), calculated for $C_{51}H_{72}O_2$ 716.5532, observed M^+ [716.4231]); demethyl-MK, ($DMMK_8$, APCI(−), calculated for $C_{50}H_{70}O_2$ 702.5376, observed M^+ [702.3421]).

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Supporting Information Available: Complete elemental analyses for compounds **4a**−**f**, **9**, and **12a**−**g**. This material is available free of charge via the Internet at http://pubs.acs.org.

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