



Optimization of a novel potent and selective bacterial DNA helicase inhibitor scaffold from a high throughput screening hit



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ARTICLE INFO

Article history:

Received 26 February 2013

Revised 12 April 2013

Accepted 22 April 2013

Available online 30 April 2013

Keywords:

Optimization

DNA helicase

DNA replication

Inhibitor

ABSTRACT

Benzobisthiazole derivatives were identified as novel helicase inhibitors through high throughput screening against purified *Staphylococcus aureus* (*Sa*) and *Bacillus anthracis* (*Ba*) replicative helicases. Chemical optimization has produced compound **59** with nanomolar potency against the DNA duplex strand unwinding activities of both *B. anthracis* and *S. aureus* helicases. Selectivity index (SI = CC₅₀/IC₅₀) values for **59** were greater than 500. Kinetic studies demonstrated that the benzobisthiazole-based bacterial helicase inhibitors act competitively with the DNA substrate. Therefore, benzobisthiazole helicase inhibitors represent a promising new scaffold for evaluation as antibacterial agents.

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The increasing prevalence of antibiotic resistant strains of bacterial pathogens presents a major unmet medical need. For example, the incidence of skin and soft tissue infections with community acquired methicillin-resistant *Staphylococcus aureus* (MRSA) has increased over fivefold since 2003.¹ In addition, a recent report revealed that 28% of enterococci cultured from 25 North American intensive care units (ICUs) were resistant to vancomycin (VRE).² The development of new antibiotics against underexploited targets with novel mechanisms of action is a vital part of the solution to these problems because such antibiotics will not be affected by preexisting target based resistance mechanisms. The replicative DNA helicase is an essential component of the DNA replication pathway, acting early and catalyzing a rate limiting step in replication, but it is currently untargeted by antibacterial agents.

Replicative DNA helicase is a member of a drug-validated pathway and, along with gyrase, topoisomerase IV, and DNA polymerase III, is essential to bacteria.^{3–7} The primary structures of bacterial replicative helicases differ significantly from those of their eukaryotic and human counterparts,^{8,9} indicating that bacteria-specific inhibitors of helicase may be developed. These features make it particularly attractive as a target for the discovery of new antibacterial therapeutics.

The replicative DNA helicases from *Escherichia coli*, *S. aureus*, and *Pseudomonas aeruginosa* have been targeted previously in anti-infective screens,^{10–17} but few hits have been described, and none have progressed further in drug development due to poor potency and inadequate selectivity. Two distinct X-ray crystal structures have been reported: one shows a hexameric DnaB helicase in complex with a helicase binding fragment of primase,¹⁸ and another shows that the DnaB hexamer adopts a closed spiral staircase quaternary structure in complex with ATP mimic GDP-ALF4 and ssDNA.¹⁹ The two structures suggest that helicase may exist in both inactivated and activated forms during the bacterial DNA replication process. Structure-based approaches to target both the inactivated and activated forms of DnaB helicase may aid in the discovery of novel bacterial DNA helicase inhibitors.

We have previously discovered a coumarin-based DNA helicase inhibitor series through a high throughput screening campaign, and chemical optimization yielded compounds with antibacterial activities against several Gram-positive species including multiple clinically relevant ciprofloxacin-resistant MRSA strains.^{20,21} Herein we report chemical optimization and biological evaluation of a novel series of DNA bacterial helicase inhibitors based on a benzobisthiazole scaffold.

Benzobisthiazole derivatives were identified as novel inhibitors through high throughput screening against *S. aureus* (*Sa*) and *Bacillus anthracis* (*Ba*) helicases. The screening hit compound **1** (Fig. 1) demonstrated antihelicase activities (IC₅₀ (*Ba*) = 5 μM and IC₅₀ (*Sa*) = 12 μM) and minimal cytotoxicity (CC₅₀ > 100 μM). Initial investigation of structure-activity relationships (SARs) focused on the benzamide portion of the benzobisthiazole scaffold.

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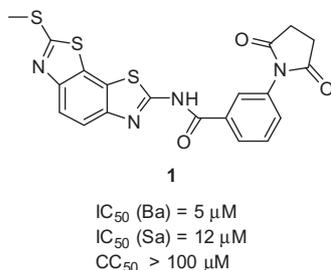


Figure 1. The structure of HTS hit 1.

Thirty-two analogs (compounds **4–35**) with various substitutions on the phenyl ring of the benzamide portion and twelve analogs (compounds **36–47**) with phenyl ring replacements were purchased from an outside vendor (Life Chemicals Inc.). Their biological activities were evaluated in a fluorescence resonance transfer (FRET)-based helicase strand unwinding assay²⁰ to measure concentration-dependent inhibition of *B. anthracis* DNA replicative helicase, and the results are summarized in Tables 1 and 2.

Substituents on the phenyl ring of the benzamide portion dramatically affected the antihelicase activity of the benzobisthiazoles (Table 1). In general, compounds with bulky substituents (compounds **2–6**) were inactive versus *B. anthracis* helicase, while smaller substituents, such as F, Cl, Br, CN, CH₃, CO₂CH₃, OCH₃, and OCH₂CH₃ were tolerated at the 3- or 4-positions (compounds **7–20**). Substituents at the 2-position of the phenyl ring were not tolerated except for the 2-CH₃ group (compound **25**). Disubstitution at the 3,4- or 3,5-positions with CH₃ or OCH₃ groups on the phenyl ring was tolerated. For example, compounds **29–32** with substituents 3,4-(CH₃)₂, 3,4-(OCH₂CH₂O), 3,4-(OCH₃)₂, and 3,5-(OCH₃)₂ displayed 1.7–3.2 μM IC₅₀ values versus *B. anthracis* helicase, while compounds with disubstitution at the 2,4- or 2,6-positions (**26–28**) exhibited weak or no inhibitory activity. Compound **33**, with 3,4,5-(OCH₃)₃ substitution on the phenyl ring, showed the best potency with an IC₅₀ value of 0.7 μM in this initial investigation of probing the substitution effect on the antihelicase activity.

The effect of replacement of the phenyl ring with various groups was also investigated in the *B. anthracis* DNA helicase assay, and the results are shown in Table 2. Replacement of the phenyl ring with alkyl, arylalkyl, naphthyl or heteroaryl groups (compounds **34–44**) significantly decreased potency, except for compound **45** with a pyrazine replacement, which exhibited modest activity (IC₅₀ = 28 μM).

The most active *B. anthracis* helicase inhibitor, compound **33**, also exhibited potent inhibitory activity versus *S. aureus* DNA helicase (IC₅₀ = 0.4 μM) without detectable cytotoxicity (CC₅₀ > 100 μM), while compound **16**, which bears a 3-OCH₃ group on the phenyl ring, inhibited *S. aureus* DNA helicase with an IC₅₀ value of 6.6 μM. To evaluate the SARs on the methylthio side of the benzobisthiazole core structure, we synthesized a series of analogs of two precursors **33** and **16**, by further transforming the methylthio group to various amines, and the synthesis is shown in Scheme 1.

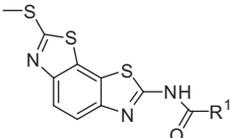
Commercially available aminobenzobisthiazole compound **46** was treated with corresponding benzoyl chlorides, to yield amides **16** and **33**.²² Treatment of compounds **16** and **33** with KMnO₄ under acidic conditions produced sulfones **47** and **48**, respectively, which were treated with amines to produce benzobisthiazole analogs **49–63**.^{23,24} To evaluate whether the amide bond was required for antihelicase activity, amino compound **46** was converted to benzylamino analog **64** through reductive amination, and an imine analog **65** was also synthesized through a condensation reaction (Scheme 2).²⁵ The biological activities of these analogs were evaluated vs both *B. anthracis* and *S. aureus* DNA replicative helicases, and cytotoxicities were measured in a serum free MTT assay.²¹ Results are summarized in Table 3.

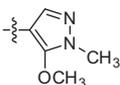
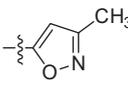
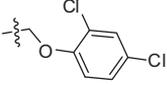
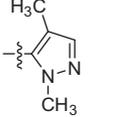
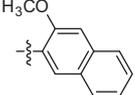
Compared to the methylthio compound **16**, methylamino analog **49** exhibited improved biological activity versus both *Ba* and *Sa* DNA helicases, and its cytotoxicity (CC₅₀) was greater than 100 μM. Increasing the size of the alkylamino group revealed that propylamino was tolerated; however, isopropylamino, cyclohexylamino and benzylamino compounds (**51**, **53**, **54**) showed significantly reduced activity, while the isobutylamino compound **52** displayed modest inhibition versus both of the helicases. Interestingly, compounds **55–58**, which bear additional polar groups, such as N(CH₃)₂, morpholino, or OH groups through a linker attaching to the aminobenzobisthiazole core, exhibited improved potency with

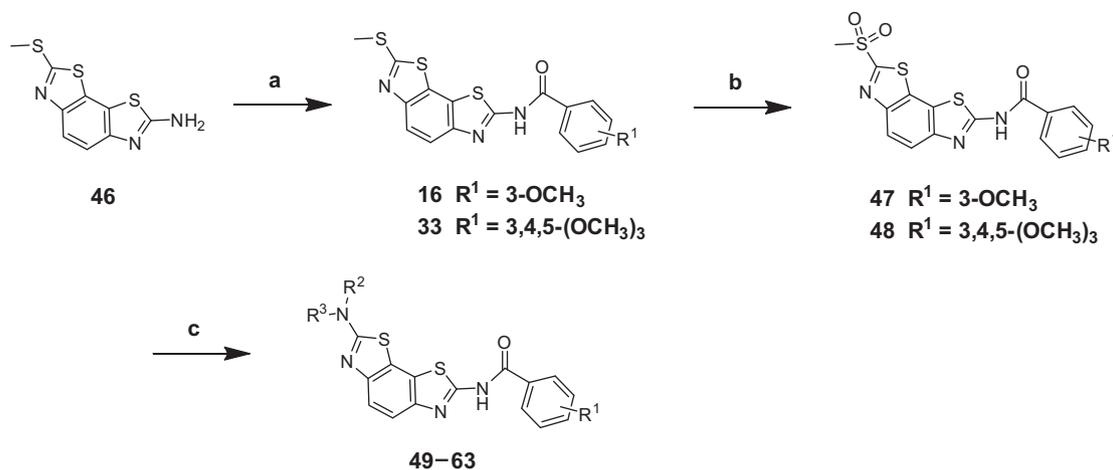
Table 1
Bacillus anthracis helicase inhibition by benzobisthiazole compounds **1–33**

Compd	R	IC ₅₀ Ba helicase (μM)	Compd	R	IC ₅₀ Ba helicase (μM)
1	3-N[-(C=O)CH ₂ CH ₂ (C=O)-]	5.0	18	3-F	3.3
2	4-N(CH ₂ CH ₃) ₂	>100	19	3-NO ₂	2.8
3	4-tBu	>100	20	3-Br	2.3
4	4-OPh	>100	21	2-OPh	>100
5	4-OiPr	>100	22	2-Cl	>100
6	4-SO ₂ N(CH ₂ CH ₃) ₂	>100	23	2-Br	>100
7	4-CH ₃	7.6	24	2-F	~50
8	4-CN	11.0	25	2-CH ₃	6.6
9	4-OCH ₂ CH ₃	6.9	26	2,4-(OCH ₃) ₂	>100
10	4-Cl	5.0	27	2,6-(OCH ₃) ₂	>100
11	4-COOCH ₃	3.7	28	2,6-F ₂	64.5
12	4-N(CH ₃) ₂	2.4	29	3,4-(CH ₃) ₂	3.2
13	4-OCH ₃	2.2	30	3,4-(OCH ₂ CH ₂ O)	3.0
14	4-N[-(C=O)CH ₂ CH ₂ (C=O)-]	1.3	31	3,4-(OCH ₃) ₂	2.1
15	3-N(CH ₃) ₂	36.0	32	3,5-(OCH ₃) ₂	1.7
16	3-OCH ₃	12.5	33	3,4,5-(OCH ₃) ₃	0.7
17	3-Cl	5.3			

Table 2
Bacillus anthracis helicase inhibition by benzobisthiazole compounds **34–45**



Compd	R ¹	IC ₅₀ Ba helicase (μM)	Compd	R ¹	IC ₅₀ Ba helicase (μM)
34	Cyclopropyl	>100	40		>100
35	CH ₂ Ph	47	41	2-Furan	102
36	CH ₂ CH ₂ Ph	>100	42	2-Thiophene	90
37		>100	43		>50
38		>100	44		>50
39		>50	45	2-Pyrazine	28

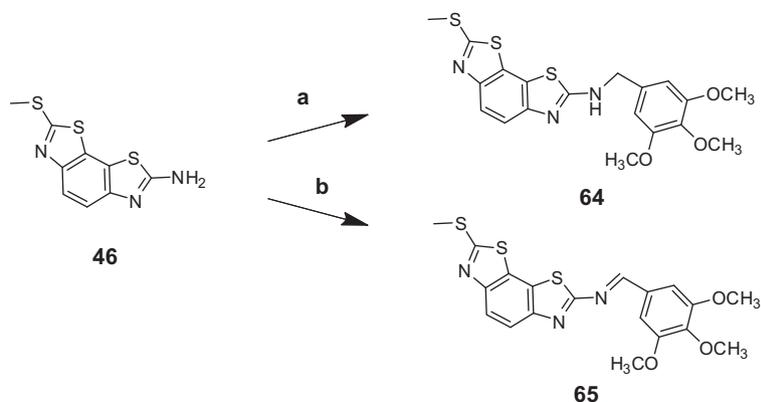


Scheme 1. Reagents and conditions: (a) benzoyl chlorides, CH₂Cl₂, yields 31–95%; (b) aq KMnO₄, glacial HOAc, dioxane, 73% for **47**, and 44% for **48**; (c) R²R³NH, DMF, 80–100 °C, yields 20–83%.

IC₅₀ values ranging between 1.0–2.3 μM and 1.0–4.2 μM versus Ba and Sa helicases, respectively. This finding suggests that the polar groups make additional, favorable interactions with the helicase enzymes.

Similarly, compared to the methylthio compound **33**, methyl-amino analog **59** also displayed improved helicase inhibitory activity (Ba helicase IC₅₀ = 0.2 μM; Sa helicase IC₅₀ = 0.2 μM) without detectable cytotoxicity (CC₅₀ > 100 μM), and selectivity indices (CC₅₀/IC₅₀) were more than 500. Analogs **60–63** all inhibited Ba and Sa helicases with IC₅₀ values between 0.2 and 0.3 μM. Furthermore, compound **63** did not show cytotoxicity (CC₅₀ > 100 μM). Compounds **64** and **65**, in which the amide linker was replaced with –NHCH₂– and –N=CH– linkers, lost activities versus both Ba and Sa helicases, suggesting that the amide bond is required for inhibitor binding, probably because of favorable hydrogen bonding interactions.

To determine the mode of inhibition for the benzobisthiazole scaffold, a dilution series of compound **49** was added to the helicase reaction in the presence of varying concentrations of the two substrates, ATP and oligonucleotide. The IC₅₀ value of compound **49** varied less than twofold (2.0–3.8 μM) in response to changes in the ATP concentration (Fig. 2A), but varied considerably (>6.5-fold) when oligonucleotide concentrations were altered (Fig. 2B). These results suggest that compound **49** is noncompetitive with ATP but competitive with the DNA substrate.²⁶ Indeed, a Dixon plot (Fig. 2C) of the DNA variation results is consistent with competitive inhibition with a K_i value of 2 μM, which is in good agreement with the IC₅₀ value at the lowest oligonucleotide concentration tested (3 nM). Similar results were also obtained for compounds **59** and **63** in kinetic studies (data not shown). These results indicate that helicase inhibitors with the benzobisthiazole scaffold act with a different mechanism of action than the previously reported coumarin-based helicase inhibitors, which



Scheme 2. Reagents and conditions: (a) 3,4,5-trimethoxybenzaldehyde, Na(OAc)₃BH, glacial HOAc, DMSO, rt, 48%; (b) 3,4,5-trimethoxybenzaldehyde, toluene, reflux, 67%.

Table 3
Bacillus anthracis and *Staphylococcus aureus* helicase inhibition by benzobisthiazole compounds **16**, **33** and **49–65**

Compd	R ¹	R ²	IC ₅₀ <i>B. anthracis</i> helicase (μM)	IC ₅₀ <i>S. aureus</i> helicase (μM)	HeLa CC ₅₀ in SFM (μM)
16	S-CH ₃	3-OCH ₃	12.5	6.6	12.4
49	NH-CH ₃	3-OCH ₃	0.5	2.2	>100
50	NH-propyl	3-OCH ₃	1.1	1.3	<0.08; 15.6
51	NH-isopropyl	3-OCH ₃	65.0	80	nd
52	NH-isobutyl	3-OCH ₃	13.0	9.0	44
53	NH-cyclohexyl	3-OCH ₃	90	>100	nd
54	NH-Bn	3-OCH ₃	75	>100	nd
55	NHCH ₂ CH ₂ N(CH ₃) ₂	3-OCH ₃	1.0	1.0	
56	NH-CH ₂ -N(CH ₂) ₄ -O	3-OCH ₃	0.5	2.2	9.6
57	-CH ₂ -N(CH ₂) ₄ -O	3-OCH ₃	2.3	3.0	>100
58	NHCH ₂ CH ₂ OH	3-OCH ₃	2.2	4.2	>100
33	S-CH ₃	3,4,5-(OCH ₃) ₃	0.7	0.4	>100
59	NH-CH ₃	3,4,5-(OCH ₃) ₃	0.2	0.2	>100
60	NHCH ₂ CH ₂ N(CH ₃) ₂	3,4,5-(OCH ₃) ₃	0.2	0.2	<0.78
61	NH-CH ₂ -N(CH ₂) ₄ -O	3,4,5-(OCH ₃) ₃	0.2	0.2	5.7
62	-CH ₂ -N(CH ₂) ₄ -N-	3,4,5-(OCH ₃) ₃	0.2	0.2	2.8
63	NHCH ₂ CH ₂ OH	3,4,5-(OCH ₃) ₃	0.2	0.3	>100
64			>100	>100	nd
65			>100	>100	nd

are noncompetitive with both ATP and DNA.²¹ However, neither the coumarin nor the benzobisthiazole inhibitors have any significant effect on the single-strand stimulated ATPase activity of *S. aureus* helicase at concentrations over 100-fold higher than their IC₅₀ values (Fig. 2D).²¹ X-ray crystallography studies have revealed two distinct hexameric DnaB helicase structures: one showed a flat structure without DNA and NTP association, and the other formed a closed spiral staircase quaternary structure in complex with DNA and ATP mimic GDP-AIF₄, suggesting that the former structure

represents an inactivated form of DnaB helicase, and the latter one represents an activated form of DnaB helicase. The coumarin type helicase inhibitors are kinetically noncompetitive with both DNA and ATP substrates, suggesting that the coumarin-based inhibitors are likely bound to the inactivated form of the helicase, while the benzobisthiazole derivatives exhibit competitive kinetics with the DNA substrate, suggesting that the benzobisthiazole-based compounds may inhibit the activated form of the bacterial helicase. Structural information for the inhibitor-DnaB helicase

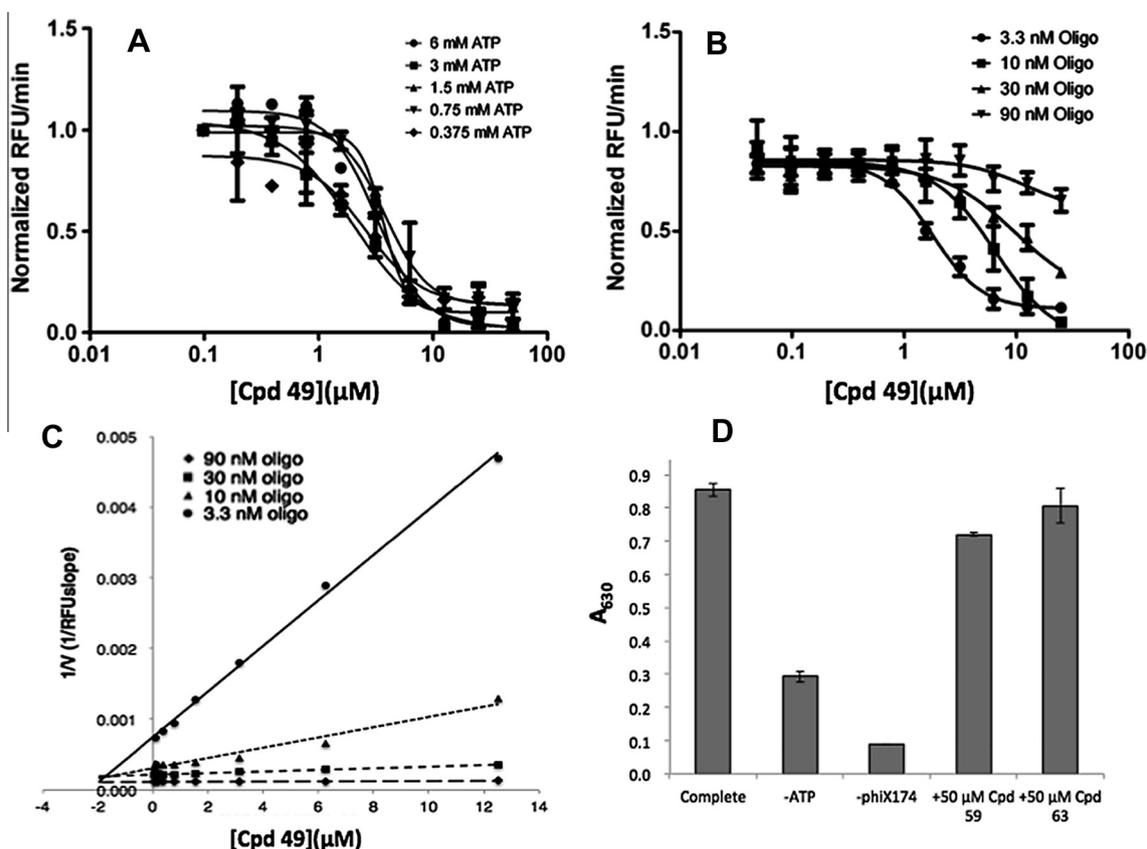


Figure 2. Mode of inhibition of benzobisthiazole **49**. A dilution series of compound **49** was examined in the FRET-based *S. aureus* helicase assay²⁰ with varying concentrations of ATP (A) or DNA (B) to determine IC₅₀ values. Data were plotted in GraphPad Prism 5.0 using 4-parameter curve fitting. (C) The results from varying DNA are also shown in a Dixon plot (1/V vs [I]). (D) Compounds **49** and **59** failed to exhibit significant inhibition of the single-stranded DNA stimulated ATPase activity of *S. aureus* helicase as detected by malachite green.²¹

complex is needed to further elucidate the mechanism of action of these small molecule helicase inhibitors and provide guidance for the discovery of novel bacterial helicase inhibitors.

In addition to potentially inhibiting replicative helicase, many benzobisthiazole analogs also inhibit the growth of bacterial cells (data not shown). However, the growth inhibition manifests primarily as a reproducible lengthened lag phase, delaying exponential growth, and is not sufficient to produce MIC values, possibly due to poor bacterial cellular permeability and/or short half-life of the parent compounds in cells. Nonetheless, incorporation of radiolabeled precursors into DNA in permeabilized preparations of *B. anthracis* cells²⁷ was reduced significantly by compounds **62** and **63** with IC₅₀ values of 10 and 17 μM, respectively (Supplementary Fig. S1). These results indicate that the benzobisthiazoles inhibit DNA synthesis in bacterial cells, and such inhibition could be responsible for the modest growth inhibition observed in live cells.

In conclusion, we have identified a novel class of potent and selective benzobisthiazole-based bacterial DnaB helicase inhibitors through high throughput screening. Subsequent structure-activity relationship analysis and chemical optimization led to substantial improvement of biological activity and identification of nanomolar compounds with selectivity indices of more than 500. To the best of our knowledge, these compounds are the most potent bacterial replicative helicase inhibitors reported to date. Further optimization of the benzobisthiazole-based helicase inhibitors may provide a novel small molecule drug for antibacterial therapy.

Acknowledgments

We thank Ms. Shawna M. Rotoli and Dr. Subhasis B. Biswas (Department of Molecular Biology, University of Medicine and

Dentistry of New Jersey (UMDNJ)) and Dr. Esther E. Biswas-Fliss (UMDNJ and Department of Bioscience Technologies, Jefferson School of Health Professions, Thomas Jefferson University) for providing *B. anthracis* helicase, for radiometric assays of the *B. anthracis* helicase inhibition potency, and for helpful discussions. We thank Mr. Steven C. Cardinale at Microbiotix for assistance with the malachite green-based ATPase assay. This work was supported by the National Institutes of Health/National Institute of Allergy and Infectious Diseases (AI064974). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2013.04.055>.

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22. *Synthetic procedures for preparation of compounds 16 and 33.* Compound **46** (10 g, 39.5 mmol) was suspended in pyridine (200 mL) and cooled to 0 °C in an ice bath. Diisopropylethylamine (11.18 g, 86 mmol) was added, followed by dropwise addition of 3-methoxybenzoyl chloride (6.8 g, 39.9 mmol). After stirring at room temperature for 3 days the solvent was evaporated. The crude product was sonicated with ethyl acetate (200 mL) and water (100 mL) for 10 min and filtered. The precipitate was stirred in chloroform (400 mL) at room temperature and filtered. The filtrate was washed thrice with water, brine, dried over sodium sulphate and evaporated to give a white solid **16** (9.4 g, yield 62%), mp = 237–238 °C, R_f = 0.71 (2:98::MeOH:CH₂Cl₂); ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.08 (s, 1H), 7.93 (d, *J* = 8.7 Hz, 1H), 7.86 (d, *J* = 8.7 Hz, 1H), 7.73 (d, *J* = 2.7 Hz, 2H), 7.48 (t, *J* = 8.1 Hz, 1H), 7.23 (d, *J* = 8.1 Hz, 1H), 3.87 (s, 3H), 2.84 (s, 3H). LC/MS *m/z* 388.3 (M+1)⁺. Compound **33** was synthesized using the same procedure. Yield 96%, mp = 231–232 °C, R_f = 0.76 (5:95::MeOH:CH₂Cl₂); ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.05 (s, 1H), 7.94 (d, *J* = 8.7 Hz, 1H), 7.85 (d, *J* = 8.4 Hz, 1H), 7.53 (s, 2H), 3.90 (s, 6H), 3.77 (s, 3H), 2.84 (s, 3H).
23. *Synthetic procedures for preparation of compounds 47 and 48.* Compound **16** (3.0 g, 10.0 mmol) was suspended in a mixture of glacial acetic acid (100 mL) and dioxane (40 mL). A solution of KMnO₄ (6.0 g, 38.0 mmol) in water (15 mL) was added dropwise while stirring. After stirring at 55 °C for 24 h the mixture was poured into a solution of NaHSO₃ (15 g in 250 mL) and stirred for 15 min. The resulting precipitate was collected by filtration, washed with water and dried at 55 °C under vacuum overnight to give the desired product. The crude product was refluxed with MeOH (250 mL) for 1 h and filtered while hot. The precipitate was washed with MeOH and dried at 55 °C overnight under vacuum to give compound **47** (3.15 g, yield 73%), mp = 284–285 °C, R_f = 0.35 (1:1::EtOAc:Hexane); ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.26 (s, 1H), 8.27 (d, *J* = 8.7 Hz, 1H), 8.06 (d, *J* = 9.0 Hz, 1H), 7.73–7.72 (m, 2H), 7.48 (t, *J* = 8.4 Hz, 1H), 7.23 (dd, *J* = 2.1, 8.1 Hz, 1H), 3.87 (s, 3H), 3.65 (s, 3H). LC/MS *m/z* 420 (M+1)⁺. Compound **48** was synthesized using the same procedure. Yield 44%, mp = 160–161 °C, R_f = 0.12 (1:1::EtOAc:Hexane); ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.27 (s, 1H), 8.33 (d, *J* = 8.7 Hz, 1H), 8.11 (d, *J* = 8.7 Hz, 1H), 7.56 (s, 2H), 3.91 (s, 6H), 3.77 (s, 3H), 3.63 (s, 3H). LC/MS *m/z* 479.8 (M+1)⁺.
24. *General synthetic procedures for preparation of compounds 49–63.* Compounds **47** and **48** (6.3 mmol) was treated with 20 equiv of corresponding amine in a sealed tube at 70 °C for 24 h. Then the reaction mixture was evaporated to dryness and dried at 50 °C under vacuum overnight. The resulting crude mixture was purified by high-pressure liquid chromatography to obtain compounds **49–63**. Characterization data for compound **49**: yield 62%, mp = 301–302 °C, R_f = 0.1 (1:1::EtOAc:Hexane); ¹H NMR (300 MHz, DMSO-*d*₆) δ 13.17 (s, 1H), 8.73 (s, 1H), 7.74 (m, 3H), 7.56 (d, *J* = 9.0 Hz, 1H), 7.48 (t, *J* = 9.0 Hz, 1H), 7.23 (dd, *J* = 8.1 Hz, 1H), 3.88 (s, 3H), 2.52 (s, 3H). LC/MS *m/z* 371.2 (M+1)⁺. Characterization data for compound **59**: yield 38%, mp = 238–239 °C, R_f = 0.87 (80:18:2::CHCl₃:CH₃OH:CH₃NH₂); ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.24 (br, 1H), 7.67 (d, *J* = 9.0 Hz, 1H), 7.54 (s, 1H), 7.51 (d, *J* = 0.9 Hz, 2H), 3.88 (s, 6H), 3.74 (s, 3H), 2.97 (s, 3H). LC/MS *m/z* 431.1 (M+1)⁺.
25. Characterization data for compounds **64** and **65**. For compound **64**: yield 48%, mp = 168–169 °C, R_f = 0.38 (50:50::EtOAc:*n*-Hexanes). ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.67 (s, 1H), 7.72 (d, *J* = 8.7 Hz, 1H), 7.51 (d, *J* = 8.7 Hz, 1H), 6.73 (s, 2H), 4.54 (d, *J* = 4.8 Hz, 2H), 3.75 (s, 6H), 3.62 (s, 3H), 2.78 (s, 3H). LC/MS *m/z* 434.1 (M+1)⁺. For compound **65**: yield 67%, mp = 207–208 °C, R_f = 0.68 (5:95::MeOH:CH₂Cl₂); ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.13 (s, 1H), 7.99 (s, 2H), 7.46 (s, 2H), 3.89 (s, 6H), 3.79 (s, 3H), 2.85 (s, 3H). LC/MS *m/z* 431.6 (M+1)⁺.
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