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

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Abstract

Benzobisthiazole derivatives were identified as novel helicase inhibitors through high throughput screening against purified *S. aureus* (*Sa*) and *B. 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 = CC_{50}/IC_{50}) 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.

Keywords

Optimization; DNA helicase; DNA replication; Inhibitor

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 five-fold 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

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developed. These features make it particularly attractive as a target for the discovery of new antibacterial therapeutics.

The replicative DNA helicases from *E. coli, S. aureus,* and *P. 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 *B. anthracis* (*Ba*) helicases. The screening hit compound **1** (Fig. 1) demonstrated antihelicase activities (IC_{50} (Ba) = 5 μM and IC_{50} (Sa) = 12 μM) and minimal cytotoxicity (CC_{50} > 100 μM). Initial investigation of structure-activity relationships (SARs) focused on the benzamide portion of the benzobisthiazole scaffold. 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 assay[20] to measure concentration-dependent inhibition of *Bacillus 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 *vs B. anthracis* helicase, while smaller substituents, such as F, Cl, Br, CN, CH_3, CO_2CH_3, OCH_3, and OCH_2CH_3 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-CH_3 group (compound **25**). Disubstitution at the 3,4- or 3,5-positions with CH_3 or OCH_3 groups on the phenyl ring was tolerated. For example, compounds **29**–**32** with substituents 3,4-$(CH_3)_2$, 3,4-(OCH_2CH_2O), 3,4-$(OCH_3)_2$, and 3,5-$(OCH_3)_2$ displayed 1.7–3.2 μM IC_{50} values *vs 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-$(OCH_3)_3$ substitution on the phenyl ring, showed the best potency with *an* IC_{50} 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 (IC_{50} = 28 μM).

The most active *B. anthracis* helicase inhibitor, compound **33,** also exhibited potent inhibitory activity *vs S. aureus* DNA helicase (IC_{50} = 0.4 μM) without detectable cytotoxicty (CC_{50} >100 μM), while compound **16**, which bears a 3-OCH_3 group on the phenyl ring, inhibited *S. aureus* DNA helicase with an IC_{50} 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 $KMnO_4$ 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 *Bacillus 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 vs both *Ba* and *Sa* DNA helicases, and its cytotoxicity (CC_{50}) 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 vs both of the helicases. Interestingly, compounds **55**–**58**, which bear additional polar groups, such as $N(CH_3)_2$, morpholino, or OH groups through a linker attaching to the aminobenzobisthiazole core, exhibited improved potency with IC_{50} values ranging between 1.0–2.3 μM and 1.0–4.2 μM vs 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**, methylamino analog **59** also displayed improved helicase inhibitory activity (Ba helicase IC_{50} = 0.2 μM; Sa helicase IC_{50} = 0.2 μM) without detectable cytotoxicity (CC_{50} >100 μM), and selectivity indices (CC_{50}/IC_{50}) were more than 500. Analogs **60**–**63** all inhibited Ba and Sa helicases with IC_{50} values between 0.2–0.3 μM. Furthermore, compound **63** did not show cytotoxicity (CC_{50} >100 μM). Compounds **64** and **65**, in which the amide linker was replaced with -$NHCH_2$- and -N=CH- linkers, lost activities vs 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 IC_{50} value of compound **49** varied less than 2-fold (2.0 to 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 Ki value of 2 μM, which is in good agreement with the IC_{50} 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 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 IC_{50} 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 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 potently 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* cells [27] was reduced significantly by compounds **62** and **63** with IC_{50} values of 10 and 17 μM, respectively (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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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 (11.1 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 minutes 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:CH_2Cl_2); ^{1}H 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:CH_2Cl_2); ^{1}H 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 $KMnO_4$ (6.0 g, 38.0 mmol) in water (15 mL) was added dropwise while stirring. After stirring at 55 °C for 24 hours the mixture was poured into a solution of $NaHSO_3$ (15 g in 250 mL) and stirred for 15 minutes. 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 hour 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); ^{1}H 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); ^{1}H 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 eq. of corresponding amine in a sealed tube at 70 °C for 24 hour. 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); ^{1}H 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::$CHCl_3$:CH_3OH:CH_3NH_2); ^{1}H 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), ^{1}H 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:CH_2Cl_2); ^{1}H 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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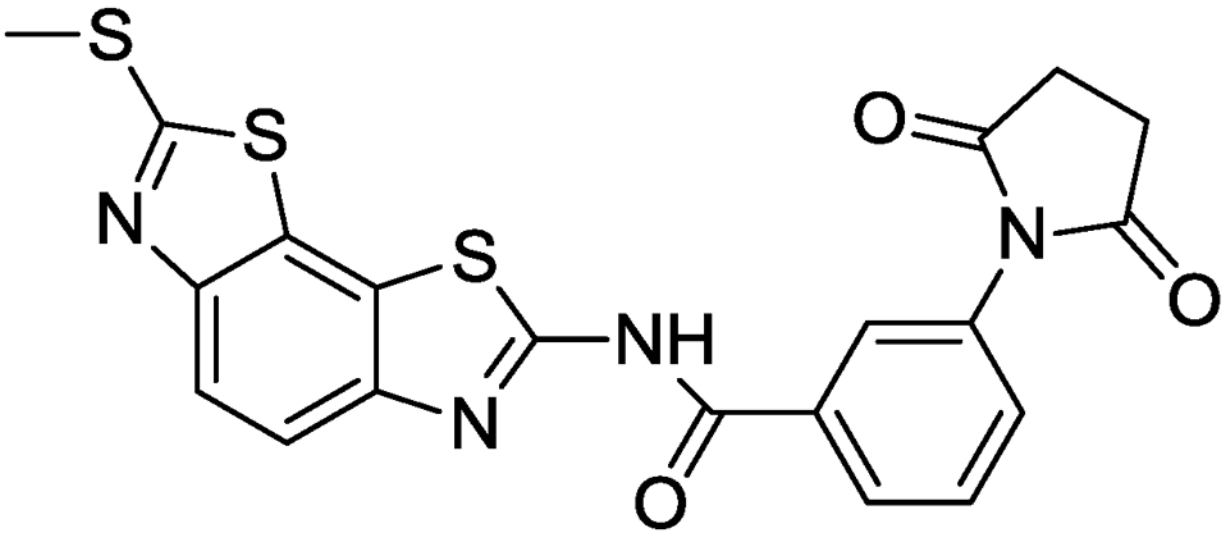


1

IC_{50} (Ba) = 5 μM

IC_{50} (Sa) = 12 μM

CC_{50} > 100 μM

Figure 1.
The structure of HTS hit **1**.

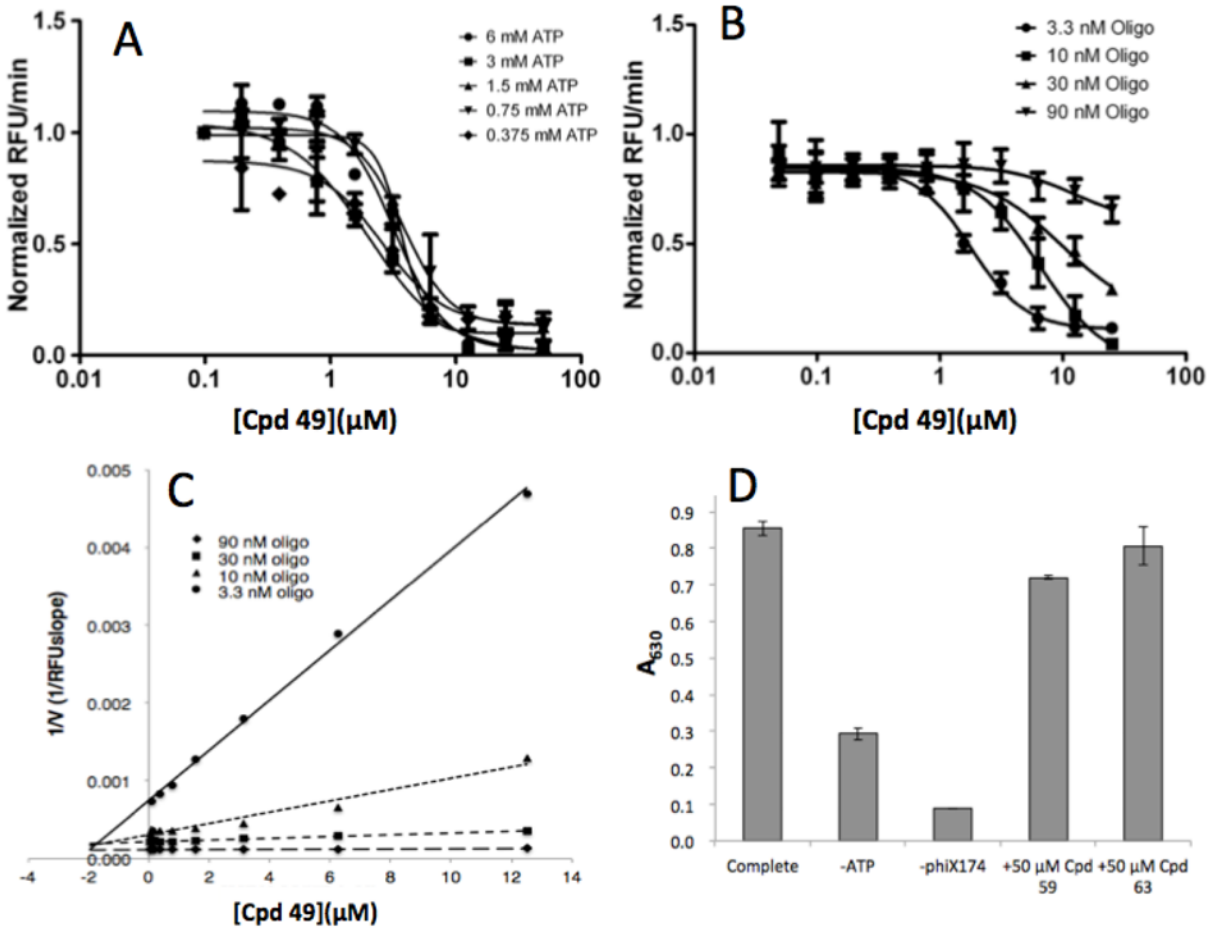
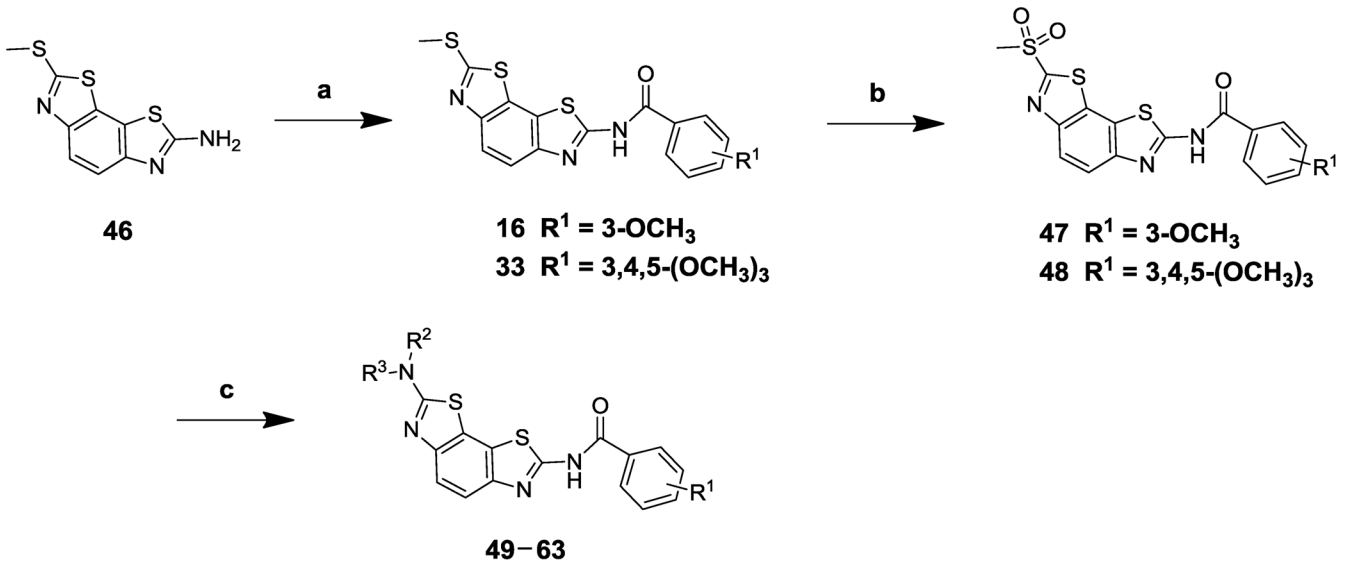


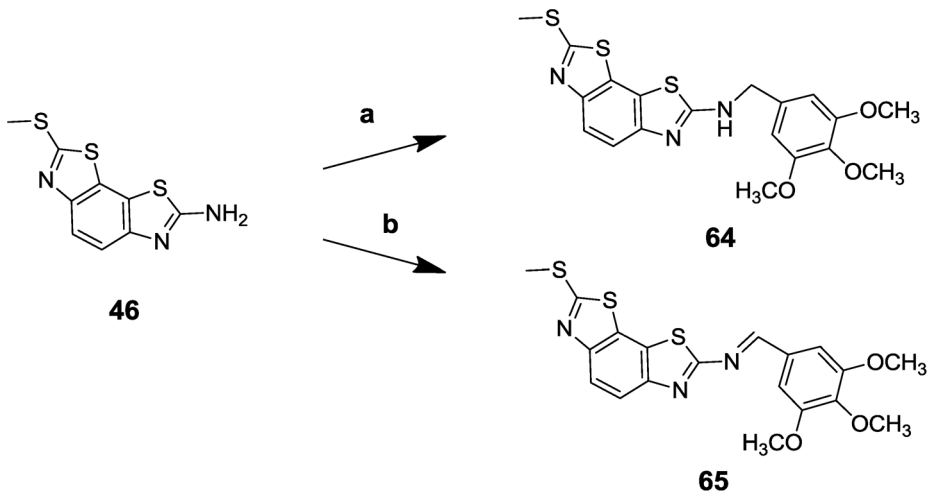
Figure 2.

Mode of inhibition of benzobisthiazole **49**. A dilution series of compound **49** was examined in the FRET-based *S. aureus* helicase assay[20] with varying concentrations of ATP (**A**) or DNA (**B**) to determine IC_{50} 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]



Scheme 1.

Reagents and conditions: a, benzoyl chlorides, CH_2Cl_2, yields 31–95%; b, aq. $KMnO_4$, glacial HOAc, dioxane, 73% for **47**, and 44% for **48**; c, R^2R^3NH, DMF, 80–100 °C, yields 20–83%.

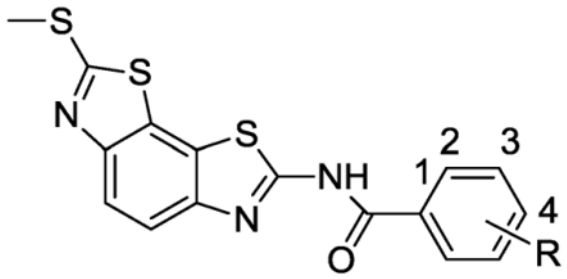


Scheme 2.

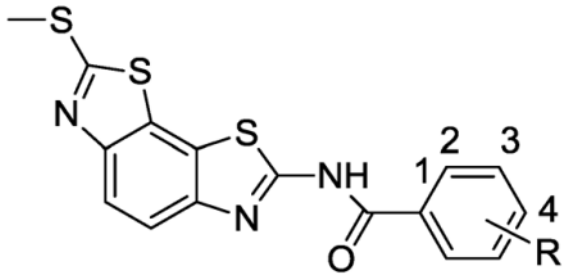
Reagents and conditions: a, 3,4,5-trimethoxybenzaldehyde, $Na(OAc)_3BH$, glacial HOAc, DMSO, r.t., 48%; b, 3,4,5-trimethoxybenzaldehyde, toluene, reflux, 67%.

Table 1

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



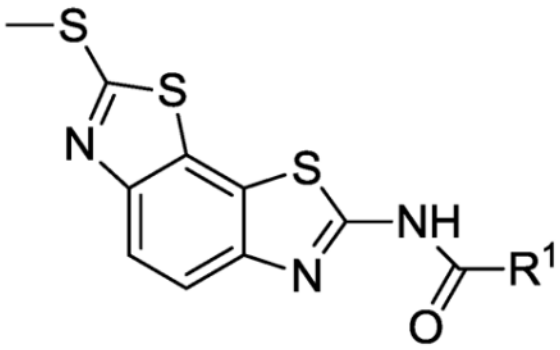
Compd.	R	IC_{50} Ba Helicase (μM)
1	3-N[-(C=O)CH_2CH_2(C=O)-]	5.0
2	4-N(CH_2CH_3)$_2$	>100
3	4-*t*Bu	>100
4	4-OPh	>100
5	4-O*i*Pr	>100
6	4-SO_2N(CH_2CH_3)$_2$	>100
7	4-CH_3	7.6
8	4-CN	11.0
9	4-OCH_2CH_3	6.9
10	4-Cl	5.0
11	4-$COOCH_3$	3.7
12	4-N(CH_3)$_2$	2.4
13	4-OCH_3	2.2
14	4-N[-(C=O)CH_2CH_2(C=O)-]	1.3
15	3-N(CH_3)$_2$	36.0
16	3-OCH_3	12.5
17	3-Cl	5.3
18	3-F	3.3
19	3-NO_2	2.8
20	3-Br	2.3
21	2-OPh	>100
22	2-Cl	>100
23	2-Br	>100
24	2-F	~50
25	2-CH_3	6.6
26	2,4-(OCH_3)$_2$	>100
27	2,6-(OCH_3)$_2$	>100
28	2,6-F_2	64.5
29	3,4-(CH_3)$_2$	3.2

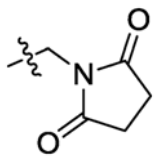
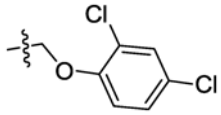
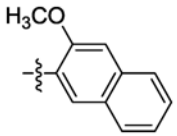


Compd.	R	IC_{50} Ba Helicase (μM)
30	3,4-(OCH_2CH_2O)	3.0
31	3,4-$(OCH_3)_2$	2.1
32	3,5-$(OCH_3)_2$	1.7
33	3,4,5-$(OCH_3)_3$	0.7

Table 2

Bacillus anthracis and *Staphyloccocus aureus* helicase inhibition by benzobisthiazole compounds **34**–**45**.

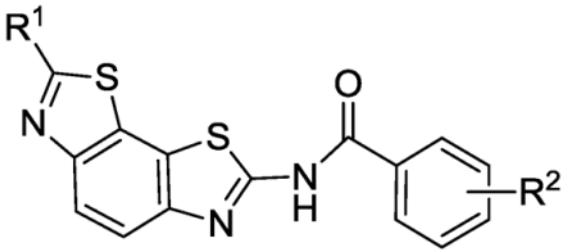


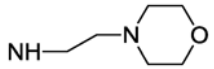
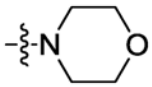
Compd.	R^1	IC_{50} Ba Helicase (μM)
34	cyclopropyl	>100
35	CH_2Ph	47
36	CH_2CH_2Ph	>100
37		>100
38		>100
39		>50

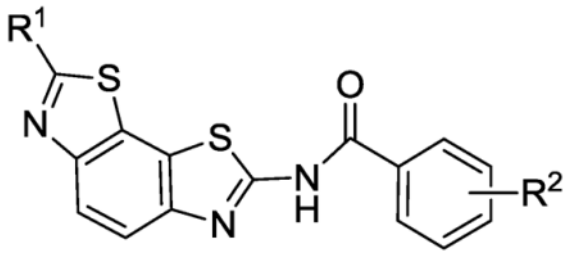
Compd.	R^1	IC_{50} Ba Helicase (μM)
40		>100
41	2-furan	102
42	2-thiophene	90
43		>50
44		>50
45	2-pyrazine	28

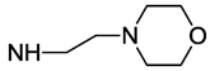
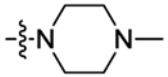
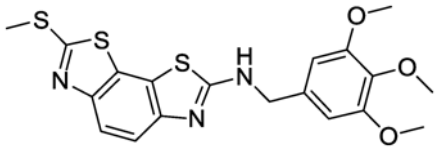
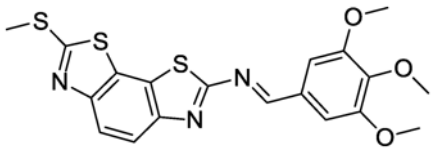
Table 3

Bacillus anthracis and *Staphyloccocus aureus* helicase inhibition by benzobisthiazole compounds **16**, **33** and **49**–**65**.



Compd.	R^1	R^2	IC_{50} *B. anthracis* Helicase (μM)	IC_{50} *S. aureus* 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	n.d.
52	NH-isobutyl	3-OCH_3	13.0	9.0	44
53	NH-cyclohexyl	3-OCH_3	90	>100	n.d.
54	NH-Bn	3-OCH_3	75	>100	n.d.
55	$NHCH_2CH_2N(CH_3)_2$	3-OCH_3	1.0	1.0	
56		3-OCH_3	0.5	2.2	9.6
57		3-OCH_3	2.3	3.0	>100
58	$NHCH_2CH_2OH$	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	$NHCH_2CH_2N(CH_3)_2$	3,4,5-$(OCH_3)_3$	0.2	0.2	<0.78



Compd.	R^1	R^2	IC_{50} *B. anthracis* Helicase (μM)	IC_{50} *S. aureus* Helicase (μM)	HeLa CC_{50} in SFM (μM)
61		3,4,5-$(OCH_3)_3$	0.2	0.2	5.7
62		3,4,5-$(OCH_3)_3$	0.2	0.2	2.8
63	$NHCH_2CH_2OH$	3,4,5-$(OCH_3)_3$	0.2	0.3	>100
64			>100	>100	n.d.
65			>100	>100	n.d.