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PII: S0040-4020(15)30173-3

DOI: 10.1016/j.tet.2015.10.073

Reference: TET 27245

To appear in: *Tetrahedron*

Received Date: 13 August 2015

Revised Date: 25 October 2015

Accepted Date: 26 October 2015

Please cite this article as: Zheng Y, Zhou J, Cooper Jr. SM, Opoku-Temeng C, De Brito AM, Sintim HO, Structure-activity relationship studies of c-di-AMP synthase inhibitor, bromophenol-thiohydantoin, *Tetrahedron* (2015), doi: 10.1016/j.tet.2015.10.073.

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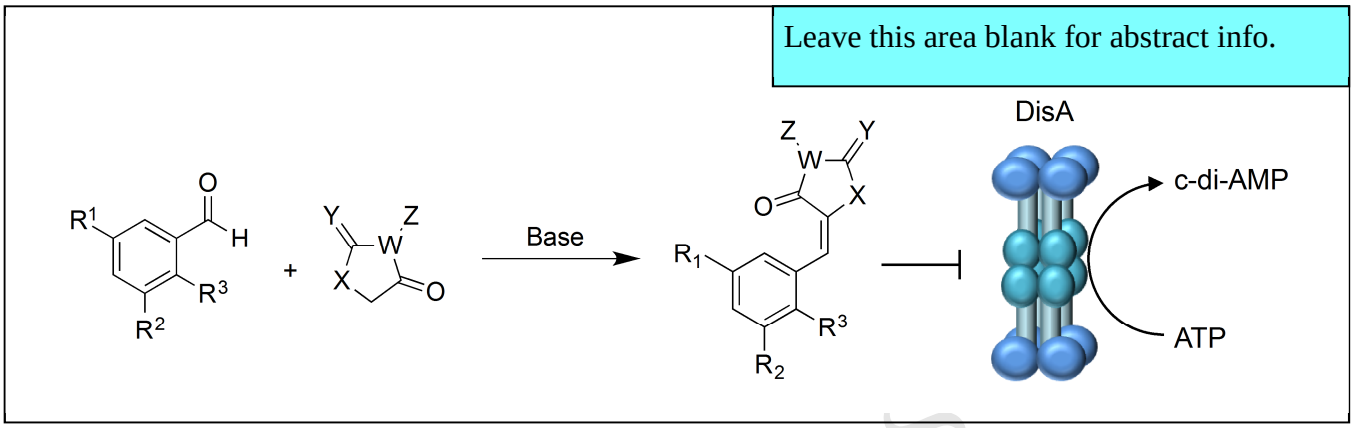

Tetrahedron
journal homepage: www.elsevier.com

Structure-activity relationship studies of c-di-AMP synthase inhibitor, bromophenol-thiohydantoin

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
c-di-AMP
DisA
inhibitor
bromophenol-TH
SAR

ABSTRACT

C-di-AMP, a bacterial second messenger, regulates various processes in Gram-positive bacteria and mycobacteria. Small molecule inhibitors of c-di-AMP metabolic enzymes could affect bacterial growth and viability. A medium throughput screening identified bromophenol-thiohydantoin (BTH) as the first inhibitor of c-di-AMP synthase, DisA. Herein, we performed SAR studies of bromophenol-thiohydantoin to identify the salient features on BTH that are important for DisA inhibition. Seemingly minor substitution changes (for example aromatic bromo to chloro substitutions) resulted in dramatic changes in ligand potency. Bromophenol TH is specific for c-di-AMP synthase and did not inhibit RocR (c-di-GMP PDE), YybT (c-di-AMP PDE) or WspR (c-di-GMP synthase).

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1. Introduction

Multidrug-resistant bacteria continue to cause a threat to public health, despite decades of antibiotics development.[1] The US CDC in a report, emphasized the continuous need for new antibiotics to keep up with the ever evolving antibacterial resistance.[2] Efforts have been put into the discovery of new types of antibiotic targets. Enzymes that make or degrade bacterial cyclic dinucleotide (cdn) second messengers or proteins/RNA receptors of cdn have emerged as new antibacterial targets, because cyclic dinucleotides regulate various cellular processes in different bacterial species, including virulence, biofilms and cell wall synthesis.[3] Since its discovery in 2008,[4] c-di-AMP has been found in many Gram-positive bacteria and mycobacteria, including human pathogens *S. aureus*,[5] *M. tuberculosis*,[6] *L. monocytogenes*,[7] and other bacteria.[8] C-di-AMP is essential for bacterial survival.[7,9,10] In some bacteria, c-di-AMP has been shown to regulate DNA integrity scanning,[4] potassium ion transport,[11] bacterial cell wall homeostasis,[12] fatty acid synthesis[13] and host type I interferon response induction[14]. Attempts to delete c-di-AMP synthase, diadenylate cyclase (DAC), from the genome of important human pathogens such as *S. aureus* have been unsuccessful,[15] implying that inhibitors of c-di-AMP synthase could have antibacterial properties.

Motivated by the potential utility of DAC inhibitors as important probes to study c-di-AMP signaling in bacteria or as new generation antibiotics we embarked on the development of a high throughput assay, using coralyne, to discover such inhibitors.[16] Using the coralyne assay, we identified the first c-di-AMP synthase inhibitor, 5-(3, 5-dibromo-2-hydroxylbenzylidene)-2-thioxoimidazolidin-4-one (referred to as bromophenol thiohydantoin or bromophenol-TH or BTH, see Figure 1 for structure).[17] BTH contains a halogen (Br), phenolic and 2-thiohydantoin (or 2-thioxoimidazolidin-4-one) moieties. To guide the development of BTH-based/inspired DAC inhibitors, we initiated a structure–activity relationship (SAR) study to tease out the salient features of BTH that are important for DisA inhibition. Herein, we present the synthesis of 19 BTH analogs, and investigate the importance of the various functionalities found on BTH for DAC inhibition.

2. Experimental section

2.1 Synthesis of BTH analogs

Three different synthetic protocols were explored for the synthesis of the analogs and the one that gave the best yield was chosen for making adequate amounts for characterization and biological evaluation.

General synthesis procedure **A**: A mixture of compound **20**, aldehyde (0.5 mmol, 1 equiv. **20**), compound **21** (1.2 equiv.), and NaOAc (3 equiv.) in glacial AcOH (4 mL) was stirred at reflux (12 h) until no starting material remained, assessed by TLC (SiO_2, hexane/ethyl acetate, 1:1, v/v). The reaction mixture was cooled down to room temperature, followed by the addition of iced water. The resulting precipitate was filtered and washed successively with water and Et_2O (10 mL). The product was subjected to column purification (hexane/ethyl acetate, 0→35%).

General synthesis procedure **B**: A mixture of aldehyde (0.5 mmol, 1 equiv. **20**), compound **21** (1.2 equiv.), and few drops of piperidine in EtOH (4 mL) was stirred at 80-90 ^{o}C (4-12 h) until no starting material remained, assessed by TLC (SiO_2, hexane/ethyl acetate, 1:1, v/v). The reaction mixture was cooled down to room temperature. The resulting precipitate was filtered and washed successively with water and Et_2O (10 mL).

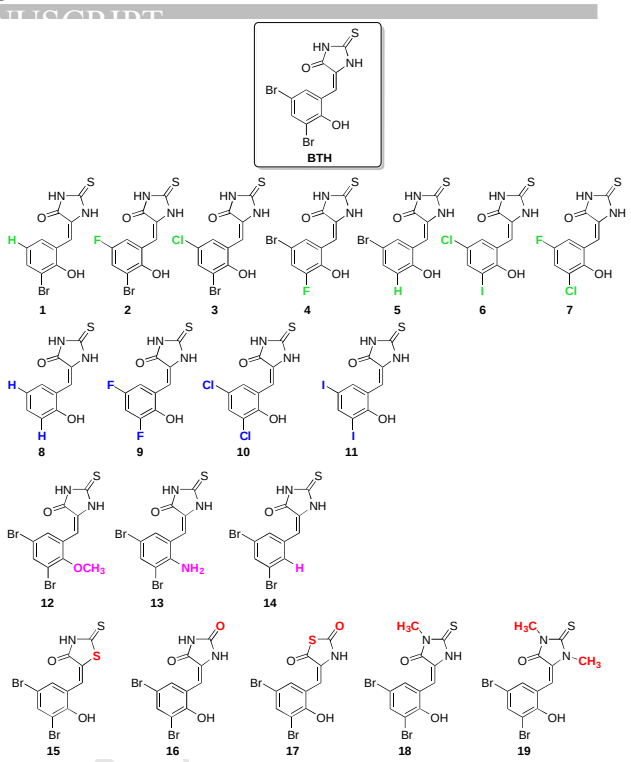


Figure 1. Structures of BTH and analogs synthesized for this study. Nineteen BTH analogs are grouped into four categories, accoding to their substitution types.

General synthesis procedure **C**: A mixture of aldehyde (0.5 mmol, 1 equiv. **20**), compound **21** (1.1 equiv.), and NH_4OAc (1.5 equiv.) in toluene (4 mL) was stirred at 80-90 ^{o}C (12 h) until no starting material remained, assessed by TLC (SiO_2, hexane/ethyl acetate, 1:1, v/v). The reaction mixture was cooled down to room temperature. The resulting precipitate was filtered and washed successively with water and Et_2O (10 mL).

5-(3-bromo-2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 1)

Following synthesis procedure A, compound **1** was obtained as brown solid. Compound **1** (38 mg, 50% yield) ^{1}H NMR (500 MHz, MeOD) δ 7.52 (dd, *J* = 7.9, 1.1 Hz, 1H), 7.46 (d, *J* = 7.6 Hz, 1H), 6.87 (t, *J* = 7.9 Hz, 1H), 6.75 (s, 1H). ^{13}C NMR (126 MHz, MeOD) δ 180.04, 167.60, 153.54, 135.17, 131.06, 130.24, 124.22, 122.78, 112.74, 108.71. HRMS (ESI$^-$) *m/z* calcd for $C_{10}H_6BrN_2O_2S$ $[M-H]^-$ 296.9333, found 296.9350.

5-(3-bromo-5-fluoro-2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 2)

Following synthesis strategy A, compound **2** was obtained as yellow solid. Compound **2** (68 mg, 29% yield) ^{1}H NMR (500 MHz, MeOD) δ 7.34 (s, 1H), 7.18 (s, 1H), 6.60 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 177.94, 167.02, 156.30, 154.39, 152.99, 129.99, 123.79, 121.28, 121.08, 116.87, 116.70, 113.55, 109.10. HRMS (ESI$^-$) *m/z* calcd for $C_{10}H_5BrFN_2O_2S$ $[M-H]^-$ 314.9239, found 314.9251.

5-(3-bromo-5-chloro-2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 3)

Following synthesis strategy A, compound **3** was obtained as brown solid. Compound **3** (28 mg, 12% yield) ^{1}H NMR (600 MHz, MeOD) δ 7.55 (d, *J* = 2.3 Hz, 1H), 7.48 (d, *J* = 2.4 Hz, 1H), 6.64 (s, 1H). ^{13}C NMR (151 MHz, MeOD) δ 178.09,

165.50, 151.50, 131.99, 129.08, 128.37, 124.07, 123.32, 111.48, 105.34. HRMS (ESI$^-$) *m/z* calcd for $C_{10}H_5BrClN_2O_2S$ [M-H]$^-$ 330.8944, found 330.8963

5-(5-bromo-3-fluoro-2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 4)

Following synthesis strategy B, compound **4** was obtained as yellow solid. Compound 4 (132 mg, 57% yield) ^{1}H NMR (400 MHz, DMSO) δ 7.36 (s, 1H), 7.30 (d, *J* = 10.3 Hz, 1H), 6.33 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 175.39, 166.19, 154.37, 152.77, 149.94, 130.98, 129.69, 125.29, 125.26, 118.88, 118.73, 108.95, 104.01. HRMS (ESI$^-$) *m/z* calcd for $C_{10}H_5BrFN_2O_2S$ [M-H]$^-$ 314.9239, found 314.9217.

5-(5-bromo-2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 5)

Following synthesis strategy B, compound **5** was obtained as yellow solid. Compound **5** (156 mg, 67% yield) ^{1}H NMR (600 MHz, DMSO) δ 7.39 (d, *J* = 2.5 Hz, 1H), 7.16 (dd, *J* = 8.7, 2.6 Hz, 1H), 6.57 (d, *J* = 8.7 Hz, 1H), 6.01 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 176.18, 168.36, 158.56, 137.42, 134.49, 132.17, 124.56, 120.73, 108.95, 107.74. HRMS (ESI$^-$) m/z calcd for $C_{10}H_6BrN_2O_2S$ [M-H]$^-$ 296.9333, found 296.9356.

5-(5-chloro-2-hydroxy-3-iodobenzylidene)-2-thioxoimidazolidin-4-one (compound 6)

Following synthesis strategy B, compound **6** was obtained as yellow solid. Compound **6** (120 mg, 56% yield) ^{1}H NMR (600 MHz, DMSO) δ 7.53 (s, 1H), 7.19 (s, 1H), 6.09 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 172.79, 166.19, 162.94, 138.04, 133.27, 130.73, 121.58, 115.99, 112.63, 95.29. HRMS (ESI$^-$) m/z calcd for $C_{10}H_5ClIN_2O_2S$ [M-H]$^-$ 378.8805, found 378.8813.

5-(3-chloro-5-fluoro-2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 7)

Following synthesis strategy B, compound **7** was obtained as yellow solid, a mixture of E/Z isomers. Compound **7** (153 mg, 61% yield) ^{1}H NMR (600 MHz, DMSO) δ 8.19 (dd, *J* = 10.3, 3.1 Hz, 0.1H), 7.38 (dd, *J* = 8.0, 3.1 Hz, 0.1H), 7.20 (dd, *J* = 8.0, 3.2 Hz, 1H), 7.09 (dd, *J* = 9.9, 3.1 Hz, 1H), 6.86 (s, 0.1H), 6.17 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 176.07, 175.23, 166.85, 163.54, 154.78, 154.13, 153.21, 152.64, 151.10, 148.38, 133.48, 130.94, 123.47, 123.41, 123.17, 123.09, 123.06, 123.00, 121.58, 121.50, 117.57, 117.35, 117.18, 116.89, 116.75, 115.09, 114.93, 111.49, 110.00. HRMS (ESI$^-$) m/z calcd for $C_{10}H_5ClFN_2O_2S$ [M-H]$^-$ 270.9744, found 270.9723.

5-(2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 8)

Following synthesis strategy A, compound **8** was obtained as brown solid. Compound **8** (213 mg, 59% yield) ^{1}H NMR (500 MHz, MeOD) δ 7.36 (d, *J* = 7.5 Hz, 1H), 7.21 (t, *J* = 7.4 Hz, 1H), 6.99 – 6.73 (m, 2H), 6.64 (s, 1H). ^{13}C NMR (126 MHz, MeOD) δ 180.41, 168.49, 158.36, 133.73, 132.72, 129.55, 121.82, 120.80, 118.03, 112.52. HRMS (ESI$^-$) m/z calcd for $C_{10}H_5N_2O_2S$ [M-H]$^-$ 219.0228, found 219.0240.

5-(3,5-difluoro-2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 9)

Following synthesis strategy A, compound **9** was obtained as brown solid. Compound **9** (72 mg, 89% yield) ^{1}H NMR (500 MHz, MeOD) δ 7.05 (d, *J* = 9.2 Hz, 1H), 6.99 (ddd, *J* = 11.0, 8.3, 2.9 Hz, 1H), 6.63 (s, 1H). ^{13}C NMR (126 MHz, MeOD) δ 177.80, 166.09, 155.31 (d, *J* = 11.8 Hz), 153.42 (d, *J* = 11.7 Hz), 152.91 (d, *J* = 12.8 Hz), 151.00 (d, *J* = 12.8 Hz), 141.64 (d, *J* = 13.7 Hz), 128.93 (s), 122.97 (dd, *J* = 9.4, 4.1 Hz), 111.46 (s), 111.28 (d, *J* = 3.2 Hz), 106.64 (s), 104.77 (s), 104.56 (d, *J* = 3.8 Hz), 104.36 (s). HRMS (ESI$^-$) m/z calcd for $C_{10}H_5F_2N_2O_2S$ [M-H]$^-$ 255.0040, found 255.0049.

5-(3,5-dichloro-2-hydroxybenzylidene)-2-thioxoimidazolidin-4-one (compound 10)

Following synthesis strategy A, compound **10** was obtained as brown solid. Compound **10** (58 mg, 78% yield) ^{1}H NMR (500 MHz, MeOD) δ 7.19 (s, 1H), 7.01 (s, 1H), 6.30 (s, 1H). ^{13}C NMR (126 MHz, MeOD) δ 180.47, 168.16, 163.51, 133.26, 131.80, 128.79, 128.12, 125.62, 117.68, 116.00. HRMS (ESI$^-$) *m/z* calcd for $C_{10}H_5Cl_2N_2O_2S$ [M-H]$^-$ 286.9449, found 286.9476.

5-(2-hydroxy-3,5-diiodobenzylidene)-2-thioxoimidazolidin-4-one (compound 11)

Following synthesis strategy B, compound **11** was obtained as yellow solid. Compound **11** (100 mg, 50% yield) ^{1}H NMR (600 MHz, DMSO) δ 7.69 (s, 1H), 7.36 (d, *J* = 1.9 Hz, 1H), 6.05 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 172.31, 166.00, 164.21, 145.84, 142.32, 129.76, 123.86, 112.94, 97.38, 71.81. HRMS (ESI$^-$) *m/z* calcd for $C_{10}H_5I_2N_2O_2S$ [M-H]$^-$ 470.8161, found 470.8165.

5-(3,5-dibromo-2-methoxybenzylidene)-2-thioxoimidazolidin-4-one (compound 12)

Following synthesis strategy C, compound **12** was obtained as brown solid, a mixture of E/Z isomers. Compound **12** (25 mg, 12% yield) ^{1}H NMR (400 MHz, DMSO/D_2O) δ 8.69 (s, 1H), 8.57 (s, 0.26H), 7.85 (s, 0.3H), 7.68 (d, *J* = 1.9 Hz, 1H), 6.66 (s, 0.28H), 6.16 (s, 1H), 4.08 (s, 1H), 3.73 (s, 3H).^{13}C NMR (151 MHz, DMSO/D_2O) δ 183.85, 183.38, 182.96, 174.48, 172.03, 170.35, 153.68, 133.00, 132.45, 131.93, 117.44, 116.94, 98.03, 50.25. HRMS (ESI$^-$) *m/z* calcd for $C_{11}H_7Br_2N_2O_2S$ [M-H]$^-$ 388.8595, found 388.8583.

5-(2-amino-3,5-dibromobenzylidene)-2-thioxoimidazolidin-4-one (compound 13)

Following synthesis strategy B, compound **13** was obtained as yellow solid, a mixture of E/Z isomers. Compound **13** (86 mg, 30% yield) ^{1}H NMR (500 MHz, DMSO/D_2O) δ 9.39 (s, 0.3H), 7.91 (s, 0.3H), 7.89 (s, 0.3H), 7.58 (d, *J* = 2.0 Hz, 1H), 7.41 (s, 1H), 6.43 (s, 1H). ^{13}C NMR (126 MHz, DMSO/D_2O) δ 181.92, 179.82, 166.35, 159.34, 144.82, 135.60, 134.09, 132.50, 132.38, 131.87, 130.67, 130.26, 123.89, 121.09, 119.50, 115.56, 110.34, 109.95, 108.15, 107.78. HRMS (ESI$^-$) *m/z* calcd for $C_{10}H_6Br_2N_3OS$ [M-H]$^-$ 373.8598, found 373.8589.

5-(3,5-dibromobenzylidene)-2-thioxoimidazolidin-4-one (compound 14)

Following synthesis strategy B, compound **14** was obtained as yellow solid. Compound **14** (45 mg, 20% yield) ^{1}H NMR (600 MHz, DMSO) δ 8.22 (s, 2H), 7.58 (s, 1H), 5.95 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 183.21, 183.09, 170.83, 140.49, 130.56, 130.50, 122.27, 103.54. HRMS (ESI$^-$) *m/z* calcd for $C_{10}H_5Br_2N_2OS$ [M-H]$^-$ 358.8489 found 358.8506.

5-(3,5-dibromo-2-hydroxybenzylidene)-2-thioxothiazolidin-4-one (compound 15)

Following synthesis strategy A, compound **15** was obtained as brown solid. Compound **15** (28 mg, 13% yield) ^{1}H NMR (500 MHz, MeOD) δ 7.83 (s, 1H), 7.75 (d, *J* = 2.2 Hz, 1H), 7.42 (d, *J* = 2.2 Hz, 1H). ^{13}C NMR (126 MHz, MeOD) δ 195.71, 170.06, 153.56, 137.18, 131.22, 128.96, 125.82, 125.50, 113.12, 112.51.

HRMS (ESI⁻) *m/z* calcd for $C_{10}H_4Br_2NO_2S_2$ $[M-H]^-$ 391.8050, found 391.8072.

5-(3,5-dibromo-2-hydroxybenzylidene)imidazolidine-2,4-dione (compound 16)

Following synthesis strategy A, compound **16** was obtained as brown solid. Compound **16** (52 mg, 25% yield) ^{1}H NMR (400 MHz, MeOD) δ 7.61 (d, *J* = 2.3 Hz, 1H), 7.50 (d, *J* = 2.3 Hz, 1H), 6.64 (s, 1H). ^{13}C NMR (151 MHz, MeOD) δ 167.28, 157.11, 154.27, 136.19, 132.80, 130.84, 126.27, 114.05, 112.25, 105.95. HRMS (ESI⁻) *m/z* calcd for $C_{10}H_5Br_2N_2O_3$ $[M-H]^-$ 358.8667, found 358.8685.

4-(3,5-dibromo-2-hydroxybenzylidene)thiazolidine-2,5-dione (compound 17)

Following synthesis strategy C, compound **17** was obtained as brown solid. Compound **17** (146 mg, 67 % yield) ^{1}H NMR (600 MHz, MeOD) δ 8.06 (s, 1H), 7.56 (d, *J* = 2.4 Hz, 1H), 7.40 (d, *J* = 2.4 Hz, 1H). ^{13}C NMR (151 MHz, DMSO) δ 173.64, 172.65, 157.59, 134.04, 128.65, 125.05, 124.81, 124.28, 113.57, 105.24. HRMS (ESI⁻) *m/z* calcd for $C_{10}H_4Br_2NO_3S$ $[M-H]^-$ 375.8279, found 375.8282.

5-(3,5-dibromo-2-hydroxybenzylidene)-3-methyl-2-thioxoimidazolidin-4-one (compound 18)

Following synthesis strategy B, compound **18** was obtained as yellow solid. Compound **18** (201 mg, 89 % yield) ^{1}H NMR (600 MHz, DMSO) δ 7.46 (d, *J* = 2.6 Hz, 1H), 7.29 (d, *J* = 2.6 Hz, 1H), 6.25 (s, 1H), 3.10 (s, 3H). ^{13}C NMR (151 MHz, DMSO) δ 173.41, 164.62, 160.61, 135.29, 135.12, 129.68, 124.54, 116.92, 113.65, 102.85, 27.00. HRMS (ESI⁻) *m/z* calcd for $C_{11}H_7Br_2N_2O_2S$ $[M-H]^-$ 388.8595, found 388.8571.

5-(3,5-dibromo-2-hydroxybenzylidene)-1,3-dimethyl-2-thioxoimidazolidin-4-one (compound 19)

Following synthesis strategy B, compound **19** was obtained as yellow solid. Compound **19** (156 mg, 67% yield) ^{1}H NMR (600 MHz, DMSO) δ 8.63 (d, *J* = 2.5 Hz, 1H), 7.44 (d, *J* = 2.6 Hz, 1H), 7.21 (s, 1H), 3.50 (s, 3H), 3.20 (s, 3H). ^{13}C NMR (151 MHz, DMSO) δ 173.99, 163.12, 161.15, 135.74, 131.56, 125.33, 122.68, 119.73, 116.45, 100.24, 30.35, 27.82.HRMS (ESI⁻) *m/z* calcd for $C_{12}H_9Br_2N_2O_2S$ $[M-H]^-$ 402.8751, found 402.8790.

2.2 Protein purification

BL21(DE3) cells with protein expression plasmids were cultured in LB medium with 100 μg/ml ampicillin or 50 μg/ml kanamycin at 37 °C. Expression was induced by addition of 1 mM IPTG. After induction at 30 °C for 6 hours (WspR D70E and YybT) or at 16 °C overnight (DisA and RocR), cells were harvested by centrifugation at 4,000 rpm for 15 min. Cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl for WspR D70E, RocR and YybT, and 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl for DisA) and lysed by sonication. Proteins were purified by GE Hitrap Nickel columns and dialyzed into lysis buffer.

2.3 The coralyne assay

BTH and analogs were stored as 100 mM stock solutions in DMSO. 300 μM ATP, 10 μM coralyne, 3 mM KI, 50 μM BTH/analogs and 1 μM DisA were mixed in reaction buffer, which contains 40 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM $MgCl_2$. Fluorescence measurements were performed on a Molecular Devices SpectraMax M5e microplate reader (λ_{ex}=420 nm and λ_{em}=475 nm). Reactions were monitored for 30 min with 2 min interval.

*2.4 IC_{50} measurements of BTH, compound **5** and compound **6***

300 μM ATP, 33 nM ^{32}P-ATP and various concentrations of BTH/analogs were mixed in reaction buffer. Reaction was initiated by addition of 1 μM DisA and performed at 30 °C for 1 hour. Aliquots of the reaction mixtures were spotted on a TLC plate (EMD Millipore TLC Cellulose). TLC running buffer was saturated $(NH_4)_2SO_4$: 1.5 M KH_2PO_4 = 1:1.5.

2.5 Assay to determine if BTH is a covalent inhibitor

10 μM DisA was incubated with 100 μM BTH or DMSO on ice for 90 min. BTH/DMSO was removed by filtration. DisA was washed by reaction buffer for three times. DisA was then incubated with 300 μM ATP, 10 μM coralyne and 3 mM KI. Reaction was monitored by fluorescence emission on a Molecular Devices SpectraMax M5e microplate reader (λ_{ex}=420 nm and λ_{em}=475 nm). The reaction was monitored for 30 min with 5 min interval.

2.6 Effect of BTH on YybT, WspR D70E and RocR activity

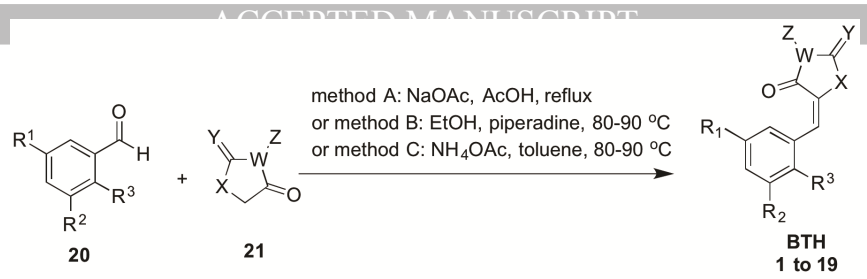
1.5 μM YybT reacted with 50 μM c-di-AMP and 16 nM ^{32}P-c-di-AMP in the presence or absence of 100 μM BTH in reaction buffer (100 mM Tris-HCl, pH 8.3, 20 mM KCl, 0.5 mM $MnCl_2$ and 1 mM DTT) at 37 °C. The reaction was stopped after 30 min. 1 μM WspR D70E reacted with 300 μM GTP and 33 nM ^{32}P-GTP in the presence or absence of 100 μM BTH in reaction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 5 mM $MgCl_2$) at 37 °C. Reaction was stopped after 2 hours. 1.2 μM RocR reacted with 50 μM c-di-GMP and 16 nM ^{32}P-c-di-GMP in reaction buffer (100 mM Tris-HCl, pH 8.0, 20 mM KCl and 25 mM $MgCl_2$) at 37 °C. Reaction was stopped after 30 min. All the reactions were monitored by TLC.

3 Results and Discussion

BTH, is easily prepared via the condensation of 3, 5-dibromosalicylic aldehyde and 2-thiohydantoin.[18-20] Conveniently, this ease of preparation allows for facile preparation of analogs *via* condensation reactions with various commercially available halo-substituted salicylic aldehydes and hydantoins, thiohydantoins or rhodanines. (Scheme 1). Electronic and steric derivations are possible by varying the halogens present on the aromatic ring.

Four types of BTH analogs (A, B, C, D, see Figure 1) were prepared. In type A, we synthesized analogs containing mixed halogens whereas type B consisted of only one type of halogens. In type C, we investigated the role of the phenolic group in BTH by replacing the OH with hydrogen, amino or methoxy groups. Type D explored how subtle changes to the heterocyclic ring of BTH would affect DisA inhibition.[21-23]

3.1 Inhibition of DisA by BTH analogs



Scheme 1. Synthetic strategy for preparing BTH and analogs. Conditions for method A, B and C are listed above the arrow. The method that gave the best yield was chosen for each compound.

The coralyne assay[16] (Supplementary Material, Figure S1) was performed to identify the DisA inhibition effects of BTH analogs. In the absence of c-di-AMP, iodide quenches corlayne fluorescence. As DisA synthesizes c-di-AMP, the dinucleotide forms a complex with coralyne and protects coralyne from quenching effects. Coralyne fluorescence emission intensity correlates with c-di-AMP concentration. In the presence of inhibitors, the concentration of c-di-AMP would be lower and hence the fluorescence of coralyne would be reduced.[16] The coralyne assay indicated that BTH was the strongest inhibitor, followed by compound **15** in group D and compound **3** in group A. Other BTH analogs did not show significant inhibition of DisA. IC_{50} of BTH ($6.7 \pm 0.6 \times 10^{-5}$ M), compound **15** ($12.4 \pm 1.0 \times 10^{-5}$ M) and compound 3 ($17.9 \pm 1.7 \times 10^{-5}$ M) were obtained using radiolabeled ^{32}P-ATP (Figure 2). In group A, BTH, compound **1**, compound **2** and compound **3** have 3'-bromo and their inhibition effects increase as 5'-halogen size increases. It appears that the bromo substitution at the 3-position is important as deleting this substitution (compound **5**) resulted in loss of inhibition. Compound **6** or **7**, which replaced the dibromo with mixed halogens (Cl, F or I) were also inactive, highlighting the need for bromo substitution.

The diiodinated analog compound **11** of group B showed more inhibition than the non-halogenated analog compound **8**, difluorinated compound **9** and dichlorinated analog compound **10**, but it was still less active than BTH (see Supplementary Material, Figure S1). The hydroxyl group in BTH was replaced with a methoxy (compound **12**), amino (compound **13**) and hydrogen (compound **14**) to investigate if the phenolic moiety played a role in inhibition. In all of the three cases, replacement of the phenolic group with the aforementioned moieties completely diminished any inhibition effects. In group D, substitution of the nitrogen atom for sulfur atom at 1' position (2-thioxoimidazolidin-4-one to 2-thioxothiazolidin-4-one, compare compound **15** with BTH) has minor impact on inhibition, whereas replacement of the thiocarbonyl by carbonyl (2-thioxoimidazolidin-4-one to imidazolidine-2,4-dione, compare compound **16** with BTH) strongly reduced the inhibition effects. Substitution of the N-3 of the thiohydantoin group also eliminated inhibition by the BTH analog (compare compound **18** with BTH), Supplementary Material, Figure S1. compound **19**, the dimethyl thiohydantoin analog was also not active.

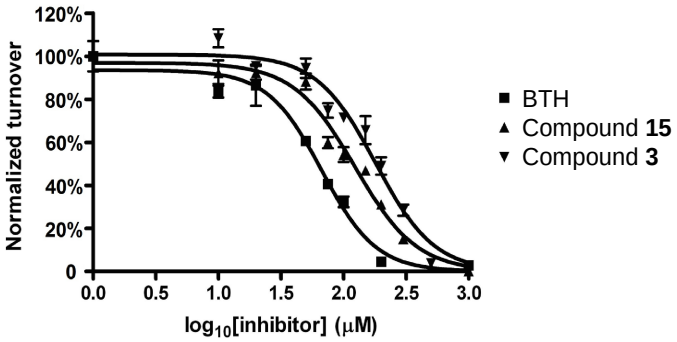


Figure 2. Enzymatic proficiency of DisA, 1 μM, (converting ^{32}P-ATP into ^{32}P-c-di-AMP) in the presence of increasing amounts of inhibitors BTH, compound **15** and compound **3**.

3.2 BTH is a non-covalent inhibitor

BTH is reported to be a non-competitive inhibitor[17], although the binding site for inhibitors within DisA is currently unknown. BTH contains an enone moiety and hence could act via covalent inhibition. To ascertain if covalent inhibition was operative, DisA was incubated with high concentration of BTH and then unbound BTH was removed by filtration. The remaining DisA was then washed three times with reaction buffer and then assayed for activity. The BTH-treated DisA retained enzymatic activity (similar to DMSO-treated DisA, see Supplementary Material, Figure S2), revealing that BTH and analogs are not covalent inhibitors.

3.3 BTH did not affect YybT, WspR D70E and RocR activity

We were curious to know the selectivity of BTH and analogs towards cyclic dinucleotide metabolism enzymes. We therefore tested BTH against a few cyclic dinucleotide-related enzymes, namely c-di-AMP PDE YybT from *B. subtilis*[24], c-di-GMP DGC WspR D70E[25] and c-di-GMP PDE RocR[26] from *P. aeruginosa*. Interestingly 100 μM of BTH did not affect any of these enzymes (Supplementary Material, Figure S3), implying that BTH is indeed specific for c-di-AMP synthase, DisA.

4 Conclusion.

Cyclic dinucleotides have emerged as interesting second messengers in both prokaryotes and eukaryotes and there has been an explosion of research activities to unravel the intricacies of signaling regulated by these nucleotides. There is however a paucity of small molecules that inhibit the metabolic enzymes of cyclic dinucleotides and such molecules could have important roles in elucidating cdn signaling and/or become next generation antibacterial agents. In this paper, we have demonstrated that bromophenol TH, the first reported inhibitor of c-di-AMP synthase is sensitive to modifications and most changes to this inhibitor abrogated inhibition. Interestingly, seemingly minor changes (for example changing an aromatic bromo to a chloro or iodo substituent) resulted in drastic inhibition profile, highlighting that halogen substitution can be used to cause dramatic changes in enzyme affinity for a ligand. Despite the presence of an enone moiety, bromophenol TH is not a covalent inhibitor of DisA. Future work will involve the search of other heterocyclic groups, apart from hydantoin or rhodanines, which could be appended to the bromophenol moiety with the ultimate goal of discovering more potent inhibitors of DisA.

Acknowledgments

We sincerely thank Dr. Karl-Peter Hopfner and Dr. Angelika Gründling for providing DisA plasmids and Dr. Zhaoxun Liang for providing RocR and YybT plasmids. Funding for this work was provided by the National Science Foundation (CHE1307218), Camille Dreyfus Foundation and Kraybill Biochemistry Fellowship (YZ).

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Supplementary Material

The coralyne assay to determine the inhibition profile of analogs, determination of whether BTH is a covalent inhibitor and the activity of BTH towards other cyclic dinucleotide-related enzymes.