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Synthesis of cyclic di-nucleotidic acids as potential inhibitors targeting diguanylate cyclase

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ABSTRACT

Five analogs of cyclic di-nucleotidic acid including c-di-GMP were synthesized and evaluated for their biological activities on Slr1143, a diguanylate cyclase of *Synechocystis* sp. Slr1143 was overexpressed from the recombinant plasmid which contained the gene of interest and subsequently purified by affinity chromatography. A new HPLC method capable of separating the compound and product peaks with good resolution was optimized and applied to the analysis of the compounds. Results obtained show that cyclic di-inosinylic acid **1b** demonstrates a stronger inhibition on Slr1143 than c-di-GMP and is a potential inhibitor for biofilm formation.

1. Introduction

Bacteria biofilm[1,2] has long been a cause of concern for medical specialists and researchers as many chronic bacterial infections are a result of its formation. Some of the pathogenic bacteria involved are *Vibrio cholerae*,[3,4] *Yersinia pestis*, *Pseudomonas aeruginosa*,[5] *Staphylococcus aureus*,[6] etc. For instance, *S. aureus* is an important human and animal pathogen that is found on the skin and mucosal surfaces of humans, specifically in the anterior nares. It is the primary and most common cause of surgical infections and nosocomial bacteremia due to biofilm-based infections,[6,7] which can increase hospital stays by durations of up to 2–3 days, incurring billions of added cost per year. In addition, biofilms are resistant to antimicrobial and antibiotics for a myriad of reasons. The altered living conditions of the biofilm (low pH, low pO_2, high pCO_2, low hydration level, etc) may result in low metabolic activity and hence low antimicrobial activity.[1,8] Furthermore, the antimicrobial agents may be trapped as waste or chelated by inactivating enzymes. Horizontal gene transfer within bacteria in biofilms also allows bacteria to gain resistance.[1] Quorum sensing signaling systems allow synchronization of the target gene expression within the biofilm, allowing the bacteria to evade the effects of antimicrobial agents.[9,10] In addition, a fraction of bacteria may differentiate into persisters cells which have extremely low metabolic rate and are non-growing. These cells are resistant and may regenerate the biofilm once the therapy has ended.[11]

Current therapies to reduce the rate of biofilm infections include prophylactic use of antibiotics and microbiocides through methods such as device coatings, device immersion, and surgical site irrigation.[12] Quorum quenching enzymes or inhibitors,[13] as well as antimicrobials to destroy persister cells, have been developed over the years. However despite these efforts, biofilms have continued to gain resistance to antimicrobial and antibiotics,[1,8–10] hence alternative therapeutic methods need to be explored.

Cyclic purine ribonucleotides such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are well-studied examples of second messengers in cellular signaling and function. Regulation of the intracellular levels of these cyclic purine ribonucleotide molecules is responsible for the bacterial response to external stimuli such as change in temperature, light, pH, oxygen levels, and nutrients. Although cGMP is commonly involved in cellular signaling in eukaryotic cells, prokaryotes do not seem to use it as a signaling molecule. Instead they utilize an alternative, cyclic guanosine monophosphate (c-di-GMP), also known as cyclic (3′–5′)diguanylic acid in cellular signaling. Synthesis of c-di-GMP involves the conversion of two guanosine triphosphate (GTP) molecules by diguanylate cyclases (DGCs) whereas the degradation of c-di-GMP is achieved via hydrolytic cleavage of the cyclic compound into guanosine monophosphate (GMP) by phosphodiesterases (PDEs) through the GGDEF domain of DGCs and the EAL domains in PDEs, respectively.[14] Recent works have established that c-di-GMP is a ubiquitous signaling molecule in bacteria but not in eukaryotic cells or archaea.[15] It has the ability to regulate motility and virulence gene expression of bacteria and, at high intracellular concentrations of c-di-GMP, biofilm formation and exopolysaccharide movement.[16] In addition, increases

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in the extracellular concentration of c-di-GMP was discovered to inhibit the growth of biofilm in methicillin resistant *S. aureus*, human and bovine intramammary mastitis isolates of *S. aureus* and human epithelial cells HeLa.[6] Subsequent research also proved that c-di-GMP is an effective immunomodulator and vaccine adjuvant against pneumococcal infection.[17]

Since c-di-GMP has shown to possess numerous biological potentials and is also able to control biofilm formation via regulation of c-di-GMP levels, we were interested to study the effects of other cyclic dinucleotides as potential antimicrobial agents. Hence we herein report the synthesis of five cyclic dinucleotides with the same backbone structure as c-di-GMP and their inhibitory activities against Slr1143, a diguanylate cyclase of *Synechocystis* sp.

2. Results and discussion

2.1. Synthesis of c-di-GMP and its analogues

There are two general approaches towards the synthesis of c-di-GMP (Fig. 1). The first approach synthesizes the cyclic backbone first with the base introduced downstream of the synthesis, as demonstrated by Giese and co-workers.[18] Although this method allows base derivatives of c-di-GMP to be made from a common synthetic intermediate, the analogues prepared can only have identical bases on both riboses. The second approach utilizes a nucleoside as the starting material. Hence besides obtaining analogues with identical nucleosides, those with different combinations of nucleosides can also be prepared. This allows a more diverse library of cyclic dinucleotides to be formed. Thus for our synthesis, we have chosen to utilize the second approach and the synthetic route used (Scheme 1) was adapted and optimized from the procedure reported earlier by Hyodo et al.[19]

2′-*O*-TBDMS-protected nucleoside **3** was prepared by regioselectively protecting the 3′- and 5′-hydroxyl groups[20] of **2** before protecting the 2′-OH with TBDMSCl. To prepare **3a**, guanosine **2a** was first suspended in DMF at 0 °C. The di-*tert*-butylsilanediyl ditriflate then added preferentially reacted with the 5′-OH then with the 3′-OH to form the protected diol that was soluble in DMF. This allowed more guanosine to dissolve, resulting in a clear solution 45 min later. Imidazole was then added to neutralize the triflic acid formed and to serve as a nucleophilic catalyst for the protection of the 2′-OH by TBDMSCl to form a white precipitate **3a**. The free amine on the purine ring of **3a** was subsequently protected with a dimethylformamidine group to provide **4a**. An imine protecting group was chosen because it is susceptible to strong bases and could be cleaved at the end of the synthesis together with the β-cyanoethyl protecting group. For uridine **2b**, it was soluble in DMF but not inosine **2c**. However interestingly, **3b** and **3c** did not precipitate out of the reaction mixture and thus had to be purified by extraction and column chromatography.

To allow the 3′-OH to be phosphorylated, the silyl diol protecting group was cleaved with HF–pyridine complex to free the 3′- and 5′-OH groups. The product after extraction was of high purity (as observed from TLC) and was directly protected at the 5′-OH with a dilute concentration (so as to avoid the protection of the 3′-OH) of DMTrCl under anhydrous condition to give **5**. Unlike **4a** and **3b**, treatment of **3c** with HF–pyridine complex gave a detritylated product which was insoluble in DCM. Hence workup was simplified as only filtration was needed and the solid was washed thoroughly with saturated $NaHCO_3$ to neutralize the acid and remove the residual pyridine.

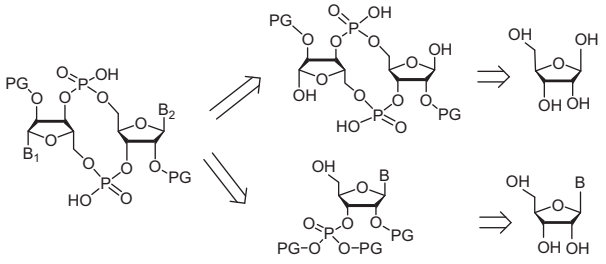


Figure 1. Retrosynthetic analysis of two major approaches for synthesizing c-di-GMP.

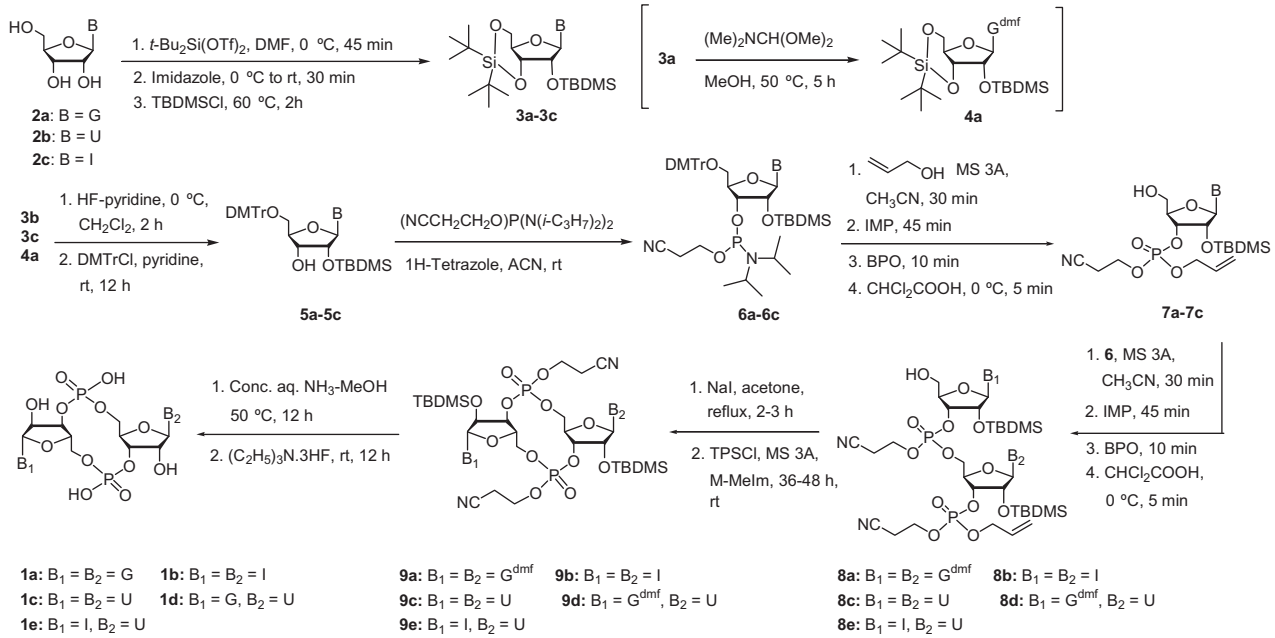
2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite **11** was initially synthesized (Scheme 2) and coupled with the free 3′-OH on **5** as according to Hyodo et al.[19] However **11** proved to be difficult to handle as it is highly moisture-sensitive. Furthermore, extractive workup performed after the phosphitylation reaction utilizing excess **11** caused the monochlorophosphoramidite to be hydrolyzed to monohydroxyphosphoramidite which was difficult to remove by column chromatography as it tails seriously and eventually eluted together with **6**. To circumvent this problem, we explored using 2-cyanoethyl bis-*N*,*N*-diisopropylphosphordiamidite **12**.[21] Coupling of **5** with **12** was achieved in the presence of 1*H*-tetrazole in anhydrous acetonitrile. 1*H*-Tetrazole besides acting as a base to extract the proton from the 3′-OH also converts diisopropylamine to diisopropylammonium tetrazolide salt, which precipitated out of the reaction mixture, thus simplifying the purification process. Although both **11** and **12** gave similar yields of **6** (~80%), **12** is not moisture-sensitive and thus purification of the reaction mixture was much easier.

Next, the diisopropylamine functionality on **6** was displaced by allyl alcohol with the aid of imidazolium perchlorate (IMP). Subsequent oxidation of the diribonucleoside phosphite to diribonucleoside phosphate was achieved with butanone peroxide (BPO) and finally the DMTr protecting group was cleaved with dichloroacetic acid to provide **7** in high yields. With **7** in hand, the compound was coupled to analogs of **6** in the presence of promoter IMP and molecular sieves as moisture scavenger.[22] This was followed by oxidation and detritylation to give **8** which was then treated with NaI in refluxing acetone to provide the alkoxide with allyl iodide as the side product. The alkoxide intermediate was highly hygroscopic and could only be successfully isolated when it was precipitated from cold anhydrous acetone. Treatment of alkoxide with *N*-methylimidazole (*N*-MeIm) as the nucleophilic catalyst and 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) as the condensing agent according to the procedure of Hyodo et al.[19] facilitated the cyclization of **8**. However the yields obtained were generally low and the cyclization reaction did not go to full completion even after 60 h. In addition, long reaction time also resulted in the formation of many side products (as shown by TLC analysis). Thus to circumvent these problems, we proceeded to modify the procedure by adding moisture scavenger (molecular sieves 3 Å) to the reaction mixture and using 5–7 equiv of TPSCl and *N*-MeIm. The reaction was completed in 36 h with minimal side products and the cyclized product **9** could be isolated more easily. As the reaction is diluted, the molecules are not in proximity, hence intramolecular reaction is promoted and minimal dimers of 8 were obtained. The major compound obtained is the cyclized product **9a–d**. However, the low yield of **9a–d** may be due to the presence of uncyclized products and disintegration of the dimers to form monomers.

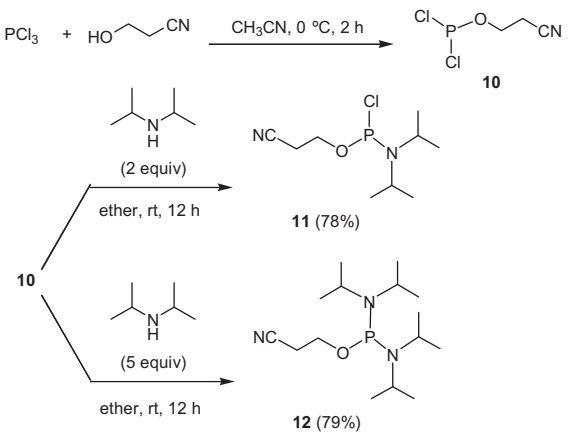
Treatment of **9** with a 1:1 mixture of concentrated aqueous ammonia and methanol followed by triethylammonium fluoride resulted in the deprotection of the dimethylformamidine, β-cyanoethyl, and silyl groups. The resulting crude product was purified by reverse-phase HPLC to provide **1a–e** in 10–15% overall yields.

2.2. Overexpression and purification of Slr1143

Gomelsky and co-workers have recently isolated PCR-amplified bacterial DNA fragments coding for the GGDEF domain-containing



Scheme 1. Synthesis of cyclic di-nucleotidic acid **1** (IMP = imidazolium perchlorate).



Scheme 2. Synthesis of **11** and **12**.

proteins and cloned them into vector pMAL-c2x (New England Biolabs) in strain *Escherichia coli* DH5α.[15] To evaluate the inhibitory activities of compounds **1a–e** against diguanylate cyclase, we obtained the recombinant bacterial cells from him and overexpressed Slr1143, a 344 amino acid diguanylate cyclase from oxygenic phototroph *Synechocystis sp. (cyanobacteria*), from it.

The cells were lysed by adding lysozyme (1 mg/mL of buffer) and incubating the cell mixture at 37 °C for 30 min. To further disrupt the cells to facilitate the release of the Slr1143 into the supernatant, the cells were sonicated for 10 min, 10 s on, 15 s off, on ice. Thereafter the crude cell extract was centrifuged and the supernatant obtained was subjected to affinity chromatography to obtain the pure Slr1143 protein.

2.3. Screening of compounds 1a–e activities against Slr1143

Prior to the enzymatic assay, a Michaelis–Menten graph was plotted to determine if Slr1143 was inactivated by an allosteric inhibitor like other DGCs.[23] To obtain this graph, different concentrations of substrate GTP were introduced to a mixture of pre-incubated Slr1143 (Final concentration: 1 μM) and assay buffer (50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM EDTA, and 50 mM NaCl) for 2 min. The reaction mixture was aliquoted out every 30 s and quenched by heating at 95 °C for 3 min. The experiment was repeated twice and a graph of product (c-di-GMP) peak area was plotted against time to obtain the rate of reaction. Subsequently, the average rate of reaction was plotted against the GTP concentration. From the results obtained (Fig. 2), it was observed that the rate of reaction initially increases before undergoing a decline. This indicates that Slr1143 exhibits allosteric product inhibition. At low concentrations of GTP, the amount of c-di-GMP formed was too low to inhibit the enzyme allosterically. As the GTP concentration increases, the frequency of collision between Slr1143 and the GTP increases, thus increasing in the rate of reaction. However the rate of reaction reaches a maximum and thereafter, the amount of c-di-GMP synthesized will be high enough to inhibit the activity of Slr1143, resulting in a reduction of the rate of reaction. From Figure 2, the optimum substrate concentration at which the enzyme activity exhibits the maximum rate was found to be

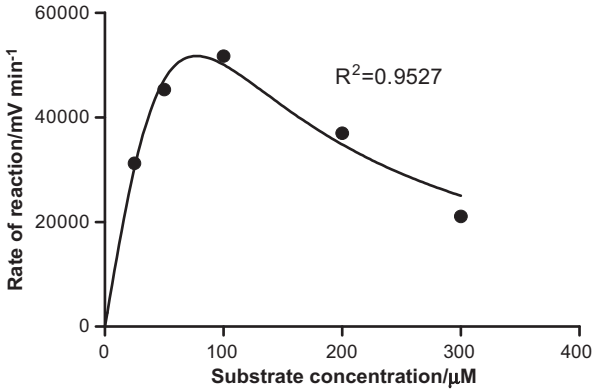


Figure 2. Activity of Slr1143 at 30 °C in the presence of different substrate (GTP) concentration. Final enzyme concentration: 1 μM. Enzymatic assay buffer: 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM EDTA, and 50 mM NaCl. HPLC eluent pair: 20 mM triethylammonium bicarbonate buffer, pH 7.0, methanol. Reaction time: 2 min.

~100 μM. Hence for our enzymatic assay, 100 μM of the respective compound **1a**–**e** and 100 μM of GTP were used.

For the enzymatic assay, Slr1143 was incubated with the substrate, GTP, and the respective compound **1a**–**e** for 2 min at 37 °C. Thereafter, the reactions were quenched by heating at 95 °C and the mixtures were filtered using a 0.2 μm HPLC filter and separated by reverse-phase HPLC.

Phosphate buffers are commonly used for the separation of GTP and c-di-GMP peaks with HPLC.[14c,15,24] However when we applied it to our HPLC analysis, we encountered problems of noisy baseline and column clogging. We attempted to use standard buffer systems of CH_3CN/H_2O and 0.1% TFA in CH_3CN/0.1% TFA in H_2O but in these running buffers, the GTP peak was not observed at 254 nm. Hence we tried triethylammonium bicarbonate[25] as it is a liquid at room temperature and would thus circumvent the problem of potential column clogging. A smoothened baseline was observed and after optimizing the analysis conditions, a good resolution of the peaks was obtained and the running time was reduced from 50 min to 30 min (Fig. 3). A negative peak was also observed in all HPLC analysis at 15 min and this is attributed to the elution of EDTA in the enzymatic buffer. This optimized analysis condition was used in the screening of compounds **1a**–**e**. The product peak area was determined by the LC solution Ver 1.2 software. To determine the inhibitory activity of the compounds, c-di-GMP was assayed relative to the control. Results obtained (Fig. 4) shows that compound **1b** is a much stronger inhibitor of Slr1143 than c-di-GMP **1a**, providing an inhibition of 60% at 100 μM with an IC_{50} value of 68.9 μM (Fig. 5). Interestingly, compound **1c**, where both bases are replaced with uracil, and compound **1d**, where only one of the bases is replaced with uracil, were found to activate Slr1143. Presently we have not determined how compounds **1c** and **1d** activate Slr1143 although earlier studies have identified determinants involved in diguanylate cyclase activation.[26]

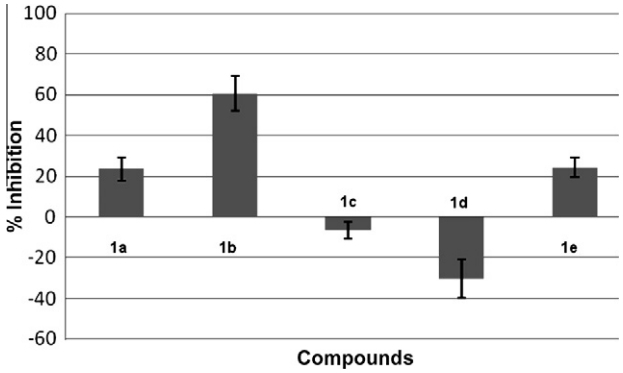


Figure 4. Effect of compounds **1a**–**e** on the enzymatic activity of Slr1143. 100 μM of substrate, GTP was added to a mixture of 1 μM of enzyme in assay buffer and 100 μM **1a**–**e**. The reaction was quenched by heating after 2 min and analyzed by HPLC. A reaction mixture without compound was used as a control.

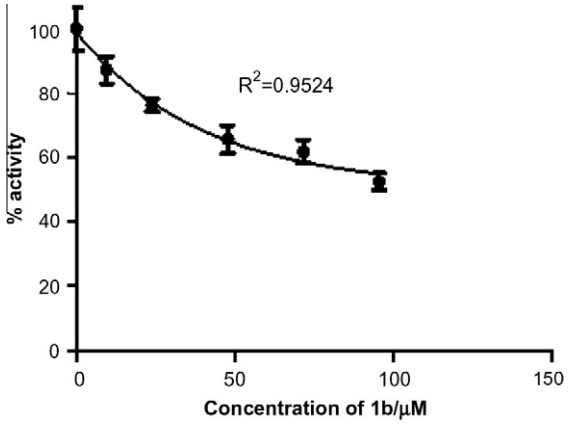


Figure 5. Effect of the concentration of compound **1b** (cyclic di-inosinylic acid) on the activity of Slr1143. 100 μM of substrate, GTP was added to a mixture of 1 μM of enzyme in assay buffer and 10, 25, 50, 75 and 100 μM of **1b**. A reaction mixture without compound was used as a control. The reaction was quenched by heating after 2 min and analyzed by HPLC.

2.4. Inhibitory activity of 1b

It is well reported that bacterial diguanylate cyclase has a feedback control system via allosteric non competitive product inhibition to regulate the cellular concentration of c-di-GMP.[23,27] Dimeric base-intercalated c-di-GMPs are known to act as intersubunit cross linker, linking a primary inhibition site (I_p) with a secondary inhibition site (I_s) of an adjacent dimer, locking the enzyme in the inactive state. Thus the two active half sites which contain the monomer could not come within proximity to form the dimer, c-di-GMP. This feedback control, which is also known as inhibition by domain immobilization, avoids excessive GTP consumption, helps to set an upper limit for c-di-GMP accumulation and might buffer against stochastic variations in cellular c-di-GMP content.[23,27]

In this study, we acknowledge the varied effects analogs of c-di-GMP may have on the bioactivity of the enzyme diguanylate cyclase. Compound **1b** is structurally similar to c-di-GMP, except that it has a hypoxanthine base instead of a guanine, has shown to have an inhibitory activity, stronger than that of c-di-GMP. This could be attributed to the lack of amino group in **1b** which could result in a tighter dimeric base-intercalation and better fit within the allosteric sites than **1a**. As dissociation constant decreases, more enzyme

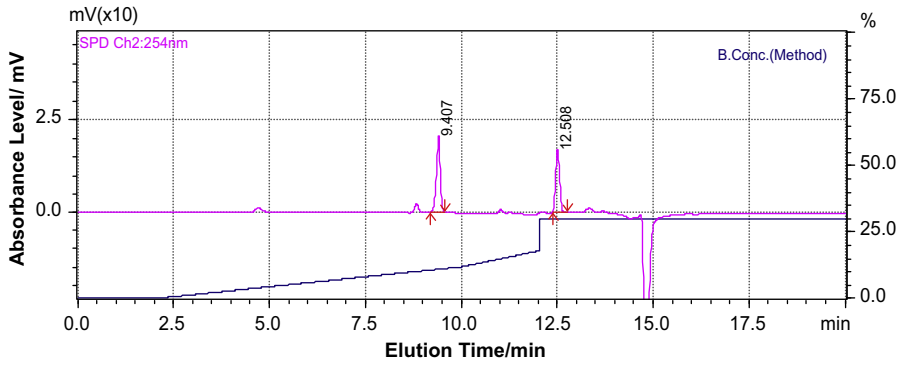


Figure 3. HPLC chromatogram obtained after optimizing the conditions to obtain a good separation. HPLC eluent pair: 20 mM triethylammonium bicarbonate buffer, pH 7.0 and methanol. Absorbance is measured at 254 nm. c-di-GMP has a longer retention time (12 min) and is eluted out later than GTP (9 min).

remain immobilized and inactivated, thus providing a stronger inhibitory activity.

3. Conclusion

In summary, five cyclic di-nucleotidic acids were synthesized. The compounds were evaluated for their inhibitory activities against Slr1143 which was overexpressed in *E. coli*, isolated and purified. Results obtained show that cyclic di-inosinylic acid **1b** provides a greater inhibition on Slr1143 than c-di-GMP. Going forward, the inhibitory activity of **1b** could be further analyzed by assaying on diguanylate cyclase of other bacteria species to determine its potential as an inhibitor of biofilm formation.

4. Experimental

4.1. General

All solvents and reagents were purchased from commercial sources and used without further purification. Powdery molecular sieves (MS) 3 Å were used without further treatment. Imidazolium perchlorate[22] and 2-cyanoethyl bis-*N*,*N*-diisopropylphosphoramidite[21] were prepared according to reported methods. Reactions were monitored by thin layer chromatography (TLC) using precoated plates (Merck Silica Gel 60, F254) and visualized with UV light or by charring with ninhydrin or phosphomolybdic acid. Flash column chromatography was performed with silica (Merck, 230–400 mesh). ^{1}H NMR and ^{31}P NMR spectra were recorded at 298 K on either a Bruker ACF300, Bruker AMX500 or Bruker DPX-300 NMR spectrometer calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). The number of protons (*n*) for a given resonance was indicated as *n*H. ESI mass spectra were acquired using Finnigan LCQ or Finnigan TSQ7000 spectrometer. HPLC analysis was carried out using a Phenomenex Luna 3μ-C18 column [4.6 (diameter) × 50 (height) mm]. Semi-preparative HPLC was achieved using a COSMOSIL 5C18-AR-300 column [10 (diameter) × 250 (height) mm].

All methods for the preparation of media and solutions can be obtained from the New England Biolabs pMAL™ Protein Fusion and Purification System Instruction Manual. Plasmid coding for Slr1143 was kindly provided by Gomelsky.[15] Enzymatic assay buffer was prepared according to the procedure reported by Gomelsky and co-workers.[15] Slr1143 was overexpressed and isolated. The protein concentration was determined to be 2.069 mg/mL by Bradford assay. HPLC analysis was carried out using a Phenomenex Luna 3μ-C18 column [4.6 (diameter) × 50 (height) mm].

4.2. Synthesis of cyclic di-nucleotidic acid 1

4.2.1. General procedure for the synthesis of 3

To a stirred suspension of the respective nucleoside **2** (15 mmol) in DMF (30 mL) at 0 °C was added di-*tert*-butylsilandiyl ditriflate (6.6 mL, 18 mmol) dropwise over 15 min. After stirring at the same temperature for 30 min, imidazole (5.1 g, 75 mmol) was added and the resulting mixture was stirred at 0 °C for another 5 min and then at room temperature for 45 min. Thereafter *tert*-butyldimethylchlorosilane (3.4 g, 22.5 mmol) was added and the reaction mixture was stirred at 60 °C for 2 h. For **3a**, the compound precipitated from the reaction mixture and was filtered, washed with cold methanol and dried in vacuo. For **3b** and **3c**, the reaction mixture remained clear at the end of the reaction. Thus the DMF was partially removed in vacuo and the remaining reaction mixture was partially partitioned between water (30 mL) and ether (30 mL). The layers were separated and the aqueous layer was extracted with ether (3 × 20 mL). The combined organic extract was washed with brine (50 mL), dried over anhydrous Na_2SO_4, concentrated in vacuo and purified by column chromatography (1:30 EtOAc/CH_2Cl_2 to 1:20 EtOAc/CH_2Cl_2 for **3b** and 1:50 MeOH/CH_2Cl_2 to 1:40 MeOH/CH_2Cl_2 for **3c**).

4.2.1.1. 2′-*O*-(*tert*-Butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilanediyl)guanosine 3a. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.07 (s, 3H), 0.09 (s, 3H), 0.86 (s, 9H), 1.01 (s, 9H), 1.06 (s, 9H), 3.93–4.01 (m, 2H), 4.26–4.35 (m, 2H), 4.57 (d, J = 5.1 Hz, 1H), 5.72 (s, 1H), 6.35 (s, br, 2H), 7.91 (s, 1H), 10.65 (s, 1H); $C_{24}H_{44}O_5N_5Si_2^+$ (M+H$^+$) calcd *m/z* 538.2876, found *m/z* 538.2895. Yield = 72%.

4.2.1.2. 2′-*O*-(*tert*-Butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilanediyl)uridine 3b. ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 0.17 (s, 3H), 0.21 (s, 3H), 0.96 (s, 9H), 1.06 (s, 18H), 4.10–4.16 (m, 3H), 4.46 (d, J = 4.2 Hz, 1H), 4.52 (d, J = 4.2 Hz, 1H), 5.62 (d, J = 8.1 Hz, 1H), 5.77 (s, 1H), 7.60 (d, J = 8.1 Hz, 1H), 10.27 (s, br, 1H); $C_{23}H_{42}O_6N_2NaSi_2^+$ (M+Na+) calcd *m/z* 521.2474, found *m/z* 521.2490. Yield = 67%.

4.2.1.3. 2′-*O*-(*tert*-Butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilanediyl)inosine 3c. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.06 (s, 3H), 0.08 (s, 3H), 0.85 (s, 9H), 0.98 (s, 9H), 1.06 (s, 9H), 4.00–4.08 (m, 2H), 4.35–4.36 (m, 1H), 4.50–4.55 (m, 1H), 4.58–4.59 (m, 1H), 5.93 (s, 1H), 8.04 (s, 1H), 8.28 (s, 1H), 12.43 (s, br, 1H); $C_{24}H_{42}O_5N_4NaSi_2^+$ (M+Na$^+$) calcd *m/z* 545.2586, found *m/z* 545.2612. Yield = 80%.

4.2.2. Synthesis of N^2-(dimethylaminomethylene)-2′-*O*-(*tert*-butyldimethylsilyl)-3′,5′-*O*-(di-*tert*-butylsilanediyl) guanosine 4a

N,*N*-Dimethylformamide dimethyl acetal (6.96 mL, 52 mmol) was added to a suspension of **3a** (13 mmol) in methanol (80 mL). The reaction mixture was stirred at 50 °C for 5 h. After the solvent was removed in vacuo, hexane was added to precipitate out a white solid under cooling. The solid was collected by filtration, washed with cold hexane and dried in vacuo to afford pure **4a**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.08 (s, 3H), 0.11 (s, 3H), 0.87 (s, 9H), 1.01 (s, 9H), 1.05 (s, 9H), 3.04 (s, 3H), 3.12 (s, 3H), 3.93–4.08 (m, 2H), 4.31–4.40 (m, 2H), 4.59 (d, J = 4.9 Hz, 1H), 5.88 (s, 1H), 7.99 (s, 1H), 8.48 (s, 1H), 11.40 (s, 1H); $C_{27}H_{49}O_6N_2Si_2^+$ (M+H$^+$) calcd *m/z* 593.3298, found *m/z* 593.3311. Yield = 96%.

4.2.3. General procedure for the synthesis of 5

To a solution of **3b**, **3c** or **4a** (10 mmol) in CH_2Cl_2 (40 mL) at 0 °C was added dropwise over 15 min a chilled solution of hydrogen fluoride–pyridine complex (3.96 g, 40 mmol) in pyridine (5 mL). The reaction mixture was stirred at 0 °C for 2 h. Thereafter, the reaction mixture was washed with saturated $NaHCO_3$ solution (2 × 30 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extract was washed with saturated NaCl (40 mL), dried over anhydrous Na_2SO_4, concentrated and dried in vacuo to give the detritylated product of **3b** and **4a** as white solids. For detritylated **3c**, it precipitated out of the reaction mixture. Saturated $NaHCO_3$ solution was added to the stirred reaction mixture till alkaline. The white solid of detritylated **3c** was collected by filtration and washed with water followed by cold CH_2Cl_2. The resulting dried solid of detritylated **3b**, **3c** and **4a** were then, respectively, dissolved in anhydrous pyridine (100 mL). Dimethoxytrityl chloride (6.78 g, 20 mmol) was added and the reaction mixture stirred at room temperature for 12 h. The reaction was quenched by addition of MeOH (3 mL). Concentration of the reaction mixture gave a viscous liquid which was washed with saturated $NaHCO_3$ and the aqueous layer was then extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extract was washed with saturated NaCl, dried

over anhydrous Na_2SO_4 and concentrated in vacuo. The resulting residue was purified by flash column chromatography using a elution gradient from 1:1 EtOAc/hexane to 1:20 MeOH/CH_2Cl_2 to afford pure **5a–c**.

4.2.3.1. N^2-(Dimethylaminomethylene)-2′-*O*-(*tert*-butyldimethyl silyl)-5′-*O*-(*p,p*′-dimethoxytrityl)guanosine 5a. ^{1}H NMR (300 MHz, CD_3OD) δ −0.06 (s, 3H), 0.03 (s, 3H), 0.82 (s, 9H), 2.95 (s, 3H), 3.02 (s, 3H), 3.32–3.46 (m, 2H), 3.70 (s, 6H), 4.17–4.18 (m, 1H), 4.36–4.39 (m, 1H), 4.71 (t, J = 5.0 Hz, 1H), 6.00 (d, J = 5.0 Hz, 1H), 6.79 (d, J = 8.9 Hz, 4H), 7.16–7.44 (m, 9H), 8.02 (s, 1H), 8.49 (s, 1H); HRMS (ESI$^+$) $C_{40}H_{51}O_7N_6Si^+$ (M+H$^+$) calcd m/z 755.3583, found m/z 755.3601. Yield = 89%.

4.2.3.2. 2′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(*p,p*′-dimethoxytrityl) uridine 5b. ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 0.14 (s, 6H), 0.92 (s, 9H), 3.46–3.47 (m, 2H), 4.04 (s, 6H), 4.11 (m, 6H), 4.32–4.33 (m, 2H), 5.25 (d, J = 8.1 Hz, 2H), 5.89 (s, 1H), 6.84 (d, J = 8.7 Hz, 4H), 7.20–7.42 (m, 9H), 8.00 (d, J = 8.1 Hz, 1H); HRMS (ESI$^+$) $C_{36}H_{44}O_8N_2NaSi^+$ (M+Na$^+$) calcd m/z 683.2759, found m/z 683.2766. Yield = 90%.

4.2.3.3. 2′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(*p,p*′-dimethoxytrityl) inosine 5c. ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 0.54 (s, 3H), 0.64 (s, 3H), 1.44 (s, 9H), 3.39–3.42 (m, 2H), 4.02–4.04 (m, 2H), 4.37 (s, 6H), 4.82–4.85 (m, 1H), 5.00–5.03 (m, 1H), 5.52–5.55 (m, 1H), 6.64–6.66 (m, 1H), 7.38–7.48 (m, 4H), 7.79–8.11 (m, 9H), 8.63 (s, 1H), 8.73 (s, 1H); HRMS (ESI$^+$) $C_{37}H_{44}O_7N_4NaSi^+$ (M+Na$^+$) calcd m/z 707.2871, found m/z 707.2884. Yield = 89%.

4.2.4. General procedure for synthesis of 6

To a solution or suspension of the respective compound **5** (5 mmol) in anhydrous MeCN (30 mL) was added 1*H*-tetrazole (0.35 g, 5 mmol). Thereafter, $NCCH_2CH_2OP(N(i\text{-}C_3H_7)_2)_2$ **12** (3.0 g, 10 mmol) was added dropwise at room temperature and the reaction mixture was stirred for 12 h. The white solid that formed was removed by filtration and washed with EtOAc. The filtrate was concentrated, diluted with EtOAc (30 mL) and washed with saturated $NaHCO_3$. The aqueous layer was extracted with EtOAc (3 × 30 mL) and the combined organic extract was then washed with saturated NaCl, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The resulting residue was purified by flash column chromatography using 1:2 EtOAc/hexane to afford **6b**. Elution using a gradient of 3:2 EtOAc/hexane to 2:1 EtOAc/CH_2Cl_2 afforded purified **6a** and **6c**, respectively.

4.2.4.1. N^2-(Dimethylaminomethylene)-2′-*O*-(*tert*-butyldimethyl silyl)-5′-*O*-(*p,p*′-dimethoxytrityl)guanosine 3′-[(2-cyano-ethyl)*N,N*-diisopropylaminophosphoramidite] 6a. ^{1}H NMR (300 MHz, CD_3OD) δ −0.13, −0.09 (2s, 3H), 0.03, 0.04 (2s, 3H), 0.80, 0.83 (2s, 9H), 1.03–1.23 (m, 12H), 2.40–2.44 (m, 1H), 2.72–2.78 (m, 1H), 2.94, 2.96, 3.08 (3s, 6H), 3.47–3.68 (m, 4H), 3.77, 3.78 (2s, 6H), 3.84–4.01 (m, 1H), 4.29–4.40 (m, 1H), 4.46–4.51 (m, 1H), 4.88–4.97 (m, 1H), 5.99–6.05 (m, 1H), 6.81–6.87 (m, 4H), 7.21–7.48 (m, 9H), 8.02, 8.04 (2s, 1H), 8.48, 8.51 (2s, 1H); ^{31}P NMR (121.5 MHz, CD_3OD) δ 150.21, 151.72; HRMS (ESI$^+$) $C_{49}H_{68}O_8N_8PSi^+$ (M+H$^+$) calcd m/z 955.4662, found m/z 955.4658. Yield = 79%.

4.2.4.2. 2′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O*-(*p,p*′-dimethoxytrityl) uridine 3′-[(2-cyanoethyl) *N,N*-diisopropylamino phosphoramidite] 6b. ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ 0.27, 0.29 (2s, 6H), 1.03, 1.04 (2s, 9H), 1.16–1.31 (m, 12H), 2.69 (t, J = 6.0 Hz, 1H), 2.84–2.88 (m, 1H), 3.57–3.82 (m, 5H), 3.87 (s, 6H), 3.94–4.02 (m, 1H), 4.38–4.46 (m, 1H), 4.50–4.57 (m, 1H), 4.62–4.68 (m, 1H), 5.41 (dd, J = 6.6, 8.1 Hz, 1H), 6.04–6.11 (m, 1H), 6.99–7.03 (m, 4H), 7.35–7.62 (m, 9H), 8.02 (dd, J = 8.2, 16.8 Hz, 1H) 10.27 (s, br, 1H); ^{31}P NMR (121.5 MHz, $(CD_3)_2CO$) δ 150.80, 151.22, 151.43; HRMS (ESI$^+$) $C_{45}H_{61}O_9N_4NaPSi^+$ (M+Na$^+$) calcd m/z 883.3838, found m/z 883.3850. Yield = 84%.

4.2.4.3. 2′-*O*-(*tert*-Butyldimethylsilyl)-5′-*O* (*p,p*′-dimethoxytrityl) inosine 3′-[(2-cyanoethyl) *N,N*-diisopropylamino phosphoramidite] 6c. ^{1}H NMR (300 MHz, $(CD_3)_2CO$) δ −0.10 (s, 3H), 0.05, 0.06 (2s, 3H), 0.83 (s, 9H), 1.14–1.34 (m, 12H), 2.55–2.63 (m, 2H), 2.79–2.83 (m, 1H), 3.43–3.48 (m, 1H), 3.55–3.62 (m, 2H), 3.66–3.72 (m, 2H), 3.79 (s, 6H), 4.01–4.08 (m, 1H), 4.50 (m, 2H), 5.10 (m, 1H), 6.07–6.09 (m, 1H), 6.88–6.92 (m, 4H), 7.24–7.57 (m, 9H), 8.10–8.11 (m, 1H), 8.16–8.19 (m, 1H); ^{31}P NMR (121.5 MHz, $(CD_3)_2CO$) δ 149.35, 150.90; HRMS (ESI$^+$) $C_{46}H_{61}O_8N_6NaPSi^+$ (M+Na$^+$) calcd m/z 907.3950, found m/z 907.3962. Yield = 87%.

4.2.5. General procedure for preparation of 7

To a solution of the respective compound **6** (2.5 mmol) in anhydrous MeCN (9 mL) was added powdery MS 3 Å (115 mg) and allyl alcohol (0.20 mL, 3 mmol). The resulting mixture was stirred at room temperature for 30 min. Imidazolium perchlorate (0.85 g, 5 mmol) was then added and stirring was continued for an additional 45 min. Thereafter, a 31% solution of 2-butanone peroxide/dimethyl phthalate in toluene (1.2 mL) was added and the reaction mixture was stirred for 10 min. After which the MS 3 Å was removed by filtration through a small pad of Celite 545 and the filtrate was diluted with EtOAc (20 mL) and then washed with saturated $NaHCO_3$. The aqueous layer was extracted with EtOAc (3 × 20 mL) and the combined organic extract was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and concentrated in vacuo to obtain a viscous liquid. This liquid was then dissolved in CH_2Cl_2 (20 mL), cooled to 0 °C and dichloroacetic acid (4.12 mL, 50 mmol) was then added dropwise to the reaction mixture. Thereafter, the reaction mixture was stirred for 5 min and then saturated $NaHCO_3$ was added till the pH was alkaline. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL) and the combined organic extract was washed with saturated NaCl (30 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuo. The resulting residue was purified by flash column chromatography using an elution gradient of 1:30 MeOH/CH_2Cl_2 to 1:10 MeOH/CH_2Cl_2 to afford **7a–c**, respectively.

4.2.5.1. N^2-(Dimethylaminomethylene)-2′-*O*-(*tert*-butyldimethyl silyl)guanosine 3′-(allyl-2-cyanoethyl phosphate) 7a. ^{1}H NMR (300 MHz, CD_3OD) δ −0.18, −0.17 (2s, 3H), −0.01, 0.00 (2s, 3H), 0.78, 0.79 (2s, 9H), 2.92–2.94 (m, 2H), 3.11 (s, 3H), 3.20 (s, 3H), 3.85–3.88 (m, 2H), 4.31–4.34 (m, 2H), 4.42–4.44 (m, 1H), 4.66–4.72 (m, 2H), 4.98–5.02 (m, 2H), 5.31–5.48 (m, 1H), 5.43–5.50 (m, 1H), 5.98 (d, J = 6.42 Hz, 1H), 6.05–6.11 (m, 1H), 8.14 (s, 1H), 8.59 (s, 1H); ^{31}P NMR (121.5 MHz, CD_3OD), δ −1.02, −0.99; HRMS (ESI$^+$) $C_{25}H_{41}O_8N_7PSi^+$ (M+H$^+$) calcd m/z 626.2518, found m/z 626.2507. Yield = 85%.

4.2.5.2. 2′-*O*-(*tert*-Butyldimethylsilyl)uridine 3′-(allyl 2-cyanoethyl phosphate) 7b. ^{1}H NMR (300 MHz, CD_3OD) δ 0.09, 0.10, 0.13, 0.14 (4s, 6H), 0.91 (s, 9H), 2.93 (t, J = 5.9 Hz, 2H), 3.82–3.85 (m, 2H), 4.28–4.37 (m, 3H), 4.54–4.58 (m, 1H), 4.65–4.71 (m, 2H), 5.31–5.35 (m, 1H), 5.42–5.49 (m, 1H), 5.77 (d, J = 8.1 Hz, 1H), 5.99–6.09 (m, 2H), 8.05 (d, J = 8.1 Hz, 1H); ^{31}P NMR (121.5 MHz, CD_3OD) δ −1.83; HRMS (ESI$^+$) $C_{21}H_{34}O_9N_3NaPSi^+$ (M+Na$^+$) calcd m/z 554.1694, found m/z 554.1706. Yield = 78%.

4.2.5.3. 2′-*O*-(*tert*-Butyldimethylsilyl)inosine 3′-(allyl 2-cyanoethyl phosphate) 7c. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.22, 0.25 (2s, 6H), 0.93, 0.96 (2s, 9H), 2.78–2.87 (m, 1H), 2.89–2.94 (m, 1H), 3.84–3.91 (m, 1H), 4.14–4.19 (m, 1H), 4.33–4.34 (m, 2H), 4.42–4.46 (m, 2H), 4.65–4.67 (m, 2H), 5.28–5.44 (m, 4H), 5.94–

6.10 (m, 2H), 8.51 (s, 1H), 9.87–9.96 (m, 1H), 13.59 (s, br, 1H); ^{31}P NMR (202.5 MHz, $CDCl_3$) δ −1.23, −1.08; HRMS (ESI^+) $C_{22}H_{34}O_8N_5NaPSi^+$ ($M+Na^+$) calcd *m/z* 578.1807, found *m/z* 578.1820. Yield = 80%.

4.2.6. General procedure for preparation of 8

A mixture of the respective compound **6** (0.9 mmol) and the respective compound **7** (0.9 mmol) were dissolved in anhydrous MeCN (7 mL). MS 3 Å (80 mg) was then added and the reaction mixture was stirred at room temperature for 30 min. Thereafter, imidazolium perchlorate (0.30 g, 1.8 mmol) was added and stirring was continued for an additional 45 min. To the resulting mixture was added a 31% solution of 2-butanone peroxide/dimethyl phthalate in toluene (1.2 mL) and the reaction mixture was stirred for 10 min. After which, the MS 3 Å was removed by filtration through a small pad of Celite 545 and the filtrate was concentrated to obtain a viscous liquid. This liquid was then dissolved in CH_2Cl_2, cooled to 0 °C and dichloroacetic acid (1.48 mL, 18 mmol) was added dropwise to the reaction mixture. Thereafter, the reaction mixture was stirred for 5 min and then saturated $NaHCO_3$ was added till the pH was alkaline. The aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL) and the combined organic extract was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The resulting residue was purified by flash column chromatography using an elution gradient of 1:30 MeOH/CH_2Cl_2 to 1:5 MeOH/CH_2Cl_2 to afford compound **8**.

4.2.6.1. Guanylyl (3′–5′)guanosine 3′-phosphate 8a. ^{1}H NMR (300 MHz, $CDCl_3$) δ−0.23–0.09 (m, 12H), 0.75–0.85 (m, 18H), 2.80–2.82 (m, 4H), 3.09, 3.12, 3.19, 3.23 (4s, 9H), 3.72–3.82 (m, 2H), 4.31–4.63 (m, 10H), 4.89–5.04 (m, 4H), 5.29–5.45 (m, 2H), 5.78–6.02 (m, 3H), 7.80–7.91 (m, 2H), 8.40 (s, 1H), 8.61 (s, 1H); ^{31}P NMR (121.5 MHz, $CDCl_3$), δ −1.68, −1.41, −1.13, −0.88; HRMS (ESI^+) $C_{44}H_{68}O_{14}N_{14}NaP_2Si_2^+$ ($M+Na^+$) calcd *m/z* 1157.3945, found *m/z* 1157.3958. Yield = 57%.

4.2.6.2. Inosinylyl(3′–5′)inosine 3′-phosphate 8b. ^{1}H NMR (300 MHz, MeOD) δ −0.21–0.09 (m, 12H), 0.74–0.97 (m, 18H), 2.89–2.95 (m, 4H), 3.77–3.78 (m, 2H), 4.33–4.37 (m, 4H), 4.96–4.99 (m, 2H), 5.17 (m, 1H), 5.33–5.36 (m, 1H), 5.44–5.50 (m, 1H), 6.04–6.09 (m, 3H), 7.61–7.67 (m, 2H), 7.71–7.75 (m, 2H), 8.15 (d, *J* = 5.8 Hz, 1H) 8.31 (s, 1H), 8.42 (s, 1H); ^{31}P NMR (202.5 MHz, $CDCl_3$) δ −1.64, −1.51, −1.13, −1.03, −0.95; HRMS (ESI^+) $C_{41}H_{62}O_{15}N_{10}NaP_2Si_2^+$ ($M+Na^+$) calcd *m/z* 1075.3302, found *m/z* 1075.3303. Yield = 60%.

4.2.6.3. Uridylyl(3′–5′)uridine 3′-phosphate 8c. ^{1}H NMR (300 MHz, $(CD_3)CO$) δ 0.12–0.16 (m, 12H), 0.91 (s, 18H), 2.95–3.01 (m, 6H), 3.88 (s, 2H), 4.27–4.68 (m, 12H), 4.94–4.96 (m, 2H), 5.33 (d, 1H, *J* = 10.4 Hz), 5.44 (d, 1H, *J* = 15.8 Hz), 5.66–5.72 (2H, m), 5.91–5.98 (m, 3H), 7.74 (d, 1H, *J* = 8.2 Hz), 8.00 (d, 1H, *J* = 5.4 Hz); ^{31}P NMR (121.5 MHz, $(CD_3)CO$) δ −0.99, −1.20 (m); HRMS (ESI^+) $C_{39}H_{62}N_6NaO_{17}P_2Si_2^+$ ($M+Na^+$) calcd *m/z* 1027.3078, found *m/z* 1027.3092 Yield = 79%.

4.2.6.4. Guanylyl(3′–5′)uridine 3′-phosphate 8d. ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.25–0.12 (m, 12 H), 0.78–0.89 (m, 18H), 2.82 (m, 4H), 3.11 (s, 3H), 3.19 (s, 3H), 3.72–3.91 (m, 4H), 4.29–4.63 (m, 10H), 4.93–5.07 (m, 3H), 5.32–5.43 (m, 2H), 5.68–5.78 (m, 2H), 5.95 (m, 2H), 7.47 (m, 1H), 7.86 (s, 1H), 8.43 (s, 1H), 9.15 (m, 1H); ^{31}P NMR (202.5 MHz, $CDCl_3$) δ −1.96, −1.80, −1.31, −0.99, −0.88; HRMS (ESI^+) $C_{43}H_{69}O_{16}N_{10}P_2Si_2^+$ ($M+H^+$) calcd *m/z* 1099.3901, found *m/z* 1099.3899. Yield = 49%.

4.2.6.5. Inosylyl(3′–5′)uridine 3′-phosphate 8e. ^{1}H NMR (300 MHz, MeOD) δ −0.18–0.16 (m, 12H), 0.84 (dd, 18H, *J* = 2.4, 43.5 Hz), 1.99 (s, 1H), 2.90–2.98 (m, 4H), 3.86 (m, 2H), 4.31–4.71 (m, 11H), 5.03 (d, 2H, *J* = 4.5 Hz), 5.33 (d, 1H, *J* = 10.4 Hz), 5.45 (d, 1H, *J* = 17.1 Hz), 6.00–5.78 (m, 2H), 6.01–6.09 (m, 2H), 7.68–7.73 (m, 1H), 8.12 (s,1H), 8.40 (s, 1H); ^{31}P NMR (202.5 MHz, CD_3OD) δ 8.04, 8.68; HRMS (ESI^+) $C_{40}H_{62}O_{16}N_8NaP_2Si_2^+$ ($M+H^+$) calcd *m/z* 1051.3190, found *m/z* 1051.3220. Yield = 53%.

4.2.7. General procedure for preparation of 9

To a solution of the respective **8** (0.24 mmol) in anhydrous acetone (7.2 mL) was added sodium iodide (0.36 g, 2.4 mmol). The resulting mixture was stirred under reflux for 2–3 h, concentrated and chilled anhydrous acetone was then added. The white precipitate that formed was filtered, washed with chilled anhydrous acetone and subsequently dissolved in MeOH. Thereafter, the MeOH was removed in vacuo to obtain a colorless crystalline solid which was then suspended in anhydrous THF (40 mL). MS 3 Å (50 mg), *N*-methylimidazole (0.96 mL, 12 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (3.63 g, 12 mmol) were added and the resulting mixture was stirred at room temperature for 36–48 h. Water (18 mL) was then added and stirring was continued for an additional 1 h. The reaction mixture was concentrated in vacuo and the residual material was dissolved in EtOAc, washed with saturated NaCl and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic extract was dried over anhydrous Na_2SO_4, concentrated in vacuo and the resulting residue was purified by flash column chromatography using an elution gradient of 1:30 MeOH/CH_2Cl_2 to 1:10 MeOH/CH_2Cl_2 to afford the respective **9**.

4.2.7.1. Fully protected cyclic (3′–5′)diguanylic acid 9a. ^{1}H NMR (300 MHz, CD_3OD) δ −0.17–0.06 (m, 12H), 0.79–0.83 (m, 18H), 2.91–2.95 (m, 4H), 3.13 (s, 6H), 3.21 (s, 6H), 4.08–4.13 (m, 2H), 4.29–4.38 (m, 4H), 4.66–4.73 (m, 4H), 5.31 (m, 1H), 5.34 (m, 1H), 5.42 (m, 1H), 5.47 (m, 1H), 5.96 (d, *J* = 6.6 Hz, 2H), 8.62 (s, 2H), 8.90 (s, 2H); ^{31}P NMR (202.5 MHz, CD_3OD) δ −1.72, −1.69; HRMS (ESI^+) $C_{44}H_{68}O_{14}NaP_2Si_2^+$ ($M+Na^+$) calcd *m/z* 1157.3945, found *m/z* 1157.3958. Yield = 40%.

4.2.7.2. Fully protected cyclic (3′–5′)diinosinic acid 9b. ^{1}H NMR (500 MHz, MeOD) δ −0.14 (s, 6H), 0.11 (s, 6H), 0.78 (s, 18H), 2.98–3.00 (m, 4H), 4.24–4.28 (m, 2H), 4.58 (dd, *J* = 4.4, 10.7 Hz, 4H), 4.74–4.78 (m, 4H), 5.33 (dd, *J* = 5.1, 8.2 Hz, 2H), 5.41–5.45 (m, 2H), 6.04 (d, *J* = 7.6 Hz, 2H), 8.12 (s, 2H), 8.26 (s, 2H); ^{31}P NMR (202.5 MHz, CD_3OD) δ 0.88; HRMS (ESI^+) $C_{38}H_{56}O_{14}N_{10}NaP_2Si_2^+$ ($M+Na^+$) calcd *m/z* 1017.2884, found *m/z* 1017.2842. Yield = 45%.

4.2.7.3. Fully protected cyclic (3′–5′)diuridylic acid 9c. ^{1}H NMR (300 MHz, MeOD) δ 0.13 (s, 6H), 0.19 (s, 6H), 0.91 (s, 9H), 0.94 (s, 9H), 2.95 (t, *J* = 5.9 Hz, 4H), 4.24–4.68 (m, 10H), 5.70–5.76 (d, 2H), 5.82 (d, *J* = 6.1 Hz, 2H), 7.68 (d, *J* = 8.2 Hz, 2H); ^{31}P NMR (121.5 MHz, CD_3OD) δ −0.66; HRMS (ESI^+) $C_{36}H_{56}N_6O_{16}NaP_2Si_2^+$ ($M+Na^+$) calcd *m/z* 969.2659, found *m/z* 969.2688. Yield = 30%.

4.2.7.4. Fully protected cyclic (3′–5′)guanylic/uridylic acid 9d. ^{1}H NMR (300 MHz, MeOD) δ −0.10, 0.10, 0.20 (3s, 12H), 0.79, 0.90 (2s, 18H), 2.91–3.00 (m, 4H), 3.20 (s, 6H), 4.10–4.68 (m, 12H), 5.13–5.19 (m, 1H), 5.32–5.38 (m, 1H), 5.76 (d, 1H, *J* = 8.1 Hz), 5.86–5.94 (m, 2H), 7.66 (d, *J* = 8.1 Hz, 1H), 7.95 (s, 1H), 8.69 (s, 1H); ^{31}P NMR (121.5 MHz, CD_3OD) δ 0.34, 0.68; HRMS (ESI^+) $C_{40}H_{62}N_{10}O_{15}NaP_2Si_2^+$ ($M+Na^+$) calcd *m/z* 1063.3, found *m/z* 1063.3. Yield = 39%.

4.2.7.5. Fully protected cyclic (3′–5′)inosylyl/uridylic acid 9e. ^{1}H NMR (300 MHz, MeOD) δ −0.14–0.19 (m, 12H), 0.78–1.68 (m, 18H), 2.95–3.00 (m, 4H), 4.21–4.76 (m, 11H), 4.90–5.37 (m, 3H), 5.74 (d, *J* = 8.3 Hz, 1H), 5.85 (d, *J* = 7.3 Hz, 1H), 6.03 (d,

1H, J = 7.0 Hz), 7.66 (d, J = 8.0 Hz, 1H), 8.11 (s, 1H), 8.25 (s, 1H); ^{31}P NMR (202.5 MHz, $(CD_3)_2CO$) δ 0.88–3.71 (m); HRMS (ESI^+) $C_{37}H_{56}O_{15}N_8NaP_2Si_2^+$ calcd m/z 993.2771, found m/z 993.2804. Yield = 30%.

4.2.8. General procedure for preparation of 1

To a solution of the respective **9** (0.05 mmol) in MeOH (8 mL) was added concentrated aqueous ammonium hydroxide (8 mL) and the resulting mixture was stirred at 50 °C for 12 h. After which the reaction mixture was concentrated and dried in vacuo to obtain a residual material which was dissolved in triethylammonium fluoride (1.0 mL) and stirred at room temperature for 12 h. After which, a 1 M ammonium acetate buffer solution (10 mL) was added and the reaction mixture was stirred vigorously at 40 °C to precipitate a pale yellow solid. After the removal of the precipitate, the aqueous solution was subjected to semi-preparative HPLC using a COSMOSIL 5C18-AR-300 column [20 (diameter) × 250 (height) mm]. Elution was carried out under the following conditions to obtain **1a–e**, respectively: [A = water with 1% TFA, B = 20:80 mixture of water and MeCN with 1% TFA] gradient: 0–3 min: 100% A, 3–35 min: (linear gradient) 100% A to 85% A/15% B, 35–45 min: 100% B, 45–55 min: 100% A; detection at 254 nm; flow rate 3 mL/min. Relevant fractions were collected, dried and subsequently washed with MeCN. Centrifugation afforded a white solid which was dissolved in 1 M aqueous ammonium acetate. Concentration in vacuo afforded the respective diammonium salt of **1** with average overall yield of 10–15%.

4.2.8.1. Cyclic (3′–5′)diguanylic acid 1a. ^{1}H NMR (500 MHz, D_2O), δ 4.01–4.04 (m, 2H), 4.32–4.40 (m, 4H), 4.83 (s, 2H), 5.04 (m, 2H), 5.81 (s, 2H), 7.95 (s, 2H); ^{31}P NMR (202.5 MHz, D_2O) δ −1.05; HRMS (ESI^-) $C_{20}H_{23}O_{14}N_{10}P_2^-$ ($M-H^-$) calcd m/z 689.0865, found m/z 689.0849. Yield = 84%.

4.2.8.2. Cyclic (3′–5′)diinosylic acid 1b. ^{1}H NMR (500 MHz, D_2O) δ 3.92–3.98 (m, 2H), 4.12 (m, 2H), 4.51–4.60 (m, 4H), 6.09 (s, 1H), 6.21 (s, 1H), 8.10 (s, 1H), 8.30 (s, 1H), 8.63 (s, 2H); ^{31}P NMR (202.5 MHz, D_2O) δ −1.04; HRMS (ESI^+) $C_{20}H_{23}O_{14}N_8P_2^+$ ($M+H^+$) calcd m/z 661.0804, found m/z 661.0828. Yield = 85%.

4.2.8.3. Cyclic (3′–5′)diuridylic acid 1c. ^{1}H NMR (300 MHz, MeOD) δ 3.97–3.94 (m, 2H), 4.40–4.28 (m, 10H), 4.56–4.48 (m, 4H), 5.5–5.70 (m, 4H), 7.90 (d, 2H, J = 7.7 Hz); ^{31}P NMR (121.5 MHz, D_2O) δ −1.08; HRMS (ESI^-) $C_{18}H_{20}O_{16}N_4P_2^-$ ($M-H^-$) calcd m/z 611.0433, found m/z 611.0452. Yield = 90%.

4.2.8.4. Cyclic (3′–5′)guanylic/uridylic acid 1d. ^{1}H NMR (500 MHz, D_2O) δ 4.08 (m, 2H), 4.41–4.52 (m, 4H), 5.34 (m, 2H), 5.60 (m, 1H), 5.93 (m, 1H), 7.49 (s, 1H), 7.92 (m, 2H), 8.45 (m, 1H), 8.70 (s, 1H); ^{31}P NMR (202.5 MHz, D_2O) δ −0.89; MS (ESI^+) $C_{19}H_{23}N_7O_{15}NaP_2^+$ ($M+Na^+$) calcd m/z 673.0547, found m/z 673.0568. Yield = 82%.

4.2.8.5. Cyclic (3′–5′) inosylic/uridylic acid 1e. ^{1}H NMR (500 MHz, D_2O) δ 4.05 (m, 2H), 4.51–4.36 (m, 7H), 5.42 (s, 1H), 6.16 (s, 1H), 7.89 (s, 1H), 8.16 (s, 1H); ^{31}P NMR (202.5 MHz, D_2O) −0.97 (s); HRMS (ESI^-) $C_{19}H_{21}O_{15}N_6P_2$ calcd m/z 635.0540, found m/z 635.0535. Yield = 92%.

4.3. Overexpression and purification of Slr1143 diguanylate cyclase as an MBP (maltose binding protein) fusion

4.3.1. Large scale overexpression of protein

One liter rich LB broth with glucose (2 g) and ampicillin (100 μg/mL) was inoculated with 10 mL of overnight culture of *E. coli* DH5α and incubated at 37 °C to an OD of 0.6–0.8 at 600 nm. Three milliliters of 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to the subculture to a final concentration of 0.3 mM and the culture media was further incubated for 2 h to induce the expression of protein. After which, the cells were centrifuged at 4 °C for 20 min at 4000*g* and the supernatant was discarded. Twenty five milliliters of column buffer was added to resuspend the cells before Halt Protease Inhibitor Cocktail (*Pierce*) was added and the mixture was kept at −20 °C overnight. Lysozyme was added at 1 mg/mL of buffer and incubated for 30 min. To further disrupt the cells to facilitate the release of protein into the supernatant, the cells were sonicated for 10 min, 10 s on, 15 s off, on ice. The crude mixture was then centrifuged at 9000*g* for 30 min. The supernatant was subsequently incubated with 1 mL of amylose beads (*New England Biolabs*), which were washed beforehand according to the manufacturer's instructions, for 1 h at 4 °C. The targeted protein would bind to the amylase beads.

4.3.2. Purification of Slr1143 diguanylate cyclase

The amylose beads were poured into an affinity column and the flow through was collected. As the targeted protein would bind to the amylase beads, washing with 10 column volumes of column buffer would remove all unwanted protein. The fusion protein was subsequently eluted with 10 mM of maltose in column buffer. Small fractions of 0.6 mL of eluant were collected and SDS–PAGE was carried out on all the fractions to determine which fractions contained the fusion protein. Glycerol was added at a concentration of 20% before a Bradford assay was performed to determine the concentration of the protein. Subsequently, the protein was aliquoted into smaller fractions of 20 μl for storage at −78 °C.

4.3.3. Analysis of the activity of the Slr1143 diguanylate cyclase using different GTP concentrations

Enzyme (2 μl, 1 μM) (kept on ice) in enzymatic assay buffer (50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM EDTA, and 50 mM NaCl) was incubated at 30 °C for 5 min. Following which, GTP (kept on ice) was added to the desired concentration (total volume = 200 μl) and the reaction mixture was incubated at 30 °C. Fifty microliters of the reaction mixture was pipetted out at 0.5, 1, 1.5 and 2 min and quenched by heating to 95 °C. Each sample was filtered with a 0.2 μm HPLC filter before 10 μl was analyzed by HPLC. The experiment was then repeated for other GTP concentrations.

4.3.4. Effect of compounds 1a–e on the activity of Slr1143 diguanylate cyclase

Five microliters of a 1 mM concentration of the respective compound **1** in enzymatic assay buffer was added to a mixture of Slr1143 (1 μM, 2 μl) (kept on ice) in enzymatic assay buffer (38 μl; enzymatic assay buffer: 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM EDTA, and 50 mM NaCl) to a final concentration of 100 μM in 50 μl and the mixture was incubated at 30 °C for 5 min. Following that, 5 μl of 1 mM GTP (kept on ice) was added to a final concentration of 100 μM and the reaction was quenched at 2 min by heating at 95 °C. Each sample was filtered with a 0.2 μm HPLC filter before 10 μl was analyzed by HPLC. For elution condition for the HPLC analysis was: Eluent A: 20 mM triethylammonium bicarbonate buffer; Eluent B: methanol; 0–2 min: A: 100%, 2–10 min: B: 12% 10–12 min B: 18% 12–20 min B: 30%.

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Supplementary data

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