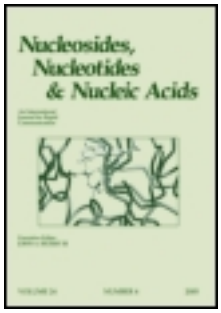

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Synthesis of 3′,5′-cyclic diguanylic acid (cdiGMP) Using 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl as a Protecting Group for 2′-hydroxy Functions of Ribonucleosides

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SYNTHESIS OF 3′,5′-CYCLIC DIGUANYLIC ACID (cdiGMP) USING 1-(4-CHLOROPHENYL)-4-ETHOXYPIPERIDIN-4-YL AS A PROTECTING GROUP FOR 2′-HYDROXY FUNCTIONS OF RIBONUCLEOSIDES

Hongbin Yan and Aimé López Aguilar □ *Department of Chemistry, Brock University, St. Catharines, Ontario, Canada*

□ *We herein report a convenient synthesis of 3′,5′-cyclic diguanylic acid via the modified H-phosphonate approach. The 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep) group was used as protecting group for the 2′-hydroxy functions of ribonucleosides. Complete unblocking of the fully protected 3′,5′-cyclic diguanylic acid gave cdiGMP as a homogeneous compound in an excellent yield.*

Keywords Biofilm; bacterial signaling pathway; H-phosphonate; cyclic ribonucleotide; Cpep

INTRODUCTION

Biofilms are assemblies of microorganisms closely associated with a surface and enclosed in a matrix of materials, such as polysaccharides, nucleic acids, and proteins. Compared with organisms in their planktonic phenotype, those in the biofilm state are very closely associated with each other; as a consequence, microorganisms existing in biofilms are remarkably more resistant to selection pressure, such as antibiotic treatment. It is known that in biofilm formation, a process called quorum sensing is involved. Thus, as the densities of microorganism change, certain compounds are released from bacterial cells to interact with other cells by attaching to and activating specific cell receptors. When the level of these compounds reaches a certain threshold, signal transduction leads to the activation of genes that control a variety of functions, including biofilm development and regulation. 3′,5′-Cyclic diguanylic acid (cdiGMP) is an important signalling

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molecule that regulates quorum sensing; its signaling function was first revealed in a bacterium associated with grapes—*Acetobacter xylinum*,[1] and it is emerging as an important bacterial second messenger in regulating bacterial growth on surfaces (for reviews on the role of cdiGMP in biofilm regulation, see ref.[2,3]). cdiGMP Concentration is believed to be regulated by cdiGMP synthetase and phosphodiesterase. Proteins that contain these synthetase and phosphodiesterase domains may contribute to the regulation of biofilm formation by controlling cdiGMP concentration.[4] This effect was demonstrated in *Vibrio cholerae* and other microorganisms.[5,6] It recently was shown that cdiGMP can serve as an inhibitor for *Staphylococcus aureus* cell-cell interactions and biofilm formation.[7,8] Additionally, the role of cdiGMP in the virulence of *Pseudomonas aeruginosa*[9] and the response of the transcriptional profile of *Escherichia coli* to high levels of cdiGMP[10] have been reported. Thus, a convenient synthetic methodology of this compound is of importance in order to further evaluate its roles in the regulation of bacterial functions.

So far, three methods have been reported for the synthesis of cdiGMP.[11–13] These procedures involved the use of *tert*-butyldimethyl silyl (TBDMS) group[12,13] or tetrahydrapyranyl[11] for the protection of the 2′-hydroxy functions. The use of TBDMS[14] suffers from a notable disadvantage in that it can readily undergo base-catalyzed migration. The deprotection of 2′-*O*-tetrahydrapyranyl-uridine requires relatively drastic acidic conditions (pH 2.0, 16 hours at room temperature), under which conditions ribonucleotides may undergo migration and degradation. Additionally, the tetrahydrapyranyl group is chiral; therefore, its use leads to diastereoisomeric mixtures of products.[15]

Other protecting groups such as TOM,[16] ACE,[17] and cyanoethoxymethyl[18] groups (Figure 1) also are used for the protection of the 2′-hydroxy functions. However, introduction of the TOM and cyanoethoxymethyl groups at *O*-2′ position of ribose is not regiospecific. In addition to this, unblocking of cyanoethoxymethyl is relatively difficult. The ACE protecting group requires the use of relatively expensive starting materials.

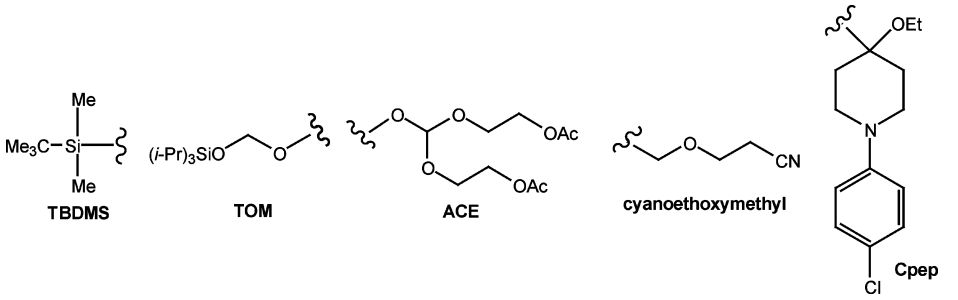


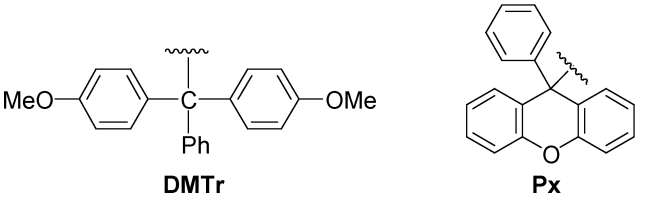
FIGURE 1 Some protecting groups for the 2′-OH of ribonucleosides.

RESULTS AND DISCUSSION

The 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep) group has unique hydrolytic properties which make it the potential protecting group of choice in solid-phase RNA synthesis.[19] Its apparent advantages are as follows: (i) only cheap starting materials are used in the preparation of the monomeric building blocks; (ii) 2′-*O*-Cpep protection of 2′-hydroxy functions proceeds readily and regiospecifically; (iii) the Cpep group cannot migrate and remains intact until the final deblocking step; (iv) as Cpep-protected RNA sequences are stable to base and resistant to ribonucleases, they can readily be purified and stored; (v) removal of the Cpep protecting groups in the final deblocking step proceeds readily under very mild acidic conditions (such as pH 4.0 at 35–40°C for 5 hours) that do not promote cleavage or migration of the internucleotide linkages. This chemistry was successfully used to assemble the 3′-terminal decamer of yeast $tRNA^{Ala}$ in solution.[20] For these reasons, the Cpep was chosen as the protecting group for the 2′-hydroxy functions in this study.

In conjunction with the Cpep group, an acid-sensitive protecting group also is required for the 5′-hydroxy functions of ribonucleosides. The advantage of using the 9-phenyl-xanthen-9-yl (or the pixyl as in Figure 2) as the protecting group for 5′-hydroxy functions versus the 4,4′-dimethoxytrityl group was recently demonstrated.[21] Because the pixyl group is about 3 times more readily removable under acidic conditions compared with the DMTr group, its use shortens the exposure of Cpep groups to acids. This feature consequently ensures that the Cpep groups remain intact during deprotection of the 5′-hydroxy protecting group using trifluoroacetic acid. Thus, the pixyl group is completely deprotected using trifluoroacetic acid in the presence of pyrrole in 10 seconds. It is worthwhile to note that 5′-pixylated nucleosides and nucleotides are stable compounds and can be readily purified by column chromatography without notable loss of the pixyl groups.

The guanine base residues were "doubly" protected with O^6-(2,5-dichlorophenyl)- and N^2-phenyl acetyl groups (Figure 3). Protection of the O^6-carbonyl function is necessary to both increase the solubility of guanosine and prevent possible side-reaction with phosphorylating agents, such as diphenyl chlorophosphate, which is used in the cyclization reaction



DMTr **Px**

FIGURE 2 DMTr and Pixyl groups.

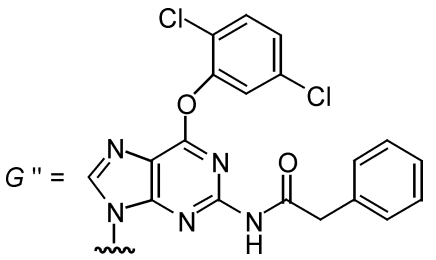


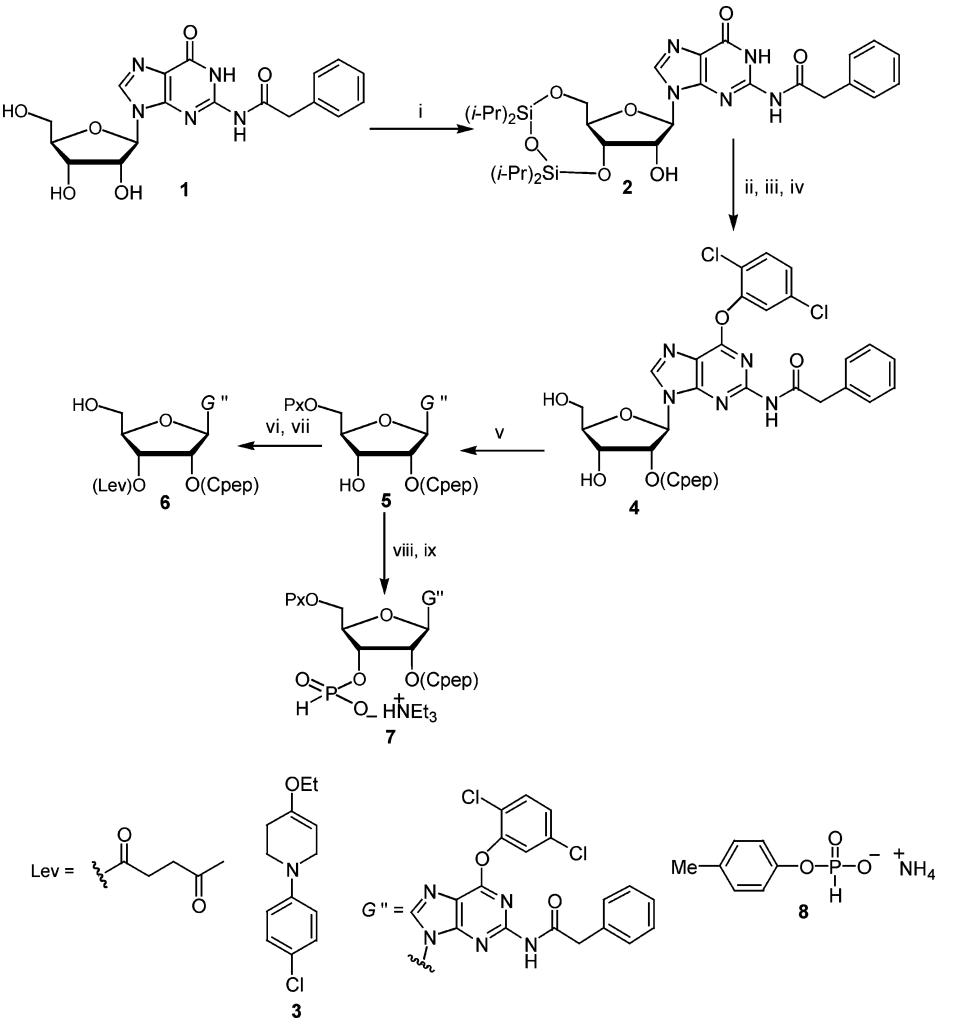
FIGURE 3 "Double" protection of guanine base residue.

as an activator.[22] The O^6-(2,5-dichlorophenyl) is readily removable by treatment with 2-nitro-benzaldoximate followed by aqueous ammonia.

The introduction of Cpep group at the *O*-2′ positions of ribose is straightforward and involves only relatively inexpensive starting material.[17] 2-*N*-Phenylacetylguanosine **1**[23] first reacted with the 1,1-dichloro-1,1,3,3-tetraisopropyldisiloxane (the Markiewicz reagent)[24] to give the 3′,5′-*O* protected guanosine **2**. After the O^6- of guanine residue was protected by 2,5-dichlorophenol,[22] the Cpep group was introduced by treatment with the enolether **3** under acidic conditions. After the "Markiewicz protecting group" was removed by treatment with tetraethylammonium fluoride, the "doubly" protected guanosine **4** with its 2′-hydroxy function protected with Cpep group was obtained in 75% overall yields from **1** (Scheme 1). This 2′-*O*-Cpep protected guanosine **4** was then transformed into the two components **5** and **6** that are required for the assembly of dinucleotides (Scheme 1, steps v, vi, and vii).

The modified H-phosphonate approach[25] which involves an in situ treatment of H-phosphonate diesters with a sulfur transfer reagent has been shown to be a valuable method towards the synthesis of both oligonucleotide phosphates and phosphorothioates.[26] Triethylammonium 2′-*O*-[1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-6-*O*-(2,5-dichlorophenyl)-2-*N*-phenylacetyl-5′-*O*-(xanthen-9-yl)guanosine 3′ H-phosphonate **7** was prepared in 95% yield by treatment of 2′-*O*-[1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-6-*O*-(2,5-dichlorophenyl)-2-*N*-phenylacetyl-5′-*O*-(xanthen-9-yl)guanosine **5** with *p*-tolyl H-phosphonate **8** (Scheme 1, steps viii and ix).[27]

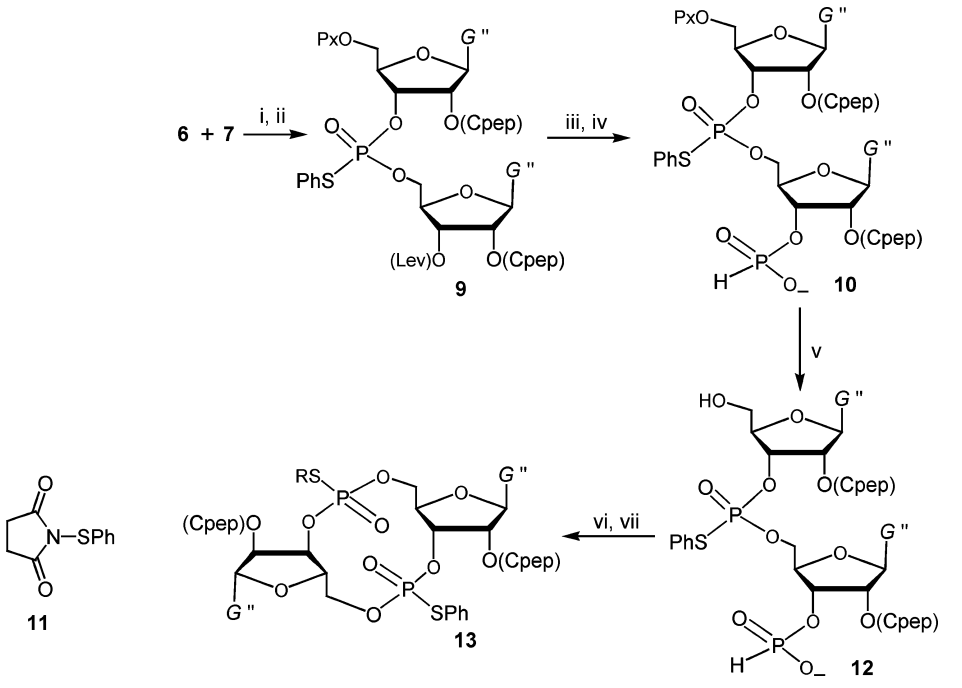
The overall strategy for the synthesis of fully protected cdiGMP **13** is represented in Scheme 2. Thus, coupling of the H-phosphonate **7** with **6** was activated by pivaloyl chloride. This was followed by treatment with 1-phenylsulfanyl-pyrrolidine-2,5-dione **11**[28] in situ to give the fully protected linear diguanylic acid **9**. Removal of the 3′-levulinyl group from **9** is effected by treatment with hydrazine hydrate in aqueous acetic acid–pyridine solution. Phosphitylation of the product gives fully protected diguanylic acid H-phosphonate **10**. Upon removal of the 5′-pixyl group, cyclization



SCHEME 1 *Reagents and conditions* i, $(i\text{-Pr})_2Si(Cl)OSi(i\text{-Pr})_2(Cl)$, C_5H_5N; ii, **3**, CF_3COOH, CH_2Cl_2; iii, 2,5-dichlorophenol, C_5H_5N, *N*-methylpyrrolidine, mesitylenesulfonyl chloride; iv. $Et_4N^+ + F^-$, CH_3CN; v, PxCl, C_5H_5N; vi, $(Lev)_2O$, C_5H_5N; vii, CF_3COOH, pyrrole, CH_2Cl_2; viii, **8**, $(CH_3)_3COCl$, C_5H_5N; ix, *aq*. work-up.

of H-phosphonate **12** was carried out under high dilution conditions to furnish fully protected cyclic product **13** in 73% yield, using diphenyl chlorophosphate as an activating agent.

The linear diguanylic acid H-phosphonate **12** was characterized by ^{31}P NMR (Figure 4). As expected, two sets of peaks were observed, each of which gave an integral representing one phosphorus. The resonance at ca. 2.9 ppm represents the H-phosphonate phosphorus center and is split by the proton that is attached to it with a coupling constant of 626.9 Hz.



SCHEME 2 *Reagents and conditions* i. $(CH_3)_3COCl$, C_5H_5N; ii, **11**, C_5H_5N; iii, $NH_2NH_2{\cdot}H_2O$, CH_3COOH, H_2O, C_5H_5N; iv, **8**, $(CH_3)_3COCl$, C_5H_5N, 0°C; v, CF_3COOH, pyrrole, CH_2Cl_2; vi, $(PhO)_2P(O)Cl$, CH_2Cl_2, C_5H_5N, -40°C; vii, **11**, C_5H_5N.

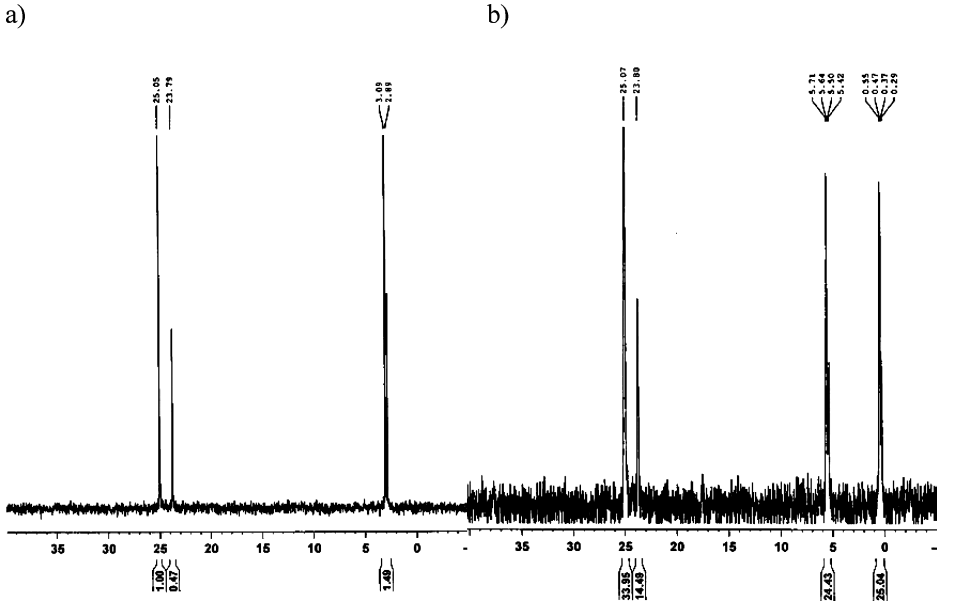


FIGURE 4 ^{31}P NMR spectra of linear diguanylic acid H-phosphonate **10**. a) ^{1}H decoupled; b) ^{1}H undecoupled.

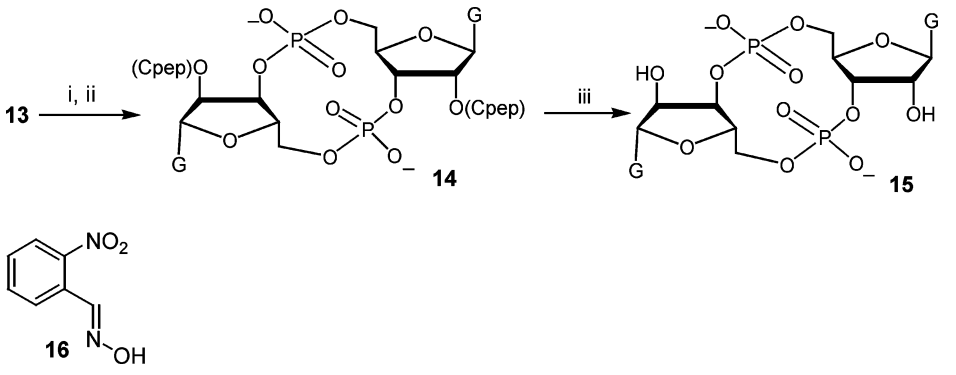
^{31}P NMR spectrum of the fully protected cdiGMP **13** revealed three distinguishable peaks (25.46, 28.73, and 28.94 ppm). Presumably, they represent the four diastereoisomers ([*R, R*], [*R, S*], [*S, S*], and [*S, R*]) due to the chirality of the internucleotide phosphorothioate linkages.

Unblocking of cdiGMP **13** involves three steps. First, an oximate treatment displaces the P-SPh groups as well as the O^6-phenyl protecting groups on guanine base residues (Scheme 3, step i). This is followed by ammonolysis to give the partially, that is, Cpep-protected cdiGMP **14** (Scheme 3, step ii). Its molecular weight in the form of M− was found by ESI-MS to be 1163.3 (calculated 1163.27). This intermediate also was characterized by ^{1}H and ^{31}P NMR. Since the internucleotide phosphorothioate linkages in the fully protected compound **13** are now converted to phosphate diesters, only one phosphorus peak was observed in the ^{31}P NMR spectrum at 0.38 ppm.

Unblocking of the Cpep protecting groups (Scheme 3, step iii) was effected by treatment with triethylammonium formate buffer [pH 2.52]–DMA (2:3 v/v) (apparent pH 4.0) at 40°C for 5 hours to give fully unblocked cdiGMP **15** in 95% yield. The fully unblocked cdiGMP **15** was characterized by ^{1}H and ^{31}P NMR (Figure 5). ^{1}H NMR spectroscopy recorded at 40°C (Figure 5a) revealed all the protons of cdiGMP **15**. Formation of homogeneous product was indicated by the ^{1}H and ^{31}P NMR spectra. HR-MALDI for $[M-H]^-$ was found to be 689.1095 (calculated 689.0870). Reverse phase HPLC analysis (Figure 6b) also confirmed the homogeneity of cdiGMP **15**.

CONCLUSION

Using Cpep as the protecting group for 2′-hydroxy functions, homogeneous cdiGMP was successfully synthesized in good yield. This method can be extended to the preparation of other cyclic nucleotide analogues that are under investigation for their roles in bacterial biofilm regulation.



SCHEME 3 *Reagents and conditions* i. **16**, $(CH_3)_2NC(=NH)N(CH_3)_2$, CH_3CN; ii, *aq.* NH_3, 55°C; iii, $(CH_3)_2NC(O)CH_3$, NEt_3-HCOOH buffer (pH2.52), 40°C, 5 hours.

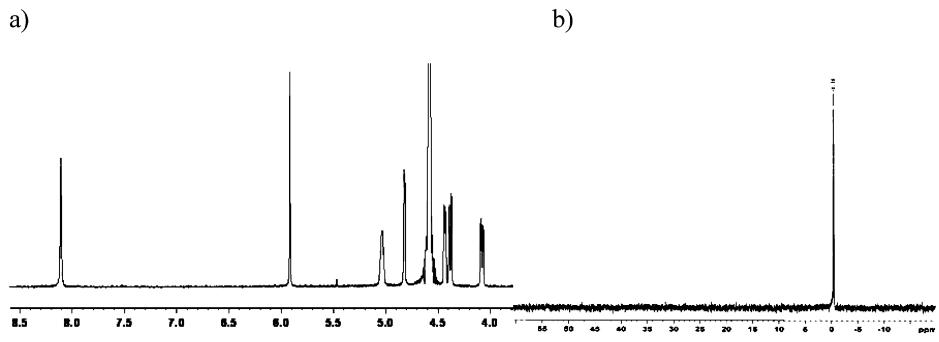


FIGURE 5 a) ^{1}H (recorded at 40°C) and b) ^{31}P NMR spectra of the fully-deprotected cdiGMP **15** in D_2O.

Experimental

^{1}H and ^{31}P NMR spectra were measured at 300 and 90 MHz on a Bruker Avance (at the Department of Chemistry, Brock University) 300 spectrometer, respectively, unless stated otherwise; tetramethylsilane was used as an internal standard, and J values are given in Hz. Reverse phase high-performance liquid chromatography (HPLC) was carried out on a 4.6 × 150 mm Acclaim PA C_{18} 3μ column: the column was eluted with triethylammonium acetate (TEAA) buffer (0.1 *M*, pH7.0)—acetonitrile mixtures [programme A: linear gradient of TEAA buffer-acetonitrile (100 :

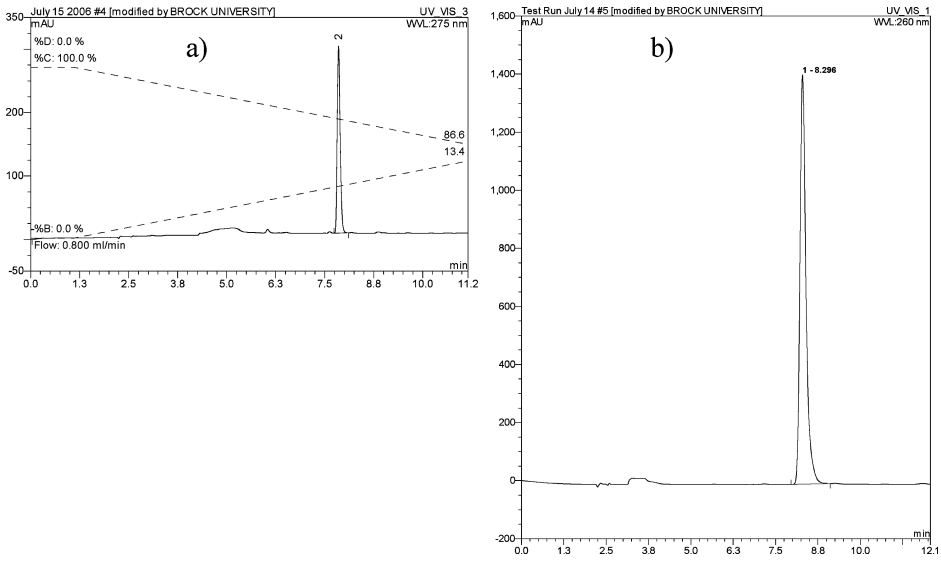


FIGURE 6 Reverse phase HPLC profile of **14** (profile a, program A: linear gradient of 0.1 *M* linear gradient of triethylammonium acetate [TEAA] buffer-acetonitrile (100 : 0 v/v to 80 : 20 v/v) over 10 minutes and then isocratic elution) and **15** (profile b, programme B: TEAA buffer–acetonitrile (100: 00 v/v to 98 : 2 v/v) over 10 minutes and then isocratic elution).

0 v/v to 80 : 20 v/v) over 10 minutes and then isocratic elution; programme B: linear gradient of TEAA buffer–acetonitrile (100 : 00 v/v to 98 : 2 v/v) over 10 minutes and then isocratic elution]. Merck silica gel 60 F_{254} TLC plates were developed in solvent system: [dichloromethane–methanol (95:5 v/v)]. Merck Kieselgel H (Art 7729 and 9385) was used for short column chromatography. Pyridine, 1-methylpyrrolidine, and acetonitrile were dried by heating with calcium hydride, under reflux, and then distilled; *N*,*N*,*N*′*N*′-tetramethylguanidine was dried by distillation over calcium hydride under reduced pressure; dichloromethane was dried over phosphorous pentoxide and distilled; and toluene was dried by heating, under reflux, with sodium and benzophenone, and then distilled. 1,1-Dichloro-1,1,3,3-tetraisopropyldisiloxane, 9-phenyl-xanthene-9-ol, and 2-*N*-phenylacetylguanosine were purchased from Rasayan, Inc. and were used without further purification.

2′*-O-*[1-(4-Chlorophenyl)-4-ethoxypiperidin-4-yl]*-6-O-*(2,5-dichlorophenyl)*-2-N-*phenylacetylguanosine 4

2′-*O*-[1-(4-Chlorophenyl)-4-ethoxypiperidin-4-yl]-2-*N*-phenylacetyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxyl)guanosine **2**[19] (6.800 g, 7.713 mmol) was azeotroped with dry pyridine (2 × 10 ml) and then redissolved in pyridine (30 ml) and cooled (ice-water bath). Mesitylenesulfonyl chloride (2.700 g, 12.34 mmol) and *N*-methylpyrrolidine (8.2 ml, 78.9 mmol) followed after 15 minutes, 2,5-dichlorophenol (4.10 g, 25.19 mmol) were added. The reaction mixture was then allowed to warm up to room temperature. After 2 hours, the products were partitioned between dichloromethane (150 ml) and saturated aqueous sodium hydrogen carbonate (2 × 100 ml). The layers were separated and the aqueous layers were back-extracted with dichloromethane (2 × 50 ml). The combined dried ($MgSO_4$) organic layers were concentrated under reduced pressure. The residue was taken up with acetonitrile (40 ml) and a solution of tetraethylammonium fluoride in acetonitrile (40 ml, 1 *M*, pH 8.0) was added. After 30 minutes, the products were evaporated under reduced pressure and the residue was partitioned between dichloromethane (200 ml) and saturated aqueous sodium hydrogen carbonate (100 ml). The layers were separated and the aqueous layer was back-extracted with dichloromethane (2 × 50 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane-methanol (98:2 v/v) were pooled and evaporated under reduced pressure to give the *title compound* as a colorless froth (5.500 g, 90.9%). R_f: 0.35. ESI-MS found $[M-H]^- = 781.1$. $^{12}C_{37}{}^{1}H_{36}{}^{35}Cl_3{}^{14}N_6{}^{16}O_7{}^-$ requires: 781.1711.

δ_H[$(CD_3)_2SO$] include the following peaks: 0.68 (3 H, t, $-CH_2CH_3$ (Cpep), $J = 6.9$), 1.64 (2 H, m, CH_2 (Cpep)), 1.85 (2 H, m, CH_2 (Cpep)), 2.73 (2 H, m, CH_2 (Cpep)), 2.96 (1 H, m, CH_2 (Cpep)), 3.07 (1 H, m, CH_2 (Cpep)), 3.32 (4 H, m, $-CH_2CH_3$ (Cpep)), 3.65 (1 H, m, H-5′), 3.72 (1 H, m, H-5″), 4.00 (1 H, br, H-4′), 4.19 (1 H, br, H-3′), 4.93 (1 H, dd, H-2′, $J =$ 5.1 and 6.6), 5.12 (1 H, t, 5′-OH, $J = 5.4$, ex), 5.22 (1 H, d, 3′-OH, $J = 4.8$, ex), 6.09 (1 H, d, H-1′, $J = 6.6$), 7.14–7.44 (7 H, m), 7.66 (1 H, d, $J = 8.7$), 7.76 (1 H, d, $J = 2.4$), 8.58 (1 H, dd, $J = 5.7$ and 1.5), 8.71 (1 H, s, H-8), 10.63 (1 H, s, ex, NH).

2′-O-[1-(4-Chlorophenyl)-4-ethoxypiperidin-4-yl]-6-O-(2,5-dichlorophenyl)-2-N-phenylacetyl-5′-O-(xanthen-9-yl)guanosine 5

4 (5.40 g, 6.89 mmol) Was evaporated with dry pyridine (2 × 10 ml). The residue was redissolved in anhydrous pyridine (40 ml) followed by addition of 9-chloro-9-phenylxanthene[29] (2.50 g, 8.54 mmol). After 30 minutes, a mixture of methanol–*N*-methylmorpholine (4 ml, 1:1 v/v) was added. After a further period of 10 minutes, the products were partitioned between dichloromethane (100 ml) and saturated aqueous sodium hydrogen carbonate (80 ml). The layers were separated and the aqueous layer was back-extracted with dichloromethane (2 × 10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (98.5:1.5 v/v) were combined and concentrated under reduced pressure to give **5** as a colorless froth (6.31 g, 88%). R_f: 0.45.

δ_H[$(CD_3)_2SO$] include the following peaks: 0.76 (3 H, t, $-CH_2CH_3$ (Cpep), $J = 6.9$), 3.63 (1 H, d, $-CH_2Ph$, $J = 15.0$), 3.70 (1 H, d, $-CH_2Ph$, $J = 15.0$), 4.12 (1 H, br, H-4′), 4.25 (1 H, br, H-3′), 5.01 (1 H, t, H-2′, $J = 5.6$), 5.23 (1 H, d, 3′-OH, $J = 6.0$, ex), 6.10 (1 H, d, H-1′, $J = 6.0$), 7.66 (1 H, d, $J = 8.7$), 8.50 (1 H, s, H-8), 8.58 (2 H, dd, $J = 5.8$ and 1.6), 10.54 (1 H, s, ex, NH).

2′-O-[1-(4-Chlorophenyl)-4-ethoxypiperidin-4-yl]-6-O-(2,5-di-chlorophenyl)-2-N-phenylacetyl-5′-O-(xanthen-9-yl)guanosine 3′ H-phosphonate, triethylammonium salt 7

p-Tolyl H-phosphonate **8** (1.14 g, 6.02 mmol) was evaporated with triethylamine (1.70 ml, 12.20 mmol) and methanol (10 ml). To the residue was added **5** (2.080 g, 2.0 mmol). The mixture was azeotroped with dry pyridine (2 × 5 ml). The residue was redissolved in anhydrous pyridine (25 ml) and cooled to 0°C (ice-water bath). Pivaloyl chloride (0.92 ml, 7.47 mmol) was added over a period of 5 minutes. After 1 hour, water (5 ml) was added and the reactants were allowed to warm up to room temperature. After 1 hour, the products were partitioned between dichloromethane (100

ml) and saturated aqueous sodium hydrogen carbonate (2 × 80 ml). The combined aqueous layers were back extracted with dichloromethane (2 × 20 ml). The combined organic layers were further washed with triethylammonium phosphate buffer (0.5 *M*, pH 7.0, 80 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (20 ml). The combined dried ($MgSO_4$) organic layers were concentrated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (93:7 v/v) were combined and concentrated under reduced pressure to give **7** as a colorless froth (2.20 g, 91%). ESI-MS found M^-=1101.22. $^{12}C_{56}{}^{1}H_{49}{}^{35}Cl_3{}^{14}N_6{}^{16}O_{10}{}^{31}P^-$ requires: 1101.2313.

δ_H[$(CD_3)_2SO$] include the following peaks: 0.76 (3 H, t, $-CH_2CH_3$ (Cpep), J = 6.9), 1.11 (9 H, t, $NH^+(CH_2CH_3)_3$, J = 2.7), 4.35 (1 H, br, H-4′), 4.75 (1 H, dd, H-3′, J = 8.4 and J 3.9), 5.17 (1 H, dd, H-2′, J = 7.2 and 4.2), 5.79 (0.5 H, s, P*H*), 6.13 (1 H, d, H-1′, J = 7.2), 6.85 (2 H, d, J = 9.0), 6.97 (1 H, t, J = 7.5), 7.15–7.32 (17 H, m), 7.40 (1 H, dd, J = 6.5 and 2.4), 7.64 (1 H, d, J = 8.7), 7.74 (0.5 H, s, P*H*), 7.75 (1 H, s), 8.45 (1 H, s, H-8), 10.71 (1 H, s, ex, NH).

δ_P[$(CD_3)_2SO$]: 0.18 ($^1J_{P\text{-}H}$ = 564.9)

***2′*-O-*[1-(4-Chlorophenyl)-4-ethoxypiperidin-4-yl]*-6-O-*(2,5-dichlorophenyl)-3′*-O-*levulinyl*-2-N-*phenylacetyl*-guanosine 6**

Compound **5** (1.50 g, 1.442 mmol) was dried by azeotroping with dry pyridine (2 × 5 ml), and then redissolved in dry pyridine (10 ml). Levunilic anhydride (0.628 g, 2.932 mmol) was then added, and the reaction was allowed to proceed for 15 hours. Methanol (2 ml) was added. After another 10 minutes, the products were concentrated to dryness under reduced pressure. The residue was co-evaporated with toluene (3 × 5 ml), and then redissolved in dichloromethane (10 ml). Pyrrole (1.00 ml, 14.41 mmol) followed by trifluoroacetic acid (0.67 ml, 9.02 mmol) were added. After 30 seconds, *N*-methylmorpholine (1.00 ml, 9.09 mmol) was added. The products were poured into saturated aqueous sodium hydrogen carbonate (15 ml). The layers were separated and the aqueous layer was back-extracted with dichloromethane (20 ml). The organic layers were then combined, dried ($MgSO_4$), and concentrated. The residue was purified by short column chromatography. The appropriate fractions, which were eluted with dichloromethane–methanol (98:2 v/v) were combined and concentrated under reduced pressure to give **6** as a colorless froth (1.143 g, 90%). R_f: 0.43. ESI-MS found $[M\text{-}H]^-$ = 879.2. $^{12}C_{42}{}^{1}H_{42}{}^{35}Cl_3{}^{14}N_6{}^{16}O_9$ requires: 879.207.

δ_H[$(CD_3)_2SO$] include the following peaks: 0.61 (3 H, t, $-CH_2CH_3$ (Cpep), J = 6.9), 2.95 (2 H, m, CH_2 (Cpep)), 3.19 (2 H, m, CH_2 (Cpep)), 4.14 (1 H, H-4′), 5.26 (1 H, H-2′), 5.29 (1 H, H-3′), 5.34 (1 H, ex, 5′-OH),

6.12 (1 H, d, H-1′, $J = 7.5$), 6.83 (1 H, d, $J = 9.0$), 7.16–7.31 (7 H, m), 7.43 (1 H, dd, $J = 8.7$ and 2.4), 7.66 (1 H, d, $J = 8.7$), 7.76 (1 H, d, $J = 2.4$), 8.74 (1 H, s, H-8), 10.68 (1 H, s, ex, NH).

Px-*G′p(s′)G′*-Lev* 9

Compounds **6** (0.463 g, 0.525 mmol) and **7** (0.750 g, 0.622 mmol) were dried by azeotroping with dry pyridine (2 × 5 ml). The residue was redissolved in anhydrous pyridine (5 ml) and cooled (ice-water bath). Pivaloyl chloride (0.16 ml, 1.30 mmol) was added. After 5 minutes, 1-phenylsulfanyl-pyrrolidine-2,5-dione **11** (0.310 g, 1.496 mmol) was added. The reactants were then warmed up to room temperature. After 30 minutes, water (0.2 ml) was added. After 5 minutes, the products were partitioned between dichloromethane (25 ml) and saturated aqueous sodium hydrogen carbonate (25 ml). The layers were separated and the aqueous layer was back extracted with dichloromethane (2 × 5 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (98.5:1.5 v/v) were combined and concentrated under reduced pressure to give **9** as a colorless froth (1.02 g, 93.5%). R_f: 0.52.

$\delta_P[(CD_3)_2SO]$: 22.10, 22.73

Preparation of HO-*G′p(s′)G′*p(H) 12

Px-*G′p(s′)G′*-Lev **9** (0.900 g, 0.433 mmol) was dissolved in pyridine (6 ml) followed by addition of a mixture of hydrazine monohydrate (0.20 ml, 4.12 mmol), acetic acid (2.4 ml), water (0.4 ml) and pyridine (9 ml) at room temperature. After 15 minutes, pentan-2,4-dione (0.85 ml) was added. Stirring was continued for 10 minutes and the products were partitioned between dichloromethane (30 ml) and saturated aqueous sodium hydrogen carbonate (3 × 30 ml). The layers were separated and the aqueous layers were back-extracted with dichloromethane (2 × 50 ml). The combined dried ($MgSO_4$) organic layers were concentrated under reduced pressure. The residue was dissolved in dichloromethane (1.0 ml) and added drop-wise to diethyl ether (100 ml) under stirring. The precipitate was collected by filtration to give a colorless solid (0.80 g). This material (abbreviated as Px-*G′p(s′)G′*p(H) **10**) was used for the next step without further purification.

*A system of abbreviations[30] is followed for protected oligonucleotides in which nucleoside residues and internucleotide linkages are italicized if they are protected in some defined way. In the present context, *G′* represents guanine protected on *O*-6 with a 2,5-dichlorophenyl group and *N*-2-protected with a phenylacetyl group; and *p(s′)* represents an *S*-phenyl-protected phosphorothioate.

p-Tolyl H-phosphonate **8** (0.222 g, 1.174 mmol) was evaporated with triethylamine (0.50 ml, 3.59 mmol) and methanol (1.0 ml). To the residue was added Px-*G′p(s′)G′*—OH **10** (0.780 g, 0.394 mmol). The mixture was azeotroped with dry pyridine (2 × 5 ml). The residue was redissolved in anhydrous pyridine (8 ml) and cooled to 0°C (ice-water bath). Pivaloyl chloride (0.15 ml, 1.22 mmol) was added in one portion. After 30 minutes, water (1.0 ml) was added. The reactants were stirred at room temperature for 1 hour. The products were then partitioned between dichloromethane (30 ml) and saturated aqueous sodium hydrogen carbonate (30 ml). The combined aqueous layers were back-extracted with dichloromethane (2 × 10 ml). The organic phases were combined, dried ($MgSO_4$) and concentrated under reduced pressure, followed by co-evaporation with toluene (3 × 10 ml). The residue was redissolved in dichloromethane (15 ml). Pyrrole (0.41 ml, 5.91 mmol) followed by trifluoroacetic acid (0.23 ml, 3.10 mmol) were added. After 1 minute, *N*-methylmorpholine (0.48 ml) was added. The products were then extracted with saturated aqueous sodium hydrogen carbonate (20 ml). The layers were separated and the aqueous layers were back extracted with dichloromethane (2 × 10 ml). The combined organic layers were further washed with triethylammonium phosphate buffer (0.5 *M*, pH 7.0, 20 ml). The layers were separated and the aqueous layer was back-extracted with dichloromethane (10 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (92:8 v/v) were pooled and concentrated under reduced pressure to give H-phosphonate **12** as a colorless froth (0.560 g, 70% for three steps). ESI-MS found $M^- = 1781.2$. $^{12}C_{80}{}^{1}H_{77}{}^{35}Cl_6{}^{14}N_{12}{}^{16}O_{17}{}^{31}P_2{}^{32}S^-$ requires: 1781.2857.

$\delta_P[(CD_3)_2SO]$: 2.89, 3.09 (1 P), 23.79, 25.05 (1 P, $^1J_{P\text{-}H} = 626.9$).

Preparation of Fully Protected cdiGMP 13

HO-*G′p(s′)G′*p(H) **12** (0.190 g, 0.100 mmol) was co-evaporated with dry pyridine (2 × 3 ml) and dissolved in anhydrous dichloromethane (10 ml). This solution was then added dropwise over a period of 20 minutes to a cooled solution of diphenyl chlorophosphate (0.41 ml, 1.98 mmol) in dry pyridine (10 ml) at −40°C. After 20 minutes, 1-phenylsulfanyl-pyrrolidine-2,5-dione **11** (0.453 g, 2.18 mmol) was added and the reactants were allowed to warm up to room temperature over 30 minutes. Water (0.5 ml) was then added and after a further period of 5 minutes, the products were partitioned between dichloromethane (15 ml) and saturated aqueous sodium hydrogen carbonate (2 × 10 ml). The layers were separated and the aqueous layers were back extracted with dichloromethane (2×5 ml). The combined dried ($MgSO_4$) organic layers were concentrated under reduced pressure and the

residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (98.5:1.5–98:2 v/v) were pooled and concentrated under reduced pressure to give the fully protected cdiGMP **13** as a colorless froth (0.138 g, 73%). R_f: 0.51 and 0.45.

δ_P[$(CD_3)_2SO$]: 25.46, 28.73, 28.94.

Preparation of 2′-*O*-Cpep Cyclic bis(3′→5′)diguanylic Acid 14 by Partial-Unblocking of Fully Protected Cyclic Diguanydic Acid 13

Fully-protected cdiGMP **13** (30 mg, 15.99 μmol) was azeotroped with dry toluene (2 × 2 ml) and the residue was dissolved in dry acetonitrile (2 ml). 2-Nitrobenzaldoxime **16** (38.3 mg, 0.231 mmol) followed by *N*,*N*,*N*′,*N*′-tetramethylguanidine (26 μl, 0.21 mmol) were added. The reactants were stirred at room temperature for 16 hours and were then concentrated under reduced pressure. To the residue was added concentrated aqueous ammonia (33%, *d* 0.88, 2.0 ml) and the mixture was heated at 55°C for 15 hours. After the products had been cooled, they were concentrated to dryness followed by co-evaporation with ethanol (2× 2 ml) under reduced pressure. The residue was dissolved in methanol (1 ml) and precipitated with diethyl ether (30 ml). The precipitate was collected by centrifugation. This precipitation-centrifugation process was repeated for one more time and the solid residue was dried in vacuo to give 2′-*O*-Cpep cyclic bis(3′→5′)diguanylic acid **14** as a colorless solid (17.0 mg, 89.5%). ESI-MS found M^-=1163.3. $^{12}C_{46}{}^{1}H_{55}{}^{35}Cl_2{}^{14}N_{12}{}^{16}O_{16}{}^{31}P_2{}^-$ requires: 1163.2711. R_t (Programme A): 7.8 minutes.

δ_P[D_2O]: −0.38.

δ_H[D_2O, 600 MHz] include the following peaks: 0.67 (6 H, s, $-CH_2CH_3$ (Cpep)), 3.45 (2 H, br), 3.99 (2 H, br, H-5′), 4.21 (2 H, br, H-5″), 4.55 (2 H, br, H-4′), 4.85 (2 H, br, H-2′), 5.00 (2 H, br, H-3′), 5.86 (2 H, br, H-1′), 6.79 (4 H, br, Cpep), 7.11 (2 H, s, br, Cpep), 7.12 (2 H, s, br, Cpep), 7.88 (2 H, s, H-8).

Cyclic bis(3′→5′)diguanylic Acid 15 (cdiGMP)

Substrate **14** (10 mg, 8.60 μmol) was dissolved in dimethylacetamide (1.8 ml) followed by addition of triethylammonium formate buffer (0.5 *M*, pH 2.52, 1.2 ml, prepared with sterile water). The reactants were then sealed and heated at 40°C. After 5 hours, the products were cooled, and the pH was adjusted to ca. 7.0 with triethylamine. The products were then extracted with chloroform (4 × 2.0 ml). The organic layers were discarded. The aqueous layer was evaporated under reduced pressure at room temperature to a volume of *ca.* 0.2 ml. To the residue was added

n-butanol (2.0 ml). The mixture was vortexed vigorously and then chilled at –78°C (dry ice–acetone bath) for 10 minutes. Then it was centrifuged for 10 minutes. The supernatant was discarded and the pellet was redissolved in sterile water (0.2 ml). The above precipitation–centrifugation process was repeated two more times. The final pellet was then dried in vacuo. This material was dissolved in sterile water (1.0 ml) and passed through an Amberlite (IR120, Na^+ form) cation-exchange column (0.5 × 2.0 cm). The fractions which contained oligonucleotide were pooled and freeze-dried to give the fully unblocked cdiGMP **15** as a colorless froth (6.0 mg, 94.9%). HR-MALDI found $M^- = 689.1095$. $^{12}C_{20}{}^{1}H_{23}{}^{14}N_{10}{}^{16}O_{14}{}^{31}P_2{}^-$ requires 689.0870. R_t (Programme B): 8.3 minutes.

δ_H[$(CD_3)_2SO$, 600 MHz 40°C]: 4.08 (2 H, dd, H-5′, *J* 11.6 and 3.7), 4.38 (2 H, d, H-5″, *J* 11.5), 4.44 (2 H, d, H-4′, *J* 8.3), 4.82 (2 H, d, H-2′, *J* 3.6), 5.04 (2 H, m, H-3′), 5.92 (2 H, s, H-1′), 8.11 (2 H, s, H-8).

δ_P[D_2O, 242 MHz]: −1.55.

REFERENCES

1. Ross, P.; Weinhouse, H.; Aloni, Y.; Michaeli, D.; Weinberger-Ohana, P.; Mayer, R.; Braun, S.; de Vroom, E.; Van Der Marel, G.A.; van Boom, J.H.; Benziman, M. Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* **1987**, 325, 279–281.
2. Jenal, U. Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria?. *Curr. Opin. Microbiol.* **2004**, 7, 185–191.
3. Römling, U.; Gomelsky, M.; Galperin, M.Y. C-di-GMP: the dawning of a novel bacterial signalling system. *Mol. Microbiol.* **2005**, 57, 629–639.
4. Simm, R.; Morr, M.; Kader, A.; Nimtz, M.; Römling, U. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol. Microbiol.* **2004**, 53, 1123–1134.
5. Tischler, A.D.; Camilli, A. Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. *Mo. Microbiol.* **2004**, 53, 857–869.
6. Thormann, K.M.; Duttler, S.; Saville, R.M.; Hyodo, M.; Shukla, S.; Hayakawa, Y.; Spormann, A.M. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. *J. Bacteriol.* **2006**, 188, 2681–2691.
7. Karaolis, D.K.R.; Rashid, M.H.; Chythanya, R.; Luo, W.; Hyodo, M.; Hayakawa, Y. c-di-GMP (3′-5′-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. *Antimicrob. Agents Chemother.* **2005**, 49, 1029–1038.
8. Brouillette, E.; Hyodo, M.; Hayakawa, Y.; Karaolis, D.K.; Malouin, F. 3′,5′-Cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection. *Antimicrob. Agents Chemother.* **2005**, 49, 3109–3113.
9. Kulasakara, H.; Lee, V.; Brencic, A.; Liberati, N.; Urbach, J.; Miyata, S.; Lee, D.G.; Neely, A.N.; Hyodo, M.; Hayakawa, Y.; Ausubel, F.M.; Lory, S. Analysis of Pseudomonas aeruginosa diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′-5′)-cyclic-GMP in virulence. *Proc. Natl. Acad. Sci. USA* **2006**, 103, 2838–2844.
10. Méndez-Ortiz, M.M.; Hyodo, M.; Hayakawa, Y.; Membrillo-Hernández, J. Genome-wide transcriptional profile of Escherichia coli in response to high levels of the second messenger 3′,5′-cyclic diguanylic acid. *J. Biol. Chem.* **2006**, 281, 8090–8099.
11. Ross, P.; Mayer, R.; Weinhouse, H.; Amikam, D.; Huggirat, Y.; Benziman, M.; de Vroom, E.; Fidder, A.; de Paus, P.; Sliedregt, L.A.J.M.; Van Der Marel, G.A. The cyclic diguanylic acid regulatory system of cellulose synthesis in Acetobacter xylinum. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives. *J. Biol. Chem.* **1990**, 265, 18933–18943.

12. (a) Hayakawa, Y.; Nagata, R.; Hirata, A.; Hyodo, M.; Kawai, R. A facile synthesis of cyclic bis(3′→5′) diguanylic acid, *Tetrahedron* **2003**, 59, 6465–6471. (b) Hyodo, M.; Hayakawa, Y. An improved method for synthesizing cyclic bis(3′-5′)diguanylic acid (c-di-GMP). *Bull. Chem. Soc. Jpn.* **2004**, 77, 2089–2093.
13. Zhang, Z.; Gaffney, B.L.; Jones, R.A. c-di-GMP displays a monovalent metal ion-dependent polymorphism. *J. Am. Chem. Soc.* **2004**, 126, 16700–16701.
14. Ogilvie, K.K.; Sadana, K.L.; Thompson, A.E.; Quillian, M.A.; Westmore, J.B. The use of silyl groups in protecting the hydroxyl functions of ribonucleosides. *Tetrahedron Lett.* **1974**, 15, 2861–2863.
15. Griffin, B.E.; Jarman, M.; Reese, C.B. The Synthesis of oligoribonucleotides—IV: Preparation of dinucleoside phosphates from 2′,5′-protected ribonucleoside derivatives. *Tetrahedron* **1968**, 24, 639–662.
16. Pitsch, S.; Weiss, P.A.; Jenny, L.; Stutz, A.; Wu, X. Reliable chemical synthesis of oligoribonucleotides (RNA) with 2′-*O*-[(triisopropylsilyl)oxy]methyl(2′-*O*-tom)-protected phosphoramidites. *Helv. Chim. Acta* **2001**, 84, 3773–3795.
17. Scaringe, S.A.; Wincott, F.E.; Caruthers, M.H. Novel RNA synthesis method using 5′-*O*-silyl-2′-*O*-orthoester protecting groups. *J. Am. Chem. Soc.* **1998**, 120, 11820–11821.
18. Ohgi, T.; Masutomi, Y.; Ishiyama, K.; Kitagawa, H.; Shiba, Y.; Yano, J. A new RNA synthetic method with a 2′-*O*-(2-cyanoethoxymethyl) protecting group. *Org. Lett.* **2005**, 7, 3477–3480.
19. Lloyd, W.; Reese, C.B.; Song, Q.L.; Vandersteen, A.M.; Visintin, C.; Zhang, P.Z. Some observations relating to the use of 1-aryl-4-alkoxypiperidin-4-yl groups for the protection of the 2′-hydroxy functions in the chemical synthesis of oligoribonucleotides. *J. Chem. Soc., [Perkin 1]* **2000**, 1, 165–176.
20. Reese, C.B.; Yan, H. [unpublished results]
21. Reese, C.B.; Yan, H.B. Alternatives to the 4,4′-dimethoxytrityl (DMTr) protecting group. *Tetrahedron Lett.* **2004**, 45, 2567–2570.
22. Reese, C.C.; Skone, P.A. The protection of thymine and guanine residues in oligodeoxyribonucleotide synthesis. *J. Chem.Soc.[Perkin 1]* **1984**, 1, 1263–1271.
23. Vaman Rao, M.; Reese, C.B.; Schehlmann, V.; Pak Sang, Y. Use of the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) protecting group in the solid-phase synthesis of oligo- and polyribonucleotides. *J. Chem. Soc. [Perkin 1]* **1993**, 1, 43–55.
24. Markiewicz, W.T. Tetraisopropyldisiloxane-1,3-diyl, a group for simultaneous protection of 3′-hydroxy and 5′-hydroxy functions of nucleosides. *J. Chem. Res. Synop.* **1979**, 1, 24–25.
25. Reese, C.B.; Song, Q.L. The H-phosphonate approach to the solution phase synthesis of linear and cyclic oligoribonucleotides. *Nucleic Acids Res.* **1999**, 27, 963–971.
26. Reese, C.B.; Yan, H.B. Solution phase synthesis of ISIS 2922 (Vitravene) by the modified H-phosphonate approach. *J. Chem. Soc [Perkin 1]* **2002**, 23, 2619–2633.
27. Ozola, V.; Reese, C.B.; Song, Q.L. Use of ammonium aryl H-phosphonates in the preparation of nucleoside H-phosphonate building blocks. *Tetrahedron Lett.* **1996**, 37, 8621–8624.
28. LaLonde, R.T.; Eckert, T.S. Synthesis of α-thiohemiaminal derivatives of deoxynupharidine. *Can. J. Chem.* **1981**, 59, 2298–2302.
29. Gomberg, M.; Cone, L.H. Triphenylmethyl. XVIII. Quinocarbonium Salts. *Justus Liebigs Ann. Chem.* **1909**, 370, 142–208.
30. Chaudhuri, B.; Reese, C.B.; Węcławek, K. Rapid oligodeoxyribonucleotide synthesis by the "filtration" method. *Tetrahedron Lett.* **1984**, 25, 4037–4040.