Bioorganic & Medicinal Chemistry Letters 18 (2008) 5631–5634

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and immunostimulatory properties of the phosphorothioate analogues of cdiGMP

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ARTICLE INFO

Article history:
Received 24 July 2008
Revised 23 August 2008
Accepted 26 August 2008
Available online 29 August 2008

Keywords:
cdiGMP
Vaccine adjuvant
Immunostimulation
Phosphorothioate

ABSTRACT

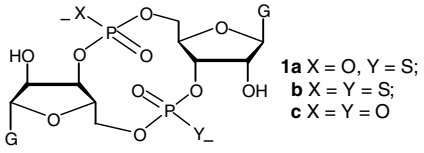
The synthesis of mono- and bisphosphorothioate analogues of 3′,5′-cyclic diguanylic acid (cdiGMP) via the modified H-phosphonate chemistry is reported. The immunostimulatory properties of these analogues were compared with those of cdiGMP.

3′,5′-Cyclic diguanylic acid (cdiGMP, **1c**) has recently been recognized as an important bacterial second messenger.[1–3] It has also been shown to possess extraordinary immunostimulatory properties and is therefore evaluated as a potential vaccine adjuvant candidate.[4–7]

We previously reported a convenient synthesis of cdiGMP.[8] In order to explore the structure–immunostimulation relationship of cdiGMP, we synthesized the phosphorothioate analogues of cdiGMP (Fig. 1), where either one (cdiGMP-S1 **1a**)[9] or two (cdiGMP-S2 **1b**) sulfur atoms replace the non-bridging oxygen at the internucleotide linkages.

The 2′- and 5′-hydroxyls of guanosine were protected with the 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep)[10] and the 9-phenyl-xanthen-9-yl (or the pixyl)[11,12] groups, respectively (Fig. 2). Guanine was 'doubly'-protected at both *O*-6 and *N*-2, as is shown in Figure 2. The modified H-phosphonate approach[13,14] was used due to its flexibility in the preparation of both phosphates and phosphorothioates.

The synthesis of the phosphorothioates via the modified H-phosphonate approach is illustrated in Scheme 1. In situ treatment of H-phosphonate diesters with a sulfur-transfer reagent *S*-(2-cyanoethyl)phthalimide **9** gave phosphorothioate triester **4**, which was further transformed into linear dimer H-phosphonate **6**. Cyclization of this linear dimer H-phosphonate **6** took place under high dilution conditions to furnish the fully protected cyclic dinucleotide phosphorothioate trieters **7a** and **7b** in good yields (75–80%).



1a X = O, Y = S;
b X = Y = S;
c X = Y = O

Figure 1. cdiGMP **1c** and its phosphorothioate analogues.

A four-step deprotection protocol (Scheme 2) was used to give the fully deprotected cdiGMP-S1 **1a** and cdiGMP-S2 **1b** in good yields (70–75%).[15] Removal of the *S*-(2-cyanoethyl)- group by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) under anhydrous conditions was followed by treatment with 2-nitrobenzaldoxime **12** and ammonolysis in the presence of mercaptoethanol (Scheme 2, steps i–iii). The resulting partially protected cyclic dimers **11** were then further deprotected in a triethylammonium formate buffer that contains methanol (Scheme 2, steps iv and v). The ^{1}H and ^{31}P NMR spectra of **1a** and **1b** are shown in Figure 3. Resonance at ca. 55 and −1 ppm in the ^{31}P NMR spectra correspond to phosphorothioate and phosphate, respectively. It is noted that the two sets of phosphorous signals in cdiGMP-S1 **1a** integrate equally (panel b) and that there is no signal at ca. 0 ppm in the cdiGMP-S2 **1b** (panel d).

The fully deprotected cdiGMP-S1 **1a** and S2 **1b** were also analyzed by reverse phase HPLC on a Dionex Acclaim PA C_{18} column (Fig. 4).

We then carried out preliminary evaluation of the immunostimulatory properties of cdiGMP, cdiGMP-S1, and cdiGMP-S2. In

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doi:10.1016/j.bmcl.2008.08.088

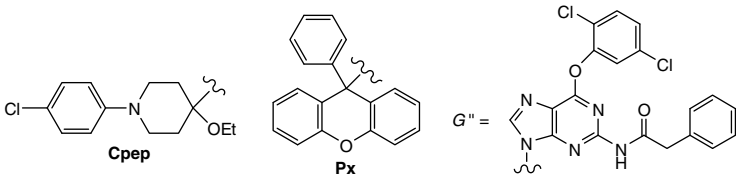


Figure 2. Protecting groups used in this study.

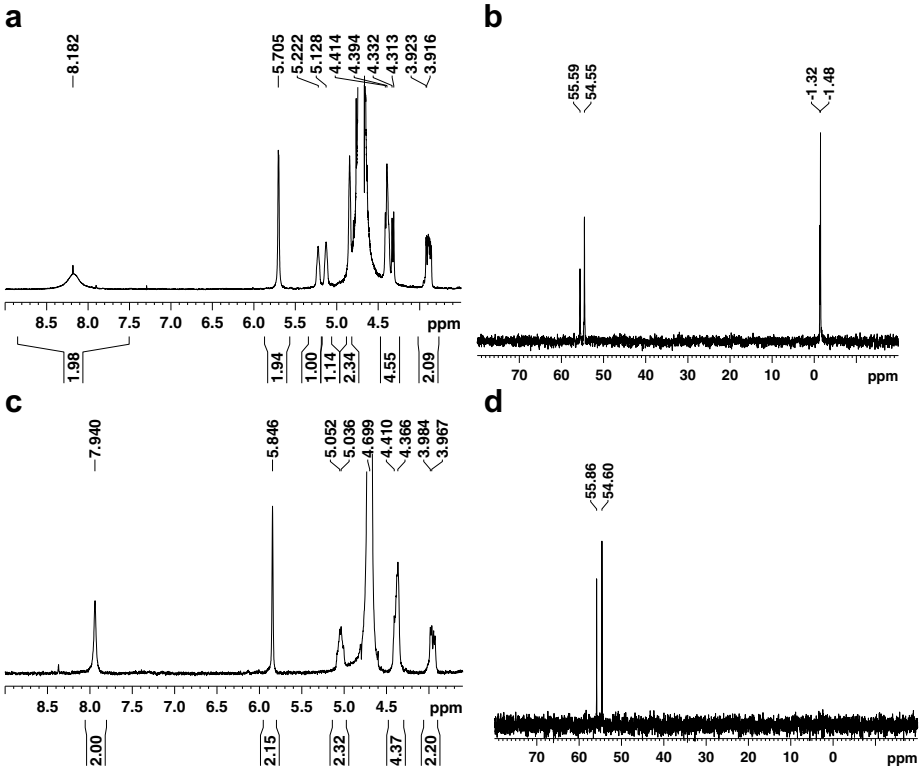
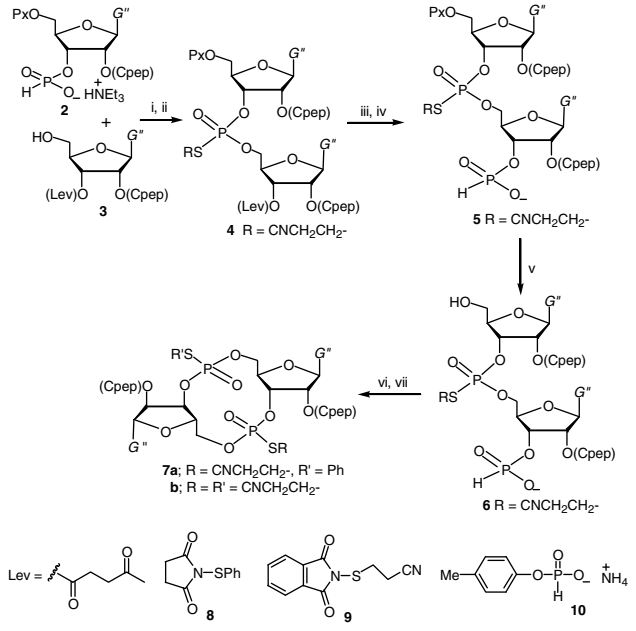
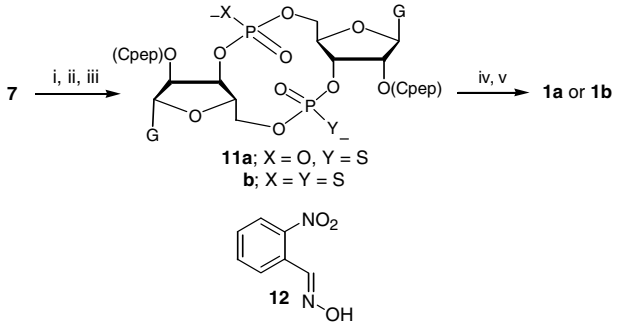


Figure 3. ^{1}H and ^{31}P NMR spectra of **1a** (a and b) and **1b** (c and d).



Scheme 1. Synthesis of fully protected cdiGMP-S1 and S2. (i) $(CH_3)_3COCl$, C_5H_5N; (ii) **9**, C_5H_5N; (iii) $NH_2NH_2{\cdot}H_2O$, CH_3COOH, H_2O, C_5H_5N; (iv) **10**, $(CH_3)_3COCl$, 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) **8** or **9**, C_5H_5N.

the first experiment, groups of five female 8-week-old C57BL/6 mice were intranasally administered with 0, 5, 10, and 69 μg of cdiGMP in injectable phosphate buffered saline (PBS). The mice were killed 24 h later and their lungs were lavaged with PBS supplemented with 3 mM EDTA (1 ml, 5×). The total and differential cell counts as well as a panel of 21 chemokines and cytokines were measured. As can be seen in Figure 5, intranasal instillation of cdiGMP induced a dose-dependent recruitment of inflammatory cells into the bronchoalveolar spaces with the majority of recruited



Scheme 2. Unblocking of fully protected cdiGMP-S1 and S2. (i) DBU, $(CH_3)_3SiCl$, CH_3CN; (ii) **12**, DBU, CH_3CN; (iii) aq NH_3, $HSCH_2CH_2OH$, 55 °C; (iv) CH_3OH, NEt_3-HCOOH buffer (pH 3.75), 40 °C, 4 h; (v) Amberlite IR-120, Na^+ form.

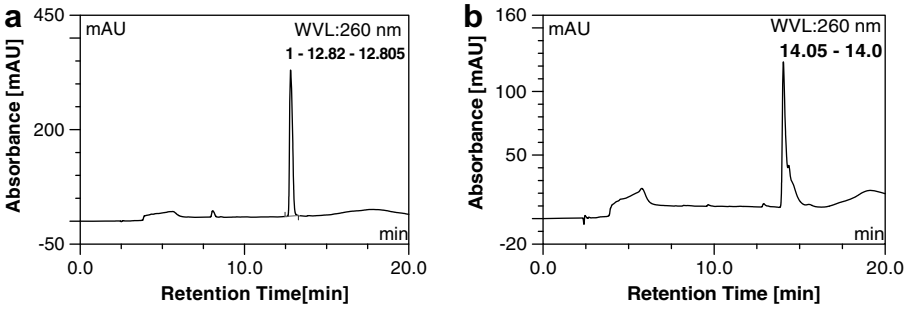


Figure 4. HPLC profiles of **1a** (a) and **1b** (b) (Programme: linear gradient of 0.1 M triethylammonium acetate buffer–acetonitrile (100:0–90:10 v/v) over 10 min and then isocratic elution).

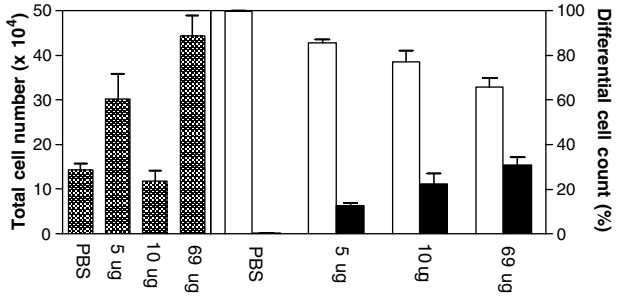


Figure 5. Dose-dependent recruitment of inflammatory cells into the bronchoalveolar spaces by cdiGMP.

cells being neutrophils (solid bars in the right panel). This effect was particularly evident when 69 μg of cdiGMP was used.

Similarly, Luminex analysis of the lavage fluid showed a significant dose-dependent induction of key proinflammatory cytokines and chemokines such as IL-12(p40), IL-1beta, IL-6, KC, MCP-1, MIP-1beta, RANTES, and TNF-alpha (Fig. 6) whereas there was no change in the levels of GM-CSF, IFN-gamma, IL-10, IL-13, IL-17, IL-1alpha, IL-2, IL-3, IL-4, IL-5, IL-9, or VEGF. These data suggests that cdiGMP is immunostimulatory at one of the mucosal sites and has the potential to function as a mucosal adjuvant.

When the same experiments were carried out with cdiGMP-S1 and cdiGMP-S2, these two analogues appear to retain their immunostimulatory function by induction of pulmonary neutrophil recruitment (Fig. 7) and local proinflammatory cytokine/chemokine responses (Fig. 7). However, the inflammatory responses elicited by the two analogues, particularly the cdiGMP-S1, were substantially milder than cdiGMP (Figs. 7 and 8), in that fewer neutrophils and lower amounts of chemokine KC, MIP-1beta, and RANTES were induced in cdiGMP-S1-treated than in cdiGMP-treated

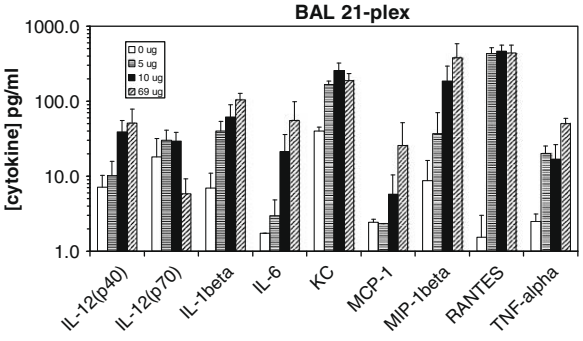


Figure 6. Dose-dependent induction of key proinflammatory cytokines and chemokines by cdiGMP.

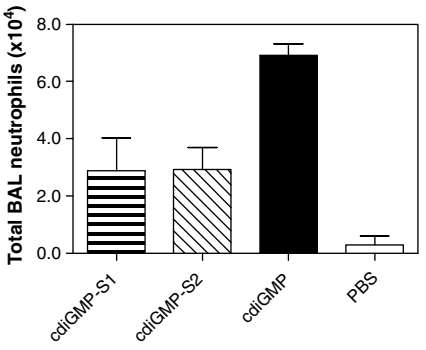


Figure 7. Induction of pulmonary neutrophil recruitment by cdiGMP and its analogues (cdiGMP-S1 and cdiGMP-S2).

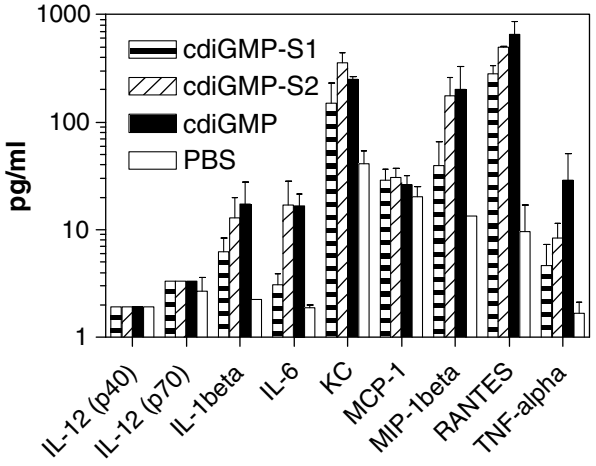


Figure 8. Induction of key proinflammatory cytokines and chemokines by cdiGMP and its analogues (cdiGMP-S1 and cdiGMP-S2).

mice. Although the induction of proinflammatory responses is a prerequisite for an adjuvant to induce effective immune responses, excessive tissue inflammation is also detrimental to the host. Thus, it is possible that these analogues are superior to cdiGMP as mucosal adjuvants with fewer side effects. More detailed immunological evaluations of these analogues are currently underway.

Acknowledgments

This work is supported by Research Corporation, Canada Foundation for Innovation, Natural Sciences and Engineering Research Council of Canada, and National Research Council Canada (A-base). The authors thank Tim Jones and Razvan Simionescu for mass

spectrometric and NMR analysis, respectively, and Mr. Greg Harris for Luminex assay.

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15. Deprotection of the fully protected cdiGMP-S2 **7b**: Fully protected cdiGMP-S2 **7b** (50 mg, 27 μmol) was dried by evaporation with dry pyridine (2× 1 ml). The residue was dissolved in dry acetonitrile (2 ml) followed by addition of trimethylsilyl chloride (20 μl) and DBU (0.10 ml, 0.67 mmol). After 30 min, the products were concentrated under reduced pressure and the residue was redissolved in dry acetonitrile (2 ml) followed by addition of 2-nitrobenzaldoxime (92 mg, 0.55 mmol). The reaction was allowed to proceed overnight at room temperature, and the products were evaporated to dryness under reduced pressure. The residue was taken up with a mixture of 2-mercaptoethanol (0.1 ml) and aqueous ammonium hydroxide (1.0 ml) and incubated in a sealed vial at 55 °C overnight. Upon cooling, the products were evaporated under reduced pressure and were then co-evaporated with absolute ethanol (2× 2 ml). The residue was dissolved in methanol (1.5 ml) and precipitated with a mixture of ethyl acetate and diethyl ether (30 ml, 1:3 v/v). The precipitate was collected by centrifugation at 5000 RPM. This precipitation–centrifugation process was repeated two more times. The dried residue was suspended in methanol (3.0 ml) followed by addition of triethylammonium formate buffer (4.5 ml, pH 3.75, 0.5 M) and incubated at 40 °C. After 6 h, the pH of the reaction mixture was adjusted to neutral by addition of triethylammonium acetate buffer (pH 10). The products were then lyophilized. The residue was taken up with water (300 μl) followed by addition of *n*-butanol (3.0 ml). The mixture was vortexed vigorously, frozen in liquid nitrogen, and then centrifuged at 10,000 rpm. The pellet was collected and redissolved in water and precipitated with *n*-butanol two more times in the same way as described above. The final pellet was dissolved in water (1.0 ml) and passed through an Amberlite ionic exchange column (Na^+ form, 1× 10 cm). The appropriate fractions were combined and lyophilized to give the sodium salt of cdiGMP-S2 1b as a loose colorless solid (15 mg, 72%). ESI-MS found M^- = 721.06. $^{12}C_{20}{}^{1}H_{23}{}^{14}N_{10}{}^{16}O_{12}{}^{31}P_2{}^{32}S_2{}^-$ requires: 721.04.