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Synthesis of c-di-GMP Analogs with Thiourea, Urea, Carbodiimide, and Guanidinium Linkages

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Abstract

The first syntheses of neutral thiourea, urea, and carbodiimide analogs, along with two guanidinium analogs, of the bacterial signaling molecule cyclic diguanosine monophosphate (c-di-GMP) are reported. The key intermediate, obtained in nine steps, is a 3'-amino-5'-azido-3',5'-dideoxy derivative. The 5'-azide serves as a masked amine from which the amine is obtained by Staudinger reduction, while the 3'-amine is converted to an isothiocyanate that, while stable to chromatography, and Staudinger conditions, nevertheless reacts well with the 5'-amine.

The bacterial signaling molecule cyclic diguanosine monophosphate (c-di-GMP) is responsible for regulating bacterial responses to a variety of environmental factors, including aggregation into the biofilm state.^{1–6} Binding of c-di-GMP as a monomer and as a self-intercalated dimer to the PilZ domain proteins has been demonstrated.^{1, 2, 7, 8}

Activation of two different classes of riboswitches in noncoding regulatory mRNA domains also has been identified upon binding c-di-GMP.^{9–12} Finally, c-di-GMP, among other cyclic dinucleotides, plays a role in triggering an innate immune response^{13, 14} through a transmembrane protein named STING in the innate immune sensing pathway, where a specific receptor for cyclic dinucleotides has been identified.¹⁵

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Supporting Information **Available** Synthetic procedures, HPLC, ¹H, ¹³C NMR, and UV spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

A number of synthetic routes to c-di-GMP and its thiophosphate analogs have been reported.¹⁶⁻¹⁹ Two analogs with a non-phosphate backbone have been prepared, one a methylphosphonate,²⁰ the other a carbamate,²¹ but each lacks a 2'-hydroxyl group. An analog with a 2'-fluoro in place of the 2'-hydroxyl, with a phosphate backbone, was reported most recently.²² The goal of the work reported below was to prepare c-di-GMP analogs with urea or urea related backbone linkages that should be stable to the bacterial phosphodiesterases that regulate c-di-GMP. The syntheses start with introduction of nitrogen atoms to the guanosine 3' and 5' positions. The first steps are to prepare the 5'-azido-5'-deoxy derivative **3**, as shown in Scheme 1.

The conversion of **4** to the 3'-amino-5'-azido derivative **9**, shown in Scheme 2, proceeded analogously to the preparation of 3'-amino-3'-deoxyguanosine reported by Zhang, although by using extensively altered conditions, and some different reagents, the reaction times were significantly reduced.²⁶ Catalytic DMAP in methanol with a few equiv of TEA effected clean removal of the acetyl group from **4**. The reaction of **5** with benzylisocyanate in acetonitrile then proceeded in 2 h to give **6**. After investigating numerous reagents for cyclization to **7**, *t*BuONa in THF was found to give complete conversion in 45 min. Saponification of **7** to **8** by addition of 10 N NaOH to a methanol solution of **7** proceeded in 2 h. It is somewhat surprising that the *N*²-dmf group survived these strongly basic conditions with only minimal loss. After neutralization of the reaction mixture, **8** was isolated by extraction. The steps from **4** to **8** were carried out in one flask, without isolation of intermediates, and **8** did not need purification before conversion to **9**.

The *N*²-dimethylformamidinium (dmf) derivative of guanosine, **1**, was prepared by standard methods as described in detail in the Supplementary Information. Preparation of **2** and **3** followed procedures reported for guanosine by Martin,²³ by Dean,²⁴ respectively. The major differences in this case were that heating was not required for reaction of **2** with sodium azide, and that **3** was readily isolated simply by addition of methanol to the reaction mixture. The *N*²-dmf group was used in this synthesis as it has been shown to be essential for the reaction of guanosine with α -acetoxyisobutyryl bromide.²⁵ The reaction of **3** with this reagent proceeded analogously to that reported for **1**, with no degradation of the azido group under the acidic reaction conditions. In addition to the desired product, **4**, a small amount of the 2'-Br isomer was produced, in the ratio of 92:8, by LC-MS. These isomers were not separable by silica chromatography, but **4** was readily crystallized from methylene chloride, which efficiently removed the 2'-Br isomer. No chromatography was required for the preparation of compounds **1-4**, so that these reactions were conveniently carried out starting with 20 g of guanosine to give **4** in an overall yield of 38%.

Because of the 5'-azide it was not possible to use reduction to debenzylate the 3'-amino group in **8**, and instead oxidation using diisopropylazodicarboxylate (DIAD) was employed.²⁷ This is a slow reaction that required overnight to give the corresponding imine (not shown). Hydrolysis to **9** was effected using 1 N HCl, within ten min, again with minimal loss of the *N*²-dmf group. After neutralization of the reaction mixture with NaHCO₃, **9** was isolated by extraction, in this case remaining in the aqueous phase while excess reagent was removed in the organic phase. The purification of **9** was carried out by reversed phase chromatography using 10 mM aqueous ammonium bicarbonate and acetonitrile, to give **9** in a yield of 30% from **4**. Although the *N*²-dmf group survives limited time treatment with NaOH or HCl, it is slowly hydrolyzed by the ammonium bicarbonate eluant, so that solutions of **9** should not be allowed to stand for long periods of time after purification.

The derivatization of **9** for synthesis of the cyclic dimers required protection of the 2'-hydroxyl, conveniently done by reaction with *tert*-butyldimethylsilyl chloride, as shown in

Scheme 3. Addition of the TBS group makes **10** again amenable to silica chromatography, and all of the subsequent intermediates were purified on silica using gradients of methanol (with 0.5% TEA for those with a free amino group or the acid labile monomethoxytrityl group) and methylene chloride. The strategy for synthesis of the linear and cyclic dimers was to elaborate the 3'-amino group into an isothiocyanate, and to couple this to a 5'-amino group obtained by Staudinger reduction of the 5'-azide. The 3'-isothiocyanate is stable to silica chromatography, so that intermediates **11** and **14** are easily handled, but it does react well with the 5'-amino group. Thus the 5'-azide functions as a stable masked amino group that can be converted to the amine without harming the 3'-isothiocyanate of **14**.

Formation of the 3'-isothiocyanate derivative **11** was carried out by reaction of **10** with carbon disulfide followed by reaction of the resulting dithiocarbamate (not shown) with tosyl chloride or benzenesulfonyl chloride.²⁸

This was done as a two step procedure using a ten fold excess of CS₂ in the first step, that was readily removed on a rotary evaporator before reaction with the sulfonyl chloride. The 5'-amino nucleoside **12** was obtained by Staudinger reduction after protection of the 3'-amino group of **10** by reaction with monomethoxytrityl chloride. Condensation of **11** and **12** in THF at room temperature gave clean conversion to the linear dimer **13** within 17 h in 93% yield. The monomethoxytrityl group was removed using dichloroacetic acid (DCA) and the amino group converted to an isothiocyanate to give **14** by the same two step procedure used for preparation of **11**.

Cyclization of the linear dimer **14** to the cyclic dimer **15** was effected by a two step sequence starting with reaction of **14** with triphenyl phosphine to give the azine (not shown, but sufficiently stable to be clearly visible by LC-MS), followed by dilution of the reaction mixture with THF/water/TEA, and heating at 60 °C for 2 h. Although LC-MS showed that the protected cyclic dimer **15** was the only significant product, there were a number of small impurities visible, possibly oligomers from intermolecular reaction, even under the dilute conditions of the cyclization. It was again possible to purify **15** by silica chromatography, using a steep gradient of methanol in methylene chloride. The isolated yield for **15** was only 40%, presumably because of competing intermolecular reactions or degradation.

The deprotection of **15** to the cyclic thiourea **16** was effected using 2 N NaOH in methanol/water (1:1). Under these conditions the TBS groups were removed in minutes, at room temperature, while the N²-dmf groups required heating at 60 °C for 2 h to effect removal, consistent with the surprising stability noted earlier. Neutralization of the reaction mixture with either 1 N HCl or with acetic acid caused precipitation of **16**, which was isolated by filtration in quantitative yield. Of the many potential routes for conversion of thioureas to ureas,²⁹ reaction of **16** with DMSO and catalytic iodine, at 80 °C, was employed.³⁰ This is a simple, if slow, procedure that does not involve metals or unusual conditions, and gave clean conversion to **17**, in 37% yield.

The reaction of **16** with iodine, this time in DMF at room temperature with triethylamine, was also effective for preparation of the carbodiimide **18**.³¹ Although this reaction is reported to require aryl thioureas,³¹ it worked well for preparation of **18**. The reaction of carbodiimides with amines for synthesis of guanidines is well known,³² and aqueous methylamine and aqueous ammonia gave **19a** and **19b**, respectively, although slowly and in modest yields.

The carbodiimide **18** proved to be sufficiently stable to be handled and purified using the same conditions used for **17** and **19a/b**. All of these compounds have poor solubility in water, but are soluble in 0.1 N NaOH. Purification of each was done by RP chromatography

using 0.1 N NaOH and methanol. Neutralization of the product fractions using CO₂ gas gave each compound as a white solid easily isolated by filtration. The preparations of **17**, **18**, and **19a/b** were carried out on small scales only and were not optimized.

Recent reports of activation of the innate immune system with the 2'/3' isomers of cyclic GMP-AMP (cGAMP)³³⁻³⁵ provide a new impetus for preparation of cyclic dinucleotides (CDNs) and their analogs. The compounds reported here are the first examples of a new class of CDN analogs that possess a urea, or urea related, backbone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1. Synthesis of Key Intermediate 4

Scheme 2. Synthesis of 3'-amino-5'-azido derivative 9

Scheme 3. Synthesis of the linear dimer 14

Scheme 4. Syntheses of thiourea 16 and urea 17

Scheme 5. Syntheses of carbodiimide **18** and guanidines **19a** and **19b**