Guanosine Tetraphosphate and Its Analogues. Chemical Synthesis of Guanosine 3′,5′-Dipyrophosphate, Deoxyguanosine 3′,5′-Dipyrophosphate, Guanosine 2′,5′-Bis(methylenediphosphonate), and Guanosine 3′,5′-Bis(methylenediphosphonate)†

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ABSTRACT: A new procedure for the synthesis of the pyrophosphate bond has been employed in the preparation of nucleoside dipyrophosphates from nucleoside 3′,5′-diphosphates. The method makes use of a powerful phosphorylating agent generated in a mixture of cyanoethyl phosphate, dicyclohexylcarbodiimide, and mesitylenesulfonyl chloride in order to avoid possible intramolecular reactions between the two phosphate groups on the sugar ring. That such reactions can readily occur was shown by the facile cyclization of deoxyguanosine 3′,5′-diphosphate to P^1,P^2-deoxyguanosine 3′,5′-cyclic pyrophosphate in the presence of dicyclohexylcarbodiimide alone. The phosphorylation reagent was initially tested in the conversion of deoxyguanosine 3′,5′-diphosphate to the corresponding 3′,5′-dipyrophosphate and was then used to phosphorylate 2′-*O*-(α-methoxyethyl)guanosine 3′,5′-diphosphate, which had been prepared from 2′-*O*-(α-methoxyethyl)guanosine. In the latter case, the addition of the two β phosphate groups was accomplished in 40% yield. Removal of the methoxyethyl group from the phosphorylated product gave guanosine 3′,5′-dipyrophosphate, which was shown to be identical with guanosine tetraphosphate prepared enzymatically from a mixture of GDP and ATP. A modification of published procedures was also necessary to effect the synthesis of guanosine bis(methylenediphosphonate). Guanosine was treated with methylenediphosphonic acid and dicyclohexylcarbodiimide in the absence of added base. The product consisted of a mixture of guanosine 2′,5′- and 3′,5′-bis(methylenediphosphonate), which was resolved by anion-exchange chromatography. The 2′,5′ and 3′,5′ isomers are interconvertible at low pH, with the ultimate formation of an equilibrium mixture having a composition ratio of 2:3. The predominant constituent of this mixture has been unequivocally identified as the 3′,5′ isomer by synthesis from 2′-*O*-tetrahydropyranylguanosine.

Current interest in the biological functions of guanosine 3′,5′-dipyrophosphate requires that chemical procedures be developed for the synthesis of this unusual nucleotide and structurally related compounds. Up to the present time, much of the guanosine tetraphosphate in use has been obtained by using *Escherichia coli* ribosomes (Cashel, 1974) or a purine nucleotide pyrophosphotransferase from *Streptomyces* (Murao et al., 1974) to effect enzymatic transfer of the pyrophosphate moiety from ATP to the 3′-hydroxyl group of GDP. A number of complementary chemical methods for the synthesis of ppGpp[1] have been introduced, most of which are based on the simultaneous addition of two β-phosphate groups to guanosine 3′,5′-diphosphate derivatives that are protected at the 2′-hydroxyl position by an easily removable blocking group. Thus, in separate investigations, 2′-*O*-(α-ethoxyethyl)-, 2′-*O*-(α-methoxyethyl)-, and 2′-*O*-acetylguanosine 3′,5′-diphosphates have been converted to the tetraphosphate by means of the anion-exchange procedure and two variations on the phosphorimidazolidate method, respectively, with yields of 4–13% based on pGp as the starting material (Simoncsits and Tomasz, 1974; Kozarich et al., 1975; Cook and Holman, 1975). We report here syntheses of two analogues that are structurally similar to ppGpp and also a more efficient synthetic method for the tetraphosphate itself. The preparation of one of the analogues, deoxyguanosine 3′,5′-dipyrophosphate, has recently been described by Hamel et al. (1975), whose relatively intricate reaction scheme was necessitated by the aim of providing a route to both ppdGpp and pppdGpp. The other analogue, guanosine 3′,5′-bis(methylenediphosphonate), differs from ppGpp by the replacement of each of the two internal pyrophosphate oxygen atoms with a methylene group. This compound may be of some value in the study of the biological action of ppGpp since the substitution by methylene groups should result in only minor alterations to the geometry of the phosphorus-containing side chains (Larsen et al., 1969), while conferring on the molecule resistance to the action of the various phosphohydrolases.

The synthesis of ppdGpp (**3**, Scheme I) was undertaken in order to investigate problems associated with attempts to generate pyrophosphate linkages simultaneously at two closely spaced positions on the sugar ring, in the absence of complications that might arise from the presence of the neighboring 2′-hydroxyl group in the corresponding ribonucleotide. Deoxyguanosine (**1**) was converted to its 3′,5′-diphosphate (**2**) in 50% yield, using cyanoethyl phosphate and dicyclohexylcarbodiimide under conditions similar to those employed by Tener (1961) for the preparation of deoxycytidine 3′,5′-diphosphate. The product was identical with a sample of **2** produced by micrococcal nuclease digestion of pdG-dT. It was anticipated that, in the further phosphorylation of this diphosphate by standard methods, intramolecular reactions between activated phosphate or polyphosphate moieties at the 3′ and 5′ positions might seriously compete with the desired addition of the two β-phosphate residues and, indeed, the re-

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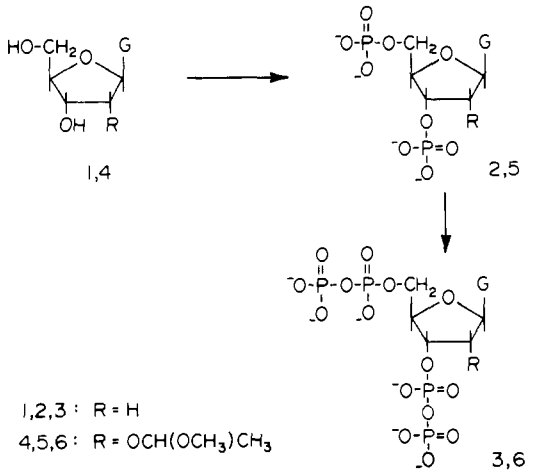
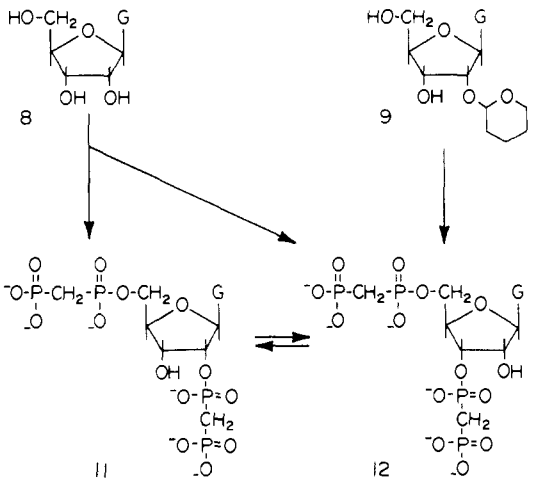
[1] Abbreviations used: ppGpp, guanosine 3′,5′-dipyrophosphate; pGp, guanosine 3′,5′-diphosphate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

Scheme I



Scheme II



sults of preliminary experiments indicated that cyclic di- and triphosphates could readily be formed. For example, it was shown that exposure of the diphosphate **2** to dicyclohexylcarbodiimide in pyridine solution results in the formation, in 50% yield, of a product which was resistant to the action of alkaline phosphatase and has been assigned the structure P^1, P^2-deoxyguanosine 3′,5′-cyclic pyrophosphate.[2]

Accordingly, a strategy was developed whereby the nucleoside 3′,5′-diphosphate would be attacked by a large excess of a highly active preformed phosphorylating agent that was expected to react with both the 3′- and 5′-phosphate groups so rapidly that the competing cyclization reaction would be minimized. It was necessary that the reagent should consist of an activated phosphomonoester in order to preclude the formation of tri- and higher order polyphosphates at either position of the deoxyguanosine residue. Cyanoethyl phosphate was considered to be suitable for this purpose since the cyanoethyl group could be easily removed at the conclusion of the synthesis by mild alkaline treatment. While it is known that phosphomonoesters of this type can be activated by dicyclohexylcarbodiimide or, more effectively, by arylsulfonyl chlorides and that the resulting species exhibit a high reactivity toward alcohols (Jacob and Khorana, 1964), such phosphorylating agents have not been previously employed for the synthesis of nucleoside pyrophosphates. The reagent was prepared by allowing cyanoethyl phosphate to react briefly with excess dicyclohexylcarbodiimide in anhydrous pyridine to yield a mixture of condensed cyanoethyl phosphates (Weimann and Khorana, 1962; Zarytova et al., 1976), which was then superactivated by adding mesitylenesulfonyl chloride. The pretreatment with carbodiimide serves to remove traces of water and reduces the amount of arylsulfonyl chloride required; reagents of the latter class have been described as exerting a deleterious influence on the yields of reactions involving guanine nucleotides (Catlin and Cramer, 1973; Katagiri et al., 1974). The mixture was used to phosphorylate the diphosphate **2** and, after treatment of the product with aqueous pyridine to destroy the excess reagent and then with alkali to remove the cyanoethyl groups, deoxyguanosine 3′,5′-dipyrophosphate (**3**) was isolated in 20% yield by paper and ion-exchange chromatography. The nucleotide had an ultraviolet spectrum similar to that of deoxyguanosine and contained four phosphorus atoms per molecule. It was completely degraded to deoxyguanosine by alkaline phosphatase, indicating that its structure did not include phosphate groups in a cyclic arrangement. On digestion with snake venom phosphodiesterase, the ppdGpp behaved in a manner similar to that of the naturally occurring ribonucleotide, ppGpp, which is degraded in two stages: an initial cleavage producing pGpp, followed by a second, slower hydrolysis to pGp (Cashel and Kalbacher, 1970).

The same sequence of phosphorylations was also used to synthesize guanosine 3′,5′-dipyrophosphate from 2′-*O*-(α-methoxyethyl)guanosine. In this case, the addition of the two β-phosphate groups was accomplished in 40% yield. The protected nucleoside (**4**), prepared by the method of Bennett and Gilham (1975), was converted to 2′-*O*-(α-methoxyethyl) guanosine 3′,5′-diphosphate (**5**) in 50% yield, and this intermediate was then allowed to react with the phosphorylation mixture described above. The resulting 2′-*O*-(α-methoxyethyl)guanosine 3′,5′-dipyrophosphate (**6**) was subsequently treated with dilute hydrochloric acid to yield the tetraphosphate, ppGpp (**7**). Analysis of the product confirmed the presence of four phosphorus atoms in the molecule, and its ultraviolet spectrum indicated that the guanine chromophore had not been modified. It was degraded to guanosine by alkaline phosphatase and to guanosine 3′,5′-diphosphate by yeast inorganic pyrophosphatase. The compound exhibited the reported behavior upon treatment with snake venom phosphodiesterase. It was also labile to alkali, generating, as expected, guanosine 2′(3′)-phosphate 5′-pyrophosphate through attack of the 2′-hydroxyl group on the α-phosphate moiety of the 3′-pyrophosphate side chain with concomitant release of the β-phosphate group. Finally, the synthetic tetraphosphate was shown to be chromatographically indistinguishable from a sample of enzymatically synthesized ppGpp. The relatively good yield obtained in the conversion of the diphosphate to the tetraphosphate suggests that this method of phosphorylation may be useful for large scale chemical preparation of ppGpp via an appropriately $O^{2'}$-protected intermediate derived from commercially available pGp.

The synthesis of guanosine 3′,5′-bis(methylenediphosphonate) (**12,** Scheme II) was initially attempted using conditions that had been successfully employed for preparation of the 5′-methylenediphosphonates of adenosine (Myers et al., 1965) and 2-chloroadenosine (Gough et al., 1972) from their respective 2′,3′-*O*-isopropylidene derivatives. These syntheses involved reaction of the nucleoside in pyridine at 60 °C with

[2] Knorre et al. (1976) have recently identified P^1,P^2-thymidine 3′,5′-cyclic pyrophosphate as the major product in a reaction of thymidine 3′,5′-diphosphate with dicyclohexylcarbodiimide.

methylenediphosphonic acid and dicyclohexylcarbodiimide in the presence of a large excess of tri-*n*-butylamine. However, this procedure, when applied to guanosine, gave little or no product beyond the monosubstitution stage. It was reasoned that a more active reagent might be formed in an environment in which the carbodiimide would be strongly protonated. The reaction was therefore carried out with the methylenediphosphonic acid and dicyclohexylcarbodiimide dissolved in anhydrous dimethylformamide at 25 °C in the absence of added base. Under these conditions, a powerful phosphorylating agent was produced with the result that all the hydroxyl groups of guanosine were subject to attack. After treatment with formic acid to break down P–O–P linkages (van Wazer, 1958), presumed to be present in the form of condensed polymethylenediphosphonates, the reaction mixture was fractionated by ion-exchange chromatography. The major products, which were seen as two closely adjoining peaks with retention volumes of 125 and 133 ml (Table I), were obtained in amounts corresponding to a ratio of 2:3, respectively. The two species were isolated separately and characterized by elemental analysis as isomers of guanosine bis(methylenediphosphonate). These two products are interconvertible in that treatment of either isomer with formic acid generates its congener until the 2:3 equilibrium mixture is reestablished. Such behavior, together with the assumption that the 5′-hydroxyl group would have been the most readily phosphorylated during the synthesis, suggested that the two compounds were in fact the 2′,5′ isomer (**11**) and the 3′,5′ isomer (**12**). It is recognized that nucleoside 2′(3′)-phosphates isomerize at low pH, forming, at least in the case of adenosine 2′(3′)-phosphate, an equilibrium mixture containing about 40% of the 2′ and 60% of the 3′ isomer (Khym and Cohn, 1954). This distribution, taken in conjunction with the known retention volumes of nucleoside 2′- and 3′-phosphates on anion-exchange columns, where the 2′ invariably precedes the corresponding 3′ isomer (Asteriadis et al., 1976), leads to the designation of the bis(methylenediphosphonate) with $\overline{V}$ = 125 ml as the 2′,5′ isomer and the derivative with $\overline{V}$ = 133 ml as the 3′,5′ isomer. It is interesting to note that these methylenediphosphonate analogues display substantially lower retention volumes than that of ppGpp, an observation which presumably reflects the higher pK_a values of secondary P–OH ionizations in monoesterified methylenediphosphonates, relative to those in the corresponding pyrophosphates (Myers et al., 1965).

In order to establish unequivocally the identities of the two substances, it was necessary to carry out an unambiguous synthesis of one of them. 2′-*O*-Tetrahydropyranylguanosine (**9**), which is less acid labile than the α-methoxyethyl derivative (**4**), was the preferred starting material. The protected nucleoside was treated for a short time with methylenediphosphonic acid and dicyclohexylcarbodiimide. A portion of the reaction mixture was removed for analysis and shown to contain 53% of the guanosine in the form of the bis(methylenediphosphonate). During the isolation of the remainder of the product, precautions were taken to avoid loss of the tetrahydropyranyl group. Thus, the pyrophosphate linkages were cleaved by the use of a mixture of acetic anhydride and pyridine (Khorana et al., 1962), followed by treatment with ammonium hydroxide to remove acetyl groups. Paper chromatographic purification of the product resulted in a 42% yield of 2′-*O*-tetrahydropyranylguanosine 3′,5′-bis(methylenediphosphonate) (**10**). The relatively mild acidic conditions employed for complete removal of the tetrahydropyranyl group (Smith et al., 1962) were shown to cause only 15% isomerization of **12**, and subsequent exposure of **10** to this treatment produced an 87% yield of the isomer with $\overline{V}$ = 133 ml, thereby defining it as the 3′,5′-bis(methylenediphosphonate) of guanosine.

TABLE I: Chromatographic Data for Guanosine and Deoxyguanosine Derivatives.

Compound	$\overline{V}$ (ml) System I[a]	$\overline{V}$ (ml) System II[b]	R_f[c]
dG (**1**)			2.04
pdG	72		1.16
ppdG	108		1.12
pppdG	132		1.08
pdGp (**2**)	120		0.62
P^1,P^2-Deoxyguanosine 3′,5′-cyclic pyrophosphate	156		1.49
ppdGpp (**3**)	150	147	0.60
G(MeOEt) (**4**)			2.65
pG	75	75	1.00
ppG	115	119	0.93
pppG	142	153	0.85
pGp	138	124	0.68
pG(MeOEt)p (**5**)	107		1.11
ppG(MeOEt)pp (**6**)	148		1.00
ppGpp (**7**)	174	194	0.60
G (**8**)			1.78
G(Thp) (**9**)			2.80
2′-*O*-Tetrahydropyranyl-guanosine 3′,5′-bis-(methylenediphosphonate) (**10**)		125	1.00
Guanosine 2′,5′-bis-(methylenediphosphonate) (**11**)		125	0.65
Guanosine 3′,5′-bis-(methylenediphosphonate) (**12**)		133	0.56

[a] Chromatographic retention volumes obtained on a column (75 × 0.4 cm) of Dowex 1-X2 ion-exchange resin by elution at 12–15 ml/h with 300 ml of 40% ethanol containing a linear gradient of 0.1–0.4 M NH_4Cl (pH 8.0). [b] Chromatographic retention volumes obtained on a column (100 × 0.4 cm) of Dowex 1-X2 ion-exchange resin by elution at 12–15 ml/h with 200 ml of water containing a linear gradient of 0.2–0.5 M NH_4Cl (pH 8.0). [c] Values are relative to the R_f of guanosine 5′-phosphate and were determined by descending chromatography on Whatman 3MM paper using solvent system A: *n*-propyl alcohol–concentrated NH_4OH–water (55:10:35, v/v/v).

Experimental Section

Materials and Methods. Guanosine and deoxyguanosine, their mono-, di-, and triphosphates, and methylenediphosphonic acid were obtained from Sigma Chemical Co., St. Louis, Mo. Guanosine 3′,5′-diphosphate was the product of P-L Biochemicals, Milwaukee, Wis., and the dinucleotide pdG-dT was purchased from Collaborative Research, Waltham, Mass. 2′-*O*-(α-Methoxyethyl)guanosine was prepared as previously described (Bennett and Gilham, 1975). The ion-exchange resins, Dowex 1-X2 and Dowex 50W-X8, and beaded polyacrylamide were purchased as AG 1-X2 (−400 mesh), AG 50W-X8 (100–200 mesh), and Bio-Gel P-2 (200–400 mesh) from Bio-Rad Laboratories, Richmond, Calif. Pyridine and dimethylformamide were dried by storage over calcium hydride and Linde Molecular Sieve type 4A, respectively. Barium cyanoethyl phosphate (Calbiochem, La Jolla, Calif.) was converted to its pyridinium salt as described by

Tener (1961). It was freed from water by repeated evaporations of pyridine and stored as a 1 mmol/ml solution in dry pyridine. Nucleotides were converted to their anhydrous tri-*n*-hexylammonium salts by passing them through a small column of AG 50W-X8 (pyridinium) resin, adding the appropriate quantity of tri-*n*-hexylamine, and repeatedly evaporating their pyridine solutions to dryness. Yeast inorganic pyrophosphatase (type III) was obtained from Sigma Chemical Co., and micrococcal nuclease (grade NFCP), *Escherichia coli* alkaline phosphatase (grade BAPF), and snake venom phosphodiesterase (grade VPH) were purchased from Worthington Biochemical Corp., Freehold, N.J. The venom enzyme was treated according to the procedure of Sulkowski and Laskowski (1971) to remove any 5′-nucleotidase activity. A sample of enzymatically prepared guanosine 3′,5′-dipyrophosphate labeled with ^{32}P was kindly supplied by Dr. M. Cashel.

All evaporations were carried out in vacuo at temperatures below 35 °C. Paper chromatography was performed on Whatman 3MM paper by the descending technique, with solvent systems: (A) *n*-propyl alcohol–concentrated NH_4OH–water (55:10:35, v/v/v); (B) isopropyl alcohol–concentrated NH_4OH–water (7:1:2, v/v/v); (C) isopropyl alcohol–concentrated NH_4OH–water (6:1:3, v/v/v); (D) a mixture of water (100 ml) and $(NH_4)_2SO_4$ (40 g) adjusted to pH 8.5 with concentrated NH_4OH. Anion-exchange chromatography was carried out as described by Asteriadis et al. (1976), and, unless otherwise specified, the fractions from ion-exchange columns were desalted by concentrating them to a small volume and passing the resulting solution through a column (60 × 1 cm) of Bio-Gel P-2, using 20% ethanol as the eluting solvent. Phosphorus analyses were effected by the procedure of Ames and Dubin (1960). On paper chromatograms, phosphate and pyrophosphate were detected by the method of Runeckles and Krotkov (1957).

Enzyme Digestions. Alkaline Phosphatase. The sample (1–4 A_{252} units) in water (0.1 ml) was mixed with 0.04 M Tris-Cl–0.02 M $MgCl_2$, pH 8.0 (0.1 ml), and incubated at 37 °C for 4 h with 0.6 unit of alkaline phosphatase where the unit of activity is defined according to Garen and Levinthal (1960).

Snake Venom Phosphodiesterase. The sample (1–8 A_{252} units), dissolved in 0.2 M Tris-Cl–0.04 M magnesium acetate, pH 9.0 (0.1 ml), was added to 0.1 ml of snake venom phosphodiesterase solution (1.0 mg/ml) and kept at 37 °C for 2–3 h. For more exhaustive digestion, the sample was dissolved in 0.25 ml of the buffer and treated with 0.25 ml of the enzyme solution for 16 h at 37 °C.

Inorganic Pyrophosphatase. The sample (1–2 A_{252} units) was dissolved in a mixture of 0.1 M zinc acetate (20 μl), 0.04 M sodium succinate, pH 5.9 (0.2 ml), and yeast inorganic pyrophosphatase (20 μg in 0.2 ml of water). The digestion was allowed to proceed at 37 °C for 10 h.

Deoxyguanosine 3′,5′-Diphosphate (**2**). Deoxyguanosine (**1**, 50 mg, 0.19 mmol), pyridinium cyanoethyl phosphate (2 mmol), and dicyclohexylcarbodiimide (0.8 g, 3.9 mmol) were combined in anhydrous pyridine (6 ml) and the reaction mixture was shaken for 3 days. Water (0.5 ml) was then added and, after 30 min, the mixture was evaporated to dryness in vacuo. The residue was resuspended in water (10 ml) and again taken to dryness. It was then treated with 0.4 M LiOH (15 ml) and heated under reflux for 1 h. The mixture was cooled and filtered, and the pH of the filtrate was adjusted to 6 by the addition of Dowex 50W-X8 (H^+) ion-exchange resin. The resin was removed by filtration, and the filtrate was concentrated to a small volume and applied to Whatman 3MM paper (80 cm). After chromatography using solvent C, the product was obtained as a band with an R_f value of 0.2 relative to that of deoxyguanosine. Further purification on paper with solvent B gave **2** in 50% yield. The product was chromatographically indistinguishable from a sample of pdGp prepared from pdG-dT. The dinucleotide (20 A_{260} units) was dissolved in 0.2 ml of 0.1 M Tris-Cl–0.02 M $CaCl_2$ (pH 9.0) containing 1000 units of micrococcal nuclease and the mixture was incubated at 37 °C for 20 h. The diphosphate was isolated by paper chromatography using solvent A. The unit of activity of the nuclease corresponds to that defined by the assay conditions of Heins et al. (1966).

Reaction of Deoxyguanosine 3′,5′-Diphosphate with Dicyclohexylcarbodiimide. The tetrakis(tri-*n*-hexylammonium) salt of **2** (0.066 mmol) was dissolved in dry pyridine (2.5 ml) and treated with dicyclohexylcarbodiimide (1 mmol). After 3 days, water (0.5 ml) was added and the insoluble dicyclohexylurea was removed by filtration. The filtrate was applied to Whatman 3MM paper (80 cm), and chromatography with solvent C gave P^1,P^2-deoxyguanosine 3′,5′-cyclic pyrophosphate (48%) as a band with R_f 1.9 relative to that of pdGp. Phosphorus analysis gave a value of 2.2 P atoms per deoxyguanosine residue. The cyclic pyrophosphate was resistant to the action of alkaline phosphatase but was degraded by snake venom phosphodiesterase yielding pdGp as the only product.

Deoxyguanosine 3′,5′-Dipyrophosphate (**3**). Pyridinium cyanoethyl phosphate (1 mmol) in dry pyridine (1 ml) was treated with dicyclohexylcarbodiimide (515 mg, 2.5 mmol) and, after 10 min, mesitylenesulfonyl chloride (44 mg, 0.2 mmol) was added. After a further 10 min, the phosphorylation mixture was added to a solution of the tetrakis(tri-*n*-hexylammonium) salt of **2** (200 A_{252} units, 14.7 μmol) in dry pyridine (1 ml). The reaction was allowed to proceed for 1 h and was then terminated by the addition of water (1 ml). After a further 60 min, the mixture was filtered and the filtrate was evaporated to dryness in vacuo. The residue was treated with 2 M NaOH (2 ml) at 0 °C for 15 min and then the pH of the solution was adjusted to 7 by the addition of Dowex 50W-X8 (H^+). The resin was removed and the solution, after evaporation to a small volume, was applied to Whatman 3MM paper (60 cm). Chromatography with solvent C gave **3** as a band having an R_f value of 0.8 relative to that of pdGp. The product was eluted with water and was further purified by ion-exchange chromatography using system I (Table I). The combined fractions corresponding to the major peak ($\bar{V}$ = 150 ml) contained 40 A_{252} units (20%) of **3**, and these were desalted using Bio-Gel P-2. Phosphorus analysis showed that the substance contained 4.2 P atoms per deoxyguanosine residue. Alkaline phosphatase degraded it to deoxyguanosine, while partial digestion with snake venom phosphodiesterase yielded a product ($\bar{V}$ = 135 ml in ion-exchange system I) that was assigned the structure pdGpp. More extensive digestion with the phosphodiesterase gave pdGp, and paper chromatographic analysis of the reaction mixture with solvent C showed that inorganic phosphate was the only other product formed. In this chromatographic system the phosphate anion and the pyrophosphate anion have R_f values of 1.2 and 0.7, respectively, relative to that of guanosine 5′-phosphate. Under the same digestion conditions guanosine 5′-triphosphate was degraded to guanosine 5′-phosphate and inorganic pyrophosphate.

2′-O-(α-Methoxyethyl)guanosine 3′,5′-Diphosphate (**5**). Pyridinium cyanoethyl phosphate (5 mmol) and 2′-*O*-(α-methoxyethyl)guanosine (**4**, 0.33 mmol), dissolved in dry

pyridine (15 ml), were treated with dicyclohexylcarbodiimide (2 g, 9.7 mmol), and the mixture was shaken for 48 h. Water (1 ml) was then added and, after 1 h, the suspension was cooled to 0 °C and treated with 2 M NaOH (30 ml). After the mixture had been kept at 0 °C for 40 min, it was adjusted to pH 8 with Dowex 50W-X8 (H^+) resin and filtered. The filtrate was applied to a column (50 × 2.5 cm) of DEAE-cellulose (Whatman DE 23, HCO_3^- form), which was then eluted with 6 l. of 5% *n*-butyl alcohol containing a linear gradient of 0–0.5 M triethylammonium bicarbonate (pH 7.5), at a flow rate of 1 ml/min. The product, **5** (2275 A_{252} units, 50%), was recovered between elution volumes of 2 and 2.7 l., and was rendered free of salt by repeated evaporation of its aqueous solution. A portion of the product was adjusted to pH 3.5 with dilute hydrochloric acid and kept for 4 h at 37 °C to yield guanosine 3′,5′-diphosphate that had chromatographic characteristics identical with those of a commercial sample of pGp.

Guanosine 3′,5′-Dipyrophosphate (**7**). Pyridinium cyanoethyl phosphate (1 mmol) in dry pyridine (1 ml) was treated with dicyclohexylcarbodiimide (520 mg, 2.5 mmol) and, after 10 min, mesitylenesulfonyl chloride (44 mg, 0.2 mmol) was added. After a further 10 min the phosphorylation reagent was mixed with a solution of the tetrakis(tri-*n*-hexylammonium) salt of **5** (140 A_{252} units, 10 μmol) in dry pyridine (1 ml). The reaction was allowed to proceed for 50 min, and water (0.5 ml) was then added. After 1 h, the mixture was treated with 2 M NaOH (5 ml) at 0 °C for 20 min, then brought to pH 8 with Dowex 50W-X8 (H^+) and filtered. The filtrate and washings, reduced to a small volume in vacuo, were applied to Whatman 3MM paper (80 cm). Chromatography with solvent A gave the product as a band with R_f value equal to that of pG. The α-methoxyethyl derivative was eluted with dilute ammonia and subjected to ion-exchange chromatography in system I. The fractions corresponding to the major peak ($\bar{V}$ = 150 ml) were combined and concentrated and finally desalted to yield 56 A_{252} units (40%) of **6.** A portion of this material was treated with dilute hydrochloric acid (pH 3.5) for 5 h at 37 °C. Ion-exchange chromatography then gave a peak ($\bar{V}$ = 174 ml) containing the tetraphosphate **7** (91%), which was desalted as before. The product had the uv spectral characteristics of guanosine, and phosphorus analysis showed that it contained 4.2 phosphate groups per guanosine residue. Incubation with alkaline phosphatase produced a compound with the R_f value of guanosine (solvent B). Degradation with inorganic pyrophosphatase in the presence of Zn^{2+} ions gave a product (pGp) with $\bar{V}$ = 140 ml in ion-exchange chromatographic system I. Snake venom phosphodiesterase digestion gave products with $\bar{V}$ = 152 ml (pGpp) and $\bar{V}$ = 140 ml (pGp). Treatment of **7** with 0.3 M KOH at 37 °C for 2 h caused 50% degradation to a product (ppGp) with $\bar{V}$ = 152 ml while snake venom digestion as above, followed by alkaline treatment (0.3 M NaOH, 4 h, 37 °C), produced only pGp. A few A_{252} units of the synthetic ppGpp was cochromatographed in ion-exchange system I with a sample of enzymatically prepared tetraphosphate labeled with ^{32}P. The fractions corresponding to **7** exhibited a constant cpm/A_{252} ratio across the peak.

Guanosine 2′,5′-Bis(methylenediphosphonate) (**11**) *and Guanosine 3′,5′-Bis(methylenediphosphonate)* (**12**). Methylenediphosphonic acid (880 mg, 5 mmol) was dissolved in dry dimethylformamide (20 ml) and treated with dicyclohexylcarbodiimide (10.3 g, 50 mmol). After 1.5 h, guanosine (283 mg, 1 mmol) was added and the reaction mixture was stirred at 25 °C for 24 h. It was then poured into a mixture of 100 ml of light petroleum and 200 ml of 25% pyridine in water. The aqueous layer was removed and the light petroleum was washed with water (2 × 100 ml). The combined aqueous extracts were evaporated to dryness in vacuo and the residue was treated with 88% formic acid (150 ml) for 16 h at 25 °C. After removal of the acid by repeated evaporations with water, the residue was suspended in water (50 ml) and filtered. The filtrate was diluted to a total volume of 100 ml. Analysis of a portion of this solution using ion-exchange chromatographic system II displayed two major peaks corresponding to the 2′,5′ isomer **11** ($\bar{V}$ = 125 ml) and the 3′,5′ isomer **12** ($\bar{V}$ = 133 ml), in yields of 14 and 21%, respectively. Larger amounts of the mixture (up to ¼ of the total) were fractionated on a column (100 × 0.7 cm) of Dowex 1-X2 (−400 mesh), using 400 ml of water containing a linear gradient of 0.2–0.5 M NH_4Cl (pH 8) as eluting solvent. Fractions corresponding to the two isomers were pooled separately and desalted; each isomer was converted to its sodium salt by passing it through a column of Dowex 50W-X8 (Na^+) and then lyophilized. Anal. Calcd for $C_{12}H_{15}N_5O_{15}P_4Na_6 \cdot 6H_2O$: C, 17.17; H, 3.24; N, 8.35; P, 14.76. Found (**11**): C, 17.16; H, 3.45; N, 8.30; P, 15.00. (**12**): C, 16.92; H, 3.41; N, 8.20; P, 14.99.

Interconversion of Guanosine 2′,5′-Bis(methylenediphosphonate) and Guanosine 3′,5′-Bis(methylenediphosphonate). The 2′,5′ isomer, **11** (0.5 mg), was dissolved in 88% formic acid (100 μl) and the solution was allowed to stand at 25 °C for 20 h. The formic acid was removed by evaporation in vacuo and the residue was subjected to ion-exchange chromatography in system II. The products consisted of **11** and **12** in amounts corresponding to a ratio of 2:3. Under the same conditions, the 3′,5′-bis(methylenediphosphonate) produced an identical distribution of the two isomers.

2′-O-Tetrahydropyranylguanosine (**9**). $O^{3'}$-Acetyl-N^2,$O^{5'}$-dibenzoyl-$O^{2'}$-tetrahydropyranylguanosine (0.25 mmol), prepared by the method of Neilson et al. (1973), was dissolved in 20 ml of methanol that had been saturated with ammonia at 0 °C and the mixture was allowed to stand at 25 °C in a sealed bottle for 4 days. The solvent was then removed in vacuo. Chromatography of the residue on 50 g of silicic acid (Mallinckrodt Silicar CC-7), using methanol–pyridine–chloroform (10:1:89, v/v/v) as eluting solvent, gave 57 mg (62%) of the two diastereomers of **9.** On paper chromatography with solvent D the product ran as a single spot with an R_f value of 1.8 relative to that of 2′,3′-*O*-isopropylideneguanosine. This solvent system readily separates **9** from its constitutional isomer 3′-*O*-tetrahydropyranylguanosine, which has an R_f value of 0.8 relative to that of isopropylideneguanosine. The 3′ derivative was prepared by tetrahydropyranylation of $O^{2'}$, $O^{5'}$,N^2-triacetylguanosine followed by removal of the acetyl groups with alkali (G. R. Gough, unpublished results).

Synthesis of Guanosine 3′,5′-Bis(methylenediphosphonate) (**12**) *from 2′-O-Tetrahydropyranylguanosine.* Methylenediphosphonic acid (22 mg, 0.125 mmol) was dissolved in anhydrous dimethylformamide (1 ml) and treated with dicyclohexylcarbodiimide (260 mg, 1.25 mmol). After 1.5 h, the suspension was added to dry 2′-*O*-tetrahydropyranylguanosine (25 μmol) and the reaction was allowed to proceed at 25 °C for 6 h. Pyridine (2 ml) was then added and the mixture was poured into light petroleum (10 ml) over water (8 ml). The phases were stirred together for 1 h and then the aqueous layer was separated and combined with subsequent water washes (2 × 10 ml). An aliquot (10%) of the solution was removed, evaporated to dryness, and treated with 88% formic acid for 16 h at 25 °C. After evaporation of the formic acid, the residue was subjected to paper chromatography with solvent A. The area of the chromatogram corresponding to the location of guanosine 2′(3′),5′-bis(methylenediphosphonate) was cut out

and eluted, yielding 18.1 A_{252} units (53%) of **11** and **12.** The remaining 90% of the aqueous extract was treated with 0.68 M tetraethylammonium hydroxide (1 ml) and the mixture was evaporated in vacuo to an oil. Water was removed by several additions and evaporations of anhydrous pyridine and then the residue was taken up in pyridine (2 ml) and treated with acetic anhydride (1 ml). The mixture was set aside for 3 days, and the solvents were then removed under high vacuum. Following addition and evaporation of pyridine (3 ml) and 50% aqueous pyridine (2 × 3 ml), the residue was dissolved in concentrated NH_4OH (30 ml) and allowed to stand for 3 days. The solvent was removed in vacuo and the product was applied to Whatman 3MM paper (80 cm). After chromatography with solvent A, the band with R_f value of 1.0 relative to that of guanosine 5′-phosphate was cut out and eluted, yielding 128 A_{252} units (42%) of 2′-*O*-tetrahydropyranylguanosine 3′,5′-bis(methylenediphosphonate) (**10**). A portion (30 A_{252} units) of this material was treated with 80% acetic acid at 25 °C for 4 h and the solution was then evaporated to dryness in vacuo. Ion-exchange chromatography of the residue using system II showed the product to consist of the 3′,5′ isomer, **12** (87%), together with a 13% yield of the 2′,5′-isomer, **11.** Pure **12,** subjected to the same acid treatment, underwent 15% isomerization to **11.**

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