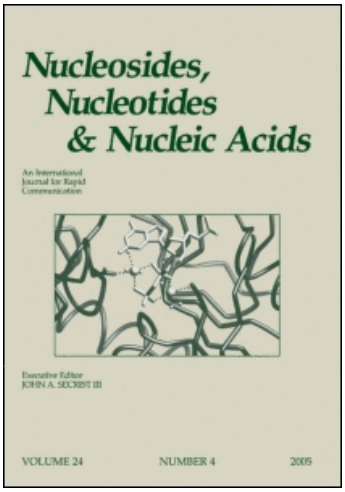

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SYNTHESIS AND PROPERTIES OF S-PHOSPHATES OF SOME ANTIVIRAL ACYCLONUCLEOSIDES

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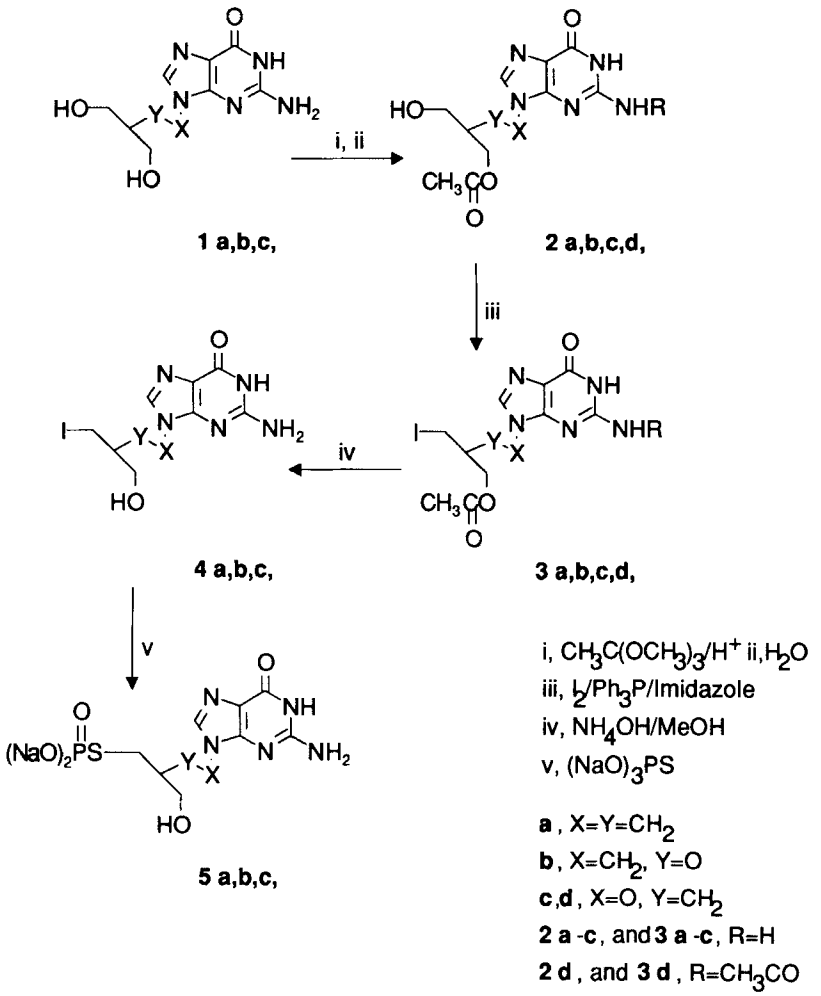
Abstract: Suitably protected penciclovir, ganciclovir and 9-[3-hydroxy-2-(hydroxymethyl)propoxy]guanine were converted into their iodo-derivatives which in turn were reacted with trisodium thiophosphate to give the corresponding S-phosphates in good yields.

A number of acyclonucleosides display potent and selective activity against herpes viruses type 1 and 2[1-5] and cytomegalovirus.[6-8] A common feature in the mode of action of these analogues is their ability to be phosphorylated [9,10] by virally encoded kinases to their monophosphates. Subsequent phosphorylations by cellular kinases form the respective triphosphates which act as competitive inhibitors of the virus encoded DNA polymerase.

In continuation of our studies on nucleotide analogues as potential antiviral agents we have undertaken the synthesis of novel S-phosphates derived from 9-[4-hydroxy-3-(hydroxymethyl)butyl]guanine (penciclovir) **1a**, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (ganciclovir) **1b** and 9-[3-hydroxy-2-(hydroxymethyl)propoxy]guanine **1c**.

Replacement of the bridging oxygen in phosphate esters of nucleosides with sulphur has been shown to be a useful structural modification. A number of nucleoside S-phosphates have proved valuable tools for the study of the enzymes affecting the hydrolysis of nucleotides[11-13] as well as the substrate-enzyme interactions.[14-17] Analogues such as adenosine and inosine 5'-S-phosphates were found completely resistant[13] to the action of 5'-nucleotidase whereas calf-intestine mucosa alkaline phosphatase hydrolysed the thiophosphoesters at much reduced rates[13] in comparison



Scheme 1

with the corresponding 5'-O-monophosphates. It was also demonstrated that analogues of ATP and UTP bearing the $C^{5'}$-S-P ester bond were weak competitive inhibitors[17] rather than substrates of E.coli RNA polymerase.

Results and Discussion

The synthesis of S-phosphates **5a-c** entailed prior preparation of the appropriate iodo-acyclonucleosides **4a-c** and their subsequent reaction with trisodium thiophosphate.

The synthesis of 9-[4-acetoxy-3-(hydroxymethyl)butyl]guanine **2a** reported previously,[4] required transient protection of the exocyclic amino and hydroxyl functions with the monomethoxytrityl group. We found that the protection of one of the two equivalent hydroxyl functions in 9-[4-hydroxy-3-(hydroxymethyl)butyl]guanine (penciclovir) **1a** and 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine **1b** with the acetyl group could be achieved by reaction of **1a** or **1b** with an excess of trimethyl orthoacetate followed by acidic hydrolysis of the cyclic orthoester intermediate. The resulting compounds **2a** and **2b** were isolated in 79% and 74% yield respectively as crystalline solids. The similar reaction of 9-[3-hydroxy-2-(hydroxymethyl)propoxy]guanine[18] **1c** with trimethyl orthoacetate was less selective. Presumably, due to the electronic effect of the oxygen in the N^9-O bond, partial acetylation of the exocyclic amino group also occurred. Thus, the expected monoacetylated compound **2c** was isolated in 64% yield whereas the N^2,O-bis-acetylated product **2d** was obtained in 21% yield.

Replacement of the primary hydroxyl group in nucleosides by halogen has been studied extensively and many synthetic methods are available.[19,20] Because of our previous experience,[21] carbon tetrabromide and triphenylphosphine were chosen initially to carry out the conversion of **2a** into its bromo-derivative . However, isolation of the desired product proved difficult since a mixture of several products was formed, owing to side reactions of the purine ring. This problem was circumvented by the use of triphenyl phosphine, imidazole and iodine in toluene-acetonitrile.[22] As a result, the monoacetylated compounds **2a** or **2b** were converted into the corresponding iodo-derivatives **3a** and **3b** in 67% and 65% yield respectively. When an attempt was made to replace the hydroxyl group of compound **2c** with iodine using the same procedure, the reaction proceeded sluggishly and extension of the reaction time up to 45 min led to the formation of side products and a poor yield of the expected compound **3c**. However, iodination of the N^2,O-bis-acetylated compound **2d** under similar conditions was complete after 8 min and the iodo-derivative **3d** was obtained as a crystalline solid in 60% yield after column chromatography on silica gel. Apparently, the acetylation of the exocyclic amino function reduced substantially the nucleophilicity of the N^3 atom thus eliminating the undesired reactions which were reported[23-25] for similar displacements carried out with unprotected purine ribo- and deoxynucleosides. Deacetylation of compounds **3a-d** was carried out with aqueous ammonia in methanol at room temperature to afford the iodo-acyclonucleosides **4a-c** in 70-75% yield. Reaction of compounds **4a-c** with trisodium thiophosphate[26] in water afforded the corresponding S-phosphates **5a-c** in 60-65% yield after purification on a reverse phase C_{18} column.

Compounds **5a-c** were subjected to the action of alkaline phosphatase and each of them was hydrolysed with the formation of a putative thioacyclonucleoside.[13] S-phosphates **5a-c** proved unstable at acidic pH with the rate of hydrolysis being similar for each of the three thiophosphoesters. Thus, **5a-c** were completely hydrolysed with 0.01 M hydrochloric acid (pH=2.0) at 23°C within 8 h with the formation of mixtures of products. The compounds were relatively stable at pH 7.0, only 10% of each S-phosphate underwent hydrolysis within 24 h, and no hydrolysis was observed at pH 9.2 within 96 h.

The S-phosphates prepared in this study were tested at concentrations up to 100 $\mu g\ ml^{-1}$ for antiviral activity in cell cultures. Unlike their acyclonucleoside precursors none of the compounds proved active against herpes simplex virus type 1 and 2 and cytomegalovirus.

Experimental

NMR spectra were recorded on JEOL GX270 and Bruker AM 400 spectrometers. Mass spectroscopy was performed using a JEOL SX-102 instrument operating at 70 eV. M.p.s. were determined using a Reichert-Koffler apparatus and are uncorrected. Elemental analyses were carried out on a CC440 Elemental Analyser. All compounds were homogenous by TLC on silica gel 60 F_{254} coated glass plates or on cellulose F coated aluminium sheets. Column chromatography was performed on Merck 7736 60H silica gel. H.p.l.c. was carried out on Waters 6000A/660 equipment using a μ-Bondapak C18 column or a Spherisorb ODS column.

9-[4-Acetoxy-3-(hydroxymethyl)butyl]guanine (**2a**)

A mixture of 9-[4-hydroxy-3-(hydroxymethyl)butyl]guanine **1a** (2.28 g, 9 mmol), trimethyl orthoacetate (2 mL, 16 mmol) and trifluoroacetic acid (0.76 mL, 9.9 mmol) in DMF (15 mL) was stirred at room temperature for 2 h. Water (2 mL) was then added and after 20 min the solution was neutralised to pH 7.0 with dilute NH_4OH. The solvents were evaporated and the residue was coevaporated with ethanol-toluene (1:1, 3 x 30 ml). The product was purified by column chromatography on silica gel eluting with chloroform-ethanol (gradient 5% to 30% ethanol in chloroform) to afford 2.1 g (79%) of **2a** as a colourless crystalline solid:mp 204°C ($EtOH/H_2O$) [lit mp 204°C].[4] ^1H NMR data agreed with the literature values.[4]

9-[(1-Acetoxy-2-hydroxymethyl)ethoxymethyl]guanine (**2b**)

A mixture of **1b** (1g, 3.92 mmol), trimethyl orthoacetate (1 mL, 7.8 mmol) and trifluoroacetic acid (0.33 mL, 4.3 mmol) in DMF (10 mL) was stirred at room temperature for 4 h. After the work-up as for **2a**, the compound was crystallised from ethanol-water to afford 0.86 g (74%) of **2b** as colourless crystals: mp 214-218°C; ^{1}H NMR (Me_2SO-d_6) δ 1.85 (3H, s, CH_3CO), 3.39 (2H, m, $\underline{CH_2}OH$), 3.78 (1H, m, CH), 3.97 (2H, m, $C\underline{H_2}OCOCH_3$), 4.83 (1H, t, J=5.5 Hz, D_2O exchangeable, OH), 5.41 (2H, s, CH_2N), 6.48 (2H, s, D_2O exchangeable, NH_2), 7.80 (1H, s, CH), 10.60 (1H, s, D_2O exchangeable, NH). Anal. Calcd for $C_{11}H_{15}N_5O_5.0.5\ H_2O$: C, 43.14; H, 5.26; N, 22.87. Found: C, 43.02; H, 5.19; N, 22.92.

9-[3-Acetoxy-2-(hydroxymethyl)propoxy]guanine (**2c**) and 9-[3-Acetoxy-2-(hydroxymethyl)propoxy]-N^2-acetylguanine (**2d**)

A mixture of **1c** (0.36 g, 1.41 mmol), trimethylorthoacetate (0.3 mL, 2.4 mmol) and trifluoroacetic acid (0.13 mL, 1.69 mmol) in DMF (10 mL) was stirred at room temperature for 40 min. After the work-up as for **2a**, the mixture of products was purified by column chromatography on silica gel. Elution of the column with chloroform-ethanol (gradient 1% to 10% ethanol in chloroform) afforded 0.10 g (21%) of **2d** as a colourless crystalline solid: mp 130-131°C. ^{1}H NMR (Me_2SO-d_6) δ 2.02 (3H, s, CH_3CO), 2.19 (4H, m, CH_3CO and CH), 3.55 (2H, dd, J=4.7 Hz, $\underline{CH_2}OH$), 4.16 (2H, d, J=6.0 Hz, CH_2OCO), 4.36 (2H, m, CH_2O), 4.79 (1H, t, J=5.22 Hz, D_2O exchangeable, OH), 8.27 (1H, s, CH), 11.93 (2H, br d, D_2O exchangeable NH and NH). Found: (CI), MH^+, 340.1258; $C_{13}H_{17}N_5O_6$ requires: MH^+, 340.1257. Anal. Calcd for $C_{13}H_{17}N_5O_6.H_2O$: C, 43.70; H, 5.35; N, 19.60. Found: C, 43.61; H, 5.37; N, 19.72.

Further elution of the column with chloroform-ethanol (gradient 10% to 30% ethanol in chloroform) afforded 0.27 g (64%) of **2c** as a colourless crystalline solid: mp 178-179°C. ^{1}H NMR (Me_2SO-d_6) δ 2.02 (3H, s, CH_3CO), 2.18 (1H, m, CH), 3.54 (2H, dd, J=5.5 Hz, $C\underline{H_2}OH$), 4.15 (2H, d, J=6.0 Hz, CH_2OCO), 4.29 (2H, m, CH_2O), 4.78 (1H, t, J=5.3 Hz, D_2O exchangeable, OH), 6.58 (2H, br s, D_2O exchangeable, NH_2), 7.94 (1H, s, CH), 10.67 (1H, s, D_2O exchangeable, NH). Found: (FAB), MH^+, 298.1150; $C_{11}H_{15}N_5O_5$ requires MH^+, 298.1157. Anal. Calcd for $C_{11}H_{15}N_5O_5.0.4\ H_2O$: C, 43.40; H, 5.23; N, 23.00. Found: C, 43.34, H, 5.26; N, 23.10.

9-[4-Acetoxy-3-(iodomethyl)butyl]guanine (**3a**)

Triphenylphosphine (1.18 g, 4.5 mmol), imidazole (0.61 g, 9 mmol) and iodine (1.14 g, 4.5 mmol) were added to a vigorously stirred suspension of **2a** (0.89 g, 3 mmol) in toluene-acetonitrile (2:1, 50 mL) at 80°C. The reaction mixture was vigorously stirred at 80°C for 25 min and the solution was then allowed to cool to room temperature. Toluene (80 mL) and 8 M methanolic ammonia (0.38 mL) were added and the resulting mixture was stirred at room temperature for 30 min. A solid was collected, washed with acetone-diethyl ether (1:1) and crystallised from ethanol-water to give 0.82 g (67%) of **3a** as a slightly yellow crystalline solid: mp 163°C. ^{1}H NMR (Me_2SO-d_6) δ 1.52 (1H, m, CH), 1.79 (2H, m, CH_2), 2.00 (3H, s, CH_3CO), 3.39 (2H, d, J=4.7 Hz, CH_2I), 3.95 (4H, m, CH_2N, CH_2OCO), 6.40 (2H, s, D_2O exchangeable, NH_2), 7.70 (1H, s, CH), 10.52 (1H, s, D_2O exchangeable, NH). Anal. Calcd for $C_{12}H_{16}N_5O_3I.0.25\ H_2O$: C, 35.18; H, 4.05; N, 17.09. Found: C, 34.93; H, 3.82; N, 17.01.

9-[(1-Acetoxy-2-iodomethyl)ethoxymethyl]guanine (**3b**)

Triphenylphosphine (0.39 g, 1.5 mmol), imidazole (0.20 g, 3 mmol) and iodine (0.38 g, 1.5 mmol) were added to a vigorously stirred suspension of **2b** (0.30 g, 1 mmol) in toluene-acetonitrile (2:1, 25 mL) at 80°C. After 10 min the reaction mixture was worked-up as for **3a** and the compound was crystallised from methanol-water to give 0.26 g (65%) of **3b** as colourless crystals: mp 197°C. ^{1}H NMR (Me_2SO-d_6) δ 1.88 (3H, s, CH_3CO), 3.32 (2H, m, CH_2I), 3.82 (1H, m, CH), 4.00 (2H, m, CH_2OCOCH_3), 5.43 (2H, s, CH_2N), 6.48 (2H, s, D_2O exchangeable, NH_2), 7.83 (1H, s, CH), 10.62 (1H, s, D_2O exchangeable, NH). Anal. Calcd for $C_{11}H_{14}N_5O_4I.0.25\ H_2O$: C, 32.09, H, 3.54; N, 17.01. Found: C, 31.75; H, 3.50; N, 17.01.

9-[3-Acetoxy-2-(iodomethyl)propoxy]guanine (**3c**)

Triphenylphosphine (0.26 g, 1 mmol), imidazole (0.14 g, 2 mmol) and iodine (0.25 g, 1 mmol) were added to a vigorously stirred suspension of **2c** (0.20 g, 0.67 mmol) in toluene-acetonitrile (2:1, 20 mL) at 80°C. After 45 min the reaction mixture was worked-up as for **3a** and the compound was purified by column chromatography on silica gel, eluting with chloroform-methanol (gradient 1% to 20% methanol in chloroform) to afford 58 mg (21%) of **3c** as a slightly yellow solid: mp 190-193°C after crystallisation from ethanol-water. ^{1}H NMR (Me_2SO-d_6) δ 2.04 (3H, s, CH_3CO), 2.20

(1H, m, CH), 3.45 (2H, d, J=5.6 Hz CH_2I), 4.13 (2H, m, CH_2OCO), 4.28 (2H, m, CH_2O), 6.54 (2H, br s, D_2O exchangeable, NH_2), 7.96 (1H, s, CH), 10.65 (1H, s, D_2O exchangeable, NH). Found: (FAB), MH^+, 408.0178, $C_{11}H_{14}N_5O_4I$ requires: MH^+, 408.0169. Anal. Calcd for $C_{11}H_{14}N_5O_4I$: C, 32.45; H, 3.46; N, 17.20. Found: C, 32.11, H, 3.44; N, 17.27.

9-[3-Acetoxy-2-(iodomethyl)propoxy]-N^2-acetylguanine (**3d**)

Triphenylphosphine (0.20 g, 0.75 mmol), imidazole (0.10 g, 1.5 mmol) and iodine (0.19 g, 0.75 mmol) were added to a vigourously stirred suspension of **2d** (0.17 g, 0.5 mmol) in toluene-acetonitrile (2:1, 20 mL) at 80°C. After 8 min the reaction mixture was cooled to room temperature. Chloroform (40 mL) was added and the resulting solution was washed with saturated aqueous $NaHCO_3$ (1 x 20 mL), dried ($MgSO_4$) and evaporated to dryness. The residue was purified by column chromatography on silica gel, eluting with chloroform-ethanol (99:1) to afford 0.13 g (60%) of **3d** as a colourless crystalline solid: mp 170-171°C. ^1H NMR (Me_2SO-d_6) δ 2.04 (3H, s, CH_3CO), 2.19 (3H, s, CH_3CO), 2.22 (1H, m, CH), 3.46 (2H, d, J=6.07 Hz, CH_2I), 4.15 (2H, m, CH_2O), 4.36 (2H, m, CH_2O), 8.30 (1H, s, CH), 11.76 (1H, br s, D_2O exchangeable, NH), 12.06 (1H, br s, D_2O exchangeable, NH). Found: (CI), MH^+, 450.0276; $C_{13}H_{16}N_5O_5I$ requires: MH^+, 450.0275. Anal. Calcd for $C_{13}H_{16}N_5O_5I$: C, 34.76; H, 3.59; N, 15.59. Found: C, 34.73; H, 3.62; N, 15.62.

9-[4-Hydroxy-3-(iodomethyl)butyl]guanine (**4a**)

A solution of **3a** (0.27 g, 0.67 mmol) in concentrated ammonia-methanol (1:2, 50 mL) was stirred at room temperature for 6 h. The solvent was evaporated and the residue was coevaporated with methanol (3 x 30 mL). The resulting solid was triturated with chloroform-acetonitrile (4:1, 3 x 25 mL) and afterwards crystallised from ethanol-water to give 0.17 g (70%) of **4a** as colourless crystals: mp 240-245°C. ^1H NMR (Me_2SO-d_6) δ 1.21 (1H, m, CH), 1.70(2H, m, CH_2), 3.32 (2H, m, $C\underline{H}_2OH$), 3.41 (2H, d, J=4.7 Hz, CH_2I), 3.99 (2H, m, CH_2N), 4.69 (1H, t, J=5.2 Hz, D_2O exchangeable, OH), 6.41 (2H, s, D_2O exchangeable, NH_2), 7.70 (1H, s, CH), 10.52 (1H, br.s, D_2O exchangeable, NH). Found: (FAB), MH^+, 364.0288. $C_{10}H_{14}N_5O_2I$ requires MH^+, 364.0271. Anal. Calcd for $C_{10}H_{14}N_5O_2I$: C, 33.07; H, 3.89; N, 19.28. Found: C, 33.03; H, 3.96; N, 19.22.

9-[(1-Hydroxy-2-iodomethyl)ethoxymethyl]guanine (**4b**)

Compound **4b** was prepared from **3b** (0.69 mmol) according to the same procedure as described for **4a**; rection time 8h. After the work-up as for **4a** the compound was crystallised from ethanol-water to give 0.19 g (75%) of **4b** as colourless crystals: m.p. 189-190°C. ^{1}H NMR (Me_2SO-d_6) δ 3.17-3.52 (5H, m, CH, $C\underline{H}_2OH$, CH_2I), 4.86 (1H, br s, D_2O exchangeable, OH), 5.42 (2H, s, CH_2N), 6.48 (2H, br s, D_2O exchangeable, NH_2), 7.82 (1H, s, CH), 10.61 (1H, br s, D_2O exchangeable, NH). Found: (FAB), MH^+, 366.0055. $C_9H_{12}N_5O_3I$ requires MH^+, 366.0063. Anal. Calcd for $C_9H_{12}N_5O_3I$. H_2O: C, 28.21; H, 3.68; N, 18.27. Found: C, 27.95; H, 3.58; N, 18.11.

9-[3-Hydroxy-2-(iodomethyl)propoxy]guanine (**4c**)

Compound **4c** was prepared from **3c** or **3d** (0.3 mmol) according to the same procedure as described for **4a**. After the work-up as for **4a** the compound was purified by column chromatography on silica gel eluting with chloroform-ethanol (gradient 5% to 25% ethanol in chloroform) to give **4c**; yield 80 mg (73%) from **3c** and 73 mg (67%) from **3d**. Crystallisation of **4c** from ethanol-water afforded a colourless solid: mp. 248-250°C. ^{1}H NMR (Me_2SO-d_6) δ 1.90 (1H, m, CH), 3.46 (2H, d, J=5.6 Hz, CH_2I), 3.50 (2H, m, $C\underline{H}_2OH$), 4.25 (2H, m, CH_2O), 4.85 (1H, t, J=5.3 Hz, D_2O exchangeable, OH), 6.56 (2H, br s, D_2O exchangeable, NH_2), 7.96 (1H, s, CH), 10.65 (1H, br s, D_2O exchangeable, NH). Found: (FAB), MH^+, 366.0077; $C_9H_{12}N_5O_3I$ requires: MH^+, 366.0063. Anal. Calcd for $C_9H_{12}N_5O_3I.1.2H_2O$ C, 27.95; H, 3.75; N, 18.10. Found: C, 27.74; H, 3.58; N, 18.06.

9-[1-Hydroxy-3-(thiomethyl)butyl]guanine S-phosphate Di-Sodium Salt (**5a**)

A mixture of **4a** (0.24 g, 0.66 mmol) and trisodium thiophosphate (1.26 g, 7 mmol) in water (17 mL) was stirred at 22°C for 30 h. Water (35 mL) and methanol (100 mL) were added to the reaction mixture, the resulting solution was cooled to 0°C and an excess of trisodium thiophosphate was removed by centrifugation. The pH of the supernatant was adjusted to 8.5 with dilute aqueous NaOH. The solvent was evaporated and the residue was dissolved in water (1 mL) and applied to a reverse phase C_{18} column. The column was eluted with water (pH 7.0) and the appropriate fractions were combined. The pH of the solution was adjusted to 8.5 with dilute aqueous NaOH, concentrated and lyophilized to give 0.16 g (60%) of **5a** as a colourless powder. ^{1}H

NMR (D_2O) δ 1.73 (3H, m, CH, CH_2), 2.72 (2H, m, CH_2S), 3.51 (2H, d, J=5.3 Hz, $C\underline{H}_2OH$), 4.00 (2H, m, CH_2N), 7.71 (1H, s, CH), ^{31}P NMR (D_2O) δ_p 17.18 p.p.m. Found: (FAB), MH^+, 394.0320; $C_{10}H_{14}N_5O_5PSNa_2$ requires MH^+, 394.0327.

9-[(1-Hydroxymethyl-2-thio)ethoxymethyl]guanine S-phosphate Di-Sodium Salt (**5b**)

Compound **5b** was prepared by reaction of **4b** (1.37 mmol) with trisodium thiophosphate according to the same procedure as described for **5a**. After the usual work-up **5b** was obtained as a colourless powder; yield 0.35 g (65%). ^1H NMR (D_2O) δ 2.66 (2H, m, CH_2S), 3.33-3.47 (2H, m, $C\underline{H}_2OH$), 3.68 (1H, m, CH), 5.37 (2H, s, CH_2N), 7.74 (1H, s, CH). ^{31}P NMR (D_2O) δ_p 16.62 p.p.m. Found: (FAB), MH^+, 396.0120; $C_9H_{12}N_5O_6PS\,Na_2$ requires: MH^+, 396.0120.

9-[3-Hydroxy-2-(thiomethyl)propoxy]guanine S-phosphate Di-Sodium Salt (**5c**)

Compound **5c** was prepared by reaction of **4c** (0.14 mmol) with trisodium thiophosphate according to the same procedure as described for **5a**. After the usual work-up **5c** was obtained as a fluffy colourless powder; yield 35 mg (64%). ^1H NMR (D_2O) δ 2.10 (1H, m, CH), 2.70 (2H, m, CH_2S), 3.64 (2H, t, J=4.5 Hz, $C\underline{H}_2OH$), 4.20(2H, d, J=6.23 Hz, CH_2N), 7.83 (1H, s, CH). ^{31}P NMR (D_2O) δ_p 17.26 p.p.m. Found: (FAB), MH^+, 396.0120; $C_9H_{12}N_5O_6PSNa_2$ requires: MH^+, 396.0120.

Hydrolysis of S-phosphates **5a-c** by Alkaline Phosphatase

The substrates (0.3 μmol) in water (10 μl) were incubated with 0.1 M Tris buffer pH 8.0 (30 μl) and alkaline phosphatase (from Escherichia coli, Sigma; 5 μl) and the mixtures were monitored by h.p.l.c. (Spherisorb, S5 0DS S2 column, isocratic elution with 0.1 M triethylammonium acetate buffer pH 7.0-acetonitrile (85:15). In all cases no substrate could be detected in the reaction mixture after 15 min. A control experiment without the enzyme indicated no change in the starting material.

Acidic Hydrolysis of S-phosphates **5a-c**

The substrates (1 mg) were dissolved in 2.0 ml of hydrochloric acid pH 2.0 or buffer pH 7.0 and 9.2. Hydrolysis reactions were carried out at 23°C and progress of the reactions was monitored by h.p.l.c. (Spherisorb, S5 ODS S2 column, isocratic elution with 0.1 M triethylammonium acetate buffer pH 7.0-acetonitrile (85:15) or (95:5).

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