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Synthesis and separation of diastereomers of deoxynucleoside 5'-O-(1-thio)triphosphates

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ABSTRACT

Treatment of unprotected nucleosides with an excess of phosphorous acid and stoichiometric proportions of N,N'-di-p-tolylcarbodiimide in anhydrous pyridine gives predominantly deoxynucleoside monophosphites and minor amounts of 5':3'-diphosphites; for deoxyadenosine and deoxyguanosine, the monophosphite products are exclusively 5'-phosphites, whereas for deoxycytidine and thymidine, the yields of the 5'-phosphites are 85% and 92% respectively. Sulfurization of these deoxynucleoside monophosphites with sulfur in the presence of trialkylamines and trimethylsilyl chloride in dry pyridine nearly quantitatively produces deoxynucleoside phosphorothioates. Condensation of these phosphorothioates with pyrophosphate forms diastereomers of the α-thio-derivatives of deoxynucleoside triphosphate. The individual diastereomers of each deoxynucleoside 5'-O-(1-thio)triphosphate can be separated, on a preparative scale, by ion exchange chromatography.

INTRODUCTION

Deoxynucleoside 5'-O-(1-thio)triphosphates have many important applications. They have been employed as: substrates for an *in vitro* mutagenic reaction (1), precursors for the *in vitro* synthesis of viral DNAs (2,3), and probes for studying the stereochemistry of the DNA polymerase reaction (4) as well as a variety of other enzymes (5). Some interesting recent applications include the synthesis of a ^{35}S-labelled cDNA of rabbit globin mRNA (6) and the use of dATP[α-^{35}S] for the nick-translation of plasmid DNA (6).

The four deoxynucleoside 5'-O-(1-thio)triphosphates have been prepared analogously to ATPαS (7,8). The synthetic scheme employed was pyrophosphorylation of the deoxynucleoside 5'-O-thiomonophosphates, which were prepared by hydrolysis of the products obtained from the reaction of unprotected deoxynucleosides with thiophosphoryl chloride in triethyl phosphate. However, this method is not attractive for the synthesis of [^{35}S]-(dNTPαS) because of the limited availability of [^{35}S]-($PSCl_3$) and the generally low yield in the thiophosphorylation step. Sulfurization of thymidine 5'-phosphite and 5'-O-tritylthymidine 3'-phosphite with sulfur has been reported to produce nearly quantitatively, the corresponding 5' and 3' thiomonophosphates (9). We have extended

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and improved a route through the 5'-phosphite to prepare the four deoxynucleoside 5'-O-thiomonophosphates. This method has the flexibility to permit the introduction of radiolabeled ^{35}S to yield [^{35}S]-(dNMPS), a necessary precursor of [^{35}S]-(dNTPαS).

RESULTS AND DISCUSSION

Nucleoside phosphites can be synthesized by: condensation of suitably protected nucleosides with the mixed anhydride generated from benzyl hydrogen phosphite and diphenyl hydrogen phosphate followed by subsequent scission of the resulting nucleoside benzyl phosphite (10-13); or, by treatment of the nucleoside component either with phosphorous acid triesters and acid catalysts in dimethylformamide followed by hydrolysis (14,15) or with phosphorus acid in the presence of condensing agents (16-18). We have used the latter approach to prepare all four deoxynucleoside 5'-phosphites. The results are summarized in Table 1. The reaction of four unprotected deoxynucleosides with excess phosphorous acid and a stoichiometric proportion of N,N'-di-p-tolyl-carbodiimide gives, in addition to the unreacted deoxynucleoside, the desired monophosphite and lesser amounts of the diphosphite. The components of the product mixture could be separated, on a preparative scale, by chromatography on a column of DEAE-Cellulose. One exception was deoxyguanosine 5'-phosphite that could not be resolved from inorganic phosphorous acid on a column of DEAE-Cellulose, but the separation was readily effected on a Dowex AG 1 x 8 column. Surprisingly, deoxyadenosine and deoxyguanosine gave exclusively the 5'-isomer of the monophosphite, while thymidine and deoxycytidine gave a mixture predominantly of the 5'-isomer with some 3'-isomer. Resolution of the 5'-isomers from the 3'-isomers was not achieved on a DEAE-Cellulose column

Table 1
Phosphitylation of Nucleosides with Phosphorus Acid

Nucleoside	Yield* of Nucleoside Phosphite		
	5'-mono	3'-mono	5':3'-di
deoxyadenosine	64	no detectable	8
deoxyguanosine	58	no detectable	6
thymidine	53	5	17
deoxycytidine	30	5	3

*Yields, express as the mole % of the initial amount of nucleoside, were determined by UV absorbance at 260 nm assuming the absorptivity of the nucleoside phosphites to be identical to that of the nucleoside phosphates.

under our conditions. The content of the 5'- and 3'-isomers was estimated by ^{31}P NMR. In the proton-coupled phosphorous NMR spectrum, the primary (5')-phosphite esters ($P-O-CH_2-$) afford a triplet resonance signal, while the secondary (3')-esters (P-O-CH) yield a doublet (19,20); the phosphites have a strong coupling constant between the phosphorous atom and the phosphorous bound proton (20). The chemical shifts and coupling constants of the obtained nucleoside phosphites are given in Table 2.

Oxidation of the thymidine 5'- and 3'-phosphites to the thymidine phosphorothioates in pyridine by sulfur, employing trimethylsilyl nucleoside phosphites as intermediates, was first described by Hata and coworkers (9). We have established a general procedure to prepare all four deoxynucleoside phosphorothioates by using sulfur as an oxidizing agent. The basic scheme for the oxidation of the deoxynucleoside phosphites by sulfur involves their conversion to the trimethylsilyl derivatives, that are highly reactive

Table 2

^{31}P NMR Spectra of Nucleoside Phosphites

Nucleoside Phosphite	δ(ppm)[a]	J_{pH}(Hz)[b]	J_{poch}(Hz)[c]
deoxyadenosine 5'-phosphite	6.59	638(d)	6.10(t)
deoxyadenosine 5':3'-diphosphite	6.63	638(d)	6.10(t)
	5.00	644(d)	10.1 (d)
thymidine 5'-phosphite	6.42	637(d)	6.10(t)
thymidine 3'-phosphite	4.95	643(d)	9.15(d)
thymidine 5':3'-diphosphite	6.45	637(d)	6.10(t)
	4.98	643(d)	9.76(d)
deoxycytidine 5'-phosphite	6.51	638(d)	6.10(t)
deoxycytidine 3'-phosphite	4.91	641(d)	9.15(d)
deoxycytidine 5':3'-diphosphite	6.55	636(d)	6.10(t)
	4.97	642(d)	9.76(d)
deoxyguanosine 5'-phosphite	6.57	640(d)	7.30(t)
deoxyguanosine 5':3'-diphosphite	6.65	639(d)	7.00(t)
	4.94	642(d)	9.83(d)

[a]Values of chemical shift are expressed relative to 1 M H_3PO_4 in D_2O (31) and were measured in 50% D_2O and 50% 1.0 M Tris·HCl-0.05 M EDTA at pH 8.8.
[b]Coupling constant between the phosphorous atom and the phosphorous bound proton.
[c]Coupling constant between the phosphorous atom and the protons on the nearest carbon atom.

nucleophiles because the lone electron pair is localized on the trivalent phosphorous atom, followed by their sulfurization and hydrolysis. During several experiments, we encountered solubility problems with the triethylammonium salts of the deoxycytidine and deoxyguanosine phosphites in pyridine. However, this was solved by converting the triethylammonium salts to tri-n-octylammonium salts. The sulfurization of the trimethylsilyl deoxynucleoside phosphites proceeds rapidly and nearly quantitatively. PEI-Cellulose TLC analysis (developing solution: 0.75 M KH_2PO_4, pH 3.6) ten minutes after addition of sulfur showed that the reactions were already complete, in all cases. The reaction products, deoxynucleoside phosphorothioates, were purified by chromatography on a column of DEAE-Cellulose. The ^{31}P NMR analysis of these phosphorothioates is shown in Table 3.

Deoxynucleoside 5'-O-(1-thio)triphosphates were prepared, according to method used for the synthesis of ATPαS from AMPS (8), by condensation of the deoxynucleoside 5'-O-thiomonophosphates with pyrophosphate. With some modi-

Table 3

^{31}P NMR Spectra of Nucleoside Thiophosphate Analogues

	Chemical shift (ppm)[a]			Coupling constant (Hz)	
Compound[c]	α-P	β-P	γ-P	$J_{\alpha\beta}$	$J_{\beta\gamma}$
dAMPS	43.24(s)[b]				
dGMPS	43.24(s)				
dCMPS	43.17(s)				
(3')dCMPS	43.07(s)				
TMPS	43.12(s)				
(Sp)dATPαS	43.18(d)	-22.72(q)	-5.94(d)	27.15	20.06
(Rp)dATPαS	42.85(d)	-22.70(q)	-5.94(d)	27.95	20.06
(Sp)dGTPαS	43.20(d)	-22.73(q)	-5.95(d)	27.67	20.29
(Rp)dGTPαS	42.89(d)	-22.73(q)	-5.95(d)	28.13	20.29
(Sp)dCTPαS	43.03(d)	-22.67(q)	-5.96(d)	27.57	19.98
(Rp)dCTPαS	42.84(d)	-22.70(q)	-5.96(d)	28.17	19.98
(Sp)TTPαS	42.98(d)	-22.74(q)	-5.96(d)	27.78	19.68
(Rp)TTPαS	42.48(d)	-22.77(q)	-5.96(d)	28.33	19.68

[a]Values are express relative to 1 M H_3PO_4 in D_2O (31) and were measured in 50% D_2O and 50% 1.0 M Tris·HCl-0.05 M EDTA at pH 8.8. [b]S, singlet; d, doublet; q, quartet. [c]Abbreviations used: dNMPS, deoxynucleoside 5'-O-thiomonophosphate; dNTPαS, deoxynucleoside 5'-O-(1-thio)triphosphate; (3')dCMPS, deoxycytidine 3'-O-thiomonophosphate.

fications, we have been able to obtain a 40% and 65% yield for dATPαS and dGTPαS respectively. In Table 3 are given the ^{31}P NMR analysis of the obtained deoxynucleoside 5'-O-(1-thio)triphosphates.

Since the α-phosphorous atom of dNTPαS is chiral, it exists in either the Sp or Rp configuration. We have separated the diastereomers of each of the four deoxynucleoside 5'-O-(1-thio)triphosphates, on a preparative scale, by ion exchange chromatography (DEAE-Sephadex A-25). The isomeric purity of the individual diastereomer was checked by reverse-phase HPLC (Partisil PXS 10/25 ODS-2, Whatman; the mobil phase was 40 mM KH_2PO_4 buffer with pH 6.0) and ^{31}P NMR. The order of the eluted diastereomers of dNTPαS from a DEAE-Sephadex A-25 column was found to be the same as that in a reverse-phase HPLC (the first eluted diastereomers were shown to have the Sp configuration at phosphorus; the second, Rp, in all cases). The elution profiles for the resolution of the diastereomers of deoxyguanosine 5'-O-(1-thio)triphosphate from a DEAE-Sephadex A-25 column and reverse-phase HPLC, for example, are shown in Figure 1.

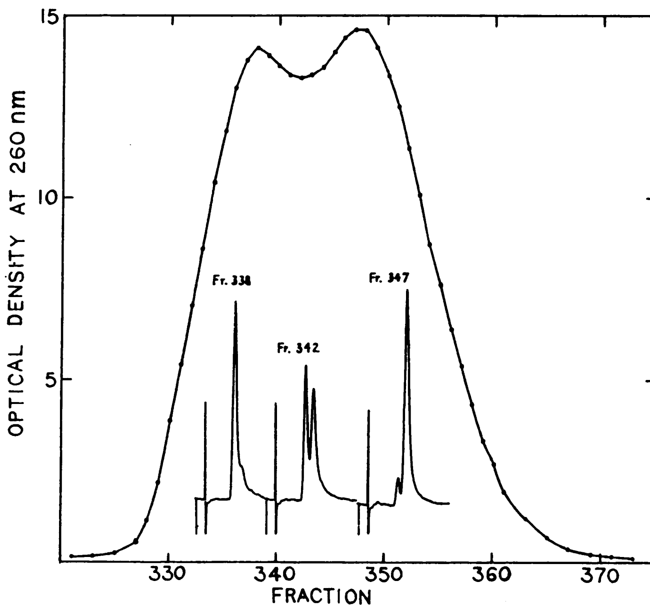

Figure 1. Resolution of 300 μmole of the diastereomers of dGTPαS by chromatography on DEAE-Sephadex A-25 (105 x 2.5 cm). The first eluted stereoisomer is the Sp form, the second one, the Rp form. Isomeric purity was checked by reverse-phase HPLC (insert). The retention times for the Sp and the Rp diastereomers were 3.5 min and 4.2 min respectively; the elution was carried out with 40 mM KH_2PO_4 (pH 6.0) at a flow rate of 1.5 ml/min.

The configurations at Pα in α-thionucleotides can be determined by enzymic methods and ^{31}P nuclear magnetic resonance (21). These methods are used to determine relative configurations, all configurations being correlated with that of (Rp) ATPαS, which has been correlated with the absolute configuration of (Rp) 5'-O-adenosyl 3'-O-uridylphosphorothioate, (Rp) adenosine 5'-O-phosphorothioate O-p-nitrophenyl ester, and the S enantiomer of O-p-nitrophenyl phenylphosphorothioate (22-27). The chemical shift difference between the Sp form and the Rp form for α-thionucleotides is 0.2 - 0.4 ppm for Pα, with the Sp form more down-field (21,27-29). In making configurational assignments by the ^{31}P NMR technique, we mixed about one third of the first eluted stereoisomer with two thirds of the second eluted stereoisomer. The ^{31}P NMR spectra showed that all the first eluted stereoisomers have a chemical shift more down-field than the second eluted stereoisomers (Table 3). Hence, these first eluted diastereomers have the Sp configuration at the α-phosphorous atom, and the second eluted ones have the Rp configuration.

Our synthesis of the deoxynucleoside 5'-O-(1-thio)triphosphates by sulfurization of deoxynucleoside 5'-phosphites followed by pyrophosphorylation has the following advantages: 1) enzymic methods are not readily available for preparing the dG, T and dC α-thiotriphosphates; 2) the Rp and Sp diastereomers can be separated, if required, by chromatography on Sephadex; and 3) ^{35}S can be readily introduced because of the quantitative yield on the sulfurization step.

EXPERIMENTAL

Deoxyadenosine, deoxyguanosine, thymidine and deoxycytidine were purchased from Sigma. DEAE-Sephadex was from Pharmacia, DEAE-Cellulose DE-52 was from Whatman, Dowex-50W 50 x 2 -400 was from Sigma, and Dowex AG 1-X8 (100-200 mesh) was from Bio-Rad. 3'-O-Acetylthymidine was prepared according to the method of Michelson and Todd (30). Phosphorous acid, N,N'-di-p-tolylcarbodiimide, and diphenyl phosphorochloridate were purchased from Aldrich. All other reagents, buffers, and inorganic salts were reagent grade. Doubly deionized distilled water was used throughout. The deuterium oxide (99.8%) was obtained from Merck Sharp & Dohme.

^{31}P nuclear magnetic resonance spectra were recorded on a Bruker WH 360 spectrometer operating in the Pulsed Fourier Transform mode at 145.8 MHz. Chemical shifts were given in ppm with 1 M H_3PO_4 in D_2O as external standard (31). Chromatography was monitored on a Gilford 240 spectrophotometer at 260 nm. The amounts of the nucleotides and their analogues were estimated by a spectro photometric method assuming their absorptivities to be identical to that of

the corresponding deoxynucleoside phosphate. The employed molar absorbance coefficients for the 5'-phosphates of deoxyadenosine, deoxyguanosine, thymidine and deoxycytidine were $\varepsilon_{260\ nm} = 15.3 \times 10^3\ M^{-1}\ cm^{-1}$, $12.8 \times 10^3\ M^{-1}\ cm^{-1}$, $9.13 \times 10^3\ M^{-1}\ cm^{-1}$ and $7.30 \times 10^3\ M^{-1}\ cm^{-1}$ respectively (32). All HPLC experiments were performed with a Altex (Model 100) liquid chromatography system fitted with a reverse column (Partisil PXS 10/25 ODS-2, Whatman) and a 254 nm absorbance detector (Altex Model 153). The elution was carried out with 40 mM KH_2PO_4 (pH 6.0) at a flow rate of 1.5 ml/min.

Deoxyadenosine 5'-phosphite

To 10 ml of dry pyridine were added anhydrous deoxyadenosine (0.26 g; 1 mmol; dried at 100°C/0.05 mm Hg for 8 hours) and phosphorous acid (0.12 g; 1.4 mmole). After 10 minutes of stirring at room temperature, N,N'-di-p-tolylcarbodiimide (0.32 g; 1.4 mmol) was added, and the reaction mixture was then stirred at room temperature for 3 days. Deionized water (50 ml) was added with stirring for another 30 minutes. The precipitate of di-p-tolylurea was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure at 35°C. The residue was treated with 8 ml of 1 M triethylammonium bicarbonate (pH 7.5) and the solution was evaporated to dryness again as before. The excess of triethylamine and a trace of insoluble material were removed by repeated coevaporation with deionized water and filtration. The residue was dissolved in 10 ml of deionized water, and the solution was then chromatographed on a column (50 x 4.5 cm) of DEAE-Cellulose with a linear gradient formed from 2.0 liters each of deionized water and 0.40 M triethylammonium bicarbonate (pH 7.5). The elution was carried out at a flow rate of 1.5 ml/min and 20 ml fractions collected. The 0.19-0.22 M buffer fraction yielded 0.64 mmole of the deoxyadenosine 5'-phosphite triethylammonium salt. The 0.28-0.30 M buffer fraction afforded 0.08 mmole of the deoxyadenosine 5':3'-diphosphite triethylammonium salt.

Thymidine 5'-phosphite

(a) The reaction of thymidine with phosphorous acid.

A mixture of thymidine (0.24 g; 1.0 mmole; dried at 100°C/0.05 mm Hg for 8 hours), anhydrous pyridine (10 ml), phosphorous acid (0.12 g; 1.4 mmole), N,N'-di-p-tolylcarbodiimide (0.32 g; 1.4 mmole) was worked up as in the case of deoxyadenosine. The chromatography was performed on a column (39 x 4 cm) of DEAE-Cellulose with a linear gradient formed from 1.5 liter each of deionized water and 0.60 M triethylammonium bicarbonate (pH 7.5) at a flow rate of 1.3 ml/min and 20 ml fractions collected. The buffer fractions (ca. 0.20 M) yielded 0.58 mmole of the mixture of the triethylammonium salts of thymidine 5'-phosphite (92%) and thymidine 3'-phosphite (8%). The buffer fractions (ca.

0.27 M) afforded 0.17 mmole of the thymidine 5':3'-diphosphite triethylammonium salt.

(b) The reaction of 3'-O-acetylthymidine with triphenyl phosphite (14).

To 2.5 ml of dry dimethylformamide were added anhydrous 3'-O-acetylthymidine (0.43 g; 1.5 mmole; dried at 110°C for 2 hrs), triphenyl phosphite (1.4 g; 4.5 mmole) and 6.1 M hydrogen chloride in dimethylformamide (0.55 ml). The reaction mixture was stirred at room temperature for 5 hours. Dilute (1:4) aqueous ammonia (50 ml) was then added. After 2 hours of vigorous stirring, the mixture was washed with three 25 ml portions of ether. The aqueous layer was evaporated to dryness under reduced pressure at 35°C. The residue was then dissolved in dilute (1:1) aqueous ammonia (25 ml), and the solution was stirred at room temperature for 10 hours and evaporated to dryness as above. The residue was dissolved in 10 ml of deionized water, and the solution was chromatographed on a column (45 x 4.5 cm) of DEAE-Cellulose with a linear gradient composed of 1.0 liter each of deionized water and 0.40 M triethylammonium bicarbonate (pH 7.5). The elution was performed at a flow rate of 1.3 ml/min and 20 ml fractions collected. The 0.23–0.33 M buffer fraction yielded 1.2 mmole of the thymidine 5'-phosphite triethylammonium salt.

Deoxycytidine 5'-phosphite

A mixture of deoxycytidine (0.45 g; 2.0 mmole; dried at 110°C for 25 hours), anhydrous pyridine (12 ml), phosphorous acid (0.24 g; 2.8 mmole) and N,N'-di-p-tolylcarbodiimide (0.64 g; 2.8 mmol) was worked up as in the case of deoxyadenosine. After stirring at room temperature for 3 days, an oily precipitate formed, which was collected and washed with three 1 ml portions of cold pyridine. Deionized water (50 ml) was added, and the mixture was stirred for 30 minutes and filtered. The filtrate was worked up as before. The residue was dissolved in 20 ml of deionized water, and the solution was chromatographed on a column (42 x 4 cm) of DEAE-Cellulose and eluted with a linear gradient composed of 1.0 liter each of deionized water and 0.40 M triethylammonium bicarbonate (pH 7.5) at a flow rate of 1.3 ml/min. Fractions (20 ml) were collected, the 0.21–0.26 M buffer fraction yielded 0.70 mmol of a mixture of the triethylammonium salts of deoxycytidine 5'-phosphite (85%) and 3'-phosphite (15%). The 0.32–0.35 M buffer fraction afforded 0.07 mmole of the deoxycytidine 5':3'-diphosphite triethylammonium salt.

Deoxyguanosine 5'-phosphite

To 40 ml of dry dimethylformamide were added anhydrous deoxyguanosine (1.34 g; 5.0 mmol; dried at 100°C/0.05 mm Hg for 2 days). After 10 minutes of stirring at room temperature, anhydrous pyridine (7 ml) and phosphorous acid (0.59 g; 7.2 mmole) were added, and the mixture was stirred for another 10

minutes. N,N'-di-p-tolylcarbodiimide (1.6 g; 7.2 mmole) was then added, and the reaction mixture was stirred at room temperature for 3 days. The mixture was filtered, and deionized water (250 ml) was added to the filtrate with stirring for 30 minutes. The precipitate of di-p-tolylurea was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure at a temperature about 35°C. The residue was treated with 20 ml of 1 M triethylammonium bicarbonate (pH 7.5). The excess triethylamine and a trace of insoluble material were removed by repeated coevaporation with deionized water and filtration. The residue was dissolved in 20 ml of deionized water, and the solution was applied to a column (5 x 2.5 cm) of Dowex AG 1 x 8 (HCO_3^- form; 100-200 mesh) in 5 ml aliquots at a flow rate of 0.67 ml/min. The elution was first conducted with a linear gradient composed of 250 ml each of deionized water and 0.50 M triethylammonium bicarbonate (pH 7.5) followed by 1.5 liters of 2.0 M triethylammonium bicarbonate (pH 7.5) both at a flow rate of 1.3 ml/min. The fractions (20 ml) after the 20th were collected. The combined solution was evaporated to dryness, and the excess of triethylamine was removed by repeated coevaporation with deionized water under reduced pressure at a temperature about 35°C. The residue was dissolved in 30 ml of deionized water, and the solution was loaded on a column (56 x 4.5 cm) of DEAE-Cellulose in aliquots of 15 ml. The chromatography was carred out at a flow rate of 1.3 ml/min with a linear gradient formed from 2.0 liters each of deionized water and 0.60 M triethylammonium bicarbonate (pH 7.5) and 20 ml fractions collected. The 0.21-0.28 M buffer fraction yielded 2.7 mmole of the deoxyguanosine 5'-phosphite triethylammonium salt. The 0.33-0.37 M buffer fraction furnished 0.30 mmole of the deoxyguanosine 5':3'-diphosphite triethylammonium salt.

Deoxyadenosine 5'-O-thiomonophosphate (dAMPS)

The deoxyadenosine 5'-phosphite triethylammonium salt (2.9 mmole), after evaporation with four 30 ml portions of anhydrous pyridine (warmed until it completely dissolved), was dissolved in 40 ml of anhydrous pyridine. Anhydrous triethylamine (2.08 ml; 14.5 mmole) and trimethylsilyl chloride (1.85 ml; 14.5 mmole) were then added. The mixture was stirred at room temperature for 10 minutes, and dry pulverized sulfur (185 mg; 5.8 mmole) was then added. After 1 hour, the precipitate of triethylammonium chloride was removed by filtration, deionized water (10 ml) was added to the filtrate, and the solution was evaporated to dryness under reduced pressure at 30°C. Water (60 ml) and ether (15 ml) were added, and the mixture stirred for 10 minutes. The aqueous layer was separated, and then filtered through a Millipore filter. The filtrate was washed with four 15 ml portions of ether, and the aqueous layer was separated and concentrated to 30 ml. The solution was applied to a column (52 x 4.5 cm)

of DEAE-Cellulose in aliquots of 15 ml. The elution was carried out with a linear gradient composed of 2.0 liters each of deionized water and 0.60 M triethylammonium bicarbonate (pH 7.5) at a flow rate of 1.3 ml/min and 20 ml fractions collected. The 0.34-0.45 M buffer fraction yielded 2.8 mmole of the deoxyadenosine 5'-O-thiomonophosphate triethylammonium salt.

Thymidine 5'-O-thiomonophosphate (TMPS)

A mixture of the thymidine 5'-phosphite triethylammonium salt (0.81 mmole), anhydrous pyridine (10 ml), triethylamine (0.58 ml; 4.0 mmole), trimethylsilyl chloride (0.52 ml; 4.0 mmole) and pulverized sulfur (52 mg; 1.6 mmole) was worked up as before for the preparation of deoxyadenosine 5'-O-thiomonophosphate. Chromatography of the products was carried out at a flow rate of 1.1 ml/min on a column (40 x 4 cm) of DEAE-Cellulose with a linear gradient derived from 0.90 liters each of deionized water and 0.60 M triethylammonium bicarbonate (pH 7.5) and 20 ml fractions collected. The 0.40-0.51 M buffer fraction yielded 0.78 mmole of the thymidine 5'-O-thiomonophosphate triethylammonium salt.

Deoxycytidine 5'-O-thiomonophosphate (dCMPS)

A mixture of 1.26 mmole of the triethylammonium salts of deoxycytidine 5'-phosphite (85%) and 3'-phosphite (15%) in 10 ml of water was converted to the pyridinium salt by passing it through a column (45 x 3 cm) of pyridinium Dowex 50 x 2 (200-400 mesh) ion exchange resin. The salt was eluted with 350 ml of deionized water at a flow rate of 0.8 ml/min. The eluate was evaporated to dryness under reduced pressure at 35°C. The residue was further dried at 35°C/0.05 mm Hg for 1 hour. Anhydrous methanol (25 ml) and tri-n-octylamine (0.56 ml; 1.26 mmole) were then added. The mixture was stirred until a clear solution was obtained. After evaporation, the residue was further dried at 35°C/0.05 mm Hg for 2 hours, and then evaporated with four 15 ml portions of anhydrous pyridine. The residue was dissolved in anhydrous pyridine (25 ml), and then anhydrous triethylamine (0.91 ml; 1.3 mmole) and trimethylsilyl chloride (0.81 ml; 1.3 mmole) were added. After stirring the mixture at room temperature for 30 minutes, dry pulverized sulfur (89 mg; 2.5 mmole) was added. The reaction mixture was stirred at room temperature for another 1 hour and worked up as before for the preparation of deoxyadenosine 5'-O-thiomonophosphate. The chromatographic isolation of the deoxycytidine 5' & 3'-phosphorothioates was accomplished on a column (55 x 4.5 cm) of DEAE-Cellulose eluted at a flow rate of 1.3 ml/min with a linear gradient of 2.0 liters each of deionized water and 1.0 M triethylammonium bicarbonate (pH 7.5) and 20 ml fractions collected. The 0.41-0.53 M buffer fraction afforded 1.24 mmole of a mixture of the triethylammonium salts of deoxycytidine 5'-O-thiomonophosphate (85%) and 3'-O-thiomonophosphate (15%).

Deoxyguanosine 5'-O-thiomonophosphate (dGMPS)

The deoxyguanosine 5'-phosphite triethylammonium salt (2.0 mmole) was converted to the tri-n-octylammonium salt as before for the deoxycytidine monophosphite triethylammonium salts. To the anhydrous deoxyguanosine 5'-phosphite tri-n-octylammonium salt (2.0 mmole; evaporated with four 30 ml portions of anhydrous pyridine) were added tri-n-octylamine (4.5 ml in 15 ml of anhydrous pyridine; 10 mmole; evaporated with four 20 ml portions of anhydrous pyridine) and trimethylsilyl chloride (1.3 ml; 10 mmol). After stirring the mixture at room temperature for 10 minutes, dry pulverized sulfur (128 mg; 4 mmole) was added, and the mixture was stirred at room temperature for another one hour. Triethylamine (1.7 ml; 12 mmole) was then added to the reaction mixture which was stirred at room temperature for 20 minutes. The precipitate of triethylammonium chloride was removed by filtration, and deionized water (10 ml) was added to the filtrate. The solution was evaporated to 15 ml under reduced pressure at 30°C. Deionized water (150 ml) and ether (30 ml) were added. After 10 minutes of stirring, the aqueous layer was separated, and then filtered through a Millipore filter. The filtrate was washed with three 20 ml portions of ether. Triethylammonium bicarbonate (30 ml; 1 M, pH 7.5) was added to the separated aqueous layer, and the solution was then evaporated to dryness as before. The excess triethylamine and a trace of insoluble material were removed by repeated evaporation with deionized water and filtration. The residue was then dissolved in 30 ml of deionized water and the resulting solution was chromatographed on a column (55 x 4.5 cm) of DEAE-Cellulose and eluted with a linear gradient composed of 2.0 liters each of deionized water and 0.80 M triethylammonium bicarbonate (pH 7.5) at a flow rate of 1.3 ml/min and 20 ml fractions collected. The 0.41-0.55 M buffer fraction yielded 1.6 mmole of the deoxyguanosine 5'-O-thiomonophosphate triethylammonium salt.

Deoxyadenosine 5'-O-(1-thio)triphosphate (dATPαS)

The deoxyadenosine 5'-O-thiomonophosphate triethylammonium salt (1.5 mmole) was converted to the pyridinium salt by passing 10 ml of the solution through a column (45 x 3) of pyridinium Dowex 50 x 2 (200-400 mesh) ion exchange resin. The salt was eluted with 350 ml of deionized water at a flow rate of 0.8 ml/min. The eluate was evaporated to dryness under reduced pressure at 30°C. The residue was further dried at 30°C/0.05 mm Hg for 1 hour. Anhydrous methanol (25 ml) and tri-n-octylamine (0.66 ml; 1.5 mmole) were added, and the mixture stirred until a clear solution was obtained. After removal of methanol by evaporation, the residue was further dried at 30°C/0.05 mm Hg for 2 hours, and then evaporated with three 20 ml portions of dry dimethylformamide. The residue was dissolved in anhydrous dioxane (6 ml), and diphenyl phosphoro-

chloridate (0.45 ml; 2.2 mmole) and dry tri-n-butylamine (0.75 ml; 3 mmole) then were added. After stirring the mixture at room temperature for 3 hours, the solvent was removed by evaporation. The oily residue was washed with three 20 ml portions of anhydrous ether (ether:petroleum ether = 1:3) and evaporated to dryness.

Tetrasodium pyrophosphate decahydrate (6.7 g; 15 mmole) was converted to the pyridinium salt by passing 75 ml of the solution through a column (45 x 3 cm) of pyridinium Dowex 50 x 2 ion exchange resin (200-400 mesh). The salt was eluted with 300 ml of deionized water at a flow rate of 0.9 ml/min. The eluate was evaporated to dryness under reduced pressure at 35°C. The residue was further dried at 35°C/0.05 mm Hg for 2 hours. Anhydrous pyridine (20 ml) and dry tri-n-butylamine (6.6 ml; 30 mmole) were added to the residue, and the mixture was stirred until a clear solution was obtained. After evaporation with three 20 ml portions of anhydrous pyridine, the residue was dissolved in anhydrous pyridine (9 ml), and the solution was added to the activated deoxyadenosine 5'-phosphorothioate prepared above.

After stirring at room temperature for 2 hours, the reaction mixture was evaporated to dryness under reduced pressure at 30°C, and deionized water (60 ml) and ether (15 ml) were added. After stirring for 10 minutes, the aqueous layer was separated, washed with three 15 ml portions of ether, and concentrated to 30 ml. The solution was chromatographed on a column (53 x 4.5 cm) of DEAE-Cellulose and eluted with a linear gradient composed of 2.0 liters each of deionized water and 0.6 M triethylammonium bicarbonate (pH 7.5) at a flow rate of 1.3 ml/min and 20 ml fractions collected. The 0.49 M-0.59 M buffer fraction yielded 0.62 mmole of the deoxyadenosine 5'-O-(1-thio)triphosphate triethylammonium salt.

Separation of the Diastereomers of Deoxyadenosine 5'-O-(1-thio)triphosphate

The deoxyadenosine 5'-O-(1-thio)triphosphate triethylammonium salt (0.30 mmole) obtained from the above was dissolved in 2 ml of 0.20 M triethylammonium bicarbonate (pH 7.5). The solution was chromatographed on a column (99 x 2.5 cm) of DEAE-Sephadex A-25, preequilibrated with 0.20 M triethylammonium bicarbonate (pH 7.5). The elution was carried out at 4°C with a linear gradient formed from 2.0 liters each of 0.20 M and 1.0 M triethylammonium bicarbonate (pH 7.5) at a flow rate of 0.54 ml/min and 10 ml fractions collected. The 0.74-0.76 M buffer fraction yielded 84 μmole of the Sp form at about 95% isomeric purity. The 0.78-0.80 M buffer fraction afforded 85 μmole of the Rp form at about 95% isomeric purity. The pure isomers were obtained by rechromatography of the separated diastereomers.

Thymidine 5'-O-(1-thio)triphosphate (TTPαS)

This compound was prepared as described by Eckstein and Goody (8), with 0.78 mmole of TMPS and appropriate scaleup in conditions. Purifications was achieved by chromatography on a column (50 x 4.5 cm) of DEAE-Cellulose eluted at a flow rate of 1.3 ml/min with a linear gradient formed from 1.5 liters each of deionized water and 0.60 M triethylammonium bicarbonate. Fractions (20 ml) were collected. The 0.53-0.60 M fraction yielded 0.29 mmole of the thymidine 5'-O-(1-thio)triphosphate triethylammonium salt.

Separation of the Diastereomers of Thymidine 5'-O-(1-thio)triphosphate

The thymidine 5'-O-(1-thio)triphosphate triethylammonium salt (0.29 mmole) obtained from the above was dissolved in 2 ml of deionized water. The solution was chromatographed on a column (108 x 2.5 cm) of DEAE-Sephadex A-25 employing a linear gradient formed from 2.0 liters each of deionized water and 1.0 M triethylammonium bicarbonate (pH 7.5). The elution was carried out at 4°C, at a flow rate of 0.67 ml/min and 10 ml fractions collected. The 0.85-0.87 M buffer fraction yielded 81 µmole of the Sp form at ca. 90% isomeric purity. The 0.88-0.90 M buffer fraction afforded 86 µmole of the Rp form at ca. 90% isomeric purity. A second chromatography of the separated diastereomers yielded the pure isomers.

Deoxycytidine 5'-O-(1-thio)triphosphate (dCTPαS)

This compound was prepared as described by Eckstein and Goody (8), employing 1.3 mmole of dCMPS (85% of the 5'-isomer and 15% of the 3'-isomer) and an appropriate scale-up in conditions. Purification was by chromatography over a column (49 x 4.5 cm) of DEAE-Cellulose with a linear gradient composed of 2.0 liters each of deionized water and 1.0 M triethylammonium bicarbonate (pH 7.5). The elution was carried out at a flow rate of 1.3 ml/min and 20 ml fractions collected. The 0.60-0.68 M buffer fraction yielded 0.28 mmole of a mixture of the triethylammonium salts of deoxycytidine 5'-O-(1-thio) triphosphate (85%) and deoxycytidine 3'-O-(1-thio)triphosphate (15%).

Separation of the Diastereomers of Deoxycytidine 5'-O-(1-thio)triphosphate

The 5'-,3'-deoxycytidine α-thiotriphosphates (0.28 mmole) obtained from the above were dissolved in 2 ml of deionized water. The solution was chromatographed on a column (105 x 2.5 cm) of DEAE-Sephadex with a linear gradient composed of 2.0 liters each of deionized water and 1.0 M triethylammonium bicarbonate (pH 7.5). The elution was performed at 4°C, at 0.64 ml/min and 10 ml fractions collected. The 0.74-0.76 M buffer fraction yielded 60 µmole of the Sp form of deoxycytidine 5'-O-(1-thio)triphosphate at about 85% isomeric purity. The 0.77-0.79 M buffer fraction afforded 63 µmole of the Rp

form at ca. 85% isomeric purity. The pure isomers were obtained by rechromatography of the separated diastereomers.

Deoxyguanosine 5'-O-(1-thio)triphosphate

The deoxyguanosine 5'-O-thiomonophosphate triethylammonium salt (1.5 mmole) was converted to the tri-n-octylammonium salt exactly as for deoxyadenosine 5'-phosphorothioate. After the methanol was removed by evaporation, the residue was further dried at 30°C/0.05 mm Hg for 2 hours, and then evaporated with four 20 ml portions of anhydrous dioxane (warmed at 45°C to increase its solubility; the oily residue remained mainly undissolved). Anhydrous dioxane (8 ml) was added to the residue, and the mixture was stirred at room temperature for 30 minutes. Diphenyl phosphorothloridate (0.45 ml; 2.2 mmole) and dry tri-n-butylamine (0.75 ml; 3 mmole) were then added to the mixture. In about 1 hour, the mixture became clear. The reaction mixture was stirred at room temperature for another 2 hours. After removal of the solvent by evaporation, the oily residue was washed with three 20 ml portions of anhydrous ether (ether:petroleum ether, 1:3) and evaporated to dryness.

Tetrasodium pyrophosphate, converted to the tri-n-butylammonium salt as above, was dissolved in anhydrous pyridine (9 ml) and added to the activated deoxyguanosine 5'-phosphorothioate. The reaction mixture was stirred at room temperature for 2 hours. The rest of the dGTPαS isolation was identical to that described for dATPαS. Purification was by chromatography over a column (53 x 4.5 cm) of DEAE-Cellulose employing a linear gradient composed of 2.0 liters each of deionized water and 1.0 M triethylammonium bicarbonate (pH 7.5) at a flow rate of 1.3 ml/min and collecting 20 ml fractions. The 0.53-0.80 M buffer fraction was pooled and the excess triethylamine was removed by repeated evaporation with deionized water. The residue was dissolved in 15 ml of deionized water and rechromatographed. The 0.50-0.62 M buffer fraction afforded 0.35 mmole of a mixture of the triethylammonium salts of deoxyguanosine 5'-O-thiomonophosphate (ca. 50%) and 5'-O-(1-thio)triphosphate (ca. 50%). The 0.62-0.75 M buffer fraction yielded 0.78 mmole of the deoxyguanosine 5'-O-(1-thio)triphosphate triethylammonium salt.

Separation of the Diastereomers of Deoxyguanosine 5'-O-(1-thio)triphosphate

The deoxyguanosine 5'-O-(1-thio)triphosphate triethylammonium salt (0.30 mmole) obtained from the above was dissolved in 2 ml of 0.30 M triethylammonium bicarbonate (pH 7.5). The resulting solution was chromatographed on a column (105 x 2.5 cm) of DEAE-Sephadex A-25, which has been preequilibrated with 0.30 M triethylammonium bicarbonate (pH 7.5). Elution was carried out at 4°C, at a flow rate of 0.36 ml/min with a linear gradient composed of 2.0 liters each of 0.30 M and 1.0 M triethylammonium bicarbonate

(pH 7.5) and 10 ml fractions collected. The 0.82-0.84 M buffer fraction yielded 82 μmole of the Sp form at 90% isomeric purity. The 0.87-0.90 M fraction afforded 85 μmole of the Rp form at about 90% isomeric purity. The pure isomers were obtained by rechromatography of the separated diastereomers.

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