Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 150-155

A SIMPLIFIED PROCEDURE FOR SYNTHESIZING NUCLEOSIDE 1-THIOTRIPHOSPHATES: $dATP_{\alpha}S$, $dGTP_{\alpha}S$, $UTP_{\alpha}S$, AND $dTTP_{\alpha}S$

Abolfazl Arabshahi and Perry A. Frey*

Institute for Enzyme Research, The Graduate School, and Department of Biochemistry,
College of Agricultural and Life Sciences, University of Wisconsin-Madison,
Madison, Wisconsin 53705

Received August 8, 1994

SUMMARY. A procedure for synthesizing a nucleoside 1-thiotriphosphate in a single reaction vessel beginning with the nucleoside, $PSCl_3$, and PP_i is described. Reaction of the dried nucleoside with $PSCl_3$ in anhydrous triethylphosphate is followed by addition of the tetrabutylammonium salt of PP_i. Addition of excess triethylamine precipitates the nucleotides. The crude product is dissolved in water, and the nucleoside 1-thiotriphosphate is purified in 24% to 50% yield by chromatography. The method is applied to the synthesis of $dATP_{\alpha}S$, $dGTP_{\alpha}S$, $UTP_{\alpha}S$ and $dTTP_{\alpha}S$.

Nucleoside 1-thiotriphosphates are valuable nucleotide analogues that are used in studies of the mechanisms of action phosphotransferases and nucleotidyltransferases, in studies of allosteric regulation by nucleotides, in studies of signal transduction, in studies of nucleic acid processing, and in nucleotide sequencing of nucleic acids (1, 2). These compounds can be synthesized by a number of routes that have been described in the literature that are multistep methods entailing the synthesis of intermediates that are later converted into the final products or that require the use of blocked nucleosides. We describe here a method that is generally applicable to the synthesis of nucleoside 1-thiotriphosphates in a "one pot" procedure that can be carried out in a single day starting with the nucleoside, thiophosphoryl trichloride, and PP_i. The syntheses of two purine nucleoside and two pyrimidine nucleoside 1-thiotriphosphates are described in detail.

METHODS

Materials—The following chemicals were purchased from Sigma Chemical Co.: thymidine, cytidine, 2' deoxyadenosine, 2' deoxyguanosine, SP-Sephadex and DEAE-Sephadex A-25. Uridine, D_2O, collidine, triethyl phosphate, tri-n-butylamine, tri-n-octylamine, thiophosphoryl trichloride, pyridine, calcium hydride, and 4-Å molecular sieves were purchased from Aldrich Chemical Co. Sodium pyrophosphate, triethylamine, and 1-propanol were purchased from Fisher Scientific, and TLC plates from Whatman Co.

Purification of solvents—Triethyl phosphate was mixed with calcium hydride, stirred overnight, and distilled *in vacuo* into a clean, dry flask after discarding the first 20%. It was stored in a sealed flask over 4-Å molecular sieves in the dark. Tri-n-octylamine was distilled *in*

*To whom correspondence should be addressed. Fax:(608)265-2904.

0006-291X/94 $5.00

vacuo and stored in a sealed container inside a desiccator over P_2O_5. Collidine and thiophosphoryl trichloride were distilled and stored prior to use. Tri-n-butylamine was stirred with calcium hydride for 20 h, redistilled under reduced pressure and stored under N_2 at 5 °C.

Chromatography—The progress of the synthesis reactions was monitored by thin layer chromatography using Whatman flexible silica gel plates containing a fluorescent indicator and a mobile phase consisting of 6:3:1 n-propanol : ammonium hydroxide : water. Nucleotides were purified by anion exchange chromatography. A column of DEAE-sephadex A-25 (1.5 x 30 cm) in the bicarbonate form was prepared. After application of the nucleotide sample, the column was eluted by a 1.5 L linear gradient of triethylammonium bicarbonate at pH 7.6 increasing in concentration from 50 mM to 1M. Fractions were collected, and those containing nucleotides were identified by measurements of A_{260}. Peak fractions were pooled and the buffer salts removed by rotary evaporation in *vacuo*.

Phosphate assay—Pyrophosphate was assayed by enzymatic hydrolysis to P_i utilizing inorganic pyrophosphatase, followed by determination of P_i at 578 nm after addition of a solution of ammonium molybdate, sulfuric acid, and reducing reagents. Organic phosphate in synthetic nucleotides was measured by ashing the samples and measuring P_i by the malachite green method (3). Ashing was carried out on the sample in a test tube with magnesium nitrate solution by swirling the solution over an open flame, followed by addition of HCl and heating at 100 °C for 15 min. After cooling to room temperature and neutralizing with NaOH, a solution of ammonium molybdate and malachite green in HCl was added and the A_{660} measured.

Preparation of tetra(tri-n-butylammonium)pyrophosphate—A column of SP-Sephadex (3.5 x 48cm) was prepared in the pyridinium form. A solution of 0.1 M $Na_4P_2O_7$ (16 ml) was loaded on the column, eluted with water, and PP_i in the effluent was identified by enzymatic assay. Fractions containing PP_i were pooled and divided into four equal portions. Four moles of freshly redistilled tri-n-butytlamine per mole of PP_i were added to each portion. These mixtures were immediately evaporated to dryness by rotary evaporation *in vacuo*, depositing tetra(tri-n-butylammonium)-PP_i as a thin film on the wall of the evaporation flask. To remove traces of water, the residue was dried *in vacuo* in a desiccator over P_2O_5 for 48 h at 25 °C.

UV-Vis spectrophotometry—Spectrophotometric measurements were made using a Hewlett Packard 8452A diode array spectrophotometer.

^{31}P NMR—^{31}P-NMR analysis was carried out on a Bruker 500 MHz spectrometer at 25 °C, field frequency locked on the resonance of deuterium in the solvent. ^{31}P NMR spectra of nucleotides were obtained on 0.5 ml samples consisting of 2mM to 5 mM nucleotide in a solution of 50 mM EDTA at pH 9.2 in 40% D_2O. Samples were contained in 5 mm Wildmad or Aldrich NMR tubes. All chemical shifts were related to that of 85% H_3PO_4 (external reference).

Synthesis of dGTP$_\alpha$S—2'-Deoxyguanosine was dried over P_2O_5 *in vacuo* at 110 °C overnight, and 0.4 mmol was dissolved in 2 ml anhydrous triethyl phosphate in a dry 25 ml reaction flask. To dissolve the deoxyadenosine it was necessary to heat the flask carefully over an open flame until the suspension became clear. Tri-n-octylamine (0.41 mmol) was added and the solution cooled to 0 °C. Thiophosphoryl trichloride (0.44 mmol) was added and the mixture stirred at 4 °C for 60 min. Tetra(tri-n-butylammonium)-PP_i (0.41 mmol), which had been dissolved in 3.7 ml of anhydrous triethylphosphate, was added to the reaction flask at 25 °C. After stirring the solution at 25 °C for 30 min, 15 mmols of anhydrous triethylamine was added to precipitate the phosphates as a white solid. The solvent was filtered and the crude product dissolved in 30 ml of water. If the pH was below 7 additional triethylamine was added, but the solution was generally alkaline. The product was purified by ion exchange chromatography, and dGTP$_\alpha$S emerged as a peak of A_{260} at 0.5 M triethylammonium bicarbonate in a yield of 26.5%. ^{31}P-NMR: two doublets, 43.88 ppm and 44.22 ppm, P_α (R_P- and S_P-epimers); doublet of doublets, –21.46 ppm, P_β; doublet at –4.69 ppm, P_γ; coupling constants $J_{\alpha\beta}$ = 27.47 Hz and $J_{\beta\gamma}$ = 20.24 Hz. UV spectrum: typical of guanine nucleotides, maximum at 250 nm and shoulder at 275 nm, A_{250}/A_{260} = 1.11 and A_{280}/A_{260} = 0.67. Mole ratio organic phosphate/guanine was 3.0 and R_f for TLC 0.06.

Synthesis of dATP$_\alpha$S—2'-Deoxyadenosine was dried over P_2O_5 *in vacuo* at 110 °C overnight, and 0.2 mmol was dissolved in a 0.75 ml anhydrous triethyl phosphate in a 25 ml reaction flask. The flask was heated carefully over a flame until the suspension became clear. Tri-n-octylamine (0.21mmol) was added to the clear solution before cooling it to 4 °C. Thiophosphoryl trichloride (0.2 mmol) was added to the cold solution and the reaction mixture was stirred at 4 °C for 60 min. Tetra(tri-n-butylammonium)-PP_i (0.21 mmol) which had been dissolved in 1.9 ml of anhydrous triethyl phosphate was added to reaction mixture at 25 °C. After stirring for 30 min,

7.5 mmols of anhydrous triethylamine was added to precipitate the phosphates. The solvent was filtered and the crude product dissolved in 15 ml of water. If the pH was less than 7 additional triethylamine was added, but the solution was generally alkaline. The aqueous solution of product was purified by ion exchange chromatography. $dATP_{\alpha}S$ emerged as a major peak at 0.58 M triethylammonium bicarbonate, yielding 42% product. ^{31}P-NMR: two doublets at 43.75 ppm and 44.13 ppm, P_{α} (S_P and R_P epimers); doublet of doublets at –21.53 ppm, P_{β}; doublet at -4.80 ppm, Pγ; coupling constants $J_{\alpha\beta}$ = 28.3 Hz and $J_{\beta\gamma}$= 20.17 Hz. These NMR results are in agreement with the values reported by Sheu and Frey (1977). UV spectrum: typical of adenine nucleotides, A_{250}/A_{260} = 0.83 and A_{280}/A_{260} = 0.33. Mole ratio organic phosphate/adenine was 3.0 and R_f for TLC 0.08.

Synthesis of $UTP_{\alpha}S$—Uridine was dried over P_2O_5 *in vacuo* at 110 °C overnight, and 0.2 mmol was dissolved in a 0.3 ml anhydrous triethyl phosphate in a clean dry flask. To dissolve the uridine it was necessary to heat the flask carefully with a heat-gun until the suspension became clear. Thiophosphoryl trichloride (0.5 mmol) was added to the solution which had been cooled to 4 °C. Collidine (0.2 mmol) was added to the reaction mixture and this created some white precipitate. The reaction mixture was warmed to 25 °C and stirred for 60 min and then an additional 60 min under a stream of N_2 to remove some of the excess $PSCl_3$ in the reaction mixture. Tetra(tri-n-butylammonium)PP_i (0.4 mmol) which had been dissolved in 2.5 ml of anhydrous triethyl phosphate was added to this reaction flask. In this stage very carefully the reaction flask was warmed by a flow of warm air from a heat-gun. After 30 min 7.5 mmols of anhydrous triethylamine was added to precipitate the phosphates,the solvent was filtered, and the crude product dissolved in 15 ml of water. If the pH was below 7 additional triethylamine was added, but the solution was generally alkaline. The product was purified by ion exchange chromatography, and $UTP_{\alpha}S$ emerged at 0.5 M triethylammonium bicarbonate, yielding 27% product. 31P-NMR: two doublet at 43.98 ppm and 43.39 ppm, P (S_P and R_P epimers), doublet of doublets at –21.73 ppm, P_{β}; doublet at –5.03 ppm, P_{γ}; coupling constants $J_{\alpha\beta}$ = 28.9 Hz and $J_{\beta\gamma}$ = 21.9 Hz. These NMR results are in agreement with literature values (4). UV spectrum: typical of uridine nucleotides, A_{250}/A_{260} = 0.73 and A_{280}/A_{260} = 0.42. Mole ratio organic phosphate/uridine was 3.0 and R_f for TLC 0.05.

Synthesis of $dTTP_{\alpha}S$—Thymidine was dried over P_2O_5 *in vacuo* at 110 °C overnight, and 0.1 mmol was dissolved in a 0.3 ml anhydrous triethyl phosphate in a clean dry flask. To dissolve the thymidine it was necessary to heat the flask carefully with a heat-gun until the suspension became clear. Thiophosphoryl trichloride (0.4 mmol) was added to the solution, which had been cooled to 4 °C. Collidine (0.15 mmol) was added to the reaction mixture and produced a small precipitate. The mixture was stirred for 60 min at 25 °C and then for another 60 min under flowing N_2. Tetra(tri-n-butylammonium)PP_i (0.4 mmol) dissolved in 2.5 ml of anhydrous triethyl phosphate was added to the flask, which was then carefully warmed with a stream from the heat-gun. After 30 min, 5 mmol of anhydrous triethylamine was added to precipitate the phosphates, the solvent was filtered and crude product dissolved in 7.5 ml of water. The pH was generally above 7 but was adjusted by addition of triethylamine when necessary. The product was purified by ion exchange chromatography, and $dTTP_{\alpha}S$ emerged at 0.55 M triethylammonium bicarbonate in 26% yield. ^{31}P-NMR; two doublets at 43.93 ppm and 43.34 ppm, P_{α} (S_P and R_P epimers); doublet of doublets at –21.76 ppm, P_{β}; doublet at –5.08 ppm, P_{γ}; coupling constants $J_{\alpha\beta}$ = 28.73 Hz and $J_{\beta\gamma}$ = 21.46 Hz. UV spectrum: typical of thymidine nucleotides, A_{250}/A_{260} = 0.66 and A_{280}/A_{260} = 0.80. Mole ratio organic phosphate/uridine was 3.0 and R_f for TLC 0.07.

RESULTS

The procedure for synthesizing nucleoside 1-thiotriphosphates described in METHODS for $dATP_{\alpha}S$, $dGTP_{\alpha}S$, $UTP_{\alpha}S$, and $dTTP_{\alpha}S$ is carried out in one operation within three hours in a single reaction vessel beginning with a nucleoside, thiophosphoryl trichloride, and PP_i. The product is purified by anion exchange chromatography in an overall yield of 25% to 50%, depending on the nucleoside. Yields as high as 60% have been attained in this laboratory in the synthesis of $ATP_{\alpha}S$. The elution profile for the purification of $dATP_{\alpha}S$ is shown in Figure 1.

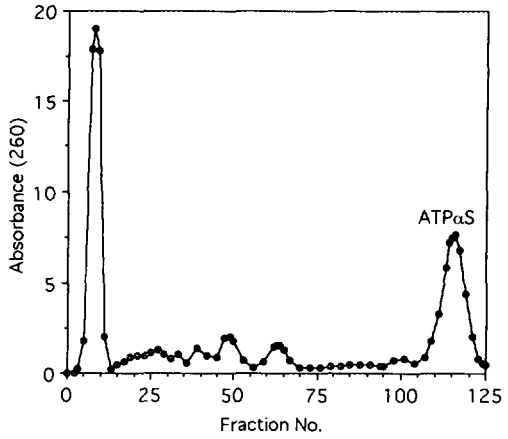
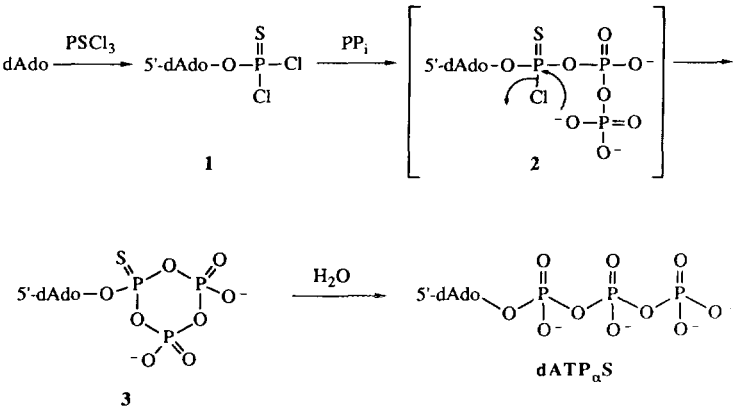


Figure 1. Chromatographic purification of $dATP_{\alpha}S$. The procedure is described in **METHODS**.

The chemical route for $dATP_{\alpha}S$ is illustrated in Scheme I. Reaction of 2'-deoxyadenosine with thiophosphoryl trichloride in triethylphosphate leads to regioselective reaction of the 5'-hydroxyl group, to produce 2'-deoxyadenosine-5'-thiophosphorodichloridate **1**. This reacts *in situ* with PP_i, which is added as its tetra-(tri-n-butylammonium salt, to form initially 2'-deoxyadenosine-5'-1-chloro-1-thiotriphosphate **2**. The latter intermediate quickly reacts internally in a displacement of the 1-chloro substituent by the P_{γ}-phosphate group, to produce 2'-deoxyadenosine-5'-1-thio-*cyclo*-triphosphate **3** as its tri-n-butylammonium salt. This is precipitated by the addition of excess triethylamine, which by amine exchange forms the insoluble triethylammonium salt. Upon being dissolved in water, the 1-thio-*cyclo*-triphosphate undergoes hydrolysis to 2'-deoxyadenosine-5'-1-thiotriphosphate, which is purified by chromatography.

The nucleoside 1-thiotriphosphates are produced as mixtures of P_{α}-epimers (S_P + R_P) as shown by the ^{31}P NMR spectra (5). For some applications the mixture of epimers is satisfactory. When pure epimers are required, they can be separated by reverse phase high performance liquid



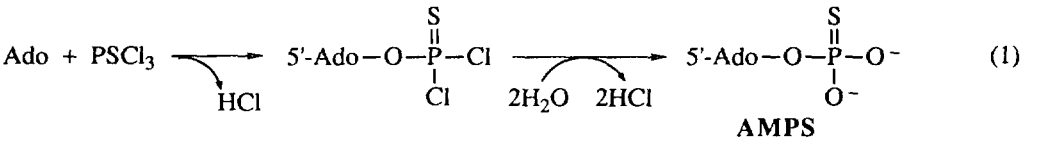
Scheme I

chromatography (6). Micromole amounts can easily be separated into pure epimers by use of a semipreparative reverse phase column equilibrated and eluted with 50 mM KP_i buffer at pH 6.0.

The conditions for the first step in the syntheses of nucleoside 1-thiotriphosphates differ significantly. Purine nucleosides react with thiophosphoryl trichloride at 4 °C, whereas pyrimidine nucleosides react sluggishly at low temperatures in the first step. Therefore, the reaction of pyrimidine nucleosides is carried out at a higher temperature, for a longer time, and in the presence of added collidine. Guanine and adenine nucleosides react satisfactorily at 4 °C, and tri-n-octylamine is added in the reactions of both nucleosides to absorb the HCl produced in the first step. Adenine nucleosides react slowly in the absence of added tertiary amines, but faint cloudiness is caused by the formation of HCl, which reduces their solubility in triethyl phosphate.

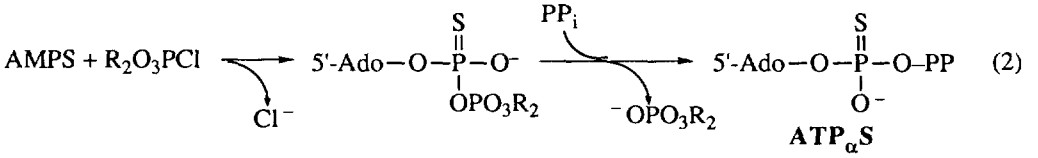
DISCUSSION

The present procedure for the synthesis of nucleoside-5'-1-thiotriphosphates was inspired by the analogous method introduced by Ho and Frey for the synthesis of $ATP_{\beta}S$ (7). Other methods for nucleoside 1-thiotriphosphates are multistep procedures that entail the isolation of intermediates or the use of blocked reactants that have to be deblocked (5, 8-10). The one most closely related to the present method begins with thiophosphorylation of a nucleoside by thiophosphoryl trichloride (5, 8), as for adenosine in eq. 1.



(1)

The adenosine-5'-thiophosphorodichloridate formed initially is hydrolyzed to AMPS and purified.In a second operation, AMPS is activated by reaction with a dialkyl- or diarylphosporochloridate such as diphenylphosphorochloridate in eq. 2. The activation product is



(2)

then converted to $ATP_{\alpha}S$ by reaction with PP_i. The present method avoids the hydrolysis of adenosine-5'-thiophosphorodichloridate, the isolation of AMPS, and the reactivation of AMPS for coupling with PP_i.

An alternative method that entails the isolation of intermediates and separate reaction steps utilizes phosphite chemistry (9). Adenosine-5'-phosphite is synthesized, sulfurized to AMPS, and coupled with PP_i to form $ATP_{\alpha}S$. Another synthetic route by way of phosphite intermediates is a one pot procedure that requires the use of blocked nucleosides (10).

ACKNOWLEDGMENTS

This research was supported by Grant No. GM30480 from the National Institute for General Medical Sciences. This study made use of the National Magnetic Resonance Facility at Madison, which is supported by NIH grant RR02301 from the Biomedical Research Technology Program, National Center for Research Resources. Equipment in the facility was purchased with funds from the University of Wisconsin, the NSF Biological Instrumentation Program (grant DMB-8415048), NIH Biomedical Research Technology Program (grant RR02301), NIH Shared Instrumentation Program (grant RR02781), and the U.S. Department of Agriculture.

REFERENCES

(1) Frey, P.A. (1989)*Advances in Enzymology and Related Topics in Molecular Biology* **62**, 119-201.
(2) Eckstein, F. (1985?) *Ann. Rev. Biochem.* **54**, 367-402.
(3) Goody, R. S., and Eckstein, F. (1971) *J. Am. Chem. Soc.* **93**, 6252-6257.
(4) Sheu, K.-F. R. and Frey, P. A. (1978) *J. Biol. Chem.* **253**, 3378-3380.
(5) Sheu, K.-F. R., and Frey, P.A. (1977) *J. Biol. Chem.* **252**, 4445-4448.
(6) Sammons, R. D., and Frey, P. A. (1982) *J. Biol. Chem.* **257**, 1138-1141.
(7) Ho, H.-T., and Frey, P.A. (1984) *Biochemistry* **23**, 1978-1983. (U. S. Pat. 4728730)
(8) Eckstein, F., and Goody, R. S. (1976) *Biochemistry* 15, 1685-1691.
(9) Chen, J. T., and Benkovic, S. J. (1983) *Nucleic Acids Res.* **11**, 3737-3751.
(10) Ludwig, J., and Eckstein, F. (1989) *J. Org. Chem.*, 631-635.