JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Labelled Cpd Radiopharm 2002; **45**: 19–28.
DOI: 10.1002/jlcr.531

Research Article

Synthesis of deoxyadenosine-5′-(^{35}S)-thiomonophosphate [dAMP(^{35}S)] of high specific activity – a key intermediate for the synthesis of Sp-deoxyadenosine-5′-(α-^{35}S)-thiotriphosphate [Sp-dATP (α-^{35}S)]

K. M. Mathew*, S. Ravi†, C. V. Sontakke and H. Chander‡
LC/ILCJP, Board of Radiation and Isotope Technology, BARC Vashi Complex, Navi Mumbai 400 705, India

Summary

Unprotected deoxyadenosine **1** was treated with an excess of phosphorus acid and stoichiometric proportions of *N*, *N*′-di-*p*-tolylcarbodiimide in anhydrous pyridine to give deoxyadenosine-5′-monophosphite **2**. The latter was activated with trimethylsilyl chloride followed by sulphurisation with elemental ^{35}S (specific activity > 1000 Ci/mmol) in toluene solution to give deoxyadenosine-5′-(^{35}S)-thiomonophosphate [dAMP(^{35}S)] **3**. Enzymatic conversion of deoxyadenosine-5′-(^{35}S)-thiomonophosphate to Sp-deoxyadenosine-5′-(α-^{35}S)-thiotriphosphate [Sp-dATP (α-^{35}S)] **5** was carried out following a standard reaction protocol. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: deoxyadenosine; deoxyadenosine-5′-monophosphite; sulphurisation deoxyadenosine-5′-(^{35}S)-thiomonophosphate [dAMP(^{35}S)]; deoxynucleotide-5′-thiomonophosphate (dNMPS); Sp-deoxyadenosine-5′-(α-^{35}S)-thiotriphosphate [Sp-dATP (α-^{35}S)]; deoxynucleotide-5′-thiotriphosphate (dNTPS)

*Correspondence to: K.M. Mathew, LC/ILCJP, Board of Radiation and Isotope Technology, BARC Vashi Complex, Navi Mumbai 400 705, India. E-mail: mathew@apsara.barc.ernet.in
† E-mail: ravisesh@apsara.barc.ernet.in
‡ E-mail: harishch@magnum.barc.ernet.in

Received 25 June 2001
Revised 13 September 2001
Accepted 3 October 2001

Introduction

Deoxyadenosine-5′-(α-^{35}S)-thiotriphosphate [dATP (α-^{35}S)] is an analogue of deoxyadenosine-5′-triphosphate (dATP) in which a non-bridging oxygen atom of α-phosphorus is replaced by a sulphur atom. Such a substitution leads to an additional centre of chirality into the nucleotide and thus to the existence of a pair of diastereomers. This facilitates determination of the stereochemical course of an enzymatic reaction and its importance can be seen from the large number of review articles dealing with this subject.[1–9] These phosphorothioates are also stable towards hydrolytic cleavage relative to the corresponding phosphates.[9] It has been reported.[10–16] that the Sp-diastereomer of dATP(α-S) is polymerised by DNA polymerase in the presence of a primer template to produce DNA chain containing phosphorothioate internucleotide linkages. The kinetic constants for the polymerisation of Sp-dATP (α-S) are similar to those for dATP, while the Rp-diastereomer is not a substrate for these enzymes. Incorporation of deoxyadenosine-5′-(α-^{35}S)-thiotriphosphate [dATP (α-^{35}S)], instead of (α-^{32}P)dATP, as the radioactive label in dideoxynucleotide sequence reactions has three apparent advantages. (i) The major one is the very sharp definition of bands on a sequencing gel autoradiography and thus increased resolution, which is due to the short path length of β particles emitted by ^{35}S. The strong emission of ^{32}P gives diffuse and broad bands. (ii) The weak emission of ^{35}S reduces the radiation dose received during handling of the labelled material, and (iii) the reaction products can be stored at -20°C for periods of 1 week or more before use in electrophoresis with minimal damage to the labelled DNA molecules. Whereas decay of an incorporated ^{32}P atom will break the sugar-phosphate backbone, this does not usually seem to be the case during decay of an incorporated ^{35}S atom.

Deoxynucleotide-5′-thiomonophosphate (dNMPS) has been prepared by hydrolysis of the products obtained from the reaction of unprotected deoxynucleosides with thiophosphoryl chloride in triethyl phosphate. This procedure is not attractive for the ^{35}S-deoxynucleotide-5′-thiotriphosphate (^{35}S-dNTPS) because of limited availability if high specific active (^{35}S)-$PSCl_3$ and low yield in the thiophosphorylation steps.[17–20] The reaction of 2-chloro-4H-1,3,2-benodioxaphosphorin-4-one (salicylphosphorochloridite) with the 5′-hydroxy group of the nucleoside to give an intermediate, which on subsequent reaction with pyrophosphate followed by oxidation with sulphur and hydrolysis to

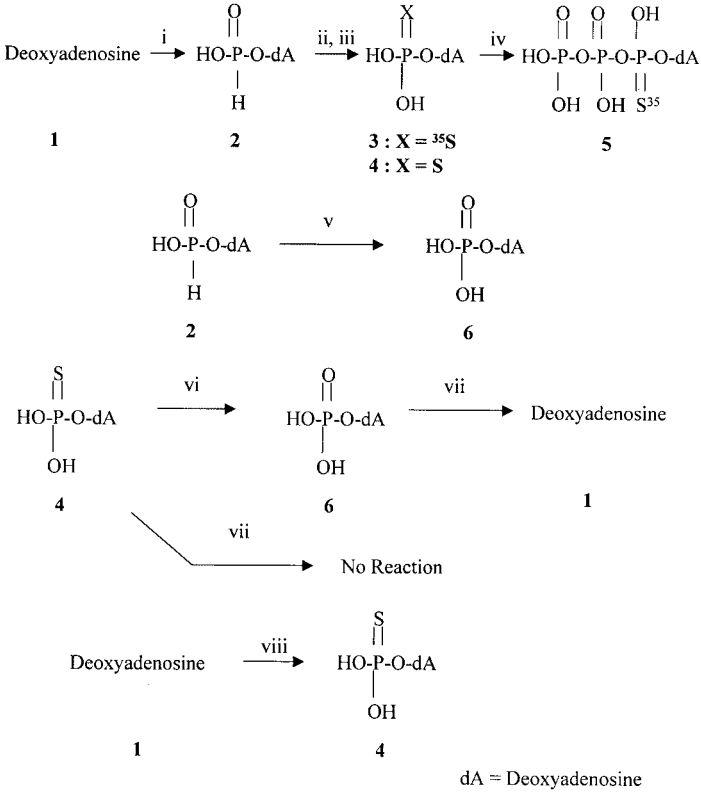
give a diastereomeric mixture of dNTPS, has been reported by Ludwig *et al.*[21] The procedure reported by Chen *et al.*[22] involves sulphurisation of the trimethylsilyl derivative of deoxyadenosine monophosphite with an excess of pulverised sulphur. This procedure as such is not suitable for a radioactive preparation, because of (i) potential loss of radioactive sulphur while pulverisation, (ii) twice the amount of sulphur has to be used in the reaction and (iii) the scale of operation is in millimoles, and the yield of reaction product may be drastically low when the reaction is carried out at nanomole levels.

Therefore, we have modified this approach for the radioactive preparation of deoxyadenosine-5′-(^{35}S)-thiomonophosphate [dAMP(^{35}S)] **3** of high specific activity (> 1000 Ci/mmol) – an intermediate for the synthesis of Sp-dATP(α-^{35}S) **5**. The salient features of our synthesis are (a) ^{35}S elemental sulphur was used in toluene thereby avoiding the loss of sulphur due to pulverisation. This also reduces the radiolytic decomposition of radioactive sulphur and (b) deoxyadenosine-5′-monophosphite was used in excess compared to radioactive elemental sulphur to give a high yield (85%) of dAMP(^{35}S).

Deoxyadenosine-5′-monophosphite **2** was prepared by condensing phosphorus acid with deoxyadenosine **1** in the presence of *N*,*N*′-di-*p*-tolylcarbodiimide.[22] The formation of deoxyadenosine-5′-monophosphite **2** was confirmed by oxidising it to deoxyadenosine-5′-monophoshpate **6** using $KmnO_4$. The reaction products were analysed by HPLC. Attempts were made to optimise the yield of **3** by increasing the reaction volume by addition of various solvents such as pyridine, triethylamine, dimethylformamide, methylene chloride and toluene. The yield of dAMP(^{35}S) was 85% when toluene was used as a solvent whereas in other solvents the yield was in the range 5–10%.

Deoxyadenosine-5′-phosphite **2** was activated with trimethylsilyl chloride and excess of activated intermediate was allowed to react with [^{35}S] elemental sulphur in toluene. The reaction products were analysed by HPLC and TLC. The formation of dAMPS **4** was confirmed by desulphurisation[23] with 1-[3′-(dimethylamino)propyl]-3-ethylcarboiimide to give dAMP **6** which in turn, on enzymatic reaction with alkaline phosphatase, afforded deoxyadenosine **1**. The reaction products obtained were analysed by HPLC and TLC. It may be noted that dAMPS **4** is resistant to alkaline phosphatase and is not degraded under these conditions.[24] The reaction products were analysed by HPLC. The presence of dAMP(^{35}S) **3** was also further

confirmed by comparison with a cold specimen sample of dAMPS **4** obtained via the $PSCl_3$ route[20] and both the products were found to be identical. The required intermediate **3** was purified by column chromatography on DEAE Cellulose column and was enzymatically converted to Sp-dATP(α-^{35}S) **5** using a standard reaction protocol,[20] in 70% yield. The reaction products were analysed by TLC. The presence of compound **5** was also confirmed by incorporating it into a nucleotide chain by Nick Translation reaction[25] and comparison with a chain obtained from an authentic sample of **5** obtained from M/s Amersham Pharmacia Biotech. Both the samples showed identical features (Scheme 1).



i) Phosphorus acid, N,N'-di-p-tolylcarbodiimide , pyridine ii) trimethylsilyl chloride, triethylamine, pyridine, ^{35}S elemental sulphur in toluene / elmental sulphur in toluene iii) hydrolysis iv) ATP, phosphoenol pyruvate, pyruvate kinase, adenylate kinase v) $KMnO_4$ vi)1-[3'dimethylaminopropyl]3-ethylcarbodiimide vii) alkaline phosphatase viii) pyridine, triethyl phosphate, thiophosphoryl chloride

Scheme 1.

Experimental

Chemicals and methods

Deoxyadenosine, deoxyadenosine-5′-monophosphate, trimethylsilyl chloride, *N*,*N*′-di-*p*-tolylcarbodiimide, Tris-HCl, 1,4-dithiothreitol, phosphorus acid thiophosphoryl chloride were obtained from Aldrich. Adenylate kinase, phosphoenol pyruvate, pyruvate kinase, DEAE Cellulose, DEAE Sephadex A-25 and 1-[3′(dimethylaminopropyl)]-3-ethylcarbodiimide were obtained from Sigma. (^{35}S)Elemental sulphur (specific activity > 1000 Ci/mmol) and dATP(α-^{35}S) were obtained from M/s Amersham Pharmacia Biotech. (^{35}S)Elemental sulphur was purified by HPLC and used for the reaction. The solvents were freshly dried by standard procedures just prior to use. Thin layer chromatography was carried out on Merck silica gel plates containing fluorescent indicator with a solvent system: 1-propanol/ammonia/water, 6:3:1 (v/v/v). Nucleotides were visualised with UV light. Phosphorothioate nucleotides were visualised with UV light as well as by spraying with iodine azide-reagent.[26] Radioactive nucleotides were identified by autoradiography. A Shimadzu HPLC was used with a UV-Visible detector of LDC Milton Roy make.

Deoxyadenosine-5′-monophosphite **2**. To anhydrous deoxyadenosine **1** (260 mg, 1 mmol) dried at 100°C/0.5 mm Hg for 8 h was added anhydrous pyridine (10 ml) and phosphorus acid (120 mg, 14 mmol) and the mixture stirred for 10 min at room temperature. *N*,*N*′-di-*p*-tolylcarbodiimide (320 mg, 1.4 mmol) was added and stirring continued for 3 days at room temperature. Deionised water (50 ml) was added and again the solution stirred for 30 min. The reaction mixture was filtered to remove di-*p*-tolylurea and the filtrate was rotary evaporated to dryness under reduced pressure at 35°C. The residue was taken up in 1 M triethylammonium bicarbonate (pH 7.5) (8 ml) and rotary evaporated to dryness. Excess of triethylamine was removed by repeated co-evaporation with deionised water under reduced pressure. The residue left was then dissolved in deionised water (10 ml) and the solution chromatographed on a column (1 cm × 40 cm) of DEAE-cellulose. The column was eluted with a linear gradient formed from 2.5 l of deionised water and 2.0 l of 0.4 M triethylammonium bicarbonate (pH 7.5). The eluate was monitored for absorbance at 259 nm. Fractions were collected and analysed by HPLC. The results are presented in Table 1. The fractions corresponding to deoxyadeno-

Table 1. HPLC analysis of phosphites and 5′ phosphate

Compound	Retention time (min)
Deoxyadenosine-3′,5′-diphosphite	6.1
Deoxyadenosine-5′-monophosphate	7.0
Deoxyadenosine-5′-monophosphite	9.8
Deoxyadenosine	14.7

sine-5′-monophosphite were pooled. The yield was 65% (0.65 mmol) (0.19–0.21 M buffer fraction yielded the deoxyadenosine-5′-monophosphite whereas deoxyadenosine-3′, 5′-diphosphite was eluted with 0.28–0.3 M buffer).

The presence of deoxyadenosine-5′-monophosphite **2** was also confirmed by oxidising to deoxyadenosine-5′-monophosphate **6** followed by HPLC (Table 1).

Deoxyadenosine-5′-monophosphate **6**. Deoxyadenosine-5′-monophosphate **2** (32 mg) was dissolved in water (5 ml) containing sodium hydrogen carbonate (10 mg). The solution was cooled in ice and 0.155% aqueous $KMnO_4$ (5 ml) was added over the course of 45 min with stirring. The reaction mixture was filtered and was analysed by HPLC. Results are presented in Table 1.

Column: C-18 ODS (Shandon, 4 mm × 25 cm), mobile phase: 300 mM Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$: methanol::90:10 (v/v), detector UV-259 nm, flow rate 1.0 ml/min.

Purification [^{35}S] elemental sulphur. [^{35}S]Elemental sulphur was purified by HPLC using methanol/water 20:1 (v/v) as mobile phase on a column of C-18 ODS (Shandon, 4 mm × 25 cm) with a flow rate of 1 ml/min.[27] The column was monitored by a UV wavelength of 264 nm. [^{35}S] elemental sulphur (S_8, eluting at 9.7 min, was collected and was used for the reaction).

Deoxyadenosine-5′-((^{35}S)thiomonophosphate (dAMP^{35}S) **3**. Deoxyadenosine-5′-monophosphite triethylammonium salt (23.13 μmol) was repeatedly (four times) rotary evaporated with anhydrous pyridine (1 ml). Anhydrous pyridine (645 μl) was added to the residue followed by addition of anhydrous triethylamine (16.8 μl, 117.5 μmol) and trimethylsilyl chloride (15 μl, 117.5 μmol). The reaction mixture was stirred at room temperature for 45 min. The reaction mixture of 33 nmol (1 μl) was added to 5 mCi (5 nmol, 175 ng) of ^{35}S elemental sulphur in toluene (100 μl) and the whole stirred for 1 h. The reaction mixture was quenched with deionised water (1 ml). The sample was repeatedly rotary

Table 2. HPLC analysis of deoxyadenosine-5′-(^{35}S)thiomonophosphate

Compound	Retention time (min)
Deoxyadenosine-5′-thiomonophosphate	5.2
Deoxyadenosine-5′-(^{35}S)thiomonophosphate	5.2
Deoxyadenosine-5′-monophosphate	7.2
Deoxyadenosine-5′-monophosphite	9.3
Deoxyadenosine	14.2

evaporated with deionised water under reduced pressure to completely remove the pyridine. The residue was taken up in deionised water (1 ml) and loaded on a DEAE-Cellulose column (1 cm × 40 cm). Elution was carried out with a linear gradient formed of 2 l each of deionised water and 0.6 M triethylammonium bicarbonate buffer. The flow rate was kept at 1 ml/min. The column was monitored for both radioactivity and UV absorbance at 259 nm. Fractions were collected and analysed by HPLC (Table 2). The fraction corresponding to deoxyadenosine-5′-(^{35}S)-thiomonophosphate [dAMP(^{35}S)] was pooled, giving a yield of 80% (4 mCi, 4 nmol). Buffer fraction of 0.34–0.45 M yielded deoxyadenosine-5′-(^{35}S)-thiomonophosphate [dAMP(^{35}S)] **3**.

Formation of dAMP(^{35}S) was confirmed by preparing a cold sample of dAMPS 4 with 600 nmol of elemental sulphur using the above procedure.

Analysis of deoxyadenosine-5′-thiomonophosphate (dAMPS)

dAMPS in the reaction mixture was identified by using TLC (results presented in Table 4) and also by desulphurisation followed by reaction with alkaline phosphatase to give deoxyadenosine.

dAMPS **4** (100 nmol) in 0.5 M Tris–HCl buffer (pH = 7.5) (0.5 ml) was stirred with 1-[3′(dimethylaminopropyl]-3-ethylcarbodiimide at room temperature for 1 h. The dAMP formed was analysed by HPLC and TLC (Tables 2 and 3). To the reaction mixture, three units of alkaline phosphatase were added and stirred at room temperature. Under these conditions dAMP was converted to deoxyadenosine. The reaction mixture was analysed by HPLC and TLC and the results are presented in Tables 2 and 3.

Deoxyadenosine-5′-thiomonophosphate (dAMPS) **4**. To anhydrous deoxyadenosine **1** (2 mmol, 535 mg) dried at 100°C/0.5 mm Hg for 8 h was added anhydrous triethylphosphate (10 ml) and anhydrous pyridine (4.1 mmol, 330 µl) and the mixtures stirred at 100°C until almost all the

deoxyadenosine dissolved ($\sim$10 min). After the solution has been cooled in an ice bath, freshly distilled thiophosphoryl chloride (8 mmol, 800 µl) was added and the reaction mixture stirred at 0°C for 1 h. Unreacted thiophoshoryl chloride was then removed under pressure at 30°C. The residue left was again cooled in an ice bath and a solution of aqueous pyridine (8 mmol, 660 µl in 500 µl water) was added. The pH was then raised from 5 to 9 by addition of 1 M NaOH and the solution loaded on to a column (2.3 × 41 cm) of DEAE-Sephadex-A25. Elution was carried out at 4°C with a linear gradient of 1.5 l of 0.1 M triethylammonium bicarbonate buffer (pH 7.5) and 1.5 l of 0.4 M triethylammonium bicarbonate buffer (pH 7.5). The eluate was monitored for absorbance at 259 nm. Fractions were collected and analysed by HPLC and TLC (Tables 2 and 3). The fraction corresponding to deoxyadenosine-5′-thiomonophosphate (dAMPS) were pooled. The product was identical with that obtained in the previous synthesis.

Column: C-18 ODS (Shandon, 4 mm × 25 cm), mobile phase: 300 mM Tris–HCl (pH 8.0) containing 20 mM $MgCl_2$: methanol::90:10 (v/v), detector UV-259 nm, flow rate 1.0 ml/min.

Sp-Deoxyadenosine-5′-(α-^{35}S)thiotriphosphate [Sp-dATP (α-^{35}S)] **5**. A mixture of dAMP(^{35}S) **3** (3 nmol, 3 mCi), ATP (3 nmol), phosphoenol pyruvate (1 nmol), magnesium chloride (1 nmol), potassium chloride (60 nmol), Tris–HCl (pH 8.0) (42 nmol), 1,4-dithiothreitol (1 nmol), adenylate kinase (790 units/ml) and pyuravate kinase (35 units/ml) was stirred at room temperature. The progress of the reaction was monitored by TLC. The reaction mixture was chromatographed on a column (1 cm × 30 cm) of DEAE-Sephadex A-25 (bicarbonate form) and was eluted at 4°C with a linear gradient of 400 ml of 0.2 M

Table 3. Thin layer chromatography of deoxyadenosine-5′-thiomonophosphate

Compound	R_f Value
Deoxyadenosine	0.60
Deoxyadenosine-5′-thiomonophosphate	0.45
Deoxyadenosine-5′-monophosphate	0.19

Table 4. Thin layer chromatography of dAMP(^{35}S) and Sp-dATP[α-^{35}S]

Compound	R_f value
dAMP(^{35}S)	0.45
Sp-dATP[α-^{35}S]	0.12

triethylammonium bicarbonate buffer (pH 7.5) and 0.75 M triethylammonium bicarbonate buffer (pH 7.5). The eluant from the column was checked by TLC (the results are presented in Table 4). The fractions corresponding to Sp-dATP(α-^{35}S) were pooled, giving a yield of 70% (2.1 mCi).

The product **4** was incorporated into a nucleotide chain by Nick translation reaction and its performance compared with authentic dATP[α-^{35}S] obtained from M/s Amersham Pharmacia Biotech. Both the samples gave identical incorporation.

Acknowledgements

The authors wish to express their thanks to Dr N. Ramamoorthy, Chief Executive, BRIT, Dr N. Sivaprasad, General Manager, ILCJP, BRIT and Mr A.U. Garana, Deputy General Manger, LC/ILCJP, BRIT for their support and encouragement.

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