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Synthesis of S-Benzyl-[$^{18}O_3$]phosphorothioate and Adenosine 5'-[$^{18}O_3$]phosphate

Richard C. Bethell and Gordon Lowe*

The Dyson Perrins Laboratory, Oxford University, South Parks Road, Oxford OX1 3QY.

SUMMARY

Several synthetic routes to the two title compounds are presented. S-Benzyl-[$^{18}O_3$]phosphorothioate was synthesised in 68% yield from phosphorus trichloride. Adenosine 5'-[$^{18}O_3$]phosphate was synthesised in two steps from adenosine in 44% yield.

INTRODUCTION

During the course of work aimed at developing a new synthesis of adenosine 5'[γ(R)-^{17}O,^{18}O-thio]-triphosphate, S-benzyl phosphorothioate and adenosine 5'-phosphate were required which were efficiently labelled with ^{17}O or ^{18}O. Isotopically enriched water is the most suitable source of label and the most common approach to the synthesis of oxygen-labelled phosphates and phosphorothioates has made use of the hydrolysis of phosphorus halide bonds.

* To whom correspondence should be addressed

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RESULTS AND DISCUSSION

Initial attempts to synthesize S-benzyl-[$^{18}O_3$]phosphorothioate were based on the approach outlined in Scheme 1. Trisodium thiophosphate may be prepared in good yield from thiophosphoryl chloride by the method of Yasuda and Lambert (1) with the modification of Washburn and Hayes (2). The crude reaction product was converted to S-benzyl phosphorothioate (in 65% yield from thiophosphoryl chloride) by reaction with benzyl bromide under phase transfer conditions. However, this route is inefficient in the use of labelled water since 2 mmoles of thiophosphoryl chloride require approximately 3 g of water in order to maintain the sodium hydroxide in solution and the pH at 11 or above. The reaction is further complicated by the need to make up the sodium hydroxide solution from sodium and the labelled water. In order to control the reaction, and in an attempt to reduce the quantity of water required, sodium hydroxide in aqueous tetrahydrofuran was investigated. This, however, led to a reduction in the yield of trisodium thiophosphate.

$$PSCl_3 \xrightarrow{i} Na_3SP^{18}O_3 \xrightarrow{ii} PhCH_2.SP^{18}O_3$$

Scheme 1. Reagents i $Na^{18}OH$, $H_2{}^{18}O$

ii $PhCH_2Br, Bu_4N^+Cl^-, H_2O, CH_2Cl_2$

In pursuing alternative strategies by which label might be more efficiently incorporated into S-benzyl phosphorothioate, attention turned to the work of Hata and Sekine (3) who had demonstrated that phosphorous acid, and its monoesters, could be converted easily and efficiently into thiophosphate or S-alkyl thiophosphates by way of trimethylsilyl protected intermediates. Phosphorous acid may be prepared in labelled form by the hydrolysis of phosphorus trichloride with a slight excess of labelled water, although care must be taken to prevent acid-catalysed exchange with moisture. In practice this presents no problem because on completion of the reaction the labelled phosphorous acid is taken up in pyridine ready for the next stage. One method of synthesising S-benzyl phosphorothioate

would be to convert tris(trimethylsilyl)-phosphite (or its equivalent) to phosphorothioate with elemental sulphur and then react this with benzyl bromide under phase transfer conditions. However, a much more direct method is to treat tris(trimethylsilyl)phosphite with bis-benzyl disulphide, thereby producing S-benzyl phosphorothioate directly. This is outlined in Scheme 2.

$$PCl_3 \xrightarrow{i} H_3P^{18}O_3 \xrightarrow{ii} (Me_3Si^{18}O)_3P \xrightarrow{iii} PhCH_2.SP^{18}O_3$$

Scheme 2. Reagents i $H_2{}^{18}O$

ii Me_3SiCl, Et_3N

iii(a) $(PhCH_2.S)_2$, (b) aqueous NaOH

This method requires only 1.5 equivalents of water to ensure complete hydrolysis of the phosphorus trichloride and the ^{31}P n.m.r. spectrum of the crude S-benzyl phosphorothioate showed that the conversion from phosphorus trichloride was quantitative. Slow hydrolysis during purification by ion-exchange chromatography, even when the buffers are well above pH8, however, does lead to some loss of material. S-o-nitrobenzyl- and S-p-nitrobenzyl phosphorothioate have also been synthesized by this method. The method is probably general for the synthesis of S-alkyl and S-aryl phosphorothioates. When [^{18}O]water was used the ^{18}O incorporation was measured by ^{31}P n.m.r. spectroscopy using the isotope shift (4). Only two species were observed, namely S-benzyl-[$^{18}O_3$]-phosphorothioate and S-benzyl-[$^{18}O_2$]phosphorothioate in the ratio of 95:5, indicating that the label per site was about 98 atom%. Since 98.4 atom % [^{18}O]water was used in the synthesis, no loss of label had occurred.

Synthesis of adenosine 5'-[$^{18}O_3$]phosphate demands a different strategy from that used for S-benzyl [$^{18}O_3$]-phosphorothioate, since there is no analogous reaction whereby phosphate monoesters may be synthesized. Yoshikawa *et al.* (5), as well as Sowa and Ouchi (6), have reported methods whereby unprotected nucleosides are directly phosphorylated at the

5'-hydroxyl by phosphoryl chloride in differing solvent systems. In our hands, however, the phosphorylation procedure of Sowa and Ouchi (6) yielded only small quantities of a mixture of 2'-,3'-, and 5'-phosphorylated products, along with some diphosphorylated species, and our experiments with the phosphorylation procedure of Yoshikawa et al.(5) confirmed the work of Dawson et al. (7) who showed that the method does not result in the sole phosphorylation of the 5'-hydroxyl group. 2',3'-Di-O-acetyl adenosine, does allow exclusive phosphorylation at the 5'-hydroxyl group, but the yield after deacetylation with ammonia in methanol was low and variable.

The route to adenosine 5'-[$^{18}O_3$]phosphate outlined in Scheme 3 was considered next. Although the method is less direct, experiments with unlabelled material showed that the route gave AMP in 53% overall yield. However, trial studies using [^{18}O]water were not satisfactory, ^{31}P n.m.r. spectroscopy showing that approximately 50% of the ^{18}O was exchanged. Since the method of production of isotopically enriched phosphorous acid was not accompanied by label loss, it must be caused by reaction of the product, as it is formed, with excess dicyclohexylcarbodiimide which on work up is displaced by [^{16}O]water. Although a 1:1:1 ratio of phosphorous acid, 2',3'-diacetyl adenosine and dicyclohexylcarbodiimide was used, adenosine 5'-phosphite is obtained in only 65% yield, suggesting that dicyclohexylcarbodiimide does react with adenosine 5'-phosphite.

$PCl_3 \xrightarrow{i} H_3P^{18}O_3 \xrightarrow{ii} {}^{18}O_2PH.OAdo \xrightarrow{iii} {}^{18}O_2SP.OAdo \xrightarrow{iv} {}^{18}O_3P.OAdo$

Scheme 3

Reagents i $H_2{}^{18}O$

ii(a) $C_6H_{11}.N{=}C{=}N.C_6H_{11}$, 2',3'-diacetyl-Ado

(b) aq. NH_3

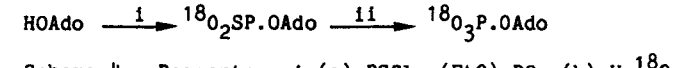
iii(a) Me_3SiCl, Et_3N

(b) S_8

(c) H_2O

iv $Br_2, H_2{}^{18}O$

By contrast with the reaction of adenosine with phosphoryl chloride in various solvents, thiophosphoryl chloride in triethyl phosphate does appear to react exclusively at the 5'-hydroxyl group. Hydrolysis of the product with [^{18}O]water gives adenosine 5'-[$^{18}O_2$]phosphorothioate (8) (Scheme 4). The enhanced regioselectivity is presumably due to the lower reactivity of thiophosphoryl chloride as compared with phosphoryl chloride. The conversion of adenosine 5'-[$^{18}O_2$]phosphorothioate to adenosine 5'-[$^{18}O_3$]phosphate was achieved by the use of bromine in [^{18}O]water (9), in 85% yield.



Scheme 4 Reagents i (a) $PSCl_3$,$(EtO)_3PO$; (b) $H_2{}^{18}O$
ii Br_2,$H_2{}^{18}O$

EXPERIMENTAL

S-Benzyl phosphorothioate from thiophosphoryl chloride.

Thiophosphoryl chloride (0.22 ml, 2 mmoles) was added with stirring to a solution of sodium hydroxide (0.6 g, 15 mmoles) in water (3 ml). The solution was heated to 90°C for 2h and then cooled to room temperature. Tetrabutylammonium chloride (50 mg) was added to the solution followed by benzyl bromide (1.5 ml) in dichloromethane (2 ml). The two phase system was stirred vigorously for 15 h, the phases separated and the organic layer washed with 30 mM-triethylammonium bicarbonate buffer (5 ml). The combined aqueous extracts were diluted and applied to a column of DEAE A-25 Sephadex (100 ml) and eluted with a gradient of triethylammonium bicarbonate buffer (30 mM- 300 mM, pH 8.2). Fractions containing S-benzyl phosphorothioate eluted at about 120 mM buffer concentration and were detected by spotting on silica t.l.c. plates and spraying with a solution of platinium chloride (1 g in 200 ml of 1M-HCl). The fractions containing S-benzyl phosphorothioate gave a yellow colour, and were combined and evaporated under reduced pressure. The remaining buffer salts were

removed by addition and evaporation (several times) of methanol. S-Benzyl phosphorothioate (1.3 mmoles, 65%) had δ_P (CD_3OD) + 13.4 (t, J_{PH} 8.6 Hz); δ_H (CD_3OD) 7.2 (5H, m, ArH), 4.0 (2H, d, J_{PH} 8.6Hz), 3.1 (q, J 7.3 Hz, NCH_2CH_3), 1.3 p.p.m. (t, J 7.3 Hz; $NCH_2.CH_3$).

S-Benzyl [$^{18}O_3$]phosphorothioate

Freshly distilled phosphorus trichloride (0.44 ml, 500μmoles) was placed in a flask in a drybox and [^{18}O]water (0.45 ml, 2.25mmoles, 98.4 atom % ^{18}O) added over several minutes with stirring. The reaction mixture was heated to 50°C, stirred at this temperature for 15 min., allowed to cool to room temperature, after which the flask was sealed and stirred for a further 20h. The [$^{18}O_3$]phosphorous acid was dried by the addition of dry pyridine (5 ml) followed by its evaporation under reduced pressure. This procedure was carried out three times. The pyridinium hydrogen phosphonate was dissolved in pyridine (5 ml) and to this solution was added dry triethylamine (0.23 ml, 1.65 mmoles) and chlorotrimethylsilane (0.21 ml, 1.65 mmoles). The reaction mixture was stirred for 10 min., after which bis-benzyl disulphide (0.15 g, 600 μmoles) (3) was added. The reaction was stirred for 16 h., filtered, and to the resulting solution was added water (2 ml). This was then evaporated under reduced pressure and the residue taken up in a sodium hydroxide solution (0.08 g in 40 ml). The aqueous solution, after being washed with ether, was diluted and purified by ion-exchange chromatography as above. S-Benzyl [$^{18}O_3$]phosphorothioate (340 μmoles) showed an isotopic shift of 0.025 p.p.m. per ^{18}O and contained 99 atom % ^{18}O.

Adenosine 5'-[$^{18}O_3$]phosphate

Adenosine (0.534 g, 2 mmoles) dried *in vacuo* over phosphorus pentoxide for 20h, was added to dry triethyl phosphate (4 ml). The suspension was stirred vigorously for 15 min. at 100°C, then cooled rapidly to 0°C, whereupon freshly distilled thiophosphoryl chloride (0.6 ml, 5.8 mmoles) was added and the mixture stirred overnight at 4°C. Excess thiophosphoryl chloride was removed by distillation for 3 h at 35°C and 2mm Hg. Sodium acetate (0.5 g, 6mmoles), dried *in vacuo* over

phosphorus pentoxide for 20h, was added, followed by [^{18}O]water (450 µl 99 atom % ^{18}O), and the mixture stirred at room temperature for 4h. The reaction mixture was diluted, its pH adjusted to pH 8.1, and was applied to a column (150 ml) of DEAE A-25 Sephadex. It was eluted with a linear gradient of 100 mM-400 mM triethylammonium bicarbonate buffer (pH= 8.1), to give adenosine 5'-[$^{18}O_2$]phosphorothioate (pure by n.m.r.) in 64%, δ_P + 40.2 p.p.m. with an isotope shift of 0.0332 p.p.m. per ^{18}O and 94 atom % per site. Adenosine 5'-[$^{18}O_2$]phosphorothioate (1.18 mmoles) (dried in vacuo overnight) was dissolved on [^{18}O]water (0.5 ml, 99 atom % ^{18}O) and cooled in an ice-water bath. Bromine (164 µl) was added and the mixture stirred for 5 min. The reaction was quenched by the addition of sodium metabisulphite, which had been dried in vacuo over phosphorus pentoxide. Water was added and the pH of the resulting solution was adjusted to 8 by the addition of triethylamine. The product was purified by ion-exchange chromatography, the elution being carried out with a linear gradient of 30 mM-300 mM triethylammonium bicarbonate buffer (pH=7.8). The yield of the title compound (pure by n.m.r.) was 880 µmoles and had δ_P + 0.7, isotope shift of 0.0230 p.p.m. per ^{18}O and 94 atom % ^{18}O per site.

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