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SYNTHESIS OF INORGANIC P^1-[(S)-^{16}O,^{17}O,^{18}O]PYROPHOSPHATE

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SUMMARY

A novel synthetic approach has been developed for the synthesis of inorganic pyrophosphate which allows the isotopes ^{17}O and ^{18}O to be incorporated at P^1 to give chirally labelled material.

INTRODUCTION

Chiral [^{16}O,^{17}O,^{18}O] phosphate esters and anhydrides are now well recognised as being useful tools for the determination of the stereochemical course of enzyme-catalysed phosphoryl and nucleotidyl transfer reactions (1-3), and methods for their synthesis (4,5) and stereochemical analysis (6,7) have been devised. A wide variety of reactions in mechanistic chemistry and enzymology have been investigated using such molecules.

During the course of our studies in this area we required P^1[(S)-^{16}O,^{17}O,^{18}O] pyrophosphate 2 which is a novel molecule, chiral by virtue of isotopic substitution, and related to inorganic pyrophosphate 1. We now report the synthesis of this compound. The unusual effects which result from the introduction of ^{18}O and the magnetically active ^{17}O nucleus (I=5/2) into this molecule have already been discussed (8).

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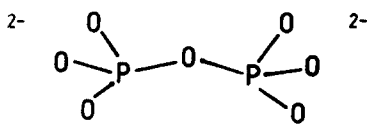

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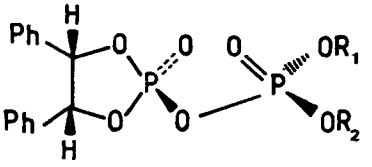
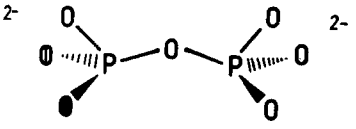


3 $R_1 = R_2 = -CH_2Ph$

4 $R_1 = -CH_2Ph$, $R_2 = H$

5 $R_1 = -CH_2CH_2CN$ $R_2 = H$



2

⓪ ≡ ^{17}O

● ≡ ^{18}O

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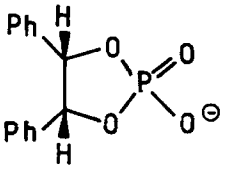


EXPERIMENTAL

^{17}O Water (44 atom %) was obtained from the Monsanto Research Corporation, Miamisburg, Ohio. (1R,2S)-[1-^{18}O]-1,2-dihydroxy-1,2-diphenylethane (99% enriched in ^{18}O) was a kind gift of Dr. P.M. Cullis.

2-Cyanoethylphosphate was purchased from Sigma, London. Pyridine was refluxed with potassium hydroxide, distilled and stored over KOH pellets. Dioxan was passed down an alumina column, refluxed with sodium and benzophenone until a permanent blue colour appeared, distilled, and stored over sodium wire under dry nitrogen.

The Briggs phosphate test (9) was adapted for the detection of pyrophosphate as follows. Aliquots (250 µl) of fractions from the ion-exchange columns were transferred to individual vials and evaporated to dryness in vacuo overnight over conc. sulphuric acid. M Sulphuric acid (100 µl) was then added and the mixtures incubated at 90°C overnight to destroy the pyrophosphate. Water (250 µl) was added, followed by ammonium molybdate solution (500 µl, 2.5 g in 20 ml water and 7.5 ml conc. sulphuric acid), hydroquinone solution (250 µl, 0.5 g in 100 ml water and one drop of conc. sulphuric acid) and sodium sulphate solution (250 µl,

4 g in 20 ml water) and the vials sealed and left for several days. Generally, after 48 h the blue colouration of phosphate could be discerned.

Gradients for ion-exchange chromatography were provided by an LKB 1130 Ultrograd mixer and an LKB Perpex peristaltic pump. The column effluent was monitored at 254 nm by an LKB Uvichord II detector, and fractions were collected using an LKB 7000 Ultrorac collector.

^{31}P NMR spectroscopy was performed at 36.43 MHz on a Bruker WH90 spectrometer, operating in the Fourier transform mode, with broad band proton decoupling. Samples in D_2O were contained in 5 mm or 10 mm precision NMR tubes, and samples in other solvents were contained in 8 mm tubes which were mounted coaxially inside 10 mm tubes containing D_2O to provide the lock signal. Chemical shifts are referred to external trimethylphosphate and are positive when downfield from this standard.

Dibenzyl hydrogen phosphate

Dibenzyl hydrogen phosphate was prepared by the method of Coulson (10). Bromine (2.87 ml, 56 mmole) in CCl_4 (12 ml) was added dropwise over 3h to a rapidly stirred mixture of dibenzyl phosphite (18 g, 68 mmole) CCl_4 (20 ml), pyridine (16 ml) and water (48 ml) cooled in an ice bath. The mixture was stirred for a further 1h at ambient temperature and the product isolated. Dibenzyl hydrogen phosphate was obtained as small colourless needles from CCl_4-hexane, m.p. 78-79°C (lit. 78-79°C); ^1H n.m.r. $\delta(CCl_4)$ 4.96 (d,4H,-$C\underline{H}_2$-$^3J_{PH}$ = 7 Hz) 7.22 (s,10H,$\underline{Ar}$), 12.24 (s,1H,P-O$\underline{H}$); ^{31}P n.m.r. δ_P (THF-3.15 (s,(PhCH$_2$O)$\underline{P}$-, ^1H decoupled, q, $^3J_{PH}$ = 4 Hz).

Trans-(4R,5S)-2(dibenzylphospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane 3

The phosphorylating agent, trans-(4R,5S)-2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane 6 (1 mmole) (11) was generated as previously described (5), the pyridine carefully removed *in vacuo*, excluding moisture from the system and releasing the vacuum under nitrogen. The residue was suspended in dry benzene. Dibenzyl phosphate

(280 mg, 1 mmole) which had been thoroughly dried in vacuo over P_2O_5 was dissolved in dry benzene (10 ml) and this volume was evaporated down four times to ensure dryness. It was then dissolved in dry benzene (3 ml) and added to the cooled stirred solution of 6 together with an equivalent of pyridine (45 µl). After 1 h at ambient temperature the suspension of pyridinium chloride was allowed to settle and a sample removed for ^{31}P n.m.r. δ_P (C_6H_6)D_2O lock, -16.2 (d, $(PhCH_2O)_2\underline{P}O$-$^2J_{PP}$ = 21 Hz), -15.5 (s,[$(PhCH_2O)_2\underline{P}O]_2O$), -4.2, (s,$(PhCH_2O)_2\underline{P}$-), -1.5 p.p.m. (d, ring P, $^2J_{PP}$ = 21 Hz, ^1H undecoupled signal, $^3J_{PH}$ = 8.0 Hz).

Benzyl dihydrogen phosphate

The title compound was prepared according to Cramer and Weimann (12). Crystalline phosphoric acid (0.196 g, 2 mmole), benzyl alcohol (10 g), triethylamine (0.404 g, 4 mmole) and trichloroacetonitrile (1.44 g, 1 mmole) were added together and after 4 h at 75°C cyclohexylamine (2 g, 20 mmole) was added, the excess benzyl alcohol distilled off and the product isolated as its bis-cyclohexylammonium salt from acetone-water. Yield 77%, m.p. 233°C (lit. 233°C); ^{31}P n.m.r. δ_P (dioxan, bis-Bu_3NH^+ salt)D_2O lock, s, -3.05 p.p.m.

Trans-(4R,5S)-2(benzylphospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane 4

The phosphorylating reagent 6 was prepared as previously described (5) and the pyridine removed in vacuo. The residue was dissolved in dry dioxan (4 ml). The pyridinium chloride did not dissolve. Benzyl phosphate free acid (189 mg, 1 mmole - from the cyclohexylammonium salt and amberlite IR120 (H^+) resin) was dissolved in dry dioxan and tributylamine added (185 mg, 1 mmole), the salt dried by coevaporation of dry dioxan and the mixture quickly added in one portion to the phosphorochloridate 6 together with further tributylamine (1 mmole). The mixture was left stirring for 3 h and the ^{31}P n.m.r. spectrum recorded δ_P (dioxan)D_2O lock -15.8 (d, $PhCH_2O\underline{P}$-, J_{PP} = 22.06 Hz), -3.65, (s, $PhCH_2O\underline{P}O_3^-$) 0.00 (d, 5-ring P, $^2J_{PP}$ = 22.06 Hz, ^1H undecoupled $^3J_{PH}$ = 8.0 Hz).

Attempted deprotection of 4

The reaction mixture was divided into two portions. One half was transferred to a hydrogenation flask containing ethyl acetate (10 ml) and 10% Pd/C (180 mg) and hydrogenolysed overnight. After filtration the mixture was extracted with sodium bicarbonate solution which was evaporated and the residue dissolved in D_2O. ^{31}P n.m.r. δ_P (D_2O) -7.57 (s, $\underline{PP_i}$), 0.24 (s, $\underline{P_i}$), 0.24 (s, $\underline{P_i}$), 12.88 (s, 5-ring phopshate).

The other half was deprotected in sodium/liquid ammonia in a similar fashion as that described for (5). ^{31}P n.m.r. δ_P (D_2O) -7.51 (s, $\underline{PP_i}$), 3.06 (s, $\underline{P_i}$).

Trans-(4R,5S)-2(2-cyanoethylphospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane 5

2-Cyanoethylphosphate dihydrate (322 mg, 1 mmole) was converted to the pyridinium salt with Dowex 50 W (pyridinium form) and suspended in methanol (5 ml). Tri-n-octylamine (353 mg, 1 mmole) was added and the mixture stirred until solution was obtained. The pyridinium salt was dried by repeated evaporation of dry pyridine, and was then dissolved in dry dioxan (3 ml) with tri-n-butylamine (185 mg, 1 mmole) and this solution added all at once with a syringe to a stirred solution of trans-(4R,5S)-2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane (1 mmole) 6 prepared according to Ukita (11). The phosphorochloridate had been prepared in dry pyridine which had been evaporated and replaced by dry dioxan (5 ml) under strictly anhydrous conditions. Pyridinium chloride formed during the preparation was insoluble in dioxan. The mixture was left to stir for 3 h, when complete reaction was observed, as judged by ^{31}P n.m.r. spectroscopy. δ_P (dioxan)D_2O lock -15.50 (d,-$\underline{P}$O$\underline{P}$OCH$_2$-, $^2J_{PP}$ = 22.8 Hz), -0.75 (d, $\underline{5\ ring\ P}$, $^2J_{PP}$ = 22.06 Hz, ^1H undecoupled $^3J_{PH}$ = 7.5 Hz).

Inorganic pyrophosphate 1

Liquid ammonia (ca. 60 ml) was distilled from sodium metal in a stream of dry nitrogen, and sodium metal (30 mmole, 80 cm wire) added

under anhydrous conditions in a dry nitrogen atmosphere. The dioxan mixture containing the protected pyrophosphate was added rapidly by syringe to the mechanically stirred deep-blue solution, and after exactly 10 min the mixture was quenched by addition of solid ammonium chloride until the blue colour had been discharged. The ammonia was allowed to evaporate in a stream of dry nitrogen to leave a white solid which was partitioned between chloroform and water. The aqueous layer was evaporated to dryness to give crude product as a white solid. ^{31}P n.m.r. spectroscopy showed peaks characteristic of inorganic pyrophosphate and phosphate.

The crude product was dissolved in water (300 ml) and the pH adjusted to 10.5 with HCl. The sample was applied at a flow rate of 80 ml h^{-1} to a column of DEAE Sephadex A-25 (200 ml) equilibrated in 100 mM TEAB. Products were eluted by a 48 h linear gradient of 100-600 mM TEAB pH 10.5, and fractions were collected at 15 min intervals. Samples (250 µl) were withdrawn and examined by an adaptation of the Briggs phosphate test (9). Pyrophosphate was located in fractions 26 - 38 which were evaporated to dryness, and excess TEAB removed by coevaporation with dry methanol. The resulting gum was dissolved in water and the solution stirred with Dowex 50 W (sodium form) for 30 min. The water was lyophilised to give tetrasodium pyrophosphate 1 as a fine white powder (130 mg, 50%). (D_2O) -9.2 (s, $\underline{PP_i}$).

(2R,4S,5R)-2(2-Cyanoethylphospho)-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane 5a

This material was prepared as described for the unlabelled compound except that 2-cyanoethylphosphate was reacted with (2R,4S,5R)-2-chloro-[2-^{17}O]-oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane 6a prepared by the method of Cullis and Lowe (5).

P^1-[(S)-^{16}O,^{17}O,^{18}O]-Pyrophosphate 2

The deprotection of the protected labelled pyrophosphate was carried out as described for the unlabelled compound, and the P^1-[(S)-^{16}O,^{17}O,^{18}O]-pyrophosphate 2 purified by ion-exchange

chromatography as above. δ_P (D_2O) -8.98 [d, $P_2(-\underline{P}O_3)$ residue of P_1-[(S)-^{16}O,^{17}O,^{18}O]-PPi, P^1 residue not observable, $^2J_{PP}$ = 21.1 Hz]; -9.0 (s, unresolved central lines of AB systems - 8.74, 9.26; $^2J_{PP}$ = 21.1 Hz).

RESULTS AND DISCUSSION

During our work on the application of chiral [^{16}O,^{17}O,^{18}O]phosphate esters to stereochemical problems in enzyme catalysed phosphoryl transfer reactions (3) we developed a synthesis of P^1-[^{16}O,^{17}O,^{18}O]-pyrophosphate. This molecule was expected to have unusual chiroptical and magnetic properties by virtue of its unique asymmetry. Whereas in many [^{16}O,^{17}O,^{18}O]-phosphates prepared to date there is usually already considerable asymmetry in other parts of the molecule, the introduction of this type of isotopic chirality alone in a totally symmetrical molecule has not previously been accomplished.

Much of the classical chemistry developed for the chemical synthesis of pyrophosphate systems in nucleotides has been well reviewed (13,14). We approached the synthesis of this molecule from the standpoint of our general method (5) using initially the phosphorochloridate, trans-(4R,5S)-2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholane 6 for the preparation of the unlabelled compound. The problems were to find a suitable phosphate derivative and an appropriate deprotection method. A wide variety of conditions was investigated, since the usual conditions for phosphorylation, namely generation and *in situ* reaction of the phosphorochloridate 6 in pyridine were found to produce a number of side reactions.

When dibenzyl phosphate was allowed to react with 6 in pyridine and the reaction mixture monitored by ^{31}P nmr spectroscopy an appreciable quantity of the desired pyrophosphate tetraester trans-2(dibenzyl-phospho)-2-oxo-4,5-diphenyl-1,3,2-dioxaphopsholane 3 was formed. However, the spectrum showed the presence of several other products and an intense singlet at -15.5 ppm, assigned to tetrabenzyl pyrophosphate. This is an analogous reaction to that reported by Corby *et al* (15) in which P^1-dibenzyl-P^2-diphenylpyrophosphate was observed to react with dibenzyl

phosphate to produce tetrabenzyl pyrophosphate. Moreover it was also found that P^1-di-p-methoxyphenyl-P^2-diphenyl-pyrophosphate disproportionated in pyridine solution which may account for the side reactions observed here.

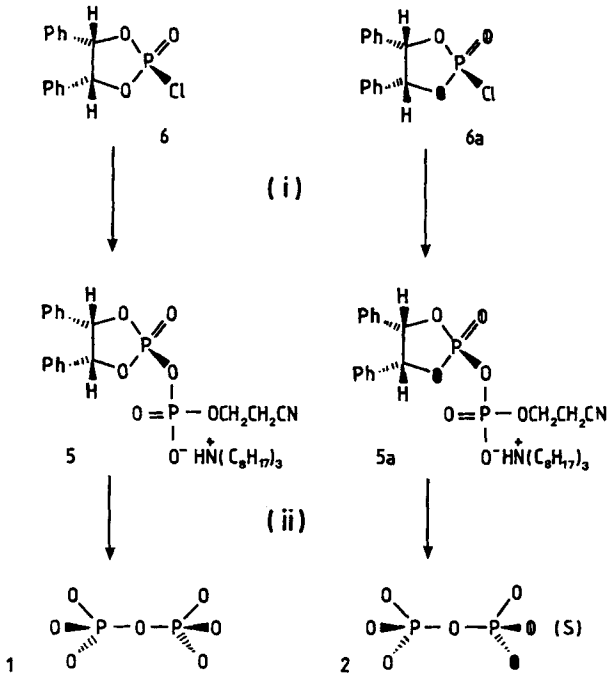
Initial experiments employing dibenzylphosphate as nucleophile in dry benzene showed that the desired intermediate pyrophosphate tetraester 3 could be formed in excellent yield. However, presumably on account of the increase in reactivity over tetrabenzylpyrophosphate associated with the presence of the 5-membered ring (16), the tetraester 3 was exceptionally sensitive to water and was not therefore suitable. Indeed, addition of a few drops of water to a solution of 3 in dry benzene caused complete destruction of the anhydride within 5 min as monitored by ^{31}P n.m.r. spectroscopy.

Since a pyrophosphate triester such as 4 might be expected to be more resistant to nucleophilic attack, the preparation of 4 was investigated using benzyl phosphate as the nucleophile. Reaction with 6 in dioxan gave an almost quantitative yield of 4 and addition of water did not cause hydrolytic scission of the anhydride system. Although triester 4 was considerably more stable, the ^{31}P nmr spectrum of the products obtained after attempted removal of blocking groups by hydrogenolysis showed the presence of only small amounts of inorganic pyrophosphate at δ_P-7.6. The main product was orthophosphate at δ_P-0.24 ppm. It appeared that rapid removal of the benzyl group had left a P^1P^1 pyrophosphate diester which had decomposed. This was confirmed by the presence of a major peak at δ_P+12.9 ppm which was assigned to the 5-ring diester 7.

An alternative deprotection reaction, namely sodium metal in liquid ammonia has the advantage of anhydrous conditions. Arris et al (17) have studied the deprotection of benzyl pyrophosphates by this procedure and have found that the di- and triesters give inorganic pyrophosphate, but the tetraester gives mainly orthophosphate and some inorganic pyrophosphate. When 4 was deprotected by sodium in liquid ammonia only two resonances could be observed in the ^{31}P n.m.r. spectrum, the major one at δ_P+3.06 being assigned to inorganic phosphate, and the other at δ_P-7.51

to inorganic pyrophosphate. It seemed that a similar kind of deprotection problem was being encountered in the sodium-liquid ammonia reduction, probably due to the facile formation of benzyl radicals, and therefore what was required was a protecting group on P^2 which would either be completely stable to the deprotection conditions or be removed at a rate much slower than that of the 5-ring system. The choice of the ß-cyanoethyl protecting group appeared to be appropriate in this instance for it would be removed by mildly basic conditions and be completely stable to hydrogenolysis.

2-Cyanoethyl phosphate as its tri-n-octylammonium salt was treated with the phosphorochloridate 6, as previously. Dry dioxan was used as solvent and ^{31}P n.m.r. spectroscopy indicated an excellent yield of the protected pyrophosphate trans-2-(2-cyanoethylphospho)-2-oxo-4,5-diphenyl-



Synthesis of pyrophosphate and P^1-[(S)-^{16}O,^{17}O,^{18}O] pyrophosphate

Reagents (i) $NCCH_2CH_2OPO_3H^{\ominus}{}^{\oplus}HN(C_8H_{17})_3$ in dioxan.

(ii) Na - liq. NH_3.

1,3,2-dioxaphospholane 5. The trans stereochemistry of the 5-membered ring system was confirmed by the measurement of $^{3}J_{PH}$ = 7.5 Hz (5).

Hydrogenolysis under anhydrous conditions failed to give a satisfactory yield of inorganic pyrophosphate, but reductive cleavage using sodium in liquid ammonia was much more successful. The 2-cyanoethyl group was also removed by this procedure, possibly during the aqueous alkaline conditions of the work-up. Purification of the product by ion-exchange chromatography gave inorganic pyrophosphate in 50% yield.

This method was then applied to the synthesis of chirally labelled pyrophosphate. 2-Cyanoethylphosphate was reacted with (2R,4S,5R)-2-chloro[2-^{17}O]oxo-4,5-diphenyl-[1-^{18}O]-1,3,2-dioxaphospholane 6a to give 5a.

Deprotection with sodium in liquid ammonia gave P^{1}-[(S)-^{16}O,^{17}O,^{18}O]-pyrophosphate 2.

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