Synthesis of \((R_p,R_p)-P^1,P^4\text{-}\text{Bis}(5'^\prime\text{-}\text{adenosyl})\text{-}1[\text{thio}^{18}\text{O}^{18}\text{O}],4[\text{thio}^{18}\text{O}^{18}\text{O}])\text{tetraphosphate from } (S_p,S_p)-P^1,P^4\text{-}\text{Bis}(5'^\prime\text{-}\text{adenosyl})\text{-}1[\text{thio}^{18}\text{O}^{18}\text{O}],\text{4[thio}^{18}\text{O}^{18}\text{O}])\text{tetraphosphate with Retention at Phosphorus and the Stereochemical Course of Hydrolysis by the Unsymmetrical Ap4A Phosphodiesterase from Lupin Seeds*}

(Received for publication, May 20, 1988)

Ruth M. Dixon and Gordon Lowe
From The Dyson Perrins Laboratory, Oxford University, South Parks Road, Oxford OX1 3QY, United Kingdom

The three stereoisomers of \(P^1,P^4\text{-}\text{Bis}(5'^\prime\text{-}\text{adenosyl})\text{-}1,4\text{-dithiotetraphosphate} \) have been synthesized and their \(^{31}\text{P} \) NMR spectra investigated. The effect of temperature on the circular dichroic spectrum of the \((S_p,S_p)-\text{stereoisomer} \) shows that unstacking of the molecular occurs as the temperature is raised. Treatment of the \((S_p,S_p)-\text{stereoisomer} \) with cyanogen bromide in \(^{18}\text{O}\text{water} \) leads to substitution of sulfur by \(^{18}\text{O} \) with predominant retention of configuration at \(P^1 \) and \(P^4 \). The \((S_p,S_p)-P^1,P^4\text{-}\text{Bis}(5'^\prime\text{-}\text{adenosyl})\text{-}1[\text{thio}^{18}\text{O}^{18}\text{O}],4[\text{thio}^{18}\text{O}^{18}\text{O}]\text{tetraphosphate} \) was synthesized and on treatment with cyanogen bromide in \(^{18}\text{O}\text{water} \) gave \((R_p,R_p)-P^1,P^4\text{-}\text{Bis}(5'^\prime\text{-}\text{adenosyl})\text{-}1[\text{thio}^{18}\text{O}^{18}\text{O}],4[\text{thio}^{18}\text{O}^{18}\text{O}]\text{AMP} \). The reaction therefore proceeds with inversion of configuration at phosphorus, indicating that the enzyme-catalyzed displacement by water occurs by a direct “in-line” mechanism.

\(^{31}\text{P} \), \(^{31}\text{P} \text{-Bis}(5'^\prime\text{-}\text{adenosyl})\text{-tetraphosphate} (\text{Ap4A}), \) which was first reported by Zamecnick et al. (1), is ubiquitous in living cells (2). It appears to play an important role in protein biosynthesis (3), the intracellular level being directly related to the proliferative activity of the cell and results in the stimulation of DNA synthesis (4). Moreover, Ap4A associates tightly but non-covalently with DNA polymerase \(\alpha \) (5) and acts as a primer for DNA synthesis in vitro (6, 20, 21). In Salmonella, the Ap4A concentration increases to 100 \(\mu \text{M} \) in response to the bacteriostatic quinone, 6-amino-7-chloro-5,8-dioxoquinoline, and Ap4A has been proposed as an “alarmone,” i.e. a substance whose intracellular concentration increases dramatically in response to stress (7).

Many of the aminocyl-tRNA synthetases show weak Ap4A synthetase activity, but for a few this is markedly enhanced in the presence of \(\text{Zn}^{2+} \) (8, 22, 23). Since Ap4A is a pleiotropic regulator or signal nucleotide for cellular metabolism its concentration must be capable of being lowered as well as raised. Not surprisingly, therefore, Ap4A phosphodiesterases have been found which hydrolyze Ap4A to ATP and AMP (9, 10, 24–26), and ADP (11). With a view to investigating the stereochemical course of unsymmetrical Ap4A phosphodiesterases, Ap4A made chiral at \(P^1 \) and \(P^4 \) was required.

EXPERIMENTAL PROCEDURES

Adenylate kinase, pyruvate kinase, and hexokinase (used in the preparation of \((S_p)-\text{ATP} \) and \((S_p)-\text{ADP} \)) were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom). Ap4A phosphodiesterase (EC 3.6.1.17) from lupin seeds (10) was a gift from Dr. A. Guranowski (Institute of Biochemistry, Agricultural University, Poznan, Poland) and was stored at \(-20^\circ C \) adsorbed on to Sephadex G-50. Snake venom phosphodiesterase Type IV (from Crotalus atrox) was obtained from Sigma Chemical Co. Ltd. Deionized, distilled water used in the preparation of all the buffers was obtained from a Milli-Q water purification system (Millipore Ltd., Harrow, Middlesex, U.K.). \(^{18}\text{O} \text{Water} \) (99 atom % \(^{18}\text{O} \)) was obtained from Amersham International plc (Amersham, U.K.), and \(^{18}\text{O} \text{water} (25.5\% \text{^{18}O}, \text{55.9\%} \text{^{16}O}, \text{and} \text{20.6\%} \text{^{18}O}) \) was obtained from Monsanto Research Corporation (Miamisburg, Ohio).

Analysis of nucleotides was performed by ion-exchange (Mono Q) chromatography on a fast protein liquid chromatography system (Pharmacia, Hounslow, U.K.). This system was used for the separation of the diastereoisomers of \(P^1,P^4\text{-}\text{Bis}(5'^\prime\text{-}\text{adenosyl})\text{-1,4-dithiotetraphosphate} \). NMR spectra were recorded with quadrature detection at 500 MHz (\(^{1}H \)) or 202 MHz (\(^{31}\text{P} \)) on a Bruker AM 500 Fourier transform spectrometer under Aspect 3000 control. Field frequency locking was provided by the deuterium resonance of D\(_2\)O. Chemical shifts are referred to internal 3-(trimethylsilyl)propionic acid (\(^{1}H \)) or external trimethyl phosphate in D\(_2\)O (\(^{31}\text{P} \)).

The measured chemical shifts and coupling constants were confirmed by spectral simulation with PANIC, a microcomputer version of the LAOCOON program.

Circumstantial spectra were measured on a JASCO J40C instrument by Dr. Alex S. Drake and Dr. P. M. Udvardi of the National CD Service, Birbeck College, London. (\((S_p)-\text{ADP} \)) was converted into its tri-n-octylammonium salt and dried by coevaporation of dioxan (3 × 1 ml) and pumped at high vacuum (0.1 mm, 2 h). It was dissolved in dioxan (8 ml) under a dry \(\text{N}_2 \) atmosphere. Tri-n-butylamine (0.162 ml, 680 \(\mu\text{mol} \)) and diphenyl chlorophosphate (0.035 ml, 170 \(\mu\text{mol} \), 0.5 equivalent) were added and stirred for 3 h, protected from moisture. The dioxan was evaporated, and dry pyridine (2 ml) was added and evaporated. Dry pyridine (8 ml) was added and the mixture stirred for a further 3 h. The pyridine was evaporated and the residue dissolved in triethylammonium bicarbonate buffer (0.1 M, pH 7.5). The solution was washed with ether and the ether backwashed with water. The combined aqueous extracts were adjusted to pH 7.5 with triethylamine and diluted. The solution was applied to a column (1.5 × 25 cm) of DEAE-Sephadex A-25 that had been equilibrated with triethylammonium bicarbonate buffer (TEAB; 0.5 M, pH 7.5). The column was eluted with a linear gradient of TEAB (0.5–10 M, pH 7.5). The product (60 \%mol, 35%) appeared
The mixture of diastereoisomers was made by activating the tri-n-octylammonium salt of AMPS (200 pmol) (19) with diphenyl chlorophosphate (0.042 ml, 200 pmol) in dioxan (6 ml) in the presence of tri-n-butylamine (0.048 ml, 400 pmol). After 3 h, protected from moisture, the dioxan was evaporated and (Sp,Sp)-ATPaS (200 pmol) (12) in dry pyridine (6 ml) was added and stirred for 3 h. The solvent was evaporated and the residue extracted and chromatographed as above. (The (S,,S,)- and (R,,S,)-diastereoisomers could be partially separated on a column (15 x 100 cm) of DEAE-Sephadex A-25, with a linear gradient of TEAB buffer (0.3-0.8 M, pH 7.5). Pure samples were obtained by fast protein liquid chromatography with a Mono Q ion-exchange column.

(S,,S,)- and (R,,S,)-P,,P-Bis(5'-adenosyl)-1,4-dithiotetraphosphate—This mixture of diastereoisomers was made in the same way as the (Sp,Sp)-isomer, using a mixture of (R,,R,)- and (S,,S,)-ATPaS (13).

Replacement of Sulfur by O in (S,,S,)-P,,P-Bis(5'-adenosyl)-1,4-dithiotetraphosphate (2)—(Sp,Sp)-P,,P-Bis(5'-adenosyl)-1,4-dithiotetraphosphate (5 pmol) was dried by coevaporation of dioxan (3 ml) and by pumping under vacuum (<0.1 mm Hg, 2 h). Under an atmosphere of dry nitrogen, [63,14C]water (90, 50 atom %, 400 pmol) was introduced, followed by cyanogen bromide (5.3 mg, 50 mmol), and the mixture was stirred for 9 min. The reaction was stopped by the addition of mercaptoethanol (5 pl) and the solution chromatographed on DEAE-Sephadex A-25 (1 x 10 cm column), eluted with TEAB buffer (50-300 mM, pH 7.5). [1',O2]AMP (4.4 pmol, 75%) was isolated and analyzed by 31P NMR spectroscopy (Fig. 2) after cyclization and methylation. It is possible that the (R,,S,)-stereoisomer arose owing to dismutation of the ADPaS to AMPS and ATPaS which would give rise to both the (S,,S,)- and (R,,R,)-stereoisomers.

When a mixture of (Sp,Sp)- and (S,,S,)-ADPaS (13) were coupled in the same way, all three stereoisomers were formed. Replacement of Sulfur by O in (S,,S,)-P,,P-Bis(5'-adenosyl)-1,4-dithiotetraphosphate—Bromine/water and N-bromosuccinimide both reacted slowly with (Sp,Sp)-P,,P-bis(5'-adenosyl)-1,4-dithiotetraphosphate and gave a mixture of products which contained only a small amount of ApA and some P,,P-bis(5'-adenosyl)-1-thiotetraphosphate. Cyanogen bromide in the absence of buffer gave a much cleaner product and after 9 min about 70% ApA was formed, the minor products being identified as P,,P-bis(5'-adenosyl)-1-thiotetraphosphate, ApA, ATP, AMPS, and AMP. When the cyanogen bromide reaction was run in [15O]water the [15O]ApA contained 70% of the label at P and 30% at P. The P,,P-bis(5'-adenosyl)-[15O]-1-thiotetraphosphate was found to contain 78% label at P, 8% at P, and 14% at P. Clearly, the central phosphate residues can participate in the reaction leading to cyclo-triphosphate and cyclo-diphosphate as transient intermediates. If the label at P and P was introduced exclusively by way of these cyclic intermediates overall retention of stereochemistry would be expected (14).

The [15O2]ApA (3) was hydrolyzed with snake venom phosphodiesterase in [15O]water and the [15O][15O]AMP (4) analyzed for chirality at phosphorus after cyclization and methylation (18). The 31P NMR spectrum (Fig. 1) shows it to
Mechanism of Action of ApA Phosphodiesterase

Fig. 1. 31P NMR spectrum of the equatorial and axial triesters derived by cyclization and methylation of 5'-[160,170,180]AMP (4) obtained by hydrolyzing (S,S)-P',P4-bis(5'-adenosyl)-1,4-[14]O2-tetraphosphate with snake venom phosphodiesterase in [170]water. The ratio of the 160ax:180eq triesters to the 180,160eq triesters shows that the 5'-[160,170,180]AMP has the predominant (S,) configuration.

FIG. 1. "P NMR spectrum of the equatorial and axial triesters derived by cyclization and methylation of 5'-[160,170,180]AMP (4) obtained by hydrolyzing (S,S)-P',P4-bis(5'-adenosyl)-1,4-[14]O2-tetraphosphate with snake venom phosphodiesterase in [170]water. The ratio of the 160ax:180eq triesters to the 180,160eq triesters shows that the 5'-[160,170,180]AMP has the predominant (S,) configuration.

be predominantly of the (S,) configuration. If the [170]water and the P',160 site had been fully enriched only one isoto-pomer of the esters would be observed in the 31P NMR spectrum owing to scalar relaxation caused by the 31O when directly bonded to phosphorus (16, 27, 28). In fact, the isotopic composition of the [170]water was known (see "Experimental Procedures") and the ApA was 70% 160 at P' and P, so all four isotopomers containing 160 and 180 were observed. The stereochemical evidence is provided by the ratio of the two mono-160 esters. Since an unknown amount of [160]water was introduced with the snake venom phosphodiesterase causing the composition of the "[170]water," it was not possible to quantify the stereochemical analysis. However, since snake venom phosphodiesterase is known to hydrolyse phosphate diesters with retention of configuration at phosphorus (17, 29), the sulfur displacement has occurred with participation of the neighboring phosphate groups leading to predominant retention of configuration, as was observed with adenosine 5'-[(S)-thio-γ-benzyl]triphosphate (14).

Replacement of Sulfur by 160 in (S2,S2)-P',P4-Bis(5'-adenosyl)1,4-[160,180]tetraphosphate—ApA chirally labeled at P' and P with both 160 and 180 was synthesized (Scheme 2). (S2,S2)-P',P4-Bis(5'-adenosyl)-1,4-[160,180]tetraphosphate (6) was prepared from (S2)-α-180 ADPβS (5) (18) by the same procedure used for the unlabeled material and purified chromatographically. Treatment of (S2,S2)-P',P4-bis(5'-adenosyl)-1[thio-160]4[thio-180]tetraphosphate (7) with cyanogen bromide in [170]water gave predominantly (R,S)-P',P4-bis(5'-adenosyl)-1[170,180]4-[170,180]tetraphosphate (8). Hydrolysis with snake venom phosphodiesterase in water gave [180,170,180]AMP (8) which had predominantly the (S,) configuration (Fig. 2), confirming that the displacement of sulfur occurred with predominant
Mechanism of Action of Ap₄A Phosphodiesterase

Fig. 2. ³¹P NMR spectrum of the equatorial and axial triesters derived by cyclization and methylation of 5'-[¹⁶O,¹⁸O]AMP (8) obtained by hydrolyzing (R₃₃,R₄₄)-P¹₄₅₆₁₆₇₈₉ₐ₉₉₈₉₉₉₈₉₈₉₈₉₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉₈₉ূ

![31P NMR spectrum of the equatorial and axial triesters derived by cyclization and methylation of 5'-[¹⁶O,¹⁸O]AMP (8) obtained by hydrolyzing (R₃₃,R₄₄)-P¹₄₅₆₁₆₇₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉২

Table I

| ³¹P NMR data for Ap₄A and the stereoisomers of P¹₄₅₆₁₆₇₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉২
|-----------------|-----------------|-----------------|-----------------|
| ppm             | Hz              | ppm             | Hz              |
| Ap₄A            | 14.25           | 18.1            | 16.0            |
| (S₃₃,S₄₄)-Ap₄A | 19.13           | 25.9            | 17.5            |
| (R₃₃,R₄₄)-Ap₄A | 39.96           | 26.3            | 17.3            |
| (S₃₃,S₄₄)-Ap₄A | 40.04           | 25.9            | 17.4            |
| (R₃₃,S₄₄)-Ap₄A | 40.24           | 25.6            | 17.4            |

TABLE I

³¹P NMR data for Ap₄A and the stereoisomers of P¹₄₅₆₁₆₇₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉₈₉₉২

 retention of configuration.

³¹P NMR Spectra of Ap₄A and its Dithio Analouges—Ap₄A, (S₃₃,S₄₄)- and (R₃₃,R₄₄)-P¹₄₅₆₁₆₇২. The ³¹P NMR spectrum of the (R₃₃,S₄₄)-stereoisomer is more complex because it is an ABXY spectrum. It was, however, possible to analyze the spectrum by use of the simulation program PANIC. The chemical shifts and coupling constants for this and the other stereoisomers are shown in Table I together with those of Ap₄A.

FIG. 3. The circular dichroic spectra of the (S₃₃,S₄₄)-stereoisomer of P¹₄₅₆₁₆₇২. The Circular Dichroic Spectra of Ap₄A and Its Dithio Analouges—Ap₄A was first detected because of its circular dichroic spectrum which in contrast to AMP, ADP, and ATP shows a
positive ellipticity at shorter wavelengths and a negative ellipticity at longer wavelengths. This has been attributed to it adopting a conformation in which the adenine rings are stacked with their α-faces facing each other. The CD spectra of Ap4A and the stereoisomers of P',P'-bis(5'-adenosyl)-1,4-dithiotetraphosphate are independent of pH in the range 6.0–9.0. The (S,S)-P',P'-bis(5'-adenosyl)-1,4-dithiotetraphosphate stereoisomer at pH 9.0 showed a loss of amplitude as the temperature was raised from 9 to 89 °C (Fig. 3); an isosbestic point occurs at 270 nm. This suggests, as expected, that the stacking of the adenine rings is disrupted as the temperature is raised. A plot of the change in ellipticity at 280 nm against $1/T \ (K)$ gives a straight line. Ap4A and (S,S)-P',P'-bis(5'-adenosyl)-1,4-dithiotetraphosphate show different behavior in their CD spectra when titrated with Cd2+. When Ap,A was titrated with Cd2+ over the range 0–250 μM CdCl2, a loss of amplitude of the peak and trough was observed but their positions did not alter. (S,S)-P',P'-bis(5'-adenosyl)-1,4-dithiotetraphosphate showed similar loss of amplitude, but in addition the long wavelength trough moved to shorter wavelength (about 10 nm) suggesting that a different conformational change occurs when Cd2+ binds presumably to the sulfur atoms. When (S,S)-P',P'-bis(5'-adenosyl)-1,4-dithiotetraphosphate at pH 9.0 is titrated with ZnCl2, again the CD spectrum becomes less intense but the position of the peak and trough does not alter.

The Stereochemical Course of Hydrolysis of Ap4A with Ap4A Phosphodiesterase (from Lupin Seeds)—(R,R)-P',P'-Bis(5'-adenosyl)-1[15O,18O],4[15O,18O]tetraphosphate (7) (note it has predominantly the (R) configuration at P' and P) was hydrolyzed with Ap4A phosphodiesterase in water to give labeled ATP and [15O,18O,18O]AMP (Scheme 3). After purification the [18O,18O,18O]AMP was analyzed for chirality by 31P NMR spectroscopy (Fig. 4) after cyclization and methylation (15). From the ratio of the intensities of the two mono-O isotopomers of the axial and equatorial triesters it is clear that the [18O,18O,18O]AMP has predominantly the (R) configuration. Comparison of the relative intensities from Fig. 4 with those from Fig. 2 (see Table II) shows that Ap4A phosphodiesterase catalyzes the hydrolysis of Ap4A with inversion of configuration at Pα, since snake venom phosphodiesterase is known to catalyse the hydrolysis of phosphodiesters with retention (17, 29). This result indicates that the Ap4A phosphodiesterase catalyzes the hydrolysis of Ap4A at P' by a direct “in-line” mechanism.

**Table II**

Comparison of the observed relative peak intensities of the 31P NMR resonances of the diastereoisomeric triesters derived by cyclization and methylation of the 5'-[18O,18O]AMP obtained by hydrolyzing (S,S)-P',P'-bis(5'-adenosyl)-1[15O,18O],4[15O,18O]tetraphosphate with snake venom phosphodiesterase (SVPDE) (from Fig. 2) and Ap4A phosphodiesterase from lupin seeds (ApAPDE) (from Fig. 4) in ordinary water.

| Labelled triester | Equatorial triester | Axial triester |
|------------------|---------------------|----------------|
|                  | SVPDE               | ApAPDE         |
| SVPDE            |                     |                |
| MeO·P=O          | 0.77                | 0.77           | 0.77 | 0.77 |
| Me·P=O           | 1.00                | 0.76           | 0.77 | 1.00 |
| Me·P=O           | 0.78                | 1.00           | 1.00 | 0.67 |
| Me·P=O           | 0.20                | 0.30           | 0.24 | 0.16 |

**Scheme 3.** The stereochemical course of hydrolysis by Ap4A phosphodiesterase from lupin seeds. The reaction proceeds with inversion of configuration at phosphorus.

**Fig. 4.** 31P NMR spectrum of the equatorial and the axial triesters derived from cyclopentanone and methylation of 5'-[18O,18O,18O]AMP (9) obtained by hydrolyzing (R,R)-P',P'-bis(5'-adenosyl)-1[18O,18O],4[18O,18O]tetraphosphate (Scheme 3) with Ap4A phosphodiesterase in ordinary water. The ratio of the 18O,18O,18O triesters to the 18O,18O,18O,18O,18O triesters shows that the 5'-[18O,18O,18O]AMP has the predominant (R) configuration, and hence the hydrolysis has taken place with inversion of configuration at phosphorus.
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