A Simple Method for the Configurational Analysis of a Deoxynucleoside 5'-[^{16}O,^{18}O,S]Phosphorothioate

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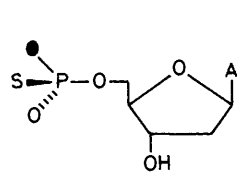
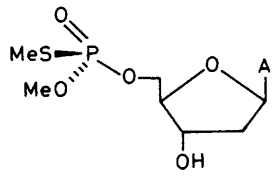
Methylation of 2'-deoxyadenosine 5'-phosphorothioate gives two diastereoisomeric triesters whose configurations can be assigned by ^{31}P n.m.r. spectroscopy by methylation of an asymmetric mixture of the diastereoisomers of 2'-deoxyadenosine 5'-*O*-methylphosphorothioate after configurational assignment; this can be used as a simple method for stereochemical analysis of 2'-deoxyadenosine 5'-[^{16}O,^{18}O]phosphorothioate.

Recent advances in our understanding of the mechanistic enzymology of phosphoryl transfer reactions have relied heavily on a stereochemical approach in which the phosphorus centre is made chiral either by virtue of isotopic substitution with the stable isotopes of oxygen,[1] or by using one oxygen isotope and sulphur in a chiral phosphorothioate analogue.[2] Central to the success of these methods has been the development of stereospecific syntheses of chiral biophosphates[1,2] and methods for their stereochemical analysis.[3] Using these tools a large number of enzymes have been investigated.

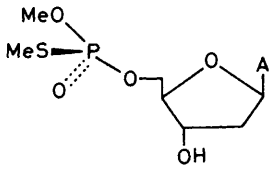
Advances in the techniques of oligonucleotide synthesis[4] now make an extension of this stereochemical approach to the large group of DNA restriction endonuclease enzymes[5] possible by enabling small-substrate DNA fragments containing endonuclease recognition sequences to be synthesised. In view of the relatively large amounts of material which would be needed for such investigations using the oxygen chiral phosphate method, the best way to tackle the problem appears to lie with the earlier-developed sulphur-substrate analogue approach. However, whilst the synthetic side of this approach is well developed, and indeed, increasing interest is being shown in the synthesis of oligonucleotides containing phosphorothioate groups,[6] present methods for the stereochemical analysis of chiral phosphorothioates severely limit its flexibility. The method relies on the stereospecific cleavage of one diastereoisomer of an oligonucleotide phosphorothioate in ^{18}O-labelled water by an enzyme, and after degradation to mononucleotides stereochemical analysis of the resulting 2'-deoxynucleoside 5'-[^{16}O,^{18}O]phosphorothioate monoester.[7] An enzymic method for the stereospecific phosphorylation of adenosine 5'-phosphorothioate exists,[8] which has been used for stereochemical analysis of labelled 2'-deoxyadenosine 5'-phosphorothioate by mass spectrometry and n.m.r. spectroscopy,[2,9] but this method rests on the specificity of adenylate kinase and is not applicable to other systems. Since restriction enzymes are capable of cleaving next to any nucleoside residue in DNA, in order to take advantage of the wide range of enzymes available one must be able to determine the configuration at phosphorus of any such labelled deoxynucleoside 5'-phosphorothioate.



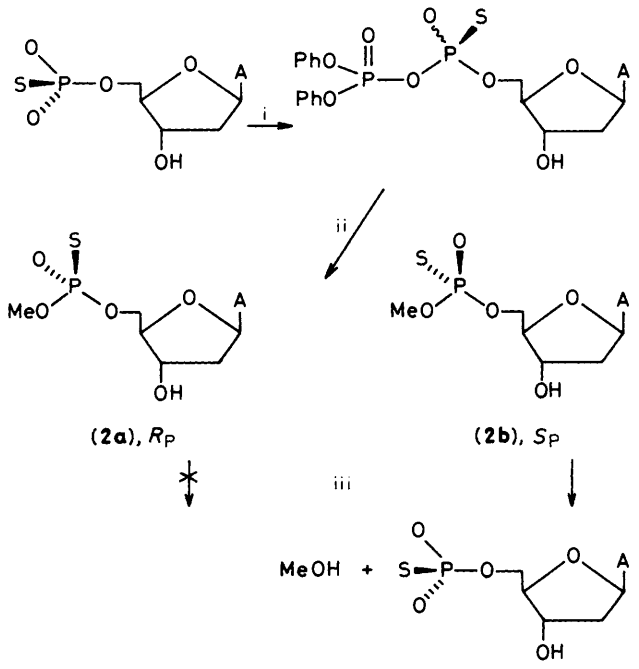
(1) ● = ^{18}O



(3a), R_P



(3b), S_P



Scheme 1. A = Adenine. *Reagents:* i, $(PhO)_2POCl$–dioxane; ii, MeOH–pyridine; iii, snake venom phosphodiesterase.

We report here our progress towards the development of a general chemical method for the configurational analysis of such phosphorothioates by outlining a simple configurational analysis of 2′-deoxyadenosine 5′-[^{16}O,^{18}O]phosphorothioate (**1**).

Nucleoside 5′-*O*-methylphosphorothioates can be synthesised from the parent 5′-monophosphorothioates by activation with diphenyl phosphorochloridate and subsequent reaction with methanol. 2′-Deoxyadenosine 5′-*O*-methylphosphorothioate (**2a,b**) was synthesised in this fashion (Scheme 1), the generation of a chiral centre resulting in the formation of two diastereoisomers which could be distinguished by ^{31}P n.m.r. spectroscopy (diastereoisomer separation in dimethylformamide, DMF, 0.126 p.p.m.)† and h.p.l.c. The configurations of these diastereoisomers were assigned by partial digestion of a *ca.* 1 : 1 mixture with snake venom phosphodiesterase, an enzyme known to cleave preferentially one diastereoisomer of phosphorothioate esters and anhydrides,[10,11] usually the R_p compound. However, in view of the known specificity of the enzyme for sulphur-containing substrates [(S_p)-*O*-*p*-nitrophenyl phenylphosphonothionate is cleaved[11]], and the sequence rules governing the *R,S* notation, we assign the S_p configuration to the diastereoisomer of (**2**) which is cleaved, since the 5′-adenosyl moiety exceptionally has a higher priority than the group to be removed, in this case methoxy. Methylation of the now asymmetric mixture of (**2a,b**) with methyl iodide produced the 2′-deoxyadenosine 5′-phosphorothioate-*S*-methyl-*O*-methyl triesters (**3a,b**), which are also distinguishable by ^{31}P n.m.r. spectroscopy (diastereoisomer separation in 3 : 1 DMF–MeOH 0.072 p.p.m.)† and whose configurations at phosphorus could be assigned by examining the relative intensities of the corresponding resonances in the ^{31}P n.m.r. spectrum and assigning the smaller resonance to the S_p diastereoisomer (Figure 1).

† Chemical shifts of (**2a,b**) and (**3a,b**) relative to H_3PO_4, 57.7 and 30.3 p.p.m. respectively.

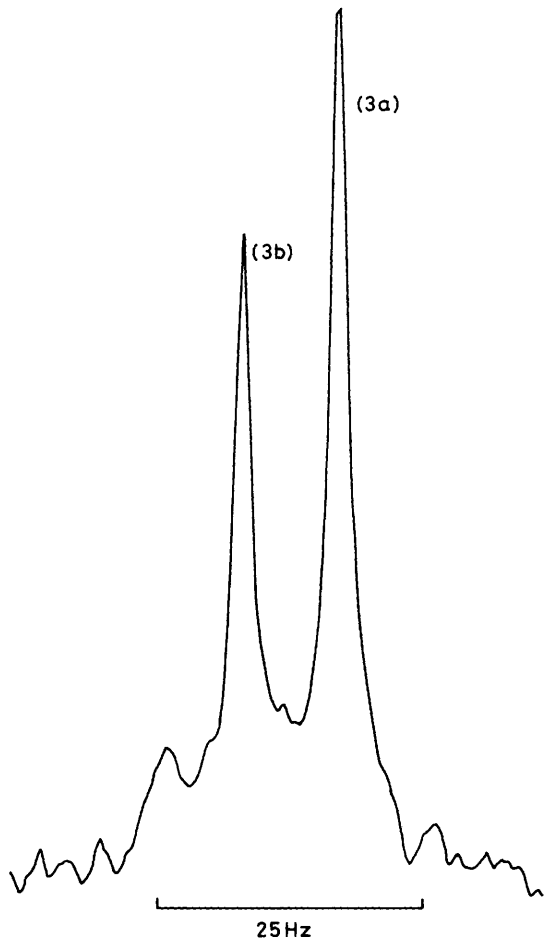
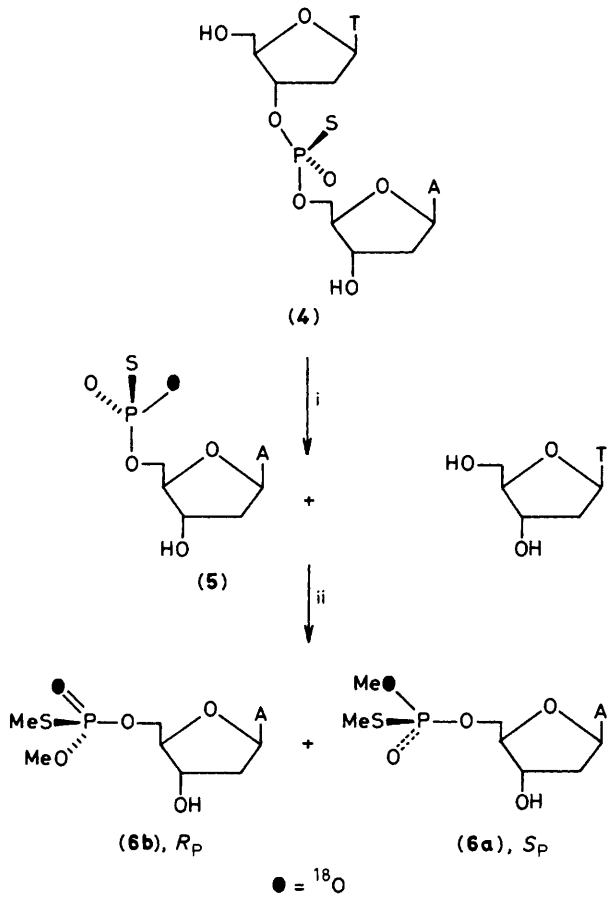


Figure 1. 121.5 MHz ^{31}P n.m.r. spectrum of the diastereoisomers of 2′-deoxyadenosine 5′-*S*-methyl-*O*-methylphosphorothioate (**3a,b**) (*ca.* 4 mM solution in 3 : 1 DMF–CD_3OD) after methylation of the diastereoisomeric mixture of 2′-deoxyadenosine 5′-*O*-methylphosphorothioate (**2a,b**) obtained by partial digestion with snake venom phosphodiesterase. The ^{31}P n.m.r. parameters were: sweep width, 5500 Hz; pulse width, 15 μs; acquisition time, 2.98 s; collected in 32K; no. of transients, 19000; line broadening, 1.0 Hz.

Diastereoisomers (**3a,b**) are available directly from 2′-deoxyadenosine 5′-phosphorothioate by treatment of the monotriethylammonium salt with diazomethane. This forms the basis of our method of analysis. Since the configurations of (**3a,b**) have already been assigned as detailed, then the position of an ^{18}O isotope in a 2′-deoxyadenosine 5′-[^{16}O,^{18}O]phosphorothioate of unknown configuration at phosphorus can be determined by examination of the isotope shifts[12] in the ^{31}P n.m.r. spectrum of the resulting triester. These are dependent on bond order.[13] In one diastereoisomer the ^{18}O will be in a bridging position and will show a small isotope shift, and in the other it will be in a non-bridging position and will show a larger isotope shift.

We demonstrate this argument by the methylation of a sample of 2′-deoxyadenosine 5′-(S_p)-[^{16}O,^{18}O]phosphorothioate (**5**) obtained from the stereospecific cleavage of (S_p)-5′-*O*-(2′-deoxyadenosyl)-3′-*O*-thymidylphosphorothioate (**4**) in ^{18}O-labelled water using nuclease P1 (Scheme 2). This cleavage is known to proceed with inversion of configuration at phosphorus.[14] The ^{31}P n.m.r. spectrum of the methylated material is shown in Figure 2 (unlabelled deoxyadenosine 5′-[^{16}O]phosphorothioate was added to the sample



Scheme 2. A = Adenine; T = thymine. *Reagents:* i, Nuclease P1–$H_2{}^{18}O$; ii, CH_2N_2–MeOH.

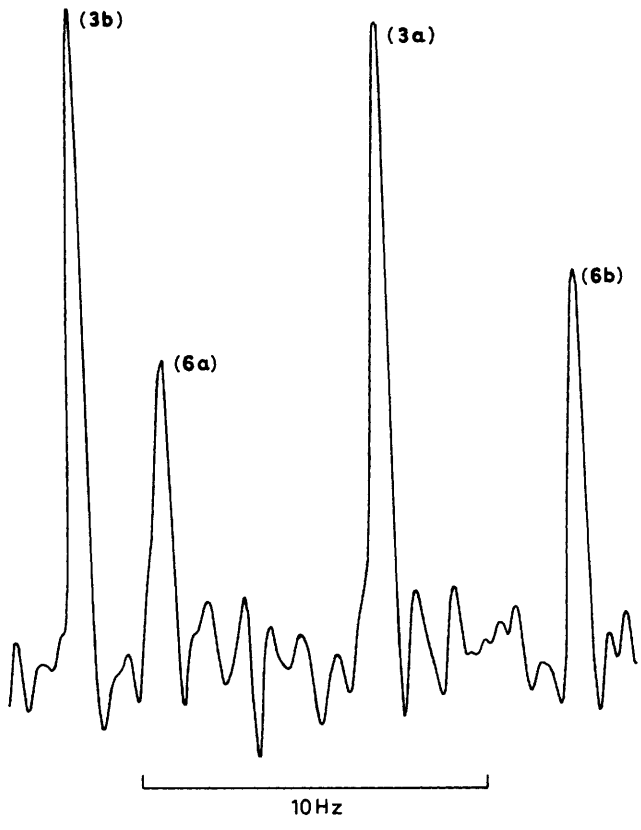


Figure 2. 121.5 MHz ^{31}P n.m.r. spectrum of the diastereoisomers of 2′-deoxyadenosine 5′-[^{16}O]- and -[^{18}O]-phosphorothioate-*S*-methyl-*O*-methyl ester (*ca.* 10 mM, in 3 : 1 DMF–CD_3OD), obtained from the methylation of a mixture of 2′-deoxyadenosine 5′-[^{16}O]- and -[^{16}O,^{18}O]-phosphorothioate in methanol with ethereal diazomethane. The ^{31}P n.m.r. parameters were: sweep width, 2000 Hz; pulse width, 8 μs; acquisition time, 8.4 s; collected in 16K; no. of transients, 6600; line broadening, −0.5 Hz; Gaussian broadening, 0.1 Hz.

before methylation to provide a reference). Two isotope shifts are observable relative to unlabelled material, a small one (2.3 Hz) on the downfield resonance and a larger one (5.7 Hz) on the upfield resonance. These shifts can be assigned to the isotopomers (**6a**) and (**6b**) respectively. The assignments are shown on the spectrum, and are identical to those which would have been predicted on the basis of the foregoing stereochemical argument had the ^{18}O position not been known.

Thus, since this approach can easily be extended to other systems it offers a simple and potentially general chemical method for the stereochemical analysis of small quantities of both deoxy- and ribo-nucleoside 5′-[^{16}O,^{18}O]phosphorothioates. Assignments of other nucleoside phosphorothioate systems will form the basis of a full report at a later date.

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