Facile Synthesis and ^{31}P NMR Spectra of a Double-Labeled Oligonucleotide d(Ap(^{17}O)Gp(^{18}O)Cp(^{16}O)T)

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^{31}P NMR has been shown to provide important structural and dynamic on nucleic acids.[1-5] In small oligonucleotides it is often possible to observe separate ^{31}P signals for each of the phosphate diesters, and thus ^{31}P is potentially able to probe the conformational dynamics along the entire sugar phosphate backbone. However, to obtain the greatest amount of information from the ^{31}P NMR spectra of oligonucleotides it is imperative that a rapid and convenient method be available to label the phosphates and hence identify the ^{31}P signals. Recently both thiophosphoryl labeling[6,7] and ^{17}O phosphoryl labeling[8,9] have been introduced. Since thiophosphoryl labeling introduces a new chiral center resulting in diasteromeric mixtures, and since sulfur substitution can perturb the structure of the nucleic acid,[6] oxygen labeling is generally preferable. Labeling of the phosphoryl oxygen with ^{17}O is quite attractive since the additional ^{17}O scalar relaxation of the second kind will cause extensive line broadening of the directly bonded ^{31}P nucleus NMR signal.[10] Petersheim et al.[9] have demonstrated the power of this technique in labeling one of the three phosphates in d(CpGpCpG) with ^{17}O using the solution-phase phosphotriester methodology and showing the expected effect on the ^{31}P NMR spectrum. ^{18}O isotopic substitution can also be used because this isotope is known to provide a small upfield isotopic shift on the ^{31}P signal.[11,12] As shown in this report, it is thus possible to unambiguously assign all three phosphate ^{31}P signals of the oligonucleotide tetramer d(ApGpCpT) by site-specific introduction of the three different oxygen isotopes in the three different phosphate diesters. Most importantly we show how multimilligram quantities of the labeled oligonucleotide may be readily obtained by a simple modification and scale-up of the solid-phase phosphoramidite oligonucleotide synthetic method.[13-15]

A manual modification of the automated solid-phase phosphoramidite method[15] was used for the synthesis of unlabeled d(ApGpCpT) and the phosphoryl labeled d(Ap(^{17}O)Gp(^{18}O)-Cp(^{16}O)T) tetramers. For the unlabeled tetramer, 8 μmol of Applied Biosystems (dimethoxytrityl)deoxythymidine bound to porous silica (40 μmol nucleotide/g silica) was introduced under nitrogen to a specially constructed sintered glass filter unit. Detritylation was accomplished with 3% trichloroacetic acid in CH_2Cl_2. All nonaqueous solvents were rigorously dried and purified prior to use. An 8-fold excess of tetrazole-activated *N*-benzoyl-5'-(dimethoxytrityl)deoxycytidine was coupled to the free 5'-hydroxyl of the support bound thymidine. Capping of any unreacted thymidine was accomplished with the addition of (dimethylamino)pyridine, acetic anhydride, and 2,6-lutidine. This

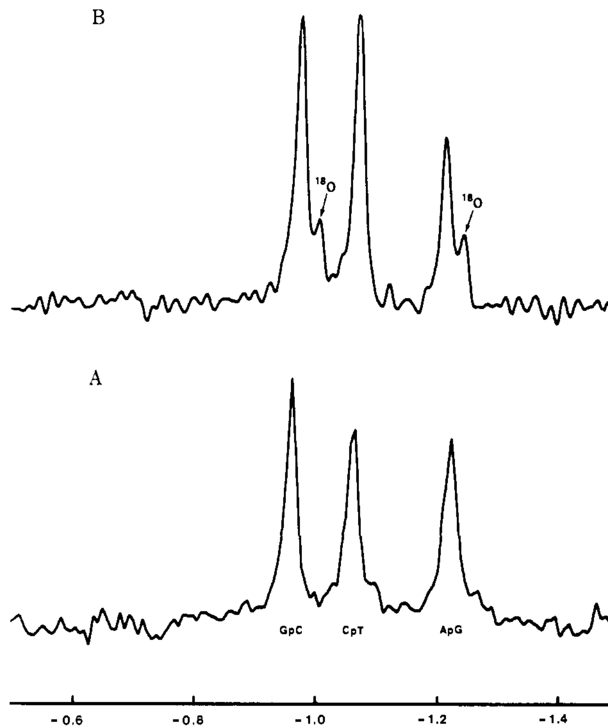

Figure 1. (A) ^{31}P NMR spectrum of d(ApGpCpT) and (B) d(Ap(^{17}O)Gp(^{18}O)Cp(^{16}O)T) at 32.4 MHz, 30 °C, in 1 mM EDTA, pH 7, D_2O on a Bruker WP-80 spectrometer.

protected phosphite dimer was oxidized with H_2O and iodine. The cycle was repeated twice with the detritylation step, and addition of the appropriate protected dimethoxytrityl nucleoside phosphoramidite yielded the fully protected phosphate triester tetramer. Substitution of oxygen-labeled water at the appropriate iodine-/water oxidation step introduced the phosphoryl oxygen label. The total time required for the synthesis of the support-bound fully protected (labeled or unlabeled) tetramer was 5 h, and the average coupling efficiency monitored by the amount of liberated dimethoxytrityl cation was >95%.

The support-bound tetramer was treated with thiophenol and NH_4OH. The phosphate diester tetramer liberated from the support was purified by preparative C-18 reverse-phase HPLC. The partially protected tetranucleotide (>80% of the crude mixture was pure tetranucleotide) was collected and detritylated with 80% acetic acid, desalted on a sephadex G50-40 column with 10 mM triethylammonium bicarbonate as the eluant, and lyophilized. The purity of the tetramer was confirmed by HPLC (overall yield based on support bound thymidine was 55-60%).

The isotopic enrichment of the water for ^{17}O labeling was 51.1% ^{17}O and 39% ^{18}O, and for ^{18}O labeling was 49.3% ^{18}O and 0.3% ^{17}O. Shown in Figure 1A is the spectrum of the unlabeled tetramer. The three ^{31}P signals have identical chemical shifts to the phosphate diester signals of an authentic sample of d(pApGpCpT) from Collaborative Research and a ^1H NMR spectrum confirms the tetramer structure. The ^{31}P NMR spectrum of the labeled tetramer (Figure 1B) allows the immediate unambiguous identification of the signals. Thus, the upfield signal at −1.22 ppm is reduced in intensity relative to the other main signals at −1.067 and −0.965 ppm. The ^{17}O directly bound to the phosphorus will broaden the high-resolution ^{31}P signal below detection, and only the ^{18}O labeled and unlabeled signals will be observable. Because ^{17}O was introduced into the ApG phosphate, the −1.22 ppm signal may be assigned to ApG. Since the ^{17}O water also is enriched with ^{18}O, an ^{18}O isotope shifted ^{31}P signal is also found 0.032 ppm upfield of the unlabeled ApG signal. Similarly the observation of the ^{18}O isotope shifted signal upfield of the −0.965 ppm signal identifies the Gp(^{18}O)C phosphate (note that the combined integrated intensity of the labeled and unlabeled GpC signals is the same as the integrated intensity of the *single* middle signal at −1.067 ppm, which thus is identified as the CpT phosphate). This

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level of enrichment should have provided a 1:1 peak area ratio for the Gp(^{18}O)C and Gp(^{16}O)C peaks. As shown in Figure 1, however, the ratio of the ^{18}O isotope shifted signal to the unlabeled GpC signal is only 0.20:1. Similarly while the ratio of the ^{17}O-labeled signal of ApG (obtained by difference) to the ^{18}O-labeled signal of ApG fits nicely with the original ^{17}O and ^{18}O enrichement of the water, the ^{16}O-labeled signal is much larger than would be expected on basis of the 9.9% ^{16}O content of the ^{17}O water. Thus, as also noted by Seela et al.,[8] the phosphite must undergo oxidation by molecular oxygen, or extraneous water (all ^{16}O of course) in the iodine oxidation step must still be present in our dried nonaqueous solvents. This does not present any problem, however, for our signal identification methodology.

As pointed out by Petersheim et al.[9] oxygen labeling of the nucleic acids is clearly a superior method for ^{31}P signal assignment compared to methods dependent upon correct (and often difficult) assignment first of the ^{1}H NMR signals.[16,17] Perhaps even greater utilization of this phosphoryl oxygen-labeling method will develop since rapid and convenient introduction of the labels requires no special capability other than the scale-up of the highly efficient solid-phase phosphoramidite synthesis as shown here. Using the two-dimensional ^{31}P/^{1}H correlated spectral methods,[16] we can now unambiguously identify the ^{1}H NMR signals coupled to the *assigned* ^{31}P signals. Because of spectral overlap, even with 2-D methods, ^{1}H signal assignments in oligonucleotides much longer than tetramers will be difficult.[18] However, this should not be a problem using the ^{31}P-labeling method combined with 2-D NMR techniques. It should also be noted that we have been able to identify all three ^{31}P signals from a doubly labeled tetramer. Since we can introduce different ratios of the three oxygen isotopes at each cycle of the phosphite oxidation, there should be no difficulty in assigning at least six or more phosphate signals in each preparation of a labeled hexamer or even larger oligomer.

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Additions and Corrections

A Simple Model for the Interaction Potentials in Electron-Transfer Reactions: Application to the H_2^+/H_2 System [*J. Am. Chem. Soc.* **1983**, *105*, 6775.]. CHYUAN-YIH LEE and ANDREW E. DEPRISTO*

Page 6778: Equations 44e and 44f are incorrect. They should read:

$$V_{DD}^{(e,B+)} = \sum_{i=1}^{2}\sum_{j=3}^{4} \frac{-1}{4R_{ij}} + \frac{C_A^2}{2R_{ij}}(1 + \xi R_{ij}) \exp(-2\xi R_{ij}) \quad (44e)$$

$$V_{XX}^{(e,A+)} = \sum_{i=1}^{2}\sum_{j=3}^{4} \frac{-1}{4R_{ij}} + \frac{C_B^2}{2R_{ij}}(1 + \xi R_{ij}) \exp(-2\xi R_{ij}) \quad (44f)$$

The correct equations, above, were used in all the calculations presented in the article.