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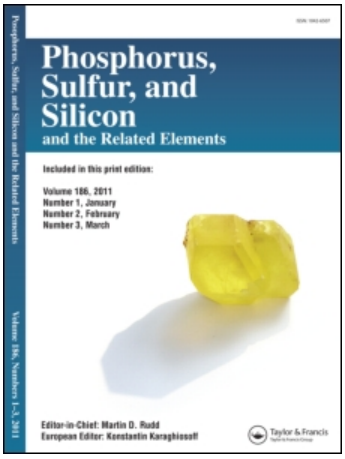

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Oxygen Isotope and Sulfur Labeling of Phosphoryl Groups and 2-Dimensional NMR Methodology for Assignment of ^{31}P and ^{1}H Signals of Oligonucleotides

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OXYGEN ISOTOPE AND SULFUR LABELING OF PHOSPHORYL GROUPS AND 2-DIMENSIONAL NMR METHODOLOGY FOR ASSIGNMENT OF ^{31}P AND ^{1}H SIGNALS OF OLIGONUCLEOTIDES.

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Abstract Because of spectral overlap, even with 2-D NMR methods, ^{1}H and ^{31}P signal assignments in oligonucleotides much longer than tetramers is difficult. However, by chemically introducing site-specific ^{17}O, ^{18}O, and S labeling in the phosphoryl groups of oligonucleotides, it is possible to unambiguously assign the ^{31}P peaks. Thus, it is possible to assign all three phosphate ^{31}P signals of the oligonucleotide tetramer d(ApGpCpT) by site-specific introduction of the three different oxygen isotopes into the three different phosphate diesters. Using two-dimensional $^{31}P/^{1}H$ correlated spectral methods we can also unambiguously identify the ^{1}H NMR signals coupled to the assigned ^{31}P signals. In the latter, only those protons which are scalar coupled to the ^{31}P nucleus are observed in the 2-D heteronuclear spectrum. Finally by $^{1}H/^{1}H$ COSY and NOESY we can identify the other protons of the oligonucleotides. This methodology is not dependent upon any assumed B-DNA structure as is required in other recent 2-D oligonucleotide assignment techniques. Assignment of signals in the actinomycin D intercalating d(ApGpCpT) tetramer complex, d(CGCAGAATTCGCG), and lac operator pseudo-fragment, d(TGTGAGCGCTCACA), are described.

Nuclear magnetic resonance spectroscopy is now able to provide detailed 3-dimensional structures of oligonucleotide duplexes and nucleic acid complexes. Unfortunately, of the six torsional angles that largely define the backbone structure, only the four involving the deoxyribose ring have been shown to be directly amenable to analysis by NMR techniques. We have proposed that ^{31}P NMR spectroscopy is potentially capable of providing information on the

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most important remaining two torsional angles involving the phosphate ester bonds. Our studies[1-3] indicated that a phosphate diester monoanion in a gauche, gauche (g,g) conformation should have a ^{31}P chemical shift 1.5-2.5 ppm upfield from an ester in a non-g,g conformation.

Definition of phosphate diester torsional angles ω,ω': −g,−g (left); −g,t (right).

Our earlier ^{31}P NMR studies on poly- and oligonucleic acids[1-5] supported our suggestion that the base stacked, helical structure with a gauche, gauche phosphate ester torsional conformation should be upfield from the random coil conformation.

$(dAGCT)_2$

dCGCAGAATTC-GCG
dGCG-CTTAAGACGC

1

dCGTGAATTCGCG
dGCGCTTAAGTGC

2

dTGTGAGCGCTCACA
dACACTCGCGAGTGT

3

4 (lac operator pseudo fragment)

We have recently shown that a ^{17}O- phosphorylation scheme promises to provide an important methodology for ^{1}H (and ^{13}C NMR) signal identification[5]. Using the solid-phase phosphoramidite method, we have synthesized oligodeoxynucleotides 1 - 4. We can readily introduce ^{17}O (or/^{18}O-) labels in the phosphoryl groups by replacing the I_2/H_2O in the oxidation step of the phosphite by $I_2/H_2{}^{17}O$ (40%)or $H_2{}^{18}O$[5,7]. Similarly, Stec et al.[8] have replaced one of the nucleoside phosphates by a nucleoside thiophosphate by using sulfur/2,6-lutidine in the oxidation step at the appropriate cycle. By synthesizing the corresponding mono-^{17}O phosphoryl labeled oligonucleotide (each phosphate is separately substituted along the chain), we can identify the ^{31}P signal of that phosphate diester. The quadrupolar ^{17}O nucleus (generally ca. 40% enriched) broadens the ^{31}P signal of the directly attached ^{17}O-labeled phosphate and we only observe the high-resolution signal of the remaining 60% non-quadrupolar broadened phosphate at the ^{17}O-labeled site.[5-7] In this way each synthesized oligonucleotide with a different monosubstituted ^{17}O-phosphoryl group allows identification of all phosphate ^{31}P signals.

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Since we know the nucleoside to which the ^{31}P signal is associated by P = S or P = $^{17}O/^{18}O$ labeling, we are able to identify the ^{1}H signals coupled to this phosphorus atom. (H3', H5', H5'' via $^{31}P/^{1}H$ heteronuclear COSY, and by $^{1}H/^{1}H$ COSY, we can identify H4', H2', and H1'). Recent advances in NOE techniques, particularly 2-D NOESY,[9,10] have shown that NOE's between the sugar and stacked base pairs can allow the identification of the base ^{1}H and most of the sugar ^{1}H signals. These sequential resonance assignment methods via 2D NOESY and COSY, however, generally require knowing the structure in advance (i.e. assuming a B-DNA double helix geometry). We have been able to unambiguously make some of the ^{1}H signal identifications by a $^{31}P/^{1}H$ heteronuclear COSY (plus $^{1}H/^{1}H$ COSY, NOESY) 2-dimensional NMR techniques without recourse to any assumed initial geometry.[5] This is particularly important if the double helix possesses unusual geometry. We have now applied this methodology to oligonucleotides 1 - 4.

Patel and coworkers[11] have shown that base loop out and mismatch in duplexes 2 and 3 provide very interesting ^{31}P spectral shifts. Whereas the ^{31}P spectral dispersion is < .6 ppm in normal B-DNA double helices 1 and 4, new signals are shifted upfield and downfield from the ''normal'' double helical phosphate ^{31}P signals with a total spread > 1 ppm in duplexes 2 and 3. By ^{17}O and S labeling we have been able to assign these perturbed ^{31}P signals to phosphates in non-Watson-Crick regions of the duplexes.

Assignment of most of the ^{1}H and all 13 of the ^{31}P signals of the tetradecamer 4 has followed the ^{17}O-labeling, 2-D NMR methodology.[12] Thus as shown in Figure 1, the ^{31}P NMR spectra of

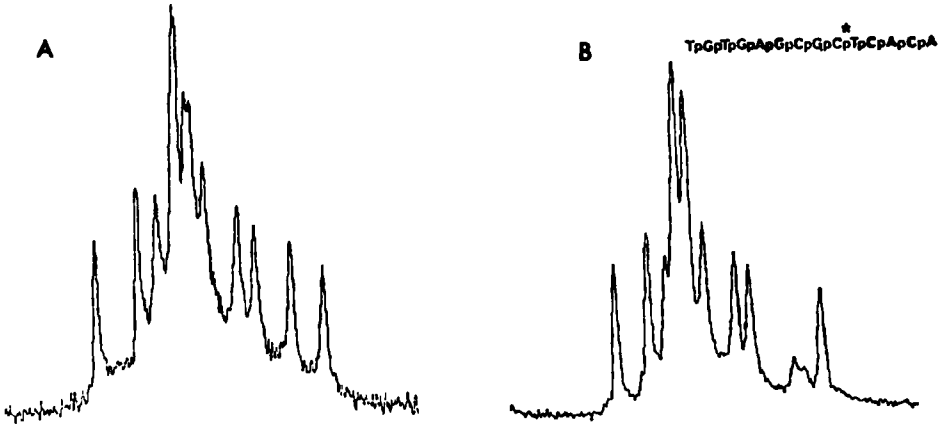

FIGURE 1 P-31 NMR spectra of 14-mer 4 (A) and O-17 labeled 14-mer at indicated position.

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various regio-specifically labeled phosphates in the duplex 4 allow clear assignment of the ^{31}P signals. The 2D $^{31}P/^{1}H$ two-dimensional chemical shift correlated spectrum of 4 also provides identification of the 3'- and 5'- deoxyribose coupled protons to the identified ^{31}P signals of the phosphates. The other ^{1}H signals have been identified through $^{1}H/^{1}H$ COSY and $^{1}H/^{1}H$ NOESY.

By using ^{17}O and thiophosphoryl-labeling of the oligonucleotides we can also determine the site and detailed structure of drug binding to oligonucleotides. Thus as confirmed by ^{31}P NMR studies of Petersheim et al.[6] and our laboratory[5] actinomycin D has specificity for G-C base pairs and as Patel[13] had earlier shown two ^{31}P signals are shifted 1.5-2.6 ppm downfield from the other signals in the actinomycin D complexes of d(CGCG) and d(ATGCAT).

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