THE CHEMICAL AND ENZYMATIC SYNTHESES OF SPECIFICALLY DEUTERATED DINUCLEOSIDE MONOPHOSPHATES*

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SUMMARY

Proton magnetic resonance (pmr) studies of nucleic acid struc--ture are currently hampered by extensive overlapping of signals, particularly in the ribose region. An approach with considerable promise involves the preparation of oligomers with specifically deuterated nucleotidyl units at known positions. In this publi--cation, we report the synthesis of five dinucleoside monophos--phates in which the 3'-nucleotidyl unit is deuterated. These compounds were prepared by standard chemical procedures and an enzymatic approach using ribonuclease T_1. The deuterated dimers give pmr spectra which are much simpler than their protio analogs, thus permitting unambiguous peak assignments.

INTRODUCTION

Proton magnetic resonance (pmr) spectroscopy plays an important role in conformational studies of nucleic acids and their constituents in solution. Current advances in instrumentation have made possible complete analyses of pmr spectra for mononucleotides in solution[1,2] thereby providing information

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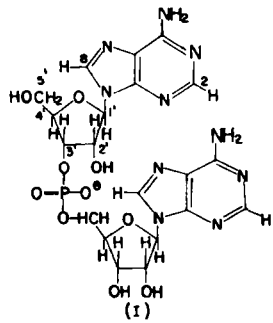
relating to major conformational features, including rotational isomerism about the glycosidic bond (*syn* vs. *anti* orientations)[3,4], puckering of the ribose ring[5], and orientation of exocyclic groups[6-8]. In principle, similar information should also be obtainable for dinucleoside monophosphates and longer oligomers. However, in practice this is not realized because extensive overlapping of signals prevents an unequivocal proton assignment. Studies on dinucleoside monophosphates have, therefore, been limited to the base and anomeric protons where reasonable indirect assignments can be made[9-11].

Recently, we have been able to make the first complete proton resonance assignment for a dinucleoside monophosphate, ApA[12] (I). This was accomplished at 220 MHz with the aid of 2,1',2',3',4',5',5'-heptadeuterioadenylyl-(3'-5')-adenosine (*ApA), a dinucleoside monophosphate which has the Ap-unit deuterated. From the derived spectral data, it was possible to deduce important conformational features for the dinucleotide in solution. The preparation of oligonucleotides with deuterated nucleotidyl units in known positions is



(I)

thus essential in nucleic acid structure determination. In this communication, we describe two alternative procedures, one involving direct chemical synthesis and the other an enzymatic synthesis, for preparing selectively labelled

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*Deuterated

dinucleoside monophosphates. Both approaches were used to prepare dimers in which the 3'-nucleotidyl unit is fully deuterated. For pmr analysis, it was necessary that only one of the units be deuterated; therefore, no dimers in which the 5'-nucleotidyl unit was labelled were prepared.

EXPERIMENTAL

Reagents

Adenosine, cytidine, uridine, 2',3'-isopropylideneuridine, 2'-and 3'-isomers of adenosine-,cytidine-, uridine- and guanosine-monophosphate, GMP-2', 3'-cyclic phosphate (Gp!), ApA, ApU, GpC, GpU, and GpA were purchased from Sigma Chemical Company, St. Louis, Missouri. Ribonuclease T_1 was purchased from Calbiochem Company, San Diego, California. Dicyclohexylcarbodiimide was purchased from Schwarz/Mann, Orangeburg, New York.

Chromatography and Instrumentation

Paper chromatography was carried out on Whatman No. 3MM paper by the descending techniques using the following solvent systems: Solvent A, 1-propanol: concentrated ammonium hydroxide: H_2O (55:10:35, v/v); Solvent B, saturated ammonium sulfate: 1 M sodium acetate: isopropanol (80:18:2, v/v). Pmr spectra were recorded on a Varian HR-220 spectrometer at probe temperature (17-18). Ultraviolet readings were obtained with a Beckman DU spectrophotometer.

Isolation of Deuterated Mononucleotides

One-hundred and ten grams of frozen lysed *Synechococcus lividus* algae cells grown in D_2O[+] were allowed to come to room temperature and the cells were then stirred for 20 minutes in 600 ml of 7% trichloroacetic acid (TCA) solution at 0° . The aqueous phase was removed by centrifugation at 8000 rpm for 15 minutes followed by decantation. Following repetition of the above procedure with 600 ml of 1% TCA, the residue was transferred to a 1 liter flask, 400 ml

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[+]Kindly supplied by Drs. H. L. Crespi and J. J. Katz of the Chemistry Division, Argonne National Laboratory.

of ethanol and 10 ml of ether added, and the mixture brought to a boil. After cooling, the mixture was filtered through a sintered glass funnel layered with celite. The remaining solid was washed with ether and dried. The dried mass was transferred to a 500 ml boiling flask and refluxed in 50% MeOH:$CHCl_3$ (300 ml) for 30 minutes. After cooling, the solid was collected by filtration (sintered glass funnel layered with celite), and washed with large quantities of ether. The dried solid was then transferred with shaking into 300 ml of 1 N KOH and the resulting alkaline solution was incubated at 37° for 20 hours. Neutralization with 35% $HClO_4$ yielded a mixture containing solid material. The latter was removed by filtration and the solution was made acidic to precipitate out protein and DNA. Separation of most of the $KClO_4$ from the neutralized solution was achieved by allowing the filtered solution to stand at 5° for several days and then decanting to remove the $KClO_4$ which precipitated.

At this point the solution was still highly colored (dark green). It was therefore diluted to about 2 liters and the entire volume passed through a DEAE cellulose column. After washing with water the mononucleotides were eluted with 0.1 N NH_4HCO_3, leaving most of the colored material on the column. The NH_4HCO_3 was then removed by repeated evaporation with H_2O, and the mixture of 2',3'-nucleotides was applied to a 90 cm X 3.5 cm Dowex-50 NH_4^+ column[13] which had been previously equilibrated with 0.25 M ammonium formate, pH 4.1, and eluted with this same solvent system. Five to 6 ml fractions were collected and the order of elution of the nucleotides is as follows.

Fraction No.	A_{280}/A_{260}	TOD_{260} Pooled Fractions	Nucleotides
52-65	.31- .33	1353	2'- and 3'-*UMP
68-81	.61- .69	2580	2'- and 3'-*GMP
84-93	.19- .22	3080	2'- and 3'-*AMP
100-115	1.1 -1.4	1657	2'- and 3'-*CMP

2'- and 3'-*AMP isomers were separated by preparative paper chromatography

using solvent system B. 3'-*AMP (1655 OD_{260} units) was desalted by absorption onto charcoal, washing with water, and elution with 2% NH_4OH in 50% ethanol.

2'- and 3'-*GMP were converted to 2',3'-cyclic *GMP following the method described by Michelson[14].

Synthesis of deuterated dinucleoside monophosphates

Chemical Synthesis

2,1',2',3',4',5',5'-Heptadeuterioadenylyl-(3'-5')-adenosine, (*ApA)

The pyridinium salt of 2,1',2',3',4',5',5'-heptadeuterio-N,$O^{2'}$, $O^{5'}$-tribenzoyladenosine-3'-phosphate[15] (0.1 mmole), N,N^1,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine [16] (0.12 mmoles), and pyridinium Dowex-50 (500 mg) were dried by repeated evaporation with anhydrous pyridine and the highly anhydrous mixture dissolved in 3 ml of pyridine. Dicyclohexylcarbodiimide (DCC), 0.4 g, was then added and the reaction mixture was sealed and stirred in the dark for 4 days. Fifty percent aqueous pyridine (20 ml) was added to terminate the reaction and the resulting mixture was stirred for three hours. This procedure was followed by pentane extraction to remove any unreacted DCC. After filtering to remove insoluble material the aqueous pyridine solution was evaporated to dryness. Blocking groups were removed by treating the dry residue overnight with 50 ml of 15 M methanolic ammonia at 0°. After removal of the solvent by evaporation the residue was dissolved in approximately 2 ml of water and streaked onto Whatman No. 3 MM paper. The paper was developed overnight in solvent system A and the streak corresponding to ApA was cut out and eluted with water. The eluted material was then directly applied to a DEAE cellulose column and after washing with a large volume of water, 310 OD_{260} units of *ApA were eluted with 0.025 M NH_4HCO_3 solution. The salt was removed by evaporation with water and the compound further characterized by alkaline hydrolysis to monomer units.

2,1',2',3',4',5',5'-Heptadeuterioadenylyl-(3'-5')-uridine, *ApU

2',3'-Isopropylideneuridine (0.075 mmole), the pyridinium salt of 2,1',2',3',4',5',5'-heptadeuterio-N, $O^{2'}$, $O^{5'}$-tribenzoyladenosine-3'-phosphate (0.056 mmoles), and 250 mg of pyridinium Dowex-50 were rendered anhydrous by repeated evaporation with anhydrous pyridine. The dried residue was dissolved in 2 ml of pyridine and to this was added 200 mg of DCC. The reaction, extraction, and filtration procedures were identical to those employed in the preceding synthesis. After removal of the methanolic-ammonia by evaporation the residue was then treated with 25 ml of 88% formic acid, and left to stand for 100 minutes. The formic acid was then evaporated under reduced pressure and the remaining reaction mixture dissolved in 2 ml of water and streaked onto Whatman 3 MM paper. Since solvent system A did not completely resolve *ApU from *Ap- both streaks were cut out, eluted, and placed on a DEAE cellulose column. After washing with water 85 OD_{260} units of *ApU were eluted with 0.025 M NH_4HCO_3; a higher salt concentration was necessary to elute *Ap-. *ApU was characterized by alkaline hydrolysis to 2' and 3'-*AMP and uridine.

Enzymatic Synthesis

1',2',3',4',5',5'-Hexadeuterioguanylyl-(3'-5')-cytidine, *GpC

The procedure followed is essentially that previously published by Mohr and Thach[17] with the exception that Gp! used in this work was fully deuterated in the ribose ring. The reaction vessel was a calibrated 15 ml centrifuge tube fitted with a 24/40 standard taper joint. To this tube were added 240 OD_{260} units of Gp!(d_6), 21.4 mg of cytidine, and 0.2 ml of 0.01 M phosphate buffer, pH 7.0. After cooling to 0°, 100 units of ribonuclease T_1 (10 µl) were added and the flask was attached to a rotary evaporator. The solution was evaporated to a volume of about 0.08 ml at 0° then removed from the evaporator and shaken further; the total reaction time being

approximately 90 minutes. The volume was then made up to 0.6 ml with cold water. This cold solution was streaked onto Whatman 3 MM paper, sprayed quickly with solvent system A, and the chromatogram immediately placed into the solvent chamber. Material with an R_f value identical with an authentic sample of GpC was collected and eluted with water. The *GpC was placed on a DEAE cellulose column, washed with water, and eluted with 0.025 M NH_4HCO_3, yielding 95 OD_{260} units of *GpC. The salt was removed by repeated evaporation with water.

1',2',3',4',5',5'-Hexadeuterioguanylyl-(3'-5')-uridine, *GpU

The synthetic procedure is identical with that for *GpC except cytidine was replaced by 31.0 mg of uridine. The yield was comparable to that obtained for *GpC.

1',2',3',4',5',5'-Hexadeuterioguanylyl-(3'-5')-adenosine, *GpA

Because of the limited solubility of adenosine in aqueous solution, a slightly different procedure was followed in the synthesis of *GpA. *Gp (200 OD_{260} units), 10 mg of adenosine and 0.1 ml of 0.01 M phosphate buffer were heated to dissolve the adenosine. The solution was quickly cooled to 0° and 100 units of Ribonuclease T_1 was added. The reaction mixture was shaken at 0° for 90 minutes and the *GpA then isolated as described previously. A small amount of adenosine precipitated during the course of the reaction and could account for the low yield of 25 OD_{260} units for *GpA. Low yields were also reported by Mohr and Thach[17] and this was attributed to the low solubility of adenosine in aqueous solution.

RESULTS AND DISCUSSION

Two approaches were used in the present work to synthesize specifically ^2H labelled dinucleoside monophosphates. The chemical approach followed procedures developed by Khorana and coworkers and was used to prepare *ApA and *ApU, in which the 3'-nucleotide is fully deuterated. Both dimers were

chromatographically identical with respective reference samples of ApA and ApU. A further check of the incorporation of the deuterated 3'-nucleotide *Ap- in the dimer was made by measuring the dimer proton resonance spectrum at 220 MHz and comparing it with the corresponding spectrum for fully protonated ApA recorded under the same conditions of concentration, pD, and temperature. The result confirmed the absence of signals corresponding to the 3'-nucleotide in the *ApA spectrum and indicated, moreover, that incorporation of the deuterated nucleotide occurs without any exchange-loss of deuterium atoms in the ribose or adenine rings (except for H-8) in the course of the synthesis reactions. Similar results were obtained for *ApU.

The observation of an H-8 signal for *Ap- and other deuterated purine nucleotides in dimers is not unexpected. Earlier work has shown purine H-8 protons to be quite labile even at neutral pH[18]. The presence of an H-8 signal for *Ap- of the dimers (spectrum published in reference 12) can therefore be attributed to an exchange of ^{2}H-8 with available solvent protons, most likely in the nucleotide extraction steps. H-2 is much less labile, and conditions encountered at various steps of the extraction and synthesis are not drastic enough to produce any loss of ^{2}H-2.

The overall yield of the synthesis procedure is somewhat lower than reported for ApA[19]. Nevertheless, an amount of purified material sufficient for meaningful NMR measurements was readily obtained from approximately 100 grams of lysed deuterated algal cells.

Although the chemical approach produced acceptable material, the lengthy procedures and time span involved led us to consider an alternative and more rapid biosynthetic route described by Mohr and Thach[17]. The enzymatic synthesis is procedurally straightforward, requires less starting material, and is carried out under mild solvent conditions. This approach was used to synthesize a number of dimers containing a guanyl residue in the 3' position.

In each case the products were chromatographically identical with corresponding protonated analogs, and pmr measurements confirmed incorporation of the deuterated 3'-nucleotide. Figure I shows the spectrum for enzymatically synthesized *GpC along with the spectrum for GpC. It is apparent that only one set of nucleotide signals, i.e., those due to -pC are observed for *GpC. Comparison of the two spectra furthermore permits an

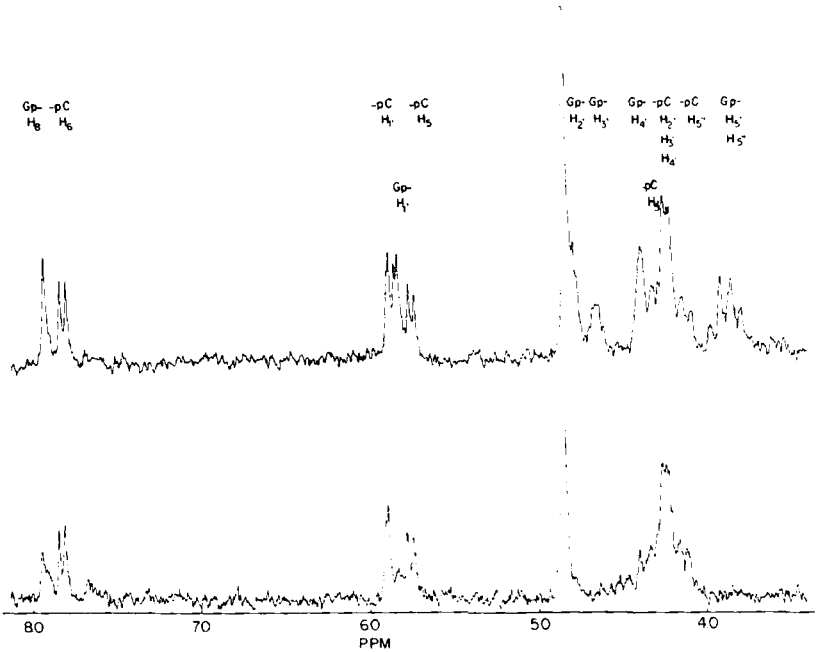

FIGURE LEGEND

220 MHz pmr spectra of GpC (upper) and *GpC (lower) in D_2O at 18°. Solutions were prepared in 100% D_2O and samples were contained in 5 mm capillary spinning tubes. Both spectra were measured for 0.02 M solutions, pD = 6.8 and instrumental settings were the same in each case. The hydroxyl and amino proton resonances were not observed because of exchange with the solvent.

identification of signals corresponding to the individual nucleotidyl units of GpC. When combined with results of ^{1}H-^{1}H and ^{1}H-^{31}P decoupling experiments, a complete assignment of the GpC spectrum can be achieved as shown in Fig. 1. As is the case for chemically synthesized molecules, no exchange of ^{2}H atoms, other than at the C-8 position, was observed in any enzymatically prepared dimers.

A further advantage of the enzymatic procedure over the chemical is that unused nucleotide starting materials are readily recoverable intact in the former method--an important consideration when dealing with small amounts of deuterated nucleotides. On balance the enzymatic method using ribonuclease T_1 has an advantage over the direct synthetic approach although its adaptability is limited to 3'-guanyl dimers.

CONCLUSIONS

The present work shows that dinucleoside monophosphates containing a deuterated nucleotidyl unit at a known position can be prepared successfully, either by direct chemical synthesis or by biosynthetic procedures, in amounts suitable for spectroscopic studies. The synthesis of such labelled dimers makes possible complete and unambiguous signal assignments in proton spectra for these molecules. Moreover, the technique of selectively deuterating one (or more) of the nucleotidyl units can be extended in principle to higher oligomers and efforts are currently underway in this direction.

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