SYNTHESIS AND CONFORMATIONAL ANALYSIS OF DEUTERIUM-LABELED ADENYLYL-(3'-5')-ADENOSINE AND RELATED SUBSTANCES

BY

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A thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(Pharmacy)

at the

UNIVERSITY OF WISCONSIN-MADISON

1980

To My Parents

and My Wife

their love has made

everything possible

for me

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and gratitude:

To my advisor, Professor Phillip A. Hart, whose concern, encourgement, support, and guidance have made this thesis possible. I have profitted greatly from his good example, both as a person and as a scientist;

To my parents for their unfailing love, support, advice, and encourgement throughout the years - they gave unselfishly so that I could always have the best opportunities;

To my sisters and brother, especially Jo-Ying and Jo-Yung, for their love and interest;

To Winkle and Julia Lee for their help and concern;

To Dr. Charles Anderson and Shu-Fen Lin for their friendship;

To Miwako Fujimoto for her excellent typing and, Dr. Yoshinori Fujimoto for his assistance in this respect;

And, most of all, to my wife for her deepest love which was essential for the completion of this study.

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ABBREVIATIONS

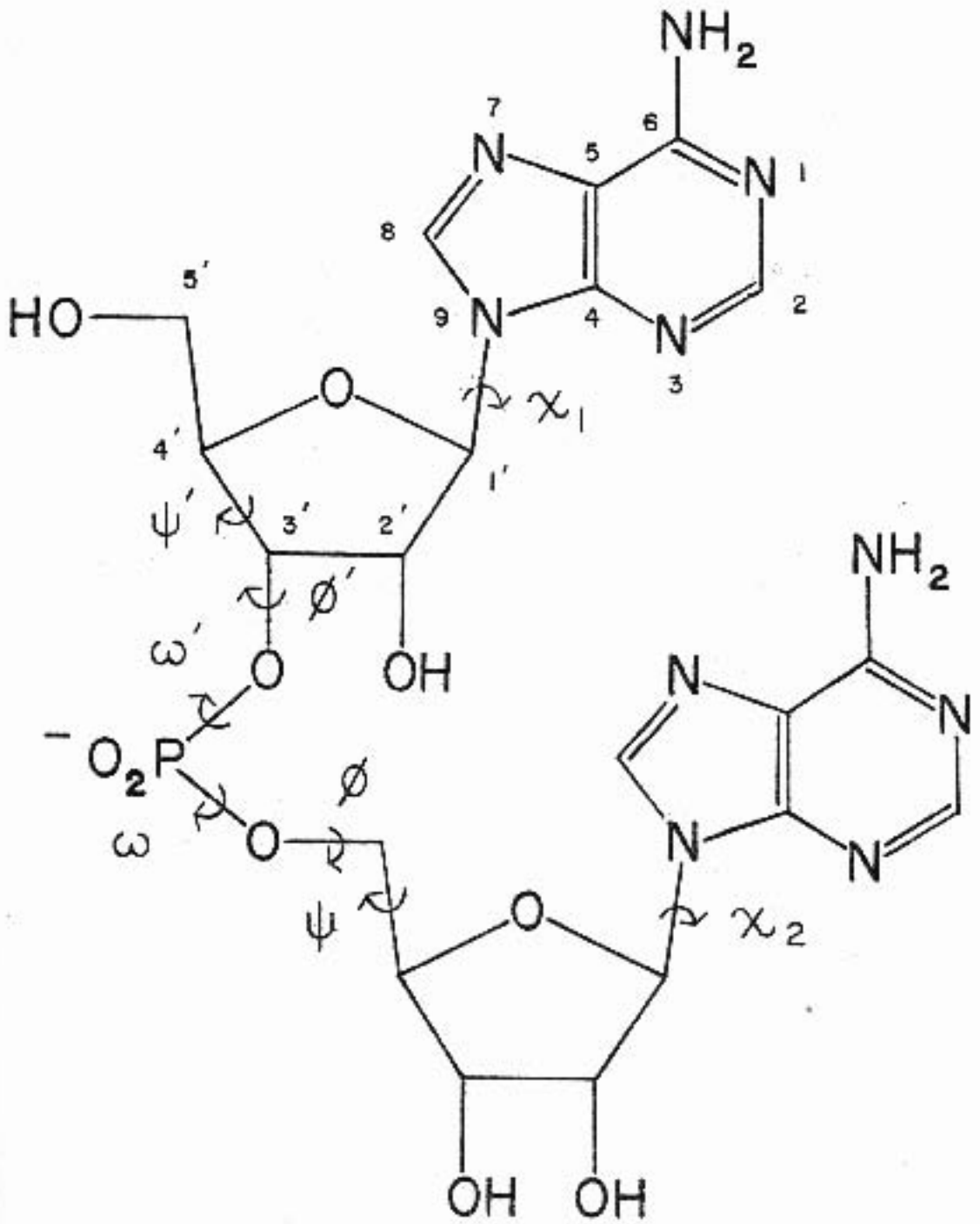
3'-AMP	adenosine 3'-phosphate
5'-AMP	adenosine 5'-phosphate
ApA	adenylyl-(3'-5')-adenosine
d_3-ApA	adenylyl-(3'-5')-4',5'-[$^2H_3$]adenosine
ApApA	adenylyl-(3'-5')-adenylyl-(3'-5')-adenosine
d_3-Ap(iA)	adenylyl-(3'-5')-2',3'-O-isopropylidene-4',5'-[$^2H_3$]adenosine
CD	circular dichroism
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetate
G	gauss
3'-GMP	guanosine 3'-phosphate
5'-GMP	guanosine 5'-phosphate
LAH	lithium aluminum hydride
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
TBAF	tetra-n-butylammonium fluoride
TBDMS	t-butyldimethylsilyl
UV	ultraviolet

I. Introduction

It is increasingly clear that nucleic acids, both single- and double-stranded, are flexible, rather than rigid, biopolymers in the solution state. The extent of conformational fluctuations that characterize this flexibility are, no doubt, highly variable from one specific nucleic acid to another. The existence of this flexibility, however, makes the investigation of conformational properties of nucleic acids exceedingly difficult. Adding to this difficulty is the likelihood, indeed the certainty, that polymer conformation is not a linear combination of the conformations of its monomeric units. Nevertheless, there must be certain conformational parameters of the monomer or small oligomer, which are either rather insensitive toward various conditions or can be so well studied, that they can be extrapolated to the polymeric system where they can, at least, serve to suggest an approximate overall conformation when combined with established or plausible polymer properties. The geometry of an amide bond and how knowledge of it contributes to our understanding of protein conformation is an example. Most conformational

analyses of nucleosides, nucleotides, and oligonucleotides that have been done were motivated by the hope of finding those conformational parameters and applying them to describe structures of nucleic acids.

This thesis concerns elements of the structure of adenylyl-(3'-5')-adenosine (ApA) and closely related substances. The chemical structure, torsion angle notation, and numbering scheme of ApA are given in Figure 1. The structure is usually partitioned into two parts for easy reference: Ap- (a 3'-unit) designates a adenosyl unit with the phosphate at C3' and pA- (a 5'-unit) designates a unit with the phosphate at C5'. The sugar-phosphate backbone linkage is comprised of the C4'-C3'-O3'-P-O5'-C5'-C4' bond network and denoted by torsion angles ψ', ϕ', ω', ω, ϕ, and ψ. Numerical designations for the dihedral angles, shown in Figure 1, utilized in the present work are consistent with that proposed by Sundaralingam *et al.*[1] (Table I). The commonly used, although less versatile, gauche-trans designations of backbone torsion angles are given as well. For the glycosyl torsion angle χ, two major domains describing the disposition of the base relative to the sugar are loosely referred to as syn ($0^{\circ}\pm90^{\circ}$) and anti ($180^{\circ}\pm90^{\circ}$). The furanose ring


NH2
7
6
5
N
N
1
8
HO
5'
9
N
4
N
2
3
O
χ1
4'
1'
ψ'
3'
2'
ø'
ω'
O
OH
NH2
N
N
-O2P
ø
ω
O
N
N
ψ
O
χ2
OH
OH

ApA

Figure 1

Table I

Dihedral Angle Definitions

Angles*	Atoms (A-B-C-D)**	Specific designations		
ϕ'	C4'-C3'-O3'-P	60°	180°	-60°
	P-O3'-C3'-H3'	t	g^-	g^+
ω'	C3'-O3'-P-O5'	60°	180°	-60°
	C3'-O3'-P-O5'	g^+	t	g^-
ω	O3'-P-O5'-C5'	60°	180°	-60°
	O3'-P-O5'-C5'	g^+	t	g^-
ϕ	P-O5'-C5'-C4'	60°	180°	-60°
	P-O5'-C5'-H5',5"	g't'	g'g'	t'g'
ψ	O5'-C5'-C4'-C3'	60°	180°	-60°
	H5',5"-C5'-C4'-H4'	gg	gt	tg
χ_1, χ_2	O4'-C1'-N9-C4	0°		180°
		syn		anti

*The backbone torsion angle ψ' (C5'-C4'-C3'-O3'), which resides in the furanose ring, is better represented by the puckering modes (see text).

**The angle A-B-C-D is measured as positive by clockwise rotation of D with respect to A, looking down the B-C bond. The counterclockwise rotation is negative.

conformation,* which plays an important part in the conformational states of nucleotides, has been commonly described in terms of puckering modes. Among them, C3'-endo (^{3}E) and C2'-endo (^{2}E) puckers are most favorable in which C3' and C2' are displaced separately from the plane containing the remaining furanose atoms and are on the same side as C5'. The abbreviation E refers to the envelope comformation of the five-membered ring.

Conformational properties of dinucleoside monophosphates (e.g. ApA) have been extensively studied as they represent the simplest repeating chemical and structural units of polynucleotides and nucleic acids.** Both semiemperical potential energy[3-8] and quantum mechanical[9-12] methods have been used in theoretical calculations. Although no solid state studies of ApA has been reported, X-ray crystallographic data of ApApA is available.[13] Extensive studies of ApA in aqueous solution

*A description based on the concept of pseudorotation has been described by Altona and Sundaralingam (ref. 2).

**Although dinucleoside triphosphates (e.g. pApAp) have been shown (ref. 3,4) to be better models of nucleic acid structure than dinucleoside monophosphates (because of the presence of adjacent phosphates), they are more difficult to synthesize. It will be possible to assess the multiple phosphodiester effect by eventually studying trinucleoside diphosphates (e.g. ApApA) and tetranucleoside triphosphates (e.g. ApApApA).

have been made by UV, CD[14,15] and NMR (^{1}H, ^{31}P, ^{13}C) [16-20,21,22-24] spectroscopy. The latter method is unique for its sensitivity to conformational variation about individual bonds. It is, therefore, useful to summarize the large body of work on ApA which has been reported from other laboratories.

The following summary emphasizes NMR studies of ApA because it is these studies that consistently provide the greatest details pertinent to the conformation about specific bonds of ApA in solution.* The X-ray and theoretical studies will be mentioned as needed. Attention is focussed on the two glycosyl torsion angles χ_1 and χ_2, the two ribose puckers, and the five backbone torsion angles ϕ', ω', ω, ϕ, and ψ. Three and four-bond scalar coupling constants and dimerization chemical shifts are the most frequently analyzed data. In the latter analysis chemical shifts (usually proton) of ApA (the dimer) are compared with those of its constituent monomeric units (3'-AMP and 5'-AMP) and the differences are termed dimerization chemical shifts.

Quantitative determinations of the distribution of ribose puckers, staggered rotamers about ψ (C5'-C4'),

*Much more detailed summaries can be found in quoted references.

and ϕ (O5'-C5') have been made based upon vicinal coupling constants $^3J_{1'2'}$, and $^3J_{3'4'}$, $^3J_{4'5'}$,* and $^3J_{4'5''}$, and $^3J_{P,5'}$, $^3J_{P,5''}$, and $^3J_{P,C4'}$, respectively.[18-21,23,25] These results are summarized in Table II and they indicate a preference for the two riboses to assume the ^3E pucker and a high proportion of the gg ($\psi = 60^\circ$) and g'g' ($\phi = 180^\circ$) rotamers in neutral aqueous solution. A rotamer analysis of ϕ' (C3'-O3') is less straightforward. An analysis based on $^3J_{P,C4'}$ and $^3J_{P,C2'}$ reported by Alderfer and Ts'o[24] modifies a similar previous analysis[23] and leads to the conclusion that ϕ' rotamers of 205° (g^- domain) and 275° (g^+ domain) are favored nearly equally. The analysis arbitrarily excludes the t rotamer (60°) on the basis of model studies, theoretical calculations, and X-ray data for similar systems. Phosphorus-hydrogen coupling constants suggest that the g^- rotamer is preferred[21] and this inference is supported in particular by values of $^4J_{P,2'}$ (~1Hz). Four-bond phosphorus-hydrogen coupling constants are a maximum (2.5-2.7 Hz) when the relevant atoms adopt a coplanar W arrangement. In the case of phosphorus and H2' the optimum conformation for maximum $^4J_{P,2'}$ is

*The H5' refers to that geminal proton on C5' which is trans to C3' in the gg conformation.

Table II

Population Distribution (%) of Ribose Puckers and Staggered Rotamers about ψ and φ Bonds for ApA in Aqueous Solution (0.005-0.1 M, pD 6.7-7.4, 12-28°C)

	^3E (Ap-)*	^3E (-pA)*	gg	g'g'	Ref.
Ribose	62	59			18
	58	61			19
	62	58			20
	65	65			25
ψ			75		18
			74		19
			79		25
φ				86	18
				90	19
				88	21
				80	23
				83	25

*$^3E + {}^2E = 100\%$

achieved only when the sugar is ^3E and ϕ' is g^+. Further indirect evidence supports the above conclusion.[19]

Existing analyses of the glycosyl torsion angles of ApA (χ_1 and χ_2) based on the observed dimerization chemical shifts of H2, H8, and ribose protons afford the qualitative conclusion that ApA prefers anti-anti (χ_1-χ_2), partially stacked conformations.[18] The latter claim is evident from earlier CD[26,27] and NMR[16] studies. Since the ribose coupling constants are highly sensitive to stacking interactions, the extent of stacking of ApA in solution has been estimated from $^3J_{3',4'}$.[19,25] Assuming a pure ^3E ribose pucker is adopted in the fully stacked conformation, an about 40% base-stacked population has been proposed for ApA under the condition* in which intermolecular effects are minimal.

Direct evaluation of conformational preferences about ω' (O3'-P) and ω (P-O5') has proved to be difficult because of lack of appropriate coupling constants ($^3J_{O,C}$ in this case) and clear-cut phosphorus chemical shift dependence on structural and conformational features.[21,28-30] Quantitative estimates of favorable orientations about the ω' and ω bonds from chemical

*The data were obtained in a concentration below 0.03 M at 20°C and neutral pH.

shift data of base and ribose protons have been made in the light of preferred conformations suggested by model studies, crystal structure data, and theoretical calculations.[18,19] From this analysis, the existence of at least two stacked ApA conformations (ω',ω in g^-,g^- and g^+,g^+ domains for right-handed helical stack and left-handed loop stack respectively) in solution have been suggested.

In summary, at neutral pH and low temperature the stacked form of ApA has a predominant anti-anti, ^3E-^3E, g^-, gg, g'g' conformation in dilute aqueous solution and ApA exists as an equilibrium between stacked and unstacked forms. This description is substantiated by both single crystal X-ray data of ApApA[13] and theoretical calculations.[8,12] As will be detailed below, the ω' and ω bonds are assumed to be the major source of conformational flexibility. The remaing torsion angles are thought to be relatively inflexible, in accord with the rigid nucleotide concept proposed by Sundaralingam.[31]

It has been demonstrated from various experimental ApA studies that conformational distributions of the glycosyl torsion angles, the ribose puckers, and the backbone torsion angles (φ', ϕ, and ψ) are strongly coordinated. Available X-ray data of mononucleosides

and mononucleotides indicate an apparent correlation between the glycosyl torsion angle χ and the ribose conformation; that is, a χ value of 180° to ~225° (low anti) is associated with a favored ^{3}E ribose pucker, while values >225° (high anti) correlate with a ^{2}E ribose pucker.[2] A similar correlation is proposed for ApA in solution.[16]

Calculations based on coupling constants suggest that for ϕ' in ApA, a g^{-} orientation is preferred when the Ap- ribose is in the ^{3}E form and that the ^{2}E pucker favors a g^{+} orientation.[19,24] From ^{1}H NMR studies of ribose dinucleoside monophosphates such as ApA, it is known that both ribose pucker conformations are affected by stacking interactions.[16,19] Cozzone and Jardetzky have indicated in their studies of ApA and other dinucleotides that the favored gg distribution of ψ is coupled with base stacking.[21] In addition, a direct correlation between the preferred conformations gg and g'g' of ψ and ϕ torsion angles has emerged from an examination of NMR solution studies of dinucleoside monophosphates including ApA, and these charges are coupled with other conformational variations (e.g. the ribose pucker) during the base-stacking process.[33]

Although conformation states of ApA stay relatively

unchanged* in the pH range of 2-12,[21,32] substantial unstacking of the bases is observed when the temperature is increased. Since the phosphodiester bonds ω' and ω are thought to present the major degree of freedom in ApA, unstacking is thought to be correlated with rotations about ω' and ω.[18] These then are accompanied by interdependent changes of other conformational parameters; that is an increase of glycosyl torsion angles χ_1 and χ_2 to the high anti domain and a shift of the ribose conformation in favor of the ^2E pucker. Coupling to these changes, the g^-, $g'g'$, and gg conformation of backbone torsion angles ϕ', ϕ, and ψ respectively are depopulated to various extents (but are still favored at 75-80°C[21,25]). It is worth noting that the elevation of the temperature produces an effect on the ApA conformation directly opposite to those changes observed upon the dimerization process. The conformations of individual Ap- and -pA units of ApA in the relatively unstacked form are quite similar to those of the respective mononucleotide constituents (3'-AMP and 5'-AMP).

It is evident from the above brief summary of solution studies of the ApA conformation that certain

*Except at pH ~4 where a slight change is related to the protonation of the adenine bases.

torsion angles are difficult to analyze. The distribution of rotamers about ϕ' has been studied by several NMR methods, but ambiguities remain. Analysis of the glycosyl torsion angles can be qualitative at best using dimerization chemical shifts alone and there are no direct methods available to provide unambiguous information about the phosphodiester bonds ω' and ω. Proton-proton ($^1H\{^1H\}$)* and phosphorus-proton ($^{31}P\{^1H\}$) nuclear Overhauser effects (NOE's), interpreted quantitatively, have provided confomation information about χ and ϕ' of 3'-AMP and about χ, ϕ, and ψ of 5'-AMP.[34,35] It is the interest of this laboratory to extend these methods to the conformational analysis of ApA, similar dimers, and higher oligomers. Furthermore, work is in progress to analyze the elusive ω', ω torsion angles <u>via</u> magnetic field dependent spin-lattice and spin-spin relaxation times (for both ^{31}P and ^1H) and total $^{31}P\{^1H\}$ NOE's.** The NOE studies require adequate chemical shift separation so that unambiguous proton irradiations can be performed and the relaxation studies can be conducted

*This compact double irradiation symbolism is conventional. The bracketed nucleus is irradiated and the unbraketed nucleus is observed.

**P. A. Hart and C. F. Anderson, work in progress.

only if the number of phosphorus-hydrogen dipole-dipole interactions is known. Adequate chemical shift separation is seen at higher fields, but at those fields (e.g. 63 kG) the extreme narrowing condition is violated, causing a decrease in observable NOE's.

The need to work at a moderate field (e.g. 21 kG) prompts the need to prepare selectively deuterated ApA so that the above requirements can be met. We have chosen to synthesize 4'- and/or 5'-deuterated monomeric units which can then be elaborated to the corresponding dimers. The following sections of this thesis describe the synthesis of trideuterated ApA and related dimers and NMR studies of these compounds.

II. Synthesis of Deuterium-labeled ApA

A. Monomer Labeling

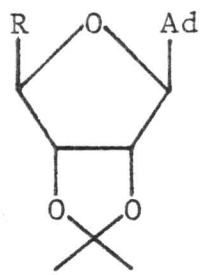
1. Background and strategy

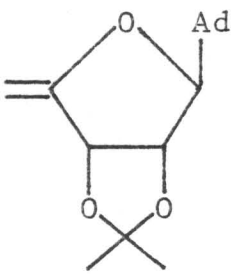
The preparation of 2',3'-O-isopropylidene adenosine (1), deuterated at C5', has been reported.[36] The blocked nucleoside was converted to the carboxylic acid (2) by alkaline potassium permanganate oxidation and the latter was treated in three steps with ethyl chloroformate and triethylamine, sodium azide, and sodium borodeuteride to give 5'-deuterated (3) in an overall yield of 33%. Recently an extension of this approach leading to (5'R)-[$^{2}H_{1}$]adenosine (4) (60% optical purity) from (3) in 3.5% overall yield *via* the deuterated aldehyde (5) has been reported.[37] The aldehyde was obtained by the method of Moffatt *et al*. described later. Although the route from (1) to (3) satisfies the need for 5' deuterium incorporation, it is not efficient and it lacks the versatility needed for 4' deuterium exchange. This makes it necessary to develop a versatile procedure in which the deuteration at both C4' and C5'

can be included. Three compounds present themselves as possible candidates for both 4' and 5' deuterium incorporation. They are, 2',3'-O-isopropylidene adenosine 5'-aldehyde (6), 5'-carboxylate methyl ester (7), and 5'-deoxy-4'-adenosinene (8) (Scheme 1). The first two offer the possibility of a base-catalyzed exchange reaction followed by reduction, whereas the third offers the possibility of deuterated borane addition followed by oxidation.

The 5'-aldehyde (6), first prepared from (1) by Pfitzner and Moffatt,[38] is difficult to isolate. It readily epimerizes at C4' or decomposes *via* elimination of the isopropylidene group.[39] The aldehyde can be isolated as its 1,3-diphenylimidazolidine derivative and can be regenerated *via* mild acidic treatment,[40] but the imidazolidine is not a satisfactory intermediate for C4' deuterium exchange. Thus, the aldehyde is an unsatisfactory candidate both because it is difficult to prepare in high yield and because it is labile under the conditions very likely needed for deuterium exchange. For example, Jones *et al.*[41] reported a rapid interconversion between (9) and (10) in the presence of triethylamine (Scheme 2). Furthermore, the C4' position is so labile that facile conversion of ribonucleoside 5'-

Scheme 1





Ad = Adenine (8)

(1) R = CH_2OH

(2) R = CO_2H

(3) R = CD_2OH

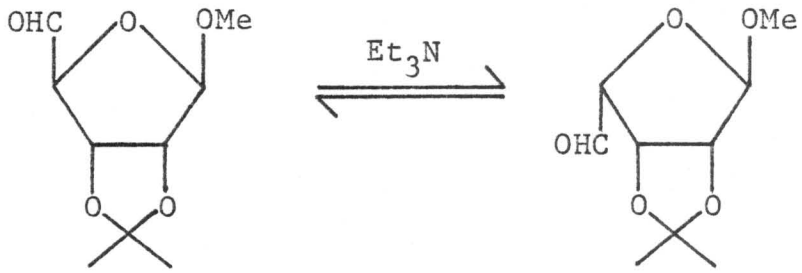
(4) R = CHDOH

(5) R = CDO

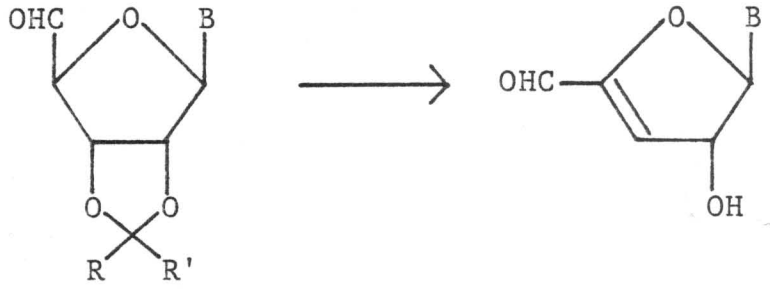
(6) R = CHO

(7) R = CO_2Me

Scheme 2, 3



(9) (10)



(11) (12)

R = H, alkyl or aryl; R' = alkyl or aryl

B = purine or pyrimidine bases

aldehyde (11) into its corresponding 3'-deoxy unsaturated compound (12) has been observed (Scheme 3).[42]

The olefin (8), prepared as the benzoyl derivative in three steps *via* the corresponding 5'-O-mesylate,[43] is expected to transform to the 4'-deuterated alcohol following hydroboration and oxidation. However, the uncertainty in the stereospecificity of the deuterium incorporation made the proposed route involving (8) sufficiently speculative to prompt a thorough study of the usefulness of 5'-ester (7). Because the ester was found to satisfy our requirements well, the olefin (8) was never investigated.

In the following section, the preparation of (7) and its use in the synthesis of the selectively 4'- and/or 5'-deuterated isopropylidene adenosines are presented and discussed.

2. Results and discussion

Specific conversion of the 5'-hydroxyl group of nucleosides to the carboxylic acid has been reported for adenosine, uridine, thymidine, and guanosine *via* reaction with oxygen in the presence of a platinum catalyst.[44] Potassium permanganate was a successful oxidant

only in the case of isopropylidene adenosine (1). The latter observation can probably be rationalized by the observed unique stability of adenine (among the common bases) to this oxidizing agent.[45]

Among various methods available,[36,46,47] our preparation of the 5'-carboxylic acid (2) proceeded largely according to the method of Schmidt et al.[36] Oxidation was carried out by treating the commercially available 5'-alcohol (1) in water with excess potassium permanganate at pH 12 maintained by the presence of potassium hydroxide. Workup of the reaction mixture after one to three days at room temperature yielded (2) as an amorphous white power (61-72% yield). Similar results were obtained on a larger scale (33-54 mmol).

Esterification of this 5'-acid (2) with ethereal alcoholic diazomethane has been reported by Harper and Hampton.[46] Following their method, we were able to prepare the corresponding methyl ester (7) on a scale greater than twice that reported. The reaction was conducted at $0^{\circ}C$ using a large amount (23 equiv.) of diazomethane to afford (7) in 93% yield after recrystallization from methanol. In spite of the large scale preparation of potentially explosive and hazardous diazomethane (ca. 7.2 g was involved in this procedure)

this procedure offers advantages in ease and yield for the preparation of (7) in comparison with other methods using dimethyl sulfate in dimethylforamide (DMF) or via the acid chloride in the presence of anhydrous methanol.[48] Nucleoside methyl carboxylates have also been prepared by acid-catalyzed esterification.[49] However, in these cases 2',3'-cis-diol on the nucleosides was not protected by acid-sensitive groups.

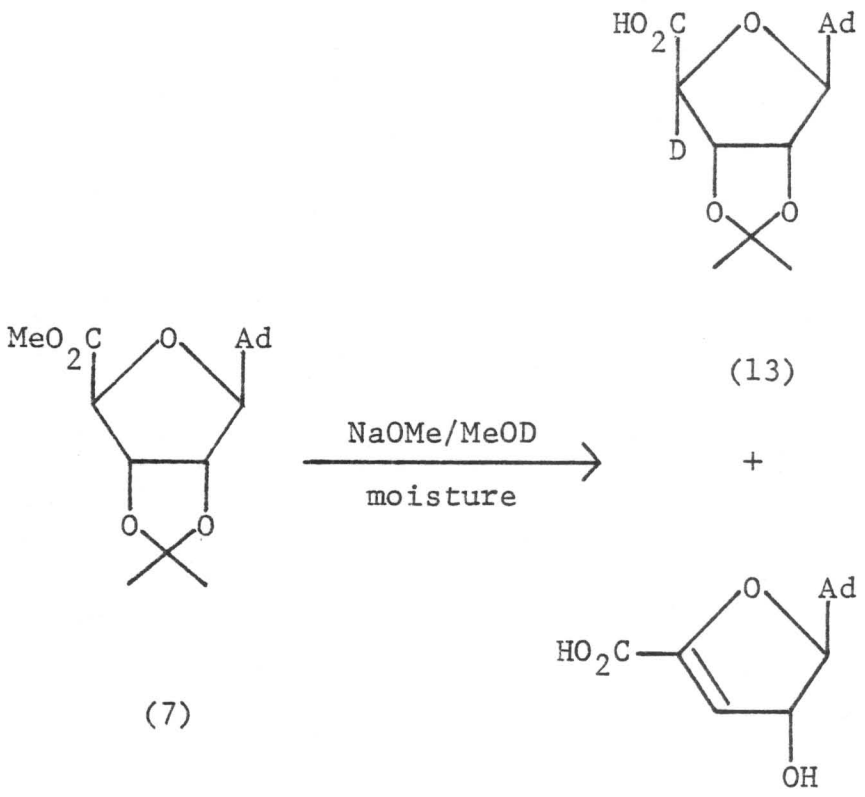
With a carboxyl group attached at C4' of the 5'-methyl ester (7), deuterium exchange at this position becomes feasible. Our first attempt involved the treatment of (7) with 50 equiv. of sodium methoxide in MeOD at reflux for half an hour. The major product isolated was identified as the 4'-deuterated acid (13). Evidently, the hydrolysis of (7) proceeded concurrently during the course of reaction due to the presence of moisture. Although (13) is not the 4'-deuterated ester we originally desired, its complete exchange at 4' position coupled with the reasonable yield (51%) prompted us to study this reaction further. It was found that when the proportion of sodium methoxide decreased, a more polar substance (14) was the major product. ^{1}H NMR analysis showed that the isopropylidene group was absent from (14) along with the disappearance of the proton at

C4'. Although an acidification (pH 4-5) step was employed in the workup, (14) was not identical to 4'-deuterated adenosine. The acidic product was tentatively identified as 3'-deoxy-3'-adenosinene 5'-carboxylic acid which shows a characteristic C3' vinyl proton (δ 6.35 ppm) on the sugar moiety (Scheme 4). β-elimination of this type has been reported for similar subsrances commonly used in the area of carbohydrates for the preparation of unsaturated sugars.[50]

Because the 5'-carboxylic acid (2) partially exchanged at C4' was isolated from an early experiment, it was of interest to know whether it could be exchanged directly. We noted that it was resistant to both exchange and β-elimination in the presence of sodium methoxide and MeOD for up to five hours at room temperature whereas about 20% elimination occured at reflux temperature after half an hour. In the latter case only about 60% deuterium incorporation at C4' was observed. With this result in mind, an investigation was begun to determine the conditions required to differentiate exchange and β-elimination in the 5'-methyl ester (7).

Our initial approach to this problem was to determine how the β-elimination would be affected by varying the temperature of this base-catalyzed exchange

Scheme 4



(13)

(7)

(14)

Ad = Adenine

reaction. To facilitate our study with respect to monitoring the exchange at the 4' position of the methyl ester (7), we chose to run the reaction with sodium methoxide as the base to avoid the occurence of transesterification.[42] Dimethyl sulfoxide (DMSO) was added to improve the solubility of (7). The reaction of (7) with sodium methoxide (5-6 equiv.) in CD_3OD-DMSO-d_6 (1:1) was monitored by ^1H NMR spectroscopy. The observations were that: (a) at 45°C, the C4' proton was lost along with about 65% of the isopropylidene group after six minutes; (b) at 25°C, the C4' proton as well as about 55% of the isopropylidene group was gone after the same period of time; and (c) at 25°C and two minutes, about 60% of the C4' proton and 5% of the isopropylidene group were eliminated. Since the C4' proton was undergoing the deuterium exchange and the disappearence of those methyl protons on the isopropylidene group was indicative of the β-elimination, the above results led us to believe that the relative rates of these two reactions could be further differentiated in favor of the exchange at lower temperature.

Thus, the base-catalyzed exchange reaction of the 5'-methyl ester (7) with freshly prepared sodium methoxide (7-8 equiv.) in anhydrous MeOD-DMSO (2:1)

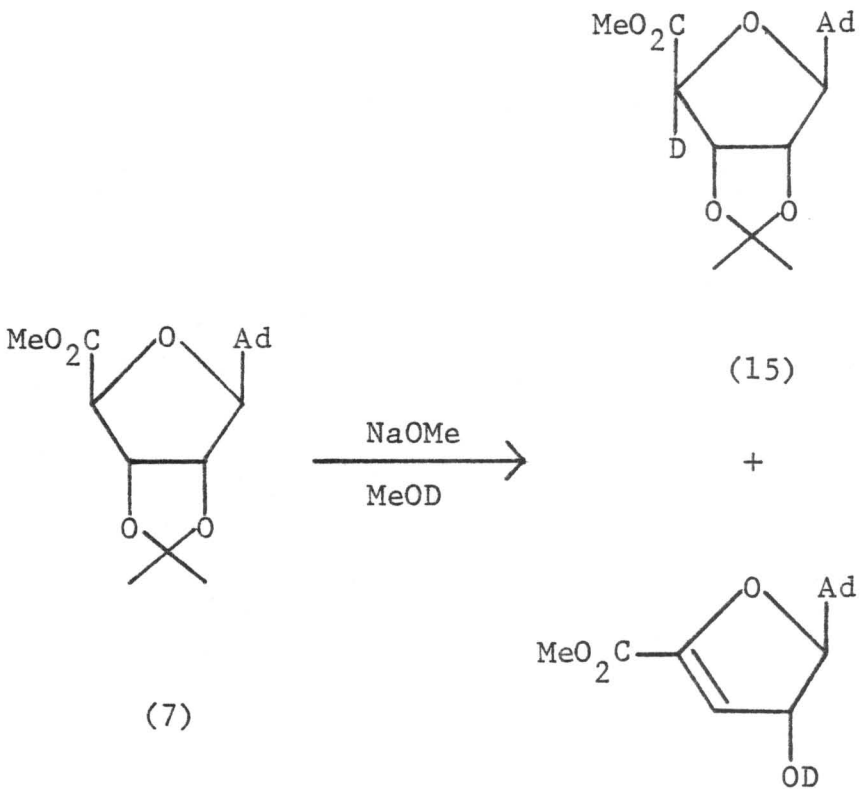
at -23°C was allowed to proceed for one hour. Under these conditions exchange at C4' (15) was complete and about 14% of the β-elimenation product (16) was detected (Scheme 5). Other bases such as triethylamine and magnesium methoxide were tried under various conditions but they were inferior to sodium methoxide.

Epimerization at the 4' position is another possible side reaction that needs to be considered. However, this alternative has been shown to be negligible at even higher temperature (ambient temperature instead of -23°C)* and there was no evidence for epimerization in our preparation of (15).

With the 4'-deuterated 5'-methyl ester (15) available, the next step is to introduce deuterium by reduction of the 5'-ester. Although lithium aluminum hydride (LAH) has been shown to reduce N^2-acyladenine[51] nad N^2-acylguanine[52] without affecting the purine nucleus, it is not suitable for the reduction of esters (7) and (15) for the reason that they are barely soluble in ethereal solvents. Only 50% yield was reported for the reduction of isopropyl 3'-deoxyadenosine 5'-carboxylate in tetrahydrofuran (THF) with sodium bis(2-

*In this experiment (7) was treated with sodium isopropoxide (ref. 42).

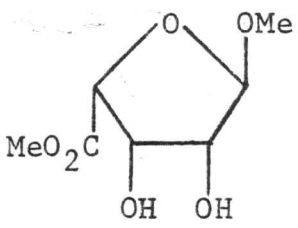
Scheme 5



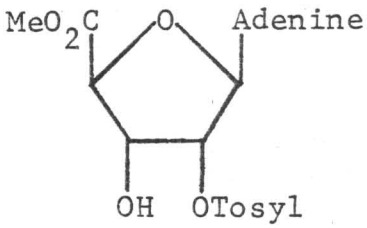
Ad = Adenine

methoxyethoxy)aluminum hydride.[42] This observation further indicates that other types of reducing agents should be employed to enable the reduction to be run in more polar solvent systems.

Unlike LAH, sodium borohydride could be used in hydroxylic solvents and is readily available in its deuterated form. It is generally accepted that sodium borohydride is a much milder reducing agent than LAH and does not reduce carboxylate esters. However, a number of reports have appeared in which reduction of esters to primary alcohols was observed.[53,54] Using sodium borohydride, Hulyalkar and Perry[54] described the reduction of (17) in ethanol and Schmidt et al.[54] demonstrated the conversion of (18) to 2'-O-tosyladenosine in DMF-water. In the latter report, the authors also suggested that the proximate 3'-hydroxyl group on (18) took part in the reduction by complex formation with the reducing agent



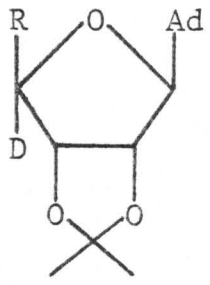
(17)



(18)

and subsequent intramolecular transfer of hydride ions to the methyl ester. They based their assumption on the observation that the reduction did not occur if the 3'-hydroxyl group was replaced by a tosyl group. Similar neighboring-group participation[55] as well as electron-withdrawing substituents α to the carboxyl group[56] have been shown by others to be the factors which enhance such abnormal reduction of the esters by sodium borohydride. However, Brown and Rapoport demonstrated that no special features are necessary for the reduction of esters, in methanol, to occur at least to some extent, by using an excess of the reducing agent.[53]

We found that 5'-methyl esters (7) and (15) were reduced by sodium borohydride or sodium borodeuteride (10 equiv.) to give 4'- and/or 5'-deuterated isopropylidene adenosines (3), (19), and (20) in excellent yield (89%). Unsatisfactory results were obtained when the proportion of reducing agent was decreased. The



(19) R = CH_2OH

(20) R = CD_2OH

Ad = Adenine

reduction was carried out in aqueous methanol at room temperature overnight and deuterated solvents were used to eliminate unwanted back-exchange at the 4' position (ca. 15%) during the reduction. On one occasion, the reduction was attempted by treating the esters, suspended in THF, with lithium borohydride for four days, but only 42% of the reduction product was isolated.

It is probable that no nearby groups participated in the reduction of esters such as (15) since the 3'-hydroxyl group was protected by an isopropylidene group. However, the possibility that the effective reducing agent under our reaction condition was trimethoxyborohydride cannot be ruled out. This formation of the more reactive trimethoxyborohydride in situ has been suggested[55] to be responsible for the reduction of the esters in other cases when the reduction was run with a large excess of sodium borohydride in methanol. As for sodium trimethoxyborohydride, its selectivity in reduction of the esters has been well demonstrated.[57]

The goal of preparing isopropylidene adenosines (3), (19), and (20) selectively deuterated at C4' and C5' has been reached. We discuss in the following section how the deuterated nucleosides were carried to the dinucleoside monophosphate stage.

B. Dimer Synthesis

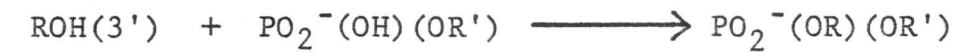
1. Background

There are generally two methods available for the formation of an internucleotide bond between nucleosides (Scheme 6)*. One of them, which was introduced earlier and developed by Khorona et al., is the phosphodiester approach.[58] It involves the condensation between an appropriately blocked nucleoside and a phosphate monoester component to form a new phosphodiester bond. Dicyclohexyl carbodiimide was the condensing agent originally used, it was later replaced by the more effective arylsulfonic acid derivatives triisopropylbenzenesulfonyl chloride or tetraazolide.[63] Although this approach is useful and reliable, it suffers from several inherent limitations: (a) the synthetic intermediates are ionic species (phosphate diesters), thus the routine isolation techniques of organic chemistry such as solvent extraction and silica gel chromatography are either not applicable or are difficult ; (b) the intermediate phosphodiester functions are nucleophilic,

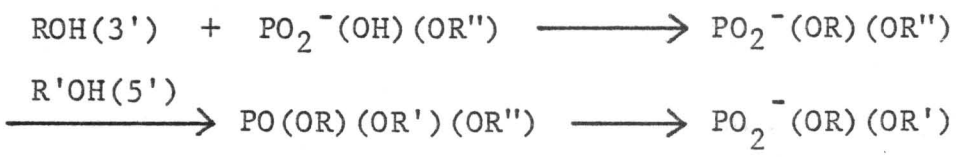
*For recent reviews of chemical synthesis of oligo- and polynucleotides, see references 59-62.

Scheme 6[60]

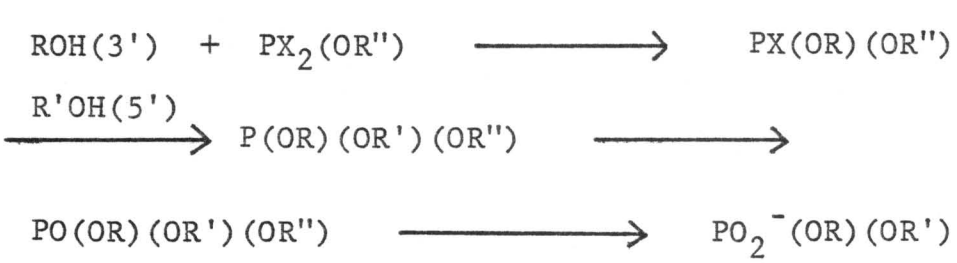
Phosphodiester approach:



Phosphotriester approach:



Modified triester approach <u>via</u> phosphite:



they become activated by the condensing agent and subsequent reaction leads to the uncontrolled cleavage of internucleotide bonds; and (c) the subsequent treatment of phosphodiesters becomes treacherous for the ribonucleotides (bearing a 2'-hydroxyl group which is absent in deoxyribose series) as it is absolutely essential to avoid any 2'-3' phosphodiester bond migration. Although the difficulties described in (a) and (b) are alleviated to some extent by the use of reverse-phase chromatography and excess phosphate monoesters for successive chain elongation respectively, the phosphodiester approach is still far from ideal for the practical synthesis of oligonucleotides

Undoubtly, the disadvantages associated with the phosphodiester functions would be removed by masking them as neutral species. This has been accomplished in the phosphotriester approach in which the phosphate monoester component and the internucleotide bond were protected *via* esterification. The 2,2,2-trichloroethyl group[64] is the most suitable blocking group for this purpose, because of its stability under usual reaction conditions and because it can be readily and selectively removed.

Two important modifications of the phosphotriester

approach have been introduced which significantly improve the internucleotide coupling reaction and allow for large scale manipulation. The groups of Narang and Cramer reported separately the use of mononucleosides containing a fully masked phosphate group as the building blocks for subsequent chain elongation.[65] Accordingly, the necessity for a phosphorylation step at each condensation stage is eliminated and thus simplifies the approach. The other major advance was made by Letsinger and coworkers who used a bifunctional trichloroethyl phosphorodichloridite (21) to couple two nucleoside components. Rapid oxidation of the resultant phosphite intermediate with iodine gave the corresponding fully protected phosphotriester.[66] This method offers several advantages: the short reaction time, mild reaction conditions, and favorable yields.

Both phosphodiester and phoshotriester approaches have been utilized in the synthesis of medium-length oligonucleotides on polar cross-linked polyamide supports.[67] Chain extension using stepwise addition of a single nucleotide[68] as well as one trinucleotide block[69] were reported. However, only limited success has been attained in this area thus far because of the significant non-quantitative nature of the internucleo-

tide coupling reaction. Since we are only interested in synthesizing selectively labeled dinucleotides and short oligonucleotides, the solid-phase method was not explored.

Toward our synthesis of deuterated ApA, the phosphite triester approach was adopted bacause it is a mild procedure, yields are good, and no protection of the 6-amino group is required.[70] The course of this study is detailed in the next section.

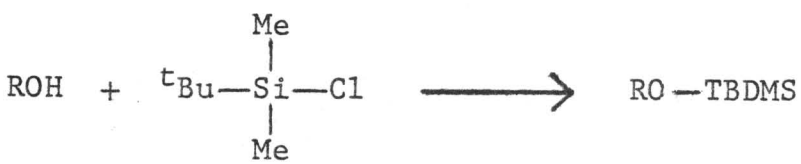
2. Results and discussion

Since the selectively 4'- and/or 5'-deuterated mononucleosides (3), (19), and (20) were already properly protected at both the 2' and 3' positions by an isopropylidene group, the next task was to synthesize another mononucleoside component which had its 2'- and 5'-hydroxyl groups suitably protected. By using a phosphorylating agent such as phosphorodichloridite (21), the free 5'-hydroxyl group of the former would be expected to couple with the free 3'-hydroxyl group of the latter to form a dinucleoside monophosphate with a 3'-5' internucleotide phosphodiester bond.

There are two general criteria applied to the se-

lection of protecting groups for the 2'- and 5'-hydroxyl functions: (a) stability under the phosphorylative coupling reaction and (b) ready removability from the coupled product under conditions mild enough to prevent cleavage or migration of the internucleotide linkage. Both acid-labile (e.g. acetal, trityl) and base-labile (acyl) protecting groups have been commonly used.[59,62,71] In addition, use of a photolabile group (2-nitrobenzyl) as a protection for the 2'-hydroxyl function has been reported.[72,73]

Our preparation of suitably 2',5'-protected nucleosides was aided by the recently developed trialkylsilyl hydroxyl-protecting groups. Although unhindered silyl groups such as the trimethylsilyl group are too susceptible to solvolysis to be useful for our purpose, the t-butyldimethylsilyl (TBDMS) group, originally introduced by Corey and Venkateswarlu[74] in the synthesis of prostaglandins, has been found to be ideally suited to nucleoside protection. Indeed, the TBDMS group possesses.



desirable properties for many other synthetic transformations involving multi-functional compounds.[75] The major advantages of this protecting group are: (a) it is easy to introduce via TBDMS chloride; (b) the solubility of silylated compounds in less polar solvents is increased; (c) TBDMS ethers are stable to aqueous or alcoholic bases, hydrogenolysis, mild zinc reduction, and phosphorylation; and (d) the TBDMS group can be removed easily with naked fluoride ion or aqueous acid.[74] Ogilvie and coworkers first used this group for the protection of primary and secondary hydroxyl functions on deoxyribonucleosides[76] and ribonucleosides.[77,78]

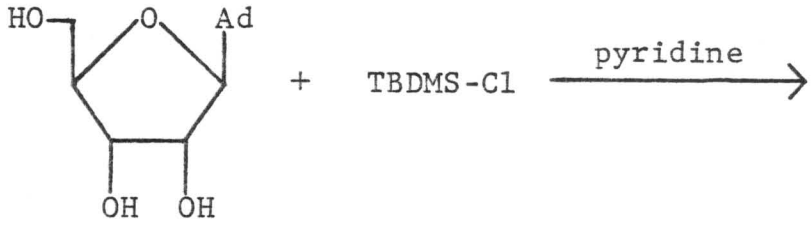
The TBDMS derivatives of the corresponding alcohols are usually prepared by treating them with TBDMS chloride in DMF using imidazole as the catalyst. Although this method was found to be satisfactory for protecting primary and secondary hydroxyl groups on nucleosides,[79,80] other procedures need to be employed for the silylation of tertiary hydroxyl groups. TBDMS chloride in the presence of lithium sulfide[81] and TBDMS perchlorate[82] are required in these hindered systems. The former also provides a mild and non-basic reagent for the silylation of base-labile compounds.

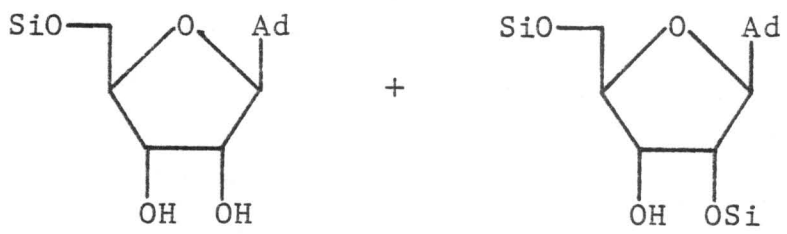
The TBDMS chloride reagent, which can be readily

prepared from t-butyllithium and dimethyldichlorosilane,[74] is commercially available. Our preparation of 2',5'-di(TBDMS)adenosine (22) proceeded largely according to the described method[79] with a modified workup procedure. It was carried out in pyridine which serves both as solvent and catalyst. Although the reaction in this system was slower than in the DMF-imidazole system, it has been reported[79] that the pyridine system affords a more favorable ratio of 2',5'- to 3',5'-disilylated adenosine. However, this improved selectivity could not be reproduced during the present studies. Generally, the 2',5' isomer (22) was only slightly favored over the 3',5' isomer (23) (Scheme 7).

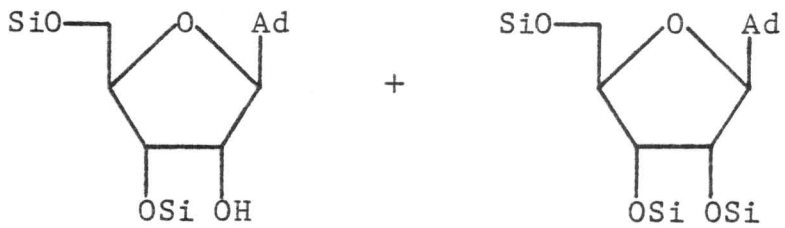
Depending on the scale, both preparative thin layer and column chromatography on silica gel were used for the isolation of 2',5'-disilylated adenosine (22) in solvent systems such as ether and benzene-ethyl acetate. Pure (22) becomes increasingly difficult to obtain when the contact time with the adsorbent increases. Evidently, 2' to 3' isomerization of the O-TBDMS group occurs, promoted by silica gel.[79,83] On the one hand, this means that the chromatographic separation needs to be accomplished without delay. On the other hand, this inherent lability proves useful in convert-

Scheme 7





(22)



(23)

Si = TBDMS = t-butyldimethylsilyl

Ad = Adenine

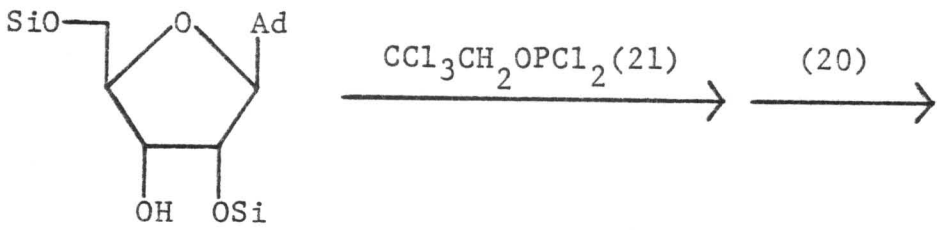
ing the less desirable 3',5'-disilylated adenosine (23) to the desired 2',5' isomer (22). Moreover, the isomerization occurs in methanolic solution upon long standing at room temperature and at reflux temperature pure (23) was converted to a one to one mixture of (23) and (22) in eight hours. Since the completion of our study, a similar observation has been reported.[80,84]

Thus, the effective yield of 2',5' isomer (22) was significantly improved (64-84%) by interconversion of the 2'/3' TBDMS groups. The isomerization step is particularly important when valuable deuterated materials (e.g. 4'-[$^{2}H_{1}$]adenosine (24)*) are involved.

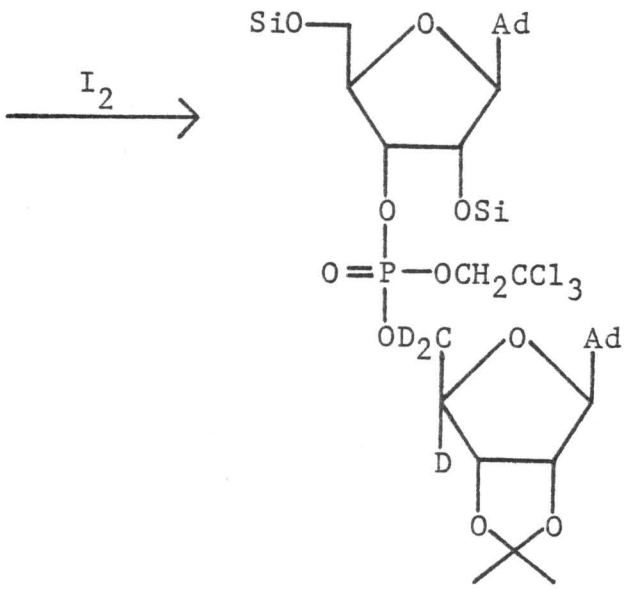
Conversion of the blocked nucleosides to blocked dinucleoside monophosphates (e.g. (25); Scheme 8) was accomplished using the phosphite triester approach. A common complication in using symmetrical coupling agents like phosphorodichloridite (21) to prepare unsymmetrical condensation products is that self-condensation occurs as a side reaction. In our cases for example: (a) if excess 3'-hydroxyl nucleoside (22) were used initially, a 3'-3' linked dimer would be formed in addition to the desired 3'-5' linked dimer (25) and (b) if excess (21)

*It is readily prepared from (19) in 95% yield by acidic hydrolysis.

Scheme 8



(22)



(25)

Ad = Adenine

Si = TBDMS

were present, it would couple the 5'-hydroxyl nucleoside (20) added subsequently to yield the unwanted 5'-5' linked dimer. This problem was generally avoided by using a slight excess of the first nucleoside (22) added to assure complete consumption of coupling agent (21). The amount of second nucleoside (20) added for the condensation reaction with the active intermediate, formed from (21) and (22), was usually less than the stoichiometric proportion. Thus, all of (20), the more valuable nucleoside, would be converted in the reaction to the desired 3'-5' phosphodiester compound (25). Molar ratios of approximately 1.1-2.0 to 1.0 have been used for the phosphorylative coupling of the initially added nucleoside (with 3'-hydroxyl open) to the second nucleoside (with 5'-hydroxyl open) added subsequently.[66,79,85] In our experiments, relative ratios of 1.9-2.2 to 1.0 were employed for the preparation of fully blocked ApA. The yields of these condensation reactions were consistently high (>75%), especially when lutidine was replaced by collidine as the base. The former was originally used by Letsinger[66] in his development of the phosphorylative coupling reaction. It gave good but less reproducible results probably because of poor reagent solubility at the reaction temperature (-78°C). The

use of collidine, on the other hand, gave improved and consistent yields.

Reaction of the blocked nucleoside (22) with phosphrodichloridite (21) in tetrahydrofuran/collidine was accomplished at -78°C for forty minutes. 4',5'-Deuterated isopropylidene adenosine (20) was added, and following a further two-hour reaction period, the reaction mixture was allowed to warm to room temperature. Oxidation with iodine at room temperature gave the fully protected 3'-5' ApA (25). Following an identical procedure, (26) was prepared from the corresponding 2',5'-di(TBDMS)-4'-[$^{2}H_{1}$]adenosine (27) and 5'-deuterated isopropylidene adenosine (3). The coupling products were separated from the first nucleoside component, and if not fully consumed, the second nucleoside component by silica gel chromatography on thick layer plates in ethyl acetate-ethanol (9:1). It is important that the 2', 5'-disilylated adenosines (22) and (27) used are free of 3',5' isomer contamination, because the 2'-5' linked impurities thus formed are difficult to separate from their 3'-5' linked counterparts. The condensation of (22)-(21)-(20) and (27)-(21)-(3) in a relative proportion of 2.2-2.2-1.0 gave yields of 83% or higher.

There are a number of ways of removing the three

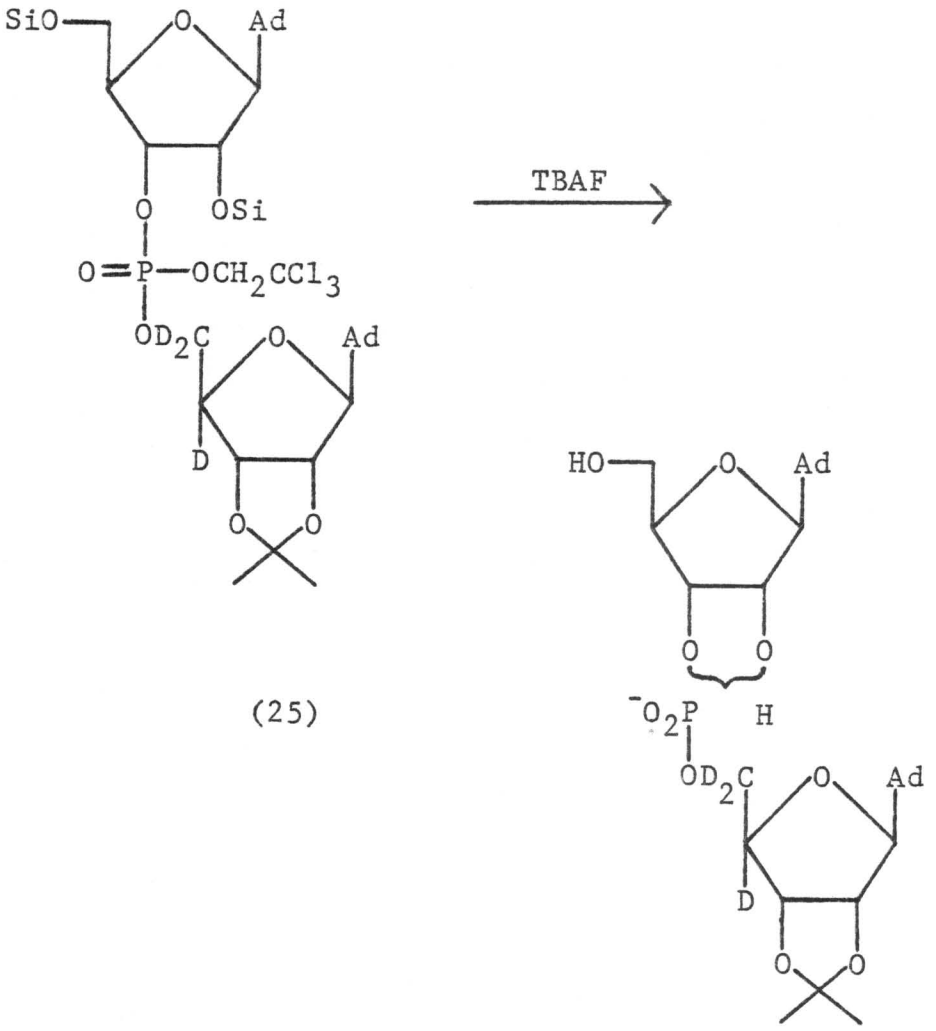
distinct types of blocking groups from the protected dinucleoside monophosphates. Whatever methods are adopted, it is essential that no or minimal internucleotide chain cleavage nor phosphodiester bond migration occur. Furthermore, methods were needed that would leave the isopropylidene group intact because we wished to know the influence of that group on glycosyl tortion angle and phosphodiester backbone conformation of ApA. As well, the acidic conditions used for removing the ketal, may also remove a 2'-silyl group from the 3'-nucleotidyl unit of blocked ApA and promote the unwanted phosphodiester bond migration.

In their introduction of the TBDMS hydroxyl-protecting group, Corey and Venkateswarlu described the removal of such a group by treatment with 2-3 equiv. of tetra-n-butylammonium fluoride (TBAF) in an aprotic medium.[74] Thus, the silyl group can be removed without affecting other acid- or base-labile protecting groups. Additionally, Ogilvie et al. reported that TBAF not only removes silyl groups but the phosphate protecting group as well.[85] Therefore, all the protecting groups on the blocked dinucleoside monophosphates, (25) and (26), except the isopropylidene group can be removed readily using fluoride ion in tetrahydrofuran at room temperature.

TBAF, as a convenient source of active fluoride ion, was prepared by neutralization of 40% aqueous tetra-n-butylammonium hydroxide with dilute hydrofluoric acid followed by complete removal of water. The quaternary ammonium fluoride is hygroscopic and, like other quaternary salts, is known to hydrate easily and extensively.[86] Azeotropic evaporation with benzene-acetonitrile and lyophilization followed by final drying over phosphorus pentoxide for a few days in a vacuum desiccator are the means usually adopted to ensure an anhydrous product. In most of our deblocking experiments TBAF was employed either in powder form or as a solution (0.1-0.5 M) in tetrahydrofuran. To test the effectiveness of our product, a mixture of disilylated adenosines, (22) and (23), was treated at room temperature with 5 equiv. of 0.5 M TBAF solution in tetrahydrofuran for forty five minutes. Adenosine was isolated in 91% yield, in agreement with other observations in similar systems.[76]

Treatment of the blocked dinucleoside monophosphate (25) with 7-8 equiv. of the above TBAF solution in tetrahydrofuran resulted in the formation of two very similar (by chromatography) major products in about equal amounts (Scheme 9). One of these was ultimately shown to be isopropylidene-blocked ApA (28) and there is

Scheme 9



(25)

(28) 3'-5' linked dimer

(29) 2'-5' linked dimer

Ad = Adenine; Si = TBDMS

very good evidence that the second material is the corresponding 2'-5' linked isomer (29). This result differs from earlier reports on similar systems in which only 3'-5' linked dimers are produced in excellent if not quantitative yield.[79,85] Variation of the amounts of TBAF (6-66 equiv.), the reaction temperature (-42°C to room temperature), and the reaction time (1-7 h) produced no significant changes in the observed product ratio.

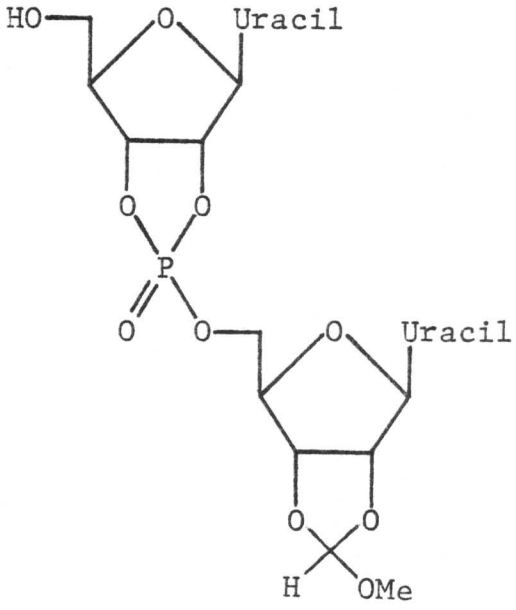
Recently, Clark discovered that TBAF may be rendered apparently anhydrous by adsorbing it on the surface of silica gel which provides many hydroxyl functions acting as hydrogen bond acceptors of fluoride ion.[87] The TBAF-silica reagent (1 mmol TBAF/g), which can be readily prepared according to the described procedure, was employed in the deblocking reaction mentioned above. Still, an approximately one to one ratio of two major products was observed. Moreover, running the deprotection in different aprotic medium (THF, DMF, acetonitrile) altered only the rate of the reaction but not the relative ratio of the products. Since the completion of our work, several other fluoride reagents were reported which involved the use of tetra-n-butylammonium chloride and potassium fluoride dihydrate,[88] aqueous

Table VI

$^{1}H\{^{1}H\}$ NOE Studies of ApA. H2 Enhancements

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Ap- H2		-pA H2	
	6°C	22°C	6°C	22°C	6°C	22°C
-pA H1'	-5664	-5638	0	0	1	1
Ap- H1'	-5675	-5653	-3	5	-2	5
Ap- H2',H3'		-5756		5		3
-pA H2',H3'	-5789	-5768	4	2	5	3
-pA H4',H5' & Ap- H4'	-5804	-5784	7	1	8	3
-pA H5" & Ap- H5',H5"	-5846	-5829	2	-3	3	1

2'-3' phosphodiester bond migration has occurred during TBAF mediated deprotection.[92] Additional evidence was provided by van Boom et al. in their study of a similar dinucleoside monophosphate, uridilyl-(3'-5')-uridine.[93] They proposed that the 2'- and 5'-hydroxyl protecting groups were first removed by TBAF to give the corresponding alkoxide ions. Due to the close proximity of the 2'-alkoxide ion to the phosphodiester function, a 2',3'-cyclic intermediate (30)[94] would be formed which can be reopened nonspecifically by nucleophilic attack of fluoride ion at phosphorus and results in the forma-

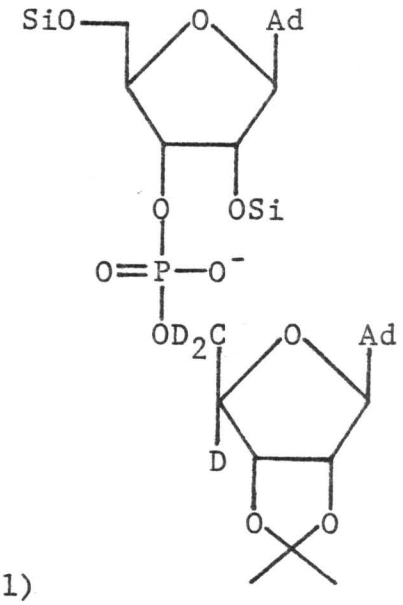


(30)

tion of 3'-5' and 2'-5' linked isomers. For this reason, an additional step needs to be included in the deblocking procedure so that the trichloroethyl group on the phosphotriester is removed before the silyl groups.

Activated zinc has been employed to remove the trichloroethyl group. This is the same reagent that was used earlier and later replaced by TBAF when the latter was introduced by Ogilvie et al. in oligonucleotide synthesis.[85] For successful cleavage of the trichloroethyl group, the zinc surface needs to be highly activated. In our experiments, zinc was prepared by various methods[95,96] and eventually the one by van Boom et al.[96] was adopted for its effectiveness. The deblocking reaction of the fully protected dinucleotides such as (25) was carried out in pyridine at room temperature for three hours with freshly prepared zinc and catalytic amounts of 2,4,6-triisopropylbenzenesulfonic acid. The time required for completing the reaction varied from experiment to experiment due to different concetration of reagents or variation in the activity of the zinc. Without isolation of the assumed product (31), the residue obtained from the zinc mediated deprotection was treated directly with TBAF (5 equiv.) as before to afford the 3'-5' linked isopropylidene protected

dinucleotide (28). No undesirable 2'-5' linked isomer (29) was detected in the reaction mixture. It is worth noting that Ogilvie et al. have recently adopted the two-step deblocking procedure in preference to the single-step TBAF mediated process.[80]



Ad = Adenine

Si = TBDMS

(31)

The remaining isopropylidene group on (28) was removed by mild acidic hydrolysis to avoid, if possible, any 2'-3' phosphodiester bond migration and cleavage of the internucleotide linkage. The reaction was run most successfully in aqueous acetic acid at pH 2.1-2.5

and followed closely by thin layer chromatography (i-PrOH-conc. NH_4OH-H_2O, 8:1:1). By maintaining the solution over one day at 53-54°C, optimal yield (81%) of trideuterated ApA was obtained with minimal side reactions. The only other example of mild hydrolytic removal of an isopropylidene group on dinucleotides was carried out with aqueous formic acid at pH 2.5 and 37°C for three days.[97] In this report, however, no yield was given and the starting material was a mixture of two dinucleotide isomers with 3'-5' and 2'-5' phosphodiester linkages respectively.

Although the isopropylidene group is relatively resistent to acidic hydrolysis under mild conditions, its use as the 2',3'-cis diol protecting group in our studies is justified on the basis of our interest in the influence of the isopropylidene group on ApA conformation mentioned previously. Otherwise, when a direct synthesis of deuterated ApA is required, use of more acid-labile groups (e.g. methoxymethylidene[98]) would be favored.

Isolation of oligonucleotides of various chain lengths is usually performed by open column ion-exchange chromatography (e.g. DEAE-cellulose, DEAE-Sephadex)[99] and gel filtration (e.g. Sephadex).[100] The usefulness

of the former is limited by its long elution time and use of buffers and the latter is not feasible for short-chain nucleotides. Recent developments in separation methods involve high-pressure ion-exchange[101] and reverse-phase[102] chromatography on chemically bonded phase (e.g. octadecylsilyl-derivatized silica gel) which offer major advantages with respect to speed and efficiency.

We have developed a simple and economical workup procedure for the reaction mixture obtained from acidic hydrolysis of (28) in which a short silica gel column is employed.* The residue obtained after the removal of acetic acid and adjustment of pH to 7-8 (with dil. NH_4OH) was chromatographed on the column packed with silica gel (4.0 g) in ethyl acetate-methanol (4:1) which had been pre-washed thoroughly in a Soxhlet extractor with ethyl acetate-methanol (1:1) and methanol. The column was eluted with a gradient of ethyl acetate-methanol (4:1 to 1:1) to obtain adenylyl-(3'-5')-4',5'-[$^2H_3$]adenosine (d_3-ApA) in 81% yield contaminated by a small amount ($\sim$5%) of (28).

*Isolation of milligram quantities of short nucleotides such as ApA by preparative reverse-phase thin-layer plates commercially available just recently appears to be very promising.

The identity of d_3-ApA obtained in this fashion was confirmed by comparison with an authentic nondeuterated sample on the basis of chromatographic behavior and NMR spectral analysis. When no standards are available, synthetic dinucleoside monophosphates can be partially identified by subjecting them to enzymatic degradation. Snake venom phosphodiesterase (VPD), spleen phosphodiesterase (SPD), and ribonuclease (RNase) are most commonly used for this purpose, which digest 3'-5' linked nucleotides completely to mononucleoside and mononucleotide residues.[103] VPD also degrades 2'-5' and 5'-5' linked nucleotides.[104]

$$XpYpZ \xrightarrow{VPD} X + pY + pZ$$

$$XpYpZ \xrightarrow{SPD} Xp + Yp + Z$$

$$XpApYpZ \xrightarrow{RNase\ U_2} XpAp + YpZ$$

X, Y, Z = Nucleosides; A = Adenosine

C. Experimental

General procedures. Melting points (mp) were taken on a Kofler block and are uncorrected. The pH measurements were made with a Corning 61A pH meter. Infrared (IR) spectra were carried out on a Perkin-Elmer 257 spectrophotometer. The nuclear magnetic resonance (NMR) spectrophotometer employed for most measurements was a Bruker HX90E operating at 90 MHz for ^{1}H resonance and at 36.4 MHz for ^{31}P resonance. Occasionally, ^{1}H NMR spectra were recorded on Varian EM390 and Bruker WH270 instruments. Chemical shifts of protons are reported as δ values in ppm relative to residual absorption of DMSO-d_6 (2.50 ppm). Deta for individual protons are recorded in the following order: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants, and interpretation.

Routine thin layer chromatography (tlc) was conducted on Brinkmann silica gel G/UV_{254} (0.25 mm thick; coated on plastic sheets) and R_f values were reported by using 2.5x7.0 cm strips. Preparative tlc (PLC) was performed on 1.0-1.5 mm thick layer silica gel (E. Merck PF254) plates (20x20 cm) or pre-coated 0.25 mm thin

layer silica gel (E. Merck 60 F254) plates. Brinkmann silica gel 60 (70-270 mesh) was utilized for column chromatography. Solvent systems frequently used for tlc studies were: solvent A, n-propanol-conc. ammonium hydroxide-water (20:12:3); solvent B, i-propanol-conc. ammonium hydroxide-water (8:1:1); solvent C, (7:1:2); solvent D, ethyl acetate-acetic acid (19:1); solvent E, ethyl acetate-methanol (4:1); solvent F, ethyl acetate-ethanol (9:1).

In the following reactions, the solvent was dried and distilled, when necessary, by the standard procedure.[105] Dioxane was rendered peroxide-free by refluxing 30 min with 0.5% cuprous chloride solution. To obtain anhydrous solvents for moisture-sensitive reactions, the distillation was usually conducted in a round-bottomed flask fitted with a pressure-equalizing dropping funnel, a condenser, and a nitrogen inlet. The distillate was collected in the dropping funnel. Anhydrous methanol was prepared by refluxing over magnesium methoxide followed by distillation. Pre-drying over potassium hydroxide and distilling subsequently from calcium hydroxide and lithium aluminum hydride gave dry pyridine and tetrahydrofuran, respectively. In most cases, freshly distilled anhydrous solvents were

used immediately. However, on several occasions, they were stored over molecular sieves before use (e.g. THF, MeOD, and DMSO).

Deuterated compounds (%D) used in our experiments were deuterium oxide (99.8%), methanol-d, -d_4 (99.5%), dimethyl sulfoxide-d_6 (99.5%), and benzene-d_6 (99%) obtained from Aldrich Chemical Co., and sodium boro-deuteride (99%) purchased from Stohler Isotope Chemicals.

Reagents employed in the synthesis were commercially available if no preparation was given, and were used directly as received. Nucleosides and nucleotides employed in moisture-sensitive reactions were dried by dissolving in anhydrous pyridine and evaporating the pyridine three times.

For reactions requiring an inert atmosphere, nitrogen was used which had been pre-dried by passing through conc. sulfuric acid and potassium hydroxide. All glassware used in these experiments was dried thoroughly either in the oven (155°C) for several hours and then allowed to cool to room temperature under nitrogen or in a vacuum desiccator over phosphorus pentoxide. Hypodermic syringes were utilized in transfering solvents and reagents for these moisture-sensitive reactions.

The reaction temperatures refer to those of the

bath. Nucleosides and nucleotides when subject to tlc and PLC studies were detected by using an ultraviolet light source. All evaporations were done on a rotary evarorator under reduced pressure. Unless otherwise specified, drying of solid samples was carried out under vacuum in a desiccator over phosphorus pentoxide at room temperature.

9-(2',3'-O-Isopropylidene-β-D-ribofuranosyluronic acid)adenine (2).[36] Isopropylidene adenosine (1) (16.4 g, 54 mmol) was dissolved in warm water (5.3 L) before cooling it to room temperature. Potassium hydroxide (9.0 g, 160 mmol) was added, to maintain the pH of the solution about 12, followed by portionwise addition of potassium permanganate (17.2 g, 109 mmol) over a 2-h period. The reaction mixture was stirred at room temperature for 1-3 days. After this time, complete disappearance of the starting material was observed by tlc (EtOAc-HOAc, 20:1). The excess permanganate was destroyed by dropwise addition of 30% hydrogen peroxide until no pink ring was observed in the spot test. The manganase oxide precipitate was removed by filtration. The clear filtrate was reduced in volume to 450 mL under reduced pressure then the pH of the concentrate

(at 0°C) was adjusted to 4.5-4.6 with 1.0 N hydrochloric acid. The white precipitate was collected and dried to give pure (2) (11.9 g) in 69% yield as an amphorus powder: mp 272°C (slow dec., lit.[36] 276°C); IR (KBr) 1709 cm^{-1} (carboxyl); NMR (DMSO-d_6) δ 8.18 (1H, s, H8), 8.01 (1H, s, H2), 7.20 (2H, broad s, NH_2), 6.25 (1H, s, H1'), 5.41 (2H, s, H2' and H3'), 4.60 (1H, s, H4'), 1.43 and 1.26 ppm (ea. 3H, ea. s, 2 CH_3); R_f 0.60 (solvent A), 0.25 (solvent D).

9-(2',3'-O-Isopropylidene-β-D-ribouronic acid methyl ester)adenine (7).[46] The 5'-carboxylic acid (2) (2.40 g, 7.5 mmol) was dissolved in warm peroxide-free dioxane-methanol (1:1, 2.0 L) before the resulting solution was brought to 0°C in an ice bath. Upon gentle stirring, a cold solution containing a large excess of diazomethane (ca. 7.2 g, 171 mmol; prepared from 51.6 g Diazald by standard procedure) in ether-ethanol (0.75 L) was added cautiously. After 1 h of reaction, the formation of a less polar material was detected by tlc (EtOAc-MeOH, 4:1). Glacial acetic acid was added dropwise to the reaction mixture until the distinct color of diazomethane disappeared. The solvent was removed under reduced pressure and the resulting residue was dissolved in hot

methanol (800 mL). After reducing the methanolic solution to a small volume, it was allowed to cool to room temperature. Crystals deposited after the solution had remained at -15°C overnight were collected and washed with cold methanol. These together with those fine crystals obtained from repeated crystallization of the mother liquor gave a combined 93% yield of (7) (2.33 g) as colorless needles: R_f 0.53 (solvent E). This compound has an identical ^1H NMR spectrum to that reported.[46]

The ester (0.64 g) has also been prepared from (2) in 66% yield *via* the acid chloride essentially according to the procedure of Prasad *et al*.[48]

Preparation of NaOMe/MeOD: To a piece of freshly cut sodium (0.52 g, 22.5 mmol) placed in a dry 25 mL two-necked flask fitted with a pressure-equalizing dropping funnel, a reflux condenser, and a nitrogen inlet was added anhydrous MeOD (9.0 mL) at a rate to maintain vigorous reflux. After completion of the reaction, the resulting solution was allowed to cool to room temperature and was used immediately for the deuterium exchange reaction.

Deuterium exchange of (7). To a solution of the 5'-ester (7) (43 mg, 0.13 mmol) in MeOD (3.0 mL) at room temperature was added an excess of freshly prepared NaOMe/MeOD (6.5 mmol/2.0 mL). The reaction mixture was heated to the reflux temperature and reflux was continued for 30 min. The resulting solution, in which no starting material was present (tlc; $CHCl_3$-n-BuOH, 4:1), was reduced to a small volume and acidified to pH 4.6 with 1.0 N hydrochloric acid. The precipitate thus formed was filtered, washed with cold water, and dried to yield 14 mg of (13), a 4'-deuterated form of (2), in 51% yield as a white solid: NMR (DMSO-d_6) δ 8.07 (1H, s, H2), 7.19 (2H, broad s, NH_2), 6.31 (1H, s, H1'), 5.47 (2H, unresolved s, H2' and H3'), 1.53 and 1.35 ppm (ea. 3H, ea. s, 2 CH_3). (13) has identical mp and tlc behavior as the non-deuterated sample (2).

A more polar side product was also obtained from the above reaction which was characterized as 3'-deoxy-3'-adenosinene 5'-carboxylic acid (14): NMR (DMSO-d_6) δ 8.29 (1H, s, H8), 8.17 (1H, s, H2), 7.37 (2H, broad s, NH_2), 6.35 (1H, d, $J_{2'3'}$ = 3.9 Hz, H3'), 6.14 (1H, d, $J_{1'2'}$ = 2.4 Hz, H1'), 5.51 ppm (1H, t, H2'); R_f 0.57 (solvent A), 0.13 (solvent D).

Deuterium exchange of (2). The 5'-acid (2) (287 mg, 0.90 mmol) in MeOD (6.0 mL) was treated with sodium methoxide (15.2 mmol) essentially the same as for (7) and the starting material was recovered in 63% yield. ^{1}H NMR analysis of the recovered 5'-acid showed about 60% deuterium incorporation at C4'.

Studies of the deuterium exchange of (7) at 25°C and 45°C by ^{1}H NMR (90 MHz). A. To a solution of the 5'-ester (7) (4.0 mg, 0.012 mmol), dissolved in anhydrous CD_3OD-DMSO-d_6 (0.15 mL-0.12 mL) in a 5 mm nmr tube was added freshly prepared NaOMe/CD_3OD (ca. 0.065 mmol/0.09 mL). A total of eleven NMR spectra were taken at 45°C (probe temperature) in which one spectrum was run before the addition of the base and the rest were obtained at 3-min intervals. Resonances at δ 4.81 (H4'), 1.52 and 1.35 ppm (isopropylidene CH_3) were monitored closely.

B. The above procedure was repeated at 25°C with (7) (2.5 mg, 0.007 mmol) and sodium methoxide (0.043 mmol) in CD_3OD-DMSO-d_6 (0.16 mL-0.12 mL). The first spectrum was taken two minutes after the addition of the base, which was followed by nine spectra run at 1-min intervals. Again, resonances of H4' and two methyl

protons were monitored.

The results of A and B were that: (a) at 45°C, the H4' was lost along with about 65% of the isopropylidene group after 6 min; (b) at 25°C, the H4' as well as about 55% of the isopropylidene group was gone after 6 min; and (c) at 25°C and 2 min, about 60% of the H4' and 5% of the isopropylidene group were eliminated.

Deuterium exchange of (7) at low temperature. To a solution of the 5'-ester (7) (1.01 g, 3.01 mmol) dissolved in warm anhydrous DMSO (96 mL) was added, at room temperature, MeOD (40 mL). After placing the solution in a dry ice-CCl_4 bath (-23°C), freshly prepared NaOMe/MeOD (22.5 mmol/8.0 mL) was added and the resulting solution was stirred under a nitrogen atmosphere for 1 h. After neutralizing the reaction mixture with dry glacial acetic acid, it was partitioned between ethyl acetate (1.0 L) and water (200 mL). The aqueous phase was separated and the organic phase was extracted four times with water (ea. 200 mL), dried (anh. K_2CO_3), and evaporated to dryness. Crystallization from methanol gave a total 68% yield of 4'-deuterated 5'-ester (15) as colorless needles: NMR (DMSO-d_6) δ 8.25 (1H, s, H8), 8.05 (1H, s, H2), 7.31 (2H, broad s, NH_2), 6.38 (1H, s,

H1'), 5.60 (1H, d, $J_{2'3'}$ = 6.0 Hz, H2'), 5.43 (1H, d, H3'), 3.3 (obscured by HDO, CO_2CH_3), 1.52 and 1.35 ppm (ea. 3H, ea. s, 2 CH_3). (15) has identical mp and tlc behavior as those of non-deuterated (7). The mother liquor was evaporated to dryness and the residue was subjected to PLC ($CHCl_3$-n-BuOH, 4:1; two developments) to yield 118 mg of (16) which was characterized as methyl 3'-deoxy-3'-adenosinene 5'-carboxylate: NMR (DMSO-d_6) δ 8.31 (1H, s, H8), 8.15 (1H, s, H2), 7.37 (2H, broad s, NH_2), 6.39 (1H, d, $J_{2'3'}$ = 4.0 Hz, H3'), 6.26 (1H, d, $J_{1'2'}$ = 2.4 Hz, H1'), 5.54 (1H, t, H2'), 3.74 ppm (3H, s, CO_2CH_3); R_f 0.39 (solvent E).

Reduction of the methyl esters (7) and (15). To a stirred solution of 4'-deuterated 5'-ester (15) (1.26 g, 3.76 mmol) suspendes in MeOD-D_2O (1:1, 250 mL) was added portionwise sodium borohydride (1.43 g, 37.8 mmol) at 0°C. On completion of the addition, the reaction mixture was allowed to warm to room temperature and stir overnight in an atmosphere of nitrogen. The resulting clear solution was evaporated to dryness under reduced pressure and the residue thus obtained was taken into methanol-water (3:1, 34 mL). The aqueous methanolic solution was filtered, concentrated, and stored at 5°C

overnight. The precipitate was collected and dried to to give 1.02 g (89%) of 4'-deuterated (19) as a white solid: NMR (DMSO-d_6) δ 8.34 (1H, s, H8), 8.15 (1H, s, H2), 7.34 (2H, broad s, NH_2), 6.11 (1H, d, $J_{1'2'}$ = 3.0 Hz, H1'), 5.34 (1H, q, $J_{2'3'}$ = 6.3 Hz, H2'), 4.96 (1H, d, H3'), 3.54 and 3.52 (ea. 1H, ea. s, H5' and H5''), 1.54 and 1.32 ppm (ea. 3H, ea. s, 2 CH_3); R_f 0.68 (solvent C), 0.38 (solvent E). (19) has identical mp and tlc behavior as those of the non-deuterated sample.

Following an identical procedure, the 5'-deuterated compound (3) and the 4',5'-deuterated compound (20) were obtained by the reduction of (7) and (15) respectively with sodium borodeuteride. Additionally, (19) (12 mg, 42%) has been obtained by the reduction of suspended (15) (30 mg) in 6.0 mL tetrahydrofuran with lithium borohydride (5 mg) at room temperature for four days.

Preparation of 4'-[$^2H_1$]adenosine (24). Isopropylidene 4'-[$^2H_1$]adenosine (19) (861 mg, 3.2 mmol) was treated with 0.9 N hydrochloric acid (29 mL) at room temperature for 1 h. After this time, tlc (EtOAc-MeOH, 3:1) showed the completion of the reaction and the reaction mixture was then neutralized (dil. NH_4OH), concentrated, and stored at 5°C overnight. The resulting

precipitates were collected and precipitation was repeated on the mother liquor. Together with the second crop, a total of 713 mg of (24) was obtained in 95% yield as a white solid. (24) was characterized by comparison with an authentic non-deuterated sample; the former lacks the H4' resonance (δ 3.96 ppm).

Silylation of adenosine and 4'-[$^2H_1$]adenosine (24) with t-butyldimethylsilyl chloride (TBDMS-Cl).[79] The TBDMS-Cl reagent (462 mg, 3.1 mmol) was added to a stirred, turbid solution of adenosine (271 mg, 1.0 mmol) in dry pyridine (4.0 mL) at room temperature. The reaction mixture was protected from moisture with a drying tube (anh. $CaSO_4$) and stirred for 2 days. The now clear reaction mixture was partitioned between ethyl acetate (40 mL) and water (20 mL). The organic extracts were washed with water (4x20 mL), dried (anh. K_2CO_3), and evaporated to dryness. The residue was chromatographed on a 2.1x23 cm silica gel column (30 g). The column was eluted with a gradient of benzene and ethyl acetate and pure fractions (ea. 10 mL) were pooled and mixed fractions were further purified by PLC in ether (three developments) or benzene-ethyl acetate (1:3, two developments). The overall yields of four

different silylated products 5'-mono(TBDMS)-, 3',5'-di(TBDMS)- (23), 2',5'-di(TBDMS)- (22), and 2',3',5'-tri(TBDMS)adenosine were 6, 41, 43 and 3%, respectively. The R_f values of these four products in the above order are 0.02, 0.15, 0.25, and 0.43 (ether) and 0.08, 0.46, 0.64, and 0.77 (EtOAc).

The desired product (22) was obtained as an amorphous white powder: mp 171-174°C (lit.[79] 174-177°C). It has also been prepared from adenosine on the same scale by running the silylation in dimethylforamide (1.5 mL) in the presence of imidazole (340 mg) at room temperature for 3h. However, the yield (39%) was slightly lower in this case. Following an identical procedure, 2',5'-di(TBDMS)-4'-[$^2H_1$]adenosine (27) was prepared from (24).

Interconversion of disilylated (22) and (23) by 2'-3' isomerization. Pure 3',5'-di(TBDMS)adenosine (23) (132 mg) was allowed to reflux in methanol (20 mL) for 8 h until an equilibrium was reached (as observed by tlc in EtOAc). The resulting reaction mixture was worked up, by PLC as previously described, to obtain both (23) and its 2',5'-isomer (22) in about a 1:1 ratio (64 mg vs. 67 mg).

Preparation of the coupling reagent, 2,2,2-trichloroethyl phosphorodichloridite (21).[106] To a dry 50 mL two-necked flask containing phosphorus trichloride (7.8 mL, 90 mmol), stirred and maintained under a nitrogen atmosphere at 0°C, was added dropwise 2,2,2-trichloroethanol (5.8 mL, 60 mmol) from a pressure-equalizing funnel over a period of 20 min. After completion of the addition, the reaction mixture was allowed to warm to room temperature and was allowed to react for 27 h. The hydrogen chloride gas produced during the reaction was trapped by potassium hydroxide pellets. The resulting solution was subjected to vacuum distillation to yield 8.5 g (57%) of (21) as a colorless liquid: bp 57°C/0.7 mm (lit.[106] bp 42°C/0.1 mm); ^{31}P NMR δ -41 ppm (rel. to 0.925 M H_3PO_4).

Preparation of fully protected ApA, (25) and (26). To a dry 50 mL flask, equipped with a magnetic stirring bar and an inlet of nitrogen, was added successively through a rubber septum, tetrahydrofuran (0.30 mL), collidine (350 µL, 2.65 mmol), and $CCl_3CH_2OPCl_2$ (100 µL, 0.68 mmol). The flask was placed in an acetone-dry ice bath and was stirred under nitrogen. After cooling the solution to -78°C, a solution of 2',5'-disilylated

adenosine (27) (340.4 mg, 0.68 mmol) in tetrahydrofuran (2.3 mL) was added over a period of 2-3 min and the resulting white suspension was stirred for 40 min. This was followed by the addition of isopropylidene adenosine (3) (101.0 mg, 0.33 mmol) in tetrahydrofuran (20.6 mL) and the stirring was continued at -78°C for 2 h. Then the cold bath was removed and the reaction mixture was allowed to warm to room temperature. Iodine (170.5 mg, 0.67 mmol) and collidine (177 μL, 1.34 mmol) in a solution of tetrahydrofuran-water (2:1) (10.5 mL) was added and stirring was continued for 15 min. The solvent was removed under reduced pressure and the resulting syrupy residue was dissolved in about 30 mL of n-butanol-chloroform (1:9). The solution was washed with 2% aqueous sodium bisulfite (5.0 mL) and water (30 mL). The aqueous phase was back-extracted twice with chloroform (ea. 25 mL). The combined organic extracts were dried (anh. K_2CO_3), concentrated, and purified by PLC. The thick layer plates were developed (EtOAc and EtOAc-EtOH, 9:1) and the major band was eluted to give, after evaporation, 265.5 mg (83%) of (26) as a foamy solid: mp 117-121°C; R_f 0.48 (THF), 0.19 (solvent F).

The resultant phosphotriester was a mixture of two

diastereomers. On one occasion, precipitation in ether-ethyl acetate resulted in an increase of the relative amount of one stereomer vs. the other.

Both (25) and its non-deuterated form have been prepared by the identical procedure as described for (26). However, in the latter case, 2,6-lutidine was utilized instead of collidine and the yield of that coupling reaction was slightly lower (75%).

Preparation of tetra-n-butylammonium fluoride (TBAF). A stirred 40% aqueous solution of tetra-n-butylammonium hydroxide (2.0 mL) was neutralized by the dropwise addition of 10% aqueous hydrofluoric acid. After removal of the solvent under reduced pressure, several portions of benzene-acetonitrile were added and evaporated from the residue. (Alternatively, the neutral solution was lyophilized.) The resulting solid was dried further (over P_2O_5) under vacuum for 20-60 h to give, after shaking, 836 mg of very hygroscopic powder.

Preparation of the TBAF-silica reagent. The procedure of Clark[87] was followed in which 0.60 g of TBAF and 1.70 g of 70-270 mesh silica gel was used to give 2.30 g of the reagent (ca. 1 mmol TBAF/g TBAF-silica).

Deblocking of disilylated adenosines (22) and (23) with TBAF. A mixture of dry (22) and (23) (41 mg, 0.083 mmol) was treated with 0.5 M TBAF/THF (0.80 mL). Complete deblocking was observed by tlc (EtOAc) after stirring 45 min at room temperature. The solvent was removed under reduced pressure and the resulting residue was purified by PLC (EtOAc-EtOH, 4:1). The major band was eluted to give, after removal of the solvent, a gel which was subsequently broken down by methanol. Thus, adenosine (20 mg, 91%) was obtained as a white precipitate, which was identical to an authentic sample (tlc and NMR).

Deblocking of fully protected (25) with TBAF. Dry (25) (112.1 mg, 0.11 mmol) in tetrahydrofuran (3.0 mL) was treated with TBAF (375.7 mg, 1.44 mmol) at room temperature for 1 h. The reaction mixture was shown by tlc (i-PrOH-conc. NH_4OH-H_2O, 8:1:1) to contain two major products in about a 1:1 ratio. After concentration, the reaction mixture was chromatographed on thick layer plates (EtOAc-EtOH, 19:1 and tlc solvent). Two major bands were observed and they were eluted separately (EtOAc-MeOH, 1:1). Since a relatively polar solvent was employed for elution, some impurities from

silica gel were carried though. Further purifications of the reaction products were conducted as follows. The residue of either major product (50-100 mg from several runs) was applied to a 2.1x27 cm silica gel column (40 g) which had been washed previously with ethyl acetate-methanol (1:1). The column was eluted using a gradient of ethyl acetate-methanol (4:1 to 1:1) with a 1.2 mL/min flow rate. Fractions of 25 mL were collected and those containing the desired material were pooled and evaporated. One of the major products, which is only slightly less polar than the other one (R_f 0.42 vs. 0.37 in solvent B), was identified as isopropylidene protected ApA (28) on the basis of its subsequent hydrolytic conversion to free ApA. The identity of the other major product was rationalized to be the 2'-5' linked isomer (29) according to other evidences that van Boom *et al.*[93] obtained similar results in their study of uridilyl-(3'-5')uridine.

It is interesting to note that the nature of the above TBAF deblocking reaction does not vary significantly by varying the reaction conditions such as (a) TBAF forms (solid powder, silica-bound, and THF solution); (b) amounts of TBAF used (6-66 equiv.); (c) reaction temperature (-42°C to room temperature);

(d) reaction time (1-7 h); and (e) solvents (THF, DMF, and acetonitrile).

Activation of Zn. Zn dust (150 g) was treated according to the procedure described by van Boom et al.[96] except that additional amounts of water (3x250 mL) were used in the preliminary washing to neutrality.

Two-step deblocking of fully protected (25). Activated zinc (0.41 g, 6.3 mmol), freshly prepared, was added to a well-stirred solution of (25) (86.7 mg, 0.088 mmol) and 2,4,6-triisopropylbenzenesulfonic acid[96] (6.7 mg, 0.024 mmol) in anhydrous pyridine (1.5 mL). The deblocking was followed by tlc (EtOAc-MeOH, 9:1). No starting material was left after 3 h at room temperature. The reaction mixture was then filtered to remove zinc. Following the dilution of the filtrate with ethyl acetate (15 mL), the resulting solution was washed successively with a dilute aqueous solution of ammonium bicarbonate (pH 7.7) and water (ea. 5 mL). The aqueous phase was back-extracted with ethyl acetate (10 mL). The combined organic extracts were evaporated to dryness and further dried by co-evaporation with anhydrous pyridine three times.

The residue thus obtained was treated with TBAF (133.3 mg, 0.51 mmol) in dry tetrahydrofuran (7.0 mL) at room temperature for 0.5 h. After removal of the solvent, the resulting residue was shown by tlc (i-PrOH-conc. NH_4OH-H_2O, 7:1:2) to contain only the desired 3'-5' linked dinucleotide (28). No 2'-5' linked isomer (29) observed previously in the one-step deblocking procedure was detected in this case.

Adenylyl-(3'-5')-4',5'-[$^2H_3$]adenosine (d_3-ApA). An aqueous solution (2.5 mL) of the isopropylidene dinucleotide (28) (11.5 mg, 0.018 mmol) was acidified with glacial acetic acid to pH 2.1 and maintained at 53-54°C under magnetic stirring. The progress of the reaction was followed closely by tlc (i-PrOH-conc. NH_4OH-H_2O, 8:1:1). A very small amount of starting material still remained in the reaction mixture after a total of 32 h. However, at this time trace impurities started to appear. Therefore, the reaction was removed from the oil bath and was diluted with water (2.5 mL). Several portions of water were added during the ensuing evaporation to drive off the acetic acid. The resulting residue obtained was chromatographed on a short silica gel column (1.0x14 cm). The silica gel (4.0 g) had been

washed thoroughly in a Soxhlet extractor with methanol and methanol-ethyl acetate (1:1) separately before packing. Gradient elution was performed using methanol-ethyl acetate (4:1-1:1). With a flow rate of 1 mL/min, fractions of 20 mL were collected and examined by tlc (as above). The pure fractions were pooled and mixed fractions were rechromatographed. Combined eluents which contain the major product were evaporated to dryness to yield 8.8 mg (81%) of a white solid which was characterized as d_3-ApA by comparison (tlc and NMR) with an authentic nondeuterated sample.

III. Proton-Proton and Phosphorus-Proton Nuclear Overhauser Effect Studies

A. Introduction

Complete decoupling of nuclear spins has been widely used for spectral assignments and structural elucidations in which a high power second magnetic field is applied. The power requirement for spin saturation, however, is relatively lower and in cases when two nuclei are related by a mutual relaxation process, a spin saturation of one nucleus will be reflected by the corresponding change of integrated intensity of the other. It is this phenomenon that constitutes the nuclear Overhauser effect (NOE). Since this effect was first observed between protons by Anet and Bourn,[107] the NOE technique has been developed and applied to studies of molecular geometry and conformation distribution of both rigid and non-rigid molecules.[108,109]

The NOE can be described quantitatively as the fractional enhancement of the resonance of spin d when the resonances of spin s are saturated, or

$$f_{\underline{d}}(\underline{s}) = \frac{\text{area of } \underline{d} \text{ when } \underline{s} \text{ is saturated - equilibrium area of } \underline{d}}{\text{equilibrium area of } \underline{d}}$$

Before any useful information can be extracted from the enhancement, the contributing relaxation mechanisms (e.g. dipole-dipole, chemical shift anisotropy, spin-rotation, scalar) must be determined. For proton and phosphorus nuclei in small molecules such as mono-, di-, and short oligonucleotides, the dipole-dipole relaxation has been shown to be predominant at a field of 21 kG. The chemical shift anisotropy, which is field dependent, contributes to the relaxation of phosphorus nuclei at high magnetic field; it is therefore preferable to run the NOE experiment in the range 15-25 kG for optimal NOE's and acceptable sensitivity. To obtain the maximum observable NOE, it is also required that the extreme narrowing condition is met, that is, $\omega_0\tau_c \ll 1$, where ω_0 is the Larmor frequency of the observed nucleus and τ_c is the time constant for overall molecular reorientation. For $^1H\{^1H\}$ and $^{31}P\{^1H\}$ NOE measurements performed at 21 KG, the extreme narrowing condition is usually satisfied for small nucleotides (MW<1000) as the overall

motion is sufficiently short ($\tau_c < 10^{-9}$ sec)[110] so that the internal motion will have no or negligible effect on measured NOE's within the experimental error. Additionally, for maximum NOE enhancements to be observed it is essetial that paramagnetic impurities be excluded from the sample, as the electron-nuclear dipole-dipole interaction can be as much as 1000 times greater than the nuclear-nuclear interaction.

In the case when the extreme narrowing condition is met, the maximum enhancements of $f_H(H)$ and $f_P(H)$ will be found to be 0.50 and 1.24 respectively when the interacting nuclei (uncoupled or loosely coupled) are relaxed via the dipole-dipole mechanism, and the observed NOE enhancement is inversely proportional to the sixth power of the internuclear distance between the nuclei involved. As described earlier, the observable NOE will be attenuated because the above conditions cannot be fully satisfied in practice. However, it is important to note that these effects, such as small contributions of other relaxation mechanisms in addition to dipole-dipole interactions, need not complicate the analysis of NOE measurements to obtain structural information.[35]

NOE's have been exploited for the establishment of

spatial proximity between interacting nuclei in small molecules (MW<1000). For example, it has been demonstrated that $^{1}H\{^{1}H\}$ and $^{31}P\{^{1}H\}$ NOE measurements are useful tools for conformational analysis of mononucleotides in which the former allows a direct determination of the syn-anti distribution of glycosyl torsion angle χ and the latter provides information regarding the preferred rotamer distributions of sugar-phosphate backbone torsion angles ϕ', ϕ, and ψ.[34,35,111,112] Among these torsion angles, χ and ϕ' are difficult to study directly by other methods. A summary of typical NOE measurements and corresponding rotamer distributions from these studies is presented in Table III and IV. However, as molecular weight increases, the extreme narrowing condition is progressively violated and the NOE exhibits greater dependence upon field strength, overall motion, and internal motion in addition to its dependence upon the geometry. Moreover, these effects along with increasing difficulties of resonance resolution and assignment cause the NOE studies to be less selective and therefore more qualitative. For conformational analysis of oligonucleotides beyond the trimer level (MW ~1000), the proton and phosphorus spin-lattice relaxation time, T_1, measurements at various field strengths will

Table III

Examples of NOE-derived X Distributions
Based on Experimental Enhancements
at Neutral pH and Room Temperature[34,111,112]

Nucleosides and Nucleotides	Protons irradiated	H8 NOE Enhancements (%)	X Distributions (%) Syn	Anti
i-Adenosine*	H1'	22	84	16
	H2'	12		
i-Guanosine	H1'	16	76	24
	H2'	16		
3'-AMP	H1'	20	72	28
3'-GMP	H1'	18	72	28
5'-AMP	H1'	4	30	70
	H2'	20		
5'-GMP	H1'	10	53	47
	H2'	18		

*i = 2',3'-O-isopropylidene

Table IV

Examples of NOE-derived ϕ' and ϕ, ψ Distributions Based on Experimental Enhancements at Neutral pH and Room Temperature[35]

Nucleotides	Protons irradiated	Phosphorus NOE Enhancements (%)	Distributions of Rotamers (%)*	
			ϕ'	ϕ,ψ
			g^-	$g'g',gg$
3'-AMP	H2',H3'	34	77	
	H4'	15		
	H5',H5"	-6.5		
5'-AMP	H5',H5"	47		72
	H4'	14		

*Computations were done by assuming a $^3E/^2E$ (36/64) ribose conformation.

complement the NOE data in providing valuable information, particularly, to the phosphodiester geometry.*

B. Experimental

1. Sample preparation

Non-deuterated ApA was purchased from Boehringer-Mannheim Biochemicals. All samples were lyophilized at least twice from 99.8% D_2O, taken up in "100%" D_2O (Aldrich Chemical Co.), passed through 1-2 cm columns of Chelex 100 (Bio-Rad Laboratories) previously adjusted to pD 7-8** and equilibrated with "100%" D_2O,*** and made up in appropriate volumes to a concentration of 0.01-0.05 M. These pre-treated aqueous samples were further degassed by a special technique. The sample (except d_3-ApA) was placed in one leg of an inverted "V" tube connected at its vertex to a vacuum line via

*P.A. Hart and C.F. Anderson, work in progress.

**The pD (pH+0.4) of the column was adjusted with 40% NaOD in D_2O(Merck & Co., Inc.).

***This step was included to remove any possible paramagnetic impurities from the samples.

a standard-taper connector. On the other leg was fused a Wilmad 10 mm cylindrical insert (Figure 2). After three freeze-pump-thaw cycles were completed at high vacuum (10^{-5}-10^{-6} mm), the assembly was tipped whereupon the degassed aqueous solution flowed into the insert while it was still under vacuum. The insert was then sealed near the top of its neck (2 mm) and removed from the assembly. The d_3-ApA sample was prepared in a Wilmad 5 mm cylindrical microcell (Figure 3) by a similar technique. All glassware that was to contact the samples after Chelex treatment was soaked in basic EDTA, rinsed with methanol, and dried. For each sample, both proton and phosphorus spectroscopy were done on the same sample configuration.

2. NOE measurements

All NOE measurements were made with a Bruker HX90E spectrometer modified for quadrature detection (^{2}H lock). Phosphorus resonance was observed at 36.4 MHz and proton resonance at 90 MHz. Generally, an observing pulse of 90° was used and the pulse delay was determined empirically for maximum signal intensity. A 90° pulse was chosen because slight variability in pulse width (a

Figure 2

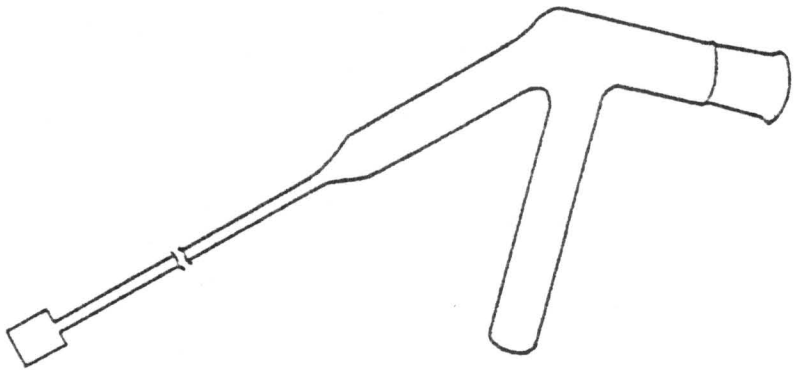
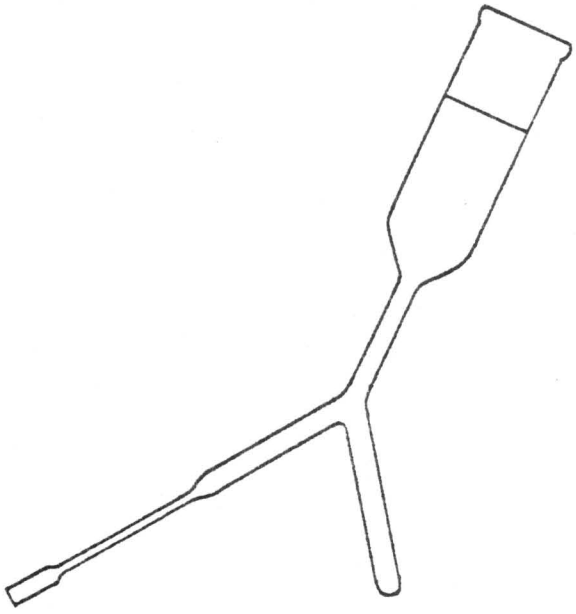


Figure 3



critical parameter) is not so serious at larger pulse widths. Free induction decays were transmitted to and transformed by a Nicolet 1080 computer. Exponential multiplication of the free induction decays resulting in 0.25-0.50 Hz line broading were routinely applied. Digital resolution was always 0.25 Hz. Sample temperature was maintained to $\pm 0.2^{\circ}C$ using a Bruker B-ST 100/700 temperature controller.

Ideally, every spectrum corresponding to irradiation at a specific resonance frequency should be accompanied by an off-resonance experiment in which the irradiation frequency is set to correspond to a transparent region of the spectrum. That procedure is too time consuming in practice, particularly when phosphorus is the observed nucleus. If all instrument parameters and the temperature are carefully controlled that procedure is unnecessary. It suffices to collect "off" resonance data for every two or three "on" resonance experiments such that the latter are bracketed by the former.

The transformed free induction decays were phased as nearly perfectly and reproducibly as possible and prescribed regions were integrated electronically. If all precautions have been carefully exercised, 1-2%

experimental precision is possible, 1-5% is usual, and when the signal to noise ratio is less than ten, precision can be as poor as 10%. The experimental precision of the data reported in this thesis is in the 1-5% range.

C. Results and Discussion of the NOE Experiments

In what follows, the experimental NOE results will be interpreted qualitatively under the heading of each relevant torsion angle(s) for ApA, d_3-ApA, and d_3-Ap(iA),* (28). To make quantitative conformational analyses practical, more extensive deuterium substitution is required to eliminate Ap- and -pA H2',H3' overlap.

χ_1, χ_2 The 6-22°C temperature range was chosen because it had previously been observed[26] that the amplitude of the CD curves in the region characteristic of base-stacking was significantly reduced. The proton-proton NOE's recorded in Table V and VI show, among other things, that the syn distribution for the glycosyl torsion angle χ_1 is significantly more populated at

*iA = 2',3'-O-isopropylidene adenosine

Table V

$^{1}H\{^{1}H\}$ NOE Studies of ApA. H8 Enhancements

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Ap- H8		-pA H8	
	6°C	22°C	6°C	22°C	6°C	22°C
-pA H1'	-5664	-5638	2	5	-1	10
Ap- H1'	-5675	-5653	3	14	-2	10
Ap- H2',H3'		-5756		16		12
-pA H2',H3'	-5789	-5768	6	9	20	26
-pA H4',H5' & Ap- H4'	-5804	-5784	4	2	12	17
-pA H5" & Ap- H5',H5"	-5846	-5829	4	1	2	7

Table VI

$^{1}H\{^{1}H\}$ NOE Studies of ApA. H2 Enhancements

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Ap- H2		-pA H2	
	6°C	22°C	6°C	22°C	6°C	22°C
-pA H1'	-5664	-5638	0	0	1	1
Ap- H1'	-5675	-5653	-3	5	-2	5
Ap- H2',H3'		-5756		5		3
-pA H2',H3'	-5789	-5768	4	2	5	3
-pA H4',H5' & Ap- H4'	-5804	-5784	7	1	8	3
-pA H5" & Ap- H5',H5"	-5846	-5829	2	-3	3	1

higher temperature. This conclusion is supported by the larger Ap- H8{Ap- H1'} NOE result (Table V). Table III of the introduction indicates clearly that relatively greater populations of syn conformers are associated with larger H8-H1' interactions. The relatively small enhancement of -pA H8 when Ap- H1' is irradiated confirms that the two H1' resonances can be irradiated uniquely and separately even though they are only 11-15 Hz apart. Therefore, the -pA H8{-pA H1'} NOE result is significant and indicates a higher proportion of the syn conformer about χ_2 also at higher temperature. The remaining enhancements recorded in Table V and VI are ambiguous because of the very complex inter-monomer enhancements that are possible but also because there is considerable resonance overlap in the non-deuterated sample. These experiments were not repeated with the deuterated material because some chemical shift overlap remains but also because the inter-monomer effects remain. It will be interesting to devote further study to the small but significant purine H2 enhancements seen in Table VI because these almost certainly indicate inter-monomer effects only. Purine H2 enhancements are not observed in mononucleotides.[34,112]

For d_3-Ap(iA), (28), the proton-proton NOE data

(Table VIII and IX) were recorded at 26°C and 46°C instead of 6°C and 22°C as for d_3-ApA because noticeable aggregation occurs below ca. 25°C. Table VIII shows no detectable change in Ap- H8{Ap- H1'} NOE in the temperature range of 26-46°C. However, a small increase of the -pA H8{-pA H1'} NOE was observed in this temperature range. Evidently, χ_1 and χ_2 assume significant populations of <u>syn</u> conformers at either 26°C or 46°C and the populations are not very sensitive to the elevation of temperature except that a slightly higher <u>syn</u> distribution for χ_2 of d_3-Ap(iA) was observed at higher temperature. Likewise, the remaining enhancements recorded in Table VIII exibit little sensitivity toward the temperature variation. As mentioned earlier, the intermonomer cross interactions also play a significant role in these observations. Again, both H2 enhancements for d_3-Ap(iA) were observed. Since the NOE experiments cannot be carried out at lower temperature, it is not known if the <u>syn</u> rotamer probabilities decrease at lower temperature as was observed for d_3-ApA.

<u>ϕ'</u> The data applicable to this torsion angle for d_3-ApA are phosphorus-proton NOE's recorded in Table VII. In order to compare the data taken at 5°C and 26°C,

Table VII

$^{31}P\{^{1}H\}$ NOE Studies of d_3-ApA

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Phosphorus	
	5°C	26°C	5°C	26°C
All	Broad band		46	78
-pA H1'		-5633		0
Ap- H1'		-5644		2
Ap- H2',H3' & -pA H2'	-5777	-5750	10	17
-pA H3'	-5792	-5763	10	14
Ap- H4'	-5808	-5781	8	12
Ap- H5',H5"		-5823		-1

Table VIII

$^{1}H\{^{1}H\}$ NOE Studies of (3'-5')-d_3-Ap(iA).

H8 Enhancements

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Ap- H8		-p(iA) H8	
	26°C	46°C	26°C	46°C	26°C	46°C
-p(iA) H1'	-5616	-5599	5	4	7	10
Ap- H1'	-5651	-5631	10	10	2	-1
-p(iA) H2'	-5692	-5674	4	4	15	15
-p(iA) H3'	-5710	-5688	0	3	3	6
Ap- H2',H3'	-5757	-5740	11	12	10	10
Ap- H4'	-5793	-5772	3	5	5	4
Ap- H5',H5"	-5835	-5820	3	2	4	2

Table IX

$^{1}H\{^{1}H\}$ NOE Studies of (3'-5')-d_3-Ap(iA).

H2 Enhancements

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Ap- H2		-p(iA) H2	
	26°C	46°C	26°C	46°C	26°C	46°C
-p(iA) H1'	-5616	-5599	3	2	1	1
Ap- H1'	-5651	-5631	1	2	0	0
-p(iA) H2'	-5692	-5674	4	6	5	3
-p(iA) H3'	-5710	-5688	-2	6	0	3
Ap- H2',H3'	-5757	-5740	5	6	4	3
Ap- H4'	-5793	-5772	2	7	4	3
Ap- H5',H5"	-5835	-5820	2	2	3	2

it is necessary to scale the results according to the relative values of the total NOE enhancements (46% at 5°C and 78% at 26°C). These numbers differ because the nucleotide aggregates at lower temperature. The result of this aggregation is a violation of the extreme narrowing condition to which $^{31}P\{^{1}H\}$ NOE's are more sensitive than $^{1}H\{^{1}H\}$ NOE's. The scaled 5°C NOE data are 17.0, 16.5, and 12.9 to be compared with 17, 14, and 12 at 26°C. It is clear that the NOE profiles are not temperature sensitive. Thus, we conclude that ϕ' does not change significantly over the temperature that produces significant changes in χ_1 and χ_2. Of further significance is the substantial P{-pA H3'} NOE that was not observed in 5'-AMP.[35] This point will be discussed later. It is tempting to compare the enhancements seen in the dimer with those reported earlier for 3'-AMP[35] (summarized in Table IV). The relevant enhancements are those associated with irradiations of Ap- H2',H3', H4', and H5',H5". Before trying to make a comparison between the dimer and the monomer data, it should be noted that a clear $^{4}J_{p,2'}$ is observed for ApA whereas that coupling is too small to be observed in 3'-AMP. As stated earlier this four-bond coupling is characteristic of the g^{+} conformer. Furthermore, the

negative P{Ap- H5',H5"} NOE of 3'-AMP was a critical factor in establishing a small proportion of the t rotamer in ϕ' equilibrium. That negative NOE is virtually missing in ApA. Therefore, although the enhancement profiles are qualitatively similar, it is fairly certain that further experimentation will show that ϕ' of ApA is significantly different from that of 3'-AMP.

As was done for d_3-ApA, the analysis of the NOE data recorded for d_3-Ap(iA) (Table X) has taken into account the effect of aggregation. Therefore, $^{31}P\{^1H\}$ NOE's at 26°C were scaled to the values of 3.5, 6.4, 49.6, 29.3, and 5.8 according to the total NOE's at 46°C relative to 26°C. It is clear that the NOE profiles of d_3-ApA and d_3-Ap(iA) are different at 26°C. Based on the observed NOE for Ap- H2',H3', it is tempting to think that the relative g^- population is higher in the latter than in the former based on the typical value recorded for 3'-AMP in Table IV. However, the small positive P{Ap- H5',H5"} NOE and relatively large P{Ap- H4'} NOE observed for d_3-Ap(iA) in comparison to those of d_3-ApA hinted that a certain extent of g^+ and t rotamers are present. Therefore, further discussion of these experimental results should be done with caution. This also applies to the rationalization of the

Table X

$^{31}P\{^{1}H\}$ NOE Studies of (3'-5')-d_3-Ap(iA)

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Phosphorus	
	26°C	46°C	26°C	46°C
All	Broad band		600	84
Ap -H1'	-5651	-5631	3	1
-p(iA) H3'	-5710	-5688	5	9
Ap- H2',H3'	-5757	-5740	36	39
Ap- H4'	-5793	-5772	21	24
Ap- H5',H5"	-5835	-5820	4	3

significant enhancement changes upon temperature recorded for d_3-Ap(iA). The NOE profile of d_3-Ap(iA) at 46°C is somewhat similar to that of 3'-AMP (Table IV), but significant differences remain for Ap- H4' and H5',H5" enhancements, especially the latter in which a negative 0.065 value was recored for 3'-AMP. It is worth noting that the P{-p(iA) H3'} NOE observed for d_3-Ap(iA) is smaller than that of d_3-ApA, but is still significant. It can be concluded tentatively that the conformation indicated by this P-pA H3' interaction is still present in d_3-Ap(iA).

<u>ϕ, ψ</u> The model we have chosen to examine provides virtually no information about ϕ and ψ because the critical 4', 5', and 5" positions are deuterated and in the analysis of 5'-AMP, the H4' enhancement was a critical factor. As noted above, however, the substantial -pA H3' enhancement indicates that a different rotamer distribution for ApA about ϕ and/or ψ compared to 5'-AMP.

Since d_3-Ap(iA) is also deuterated at 4', 5', and 5" positions, the only indication of ϕ and ψ profiles is from the observed P{-pA H3'} NOE. As mentioned earlier, the small enhancement value for d_3-Ap(iA)

suggests that its ϕ and ψ distributions are significantly different from those of d_3-ApA and 5'-AMP. In addition, these distributions are relatively temperature sensitive in the range of 26-46°C, as indicated by the change in the P{-pA H3'} NOE.

D. Conclusions

Partial answers have been provided to the questions set out in the introduction. Those are: (a) how many protons interact with the backbone phosphorus? (b) what is the ϕ' conformation? and (c) what torsion angle changes accompany the destacking seen from 5°C to 25°C?[26] It is evident from the NOE studies of d_3-ApA that Ap- H3',H4' as well as -pA H3' interact with the phosphorus. It is likely that Ap- H2' interacts and it is certain that -pA H5',H5" would interact in the non-deuterated model. Therefore, five and perhaps six protons interact with the backbone phosphorus; not three, as is commonly assumed.[110]

It can be safely concluded that the ϕ' rotamer distribution in d_3-ApA is different from that of 3'-AMP because no negative P{Ap- H5',H5"} NOE was seen and

because four-bond J coupling of the phosphorus and Ap-H2' can be observed. Within our experimental error and within the sensitivity of the NOE measurement, the distribution is insensitive to temperature in the range of 5-26°C. Therefore, assuming that the ϕ and ψ rotamer distributions are also relatively stable in this temperature range, the hypothesis that the phosphodiester ω' and ω bonds are the most flexible backbone bonds is supported.

In addition to the above conclusions, it is also true that the -pA 2',3'-O-isopropylidene group changes the various rotamer distributions and because the protons of this model are much better separated, further detailed study of it is warranted.

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V. Appendices

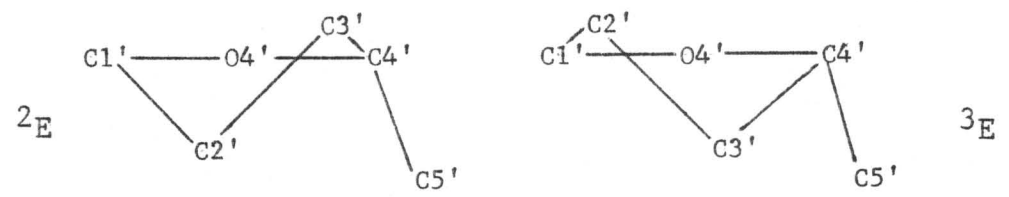

Figure 4. Two Preferred Ribose Conformations of ApA

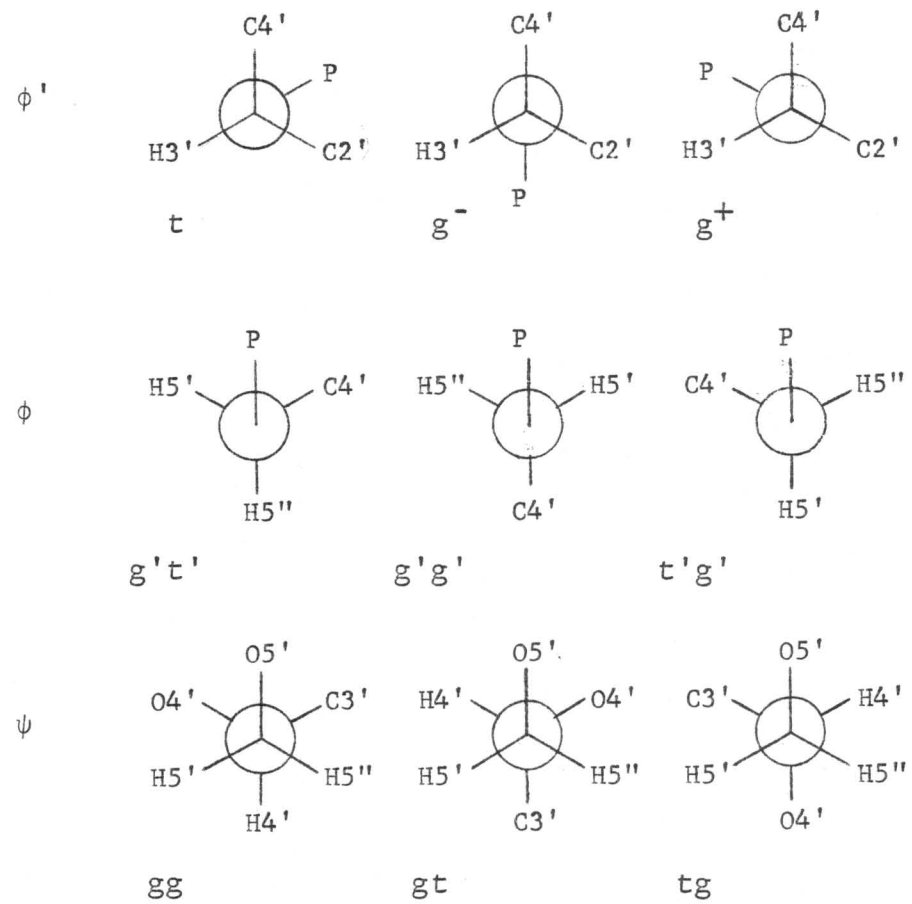

Figure 5. Classical Staggered Rotamers about ϕ', ϕ, and ψ Bonds of ApA

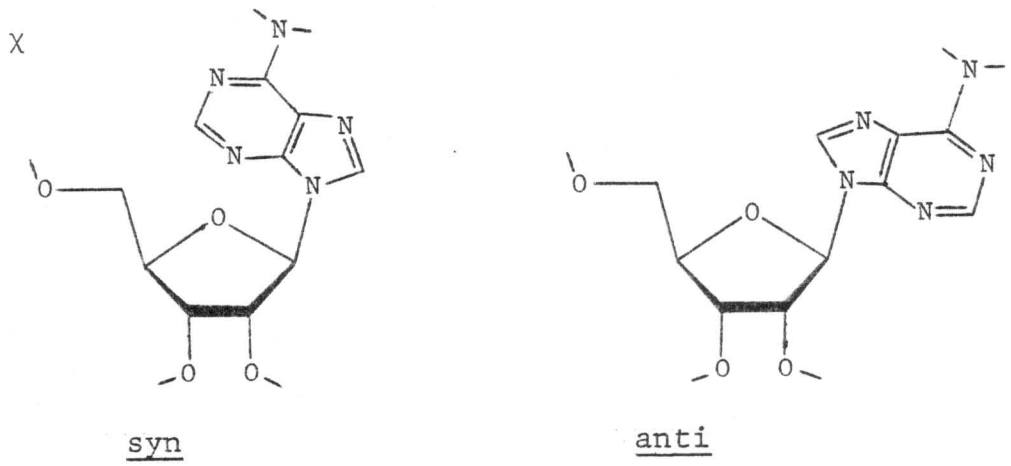

Figure 6. Anti and Syn Conformations about the Glycosyl Bonds χ_1 and χ_2 of ApA

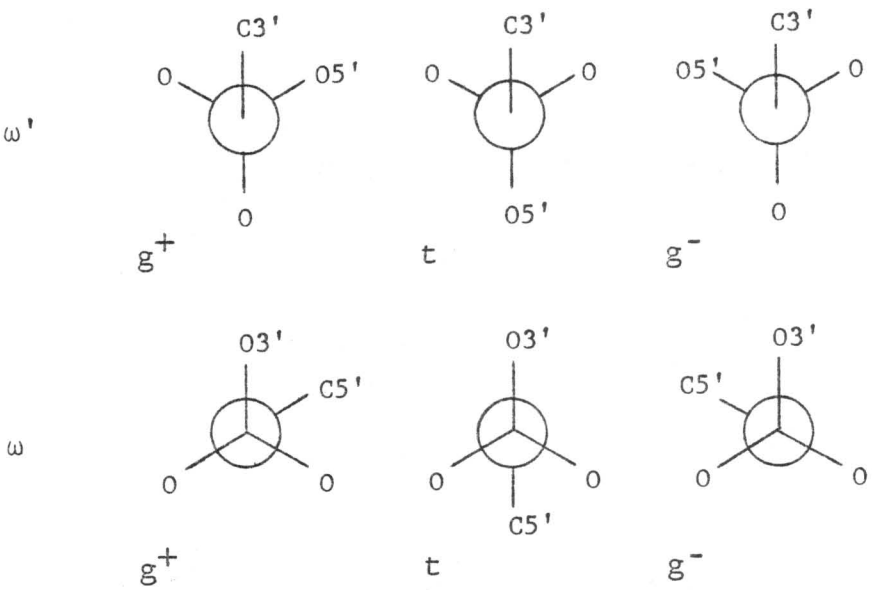

Figure 7. Classical Staggered Rotamers about ω' and ω Bonds of ApA

Table XI

$^{1}H\{^{1}H\}$ NOE Studies of (2'-5')-d_3-Ap(iA)

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Both H8 base protons	
	7°C	26°C	7°C	26°C
Ap- H1'	-5646	-5621	10	2
-p(iA) H1'	-5654	-5631	5	2
Ap- H2',H3'	-5741	-5722	13	13
-p(iA) H2',H3'	-5773	-5756	10	9
Ap- H4'	-5807	-5789	1	1
Ap- H5',H5"	-5848	-5828	2	-1

Table XII

$^{31}P\{^{1}H\}$ NOE Studies of (2'-5')-d_3-Ap(iA)

Protons irradiated	Frequencies irradiated (Hz)		NOE enhancements (%) Phosphorus	
	7°C	26°C	7°C	26°C
All	Broad band		53	88
Ap- H1'		-5623		7
-p(iA) H1'		-5632		7
Both H1'	-5650		2	
Ap- H2',H3'		-5721		30
HDO		-5741		26
Ap- H2',H3' & HDO	-5741		30	
-p(iA) H2',H3'	-5773	-5756	17	5
Ap- H4'	-5807	-5789	10	5
Ap- H5',H5"	-5848	-5828	3	2