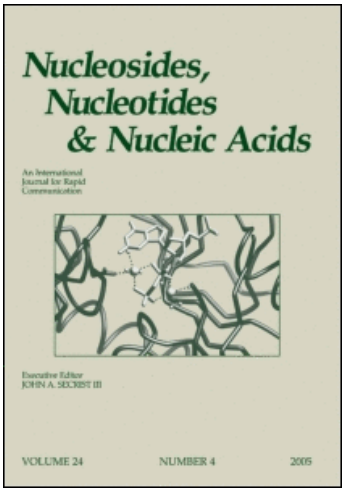

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SYNTHESIS AND FAB MASS SPECTROSCOPICAL STUDIES ON 2',5'-DINUCLEOSIDE-MONOPHOSPHATES†

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ABSTRACT : Synthesis and fast atom bombardment studies on a few 2',5'- and 3',5'-linked dinucleoside-monophosphates are presented. The results indicate that both the isomers exhibit similar fragmentation pattern.

INTRODUCTION

In natural nucleic acids, there is an overwhelming preponderance of 3',5'-linked sugar-phosphate residues, although 2',5'-linked nucleic acids are found in interferon-treated cells[1] and during intron splicing.[2] The question why nucleic acids prefer 3',5'- over 2',5'-linkages has been the subject of considerable theoretical[3-5] and experimental studies,[6-13] in recent years. The available data suggest that the preference for 3',5'-linkages is due to their ability to form more stable duplex structure which is essential for information storage and replication. To better understand the reasons for observed preferential properties, we have initiated a systematic comparative study of 3',5'- *versus* 2',5'- linked oligonucleotides of identical base sequences. Earlier we have observed that 3'-deoxyribonucleosides, the monomeric units for making 2',5'-linked oligonucleotides, have distinctly different conformation as compared to 2'-deoxyribo-nucleosides. In the case of former, sugar puckering and the

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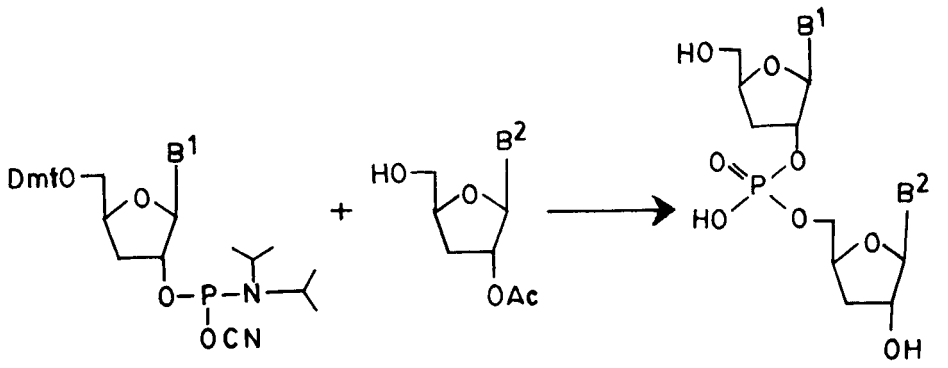
glycosyl torsion angle are predominantly in *S* form and *anti* configuration as compared to the *N* form and *syn/anti* equilibrium respectively in the case of latter.[14] These differences could have a direct bearing on the physicochemical properties of the 2',5'-linked oligonucleotides. Therefore we thought it worthwhile to investigate whether 2',5'-linked oligonucleotides behave differently in their fragmentation pattern under FAB conditions. Secondly whether this technique can be used for the sequencing of 2',5'-linked oligonucleotides. In this paper we report the synthesis and fragmentation pattern of a few 2',5'-linked dimers as prototypes and the results are compared with the corresponding 3',5'-linked dimers.

RESULTS AND DISCUSSION

The 3'-deoxyribonucleosides were obtained by the method reported earlier from this laboratory.[15] After suitable derivatization by the transient protection method,[16] they have been used for the synthesis of dimers. The desired phosphoramidites (**1a-1c**) were prepared from the suitably protected nucleosides by reacting with the 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite and 1-*H* tetrazole in acetonitrile. Phosphitylation was complete in 2 h as monitored by tlc. After workup and purification the phosphoramidites were obtained in 80% yield. In the second step these phosphor-amidites (**1a-1c**) were coupled with the appropriate hydroxyl components (**2a/2b**), using 1-*H* tetrazole as an activator followed by *in situ* oxidation using aq. iodine solution (**SCHEME 1**). The fully protected dimers thus obtained were deblocked and purified by reversed-phase HPLC to give 2',5'-linked CG (**3**), GC (**4**) and TC (**5**). The corresponding 3',5'-dimers were synthesized by the phosphotriester method using BOPDC as a coupling reagent.[17] Subsequently the protecting groups were deblocked and the dimers were purified by the reverse phase HPLC to give 3',5'-linked CG (**6**), GC (**7**) and TC (**8**).



1 a)B^1= C^{bz} 2 a)B^2= G^{ibu} 3)B^1= C, B^2= G
b)B^1= G^{ibu} b)B^2= C^{bz} 4)B^1= G, B^2= C
c)B^1= T 5)B^1= T, B^2= C

Abbreviations : C^{bz}= *N*⁴-benzoylcytosine, G^{ibu}= *N*²-isobutyryl-guanine, Dmt = 4,4'-dimethoxytrityl, Ac = acetyl

SCHEME 1

The dimers (3-8) were characterized by the enzymatic hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase. The hydrolyzed products of the dimers 3-5 comigrated with the corresponding 3'-deoxyribonucleosides, whereas the hydrolysates of dimers 6-8 comigrated with the corresponding 2'-deoxyribonucleosides [Retention time (in min) = 9.27 (3'-drC), 13.61 (3'-drG), 11.82 (3'-drT), 7.42 (2'-drC), 11.68 (2'-drG) and 10.7 (2'-drT); gradient system = 0-15% *B* in *A* (20 min); flow = 1 mL/min; where *A* = 0.1 M TEAA (pH =7.3), *B* = acetonitrile].

FAB MASS STUDIES

The negative ion fast atom bombardment (FAB) mass spectra of 3-8 were recorded using the matrix *m*-nitrobenzylalcohol (NBA) as this was found to give better spectra for a longer duration. Although the abundant $[M-H]^-$ ions were observed in the spectra of all dimers, not all peaks corresponding to the

various bond cleavages were discernible from the FAB mass spectrum, because of the interference from matrix peaks.[18] Therefore, the collision-induced decomposition (CID) spectra of the $[M-H]^-$ ions of 3-8 were recorded to enhance the specificity of the fragmentation using linked scanning at a constant ratio of magnetic (B) and electric (E) fields (constant B/E).[19] As a representative example, the FAB mass spectra and the CID spectra of 4 is shown in FIGURE 1.

The ion abundances in the CID spectra of the $[M-H]^-$ ions of 3-8 are given in TABLE 1. The general fragmentation of the 2',5'-linked dimers is shown in SCHEME 2. As observed in the case of 3',5'-linked oligonucleotides,[20-23] the major fragmentation pathways are, the cleavage of C2'-O (**a**), the cleavage of P-5'O bond (**b**) and the cleavage of glycosyl bond (**c** and **d**). All these cleavages are followed by the hydrogen transfer from the sugar moiety resulting in the formation of $[M-drB^1]^-$, $[drB^1p]^-$, $[M-B^1]^-$ and $[M-B^2]^-$ respectively. The other fragment ions observed, correspond to sugar-phosphate anions (m/z 195) and heterocyclic base anions (m/z 148, 109 and 124 corresponding to guanine, cytosine and thymine anions respectively).

Analogous to the behavior of 3',5'-linked dimers, 2',5'-linked dimers show the preference for cleavage **a** because the the O-C2' bond is more labile (connected to a secondary carbon atom), than the O-C5' (connected to a primary carbon atom of the sugar moiety).[21] Also the loss of the base from 5' terminus (cleavage **c**) was found to be more facile than that from the 3'-terminus (cleavage **d**),[24] in both 2',5'-linked as well as 3',5'-linked dimers. In the present case this is true for dimers 4, 5, 7 and 8 only. In the case of CG and GC dimers, the loss of G is preferred to that of C.

The above data clearly indicate that 2',5'-linked dimers exhibit fragmentation pattern identical to the corresponding 3',5'-isomers under negative ion FAB mass spectral conditions. This technique can also be used for the sequencing of 2',5'-linked oligonucleotides.

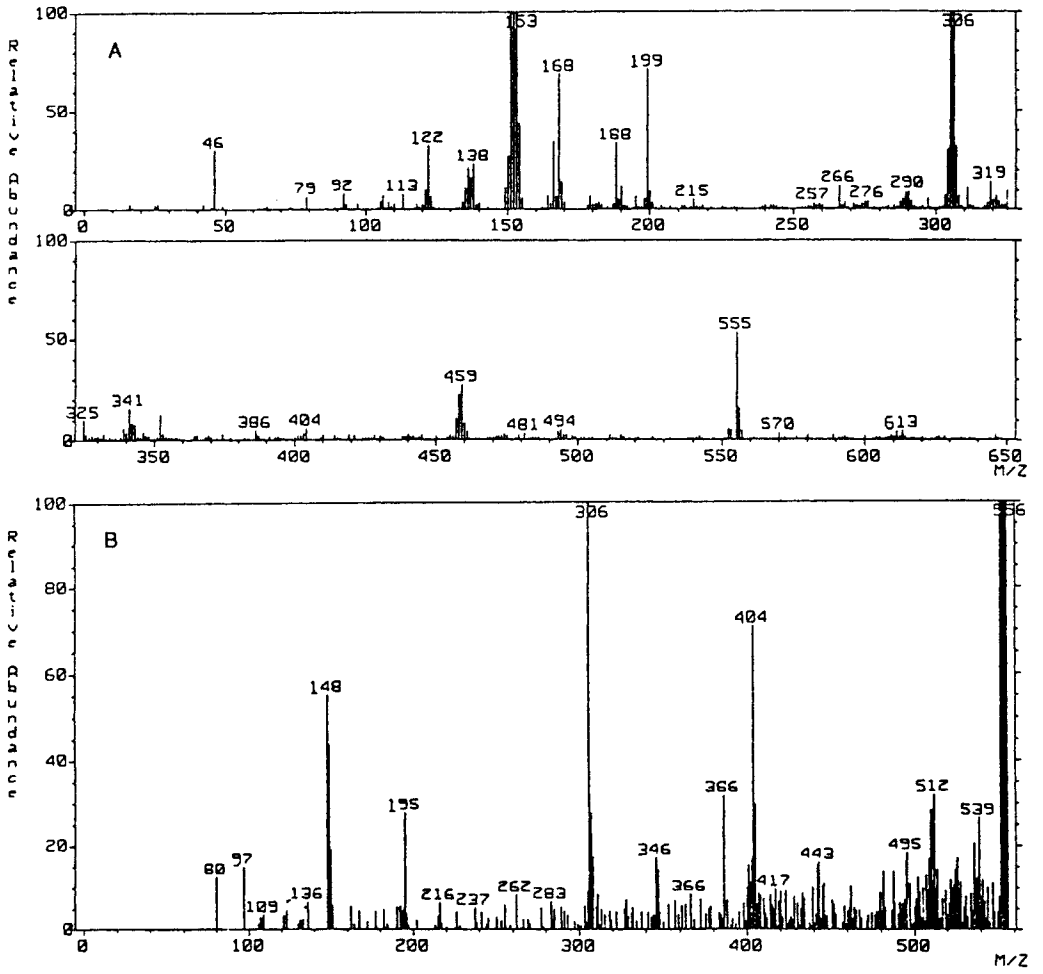


FIGURE 1 : A) FAB mass spectra and B) CID spectra of **4**

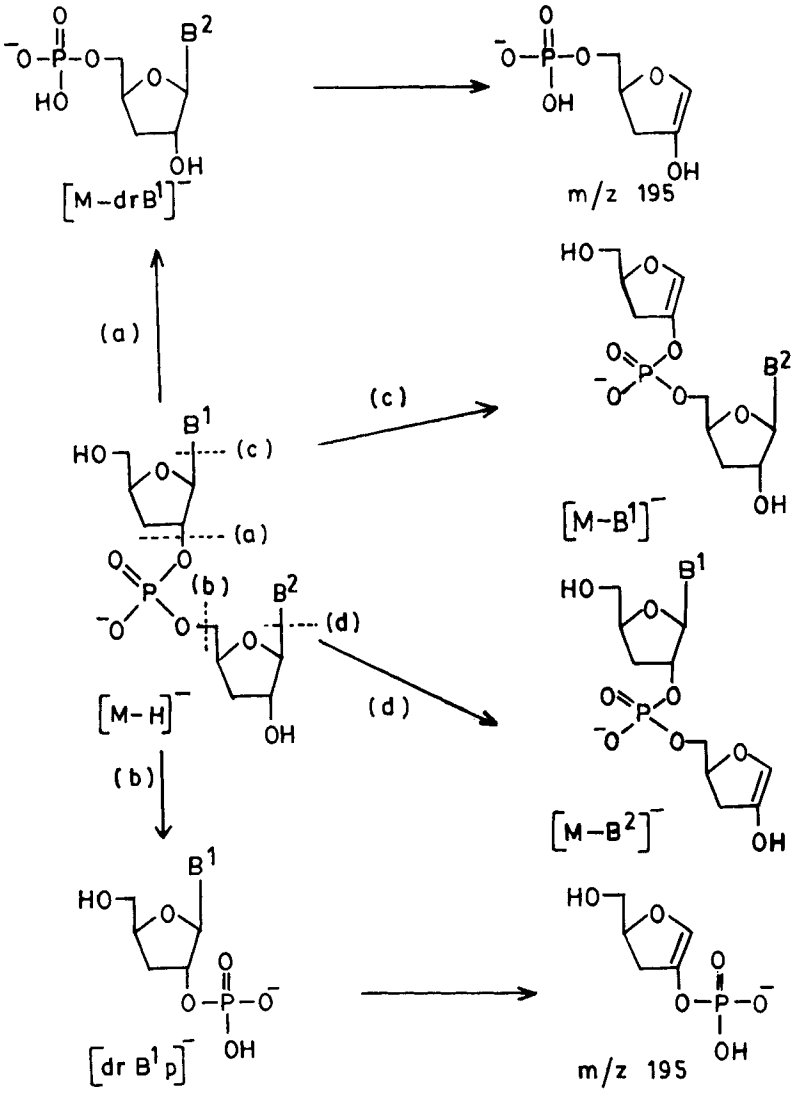
Table 1 : Ion abundances (%) in the CID spectra of the $[M-H]^-$ ions of dimers 3-8.

Fragments (parent-ion m/z)	3 555	4 555	5 530	6 555	7 555	8 530
$[M-drB^1]^-$	49	100	67	54	74	57
$[drB^1p]^-$	53	17	16	40	62	39
$[M-B^1]^-$	77	70	100	94	100	100
$[M-B^2]^-$	100	16	5	100	35	20
m/z 195	71	27	30	31	33	18

EXPERIMENTAL

All reactions were performed in freshly distilled and dried solvents and reagents. 2-Cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite was obtained from Sigma chemical co., St.Louis, USA. 1*H*-tetrazole and 1-methylimidazole were purchased from Aldrich, Milwaukee, USA and 4-dimethylaminopyridine from Fluka, Buchs, Switzerland. The tlc was performed on readymade plates (MERCK DC-Alufolien Kieselgel 60 F_{254}, 0.2 mm, E.Merck (India) ltd., Mumbai, India) using different solvent systems. Purification of fully deblocked dimers was carried out on Waters HPLC system using a reversed-phase column (MERCK 50983-Lichrosopher 100 RP-18, 5 µm, 250 x 4 mm, E.Merck (India) ltd., Mumbai, India). All UV measurements were carried out on Lambda 15 UV/VIS spectrophotometer (Perkin Elmer ltd. Bucks, England). NMR spectra were recorded on DRX-300 MHz spectrometer (Bruker, Zurich, Switzerland).

The FAB mass spectra were recorded on a Jeol SX-102/DA-6000 double focusing spectrometer with reverse geometry using a 6 kV Xenon beam (10 mA). *m*-Nitrobenzylalcohol (NBA) was used as a matrix. The sample was dissolved in 1 % aq. NH_3 and 1 µL of the solution containing 2.0 A_{260} unit of the sample



SCHEME 2

was placed on the FAB probe tip containing 1 μL of the matrix. The CID spectra were recorded on the same instrument using the constant B/E linked scan technique. The pressure of the Helium in the first field free region collision cell was adjusted so that the parent ion signal was reduced to 50%. Each B/E scan spectrum reported in this paper is an average of 6-7 scans.

***N*-Acyl-5'-*O*-dimethoxytrityl-3'-deoxyribonucloside-2'-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidites** (1a, 1b, and 1c).

Predried *N*-acyl-5'-*O*-dimethoxytrityl-3'-deoxyribonucleoside (0.5 mmol) was dissolved in dry MeCN (5 mL). To this was added 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphorodiamidite (0.32 mL, 1 mmol) followed by 0.5 M solution of 1-*H* tetrazole in dry MeCN (1 mL), dropwise with stirring. The mixture was stirred at rt for additional 2 h. It was diluted with 2 % TEA solution in $CHCl_3$ (50 mL) and washed successively with aq. $NaHCO_3$, H_2O and brine. The organic layer was passed through anhydrous Na_2SO_4 and concentrated to a foam. It was dissolved in dry $CHCl_3$ and precipitated in hexane. The precipitate was filtered and dried in a vacuum desiccator. Physical data of the compounds 1a-c are given below.

Phosphoramidite	Yield(%)	Rf*	^{31}P NMR** ($CDCl_3$) δ
1a	79	0.65	150 , 151.3
1b	80	0.67	148.8, 150.5
1c	82	0.59	149.8, 151

* Rf was checked in TEA/ EtOAc/ CH_2Cl_2 (1:2:7).
** Phosphoric acid was used as an external standard.

***N*-Acyl-2'-*O*-acetyl-3'-deoxyribonuclosides (2a and 2b).**

To a stirred solution of predried *N*-acyl-5'-*O*-dimethoxytrityl-3'-deoxyribonucleoside (10 mmol) in dry pyridine (50 mL) was added distilled Ac_2O (1.9 mL, 20 mmol), dropwise at 5° C and the mixture was stirred at rt for 4-6 h. After completion of the reaction, chilled H_2O (1 mL) was added. The reaction mixture was concentrated and the residue was dissolved in $CHCl_3$ (100 mL). It was washed successively with

aq. $NaHCO_3$, H_2O and brine, passed over anhydrous Na_2SO_4 and concentrated to a foam. The residue thus obtained was dissolved in an ice cooled solution of 2% BSA in 3:7 MeOH:$CHCl_3$ (87 mL) and the mixture was stirred at 5° C for 30 min. After complete dedimethoxytritylation, the excess of BSA was quenched with aq. $NaHCO_3$ and the reaction mixture was concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ and washed successively with aq. $NaHCO_3$, H_2O and brine. The organic layer was dried over anhydrous $NaHCO_3$ and concentrated to a foam. The crude product was purified by silica gel column chromatography, using a linear gradient of 0-5% MeOH in $CHCl_3$. Appropriate fractions were pooled and concentrated to a foam. It was dissolved in $CHCl_3$, precipitated in hexane and dried to give the desired products **2a** and **2b** as white solid. Physical data are given below.

Protected nucleosides	Yield (%)	Rf*	FAB $[M-H]^-$ m/z
2a	78	0.32	378
2b	81	0.35	372

* Rf was checked in 5 % MeOH in $CHCl_3$

Synthesis of 2',5'-Linked Dimers (3, 4 and 5).

To a stirred solution of predried protected phosphoramidite (**1a/1b/1c**, 0.25 mmol) and the appropriate hydroxy component (**2a/2b**, 0.2 mmol) in dry MeOH (5 mL) was added a solution of 0.5 M 1-*H* tetrazole in dry MeCN (1.5 mL). The mixture was stirred at rt for 2 h. After completion of reaction, TEA (1.4 mL, 10 mmol) followed by 0.1 M I_2 solution in 2:1 MeCN:H_2O (5 mL) was added and the mixture was stirred for additional 2 h. It was diluted with $CHCl_3$ (50 mL) and washed successively with aq. $Na_2S_2O_3$, aq. $NaHCO_3$, H_2O and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to a foam. It was dissolved in a mixture of pyridine/20 % aq. NH_3 (1:1, 1 mL) and kept at 5°C. After 48 h, the mixture was concentrated under reduced pressure, dissolved in 25 % aq. NH_3 (1 mL) and kept for another 24-48 h at rt.

The reaction mixture was concentrated and the residue was stirred with 80 % aq. AcOH for 30 min to remove the 5'-dimethoxytrityl group. The AcOH was completely removed by coevaporation with MeOH on a Speedvac (Savant instruments inc. Farmingdale, USA). The residue was dissolved in H_2O (0.5 mL) and washed with $CHCl_3$ (4 x 0.5 mL). The aq. layer was concentrated and the crude product was purified by HPLC.

Synthesis of 3',5'-Linked Dimers (6, 7 and 8).

A mixture of *N*-acyl-5'-*O*-dimethoxytrityl-2'-deoxyribonucleoside-3'-*O*-(2-chlorophenyl)-triethylammonium-phosphate[19] (1.2 mmol) with appropriate *N*-acyl-3'-*O*-acetyl-2'-deoxyribonucleoside (1 mmol) and *N*-methylimidazole (0.2 mL, 2.4 mmol) was coevaporated with dry pyridine (2 x 5 mL). The dried mixture was dissolved in dry pyridine (5 mL) and BOP-Cl (0.6 g, 2.4 mmol) was added to it. The reaction mixture was stirred at rt for 30 min. After completion of reaction, the solvent was removed under reduced pressure and the residue was dissolved in $CHCl_3$ (20 mL). It was washed successively with aq. $NaHCO_3$, H_2O and brine, dried over anhydrous Na_2SO_4 and concentrated to a foam. The crude product was purified by silica gel column chromatography, using a linear gradient of 0-5% MeOH in $CHCl_3$. Appropriate fractions were pooled and concentrated to a foam. It was dissolved in $CHCl_3$, precipitated in hexane and dried to give the protected dimers in 70-80% yield. These were deblocked as described above and purified by HPLC.

HPLC Purification

Completely deblocked dimers were purified to homogeneity by reversed-phase HPLC using different gradients (details given below). Appropriate fractions of the purified dimers were pooled and concentrated.

Dimers	:	3	4	5	6	7	8
Rt (in min)	:	16.4	17.3	16.9	16.2	16.3	22.4
Gradient used	:	I	II	I	I	I	III

Gradient systems:
I) 0-15 % *B* in *A* (20 min), 15-50 % *B* in *A* (10 min)
II) 0-15 % *B* in *A* (25 min), 15-50 % *B* in *A* (10 min)
III) 0-15 % *B* in *A* (30 min), 15-50 % *B* in *A* (10 min)
[*A* = 0.1 M aq. TEAA, pH = 7.3; *B* = Acetonitrile; Flow = 1 mL/min]

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