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SPECTROSCOPIC AND ENZYMATIC CHARACTERIZATION OF 2'-5'AND 3'-5' RNA HEXAMERS AACCUU SYNTHESISED BY PHOSPHOTRIESTER APPROACH IN SOLUTION USING 2'-t-BUTYLDIMETHYLSILYL PROTECTION+

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ABSTRACT: Comparative ^{1}H, ^{31}P NMR and CD spectroscopic results and enzymatic cleavage of single stranded RNA hexamers which have identical base sequence (AACCUU) but are regioisomeric at the internucleotide phosphate linkages (2'-5', 3'-5' and their covalent hybrid) are presented. The ^{31}P NMR results revealed significant differences in local phosphate backbone conformation among these isomers, with 2'-5' isomer exhibiting maximum heterogeneity as compared to the 3'-5' isomer, the analogous DNA hexamer and the covalent hybrid hexamer. In contrast to this, there are no appreciable differences in the overall base-base stacking as seen in the CD spectra of 2'-5' and 3'-5' isomers. All RNA hexamers were synthesised by solution phase phosphotriester chemistry with t-butyldimethylsilyl (TBDMS) as 2'-O-protecting group. The fully protected oligorobomers were deprotected in two steps: (i) saturated anhydrous MeOH-NH_3 for phosphate and amino deprotections and (ii) TBAF for removal of 2'-O-TBDMS group. Use of sat. MeOH-NH_3 (instead of aq. NH_3) prevents 2'-3' internucleotide phosphate migrations, chain fragmentations and 5'-terminal modifications by neighbouring group participation (NGP). The retention of isomeric integrity and absence of 5'-terminal modification in the final products was established by digestion with several nucleases.

INTRODUCTION

The chemical synthesis and structural studies on RNA has assumed importance due to its role in protein synthesis[1] and the recent discovery of its catalytic activity[2]. Apart from the naturally occurring 3'-5' linked RNA, there has been considerable interest in its regioisomer 2'-5' RNA[3] and unusual branched structures derived from these linkages[4]. The major challenge in the chemistry of RNA synthesis[5] has been (i) regiospecific monoprotection of ribose cis diol system by a compatible group, (ii) efficient and rapid coupling strategies and (iii) final deprotection tactics to avoid or minimise the accompanying cleavage and migration of internucleotidic phosphate bonds. Despite these stringent pre-requisites, considerable progress has been made in the chemical synthesis of oligoribonucleotides in the past. Sequences of increasing lengths such as the 5'-half of initiator t-RNA[6] from

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B.Subtilis and that corresponding to *E. Coli* methionine t-RNA[7] have been synthesised using solid phase phosphoramidite chemistry with t-butyldimethylsilyl groupfor 2'-hydroxyl protection. Recently, the synthesis of the 3'-terminal half of yeast alanine t-RNA by solution phase phosphotriester chemistry using the acid-labile MTHP group for 2'-hydroxyl protection has been reported[8]. These advances in achieving the synthesis of long RNA fragments remain rare, since easy and routine procedures for making oligoribonucleotides of even moderate sizes, in milligram amounts needed for biophysical studies[9] (NMR and X-ray crystallography) are still lacking.

Continuing our interest[10] in the synthesis and structural studies of oligonucleotides, here we demonstrate the distinct differences in the spectroscopic properties and ribonuclease susceptibility patterns of ribohexamers AACCUU possessing homo 2'-5' and 3'-5' linkages. We noticed significant variations in the backbone conformations of these hexamers which have identical base sequences and differ only in the nature of internucleotidic phosphate linkages. We have also observed that 2'-5'linkages are resistant to pancreatic ribonuclease while 3'-5' linkages are not and as we demonstrate here, this specificity can be employed to establish the regioisomeric integrity of the synthesised ribomers. The oligoribomers (2'-5', 3'-5' and a covalent hybrid) were synthesised by solution phase phosphotriester chemistry using TBDMS as 2'-hydroxyl protecting group. A simple two step deprotection procedure lead to ribohexamers in high isomeric purity as established by enzymatic and spectroscopic studies.

RESULTS AND DISCUSSION

Chemical synthesis of protected RNA fragments

Synthesis of protectected phosphodiester monomers 1: Among the various groups that have been used for 2'-protection[11-13], t-butyldimethylsilyl has the potential to emerge as a general protecting group for 2'-hydroxyl function as it has found applicability in both phosphoramidite[6,7] and H-phosphonate methods[12]. Although this group has been initiated into the phosphotriester method[14], there have been no subsequent pursuits. The N,O-protected ribophosphodiesters **1** were synthesised from the corresponding nucleosides by a sequential protection strategy as reported elsewhere[15]. The phosphorylations of protected nucleosides **2**, were carried out using 2-chlorophenyl dichlorophosphate reagent in pyridine containing stoichiometric amounts of water. This reaction, successfully employed earlier in deoxyribonucleotide synthesis[16,17], proved equally efficient in ribonucleotide synthesis. Both 2'-silyl and 3'-silyl derivatives of all ribunucleosides were individually converted by this method to the respective ribophosphodiesters **1**. A crude product analysis indicated no side products either due to base modifications or due to symmetric 3'-3' dimers were observed. Further, 2'-3' migrations of silyl groups during phosphorylation were also absent. The ribophosphodiesters **1** were isolated as stable triethylammonium salts after purification by silica gel chromatography and their purity and isomeric integrity was established by ^{1}H and ^{31}P NMR[15] .

Assembly of 3'-5' and 2'-5' oligoribonucleotides: The general strategy used for synthesis of 2'-5' and 3'-5' oligoribonucleotides is illustrated in

Scheme 1 for the particular synthesis of r(AACCUU). Each dimer block was obtained by a cycle consisting of condensation-chromatographic purification and detritylation steps. The three dimer blocks r(DMT-$A^{Bz}pA^{Bz}p'$) **4**, r(DMT-$C^{Bz}pC^{Bz}p'$) **5**, and r(DMT-UpU-Bz_2) **6**, required for the final assembly were synthesised from the appropriate 5'-hydroxy (**3**) and 3'-phosphate (**1**) components. The 3'-O-phosphodiesters, **8** and **9** were generated from protected phosphotriesters **5** and **4** respectively by treatment with DBU to result in loss of p-nitrophenylethyl group.

The condensation reactions, done in pyridine containing mesitylenesulphonyl-3-nitrotriazole (MSNT) and N-methylimidazole (NMI), were complete within 15min. The products were separated from excess monomer on a short silica gel column using DCM as eluent. The resulting dimers were deprotected at 5' or 3' ends as shown in Scheme 1, by acid or DBU treatment respectively. The 5'-O-detritylation of the condensation product **6** was achieved by treatment with 2% dichloroacetic acid (DCA) in dichloromethane (DCM). The reaction product, the 5'-hydroxy dinucleotide, **7** was separated from the liberated tritanol by a simple precipitation from DCM/Hexane. The overall yield for each condensation-chromatography-detritylation cycle was 80%.

B = U, C^{Bz}, A^{Bz}, or G^{Bz}

1a, R_1 = DMT, R_3 = TBDMS, R_2 = (2-chlorophenyl)-O-P(=O)-O$^-Et_3NH^+$

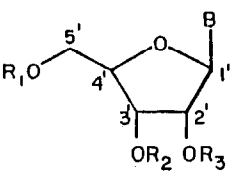
1b, R_1 = DMT, R_2 = TBDMS, R_3 = (2-chlorophenyl)-O-P(=O)-O$^-Et_3NH^+$

2a, R_1 = DMT, R_2 = H, R_3 = TBDMS

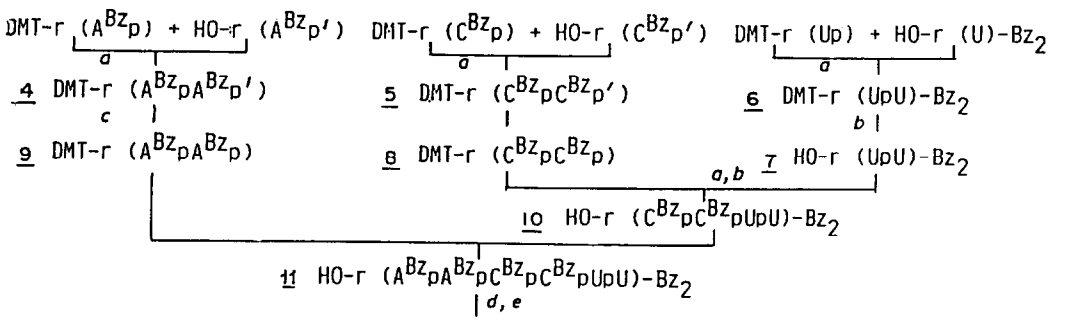
2b, R_1 = DMT, R_2 = TBDMS, R_3 = H

3a, R_1 = H, R_2 = R_3 = Bz

3b, R_1=H, R_2/R_3 = TBDMS, R_3/R_2=(2-chlorophenyl)-O-P(=O)-OCH$_2$CH$_2$(4-nitrophenyl),



SCHEME 1



Reagents: a, Pyridine, MSNT, NMI (15min), Work-up (15min) and Silica gel chromatography (30min). b, 2% DCA in DCM (10min), Work-up (15min) and precipitation (30min) c, DBU. d, Methanolic NH_3. e, 1M TBAF/THF.

The dimer blocks 7 and 8 were then coupled, purified and detritylated (as in Scheme 1) to obtain the ribotetranucleotide 10. This tetramer and the dimer 9 on a similar reaction cycle gave the protected hexaribonucleotide 11. The overall yields after 2+2 coupling cycle to give tetramers followed by 2+4 coupling cycle to generate the hexamer were about 55%. The stoichiometry of reagents and reactants used at the various coupling steps are indicated in Table-I (see experimental). The average time involved per cycle (condensation-chromatography-detritylation-or DBU treatment and precipitation) is only about 2hr. (Scheme 1) and the protected hexamers were easily obtained by a block coupling approach in about 10-12hr.

All dimer couplings and subsequent condensations to obtain protected tetra and hexaribomers proceeded neatly and quantitatively in pyridine and in presence of MSNT and NMI. The reactions were essentially complete in 15-20min., as fast as the corresponding DNA reactions[16,17], indicating the absence of any steric hindrance imposed by the TBDMS group. The condensation product is highly non-polar due to the presence of bulky hydrophobic groups such as DMT and TBDMS enabling its easy separation from excess 3'-phosphate components on silica gel column by a simple elution with DCM. Subsequently, the detritylated intermediate oligonucleotide products could be isolated as pure stable amorphous solids by precipitation from DCM/Hexane. Using appropriate reactants and an identical strategy, protected 2'-5' linked r'(AAC-CUU) was obtained. The fully blocked covalent hybrid r(AA)r'(CCUU) was obtained by coupling of the dinucleotide 9 to the tetramer (protected) r'-OH($C^{Bz}p'C^{Bz}p'Up'U$)-Bz_2.

Deprotection and purification of oligoribonucleotides

The main problems associated with the unblocking of fully protected ribophosphotriesters are (i) deprotection of 2'-hydroxy function prior to that of phosphate leading to degraded RNA products and (ii) 5'-terminal modification and formation of unwanted 5'-5' and 3'-3' internucleotide linkages arising from NGP[18]. Hence the choice and sequence of deblocking conditions used for obtaining fully deprotected RNA is quite critical.

Normally, in phosphotriester approach, oximate reagent[18a] is used for removal of o-chlorophenyl groups from phosphate residues. This works well when the 2'-hydroxy protecting group is acid labile (THP)[11] or photolabile (o-nitrobenzyl)[13]. However, in our present studies, where we have used TBDMS group for 2'/3'-protection, we observed that oximate ion promotes significant TBDMS cleavage prior to phosphate deprotection. In dioxane:water, the oximate induced cleavage of TBDMS was 100% in 5hr., and in anhydrous pyridine it was 25% during the same period. The complete removal of o-chlorophenyl by oximate occurs at 55°C in 17hr. Thus desilylation occurs much earlier than phosphate deprotection leading to RNA degradation during this and the subsequent ammonolysis step. Hence we investigated alternative procedures for removal of o-chlorophenyl groups without bringing about any desilylations.

It has earlier been established that use of either hydroxide ions (0.1N NaOH)[18b] or aq. NH_3 (15N in dioxan:water,4:1)[18c] for deprotection of phosphates generated terminal 3'-5' cyclic phosphotriester intermediates via NGP of terminal 5'/3'-hydroxyls. The hydrolysis of these intermediates then lead to either unwanted 5'-5'/3'-3'internucleotide bonds or 5'-terminal

modification to obtain 5'-amino derivatives. Saturated anhydrous MeOH-NH_3 has recently been shown to be superior to aq. NH_3 in terms of 2'-O-TBDMS group stability in RNA synthesis by phosphoramidite approach. In view of this, we investigated the applicability of sat. MeOH-NH_3 for unblocking of RNA chlorophenyl phosphotriesters.

The model dinucleotide Z was treated with sat. MeOH-NH_3 in a sealed tube and heated at 50°C for (i) 1hr. and (ii) 12hr. The reactions were

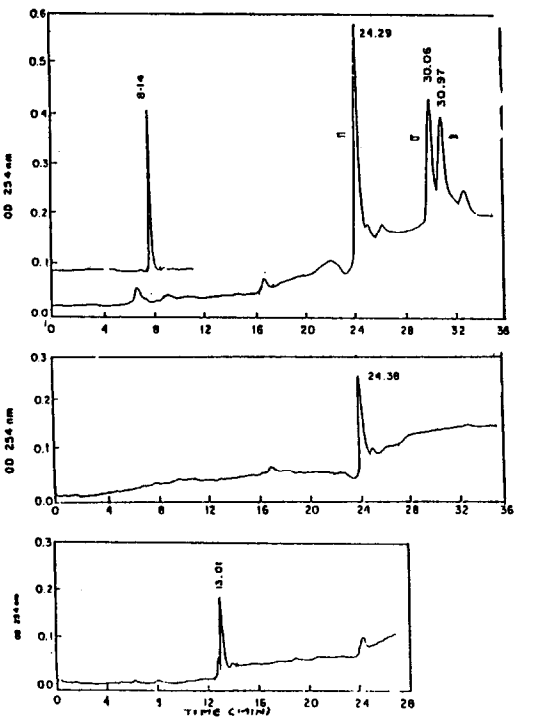


Figure 1. HPLC analysis of deprotection of Z with sat. methanolic NH_3, (a) after 1hr. at 50°C, (b) after 12hr. at 50°C and (c) after TBAF treatment of (b) for 20min. Inset in (a) is uridine. Solvents: A, 50mM aq.NH_4OAc; B, A+30% CH_3CN; Flow rate: 1ml/min. Gradient: A to B in 20min and100% B for 15min.

analysed by HPLC (Figure 1) and the products were compared with appropriate standards. After 1hr. (Figure 1a) three products were obtained and these were identified as those due to partial hydrolysis of 3'-terminal benzoyl groups (peaks a and b, 2'/3'-monobenzoyl derivatives) and that due to removal of both benzoyl and chlorophenyl groups (peak c), i.e., r(OH-U^{OSi}pU)-$(OH)_2$. These chromatograms indicate that within 1hr., the terminal O-benzoyl groups were fully deblocked, accompanied by partial removal of o-chlorophenyl from phosphate (a slower process). After 12hr. (Figure 1b), peaks a and b totally disappear and only peak c is seen, indicating the complete hydrolysis of both benzoyl and o-chlorophenyl groups. The product of Figure 1b was treated with 1M TBAF in THF for 20min. and the HPLC of the resultant is depicted in Figure 1c. Only one major peak was seen without any side products. This product was identified as 3'-5' r(HO-UpU) by its complete susceptibility to the enzyme calf spleen phosphodiesterase, confirming the presence of exclusive 3'-5' phosphodiester bond and a free 5'-hydroxyl[18c]. Its identity was further established by comparison with a reference sample obtained from Sigma.

If any internucleotide cleavage due to desilylation had occurred during sat. MeOH-NH_3 treatment, one of the expected products would be uridine.

It is seen from the figure (b) that no uridine was observed after MeOH-NH_3 treatment for 12hr. at 50°C, ruling out any desilylation prior to phosphate deprotection that could lead to chain cleavage or migration. These results conclusively establish that (i) sat. MeOH-NH_3 removes chlorophenyl groups from phosphates, (ii) leaves TBDMS unaffected and (iii) unlike aq. NH_3, sat. MeOH-NH_3 does not bring about any 5'-modifications even in presence of free 5'-OH.

It has been observed[19] that sat. MeOH-NH_3 at room temperature for 12hr. brought about complete N-debenzoylations, even for G, without any noticeable desilylations. Our results indicate that even at higher temperatures needed to effect complete phosphate deprotection, sat. MeOH-NH_3 is quite safe for use with 2'-TBDMS groups. We emphasise that this stability factor is true for sat. methanolic ammonia only and does not hold good for aq. NH_3 or its solution in dioxane, ethanol or pyridine that have been used for phosphate and N-deprotections[14,19].

The fully protected oligoribonucleotides were therefore completely deblocked in only two steps. The first step involved global deprotection of phosphate and amino groups using sat. MeOH-NH_3 and the resulting residue was treated with TBAF(1M) in dry THF to effect desilylation in the second step. FPLC (Figure 2a-c) of the resultant crude indicated the required oligonucleotide to be more than 90% pure. Our present results indicate that sat. methanolic ammonia is ideal for simultaneous removal of all benzoyl groups (N and O) and chlorophenyls (phosphates) without harming 2'/3'-TBDMS. The resulting product was then purified by ion-exchange chromatography over DEAE Sephadex A-25, followed by gel filtration on Sephadex G-15 to yield pure hexaribonucleotides (Figure 2a-c). The FPLC patterns show 3'-5' RNA to be more hydrophobic than the equivalent 2'-5' RNA, substantiating an earlier observation[21] arising from their differential elution behaviour on Sephadex LH20.

Structural characterization of oligoribonucleotides

Enzymatic hydrolysis: Although the monomers used for synthesis were shown[15] to be isomerically homogeneous by ^{31}P NMR, possibilities of internucleotide migrations during the various condensation and deprotection steps cannot be overlooked. Despite the fact that the three sequences are chromatographically pure with different retention times, the regio-isomeric integrity and homogeneity at all phosphodiester bonds needs to be established unambiguously. This was done by studying their susceptibility to the enzymes pancreatic ribonuclease, calf spleen phosphodiesterase and RNase U_2. These enzymes are specific only to 3'-5' phosphodiester linkages and not to 2'-5'.

The 3'-5', 2'-5' and the covalent hybrid oligoribonucleotides were subjected to hydrolysis with the above enzymes separately and the reactions were followed by FPLC (Figure 1). As expected, the 2'-5' sequence remained totally unaffected in all cases whereas the 3'-5' sequence got completely hydrolysed (Figure 1e). In case of the hybrid, hydrolysis with the enzymes was effective only on the 3'-5' linkage to produce a tetramer product consisting of 2'-5' linkages (Figure 2f). This feature of hybrid sequence when considered along with the results on 2'-5' and 3'-5' ribomers confirms the non-migration of inter-nucleotidic phosphate bonds among 2' and 3'-hydroxyls during the various condensation-deprotection steps. It also

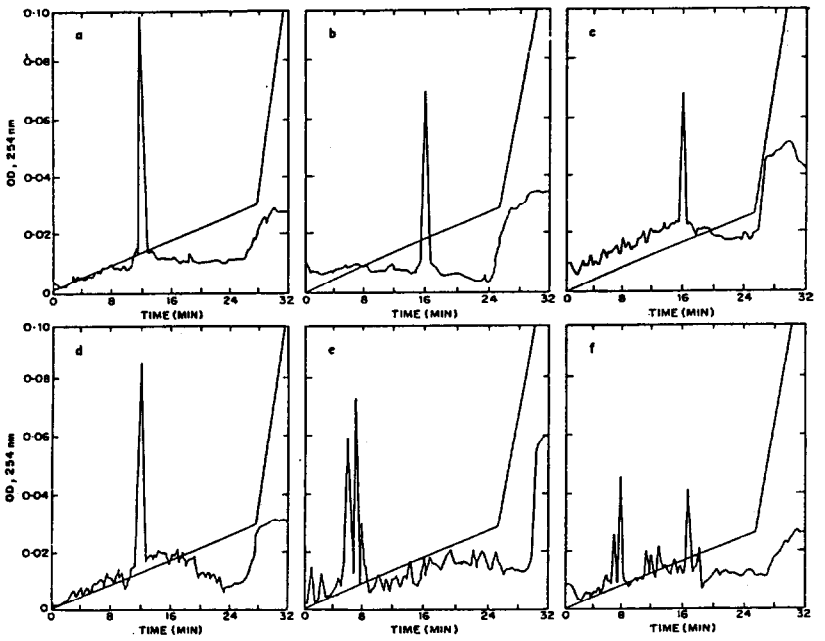


Figure 2. Reverse Phase (PepRPC) FPLC chromatogram of (a) r'(AACCUU), (b) r(AACCUU) and (c) r(AA)r'(CCUU). (d), (e) and (f) are the ribonuclease digestion of (a), (b) and (c) respectively. Solvents: A, 100mM TEAA, B, 100mM TEAA +30% CH_3CN, Gradient: 0%B to 30%B in 30 min. Flow rate: 1ml/min.

establishes unambiguosly the isomeric integrity of all synthesised sequences. The susceptibility of 3'-5' RNA and the covalent hybrid to calf spleen phosphodiesterase further confirms the absence of 5'-modifications. With 1M alkali, all sequences hydrolysed completely to yield a mixture of nucleosides and nucleotides indicating that they are all oligoribonucleotides.

^{1}H NMR: Figure 3a and 3b show the partial 300MHz ^{1}H NMR spectra of r'(AACCUU) and r(AACCUU) respectively, exhibiting resonances due to different base and sugar H1 protons, in the relevant chemical shift regions. It is seen that the base resonances of 2'-5' RNA appear dramatically downfield (beyond 8.0ppm) compared to those of 3'-5' RNA in the region 7.0-8.4ppm. The region 5.6-6.4ppm in NMR spectra of r(AACCUU) consists of H5 protons of the bases C and U seen as doublets, along with sugar H1' protons as multiplets. It is noticed from the spectra of r'(AACCUU) that only doublets due to H5 of C and U are observed and the multiplets due to sugar H1' protons are not seen in this region. In fact, they are shifted upfield compared to those of 3'-5' RNA and are masked by the residual solvent water signals around 5ppm. These features may point to an increased base stacking for 2'-5' RNA than the 3'-5' isomer. In both RNA's, the resonance lines are sharp, ruling out any significant aggregation effects. However, a more definitive evidence for this and other conformational differences awaits total assignments of resonances by two-dimensional NMR, currently under progress.

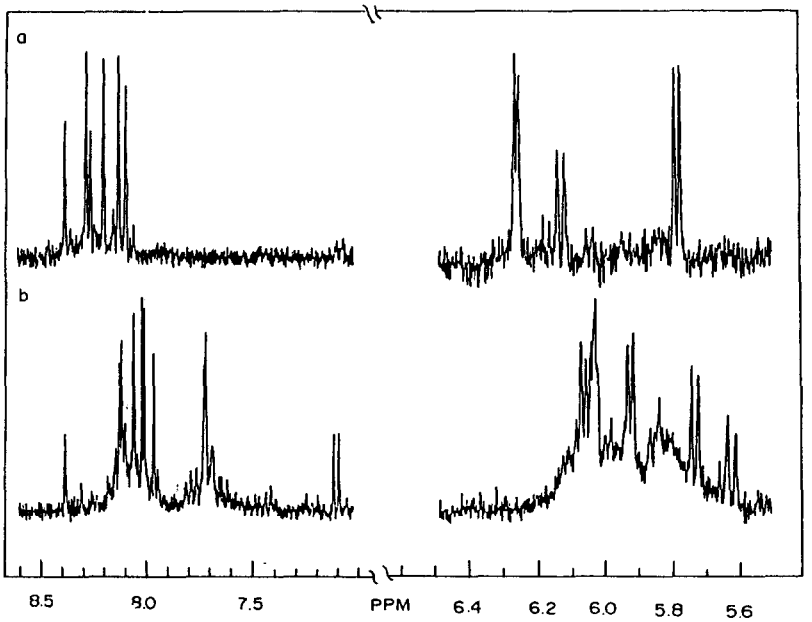


Figure 3. Partial ^{1}H NMR (300MHz) spectrum of (a) r'(AACCUU) and (b) r(AACCUU) in 10mM phosphate buffer.

^{31}P NMR: Figure 4 shows the ^{31}P NMR spectra of different single strands of oligoribohexamers along with the corresponding DNA hexamer. Distinct differences are noticed among the spectral profiles of the various oligonucleotides. In case of 2'-5' RNA (Figure 4a), three peaks are seen separately at -0.369, -0.552 and -0.788ppm; only two peaks are seen for 3'-5' RNA (Figure 4b) at comparatively lower fields at -0.296 and -0.455ppm. In contrast to these, both DNA hexamer (Figure 4c) and the RNA hybrid (Figure 4d) show broad resonance centered at -0.578 and -0.610ppm respectively and each spread over 0.6ppm range. The average ^{31}P shift of DNA hexamer is upfield to that of the RNA hybrid by 0.04ppm. However, the most upfield signals are noticed in case of 2'-5' RNA.

^{31}P chemical shifts can potentially provide a probe of conformation of phosphate ester backbone in nucleic acids[23]. Due to the predominating influence of stereoelectronic effects on ^{31}P chemical shifts, it has been observed that (i) phosphate diesters in a g^{-},g^{-} conformation exhibit upfield ^{31}P shifts compared to those in g^{-},t or t,t forms[24], (ii) phosphate esters in a base stacked helical structure g,g are upfield to those in random coil forms which have non-gauche forms, (iii) phosphates in the middle of a chain prefer stereoelectronically favoured g^{-},g^{-} conformation whereas those at the ends can assume g,t forms and (iv) the environmental effects on ^{31}P chemical shifts of nucleic acids are generally smaller than the intrinsic conformational factors.

Extensions of these general observations about ^{31}P chemical shift-conformation correlations to the present cases reveal interesting information on the backbone structural perturbation caused solely due to

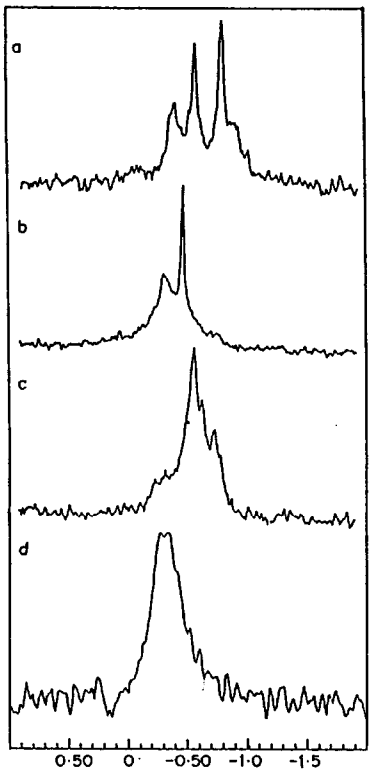


Figure 4. ^{31}P NMR spectrum of (a) r'(AACCUU), (b) r(AACCUU), (c) d(AACCUU) and (d) r(AA)r'(CCUU) in 20% D_2O. Chemical shifts are referenced to external H_3PO_4),

differences in the regiospecificity of the phosphodiester linkages. The multiple resonances seen in 2'-5'RNA indicate that conformationally this is more heterogeneous than the corresponding 3'-5' isomer or its hybrid and the DNA hexamer. Further, the general upfield shifts seen for 2'-5' RNA suggest that its phosphate esters are predominantly in a g,g conformation. As indicated by their relatively lower field shifts, the phosphates in 3'-5' RNA are disposed more in g,t or t,t forms. In contrast to both these ribomers, the constituent phosphates of the DNA hexamer and the 2'-5'/3'-5' RNA hybrid are in closely related conformational states, leading to a broad peak. The heterogeneity observed in phosphate backbone conformation of either 2'-5' or 3'-5' RNA is clearly absent in case of the hybrid and DNA, suggesting a more random coil state. It has been previously suggested that 2'-5' linkages in RNA lead to more base overlap at the dinucleotide steps[25]. This may perhaps be the reason for the appearence of high field components in its ^{31}P NMR since base overlap favours a g,g conformation for the phosphate.

The observed differences in the fine structure ^{31}P chemical shifts of above oligomers which have identical base sequences may therefore be attributed to the conformational dissimilarity arising entirely due to the

difference in nature of the linkages. Further work is in progress to understand the consequence of the above conformational differences seen in single strand forms on their ability to form duplexes by temperature dependent hybridization studies.

Circular dichroism: Circular dichroism is very sensitive to conformational changes in nucleic acids[26], especially to alterations of base tilt, twist, stacking and handedness. Studies on CD of several short oligonucleotides (DNA and RNA) have revealed that most oligomers in a single stranded form exist in a conformation in which bases are stacked resembling a single strand of a double helix. The geometric relationships between two stacked bases in isomeric dimers are known to be strongly influenced by the disposition of the phosphodiester linkage they possess[26,27]. The CD spectra of single stranded ribohexamers r(AACCUU) and r'(AACCUU) at ambient temperature is depicted in Figure 5. The spectral range from 220nm to 300nm covers the optical activity of the major transition band near 260nm. The observance of significant absolute ellipticity (positive and negative) is indicative of considerable base-base stacking in these hexamers, even in single stranded form. All have a positive maximum at 268nm, a negative minimum at 245nm and a positive maximum at 215nm. This pattern of dependence of ellipticity on wavelength suggests a right hand turn for the screw axis of the base stack[28]. While the spectra of both 3'-5' and 2'-5' hexamers are similar with respect to $_{max}$, maximum positive ellipticity (peak) and the crossover wavelength at 254nm, there is a slight difference in the magnitude of the negative ellipticity (trough). The absolute values of ellipticity at peak and trough are marginally different for both the isomers indicating a slight non-conservative nature of the CD profiles. The peak intensity at 218nm for 3'-5' RNA is considerably enhanced over that of 2'-5' RNA. The peak (268±2nm) and trough (248±2nm) positions of these hexamers are shifted lower by 5 to 7nm compared to those of the dinucleotides r(AA) and r'(AA)[27].

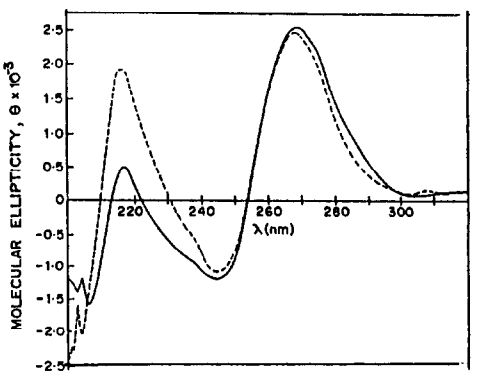


Figure 5: CD spectra of (a) r(AACCUU) and (b) r'(AACCUU) in 2mM sodium cacodylate, pH7.40, EDTA (0.1mM) and NaCl (100mM).

Extensive CD studies on dinucleotides have indicated that 2'-5' RNA have substantial degree of stacking compared to 3'-5' RNA[27]. However in the present hexamers, the extent of overall base-base overlap seen by CD is almost equal for 2'-5' and 3'-5' isomers. Thus the better base stacking seen

in 2'-5' isomers during dinucleotide steps may not accumulate cumulatively to effect perceptible differences at the hexameric level. CD represents the overall stacking behaviour and from present results it is not possible to delineate the contribution from various local stacking effects. However, in ^{31}P NMR, local conformational effects can be identified and as reported in above sections, our ^{31}P NMR results clearly support an increased base overlap locally in 2'-5' RNA hexamers. The temperature dependent studies of CD profiles of these hexamers and their duplexes with complementary DNA sequences have been carried out in order to understand the stacking behavioural differences among these and the results will be reported elsewhere[29].

CONCLUSIONS

^{1}H, ^{31}P NMR and CD spectroscopic results of the three regioisomeric RNA hexamers, r(AACCUU), r'(AACCUU) and a covalent hybrid r(AA)r'(CCUU) are presented. The ^{31}P NMR results indicated dissymmilarities among these in phosphate backbone conformation, with 2'-5' isomer exhibiting considerable local heterogeneity. No appreciable differences were noticed in the overall base-base stacking as seen in the CD spectra. They exhibited different behaviour towards the enzyme pancreatic ribonuclease, with 3'-5' RNA being consumed and 2'-5' RNA being resistant.

The fully protected RNA hexamers were synthesised by solution phase phosphotriester chemistry with the use of hydrophobic protecting groups on 5' (DMT) and 2'/3' (TBDMS) hydroxyls of ribose moiety. A simple deprotection procedure, consisting of treatment with sat. methanolic ammonia to remove both phosphate and amino protectors without affecting the TBDMS groups, which were later deprotected by TBAF treatment, gave high purity oligoribomers. Unlike aqueous NH_3, sat. MeOH-NH_3 does not bring about any 5'-modifications even with a free terminal 5'-OH. The regioisomeric purity of internucleotidic phosphate bonds in the above individual sequences was established by their specific response to hydrolysis with different nucleases. The availability of 2'-5' RNA and its nuclease resistant property may have practical chemotheraputic importance in its use as prodrugs for delivery and targetting of antiviral and antitumour agents, including AZT[3]. Comparative conformational and structural studies (2D NMR, CD and hybridization) of 3'-5' and 2'-5' RNA synthesised as above are under progress and are expected to throw light on the reason for the exclusive predominance of 3'-5' RNA over 2'-5' in nature.

EXPERIMENTAL PROCEDURE

Chemicals: All N,O-protected ribonucleosides (2 and 3) were synthesised according to procedures already reported[15]. *o*-Chlorophenyl dichlorophosphate and NMI were procured from Fluka, Switzerland. MSNT was synthesised as reported[5b]. Pyridine and DCM were purified according to standard procedures. DEAE Sephadex A-25, Sephadex G-10 and G-15 were obtained from Pharmacia (Sweden) and used after autoclaving. At all stages of deprotection and purifications, extreme caution was exercised to exclude contamination by extraneous ribonuclease by use of sterile glassware and reagents prepared in autoclaved water.

Chromatography: All column chromatographic purifications were done over silica gel (100-200 mesh, Loba-Chemie) by the short column method and moni-

tored over Keiselgel $60F_{254}$ (E.Merck 5554) pre-coated TLC plates. The spots were visualised by a UV lamp, followed by spraying with 60% perchloric acid in ethanol. The trityl compounds showed orange spots and those without trityl group exhibited black spots on spraying and heating. All flash chromatography separations were done on Eyela (Japan) system using silica gel (E.Merck 9385). FPLC analysis was carried out on a Pharmacia LCC-500 liquid chromatograph equipped with a gradient mixing system, uv detector (254nm) and a photometer output recorder. The HPLC analysis were performed on a Waters machine.

Spectroscopy: All UV-visible spectra were recorded on a Hitachi 330 spectrophotometer. NMR spectra were obtained on MSL300 Bruker spectrometer equipped with an Aspect 3000 computer and detection of ^{1}H at 300MHz and ^{31}P at 121MHz. For ^{1}H NMR, the hexamers (1mg, 1.1mM) were dissolved in 10mM phosphate buffer (0.5ml, pH6.6), containing EDTA (0.1mM), lyophilised thrice with D_2O and finally redissolved in 0.5ml of D_2O. The ^{31}P NMR of the hexamers (1mg, 1.1mM) were recorded in 20% D_2O. The CD spectra were recorded at ambient temperature on a Jasco J500 spectropolarimeter.

General procedure for preparation of 3'-O-phosphoryl-N,O-protected ribonucleosides 1: N-acyl-5'-O-dimethoxytrityl-2'/3'-O-t-butyldimethyl silyl ribonucleoside **2** (2mmol) was suspended in anhydrous pyridine (20ml) and the mixture was evaporated to a final volume of 10ml. *o*-chlorophenyl dichlorophosphate (1.6ml, 10mmol) was added to pyridine (20ml) contained in a glass reaction vessel fitted with a sintered disc and a stopcock. While cooling, water (180ul, 10mmol) was added slowly into the reaction vessel. On keeping the reaction mixture aside for 10min., pyridine hydrochloride separated out. This was filtered into the reaction vessel containing the dried substrate. The mixture was concentrated to 10ml and after 30min stirring at room temperature, phosphorylation was found to be complete as shown by TLC. The reaction was quenched by the addition of TEAB (15ml, 1M) at $0^{o}C$. Extraction with DCM (3x75ml) followed by recovery yielded a foam which was directly chromatographed over silica gel (35gm). Elution with 1%TEA in DCM and increasing amounts of methanol (upto 5%), gave ribomonomers **1** as their triethylammonium salts (80-90%yield). ^{31}P NMR: **1**(B=U), **a**,-5.52; **b**, -5.49; **1**($B=C^{Bz}$), **a**,-5.41, **b**,-5.5; **1**($B=A^{Bz}$), **a**, -5.52, **b**,-5.57; **1**($B=G^{Bz}$), **a**, -5.39, **b**, -5.38 ppm.

N-acyl-2'O-t-butyldimethylsilyl-3'O-(2-chlorophenyl-4-nitrophenylethyl) phosphotriesters 3: A mixture of the appropriate ribophosphodiester **1** (0.1mmol) and 4-nitrophenylethanol (68mg, 2mmol) was dried by coevaporation with pyridine. The resulting gum was dissolved in pyridine (1ml) and MSNT (242mg, 0.8mmol) and NMI (133ul, 1.6mmol) were added to it. The reaction mixture was stirred at room temperature for 15-20min. after which 5% aq.$NaHCO_3$ (5ml) was added and the mixture extracted with DCM (3x10ml). The removal of solvent furnished a gum that was treated with 2%DCA in DCM (20ml) to result in an orange red solution. The usual work-up and concentration gave a gum which on purification by silica gel chromatography yielded the 5'-hydroxy derivative (3) in 98% yield.

r(HO-UpU)-Bz_2: 2',3'-di-O-benzoyl uridine (150mg, 0.3mmol) was coevaporated in pyridine (1ml) along with DMT-r(Up) (377mg, 0.4mmol). This was then NMI (265ul, 3.2mmole). After 15min., the reaction was quenched with aq.

$NaHCO_3$ and extracted into DCM (3x10ml). The organic layer was washed with saturated brine, dried and concentrated to a foam. This was chromatographed on a short column (1cmx6cm) of silica gel (5gm) using DCM containing 0.5%TEA eas eluent and monitored by TLC. The appropriate fractions were pooled and concentrated to yield a foam of **6** (400mg, 92% yield).

The above dinucleotide was detritylated using 2%DCA in DCM as described before. The organic layer on concentration gave a foam which was redissolved in DCM (2ml) and added slowly while cooling to a stirred solution of dry petroleum ether (25ml). The desired product which precipitated out as a white amorphous solid was collected by centrifugation and dried to yield **7** (270mg, 90% yield).

TABLE-I: Reactant stochiometry for synthesis of r(AACCUU)

5'-Component (mg, mmol)	*3'-Component* (mg, mmol)	*MSNT* (mg, mmol)	*NMI* (ul, mmol)	*Product* (mg, %yield)
DMT-r(Up) 377, 0.40	*HO-r(U)-Bz$_2$* 150, 0.30	485, 1.60	265, 3.20	*DMT-r(UpU)-Bz$_2$* 400, 92
DMT-r(C^{Bz}p) 340, 0.32	*HO-r(C^{Bz}p')* 220, 0.28	394, 1.30	215, 2.60	*DMT-r(C^{Bz}pC^{Bz}p')* 440, 92
DMT-r(A^{Bz}p) 190, 0.18	*HO-r(A^{Bz}p')* 118, 0.15	253, 0.81	135, 1.62	*DMT-r(A^{Bz}pA^{Bz}p')* 218, 90
DMT-r(C^{Bz}pC^{Bz}p) 384, 0.22	*HO-r(UpU)-Bz$_2$* 200, 0.22	280, 0.88	160, 1.76	*HO-r(C^{Bz}pC^{Bz}pUpU)-Bz$_2$* 390, 76
DMT-r(A^{Bz}pA^{Bz}p) *40, 0.034*	*HO-r(C^{Bz}pC^{Bz}pUpU)-Bz$_2$* *38, 0.02*	*73, 0.24*	*40, 0.48*	*HO-r(A^{Bz}pA^{Bz}pC^{Bz}pC^{Bz}pUpU)-Bz$_2$* *46, 75*

r(DMT-C^{Bz}pC^{Bz}p): The condensation of r(DMT-C^{Bz}p)(340mg, 0.32mmol) with r(HO-C^{Bz}p') (220mg, 0.28mmol) as described above, followed by treatment with DBU (236ul, 1.6mmol) gave **5** (440mg, 92%). Similarly **4** was obtained from reaction of DMT-r(A^{Bz}p) (190mg, 0.18mmol) and OH-r(Ap') (118mg, 0.15mmol) as in the above case.

r(HO-C^{Bz}pC^{Bz}pUpU)-Bz$_2$ (2+2 block coupling): HO-r(UpU)-Bz$_2$ (200mg, 0.17mmol) was condensed with DMT-r(C^{Bz}pC^{Bz}p) (384mg, 0.22mmol) using MSNT (280mg, 0.88mmol) and NMI (135ul, 1.62mmol) in dry pyridine (1.7ml). The work-up, purification and detritylation similarly as above gave **10** (76% yield).

r(HO-A^{Bz}pA^{Bz}pC^{Bz}pC^{Bz}pUpU)-Bz$_2$ (2+4 block coupling): The protected tetramer **10** (38mg, 0.02mmol) was mixed with the dimer **9** (40mg, 0.034mmol) and coevaporated twice with pyridine. The gum was then dissolved in pyridine (0.2ml) and treated with MSNT (73mg, 0.24mmol) and NMI (0.48mmol). The reaction product after work-up was purified by chromatography using a column (1cmx5cm) of silica gel packed under centrifugation (3000rpm). The sample was loaded and elutions were done under centrifugation using DCM containing TEA (0.5%) in 1ml aliquots and increasing amounts of acetone (upto 5%). The

column elutants were monitored by TLC and the required product tions were pooled and concentrated. It was then detritylated anc ed as before to obtain the protected hexamer **11** (37mg, 66% yielc

The protected 2'-5'RNA hexamer r'(HO-$A^{Bz}pA^{Bz}pC^{Bz}pC^{Bz}$pUpU)-covalent hybrid r(HO-$A^{Bz}pA^{Bz}$p)r'($C^{Bz}pC^{Bz}$pUpU)-Bz_2 were synth appropriate precursor protected nucleotides by a similar blc approach and in similar yields.

General method for deprotection and purification of oligonucle complete deprotection of all amino and phosphate groups in the tides was done in a single step by treatment with anhydrous satu nolic ammonia (15ml) in a sealed flask at room temperature followed by heating at 60°C for 4hr. The solution was coole solvent was slowly evaporated. In the case eof the oligodeox tides the resultant product was directly purified by ion-excha tography over DEAE Sephadex A-25. The oligoribonucleotides pr then subjected to 1M TBAF in THF for 5hr. at room temperature desilylation. The reaction was quenched with TEAA and the lyophilised.

The crude deblocked oligonucleotides were dissolved in (1ml) and directly loaded onto a column (2cmx20cm) of DEAE Sep previously swollen and autoclaved in the same solvent. The he eluted using a linear gradient (400ml) of 0.05M to 0.5M TEAA at of 1ml/min. The fractions corresponding to the major peak contai quired compound were pooled, lyophilised and then subjected to tion over Sephadex G-15 using sterile water as eluant. The void on lyophilisation afforded the required compounds as colourl (10mg) which on rechecking by FPLC was about 90-95% pure (Figure

Enzymatic and alkaline hydrolysis of ribonucleotides

Enzymatic hydrolysis: The hexaribonucleotides ($1A_{260}$) were dissc buffer (40mM, 10ul, pH7.8) containing NaCl (50mM) and EDTA (0. was treated with pancreatic ribonuclease (1mg in 100ul buffer) a for 1hr. at 37°C.

Alkali hydrolysis: The oligoribonucleotides ($1A_{260}$) were treate sodium hydroxide (1M, 50ul) at 37°C for 2hr. These reaction pr analysed by FPLC over pep-RPC column using the solvent syste before (Figure 1d-1f).

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REFERENCES

1. (a) Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K son, J. D. , *"Molecular biology of cell"*, 1983, Garland, Ne

Saenger, W. "*Principles of nucleic acids structure*", 1984, Springer-Verlag, New York.

2. Cech, T. R. *Science*, 1987, 236, 1532.
3. (a) Ogilvie, K. K. and Theriault, *Tetrahedron Lett.*, 1979, 20, 2111. (b) Charubala, R. and Pfleiderer, W., *Tetrahedron Lett.*, 1982, 23, 4789.(c) Hayakawa, Y., Uchiyama, M., Nobori, T., and Noyori, R., *Tetrahedron Lett.*, 1985, 26, 761. (d) Yoshida, S. and Takaku, H. *Chem. Pharm. Bull.*, 1986, 34, 2456. (e) Srinivasan, A. R. and Olson, W. K., *Nucleic Acids Res.*, 1986, 14, 5461. (f) Herdwijn, P., Charubala, R. and Pfleiderer, W. *Helv. Chim. Acta*, 1989, 72, 1729. (g) Herdwijn, P., Charubala, R., Clerq, E. D., and Pfleiderer, W. *Helv. Chim. Acta*, 1989, 72, 1739.
4. Padgett, R. A., Grabowski, P. J., Kornarska, M. M., Seiler, S. and Sharp, P. A. *Ann. Rev. Biochem.*, 1986, 55, 1119.
5. (a) Ohtuska, E and Iawai, S., in Narang, S. (Ed), *"Synthesis and applications of DNA and RNA"*, 1987, Academic press, Orlando. (b) Gait,M.J. (ed) *"Oligonucleotide synthesis: a practical approach"*, 1986, IRL Press, Oxford. (c) Mizuno, Y., "*The organic chemistry of nucleic acids*", 1986, Elsevier, Amsterdam.
6. Chaix, C., Duplaa, A. M., Molka, D., and Teole, R., *Nucleic Acids Res.*, 1989, 17, 7381.
7. (a) Usman, N., Ogilvie, K. K., Jiang, N.Y. and Cedergren, R. J., J. Am. Chem. Soc., 1987, 109, 7845. (b) Ogilvie, K. K., Usman, N., Nicoghosian, K. and Cedergren, R.J., *Proc. Natl. Acad. Sci. USA*, 1988, 85, 5764.
8. (a) Brown, J. M., Christodolou, C., Jones, S. S., Modak, A. S., Reese, C. B., Sibanda, S. and Ubasawa, A., *J.Chem.Soc. Perkin Trans. I*, 1989, 1735. (b) Brown, J. M., Christodolou, C., Modak, A. S., Reese, C. B., and Serafinowska, A., *J.Chem.Soc. Perkin Trans. I*, 1989, 1751.
9. (a) deVroom, E., Fidder, A., Marugg, J. E., van der Marcel, G. A and van Boom, J. H., *Nucleic Acids Res.*, 1986, 14, 5885. (b) Dock-Bregeon, A. C., Chevrier, B., Podjarny, A., Johnson, J., de Bear, C., Gough, G. R., Gilham, P. T. and Moras, D. *J.Mol.Biol.*, 1989, 209, 459.
10. (a) Ganesh, K. N. and Rajendrakumar, G. V. *Tetrahedron*, 1990, 1311. (b) Singh, D., Kumar, V. A. and Ganesh, K. N., *Nucleic Acids Res.*, 1990, 18, 3339.
11. (a) Sakatsume, O., Ohtsuki, M., Takaku, H., and Reese, C. B., *Nucleic Acids Res.*, (Symposium series no 20), 1988, 77. (b) Lehmann, C., Xu, Y-Z., Christodolou, C., Tan, Z-K and Gait, M. J., *Nucleic Acids Res.*, 1989, 17, 2379.
12. (a) Ogilvie, K. K., Nemer, M. J., and Gilen, M. F., *Tetrahedron Lett.*, 1984, 25, 1669. (b) Garegg, P. J., Lindh, I., Regberg, T., Stawinski, J., and Stromberg, R., *Tetrahedron Lett.*, 1986, 27, 4055.
13. Tanaka, T., Tamatsukuri, S. and Ikehara, M., *Nucleic Acids Res.*, 1986, 14, 6265.
14. (a) Sung, W. L. and Narang, S. A., *Can. J. Chem.*, 1982, 60, 111. (b) von Gunter, S., Flocheszi, D., Varma, R. S., Charubala, R., Uhlmann, E. and Pfleiderer, W., *Helv. Chim. Acta*, 1981, 64, 1704.
15. Vidhya, G and Ganesh, K. N., *Proc. Ind. Acad. Sci. (Chem. Sci.)*, 1989, 101, 401.

16. Efimov, V. A., Reverdatto, S. V and Chakmakhcheva, O. G., ***Nucleic Acids Res.***, 1982, 10, 6675.
17. Rajendrakumar, G. V., Sivakamasundari, N and Ganesh, K. N., ***Proc. Ind. Acad. Sci. (Chem. Sci.)***, 1987, 99, 341.
18. (a) Reese, C. B. and Zard, L., ***Nucleic Acids Res.***, 1981, 9, 4611. (b) van Boom, J. H., Burgers, P. M. J., van Deursen, P. H., De Rooij, J. F. M. and Reese, C. B., ***JCS Chem. Comm.***, 1976, 167. (c) De Rooij, J. F. M.. Hazeleger, W-.G., Burgers, P. M. J. and van Boom, J. H., ***Nucleic Acids Res.***, 1979, 91, 2237.
19. Wu, T and Ogilvie, K. K., ***Tet. Lett***, 1988 29, 4249.
20. Stawinsky, J., Stromberg, R., Thelin, M., and Westman, E., ***Nucleic Acids Res.***, 1988, 16, 9285.
21. Sussman, J. L., Barzilay, I., Keren-ur, M. and Lapidot, Y., ***Biochim. Biophys. Acta***, 1973, 308, 189.
22. While this manuscript was in preparation, the following paper appeared with a similar conclusion using the enzyme ribonuclease T_2; Wang, Y. Y., Lyttle, M. H. and Borer, P. N., ***Nucleic Acids Res.***, 1990, 18, 3347.
23. Chen, C. W. and Cohen, J. S. in ^{31}P ***NMR*** (Ed. Gorenstein, G.), 1984, Academic press, New York, 233.
24. The convention for representing g and t forms of phosphodiester linkage in nucleic acids is followed as in reference 1(b).
25. Kondo, N. S., Holmes, H. M., Stempel, L. M., and Ts'O, P. O. P., ***Biochemistry***, 1970, 9, 3479.
26. (a) Cantor, C. R. and Schimmel, P. R., "**Biophysical Chemistry**", **Part 3, (1980)**, W.H. Freeman & Co, New York. (b) Gratzer, W. B., in "**Procedures in nucleic acids chemistry**", Vol II, 3.
27. Doornbas, J., Den Hartog, J. A. J., Van Boom, J. H. and Altona, C., ***Eur.J.Biochem.***,1981, 116, 403.
28. Uesugi, S., Okhubo, M., Ohtsuka, E., Ikehara, M., Kobayashi, Y., Kyogoku, Y., Westerink, H. P., Van der Mare, G. A., van Boom, J. H. and Haasnoot, C. A., ***J. Biol. Chem.***, 1989, 259, 1390.
29. Vidhya, G and Ganesh, K. N., manuscript under preparation.
30. *Abbreviations*: **DMT**, 4,4'-dimethoxytriphenylmethyl; **Pixyl**, phenylxanthyl; **Pac**, phenoxyacetyl; **Bz**, benzoyl; **TBDMS**, t-butyldimethylsilyl; **DCM**, dichloromethane; **THF**, tetrahydrofuran; **TEAB**, triethyl bicarbonate; **TEAA**, triethyl ammonium acetate; **TBAF**, tetrabutyl ammonium fluoride; **FPLC**, Fast protein liquid chromatography (Pharmacia); **HPLC**, High performance liquid chromatography; **r**, 3'-5' RNA; **r'**, 2'-5' RNA; **p**, *o*-chlorophenyl phosphodi/triester; **p'**, 4-nitrophenylethyl phosphotriester