

## Chemical synthesis of oligonucleotides. 3<sup>†</sup>: Synthesis and characterization of N,O-protected ribophosphoesters for applications in RNA synthesis<sup>††</sup>

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MS received 11 May 1989; revised 25 September 1989

**Abstract.** The chemical synthesis of RNA, in contrast to that of DNA, poses problems due to (i) additional requirement of 2'-hydroxyl protection of the ribose moieties, and (ii) high lability of inter-ribophosphate bonds. Herein we report the synthesis and characterisation of N, O-protected ribophosphoesters  $\mathbf{1}$  which are key monomeric derivatives in phosphotriester methodology for RNA synthesis. Both the isomeric 2' and 3'-O-phosphates have been obtained and characterised. The utility of the *t*-butyl dimethylsilyl group for 2'-hydroxyl protection in the phosphotriester method is demonstrated by the synthesis of *r*(AUAU), *r*(UAUA) and *r*(CACA).

**Keywords.** Oligoribonucleotide synthesis; phosphotriester; *t*-butyl dimethyl silyl group.

### 1. Introduction

RNA is involved in a wide range of functions in a living cell. Besides its known important role in protein synthesis, several unique features of RNA such as self-splicing (Padgett *et al* 1986) and self-cleavage (Cech 1987) abilities have been recently discovered. RNA also exhibits a variety of three dimensional structures based primarily on stem-loop and cruciform motifs as in *t*-RNA and *r*-RNA (Saenger 1984). Recently unusual branched structures in RNA have been recognized (Padgett *et al* 1986). Our increasing knowledge of RNA biology has provided the main impetus for understanding its structure and chemical reactivity. This has generated a growing need for synthetic methodologies to obtain sequence-specific RNA fragments.

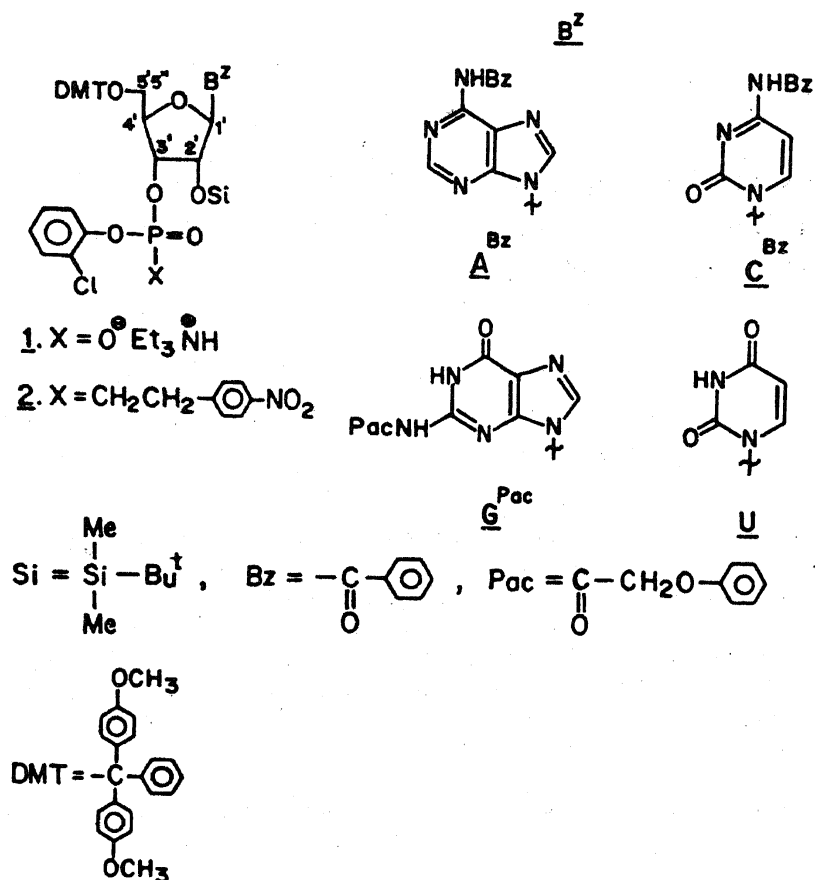
### 2. Problems in RNA synthesis

The chemical synthesis of RNA in contrast to that of DNA, poses problems due to the additional requirement of the 2'-hydroxyl protection (Reese 1978). The choice of the protecting group is governed by its compatibility with the N,O-protection system in terms of selective insertion and removal, and the chemistry of inter-ribonucleotide

<sup>†</sup> Part 2, Rajendrakumar *et al* (1987)

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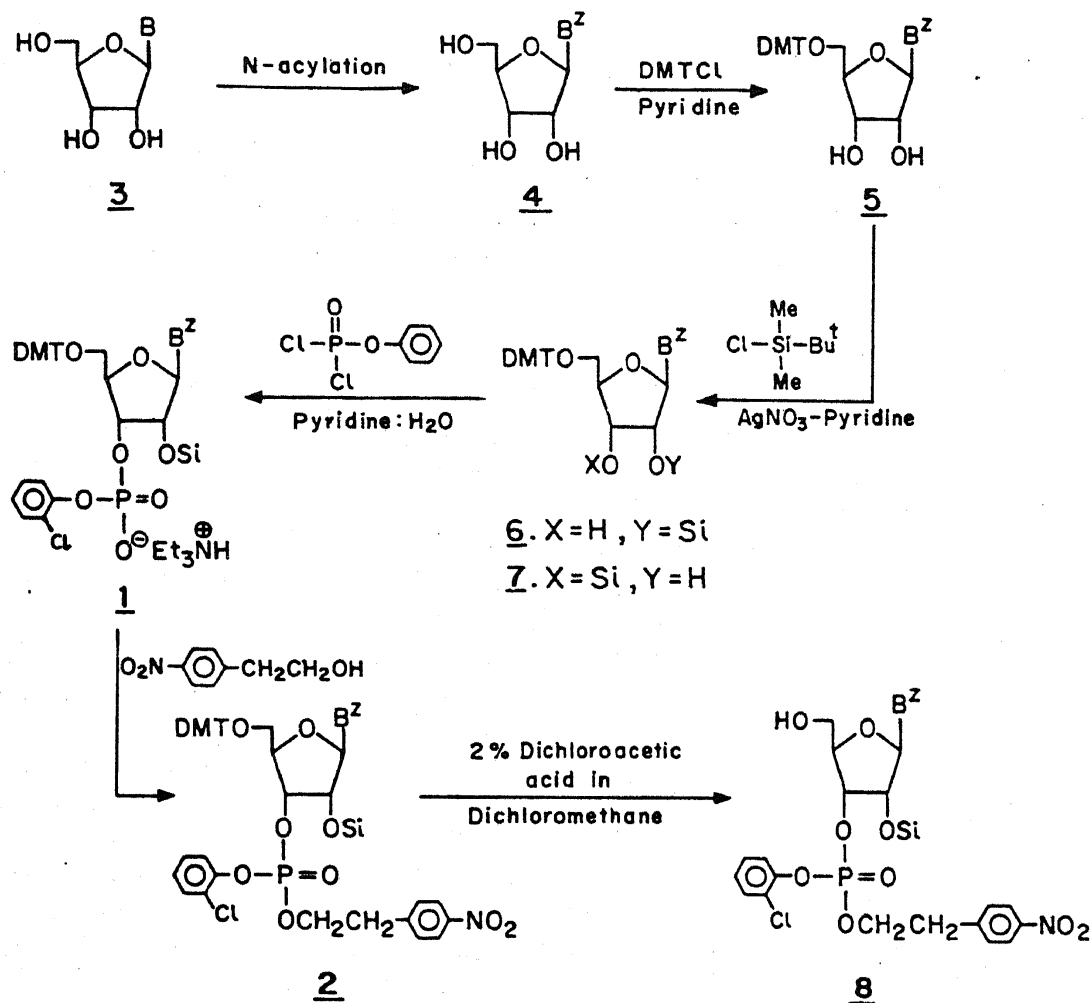


bond formation. Further, the choice of this protecting group is constrained by the high lability of inter-ribophosphate bonds which may cause isomerization or cleavage of phosphodiester linkages during removal of the 2'-protection. Despite these problems, the synthesis of oligoribonucleotides by several chemical strategies have been achieved (Kwiatkowski *et al* 1983; Sung and Narang 1982; Stawinski *et al* 1988), the most recent being that of methionine *t*-RNA by the solid phase phosphoramidite method (Ogilvie *et al* 1988). However, rapid and convenient procedures for routine synthesis of RNA, particularly in large amounts as required for NMR and X-ray crystallographic studies are still lacking. Here we report optimised procedures for the synthesis and characterization of ribophosphodiester monomers (1) and triesters (2) which are needed as monomeric units for RNA synthesis by the phosphotriester method. Among the various routes for oligonucleotide synthesis, the phosphotriester method alone offers the unique advantage of being applicable both in the solution phase as well as on solid supports. The *t*-butyldimethylsilyl group is effective for 2'-hydroxyl protection and the protected nucleotides (1) can be successfully used to construct oligoribonucleotides by either solution phase (sequential- and block-coupling) or solid phase phosphotriester techniques.

### 3. Results and discussion

#### 3.1 *N*-Acyl-5'-*O*-(4,4'-dimethoxytrityl) ribonucleosides

The N, O-protected ribophosphoesters (1, 2) were synthesised from the corresponding free nucleosides (3) by a sequential protection strategy (scheme 1). The first step is the



Scheme 1.

protection of the exocyclic amino groups of bases. Depending on the nature of the base, this is achieved by either the peracylation-selective O-acyl hydrolysis (Schaller *et al* 1963) or the transient protection method (Ti *et al* 1982). It may be mentioned that the literature on N-protection of ribonucleosides is scarce and the available procedures are quite cumbersome with low degrees of routine reproducibility. This is perhaps due to the fact that the selectivity of the hydrolysis of O-benzoyl over N-benzoyl functions in perbenzoylated ribonucleosides is highly dependent on the nature of the nucleoside base residue. The selectivity is also critically sensitive to the conditions of the hydrolysis reactions. Often the reactions result in either total hydrolysis to free nucleosides or alkali-promoted depurinations to generate the bases. Taking these problems into consideration, we have optimised the reaction conditions for N-protection of individual ribonucleosides. The present procedures have led to reproducible yields of N-acyl ribonucleosides.

We have found that in the case of adenosine (3, B=A) perbenzoylation followed by selective hydrolysis of O-benzoyl group with sodium methoxide in methanol (0.75 M, 5°C, 20 min) gave pure N-benzoyl adenosine (4, B<sup>z</sup>=A<sup>Bz</sup>) in quantitative yields. For the preparation of N-benzoyl cytidine (4, B<sup>z</sup>=C<sup>Bz</sup>) the one-pot transient protection method as used in case of deoxynucleosides (Ti *et al* 1982; Rajendrakumar *et al* 1985) was better than the perbenzoylation procedure. Either of these methods was

unsatisfactory for N-protection of guanosine (3, B=G) as benzoyl or isobutyryl derivatives. The recently reported N-protection of guanosine by phenoxyacetyl group (Wu and Ogilvie 1988) was found to be better in terms of yields and purity. This was achieved through a transient protection method using phenoxyacetylchloride in conjunction with 1-hydroxybenzotriazole as the N-acylating agent to yield N-phenoxyacetyl guanosine (4, B<sup>z</sup>=G<sup>Pac</sup>). The N-acyl nucleoside derivatives (4) and uridine (3, B=U) were converted to the corresponding 5'-O-trityl derivatives (5) and purified by silica gel chromatography.

### 3.2 *N-Acyl-5'-O-(4,4'-dimethoxytrityl)-2'(3')-O-(t-butyldimethylsilyl)-ribonucleosides*

*t*-Butyldimethylsilyl protecting group introduced into RNA chemistry by Ogilvie *et al* (1977) has proved to be versatile due to its tolerance for different methods of RNA synthesis. Monosilylations of protected ribonucleosides were carried out using stoichiometric amounts of AgNO<sub>3</sub>-pyridine as catalyst. This produces a mixture of 2' (6) and 3' (7) O-silyl derivatives with very low amounts of 2',3'-bis silyl ribonucleosides. The earlier method of introduction of silyl groups using imidazole as a catalyst in dimethylformamide (Sung and Narang 1982) resulted in large amounts of 2',3'-bis silyl derivatives and hence the AgNO<sub>3</sub>-pyridine method is preferred. The relative amounts of 2' and 3' silyl isomers in the product mixture also depend on the nature of the base residue and the reaction conditions. These isomers have very close solubilities and chromatographic mobilities ( $\Delta r_f < 0.1$ ). Their effective separation is possible only by careful flash chromatography over fine grade silica gel. Further, migrations of silyl groups among the 2' and 3' positions, to produce an equilibrium composition occur in the presence of alcohols and triethylamine. We have found that dichloromethane with varying amounts of acetone is a good solvent system for efficient separation of the two isomeric silyl derivatives. In case of guanosine, it was noticed that the N-phenoxyacetyl derivatives allow a better separation of 2' and 3' *t*-butyldimethylsilyl derivatives than the corresponding N-benzoyl or N-isobutyryl derivatives.

It is very important to ensure the purity of the 2' and 3'-silyl derivatives at this stage, since after phosphorylation, their chromatographic resolution is very difficult. The isomeric purity of the silyl derivatives was established by TLC on both normal and reverse phase silica gel plates using various solvent systems. It has been reported that in silica gel TLC, 2'-silyl derivatives (6) are generally faster moving than 3'-silyl derivatives (7). This order of mobility gets inverted on reverse phase TLC plates. Their final identity as regards their structure was supported by NMR spectroscopy.

### 3.3 *Phosphorylation of N-acyl-5'-O-(4,4'-dimethoxytrityl)-2'(3')-O-t-butyldimethylsilyl ribonucleosides*

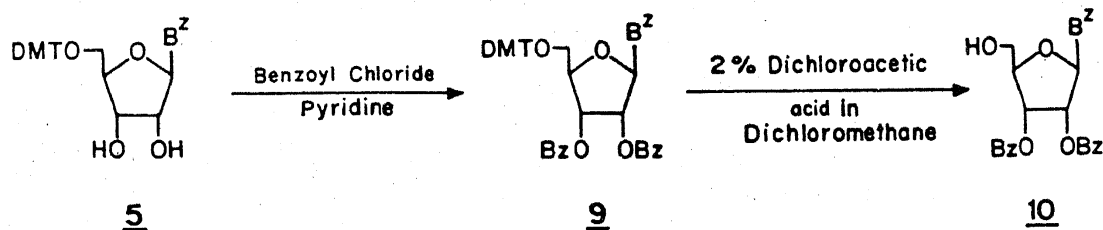
The phosphorylations of protected nucleosides (6 and 7) were carried out using 2-chlorophenyl phosphorodichloridate reagent in pyridine containing stoichiometric amounts of water. This reaction which has been successfully employed earlier in deoxyribonucleotide synthesis (Efimov *et al* 1982; Rajendrakumar *et al* 1985) proved to be equally good for ribonucleotide synthesis. The products of the reaction are N,O-protected ribo-3'-O-phosphates (1) which are suitable precursors for oligoribonucleotide synthesis by the phosphotriester procedure. In alternative methods of phosphorylation using either triazole or 1-hydroxybenzotriazole

activations, significant amounts of by-products have been reported (Reese and Richards 1985; de Vroom *et al* 1986). These arise by the modifications of 6-O in guanosine and 4-O in uridine. In the present method, no such by-products were noticeable. In addition to the non-observance of base-modified by-products, no 2'-3' migrations of silyl groups occurred during phosphorylation. Both 2'-silyl (6) and 3'-silyl (7) derivatives of all ribonucleosides were individually converted by this method to their respective ribophosphodiester. These were isolated as stable solids after purification by silica gel chromatography and characterised by both  $^1\text{H}$  and  $^{31}\text{P}$  NMR spectroscopy.

Sung *et al* (1982) have previously reported RNA synthesis in solution using ribophosphodiester such as 1. But they have generated these compounds *in situ* and without isolating them have carried out the condensation with a second nucleoside. von Gunter *et al* (1981) have also reported similar ribophosphodiester blocks but with *p*-nitrophenylethyl as a permanent protecting group on the phosphates. Our present synthesis and isolation of N, O-protected ribophosphodiester (1) as stable salts has not only permitted unambiguous characterization of the isomeric purity of these compounds but also paves the way for their easy utilization in solid phase procedures. In addition, isolation of the protected ribophosphates (both 3' and 2'-O-phosphates) as stable solids helps in storage and handling, and in large scale operations.

#### 3.4 Synthesis of 3'-terminal blocks for RNA synthesis

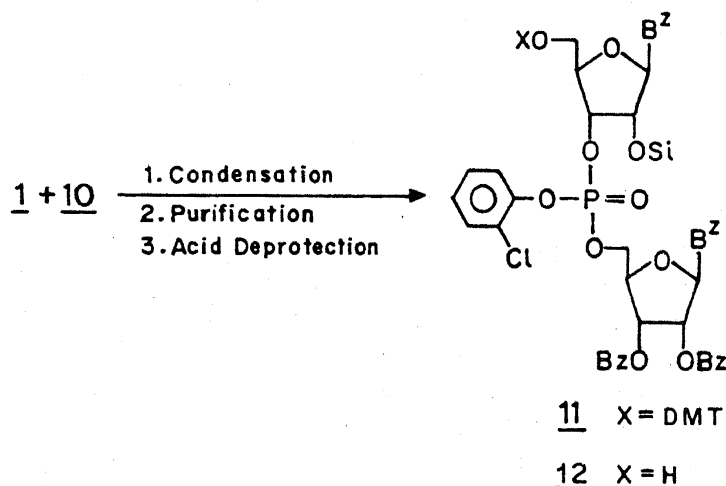
The N,2,3-triacyl ribonucleosides (10) possessing a free 5'-hydroxy group would enable RNA synthesis in the 3'-5' direction by a sequential extension method. These 3'-terminal blocks were prepared (scheme 2) by benzylation of N-acyl-5'-O-(dimethoxytrityl) ribonucleosides followed by removal of the 5'-DMT group by acid treatment. The resulting RNA fragments after complete deblocking will eventually have a free 3'-hydroxyl group. Alternatively, a terminal block such as 8 would give RNA fragments with a phosphate group at the 3'-end. The polynucleotides synthesised with terminal blocks such as 8 on treatment with the base DBU undergo loss of the *p*-nitrophenylethyl protecting group and generate the terminal 3'-O-phosphodiester function. They can then be used as the 5'-component in a block condensation strategy. The nucleotidetriester (8) was synthesised by reaction of 1 with *p*-nitrophenylethanol in the presence of a condensing agent such as mesitylenesulphonyl chloride to obtain the ribophosphotriester (2). This was then detritylated by acid to generate the 5'-hydroxyl ribonucleoside phosphotriester (8).



Scheme 2.

#### 4. Application to RNA synthesis

The 3'-terminal block such as (8) or (10) was condensed with the ribonucleoside 3'-phosphodiester (1) in the presence of mesitylenesulphonyl chloride to generate a ribodinucleotide (11). This product was purified by a fast silica gel column and was detritylated by 2% dichloroacetic acid in dichloromethane to generate (12). The cycle of condensation and acid deprotection was repeated the required number of times on 12 to synthesise oligoribonucleotides of the desired length. By sequential addition, we have conveniently synthesised tetra- to hexamers whereas by the block condensation method higher oligomers can be obtained. After the final deprotection step, RNA fragments obtained were analysed by HPLC on ion exchange and reverse phase columns. The HPLC profiles (figure 1) clearly indicate the absence of any fragments arising due to the degradation or migration of internucleotide phosphodiester bonds. The detailed method of RNA synthesis using the phosphodiesters (1) by blockwise condensation in solution and solid phase synthesis will be reported elsewhere.



Scheme 3.

#### 5. NMR spectroscopy of substituted ribonucleosides and ribonucleotides

Based on studies of a series of substituted ribonucleosides, Fromageot *et al* (1966) have observed that in the  $^1\text{H}$  NMR of a pair of 2' and 3' isomers, the anomeric proton (H1') of 2'-isomer resonates at lower field relative to the 3'-isomers. Further  $J_{1,2'}$  is greater for 3'-isomers than that for 2'-isomers. In most of the examples reported, the substituents have either strong inductive effects or through-space shielding effects which cause the observed differences. However, the *t*-butyldimethylsilyl group has weak inductive and shielding effects and the pattern of chemical shifts noticed (table 1) indicate that the silyl derivatives do not generally conform to reported observations. Guanosine derivatives (6, 7,  $\text{B}^z = \text{G}^{\text{Pac}}$ ) show H-1' chemical shifts according to the general rule. Uridine (6, 7,  $\text{B}^z = \text{U}$ ) and adenosine (6, 7,  $\text{B}^z = \text{A}^{\text{Bz}}$ ) derivatives do not show significant differences in H-1' chemical shifts among the 2' and 3' silyl derivatives. In case of cytidine (6, 7,  $\text{B}^z = \text{C}^{\text{Bz}}$ ), a reverse trend is observed.  $J_{1,2'}$  are all

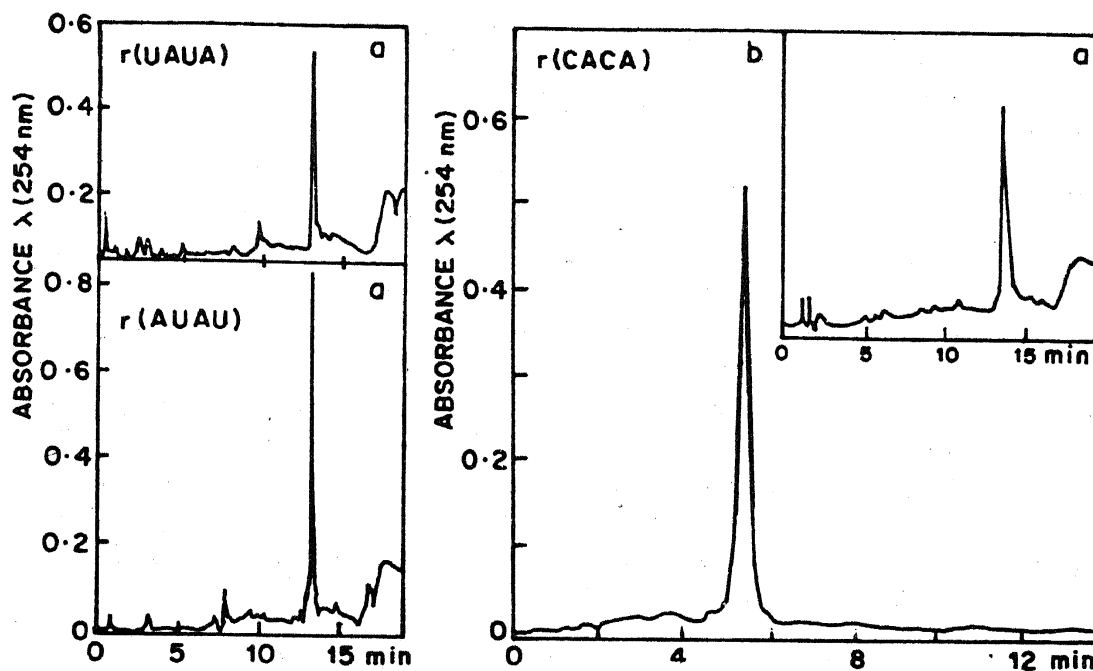


Figure 1. (a) Ion-exchange: Column, Waters Partisil SAX, elution gradient from 0.05 M  $\text{KH}_2\text{PO}_4$  to 0.5 M  $\text{KH}_2\text{PO}_4$ /acetonitrile (9:1) over 30 min. (b) Reverse phase: Bondapak C-18, elution gradient from 0.05 M  $\text{NH}_4\text{OAc}$  to 0.5 M  $\text{NH}_4\text{OAc}$ /acetonitrile (6:4) over 30 min. Flow rate: 2 ml/min.

uniformly of the same magnitude (4–5 Hz) for the silyl ribonucleosides. On the other hand, the chemical shifts for H-2' and H-3' exhibit a pattern in a rather predictable way – the silylation of hydroxyl groups always shifts the corresponding methine protons downfield.

Interesting features were also noticed in the chemical shifts of H-5', 5'' and the alkyl protons of the silyl group. The H-5' and H-5'' are non-equivalent ( $J_{\text{gem}}$ ) in all 3'-silyl derivatives, the difference in shifts ranging from 0.2 to 0.4 ppm. This was absent in the case of all 2'-silyl derivatives with the exception of that of adenosine where the non-equivalence and geminal coupling of H-5' and H-5'' in 2'-silyl isomers is lower than that of 3'-isomers. The two methyls of the *t*-butyl dimethylsilyl group are also non-equivalent in all ribonucleosides. The methyl protons of the 3'-silyl isomers are generally shielded relative to the 2'-isomers. This difference is more significant for pyrimidines than for purines. In the 3'-O-phosphodiester of the 2'-silyl isomers (1), large downfield shifts (0.8 ppm) are noticed as expected for H-3'. Even H-2' and H-4' show downfield shifts but are of lower magnitudes (0.3 ppm). These characteristic features noticed in the  $^1\text{H}$  NMR of silyl ribonucleosides (6, 7) and ribonucleotides (1) for the various sugar protons and their internal consistencies provide ample support for distinguishing the various 2' and 3' silyl derivatives.

$^{31}\text{P}$  NMR chemical shifts are sensitive to the inductive and stereoelectronic effects of the substituents on phosphorus. The  $^{31}\text{P}$  NMR data of substituted 3' and 2'-O-phosphodiester of the four ribonucleosides are shown in table 2. All exhibited a single resonance in  $^{31}\text{P}$  NMR under  $^1\text{H}$  decoupling conditions, indicating the phosphate purity of these compounds. The chemical shift variations among the two isomers do not conform to any particular pattern. Further, the difference in  $^{31}\text{P}$  chemical shifts

**Table 1.**  $^1\text{H}$  NMR spectra data of N,O-protected 2'(3') silyl ribonucleosides and ribonucleotides<sup>†</sup>.

Compound	H-1'	H-2'	H-3'	H-4'	H-5'5''	H-5	H6/H8/H2	Si-Bu'	Si-CH <sub>3</sub>
6, B=U	5.90 <i>d</i> , 4 Hz	4.31 <i>m</i>	4.09 <i>m</i>	4.31 <i>m</i>	3.47 <i>m</i>	5.27 <i>d</i> , 8 Hz	7.89	0.89	0.15 0.07
7, B=U	5.91 <i>d</i> , 4 Hz	4.09 (overlapping <i>m</i> )			3.24 3.46	5.33 <i>d</i> , 8 Hz	7.82 <i>d</i> , 8 Hz	0.81	-0.07 0.04
6, B=C <sup>Bz</sup>	5.93 <i>s</i> , <i>br</i>	4.31 <i>m</i>	4.09 <i>m</i>	4.31 <i>m</i>	3.58 <i>s</i> , <i>br</i>	*	8.51 <i>d</i> , 8 Hz	0.92	0.18 0.31
7, B=C <sup>Bz</sup>	6.09 <i>d</i> , 4 Hz	4.20 <i>m</i>	4.40 <i>m</i>	4.20 <i>m</i>	3.36 3.73	*	8.42 <i>d</i> , 8 Hz	0.84	-0.05 0.05
6, B=A <sup>Bz</sup>	6.07 <i>d</i> , 5 Hz	4.29 (overlapping <i>m</i> )			3.46 3.55	—	H8, 8.74 H2, 8.25	0.80	-0.13 0.00
7, B=A <sup>Bz</sup>	6.07 <i>d</i> , 5 Hz	4.18 <i>m</i>	4.61 <i>d</i> , 4 Hz	4.18 <i>dd</i> , 4 Hz	3.24 3.63	—	H8, 8.78 H2, 8.27	0.88	0.02 0.11
6, B=G <sup>Pac</sup>	5.70 <i>d</i> , 4 Hz	4.20 (overlapping <i>m</i> )			3.60 <i>m</i>	—	7.60 <i>s</i>	0.84	0.00 -0.16
7, B=G <sup>Pac</sup>	5.45 <i>s</i> , <i>br</i>	4.08 <i>s</i> , <i>br</i>	4.18 <i>s</i> , <i>br</i>	4.08 <i>s</i> , <i>br</i>	3.40 3.20	—	7.54 <i>s</i>	0.85	0.02 -0.06
1, B=U	6.00 <i>d</i> , 6 Hz	4.52 <i>m</i>	4.94 <i>m</i>	4.52 <i>m</i>	3.44 <i>s</i> , <i>br</i>	5.17 <i>d</i> , 8 Hz	7.78 <i>d</i> , 8 Hz	0.85	0.09 0.13
1, B=C <sup>Bz</sup>	6.00 <i>d</i> , 3 Hz	4.54 <i>m</i>	4.82 <i>m</i>	4.53 <i>m</i>	3.53 <i>d</i> , <i>br</i>	*	8.44 <i>d</i> , 7 Hz	0.88	0.22 0.17
1, B=A <sup>Bz</sup>	5.67	4.35 <i>s</i> , <i>br</i>	4.57 <i>dt</i>	4.20 <i>dd</i>	3.98 <i>s</i> , <i>br</i>	—	H8, 8.63 H6, 8.55	0.88	0.04 0.15
1, B=G <sup>Pac</sup>	6.00 <i>s</i> , <i>br</i>	4.90 <i>m</i>	5.05 <i>m</i>	4.42 <i>m</i>	3.19 3.42	—	7.54	0.75	0.09 0.04

<sup>†</sup> All spectra recorded on Bruker WH 90 NMR spectrometer. Solvent: CDCl<sub>3</sub>. All values in  $\delta$ . *s* = singlet, *d* = doublet, *dd* = double doublet, *dt* = double triplet, *br* = broad.

\* Overlapping with the aromatic protons (6.8–7.8).

**Table 2.**  $^{31}\text{P}$  chemical shifts of ribophosphotriesters<sup>†</sup>.

Compound	3'-O-phosphate	2'-O-phosphate
1, B=U	-5.52	-5.49
1, B=C <sup>Bz</sup>	-5.41	-5.50
1, B=A <sup>Bz</sup>	-5.52	-5.57
1, B=G <sup>Pac</sup>	-5.39	-5.38

<sup>†</sup> All measured on Bruker MSL 300 NMR spectrometer, operating at 121.4 MHz for  $^{31}\text{P}$ . Solvent: CDCl<sub>3</sub>; reference: 85% H<sub>3</sub>PO<sub>4</sub> (external).



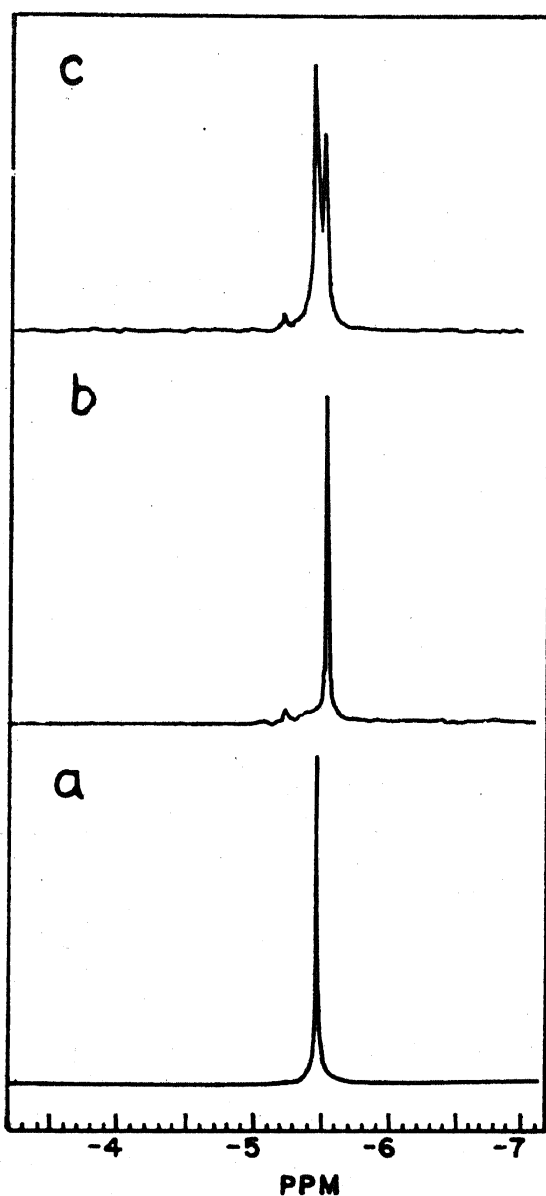


Figure 2.  $^1\text{H}$ -decoupled  $^{31}\text{P}$  NMR of (a) **6**, X = 2-chlorophenyl phosphate and Y = Si, (b) **7**, X = Si and Y = 2-chlorophenyl phosphate, and (c) mixture of **6** and **7** as above.

among the 2' and 3' isomers is small ( $< 0.05$  ppm) except in the case of cytidine derivatives (0.09 ppm). Because of such small changes, the unambiguity of the results was confirmed by recording the  $^{31}\text{P}$  NMR of a sample of pre-mixed 2' and 3' phosphates (figure 2) which indicated two separate resonances. Thus, these  $^{31}\text{P}$  NMR results not only provide clear proof of the purity of the synthesised ribophosphodiester (1), but also rule out 2'-3' migrations of the silyl protecting group during phosphorylating conditions. The isomeric integrity of the various phosphodiester thus stands established.

## 6. Experimental

The ribonucleosides were obtained from SRL, Bombay. 4,4'-Dimethoxytrityl chloride, phenoxyacetyl chloride, and 2-chlorophenyl phosphorodichloridate were synthesised according to literature procedures. 2,4,6-Mesitylene sulphonyl chloride (Aldrich) was recrystallised from hexane just before use. *t*-Butyldimethyl silyl chloride (Aldrich) was used without purification. Trimethylchlorosilane *N*-methyl imidazole and dichloroacetic acid were procured from Fluka, Switzerland. Pyridine was refluxed over anhydrous KOH and distilled twice. Acetonitrile and dichloromethane were purified by distillation over phosphorous pentoxide. Tetrahydrofuran was purified by distillation over sodium and benzophenone. All column chromatographic purifications were done over silica gel (100–200 mesh, Loba-Chemie) by the short column method and monitored over precoated Keiselgel 60F<sub>254</sub> TLC plates (E Merck, Cat. No. 5554). The spots were visualised by a UV hand lamp followed by spraying with 60% perchloric acid in ethanol (3:2) for trityl detection. Compounds without the trityl group show block spots on spraying followed by heating. Flash chromatography was done using an Eyla system on silica gel (E Merck, Cat. No. 9385).

### 6.1 *N*<sup>6</sup>-benzoyl adenosine (4, B<sup>z</sup>=A<sup>Bz</sup>)

Dry adenosine (3, B=A, 10 g, 37 mmol) was suspended in dry pyridine (120 ml) and treated with benzoyl chloride (24 ml, 200 mm) with stirring under cold conditions (5°C). The reaction mixture was stirred at room temperature (3 h) poured into ice cold water (150 ml) and extracted into CH<sub>2</sub>Cl<sub>2</sub> (3 × 100 ml). The organic extract on concentration yielded a solid foam (25 g) identified as 6(N,N),2',3',5'-pentabenzoyl adenosine m.p. 183–185°C. This solid was dissolved in pyridine:methanol (3:1, 200 ml) and treated with sodium methoxide in methanol (0.5 M, 50 ml) at 0°C. The reaction as monitored on TLC was complete within 20 min and the reaction mixture was adjusted to pH 6.0 by addition of Dowex resin (H<sup>+</sup> form) in 20 ml water. The resin was filtered and the filtrate on concentration gave a gum which crystallised from methanol:water (2:1) to give *N*-benzoyl adenosine (4, B=A<sup>Bz</sup>, 11 g, 80%), m.p. 152°C (von Gunter *et al* 1981).

### 6.2 4-*N*-benzoyl cytidine (4, B<sup>z</sup>=C<sup>Bz</sup>)

Cytidine (3, B=C, 2.43 g, 10 mmol) dried by coevaporation with pyridine was suspended in dry pyridine (75 ml) and treated with chlorotrimethyl silane (8.3 g, 75 mmol). The mixture was stirred at room temperature for 2 h and to this was added benzoyl chloride (4.0 ml, 30 mmol). After one hour, the reaction was quenched by addition of a mixture of dichloromethane:water (1:1, 30 ml). The organic layer was separated and concentrated under vacuum to yield white crystals of 4-*N*-benzoyl cytidine (4, B<sup>z</sup>=C<sup>Bz</sup>, 3.2 g, 90%), m.p. 235–238°C (von Gunter *et al* 1981).

### 6.3 2-*N*-phenoxyacetyl guanosine (4, B<sup>z</sup>=G<sup>Pac</sup>)

Guanosine (3, B=G, 5.66 g, 20 mmol), dried by coevaporation with pyridine was suspended in dry pyridine (100 ml). Chlorotrimethyl silane (22 ml, 150 mmol) was

added dropwise and the reaction mixture was stirred for 30 min at room temperature. During this period, 1-hydroxybenzotriazole (4.3 g, 32 mmol) was dried by evaporation with acetonitrile (15 ml) and subsequently suspended in a mixture of acetonitrile and pyridine (1:1, 30 ml), to which was slowly added phenoxyacetylchloride (4.2 ml, 30 mmol). After 5 min, the persilylated guanosine reaction mixture prepared earlier was added dropwise at 0°C to the phenoxyacetylhydroxy benzotriazole complex. The mixture was stirred overnight at ambient temperature after which it was cooled to 5°C. Water (15 ml) followed by ammonia (15 ml) was added dropwise and the reaction mixture was concentrated to a gum. This was dissolved in water (300 ml) and washed with dichloromethane (100 ml). The solid that separated out at the interface was filtered and dried to yield an amorphous white powder of 2-N-phenoxyacetyl guanosine (4, B<sup>z</sup>=G<sup>Pac</sup>, 6.8 g, 85%).

#### 6.4 *N*-acyl-5'-O-(4,4'-dimethoxytrityl) ribonucleosides (5)

The *N*-acyl ribonucleoside (4, 10 mmol) was dried by coevaporation with anhydrous pyridine (20 ml). The resulting gummy foam was dissolved in pyridine (20 ml), cooled to 0°C and treated with 4,4'-dimethoxytrityl chloride (4.1 g, 12 mmol) in 4 lots over 8 h and stirred for an additional 2 h. TLC analysis then indicated the completion of the reaction. Methanol (5 ml) was added to the reaction mixture and was concentrated to a gum under reduced pressure. Sodium bicarbonate (5%, 20 ml) was then added and the resulting mixture was extracted with chloroform (3 × 50 ml). The dried organic layer on concentration gave a pale yellow foam. This was chromatographed on silica gel (50 g, column i.d. 4 cm) and eluted with dichloromethane (containing 1% triethylamine) with increasing amounts of methanol. The required product was eluted with 5–6% methanol as monitored by TLC and the appropriate fractions were combined and concentrated to a foam to yield the dimethoxytrityl derivative in about 80% yield. Using this procedure, 5'-dimethoxytrityl uridine (5, B<sup>z</sup>=U), 6-N-benzoyl-5-O-dimethoxytrityl adenosine (5, B<sup>z</sup>=A<sup>Bz</sup>), 4-N-benzoyl-5'-O-dimethoxytrityl cytidine (5, B<sup>z</sup>=C<sup>Bz</sup>) and 2-N-phenoxyacetyl-5-O-dimethoxytrityl guanosine (5, B<sup>z</sup>=G<sup>Pac</sup>) were prepared.

#### 6.5 *N*-acyl-5'-O-(4,4'-dimethoxytrityl)-2'(3')-O-(*t*-butyl dimethyl silyl) ribonucleosides (6, 7)

*N*-acyl-5'-O-(4,4'-dimethoxytrityl) ribonucleoside (5, 7.5 mmol) was dissolved in dry tetrahydrofuran (75 ml) to which pyridine (2.25 ml, 27.75 mmol) and powdered silver nitrate (1.5 g, 9 mmol) were added. The solution was stirred (5 min) till all the silver nitrate dissolved. *t*-Butyldimethylsilyl chloride (1.8 g, 9.7 mmol) was added to the reaction mixture and stirred at room temperature for 5 h after which TLC indicated the completion of the reaction. The reaction mixture was filtered into 5% aqueous sodium bicarbonate (75 ml), and extracted into dichloromethane (3 × 75 ml). The dried, organic extract on evaporation yielded a mixture of 2' and 3' silyl derivatives (6, 7) as a foam. This mixture (2 g) was separated into individual components using silica gel flash column chromatography in a column of 4 cm i.d. The silica gel column was packed in petroleum ether:dichloromethane (1:1) containing 0.5% triethylamine and eluted initially with increasing amounts of dichloromethane followed by increasing amounts of acetone in dichloromethane (0.5% triethylamine). As monitored by TLC, 2'-silyl derivatives (6) eluted first, followed by 3'-silyl derivatives

(7), which were characterized by a combination of reverse phase TLC and  $^1\text{H}$  NMR (see table 1).

#### 6.6 General procedure for phosphorylation

N-acyl-5'-O-dimethoxytrityl-2'(3')-O-*t*-butyldimethylsilyl ribonucleoside (6, 7, 2 mmol) was suspended in anhydrous pyridine (20 ml) and the mixture was evaporated to a final volume of 10 ml. 4-Chlorophenyl phosphorodichloridate (10 mmol) was added to pyridine (20 ml) contained in a glass reaction vessel fitted with a sintered disc and a stopcock, and while cooling water (10 mmol, 180 ml) was added slowly into the reaction vessel. On keeping aside the mixture at room temperature for 10 min pyridine hydrochloride separated out. It was filtered into the reaction flask containing the nucleosides in pyridine under a nitrogen atmosphere. The mixture was concentrated to 10 ml and after 30 min of stirring at room temperature, phosphorylation was found to be complete as shown by TLC. The reaction was quenched by addition of 1 M triethylammonium carbonate (TEAB, 15 ml) at  $0^\circ\text{C}$ . It was extracted into chloroform ( $3 \times 75$  ml) washed with 0.1 M TEAB and coevaporated to a foam. This was then chromatographed over silica gel (35 g) by short column method using 1% triethylamine in dichloromethane and increasing amounts of methanol. The phosphorylated product eluted as triethyl ammonium salt with 5% methanol in dichloromethane. The appropriate fractions were pooled and concentrated to yield the ribophosphodiester (1) which were characterized by a combination of  $^1\text{H}$  and  $^{31}\text{P}$  NMR (tables 1 and 2).

#### 6.7 N, 2', 3'-tribenzoyl ribonucleosides (10)

N-benzoyl-5'-O-dimethoxytrityl ribonucleoside (5, 3 mmol) was dissolved in pyridine (9 ml) and benzoyl chloride (1.3 ml, 12 mmol) was added dropwise while cooling. The reaction mixture was stirred for 2 h at room temperature, followed by pouring into ice-cold water (100 ml). The gum which separated was extracted into dichloromethane ( $3 \times 50$  ml) and the organic layer was concentrated to a foam. This was then treated with 3%-*p*-toluene sulphonic acid in dichloromethane:methanol (7:3) when an orange-red colour resulted. After checking with TLC for completion of the reaction (5–10 min), aq. sodium bicarbonate (15 ml) was added. Extraction with dichloromethane followed by solvent evaporation yielded a gum which on chromatographic purification yielded N, 2', 3'-tribenzoyl ribonucleosides (10, 90%).

#### 6.8 N-acyl-2'-O-(*t*-butyldimethylsilyl)-3'-O-(2-chlorophenyl, 4-nitrophenylethyl)-phosphotriester (8)

A mixture of the phosphodiester (1, 0.1 mmol) and 4-nitrophenylethanol (0.2 mmol) was dried by coevaporation with pyridine. The resulting gum was dissolved in pyridine (1 ml) and mesitylene sulphonyl chloride (0.4 mmol) and N-methyl imidazole (0.8 mmol) were added to it. The reaction mixture was stirred at room temperature for 15–20 min after which 5% aq. sodium bicarbonate (5 ml) was added and the mixture extracted with dichloromethane ( $3 \times 10$  ml). The removal of solvent furnished a gum (2) that was treated with 2% dichloroacetic acid in dichloromethane (10 ml) resulting 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 (8) in 90% yield.

## 7. Conclusions

We have reported convenient and reproducible methods for the synthesis of N,O-protected ribonucleosides and corresponding ribonucleotides. The difficult separation of the 2' and 3'-*t*-butyl dimethylsilyl derivatives has been achieved by the flash chromatography technique. The phosphorylation of these compounds generate precursors for RNA synthesis by phosphotriester methodology. The utility of the ribophosphodiester (1) in the synthesis of various RNA tetramers has been demonstrated. Further work in using these for RNA synthesis by block condensation and solid phase synthesis is currently in progress.

## Acknowledgements

One of us (VG) thanks the Council of Scientific and Industrial Research for the grant of a fellowship.

## References

- Cech T R 1987 *Science* **236** 1532  
de Vroom E, Fidder A, Marugg J E, van der Marel G A and van Boom J H 1986 *Nucleic Acids Res.* **14** 5885  
Efimov V A, Reverdatto S V and Chakmakhcheva O G 1982 *Nucleic Acids Res.* **10** 6675  
Fromageot H P M, Griffin B E, Reese C B, Sulston J E and Trentham D R 1966 *Tetrahedron* **22** 705  
Kwaiatkowski J, Heikkila, Bjorkman S and Chattopadhyaya J B 1983 *Chem. Scr.* **22** 30  
Ogilvie K K, Theriault N and Sandana K L 1977 *J. Am. Chem. Soc.* **99** 7741  
Ogilvie K K, Usman N, Nicoghosian K and Cedergren R J 1988 *Proc. Natl. Acad. Sci., USA* **85** 5764  
Padgett R A, Grabowski P J, Kornarska M M, Seiler S and Sharp P A 1986 *Annu. Rev. Biochem.* **55** 1119  
Rajendrakumar G V, Sivakamisundari N and Ganesh K N 1985 *Proc. Indian Acad. Sci. (Chem. Sci.)* **95** 357  
Rajendrakumar G V, Sivakamisundari N and Ganesh K N 1987 *Proc. Indian Acad. Sci. (Chem. Sci.)* **99** 341  
Reese C B 1978 *Tetrahedron* **34** 3143  
Reese C B and Richards K H 1985 *Tetrahedron Lett.* **26** 2245  
Saenger W 1984 *Principles of nucleic acid structure* (New York: Springer-Verlag)  
Schaller H, Weimann G, Leerch B and Khorana H G 1963 *J. Am. Chem. Soc.* **85** 3821  
Stawinski J, Stromberg R, Thelin M and Westman E 1988 *Nucleic Acids Res.* **16** 9285  
Sung W L and Narang S A 1982 *Can. J. Chem.* **60** 111  
Ti G S, Gaffney B L and Jones R A 1982 *J. Am. Chem. Soc.* **104** 1316  
von Gunter S, Flocherzi D, Varma R S, Charubala R, Uhlmann E and Pfeleiderer W 1981 *Helv. Chim. Acta* **64** 1704  
Wu T and Ogilvie K K 1988 *Tetrahedron Lett.* **29** 4249