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Solid phase synthesis of oligoribonucleotides using the 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (Ctmp) group for the protection of the 2'-hydroxy functions and the H-phosphonate approach

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ABSTRACT

The solid phase synthesis of oligoribonucleotides using the H-phosphonate approach and the 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (Ctmp) and dimethoxytrityl (DMTr) groups, respectively, for the protection of the 2'- and 5'-hydroxy functions is described. The use of a new reagent, tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite for the preparation of nucleoside H-phosphonate units is also discussed in detail.

INTRODUCTION

In recent years, oligonucleotide synthesis has been markedly facilitated by the introduction of the phosphoramidite [1,2] and H-phosphonate [3−6] approaches on solid supports. These approaches have made rapid preparation of long chain deoxyribo-oligonucleotides possible. Some advantages of the H-phosphonate approach over the phosphoramidite approach for the synthesis of deoxyribooligonucleotides have recently become apparent. [4,7] However, this approach introduces some problems, such as the need for a convenient procedure for the preparation of nucleoside 3'-H-phosphonate units and the stability of coupling reagent [8,9] which still remain to be solved.

The chemical synthesis of oligoribonucleotides on a solid support is more complex than the synthesis of deoxyribooligo-nucleotides, primarily because of the need to protect the 2'-hydroxy functions of the ribonucleoside building blocks. The choice of the protecting group for the 2'-hydroxy functions is a crucial factor in oligoribonucleotide synthesis and it is essential that this protecting group should be completely stable under the conditions required for 5'-deprotection. Recent studies have indicated [10,11] that both the tetrahydropyranyl (Thp) and methoxytetrahydropyranyl (Mthp) groups are unstable under the acidic conditions required to remove the DMTr or Px groups in oligoribonucleotide synthesis on a solid support. Therefore, a more stable protecting group is needed, which will survive the conditions required for 5'-deprotection, but which will be cleaved under conditions similar to those required for the removal of the Mthp group in the region of pH 2.0 at room temperature. It is clear from recent studies [12,13] that the 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl(Ctmp) group meets these requirements and is suitable for the protection of the 2'-hydroxy functions in the solid phase synthesis of oligoribonucleotides.

Ogilvie [14] and Tanaka [7,15] have studied the use of the *tert*-butyldimethylsilyl and *o*-nitrobenzyl groups, respectively, for the protection of the 2'-hydroxy functions in solid phase RNA synthesis and have shown that both of these protecting groups are stable under the acidic conditions required for the removal of 5'-*O*-DMTr groups.

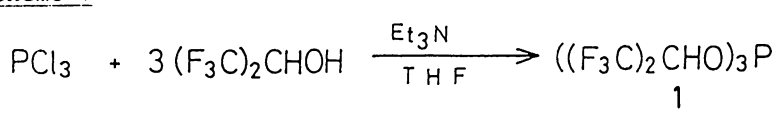
In this paper, we wish to report the solid phase synthesis of oligoribonucleotides by the H-phosphonate approach using the combination of Ctmp and DMTr groups, respectively, for the protection of the 2'- and 5'-O-hydroxy functions. We also describe the use of a new phosphitylating reagent, tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (*1*).

RESULTS AND DISCUSSION

A new phosphitylating reagent

The deoxyribonucleoside 3'-H-phosphonates are key intermediate in the synthesis of deoxyribo-oligonucleotides by the H-phosphonate approach. However, only a few methods have been reported for the synthesis of H-phosphonate units [4,16–19]. One method, reported by Garegg et al. [4], involves the use of tris(imidazolo)phosphine, an unstable reagent that is generated *in situ*. In a previous paper [20], we reported that bis-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphonate (*4*) was a useful reagent for the preparation of H-phosphonate units. We now report that tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (*1*) is much more effective reagent for the preparation of H-phosphonate units (*3*). The latter reagent (*1*) is easily prepared in good yield by treating PCl_3 with 1,1,1,3,3,3-hexafluoro-2-propanol and tri-ethylamine using a modification of the procedure reported by Denney et al. [21]. Compound *1* can be stored unchanged in a screw-cap vial in a refrigerator for 9 months.

Scheme 1



$$PCl_3 + 3\,(F_3C)_2CHOH \xrightarrow[THF]{Et_3N} ((F_3C)_2CHO)_3P$$

1

Synthesis of ribonucleoside 3'-H-phosphonate units (3)

The required ribonucleoside 3'-H-phosphonate building blocks (*3*) were readily prepared by allowing the corresponding *N*-acyl-5'-*O*-(dimethoxytrityl)-2'-*O*-{1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl}-ribonucleosides (*2*) to react with two molecular equivalents of (*1*) in anhydrous pyridine at room temperature for 10 min, followed by a hydrolytic work-up and chromatography of the products. Isolated yields and ^{31}P-NMR spectroscopic data are listed in Table 1. This result indicates that the use of (*1*) rather than as a phosphitylating reagent considerably shortens the reaction time for the preparation of protected ribonucleoside 3'-H-phosphonates (*3*). When the phosphonylation of (*2*) was

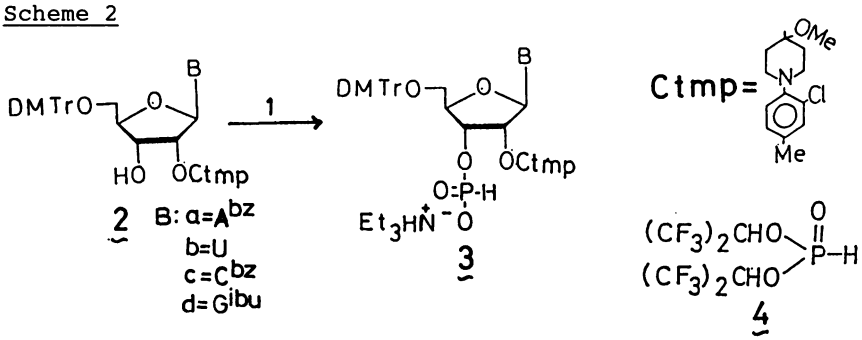
Table 1. Isolated Yields and ^{31}P-NMR Spectra (in $CDCl_3$) of Ribonucleoside 3'-H-Phosphonates (3a-d)

Compd. No.	Yields (%)	^{31}P-NMR Chemical Shift (ppm)
3a	90	3.10
3b	95	3.23
3c	95	3.10
3d	97	3.00

The chemical shifts are reported relative to 85% H_3PO_4 in as external standard.

carried out with reagent (*4*) in the presence of tetrazole in dry pyridine, the reaction was complete in 20 min. However, the corresponding ribonucleoside 3′-H-phosphonates (*3*) were obtained in relatively unsatisfactory (66−75%) yields after separation by silica gel chromatography.

Scheme 2



Oligoribonucleotide synthesis

The utility of ribonucleoside 3′-H-phosphonates (*3*) in which the 2′-hydroxy functions are protected with the Ctmp group is now demonstrated by the synthesis of the octadecaribonucleotide, AGUAUAAGAGGACAUAUG, the leader sequence of Qβ-A protein m-RNA [22, 23].

Scheme 3

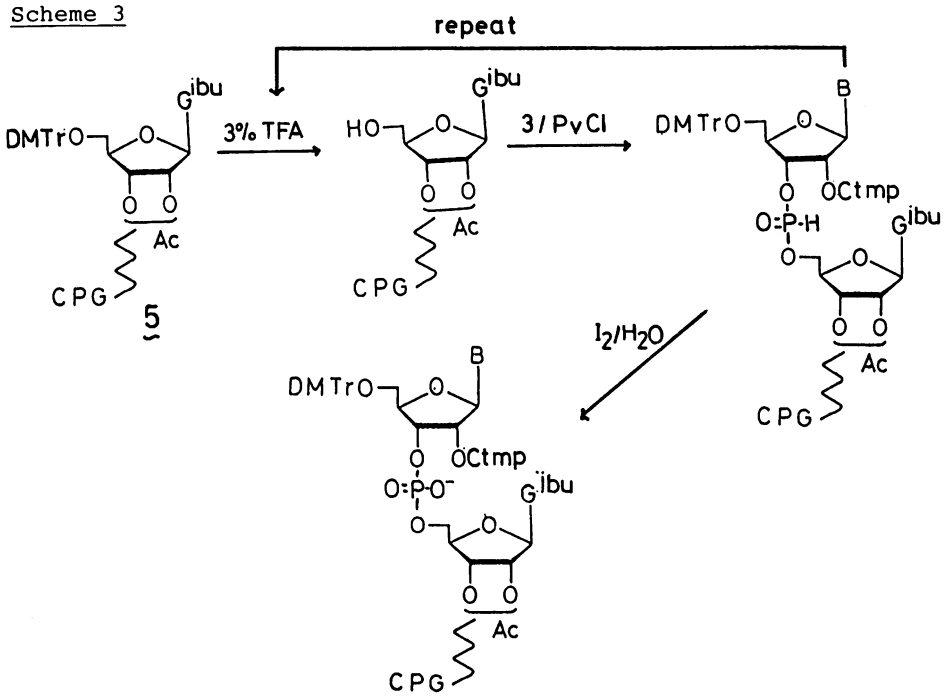


Table 2. The Synthetic Cycle

Step	Reagents and solvents (flow rate 1 ml/min)	Time (min)
1. detritylation	3% trifluoroacetic acid in CH_2Cl_2	1
2. washing	acetonitrile	2
3. washing	acetonitrile-pyridine (1:1, v/v)	2
4. coupling	monomer units (40 mM solution) and pivaloyl chloride (200 mM) in acetonitrile-pyridine (1:1, v/v)	5
5. washing	acetonitrile-pyridine (1:1, v/v)	1
6. washing	acetonitrile	2
7. washing	CH_2Cl_2	2

We first examined the preparation of a nucleoside-bound resin by a modification of the procedure reported by Tanaka et al. [7]. 2′(3′)-*O*-Monoacetyl-N^2-isobutyrylguanosine, prepared by the acetylation of N^2-isobutyryl-3', 5′-O-(tetraisopropyldisiloxan-1,3-diyl)guanosine, was converted to the corresponding 5′-*O*-(DMTr)-3′(2′)-succinate. The latter material was allowed to react with controlled pore glass (CPG), functionalized with amino groups, in the presence of DCC to give the loaded resin (*5*), The extent of nucleoside loading was 24 μmol/g, as estimated from DMTr cation release, following treatment with 3% trifluoroacetic acid in CH_2Cl_2.

In a previous paper [20], we tested the reaction conditions required for high coupling yields in the synthesis of deoxyribooligonucleotides *via* the H-phosphonate approach on a solid support, and found that the coupling reaction could be carried out effectively at very high concentrations of the reacting species. Therefore, we have applied these conditions to the synthesis of oligoribonucleotides. The synthesis of the octa-decamer,

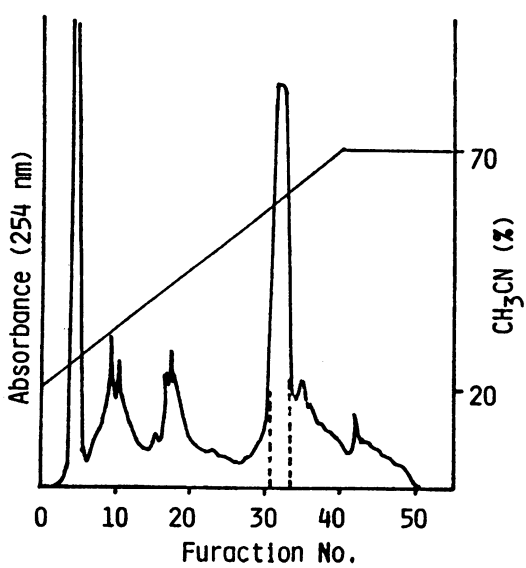


Fig. 1 Isolation of 2′,5′-protected oligoribonucleotide, AGUAUAAGAGGAACAAUG on the reverse phase C-18 column.

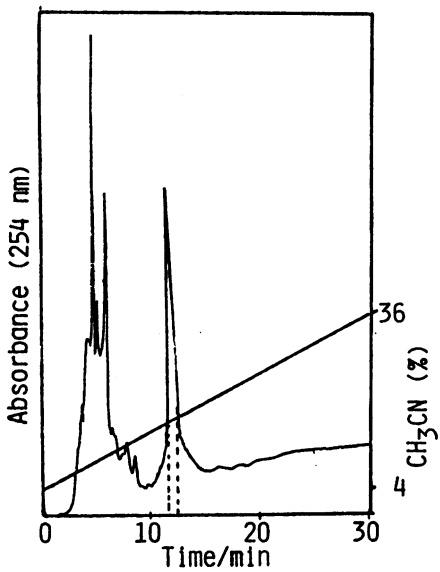


Fig. 2 Purification of crude AGUAUAAGAGGAACAAUG by the reverse phase C-18 HPLC.

AGUAUAAGAGGACAUAUG, was performed with an automatic synthesizer (Biosearch-SOME ONE) using 1 μmol of guanosine-CPG. The synthetic cycle is indicated in Scheme 3 and Table 2. The extent of coupling in each cycle was monitored by the spectrophotometric assay of the DMTr cation. The time required for each elongation cycle was 15 min, and the average coupling yield was ca. 95%. After the chain assembly was

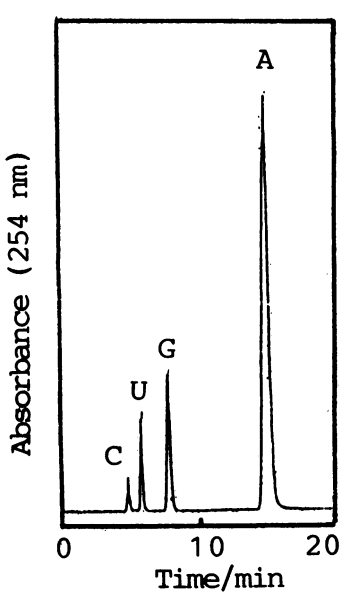


Fig.3 HPLC separation of four common ribonucleosides after digestion of AGUAUAAGAGGAACAAUG with snake venom phosphodiesterase and alkaline phosphatase. Elution was carried out with 5% CH_3CN in 0.1 M TEAA (pH 7.0).

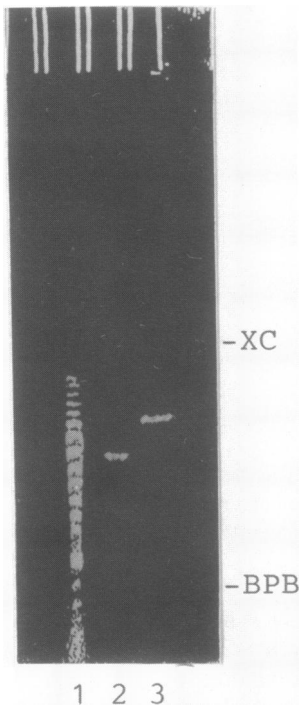


Fig. 4 20% Polyacrylamide gel electrophoresis in 7 M urea of 18-mer. Lane 1: oligo-U chain length standard; Lane 2: U_{16} chain length standard; Lane 3: UAAGAGGAACAAUG.

complete, the resulting H-phosphonate oligomer was oxidized to the corresponding oligoribonucleotide by reaction with 0.1 M I_2 in THF-pyridine-H_2O (44:3:3, v/v). Then the resin was treated with conc. aqueous ammonia at 55°C for 12 h to remove *N*-acyl protecting groups, and to release the nucleotide material from the resin. The partially-protected oligoribonucleotide was fractionated by chromatography on a reverse phase silica gel which was eluted with a linear gradient of CH_3CN in 0.1 M triethylammonium acetate (pH 7.0) (Fig. 1). The desired product was then treated with 0.01 N HCl (pH 2.0) at room temperature for 36 h. Finally, the octadecamer, AGUAUAAGAGGACAUAUG, was purified by reverse-phase HPLC (Fig. 2). The main peak was partitioned and 1.8 0D A_{260} unit was obtained by injecting one-ten of crude product. The overall yield from guanosine-loaded CPG was ca. 7.9%. The ratio of the constituent nucleosides was determined by reverse-phase HPLC, following digestion of the unblocked oligomer with snake venom phosphodiesterase and alkaline phosphatase and was found to agree with the calculated value (Fig. 3). The chain-length of the synthetic octadecamer was confirmed by 20% polyacrylamide gel electrophoresis in the presence of 7 M urea (Fig. 4).

It may be concluded that the ribonucleoside derivatives with Ctmp and DMTr groups on the 2′- and 5′-hydroxyl functions, respectively, can be used very effectively in the synthesis of oligoribonucleotides by the H-phosphonate approach, in an automatic synthesizer. Further, the new phosphitylating reagent (*1*) has proved to be very effective in the preparation of ribonucleoside 3′-H-phosphonate units. No side-reactions were observed.

EXPERIMENTAL

General methods

N-Acyl-5′-*O*-dimethoxytrityl-2′-*O*-{1-[(2-chloro-4-methoxy)-phenyl]--4-methoxypiperidin-4-yl} nucleosides (*2a-d*) were prepared as described previously [12].

Thin layer chromatography (TLC) was carried out on Merck Kieselgel $60F_{254}$ plates which were developed in system A (CH_2Cl_2-MeOH, 9:1, v/v). Reverse phase TLC, was carried out on Merck silanized silica gel; [RP-8F $60F_{254}$] plates with a mixture of acetone and 0.02 M triethylammonium acetate (TEAA) (6:4, v/v) as the eluting agent. Column chromatography was carried out on silica gel(BW-300; Fuji Davison Co. Ltd.) and alkylated silica gel (C-18, Waters Associates Inc.).

The chain elongation steps were carried out in a Biosearch-SAM ONE synthesizer using a CPG column containing 1 μmol of partially-protected guanosine.

^{1}H-NMR spectra were recorded on a JEOL JNMPS 100 spectrometer with TMS as an internal standard. Ultraviolet spectra were recorded on a Shimazu UV-160 spectrometer.

Reverse phase HPLC was performed on a Shimazu LC-6A system using a TSK gel oligo-DNA RP, with a linear gradient of CH_3CN in 0.1 M TEAA (pH 7.0).

Snake venom phosphodiesterase and alkaline phosphatase were purchased from Boehringer Mannheim.

Tris-(1,1,1,3,3,3-hexafluoro-2-propyl)phosphite (1)

To a solution of phosphorus trichloride (10.05 ml, 100 mmol) and triethylamine (44.10 ml, 316.7 mmol) in dry THF (73 ml) at −20°C, 1,1,1,3,3,3-hexafluoro-2-propanol (33.30 ml, 316.7 mmol) was added. The mixture was allowed to warm up to room temperature, and was stirred for an additional 5 h. Petroleum ether (50 ml) was then added. The products were kept overnight at 4°C and were then filtered. The filtrate was concentrated, and the residue was distilled under reduced pressure. The main fraction (48.6 g, 73%) was obtained as a colorless liquid: b.p. 60°C/25 mmHg (lit. (21) 87°C/47 mmHg); ^{31}P-NMR ($CDCl_3$, 85% H_3PO_4) δ 141.09. ^{1}H-NMR ($CDCl_3$) δ 4.80 (dh, $J_{H,P}$=9.5 Hz, $J_{H,F}$=5.1Hz).

Preparation of nucleoside 3′-H-phosphonates (3)

After coevaporation with dry pyridine, N^6-benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-{1-[(chloro-4-methyl)phenyl]-4-methoxypi-peridin-4-yl}adenosine (*2a*) (2.109 g, 2.31 mmol) was dissolved in dry pyridine (16 ml) and tris-(1,1,1,3,3,3-hexafluoro-2-propyl) phosphite (*1*) (1.38 ml, 4.62 mmol) was added. The reaction was complete in 10 min, and a mixture of 1 M triethylammomium bicarbonate (TEAB) and triethylamine (50:1, v/v) was added to the reaction mixture. After 30 min, the product was extracted with CH_2Cl_2 (2×50 ml), washed with 1 M TEAB and dried (Na_2SO_4). The CH_2Cl_2 layer was evaporated and the residue was applied to a silica gel column and eluted with a stepwise gradient of MeOH (0−3%) in CH_2Cl_2 containing triethylamine (2%). The appropriate fractions were pooled, washed with 1 M TEAB and dried (Na_2SO_4). The CH_2Cl_2 layer was evaporated *in vacuo* to give the corresponding H-phosphonate unit (*3a*) (2,23 g, 90%).

The other nucleoside 3′-H-phosphonates (*3b−d*) were similarly obtained in good yields (Table 1).

Preparation of nucleoside resin

N^2-Isobutyryl-5′-*O*-dimethoxytrityl-2′(3′)-*O*-succinoyl-guanosine (180 mg, 0.2 mmol), prepared according to published procedure (7), was treated (24) with alkylamino-CPG (500 mg) and DCC (206 mg, 1 mmol) in the presence of 4-(dimethylamino)-pyridine (10 mg, 0.1 mmol) in dry DMF (1 ml). After the mixture had been shaken at 30°C for 72

h, the resin was filtered off and washed with DMF (10 ml), pyridine (10 ml), MeOH (10 ml), and ether (10 ml). The unreacted amino groups were capped by treatment with acetic anhydride-pyridine (1:9, v/v) in the presence of a catalytic amount of 4-(dimethylamino)pyridine for 30 min. The resin was filtered off and washed with pyridine (15 ml) and ether (10 ml). The quantity of guanosine loaded on CPG was estimated to be 24 μmol/g by measuring the DMTr cation released.

Oligoribonucleotide synthesis

The oligoribonucleotide was synthesized in an automatic synthesizer using protected guanosine-loaded CPG (41.7 mg, 1 μmol) and the synthetic cycle indicated in Table 2. After the synthesis, the resin was treated with 0.1 M I_2 in THF-pyridine-H_2O (44:3:3, v/v) (1.5 ml) at room temperature for 30 min. The resin was washed with pyridine, CH_2Cl_2, ether and then treated with concentrated ammonia (2 ml) at 55°C for 12 h. The resin was filered off and washed with H_2O (1 ml). The filtrate was evaporated and the residue was applied to a reverse phase C-18 column (0.5×10 cm). Elution was performed with a linear gradient of CH_3CN (20−80%) in 0.1 M TEAA (Fig. 1). The appropriate fractions were pooled and evaporated *in vacuo*. The residue was dissolved in 0.01 N HCl (5 ml) and the pH was adjusted to 2.0 by addition of 0.1 N HCl. The mixture was stirred at room temperature for 36 h, neutralized with dil. ammonia, and passed through a Sephadex G-25 column for desalting. Elution was performed with 0.1 M TEAB buffer (pH 7.0). The appropriate fractions were collected and evaporated. The residue was purified by reverse phase HPLC. Elution was performed with a linear gradient of CH_3CN (4−36 %) in 0.1 M TEAA for 20 min at a flow rate of 1.0 ml/min (Fig. 2). The ratio of nucleosides were estimated by the reverse phase HPLC after digestion of AGUAUAAGAGGAACAAUG with snake venom phosphodiesterase and alkaline phosphatase and found to be C:A:G:U=1.00:8.35.4.88:3.81(theoretical, 1:8:5:4). The chain length of the latter purified material was established by electrophoresis on 20% polyacrylamide gel (Fig 4.).

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