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1,1,1,3,3,3-HEXAFLUORO-2-PROPYL GROUP AS A NEW PHOSPHATE PROTECTING GROUP FOR OLIGORIBONUCLEOTIDE SYNTHESIS IN THE PHOSPHOTRIESTER APPROACH

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Abstract: The 1,1,1,3,3,3-Hexafluoro-2-propyl group can be used as a new class of phosphate protecting group for the protecting group of internucleotidic bonds in the oligonucleotide synthesis by the phosphotriester approach. This protecting group is removed easily by treatment with 0.3 M N^1,N^1,N^3,N^3-tetramethylguanidinium syn-2-pyridinealdoximate in pyridine-water (9:1, v/v). The butylthiocarbonyl group was chosen for the protecting group of the O^6-amide and N^2-amino functions of guanosine and the N^3-imide group of uridine. The fully protected monomer unit (10) was prepared by the reaction of the phosphorylating agent (1) with 7a. These monomer units have successfully been utilized for the synthesis of UGUCGGUC, the box 9R sequence of r-RNA precursor of Tetrahymena.

In recent years, the oligonucleotide synthesis has markedly been facilitated by introduction of the phosphoramidite[1] and H-phosphonate[2] approaches on solid supports. However, when oligonucleotides is required in relatively large quantity, the phosphotriester approach is more effective than the phosphoramidite and H-phosphonate approaches.

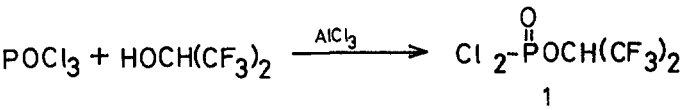
Recently, we have described a new phosphate protecting group, 1,1,1,3,3,3-hexafluoro-2-propyl (HFP) group, for the synthesis of deoxyribonucleotides by the phosphoramidite approach.[3] In this paper, we wish to report the synthesis of oligoribonucleotides in the phosphotriester approach using the HFP group as a phosphate protecting group for the internucleotidic bonds.

RESULTS AND DISCUSSION

A new phosphorylating agent

A number of phosphate protecting groups have been proposed for the synthesis of oligonucleotide.[4] The 2-chlorophenyl[5] and 4-chlorophenyl[6] groups as phosphate protecting groups have been used frequently in the phosphotriester approach. These groups could be removed selectively from internucleotidic bonds by treatment with oximate ions[7] without cleavage of internucleotidic bonds. Recently, we have tried to apply a new phosphorylating agent, 1,1,1,3,3,3-hexafluoro-2-propyl phosphorodichloridate (1) bearing the HFP group as the phosphate protecting group to the syn-

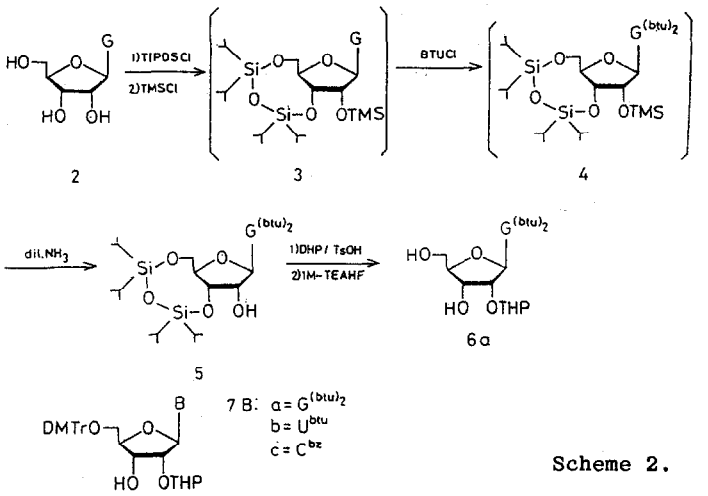
thesis of oligoribonucleotides. This agent 1 is easily prepared in good yield by treatment of 1,1,1,3,3,3-hexafluoro-2-propanol with $POCl_3$ in the presence of $AlCl_3$ according to the published procedure[8] (Scheme 1). By the use of 1, the new ribonucleotide units were prepared and applied to the oligoribonucleotide synthesis.



Scheme 1.

Synthesis of ribomononucleotide units

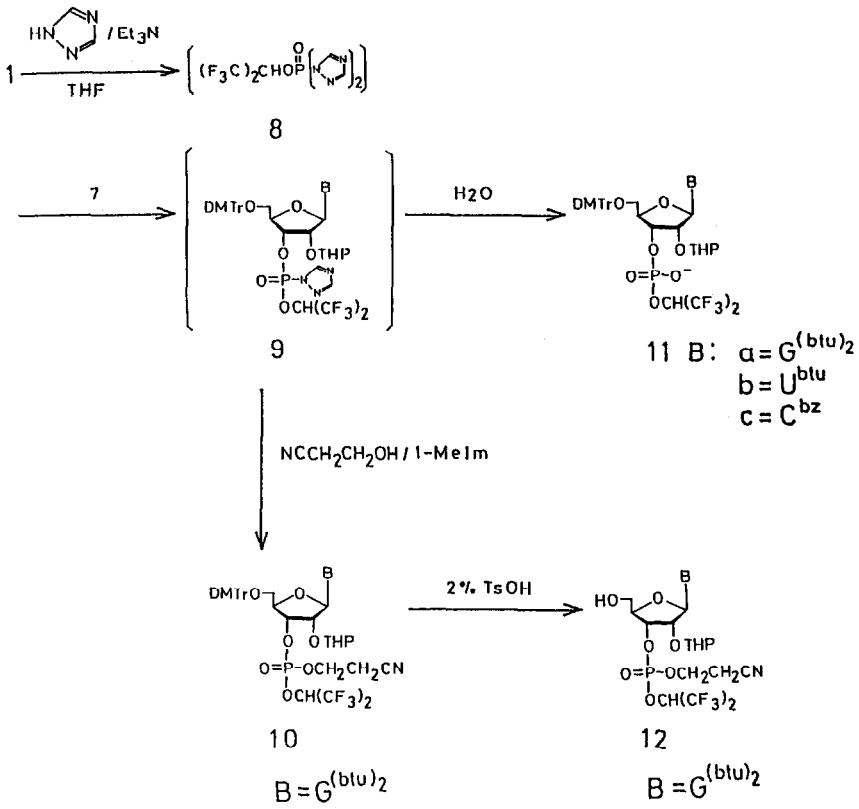
The phosphodiester derivatives (11a-c) and 5'-hydroxyl nucleoside-3'-phosphotriester derivative (12) are the key intermediates in the oligoribonucleotide synthesis by the phosphotriester approach. The benzoyl and (butylthio)carbonyl groups were used for protecting groups of cytidine and uridine[9], respectively. Further, the (butylthio)carbonyl group was also used for protection of the O^6-amide and N^2-amino groups of guanosine.[10] It is now found that O^6,N^2-bis-(butylthio)-carbonyl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)guanosine (5) as a key



Scheme 2.

intermediate in the oligoribonucleotide synthesis is prepared easily by a one-pot reaction from guanosine than the procedure described previously[10]. Guanosine derivative (5) was prepared as follows: Guanosine was first treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($TIPDSCl_2$) in the presence of imidazole in DMF and then trimethylchlorosilane (TMSCl) was added. After 15 min, the mixture was treated with (butylthio)carbonyl chloride (BTCCl) and diisopropylethylamine for 24 h. The mixture was quenched with dil. ammonia. Treatment of 4 with dil ammonia over 3 h afforded de(butylthio)carbonylated guanosine as a side reaction

product in 15% yield. The product 5[11] was obtained in 78% yield from guanosine after separation by silica gel column chromatography.
The 5'-hydroxyl group was protected with the dimethoxytrityl (DMTr) group.



Scheme 3.

Phosphorylation of nucleoside derivatives (7) was carried out as follows: The phosphorylating agent 1 was treated with 1,2,4-triazole in THF for 1 h to give 8. Nucleoside derivatives (7) were added and the mixture was stirred for 1 h, followed by treatment with β-cyanoethyl alcohol and 1-methylimidazole as a catalyst[12]. After the usual workup, chromatography afforded the phosphotriester unit (10)(89%). On the other hand, the intermediates (9) were treated with triethylamine-water to give the corresponding phosphodiester derivatives (11a-c) in good yields.[13] The phosphodiester components were used in the next coupling reaction without further purification.

Synthesis of oligoribonucleotide

To prepare 5'-terminal hydroxyl components, 2% TsOH in CH_2Cl_2-MeOH (7:3, v/v) was used for removal of the DMTr group from 10. The 5'-terminal hydroxyl components were isolated as pure solids by precipitation and then were used for the next coupling reactions without further purification. The time of complete de-

blocking depended on the chain length of oligomers.[14] The selective removal of the β-cyanoethyl group from the fully protected dimer and trimer could be achieved by using triethylamine in CH_3CN-H_2O[15] to give the 3'-phosphodiester components in good yields which were used in the next coupling reaction without further purification. The HFP group was found to be stable under these operations.

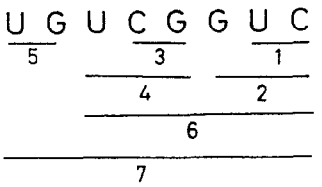


Fig. 1 Strategy for the synthesis of the octaribonucleotide.

Table I. The conditions and results of fragment condensations for the synthesis of the fully protected oligoribonucleotide

3'-Diester compd. (equiv.)	CH_3CN-Et_3N-H_2O(3:2:1) Time/min	5'-OH compd.	QSCl---MeIm equiv.	MeIm equiv.	Time (h)	Frag. No.	Yield (%)	Removal of DMTr group with 2% TsOH at 0 °C Time/min	Yield (%)
Up (1.5)		C	3.0	6.0	1	1	80		
Gp (1.5)		UC	3.0	6.0	1	2	82	15	92
Cp (1.5)		Gp	3.2	6.4	3	3	74	10	86
Up (1.5)		CGp	3.0	6.0	3	4	72		
Up (1.5)		Gp	3.0	6.0	1	5	73		
UCGp (2.0)	25	GUCp	4.5	9.0	5	6	77	19	90
UGp (2.0)	30	UCGGUC	4.5	9.0	3	7	75		

The 5'-terminal hydroxyl and 3'-phosphodiester components (12 and 11) thus obtained were applied to the synthesis of UGUCGGUC, the box 9R sequence of r-RNA precursor of Tetrahymena[16] by fragment condensation method as shown in Fig. 1. Condensation of 11 and 12 was performed by using 8-quinolinesulfonyl chloride (QSCl) and 1-methylimidazole (MeIm). The conditions and results of the fragment condensations are summarized in Table I. As the oligomer chain was elongated, the rate of coupling reaction became slower and the yield became slightly lower. However, TLC analysis showed that all the condensations were completed under the conditions described above and independent of the chain length.

Deprotection of oligomers

In order to study of possible occurrence of both internucleotidic cleavage and

phosphoryl migration under removal conditions, 5'-DMTr(but)Utp(HFP)bzC(OBz)$_2$ was treated with 0.3 M N^1,N^1,N^3,N^3-tetramethylguanidinium syn-2-pyridinealdoximate (in 90% aqueous pyridine) at room temperature for 8 h, followed by conc. ammonia at 60 °C for 2 h and 0.01 N HCl (pH 2.0) at room temperature for 6 h. The unblocked UpC is analyzed by reversed phase HPLC as shown in Fig. 2. Integration of the main peak in Fig. 2a reveals that the dimer, UpC account for 96% of the total absorbance at 254 nm. Therefore, we did not observe the peak of uridylyl (2'-5') cytidine as a side product. The results thus indicate that the HFP group

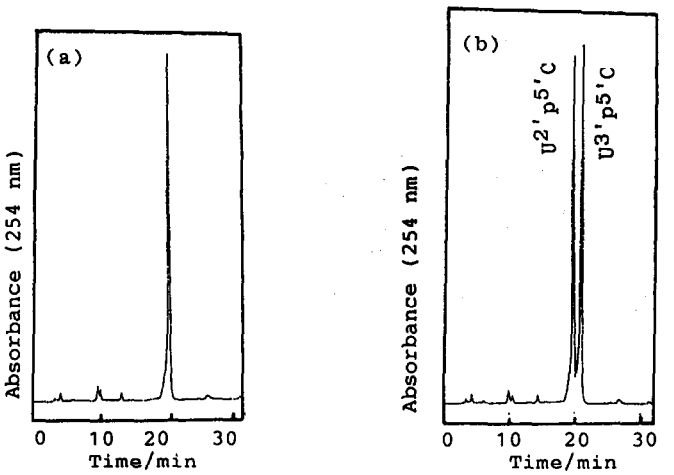


Fig. 2. HPLC analysis of the crude mixture containing $U^{3'}p^{5'}C$ after deprotection (a), using a TSK gel oligo-DNA RP column with a linear gradient of 50% aqeous methanol (from 0 to 50% during 30 min) in 0.05 M ammonium phosphate (pH 7.0), and after addition of $U^{2'}p^{5'}C$ (b), using the same gradient.

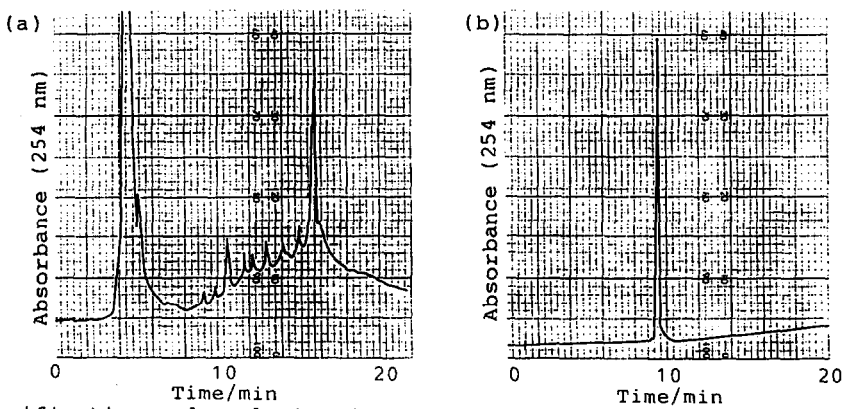


Fig. 3. Purification and analysis of the 8 mer.
(a) The 8 mer was purified by anion-exchange HPLC using a TSK gel DEAE-2SW column with a linear gradient of ammonium formate (from 0.1 M to 0.9 M during 30 min) in 20% aqueous acetonitrile. (b) The purified 8 mer was analyzed by reversed phase HPLC using a TSK gel oligo-DNA RP column with a linear gradient of acetonitrile (from 5 to 11% during 20 min) in 0.1 M TEAA (pH 7.0).

is likely to be suitable for the phosphate protecting group in the synthesis of oligoribonucleotides by both the phosphoramidite and phosphotriester approaches.

The fully protected oligomer was treated with 0.3 M N^1,N^1,N^3,N^3-tetramethylguanidinium syn-2-pyridinealdoximate (in 90% aqueous pyridine) at room temperature for 24 h and then with conc. ammonia at 60 °C for 6 h to remove the HFP group and acyl groups. Finally, the DMTr and THP groups were deblocked by treatment with 0.01 N HCl at room temperature for 24 h. The unblocked oligomer was purified by TSK-gel DEAE-2SW (Fig. 3a). The main peak was found to be homogeneous by the reversed phase C-18 HPLC (Fig. 3b) and by electrophoresis (Fig. 4). The ratio of nucleoside components was determined by reversed phase C-18 HPLC following digestion of the unblocked oligomer with snake venom phosphodiesterase and alkaline phosphatase (Fig. 5) and was found to agree with the correct ratios (3U:3G:2C).

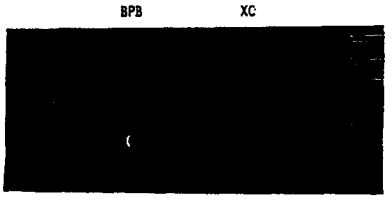


Fig. 4. Electrophoresis on 20% polyacrylamide gel of UGUCGGUC, synthesized by present phosphotriester approach.

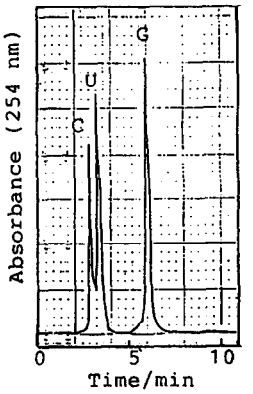


Fig. 5 Analysis of the products obtained after hydrolysis of UGUCGGUC with snake venom phosphodiesterase and alkaline phosphatase on a TSK gel oligo-DNA RP column. Elution was performed with 2% acetonitrile in 0.1 M TEAA (pH 7.0). The flow rate was 1.0 ml/min.

It should be concluded that a new phosphorylating agent, 1,1,1,3,3,3-hexafluoro-2-propyl phosphorodichloridate is obtained easily in good yield by distillation under lower vacuum than other phosphorylating agents. This agent can be used for the preparation of ribomononucleotide units as key intermediates in the synthesis of oligoribonucleotides by the phosphotriester approach. Further, the 1,1,1,3,3,3-hexafluoro-2-propyl group would provide promising phosphate protecting group for the synthesis of oligoribonucleotides by both the phosphoramidite and phosphotriester approaches.

EXPERIMENTAL

N-Acyl-5'-O-dimethoxytrityl-2'-O-tetrahydropyrapyl nucleosides (7a-c) were prepared as described previously[10,14].

Thin layer chromatography (TLC) was performed on Kieselgel $60F_{254}$ plates (Merck) with solvent A (CH_2Cl_2-MeOH, 9:1, v/v) and solvent B (CH_2Cl_2-MeOH, 95:5,

v/v). For reversed phase thin layer chromatography (RTLC) using silanized silica gel, RP-8 F $60F_{254}$ (Merck) was performed with a mixture of acetone and 0.02 M triethylammonium acetate (TEAA) (6:4, v/v). Column chromatography was performed with silica gel (BW-300; Fuji Davison Co. Ltd.).

^{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.

Reversed phase HPLC was performed on a Shimazu LC-6A system using a TSKgel oligo-DNA RP using the following solvent systems: System A: a linear gradient of CH_3CN in 0.1 M TEAA (pH 7.0); System B: a linear gradient of MeOH-H_2O (1:1, v/v, 0-50%) in 0.05 M ammonium phosphate (pH 7.0). Anion exchange column chromatography was performed on TSKgel DEAE-2SW with a linear gradient of ammonium formate (from 0.1 M to 1.5 M) in 20% CH_3CN.

Snake venom phosphodiesterase and alkaline phosphatase were purchased from Boehringer Mannheim. Uridylyl (2'-5') cytidine was purchased from Sigma Chem. Co..

1,1,1,3,3,3-Hexafluoro-2-propyl phosphorodichloridate (1)

A mixture of 1,1,1,3,3,3-hexafluoro-2-propanol (10.52 ml, 100 mmol) ,$POCl_3$ (25 ml, 268 mmol), and $AlCl_3$ (266 mg) was heated under reflux for 6 h. It was concentrated and the residue was distilled under reduced pressure. The main fraction (22.8 g, 81%) was obtained as a colorless liquid: bp 35 °C/15 mmHg; ^{31}P-NMR ($CDCl_3$) δ 5.34 ppm; ^{1}H-NMR ($CDCl_3$) δ 6.03-5.29 (m, 1H, $CH(CF)_3$).

O^6,N^2-Bis(butylthio)carbonyl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-guanosine (5)

Guanosine (2) (2.83 g, 10 mmol) was treated with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (3.5 ml, 11 mmol) in the presence of imidazole (2.72 g, 40 mmol) in DMF-pyridine (5:1, v/v, 50 ml). After 6 h, the solvent was removed by evaporation and the residue was dissolved in pyridine (50 ml), and then TMSCl (2.5 ml, 20 mmol) was added. After 15 min, the mixture was treated with (buthylthio)-carbonyl chloride (BTCCl) (8.4 ml, 60 mmol) and diisopropylethylamine (10.45 ml, 60 mmol) for 24 h. The mixture was quenched with 0.1 N ammonia. The product was extracted with CH_2Cl_2 (100 ml X 2), washed with 5% $NaHCO_3$ and dried over Na_2SO_4. The CH_2Cl_2 layer was evaporated and the residue was applied to a column of silica gel and eluted with a stepwise gradient of MeOH (0-2%) in CH_2Cl_2. The appropriate fractions were pooled and evaporated in vacuo. The product 5 was obtained in 78% (5.83 g) yield. UV λmax (MeOH): 280 (sh), 258 nm, λmin (MeOH): 235 nm. ^{1}H-NMR ($CDCl_3$) δ 8.50 (s, 1H, H-8), 6.21 (d, 1H, $J_{1',2'}$=6 Hz, H-1'), 4.61 (m, 1H, H-2'), 4.39 (m, 1H, H-3'), 4.21 (m, 1H, H-4'), 3.45 (brs, 2H, H-5'), 3.05 (brs, 4H, SCH_2), 2.01-1.09 (m, 42 H, CH_2, $(CH_3)_2CHSi$). Found: C, 50.81; H, 7.45; N, 9.18; $C_{32}H_{55}N_5O_8S_2Si_2$ requires: C, 50.64; H, 7.31; N, 9.22.

The compound 6a was obtained in 82% yield from the reaction of 5 and 2,3-dihydropyran[14].

Phosphorylation of the partially protected ribonucleosides (7)

1,1,1,3,3,3-Hexafluoro-2-propyl phosphorodichloridate (1) (1.14 g, 3.99 mmol) was added to a dry THF (11 ml) solution of 1,2,4-triazole (0.76 g, 10 mmol) and Et_3N (1.61 ml, 11 mmol) at 0 °C. The reaction mixture was stirred at 22 °C for 45 min. The precipitated triethylammonium hydrochloride was removed by filtration and the filtrate was concentrated (to 7 ml) under reduced pressure. To this solution was added nucleoside derivatives (7) (2.0 mmol) and the mixture was stirred for 1.5 h. At this stage, two different workup procedures led to the formation of either 10 (method a) or 11a-c (method b). Method a: β-Cyanoethanol (1.35 ml, 19.9 mmol) and 1-methylimidazole (MeIm) (1.7 ml, 21 mmol) were added to solution and the mixture was further stirred for 2 h. The solution was extracted with CH_2Cl_2 (10 ml X 2) and washed with H_2O, dried over Na_2SO_4. The solution was evaporated and the residue was flash-chromatographed on silica gel (eluted with a stepwise gradient of MeOH (0-3%) in CH_2Cl_2) to give 10 (1.97 g, 83%) as a white solid; UV λmax (MeOH): 280 (sh), 274 nm, λmin (MeOH): 232 nm. ^{1}H-NMR ($CDCl_3$) δ 8.65-7.40 (m, 14H, Ar-H, H-8), 6.70 (d, 1H, $J_{1',2'}$-6 Hz, H-1'), 6.43-4.20 (m, 7H, H-2', H-3', H-4', $CH(CF_3)_2$, acetal of THP, $POCH_2$), 4.01 (s, 6H, CH_3O), 3.51-1.53 (m, 24H, H-5', O-methylene and C-methylene of THP, $(CH_2)_3CH_3$, CH_2CN), 1.03 (brs, 6H, CH_3). Found: C, 54.59; H, 5.41; N, 5.68; $C_{52}H_{59}N_5O_{13}PS_2F_6 \cdot 1/2C_6H_{14}$ requires: C, 54.48; H, 5.34; N, 5.76.

Method (b): Pyridine-1 M TEAB (1:1, v/v) was added to the solution and the mixture was stirred for 10 min. The mixture was extracted with CH_2Cl_2 (10 ml X 2) and washed with 0.1 M TEAB, dried over Na_2SO_4 and evaporated. The residue was dissolved in a minimum volume of CH_2Cl_2 and precipitated by dropwise addition into n-hexane to give the corresponding phosphodiesters (11a-c) (81-85%) as white solids.

11a: ^{31}P-NMR ($CDCl_3$, 85% H_3PO_4) δ -2.98; 11b: ^{31}P-NMR ($CDCl_3$, 85% H_3PO_4) δ -3.09; 11c: ^{31}P-NMR ($CDCl_3$, 85% H_3PO_4) δ -3.01.

General procedure for detritylation from the fully protected oligoribonucleotides

The fully protected oligoribonucleotides were dissolved in 2% TsOH in a mixture of CH_2Cl_2 and MeOH (7:3, v/v) at 0 °C. The solution was kept at 0 °C for the time as listed in Table I. It was neutralized with a mixture of pyridine and water (1:1, v/v) and extracted several times with CH_2Cl_2. The CH_2Cl_2 extracts was washed with water, dried over Na_2SO_4, and evaporated. The residue was precipitated by dropwise addition into a mixture of n-hexane and ether (9:1, v/v), which was used as 5'-hydroxyl components in the subsequent condensation without further purification.

General procedure for the synthesis of fully protected oligoribonucleotides

An appropriate fully protected ribonucleotide was dissolved in a mixture of CH_3CN-Et_3N-H_2O (3:1:1, v/v). The reaction was checked by TLC analysis in the solvent A. The solution was concentrated under reduced pressure, dried by evaporation with several portions of dry pyridine. The residue was treated with a 5'-hydroxyl component in the presence of 8-quinolinesulfonyl chloride (QSCl)[17] and N-methylimidazole (MeIm)[18] in dry pyridine. The mixture was allowed to stand

until the reaction was completed. The 0.05 M TEAB was added and the product was extracted with CH_2Cl_2 and then dried over Na_2SO_4. The CH_2Cl_2 extracts was evaporated and the residue was applied to a column of silica gel. The appropriate fractions (eluted with a stepwise gradient of MeOH in CH_2Cl_2) were collected and evaporated to give the coupling product. The results are summarized in Table I.

Deprotection of the fully protected oligomer

The dimer, DMTr(but)Utp(HFP)bzC(OBz)$_2$ (1.5 μmol, 2.34 mg) was dissolved in 450 μL of 0.3 M N^1,N^1,N^3,N^3-tetramethylguanidnium syn-2-pyridinealdoximate in pyridine-water (9:1, v/v). The resulting homogeneous solution was allowed to stand at room temperature for 8 h and then evaporated under pressure. The residue was dissolved in conc. ammonia (2 ml) and the flask was kept at 60 °C for 2 h. The solution was concentrated and chromatographed on Dewex 50W-X2 (pyridnium form) with 50% aqueous pyridine. The solution was evaporated in vacuo and the residue was dissolved in 0.01 N HCl (2 ml) and the pH was lowered to 2.0 by the addition of 0.1 N HCl. After the reactants have been allowed to stand at room temperature for 6 h, dil ammonia was added until the pH 6.7. The product was then washed with ether and analyzed by reversed phase HPLC (Fig. 2).

The octamer (2.25 μM, 16.2 mg) was dissolved in 900 μL of 0.3 M N^1,N^1,N^3,N^3-tetramethylguanidinium syn-2-pyridinealdoximate in pyridine-water (9:1, v/). The mixture was kept at room temperature for 24 h. Then conc. ammonia (5 ml) was added and the flask was kept at 60 °C for 6 h. The mixture was cooled and applied to Dowex 50W-X2 (pyridinium form). The column was washed with 50% pyridine (50 ml). The solution was concentrated to ca. 20 ml and washed with ethyl acetate. The solution was evaporated in vacuo and the residue was dissolved in 0.01 N HCl (10 ml), then the solution was adjusted to pH 2.0 by addition of 0.1 N HCl and kept at room temperature for 24 h. The mixture was neutralized with dil ammonia, and applied to a Sephadex G-10 column (1 X 35 cm) for desalting. The product was eluted with 0.1 M TEAB buffer and purified by TSKgel DEAE-2SW (Fig. 3a). The main peak in Fig. 3a was partitioned and 9.2 OD (47%) was obtained by injecting one-ten of crude product. The purified octamer was checked by the reversed phase HPLC (Fig. 3b) and by gel electrophoresis (Fig. 4). The nucleoside components was analyzed by the reversed phase HPLC after hydrolysis of UGUCGGUC with snake venom phosphodiesterase and alkaline phosphatase and was found to be C:U:G=2.00:2.98:3.04 (theoretical, 2:3:3).

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