New and Convenient Protection System for Pseudouridine, highly Suitable for Solid-Phase Oligoribonucleotide Synthesis

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The pivaloyloxymethyl (Pom) group has been used simultaneously to protect both nitrogens in the uracil ring system of pseudouridine. This enabled the synthesis of a 2'-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-protected (Fpmp protected) pseudouridine building block suitable for solid-phase oligoribonucleotide synthesis by the phosphoramidite method as well as a useful 2'-*O*-methylpseudouridine building block. Both compounds were incorporated in high yield into synthetic oligonucleotides, the Pom groups being cleaved during an extended deblock with ammonia.

The *C*-nucleoside pseudouridine (Ψ) or 5-(β-D-ribofuranosyl)-uracil is an important constituent of transfer RNAs (tRNAs) and small nuclear ribonucleic acids (snRNAs). Our interests in RNA processing and in the structure and function of the small nuclear ribonucleoprotein particles (snRNPs) particularly as studied by 2'-*O*-alkyloligoribonucleotide antisense technology[1-8] prompted our interest in pseudouridine- and 2'-*O*-methylpseudouridine-containing polymers.

The U1, U2, U4/U6 and U5 snRNPs are localised in the nucleoplasm and are involved in the maturation of pre-messenger RNAs, as essential components of the spliceosome. Human U2 snRNA contains 13 pseudouridines as well as ten 2'-*O*-methylribonucleosides[9] and the 116-residue-long human U5 snRNA contains three pseudouridines and five 2'-*O*-methylribonucleosides, of which two pseudouridines and three 2'-*O*-methylribonucleosides are located in the short C loop.[10]

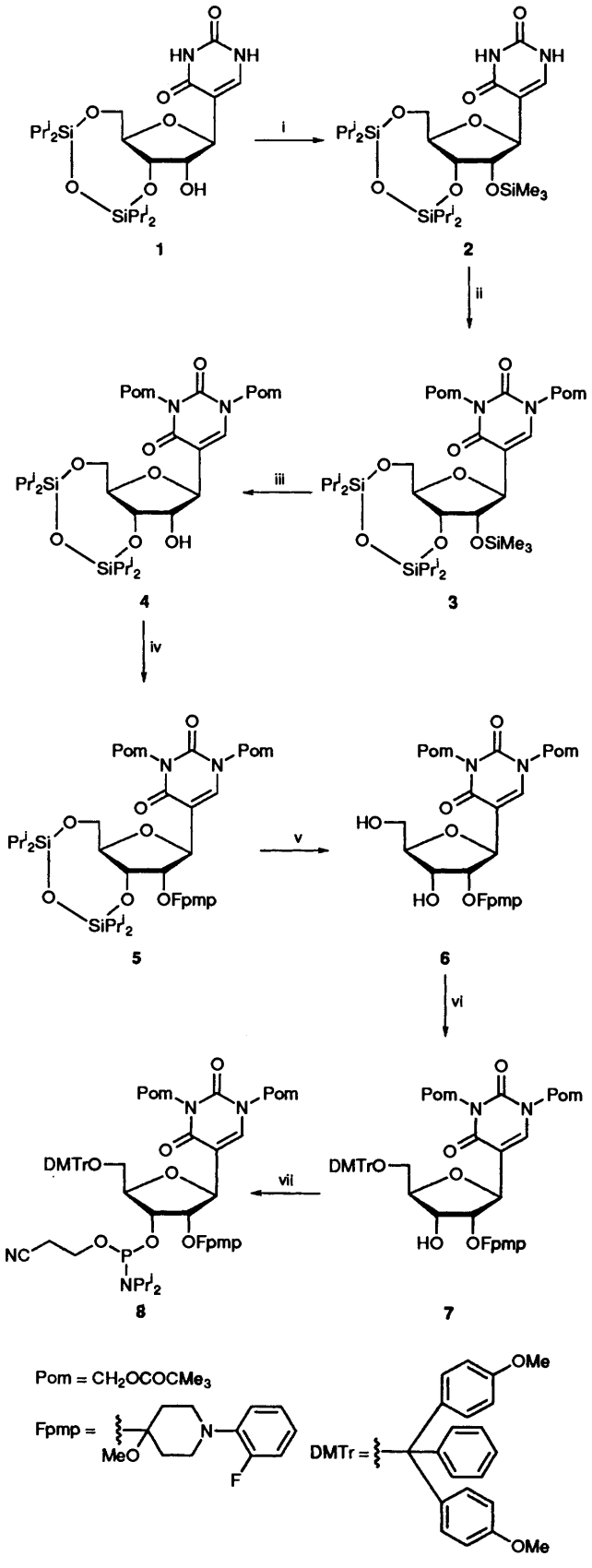
There is a single report in the literature mentioning the chemical synthesis of 2'-*O*-methylpseudouridine, which was prepared by methylation of the free nucleoside with diazomethane in the presence of tin(II) chloride in methanol.[11] This analogue occurs in RNA in nature in trace amounts[12] and has to our knowledge never been incorporated into oligonucleotides. Few workers have reported polynucleotide synthesis incorporating pseudouridine. In 1983 Ohtsuka *et al.* reported the synthesis of TΨC in 8% yield using phosphodiester chemistry in solution; moreover, the nucleobase moiety of the pseudouridine was protected with a 1-*N*-benzoyl group.[13] Recently, Reese and co-workers performed a synthesis of the 3'-terminal half of yeast $tRNA^{ala}$, which contains a single pseudouridine, using solution phosphotriester chemistry.[14] It was found that side reactions involving 2-*N*-benzoylguanine and uracil residues occurred during coupling in the phosphotriester method[15] and it can be expected that modifications will occur on the lactam functions of uridine, guanosine, inosine and pseudouridine (pK_as of the heterocyclic bases are 9.2, 9.2, 8.8 and 8.9, respectively) during the synthesis of long oligoribonucleotides by the phosphoramidite method. Such problems are avoided by the use of extra protecting groups which also have the advantage of increasing the lipophilicity of the various compounds, thus improving solubility and easing purification. During the course of our work Hall and McLaughlin[16] and Gasparutto *et al.*[17] reported the sysnthesis and use of 2'-*O*-(*tert*-butyldimethylsilyl) (TBDMS)-protected pseudouridine 3'-*O*-phosphoramidite building blocks with no additional base protection. The latter group used their monomer in the elegant solid-phase synthesis of an *E. coli* $tRNA^{ala}$ containing its minor bases.[17]

Reese *et al.*[14] protected the N-1 of pseudouridine with the 4-bromobenzenesulfonyl (brosyl) group. However, this group is not cleaved by ammonolysis and must be removed by oximate.[18] Just as our work was largely complete, an alternative protecting-group strategy for the solid-phase synthesis of oligoribonucleotides was published by Bergmann and Pfleiderer.[19] The new strategy uses the base-labile 2-dansylethoxycarbonyl group for protection of the 5'-hydroxy function and the acid-labile 4-methoxytetrahydropyran-4-yl group for protection of the 2'-hydroxy function, and the β-eliminating 2-(4-nitrophenyl)ethyl and 2-(4-nitrophenyl)ethoxycarbonyl groups for protection of the lactam and exocyclic amino functions of the nucleobases, respectively. The authors prepared a 2-*O*,4-*O*-bis[2-(4-nitrophenyl)ethyl]-protected pseudouridine 3'-*O*-phosphoramidite; however, the overall yield was only 13% based on pseudouridine. When the aglycone was left unprotected the yield of phosphoramidite monomer was 16.6% and several problems were encountered with side reactions during synthesis of the monomer.[19]

For compatibility with standard β-cyanoethyl phosphoramidite chemistry we required a protecting group that could be cleaved by ammonia. The ammonia-labile Pom protecting group[20] has been used by Reese for protection of the N-1 of inosine[14] and by Sekine and Hata[21] for protection of the N-3 of uridine. In the latter case, reaction of 3',5'-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-(tetrahydropyran-2-yl)uridine with sodium hydride–dimethylformamide (DMF) followed by chloromethyl pivalate afforded the 3-*N*-Pom derivative in a moderate 62% yield. We thought that the Pom group could be a suitable protecting group for pseudouridine and we describe here the synthesis of a pseudouridine monomer and a novel 2'-*O*-methylpseudouridine monomer fully compatible with standard β-cyanoethyl phosphoramidite methodology.

Results and Discussion

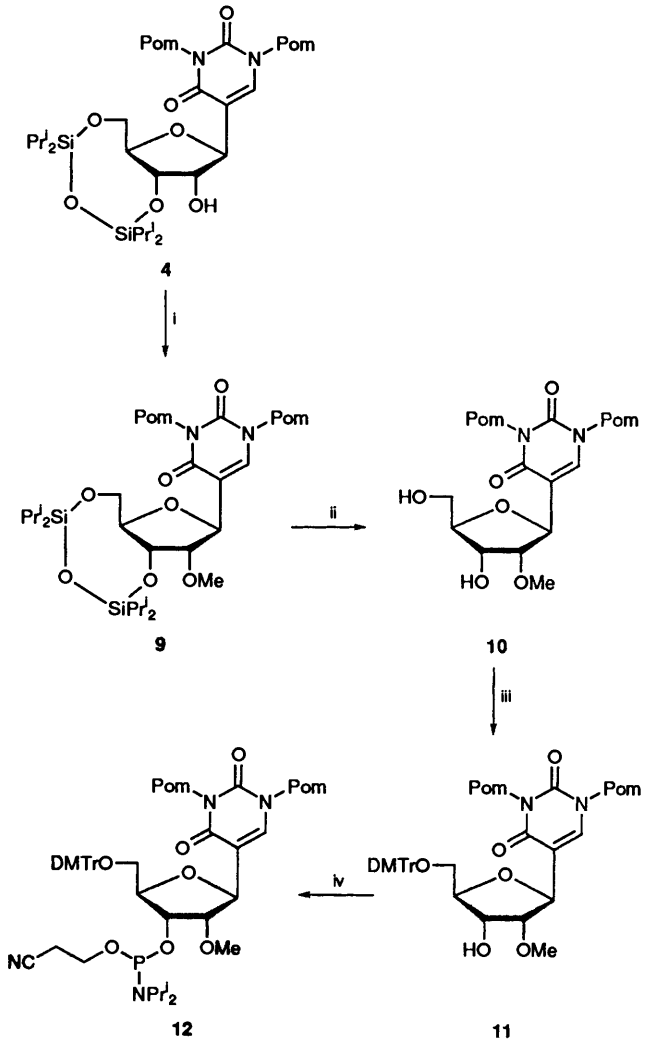
Monomer Synthesis.—Our route to the 2'-*O*-Fpmp-protected pseudouridine building block, compound **8**, is illustrated in Scheme 1. Pseudouridine was first protected with the Markiewicz disiloxane reagent[22] to afford compound **1** in 85% yield. The 2'-hydroxy group of compound **1** was then transiently protected by trimethylsilylation to give compound **2** in quantitative yield; this was a rather slow reaction, requiring 12 h to reach completion. Compound **2** was then allowed to react overnight with 5 mol equiv. of chloromethyl pivalate in DMF in the presence of 10 mol equiv. of anhydrous potassium carbonate. This reaction yielded the highly lipophilic bis(Pom) derivative **3** in 94% yield. Subsequent removal of the 2'-*O*-



Scheme 1 Reaction scheme for the preparation of the 2′-*O*-Fpmp-protected pseudouridine building block. *Reagents:* i, Me_3SiCl, Et_3N, CH_2Cl_2; ii, $Me_3CCO_2CH_2Cl$, K_2CO_3, DMF; iii, PTSA monohydrate, THF; iv, 1-(2-fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine, 2,4,6-trimethylbenzenesulfonic acid, MeCN–THF; v, TBAF, THF; vi, 4,4′-dimethoxytrityl chloride, pyridine; vii, chloro-2-cyanoethoxy-*N*,*N*-diisopropylaminophosphine, Pr^i_2NEt, $(CH_2Cl)_2$.

trimethylsilyl group required 10 min with toluene-4-sulfonic acid (PTSA) and afforded intermediate **4** in good yield. Protection of the 2′-hydroxy moiety of compound **4** with a large excess of 1-(2-fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine in the presence of mesitylenesulfonic acid gave compound **5** in 94% yield. Desilylation of compound **5** by reaction with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) was kept very short (70 s) to avoid loss of the Pom groups, one of which is relatively labile. Under these conditions the desired intermediate **6** was obtained in 75% yield. Subsequent reaction with dimethoxytrityl chloride (DMTrCl) afforded compound **7**, which was then phosphitylated[23] to afford the pure pseudouridine building block **8**. The overall yield of monomer **8** starting from pseudouridine was 32% for the eight-step synthesis. The synthesis of the alternative 2′-*O*-TBDMS-protected pseudouridine monomers gave rather low yields of 21%[16] and 19%[17] mainly because the silylation procedure generated an almost equal mixture of the 2′-*O*- and 3′-*O*-silyl ethers as well as some bis(silyl) material; moreover, the yields of the phosphitylation were only moderate, probably because the nucleobase was not protected.

The reaction scheme for the preparation of the 2′-*O*-methylpseudouridine building block, compound **12**, is illustrated in Scheme 2. The useful intermediate **4** was, in addition, readily methylated on the 2′-hydroxy group using standard



Scheme 2 Reaction scheme for the synthesis of the 2′-*O*-methylpseudouridine building block. *Reagents:* i, MeI, Ag_2O, MeCN; ii, TBAF, THF; iii, 4,4′-dimethoxytrityl chloride, pyridine; iv, chloro-2-cyanoethoxy-*N*,*N*-diisopropylaminophosphine, Pr^i_2NEt, $(CH_2Cl)_2$.

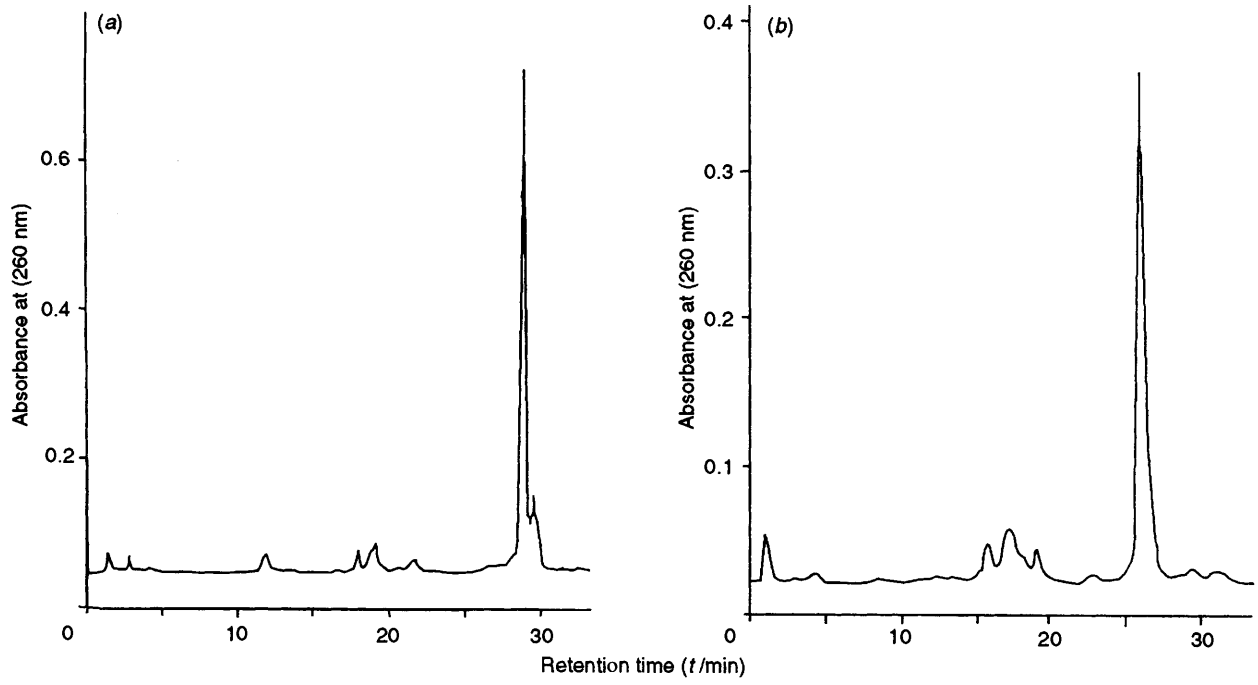


Fig. 1 Analytical reversed-phase HPLC profiles of crude 5′-*O*-dimethoxytrityl, 2′-*O*-Fpmp-protected oligonucleotides on an 8 mm × 100 mm μBondapak C_{18} cartridge (10 μ); buffer A: 95% 0.1 mol dm^{-3} aq. triethylammonium acetate (pH 7) and 5% acetonitrile; buffer B: 30% 0.1 mol dm^{-3} aq. triethylammonium acetate (pH 7) and 70% acetonitrile; gradient 20–80% buffer B in 35 min, flow rate 2 cm^3 min^{-1}. Panel (a): 5′-UCGmCCUUmUΨACmΨA, panel (b): 5′-UCGmCCUUmUΨmACmΨmA.

conditions with methyl iodide and silver oxide[24] in acetonitrile. No side products were observed, due to the complete protection of the uracil ring. The desired 2′-*O*-methyl ether **9** was obtained in 92% yield; in comparison, the previously reported methylation of unprotected pseudouridine using the highly toxic and explosive reagent, diazomethane, which generates both the 2′-*O*- and 3′-*O*-methyl ethers, gave only a 27% yield of isolated 2′-*O*-methyl ether.[11] Subsequent desilylation, dimethoxytritylation and phosphitylation were carried out as above for the 2′-*O*-Fpmp derivative. The new building block was obtained in a respectable overall yield of 30% starting from pseudouridine.

Free pseudouridine readily undergoes isomerisation when heated in strong acid or base, due to the presence of an allyl ether moiety, giving the α- and β-furanosyl compounds and the α- and β-pyranosyl compounds.[25] Of course, when the 5′-hydroxy group of pseudouridine is esterified, isomerisation to the pyranose is not possible and only the anomerisation at C-1′ can occur. Moreover, under mild conditions, *e.g.* 1 mol dm^{-3} aq. HCl at room temperature for 24 h, only 5% isomerisation of β-pseudouridine to the α-isomer was observed by Lerch *et al.*[26] Thus, under the very mild conditions required to remove 2′-*O*-Fpmp groups from a synthetic partially protected oligoribonucleotide (pH 2–2.5 for 24 h at room temperature) we did not expect to see any isomerisation at the C-1′ of the pseudouridine residues. Anomerisation at C-1′ during the ammonia deprotection step was a distinct possibility, although Brown *et al.* deprotected a synthetic 37-residue oligoribonucleotide containing a single internal pseudouridine residue by using conc. aq. ammonia for up to 72 h at room temperature and made no comment about isomerisation.[14] However, this point has recently been clarified, since Gasparutto *et al.* reported that when pseudouridine was treated with conc. aq. ammonia at 55 °C for 24 h there was no trace of isomerisation.[17] Our own findings which follow are in complete agreement with this. In order to check whether the pseudouridine in an oligonucleotide had been completely deprotected without isomerisation we prepared and purified (by HPLC) CmΨCm by using the 2′-*O*-Fpmp-protected monomer, compound **8**. We then used enzymes to analyse whether there had been any anomerisation at C-1′ of the internal pseudouridine residue during the deprotection steps. Pseudouridine incorporated in an oligonucleotide is accepted as a substrate by pancreatic ribonuclease; however, the β-D-*ribo* configuration is required for cleavage.[27] Thus, the chemically synthesized CmΨCm was completely cleaved into CmΨp and Cm by treatment with bovine pancreatic RNase. Incubation of CmΨCm with a mixture of snake venom phosphodiesterase and alkaline phosphatase gave only Cm and Ψ, identical with co-injected authentic samples of the two nucleosides, in the ratio 2:1. Thus, it is quite clear from the enzymic analysis that the stereochemical integrity of internal pseudouridine residues is maintained during the deprotection of oligoribonucleotides synthesized by the Fpmp route.

The new pseudouridine building block **8** was further used in solid-phase phosphoramidite chemistry to prepare a tridecamer oligonucleotide, *viz.* 5′-UCGmCCUUmUΨACmΨA, corresponding to residues 35–47 of human U5 snRNA. Upon completion of assembly the controlled-pore glass carrier was treated with conc. ammonia for 48 h at 60 °C to cleave the oligomer from the carrier and remove the β-cyanoethyl, acyl and Pom protecting groups. The prolonged reaction with ammonia was necessary to remove completely the Pom groups, and this is not detrimental to the oligonucleotide as the Fpmp protecting group is completely stable to aq. ammonia. The crude 5′-*O*-dimethoxytrityl, 2′-*O*-Fpmp-protected oligomer was then analysed (see Fig. 1, panel a) and purified by reversed-phase HPLC on a μ-Bondapak C_{18} cartridge. The product peak was then lyophilised and the residue treated with 10 mmol dm^{-3} aq. HCl to remove the remaining protecting groups. The overall yield of pure material was 30% based on the controlled-pore glass support used for the synthesis. As expected, this oligonucleotide hybridised cleanly to the antisense biotinylated oligo-(2′-*O*-allylribonucleotide) containing 2,6-diaminopurine riboside that is used to deplete human U5 snRNP from HeLa cell splicing extracts.[28] A modified version of the U5 oligo-

nucleotide was also prepared using the 2′-*O*-methylpseudouridine monomer instead of the pseudouridine monomer and the reversed-phase HPLC analysis of the crude 5′-*O*-dimethoxytrityl, 2′-*O*-Fpmp-protected oligomer is shown in Fig. 1, panel b.

In order to compare the hybridisation properties of antisense probes containing 2′-*O*-methylpseudouridine or 2′-*O*-methyluridine with an oligoribonucleotide target sequence the melting temperatures of the hybrids formed between 5′-GA GGCUUAUCCA (*ribo*-1) and 5′-ΨmGmGmAmΨmAmAmGmCmCmΨmCm (methylΨ*ribo*-2) and between 5′-GAG GCUUAUCCA (*ribo*-1) and 5′-UmGmGmAmUmAmAmGmCmCmUmCm (methyl*ribo*-2) were measured and found to be 65.3 and 62.4 °C, respectively. The hybrid formed between 5′-GAGGCUUAUCCA (*ribo*-1) and 5′-UGGAUAAGCCUC (*ribo*-2) melted at 57.9 °C. Thus the use of 2′-*O*-methylpseudouridine to replace 2′-*O*-methyluridine in oligo-(2′-*O*-methylribonucleotide) antisense probes gives an increase in hybrid stability with an RNA target sequence of about 1 °C per substitution which could prove useful when the probes are very short and U-rich.

Experimental

General Materials and Procedures.—β-Pseudouridine was obtained from Yamasa Shoyu Co., Ltd. (Nihonbashi, Chuo-ku, Tokyo, Japan), 2′-*O*-methylcytidine was from Sigma (Deisenhofen, Germany), 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane was from Fluka AG (Buchs, Switzerland) and chloro-2-cyanoethoxy-*N*,*N*-diisopropylaminophosphine was from Bio-Syntech (Hamburg, Germany).

1-(2-Fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine was prepared using the method of Reese and Thompson.[29] 2′-*O*-Fpmp-protected ribonucleotide building blocks for U, C, G and A were prepared as previously described.[30] 2′-*O*-Methylribonucleotide monomers were also prepared as described previously.[31] All other reagents used were of the highest purity commercially available. Anhydrous high-purity solvents were purchased from Romil Chemicals Ltd. (Loughborough, Leicestershire, UK).

Pancreatic ribonuclease, calf intestine alkaline phosphatase and snake venom phosphodiesterase were obtained from Boehringer Mannheim (Mannheim, Germany).

Column chromatography was performed on Kieselgel 60 (Fluka, Neu-Ulm, Germany) and ascending-mode TLC was performed on aluminium foil-supported silica gel containing a 254 nm fluor.

^{13}C and ^{31}P NMR spectra were recorded on a Bruker AM250 spectrometer using internal tetramethylsilane and external trimethyl phosphate as the respective references. ^{13}C NMR spectroscopy data are reported with broad-band proton-noise decoupling. The sugar carbons, apart from C-5′, cannot be unambiguously assigned using only the off-resonance data. To ensure correct assignments ^{1}H–^{1}H and ^{13}C–^{1}H COSY spectra were recorded for each of the compounds described here. The assignments of the proton signals from the homonuclear COSY spectrum is straightforward in the pseudouridine system, using 1′-H, which shows connectivity to 6-H as a starting point. The ^{13}C–^{1}H COSY spectrum then enables correct assignment of the carbon signals. ^{31}P NMR spectroscopic data were recorded using broad-band proton-noise decoupling. ^{13}C NMR spectroscopic data for the 5′-*O*-dimethoxytrityl compounds and the 3′-*O*-phosphoramidites are not included, but are available upon request.

Thermal denaturation curves were measured on a Cary 3 UV-visible spectrophotometer (Varian, Australia) equipped with a thermostatted multicell block. Oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380B-02 (Foster City, California) using β-cyanoethyl phosphoramidite chemistry.[23]

Oligonucleotides containing pseudouridine and 2′-*O*-methylpseudouridine were analysed by Electrospray Ionisation Mass Spectroscopy (ESMS).[32] Experiments were performed on an API III triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Ontario, Canada) equipped with an electrospray ionisation source and operated in the negative-ion mode. Light petroleum refers to the fraction with distillation range 40–60 °C.

Synthesis of Monomers and Intermediates.—3′,5′-O-(*Tetraisopropyldisiloxane*-1,3-*diyl*)*pseudouridine* **1**. Pseudouridine (4.73 g, 19.4 mmol) and imidazole (6.15 g, 90.3 mmol) were dried by evaporation of anhydrous DMF (250 cm^3) under reduced pressure. The residual gum was redissolved in anhydrous DMF (250 cm^3) and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (7.57 g, 24.1 mmol) was added to the stirred solution with exclusion of moisture. Silica gel TLC showed complete reaction after 2 h. Solvent was removed under reduced pressure, the residue was dissolved in dichloromethane (200 cm^3), and the solution was washed with 5% aq. sodium hydrogen carbonate (2 × 250 cm^3). The organic phase was dried (Na_2SO_4), then filtered, and solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (100 g), and eluted with a gradient of 0–7% ethanol in dichloromethane. The title compound was obtained as a solid foam (8.07 g, 85.5%) of R_f 0.29 on silica gel TLC in ethanol–dichloromethane (1:19 v/v); $\delta_C(CDCl_3)$ 163.16 (C-4), 152.49 (C-2), 138.95 (C-6), 112.42 (C-5), 80.82 (C-4′), 80.08 (C-1′), 74.46 (C-2′), 70.91 (C-3′), 61.34 (C-5′), 17.32–16.85 ($CHMe_2$) and 13.30, 12.98, 12.62 and 12.50 ($CHMe_2$).

3′,5′-O-(*Tetraisopropyldisiloxane*-1,3-*diyl*)-2′-O-(*trimethylsilyl*)*pseudouridine* **2**. Compound **1** (7.97 g, 16.4 mmol) was dissolved in dry dichloromethane (180 cm^3), and triethylamine (11.2 cm^3, 82.4 mmol) and chlorotrimethylsilane (6.17 cm^3, 48.6 mmol) were added to the stirred solution with exclusion of moisture at 0 °C. The reaction was left to proceed overnight at room temperature whereupon TLC showed completion. The mixture was then poured into vigorously stirred, ice-cold 1 mol dm^{-3} aq. sodium hydrogen carbonate (250 cm^3). The organic phase was separated, dried (Na_2SO_4), and filtered, and solvent was removed under reduced pressure. The crude product was practically pure as judged by TLC and used in the next reaction without purification. The title compound was obtained as a solid reddish foam (9.17 g, 100%) of R_f 0.18 on silica gel TLC in light petroleum–ethyl acetate (3:1 v/v) and R_f 0.64 in ethanol–dichloromethane (1:9 v/v); $\delta_C(CDCl_3)$ 162.83 (C-4), 152.78 (C-2), 138.23 (C-6), 113.76 (C-5), 80.93 (C-1′), 79.64 (C-4′), 75.74 (C-2′), 69.31 (C-3′), 60.12 (C-5′), 17.40–16.93 ($CHMe_2$), 13.43, 12.97, 12.92 and 12.68 ($CHMe_2$) and 0.31 ($SiMe_3$).

N-1,N-3-*Bis*(*pivaloyloxymethyl*)-3′,5′-O-(*tetraisopropyldisiloxane*-1,3-*diyl*)-2′-O-(*trimethylsilyl*)*pseudouridine* **3**. Compound **2** (9.07 g, 16.2 mmol) was dissolved in anhydrous DMF (200 cm^3). Anhydrous potassium carbonate (22.57 g, 163.3 mmol) and fresh chloromethyl pivalate (11.77 cm^3, 81.6 mmol) were added to the stirred solution with exclusion of moisture. The reaction was left to proceed overnight at room temperature whereupon TLC showed completion. The mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was dissolved in ethyl acetate (250 cm^3) and the solution was washed with 5% aq. sodium hydrogen carbonate (2 × 250 cm^3). The organic layer was dried (Na_2SO_4), and filtered, and solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (200 g) with light petroleum–ethyl acetate (3:1 v/v) as eluent. Pure title compound was obtained as a foam (12 g, 94%) of R_f 0.71 on silica gel TLC in light petroleum–ethyl acetate (3:1 v/v);

δ_C($CDCl_3$) 177.53 and 177.20 (Me_3CCO), 160.49 (C-4), 150.08 (C-2), 139.97 (C-6), 113.73 (C-5), 81.04 (C-1'), 79.78 (C-4'), 75.99 (C-2'), 71.83 (CH_2 $OCOCMe_3$), 69.74 (C-3'), 64.61 (CH_2 $OCOCMe_3$), 60.25 (C-5'), 38.86 and 38.77 (Me_3CCO), 26.93 and 26.85 (Me_3CCO), 17.49–16.94 ($CHMe_2$), 13.43, 13.01, 12.91 and 12.87 ($CHMe_2$) and 0.34 ($SiMe_3$).

N-1,N-3-*Bis(pivaloyloxymethyl)*-3'-5'-O-(*tetraisopropyldisiloxane*-1,3-*diyl)pseudouridine* **4**. Compound **3** (5.46 g, 6.94 mmol) was dissolved in THF (100 cm^3). PTSA monohydrate (1.98 g, 10.4 mmol) was added to the stirred solution and after 10 min the reaction was quenched by addition of triethylamine (2.1 cm^3, 15 mmol). The solution was diluted with ethyl acetate (300 cm^3) and then was washed with 5% aq. sodium hydrogen carbonate (2 × 250 cm^3). The organic phase was dried (Na_2SO_4), and filtered, and solvent was removed under reduced pressure. The product was purified by silica gel column chromatography with light petroleum–ethyl acetate (3:1 v/v) as eluent. Pure title compound was obtained as a solid foam (4.2 g, 84.7%) of R_f 0.36 on silica gel TLC in light petroleum–ethyl acetate (3:1 v/v); δ_C($CDCl_3$) 177.70 and 176.97 (Me_3CCO), 160.32 (C-4), 149.93 (C-2), 140.83 (C-6), 112.27 (C-5), 80.92 (C-4'), 80.62 (C-1'), 74.30 (C-2'), 71.35 (C-3'), 71.17 and 64.50 ($CH_2OCOCMe_3$), 61.48 (C-5'), 38.66 and 38.59 (Me_3CCO), 26.76 and 26.67 (Me_3CCO), 17.26–16.78 ($CHMe_2$) and 13.16, 12.90, 12.54 and 12.44 ($CHMe_2$).

2'-O-[1-(2-*Fluorophenyl*)-4-*methoxypiperidin*-4-*yl*]-N-1,N-3-*bis(pivaloyloxymethyl)*-3',5'-O-(*tetraisopropyldisiloxane*-1,3-*diyl)pseudouridine* **5**. Compound **4** (4.2 g, 5.87 mmol) and 1-(2-fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine (11 g, 53.2 mmol) were dissolved in dry acetonitrile. 2,4,6-Trimethylbenzenesulfonic acid dihydrate (450 mg, 1.9 mmol) was dried by evaporation of dry THF (2 × 50 cm^3) under reduced pressure, then was redissolved in dry THF (50 cm^3), and the solution was added to the above mixture. The reaction mixture was stirred overnight at room temperature, whereupon silica gel TLC in light petroleum–ethyl acetate (5:1 v/v) showed almost total consumption of starting material to give a new spot of R_f 0.33. The reaction was quenched with triethylamine (2.8 cm^3, 20 mmol) and solvent was removed under reduced pressure. The residue was dissolved in dichloromethane (250 cm^3) and the solution was washed with 1 mol dm^{-3} aq. sodium hydrogen carbonate. The organic phase was dried (Na_2SO_4), then filtered, and solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (280 g) packed in light petroleum–ethyl acetate (6:1 v/v). The column was eluted with a gradient from 14.3 to 16.7 vol.-% ethyl acetate in light petroleum. Pure product fractions were pooled, and solvent was removed under reduced pressure to give the title compound as a solid foam (5.08 g, 93.8%); δ_C($CDCl_3$) 177.60 and 177.12 (Me_3CCO), 160.32 (C-4), 157.47 and 153.57 (C–^{19}F), 149.92 (C-2), 141.39 (C-6), 140.26 and 140.13 (fluorophenyl C-1), 124.15 (fluorophenyl C-5), 121.96 and 121.83 (fluorophenyl C-4), 119.09 (fluorophenyl C-6), 116.00 and 115.66 (fluorophenyl C-3), 113.27 (C-5), 99.50 (piperidine C-4), 80.06 (C-4'), 79.79 (C-1'), 73.20 (C-2'), 71.65 ($CH_2OCOCMe_3$), 69.18 (C-3'), 64.62 ($CH_2OCOCMe_3$), 60.17 (C-5'), 48.33 (OMe), 47.96 (piperidine C-2 and C-6), 38.79 and 38.72 (Me_3CCO), 34.20 and 33.84 (piperidine C-3 and C-5), 26.89 and 26.79 (Me_3CCO), 17.47–18.87 ($CHMe_2$) and 13.32 and 12.92 ($CHMe_2$).

2'-O-[1-(2-*Fluorophenyl*)-4-*methoxypiperidin*-4-*yl*]-N-1,N-3-*bis(pivaloyloxymethyl)pseudouridine* **6**. Compound **5** (5.08 g, 5.5 mmol) was dissolved in dry THF (100 cm^3) and stirred with 1.1 mol dm^{-3} TBAF in THF (10 cm^3) for 70 s only. The reaction was immediately quenched by addition of pyridine–methanol–water (50 cm^3; 3:1:1 by vol.). Pyridinium Dowex 50 W × 2 – 200 resin (25 g) suspended in pyridine–methanol–water (50 cm^3; 3:1:1 by vol.) was added and the mixture was stirred for 20 min. The resin was filtered off and washed, and the combined filtrate and washings were concentrated under reduced pressure. Residual pyridine and water were removed by coevaporation with toluene. Chromatography of the crude product on silica gel (100 g) and elution with dichloromethane followed by a gradient of 0–5% ethanol in dichloromethane afforded the title compound as a solid foam (2.80 g, 74.9%), R_f 0.46 on silica gel TLC in ethanol–dichloromethane (1:19 v/v); δ_C($CDCl_3$) 178.23 and 177.01 (Me_3CCO), 161.16 (C-4), 157.50 and 153.58 (C–F), 149.61 (C-2), 144.08 (C-6), 139.76 and 139.62 (fluorophenyl C-1), 124.28 (fluorophenyl C-5), 122.63 and 122.50 (fluorophenyl C-4), 119.22 (fluorophenyl C-6), 116.13 and 115.80 (fluorophenyl C-3), 110.07 (C-5), 99.91 (piperidine C-4), 86.15 (C-4'), 79.53 (C-1'), 72.55 (C-3'), 71.32 (C-2'), 70.65 ($CH_2OCOCMe_3$), 64.60 ($CH_2OCOCMe_3$), 63.08 (C-5'), 48.10 (OMe), 47.82 (piperidine C-2 and C-6), 38.66 (Me_3CCO), 34.02 and 33.18 (piperidine C-3 and C-5) and 26.78 and 26.61 (Me_3CCO).

5'-O-*Dimethoxytrityl*-2'-O-[1-(2-*fluorophenyl*)-4-*methoxypiperidin*-4-*yl*]-N-1,N-3-*bis(pivaloyloxymethyl)pseudouridine* **7**. Compound **6** (2.8 g, 4.12 mmol) was dried by evaporation of pyridine (2 × 50 cm^3) under reduced pressure. The residue was dissolved in anhydrous pyridine (50 cm^3) and 4,4'-dimethoxytrityl chloride (1.75 g, 5.15 mmol) was added to the stirred mixture with exclusion of moisture. TLC showed complete reaction after 2 h. The reaction was quenched with methanol (1 cm^3) and solvent was removed under reduced pressure. The crude product was worked up in the usual fashion and was then purified by silica gel column chromatography, with light petroleum–ethyl acetate–chloroform (6:3:1 by vol.) containing 1% triethylamine as eluent. Pure title compound was obtained as a solid, off-white foam (3.77 g, 93.2%) of R_f 0.33 on silica gel TLC in light petroleum–ethyl acetate (2:1 v/v) containing 1% triethylamine.

2-*Cyanoethyl* 5'-O-*dimethoxytrityl*-2'-O-[1-(2-*fluorophenyl*)-4-*methoxypiperidin*-4-*yl*]-N-1,N-3-*bis(pivaloyloxymethyl)pseudouridine*-3'-*yl*-N,N-*diisopropylphosphoramidite* **8**. Compound **7** (3.77 g, 3.84 mmol) was dissolved in dry 1,2-dichloroethane (50 cm^3) containing *N*,*N*-diisopropylethylamine (1.34 cm^3, 7.68 mmol) under argon, and chloro-2-cyanoethoxy-*N*,*N*-diisopropylaminophosphine (0.93 cm^3, 4.22 mmol) was added dropwise to the stirred solution. The reaction was left to proceed overnight under anhydrous conditions whereupon silica gel TLC showed complete reaction. Dichloromethane (100 cm^3) was added and the solution was washed successively with 1 mol dm^{-3} aq. sodium hydrogen carbonate (2 × 150 cm^3) and saturated brine (150 cm^3). The organic phase was dried (Na_2SO_4), then filtered, and solvent was removed under reduced pressure. Chromatography of the residue on silica gel (100 g) and elution with light petroleum–ethyl acetate (3:1 v/v) containing 1% triethylamine afforded the pure product as a solid foam (3.22 g, 70.9%) of R_f 0.24 on TLC in light petroleum–ethyl acetate (3:1 v/v) containing 1% triethylamine; δ_P(ethyl acetate, concentric external D_2O lock) 146.73 and 146.07.

2'-O-*Methyl*-N-1,N-3-*bis(pivaloyloxymethyl)*-3',5'-O-(*tetraisopropyldisiloxane*-1,3-*diyl)pseudouridine* **9**. Compound **4** (1.22 g, 1.71 mmol) was dissolved in anhydrous acetonitrile (10 cm^3). Silver oxide (3 g, 12.9 mmol) and iodomethane (5 cm^3, 80 mmol) were added and the sealed mixture was stirred for 96 h at room temperature. Silica gel TLC showed complete reaction. The mixture was diluted with acetonitrile, then filtered, and solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel and elution with light petroleum–ethyl acetate (3:1 v/v). Pure product was obtained as a foam (1.14 g, 91.7%) of R_f 0.55 on silica gel TLC in light petroleum–ethyl acetate (3:1 v/v); δ_C($CDCl_3$) 177.50 and 177.09 (Me_3CCO), 160.51 (C-4), 149.97 (C-2), 140.22 (C-6),

113.28 (C-5), 83.59 (C-2'), 79.90 (C-4'), 78.31 (C-1'), 71.67 ($CH_2OCOCMe_3$), 70.11 (C-3'), 64.50 ($CH_2OCOCMe_3$), 59.99 (C-5'), 58.82 (OMe), 38.79 and 38.73 (Me_3CCO), 26.88 and 26.79 (Me_3CCO), 17.42, 17.27, 17.10 and 16.89 ($CHMe_2$) and 13.42, 12.94, 12.70 and 12.48 ($CHMe_2$).

2'-O-*Methyl*-N-1,N-3-*bis*(*pivaloyloxymethyl*)*pseudouridine* **10**. Compound **9** (1.14 g, 1.56 mmol) was dissolved in dry THF (5 cm^3) and treated with 1.1 mol dm^{-3} TBAF in THF (3.25 cm^3) for 1.2 min only, quenched, and worked up as described for the preparation of compound **6** above. The crude product was purified by silica gel column chromatography and elution with a gradient of ethanol from 0–5% in dichloromethane. The title compound was obtained as a foam (595 mg, 78.4%) of R_f 0.33 on silica gel TLC in ethanol–dichloromethane (1:19 v/v); $\delta_C(CDCl_3)$ 178.29 and 177.13 (Me_3CCO), 160.96 (C-4), 149.88 (C-2), 142.50 (C-6), 111.25 (C-5), 84.32 (C-4'), 82.72 (C-2'), 77.95 (C-1'), 70.72 ($CH_2OCOCMe_3$), 69.85 (C-3'), 64.57 ($CH_2OCOCMe_3$), 61.95 (C-5'), 58.12 (OMe), 38.77 and 38.70 (Me_3CCO) and 26.82 and 26.70 (Me_3CCO).

5'-O-*Dimethoxytrityl*-2'-O-*methyl*-N-1,N-3-*bis*(*pivaloyloxymethyl*)*pseudouridine* **11**. Compound **10** (540 mg, 1.11 mmol) was dimethoxytritylated according to the procedure used for preparing compound **7** above. Crude product was purified by column chromatography on silica gel and elution with a gradient from 0 to 5% ethanol in triethylamine–chloroform (1:199 v/v). The product was obtained as a pale yellow foam (860 mg, 98.2%) of R_f 0.26 on silica gel TLC in triethylamine–ethanol–chloroform (1:2:197 by vol.).

2-*Cyanoethyl* 5'-O-*dimethoxytrityl*-2'-O-*methyl*-N-1,N-3-*bis*(*pivaloyloxymethyl*)*pseudouridin*-3'-*yl* N,N-*diisopropylphosphoramidite* **12**. Compound **11** (860 mg, 1.09 mmol) was phosphitylated according to the procedure used to prepare compound **8** above. The product was purified by silica gel column chromatography with light petroleum–ethyl acetate (3:1 v/v) containing 0.5% triethylamine as eluent. Pure title compound was obtained as a solid foam (670 mg, 62%) of R_f 0.46 and 0.42 on silica gel TLC in the above solvent system; $\delta_P(CH_2Cl_2$, concentric external D_2O lock) 146.81 and 145.50.

Polymer Assembly, Deprotection and Purification.—In order to test out the 2'-*O*-Fpmp-protected pseudouridine monomer **8**, the trimer Cm*Ψ*Cm (Cm represents 2'-*O*-methylcytidine) was synthesized 'trityl on', on a 1 μmol scale. Nucleotide monomers were dried overnight *in vacuo* over phosphorus pentaoxide and potassium hydroxide pellets, and were then made up as 0.1 mol dm^{-3} solutions in anhydrous acetonitrile (< 30 p.p.m. water). A standard β-cyanoethyl phosphoramidite DNA cycle was used with the condensation wait time increased to 15 min with 1*H*-tetrazole as the activator. Upon completion of the assembly, the carrier-bound trimer was first deblocked with 30% aq. ammonia–ethanol (3:1 v/v) for 48 h at 60 °C (one Pom group is cleaved rapidly, the other one rather slowly). When cool, solvent was removed under reduced pressure at room temperature and the residue was purified by reversed-phase HPLC on μ-Bondapak C_{18} using a gradient of acetonitrile in 0.1 mol dm^{-3} aq. triethylammonium acetate, pH 7. The product peak was eluted at an acetonitrile concentration of ~45% and was lyophilised on a Speedvac.

The DMTr-CmΨ_{Fpmp}Cm from above was then dissolved in 10 mmol dm^{-3} sterile aq. hydrochloric acid (2 cm^3) and kept for 24 h at room temperature to remove the remaining protecting groups. The reaction was quenched with sterile 1 mol dm^{-3} Tris-HCl buffer (0.1 cm^3; pH 7.5) and the solution was lyophilised. The residue of Cm*Ψ*Cm was purified by reversed-phase HPLC as a single peak eluted at an acetonitrile concentration of ~11%. This peak was lyophilised to give a residue.

The highly modified U5 oligomer, 5'-UCGmCCUUmU*Ψ*ACm*Ψ*A was also synthesized using the 'trityl on' manual ending procedure. Gm, Um and Cm represent 2'-*O*-methylguanosine, 2'-*O*-methyluridine and 2'-*O*-methylcytidine respectively. Upon completion of the assembly the carrier-bound fully protected oligonucleotide was treated with 25% aq. ammonia for 48 h at 60 °C in a sealed sterile vial. Solvent was removed under reduced pressure and the crude oligomer was purified by reversed-phase HPLC on a μ-Bondapak C_{18} cartridge using a gradient of acetonitrile in 0.1 mol dm^{-3} aq. triethylammonium acetate, pH 7, as eluent. The product peak was lyophilised and desalted by passage through a G15 Sephadex column (30 cm^3), eluted with sterile distilled water. A 3 cm^3 fraction following the void volume was collected and acidified with sterile aq. hydrochloric acid (0.25 cm^3, 0.1 mol dm^{-3}). The solution was incubated at 22 °C for 20 h to cleave the 5'-*O*-dimethoxytrityl group and the Fpmp protecting groups. The solution was then neutralised by addition of 2 mol dm^{-3} Tris acetate buffer (75 mm^3, pH 7.9) and the oligonucleotide was recovered as a pellet by several extractions with butan-1-ol according to the procedure of Cathala and Brunel.[33] The yield of pure oligonucleotide was 30% based on the amount of support used in the synthesis. The relative molecular mass of the oligomer was found to be 4040.2 by ESMS (Calc. for $C_{123}H_{157}N_{39}O_{93}P_{12}$: M, 4041.48). A version of the U5 oligomer was also synthesized with *Ψ*m replacing *Ψ* following the above procedure and the relative molecular mass was found to be 4068.2 by ESMS (Calc. for $C_{125}H_{161}N_{39}O_{93}P_{12}$: M, 4069.53).

In addition, 5'-GAGGCUUAUCCA (*ribo*-1), 5'-UGGAUAAGCCUC (*ribo*-2), 5'-UmGmGmAmUmAmAmGmCmCmUmCm (methyl*ribo*-2) and 5'-*Ψ*mGmGmAm*Ψ*mAmAmGmCmCm*Ψ*mCm (methyl*Ψribo*-2) were synthesized using standard 2'-*O*-Fpmp-protected ribonucleoside monomers and 2'-*O*-methylribonucleoside monomers and the new 2'-*O*-methylpseudouridine monomer described above. The relative molecular mass of purified methyl*Ψribo*-2 was found to be 3962.4 by ESMS (Calc. for $C_{126}H_{166}N_{45}O_{82}P_{11}$: M, 3963.66).

*Enzymic Analysis of Cm*Ψ*Cm.*—An aliquot of Cm*Ψ*Cm (~1 A_{260} unit) from above was dissolved in Tris-acetate buffer (50 mm^3; pH 7.5) and pancreatic RNase solution (5 mm^3; 10 mg cm^{-3}) was added. The solution was incubated for 20 h at room temperature and an aliquot was then analysed by reversed-phase HPLC on a μ-Bondapak C_{18} cartridge, using a gradient from 0–14% acetonitrile in 0.1 mol dm^{-3} aq. triethylammonium acetate, pH 7, over a period of 30 min.

Likewise, an aliquot of Cm*Ψ*Cm (~2 A_{260} units) from above was dissolved in alkaline phosphatase buffer (50 mm^3) to which was added snake venom phosphodiesterase solution (5 mm^3; 11 mg cm^{-3}). The solution was incubated for 5 h at room temperature and then alkaline phosphatase (2 units) was added and the incubation was continued overnight. An aliquot was then analysed by reversed-phase HPLC as described above, and in addition co-injections with authentic β-pseudouridine and 2'-*O*-methylcytidine were made.

Thermal Analysis of Duplexes.—Melting curves were performed with 3 μmol dm^{-3} of each strand in Tris hydrochloride buffer (10 mmol dm^{-3}; pH 7) containing KCl (0.1 mol dm^{-3}) and EDTA (1 mmol dm^{-3}) for the following three duplexes: i, *ribo*-1 + *ribo*-2; ii, *ribo*-1 + methyl*ribo*-2; iii, *ribo*-1 + methyl*Ψribo*-2. The samples were preannealed by heating to 90 °C, then cooling slowly to room temperature. Melting curves were obtained by measuring the increase of absorbance at 260 nm with temperature using a temperature gradient of 0.3 °C min^{-1} starting at 25 °C and finishing at 90 °C. The melting temperatures were obtained by taking the first derivative of the curves.

Conclusions.—The results that we have presented show that the solid-phase synthesis of oligoribonucleotides using 2′-*O*-Fpmp protection is an excellent way of incorporating rare and expensive nucleosides such as pseudouridine. The pivaloyloxymethyl group has been established as a useful group for the complete protection of the nucleobase of pseudouridine, which was demonstrated by the excellent 2′-*O*-methylation reaction. The stability of the Fpmp group to both fluoride and ammonia enables optimal synthesis of the monomers and in addition enables the partially protected oligoribonucleotides to be readily purified by reversed-phase HPLC. Moreover, the removal of the 5′-*O*-dimethoxytrityl group and the 2′-*O*-Fpmp groups is achieved under homogeneous aqueous conditions, rendering the method particularly useful for large-scale syntheses. Further improvements to the solid-phase synthesis of oligoribonucleotides using the 2′-*O*-Fpmp protecting group have recently been reported by Rao *et al.*, demonstrating the synthesis of a heptatriacontamer (37-mer) in good yield.[34]

The Pom group would not be as suitable for use with a 2′-*O*-(*tert*-butyldimethylsilyl)-protected pseudouridine since the necessarily long deprotection time with aq. ammonia or ethanolic ammonia would cause substantial premature loss of silyl protecting groups, leading to highly undesirable strand scission.[35]

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