Z-RNA. The synthesis of 2'-O-[13C]methyl- and 5-methyl-analogs of ribo-CGCCG*

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Chemical synthesis of 2'-O-[13C]methyl-riboribose and 5-methyl-ribo-CGCCG using support-aided phosphoramidite method is presented. 2'-O-Methyl guanosine derivative was separated from its 3'-O-methyl counterpart using transient 5',3'-O-silylation with 1,3-dichloro-1,1,3,3-tetraisopropylsiloxane (Markiewicz reagent). The hexamers were obtained in a purity suitable for NMR studies.

It has been proved that RNA can adopt a left-handed double helical conformation similar to that of Z-DNA. Hall et al. [1] reported that in 6 M sodium perchlorate under elevated temperatures poly(r(CG)) converts from the canonical right-handed A form to a left-handed double helix-Z-RNA. We have found [2] a similar transition for the ribo-CGCCG oligonucleotide duplex at salt concentrations above 2.3 M magnesium chloride or 5.0 M lithium chloride. It is almost certain that such an RNA transition does not occur in the living cell. However, it seems of interest to propose the process of A->Z transition as an experimental model for evaluation of the structural features of CG tracts in double helical RNAs and their modified counterparts. This will concern especially the role of 2'-hydroxyl function in maintaining the intra- and intermolecular hydrogen bonding network. Till now no X-ray data on such structures exist. Our recent NMR data [3] clearly indicate involvement of 2'-hydroxyl groups in maintaining the Z-RNA structure and reveal close contacts of 5-pyrimidine site with neighbouring guanosine units. The importance of those indications should be evaluated on appropriately modified structural models.

In this paper we present the synthesis of modified analogs of ribo-CGCCG duplex, namely, 2'-O-[13C]methyl CGGCCG and m5CGm5CGm5CG obtained in view of further 1H and 13C NMR structural studies.

MATERIALS AND METHODS

Nucleosides were purchased from Pharma Waldhoff. Solvents and reagents were of pure grade from POCh (Gliwice, Poland), if not specified otherwise. Pyridine and acetonitrile were distilled over P2O5 and then CaH2 and stored over 4A molecular sieves. Ethyl acetate, methylene chloride and chloroform were freshly distilled over anhydr. potassium carbonate.

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1Abbreviations used throughout the text: m5C, 5-methylcytidine; THF, tetrahydrofuran; TEAHF, triethylammonium hydroxide; TBAF, tetraethylammonium fluoride; CFG, controlled pore glass; LCAA-CFG, long chain alkylamine-modified controlled pore glass; TEAA, triethylammonium acetate; tBDMSi, t-butylmethylsilyle; TIFSiDCI, 1,3-dichloro-1,1,3,3-tetraisopropylsilioxane.
then chloroform and methylene chloride were stabilised with 0.5% 2-propanol. Benzene was distilled from sodium wire. Dioxane, THF, tritielyamine (Fluka), and N,N-diisopropylamine (Fluka) were distilled from CaH2 and stored over 4Å molecular sieves. Bis(N,N-diisopropylamino)(2-cyanoethoxy)phosphine, prepared according to [4], was vacuum distilled and stored protected from moisture in freezer at -20°C. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane was prepared according to [5]. 13C-Labelled methyl iodide (99% enrichment) was from Aldrich. Tetrazole was vacuum sublimed. Acetonitrile for automated oligonucleotide synthesis was from Romil Chemicals and contained less than 10 ppm of water. Other reagents for automated synthesis were from Applied Biosystems. Substrates for dimethoxytritylation and silylation were rendered anhydrous by repeated (3×) evaporation with anhydrous pyridine. TLC was carried out on Merck silica gel HF glass plates, using chloroform-methanol (9:1, v/v) as eluent, unless otherwise specified. Column chromatography was carried out on Merck silica gel H, using step gradient of methanol in chloroform or ethyl acetate in hydrocarbons in concentrations specified, or on Merck silanised silica gel type 60 in water-acetonitrile systems. HPLC separations were performed on Waters HPLC with UV detector system. Columns used were 7.8 x 300 mm steel column filled with PRP-1 phase (Hamilton) and NovaPak C-18 25 x 100 mm cartridge (Waters) for preparative runs and Nova-Pak C-18 8 x 100 mm cartridge (Waters) for analytical runs. Elution was performed with solutions of 0.1 M ammonium acetate in 5% acetonitrile, pH 7 (buffer A) and 0.1 M ammonium acetate in 50% acetonitrile, pH 7 (buffer B). NMR spectra were measured on Varian Unity (1H, 300 MHz, 13C, 75.4 MHz and 31P, 36.2 MHz) spectrometer. Chemical shifts are reported in ppm relative to TMS (internal standard) for 1H and 13C, and 85% phosphoric acid (external standard) for 31P. UV spectra were measured on Beckmann DU-65 instrument. Elemental analyses were performed on crystalline compounds only (Perkin Elmer analyser model 240) and differed not more than by 0.3% from calculated values. 5'-O-Dimethoxytrityl-2'-O-t-butyldimethylsilyl-N2'-isobutyryl guanosine 16 (7, 8) were prepared according to reported procedures.

5'-O-Dimethoxytrityl-2'(3')-O-methyl-N2'-isobutyryl guanosine (mixture of 2a and 2b). 5'-O-Dimethoxytrityl-N2'-isobutyryl guanosine 1 [6] (2.34 g, 3.75 mmol) and 5n(II) chloride dihydrate (84 mg, 0.375 mmol) in methylene chloride (300 ml) were stirred together and cooled to 0°C. Diazomethane in methylene chloride (approx. 0.5 M) was added in portions (2 × 6 ml) at 30 min intervals. After 3 h TLC showed no starting material. The reaction mixture was concentrated under reduced pressure and oily residue was chromatographed on silica gel and eluted with chloroform-methanol. The appropriate fractions (emerging at 2 - 2.5% methanol) were pooled and concentrated, giving a mixture of 2'(3')-O-methyl isomers 2a(2b) as colourless foam (1.9 g, 75%). 1H NMR (CDCl3): 8.15-7.10 (m, 14H, H8, DMT), 5.88 (d, H1' of 2a), 5.72 (d, H1' of 2b), 5.19 (m, H2' of 2b), 4.64 (m, H2' of 2a), 3.07-4.53 (m, remaining ribose protons), 3.77 (s, DMT OCH3), 3.63 (s, 2'-OCH3 of 2a), 3.48 (s, 3'-OCH3 of 2b), cf. [9] for assignments.

5',3'-O-Tetraisopropyldisiloxane-1,3-diyl-2'-O-methyl-N2'-isobutyryl guanosine 4. The mixture of isomers 2a, 2b (1.925 g, 3 mmoles) was treated with 80% acetic acid (5 ml) for 4 h. Solvents were evaporated and the residue was partitioned between chloroform and water (20 + 20 ml). The organic layer was back-extracted with water (20 ml) and the combined aqueous phases were evaporated in vacuo. The white solid composed of a mixture of isomers 3a, 3b (1.1 g, 3 mmol) was dried, then dissolved in dry pyridine (30 ml) and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (1.580 mg, 5.03 mmol) was added with stirring. After 2 h, satd. sodium bicarbonate (30 ml) and chloroform (30 ml) were added and the phases were separated. The aqueous phase was extracted with chloroform (2 × 30 ml) and the combined organic layers were dried (sodium sulphate) and evaporated to a foam. Silica gel chromatography (elution with benzene-ethyl acetate from 20% to 50% of ethyl acetate) gave two compounds: the major one 4 (880 mg, 48%, foam), TLC Rp = 0.33 (benzene-ethyl acetate, 1:1). 1H NMR (CDCl3): 8.02 (s, 1H, H8), 5.86 (s, 1H, H1'), 4.53 (dd, 1H, J2'=4.8Hz), J3'=9.3Hz, H3'), 4.22 (dd, 1H, J5'=13.3Hz, H5'), 4.12 (dd, 1H, J3'=9.3Hz, J4'= 2.4Hz, H4'), 4.02 (dd, 1H, J5'=13.3Hz, J4'=...
2.4 Hz, H5'), 3.81 (dd, 1H, J2=3 4.3 Hz, H2'), 3.65 (s, 3H, OCH3), 2.63 (m, J 6.5 Hz, 1H, CH isobuteryl), 1.25 (6H, J 6.5 Hz, CH3 isobuteryl), 0.98 (m, 28 H, Si-isopropyl) and the minor one of putative structure 5, (370 mg, solid), TLC Rp = 0.74.

5'-Dimethoxytrityl-2'-O-methyl-2'-isobutyryl guanosine 2a. Compound 4 (880 mg, 1.443 mmol) was stirred with 1 M solution of triethylammonium hydrofluoride in THF (TEA:HF/THF) [5] (6 ml, 6 mmol) overnight, after which time TLC showed no starting material. Satd. sodium bicarbonate (3 ml) and pyridine (3 ml) were added and the resulting solution was evaporated in vacuo. The residue was coevaporated with pyridine (3 x 5 ml), treated with pyridine (15 ml) and inorganic solids were filtered and washed with pyridine (10 ml). The combined filtrates were evaporated giving a colourless oil of 3a (523 mg, 1.42 mmol), which was used directly for the next step. The oil was dissolved in anhyd. pyridine (7.5 ml) and dimethoxytrityl chloride (600 mg, 1.77 mmol) was added. After 3 h the reaction was completed. Chloroform (25 ml) and satd. sodium bicarbonate (25 ml) were added and the two phases were separated. The organic layer was extracted twice with chloroform (15 ml) and combined extracts were dried (sodium sulphate) and evaporated to a yellow foam. The foam was separated on silica gel with chloroform:3% methanol and after evaporation gave a white foam, which was dissolved in chloroform (4 ml) and added dropwise with vigorous stirring to hexane (250 ml). White precipitate of 2a was filtered and dried (790 mg, 1.18 mmol, overall yield from 1 31%). 1H NMR (CDCl3): 8.12 (s, 1H, H8), 7.10 - 7.95 (m, 13 H, DMTr), 5.88 (d, 1H, J1=2 6.3 Hz, H1'), 4.64 (dd, 1H, J1=2 6.3 Hz, J2=3 5.5 Hz, H2'), 4.53 (dd, 1H, J2=3 5.5 Hz, J3=4 4.3 Hz, H3'), 4.19 (m, 1H, H4'), 3.50 (dd, 1H, J4=5 3.5 Hz, J5=6 11.6 Hz, H5'), 3.20 (dd, 1H, J4=5 3.7 Hz, J5=6 11.6 Hz, H5'), 3.63 (s, 3H, OCH3), 2.73 (m, 1H, J 6.0 Hz CH-isobutyryl), 1.75 (d, 6H, J 6.0 Hz, CH3-isobutyryl).

3',5'-Teitraisopropylsiloxane-1,3-diyl-2'-O-[13C]methyl-2'-benzoyl cytidine 3. Compound 6 [5] (1 g, 1.6 mmol) was dissolved in benzene (15 ml), silver oxide (1.1 g, 4.8 mmol, freshly prepared from silver nitrate and sodium hydroxide, washed with water until neutral, then with ethanol and ether, dried in vacuo) was added, and to the stirred suspension [13C]methyl iodide (1.9 ml, 32 mmol) was injected through septum. The suspension was stirred overnight at room temperature. The reaction mixture was adsorbed on the top of silica gel column, washed with chloroform and with 1% methanol-chloroform to give 7 as a foam (650 mg, 63.5%), which was used directly for the next step. 1H NMR (CDCl3): 8.33 (m, 1H, H6), 7.89 - 7.52 (m, 5H, Bz), 5.86 (m, 2H, H1' and H5'), 4.30 (m, 1H, H3'), 4.22 (m, 1H, H5'), 4.17 (m, 1H, H4'), 3.99 (m, 1H, H5'), 3.83 (m, 1H, H2'), 3.73 (s, 3H, OCH3, 13C satellites at 3.97 and 3.49), 1.05 (m, 28 H, Si-isopropyl).

2'-O-[13C]Methyl-N4-benzoyl cytidine 8. Compound 7 (300 mg, 0.82 mmol) was rendered dry by repeated evaporation with dry dioxane (2 x 10 ml, TEA:HF/THF [5] (6 ml, approx. 6 mmol) was added and after 5 h the mixture was treated with sodium bicarbonate (10 ml), shaken, pyridine (10 ml) was added, after filtration the supernatant was evaporated and the residue was coevaporated with pyridine (2 x 2 ml) and then with toluene (2 ml). The residue was chromatographed on silica gel and eluted with chloroform-methanol. The desired compound 8 was eluted with 4% methanol (208 mg, 70%). 1H NMR (CDCl3): 8.42 (d, 1H, J 7.4 Hz, H6), 7.5 - 7.9 (m, 6H, Bz, H5), 5.88 (d, 1H, J1=2 1.9 Hz, H1'), 4.35 (m, 1H, H3'), 4.12 (m, 1H, H4'), 4.02 - 4.10 (m, 2H, H5'), 3.96 (m, 1H, H2'), 3.69 (s, 3H, OCH3, 13C satellites at 3.93 and 3.45). 13C NMR (CDCl3): prominent peak at 58.907.

5'-O-Dimethoxytrityl-2'-O-[13C]methyl-N4-benzoyl cytidine 9. Compound 8 (220 mg, 0.61 mmol) was dissolved in pyridine (2 ml) and dimethoxytrityl chloride (226 mg, 0.66 mmol) was added. The mixture was left overnight with stirring. Satd. sodium bicarbonate (4 ml) was added, together with chloroform (10 ml), the mixture was shaken, the chloroform layer separated and evaporated. The residue was separated on silica gel and eluted with chloroform containing 1% triethylamine. The compound 9 was obtained as a foam (385 mg, 95%). 1H NMR (CDCl3): 8.60 (d, 1H, J7=4 4.4 Hz, H6), 7.90 - 6.90 (m, 19H, Bz, DMTr, H5), 6.04 (s, 1H, H1'), 4.47 (d, 1H, J3=4 9.2 Hz, H3'), 4.05 (m, 1H, J4=5 9.2 Hz, J5=6 2.2 Hz, J4=5 2.6 Hz, H4'), 3.81 (s, 1H, H2'), 3.75 (s, 3H, OCH3, 13C satellites at 3.50 and 4.00), 3.63 (dd, 1H, J5=6 11.4 Hz, J4=5 2.2 Hz, H5'), 3.56 (dd, 1H, J5=6 11.4 Hz, J4=5 2.6 Hz, H5').
**N^4-Benzoyl-5-methylcytidine 11.** 5-Methylcytidine 10 of m.p. 211 - 213°C (lit. [10] 210 - 211°C) (3.1 g, 12 mmol) was dissolved in absolute ethanol (100 ml) and benzoic anhydride (5.45 g, 24 mmol) was added. The mixture was boiled under reflux for 2 h. After concentration in vacuo to about 30 ml the mixture was left overnight for crystallization. Yield of pure product 11 2.34 g (54%). M.p. 192 - 193°C. NMR (CDCl₃): 7.42 - 8.32 (m, 6H, Bz, H6), 5.67 (d, 1H, J₁= 4.5Hz, H1'), 4.47 (dd, 1H, J₁= 4.5Hz, J₂= 6Hz, H2'), 4.39 (m, 1H, H3'), 4.26 (m, 1H, H4'), 4.02 (dd, 1H, J₄= 3Hz, J₅= 12Hz, H5'), 3.85 (dd, 1H, J₄= 3Hz, J₅= 12Hz, H5'), 2.12 (s, 3H, 5-methyl). UV (methanol): λ_{max} 280 nm.

**5′-O-Dimethoxytrityl-N^4-benzoyl-5-methylcytidine 12.** Dry N^4-benzoyl-5-methylcytidine 11 (2.3 g, 6.3 mmol) was dissolved in pyridine (50 ml) and 4,4′-dimethoxytrityl chloride (3.4 g, 10 mmol) was added. The mixture was stirred at room temperature for 18 h (progress of the reaction was controlled by TLC). After evaporation of the solvent, the residue was dissolved in methylene chloride, washed with satd. sodium bicarbonate and water. Removal of solvent in vacuo yielded a foam which was purified by column chromatography on silica gel (methylene chloride-2% methanol) to give product 12, 3.1 g (65%). NMR (CDCl₃): 8.32 - 6.89 (m, 19H, Bz, DMTTr, H6), 5.95 (d, 1H, J₁= 4.5Hz, H1'), 4.42 (dd, 1H, J₁= 4.5Hz, J₂= 6Hz, H2'), 4.39 (m, 1H, H3'), 4.16 (m, 1H, H4'), 3.55 (dd, 1H, J₄= 2Hz, J₅= 11Hz, H5'), 3.45 (dd, 1H, J₄= 3Hz, J₅= 11Hz, H5'), 2.09 (s, 3H, 5-CH₃).

**5′-O-Dimethoxytrityl-2′-O-t-butyldimethylsilyl-N^4-benzoyl-5-methylcytidine 13.** Compound 12 (2.15 g, 3.2 mmol) and imidazole (0.88 g, 12.95 mmol) were dried and dissolved in anhydrous pyridine (40 ml). t-Butyldimethylsilyl chloride (0.58 g, 3.84 mmol) was added and the mixture was stirred at room temperature for 20 h (progress of the reaction controlled by TLC). Removal of solvent in vacuo yielded a residue which was dissolved in methylene chloride and washed with phosphate buffer, pH 7.5; then the solvent was removed in vacuo, giving a colourless foam (2.5 g). Chromatography on silica gel (hexane-methylene chloride-ethyl acetate, 5:3:0 to 0.5) gave the isomer 2′-O-tBDMSi 13 (1.03 g, 1.37 mmol, 43%), TLC Rf = 0.33 (hexane-methylene chloride-ethyl acetate, 6:3:1). ¹H NMR (CDCl₃): 8.31 - 6.84 (m, 19H, Bz, DMTTr and H6), 6.07 (d, 1H, J₁= 4.9Hz, H1'), 4.50 (t, 1H, J₁= 4.9Hz, H2'), 4.32 (m, 1H, H3'), 4.18 (m, 1H, H4'), 3.56 (dd, 1H, J₄= 2.2Hz, J₅= 10.9Hz, H5'), 3.40 (dd, 1H, J₄= 2.1, J₅= 10.9Hz), 2.71 (s, 1H, OH), 1.54 (s, 3H, 5-CH₃), 0.93 (s, 9H, t-Bu), 0.17, 0.16 (2 × s, 2 × 3H, Si-CH₃), and the isomer 2′-O-tBDMSi 14 (0.93 g, 1.24 mmol, 39%), TLC Rf = 0.2 (hexane-methylene chloride-ethyl acetate, 6:3:1).

**General procedures for synthesis of 3′-O-phosphoramidites.**

**5′-O-Dimethoxytrityl-2′-O-methyl-N^2-isobutyryl guanosine 3′-O-(2-cyanoethoxy)(N,N-diisopropylamino)phosphoramidite 15.** Compound 2a (334 mg, 0.5 mmol) and N,N-diisopropylammonium tetrazolide (43 mg, 0.25 mmol) were placed in a 25 ml flask equipped with septum and venting needle. The flask was dried in a vacuum dessicator at 1 torr for 4 h, then dry argon gas was let into dessicator. Acetonitrile (2.5 ml) was added to the flask and, after dissolution of solids, bis(N,N-diisopropylamino)(2-cyanoethoxy)phosphine (166 ml, 166 mg, 0.55 mmole) was injected. The solution was left at room temperature overnight. Triethylamine (1 ml) followed by said sodium chloride (5 ml) and ethyl acetate (5 ml) were added and the layers were separated. The aqueous layer was extracted with ethyl acetate (5 ml), combined extracts were dried (sodium sulphate) and evaporated to a foam. Chromatography on silica gel with hexane-ethyl acetate-triethylamine (30:70:1 to 10:90:1) as the eluent gave 15 as a foam which was dissolved in benzene-1% triethylamine (6 ml/mmol), filtered through Millipore filter and freeze-dried, leaving a white powder (353 mg, 81%). ³¹P NMR (CH₃CN): 149.95; 149.82.

**5′-O-Dimethoxytrityl-2′-O-methyl-N^4-benzoyl cytidine 3′-O-(2-cyanoethoxy)(N,N-diisopropylamino)phosphoramidite 17.** 5′-O-Dimethoxytrityl-2′-O-methyl-N^4-benzoyl cytidine 16 [8] (920 mg, 1.386 mmole) was phosphorylated in the same manner as 2a. The crude phosphoramidite was purified by reverse phase chromatography on silanised silica gel with acetonitrile-water-triethylamine (40:60:3 to 60:40:3), as the eluent. Fractions containing the product were concentrated under reduced pressure to remove acetonitrile and the residue was extracted with ethyl acetate, evaporated to a foam, dissolved in benzene-1%
triethylamine (6 ml/mmol), filtered through Millipore filter and freeze-dried. Yield 780 mg of 6b (0.903 mmol, 65%). $^{31}$P NMR (CD$_3$CN): 149.82; 149.28.

$^{13}$C-Benzoyl-2'-O-$^{13}$C-methyl-5'-O-dimethoxytrityl cytidine 3'-O-(2-cyanoethoxy)-(N,N-diisopropylamino)phosphoramidite 18. Compound 9 (380 mg, 0.5 mmole) was phosphorylated in the same manner as 2a and gave the amide 18 as a foam (396 mg, 80%), which was dissolved in benzene-1% triethylamine (6 ml/mmoll) filtered through Millipore filter and freeze-dried. $^{31}$P NMR (CDCl$_3$): 150.67; 150.13.

5'-O-Dimethoxytrityl-2'-O-t-butyldimethylsilyl-N'-isobutyryl guanosine 3'-O-(2-cyanoethoxy)-(N,N-diisopropylamino)phosphoramidite 20. 5'-O-Dimethoxytrityl-2'-O-t-butyldimethylsilyl-N'-isobutyryl guanosine 19 [6] (377 mg, 0.5 mmol) and tetrazole (33.2 mg, 0.475 mmol) were dried together in a flask equipped with septum and venting needle (1 torr, 6 h). Dry argon was let into the desiccator and anhydrous acetonitrile (2.5 ml) was added to the flask. Bis(N,N-diisopropylamino)(2-cyanoethoxy)phosphine (175 ml, 175 mg, 0.58 mmol) was added through the septum and the reaction mixture was left overnight. TLC on Whatman C-18 reverse phase (acetonitrile-water-triethylamine, 14:5:1) showed only traces of starting material (silica gel TLC showed the substrate and product spots very close to each other and control of the reaction progress was dubious). The reaction was quenched with triethylamine (1 ml) and satd. sodium chloride (5 ml) and the product was extracted with ethyl acetate (10 ml). The aqueous phase was extracted with ethyl acetate (2 x 10 ml), combined extracts were evaporated and the resulting foam was chromatographed on silica gel with hexane-ethyl acetate 1:1 containing 0.1% pyridine as the eluent. The product 20 was obtained as a foam, which was dissolved in benzene-1% triethylamine (6 ml/mmol) filtered through the Millipore filter and freeze-dried. Yield 190 mg of 20 (0.2 mmol, 40%). $^{31}$P NMR (CH$_3$CN): 148.91, 151.00.

5'-O-Dimethoxytrityl-2'-O-t-butyldimethylsilyl-N'-benzoyl-5-methyl cytidine 3'-O-(2-cyanoethoxy)(N,N-diisopropylamino)phosphoramidite 21. Compound 13 (389 mg, 0.5 mmol) was phosphorylated in the same manner as 19 with tetrazole (33.25 mg, 0.475 mmol) and bis(N,N-diisopropylamino)(2-cyanoethoxy)-phosphine (187 mg, 0.62 mmol) and after chromatography (hexane-ethyl acetate-triethylamine, 85:15:1 to 75:25:1) and freeze-drying 21 was obtained as a white powder (389 mg, 0.39 mmol, 77%). $^{31}$P NMR (CH$_3$CN): 149.16, 150.62.

**Synthesis of nucleotide-loaded solid supports**

Modified supports were prepared according to a standard procedure [11], starting from protected nucleosides 2a and 14. The amount of the nucleoside attached to the support LCAA-CPG was estimated as 27 μmol/g for 2'-O-methyl G and 31 μmol/g for 2'-O-t-butyldimethylsilyl G.

**Synthesis of modified RNA oligomers**

The oligomers were synthesised on Applied Biosystems synthesizer PCR-Mate DNA/RNA model 391. Standard Applied Biosystems protocols for 1μmol scale were used except prolonged condensation time (STEP 13) which was 60 s for 2'-O-methyl derivatives and 600 s for 2'-O-silylated synths. Average coupling yield (judged from the extinction of trityl cation released) was 98% per step for 2'-O-methyl and 96.1% per step for 2'-O-silyl.

2'-O-$^{13}$C-Methyl-CGGCGG

The $^{13}$C-labelled oligomer-loaded CPG support collected from six 1 μmol synthetic runs was treated with concn. ammonia (1 ml) for 30 min in Eppendorf tube, the solid was centrifuged off, the supernatant transferred to a sealed vial, the support treated twice more with 0.5 ml portions of concd. ammonia for 30 min, and supernatants pooled with the first one. The vial was tightly capped and heated at 55°C for 16 h. After cooling the vial was opened, the residue was evaporated under diminished pressure and the resulting solid was dissolved in deionised water (0.5 ml) and filtered through Millipore filter. The tube and filter were washed with additional 1 ml water and the resulting solution (200 A$_{260}$ units) was separated on HPLC and eluted with a gradient starting from 100% buffer A for 5 min, then up to 70% buffer A, 30% buffer B for 35 min, flow 5 ml/min, Nova-Pak 25 x 100 mm cartridge. The fraction containing the main peak (retention time 24.8 min) was evaporated to dryness, dissolved in 1 ml deionised water and desalted on a column of Sephadex G-25 (20 x 170 mm). Evaporation of the appropriate fraction gave
the hexamer as a white solid (85 A_{260} units). UV (pH 7): \lambda_{\text{max}} 257 \text{ nm}.

The unlabelled hexamer (from 8 \times 1 \mu\text{mol} runs) was deprotected and purified in exactly the same way, giving 100 A_{260} units of pure oligomer. The two samples were combined and prepared for NMR studies. Due to stability of 2'-O-methylated RNAs to enzymatic and chemical digestion, the hexamer was characterised by its \textsuperscript{1}H NMR spectrum (D_2O, external DSS as reference): 8.03 (d, 1H, J 7.5\text{Hz}, H6), 7.83 (s, 1H, H8), 7.73 (d, 1H, J 7.5\text{Hz}, H6), 7.67 (d, 1H, J 7.5\text{Hz}, H6), 7.58 (s, 1H, H8), 7.55 (s, 1H, H8), 6.02 - 6.00 (m, 2H, H1' + H5), 5.92 (s, 2H, 2 \times H3'), 5.71 (s, 1H, H1'), 5.69 (s, 2H, 2 \times H1'), 5.30 (d, 1H, J 7.5\text{Hz}, H5), 5.25 (d, 1H, J 7.5\text{Hz}, H5), 4.56 - 3.74 (m, 30 H, ribose), 3.66, 3.62, 3.53, 3.50, 3.46, 3.33 (6 \times s, 6 \times 3H, 6 \times \text{OCH}_3), \text{C}^{13} \text{ satellites at 3.74 and 3.26 (10%) and 3.70 and 3.22 (20%)}, corresponding to peaks at 3.50 and 3.46. \text{C}^13 \text{NMR: two prominent peaks at 59.37 (smaller) and 59.28 (larger).}

m\textsuperscript{5}CGm\textsuperscript{5}CGm\textsuperscript{5}CG

The oligomer-loaded CPG was treated with cold (0°C) concd. ammonia-ethanol (1:3) for 30 min, the supernatant was transferred to a sealed vial, the support was treated with two additional portions of ethanolic ammonia for 30 min and supernatants were pooled. The vial was tightly capped and heated at 55°C for 24 h. After cooling, the solvents were evaporated to dryness and the residue was dissolved in 1 M TBAP/THF (1 ml). After 24 h the reaction was quenched by addition of 0.1 M TEAA buffer, pH 7.5 (1 ml), the solution was concentrated to approx. 0.5 ml and applied on Biogel P-2 column (20 \times 200 mm). The oligomer was eluted with sterile water and the oligomer fraction was passed through Dowex 50 \times 8 column (10 \times 30 mm) in ammonium form. This procedure was repeated for each of 1 \mu\text{mol} synthesis runs separately (3 \times). Typical yield of desalted oligomer was 23 - 25 A_{260} units per synthesis. The appropriate fractions were pooled, evaporated, purified by HPLC (conditions as for the 2'-O-methyl hexamer, retention time 18.8 min) and desalted as above, giving 60 A_{260} units of pure oligomer. UV (pH 7, \lambda_{\text{max}} 260 \text{ nm}). An aliquot (2 A_{260} units) was digested with the mixture of ribonuclease P1 and alkaline phosphatase, pH 7.0 (Tris/HCl) and the mixture analysed by HPLC, aided with an diode array UV detector, showing the expected 1:1 ratio of nucleosides (m\textsuperscript{5}C.G).

RESULTS AND DISCUSSION

Both modified oligoribonucleotides were obtained \textit{via} solid support-aided phosphite phosphotriester synthesis using an automated DNA/RNA synthesizer [12]. In the course of the synthesis two major issues had to be taken into account: (i) synthesis of modified nucleoside (2-cyanooethyl) phosphorimidates 15, 17, 18, 20 and 21 as synthons for oligomer assembly, and (ii) improvement in the purification procedures concerning 2'-O-t-butyldimethylsilyl protected hexamer (m\textsuperscript{5}CG)\textsubscript{3} in view of its further use for NMR studies.

\textbf{Modified ribonucleoside 3'-O-phosphorimidates 15, 17, 18, 20 and 21}

Due to intrinsic properties of the aglycone, 2'-O-methylation of guanosine is troublesome. Methylolation of 5'-O-dimethoxytrityl-N\textsuperscript{2}-isobutyryl guanosine 1, using diazomethane - Sn(II) chloride method [8] gave a moderate yield of both 2'- and 3'-O-methyl isomers (2a, 2b) along with side products. Straightforward separation of this mixture on a large scale is uneconomical. The following procedure was developed to obtain 2a (Scheme 1).

To isolate the desired 2'-isomer 2a, selective and simultaneous protection of 5'- and 3'-OH groups of ribonucleoside moieties with dichloro-tetraisopropyl-disiloxane (Markiewicz reagent) [5] was applied. Isomeric mixture was subjected to 5'-O-detrylylation with acetic acid and subsequent silylation with the above reagent to obtain two products: a major one, the 3'-5' cyclic silylated derivative 4 arising from the 2'-OCH\textsubscript{3} isomer and a minor one arising from the 3'OH isomer bearing open-chain 2',5'-disilyl substituents 5. The presence of an additional lipophilic group in the latter compound caused a large difference in its chromatographic properties and, even on a large scale, simple chromatography on silica gel gave pure isomer of 2'-OCH\textsubscript{3} guanosine as a silyl derivative 4. Desilylation of 4 with triethylammonium hydrofluoride [5] and subsequent dimethoxytritylation gave the desired pure protected nucleoside 2a, the 3'-OH component for subsequent phosphitylation. We propose the
above procedure as advantageous one over that recently reported [13] or multistep the procedure recently offered, applying guanosine lactam-protected intermediates [14].

The appropriate $^{13}$C-labelled 2'-O-methyl cytidine 3'-OH component 9 was prepared according to Scheme 2.

5',3'-Tetraisopropylidilsilyloxy-N$^9$-benzoyl cytidine 6 [5] was subjected to 2'-O-methylation with $[^{13}$C]methyl iodide and silver oxide in anhydrous benzene. The procedure was modified from that earlier discussed [7] to use a minimal amount of methyl iodide and gave the 2'-O-$[^{13}$C]methylated product with 63% yield. Further steps were conducted as in case of guanosine component. Unlabelled 2'-O-methylcytidine 3'-OH component 16 was prepared by the diazomethane - Sn(II) chloride method [8].

For preparation of the 5-methylcytidine derivative 13, 5-methyluridine was synthesised from 5-methyluracil by a standard procedure [15]. Two routes of its transformation into 5-methylcytidine were tested. First we tried the method via the 4-(1,2,4-triazolyl) derivatives [16]. Although the triazole intermediate was widely used as an intermediate to various 4-substituted pyrimidines [16], we found that the ammonolysis of the 4-(1,2,4-triazolyl)-5-methyl derivative always led to a mixture of 4-amino and 4-oxo products, irrespective of changing reaction conditions (not shown). When n-butyramine was used as the nucleophile in a

I, Ag₂O/I₃CCH₃; II, TEAHF/THF; III, DMTCl/py.

The model experiment, the amination smoothly gave the N⁴-butylic derivative as the sole product. Therefore the previously published procedure using 4-thio derivative was used. Thiation of 2,3,5-tri-O-acetyl-5-methyluridine with phosphorus pentasulfide gave the 4-thio analogue, which upon treatment with concentrated aqueous ammonia under pressure gave the desired 5-methylcytidine as the sole product in a good yield. During its N⁴-benzoylation via the "transition protection" method [17] (O-silylation, N-benzoylation, O-desilylation), the initially formed N⁴-benzoyl-5-methyl cytidine was largely hydrolysed back to the parent nucleoside during the preparation. Finally, the benzoylation with benzoic anhydride in ethyl alcohol, as described for 5-fluoro-2’-deoxyctydine [18], gave the desired product with a good yield. The 5’-O-dimethoxytritylation and 2’(3’)-O-silylation of m⁵C was accomplished according to a standard procedure [6] and the desired 5’-O-dimethoxytrityl-2’-O-t-butyldimethylsilyl-N⁴-benzoyl-5-methylcytidine was isolated in 18% yield from the nucleoside.

The silylated nucleosides 13 and 14 were obtained by a described procedure [6] and purified by silica gel chromatography.

In all cases the protected nucleosides were phosphitylated with bis(N,N-diisopropylamino)(2-cyanoethoxy)phosphine, according to Barone et al. [4] except that tetrazole was used as a catalyst in the case of silylated derivatives in order to avoid silyl group isomerisation [19] (Scheme 3).

The purity of phosphoramidites was controlled by 31P NMR. For the analysis of 2’-O-silylated phosphoramidites formation RP-18 TLC plates were used.

Synthesis of 2’-O-[13C]methyl-CGGCGG and m⁵CGm⁵CGm⁸CG

The hexamers were assembled using Applied Biosystems automatic synthesizer in 1 μmol scale (several runs) with modified standard protocols (see Materials and Methods). To achieve varying abundance of isotope the labelled 2’-O-methyl group in 2’-O-methylated hexamer, the isotope-labelled and non-labelled cytidine phosphoramidites were used applying additionally port X (for the modified base) of the synthesizer. This procedure allows to get relative proportions of the label on 2’-O-methyl (CGCGCG) as (5’ to 3’): 100% labelled, 50% labelled and 0% labelled (natural abundance). This isotope distribution will allow for easy identification of cytidine 2’-O-methyls in the 1H and 13C NMR spectra.

Deprotection and purification of 2’-O-[13C]methyl CGCGCG

2’-O-[13C]Methyl hexamer was cleaved off the LCAA-CPG support and treated with concn. ammonia for 16 h at 55°C. After cooling, the solution was evaporated to dryness, the residue was dissolved in deionised water, filtered through Millipore filter, purified by
Scheme 3. Preparation of protected 3'-O-ribonucleoside phosphoramidites.
I, (iPr2)NH-tetrazole; II, tetrazole.

HPLC and desalted on Sephadex G-25. Its structure was confirmed by $^1$H NMR.

Deprotection and purification of m$^5$CGm$^5$CG

2′-O-Silylated hexamer was cleaved off LCAA-CPG support with cold 35% ammonia/ethanol (3:1) and left at 55°C for 16 h [20]. The residue after evaporation of solvents was dissolved in 1 M TBAF/THF (approx. 160 eq. per silyl group). After 16 h the reaction was quenched with 0.1 M TEA buffer and procedure continued. The mixture after desilylation contained, in addition to the desired hexamer, a large excess of deblocking reagent, tetrabutylammonium fluoride. In view of further NMR applications, the tetrabutylammonium cations should be completely removed from the sample. This problem was not encountered earlier since most of the RNA syntheses using 2′-O-silyl protection were aimed at functional RNA studies [6, 21 - 23]. We tested the efficiency of different routes to achieve this separation, verifying it by estimation of the remaining tetrabutylammonium cation with $^1$H NMR. The newly reported [24] reverse phase HPLC on PRP-1 polystyrene phase proved to be unsatisfactory for such a short oligomer. Because of relatively low molecular weight of the oligomer we had to exclude dialysis; finally, the gel filtration chromatography on Biogel P-2 turned out to be the method of choice. The sample after Biogel P-2 and Dowex 50W × 8 (ammonium form) chromatography was homogeneous and showed a negligible level of tetrabutylammonium cation by $^1$H NMR. The sample could then be finally purified by reverse phase HPLC. The purified m$^5$CGm$^5$CG hexamer was digested to reveal the expected 1:1 ratio of m$^5$C to G.

This is worth to note that the fluoride ion acting as a very reactive desilylating agent in anhydrous THF, retains some of the activity even when diluted with aqueous buffer. This fact can lead to cleaving off the octadecylysilyl groups from the column stationary phase during the HPLC separation, especially in large-scale synthesis, when the amount of fluoride to be applied is significant.

REFERENCES


