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

Ewa Biała[a], Jan Milecki[b], Andrzej Kowalewski[a], Mariusz Popenda[a], Wiesław Z. Antkowiak[b] and Ryszard W. Adamiak[a]**

[a]*Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61–704 Poznań, Z. Noskowskiego 12/14,*
[b]*Faculty of Chemistry, Adam Mickiewicz University, Poznań, Poland*

Chemical synthesis of 2'-*O*-[^{13}C]methyl-rCGCGCG and 5-methyl-rCGCGCG 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-tetraisopropyldisiloxane (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-CGCGCG 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-CGCGCG duplex, namely 2'-*O*-[^{13}C]methyl CGCGCG and m^{5}CGm^{5}CGm^{5}CG obtained in view of further ^{1}H and ^{13}C 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 P$_2$O$_5$ and then CaH$_2$ and stored over 4A molecular sieves. Ethyl acetate, methylene chloride and chloroform were freshly distilled over anhydr. potassium carbonate,

**To whom correspondence should be addressed.
[1]Abbreviations used throughout the text: m^{5}C, 5-methylcytidine; THF, tetrahydrofuran; TEAHF, triethylammonium hydrofluoride; TBAF, tetrabutylammonium fluoride; CPG, controlled pore glass; LCAA-CPG, long chain alkylamine-modified controlled pore glass; TEAA, triethylammonium acetate; tBDMSi, t-butyldimethylsilyl; TiPSiDCl, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane.

then chloroform and methylene chloride were stabilised with 0.5% 2-propanol. Benzene was distilled from sodium wire. Dioxane, THF[1], triethylamine (Fluka), and N,N-diisopropylamine (Fluka) were distilled from CaH_2 and stored over 4A 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-tetraisoproyldisiloxane was prepared according to [5]. ^{13}C-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 anhydr. 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 × 300 mm steel column filled with PRP-1 phase (Hamilton) and Nova-Pak C-18 25 × 100 mm cartridge (Waters) for preparative runs and Nova-Pak C-18 8 × 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 (^{1}H, 300 MHz, ^{13}C, 75.4 MHz and ^{31}P, 36.2 MHz) spectrometer. Chemical shifts are reported in ppm relative to TMS (internal standard) for ^{1}H and ^{13}C, and 85% phosphoric acid (external standard) for [31]P. 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-N[2]-isobutyryl guanosine 14 [6] (cf. Scheme 3, below) and 5'-O-dimethoxytrityl-2'-O-methyl-N[4]-benzoyl

cytidine 16 [7, 8] were prepared according to reported procedures.

5'-O-Dimethoxytrityl-2'(3')-O-methyl-N[2]-isobutyryl guanosine (mixture of 2a and 2b). 5'-O-Dimethoxytrityl-N[2]-isobutyryl guanosine 1 [6] (2.34 g, 3.75 mmol) and Sn(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%). ^{1}H NMR (CDCl3): 8.15-7.10 (m, 14H, H8, DMTr), 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, DMTr 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-N[2]-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 R_F = 0.33 (benzene-ethyl acetate, 1:1).^{1}H NMR (CDCl3): 8.02 (s, 1H, H8), 5.86 (s, 1H, H1'), 4.53 (dd, 1H, J2'3' 4.8Hz, J3'4' 9.3Hz, H3'), 4.22 (d, 1H, J5'5'' 13.3Hz, H5'), 4.12 (dd, 1H, J3'4' 9.3Hz, J4'5'' 2.4Hz, H 4'), 4.02 (dd, 1H, J5'5'' 13.3Hz, J4'5''

2.4Hz, H5″), 3.81 (d, 1H, J$_{2'3'}$ 4.3Hz, H2′), 3.65 (s, 3H, OCH$_3$), 2.63 (m, J 6.5Hz, 1H, CH isobutyryl), 1.25 (d, 6H, J 6.5Hz, CH$_3$ isobutyryl), 0.98 (m, 28 H, Si-isopropyl) and the minor one of putative structure 5, (370 mg, solid), TLC R$_F$ = 0.74.

5′-Dimethoxytrityl-2′-O-methyl-N^2-isobutyryl guanosine 2a. Compound 4 (880 mg, 1.443 mmol) was stirred with 1 M solution of triethylammonium hydrofluoride in THF (TEAHF/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 × 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%). ^{1}H NMR (CDCl$_3$): 8.12 (s, 1H, H8), 7.10 - 7.95 (m, 13 H, DMTr), 5.88 (d, 1H, J$_{1'2'}$ 6.3 Hz, H1′), 4.64 (dd, 1H, J$_{1'2'}$ 6.3Hz, J$_{2'3'}$ 5.5Hz, H2′), 4.53 (dd, 1H, J$_{2'3'}$ 5.5Hz, J$_{3'4'}$ 4.3Hz, H3′), 4.19 (m, 1H, H4′), 3.50 (dd, 1H, J$_{4'5'}$ 3.5Hz, J$_{5'5''}$ 11.6Hz, H5′), 3.20 (dd, 1H, J$_{4'5''}$ 3.7Hz, J$_{5'5''}$ 11.6Hz, H5″), 3.63 (s, OCH$_3$), 2.73 (m, 1H, J 6.0Hz CH-isobutyryl), 1.75 (d, 6H, J 6.0Hz, CH$_3$-isobutyryl).

3′,5′-Tetraisopropyldisiloxane-1,3-diyl-2′-O-[^{13}C]methyl-N^4-benzoyl cytidine 7. 3′,5′-Tetraisopropyldisiloxane-1,3-diyl-N^4-benzoyl cytidine 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 [^{13}C]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. ^{1}H NMR (CDCl$_3$): 8.33 (m, 1H, H6), 7.89 - 7.52 (m, 5 H, 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, OCH$_3$, ^{13}C satellites at 3.97 and 3.49), 1.05 (m, 28 H, Si-isopropyl).

2′-O-[^{13}C]Methyl-N^4-benzoyl cytidine 8. Compound 7 (500 mg, 0.82 mmol) was rendered dry by repeated evaporation with dry dioxane (2 × 10 ml), TEAHF/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 × 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%). ^{1}H NMR (CDCl$_3$): 8.42 (d, 1H, J 7.4Hz, H6), 7.5 - 7.9 (m, 6H, Bz, H5), 5.88 (d, 1H, J$_{1'2'}$ 1.9Hz, H1′), 4.35 (m, 1H, H3′), 4.12 (m, 1H, H4′), 4.02 - 4.10 (m, 2H, H5′, H5″), 3.96 (m, 1H, H2′), 3.69 (s, 3H, OCH$_3$, ^{13}C- satellites at 3.93 and 3.45). ^{13}C NMR (CDCl$_3$): prominent peak at 58.907.

5′-O-Dimethoxytrityl-2′-O-[^{13}C]methyl-N^4-benzoyl cytidine 9. Dry 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%). ^{1}H NMR (CDCl$_3$): 8.60 (d, 1H, J 7.4Hz, H6), 7.90 - 6.90 (m, 19H, Bz, DMTr, H5), 6.04 (s, 1H, H1′), 4.47 (d, 1H, J$_{3'4'}$ 9.2Hz, H3′), 4.05 (m, 1H, J$_{3'4'}$ 9.2Hz, J$_{4'5'}$ 2.2Hz, J$_{4'5''}$ 2.6Hz, H4′), 3.81 (s, 1H, H2′), 3.75 (s, 3H, OCH$_3$, ^{13}C-satellites at 3.50 and 4.0), 3.63 (dd, 1H, J$_{5'5''}$ 11.4Hz, J$_{4'5'}$ 2.2Hz, H5′), 3.56 (dd, 1H, J$_{5'5'}$ 11.4Hz, J$_{4'5''}$ 2.6Hz, H5″).

N^4-Benzoyl-5-methylcytidine 11. 5-Methyl-cytidine 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, $J_{1'2'}$ 4.5Hz, H1'), 4.47 (dd, 1H, $J_{1'2'}$ 4.5Hz, $J_{2'3'}$ 6Hz, H2'), 4.39 (m, 1H, H3'), 4.26 (m, 1H, H4'), 4.02 (dd, 1H, $J_{4'5'}$ 2Hz, $J_{5'5''}$ 12Hz, H5'), 3.85 (dd, 1H, $J_{4'5''}$ 3Hz, $J_{4'5'}$ 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, DMTr, H6), 5.95 (d, 1H, $J_{1'2'}$ 4.5Hz, H1'), 4.42 (dd, 1H, $J_{1'2'}$ 4.5Hz, $J_{2'3'}$ 6.1Hz, H2'), 4.39 (m, 1H, H3'), 4.16 (m, 1H, H4'), 3.55 (dd, 1H, $J_{4'5'}$ 2Hz, $J_{5'5''}$ 11Hz, H5'), 3.45 (dd, 1H, $J_{4'5''}$ 2.3Hz, $J_{5'5''}$ 11Hz, H5''), 2.09 (s, 3H, 5-CH3).

5'-O-Dimethoxytrityl-2'-O-t-butyldimeth-ylsilyl-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 R_F = 0.33 (hexane-methylene chloride-ethyl acetate, 6:3:1), ^{1}H NMR (CDCl3):

8.31 - 6.84 (m, 19H, Bz, DMTr and H6), 6.07 (d, 1H, $J_{1'2'}$ 4.9Hz, H1'), 4.50 (t, 1H, $J_{1'2'}$ 4.9Hz, H2'), 4.32 (m, 1H, H3'), 4.18 (m, 1H, H4'), 3.56 (dd, 1H, $J_{4'5'}$ 2.2Hz, $J_{5'5''}$ 10.9Hz, H5'), 3.40 (dd, 1H, $J_{4'5''}$ 2.1, $J_{5'5''}$ 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 3'-O-tBDMSi 14 (0.93 g, 1.24 mmol, 39%), TLC R_F = 0.2 (hexane-methylene chloride-ethyl acetate, 6:3:1).

General procedures for synthesis of 3'-O-phos-phoramidites.

5'-O-Dimethoxytrityl-2'-O-methyl-N^2-iso-butyryl 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 satd. 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%). ^{31}P NMR (CH3CN): 149.95; 149.82.

5'-O-Dimethoxytrityl-2'-O-methyl-N^4-ben-zoyl cytidine 3'-O-(2-cyanoethoxy)(N,N-di-isopropylamino)phosphoramidite 17. 5'-O-Di-methoxytrityl-2'-O-methyl-N^4-benzoyl cytidine 16 [8] (920 mg, 1.386 mmole) was phosphitylated 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 (CH3CN): 149.82; 149.28.

N^4-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 phosphitylated in the same manner as 2a and gave the amidite 18 as a foam (396 mg, 80%), which was dissolved in benzene-1% triethylamine (6 ml/mmol) filtered through Millipore filter and freeze-dried. ^{31}P NMR (CDCl3): 150.67; 150.13.

5'-O-Dimethoxytrityl-2'-O-t-butyldimethylsilyl-N^2-isobutyryl guanosine 3'-O-(2-cyanoethoxy)(N,N-diisopropylamino)phosphoramidite 20. 5'-O-Dimethoxytrityl-2'-O-t-butyldimethylsilyl-N^2-isobutyril 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 dessicator 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 × 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 (CH3CN): 148.91, 151.00.

5'-O-Dimethoxytrityl-2'-O-t-butyldimethylsilyl-N^4-benzoyl-5-methyl cytidine 3'-O-(2-cyanoethyl)(N,N-diisopropylamino)phos-p horamidite 21. Compound 13 (389 mg, 0.5 mmol) was phosphitylated 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 (CH3CN): 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 synthons. 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-CGCGCG

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 Eppendorff 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 A260 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 × 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 × 170 mm). Evaporation of the appropriate fraction gave

the hexamer as a white solid (85 A_{260} units). UV (pH 7): λ_{max} 257 nm.

The unlabelled hexamer (from 8×1 μmol 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 ^{1}H NMR spectrum (D_2O, external DSS as reference): 8.03 (d,1H, J 7.5Hz, H6), 7.83 (s, 1H, H8), 7.73 (d, 1H, J 7.5Hz, H6), 7.67 (d, 1H, J 7.5.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 × H1'), 5.71 (s, 1H, H1'), 5.69 (s, 2H, 2 × H1'), 5.30 (d, 1H, J 7.5Hz, H5), 5.25 (d, 1H, J 7.5Hz, H5), 4.56 - 3.74 (m, 30 H, ribose), 3.66, 3.62, 3.53, 3.50, 3.46, 3.33 (6 × s, 6 × 3H, 6 × OCH$_3$, ^{13}C satellites at 3.74 and 3.26 (10%) and 3.70 and 3.22 (20%), corresponding to peaks at 3.50 and 3.46). ^{13}C NMR: two prominent peaks at 59.37 (smaller) and 59.28 (larger).

$m^5CGm^5CGm^5CG$

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 TBAF/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 × 200 mm). The oligomer was eluted with sterile water and the oligomer fraction was passed through Dowex 50 × 8 column (10 × 30 mm) in ammonium form. This procedure was repeated for each of 1 μmole synthesis runs separately (3×). 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, λ_{max} 260 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^5C:G).

RESULTS AND DISCUSSION

Both modified oligoribonucleotides were obtained 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-cyanoethyl) phosphoramidites 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^5CG)$_3$ in view of its further use for NMR studies.

Modified ribonucleoside 3'-O-phosphoramidites 15, 17, 18, 20 and 21

Due to intrinsic properties of the aglycone, 2'-O-methylation of guanosine is troublesome. Methylation of 5'-O-dimethoxytrityl-N^2-isobutyril 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-detritylation 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$_3$ isomer and a minor one arising from the 3'-OCH$_3$ 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$_3$ 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
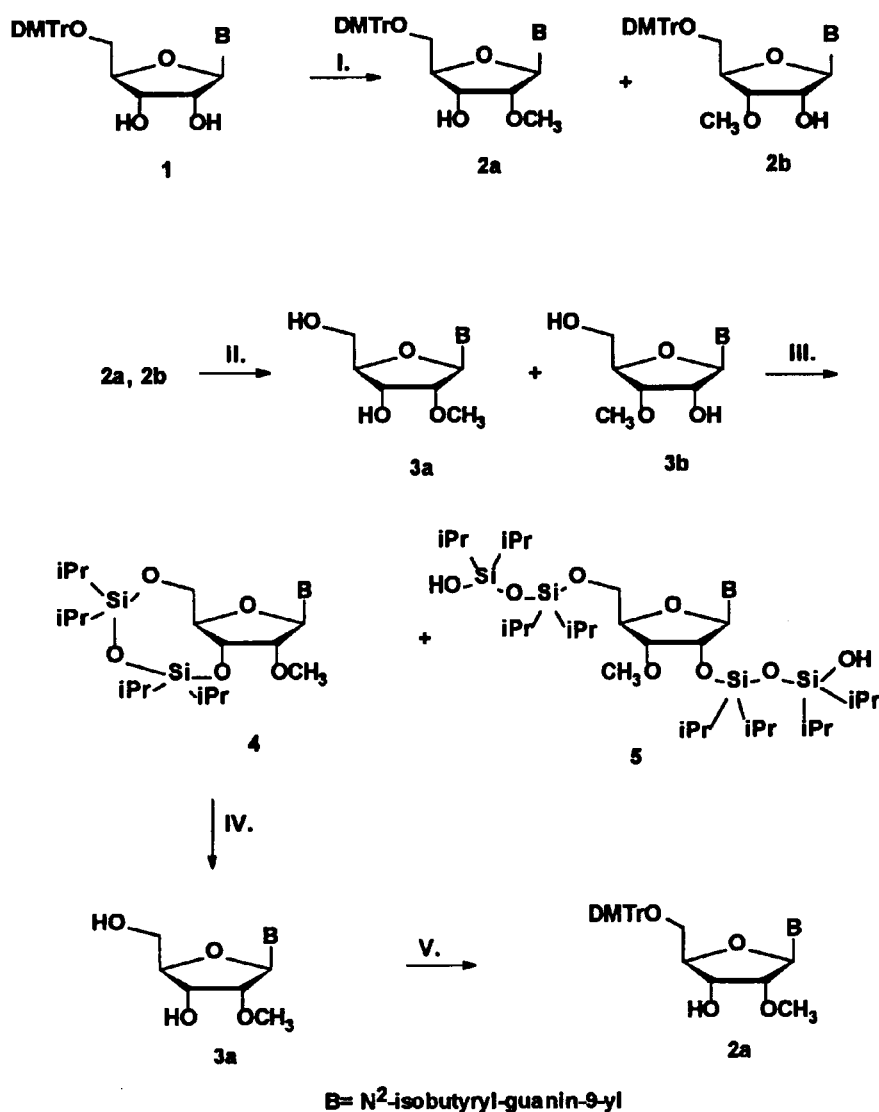
Scheme 1. *Preparation of pure 5'-O-dimethoxytrityl-2'-O-methyl-N²-isobutyril guanosine.*
I, SnCl₂-2H₂O, CH₂N₂/CH₂Cl₂; II, 80% AcOH; III, TiPSiDCl/Py; IV, TEAHF/THF; V, DMTrCl/Py.

above procedure as advantageous one over that recently reported [13] or multistep the procedure recently offered, applying guanosine lactam-protected intermediates [14].

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

5',3'-Tetraisopropyldisilyloxy-N⁴-benzoyl cytidine **6** [5] was subjected to 2'-O-methylation with [¹³C]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-[¹³C]methylated product with 63% yield. Further steps were conducted as in case of guanosine component. Unlabelled 2'-O-methylcy-

tidine 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-butylamine was used as the nucleophile in a

B= N⁴-benzoyl-cytosin-1-yl
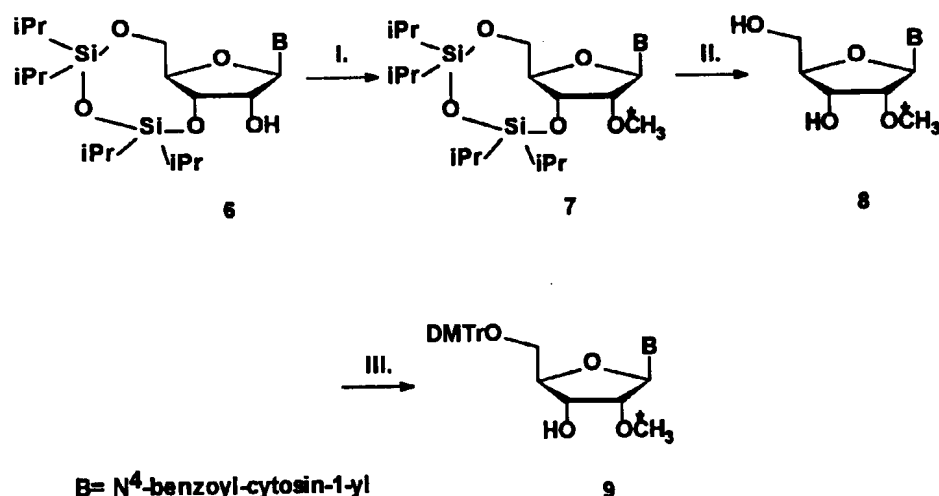
Scheme 2. Preparation of 5'-O-dimethoxytrityl-2'-O-[^{13}C]methyl-N^4-benzoyl cytidine.
I, Ag$_2$O/[^{13}C]CH$_3$I; II, TEAHF/THF; III, DMTrCl/Py.

model experiment, the amination smoothly gave the N^4-butyl derivative as the sole product. Therefore the previously published procedure using 4-thio derivative [10] was used. Thiation of 2,3,5-tri-O-acetyl-5-methyluridine with phosphorus pentasulfide gave the 4-thio analoge, which upon treatment with concentrated aqueous ammonia under pressure gave the desired 5-methylcytidine as the sole product in a good yield. During its N^4-benzoylation *via* the "transition protection" method [17] (O-silylation, N-benzoylation, O-desilylation), the initially formed N^4-benzoyl-5-methyl cytidine was largely hydrolysed back to the parent nucleoside during the preparation. Finaly, the benzoylation with benzoic anhydride in ethyl alcohol, as described for 5-fluoro-2'-deoxycytidine [18], gave the desired product with a good yield. The 5'-O-dimethoxytritylation and 2'(3')-O-silylation of m^{5}C was accomplished according to a standard procedure [6] and the desired 5'-O-dimethoxytrityl-2'-O-t-butyldimethylsilyl-N^4-benzoyl-5-methylcytidine 13 was isolated in 18% overall 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 deri-

vatives in order to avoid silyl group isomerisation [19] (Scheme 3).

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

Synthesis of 2'-O-[^{13}C]methyl-CGCGCG and m^{5}CGm^{5}CGm^{5}CG

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 ^{1}H and ^{13}C NMR spectra.

Deprotection and purification of 2'-O-[^{13}C]methyl CGCGCG

2'-O-[^{13}C]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
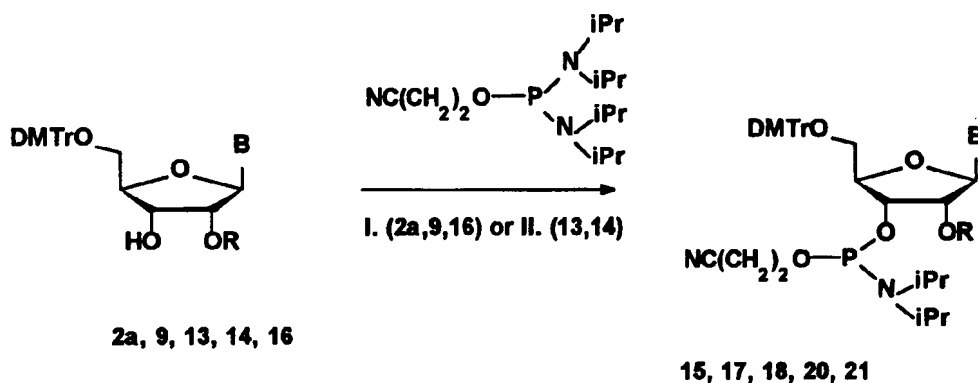
2a, 15   B= N^2-isobutyryl-guanin-9-yl,   R=CH$_3$

9, 18   B= N^4-benzoyl-cytosin-1-yl,   R=(^{13}C)CH$_3$

16, 17   B= N^4-benzoyl-cytosin-1-yl,   R=CH$_3$

14, 20   B= N^2-isobutyryl-guanin-9-yl,   R=tBuMe$_2$Si

13, 21   B= N^4-benzoyl-5-methyl-cytosin-1-yl,   R=tBuMe$_2$Si

Scheme 3. Preparation of protected 3'-O-ribonucleoside phosphoramidites.
I, (iPr$_2$)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}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 TEEA buffer and procedure continued. The mixture after desilylation contained, in addition to the desired hexamer, a large excess of deblocking reagent, tetra-n-butylammonium 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}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 octadecylsilyl 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.

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