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Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides

Hideo Inoue, Yoji Hayase, Akihiro Imura, Shigenori Iwai, Kazunobu Miura and Eiko Ohtsuka

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

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ABSTRACT

2'-O-Methyl derivatives of the common ribonucleosides except for guanosine were synthesized via the 2'-O-methylation of appropriately-protected nucleosides with CH_3I in the presence of Ag_2O. The 2'-O-methylguanosine derivative was prepared by the monomethylation of a 2',3'-cis-diol system with diazomethane. These derivatives were converted to protected 2'-O-methylribonucleoside 3'-phosphates and used for oligonucleotide synthesis on polymer supports. Thus, oligo(2'-O-methylribonucleotides) having the sequence identical to the consensus sequence of the 5'-splice junction CAGGUAAGU and its complement were synthesized in a stepwise manner using the phosphotriester method. Thermal stabilities (Tm's) of the duplex of these 2'-O-methyl ribo-oligomers and eight related duplexes containing ribo- or deoxyribo-oligomers were examined. It was found that the 2'-O-methyl oligoribonucleotides can be utilized as an alternative to an oligoribonucleotide probe in RNA hybridizations as the hybrid formed has a high, or a higher Tm, the probe is much easier to synthesize and it is less likely to be enzymatically degraded.

INTRODUCTION

Because 2'-O-methyl ethers of common ribonucleosides have been found as minor components of RNAs [1], nucleosides, nucleotides [2] and oligoribonucleotides [3] containing these residues have been synthesized in connection with studies on the chemical behaviour, structural and biochemical significance of 2'-O-methylnucleoside residues. Various polymers containing 2'-O-methylribonucleosides have also been prepared and have served as useful tools to examine the role of the 2'-hydroxyl group in stabilizing RNA structure [2]. However, the effect of 2'-O-methylation on the thermal stabilities of the duplexes of ribopolymers with defined sequences has remained uninvestigated.

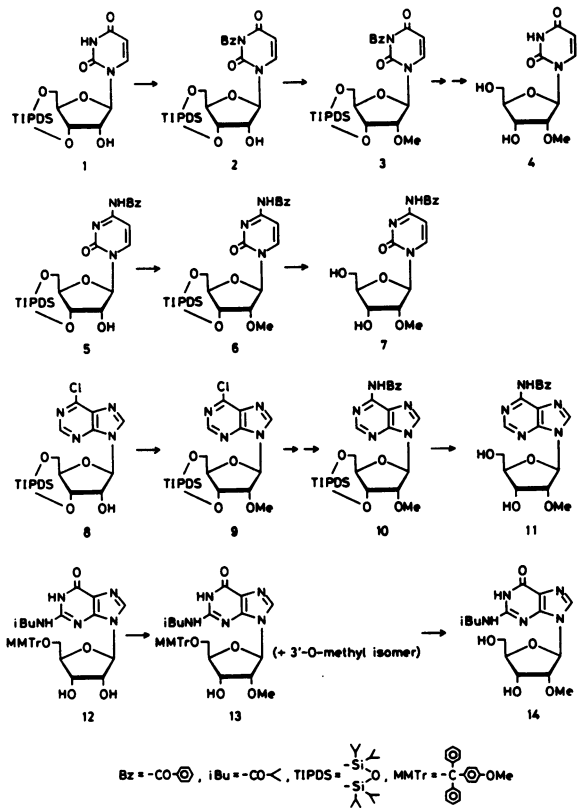
In the course of our studies to search for efficient hybridization probes, we have recently demonstrated that an oligodeoxyribonucleotide containing deoxyinosine is very useful as a mixed probe substitute [4] and that an oligodeoxyribonucleotide containing a pyridopyrimidine nucleoside, as a modified deoxycytidine can be a good hybridization probe because of the ability of the unique base enhancing stability of the hybrid [5]. The present investigation is aimed at developing RNA hybridization probes and this paper deals with the synthesis and hybridization properties of oligo(2'-O-methylribonucleotides) of defined sequence [6].

Since a ribo-oligonucleotide seemed to be preferable to a deoxyribo-oligonucleotide as a probe in view of the thermal stability of hybrids containing RNA [7], we chose oligo(2'-O-methylribonucleotides) as target oligonucleotides. Although oligoribonucleotide synthesis is still inconvenient, 2'-O-methyl oligomers can be easily synthesized in a manner similar to oligodeoxyribonucleotide synthesis providing that the starting 2'-O-methylnucleosides are readily accessible. Furthermore, the use of this oligomer may be enhanced by the findings that homo- and co-polymers containing 2'-O-methyl ribonucleosides are stable to alkaline treatment and RNase digestion, and resistant to digestion by certain nucleolytic enzymes [8,9]. The present 2'-O-methyl oligomers have sequences identical and complementary to the consensus sequence CAGGUAAGU for the 5'-donor sites of splice junctions [10] in precursor mRNAs. The synthesis of the necessary 2'-O-methyl ribonucleosides and their nucleotide units is also reported in this paper.

RESULTS AND DISCUSSION

Synthesis of N-acylated 2'-O-methylribonucleosides

For the synthesis of 2'-O-methylribonucleosides, the most widely used method is the monomethylation of the 2',3'-cis-diol system of a free ribonucleoside with diazomethane in the absence [11-15] or presence [16-19] of $SnCl_2$. However, the reaction gives a mixture of 2'- and 3'-O-methyl isomers and the separation of the products usually requires tedious ion-



Scheme 1

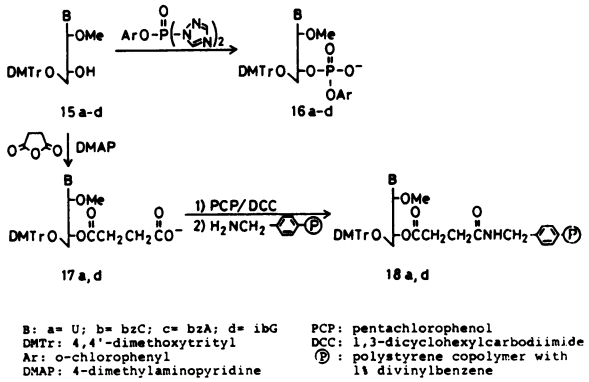
exchange chromatography. To circumvent these problems, the present syntheses mostly involved alkylation with CH_3I [20] of 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)(TIPDS)-ribonucleoside derivatives [21] (Scheme 1).

Treatment of 3',5'-O-TIPDS-uridine (1) with benzoyl chloride (BzCl, 1.1 equiv.) in N,N-dimethylacetamide in the presence of triethylamine (1.3 equiv.) [22] selectively gave the N^3-benzoylated derivative (2) in 70.5% yield. Then, 2 was treated with CH_3I (25 equiv.) in benzene in the presence of Ag_2O (3 equiv.) at 40°C overnight to give the N^3-benzoyl-2'-O-methyl derivative (3, 84.5%). Debenzoylation of 3 with dil. NH_4OH followed by removal of the TIPDS group with 0.5 N HCl afforded 2'-O-methyluridine (4) in 84% yield. Although similar synthetic methods for 4 have been recently reported

[23,24], our route is more efficient. Similarly, 3',5'-O-TIPDS-N^4-benzoylcytidine (5) was converted to the corresponding 2'-O-methyl derivative (6, 69%) by treatment with CH_3I-Ag_2O for 4 hr. If allowed to react longer, methylation also took place at the base moiety. Removal of the TIPDS group afforded N^4-benzoyl-2'-O-methylcytidine (7) in 52.5% yield.

N^6-benzoyl-2'-O-methyladenosine was synthesized by starting from 6-chloro-9-β-D-ribofuranosylpurine, since methylation of a TIPDS-protected N^6-benzoyladenosine preferentially occurred at the base moiety. Treatment of the TIPDS derivative (8) of 6-chloropurine riboside in the absence of solvent with CH_3I in the presence of Ag_2O for 50 min at 40°C gave the desired 2'-O-methyl derivative (9) in 66.3% yield. Compound 9 readily reacted with liq. ammonia in CH_2Cl_2 at room temperature to give the TIPDS derivative of 2'-O-methyladenosine (66%), which was converted into the N^6-benzoyl derivative (10, 89%). Deprotection of the TIPDS group with tetrabutylammonium fluoride gave N^6-benzoyl-2'-O-methyladenosine (11) in 92% yield. It should be noted that the intermediate 9 can be useful for preparing other 6-substituted purine 2'-O-methylribosides.

For the synthesis of N^2-isobutyryl-2'-O-methylguanosine (14), 2'-O-methylation of N^2-isobutyryl-3',5'-O-TIPDS-guanosine and its derivatives with protection of the N^1-imino function was attempted by the above method. However, in each case, methylation at the base moiety occurred concomitantly. Therefore, the diazomethane procedure was employed for sugar-monomethylation of the 5'-O-monomethoxytrityl (MMTr) derivative (12) of N^2-isobutyrylguanosine. The introduction of the highly lipophilic group makes it possible to separate 2'- and 3'-O-methyl isomers on a silica gel column [17,19]. Compound 12 was treated with diazomethane in dimethylformamide in the presence of $SnCl_2$ at 0° C. A mixture of methyl ethers was obtained in 54 % yield after column chromatography using $CHCl_3$-MeOH solutions. The ^{1}H-NMR analysis showed the ratio of 2'- and 3'-O-methyl isomers to be 3:1. In order to isolate the 2'-O-methyl isomer (13), further chromatography was performed on a silica gel column using $CHCl_3$-CH_3CN-MeOH mixture. The desired isomer (13, 30% from 12), which contained a trace amount of the other

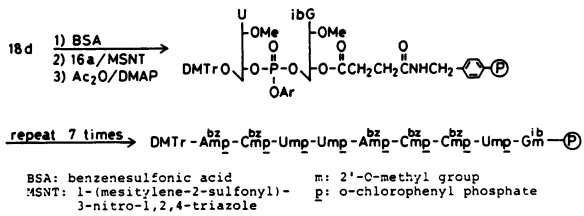


Scheme 2

isomer, was obtained and removal of the MMTr group with 80% AcOH gave a 60% yield of N^2-isobutyryl-2'-O-methylguanosine (14) as crystals.

Synthesis of oligo(2'-O-methylribonucleotides)

For the synthesis of oligo(2'-O-methylribonucleotides), we applied the phosphotriester solid-phase method [25,26]. Thus, 2'-O-methylribo nucleoside derivatives (4, 7, 11 and 14) were converted to the 5'-O-dimethoxytrityl (DMTr) derivatives (15), which were treated with o-chlorophenylphosphoroditriazolide [27] to give the 2'-O-methylribonucleoside-3'-(o-chlorophenyl-phosphate) derivatives (16). Compounds 15a and 15d were also converted to the 3'-succinates and separately linked to an aminomethylene polystyrene resin (Scheme 2). Then, we constructed the fully protected 2'-O-methyl nonamers having the sequence CAGGUAAGU and its complementary sequence ACUUACCUG by repeated additions of mononucleotide units (16) to the nucleoside-bound resin (18). 1-(Mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) was used as the condensing reagent [28]. The average of the coupling yield in both cases was ca. 85% which was somewhat lower than that for the corresponding deoxy-nonanucleotide. The reason would be mainly due to the steric hindrance of the 2'-O-methyl group with the neighboring 3'-hydroxyl function. As an example, the synthesis of the protected AmCmUmUmAmCmCmUmGm on the polymer support is shown in Scheme 3. The product was treated with N^1,N^1,N^3,N^3-tetramethyl-



Scheme 3

guanidinium syn-pyridine-2-aldoximate [29] to cleave the succinyl ester as well as the o-chlorophenyl esters and with concentrated ammonia to remove N-acyl groups. The 5'-O-DMTr-2'-O-methylnonamer obtained was purified by reversed phase chromatography at low pressure (Fig. 1a). After removal of the DMTr residue with 80% AcOH, completely deblocked 2'-O-methyl-nonamer was analysed by reversed-phase HPLC and fractionated as shown in Fig. 1b. The purity of the product was confirmed by anion-exchange HPLC analysis and its sequence (ACUUACCUG) was

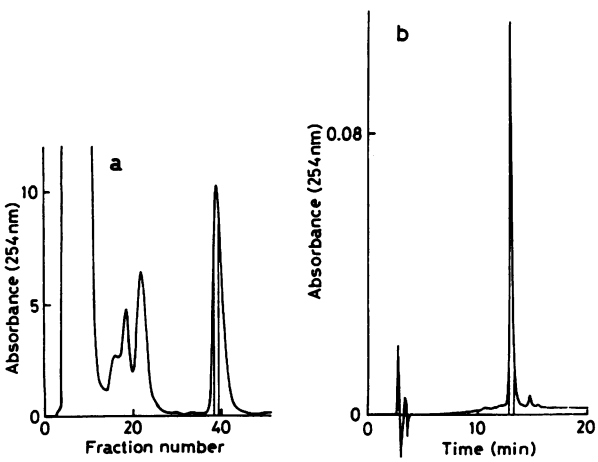


Fig. 1 a) Reversed phase chromatography of 5'-dimethoxytrityl 9 mer (AmCmUmUmAmCmCmUmGm) using a column (0.7 x 12 cm) of C-18 silica gel (35-105 u, Waters Associates) with a linear gradient of acetonitrile (5-35%) in 50 mM triethylammonium bicarbonate (total, 200 ml). Fractions of 3 ml were collected every 4 min. b) Reversed phase HPLC of the dedimethoxytritylated 9 mer (fraction 38) using a column (0.46 x 30 cm) of Nucleosil $5C_{18}$ (Macherey-Nagel) with a linear gradient of acetonitrile (from 13 to 21% during 20 min) in 0.1 M triethylammonium acetate. The flow rate was 1 ml/min.

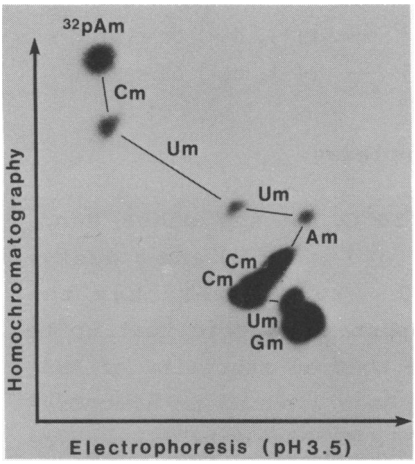


Fig. 2 Mobility shift analysis of the 9 mer. Venom phosphodiesterase was used for the partial digestion of the 5'-labeled oligonucleotide.

confirmed by mobility shift analysis [30,31] as shown in Fig. 2. The other nonamer, CmAmGmGmUmAmAmGmUm was prepared in a similar manner as above.

We also synthesized deoxyribo- and ribo-oligonucleotides having the sequences identical those for the 2'-O-methyl-oligomers. The synthesis of the deoxyoligomers followed the reported method [26] and the ribooligomer synthesis was carried out by our newly-developed method which involved a solid-phase synthesis in the 3'-direction [32].

Thermal stabilities of duplexes containing the 2'-O-methyl oligomers

The thermal stabilities of duplexes containing 2'-O-methyl nonaribonucleotides and related duplexes were evaluated by determination of the UV-absorbance-temperature profiles. We now refer to the synthetic oligomers as (+) for the oligomers having the sequence identical to the consensus sequence of the 5'-splice junctions, and as (-) for the oligomers containing the complementary sequence. In addition, 2'-O-methylribo-, deoxyribo- and ribo-oligomers are abbreviated as m, d and r, respectively (Fig. 3).

All nine duplexes showed cooperative melting and the Tm values are listed in Table. The highest Tm value (54.3°C) was observed for the 2'-O-methyloligomer-containing riboduplex r(+)·m(-), and the other riboduplexes m(+)·m(-), r(+)·r(-) and

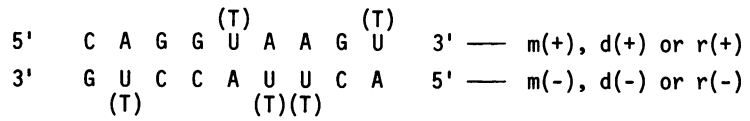


Fig. 3 Sequences of nonanucleotide duplexes.

m(+)·r(-) showed Tm values around 50°C. On the other hand, hybrids containing a deoxyoligomer (m·d and r·d) and a deoxyduplex d(+)·d(-) had Tm values 10 - 20°C lower than the corresponding riboduplexes. It is important to note that up to date, such a comparative study on the thermal stability of RNA-RNA duplexes and RNA-DNA hybrids has been limited to homopolymers [7] or homo-like oligomers [33]. When the Tm values are compared, it can be seen that because the (+) strand is purine-rich there is a base-composition dependency of oligomer sequences and an effect of the 2'-O-methyl groups on the thermal stabilities of these duplexes. The hybrids r(+)·d(-) and m(+)·d(-) showed Tm values of 41.0°C and 38.0°C, respectively, whereas lower Tm values (33.0°C and 34.8°C) were observed for the opposite r(-)·d(+) and m(-)·d(+), respectively. It can be concluded that the hybrid is more stabilized when the ribo- or 2'-O-methylribo-oligomer is located in the (+) strand which has a purine-rich sequence. The data also revealed that introduction of 2'-O-methyl groups into the (+) strand slightly decreased the stability of the hybrid r(+)·d(-), and the presence of 2'-O-methyl groups in the (-) strand gave the reverse change. Similar effects of 2'-O-methyl residues were observed for the stability of hetero riboduplexes m(+)·r(-) and m(-)·r(+).

	d(+)	r(+)	m(+)
d(-)	35.8 °C	41.0 °C	38.0 °C
r(-)	33.0	50.1	49.0
m(-)	34.8	54.3	51.5

Table: Tm values at 260 nm of the duplexes in 0.01 M sodium cacodylate buffer, pH 7.0 containing 0.1 M NaCl at a sample concentration of 0.8 A_{260}.

Of course, the deoxyribonucleotides contain thymine, not uracil, and the (+) strand contains two of these residues whereas the (-) strand contains three. It is known [34] that the methyl in thymine stabilizes the helix and so (i) all duplexes containing a deoxy strand will show an elevated Tm and (ii) those containing a (-) deoxy strand will have a relatively higher Tm than those containing a (+) deoxy strand. The quantitative effect of this U → T substitution is not known but as all duplexes containing a deoxy strand already have the lowest Tm's, this does not affect the conclusions of this study.

Solid state- [35] and solution-studies [36,37] on single-stranded 2'-O-methyl polymers and their double-stranded complexes have indicated that there is no significant conformational change on 2'-O-methylation of the corresponding parent polymer or complex. However, the limited studies available only concern homopolynucleotides and the work on such duplexes give no information concerning the effect of base-composition or sequence on duplex stability [38-42]. Our results show for the first time that the 2'-O-methyl residues make little contribution to the stability of the nonaribonucleotide duplexes (even of the duplexes with the deoxyribooligomer). This would be explained as the result of the counteraction of stabilizing (stacking)- and destabilizing (steric)-effects of the 2'-O-methyl group.

In conclusion, oligo(2'-O-methylribonucleotides) are capable of forming stable duplexes with RNA fragments to a degree similar to their oligonucleotide counterparts. Therefore, they can be utilized as RNA hybridization probes or other biochemical tools [43], having chemical and biological properties distinct from those of an oligoribonucleotide-probe. The 2'-O-methyl nonaribonucleotides synthesized will be used in the biochemical study on the mechanism of the splicing of precursor mRNAs.

EXPERIMENTAL SECTION

Melting points were determined on a Yanagimoto MP-3 micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra were obtained with a JEOL FX-100FT spectrometer and the

chemical shifts are expressed in δ values (ppm) relative to tetramethylsilane as an internal standard. UV spectra were recorded on a Shimadzu UV-240 spectrophotometer. UV absorption-temperature profiles were recorded on a Beckman DU-8B spectrophotometer. Mass (MS) spectra were obtained with a JEOL D-300 spectrometer. Elemental analyses were performed by the Center of Instrumental Analysis, Hokkaido University.

TLC was performed on a Silica gel $60F_{254}$ plates (Merck) with $CHCl_3$-MeOH unless otherwise stated. For reversed-phase TLC (RTLC), Silica gel 60 F_{254} silanized (Merck) was used with acetone-20 mM triethylammonium acetate (TEAA) buffer (pH 7.0). Protected nucleosides and nucleotides were purified using a column of Wakogel C-200 (Wako Pure Chemical) or preparative C18 (Waters Associates) unless otherwise stated.

Reversed phase column chromatography for oligonucleotides was performed using a pump (Eldex, Model E), a UV monitor (Gilson, Model 111B) and a fractionater (Gilson, Model FC-80K). HPLC was performed using a Gilson MS-3 apparatus. The columns and solvents are described in the legends to Fig. 1a,b. Purified oligonucleotides were analyzed by anion-exchange HPLC using a TSK gel DEAE-2SW column (0.46 x 25 cm, Toyo Soda Manufacturing) with a linear gradient of ammonium formate in 20% aqueous acetonitrile.

Mobility shift analysis of the 2'-O-methyl nonamers was performed according to the reported procedure [31] except two-fold amount of snake venom phosphodiesterase was used for the partial digestion.

N^3-Benzoyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)uridine (2) and its 2'-O-methyl derivative (3)

Benzoyl chloride (3.94 ml, 33.9 mmol) was added in portions to an ice-cooled mixture of 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)uridine (1, 15.0 g, 30.8 mmmol)[21] and Et_3N (5.55 ml, 39.8 mmol) in N,N-dimethylacetamide (150 ml). After being stirred at room temperature for 3 hr, the solution was concentrated in vacuo and partitioned between AcOEt and H_2O. The organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated in vacuo. Chromatography of the residue on a column of silica gel (C-300, 350 g) with 0.2% AcOEt-$CHCl_3$ as an

eluent gave 12.8 g (70.5%) of 2 as a foam. UV (MeOH): λmax 252 nm, λmin 221 nm. NMR ($CDCl_3$): 8.0-7.4 (6H, m, 6-H and Bz), 5.80 (1H, d, 5-H, J= 8.3 Hz), 5.76 (1H, s, 1'-H), 4.5-3.9 (5H, m, 2',3',4',5'-H), 2.79 (1H, d, 2'-OH), 1.1 (28H, m, TIPDS). MS m/e: 547 (M-isoPr). Anal. Calcd for $C_{28}H_{42}N_2O_8Si_2$: C, 56.92; H, 7.16; N, 4.74. Found: C, 56.74; H, 7.19; N, 4.74.

A mixture of 2 (7.35 g, 12.4 mmol), CH_3I (19.4 ml, 312 mmol) and Ag_2O (8.26 g, 35.6 mmol) in benzene (70 ml) was stirred at 40°C overnight. The reaction mixture was filtered and the filtrate was concentrated in vacuo. The residue was partitioned between $CHCl_3$ and H_2O, then the organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated to dryness. Chromatography of the residue on a column of silica gel (200 g) with 0.5% MeOH-$CHCl_3$ as an eluent gave 6.34 g (84.5%) of 3 as a foam. UV (MeOH): λmax 252 nm, λmin 221 nm. NMR ($CDCl_3$): 8.1-7.3 (6H, m, 6-H and Bz), 5.80 (1H, d, 5-H, J= 7.1 Hz), 5.76 (1H, s, 1'-H), 3.60 (3H, s, 2'-OCH_3), other sugar and TIPDS protons. MS m/e: 561 (M-isoPr). Anal. Calcd for $C_{29}H_{44}N_2O_8Si_2$: C, 57.59; H, 7.33; N, 4.63. Found: C, 57.18; H, 7.30; N, 4.80.

2'-O-Methyluridine (4) [13,15,16,20,23,24]

A solution of 3 (6.32 g, 10.4 mmol) in dioxane (90 ml)-conc. NH_4OH (11 ml) was stirred at room temperature for 4.5 hr. The reaction mixture was concentrated to dryness and the residue was partitioned between $CHCl_3$ and H_2O. The organic layer was washed with H_2O, then with 0.5% $NaHCO_3$ solution, and dried (Na_2SO_4). The solvent was removed in vacuo and the residue was chromatographed on a column of silica gel (150 g). The eluate with 1% MeOH-$CHCl_3$ was evaporated to give 4.98 g (95.6%) of 2'-O-methyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)uridine as a foam. UV (MeOH): λmax 261 nm, λmin 232 nm. MS m/e: 457 (M-isoPr). Anal. Calcd for $C_{22}H_{40}N_2O_7Si_2$: C, 52.77; H, 8.05; N, 5.59. Found: C, 52.80; H, 8.02; N, 5.62.

The product (2.89 g, 5.77 mmol) was dissolved in a mixture of dioxane (30 ml) and 1N HCl (30 ml). After being stirred for 3.5 hr at room temperature, the solution was neutralized with Dowex 1 (bicarbonate form) resin and filtered. The resin was washed with H_2O-MeOH (10:1 v/v) and the combined filtrates were concentrated to dryness. The residue was partitioned between

$CHCl_3$ and H_2O-MeOH (10:1 v/v) and the aqueous layer was concentrated to leave a foam, which was crystallized from AcOEt-MeOH to yield 1.31 g (87.9%) of 4, mp 156-159°C (lit. mp 159-161°C)[16]. UV (MeOH): λmax 261 nm, λmin 230 nm, NMR (DMSO-d_6): 11.37 (1H, br s, N^3-H), 7.94 (1H, d, 6-H, J= 8.3 Hz), 5.86 (1H, d, 1'-H, J= 5.1 Hz), 5.65 (1H, br d, 5-H, J= 8.3 Hz), 5.2 (2H, m, 3',5'-OH), 4.2-3.5 (5H, m, 2',3',4',5'-H), 3.36 (3H, s, 2'-OCH_3). MS m/e: 258 (M). Anal. Calcd for $C_{10}H_{14}N_2O_6$: C, 46.51; H, 5.46; N, 10.84. Found: C, 46.59; H, 5.39; N, 11.11.

N^4-Benzoyl-2'-O-methyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)cytidine (6)

A mixture of N^4-benzoyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)cytidine (5, 9.82 g, 16.6 mmol)[21], CH_3I (20.7 ml, 333 mmol) and Ag_2O (11.57 g, 49.9 mmol) in benzene (100 ml) was stirred at 40°C for 4 hr. The reaction mixture was filtered and the filtrate was concentrated in vacuo. The residue was partitioned between $CHCl_3$ and H_2O, then the organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated to dryness. Chromatography of the residue on a column of silica gel (300 g) with 0.5% MeOH-$CHCl_3$ as an eluant gave 6.92 g (69.0%) of 6 as a foam. UV (MeOH); λmax 259, 303 nm, λmin 284 nm. NMR ($CDCl_3$): 8.8 (1H, br s, N^4-H), 8.40 (1H, d, 6-H, J= 7.6 Hz), 8.0-7.4 (6H, m, 5-H and Bz), 5.86 (1H, s, 1'-H), 4.4-3.8 (5H, m, 2',3',4',5'-H), 3.74 (3H, s, 2'-OCH_3) and TIPDS protons. MS m/e: 603 (M). Anal. Calcd for $C_{29}H_{45}N_3O_7Si_2$: C, 57.68; H, 7.51; N, 6.96. Found: C, 57.27; H, 7.39; N, 7.09.

N^4-Benzoyl-2'-O-methylcytidine (7) [3b,3c,44]

Compound 6 (6.90 g, 11.4 mmol) was added to a mixture of dioxane (70 ml), MeOH (20 ml) and 1N HCl (70 ml). The whole was stirred at room temperature for 6 hr, neutralized with Dowex 1 (bicarbonate form) resin and filtered. The resin was washed with MeOH and H_2O, then the combined filtrates were evaporated to dryness. The residue was crystallized from EtOH to give 2.16 g (52.5%) of 7, mp 178-180°C (lit. mp 180-181°C)[43]. UV (MeOH): λmax 259, 303 nm, λmin 284 nm. NMR (DMSO-d_6): 11.22 (1H, br s, N^4-H), 8.55 (1H, d, 6-H, J= 7.6 Hz), 8.1-7.2 (6H, m, 5-H and Bz), 5.89 (1H, d, 1'-H, J= 2.2

Hz), 5.2 (2H, m, 3',5'-OH), 4.2-3.6 (5H, m, 2',3',4',5'-H), 3.48 (3H, s, 2'-OCH_3). MS m/e 361 (M). Anal. Calcd for $C_{17}H_{19}N_3O_6 \cdot 0.5\ H_2O$: C, 55.13; H, 5.44; N, 11.35. Found: C, 55.45; H, 5.35; N, 11.40.

6-Chloro-9-[3,5-O-(tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl]purine (8) and its 2'-O-methyl derivative (9)

1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (14 ml, 44 mmol) was added to an ice-cooled pyridine (100 ml) solution of 6-chloro-9-(β-D-ribofuranosyl)purine (10.47 g, 36.5 mmol) which was prepared via 6-chlorination of triacetylinosine with $SOCl_2$-DMF. After being stirred at room temperature for 3 hr, MeOH (10 ml) was added to the reaction mixture. The whole was concentrated in vacuo and partitioned between $CHCl_3$ and H_2O. The organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated in vacuo. After removal of pyridine by coevaporation with toluene, the residue was chromatographed on a column of silica gel (500 g). The eluate with 1% MeOH-$CHCl_3$ was concentrated and the residue was crystallized from hexane to give 12.96 g (67.1%) of 8, mp 109-110°C. UV (MeOH); λmax 264 nm, λmin 226 nm. NMR($CDCl_3$): 8.70, 8.32 (1H each, s, 2,8-H), 6.07 (1H, s, 1'-H), 5.1-4.0 (5H, m, 2',3',4',5'-H), 3.26 (1H, br s, 2'-OH) and TIPDS protons. MS m/e: 485 (M-isoPr). Anal. Calcd for $C_{22}H_{37}ClN_4O_5Si_2$: C,49.93; H, 7.05; Cl, 6.70; N, 10.59. Found: C, 49.96; H, 7.03; Cl, 6.70; N, 10.60.

A mixture of 8 (9.42 g, 17.8 mmol), CH_3I (100 ml) and Ag_2O (20.7 g, 89.3 mmol) was stirred at 40°C for 50 min. The reaction mixture was filtered and the filtrate was concentrated in vacuo. The residue was partitioned between $CHCl_3$ and H_2O, then the organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated to dryness. The residue was chromatographed on a column of silica gel (400 g). The eluate with 1% MeOH-$CHCl_3$ was concentrated and the residue was crystallized from EtOH to give 6.41 g (66.3%) of 9, mp 153-154°C. UV (MeOH): λmax 264 nm, λmin 227 nm. NMR ($CDCl_3$): 8.73, 8.45 (1H each, s, 2,8-H), 6.10 (1H, s, 1'-H), 3.72 (3H, s, 2'-OCH_3), other sugar and TIPDS protons. MS m/e: 542 (M). Anal. Calcd for $C_{23}\ H_{39}\ Cl\ N_4\ O_5\ Si_2$: C, 50.86; H, 7.24; Cl, 6.53; N, 10.31. Found: C, 50.80; H, 7.13; Cl, 6.50; N, 10.47.

N^6-Benzoyl-2'-O-methyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)adenosine (10)

A solution of 9 (4.45 g, 8.19 mmol) in CH_2Cl_2 (30 ml)-liq.NH_3 (30 ml) was kept at room temperature for 24 hr in a sealed steinless steel tube. The reaction mixture was concentrated and the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with dil. $NaHCO_3$ solution, then with H_2O, and dried (Na_2SO_4). After removal of the solvent in vacuo, the residue was chromatographed on a column of silica gel (120 g). The eluate with 1.5% MeOH-$CHCl_3$ was concentrated and the residue was crystallized from hexane-AcOEt to give 2.83 g (66%) of 2'-O-methyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)adenosine, mp 159-160°C. UV (MeOH): λmax 259 nm, λmin 233 nm. NMR ($CDCl_3$): 8.32, 8.12 (1H each, s, 2,8-H), 6.03 (1H, s, 1'-H), 5.76 (2H, br s, 6-NH_2), 4.8-3.9 (5H, m, 2',3',4',5'-H), 3.71 (3H, s, 2'-OCH_3) and TIPDS protons. MS m/e: 523 (M). Anal. Calcd for $C_{23}H_{41}N_5O_5Si_2$: C, 52.74; H, 7.89; N, 13.37. Found: C, 52.68; H, 7.94; N, 13.32.

The product (2.0 g, 3.8 mmol) was dissolved in pyridine (20 ml) and benzoyl chloride (0.57 ml, 4.9 mmol) was added to the solution cooled in an ice-bath. After being stirred at room temperature for 2 hr, H_2O (5 ml) was added to the reaction mixture. The whole was partitioned between $CHCl_3$ and satd. $NaHCO_3$ solution, then the organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated to dryness. Chromatography of the residue on a column of silica gel (60 g) with 1% MeOH-$CHCl_3$ as an eluent gave 2.13 g (89%) of 10 as a foam. UV (MeOH): λmax 279 nm, λmin 247 nm. NMR ($CDCl_3$): 9.25 (1H, br s, N^6-H), 8.80, 8.34 (1H each, s, 2,8-H), 8.1-7.4 (5H, m, Bz), 6.10 (1H, s, 1'-H), 3.73 (3H, s, 2'-OCH_3), other sugar and TIPDS protons. MS m/e: 627 (M). Anal. Calcd for $C_{30}H_{45}N_5O_6Si_2$: C, 57.39; H, 7.22; N, 11.15. Found: C, 57.06; H, 7.15; N, 11.18.

N^6-Benzoyl-2'-O-methyladenosine (11) [3f]

Compound 10 (0.90 g, 1.4 mmol) was dissolved in THF (9 ml), and tetrabutylammonium fluoride (1M THF solution, 0.7 ml) was added. After 50 min, the solution was diluted with pyridine-MeOH-H_2O (3:1:1 v/v) 20 ml and Dowex 50 (pyridinium form) resin (ca. 3 ml) was added. The mixture was stirred for

5 min, then the resin was filtered off and the filtrate was concentrated in vacuo. Chromatography of the residue on a column of silica gel (40 g) with 2.5% MeOH-$CHCl_3$ as an eluent gave 0.51 g (92%) of 11 as a foam. UV (MeOH): λmax 279 nm, λmin 247 nm. NMR (DMSO-d_6): 11.21 (1H, br s, N^6-H), 8.76, 8.75 (1H each, s, 2,8-H), 8.1-7.4 (5H, m, Bz), 6.17 (1H, d, 1'-H, J= 5.1 Hz). 5.32 (1H, d, 2'-OH), 5.17 (1H, t, 5'-OH), 4.5-3.5 (5H, m, 2',3',4',5'-H), 3.37 (3H, s, 2'-OCH_3). MS m/e : 385 (M). Anal. Calcd for $C_{18}H_{19}N_5O_5$: C, 56.10; H, 4.97; N, 18.17. Found: C, 55.96; H, 5.03; N, 17.93.

N^2-Isobutyryl-5'-O-(4-methoxytrityl)-2'-O-methylguanosine (13) [3d]

N^2-Isobutyryl-5'-O-(4-methoxytrityl)guanosine (12, 6.0 g, 9.6 mmol)[45] and $SnCl_2$ (0.38 g, 2 mmol) was dissolved in DMF (480 ml) and cooled in an ice-bath. To the stirred solution, diazomethane (1,2-dimethoxyethane solution, 25 ml) [16] was added in portions over a period of 3 hr. After additional stirring for 2 hr at 0°C, conc. NH_4OH (0.5 ml) was added to the reaction mixture and the solvent was removed in vacuo. The residue was partitioned between $CHCl_3$ and H_2O, then the organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated to dryness. Chromatography on a column of silica gel (200g) with 2% MeOH-$CHCl_3$ as an eluent gave 3.31 g (54%) of a foam, which was shown to be a mixture of the title compound 13 (75%) and its 3'-O-methyl isomer (25%) by NMR ($CDCl_3$) analysis [16,19]: 5.87 (d, 1'-H, J= 6.1 Hz) and 3.46 (s, sugar CH_3) for 13; 5.71 (d, 1'-H, J= 6.4 Hz) and 3.42 (s, sugar CH_3) for the 3'-O-methyl isomer. Flash chromatography of the mixture (2.5 g) on a column of silica gel (C-300, 120 g) with $CHCl_3$-CH_3CN-MeOH (60:24:1 v/v) as an eluent gave 1.4 g of 13, which contained a trace amount of the 3'-O-methyl isomer. Rf value of 13 in TLC ($CHCl_3$-CH_3CN-MeOH, 20:8:1 v/v): 0.29 (Rf 0.21 for the 3'-O-methyl isomer). This material was used for the next step without further purification.

N^2-Isobutyryl-2'-O-methylguanosine (14) [3d]

A solution of 13 (1.4 g, 2.2 mmol) in 80% aq. AcOH was stirred at 50°C for 1 hr. The reaction mixture was concentrated to dryness and the residue was partitioned between

$CHCl_3$ and H_2O. The aqueous layer was washed with $CHCl_3$ and evaporated in vacuo. The resulting solid was crystallized from H_2O to give 0.49 g (60%) of 14, mp 208-210°C. UV (H_2O): λmax 260 nm (280 nm sh), λmin 227 nm. NMR (DMSO-d_6): 12.0, 11.7 (1H each, br s, N^1,N^2-H), 8.29(1H, s, 8-H), 5.90 (1H, d, 1'-H, J= 5.9 Hz), 5.24 (1H, d, 2'-OH), 5.08 (1H, t, 5'-OH), 4.4-3.5 (5H, m, 2',3',4',5'-H), 3.33 (3H, s, 2'-OCH_3), 2.77 (1H, m, isoPr methine), 1.12 (6H, d, isoPr CH_3x2). MS m/e: 367 (M). Anal. Calcd for $C_{15}H_{21}N_5O_6 \cdot 0.75\ H_2O$: C, 47.30; H, 5.95; N, 18.39. Found: C, 47.44; H, 5.99; N, 18.15.

Preparation of 2'-O-methylribonucleotide unit (16)

Compounds 4,7,11 and 14 were dimethoxytritylated by the standard tritylation method and the products (15a-d) were phosphorylated with o-chlorophenyl phosphoroditriazolide according to the method described [27]. After the work-up, the nucleotides (16a-d) were purified using a reversed phase column (4 x 6 cm) with a gradient of acetone in 0.2% pyridine and precipitated in hexane-ether (1:1 v/v containing 1% Et_3N). The yields as the triethylammonium salts and Rf values in RTLC (acetone-20 mM TEAA, 6:4 v/v) were as follows: 16a, 81%, Rf 0.69; 16b, 97%, Rf 0.59; 16c, 72%, Rf 0.62; 16d, 68%, Rf 0.65.

Preparation of 2'-O-methylribonucleoside resin (18)

In a similar manner to deoxynucleoside-resin preparation [25], compound 15a and 15d were converted to the 3'-succinates (17a,d) and separately linked to an aminomethylated (0.23 mmol/g or 0.13 mmol/g) polystyrene (1% cross linked, Peptide Institure). The nucleoside content of the product (18) was 0.20 mmol/g for 18a and 0.12 mmol/g for 18d.

Synthesis of nona(2'-O-methyl)ribonucleotides AmCmUmUmAmCmCmUmGm and CmAmGmGmUmAmAmGmUm

The 2'-O-methyl nonamers were synthesized in a similar manner to oligodeoxyribonucleotide synthesis by the phosphotriester solid-phase method [26]. Five μmol of the nucleoside resin (18) was used as the starting material and in each condensation step, ca. 25 μmol of the protected nucleotide (16) and MSNT (100 μmol) were employed. Overall yields of the 2'-O-methyl nonamers were ca. 10%.

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