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This paper is dedicated to Professor Maciej Wiewiórowski

Oligonucleotide synthesis using the 2-(levulinyloxymethyl)-5-nitrobenzoyl group for the 5′-position of nucleoside 3′-phosphoramidite derivatives

Kazuo Kamaike, Hiroaki Takahashi, Kimio Morohoshi, Noriyasu Kataoka, Takashi Kakinuma and Yoshiharu Ishido✉

Laboratory of Pharmaceutical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

Key words: 2-(levulinyloxymethyl)-5-nitrobenzoyl group, 2-(levulinyloxymethyl)benzoyl group, base-labile protecting group, solid-phase oligonucleotide synthesis, CpCpA terminus triplet of tRNAs, ^{15}N-labeled oligoribonucleotide, 2′-*O*-(β-D-ribofuranosyl)adenosine

A comparative study on the utility of 2-(levulinyloxymethyl)-5-nitrobenzoyl (LMNBz) and 2-(levulinyloxymethyl)benzoyl (LMBz) protecting groups for the 5′-positions of nucleoside 3′-phosphoramidite derivatives in the oligonucleotide synthesis is presented in terms of the syntheses of TpTpT, TpTpTpT, and UpCpApGpUpUpGpG. In addition we describe the synthesis, using the LMNBz protecting group, of the CpCpA terminus triplet of tRNAs bearing exocyclic amino groups with ^{15}N-labeling, and the trimer Gp[A*]pG containing 2′-*O*-(β-D-ribofuranosyl)adenosine ([A*]), the latter of which is found at position 64 in the yeast initiator tRNA^{Met}.

The current methodology of the automated solid-phase approach to oligonucleotides on controlled pore glass (CPG) support apparently seems to be used successfully, but has

✉Author for correspondence.

Abbreviations: LMNBz, 2-(levulinyloxymethyl)-5-nitrobenzoyl; LMBz, 2-(levulinyloxymethyl)benzoyl; CPG, controlled pore glass; DABCO, 1,4-diazabicyclo[2.2.2]octane; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, *N,N′*-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMTr, 4,4′-dimethoxytrityl; Thp, tetrahydropyran-2-yl; TBAF, tetrabutylammonium fluoride; TEAA, triethylammonium acetate; THF, tetrahydrofuran; Thf, tetrahydrofuran-2-yl; TBDMS, *tert*-butyldimethylsilyl; U, uridine; C^{Bz}, N^4-benzoylcytidine; C^{An}, N^4-anisoylcytidine; TPSCI, 2,4,6-triisopropylbenzenesulfonyl chloride; [4-^{15}N]C^{An}, N^4-anisoyl[4-^{15}N]cytidine; A^{Bz}, N^6-benzoyladenosine; [6-^{15}N]A^{Bz}, N^6-benzoyl[6-^{15}N]adenosine; G^{iBu}, N^2-isobutyrylguanosine; A*, 2′-*O*-(5′′-*O*-phosphoryl-β-D-ribofuranosyl)adenosine; [A*], 2′-*O*-(β-D-ribofuranosyl)adenosine; I, inosine; [I*], 2′-*O*-(β-D-ribofuranosyl)inosine.

been hampered by a series of problems. In the case of oligodeoxyribonucleotide synthesis, the depurination reaction occurs inevitably under the acidic conditions used for the removal of the 5′-*O*-(4,4′-dimethoxy-trityl) (DMTr) protecting group [1, 2]. In the case of oligoribonucleotide synthesis, on the other hand, the bulky *tert*-butyldimethylsilyl (TBDMS) protecting group is widely used for the 2′-hydroxyl group of ribonucleosides in place of the tetrahydropyran-2-yl (Thp) group [3], which is somewhat affected upon the removal of the 5′-*O*-DMTr group under acidic conditions [4–6]. The 2′-*O*-TBDMS group, however, brings about an unfavorable steric effect on the introduction of the phosphityl function to the 3′-hydroxyl groups [7] and on coupling reactions with the ribonucleoside phosphoramidite units bearing free 5′-hydroxyl groups [7], in addition to its incomplete removal at the final stage of assembly even by a fluoride ion [8]. Consequently, various base-labile protecting groups for the 5′-hydroxyl groups of ribonucleoside and 2′-deoxyribonucleoside 3′-phosphoramidites have been reported [9–12], as exemplified by the levulinyl group [13–16] and the 2-(acetoxy- and -benzoyloxymethyl)benzoyl groups [17, 18], the latter of which are used for the exocyclic amino group of nucleobase moieties. The former is characterized by facile unmasking through 0.5 M hydrazine hydrate in acetic acid/pyridine at room temperature for 2 min in the case of the liquid-phase approach [13] or 10–15 min without damaging the 2-cyanoethyl phosphate protecting group in the case of the solid-phase approach [15, 16]. Based on the chemistry of these protecting groups, we assumed a possibility to replace the acetyl and benzoyl groups of the latter with the levulinyl group, i.e. a potential utility of the 2-(levulinyloxymethyl)benzoyl (LMBz) protecting group for the 5′-hydroxyl groups of nucleoside 3′-phosphoramidites in the oligonucleotide synthesis.

A comparative study of these protecting groups for the synthesis of oligonucleotides, as exemplified by synthesis of TpTpT, TpTpTpT, and UpCpApGpUpUpGpG, clearly proved particular feasibility of the LMNBz protecting group over LMBz [19]. Consequently, synthetic studies on CpCpA and Gp[A*]pG were carried out in terms of the LMNBz protecting group [20]. The results thus obtained will be described in full herein.

MATERIALS AND METHODS

General procedures. Melting points were determined by a Yanagimoto micro-melting-point apparatus and are uncorrected. TLC was conducted on Merck silica gel F_{254} and was developed with 9:1 chloroform/methanol (Solvent A). Column chromatography was performed on silica gel (Wakogel C-300, purchased from Wako Pure Chemical Industries, Ltd.) by the use of chloroform/methanol. High performance liquid chromatography (HPLC) was conducted on μBONDASPHERE 5μ C18 (3.9 mm ID × 150 mm L) for purification of oligonucleotides and analyses of the digestion of oligonucleotides with snake venom phosphodiesterase and alkaline phosphatase. ^{1}H-NMR spectra were recorded on a Varian GEMINI 300 apparatus. ^{31}P-NMR and ^{13}C-NMR spectra were recorded on a Brucker AM 400 apparatus with 85% H_3PO_4 as an external standard. ^{15}N-NMR spectra were recorded on a Brucker AM 500 apparatus with liquid $^{15}NH_3$ as an external standard. Mass spectra were recorded on a VG AutoSpecE apparatus. Elemental analyses were achieved with a Perkin-Elmer 240-002 apparatus.

***2-(Levulinyloxymethyl)benzoic acid* (3).** A solution of phthalide (1) (5.36 g, 40 mmol) and potassium hydroxide (2.47 g, 44 mmol) in 85:15 methanol/water (20 mL) was heated at reflux for 2 h [17]. After evaporation, the residue was dissolved in water (100 mL) and was washed with diethyl ether (50 mL × 3). The aqueous solution was acidified (about pH 2) with conc. HCl. The resultant precipitates were gathered by filtration, and were washed

with chilled water (20 mL) to give a white powder of 2-(hydroxymethyl)benzoic acid (**2**) (5.51 g, 91% yield); m.p. 68–69°C; ^{1}H-NMR ($CDCl_3$): δ 4.85 (s, 2H, PhCH_2), 7.44 (t, 1H, J = 7.6 Hz, Ph-*H*), 7.49–7.60 (m, 2H, Ph-*H* × 2), and 8.13 (d, 1H, J = 7.8 Hz, Ph-*H*).

Separately, to a solution of levulinic acid (4.23 mL, 41.5 mmol) in dried 1,4-dioxane (41.5 mL) was added *N,N'*-dicyclohexylcarbodiimide (DCC) (4.28 g, 20.75 mmol). The mixture was stirred for 3 h at room temperature. After removing the precipitate by filtration, to the filtrate was added **2** (2.10 g, 13.8 mmol), and the mixture was stirred for 1 h at room temperature in the presence of 1-methylimidazole (1.66 mL, 20.7 mmol). After evaporation, the residue was dissolved in a saturated aqueous sodium carbonate solution (100 mL) and the solution was washed with diethyl ether (50 mL × 3). The aqueous layer was acidified (about pH 2) with conc. HCl. The resultant precipitate was gathered by filtration, and was washed with chilled water (20 mL) to give a white powder of **3** (2.05 g, 59% yield); m.p. 104–104.5°C; ^{1}H-NMR ($CDCl_3$): δ 2.21 (s, 3H, COCH_3), 2.70 (t, 2H, J = 6.1 Hz, CH_2), 2.82 (t, 2H, J = 6.1 Hz, CH_2), 5.59 (s, 2H, PhCH_2), 7.43 (t, 1H, J = 7.8 Hz, Ph-*H*), 7.53–7.64 (m, 2H, Ph-*H* × 2), and 8.13 (d, 1H, J = 7.8 Hz, Ph-*H*). *Anal.*, calc. for $C_{13}H_{14}O_5$: C, 62.39; H, 5.64; found: C, 62.34; H, 5.62.

***2-(Levulinyloxymethyl)-5-nitrobenzoic acid* (4).** A solution of **3** (3.76 g, 15 mmol) in 98% H_2SO_4 (15 mL)/61% HNO_3 (15 mL) was stirred at 0°C for 30 min. The resulting mixture was added to 5% aqueous sodium hydrogen carbonate solution (50 mL) and washed with diethyl ether (50 mL × 2). The aqueous layer was acidified (about pH 2) with conc. HCl and extracted with chloroform (50 mL × 2). The organic layer was washed with water (50 mL × 3), dried over anhydrous magnesium sulfate, and then evaporated to give crude **4**, which was purified by crystallization from diethyl ether (2.25 g, 50% yield). The filtrate was evaporated to dryness, and the residue was subjected to chromatographic separation on a column of silica gel by the use of n-hexane/ethyl acetate system. Crystallization from diethyl ether gave a second crop of **4** (513 mg, 12% yield); m.p. 107–108°C; ^{1}H-NMR ($CDCl_3$): δ 2.22 (s, 3H, COCH_3), 2.73 (t, 2H, J = 6.5 Hz, CH_2), 2.85 (t, 2H, J = 6.5 Hz, CH_2), 5.67 (s, 2H, PhCH_2), 7.82 (d, 1H, J = 8.8 Hz, Ph-*H*), 8.44 (dd, 1H, J = 2.4 Hz and J = 8.8 Hz, Ph-*H*), 8.93 (d, 1H, J = 2.4 Hz, Ph-*H*), and 9.31 (br s, 1H, COO*H*). *Anal.*, calc. for $C_{13}H_{13}NO_7$: C, 52.89; H, 4.44; N, 4.74; found: C, 52.89; H, 4.56; N, 4.80.

5'-O-[2-(Levulinyloxymethyl)benzoyl]-2'-O-(tetrahydropyran-2-yl)uridine [9 (B = U, R = OThp, R' = LMBz)]. 2'-*O*-(Tetrahydropyran-2-yl)uridine [**6** (B = U)] (627 mg, 1.91 mmol; the more polar diastereoisomer) [21] and **3** (526 mg, 2.1 mmol) were, after azeotropic evaporation from pyridine (5 mL × 3), dissolved in pyridine (19.1 mL), and 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) (1.27 g, 4.2 mmol) was added to the solution, which was then stirred at room temperature for 1 day. The resulting mixture was quenched with water (10 mL) with stirring, and extracted with chloroform (50 mL × 2). The extracts were combined and washed with saturated aqueous sodium hydrogen carbonate solution (50 mL × 2) and then with water (50 mL). After drying over anhydrous magnesium sulfate the organic layer was evaporated to dryness, and the residue was subjected to chromatographic separation on a column of silica gel by the use of chloroform/methanol system to give **9** (B = U, R = OThp, R' = LMBz) (640 mg, 60% yield); ^{1}H-NMR ($CDCl_3$): δ 1.50–1.84 (m, 6H, CH_2 × 3 of Thp group), 2.20 (s, 3H, COCH_3 of LMBz group), 2.65 (t, 2H, J = 6.4 Hz, CH_2 of LMBz group), 2.79 (t, 2H, J = 6.4 Hz, CH_2 of LMBz group), 3.46–3.53, 3.77–3.84 (2m, 2H, OCH_2C of Thp group), 4.20–4.25 (m, 1H, H-4'), 4.36 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 6.0 Hz, H-3'), 4.41 (dd, 1H, H-2'), 4.54 (dd, 1H, $J_{4',5'}$ = 4.8 Hz, $J_{5',5''}$ = 12.2 Hz, H-5'), 4.69 (dd, 1H, $J_{4',5''}$ = 3.0 Hz, H-5"), 4.76–4.78 (m, 1H, OC*H*O of Thp group), 5.51

(s, 2H, PhCH_2), 5.53 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.89 (d, 1H, $J_{1',2'}$ = 3.2 Hz, H-1'), 7.32 (d, 1H, H-6), 7.42 (t, 1H, J = 7.7 Hz, Ph-*H*), 7.50–7.60 (m, 2H, Ph-*H* × 2), 7.97 (d, 1H, J = 7.9 Hz, Ph-*H*), and 8.36 (br s, 1H, N^3-*H*). *Anal.*, calc. for $C_{27}H_{32}N_2O_{11}$: C, 57.85; H, 5.75; N, 4.99; found: C, 57.59; H, 5.66; N, 4.88.

N^4-Anisoyl-5'-O-[2-(levulinyloxymethyl)benzoyl]-2'-O-(tetrahydropyran-2-yl) cytidine [9 (B = C^{An}, R = OThp, R' = LMBz)]. Compound **9** (B = C^{An}, R = OThp, R' = LMBz) was obtained in 61% yield (338 mg) by treating N^4-anisoyl-2'-*O*-(tetrahydropyran-2-yl)cytidine [**6** (B = C^{An})] (369 mg, 0.8 mmol; the more polar diastereoisomer) [21] with **3** (220 mg, 0.88 mmol) in the presence of TPSCl (533 mg, 1.76 mmol) in pyridine (8 mL) for 2 days and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.50–1.90 (m, 6H, CH_2 × 3 of Thp group), 2.17 (s, 3H, COCH_3 of LMBz group), 2.64 (t, 2H, J = 6.6 Hz, CH_2 of LMBz group), 2.77 (t, 2H, J = 6.6 Hz, CH_2 of LMBz group), 3.50–3.56, 3.85–3.95 (2 m, 2H, OCH_2C of Thp group), 3.87 (s, 3H, OCH_3), 4.32–4.45 (m, 2H, H-3' and 4'), 4.50 (dd, 1H, $J_{2',3'}$ = 4.8 Hz, H-2'), 4.63 (dd, 1H, $J_{4',5'}$ = 4.2 Hz, $J_{5',5''}$ = 12.4 Hz, H-5'), 4.72 (dd, 1H, $J_{4',5''}$ = 2.4 Hz, H-5"), 4.93–4.95 (m, 1H, OC*H*O of Thp group), 5.51 (s, 2H, PhCH_2), 6.03 (d, 1H, $J_{1',2'}$ = 1.9 Hz, H-1'), 6.98 (d, 2H, J = 9.0 Hz, Ph-*H* × 2 of An group), 7.42–7.60 (m, 4H, Ph-*H* × 3 and H-5), 7.86–7.93 (m, 3H, Ph-*H* × 3), 7.98 (d, 1H, $J_{5,6}$ = 7.7 Hz, H-6), and 8.86 (br s, 1H, N^4-*H*). *Anal.*, calc. for $C_{35}H_{39}N_3O_{12}$ · 0.25 H_2O: C, 60.21; H, 5.67; N, 6.06; found: C, 60.14; H, 5.67; N, 5.91.

N^6-Benzoyl-5'-O-[2-(levulinyloxymethyl)benzoyl]-2'-O-(tetrahydropyran-2-yl) adenosine [9 (B = A^{Bz}, R = OThp, R' = LMBz)]. Compound **9** (B = A^{Bz}, R = OThp, R' = LMBz) was obtained in 61% yield (420 mg) by treating N^6-benzoyl-2'-*O*-(tetrahydropyran-2-yl)adenosine [**6** (B = A^{Bz})] (456 mg, 1.0 mmol; the more polar diastereoisomer) [21] with **3** (275 mg, 1.1 mmol) in the presence of TPSCl (666 mg, 2.2 mmol) in pyridine (10 mL) for 1 day and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.40–1.85 (m, 6H, CH_2 × 3 of Thp group), 2.17 (s, 3H, COCH_3 of LMBz group), 2.63 (t, 2H, J = 6.9 Hz, CH_2 of LMBz group), 2.76 (t, 2H, J = 6.9 Hz, CH_2 of LMBz group), 3.18–3.26, 3.53–3.60 (2m, 2H, OCH_2C of Thp group), 4.34–4.38 (m, 1H, H-4'), 4.57 (dd, 1H, $J_{4',5'}$ = 5.3 Hz, $J_{5',5''}$ = 12.2 Hz, H-5'), 4.64–4.67 (m, 1H, OC*H*O of Thp group), 4.75 (dd, 1H, $J_{4',5''}$ = 3.5 Hz, H-5''), 4.80 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 6.0 Hz, H-3'), 5.00 (dd, 1H, H-2'), 5.44, 5.51 (2 d, 2H, J = 14.1 Hz, PhCH_2), 6.20 (d, 1H, $J_{1',2'}$ = 3.8 Hz, H-1'), 7.35 (t, 1H, J = 7.8 Hz, Ph-*H*), 7.46–7.61 (m, 5H, Ph-*H* × 5), 7.96 (d, 1H, J = 7.8 Hz, Ph-*H*), 8.02 (d, 2H, J = 7.1 Hz, Ph-*H* × 2 of Bz group), 8.08 (s, 1H, H-8), 8.66 (s, 1H, H-2), and 9.22 (br s, 1H, N^6-*H*). *Anal.*, calc. for $C_{35}H_{37}N_5O_{10}$ · 0.5 H_2O: C, 60.34; H, 5.50; N, 10.05; found: C, 60.44; H, 5.41; N, 10.01.

N^2-Isobutyryl-5'-O-[2-(levulinyloxymethyl)benzoyl]-2'-O-(tetrahydropyran-2-yl) guanosine [9 (B = G^{iBu}, R = OThp, R' = LMBz)]. Compound **9** (B = G^{iBu}, R = OThp, R' = LMBz) was obtained in 64% yield (430 mg) by treating N^2-isobutyryl-2'-*O*-(tetrahydropyran-2-yl)guanosine [**6** (B = G^{iBu})] (438 mg, 1.0 mmol; the more polar diastereoisomer) [21] with **3** (0.275 g, 1.1 mmol) in the presence of TPSCl (666 mg, 2.2 mmol) in pyridine (10 mL) for 20 h and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.21, 1.24 (2d, 6H, J = 6.9 Hz, CH(CH_3)$_2$), 1.80–1.94 (m, 6H, CH_2 × 3 of Thp group), 2.19 (s, 3H, COCH_3 of LMBz group), 2.61–2.81 (m, 5H, CH_2 × 2 of LMBz group and C*H*(CH$_3$)$_2$), 3.23–3.29, 3.46–3.53 (2 m, 2H, OCH_2C of Thp group), 4.38–4.44 (m, 1H, H-4'), 4.58–4.66 (m, 2H, H-5' and OC*H*O of Thp group), 4.74 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 6.0 Hz, H-3'), 4.80–4.85 (m, 2H, H-2' and 5''), 5.45 (s, 2H, PhCH_2), 6.00 (d, 1H, $J_{1',2'}$ = 4.1 Hz, H-1'), 7.32–7.37 (m, 1H, Ph-*H*), 7.47–7.57 (m, 2H, Ph-*H* × 2), 7.72 (s, 1H, H-8), 7.91 (d, 1H, J = 7.7 Hz, Ph-*H*), 9.41 (br s, 1H, N^1-*H*), and 12.11

(br s, 1H, N^2-*H*). *Anal.*, calc. for $C_{32}H_{39}N_5O_{11} \cdot 0.5H_2O$: C, 56.63; H, 5.94; N, 10.32; found: C, 56.43; H, 5.80; N, 10.17.

5′-O-[2-(Levulinyloxymethyl)benzoyl]thymidine [9 (B = T, R = H, R′ = LMBz)]. Compound **9** (B = T, R = H, R′ = LMBz) was obtained in 67% yield (478 mg) by treating thymidine (**5**) (363 mg, 1.5 mmol) with **3** (413 mg, 1.65 mmol) in the presence of TPSCl (1.00 g, 3.3 mmol) in pyridine (15 mL) for 1 day and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.69 (s, 3H, CH_3 of thymine), 2.21 (s, 3H, $COCH_3$ of LMBz group), 2.16–2.25 (m, 1H, H-2′), 2.42–2.50 (m, 1H, H-2″), 2.63 (t, 2H, J = 6.4 Hz, CH_2 of LMBz group), 2.78 (t, 2H, CH_2 of LMBz group), 4.21–4.25 (m, 1H, H-4′), 4.49–4.54 (m, 1H, H-3′), 4.55 (dd, 1H, $J_{4',5'}$ = 3.8 Hz, $J_{5',5''}$ = 12.1 Hz, H-5′), 4.66 (dd, 1H, $J_{4',5''}$ = 4.4 Hz, H-5″), 5.42, 5.53 (2 d, 2H, J = 13.4 Hz, PhCH_2), 6.30 (t, 1H, $J_{1',2'} = J_{1',2''}$ = 6.5 Hz, H-1′), 7.21 (s, 1H, H-6), 7.42 (t, 1H, J = 7.6 Hz, Ph-*H*), 7.50–7.61 (m, 2H, Ph-*H* × 2), 7.95 (d, 1H, J = 7.7 Hz, Ph-*H*), and 8.48 (br s, 1H, N^3-*H*). *Anal.*, calc. for $C_{23}H_{26}N_2O_9 \cdot 0.25 H_2O$: C, 57.68; H, 5.58; N, 5.85; found: C, 57.61; H, 5.50; N, 5.82.

5′-O-[2-(Levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)uridine [9 (B = U, R = OThp, R′ = LMNBz)]. Compound **9** (B = U, R = OThp, R′ = LMNBz) was obtained in 63% yield (229 mg) by the reaction of **6** (B = U) (197 mg, 0.6 mmol; the more polar diastereoisomer) [21] with **4** (195 mg, 0.66 mmol) in the presence of TPSCl (400 mg, 1.32 mmol) in pyridine (6 mL) for 2 h and subsequent work-up as described above; m.p. 171–172°C (from methanol); ^{1}H-NMR ($CDCl_3$): δ 1.52–1.85 (m, 6H, CH_2 × 3 of Thp group), 2.21 (s, 3H, $COCH_3$ of LMNBz group), 2.70 (t, 2H, J = 6.7 Hz, CH_2 of LMNBz group), 2.80 (t, 2H, CH_2 of LMNBz group), 3.45–3.50, 3.77–3.83 (2m, 2H, OCH_2C of Thp group), 4.12–4.17 (m, 1H, H-4′), 4.48–4.75 (m, 5H, H-2′, 3′, 5′, 5″, and OC*H*O of Thp group), 5.63 (s, 2H, PhCH_2), 5.66 (d, 1H, $J_{5,6}$ = 8.1 Hz, H-5), 5.67 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1′), 7.22 (d, 1H, H-6), 7.78 (d, 1H, J = 8.6 Hz, Ph-*H*), 8.40 (dd, 1H, J = 2.4 Hz and J = 8.6 Hz, 2H, Ph-*H*), 8.59 (br s, 1H, N^3-*H*), and 8.86 (d, 1H, J = 2.4 Hz, Ph-*H*). *Anal.*, calc. for $C_{27}H_{31}N_3O_{13} \cdot 0.1 H_2O$: C, 53.39; H, 5.18; N, 6.92; found: C, 53.25; H, 5.20; N, 6.96.

N^4-Anisoyl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)cytidine [9 (B = C^{An}, R = OThp, R′ = LMNBz)]. Compound **9** (B = C^{An}, R = OThp, R′ = LMNBz) was obtained in 64% yield (282 mg) by treating **6** (B = C^{An}) (277 mg, 0.6 mmol; the more polar diastereoisomer) [21] with **4** (195 mg, 0.66 mmol) in the presence of TPSCl (400 mg, 1.32 mmol) in pyridine (6 mL) for 2 h and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.50–1.88 (m, 6H, CH_2 × 3 of Thp group), 2.19 (s, 3H, $COCH_3$ of LMNBz group), 2.67 (t, 2H, J = 6.7 Hz, CH_2 of LMNBz group), 2.81 (t, 2H, J = 6.7 Hz, CH_2 of LMNBz group), 3.05 (d, 1H, $J_{3',3'\text{-OH}}$ = 8.4 Hz, 3′-OH), 3.45–3.53, 3.85–3.92 (2 m, 2H, OCH_2C of Thp group), 3.88 (s, 3H, OCH_3), 4.26–4.31 (m, 1H, H-4′), 4.44–4.83 (m, 5H, H-2′, 3′, 5′, 5″, and OC*H*O of Thp group), 5.62 (s, 2H, PhCH_2), 5.85 (d, 1H, $J_{1',2'}$ = 2.1 Hz, H-1′), 6.95–7.00 (m, 1H, H-5), 6.98 (d, 2H, J = 8.8 Hz, Ph-*H* × 2 of An group), 7.78 (d, 2H, J = 8.8 Hz, Ph-*H* × 2 of An group), 7.80–7.85 (m, 4H, H-6), 7.87 (d, 1H, J = 8.6 Hz, Ph-*H* of LMNBz group), 8.41 (dd, 1H, J = 2.4 Hz and J = 8.6 Hz, Ph-*H* of LMNBz group), 8.70 (br s, 1H, N^4-*H*) and 8.82 (d, 1H, J = 2.4 Hz, Ph-*H* of LMNBz group). *Anal.*, calc. for $C_{35}H_{38}N_4O_{14} \cdot 0.3 H_2O$: C, 56.50; H, 5.23; N, 7.53; found: C, 56.28; H, 5.28; N, 7.36.

N^6-Benzoyl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)adenosine [9 (B = A^{Bz}, R = OThp, R′ = LMNBz)]. Compound **9** (B = A^{Bz}, R = OThp, R′ = LMNBz) was obtained in 65% yield (283 mg) by treating **6** (B = A^{Bz}) (273 mg, 0.6 mmol; the more polar diastereoisomer) [21] with **4** (195 mg, 0.66 mmol) in the presence of TPSCl (400 mg, 1.32 mmol) in pyridine (6 mL) for 2 h and subsequent work-up as de-

scribed above; ^{1}H-NMR ($CDCl_3$): δ 1.43–1.84 (m, 6H, CH_2 × 3 of Thp group), 2.19 (s, 3H, $COCH_3$ of LMNBz group), 2.67 (t, 2H, J = 6.7 Hz, CH_2 of LMNBz group), 2.76 (t, 2H, J = 6.7 Hz, CH_2 of LMNBz group), 2.92 (d, 1H, $J_{3',3'\text{-OH}}$ = 7.3 Hz, 3'-OH), 3.22–3.28, 3.60–3.66 (2m, 2H, OCH_2C of Thp group), 4.35–4.40 (m, 1H, H-4'), 4.60–4.66, 4.80–4.86 (m, 4H, H-3', 5', 5'', and OC*H*O of Thp group), 5.01 (dd, 1H, $J_{1',2'}$ = 3.3 Hz and $J_{2',3'}$ = 5.6 Hz, H-2'), 5.53, 5.61 (2 d, 2H, J = 16.0 Hz, $PhCH_2$), 6.19 (d, 1H, H-1'), 7.50–7.62 (m, 3H, Ph-*H* × 3 of Bz group), 7.77 (d, 1H, J = 8.6 Hz, Ph-*H* of LMNBz group), 8.02 (d, 2H, J = 7.1 Hz, Ph-*H* × 2 of Bz group), 8.07 (s, 1H, H-8), 8.39 (dd, J = 2.4 Hz and J = 8.6 Hz, Ph-*H* of LMNBz group), 8.59 (s, 1H, H-2), 8.78 (d, 1H, J = 2.4 Hz, Ph-*H* of LMNBz group), and 9.02 (br s, 1H, N^6-*H*). *Anal.*, calc. for $C_{35}H_{36}N_6O_{12} \cdot 0.4\ H_2O$: C, 56.82; H, 5.01; N, 11.36; found: C, 57.10; H, 5.12; N, 11.07.

N^2-Isobutyryl-5'-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2'-O-(tetrahydropyran-2-yl)guanosine [9 (B = G^{iBu}, R = OThp, R' = LMNBz)]. Compound **9** (B = G^{iBu}, R = OThp, R' = LMNBz) was obtained in 72% yield (310 mg) by treating **6** (B = G^{iBu}) (262 mg, 0.6 mmol; the less polar diastereoisomer) [21] with **4** (195 mg, 0.66 mmol) in the presence of TPSCl (400 mg, 1.32 mmol) in pyridine (6 mL) for 2 h and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.26, 1.29 (2d, 6H, J = 7.2 Hz, $CH(CH_3)_2$), 1.67–1.85 (m, 6H, CH_2 × 3 of Thp group), 2.20 (s, 3H, $COCH_3$ of LMNBz group), 2.64–2.75 (m, 1H, $CH(CH_3)_2$), 2.66 (t, 2H, J = 6.2 Hz, CH_2 × 2 of LMNBz group), 2.80 (t, 2H, J = 6.2 Hz, CH_2 × 2 of LMNBz group), 3.46–3.55, 3.92–4.01 (2m, 2H, OCH_2C of Thp group), 3.69 (d, 1H, $J_{3',3'\text{-OH}}$ = 3.5 Hz, 3'-OH), 4.49–4.55, 4.73–4.78 (2m, 6H, H-2', 3', 4', 5', 5'', and OC*H*O of Thp group), 5.52 (s, 2H, $PhCH_2$), 5.90 (d, 1H, $J_{1',2'}$ = 5.2 Hz, H-1'), 7.72 (s, 1H, H-8), 7.78 (d, 1H, J = 8.6 Hz, Ph-*H*), 8.39 (dd, 1H, J = 2.4 Hz and J = 8.6 Hz, Ph-*H*), 8.77 (d, 1H, J = 2.4 Hz, Ph-*H*), 9.13 (br s, 1H, N^1-*H*), and 12.06 (br s, 1H, N^2-*H*). *Anal.*, calc. for $C_{32}H_{38}N_6O_{13} \cdot 1.5\ H_2O$: C, 51.82; H, 5.57; N, 11.33; found: C, 51.74; H, 5.33; N, 11.05.

5'-O-[2-(Levulinyloxymethyl)-5-nitrobenzoyl]thymidine [9 (B = T, R = H, R' = LMNBz)]. Compound **9** (B = T, R = H, R' = LMNBz) was obtained in 71% yield (185 mg) by the reaction of **5** (121 mg, 0.5 mmol) with **4** (162 mg, 0.55 mmol) in the presence of TPSCl (333 mg, 1.1 mmol) in pyridine (5 mL) for 2 h and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ1.80 (s, 3H, CH_3 of thymine), 2.21 (s, 3H, $COCH_3$ of LMNBz group), 2.23–2.50 (m, 1H, H-2'), 2.42–2.50 (m, 1H, H-2''), 2.68 (t, 2H, J = 6.5 Hz, CH_2 of LMNBz group), 2.83 (t, 2H, J = 6.5 Hz, CH_2 of LMNBz group), 4.23–4.28 (m, 1H, H-4'), 4.53–4.69 (m, 3H, H-3', 5', and 5''), 5.55, 5.62 (2d, 2H, J = 15.6 Hz, $PhCH_2$), 6.22 (t, 1H, $J_{1',2'} = J_{1',2''}$ = 6.6 Hz, H-1'), 7.17 (s, 1H, H-6), 7.79 (d, 1H, J = 8.7 Hz, Ph-*H*), 8.41 (d, 1H, J = 2.5 Hz and J = 8.7 Hz, Ph-*H*), 8.48 (br s, 1H, N^3-*H*), and 8.81 (d, 1H, J = 2.5 Hz, Ph-*H*). *Anal.*, calc. for $C_{23}H_{25}N_3O_{11}$: C, 53.18; H, 4.85; N, 8.09; found: C, 52.88; H, 4.84; N, 8.11. m.p. 162–163°C (from methanol).

5'-O-[2-(Levulinyloxymethyl)benzoyl]-2'-O-(tetrahydropyran-2-yl)uridine 3'-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = U, R = OThp, R' = LMBz)]. Compound **9** (B = U, R = OThp, R' = LMBz) (336 mg, 0.6 mmol; the more polar diastereoisomer) was, after azeotropic evaporation from pyridine (2 mL × 3), dissolved in dried acetonitrile (6 mL), and *N*-ethyldiisopropylamine (0.16 mL, 0.9 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite [22] (0.20 mL, 0.9 mmol) were added to the solution, which was then stirred at room temperature for 1 h. The resulting mixture was quenched with water (1 mL) with stirring. The solution was diluted with ethyl acetate (50 mL) and washed with saturated aqueous sodium hydrogen carbonate solution (25 mL × 2) and water (25 mL). After drying over anhydrous magnesium sulfate the organic layer was evaporated to dryness, and

the residue was subjected to chromatographic separation on a column of silica gel by the use of n-hexane/ethyl acetate system to give **10** (B = U, R = OThp, R′ = LMBz) (383 mg, 84% yield); ^{31}P-NMR ($CDCl_3$): δ 149.88 and 150.42.

N^4-Anisoyl-5′-O-[2-(levulinyloxymethyl)benzoyl]-2′-O-(tetrahydropyran-2-yl)cytidine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = C^{An}, R = OThp, R′ = LMBz)]. Compound **10** (B = C^{An}, R = OThp, R′ = LMBz) was obtained in 76% yield (327 mg) by treating **9** (B = C^{An}, R = OThp, R′ = LMBz) (331 mg, 0.5 mmol; the more polar diastereoisomer) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.17 mL, 0.75 mmol) in the presence of *N*-ethyldiisopropylamine (0.13 mL, 0.75 mmol) in dried acetonitrile (5 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ 149.75 and 150.57.

N^6-Benzoyl-5′-O-[2-(levulinyloxymethyl)benzoyl]-2′-O-(tetrahydropyran-2-yl)adenosine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = A^{Bz}, R = OThp, R′ = LMBz)]. Compound **10** (B = A^{Bz}, R = OThp, R′ = LMBz) was obtained in 91% yield (403 mg) by treating **9** (B = A^{Bz}, R = OThp, R′ = LMBz) (344 mg, 0.5 mmol; the more polar diastereoisomer) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.17 mL, 0.75 mmol) in the presence of *N*-ethyldiisopropylamine (0.13 mL, 0.75 mmol) in dried acetonitrile (5 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ 149.67 and 150.22.

N^2-Isobutyryl-5′-O-[2-(levulinyloxymethyl)benzoyl]-2′-O-(tetrahydropyran-2-yl)guanosine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = G^{iBu}, R = OThp, R′ = LMBz)]. Compound **10** (B = G^{iBu}, R = OThp, R′ = LMBz) was obtained in 75% yield (415 mg) by treating **9** (B = G^{iBu}, R = OThp, R′ = LMBz) (375 mg, 0.55 mmol; the more polar diastereoisomer) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.19 mL, 0.83 mmol) in the presence of *N*-ethyldiisopropylamine (0.14 mL, 0.83 mmol) in dried acetonitrile (5.5 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ149.10 and 149.65.

5′-O-[2-(Levulinyloxymethyl)benzoyl]thymidine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = T, R = H, R′ = LMBz)]. Compound **10** (B = T, R = H, R′ = LMBz) was obtained in 66% yield (265 mg) by treating **9** (B = T, R = H, R′ = LMBz) (285 mg, 0.6 mmol) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.20 mL, 0.9 mmol) in the presence of *N*-ethyldiisopropylamine (0.16 mL, 0.9 mmol) in dried acetonitrile (6 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ149.00 and 149.16.

5′-O-[2-(Levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)uridine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = U, R = OThp, R′ = LMNBz)]. Compound **10** (B = U, R = OThp, R′ = LMNBz) was obtained in 90% yield (287 mg) by treating **9** (B = U, R = OThp, R′ = LMNBz) (242 mg, 0.4 mmol; the more polar diastereoisomer) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.14 mL, 0.6 mmol) in the presence of *N*-ethyldiisopropylamine (0.11 mL, 0.6 mmol) in dried acetonitrile (4 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ149.79 and 150.18.

N^4-Anisoyl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)cytidine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = C^{An}, R = OThp, R′ = LMNBz)]. Compound **10** (B = C^{An}, R = OThp, R′ = LMNBz) was obtained in 77% yield (253 mg) by treating **9** (B = C^{An}, R = OThp, R′ = LMNBz) (259 mg, 0.35 mmol; the more polar diastereoisomer) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.12 mL, 0.53 mmol) in the presence of *N*-ethyldiisopropylamine (90 μL, 0.53 mmol) in dried acetonitrile (3.5 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ 149.68 and 150.38.

N^6-Benzoyl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)adenosine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = A^{Bz}, R = OThp, R′ = LMNBz)]. Compound **10** (B = A^{Bz}, R = OThp, R′ = LMNBz) was obtained in 80% yield (263 mg) by treating **9** (B = A^{Bz}, R = OThp, R′ = LMNBz) (256 mg, 0.35 mmol; the more polar diastereoisomer) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.12 mL, 0.53 mmol) in the presence of *N*-ethyldiisopropylamine (90 μL, 0.53 mmol) in dried acetonitrile (3.5 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ 150.51 and 150.87.

N^2-Isobutyryl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)guanosine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = G^{iBu}, R = OThp, R′ = LMNBz)]. Compound **10** (B = G^{iBu}, R = OThp, R′ = LMNBz) was obtained in 80% yield (291 mg) by treating **9** (B = G^{iBu}, R = OThp, R′ = LMNBz) (286 mg, 0.4 mmol; the less polar diastereoisomer) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.14 mL, 0.6 mmol) in the presence of *N*-ethyldiisopropylamine (0.11 mL, 0.6 mmol) in dried acetonitrile (4 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ150.06 and 150.50.

5′-O-[2-(Levulinyloxymethyl)-5-nitrobenzoyl]thymidine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = T, R = H, R′ = LMNBz)]. Compound **10** (B = T, R = H, R′ = LMNBz) was obtained in 65% yield (186 mg) by treating **9** (B = T, R = H, R′ = LMNBz) (208 mg, 0.4 mmol) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.14 mL, 0.6 mmol) in the presence of *N*-ethyldiisopropylamine (0.11 mL, 0.6 mmol) in dried acetonitrile (4 mL) and subsequent work-up as described above; ^{31}P-NMR ($CDCl_3$): δ149.13 and 149.29.

3′-O-[3-(Carboxy)propionyl]-N^2-isobutyryl-5′-O-[2-(levulinyloxymethyl)benzoyl]-2′-O-(tetrahydropyran-2-yl)guanosine. A solution of **9** (B = G^{iBu}, R = OThp, R′ = LMBz) (205 mg, 0.3 mmol; the more polar diastereoisomer) in pyridine (1 mL) was treated with succinic anhydride (45 mg, 0.45 mmol) and 4-dimethylaminopyridine (DMAP) (18 mg, 0.15 mmol) at room temperature for 12 h with stirring [23]. The resulting mixture was quenched by treating with chilled water (1 mL) at room temperature for 30 min with stirring and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel by the use of chloroform/methanol system to give 3′-*O*-[3-(carboxy)propionyl]-N^2-isobutyryl-5′-*O*-[2-(levulinyloxymethyl)benzoyl]-2′-*O*-(tetrahydropyran-2-yl)guanosine (234 mg, quantitative yield); ^{1}H-NMR ($CDCl_3$): δ 1.21, 1.24 (2d, 6H, J = 6.9 Hz, CH(CH_3)$_2$), 1.37–1.65 (m, 6H, CH_2 × 3 of Thp group), 2.17 (s, 3H, COCH_3 of LMBz group), 2.62–2.81 (m, 9H, CH_2 × 4 and CH(CH_3)$_2$), 3.16 –3.20 (m, 2H, OCH_2C of Thp group), 4.55–4.75 (m, 4H, H-4′, 5′, 5″, and OCHO of Thp group), 5.07 (t, 1H, $J_{1',2'}$ = $J_{2',3'}$ = 5.3 Hz, H-2′), 5.45 (s, 2H, PhCH_2), 5.67 (t, 1H, H-3′), 5.93 (d, 1H, H-1′), 7.31–7.36 (m, 1H, Ph-*H*), 7.48–7.58 (m, 2H, Ph-*H* × 2), 7.76 (s, 1H, H-8), 7.86 (d, 1H, J = 7.8 Hz, Ph-*H*), 9.37 (br s, 1H, N^1-*H*), and 12.07 (br s, 1H, N^2-*H*).

3′-O-[3-(Carboxy)propionyl]-5′-O-[2-(levulinyloxymethyl)benzoyl]thymidine. 3′-*O*-[3-(Carboxy)propionyl]-5′-*O*-[2-(levulinyloxymethyl)benzoyl]thymidine was obtained in 93% yield (160 mg) by treating **9** (B = T, R = H, R′ = LMBz) (142 mg, 0.3 mmol) in pyridine (1 mL) with succinic anhydride (45 mg, 0.45 mmol) in the presence of DMAP (18 mg, 0.15 mmol) for 12 h and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.65 (s, 3H, CH_3 of thymine), 2.19 (s, 3H, COCH_3 of LMBz group), 2.23–2.28 (m, 1H, H-2′), 2.51-2.59 (m, 1H, H-2″), 2.63–2.81 (m, 8H, CH_2 × 4), 4.38–4.42 (m, 1H, H-4′), 4.60–4.62 (m, 2H, H-5′ and 5″), 5.37–5.39 (m, 1H, H-3′), 5.52 (s, 2H, PhCH_2), 6.32 (dd, 1H, $J_{1',2'}$ = 8.3 Hz, $J_{1',2''}$ = 5.8 Hz, H-1′), 7.23 (s, 1H, H-6),

7.37–7.42 (m, 1H, Ph-*H*), 7.54–7.62 (m, 2H, Ph-*H* × 2), 7.93 (d, 1H, *J* = 7.8 Hz, Ph-*H*), and 9.74 (br s, 1H, N^3-*H*).

3′-O-[3-(Carboxy)propionyl]-N^2-isobutyryl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)guanosine. 3′-*O*-[3-(Carboxy)propionyl]-N^2-isobutyryl-5′-*O*-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-*O*-(tetrahydropyran-2-yl) guanosine was obtained in 98% yield (240 mg) by treating **9** (B = G^{iBu}, R = OThp, R′ = LMNBz) (214 mg, 0.3 mmol; the less polar diastereoisomer) with succinic anhydride (45 mg, 0.45 mmol) in pyridine (1 mL) in the presence of DMAP (18 mg, 0.15 mmol) in pyridine (1 mL) for 12 h and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.25, 1.26 (2d, 6H, *J* = 6.9 Hz, $CH(CH_3)_2$), 1.45–1.76 (m, 6H, CH_2 × 3 of Thp group), 2.20 (s, 3H, $COCH_3$ of LMNBz group), 2.63–2.83 (m, 9H, CH_2 × 4 and $CH(CH_3)_2$), 3.43–3.50, 3.70–3.78 (2m, 2H, OCH_2C of Thp group), 4.50–4.65 (m, 4H, H-4′, 5′, 5″, and OC*H*O of Thp group), 5.19 (t, 1H, $J_{1',2'} = J_{2',3'}$ = 5.8 Hz, H-2′), 5.45 (s, 2H, $PhCH_2$), 5.60–5.63 (m, 1H, H-3′), 6.12 (d, 1H, H-1′), 7.70 (d, 1H, *J* = 8.6 Hz, Ph-*H*), 7.80 (s, 1H, H-8), 8.34 (dd, 1H, *J* = 2.5 Hz and *J* = 8.6 Hz, Ph-*H*), 8.69 (d, 1H, *J* = 2.5 Hz, Ph-*H*), 9.72 (br s, 1H, N^1-*H*), and 12.16 (br s, 1H, N^2-*H*).

3′-O-[3-(Carboxy)propionyl]-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl] thymidine. 3′-*O*-[3-(Carboxy)propionyl]-5′-*O*-[2-(levulinyloxymethyl)-5-nitrobenzoyl]thymidine was obtained in a quantitative yield (190 mg) by treating **9** (B = T, R = H, R′ = LMNBz) (160 mg, 0.31 mmol) in pyridine (1 mL) with succinic anhydride (45 mg, 0.45 mmol) in the presence of DMAP (18 mg, 0.15 mmol) for 6 h and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ1.77 (s, 3H, CH_3 of thymine), 2.19 (s, 3H, $COCH_3$ of LMNBz group), 2.25–2.36 (m, 1H, H-2′), 2.48–2.59 (m, 1H, H-2″), 2.62–2.71 (m, 6H, CH_2 × 3), 2.82 (t, 2H, *J* = 6.5 Hz, Ph-*H* of LMNBz group), 4.38–4.42 (m, 1H, H-4′), 4.56–4.70 (m, 2H, H-5′ and 5″), 5.33–5.36 (m, 1H, H-3′), 5.60 (s, 2H, $PhCH_2$), 6.25 (dd, 1H, $J_{1',2'}$ = 8.2 Hz, $J_{1',2''}$ = 5.9 Hz, H-1′), 7.22 (s, 1H, H-6), 7.80 (d, 1H, *J* = 8.7 Hz, Ph-*H*), 8.40 (dd, 1H, *J* = 2.4 Hz and *J* = 8.7 Hz, Ph-*H*), 8.78 (d, 1H, *J* = 2.4 Hz, Ph-*H*), and 9.97 (br s, 1H, N^3-*H*).

Functionalization of the CPG support with a nucleoside 3′-(carboxy)propionate derivative (11) [24].

1) 3′-*O*-[3-(Carboxy)propionyl]-N^2-isobutyryl-5′-*O*-[2-(levulinyloxymethyl)benzoyl]-2′-*O*-(tetrahydropyran-2-yl)guanosine (156 mg, 0.2 mmol; the more polar diastereoisomer) and 1-hydroxybenzotriazole (41 mg, 0.3 mmol) were, after azeotropic evaporation from pyridine (2 mL × 3), dissolved in dried 1,4-dioxane (2 mL), to which was added DCC (62 mg, 0.3 mmol). After stirring for 2 h at room temperature, the resulting precipitates were removed by filtration, and the filtrate was poured onto the CPG support (Long Chain Amino-Alkyl Controlled-Pore Glass, 500 Å, 80–120 mesh; purchased from Funakoshi) (400 mg), which was in advance subjected to azeotropic evaporation from pyridine (2 mL × 3). The mixture was shaken for 30 min at room temperature in the presence of 1-methylimidazole (80 μL, 1 mmol). CPG loaded with N^2-isobutyryl-5′-*O*-[2-(levulinyloxymethyl)benzoyl]-2′-*O*-(tetrahydropyran-2-yl)guanosine derivative [**11** (B = G^{iBu}, R = OThp, R′ = LMBz)] was filtered off and washed successively with 1,4-dioxane (5 mL), acetonitrile (5 mL), methanol (5 mL), and methylene chloride (5 mL). Unreacted amino groups of the CPG support were capped by treating with 1:1:8 acetic anhydride/2,6-lutidine/THF (1.5 mL) and 1:9 1-methylimidazole/THF (1.5 mL) for 30 min at room temperature, followed by successive washing of CPG **11** (B = G^{iBu}, R = OThp, R′ = LMBz) (255 mg) with 1,4-dioxane (5 mL), acetonitrile (5 mL), methanol (5 mL), and finally methylene chloride (5 mL).

Guanosine content in CPG **11** (B = G^{iBu}, R = OThp, R′ = LMBz) was determined to be 32.5 μmol/g by UV spectroscopy [guanosine, λ_{max} 253 nm in H_2O (ε = 13600) [25]] after deprotection of the LMBz group by consecutive treatments with 0.5 M $NH_2NH_2 \cdot H_2O$ in 4:1 pyridine/acetic acid at room temperature for 15 min and with 0.5 M imidazole in acetonitrile at room temperature for 5 min. Then **6** (B = G^{iBu}) was detached by treating with conc. aqueous ammonia at room temperature for 3 h, the N^2-iBu group was removed by treating with conc. aqueous ammonia at 55°C for 6 h, and the finally 2′-*O*-Thp group was removed by treating with HCl (pH 2.0) at room temperature for 1 day using 9.8 mg of CPG **11**.

2) 3′-*O*-[3-(Carboxy)propionyl]-5′-*O*-[2-(levulinyloxymethyl)benzoyl]thymidine (160 mg, 0.278 mmol) was introduced to the CPG support (556 mg) as described above to give the CPG loaded with 5′-*O*-[2-(levulinyloxymethyl)benzoyl]thymidine derivative [**11** (B = T, R = H, R′ = LMBz)] (526 mg). Thymidine content in CPG **11** (B = T, R = H, R′ = LMBz) was determined to be 52.8 μmol/g by UV spectroscopy [thymidine, λ_{max} 267 nm in H_2O (ε = 9650) [26]] after deprotection of the LMBz group and removing **5** from CPG **11** (B = T, R = H, R′ = LMBz) (40 mg).

3) 3′-*O*-[3-(Carboxy)propionyl]-N^2-isobutyryl-5′-*O*-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-*O*-(tetrahydropyran-2-yl)guanosine (204 mg, 0.25 mmol) was introduced to the CPG support (500 mg) as described for the synthesis of CPG **11** (B = G^{iBu}, R = OThp, R′ = LMBz) to give CPG **11** (B = G^{iBu}, R = OThp, R′ = LMNBz). Guanosine content of CPG **11** (B = G^{iBu}, R = OThp, R′ = LMNBz) was determined to be 48.8 μmol/g by UV spectroscopy after deprotection of the LMNBz group, removing **6** (B = G^{iBu}), and deprotection of the iBu and Thp groups, using of 9.8 mg CPG **11** (B = G^{iBu}, R = OThp, R′ = LMNBz).

4) 3′-*O*-[3-(Carboxy)propionyl]-5′-*O*-[2-(levulinyloxymethyl)-5-nitrobenzoyl]thymidine (190 mg, 0.307 mmol) was introduced to the CPG support (614 mg) as described for CPG **11** (B = G^{iBu}, R = OThp, R′ = LMBz) to give CPG containing 5′-*O*-[2-(levulinyloxymethyl)-5-nitrobenzoyl]thymidine derivative (637 mg) [**11** (B = T, R = H, R′ = LMNBz)]. Thymidine content of CPG **11** (B = T, R = H, R′ = LMNBz) was determined to be 53.6 μmol/g) by UV spectroscopy after deprotection of the LMNBz group and removing **5** from CPG **11** (B = T, R = H, R′ = LMNBz) (10 mg).

Synthesis of TpTpT (15) and TpTpTpT (16). After chain elongation with a simple syringe-based reaction system [2, 27] using a column of CPG **11** (B = T, R = H, R′ = LMBz) or CPG **11** (B = T, R = H, R′ = LMNBz) and **10** (B = T, R = H, R′ = LMBz) or **10** (B = T, R = H, R′ = LMNBz) (Table 2), the resulting oligothymidylic acid was detached from the CPG support by treating with conc. aqueous ammonia (4 mL) for 3 h at room temperature. After evaporation of aqueous ammonia, the residue was dissolved in water (10 mL) and washed with ethyl acetate (5 mL × 3). The aqueous layer was evaporated and the residual deprotected oligothymidylic acid was purified by reverse-phase HPLC (see Figs. 1, 2, 3, and 4). The main peak was collected [yield of **15**, 4.8 A_{260} units from CPG **11** (B = T, R = H, R′ = LMBz) (23 mg, 1.23 μmol) and 18.0 A_{260} units from CPG **11** (B = T, R = H, R′ = LMNBz) (20 mg, 1.07 μmol); yield of **16**, 5.6 A_{260} units from CPG **11** (B = T, R = H, R′ = LMBz) (23 mg, 1.23 μmol) and 16.8 A_{260} units from CPG **11** (B = T, R = H, R′ = LMNBz) (20 mg, 1.07 μmol)].

Synthesis of the octaribonucleotide UpCpApGpUpUpGpG (17). After chain elongation with a simple syringe-based reaction system using a column of CPG **11** (B = G^{iBu}, R = OThp, R′ = LMBz) or CPG **11** (B = G^{iBu}, R = OThp, R′ = LMNBz) and 5′-*O*-LMBz-[**10** (R = OThp, R′ = LMBz)] or 5′-*O*-LMNBz-2′-*O*-Thp-ribonucleoside 3′-phosphoramidite units [**10** (R = OThp, R′ = LMNBz)] (Table 2), the resulting octamer was detached from the CPG support by treatment with conc. aqueous ammonia (4 mL) for 3 h at room temperature. The resulting solution was heated in a sealed

vial at 55°C for 6 h. After evaporation, the residue was dissolved in water (10 mL) and washed with ethyl acetate (5 mL × 3). The aqueous layer was evaporated and the residue was dissolved in HCl (pH 2, 5 mL). The solution was left to stand for 1 day at room temperature, neutralized with 0.2 M aqueous ammonia, and washed with ethyl acetate (5 mL × 3). The deprotected oligonucleotide was purified by reverse-phase HPLC (see Figs. 5 and 6 (I)). The main peak was collected [yield of **17**, 41.1 A_{260} units from CPG **11** (B = G^{iBu}, R = OThp, R' = LMNBz) (40 mg, 1.95 μmol), see Figs. 6 (II) and 7].

C:U:G:A ratio obtained for the octamer was 1.0 : 3.1 : 3.0 : 0.9, see Fig. 8).

N^4-Anisoyl-2'-O-(tetrahydropyran-2-yl)[4-^{15}N]cytidine {7 (B = [4-^{15}N]C^{An})}. 3',5'-Di-*O*-benzoyl-2'-*O*-(tetrahydropyran-2-yl)-[4-^{15}N]cytidine [28, 29] (815 mg, 1.52 mmol) was, after azeotropic evaporation from pyridine (5 mL × 3), dissolved in dried pyridine (7.5 mL), and anisoyl chloride (389 mg, 2.28 mmol) was added to the solution, which was then stirred at room temperature for 1 h. The mixture was quenched with water (2 mL), diluted with chloroform (40 mL) and washed with 5% aqueous $NaHCO_3$ (20 mL × 2) and

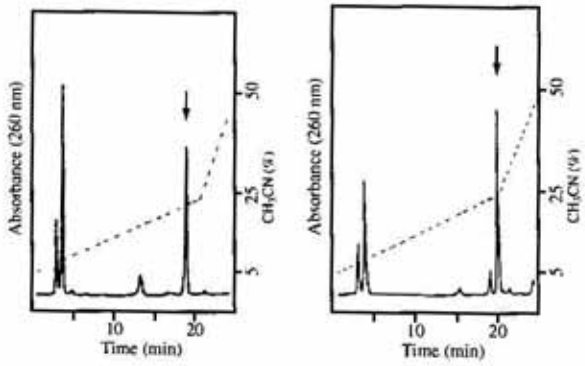


Figure 1.

Figure 2.

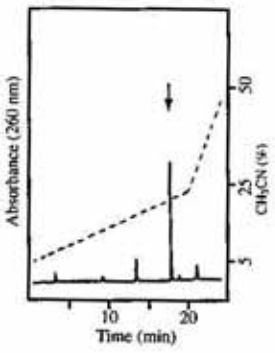


Figure 3.

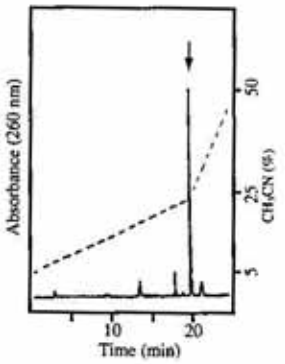


Figure 4.

Figure 1. Yield of TpTpT; 4.8 A_{260} units from 11 (B = T, R = H, R' = LMBz; 1.23 μmol).

Figure 2. Yield of TpTpTpT; 5.6 A_{260} units from 11 (B = T, R = H, R' = LMBz; 1.23 μmol).

Figure 3. Yield of TpTpT; 18 A_{260} units from 11 (B = T, R = H, R' = LMNBz; 1.07 μmol).

Figure 4. Yield of TpTpTpT; 16.8 A_{260} units from 11 (B = T, R = H, R' = LMNBz; 1.07 μmol).

Reversed-phase HPLCs of crude products of TpTpT and TpTpTpT preparations using the 5'-*O*-LMBz-thymidine 3'-phosphoramidite derivative 10 (B = T, R = H, R' = LMBz) (Figs. 1 and 2) and the 5'-*O*-LMNBz-thymidine 3'-phosphoramidite derivative 10 (B = T, R = H, R' = LMNBz) (Figs. 3 and 4), respectively.

Enzymatic digestion of UpCpApGpUpUpGpG (17). A solution of **17** (1.0 A_{260} unit) in 70 μl of 30 mM Tris/HCl (pH 8.0) and 6 mM $MgCl_2$ was treated with snake venom phosphodiesterase (1 mg/0.5 mL, Boehringer Mannheim) (6 μL) and alkaline phosphatase (1 u/mL, Boehringer Mannheim) (6.4 μL) for 24 h at 37°C [12, 15, 16]. Digestion products were analyzed by reversed-phase HPLC with 2% acetonitrile/0.04 M triethylammonium acetate (TEAA) (pH 7.0) as eluent (the

water (20 mL). The organic layer was evaporated and the residue was dissolved in 1:2 pyridine/ethanol (7.2 mL). Six milliliters of 2 M aqueous NaOH was added to the above solution under cooling in an ice-bath. After stirring for 10 min at 0°C, the resulting mixture was neutralized with Dowex 50Wx8 (H^+ form). The resin was filtrated off and washed with 1:2 pyridine/ethanol solution (about 50 mL). The filtrate and the washings were combined and evaporated. The residue was then

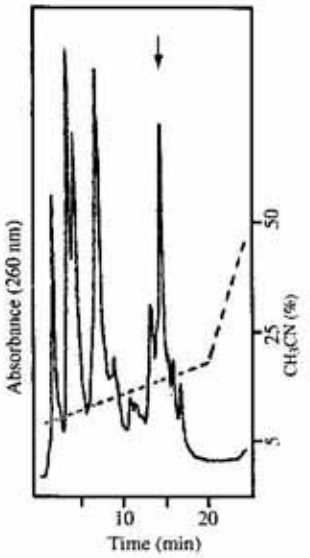


Figure 5.

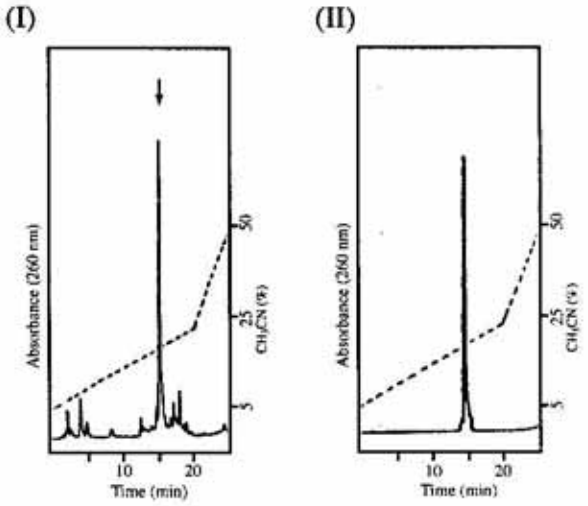


Figure 6.

Figure 5. Reversed-phase HPLC of crude products from the preparation of the octamer (UpCpApGpUpUpGpG) using 5′-*O*-LMBz-2′-*O*-Thp-ribonucleoside 3′-phosphoramidite derivatives 10 (R = OThp, R′ = LMBz).

Figure 6. (I) Reversed-phase HPLC of crude products from the preparation of the octamer (UpCpApGpUpUpGpG) using 5′-*O*-LMNBz-2′-*O*-Thp-ribonucleoside 3′phosphoramidite derivatives 10 (R = OThp, R′ = LMNBz). (II) Reversed-phase HPLC of the octamer isolated from the mixture of (I). Yield of the octamer; 41.1 A_{260} units from 11 (B = G^{iBu}, R = OThp, R′ = LMNBz; 1.95 μmol).

Conditions of reversed-phase HPLCs (Figs. 1, 2, 3, 4, 5 and 6): column μBONDASPHERE 5 μ C18 (3.9 mm ID × 150 mm L); elution buffer acetonitrile/0.1 M TEAA (pH 7); flow rate 1 mL/min; detected by UV at 260 nm.

subjected to chromatographic separation on a column of silica gel by the use of chloroform/methanol system to give **7** (B = [4-^{15}N]C^{An}) (the less polar diastereoisomer, 123 mg, and the more polar diastereoisomer, 508 mg, 90% yield); ^{1}H-NMR ($CDCl_3$): δ for the less polar diastereoisomer 1.45–1.90 (m, 6H, CH_2 × 3 of Thp group), 3.46–3.56 (m, 1H, OCH_2C of Thp group), 3.80–3.94 (m, 2H, H-5′ and OCH_2C of Thp group), 3.86 (s, 3H, OCH_3), 4.06 (dd, 1H, $J_{4',5''}$ = 1.8 Hz, $J_{5',5''}$ = 12.5 Hz, H-5′′), 4.14–4.17 (m, 1H, H-4′), 4.30 (dd, 1H, $J_{2',3'}$ = 5.0 Hz, $J_{3',4'}$ = 6.1 Hz, H-3′), 4.59 (dd, 1H, $J_{1',2'}$ = 3.1 Hz, H-2′), 4.75–4.78 (m, 1H, OC*H*O of Thp group), 5.74 (d,1H, H-1′), 6.96 (d, 2H, J = 9.0 Hz, Ph-*H* × 2 of An group), 7.48–7.65 (m, 1H, H-5), 7.87 (d, 1H, J = 9.0 Hz, Ph-*H* × 2 of An group), and 8.08 (d, 1H, $J_{5,6}$ = 7.7 Hz, H-6); ^{15}N-NMR ($CDCl_3$): δ for the less polar diastereoisomer 139.50 (N^4); EI mass spectrum, *m/z* 462.0 (M^+), and ^{1}H-NMR ($CDCl_3$): δ for the more polar diastereoisomer 1.38–1.86 (m, 6H, CH_2 × 3 of Thp group), 3.30–3.41 (m, 1H, OCH_2C of Thp group), 3.72–3.88 (m, 2H, H-5′and OCH_2C of Thp group), 3.86 (s, 3H, OCH_3), 3.98 (dd, 1H, $J_{4',5''}$ = 1.8 Hz, $J_{5',5''}$ = 12.5 Hz, H-5′′), 4.14–4.16 (m, 1H, H-4′), 4.50 (t, 1H, $J_{2',3'}$ = $J_{3',4'}$ = 5.2 Hz, H-3′), 4.74 (dd, 1H, $J_{1',2'}$ = 4.1 Hz, H-2′), 4.78–4.80 (m, 1H, OC*H*O of Thp

Figure 7. Electrophoresis of the octamer (17) (Fig. 6 II) on a 20% polyacrylamide gel containing 7 M urea, visualized by UV shadowing.

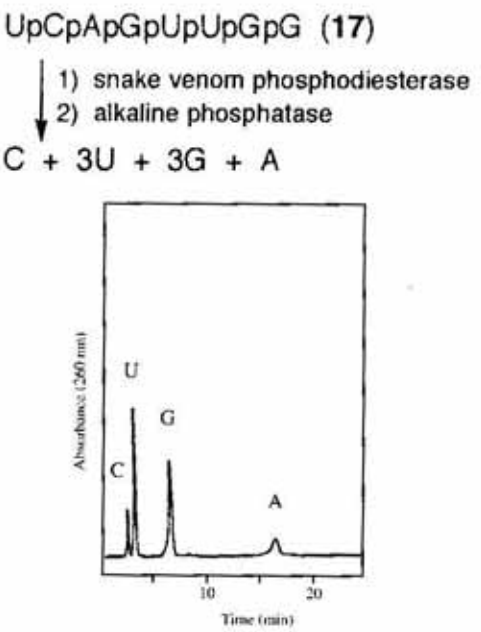


Figure 8. Reversed-phase HPLC of products obtained by digestion of the completely unmasked octamer (17) (Fig. 6 II) with snake venom phosphodiesterase and alkline phosphatase.

Conditions of reversed-phase HPLC: column μBONDASPHERE 5 μ C18 (3.9 mm ID × 150 mm L); elution buffer 2% acetonitrile/0.04 M TEAA (pH 7); flow rate 1 mL/min; detected by UV at 260 nm.

group), 5.79 (d,1H, H-1'), 6.95 (d, 2H, J = 8.9 Hz, Ph-*H* × 2 of An group), 7.46–7.60 (m, 1H, H-5), 7.85 (d, 2H, J = 8.9 Hz, Ph-*H* × 2 of An group), and 8.06 (d, 1H, $J_{5,6}$ = 7.4 Hz, H-6); ^{15}N-NMR ($CDCl_3$): δ for the more polar diastereoisomer 140.03 (N^4); EI mass spectrum, m/z 462.1 (M^+).

N*^{4}*-Anisoyl-5'-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2'-O-(tetrahydropyran-2-yl)[4-^{15}N]cytidine {9 (B = [4-^{15}N]C^{An}, R = OThp, R' = LMNBz)}. Compound **9** (B = [4-^{15}N]C^{An}, R = OThp, R' = LMNBz) was obtained in 62% yield (229 mg) by the reaction of **7** (B = [4-^{15}N]C^{An}) (231 mg, 0.5 mmol; the more polar diastereoisomer) with **4** (162 mg, 0.55 mmol) in the presence of TPSCl (333 mg, 1.1 mmol) in pyridine (5 mL) for 3 h and subsequent work-up as described in the synthesis of **9** (B = U, R = OThp, R' = LMBz); ^{1}H-NMR ($CDCl_3$): δ 1.52–1.90 (m, 6H, CH_2 × 3 of Thp group), 2.19 (s, 3H, $COCH_3$ of LMNBz group), 2.69 (t, 2H, J = 6.1 Hz, CH_2 of LMNBz group), 2.81 (t, 2H, J = 6.1 Hz, CH_2 of LMNBz group), 3.45–3.52, 3.84–3.91 (2m, 2H, OCH_2C of Thp group), 3.87 (s, 3H, OCH_3), 4.27–4.84 (m, 6H, H-2', 3', 4', 5', 5'', and OC*H*O of Thp group), 5.62 (s, 2H, $PhCH_2$), 5.85 (d, 1H, $J_{1',2'}$ = 2.0 Hz, H-1'), 6.97 (d, 2H, J = 8.8 Hz, Ph-*H* × 2 of An group), 7.33–7.50 (m, 1H, H-5), 7.74–7.87 (m, 4 H, H-6 and Ph-*H* × 3 of An and LMNBz groups), 8.40 (dd, 1H, J = 2.5 Hz, J = 8.6 Hz, Ph-*H* of LMNBz group), and 8.82 (d, 1H, J = 2.5 Hz, Ph-*H* of LMNBz group).

N*^{6}*-Benzoyl-2'-O-(tetrahydropyran-2-yl)[6-^{15}N]adenosine {7 (B = [6-^{15}N]A^{Bz})}. 6-(3-Nitro-1,2,4-triazol-1-yl)-9-[3',5'-di-*O*-benzoyl-2'-*O*-(tetrahydropyran-2-yl)-β-D-ribofuranosyl]-9*H*-purine [28, 29] (1.642 g, 2.5 mmol) was dissolved in dried methylene chloride (5 mL) and [^{15}N]phthalimide (741 mg, 5 mmol; 99.6% ^{15}N-enriched, purchased from Shoko Co. Ltd.) and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) (1.1 mL, 7.5 mmol) were added to the solution. After stirring for 2 days at room temperature, the mixture was quenched with water (4 mL), diluted with chloroform (50 mL), and washed with 5% aqueous $NaHCO_3$ (25 mL × 2) and water (25 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered and evaporated. The residue was dissolved in 1:2 pyridine/ethanol (25 mL). The solution was added to 2 M aqueous NaOH (10 mL). After stirring

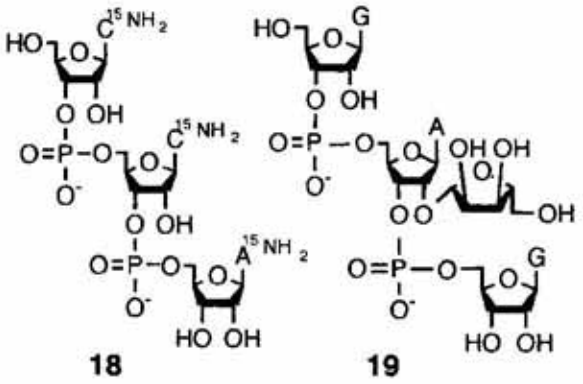


Figure 9.

for 1 day at 50°C, to the resulting mixture 2 M aqueous NaOH (10 mL) was added again and then stirred for another day at 50°C. The reaction mixture was neutralized with Dowex 50Wx8 (H^+ form). The resin was filtered off and washed with 1:2 pyridine/ethanol (about 50 mL). The filtrate and the washings were combined and evaporated. The residue was then subjected to chromatographic separation on a column of silica gel by the use of chloroform/methanol system to give a mixture of the diastereoisomers of 2′-*O*-(tetrahydropyran-2-yl)[6-^{15}N]adenosine (870 mg, quantitative yield).

2′-*O*-(Tetrahydropyran-2-yl)[6-^{15}N]adenosine was, after azeotropic evaporation from pyridine (5 mL × 3), dissolved in dried pyridine (12.5 mL), and benzoyl chloride (1.45 mL, 12.5 mmol) was added to the solution, which was then stirred at room temperature for 3 h. The mixture was quenched with water (5 mL), diluted with chloroform (50 mL) and washed with 5% aqueous $NaHCO_3$ (25 mL × 2) and water (25 mL). The organic layer was evaporated and the residue was dissolved in 1:2 pyridine/ethanol (12.5 mL). The solution was added to 2 M aqueous NaOH (6.3 mL) under cooling in an ice-bath. After stirring for 10 min at 0°C , the resulting mixture was neutralized with Dowex 50Wx8 (H^+ form). The resin was filtered off and washed with 1:2 pyridine/ethanol solution (about 50 mL). The filtrate and the washings were combined and evaporated. The residue was then subjected to chromatographic separation on a column of silica gel by the use of chloroform/methanol system to give **7** (B = [6-^{15}N]A^{Bz}) (the less polar diastereoisomer, 369 mg, and the more polar diastereoisomer, 608 mg, 86% yield); ^{1}H-NMR ($CDCl_3$): δ for the less polar diastereoisomer 1.39–1.82 (m, 6H, CH_2 × 3 of Thp group), 3.42–3.50 (m, 1H, OCH_2C of Thp group), 3.74–3.80 (m, 1H, H-5′), 3.96–4.02 (m, 2H, H-5″ and OCH_2C of Thp group), 4.33–4.40 (m, 2H, H-4′ and OC*H*O of Thp group), 4.55 (d, 1H, $J_{2',3'}$ = 4.7 Hz, H-3′), 4.88 (dd, 1H, $J_{1',2'}$ = 7.7 Hz, H-2′), 5.98 (d, 1H, H-1′), 7.50–7.65 (m, 3H, Ph-*H* × 3 of Bz group), 8.01–8.05 (m, 2H, Ph-*H* × 2 of Bz group), 8.07 (s, 1H, H-2), 8.79 (s, 1H, H-8), and 9.73 (br d, 1H, $J_{15N,H}$ = 88.7 Hz, $^{15}N^6$-*H*); ^{15}N-NMR ($CDCl_3$): δ for the less polar diastereoisomer 129.41 (N^6); EI mass spectrum, *m/z* 456.0 (M^+), and ^{1}H-NMR ($CDCl_3$): δ for the more polar diastereoisomer 1.30–1.74 (m, 6H, CH_2 × 3 of Thp group), 2.91–2.99, 3.254–3.31 (2m, 2H, OCH_2C of Thp group), 3.74–3.79 (m, 1H, H-5′), 3.96 (dd, 1H, $J_{4',5''}$ = 1.4 Hz, $J_{5',5''}$ = 12.9 Hz, H-5″), 4.30–4.2 (m, 1H, H-4′), 4.40 (dd, 1H, *J* = 2.3 Hz, *J* = 5.9 Hz, OC*H*O of Thp group), 4.57 (d, 1H, $J_{2',3'}$ = 4.9 Hz, H-3′), 5.07 (dd, 1H, $J_{1',2'}$ = 7.3 Hz, H-2′), 6.00 (d, 1H, H-1′), 7.47–7.62 (m, 3H, Ph-*H* × 3 of Bz group), 8.00–8.03 (m, 2H, Ph-*H* × 2 of Bz group), 8.04 (s, 1H, H-2), 8.75 (s, 1H, H-8), and 9.30 (br d, 1H, $J_{15N,H}$ = 89.0 Hz, $^{15}N^6$-*H*); ^{15}N-NMR ($CDCl_3$): δ for the more polar diastereoisomer 128.82 (N^6); EI mass spectrum, *m/z* 456.0 (M^+).

N*^{6}*-Benzoyl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)[6-^{15}N]adenosine {9 (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz)}. Compound **9** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz) was synthesized in 55% yield (160 mg) by the reaction of **7** (B = [6-^{15}N]A^{Bz}) (182 mg, 0.4 mmol; the less polar diastereoisomer) with **4** (130 mg, 0.44 mmol) in the presence of TPSCl (267 mg, 0.88 mmol) in pyridine (4 mL) for 3 h as described above; ^{1}H-NMR ($CDCl_3$): δ

1.48–1.86 (m, 6H, CH_2 × 3 of Thp group), 2.17 (s, 3H, $COCH_3$ of LMNBz group), 2.66 (t, 2H, J = 6.6 Hz, CH_2 of LMNBz group), 2.79 (t, 2H, J = 6.6 Hz, CH_2 of LMNBz group), 3.52–3.60, 3.97–4.03 (2m, 2H, OCH_2C of Thp group), 4.44–4.66 (m, 4H, H-3′, 4′, 5′, and OC*H*O of Thp group), 4.79 (dd, 1H, $J_{4',5''}$ = 3.3 Hz, $J_{5',5''}$ = 12.1 Hz, H-5″), 5.18 (t, 1H, $J_{1',2'} = J_{2',3'}$ = 5.0 Hz, H-2′), 5.54, 5.61 (2 d, 2H, J = 15.8 Hz, $PhCH_2$), 6.09 (d, 1H, H-1′), 7.47-7.61 (m, 3H, Ph-*H* × 3 of Bz group), 7.77 (d, 1H, J = 8.7 Hz, Ph-*H* of LMNBz group), 7.98–8.03 (m, 2H, Ph-*H* × 2 of Bz group), 8.12 (s, 1H, H-2), 8.38 (dd, 1H, J = 2.3 Hz, J = 8.7 Hz, Ph-*H* of LMNBz group), 8.52 (s, 1H, H-8), 8.79 (d, 1H, J = 2.3 Hz, Ph-*H* of LMNBz group) and 9.21 (br d, 1H, $J_{^{15}N,H}$ = 89.0 Hz, $^{15}N^6$-*H*).

***N*^{4}-Anisoyl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)[4-^{15}N]cytidine 3′-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = [4-^{15}N]C^{An}, R = OThp, R′ = LMNBz)].** Compound **10** (B = [4-^{15}N]C^{An}, R = OThp, R′ = LMNBz) was obtained in 80% yield (187 mg) by treating **9** (B = [4-^{15}N]C^{An}, R = OThp, R′ = LMNBz) (184 mg, 0.25 mmol; the more polar diastereoisomer) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (66 μL, 0.375 mmol) in the presence of *N*-ethyldiisopropylamine (85 μL, 0.375 mmol) in dried acetonitrile (2.5 mL) and subsequent work-up as described in the synthesis of **10** (B = U, R = OThp, R′ = LMBz); ^{31}P-NMR ($CDCl_3$): δ 149.63 and 150.34.

3′-O-[3-(Carboxy)propionyl]-N^6-benzoyl-5′-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-O-(tetrahydropyran-2-yl)[6-^{15}N]adenosine. 3′-*O*-[3-(Carboxy)propionyl]-*N*^{6}-benzoyl-5′-*O*-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-*O*-(tetrahydropyran-2-yl)[6-^{15}N]adenosine was obtained in 79% yield (125 mg) by treating **9** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz) (139 mg, 0.19 mmol; the less polar diastereoisomer) in pyridine (0.7 mL) with succinic anhydride (30 mg, 0.3 mmol) in the presence of DMAP (12 mg, 0.1 mmol) for 12 h and subsequent work-up as described above; ^{1}H-NMR ($CDCl_3$): δ 1.42–1.75 (m, 6H, CH_2 × 3 of Thp group), 2.18 (s, 3H, $COCH_3$ of LMNBz group), 2.65-2.83 (m, 8H, CH_2 × 4), 3.40–3.49 and 3.71–3.80 (m, 2H, OCH_2C of Thp group), 4.54–4.72 (m, 4H, H-4′, 5′, 5″, and OC*H*O of Thp group), 5.37 (t, 1H, $J_{1',2'} = J_{2',3'}$ = 5.7 Hz, H-2′), 5.57 (s, 2H, $PhCH_2$), 5.60 (dd, 1H, $J_{3',4'}$ = 4.0 Hz, H-3′), 6.16 (d, 1H, H-1′), 7.49-7.61 (m, 3H, Ph-*H* of Bz group), 7.77 (d, 1H, J = 8.7 Hz, Ph-*H* of LMNBz group), 8.02-8.04 (m, 2H, Ph-*H* × 2 of Bz group), 8.39 (dd, 1H, J = 2.3 Hz, J = 8.7 Hz, Ph-*H* of LMNBz group), 8.61 (s, 1H, H-8), and 8.81 (d, 1H, J = 2.3 Hz, Ph-*H* LMNBz group).

Functionalization of the CPG support with [6-^{15}N]adenosine 3′-(3-carboxy) propionate Derivative [11 (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz)]. 3′-*O*-[3-(Carboxy)propionyl]-*N*^{6}-benzoyl-5′-*O*-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-*O*-(tetrahydropyran-2-yl)[6-^{15}N]adenosine (125 mg, 0.15 mmol; the less polar diastereoisomer) was introduced to the CPG support (450 mg) to give CPG containing *N*^{6}-benzoyl-5′-*O*-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2′-*O*-(tetrahydropyran-2-yl)[6-^{15}N]adenosine derivative [**11** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz)] (477 mg) as described above for the synthesis of CPG **11** (B = G^{iBu}, R = OThp, R′ = LMBz). [6-^{15}N]Adenosine content of CPG **11** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz) was determined to be 21 μmol/g by UV spectroscopy {adenosine, λ_{max} 260 nm in H_2O (ε = 14900) [30]} after deprotection of the LMNBz group, detachement of **7** (B = [6-^{15}N]A^{Bz}), and deprotection of the Bz and Thp groups, using 10.7 mg of CPG **11** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz).

Synthesis of the trimer C^{15NH2}pC^{15NH2}pA^{15NH2} (18). After chain elongation with a simple syringe-based reaction system [2, 26] using a column of CPG **11** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz) and **10** (B = [4-^{15}N]C^{An}, R = OThp, R′ = LMNBz) (Table 2), the trimer was cleaved by treatment with

conc. aqueous ammonia (4 mL) for 3 h at room temperature. The resulting solution was heated in a sealed vial at 55°C for 6 h. After evaporation, the residue was dissolved in water (10 mL) and was washed with ethyl acetate (5 mL × 3). The aqueous layer was evaporated and the residue was dissolved in hydrochloric acid (pH 2.0, 5 mL). The solution was left for 1 day at room temperature, neutralized with 0.2 M aqueous ammonia, and washed with ethyl acetate (5 mL × 3). The deprotected trimer was purified by reverse-phase HPLC (see Fig. 10). The main peak was collected {yield of **18**, 60 A_{260} units from CPG **11** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz) (71.4 mg, 3 μmol)} (see Figs. 10 (II) and 11).

Enzymatic digestion of the trimer 18. A solution of the fully deprotected trimer **18** (1.0 A_{260} unit) in 70 μL of 30 mM Tris/HCl (pH 8.0) and 6 mM $MgCl_2$ was treated with snake venom phosphodiesterase (1 mg/0.5 mL, Boehringer Mannheim) (6 μL) and alkaline phosphatase (1 u/mL, Boehringer Mannheim) (6.4 μL) for 24 h at 37°C [12, 15, 16]. Digestion products were analyzed by reversed-phase HPLC with of 2% acetonitrile/0.04 M TEAA (pH 7.0) as eluent {The C:I ratio obtained for the trimer **18** was 2:1 (see Fig. 12). Inosine was produced from adenosine (retention time, 16.5 min) by contamination with adenosine deaminase [16].}

O-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl) trichloroacetimidate (21). To a solution of 2,3,5-tri-*O*-benzoyl-D-ribofuranose (**20**) [31] (1.514 g, 3 mmol) and trichloroacetonitrile (2 mL, 20 mmol) in methylene chloride (35 mL), chilled in an ice-bath, DBU (44 μL, 0.3 mmol) was added with stirring. The solution was then stirred at room temperature for 50 min. The resulting mixture was evaporated, and the residue was subjected to chromatographic separation on a column of silica gel by the use of toluene/ethyl acetate system. Compounds **21** (1.56 g, 80% yield) and α-isomer (**22**) (191 mg, 10% yield) were obtained as the 1st and 2nd fraction, respectively.

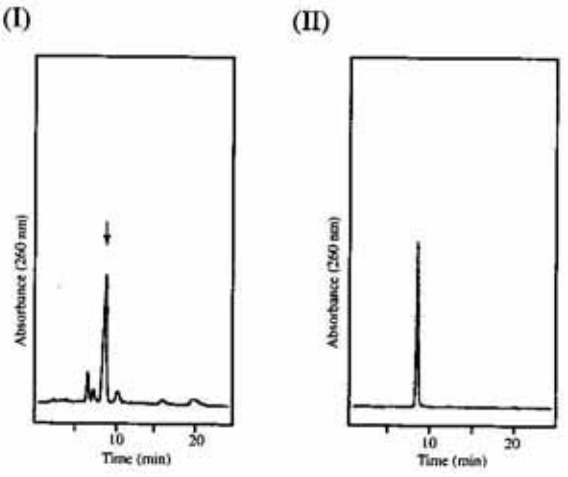


Figure 10. (I) Reversed-phase HPLC of crude products preparation of the trimer (18) using 5′-*O*-LMNBz-2′-*O*-Thp-cytidine 3′-phosporamidite derivative 10 (B = [4-^{15}N]C^{An}, R = OThp, R′ = LMNBz). (II) Reversed-phase HPLC of the trimer (18) isolated from the mixture of (I). [Yield of 60 A_{260} units from 11 (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz; (3 μmol)].

Condition: column μBONDASPHERE 5 μ C18 (3.9 mm ID × 150 mm L); elution buffer 5% acetonitrile/0.1 M TEAA (pH 7); flow rate 1 mL/min; detected by UV at 260 nm.

Compound **21** had m.p. 116–118°C (from toluene); ^{1}H-NMR ($CDCl_3$): δ 4.59–4.75 (m, 2H, H-5 and 5′), 4.88 (dd, 1H, $J_{4,5}$ = 4.7 Hz, $J_{4,5'}$ = 5.7 Hz, H-4), 5.93 (dd, 1H, $J_{2,3}$ = 4.9 Hz, $J_{3,4}$ = 6.8 Hz, H-3), 5.98 (d, 1H, H-2), 6.62 (s, 1H, H-1), 7.30–7.59, 7.85–8.05 (m, 15H, Ph-*H* × 15 of Bz group), and 8.69 (br s, 1H, -OC(=N*H*)CCl_3). *Anal.*, calc. for $C_{28}H_{22}O_8NCl_3$: C, 55.42; H, 3.65; N, 2.31; found: C, 55.72; H, 3.61; N, 2.40.

Compound **22** had ^{1}H-NMR ($CDCl_3$): δ 4.65–4.76 (m, 2H, H-5 and 5′), 4.88 (m, 1H, H-4), 5.66 (dd, 1H, H-2), 5.93 (dd, 1H, H-3), 6.89 (d, 1H, $J_{1,2}$ = 4.5 Hz, H-1), 7.18–7.61, 7.90–7.90, 8.09–8.16 (m, 15H, Ph-*H* × 15 of Bz group), and 8.64 (br s, 1H, -OC(=N*H*)CCl_3).

N^6-Benzoyl-2′-O-(2″,3″,5″-tri-O-benzoyl-β-D-ribofuranosyl)-3′,5′-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-di-

yl)adenosine (24) [32]. Compound **21** (2.25 g, 3.50 mmol) and N^6-benzoyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (**23**) [33] (2.00 g, 3.27 mmol) were dissolved in THF (50 mL) in the presence of molecular sieves 4A (about 300 mg). To the solution, chilled in an ice-bath, trifluoromethanesulfonic acid (0.29 mL, 3.3 mmol) was added with stirring, and the reaction mixture was then stirred for 20 min. The resulting mixture was quenched with a saturated aqueous sodium hydrogen carbonate solution (50 mL), and was extracted with chloroform (50 mL × 2). The extracts were combined and washed with water (50 mL). The organic layer was evaporated to dryness after drying over anhydrous magnesium sulfate, and the residue was subjected to chromatographic separation on a column of silica gel by the use of chloroform/methanol system to give **24** (3.21 g, 93% yield) (Entry 4 in Table 3); ^{1}H-NMR ($CDCl_3$): δ 0.93–1.09 (m, 28H, C*H*(CH_3)$_2$ × 4 of TIPDS group), 3.99–4.20 (m, 3H, H-4' and 5' × 2), 4.64–4.81 (m, 3H, H-4'' and 5'' × 2), 4.89 (d, 1H, $J_{2',3'}$ = 4.6 Hz, H-2'), 4.96 (dd, 1H, $J_{3',4'}$ = 9.2 Hz, H-3'), 5.82 (s, 1H, H-1"), 5.88 (d, 1H, $J_{2'',3''}$ = 4.8 Hz, H-2''), 5.96 (t, 1H, H-3''), 6.08 (s, 1H, H-1'), 7.30–7.61, 7.86–8.03 (m, 20H, Ph-*H* × 20 of Bz group), 8.13 (s, 1H, H-2), and 8.73 (s, 1H, H-8). ^{13}C-NMR ($CDCl_3$): δ 166.62, 166.03, 165.99, 165.59, 165.10, 151.39, 149.94, 149.87, 134.34, 134.06, 134.01, 133.94, 133.75, 133.29, 130.32, 130.24, 130.15, 129.68, 129.42, 129.30, 129.23, 129.15, 129.04, 128.94, 128.41 (Aromatic carbons), 152.80 (C-2), 141.85 (C-8), 105.73 (C-1''), 88.85 (C-1'), 81.40 (C-4'), 79.59 (C-4''), 78.54 (C-2'), 75.56 (C-2''), 72.56 (C-3''), 69.86 (C-3'), 65.34 (C-5''), 59.74 (C-5'), 18.00, 17.89, 17.85, 17.76, 17.69, 17.63, 17.47, 17.36 (-CH_3 × 8), 13.90, 13.49, 13.34, and 13.17 (-CH_2 × 4). *Anal.*, calc. for $C_{55}H_{63}N_5O_{13}Si_2$: C, 62.42; H, 6.00; N, 6.62; found: C, 62.30; H, 5.94; N, 6.58. Low-resolution FAB-MS, *m/z* 1058.7 $(M+H)^+$.

N^6-Benzoyl-2'-O-(2'',3'',5''-tri-O-benzoyl-β-D-ribofuranosyl)adenosine (8)

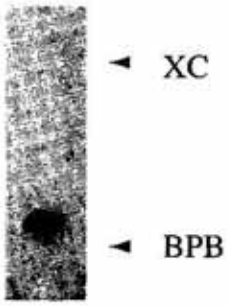


Figure 11. Electrophoresis of the trimer (18) on 20% polyacrylamide gel containing 7 M urea, visualized by UV-shadowing.

[32]. Compound **24** (0.847 g, 0.8 mmol) was dissolved in THF (1.6 mL), and 1 M tetrabutylammonium fluoride (TBAF) (1.6 mL) was added to the solution, which was then stirred at room temperature for 30 min. The resulting mixture was diluted with chloroform (50 mL) and washed with water (25 mL × 2). The organic layer was evaporated to dryness after drying over anhydrous magnesium sulfate, and the residue was subjected to chromatographic separation on a column of silica gel by the use of chloroform/methanol system to give **8** (640 mg, 98% yield); ^{1}H-NMR ($CDCl_3$–D_2O): δ 3.75 (dd, 1H, $J_{5',5'}$ = 13.1 Hz, $J_{5',4'}$ = 1.6 Hz, H-5'), 3.97 (m, 1H, $J_{5',5'}$ = 13.1 Hz, $J_{5',4'}$ = 1.6 Hz, H-5'), 4.09 (dd, 1H, $J_{5'',4''}$ = 4.1 Hz, $J_{5'',5''}$ = 11.9 Hz, H-5''), 4.30 (br s, 1H, H-4'), 4.47 (dd, 1H, $J_{4',5'}$ = 4.2 Hz, H-4''), 4.54 (dd, 1H, $J_{5'',4''}$ = 4.1 Hz, $J_{5'',5'}$ = 11.9 Hz, H-5''), 4.58 (d, 1H, $J_{3',2'}$ = 4.7 Hz, H-3'), 5.19 (d, 1H, $J_{1'',2''}$ = 2.3 Hz, H-1''), 5.21 (dd, 1H, $J_{1',2'}$ = 7.4 Hz, $J_{2',3'}$ = 4.7 Hz, H-2'), 5.64 (dd, 1H, $J_{1'',2''}$ = 2.4 Hz, $J_{2'',3''}$ = 5.4 Hz, H-2''), 5.72 (dd, 1H, $J_{2'',3''}$ = 5.14 Hz, H-3''), 6.04 (d, 1H, $J_{1',2'}$ = 7.4 Hz, H-1'), 8.07–8.02, 7.91–7.68, 7.55–7.33 (m, 20H, Ph-*H* × 20 of Bz group), 8.14 (s, 1H, H-2), and 8.80 (s, 1H, H-8). ^{13}C-NMR ($CDCl_3$): δ 165.94, 165.45, 165.37, 164.52, 150.32, 133.73, 133.60, 133.48, 132.77, 129.75, 129.69, 129.64, 129.54, 129.44, 129.10, 128.90, 128.79, 128.59, 128.53, 128.45, 128.42, 128.12, 128.07, 128.01, 127.90 (Aromatic carbons), 152.18 (C-2), 144.02 (C-8), 106.62 (C-1''),

89.15 (C-1'), 87.21 (C-4'), 80.91 (C-2'), 80.23 (C-4''), 75.85 (C-2''), 72.23 (C-3''), 71.51 (C-3'), 63.96 (C-5''), and 63.09 (C-5'). *Anal.*, calc. for $C_{43}H_{37}N_5O_{12} \cdot 0.5\,H_2O$: C, 62.62; H, 4.64; N, 8.49; found: C, 62.82; H, 4.48; N, 8.48. Low-resolution FAB-MS, *m/z* 816.7 $(M+H)^+$.

***N*^{6}-Benzoyl-5'-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2'-O-(2'',3'',5''-tri-O-benzoyl-β-D-ribofuranosyl)adenosine [9 (B = A^{Bz}, R = ORfBz$_3$, R' = LMNBz)].** Compound **9** (B = A^{Bz}, R = ORfBz$_3$, R' = LMNBz) was obtained in 68% yield (446 mg) by the reaction of **8** (0.489 g, 0.6 mmol) and **4** (195 mg, 0.66 mmol) in the presence of TPSCl (400 mg, 1.32 mmol) in pyridine (6 mL) for 2 h and subsequent work-up as described in the synthesis of **9** (B = U, R = OThp, R' = LMBz); ^{1}H-NMR ($CDCl_3$): δ 2.17 (s, 3H, $COCH_3$ of LMNBz group), 2.67 (t, 2H, J = 6.7 Hz, CH_2 of LMNBz group), 2.80 (t, 2H, J = 6.7 Hz, CH_2 of LMNBz group), 4.19–4.24 (m, 1H, H-4'), 4.37 (dd, 1H, $J_{4'',5''}$ = 4.3 Hz, $J_{5'',5''}$ = 12.1 Hz, H-5''), 4.56 (dd, 1H, $J_{4'',5''}$ = 5.1 Hz, $J_{5'',5''}$ = 12.1 Hz, H-5''), 4.61–4.91 (m, 4H, H-3', 4', and 5'' × 2), 5.26 (t, 1H, $J_{1',2'} = J_{2',3'}$ = 4.8 Hz, H-2'), 5.49 (d, 1H, $J_{1'',2''}$ = 1.7 Hz, H-1''), 5.53, 5.56 (2s, 2H, PhCH_2 of LMNBz group), 5.79 (dd, 1H, $J_{2'',3''}$ = 5.1 Hz, H-2''), 5.85 (t, 1H, H-3''), 6.18 (d, 1H, H-1'), 7.34–7.61 (m, 12H, Ph-*H* of Bz group), 7.75 (d, 1H, J = 8.6 Hz, Ph-*H* of LMNBz group), 7.90–8.09 (m, 9H, Ph-*H* × 8 of Bz group and H-2), 8.38 (dd, 1H, J = 2.3 Hz, J = 8.6 Hz, Ph-*H* of LMNBz group), 8.52 (s, 1H, H-8), 8.73 (d, 1H, J = 2.3 Hz, Ph-*H* of LMNBz group), and 9.03 (br s, 1H, N^6-*H*). *Anal.*, calc. for $C_{56}H_{48}N_6O_{18} \cdot 0.2\,H_2O$: C, 61.33; H, 4.45; N, 7.66; found: C, 61.15; H, 4.49; N, 7.63.

***N*^{6}-Benzoyl-5'-O-[2-(levulinyloxymethyl)-5-nitrobenzoyl]-2'-O-(2'',3'',5''-tri-O-benzoyl-β-D-ribofuranosyl)adenosine 3'-(2-cyanoethyl) N,N-diisopropylphosphoramidite [10 (B = A^{Bz}, R = ORfBz$_3$, R' = LMNBz)].** Compound **10** (B = A^{Bz}, R = ORfBz$_3$, R' = LMNBz) was obtained in 85% yield (199 mg) by treating **9** (B = A^{Bz}, R = ORfBz$_3$, R' = LMNBz) (199 mg, 0.2 mmol) with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (68 μL, 0.3 mmol) in the presence of *N*-ethyldiisopropylamine (53 μL, 0.3 mmol) in dried acetonitrile (2 mL) and subsequent work-up as described in the synthesis of **10** (B = U, R = OThp, R' = LMBz); ^{31}P-NMR ($CDCl_3$): δ 150.02 and 150.54.

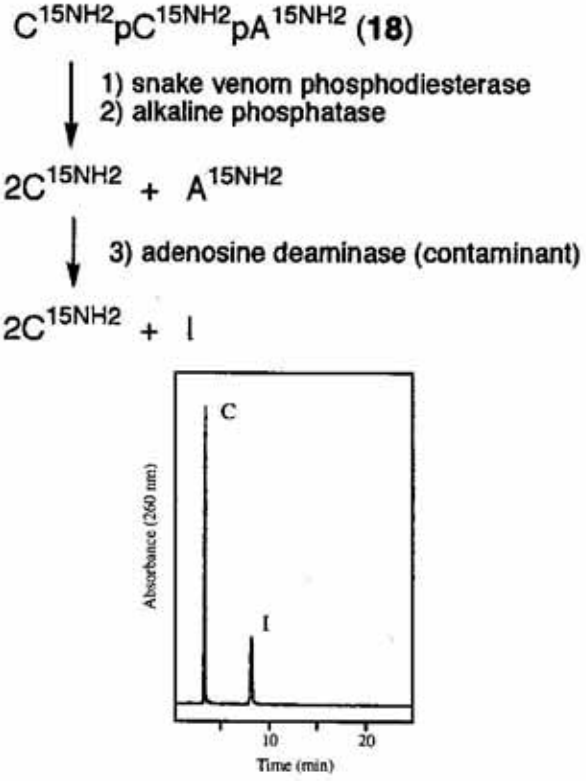


Figure 12. Reversed-phase HPLC of products obtained by digestion of the completely unmasked trimer (18) with snake venom phosphodiesterase and alkaline phosphatase.

Conditions: column μBONDASPHERE 5 μ C18 (3.9 mm ID × 150 mm L); elution buffer 2% acetonitrile/0.04 M TEAA (pH 7); flow rate 1 mL/min; detected by UV at 260 nm.

Synthesis of Gp[A*]pG (19). After chain elongation with a simple syringe-based reaction system [2, 27] using a column of CPG **11** (B = G^{iBu}, R = OThp, R' = LMNBz), **10** (B = A^{Bz}, R = ORfBz$_3$, R' = LMNBz) and **10** (B = G^{iBu}, R = OThp, R' = LMNBz) (Table 2), the trimer was cleaved by treatment with conc. aqueous ammonia (4 mL) for 3 h at room tem-

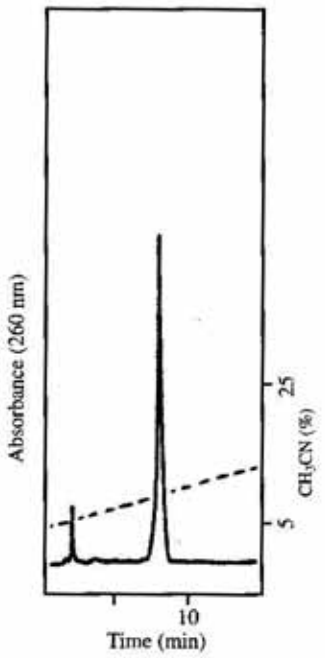


Figure 13. Reversed-phase HPLC of crude products preparation of the trimer (19) using 5′-*O*-LMNBz-ribonucleoside 3′-phosphoramidite derivatives 10 (R = OThp, $ORfBz_3$, R′ = LMNBz) [Yield of 33 A_{260} units from 11 (B = G^{iBu}, R = OThp, R′ = LMNBz; (1.95 μmol)].

Condition: column μBONDASPHERE 5 μ C18 (3.9 mm ID × 150 mm L); elution buffer acetonitrile/0.1 M TEAA (pH 7); flow rate 1 mL/min; detected by UV at 260 nm.

perature. The resulting solution was heated in a sealed vial at 55°C for 6 h. After evaporation, the residue was dissolved in water (10 mL) and washed with ethyl acetate (5 mL × 3). The aqueous layer was evaporated and the residue was dissolved in hydrochloric acid (pH 2.0, 5 mL). The solution was left for 1 day at room temperature, neutralized with 0.2 M aqueous ammonia, and washed with ethyl acetate (5 mL × 3). The deprotected trimer was purified by reverse-phase HPLC (see Fig. 13). The main peak was collected [yield of **19**, 33 A_{260} units from CPG **11** (B = G^{iBu}, R = OThp, R′ = LMNBz) (40 mg, 1.95 μmol)] (see Fig. 14).

Enzymatic digestion of Ap[A*]pG (19). A solution of **19** (1.0 A_{260} unit) in 70 μL of 30 mM Tris/HCl (pH 8.0) and 6 mM $MgCl_2$ was

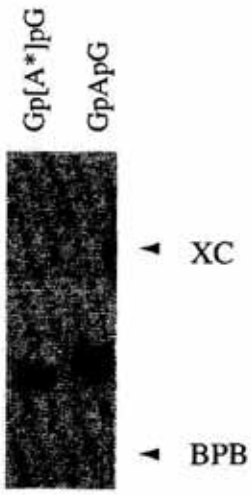


Figure 14. Electrophoresis of the trimer (19) on 20% polyacrylamide gel containing 7 M urea, visualized by UV-shadowing.

treated with snake venom phosphodiesterase (1 mg/0.5 mL, Boehringer Mannheim) (6 μL) and alkaline phosphatase (1 u/mL, Boehringer Mannheim) (6.4 μL) for 24 h at 37°C [12, 15, 16]. Digestion products were analyzed by reversed-phase HPLC with 2% acetonitrile/0.04 M TEAA (pH 7.0) as eluent. {The G:[I*] ratio obtained for the trimer Gp[A*]pG (**19**) was 2:1 (see Fig. 15). 2′-*O*-(β-D-Ribofuranosyl)inosine ([I*]) was produced from [A*] (retention time, 22 min) by contamination with adenosine deaminase [16]}.

RESULTS AND DISCUSSION

A study on the utility of LMBz in comparison to LMNBz as the protecting groups for the 5′-hydroxyl groups of nucleoside phosphoramidites in oligonucleotide synthesis

In the first place, we undertook a comparative study on utility of the LMBz with LMNBz protecting groups in terms of the syntheses of TpTpT (**15**), TpTpTpT (**16**), and UpCpApGpUpUpGpG (**17**).

The protecting reagents, 2-(levulinyloxymethyl)benzoic acid (**3**) and the corresponding 5-nitro derivative (**4**) were prepared starting

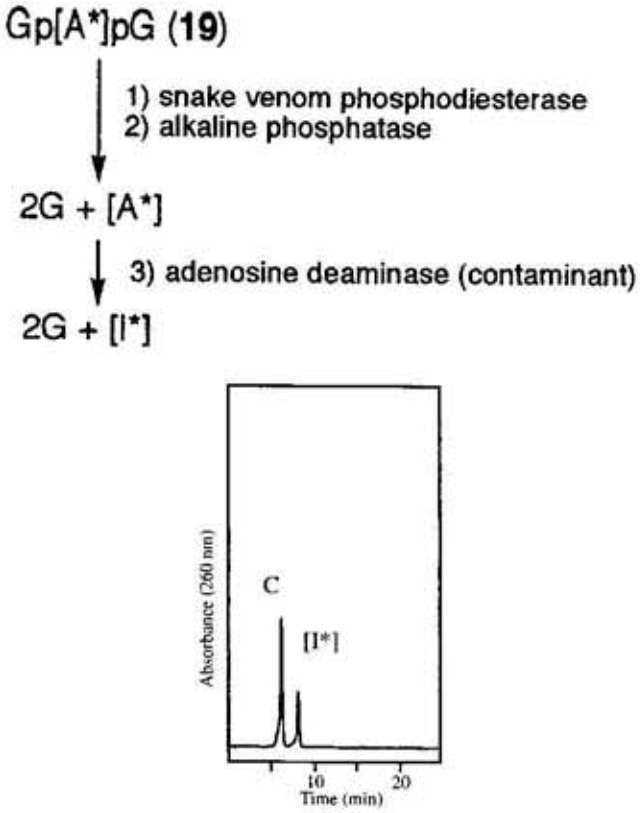


Figure 15. Reversed-phase HPLC of products obtained by digestion of the completely unmasked trimer (19) with snake venom phosphodiesterase and alkaline phosphatase.

Condition: column μBONDASPHERE 5 μ C18 (3.9 mm ID × 150 mm L); elution buffer 2% acetonitrile/0.04 M TEAA (pH 7); flow rate 1 mL/min; detected by UV at 260 nm.

from phthalide (**1**) by the sequence of reactions as shown in Scheme 1, i.e. alkaline hydrolysis giving 2-(hydroxymethyl)benzoic acid (**2**) [17], crystallization of **2** at about pH 2 (conc. HCl) in 91% yield, levulinylation with levulinic anhydride-1-methylimidazole giving **3** in 59% yield, and subsequent nitration with HNO_3/conc. H_2SO_4 giving **4** in 62% yield. The LMBz and LMNBz groups were then introduced at the 5′ position of thymidine (**5**) and 2′-*O*-Thp-ribonucleosides (**6**) by treatment with **3** or **4** (1.1 mol. equiv.), respectively, in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) (2.2 mol. equiv.) in pyridine at room temperature to give the corresponding 5′-*O*-levulinyl derivatives (**9**) in the yields over 60% regardless of the structure of the nucleosides, and the results are summarized in Table 1. It is of interest that the reactions with **4** (2–3 h) were considerably faster than those with **3** (1 day). In both cases, the yields are not quite satisfactory, but are rather improved compared with those [30–46% for 2′-*O*-Thf-U, -C^{Bz}, and -A^{Bz} except for 2′-*O*-Thf-G^{iBu} (68% yield)] obtained by 5′-*O*-levulinylation using levulinic acid (4 mol. equiv.), 2-chloro-1-methylpyridinium iodide (2 mol. equiv.), and 1,4-diazabicyclo[2.2.2]octane (DABCO) (4.3 mol. equiv.) [15, 16].

Compounds **9** were then subjected to phosphitylation reactions with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.5 mol. equiv.) [22] and *N*-ethyldiisopropylamine (1.5 mol. equiv.) in acetonitrile, respectively, to give the corresponding 3′-(2-cyanoethyl) *N,N*-diisopropylphosphoramidites (**10**) in 65% to 91% yields as shown in the second step in Scheme 2; the results are summarized in Table 1.

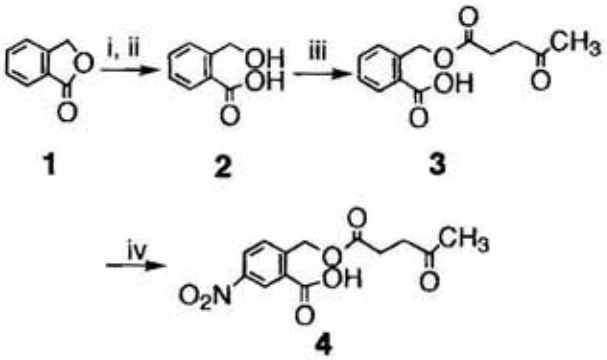
Subsequently, the CPG support was functionalized with the 3′-(3-carboxy)propionates of 5′-*O*-LMBz and -LMNBz derivatives [**9** (B = T, R = H; B = G^{iBu}, R = OThp)] in the usual manner [23, 24] to give functionalized CPG **11**[(B = T, R = H, R′ = LMBz); (B = T, R = H, R′ = LMNBz); (B = G^{iBu}, R = OThp, R′ = LMBz); (B = G^{iBu}, R = OThp, R′ = LMNBz)]. These materials were used then for oligonucleotide synthesis through the reaction cycle

Table 1. Syntheses of the 5′-*O*-LMBz- and -LMNBz-nucleoside derivatives (9) and the corresponding 3′-phosphoramidite derivatives (10)

B	R	R′	Yield of **9** (%)	Yield of **10** (%)
U	OThp	LMBz	60	84
C^{An}			61	76
A^{Bz}			61	91
G^{iBu}			64	75
T	H		67	66
U	OThp	LMNBz	63	90
C^{An}			64	77
A^{Bz}			65	80
G^{iBu}			72	80
T	H		71	65

involving a two-step unmasking procedure for the LMBz and LMNBz protecting groups, as shown in Scheme 3 and Table 2, by manual, and not automated, synthesis. Incidentally, treatment of an intermediate **14** under the conditions of the two-step unmasking procedure (see steps i and ii in Scheme 3 and step 1–4 in Table 2 [15, 16, 34]) brought about no effect at all on the 2-cyanoethyl group on the phosphotriester resulting from the oxidation reaction (see step v in Scheme 3).

Oligothymidylic acids (**15** and **16**) were then synthesized starting from **11**[(B = T, R = H, R′ = LMBz); (B = T, R = H, R′ = LMNBz)],



Scheme 1.

Conditions: (i) KOH, 85% MeOH aq., reflux, 2 h; (ii) adjust to about pH 2 with conc. HCl; (iii) levulinic anhydride, 1-methylimidazole, 1,4-dioxane, 1 h; (iv) H_2SO_4/HNO_3, 0°C, 1 h.

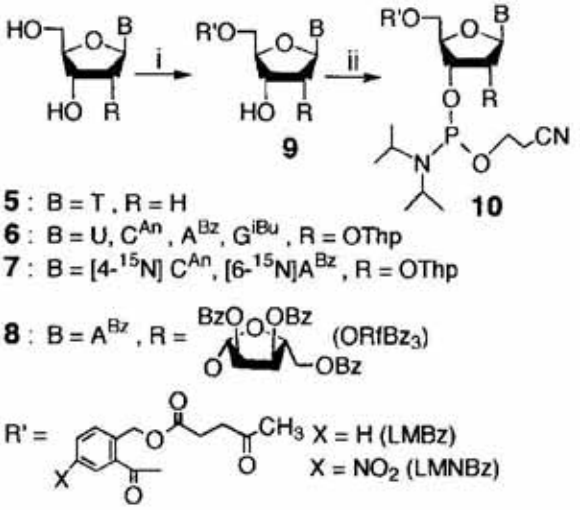
respectively, and conspicuous superiority of LMNBz protection over LMBz protection was proved from reversed-phase HPLC chromatograms obtained by comparing Figs. 1 and 3 for the trimer **15**, and Figs. 2 and 4 for the tetramer **16**. The octaribonucleotide **17** was similarly synthesized starting from **11** [(B = G^{iBu}, R = OThp, R′ = LMBz); (B = G^{iBu}, R = OThp, R′ = LMNBz)], respectively, and similar superiority of LMNBz protection over LMBz protection was also proved by comparing Figs. 5 and 6 (I). It is significant that we should be able to easily isolate an oligonucleotide in pure form from the resulting mixtures as demonstrated here in Fig. 6 (II). The yield of completely unmasked octamer **17** was 41.1 A_{260} units from **11** (B = G^{iBu}, R = OThp, R′ = LMNBz) loading guanosine (1.95 μmol). The octamer **17** gave a clear single band in electrophoretic profile as shown in Fig. 7, and its successive enzymatic digestion with snake venom phosphodiesterase and alkaline phosphatase gave the reversed-phase HPLC chromatogram in Fig. 8 showing a reasonable proportion of 4 nucleosides for its structure.

The superiority of LMNBz protection over LMBz protection led us to conduct further syntheses of CpCpA triplet, which is found in all tRNAs as the terminus dangling structure, and of the Gp[A*]pG triplet, whose central nu-

cleotide is found at the 64 position of the yeast initiator $tRNA^{Met}$ between two guanylic acid units at positions 63 and 65 [35, 36] (Fig. 9).

Synthesis of CpCpA (18) bearing ^{15}N-labeled exocyclic amino groups

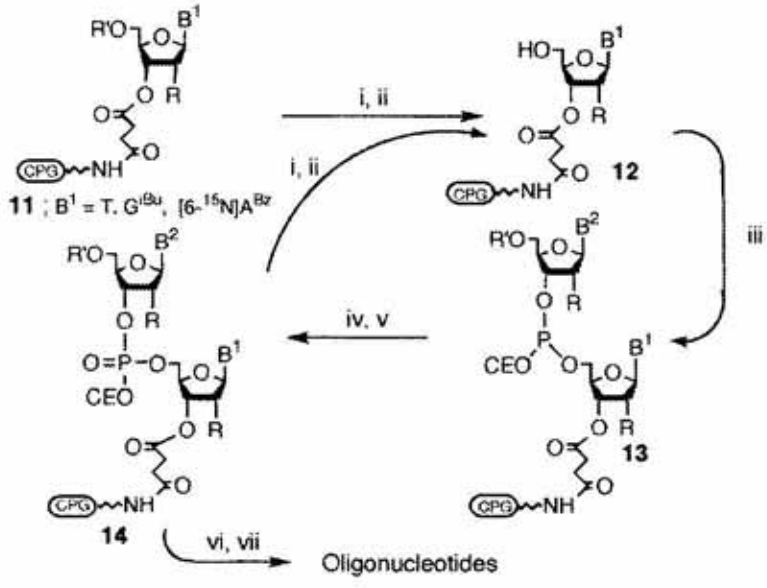
An acyl group attached to the exocyclic amino groups of adenosine and cytidine derivatives, in contrast with those of guanosine, has been demonstrated to be active enough to esterify a series of hydroxyl compounds ranging from mononitrophenols to ordinary alcohols [37]. Such unusual activity of the aminoacyl groups led us to a question,"Why did nature choose these particular ribonucleosides as constituents of the triplet located at the 3′-termini of tRNAs?" The amino groups involved in the terminal triplet of tRNA might be anticipated to play an unexpected important role in the process of accepting an amino acid in protein biosynthesis. Generally, NMR spectroscopic analysis of specifically labeled nucleic acids has been useful in probing local structural phenomena ranging from thermally induced local melting to the behavior of mismatched base pairs, and to the delicate structural changes triggered by enzyme recognition or drug binding [38–41]. Therefore, the synthesis of CpCpA bearing ^{15}N-labeling on their exocyclic amino groups was conducted for the study on potential functions of the CpCpA terminus of tRNA.



Scheme 2.

Conditions: (i) **3** or **4**, TPSCl, pyridine, 1 day (LMBz) or 2–3 h (LMNBz); (ii) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, iPr_2NEt, CH_3CN.

On the other hand, an efficient method for the synthesis of cytidine and adenosine derivatives bearing a ^{15}N-labeled exocyclic amino group, respectively, from uridine and inosine has been reported [28, 29]. 3′,5′-Di-*O*-benzoyl-2′-*O*-(tetrahydropyran-2-yl)[4-^{15}N] cytidine [28, 29] was subjected to N^4-anisoylation and *O*-debenzoylation as usual to give N^4-anisoyl-2′-*O*-(tetrahydropyran-2-yl)[4-^{15}N]cytidine {**7** (B = [4-^{15}N]C^{An})}, which is one of the intermediates for the triplet synthesis, in 90%



Scheme 3.

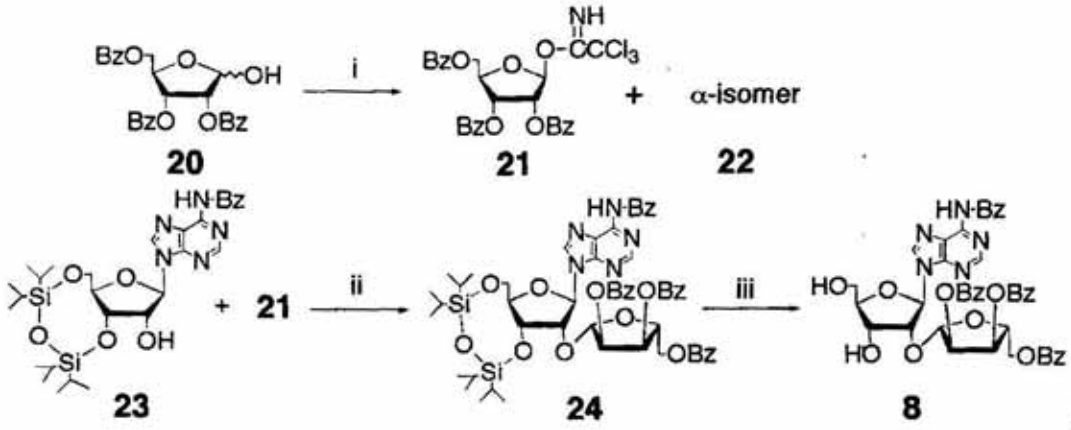
Conditions: (i) 0.5 M $NH_2NH_2 \cdot H_2O$, 1:4 CH_3COOH/pyridine, 15 min; (ii) 0.5 M imidazole, CH_3CN, 5 min; (iii) 50 mM **10** – 0.25 M 1 *H*-tetrazole, CH_3CN, 10 min; (iv) $(CH_3CO)_2O$-2,6-lutidine-1-methylimidazole, THF, 1 min; (v) 0.1 M I_2, H_2O/pyridine/THF, 1 min; (vi) conc. NH_4OH, room temp., 3 h – 55°C, 6 h; (vii) pH 2 HCl aq., 1 day.

Table 2. Reaction cycle for chain elongation[1]

Step	Reagent	Time (min)
Deprotection of the 5'-teriminal LMBz and LMNBz group		
1.	0.5 M $NH_2NH_2 \cdot H_2O$ in 1:4 acetic acid/pyridine (0.8 mL)	15
2.	washing with acetonitrile (3 mL)	
3.	0.5 M imidazole in acetonitrile (0.8 mL)	5
4.	washing with acetonitrile (3 mL)	
Coupling		
5.	50 mM 5'-*O*-LMBz and -LMNBz-nucleoside 3'-phosporamidite (**10**) and 0.25 M 1*H*-tetrazole in acetonitrile (0.6 mL)	10
6.	washing with acetonitrile (3 mL)	
Capping		
7.	5% acetic anhydride/5% 2,6-lutidine/5% 1-methylimidazole in THF (0.8 mL)	1
8.	washing with acetonitrile (3 mL)	
Oxidation		
9.	0.1 M I_2 in 3% H_2O – 19% pyridine – THF (0.6 mL)	1
10	washing with acetonitrile (3 mL)	

[1]Reactions were carried out by manual synthesis on the ABI column using 1–2 μmol of a nucleoside loaded on the CPG.

yield. Moreover, N^6-benzoyl-2'-*O*-(tetrahydropyran-2-yl)[6-^{15}N]adenosine {**7** (B = [6-^{15}N]A^{Bz})} was prepared in 86% yield without isolating each intermediate from 6-(3-nitro-1,2,3-triazol-1-yl)-9-[3',5'-di-*O*-benzoyl-2'-*O*-(tetrahydropyran-2-yl)-β-D-ribofuranosyl]-9*H*-purine [28, 29] by consecutive steps involving labeling with [^{15}N]phthalimide–1,8-diaza-



Scheme 4.

Table 3. Synthesis of 2'-*O*-(β-D-ribofuranosyl)adenosine derivative (24)

Entry	Glycosyl activator (mol. equiv.)	**21** (mol. equiv.)	Time	Temp.	Yield of **24** (%)
1	TMSOTf (1.1)	1.31	60 min	0°C	64
2	TMSOTf (0.2)	1.03	20 h	r.t.*	trace
3	$BF_3 \cdot OEt_2$ (1.0)	1.06	60 min	r.t.*	57
4	TfOH (1.0)	1.14	20 min	0°C	93

*r.t., room temperature.

bicyclo[5.4.0]undec-7-ene (DBU), unmasking of the N^6-phthaloyl and *O*-benzoyl groups, N^6,$O^{3'}$,$O^{5'}$-tribenzoylation, and unmasking of the *O*-benzoyl groups.

The introduction of the LMNBz protecting group to the 5′-position of 2′-*O*-Thp-ribonucleosides [**7** (B = [4-^{15}N]C^{An}; [6-^{15}N]A^{Bz})] was performed by treatment with **4** in the presence of TPSCl in pyridine at room temperature to give the 5′-*O*-LMNBz-nucleoside derivatives [**9** (B = [4-^{15}N]C^{An}; [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz)] in 62% and 55% yields, respectively. The 5′-*O*-LMNBz derivative **9** (B = [4-^{15}N]C^{An}, R = OThp, R′ = LMNBz) was then subjected to the phosphitylation reaction with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite–*N*-ethyldiisopropylamine in acetonitrile to give the corresponding 3′-(2-cyanoethyl) *N*,*N*-diisopropylphosphoramidite {**10** (B = [4-^{15}N]C^{An}, R = OThp, R′ = LMNBz)} in 80% yield (Scheme 2).

Prior to the assembly of the CpCpA triplet structure, the CPG support was functionalized with **9** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz) in the usual manner [23, 24] to give CPG **11** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz). The CPG support was used for the assembly by manual synthesis through the reaction cycle shown in Scheme 3 and Table 2. Starting with **11** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz), the synthesis of the CpCpA triplet (**18**) bearing ^{15}N-labeled exocyclic amino groups was efficiently performed using **10** (B = [4-^{15}N]C^{An}, R = OThp, R′ = LMNBz) [60 A_{260} units were obtained from 3 μmol of **11** (B = [6-^{15}N]A^{Bz}, R = OThp, R′ = LMNBz); Fig. 10]. Its electrophoretic profile gave a clear single band as shown in Fig. 11 and its enzymatic degradation gave satisfactory data for proving its structure as shown in Fig. 12.

Synthesis of Gp[A*]pG (19)

In yeast initiator $tRNA^{Met}$, 2′-*O*-(5″-*O*-phosphoryl-β-D-ribofuranosyl)adenosine (A*) is located at position 64, and this modification is deduced to play an important role in the tRNA exerting its biological function as the initiator [42]. Confronting such an interesting information, incidentally, Markiewicz and his co-workers have already reported the synthesis of 2′-*O*-(2″,3″,5″-tri-*O*-benzoyl-β-D-ribofuranosyl)adenosine derivative (**24**) in 62% yield by the tin tetrachloride-catalyzed coupling reaction of 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-N^6-benzoyladenosine (**23**) with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in 1,2-dichloroethane, and of the [A*]pG unit from **24** [32].

Correlation of such an interesting structure and its biological function also prompted us to extend our present methodology to the synthesis of the trimer Gp[A*]pG (**19**).

In carbohydrate chemistry, anomeric trichloroacetimidates of glycopyranosides were recognized as useful glycosylating agents towards nucleophiles [43, 44]. We performed the preparation of **24** by the condensation of **23** with *O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)trichloroacetimidate (**21**), which was prepared from 2,3,5-tri-*O*-benzoyl-D-ribofuranose (**20**) by the Schmidt's procedure [45], as a β-D-ribofuranosyl donor in the presence of an activator such as trimethylsilyl triflate (TMS-OTf), boron trifluoride ethyl ether complex ($BF_3 \cdot Et_2O$), or trifluoromethansulfonic acid (TfOH) (see Scheme 4 and Table 3). Condensation of **23** with *O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)trichloroacetimidate (**21**) in the presence of TfOH in dichloromethane at 0°C for 20 min gave the desired **24** in 93% yield (Entry 4 in Table 3).

In order to synthesize the phosphoramidite derivative **10** [B = A^{Bz}, R = *O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl), R′ = LMNBz], the tetraisopropyldisiloxane-1,3-diyl group of **24** was removed by treatment with TBAF in THF to give **8** in 98% yield.

Introduction of the LMNBz protecting group to the 5′-position of **8** was accomplished by treatment with **4** in the presence of TPSCl in pyridine to give the 5′-*O*-LMNBz derivative **9** [B = A^{Bz}, R = *O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl), R′ = LMNBz] in 68% yield,

as described above. The 5′-*O*-LMNBz derivative **9** [B = A^{Bz}, R = *O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl), R′ = LMNBz] was then subjected to phosphitylation reaction with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite–*N*-ethyldiisopropylamine in acetonitrile to give the corresponding 3′-(2-cyanoethyl) *N,N*-diisopropylphosphoramidite **10** [B = A^{Bz}, R = *O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl), R′ = LMNBz] in 74% yield (Scheme 2).

CPG **11** (B = G^{iBu}, R = OThp, R′ = LMNBz) [23, 24] was then used for the assembly through the reaction cycle as shown in Scheme 3 and Table 2, as described above. Starting with **11** (B = G^{iBu}, R = OThp, R′ = LMNBz), the synthesis of Gp[A*]pG (**19**) was efficiently performed using the 5′-*O*-LMNBz-ribonucleoside 3′-phosphoramidites **10** [B = G^{iBu}, R = OThp, R′ = LMNBz; B = A^{Bz}, R = *O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl), R′ = LMNBz] (33 A_{260} units were obtained from 1.95 μmol of **11** (B = G^{iBu}, R = OThp, R′ = LMNBz); Figs. 13 and 14). Enzymatic degradation of the trimer **19** gave satisfactory data for proving its structure (Fig. 15).

In conclusion, the LMNBz protecting group for the 5′-hydroxyl groups of nucleoside 3′-phosphoramidites is proved to be basically useful for oligonucleotide synthesis on CPG support, and expected to be free from the incompatibility due to the use of DMTr protecting group, although the synthetic processes have yet to be fully automated.

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