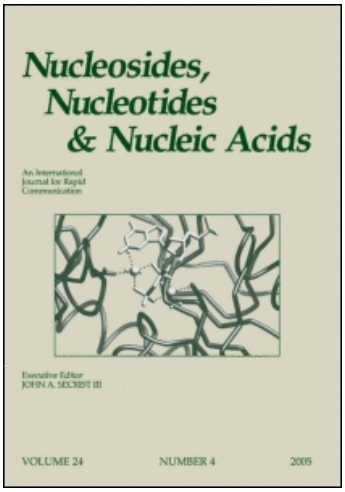

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PREPARATION OF OLIGODEOXYNUCLEOTIDES CONTAINING 5-(*N*-METHYLPIPERAZINYL) AND 5-BENZYLOXYMETHYL URACILS

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PREPARATION OF OLIGODEOXYNUCLEOTIDES CONTAINING 5-(*N*-METHYLPIPERAZINYL) AND 5-BENZYLOXYMETHYL URACILS

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ABSTRACT

Deprotected compounds **1** and **9** were allowed to react with 4,4′-dimethoxytrityl chloride in pyridine to give 5′-*O*-DMT nucleosides **2** and **10.** The 3′-phosphoramidites **4** and **11** were incorporated into oligodeoxynucleosides (ODNs). The hybridization properties of the modified ODNs with their complementary DNA strands were studied. Interesting results were obtained when **11** was inserted as a bulged nucleoside into TWAs, duplexes, and triplexes.

Key Words: Nucleosides; 5′-*O*-DMT nucleosides; Nucleoside-3′-phosphoramidites; Oligodeoxynucleotide.

INTRODUCTION

Oligodeoxynucleotides (ODNs) have received a great interest during recent years because they could interfere with expression of selected genes through antisense agents (1). The major obstacles to the wider application of antisense nucleotides include limitation in their cellular uptake, their stability, and their distribution inside the cells, as well as their binding to the target DNA or RNA (2). Several attempts have been made to overcome some of these problems by

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synthesizing ODNs with modifications in the backbone, the sugar or the nucleobase part of the ODNs (3–6). Matteucci and coworkers (7) have studied the thermal denaturation of duplexes containing C5 heteroaryl uridine modifications. The results showed enhanced thermal stability on hybridization to complementary RNA relative to the corresponding thymidine ODNs (7). The stability of ODN duplexes containing aminouracil and its *N*-acetyl derivatives were also studied (8). Moreover the stability of ODNs containing a thymidine modified at the C5 methyl with the triethylester of EDTA was studied by high-resolution gel electrophoresis (9). The properties of ODNs containing uridine modified at C5 with propyne (10), aminolinker carrying intercalators (11), or lipophilic compounds (12) are described. Synthesis and tests of the alkylating ODN derivatives containing cholesterol moiety at the 3′- and 5′-terminals are reported (13–17). The C5 position of pyrimidines is useful because it offers major groove modifications without interfering with hydrogen bonding. In this paper we describe the synthesis and incorporation of 5-(*N*-methylpiperazinyl)-2′-deoxyuridine and 5-benzyloxymethyl-2′-deoxyuridine into oligonucleotides in order to observe an eventual stabilizing effect due to charge neutralization by the protonated piperazine ring in the former compound and to observe the effect of the increased lipophilicity by the benzyloxymethyl group in the latter compound.

RESULTS AND DISCUSSION

The starting material 5-bromo-2′-deoxyuridine (**1**) was protected by treatment with 4,4′-dimethoxytrityl chloride (DMTr-Cl) in pyridine to give 5-bromo-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**2**) in 82% yield (18). On refluxing **2** with *N*-methylpiperazine in 1,4-dioxane, 5′-*O*-(4,4′-dimethoxytrityl)-5-(*N*-methylpiperazinyl)-2′-deoxyuridine (**3**) was formed in 32% yield after silica gel chromatography. Its ^{1}H and ^{13}C NMR spectra were in good agreement with those of similar compounds (19,20). The phosphoramidite **4** was obtained by reaction with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite [$NCCH_2CH_2OP(Cl)NPr^{i}_{2}$] in anhydrous dichloromethane in 71% yield after column chromatographic purification and precipitation from petroleum ether as described by Sinha et al. (21) The purity of the phosphoramidite was 100% according to ^{31}P-NMR (Scheme 1).

Methyl 2-deoxy-3,5-di-*O*-toluyl-D-pentofuranoside (6) was prepared from 2-deoxy-D-ribose **5** as described (22,23). Condensation of **6** with silylated 5-benzyloxymethyl uracil (**7**) (24), using the trimethylsilyl trifluoromethanesulfonate (TMS triflate) method of Vorbrüggen et al. (25) in dry acetonitrile at −35°C for 2 h, gave an anomeric mixture of the protected nucleoside. After purification by silica gel chromatography, the β-anomer **8** was obtained as the major product in 60% yield. Deprotection of **8** using MeONa/MeOH at room temperature gave 5-benzyloxymethyl-2′-deoxyuridine (**9**) in 100% yield. Reaction of **9** with 4,4′-dimethoxytrityl chloride in dry pyridine afforded 5-benzyloxymethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**10**) in 63% yield. Reaction of **10** with 2-cyanoethyl

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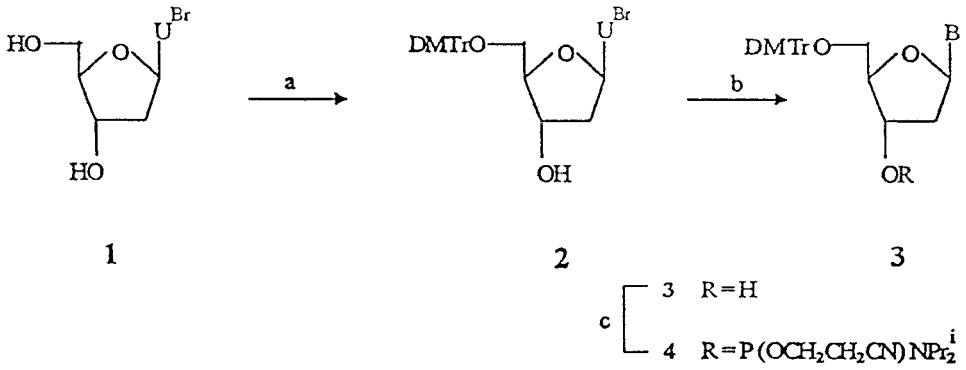
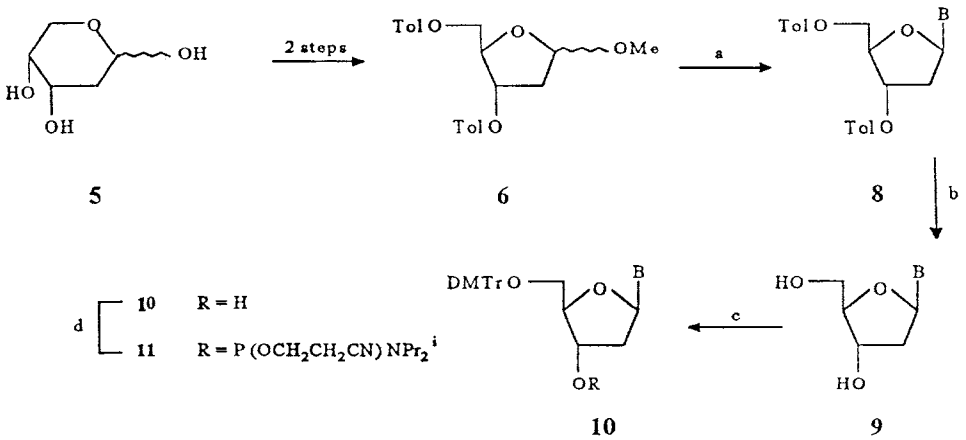


Scheme 1. a) DMTr-Cl, pyridine, r.t.; b) *N*-methylpiperazine, 1,4-dioxane; c) $NCCH_2CH_2OP(Cl)NPr^i_2$, CH_2Cl_2, $EtNPr^i_2$, U^{Br} = 5-bromouracil-1-yl, B = 5-(*N*-methylpiperazinyl)uracil.

N,N-diisopropylphosphoroamidochloridite [$NCCH_2CH_2OP(Cl)NPr^i_2$] as described by Sinha et al. (21) gave the corresponding phosphoramidite **11** in 76% yield. The purity of the amidite **11** was 100% according to ^{31}P-NMR (Scheme 2). The β-anomer assignment of compound **10** was made by comparison of ^{1}H and ^{13}C NMR spectra with previously published data of similar compounds (20,25).

DNA Synthesis

According to the phosphoramidite methodology (26,27), the ODNs were synthesized on a Pharmacia Gene Assembler Special synthesizer on a 0.2 mole scale. The coupling efficiencies for the modified phosphoramidites **4** and **11** were 99 and 60%, respectively. Removal from solid support and deprotection was carried out at



Scheme 2. a) Silylated 5-benzyloxymethyluracil (7), TMS triflate, MeCN, −35°C; b) MeONa/MeOH, r.t.; c) DMTr-Cl, pyridine, r.t.; d) $NCCH_2CH_2OP(Cl)NPr^i_2$, CH_2Cl_2, $EtNPr^i_2$, B = 5-benzyloxymethyluracil.

room temperature for 4 days in 25% ammonia. All ODNs were desalted using the Pharmacia NAP-10 columns. The identities of the oligomers containing one modified nucleoside **11** were proven by Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectroscopy, giving a relative molecular mass 5444.99 (cald. *m/z* 5444.45).

UV-Thermal Denaturation

The hybridization properties of the modified ODNs with their complementary DNA strands were studied. The ability of ODNs to hybridize to their complementary DNA strand was examined by UV melting measurements at 2.5 *M* concentrations of the ODNs containing **4** at pH 7.2 in 20 m*M* Na_2HPO_4 and 140 m*M* NaCl. It is evident that the partial charge neutralization in the ODN caused by introducing a piperazinyl group at 5′-position of the uracil did not result in increased stabilization of the duplexes. In fact, entries **6** and **7** (Tab. 1) show that the T_m is decreased by 11.0 or 20.4°C, respectively, when the modified base **4** is introduced into the ODN three or four times, whereas a typical 6°C decrease in T_m is observed when only one modified base is introduced into the DNA (entries 2 and 3). Only in the region at the 5′-end is the decrease in T_m moderate (entries 4 and 5).

Interesting results were obtained when **11** was inserted as a bulged nucleoside into TWJs, duplexes, and triplexes as we selected a hairpin which has been previously investigated (28). A considerable stabilization was observed when the C5 benzyloxymethyl modified uridine was bulged in the target ODN (Tab. 2). A promising stabilization was observed when the bulge **11** was inserted in the middle of the TWJ. The largest increase in T_m was 3.4°C (entry 10) when the C5 benzyloxymethyl uridine was inserted to the middle of targeting sequence. Whereas T_m increased 1°C (entry 9) when the bulged nucleoside **11** was adjacent to the stem towards the 3′-end of the target while it was 1.4°C (entry 11) on the other side. Larger effects have been observed when using strong intercalators and we may conclude that a lipophilic effect atoms does not result in substancial stabilization of TWJ (20).

Table 1. Hybridization Data (T_m°C) of ODNs with the Complementary Strand 5′-AAA AAA GAA AGG GA-3′

Entry	ODN	T_m (°C)	ΔT_m (°C)
1	5′-TCC CTT TCT TTT TT-3′	49.6	–
2	5′-TCC CTT TCT TTT XT-3′	43.8	−5.8
3	5′-TCC CTT XCT TTT TT-3′	43.0	−6.6
4	5′-XCC CTT TCT TTT TT-3′	46.6	−3.0
5	5′-XCC CTT TCT TTT XT-3′	47.4	−2.2
6	5′-XCC CTT XCT TTT XT-3′	36.6	−11.0
7	5′-XCC CTT XCX TTT XT-3′	29.2	−20.4

X is the Modified Nucleoside using the Amidite **4** with the 5-(*N*-methylpiperazinyl) Group.

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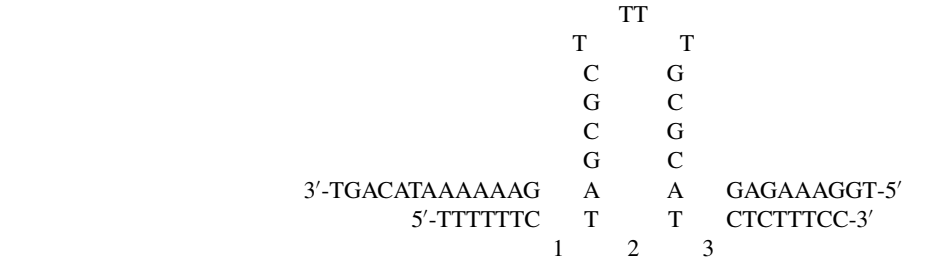
Table 2. Hybridization Data (T_m°C) when Hybridized at the Foot with a Complementary DNA Which Has Been Inserted at Position 1–3, using **11** with the Benzyloxymethyl Group



Entry	ODN	Insertion Position	T_m (°C)	ΔT_m (°C)
8	5′-TTT TTT CTT CTC TTT CC-3′	–	27.6	–
9	5′-TTT TTT CXT TCT CTT TCC-3′	1	28.6	1
10	5′-TTT TTT CTX TCT CTT TCC-3′	2	31.0	3.4
11	5′-TTT TTT CTT XCT CTT TCC-3′	3	29.0	1.4

When the stabilization of the TWJ was compared with the duplex obtained by the deletion of the stem for the duplex, we observed a decrease in T_m (around 8°C) (Tab. 3). In fact, T_m is decreased by 7.1–8.8°C (entry 13, 14, or 15 respectively) when the C5 benzyloxymethyl modified nucleoside was bulged into the ODN as compared to the natural one (entry 12).

To determine the thermodynamic stability of the DNA triplexes, we determined the melting point curves. The ability of the ODNs to form triplexes was examined at pH 6.3 in 10 m*M* CH_3COONa and 0.5 *M* NaCl. Destabilization was observed (Tab. 4) when ODNs with 5-benzyloxymethyl-2′-deoxyuridine inserted as a bulge in three different positions in the middle of the triplex forming ODN. The T_m was lowered by 8.9–11.3°C (entry 18, 19, or 20 respectively).

EXPERIMENTAL

NMR spectra were recorded at 250 MHz for ^{1}H NMR, 62.9 MHz for ^{13}C-NMR, and 101.3 MHz for ^{31}P NMR on a Bruker AC-250 FT spectrometer, δ-values are in ppm relative to tetramethylsilane as internal standard (^{1}H-NMR and

Table 3. Hybridization Data (T_m°C) of ODNs with the Complementary Strand 3′-TGACAT AAAAAA GAAGAG AAAGGT-5′

Entry	ODN	T_m (°C)	ΔT_m (°C)
12	5′-TTT TTT CTT CTC TTT CC-3′	48.0	–
13	5′-TTT TTT CXT TCT CTT TCC-3′	39.2	−8.8
14	5′-TTT TTT CTX TCT CTT TCC-3′	40.4	−7.6
15	5′-TTT TTT CTT XCT CTT TCC-3′	40.8	−7.2

X is the modified nucleoside using the amidite **11**.

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Table 4. Hybridization Data (T_m°C) of Triplexes with Insertions into the Triplex Forming ODN using **11** at pH 6.3

Entry	ODN	T_m (10–70°C)	ΔT_m (70–10°C)
16	5′-TGACATAAA AAA GAA GAG AAAGGT-3′ 3′-ACT GTA TTT TTT CTT CTC TTT CCT-3′	60[a]	59.6[a]
17	5′-TTT TTT CTT CTC TTT CC-3′	37.3	36.5
18	5′-TTT TTT CXT CTC CTT TCC-3′	28.4	24.4
19	5′-TTT TTT CTX CTC CTT TCC-3′	26.0	18.8
20	5′-TTT TTT CTT XCT CTT TCC-3′	26.0	24.4

[a]Duplex melting point.

^{13}C-NMR), relative to 85% H_3PO_4 as external standard in ^{31}P-NMR. Positive FAB mass spectra were recorded on a Kratos MS 50 RF spectrometer. Analytical silica gel TLC was performed on Merck precoated $60F_{245}$ plates. The silica gel (0.040–0.063 mm) used for column chromatography was purchased from Merck.

MALDI mass spectra were obtained on a Bruker Reflex mass spectrometer. Melting experiments were carried out on a Perkin-Elmer UV–vis spectrometer Lamda 2 fitted with a PTP-6-Peltier temperature programming element. The absorbance 260 nm was increased 1°C/min in 1 cm cuvette. DNA synthesis were performed on a Pharmacia Gene Assembler Special® DNA-synthesizer. Purification of 5′-*O*-DMT-ON oligonucleotides was accomplished using disposable oligopurification cartridges (Cop, Cruachem) and desalting using NAP-10 columns (Pharmacia).

5′-*O*-(4,4′-Dimethoxytrityl)-5-(*N*-methylpiperazinyl)-2′-deoxyuridine (3). Compound **2** (1.5 g, 2.5 mmol) and *N*-methylpiperazine (1.0 g, 10 mmol) in 1,4-dioxane (20 mL) were refluxed overnight. The solution was concentrated under vacuum and the resulting residue partitioned between CH_2Cl_2 (75 mL) and water (100 mL). The organic layer was dried over Na_2SO_4, evaporated and the residue chromatographed on a silica gel column (50 g) with 10–40% MeOH/ether to give compound **3**: Yield 0.50 g (32%). ^{1}H-NMR ($CDCl_3$): δ 2.21 (s, 3H, N-CH_3), 2.34 (brs, 6H, 2 × CH_2, H2′), 2.78 (m, 4H, 2 × CH_2), 3.36 (m, 2H, H5′), 3.77 (s, 6H, 2 × CH_3O), 4.02 (m, 1H, H4′), 5.50 (m, 1H, H3′), 6.32 (t, 1H, $J = 7.8$ Hz, H1′), 6.81 (d, 4H, $J = 9.0$ Hz, H_{Arom}), 6.87 (s, 1H, H6), 7.19–7.42 (m, 9H, H_{arom}). ^{13}C-NMR ($CDC1_3$): δ 40.13 (C2′), 45.67 (N-CH_3), 49.38 (2 × CH_2), 54.40 (2 × CH_2), 55.09 (2 × CH_3O), 63.58 (C5′), 71.85 (C3′), 84.63, 85.50 (C1′ and C4′), 86.39 (C_{arom}), 113.12 (C_{arom}), 123.99 (C5), 126.91 (C6), 127.79, 129.95, 135.44, 135.50, 144.31 (C_{arom}), 149.30 (C2), 158.52 (C_{arom}), 160.55 (C4′). FAB MS ($CHCl_3$ + 3-nitrobenzyl alcohol) *m/z* 629 ($M + H^+$).

5′-*O*-(4′,4-Dimethoxytrityl)-5-(*N*-methylpiperazinyl)-2′-deoxyuridine 3′-*O*-(*N*,*N*-diisopropyl)-2-cyanoethyl phosphoramidite (4). The nucleoside **3** (0.4 g, 0.64 mmol) was dried by coevaporation with anhydrous MeCN (2 × 2 mL) and dissolved under nitrogen in anhydrous CH_2Cl_2 (1.8 mL) and *N,N*-diisopropylethylamine (0.56 mL), 2-cyanoethyl *N,N*-diisopropyl-chlorophosphoramidite (0.26 mL,

1.21 mmol) was added dropwise. After 1 h the reaction mixture was quenched by adding MeOH (1.4 mL) followed by addition of EtOAc (20 mL). The solution was washed with saturated aqueous $NaHCO_3$ (3×20 mL) and with saturated aqueous NaCl (3×20 mL), dried over Na_2SO_4 and concentrated in vacuo. The oil was dissolved in toluene (1 mL) and added dropwise to cold (-30°C) petroleum ether (200 mL) to give compound **4** as a white powder. Yield: 370 mg (73%), ^{31}P-NMR ($CDCl_3$): 149.48, 149.92 ppm.

5-Benzyloxymethyl-3′,5′-di-*O*-toluyl-2′-deoxyuridine (8). To a stirred solution of compound **6** (2.0 g, 5.2 mmol) and *O,O′*-bis (trimethylsilyl)-5-benzyloxymethyl uracil (**7**) (1.16 g, 5 mmol) in anhydrous acetonitrile (20 mL) was added TMS triflate (1.2 mL, 6.2 mmol) dropwise at -35°C. After complete addition, the reaction mixture was stirred for 2 h. The reaction mixture was diluted with CH_2Cl_2 (200 mL) and washed with a cold saturated aqueous of $NaHCO_3$ (100 mL). The aqueous solution was extracted with CH_2Cl_2 (2×100 mL). The organic layer was dried over Na_2SO_4 and evporated *in vacuo* to give a crude dark brown product, which was subjected to silica gel column chromatography with ether: petroleum ether (1:1) to afford compound **8**. Yield 1.75 g (58%), ^{1}H-NMR ($CDCl_3$): δ 2.35 (s, 3H, CH_3), 2.38 (m, 1H, H2′), 2.41 (s, 3H, CH_3), 2.67 (m, 1H, H2′), 4.09 (m, 1H, H4′), 4.38 (s, 2H, CH_2O), 4.53 (m, 2H, OCH_2), 4.66 (m, 2H, H5′), 5.58 (m, 1H, H3′), 6.42 (m, 1H, H1′), 7.18–7.31 (m, 9H, H_{arom}), 7.63 (s, 1H, H6), 7.87–7.95 (m, 4H, H_{arom}), 9.38 (brs, 1H, NH). ^{13}C-NMR ($CDCl_3$): δ 21.57, 21.64 ($2 \times CH_3$), 38.12 (C2′), 64.26 (C5′ CH_2O), 72.84 (OCH_2), 74.85 (C3′), 84.86 (C1′ and C4′), 112.57 (C5), 126.27–129.78 (C_{arom}), 136.73 (C6), 137.69–144.43 (C_{arom}), 150.14 (C2), 162 (C4), 165.94, 165.97 ($2 \times$ CO). MS FAB ($CHCl_3$ + 3-nitrobenzyl alcohol) *m/z* 607 ($M + Na^+$).

5-Benzyloxymethyl-2′-deoxyuridine (9). Compound **8** (1.46 g, 2.5 mmol) was dissolved in MeOH (30 mL) and then MeONa (810 mg, 15 mmol) was added. The reaction mixture was stirred at room temperature for 2 h, NH_4Cl (803 mg, 15 mmol) was added and the stirring was continued for 30 min. The reaction mixture was evaporated to dryness and purified on silica gel column eluted by 0–5% MeOH/CH_2Cl_2 to give **9**. Yield 0.87 g (100%). ^{1}H-NMR (DMSO-d_6): δ 2.12 (m, 2H, H2′), 3.39 (m, 1H, H5′), 3.80 (m, 1H, H5′), 4.18 (s, 1H, H4′), 4.26 (m, 2H, CH_2O), 4.50 (s, 2H, OCH_2), 5.08, 5.23 ($2 \times$ brs, 3H, H2′ + OH), 6.18 (m, 1H, H′), 7.30–7.34 (m, 5H, H_{arom}), 7.94 (s, 1H, H6), 10.95 (brs, 1H, NH). ^{13}C-NMR (DMSO-d_6): δ 39.58 (C2′), 61.16 (OCH_2), 64.26 (C5′), 70.24 (C3′), 71.25 (CH_2O), 84.09 (C1′), 87.31 (C4′), 110.40 (C5), 127.19, 127.26, 128.06, 138.93 (C_{arom}), 138.31 (C6), 150.16 (C2), 162.56 (C4). MS FAB ($CHCl_3$ + 3-nitrobenzyl alcohol) *m/z* 371 ($M + Na^+$). $C_{17}H_{20}N_2O_6$: Calcd. C, 58.60%, H, 5.79%, N, 8.01%. Found C, 58.57%, H, 5.81%, N, 8.02%.

5-Benzyloxymethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyuridine (10). 4,4′-Dimethoxytrityl chloride (714 mg, 2.1 mmol) was added to a stirred suspension of compound **9** (696 mg, 2 mmol) in pyridine (20 mL) and the reaction mixture was stirred at room temperature 2 h. After addition of methanol (5 mL), the solvent was evaporated in vaccuo and the residue partitioned between CH_2Cl_2 (300 mL) and

water (200 mL). The organic phase was dried over Na_2SO_4 and evaporated under pressure. The purification was carried out on silica gel column chromatography, using 0–2% CH_3OH/CH_2Cl_2 as eluent to afford compound **10**. Yield 819 mg (62%). ^{1}H-NMR ($CDCl_3$): δ 2.26 (m, 1H, H2′), 2.40 (m, 1H, H2′), 3.38 (m, 2H, H5′), 3.71 (s, 3H, CH_3O), 3.73 (s, 3H, CH_3O), 4.04 (m, 1H, H4′), 4.25 (s, 2H, CH_2O), 4.49 (s, 2H, OCH_2), 5.59 (brs, 1H, H3′), 6.35 (m, H, H1′), 6.80 (d, 4H, $J = 8.5$ Hz, H_{arom}), 7.18–7.42 (m, 14H, H_{arom}), 7.79 (s, 1H, H6). ^{13}C-NMR ($CDCl_3$): δ 40.83 (C2′), 55.02 (2 × CH_3O), 63.41 (C5), 64.19 (CH_2O), 71.81 (C3′), 72.68 (OCH_2), 84.87 (C1′), 85.99 (C4′), 86.64 (C_{arom}), 111.98 (C5′), 113.12, 126.85, 135.33 (C_{arom}), 135.46 (C6), 137.80, 144.41 (C_{arom}), 150.33 (C2), 158.49 (C_{arom}), 162.76 (C4). MS FAB ($CHCl_3$ + 3-nitrobenzyl alcohol) *m/z*: 673 ($M + Na^+$).

5-Benzyloxymethyl-5′-*O*-(4,4′-Dimethoxytrityl-2′-deoxyuridine 3′-*O*-(*N*, *N*-diisopropyl)-2′-cyanoethyl phosphoramidite (11). Compound **10** (650 mg, 1 mmol) was dried by coevaporation with anhydrous MeCN (2 × 20 mL) and dissolved under argon atmosphere in anhydrous CH_2Cl_2 (5 mL). *N,N*-Diisorpropylethylamine (0.5 mL) was added followed by dropwise addition of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.1 mL, 1.2 mmol). After 2 h when analytical TLC showed no more starting material, the reaction was quenched with methanol (2 mL) and diluted with ethyl acetate (15 mL). The mixture was washed with saturated aqueous solution of $NaHCO_3$ (2 × 20 mL) and with saturated aqueous NaCl (2 × 20 mL), dried over Na_2SO_4, then evaporated under vacuum. The residual gum was purified by column chromatography eluted by (60:25:10:5) CH_2Cl_2:pet. ether:AcOEt:Et_3N. After evaporation, the resulting gum was redissolved in anhydrous toluene (2 mL) and precipitated in ice-cold petroleum ether (200 mL). The product was collected by filtration and dried under vacuum to give compound **11**. Yield 631 mg (76%) as a white fine powder. ^{31}P NMR ($CDCl_3$): 149.37, 149.81 ppm.

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