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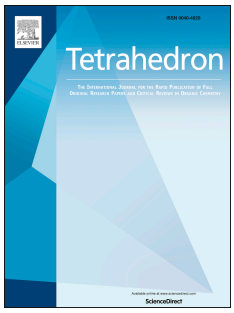

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Graphical Abstract

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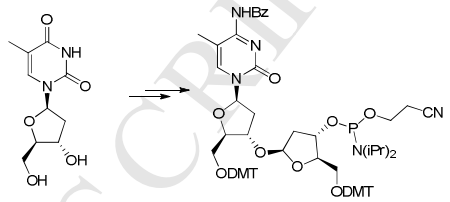

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ABSTRACT

Synthesis of impurity reference compound of anti-tumor drug ISIS 183750 was achieved. In this process, a general method for synthesis of 2-deoxy ribosyl disaccharide nucleosides was established for the first time.

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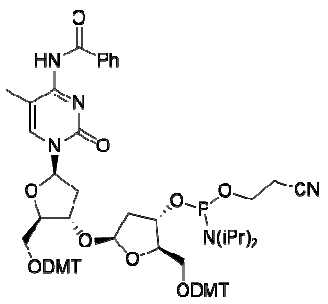
More than hundred disaccharide nucleosides have been isolated from various natural sources.[1] They display antibacterial, anti-mycotic, herbicidal, insecticidal, antitumor, and antiviral properties,[2] are structural elements of biopolymers, such as tRNA and poly(ADP-ribose),[3] and can be formed during nucleoside metabolism.[4] Recent studies indicated some disaccharide analogues of thymidine were potential inhibitors of PARPs, which are considered to be promising targets in designing drugs for the treatment of stroke, ischemia, diabetes, arthritis, colitis, cancer, viruses and other inflammatory disorders.[5] Most antiviral drugs are derivatives of nucleosides or nucleotides, anchoring one or two carbohydrate moieties on their sugar portion may increase the activity in comparison with the original drugs.[6] Therefore, disaccharide nucleoside synthesis methods are expected to have wide-spread applications in drug discovery.

Many strategies were developed for the synthesis of disaccharides nucleosides by using aldohexoses and pentose.[7] As a component of DNA, 2-deoxyribose derivatives have an important role in biology. The DNA (deoxyribonucleic acid) molecule, which is the main repository of genetic information in life, consists of a long chain of deoxyribose-containing units called nucleotides, linked via phosphate groups. However, 2-deoxy ribose related disaccharide nucleosides have not reported yet.

Compound **1** is an impurity found in the production of ISIS183750, which is a synthetic oligomer of 20 nucleotides that are connected sequentially by phosphorothioate linkages and a second-generation antisense oligonucleotide drug for advanced lung cancer,[8] and it is needed as a reference compound. In this paper, we would like to report its synthesis while establishing a strategy for synthesizing the 2-deoxy ribose related disaccharide nucleosides and their phosphoramidites.

The central portion of the oligonucleotide ISIS 183750 is composed of ten 2′-deoxynucleotides, and synthesized by introducing DNA or MOE monomers sequentially on solid support, the disaccharide nucleoside phosphoramidite **1** was likely formed during the glycosylation and phosphorylation stages of the monomer synthesis.



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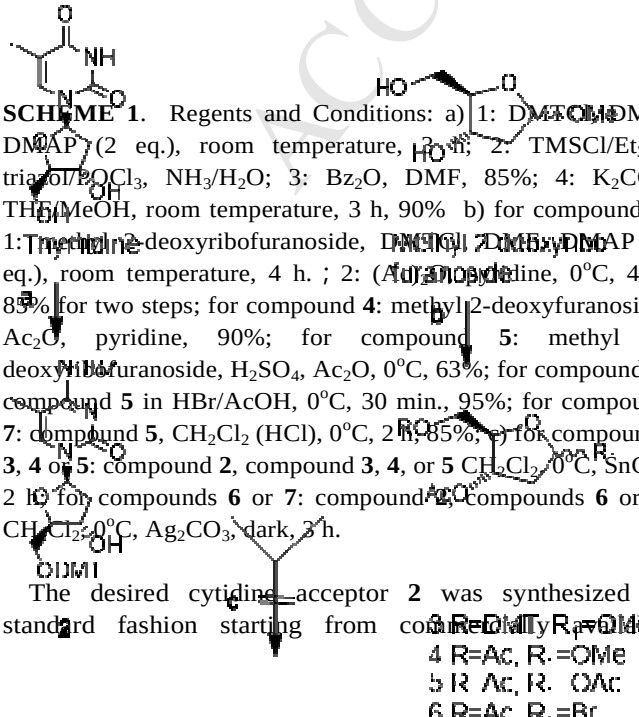
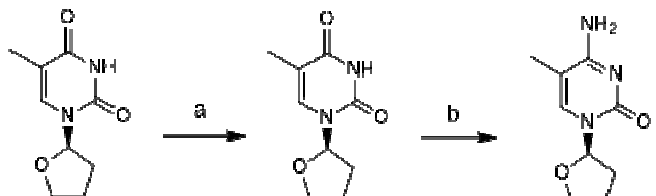
Current reports for the synthesis of disaccharide nucleosides may be classified into two methods. In the first method, *N*-glycosylation of properly protected disaccharide with a heterocyclic base is employed and this method is not high yielding as disaccharide glycosylation donors are not very stable.[9] The second method involves coupling reaction between a nucleoside and a properly protected monosaccharide donor.[10] As our target compound **1** contains a natural nucleoside, direct glycosylation of the properly protected thymidine **2** with a *p*-dimethoxytrityl (DMT) protected monosaccharide donor **3** under classic glycosylation conditions was considered first as shown in the **Scheme 1**.



SCHEME 1. Regents and Conditions: a) 1: DMTCl (DMF, DMAP) (2 eq.), room temperature, 3 h; 2: TMSCl/Et_3N, triazol/$POCl_3$, NH_3/H_2O; 3: Bz_2O, DMF, 85%; 4: K_2CO_3, THF/MeOH, room temperature, 3 h, 90% b) for compound **3**, 1: methyl 2-deoxyribofuranoside, DMTCl, DMF, DMAP (2 eq.), room temperature, 4 h. ; 2: (Acetoxyl)-methyl-2-deoxyribofuranoside, 0°C, 4 h, 85% for two steps; for compound **4**: methyl 2-deoxyfuranoside, Ac_2O, pyridine, 90%; for compound **5**: methyl 2-deoxyribofuranoside, H_2SO_4, Ac_2O, 0°C, 63%; for compound **6**: compound **5** in HBr/AcOH, 0°C, 30 min., 95%; for compound **7**: compound **5**, CH_2Cl_2 (HCl), 0°C, 2 h, 85%; c) for compounds **3**, **4** or **5**: compound **2**, compound **3**, **4**, or **5** CH_2Cl_2, 0°C, $SnCl_4$, 2 h; for compounds **6** or **7**: compound **2**, compounds **6** or **7**, CH_2Cl_2, 0°C, Ag_2CO_3, dark, 3 h.

The desired cytidine acceptor **2** was synthesized in standard fashion starting from commercially available thymidine, after sequentially treating it with TMSCl/Et_3N, triazol/$POCl_3$, NH_3/H_2O and DMT-Cl. Protection of the 5-OH of methyl 2-deoxyribofuranoside with DMT group, followed by acetylation, gave the monosaccharide glycosylation donor **3**. The glycosylation between compound **2** and **3** was attempted under standard conditions which resulted in a complicate mixture of products. After work up and careful purification, no desired disaccharide nucleoside was isolated. Similarly , we also tried other monosaccharide donors **4**, **5**, **6** or **7** under different glycosylation conditions, the results were same. This may be due to the DMT protecting groups in compounds **2** and **3** which are not stable under the attempted glycosylation conditions, and based on the LCMS analysis, only trace amount of disaccharide without DMT protecting group was detected. Hence , it's felt we should employ protecting groups stable under acidic conditions, such as *t*-butyldiphenylsilyl ether (TBDPS).

Accordingly , thymidine was protected with TBDPS at 5'-OH position to provide the compound **8**, and in three steps, thymidine derivative **8** was transferred to the cytidine derivative **9**, which was coupled with per-*O*-acetyl 2-deoxy methyl-ribofuranoside **5** in the presence of $SnCl_4$. After work up, and careful purification, an unstable coupling product mixture was isolated. Based on their NMR and MS data, the compounds were not the desired products instead they were found to be compounds **10α** and **10β** which are *N*-glycosylated products instead of the 3'-OH in the nucleoside acceptor **9**. (**Scheme 2**)



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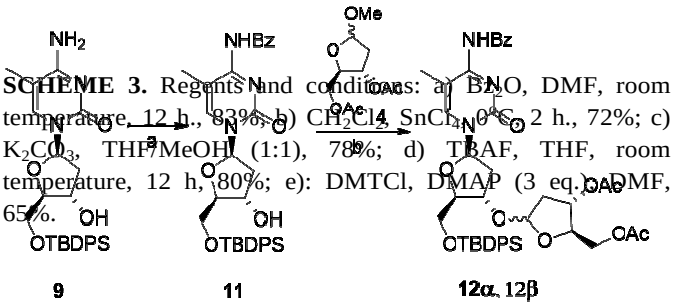
SCHEME 2. Reagents and Conditions: a) TBDPSCl, pyridine, 90%; b) TMSCl/Et_3N, triazol/$POCl_3$, NH_3/H_2O; 85%; c) CH_2Cl_2, 0°C, $SnCl_4$, 2 h.

In order to avoid the *N*-glycosylation products, the NH_2 group in **9** was protected with Bz group to give the nucleoside acceptor **11**. Under $SnCl_4$ conditions, compound **11** was coupled with the glycosylation donor **4** in dry dichloromethane to give a mixture (1:1) of disaccharide nucleosides **12α** and **12β** in more than 70% yield. We also tried the coupling reactions between **11** and other monosaccharide donors **5**, **6** or **7** under different glycosylation conditions and the results were similar based on the TLC (**Scheme 3**).

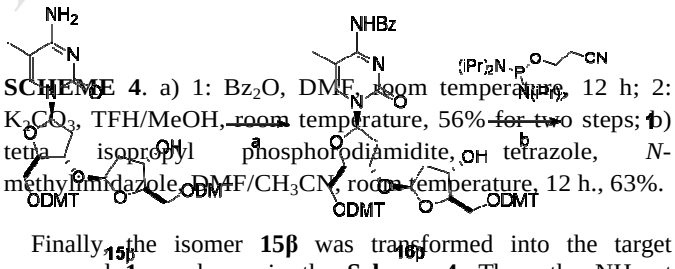
The separation of anomeric diastereomers **12α** and **12β** was found to be very difficult. All attempted methods, silica gel column chromatography, preparative TLC, HPLC and reverse phase C-18 column chromatography and SCF separation, didn't give satisfactory separation. Even the corresponding hydroxyl derivatives **13α** and **13β**, which were obtained by subjecting compounds **12α** and **12β** to weak basic conditions, also couldn't be purified satisfactorily.



SCHEME 3. Regents and conditions: a) Bz_2O, DMF, room temperature, 12 h., 83%; b) CH_2Cl_2, $SnCl_4$, 0°C, 2 h., 72%; c) K_2CO_3, THF/MeOH (1:1), 78%; d) TBAF, THF, room temperature, 12 h, 80%; e): DMTCl, DMAP (3 eq.), DMF, 65%.

The mixture of compounds **13α** and **13β** was then treated with TBAF in THF to give the unprotected disaccharide nucleosides **14α** and **14β**, which also could not be separated. Then DMT-ethers (**15α** and **15β**) of **14α** and **14β** were prepared by treating them with DMTCl in DMF in the presence of DMAP. Among all the methods studied for their separation, only the supercritical fluid chromatography method was found to be suitable.

The desired isomer **15β** was characterized by different 2D NMR studies such as 2D H-H COSY and C-H COSY apart from assigning each proton in its ^{1}H-NMR spectrum. The triplet at 5.15 ppm was assigned for H-1 in the monosaccharide part while the multiplet at 6.04 ppm was assigned for H-1' in the nucleoside part. The multiplet at 4.05 ppm was assigned for H-4 in monosaccharide, and the multiplet at 4.07 ppm was assigned for H-4' in the nucleoside part. In its 2D NOESY spectrum, the H-1 triplet at 6.04 ppm was found to have interaction with the H-4 at 4.05 ppm, and the H-1' multiplet at 5.15 ppm was found to interact with that of H-4' at 4.07 ppm, indicating that both sugars have the β configurations and indeed this compound is **15β** as desired. By using the similar studies, the structure of compound **15α** was also confirmed.



SCHEME 4. a) 1: Bz_2O, DMF, room temperature, 12 h; 2: K_2CO_3, TFH/MeOH, room temperature, 56% for two steps; b) tetra isopropyl phosphorodiamidite, tetrazole, *N*-methylimidazole, DMF/CH_3CN, room temperature, 12 h., 63%.

Finally, the isomer **15β** was transformed into the target compound **1** as shown in the **Scheme 4**. Thus, the NH_2 at heterocyclic part in compound **15β** was protected with Bz group in a two-step sequence to give the compound **16β**. The compound **16β** was then treated with tetraisopropylphosphorodiamidite, tetrazole and *N*-methylimidazole in dry DMF, to give the phosphorylated product. After HPLC purification, the desired phosphoramidite **1**, with more than 92% purity was obtained in 62% yield. Its structure was confirmed by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and mass spectra.

In conclusion, a partially protected disaccharide nucleoside phosphoramidite **1** was successfully synthesized as the impurity reference compound for the antisense anti-tumor drug ISIS183750. We believe this method allows synthesis of compound **1** in sufficient quantities and it's also believed that the synthetic strategy permits synthesis of 2-deoxy ribose related disaccharide nucleosides and the corresponding phosphoramidites in an expedient fashion.

Experiments

NMR spectra were obtained with a Bruker AV-400 (400 MHz), the ^{1}H NMR chemical shifts were measured relative to tetramethylsilane, $CDCl_3$ or DMSO-d_6 as the internal reference, while the ^{13}C NMR chemical shifts were recorded with $CDCl_3$ or

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DMSO-d_6 as the internal standard. Mass spectra were obtained on Agilent LCMS instrument. Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. Anhydrous solvents were dried by heating at reflux for at least 24 h over CaH_2, dichloromethane, DMF, tetrahydrofuran, toluene, and diethyl ether were freshly distilled prior to use. Unless otherwise indicated, all syntheses and manipulations were carried out under dry nitrogen atmosphere.

2-Deoxy 3，5-di-*O*-acetyl methyl ribofuranoside (**4**) was prepared according to the procedures reported by Iglesias.[11]

2'-Deoxy 5'-*O*-TBDPS 5-methyl uridine (**8**): To a solution of 2'-deoxy 5-methyl uridine (14.71 g, 0.061 mol) in dry pyridine (88 mL) was added imidazole (8.30 g, 0.122 mol), followed by addition of TBDPSCl (26.83 g, 0.098 mol). The reaction mixture was stirred at room temperature under N_2 overnight, and quenched by adding water. The solvent was evaporated, and the residue was purified by flash column (CH_2Cl_2 : CH_3OH = 20:1) to give the compound **8** as a solid (22 g, yield: 75.21%), ^{1}H NMR ($CDCl_3$): δ8.50 (s, 1H), 7.50 (m, 5H), 7.20 (m, 7H), 6.20 (m, 1H), 4.40 (d, 1H), 4.00 (d, 1H), 3.75 (m, 1H), 3.65 (m, 1H), 2.40 (m, 1H), 1.90 (m, 1H), 1.60 (s, 3H), 0.80 (s, 9H); ESIMS: m/z 481 $[M+H]^+$.

2'-Deoxy 5'-*O*-TBDPS 5-methyl cytidine (**9**): To a solution of compound **8** (72 g, 0.15mol) in anhydrous acetonitrile (480 mL) was added Et_3N (242.4 g, 2.4 mol) at -15°C under N_2. After stirring for 20 min., TMSCl (48.6 g, 0.45 mol) was added, after another 1h, triazole (82.8 g, 1.2 mol) and $POCl_3$ (45.6 g, 0.3 mol) were added at -15°C. This mixture was stirred at this temperature for 2h, quenched with addition of 240 mL of water at -15°C. The mixture was extracted with EtOAc, and the aqueous phase was re-extracted with EtOAc. The solvent was evaporated, and the residue was dissolved in the mixture of 1,4-dioxane (600 mL) and aqueous ammonia (30 mL). The mixture was stirred at room temperature under N_2 overnight. After evaporation, the product was purified by flash column (CH_2Cl_2 : CH_3OH = 10:1) to give the compound **9** as a solid (63 g, yield: 87.5%), ^{1}H NMR ($CDCl_3$): δ7.65 (m, 5H), 7.40 (m, 6H), 6.50 (m, 1H), 4.50 (m, 1H), 4.10 (d, 1H), 4.00 (dd, 1H), 3.80 (dd, 1H), 2.65 (m, 1H), 2.10 (m, 1H), 1.60 (s, 3H), 1.09 (s, 9H); ESIMS: m/z 480 $[M+H]^+$.

2'-Deoxy 5'-*O*-TBDPS *N*-benzoyl 5-methyl cytidine (**11**): To a solution of the compound **9** (63 g, 0.13 mol) in anhydrous DMF (480 mL) was added Bz_2O (32.57 g, 0.14 mol). The mixture was stirred at room temperature under N_2 overnight, the solvent was removed, and the residue was diluted with EtOAc (200 mL), the resulting solution was washed with water (100 mL×2), dried with anhydrous Na_2SO_4. After filtration and concentration, the residue was purified by flash silica gel column (EtOAc : PE = 3:1) to give the compound **11** as a solid (63 g, yield: 82.89%), ^{1}H NMR (DMSO-D_6): δ13.00 (s, 1H), 8.10 (m, 2H), 7.70 (m, 1H), 7.60 (m, 4H), 7.50 (m, 1H), 7.40 (m, 8H), 6.20 (m, 1H), 5.40 (m, 1H), 4.30 (m, 1H), 3.95 (m, 2H), 3.80 (m, 1H), 2.25 (m, 2H), 1.70 (s, 3H), 1.00 (s, 9H); ESIMS: m/z 585 $[M+H]^+$.

2'-Deoxy 5'-*O*-TBDPS 3'-*O*-(2"-deoxy 5",3"-*O*-diacetyl ribofuranosyl)-*N*-benzoyl 5-methyl cytidine (**12α**, **12β**): To a solution of the compound **4** (2.66 g, 11.49 mmol) in anhydrous CH_2Cl_2 (100 mL) was added dropwise $SnCl_4$ (3.53 mL) at 0°C. The mixture was stirred at 0℃ under nitrogen for 15 min., compound **11** (6.7 g, 11.49 mmol) was added, and the reaction was stirred at 0℃ for 2h, quenched with saturated sodium bicarbonate solution, extracted with dichloromethane (500 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated. The residue was purified by flash column (CH_2Cl_2 : CH_3OH = 20:1) to give the compounds **12α** and **12β** as a solid (6.43 g, yield: 71.52%), ^{1}H NMR ($CDCl_3$): δ8.30 (m, 2H), 7.70-7.60 (m, 5H), 7.50-7.30 (m, 10H), 6.30-6.50 (m, 1H), 5.30-5.00 (m, 2H), 4.50 (m, 1H), 4.30-3.90 (m, 5H), 2.55-2.35 (m, 2H), 2.20-2.10 (m, 2H), 2.10 (s, 3H), 2.00 (s, 3H), 1.70 (s, 3H), 1.10 (s, 9H); ESIMS: m/z 785 $[M+H]^+$.

2'-Deoxy 3'-*O*-(2"-deoxy ribofuranosyl) 5'-*O*-TBDPS 5-methyl cytidine (**13α**, **13β**): To a solution of the compounds **12α**, **12β** (25.5 g, 32.57 mmol) in the mixture of THF (150 mL) and methanol (150 mL) was added anhydrous potassium carbonate (6.75 g, 48.85 mmol). The mixture was stirred at room temperature under N_2 overnight, the solvent was removed, and the residue was purified by flash silica column (CH_2Cl_2: CH_3OH = 10:1) to give compounds **13α**, **13β** as a solid (15.23 g, yield: 78.59%). ^{1}H NMR (DMSO-D_6): δ7.95 (m, 1H), 7.60 (m, 5H), 7.40 (m, 9H), 6.80 (m, 1H), 6.10 (m, 1H), 5.20 (m, 1H), 5.10 (m, 1H), 4.90 (m, 1H), 4.80-4.30 (m, 3H), 4.10-3.60 (m, 7H), 3.60-3.20 (m, 10H), 2.4-1.9 (m, 4H), 1.50 (two s, 3H), 1.00 (s, 9H); ESIMS: m/z 596 $[M+H]^+$.

2'-Deoxy 3'-*O*-(2"-deoxy ribofuranosyl) 5-methyl cytidine (**14α**, **14β**): To a solution of the compounds **13α**, **13β** (15.23 g, 25.60 mmol) in THF (200 mL) was added TBAF (33.28 g, 12.8 mmol). The mixture was stirred at room temperature under N_2 overnight. The solvent was removed, and the residue was purified by flash column (CH_2Cl_2 : CH_3OH = 10:1) to give compounds **14α**, **14β** as a white solid (7.36 g, yield: 80.61%), ^{1}H NMR ($CDCl_3$): δ7.60 (m, 1H), 7.35 (m, 1H), 6.80 (m, 1H), 6.20 (m, 1H), 5.20 (m, 1H), 5.00 (m, 1H), 5.85 (m, 1H), 4.35 (m, 1H), 4.10 (m, 1H), 3.90 (m, 1H), 3.80 (m, 1H), 3.70 (m, 1H), 3.40-3.60 (m, 3H), 2.30-1.90 (m, 4H), 1.70 (s, 3H); ESIMS: m/z 358 $[M+H]^+$.

2'-Deoxy 3'-*O*-(2"-deoxy 5"-*O*-DMT α-D-ribofuranosyl) 5'-*O*-DMT 5-methyl cytidine (**15α**) and 2'-deoxy 3'-O-(2"-deoxy 5"-*O*-DMT β-D-ribofuranosyl) 5'-*O*-DMT 5-methyl cytidine (**15β**): To a solution of the compounds **14α**, **14β** (2.05 g, 5.74 mmol) in anhydrous DMF (15 mL) was added DMTCl (3.88 g, 11.48 mmol) and DMAP (1.75 g, 14.35 mmol). The mixture was stirred at room temperature under N_2 for four days. The reaction mixture was purified by supercritical fluid chromatography separation to give the products **15β** (0.9 g, 32.67%) and **15α** (0.9 g, 32.67%) as solids. Spectral data for compound **15β**, ^{1}H NMR (DMSO-D_6): δ8.09 (d, 0.5H), 7.40-7.10 (m, 22H), 6.75-6.90 (m, 20H), 6.58 (d, 0.5H), 6.04 (t, 1H), 5.15 (d, 1H), 5.10 (d, 1H), 4.49 (m, 1H), 4.10 (m, 1H), 3.90 (m, 1H), 3.85 (m, 1H), 3.72 (s, 3H), 3.71(s, 3H), 3.70 (s, 3H), 3.69 (s, 3H), 3.15 (m, 2H), 3.08 (m, 1H), 2.97-1.80 (m, 4H), 1.50 (s, 3H); ^{13}C NMR (DMSO-D_6): 103.5, 102.0, 86.5, 86.0, 85.8, 83.0, 77.5, 71.2, 65.5, 55.5, 37.0, 31.5, 12.9; ESIMS: m/z 962 $[M+H]^+$; spectral data for compound **15α**, ^{1}H NMR (DMSO-D_6): δ8.10 (d, 0.37H), 7.50 (m, 1H), 7.35-7.10 (m, 21H), 6.80-6.70 (m, 10H), 6.55 (m, 0.45H), 6.20 (t, 1H), 5.50 (m, 1H), 4.93 (d, 1H), 4.53 (m, 1H), 4.15 (m, 1H), 3.85 (m, 1H), 3.75 (m, 1H), 3.64 (s, 3H), 3.63 (s, 3H), 3.62 (s, 3H), 3.61 (s, 3H), 3.23 (m, 1H), 3.00 (m, 1H), 2.90 (m, 1H), 2.40-2.10 (m, 3H), 1.65 (m, 1H), 1.35 (s, 3H); ^{13}C NMR (DMSO-D_6): 102.0, 87.0, 86.0, 85.2, 84.3, 77.8, 71.2, 64.5, 55.5, 42.0, 39.0, 13.9; ESIMS: m/z 962 $[M+H]^+$.

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2'-Deoxy 3'-*O*-(2"-deoxy 5"-*O*-DMT β-D-ribofuranosyl) 5'-O-DMT *N*-benzoyl 5-methyl cytidine (**16β**): To a solution of the compound **15β** (1.4 g, 1.45mmol) in anhydrous DMF (9 mL) was added Bz_2O (0.64 g, 2.9mmol), the mixture was stirred at room temperature under N_2 overnight. After evaporation of the solvent, the residue was dissolved in the mixture of THF and methanol (1:1, 100 mL), 80 mL of saturated potassium carbonate solution was added, and the reaction mixture was stirred at room temperature and monitored by TLC. When the starting material was almost consumed, aqueous citric acid solution (pH=6) was added to neutralize the solution. After the solvent was removed, the residue was dissolved in EtOAc (10 mL). The solution was washed with water (5 mL×2), evaporated, and the residue was purified by preparatory HPLC to give the compound **16β** as a white solid (0.71 g), ^{1}H NMR (DMSO-D_6): δ8.15 (m, 2H), 7.60-7.10 (m, 23H), 6.90-6.80 (m, 8H), 5.97 (t, 1H), 5.20-5.10 (m, 2H), 4.45 (m, 1H), 4.10 (m, 1H), 3.98 (m, 1H), 3.90 (m, 1H), 3.70-3.60 (4s, 12H), 3.30 (m, 2H), 3.20 (m, 2H), 3.10 (m, 1H), 2.95 (m, 1H), 2.20-1.80 (m, 4), 1.70 (s, 3H); ESIMS: m/z 1067 $[M+H]^+$.

2'-Deoxy 3'-*O*-[2"-deoxy 5"-*O*-DMT 3"-*O*-(di-*N*-isopropylamino *O*-propanenitrile phosphinoamidite) β-D-ribofuranosyl] 5'-*O*-DMT *N*-benzoyl 5-methyl cytidine (**1**): The solution of compound **16β** (200 mg, 0.188 mmol) in 1 mL of DMF was co-evaporated with anhydrous toluene (3 mL×2), followed by the addition of ditetraisopropylamino-O-propanenitrile phosphinoamidite (452 mg, 1.5 mmol) and tetrazole (1.25 mL, 0.45 M in MeCN, 0.56 mmol) at room temperature under nitrogen. After 5 minutes, 30 μL of N-methylimidazole was added, and the mixture was stirred at room temperature overnight. The crude product was purified by HPLC to give the compound **1** as a white foam (150 mg, yield: 63%) with 95% purity (based on HPLC). ^{1}H NMR ($CDCl_3$): δ12.86 (brs, 1H,), 8.13 (brs, 2H), 7.79 (brs, 1H), 7.55 (t, J = 7.2 Hz, 1H), 7.46 (t, J = 7.6 Hz, 2H), 7.30~7.42 (m, 4H), 7.10~7.30 (m, 14H), 6.78~6.90 (m, 8H), 5.99 (t, J = 6.4 Hz, 1H), 5.25 (m, 1H), 4.30~4.48 (m, 2H), 3.94~4.10 (m, 2H), 3.60~3.70 (m, 12H), 3.40~3.60 (m, 3H), 3.22 (m, 2H), 3.07~3.18 (m, 1H), 2.99 (m, 1H), 2.70 (t, J = 5.6 Hz, 1H), 2.61 (t, J = 5.6 Hz, 1H), 2.02~2.30 (m, 3H), 1.68 (s, 3H), 1.04 (m, 12H); ^{31}P NMR ($CDCl_3$): 147.85, 147.52; ESIMS: m/z: 1267 $[M+H]^+$.

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