Bioconjugate Chemistry

TECHNICAL NOTE

pubs.acs.org/bc

Solid-Supported 2′-*O*-Glycoconjugation of Oligonucleotides by Azidation and Click Reactions

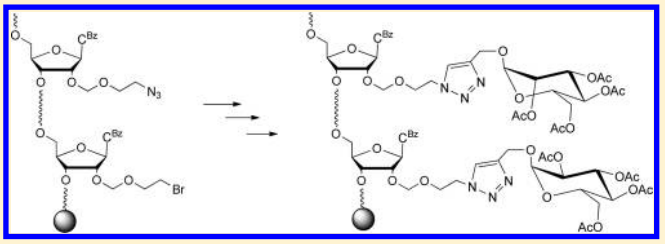
Anu Kiviniemi,*[,†] Pasi Virta,[†] Mikhail S. Drenichev,[‡] Sergey N. Mikhailov,*[,‡] and Harri Lönnberg[†]

[†]Department of Chemistry, University of Turku, FIN-20014 Turku, Finland

[‡]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov Street 32, Moscow 119991, Russia

Supporting Information

ABSTRACT: 2′-*O*-[(2-Bromoethoxy)methyl]cytidine and 2′-*O*-[(2-azidoethoxy)methyl]cytidine have been prepared and introduced as appropriately protected 3′-phosphoramidite (**1**) and 3′-(*H*-phosphonate) (**2**) building blocks, respectively, into 2′-*O*-methyl oligoribonucleotides. The support-bound oligonucleotides were subjected to two consecutive conjugations with alkynyl-functionalized monosaccharides. The first saccharide was introduced by a Cu(I) promoted click reaction with **2** and the second by azidation of the 2-bromoethoxy group of **1** followed by the click reaction. The influence of the 2′-glycoconjugations on hybridization with DNA and 2′-*O*-methyl RNA targets was studied. Two saccharide units within a 15-mer oligonucleotide had a barely noticeable effect on the duplex stability, while introduction of a third one moderately decreased the melting temperature.



INTRODUCTION

Among various oligonucleotide conjugates, the 2′-conjugates have received special interest for the reason that upon formation of an A- or B-type duplex, the conjugate group is accommodated in the minor or major grooves, respectively, and hence, the duplex is not markedly destabilized.[1,2] Introduction of reactive functionalities in the 2′-position of nucleosides, incorporation of the resulting monomers into oligonucleotides, and their subsequent reactions with appropriately functionalized conjugate groups has been rather recently reviewed.[3] Somewhat surprisingly, such a stepwise protocol has only once been utilized for solid phase-synthesis of 2′-glycoconjugates, potentially useful for the delivery and targeting of therapeutic oligonucleotides,[4−8] although several procedures for the derivatization of other intrachain positions, including the C4′-position,[9,10] internucleosidic phosphoramidate linkage,[11,12] or intrachain non-nucleosidic units,[13−18] have been described. Very recently,[19] saccharide ligands have been conjugated by Cu(I) promoted click reaction[20,21] to 2′-*O*-(2-propynyl) groups on-support. Otherwise 2′-glycoconjugates have been obtained by converting prefabricated 2′-*O*-glycosyl nucleosides with all four natural heterocyclic bases[22] into phosphoramidite building blocks and introducing them into oligonucleotides on a solid-support.[23−26] The *cis* diol of the 2′-*O*-glycosyl group, when oxidized to dialdehyde with sodium periodate, has been shown to allow affinity labeling of different enzymes.[27−29]

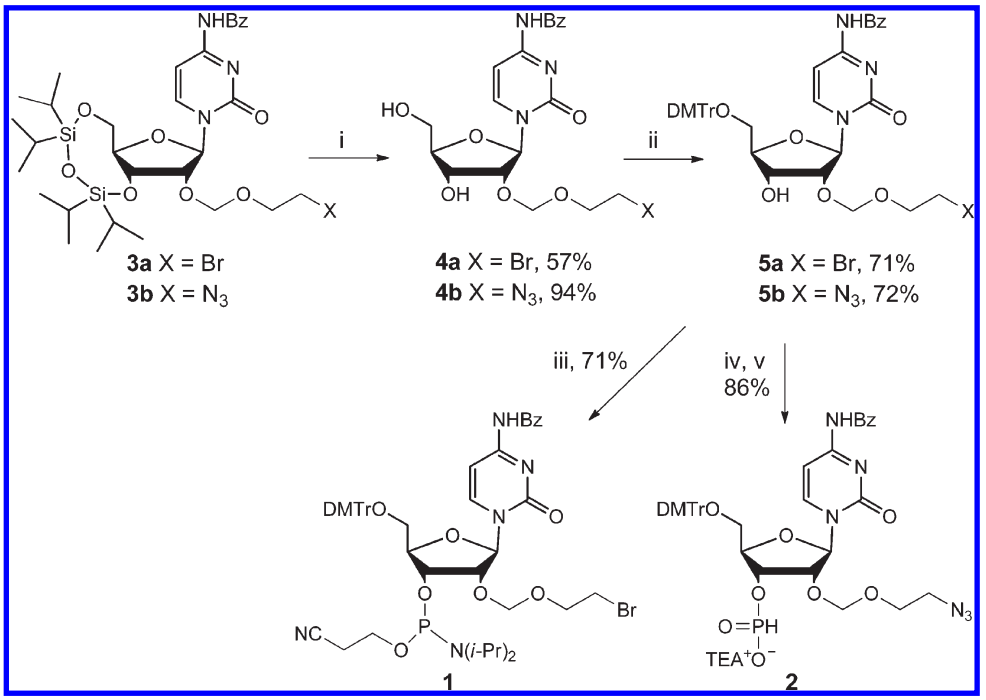
We now report on a solid-supported conjugation method that enables the attachment of two different carbohydrate ligands to the 2′-position at desired sites within the chain. For this purpose, two building blocks, viz. 2′-*O*-[(2-bromoethoxy)methyl]-5′-*O*-(4,4′-dimethoxytrityl)cytidine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (**1**) and 2′-*O*-[(2-azidoethoxy)methyl]-5′-*O*-(4,4′-dimethoxytrityl)cytidine 3′-(*H*-phosphonate) (**2**) monomers, were prepared and incorporated into 2′-*O*-methyl oligoribonucleotides. The azido group of the nucleoside units **2** was first subjected to a click reaction,[20,21] with a propynyl functionalized monosaccharide on-support. The bromo substituent of unit **1** was then displaced with azide ion on-support, as shown previously by Pourceau et al.,[14] and another click reaction was carried out. The hybridization efficiency of the conjugates with cDNA- and 2′-*O*-Me-RNA-oligonucleotides was assessed by UV-melting studies and compared to the hybridization of the corresponding unmodified sequence.

EXPERIMENTAL PROCEDURES

General Remarks. Synthetic reactions were monitored by TLC (Merck DC Kieselgel 60F_{254}), using UV light, iodine vapors, or heating with 10% H_2SO_4 (aq) for detection. Column chromatography was performed on Silica gel 60 (Fluka, 0.040−0.063 mm). The NMR spectra were recorded at 400 or 500 MHz. Chemical shifts are given in parts per million (ppm) and measured relative to the solvent signals (^{1}H and ^{13}C). Appropriate 2D NMR methods (COSY and HSQC) were used for peak assignment. Solvents were dried with 4 Å molecular sieves (DCM, pyridine and MeCN) or by distillation and storage over CaH_2 (Et_3N). The mass spectra were recorded using ESI ionization methods. RP HPLC analysis of the oligonucleotide conjugates was performed on a Thermo ODS Hypersil C18 (250 × 4.6 mm, 5 μm) analytical column, applying a gradient elution from 0 to 50% MeCN in

Received: February 25, 2011
Revised: April 11, 2011
Published: May 03, 2011

Scheme 1[a]



[a] Reagents: (i) Bu_4NF, THF; (ii) DMTrCl, pyridine; (iii) 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, Et_3N, DCM; (iv) diphenyl *H*-phosphonate, pyridine; (v) Et_3N, H_2O.

0.1 mol L^{-1} TEAA (aq, Et_3NHOAc) buffer over 60 min, at a flow rate of 1 mL min^{-1}, and detection at 260 nm.

2′-*O*-(2-Bromoethoxymethyl)-*N*^{4}-benzoylcytidine (4a). Nucleoside **3a** (1.30 g, 1.79 mmol), prepared as described earlier,[30] was dissolved in Bu_4NF trihydrate in THF (0.25 mol L^{-1}; 4.8 mL), kept for 10 min at 20 °C, evaporated to dryness, coevaporated with chloroform (2 × 10 mL), and applied onto a column with silica gel (25 g). The column was washed with DCM (100 mL), DCM-EtOH (97:3, v/v), and then eluted with DCM-EtOH (95:5) to obtain **4a** (490 mg, 57%) as a white amorphous foam (Scheme 1). ^{1}H NMR [400 MHz, $CDCl_3/CD_3OD$ (9:1)] δ_{ppm} 8.47 (d, 1H, J = 7.5 Hz, H-6), 7.98−7.40 (m, 6H, Bz, H-5), 5.88 (d, 1H, J = 2.2 Hz, H-1′), 5.02 (d, 1H, J = 6.5 Hz, OCHHO), 4.92 (d, 1H, J = 6.5 Hz, OCHHO), 4.33 (dd, 1H, $J_{2',3'}$ = 5.0 Hz, H-2′), 4.26 (dd, 1H, $J_{3',4'}$ = 7.5 Hz, H-3′), 4.07 (dt, 1H, $J_{4',5'a}$ = $J_{4',5'b}$ = 2.2 Hz, H-4′), 3.99 (dd, 1H, $J_{5'a,5'b}$ = 12.8 Hz, H-5′a), 3.89 (dd, 2H, J = 6.2 Hz, 5.9 Hz, OCH_2CH_2Br), 3.81 (dd, 1H, H-5′b), 3.44 (dd, 2H, OCH_2CH_2Br). ^{13}C NMR ($CDCl_3/CD_3OD$ (9:1)) δ_{ppm} 167.1 (C═O), 163.1 (C-4), 146.0 (C-6), 133.3, 129.0, 128.0 (Ph), 97.3 (C-5), 95.3 (OCH_2O), 90.9 (C-1′), 84.7 (C-4′), 79.9 (C-2′), 68.8 (C-3′), 68.0 (OCH_2CH_2Br), 60.1 (C-5′), 30.5 (OCH_2CH_2Br).

2′-*O*-(2-Azidoethoxymethyl)-*N*^{4}-benzoylcytidine (4b). Analogous to the preparation of **4a**, the desilylation of **3b** (1.12 mg, 1.63 mmol) yielded **4b** (683 mg, 94%) as a white amorphous foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ_{ppm} 8.48 (d, 1H, J = 7.5 Hz, H-6), 8.00−7.40 (m, 6H, Bz, H-5), 5.88 (d, 1H, J = 2.5 Hz, H-1′), 5.03 (d, 1H, J = 6.5 Hz, OCHHO), 4.93 (d, 1H, OCHHO), 4.48 (dd, 1H, J = 5.3 Hz, H-2′), 4.41 (dd, 1H, J = 6.5 Hz, H-3′), 4.16 (ddd, 1H, $J_{4',5'a}$ = 1.9 Hz, $J_{4',5'b}$ = 1.5 Hz, H-4′), 4.08 (dd, 1H, $J_{5'a,5'b}$ = 12.5 Hz, H-5′a), 3.92 (dd, 1H, H-5′b), 3.82−3.71 (m, 2H, $OCH_2CH_2N_3$), 3.46−3.37 (m, 2H, $OCH_2CH_2N_3$). ^{13}C NMR (MHz, $CDCl_3$) δ_{ppm} 167.2 (C═O), 163.1 (C-4), 155.4 (C-2), 146.2 (C-6), 133.3, 129.0, 128.0 (Bz), 97.3 (C-5), 95.5 (OCH_2O), 91.2 (C-1′), 84.9 (C-4′), 79.9 (C-2′), 68.2 (C-3′), 67.4 ($OCH_2CH_2N_3$), 60.4 (C-5′), 50.9 ($OCH_2CH_2N_3$).

2′-*O*-(2-Bromoethoxymethyl)-5′-*O*-(4,4′-dimethoxytrityl)-*N*^{4}-benzoylcytidine (5a). Nucleoside **4a** (357 mg, 0.74 mmol) was dried by coevaporation with pyridine (2 × 20 mL). The residue was dissolved in dry pyridine (10 mL), 4,4′-dimethoxytrityl chloride (351 mg, 1.04 mmol) was added, and the resulted solution was kept in the dark for 16 h at 20 °C. MeOH (1 mL) was added and after 30 min, the mixture was concentrated in vacuo to near dryness. The residue was dissolved in DCM (50 mL) and washed with a 10% aqueous solution of sodium bicarbonate (20 mL) and water (2 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4, the solvent was removed in vacuo, and the residue was coevaporated with toluene (2 × 10 mL) and purified by column chromatography on silica gel (20 g). The column was washed with a mixture of DCM, hexane, and Et_3N (75:23:2, v/v/v, 200 mL), followed by DCM, and the product was eluted with a mixture of DCM and Et_3N (98:2, v/v) to give **5a** (412 mg, 71%) as a foam. ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 9.63 (br s, 1H, NH), 8.53 (d, 1H, J = 7.5 Hz, H-6), 8.00−7.94 (m, 2H, Bz), 7.60 (m, 1H, Bz), 7.52−7.46 (m, 4H, Bz, DMTr), 7.41−7.32 (m, 6H, DMTr), 7.29−7.22 (m, 2H, DMTr, H-5), 6.94−6.88 (m, 4H, DMTr), 5.92 (s, 1H, H-1′), 5.17 (d, 1H, J = 6.5 Hz, OCHHO), 4.95 (d, 1H, J = 6.5 Hz, OCHHO), 4.58 (m, 1H, H-3′), 4.38 (d, 1H, J = 5.0 Hz, H-2′), 4.15 (m, 1H, H-4′), 4.00−3.89 (m, 2H, OCH_2CH_2Br), 3.78 (s, 6H, DMTr-OMe), 3.56−3.46 (m, 4H, OCH_2CH_2Br, H-5′). ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 167.2 (Bz-C═O), 163.0 (C-4), 158.8, 158.8 (DMTr), 154.9 (C-2), 144.9 (C-6), 144.5, 136.0, 135.6 (DMTr), 133.5, 132.9 (Bz), 130.2, 130.0 (DMTr), 128.7, 128.3, 128.1, 128.1 (DMTr, Bz), 127.1, 113.3 (DMTr), 96.5 (C-5),

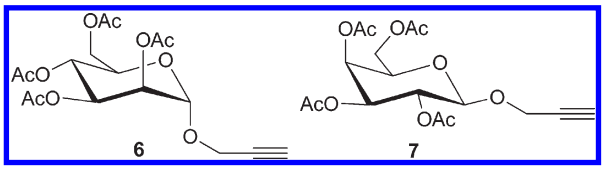
94.3 (OCH_2O), 89.8 (C-1′), 86.8 (DMTr-C_q), 82.6 (C-4′), 78.9 (C-2′), 68.3 (OCH_2CH_2Br), 68.0 (C-3′), 61.2 (C-5′), 55.0 (DMTr-OMe), 31.5 (OCH_2CH_2Br). HRMS(ESI): $[MH]^+$ $C_{40}H_{41}BrN_3O_9$ requires 786.2021, found 786.2019.

2′-*O*-(2-Azidoethoxymethyl)-5′-*O*-(4,4′-dimethoxytrityl)-β-D-*N*⁴-benzoylcytidine (5b). Analogous to the preparation of **5a**, the tritylation of nucleoside **4b** (778 mg, 1.74 mmol) yielded **5b** (940 mg, 72%) as a foam. ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 9.29 (br s, 1H, NH), 8.48 (d, 1H, J = 7.5 Hz, H-6), 8.00−7.95 (m, 2H, Bz), 7.66 (m, 1H, Bz), 7.57−7.52 (m, 2H, Bz), 7.51−7.48 (m, 2H, DMTr), 7.41−7.35 (m, 6H, DMTr), 7.30 (m, 1H, DMTr), 7.18 (br d, 1H, J = 6.5 Hz, H-5), 6.96−6.91 (m, 4H, DMTr), 5.91 (d, 1H, J = 1.0 Hz, H-1′), 5.16 (d, 1H, J = 7.0 Hz, OCHHO), 4.94 (d, 1H, J = 6.5 Hz, OCHHO), 4.52 (m, 1H, H-3′), 4.32 (d, 1H, J = 5.0 Hz, H-2′), 4.11 (ddd, 1H, J = 9.0 Hz, 2.5 Hz, 2.5 Hz, H-4′), 3.88−3.76 (m, 8H, DMTr-OMe, $OCH_2CH_2N_3$), 3.53−3.37 (m, 5H, H-5′, $OCH_2CH_2N_3$, 3′−OH). ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 167.2 (Bz-C=O), 162.9 (C-4), 158.8, 158.8 (DMTr), 154.8 (C-2), 144.9 (C-6), 144.5, 136.0, 135.6 (DMTr), 133.5, 132.9 (Bz), 130.2, 130.0 (DMTr), 128.7 (Bz), 128.2, 128.1, 128.1 (DMTr, Bz), 127.1, 113.3 (DMTr), 96.3 (C-5), 94.4 (OCH_2O), 89.8 (C-1′), 86.7 (DMTr-C_q), 82.6 (C-4′), 79.0 (C-2′), 68.0 (C-3′), 66.9 ($OCH_2CH_2N_3$), 61.2 (C-5′), 55.0 (DMTr-OMe), 50.7 ($OCH_2CH_2N_3$). HRMS(ESI): $[MH]^+$ $C_{40}H_{41}N_6O_9$ requires 749.2930; found, 749.2935.

2′-*O*-(2-Bromoethoxymethyl)-5′-*O*-(4,4′-dimethoxytrityl)-β-*N*⁴-benzoylcytidine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (1). Compound **5a** (0.29 g, 0.37 mmol) was predried over P_2O_5 in a vacuum desiccator and dissolved in DCM (5 mL). Et_3N (260 μL, 1.85 mmol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (99 μL, 0.44 mmol) were added under nitrogen. After 2 h, the mixture was filtered through a short dried silica gel column (60−70% EtOAc in hexane and 0.1% Et_3N). The mixture of phosphoramidite **1** R_P- and S_P-diastereomers (0.26 g, 71%) was obtained as a colorless oil. ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 9.22 (br, 1H, NH), 8.50 [d, 0.5H, J = 7.5 Hz, H-6 (*diast. I*)], 8.45 [d, 0.5H, J = 7.0 Hz, H-6 (*diast. II*)], 8.00−7.94 (m, 2H, Bz), 7.66 (m, 1H, Bz), 7.58−7.48 (m, 4H, Bz, DMTr), 7.43−7.34 (m, 6H, DMTr), 7.31 (m, 1H, DMTr), 7.11 [br, 0.5H, H-5 (*diast. I*)], 7.04 [br, 0.5H, H-5 (*diast. II*)], 6.96−6.90 (m, 4H, DMTr), 5.99 (br s, 1H, H-1′), 5.09 [d, 0.5H, J = 6.5 Hz, OCHHO (*diast. I*)], 5.06 [d, 0.5H, J = 6.5 Hz, OCHHO (*diast. II*)], 5.01 [d, 0.5H, J = 6.5 Hz, OCHHO (*diast. II*)], 4.96 [d, 0.5H, J = 6.5 Hz, OCHHO (*diast. I*)], 4.60 (m, 1H, H-3′), 4.47 [m, 0.5H, H-2′ (*diast. I*)], 4.41 [m, 0.5H, H-2′ (*diast. II*)], 4.28 [m, 0.5H, H-4′ (*diast. I*)], 4.23 [m, 0.5H, H-4′ (*diast. II*)], 4.05−3.85 [m, 2.5H, OCH_2CH_2Br, $OCHHCH_2CN$ (0.5H *diast. II*)], 3.84−3.46 [m, 13.5H, DMTr-OMe, OCH_2CH_2CN (2 × 0.5H *diast. I*), $OCHHCH_2CN$ (0.5H *diast. II*), $NCH(CH_3)_2$, OCH_2CH_2Br, H-5′], 2.71−2.67 [m, 2 × 0.5H, OCH_2CH_2CN (*diast. II*)], 2.58−2.53 [m, 2 × 0.5H, OCH_2CH_2CN (*diast. I*)], 1.21−1.12 [m, 12 × 0.5H (*diast. I*), 6 × 0.5H (*diast. II*), $NCH(CH_3)_2$], 1.06 [d, 6 × 0.5H, J = 7.0 Hz, $NCH(CH_3)_2$ (*diast. II*)]. ^{31}P NMR (200 MHz, CD_3CN) δ_{ppm} 148.6 (*diast. I*), 150.5 (*diast. II*). HRMS(ESI): $[MH]^+$ $C_{49}H_{58}BrN_5O_{10}P$ requires 986.3099; found, 986.3078.

2′-*O*-(2-Azidoethoxymethyl)-5′-*O*-(4,4′-dimethoxytrityl)-*N*⁴-benzoylcytidine 3′-(*H*-phosphonate) Triethylammonium Salt (2). Compound **5b** (0.37 g, 0.5 mmol) was dissolved in dry pyridine (2 mL), and diphenyl *H*-phosphonate (0.7 mL, 3.6 mmol) was added. After 45 min, water (0.6 mL) and Et_3N (0.6 mL) were added, and the reaction mixture was stirred for an additional 15 min. After evaporation, the residue was partitioned between DCM (20 mL) and 5% aq $NaHCO_3$ (20 mL). The organic layer was dried with Na_2SO_4, evaporated, and purified by silica gel chromatography (5−10% MeOH in DCM + 0.1% Et_3N), yielding product **2** (0.39 g, 86%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ (1:1)) δ_{ppm} 8.53 (m, 1H, H-6), 7.99−7.94 (m, 2H, Bz), 7.61 (m, 1H, Bz), 7.55−7.49 (m, 2H, Bz), 7.47−7.40 (m, 2H, DMTr), 7.37−7.14 (m, 8.5H, DMTr, H-5, PH (0.5H)), 6.92−6.85 (m, 4H, DMTr), 6.19 (br, 0.5H, PH), 6.09 (s, 1H, H-1′), 5.05 (d, 1H, J = 7.0 Hz, OCHHO), 4.97 (d, 1H, J = 7.0 Hz, OCHHO), 4.92 (m, 1H, H-3′), 4.52 (m, 1H, H-2′), 4.35 (d, 1H, J = 7.0 Hz, H-4′), 3.87−3.76 (m, 8H, DMTr-OMe, $OCH_2CH_2N_3$), 3.64 (d, 1H, J = 11.0 Hz, H-5′a), 3.52 (d, 1H, J = 11.0 Hz, H-5′b), 3.43 (m, 2H, $OCH_2CH_2N_3$), 3.13 (br q, 6H, J = 6.5 Hz, CH_2 of Et_3N), 1.31 (t, 9H, J = 7.3 Hz, CH_3 of Et_3N). ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 1:1) δ_{ppm} 167.3 (Bz-C=O), 163.3 (C-4), 158.8 (DMTr), 156.2 (C-2), 144.8 (C-6), 143.9, 135.4, 135.1 (DMTr), 133.1, 133.0 (Bz), 130.2, 130.1 (DMTr), 128.7 (Bz), 128.3, 127.9, 127.9 (DMTr, Bz), 127.1, 113.2 (DMTr), 97.6 (C-5), 94.9 (OCH_2O), 89.4 (C-1′), 87.2 (DMTr-C_q), 82.0 (C-4′), 78.5 (C-2′), 70.1 (C-3′), 67.1 ($OCH_2CH_2N_3$), 60.9 (C-5′), 54.9 (DMTr-OMe), 50.7 ($OCH_2CH_2N_3$), 46.3 (CH_2 of Et_3N), 8.3 (CH_3 of Et_3N). ^{31}P NMR (200 MHz, $CDCl_3/CD_3OD$, 1:1) δ_{ppm} 2.53. HRMS(ESI): $[M]^-$ $C_{40}H_{40}N_6O_{11}P$ requires 811.2498; found, 811.2488.

Chart 1



Synthesis of 2′-*O*-Me-oligoribonucleotides. Oligonucleotides incorporating **1** and **2** were synthesized in a 1.0 μmol scale using commercial 500 Å CPG-succinyl-2′-*O*-Me-adenosine support. For the synthesizer, a protocol including both the phosphoramidite/tetrazole and *H*-phosphonate/pivaloyl chloride couplings was generated. For the coupling of phosphoramidite block **1** (0.13 mol L^{-1} solution in dry MeCN) and the commercial 2′-*O*-Me-nucleoside phosphoramidites, standard RNA phosphoramidite protocol was applied, with the exception of an extra detritylation step for monomer **1**. For the *H*-phosphonate monomer **2** [35 mmol L^{-1} solution in dry MeCN/pyrine (1:1, v/v)], a previously generated protocol was utilized.[9] Accordingly, the coupling time was 570 s on using pivaloyl chloride at a concentration of 100 mmol L^{-1} in dry MeCN/pyrine (1:1, v/v). An additional detritylation step was used also for monomer **2**. After the oligonucleotide assembly, the support-bound material was removed from the synthesizer, oxidized with iodine (0.1 mol L^{-1}) in wet pyridine (98:2, v/v) at rt for 1 h, washed with pyridine/MeCN/MeOH, and dried in vacuum. The oligonucleotides were released from the support and deprotected with concentrated ammonia (33% aqueous NH_3) at 55 °C overnight.

On-Support Click Conjugation. Alkynyl-functionalized ligands **6**[9] and **7**[31] (Chart 1) were conjugated to the azido groups of the support-bound oligonucleotides as follows. The ligands in MeOH (50 mmol L^{-1}, 15 equiv/azido group) were added onto the support. After that, $CuSO_4$ and tris[(1-benzyl-1,2,3-triazol-4-yl)methyl]amine (TBTA-ligand) were added as a 25 mmol L^{-1}

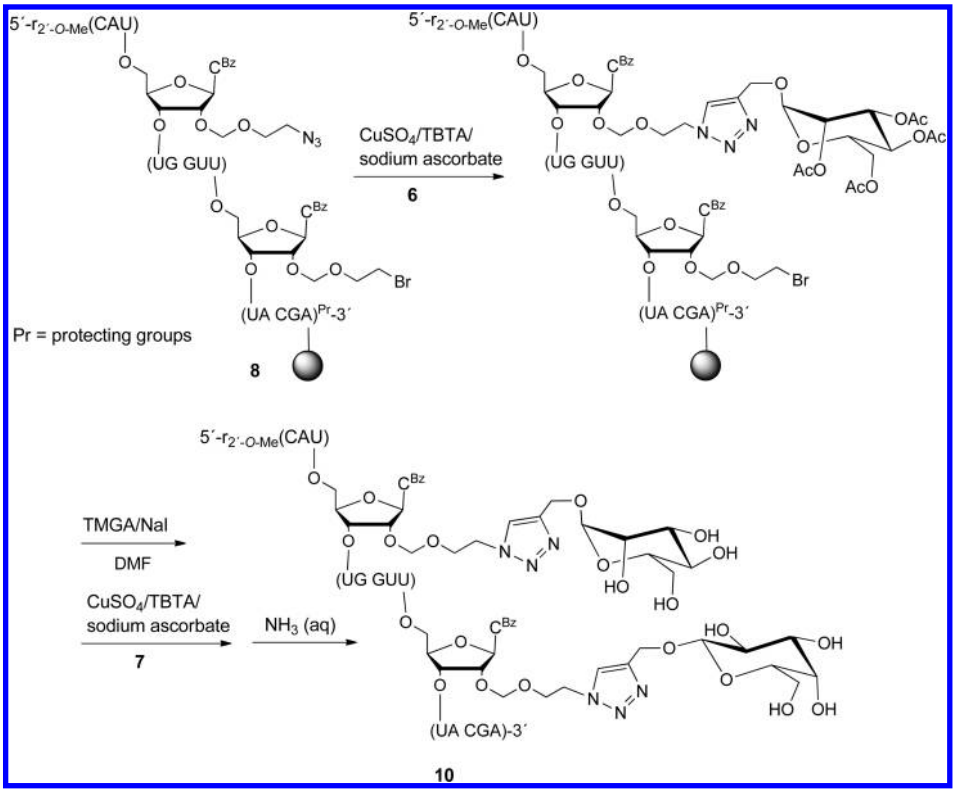


Figure 1. Solid-supported introduction of two different sugar ligands into 2′-positions within a 2′-*O*-Me-oligoribonucleotide (**10**).

solution in a 12:3:1 (v/v/v) mixture of water, DMSO, and 2-butanol (7.5 equiv/azido group). Finally, sodium ascorbate (15 equiv/azido group) as a 0.1 mol L^{-1} aqueous solution was added. The mixture was shaken at room temperature for 5 h. The support was collected by filtration and washed first with water, DMF, and MeOH. To remove the copper salt and TBTA ligand as quantitatively as possible, the support was then treated for 10 min with aqueous EDTA solution (0.1 mol L^{-1}), washed with water, and then for another 10 min with an aqueous solution of neomycin trisulfate (0.1 mol L^{-1}). Finally, the support was washed with water, DMF, DCM, MeOH, and MeCN.

On-Support Azidation. The support-bound oligonucleotides (0.5 μmol) were treated with a solution of tetramethylguanidinium azide (TMGA, 15.8 mg, 200 equiv) and NaI (15.0 mg, 200 equiv) in dry DMF (500 μL) for 1.5 h at 65 °C.[13] The support was collected by filtration and washed with DMF, DCM, water, and MeOH.

Characterization of the Conjugates. The identity of the sugar conjugates **10** and **11** was verified by ESI-MS. Observed masses are calculated from [(M-4H)/4]. ON**10** requires an isotopic mass of 5527.08; found, 5527.15. ON**11** requires an isotopic mass of 5830.19; found, 5830.18.

Melting Temperature Studies. The melting curves (absorbance versus temperature) were measured at 260 nm on a Perkin-Elmer Lambda 35 UV−vis spectrometer equipped with a multiple cell holder and a Peltier temperature controller. The temperature was changed at a rate of 0.5 °C/min (from 15 to 90 °C). The measurements were performed in 10 mmol L^{-1} potassium phosphate buffer (pH 7) containing 0.1 mol L^{-1} or 1.0 mol L^{-1} NaCl. The oligonucleotide conjugates and their cDNA or 2′-*O*-methyl RNA targets were used at a concentration of 2 μmol L^{-1}. T_m values were determined as the maximum of the first derivate of the melting curve.

RESULTS AND DISCUSSION

Synthesis of Monomeric Building Blocks. An *O*-glycosylation approach[30,32] was applied to the preparation of the 2′-*O*-(2-bromoethoxy)methyl (**3a**) and 2′-*O*-(2-azidoethoxy)methyl (**3b**) substituted cytidine monomers. Accordingly, 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-N^4-benzoylcytidine[33,34] was treated with (2-bromoethoxy)methyl acetate in the presence of tin tetrachloride at −12 °C.[30] Nucleoside **3a** was isolated in 88% yield, and conversion to the corresponding 2′-*O*-(2-azidoethoxy) methyl derivative (**3b**) by displacement of the bromo substituent with NaN_3 in DMF was virtually quantitative. The 3′,5′-cyclic silyl protecting group was removed from **3a** and **3b** with Bu_4NF in THF, and the 5′-OH of the deprotected nucleosides (**4a**,**b**) was protected with a 4,4′-dimethoxytrityl group to obtain **5a**,**b**. Finally, **5a** and **5b** were converted to 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (**1**) and 3′-(*H*-phosphonate) (**2**), respectively. Owing to the presence of the azido group, **5b** undergoes a Staudinger reaction upon phosphitylation, which prevents its conversion to a phosphoramidite reagent.[9,35]

The alkynyl-derivatized monosaccharide ligands, viz. propargyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**6**) and propargyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (**7**), used in the click conjugation were prepared from peracetylated α-D-mannopyranose and β-D-galactopyranose by boron trifluoride etherate promoted glycosidation with propargyl alcohol. The compounds exhibited ^1H and ^{13}C NMR chemical shifts identical to those reported earlier.[9,31]

Oligonucleotide Synthesis and Click Conjugation. The appropriately protected phosphoramidite (**1**) and *H*-phosphonate (**2**) building blocks were incorporated into 15-mer 2′-*O*-methyl oligoribonucleotides: 5′-(CAU $\mathbf{C^2}$UG GUU $\mathbf{C^1}$UA

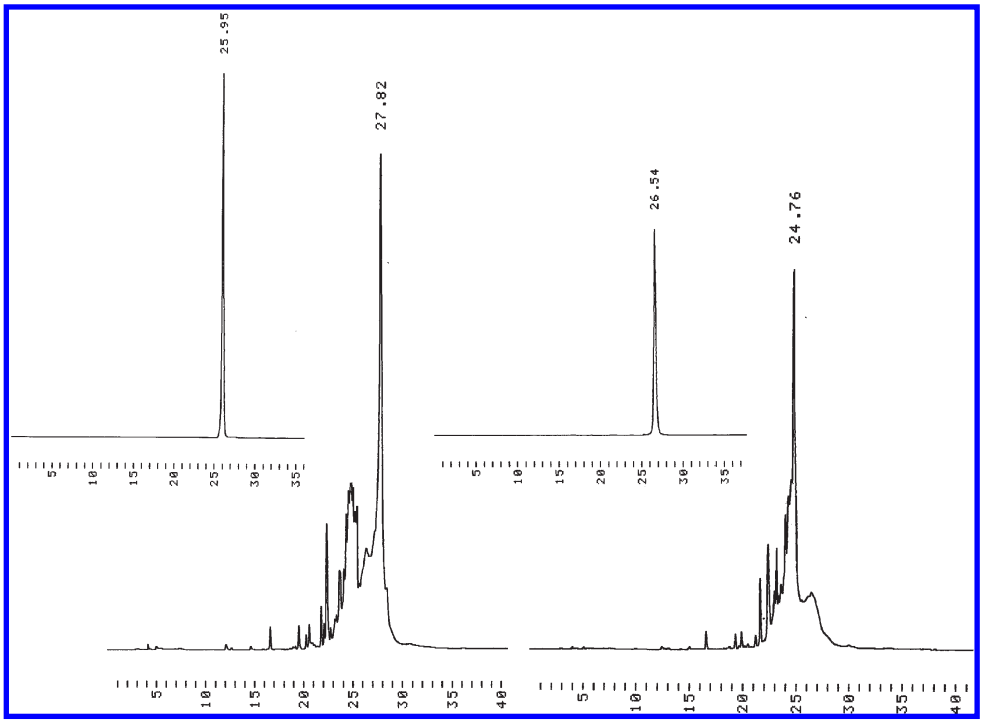


Figure 2. RP HPLC traces of the crude and purified oligonucleotide glycoconjugates **10** and **11**, respectively. For the chromatographic conditions, see the Experimental Procedures Section.

Table 1. Melting Temperatures for the Duplexes of Oligonucleotide Conjugates 10 and 11 with Their Complementary DNA and 2′-*O*-Methyl-RNA Sequences[a]

	compl. DNA T_m/°C and (ΔT_m/°C)		compl. 2′-*O*-methyl RNA T_m/°C and (ΔT_m/°C)
	0.1 mol L^{-1} NaCl	1.0 mol L^{-1} NaCl	0.1 mol L^{-1} NaCl
5′-CAU CUG GUU CUA CGA	50.1	61.7	72.0
5′-CAU *X*UG GUU *Y*UA CGA (**10**)	50.6 (+0.5)	60.6 (−1.1)	71.5 (−0.5)
5′-CAU *Y*UG GUU *X*UA *Y*GA (**11**)	47.0 (−3.1)	60.2 (−1.5)	68.2 (−3.8)

[a] ***X*** = monomer C^2 conjugated with **6**; ***Y*** = monomer C^1 conjugated with **7**.

CGA)-3′ (**8**) and 5′-(CAU $\mathbf{C^1}$UG GUU $\mathbf{C^2}$UA $\mathbf{C^1}$GA)-3′ (**9**) ($\mathbf{C^1}$ and $\mathbf{C^2}$ refer to nucleosidic units derived from **1** and **2**, respectively). The oligomers were assembled by phosphoramidite chemistry, with the exception of the insertion of **2**, which was carried out by pivaloyl chloride promoted *H*-phosphonate coupling. As also shown previously, the presence of an azido group in the growing solid-supported oligonucleotide chain does not disturb the phosphoramidite coupling of subsequent monomers.[9] The coupling yield of building block **1** was ~99%, but the *H*-phosphonate block **2** was coupled in only 85% yield.

After completion of the chain assembly, oligonucleotides **8** and **9** were conjugated with the two alkynyl-functionalized monosaccharide ligands (**6** and **7**) (Figure 1). The azido group of monomeric unit **2** was first subjected to Cu(I) promoted click reaction with propargyl α-D-mannopyanoside tetraacetate (**6**), using a mixture of $CuSO_4$ and sodium arcorbate in the presense of tris[(1-benzyl-1,2,3-triazol-4-yl)methyl]amine (TBTA-ligand) as the source of Cu(I) ion (5 h, r.t.). The bromo substituent of monomeric unit **1** was then displaced with an azide ion, as reported previously by Pourceau et al.[13,14] Both tetramethylguanidinium azide[36] (TMGA/NaI/DMF, 65 °C) and less soluble NaN_3 (NaN_3/NaI/DMF, 65 °C) were used as a source of azide ions. The second click reaction was then performed using propargyl β-D-galactopyranoside tetraacetate (**7**) as the alkyne component. The oligonucleotide conjugates obtained (**10** and **11**) were deprotected and released from the support by standard ammonolysis (33% aq NH_3, 55 °C, overnight). The crude products were purified by RP-HPLC, and their identity was verified by ESI-MS. Figure 2 shows the HPLC traces of the crude conjugates and the homogeneity of the products after purification.

Melting Temperature Studies. It has been shown previously that oligonucleotides bearing a β-D-ribofuranosyl group at an intrachain 2′-O form stable complexes with RNA (ΔT_m = 0 °C) and maintain A-type helical geometry with the additional 2′-*O*-ribose moiety located in the minor groove.[2,24] Consistent with these findings, the sugar ligands attached in the present study to 2′-*O* via a [2-(4-hydroxymethyl-1*H*-1,2,3-triazol-1-yl)ethoxy]-methyl linker only slightly destabilize the duplex. Table 1 records the melting temperatures (T_m) for the duplexes of oligonucleotide conjugates **10** and **11** with the full cDNA and 2′-*O*-methyl RNA sequences at pH 7.

The saccharide ligands accommodated quite well in the duplex structure. Oligonucleotide **10** bearing two saccharide units in an

intrachain position exhibited even a slight stabilization of the duplex with the DNA target at a low ionic strength (ΔT_m = +0.5 °C) and a slight destabilization at a high ionic strength (ΔT_m = −1.1 °C). Addition of a third saccharide ligand to obtain conjugate **11** resulted in a moderate destabilization even at a low ionic strength (ΔT_m = −3.1). At high ionic strength, the destabilization was less prominent (ΔT_m = −1.5 °C). With the 2′-*O*-Me RNA target, two saccharide ligands within conjugate **10** were similarly well tolerated (ΔT_m = −0.5 °C), but with **11**, containing three saccharides, T_m again dropped significantly (ΔT_m = −3.8 °C). A tentative explanation for the marked destabilizing influence of the third saccharide, especially at low ionic strength, is that two of the sugars are rather close to each other within conjugate **11**, viz. in positions 10 and 13.

■ CONCLUSIONS

A convenient and reliable method for solid-phase introduction of two different types of saccharide ligands in desired positions of an oligonucleotide has been developed. The underlying principle is regiospecific introduction of azido- and bromo-functionalized building blocks into the oligonucleotide upon chain assembly and their subsequent derivatization on the support. A propynyl-functionalized saccharide is first attached to the azido groups by Cu(I) promoted 1,3-dipolar cycloaddition, and the same reaction is utilized to conjugate the second propynyl saccharide after conversion of the bromo substituents to azido groups by displacement with the azide ion. For the chain assembly, conventional phosphoramidite chemistry may be applied, except for the introduction of the azido functionalized building blocks that have to be coupled as *H*-phosphonates. The saccharide ligands are quite well accommodated to the duplexes formed with both DNA and 2′-*O*-Me RNA targets

■ ASSOCIATED CONTENT

Supporting Information. ESI-MS data and melting temperature curves for oligonucleotides **10** and **11**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(A.K.) Fax: +35823336700. E-mail: anu.kiviniemi@utu.fi. (S.N.M.) Fax: +074991359751. E-mail: smikh@eimb.ru.

■ ACKNOWLEDGMENT

Financial support from the Russian Foundation for Basic Research, Russian Academy of Sciences (Program "Molecular and Cell Biology"), and the Academy of Finland is gratefully acknowledged.

■ REFERENCES

(1) Manoharan, M. (1999) 2′-Carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. *Biochim. Biophys. Acta 1489*, 117–130.

(2) Luyten, I., Esnouf, R. M., Mikhailov, S. N., Efimtseva, E. V., Michiels, P., Heus, H. A., Hilbers, C. W., and Herdewijn, P. (2000) Solution structure of a RNA decamer duplex, containing 9-[(2-*O*-(β-D-ribofuranosyl)-(β-D-ribofuranosyl]adenine, a special residue in lower eukaryotic initiator tRNAs. *Helv. Chim. Acta 83*, 1278–1289.

(3) Zatsepin, T. S., Romanova, E. A., and Oretskaya, T. S. (2004) Nucleosides and oligonucleotides containing 2′-reactive groups: synthesis and applications. *Russ. Chem. Rev. 73*, 701–733.

(4) Ugarte-Uribe, B., Pérez-Rentero, S., Lucas, R., Aviñó, A., Reina, J. J., Alkorta, I., Eritja, R., and Morales, J. C. (2010) Synthesis, cell-surface binding, and cellular uptake of fluorescently labeled glucose-DNA conjugates with different carbohydrate presentation. *Bioconjugate Chem. 21*, 1280–1287.

(5) Zhu, L., and Mahato, R. I. (2010) Targeted delivery of siRNA to hepatocytes and hepatic stellate cells by bioconjugation. *Bioconjugate Chem. 21*, 2119–2127.

(6) Lönnberg, H. (2009) Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. *Bioconjugate Chem. 20*, 1065–1094.

(7) Zatsepin, T. S., and Oretskaya, T. S. (2004) Synthesis and applications of oligonucleotide-carbohydrate conjugates. *Chem. Biodiversity 1*, 1401–1417.

(8) Yan, H., and Tram, K. (2007) Glycotargeting to improve cellular delivery efficiency of nucleic acids. *Glycoconjugate J. 24*, 107–123.

(9) Kiviniemi, A., Virta, P., and Lönnberg, H. (2008) Utilization of intrachain 4′-*C*-azidomethylthymidine for preparation of oligodeoxyribonucleotide conjugates by click chemistry in solution and on a solid support. *Bioconjugate Chem. 19*, 1726–1734.

(10) Kiviniemi, A., Virta, P., and Lönnberg, H. (2010) Solid-supported synthesis and click conjugation of 4′-*C*-alkyne functionalized oligodeoxyribonucleotides. *Bioconjugate Chem. 21*, 1890–1901.

(11) Chevolot, Y., Bouillon, C., Vidal, S., Morvan, F., Meyer, A., Cloarec, J.-P., Jochum, A., Praly, J.-P., Vasseur, J.-J., and Souteyrand, E. (2007) DNA-based carbohydrate biochips: a platform for surface glycol-engineering. *Angew. Chem., Int. Ed. 46*, 2398–2402.

(12) Bouillon, C., Meyer, A., Vidal, S., Jochum, A., Chevolot, Y., Cloarec, J.-P., Praly, J.-P., Vasseur, J.-J., and Morvan, F. (2006) Microwave assisted click chemistry for the synthesis of multiple labeled-carbohydrate oligonucleotides on solid support. *J. Org. Chem. 71*, 4700–4702.

(13) Pourceau, G., Meyer, A., Chevolot, Y., Souteyrand, E., Vasseur, J.-J., and Morvan, F. (2010) Oligonucleotide carbohydrate-centered galactosyl cluster conjugates synthesized by click and phosphoramidite chemistries. *Bioconjugate Chem. 21*, 1520–1529.

(14) Pourceau, G., Meyer, A., Vasseur, J.-J., and Morvan, F. (2009) Synthesis of mannose and galactose oligonucleotide conjugates by bi-click chemistry. *J. Org. Chem. 74*, 1218–1222.

(15) Ketomäki, K., and Virta, P. (2008) Synthesis of aminoglycoside conjugates of 2′-*O*-methyl oligoribonucleotides. *Bioconjugate Chem. 19*, 766–777.

(16) Morvan, F., Meyer, A., Jochum, A., Sabin, C., Chevolot, Y., Imberty, A., Praly, J.-P., Vasseur, J.-J., Souteyrand, E., and Vidal, S. (2007) Fucosylated pentaerythrityl phosphodiester oligomers (PePOs): automated synthesis of DNA-based glycoclusters and binding to *Pseudomonas aeruginosa* lectin (PA-IIL). *Bioconjugate Chem. 18*, 1637–1643.

(17) Karskela, M., Virta, P., Malinen, M., Urtti, A., and Lönnberg, H. (2008) Synthesis and cellular uptake of fluorescently labeled multivalent hyaluronan disaccharide conjugates of oligonucleotide phosphorothioates. *Bioconjugate Chem. 19*, 2549–2558.

(18) Katajisto, J., Virta, P., and Lönnberg, H. (2004) Solid-phase synthesis of multiantennary oligonucleotide glycoconjugates utilizing on-support oximation. *Bioconjugate Chem. 15*, 890–896.

(19) Yamada, T., Peng, C. G., Matsuda, S., Addepalli, H., Jayaprakash, K. N., Alam, M. R., Mills, K., Maier, M. A., Charisse, K., Sekine, M., Manoharan, M., and Rajeev, K. G. (2011) Versatile site-specific conjugation of small molecules to siRNA using click chemistry. *J. Org. Chem. 76*, 1198–1211.

(20) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596–2599.

(21) Tornoe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific

copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057–3064.

(22) Mikhailov, S. N., Efimtseva, E. V., Gurskaya, G. V., Zavodnik, V. E., De Bruyn, A., Rozenski, J., and Herdewijn, P. (1997) An efficient synthesis and physico-chemical properties of 2′-*O*-*β*-D-ribofuranosyl-nucleosides, minor tRNA components. *J. Carbohydr. Chem. 16*, 75–92.

(23) Efimtseva, E. V., Kulikova, I. V., and Mikhailov, S. N. (2007) Disaccharide nucleosides and their incorporation into oligonucleotides. *Curr. Org. Chem. 11*, 337–354.

(24) Efimtseva, E. V., Bobkov, G. V., Mikhailov, S. N., Van Aerschot, A., Schepers, G., Busson, R., Rozenski, J., and Herdewijn, P. (2001) Oligonucleotides containing disaccharide nucleosides. *Helv. Chim. Acta 84*, 2387–2397.

(25) Mikhailov, S. N., Efimtseva, E. V., Rodionov, A. A., Shelkunova, A. A., Rozenski, J., Emmerechts, G., Schepers, G., Van Aerschot, A., and Herdewijn, P. (2005) Synthesis of RNA containing *O*-*β*-D-ribofuranosyl-(1′′-2′)-adenosine-5′′-phosphate and 1-methyladenosine, minor components of tRNA. *Chem. Biodiversity 2*, 1153–1163.

(26) Andreeva, O. I., Golubeva, A. S., Kochetkov, S. N., Van Aerschot A., Herdewijn, P., Efimtseva, E. V., Ermolinsky, B. S., and Mikhailov, S. N. (2002) An additional 2′-ribofuranose residue at a specific position of DNA primer prevents its elongation by HIV-1 reverse trancriptase. *Bioorg. Med. Chem. Lett. 12*, 681–684.

(27) Tunitskaya, V. L., Rusakova, E. E., Memelova, L. V., Kochetkov, S. N., Van Aerschot, A., Herdewijn, P., Efimtseva, E. V., Ermolinsky, B. S., and Mikhailov, S. N. (1999) The mapping of T7 RNA polymerase active site with novel reagents - oligonucleotides with reactive dialdehyde groups. *FEBS Lett. 442*, 20–24.

(28) Efimtseva, E. V., and Mikhailov, S. N. (2002) Disaccharide nucleosides and oligonucleotides on their bases. New tools for the study of enzymes of nucleic acid metabolism. *Rev. Biochem. (Moscow) 67*, 1136–1144.

(29) Gritsenko, O. M., Koudan, E. V., Mikhailov, S. N., Ermolinsky, B. S., Van Aerschot, A., Herdewijn, P., and Gromova, E. S. (2002) Affinity modification of *Eco*RII DNA methyltransferase by the dialdehyde-substituted DNA duplexes: mapping the enzyme region that interacts with DNA. *Nucleosides, Nucleotides Nucleic Acids 21*, 753–764.

(30) Bobkov, G. V., Mikhailov, S. N., Van Aerschot, A., and Herdewijn, P. (2008) Phosphoramidite building blocks for efficient incorporation of 2′-O-aminoethoxy(and propoxy)methyl nucleosides into oligonucleotides. *Tetrahedron 64*, 6238–6251.

(31) Mereyala, H. B., and Gurrala, S. R. (1998) A highly diastereoselective, practical synthesis of allyl, propargyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-gluco, *β*-D-galactopyranosides and allyl, propargyl heptaacetyl-*β*-D-lactosides. *Carbohydr. Res. 307*, 351–354.

(32) Efimtseva, E. V., Kulikova, I. V., and Mikhailov, S. N. (2009) Disaccharide nucleosides as an important group of natural compounds. *Mol. Biol. (Russia) 43*, 301–312.

(33) Markiewicz, W. T. (1979) Tetraisopropyldisiloxane-1,3-diyl group for simultaneous protection of 3′- and 5′-hydroxy functions of nucleosides. *J. Chem. Res. (S)*, 24–25.

(34) Bobkov, G. V., Brilliantov, K. V., Mikhailov, S. N., Rozenski, J., Van Aerschot, A., and Herdewijn, P. (2006) Synthesis of oligoribonucleotides containing pyrimidine 2′-O-[(hydroxyalkoxy)methyl]-ribonucleosides. *Collect. Czech. Chem. Commun. 71*, 804–819.

(35) Coppola, C., Simeone, L., De Napoli, L., and Montesarchio, D. (2011) On the compatibility of azides in phosphoramidite-based couplings: synthesis of a novel, convertible azido-functionalized Cy-PLOS analogue. *Eur. J. Org. Chem. 6*, 1155–1165.

(36) Papa, A. J. (1966) Synthesis and azidolysis of 2-chlorotetramethylguanidine. Synthetic utility of hexa- and tetramethylguanidinium azide. *J. Org. Chem. 31*, 1426–1430.