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Solid-Phase Synthesis of Oligonucleotide Glycoconjugates Bearing Three Different Glycosyl Groups: Orthogonally Protected Bis(hydroxymethyl)-*N*,*N*′-bis(3-hydroxypropyl)malondiamide Phosphoramidite as Key Building Block

Johanna Katajisto,* Petri Heinonen, and Harri Lönnberg

Department of Chemistry, University of Turku, FIN-20014 Turku, Finland

jokrka@utu.fi

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Diethyl *O*,*O*′-(methoxymethylene)bis(hydroxymethyl)malonate (**3**) was observed to undergo a stepwise aminolysis when treated with 3-aminopropanol. This allowed convenient preparation of bis(hydroxymethyl)-*N*,*N*′-bis(3-hydroxypropyl)malondiamide bearing orthogonal levulinyl (Lev) and *tert*-butyldiphenylsilyl (TBDPS) protections at the two *N*-hydroxypropyl groups (**8**). One of the hydroxylmethyl functions was then protected with a 4,4′-dimethoxytrityl (DMTr) group, and the other one was phosphitylated to obtain a methyl *N*,*N*-diisopropylphosphoramidite (**1**). This building block was used for the synthesis of oligonucleotide glycoconjugates (**25** and **26**) carrying three different sugar units. After conventional phosphoramidite chain assembly of the sequence containing **1**, the 5′-terminal DMTr group was removed and an appropriate glycosyl 6-*O*-phosphoramidite was coupled. The remaining protections of the branching unit were removed in the order of Lev and TBDPS, and the exposed hydroxyl functions were reacted one after another with the desired glycosyl 6-*O*-phosphoramidites. Global deprotection and cleavage of the conjugate from the support were achieved by conventional ammonolysis.

Introduction

A number of important biological systems are known to interact through multiple simultaneous carbohydrate−protein binding events.[1] This kind of multipoint attachment to cell-surface lectins through several sugar ligands covalently tethered to a natural scaffold, such as a protein or lipid, ensures high affinity binding and provides the molecule with cell- or tissue-specificity.[2−6] Several multiantennary glycoclusters,[7] glycodendrimers,[8] glycopeptides,[9] cyclodextrin-based glycoclusters,[10] and glycopolymers[11] have been synthesized for this purpose. Most of them contain a single type of sugar unit attached to a polyfunctional scaffold. Many biological recognition processes, however, rely on binding of several different types of sugar moieties. Suprisingly, only few syntheses of such diverse glycoconjugates have been reported. In fact, the examples available are limited to the use of orthogonally protected sugar-[12] and pentaerythritol-based[13] templates.

Covalent conjugation of carbohydrates to oligonucleotides has also gained increasing interest during recent years as it offers a convenient way to enhance binding of antisense oligonucleotides[14] to a certain cell type and thus improve their cellular intake by endocytosis. For these purposes, noncovalent carriers and covalent con-

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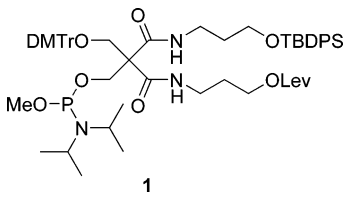
jugates have been prepared. The methods described include electrostatic complexation of an oligonucleotide with glycosylated polylysine;[15] chemical ligation to galactosylated polylysine,[16] to a cholane scaffold,[17] and to a neoglycopeptide;[18] on-support glycosylation with trichloroacetimidates[19] or unprotected carbohydrates;[20] and the use of base moiety glycosylated nucleoside phosphoramidites[21] and glycoside phosphoramidites[22] as building blocks for the chain assembly. Some recent approaches allow creation of structural diversity in the terms of the number of sugar ligands and the distance between them. These methods utilize pentaerythritol-based building blocks as a branching unit either at the 5'-terminus[23] or at an intrachain position.[24] The sugar units are coupled to these polyvalent scaffolds as phosphoramidites[23] or as aldehydes via oxime bond formation with a solid-supported aminooxy group,[24] respectively. Phosphoramidite building blocks bearing orthogonally protected functional groups may be expected to allow attachment of different types of sugar moieties in a controlled manner and, hence, the synthesis of more complex glyco-oligonucleotide conjugates. We now report on a facile method of preparation of this kind of diverse oligonucleotide glycoconjugates. A phosphoramidite derived from bis(hydroxymethyl)-*N*,*N*′-bis(3-hydroxypropyl)malondiamide that contains three orthogonally protected hydroxyl groups (**1**) and is compatible with normal chain assembly has been used as a key building block (Figure 1). Stepwise removal of the 4,4′-dimethoxytrityl (DMTr), levulinyl (Lev), and *tert*-butyldiphenylsilyl (TBDPS) protections followed by coupling of a glycosyl-derived phosphoramidite to the exposed hydroxyl group then gives the desired oligonucleotide glycoconjugate. The applicability of this approach has been demonstrated by preparing both T_6 and a heterosequence bearing three different sugar units, viz., mannose, galactose, and glucose.



1

FIGURE 1.

Results and Discussion

Synthesis of Bis(hydroxymethyl)-*N*,*N*′-bis(3-hydroxypropyl)malondiamide Phosphoramidite 1 (Scheme 1). The orthogonally protected building block **1** was synthesized starting from commercially available diethyl 2,2-bis(hydroxymethyl)malonate (**2**). Direct aminolysis of **2** or its DMTr-ether is precluded, owing to their tendency to rapidly release formaldehyde by retro-aldol condensation in the presence of primary amines.[25] Accordingly, **2** was first converted to its *O*,*O*′-methoxymethylene derivative (**3**). The next step, selective aminolysis of one of the ethyl ester functions in an excess of 3-aminopropanol in THF was the crucial step of the synthesis. A 48-h treatment at room temperature afforded exclusively monoamide **4** in a 36% yield. The remaining unreacted starting material (64%) could be easily recycled, which favorably compensated for the relatively low yield of this step. The reaction was found to be highly dependent on the conditions used. When the reaction was performed in the absence of THF, when aprotic solvents such as dichloromethane (DCM) or dichloroethane were used as cosolvents, or when the reaction mixture was heated to reflux, a mixture of mono- and dialcohol products were obtained. The free hydroxy function was then protected with a TBDPS group to afford **5**, and the remaining ester group was reacted with 3-aminopropanol (48 h at 50 °C) to yield **6**. Protection of the hydroxy function with a levulinyl group to obtain **7** was followed by acid-catalyzed hydrolysis of the ortho ester protection, yielding **8**. Selective protection of one of the hydroxymethyl groups of **8** as a DMTr ether and standard phosphitylation of the remaining hydroxyl function with methyl *N*,*N*-diisopropylphosphoramidochloridite accomplished the synthesis of **1**.

Synthesis of Glycosyl Phosphoramidites (19−21). The fully protected glycosyl phosphoramidites (**19**−**21**) were obtained easily from commercially available methyl α-D-glycopyranosides (galactose (**10**), mannose (**11**), and glucose (**12**)) via a three-step procedure (Table 1). Methyl α-D-glycopyranosides **10**−**12** were first subjected to two sequential reactions in one pot. The trityl (Tr) protecting group was introduced at the primary 6-*O*-position,[26] and the secondary hydroxyl groups were subsequently esterified with *p*-toluoyl chloride (**13**−**15**).[22b] The trityl protection was then removed with iodine in methanol (**16**−

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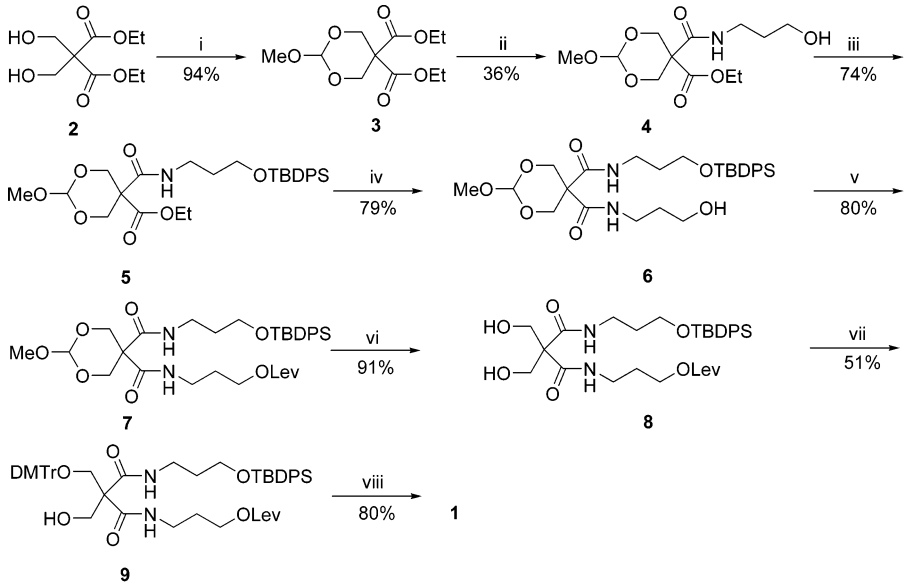
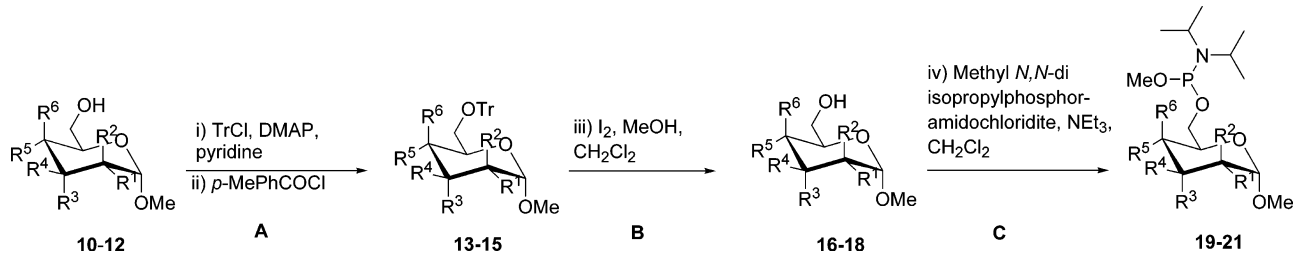
SCHEME 1[a]



[a] Conditions: (i) $(MeO)_3CH$, TsOH; (ii) 3-aminopropanol, THF; (iii) TBDPSCl, imidazole, DMF; (iv) 3-aminopropanol; (v) levulinic acid, DCC, DMAP, pyridine, dioxane; (vi) 80% AcOH; (vii) DMTrCl, pyridine; (viii) methyl *N*,*N*-diisopropylphosphoramidochloridite, NEt_3, dichloromethane.

TABLE 1. Synthesis of Glycosyl Phosphoramidites 19−21



starting material	products	step **A**[a]	step **B**[a]	step **C**[a]
10: $R^1 = R^4 = R^6 = OH$; $R^2 = R^3 = R^5 = H$	**13**, **16**, **19**: $R^1 = R^4 = R^6 = OTol$; $R^2 = R^3 = R^5 = H$	**13**: 86%	**16**: 70%	**19**: 82%
11: $R^2 = R^4 = R^5 = OH$; $R^1 = R^3 = R^6 = H$	**14**, **17**, **20**: $R^2 = R^4 = R^5 = OTol$; $R^1 = R^3 = R^6 = H$	**14**: 89%	**17**: 95%	**20**: 74%
12: $R^1 = R^4 = R^5 = OH$; $R^2 = R^3 = R^6 = H$	**15**, **18**, **21**: $R^1 = R^4 = R^5 = OTol$; $R^2 = R^3 = R^6 = H$	**15**:95%	**18**: 74%	**21**: 81%

[a] Yields were determined after purification by silica gel chromatography.

18),[27] and the exposed hydroxyl function was phosphitylated with methyl *N*,*N*-diisopropylphosphonamidic chloride to afford the desired fully protected glycosyl phosphoramidites **19**−**21**.

Synthesis of Oligonucleotide Glycoconjugates (25, 26). The compatibility of phosphoramidite **1** with the conventional oligonucleotide synthesis by the phosphoramidite strategy was first examined by assembling a T_6 sequence containing an internally linked **1** (**22** in Scheme 2). A prolonged coupling time (600 s) was applied to the nonnucleosidic building block **1**. Nucleoside-3′-yl methyl *N*,*N*-diisopropylphosphoramidites were used instead of the more common 2-cyanoethyl phosphoramidites, because it has been previously shown that the latter are not compatible with fluoride reagents required for removal of the TBDPS protection.[28] Otherwise the recommended protocols were employed. On the basis of the DMT-cation assay, an acceptable 96% coupling efficiency was obtained for **1**.[29] Next, the free 5′-terminal hydroxy group of the solid-supported conjugate **22** was phosphitylated with galactosyl 6-*O*-phosphoramidite (**19**) using a prolonged coupling time of 600 s to afford **23**. The levulinyl protection was then removed by 30 min treatment with 0.5 mol L^{-1} hydrazinium acetate, and mannosyl 6-*O*-phosphoramidite (**20**) was introduced to give **24**. In this case, the coupling was somewhat retarded compared to the coupling to the 5′-terminal hydroxyl function, in all likehood owing to the steric hindrance caused by the bulky TBDPS group. Accordingly, a longer coupling procedure consisting of three successive injections of the mixture of the 6-*O*-phosphoramidite (**20**) and activator (3 × 600 s) was applied. At this state, a small aliquot of the solid support was withdrawn from the synthesis column, and the completion of the last coupling step and the formation of the desired conjugate **24** was

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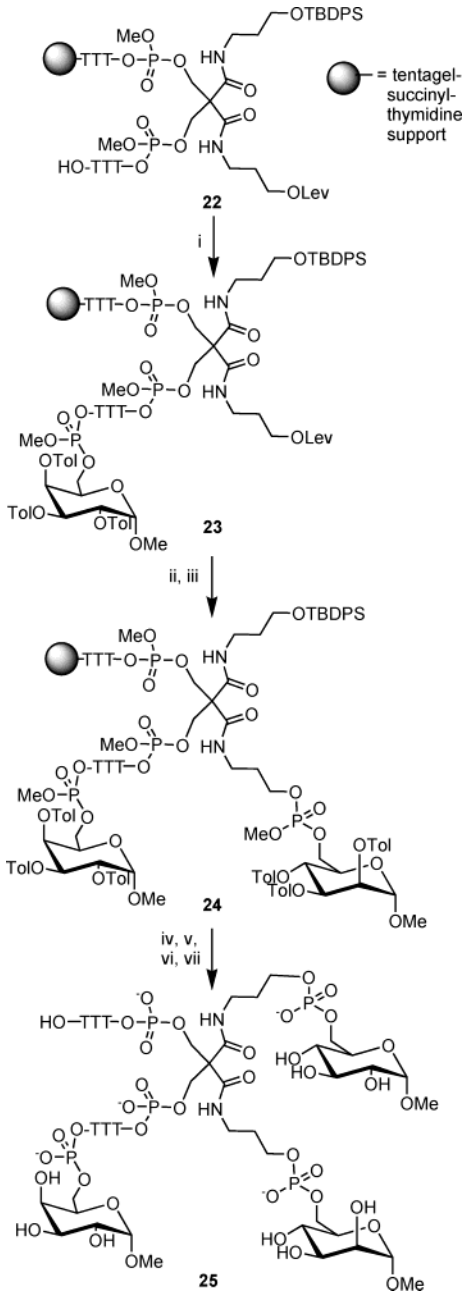
SCHEME 2[a]



[a] Conditions: (i) **19** in MeCN; (ii) hydrazinium hydrate, pyridine, AcOH (0.124/4/1); (iii) **20** in MeCN; (iv) (TEA, 3HF), THF; (v) **21** in MeCN; (vi) 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate (S_2Na_2) in DMF; (vii) aq NH_3.

verified by RP HPLC and ESI-MS analysis after deprotection and cleavage from the solid support (for the RP HPLC chromatogram of the crude product, see Supporting Information). The next step, removal of the TBDPS protection by using triethylamine trihydrofluoride,[30] turned out to be somewhat problematic, because quantitative cleavage of the TBDPS group could not be achieved despite several attemps to optimize the reaction conditions. The best results were obtained by repeating an 18-h treatment with triethylamine trihydrofluoride in THF, yielding the deprotected conjugate in 75% yield (based on the relative peak areas of the protected and deprotected conjugate in the HPLC chromatogram; for the RP HPLC chromatogram, see Supporting Information). Attempts to replace THF with solvents such as DCM, water, or MeCN were unsuccessful, as was also elevation of the reaction temperature. Finally, glucosyl 6-*O*-phosphoramidite (**21**) was coupled applying again a prolonged reaction time of 600 s. Cleavage of the methyl protections from the phosphate groups[31] and subsequent standard ammonolytic treatment (33% aqueous NH_3, 7 h at 55 °C) released the desired crude oligonucleotide glycoconjugate **25** in solution. No side reactions were detected upon the ammonolytic deprotection. The main product (**25**) was easily separated by RP HPLC from the hydrophobic diglycosylated side product bearing still the TBDPS protection (Table 2; entry 1, for the RP HPLC chromatogram of purified **25**, see Supporting Information). The identity of conjugate **25** was verified by ESI-MS (Table 2; entry 1)

TABLE 2. Properties of Oligonucleotide Glycoconjugates

entry	oligonucleotide glycoconjugate	retention time (min)[a]	obsd mass	calcd mass	yield (%)[b]
1	**25**	8.32	2872.0	2871.0	52
2	**26**	11.5	5032.7	5032.6	50

[a] On a ThermoHypersil C-18 column (4.6 mm × 150 mm, 5 μm), a linear gradient from 0 to 100% B in 30 min, flow rate 1 mL min^{-1}. Buffer A: 0.05 mol L^{-1} NH_4OAc (aq). Buffer B: A containing 65% MeCN. [b] Percentage of the conjugate in the crude reaction mixture on the basis of HPLC signal areas.

The applicability of the procedure to the construction of diverse oligonucleotide glycoconjugates was verified by preparing an 11-mer heterosequence (**26**) carrying three different sugar units essentially by the protocol described above. The branching unit (**1**) was coupled to the 5′-terminus of the solid-supported 11-mer; the protecting groups were removed in the order DMTr, Lev, TBDPS; and the exposed hydroxyl groups were phosphitylated with **19**, **20**, and **21**, respectively. The hydroxymethyl branch was, however, elongated with one T before the introduction of **19**. Interestingly, a better 87% yield was now obtained for the TBDPS deprotection step. Figure 2 shows the analytical RP HPLC chromatogram of the crude product mixture of **26**. After RP HPLC purification (for the RP HPLC chromatogram of purified **26**, see Supporting Information), the authenticity of the desired diverse oligonucleotide glycoconjugate was confirmed by ESI-MS (Table 2; entry 2).

Melting temperature (T_m) measurements showed that although the presence of a bulky glycocluster at the 5′-terminus of an 11-mer heterosequence retarded the hybridization, a reasonably stable duplex with a fully complementary 11-mer deoxyribo-oligonucleotide was still formed. The melting temperature of this duplex was 41 °C, when the concentration of **26** and its complementary 11-mer was 2 μmol L^{-1}. Omission of the glycosylated non-nucleosidic moiety from **26** increased the melting point to 48 °C.

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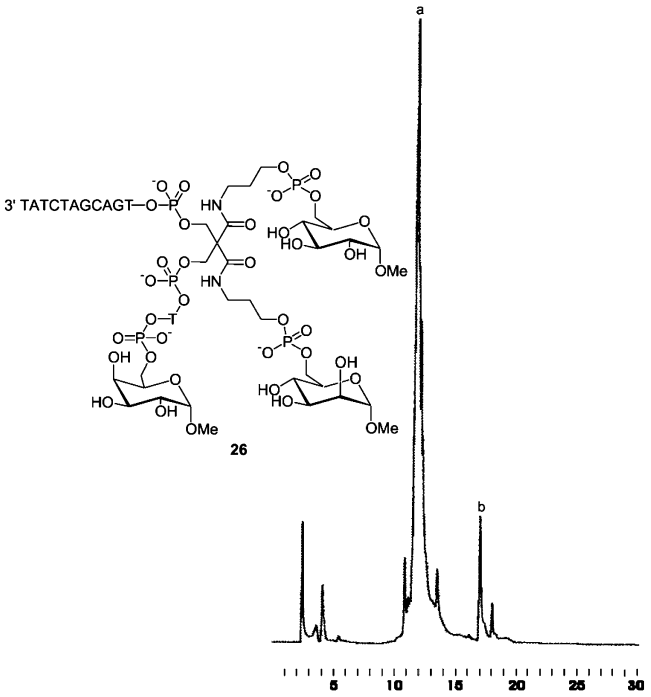

FIGURE 2. RP HPLC profile of crude **26** at 260 nm. Notation: (a) conjugate **26**, t_R = 11.5 min; (b) diglycosylated side product carrying the TBDPS protection, t_R = 16.9 min. For detailed chromatographic conditions, see footnote *a* in Table 2.

In summary, a protocol for the attachment of three different glycosyl phosphoramidites to the 5′-terminus of an oligonucleotide on a solid support has been developed. Alternatively, two different sugar moieties may be tethered to any site within a given oligonucleotide sequence and the third one to the 5′-terminus. Although the conjugates have to be subjected to conventional HPLC purification, owing to the incomplete removal of the TBDPS group, these methods allow quite convenient synthesis of combinatorial libraries.

Experimental Section

General Methods and Materials. See Supporting Information for detailed information. The NMR spectra were recorded at 200, 400, or 500 MHz in deuteriochloroform. The chemical shifts are given in ppm from internal TMS, and the coupling contants are reported in hertz. The mass spectra were recorded using EI or ESI ionization methods.

Diethyl 2-Methoxy-1,3-dioxane-5,5-dicarboxylate (3). Compound **3** was prepared according to the procedure reported previously.[24] The product was obtained as a clear oil in 94% yield. The ^{1}H and ^{13}C NMR spectra were in accordance with the published data. HRMS (ESI) [M + Na]$^+$ calcd 285.0945, obsd 285.0950.

Ethyl 2-Methoxy-5-(3-hydroxypropylcarbamoyl)-1,3-dioxane-5-carboxylate (4). Compound **3** (10.0 g, 38.1 mmol) and 3-aminopropanol (19.3 mL, 229 mmol) were dissolved in dry THF (30 mL), and the mixture was stirred at ambient temperature for 48 h. The solvent was removed in vacuo, and the residue was dissolved in DCM, washed with water, dried with Na_2SO_4, and evaporated to dryness. The oily residue was purified by silica gel chromatrography (3% to 5% MeOH in DCM), yielding compound **4** as a clear oil in a 36% yield (4.0 g): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.33, 5.31, 5.29, 5.23 (four s, total 1H), 4.45 (m, 2H), 4.08−4.30 (m, 5H), 3.65 (m, 2H), 3.43−3.51 (m, 2H), 3.42, 3.39 (two s, total 3H), 1.71 (m, 2H), 1.29 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.3, 169.0, 168.4, 168.2, 110.0, 108.6, 64.3, 62.8, 62.6, 62.3, 62.0, 59.5, 59.3, 53.2, 53.0, 52.1, 51.7, 36.7, 36.6, 32.0, 31.8, 13.9; HRMS (ESI) [M + Na]$^+$ calcd 314.1210, obsd 314.1227.

Ethyl 2-Methoxy-5-[3-(*tert*-butyldiphenylsilyloxy)-propylcarbamoyl]-1,3-dioxane-5-carboxylate (5). Compound **4** (2.60 g, 8.90 mmol) was coevaporated twice with dry MeCN. The residue was dissolved in dry DMF (30 mL) together with imidazole (1.80 g, 26.8 mmol), and *tert*-butyldiphenylsilyl chloride (2.74 mL, 10.7 mmol) was slowly added. After 16 h of treatment, the consumption of **4** was complete according to TLC (3% MeOH in DCM). Diethyl ether was added, and the mixture was washed with water and aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4 and evaporated to dryness, and the product was isolated by silica gel chromatography (3% MeOH in DCM) to afford **5** as a colorless oil in a 74% yield (5.50 g): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.65 (m, 4H), 7.42 (m, 6H), 5.30, 5.29, 5.23, 5.20 (four s, total 1H), 4.43 (m, 2H), 4.07−4.30 (m, 4H), 3.69 (m, 2H), 3.36−3.48 (m, 5H), 1.69−1.81 (m, 3H), 1.06 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.6, 168.8, 167.8, 166.6, 135.5, 132.6, 129.7, 127.7, 109.5, 108.8, 63.7, 63.2, 62.8, 62.3, 62.2, 62.1, 61.9, 61.2, 53.0, 52.9, 52.8, 52.3, 51.8, 36.9, 36.8, 31.9, 31.8, 26.8, 19.1, 13.9; HRMS (ESI) [M + Na]$^+$ calcd 552.2388, obsd 552.2380.

***N*-[3-(*tert*-Butyldiphenylsilyloxy)propyl]-*N*′-(3-hydroxypropyl)-2-methoxy-1,3-dioxane-5,5-dicarboxamide (6).** The mixture of compound **5** (3.50 g, 6.61 mmol) and 3-aminopropanol (4.5 mL, 52.9 mmol) was stirred for 48 h at 50 °C. DCM was added to the reaction solution, and the mixture was washed with water and dried with Na_2SO_4. The solvent and the excess of 3-aminopropanol were removed under reduced pressure. Separation of the crude product by silica gel chromatography (3% MeOH in CH_2Cl_2) gave **6** as a clear oil in a 79% yield (2.9 g): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.64 (m, 4H), 7.36 (m, 6H), 5.30, 5.20 (two s, total 1H), 4.48 (d, 2H, J = 11.9 Hz), 4.12 (m, 3H), 3.59−3.70 (m, 5H), 3.21−3.49 (m, 7H), 2.95 (m, 1H), 1.63−1.78 (m, 4H), 1.06 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 170.4, 169.9, 169.4, 168.8, 135.4, 133.6, 129.7, 127.7, 109.7, 65.3, 61.2, 59.3, 50.5, 36.9, 36.5, 31.9, 26.8, 19.2; HRMS (ESI) [M + Na]$^+$ calcd 581.2654, obsd 581.2659.

***N*-[3-(*tert*-Butyldiphenylsilyloxy)propyl]-*N*′-[3-(levulinyloxy)propyl]-2-methoxy-1,3-dioxane-5,5-dicarboxamide (7).** A solution of *N*,*N*-dicyclohexylcarbodiimide (DCC; 2.13 g, 10.3 mmol) was slowly added to a solution of levulinic acid (2.40 g, 20.6 mmol) in dry dioxane (40 mL). After the mixture stirred for 4 h at room temperature, dicyclohexylurea was filtered off, and compound **6** (2.90 g, 5.19 mmol) in dry pyridine (15 mL) and a catalytic amount of DMAP were added. The reaction was allowed to proceed for 2 h at room temperature. The reaction mixture was diluted with DCM, and the mixture was washed with aqueous $NaHCO_3$ and dried with Na_2SO_4. The organic phase was evaporated to dryness. Purification of the crude product on a silica gel (2% MeOH in DCM) yielded **7** as a clear oil in a 80% yield (1.20 g): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.64 (m, 4H), 7.40 (m, 6H), 4.46 (d, 2H, J = 11.2 Hz), 4.12 (m, 4H), 3.71 (m, 2H), 3.30−3.46 (m, 7H), 2.75 (t, 2H, J = 5.7 Hz), 2.58 (t, 2H, J = 5.7 Hz), 2.18 (s, 3H), 1.71−1.94 (m, 4H), 1.10 (s, 9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 206.6, 172.8, 170.9, 168.9, 135.5, 133.6, 129.6, 127.7, 109.6, 65.2, 62.0, 61.2, 58.4, 53.3, 37.9, 36.9, 36.7, 32.5, 29.8, 28.3, 27.8, 19.1; HRMS (ESI) [M + Na]$^+$ calcd 679.3021, obsd 679.3049.

2,2-Bis(hydroxymethyl)-*N*-[3-(*tert*-butyldiphenylsilyloxy)propyl]-*N*′-[3-levulinyloxy)propyl]-malondiamide (8). Compound **7** (1.20 g, 1.83 mmol) was dissolved in 80% aqueous AcOH (50 mL) and left for 2 h at room temperature. The reaction mixture was concentrated to an oil and coevaporated three times with water. Purification by silica gel chromatography (10% MeOH in DCM) afforded **8** as a clear oil in a 91% yield (1.0 g): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.64 (m, 4H), 7.40 (m, 6H), 4.10 (t, 2H, J = 5.9 Hz), 3.99 (t, 2H, J = 7.4 Hz), 3.83 (d, 4H, J = 7.4 Hz), 3.68 (t, 2H, J = 5.9 Hz), 3.42 (q, 2H, J = 7.3 and 13.2 Hz), 3.30 (q, 2H, J = 6.8 and 13.0 Hz), 2.88 (t,

2H, J = 7.8 Hz), 2.58 (t, 2H, J = 7.8 Hz), 2.18 (s, 3H), 1.73−1.85 (m, 4H), 1.04 (s, 9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 206.7, 172.8, 171.5, 135.4, 133.6, 129.7, 127.7, 64.1, 61.7, 61.1, 57.2, 38.7, 36.6, 36.3, 32.5, 29.8, 28.3, 27.9, 26.8; HRMS (ESI) $[M + H]^+$ calcd 615.3096, obsd 615.3104.

2-Hydroxymethyl-2-(4,4′-dimethoxytrityloxymethyl)-*N*-[3-(*tert*-butyldiphenylsilyloxy)propyl]-*N*′-[3-(levulinyloxy)propyl]malondiamide (9). Compound **8** (1.00 g, 1.63 mmol) was dried by repeated coevaporations with dry pyridine and dissolved in dry pyridine (20 mL). 4,4′-Dimethoxytrityl chloride (550 mg, 1.63 mmol) was added, and the solution was stirred overnight at room temperature. Pyridine was removed in vacuo, and the residue was subjected to a DCM/aq $NaHCO_3$ workup. The organic phase was dried with Na_2SO_4, the solution was evaporated, and the residue was coevaporated twice with toluene. The product was separated by silica gel chromatography (0 to 5% MeOH in DCM) to give **9** as a white solid foam in a 51% yield (760 mg): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.62−7.65 (m, 4H), 7.34−7.43 (m, 9H), 6.80 (m, 4H), 4.05 (t, 2H, J = 5.5 Hz), 3.77 (s, 6H), 3.65 (t, 1H, J = 5.3 Hz), 3.44 (d, 2H, J = 2,2 Hz), 3.21−3.42 (m, 4H), 2.71 (t, 2H, J = 7.5 Hz), 2.54 (t, 2H, J = 7.5 Hz), 2.16 (s, 3H), 1.68−1.79 (m, 4H), 1.04 (t, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 206.6, 172.7, 170.7, 170.6, 158.6, 144.3, 135.5, 133.6, 130.0, 129.7, 127.9, 127.8, 127.7, 126.9, 86.3, 65.3, 63.9, 62.0, 61.1, 58.4, 55.2, 37.9, 36.8, 36.6, 32.1, 29.8, 28.4, 27.8, 26.8, 20.0; HRMS (ESI) $[M + Na]^+$ calcd 939.4222, obsd 939.4167.

Methyl 3-[(4,4′-Dimethoxytrityl)oxy]-2-{[3-(*tert*-butyldiphenylsilyloxy)propyl]carbamoyl}-2-{[3-(levulinyloxy)propyl]carbamoyl}propyl *N*,*N*-diisopropylphosphoramidite (1). Compound **9** (148 mg, 0.16 mmol) was predried overnight in vacuo over P_2O_5. Methyl *N*,*N*-diisopropylphosphoramidochloridite (43 μL, 0.23 mmol) was added to a solution of **9** and triethylamine (112 μL, 0.81 mmol) in dry MeCN (2 mL) under nitrogen. The reaction mixture was left to stand at room temperature. The reaction was completed in 45 min, according TLC analysis (40% ethyl acetate in hexane). The reaction mixture was applied onto a short dried silica gel column and the pure compound was isolated by eluting with a mixture of ethyl acetate, hexane, and triethylamine (40:59:1, v/v/v). Phosphoramidite **1** was obtained as a white solid foam in 80% yield (140 mg): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.65 (d, 4H, J = 6.7 Hz), 7.25−7.44 (m, 15H), 6.80 (d, 4H, J = 8.1 Hz), 4.05−4.16 (m, 4H), 3.76 (s, 6H), 3.46−3.69 (m, 7H), 3.21−3.38 (m, 6H), 2.72 (t, 2H, J = 6.5 Hz), 2.56 (t, 2H, J = 6.6 Hz), 2.17 (s, 3H), 1.67−1.82 (m, 4H), 1.05−1.16 (m, 21H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 206.6, 172.7, 170.1, 169.7, 158.5, 144.4, 135.4, 133.7, 130.1, 129.7, 128.1, 127.9, 127.7, 126.9, 113.3, 86.4, 64.7, 64.5, 64.0, 62.2, 61.5, 59.1, 55.2, 50.6, 50.5, 43.0, 42.1, 37.9, 36.9, 32.3, 29.8, 28.5, 27.9, 26.9, 24.6, 20.0; ^{31}P NMR ($CDCl_3$, 400 MHz) δ 149.5.

Methyl 6-*O*-Trityl-2,3,4-tri-*O*-(*p*-toluoyl)-α-D-galactopyranoside (13). Methyl α-D-galactopyranoside (**10**) (5.00 g, 25.7 mmol) was dried by repeated coevaporations with dry pyridine and dissolved in dry pyridine (50 mL). Trityl chloride (7.90 g, 28.3 mmol) and a catalytic amount of (*N*,*N*-dimethylamino)pyridine were added. The mixture was stirred at 80 °C overnight, and then *p*-toluoyl chloride (15.3 mL, 116 mmol) was added. The reaction mixture was shaken overnight at 50 °C, slowly poured into ice-cold water, extracted with ethyl acetate, washed with saturated $NaHCO_3$, and dried over Na_2SO_4. The solution was concentrated in vacuo and coevaporated to dryness with toluene. The oily residue was purified by silica gel chromatography (DCM) to give **13** as a white amorphous solid in a 86% yield (17.4 g): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.86 (t, 4H), 7.69 (d, 2H), 7.34 (d, 6H), 7.18−7.30 (m, 13H), 7.05 (d, 2H), 5.95 (m, 2H), 5.53 (dd, 1H, J = 3.7 and 10.2 Hz), 5.25 (d, 1H, J = 3.6 Hz), 4.09 (t, 1H, J = 6.6 Hz), 3.50 (s, 3H), 3.42 (m, 1H), 3.28 (dd, 1H, J = 7.1 and 9.4 Hz), 2.46, 2.36 and 2.31 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.2, 165.6, 165.4, 146.9, 143.8, 129.9, 129.1, 128.9, 128.6, 127.9, 127.8, 126.8, 126.6, 97.5, 87.0, 69.6, 69.2, 68.3, 68.0, 61.8, 55.5, 21.7, 21.6. Anal. Calcd for $C_{50}H_{46}O_9$: C, 75,93; H, 5.86. Found: C, 77.35; H, 5.91.

Methyl 6-*O*-Trityl-2,3,4-tri-*O*-(*p*-toluoyl)-α-D-mannopyranoside (14) and Methyl 6-*O*-Trityl-2,3,4-tri-*O*-(*p*-toluoyl)-α-D-glucopyranoside (15). See Supporting Information.

Methyl 2,3,4-Tri-*O*-(*p*-toluoyl)-α-D-galactopyranoside (16). Compound **13** (17.4 g, 22.0 mmol) was stirred in a mixture of methanolic iodine (200 mL, 1% iodine in methanol) and DCM (100 mL) overnight at 60 °C. The mixture was washed twice with aqueous Na_2SO_3 (10%, m/v), dried over Na_2SO_4, and evaporated to dryness. The product was isolated on a silica gel column, eluting with a gradient of ethyl acetate (20% to 50%) in petroleum ether, which afforded **16** as a white amorphous solid in a 70% yield (8.50 g): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.01, 7.89, 7.71, 7.30, 7.18 and 7.04 (each d, each 2H), 5.97 (dd, 1H, J = 3.4 and 10.7 Hz), 5.83 (d, 1H, J = 3.5 Hz), 5.69 (dd, 1H, J = 3.6 and 10.7 Hz), 5.27 (d, 1H, J = 3.6 Hz), 4.32 (m, 1H), 3.76 (m, 1H), 3.63 (m, 1H), 3.30 (s, 3H), 2.46, 2.37 and 2.31 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.9, 166.3, 165.5, 144.6, 144.2, 143.9, 130.1, 129.9, 129.7, 129.4, 129.2, 129.0, 97.6, 70.0, 69.5, 69.2, 68.2, 60.8, 55.7, 21.8, 21.7, 21.6; HRMS (EI) $[M - H]^-$ calcd 547.2010, obsd 547.2010.

(Methyl 2,3,4-Tri-*O*-(*p*-toluoyl)-α-D-mannopyranoside (17) and Methyl 2,3,4-Tri-*O*-(*p*-toluoyl)-α-D-glucopyranoside (18). See Supporting Information.

(Methyl 2,3,4-Tri-*O*-(*p*-toluoyl)-α-D-galactopyranoside-6-yl) Methyl *N*,*N*-Diisopropylphosphoramidite 19. The phosphoramidite **19** was prepared as described for **1**, using **16** (217 mg, 0.40 mmol) as a starting material. The product was obtained as a clear oil in a 82% yield (230 mg): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.97, 7.87, 7.68, 7.27, 7.16 and 7.03 (each d, each 2H), 5.97 (m, 2H), 5.60 (dd, 1H, J = 3.4 and 10.1 Hz), 5.30 (m, 1H), 4.10 (dd, 1H, J = 6.1 and 11.7 Hz), 3.72−3.85 (m, 2H), 3.53−3.66 (m, 2H), 3.52 (s, 3H), 3.42, 3.39, 3.36 and 3.32 (each s, each 3H), 2.44, 2.35, 2.29 (each s, each 3H), 1.16 (m, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.2, 165.5, 144.3, 143.6, 129.9, 129.8, 129.2, 129.1, 128.9, 97.5, 69.6, 69.1, 68.8, 68.7, 68.3, 61.8, 61.6, 55.5, 42.8, 42.3, 24.7, 21.6; ^{31}P NMR ($CDCl_3$, 400 MHz) δ 149.2; MS (ESI) $[M + Na]^+$ calcd 1100.5, obsd 1100.5.

(Methyl 2,3,4-Tri-*O*-(*p*-toluoyl)-α-D-mannopyranoside-6-yl) Methyl *N*,*N*-Diisopropylphosphoramidite (20) and (Methyl 2,3,4-tri-*O*-(*p*-toluoyl)-α-D-glucopyranoside-6-yl) Methyl *N*,*N*-Diisopropylphosphoramidite (21). See Supporting Information.

Immobilization of Thymidine to a Tentagel Support. To 5′-*O*-(4,4′-dimethoxytrityl)thymidine (500 mg, 0.92 mmol)[32] in dry pyridine (1.6 mL) were added (*N*,*N*-dimethylamino)-pyridine (112 mg, 0.92 mmol) and succinic anhydride (147 mg, 1.47 mmol). The reaction mixture was stirred under nitrogen atmosphere at room temperature for 20 h, and then water (200 μL) was added. The solution was stirred for 1 h and evaporated to dryness, and the residue was coevaporated with toluene (3 × 2 mL). The reaction product was dissolved in DCM (80 mL) and washed with 10% aqueous citric acid and water. The organic phase was dried over Na_2SO_4 and evaporated in vacuo. The residue was dissolved in a mixture of DCM and pyridine (5 mL, 95:5, v/v), and the solution was added to the mixture of anhydrous diethyl ether and hexane (200 mL, 25:75, v/v). The mixture was centrifuged, and the precipitate was washed with the mixture of diethyl ether and hexane (25:75, v/v) and dried to give 420 mg of the 5′-*O*-(4,4′-dimethoxytrityl)thymidine hemisuccinic acid ester. The crude product (420 mg, 0.65 mmol) and *p*-nitrophenol (81 mg, 0.65 mmol) were dissolved in a mixture of dry dioxane (160 μL) and dry pyridine (40 μL). Dicyclohexylcarbodiimide (134 mg, 0.65 mmol) in dry dioxane (300 μL) was added dropwise to this solution under nitrogen. After 4 h at room temperature, the insoluble *N*,*N*-dicyclohexy-

(32) Koga, M.; Wilk, A.; Moore, M. F.; Scremin, C. L.; Zhou, L.; Beaucage, S. L. *J. Org. Chem.* **1995**, *60*, 1520−1530.

lurea formed was filtered off and washed with anhydrous dioxane. Filtrates were combined and added to 500 mg of Tentagel S-NH_2 (260 μmol/g) suspended in dry DMF (280 μL) and triethylamine (60 μL). The suspension was shaken overnight at ambient temperature. The support was then filtered and washed with DMF, dioxane, methanol, and diethyl ether. On the basis of the DMTr-cation assay,[29] a loading of 240 μmol/g was obtained. The unreacted amino groups were capped with Ac_2O in THF containing *N*-methylimidazole and lutidine. Finally the support was thoroughly washed with THF, DCM, MeOH, and diethyl ether and vacuum-dried.

Oligodeoxyribonucleotide Synthesis. The oligodeoxyribonucleotides were assembled on an Applied Biosystems 392 DNA synthesizer on a 1.0-μmol scale using the Tentagel support described above (240 μmol/g) and methyl *N*,*N*-diisopropylphosphoramidite building blocks. Phosphoramidite **1** was used as a 0.15 mol L^{-1} solution in dry MeCN, the coupling time being 600 s. After coupling of **1**, the detritylation was carried out by using two consecutive "no. 14 (acid solution) to column" steps (2 × 60 s) separated by a trityl flush step (5 s). Otherwise standard protocols were employed.

Synthesis of Oligonucleotide Glycoconjugates 25 and 26. Protected oligonucleotide sequences 5′-d(TTTXTTT)-3′ and 5′-d(TXTGACGATCTCAT)-3′, where X stands for the nonnucleosidic building block **1**, were synthesized as described above. Galactosyl 6-*O*-phosphoramidite (**19**, 0.15 mol L^{-1} solution in dry MeCN) was reacted with the free 5′-terminal hydroxyl group of the solid-supported conjugate, the coupling time being 600 s. The support-bound conjugates were next treated with 0.5 mol L^{-1} hydrazine acetate solution (0.124/4/1, $H_2NNH_2 \cdot H_2O$/pyridine/AcOH, v/v/v) for 30 min, washed with pyridine, MeOH, DCM and diethyl ether, and dried under reduced pressure. The synthesis columns were transferred back to the DNA synthesizer and mannosyl 6-*O*-phosphoramidite (**20**, 0.2 mol L^{-1} solution in dry MeCN) was introduced. Reaction time of 600 s was used, and the coupling was repeated three times. The supports were dried in vacuo and transferred to microcentrifuge tubes. The TBDPS protection was removed by shaking the resins for 18 h with a mixture of triethylamine trihydrofluoride (30 μL) and dry THF (150 μL) at room temperature. The reaction was repeated, after which the support was filtered, washed with THF, MeOH, DCM, and diethyl ether, and dried under vacuum. The supports were then transferred again back to the DNA synthesizer and glucosyl 6-*O*-phosphoramidite (**21**, 0.15 mol L^{-1} solution in dry MeCN) was coupled using a reaction time of 600 s. Next, the phosphate methyl protections were removed by treating the support with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate (S_2Na_2) (1 M solution in DMF) for 30 min. The supports were washed with DMF, MeOH, DCM, and diethyl ether, dried, and finally treated with concentrated ammonia (33% aqueous NH_3, 7 h at 55 °C) to release and deprotect the resulting oligonucleotide glycoconjugates. Evaporation, dissolution in water, HPLC purification, desalting, and characterization by ESI-MS verified the formation of the expected conjugates **25** and **26** (Table 2).

Melting Temperature Studies of 26. The melting curve (absorbance versus temperature) was measured at 260 nm on a UV−vis spectrometer equipped with a temperature controller, the heating rate being 1 °C min^{-1} (from 15 to 90 °C). The experiments were performed in 10 mmol L^{-1} potassium phosphate buffer (pH 7) containing 100 mmol L^{-1} NaCl. The concentration of the oligonucleotide conjugate **26** and its complementary sequence was 2 μmol L^{-1}.

Supporting Information Available: Experimental details and spectral data for the compounds **1**, **4**−**9**, and **13**−**21** and HPLC analytica data and ESI-MS spectra for oligonucleotide glycoconjugates **25** and **26**. This material is available free of charge via the Internet at http://pubs.acs.org.

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