Synthesis of a Pentasaccharide–Oligonucleotide Conjugate: A Novel Antithrombotic Agent

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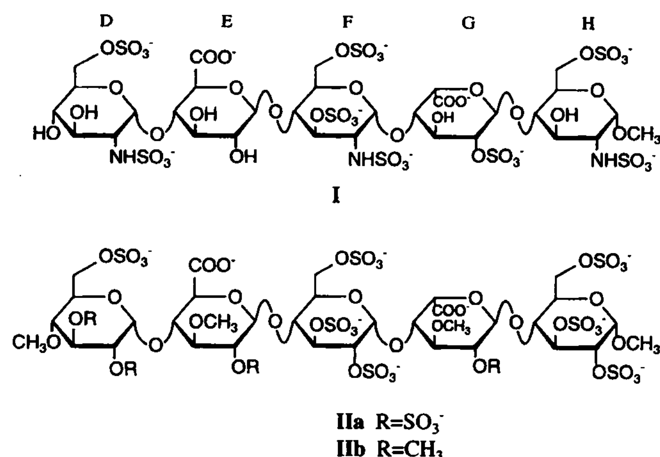
Abstract: Derivatization of the octadecathymidylate derivative **18** (T_{18} ODN) containing a free amine function with sulfo-SIAB® gave the corresponding iodoacetyl ODN **21**. Conjugation of the latter with the thiol-containing pentasaccharide **17c** gave pentasaccharide–ODN conjugate **III**, which exhibited anti-Xa and antithrombin activities of 173 $U mg^{-1}$ and 5 $U mg^{-1}$, respectively.

Keywords
antithrombotics · enzyme inhibitors · oligonucleotides · oligosaccharides · protecting groups

Introduction

The sulfated glycosaminoglycan heparin[1] enhances the inhibitory potency of antithrombin III (AT III) towards factors Xa and IIa (thrombin), two essential serine proteases in blood coagulation. For this reason heparin is frequently used for the prevention and treatment of thrombotic disorders. The catalytic role of heparin embodies a conformational and a template effect.[2] It has been established that a unique and well-defined pentasaccharide (PS) domain in heparin (synthetic counterpart: compound **I**) strongly binds to AT III, thereby inducing a conformational transition in this protease inhibitor, which enhances its inhibitory activity.[3, 4] The conformational change in AT III proved to be the sole requirement for an increased anti-Xa activity. Subsequently, several *O*-methylated/*O*-sulfated pentasaccharide analogues (e.g., **IIa–b**), which selectively stimulate the AT III-mediated inhibition of factor Xa, have been prepared.[5, 6]



In order to increase thrombin inhibition too, heparin should form a ternary complex with AT III and thrombin, as shown schematically in Figure 1. In the first step AT III strongly binds to the PS domain of heparin, designated as the AT III-binding domain (ABD), after which thrombin interacts with a remote thrombin-binding domain (TBD) of heparin. This ternary complex formation requires heparin fragments that contain at least thirteen consecutive saccharide units in addition to the PS domain.[7, 8]

In order to circumvent tedious syntheses of long sulfated oligosaccharides, a challenging program directed towards the rational design of sulfated glycoconjugates[9, 10] (Fig. 1) dis-

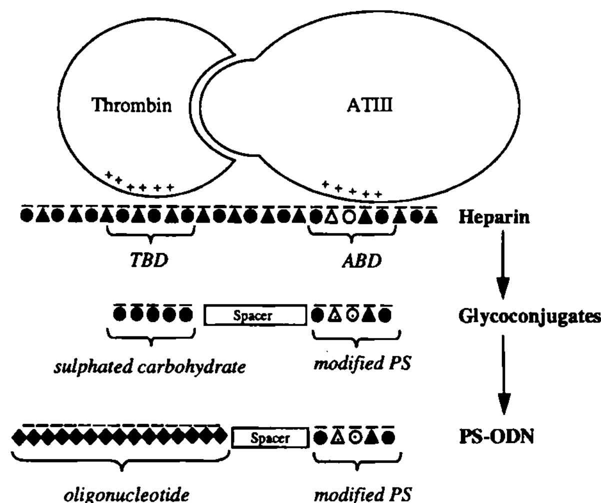


Fig. 1. Schematic representation of the ternary complex of thrombin and AT III with heparin, glycoconjugates, and the proposed PS–ODN conjugate.

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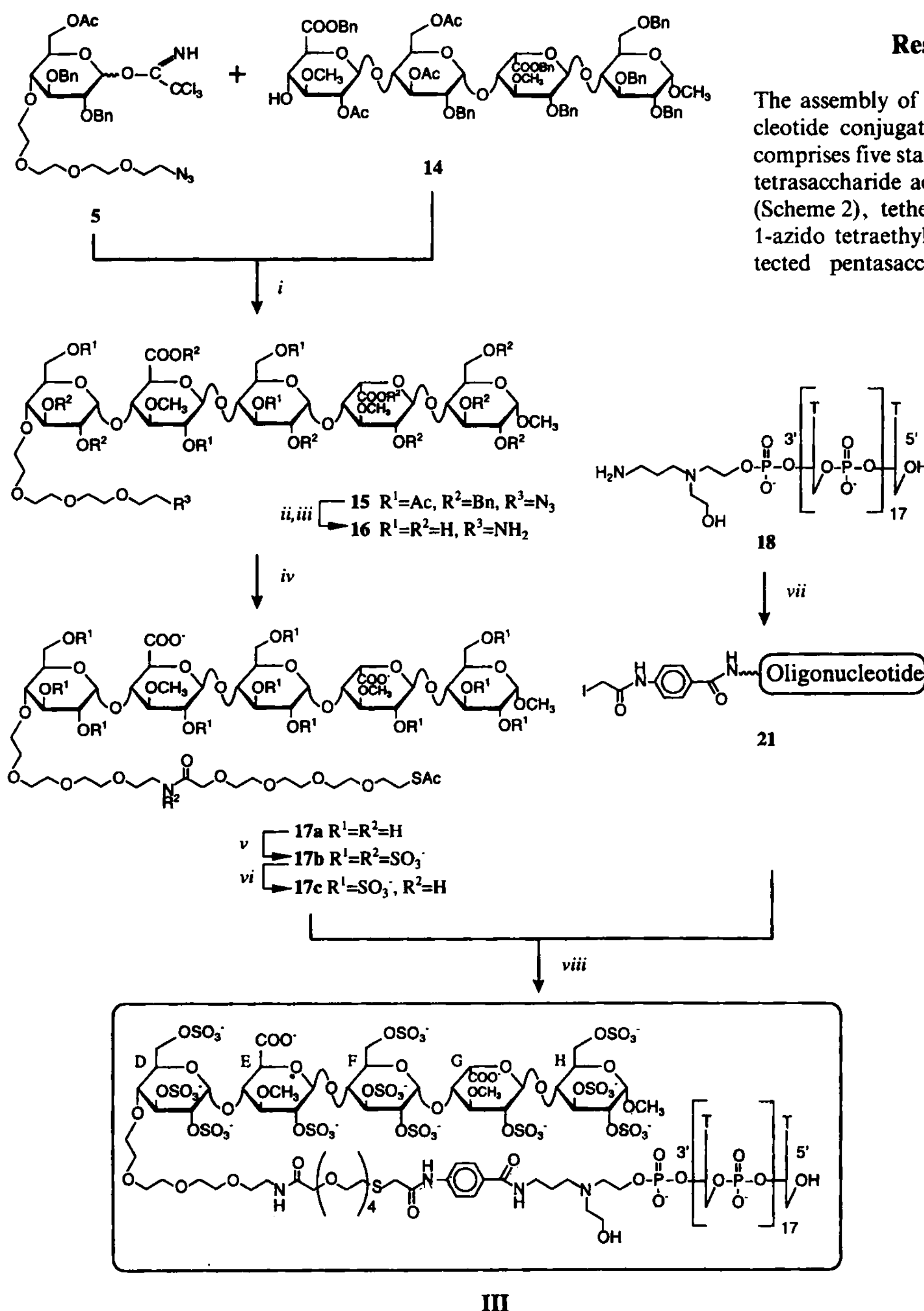
playing both anti-Xa and antithrombin activity was initiated. In these conjugates the noninteracting carbohydrates between the ABD and the TBD part of heparin were replaced by a molecular spacer. In addition the spacer was attached to the nonreducing end of the ABD, thus having the same spatial arrangement as the TBD in heparin. High antithrombin activity was observed when a spacer of at least 50 atoms was used.[9, 10]

Compared with the highly specific and strong binding of the ABD of heparin with AT III, the interaction between thrombin and the TBD of heparin is less specific in nature and about three orders of magnitude weaker.[2, 11] This phenomenon was confirmed by the fact that synthetic ABD–TBD conjugates having uncompounded persulfated oligosaccharides as the TBD still displayed high antithrombin activity.[9, 10] Crystal structure analysis of a complex between thrombin and a specific oligonucleotide, a so-called DNA aptamer, indicated that oligonucleotides may also associate with the heparin-binding site.[12] On the basis of this information we were anxious to find out whether random oligonucleotides, instead of persulfated oligosaccharides, could be used as a TBD.

We wish to report here the synthesis of a conjugate (compound **III**) in which part of the spacer moiety and the TBD of the glycoconjugates are replaced by the readily accessible octadecathymidylate (**18** in Scheme 1).



Scheme 1. i) TMSOTf, Et_2O, −20 °C, 10 min, 52%; ii) H_2, Pd–C, *t*-BuOH, H_2O, 16 h, 82%; iii) 0.4 N NaOH, CH_3OH, 12 h, 80%; iv) **13**, *N*-methylmorpholine, DMF, H_2O, 10 min, 92%; v) $(C_2H_5)_3N\cdot SO_3$, DMF, 55 °C, 16 h; vi) 0.2 N HCl 4 °C, 16 h, 70% (two steps); vii) sulfo-SIAB **20**, phosphate buffer pH = 7.5, 3 h, 73%; viii) NH_2OH, phosphate buffer pH = 7.0, 48 h, 52%.

Results and Discussion

The assembly of the target pentasaccharide–oligonucleotide conjugate **III** is presented in Scheme 1 and comprises five stages: i) glycosylation[13] of the known tetrasaccharide acceptor **14**[14] with glucosyl donor **5** (Scheme 2), tethered at its nonreducing end with a 1-azido tetraethylene glycol spacer, to give fully protected pentasaccharide **15**; ii) hydrogenolysis and saponification of **15** to give deprotected **16**; iii) condensation of the latter with the bifunctional spacer **13** (Scheme 3), followed by sulfation of the free hydroxyl functions to yield pentasaccharide **17c**; iv) synthesis of a functionalized oligonucleotide containing a thiophilic iodoacetyl moiety (i.e., compound **21**); v) one-pot conversion of **17c** into the corresponding thiol derivative and its condensation with the iodoacetyl-functionalized T_{18} oligonucleotide **21**.

The synthesis of glucosyl donor **5** (Scheme 2) commences with a one-pot–two-step conversion of methyl 2,3,6-*O*-benzyl-α-D-glycopyranoside **1**[15] into **2**, entailing treatment of **1** with tetraethylene glycol di-*p*-tosylate in the presence of sodium hydride at 40 °C, followed by addition of lithium azide to the resulting monotosyl derivative and elevation of the temperature (70 °C). Removal of the primary benzyl and anomeric *O*-methyl groups of **2** with 5% sulfuric acid in acetic anhydride[16] at −20 °C afforded **3**. Anomeric deacetylation[17] of **3** with hydrazine acetate, followed by treatment of **4** with trichloroacetonitrile in the presence of cesium carbonate[18] led to the isolation of the α/β imidate **5** in 17% overall yield (based on **1**). Trimethylsilyl trifluoromethanesulfonate

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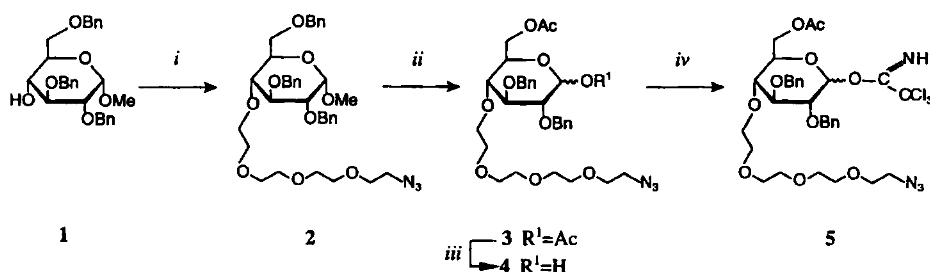
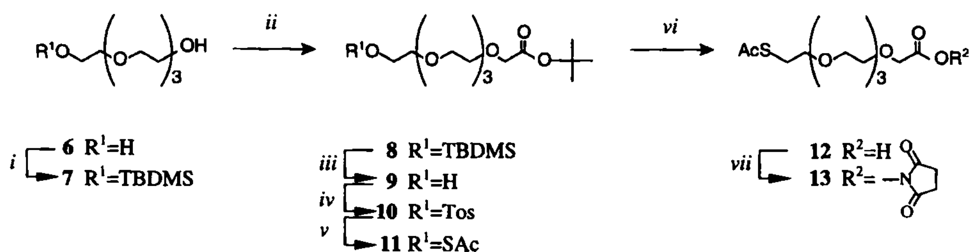


Scheme 2. i) $Tos_2(OCH_2CH_2O)_4$, NaH, LiN_3, DMF, 70 °C, 7 h, 61 %; ii) 5 % H_2SO_4 in Ac_2O, −20 °C, 10 min, 44 %; iii) NH_2NH_2, HOAc, DMF, 1 h, 80 %; iv) Cl_3CCN, Cs_2CO_3, CH_2Cl_2, 1 h, 80 %.



Scheme 3. i) TBDMS–Cl, NaH, THF, 1.5 h, 51 %; ii) *tert*-butylbromoacetate, NaH, THF, 30 min, 50 °C; iii) AcOH, H_2O, THF, (3/1/1, v/v/v), 2 h, 30 % (two steps); iv) Tos–Cl, pyridine, CH_2Cl_2, 2 h, 80 %; v) KSAc, acetone, 1 h, 80 %; vi) TFA/CH_2Cl_2, (1/9, v/v), 5 h, 95 %; vii) *N*-hydroxysuccinimide, EDCI, CH_2Cl_2, 1 h, 100 %.

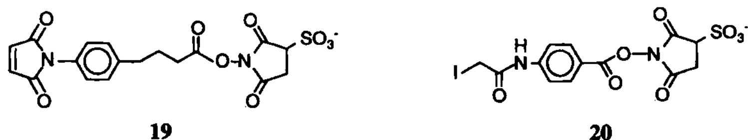
(TMSOTf)-mediated glycosylation of the known tetrasaccharide **14**[14] (Scheme 1) with donor **5** at −20 °C gave the α-linked derivative **15** in a 52 % yield together with a small amount (8 %) of the β-coupled product. Removal of benzyl protective groups by hydrogenolysis and simultaneous azide reduction of **15** and subsequent saponification of acetyl groups yielded pentasaccharide **16** in 64 % yield.

The requisite bifunctional spacer **13** was prepared from tetraethylene glycol (**6**) by the sequence of reactions as pictured in Scheme 3. Monosilylation of **6** with *tert*-butyldimethylsilyl chloride and sodium hydride in THF yielded **7**. An equimolar amount of sodium hydride was added to a heated solution (55 °C) of THF containing **7** and excess *tert*-butylbromoacetate. Desilylation by mild acid treatment of the resulting crude **8** gave homogeneous **9** after flash chromatography. Tosylation of **9** and subsequent substitution of the tosylate group in **10** with potassium thioacetate gave **11**. Acidolysis of the *tert*-butyl ester and activation of the carboxylic acid function of **12** with *N*-hydroxysuccinimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) afforded the requisite spacer **13** in 12 % overall yield (based on **6**).

Pentasaccharide **16** was elongated and functionalized by condensation with spacer **13** under the influence of *N*-methylmorpholine in a mixture of DMF/water to give **17a** (Scheme 1). Subsequent sulfation of the free hydroxyl functions in **17a** with a triethylamine/sulfur trioxide complex in DMF at 55 °C was accompanied by extensive *N*-sulfation of the amide bond (≈70 %). Fortunately this $N{-}SO_3^-$ bond in **17b** could be selectively cleaved[10] with 0.2 N HCl at 4 °C to give the desired ABD **17c** as a white fluffy solid in 64 % yield.

The modified solid support developed by Miller et al.[19] was utilized to obtain a T_{18} ODN derivative (compound **18**) containing at its 3′ position a *N*-(3-aminoprop-1-yl)-*N*-(2-hydroxyethyl)-2-aminoethyl)phosphate moiety ("Miller cap"). This 3′-amino cap not only prevents the ODN from being degraded by 3′-exonucleases present in serum, but also allows further derivatization of the ODN. The functionalized ODN **18** was synthesized on a 10 µmol scale with an automated DNA synthesizer. HPLC analysis (reversed phase) of the crude **18** obtained after ammonolysis of the immobilized ODN (25 % NH_4OH, 55 °C, 12 h) revealed the presence of an unidentified side product (20–30 %). ^1H NMR spectroscopy of the isolated side product indicated the absence of the terminal amino function; this contention was supported by its failure to react with **19** or **20**.

At this stage, oligonucleotide **18** had to be derivatized with a thiophilic linker suitable for condensation with pentasaccharide **17c**. The most commonly used thiophilic groups in conjugations are maleimides and iodo- or bromoacetates.[20] Our experience with maleimides in conjugations of ODNs with large proteins[21] urged us to apply sulfo-SMPB® (**19**, Scheme 4) in derivatization of ODN **18**. However, condensation of ODN **18** with **19** led to substantial degradation of the maleimide-derivatized ODN, as evidenced by HPLC analysis, thus explaining the very poor yield (2–5 %) of the conjugation step. This result is in sharp contrast to the result obtained by Tung et al.[22] of a conjugation between a maleimide-derivatized ODN and a cysteine-containing peptide.



Scheme 4. Sulfo-SMPB (**19**) and sulfo-SIAB (**20**).

A single thymidylate residue anchored to a 3′ Miller cap was treated with **19**. ^1H NMR spectroscopy revealed that the main product did not contain an intact maleimide function. The lability of the maleimide ODN prompted us to apply sulfo-SIAB® (**20**, Scheme 4), an iodoacetyl-type of thiophilic linker, in the derivatization step. Figure 2 shows the progress of the reaction between the T_{18} ODN and sulfo-SIAB **20** monitored by HPLC. The iodoacetyl-derivatized ODN proved to be more stable than the maleimide ODN. Thus when ODN **18** was treated with **20** (5 equiv) in a buffered solution (0.1 M Na_2HPO_4, pH = 7.5) for 3 h, HPLC analysis showed smooth conversion to the iodoacetyl derivative **21**. After purification by size exclusion chromatography on a Superdex 30 column, HPLC analysis revealed minor degradation (≈10 %) of the iodoacetyl ODN.

In the final stage, deprotection of the thioacetyl function in **17c** and conjugation of the deprotected thiol with iodoacetyl ODN **21** was effected in a one-step procedure with a 0.05 M solution of hydroxylamine in 0.1 M Na_2HPO_4, pH = 7.0.[10] The reaction was monitored by HPLC (Fig. 3), which showed that the reaction was complete after 48 h. Sephadex G-50 size exclusion chromatography afforded the conjugate **III** in 52 % yield. The homogeneity of **III** was firmly established by ^1H NMR

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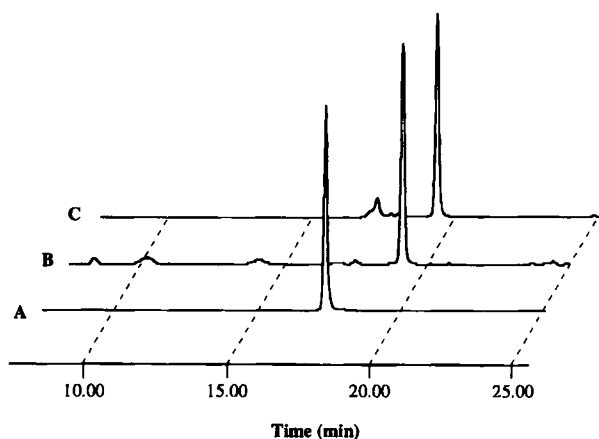

Fig. 2. Reversed-phase HPLC diagram (absorbance 254 nm) showing the formation of iodoacetyl ODN (**21**). Lane A: T_{18} ODN (**18**); Lane B: T_{18} ODN + 5 equiv sulfo-SIAB after 3 h; Lane C: iodoacetyl ODN (**21**) after purification on Superdex 30.

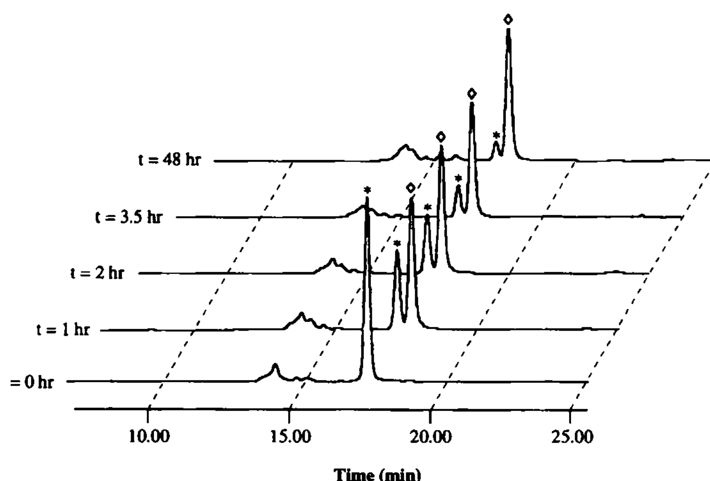

Fig. 3. Reversed-phase HPLC diagram (absorbance 254 nm) showing the progression of the conjugation of iodoacetyl ODN (**21**) (*) with **17c** in the presence of 0.05 M NH_2OH to yield conjugate **III** (◇).

spectroscopy, UV capillary electrophoresis, ES-MS analysis and HPLC analysis.

The biological activity of the conjugate was tested[23] in vitro; it was found to have a 173 $U mg^{-1}$ anti-Xa and a 5 $U mg^{-1}$ antithrombin activity. Taking into account the high molecular weight of the conjugate (M_r = 8719) and the modest charge density of the ODN, the Xa and thrombin inhibitory activities are still substantial; in comparison heparin (M_r = 15000) shows 160 $U mg^{-1}$ anti-Xa and 160 $U mg^{-1}$ antithrombin activity.

These results reveal that an ODN can associate with the heparin-binding site of thrombin. This phenomenon is in line with the interaction of a DNA aptamer with thrombin observed in a crystal structure published by Padmanabhan et al.[12] Furthermore, our result confirms that the interaction of the TBD of glycoconjugates with thrombin has less stringent requirements than the interaction of its ABD part with AT III.

Experimental Procedure

Material and methods: ^1H and ^{13}C NMR spectra were recorded with a Jeol JNM FX200 (200 and 50.1 MHz, respectively). 2D (^1H-^1H COSY, ^1H-^1H TOSCY, ^1H-^{13}C COSY) NMR spectra were recorded at 300 MHz on a Bruker DPX 300, at 360 MHz on a Bruker WM 360 or at 600 MHz on a Bruker DMX600. ^1H NMR chemical shifts (δ) in organic solvents are reported relative to tetramethylsilane. For proton spectra in aqueous solutions (D_2O) the residual HDO peak was set at δ = 4.80. Optical rotations were determined at 20 °C by means of a Propol polarimeter.

Dichloromethane, pyridine, toluene and diethyl ether were refluxed with CaH_2 for 3 h, distilled and stored over molecular sieves (4 Å). *N,N*-dimethylformamide was stirred with CaH_2 for 16 h and then distilled under reduced pressure and stored over molecular sieves (4 Å). THF was refluxed with $LiAlH_4$ and distilled directly before use. *N*-methylmorpholine was distilled with ninhydrin under a stream of nitrogen directly before use. Acetone was dried over K_2CO_3 and then distilled and stored over molecular sieves (4 Å). Tetraethylene glycol di-*p*-tosylate, trimethylsilyl trifluoromethanesulfonate, potassium thioacetate (Aldrich), sodium hydride (60% dispersion in mineral oil), hydrazine monohydrate, trichloroacetonitrile, cesium carbonate, *tert*-butyldimethylsilyl chloride, *tert*-butylbromoacetate, hydroxylamine hydrochloride, *N*-hydroxysuccinimide, EDCI (Acros), *p*-toluenesulfonyl chloride, trifluoroacetic acid (Merck), sulfo-SIAB (Pierce) were used as received.

Column chromatography was performed on Merck Kieselgel 60 (230–400 mesh). Gel permeation chromatography was accomplished on Sephadex LH 20 (Pharmacia), Superdex 30 prep. grade (Pharmacia), Sephadex G 25 and G 50 (Pharmacia). TLC analysis was performed on DC Fertigfolien (Schleicher & Schüll, F 1500, LS 254). Compounds were visualized by UV absorption (254 nm), charring with 20% H_2SO_4 in MeOH or exposure to sublimated iodine crystals. High performance liquid chromatography (HPLC) (anion exchange and reversed phase) was conducted on a Waters 600 E (system controller) single pump gradient system with a MonoQ prepacked HR 5/5 column (Pharmacia) (anion exchange) and a Supelcosil LC-18-DB column (Supelco) (reversed phase). A Waters model 484 variable wavelength UV detector was used for the detection of the ODN at 254 nm. A IBZ Messtechnik Chiralyser was used for optical rotation detection. Gradient elution in anion exchange was performed at 20 °C by building up a gradient starting with buffer A, 20% CH_3CN in H_2O, and applying buffer B, 2.0 M NaCl in 20% CH_3CN in H_2O, at a flow rate of 1 $mL min^{-1}$. Gradient elution in reversed phase was performed at 20 °C by building up a gradient starting with buffer A, 0.1 M triethylammonium acetate in H_2O, pH = 7.0, and applying buffer B, 0.1 M triethylammonium acetate in H_2O/CH_3CN, 1/1, v/v, pH = 7.0, at a flow rate of 1 $mL min^{-1}$.

Methyl 4-*O*-(1-azido-3,6,9-trioxaundecyl)-2,3,6-*O*-benzyl-α-D-glucopyranoside (2): Methyl 2,3,6-*O*-benzyl-α-D-glucopyranoside **1** (5.4 mmol, 2.5 g) was dissolved in DMF (75 mL). Tetraethylene glycol di-*p*-tosylate (16.2 mmol, 6.5 mL) and sodium hydride (325 mg, 8.1 mmol, 60 wt% suspension) were added at 40 °C. After stirring for 2 h, lithium azide (2.6 g, 54 mmol) was added and the reaction temperature was elevated to 70 °C. After 5 h, excess sodium hydride was quenched with methanol (2.5 mL). The reaction mixture was concentrated and the residue was dissolved in diethyl ether (100 mL), washed twice with 1 M $NaHCO_3$, dried with $MgSO_4$, and concentrated in vacuo. The crude product was chromatographed on silica gel (toluene/EtOAc, 4/1, v/v) to afford **2** (61% yield, 2.2 g). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 50.3 (CH_2N_3), 54.8 (OCH_3), 68.5 (C-6), 69.8, 70.4, 72.0 (CH_2O, tetraethylene glycol (TEG)), 73.9, 73.2, 75.3 (OCH_2Ph), 70.0, 78.1, 79.7, 81.7 (C-2, C-3, C-4, C-5), 97.9 (C-1), 127.3–128.2 (CH_{arom}), 138.1–138.9 (C_{arom}).

1,6-di-*O*-Acetyl-4-(1-azido-3,6,9-trioxaundecyl)-2,3-di-*O*-benzyl-α/β-D-glucopyranoside (3): To a cooled (−20 °C) solution of **2** (2.2 g, 3.2 mmol) in acetic anhydride (115 mL) was added dropwise a mixture of 5% sulfuric acid in acetic anhydride (28.6 mL). After 10 min, sodium acetate was added to neutralize the reaction mixture. The reaction mixture was diluted with ethyl acetate (300 mL) and carefully washed three times with sat. $NaHCO_3$ (100 mL), following which the water layers were extracted twice with ethyl acetate. The organic layers were combined, dried ($MgSO_4$), concentrated, and coevaporated several times with toluene. The crude mixture was purified by silica gel chromatography (toluene/EtOAc, 1/1, v/v) to give pure **3** (44% yield, 0.90 g). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 20.6, 20.82 (2 × CH_3COO), 50.4 (CH_2N_3), 62.6 (C-6), 69.8, 70.4, 72.4 (CH_2O, TEG), 73.6, 75.3 (OCH_2Ph), 71.1, 73.6, 77.6, 77.9, 78.5, 80.7, 81.1, 84.3 (α/β C-2, C-3, C-4, C-5), 89.5, 93.6 (α/β C-1), 127.4–128.2 (CH_{arom}), 137.5, 138.5 (2 × C_{arom}), 169.0, 170.4 (2 × CH_3COO).

6-*O*-Acetyl-4-(1-azido-3,6,9-trioxaundecyl)-2,3-di-*O*-benzyl-α/β-D-glucopyranose (4): Compound **3** (0.90 g, 1.4 mmol) was treated with a solution of hydrazine acetate (0.1 M) in DMF (1.54 mmol, 15.4 mL). After 1 h, TLC analysis (toluene/EtOAc, 1/2, v/v) revealed the reaction to be complete, and water (50 mL) was added to the reaction mixture. The water layer was extracted three times with CH_2Cl_2 (50 mL). The organic layer was then washed with $NaHCO_3$ (1 M, 50 mL), dried ($MgSO_4$) and concentrated in vacuo. The residue was chromatographed on silica gel (light petroleum/EtOAc, 2/8, v/v) to give **4** (80% yield, 0.67 g). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 20.6 (CH_3COO), 50.3 (CH_2N_3), 62.6 (C-6), 69.7, 70.3, 72.5 (CH_2O, TEG), 73.6, 75.3 (OCH_2Ph), 71.1–84.3 (α/β C-2, C-3, C4, C-5), 90.7, 97.6 (α/β C-1), 127.3–128.1 (CH_{arom}), 137.5, 138.6 (2 × C_{arom}), 170.6 (2 × CH_3COO).

O-[6-*O*-Acetyl-4-(1-azido-3,6,9-trioxaundecyl)-2,3-di-*O*-benzyl-α/β-D-glucopyranosyl] trichloroacetimidate (5): To a stirred solution of **4** (0.67 g, 1.1 mmol) and trichloroacetonitrile (0.55 mL) in CH_2Cl_2 was added a catalytic amount of Cs_2CO_3 (72 mg, 0.22 mmol). After 1 h, TLC analysis (2% MeOH in CH_2Cl_2) indicated complete conversion of **4** into **5**. The reaction mixture was filtered and the filtrate

was concentrated under reduced pressure. Column chromatography (2% MeOH in CH_2Cl_2) of the residue yielded pure **5** (92% yield, 0.82 g). ^{1}H NMR ($CDCl_3$): δ = 2.04–2.07 (2×s, 3H, CH_3COO α/β), 3.35 (t, 2H, CH_2N_3), 3.42–3.51 (m, 1H, H-4 α/β), 3.53–3.65 (7×t, 14H, TEG), 3.65–3.78 (m, 1H, H-2 α/β), 3.95–4.09 (m, 2H, H-3/H-5 α/β), 4.23–4.43 (m, 2H, H-6a, H-6bα/β), 4.68–4.93 (2×m, 4H, OCH_2Ph α/β), 5.79 (d, $J_{1,2}$ = 7.7 Hz, βH-1), 6.45 (d, $J_{1,2}$ = 3.6 Hz, αH-1), 7.25–7.36 (2×m, 10H, H_{arom}), 8.61, 8.70 (2×s, 1H, $NH_{imidate}$ α/β); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 20.7 (CH_3COO), 50.4 (CH_2N_3), 62.6, 62.7 (α/β C-6), 69.8, 70.3, 72.5 (CH_2O, TEG), 73.6, 75.3 (OCH_2Ph), 71.3, 73.6, 77.6, 77.9, 79.0, 80.5, 80.8, 84.1 (α/β C-2, C-3, C-4, C-5), 93.8, 97.9 (α/β C-1), 127.3–128.2 (CH_{arom}), 137.7, 138.3 (2×C_{arom}), 160.7, 160.9 (OC(NH)CCl_3), 170.3 (2×CH_3COO).

1-*O*-*tert*-Butyldimethylsilyl-3,6,9-trioxaundecanol (7): To a cooled (0 °C) solution of **6** (8.5 g, 44 mmol) in THF (125 mL) was added in portions sodium hydride (1.76 g, 35.2 mmol, 60 wt% suspension). After 1 h, the ice bath was removed and a solution of *tert*-butyldimethylsilyl chloride (5.4 g, 35.2 mmol) in THF (25 mL) was added dropwise. After 30 min, methanol (5 mL) was added to quench the reaction. The reaction mixture was concentrated under reduced pressure, diluted with EtOAc (500 mL) and washed three times with water (200 mL). The organic phase was dried with $MgSO_4$ and concentrated in vacuo. Purification by silica gel column chromatography (light petroleum/EtOAc, 1/1, v/v) afforded **7** (64% yield, 6.9 g). ^{1}H NMR ($CDCl_3$): δ = 0.05 (s, 6H, $Si(CH_3)_2$), 0.89, (s, 9H, $SiC(CH_3)_3$), 3.55–3.77 (m, 16H, TEG); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = −5.5 ($Si(CH_3)_2$), 18.2 ($SiC(CH_3)_3$), 25.6 ($SiC(CH_3)_3$), 61.1, 62.3, 69.9, 70.2, 72.2 (CH_2O, TEG).

14-*S*-Acetyl-14-mercapto-3,6,9,12-tetraoxatetradecanoic acid *tert*-butyl ester (11): A solution of **7** (6.9 g, 22.4 mmol) and *tert*-butylbromoacetate (18 mL, 112 mmol) in THF (100 mL) was heated to 55 °C. Sodium hydride (0.81 g, 20.2 mmol, 60 wt% suspension) was added and the reaction mixture was stirred for 15 min. The reaction was quenched with methanol (5 mL) and neutralized with aqueous acetic acid. The reaction mixture was diluted with EtOAc (500 mL) and washed three times with water (200 mL); the organic layer was dried ($MgSO_4$), concentrated, and coevaporated several times with toluene. The crude product **8** thus obtained was dissolved in a mixture of acetic acid, THF and water (150 mL, 3/1/1, v/v/v) and stirred for 2 h. The reaction mixture was neutralized with NaOH (6N) and extracted three times with EtOAc (150 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo. The residue was chromatographed on silica gel ($MeOH/CH_2Cl_2$, 1/1, v/v) to give **9** (30% yield, 2.1 g). *p*-Toluenesulfonyl chloride (1.92 g, 10.1 mmol) was added to a solution of **9** (2.1 g, 6.7 mmol) in pyridine/dichloromethane (30 mL, 3/5, v/v). After 2 h, TLC analysis (diethyl ether) showed complete disappearance of **9**. The reaction mixture was diluted with water (50 mL) and extracted three times with CH_2Cl_2 (75 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. The crude product was purified by silica gel chromatography (diethyl ether) to give **10** (80% yield, 2.49 g). Compound **10** (2.49 g, 5.4 mmol) was dissolved in dry acetone (100 mL) and potassium thioacetate (1.51 g, 10.8 mmol) was added. After stirring for 1 h, TLC analysis (diethyl ether) indicated complete conversion of **10** into **11**. The reaction mixture was diluted with EtOAc (200 mL) and washed twice with $NaHCO_3$ (1 M, 100 mL). The water layer was extracted twice with EtOAc (100 mL) and the combined organic layers were washed with brine (100 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography (diethyl ether) gave compound **11** (80% yield, 1.58 g). ^{1}H NMR ($CDCl_3$): δ = 1.47 (s, 9H, $(CH_3)_3CO$), 2.33 (s, 3H, $CH_3C(O)S$), 3.09 (t, CH_2S), 3.57–3.77 (m, 16H, TEG), 4.00 (s, 2H, $CH_2C(O)$); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 27.6 ($(CH_3)_3CO$), 28.0 (CH_2S), 29.8 ($CH_3C(O)S$), 67.6, 69.0, 69.5, 69.8, 70.2 (CH_2O, TEG, $CH_2C(O)$), 80.8 ($(CH_3)_3CO$), 169.2 ($CH_2C(O)$), 194.8 ($CH_3C(O)S$).

14-*S*-Acetyl-14-mercapto-3,6,9,12-tetraoxatetradecanoic acid succinimide ester (13): A solution of 10% trifluoroacetic acid in CH_2Cl_2 (20 mL) was added to compound **11** (1.58 g, 4.32 mmol). After 3 h, TLC analysis (CH_2Cl_2/MeOH/AcOH, 94/5/1, v/v/v) indicated complete conversion of **11** into **12**. The reaction mixture was coevaporated four times with toluene. Flash chromatography (CH_2Cl_2/MeOH/AcOH, 94/5/1, v/v/v) gave **12** as a colorless oil (95% yield, 1.27 g). To a solution of compound **12** (440 mg, 1.42 mmol) in CH_2Cl_2 (16 mL), *N*-hydroxysuccinimide (179 mg, 1.55 mmol) and EDCI (300 mg, 1.55 mmol) were added. After 1 h, the reaction mixture was diluted with CH_2Cl_2 (50 mL), washed three times with ice water (20 mL), dried ($MgSO_4$), and concentrated to give **13** (100% yield, 578 mg), which was used without further purification. ^{1}H NMR ($CDCl_3$): δ = 2.33 (s, 3H, $CH_3C(O)S$), 2.86 (s, 4H, succ), 3.09 (t, CH_2S), 3.57–3.83 (m, 16H, TEG), 4.53 (s, 2H, $CH_2C(O)$); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 25.1 (CH_2, succ), 28.0 (CH_2S), 29.8 ($CH_3C(O)S$), 65.9, 69.1, 69.7, 70.0, 70.6 (CH_2O, TEG, $CH_2C(O)$), 165.6 ($CH_2C(O)$), 168.6 (C=O, succ), 194.8 ($CH_3C(O)S$).

Protected pentasaccharide (15): Coupling of tetrasaccharide **14** [14] (301 mg, 0.21 mmol) with **5** (186 mg, 0.25 mmol) was accomplished in anhydrous diethyl ether (6.5 mL). After stirring for 1 h in the presence of activated molecular sieves 4 Å (200 mg), the solution was cooled (−20 °C) and a stock solution (0.34 mL) of trimethylsilyl trifluoromethanesulfonate (50 µL) in diethyl ether (3 mL) was added dropwise to the reaction mixture under a flow of argon. After 10 min, solid $NaHCO_3$ (0.5 g) was added, and the reaction mixture was filtered. The filtrate was diluted with diethyl ether (50 mL), washed twice with $NaHCO_3$ (1 M, 25 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was first purified on Sephadex LH 20 (CH_2Cl_2/MeOH, 2/1, v/v) and subsequently by silica gel purification (100/0 to 98/2 CH_2Cl_2/MeOH) to give pure α-coupled product **15** (52% yield, 220 mg). $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 20.7–20.8 (4×CH_3COO), 50.5 (CH_2N_3), 55.0, 57.8, 58.8 ($CH_3O_{E,G,H}$), 61.4, 62.3 (C-$6_{D,F}$), 66.3 (C-6_H), 69.1, 70.1, 71.0, 71.6, 74.2, 74.8, 75.7, 76.0, 76.2, 77.9, 79.7, 79.9, 80.9, 83.4 (C-2_{D-H}, C-3_{D-H}, C-4_{D-H}, C-5_{D-H}), 66.6, 67.8, 68.0, 69.8, 70.5, 72.3, 72.9, 73.1, 73.2, 74.5, 75.3 (OCH_2Ph, TEG), 96.9 (C-1_D), 97.5, 97.8 (C-$1_{G,H}$), 97.8 (C-1_F), 99.0 (C-1_E), 126.9–128.7 (CH_{arom}), 134.5, 135.3 (C_{arom} of $COOBn_{E,G}$), 137.6–139.0 (C_{arom}), 167.5, 168.6 ($COOBn_{E,G}$), 169.7, 170.2, 170.6, 170.9 (CH_3COO).

Deprotected pentasaccharide (16): Compound **15** (220 mg, 0.13 mmol) was dissolved in *tert*-butanol (35 mL), water (3 mL), and a few drops of acetic acid. The solution was stirred under a hydrogen atmosphere in the presence of 10% Pd/C (220 mg). After 16 h, TLC analysis (EtOAc/pyridine/AcOH/water, 5/7/4/1.6, v/v/v/v) showed the presence of one product. The reaction mixture was filtrated and the filtrate was concentrated in vacuo. The debenzylated pentamer was dissolved in methanol (1.4 mL) and treated with NaOH (0.4 N, 4.0 mL) for 12 h. The reaction mixture was neutralized with HCl (0.1 N), concentrated to a small volume and desalted on a Sephadex G-25 column, which was eluted with CH_3CN/H_2O (1/9, v/v). The appropriate fractions were pooled and concentrated to afford **16** (69.4 mg, 64% overall yield). $[\alpha]_D$ = +86.2 (*c* = 1, H_2O); ^{1}H NMR (D_2O, 600 MHz, HH-COSY, TOCSY): δ = 3.60, 3.49, 3.38 (3×s, 3H, $CH_3O_{E,G,H}$), 3.20 (t, 2H, *J* = 5.2 Hz, CH_2NH_2), 3.75 (c, 2H, $CH_2CH_2NH_2$), 3.71 (brs, 12H, OCH_2 TEG), ring D: 5.33 (d, 1H, $J_{1,2}$ = 4.0 Hz, H-1), 3.48 (c, 1H, H-2), 3.75 (c, 1H, H-3), 3.31 (t, $J_{4,3} \approx J_{4,5}$ = 10.3 Hz, H-4), 3.72 (c, 1H, H-5), ring E: 4.47 (d, 1H, $J_{1,2}$ = 7.4 Hz, H-1), 3.47 (c, 1H, H-2), 3.53 (c, 1H, H-3), 3.79 (c, 1H, H-4), 3.82 (c, 1H, H-5), ring F: 5.11 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1), 3.54 (dd, 1H, $J_{2,1}$ = 3.8 Hz, $J_{2,3}$ = 1.9 Hz, H-2), 3.78 (c, 1H, H-3), 3.83 (c, 1H, H-4), 3.59 (c, 1H, H-5), ring G: 4.78 (d, 1H, $J_{1,2}$ = 3.8 Hz, H-1), 3.56 (d, 1H, $J_{2,1}$ = 3.8 Hz, H-2), 3.69 (c, 1H, H-3), 3.60 (c, 1H, H-4), 3.74 (c, 1H, H-5), ring H: 4.93 (d, 1H, $J_{1,2}$ = 2.6 Hz, H-1), 3.76 (c, 1H, H-2), 3.69 (c, 1H, H-3), 4.19 (t, 1H, $J_{4,3} \approx J_{4,5}$ = 2.6 Hz, H-4), 4.65 (c, 1H, H-5); $^{13}C\{^1H\}$ NMR (D_2O, 300 MHz, CH-COSY): δ = 39.8 (CH_2NH_2), 55.6, 58.6, 60.6 ($CH_3O_{E,G,H}$), 60.0, 60.5, 60.8 (C$6_{D,F,H}$), 67.1 ($CH_2CH_2NH_2$), 70.2, 70.3, 70.4, 70.8, 71.6 (OCH_2 TEG), 68.2, 69.7, 71.2, 71.3, 71.7, 72.1, 72.5, 72.9, 74.0, 74.6, 76.9, 77.3, 78.1, 78.3, 78.5 (C-2_{D-H}, C-3_{D-H}, C-4_{D-H}, C-5_{D-H}), 96.3 (C-1_F), 98.6 (C-1_D), 99.7 (C-1_G), 102.1 (C-1_H), 102.8 (C-1_E), 175.7, 176.0 ($COO_{E,G}$); ES-MS: $[M+H]^+$ = 1074, $[M+Na]^+$ = 1096, $[M+2Na]^+$ = 1118 (calcd. 1073).

Sulfated and thioacetyl-tethered pentasaccharide (17c): To a solution of **16** (16.4 mg, 15.3 µmol) and **13** (8.97 mg, 22.0 µmol) in a mixture of H_2O/DMF (1/2, v/v, 450 µL) was added *N*-methylmorpholine (5 µL). After stirring for 5 min, the reaction mixture was directly applied onto a RP-18 column, which was eluted with H_2O/MeOH (90/10 to 60/40). The appropriate fractions were pooled, concentrated to a small volume, and applied to a Dowex 50 WX4-H^+ ion-exchange column in water. The eluate was concentrated and coevaporated three times with DMF and dissolved in DMF (1 mL). Under a nitrogen atmosphere triethylamine sulfur trioxide complex (0.173 g, 5 equiv for each hydroxyl group) was added and the mixture was stirred at 55 °C for 16 h. The mixture was cooled to 0 °C and aqueous $NaHCO_3$ was added (5 equiv for each equiv of triethylamine sulfur trioxide complex). The mixture was stirred for 1 h, concentrated to a small volume and applied onto a Sephadex G-25 column, which was eluted with CH_3CN (10%) in H_2O. The appropriate fractions were pooled and concentrated to a small volume which was passed through a column of Dowex 50 WX4 (Na^+ form) eluted with water. The eluate was concentrated and redissolved in HCl (0.2 N, 1 mL) and allowed to stand for 16 h at 4 °C. HPLC analysis (MonoQ column) indicated that the N–SO_3^- bond was selectively cleaved. The reaction mixture was neutralized with NaOH (0.1 N), desalted on a Sephadex G-25 column, and eluted with CH_3CN (10%) in water. The appropriate fractions were pooled, concentrated and lyophilized to give **17c** as a white fluffy solid (25.1 mg, 64% overall yield). $[\alpha]_D$ = +24.4 (*c* = 0.3, H_2O); ^{1}H NMR (D_2O, 360 MHz, HH-COSY): δ = 2.58 (s, 3H, CH_3CO), 3.13 (t, 2H, *J* = 6.2 Hz, CH_2SAc), 3.49 (t, 2H, *J* = 5.4 Hz, CH_2NH), 3.45, 3.59, 3.66 (3×s, 3H, $CH_3O_{E,G,H}$), 3.68–3.80 (c, OCH_2 TEG), 4.11 (s, 2H, $OCH_2C(O)NH$), ring D: 5.59 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 4.25 (dd, 1H, $J_{2,1}$ = 3.5 Hz, $J_{2,3}$ = 10 Hz, H-2), 4.64 (c, 1H, H-3), 3.66 (dd, 1H, $J_{4,3} \approx J_{4,5}$ = 7.9 Hz, H-4), 4.01 (c, 1H, H-5), 4.18, 4.26 (c, 2H, H-6a, H-6b), ring E: 4.74 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1), 4.27 (c, 1H, H-2), 3.66 (c, 1H, H-3), 3.96 (dd, H1, $J_{4,3} \approx J_{4,5}$ = 9.7 Hz, H-4), 3.82 (d, 1H, $J_{5,4}$ = 9.7 Hz, H-5), ring F: 5.49 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1), 4.32 (c, 1H, H-2), 4.57 (c, 1H, H-3), 3.94 (c, 1H, H-4), 4.05 (c, 1H, H-5), 4.56, 4.35 (c, 2H, H-6a, H-6b), ring G: 5.15 (c, 1H, H-1), 4.42 (dd, 1H, $J_{2,1}$ = 3.6 Hz, $J_{2,3}$ = 9.7 Hz, H-2), 3.80 (c, 1H, H-3), 4.28 (c, 1H, H-4), 4.93 (d, 1H, $J_{4,5}$ = 4.1 Hz, H-5), ring H: 5.14 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.35 (c, 1H, H-2), 4.58 (t, 1H, $J_{3,2} \approx J_{3,4}$ = 8.4 Hz), 4.00 (c, 2H, H-4, H-5), 4.48, 4.38 (c, 2H, H-6a, H-6b).

Condensation coupling of pentasaccharide 17c with T_{18} ODN (18): To a solution of oligonucleotide **18** (20.8 mg, 3.45 µmol) in 0.1 M NaH_2PO_4 buffer (pH = 7.5, 2.5 mL) was added **20** (8.7 mg, 17.3 µmol). After stirring for 3 h, HPLC analysis (RP column) indicated complete derivatization of **18**. The reaction mixture was applied to a Superdex 30 column, which was eluted with MeOH in water (10%). The appropriate fractions were pooled and concentrated under reduced pressure at 25 °C. The derivatized ODN **21** and pentasaccharide **17c** (6.9 mg, 2.76 µmol) were dissolved in a degassed hydroxylamine solution (0.05 M, 1.5 mL) in NaH_2PO_4 buffer

(0.1 M, pH = 7.0). The reaction was monitored by HPLC analysis (RP column and MonoQ) which indicated the formation of a conjugate (65%) after 1 h. After 48 h, the reaction showed no further progression, and the solution was applied onto a Sephadex G-50 column which was eluted with NaCl (0.05 M) in CH_3CN in H_2O (10%). The conjugate-containing fractions were pooled, concentrated to a small volume and desalted on a Superdex 30 column, which was eluted with MeOH in H_2O (10%). Concentration and lyophilization of the appropriate fractions gave conjugate **III** (12 mg, 52% yield). $[\alpha]_D = +30.2$ ($c = 0.2$, H_2O); ^1H NMR (D_2O, 600 MHz, HH-COSY): $\delta = 2.82$ (t, 2H, $J = 6.1$ Hz, CH_2SR), 2.85–3.11 (m, $(CH_2)_3N$, Miller cap), 3.57 (t, 2H, $J = 5.4$ Hz, CH_2NH), 3.39, 3.43, 3.52 (3 × s, 3H, $CH_3O_{E,G,H}$), 3.68–3.58 (c, OCH_2 TEG), 3.99 (s, 2H, $OCH_2C(O)NH$), ring D: 5.53 (d, 1H, $J_{1,2} = 2.9$ Hz, H-1), 4.11 (c, 1H, H-2), 4.56 (c, 1H, H-3), 3.57 (c, 1H, H-4), 3.89 (c, 1H, H-5), 4.13, 4.27 (c, 2H, H-6a, H-6b), ring E: 4.69 (c, 1H, H-1), 4.22 (c, 1H, H-2), 3.62 (c, 1H, H-3), 3.91 (c, H1, H-4), 3.75 (c, 1H, H-5), ring F: 5.39 (d, 1H, $J_{1,2} = 3.4$ Hz, H-1), 4.28 (c, 1H, H-2), 4.65 (c, 1H, H-3), 3.85 (t, 1H, $J_{4,3} \approx J_{4,5} = 9.8$ Hz, H-4), 4.11 (c, 1H, H-5), ring G: 5.18 (brs, 1H, H-1), 4.33 (c, 1H, H-2), 3.76 (c, 1H, H-3), 4.11 (c, 1H, H-4), 4.79 (c, 1H, H-5), ring H: 5.07 (d, 1H, $J_{1,2} = 3.3$ Hz, H-1), 4.32 (c, 1H, H-2), 4.56 (c, 1H, H-3), 4.57 (c, 1H, H-4), 3.94 (c, 1H, H-5), 4.39, 4.31 (c, 2H, H-6a, H-6b), T_{18} ODN: 1.80 (s, 54H, CH_3), 2.25, 2.45 (2 × m, 36H, H-2′, H-2″), 4.02 (2 × m, 36H, H-5′, H-5″), 4.19 (brs, 18H, H-4′), 4.82 (brs, 18H, H-3′), 6.16 (t, 18H, $J_{1,2} = 6.5$ Hz, H-1′), 7.56 (s, 18H, H-6), 7.72, 7.53 (2 × d, $J = 6.1$ Hz, CH_{arom} sulfo-SIAB); ES-MS: $[M - H]^- = 8003$, $[M - 2H + NH_4^+]^- = 8021$ (calcd. 8002).

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