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Apolar carbohydrates as DNA capping agents

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Dedicated to Professor Soledad Penadés on her 70th birthday.

Abstract. Mono- and disaccharides have been shown to stack on top of DNA duplexes stabilizing sequences with terminal C–G base pairs. Here we present an apolar version of glucose and cellobiose as new capping agents that stack on DNA increasing considerably its stability with respect to their natural polyhydroxylated mono- and disaccharide DNA conjugates.

Non-covalent forces direct molecular interactions between biomolecules and their combination and interplay in biology rules life. DNA being the central molecule of life also gives the chance to study molecular interactions in aqueous media. Aromatic π–π stacking interactions have extensively been studied using DNA as a model. Both natural[1] and non-natural[2–4] aromatic bases attached to the 30-end or 50-end of double stranded DNA have shown enhanced stabilization of DNA duplexes, acting as capping agents. These molecular ''caps'' are usually planar aromatic rings of different size and shape that take advantage of π–π stacking interactions.[5–8] The only non-planar compounds described to stack on DNA are steroids such as cholic acid which showed a high increase in DNA stability via CH–π interactions.[9] Recently, binaphthyl and phenylcyclohexyl nucleosides[10,11] with nonplanar aromatic bases have been included inside DNA but no data as capping entities were reported.

Our group has studied carbohydrate–aromatic stacking interactions using carbohydrate oligonucleotide conjugates (COCs) with dangling-ends as a model. First, we evaluated monosaccharide–phenyl interactions as a double dangling motif at the edge of a duplex of DNA.[12] We found that stabilization varies from -0.15 to -0.40 kcal mol^{-1} and depends on the number of hydroxyl groups and stereochemistry. Recently, we have shown that highly polar carbohydrates can act as DNA capping molecules. Sugar stacking is observed for mono- and disaccharides on top of C–G or T–A base pairs as the edge of theDNAduplex.[13] Nevertheless, stabilization of the DNA double helix is only observed with C–G or G–C terminal base pairs.

Herein, we report the synthesis of oligonucleotides with permethylated mono- and disaccharides covalently linked to their 50-end. These apolar carbohydrates act as new capping molecules capable of stacking on double-stranded DNA (Fig. 1). Permethylated glucose and cellobiose were found to stabilize DNA duplexes much more than natural glucose and cellobiose.

Synthesis of the permethylated carbohydrate oligonucleotide conjugates started with the preparation of the corresponding permethylated glucose and cellobiose phosphoramidite derivatives (**5** and **10**, respectively) (Scheme 1). Glycosylation of the O-benzyl protected ethylene glycol spacer followed by deprotection of the acetyl groups yielded intermediate **2**. Methylation under standard conditions produced compound **3** in good overall yield (70%, 3 steps). Further hydrogenation and standard phosphoramidite preparation proceeded uneventfully to yield permethylated glucose phosphoramidite **5** (76%, 2 steps). A similar synthetic strategy was followed to prepare permethylated cellobiose phosphoramidite **10** (48% yield, 5 steps).

Preparation of the apolar saccharide oligonucleotide conjugates was carried out by standard solid phase oligonucleotide synthesis using compounds **5** or **10** at the last coupling step. Both apolar carbohydrates were attached to self-complementary sequences CGCGCG, GGCGCC, AGCGCT and TGCGCA. Solutions of the COCs were subjected to UV melting analysis and thermodynamic parameters were calculated (Table 1).

Conjugates containing permethylated glucose and cellobiose on sequences terminated on a C–G base pair (conjugates **15** and **19**) increased considerably their melting points (7.8 ºC and 8.3 ºC, respectively) over those of the natural control sequence **11**.

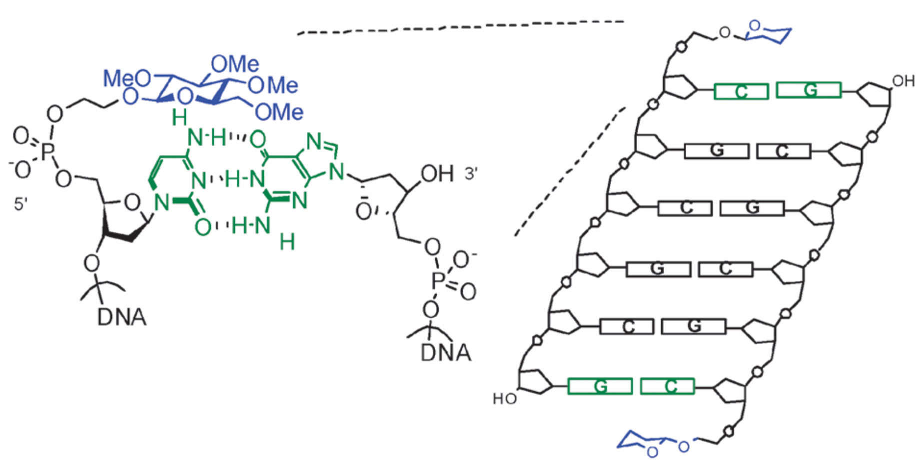
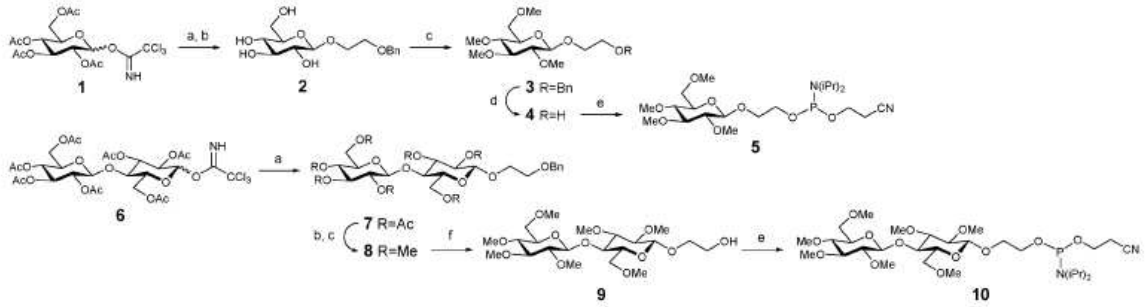


Fig. 1 Schematic drawing of COCs with dangling-ends and details of one of them (permethylated glucose stacking on top of a C–G base pair).



Scheme 1 Synthesis of permethylated glucose and cellobiose phosphoramidites **5** and **10**. Reaction conditions: (a) $BnOCH_2CH_2OH$, BF_3-OEt_2, CH_2Cl_2; (b) Na_2CO_3, MeOH; (c) MeI, NaH, DMF; (d) H_2, $Pd(OH)_2$, THF–MeOH; (e) 2-cyanoethyl-*N*,*N*′-diisopropylamino-chlorophosphoramidite, DIEA, CH_2Cl_2; (f) H_2, $Pd(OH)_2$, AcOEt–MeOH.

Table 1. Thermodynamic parameters for COC's

X-DNA sequences[a,b,c,d]	T_m [e] / ºC	-ΔHº	-ΔSº	-ΔGº$_{37}$	-$\Delta\Delta G$º$_{37}$
X = none[f]					
CGCGCG **11**	40.9	46.5	123	8.2	--
AGCGCT **12**	33.5	40.3	107	7.1	--
X = glucose-C2[f]					
CGCGCG **13**	44.0	52.1	140	8.7	-0.5
AGCGCT **14**	33.6	37.3	98	7.0	0.1
X = glc(Me)-C2[f]					
CGCGCG **15**	48.7	55.0	147	9.4	-1.2
AGCGCT **16**	34.5	44.8	121	7.2	-0.2
X = cellobiose-C2[f]					
CGCGCG **17**	45.9	49.2	130	8.9	-0.7
AGCGCT **18**	34.4	39.1	103	7.1	0.0
X = cellob(Me)[f]					
CGCGCG **19**	49.2	55.0	146	9.7	-1.5
AGCGCT **20**	37.7	43.9	117	7.7	-0.6

[a] –C2– states for $-CH_2-CH_2-OPO_2^-$–. [b] Buffer: 10 mM Na phosphate, 1 M NaCl, pH 7.0. [c] Estimated errors are: T_m ± 0.8 ºC and ±6% in ΔGº. [d] Units for ΔHº and ΔGº are kcal mol^{-1} and for ΔSº are cal K^{-1} mol^{-1}. [e] Average value of three experiments measured at 5 μM conc. [f] From ref. 13.

When conjugates with apolar glucose **15** and apolar cellobiose **19** are compared with their corresponding natural hydroxylated versions glucose–DNA conjugate **13** and cellobiose– DNA conjugate **17**, T_m's are increased by 4.7 ºC and 3.3 ºC, respectively. A similar trend is observed when ΔG values are compared; conjugates **15** and **19** stabilize CGCGCG duplexes by -1.2 and -1.5 kcal mol^{-1}, respectively, with respect to unmodified CGCGCG. This stabilization is similar to that found for a benzene nucleoside in the same context.[2] As a result, the duplex stabilizations of conjugates with the apolar version of glucose **15** and cellobiose **19** are 2.4 and 2.1 times more stable, respectively, than their corresponding conjugates with natural glucose **13** and cellobiose **17**. The smaller increase in cellobiose may be due to the fact that the increased surface of the apolar version of

cellobiose could be too large to fully stack on top of the C–G base pair. Similar results were found when the apolar sugars were attached to the GGCGCC sequence (see ESIz, Table S3).

In the case of the AGCGCT sequence, both conjugates with permethylated glucose 16 and cellobiose 20 show an increase in T_m (1 ºC and 4.2 ºC, respectively) and in free energy (-0.1 and -0.6 kcal mol^{-1}, respectively) with respect to the natural sequence **12**. Once again, similar results were found when the apolar carbohydrates were attached to the TGCGCA sequence (see Table S3, ESI). This decrease of COC stabilization on sequences with A–T or T–A base pairs at the edge of the duplex with respect to the sequences with C–G or G–C base pairs was also observed for COCs with the natural mono- and disaccharides. This effect may be due to the larger entropy cost of reducing the fraying in the more flexible terminal A–T base pair that counteracts the stabilization obtained with the stacking of the apolar sugar.[13]

The structures of the conjugates containing the permethylated glucose unit **15** and **16** were studied by NMR spectroscopy. Proton assignment was carried out following standard procedures. The DNA duplex structures are barely distorted by the presence of the apolar sugars as can be inferred by comparison of the DNA chemical shifts of the conjugates and the control sequences (see ESI, Fig. S2). Chemical shift changes are mostly observed in the neighboring residues of the permethylated glucose (C1 in the CGCGCG sequence and A1 in the AGCGCT sequence), indicating that the carbohydrate is interacting mainly with the terminal residues. This capping interaction is also supported by a significant number of NOEs (see Fig. 2 and Table S2, in ESI). The number and intensities of these NOE contacts are comparable with those observed in the disaccharide conjugates studied in our previous work.[13] Strong and medium NOEs are observed between several protons of the terminal base-pairs with H3 and H5 of the apolar glucose unit, suggesting that the permethylated glucose interacts with the terminal base-pair of the duplex predominantly through its a face. In the case of conjugate **16** some low intensity NOEs are also observed with H4 proton. These NOEs may arise from spin-diffusion or from minor species with different carbohydrate conformations, and were not used in the structural calculations. Interestingly, many of the DNA-permethylated glucose NOEs involve exchangeable protons of the terminal base-pair. In both conjugates, these protons exhibit narrow signals, indicating that they are protected from water exchange. As in the case of the natural disaccharide–DNA conjugates studied previously, the capping carbohydrate reduces strongly the internal dynamics of the terminal base-pairs. This effect is especially pronounced in conjugate **16**, where the terminal base-pair is AT.

Restrained molecular dynamics calculations were carried out with the AMBER program. Resulting structures are shown in Fig. 3. In both conjugates **15** and **16**, the carbohydrate and the linker adopt a similar and well-defined structure. Permethylated glucoses stack on top of the terminal base-pair, with their α sides oriented towards the nucleobases. Carbohydrate conformation is the usual $^4C_1$ chair. Permethylation increases the carbohydrate size and allows for an enhanced stacking interaction in which a single monosaccharide covers most of the terminal base-pair

surface (Fig. 3). Although the main features of both conjugates are quite similar, minor differences are observed (see Fig. 3, top). These differences are probably due to the different adjacent nucleobase, purine in the case of conjugate **15** and pyrimidine for **16**.

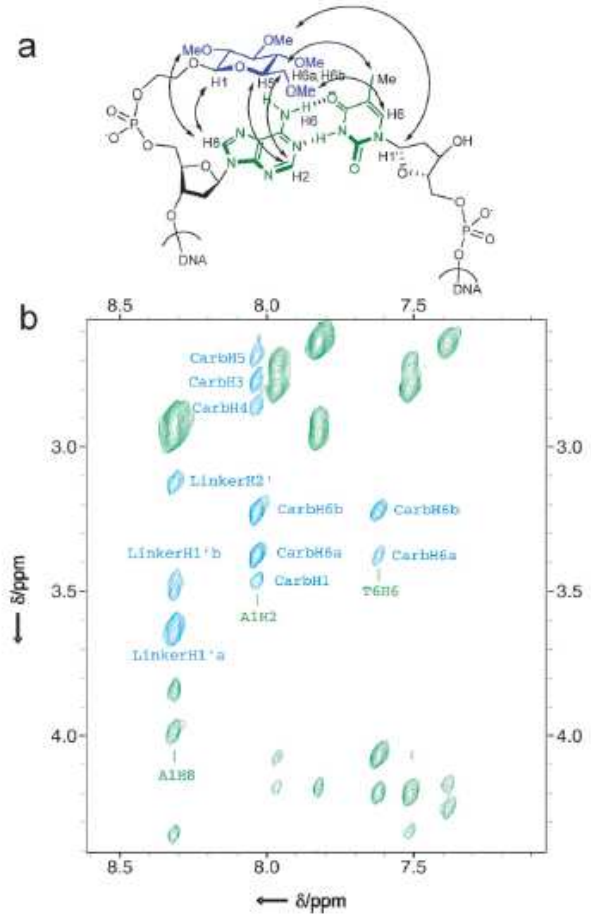


Fig. 2 (a) Schematic drawing of conjugate **16** with arrows indicating important observed NOEs; (b) selected region of NOESY spectra for conjugate **16** (carbohydrate–DNA contacts are shown in cyan).

These results are noteworthy since hydrophobic mono- and disaccharides attached to DNA show a relevant increase in stabilization of DNA duplexes especially with terminal C–G or G–C base pairs. In this context, the stability of DNA with apolar sugars 5'-caps is approaching to that found with the traditional aromatic caps. Further improvement may be obtained modulating the hydrophobicity of the carbohydrate. NMR studies confirmed that permethylated sugars stack on top of duplex DNA similarly to other aromatic moieties. Finally, our results have implications in molecular recognition and may be useful in drug design and in the assembly of supramolecular structures.

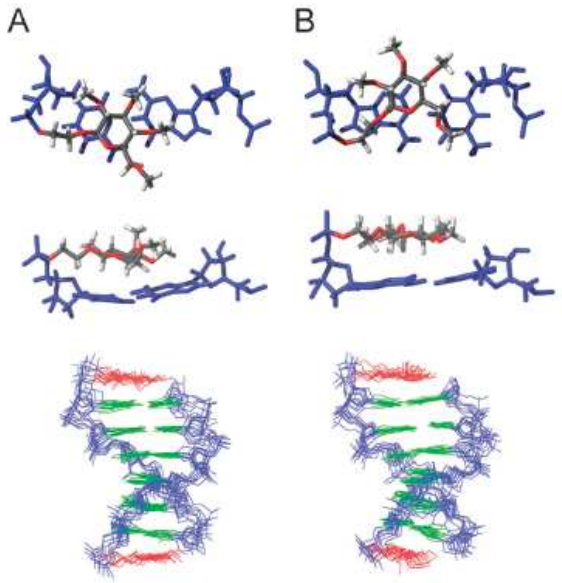


Fig. 3 Structures of conjugate **15** (A), and conjugate **16** (B). Top: details of the stacking. Bottom: superposition of ten calculated structures.

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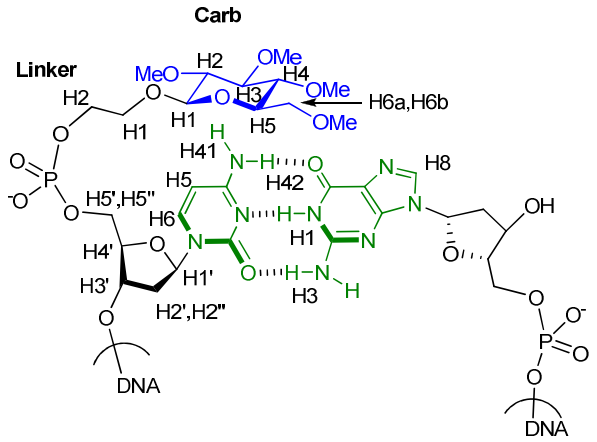
Supplementary Information

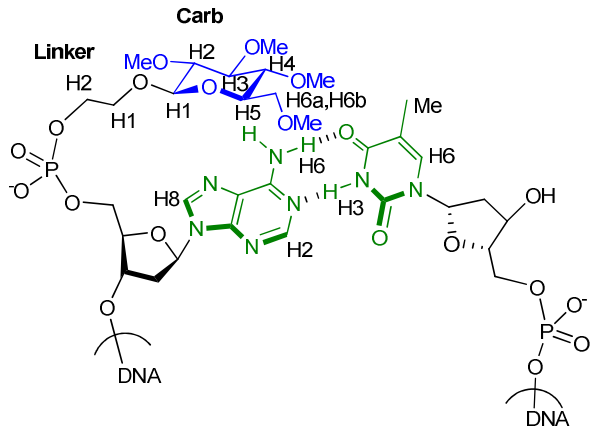
Contents

Table S1: NMR proton assignments of carbohydrate oligonucleotide conjugates **15** and **16** (5 ºC).



Conjugate 15: Glc(Me)-C2-CGCGCG

	H1’	H2’/H2”	H3’	H4’	H5’/H5”	H5	H6/H8	H1/H3	H41/H42
C1	5.40	2.35/2.46	4.85	4.12	3.97/3.91	5.97	7.72	--	7.35/8.64
G2	5.97	2.75		4.38	4.03/4.13	--	8.00	13.19	--
C3	5.77	2.11/2.47	4.91	4.24	4.06/4.14	5.48	7.42	--	6.62/8.49
G4	5.96	2.68/2.77		4.40	4.06/4.14	--	7.97	13.14	--
C5	5.75	1.73/2.23	4.83	4.13	4.07/4.24	5.53	7.36	--	6.81/8.57
G6	6.22	2.75/2.38	4.73	4.24	4.06	--	8.02	13.30	--
	LH1a/H1b	LH2a/H2b	H1	H2	H3	H4	H5	H6a/H6b	Met
glc	3.83/3.93	3.70/3.56	3.97	2.80	2.98	2.91	2.98	3.35/3.23	3.42



Conjugate 16: Glc(Me)-C2-AGCGCT

	H1’	H2’/H2”	H3’	H4’	H5’/H5”	H5/H2	H6/H8	H1/H3	H41/H42
A1	6.01	2.94/2.92	4.85	4.32	3.84/3.98	8.04	8.29	--	--
G2	5.80	2.63		4.41	4.17	--	7.83	12.91	--
C3	5.82	2.09/2.47	4.90	4.25	4.17	5.36	7.38	--	6.46/8.30
G4	5.99	2.79/2.71	4.80	4.42	4.07	--	7.97	13.08	--
C5	6.07	2.08/2.52	4.80	4.21	4.07	5.50	7.51	--	6.85/8.42
T6	6.34	2.37/2.25	4.64	4.07	4.20	1.76-Me	7.62	--	--
	LH1a/H1b	LH2a/H2b	H1	H2	H3	H4	H5	H6a/H6b	Met
glc	3.62/3.47	3.12	3.45	2.62	2.76	2.85	2.69	3.22/3.37	3.22

Control 12: AGCGCT

	H1’	H2’/H2”	H3’	H4’	H5’/H5”	H5/H2	H6/H8	H1/H3	H41/H42
A1	5.98	2.54/2.70	4.86	4.23	3.71		8.07		
G2	5.83	2.70		4.41	4.13/4.24		7.91	12.97	
C3	5.80	2.08/2.45	4.88	4.24	4.08/4.17	5.38	7.38		6.50/8.32
G4	5.97	2.78/2.69		4.41	4.08/4.19		7.96	13.07	
C5	6.13	2.26/2.52	4.80	4.26	4.08/4.20	5.52	7.52		6.82/8.41
T6	6.30	2.31	4.20	4.09	4.23	1.77-Me	7.61		

Table S2: Structurally relevant carbohydrate-DNA NOE contacts for the carbohydrate oligonucleotide conjugates glc(Me)-C2-CGCGCG **15** and glc(Me)-C2-AGCGCT **16. (**Strong NOE: s, medium NOE: m, weak NOE: w, very weak NOE: vw).

15	
Carb	Linker
C1H5-H3/GlcH5: w	C1H5-LH2': s
C1H4'-GlcMe: s	C1H5-LH1': m
C1H4'-GlcH3/H5: w	C1H6-LH2': m
C1H1'-GlcH3/H5: vw	C1H6-LH1': m
C1H41-GlcH3: w	C1H4'-LH2': vw
C1H42-GlcH3: w	
C1H42-GlcMe: w	
*G6H8-GlcH3/H5: m	
G6H1'-GlcMe: s	
*G6H1'-GlcH3/H5: m/s	
G6H3-GlcH3: s	
G6H3-GlcMe: vw	

A1H4'-GlcMe: w
T6Me-GlcH6a: w
T6Me-GlcH6b: m
T6H6-GlcMe: m
T6H6-GlcH6a: w
T6H6-GlcH6b: w
T6H1'-GlcMe: w
T6H1'-GlcH6a: w
T6H1'-GlcH6b: m
T6H2'/H2''-GlcH6a: m
T6H2'/H2''-GlcH6b: m

16	
Carb	Linker
A1H2-GlcMe: m	A1H8-LH1'a: s
A1H2-GlcH6a: m	A1H8-LH1'b: s
A1H2-GlcH6b: m	A1H8-LH2': m
A1H2-GlcH1: w	A1H5'/H5''- LH2': w
A1H2-GlcH3: m	
*A1H2-GlcH4: w	
A1H2-GlcH5: m	
A1H8-GlcMe: w	
A1H8-GlcH1: m	
*A1H8-GlcH2: w	

*Not used in structure calculations

Figure S1: Schematic drawings of conjugates **15** and **16** indicating relevant carbohydrate-DNA NOEs.

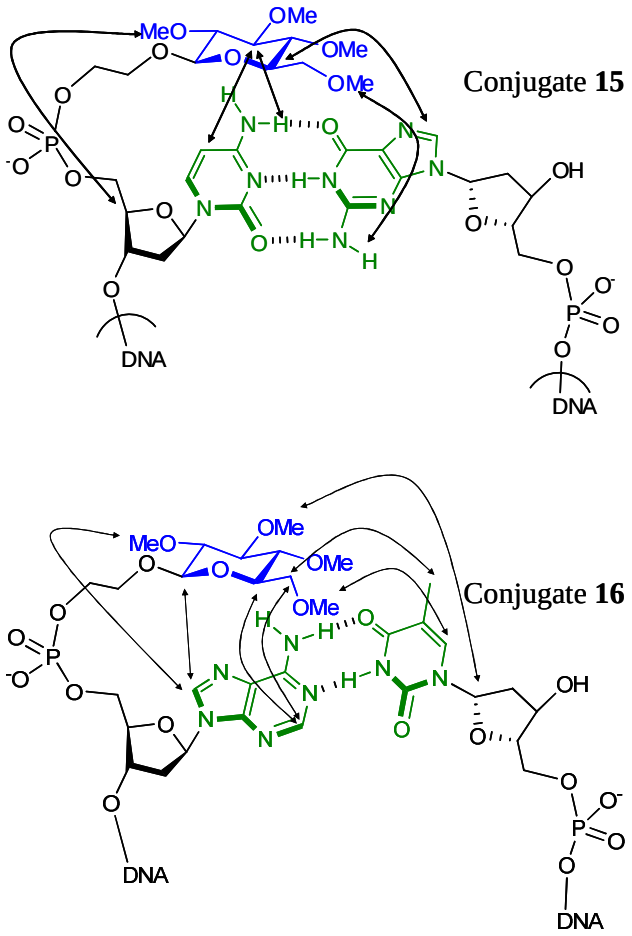
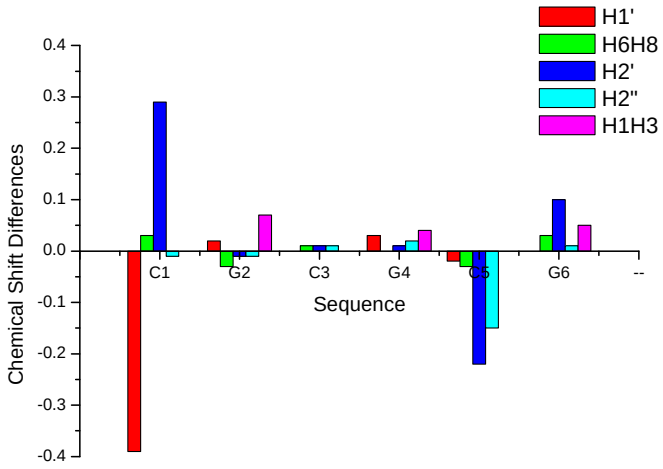


Figure S2: Changes in proton chemical shifts along the sequence for the carbohydrate-oligonucleotide conjugates **15** and **16** with respect to DNA controls **11** and **12**, respectively.

Conjugate **15**: glc(Me)-C2-CGCGCG.



Conjugate **16**: glc(Me)-C2-AGCGCT**.**

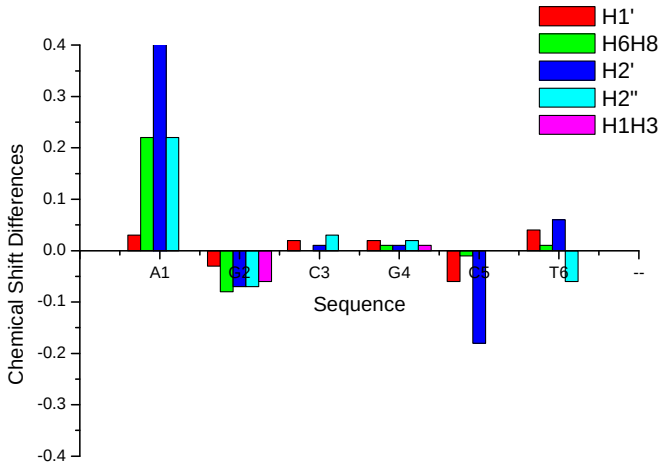
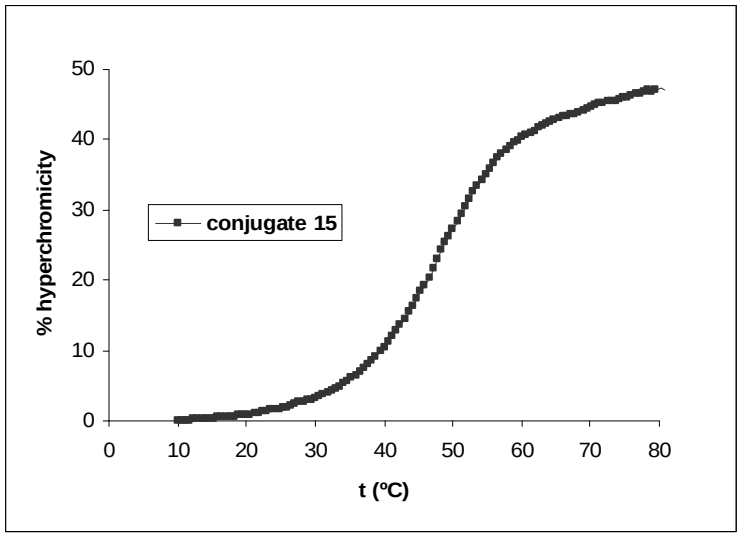


Figure S3: Melting curves for: Glc(Me)-C2- CGCGCG **15** and Glc(Me)-C2- AGCGCT **16**.


% hyperchromicity
t (ºC)
conjugate 15
0
10
20
30
40
50
60
70
80

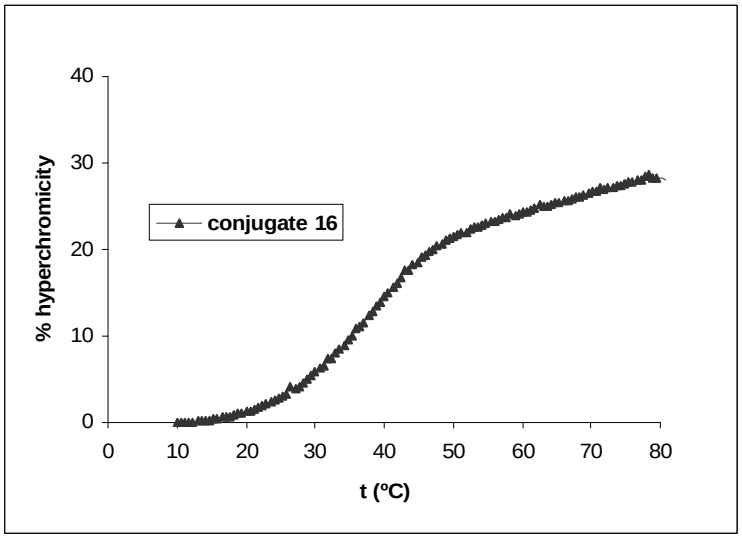
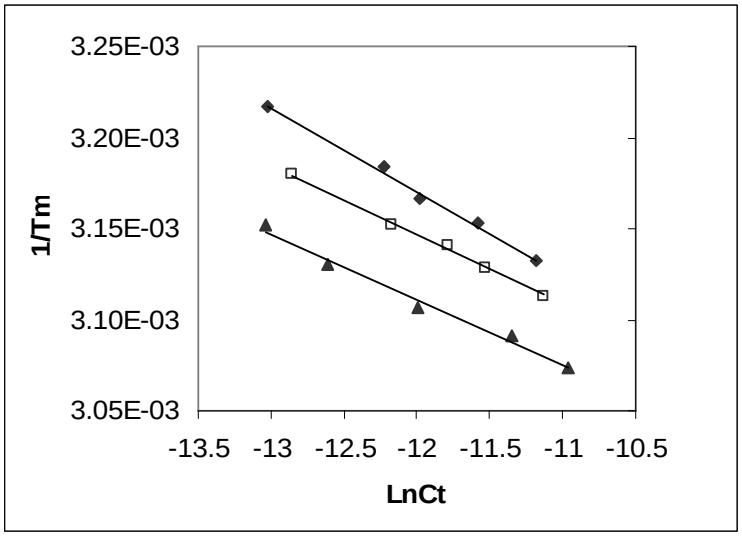
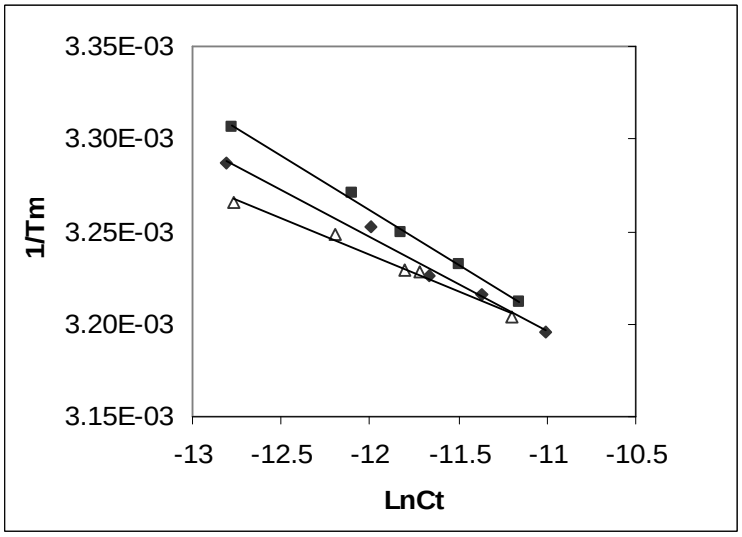

% hyperchromicity
t (ºC)
conjugate 16
0
10
20
30
40
50
60
70
80

Figure S4: Van't Hoff curves for DNA controls **11** and **12** and for DNA carbohydrate-oligonucleotide conjugates **13, 14, 15, 16, 17, 18, 19**, **25**, **26**, **29** and **30**.

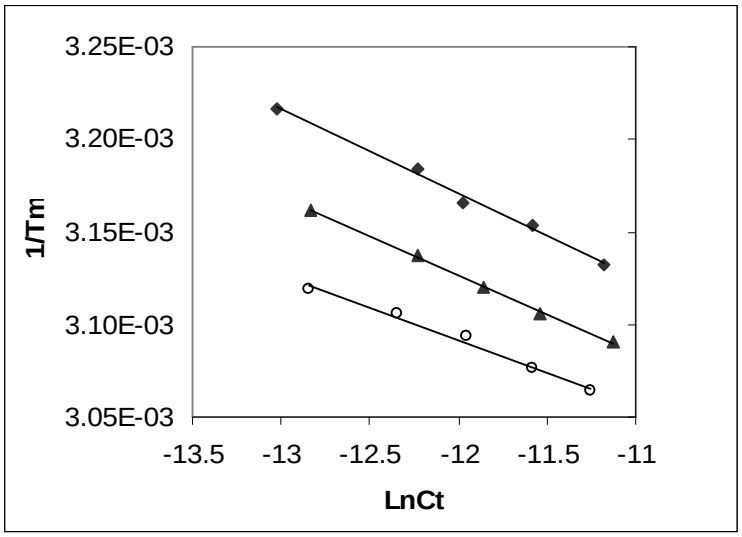
DNA control **11**: CGCGCG (◆), conjugate **13**: glc-C2-CGCGCG (□) and conjugate **15**: glc(Me)-C2-CGCGCG (▲)**.**



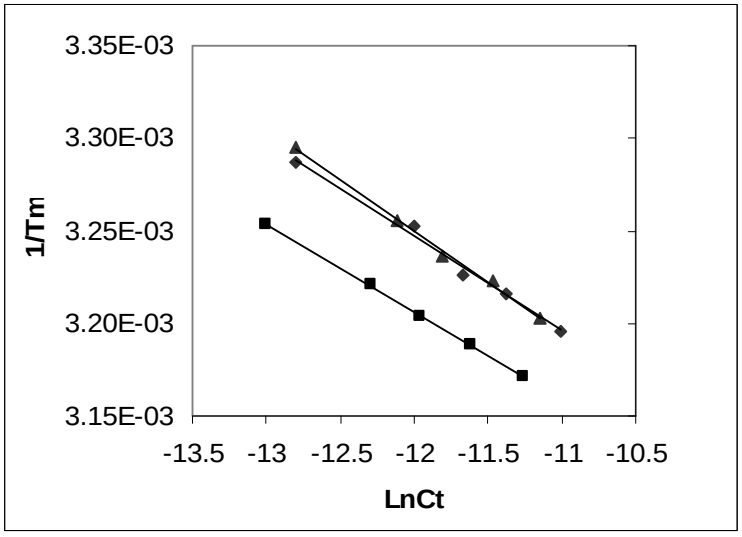
DNA control **12**: AGCGCT (◆), conjugate **14**: glc-C2-AGCGCT (■) and conjugate **16**: glc(Me)-C2-AGCGCT (△)**.**



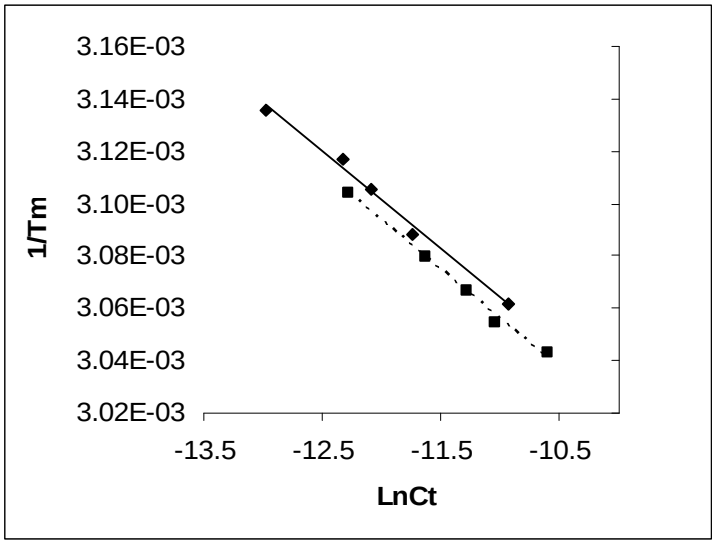
DNA control **11**: CGCGCG (◆), conjugate **17**: cellob-C2-CGCGCG (▲) and **19**: cellob(Me)C2-CGCGCG (○)**.**



DNA control **12**: AGCGCT (◆), conjugate **18**: cellob-C2-AGCGCT (▲) and conjugate **20**: cellob(Me)-C2-AGCGCT (■)**.**



Conjugate **25**: glc(Me)-C2-GGCGCC (◆) and conjugate **29**: glc(Me)-C2-GGCGCC (■)**.**



Conjugate **26**: glc(Me)-C2-TGCGCA (•) and conjugate **30**: cellob(Me)C2-TGCGCA (○)**.**

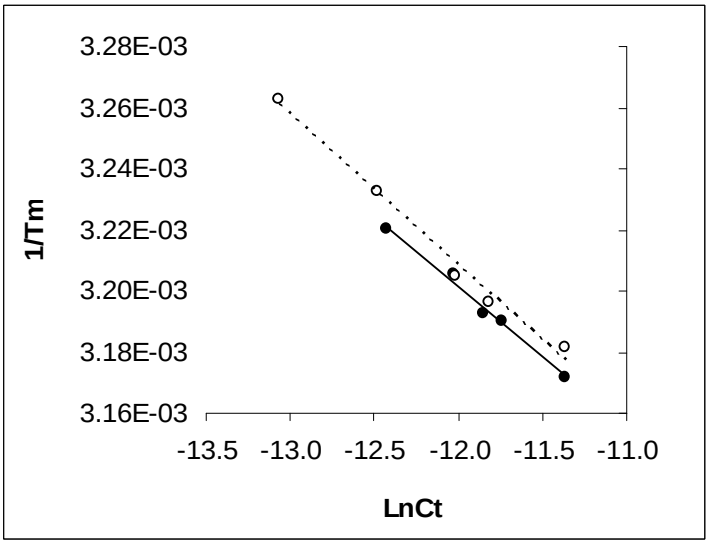


Table S3: Tm's and thermodynamic data for COCs **23** to **30** and their corresponding controls **21** and **22**.

X-DNA sequence *a,b,c,d*	Tm (°C) [e]	$-\Delta H^\circ$	$-\Delta S^\circ$	$-\Delta G_{37}$	$\Delta\Delta G_{37}^\circ$
X= none [f]					
GGCGCC **21**	37.6	45.6	122	7.8	-
TGCGCA **22**	34.8	37.8	99	7.2	-
X=glucose-C2 [f]					
GGCGCC **23**	42.2	46.7	124	8.3	-0.5
TGCGCA **24**	34.2	47.8	131	7.2	0.0
X=glc(Me)-C2					
GGCGCC **25**	48.7	53.5	142	-9.5	-1.7
TGCGCA **26**	37.4	44.7	119	-7.7	-0.5
X=cellobiose-C2 [f]					
GGCGCC **27**	44.2	51.9	139	8.7	-0.9
TGCGCA **28**	35.2	43.9	118	7.2	0.0
X=cellob(Me)-C2					
GGCGCC **29**	50.7	54.0	143	-9.6	-1.8
TGCGCA **30**	37.6	43.1	114	-7.6	-0.4

[a] -C2- states for $-CH_2-CH_2-OPO_2^-$-. [b] Buffer: 10 mM Na●phosphate, 1M NaCl, pH 7.0. [c] Estimated errors are: Tm ±0.7 °C and ±6% in ΔG°. [d] Units for ΔH° and ΔG° are kcal/mol and for ΔS° are cal/K.mol. [e] Average value of three experiments measured at 5 μM conc. [f] From ref. 6

Supplementary Methods

Synthesis. General information.

All chemicals were obtained from chemical suppliers and used without further purification, unless otherwise noted. All reactions were monitored by TLC on precoated Silica-Gel 60 plates F254, and detected by heating with Mostain (500 ml of 10% H_2SO_4, 25g of $(NH_4)_6Mo_7O_{24}\bullet 4H_2O$, 1g $Ce(SO_4)_2\bullet 4H_2O$). Products were purified by flash chromatography with silica gel60 (200-400 mesh).

NMR spectra were recorded on either a Bruker AVANCE 300 or ARX 400 or Bruker Advance DRX 500 MHz [300 or 400 MHz (1H), 75 or 100 (^{13}C), at room temperature for solutions in $CDCl_3$, D_2O or CD_3OD]. Chemical shifts are referred to the solvent signal and are expressed in ppm. 2D NMR experiments (COSY, TOCSY, ROESY, and HMQC) were carried out when necessary to assign the corresponding signals of the new compounds. High resolution FAB (+) mass spectral analyses was obtained on a Micromass AutoSpec-Q spectrometer.

Preparation and characterization of compounds 2-5 and 7-10.

2-Benzyloxyethyl-β-D-glucopyranoside (2)

To a solution of the tetraacetyl glucopyranosyl trichloroacetimidate **1**[1] (600 mg, 1.21 mmol) in anhydrous CH_2Cl_2 (10 mL) and 2-benzyloxyethanol (520 μL, 3.65 mmol), $BF_3.OEt_2$ (20 μl, 0.24 mmol) was then added. After stirring at room temperature for 1 h under argon atmosphere, NEt_3 (0.2 ml) was then added. Solvents were then removed and the crude was purified by silica gel column chromathography using as eluent (Hex-AcOEt, 2:1-2:3) to give the glycosyl derivative as a syrup. This product (560 mg, 1.16 mmol) was dissolved in dry MeOH (10 mL) and Na_2CO_3 (40 mg, 0.35 mmol) was then added. The reaction mixture was stirred for 1 h and IR-120 was then added to neutralize. Solvent was then removed and the crude was purified by silica gel column chromathography using as eluent (AcOEt:MeOH, 1:0-1:1) to give **2** (290 mg, 80%, 2 steps) as a syrup. $[\alpha]_D^{20}$ -16.2 (c 1 in MeOH); ^1H NMR (CD_3OD, 300 MHz) δ (ppm) 7.39-7.29 (m, 5 H, Ph), 4.58 (d, 1H, *J* = 7.7 Hz, H-1), 3.89-3.85 (m, 1H,), 3.80-3.65 (m, 4H,), 3.38-3.20 (m, 4H,), OC*H*$_2$CH$_2$O-). ^{13}C NMR (CD_3OD, 75 MHz) δ (ppm)

141.9 (Cq arom), 131.9, 131.7, 131.4 (Carom), 106.9 (C_1), 80.5, 80.4, 77.6, 76.8, 74.1, 73.2, 72.1, 65.3. HRMS (ES^+) Calcd. for $C_{15}H_{22}NaO_7$ (M+Na): 337.1263, found; 337.1264.

2-Benzyloxyethyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside (3)

To a solution of compound **2** (600 mg, 1.91 mmol) in anhydrous DMF (10 mL) at 0ºC, NaH (275 mg, 11.46 mmol) was added. The reaction mixture was stirred for 10 min and MeI (952 μL, 15.28 mmol) was added. After 18 h, 2-propanol was then added dropwise and finally NH_4Cl sat (50 mL) was also added. The organic phase was extracted with ethyl acetate (2x100 mL) and washed with sodium bisulfate solution (50 mL) and brine (50 mL). Solvents were then removed and the crude was purified by silica gel column chromathography using as eluent (Hex-AcOEt, 2:1-1:2) to give **3** (620 mg, 88%) as a syrup. $[\alpha]_D^{20}$ -21.5 (c 1 in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 7.26-7.18 (m, 5 H, Ph), 4.48 (s, 2 H, CH_2Ph), 4.21 (d, 1H, *J* = 7.8 Hz, H_1), 3.96-3.93 (m, 1H, OCH_2CH_2OBn), 3.68-3.57 (m, 3H, OCH_2CH_2OBn), 3.54 (s, 3 H, CH_3O), 3.52-3.47 (m, 5 H, H_6, $H_{6'}$, CH_3O), 3.30 (s, 3 H, CH_3O), 3.19-3.16 (m, 1 H, H_5), 3.08-3.05 (m, 2 H, H_3, H_4), 2.97-2.94 (m, 1H, H_2). ^{13}C NMR (CD_3OD, 75 MHz) δ (ppm) 138.2 (Cq arom), 128.4, 127.6, 127.5 (Carom), 103.5 (C_1), 86.3(C_3), 83.6 (C_2), 79.3 (C_4), 74.5 (C_5), 73.1 (CH_2Ph), 71.3 (C_6), 69.2, 68.9 (OCH_2), 60.7 , 60.4, 60.3, 59.3 (CH_3O). HRMS (ES^+) Calcd. for $C_{19}H_{30}NaO_7$ (M+Na): 393.1989, found; 393.1880.

2-Hydroxyethyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside (4)

A solution of compound **3** (500 mg, 1.349 mmol) in ethyl acetate-MeOH (1:1, 5mL) and $Pd(OH)_2$ in catalytic amount was stirred under an atmosphere of hydrogen for 18 h. The mixture was filtered off over celite and solvents were removed. The crude was purified by silica gel column chromathography using as eluent (Hex-AcOEt, 1:3) to give **4** (320 mg, 82%) as a syrup. $[\alpha]_D^{20}$ -2.5 (c 1 in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 4.20 (d, 1H, *J* = 7.8 Hz, H_1), 3.82-3.61 (m, 4H, OCH_2CH_2OBn), 3.57 (s, 4 H, $H_{6'}$, CH_3O), 3.51 (s, 3 H, CH_3O), 3.49-3.43 (m, 4 H, $H_{6'}$, CH_3O), 3.30 (s, 3 H, CH_3O), 3.29-3.24 (m, 1 H, H_5), 3.13-3.02 (2 t, 2 H, J = 9.0 Hz, H_3, H_4), 2.94 (t, 1 H, J = 9.0 Hz, H_2). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm); 103.8 (C_1), 86.4 (C_3), 83.6 (C_2), 79.5

(C_4), 74.1 (C_5), 73.4, 71.4, 62.3, 60.7, 60.4, 60.3, 59.2 (CH_3O). HRMS (ES^+) Calcd. for $C_{12}H_{24}NaO_7$ (M+Na): 303.1420, found; 303.1431.

2-(2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyloxy)ethyl (2-cyanoethyl) (*N*,*N*-diisopropyl) phosphoramidite (5)

DIEA (0.695 mL, 4.0 mmol) and 2-cyanoethyl-N, N'-diisopropylamino-chlorophosphoramidite (334 μL, 1.5 mmoL) were added to a solution of compound **4** (280 mg, 1.0 mmol) in anhydrous CH_2Cl_2 (5 mL) at room temperature under an argon atmosphere. After 1.0 h no starting material was observed. Solvent was then removed and the crude was purified by silica gel column chromatography by using Hex/EtOAc (1:1 with 5% of NEt_3) to give compound **5** (450 mg, 93%) as a syrup. ^{1}H NMR ($CDCl_3$, 300 MHz) (mix of isomers) δ (ppm) 4.19 (d, 1H, *J* =7.8 Hz, H_1), 3.93-3.44 (m, 6H, OCH_2CH_2O, $OC\underline{H_2}CH_2CN$), 3.61-3.44 (m, 12 H, 3x CH_3O, H_6, $H_{6'}$, 2x$CH_{isopropyl}$), 3.31 (s, 3 H, CH_3O), 3.18-3.16 (m, 1H, H_5), 3.08-3.05 (m, 2 H, H_4, H_3), 2.93-2.90 (m, 1 H, H_2), 2.59-2.55 (m, 2 H, -$OCH_2C\underline{H_2}CN$), 1.11 (d, 12H, *J*=6.5 Hz, 4$CH_{3isopropyl}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm); 117.6 (CN), 103.4 (C_1), 86.2 (C_3), 83.6 (C_2), 79.1 (C_4), 74.4 (C_5), 71.2, 71.3, 69.3, 62.3, 60.6, 60.3, 60.2, 59.1, 58.3, 42.9, 24.6, 24.5, 24.4, 20.2. HRMS (ES^+) Calcd. for $C_{21}H_{41}N_2O_8PNa$ (M+Na): 503.2498, found; 503.2483.

2-Benzyloxyethyl 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β- D-glucopyranosyl)-β-D-glucopyranoside (7)

To a solution of the heptaacetyl cellobiose trichloroacetimidate **6**[1] (900 mg, 1.15 mmol) in anhydrous CH_2Cl_2 (10 mL) and 2-benzyloxyethanol (250 μL, 1.72 mmol), $BF_3.OEt_2$ (15 μl, 0.11 mmol) was then added. After stirring at room temperature for 2 h under argon atmosphere, NEt_3 (0.1 ml) was then added. Solvents were then removed and the crude was purified by silica gel column chromathography using as eluent (Hex-AcOEt, 2:1-2:3) to give **7** (700 mg, 79%) as a syrup. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 7.32-7.28 (m, 5H, Ph), 5.19-5.09 (m, 2H, $H_{3A,}$ H_{3B}), 5.01 (t, 1H, *J* = 9.6 Hz, H_{4B}), 4.93-4.87 (m, 2H, $H_{2A,}$ H_{2B}), 4.56 (2d, 2H, *J* = 7.8 Hz, H_{1A}, H_{1B}), 4.54-4.47 (m, 3H, OCH_2, H_{6A}), 4.35 (dd, 1H, *J* = 4.5, 12.6 Hz, H_{6B}), 4.10-3.91 (m, 3H, OCH_2, $H_{6'A}$, $H_{6'B}$), 3.79-3.55 (m, 6H, OCH_2, H_{5A}, H_{5B}, H_{4A}), 2.09-1.96 (6s, 21H. OCOCH_3). ^{13}C NMR ($CDCl_3$, 75 MHz)

δ (ppm) 170.5, 170. 3, 170.2, 169.8, 169.6, 169.3, 169.0 (C=O), 138.1 (Cqarom), 128.4, 127.6, 127.5 (Carom), 100.8 (C_{1A}), 100.7 (C_{1B}), 76.4, 73.2, 72.9, 72.6, 72.5, 71.9, 71.6, 71.5, 69.2, 69.1, 67.8, 61.8, 61.5, 21.0, 20.8, 20.6, 20.5. HRMS (ES^+) Calcd. for $C_{35}H_{46}O_{19}Na$ (M+Na): 793.2531, found; 793.2520.

2-Benzyloxyethyl 2,3,6-tri-*O*-methyl-4-*O*-(2,3,4,6-tetra-*O*-methyl-β- D-glucopyranosyl)-β-D-glucopyranoside (8)

Compound **7** (560 mg, 0.72 mmol) was dissolved in dry MeOH (10 mL) and Na_2CO_3 (23 mg, 0.21 mmol) was then added. The reaction mixture was stirred for 2 h and IR-120 was then added to neutralize. Solvent was then removed and the crude was used for next step without any further purification. To a solution of the crude (300 mg, 0.62 mmol) in anhydrous DMF (6 mL) at 0ºC, NaH (151 mg, 6.3 mmol) was added. The reaction mixture was stirred for 10 min and MeI (549 μL, 8.81 mmol) was then added. After 24 h stirring at room temperature, 2-propanol was then added dropwise and NH_4Cl sat (25 mL). Organic phase was extracted with ethyl acetate (2x50 mL) and washed with sodium bisulfate solution (50 mL) and brine (50 mL). Solvents were then removed and the crude was purified by silica gel column chromathography using as eluent (Hex-AcOEt, 1:1-1:6) to give **8** (300 mg, 84%) as a syrup. ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm) 7.08-7.03 (m, 5H, Ph), 4.31 (s, 2H, CH_2Ph), 4.07 (2d, 2H, J = 7.8 Hz, H_{1A}, H_{1B}), 3.81-3.74 (m, 1H, OCH_2), 3.52-3.27 (m, 23H, H_{6A}, $H_{6'A}$, OCH_2, H_{5A} H_{5B}, 5xCH_3O), 3.16-3.09 (m, 7H, 2xCH_3O, H_{6B},), 3.03-2.85 (m, 4H, $H_{6'B}$, H_{3B}, H_{3A}, H_{4A}), 2.82 (dd, 1H, J = 7.8, 9.0 Hz, H_{2B}), 2.68 (t, 1H, J = 8.7 Hz, H_{2A}). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm) 139.2 (Cqarom), 129.2, 128.6, 128.5 (Carom), 104.4 (C_{1A}), 104.1 (C_{1B}), 87.9, 85.4, 85.0, 84.0, 80.3, 78.6, 75.6, 74.1, 72.1, 71.6, 70.3, 69.8, 61.6, 61.5, 61.3, 61.2, 61.1, 60.2, 60.0. HRMS (ES^+) Calcd. for $C_{21}H_{40}O_{12}Na$ (M+Na): 507.2417, found; 507.2433.

2-Hydroxyethyl 2,3,6-tri-*O*-methyl-4-*O*-(2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl)-β-D-glucopyranoside (9)

A solution of compound **8** (300 mg, 0.52 mmol) in ethyl acetate-MeOH (1:1, 5mL) and $Pd(OH)_2$ in catalytic amount was stirred under an atmosphere of hydrogen for 24 h. The

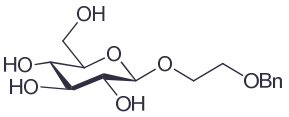
mixture was filtered off over celite and solvents were removed. The crude was purified by silica gel column chromathography using as eluent (Hex-AcOEt, 1:6→0:1) to give **9** (209 mg, 83%) as a syrup. ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm) 4.24-4.22 (2d, 2H, *J* = 7.8 Hz, H_{1A}, H_{1B}), 3.85-3.77 (m, 2H, OCH_2), 3.68-3.52 (m, 16H, H_{6A}, $H_{6'A}$, OCH_2, H_{6B}, $H_{6'B}$, H_{4B}, 3xCH_3O), 3.49, 3.47 (2s, 6H, 2xCH_3O), 3.40-3.38 (m, 1H, H_{5B}), 3.22-3.06 (m, 5H, H_{5A}, H_{3B}, H_{3A}, $H_{4A,}$ OH), 3.00 (dd, 1H, *J* = 7.8, 9.0 Hz, H_{2B}), 2.68 (t, 1H, *J* = 8.7 Hz, H_{2A}). ^{13}C NMR ($CDCl_3$, 125 MHz) δ (ppm 103.8 (C_{1A}), 103.1 (C_{1B}), 86.9, 85.5, 84.0, 83.1, 79.2, 77.9, 74.6, 74.3, 73.4, 71.1, 70.8, 62.3, 60.7, 60.5, 60.4, 60.3, 60.2, 59.2, 59.0. HRMS (ES^+) Calcd. for $C_{21}H_{40}O_{12}Na$ (M+Na): 507.2417, found; 507.2433.

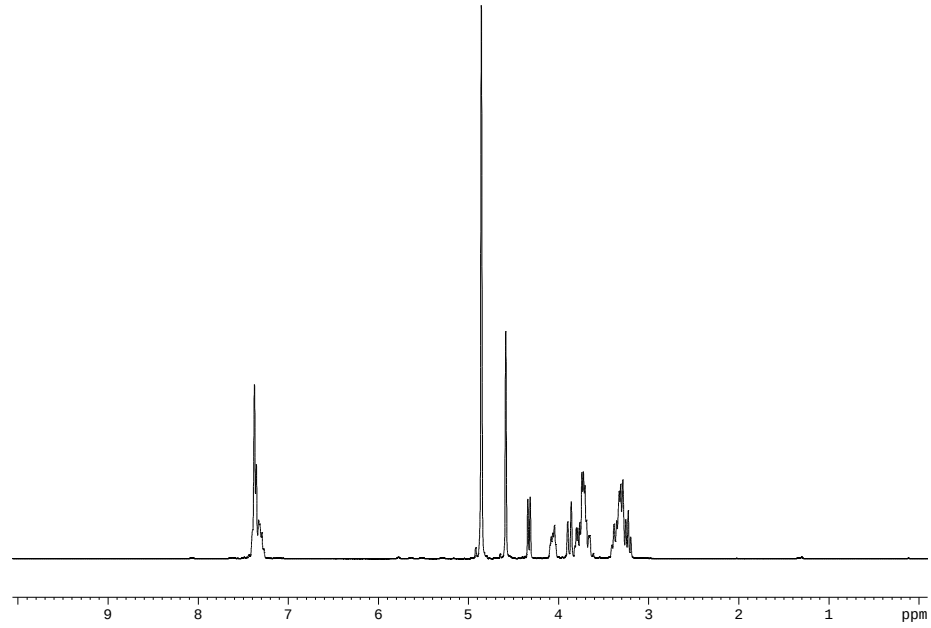
2-[(2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-glucopyranosyloxy)]ethyl (2-cyanoethyl) (*N*,*N*-diisopropyl) phosphoramidite (10)

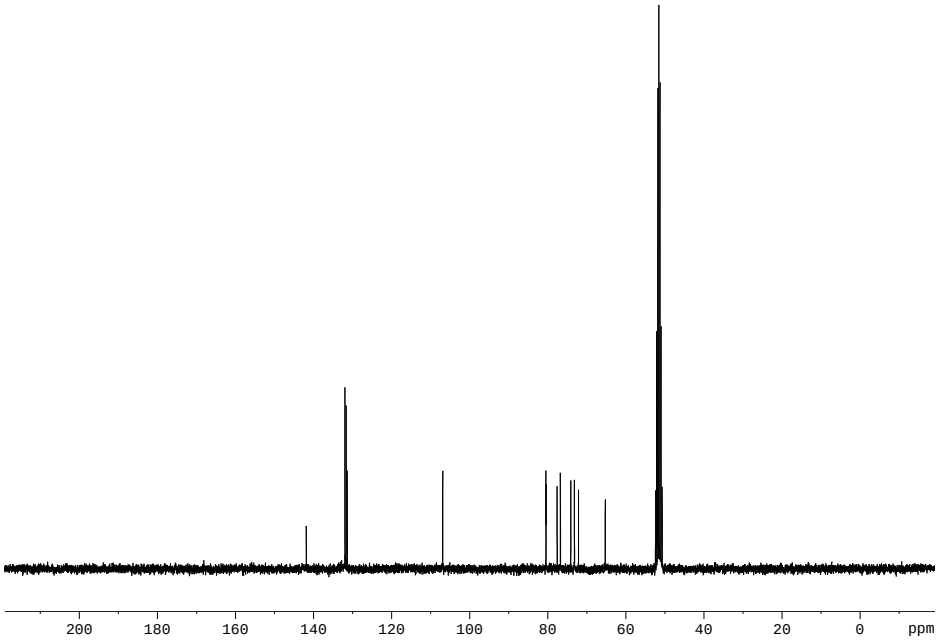
DIPEA (115 μL, 0.66 mmol) and 2-cyanoethyl-N,N'-diisopropylamino-chlorophosphoramidite (55 μL, 0.24 mmoL) were added to a solution of compound **9** (80 mg, 0.16 mmol) in anhydrous CH_2Cl_2 (3 mL) at room temperature under an argon atmosphere. After 1.0 h no starting material was observed. Solvent was then removed and the crude was purified by silica gel column chromatography by using Hex/EtOAc (1:2 with 5% of NEt_3) to give compound **10** (100 mg, 88%) as a syrup. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 4.25-4.20 (m, 2H, H_{1A}, H_{1B}), 3.95-3.41 (m, 27H, H_{6A}, H_{6B}, $H_{6'A}$, 2xOCH_2, O*C*$H_2$$CH_2CN$, 2x$CH_{isopropyl}$, $H_{6'B}$, 5xCH_3O), 3.34-3.30 (m, 7H, H_{5B}, 2xCH_3O), 3.22-3.01 (m, 4H, H_{3A}, H_{3B}, H_{4A}, H_{5A}), 2.95 (t, 1H, *J* = 9.0 Hz, H_{2B}), 2.86 (t, 1H, *J* = 9.0 Hz, H_{2A}), 2.22-2.39 (m, 2H, C$\underline{H_2}$CN), 1.09 (d, 12H, *J*=6.5 Hz, 4$CH_{3isopropyl}$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm) 117.9 (CN), 103.5 (C_{1A}), 103.1 (C_{1B}), 86.9, 84.3, 84.0, 83.0, 79.3, 77.5, 74.6, 71.1, 70.5, 69.4, 60.7, 60.6, 60.4, 60.3, 60.2, 59.3, 59.0, 58.4, 43.0, 24.7, 24.6, 24.5, 20.3. HRMS (ES^+) Calcd. for $C_{28}H_{46}O_{12}Na$ (M+Na): 597.2887, found; 597.2882.

Proton and carbon NMR spectra of compounds 2-5 and 7-10.

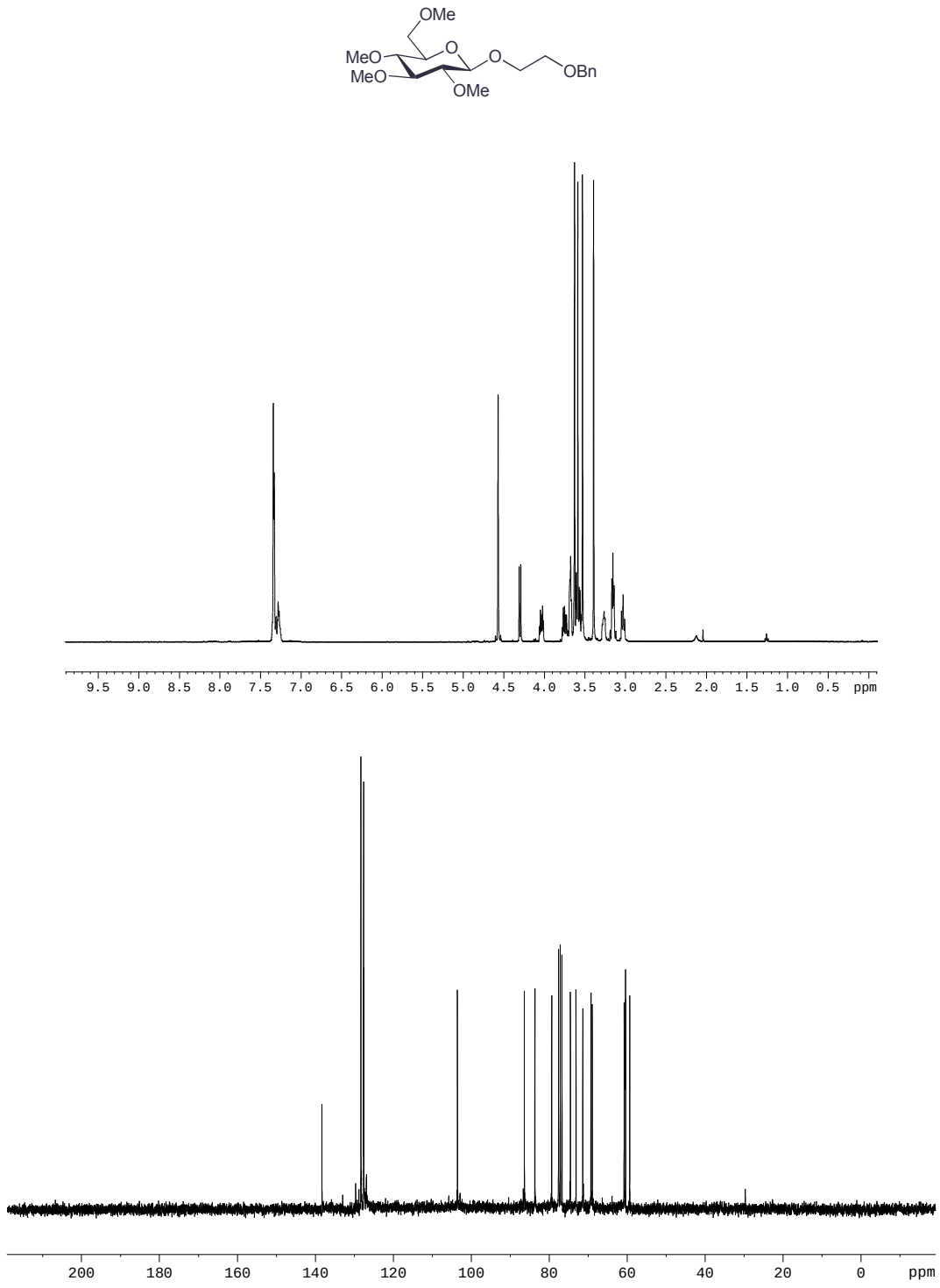
Compound 2







Compound 3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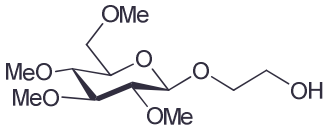
O
MeO
MeO
OMe
O
OBn
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
200 180 160 140 120 100 80 60 40 20 0 ppm

Compound 4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MeO
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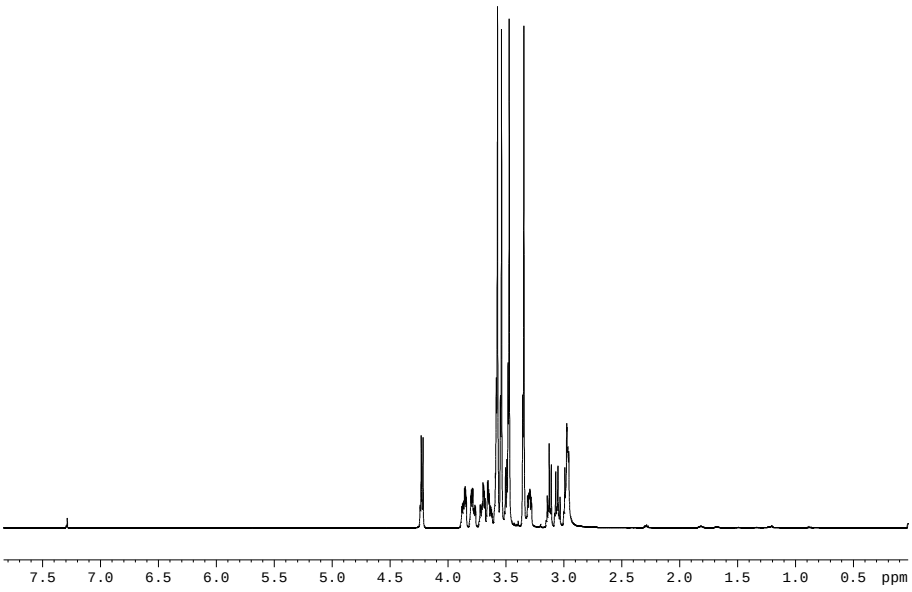
OH
OMe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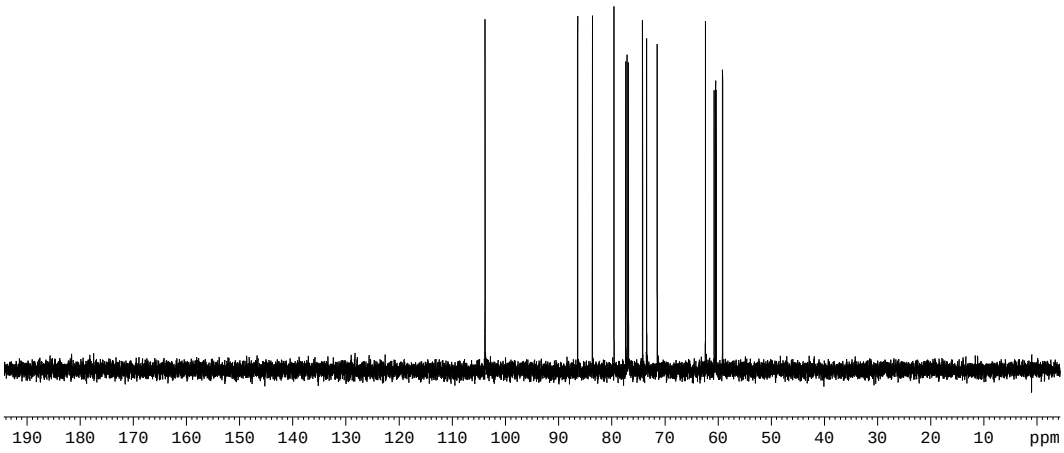
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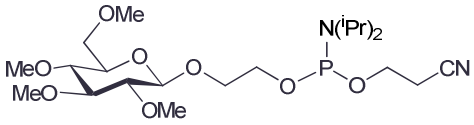
Compound 5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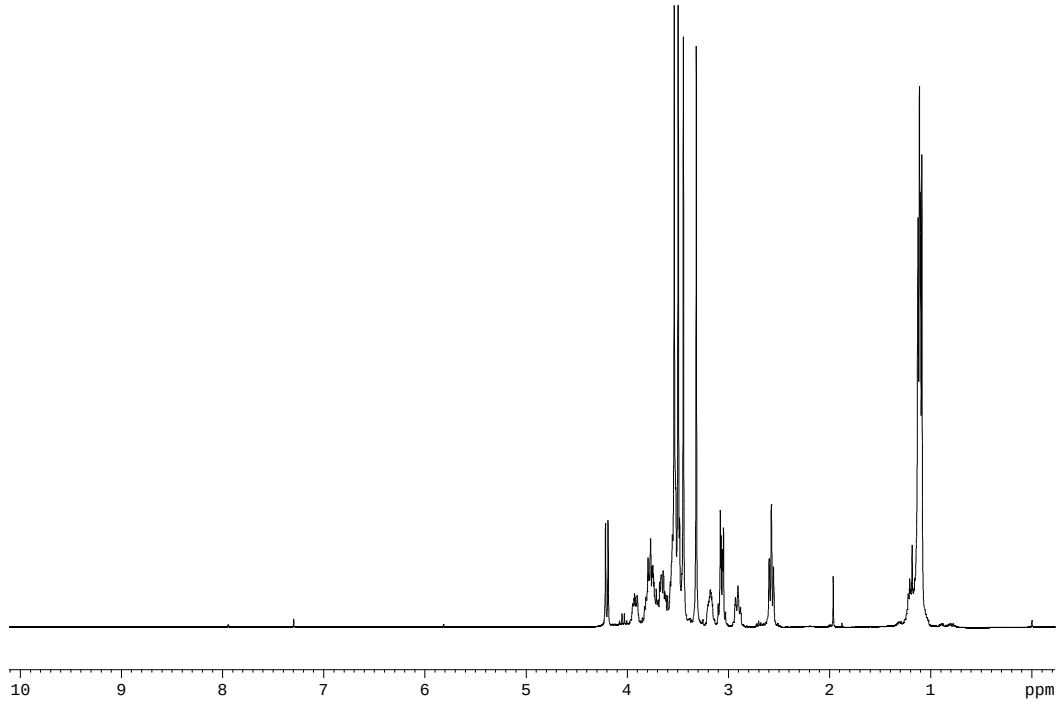
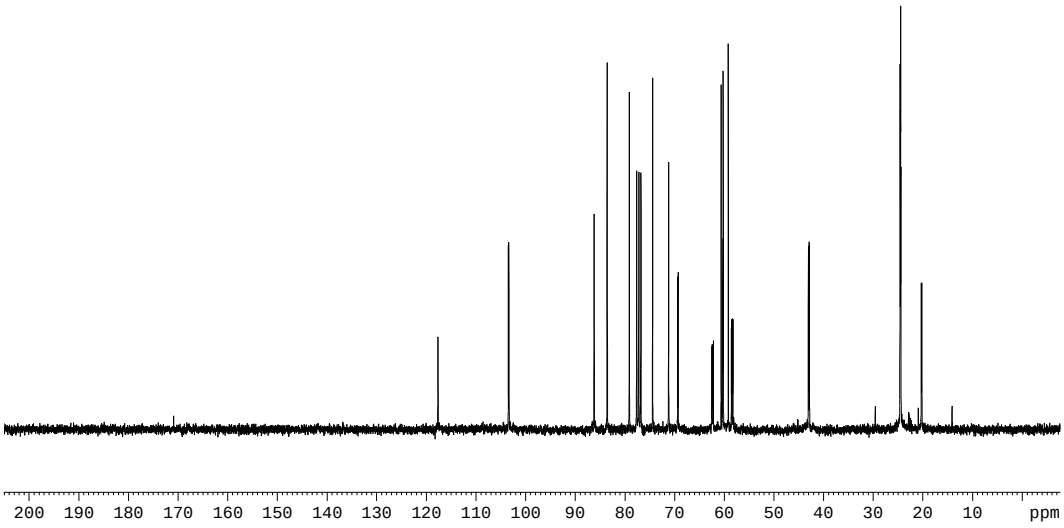
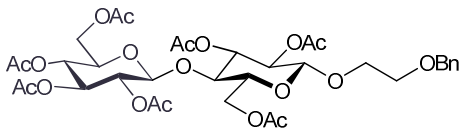
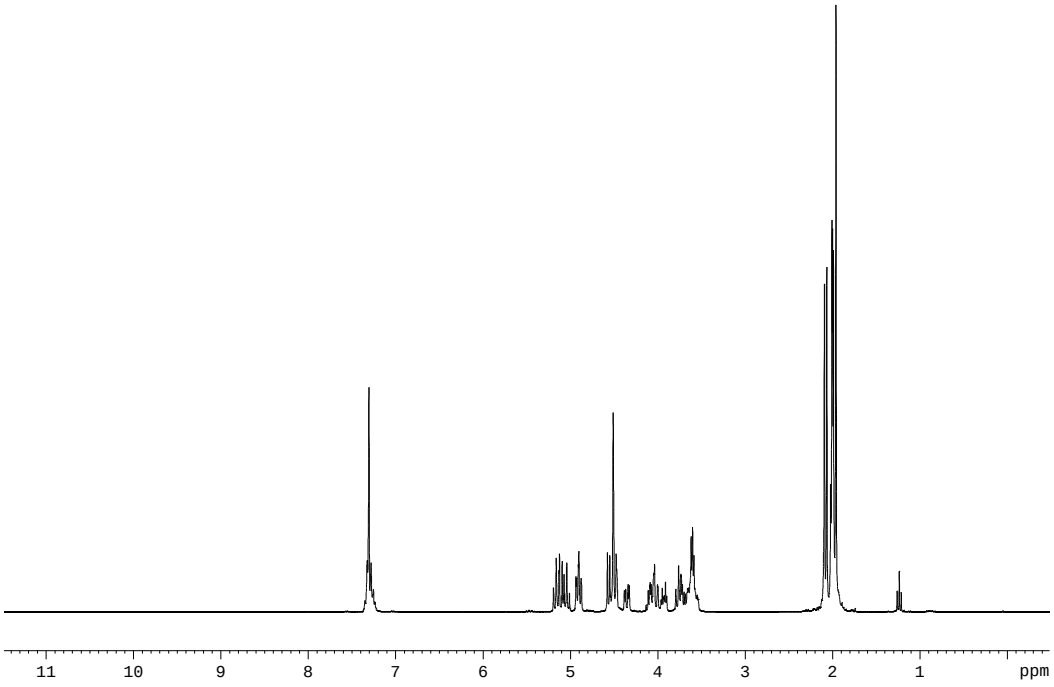
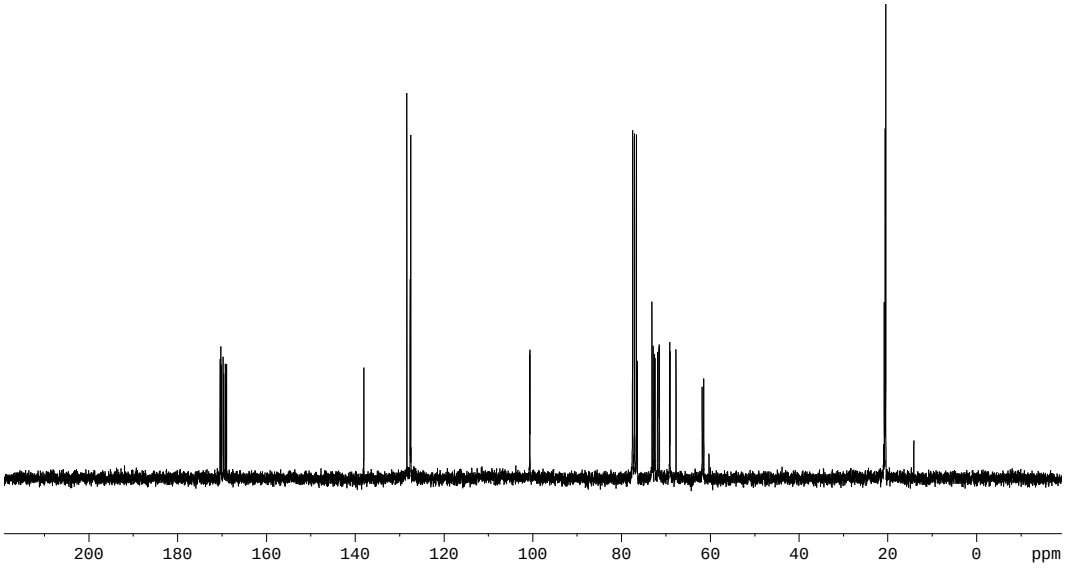

10 9 8 7 6 5 4 3 2 1 ppm


200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

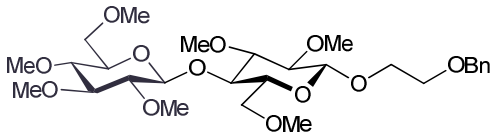
Compound 7

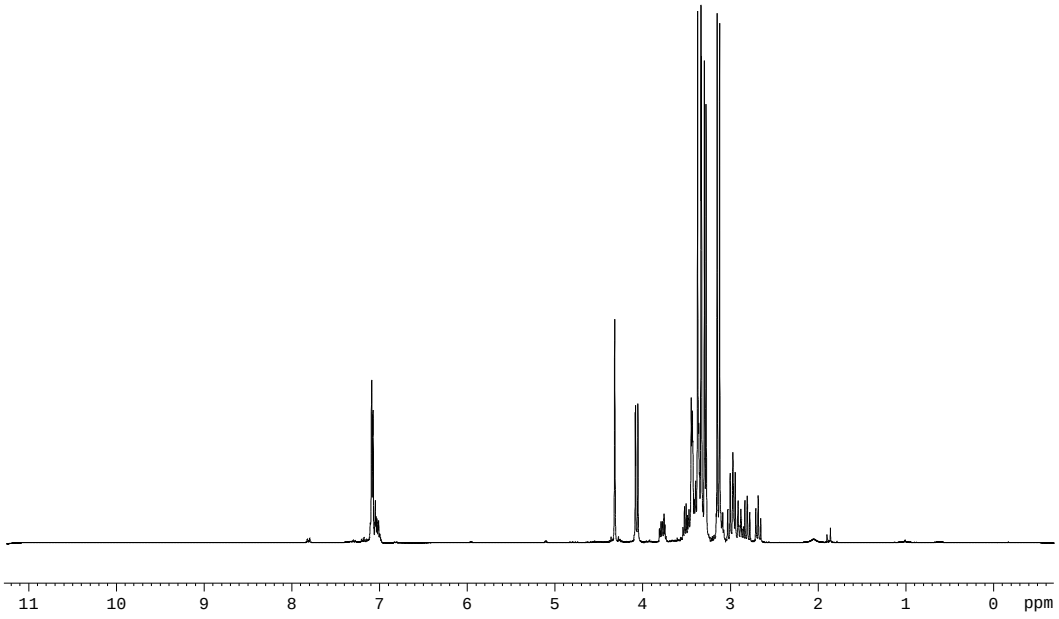

OAc
AcO
AcO
O
OAc
O
AcO
OAc
O
O
OAc
O
OBn

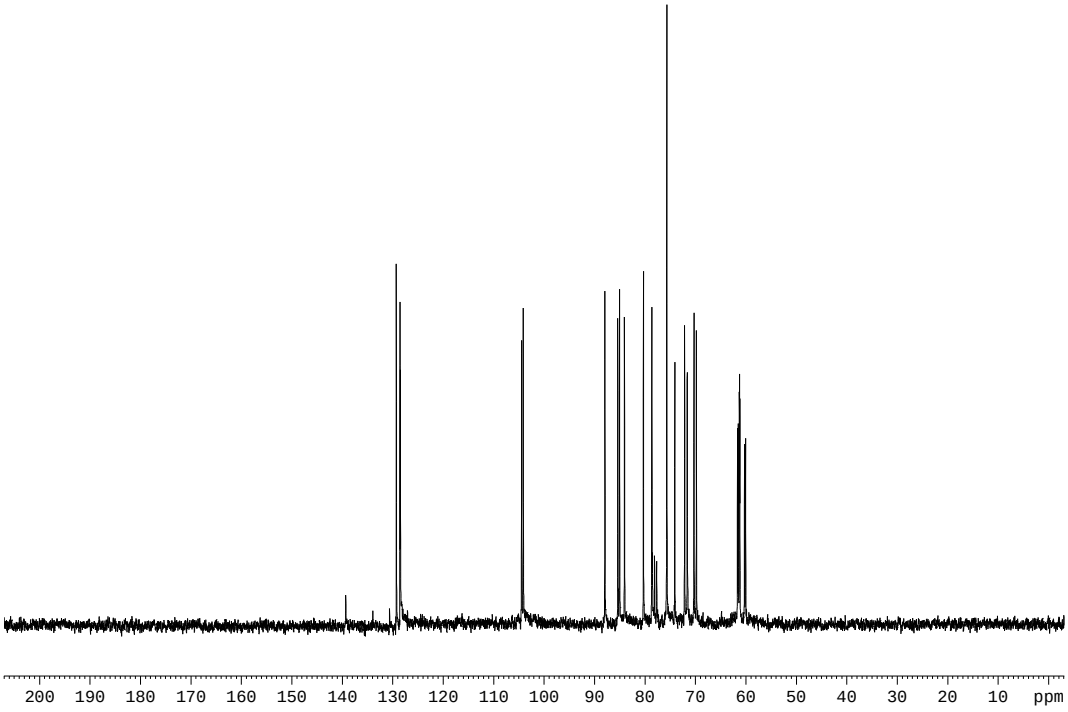

11
10
9
8
7
6
5
4
3
2
1
ppm


200
180
160
140
120
100
80
60
40
20
0
ppm

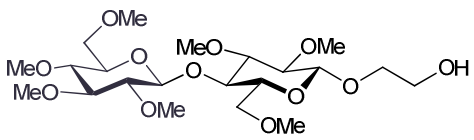
Compound 8

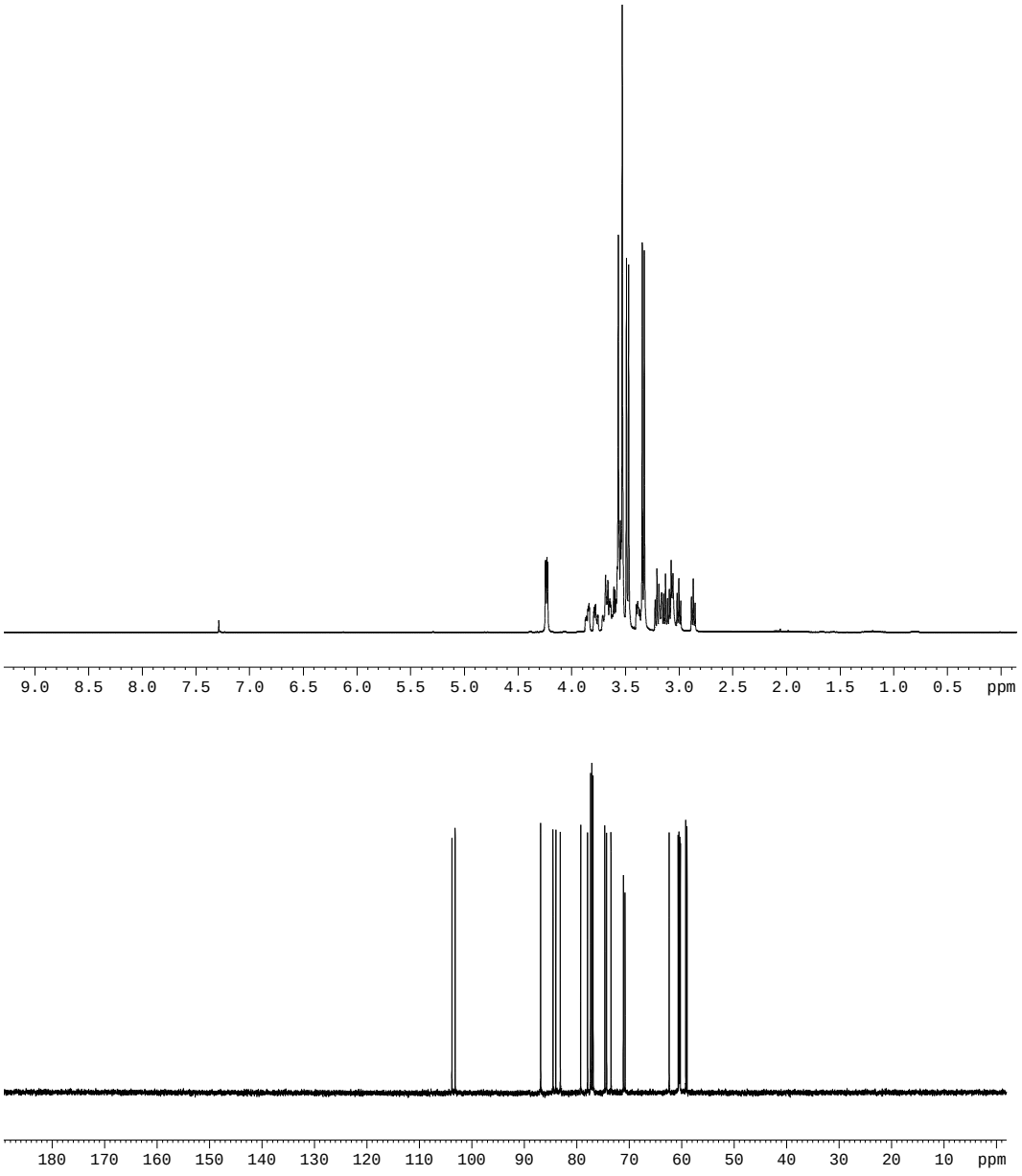

OMe
MeO
O
MeO
OMe
MeO
O
OMe
O
OBn
O
OMe


11 10 9 8 7 6 5 4 3 2 1 0 ppm

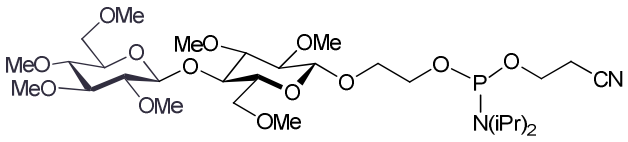

200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

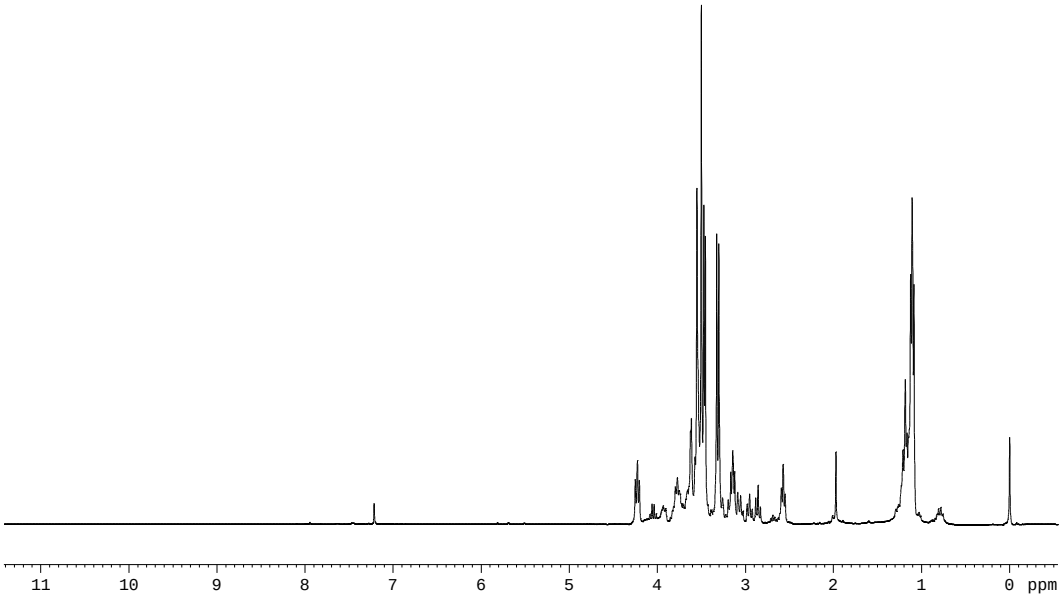
Compound 9

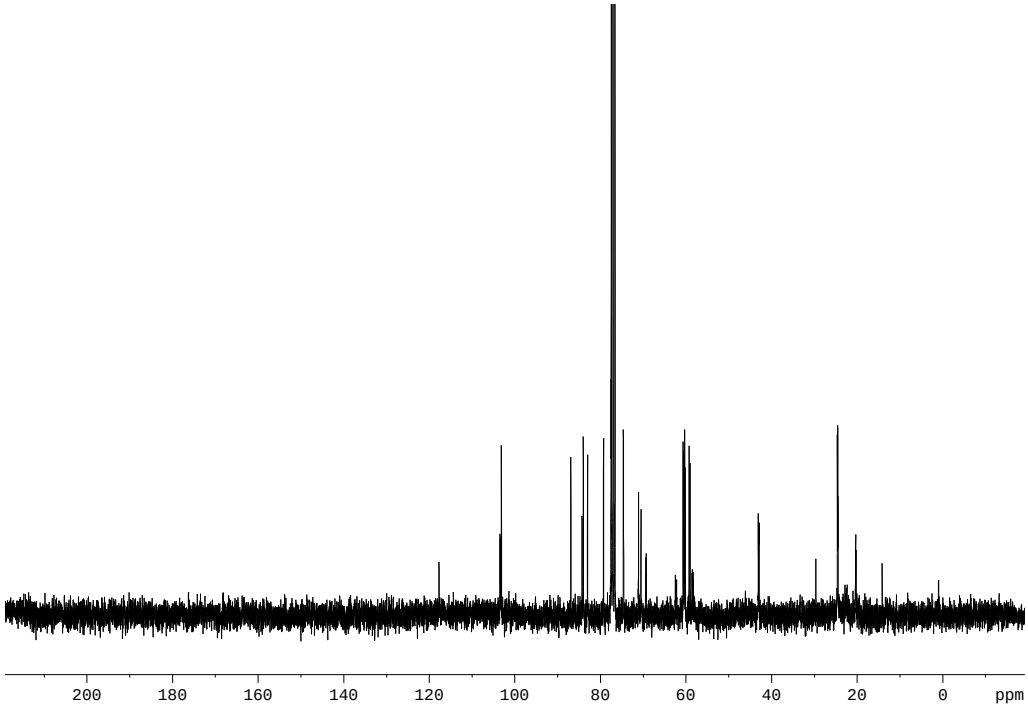

OMe
MeO
MeO
O
OMe
O
MeO
OMe
O
O
OMe
O
OH


9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

Compound 10


OMe
MeO
MeO
OMe
MeO
OMe
OMe
O
O
O
O
O
O
O
P
N(iPr)2
CN


11
10
9
8
7
6
5
4
3
2
1
0
ppm


200
180
160
140
120
100
80
60
40
20
0
ppm

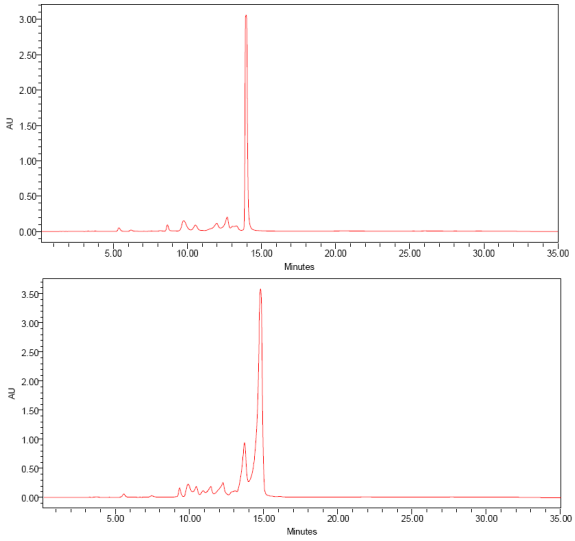
Synthesis of carbohydrate–oligonucleotide conjugates

Carbohydrate-oligonucleotide conjugates **15**, **16**, **19** and **20** were synthesized on an Applied Biosystems 394 synthesizer by using standard β-cyanoethylphosphoramidite chemistry. Conjugate **19, 20, 25, 26, 29** and **30** were prepared by *Biomers* following the same methodology. Oligonucleotide conjugates were synthesized either on low-volume 200 nmols (LV200) or 1.0 μmol scale and using the DMT-off procedure. Oligonucleotide supports were treated with 33% aqueous ammonia for 16 h at 55ºC, then the ammonia solutions were evaporated to dryness and the conjugates were purified by reversed-phase HPLC in a Waters Alliance separation module with a PDA detector. HPLC conditions were as follows: Nucleosil 120 C18, 250x8 mm, 10 μm column; flow rate: 3 mL/min. A 27 min linear gradient 0-30%B (solvent A: 5% CH3CN/ 95% 100 mM triethylammonium acetate (TEAA; pH 6.5); solvent B: 70% CH3CN/30% 100 mM TEAA (pH 6.5)).

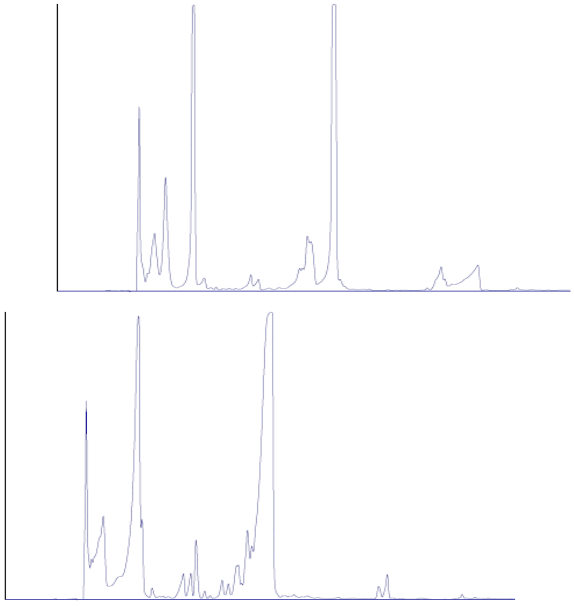
Thermodynamic measurements

Self-complementary oligonucleotides and COCs were hybridized by heating the sample at 90ºC for 3 min and letting cool down to room temperature during 3h. Melting curves for the DNA conjugates were measured in a Perkin–Elmer Lambda 750 UV/Vis spectrophotometer at 280 nm while the temperature was raised from 10 to 80 ºC at a rate of 1.0 ºCmin-1. Curve fits were excellent, with c2 values of 10^6 or better, and the Van't Hoff linear fits were quite good (r^2=0.97) for all oligonucleotides. Thermodynamic parameters were calculated from the average values obtained from melting curve fitting (using Meltwin software) and linear Van't Hoff plots of 1/Tm vs ln ([conjugate]). ΔH, ΔS, and ΔG errors were calculated as described previously.[2, 3]

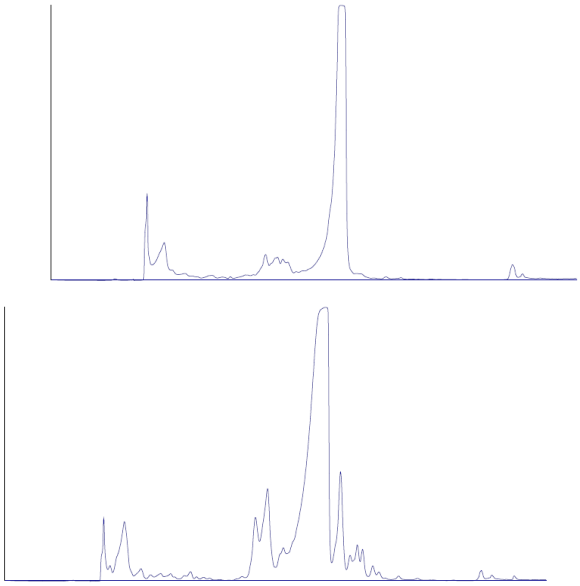
HPLC chromatograms of carbohydrate oligonucleotide conjugates 15, 16, 18, 19 and 20.



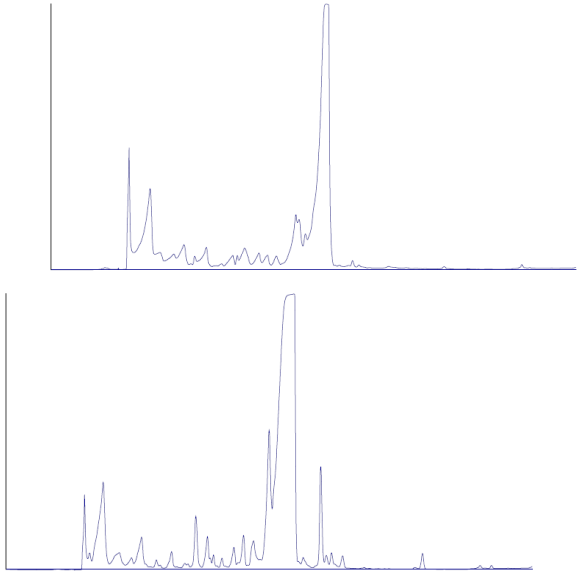
Conjugate glc(Me)-C2-CGCGCG (15) **Conjugate glc(Me)-C2-AGCGCT (16)**



Conjugate cellob(Me)-C2-CGCGCG (19) **Conjugate cellob(Me)-C2-AGCGCT (20)**



Conjugate glc(Me)-C2-GGCGCC (25) **Conjugate glc(Me)-C2-TGCGCA (26)**



Conjugate cellob(Me)-C2-GGCGCC (29) **Conjugate cellob(Me)-C2-TGCGCA (30)**

Maldi-TOF mass spectra of carbohydrate oligonucleotide conjugates.

MALDI-TOF spectra were performed using a Perseptive Voyager DETMRP mass spectrometer, equipped with nitrogen laser at 337 nm using a 3ns pulse. The matrix used contained 2,4,6-trihydroxyacetophenone (THAP, 10 mg/ml in ACN/ water 1:1) and ammonium citrate (50 mg/ ml in water).

Carbohydrate oligonucleotide conjugates	[M-H] calc.	[M-H] exp.
β-D-glc(Me)-C2-CGCGCG (**15**)	2133.5	2133.5
β-D-glc(Me)-C2-AGCGCT (**16**)	2132.5	2132.5
β-D-cellob(Me)-C2-CGCGCG (**19**)	2338.3	2339.0
β-D-cellob(Me)-C2-AGCGCT (**20**)	2337.3	2337.0
β-D-glc(Me)-C2-GGCGCC (**25**)	2133.5	2142.0
β-D-glc(Me)-C2-TGCGCA (**26**)	2132.5	2140.0
β-D-cellob(Me)-C2-GGCGCC (**29**)	2338.3	2344.0
β-D-cellob(Me)-C2-TGCGCA (**30**)	2337.3	2342.0

NMR spectroscopy and structure calculations.

Samples of the conjugates were purified by HPLC, ion-exchanged with Dowex 50W resin and then suspended in 500 µL of either D_2O or H_2O/D_2O 9:1 in phosphate buffer, 100 mM NaCl, pH 7. NMR spectra were acquired in Bruker Avance spectrometers operating at 600 or 800 MHz, and processed with Topspin software. DQF-COSY, TOCSY and NOESY (mixing times of 150 and 300ms) experiments were recorded in D_2O at temperatures ranging from 5 ºC to 25 ºC. NOESY spectra in H_2O were acquired with 100 ms mixing time at 5 ºC to reduce the exchange with water. The spectral analysis program Sparky[4] was used for semiautomatic assignment of the NOESY cross-peaks and quantitative evaluation of the NOE intensities. Distance constraints with their corresponding error bounds were incorporated into the AMBER[5] potential energy by defining a flat-well potential term. Restrained molecular dynamics calculations were carried out following protocols described in our previous study.[6] The structures were refined including explicit solvent, periodic boundary conditions and the Particle-Mesh-Ewald method to evaluate long-range electrostatic interactions. Force field parameters for the carbohydrate moieties were taken from GLYCAM[7], and the TIP3P model was used to describe water molecules.[8] Analysis of the representative structures as well as the MD trajectories was carried out with the program MOLMOL[9] and the analysis tools of AMBER.

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