Immobilized carbohydrates for the preparation of 3'-glycoconjugated oligonucleotides

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ABSTRACT:

A detailed protocol for the preparation 3'-glycoconjugated oligonucleotides, based on one-pot immobilization of 4,4'-dimethoxytrityl-protected carbohydrates to a solid support, followed by on-support peracetylation and automated assembly of oligonucleotides, is described. Compared to an appropriate building block approach and post-synthetic manipulation of oligonucleotides, this protocol may simplify the synthesis scheme and increase overall yield of the conjugates. Furthermore, the immobilization to a solid support typically increases the stability of reactants, considering prolonged storing, and makes the further processing convenient. Automated assembly on these carbohydrate-modified supports using conventional phosphoramidite chemistry produces 3'-glycoconjugated oligonucleotides in relatively high yields and purity.

Basic Protocol 1: (Synthesis of DMTr-protected carbohydrates)
Basic Protocol 2: (Synthesis of carbohydrate monosuccinates and their immobilization to LCAA CPG support)
Basic Protocol 3: (Oligonucleotide synthesis using immobilized carbohydrates)

KEYWORDS:

Oligonucleotide-glyco-conjugates, solid-supported reactants, delivery of oligonucleotides

INTRODUCTION:

Glyco-conjugated oligonucleotides have received marked interest as diagnostic tools for hybridization-based glyco-arrays and, in particular, to improve the delivery properties of oligonucleotide therapeutics. The well-known example is *N*-acetyl galactosamine (GalNAc)-concept that can be used to deliver therapeutic oligonucleotides efficiently to hepatocytes via asialoglycoprotein receptor. For the synthesis of these biomolecular hybrids, diverse set of conjugation strategies are available: for example, using of appropriate phosphoramidite building blocks or azide/alkyne-modified sugar agents, amenable to the standard phosphoramidite coupling cycle or Huisgen 1,3-dipolar cycloaddition (''click''-reaction), respectively. However, the laborious protecting group manipulation may make oligosaccharides expensive conjugation agents and an excess of these agents is sometimes needed to drive the conjugation to completion. Therefore, the building block approach may be inefficient in some cases, and even pointless, if the conjugation site is in the 3'-terminus of the oligonucleotide sequence. This protocol, based on previously published work (Kiviniemi & Virta, 2011 and Österlund et al., 2017) describes an alternative approach for the preparation 3'-glycoconjugated oligonucleotides: One-pot immobilization of 4,4'-dimethoxytrityl-protected carbohydrates, as polyols to a solid support, followed by on-support peracetylation and automated assembly of oligonucleotides on these carbohydrate-modified solid supports. A detailed procedure for the preparation of these supports and the corresponding oligonucleotides, 3'-conjugated with galactose, glucose, sucrose, ribostamycin, neomycin B (mono-, di-, tri- and tetrasaccharides, the latter two are aminoglycosides) and one trivalent *N*-acetylgalactosamine cluster is described.

Figure 1. Synthesis of 4,4'-dimethoxytrityl-protected carbohydrates

Strategic planning

The immobilized carbohydrates should contain one DMTr group. This protection is removed as a first step of the automated oligonucleotide synthesis and it dictates the site of the assembled oligonucleotides on the carbohydrate unit (Protocol 3).

Moreover, it can be utilized to determine the initial loading of the solid supports by simple DMTr-cation assay. The simplicity of the protecting group scheme, how the DMTr group can be selectively introduced to carbohydrates, defines the pool of carbohydrates that can be selected for the immobilization (Protocol 2, Figure 2). Example procedures for the synthesis of different DMTr-protected carbohydrates are described here (Protocol 1, Figure 1). Simple protecting group manipulation, followed by 6-O-DMTr-protection can be applied for D-glucose (**1**) and sucrose (**9**). For the synthesis of protected aminoglycosides (**3** and **4**), one-pot trifluoroacetylation can be carried out, which is then followed by 6''-O-DMTr-protection. In each case (**1**-**4**), the primary hydroxyl group can be selectively protected in the presence of secondary ones in a high isolated yield. Alternatively, an appropriate DMTr-protected hydroxyalkylarm (**5**) or a branching unit (**6**) can be introduced into the carbohydrate units via a few synthetic steps. 3-*O*-hydroxypropyl-β-D-galactopyranoside (**12**) is prepared by a Lewis acid-catalyzed glycosylation of commercially available β-D-galactose penta-acetate (**14**) using an excess of propane-1,3-diol, and converted then to 3-*O*-DMTr-propyl β-D-galactopyranoside (**5**). DMTr-protected tri-*O*-propargyl pentaerytritol (**18**) is exposed to Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition (i.e. click reaction) with (3-azidopropyl)-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-galactopyranoside (**19**) to obtain the triantennary *N*-acetylgalactosamine cluster (**6**). All starting materials (**7**, **11**, **12**, **13**, **14**, **17** and **19**) for these protected carbohydrate derivatives are commercially available. The reason why these particular carbohydrates are selected is based on their biological relevance and structural heterogeneity. The same protecting group schemes may be applied for many other structurally related carbohydrates.

Synthesis of DMTr-protected carbohydrates

Synthesis of 1-O-tert-butyldimethylsilyl-6-O-(4,4'-dimethoxytrityl)-β-D-glucose (1)

The anomeric hydroxyl group of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose (**7**) is protected by a TBDMS group (**8**). The acetyl groups are removed by NaOMe-catalyzed methanolysis, and the exposed primary hydroxyl group (6-OH) is selectively protected by DMTr group (**1**). The deacetylation and DMTr-protection steps can be performed one pot and the final product (**1**) is purified by silica gel chromatography.

Materials:

2,3,4,6-tetra-O-acetyl-D-glucopyranose, p.a. grade
N,N-dimethylformamide (DMF), anhydrous
Imidazole, p.a. grade
tert-Butyldimethylsilyl chloride (TBDMSCl), reagent grade
Ethyl acetate, p.a. grade
Petroleum ether, p.a. grade
Sulfuric acid, p.a. grade
Sodium chloride (NaCl)
Sodium sulfate (Na_2SO), anhydrous
Sodium hydride (NaH), 60 m-% dispersion in mineral oil (Sigma-Aldrich)
Methanol (MeOH), anhydrous
Dichloromethane (DCM), anhydrous
Pyridine, anhydrous
4,4´-dimethoxytrityl chloride, 95% (Sigma-Aldrich)
Sodium bicarbonate ($NaHCO_3$), p.a. grade

250-mL round-bottom flask
Magnetic stir bar
Hot plate magnetic stirrer
Thin-layer chromatography (TLC) plate (Silica gel 60 F254 aluminum sheets, Merck)

250-mL separating funnel
Glass funnel
Glass wool
Rotary evaporator equipped with a vacuum pump
Chromatography column with fritted disc and PTFE stopcock
Silica gel (SiO_2, NORMASIL 60® 40-63 µm, VWR)
Dowex® 50W, X8 (Sigma-Aldrich)
Silicone oil for oil bath (–50°C to +200°C, Sigma-Aldrich)

Introduce tert-butyldimethylsilyl protecting group

1. Weigh 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose (1.92 g, 5.5 mmol) in a flame-dried 250-mL round-bottom flask equipped with a magnetic stir bar. Add anhydrous DMF (20 mL), seal the flask with a cap and stir with a magnetic stirrer until a homogenous solution is obtained.
2. Into the solution, add imidazole (0.38 g, 5.5 mmol) and TBDMSCl (0.83 g, 5.5 mmol). Seal the flask with a cap and place it in an oil bath heated to 50 °C. Continue stirring at 50 °C for 3 h.
The reaction can be monitored by TLC. Elute the plates with ethyl acetate (50%, v/v) in petrol ether and then drip 10 m-% aqueous sulfuric acid on the plates and heat on a hot plate until visible spots appear.
3. Add ethyl acetate (150 ml) to the reaction mixture and transfer it to a 250-mL separating funnel. Wash the organic layer twice with brine (30 mL).
4. Dry the organic layer over anhydrous Na_2SO_4 and filter off the drying agent. Concentrate the filtrate under reduced pressure using a rotary evaporator.

> *DMF should be carefully removed by evaporation as residues may lead to fronting during silica gel chromatography in the next step.*

5. Purify the crude product by silica gel chromatography eluting with a gradient of ethyl acetate (25-50%) in petrol ether.
6. Characterize the product (1.5 g, 78%, obtained as a colorless oil) by ^{1}H NMR, ^{13}C NMR, and HRMS.

> *2,3,4,6-O-tetra-acetyl-1-O-tert-butyldimethylsilyl-β-D-glucose (**8**). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.20 (dd, 1H, J = 9.6, 9.6 Hz), 5.06 (dd, 1H, J = 9.6, 9.6 Hz), 4.97 (dd, 1H, J = 9.8 Hz, 7.7 Hz), 4.75 (d, J = 7.7 Hz), 4.21 (dd, 1H, J = 12.2, 5.9 Hz), 4.14 (dd, 1H, J = 12.1, 2.6 Hz), 3.72 (m, 1H), 2.08 (s, 3H), 2.040 (s, 3H), 2.036 (s, 3H), 2.01 (s, 3H), 0.89 (s, 9H), 0.13 (s, 3H), 0.11 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.6, 170.3, 169.5, 169.2, 95.8, 73.2, 72.8, 71.8, 68.9, 62.4, 25.4, 20.7 (3), 20.6, 17.9, -4.3, -5.4; HRMS-ESI (m/z) calc'd [M+Na] for $C_{20}H_{34}NaO_{10}Si$: 485.1819, found 485.1826.*

Remove acetyl groups and introduce DMTr protecting group

1. Prepare a solution of NaOMe in MeOH (0.01 M) by dissolving NaH (60 *m*-% dispersion in mineral oil, 12.0 mg, 0.5 mmol) in anhydrous MeOH (50 mL).
2. In a flame-dried 250-mL round bottom flask equipped with a magnetic stir bar, dissolve **8** (0.45 g, 0.97 mmol) in a minimal amount of dry DCM.

3. Into the round bottom flask, add the NaOMe/MeOH solution (50 ml, 0.01 M) prepared in step 1 and seal the flask with a cap. Stir with a magnetic stirrer in room temperature for 30 min.

The reaction can be monitored by TLC. Elute the plates with ethyl acetate (50%, v/v) in petrol ether and then drip 10 m-% aqueous sulfuric acid on the plates and heat on a hot plate until visible spots appear.

3. Neutralize the reaction solution by adding Dowex H^+ until pH 6-7 is reached, then, add a few drops of dry pyridine.
4. Filter the reaction mixture through glass wool, collect the filtrate and evaporate it to dryness under reduced pressure.
5. Dissolve the evaporation residue in anhydrous pyridine (10 mL). Stir with a magnetic stirrer until a homogenous solution is formed.
6. Into the solution, add 4,4´-dimethoxytrityl chloride (0.52 g, 1.5 mmol) while stirring vigorously. After the addition, continue stirring at room temperature for 1 h.

*The reaction can be monitored by TLC. Elute the plates with ethyl acetate (50%, v/v) in petrol ether. DMTr-containing compounds are visible under UV light (254 nm). In addition, these spots turn to yellow when heated. The spot containing the starting material (**8**) is revealed by dripping 10 m-% aqueous sulfuric acid on the plates and heating on a hot plate.*

7. Concentrate the reaction solution under reduced pressure. Dissolve the evaporation residue in ethyl acetate (150 mL) and wash the organic layer with saturated aqueous solution of $NaHCO_3$ (30 mL) and brine (30 mL).
8. Dry the organic layer over anhydrous Na_2SO_4 and filter off the drying agent. Evaporate the solvents under vacuum. Co-evaporate the residue twice with toluene.

Residual pyridine may lead to fronting during silica gel chromatography in the next step. Co-evaporation from toluene should prevent this.

9. Purify the crude product by silica gel chromatography eluting with a gradient of ethyl acetate (50-80%) in petrol ether.
10. Characterize the product (0.32 g, 72%, obtained as a white foam) by ^{1}H NMR, ^{13}C NMR, and HRMS.

*1-O-tert-butyldimethylsilyl-6-O-(4,4'-dimethoxytrityl)-β-D-glucose (**1**). ^{1}H NMR (500 MHz, CD3OD): δ 7.50 (m, 2H), 7.36 (m, 4H), 7.27 (m, 2H), 7.19 (m, 1H), 6.83 (m, 4H), 4.57 (d, 1H, J = 7.5 Hz), 3.78 (s, 6H), 3.45–3.42 (m, 2H), 3.32 (3, 1H), 3.25–3.21 (m, 2H), 3.16 (dd, 1H, J = 9.1, 7.6 Hz), 1.00 (s, 9H), 0.24 (s, 3H), 0.23 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD): δ 158.6, 145.3, 136.2, 136.1, 130.0 (2), 128.1, 127.2, 126.2, 112.5, 97.9, 85.7, 76.8, 75.8, 75.6, 70.9, 63.4, 54.3, 25.0, 17.7, -5.32, -6.05. HRMS-ESI (m/z) calc'd $[M-H]^-$ for $C_{33}H_{43}O_8Si$: 595.2727, found 595.2712.*

Synthesis of 6-O-DMTr-2,3,1',3',4',6'-hexa-O-benzoylsucrose (2)

Introductory paragraph:

Sucrose (**9**) is first protected with isopropylidene and benzoyl groups (**10**) according to published procedure (Pelletier et al, 2016). The isopropylidene protection is removed by acid-catalyzed hydrolysis (**11**) and then the exposed primary hydroxyl group is protected by DMTr-group.

Materials:

Sucrose
2,2-dimethoxypropane (2,2-DMP)
Para-toluenesulfonic acid hydrate (p-TSA•H_2O)
4,4'-Dimethoxytrityl chloride
4-dimethylaminopyridine (DMAP)
Acetone
Acetic acid (AcOH)
Amberlite IR-67 resin
Aqueous saturated sodium bicarbonate solution ($NaHCO_3$)
Benzoyl chloride (BzCl)
Dichloromethane (DCM)
Dimethylformamide, anhydrous (DMF)
Brine (saturated NaCl in H_2O)
Pyridine, anhydrous
Ethyl acetate
Aqueous saturated sodium bicarbonate solution ($NaHCO_3$)
Sodium bicarbonate ($NaHCO_3$)
Sodium sulfate
Petroleum ether
Water (H_2O)

70/100 °C oil bath
100-ml separatory funnel
250-ml round-bottom flask
250-ml separatory funnel
500-ml Erlenmeyer flask
Büchner flask
Sintered funnel
Ice-water bath
Magnetic stirrer
Magnetic stirbar
Rotary evaporator equipped with a vacuum pump
Thermometer
Thin-layer chromatography (TLC) plates
TLC chamber
UV lamp, 254 nm

Introduce isopropylidene and benzoyl protecting groups

1. To a flame-dried 250-ml round bottom flask equipped with a magnetic stir bar, add sucrose (3.02 g, 7.82 mmol).
2. Add anhydrous DMF (31 mL).
3. Heat the suspension to 100 °C and stir until the sucrose has completely dissolved.
4. Cool the solution back to ambient temperature.
5. Add 2,2-DMP (14.0 mL, 11.8 g, 113 mmol) and p-TSA (50.3 mg, 0.26 mmol).
6. Stir the reaction solution at ambient temperature for an hour.
7. Quench the reaction by adding approximately 400 milligrams of IR-67 resin.
8. Filter the solution over a sintered funnel.
9. Evaporate the filtrate *in vacuo* using a rotary evaporator to yield a clear, thick oil.
10. Dissolve the oil in anhydrous pyridine (50 mL) in a 250-ml round-bottom flask equipped with a magnetic stir bar.
11. Cool the solution down to 0 °C in an ice-water bath.
12. Add BzCl (8.8 g, 7.2 mL, 62 mmol) dropwise while stirring.
13. Stir overnight, allowing the solution to slowly warm to ambient temperature.
14. Precipitate the product by pouring it slowly into a 500 mL Erlenmeyer flask containing a stirred suspension of crushed ice, water (200 mL) and sodium bicarbonate ($NaHCO_3$, 9.0 g).
15. Filter the precipitate over a sintered funnel and wash the precipitate with cold water (100 mL).
16. Dissolve the precipitate in DCM (60 mL) and wash it with brine (3x20 mL) in a separatory funnel.
17. Dry the organic layer over anhydrous Na_2SO_4, filter off the drying agent and evaporate the solvent *in vacuo* using a rotary evaporator.
18. Purify by chromatography on silica gel using 25% ethyl acetate in petroleum ether as the eluent.
19. Collect fractions containing the desired product and evaporate *in vacuo* using a rotary evaporator to yield 4,6-isopropylidenesucrose hexabenzoate (**10**) as an orange oil (2.07 g, 2.06 mmol, 26 % yield).
20. Characterize product by ^{1}H NMR and ^{13}C NMR.

*4,6-isopropylidenesucrose hexabenzoate (**10**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.19 (d, J = 7.9 Hz, 2H), 8.09–7.98 (m, 6H), 7.95 (d, J = 7.8 Hz, 2H), 7.82 (d, J = 8.2 Hz, 2H), 7.67–7.30 (m, 16H), 7.12 (t, J = 7.7 Hz, 2H) 6.04 (d, J = 3.7 Hz, 1H) 6.02-5.98 (m, 2H), 5.90 (t, J = 9.8 Hz, 1H), 5.28 (dd, J = 3.6, 10 Hz, 1H) 4.66–4.47 (m, 5H), 4.23 (dt, J = 9.8, 4.9 Hz, 1H), 3.93 (app t, J = 9.7 Hz, 1H), 3.84 (dd, J = 10.8, 5.3 Hz, 1H), 3.66 (app t, J = 10.6 Hz, 1H) 1.48 (s, 3H), 1.34 (s, 3H) ^{13}C NMR (125 MHz, $CDCl_3$): 166.0, 165.9, 165.5, 165.4, 133.7, 133.6, 133.2, 133.1, 133.0, 132.9, 130.3, 129.9, 129.8 (2), 129.7, 129.6, 129.4, 128.8, 128.7 (2), 128.5, 128.4, 128.3, 104.2, 99.8, 91.1, 78.2, 77.1, 75.8, 72.0, 69.8, 65.1, 65.0, 64.1, 61.9, 28.9, 19.0.*

Remove isopropylidene protecting group

1. To a 250-ml round-bottom flask equipped with a stir bar, add 4,6-isopropylidenesucrose hexabenzoate (**10**) (0.90 g, 0.89 mmol).
2. Add acetone (10 mL) and acetic acid (40 mL).
3. While stirring, heat the solution to 70 °C.
4. At 70 °C, slowly add water (10 mL).
5. Stir for 20 minutes.

The reaction can be monitored by TLC. Elute the plates with ethyl acetate (50%, v/v) in petrol ether. Both the starting material and the product are visible under UV light (254 nm).

6. Cool the reaction mixture down to ambient temperature.
7. Evaporate the reaction mixture *in vacuo* using a rotary evaporator.
8. Dissolve the residue in 1/1 (v/v) mixture of DCM and water (40 mL).
9. Extract the solution with DCM (2 × 20 mL).
10. Combine the organic layers and wash them with water (2 × 20 mL) and saturated aqueous $NaHCO_3$ (2x20 mL).
11. Dry the organic layer over anhydrous Na_2SO_4, filter off the drying agent and evaporate the solvent *in vacuo* using a rotary evaporator.
12. Evaporate the organic layer *in vacuo* using a rotary evaporator to yield 2,3,1',3',4',6'-hexa-O-benzoylsucrose (**11**) as a white solid (0.75 g, 0.78 mmol, 87 % yield). The product was carried over to the next step without further purification.
13. Characterize product by TLC (on silica gel), ^{1}H NMR, ^{13}C NMR, and HRMS.

*2,3,1',3',4',6'-hexa-O-benzoylsucrose (**11**) ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.16 (d, J = 7.4 Hz, 2H), 8.08–8.00 (m, 4H), 7.99–7.94 (m, 4H), 7.82 (d, J = 7.4 Hz, 2H), 7.66–7.27 (m, 16H), 7.17 (t, J = 7.8 Hz, 2H), 6.06 (d, J = 6.0 Hz, 1H), 6.02 (d, J = 3.5 Hz, 1H), 5.98 (t, J = 6.4 Hz, 1H), 5.77 (t, J = 9.9 Hz, 1H), 5.35 (dd, J = 10.3, 3.5 Hz, 1H), 4.93 (dd, J = 11.8, 7.1 Hz, 1H), 4.72 (dd, J = 11.8, 4.7 Hz, 1H), 4.67–4.60 (m, 2H), 4.55 (d, J = 12.0 Hz, 1H), 4.26–4.20 (m, 1H), 3.96–3.80 (m, 3H), 3.00 (b, 1H), 2.36 (b, 1H) ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.2, 166.4, 165.9, 165.6, 165.5, 165.4, 133.8, 133.7, 133.3 (3), 133.2, 130.2, 130.0, 129.9, 129.8 (2), 129.7, 129.5, 129.3, 129.2, 128.8, 128.7, 128.6, 128.5, 128.4 (2), 128.3, 104.3, 90.9, 79.3, 76.3, 74.0, 73.4, 70.7, 69.8, 64.7, 64.6, 61.9, 60.4. HRMS-ESI (m/z) calc'd $[M+K]^+$ for $C_{54}H_{46}KO_{17}$: 1005.2332, found 1005.2327.*

Introduce 4,4'-dimethoxytrityl protecting group

1. To a flame-dried 250-ml round-bottom flask equipped with a magnetic stir bar, add 2,3,1',3',4',6'-hexa-*O*-benzoylsucrose (0.75 g, 0.78 mmol).
2. Add anhydrous pyridine (25 ml) and dissolve the 2,3,1',3',4',6'-hexa-O-benzoylsucrose.
3. Add a small crystal of 4-dimethylaminopyridine to the reaction mixture.
4. While stirring, add 4,4'-dimethoxytrityl chloride (0.30 g, 0.88 mmol) to the 100-ml round-bottom flask.
5. Stir reaction mixture overnight in ambient temperature.

The reaction can be monitored by TLC. Elute the plates with ethyl acetate (50%, v/v) in petrol ether. Both the starting material and the product are visible under UV light (254 nm). In addition, the spot containing the product turns yellow when the plate is heated or exposed to sulfuric acid.

6. Quench the reaction with 100 ml saturated $NaHCO_3$ solution. Extract thrice with 30 ml ethyl acetate.

7. Dry the organic layer over anhydrous Na_2SO_4, filter off the drying agent and evaporate the solvent *in vacuo* using a rotary evaporator. Purify by chromatography on silica gel using 30% ethyl acetate in petroleum ether as the eluent.
8. Collect fractions containing the desired product and evaporate *in vacuo* using a rotary evaporator to yield 6-*O*-dimethoxytrityl-2,3,1',3',4',6'-hexa-*O*-benzoylsucrose (**2**) as a white foam (0.37 g, 0.33 mmol, 42 % yield)
9. Characterize product by TLC (on silica gel), ^{1}H NMR, ^{13}C NMR, and HRMS.

*6-O-dimethoxytrityl-2,3,1',3',4',6'-hexa-O-benzoylsucrose (**2**) ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.04–8.01 (m, 2H), 7.95–7.99 (m, 8H), 7.81–7.79 (m, 2H), 7.57–7.45 (m, 6H), 7.43–7.39 (m, 5H), 7.36–7.24 (m, 15H), 7.10–7.19 (m, 4H), 6.80 (dd, J = 1.90, 8.95 Hz, 4H), 6.04 (d, J = 3.5 Hz, 1H), 5.97 (d, J = 6.35 Hz, 1H), 5.92–5.87 (m, 2H), 5.33 (dd, J = 3.55, 10.4 Hz, 1H), 4.72–4.50 (m, 5H), 4.31–4.26 (m, 1H), 4.04 (ddt, J = 4.45, 9.6 Hz, 1H), 3.72–3.71 (app d, 6H), 3.35–3.24 (m, 2H), 2.93 (d, J = 4.55 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.6, 166.1, 166.0, 165.4, 165.3, 158.5, 144.6, 135.8, 135.7, 133.6, 133.5, 133.2, 133.1 (2), 130.2, 130.1, 129.9 (2), 129.8 (2), 129.6, 128.4, 128.8 (3), 128.6, 128.5, 128.3 (2), 128.2, 127.9, 126.8, 113.2, 104.3, 91.0, 86.6, 78.8, 77.2, 76.0, 73.1, 71.7, 71.1, 70.9, 64.7, 64.4, 55.1. HRMS-ESI (m/z) calc'd $[M+Na]^+$ for $C_{75}H_{64}NaO_{19}$: 1291.3892, found 1291.3922.*

Synthesis of 6''-O-DMTr-N-trifluoroacetyl-protected aminoglycosides (3 and 4)

Introductory paragraph:

Neomycin (**11**) and ribostamycin (**10**) sulfates are first *N*-trifluoroacetylated, and then the primary hydroxyl group is selectively protected in the presence of secondary ones by DMTr-group. Between these two reaction steps only $NaHCO_3$-EtOAc-extraction is needed. The final products (**3** and **4**) are purified by silica gel chromatography.

Materials:

Neomycin trisulfate
Ribostamycin disulfate salt
Methanol
Triethylamine
Methyl trifluoroacetate
Saturated aqueous sodium bicarbonate ($NaHCO_3$) solution
Ethyl acetate
Sodium sulfate (Na_2SO_4), anhydrous
Pyridine, dry
4,4'-dimethoxytrityl chloride (DMTrCl)
Silica gel
Dichloromethane

50-mL round-bottom flasks with ground-glass stoppers
Magnetic stir bars
Magnetic stirring plate
Rotary evaporator
100-mL separatory funnel

Glass funnel
Filter paper
Sintered glass column

Introduce trifluoroacetyl protecting group

1. Weigh the aminoglycoside in 50-mL round-bottom flask and add 10 ml methanol.

   For neomycin trisulfate: 2.0 g (2.2 mmol)
   For ribostamycin disulfate salt: 0.25 g (0.38 mmol)

2. Add triethylamine (3.6 mL, 26 mmol for neomycin, 0.70 mL, 5.1 mmol for ribostamycin) and methyl trifluoroacetate (2,6 mL, 26 mmol for neomycin, 0.50 mL, 5.0 mmol for ribostamycin).
3. Stir overnight at room temperature using a magnetic stirring plate and a magnetic stir bar.
4. Evaporate to dryness with rotary evaporator
5. Add 20 ml saturated $NaHCO_3$ solution and extract with ethyl acetate (3 × 20 ml) in a 100-ml separatory funnel (works for both reaction mixtures)
6. Combine the organic layers and dry over anhydrous Na_2SO_4. Filter off the drying agent and evaporate the solvent *in vacuo* using a rotary evaporator.

Introduce 4,4'-dimetoxytrityl protecting group

7. Add 5 ml anhydrous pyridine and evaporate to dryness with rotary evaporator.
8. Dissolve the residue in 5 ml anhydrous pyridine.
9. Add 4,4'-dimetoxytrityl chloride to the mixture.
   For neomycin: add 0,80 g (2,4 mmol) 4,4'-dimethoxytrityl chloride
   For ribostamycin: add 0.139 g (0.41 mmol) 4,4'-dimethoxytrityl chloride
10. Stir overnight at room temperature using a magnetic stirring plate and a magnetic stir bar.
11. Add 20 ml saturated $NaHCO_3$ and extract with ethyl acetate (3 × 20 ml) in a 100-ml separatory funnel.
12. Combine the organic layers and dry over Na_2SO_4. Filter off the drying agent and evaporate the solvent *in vacuo* using a rotary evaporator.
13. Purify by silica gel column chromatography using a sintered glass column

    *For **3** use 10% MeOH and 1% Et_3N in CH_2Cl_2 as an eluent*
    *For **4** use 5% MeOH and 0.2% Et_3N in CH_2Cl_2 as an eluent*

14. Combine fractions containing the desired product and evaporate the solvent on a rotary evaporator.
15. Characterize the product by ^1H NMR, ^{13}C NMR, and HRMS.

*5'-O-(4,4'-Dimethoxytrityl)-1,3,2',6'-tetra-N-trifluoroacetylribostamycin (**3**). Yield of white foam 0.26 g (59%). ^1H NMR (CD_3OD, 500 MHz) δ: 7.46 (m, 2H), 7.34 (m, 4H), 7.29 (m, 2H), 7.20 (m, 1H), 6.86 (m, 4H), 5.67 (d, 1H, J = 2.7 Hz), 5.22 (d, 1H, J = 4.0 Hz), 4.18–4.10 (m, 3H), 4.02–3,97 (m, 3H), 3.92 (m, 1H), 3.81 (m, 1H), 3.78 (s, 6H), 3.76–3.64 (m, 4H), 3.60 (m, 2H), 3.30–3.25 (m, 2H), 3.23 (dd, 1H, J = 10.2, 3.8 Hz), 2.01 (ddd, 1H, J = 12.9, 4.2, 4.2 Hz), 1.75 (ddd, 1H, J = 12.8, 12.8 Hz). ^{13}C*

NMR (CD_3OD, 125 MHz) δ: 158.7, 145.0, 136.0, 135.8, 130.0 (2), 128.0, 127.4, 126.3, 112.7, 109.2, 96.6, 86.7, 83.2, 76.4, 75.5, 72.9, 72.1, 70.9, 70.2, 70.1, 63.1, 54.3, 53.8, 49.7, 49.2, 40.5, 31.5. HRMS-ESI (m/z) calc'd $[M+Na]^+$ for $C_{46}H_{48}F_{12}N_4NaO_{16}$: 1163.2771, found 1163.2786.

*5'-O-(4,4'-Dimethoxytrityl)-1,3,2',6',2''',6'''-hexa-N-trifluoroacetylneomycin (**4**). Yield of white foam 2.35 g (73%). ^1H NMR (CD_3OD, 500 MHz) δ: 7.46 (m, 2H), 7.36–7.30 (m, 6H), 7.21 (m, 1H), 6.90 (m, 4H), 5.78 (d, 1H, J = 3.3 Hz), 5.16 (d, 1H, J = 4.6 Hz), 5.09 (d, 1H, J = 1.5 Hz), 4.29 (dd, 1H, J = 4.8, 4.8 Hz), 4.21–4.20 (m, 2H), 4.14 (m, 1H), 4.11 (dd, 1H, J = 6.7, 6.7 Hz), 4.01 (m, 1H), 3.97 (m, 1H), 3.92 (m, 1H), 3.87 (dd, 1H, J = 9.9, 8.8 Hz), 3.80 (s, 6H), 3.75–3.63 (m, 5H), 3.60 (dd, 1H, J = 13.5, 7.4 Hz), 3.56–3.51 (m, 2H), 3.35–3.31 (m, 3H), 3.19 (dd, 1H, J = 10.5, 3.1 Hz), 2.00 (ddd, 1H, J = 12.9 Hz, 4.2, 4.2 Hz), 1.77 (ddd, 1H, J = 12.7, 12.7, 12.7 Hz).. ^{13}C NMR (CD_3OD, 125 MHz) δ: 157.9 (2), 145.0, 136.0, 135.6, 130.2, 129.9, 128.0, 127.4, 126.3, 112.7, 109.0, 97.6, 96.3, 87.4, 86.3, 81.9, 76.9, 75.6, 74.8, 73.0, 72.1, 71.5, 70.3, 69.8, 69.2, 67.1, 62.5, 54.3, 53.5, 51.3, 49.6, 49.1, 40.4, 39.6, 31.5. HRMS-ESI (m/z) calc'd $[M+Na]^+$ for $C_{56}H_{58}F_{18}N_6NaO_{21}$: 1515.3265, found 1515.3239.*

Synthesis of 3-*O*-DMTr-propyl β-D-galactopyranoside (5)

D-galactose pentaacetate is first exposed to $BF_3{\cdot}Et_2O$-catalyzed glycosylation with an excess of 1,3-propanediol to obtain 3-hydroxypropyl tetra-*O*-acetyl-β-D-galactopyranoside (**15**). The introduced 3-hydroxypropyl arm is protected by DMTr-group (**16**) and the acetyl groups are removed by aqueous ammonia (**5**).

Materials:

1,3-Propanediol
Anhydrous dichloromethane (CH_2Cl_2)
4 Å molecular sieves
D-galactose pentaacetate
Dry nitrogen gas (N_2)
Boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2O$)
Ethyl acetate, p.a grade
Hexane, p.a. grade
Dichloromethane, technical grade
10 m-% aqueous sulfuric acid
Vacuum desiccator with phosphorus pentoxide as drying agent
Saturated aqueous $NaHCO_3$ solution
Sodium sulfate (Na_2SO_4), anhydrous
Anhydrous pyridine
4,4'-Dimethoxytrityl chloride (DMTrCl)
Triethylamine (TEA), technical grade
Methanol (MeOH), technical grade
Concentrated aqueous ammonia solution

50-mL Erlenmeyer flask with a ground-glass stopper

50-mL, 25-mL and 10-mL round-bottom flasks with ground-glass stoppers
Rubber septa
Rotary evaporator
Magnetic stir bars
Magnetic stirring plate
Vacuum source (membrane pump, vacuum oil pump or water aspirator pump)
Separatory funnels
UV-lamp, 254 nm
Thin-layer chromatography (TLC) plate (Silica gel 60 F254 aluminum sheets, Merck)
Hot plate for heating TLC plates
4 × 25-cm sintered glass column

Introduce 1-O-(3-hydroxypropyl)-group to a peracetylated D-Galactose

1. Prepare 1,3-propanediol solution in DCM and dry the solution with molecular sieves: Transfer 1,3-propanediol (5.0 mL, 70 mmol) to a flame-dried 50-mL Erlenmeyer flask equipped with a ground joint. To this flask, add anhydrous dichloromethane (50 mL) and activated molecular sieves (4 Å). Let the solution dry for 72 h.
2. Weigh β-D-Galactose pentaacetate [**14**, 1.0 g, 2.6 mmol, 1.0 equivalent (equiv)] to a flame-dried 50-mL round-bottom flask equipped with a magnetic stir bar.
3. To this round-bottom flask, add 11 mL of the 1,3-propanediol solution prepared in steps 1-3 (1.0 mL, 14 mmol, 5.4 equiv 1,3-propanediol).
4. Place the mixture under dry N_2 atmosphere and cool down the mixture to 0 °C in an ice bath with magnetic stirring.
The N_2 atmosphere can be applied through a rubber septum that seals the flask and connects to the source of N_2.
5. Under N_2 atmosphere, inject $BF_3{\cdot}Et_2O$ (0.65 mL, 5.3 mmol, 2.0 equiv) to the mixture through a rubber septum dropwise.
6. Let the reaction mixture stir 18 h at room temperature.

> *The reaction can be monitored by TLC. Neutralize the samples by triethylamine. TLC analysis indicates the end of the reaction by disappearance of the starting material. Elute the plates with hexane (40%, v/v) in ethyl acetate and then drip 10 m-% aqueous sulfuric acid on the plates and heat on a hot plate until visible spots appear.*

7. Dilute the reaction mixture with dichloromethane and wash with saturated aqueous $NaHCO_3$ in a separatory funnel.
8. Dry the organic layer over anhydrous Na_2SO_4, filter off the drying agent and evaporate the solvent *in vacuo* using a rotary evaporator.
9. Evaporate the solvent under reduced pressure using rotary evaporator and purify the residue by column chromatography on silica gel (30% hexane in ethyl acetate). Evaporate the product fractions to dryness using rotary evaporator. Co-evaporate the residue twice with dichloromethane and dry the residue under vacuum to yield 3-hydroxypropyl tetra-*O*-acetyl-β-D-galactopyranoside (**15**) (0.11 g, 10%).

10. Characterize product by ^{1}H NMR, ^{13}C NMR and HRMS.

> *3-hydroxypropyl tetra-O-acetyl-β-D-galactopyranoside (**15**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.37–5.35 (m, 1H), 5.17 (dd, J = 10.5, 8.0 Hz, 1H), 5.00 (dd, J = 10.5, 3.5 Hz, 1H), 4.46 (d, J = 8.0 Hz, 1H), 4.08–4.18 (m, 2H), 3.97–4.03 (m, 1H), 3.63–3.55 (m, 1H), 3.87–3.93 (m, 1H), 3.74–3.63 (m, 2H), 2.13 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H), 1.85–1.77 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.4, 170.2, 169.7, 101.3, 70.8 (2), 68.8, 67.8, 67.1, 61.3, 60.2, 32.1, 20.7, 20.64, 20.55. HRMS-ESI (m/z): calc'd $[M+Na]^+$ for $C_{17}H_{26}NaO_{11}^+$: 429.1367, found 429.1346.*

*Note: there are also alternative and better yielding procedures available for the preparation of **15** (Ma, T. W. et al. 1994 and Böhrsch, V. et al. 2012).*

Introduce 4,4'-dimethoxytrityl group

12. Dissolve compound **15** (0.11 g, 0.27 mmol, 1.0 equiv) in dry pyridine (10 mL) in a 50-mL round-bottom flask equipped with a magnetic stir bar.
13. Add 4,4'-dimethoxytritylchloride (0.12 g, 0.35 mmol, 1.3 equiv) to the solution in small portions.
14. Stir the reaction mixture 18 hours at room temperature.

> *The reaction can be monitored by TLC. TLC analysis indicates the end of the reaction by disappearance of the starting material. Elute the plates with hexane (30%, v/v) in ethyl acetate and then drip 10 m-% aqueous sulfuric acid on the plates and heat on a hot plate until visible spots appear. Note that 4,4'-dimethoxytrityl group makes the product UV active, and the product spot can be visualized on TLC plate under UV light (254 nm).*

15. Evaporate the solvent under reduced pressure using rotary evaporator, dissolve the residue in dichloromethane and wash with saturated aqueous $NaHCO_3$ in a separatory funnel.
16. Separate the organic layer and dry it over anhydrous Na_2SO_4. Filter off the drying agent and evaporate the solvent *in vacuo* using a rotary evaporator.17. Evaporate the solvent under reduced pressure using rotary evaporator and purify the residue by column chromatography on silica gel (40% hexane and 0.1% triethylamine in ethyl acetate). Evaporate the product fractions to dryness using rotary evaporator. Co-evaporate the residue twice with dichloromethane and dry the residue under vacuum to yield 3-*O*-(4,4'-dimethoxytrityl)propyl tetra-*O*-acetyl-β-D-galactopyranoside (**16**) (0.12 g, 62%).
18. Characterize product by ^{1}H NMR, ^{13}C NMR and HRMS.

> *3-O-(4,4'-dimethoxytrityl)propyl tetra-O-acetyl-β-D-galactopyranoside (**16**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.44 (d, J = 8.0 Hz, 2H), 7.34–7.16 (m, 7H), 6.85 (d, J = 8.9 Hz, 4H), 5.40 (d, J = 3.3 Hz, 1H), 5.19 (dd, J = 10.4, 8.0 Hz, 1H), 5.01 (dd, J = 10.4, 8.0 Hz, 1H), 4.48 (d, J = 8.0 Hz, 1H), 4.23–4.13 (m, 2H), 4.07–4.02 (m, 1H), 3.74–3.69 (m, 1H), 3.90 (t, J = 6.7 Hz, 1H), 3.81 (s, 6H), 3.24–3.10 (m, 2H), 2.17 (s, 3H), 2.05 (s, 3H), 2.00 (s, 3H), 1.90 (s, 3H), 1.90–1.86 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.4, 170.3, 170.2, 158.4, 145.2, 136.4, 130.0, 128.2, 128.1, 127.8, 126.7, 113.1, 101.2,*

85.9, 71.0, 70.6, 68.9, 67.2, 67.1, 61.3, 59.9, 55.2, 30.3, 20.7, 20.6. HRMS-ESI (m/z): calc'd [M+Na]$^+$ for $C_{38}H_{44}O_{13}Na^+$: 731.2674, found 731.2705.

Remove the acetyl groups

18. Dissolve compound **16** (0.12 g, 0.17 mmol, 1.0 equiv) in MeOH (10 mL) in a 50-mL round-bottom flask equipped with a magnetic stir bar.
19. Add concentrated aqueous ammonia (1 mL) to the solution and let the reaction mixture stir 3 h at room temperature.

The reaction can be monitored by TLC. TLC analysis indicates the end of the reaction by disappearance of the starting material. Elute the plates with methanol (10%, v/v) in dichloromethane and heat on a hot plate until visible spots appear. Additionally, both starting material and product can be visualized on TLC plate under UV light (254 nm).

20. Evaporate the solvents under reduced pressure using rotary evaporator and purify the residue by column chromatography on silica gel (10% methanol and 0.5% triethylamine in dichloromethane). Evaporate the product fractions to dryness using rotary evaporator. Co-evaporate the residue twice with dichloromethane and dry the residue under vacuum to yield 3-*O*-(4,4'-dimethoxytrityl)propyl-β-D-galactopyranoside (**5**) (0.087 g, 94%).
21. Characterize product by ^{1}H NMR, ^{13}C NMR and HRMS.

*3-O-DMTr-propyl β-D-galactopyranoside (**5**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.40 (d, J = 7.7 Hz, 2H), 7.35–7.23 (m, 6H), 7.17 (t, J = 7.2 Hz, 1H), 6.80 (d, J = 8.6 Hz, 4H), 4.21 (d, J = 7.2 Hz, 1H), 4.08–3.97 (m, 3H), 3.85–3.78 (m, 2H), 3.75 (s, 6H), 3.72–3.64 (m, 1H), 3.63–3.52 (m, 2H), 3.47 (t, J = 5.5 Hz, 1H), 3.18–3.08 (m, 2H), 1.94–1.83 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 158.4, 145.2, 136.4, 130.0, 128.2, 127.8, 126.7, 113.1, 103.1, 85.9, 74.2, 73.5, 71.6, 68.9, 67.4, 61.6, 60.1, 55.2, 30.3. HRMS-ESI (m/z): calc'd [M+Na]$^+$ for $C_{30}H_{36}O_9Na^+$: 563.2252, found 563.2246.*

Synthesis of trivalent N-acetyl galactosamine cluster (6).

Introductory paragraph:

Tri-O-propargyl pentaerytritol (**17**) is protected by DMTr-group (**18**) and exposed to Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition with (3-azidopropyl)-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-galactopyranoside (**19**). The resultant *N*-acetyl galactosamine cluster (**6**) is purified by silica gel chromatography.

Materials:

tri-*O*-propargyl pentaerytritol
(3-azidopropyl)-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-galactopyranoside
1,4-dioxane
7 N ammonia in methanol (NH_3 in MeOH)
Tri-*O*-propargyl pentaerythritol
Copper iodide (CuI)
Diethyl ether

n-hexane
Dichloromethane, technical (DCM)
NAc-galactose-azide
Pyridine, anhydrous
Aqueous saturated sodium bicarbonate solution ($NaHCO_3$)
4,4'-dimethoxytrityl chloride
Ethyl acetate
4-dimethylaminopyridine (DMAP)
Methanol, technical (MeOH)

> *Note: tri-O-propargyl pentaerytritol (**17**) and (3-azidopropyl)-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-galactopyranoside (**19**) are commercially available, but they can be prepared following previously published procedures as well (Calvo-Flores et al. 2000, Tellers et al. 2013).*

2-ml Eppendorf tube
10-ml round-bottom flask
50-ml round-bottom flask
100-ml separatory funnel
50 °C oil bath
Eppendorf concentrator
Hot plate with magnetic stirrer
Magnetic stirbar
Thermometer
Thin-layer chromatography (TLC) plates
TLC chamber
UV lamp, 254 nm
Rotary evaporator equipped with vacuum pump

Introduce 4,4'-dimethoxytrityl group

1. To a flame-dried 50-ml round-bottom flask equipped with a magnetic stir bar, add 0.16 g (0.63 mmol) of tri-*O*-propargyl pentaerythritol (**17**).
2. Add 5 ml of anhydrous pyridine and dissolve the tri-O-propargyl pentaerythritol.
3. Add small crystal of 4-dimethylaminopyridine to the reaction mixture.
4. Add 0.25 g (0.75 mmol, 1.2 eq) of 4,4'-dimethoxytrityl chloride.
5. Stir the reaction mixture overnight in ambient temperature.
6. Quench the reaction with saturated $NaHCO_3$ solution (30 ml) and extract thrice with 20 ml ethyl acetate.
7. Combine the organic layers and dry over Na_2SO_4. Filter off the drying agent and evaporate the solvent *in vacuo* using a rotary evaporator.
8. Purify by chromatography on silica gel using 20 % diethyl ether in hexane as the eluent.
9. Collect fractions containing the desired product and evaporate *in vacuo* using a rotary evaporator to yield the 4,4'-dimethoxytritylated product (**18**) as a clear oil. (0.10 g, 0.19 mmol, 30 % yield)

> *Note that the 4,4'-dimethoxytrityl alcohol from partially hydrolyzed DMTrCl can be difficult to remove by chromatography.*

10. Characterize product by ^{1}H NMR, ^{13}C NMR, and HRMS.

*1-(4,4'-dimethoxytrityloxy)-2,2'-bis(propynyloxymethyl)-3-propynyloxypropane (**18**):* ^{1}H NMR (500 MHz, $CDCl_3$): *δ 7.38-7.26 (m, 5H), 7.22-7.17 (m, 4H), 6.88-6.82 (m, 4H), 4.17 (d, J = 2.4 Hz, 6H), 3.84-3.79 (m, 6H), 3.73 (d, J = 5.45 Hz, 2H), 3.60 (s, 6H), 2.45 (t, J = 2.4 Hz, 3H)***,** *^{13}C NMR (125 MHz, CDCl3): δ 158.7, 147.3, 139.4, 129.1, 127.9, 127.8, 127.1, 113.2, 79.7, 74.5, 70.2, 65.1, 58.8, 55.3, 44.7, HRMS-ESI (m/z) calc'd $[M+Na]^+$ for $C_{35}H_{36}O_6Na$: 575.2392, found 575.2397.*

Perform Huisgen 1,3-dipolar cycloaddition (''click'' reaction)

1. Dissolve 76 mg (0.14 mmol) of 4,4'-dimethoxytritylated tri-*O*-propargyl pentaerythritol (**18**) and 0.30 g (0.71 mmol, 5.2 eq) of (3-azidopropyl)-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-D-galactopyranoside (**19**) into 2 milliliters of 1,4-dioxane in a 10-ml round bottom flask.
2. Add small crystal of copper iodide to the reaction mixture and stir overnight in an oil bath heated to 50 °C.
3. Evaporate reaction mixture *in vacuo* using a rotary evaporator.
4. Purify by chromatography on silica gel using 5 % methanol in dichloromethane as the eluent.
5. Collect fractions containing the desired product and evaporate *in vacuo* using a rotary evaporator to yield **6** as white crystals (0.17 g, 0.33 mmol, 68 % yield).
6. Characterize product by ^{1}H NMR, ^{13}C NMR, and HRMS.

*Trivalent N-acetyl galactosamine cluster (**6**): ^{1}H NMR (500 MHz, CD_3OD): δ 7.84-7.81 (m, 3H), 7.37-7.33 (m, 2H), 7.27-7.17 (m, 7H), 6.80 (d, J = 8.45 Hz, 4H), 5.38-5.34 (m, 3H), 5.09 (dd, J = 3.35, 11.25 Hz, 3H), 4.61-4.55 (m, 3H), 4.52-4.39 (m, 12H), 4.20-4.09 (m, 9H), 4.02 (t, J = 6.6 Hz, 3H), 3.84 (quin, J = 5 Hz, 3H), 3.79 (s, 6H) 3.56-3.46 (m, 9H), 3.33 (quin, J = 1.6 Hz, 4H) 3.09-3.03 (m, 2H), 2.15-2.07 (m, 15H), 2.00 (s, 9H), 1.99-1.95 (app d, 17H), ^{13}C NMR (125 MHz, CD_3OD): δ 172.3, 170.7 (2), 170.3, 158.5, 144.8, 136.1, 130.0, 127.9, 127.3, 126.2, 123.7, 112.6, 101.4, 85.5, 70.7, 70.4, 68.8, 66.8, 65.6, 64.0, 61.3, 54.4, 50.2, 48.1, 46.7, 46.5, 45.3, 30.0, 21.6, 19.2 (3), HRMS-ESI (m/z) calc'd $[M+H]^+$ for $C_{86}H_{115}N_{12}O_{33}$: 1843.7680, found 1843.7606.*

Figure 2. Immobilization of DMTr-protected carbohydrates to long chain alkylamine controlled pore glass (LCAA-CPG). Note: **20**, **22**-**25** are randomly immobilized as polyols.

SYNTHESIS OF CARBOHYDRATE MONOSUCCINATES AND THEIR IMMOBILIZATION TO LCAA-CPG SOLID SUPPORT

The 4,4'-dimethoxytritylated carbohydrates (**1**-**6**) are immobilized to long chain alkylamine controlled pore glass (LCAA-CPG) using a one-pot procedure: 1) The 4,4'-dimethoxytritylated carbohydrates are randomly succinylated (**2** yields an uniform product)

using one equivalent of succinic anhydride and catalytic amount of 4-(dimethylamino)pyridine in pyridine. 2) The resulting carbohydrate monosuccinates (main product) are then covalently attached to LCAA-CPG solid support using PyBOP-promoted amide coupling. The remaining reactive functionalities, i.e. unreacted hydroxyl groups of the carbohydrate and unreacted amino groups on the solid support, are acetylated (capping step). Finally, the loading (µmol/g) of the carbohydrate-modified solid support (**20** – **25**) is determined by DMTr-cation assay. Note: the trivalent N-acetyl galactosamine cluster (**6**) is first deacetylated and the crude deacetylated product is used for the succinylation.

Materials:

Dry pyridine
Methanol (MeOH), technical grade
Dichloromethane (DCM), technical grade
Succinic anhydride
4-Dimethylaminopyridine (DMAP)
Long chain alkylamine controlled pore glass (LCAA-CPG) solid support
Dry dimethylformamide (DMF), p.a grade
Triethylamine (TEA), technical grade
Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP)
N,*N*-Diisopropylethylamine (DIEA)
1:1 (*v*/*v*) mixture of Cap A and Cap B solutions used in oligonucleotide synthesis (acetic anhydride : 2,6-lutidine : 1-methylimidazole : tetrahydrofurane 5:5:8:82, *v*:*v*:*v*:*v*).
Dichloroacetic acid (DCA) deblock solution (3% DCA in dichloromethane)

1.5-mL Eppendorf tubes
Vortex mixer
Thin-layer chromatography (TLC) plate (Silica gel 60 F254 aluminum sheets, Merck)
Mass spectrometer
Lyophilizer (freeze-dryer)
Oligonucleotide synthesis column, Glen Research, cat. no. 20-0030-00
Vacuum source (membrane pump, vacuum oil pump or water aspirator pump)
Vacuum desiccator with phosphorus pentoxide as drying agent
Equipment for vacuum filtration with oligonucleotide synthesis columns
2-mL syringes
10-mL volumetric flask
UV/VIS spectrophotometer with cuvettes

One-pot acetyl deprotection of trivalent *N*-acetyl galactosamine cluster (6)

1. Weigh 55.3 mg (30 µmol) of the acetylated NAc-galactose cluster into a 2 mL Eppendorf tube.
2. Add approximately 1 mL of 7 N ammonia in methanol.
3. Vortex until solid is dissolved and then leave Eppendorf tube in an Eppendorf mixer for approximately two hours.
4. Follow reaction and confirm the acetyl removal by HRMS.
5. Evaporate the solvent *in vacuo* in an Eppendorf concentrator. The product can be used in immobilization to a solid support without further purification.

Succinylate the 4,4'-dimethoxytritylated carbohydrate

1. Dissolve the 4,4'-dimethoxytritylated carbohydrate (30 µmol, 1.0 equiv) in dry pyridine (300 µL) in a 1.5-mL Eppendorf tube.
2. Add succinic anhydride (3.0 mg, 30 µmol, 1.0 equiv) dissolved in dry pyridine (200 µL), and add a catalytic amount of 4-dimethylaminopyridine.
3. Close the tube and agitate until everything is dissolved. Vortex the reaction mixture at room temperature for 18 h and monitor reaction on TLC (20% methanol in dichloromethane) and mass spectrometry.

> *The formation of the monosuccinylated product can be confirmed by mass spectrometer. In addition to the monosuccinylated products, traces of di- and trisuccinylated products are observed.*

4. Lyophilize the solution to dryness and use the crude residue as such for loading the solid support.

Load LCAA-CPG solid support with the carbohydrate monosuccinate

1. Dissolve the carbohydrate succinate crude product (30 µmol) in dry DMF (500 µL).
2. Weigh LCAA-CPG solid support (150 mg) to an oligonucleotide synthesis column and seal the cap of the column.

> *To ensure that the reactive amine groups in LCAA-CPG will be deprotonated, wash the solid support with 10% triethylamine in methanol with the aid of two plastic 2-mL syringes attached to the luers of the synthesis column. Allow the solid support to be in touch with the triethylamine solution for 15 min. Remove the solvent by vacuum filtration.*

3. Dry the column under vacuum in a vacuum desiccator.
4. Transfer the LCAA-CPG solid support (150 mg, approximately 50 milligrams of support for 10 µmol of carbohydrate) to the Eppendorf tube containing the carbohydrate monosuccinate in DMF.
5. Add PyBOP (17 mg, 33 µmol, 1.1 equiv) and DIEA (10 µL, 60 µmol, 2.0 equiv) to the mixture. Add more dry DMF to cover the solid support, if needed.
7. Vortex the reaction mixture 18 h at room temperature.
8. Filtrate the reaction mixture through an oligonucleotide synthesis column using vacuum filtration. Seal the cap of the column and wash the solid support in the column with DMF and dichloromethane using vacuum filtration. Dry the column under vacuum.

Acetylate the unreacted hydroxyl groups of the carbohydrate and unreacted amino groups of the solid support (capping step)

1. Treat the solid support with 1:1 (*v*/*v*) mixture of Cap A and Cap B solutions used in oligonucleotide synthesis (acetic anhydride : 2,6-lutidine : 1-methylimidazole : tetrahydrofurane 5:5:8:82, *v*:*v*:*v*:*v*). Use two

plastic 2-mL syringes attached to the luers of the synthesis column and allow the solid support to be in touch with the capping solution for 15 min at room temperature.
2. Wash the solid support in the column with DMF and dichloromethane using vacuum filtration. Dry the column under vacuum.

Determine the loading of the solid support by DMTr-cation assay

1. Weigh approximately 5 mg of the loaded solid support to a 1.5-mL Eppendorf tube.
2. Add approximately 1 mL of DCA deblock solution (3% DCA in dichloromethane) to the Eppendorf tube. Close the tube and agitate. Transfer the solution to a 10-mL volumetric flask. Repeat this step two more times.
3. Fill the volumetric flask up to 10 mL with the DCA deblock solution.
4. Measure the absorbance of the sample at 504 nm with UV/VIS spectrophotometer. The loading (µmol/g) can be determined using the following equation:

$$loading = \frac{A_{504} \times V}{76} \times \frac{1000}{m}$$

where A_{504} is the absorbance at 504 nm, V is the volume of the volumetric flask in milliliters and m is the mass of the weighed support in milligrams. As an example, 6.4 mg of the galactose-modified support gave the absorbance 0.7949, and the loading was hence 16 µmol/g.

OLIGONUCLEOTIDE SYNTHESIS USING IMMOBILIZED CARBOHYDRATES

The 3'-glycoconjugated oligonucleotides are synthesized on the carbohydrate-loaded LCAA-CPG solid support with automated DNA/RNA synthesizer. After automated synthesis, the oligonucleotides are released from the solid support and the protecting groups are removed. Different release/deprotection conditions are used for aminoglycoside-conjugated (derived from **22** and **23**), TBDMS-glucose-conjugated (derived from **20**) and other carbohydrate-conjugated oligonucleotides. For aminoglycoside-oligonucleotides, a two-step procedure is used (1, NaOMe/MeOH; 2, ammonolysis) to avoid O→N-acyl migration on the aminoglycoside moieties. For TBDMS-glucose-oligonucleotides, a two-step procedure is used (1, ammonolysis; 2, $Et_3N{\cdot}3HF/Et_3N$/DMSO) to remove the TBS-protection after release from the support, followed by desalting with reversed-phase (RP) cartridge. For other carbohydrate-oligonucleotides, a standard ammonolysis is used for the release and deprotection. Finally, the oligonucleotides are purified by RP HPLC and the authenticity of the products is confirmed by mass spectrometry (electrospray ionization, ESI).

Materials:

Concentrated aqueous ammonia (28% to 30%)
Milli-Q water
0.1 M NaOMe in MeOH solution
1.0 M aqueous ammonium chloride solution
Triethylamine trihydrofluoride
Triethylamine, p.a. grade
Dimethyl sulfoxide (DMSO), p.a. grade
0.1 M aqueous NaOAc solution
0.1 M aqueous triethylammonium acetate buffer, pH = 7.0
60 % aqueous acetonitrile solution: acetonitrile (MeCN), p.a. grade + Milli-Q water

Buffer A: 50 mM TEAA in water, pH = 7.0 (see recipe)
Buffer B: 50 mM TEAA in 95% acetonitrile:water, pH = 7.0 (see recipe)

Oligonucleotide synthesis column, Glen Research, cat. no. 20-0030-00
An automated DNA/RNA synthesizer
Vacuum source (membrane pump, vacuum oil pump or water aspirator pump)
Vacuum desiccator with phosphorus pentoxide as drying agent
Equipment for vacuum filtration with oligonucleotide synthesis columns
1.5-mL Eppendorf tubes
1.5-mL Eppendorf tubes equipped with a screw cap
Dry bath for heating Eppendorf tubes
Microcentrifuge (Eppendorf)
10-mL round-bottom flask
Plastic 1-mL, 2-mL and 5-mL syringes and needles
Rotary evaporator
RP cartridge (Poly-Pak™, Glen Research)
15-mL centrifuge tubes
Lyophilizer (freeze-dryer)
HPLC system equipped with semipreparative RP-C18 column (e.g. Phenomenex Clarity Oligo-RP, C18, 250 mm × 10 mm, 5µm)
Electrospray ionization mass spectrometry (ESI-MS) system

Synthesize the oligonucleotide with automated DNA/RNA synthesizer

1. Weigh 50 mg of the LCAA-CPG solid support loaded with the carbohydrate to an oligonucleotide synthesis column and seal the cap of the column.

> *Note the loading of the solid support. As an example, if the loading is 20 µmol/g, 50 mg of the support equals 1.0 µmol scale.*

2. Synthesize the oligonucleotide using an automated DNA/RNA synthesizer.

> *Standard phosphoramidite coupling cycle and coupling times can be used. Herein, 5-(benzylthio)-1H-tetrazole was used as activator.*

3. After the automated oligonucleotide synthesis is complete, remove the synthesis column from the synthesizer and dry the column briefly under vacuum in vacuum desiccator.

Release the oligonucleotide from the solid support and remove the protecting groups

Please note that three different release/deprotection protocols are described, depending on the conjugated carbohydrate.

Release/deprotection by standard ammonolysis:

1. Transfer the solid-supported 3'-carbohydrate-modified oligonucleotide to a 1.5-mL Eppendorf tube equipped with a screw cap.
2. Add concentrated aqueous ammonia (approximately 800 µl) to the Eppendorf tube and seal the tube with a screw cap.
3. Incubate the mixture 5 h at 55 °C e.g. with a dry bath.
4. Centrifuge 2 min at 12,000 rpm to pellet the solid support.
5. Transfer the supernatant carefully to a 10-mL round-bottom flask with a 1-mL syringe equipped with a needle.
6. Wash the solid support twice with Milli-Q water: add 1 mL Milli-Q water to the Eppendorf tube containing the solid support and repeat steps 4–5, combining the solutions to the same round-bottom flask.
7. Evaporate the combined solution under reduced pressure using rotary evaporator.

    The evaporated residue forms a thin, transparent film on the glass wall.

8. Re-dissolve the crude product in approximately 1 mL of Milli-Q water and transfer the solution to a 1.5-mL Eppendorf tube. Centrifuge 5 min at 12,000 rpm to pellet the possible insoluble material before HPLC purification. (The desired oligonucleotides are fully soluble in water).

    To transfer the crude product more quantitatively to the Eppendorf tube, the round-bottom flask may be washed 2 or 3 times with smaller volumes of Milli-Q water, the total volume of Milli-Q water being approximately 1 mL.

Release/deprotection of TBDMS-glucose-oligonucleotide (derived from compound 20):

Steps 1.–7. are identical with *release/deprotection by standard ammonolysis*.

8. Dissolve the residue in a mixture of triethylamine trihydrofluoride (75 µL), triethylamine (60 µL) and DMSO (115 µL) and transfer the mixture to a 1.5-mL Eppendorf tube.
9. Incubate the mixture 2 h at 50 °C e.g. with a dry bath.
10. Transfer the mixture to a 15-mL centrifuge tube and add 0.1 M aqueous NaOAc solution (10 mL).
11. Load the solution to an RP cartridge with a plastic syringe.
12. Flush the cartridge with 0.1 M aqueous triethylammonium acetate (6 mL, pH = 7.0). This procedure removes salts from the cartridge.
13. Elute the desalted oligonucleotide from the cartridge by flushing the cartridge with 60% aqueous acetonitrile solution (2 mL). Collect the eluted fraction and lyophilize it to dryness.
14. Re-dissolve the crude product in approximately 1 mL of Milli-Q water and transfer the solution to a 1.5-mL Eppendorf tube. Centrifuge 5 min at 12,000 rpm to pellet the possible insoluble material before HPLC purification.

Release/deprotection of aminoglycoside-oligonucleotides (derived from compounds 22 and 23):

1. Transfer the solid-supported 3'-carbohydrate-modified oligonucleotide to a 1.5-mL Eppendorf tube equipped with a screw cap.
2. Add 0.1 M NaOMe in MeOH (1.0 mL) to the Eppendorf tube and seal the tube with a screw cap.
3. Incubate the mixture 2 h at room temperature.

4. Centrifuge 2 min at 12,000 rpm to pellet the solid support.
5. Transfer the supernatant carefully to a 10-mL round-bottom flask with a 1-mL syringe equipped with a needle.
6. Add 1.0 M aqueous ammonium chloride (105 µl) to the round-bottom flask and mix gently. Ammonium chloride is added to neutralize the solution.
7. Evaporate the solution to dryness under reduced pressure using rotary evaporator.
8. Add concentrated aqueous ammonia (approximately 800 µl) to the residue, transfer the solution to a 1.5 mL Eppendorf tube with a 1-mL syringe equipped with a needle and seal the Eppendorf tube with a screw cap.
9. Incubate the mixture 15 h at 55 °C e.g. with a dry bath.
10. Continue as in *release/deprotection by standard ammonolysis*, steps 4–8.

Purify the oligonucleotide by RP HPLC

1. Prime semipreparative RP-C18 column with 100% buffer A at 3 mL/min for 5 min.
2. Purify the oligonucleotide using a linear gradient of buffer B in buffer A from 0% to 100% over 25 min (UV detection at 260 nm, flow rate 3 mL/min).
3. Confirm the presence of the desired oligonucleotide in the fraction using ESI-MS in negative mode.
4. Lyophilize the collected product fractions. To remove buffer salts, add 1 mL Milli-Q water, lyophilize again, and repeat this procedure.

REAGENTS AND SOLUTIONS:

Buffer A and Buffer B for HPLC purification

For preparing Buffer A and Buffer B for HPLC purification, make a 2.0 M triethylammonium acetate (TEAA) stock solution: Add 279 mL triethylamine (p. a. grade, > 99%) and 600 mL Milli-Q water in a 1 L glass container a equipped with a screw cap. Stir the solution and add 114 mL acetic acid (>99%). Stir the solution for 15 h at room temperature. Adjust the pH to 7.0 using pH meter (e.g. pHenomenal pH 1100L, VWR). Filter the solution through Millipore equipment under reduced pressure and incubate in an ultrasonic bath for 15 min. Prepare Buffer A and Buffer B from this 2.0 M stock solution by diluting with Milli-Q water (Buffer A) or HPLC grade acetonitrile (Buffer B) to get 50 mM TEAA solutions. The stock solution and Buffer A and B can be stored at 4 °C for several weeks.

COMMENTARY

BACKGROUND INFORMATION:

Covalent hybrid molecules between oligonucleotides and carbohydrates (i.e. oligonucleotide glycoconjugates) have received marked interest of improving both diano- and pharmacophoric properties of oligonucleotide therapeutics. The N-acetylgalactosamine (GalNAc) concept (Valentijn, 1997) is applied by Alnylam Pharmaceuticals for the targeting of siRNAs efficiently to hepatocytes. What makes this strategy efficient is the highly expressed receptor-ligand system (asialoglycoprotein receptor) and its rapid endocytic pathway that is able to feed the slow endosomal escape pathway of the siRNA payload. Also other carbohydrates can be applied for the delivery purposes. For example, constituents of glycans: hyaluronic acid and some chondroitin sulfates bind to CD44 receptor that is over-expressed in many cancer cells of epithelial origin (Misra et al. 2011). Bleomycin disaccharide and/or carbamoyl mannose show specificity to prostate cancer cells (Schroeder, 2014). Nanoparticles decorated with D-glucose are able to penetrate through blood brain barrier via glucose transporter-1 expressed on the brain capillary endothelial cells (Min et al. 2020). However, affinities of these other sugar ligand-receptor interactions may be too low

to overcome the unfavourable delivery properties of the oligonucleotide payload. Results that are more promising may be obtained, when these sugar constituents are used to decoration of oligonucleotide-loaded nanoparticles.

Aminoglycosides, in turn, are small molecular ligands for a variety of RNA targets and they can act as groove binders for DNA- and RNA-triple helices (Arya, 2011). Thus, they may provide enhanced binding to target DNA or RNA via a co-operative binding with hybridization, when covalently conjugated to oligonucleotides (Kiviniemi & Virta, 2011, Tähtinen et al. 2015).

Entirely different application is the DNA-based carbohydrate microarray, in which oligonucleotide glycoconjugates are prepared and immobilized on a DNA microarray by double strand formation (Morvan et al. 2012). By using appropriate building blocks and duplex formation, topologies, distances and number of the carbohydrate ligands may be readily varied. This glycoarray allows the study of the interaction between carbohydrates and lectins, and identification of the critical structural parameters for the recognition.

For the synthesis of oligonucleotide glycoconjugates, diverse set of conjugation strategies are available: Appropriate phoshoramidite building blocks of sugars have been prepared and used as an integral part of the automated assembly to produce both terminal and intra-chain glycomodifications into the oligonucleotide sequence (Dubber, M. & Fréchet, 2003, Katajisto et al. 2004, Ketomäki & Virta, 2008, Granqvist et al. 2017). The efficient reaction between oxyamines and aldehydes, i.e. oximation, has been utilized. The oligonucleotide is usually modified by an amino oxy group and used for on-support oximation with aldehyde modified carbohydrates (Katajisto et al. 2004). By using this strategy, multivalent hyaluronic acid and galactose-oligonucleotide conjugates have been successively prepared (Karskela et al. 2008 and 2010, Mäkilä et al. 2014). Both alkyne and azide-modified carbohydrates have been used for Cu(I)-catalyzed Huisgen 1,3-dipolar azide-alkyne cycloaddition (''Click''-reaction) with oligonucleotides (Kiviniemi et al. 2008, 2010 and 2011). The strain promoted Cu-free version of this reaction have been used to conjugate complex oligosaccharides. The advantage of this reaction is its efficiency and orthogonality. For example, azide-modified hyaluronic acid and chondroitin sulfates with variable sulfate patterns have been synthesized and conjugated with cyclo octyne-modified oligonucleotides (Jadhav et al. 2016 and 2018).

The compatibility of the synthetic strategy depends on the application itself. For the efficient lectin-carbohydrate recognition, multiple carbohydrate ligands are required (the so-called glycocluster effect). This binding is not necessarily strongly dependent on the orientation and spacer between the sugar units. Therefore, for example, the GalNAc ligands may be introduced into the target oligonucleotide in different ways to provide efficient delivery to hepatocytes: Three repeated couplings of monovalent GalNAc-intrachain phosphoramidite modifiers, followed by standard assembly of an oligonucleotide, can be applied. One would prefer post synthetic conjugation to the oligonucleotide with prefabricated triantennary GalNAc-cluster. The third, and perhaps the most convenient way is to immobilize this same, or corresponding prefabricated construct to a solid support, which is applicable to an automated synthesis of oligonucleotides. This makes the further processing convenient and allows assembly of any oligonucleotide sequence on this same glyco-modified solid support. In fact, this is the approach, what pharma companies would prefer, as chemically fine-toned libraries of the same active oligonucleotide sequence can be readily synthesized. In the current protocol (originally described by Kiviniemi & Virta, 2011 and Österlund et al. 2018), set of different carbohydrates is immobilized to a solid support and used for the automated assembly of oligonucleotides to produce 3'-glycoconjugated oligonucleotides. A random immobilization of carbohydrates, as polyols, to a solid support via succinyl linker may be applied. No additional bifunctional branching units or complex protecting group scheme are needed.

CRITICAL PARAMETERS AND TROUBLESHOOTING

An organic chemistry laboratory, equipped with an RNA/DNA synthesizer, an RP HPLC-instrument and UV-, HR MS(ESI)- and NMR-spectrometers, is required to perform the described protocols. In addition, experience of routine organic synthesis and routine laboratory instrumentation, regarding for example TLC analysis, chromatographic purification, extraction and evaporation of solvents, are needed for liquid phase reactions. General protocols for organic chemistry laboratory safety and safety sheets of all reagents have to follow properly.

The following critical parameters must be considered for successful performance of the described procedures. The DMTr-group is prone to acid-catalyzed cleavage that may occur during the silica gel chromatography. Be sure that the filled column and the eluents are neutralized by triethyl amine to prevent this premature cleavage before you start the column chromatography. Sodium methoxide-catalyzed methanolysis, used for the acetyl deprotection, is a moisture sensitive reaction. Plausible water contamination converts the reaction to hydrolysis and destroys the alkoxide ion-promoted catalytic effect. The boron trifluoride-catalyzed glycosylation should be carried out in dry condition to prevent the hydrolysis of the D-galactose penta acetate. Thus, follow the instructions properly and be sure that the reaction conditions are dry, when mentioned in each procedure. The carbohydrates are immobilized to a solid support using a one-pot procedure, in which crude intermediate products (carbohydrate

succinates) are immobilized to a solid support. The crude reaction mixture contains hydrolyzed succinic acid and traces of succinic anhydride, which compete with the carbohydrate succinates in the PyBOP-promoted amide coupling. Thus, follow the instructions properly and use only one equivalent (or sub-stoichiometric amount compared to hydroxyl groups) of succinic anhydride for the immobilization. The special instructions for automated oligonucleotide synthesis have to follow properly. For example, partial hydrolysis of the hygroscopic phosphoramidites (if not properly handled) may lower the efficiency of the chain elongation. Before using the carbohydrate-modified solid support, be sure that the oligonucleotide synthesis works well with standard solid supports. *N*-trifluoroacetylated and *O*-acetylated aminoglycosides are prone to *O*→*N*-acetyl migration when exposed to ammonia. Detection of this side reaction may be hard when aminoglycosides are conjugated to long oligonucleotides. Thus, it is important to follow the described two-step cleavage protocol, in which the immobilized conjugate is first exposed to sodium methoxide-catalyzed methanolysis, followed by neutralization with ammonium chloride, and then to concentrated ammonia. In order to confirm the quality of the carbohydrate-modified solid supports, it may be wise to take an aliquot of the support and use it for the automated assembly of a short oligonucleotide (for example T_5), perform cleavage as described, and analyze the released product by RP HPLC and MS(ESI). With relatively short oligonucleotides, plausible side products and failures of the overall synthesis can be traced more accurately.

UNDERSTANDING RESULTS:

RP HPLC-chromatograms and MS(ESI) data of sample oligonucleotide-3'-glycoconjugates are described here. These illustrate the purity and authenticity of the products if the protocol is replicated correctly. Appropriate eluent system (cf. buffers A and B) for each compound is described in the RP HPLC profiles. MS(ESI) spectra of the products may be observed as typical multiply charged ion patterns. The correct average molecular mass of the products may be calculated from these multiply charged signals using the most intensive isotope signal of the patterns.

Figure 3. RP HPLC profile of the crude product synthesized on the D-glucose-modified LCAA-CPG support (**20**) and MS (ESI) spectrum of the isolated product. A 10-mer DNA sequence has been assembled by an automated synthesizer. Conditions: An analytical RP HPLC column (C18, 250 × 4.6 mm, 5µm), gradient elution from 0 to 40% B in A (0 – 25 min), flow rate 1.0 mL min^{-1}, detection at 260 nm.

Figure 4. RP HPLC profile of the crude product synthesized on the sucrose-modified LCAA-CPG support (**21**) and MS (ESI) spectrum of the isolated product. A decamer DNA sequence has been assembled by an automated synthesizer. Conditions: An analytical RP HPLC column (C18, 250 × 4.6 mm, 5µm), gradient elution from 0 to 40% B in A (0 – 25 min), flow rate 1.0 mL min^{-1}, detection at 260 nm.

Figure 5. RP HPLC profile of the crude product synthesized on the ribostamycin-modified LCAA-CPG support (**22**) and MS (ESI) spectrum of the isolated product. An octamer 2'-O-methyl oligoribonucleotide sequence has been assembled by an automated synthesizer. Conditions: An analytical RP HPLC column (C18, 250 × 4.6 mm, 5µm), gradient elution from 10 to 40% B in A (0 – 25 min), flow rate 1.0 mL min^{-1}, detection at 260 nm.

Figure 6. RP HPLC profile of the crude product synthesized on the neomycin-modified LCAA-CPG support (**23**) and MS (ESI) spectrum of the isolated product. An octamer 2'-O-methyl oligoribonucleotide sequence has been assembled by an automated synthesizer. Conditions: An analytical RP HPLC column (C18, 250 × 4.6 mm, 5µm), gradient elution from 10 to 40% B in A (0 – 25 min), flow rate 1.0 mL min^{-1}, detection at 260 nm.

Figure 7. RP HPLC profile of the crude product synthesized on the D-galactose-modified LCAA-CPG support (**24**) and MS (ESI) spectrum of the isolated product. A 25-mer modified oligonucleotise sequence, consisted of a bicycloonyne moiety applicable to strain promoted azide-alkyne cycloaddition, hexaethylene glycol spacer, DNA- and 2'-*O*-methyl ribonucleotide regions, has been assembled by an automated synthesizer. Conditions: A semi-preparative RP HPLC column (C18, 250 × 10 mm, 5µm), gradient elution from 0 to 80% B in A (0 – 25 min), flow rate 1.0 mL min^{-1}, detection at 260 nm.

Figure 8. RP HPLC profile of a crude product synthesized on the trivalent GalNAc-cluster-modified LCAA-CPG support (**25**) and MS (ESI) spectrum of the isolated product. A 22-mer 2'-O-methyl oligoribonucleotide sequence has been assembled by an automated synthesizer. Conditions: An analytical RP HPLC column (C18, 250 ×4.6 mm, 5µm), gradient elution from 0 to 40% B in A (0 – 25 min), flow rate 1.0 mL min^{-1}, detection at 260 nm.

TIME CONSIDERATIONS:

With basic synthetic skills, each step in the chemical synthesis can be accomplished in 1 to 2 working days, including purification. However, if the starting material is not fully converted during the given time, the reaction period can be prolonged. Release/deprotection of TBDMS-glucose-oligonucleotide and release/deprotection of aminoglycoside-modified oligonucleotides require a 3rd working day, since lyophilization (freeze-drying) takes overnight. Note that before starting the actual synthesis of 3-hydroxypropyl tetra-*O*-acetyl-β-D-galactopyranoside (**15**) according to the protocol described here, 1,3-propanediol should be dried with molecular sieves for 72 h. Purified intermediate products can be stored in well-sealed flasks at 4 °C. Concentrated crude reaction mixtures (i.e. after work-up, prior to silica gel purification) can be stored in well-sealed flasks at –20 °C for several days.

ACKNOWLEDGEMENTS

We gratefully acknowledge funding from the Academy of Finland (308931) and Business Finland (448/31/2018).

LITERATURE CITED:

Arya, D. P. (2011) New approaches toward recognition of nucleic acid triple helices. *Accounts of Chemical Research, 44*, 134-146. doi: 10.1021/ar100113q

Böhrsch, V.; Mathew, T.; Zieringer, M.; Vallée, M. R. J.; Artner, L. M.; Dernedde, J.; Haag, R.; Hackenberger, C. P. R. (2012) Chemoselective Staudinger-phosphite reaction of symmetrical glycosyl-phosphites with azido-peptides and polygycerols. *Organic & Biomolecular Chemistry*, *10*, 6211–6216. doi: 10.1039/C2OB25207D

Calvo-Flores, F. G., Isac-Garcia, J., Hernandez-Mateo, F., Pérez-Balderas, F., Calvo-Asín, Snchéz-Vaquero, E., Santoyo-Ganzález, F. (2000), 1,3-Dipolar cycloadditions as a tool for the preparation of multivalent structures. *Organic Letters, 2*, 2499-2502. doi: 10.1021/ol00617v.

Dubber, M. & Fréchet, J. M. J. (2003) Solid-phase synthesis of multivalent glycoconjugates on a DNA synthesizer. *Bioconjugate Chemistry, 14,* 239-246. doi: 10.1021/bc0256244.

Granqvist, L., Kraszewski, A., Tähtinen, V., Virta, P. (2017) Synthesis of aminoglycoside-2'-methyl oligoribonucleotide fusions. *Molecules, 22*, doi: 10.3390/molecules22050760.

Jadhav, S., Käkelä, M., Mäkilä, J., Kiugel, M., Liljenbäck, H., Virta, J., Poijärvi-Virta, P., Laitala-Leinonen, T., Kytö, V., Jalkanen, S., Saraste, A., Roivainen, A., Virta, P. (2016) Synthesis and in vivo PET imaging of hyaluronan conjugates of oligonucleotides. *Bioconjugate Chemistry*, *27*, 391-403. doi: 10.1021/acs.bioconjchem.5b00477.

Jadhav, S., Gulumkar, V., Deshpande, P., Coffey, E., Lönnberg, H., Virta, P. (2018) Synthesis of azide-modified chondroitin sulfate precursors: substrates for ''click''-conjugation with fluorescent labels and oligonucleotides. doi: 10.1021/acs.bioconjchem.8b00317.

Karskela, M., Helkearo, M., Virta, P., Lönnberg, H. (2010) Synthesis oligonucleotide glycoconjugates using sequential click and oximation ligations, *Bioconjugate Chemistry, 21*, 748-755. doi: 10.1021/bc900529g.

Karskela, M., Virta, P., Malinen, M., Urtti, A., Lönnberg, H. (2008) Synthesis and cellular uptake of fluorescently labeled multivalent hyaluronan disaccharide conjugates of oligonucleotide phosphorothioates, *Bioconjugate Chemistry, 19*, 2549-2558. doi: 10.1021/bc800260y.

Katajisto, J., Virta, P., Lönnberg, H. (2004) Solid-phase synthesis of multiantennary oligonucleotide glycoconjugates utilizing on-support oximation, *Bioconjugate Chemistry, 15*, 890-896. doi: 10.1021/bc049955n.

Katajisto, J., Heinonen, P. Lönnberg, H. (2004) Solid-phase synhesis of oligonucleotide glycoconjugates bearing three different glycosyl groups: Orthogonally protected bis(hydroxymethyl)-N,N-bis(3-hydroxypropyl)malondiamide phsophoramidite as key building block. *Journal of Organic Chemistry, 69*, 7609-7615.

Ketomäki, K., Virta, P. (2008) Synthesis of aminoglycoside conjugates of 2′-*O*-methyl oligoribonucleotides. Bioconjugate Chemistry, 19, 766–777. doi: 10.1021/bc7004279.

Kiviniemi, A., Virta, P., Lönnberg H. (2008) Utilization of intrachain 4′-*C*-azidomethylthymidine for preparation of oligodeoxyribonucleotide conjugates by click chemistry in solution and on a solid Support. *Bioconjugate Chemistry, 19*, 1726–1734. doi: 10.1021/bc800221p.

Kiviniemi, A., Virta, P. Lönnberg, H. (2010) Solid-supported synthesis and click conjugation of 4′-*C*-alkyne functionalized oligodeoxyribonucleotides. *Bioconjugate Chemistry, 21*, 1890–1901. doi: 10.1021/bc100268w.

Kiviniemi, A., Virta, P., Drenichev, M. S., Mikhailov, S. N., Lönnberg, H. (2011). Solid-supported 2-*O*-glycoconjugation of oligonucleotides by azidation and click reactions. *Bioconjugate Chemistry, 22*, 1249–1255. doi: 10.1021/bc200097g.

Kiviniemi, A. & Virta, P. (2011) Synthesis of aminoglycoside-3′-conjugates of 2′-*O*-methyl oligoribonucleotides and their invasion to a 19F labeled HIV-1 TAR model. *Bioconjugate Chemistry, 22*, 1559-1566. doi: 10.1021/bc200101r.

Ma, T. W., Min, J. M., Zhang, L. H. (1994) Synthesis of galactosyl phosphate diester derivatives of nucleosides. *Carbohydrate Research, 257,* 323-330. doi: 10.1016/0008-6215(94)80046-4

Min, H. S., Kim, H. J., Naito, M., Ogura, S., Toh, K., Hayashi, K., Kim, B. S., Fukushima, S., Anraku, Y., Miyata, K., Katoka, K. (2020) Systemic brain delivery of antisense oligonucleotides across the blood-brain barrier with a glucose-coated polymeric nanocarrier. *Angewandte Chemie, Internal Edition, 59*, 8173-8180.

Misra, S., Heldin, P., Hascall, V. C., Karamanos, N. K., Skandalis, S. S., Markwald, R. R., Ghatak, S. (2011) Hyaluronan-CD44 interactions as potential targets for cancer therapy. *The FEBS Journal. 278*, 1429 – 1443. doi: 10.1111/j.1742-4658.2011.08071x.

Morvan, F., Vidal, S., Souteyrand, E., Chevolot, Y., Vasseur, J.-J., (2012) DNA glycoculsters and DNA-based carbohydrate microarrays: From design to applications. *RSC Advances, 2*, 12043-12068. doi: 10.1039/c2ra21550k.

Mäkilä, J., Jadhav, S., Kiviniemi, A., Käkelä, M., Liljenbäck, H., Poijärvi-Virta, P., Laitala-Leinonen, T., Lönnberg, H., Roivainen, A., Virta, P. (2014) Synthesis of multi-galactose-conjugated 2′-O-methyl oligoribonucleotides and their in vivo imaging with positron emission tomography. *Bioorganic & Medicinal Chemistry*, *22*, 6806-6813. doi: 10.1016/j.bmc.2014.10.034.

Pelletier, G.; Zwicker, A.; Allen, C. L.; Schepartz, A.; Miller, S. (2016) Aqueous glycosylation of unprotected sucrose employing glycosyl fluorides in the presence of calcium ion and trimethylamine. *Journal of American Chemical Society*, *138*, 3175–3182. doi: 10.1021/jacs.5b13384.

Schroeder, B. R., Ghare, M. I., Bhattacharya, C., Paul, R., Yu, Z., Zaleski, P. A., Bozeman, T. C., Rishel, M. J., Hecht S. (2014) The Disaccharide Moiety of Bleomycin Facilitates Uptake by Cancer Cells. *The Journal of American Chemical Society, 136*, 13641-13656. doi: 10.1021/ja507255g.

Tellers, D., Colletti, S. L., Dudkin, V., Aaronson, J., Momose, A., Tucker, T. J., Yuan, Y., Calati, K. B., Tian, L., Parmar, R. G., Shaw, A. W., Wang, W., Storr, R. A., Busuek, M., Kowtoniuk, R. A. (2013), Novel tetraGalNAc containing conjugates and methods for delivery of oligonucleotides. *International patent No. WO 2013166155A1.*

Tähtinen, V., Granqvist, L., Virta, P. (2015) Synthesis of C-5, C-2' and C-4'-neomycin-conjugated triplex forming oligonucleotides and their affinity to DNA-duplexes. *Bioorganic & Medicinal Chemistry, 23*, 4472-4480. doi: 10.1016/j.bmc.2015.06.013.

Valentijn, A. R. P. M., van der Marel, G. A., Sleidgret, L. A. J. M., van Berkel, T. J. C., Biessen, E. A. L., van Boom, J. H. (1997) Solid-phase synthesis of lysine-based cluster galactosides with high affinity for the asialoglycoprotein receptor. *Tetrahedron, 53,* 759-770. doi: 10.1016/S0040-4020(96)01018-6.

Österlund, T., Korhonen, H., Virta, P. (2018) DNA-templated N(Me)-alkoxyamine glycosylation. *Organic Letters, 20*, 1496-1499. doi: 10.1021/acs.orglett.8b00113.

FIGURE LEGENDS:

Provide legends for each of the figures for the manuscript. **DO NOT INCLUDE THE FIGURES IN THE MANUSCRIPT FILE**. Figures should be submitted as separate high-resolution (266-300 dpi) tif, eps, or jpeg files. Figures may be of any size. If figures are reproduced from a published source, you must acknowledge the source appropriately in the legend and must supply us with documentation showing that permission for use was granted.

TABLES:

Table 1. Obtained loadings for the modified LCAA-CPG supports

support	obtained loading
D-glucose-modified LCAA-CPG support (**20**)	20 µmol/g
sucrose-modified LCAA-CPG support (**21**)	20 µmol/g
ribostamycin-modified LCAA-CPG support (**22**)	14 µmol/g
neomycin-modified LCAA-CPG support (**23**)	17 µmol/g
D-galactose-modified LCAA-CPG support (**24**)	16 µmol/g
GalNAc-cluster-modified LCAA-CPG support (**25**)	8.2 µmol/g

.

- **FIGURES**

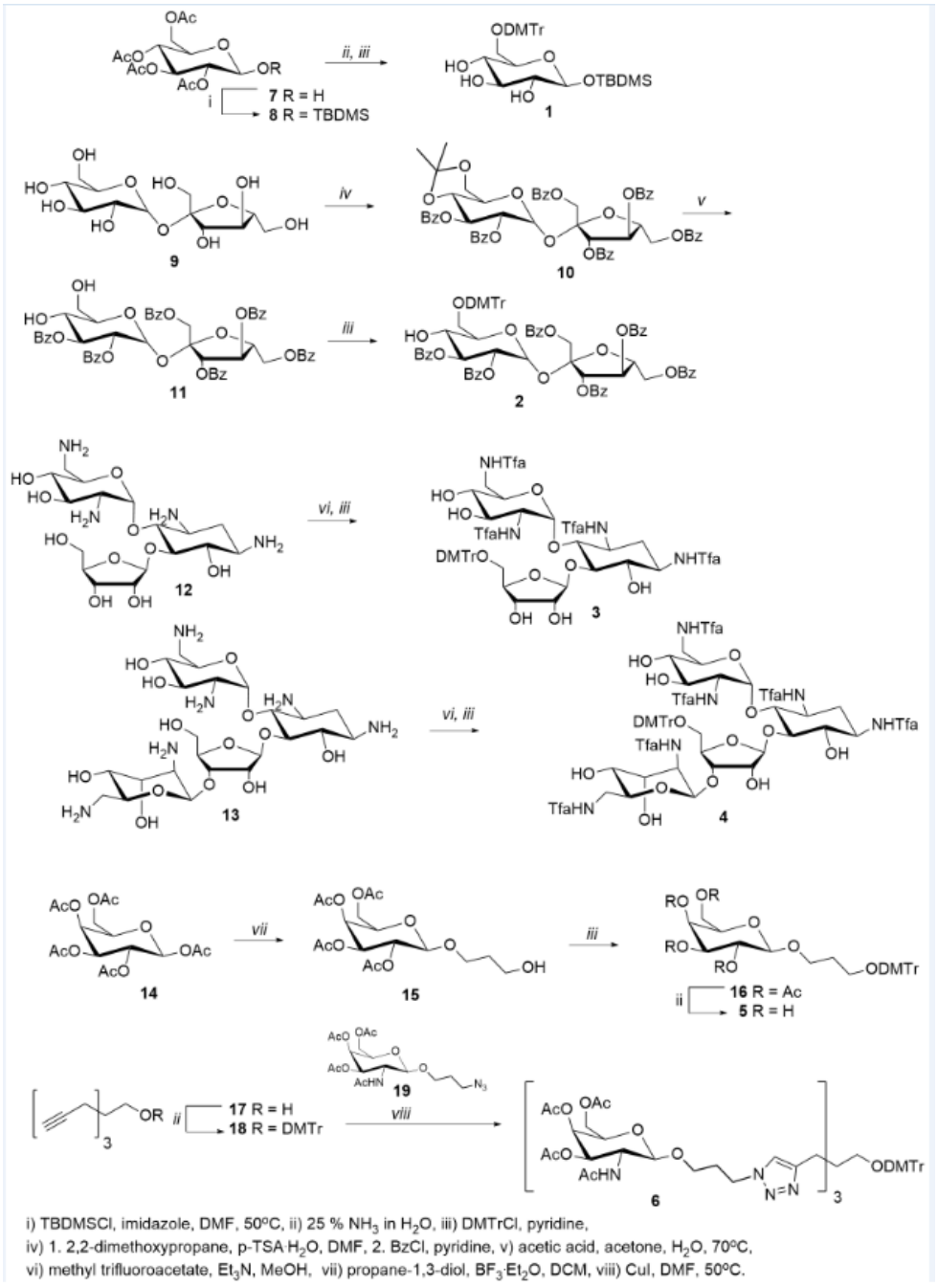
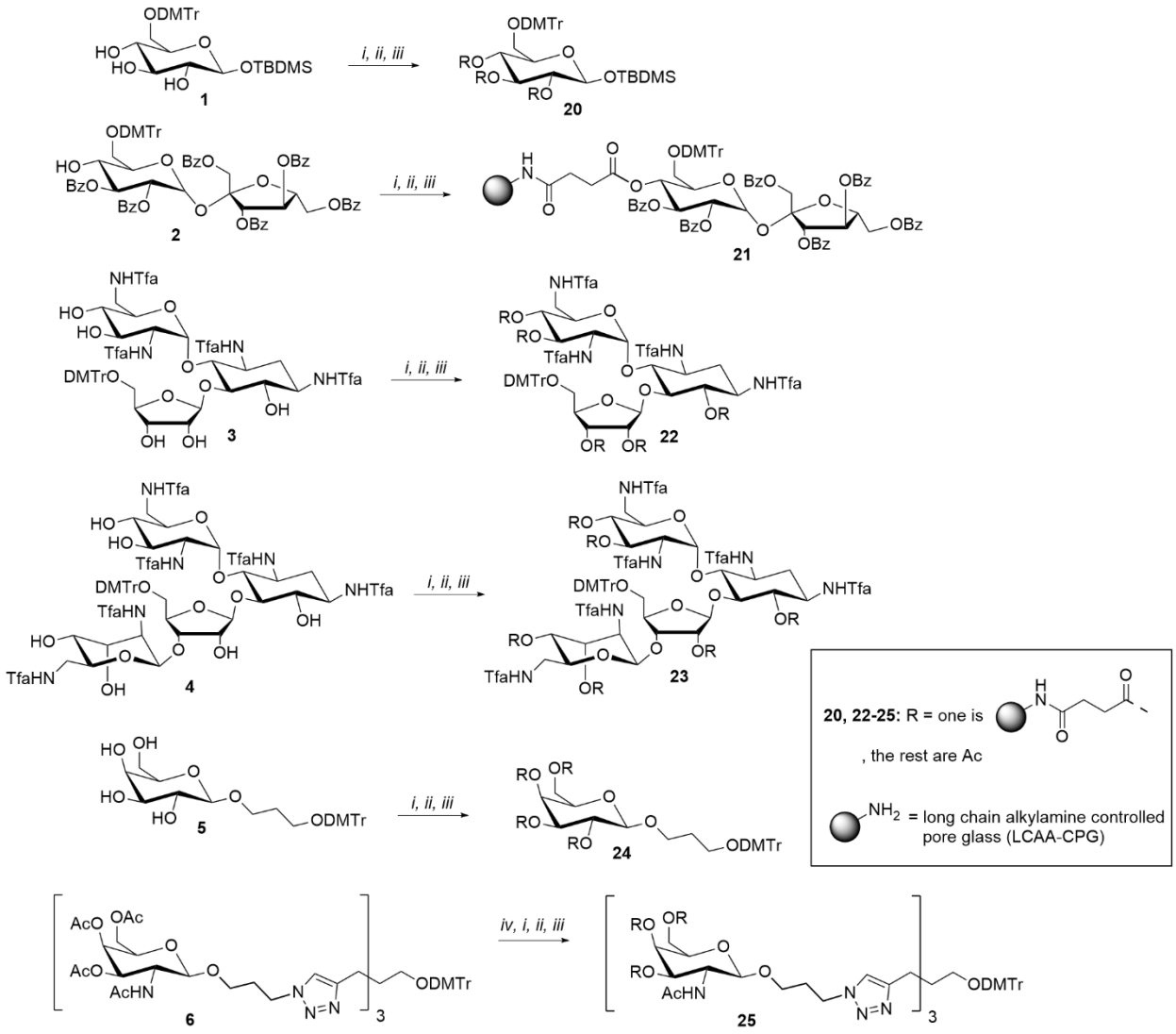

7 R = H
8 R = TBDMS
1
9
10
11
2
12
3
13
4
14
15
16 R = Ac
5 R = H
19
17 R = H
18 R = DMTr
6
i) TBDMSCl, imidazole, DMF, 50°C, ii) 25 % NH3 in H2O, iii) DMTrCl, pyridine,
iv) 1. 2,2-dimethoxypropane, p-TSA·H2O, DMF, 2. BzCl, pyridine, v) acetic acid, acetone, H2O, 70°C,
vi) methyl trifluoroacetate, Et3N, MeOH, vii) propane-1,3-diol, BF3·Et2O, DCM, viii) CuI, DMF, 50°C.

Figure 1



i) succinic anhydride, 4-(dimethylamino)pyridine, pyridine, ii) LCAA-CPG, PyBOP, DIEA, DMF,
iii) acetic anhydride, N-methylimidazole, lutidine, THF (on support), iv) 7N ammonia in methanol.

Figure 2

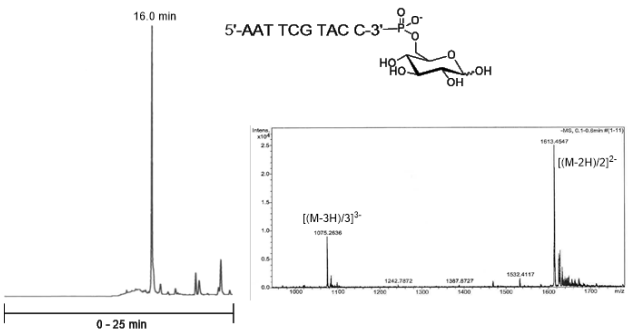


Figure 3

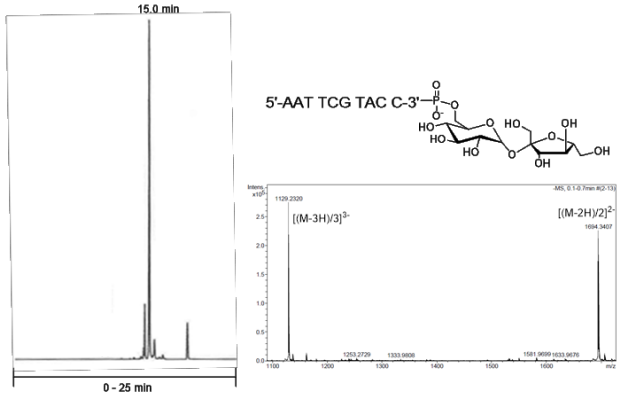


Figure 4

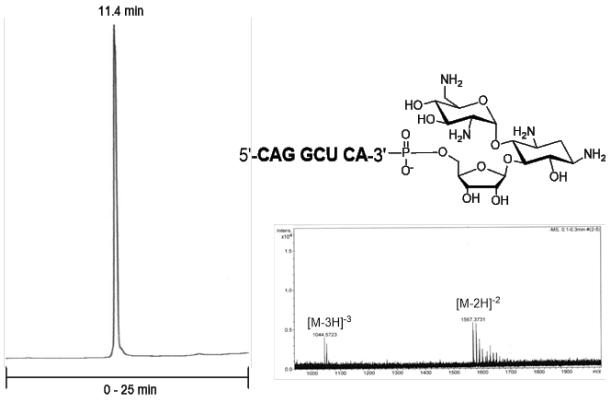


Figure 5

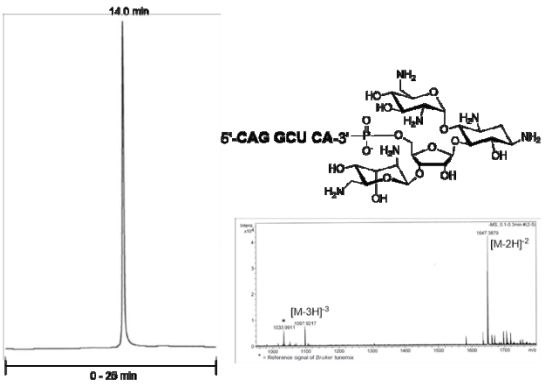


Figure 6

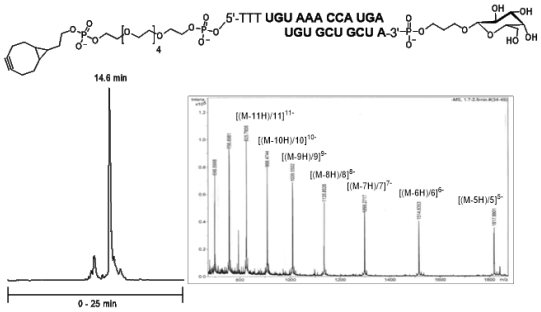


Figure 7

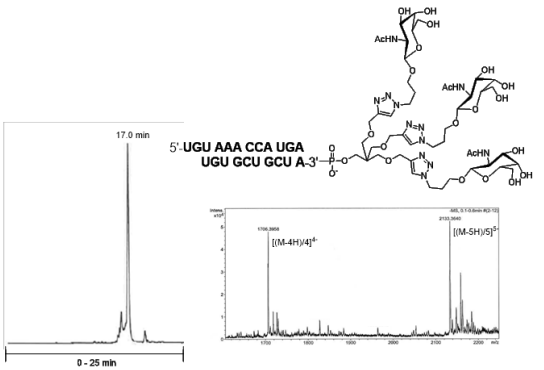


Figure 8