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Synthesis and characterization of mannosylated oligoribonucleotides

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ABSTRACT

Oligoribonucleotide (RNA)–carbohydrate conjugates bearing mono- and divalent mannosides were readily obtained using 3,4-diethoxy-3-cyclobutene-1,2-dione as the linking agent in the presence of trace amount of triethylamine. The glycoconjugates were purified by HPLC and characterized by electrospray mass spectroscopy.

1. Introduction

In recent years, nucleic acids have found a wide range of therapeutic applications such as gene therapy, antisense and antigene oligonucleotides, aptamers, and RNA interference.[1,2] One common problem that these therapeutic nucleic acids face is their poor pharmacokinetic properties, such as low bioavailability and poor selectivity. A number of approaches have been under intense investigation in order to improve the bioavailability of nucleic acids as therapeutic agents, such as the use of lipids, liposomes, peptides, viral vectors, and antibodies as delivery vehicles.[3–12]

It is known that lectins exist on cell surfaces and that they bind specifically to certain carbohydrates.[13] Sugars may therefore serve as recognition determinants to promote delivery of biologically active compounds. The concept of using lectin–glycan interactions to mediate cell targeting and cellular uptake of molecules, an approach known as glycotargeting,[14] has been under intense investigation during the past two decades.[15,16] A number of mammalian lectins with different carbohydrate specificities have been identified.[13] These lectins were found to direct their specific ligands to intracellular compartments. This effect has been demonstrated with various forms of nucleic acids of the deoxy series, for example, antisense oligonucleotides, plasmids, and chromosomal DNA.[17,18] In many of these studies, interactions between glycans and lectins improved the cellular uptake of nucleic acids. The same approach with RNAs has not been demonstrated.

We previously reported a method that allows for easy incorporation of carbohydrate moieties into the oligodeoxynucleotides (the DNA series) under mild conditions.[19] The chemistry involves the use of 3,4-diethoxy-3-cyclobutene-1,2-dione **1** (diethyl squarate)[20] as the linking reagent. First, a glycan containing an amino group reacts with diethyl squarate **1** to form an activated glycan, which further reacts with an oligonucleotide bearing a primary amine to form a glycoconjugate under slightly basic conditions. When this conjugation chemistry is applied to oligoribonucleotides (the RNA series), a few choices would have to be made to ensure successful reactions and subsequent purification. For instance, due to the instability of RNAs caused by the 2′-hydroxyls, conjugation reactions will have to be carried out under conditions that are mild enough to avoid degradation and migration of RNAs. We herein report a conjugation protocol that is suitable for the preparation of glycan–oligoribonucleotide conjugates. Thus, conjugation reactions were carried out while the oligoribonucleotides were still protected at the 2′-OH with the 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep) group. Such an approach ensures the integrity of RNA during the conjugation reactions. In this study, mannosides were chosen as the glycan because they are recognized by the macrophage mannose receptors on the surface of dendritic cells[21] that play important roles in immune responses.

2. Results and discussion

Fully protected 2-chloroethyl D-mannopyranoside **2** was prepared from mannose via the trichloroacetimidate chemistry.[22] Substitution by sodium azide gave fully protected 2-azidoethyl D-mannopyranoside **3**. Deacylation of **3** followed by the reduction

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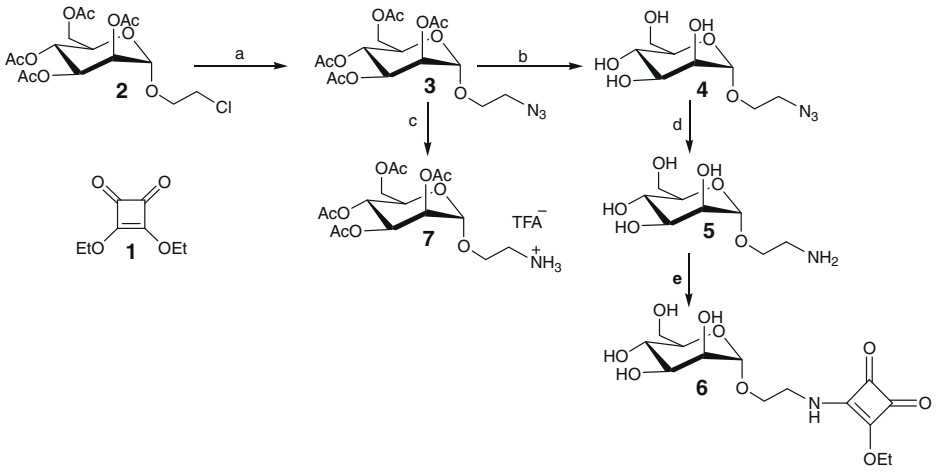
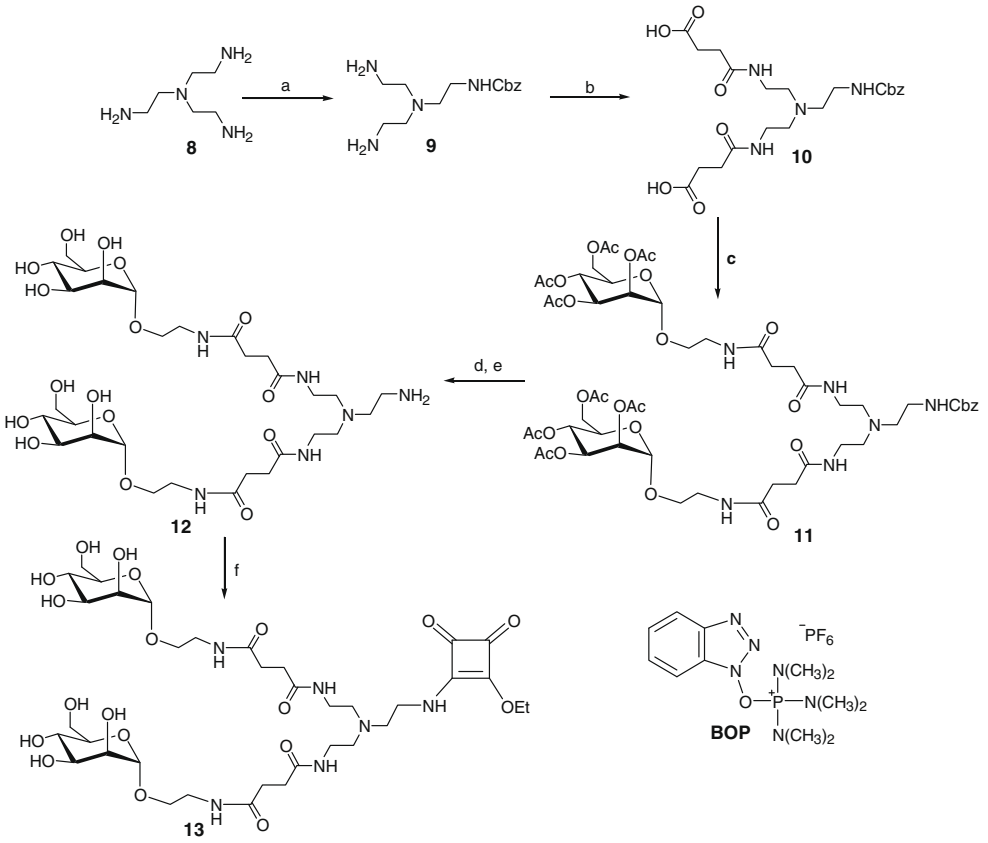


Scheme 1. Reagents and conditions: (a) NaN_3, DMF, 55 °C, 3 d; (b) NaOMe, MeOH; (c) Pd/C, H_2, MeOH, TFA; (d) Pd/C, H_2, H_2O; (e) H_2O, MeOH, NEt_3, rt, 5 min.

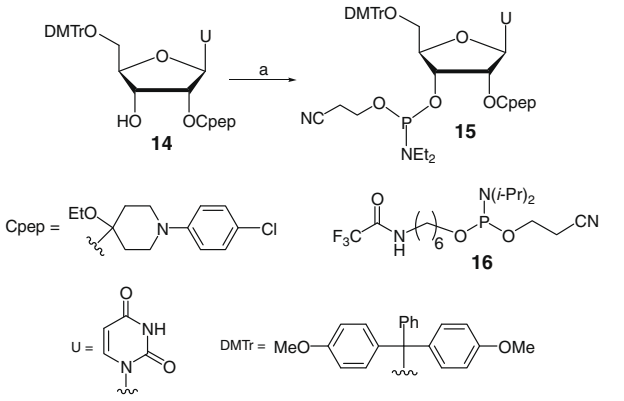


Scheme 2. Reagents and conditions: (a) $PhCH_2OCOCl$, CH_2Cl_2; (b) succinic anhydride, C_5H_5N; (c) **7**, BOP, $(i\text{-}Pr)_2NEt$, CH_2Cl_2; (d) NaOMe, MeOH; (e) Pd/C, H_2, MeOH; (f) H_2O, MeOH, NEt_3, rt, 7 min.

of the azido compound **4** gave 2-aminoethyl D-mannopyranoside **5**, which was readily transformed into its squarate analogue **6** using procedures (Scheme 1) reported previously.[19]

It is well known that interactions between lectins and their glycan ligands are relatively weak; therefore it is desirable to introduce multiple (multivalent) mannosides in order to harness the cluster glycoside effect.[23] In this study, tris(2-aminoethyl)amine **8** was used as the core structure (Scheme 2) to build multivalent mannosides. Upon protection of tris(2-aminoethyl)amine by the benzyloxycarbonyl (Cbz) group, the product **9** was condensed with succinic anhydride to furnish bis-carboxylic acid **10**. Compound **10** further reacted with the trifluoroacetate salt of 2-aminoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside[†] **7** to give the fully protected divalent mannoside **11**. Removal of *O*-acetates and Cbz groups from **11** yielded bimannoside **12**, which was subsequently

[†] Salt **7** was prepared by hydrogenation of 2-azidoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **3** in the presence of trifluoroacetic acid to avoid trans-acetylation of the reduced primary amine.

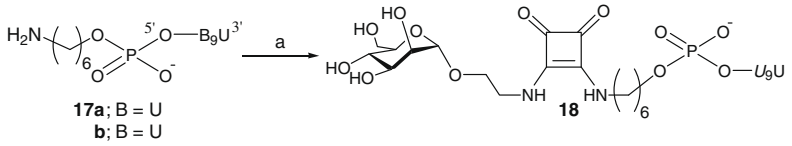


Scheme 3. Reagents and conditions: (a) $Et_2NP(Cl)OCH_2CH_2CN$, $(i\text{-}Pr)_2NEt$, CH_3CN.

The chemistry was similarly efficient when the bimannoside **13** was allowed to conjugate with U_{10}-mer **17b**. Reverse phase HPLC profiles of the reaction mixture indicated that the conjugation was complete in 9 h (Fig. 1). Conjugates **18** and **19** were purified by reverse phase HPLC and identified by ESI mass spectrometry.

The Cpep-protected conjugates **18** and **19** were then deprotected (removal of Cpep) by incubation in a mixture of DMA–triethylammonium formate buffer (pH 2.52) (6:4 v/v) to give the unprotected conjugates (**20** and **21**, Scheme 5). The final mono- and divalent conjugates **20** and **21** were identified by ESI mass spectrometry and analyzed by anion exchange chromatography on a DNAPac PA100 column (Fig. 2). These conjugates can be readily purified further by anion exchange chromatography if necessary.

When the stability of mono- and dimannosyl U_{10}-mers **20** and **21** was compared with that of unmodified U_{10}-mer in the presence



Scheme 4. Reagents and conditions: (a) **6,** MeOH, H_2O, NEt_3. *U*: uridine protected at 2′-position by Cpep; U: fully-unprotected uradine.

activated by reacting with diethyl squarate **1**. The product **13** was ready for conjugation with oligoribonucleotides.

Oligoribonucleotides were prepared by solid phase phosphoramidite chemistry using the 1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep as shown in Scheme 3)[‡] to protect the 2′-hydroxyls.[24] The Cpep chemistry was chosen because it allows for stable oligoribonucleotide 'precursor', that is, oligoribonucleotides protected only at 2′-positions with Cpep, to be prepared for conjugation with carbohydrates. The preparation of uridine phosphoramidite **15** is illustrated in Scheme 3. *N*,*N*-Diethyl phosphoramidites instead of *N*,*N*-diisopropyl phosphoramidites were used due to their higher reactivity (unpublished results).

U_{10} homodecamers were used to establish the conjugation chemistry. The decamers were modified at the 5′-termini using the standard phosphoramidite chemistry by a C_6-amino modifier **16** that is commercially available (Glen Research).

We then carried out the conjugation reactions between squarate-activated mono- and divalent mannosides and U_{10}-mer bearing a C_6-amino modifier. Two routes were attempted. First, conjugations were carried out by using amino-modified U_{10} homo decamers **17a**, in which all the Cpep-protecting groups were removed, and the conjugations took place in a triethylammonium acetate buffer (TEAA, pH 8.8, 0.1 M). Second, conjugations were performed in a DMA–TEAA buffer, and amino-modified U_{10} homo decamer **17b** (all the uridines except the 3′-terminal one were protected by Cpep at the 2′-OH, as indicated by the italic *U* in Scheme 4) was used as a precursor. Neither approach was efficient to give the conjugates in reasonable yields. In the first approach, less than 50% conversion was observed after the incubation proceeded for five days, as indicated by anion exchange chromatography on a DNA Pac PA100 column. The second route did not give any significant amount of the product after 10 days. However, in the latter approach, upon addition of trace amount of triethylamine, the reaction proceeded smoothly in aqueous methanol. Thus, when Cpep-protected U_{10}-mer **17b** was treated with squarate-activated monomannoside **6**, the reaction was complete after 5 h at room temperature as indicated by both ESI mass spectrometry and reverse phase HPLC.

[‡] Cpep-protected RNA monomers are commercially available at Rasayan Inc., CA.

of ribonuclease A and alkaline phosphatase, no significant difference was observed. Both the modified and unmodified U_{10}-oligomers were degraded by the enzymes at a similar rate under the same conditions.

3. Experimental

3.1. General methods

Reverse phase HPLC was carried out on a 250 × 4.6 mm LiChrospher 100® RP-18 5μ column: the column was eluted with triethylammonium acetate buffer (TEAA)–acetonitrile mixtures [linear gradient of TEAA buffer (0.1 M, pH 7.0)–acetonitrile (70: 30 v/v to 20: 80 v/v) over 15 min and then by isocratic elution. Flow rate 1.0 mL/min]. Anion exchange chromatography was carried out on a 250 × 4 mm DNAPac PA100 column: the column was eluted with the following program: eluent 1: TrisCl (0.1 M, pH 8.0, 10%), eluent 2: water, eluent 3: NaCl (1.0 M); flow rate: 1.5 mL/min. Concave gradient of NaCl from 0.1 M to 0.55 M over 20 min.

Dichloromethane was purified by Pure-Solv Solvent Purification Systems (Innovative Technology), and stored over activated 4 Å molecular sieves. Triethylamine, Hünig's base, and pyridine were dried by heating, under reflux, in the presence of calcium hydride and then distilled under nitrogen. (*N*-Trifluoroacetyl)aminohexyl-2-cyanoethyl *N*,*N*-diisopropylphosphoramidite, uridine, and adenosine resins for solid phase synthesis were purchased from Glen Research. The other chemicals were purchased from Aldrich and used without further purification unless stated otherwise.

3.2. 2-Chloroethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (2)

2,3,4,6-Tetra-*O*-acetyl D-mannopyranosyl α-trichloroacetimidate[25] (5.31 g, 10.8 mmol) was co-evaporated with dry toluene (2 × 10 mL) and then dissolved in dry dichloromethane (20 mL). Freshly distilled 2-chloroethanol (1.88 mL, 28.0 mmol) and 4 Å activated molecular sieve powder (1.0 g) were added, and the mixture was cooled to −78 °C (dry ice–acetone). To this solution was added a pre-cooled solution (−78 °C) of boron trifluoride diethyl etherate (2.3 mL, 18.3 mmol) in dry dichloromethane by cannula-

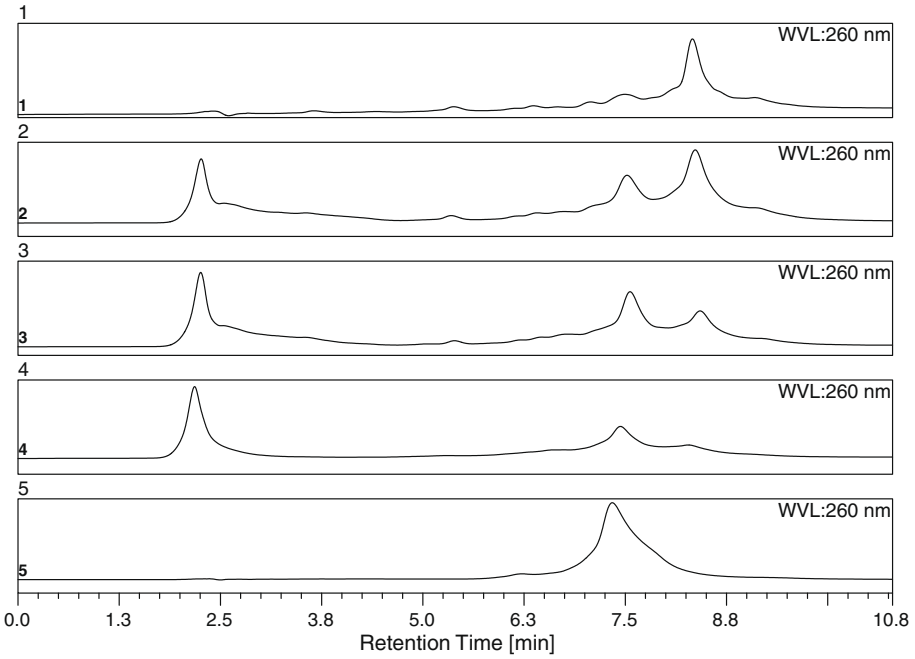


Figure 1. Stack plot of reverse phase HPLC profiles of the conjugation reaction. (1) U_{10}-mer **17b** modified at the 5′-terminus by a C_6-amino modifier; (2) conjugation reaction mixture after 3 h; (3) conjugation reaction mixture after 6 h; (4) conjugation reaction mixture after 9 h; (5) purified conjugate **19**. Samples were run on a C_{18} RP column. The peak with a t_R of 2.2 min represents the excess of squarate-activated bimannoside **13**.

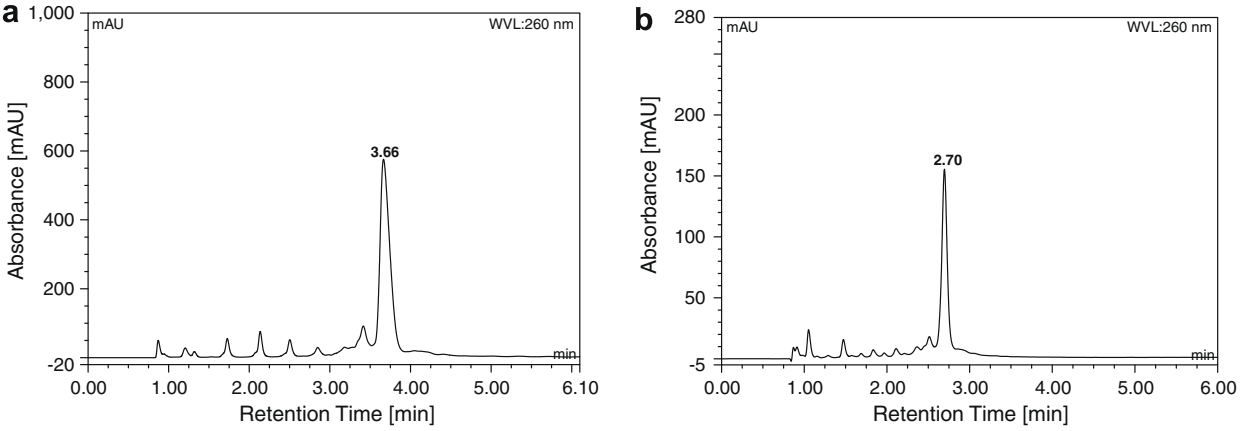


Figure 2. Anion exchange chromatography (DNAPac PA100, Dionex) profiles of fully-deprotected (a) mono mannosyl U_{10}-mer **20** and (b) dimannosyl U_{10}-mer **21**.

tion. After 1 h, the mixture was warmed up to room temperature, and then extracted with saturated aqueous sodium hydrogen carbonate (100 mL). The layers were separated and the aqueous layer was back-extracted with dichloromethane (2 × 20 mL). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel. The appropriate fractions, which were eluted with hexane–ethyl acetate (3:2 v/v) were combined and evaporated under reduced pressure to give the *title compound* as a white glass (3.17 g, 71.5%).

FAB-MS found $[M+H]^+ = 411.10556$, $C_{16}H_{24}ClO_{10}^+$ requires 411.10580.

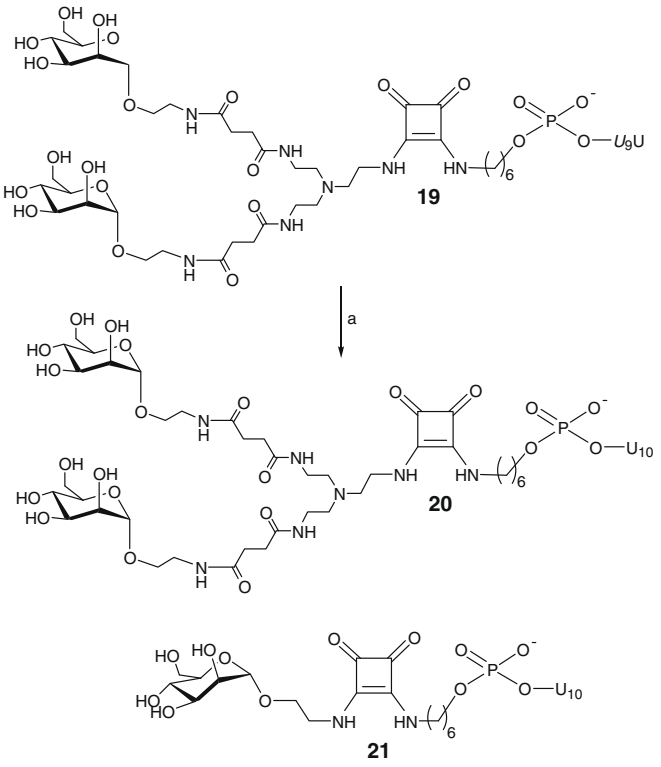
δ_H[$CDCl_3$]: 2.00 (3H, s, CH_3), 2.06 (3H, s, CH_3), 2.11 (3H, s, CH_3), 2.17 (3H, s, CH_3), 3.67 (2H, t, J = 5.6, OCH_2CH_2Cl), 3.83 (1H, dt, J = 5.3 and 10.6, OCH_2CH_2Cl), 3.93 (1H, dt, J = 5.9 and 11.3, OCH_2CH_2Cl), 4.12 (1H, m, H-5), 4.16 (1H, m, H-6), 4.30 (1H, m, H-6′), 4.88 (1H, d, J = 1.1, H-1), 5.26–5.38 (3H, m, H-2, H-3, and H-4).

δ_C[$CDCl_3$]: 20.6 (CH_3), 20.7 (CH_3), 20.9 (CH_3), 42.4 (OCH_2CH_2Cl), 62.4 (C-6), 66.0 (CH), 68.6 (OCH_2CH_2Cl), 68.9 (CH), 69.0 (C-5), 69.4 (CH), 97.8 (C-1), 169.8 (C═O), 169.9 (C═O), 170.0 (C═O), 170.6 (C═O).

R_f: 0.43 (hexane–ethyl acetate, 3:2 v/v).

3.3. 2-Azidoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (3)

2-Chloroethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **2** (2.00 g, 4.98 mmol) was dissolved in DMF (30 mL) followed by the addition of sodium azide (1.59 g, 24.5 mmol). The mixture was heated at 50 °C for 3 d. The products were then cooled to room temperature and filtered. The filtrate was concentrated under reduced pressure and the residue was purified by column chromatography on silica gel. The appropriate fractions, which were eluted with hexane–ethyl acetate (3:2 v/v) were combined and evapo-



Scheme 5. Reagents and conditions: (a) DMA–$Et_3N^+H{\cdot}HCOO^-$ buffer (pH 2.52), 40 °C, 6 h.

rated under reduced pressure to give the *title compound* as a white glass (1.94 g, 95.3%).

FAB-MS found $[M+H]^+ = 418.14481$, $C_{16}H_{24}N_3O_{10}^+$ requires 418.14617.

$\delta_H[CDCl_3]$: 2.00 (3H, s, CH_3), 2.06 (3H, s, CH_3), 2.11 (3H, s, CH_3), 2.17 (3H, s, CH_3), 3.48 (2H, m, CH_2), 3.68 (1H, m, $OCH_2CH_2N_3$), 3.88 (1H, m, $OCH_2CH_2N_3$), 4.05 (1H, m, H-5), 4.13 (1H, dd, $J = 2.3$ and 12.3, H-6), 4.30 (1H, dd, $J = 5.3$ and 12.3, H-6′), 4.88 (1H, s, H-1), 5.26–5.39 (3H, m, H-2, H-3, and H-4).

$\delta_C[CDCl_3]$: 20.6 (CH_3), 20.7 (CH_3), 20.8 (CH_3), 20.9 (CH_3), 50.3 ($OCH_2CH_2N_3$), 62.4 (CH_2, C-6), 66.0 (CH), 67.0 ($OCH_2CH_2N_3$), 68.8 (2 × CH), 69.4 (CH), 97.7 (CH, C-1), 169.7 (C=O), 169.8 (C=O), 170.0 (C=O), 170.6 (C=O).

R_f: 0.43 (hexane–ethyl acetate, 4:6 v/v).

3.4. 2-Aminoethyl α-D-mannopyranoside (5)

2-Azidoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **3** (0.61 g, 1.46 mmol) was dissolved in methanol (8.0 mL) followed by the addition of a solution of sodium methoxide in methanol (25 wt % solution in methanol, 79 μL, 0.36 mmol). After 30 min, pre-washed Amberlite IR 120 resin (H^+ form) (1.45 g) was added and stirred for 2 min. The resin was removed by filtration and the filtrate was concentrated under reduced pressure to give 2-azidoethyl α-D-mannopyranoside **4** as a colorless gum (0.283 g).

A portion of the above product (0.142 g, 0.57 mmol) was dissolved in water (10 mL) followed by the addition of Pd/C (20.0 mg, 5% Pd on charcoal). The mixture was stirred under an atmosphere of hydrogen for 6 h at room temperature. The products were filtered through a bed of Celite. The filtrate was concentrated under reduced pressure to give the *title compound* as a colorless gum (0.109 g, 66.9% over two steps).

FAB-MS found $[M+H]^+ = 224.11771$, $C_8H_{18}NO_6^+$ requires 224.11341.

$\delta_H[D_2O]$: 2.74 (2H, dd, $J = 5.5$ and 14.4, $OCH_2CH_2NH_2$), 3.44 (1H, m, $OCH_2CH_2NH_2$), 3.53–3.81 (6H, m, H-3, H-4, H-5, H-6, H-6′, and $OCH_2CH_2NH_2$), 3.87 (1H, m, H-2), 4.77 (1H, d, $J = 1.6$, H-1).

$\delta_C[D_2O]$: 39.8 ($OCH_2CH_2NH_2$), 60.9 (C-6), 66.7 (CH), 68.5 ($OCH_2CH_2NH_2$), 69.9 (C-2), 70.5 (CH), 72.7 (CH), 99.8 (C-1).

3.5. Monovalent mannosyl squarate (6)

2-Aminoethyl α-D-mannopyranoside **5** (35.5 mg, 0.16 mmol) was dissolved in distilled water (200 μL), followed by the addition of methanol (1.8 mL), 3,4-diethoxy-3-cyclobutene-1,2-dione **1** (140 μL, 0.95 mmol), and triethylamine (5 μL). After 5 min, the solvents were quickly removed under reduced pressure. The residue was diluted with distilled water (1.0 mL) and purified by size exclusion chromatography on BioGel P-2 gel (fine, 1.6 × 75 cm), eluted with milliQ water. The appropriate fractions were combined and freeze-dried to give the *title compound* as a light yellow amorphous solid (23.0 mg, 41.4%).

FAB-MS found $[M+H]^+ = 348.12946$, $C_{14}H_{22}NO_9^+$ requires 348.12765.

$\delta_H[D_2O$, recorded at 10 °C]: 1.15 (t, $J = 7.0$, $-OCH_2CH_3$), 1.17 (t, $J = 7.0$, $-OCH_2CH_3$) (these two signals integrate 3H), 3.26–3.63 (10H, m, H-2, H-3, H-4, H-5, H-6, H-6′, and CH_2), 4.44 (q, $J = 7$, $-OCH_2CH_3$), 4.47 (q, $J = 7$, $-OCH_2CH_3$) (these two signals integrate 2H), 4.58 (1H, d, $J = 6.0$, H-1) (the CH_3 and CH_2 each splits over two regions presumably due to the *pseudo*-amide nature of the compound).

R_f: 0.69 (chloroform–methanol–water, 65:35:5 v/v).

3.6. 2-(Benzyloxycarbonyl)tris(2-aminoethyl)amine (9)

Tris(2-aminoethyl)amine **8** (2.92 g, 20.0 mmol) was dissolved in dry dichloromethane (50 mL) and cooled (ice-water bath). A solution of benzyl chloroformate (1.03 mL, 7.22 mmol) in dry dichloromethane (100 mL) was added over a period of 1 h. The mixture was allowed to warm up to room temperature slowly. After an additional 1 h, the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel. The appropriate fractions, which were eluted with chloroform–methanol–ammonium hydroxide (10:4:1 v/v) were combined and evaporated under reduced pressure to give the *title compound* as a white amorphous solid (1.25 g, 61.8% based on benzyl chloroformate).

FAB-MS found $[M+H]^+ = 281.19946$, $C_{14}H_{25}N_4O_2^+$ requires 281.19775.

$\delta_H[CDCl_3]$: 2.51 (4H, t, $J = 6$, $-NCH_2CH_2NH_2$), 2.56 (2H, t, $J = 5.9$, CH_2), 2.72 (4H, t, $J = 6$, $-NCH_2CH_2NH_2$), 3.26 (2H, br, CH_2), 4.78 (4H, br, NH_2), 5.10 (2H, s, $-CH_2Ph$), 6.05 (1H, br, NH), 7.31–7.37 (5H, m, aromatic).

$\delta_C[CDCl_3]$: 39.4 (CH_2), 39.6 (CH_2), 53.7 (CH_2), 57.0 (CH_2), 66.5 (CH_2), 128.0 (CH), 128.1 (CH), 128.5 (CH), 136.8 (C), 156.7 (C=O).

R_f: 0.42 (chloroform–methanol–ammonium hydroxide, 10: 4:1 v/v).

3.7. 2,2′-Bissuccinoyl-2″-(benzyloxycarbonyl)tris(2-aminoethyl)amine (10)

2-(Benzyloxycarbonyl)tris(2-aminoethyl)amine **9** (1.88 g, 6.71 mmol) was co-evaporated with dry toluene (2 × 10 mL) and then dissolved in dry pyridine (30 mL). Succinic anhydride (1.47 g, 14.7 mmol) was added at room temperature. After 40 min, the products were concentrated under reduced pressure. The residue was dissolved in methanol (5 mL) and added dropwise to diethyl ether (300 mL). The product was collected by filtration as a white amorphous solid (2.71 g, 84.1%).

ESI-MS found $[M-H]^-$ = 479.2, $C_{22}H_{31}N_4O_8^-$ requires 479.2.

δ_H[$CDCl_3$]: 2.37 (4H, t, J = 6.0, CH_2), 2.41 (4H, t, J = 6.2, CH_2), 3.26 (6H, m), 3.39 (5H, m), 5.01 (2H, –CH_2Ph), 7.25–7.40 (5H, aromatic).

R_f: 0.48 (chloroform–methanol–water, 65:35:5 v/v).

3.8. Trifluoroacetate salt of 2-aminoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (7)

2-Azidoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **3** (600.00 mg, 1.44 mmol) was dissolved in methanol (25 mL) followed by the addition of Pd/C (42.0 mg, 5% Pd on charcoal) and trifluoroacetic acid (200 μL, 2.65 mmol). The reaction mixture was stirred under an atmosphere of hydrogen for 5 h at room temperature. The products were filtered through a bed of Celite. The filtrate was concentrated under reduced pressure and the product was used for the next reaction without further purification.

3.9. Fully protected bivalent-mannoside (11)

2,2′-Bissuccinoyl-2″-(benzyloxycarbonyl)tris(2-aminoethyl)amine **10** (0.230 g, 0.48 mmol) was dissolved in dry dichloromethane (10 mL). Then (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (0.424 g, 0.96 mmol) followed by Hünig's base (0.75 mL, 4.31 mmol) was added at room temperature. After 20 min, a solution of the trifluoroacetate salt of 2-aminoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannoside **7** (1.44 mmol) in dry dichloromethane (5 mL) was added. The reaction was allowed to proceed at room temperature for 12 h. The products were then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (95:5 v/v) were combined and evaporated under reduced pressure to give the *title compound* as a white glass (309.0 mg, 52.5%).

FAB-MS found $[M+H]^+$ = 1227.60795, $C_{54}H_{79}O_{26}N_6$ requires 1227.50441.

δ_H[$CDCl_3$] include the following signals: 2.01 (CH_3), 2.07 (CH_3), 2.12 (CH_3), 2.17 (CH_3), 4.00 (2H, br, H-5Man), 4.12 (2H, dd, J = 2.0 and 12.0, H-6Man), 4.29 (2H, dd, J = 5.0 and 12.0, H-6′Man), 4.82 (2H, s, H-1Man), 5.10 (2H, s, –CH_2Ph), 5.28–5.31 (6H, br, m, H-2, H-3, H-4Man), 7.36 (5H, aromatic).

R_f: 0.51 (dichloromethane–methanol, 90:10 v/v).

3.10. Fully deprotected bivalent-mannoside (12)

Fully protected bivalent-mannoside **11** (0.194 g, 0.158 mmol) was dissolved in methanol (3.0 mL) followed by the addition of a solution of sodium methoxide in methanol (25 wt %, 9 μL, 0.042 mmol). After 1 h, pre-washed Amberlite IR 120 resin (H^+ form, 300 mg) was added and stirred for 5 min. The resin was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was redissolved in methanol (15.0 mL) followed by the addition of Pd/C (30.0 mg, 5% Pd on charcoal). The reaction mixture was stirred in an atmosphere of hydrogen at room temperature for 3 h. The products were filtered through a bed of Celite. The filtrate was concentrated under reduced pressure and the residue was purified by size exclusion chromatography on Bio-Gel P2 gel (fine, 1.6 × 75 cm), eluted with aqueous ammonium bicarbonate buffer (30 mM). The appropriate fractions were combined and freeze-dried to give the *title compound* as a colorless glass (0.115 g, 96.2%).

FAB-MS found $[M+H]^+$ = 757.40622, $C_{30}H_{58}O_{16}N_6^+$ requires 757.38311.

δ_H[D_2O]: 2.66 (8H, s, br, CH_2), 2.80 (4H, s, br, CH_2), 2.85 (2H, s, br, CH_2), 3.05 (2H, s, br, CH_2), 3.41 (4H, s, br, CH_2), 3.50–3.53 (2H, m, H-6), 3.57–3.60 (2H, m, H-6′), 3.71–3.75 (4H, m, CH_2 and H-5), 3.78 (2H, t, J = 9.6, H-4), 3.86–3.93 (6H, m, CH_2 and H-3), 4.01 (2H, d, J = 12, CH_2), 4.07 (2H, s, H-2), 4.99 (2H, s, H-1).

δ_C[D_2O]: 31.2 (CH_2), 31.3 (CH_2), 37.2 (CH_2), 39.2 (C-6), 52.62 (CH_2), 61.2 (CH_2), 66.1 (CH_2), 67.0 (C-4), 70.3 (C-2), 70.8 (C-3), 73.1 (C-5), 99.9 (C-1), 175.0 (C=O).

R_f: 0.20 (methanol–ammonium hydroxide, 4:1 v/v).

3.11. Squarate-activated bivalent-mannoside (13)

Bivalent mannoside **12** (17.9 mg, 23.7 μmol) was dissolved in distilled water (100 μL), followed by the addition of methanol (900 μL), 3,4-diethoxy-3-cyclobutene-1,2-dione **1** (21 μL, 142 μmol), and triethylamine (3 μL). After 7 min, the solvents were quickly removed under reduced pressure. The residue was diluted with distilled water (1.0 mL) and purified by size exclusion chromatography on Bio-Gel P2 resin (fine, 1.6 × 75 cm), eluted with milliQ water. The appropriate fractions were combined and freeze-dried to give the *title compound* as a light yellow amorphous solid (11.6 mg, 55.6%).

FAB-MS found $[M+H]^+$ = 881.38400, $C_{36}H_{61}O_{19}N_6^+$ requires 881.39915.

δ_H[D_2O, recorded at 5 °C]: 1.07 (t, J = 7.2, –OCH_2CH_3), 1.08 (7, J = 7.2, –OCH_2CH_3) (these two signals integrate 3H), 2.16–2.18 (8H, m, br, CH_2), 2.36 (4H, s, br, CH_2), 2.45 (2H, s. br, CH_2), 2.94 (4H, s, br, CH_2), 2.99–3.02 (2H, m, H-6), 3.09–3.12 (2H, m, H-6′), 3.19–3.30 (6H, m, H-4, H-5, and CH_2), 3.35–3.45 (6H, m, H-3 and CH_2), 3.51 (2H, d, J = 12.0, CH_2), 3.57 (2H, s, br, H-2), 4.35 (q, J = 7.1, –OCH_2CH_3), 4.39 (q, J = 7.1, –OCH_2CH_3) (these two signals integrate 2H), 4.49 (2H, s, H-1).

R_f: 0.45 (methanol–ammonium hydroxide, 4:1 v/v).

3.12. General procedures for the synthesis of 2′-*O*-[1-(4-chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-*O*-(4,4′-dimethoxytrityl)-ribonucleoside-3′-(2-*O*-cyanoethyl) *N*,*N*-diethylphosphoramidites

2′-*O*-[1-(4-Chlorophenyl)-4-ethoxypiperidin-4-yl]-5′-*O*-(4,4′-dimethoxytrityl) nucleoside[24] (1.28 mmol) was co-evaporated with dry toluene (2 × 5 mL). The residue was redissolved in dry acetonitrile (15.0 mL) followed by the addition of *N*,*N*-diisopropylethylamine (6.32 mmol). After 5 min, *N*,*N*-diethyl-2-*O*-cyanoethyl phosphorochloridite (2.54 mmol) was added. After a further period of 30 min, the products were evaporated under reduced pressure. The residue was purified by column chromatography on silica gel to give the phosphoramidite as a colorless glass (80–85%).

3.13. Solid phase synthesis of oligoribonucleotides

Solid phase synthesis of oligoribonucleotides was performed on ABI 3400 DNA synthesizer. Standard 1.0 μmol RNA cycle conditions were used. After synthesis was complete, the resins were incubated in concentrated aqueous ammonia (28%) at 55 °C for 12 h. The supernatant was subsequently lyophilized to give partially protected (2′-Cpep-protected) oligoribonucleotides.

3.14. Man-U_9U (18)

Partially protected (all the uridines except the 3′-terminal one were protected by Cpep at the 2′-OH, as indicated by the italic *U* in Scheme 4) U_{10}-mer bearing a C_6-amino modifier at the 5′-end **17b** (0.75 μmol, 1 mol equiv) was first dissolved in methanol (200 μL), followed by the addition of an aqueous solution (70 μL) of squarate-activated monomannoside **6** (3.75 μmol, 5 mol equiv) and triethylamine (3 μL). The reaction mixture was incubated at 37 °C. After 2 h, additional aqueous solution of squarate-activated monomannoside **6** (37 μL, 2 mol equiv) was added to the reaction mixture. The reaction was allowed to proceed for another 3 h. The reaction mixture was lyophilized and the residue was purified

by semi-preparative C_{18} RP-HPLC. The appropriate fractions were collected and lyophilized to give the purified Man-U_9U **18** as a white solid.

R_t (C_{18}): 7.63 min.

ESI-MS found M^- = 5619.4, $C_{225}H_{283}Cl_9N_{31}O_{98}P_{10}^-$ requires 5618.6.

3.15. Bi-Man-U_9U (19)

Partially protected U_{10}-mer bearing a C_6-amino modifier at the 5′-end **17b** (0.5 μmol) was dissolved in methanol (300 μL), followed by the addition of an aqueous solution (73 μL) of squarate-activated bivalent-mannoside **13** (2 μmol) and triethylamine (5 μL). The reaction mixture was incubated at 37 °C. Additional portions of aqueous solution of squarate-activated bivalent-mannoside **13** (37 μL, 2 mol equiv) were added at 3 and 6 h, respectively. After 9 h, the products were lyophilized to give a white solid. The residue was purified by semi-preparative C_{18} RP-HPLC. The appropriate fractions were collected and lyophilized to give Bi-Man-U_9U **19** as a white solid.

R_t (C_{18}): 7.34 min.

ESI-MS found M^- = 6152.6, $C_{247}H_{322}Cl_9N_{36}O_{108}P_{10}^-$, requires 6152.2

3.16. General procedures for the removal of Cpep from 18 and 19

Substrate (**18** or **19**) was dissolved in DMA (300 μL) followed by the addition of triethylammonium formate buffer (TEAF, 200 μL, pH 2.52, 0.5 M). The mixture was incubated at 40 °C for 6 h. The reaction mixture was then neutralized by the addition of triethylammonium acetate buffer (pH 10.0). Chloroform (300 μL) was added, followed by vortex and centrifugation. The organic layer was discarded and the aqueous layer was further extracted with chloroform (2 × 300 μL). The aqueous layer was lyophilized to give a light yellow gel. The residue was redissolved in water (30 μL), followed by the addition of *n*-butanol (500 μL). After vortexing, the mixtures were frozen in liquid nitrogen and then centrifuged for 10 min. The butanol layer was discarded. The pellet was dissolved in water (500 μL) and lyophilized to give fully deprotected conjugate (**20** or **21**).

Man-U_{10} (**21**): R_t (DNAPac PA100): 3.66 min. ESI-MS found M^- = 3479.1, $C_{108}H_{139}N_{22}O_{89}P_{10}^-$, requires 3479.1.

Bi-Man-U_{10} (**20**): R_t (DNAPac PA100): 2.70 min. ESI-MS found M^- = 4012.6, $C_{130}H_{178}N_{27}O_{99}P_{10}^-$ requires 4012.7.

U_{10}: ESI-MS found M^- = 2998.4, $C_{90}H_{110}N_{20}O_{78}P_9^-$ requires: 2998.7. R_t (DNAPac PA100): 2.79 min.

A_{10}: ESI-MS found M^- = 3229.2, $C_{100}H_{120}N_{50}O_{58}P_9^-$ requires: 3229.1. R_t (DNAPac PA100): 2.95 min.

3.17. Enzymatic stability of U10, Man-U10, and Bi-Man-U10

0.183 ODU of substrate (unmodified U10-mer, monomanosyl-U10-mer **21**, and divalent mannosyl-U10-mer **20**) was dissolved in 200 μL of Tris chloride buffer (pH 8.0). Rnase A (100 × dilution of stock solution, 5 μL) and alkaline phosphatase (100 × dilution of stock solution, 5 μL) were added. The stock solutions of Rnase A were prepared by dissolving Rnase A (0.092 unit) in 0.1 M Tris chloride buffer (pH 8.0, 1.0 mL) and bacterial alkaline phosphatase was obtained by dissolving ca. 2 units of the enzyme in 1.0 mL of the same buffer. The mixture was incubated at 37 °C. Aliquots (20 μL) were withdrawn at the following time, 0, 2, 5, 10, 15, 20, 40, and 60 min. To the aliquots was added an aqueous solution of aluminon (5 μM, 20 μL) and the mixtures were frozen in liquid nitrogen immediately. All aliquots were analyzed with anion exchange HPLC (DNAPac PA100).

4. Conclusion

In summary, an efficient chemistry that is suitable for the preparation of carbohydrate–RNA conjugates by using the squarate linker was demonstrated. This chemistry is currently used to prepare carbohydrate-siRNA conjugates for bioavailability studies.

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