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Preparation of carbohydrate–oligonucleotide conjugates using the squarate spacer

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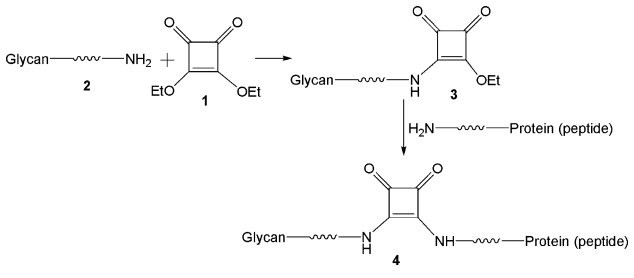
Abstract—Attachment of carbohydrates to oligonucleotides has proven to induce receptor-mediated endocytosis. A facile method for the formation of covalent linkages between glycans and oligonucleotides is herein described. Thus, use of 3,4-diethoxy-3-cyclobutene-1,2-dione as a linking reagent provides easy conjugation between carbohydrates bearing an amino group at the reducing end and oligonucleotides bearing an aminoalkyl modification.

In recent years, nucleic acids have been investigated as potential therapeutic agents in various forms, such as gene therapy, antisense oligonucleotides, antigene oligonucleotides, aptamers, and most recently RNA interference.[1] However, the effectiveness of these nucleic acids as therapeutic agents has been hampered by a number of limitations. Because nucleic acids are relatively large molecules bearing multiple negative charges, their cellular uptake is not efficient, which results in poor bioavailability. In addition, their cell targeting is not specific. A variety of structural modifications on nucleic acids have been developed which confer novel properties, such as enhanced resistance to nucleases, increased ability to hybridize with complementary sequences, and higher internalization efficiency through cell membranes. Additionally, use of delivery vehicles, such as lipids, liposome, peptide, virus and viral vectors, and antibodies,[1] have also shown promise in addressing the bioavailability of therapeutic nucleic acids.

One useful method that has received a lot of attention is to utilize the carbohydrate-binding proteins—lectins—that reside on cell membranes of certain cell types.[2] These lectins show specific binding affinity toward carbohydrate of defined structures. Upon binding, carbohydrates are internalized through receptor-mediated endocytosis. When the carbohydrate is attached to a drug molecule, the drug–carbohydrate conjugate can be transported into the cell with increased efficiency. This approach, which is called glycotargeting,[3] has been shown to improve both the internalization efficiency and specificity of cell targeting. A number of conjugation chemistry have been reported in the literature,[4,5] however, in our efforts to address the drug delivery issue of small interference RNAs (siRNA), we requested a method that allows for easy incorporation of carbohydrate moieties into the RNA molecules under mild conditions. 3,4-Diethoxy-3-cyclobutene-1,2-dione **1** (or diethyl squarate) has been shown to be an efficient linking reagent in the formation of glycopeptides and glycoproteins.[6] By using 3,4-diethoxy-3-cyclobutene-1,2-dione **1** as a linking reagent, a glycan moiety **2** can be readily attached first to the squarate to form the activated glycan **3**, which can further react with a protein or peptide to form a glycoconjugate **4** (Scheme 1). Because it is relatively straightforward to introduce an amino group into oligonucleotide using either the phospho-



Scheme 1. Squarate linker in glycoprotein synthesis.

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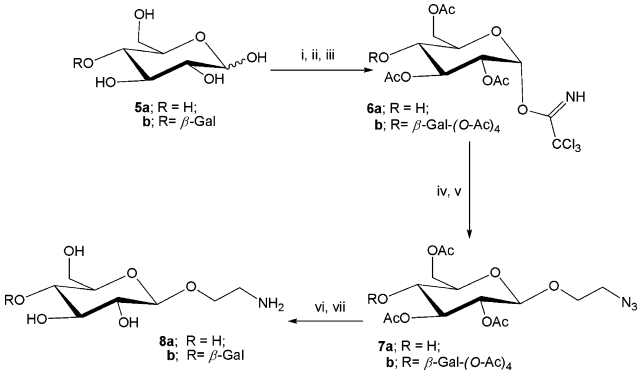
ramidite or H-phosphonate chemistry, we decided to explore the use of squarate as linker in the preparation of glycosylated oligonucleotides. Once the glycan is successfully linked to oligonucleotides through the squarate spacer, further elaboration of the conjugate can be effected by glycosyltransferases.[7] This approach will provide easy access to oligonucleotides with complex glycan structures that are required in glycotargeting.

For method development purpose, we chose to use β-(2-aminoethyl)glucoside **8a** and β-(2-aminoethyl)lactoside **8b** as glycans because these two glycosides can be readily obtained by catalytic hydrogenation of corresponding β-(2-azidoethyl)glycosides **7** (Scheme 2),[8–10] which in turn were conveniently prepared in good overall yields from glucose **5a** (or lactose **5b**) using the trichloroacetimidate chemistry.[11]

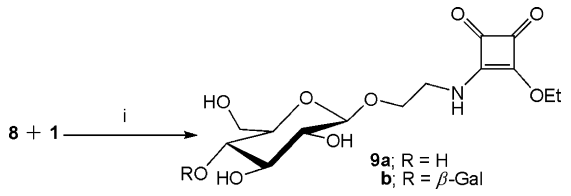
The reactions between the β-(2-aminoethyl)glycosides **8** and 3,4-diethoxy-3-cyclobutene-1,2-dione **1** were carried out in aqueous methanol solution in the presence of trace amount of triethylamine (Scheme 3).[12,13] The reactions were found to be complete within 5–10 min as indicated by TLC ($CHCl_3$–MeOH–H_2O, 65:35:5 v/v). The products were readily purified by size exclusion chromatography on Bio-Gel P2 fine gel (Bio-Rad), eluted with ammonium bicarbonate buffer. Lyophilization of the fractions that contain the products typically gives the products in good yield. The squarate-activated glycosides **9** appear to be colorless hygroscopic froth, and were fully characterized by reverse phase HPLC, ^1H and ^{13}C NMR, and mass spectroscopy.

As can be seen in the COSY–NMR spectrum of lactoside **9b** in Figure 1, single β-anomer was obtained, which is indicated by a doublet at 4.35 ppm, representing the β-anomeric proton of lactoside.

We then carried out the conjugation reaction between squarate-activated glycan **9** and 5′-deoxy-5′-aminothymidine **10**[14] and dinucleotide **12** bearing an amino modification at the 5′-terminus (Scheme 4).[15] The amino-modified dinucleotide **12** was prepared by the modified H-phosphonate chemistry in solution.[16,17] The conjugation was carried out in a mixture of sodium hydrogen carbonate (20 mM) and sodium tetraborate buffer (20 mM) (Scheme 4). The reactions were followed by reverse phase HPLC on a 4.6 × 150 mm Acclaim PA C_{18} 3μ column, eluted with a mixture of triethylammonium acetate (TEAA) buffer (0.1 M, pH 7.0)—acetonitrile under a linear gradient of TEAA–acetonitrile (100:0 to 85:15 v/v over 15 min and then isocratic



Scheme 2. Preparation of 2′-aminoethylglucoside **8a** and lactoside **8b**. Reagents and conditions: (i) $(Ac)_2O$, C_5H_5N, rt; (ii) $NH_2NH_2{\cdot}H_2O$, AcOH, DMF, rt, 45 min; (iii) CCl_3CN, DBU, CH_2Cl_2; (iv) $BF_3{\cdot}Et_2O$, $HOCH_2CH_2Cl$, CH_2Cl_2; (v) NaN_3, DMF, 55 °C, 3d; (vi) NaOMe, MeOH; (vii) Pd/C, H_2, H_2O.



Scheme 3. Activation of glycan **8** by diethyl squarate **1**. Reagents and conditions: (i) H_2O, MeOH, NEt_3, rt, 5 min.

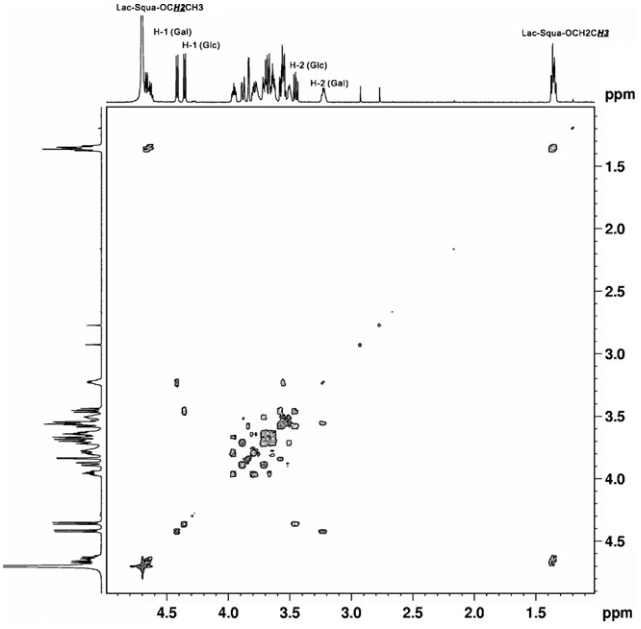
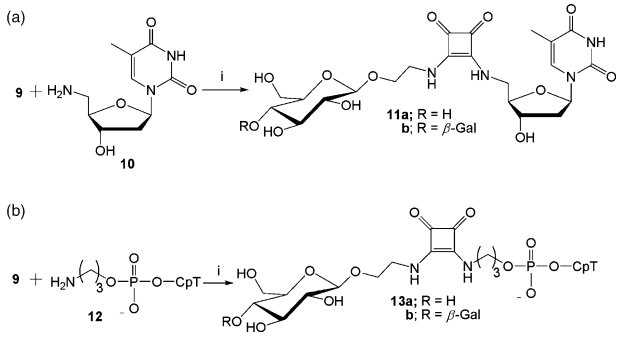


Figure 1. COSY–NMR spectrum of squarate-activated lactoside **9b**.



Scheme 4. Conjugation reaction (a,b). Reagents and conditions: (i) $NaHCO_3$, $Na_2B_4O_7$, pH 8.5.

Table 1. Glycosylated oligonucleotides

Entry	Mass (ESI$^-$)		Yield (%)
	Observed	Calculated	
11a	541.191	541.178	86
11b	703.249	703.231	84
13a	968.233	968.232	91
13b	1130.266	1130.286	95

elution). After incubation at room temperature for 24 h, the reactions were found to be complete.

The conjugation products **11** and **13** were readily isolated in good yields (Table 1) by size exclusion chromatography on Bio-Gel P2 fine gel, eluted with ammonium bicarbonate buffer (5 mM). These products were characterized, where appropriate, by ^{1}H, and ^{31}P NMR, COSY, reverse phase HPLC, and mass spectrometry. As expected, the lactoside conjugate **13b** showed two peaks at 0.28 and −1.05 ppm, respectively, in ^{31}P NMR, and each peak integrated one phosphorus (Fig. 2).

In C_{18} reverse phase HPLC, the squarate-activated glycan **9**, 5′-deoxy-5′-aminothymidine **10** (or dinucleotide **12**), and the conjugation products **11** and **13** were very well resolved. Figure 3 shows the HPLC profiles of the 5′-amino-modified dinucleotide **12** (bottom profile), squarate-activated lactoside **9b** (middle profile), and the isolated conjugate **13b** (top profile).

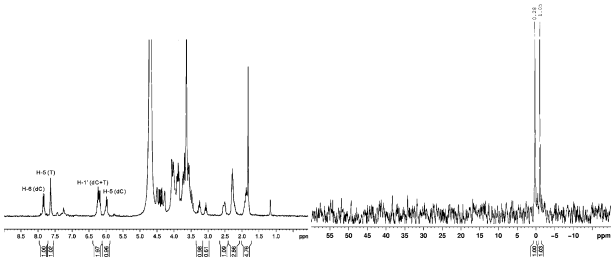


Figure 2. ^{1}H (left) and ^{31}P (right) NMR spectra of conjugate **13b**.

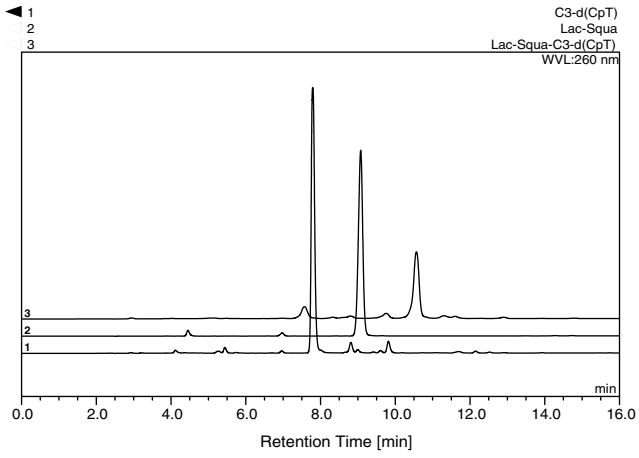


Figure 3. Stack of HPLC profiles of amino-modified d(CpT) dimer **12** (bottom), squarate-activated lactoside **9b** (middle), and conjugation product **13b** (top).

The exact mass of the conjugates was determined by electrospray mass spectroscopy to detect negative ions. All the observed masses are in close agreement with their theoretical values (Table 1).

In conclusion, we have demonstrated a facile method for the preparation of glycan–oligonucleotide conjugates that utilizes diethyl squarate as the linking reagent. Further elaboration of these conjugates by glycosyltransferases will open an avenue for the incorporation of complex glycans into oligonucleotides. It is conceivable that modification of therapeutic oligonucleotides by these complex glycans may serve to improve the bioavailability of nucleic acids as therapeutic agents.

Acknowledgments

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13. *Preparation of squarate-activated lactoside* **9b**. β-(2-Aminoethyl)-lactoside **8b** (20 mg, 0.0519 mmol) was dissolved in distilled water (50 μl), followed by addition of methanol (0.8 ml), 3,4-diethoxy-3-cyclobutene-1,2-dione **1** (50 μl, 0.338 mmol), and triethylamine (3 μl). After 5 min, the solvents were quickly removed by a flow of air. The residue was diluted with distilled water (1.0 ml) and purified by size exclusion column on Bio-Gel P2 (fine, 1.6 × 75 cm), eluted with aqueous ammonium bicarbonate buffer (5 mM). The appropriate fractions were combined and freeze-dried to give the *title compound* as a hygroscopic solid (23 mg, 87%).

ESI – MS found $M^- = C_{20}H_{30}NO_{14}^-$ 508.1789.
$^{12}C_{20}{}^{1}H_{30}{}^{14}N{}^{16}O_{14}^-$ requires : 508.1672.
Selected δ_H[D_2O, 500 MHz]: 1.35 (t, $J = 7.2$), 1.36 (t, $J = 7.2$) (these two triplets integrate three protons—CH_3CH_2O–Squarate), 3.22 (1H, m, H-2_Gal), 3.45 (1H, dd, $J = 7.8$ and 9.9, H-2_Glc), 4.35 (1H, d, $J = 7.8$, H-1_Glc), 4.41 (1H, d, $J = 8.4$, H-1_Gal), 4.65 (2H, hidden under HOD, CH_3CH_2O–Squarate).
δ_C[D_2O, 150 MHz]: 14.98 and 15.07 (CH_3CH_2O–Squarate), 44.13, 44.21, 60.10, 61.03, 68.56, 68.86, 69.14, 70.62, 70.66, 70.96, 72.54, 72.84, 74.35, 74.40, 74.79, 75.37, 78.35, 78.41, 102.14, 102.95, 173.75, 173.79, 177.21, 177.49, 183.44, 183.51, 188.98.
R_t: 9.08 min (linear gradient of triethylammonium acetate buffer–acetonitrile (100:0 to 85:15 v/v) over 15 min and then isocratic elution).

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15. *Preparation of conjugate* **13b**. Squarate-activated lactoside **9b** (5.4 mg, 10.6 μmol) and amino-modified dinucleotide **12** (3.5 mg, 5.3 μmol) were dissolved in a mixture of sodium hydrogen carbonate (0.10 ml, 20 mM) and sodium tetraborate buffer (0.10 ml, 20 mM). After the reaction mixture has been shaken for 24 h at room temperature, the products were purified by size exclusion column on Bio-Gel P2 (fine, 1.6 × 75 cm), eluted with aqueous ammonium bicarbonate (5 mM). The appropriate fractions were combined and freeze-dried to give the conjugate as a colorless solid (5.7 mg, 95%).
ESI – MS found $M^- = C_{40}H_{58}N_7O_{27}P_2^-$ 1130.266.
$^{12}C_{40}{}^{1}H_{58}{}^{14}N_7{}^{16}O_{27}{}^{31}P_2^-$ requires : 1130.286.
δ_P[D_2O, 121 MHz]: 0.28 (1 P), −1.05 (1 P).
δ_H[D_2O, 300 MHz] includes the following peaks:1.82 (3H, s, CH_3_Thy), 5.97 (1 H, d, $J = 7.5$, H-5_Cyt), 6.22 (2 H, m, H−1′_Thy + H−1′_Cyt), 7.63 (1H, s, H−6_Thy), 7.83 (1H, d, $J = 7.5$, H-6_Cyt).
R_t: 10.54 min (linear gradient of triethylammonium acetate buffer–acetonitrile (100:0 to 85:15 v/v) over 15 min and then isocratic elution).

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