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Carbohydrate Research 313 (1998) 15–20

CARBOHYDRATE RESEARCH

Studies towards neoglycoconjugates from the monosaccharide determinant of *Vibrio cholerae* O:1, serotype Ogawa using the diethyl squarate reagent

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Received 21 July 1998; accepted 30 September 1998

Abstract

The effect of reaction time, concentration and molar excess of hapten upon the efficiency of the conjugation of carbohydrates to proteins using the diethyl squarate reagent has been studied using chicken serum albumin (CSA) as the carrier protein and a linker-equipped D-glucose derivative as the hapten. A high degree of incorporation of the latter into CSA was achieved with high efficiency, and the use of a large excess of the ligand was not necessary. Conjugation of the immunodominant monosaccharide determinant of *Vibrio cholerae* O:1, serotype Ogawa, bearing the same spacer, followed a similar pattern, showing that the nature of the carbohydrate does not substantially affect the outcome of the conjugation and that a predicted degree of antigen-loading onto carrier protein is possible to achieve.

Keywords: Vaccine; Dialkyl squarate; Immunogen; Antigen; Neoglycoconjugate; *Vibrio cholerae*

1. Introduction

Traditional vaccines based on immunogens present in killed or attenuated bacteria are often pyrogenic or have other undesirable effects. It is hoped that a new generation of vaccines prepared by chemical conjugation of antigens to suitable carriers will be free from these deficiencies. Such vaccines have already opened new horizons in vaccine research and development. Concerning neo*glyco*conjugate immunogens, it has been shown that conjugate vaccines made from antigenic O-polysaccharides [1,2], as well as from fragments thereof (e.g., ref [3].), can induce protective antibodies. A large part of our effort is directed towards developing (semi)synthetic vaccines for infectious diseases caused by bacterial pathogens. In a recent detailed study [4] done as part of our attempts towards a vaccine against cholera [5–15], we found 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-α-D-mannopyranose to con-

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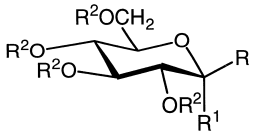
0008-6215/98/$—see front matter
PII S0008-6215(98)00261-4

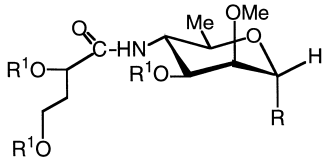
tribute about 90% of the maximum binding energy with a number of monoclonal antibodies for *Vibrio cholerae* O:1, serotype Ogawa. We therefore deemed it useful to include a neoglycoconjugate prepared from this defining hapten in our future evaluation of the immunogenicity of various conjugates made from *V. cholerae* O:1 antigens. We previously reported on the conjugation of a spacer-equipped monosaccharide determinant of *V. cholerae* O:1, serotype Ogawa to chicken serum albumin (CSA) by reductive amination [16]. Here we describe synthesis of a neoglycoconjugate prepared from that determinant monosaccharide using the squaric acid diester chemistry [17,18].

2. Results and discussion

3,4-Diethoxy-3-cyclobutene-1,2-dione [17,18] or its dimethoxy analog (squaric acid diesters) have recently been used for preparation of neoglycoconjugates [19,20], but protocols applied varied. Therefore, before conjugating the precious, spacer-equipped *V. cholerae* O:1 antigen **8**, we have studied the effect of reaction time, concentration, and molar excess of hapten to be linked over the number of amino groups present in the CSA carrier (i.e., 46 [21]) upon the outcome of the conjugation. In the preliminary experiments we used the linker-equipped D-glucose derivative **3**, bearing the same spacer as the *V. cholerae* O:1-related hapten to be conjugated later. We show here that the nature of the carbohydrate does not substantially affect the outcome of the conjugation, and that incorporation of a predicted number of haptens in a carrier protein is possible to achieve by applying conditions listed in Tables 1–3. This is important in the rational design of well-defined, synthetic glycoconjugate vaccines, which may require a defined, narrow-range loading of the antigen onto carrier proteins.

To explore optimum conditions for conjugation, neoglycoconjugates were prepared, and their molecular mass was determined by MALDI-TOF mass spectrometry. Thus, 6-methoxycarbonylhexyl β-D-glucopyranoside (**3**), prepared from 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide [22] (**1**) and methyl 6-hydroxyhexanoate [23] (**11**) via the per-*O*-benzoate **2**, was subjected to aminolysis with ethylenediamine to give **4**. Treatment of the latter with squaric acid diethyl ester (**12**) gave **5**, which was readily purified by column chromatography on silica gel. NMR and TLC showed no signs of decomposition of **5** when its aqueous





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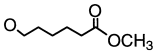
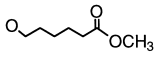
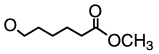
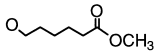
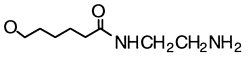
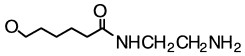
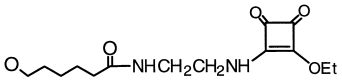
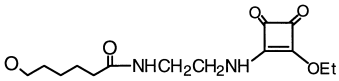
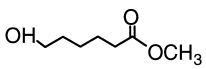
2		H	OBz	7		Ac
3		H	OH	8		H
4		H	OH	9		H
5		H	OH	10		H



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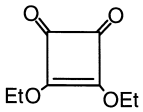

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Table 1
Effect of the concentration of **5** used upon hapten incorporation in CSA [a]

Concentration of hapten (mM)	*m/z*	Number of hapten residues incorporated/CSA	Efficiency (%)
5	75374	21	45.6
10	77446	26.2	56.9
15	79085	30.4	66.0
20	79387	31.1	67.6
25	78613	29.2	63.4

[a] Reaction time, 48 h; Hapten:L-lysine [equiv], 1:1.

Table 2
Effect of the reaction time upon incorporation of **5** in CSA [a]

Time (h)	*m/z*	Number of hapten residues incorporated/CSA	Efficiency (%)
16	77071	25	27.1
40	81724	37	40.2
64	82573	37	40.2
112	81342	36	39.1
160	81699	37	40.2

[a] Hapten concentration, 14.2 mM; Hapten:L-lysine used [equiv], 2:1.

solution was kept for several days at room temperature. To obtain conjugates, compound **5** was treated with CSA (see Experimental) under various conditions. Table 1 shows the benefit of conducting conjugation at high hapten concentrations. Maximum loading of the carrier protein with the hapten, which could not be increased by using more than 100% molar excess of hapten (Table 3), was reached after a reaction time of 1.5–2 days (Table 2).

For conjugation of the *V. cholerae* O:1 antigen, hapten **9** was prepared from the known [16] thioglycoside **6**, following the same protocol as that for **4**. At a concentration of 12.7 mM, and a ratio 2.2:1 of the hapten:lysine present in CSA, conjugation of **9** with CSA yielded a product containing ~29 residues of **10**/CSA. This is in a remarkably good agreement with the expected incorporation based on the data from the conjugation study involving an analogous derivative of D-glucose (Table 3). Preparation of related neoglycoconjugates from larger fragments of the O-polysaccharide of *V. cholerae* O:1 is in progress. Immunological studies with neoglycoconjugates prepared from various fragments of the O-PS of *V. cholerae* O:1 will be published in separate communications.

Table 3
Effect of the amount of **5** used upon its incorporation in CSA [a]

Hapten:L-lysine used [equiv]	*m/z*	Number of hapten residues incorporated/CSA	Efficiency (%)
1:4	71070	10.3	89.5
1:2	74251	18.2	79.1
1:1	77259	25.8	56.0
2:1	78756	29.5	32.0
3:1	79321	30.9	22.4

[a] Hapten concentration, 8.3 mM; Reaction time, 48 h.

General methods.—Unless stated otherwise, optical rotations were measured at ambient temperature for solutions in chloroform (c ~1), with a Perkin–Elmer automatic polarimeter, Model 341. All reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 coated glass slides (Whatman or Analtech). Detection was effected by charring with 5% (v/v) sulfuric acid in ethanol. Column chromatography was performed by gradient elution from columns of silica gel. Solvent mixtures slightly less polar than those used for TLC were used at the onset of development. Mass spectra were obtained using PerSeptive BioSystems Voyager Elite DE-STR (PE-Biosystems, Framingham, MA) MALDI-TOF instrument. The instrument was operated in the linear mode with 25 kV accelerating voltage and a 300 ns ion-extraction delay time. Samples for analysis (~0.1 mg) were dissolved in deionized water (50 μL) and applied as 1 μL droplets to separate positions in the center of the multiple-sample plate. An equal volume of matrix (saturated solution of sinapinic acid in 1:1 acetonitrile–0.5% trifluoroacetic acid) was applied over each dried sample and redried before being inserted into the mass spectrometer. CSA, purchased from Sigma Chemical Company, was purified as described previously [24], freeze-dried, and used also as a mass standard. Results from all analyses yielded a molecular mass for this protein of about 66.9 kDa. Molecular masses of the glycoconjugates were calculated using the molecular mass of CSA of 66,973 Da, predicted from the gene sequence [21]. NMR signals were recorded with a Varian Mercury spectrometer at 300 and 75 MHz for ^{1}H and ^{13}C, respectively. The assignment of signals was made by first-order analysis of the spectra, and the assignments were supported by homonuclear decoupling experiments or homonuclear and heteronuclear 2-dimensional correlation spectroscopy run with the software supplied with the spectrometers. When reporting NMR

spectral assignments, the nuclei of the 3-deoxy-L-*glycero*-teronamide side chain are noted as primed and those of the linker aglycon are noted as double-primed (even in the absence of the 3-deoxy-L-*glycero*-teronamide side chain). Solutions in organic solvents were concentrated at 40 °C/2 kPa.

5-(Methoxycarbonyl)pentyl 2,3,4,6-tetra-O-*benzoyl*-β-D-*glucopyranoside* (**2**).—A solution of silver trifluoromethanesulfonate (triflate, 1.31 g, 5.1 mmol) in toluene (20 mL) was added dropwise at −30 °C to a mixture of **1** (2.83 g, 4.3 mmol), **11** [23] (0.872 g, 7.5 mmol), 1,1,3,3-tetramethylurea (0.35 g, 3.0 mmol) and 4 Å molecular sieves (1 g), which had been stirred for 15 min. After 30 min, TLC (2:1 hexane–EtOAc) showed that all **1** was consumed. The mixture was neutralized with Et_3N, filtered, the filtrate was concentrated, and the residue was chromatographed to give amorphous **2** (2.4 g, 77%). $[\alpha]_D$ +11° (*c* 1.4); ^{1}H NMR ($CDCl_3$): δ 5.94 (t, 1 H, *J* 9.9 Hz, H-3), 5.71 (t, 1 H, *J* 9.9 Hz, H-4), 5.55 (dd, 1 H, $J_{1,2}$ 7.9, $J_{2,3}$ 9.5 Hz, H-2), 4.87 (d, 1 H, H-1), 4.67 (dd, 1 H, $J_{5,6a}$ 3.1, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.53 (dd, 1 H, $J_{5,6b}$ 5.1 Hz, H-6b), 4.19 (m, 1 H, H-5), 3.93 (m, 1 H, H-1″a), 3.60 (s, 3 H, OCH_3), 3.55 (m, 1 H, H-1″b), 2.05 (m, 1 H, H-5″a,b), 1.53 (m, partially overlapped, H-2″a,b), 1.45 (m, partially overlapped, H-4′a,b), 1.23 (m, 2 H, H-3″a,b); ^{13}C NMR ($CDCl_3$): δ 173.76 (CONH), 165.97, 165.66, 165.05, 164.90 (4 *C*OPh), 101.16 (C-1), 72.80 (C-3), 72.01 (C-5), 71.78 (C-2), 69.76 (C-1″), 69.70 (C-4), 63.04 (C-6), 51.20 (OCH_3), 33.56 (C-5″), 28.90 (C-2″), 25.17 (C-3′), 24.26 (C-4′); CIMS: *m*/*z* 742 ($[M+18]^+$). Anal. Calcd for $C_{41}H_{40}O_{12}$: C, 67.96; H, 5.52. Found: C, 68.05; H, 5.54.

5-(Methoxycarbonyl)pentyl β-D-*glucopyranoside* (**3**).—Conventional debenzoylation of **2** (Zemplén) gave **3** in virtually theoretical yield. The crystalline material showed mp 56–58 °C (from ethyl acetate) and $[\alpha]_D$ −22° (*c* 0.9, H_2O). ^{1}H NMR (D_2O): 4.42 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1), 3.94–3.86 (m, 2 H, H-6a,1″a), 3.74–3.60 (m, 5 H, H-6b,1″b, incl s, 3.67, OCH_3), 3.45 (t, partially overlapped, *J* 9.2 Hz, H-3), ~3.41 (m, partially overlapped, H-5), 3.34 (bt, partially overlapped, H-4), 3.32 (dd, 1 H, $J_{2,3}$ 9.1 Hz, H-2), 2.38 (t, 1 H, *J* 7.4 Hz, H-5″a,b), 1.66–1.56 (m, 4 H, H-2″a,b,4″a,b), 1.42–1.31 (m, 2 H, H-3″a,b); ^{13}C NMR (D_2O): δ 102.27 (C-1), 76.00 (C-5), 75.93 (C-3), 73.52 (C-2), 70.35 (C-1″), 69.78 (C-4), 60.90 (C-6), 52.19 ($COOCH_3$), 33.71 (C-5″), 28.51 (C-2″), 24.75 (C-3″), 24.51 (C-4″); CIMS: *m*/*z* 326 ($[M+18]^+$). Anal. Calcd for $C_{13}H_{24}O_8 \cdot 0.5\ H_2O$: C, 49.20; H, 7.94. Found: C, 49.22; H, 7.93.

(2-Aminoethylamido)carbonylpentyl β-D-*glucopyranoside* (**4**).—A solution of **3** (270 mg) in ethylenediamine (4 mL) was stirred at 70 °C in an atmosphere of argon until TLC (1:1:0.1 EtOAc–MeOH–concd NH_4OH) showed that the reaction was complete (~30 h). The mixture was concentrated and coevaporated with water to remove excess of the reagent. The residue was chromatographed to give amorphous **4** (239 mg, 81%). ^{1}H NMR (D_2O): δ 4.37 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1), 3.89–3.81 (m, 2 H, H-6a,1″a), 3.68–3.56 (m, 2 H, H-6b,1″b), 3.45–3.15 (m, 6 H, H-2,3,4,5,6″a,b), 2.74 (t, 2 H, *J* 7.1 Hz, H-7″a,b), 2.21 (t, 2 H, *J* 7.4 Hz, H-5″a,b), 1.62–1.51 (m, 4 H, H-2″a,b, 4″a,b), 1.36–1.26 (m, 2 H, H-3″a,b); ^{13}C NMR (D_2O): δ 102.32 (C-1), 76.05 (C-5), 75.97 (C-3), 73.28 (C-2), 70.34 (C-1″), 69.82 (C-4), 60.90 (C-6), 40.62 (C-6″), 39.76 (C-7″), 35.80 (C-5″), 28.55 (C-2″), 25.13 (C-4″), 24.77 (C-3); CIMS: *m*/*z* 337 ($[M+1]^+$).

1-[(2-Aminoethylamido)carbonylpentyl β-D-*glucopyranoside]-2-ethoxycyclobutene-3,4-dione* (**5**).—A solution of the foregoing amine **4** (80 mg, 0.24 mmol) in ethanol (10 mL) was treated at room temperature with **12** (35 mL, 0.24 mmol) until TLC (2:1 EtOAc–MeOH) showed that the reaction was complete (~6 h). After concentration, the residue was chromatographed to give amorphous **5** (85 mg, 78%). ^{1}H NMR (D_2O): δ 4.79–4.65 (m, 2 H, CH_2CH_3), 4.38 (bd, 1 H, $J_{1,2}$ 7.9 Hz, H-1), 3.88–3.80 (m, 2 H, H-6a,1″a), 3.70–3.64 (m, 2 H, H-6b,1″b), 3.61–3.52 (m, 2 H, H-7″a,b), 3.46–3.16 (m, 6 H, 2,3,4,5,6″a,b), 2.19 (t, 2 H, *J* 7.2 Hz, H-5″), 1.60–1.48 (m, 4 H, H-2″4″), 1.40 (q, 3 H, *J* 7 Hz, CH_2CH_3), 1.32–1.24 (m, 2 H, H-3″a,b); ^{13}C NMR (D_2O, typical splitting of some NMR signals due to the double bond nature of the vinylogous amide group, characteristic [18] of squaric acid amide esters): δ 102.34 (C-1), 76.03 (C-5), 75.97 (C-3), 73.30 (C-2), 70.88, 70.76 (CH_2CH_3), 70.31 (C-1″), 69.82 (C-4), 60.93 (C-6), 44.17, 44.09 (C-7″), 38.57, 38.37 (C-6″), 35.89 (C-5″), 28.59 (C-2″), 25.28 (C-4″), 24.81 (C-3″), 15.23, 15.16 (CH_2CH_3); CIMS: *m*/*z* 478 ($[M+18]^+$).

5-Methoxycarbonylpentyl 3-O-*acetyl-4-(2,4-di*-O-*acetyl-3-deoxy*-L-glycero-*tetronamido)-4,6-dideoxy-2*-O-*methyl*-α-D-*mannopyranoside* (**7**).—A mixture of **6** [16], 970 mg, 2.16 mmol), **11** [23] (473 mg, 3.24 mmol) and 4Å molecular sieves (1 g) in CH_2Cl_2 (30 mL) was stirred for 15 min. Solid NIS (680 mg, 3.02 mmol) was added, followed by a solution of AgOTf (222 mg, 0.86 mmol) in toluene

(8 mL). TLC (1:1 hexane–EtOAc) showed that the reaction was complete in ~5 min. After neutralization with Et_3N, the mixture was washed with aqueous $NaHCO_3$, water, dried, and concentrated. Chromatography of the residue gave the desired, amorphous glycoside **7** (1.14 g, ~100%), $[\alpha]_D$ +46°); ^{1}H NMR ($CDCl_3$): δ 6.30 (d, 1 H, $J_{4,NH}$ 9.5 Hz, NH), 5.19 (dd, 1 H, $J_{2,3}$ 3.1, $J_{3,4}$ 11.0 Hz, H-3), 5.07 (dd, 1 H, $J_{2',3'a}$ 4,6, $J_{2',3'b}$ 8.0 Hz, H-2′), 4.82 (d, 1 H, $J_{1,2}$ 1.9 Hz, H-1), 4.31–4.21 (m, 1 H, H-4), 4.19–4.06 (m, 2 H, H-4′a,b), 3.76–3.62 (m, 5 H, H-5, H-1″a, incl s at 3.69 for $COOCH_3$), 3.50 (s, 3 H, OCH_3), 3.49 (dd, 1 H, H-2), 3.42, 3.39 (2 t, 1 H, *J* 6.1 Hz, H-1″b), 2.34 (t, 2 H, *J* 7.3 Hz, H-5″a,b), 2.22–2.04 (m, 11 H, overlapping H-3′a,b and 3 s at 2.15, 2.12, 2.05 for 3 $COCH_3$), 1.73–1.54 (m, 4 H, H-2″a,b, H-4″a,b), 1.46–1.35 (m, 2 H, H-3″a,b), 1.21 (d, 3 H, $J_{5,6}$ 6.3 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 97.49 (C-1, $J_{C,H}$ 168.4 Hz), 77.93 (C-2), 71.13 (C-3), 70.91 (C-2′), 68.18 (C-5), 67.11 (C-1″), 59.87 (C-4′), 59.51 (OCH_3-2), 51.48 ($COOCH_3$), 51.41 (C-4), 33.82 (C-5″), 30.57 (C-3′), 28.77 (C-2″), 25.49 (C-3″), 24.35 (C-4″), 20.19, 20.70, 20.61 (3 $COCH_3$), 17.76 (C-6); CIMS: *m/z* 551 ($[M+18]^+$). Anal. Calcd for $C_{24}H_{39}NO_{12}$: C, 54.03; H,7.32; N, 2.63; Found: C, 53.93; H,7.33; N, 2.68.

5-Methoxycarbonylpentyl 4-(3-deoxy-L-glycero-*tetronamido)-4,6-dideoxy-2*-O-*methyl*-α-D-*mannopyranoside* (**8**).—Conventional deacetylation (Zemplén) of **7** (1.2 g) gave **8** as an amorphous hygroscopic solid; $[\alpha]_D$ +8° (*c* 1.1, H_2O). ^{1}H NMR (D_2O): δ 4.97 (d, 1 H, $J_{1,2}$ 1.7 Hz, H-1), 4.26 (dd, 1 H, $J_{2',3'a}$ 3.8, $J_{2',3b}$ 8.7 Hz, H-2′), 3.96 (dd, 1 H, $J_{2,3}$ 3.4, $J_{4,5}$ 10.3 Hz, 3.86–3.77 (m, 2 H, H-4,5), 3.74–3.66 (m, 6 H, H-4′a,b,1″a, incl s, 3.68, OCH_3-7), 3.57–3.47 (m, 5 H, H-2,1″b, incl s, 3.47 OCH_3-2), 2.39 (t, 2 H, *J* 7.4 Hz, H-5″a,b), 2.08–1.96, 1.89–1.77 (2 m, 1 H each, H-3′a,b), 1.67–1.56 (m, 4 H, H-2″a,b,4″a,b), 1.42–1.32 (m, 2 H, H-3″a,b), 1.15 (d, 3 H, $J_{5,6}$ 5.8 Hz, H-6); ^{13}C NMR (D_2O): δ 96.75 (C-1), 79.41 (C-2), 69.08 (C-2′), 67.94 (C-1″), 67.85 (C-3), 67.32 (C-5), 58.94 (OCH_3-2), 57.94 (C-4′), 53.46 (C-4), 52.19 (OCH_3-7), 36.08 (C-3′), 33.70 (C-5″), 28.30 (C-2″), 25.02 (C-2″), 24.13 (C-4″), 16.90 (C-6); CIMS: *m/z* 408 ($[M+1]^+$), 425 ($[M+18]^+$).

(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-glycero-*tetronamido)-4,6-dideoxy-2*-O-*methyl*-α-D-*mannopyranoside* (**9**).—Compound **8** (240 mg) was treated with ethylenediamine as described for the preparation of **4** to give amorphous **9** (250 mg, 98%). ^{1}H NMR (D_2O): δ 4.95 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 4.25 (dd, 1 H, $J_{2',3'a}$ 3.8, $J_{2',3'b}$ 8.6 Hz, H-2′), 3.93 (dd, 1 H, $J_{2,3}$ 3.4, $J_{3,4}$ 10.2 Hz, H-3), 3.85–3.64 (m, 5 H, H-4,5,4″a,b,1″a), 3.56–3.47 (m, 2 H, H-1″b, incl bdd, 3.53, H-2), 3.45 (s, 3 H, OCH_3), 3.26 (t, 2 H, *J* 6.3 Hz, H-6″a,b), 2.77 (t, 2 H, H-7″a,b), 2.24 (t, 2 H, *J* 7.3 Hz, H-5″a,b), 2.06–1.94, 1.86–1.76 (2 m, H-3′a,b), 1.62–1.54 (m, 4 H, H-2″a,b,4″a,b), 1.39–1.28 (m, 2 H, H-3″a,b), 1.13 (d, 3 H, $J_{5,6}$ 5.7 Hz, H-6); ^{13}C NMR (D_2O): δ 177.37, 177.26 (2 CO), 96.73 (C-1), 79.41 (C-2), 69.09 (C-2′), 67.97 (C-1″), 67.84 (C-3), 67.31 (C-5), 58.92 (OCH_3), 57.94 (C-4′), 53.45 (C-4), 40.67 (C-6″), 39.82 (C-7″), 36.10 (C-3′), 35.78 (C-5″), 28.35 (C-2″), 25.11 (C-4″), 25.02 (C-3″), 16.90 (C-6); CIMS: *m/z* 436 ($[M+1]^+$), 453 ($[M+18]^+$).

1-[(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-glycero-*tetronamido)-4,6-dideoxy-2*-O-*methyl*-α-D-*mannopyranoside]-2-ethoxycyclobutene-3,4-dione* (**10**).—The foregoing amine **9** (32 mg) was treated with diethyl squarate as described for the preparation of **5** to give **10** (33 mg, 80%). ^{1}H NMR (D_2O): δ 4.96 (bs, 1 H, H-1), 4.76–4.65 (m, 1 H, CH_2CH_3), 4.26 (dd, 1 H, $J_{2,3a}$ 4.0, $J_{2,3b}$ 8.7 Hz, H-2′), 3.95 (bd, 1 H, H-3), 3.83–3.77 (m, 2 H, H-4,5), 3.73–3.56 (m, 6 H, H-4′a,b,1″a,b,6″a,b), 3.54 (bdd, 1 H, H-2), 3.46 (s, 3 H, OCH_3), 3.38 (m, 2 H, H-7″a,b), 2.24–2.16 (m, 2 H, H-5″a,b), 2.07–1.95 (m, 1 H, H-3′a), 1.88–1.77 (m, 1 H, H-3b), 1.60–1.49 (m, 3 H, H-2″a,b,4″a,b), 1.46–1.39 (m, 3 H, CH_3CH_2), 1.36–1.20 (m, 2 H, H-3″a,b), 1.15 (d, 3 H, $J_{5,6}$ 5.6 Hz, H-6); ^{13}C NMR (D_2O): δ 96.75 (C-1), 79.38 (C-2), 70.88 (CH_2CH_3), 69.12 (C-2′), 67.86 (C-3), 67.33 (C-5), 58.94 (OCH_3), 57.96 (C-4′), 53.46 (C-4), 44.22 (C-7″), 39.32 (C-6″), 36.06 (C-3′), 35.85 (C-5″), 28.45 (C-2″), 25.28 (C-4″), 25.03 (C-3″), 16.89 (C-6), 15.25 (CH_2CH_3); CIMS: *m/z* 560 ($[M+1]^+$), 577 ($[M+18]^+$).

Conjugation of the squaric acid derivative ***5*** *with CSA*. In a typical experiment, a solution of **5** (13 mg, 0.028 mmol) and CSA (20.6 mg, 3.08×10^{-4} mmol) in a $KHCO_3$–$Na_2B_4O_7$ buffer (pH 9.0, 2 mL, corresponding to a ~2:1 hapten: lysine ratio at a hapten concentration of 14.1 mM) was stirred at room temperature. Samples (200 μL), periodically withdrawn at time intervals listed in Table 2, were centrifuged using 3 K Centricon filters (cut-off, 3 K), washed with deionized water (three times), and the material retained was freeze-dried Alternatively, samples containing neoglycoconjugates were dilayzed for 48 h against six changes of deionized water (2 L each), followed by freeze-drying. The products, obtained in virtually

theoretical yields, were analyzed by MALDI-TOF mass spectrometry.

Conjugation of the squaric acid derivative **10** *with CSA*. Compound **10** (14 mg, 0.025 mmol) and CSA (16.7 mg, 2.5×10^{-4} mmol) was stirred in a $KHCO_3$–$Na_2B_4O_7$ buffer (pH 9.0, 2 mL, corresponding to a 2.2:1 hapten:lysine ratio at a hapten concentration of 12.7 mM) for 2 days. After dialysis as described above and freeze drying, the MALDI-TOF analysis of the product obtained (17 mg, ~100%, *m/z* 82,003) showed that the conjugate contained ~29 hapten residues/CSA.

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