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# Conjugating oligosaccharides to proteins by squaric acid diester chemistry: rapid monitoring of the progress of conjugation, and recovery of the unused ligand

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**Abstract**

Samples that are periodically withdrawn from the mixture of a conjugation reaction can be analyzed on a picomolar scale without any work-up or pre-purification using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) in combination with the ProteinChip® System. The technique provides rapid information about the increasing molecular mass of the glycoconjugate formed, thereby allowing termination of the process when the desired incorporation of the ligand onto the carrier protein is achieved. The excess oligosaccharide used at the onset of conjugation can be recovered and used in preparation of a similar neoglycoconjugate. The overall economy of conjugations, which often involve labor-intensive linker-equipped oligosaccharides, can be markedly increased in this way. 

*Keywords:* Neoglycoconjugate; Molecular mass determination; Squaric acid derivatives; *Vibrio cholerae* O:1; SELDI-TOF MS/ProteinChip®

## 1. Introduction

The discovery by Avery and Goebel[1] that low-molecular-weight saccharides that mimic determinants of bacterial polysaccharide, when linked to protein carriers, can elicit anti-carbohydrate-specific antibodies started a quest for synthetic vaccines for bacterial diseases. During the past few years, a large number of neoglycoconjugates have been synthesized from synthetic oligosaccharides for use as experimental vaccines for infectious diseases and cancer, e.g., Refs. 2–4. In addition to their use as vaccines, neoglycoconjugates find wide application in the biomedical sciences. During this work we have focussed on two essential problems generally encountered in conjugation of oligosaccharides to proteins: (1) the recovery of the hapten normally used in excess at the onset of the reaction; and (2) the monitoring of the progress of the conjugation reaction. We have looked into possibilities to minimize these problems during conjugations involving squaric acid diester chemistry.

Larger synthetic oligosaccharides are very precious commodities, available often as products of multideca-step syntheses. Since conjugation of these molecules to proteins is often

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conducted at a high molar ligand to carrier ratio, the recovery of the unused ligand from the conjugation mixtures becomes an important concern. Therefore, we have investigated the possibility of recovering the excess oligosaccharide from the conjugation mixtures and using the material recovered in a subsequent, similar conjugation. We are aware of only one example when recovery of a hapten from conjugation mixtures has been reported,[5] but that work involved different conjugation chemistry.

One of important characteristics of glycoconjugates is the number of oligosaccharide units incorporated in the carrier as a result of conjugation. A very useful means for determining that characteristic is provided by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Depending on the resolution power of the spectrometer, the spectra obtained can be informative, not only regarding the average loading of the carrier with the carbohydrate, but also concerning the polydispersity of the conjugate.[6] Despite the high degree of sophistication the instruments have attained, the quality of MALDI-TOF mass spectra depends largely on the mode of sample preparation and the nature of the contaminants. Consequently, the method is far from routine and is normally applied only to characterize the final, highly purified product.

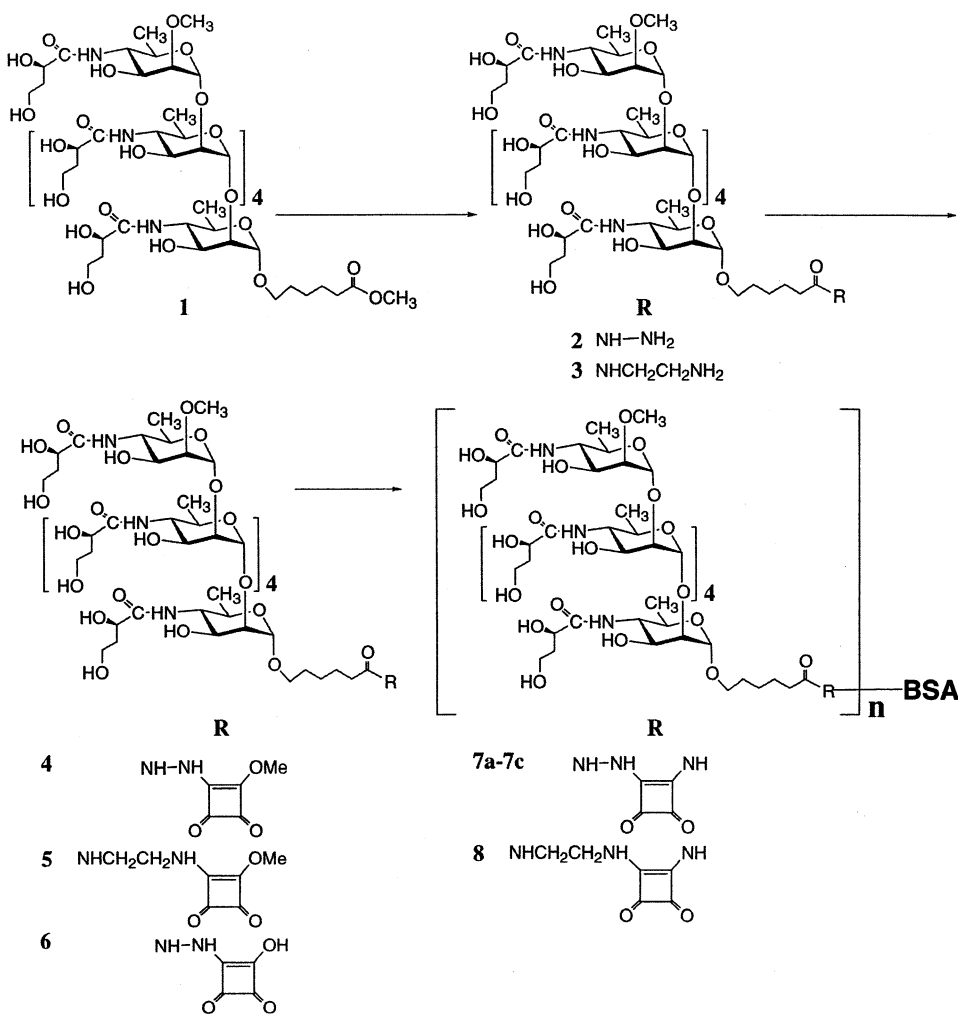
There are many methods available for conjugation of carbohydrates to proteins.[7,8] However, since the nature of both the carbohydrate and the protein affect the process, a general protocol allowing one to achieve a predicted incorporation of the saccharide cannot be proposed at this time. With the increasing use of glycoconjugates as tools in the life sciences,[9] some of which may require defined, narrow-range carbohydrate loading onto carrier proteins, there is a need for a method that allows for routine monitoring of the conjugation reaction. Ideally, such a method should be analogous to thin-layer chromatography, which is used as an analytical tool during syntheses of low molecular mass organic molecules. Thus, it should allow one to rapidly analyze samples withdrawn directly from the conjugation mixture and provide, within minutes, reliable information about the increasing molecular mass of the conjugate formed, thereby allowing termination of the process when the desired loading is achieved. Herein we report the use of the surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) in combination with the ProteinChip® System to monitor progress of the squaric acid diester-mediated conjugation reaction and show that it largely satisfies the aforementioned need. It can be reasonably assumed that crude reaction mixtures resulting from different conjugation chemistry could be analyzed in this way as well. We show that this new technology constitutes a fundamental and powerful addition to the tools available to the conjugation chemist, as it allows routine and rapid analysis of the *unprocessed* conjugation mixture on a picomolar scale.

## 2. Results and discussion

Our recent preparation of a series of glycoconjugates from bovine serum albumin (BSA, molecular mass 66,430 Da[10]) and the terminal hexasaccharide determinant of *Vibrio cholerae* O:1 required a large amount of the hexasaccharide squaric acid monoesters (e.g., **4** or **5**, Scheme 1). Therefore, at the onset of this work we have examined the possibility of recovering the excess hapten from the conjugation reaction and using it in another similar conjugation. The reactions involved BSA and a ligand **4**, prepared from the hydrazide **2**[11] (Scheme 1). The conjugation was performed using 1.33 equiv of **4**/$NH_2$ present in lysine (i.e., 59[12]), that is one of principal constituents of BSA. The reaction times were selected based on our previous experience with similar types of derivatives.[13] When the time allowed for the reaction elapsed, the mixture was subjected to ultrafiltration, and the retained material was freeze dried to afford conjugate **7a**. The material that had passed the membrane was purified using reversed-phase chromatography, freeze-dried and saved for usage in the preparation of **7c**. It produced a $^1$H NMR spectrum that was virtually superimposable with the spectrum of **4**. The same procedure,

but with shorter incubation time, was used to prepare conjugate **7b**. The combined hapten **4** recovered from the preparation of **7a** and **7b** was used in the successful preparation of conjugate **7c**. The main portion of **4** used in this preparation was that recovered from preparation of **7a** (incubation 6 days, see Section 3). The possibility that the recovered compound **4** contained some of the partially hydrolyzed material[14] i.e., **6**, can not be excluded. Due to overlapping of NMR signals, the presence of **6** could not be unequivocally ascertained. However, the amount of **6** that may have been present must have been small so that it had not affected the conjugation in any meaningful way. It should be noted that conjugates **7a**–**7c** show smaller ligand to BSA ratios (Table 1) than what would be expected from the reaction time allowed during preparation of conjugates **8** (see text below and Table 1). This mainly reflects the difference in reactivity of squaric acid derivatives **4** and **5**, which were prepared from an acid hydrazide and a primary amine, respectively. The ability to recover haptens from conjugation mixtures involving squaric acid diester chemistry improves considerably the overall economy of the conjugation process.

The relatively stringent requirements of MALDI-TOF MS for sample preparation have been largely overcome by Gayer et al.[15] and Colangelo and Orlando[16] who, independently, used a modified MALDI-TOF MS to monitor enzymatic digestion of glycoconjugates. A still more versatile tool is the SELDI-TOF mass spectrometry in combination with the ProteinChip® System, which combines active surfaces with the power of LDI-TOF MS detection and analysis. This novel technique has been recently reviewed in detail.[17] Briefly,




Scheme 1.

Table 1
Progress of conjugation of haptens **4** and **5** to BSA, to obtain neoglycoconjugates **7a**–**7c** and **8**, respectively

| Hapten | Conjugate | Reaction time | Average molecular mass (Da) | Hapten/BSA |
|---|---|---|---|---|
| **4** | **7a** | 6 days | 94,262 | 16.2 |
| | **7b** | 3 days | 83,811 | 10.1 |
| | **7c** | 32 h | 75,916 | 5.5 |
| **5** | **8** | 1 h | 73,290 | 3.9 |
| | | 3 h | 82,414 | 9.1 |
| | | 7 h | 83,942 | 10.0 |
| | | 9 h | 87,826 | 12.2 |
| | | 27 h | 100,265 | 19.3 |
| | | 54 h | 105,586 | 22.4 |
| | | 81 h | 112,417 | 26.3 |
| | | 123 h [a] | 115,212 | 27.9 |

[a] The increasing loading vs. time has not reached the plateau at this time.

very complex biological mixtures (cell extracts, sera, conditioned media etc.) containing, among other things, proteins can be applied to a variety of chemically active surfaces followed by various exhaustive washes. This is followed by MS analysis of the material retained specifically on the particular surface according to their intrinsic properties. There are obvious advantages to analyzing multicomponent mixtures in this way. Firstly, substances that normally interfere with MS analysis (lipids, salts, detergents etc.) can be removed directly from the chip surface. Secondly, the analysis, which requires only a minute amount of material, can be rapid as all binding processes take place in a monomolecular layer of active compounds.

Notwithstanding many successful applications of the SELDI-TOF MS in other fields, e.g., in biomarker discovery,[18,19] protein–protein interactions[20] and protein identification,[21] this technique has, to our knowledge, not been used as an analytical tool in synthetic glycoconjugate chemistry. According to our experience, it brings the analysis of mixtures containing glycoconjugates to a further higher level of sophistication. We tested its use during preparation of experimental immunogens described below. It allowed rapid and simple monitoring of the conjugation process on an extremely small scale, using a sample of the *crude* conjugation mixture as the analyte, and the information on the molecular mass of the neoglycoconjugate formed was obtained within $\sim 20$ min from the time of the sample withdrawal. Based on this information, the process of conjugation could be terminated when the desired carrier loading had been achieved. Consequently, while conjugation using current conjugation technology may still not be a very predictable process, the guesswork about the conjugation in progress can be minimized.

The utility of the SELDI-TOF MS was tested during preparation of neoglycoconjugates from bovine serum albumin (BSA) and hexasaccharide ligand **1**,[11] which mimics the upstream hexasaccharide determinant of the O-PS of *V. cholerae* O:1, serotype Ogawa (Scheme 1). Conjugation was effected using the squaric acid diester chemistry.[3,14,22] Accordingly, compound **1** was treated with ethylenediamine to give the amide **3**, which was converted to the squaric acid monomethyl ester **5**. The conjugation was performed at the same ligand to $NH_2$ ratio as in the experiment described above. The process was monitored with the aid of the SELDI-TOF MS, and when the desired loading was achieved, the required amount of the mixture could be withdrawn and worked up to isolate conjugates **8**. Fig. 1 shows SELDI-TOF MS spectra obtained during monitoring of the aforementioned conjugation (see also Section 3 and Table 1). Fig. 2 shows expansion of the peak in Chart D, revealing its fine structure. There, the difference between the molecular mass recorded for adjoining peaks equals, within experimental error, the expected increase of the molecular mass of the conjugate resulting

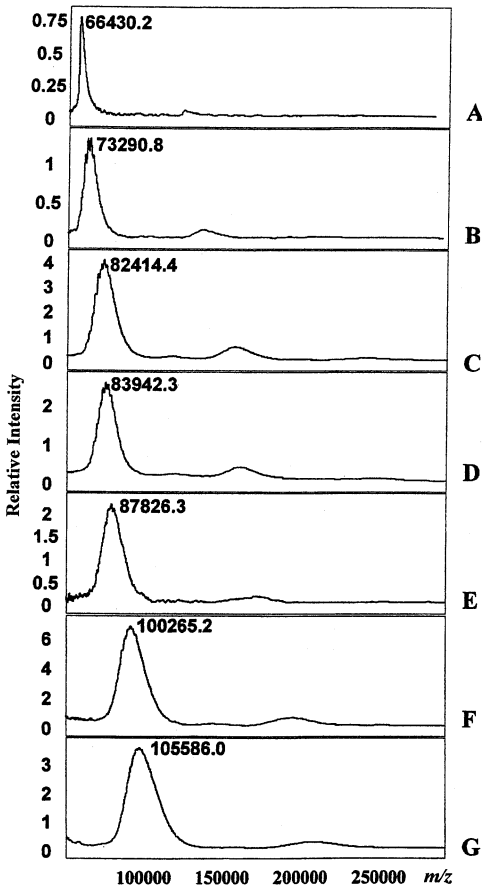



Fig. 1. The progress of conjugation of the hexasaccharide **5** (exact mass, 1780.79 Da) and BSA (molecular mass 66,430 Da) as revealed by monitoring the reaction by SELDI-TOF MS. Spectrum A was taken at the onset of the reaction ($t = 0$); spectra B–G were taken at 1, 3, 7, 9, 27, and 54 h, respectively. The spectra taken at 81 and 123 h (Table 1) are not shown. For further details, see text.

from stepwise addition of the demethoxylated hapten **5** to BSA. Thus, it can be deduced from Fig. 2 that the material analyzed consists of species containing from 5 to 15 hapten residues/BSA. The polydispersity of the material can be easily deduced in this way.

## 3. Experimental

NMR spectra (25 °C) were obtained for solutions in $D_2O$ at 300 MHz for $^1H$ and 75 MHz for $^{13}C$ with a Varian Mercury spectrometer. The reported $^1H$ and $^{13}C$ NMR chemical shifts are referenced to those of the HDO (4.78 ppm) and $CH_3OH$ (49 ppm) peaks, respectively. Assignments of signals were made by first-order analysis of spectra and by comparison with spectra of closely related substances.[3,11,23] When feasible, homo- and heteronuclear two-dimensional correlation spectroscopy was applied, using commercial software supplied with the instrument. In signal assignments, nuclei associated with the 3-deoxy-L-*glycero*-tetronic acid residues are denoted primed and those associated with the methylene groups of the linker between the anomeric O-1[I] and the squaric acid residue are denoted double-primed, the numbering starting with the group that is proximate to the anomeric oxygen. All FAB spectra were obtained with a JEOL SX-102a mass spectrome-

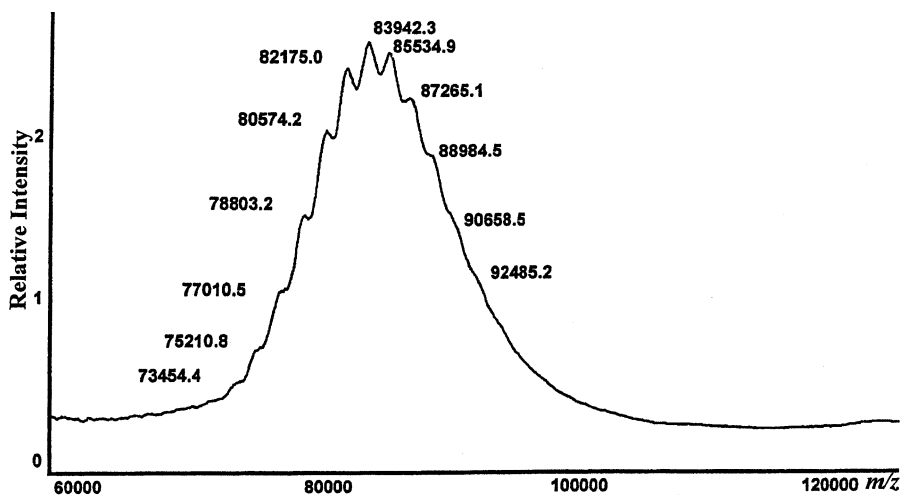



Fig. 2. The fine structure of the peak D (Fig. 1) showing the polydispersity of the neoglycoconjugate formed from the hexasaccharide **4** and BSA after 7 h of reaction time. For further details, see text.

ter utilizing a matrix of glycerol, magic-bullet (dithiotreitol–dithioerythritol) or nitrobenzyl alcohol, as appropriate. Accurate mass determinations were made with poly(ethylene glycol) mixture or CsI as reference compounds. Progress of conjugation was monitored by SELDI-TOF MS analysis using PBS-II Mass Reader in combination with the ProteinChip® System (Ciphergen Biosystems, Inc.) calibrated with BSA (66,430 Da[10]) and chicken conalbumin (77,490.0 Da). The PEAKS software™, V. 2.0, supplied with the Ciphergen instrument, was used for computer data presentation. The water used for preparation of solvents, buffers and to wash the chips before the SELDI analysis was of the HPLC grade. NP2 (normal phase) and H4 (LC aliphatic hydrophobic) chips (Ciphergen Biosystems, Inc.) were used for SELDI-TOF MS analysis of sugar ligands and coupling reactions, respectively. Sep-Pak Plus $C_{18}$ cartridges were obtained from Waters Corporation. Dialysis was performed using an Amicon ultrafiltration cell (10 mL), equipped with a PM 10 membrane, which was obtained from the Millipore Corporation.

*1-[5-Hydrazinocarbonylpentyl 4-(3-deoxy-*L-glycero-*tetronamido)-4,6-dideoxy-2-*O-*methyl-α-*D-*mannopyranosyl-(1→2)-tetrakis[4-(3-deoxy-*L-glycero-*tetronamido)-4,6-dideoxy-α-*D-*mannopyranosyl-(1→2)]-4-(3-deoxy-*L-glycero-*tetronamido)-4,6-dideoxy-α-*D-*mannopyranoside]-2-methoxycyclobutene-3,4-dione* (**4**).—A solution of **2** (41 mg, 24.96 μmol) in 0.1 M $KH_2PO_4$–NaOH buffer (pH 7.08, 4 mL) was stirred with dimethyl squarate (7.1 mg, 49.92 μmol) at rt. After 1.5 h, TLC (2.5:2.5:0.1, $CH_2Cl_2$–MeOH–$NH_4OH$) showed that the reaction was complete. The mixture was purified on Sep-Pak Plus $C_{18}$ cartridges (a series of three; elution with a stepwise gradient of water→MeOH; the squarate derivative was eluted with 20–45% MeOH in water). After lyophilization from water, the squarate derivative **4** (39 mg, 89%) was obtained as a white solid. $^{13}C$ NMR ($D_2O$) was very similar to that of **2**,[11] and the signal of the $OCH_3$ group linked to the squaric acid residue appeared at $\delta$ 60.11; FABMS: $m/z$ 1753.86 ($[M+1]^+$).

*Monitoring of the conversion of* **3**→**5**.—An aliquot of the reaction mixture (1–3 μL) was applied onto a well of the NP2 chip, dried at 133 Pa, and washed with 5 μL of water (twice). The chip was air-dried ($\sim$1 min), followed by addition on the dried spot of a solution of the matrix (0.1 M 2,5-dihydroxybenzoic acid in MeOH containing 10% of aq 2.5 mM NaCl) twice, 0.5 μL each. After drying, the chip was read at suitable laser intensity in the Ciphergen PBSII mass reader. Before the measurement, the instrument was externally calibrated using the mixture of angiotensin (molecular mass 1296.5) and endorphin (molecular mass 3755.7) supplied with the instrument.

*(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-*L-glycero-*tetronamido)-4,6-dideoxy-2-*O-*methyl-α-*D-*mannopyranosyl-(1→2)-tetrakis[4-(3-deoxy-*L-glycero-*tetronamido)-4,6-dideoxy-α-*D-*mannopyranosyl-(1→2)]-4-(3-deoxy-*L-glycero-*tetronamido)-4,6-dideoxy-α-*D-*mannopyranoside* (**3**).—A solution of **1** (29 mg) in anhyd ethylenediamine (2.5 mL) was heated in a closed tube under Ar for 2 days at 70 °C. The mixture was concentrated and co-evaporated with water to remove excess reagent. After purification of the residue on Sep-Pak Plus $C_{18}$ (a series of two cartridges, using stepwise gradient of water→MeOH for elution) and freeze-drying of an aq solution, the amide **3** was obtained as a white solid (21.3 mg, 72%). $^1H$ NMR ($D_2O$): $\delta$ 5.21 (d, 1 H, $J_{1,2}$ 1.6 Hz, H-$1^{VI}$), 5.20 (bs, 1 H), 5.17 (bs, 2 H, 5.15 (bs, 1 H), H-$1^{II-VI}$], 4.89 (bs, 1 H, H-$1^{I}$), 4.32–4.27 (m, 6 H, H-$2'^{I-VI}$), 4.18–4.05 (m, 10 H, H-$2^{II-V}$,$3^{I-VI}$), 3.98–3.84 (m, 13 H, H-$2^{I}$,$4^{I-VI}$,$5^{I-VI}$), 3.77–3.68 (m, 14 H, $2^{VI}$,4′a,$b^{I-VI}$,1″a), 3.57–3.48 (m, 4 H, H-1″b, incl s, 3.50, $OCH_3$), 3.37 (t, 2 H, $J$ 6.5 Hz, H-6″a,b), 2.93 (t, 2 H, $J$ 6.5 Hz, H-7″a,b), 2.29, 2.27 (2 t, partially overlapped, 2 H, $J$ 7.2 Hz, H-5″), 2.22–2.98 (m, 6 H, H-3′$a^{I-VI}$), 1.92–1.80 (m, 6 H, H-3′$b^{I-VI}$), 1.68–1.55 (m, 4 H, H-2″a,b,H-4″a,b), 1.20–1.12 (m, 18 H, H-$6^{I-VI}$); $^{13}C$ NMR ($CDCl_3$): $\delta$ 100.98, 100.86 (3 C, C-$1^{II-V}$), 99.07 (C-$1^{I}$), 98.56 (C-$1^{VI}$), 79.06 (C-$2^{VI}$), 77.88, 77.65, 77.33 (3 C) C-$2^{I-V}$), 68,13 (6 C, C-$2'^{I-VI}$), 68.39 (3 C), 68.06 (2 C), 67.87, 67.75, 67.67 (3 C), 67.61 (2 C), 67.52 (C-$3^{I-VI}$,$5^{I-VI}$,1″), 58.86 ($OCH_3$), 57.99 (6 C, C-$4'^{I-VI}$), 53.29 (2 C), 53.08 (4 C, C-$4^{I-VI}$),

39.68 (C-6″), 39.55 (C-7″), 36.13 (6 C, C-3′$^{I-VI}$), 35.75 (C-5″), 28.28 (C-2″), 25.05 (2 C, C-3″,4″), 16.97 (4 C), 16.93 (2 C, C-6$^{I-VI}$) ; FABMS: $m/z$ 1671.8 ($[M+1]^+$).

*1-[(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-*L*-glycero-tetronamido)-4,6-dideoxy-2-O-methyl-α-*D*-mannopyranosyl-(1→2)-tetrakis[4-(3-deoxy-*L*-glycero-tetronamido)-4,6-dideoxy-α-*D*-mannopyranosyl-(1→2)]-4-(3-deoxy-*L*-glycero-tetronamido)-4,6-dideoxy-α-*D*-mannopyranoside]-2-methoxycyclobutene-3,4-dione* (**5**).—A solution of **3** (21.3 mg, 12.74 μmol) in 0.1 M $KH_2PO_4$–NaOH buffer (pH 7.08, 3 mL) was stirred with dimethyl squarate (5.43 mg, 38.24 μmol) for 12 h at rt. SELDI-TOF MS analysis then showed absence of a peak indicating the presence of the starting material, and showed peaks at $m/z$ 1803.0 ($[M+Na]^+$), 1819.0 ($[M+K]^+$). The mixture was purified on Sep-Pak Plus $C_{18}$ cartridges (a series of three cartridges). The salts and excess reagent were eluted with water (20 mL), and the squarate derivative **5** was eluted with a stepwise gradient of water→MeOH. After lyophilization of an aq solution, the squarate derivative **5** (19 mg, 84%) was obtained as a white solid. The $^1H$ and $^{13}C$ NMR spectra ($D_2O$) were very similar to those of **3**. The $^{13}C$ spectrum showed typical splitting of some signals due to the double bond nature of the vinylogous amide group, which is characteristic[24] of squaric acid amide esters. The $^{13}C$ NMR signal of $CH_3O$ attached to the squaric acid moiety appeared as a doublet at $\delta$ 61.00; SELDI-TOF MS: $m/z$ 1803.0 ($[M+Na]^+$).

*Conjugation of squaric acid monoesters* **4** *and* **5** *to BSA*.—Conjugation of the title haptens with BSA was performed by incubation with stirring in 0.05 M borax–0.1 M $KH_2PO_4$ buffer (pH 8.98) at the hapten–BSA ratio of 80:1 and hapten concentration of 20 mM. Samples were periodically withdrawn and analyzed by SELDI-TOF MS. For results see Table 1 and Figs. 1 and 2.

*Monitoring of conjugation by SELDI-TOF MS*.—Samples of the conjugation mixtures (1 μL) were periodically withdrawn (see Table 1 and figure legends) for SELDI-TOF MS analysis. Aliquots were diluted tenfold with PBS, and 2 μL of the resulting solution were applied to the H4 chip. The chip was dried at 133 Pa and washed with water (twice, 5 μL each). A solution of the matrix (saturated sinapinic acid in 50% MeCN containing 0.5% TFA, 0.5 μL, twice) was added per spot. The chips were read at suitable laser intensity and sensitivity. Before the measurement, the instrument was externally calibrated using a mixture of BSA (molecular mass 66,430 Da) and chicken conalbumin (molecular mass 77,490 Da).

*Preparation of conjugates* **7a**–**7c** *involving ligand recovery*.—A solution of **4** (21.03 mg, 11.99 μmol) and BSA (10 mg, 0.15 μmol) in 0.05 M borax–0.1 M $KH_2PO_4$ buffer (pH 8.98, 0.6 mL) was treated as described above. After 6 days the reaction mixture was dialyzed against deionized water (80 mL), and the retained solution was lyophilized to give conjugate **7a** (12.8 mg, 91%, based on BSA). The ultrafiltrate was concentrated to 2 mL, purified on Sep-Pak Plus $C_{18}$ cartridges (a series of three) as described above, and freeze-dried to give **4** (16.8 mg, 99% based on the amount of **4** that was conjugated), which was used as described below.

A similar incubation of **4** (17 mg, 9.69 μmol) with BSA (8.1 mg, 0.12 μmol) in the buffer (pH 8.98, 485 μL) for 3 days gave conjugate **7b** (9.4 mg, 94%). The remaining **4** (12.1 mg, 80% based on the amount of **4** that was conjugated) was recovered as described above, and used as described below.

Incubation of **4** (21.03 mg, 11.99 μmol, 16.8 mg of material that was recovered from the preparations of **7a** combined with 4.23 mg of material recovered from the preparation of **7b**) with BSA (10 mg, 0.15 μmol) in the buffer (pH 8.98, 0.6 mL) gave, after 32 h and processing as described above, the conjugate **7c** (11.19 mg, 99%) and remaining **4** (15.2 mg, 76.8% based on the amount of **4** that was conjugated).

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