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Didecyl squarate—A practical amino-reactive cross-linking reagent for neoglycoconjugate synthesis

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The present paper describes the synthesis and use of the hydrophobic squaric decyl ester glycosides in neoglycoconjugate chemistry. The 2-aminoethyl glycosides of α-D-mannopyranose, lactose, globotriose, globotetraose, GM3, and sialyl Lewis^x, as well as the 2-(2-aminoethylthio)ethyl glycoside of α-D-mannopyranose, β-D-glucopyranose, and galabiose were reacted with squaric acid didecyl ester to afford the hydrophobic squaric decyl ester glycosides. These glycosides were efficient reagents for the conjugation to amino-functional microtiter plates, BSA and aminated Sepharose EAH 4B. The decyl ester moiety of the squaric decyl ester glycosides constitutes a traceless hydrophobic tag, which has the major advantage, as compared to the corresponding ethyl esters, that it enables easy purification of the glycosides with silica chromatography and that unreacted excesses glycosides from conjugation reaction mixtures can easily be recovered by means of C18 solid phase extraction.

Keywords: **neoglycoconjugates, didecyl squarate, squaric decyl ester glycosides, C18 solid phase extraction**

Introduction

Carbohydrates are involved in many biologically important recognition events [1,2]. They act as cell surface antigens in blood group systems, as cell tumor specific antigens [3], and they control growth and tissue development in embryos. Furthermore, they are involved in fertilization [4] and often function as recognition sites for pathogenic bacteria and viruses [5]. Consequently, there is a continuous need to find research tools to investigate these glycobiological phenomena. Neoglycoconjugates, i.e. carbohydrates synthetically linked to a non-carbohydrate moiety (such as a protein, an insoluble polymer bead, a surface, a fluorescence label, a chromatography matrix etc) constitute an important class of glycobiology research tools, where the biological activity of the carbohydrate is combined with the biological, chemical or physical properties of the non-carbohydrate moiety. Common methods for conjugation of carbohydrates to non-carbohydrate moieties have included the formation of amide, amine, urea, amidine, carbamate, isourea and thiourea linkages [6,7]. A attractive method is the conjugation between two amines with diethyl squarate first introduced by Tietze et al. [8,9] in neoglycoconjugate syntheses and later further developed by others [10]. The major advantages of the squaric acid ester method are that it can be used for both primary and secondary amines [8] and it uses mildly basic conditions which makes it suitable for acid sensitive molecules such as carbohydrates. Furthermore, it does not require large excess of valuable carbohydrates and the incorporation can in many cases be controlled with the number of reagent equivalents [10]. However, a difficulty with using the diethyl squaric ester is that the intermediate product obtained by reaction of a carbohydrate amine with diethyl squarate, the squaric ethyl ester glycoside, often is difficult to isolate and purify by chromatographic means. Consequently, recovery of unreacted squaric ethyl ester glycoside from conjugation reaction mixtures, particularly important in the case of complex and valuable oligosaccharides, is cumbersome. Purification [10], as well as recovery [11], of squaric ethyl ester glycosides by means of C18 solid phase extraction has been reported. However, these procedures relied on the presence of a hydrophobic linker between the carbohydrate and the squaric ethyl ester moieties. An attractive alternative strategy would be to incorporate the hydrophobicity required for C18 SPE into the squaric ester moiety *instead* of into the carbohydrate moiety, thus simplifying chromatography on silica and possibly even enable recovery of unreacted excess squaric ester glycosides by means of C18 solid phase extraction.

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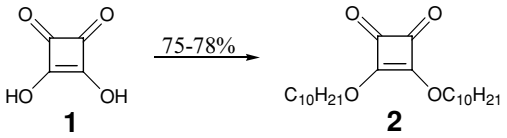


Scheme 1. 1-Decanol, toluene, reflux.

In this paper we report that squaric decyl ester glycosides are efficient coupling reagents for the preparation of glycosylated microtiter plates and Sepharose, as well as of neoglycoproteins, and that they are indeed easily purified and recovered from crude conjugation reaction mixtures using either silica chromatography or C18 solid phase extraction.

Results and discussion

Synthesis of squaric decyl ester glycosides

The synthesis of the hydrophobic squaric acid didecyl ester **2** followed the procedure described by Law and Bailey [12], with the exception that **2** was purified by flash chromatography in a yield of 75% (Scheme 1). The squaric acid didecyl ester **2** is stable unless subdued to moisture and can be stored in freezer or under dry conditions for several months.

Treatment of 2-aminoethyl glycosides the didecyl ester **2** in DMF (reactions in ethanol or methanol resulted in competing transesterfication with the solvent) afforded the corresponding squaric decyl ester glycosides **4–8** in high yields (Figure 1). Furthermore, sulfur-tagged (to facilitate differential elemental analysis) squaric decyl ester glycosides **9–11** were prepared in good yields from the corresponding 2-(2-aminoethylthio)ethyl glycosides. As hypothesized, the amphoteric squaric decyl ester glycosides were readily purified by silica flash chromatography. For example, the Gb3 squaric decyl ester glycoside **5** had an R_f value of 0.35 on TLC plates eluted with a dichloromethane/methanol/water (75:25:5) mixture, while the corresponding squaric ethyl ester trisaccharide **12** had an R_f of 0 on the same TLC plate.

Conjugation of squaric decyl ester glycosides to microtiter plates

Initially, conjugation conditions were investigated through a series of experiments with various concentrations of **3** in the presence of triethylamine, pyridine, or sodium hydrogen carbonate buffer (pH 9.0). Conjugation result was monitored with a concanavalin A-horseraddish peroxidase conjugate ELISA. The optimal condition for conjugation was at 0.01–0.2 mM of the squaric decyl ester mannoside **3** in sodium hydrogen carbonate buffer, but satisfactory results were also obtained using triethylamine as base. Furthermore, the optimal conjugation time was found to be 24 hours and capping of unreacted microtiter plate amino-groups with 20% acetic anhydride in water significantly decreased non-specific lectin binding and improved experimental reproducibility.

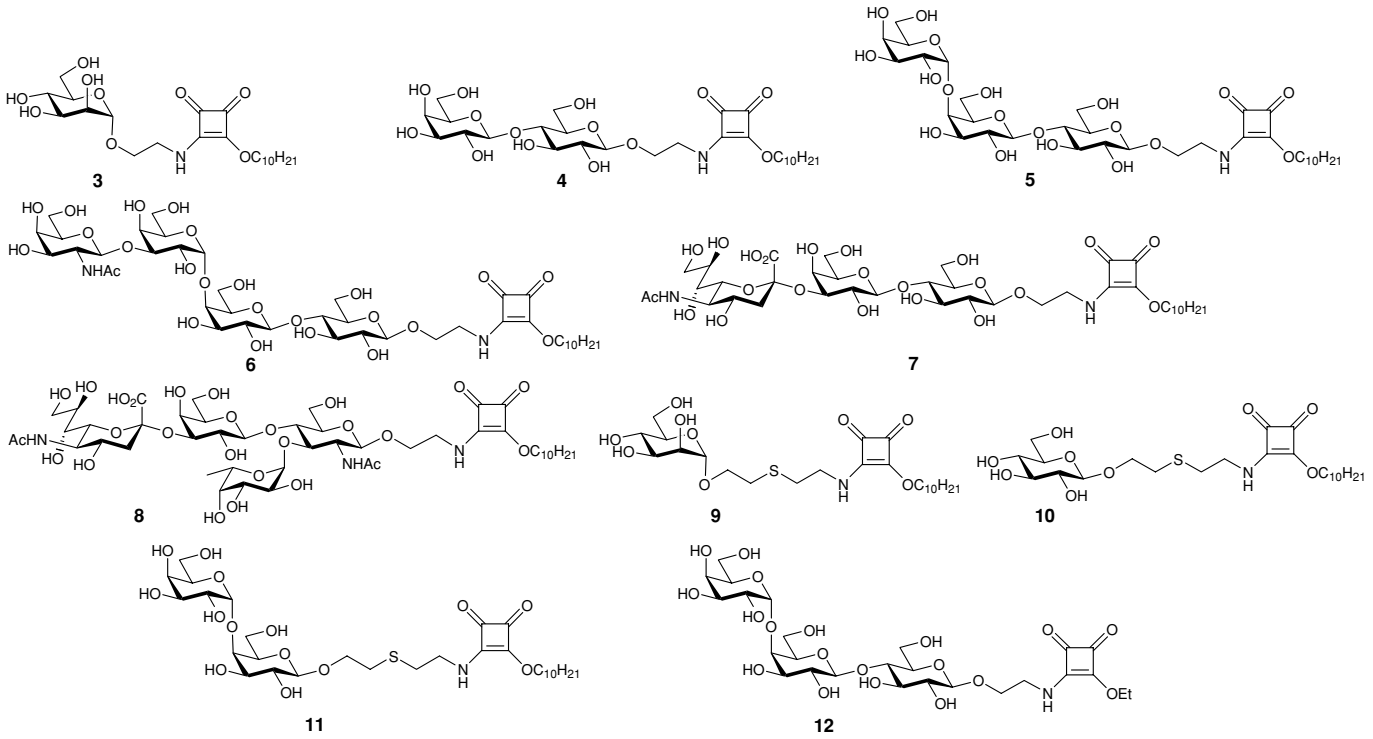
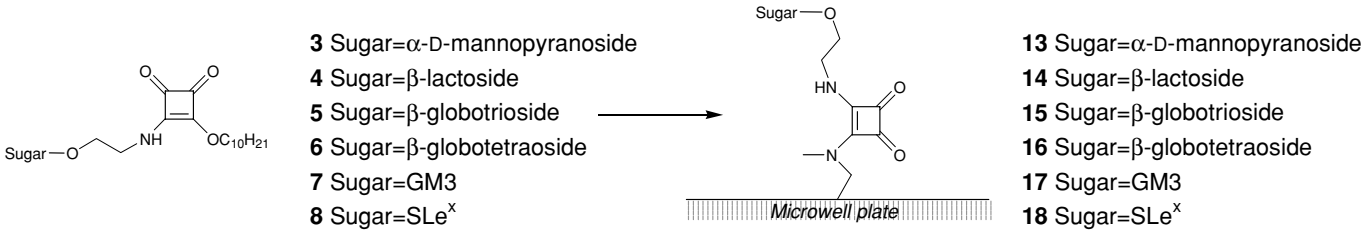


Figure 1. Squaric acid decyl ester glycosides.



Scheme 2. a) $NaHCO_3$ buffer. b) Acetic anhydride/water 1:4.

The squaric decyl ester glycosides **3–8** were then subjected to conjugation experiments with amino-functional microtiter plates (Scheme 2). The successful conjugations of **3–8**, to give the corresponding mannose-, lactose-, globotriose, globotetraose, GM3, and sialyl Lewis^x microtiter plates **13–18**, were visualized in ELISA based on lectin-horseradish peroxidase conjugates (Figure 2). The lectin binding to the microtiter plates **13–18** were compared to binding to amino-functional plates endcapped with acetic anhydride, but devoid of sugar. It is noteworthy that the sialyl Lewis^x plate **18** was recognized by both sialic acid-binding lectin conjugates (*T. vulgaris*, *L. flavus*) and a fucose-binding lectin conjugate(*T. purpureas*).

Soluble glycosides clearly inhibited lectin binding to the mannose-, globotetraose-, and sialyl Lewis^x microtiter plates **13, 17**, and **18**, which unambiguously showed that the squaric decyl ester glycosides were indeed conjugated to the microtiter wells.

One major advantage of the more hydrophobic squaric decyl ester glycosides was hypothesized to be the easy recovery of unreacted excess of the more complex and valuable squaric ester glycosides from conjugation reaction mixtures by means of C18 solid phase extraction. In order to prove this hypothesis, the recovery of unreacted excess of the squaric decyl ester globotrioside **5** from a conjugation to a microtiter plate was compared to the recovery of unreacted excess of the corresponding squaric ethyl ester globotrioside **12** in two parallel experiments. Following 24 h conjugation of the squaric decyl ester globotrioside **5** (1 mM in bicarbonate buffer, 150 L/well, i.e. a total of 11.3 mg **5**) and the squaric ethyl ester globotrioside **12** (1 mM in bicarbonate buffer, 150 L/well, i.e. a total of 11.3 mg **12**) to 96 microwells each, the reaction solutions were passed through SepPak Plus C18 cartridges. The cartridges were washed with water and unreacted excess squaric decyl ester globotrioside **5** (8.2 mg) and the squaric ethyl ester globotrioside **12** (1.2 mg) were eluted with 80% methanol. Thus, the squaric ethyl ester globotrioside **12** eluted together with the bicarbonate and water washing fractions, while the squaric decyl ester globotrioside **5** was efficiently extracted onto the C18 cartridge. In a similar manner were recovered the squaric decyl ester GM3 derivative **7** and the squaric decyl ester sialyl Lewis^x derivative **8** from

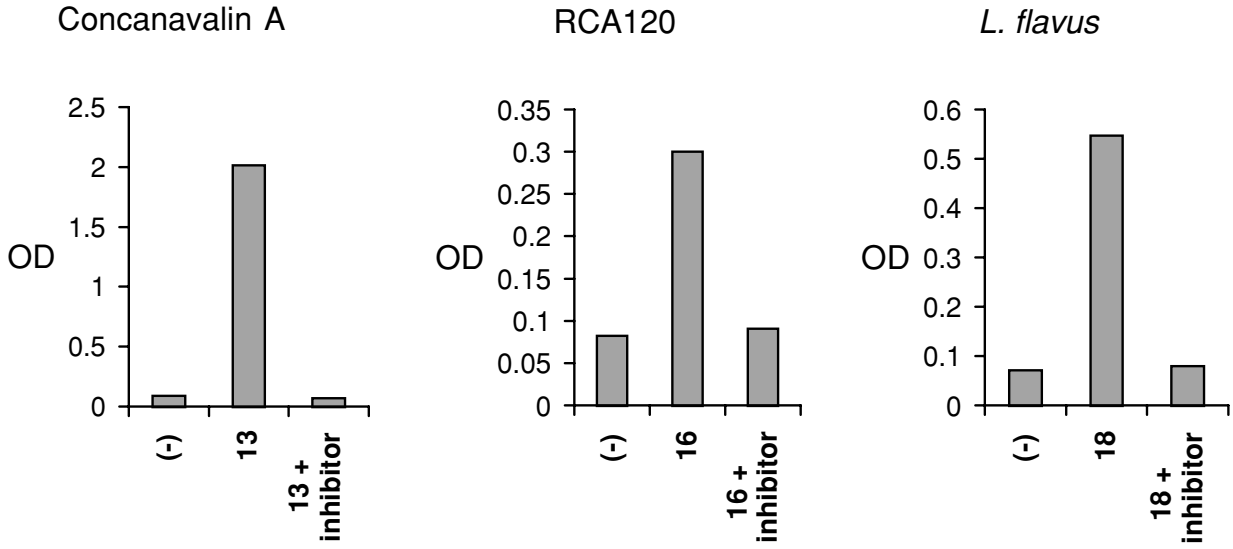


Figure 2. Typical binding experiments and competitive inhibition experiments with commercial horseradish peroxidase lectin conjugates in glycosylated microtiter plates (**13, 16**, and **18**). Amino-functionalized microtiter plates endcapped with acetic anhydride (i.e. devoid of sugar) used as controls are denoted (−). Inhibitors used were 4-nitrophenyl α-D-mannospyranoside (in plate **13**), ethyl glycoside of Gb4 (in plate **16**), and ethyl glycoside of sialyl Lewis^x (in plate **18**).

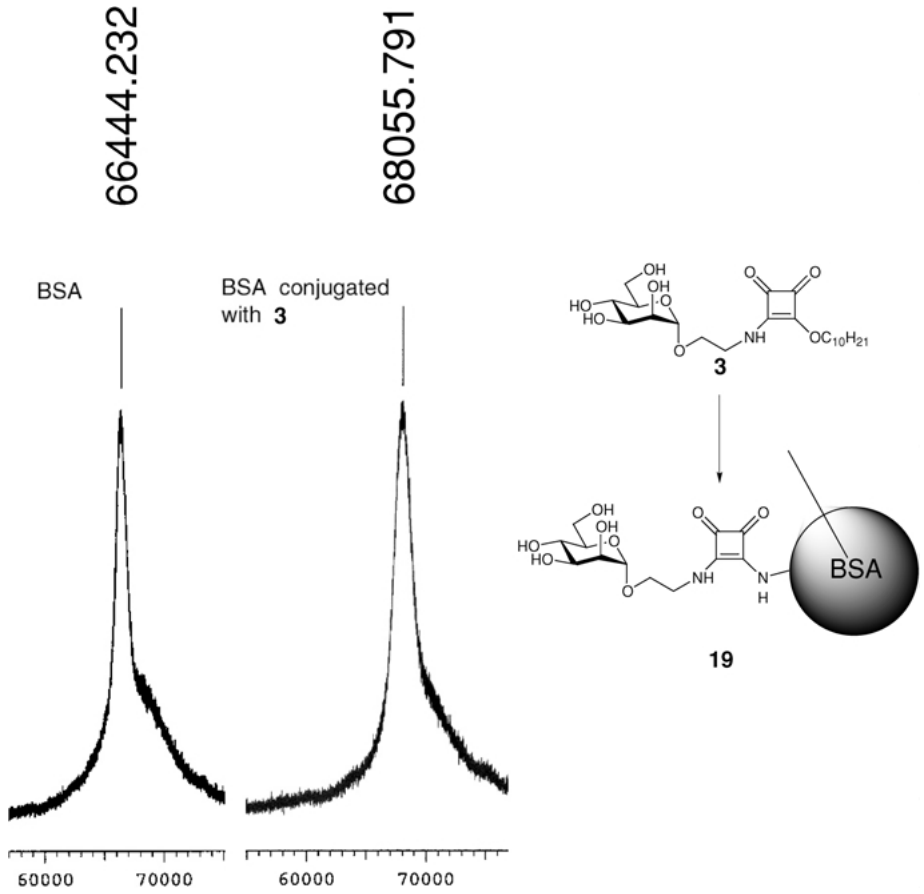


Figure 3. MALDI-TOF analysis of **3** conjugated to BSA.

conjugation to microtiter plates, thus demonstrating the simple recovery of valuable saccharides from conjugation reaction mixtures.

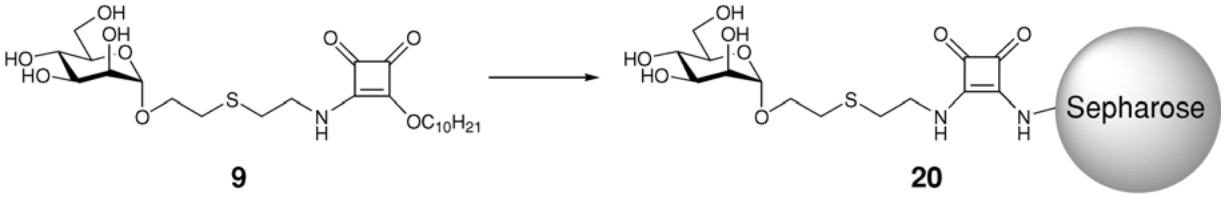
Conjugation of squaric decyl ester glycosides to BSA and Sepharose EAH 4B

The efficiency of squaric decyl ester glycosides in neoglycoconjugate synthesis was further investigated with their conjugations to BSA and amino-functional Sepharose EAH 4B. Conjugation to BSA was performed with 6 equivalents of the squaric decyl ester mannoside **3** for 24 h in sodium hydrogen carbonate buffer (pH 9.0), followed by dialysis and lyophilization. The product neoglycoprotein **19** was analyzed with MALDI-TOF MS as described [10] (Figure 3). The increase in molecular weight of the conjugated protein was 1612, which corresponds to an incorporation of 5.3 mannosides per BSA. Thus, the degree of incorporation was 84%, which is well in the range of what has been found in previous work with the less hydrophobic squaric ethyl ester glycosides [10].

Conjugation to the amino-functional Sepharose EAH 4B was investigated with a sulfur-tagged squaric decyl ester mannoside (**9**), because the sulfur tag would allow simple determination of the conjugation yield by analysis of the increase in sulfur content in the Sepharose. Thus, the conjugation was performed with 22 μmol **9** per mL drained Sepharose in sodium hydrogencarbonate buffer (pH 9.0) for 40 h, followed by filtration and washing, to give the mannosylated Sepharose **20** (Scheme 3). A sample of **20** and a sample of filtered and washed underivatized Sepharose EAH 4B were lyophilized and analyzed by elemental sulfur analysis. The increase in sulfur content due to reaction with **9** corresponded to 69 μmol mannose per gram lyophilized gel or 3.1 μmol mannose per mL drained gel.

Conclusions

Squaric decyl ester glycosides were demonstrated to be efficient reagents for conjugation of saccharides to amino-functional microtiter wells, BSA, and Sepharose EAH 4B. The use of didecyl squarate, instead of the common diethyl squarate, as an amine-reactive cross-coupling reagent allows convenient purification of squaric decyl ester glycosides and recovery of unreacted squaric decyl ester glycosides from conjugation reaction mixtures by simple C18 SPE, *independent* of the glycoside structure, because the hydrophobicity required for C18 SPE is built into the squaric ester moiety and *not* the carbohydrate. Furthermore, the hydrophobic tag (1-decanol) is released during the bioconjugation reaction, i.e. the tag is *traceless*, which is



Scheme 3. Sepharose EAH 4B, $NaHCO_3$ buffer, pH 9.0.

desirable in cases where the presence of a hydrophobic alkyl aglycon interferes with the biological activity of the glycoconjugate. These advantages are general and not limited to neoglycoconjugate synthesis and didecyl squarate **2** may very well find more general use as a homobifunctional amino-reactive reagent in bioconjugate chemistry.

Experimental

General

NMR experiments were recorded with Bruker ARX 300 MHz, Bruker DRX 400 MHz, or Varian Unity 400 MHz spectrometers at ambient temperature. ^{1}H-NMR assignments were derived from COSY experiments. The optical rotations were measured with a Perkin-Elmer 241 polarimeter. High-resolution FAB mass spectra (HRMS) were recorded with a JEOL SX-120 instrument. MALDI-TOF MS experiments were recorded with a Bruker Biflex III instrument (run in positive mode) using gentisic acid (2,5-dihydroxy benzoic acid) as matrix. Lectin-peroxidase conjugates were from Sigma-Aldrich [Concavalin A (L6397), *Arachis hypogaea* (peanut agglutinin, L7759), *Ricinus communis* (Castorbean) Toxin RCA_{120} (L2758), *Bandeira simplicifolia* (L5391), *Triticum vulgaris* (weat germ agglutinin, L3892), and *Tetragonobulus purpureas* (L5759)], EY Laboratories [*Maackia amurensis* (H7801)], and ICN [*Limax flavus* (153298)]. PBS buffer: 24 g NaCl, 0.6 g KCl, 3.46 g $NaH_2PO_4{*}H_2O$ and 0.6 g KH_2PO_4 were dissolved in 1000 mL H_2O and the pH adjusted to 7.2. Tween/PBS solution: 7.30 g Tween-20 was dissolved in 200 mL PBS buffer. Lectin solutions: 1 mg of lectin was dissolved in 1 mL Tween/PBS solution and filtered through a 0.22 μm filter. The resulting solution was then diluted between 25–50 times in Tween/PBS. Cova buffer: 117 g NaCl, 10 g $MgSO_4{*}7H_2O$ and 0.50 mL Tween were dissolved in 1000 mL PBS buffer. Citric acid buffer: 7.98 g citric acid monohydrate and 9.46 g Na_2HPO_4 were dissolved in 1000 mL H_2O and the pH adjusted to 5.0. *o*-Phenylenediamine solution: 6.0 mg *o*-phenylenediamine was dissolved in 10 mL citric acid buffer and 5 μL H_2O_2 was added. The *o*-phenylenediamine solution was used immediately. $NaHCO_3$ buffer: 20 g $NaHCO_3$ was dissolved in 1000 mL H_2O and the pH was adjusted to 9.00. Reactions were monitored by TLC on silica Gel F_{254} (E. Merck, Darmstadt, Germany). Amino-functional CovaLink microtiter plates were from Nalge Nunc International (Roskilde, Denmark). Microtiter plate absorbencies were read on an InterMed ImmunoReader NJ-2000. Isolute C18 cartridges were from International Sorbent Technology Inc.. Sepharose EAH 4B was from Amersham Pharmacia Biotech.

3,4-Didecyloxy-cyclobut-3-ene-1,2-dione (**2**)

Squaric acid **1** (1.042 g, 9.13 mmol) and decanol (7.6 mL, 39.85 mmol) were refluxed in toluene (7.5 mL) in a Dean-Stark trap for 12 h. The toluene was evaporated and the remaining decanol was distilled off under vacuum. The residue was purified by flash chromatography (heptane:etylacetate 2:1) to give **2** (2.645 g, 73%), as a clear liquid that slowly solidified; m.p. 34°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.68 (tr, 2 H, *J* 6.7 Hz, $OC\underline{H}_2CH_2$), 1.81 (dt, 2 H, *J* 6.7 Hz, $OCH_2C\underline{H}_2CH_2$), 1.43-1.28 (m, 14 H, $CH_2(C\underline{H}_2)_7CH_3$), 0.89 (t, 3H, *J* 6.6 Hz, $CH_2C\underline{H}_3$). ^{13}C-NMR (400 MHz, $CDCl_3$): δ 189.8, 184.71, 32.3, 30.3, 29.9, 30.0, 29.9, 29.7, 29.6, 25.7, 23.1, 14.5. HRMS calcd for $C_{24}H_{43}O_4$(M + H): 395.3161; found: 395.3170.

Typical procedure for the synthesis of squaric decyl ester glycosides: 2-[(2-Decyloxy-3,4-dioxocyclobut-1-enyl)amino]-ethyl α-D-mannopyranoside (**3**)

To a solution of 2-aminoethyl α-D-mannopyranoside [13] (468 mg, 1.88 mmol) in DMF (10 mL) were added **2** (250 mg, 0.63 mmol) and Et_3N (60 μL, 0.43 mmol). After 24 h, the solution was concentrated and the residue flash chromatographed (SiO_2, CH_2Cl_2: MeOH 85:15), which yielded **3** (165 mg, 83%) as a white solid; $[\alpha]_{23}^{D}$ + 10° (*c* 0.1, H_2O). ^{1}H-NMR (400 MHz, CD_3OD): δ 4.78 (d, 1 H, *J* 1.6 Hz, H-1), 4.70 (dt, 2H, *J* 14.2, 7.6 Hz, $OC\underline{H}_2CH_2CH_2$), 3.89–3.57 (m, 8H), 3.51 (dd, 1H, *J* 2.7, 3.8 Hz, H-5), 1.83 (dt, 2H, *J* 5.6, 6.3 Hz, $OCH_2C\underline{H}_2CH_2$), 1.46-1.31 (m, 14H, $(C\underline{H}_2)_7CH_3$), 0.90 (t, 3H, *J* 6.7 Hz, CH_3). HRMS calcd for $C_{22}H_{38}NO_9$ (M + H): 460.2546; found: 460.2552.

In a similar manner were prepared: 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl (β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**4**); HRMS calcd for $C_{28}H_{47}NO_{14}Na$ (M + Na): 644.2895; found 644.2894, 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl (α-D-galactopyranosyl)-(1 → 4)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**5**); HRMS calcd for $C_{34}H_{57}NO_{19}Na$ (M + Na): 806.3423; found: 806.3434, 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]-ethyl (2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1 → 3)-(α-D-galactopyranosyl)-(1 → 4)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**6**); HRMS calcd for $C_{42}H_{70}N_2O_{24}Na$

(M + Na): 1009.4216; found: 1009.4216, 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)-amino]ethyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**7**); HRMS calcd for $C_{39}H_{63}N_2O_{22}Na_2$ (M − H + 2Na): 957.3668; found: 957.3687, 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)-amino]ethyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-[(α-L-fucopyranosyl)-(1 → 3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (**8**); HRMS calcd for $C_{47}H_{76}N_3O_{26}Na_2$ (M-H + 2Na): 1144.4512; found: 1144.4504, 2-{2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio}ethyl α-D-mannopyranoside (**9**); HRMS calcd for $C_{24}H_{41}NO_9SNa$ (M + Na): 542.2400; found: 542.2413, 2-{2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio}ethyl β-D-glucopyranoside (**10**); HRMS calcd. for $C_{24}H_{41}NO_9SNa$ (M + Na): 542.2400; found: 542.2389, and 2-{2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio}ethyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranoside (**11**); HRMS calcd. for $C_{30}H_{51}NO_{14}SNa$ (M + Na): 704.2928; found: 704.2916.

Typical procedure for the conjugation of squaric decyl ester glycosides **3–8** to amino-functional microtiter plates (→**13–18**)

The squaric decyl ester glycoside solution (150 μL/well, 0.01–0.2 mM in $NaHCO_3$ buffer), was added to an amino-functional microtiter plate. After 24 h, the plates were emptied and washed three times with Cova buffert (1 + 1 + 10 min) followed by thorough washing with water. To the plates was added a solution of 20% acetic anhydride in water. After 2 h, the plates where thoroughly washed with water.

Typical procedure for the recovery of excess squaric decyl ester glycosides (**5**, **7**, and **8**) after conjugation to microtiter plates

Following a conjugation of squaric decyl ester glycosides to microtiter plates as described above, the reaction solutions were taken from the wells, combined, and passed through a C18 cartridge. The cartridge was washed with 2 × 5 mL H_2O and then eluted with 80% MeOH to give the pure recovered unreacted squaric decyl ester glycosides: (0.93 mg **5** recovered after conjugation reaction to 96 wells with of a total of 1.50 mg **5**, 0.2 mM, 100 μL/well), **7** (1.20 mg **7** recovered after conjugation reaction to 3840 wells with a total of 3.51 mg **7**, 0.01 mM, 100 μL/well), and **8** (1.20 mg **8** recovered after conjugation reaction to 3840 wells with of a total of 4.22 mg **8**, 0.01 mM, 100 μL/well).

Typical procedure for the plant lectin binding to microtiter plates **13–18**

A lectin-horseradish peroxidase conjugate solution (100 μL/well) was added and the plate was incubated at rt for 45 min. The plates were then washed with Cova buffer (1 + 1 + 10 min) followed by citric acid buffer. *o*-Phenylenediamine solution (100 μL/well) was added as substrate and the optical density at 490 nm was read.

Competitive inhibition of plant lectin binding to microtiter plates **13**, **17**, and **18**

The inhibitor solution (75 μL/well, 36 mM in Tween/PBS) was added to the first row of a microtiter plate. Tween/PBS (50 μL) was added to the rest of the wells. A volume of 25 μL from the first row was then transferred to the second row and mixed. This procedure was repeated down the rows until the second last row, from which 25 μL were discarded. This resulted in a dilution factor of three for every row. The lectin-horseradish peroxidase conjugate was then added and the binding was detected as above.

Conjugation of **3** to BSA (→**19**)

To BSA (62 mg, 0.93 μmol) in 2.5 mL of $NaHCO_3$ buffer was added **3** (2.5 mg, 5.44 μmol) and the mixture was left at ambient temperature for 24 h. The reaction mixture was then dialyzed against water (5 × 25 mL) and lyophilized affording the neoglycoprotein **19** as a white powder (61 mg). The average degree of incorporation (5.3 α-D-mannose/BSA) was determined by MALDI-TOF MS [10] using the center of the single charged protein peak.

Conjugation of the sulfur-tagged squaric decyl ester glycoside **9** to Sepharose EAH 4B (→**20**)

Sedimented Sepharose EAH 4B (0.5 mL) was suspended in $NaHCO_3$ buffer (1.5 mL) and the squaric decyl ester mannoside **9** (5.0 mg, 9.2 μmol) was added. After 24 h, the Sepharose was filtered on a sintered glass funnel and washed thoroughly with water. Samples of the Sepharose were taken before and after conjugation with **20**, washed, lyophilized, and subjected to sulfur combustion analysis. The increase in sulfur content upon conjugation reaction (0.06% → 0.22%) corresponded to an α-D-mannopyranoside content of 3.1 μmol/mL sedimented Sepharose beads.

Acknowledgments

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