Title: NOVEL METHOD FOR RECOVERY OF SQUARIC ACID MONO ESTERS IN BIOCONJUGATION CHEMISTRY

Abstract: The present invention relates to a biomolecule squaric acid monoamide conjugate of formula (I), as presented in the claims, and to a method for non-polar extraction of a biomolecule squaric acid monoamide conjugate of formula (I), as presented in the claims, obtained after a bioconjugation reaction, wherein said extraction comprises recovering said intermediate by non-polar means.
NOVEL METHOD FOR RECOVERY OF SQUIARCIC ACID MONO ESTERS IN BIOCONJUGATION CHEMISTRY

Technical field of the invention
The present invention relates to a method for non-polar extraction of novel biomolecule squaric acid mono-amide conjugates of formula (I), as presented below, obtained after a bioconjugation reaction.

Background Art
The chemical linking of a biologically active molecule (biomolecule) to another natural or artificial carrier molecule or moiety, for example, a polymer bead, a microtiter well, a protein, a fluorescence tag, an affinity matrix, is generally termed bioconjugation or bioconjugate chemistry. The resulting product is termed a bioconjugate. The properties of the biological molecule and the carrier molecule or moiety are combined, thus creating "designed" and unique characteristics of the bioconjugate. The use of bioconjugates, for example in assaying of small amount of molecules in biological samples, in drug delivery, or in modulation of biological activity, are well-known to practitioners skilled in the art and of fundamental importance for virtually every discipline within life sciences. Many biological assays, including pre-clinical drug trials, today involve the use of bioconjugates.

Conjugation of a biomolecule and a carrier molecule or moiety often involves the use of a cross-linking reagent. The cross-linking reagent possesses two reactive functional groups, of which one reacts with the biomolecule and the other with the carrier molecule or moiety. Numerous such cross-linking reagents are well known to one skilled in the art and many are commercially available.

Squaric acid diethyl ester is a highly efficient homobifunctional amine-reactive cross-linking reagent that has found widespread use for bioconjugations in general.
and in glycoconjugation (bioconjugation of carbohydrates) in particular. Glüsenkamp et al. disclosed a process for bioconjugation based on squaric acid esters (DE4341524 and DE14990550). The use of squaric acid diethyl ester in the preparation of glycoconjugates was pioneered by Tietze and Hindsgaul and later used many others. Additional publications describe squaric acid diester in conjugation chemistry.

The typical use of squaric acid diethyl ester involves an initial reaction with an amino group on the biomolecule (e.g. carbohydrate) to yield a biomolecule-squaric acid monoamide intermediate (Fig. 1). This intermediate is subsequently reacted with the carrier molecules or moiety chosen. Furthermore, the biomolecule-squaric acid monoamide intermediate is commonly used in large molar excess to the carrier molecules or moiety. Biomolecules are typically isolated or synthesized in minute amounts and thus are very valuable. Consequently, the recovery of unreacted excess of biomolecule-squaric acid monoamide intermediate is of significant importance. However, although the squaric acid diethyl ester is an efficient amino-reactive cross-linking reagent, a major disadvantage is that the biomolecule-squaric acid monoamide ethyl ester intermediate is complicated to purify, which renders recovery of unreacted biomolecule-squaric acid monoamide ethyl ester intermediate a time-consuming and complex procedure. Recovery/purification of carbohydrate-squaric acid monoamide intermediates based on C18 solid-phase extraction has been reported. However, these purification protocols rely on the presence of a hydrophobic chemical tag on the carbohydrate molecule and are thus not generally applicable to squaric acid diethyl ester bioconjugation chemistry of underivatized biomolecules, because each individual biomolecule has to be pre-derivatized with a hydrophobic tag before conjugation, which is laborious, time-consuming and costly. Another disadvantage is that the hydrophobic tag is re-
tained in the product, which can hamper the usefulness of the product in biomedical applications.

Thus, there is considerable need within the art of purification of biomolecule-squaric acid monoamide intermediates and, more important, of easy recovery of unreacted excesses of expensive biomolecule-squaric acid monoamide intermediates.

Summary of the invention

The present invention relates to a method for non-polar extraction of a biomolecule squaric acid monoamide conjugate of formula (I) obtained after a bioconjugation reaction

\[
\begin{array}{c}
\text{X} \\
\text{Y} \\
\text{Z} \\
\text{N} \\
\text{R}^1 \\
\text{R}^2 \\
\text{R}^3
\end{array}
\]

(I)

wherein

X, Y and Z are, independently, selected from the group consisting of O and S;

R\(^1\) is selected from the group consisting of an alkyl, an alkenyl, an alkynyl and an aryl group comprising at least 6 carbon atoms;

R\(^2\) is a biomolecule;

R\(^3\) is selected from the group consisting of hydrogen, an alkyl, an alkenyl, an alkynyl group comprising from 6 to 30 carbon atoms, and an aryl group comprising from 6 to 18 carbon atoms;

wherein said extraction comprises;

recovering said intermediate by non-polar means.

Brief description of the drawings

Fig. 1. Schematic procedure for conjugation of a biomolecule to a carrier molecule/matrix using squaric
acid esters as homobifunctional amine-reactive cross-linking reagents.

Fig. 2. Procedure for conjugation of 2-aminoethyl α-D-mannoside to squaric acid didecyl ester, yielding 4-[2-aminoethyl α-D-mannopyranoside] 3-decyloxy-cyclobut-3-ene-1,2-dione (1).

Fig. 3. Saccharide-squaric acid monoamide intermediates 1-9.

Fig. 4. MALDI-TOF MS of BSA before (a) and after (b) conjugation reaction with 1 showing the successful average conjugation of 5 α-D-mannopyranoside moieties per BSA molecule.

Fig. 5. Schematic procedure for the recovery of biomolecule intermediates of formula (I) subsequent bioconjugation to an amino-functionalized microtiter plate.

Fig. 6. Detection of plant lectin binding to saccharide-carrying microtiter plates prepared with saccharide-squaric acid monoamide intermediates.

Fig. 7. Inhibition of plant lectin binding to saccharide-carrying microtiter plates prepared with saccharide-squaric acid monoamide intermediates a) 1, b) 4, and c) 6.

Detailed description of embodiments of the invention

In an embodiment of the invention the method further comprises, preceding said recovering, collecting a reaction solution after said bioconjugation reaction, and bringing said reaction solution, optionally after a change, concentration, dilution and/or addition of solvent, into contact with said non-polar means.

In another embodiment of the invention said non-polar means is selected from the group consisting of a non-polar solid phase and a non-polar solution.

In a further embodiment of the invention said biomolecule is selected from the group consisting of a carbohydrate, a protein, a peptide, a nucleoside, a nucleotide, a steroid, a lipid, an alkaloid, a secondary metabolite, a saccharide, a saccharide-substituted alkyl
group, and a saccharide-substituted sulfur-containing alkyl group. Any other biomolecule known to a person skilled within the art may obviously be used as an alternative to the above mentioned ones.

In yet another embodiment of the invention said saccharide is selected from the group consisting of glucose, mannose, galactose, glucosamine, galactosamine, fucose, fructose, xylose, neuraminic acid, glucoronic acid, iduronic acid, a disaccharide, an oligosaccharide comprising at least two of the above monosaccharides, and derivatives thereof. Any other saccharide known to a person skilled within the art may obviously be used as an alternative to the above mentioned saccharides.

In another embodiment of the invention the biomolecule intermediate of the above mentioned formula (I) comprises a saccharide in the form of a glycoside with an amino-functionalized aglycon or an amino-functionalized and sulfur-containing aglycon, which saccharide is selected from the group consisting of glucose, mannose, galactose, glucosamine, galactosamine, fucose, fructose, xylose, neuraminic acid, glucoronic acid, iduronic acid, a disaccharide or an oligosaccharide comprising at least two of the above monosaccharides, and derivatives thereof. Any other saccharide known to a person skilled within the art may obviously be used as an alternative to the above mentioned saccharides.

In yet another embodiment of the invention said \( R^1 \) in the biomolecule squaric acid monoamide conjugate of formula (I) comprises from 6 to 30 carbon atoms, preferably from 10 to 20 carbon atoms. It is very convenient that \( R^1 \) may be chosen independently of the structure of the biomolecule.

In a further embodiment of the invention the nonpolar solid phase, which the intermediate is brought into contact with according to the method of the invention, is selected from the group consisting of alkylated silica (e.g. C2, C4, C6, C8, C18, cyclohexyl, cyanopropyl), ary-
lated silica (e.g. phenyl), resin-based solid-phase extraction sorbents (e.g. polystyrene resin, co-polymer resins), and mixed mode solid-phase extraction sorbents.

In one embodiment, the invention relates to an easy, manual or automated, purification and recovery of unreacted excesses of biomolecule squaric acid monoamide conjugates of the general formula (I), subsequent bioconjugation to a carrier molecule, moiety, or a matrix.

In a further embodiment, the present invention relates to the discovery that biomolecule squaric acid monoamide conjugates of formula I can readily be extracted into hydrophobic solution or solid phases, exemplified by, but not limited to, alkylated silica (e.g. C2, C4, C6, C8, C18, cyclohexyl, cyanopropyl etc), arylated silica (e.g. phenyl etc.), resin-based solid-phase extraction sorbents (e.g. polystyrene resin, co-polymer resins etc.), and mixed mode solid-phase extraction sorbents (i.e. sorbents comprising a mixture of 2 or more of the above mentioned sorbents). The biomolecule squaric acid monoamide conjugates of formula (I) can thus be recovered from complex reactions mixtures subsequent a bioconjugation reaction to amino-group containing carrier molecules or matrixes, exemplified by, but not limited to, proteins, peptides, fluorescence markers, solid polymer beads, microtiter plates, test tubes, glass surfaces, and chromatographic matrices such as silica, dextran, starch, cellulose, agarose etc. The extraction of biomolecule squaric acid monoamide conjugates of formula (I) into hydrophobic solution or solid phases is enabled by the hydrophobic properties of R1. The simplicity of extraction procedure renders it suitable for automation.

In a further embodiment of the invention, in the biomolecule squaric acid monoamide conjugate of formula (I), said X is O, said Y is O, said Z is O, and said R1 is a decyl group.

In another embodiment of the invention the biomolecule squaric acid monoamide conjugate of formula (I)
is selected from, but not limited to, the group consisting of 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl α-D-mannopyranoside (1), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl (β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (2), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl (α-D-galactopyranosyl)-(1→4)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (3), 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)-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylic acid)-(2→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (4), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylic acid)-(2→3)-(β-D-galactopyranosyl)-(1→4)-[(α-L-fucopyranosyl)-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (5), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl (5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylic acid)-(2→3)-(β-D-galactopyranosyl)-(1→4)-[(α-L-fucopyranosyl)-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (6), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio]ethyl α-D-mannopyranoside (7), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio]ethyl β-D-glucopyranoside (8), and 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio]ethyl (α-D-galactopyranosyl)-(1→4)-β-D-galactopyranoside (9).

In the present invention the term "alkyl group" is meant to comprise from 6 to 30 carbon atoms, preferably from 10 to 20 carbon atoms. Said alkyl group may be straight or branched. Said alkyl group may also form a cycle comprising from 6 to 30 carbon atoms, preferably from 10 to 20 carbon atoms. For example hexyl, heptyl, octyl etc..

In the present invention the term "alkeny group" is meant to comprise from 6 to 30 carbon atoms, preferably from 10 to 20 carbon atoms. Said alkenyl group comprises
at least one double bond. For example hexenyl, heptenyl, octenyl etc..

In the present invention the term "alkynyl group" is meant to comprise from 6 to 30 carbon atoms, preferably from 10 to 20 carbon atoms. Said alkynyl group comprises at least one triple bond. For example hexynyl, heptynyl, octynyl etc..

In the present invention the term "aryl group" is meant to comprise from 6 to 18 carbon atoms. For example phenyl, naphtyl, anthracenyl etc..

The above mentioned groups may naturally be substituted with any other known substituent within the art of organic chemistry. The groups may also be substituted with two or more of the substituents.

In a further embodiment, the invention relates to the recovery of a biomolecule squaric acid monoamide conjugate of the above mentioned formula after conjugation to a natural or artificial carrier molecule or moiety, for example, but not limited to, a polymer bead, a microtiter well, a protein, a fluorescence tag, an affinity matrix.

In still another embodiment, the present invention relates to the recovery of a biomolecule squaric acid monoamide conjugate of the above mentioned formula after conjugation to an amino-functionalized natural or artificial carrier molecule or moiety, for example, but not limited to, a polymer bead, a microtiter well, a protein, a fluorescence tag, an affinity matrix.

The present invention originates in the discovery that the use of squaric acid esters of hydrophobic alcohols in bioconjugation chemistry results in squaric acid ester monoamides carrying a hydrophobic tag that is released during the bioconjugation reaction (see fig. 1), i.e. the tag is traceless (hydrofobic tags may interfere with biological activity of the biomolecule), thus avoiding the necessity of tagging each individual biomolecule as the hydrophobic tag is present in the squaric acid es-
ter reagent. The excess of the unreacted squaric acid ester monoamide after bioconjugation reaction is easily recovered by use of the method of the invention and may be re-used in a further bioconjugation reaction.

Methodology/Experimental

General methods

NMR experiments were recorded either with a Bruker ARX 300 MHz, Bruker DRX 400 MHz, or a Varian Unity 400 MHz spectrometer at ambient temperature. $^1$H NMR assignments were derived from COSY experiments. 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 were recorded on a Bruker Biflex III instrument (run in positive mode) using gentisic acid (2,5-dihydroxy benzoic acid) as matrix. Lectin-peroxidase conjugates were purchased from Sigma-Aldrich (Concanavalin A (L6397) for 1, Arachis hypogaea (peanut agglutinin, L7759) for 2, Ricinus communis (Castorbean) Toxin RCA120 (L2758) for 2 and 4, Bandeira simplicifolia (L5391) for 3, and Triticum vulgaris (wheat germ agglutinin, L3892) for 5 and 6).

PBS buffer: 24 g NaCl, 0.6 g KCl, 3.46 g NaH$_2$PO$_4$$\cdot$H$_2$O and 0.6 g KH$_2$PO$_4$ in 1000 mL H$_2$O. The pH was adjusted to 7.2. Tween20/PBS solution: 0.10 mL Tween20 in 200 mL PBS buffer.

Lectin solutions: 1mg of lectin in 1 mL Tween20/PBS solution and filtered through a 0.22 μm filter. The resulting solution was then diluted between 25-50 times in Tween20/PBS.

Cova buffert: 117 g NaCl, 10 g MgSO$_4$$\cdot$7H$_2$O and 0.50 mL Tween20 in 1000 mL PBS buffer.

Citric acid buffer: 7.98 g citric acid monohydrate and 9.46 g Na$_2$HPO$_4$ in 1000 mL H$_2$O. The pH was adjusted to 5.0.

O-Phenylendiamine substrate solution: To 6.0 mg O-phenylendiamine in 10 mL citric acid buffer was added 5 μL H$_2$O$_2$. The solution was used immediately.
NaHCO₃ buffer: 20 g NaHCO₃ in 1000 mL H₂O. The pH was adjusted to 9.00.
4-Nitrophenyl α-D-mannopyranoside was purchased from Sigma-Aldrich. Amino-functionalized Covalink microtiter plates were from Nalge Nunc International (Roskilde, Denmark). Microtiter plates absorbencies were read on an InterMed ImmunoReader NJ-2000. Isolute C-18 cartridges were from International Sorbent Technology.

Synthesis of didecyl squarate (3,4-Didecyloxy-cyclobut-3-ene-1,2-dione)
Squaric acid (3,4-hydroxy-cyclobut-3-ene-1,2-dione, 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:ethylacetate 2:1) to give 2 (2.645 g, 73%), as a clear liquid that slowly solidified, m.p. 34°C. ¹H-NMR (400 MHz, CDCl₃): δ 4.68 (tr, 2 H, J 6.7 Hz, OCH₂CH₂), 1.81 (dt, 2 H, J 6.7 Hz, OCH₃CH₂), 1.43-1.28 (m, 14 H, CH₂(CH₂)₃CH₃), 0.89 (t, 3H, J 6.6 Hz, CH₂CH₃). ¹³C-NMR (400 MHz, CDCl₃): 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. HR FAB-MS calcd for C₂₄H₄₃O₄ (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 (1). To a solution of 2-aminoethyl α-D-mannopyranoside (17) (468 mg, 1.88 mmol) in DMP (10mL) were added didecyl squarate (250 mg, 0.63 mmol) and Et₃N (60 µL, 0.43 mmol). After 24 h, the solution was concentrated and the residue flash chromatographed (SiO₂, CH₂Cl₂:MeOH 85:15), which yielded 1 (165 mg, 83%) as a white solid; [α]D₂₅ +10° (c 0.1, H₂O). ¹H-NMR (400 MHz, CD₂OD): δ 4.78 (d, 1 H, J 1.6 Hz, H-1), 4.70 (dt, 2H, J 14.2, 7.6 Hz, OCH₂CH₂CH₂), 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,
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OCH₂CH₂CH₃), 1.46-1.31 (m, 14H, (CH₂)₂CH₃), 0.90 (t, 3H, J 6.7 Hz, CH₃). ¹³C-NMR (400 MHz, CD₃OD): δ 184.0, 188.8, 184.0, 177.4, 177.1, 174.2, 100.7, 100.5, 73.9, 73.8, 71.5, 67.5, 66.7, 66.5, 61.9, 44.4, 44.0, 32.1, 30.1
29.7, 29.7, 29.5, 29.3, 25.5, 25.4, 22.8, 13.5. HR FAB-MS calcd for C₂₂H₃₈NO₉ (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 (2). [α]D₂₃ +14° (c 0.1, H₂O). ¹H-NMR (400 MHz, CD₃OD): δ 4.59 (q, 2H, J 5.0 Hz, OCH₂(CH₂)₈), 4.26 (d, 1H, J 7.4 Hz, H-1), 4.24 (d, 1H, J 7.6 Hz, H-1'), 3.27 (dd, 1H, J 4.6, 8.2 Hz, H-2), 1.72 (dt, 2H J 6.7, 7.5 Hz, OCH₂CH₂(CH₂)₇), 1.35-1.21 (m, 14H, (CH₂)₇CH₃), 0.80 (t, 3H, J 6.7 Hz, CH₃). ¹³C-NMR (400MHz, CD₃OD): δ 104.1, 103.2, 79.6, 76.1, 75.6, 75.4, 73.9, 73.8, 71.6, 69.3, 68.8, 32.0, 30.1, 29.62, 29.60, 29.4, 29.3, 25.4, 22.7, 13.4. HR FAB-MS calcd for C₂₈H₄₇NO₁₄Na (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 (3). [α]D₂₃ + 29° (c 0.1, H₂O). ¹H-NMR (400 MHz, CD₃OD): δ 4.86 (d, 1H, J 3.6 Hz, H-1''), 4.59 (q, 2H, J 5.4 Hz, OCH₂CH₂), 4.32 (d, 1H, J 7.2 Hz, H-1''), 4.25 (d, 1H, J 7.8 Hz, H-1), 4.16 (t, 1H, J 6.0 Hz, H-5''), 3.17 (dd, 1H, J 4.8, 8.2 Hz, H-2), 4.72 (dt, 2H, J 3.0, 6.6 Hz, OCH₂CH₂), 1.38-1.17 (m, 14H, (CH₂)₇CH₃), 0.80 (t, 3H, J 6.7 Hz, CH₃). ¹³C-NMR (400 MHz, CD₃OD): δ 189.2, 188.9, 184.8 183.8, 177.5, 177.2, 173.7, 173.3, 105.9, 104.4, 104.0, 103.2, 101.7, 80.0, 78.8, 78.6, 76.3, 75.5, 75.4, 75.1, 74.2, 73.8, 73.7, 72.3, 72.2, 71.9, 71.7, 70.3, 70.1 69.6, 69.4, 69.2, 68.8, 61.7, 61.6, 60.9, 60.5, 44.7, 44.4, 32.0, 31.9, 30.1, 29.62, 29.61, 29.4, 29.3, 25.4, 22.71, 22.68, 13.4. HR FAB-MS calcd for C₃₄H₅₇NO₁₃Na (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 (4). $^1$H-NMR 400 MHz (CD$_3$OD) δ 4.89 (d, 1H, J 3.8 Hz, H-1'), 4.69 (q, 2H, J 5.3 Hz, OCH$_2$CH$_2$CH$_2$), 4.62 (d, 1H, J 8.4 Hz, H-1''), 4.41 (d, 1H, J 7.5 Hz, virtual couplings, H-1'), 4.29 (d, 1H, J 7.3 Hz, H-1), 4.27 (br t, 1H, J 6.2 Hz, H-5''), 4.16 (br d, 1H, J 1.8 Hz, H-4''), 3.42 (br dt, 1H, J 3.3, 9.5 Hz, H-5), 2.01 (s, 3H, Ac), 1.72 (q, 2H, J 7.2 Hz, OCH$_2$CH$_2$CH$_2$), 1.42-1.27 (m, 14H, (CH$_2$)$_7$CH$_3$), 0.90 (t, 3H, J 7.1 Hz, CH$_2$CH$_3$); HR FAB-MS calcd for C$_{42}$H$_{70}$N$_2$O$_{24}$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 (5). $^1$H-NMR 300 MHz (CD$_3$OD) δ 4.68 (br q, 2H, J 6.7 Hz, OCH$_2$CH$_2$CH$_2$), 4.43 (d, 1H, J 7.8 Hz, H-1'), 4.35 (d, 1H, J 7.8 Hz, H-1), 4.10 (dd, 1H, J 3.1, 9.7 Hz, H-3'), 3.27 (t, 1H, J 8.1 Hz, H-2), 2.86 (dd, 1H, J 3.9, 12.3 Hz, H-3''e), 2.01 (s, 3H, Ac), 1.82 (q, 2H, J 7.2 Hz, OCH$_2$CH$_2$CH$_2$), 1.72 (t, 1H, J 11.3 Hz, H-3′′a), 1.47-1.28 (m, 14H, (CH$_2$)$_7$CH$_3$), 0.90 (t, 3H, J 6.9 Hz, CH$_2$CH$_3$).

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 (6). $^1$H-NMR 300 MHz (CD$_3$OD) δ 5.02 (d, 1H, J 3.5 Hz, H-1'), 4.80 (br q, 1H, J 6.6 Hz, H-5''), 4.68 (br q, 2H, J 6.7 Hz, OCH$_2$CH$_2$CH$_2$), 4.50 (d, 1H, J 7.8 Hz, H-1''), 4.46 (d, 1H, J 8.2 Hz, H-1), 4.03 (dd, 1H, J 3.1, 9.7 Hz, H-3''e), 2.01 (s, 3H, Ac), 1.94 (2s, 3H, Ac), 1.81 (q, 2H, J 6.8 Hz, OCH$_2$CH$_2$CH$_2$), 1.72 (t, 1H, J 11.8 Hz, H-3''′a), 1.47-1.28 (m, 14H, (CH$_2$)$_7$CH$_3$), 1.16 (d, 3H, J 6.6 Hz, H-6''), 0.90 (t, 3H, J 6.9 Hz, CH$_2$CH$_3$).

Typical procedure for the synthesis of sulfur-containing squaric decyl ester glycosides
2-\{(2-Decyloxy-3,4-dioxocyclobut-1-enyl)amino\}ethylthio\}ethyl α-D-mannopyranoside (7). To a solution of 2-bromoethyl α-D-mannopyranoside \(10^{18}\) (180 mg, 0.395 mmol) in DMF (5 mL) under \(\text{N}_2\) were added cesium carbonate (140 mg, 0.430 mmol) and 2-(t-butoxy carbonylamino)ethanethiol (75 µL, 0.430 mmol) and the solution was stirred overnight. The reaction mixture was diluted with water (10 mL) and extracted with \(\text{CH}_2\text{Cl}_2\) (2x30 mL). The organic phase was dried (MgSO\(_4\)) and concentrated. The residue was flash chromatographed (SiO\(_2\), heptane:ethylacetate 1:2) to afford 2-[2-(t-butoxycarbonylamino)ethylthio]ethyl \(\text{2,3,4,6-tetra-0-acetyl }\) α-D-mannopyranoside (191 mg, 87%) as a clear syrup. Through a solution of a portion of the residue (53 mg, 0.096 mmol) in MeOH (25 mL) was bubbled HCl (g) at 0°C for 15 min. The solution was stirred overnight at room temperature. The solvent was evaporated and the residue dissolved in DMF (10 mL), \(\text{Et}_3\text{N}\) (27 µL, 0.192 mmol) and didecyl squarate (133 mg, 0.336 mmol) were added, and the solution was stirred for 48 h at room temperature. The solvent was evaporated and the residue was flash chromatographed (SiO\(_2\), \(\text{CH}_2\text{Cl}_2\):MeOH 85:15) to give 7 (43 mg, 87%) as a sticky solid; [\(\alpha\)]\(_D\)\(^{20}\) +10° (c 1.0, \(\text{H}_2\text{O}\)). \(^1\text{H}\) NMR (400 MHz, \(\text{CD}_3\text{OD}\)) \(\delta\) 4.79 (br s, 1H, H-1), 4.76 (t, 1H, J 6.4 Hz, \(\text{OCH}_2\text{CH}_2\text{CH}_2\)), 2.79 (m, 4H, \(\text{CH}_2\text{SCH}_2\)), 1.81 (dt, 2H, J 6.4, 7.2 Hz, \(\text{OCH}_2\text{CH}_2(\text{CH}_2)_7\)), 1.50-1.24 (m, 14H, \(\text{CH}_2\)\(_7\)\text{CH}_3), 0.89 (t, 3H, J 8.4 Hz, \(\text{CH}_3\)). \(^13\text{C}\) NMR (400 MHz, \(\text{CD}_3\text{OD}\)) \(\delta\) 173.7, 100.5, 73.7, 71.5, 71.0, 67.4, 67.3, 67.2, 61.8, 48.6, 48.3, 48.1, 47.9, 47.7, 47.5, 47.3, 44.2, 43.6, 33.1, 32.8, 32.0, 31.3, 31.0, 30.3, 29.5, 29.4, 29.2, 25.4, 25.3, 22.6, 13.4. HR FAB-MS calcd for \(\text{C}_{24}\text{H}_{42}\text{NO}_{3}\text{SNa}\) (M+Na): 542.2400, found: 542.2413.

In a similar manner were prepared:

\(\text{2-\{(2-Decyloxy-3,4-dioxocyclobut-1-enyl)amino\}ethylthio\}ethyl } \beta\text{-D-glucopyranoside (8).}\) [\(\alpha\)]\(_D\)\(^{23}\) -7° (c 0.7, MeOH). \(^1\text{H}\) NMR 400 MHz (\(\text{CD}_3\text{OD}\)) \(\delta\) 4.71 (dt, 2H,
J 6.4, 15.8 Hz, OCH₂CH₂CH₂), 4.31 (d, 1H, J 7.7 Hz, virtual couplings, H-1), 1.82 (m, 2H, OCH₂CH₂CH₂), 1.47-1.29 (m, 14H, (CH₃)₂CH), 0.90 (t, 3H, J 6.77 Hz, (CH₃)₂CH); ¹³C-NMR 400MHz (CD₃OD) δ 189.1, 188.7, 183.9, 183.8, 177.3, 177.1, 173.9, 103.5, 77.1, 74.1, 73.9, 73.8, 70.6, 69.8, 69.7, 61.8, 44.3, 43.8, 33.0, 32.8, 31.8, 31.9, 31.3, 31.0, 30.1, 29.6, 29.5, 29.3, 29.5, 25.5, 25.4, 22.7, 13.5; HR FAB-MS calcd for C₂₄H₄₁NO₃SNa (M+Na): 542.2400; found: 542.2389.

10 2-{2-[(2-Decyloxy-3,4-dioxyoclybut-1-enyl)amino]ethylthio}ethyl (β-D-galactopyranosyl)-(1→4)-α-D-galactopyranoside (9). [α]D₂⁰ +34° (c 1.0, H₂O). ¹H-NMR 400 MHz (CDCl₃:CD₃OD:D₂O) δ 4.77 (d, 1H, J 2.9 Hz, H-1'), 4.48 (m, 2H, OCH₂CH₂CH₂), 4.12 (d, 1H, J 7.5 Hz, H-1), 4.03 (t, 1H, J 5.5 Hz, H-5'), 3.30 (dd, 1H, J 7.5, 9.2 Hz, H-2), 1.58 (m, 2H, OCH₂CH₂CH₂), 1.24-1.03 (m, 14H, (CH₃)₂CH), 0.66 (t, 3H, J 6.7 Hz, (CH₃)₂CH); ¹³C-NMR 400MHz (CDCl₃:CD₃OD:D₂O) δ 188.7, 182.7, 176.9, 172.5, 103.1, 100.6, 77.6, 74.1, 73.6, 73.5, 72.7, 71.0, 70.9, 69.3, 69.1, 68.9, 68.7, 60.9, 59.3, 43.7, 43.0, 32.3, 31.3, 31.0, 30.4, 29.5, 29.0, 28.9, 28.7, 28.63, 28.58, 24.8, 24.7, 22.1, 13.3; HR FAB-MS calcd for C₃₅H₅₁NO₁₄SNa (M+Na): 704.2828; found: 704.2916.

Typical procedure for the conjugation of squaric decyl ester glycosides (1-9) to BSA

Bovine serum albumin (BSA, 62 mg, 0.93 μmol) and 1 (2.5 mg, 5.44 μmol) were dissolved in NaHCO₃ buffer (2.5 mL) and stirred in room temperature for 24 hrs. The solution was then dialyzed against 5x25 mL water and lyophilized which afforded the α-D-mannopyranoside neoglycoprotein as a white powder. The average degree of incorporation was determined by MALDI-TOF MS using the center of the single charged protein peak (Fig. 4).

Typical procedure for the conjugation of squaric decyl ester glycosides (1-9) to Sepharose EAH 4B

The sulfur-containing squaric decyl ester 7 (5.0 mg) was added to a suspension of sedimented Sepharose EAH 4B
(Pharmacia Amersham, 0.05 mL) in NaHCO$_3$ buffer (1.5 mL). The sepharose beads were after 24 h filtered on a sintered glass funnel and washed thoroughly with water. The sulfur atom present in 7 enabled differential sulfur combustion analysis: small samples of the sepharose beads were taken before and after conjugation with 7, lyophilized, and subjected to sulfur combustion analysis. The increase in sulfur content subsequent conjugation corresponded to an α-D-mannopyranoside content of 3.1 μmol/mL sedimented sepharose beads.

**Typical procedure for the conjugation of squaric decyl ester glycosides (8-9) to amino-functionalized polymeric beads**

A suspension of amino-functionalized polymer beads (500 μL, IDC amine polystyrene latex 3.0 μm, 4% aqueous suspension, Interalfacial Dynamics Corporation, Portland, USA) was further suspended in NaHCO$_3$ buffer (2 mL), mixed, centrifuged, where after the supernatant was discarded. This washing procedure was repeated 5 times. To the particles in NaHCO$_3$ buffer (2 mL) was added 8 (7.6 mg, 15 μM). The suspension was gently stirred for 15 h, then washed with water as above. Pure unreacted 8 was recovered as described below for microtiter plates.

**Typical procedure for the conjugation of squaric decyl ester glycosides (1-9) to microtiter plates**

The squaric decyl ester glycoside solution (150 μL/well, 0.02 mM in NaHCO$_3$ buffer), was added to an amino-functionalized microtiter plate(CovaLink, NUNC A/S, Roskilde, Denmark). After 24 hrs, the plates were emptied and washed three times with Cova buffert followed by a thorough washing with water. To the plates a solution of 20% acetic anhydride in water was added. After 2 hrs, the plates where thoroughly washed with water.

**Typical procedure for the recovery of squaric decyl ester glycosides (3, 5, and 6) after conjugation reaction (Fig. 5)**
Following a protocol for conjugation of squaric decyl ester glycosides to microtiter plates as described above, the reaction solutions were taken from the wells, combined, and passed through an Isolute C-18 column. The column was washed with 2x5 mL H<sub>2</sub>O and then eluted with 80% MeOH to give the pure recovered unreacted squaric decyl ester glycosides 3, 5, and 6:

- The squaric acid decyl ester glycoside 3 (8.2 mg 3 recovered after conjugation reaction to 96 wells with of a total of 11.3 mg 3, 1 mM, 150μL/well),
- The squaric acid decyl ester glycoside 3 (0.93 mg 3 recovered after conjugation reaction to 96 wells with of a total of 1.50 mg 3, 0.2 mM, 100μL/well),
- the squaric acid decyl ester glycoside 5 (1.20 mg 5 recovered after conjugation reaction to 3840 wells with a total of 3.51 mg 5, 0.01 mM, 100μL/well),
- the squaric acid decyl ester glycoside 6 (1.20 mg 6 recovered after conjugation reaction to 3840 wells with of a total of 4.22 mg 6, 0.01 mM, 100μL/well).

Examples of use of neoglycoconjugates derived with the present technology:

A) Detection of plant lectin binding to microtiter plates conjugated with squaric decyl ester glycosides (1-9) (Fig. 6)

To the microtiter plate prepared with 1, as described above, a concanavalin A-horseradish peroxidase conjugate solution was added (100 μL/well) and the plate was incubated at room temperature for 45 min. The plates were then washed three times with Cova buffert (1+1+10min) followed by once with citric acid buffer. o-Phenylenediamine solution (100 μL/well) was added as substrate and the optical density was read at 490 nm.
B) Competitive inhibition of plant lectin binding to microtiter plates conjugated with squaric decyl ester glycosides (1-9) (Fig. 7)

To a mannoside microtiter plate (prepared with 1 as described above), a solution (75 μL) of 4-nitrophenyl α-D-mannopyranoside (36 mM in TWEEN20/PBS) was added to the first row. Tween20/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 last row, from which 25 μL were discarded. This resulted in a dilution factor of three for every row. The concavalin A-horseradish peroxidase conjugate was then added and the binding was detected as above.
References.


20
CLAIMS

1. A method for non-polar extraction of a biomolecule squaric acid monoamide conjugate of formula (I) obtained after a bioconjugation reaction

\[
\begin{array}{c}
\text{X} \\
\text{Z} \\
\text{Y} \\
\text{R}^1 \\
\text{N} \\
\text{R}^2 \\
\text{R}^3
\end{array}
\]

wherein

X, Y and Z are, independently, selected from the group consisting of O and S;

R^1 is selected from the group consisting of an alkyl, an alkenyl, an alkynyl and an aryl group comprising at least 6 carbon atoms;

R^3 is a biomolecule;

R^3 is selected from the group consisting of hydrogen, an alkyl, an alkenyl, an alkynyl comprising from 6 to 30 carbon atoms, and an aryl group comprising from 6 to 18 carbon atoms;

wherein said extraction comprises: recovering said intermediate by non-polar means.

2. A method according to claim 1, wherein the method further comprises, preceding said recovering, collecting a reaction solution after said bioconjugation reaction, and bringing said reaction solution, optionally after a change, concentration, dilution and/or addition of solvent, into contact with said non-polar means.

3. A method according to claim 1 or 2, wherein said non-polar means is selected from the group consisting of a non-polar solid phase and a hydrophobic solution.

4. A method according to any one of claims 1-3, wherein said biomolecule is selected from the group con-
sisting of a carbohydrate, a protein, a peptide, a nucleoside, a nucleotide, a steroid, a lipid, an alkaloid, a secondary metabolite, a saccharide, a saccharide-substituted alkyl group, and a saccharide-substituted sulfur-containing alkyl group.

5. A method according to claim 4, wherein said saccharide is selected from the group consisting of glucose, mannose, galactose, glucosamine, galactosamine, fucose, fructose, xylose, neuraminic acid, glucoronic acid, idu-
10 ronic acid, a disaccharide, an oligosaccharide comprising at least two of the above monosaccharides, and derivatives thereof.

6. A method according to any one of claims 1-5, wherein said R^1 comprises from 6 to 30 carbon atoms.

7. A method according to claim 6, wherein said R^1 comprises from 10 to 20 carbon atoms.

8. A method according to claim 3, wherein the non-polar solid phase is selected from the group consisting of alkylated silica (preferably C2, C4, C6, C8, C18, cyclohexyl, cyanopropyl), arylated silica (preferably phenyl), resin-based solid-phase extraction sorbents (preferably polystyrene resin, co-polymer resins), and mixed mode solid-phase extraction sorbents.

9. A method according to any one of claims 1-8, wherein said X is 0.

10. A method according to claim 9, wherein said Y is 0.

11. A method according to claim 10, wherein said Z is 0.

12. A method according to claim 11, wherein said R^1 is a decyl group.

13. A method according to any one of claims 1-12, wherein the biomolecule squaric acid monoamide conjugate of formula (I) is selected from the group consisting of

\[ 2-\{(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino\}ethyl \alpha-D-mannopyranoside (1), 2-\{(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino\}ethyl (\beta-D-galactopyranosyl)-(1\rightarrow4)-\beta-D- \]
glucopyranoside (2), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethyl (α-D-galactopyranosyl)-(1→4)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (3), 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 (4), 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 (5), 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 (6), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio)ethyl α-D-mannopyranoside (7), 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio)ethyl β-D-glucopyranoside (8), and 2-[(2-decyloxy-3,4-dioxocyclobut-1-enyl)amino]ethylthio)ethyl (α-D-galactopyranosyl)-(1→4)-β-D-galactopyranoside (9).

14. A method defined in any one of claims 2-13 for recovering said biomolecule squaric acid monoamide conjugate of formula (I) after a bioconjugation reaction and re-using said intermediate in a further bioconjugation reaction.
Fig. 1

Biomolecule-squaric acid monoamide intermediate

Fig. 2

Et₃N (optional)

DMF

(1)
Fig. 4

BSA

66444.232

BSA conjugated with f

68055.791

60000  70000

60000  70000
Conjugation with excess 1 in NaHCO₃(aq.)

NHMe

Unreacted excess 1

Continue on next page
Apply reaction solution onto C18 SPE cartridge

Continued from previous page

Unreacted excess 1

a) Wash with H₂O
b) Elute recovered 1 with 80% MeOH

Recovered pure unreacted excess 1
Fig. 6

Lectin: concanavalin A

Lectin: A. hypogaea

Lectin: B. simplicifolia

Lectin: R. communis

Lectin: T. vulgaris

Lectin: T. vulgaris
Fig 7: Inhibition of plant lectins

Inhibitor: 

Lectin: Concanavalin A
b)

![Graph showing inhibition data with OD on the y-axis and Inhibitor (mM) on the x-axis.]

Inhibitor:

Lectin: Ricin communis 120
Inhibitor:

Lectin: Limax flavus
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

**IPC7: C07K 1/14, C07C 7/10**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

**IPC7: C07C, C07K**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**WPI DATA, CHEM. ABS. DATA, BIOSIS**

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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Further documents are listed in the continuation of Box C. See patent family annex.

**Date of the actual completion of the international search**

13 February 2003

**Date of mailing of the international search report**

14-02-2003

Name and mailing address of the ISA/Swedish Patent Office:

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Telephone No. +46 8 782 25 00

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### INTERNATIONAL SEARCH REPORT
Information on patent family members

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