(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: GLYCOPROTEINS AND GLYCOSYLATED CELLS AND A METHOD FOR THE PREPARATION OF THE SAME
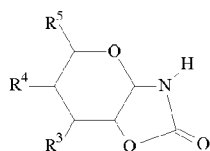
(57) Abstract: The present application discloses novel glycoproteins and a related glyco-
sylcarbamoylation methodology suitable for the preparation of glycopeptides (in particular
glycoproteins and glycosylated cells), as well as the use of such glycoproteins in medicine,
e.g. as pharmaceuticals and diagnostics or in diagnostic kits. The method for the prepara-
tion of a carbohydrate-peptide conjugate comprises reacting a cyclic carbamate (1) (wherein
R^3 and R^4 are hydroxyl, acetamido, or a carbohydrate moiety; and R^5 is hydrogen, methyl,
hydroxymethyl, acetamidomethyl, carboxyl, or X-$(CH_2)_r$-, wherein X is a carbohydrate
moiety and r is an integer selected from 0, 1, 2 and 3) with a peptide comprising at least one primary amino group.

GLYCOPROTEINS AND GLYCOSYLATED CELLS AND A METHOD FOR THE
PREPARATION OF THE SAME

FIELD OF THE INVENTION

The present invention provides novel glycoproteins and a related
5   glycosylcarbamoylation methodology suitable for the preparation of
glycopeptides (in particular glycoproteins and glycosylated cells), as well as the
use of such glycoproteins in medicine, e.g. as pharmaceuticals and diagnostics
or in diagnostic kits.

BACKGROUND OF THE INVENTION

10   Technologies suitable for the preparation of glycoconjugates such as
gycopeptides, glycoproteins, glycolipids, glycosylated cell surfaces, glycosylated
cell membranes and other glycosylated non-biological surfaces have a great
importance in drug discovery and glycobiology. Such enabling technologies play
essential roles in the development of glycopharmaceuticals by conjugating
15   immunogenic and non-immunogenic carbohydrate moieties to chemical and/or
biological entities. The most advanced technologies are usually based upon the
use of ligation chemistries, in which protecting group assistance is avoided
during the conjugation of ligating probes to target chemical and biological
entities.

20   Several ligation methodologies have been described in scientific literature
focusing on the substitution of N-terminal and lysine side-chains of peptides and
proteins in order to deliver the desired carbohydrates moieties. It is well-known
that primary amino functional groups are abundant in all kind of biological
samples such as organs, skin, fur, silk, cell surfaces and could also be easily
25   displayed on synthetic polymers. Thus, primary amine selective ligation
methodologies have the greatest potentials to provide products of many kinds
for numerous industries.

Primary amine ligation chemistries have to provide proper reactivities, chemoselectivities, often satisfactory site-selectivities in water or other aqueous solutions while eliminating the occurrence of severe by-product formation. By-product formation of primary-amine specific ligations is due to unwanted

5   reactions at numerous nucleophilic functional groups such as secondary amino, alcoholic and phenolic hydroxyl, carboxyl, etc present in both the ligating probes and the targeted multifunctional molecules/biological entities.

Several primary amine-specific ligation methodologies have been introduced in the past. These ligation processes have severe shortcomings regarding to the

10   achieved substitution degree and selectivities.

The above mentioned primary amine ligation techniques often suffer from low degree of substitution or low degree of chemoselectivity due to the use of very reactive ligating probes such as mixed anhydrides. In several cases, the use of activating agents is also necessary complicating work-up procedures and

15   lowering product purities (mixed anhydride method, reductive amination). Furthermore, in some ligation methodologies toxic or hard to remove condensation by-products could form causing serious problems in the derivatisation of sensitive biological entities (2-iminomethoxymethylthio ligation, acyl azide ligation, squaric acid ligation). In most of the cases, the developed

20   methodologies use linker systems containing artificial and/or toxic residues (coupling with aryl-isothiocyanates, squaric acid ligation) limiting the scope of ligations by the introduction of unnecessary linking moieties.

Thus, there is a demand for the development of new ligation methodologies suitable for conjugations of carbohydrates to proteins in view of the limitations

25   of present technologies. Novel methodologies have to fulfill the following criteria:

- The ligation reaction should preferably work in aqueous solutions, preferably in water

- A direct linkage between the conjugated moieties should preferably be established, thereby eliminating the use of artificial linkers.

- Natural and non-toxic linker moieties can be accepted.

- Coupling reagents should be avoided during the ligation reaction.

- Condensation by-product formation should be eliminated.

- The ligation chemistry should be capable of working in a wide pH range.

5   - The reactivity of ligating probes should support both chemoselectivity and site-selectivity while rapid conjugations could be achieved.

EP 441192 A2 discloses retroisosteric dipeptides and their use as rennin inhibitors.

WO 88/02756 A2 discloses sugar derivatives of a biologically active peptide with
10   prolonged duration of action.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed
15   before the priority date of each claim of this application.

BRIEF DESCRIPTION OF THE INVENTION

The present invention provides the desired ideal ligation procedure by linking unprotected carbohydrates directly to peptides/proteins/biological entities in water, in broad pH ranges and without the use of any coupling reagent.
20   Furthermore, the newly developed method provides an excellent chemo- and site-selectivity.

In a first aspect, the invention provides a method for the preparation of a carbohydrate-peptide conjugate, said method comprising the step of reacting a cyclic carbamate (1)

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wherein R^3 and R^4 are independently selected from the group consisting of hydroxyl, acetamido, and a carbohydrate moiety; and R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and X-$(CH_2)_r$-, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3;

with a peptide, said peptide comprising at least 30 amino acid units, and said peptide comprising at least one primary amino group.

In a second aspect, the invention provides a carbohydrate-peptide conjugate obtainable by the method according to the first aspect.

In a third aspect, the invention provides a carbohydrate-peptide conjugate comprising one or more moieties of the General Formula 1:

General Formula 1.

wherein

R^1 and R^2 together with the intervening lysine moiety represent a peptide moiety comprising at least 30 amino acid units;

R^3 and R^4 are independently selected from the group consisting of hydroxyl, acetamido, and a carbohydrate moiety;

R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and $X\text{-}(CH_2)_r\text{-}$, wherein X is a carbohydrate moiety and r is an integer selected from 0 and 1;

and pharmaceutically acceptable salts thereof.

5   .

In a fourth aspect, the invention provides a carbohydrate-peptide conjugate comprising one or more moieties of the General Formula **2**:

General Formula 2.

wherein

5   R^{11} is an amino acid side chain;

R^{12} together with $-NH-CHR^{11}-C(=O)-$ represents a peptide moiety having a total number of amino acid units of at least 30;

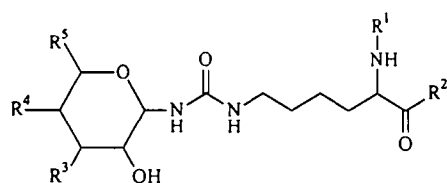R^3 and R^4 are independently selected from the group consisting of hydroxyl, acetamido, and a carbohydrate moiety;

10   R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and $X-(CH_2)_r-$, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3;

and pharmaceutically acceptable salts thereof.

In a fifth aspect, the invention provides use of a carbohydrate-peptide conjugate
15   according to any one of the second, third or fourth aspect as a pharmaceutical, a diagnostic agent, or in a diagnostic kit.

In a sixth aspect, the invention provides a cyclic carbamate of an oligosaccharide selected from (**4a**), (**4b**) and (**4c**)
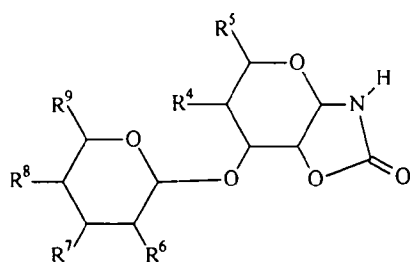
(4a)
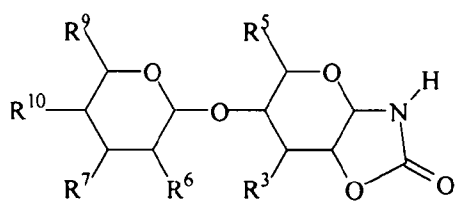
(4b)
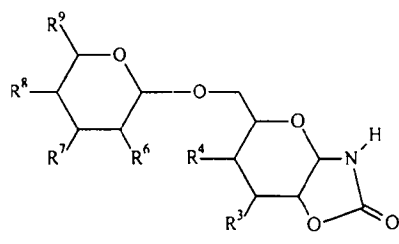
(4c)

wherein

5   R^3, R^4, R^6 and R^7 are independently selected from the group consisting of hydroxyl, acetamido, and a carbohydrate moiety;
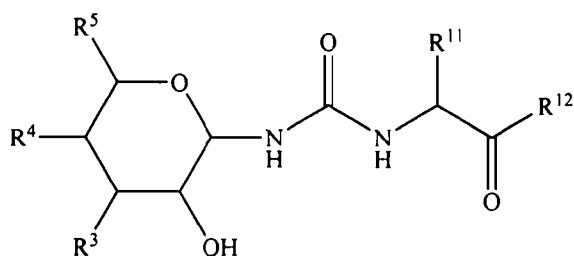
R^5 and R^9 are independently selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and X-(CH$_2$)$_r$-, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3; and

10   R^8 and R^{10} are independently selected from the group consisting of hydroxyl, C$_{1-6}$-alkoxy, C$_{1-6}$-acyloxy, acetamido, and a carbohydrate moiety;

and pharmaceutically acceptable salts thereof, with the proviso that the compound is not (4-O-β-D-glucopyranosyl-1,2-dideoxy-β-D-glucopyranoso)[1,2-d]oxazolidine-2-one or (4-O-β-D-galactopyranosyl-1,2-dideoxy-β-D-

15   glucopyranoso)[1,2-d]oxazolidine-2-one.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Specific reaction scheme of a carbohydrate cyclic carbamate ligation using a disaccharide ligating probe.

Figure 2. Specific reaction scheme of a carbohydrate cyclic carbamate ligation using a disaccharide ligating probe for the glycosylation of Human Insulin.

Figure 3. Specific reaction scheme of a carbohydrate cyclic carbamate ligation using a disaccharide ligating probe to tumor cells.

Figure 4. General conjugation of trisaccharide to cancer cells.

Figure 5. Preparation of carbohydrate cyclic carbamate of lactose.

Figure 6. Preparation of a carbohydrate N,O-cyclic carbamate via phosphinimine intermediate.

Figure 7. Preparation of a carbohydrate N,O-cyclic carbamate of an immunogenic carbohydrate.

Figure 8. Preparation of a carbohydrate N,O-cyclic carbamate via intramolecular ring formation of an acyclic carbamate.

Figure 9. Preparation of a derivatised carbohydrate N,O-cyclic carbamate.

Figure 10. Preparation of a radioactive labeled trisaccharide with N,O-cyclic carbamate.

Figures 11 and 12. Staining of cell smears with FITC-labelled GS1B4.

DETAILED DESCRIPTION OF THE INVENTION

5   As mentioned above, the present invention, i.a., relates to a method for the preparation of a carbohydrate-peptide conjugate, said method comprising the step of reacting a cyclic carbamate (**4**)

(**4**)

wherein R^3 and R^4 are independently selected from the group consisting of

10   hydroxyl, acetamido, and a carbohydrate moiety; and R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and $X-(CH_2)_r-$, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3;

with a peptide, said peptide comprising at least one primary amino group.

15   Chemical modification of biological and chemical entities is one of the most important reactions which can provide products characterized by new physical, chemical, biological and physiological properties. One of the most desired structural modifications of biopolymers and biological entities is glycosylation. Glycoconjugates are natural structures providing new properties of

20   peptides/proteins, and biological cell surfaces. For example, carbohydrate-peptide conjugates can stabilize the optimal conformation of the peptide (e.g. protein) in question thereby maintaining the desired function. Carbohydrate-peptide conjugates can also represent increased half-life, water solubility and enhanced stabilities of proteins. Covalently linked carbohydrates could also serve

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as immunodeterminants on the surface of viruses, pro-and eukaryotic cells.
Several carbohydrate moieties are known to inhibit adhesion of microorganisms,
while others act as receptors for binding of those. Thus, glycoconjugates play
important roles in viral and bacterial infections and certain derivatives could be
5   used as anti-infectives.

Hence, in the present context, the term "carbohydrate-peptide conjugate" is
intended to mean a conjugate of a carbohydrate and a peptide, e.g. as outlined
in the following by means of the conjugates of the General Formulae 1 and 2
(see further below). It should be understood that the conjugate comprises one
10   or more carbohydrate moieties and a peptide moiety. Such carbohydrate
moieties may in themselves be mono-, di- or oligosaccharides.

Indeed, the term "carbohydrate moiety" (also referred to as the glycosyl moiety)
is - when used herein - intended to encompass (but not being limited to)
derivatised and underivatised mono-, di-, oligosaccharides, N-, S- and C-
15   glycosides. A carbohydrate moiety may represent a linear or branched (often a
highly branched) structure, consisting of monosaccharide units. Some of the
more abundantly used monosaccharide units include glucose, N-acetyl-
glucosamine, mannose, galactose, neuraminic acid, N-acetyl-neuraminic acid,
etc.

20   The term "peptide" is - when used herein – intended to encompass smaller
peptides, e.g. oligopeptides having from 5 amino acid units, and up to
polypeptides and proteins having from 30 amino acid units. Typically, the
peptide/peptide moiety comprises a total of at least 30 amino acid units,
typically α-amino acids linked together by means of amide bonds (peptide
25   bonds). In more interesting embodiments, the total number of amino acid units
is typically at least 60, such as at least 100, or even at least 150. The larger
peptides/peptide moieties may even consist of two or more domains relevant for
the formation of biologically peptides/proteins, e.g. enzymes, therapeutically
relevant proteins, etc.

The cyclic carbamate (**4**) represents a key reagent for the formation of the carbohydrate-peptide conjugate.

In the cyclic carbamate, R^3 and R^4 are independently selected from the group consisting of hydroxyl, acetamido, and a carbohydrate moiety. Moreover, R^5 is

5   selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and $X-(CH_2)_r-$, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3.

It should be understood that in the most intriguing embodiments, the cyclic carbamate represent a di-, tri- or oligosaccharide, i.e. at least one of R^3, R^4 and

10   X represents a carbohydrate moiety.

As examples of interesting variants of the cyclic carbamate compounds can be mentioned compounds having the cyclic carbamate moiety at the 1,2-N,O-position of the reducing end of the di- and oligosaccharides (**4a**), (**4b**), and (**4c**):

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(**4a**)

(**4b**)

(**4c**)
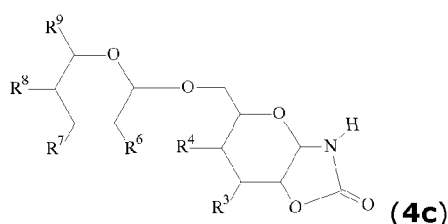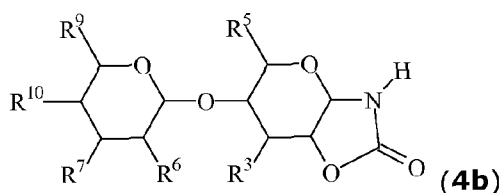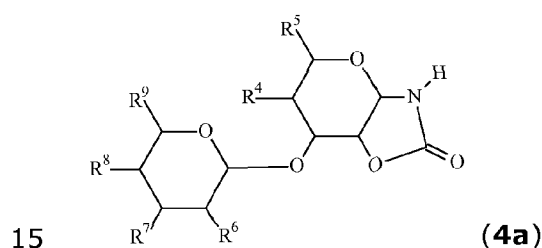
wherein R^6 and R^7 are as defined for R^3 and R^4 above, R^9 is as defined for R^5 above, and R^8 and R^{10} are independently selected from the group consisting of hydroxyl, C_{1-6}-alkoxy, C_{2-20}-acyloxy, acetamido, and a carbohydrate moiety.

The term "C_{1-6}-alkoxy" means "C_{1-6}-alkyl-oxy", where "C_{1-6}-alkyl" is intended to

5   mean a linear or branched hydrocarbon group having 1 to 6 carbon atoms, such as methoxy, ethoxy, propyloxy, *iso*-propyloxy, butyloxy, pentyloxy, and hexyloxy.

The term "C_{2-20}-acyloxy" means "C_{1-19}-alkyl-C(=O)-O-", where "C_{1-19}-alkyl" is intended to mean a linear or branched hydrocarbon group having 1 to 19 carbon

10   atoms, such as acetyloxy, ethylcarbonyloxy, propylcarbonyloxy, *iso*-propylcarbonyloxy, butylcarbonyloxy, pentylcarbonyloxy, octylcarbonyloxy, etc., as well as unsaturated variants thereof, e.g. those where "C_{2-20}-acyloxy" has the meanings ""C_{1-19}-alkylene-C(=O)-O-", "C_{1-19}-alkyl-di-ene-C(=O)-O-", "C_{1-19}-alkyl-tri-ene-C(=O)-O-", "C_{1-19}-alkyl-tetra-ene-C(=O)-O-", "C_{1-19}-alkynyl-C(=O)-

15   O-", etc., etc.

Reaction between cyclic carbamate and peptide

The peptides relevant in the present context are those having at least one primary amino group which can undergo reaction with the cyclic carbamate. It will be appreciated that some peptides include several primary amines, e.g. side

20   chain primary amines originating from lysine amino acid units and N-terminal primary amines originating from various amino acids (excluding, however, proline).

The step of reacting the cyclic carbamate (**4**) with the peptide involves contacting of the species under conditions which will facilitate reaction between

25   one or more primary amino groups and a corresponding number of cyclic carbamate molecules.

The ligation reaction can be illustrated as by the following reaction scheme where (**4**) represents the 1,2-N,O-cyclic carbamate (R^3, R^4 and R^5 defines as

above) and (**2**) represents one primary amino group (H₂N) of a peptide (E¹), and the resulting carbohydrate-peptide conjugate is represented by (**3**):
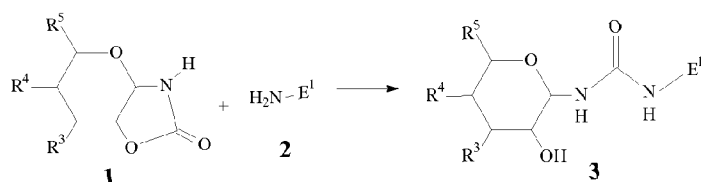
A ring-opening reaction of carbohydrate cyclic carbamates (**4**) with N-
5   nucleophiles of primary amines found in peptides/proteins, biological entities, etc. (**2**) is the key chemistry of the novel ligating process. The ligation becomes very powerful in cases, when cyclic carbamates (**4**) is characterized by trans-trans fused two-ring system. Such an extremely strained ring system prefers stabilization via ring-opening processes. The preparation of cyclic carbamates
10   such as (**4**) will be discussed later.

A chemoselective and site-selective novel ligation reaction of the corresponding cyclic carbamate of carbohydrates to chemical and biological entities expressing primary amino groups is typically carried out in either organic or aqueous solutions at temperatures ranges 0–40°C in acidic, neutral or basic reaction
15   conditions. Solvents including but not limited to methanol, water, ethanol, acetone, toluene, benzene, 1,4-dioxane, DMF, pyridine, etc and the mixtures of thereof can be used for such chemical transformations. Basic substances such as inorganic/organic bases – especially, N,N-diisopropylethylamine, triethylamine, etc - and salts of thereof might be preferred during the substitution in order to
20   control pH, catalytic procedures and site-selectivities. Acidic substances such as inorganic/organic acids – preferably HCl, acetic acid, formic acid, etc – and salts of thereof such as NaH₂PO₄ could be used. The reaction time for the substitution typically varies from 3-24 hours depending on the structures of substrates, the set temperature and the cyclic carbamates of carbohydrates. A conjugated
25   product of glycoconjugate is typically obtained in yields ranging from 80 to 95%.

It is important to emphasize that site-selectivity between N-terminal and lysine side-chain substitution could easily maintained by pH adjustment and setting proper reaction conditions. Thus, ligation reactions carried out at pH 4 – 7 usually display excellent N-terminal selectivities. Conjugations at higher pH 8 –
5 10 provide predominantly lysine side-chain modified products. The novel ligation works in water at pH 7 without the addition of any base/acid or any other activating agent. Furthermore, the resulted products have natural glycosylurea linkages, which cannot be considered to be toxic or harmful for living systems.

Hence, in one embodiment of the method according to the present invention,
10 the reaction takes place in a polar solvent, such as water.

In some embodiments of the method according to the present invention, the reaction takes place at a pH of in the range of 6.5-10.5. Alternatively, the reaction takes place at a pH in the range of 4.0-7.0 in order to facilitate selective ligation at the N-terminal, or at a pH in the range of 8.0-10.0 in order to
15 facilitate selective ligation at lysine side chains.

The method is applicable for any type of peptide, e.g. single chained peptides, multiple-chain peptides, folded peptides, aggregated peptides, cell-surface bound proteins, cell-membrane bound proteins, etc.

In one particularly interesting embodiment, the peptide is a cell-surface or cell-
20 membrane bound protein.

The method gives rise to a plethora of novel carbohydrate-peptide conjugates, preferably including those defined and described further below (see "Novel carbohydrate-peptide conjugates").

Novel carbohydrate-peptide conjugates

25 One class of novel carbohydrate-peptide conjugates are those defined by General Formula 1, in which one or more a carbohydrate moiety is linked *via* its

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glycosidic position to the ε-amino functions of lysine residues of peptides/proteins *via* a carbonyl linker.

Hence, the invention also relates to a carbohydrate-peptide conjugate comprising one or more moieties of the General Formula **1**:
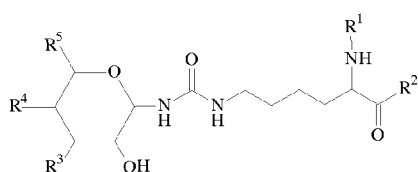
5   **General Formula 1.**

wherein

R^1 and R^2 together with the intervening lysine moiety represent a peptide moiety;

R^3 and R^4 are independently selected from the group consisting of hydroxyl,
10   acetamido, and a carbohydrate moiety;

R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and X-(CH$_2$)$_r$-, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3;

and pharmaceutically acceptable salts thereof.

15   Examples hereof are carbohydrate-peptide conjugates comprising one or more moieties of any of the General Formulae **1a**, **1b** and **1c**,
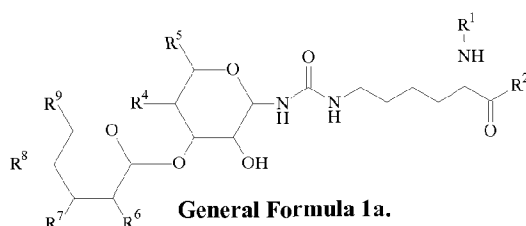
General Formula 1a.
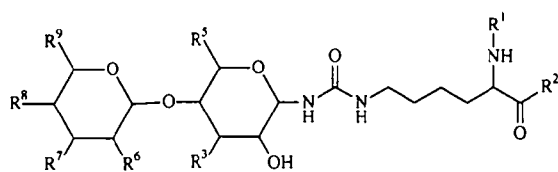
General Formula 1b.
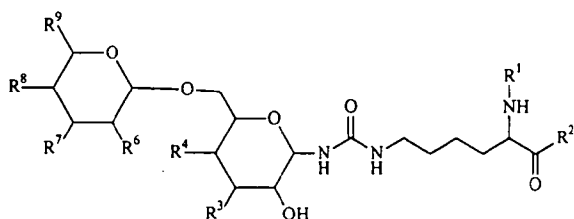
General Formula 1c.

wherein R^6 and R^7 are as defined for R^3 and R^4 above, and R^9 is as defined for R^5 above, and R^8 is selected from the group consisting of hydroxyl, C_{1-6}-alkoxy,

5   C_{2-20}-acyloxy, acetamido, and a carbohydrate moiety.

Another class of novel carbohydrate-peptide conjugates are those defined by General Formula **2**, in which a carbohydrate moiety is linked *via* its glycosidic position to the N-terminal amino function of peptides/proteins *via* a carbonyl linker.

10   Hence, the invention also relates to a carbohydrate-peptide conjugate comprising one or more moieties of the General Formula **2**:

General Formula 2.

wherein

R^{11} is an amino acid side chain;

R^{12} together with $-NH-CHR^{11}-C(=O)-$ represents a peptide moiety having a total number of amino acid units of at least 30;

R^3 and R^4 are independently selected from the group consisting of hydroxyl, acetamido, and a carbohydrate moiety;

5 R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and $X-(CH_2)_r-$, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3;

and pharmaceutically acceptable salts thereof.

Examples hereof are carbohydrate-peptide conjugates comprising one or more
10 moieties of any of the General Formulae **2a**, **2b** and **2c**,

General Formula 2a.

General Formula 2b.

General Formula 2c.
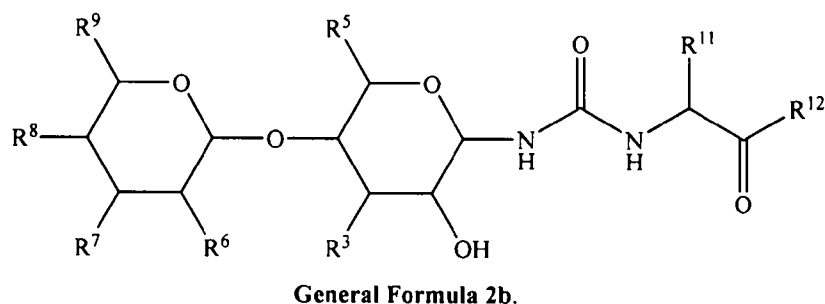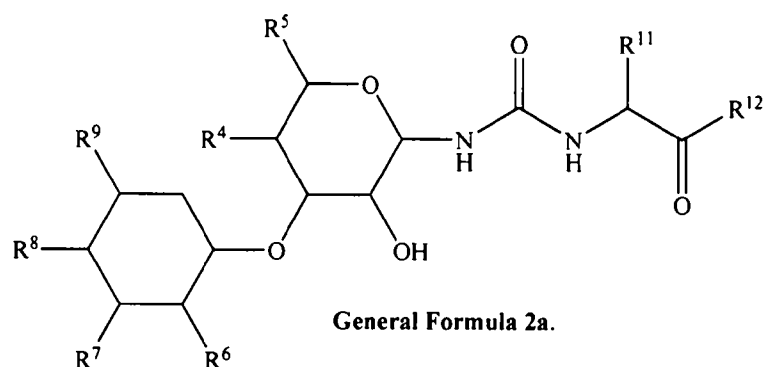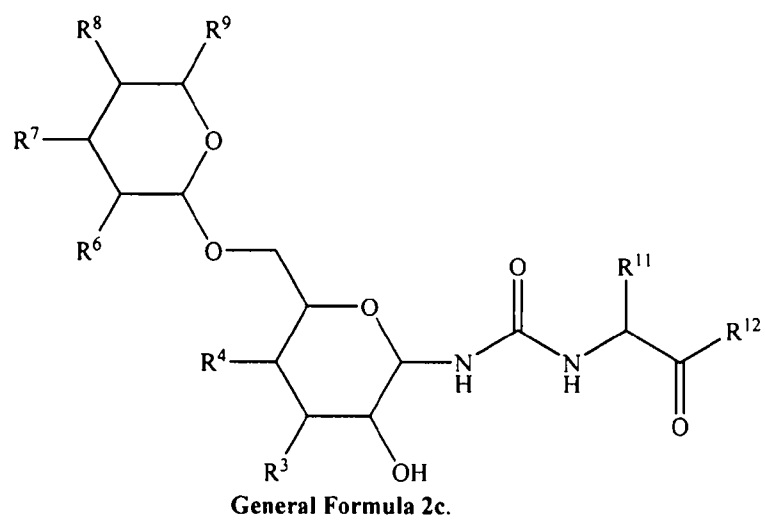
wherein R^6 and R^7 are as defined for R^3 and R^4 above, R^9 is as defined for R^5 above, and R^8 is selected from the group consisting of hydroxyl, C_{1-6}-alkoxy, C_{2-20}-acyloxy, acetamido, and a carbohydrate moiety.

It should be understood that the above classes of carbohydrate-peptide
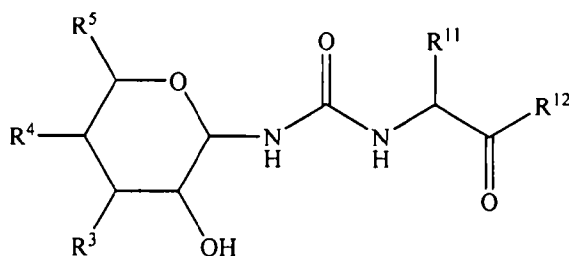5   conjugates represented by General Formula 1 and General Formula 2 are partly overlapping in that it can readily be envisaged that one or more carbohydrate moieties are linked *via* their glycosidic position to the ε-amino functions of lysine residues of the peptides *via* a carbonyl linker and that – within the same peptide - a carbohydrate moiety is linked *via* its glycosidic position to the N-terminal
10   amino function of peptides *via* a carbonyl linker. The peptide may – if consisting of two or more chains – even have two or more N-terminal linked carbohydrate moieties.

In some intriguing embodiments, the peptide is a cell-surface or cell-membrane bound protein.

15   The term "amino acid side chain" is intended to refer to the side chain group of amino acids typically included in peptides (including synthetic peptides) and is not restricted to the around 20 essential amino acids. Examples of amino acid side chains are hydrogen (representing glycine), methyl (alanine), 2-propyl (valine), 2-methyl-1-propyl (leucine), 2-butyl (isoleucine), methylthioethyl
20   (methionine), benzyl (phenylalanine), 3-indolylmethyl (tryptophan), hydroxymethyl (serine), 1-hydroxyethyl (threonine), mercaptomethyl (cysteine), 4-hydroxybenzyl (tyrosine), aminocarbonylmethyl (asparagine), 2-aminocarbonylethyl (glutamine), carboxymethyl (aspartic acid), 2-carboxyethyl (glutamic acid), 4-amino-1-butyl (lysine), 3-guanidino-1-propyl (arginine), and
25   4-imidazolylmethyl (histidine).

The term "pharmaceutically acceptable salts" is intended to include acid addition salts and basic salts. Illustrative examples of acid addition salts are pharmaceutically acceptable salts formed with non-toxic acids. Exemplary of such organic salts are those with maleic, fumaric, benzoic, ascorbic, succinic,
30   oxalic, bis-methylenesalicylic, methanesulfonic, ethanedisulfonic, acetic,

propionic, tartaric, salicylic, citric, gluconic, lactic, malic, mandelic, cinnamic,
citraconic, aspartic, stearic, palmitic, itaconic, glycolic, p-aminobenzoic,
glutamic, benzenesulfonic, and theophylline acetic acids, as well as the
8-halotheophyllines, for example 8-bromotheophylline. Exemplary of such

5   inorganic salts are those with hydrochloric, hydrobromic, sulfuric, sulfamic,
phosphoric, and nitric acids. Examples of basic salts are salts where the
(remaining) counter ion is selected from alkali metals, such as sodium and
potassium, alkaline earth metals, such as calcium, and ammonium ions
($^+N(R)_3R'$, where R and R' independently designates optionally substituted C_{1-6}-

10   alkyl, optionally substituted C_{2-20}-alkenyl, optionally substituted aryl, or
optionally substituted heteroaryl). Pharmaceutically acceptable salts are, e.g.,
those described in Remington's Pharmaceutical Sciences, 17. Ed. Alfonso R.
Gennaro (Ed.), Mack Publishing Company, Easton, PA, U.S.A., 1985 and more
recent editions and in Encyclopedia of Pharmaceutical Technology. Thus, the

15   term "an acid addition salt or a basic salt thereof" used herein is intended to
comprise such salts. Furthermore, the compounds as well as any intermediates
or starting materials may also be present in hydrate form.

Moreover, it should be understood that the compounds may be present as
racemic mixtures or the individual stereoisomers such as enantiomers or

20   diastereomers. The present invention encompasses each and every of such
possible stereoisomers (e.g. enantiomers and diastereomers) as well as
racemates and mixtures enriched with respect to one of the possible
stereoisomers.

Preparation of cyclic carbamates

25   The preparation of cyclic carbamate derivatives (**4**) (and (**4a**), (**4b**) and (**4c**))
and is based on the treatment of the corresponding glycosyl azide or the
corresponding other azido-deoxy-oligosaccharide derivative with carbondioxide
in the presence of trialkyl/aryl phosphines. Typically the reaction is carried out in
anhydrous organic solutions at temperatures ranges 0–40°C in neutral reaction

30   conditions. Solvents including but not limited to acetone, toluene, benzene, 1,4-

16

dioxane, DMF, tetrahydrofurane, etc and the mixtures of thereof can be used for such chemical transformation. The reaction time for the cyclic carbamate formation typically varies from 3-24 hours depending on the structures of substrates, the set temperature and the nature of trialkyl/arylphosphines used.

5   The cyclic carbamate products are typically obtained in high yields of 80 to 95%.

Alternatively, the preparation of cyclic carbamate derivatives (**4**) (and (**4a**), (**4b**) and (**4c**)) is based on the treatment of the corresponding glycosyl azide or the corresponding other azido-deoxy-oligosaccharide derivative with trialkyl/aryl phosphines involved in the isolation of phosphinimine derivatives. In a second

10   step, the phosphinimine derivatives are reacted with carbon dioxide providing the desired cyclic carbamates of oligosaccharides. Typically the reaction is carried out in anhydrous organic solutions at temperatures ranges 0–40°C in neutral reaction conditions. Solvents including but not limited to acetone, toluene, benzene, 1,4-dioxane, DMF, tetrahydrofurane, etc and the mixtures of

15   thereof can be used for such chemical transformation. The reaction time for the phosphinimine formation is 3 – 10 hours depending on the nature of trialkyl/arylphosphines used. The cyclic carbamate formation typically varies from 3-24 hours depending on the structures of substrates, the set temperature. The cyclic carbamate products are typically obtained in high yields of 80 to 95%.

20   Alternatively, the preparation of cyclic carbamate derivatives (**4**) (and (**4a**), (**4b**) and (**4c**)) involves the treatment of the corresponding glycosyl amine or other amino-deoxy-oligosaccharide with phosgene or other suitable phosgene derivatives such as diphosgene or triphosgene. Typically the reaction is carried out in organic or aqueous solutions at temperatures ranges 0–40°C in neutral or

25   basic reaction conditions. Solvents including but not limited to water, acetone, toluene, benzene, 1,4-dioxane, DMF, tetrahydrofurane, pyridine, etc and the mixtures of thereof can be used for such chemical transformation. The cyclic carbamate formation typically varies from 3-24 hours depending on the structures of substrates and the set temperature. The cyclic carbamate products

30   are typically obtained in high yields of 80 to 95%.

Alternatively, the preparation of cyclic carbamate derivatives (**4**) (and (**4a**), (**4b**) and (**4c**)) is based on the treatment of the corresponding acyclic carbamate derivative with base such as sodium hydride or DBU initiating an intramolecular ring-closing procedure. Typically the reaction is carried out in

5   anhydrous organic solutions at temperatures ranges 0–40°C in basic reaction conditions. Solvents including but not limited to toluene, benzene, 1,4-dioxane, DMF, tetrahydrofurane, etc and the mixtures of thereof can be used for such chemical transformation. The cyclic carbamate formation typically varies from 3-24 hours depending on the structures of substrates and the set temperature.

10 The cyclic carbamate products are typically obtained in high yields of 80 to 95%.

It is important to emphasize that the cyclic carbamate derivatives (**4**) (and (**4a**), (**4b**) and (**4c**)) show excellent stabilities in several reaction conditions allowing extensive chemical derivatisation such as O-acylation, O-alkylation, cyclic acetal, cyclic ketal formation, hydrogenolysis of the unprotected derivatives. Such a

15 unique option expands the utilities of cyclic carbamate ligating probes and could be used to ligate numerous derivatisation entities to peptides/proteins, biological and complex chemical entities.

It is believed that the cyclic carbamates of oligosaccharides (**4a**), (**4b**) and (**4c**), i.e.

20
(**4a**)

(**4b**)

18

(4c)
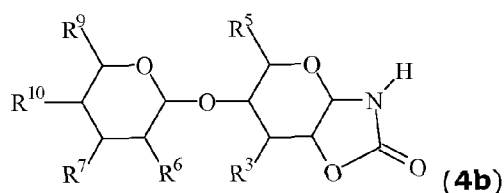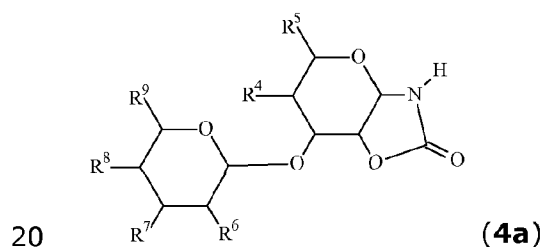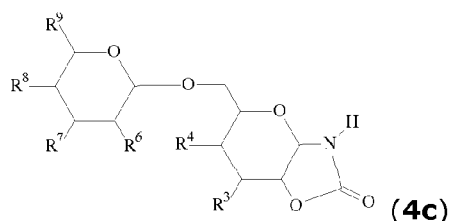
wherein R^6 and R^7 are as defined for R^3 and R^4 above, and R^9 is as defined for R^5 above. R^8 and R^{10} are independently selected from the group consisting of hydroxyl, C_{1-6}-alkoxy, C_{1-6}-acyloxy, acetamido, and a carbohydrate moiety,

5   are novel as such, and therefore the present invention also relates to such entities, which are useful, i.a. as intermediates in the preparation of the compounds of General Formulae **2a**, **2b**, **2c**, **3a**, **3b**, and **3c**.

Uses of the carbohydrate-peptide conjugates

Generally, the use of a carbohydrate-peptide conjugate as defined herein as a
10   pharmaceutical, a diagnostic agent, or in a diagnostic kit.

In particular, the carbohydrate-peptide conjugates defined and described herein including those prepared according to the method defined and described herein are believed to be offer a plethora of possibilities within medicine.

In one variant, the glycosyl moiety/moieties (the carbohydrate moiety) of the
15   carbohydrate-peptide conjugate represent(s) a non-immunogenic carbohydrate.

In one preferred embodiment several non-immunogenic carbohydrates such as dextranes, maltodextrines, maltose, cellobiose per-O-methylated oligosaccharides, thio-linked oligosaccharides could be conjugated to therapeutic peptide/protein in order to increase the half-life of those and providing beneficial
20   physical, biological and physiological properties such as increased solubility, thermal and enzymatic stability, etc.

In another variant, the glycosyl moiety (the carbohydrate moiety) of the carbohydrate-peptide conjugate represents an immunogenic carbohydrate.

Within this variant, several immunogenic carbohydrates like ABO blood antigens, Lewis type antigens, tumor specific antigens, α-Gal-epitopes, alpha-mannosyl-epitopes, polysialyc acid can be conjugated to:

5

- Peptides/proteins in order to down-regulate the specific peptide/protein by active vaccination.

- Viruses (living, inactivated or virus particles) like HIV, Hepatitis B, Herpes, Flu, Bird flu, etc. in order to prepare vaccines against the infections.

10

- Bacteria like Mycobacterium, Heliobacter, etc. in order to prepare antibacterial vaccines.

- Tumor cells in order to modify immunological properties, and to generate a strong immune response via autologous cancer vaccination.

15

- Cancer cell membranes in order to prepare autologous tumor vaccines.

In a further embodiment lipophilic oligosaccharides could be used to increase the stability of the peptide/protein by adhesion to albumin.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

20

EXAMPLES

Part 1: Ligation

Example 1:

Maltosyl cyclic carbamate **1** (40 mg) and BSA (bovine serum albumin, 50 mg) **2**
were dissolved in water (5 mL) (see Figure 1). The pH was adjusted to pH≅9.44
by the addition of triethylamine and acetic acid. The mixture was kept for 4 h at

20

room temperature, and then the reaction mixture was transferred into a dialysis membrane and dialyzed against distillated water, for 2 days, then liophylized obtaining **3** as a white powder.

Mass spectrometry: MALDI-TOF: Glycosylated BSA 68333, BSA ref: 66134.

5   *Example 2:*

Maltosyl cyclic carbamate **1** (40 mg) and BSA (bovine serum albumin, 50 mg) **2** were dissolved in water (5 mL) (see Figure 1). The pH was adjusted to pH≅8.57 by the addition of triethylamine and acetic acid. The mixture was kept for 4 h at room temperature, and then the reaction mixture was transferred into a dialysis

10   membrane and dialyzed against distillated water, for 2 days, then liophylized obtaining **3** as a white powder.

Mass spectrometry: MALDI-TOF: Glycosylated BSA 69099, BSA ref: 66134.

Example 3:

Maltosyl cyclic carbamate **1** (40 mg) and BSA (bovine serum albumin, 50 mg) **2**

15   were dissolved in water (5 mL) (see Figure 1). The pH was adjusted to pH≅8.10 by the addition of triethylamine and acetic acid. The mixture was kept for 4 h at room temperature. The reaction mixture was transferred into a dialysis membrane and dialyzed against distillated water, for 2 days, then liophylized obtaining **3** as a white powder.

20   Mass spectrometry: MALDI-TOF: Glycosylated BSA 68329, BSA ref: 66134.

Example 4:

Maltosyl cyclic carbamate **1** (40 mg) and BSA (bovine serum albumin, 50 mg) **2** were dissolved in water (5 mL) (see Figure 1). The pH was adjusted to pH≅7.45 by the addition of triethylamine and acetic acid. The mixture was kept for 4 h at

21

room temperature. The reaction mixture was transferred into a dialysis membrane and dialyzed against distillated water, for 2 days, then liophylized obtaining **3** as a white powder.

Mass spectrometry: MALDI-TOF: Glycosylated BSA 67679, BSA ref: 66134.

5   *Example 5:*

Maltosyl cyclic carbamate **1** (10 mg) and human insulin **4** (50 mg) were dissolved in water (13 mL) (see Figure 2). The pH was adjusted to pH$\cong$10.00 by the addition of diisopropyl -ethylamine and aqueous NaH_2PO_4. The mixture was kept for 2.5 h at room temperature then the reaction mixture was lyophilized

10   providing B29 glycosylated insulin **5** in more than 90% site-selectivity.

Mass spectrometry: MALDI-TOF: B-29-Glycosylated Human Insulin: 6171, Human insulin: 5805.

Example 6:

Maltosyl cyclic carbamate **1** (10 mg) and human insulin **4** (50 mg) were

15   dissolved in water (13 mL) (see Figure 2). The pH was adjusted to pH$\cong$8.00 by the addition of diisopropyl-ethylamine and aq. NaH_2PO_4. The mixture was kept for 2.5 h at room temperature then the reaction mixture was lyophilized affording the modified insulin (**5**).

Mass spectrometry: MALDI-TOF: Glycosylated Human Insulin: 6171, Human

20   insulin: 5804.

Example 7:

Maltosyl cyclic carbamate **1** (10 mg) and human insulin **4** (50 mg) were dissolved in water (13 mL) (see Figure 2). The pH was adjusted to pH$\cong$7.00 by the addition of diisopropyl -ethylamine and aq NaH_2PO_4. The mixture was kept

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for 2.5 h at room temperature then the reaction mixture was lyophilized providing B-1 Glycosylated Human Insulin **5** in more than 90% site-selectivity.

Mass spectrometry: MALDI-TOF: B-1 Glycosylated Human Insulin 6171, Human insulin: 5804.

5   *Example 8:*

α-Gal epitope-cyclic carbamate **6** (disaccharide) (25 mg) and human breast cancer-cell line **7** (2×10^6 number cell) were mixed in PBS buffer (5 mL) (see Figure 3). The mixture was kept for 3 h at 37 °C, and then the mixture was subjected to centrifugation to separate the cells from the medium.

10   The modified cells were treated with fluorescent labeled-lectin and the modification has been proven by flow-cytometry.

Example 9:

α-Gal epitope-carbamate **6** (disaccharide) (25 mg) and human breast cancer-cell line **7** (1×10^6 number cell) were mixed in PBS buffer (5 mL) (see Figure 3). The

15   mixture was kept for 3 h at 37 °C, and then the mixture was subjected to centrifugation to separate the cells from the medium.

The modified cells were treated with fluorescent labeled-lectin and the modification has been proven by flow-cytometry.

Example 10:

20   α-Gal epitope-carbamate **6** (disaccharide) (25 mg) and human breast cancer-cell line **7** (5×10^5 number cell) were mixed in PBS buffer (5 mL) (see Figure 3). The mixture was kept for 3 h at 37 °C, and then the mixture was subjected to centrifugation to separate the cells from the medium.

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The modified cells were treated with fluorescent labeled-lectin and the modification has been proven by flow-cytometry.

From Example 11 to Example 15 Compound **9** referred as "trisaccharide probe". Reference is made to Figure 4. The marked carbon atom labeled with C13

5   isotope in Example 14 and 15.

Example 11:

Conjugation of trisaccharide probe to B16BL6 cell line

<u>Cell preparation</u>

Two flasks (300 cm^2) (TPP, Trasadingen Switzerland) of confluent B16.BL6

10   melanoma cells grown in DMEM containing 10% fetal calf serum (without P/S) at
     37.5 ºC in 5% CO$_2$ were used for this experiment. Growth medium was removed
     from the cells and 25 mL of PBS (without Mg^{2+} and Ca^{2+}) (Gibco Invitrogen,
     Taastrup, Denmark) was added to the cells and immediately removed again.
     Another 10 mL of PBS was added and removed, before cells were harvested with

15   Cell Dissociation Solution C5914 (Sigma-aldrich, Saint Louis, Missouri) (3 mL per
     flask) and transferred to 10 mL tubes (TPP, Trasadingen Switzerland). The cells
     were centrifuged for 5 minutes at 300G at 20 ºC and the supernatant was
     discarded. Subsequently, cells were washed in PBS (without Mg^{2+} and Ca^{2+}),
     centrifuged as above and resuspended in PBS (without Mg^{2+} and Ca^{2+}). The

20   concentration of the cell suspension was determined.

<u>Conjugation of alpha-gal epitopes to B16.BL6 melanoma cells</u>

Five hundred mg of alpha-Gal trisaccharide-carbamate (9) was dissolved in PBS
(without Mg^{2+} and Ca^{2+}) (Gibco Invitrogen, Taastrup, Denmark) and passed
through a sterile filter (Sartorius Minisart ®, 0.20 my) (Sartorius, Goettingen,

25   Germany). Eight different concentrations of conjugate were prepared. Two mL of
     each conjugate dilution was added to a flask with 2 mL of PBS (without Mg^{2+} and

Ca^{2+}) and 600 µl of cell suspension containing 10^6 B16.BL6 cells. The final volume of the flasks was 4.6 mL. The flasks were numbered 1 to 10. Flasks 1 and 10 contained no conjugate and served as controls. The cells were incubated with the conjugate for 3 hours and 25 minutes at 37 °C and 5% CO_2.

5   After conjugation, cells from conjugation reaction 1 (non-conjugated cells), 2, 4, 6 and 8 were harvested with 10 mL of Cell Dissociation Solution C5914 (Sigma-Aldrich, Saint Louis, Missouri) and transferred to 10 mL tubes (TPP, Trasadingen Switzerland). Cells were centrifuged for 5 minutes at 300 G at 20 °C and resuspended in PBS (without Mg^{2+} and Ca^{2+}). Finally, cells were centrifuged as

10   before and resuspended in 100 µl TBS with 1% BSA. Cell concentration and viability was determined by microscopic evaluation of trypan blue stained cell samples. Cells from conjugation reaction 3, 5, 7, 9 and 10 (non-conjugated cells), were harvested the following day as described above. The cells were centrifuged once for 5 minutes at 300G at 20°C and resuspended in 100 µl TBS

15   with 1% BSA. Cell concentration and viability was determined by microscopic evaluation of trypan blue stained cell samples.

Staining of cell smears with FITC-labelled GS1B4

Smears of cells from conjugation reaction 1 (non-conjugated control cells), 2, 4, 6 and 8 were incubated for 1 hour at room temperature with FITC-labelled

20   GS1B4 diluted 1:200 in TBS. After a rinse in TBS, one drop of Fluorescent Mounting Medium (DAKO) was placed on each of the cell specimens and glass coverslips were placed on top. Binding of FITC-labelled GS1B4 to Galα1,3Gal on conjugated cells was assessed by fluorescence microscopy.

Flow cytometry analysis

25   B16.BL6 cells from conjugation reaction 1 (non-conjugated cells), 2, 4, 6 and 8 were analysed by flow cytometry. One hundred µL of cell suspension containing 1×10^5 cells were incubated for 15 minutes at 4°C with 10 µg per mL (1:100 dilution) of FITC-labelled GS1B4 in TBS containing 1% BSA. The proportion of cells stained, mean and median cell fluorescence intensity was determined by

25

flow cytometry. Afterwards, cells were washed once in PBS and measurements were repeated. Rabbit red blood cells (RRBC) known to express ~2 x 10^6 Galα1,3Gal epitopes per cell were used for comparison. The analyses of conjugated B16.BL6 cells and RRBCs were performed on different days.

5   Cell concentration and viability after conjugation

At the end of the conjugation procedure, both cell number and viability, assessed by trypan blue staining, were significantly reduced for all conjugation reactions. However, considerable variation between the different conjugation reactions was observed. A volume of 100 µl to 130 µl of cell suspension was
10   obtained from each conjugation reaction.

Table 1. Cell concentration and viability after conjugation

Reaction	Conjugate	Cell concentration	Cell viability
1	None	2.50 x 10^6 cells/mL	24.8 %
2	200 mg	3.50 x 10^6 cells/mL	58.9 %
3*	100 mg	3.08 x 10^6 cells/mL	65.6 %
4	50 mg	3.16 x 10^6 cells/mL	1.9 %
5*	20 mg	1.90 x 10^6 cells/mL	0.0 %
6	10 mg	2.26 x 10^6 cells/mL	16.8 %
7*	5 mg	2.02 x 10^6 cells/mL	34.7 %
8	2 mg	2.22 x 10^6 cells/mL	8.1 %
9*	1mg	2.12 x 10^6 cells/mL	16.0 %
10*	None	2.34 x 10^6 cells/mL	20.5 %

*) Cell concentration and viability measured 1 day after the conjugation procedure.

Table 2. Cell viability after conjugation
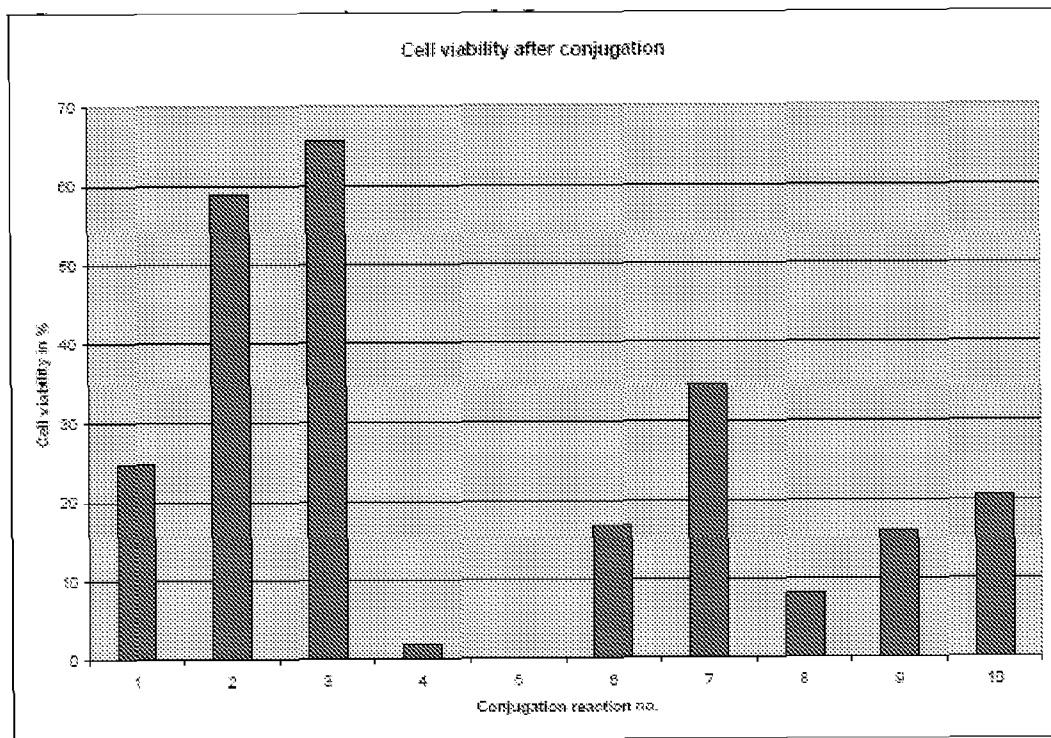
Staining of cell smears with FITC-labelled GS1B4

Galα1,3Gal epitopes, detected by binding of FITC-labelled GS1B4, were found on
cells from all conjugation reactions tested (conjugation reactions 2, 4, 6 and 8).
None of the non-conjugated cells (conjugation reaction 1) were stained.

Reference is made to Figures 11 and 12.

Flow cytometry

Galα1,3Gal epitopes, detected by binding of FITC-labelled GS1B4, were found on
B16.BL6 cells from all conjugation reactions tested (conjugation reactions 2, 6
and 8). None of the non-conjugated control cells (conjugation reaction 1) were
stained. The proportion of cells with detectable amounts of Galα1,3Gal-epitopes
was determined for each of the conjugation reactions.

Table 3. Cells with detectable amounts of Galα1,3Gal-epitopes

Conjugation reaction	Cells marked with FITC-GS1B4
0 (RRBC*)	94.23%
1 (non-conjugated cells)	0.00 %
2	95.82 %
6	62.17 %
8	56.82 %

Fluorescence intensity of cells with detectable quantities of Galα1,3Gal-epitopes on their surfaces was measured. The results strongly indicate, that the number of Galα1,3Gal-epitopes on the conjugated cells was considerably higher than the ~2 x 10^6 Galα1,3Gal epitopes known to be present on rabbit red blood cells. Mean and median values of cell fluorescence intensity for washed and unwashed cells are shown below.

Table 4. Fluorescence intensity, unwashed cells

Conjugation reaction	Mean fluorescence intensity	Median fluorescence intensity
0 (RRBC*)	38.17	36.52
1 (non-conjugated cells)	8.19	8.43
2	726.72	523.30
6	862.24	798.63
8	594.99	572.55

*) Rabbit red blood cells are known to express ~2 x 10^6 Galα1,3Gal epitopes per cell.

28

Visualisation of Galα1,3Gal epitopes on conjugated B16.BL6 melanoma cells from conjugation reaction 2. The cell membranes are heavily stained with FITC-labelled GS1B4 bound to Galα1,3Gal epitopes on the cell surfaces.

Table 5. Fluorescence intensity, washed cells

Conjugation reaction	Mean fluorescence intensity	Median fluorescence intensity
1 (non-conjugated cells)	14.9	3.68
2	447.48	321.97
6	439.04	406.79
8	264.30	259.45

5

Table 6. Fluorescence intensity, unwashed cells

Fluorescence intensity per cell

29
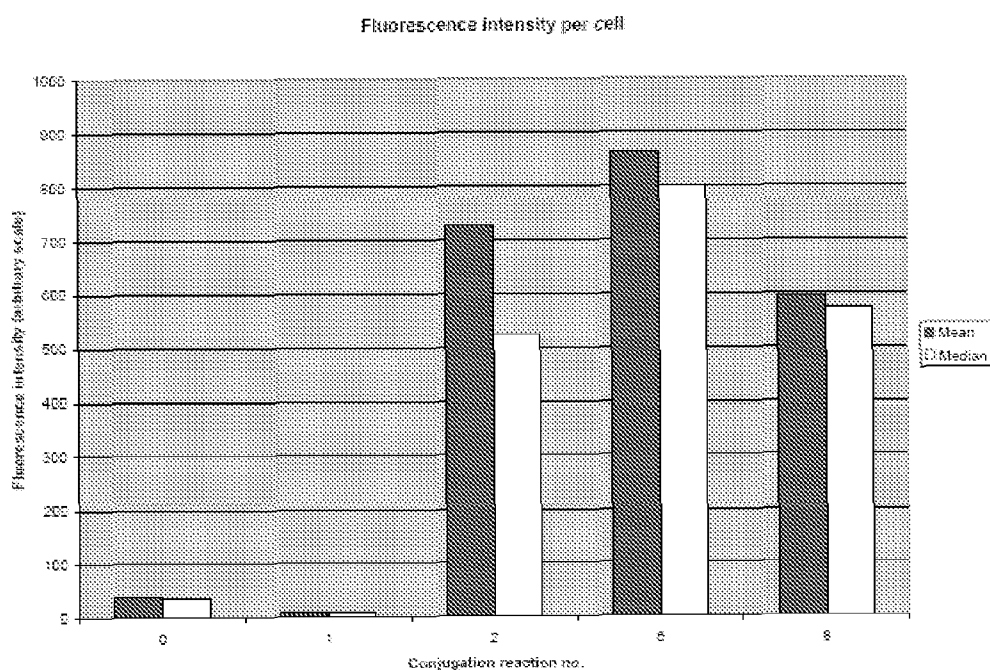
Example 12:

Conjugation of alpha-Gal trisaccharide-carbamate probe to B16BL6 cell line

Reduction of the conjugation time:

<u>Cell preparation</u>

5   See Example 11.

<u>Conjugation of alpha-gal epitopes to B16.BL6 melanoma cells</u>

The alpha-Gal trisaccharide-carbamate probe (9) was dissolved in both PBS
(without Mg $^{2+}$ and Ca $^{2+}$) (Gibco Invitrogen, Taastrup, Denmark) and HBSS (with
D-glucose) (Gibco Invitrogen, Taastrup, Denmark) and passed through sterile
10 filters (Sartorius Minisart ®, 0.20 my) (Sartorius, Goettingen, Germany). Three
different concentrations of the trisaccharide probe were prepared in both PBS
and HBSS.

Eight small flasks (25 cm^2) (TPP, Trasadingen Switzerland) were prepared, each
containing 10^6 B16.BL6 cells, and the trisaccharide probe was added. Total
15 volume of the flasks was 4.6 mL. The cells were incubated with the conjugate for
1 hour at 37 ºC and 5% CO$_2$.

Table 7. Conjugation reactions

Conjugation reaction	alpha-Gal trisaccharide-carbamate (9) added	Incubation buffer	Incubation time
P0	Control (no conjugate)	PBS	1 hour
PA	16 mg	PBS	1 hour
PB	2 mg	PBS	1 hour
PC	1 mg	PBS	1 hour
H0	Control (no conjugate)	HBSS (with D-glucose)	1 hour
HA	16 mg	HBSS (with D-glucose)	1 hour
HB	2 mg	HBSS (with D-glucose)	1 hour
HC	1 mg	HBSS (with D-glucose)	1 hour

After conjugation, the supernatant from each flask was transferred to separate
10 mL tubes (TPP, Trasadingen Switzerland). The cells were harvested with Cell
5   Dissociation Solution (2 mL per flask) (Sigma-aldrich, Saint Louis, Missouri) and
transferred to the 10 mL tubes. Cells were centrifuged for 5 minutes at 300 G at
20 ºC and resuspended in 0.5 mL of PBS or HBSS. Cell concentration and
viability was determined by microscopic evaluation of trypan blue stained cell
samples.

10 After the 1-hour incubation with the trisaccharide probe most of the cells were
viable and attached to the bottom surface of the flasks. However, it was very
difficult to harvest these cells and most of them were lost during the procedure.

Table 8. Cell viability

Conjugation reaction	Viability of cells harvested
P0	34.6 %
PA	14.3 %
PB	32.0 %
PC	37.5 %
H0	27.8 %
HA	33.3 %
HB	41.7 %
HC	41.6 %

Table 9. Cell viability
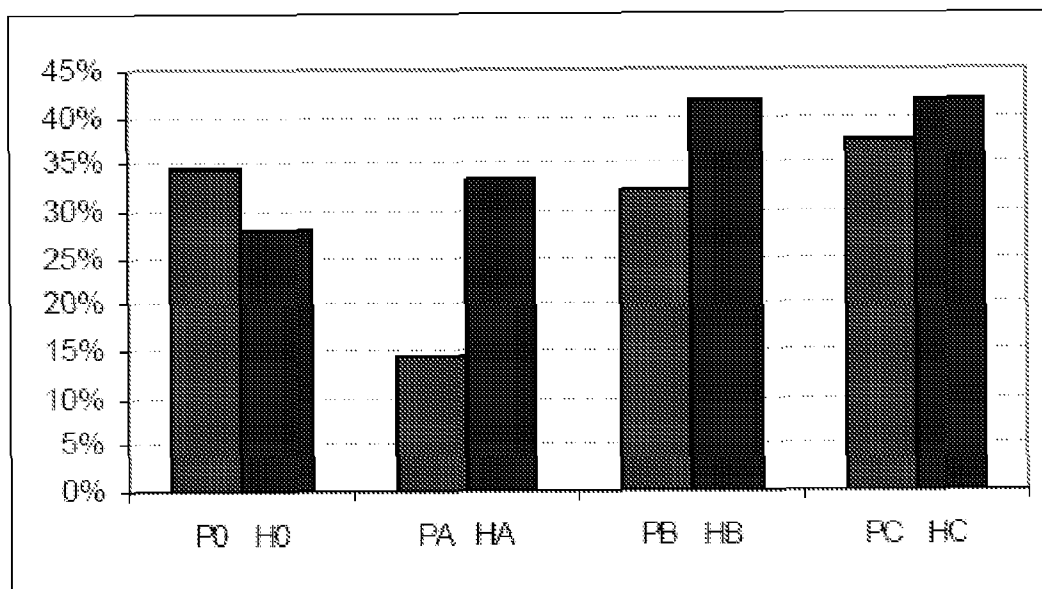
5   *Example 13:*

Conjugation of alpha-Gal trisaccharide-carbamate probe (9) to B16BL6 cell line

Reduction of the conjugation time:

Cell preparation

See Example 11.

Conjugation of alpha-gal epitopes to B16.BL6 melanoma cells

5   The alpha-Gal trisaccharide probe (9) was dissolved in HBSS (Gibco Invitrogen,
     Taastrup, Denmark) and passed through a sterile filter (0.2 μm Supor®
     Acrodisc® 13, Gelman Sciences, Ann Arbor, Michigan). Three different
     concentrations of the alpha-Gal trisaccharide-carbamate (9) were made. Five
     small flasks were prepared with 606 μL of cell suspension containing a total of
10   10^6 B16.BL6 cells in addition to 4 mL of one of the conjugate solutions. Each
     flask contained a total volume of 4.6 mL. The cells were incubated with the
     "Glycom conjugate" for either 3 or 1.5 hours at 37 °C.

Table 10. Conjugation reactions

Conjugation reaction	"Glycom conjugate"	Incubation time
1	19.2 mg	3 hours
2	19.2 mg	1.5 hours
3	1.9 mg	1.5 hours
4	58 mg	1.5 hours
5	Control (no conjugate)	1.5 hours

15   After conjugation, the supernatant from each flask was transferred to separate
     10 mL tubes (TPP, Trasadingen Switzerland). Cells were washed twice by adding
     PBS to the flasks and immediately removing it again. The cells were harvested
     with Cell Dissociation Solution (2 mL per flask) (Sigma-aldrich, Saint Louis,
     Missouri). After 5 minutes, cells were transferred to the 10 mL test tubes. The
20   cells were centrifuged for 5 minutes at 300 G at 20 °C and resuspended in 0.5

mL of PBS. Cell concentration and viability was determined by microscopic
evaluation of trypan blue stained cell samples.

Flow cytometry analysis

Cells from conjugation reaction 1:

5   One hundred µL of cell suspension containing 1 x 10^5 cells were incubated for 15
minutes at 4 °C with 10 µg per mL (1:100 dilution) of FITC-labelled GS1B4 in
PBS. Rabbit red blood cells (A) were used for comparison.

Cells from conjugation reaction 2, 3, 4 and 5:

As very few cells were available for flow cytometry analysis the exact cell
10   concentration was not determined. Instead, the amount of cells used was
estimated to 0.4 x 10^5. The cells were incubated for 15 minutes at 4°C with 4 µg
per mL of FITC-labelled GS1B4 in PBS. Rabbit red blood cells (B) were used for
comparison.

Mean and median cell fluorescence intensity was determined by flow cytometry.

15   Flow cytometry analysis

Galα1,3Gal epitopes, detected by binding of FITC-labelled GS1B4, were found on
B16.BL6 cells from all conjugation reactions. The analysis of cells from
conjugation reaction 2 and 4 showed two populations of cells with different cell
fluorescence intensities. The relatively high background staining indicated by the
20   fluorescense intensity values for the non-conjugated cells (conjugation reaction
5) could be a result of a large excess amount of FITC-labelled GS1B4 added due
to overestimation of the number of cells used.

34

Table 11. Fluorescence intensity, cells from conjugation reaction 1

Conjugation reaction	Mean fluorescence intensity	Median fluorescence intensity
RRBC (A)*	30.3	29.1
1	197.8	155.4

*) Rabbit red blood cells are known to express ~2 x 10^6 Galα1,3Gal epitopes per cell. 13

Table 12. Fluorescence intensity, cells from conjugation reaction 2, 3, 4 and 5

Conjugation reaction	Mean fluorescence intensity	Median fluorescence intensity
RRBC (B)*	25.9	23.7
2 (62,7% of the cells) **	161.63	125.2
2 (35.3% of the cells) **	2940.2	2196.8
3	167.8	66.4
4 (84,26% of the cells)	49.2	44.1
4 (15,65 % of the cells)	770.0	504.8
5 (non-conjugated cells)	39.56	32.2

*) Rabbit red blood cells are known to express ~2 x 10^6 Galα1,3Gal epitopes per cell.

**) Two groups of cells with different fluorescence intensities were observed from both conjugation reaction 2 and 4, and therefore, values are given for both groups.

Example 14:

Conjugation of the radioactive probe to B16BL6 cell line

The purpose of this experiment was to estimate the number of Galα1,3Gal
epitopes conjugated to B16.BL6 melanoma cells and to compare results obtained
5   by scintillation and flow cytometry.

Cell preparation

Nine flasks (300 cm^2)(TPP, Trasadingen Switzerland) of confluent B16.BL6
melanoma cells grown in DMEM containing 10% fetal calf serum (without P/S) at
37.5 ºC in 5% CO_2 were used for this experiment. Growth medium was removed
10   from the cells and 20 mL of HBSS containing D-glucose (without Mg^{2+} and Ca^{2+})
(Gibco, Invitrogen, Taastrup, Denmark) was added to the cells and immediately
removed again. Another 10 mL of HBSS containing D-glucose (without Mg^{2+} and
Ca^{2+}) was added and removed, before cells were harvested with Cell Dissociation
Solution C5914 (Sigma-aldrich, Saint Louis, Missouri)(10 mL per flask) and
15   transferred to 50 mL tubes (TPP, Trasadingen Switzerland). The cells were
centrifuged for 3 minutes at 300G at 20 ºC and the supernatant was discarded.
Subsequently, cells were washed twice in HBSS containing D-glucose (without
Mg^{2+} and Ca^{2+}), centrifuged as above and resuspended in HBSS containing D-
glucose (without Mg^{2+} and Ca^{2+}). The concentration of the cell suspension was
20   determined.

Conjugation of Galα1,3Gal epitopes to B16.BL6 melanoma cells

Radioactive labelled and regular trisaccharide probe was dissolved in HBSS
containing D-glucose (without Mg^{2+} and Ca^{2+}) (Gibco Invitrogen, Taastrup,
Denmark) and passed through sterile filters (0.2 μm Supor® Acrodisc® 13,
25   Gelman Sciences, Ann Arbor, Michigan). Four different concentrations of each
"Glycom conjugate" (25 mg, 5 mg, 1 mg and 0.1 mg) were made. Twelve flasks
(150 cm^2)(TPP, Trasadingen Switzerland) were prepared each containing a total
of 10 x 10^{6}B16.BL6 cells. The trisaccharide probe was added and the total

volume was adjusted to 20 mL with HBSS containing D-glucose (without

Mg^{2+} and Ca^{2+}). The cells were incubated with the "Glycom conjugate" for 1.5

hours at 37 °C. After conjugation, the supernatant from each flask was

transferred to separate 50 mL tubes (TPP, Trasadingen Switzerland). The cells

5   were harvested with Cell Dissociation Solution C5914 (5 mL per flask) (Sigma-

Aldrich, Saint Louis, Missouri and transferred to the 50 mL tubes containing the

supernatant. The cells were centrifuged for 3 minutes at 300G at 20 °C and

resuspended in 5 mL of PBS (without Mg^{2+} and Ca^{2+}). This was repeated 3 times

before cell concentration and viability was determined by microscopical

10   evaluation of trypan blue stained cell samples.

Scintillation

Cells conjugated with radioactive labelled trisaccaride probe were centrifuged for

5 minutes at 1000G at 20 °C. The supernatants were discharged and the

remaining pellets were dried. Subsequently, the pellets were dissolved in 400 µl

15   Mili-Q H_2O and 1.5 mL of scintillation fluid was added. Standards for the

scintillation were made by adding 500 pCi, 0.5 pCi and 0 pCi to the 3 samples of

control cells. The samples were stored at room temperature for 3 days, before

the cell suspensions were transferred to scintillation vials and counted.

Flow cytometry analysis

20   Cells conjugated with regular trisaccharide probe were analysed by flow

cytometry. Four hundred µl of each cell suspension containing 2 x 10^5 cells were

incubated for 15 minutes at 4 °C with 10 µg per mL of FITC-labelled GS1B4.

Mean fluorescence intensity of cells stained with GS1B4 was measured by flow

cytometry.

25   Cell concentration and viability after conjugation

During the harvesting procedure, two different fractions of cells with radioactive

labelled trisaccharide probe were accidentally combined and therefore had to be

discharged. Consequently, the results obtained from these conjugation reactions

are not comparable with the remaining. Additionally, an abnormal volume was
acquired for the suspension containing cells conjugated with 0.1 mg radioactive
labelled trisaccharide probe. As observed in the previous preliminary
experiments, the amount of cells harvested and cell viability varied both
5   between cells incubated with different concentrations of conjugate, and between
cells incubated with regular conjugate and radioactive labeled conjugate.

Table 13. Cells conjugated with regular conjugate

Conjugate added	Cell concentration*	Viability*	Cells harvested*
0 mg (control)	5.0×10^5	76.0%	2.5×10^6 (25%)
25 mg	9.0×10^5	77.8%	4.5×10^6 (45%)
5 mg	7.0×10^5	91.4%	3.5×10^6 (35%)
1 mg	13.4×10^5	79.1%	6.7×10^6 (67%)
0.1 mg	7.4×10^5	67.6%	3.7×10^6 (37%)

*) Estimated after alpha-gal conjugation

Table 14. Cells conjugated with radioactive labelled conjugate

Conjugate added	Cell concentration*	Viability*	Cells harvested*
0 mg (control 1)	13.8×10^5	87.0%	6.9×10^6 (69%)
0 mg (control 2)	12.6×10^5	90.5%	6.3×10^6 (63%)
0 mg (control 3)	11.4×10^5	87.8%	5.7×10^6 (57%)
25 mg**	2.0×10^5	90.0%	1.0×10^6 (10%)
5 mg***	10.8×10^5	81.5%	5.4×10^6 (54%)
1 mg	14.8×10^5	93.2%	7.4×10^6 (74%)
0.1 mg	13.8×10^5	91.3%	6.9×10^6 (69%)

10   *) Estimated after alpha-gal conjugation

**) Attached cells only

***) Cells from supernatant only

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Flow cytometry

As observed in the previous preliminary experiment, only a fraction of the
conjugated cells bound detectable amounts of FITC-labelled GS1B4. The mean
fluorescence intensity of cells conjugated with 1 mg was unexpectedly low,
5   whereas a positive correlation was observed between dose of conjugate and
mean fluorescence intensity for the remaining conjugation reactions.

Table 15. Mean fluorescence intensity

Conjugate added	Cells marked with GS1B4	Mean fluorescence intensity*
0 mg (control)		77.99
25 mg	31.86%	2460.67
5 mg	14.56%	1719.58
1 mg	2.36%	342.05
0.1 mg	17.64%	1025.86

*) Mean Fluorescence intensity of cells marked with GS1B4

Scintillation

10   A positive correlation was found between the scintillation counts and dose of
conjugate. However, due to the above mentioned errors made during the
harvesting procedure, the results were not comparable with the results obtained
from the flow cytometry analysis, and therefore an association between the two
quantification methods could not be established.

Table 16. Estimation of epitopes per cell

Conjugate added	Epitopes per cell
25 mg*	364.2×10^6
5 mg**	152.4×10^6
1 mg	28.3×10^6
0.1 mg	3.6×10^6

*) Attached cells only

**) Cells from supernatant only

Table 17. Epitopes per cell as a function of conjugate dose
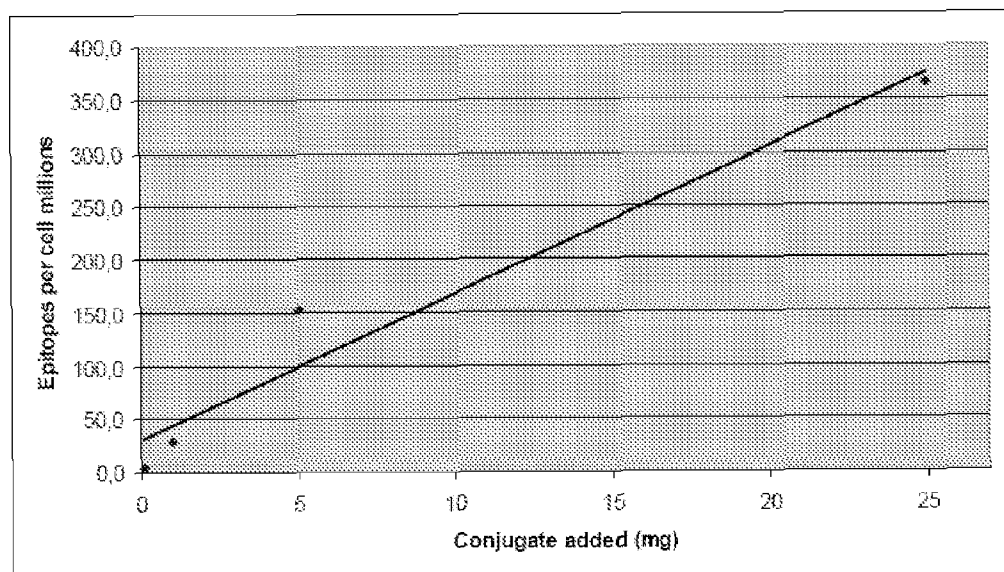
Example 15:

Conjugation of the radioactive probe to B16BL6 cell line

The purpose of this experiment was to estimate the number of Galα1,3Gal epitopes conjugated to B16.BL6 melanoma cells and to compare results obtained by scintillation and flow cytometry.

Cell preparation

Twelve flasks (300 cm^2)(TPP, Trasadingen Switzerland) of confluent B16.BL6
melanoma cells grown in DMEM containing 10% fetal calf serum (without P/S) at
37.5 °C in 5% CO$_2$was used for this experiment. Growth medium was removed

5   from the cells and 20 mL of HBSS containing D-glucose (without Mg^{2+} and Ca^{2+})
(Gibco, Invitrogen, Taastrup, Denmark) was added to the cells and immediately
removed again. Another 10 mL of HBSS containing D-glucose (without Mg^{2+} and
Ca^{2+}) was added and removed, before cells were harvested with Cell Dissociation
Solution C5914 (Sigma-aldrich, Saint Louis, Missouri)(10 mL per flask) and

10   transferred to 50 mL tubes (TPP, Trasadingen Switzerland). The cells were
centrifuged for 3 minutes at 300G at 20 °C and the supernatant was discarded.
Subsequently, cells were washed twice in HBSS containing D-glucose (without
Mg^{2+} and Ca^{2+}), centrifuged as above and resuspended in HBSS containing D-
glucose (without Mg^{2+} and Ca^{2+}). The concentration of the cell suspension was

15   determined.

Conjugation of alpha-gal epitopes to B16.BL6 melanoma cells

Radioactive labelled and regular alpha-Gal trisaccharide-carbamate (9) probe
(labeled on the cyclic carbamate carbon) was dissolved in HBSS containing D-
glucose (without Mg^{2+} and Ca^{2+}) (Gibco Invitrogen, Taastrup, Denmark) and

20   passed through sterile filters (0.2 μm Supor® Acrodisc® 13, Gelman Sciences,
Ann Arbor, Michigan). Eight different concentrations of radioactive labelled
alpha-Gal trisaccharide-carbamate (9) (1 μg, 10 μg, 100 μg, 1 mg, 5 mg, 10
mg, 15 mg and 20 mg) and 6 different concentrations of regular "Glycom
conjugate" (10 μg, 100 μg, 1 mg, 5 mg, 10 mg, 15 mg and 20 mg) were used.

25   Twelve flasks (300 cm^2) (TPP, Trasadingen Switzerland) were prepared, each
containing 10 x 10^6 B16.BL6 cells, for conjugtion with the radioactive labelled
trisaccharide probe, and 8 flasks (150 cm^2) (TPP, Trasadingen Switzerland) were
prepared, each containing 5 x 10^6 B16.BL6 cells, for conjugtion with the non-
radioactive conjugate. The Glycom conjugate was added and the total volume

30   was adjusted with HBSS containing D-glucose (without Mg^{2+}and Ca^{2+}) so that
each large flask (300 cm^2) contained a final volume of 20 mL and each medium

flask (150 cm^2) contained a final volume of 10 mL. Cells were incubated with the "Glycom conjugate" for 1.5 hours at 37 °C. After conjugation, the supernatant from each flask was transferred to separate 50 mL tubes (TPP, Trasadingen Switzerland). The cells were harvested with Cell Dissociation Solution (10 mL per

5   large flask and 5 mL per medium flask) (Sigma-aldrich, Saint Louis, Missouri) and transferred to the 50 mL tubes containing the supernatant. The cells were centrifuged for 5 minutes at 300G at 20 °C and resuspended in 5 mL of PBS (without Mg^{2+}and Ca^{2+}). This was repeated 3 times before cell concentration and viability was determined by microscopic evaluation of trypan blue stained cell

10   samples.

Scintillation

Cells incubated with radioactive labelled trisaccharide probe were centrifuged for 5 minutes at 1000G at 20 °C. The supernatant was discharged and the remaining pellets were dried. Subsequently, the pellets were dissolved in 450 μl

15   Mili-Q H$_2$O and 1.5 mL of scintillation fluid was added. Standards for the scintillation were made by adding 500 pCi, 0.5 pCi and 0 pCi to the 3 samples of control cells. The samples were stored at room temperature for 3 days, before the cell suspensions were transferred to scintillation vials and counted.

Flow cytometry analysis

20   Cells conjugated with regular trisaccharide probe were analysed by flow cytometry. Four hundred μl of each cell suspension containing 2 x 10^5 cells were incubated for 15 minutes at 4°C with 10 μg per mL of FITC-labelled GS1B4. Mean fluorescence intensity of cells stained with GS1B4 was measured by flow cytometry.

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Table 18. Cells conjugated with regular conjugate

Conjugation reaction	Conjugate added	Cell concentration*	Viability*	Cells harvested*
1	Control	3.4×10^5 (cells/mL)	94.1.0%	0.85×10^6 (17%)
2	20 mg	4.2×10^5 (cells/mL)	85.7 %	1.1×10^6 (21 %)
3	15 mg	6.0×10^5 (cells/mL)	80.0 %	1.5×10^6 (30%)
4	10 mg	2.8×10^5 (cells/mL)	71.4 %	0.7×10^6 (14%)
5	5 mg	4.4×10^5 (cells/mL)	81.8 %	1.1×10^6 (22%)
6	1 mg	4.2×10^5 (cells/mL)	66.7 %	1.1×10^6 (21%)
7	100 µg	6.0×10^5 (cells/mL)	60.1 %	1.5×10^6 (30%)
8	10 µg	4.2×10^5 (cells/mL)	57.1 %	1.1×10^6 (21%)

*) Determined after alpha-gal conjugation and after washing the cells once

Table 19. Cells conjugated with radioactive labelled conjugate

Conjugation reaction	Conjugate added	Cell concentration*	Viability*	Cells harvested*
A	Control 1	5.4×10^6 (cells/mL)	83.3 %	2.4×10^6 (24.3 %)
B	Control 2	5.7×10^6 (cells/mL)	86.3 %	2.6×10^6 (25.7 %)
C	20 mg	6.1×10^6 (cells/mL)	82.0 %	2.7×10^6 (27.5 %)
D	15 mg	4.3×10^6 (cells/mL)	88.9 %	1.9×10^6 (19.4 %)
E	10 mg	12×10^6 (cells/mL)	86.7 %	5.4×10^6 (54.0 %)
F	5 mg	5.2×10^6 (cells/mL)	82.7 %	2.3×10^6 (23.4 %)
G	1 mg	5.9×10^6 (cells/mL)	91.5 %	2.7×10^6 (26.6 %)
H	100 µg	3.8×10^6 (cells/mL)	81.6 %	1.7×10^6 (17.1 %)
1	10 µg	6.2×10^6 (cells/mL)	82.3 %	2.8×10^6 (27.9 %)
J	1 µg	5.0×10^6 (cells/mL)	84.0 %	2.3×10^6 (22.5 %)
K	Control 3	5.9×10^6 (cells/mL)	69.5 %	2.7×10^6 (26.6 %)
L	Control 4	4.6×10^6 (cells/mL)	56.5 %	2.1×10^6 (20.7 %)

*) Determined after alpha-gal conjugation and after washing the cells 4 times

<u>Flow cytometry</u>

Unexpectedly, no binding above background levels of FITC-labelled GS1B4 to cells from any of the conjugation reaction was detected. An additional amount of FITC-labelled GS1B4 was added to the cells and the measurements were

5   repeated. However, again no staining was found, and, therefore, successful alpha-gal conjugation could not be confirmed by the flow cytometry analysis.

<u>Scintillation</u>

Due to doubts on the counting of cells conjugated with 1 mg of radioactive labeled conjugate, the results obtained for these cells are omitted. For the

10   remaining conjugation reactions, a positive correlation was found between the scintillation counts and dose of conjugate. Due to the above mentioned lack of confirmation of successful alpha-gal conjugation by flow cytometry, an association between the two quantification methods could not be established.

Table 20. Cells conjugated with radioactive labelled conjugate

Conjugate added	Cells used for scintillation	Epitopes per cell
20 mg	2.4×10^6	515.2×10^6
15 mg	1.7×10^6	501.2×10^6
5 mg	2.3×10^6	175.3×10^6
1 mg	2.6×10^6	33.9×10^6
100 µg	1.7×10^6	6.8×10^6
10 µg	2.7×10^6	0.6×10^6
1 µg	2.2×10^6	1.0×10^6

15   *) Determined after alpha-gal conjugation and after washing the cells 4 times

Table 21. Epitopes per cell as a function of conjugate dose
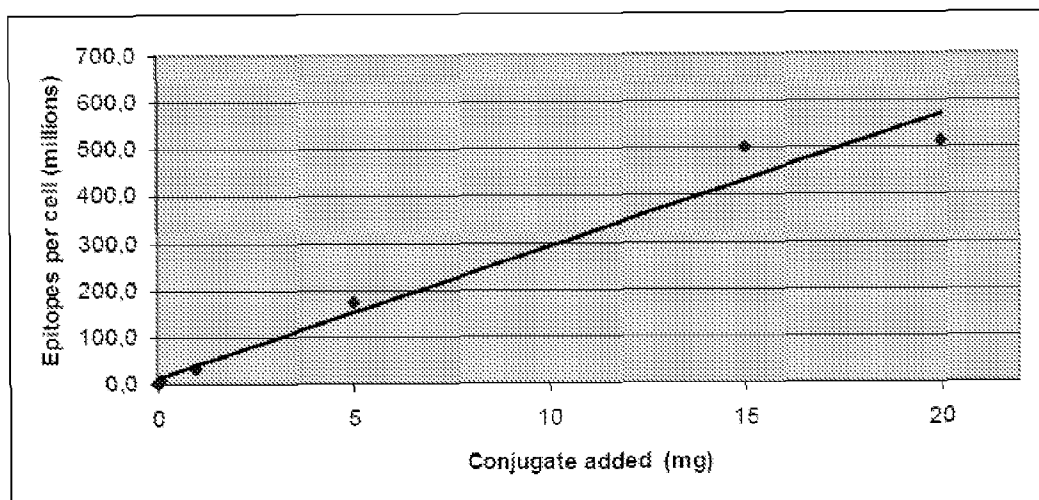
Part 2: Preparation of the cyclic carbamate containing carbohydrates

Example 16:

5   Lactosyl-azide **12** (1.5 g) was dissolved in dry DMF (40 mL) saturated with CO_2, then triphenyl phosphine (1.1 eq.) was added in dry DMF (5 mL) to the mixture over the period of 20 min (see Figure 5). CO_2 was bubbled through the mixture for 5 h, and the mixture was stirred for 8 h. The white precipitate that formed filtered and washed with cold acetone affording the desired product **13** (1 g).

10 *Example 17:*

Lactosyl-azide **12** (1.8 g) was dissolved in dry DCM (15 mL), and then triphenyl phosphin (1.1 eq.) was added to the mixture was stirred for 3 h (see Figure 6). Diethyl-ether (50 mL) was added and the white precipitate that formed filtered and washed with cold diethyl-ether affording the phosphinimine **16** (1.9 g).

15 CO_2 was bubbled through the solution of the phosphinimine derivative (1.9 g) in dry acetone (40 mL) at r.t, for 6 h. Then the formed white precipitate collected affording the desired product **13**.

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Example 18:

Triphosgene (1.1 eq.) was added to the solution of α-gal trisaccharide epitope
18 (1 g), in EtOAc and sat NaHCO$_3$, at 0 °C and the mixture was stirred
vigorously for 30 min (see Figure 7). Then the phases are separated, and the
5   organic phase collected, and concentrated. Column chromatography of the
residue afforded the product **19**.

Example 19:

Z-Cl (1.2 eq.) was added to the solution of lactosyl-amine **20** (1 g), in DMF in
the presence of DIPEA (1.3 eq.) and the mixture was stirred until TLC showed
10   the complete conversion into compound **21** (see Figure 8). Then NaOMe (1.3
eq.) was added and the mixture was stirred at room temperature. The reaction
mixture was neutralized with Amberlite IR 120 (H$^+$) and concentrated. Column
chromatography of the residue afforded the product **13**.

Example 20:

15   Acyl chloride (4 eq.) was added to a solution of glucosyl carbamate **22** (100 mg)
in DCM (3 mL) and pyridine (4 mL) then the mixture was stirred for overnight,
then concentrated and the residue chromatographed affording the product **23**
(see Figure 9).

Example 21:

20   PPh$_3$ (31.4 mg) in abs. DMF (0.2 mL) was added to a solution of trisaccharide-
azid **24** (50 mg) in abs. DMF (0.3 mL). The mixture stirred for 2 h under inert
atmosphere (see Figure 10). Then 5 mL cc H$_2$SO$_4$ added to BaCO$_3$ (labeled with
C13) (39.4 mg) and the evolved CO$_2$ transferred to the carbohydrate mixture.
And the mixture stirred at rt for 8 h. Then the mixture concentrated and
25   triturated with DCM (50 mL) and the product **25** isolated as white solid.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method for the preparation of a carbohydrate-peptide conjugate, said method comprising the step of reacting a cyclic carbamate (1)

wherein R^3 and R^4 are independently selected from the group consisting of hydroxyl, acetamido, and a carbohydrate moiety; and R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and X-(CH$_2$)$_r$-, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3;

with a peptide, said peptide comprising at least 30 amino acid units, and said peptide comprising at least one primary amino group.

2. The method according to claim 1, wherein the reaction takes place in a polar solvent, such as water.

3. The method according to any one of the preceding claims, wherein the reaction takes place at a pH of in the range of 6.5-10.5.

4. The method according to any one of the preceding claims, wherein the peptide is a cell-surface or cell-membrane bound protein.

5. The method according to any one of the preceding claims, wherein carbohydrate-peptide conjugate is a compound as defined in any one of the claims 6-13.

6. A carbohydrate-peptide conjugate obtainable by the method according to any one of the claims 1-5.

7. A carbohydrate-peptide conjugate comprising one or more moieties of the General Formula **1**:

General Formula 1.

5

wherein

R^1 and R^2 together with the intervening lysine moiety represent a peptide moiety comprising at least 30 amino acid units;

R^3 and R^4 are independently selected from the group consisting of hydroxyl,

10 acetamido, and a carbohydrate moiety;

R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and X-$(CH_2)_r$-, wherein X is a carbohydrate moiety and r is an integer selected from 0 and 1;

and pharmaceutically acceptable salts thereof.

15 8. The carbohydrate-peptide conjugate according to claim 7, which comprises one or more moieties of any of the General Formulae **1a**, **1b** and **1c**,
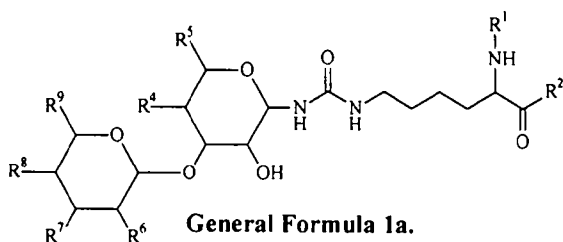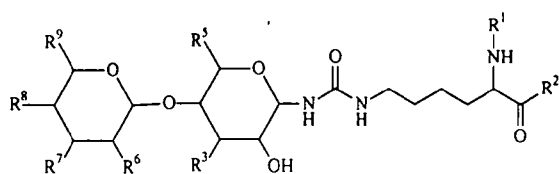
General Formula 1a.
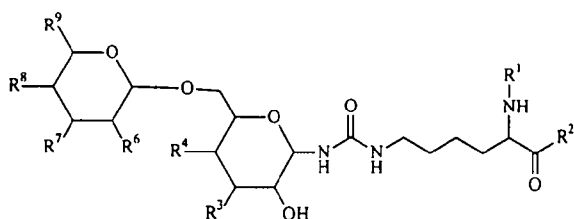
General Formula 1b.

General Formula 1c.

wherein R^6 and R^7 are as defined for R^3 and R^4 in claim 1, R^9 is as defined for R^5 in claim 1, R^8 is selected from the group consisting of hydroxyl, C_{1-6}-alkoxy,

5   C_{2-20}-acyloxy, acetamido, and a carbohydrate moiety.

9. A carbohydrate-peptide conjugate comprising one or more moieties of the General Formula **2**:
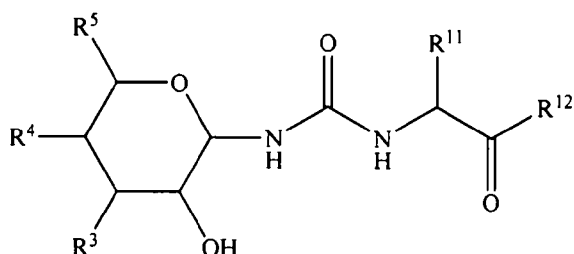
General Formula 2.

wherein

10   R^{11} is an amino acid side chain;

R^{12} together with –NH-CHR^{11}-C(=O)- represents a peptide moiety having a total number of amino acid units of at least 30;

R^3 and R^4 are independently selected from the group consisting of hydroxyl, acetamido, and a carbohydrate moiety;

R^5 is selected from the group consisting of hydrogen, methyl, hydroxymethyl, acetamidomethyl, carboxyl, and X-$(CH_2)_r$-, wherein X is a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3;

and pharmaceutically acceptable salts thereof.

5   10. The carbohydrate-peptide conjugate according to claim 9, which comprises one or more moieties of any of the General Formulae **2a**, **2b** and **2c**,

General Formula 2a.

General Formula 2b.

General Formula 2c.
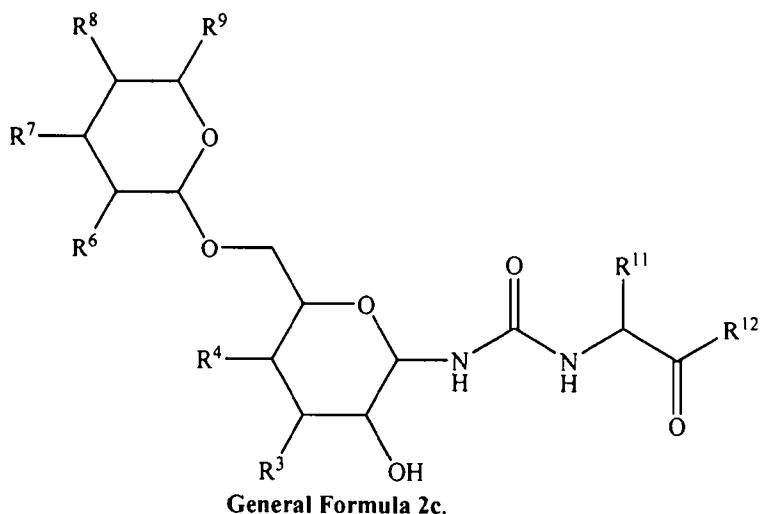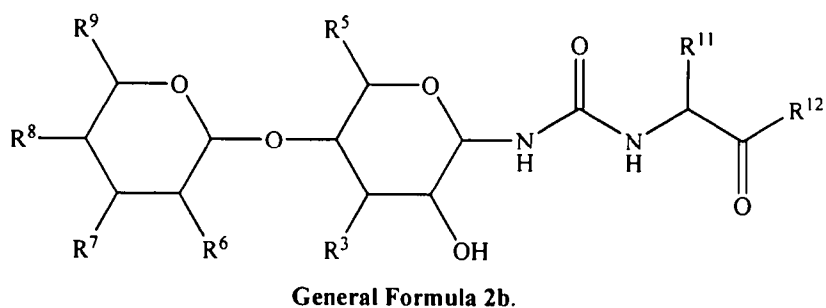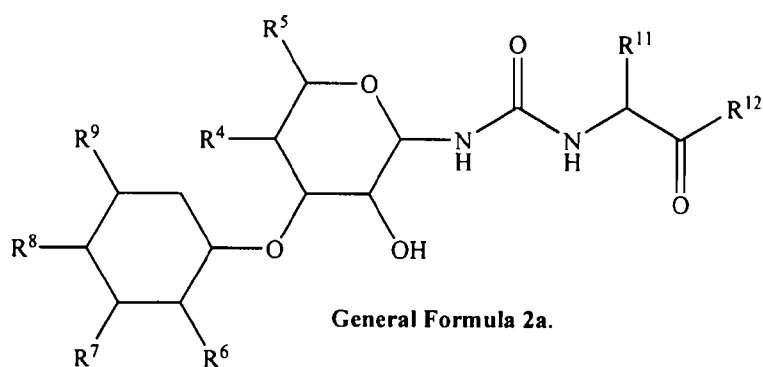
wherein R^6 and R^7 are as defined for R^3 and R^4 in claim 3, R^9 is as defined for R^5 in claim 9, and R^8 is selected from the group consisting of hydroxyl, C_{1-6}-alkoxy, C_{2-20}-acyloxy, acetamido, and a carbohydrate moiety.

11. The carbohydrate-peptide conjugate according to any one of the claims 6-10, wherein the glycosyl moiety (the carbohydrate moiety) represents a non-immunogenic carbohydrate.

12. The carbohydrate-peptide conjugate according to any one of the claims 6-10, wherein the glycosyl moiety (the carbohydrate moiety) represents an immunogenic carbohydrate.

13. The carbohydrate-peptide conjugate according to any one of the claims 6-12, wherein the peptide is a cell-surface or cell-membrane bound protein.

14. The carbohydrate-peptide conjugate as defined in any one of the claims 6-13, the peptide moiety has a total number of amino acid units of at least 100.

15. The carbohydrate-peptide conjugate as defined in any one of the claims 6-14 when used in medicine.

16. Use of a carbohydrate-peptide conjugate according to any one of the claims 6-14 as a pharmaceutical, a diagnostic agent, or in a diagnostic kit.

17. A cyclic carbamate of an oligosaccharide selected from (4a), (4b) and (4c)

(4a)

(4b)

(4c)
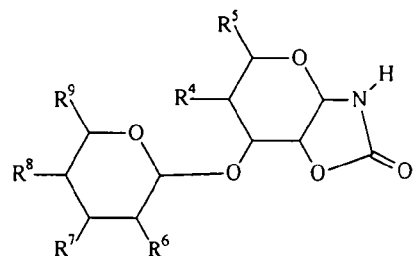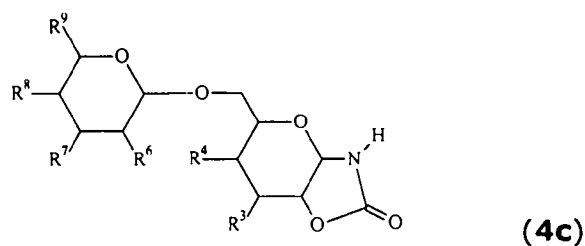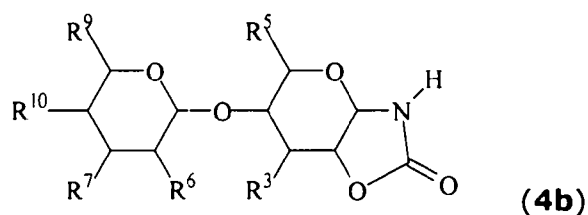
wherein

R^3, R^4, R^6 and R^7 are independently selected from the group consisting of
5   hydroxyl, acetamido, and a carbohydrate moiety;

R^5 and R^9 are independently selected from the group consisting of hydrogen,
methyl, hydroxymethyl, acetamidomethyl, carboxyl, and X-$(CH_2)_r$-, wherein X is
a carbohydrate moiety and r is an integer selected from 0, 1, 2 and 3; and

R^8 and R^{10} are independently selected from the group consisting of hydroxyl,
.0 C_{1-6}-alkoxy, C_{1-6}-acyloxy, acetamido, and a carbohydrate moiety;

and pharmaceutically acceptable salts thereof, with the proviso that the
compound is not (4-O-β-D-glucopyranosyl-1,2-dideoxy-β-D-glucopyranoso)[1,2-
d]oxazolidine-2-one or (4-O-β-D-galactopyranosyl-1,2-dideoxy-β-D-
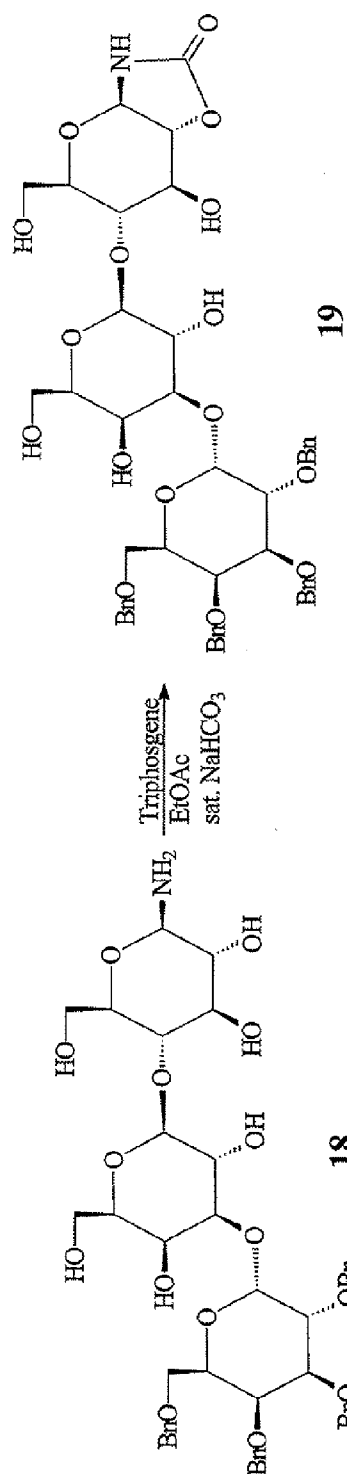glucopyranoso)[1,2-d]oxazolidine-2-one.
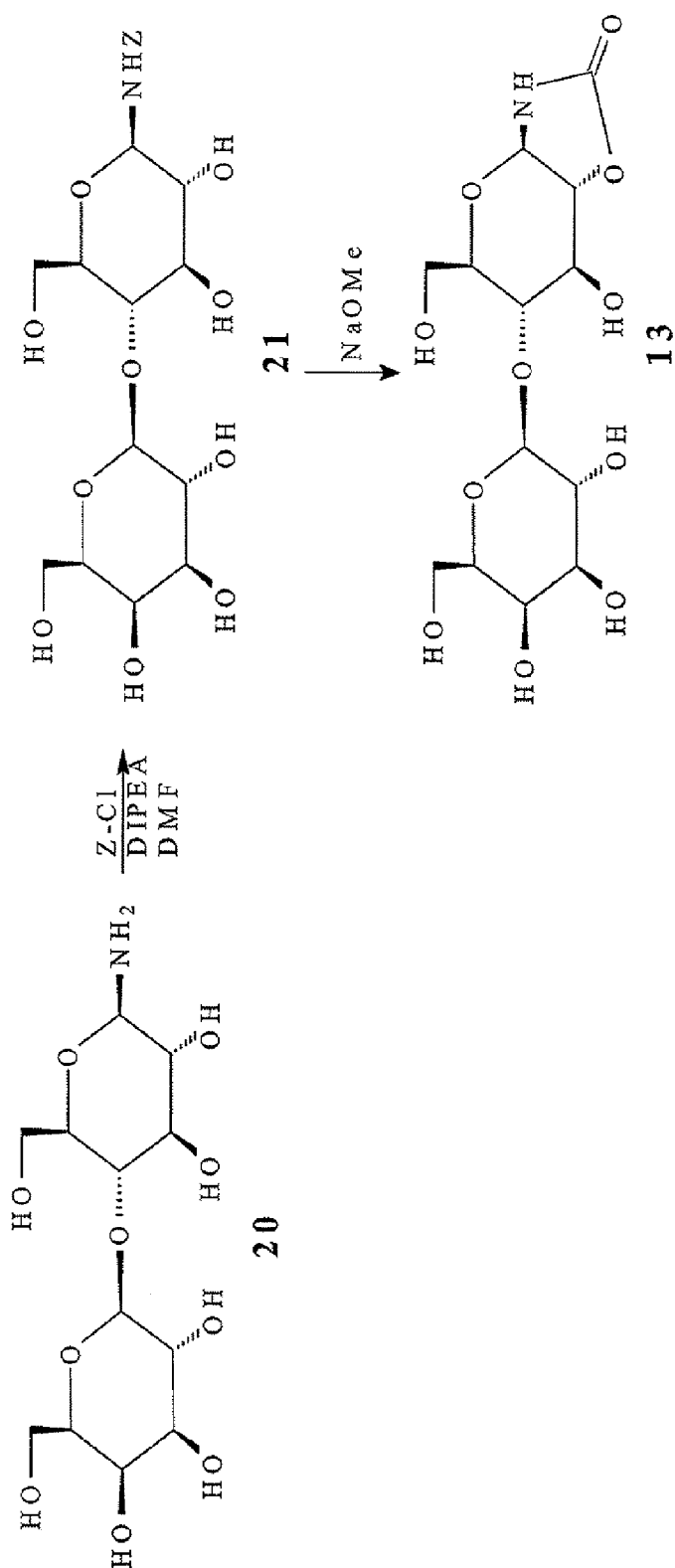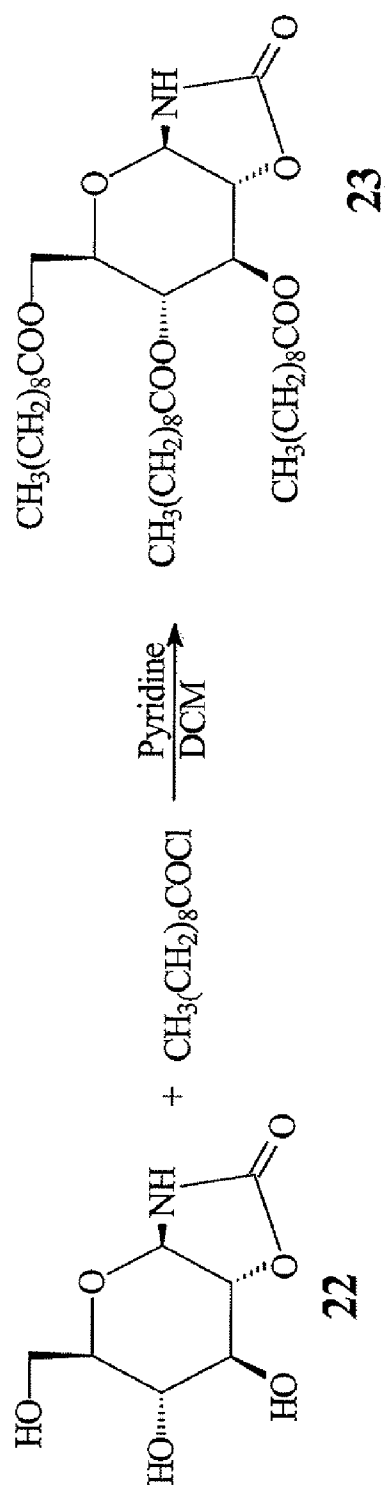
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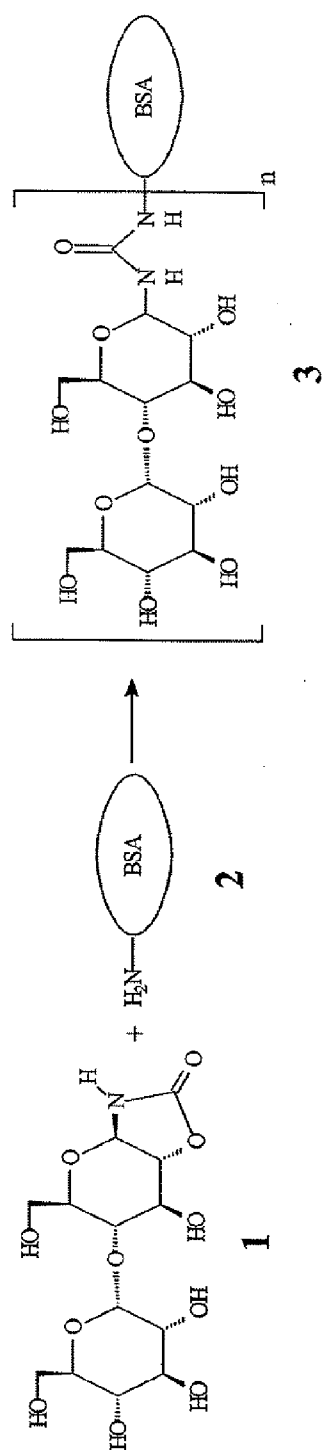
Fig. 1

Fig. 2
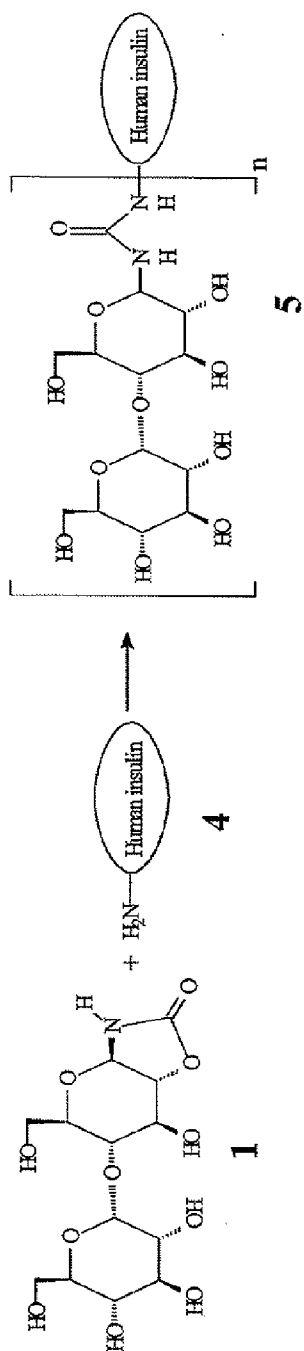
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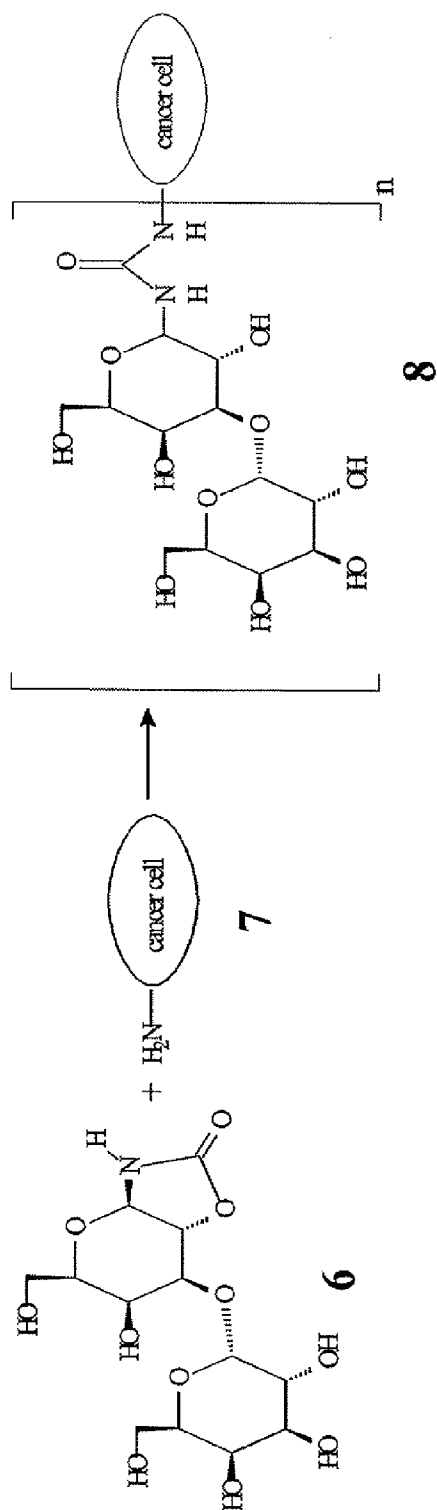
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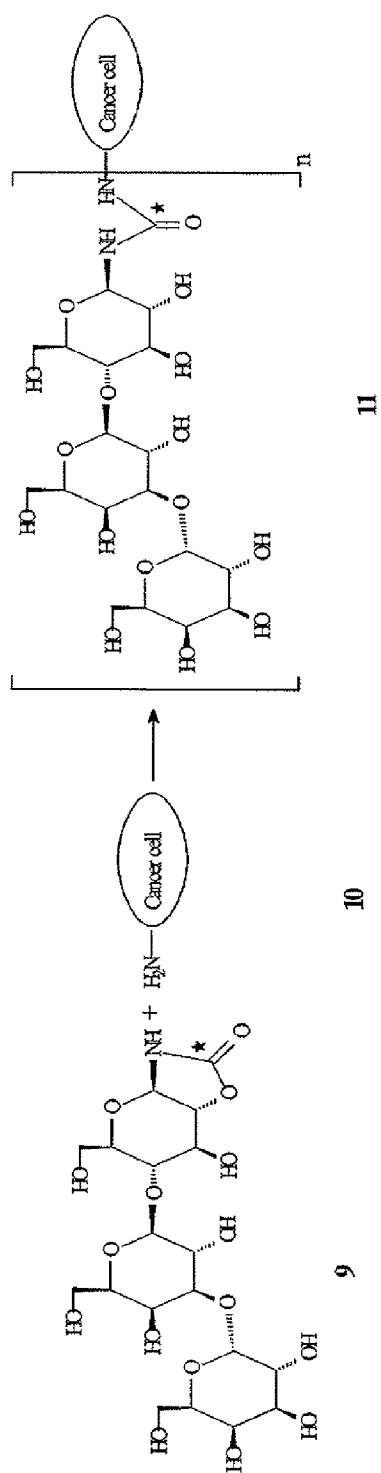
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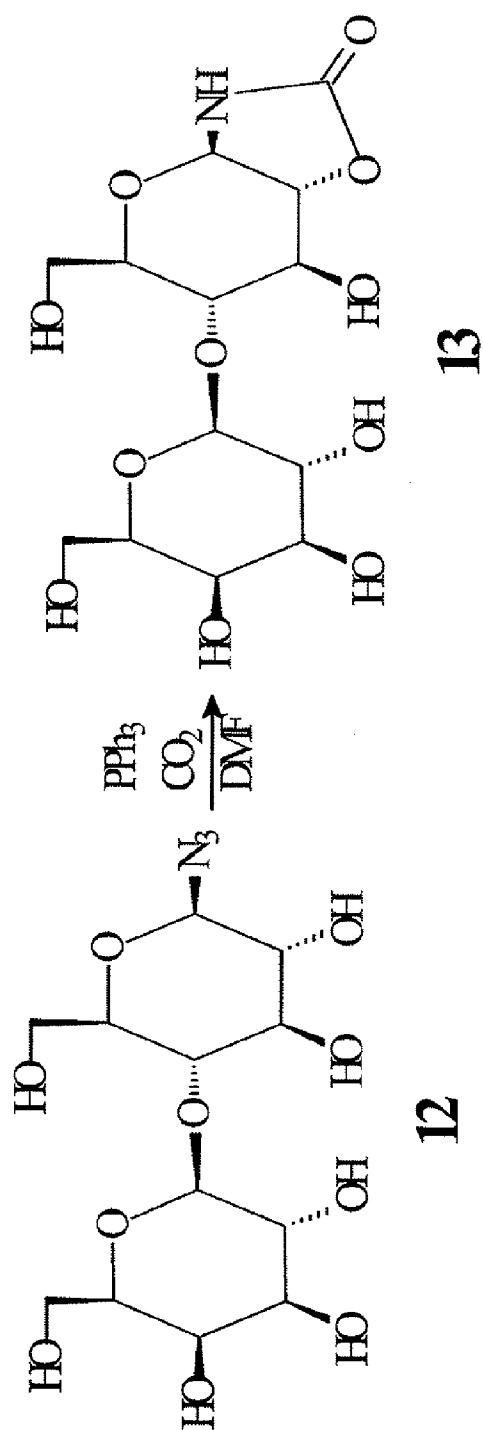
Fig. 6
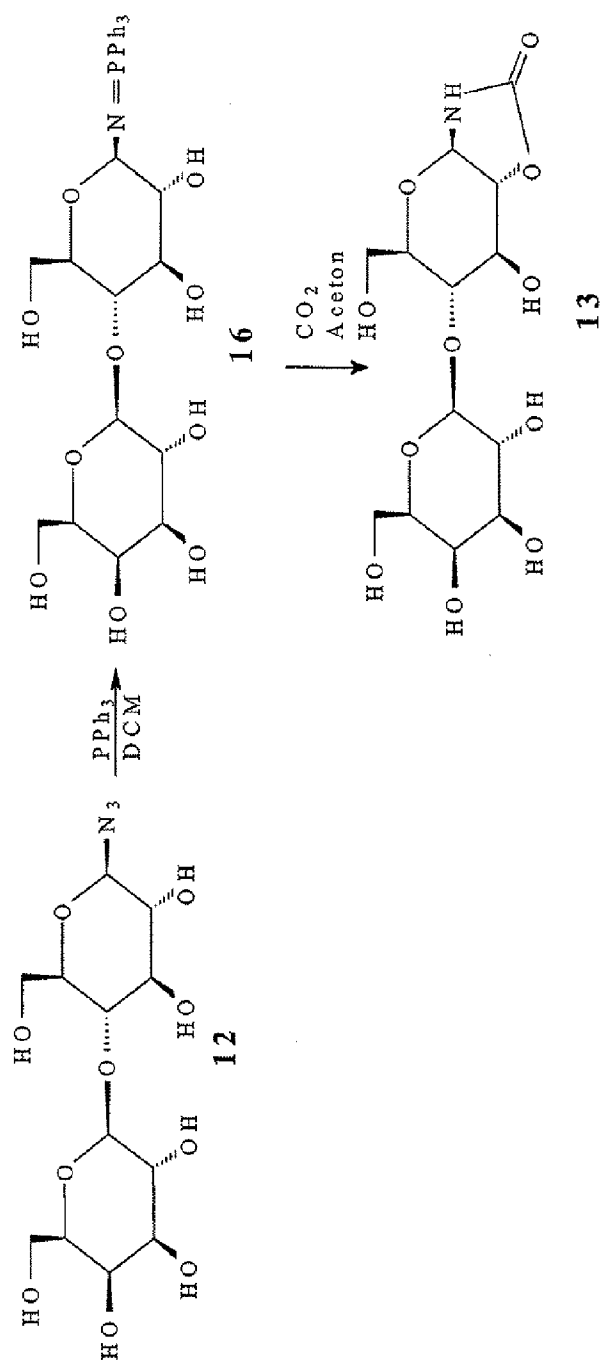
Fig. 7

Fig. 8

Fig. 9

Fig. 10

Figure 11

Figure 12