Glycoconjugate Journal 20, 11–23, 2004

Synthesis of radioactive and photoactivable ganglioside derivatives for the study of ganglioside-protein interactions

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The procedures for the preparation of radioactive and photoactivable ganglioside derivatives have been continuously developed from 1989, when for the first time the synthesis of photoactivable tritium labeled GM1 ganglioside was presented. We described previously the synthesis of photoactivable derivatives of GM3 and GM1 gangliosides, tritium-labeled at acetyl group of sugar units, and of photoactivable GM1 and GD1b gangliosides, tritium-labeled at position 6 of the external galactose. These procedures are reviewed in detail in the present paper.

The use of these ganglioside derivatives to study the ganglioside-protein interactions and to identify proteins that specifically interact with gangliosides (including GPI-anchored proteins of the outer membrane leaflet, proteins anchored to the cytoplasmic side of the plasma membrane through a fatty acyl chain, transmembrane proteins, and soluble cytoplasmic proteins) is discussed.
Published in 2004.

Keywords: **gangliosides, photolabeling, cell cultures, ganglioside-protein interactions, lipid domains**

Abbreviations: **GM3, II^{3}Neu5AcLacCer, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; Deacetyl-lysoGM3, α-Neu-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Sph; [11-^{3}H(Neu5Ac)]GM3-N$_3$, α-[11-^{3}H]Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-CerN$_3$; GM1, II^{3}Neu5AcGg$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; LysoGM1, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Sph; Deacetyl-lysoGM1, β-Gal-(1-3)-β-GalNH$_2$-(1-4)-[α-Neu-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Sph; [11-^{3}H(Neu5Ac),8-^{3}H(GalNAc)]GM1-N$_3$, β-Gal-(1-3)-β-[8-^{3}H]GalNAc-(1-4)-{α-[11-^{3}H]Neu5Ac-(2-3)}-β-Gal-(1-4)-β-Glc-(1-1)-CerN$_3$; [6-^{3}H(IV-Gal)]GM1-N$_3$, β-[6-^{3}H]Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-{α-[11-^{3}H]Neu5Ac-(2-3)}-β-Gal-(1-4)-β-Glc-(1-1)-CerN$_3$; GD1b, II^3(Neu5Ac)$_2$Gg$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; LysoGD1b, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Sph; Deacetyl-lysoGD1b, β-Gal-(1-3)-β-GalNH$_2$-(1-4)-[α-Neu-(2-8)-α-Neu-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Sph; [6-^{3}H(IV-Gal)]GD1b-N$_3$, β-[6-^{3}H]Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-CerN$_3$; Cer, ceramide, *N*-acyl-sphingosine; CerN$_3$,{(2S,3R,4E)-2-[12-(2-nitro-4-azidophenyl)amino-dodecanoyl]amino-3-hydroxy-octadec-4-ene}; Sph, sphingosine, (2S,3R,4E)-2-amino-1,3-dihydroxy-octadecene; DMF, N,N-dimethylformamide; GPI, glucosyl phosphatidyl inositol; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.**

General discussion

The ganglioside-protein interactions occurring at the level of the plasma membrane and the role played by gangliosides in modulating the functional properties of membrane proteins have been intensively studied in the past [1–7]. The more recent notion that gangliosides are key components of membrane domains enriched in sphingolipids, cholesterol, and a specific subset of proteins involved in the process of signal transduction (such as receptor tyrosine kinases, non-receptor tyrosine kinases of the Src family, adapter and regulatory molecules of tyrosine kinase signaling, heterotrimeric and small GTP-binding proteins, G protein-coupled receptors, protein kinase C isoenzymes) [8–10], has given a new interest to this topic. In fact, sphingolipid-enriched domains could provide a microenvironment within the plasma membrane for reciprocal interactions

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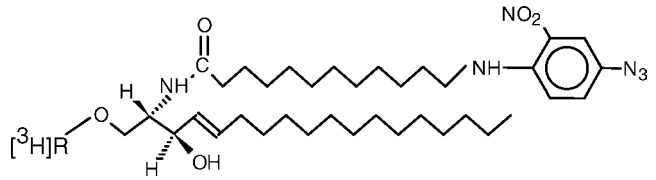

Figure 1. General structure of a radioactive and photoactivable ganglioside derivative containing a nitrophenylazide. [^{3}H]R is the tritium labeled ganglioside oligosaccharide chain.

between gangliosides and protein molecules participating in the control of signal transduction.

Several experiments on cultured cells have shown that exogenously administered gangliosides bind to cells, become components of the cell membrane, and enter the physiological sphingolipid metabolic and trafficking pathways [11–17]. Ganglioside turnover has been estimated in the order of a few hours in neuronal cells [18] and up to a few days in fibroblasts [19,20]. This allows the exogenously added ganglioside to reside some time in the plasma membranes. Thus, the administration of photoactivable gangliosides (Figure 1) to cultured cells followed by illumination allow to obtain membrane proteins cross-linked to ganglioside molecules that are inserted into the membrane and located in the protein microenvironment. As a photoactivable group, we like to use the azide. Linked to nitrophenyl, the azide group becomes very sensitive to light in solution and it must be handled with care in dark conditions (*i.e.* under red safelight). Illumination yields a nitrene group, whose reactivity leads to three main reactions: addition, insertion, and nucleophilic reactions (Figure 2). The nitrophenyl group is linked to the amino group of an aminododecanoyl chain, which is coupled to the sphingosine amino group of the ganglioside. The total length of this acyl chain is very similar to that of the natural ganglioside. Thus, after the ganglioside derivative is taken up by the cell and becomes a plasma membrane component, the azide group will be deeply inserted into the middle of the membrane lipid bilayer. Moreover, the oligosaccharide chain remains unchanged, thus

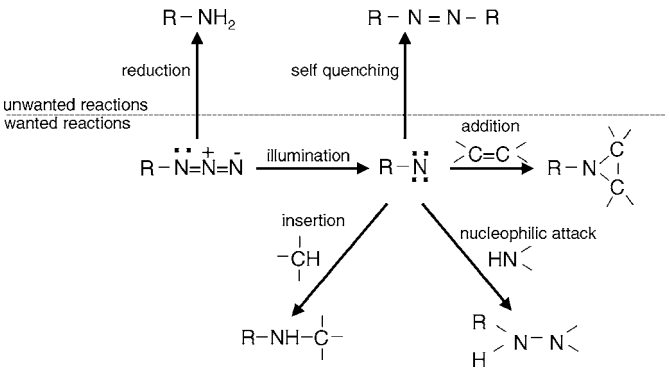

Figure 2. Scheme of the reactions involving the azide before and after illumination. The reduction of azide is a rapid reaction inside the cells in the presence of reducing proteins such as glutatione or SH enriched proteins.

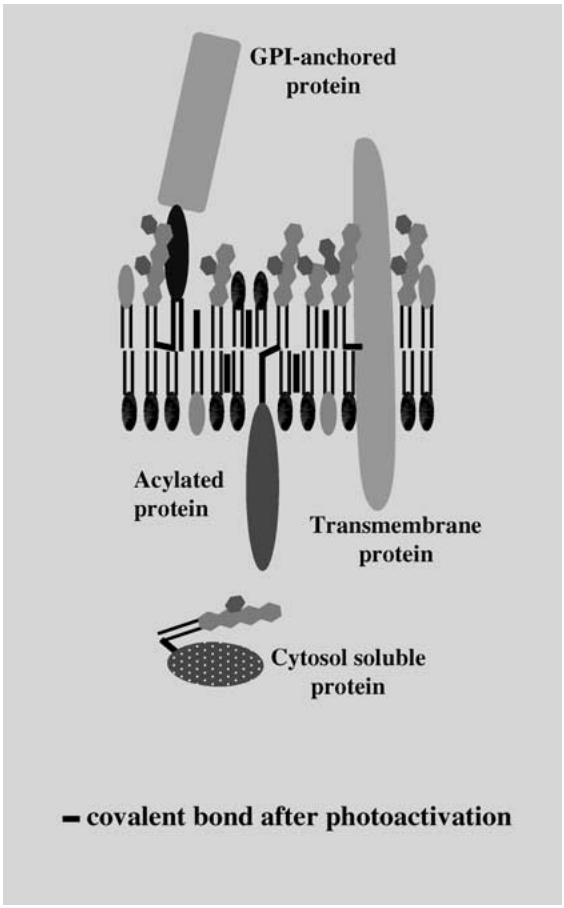

Figure 3. Cartoon representing the protein-ganglioside cross linkages that can be obtained after illumination of the photoactivable ganglioside inserted into the plasma membrane or solubly present inside the cell.

preserving any possible physiological interaction occurring between the protein and the oligosaccharide chain. According to the scheme of Figure 2, when the photoactivable ganglioside inserted in the membrane level is illuminated, the major part of it cross-links to membrane lipid molecules which are more abundant and close to the photoactivable group [1]. Nevertheless, a minor part of the nitrene-containing ganglioside, 2–5% [21], links to proteins. Figure 3 shows the three possible types of protein-ganglioside cross-linkage. Given the final azide position inside the plasma membrane, a cross-linkage might occur with the lipid moiety of a GPI-anchored protein [22], with a hydrophobic aminoacidic stretch of a transmembrane protein, or with the fatty acid anchoring a protein to the cytoplasmic side of the membrane [23,24]. Besides this, administration of photoactivable ganglioside to cells, when followed by a sufficiently long chase time, allows the ganglioside to be internalized and to enter the general metabolic and trafficking cellular processes. Thus, when illumination reaches the photoactivable ganglioside inside the cells, the activated derivatives cross-link to intracellular proteins that are in the ganglioside environment.

A soluble intracellular protein was shown to be cross-linked in ganglioside photolabeling experiments [25]; this is not surprising, since it is known that a minor part of cell gangliosides is present in the cell liquid as complexes with proteins [26,27].

Of course, to allow the detection of membrane or soluble proteins cross-linked to gangliosides, a radioactive tracer within the photoactivable ganglioside is needed. To this purpose, we use the radiochemical procedures that have been developed to introduce high specific radioactivity into a large number of different gangliosides without changing the natural structure of the oligosaccharide chains [28].

An example of the results that can be obtained using radioactive and photoactivable gangliosides to detect proteins belonging to the ganglioside environment is shown in Figure 4. Rat cerebellar granule cells differentiated in culture were incubated in the presence of a photoactivable [^{3}H]GD1b derivative for 6 h followed by a 2 h chase. After illumination and cross-linking, a sphingolipid-enriched membrane fraction was prepared by lysis in the presence of Triton X-100 and sucrose gradient centrifugation [29]. The pattern of proteins cross-linked to radioactive gangliosides in the sphingolipid membrane fraction was analyzed by two-dimensional electrophoresis followed by digital autoradiography. For comparison, Figure 4 also shows the total two-dimensional protein pattern in the sphingolipid-enriched membrane fraction after labeling with [^{35}S]methionine [29,30]. Under these experimental conditions, only a limited number of protein molecules were cross-linked to the ganglioside, if compared with the complex total protein pattern in this membrane

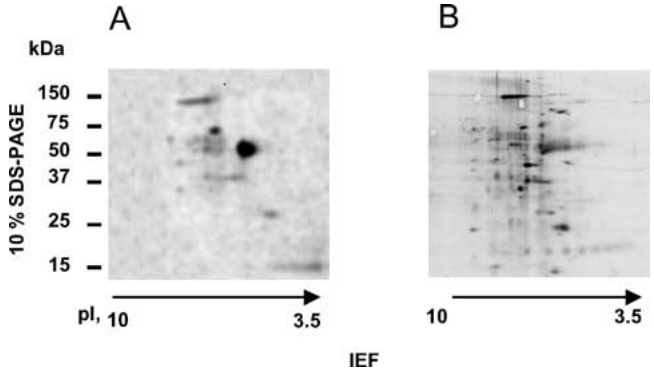

Figure 4. Two-dimensional photolabeled protein pattern in sphingolipid-enriched membrane fractions from rat cerebellar granule cells. Rat cerebellar granule cells differentiated in culture were incubated in the presence of photoactivable [^{3}H]GD1b derivative for 6 h followed by 2 h chase. After incubation and exposure to UV light, aliquots of samples (10 μg of cell protein) were analyzed by two-dimensional electrophoresis and proteins cross-linked to radioactive ganglioside were detected by digital autoradiography (80,000 dpm; time of acquisition: 48 h) (Panel A) by autoradiography (about 5000 cpm; time of exposure: 15 days). For comparison, Panel B shows the total two-dimensional protein pattern in the sphingolipid-enriched membrane fraction after steady-state metabolic labeling with [^{35}S]methionine visualized by autoradiography (about 5000 cpm; time of exposure: 15 days).

subfraction. The main radioactive protein had a molecular mass of 55 kDa and an isoelectric point of about 5.5, and was immunologically identified as tubulin. This is in agreement with previous data showing that palmitoylated tubulin is the main protein cross-linked by a photoactivable diazirine GM1 derivative in neuronal cells [23]. Two other typical proteins belonging to the sphingolipid-enriched membrane fraction from neuronal cells [30], the non-receptor protein tyrosine kinases c-Src and Lyn, were also cross-linked by the photoactivable [^{3}H]GD1b (radioactive bands at 60 and 53/57 KDa, respectively, and isoelectric points between 6 and 7) [24]. These proteins are also associated with the cell membrane via a fatty acid (myristoyl) chain. A band with a molecular mass of 135 KDa was intensely cross-linked by photoactivable [^{3}H]GD1b. As revealed by cell surface biotin-labeling experiments, this protein belongs to the plasma membrane exoplasmic leaflet, and it was identified as the GPI-anchored neural cell adhesion molecule TAG-1 [22], another typical component of the sphingolipid-enriched-membrane fraction obtained from cerebellar neurons [31].

A few remarks and recommendations on the materials

The preparation of radioactive compounds requires special attention to the purity of the materials, bearing in mind that the final amount of radioactive compound is, at most, in the order of a few milligrams. The commercial chemicals must be the purest available, checked by gas chromatography, TLC or HPLC for homogeneity, and, if necessary, further purified before use. The starting gangliosides must be as pure as possible. Purification of radioactive and photoactivable ganglioside derivatives often requires the use of solvents where radiolytic reactions are very fast, thus yielding radioactive by-products. Moreover, reaction with Pluoromitris phenylotide and purification of the corresponding ganglioside derivative need to be carried out in the dark, where the general handling is not easy. Thus, it is usually more convenient to purify the starting natural compounds and the light-stable intermediates to the highest homogeneity. The use of oil vacuum pump must be avoided and, if absolutely necessary, a liquid nitrogen trap should be used to condense the oil vapors.

The common solvents for column chromatography are distilled before use and deionised water is freshly distilled in a glass apparatus. Silica gel chromatography columns are always thoroughly washed before use with the solvent system subsequently used to elute lipid components adsorbed on the silica gel.

Some reactions require anhydrous conditions, that must be carried out in a controlled-atmosphere chamber; glass apparatus are dried overnight in an oven at 120°C, taken out just before use and connected to a tube filled with desiccant granules, and heated over a flame. The dehydration of acetone and DMF is carried out using a 3-Å and a 4-Å molecular sieve, respectively. Anhydrous triethylamine is prepared by shaking and standing over KOH pellets, anhydrous methanol and dimethylsulfoxide by refluxing repeatedly and distilling from metallic magnesium and calcium hydride, respectively.

Synthesis of [11-^{3}H(Neu5Ac)]GM3-N_3, [11-^{3}H(Neu5Ac),8-^{3}H(GalNAc)]GM1-N_3, [6-^{3}H(IV-Gal)]GM1-N_3 and [6-^{3}H(IV-Gal)]GD1b-N_3

The synthesis of radioactive and photoactivable ganglioside derivatives were carried out by combining and adapting experimental procedures developed for the synthesis of lysogangliosides [32], deacylated gangliosides [33], ganglioside analogues, and radioactive gangliosides [28]. The four general schemes for the preparation of [11-^{3}H(*Neu5Ac*)]GM3-N_3, [11-^{3}H(*Neu5Ac*),8-^{3}H(*GalNAc*)]GM1-N_3, [6-^{3}H(*IV-Gal*)]GM1-N_3 and [6-^{3}H(*IV-Gal*)]GD1b-N_3 are reported in Figures 5 to 8, respectively. In the figures, each reaction is coded with number and title as in the following description. Gangliosides with a terminal unsubstituted galactose are substrates galactose oxidase that produce the carbonylic derivative in quantitative yield. Moreover, radioactive sodium borohydrate is a stable compound and is available at any desired specific radioactivity. Thus, the galactose oxidase-[^{3}H]sodium borohydrate procedure is very effective and convenient for the tritium-labeling of gangliosides.

1. Alkaline hydrolysis. Gangliosides subjected to strong alkaline conditions yield a series of derivatives lacking in either the sugar acetyl groups or the fatty acyl chain, or both [32–38]. The yield of each derivative depends on the experimental conditions used and on the starting ganglioside structure.

1.1. Preparation of deacetyl-lysoGM3. In a round bottom flask, GM3 ganglioside was dissolved in water (16 μmoles/mL) with stirring and warming at 60°C. An equal volume of 16 M KOH, prewarmed at 60°C, is then added under strong stirring. A reflux apparatus, fitted with a connection tube for nitrogen fluxing, is adapted to the flask and the reaction allowed to proceed at 90°C under vigorous stirring. After 18 h, the solution is chilled, neutralized with 6 M HCl, dialyzed, and freeze-dried. The residue is dissolved in the smallest amount of chloroform-methanol 2:1, precipitated by the addition of 5 volumes of cold acetone, and stored overnight at 4°C. After centrifugation at 5000 rpm, acetone is discarded and the pellet dried under high vacuum. Under these experimental conditions the yield of deacetyl-lysoGM3 is quantitative.

Deacetyl-lysoGM3 is purified by silica gel column (80 μmoles on a 1 × 20 cm column) chromatography using the solvent system chloroform-methanol-5 M ammonium hydroxide, 30:50:10 by volume. The yield from the purification process is about 90%.

1.2. Preparation of deacetyl-lysoGM1. The experimental condition for the preparation of deacetyl-lysoGM1, and the results, are exactly those described for the preparation of deacetyl-lysoGM3 at paragraph *1.1*. This is a good alternative to the old alkaline hydrolysis in the presence of tetramethylammonium hydroxide [33] that gave a ganglioside derivative deacetylated at the sialic acid residue and lacking the fatty acyl chain.

1.3. Preparation of lysoGM1. A solution of GM1 in deoxygenated n-propanol (1.4 μmoles/mL) (n-propanol is deoxygenated by 30-minutes bubbling with a flux of argon, 20–30 L/min, and maintained in a closed bottle under moderate argon pressure) warmed to 90°C, is mixed in a macro-vial provided with an open-top screw-cap and a natural rubber septum, with a 90°C prewarmed 1 M KOH in deoxygenated n-propanol, to obtain a 0.2 M concentration. Two stainless-steel needles are introduced into the vial septum, one connected to the argon bottle, and the reaction mixture is maintained for 30 min under an argon flux of 5 mL/min. The needles are then removed and the reaction is continued at 90°C under continuous stirring for 6 h. The reaction mixture is then dried, and the residue dissolved in water (3.5 μmoles of starting GM1/0.1 mL), dialyzed for 3 h, and freeze-dried. The yield of reaction is about 60%. The purification of lysoGM1 is carried out by silica gel column chromatography (35 μmoles of starting GM1 on a 60 × 1.5 cm column) using chloroform-methanol-water, 60:35:5 by volume as the eluting solvent. The elution profile was monitored by TLC using chloroform-methanol-30 mM aqueous $CaCl_2$-100 mM aqueous KCl, 50:50:4:8 by vol. The yield of purification is about 90%.

1.4. Preparation of deacetyl-lysoGD1b. A solution of GD1b (1.7 μmoles/mL) in 5 M KOH, 5 M $Na_2S_2O_5$ in anhydrous methanol is stirred at 90°C for 60 h. After neutralization with acetic acid and drying, the residue is dissolved in chloroform/methanol/water 2:43:55, by volume, and purified on a RP18 silica gel column, eluted with same solvent, followed by methanol/water 1:1, methanol/water 4:1, methanol and chloroform/methanol 1:1. The chromatographic elution profile is monitored by TLC using the solvent system chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11, by volume. The yield of reaction after purification is about 80%.

2. N-acylation. The attachment of the aminododecanoic acid to the ganglioside derivative sphingosine free amino group requires that the fatty aminoacid is previously protected at the amino group and activated at the carboxyl group. Fluorenyl is a useful group to protect amines due to the easiness of its removal when necessary. To improve the yield of the *N*-acylation reaction, several reagents for the fatty acid carboxyl group activation were proposed, such as succinimide [37], 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide [33], ethylchloroformate [39], disuccinimidylcarbonate [21] and pentafluorophenol [40]. Under our conditions, pentafluorophenol gives almost quantitative yield of the *N*-acylation reaction.

In the presence of both the amino groups of sugars and sphingosine, the *N*-acylation occurs quantitatively for sphingosine, probably being facilitated by interaction between the hydrophobic sphingosine and the acyl chain [21,33].

2.1. Preparation of the fluorenyl derivative of 12-aminododecanoic acid. A solution of 9-fluorenylmethylchloroformate in tetrahydrofuran (28 μmoles/mL) is added under vigorous magnetic stirring to an equimolar quantity of 12-aminododecanoic acid in 1% aqueous $NaHCO_3$ (60 μmoles/mL). The mixture is stirred for a further 30 min at room temperature and then filtered. The solution is partitioned by adding half volume of ethyl ether and shaking the

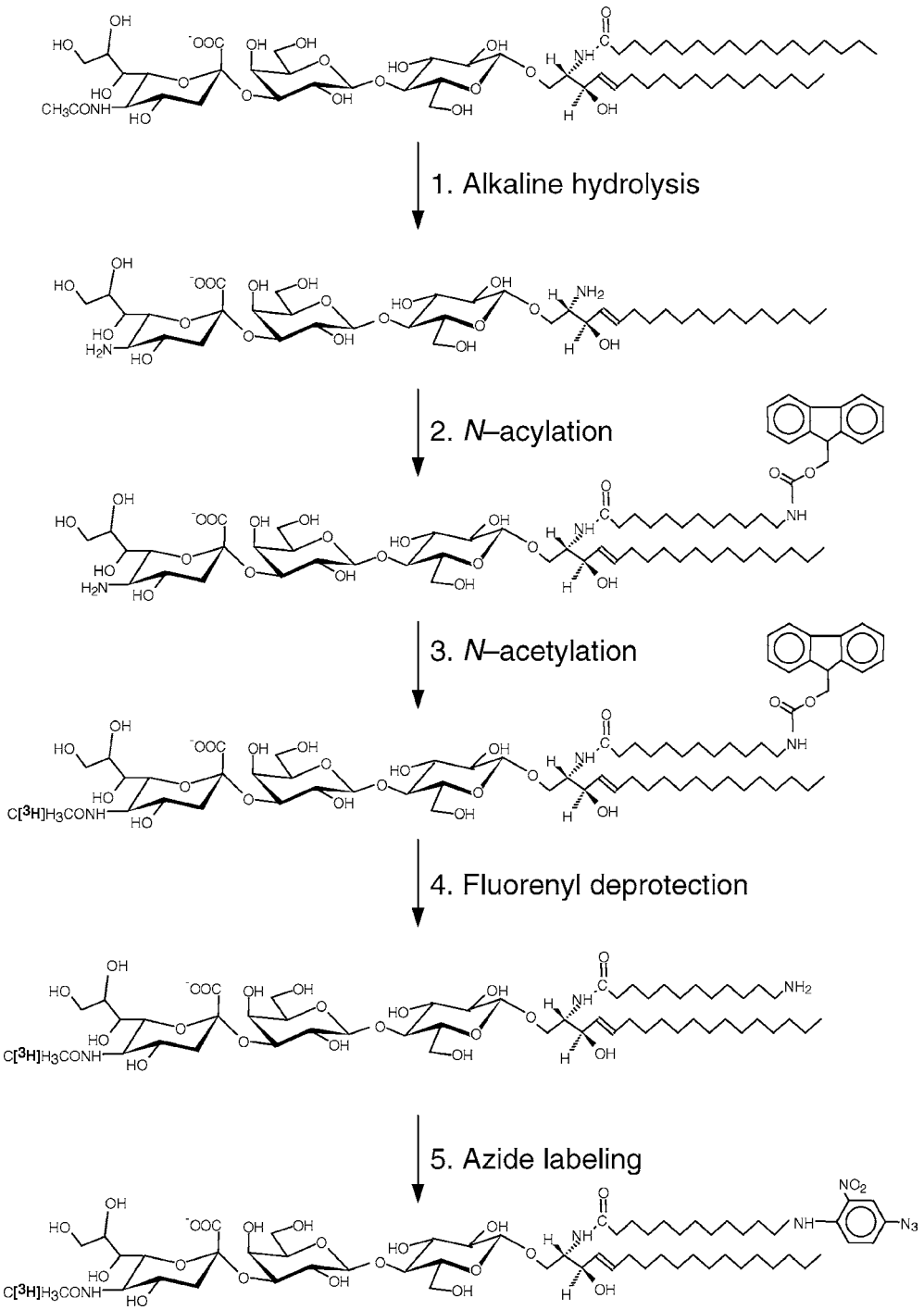

Figure 5. Scheme of the synthetic process for the preparation of [11-^{3}H*(Neu5Ac)*]GM3-N_3.

mixture. The aqueous phase is withdrawn and washed with a further half volume of ethyl ether, and the organic phase is added to the previous one. After evaporation the product is dissolved in tetrahydrofuran and precipitated with three volumes of 1 M HCl. The white precipitate is washed with water and crystallized from hexane/toluene, 80/20 by volume. The yield is about 90%.

2.2 Activation of the fluorenyl derivative of 12-aminododecanoic acid. 2-Chloro-1-methylpyridinium iodide, pentafluorophenol, fluorenyl protected 12-aminododecanoic acid and dry tributylamine, in the molar ratio of 1.2:1:1:2.4, are solubilized in this order, in 3 mL of dry dichloromethane (915 μmoles of fluorenyl protected 12-aminododecanoic acid/mL), in a three neck flask, provided with N_2 septum-inlet

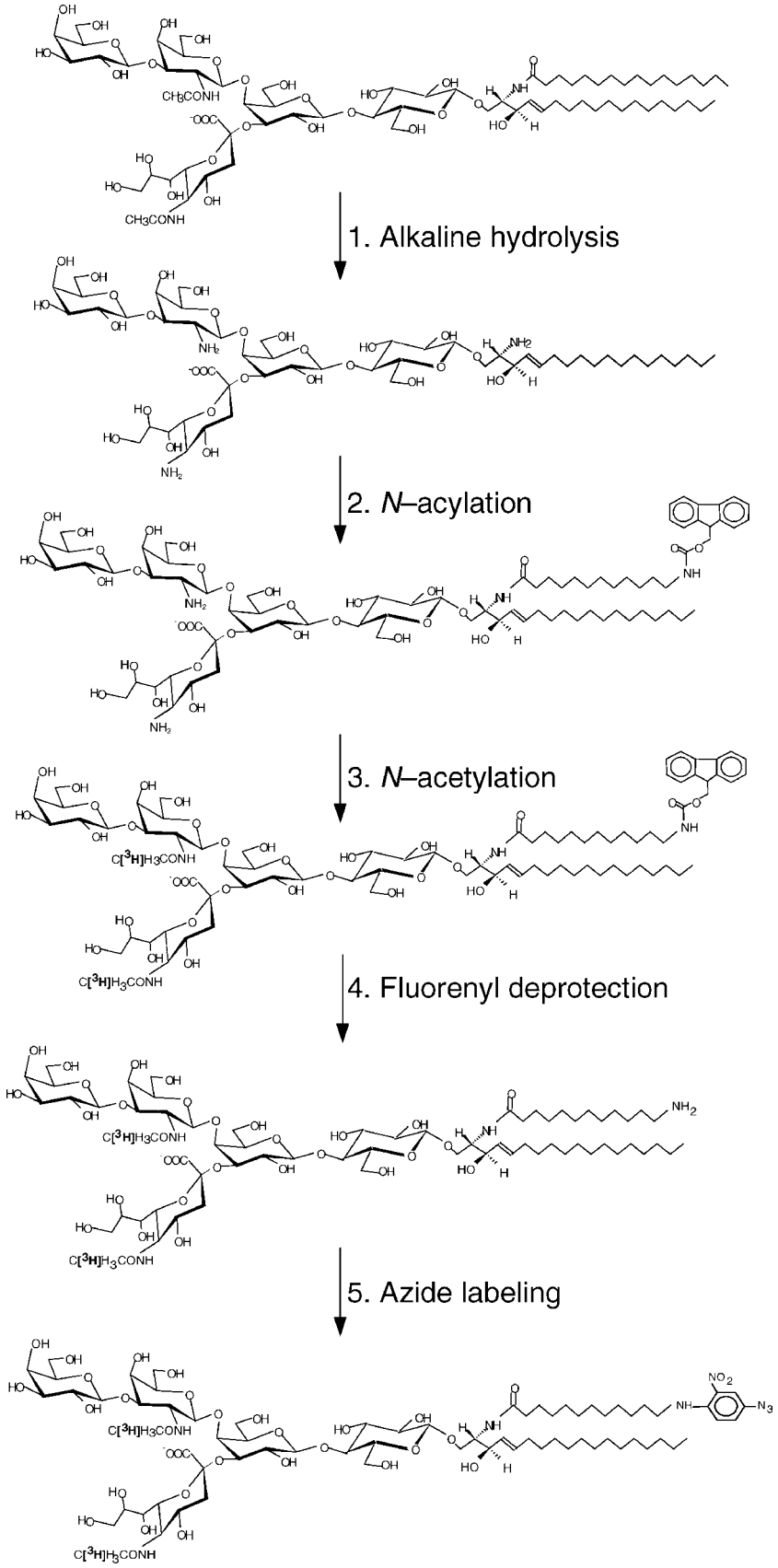

Figure 6. Scheme of the synthetic process for the preparation of [11-^{3}H*(Neu5Ac)*,8-^{3}H*(GalNAc)*]GM1-N_3.

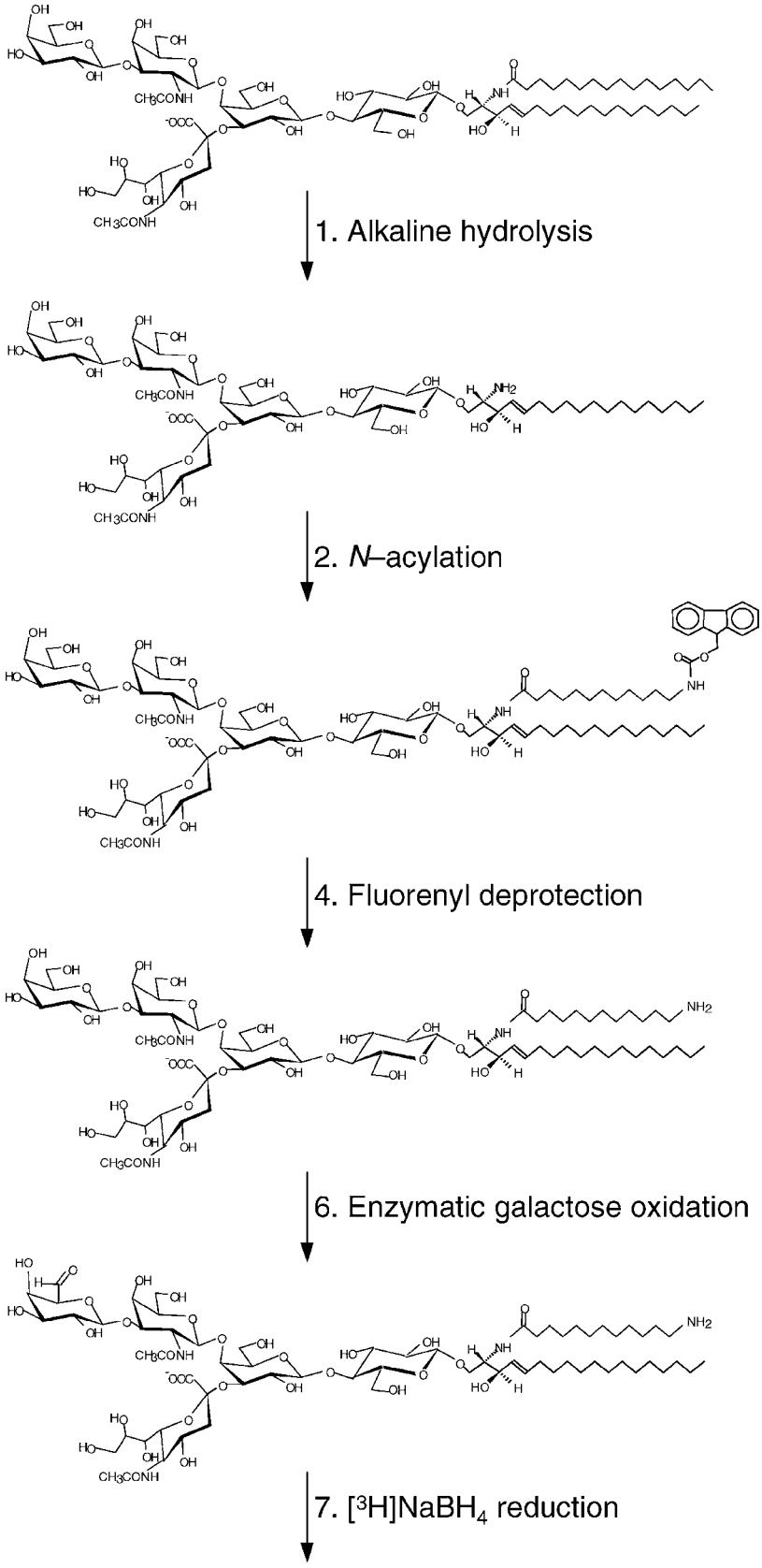

Figure 7. Scheme of the synthetic process for the preparation of [6-^{3}H*(IV-Gal)*]GM1-N_3.

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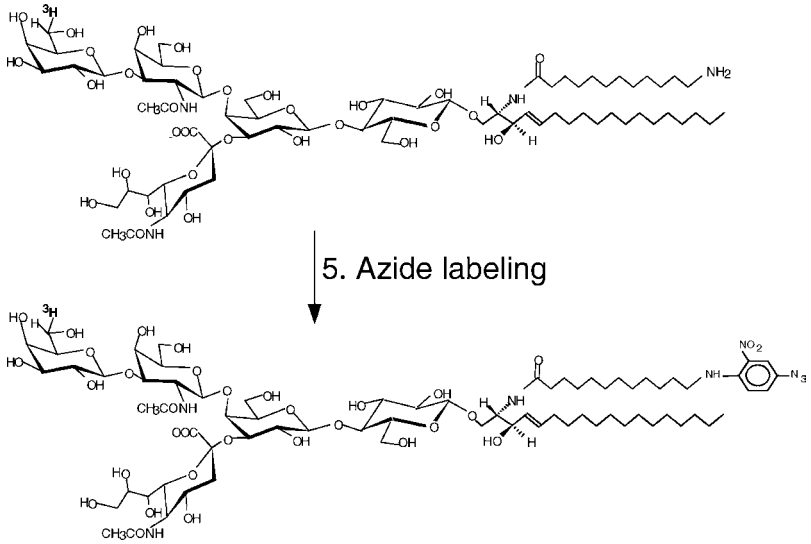

Figure 7. (*Continued.*)

adapter and a condenser. The reaction mixture is refluxed 3 h under continuous stirring, then cooled and evaporated under vacuum. The product is purified by flash chromatography with silica gel 60 column (2.5 mmoles on a 15×2.5 cm column), using 2 L hexane/ethyl acetate 19/1 by volume, 1.5 L, hexane/ethyl acetate 4/1 by volume, 0.5 L hexane/ethyl acetate 1/1 by volume. The yield is about 90%.

2.3. N-acylation of deacylated ganglioside derivatives. *N*-fluorenylmethylformate-aminolauric acid pentafluorphenylester, 1-hydroxybenzotyriazole and tributylamine in the molar ratio of 1.1:1.8:1.5 with respect to ganglioside derivative, are added to a solution of deacylated ganglioside, GM3, GM1, or GD1b, in anhydrous DMF (4 μmoles/mL). After vigorous stirring for 75 min at room temperature, the reaction mixture is dried and the residue purified on a silica gel 100 column (70 μmoles of starting deacylated ganglioside on a 70×1.4 cm column) equilibrated and eluted with chloroform/methanol/water 30:50:13, by volume. The yield of acylation, after purification, is about 80%.

3. N-acetylation. *N*-Acetylation of deacetylated ganglioside with acetic anhydride is a simple reaction. On the other hand, when radioactive acetic anhydride is used, some care is necessary since radioactive acetic anhydride samples are easily degraded to acetic acid.

3.1. N-acetylation with [^{3}H]acetic anhydride. The purified deacetyl-ganglioside is dissolved in magnesium dehydrated methanol (1 μmole/mL) and mixed with radioactive acetic anhydride to a final molar ratio of 1:1. After stirring for 30 min at room temperature, nonradioactive acetic anhydride is added, the mixture is stirred for an additional 30 min, the solution dried, and the residue rapidly dissolved in methanol or propanol. The addition of "cold" acetic anhydride can be omitted to increase the ganglioside specific radioactivity. In this case, the reaction mixture is purified by chromatography on a 50×1 cm silica gel 100 column, equilibrated, and eluted with chloroform-methanol-water, 60:35:8, by vol. A detailed information on the tritium labeling of gangliosides has been previously reported [28]. *N*-acetylation is performed using a ganglioside derivative:acetic anhydride ratio of 1:4.

3.2. N-acetylation with acetic anhydride. The deacetylated ganglioside is dissolved in water (5 μmoles/mL), and acetic anhydride is added at room temperature under vigorous stirring, until pH 5 was attained. After 3 h, the reaction product was dialyzed, lyophilized, and purified on a silica gel 100 column, equilibrated and eluted with chloroform/methanol/water 60:35:8, by vol. for GM1 and GM3, and with isopropanol/water 70:30 by volume for GD1b.

4. Fluorenyl deprotection. The ganglioside derivative is solubilized in a few mL of 32% aqueous ammonia in a screw-cap flask for 24 h under vigorous stirring at room temperature. The reaction mixture is dried and the residue dissolved in 5 mL water. The solution is washed three times with 10 mL diethylether and dried.

5. Azide labeling. The radioactive compound is dissolved in anhydrous DMF (10 μmoles/mL). An equimolar quantity of triethylamine and a two-fold molar quantity of 4-F-3-NO_2-phenylazide, dissolved in ethanol (25 μmoles of triethylamine/mL), are added, and the mixture is stirred overnight at 80°C. The reaction mixture is dried and the radioactive and photoactivable ganglioside purified on a silica gel 100 column (4 μmoles on a 100×1.2 cm column) equilibrated and eluted with chloroform/methanol/water 30:50:13, by volume. The elution profile is characterized by TLC using the solvent system chloroform/methanol/water, 30:50:13, by volume. Fractions containing the homogeneous product are dried and the residue immediately solubilized in methanol at about 10 μCi/mL, and stored at +4°C.

6. Enzymatic galactose oxidation. 8 μmoles of ganglioside containing the aminododecanoic acid as acyl chain were dissolved in chloroform/methanol 3:5, by volume. 110 mg of

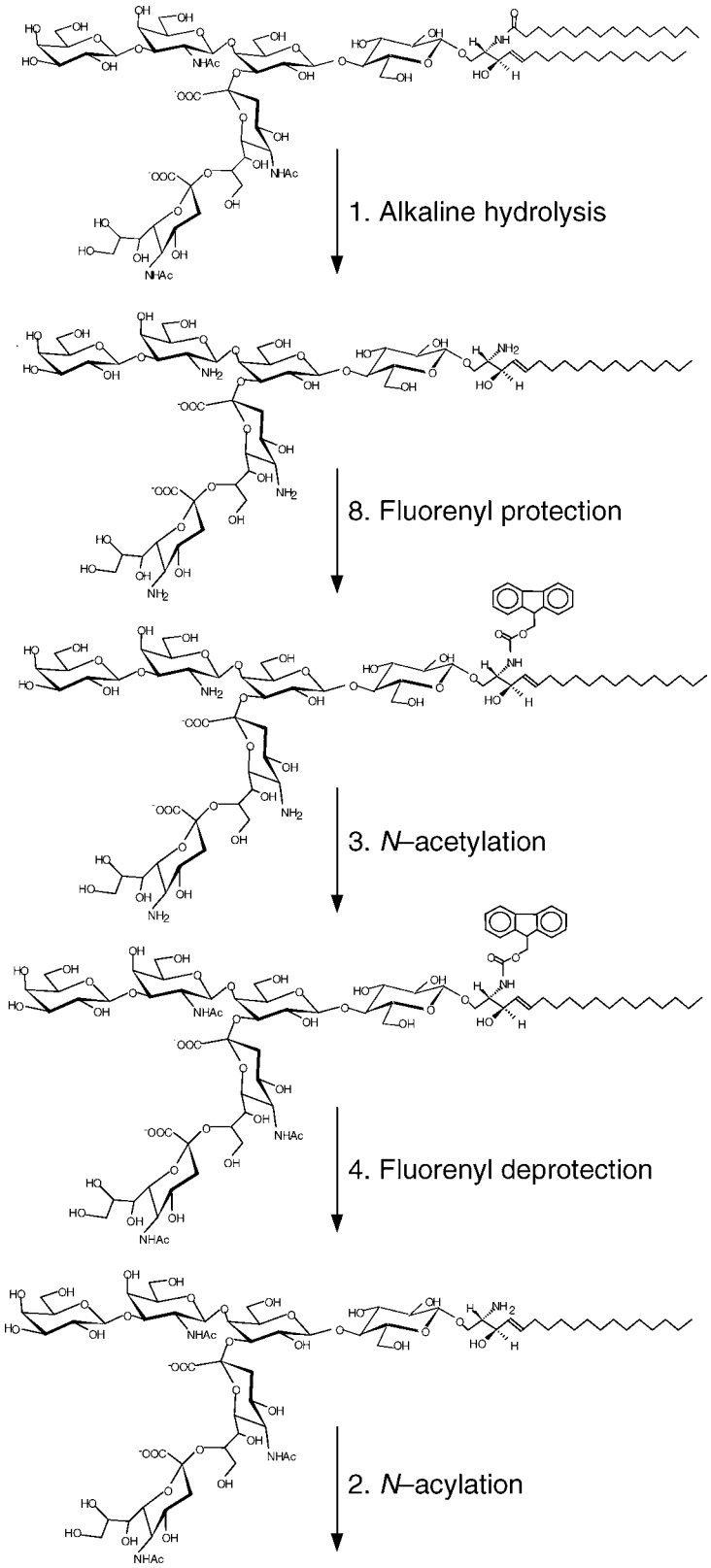

Figure 8. Scheme of the synthetic process for the preparation of [6-^{3}H*(IV-Gal)*]GD1b-N_3.

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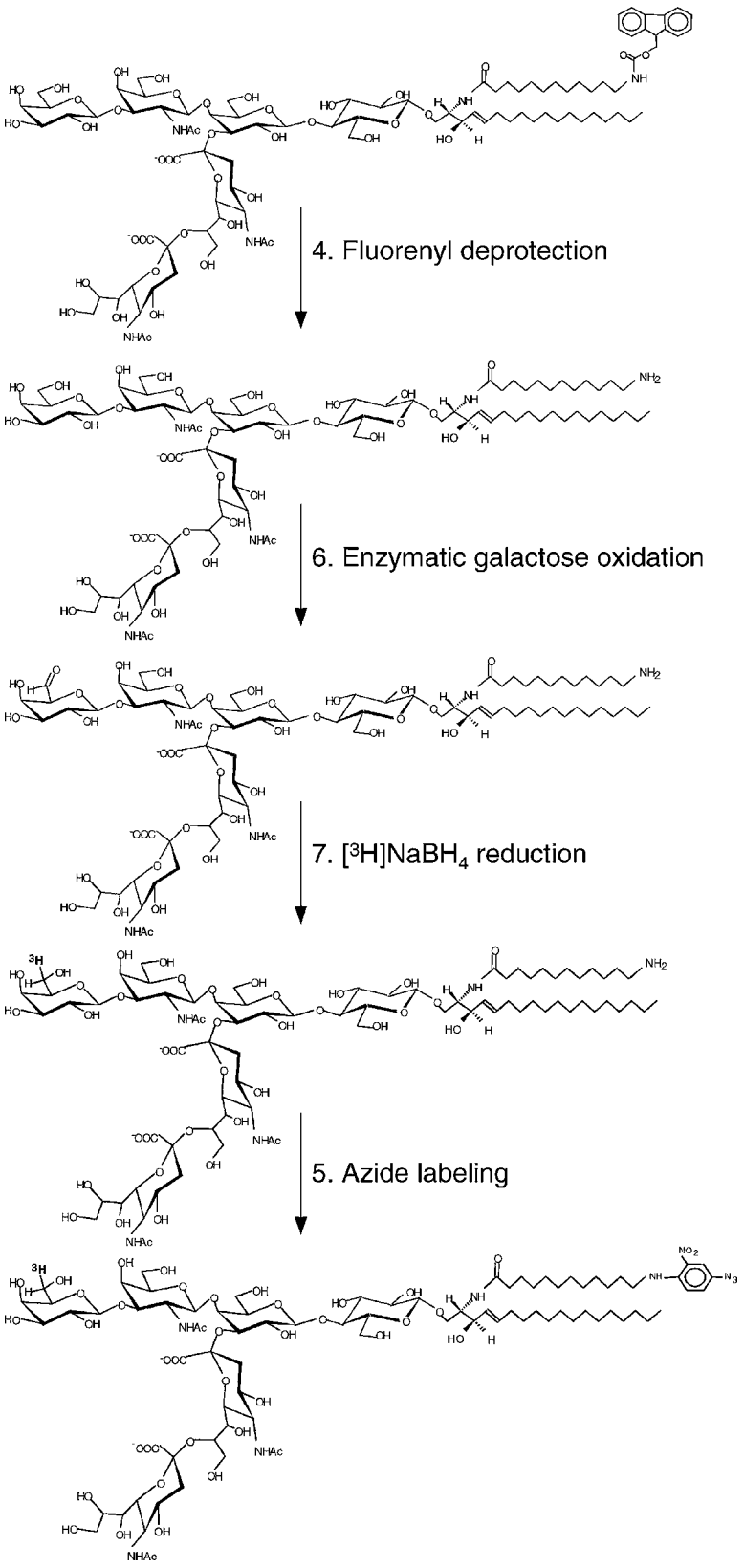

Figure 8. (*Continued.*)

Triton X-100 was added and the mixture was slowly dried. The residue was dissolved in 5 mL of 25 mM phosphate buffer, pH 7.0, 5 mM EDTA, and 450 U of galactose oxidase were added. The mixture was stirred at 37°C for 2.5 h. 450 U of galactose oxidase was further added and reaction allowed to proceed overnight in the same conditions. The reaction mixture was dried in a reaction tube provided with a screw cap and stored at −20°.

7. [^{3}H]NaBH$_4$ reduction. The galactose oxidase reaction mixture containing 7–8 μmoles of ganglioside containing the external galactose oxidated at position 6, is dissolved in n-propanol and added with 0.1 M NaOH until reaching pH 7.5. 100 mCi NaB^{3}H$_4$ (specific radioactivity 10–30 Ci/mmol) is added and reaction is allowed to proceed at room temperature for three days. The reaction mixture is dried and the radioactive ganglioside derivative is purified on a silica gel 100 column (90 × 1.4 cm), equilibrated, and eluted with chloroform/methanol/water 30:50:13. The reaction products are characterized by TLC using the solvent system chloroform/methanol/water, 30:50:13, by volume.

8. Fluorenyl protection. A solution of deacetyl-lysoGD1b in 0.5 M $NaHCO_3$ (5 μmoles/mL) is mixed with an equal volume of diethylether and cooled until the aqueous phase is frozen. After the addition of a 50% higher molar quantity of 9-fuorenylmethylchloroformate in n-hexane (40 μmoles/mL), the mixture is vigorously stirred at 10°C for 24 h. The reaction mixture is centrifuged at room temperature for 30 min and the aqueous phase is recovered.

Treatment of cell cultures with radioactive and photoactivable gangliosides: methodological remarks

In routine experiments aimed to study protein-ganglioside interactions, cells in culture are incubated with a mixture of 1.0×10^{-6} M natural ganglioside and 1.0×10^{-6} M radioactive and photoactivable ganglioside derivative. The dilution of the photoactivable ganglioside with the natural compound is essential to reduce the self-quenching process during illumination (*i.e.* the cross-linking between different photoactivable molecules, Figure 2). This phenomenon should be avoided, since it leads to the subtraction of photoactivable molecules from physiological interactions with cell membrane components and to the formation of artifactual radioactive species which might interfere with the subsequent detection of cross-linked proteins. In our experience, a one-to-one dilution of the photoactivable derivative with natural ganglioside is enough to prevent any significant self-quenching.

Incubation is performed in serum-free culture medium, the condition that allows the insertion of any exogenous ganglioside in the cell membrane in a "natural" way [41–43]. The pulse time can be varied from 2 to 6 h without significant variations in the pattern of cross-linked proteins and in the distribution of the cross-linked proteins within different regions of the plasma membrane, as indicated by studies carried out on sphingolipid-enriched membrane fractions. After 6 h, a significant variation in the pattern of cross-linked proteins can be observed, due to membrane turnover and subsequent internalization and metabolism of the photoactivable derivative. Thus, we usually carry out experiments with different chase times (up to 24 h) aimed to highlight ganglioside-protein interaction at the intracellular level. Chase incubation is performed in the complete culture medium, including serum if needed. In the case of primary cultured neurons, particularly sensitive to the culture environment, we use cell-conditioned medium.

After pulse incubation, cells are washed 5 times with medium containing 10% fetal calf serum. This step is of particular importance to remove a relevant amount of radioactive ganglioside that is weakly bound to the cells, as always in the case of exogenous ganglioside administration [14]. After removing the serum-labile quota, still a relevant amount of ganglioside derivatives remains, that is more stably associated with the cell surface but not yet inserted in the lipid bilayer. To remove it, a trypsin treatment is needed [14]. If trypsin treatment is omitted, after illumination a significant amount of ganglioside derivatives will be artifactually linked to the extracellular moiety of cell membrane components, particularly proteins. Trypsin treatment can be omitted if a sphingolipid-enriched membrane fraction is prepared. The pattern of cross-linked proteins in this fraction is not sensitive to trypsin treatment, indicating that ganglioside derivative molecules associated with this fraction are in large part "physiologically" inserted in the plasma membrane. The absence of artifact cross-linked proteins is probably also due to the high lipid-to-protein ration in this membrane fraction.

After pulse-chase, cells are further washed five times with ice cold PBS. Finally, enough PBS to cover the cell layer is added, dish lid is removed, and cells are illuminated for 45 min under UV light at 360 nm over ice. All procedures before exposure to UV light are performed under red safelight.

Acknowledgments

This work was supported by COFIN-PRIN (grants 2000 and 2001), Consiglio Nazionale delle Ricerche (PF Biotechnology), Italy, and Mizutani Foundation for Glycoscience (grant 2002).

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