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Synthesis and characterization of a carbene-generating biotinylated *N*-acetylglucosamine for photoaffinity labeling of β-(1 → 4)-galactosyltransferase

Yasumaru Hatanaka [a,*], Makoto Hashimoto [a], Shoko Nishihara [b], Hisashi Narimatsu [b], Yuichi Kanaoka [c]

[a] *Research Institute for Wakan-yaku, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-01, Japan*

[b] *Institute of Life Science Soka University, Hachioji, Tokyo 192, Japan*

[c] *Toyama Women's College, 444 Gankaiji, Toyama 930-01, Japan*

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Abstract

A photoreactive *N*-acetylglucosamine derivative, *N*-[2-[2-[2-(2-biotinylaminoethoxy)-ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoyl]-N^4-[2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]-L-aspartamide (BDGA), was synthesized as a carbene-generating biotinylated probe for UDP-galactose:*N*-acetylglucosamine β-(1 → 4)-galactosyltransferase (GalT). The photoaffinity labeling experiments of bovine GalT with BDGA under various conditions were examined based on the quantitative chemiluminescent detection of the biotinyl residue which was photochemically introduced into the GalT protein. A progressive decrease in the yield of specific photolabeling was observed upon lowering the incubation temperature from 37 °C to 20 °C or 4 °C. The amount of photoincorporation was also decreased when UMP was not included in the incubation mixture. Using a crude protein mixture of recombinant human GalT, a band corresponding to the glutathione *S*-transferase fusion GalT protein was also specifically visualized. Furthermore, combined use of BDGA photolabeling with an immobilized avidin was found to be effective for the selective retrieval of photolabeled GalT from a reaction mixture containing a large amount of unlabeled GalT protein. The results obtained clearly demonstrate that the covalent

* Corresponding author.

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biotinylation using the carbene-generating photoaffinity reagent BDGA would be useful for the analysis of acceptor substrate binding sites within the GalT protein. © 1996 Elsevier Science Ltd.

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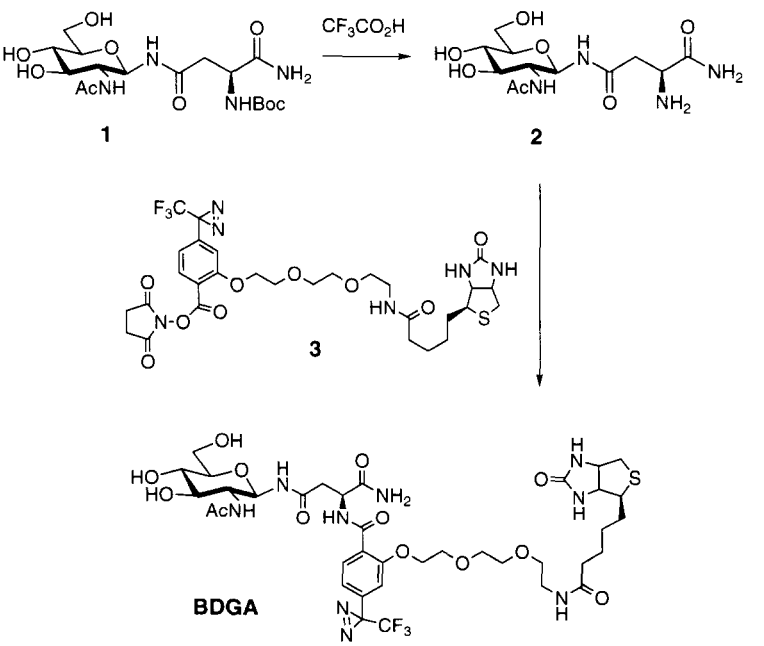
1. Introduction

The carbohydrate parts of cellular glycoconjugates comprise a tremendous diversity of structures which are now progressively appreciated [1]. The biosynthesis of carbohydrates depends on a series of highly specific enzymes, glycosyltransferases, which elongate saccharide chains sequentially by transferring glycosyl groups from donor sugar nucleotides to acceptor carbohydrates [2]. The investigation of these glycosyltransferases at the molecular level becomes increasingly important in the field of glycobiology. One of the most extensively studied glycosyltransferases, UDP-galactose:*N*-acetylglucosamine β-(1 → 4)-galactosyltransferase (GalT; EC 2.4.1.38), transfers galactose from UDP-galactose to the non-reducing terminal *N*-acetylglucosamine residues of glycoproteins and glycolipids, forming a β-(1 → 4) linkage [3]. A nitrene-generating arylazide derivative of UDP was first applied for the photoaffinity labeling of the enzyme GalT [4], because much attention had been devoted to developing various photoreactive donor nucleotide analogs [5]. Enzymes transferring the same sugar unit, however, usually utilize the same sugar nucleotide as the donor substrate. For example, GalT and α-(1 → 3)-galactosyltransferase share UDP-galactose as the common donor substrate whereas these enzymes differ in the specificity of the acceptor substrate. In addition to the conventional donor photoprobes, the development of photoreactive acceptor analogs may play a crucial role in probing the detailed molecular aspects of glycosyltransferases. In this context, several diazirino-substituted photoreactive carbohydrates were developed as carbene-generating acceptor probes for GalT [6]. A rapid intramolecular reaction is, however, reported for this type of aliphatic diazirines as an undesirable quenching process of intermediate carbenes [7]. Furthermore, the current photoprobes for glycosyltransferases usually require the introduction of radioactive markers for the detection of photolabeled GalT protein [5,6]. The synthesis of radioactive probes is usually troublesome and sometimes difficult because experiments have to be performed on a very small scale. Recent examples of biotinylated photoprobes provide a practical approach in this respect [8–11]. We have already developed a new biotinylated phenyldiazirine which is a useful photoreactive unit for the detection of photolabeled products by a highly sensitive chemiluminescent method [12]. We wish to report the synthesis and characterization of a novel photoreactive *N*-acetylglucosamine derivative, *N*-[2-[2-[2-(2-biotinylaminoethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoyl]-N^4-[2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]-L-asparta-mide (BDGA), as the first example of a biotinylated carbene-generating acceptor photoprobe for the enzyme GalT. The reagent BDGA was found to be useful for the chemiluminescent detection of photolabeled GalT, and the photochemically biotinylated GalT protein was selectively retrieved from a complex mixture containing a large amount of unlabeled protein.

2. Results and discussion

Synthesis of the photoreactive GlcNAc derivative.—For the selective introduction of the biotinylated phenyldiazirine derivative **3** (Scheme 1), the presence of a primary amino functional group within the structure of the acceptor substrate is required. The enzyme GalT has been the subject of extensive research including studies of its substrate specificity [13,14]. This enzyme appears to accept many modified *N*-acetylglucosamines, however, introduction of aromatic acyl substituents instead of the *N*-acetyl group of glucosamine may not be acceptable [15,16]. In contrast, asparagine-linked *N*-acetylglucosamine derivatives are usually good substrates for GalT [17] even though a large structural unit, pentapeptides, is present at the reducing end of the acceptor sugar [18]. The asparagine unit seems to be attractive as a short arm which links the biotinylated diazirine to the specific ligand, *N*-acetylglucosamine, without preventing GalT binding. Scheme 1 describes the synthesis of a novel photoreactive derivative of the asparagine-linked *N*-acetylglucosamine thus designed. According to the procedure of Wong [18], deprotection of **1** was performed with trifluoroacetic acid to give the TFA salt of **2** which was neutralized to give **2** as a free base. Coupling of the *N*-hydroxysuccinimide ester **3** to the amine **2** was easily accomplished at room temperature to give the desired photoaffinity probe BDGA in high yield. The reagent BDGA possesses a phenyldiazirine group which is usually a more advantageous photoreactive group than the conventional aryl azide counterparts [19]. The phenyldiazirine is connected to a biotin moiety through a hydrophilic ether spacer to provide a nonradioisotopic and



Scheme 1.

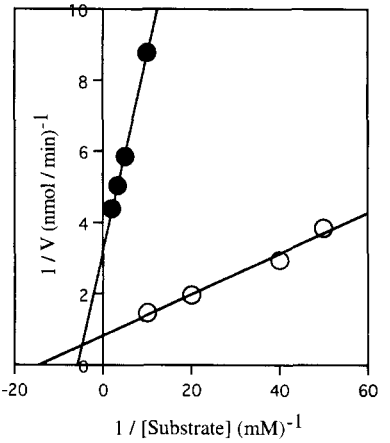


Fig. 1. Lineweaver–Burk plot of BDGA galactosylation. The enzyme reactions were carried out at 37 °C using 0.005 U GalT and various concentrations of BDGA (—○—) or **1** (—●—).

highly sensitive detection of labeled products as well as a selective method for trapping photolabeled products on an avidin-containing matrix.

Ligand characterization.—The relative rates of galactose transfer from UDP-galactose to **1** or BDGA were estimated, and both synthetic acceptors were found to be good substrates for GalT with K_m values of 0.17 mM or 0.06 mM, respectively (Fig. 1). Although the observed values are considerably lower than that of simple *N*-acetylglucosamine (2.0 mM [20]), the substitution at the reducing end of *N*-acetylglucosamine usually results in the significant improvement of binding [21]. FABMS measurements indicated that the product of the enzyme reaction had the desired molecular ion peak corresponding to the molecular formula of the galactosylated BDGA. From ^{1}H NMR spectroscopic analysis, the stereochemistry of the galactosyl bond was determined as having the β-configuration based on the large *J*-value ($J = 7.8$ Hz) determined from the H-1′ proton doublet at δ 4.32. The presence of a ^{1}H nuclear Overhauser effect between the galactose H-1′ proton and the glucosamine H-4 proton (7% enhancement of the H-4 proton) is also in consistent with the formation of β-(1 → 4) linkage [22]. Irradiation of an aqueous BDGA solution was performed at ca. 350 nm, which can selectively photolyze the diazirine ring out of the UV absorption wavelength of GalT. The changes of the diazirine bands upon photolysis are shown in Fig. 2 and a half-life of photodecomposition was determined as 2.1 min under our photolytic conditions (inset to Fig. 2).

Specific incorporation of BDGA into the GalT protein.—Bovine GalT was photolyzed with BDGA at a concentration about three times higher than its K_m value. Photolabeling was performed in the presence of UMP because the binding of GalT to *N*-acetylglucosamine is known to be enhanced in the presence of UMP or UDP [23]. After SDS–PAGE, the protein bands were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. The blotting pattern was processed with the chemiluminescent method described in our previous report for the detection of photochemically biotinylated components [12]. The silver staining pattern on the SDS–PAGE

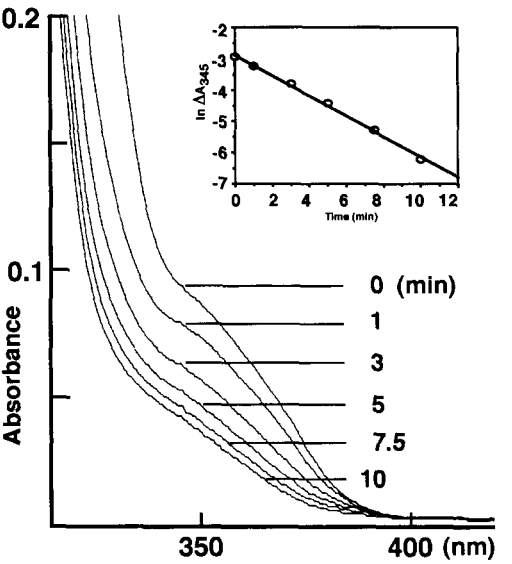


Fig. 2. Time course of BDGA photodecomposition. UV/VIS spectra of the photolyzed solution of 0.2 mM BDGA in H_2O at the times (in minutes) indicated. The inset shows the decrease in absorbance at 345 nm as the function of irradiation time in a semilogarithmic representation.

gel showed that the GalT used in the experiments gave two bands with apparent molecular weights of 42,000 and 51,000 (Fig. 3, panel A). These bands correspond to the two secretory forms which lack the first 78 or 96 amino acid residues of the full length protein [24,25], respectively. From the chemiluminescent pattern of the pho-

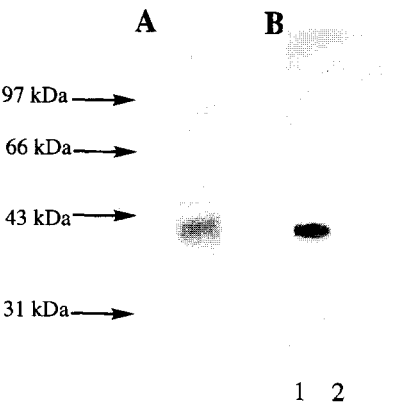


Fig. 3. Photoaffinity labeling of bovine GalT with BDGA. (A) The silver-stained gel of unlabeled bovine GalT (approximate ratio of two bands was 1:3). (B) Chemiluminescent detection pattern of GalT proteins incubated at 37 °C and photolabeled with BDGA (0.2 mM) in the absence (lane 1) or presence (lane 2) of the inhibitor **1** (100 mM). For SDS–PAGE (10%), 1.5 μg of protein per well was used.

Table 1
Yields of photolabeled GalT with BDGA

	37 °C	20 °C	4 °C	37 °C [a]	4 °C [a]
Absence of **1** (% [b])	4.1 ± 0.3	3.2 ± 0.2	1.5 ± 0.1	3.2 ± 0.2	0.7 ± 0.1
Presence of **1** (% [b])	0.1 ± 0.1	0.7 ± 0.2	0.9 ± 0.1	1.5 ± 0.2	0.7 ± 0.1
Specific labeling (% [b])	4.0 ± 0.3	2.5 ± 0.2	0.6 ± 0.1	1.7 ± 0.2	0

[a] Incubated and photolyzed without UMP.
[b] Mean values obtained from triplicate experiments.

tolyzed sample, both isoforms were labeled to the same extent as shown in lane 1 of panel B (Fig. 3). These chemiluminescent bands were remarkably diminished when the competitive acceptor substrate **1** had been included in the photolyzed sample (panel B, lane 2). This latter result is consistent with the observation that compound **1** inhibited 98% of galactose-transfer during the enzymatic galactosylation of BDGA as described in the ligand characterization. The loss of enzyme activity upon photoaffinity labeling was estimated as 6% based on the determination of V_{max} values of photolyzed GalT samples. Better yields of photoinactivation of GalT were reported using a large excess of an aryl azide donor analog [5] or diazirino-substituted acceptor saccharides [6]. However, neither report described a more direct estimation of the photolabeling yields for the SDS–PAGE-separated bands of the GalT protein. In some cases, the amount of photoincorporation was reported not to be linearly related to the extent of enzyme inactivation [5,26]. From a quantitative chemiluminescent analysis, the photolabeling yield of lane 1 was estimated as 4% (Table 1). This value agrees with the estimated amount of photoinactivation described above. To minimize undesired photolabeling other than at the binding site, the molar ratio of the reagent to the protein was set at 1:1 in our experiments. Under this condition, a significant amount of reactive intermediate could be lost by the quenching with solvent molecules if the carbene centre is poorly oriented for reaction with the protein. We have also experienced a low yield of cross-linking during the photoaffinity labeling study of sodium channels [27]. Although the yield of photolabeling is low, our results show that the labels could be incorporated within the acceptor substrate binding sites of GalT. The extent of labeling proceeded in a light dependent manner and the time course of photoincorporation is shown in Fig. 4. The photolabeling reached a maximal yield after 40 min and this value did not change upon increasing the irradiation time to 2 h. In the absence of light, BDGA is sufficiently stable under the experimental conditions and no detectable photolabeling was observed during incubation of samples at 37 °C over 2 h (Fig. 4). Using a crude protein mixture of recombinant human GalT (Fig. 5, lane 1), a band corresponding to the glutathione *S*-transferase fusion protein with an apparent molecular weight of 65 kDa was also specifically visualized by the chemiluminescent detection method (Fig. 5, lane 2).

Nature of acceptor binding.—Based on the quantitative densitometry of chemiluminescent detection, the photoaffinity labeling of the GalT protein with BDGA was further examined in order to probe the binding processes of the acceptor substrate. Upon lowering the incubation temperature from 37 to 20 °C or 4 °C, the amount of photoincorporation decreased considerably (Table 1, absence of **1**). In contrast, the

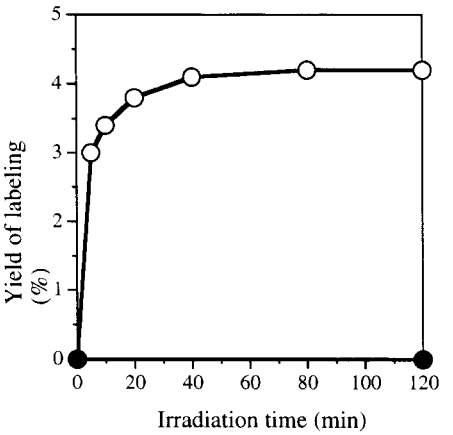


Fig. 4. Time course of label incorporation. The yields of labeling incorporated in the electroblotted GalT bands were determined by the chemiluminescent analysis of the following samples: —○— photolyzed, —●— incubated at 37 °C in the dark.

yields of photolabeling in the presence of **1** gradually increased by decreasing the temperature, as shown in Table 1. Consequently, the amount of specific photolabeling (i.e., the labelings that can be competed out with **1**) was greatly reduced by lowering the incubation temperature, as indicated in Table 1. Furthermore, the yield of photolabeling in the absence of UMP at 37 °C and 4 °C was lower than those in the presence of UMP, and the competitor **1** poorly inhibited the incorporation of the BDGA photolabel (Table 1). One possible explanation of this result could be the presence of some conformational changes which are affected by UMP [23] as well as the incubation temperature. Before the binding of substrates, the catalytic domain of GalT may be relatively exposed so as to facilitate the inclusion of substrates. Acceptor substrates could bind weakly at this stage, and this may explain why the photolabel could not be eliminated with the

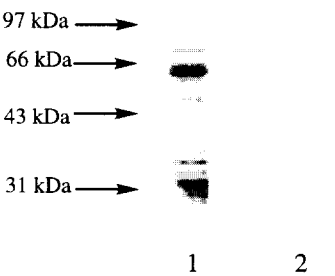


Fig. 5. Photoaffinity labeling of human GalT with BDGA. Silver staining pattern (lane 1) and chemiluminescent detection pattern (lane 2) of the crude recombinant GalT are shown. For SDS–PAGE (10%), 2 μg of protein per well was used.

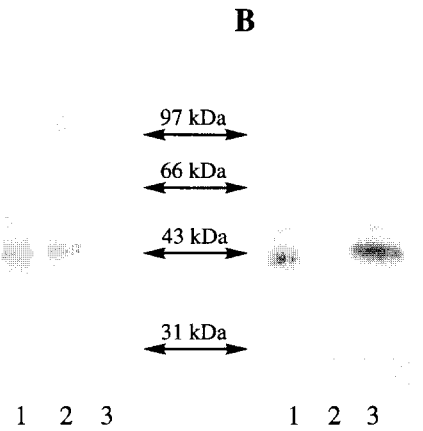


Fig. 6. Affinity purification of photolabeled GalT. A solution of photolyzed bovine GalT was chromatographed on an immobilized streptavidin column. (A) Silver-stained polyacrylamide gel (10%) of the following samples: before chromatography (lane 1), pass-through (lane 2), and extracts (lane 3). (B) Photographic result of chemiluminescent detection with the same set of samples.

competitive acceptor **1**. The binding of UMP and the acceptor substrate may induce a structural change that brings the binding domains closer together in order to facilitate subsequent glycosyl bond formation. At this stage, the photoprobe BDGA or **1** would exhibit better binding, thus resulting in the observed temperature- and UMP-dependent increment of specific labeling. The conformational changes may be promoted by binding of UMP and disturbed by lowering the incubation temperature. Recently, the X-ray crystallographic analysis of a bacteriophage β-glucosyltransferase revealed the presence of substrate-induced conformational changes [28]. Upon binding of the donor substrate, UDP-glucose, the two domains of β-glucosyltransferase experience motions resulting in a more closed and rigid conformation and increased interactions between the two domains. These structural changes may have a crucial role in effecting the highly specific nature of glycosylation. Molecules structurally different from the original substrates may bind to the recognition site but cannot induce the requisite conformational change to catalyze the enzyme reaction.

Selective retrieval of photolabeled GalT.—The photolyzed samples of bovine GalT were treated with immobilized streptavidin. The fractions were analyzed by SDS–PAGE, and their silver-staining patterns are shown in Fig. 6A. After blotting the gels, the membranes were processed by the chemiluminescent detection method and their patterns are shown in Fig. 6B. Most of the protein was recovered in the fraction that passed through the column (panel A, lane 2), whereas no detectable amount of GalT protein was contained in the SDS-treated fraction (panel A, lane 3). In contrast, no detectable chemiluminescence was observed in the lane of the unbound through fraction (panel B, lane 2) and the photoaffinity biotinylated GalT proteins were found to be selectively retrieved in the SDS fraction (panel B, lane 3). The yield was estimated as 61% from the duplicated experimental results of quantitative densitometry of the electroblotted PVDF membrane.

In conclusion, the results we have obtained demonstrate that covalent biotinylation using the novel diazirine-based photoaffinity probe, BDGA, is quite useful for radioisotope-free detection as well as specific manipulation of photolabeled GalT protein. Due to the observed low yield of photolabeling, more than 1 nmol of the GalT protein would be required to identify the crosslinking sites by the conventional Edman degradation method. The method of affinity isolation described here, however, may also be applicable for the isolation of photolabeled peptides from a tremendously complex enzyme digest, thus reducing potential difficulties arising from the conventional photoaffinity labeling method. The recent success in the identification of the hormone binding site within the insulin receptor using a biotinylated photoprobe is particulary noteworthy in this regard [29]. Because of the relative ease of the experiments, the photoprobe BDGA should be a useful tool for further exploring the detailed molecular aspects of GalT.

3. Experimental

General methods.—Methanol and dichloromethane were distilled and stored over molecular sieves. Silica gel for column chromatography was Kieselgel 60 (Merck, No. 7734, 70–230 mesh). Structural characterizations of synthetic products were performed with a Shimadzu UV-160 spectrophotometer (UV spectra), a JEOL JNM GX-400 spectrometer (^{1}H NMR spectra), and a JEOL JMS-HX110 spectrometer (fast atom bombardment mass spectra; FABMS). Bovine β-(1 → 4)-galactosyltransferase, nucleoside 5′-diphosphate kinase, UDP-glucose pyrophosphorylase, and UDP-glucose dehydrogenase, were purchased from Sigma. UDP-^{14}C-galactose was obtained from DuPont NEN and diluted with cold UDP-galactose to a specific activity of 10 mCi/mmol. Immobilized streptavidin was PIERCE UltraLink Immobilized Streptavidin Plus (specific binding 61 nmol biotinylated BSA/10 mL gel) and was pre-equilibrated with 10 mM Tris–HCl, pH 8.3 at room temperature. Biotinylated and premixed electrophoresis molecular weight standards were obtained from BioRad. Reagents for the determination of protein concentration were obtained from Pierce (BCA Protein Assay Reagent). All other reagents were commercially available and were used without further purification.

N^4-[2-(Acetamido)-2-deoxy-β-D-glucopyranosyl]-L-aspartamide (**2**).—The compound **1** [18] (434 mg, 1 mmol) was dissolved in trifluoroacetic acid (1 mL), and the solution was kept at room temperature for 30 min. After evaporation, the residue was dissolved in water (10 mL) and the solution was evaporated. This procedure was repeated three times to remove the remaining trifluoroacetic acid. The residue was dissolved in water (10 mL) and the solution was loaded on a column of Amberlite IR-120 (H^+ form, 10 mL). The column was washed with 100 mL of water and the product was eluted with 5% aqueous ammonia solution (100 mL). The eluate was lyophilized to give **2** as a colorless solid (308 mg, 92%); FABMS (glycerol) *m/z* 335 ($[M + H]^+$); high-resolution FABMS (glycerol): Calcd for $C_{12}H_{23}N_4O_7$: ($M + H^+$), 335.1567; Found: ($M + H^+$), 335.1564. The amine **2** was used in the next step without further purification.

N-[2-[2-[2-(2-Biotinylaminoethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzoyl]-N^4*-[2-(acetamido)-2-deoxy-β-*D*-glucopyranosyl]-*L*-aspartamide* (**BDGA**).—The amine **2** (33 mg, 0.1 mmol) and 2-[2-[2-(2-biotinylaminoethoxy)-ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid *N*-hydroxysuccinimide ester **3** [12] (60 mg, 0.1 mmol) were dissolved in dichloromethane–dimethylformamide (1:1, 2 mL). The solution was stirred for 14 h at room temperature. After evaporation of the solvents, the residue was dissolved in water (200 mL), and the solution was passed through a column of Dowex MB-X8 (5 mL). The aqueous solution was evaporated and the residue was chromatographed on silica gel (chloroform–methanol–water = 13:5:1) to give 79 mg (86%) of BDGA as a colorless solid; $[\alpha]_D$ + 32.0° (c = 0.5, MeOH); UV (MeOH): λ_{max} nm (ε) 294 (3200), 344 (400); ^{1}H NMR (CD_3OD): δ 7.97 (d, 1 H, $J_{CH5,CH6}$ 8.5 Hz, CH6-Ph), 7.02 (d, 1 H, CH5-Ph), 6.85 (s, 1 H, CH3-Ph), 4.96 (d, 1 H, $J_{1,2}$ 9.8 Hz, H-1), 4.45 (m, 1 H, NHC*H*CH_2S-biotin), 4.30 (m, 2 H, PhOCH_2), 4.20 (m, 1 H, NHC*H*CHS-biotin), 4.00 (m, 1 H, CHa-Asn), 3.90 (m, 2 H, H-6), 3.8–3.5 (m, 14 H, H-2, H-3, H-4, H-5, OC$H_2$$CH_2$ × 5), 3.16 (m, 1 H, SCH-biotin), 2.90 (m, 1 H, SCH_2exo-biotin), 2.80 (m, 2 H, CH_2 β-Asn), 2.70 (d, 1 H, $J_{exo,endo}$ 12.8 Hz, SCH_2endo-biotin), 2.20 (m, 2 H, $COCH_2$-biotin), 1.89 (s, 3 H, Ac), 1.7–1.5 (m, 4 H, $COCH_2$CH_2CH_2-biotin), 1.40 (m, 2 H, CH_2CH_2CH-biotin); FABMS (3-nitrobenzyl alcohol): *m/z* 920 ($[M + H]^+$); high-resolution FABMS (3-nitrobenzyl alcohol): Calcd for $C_{37}H_{53}N_9O_{13}F_3S$: ($M + H^+$), 920.3436; Found: ($M + H^+$), 920.3466.

Enzyme kinetics.—Initial rates of reaction were estimated spectrophotometrically using the method of Pierce [30]. The method was slightly modified as follows. Reactions were carried out at 37 °C in a quartz cuvette containing 10 mM $MnCl_2$, 1 mM nicotinamide adenine dinucleotide (NAD), 1 mM adenosine 5′-triphosphate, 1.6 mM glucose 1-phosphate, 0.1 mM UDP-galactose, 10 U nucleoside 5′-diphosphate kinase, 0.25 U UDP-glucose pyrophosphorylase, 0.02 U UDP-glucose dehydrogenase, and 0.005 U GalT in 1 mL of 50 mM *N*-ethylmorpholine, pH 8.0. The enzyme reaction was started by the addition of various concentrations of BDGA, and NADH formation was followed by monitoring the increase in absorbance at 340 nm. The competitive acceptor **1** was assayed similarly. The values of K_m for BDGA and **1** were calculated from Lineweaver–Burk plots of the assay results.

Product analysis of the enzyme reaction.—A solution of substrates, BDGA (4.8 mg, 5.2 μmol) and UDP-galactose (5.0 mg, 8.8 μmol), in 2.5 mL of 50 mM *N*-ethylmorpholine (pH 8.0) containing 2 mM $MnCl_2$ and GalT (0.25 U) was incubated at 37 °C for 3 days. After evaporation, the residue was chromatographed on silica gel (chloroform:methanol:water = 13:5:1), followed by lyophilization to give 5.3 mg (94%) of galactosylated BDGA as a colorless amorphous solid; ^{1}H NMR (CD_3OD:D_2O = 1:1): δ 7.85 (d, 1 H, $J_{CH5,CH6}$ 8.0 Hz, CH6-Ph), 7.00 (d, 1H, CH5-Ph), 6.81 (s, 1 H, CH3-Ph), 4.92 (d, 1 H, $J_{1,2}$ 10.0 Hz, H-1), 4.40 (m, 1 H, NHC*H*CH_2S-biotin), 4.32 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.24 (m, 3 H, PhOCH_2, NHC*H*CHS-biotin), 4.0–3.2 (m, 23 H, H-2, H-2′, H-3, H-3′, H-4, H-4′, H-5, H-5′, H-6, H-6′, OC$H_2$$CH_2$ × 5, CH α-Asn), 3.10 (m, 1 H, SCH-biotin), 2.85 (m, 1 H, SCH_2exo-biotin), 2.77 (m, 2 H, CH_2 β-Asn), 2.65 (d, 1 H, $J_{exo,endo}$ 12.8 Hz, SCH_2endo-biotin), 2.11 (m, 2 H, $COCH_2$-biotin), 1.83 (s, 3 H, Ac), 1.7–1.6 (m, 4 H, $COCH_2$CH_2CH_2-biotin), 1.30 (m, 2 H, CH_2CH_2CH-biotin);

NOE contacts: Gal H-1, GlcNAc H-4; FABMS (glycerol): *m/z* 1082 ($[M + H]^+$); high-resolution FABMS (glycerol): Calcd for $C_{43}H_{63}N_9O_{18}F_3S$: $(M + H^+)$, 1082.3970; Found: $(M + H^+)$, 1082.4040.

Inhibition of galactosylation of BDGA with **1**.—A set of 25 μL solutions containing BDGA (0.2 mM), UDP-^{14}C-galactose (0.2 mM), bovine GalT (25 mU), $MnCl_2$ (2 mM), and *N*-ethylmorpholine (50 mM, pH 8.0) was incubated with (100 mM) or without **1** at 37 °C for 1.5 h in the dark. Each mixture was then diluted with 25 μL of water and loaded on a streptavidin column (100 μL). The fraction passing through the column was loaded again on the same column; reloading was repeated 2 times in total. After washing the column with 800 μL of 10 mM Tris–HCl, pH 8.3, the gel was treated with 200 μL of denaturation buffer (50 mM sodium phosphate, pH 7.3, 2% SDS, 0.4 M urea) at 95 °C for 15 min, followed by the same treatment with 100 μL of the buffer. Supernatants were combined and subjected to liquid scintillation measurement. Incorporation of the radioactivity in the absence or presence of **1** was determined in triplicate and average values of incorporation were obtained as 25,000 dpm or 500 dpm, respectively. The results correspond to 98% inhibition of the GalT-catalyzed galactosylation.

Photolysis of BDGA.—A 0.2 mM solution of BDGA in distilled water was placed in a Pyrex vessel. After replacing the inner atmosphere with argon, photolysis was carried out at 4 °C with a black-light lamp apparatus (15 W × 2, Ultra-Violet Products Inc., San Gabriel, California, USA) with the reaction vessel at a distance of 6 cm from the surface of the light source.

Photoaffinity labeling of bovine GalT.—A sample solution (5 μL) containing bovine GalT (42 μg protein, 0.2 mM), BDGA (0.2 mM), UMP (0.5 mM), $MnCl_2$ (2 mM), and *N*-ethylmorpholine (50 mM, pH 8.0) was incubated in a glass tube (∅ 1 × 2.5 cm) at 37 °C for 10 min in the dark. The irradiation was carried out from the open end of the tube for 40 min at 0 °C with the same setup described above. As a control experiment, the same preparation of the above sample, but also containing 100 mM of **1**, was incubated and photolyzed simultaneously. Photolyzed samples were subsequently denatured in 6 mM Tris–HCl, 2% SDS, 5% β-mercaptoethanol, pH 6.8 for 30 min at room temperature before sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis (SDS–PAGE). The remaining activity of photolabeled enzyme was estimated using a control GalT which was simultaneously photolyzed without BDGA. The V_{max} values of these photolyzed GalT samples in the presence or absence of BDGA was determined as 0.247 nmol/min and 0.262 nmol/min, respectively, and the loss of enzyme activity upon photolabeling was estimated as 6% (±3%). After electrophoresis, the gels were fixed in an aqueous solution of 50% methanol–10% acetic acid before silver staining. The gels for chemiluminescent detection were not fixed and processed as described in the experimental section of 'Chemiluminescent detection of photolabeled products' (see below).

Preparation of recombinant human GalT.—The full-length human GalT cDNA subcloned in a pBluescript vector was first digested with *Acc*III which cleaves at position +227 corresponding to the *N*-terminus of the human GalT, and the *Acc*III site was replaced by a *Bam*HI site [31,32]. The cDNA fragment excised by *Bam*HI and *Eco*RI digestion, which encodes a soluble form of the GalT corresponding to the amino acid sequence +76–400 of the membrane-bound form, was inserted into *Bam*HI and

*Eco*RI sites in a pGEX-3X vector (Pharmacia), and the obtained recombined plasmid was named pGEX-GalT. pGEX-GalT encodes a fusion protein consisting of glutathione S-transferase (GST) and the soluble form of the human GalT. *E. coli* XL-1 Blue transfected with pGEX-GalT was grown in Luria-Bertani/*Amp* medium [32]. Isopropyl β-D-thiogalactopyranoside was added to the cell culture at a final concentration of 5 mM when the cells reached $OD_{600} = 0.5$. After an additional 4 h of cell growth, the cells were harvested to obtain the pGEX-GalT-directed protein. The cells were suspended in a buffer (MTPBS: 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 8.0) and washed three times with MTPBS at 4 °C. They were finally suspended in MTPBS supplemented with 2% Triton X-100 and protease inhibitors, and sonicated. The supernatant containing the solubilized fusion protein was applied to Glutathione–Sepharose (Sigma Chemical) at 4 °C [33]. The GST–GalT fusion protein adsorbed to the Glutathione–Sepharose beads was eluted with 5 mM glutathione in 50 mM Tris–HCl (pH 8.0) and used in the following experiments.

Photoaffinity labeling of recombinant human GalT.—A sample solution (12.5 μL) containing the human GalT (19 μg of protein), BDGA (0.024 mM), UMP (0.5 mM), $MnCl_2$ (2 mM), and *N*-ethylmorpholine (50 mM, pH 8.0) was incubated in a glass tube (∅ 0.5 × 1.5 cm) at 37 °C for 10 min in the dark. Irradiation and analysis of the sample were similarly performed as described for bovine GalT.

Chemiluminescent detection of photolabeled products.—Following SDS-PAGE, protein bands were electrotransferred onto a PVDF membrane (Immobilon P, Millipore) in 192 mM glycine, 25 mM Tris–HCl, 20% methanol, 0.1% SDS. Running time was 12 h at 12 mA, and a constant temperature of 4 °C was maintained. The transferred membrane was blocked for 1 h at room temperature with phosphate-buffered saline with Tween (T-PBS: 0.1 M sodium phosphate, pH 7.3, 0.15 M NaCl, 0.1% Tween-20) containing 2% skimmed milk and then washed with T-PBS (5 min, two times). After soaking the membrane in 1500 times diluted streptavidin-horseradish peroxidase conjugate (Amersham) for 1 h at room temperature, the membrane was developed using chemiluminescent detection reagents (RENAISSANCE, DuPont NEN) for 1 min. The membrane was then wrapped in a plastic sheet and exposed to Hyperfilm-ECL (Amersham) in the dark.

Quantitative densitometry.—Quantitative densitometry was performed on a MCID Image Analyzer (IMAGING Research Inc., Ontario, Canada) using biotinylated bovine serum albumin (PIERCE, ImmunoPure Biotin-LC-BSA, 8 mol biotin/mol BSA) as an external standard. After SDS–PAGE of the labeled proteins and various amounts of biotinylated BSA (1.2, 2.0, 2.7, 3.3, and 4.0 pmol as biotin molecule) on the same gel, the gel was blotted to the membrane and the blot was exposed to the film as described. The film was developed and the density of each band was measured based on pixels of the exposed area using the image analyzer's measurement scale (ROD values). A standard curve for calibration was prepared by plotting the ROD values against the mole of biotin; this curve was linear with a correlation coefficient of 0.995.

Effects of incubation temperature and UMP.—For the study of temperature effects, a pair of sample solutions, with or without **1**, was prepared as described in the photoaffinity labeling experiment. This set of samples was incubated for 10 min at 4 °C, 20 °C, or 37 °C, and photolyzed for 40 min at 0 °C. The similar pair of samples, not

containing UMP, was incubated at 4 °C or 37 °C for 10 min and photolyzed. The amounts of photolabeling were determined from quantitative chemiluminescent detection as described above.

Purification of labeled GalT with immobilized streptavidin.—Using the method described in the previous section, a sample solution containing bovine GalT (3.6 mg) was incubated with BDGA at 37 °C in the presence of UMP and photolyzed. The photolyzed sample was denatured in 6-fold volumes of 0.3 M Tris–HCl, pH 8.7 containing 7 M guanidine hydrochloride, 30 mM EDTA, and a 50-fold molar excess of β-mercaptoethanol. After 3 h at room temperature, a 500-fold molar excess of sodium iodoacetate was added directly to the denaturation mixture. Reductive carboxymethylation was performed at room temperature for 1 h in the dark under an argon atmosphere, and the sample solution was dialyzed against 10 mM Tris–HCl, pH 8.3 at 4 °C. An aliquot (1 mL, 0.8 mg protein, 19 nmol) of this sample was loaded on a streptavidin column (100 μL). The fractions passing through the column were repeatedly (2 times) loaded on the same column. The column was then successively washed with 200 μL of 20 mM sodium phosphate—0.4 M urea, pH 7.3, and 400 μL of 50 mM sodium phosphate—0.4 M urea containing 2% SDS, pH 7.3. Proteins trapped on the gel were extracted by heating the gel with 100 μL of the urea–SDS–phosphate buffer at 95 °C for 15 min and the extract was collected by centrifugation. After SDS–PAGE of the extract, the gels were subjected to silver staining or chemiluminescent detection.

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