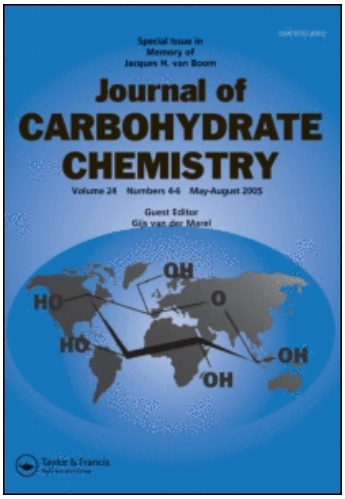

Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

Functional Fluorescence Labeling of Carbohydrates and Its Use for Preparation of Neoglycoconjugates

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To cite this Article Fukase, Koichi , Nakayama, Hideo , Kurosawa, Motohiro , Ikegaki, Toshiki , Kanoh, Takeshi , Hase, Sumihiro and Kusumoto, Shoichi(1994) 'Functional Fluorescence Labeling of Carbohydrates and Its Use for Preparation of Neoglycoconjugates', Journal of Carbohydrate Chemistry, 13: 5, 715 — 736

To link to this Article: DOI: 10.1080/07328309408011676

URL: http://dx.doi.org/10.1080/07328309408011676

J. CARBOHYDRATE CHEMISTRY, 13(5), 715-736 (1994)

FUNCTIONAL FLUORESCENCE LABELING OF CARBOHYDRATES AND ITS USE FOR PREPARATION OF NEOGLYCOCONJUGATES.[1]

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Received November 5, 1993 - Final Form February 15, 1994

ABSTRACT

New bifunctional reagents, 2-amino-6-carboxyethylpyridine and 2-amino-6-cyanoethylpyridine, were designed and synthesized in order to provide a novel procedure for preparation of neoglycoconjugates from fluorescence-labeled and purified sugar chains. Labeling of model sugar chains with these reagents was effected by reductive amination using $BH_3 \cdot Me_2NH$ to give corresponding 6-carboxyethylpyridylaminated (CEPA-) and 6-cyanoethylpyridylaminated (CNEPA-) derivatives, which were readily purified by reversed phase HPLC. The reagent parts of the labeled products possess the functional groups which then serve as linkers for coupling with matrices such as proteins and polymers. A CEPA-derivative of glucose was directly coupled with the ε-amino group of a Lys derivative to give a neoglycoprotein model. CNEPA-sugars were hydrogenated to give 6-aminopropylpyridylaminated (APPA-) derivatives, which can then be readily used for the preparation of various types of neoglycoconjugates by use of appropriate spacers as exemplified by the coupling of APPA-maltotriose with bovine serum albumin (BSA), biotin, and acrylic acid. The reaction of APPA-maltotriose with succinimidyl 3-(3-nitro-2-pyridyldithio)propionate gave the corresponding amide to be used for a disulfide formation with BSA. Condensation with biotin was effected by use of *N*-hydroxysuccinimidobiotin. The conjugation of APPA-maltotriose with acrylic acid was carried out by use of 4-acryloyloxyphenyldimethylsulfonium methylsulfate to give the corresponding acrylamide, which can be used for the preparation of sugar-acrylamide copolymers.

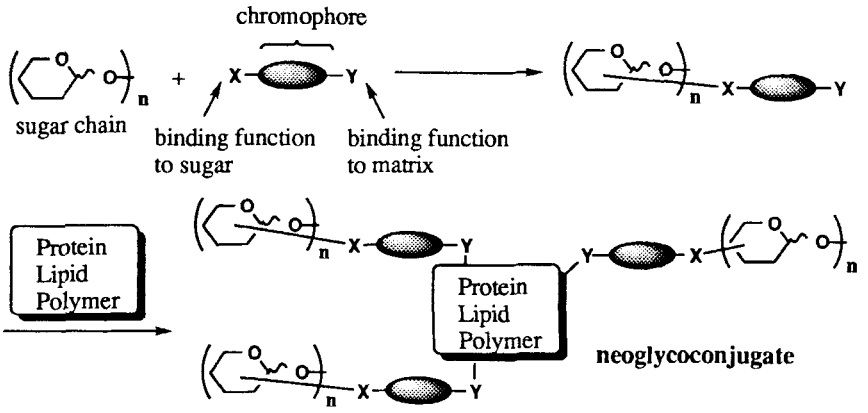


Fig. 1. Functional fluorescence labeling of a sugar chain and its application to the preparation of neoglycoconjugates.

INTRODUCTION

Recently, various types of artificial glycoconjugates have been frequently used for the elucidation of the biological functions including antigenecity of sugar chains.[2-12] These artificial glycoconjugates are termed neoglycoconjugates. The fluorescence labeling has proved to be a versatile procedure for purification and microanalysis of naturally occurring sugars, but never been applied to preparation of neoglycoconjugates because of the lack of the method for binding the purified labeled sugars to the matrices. Therefore, we developed a novel methodology for the preparation of neoglycoconjugates from fluorescence-labeled sugars by use of functional labeling reagents, which possess both the binding function to the carbohydrates and the linker moiety to the matrices such as proteins or polymers (Fig. 1).

RESULTS AND DISCUSSION

Pyridylamination using 2-aminopyridine (**1**), which was established by Hase and Ikenaka, is one of the most excellent methods for labeling of sugar chains.[13,14] Labeling reagent **1** is readily bound to the reducing terminal of sugars via Schiff base formation followed by reductive amination with BH_3•Me_2NH (Fig. 2). The resultant pyridylaminated sugars (PA-sugars) are effectively purified by reversed phase and/or size-

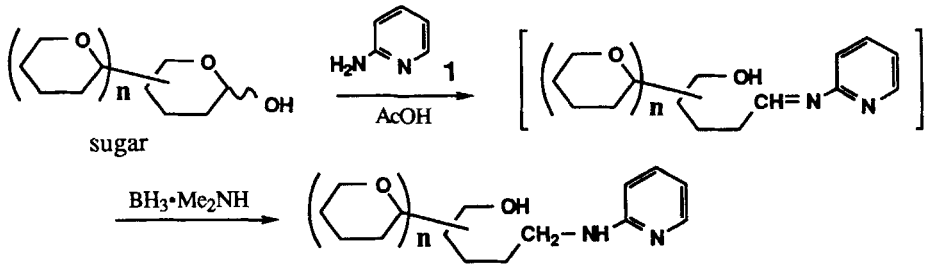


Fig. 2. Pyridylamination of a sugar with 2-aminopyridine.

fractionation HPLC. The labeled sugars less than picomole level can be detected with fluorescence spectrophotometer at an excitation wavelength of 320 nm and an emission wavelength of 400 nm. In view of such satisfactory features of PA-procedure, we decided to synthesize two functional labeling reagents based on the 2-aminopyridine molecule, i.e., 2-amino-6-carboxyethylpyridine (**2**) and 2-amino-6-cyanoethylpyridine (**3**) as shown in Fig. 3 and 4.

The ethyl ester of 2-amino-6-carboxypyridine **8** was easily prepared from commercially available 2-amino-6-methylpyridine (**4**) via *N*-acetylation, oxidation, deacetylation, and esterification according to the literature.[15] After *N*-tritylation, reduction by diisobutylaluminum hydride (DIBAL) gave an aldehyde **10**, which was condensed with ethyl malonate to give **11**. Hydrogenation of the olefinic bond, saponification of the ester, and subsequent decarboxylation gave the carboxyethyl derivative **13**. Final removal of the trityl group afforded the desired labeling reagent **2**.

Compound **3** was prepared as follows. Wittig reaction of the aldehyde **10** with $Ph_3P{=}CHCN$ gave an olefin **14** ($E : Z = 2 : 3$), the double bond of which was selectively hydrogenated to give **15**. Detritylation of **15** gave another labeling reagent **3**.

We then investigated the conditions for the fluorescence labeling of carbohydrates with the bifunctional labeling reagents, 2-amino-6-carboxyethylpyridine (**2**) and 2-amino-6-cyanoethylpyridine (**3**) thus prepared. The procedure for pyridylamination with 2-aminopyridine (**1**) was already established: a sugar derivative is treated with a solution of a large excess (ca. 100 equiv.) of 2-aminopyridine (**1**) in acetic acid at 90 °C to form a Schiff base, which is then reduced with BH_3•Me_2NH in acetic acid at 80 °C.[13,14] This method proved to cause almost no epimerization at the 2 position of reducing sugar units, almost no cleavage of even acid labile ketosidic bonds of sialic acids, and no reduction of carboxyl and amide groups.

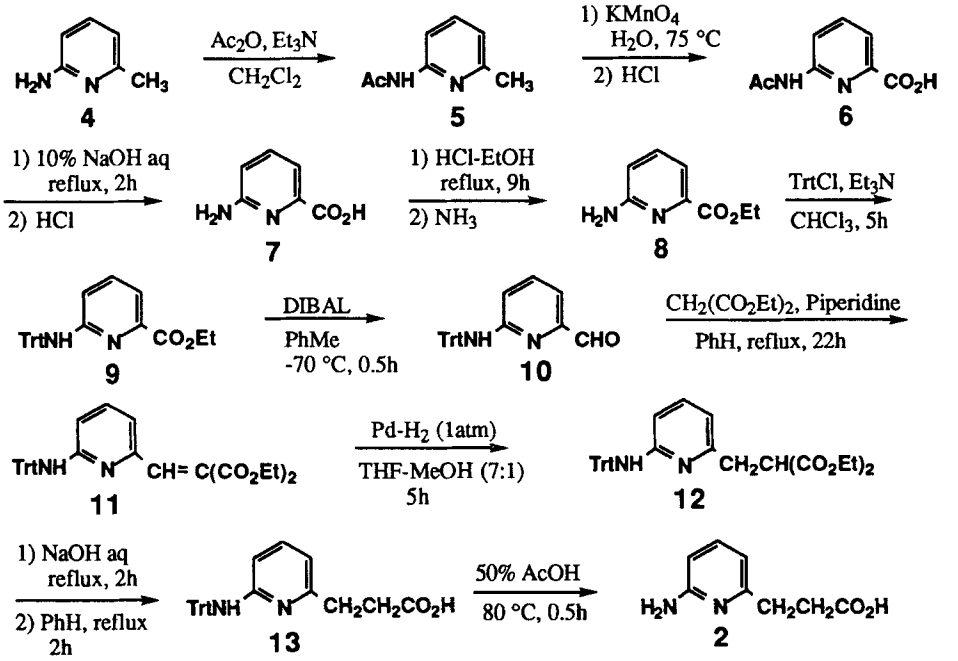


Fig. 3. Preparation of 2-amino-6-carboxyethylpyridine (2)

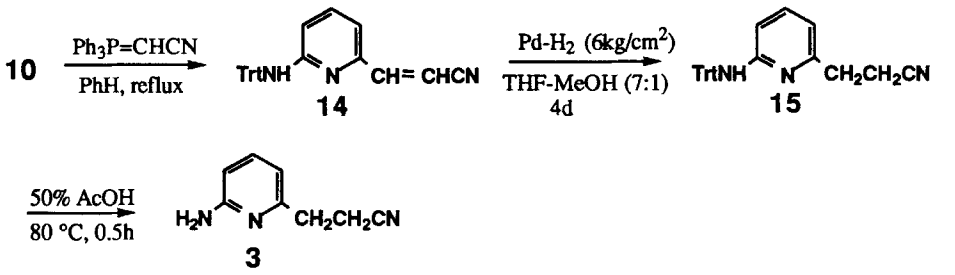


Fig. 4. Preparation of 2-amino-6-cyanoethylpyridine (3).

Labeling with the new reagents **2** and **3** was attempted under similar conditions where the cyano and carboxyl groups were stable. The amount of the labeling reagents was reduced to 20 equivalents since practically quantitative pyridylamination of glucose was effected with this amount of **1**. The reaction was carried out in much larger scales (1.0 - 20 μmol) than the standard analytical conditions of Hase et al. (0.01 - 10 nmol) in order to characterize the labeled products.

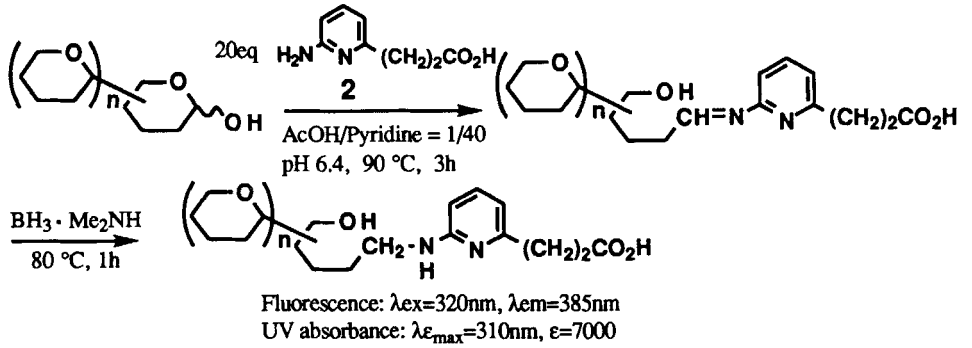


Fig. 5. Reductive amination with 2-amino-6-carboxyethylpyridine (**2**) to form CEPA-sugars.

Table 1. The results of reductive amination with 2.

product	yield	retention* time	relative intensity of fluorescence
CEPA-Glc (**16**)	93%	23.2min	1.3
CEPA-Man (**17**)	92%	23.7min	1.3
CEPA-maltose (**18**)	84%	21.4min	1.4
CEPA-maltotriose (**19**)	86%	20.9min	1.4
PA-Glc	-	24.1min	1.0

*Cosmosil 5C18AR: 20mm x 250mm, CH_3CN-0.1M NH_4OAc (pH 6.8), 0-10%(0.5%/min)-50%(2%/min), 8.0 mL/min

In the case of 2-amino-6-carboxyethylpyridine (**2**) which is less soluble in acetic acid (max. 46 mg / 200 μL) than 2-aminopyridine (**1**), a higher amount of acetic acid was required to bring the reaction mixture homogeneous than that used in the reaction with **1**. The use of more acetic acid and the presence of the additional carboxyl group in **2** render the reaction mixture acidic (pH 3). Labeling of D-glucose under such acidic conditions gave not only the desired (6-carboxyethyl-2-pyridyl)aminated glucose (**16**) (abbreviated as CEPA-Glc) but also a significant amount of the epimerized product at 2-position, i.e., CEPA-Man (**17**) (26%). We assumed that the formation of the manno-type epimer **17** was attributed to the conversion of the Schiff base into the 2-keto derivative by acid-catalyzed Amadori rearrangement and its reduction with BH_3•Me_2NH. Therefore,

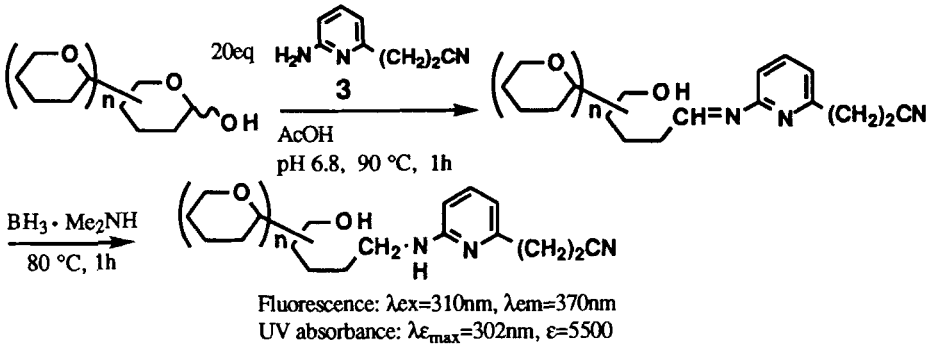


Fig. 6. Reductive amination with 2-amino-6-cyanoethylpyridine (**3**) to form CNEPA-sugars.

Table 2. The results of reductive amination with 3.

product	yield	retention* time	relative intensity of fluorescence
CNEPA-Glc (**20**)	93%	20.4min	0.51
CNEPA-maltose (**21**)	91%	19.2min	0.54
CNEPA-maltotriose (**22**)	83%	18.8min	0.54
PA-Glc	-	14.6min	1.0

*Cosmosil 5C18AR: 20mm x 250mm, CH_3CN-0.1M NH_4OAc (pH 6.8), 5-50%(1.5%/min), 8.0 mL/min

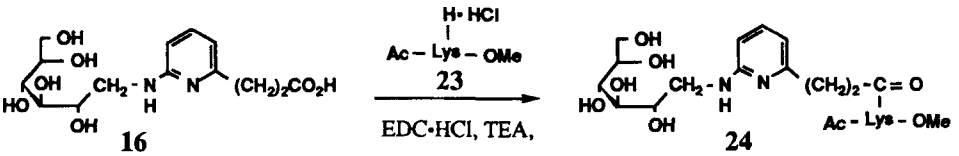
pyridine was used as a co-solvent [pyridine-acetic acid, 40:1 (v/v)] to maintain the reaction mixture essentially neutral (pH 6.4). By this modified procedure, reductive amination with **2** was achieved without epimerization though a longer reaction time (3 h) was required to complete the Schiff base formation: the reaction with **1** completed within 1 h (Fig. 5, Table 1). The resultant CEPA-sugars were readily purified by reversed phase HPLC (Cosmosil 5C18 AR). These CEPA-derivatives exhibited fluorescence at slightly shorter wavelengths of emission than that of conventional PA-derivatives (λex=320 nm, λem=400 nm).

In the case of 2-amino-6-cyanoethylpyridine (**3**) which had adequate solubility in acetic acid, formation of Schiff base was carried out without pyridine at pH 6.8 as in the reaction with 2-aminopyridine (**1**). Thus, the reductive amination with this reagent gave

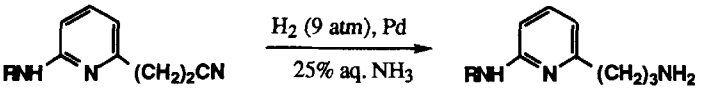
(6-cyanoethyl-2-pyridyl)aminated sugars (abbreviated as CNEPA-sugars) without epimerization in good yields (Fig. 6, Table 2). The CNEPA-sugars effectively purified by reversed phase HPLC exhibited less intensive fluorescence at shorter wavelengths on both excitation and emission than those of PA-derivatives.

Preparation of neoglycoconjugates was next investigated by use of the second functional group of the new reagents, 2-amino-6-carboxyethylpyridine (**2**) and 2-amino-6-cyanoethylpyridine (**3**) introduced in the fluorescence-labeled CEPA- or CNEPA-sugars.

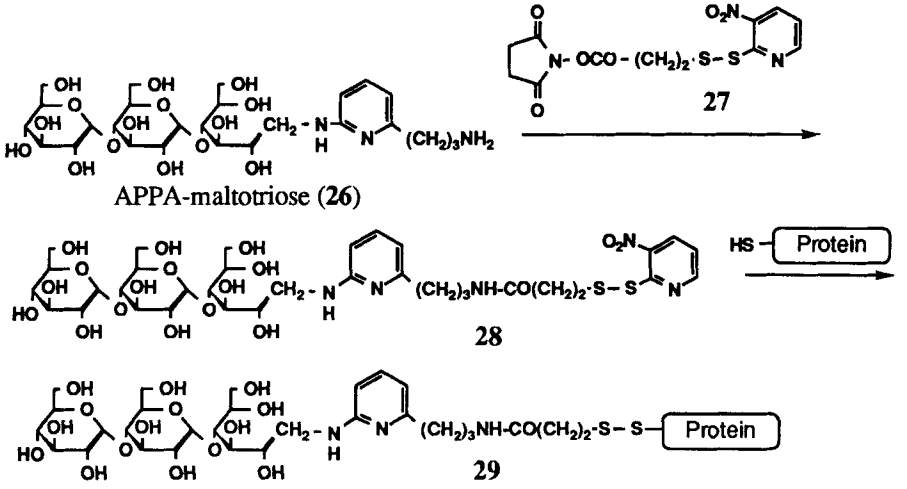
Conjugation of protein with other chemical species such as carbohydrates has been so far often effected through amide-bond formation.[2,3] Condensation of the carboxyl group of CEPA-Glc (**16**) was attempted with the ε-amino group of N^2-acetyl-L-lysine methyl ester (**23**) as a model preparation of a neoglycoprotein. By reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 1-hydroxybenzotriazol (HOBt) as coupling reagents in an aqueous solution at pH 6, the desired glycoconjugate **24** was obtained in 52% yield. The rather low yield was attributed to side reactions such as unavoidable formation of *N*-acylurea and hydrolysis of the active ester in an aqueous solution .



In order to improve the yield of glycoconjugate formation, we next examined the reduction of the nitrile function of CNEPA-sugars to a primary amine and its use for the cross-linking. Strong reducing agent such as lithium aluminum hydride could not be used for the reduction of the nitrile in the present case, since the reaction had to be carried out in an aqueous solution. We thus examined catalytic hydrogenolysis. Actually, the hydrogenation proceeded with Pd-black as a catalyst at 9 kg/cm^2 of H_2, whereas no reaction occurred at 5 kg/cm^2. In order to prevent side reactions leading to secondary amine formation during the catalytic hydrogenation of nitrile, the hydrogenation was carried out in 25% aqueous ammonia under high dilution conditions (2.3 mM of substrates). The (6-aminopropyl-2-pyridyl)aminated (abbreviated as APPA-) derivatives thus formed were readily purified by reversed phase HPLC (column: Cosmosil 5C18 AR, solvent: CH_3CN-0.1% TFA). APPA-Glc (**25**) and APPA-maltotriose (**26**) were obtained in 90% and 77% yields after isolation, respectively.



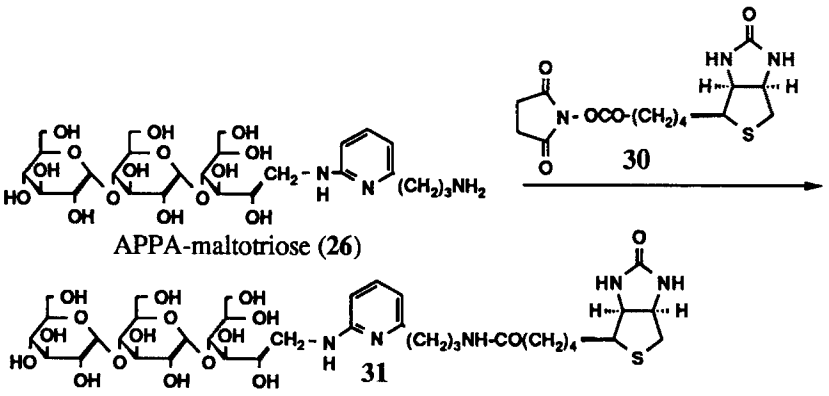
The APPA-sugars is expected to be attached to amino groups of proteins using a variety of spacers possessing two binding sites to amino groups, e.g., glyoxal, disuccinimidyl suberate, and so on.[16-20] Binding will also be possible of the APPA-sugars with mercapto groups of proteins by use of reagents such as succinimidyl 3-(2-pyridyldithio)propionate[21] or succinimidyl 6-maleimidehexanoate.[22] These reagents link to APPA-sugars giving corresponding amides containing 2-pyridyldithio or maleimide functions, which react with cysteine residues of a protein via disulfide exchange of the 2-pyridinesulfenyl group or addition of a thiol to the maleimide part of the reagents, respectively.[16-20] Matsueda et al. showed that *S*-3-nitro-2-pyridinesulfenyl (Npys) group is quite effective for the formation of disulfide bonds with mercapto groups via disulfide exchange.[23,24] We therefore prepared a bifunctional reagent, succinimidyl 3-(3-nitro-2-pyridyldithio)propionate (**27**), for conjugation of APPA sugars with proteins in the present study.



Ditrifluoroacetic acid salt of APPA-maltotriose (**26**) was coupled with **27** in a solution of tetrahydrofuran (THF)-water (2:1) at pH 9 in the presence of TEA. The product was readily purified by reversed phase HPLC (column: Cosmosil 5C18 AR, solvent: CH_3CN- aqueous 0.1% TFA). Fluorescence-labeled sugar possessing spacer **28** was thus obtained as a ditrifluoroacetic acid salt in 70% yield. We then examined the conjugation of **28** with bovine serum albumin (BSA) which possess 6 cysteine residues in the molecule. The reaction was carried out with various molar ratio of **28** to BSA in 0.5 M tris(hydroxymethyl)aminomethane - 0.005 M ethylenediaminetetraacetic acid disodium salt buffer (pH 7.5) at 4 °C. Since BSA was not eluted from reversed phase HPLC (column: Cosmosil 5C18 AR), the progress of the reaction was followed by gel-filtration HPLC [column: Asahipak GS-510, solvent: 30% CH_3CN-0.2M sodium

phosphate buffer (pH 7.0)]. Compound **28** was clearly separated from the intact BSA and the conjugated BSA **29**, though the latter two were not separated from each other in this system. The decrease in the amount of **28** or the increase in the UV absorption intensity of the BSA fractions were observed in HPLC until the reaction was completed within 30 min. The amounts of the labeled sugars linked to BSA were determined either by the increase in the intensity of UV absorption due to the conjugated BSA **29** (method 1) or by the decrease in the peak area of **28** (method 2) in HPLC. The results are shown in Table 3. The labeled sugar was bound to BSA in about a 1:1 molar ratio in each case (entry 2-3) except for the case where 1 equivalent of **28** to BSA (entry 1) was used. These results indicates that only one peripheral cysteine residue was used for conjugation under the present reaction conditions[25] and the disulfide exchange proceeded rapidly enough between the 3-nitro-2-pyridyldithio group and the particular mercapto group of the cysteine residue in BSA.

The highly specific affinity between biotin and avidin (or streptavidin) have been utilized for affinity chromatography, affinity cytochemistry, immunoassay, and so on.[26] Biotin-sugar conjugates are also useful for investigation of various biological aspects of sugar chains, such as investigation of the effects of the protein matrix on glycan processing.[12] A polymer containing both sugar and biotin moieties was constructed for ELISA of lectins.[11] Biotin was readily attached to APPA-maltotriose (**26**) by use of *N*-hydroxysuccinimidobiotin (**30**) under mild reaction conditions.[27] Such conjugates are expected to be useful for identification of lectins which interact with particular sugar chains.

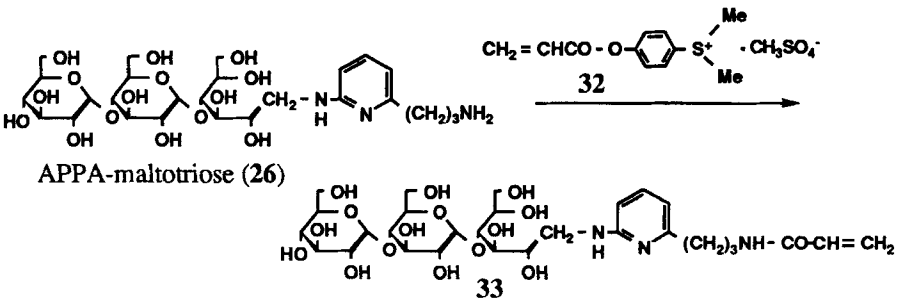


Synthetic polymers attached to particular carbohydrate moieties are also of great importance as neoglycoconjugates.[6-11] Particularly, sugar-acrylamide copolymers have been frequently used for immunological studies of sugar chains. The coupling of acrylic

Table 3. The amounts of labeled sugar linked to BSA.

entry	28 : BSA (mol / mol)	the amounts of sugars linked to BSA (method 1)	the amounts of sugars linked to BSA (method 2)
1	1 : 1	0.66	0.79
2	1.2 : 1	0.91	1.1
3	2 : 1	0.93	1.1
4	6 : 1	1.1	1.1

acid with APPA-maltotriose (**26**) was also examined. The reaction was carried out by use of water soluble active ester of acrylic acid 4-acryloyloxyphenyldimethylsulfonium methyl sulfate (**32**) in $NaHCO_3$ buffer (pH 9).[28] Acrylamide derivative **33** was thus obtained in 80% yield. Such acrylamide derivatives will be readily co-polymerized with acryl amide.



As described in this paper, the bifunctional fluorescence-labeling reagents, 2-amino-6-carboxyethylpyridine (**2**) and 2-amino-6-cyanoethylpyridine (**3**), were readily introduced to the reducing end of sugar chains by reductive amination. The resultant labeled sugars were easily purified by HPLC and successfully used for the construction of neoglycoconjugates, which are useful for the investigation of various biological functions of sugar chains. Possible applications of this methodology could also be directed to photoaffinity labeling of lectins or receptors of carbohydrates. For example, APPA-sugars will be readily coupled with photoactivable bifunctional reagents,[20] e.g., succinimidyl 4-azidobenzoate,[29] succinimidyl 4-azidobenzoylglycinate,[30] and so on. Radio-labeling of APPA-sugars will also be possible by use of appropriate radio-labeling reagents.[20]

EXPERIMENTAL

General Procedures. All melting points are uncorrected. ^1H NMR spectra were obtained with JEOL JNM-GSX 270 or JNM-EX 270 spectrometers. The chemical shifts are given in δ values either from TMS (0 ppm) as an internal standard in $CDCl_3$ solutions or from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (0 ppm) or acetone (2.22 ppm) as the internal standards in D_2O solutions. EI-MS and FAB-MS spectra were obtained with a JEOL SX-102 mass spectrometer. HPLC was carried out with a Shimadzu LC-6AD liquid chromatograph with a Cosmosil 5C18 AR (Nacalai Tesque) column or an Asahipak GS-510 (Shoko Co., Ltd) column. UV spectra were obtained with Shimadzu UV-160A UV-visible recording spectrophotometer. Fluorescent spectra were measured with Hitachi 650-40 fluorescent spectrophotometer. Silica gel column chromatography was carried out with Merck silica gel 60 (230-400 mesh) at medium pressure (2-10 kg/cm^2). Organic solutions were dried over $MgSO_4$ and concentrated *in vacuo*.

6-Acetamido-2-methylpyridine (5). To a solution of 6-amino-2-methylpyridine (**4**) (10.0 g, 92.6 mmol) and triethylamine (TEA) (12.9 mL, 92.6 mmol) in CH_2Cl_2 (100 mL) was added acetic anhydride (19.2 mL, 204 mmol) at 0 °C. The solution was stirred at room temperature for 18 h, and then concentrated. The residue was dissolved in ether and washed with an aqueous saturated $NaHCO_3$ solution and brine and worked up as usual. The crystalline residue was recrystallized from toluene and hexane: Yield 13.6 g (97.9%); mp 86-87 °C .

Anal. Calcd for $C_8H_{10}ON_2$: 63.98; H, 6.71; N, 18.65. Found: C, 64.17; H, 6.71; N, 18.57.

6-Acetamidopicolinic Acid (6). To a solution of **5** (100 g, 0.77 mol) in water (1 L) was added $KMnO_4$ powder (263 g, 1.7 mol) portionwise over 2 h at 75 °C, and the reaction mixture was stirred at 75 °C for 2 h. Solids were removed from the hot mixture by filtration, and the filtrate washed with $CHCl_3$. The aqueous layer was concentrated *in vacuo* to 500 mL, and then acidified with conc HCl to give crystals: Yield 65.7 g (54.7%); mp 213-214 °C.

Anal. Calcd for $C_8H_8O_3N_2$: C, 53.33; H, 4.48; N, 15.55. Found: C, 53.19; H, 4.52; N, 15.50.

Compound **5** was recovered from the $CHCl_3$ layer: 35.5 g (35.5%).

6-Aminopicolinic Acid (7). A solution of **6** (80.0 g, 0.444 mol) in an aqueous 10% NaOH solution (400 mL) was refluxed for 2 h. The solution was cooled to room temperature and then neutralized with conc HCl to give colorless crystals: 54.5 g (88.0%); mp 300-305 °C (dec.).

Anal. Calcd for $C_6H_6O_2N_2$: C, 52.17; H, 4.38; N, 20.28. Found: C, 51.89; H, 4.31; N, 20.05.

Ethyl 6-Aminopicolinate (8). A suspension of **7** (49.5 g, 0.359 mol) in EtOH (500 mL) saturated with HCl was refluxed for 9 h. The resultant solution was concentrated. To an aqueous solution of the residue was added excess 25% aqueous NH_3. The mixture was extracted three times with $CHCl_3$ and worked up as usual to give colorless solid: Yield 32.5 g (54.5%) ; mp 72-73 °C.

Anal. Calcd for $C_8H_{10}O_2N_2$: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.96; H, 6.01; N, 16.83.

Ethyl 6-Tritylaminopicolinate (9). To a solution of **8** (4.06 g, 24.5 mmol) in $CHCl_3$ (50 mL) were added TEA (4.08 mL, 29.3 mmol) and trityl chloride (7.47 g, 26.8 mmol), and the solution was stirred at room temperature for 5 h. The solution was washed with an aqueous saturated $NaHCO_3$ solution and brine and worked up as usual. The residue was purified by silica gel column chromatography (180 g, toluene-AcOEt=19:1) to give crystals: Yield 9.96 g (100%); mp 53-58 °C.

Anal. Calcd for $C_{27}H_{24}O_2N_2$: C, 79.39; H, 5.92; N, 6.86. Found: C, 79.55; H, 6.02; N, 6.57.

2-Tritylamino-6-formylpyridine (10). To a solution of **9** (4.06 g, 9.95 mmol) in toluene (140 mL) was added a 1 M solution of diisobutylaluminum hydride (DIBAL) in toluene (18.0 mL, 18.0 mmol) dropwise at -70 °C over 20 min under a N_2 atmosphere. After the solution was stirred at -70 °C for 30 min, ether (20 mL) and aqueous 20% acetic acid (10 mL) were added to it. The solution was stirred for 30 min, warmed to room temperature, and then excess aqueous NH_3 was added. The precipitates were removed by filtration. The filtrate was diluted with toluene, washed with brine, and worked up as usual. The residue was purified by silica gel column chromatography (30 g, $CHCl_3$) to give colorless crystals: Yield 3.06g (84.5%); mp 208-209 °C (dec.); positive EI-MS m/z 364 $(M)^+$.

Anal. Calcd for $C_{25}H_{20}ON_2$: C, 82.39; H, 5.53; N, 7.69. Found: C, 82.47; H, 5.58; N, 7.58.

Ethyl 2-Ethoxycarbonyl-3-(6-tritylamino-2-pyridyl)acrylate (11). A solution of compound **10** (2.20 g, 6.04 mmol) in benzene (60 mL), diethyl malonate (1.53 mL, 10.0 mmol) and piperidine (100 mL) were put into Dean-Stark apparatus, and the solution was refluxed for 22 h. The solution was diluted with benzene, washed with an aqueous saturated $NaHCO_3$ solution and brine, and worked up as usual. The residue was purified by silica gel column chromatography (180 g, toluene-AcOEt=50:1) to give colorless crystals: Yield 2.56 g (83.8%); mp 146-150 °C; positive EI-MS m/z 506 $(M)^+$.

Anal. Calcd for $C_{32}H_{30}O_4N_2$: C, 75.87; H, 5.97; N, 5.53. Found: C, 76.03; H, 5.97; N, 5.52.

Diethyl (6-Tritylamino-2-pyridyl)methylmalonate (12). Compound **11** (1.28 g, 2.52 mmol) was hydrogenated with Pd-black (500 mg) in THF-MeOH [7:1 (v/v), 32 mL] at room temperature under a H_2 atmosphere for 5 h. The catalyst was removed by filtration, and the filtrate was concentrated to give colorless crystals: Yield 1.27g (99.5%); mp 72-74 °C; positive EI-MS m/z 508 $(M)^+$.

Anal. Calcd for $C_{32}H_{32}O_4N_2$: C, 75.57; H, 6.34; N, 5.51. Found: C, 75.76; H, 6.34; N, 5.45.

2-Tritylamino-6-(2-carboxyethyl)pyridine (13). A suspension of **12** (1.02 g, 2.00 mmol) in aqueous 50% NaOH solution (20 mL) was refluxed for 2 h. The mixture was acidified to pH 4 with aqueous 10% citric acid at 0 °C, and then extracted with $CHCl_3$. The organic layer was washed with brine and worked up as usual. The residue was dissolved in benzene (40 mL) and refluxed for 2 h. Evaporation of the solvent gave colorless crystals: 760 mg (99.1%); mp 110-120 °C; ^1H NMR ($CDCl_3$) δ 7.35-7.21 (15H, m, Ph-, -NH-Trt), 7.13 (1H, dd, J=8Hz, J=7Hz, H-4), 6.46 (1H, d, J=7Hz, H-3 or 5), 5.88 (1H, d, J=8Hz, H-5 or 3), 3.01 (2H, t, J=6Hz, $-CH_2CH_2-$), 2.82 (2H, t, J=6Hz, $-CH_2CH_2$); positive FAB-MS m/z 409 $[(M+H)^+]$.

Anal. Calcd for $C_{27}H_{24}O_2N_2$: C, 77.67; H, 6.04; N, 6.71. Found: C, 77.78; H, 5.92; N, 6.65.

2-Amino-6-(2-carboxyethyl)pyridine (2). A mixture of **13** (697 mg, 1.71 mmol) in aqueous 50% acetic acid (10 mL) was refluxed for 30 min to give precipitates which were removed by filtration. The filtrate was washed with toluene, and the aqueous layer was concentrated *in vacuo* to give colorless crystals: yield 273 mg (96.6%); mp 166 °C (dec.); ^1H NMR (D_2O) δ 7.79 (1H, dd, J=9Hz, 7Hz, H-4), 6.81 (1H, d, J=9Hz, H-3 or 5), 6.73 (1H, d, J=7Hz, H-5 or 3), 2.96 (2H, t, J=7Hz, $-CH_2CH_2-$), 2.58 (2H, t, J=7Hz, $-CH_2CH_2-$); positive FAB-MS m/z 167 $[(M+H)^+]$.

Anal. Calcd for $C_8H_{10}O_2N_2 \cdot 0.23H_2O$: C, 56.41; H, 6.19; N, 16.45. Found: C, 56.41; H, 6.15; N, 16.49.

3-(6-Tritylamino-2-pyridyl)-2-propenenitrile (14). A solution of 2-tritylamino-6-formylpyridine (**10**) (1.00 g, 2.74 mmol) and Ph_3P=CHCN (4.13 g, 5.00 mmol) in benzene (15 mL) was refluxed for 20 min under a N_2 atmosphere. The solution was then concentrated *in vacuo*, and the residue was purified by silica gel column chromatography (50 g, toluene-AcOEt=50:1) to give **14** (1.05 g, 99%) as the mixture of *E*- and *Z*-isomers (*E*:*Z*=2:3). Both isomers were partially separated by silica gel column chromatography.

E-isomer: mp 222-224 °C (dec.); ^{1}H NMR ($CDCl_3$) δ 7.35-7.19 (15H, m, Ph-), 7.12 (1H, dd, J=8Hz, 7Hz, H-4), 7.12 (1H, d, J=16Hz, -CH=CHCN), 6.50 (1H, d, J=7Hz, H-3 or 5), 6.17 (1H, d, J=16Hz, -CH=CHCN), 6.05 (1H, s, -NH-Trt), 6.03 (1H, d, J=8Hz, H-5 or 3); positive FAB-MS m/z 388 [(M+H)$^+$].

Anal. Calcd for $C_{27}H_{21}N_3$: C, 83.69; H, 5.46; N, 10.84. Found: C, 83.36; H, 5.38; N, 10.67.

Z-isomer: mp 138-141 °C (dec.); ^{1}H NMR ($CDCl_3$) δ 7.35-7.20 (15H, m, Ph-), 7.10 (1H, m, H-4), 6.96 (2H, m, H-3 or 5, -CH=CHCN), 6.23 (1H, s, -NH-Trt), 5.87 (1H, d, J=9Hz, H-5 or 3), 5.49 (1H, d, J=12Hz, -CH=CHCN); positive FAB-MS m/z 388 [(M+H)$^+$].

Anal. Calcd for $C_{27}H_{21}N_3$: C, 83.69; H, 5.46; N, 10.84. Found: C, 83.36; H, 5.38; N, 10.67.

3-(6-Tritylamino-2-pyridyl)propionitrile (15). Compound **14** (11.7 g, 31.0 mmol) was hydrogenated with Pd-black (560 mg) as a catalyst in THF-MeOH [7:1 (v/v), 50 mL] under 6 kg/cm^2 of H_2 at room temperature for 4 d. After the catalyst was removed by filtration, the filtrate was concentrated. The crude product was purified by silica gel column chromatography (180 g, toluene-AcOEt=19:1) to give colorless crystals: yield 11.3 g (93.4%); mp 47-49 °C; ^{1}H NMR ($CDCl_3$) δ 7.35-7.19 (15H, m, Ph-), 7.04 (1H, dd, J=8Hz, 7Hz, H-4), 6.38 (1H, d, J=7Hz, H-3 or 5), 5.93 (1H, s, -NH-Trt), 5.84 (1H, d, J=8Hz, H-5 or 3), 2.80 (2H, t, J=7Hz, -CH_2CH_2CN), 2.57 (2H, t, J=7Hz, -CH_2CH_2CN); positive FAB-MS m/z 390 [(M+H)$^+$].

Anal. Calcd for $C_{27}H_{23}N_3$: C, 83.26; H, 5.95; N, 10.79. Found: C, 83.37; H, 5.92; N, 10.59.

2-Amino-6-(2-cyanoethyl)pyridine (3). Detritylation of **15** (1.07 g, 2.75 mmol) was carried out in a manner similar to the preparation of **2**. After the precipitates were removed by filtration, the filtrate was made alkaline with excess aqueous 5% NH_3. The product was extracted with $CHCl_3$ and the organic layer worked up as usual. The residue was purified by silica gel column chromatography (50 g, toluene-AcOEt=19:1) to give colorless crystals: yield 350 mg (87.0%); mp 66-68 °C; ^{1}H NMR ($CDCl_3$) δ 7.45 (1H, dd, J=8Hz, 7Hz, H-4), 6.59 (1H, d, J=7Hz, H-3 or 5), 6.46 (1H, d, J=8Hz, H-5 or 3), 4.89 (2H, bs, -NH_2), 2.97 (2H, t, J=7Hz, -CH_2CH_2CN), 2.82 (2H, t, J=7Hz, -CH_2CH_2CN); positive EI-MS m/z 147 (M$^+$).

Anal. Calcd for $C_8H_9N_3$: C, 65.29; H, 6.16; N, 28.55. Found: C, 65.28; H, 6.05; N, 28.48.

Reductive Amination Using 2-Amino-6-(2-carboxyethyl)pyridine (2): General Procedure. A mixture of a sugar (11.1 μmol) and compound **2** (36.9 mg, 222 μmol) in acetic acid-pyridine [1:40 (v/v), 540 μL] was heated at 90 °C in a sealed

tube for 3 h. After cooling to room temperature, a solution of BH_3•Me_2NH in acetic acid [(0.195 mg/μL), 200 μl, 662 μmol] was added. The mixture was heated at 80 °C in a sealed tube for 1 h, cooled to room temperature, and lyophilized. The residue was dissolved in H_2O and then purified by HPLC [column: Cosmosil $5C_{18}$ AR, 20 mm x 250 mm; solvent: CH_3CN-0.1M $CH_3CO_2NH_4$ (pH 6.9); gradient: 0% CH_3CN - 10% CH_3CN (0.5 %/min) - 50% CH_3CN (2 %/min); flow rate: 8 mL/min; detection: UV at 300 nm].

Determination of the Yields of CEPA-sugars. The yields of CEPA-sugars were determined by means of HPLC [column: Cosmosil $5C_{18}$ AR, 4.6 mm x 250 mm; solvent: CH_3CN-0.1M $CH_3CO_2NH_4$ (pH 6.9); gradient: 0% CH_3CN - 5% CH_3CN (0.16 %/min) - 45% CH_3CN (4 %/min); flow rate: 1.0 mL/min; detection: UV at 314 nm] using a solution of CEPA-Glc (**16**) in water as an external standard. The yields with various model sugars are listed in Table 1.

1-Deoxy-1-[(6-carboxyethyl-2-pyridyl)amino]-D-glucitol (CEPA-Glc) (16). ^1H NMR (D_2O, acetone) δ 7.79 (1H, dd, J=7Hz, 9Hz, H-4), 6.88 (1H, d, J=9Hz, H-3 or 5), 6.71 (1H, d, J=7Hz, H-5 or 3), 4.1-3.5 (8H, glucitol H-1 - H-6), 2.99 (2H, t, J=7Hz, -$C\underline{H}_2CH_2CO_2H$), 2.62 (2H, t, J=7Hz, -$CH_2C\underline{H}_2CO_2H$); positive FAB-MS m/z 331.0 [$(M+H)^+$].

Calcd for $C_{14}H_{22}O_7N_2$•$1.5H_2O$: C, 47.05; H, 7.05; N, 7.84. Found: C, 46.81; H, 6.86; N, 8.01.

1-Deoxy-1-[(6-carboxyethyl-2-pyridyl)amino]-D-mannitol (CEPA-Man) (17). ^1H NMR (D_2O, acetone) δ 7.76 (1H, dd, J=7Hz, 9Hz, H-4), 6.88 (1H, d, J=9Hz, H-3 or 5), 6.69 (1H, d, J=7Hz, H-5 or 3), 4.0-3.5 (8H, mannitol H-1 - H-6), 2.97 (2H, t, J=7Hz, -$C\underline{H}_2CH_2CO_2H$), 2.60 (2H, t, J=7Hz, -$CH_2C\underline{H}_2CO_2H$); positive FAB-MS m/z 331.0 [$(M+H)^+$].

Anal. Calcd for $C_{14}H_{22}O_7N_2$•$1.1H_2O$: C, 48.02; H, 6.97; N, 8.00. Found: C, 48.03; H, 6.75; N, 8.06.

***O*-α-D-Glucopyranosyl-(1→4)-1-deoxy-1-[(6-carboxyethyl-2-pyridyl)amino]-D-glucitol (CEPA-maltose) (18).** ^1H NMR (D_2O, DSS) δ 7.77 (1H, dd, J=7Hz, 9Hz, H-4), 6.85 (1H, d, J=9Hz, H-3 or 5), 6.70 (1H, d, J=7Hz, H-5 or 3), 5.13 (1H, d, J=4Hz, Glc H-1), 4.08-3.39 (14H, glucitol H-1 - H-6, Glc H-2 - H-6), 2.97 (2H, t, J=7Hz, -$C\underline{H}_2CH_2CO_2H$), 2.61 (2H, t, J=7Hz, -$CH_2C\underline{H}_2CO_2H$); positive FAB-MS m/z 493.2 [$(M+H)^+$].

Anal. Calcd for $C_{20}H_{32}O_{12}N_2$•$1.64H_2O$: C, 46.02; H, 6.81; N, 5.37. Found: C, 46.02; H, 6.70; N, 5.47.

***O*-α-D-Glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-1-deoxy-1-[(6-carboxyethyl-2-pyridyl)amino]-D-glucitol (CEPA-**

maltotriose) (19). ^{1}H NMR (D_2O, DSS) δ 7.78 (1H, dd, J=7Hz, 9Hz, H-4), 6.86 (1H, d, J=9Hz, H-3 or 5), 6.70 (1H, d, J=7Hz, H-5 or 3), 5.40 (1H, d, J=4Hz, Glc H-1), 5.14 (1H, d, J=4Hz, Glc H'-1), 4.06-3.41 (20H, glucitol H-1 - H-6, Glc H-2 - H-6, Glc H-2 - H-6), 2.97 (2H, t, J=7Hz, -C<u>H</u>$_2$CH$_2$CO$_2$H), 2.61 (2H, t, J=7Hz, -CH$_2$C<u>H</u>$_2$CO$_2$H); positive FAB-MS m/z 655.2 [(M+H)$^+$].

Anal. Calcd for $C_{26}H_{42}O_{17}N_2 \cdot 2.5H_2O$: C, 44.63; H, 6.77; N, 4.00. Found: C, 44.47; H, 6.52; N, 4.09.

Reductive Amination Using 2-Amino-6-(2-cyanoethyl)pyridine (3): General Procedure. A mixture of a sugar (9.21 μmol), compound **3** (26.2 mg, 178 μmol), and acetic acid (6.1 μL) was heated at 90 °C in a sealed tube for 1 h. After cooling to room temperature, a solution of $BH_3 \cdot Me_2NH$ in acetic acid [(0.195 mg/μL), 55.6 μL, 184 μmol] was added. The mixture was heated at 80 °C in a sealed tube for 1 h, cooled to room temperature, and lyophilized. The residue was dissolved in H_2O (5 mL) and then purified by HPLC [column: Cosmosil 5C_{18} AR, 20 mm x 250 mm; solvent: CH_3CN-0.1M $CH_3CO_2NH_4$ (pH 6.9); gradient: 5% CH_3CN - 50% CH_3CN (1.5 %/min); flow rate: 8 mL/min; detection: UV at 307 nm].

Determination of the Yields of CNEPA-sugars. The yields of CNEPA-sugars were determined by means of HPLC [column: Cosmosil 5C_{18} AR, 4.6 mm x 250 mm; solvent: CH_3CN-0.1M $CH_3CO_2NH_4$ (pH 6.9); gradient: 5% - 50% CH_3CN (1.5 %/min); flow rate: 1.0 mL/min; detection: UV at 307 nm] using a solution of CNEPA-Glc (**20**) in water as an external standard. The yields with various model sugars are listed in Table 2.

1-Deoxy-1-[(6-cyanoethyl-2-pyridyl)amino]-D-glucitol (CNEPA-Glc) (20). ^{1}H NMR (D_2O, acetone) δ 7.53 (1H, dd, J=7Hz, 8Hz, H-4), 6.65 (1H, d, J=7Hz, H-3 or 5), 6.56 (1H, d, J=8Hz, H-5 or 3), 4.0-3.3 (8H, glucitol H-1 - H-6), 2.94 (2H, m, -C<u>H</u>$_2$CH$_2$CN), 2.85 (2H, m, -CH$_2$C<u>H</u>$_2$CN); positive FAB-MS m/z 312.1 [(M+H)$^+$].

Anal. Calcd for $C_{14}H_{21}O_5N_3 \cdot 0.24H_2O$: C, 53.28; H, 6.86; N, 13.31. Found: C, 53.28; H, 6.71; N, 13.18.

***O*-α-D-Glucopyranosyl-(1→4)-1-deoxy-1-[(6-cyanoethyl-2-pyridyl)-amino]-D-glucitol (CNEPA-maltose) (21).** ^{1}H NMR (D_2O, acetone) δ 7.52 (1H, dd, J=7Hz, 8Hz, H-4), 6.65 (1H, d, J=7Hz, H-3 or 5), 6.56 (1H, d, J=8Hz, H-5 or 3), 5.12 (1H, d, J=4Hz, Glc H-1), 3.98-3.39 (14H, glucitol H-1 - H-6, Glc H-2 - H-6), 2.93 (2H, m, -C<u>H</u>$_2$CH$_2$CN), 2.86 (2H, m, -CH$_2$C<u>H</u>$_2$CN); positive FAB-MS m/z 474.2 [(M+H)$^+$].

Anal. Calcd for $C_{20}H_{31}O_{10}N_3 \cdot 1.2H_2O$: C, 48.52; H, 6.80; N, 8.49. Found: C, 48.52; H, 6.65; N, 8.43.

***O*-α-D-Glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-1-deoxy-1-[(6-cyanoethyl-2-pyridyl)amino]-D-glucitol (CNEPA-maltotriose) (22).** ^{1}H NMR (D_2O, acetone) δ 7.53 (1H, dd, J=7Hz, 8Hz, H-4), 6.65 (1H, d, J=7Hz, H-3 or 5), 6.56 (1H, d, J=8Hz, H-5 or 3), 5.39 (1H, d, J=4Hz, Glc H-1), 5.13 (1H, d, J=4Hz, Glc H'-1), 4.0-3.4 (20H, glucitol H-1 - H-6, Glc H-2 - H-6, Glc H'-2 - H'-6), 2.95 (2H, m, -C<u>H</u>$_2$CH$_2$CN), 2.85 (2H, m, -CH$_2$C<u>H</u>$_2$CN); positive FAB-MS m/z 636.3 [(M+H)$^+$].

Anal. Calcd for $C_{26}H_{41}O_{15}N_3$•1.6H_2O: C, 47.00; H, 6.70; N, 6.32. Found: C, 47.00; H, 6.59; N, 6.39.

***N*^{2}-Acetyl-*N*^{6}-3-[6-(1-deoxy-1-D-glucitolyl)amino-2-pyridyl]-propionyl-L-lysine Methyl Ester (24).** To a mixture of CEPA-Glc (**16**) (15.0 mg, 45.5 μmol), *N*^{2}-acetyl-L-lysine methyl ester hydrochloride (**23**) (10.8 mg, 45.5 μmol), and 1-hydroxybenzotriazole (6.1 mg, 45.5 μmol) in water (60 μL) were added EDC•HCl (19.0 mg, 99.5 μmol) and TEA (6.3 μL, 46 μmol). The solution was stirred at room temperature for 30 h, and then diluted with water (5 mL). The solution was applied to HPLC [column: Cosmosil 5C_{18} AR, 20 mm x 250 mm; solvent: CH_3CN-0.1M $CH_3CO_2NH_4$ (pH 6.9); gradient: 0% - 45% CH_3CN (1.5 %/min); flow rate: 8 mL/min; detection: UV at 300 nm]. The fraction eluted at 23.6 min was collected and lyophilized to give colorless powder: yield 12.0 mg (51.6%); ^{1}H NMR (D_2O, acetone) δ 7.49 (1H, dd, J=7Hz, 9Hz, H-4), 6.54 (2H, m, H-3 and 5), 4.24 (1H, dd, J=5Hz, 9Hz, Lys α-CH), 4.0-3.3 (8H, glucitol H-1 - H-6), 3.74 (3H, s, -CO_2CH_3), 3.07 (2H, t, J=7Hz, Lys ε-CH_2), 2.89 (2H, t, J=7Hz, -C<u>H</u>$_2$CH$_2$CONH-), 2.59 (2H, t, J=7Hz, -CH$_2$C<u>H</u>$_2$CONH-), 2.02 (3H, s, C<u>H</u>$_3$CONH-), 1.76-1.60 (2H, m, Lys β-CH_2), 1.33 (2H, m, Lys δ-CH_2), 1.10 (2H, m, Lys γ-CH_2) ; positive FAB-MS m/z 515.3 [(M+H)$^+$].

Reduction of Nitrile Group of CNEPA-sugars. General Procedure. A CNEPA-sugar (32 μmol) was hydrogenated with Pd-black (50 mg) in 25% aqueous ammonia (14 mL) under 9 kg/cm^2 of H_2 at room temperature for 66 h. The catalyst was removed by filtration and the filtrate concentrated. The residue was dissolved in H_2O (10 mL) and then purified by HPLC [column: Cosmosil 5C_{18} AR, 20 mm x 250 mm; solvent: CH_3CN-0.1M $CH_3CO_2NH_4$ (pH 6.9); gradient: 5% CH_3CN - 50% CH_3CN (1.5 %/min); flow rate: 8 mL/min; detection: UV at 307 nm]. The fraction containing the APPA-sugar was lyophilized, and the powder obtained was dissolved in H_2O (10 mL). The solution was rechromatographed on HPLC (column: Cosmosil 5C_{18} AR, 20 mm x 250 mm; solvent: 15% CH_3CN-0.01% TFA; flow rate: 8 mL/min; detection: UV at 307 nm) for the removal of contaminating ammonium salt.

1-Deoxy-1-[(6-aminopropyl-2-pyridyl)amino]-D-glucitol Hydrochloride (APPA-Glc) (25). Compound **25** was obtained as a hydrochloride: yield 90.0%; ^{1}H NMR (D_2O, acetone) δ 7.51 (1H, m, H-4), 6.61 (1H, d, J=7Hz, H-3 or 5), 6.56 (1H, d, J=7Hz, H-5 or 3), 4.0-3.3 (8H, glucitol H-1 - H-6), 2.96 (2H, t, J=7Hz, -$CH_2CH_2C\underline{H}_2NH_2$), 2.69 (2H, t, J=7Hz, -$C\underline{H}_2CH_2CH_2NH_2$), 1.98 (2H, t, J=7Hz, -$CH_2C\underline{H}_2CH_2NH_2$); positive FAB-MS m/z 316.0 [(M+H)$^+$].

***O*-α-D-Glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-1-deoxy-1-[[(6-aminopropyl-2-pyridyl)amino]-D-glucitol (APPA-maltotriose) (26).** Yield 76.7%; ^{1}H NMR (D_2O, acetone) δ 7.86 (1H, dd, J=7Hz, 9Hz, H-4), 6.96 (1H, d, J=9Hz, H-3 or 5), 6.78 (1H, d, J=7Hz, H-5 or 3), 5.39 (1H, d, J=4Hz, Glc H-1), 5.13 (1H, d, J=4Hz, Glc H'-1), 4.1-3.3 (20H, glucitol H-1 - H-6, Glc H-2 - H-6, Glc H'-2 - H'-6), 3.07 (2H, t, J=8Hz, -$CH_2CH_2C\underline{H}_2NH_2$), 2.88 (2H, t, J=8Hz, -$C\underline{H}_2CH_2CH_2NH_2$), 2.11 (2H, t, J=8Hz, -$CH_2C\underline{H}_2CH_2NH_2$); positive FAB-MS m/z 639.9 [(M+H)$^+$].

Anal. Calcd for $C_{26}H_{45}O_{15}N_3$•2TFA•1.6H_2O: C, 40.19; H, 5.64; N, 4.69. Found: C, 40.00; H, 5.43; N, 4.68.

3-(3-Nitro-2-pyridyldithio)propionic Acid. To a solution of 3-mercaptopropionic acid (0.59 mL, 6.8 mmol) and TEA (1.79 mL, 12.9 mmol) in CH_2Cl_2 (10 mL) was added dropwise a solution of 3-nitro-2-pyridinesulfenyl chloride[31] (1.23 g, 6.44 mmol) in CH_2Cl_2 (10 mL) at 0 °C under a N_2 atmosphere. The solution was stirred at the same temperature for 1 h and then concentrated. The residue was dissolved in ethyl acetate. The product was extracted with an aqueous saturated $NaHCO_3$ solution. After the aqueous layer was acidified to pH 1 with 6M HCl, the product was extracted with $CHCl_3$. The organic layer was dried over $NaSO_4$ and worked up as usual. The crystalline residue was recrystallized from ethyl acetate and hexane to give yellow crystals: yield 681 mg (40.5%); mp 129-135 °C; positive EI-MS m/z 260.0 (M)$^+$.

Anal. Calcd for $C_8H_8O_4N_2S_2$: C, 35.98; H, 2.95; N, 10.37. Found: C, 36.92; H, 3.10; N, 10.76.

Succinimidyl 3-(3-Nitro-2-pyridyldithio)propionate (27). To a solution of 3-(3-nitro-2-pyridyldithio)propionic acid (50.0 mg, 192 μmol) and *N*-hydroxysuccinimide (23.5 mg, 204 μmol) in CH_2Cl_2 (2.5 mL) was added EDC•HCl (43.8 mg, 230 μmol) at 0 °C. The solution was stirred at room temperature for 4 h and then diluted with ethyl acetate. The organic solution was washed with an aqueous saturated $NaHCO_3$ solution and brine, and worked up as usual. The residue was crystallized from ethyl acetate and hexane: yield 53.3 mg (77.7%); mp 141.5-144.5 °C.

Anal. Calcd for $C_{12}H_{11}O_6N_3S_2$: C, 40.33; H, 3.10; N, 11.76. Found: C, 40.27; H, 3.11; N, 11.55.

O-α-D-Glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-1-deoxy-1-[[6-(3-nitro-2-pyridyldithio)propanamidopropyl-2-pyridyl]-amino]-D-glucitol (28). To a solution of APPA-maltotriose (**26**) (9.9 mg, 11.4 μmol) and TEA (6.0 μL, 43 μmol) in water (300 μL) (pH 9.0) was added a solution of **27** (16.2 mg, 45.3 μmol) in THF (600 μL). The solution was stirred at room temperature for 6 h and then diluted with ethyl acetate and water. The aqueous layer was subjected to separation with HPLC [column: Cosmosil $5C_{18}$ AR, 20 mm x 250 mm; solvent: CH_3CN-0.1% TFA; gradient: 20% CH_3CN - 50% CH_3CN (2 %/min); flow rate: 8 mL/min; detection: UV at 300 nm]. The fraction eluted at 13.1 min was collected and lyophilized to give powder: yield 8.9 mg (70%); ^1H NMR (D_2O, acetone), δ 8.75 (1H, m, J=5Hz, Npys H-4 or 6), 8.64 (1H, m, J=8Hz, Npys H-6 or 4), 7.79 (1H, dd, J=8Hz, H-4), 7.55 (1H, dd, J=7Hz, Npys H-5), 6.85 (1H, d, J=9Hz, H-3), 6.73 (1H, d, J=7Hz, H-5), 5.38 (1H, d, J=4Hz, Glc H-1), 5.12 (1H, d, J=4Hz, Glc H'-1), 4.1-3.4 (20H, m, glucitol H-1 - H-6, Glc H-2 - H-6, Glc H'-2 - H'-6), 3.21 (2H, t, J=7Hz, -$CH_2CH_2C\underline{H}_2NH$-), 3.05 (2H, t, J=6Hz, -$COCH_2C\underline{H}_2S$-), 2.78 (2H, t, J=8Hz, -$C\underline{H}_2CH_2CH_2NH$-), 2.61 (2H, t, J=6Hz, -$COC\underline{H}_2CH_2S$-), 1.90 (2H, m, J=7Hz, -$CH_2C\underline{H}_2CH_2NH$-); positive FAB-MS m/z 882.1 [(M+H)+].

Anal. Calcd for $C_{34}H_{51}O_{18}N_5S_2 \cdot 2TFA \cdot 2.6H_2O$: C, 39.20 ; H, 4.87 ; N, 6.38. Found: C, 39.45 ; H, 5.07 ; N, 6.05.

The reaction of 28 with BSA. To a solution of **28** (269 μg, 270 nmol) in 0.5 M tris(hydroxymethyl)aminomethane - 0.005 M ethylenediaminetetraacetic acid disodium salt buffer (pH 7.5) (727 μL) was added BSA (3.02 mg, 45.0 nmol) at 4 °C. The reaction was analyzed by gel-filtration HPLC [column: Asahipak GS-510 7.6 mm x 500 mm; solvent: 30% CH_3CN-0.2 M sodium phosphate buffer (pH 7.0); flow rate: 1 mL/min; detection: UV at 300 nm]. Compound **28** was eluted at 40.8 min, whereas both BSA and sugar-BSA conjugate **29** were eluted at 14.7 min. After the reaction was completed, the solution was applied to column chromatography on Bio-Gel P-2 (12 mm x 230 mm; solvent: aqueous 1% acetic acid; detection: UV at 300 nm). The fraction containing **29** was lyophilized to give powder: yield 2.0 mg.

O-α-D-Glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-1-deoxy-1-[[(6-biotinylaminopropyl-2-pyridyl)amino]-D-glucitol (31). To a solution of APPA-maltotriose (**26**) (15.0 mg, 16.6 μmol) in aqueous 0.5% $NaHCO_3$ solution - DMF [1:1 (v/v), 500 μL] was added *N*-hydroxysuccinimidobiotin[27] (**30**) (40.0 mg, 117 μmol), and the mixture was stirred at room temperature for 3.5 h. After another portion of **30** (24.0 mg, 70.4 mmol) had been added, the mixture was further stirred at room temperature overnight. The mixture was filtered, and the filtrate was applied to HPLC [column: Cosmosil $5C_{18}$ AR, 20 mm x 250 mm; solvent: CH_3CN-0.1

M HCO_2NH_4 (pH 6.3); gradient: 5% CH_3CN - 50% CH_3CN (1.5 %/min); flow rate: 8 mL/min; detection: UV at 300 nm]. The fraction eluted at 20.9 min was collected and lyophilized to give colorless powder: yield 9.3 mg (65%); ^1H NMR (D_2O) δ 7.84 (1H, dd, J=7Hz, 9Hz, H-4), 6.92 (1H, d, J=9Hz, H-3 and 5), 6.75 (1H, d, J=7Hz, H-3 and 5), 5.39 (1H, d, J=4Hz, Glc H-1), 5.14 (1H, d, J=4Hz, Glc H'-1), 4.58 [1H, dd, J=4 Hz, 8 Hz, -CONHC<u>H</u>(CH-)CH_2S-], 4.39 [1H, dd, J=5 Hz, 8 Hz, -CONHC<u>H</u>(CH-)CH(CH_2-)S-], 4.08-3.56 (20H, glucitol H-1 - H-6, Glc H-2 - H-6, Glc H'-2 - H'-6), 3.34-3.23 (3H, m, -NHCO$CH_2CH_2CH_2CH_2$C<u>H</u>- + -$CH_2CH_2C<u>H_2</u>$NHCO-), 2.96 (1H, dd, J=5 Hz, 13 Hz, -S-C<u>H</u>H'-CH-), 2.77 (3H, m, -C<u>H_2</u>CH_2CH_2NHCO- + -S-CH<u>H</u>'-CH-), 2.23 (2H, t, J=7 Hz, -NHCOC<u>H_2</u>$CH_2CH_2CH_2$-), 1.92 (2H, t, J=7 Hz, -CH_2C<u>H_2</u>CH_2NHCO-), 1.72-1.54 (4H, m, -NHCOCH_2C<u>H_2</u>CH_2CH_2- + -NHCO$CH_2CH_2CH_2$C<u>H_2</u>-), 1.38 (2H, m, -NHCOCH_2CH_2C<u>H_2</u>CH_2-); positive FAB-MS m/z 866.7 [(M+H)$^+$].

Acryloyloxyphenyldimethylsulfonium methyl sulfate (32). To a solution of acrylic acid (142 μL, 2.08 mmol) and 4-hydroxyphenyldimethylsulfonium methyl sulfate[28] (553 mg, 2.08 mmol) in CH_3CN (9.0 mL) was added dicyclohexylcarbodiimide (429 mg, 2.08 mmol). The mixture was stirred at room temperature for 4 h, and the insoluble material was removed by filtration. The filtrate was concentrated to give an oil: yield 670 mg (quant.). The product was subjected to the following reaction without purification.

***O*-α-D-Glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-1-deoxy-1-[(6-acrylamidopropyl-2-pyridyl)amino]-D-glucitol (33).** To APPA-maltotriose (**26**) (15.0 mg, 16.6 μmol) and active ester **32** (20. 0 mg, 62.5 μmol) was added saturated $NaHCO_3$ solution (300 μL). After the solution was stirred at room temperature for 5.5 h, a solution of active ester **32** (15.0 mg, 16.6 μL) in water (100 μL) was added. The mixture was further stirred at room temperature overnight and then filtered. The filtrate was applied to HPLC [column: Cosmosil 5C_{18} AR, 20 mm x 250 mm; solvent: CH_3CN-0.1M HCO_2NH_4 (pH 6.3); gradient: 5% CH_3CN - 50% CH_3CN (1.5 %/min); flow rate: 8 mL/min; detection: UV at 300 nm]. The fraction eluted at 18.7 min was collected and lyophilized to give colorless powder: yield 9.2 mg (80.0%); ^1H NMR (D_2O) δ 7.80 (1H, dd, H-4), 6.85 (1H, d, J=9 Hz, H-3 or 5), 6.74 (1H, d, J=7 Hz, H-5 or 3), 6.17 (2H, m, -CH=C<u>H_2</u>), 5.74 (1H, dd, J=3 Hz, 9 Hz, -C<u>H</u>=CH_2), 5.39 (1H, d, J=4 Hz, Glc H-1), 5.13 (1H, d, J=4 Hz, Glc H'-1), 4.06-3.38 (20H, glucitol H-1 - H-6, Glc H-2 - H-6, Glc H'-2 - H'-6), 3.32 (2H, t, J=7Hz, -CH_2CH_2C<u>H_2</u>NH-), 2.79 (2H, t, J=7 Hz, -C<u>H_2</u>CH_2CH_2NH-), 1.96 (2H, m, -CH_2C<u>H_2</u>CH_2NH-); positive FAB-MS m/z 694.4 [(M+H)$^+$].

ACKNOWLEDGMENTS

The authors thank Drs. Kagetoshi Yamamoto and Yoshikazu Sugihara of Osaka University for giving opportunities for measurements of fluorescence spectra. Parts of this work were supported by Grand-in-Aid for Scientific Research from the Ministry of Education, Science and Culture and funds from Ono Pharmaceutical Company.

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