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Synthesis of glycosylated human interleukin-1α, neoglyco IL-1α, coupled with *N*-acetylneuraminic acid

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In order to develop glycosylated cytokine, recombinant human IL-1α was chemically modified with *N*-acetylneuraminic acid (NANA). NANA with C9 spacer, 8-(hydrazinocarbonyl)octyl 5-acetamido-3, 5-dideoxy-D-glycero-α-D-galacto-2-nonulo-pyranosidonic acid potassium salt (6), was synthesized by glycosylation of C9 spacer, 8-[2-N-(benzyloxycarbonyl)hydrazinocarbonyl]octanol, with methylthio glycoside derivatives of NANA in the presence of molecular sieves 3Å and methyl (methylthio)sulfonium trifrate in propionitrile, followed by separation of α and β anomers with a column chromatography and deprotection. Compound 6 was coupled to IL-1α by the acyl azide method. The glycosylated IL-1 was purified by anion-exchange chromatography, and NANA coupled to IL-1 was confirmed by oxidation with $NaIO_4$. Based on the molecular weight average number of carbohydrate molecules introduced per molecule of IL-1α was estimated to be 2.9.

Keywords: **neoglycoprotein, sialic acid, interleukin 1, cytokine**

Introduction

Glycoproteins are widely distributed in animals, plants and microorganisms. Recent studies revealed that neoglycoproteins, proteins chemically or enzymatically coupled with carbohydrates, are quite useful to investigate the role of carbohydrates in functions and physicochemical properties of glycoproteins. The great advantage to synthesize neoglycoproteins is that chemically synthesized carbohydrates, not only natural but also unnatural carbohydrates, can be introduced into proteins. The neoglycoproteins have been used to raise carbohydrate-directed antibodies [1], to examine the immunogenicity of carbohydrate [2–4], to detect lectins in tissue or cell surface [5] and to isolate carbohydrate-binding proteins [6]. Neoglycoproteins may also be used as soluble inhibitors of glycoconjugate-mediating processes, such as cell-cell contact [7].

Several methods have been developed to synthesize neoglycoproteins, including the imidate method [1], the reductive amination method [8] and the acyl azide method [9]. In previous studies we have chemically introduced D-mannose dimers and D-galactose monosaccharide into recombinant human interleukin-1α (rhIL-1α) by the acyl azide method [10,11] and effects of the modification on biological activities of IL-1 *in vitro* and *in vivo* were investigated [10–15]. The coupling method appeared to be applicable to biological active proteins because the reaction could be performed in mild condition, pH 9 at 0 °C.

Sialic acid is usually present at the non-reducing position of oligosaccharide in glycoproteins and glycolipids, and plays an important role in function, stability and tissue distribution of glycoproteins [16]. Sialic acid is especially important in preventing the clearance of glycoproteins from serum because asialoglycoproteins are rapidly cleared through galactose binding lectins present in the liver [17]. NANA, the constituent of sialic acid, is characterized by its carboxyl residue, which is negatively charged in physiological condition and readily reacts with other functional groups. Therefore, it is difficult to couple NANA to proteins.

Interleukin 1 (IL-1) is a cytokine produced mainly by macrophages and monocytes. IL-1 exhibits a variety of biological activities and plays an important role in immunologic and inflammatory reactions [18]. By introduction of carbohydrate into IL-1α, there are several possibilities of expected effectiveness: (I) a particular activity may be inhibited or augmented; (II) IL-1 with less side effects may be obtained; (III) an IL-1 inhibitor may be obtained; or (IV) IL-1 with a novel activity may be obtained. We reported that D-mannose dimer coupled rhIL-1α exhibited

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selective activities *in vivo* and altered tissue distribution, however D-Gal coupled rhIL-1α did not exhibit such selective activities [10–15]. In this study we synthesized and purified rhIL-1α into which NANA had been chemically introduced.

Materials and methods

General procedures

Melting points were determined with a Yanagimoto MP-S2 micro melting point apparatus and are uncorrected. Solutions were concentrated in a rotary evaporator below 50 °C under vacuum. Optical rotations were measured with a JASCO DIP-140 automatic digital polarimeter in a 0.5 dm tube. IR spectra were recorded with a Perkin-Elmer 1600 Series FTIR spectrometer. ^{1}H NMR spectra were recorded at 270 MHz with a JEOL JNM-ES-270, or at 500 MHz with a JEOL α-500 spectrometer. ^{13}C NMR spectra were recorded at 67.5 MHz with a JEOL JNM-ES-270, or at 125 MHz with a JEOL α-500 spectrometer. Tetramethylsilane was used as an internal standard. Chemical shifts are given on the δ scale. TLC was performed on precoated silica gel plates 0.25 mm thick (Kieselgel $60F_{254}$, Merck). Detection was effected with H_2SO_4 or by UV irradiation at 254 nm. Column chromatography was performed on Silica Gel BW-820MH (Fuji-Silysia Chemical Ltd., Nagoya, Japan). Solvent combinations for elution of a column chromatography and the developing solvent on TLC are given as v/v.

8-(Hydrazidocarbonyl)octanol (1)

Hydrazine monohydrate (5.2 ml, 107.0 mmol) was added to a solution of methyl 9-hydroxynonanoate [19] (2.00 g, 10.6 mmol) in dry methanol (50 ml) and the mixture was stirred overnight at room temperature. The resulting precipitates were collected by filtration, dried and crystallized from ethanol. Recrystallization from the same solvent gave **1** (1.78 g, 89 %) as colourless leaflets: m.p. 136–137°C ; IR (KBr) 3500-3000 (OH, NH, NH_2), 1638 (amide I), and 1534 cm^{-1} (amide II); ^{1}H NMR (pyridine-d_5) δ 1.08–2.00 [m, 12H, $CH_2(CH_2)_6CH_2$], 2.34 (t, 2H, J = 7.5 Hz, CH_2CONH), and 3.84 (t, 2H, J = 6.6 Hz, CH_2OH); ^{13}C NMR (pyridine-d_5) δ 26.2, 26.4, 29.6, 29.7 (X3) [$CH_2(CH_2)_6CH_2$], 33.7 (CH_2CONH), 62.1 (CH_2OH), and 173.3 (CH_2CONH).

Anal. Calcd for $C_9H_{20}N_2O_2$ (188.27): C, 57.42; H, 10.71; N, 14.88. Found: C, 57.28; H, 10.51; N, 14.86.

8-[2-N-(Benzyloxycarbonyl)hydrazinocarbonyl]-octanol (2)

Carbobenzoxy chloride (37.7 ml, 264.1 mmol) was added dropwise to a solution of **1** (9.90 g, 52.6 mmol) in dist. pyridine (150 ml) and the mixture was stirred overnight at room temperature. After a few piece of ice was added, the mixture was stirred and evaporated by repeated co-distillation with toluene to afford a syrup which was treated with water and a small amount of chloroform. The resulting precipitates were collected, dried and crystallized from acetonitrile. Recrystallization from the same solvent gave **2** (10.24 g, 60.4 %) as white crystals: m.p. 76–77°C ; IR (KBr) 3500-3120 (OH, NH), 1724 (ester), 1671 (amide I), and 1560 cm^{-1} (amide II); ^{1}H NMR (CD_3OD) δ 1.35 [m, 8H, $CH_2(CH_2)_4CH_2$], 1.52 (m, 2H, CH_2CH_2CO), 1.63 (m, 2H, CH_2CH_2OH), 2.23 (m, 2H, CH_2CONH), 3.54 (t, 2H, J = 6.7 Hz, CH_2OH), 5.14 (s, 2H, $CH_2C_6H_5$), and 7.30 (m, 5H, C_6H_5); ^{13}C NMR (CD_3OD) δ 26.5 (CH_2CH_2OH), 26.9, 30.1, 30.4 (X2) [$CH_2(CH_2)_4CH_2$], 33.6 (CH_2CH_2CO), 34.7 (CH_2CONH), 63.0 (CH_2OH), 68.2 ($CH_2C_6H_5$), 129.0, 129.2, 129.5, 129.6 (aromatic carbons), 158.5 ($COOCH_2$), and 175.9 (CH_2CONH).

Anal. Calcd for $C_{17}H_{26}N_2O_4$ (322.41): C, 63.33; H, 8.13; N, 8.69. Found: C, 63.04; H, 8.03; N, 8.63.

Methyl {8-[2-N-(benzyloxycarbonyl) hydrazinocarbonyl]octyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulo-pyranosid}onate (3) and Methyl {8-[2-N-(benzyloxycarbonyl)hydrazino-carbonyl]octyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosid}onate (4)

To a solution of **2** (1.2 g, 3.72 mmol) and methyl (methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-D-galacto-2-nonulopyranosid)onate [20] (3.89 g, 7.46 mmol) in dry propionitrile (24 ml) were added powdered molecular sieves 3 Å (MS-3A, 9.05 g) and the mixture was stirred for 1.5 h at room temperature under argon, then cooled to −50°C for 1 h. Dimethyl(methylthio)sulfonium triflate (DMTST, 7.68 g, 29.7 mmol) was added to the mixture, and it was stirred for 24 h at −20°C. The mixture was filtered and the residue was washed with chloroform. The combined filtrate and washings were washed with 1M Na_2CO_3 solution and water, dried ($MgSO_4$), and concentrated to dryness to afford a syrup which was chromatographed on a column of silica gel, eluting with $CHCl_3$-MeOH (50:1–10:1). Evaporation of the solvent afforded a mixture (2.49 g, 84 %) of **3** and **4** as an amorphous powder. The mixture was rechromatographed with the same solvent system and separated **3** and **4,** respectively. Evaporation of the solvent from the faster moving eluate afforded **3** as an amorphous powder: $[\alpha]_D^{20}$ −8.2° (C = 1.01, $CHCl_3$); IR (KBr) 3310 (NH), 1744 (ester), 1667 (amide I), and 1544 (amide II); ^{1}H NMR ($CDCl_3$) δ 1.33 [m, 8H, $CH_2(CH_2)_4CH_2$], 1.55 (m, 2H, CH_2CH_2O), 1.66 (m, 2H, CH_2CH_2CO), 1.82, 1.97, 2.02, 2.03, 2.14 (5s, 15H, $COCH_3$), 1.84 (dd, 1H, $J_{3ax,3eq}$ = 12.8 Hz, $J_{3ax,4}$ = 11.0 Hz, H-3ax), 2.24 (m, 2H, CH_2CONH), 2.45 (dd, 1H, $J_{3eq,4}$ = 4.9 Hz, H-3eq), 3.32, 3.48 (m, 2H, CH_2O), 3.79 (s, 3H, $COOCH_3$), 4.00 (dd, 1H, $J_{5,6}$ = 10.4 Hz, $J_{6,7}$ = 1.8 Hz, H-6), 4.09–4.16 (m, 2H, H-5, H-9a), 4.85 (dd, 1H, $J_{8,9b}$ = 2.4 Hz, $J_{9a,9b}$ = 12.2 Hz,

H-9b), 5.15 (s, 2H, $CH_2C_6H_5$), 5.19 (m, 1H, H-8), 5.27 (m, 1H, H-4), 5.44 (dd, 1H, $J_{7,8}$ = 3.7 Hz, H-7), and 7.34 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$) δ 20.8 (X2), 20.9, 21.0, 22.9 (5 $COCH_3$), 24.9 (CH_2CH_2CO), 25.8, 28.3, 28.4, 28.6 [$CH_2(CH_2)_4CH_2$], 29.3 (CH_2CH_2O), 33.8 (CH_2CONH), 37.5 (C-3), 49.0 (C-5), 52.7 ($COOCH_3$), 62.6 (C-9), 64.1 (CH_2O), 67.8 ($CH_2C_6H_5$), 68.7 (C-7), 69.2 (C-4), 71.7 (C-6), 72.6 (C-8), 98.5 (C-2), 128.2, 128.4, 128.6, 135.6 (aromatic carbons), 156.6 ($COOCH_2$), 167.8(C-1), 170.3, 170.6, 170.7, 171.2, 171.3 (5 $COCH_3$), and 173.2 (CH_2CONH).

Anal. Calcd for $C_{37}H_{53}N_3O_{16}$ • $1/2H_2O$(804.86): C, 55.22; H, 6.76; N, 5.22. Found: C, 55.20; H, 6.71; N, 5.16.

Evaporation of the solvent from the last effluent afforded **4** as an amorphous powder: $[\alpha]_D^{20}$ −14.3° (C = 1.00, $CHCl_3$); IR (KBr) 3312 (NH), 1750 (ester), 1664 (amide I), and 1543 (amide II); ^1H NMR ($CDCl_3$) δ 1.28 [m, 8H, $CH_2(CH_2)_4CH_2$], 1.51 (m, 2H CH_2CH_2O), 1.62 (m, 2H, CH_2CH_2CO), 1.85, 2.00, 2.02, 2.12(X2) (5s, 15H, $COCH_3$), 1.93 (dd, 1H, $J_{3ax,3eq}$ = 12.8 Hz, $J_{3ax,4}$ = 12.8 Hz, H-3ax), 2.20 (m, 2H, CH_2CONH), 2.58 (dd, 1H, $J_{3eq,4}$ = 4.3 Hz, H-3eq), 3.22, 3.73 (m, 2H, CH_2O), 3.77 (s, 3H, $COOCH_3$), 4.05 (dd, 1H, $J_{4,5}$ = 9.8 Hz, $J_{5,6}$ = 11.0 Hz, H-5), 4.10–4.14 (m, 2H, H-6, H-9a), 4.34 (dd, 1H, $J_{8,9b}$ = 2.4 Hz, $J_{9a,9b}$ = 12.2 Hz, H-9b), 4.84 (ddd, 1H, H-4), 5.14 (s, 2H, $CH_2C_6H_5$), 5.33 (dd, 1H, $J_{6,7}$ = 1.8 Hz, $J_{7,8}$ = 7.9 Hz, H-7), 5.38 (m, 1H, H-8), and 7.33 (m, 5H, C_6H_5); ^{13}C NMR ($CDCl_3$) δ 20.8 (X3), 21.2, 23.0 (5 $COCH_3$), 25.2 (CH_2CH_2CO), 25.7, 29.0 (X2), 29.1 [$CH_2(CH_2)_4CH_2$], 29.5 (CH_2CH_2O), 33.9 (CH_2CONH), 38.0 (C-3), 49.2 (C-5), 52.6 ($COOCH_3$), 62.5 (C-9), 64.9 (CH_2O), 67.6 (C-7), 67.7 ($CH_2C_6H_5$), 69.1 (C-8), 69.3 (C-4), 72.4(C-6), 98.8 (C-2), 128.1, 128.3, 128.5, 135.6 (aromatic carbons), 156.6 ($COOCH_2$), 168.7 (C-1), 170.2, 170.3, 170.6, 170.9, 171.0 (5 $COCH_3$), and 173.2 (CH_2CONH).

Anal. Calcd for $C_{37}H_{53}N_3O_{16}$ • $1/2H_2O$(804.86): C, 55.22; H, 6.76; N, 5.22. Found: C, 55.15; H, 6.68; N, 5.22.

Methyl [8-(hydrazinocarbonyl)octyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosid]onate (5)

Freshly prepared [22] Pd black obtained by reduction of $PdCl_2$ (0.5 g) in methanol (50 ml) was added to a solution of **4** (919 mg, 1.15 mmol) in glacial acetic acid (25 ml). The mixture was hydrogenated at room temperature under the atmospheric pressure for 3.5 h. The catalyst was removed by filtration and the solvent was evaporated by repeated co-distillation with toluene to afford **5** (633 mg, 83.2 %) as an amorphous powder: $[\alpha]_D^{20}$ −14.8° (C = 0.98, CH_3OH); IR (KBr) 3500–3240 (NH, NH_2), 1750 (ester), 1654 (amide I), and 1560 (amide II); ^1H NMR (CD_3OD) δ 1.33 [m, 8H, $CH_2(CH_2)_4CH_2$], 1.51 (m, 2H CH_2CH_2O), 1.64 (m, 2H, CH_2CH_2CO), 1.79 (dd, 1H, $J_{3ax,3eq}$ = 12.5 Hz, $J_{3ax,4}$ = 12.2 Hz, H-3ax), 1.83, 1.98, 2.01, 2.11, 2.13 (5s, 15H, $COCH_3$), 2.24 (t, 2H, J = 7.5 Hz, CH_2CONH), 2.61 (dd, 1H, $J_{3eq,4}$ = 4.9 Hz, H-3eq), 3.23, 3.74 (m, 2H, CH_2O), 3.81 (s, 3H, $COOCH_3$), 3.94 (dd, 1H, $J_{4,5}$ = 10.1 Hz, $J_{5,6}$ = 11.0 Hz, H-5), 4.07 (dd, 1H, $J_{8,9a}$ = 5.5 Hz, $J_{9a,9b}$ = 12.2 Hz, H-9a), 4.16 (dd, 1H, $J_{6,7}$ = 2.1 Hz, H-6), 4.31 (dd, 1H, $J_{8,9b}$ = 2.7 Hz, H-9b), 4.78 (ddd, 1H, H-4), 5.32 (dd, 1H, $J_{7,8}$ = 8.8 Hz, H-7), and 5.38 (m, 1H, H-8); ^{13}C NMR (CD_3OD) δ 20.4, 20.7, 20.9, 21.2, 22.7 (5 $COCH_3$), 26.5 (CH_2CH_2CO), 26.9, 30.1 (X2), 30.3 [$CH_2(CH_2)_4CH_2$], 30.6 (CH_2CH_2O), 34.7 (CH_2CONH), 39.2 (C-3), 50.1 (C-5), 53.1 ($COOCH_3$), 63.4 (C-9), 65.9 (CH_2O), 68.7 (C-7), 69.7 (C-8), 70.8 (C-4), 73.1 (C-6), 100.0 (C-2), 169.8 (C-1), 171.6, 171.7, 171.8, 172.4, 173.5 (5 $COCH_3$), and 175.1 (CH_2CONH).

Anal. Calcd for $C_{29}H_{47}N_3O_{14}$ • $1/2H_2O$(670.72): C, 51.93; H, 7.21; N, 6.26. Found: C, 52.14; H, 7.08; N, 6.06.

8-(Hydrazinocarbonyl) octyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosidonic acid potassium salt (6)

To a chilled solution of **5** (305 mg, 0.46 mmole) in dry methanol (20 ml) was added dropwise 0.5 M methanolic sodium methoxide (0.5 ml) and the mixture was stirred for 3 h. After neutralized with Amberlite IR-120B (H^+) resin, the mixture was filtered and concentrated to afford a syrup which was dissolved in 0.1 M potassium hydroxide solution (10 ml). The mixture was stirred for 4 h and neutralized with Amberlite IR-120B (H^+) resin. After filtration, the solution was lyophilized to afford an amorphous powder which was chromatographed on Sephadex LH-20, eluting with $CHCl_3$-MeOH (1:1). Evaporation of the solvent gave **6** (120.6 mg, 51.0%) as an amorphous mass: $[\alpha]_D^{17}$ +7.0° (C = 1.02, H_2O); IR (KBr) 3620-3120 (OH, NH, NH_2), 1750-1500 (COO, amide I, amide II); ^1H NMR (CD_3OD) δ 1.31 [m, 8H, $CH_2(CH_2)_4CH_2$], 1.57 (m, 5H, CH_2CH_2O, CH_2CH_2CO, H-3ax), 2.02(s, 3H, $COCH_3$), 2.36 (m, 2H, CH_2CONH), 2.80 (dd, 1H, $J_{3ax,3eq}$ = 11.0 Hz, $J_{3eq,4}$ = 4.6 Hz, H-3eq), 3.45, 3.70 (m, 2H, CH_2O), 3.52 (m, 1H, H-7), 3.58 (m, 1H, H-8), 3.62 (m, 1H, H-9a), 3.66 (m, 1H, H-5), 3.70 (m, 1H, H-4), 3.82 (m, 1H, H-9b), and 3.84 (m, 1H, H-6); ^{13}C NMR (CD_3OD) δ 22.7 ($COCH_3$), 26.7, 27.0, 30.1, 30.2, 30.3, 30.9 [$CH_2(CH_2)_6CH_2$], 31.7 (CH_2CONH), 42.6 (C-3), 54.2 (C-5), 64.4 (C-9), 65.1 (CH_2O), 69.4 (C-4), 70.3 (C-7), 73.1(C-6), 74.3 (C-8), 101.9 (C-2), 174.5 (C-1), 175.2 ($COCH_3$), and 175.6 (CH_2CONH).

Anal. Calcd for $C_{20}H_{36}KN_3O_{10}$ • $1/2H_2O$(526.63): C, 45.62; H, 7.08; N, 7.98. Found: C, 45.89; H, 7.10; N, 7.74.

Coupling of 6 with rhIL-1α

Recombinant human IL-1α (rhIL-1α) was provided by Dr. M. Yamada of Dainippon Pharmaceutical Co. (Osaka, Japan). Compound **6** (6.73 mg, 12.8 μmol) was dissolved in water (325 μL) and the solution chilled on ice. To the chilled solution (288 μL), cold 4 M HCl (40 μL) and 2 M sodium nitrite (20 μL) were added. After the solution was kept at room temperature for 15 min, 2 M ammonium sulfamate (20 μL) was added and the mixture was kept at

room temperature for 15 min in order to inactivate excess HNO_2. This mixture (containing the acyl azide) was added to ice-cooled 0.4 M sodium borate buffer (pH 10.0, 100 μL) and PBS (400 μL) containing 1.984 mg of rhIL-1α. The pH was quickly adjusted to 9.0 with 4 N NaOH with stirring for 60 min at room temperature. The reaction mixture was then filtrated with 0.2 μm nylon membrane filter and desalted with 20 mM Tris-HCl buffer (pH 7.0) with Hi Trap Desalting column (Pharmacia). The rhIL-1α treated with the same manner without compound **6** was used as control (mock treated) IL-1α.

Purification of NANA-introduced IL-1α

Purification was carried out at room temperature employing the FPLC system (Pharmacia). The desalted sample was loaded onto an anion-exchange chromatography column (Mono Q, Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 7.0) at flow rate of 1 ml/min, eluted with a 30 mL liner NaCl gradient (0–0.5 M) in the same buffer, and fractions (1 ml) were collected. The buffer of fractions containing NANA-introduced IL-1α was exchanged to PBS using HiTrap Desalting column and concentrated in Centriplus-3 (Amicon, *M*r 30,000 cut-off). The final yield of NANA-IL-1α was 12.4%.

Electrophoresis

Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [22] on 15% polyacrylamide gel in the presence of 0.1 % SDS using a vertical slab minigel apparatus. Protein bands were visualized with Coomassie brilliant blue at room temperature for 2 h and decolorized with 10% acetate solution overnight. Molecular weight of NANA-introduced IL-1α was determined by comparison of the electrophoretic mobility with those of standard molecular weight markers (phosphorylase b, *M*r 94,000; bovine serum albumin, *M*r 67,000; ovalbumin, *M*r 43,000; carbonic anhydrase, *M*r 30,000; soybean trypsin inhibitor, *M*r 20,100; α-lactalbumin, *M*r 14,400).

Time-of-mass spectrometry (TOF-MS) analysis

TOF-MS analysis was performed according to the procedure of the supplier using Voyage Elite (PE Biosystems, Foster, CA).

Confirmation of NANA coupling

Size fractionated proteins were transfered from gels to immobilon polyvinylidene difluoride membrane (Milipore Corporation, Bedford, MA) using a semidry apparatus (Marysol, Tokyo, Japan) for 1 h with a current of 100 mA at room temperature. The coupled NANA was detected using biotin-hydrazide and horse-radish peroxidase (HRP)-conjugated avidin following peroxidation with $NaIO_4$ according to a protocol of G.P. Sensor kit (SEIKAGAKU CO., Tokyo, Japan).

Determination of protein content

The amount of protein was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Results and discussion

Synthesis of 8- (Hydrazinocarbonyl) octyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosidonic acid potassium salt (6)

The reaction of methyl 9-hydroxynonanoate [19], and hydrazine monohydrate in methanol gave the easily crystallized acid hydrazide (**1**) as colourless leaflets in 89 % yield. ^1H NMR spectrum of **1** showed no resonance due to a methyl ester group. The protection of an acid hydrazide group in **1** was effected by the reaction of carbobenzoxy chloride in pyridine to afford **2** as white crystals in 60.4 % yield. ^1H NMR spectrum of **2** showed a singlet at δ 5.16 due to a methylene group and a multiplet at δ 7.35 due to aromatic protons in a benzyl group. A mixture of **2,** methyl (methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero-D-galacto-2-nonulopyranosid)onate [20], molecular sieves 3Å and dimethyl(methylthio)sulfonium trifrate in propionitrile was stirred for 24 h at −20 °C under argon. After purification with a column chromatography, a mixture of two compounds was obtained in 84 % yield as an amorphous powder and they were designated **3** and **4** in order of decreasing Rf value. The ratio of **3** and **4** was estimated as ~ 1:3 by comparison of intensities of H-3 equatorial protons of each sialic acid residue in ^1H NMR spectrum. From the faster moving eluate of rechromatography, compound **3** was isolated as an amorphous powder and its ^1H NMR spectrum showed H-3 eq at δ 2.45 ($J_{3ax,3eq}$ = 12.8 Hz, $J_{3eq,4}$ = 4.9 Hz) as a doublet of doublets, H-4 at δ 5.27 as a multiplet and H-7 at δ 5.44 ($J_{7,8}$ = 3.7 Hz) as a doublet of doublets, and Δ | H-9a – H9b | was 0.69-0.76 ppm. These data are characteristic for β-glycosidic linkage of NANA [23]. Evaporation of the last effluent afforded **4** as an amorphous powder. The ^1H NMR spectrum of **4** exhibited H-3 eq at at δ 2.58 ($J_{3ax,3eq}$ = 12.8 Hz, $J_{3eq,4}$ = 4.3 Hz) as a doublet of doublets, H-4 at δ 4.84 (ddd, $J_{3ax,4}$ = 12.8 Hz, $J_{4,5}$ = 9.8 Hz) and H-7 at δ 5.33 ($J_{7,8}$ = 7.9 Hz) as a doublet of doublets, and Δ | H-9a – H9b | was 0.20–0.24 ppm. These data are characteristic for α-glycosidic linkage of NANA [23]. Therefore, the stereochemistry of the newly formed glycosidic bond of **3** was assigned to be β and that of **4** to be α. Deprotection of a benzyloxycarbonyl group of **4** was effected by hydrogenolysis with freshly prepared [21] palladium catalyst in glacial acetic acid to afford **5** as an amorphous powder in

83.2 % yield. Deacetylation of **5** by Zemplen's method (sodium methoxide-methanol), followed by saponification (0.1 M potassium hydroxide solution) gave **6** as an amorphous mass in 51 % yield, after purification of Sephadex LH-20 eluting with $CHCl_3$-MeOH (1:1).

Coupling of 6 with rhIL-1α

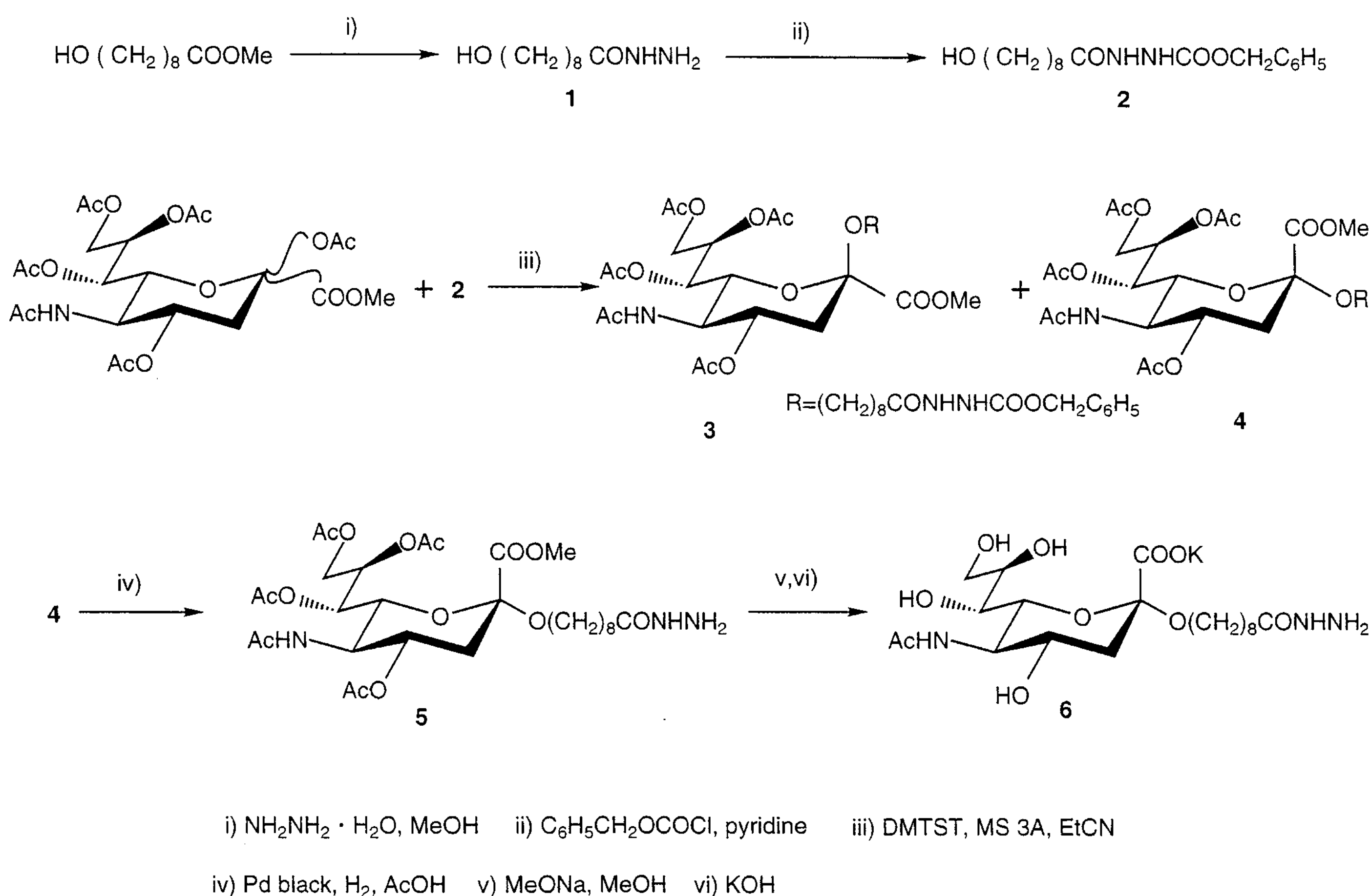
Compound **6** was transformed to an acyl azide derivative by reaction with hydrogen nitrite. The acyl azide derivative was coupled with rhIL-1α in 0.4 M sodium borate buffer (pH 10.0) for 60 min to yield a glycosylated rhIL-1α (Scheme 1). rhIL-1 treated in the same manner without the acyl azide derivative was used as control (treated) IL-1α.

Purification of NANA-introduced IL-1α

The NANA-coupled IL-1 (NANA-IL-1α) was purified employing FPLC system using an anion-exchange chromatography column. As shown in Figure 1A and B, untreated IL-1α eluted in Fr. 6, and treated IL-1α in Fr. 6 and 8. The small peak at Fr. 8 of treated IL-1 seemed to be due to the conformational changes or modification caused by the reaction condition. In contrast, glycosylated IL-1 eluted in Fr. 7 to 12 peaking at several fractions (Fig. 1C). As Fr. 8 may contain the nonglycosylated IL-1 as demonstrated in Fig. 1 B, Fr. 9 to 12 were collected as glycosylated IL-1α. The retarded elution profile of glycosylated IL-1 suggests that amino residues of IL-1 reacted with NANA-C9 and that NANA-derived negative charge was introduced into IL-1. The yield of NANA-IL-1 was 12.4%.

SDS-PAGE analysis of NANA-IL-1α and confirmation of glycosylation

Untreated, treated and NANA-IL-1s were analyzed on SDS-PAGE. As shown Figure 2A, untreated IL-1 and treated IL-1 migrated at 17.6 kDa, and NANA-IL-1 at 19.0 kDa in average. The increased M.W. of NANA-IL-1 represents NANA introduction. Based on the difference in M.W., NANA introduced into IL-1 was estimated at 2.9 mole per molar IL-1. The M.W. of NANA-IL-1 was also determined by TOF-MS analysis. NANA-IL-1 exhibited a



Scheme 1

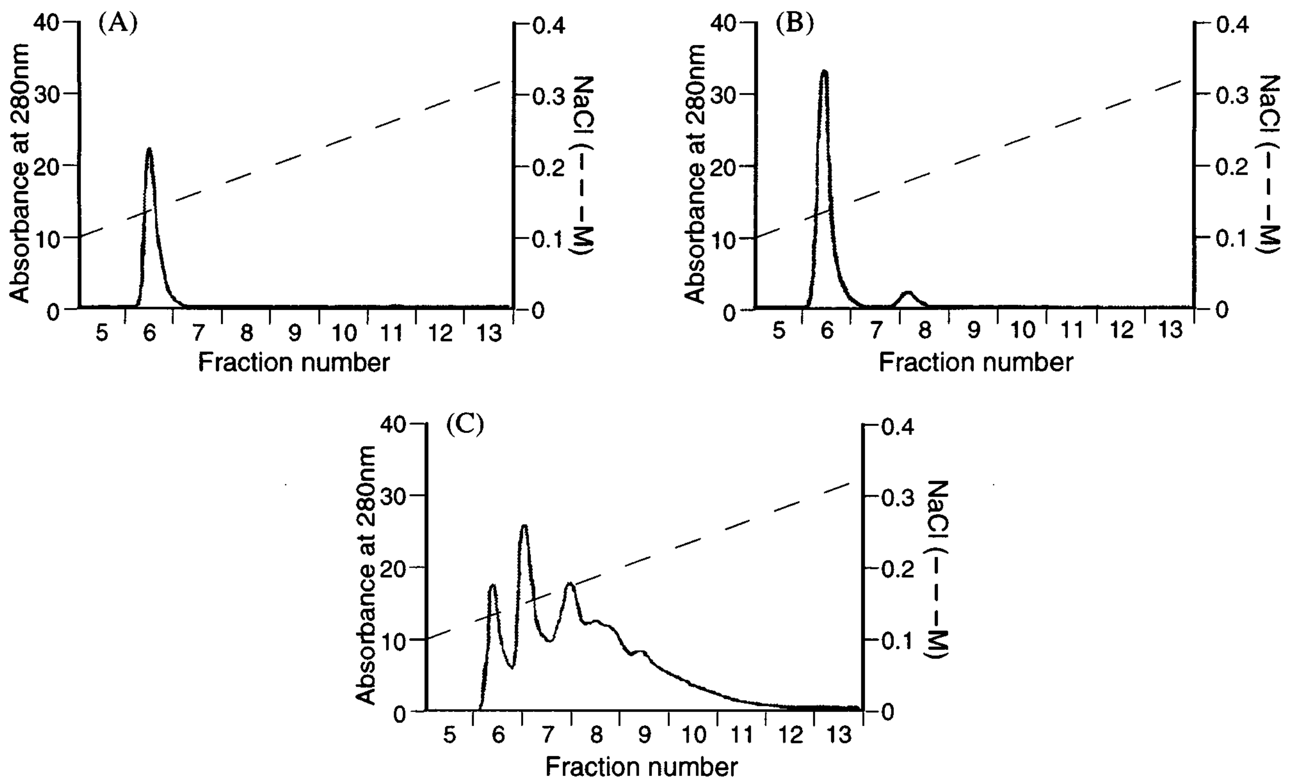
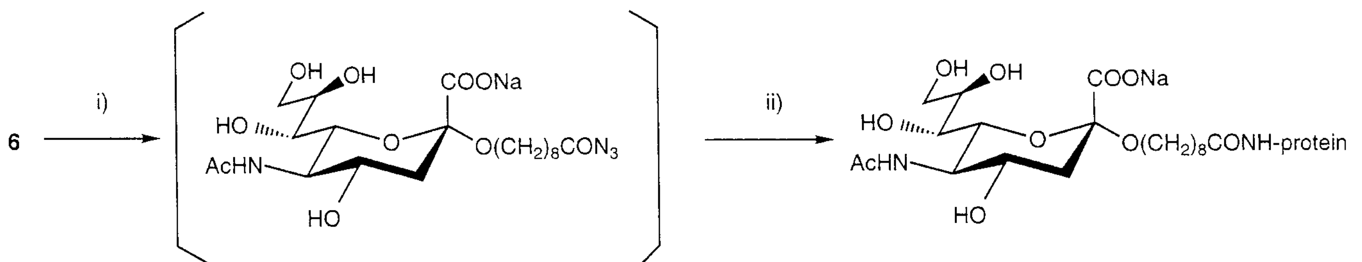


Figure 1. Purification of NANA-IL-1α. (A) Untreated rhIL-1α (250μg), (B) treated IL-1α (500μg) or (C) NANA-IL-1α (2mg) after reaction were applied to the anion-exchange chromatography column (Mono Q, Pharmacia) with FPLC system. The starting buffer was 20mM Tris-HCl (pH 7.0), and the elution buffer was the starting buffer containing 0.5M NaCl with a liner gradient. Fractions (1 ml) were collected at a flow rate of 1ml/min.

major peak at 18.6 kDa (data not shown). Therefore, NANA introduced into IL-1 was about 2.7 mole per molar IL-1, which is close to the result of SDS-PAGE analysis. By the acyl azide method, the carbohydrate derivative theoretically reacts with amino residues in IL-1 [9]. rhIL-1α contains 15 potentially reactive amino residues, 13 Lys, 1 Arg and N-terminal amno acid [24]. Therefore, about 20% of them appeared to be reacted with NANA.

To confirm that NANA was introduced into IL-1, IL-1-transferred membrane was analyzed with G.P. Sensor kit. As shown in Figure 2B, only NANA-IL-1 was markedly stained indicating that NANA was coupled to IL-1. The faint staining of untreated and control IL-1s was nonspecific because nonglycosylated molecular weight markers were also stained in the same density (data not shown). Fr. 7 and 8 of Mono Q chromatography of NANA-IL-1 (Fig. 1C) were also collected and analyzed by SDS-PAGE and G.P. Sensor kit. IL-1 in these fractions was slightly larger than untreated IL-1, and could be stained by G.P. Sensor, indicating that these fractions also contained NANA-introduced IL-1, but the number of NANA is lower.

It is difficult to couple NANA to proteins because of its carboxyl residue. However, we succeeded in the synthesis of NANA-IL-1 by using C9 spacer-conjugated NANA. This



Scheme 2

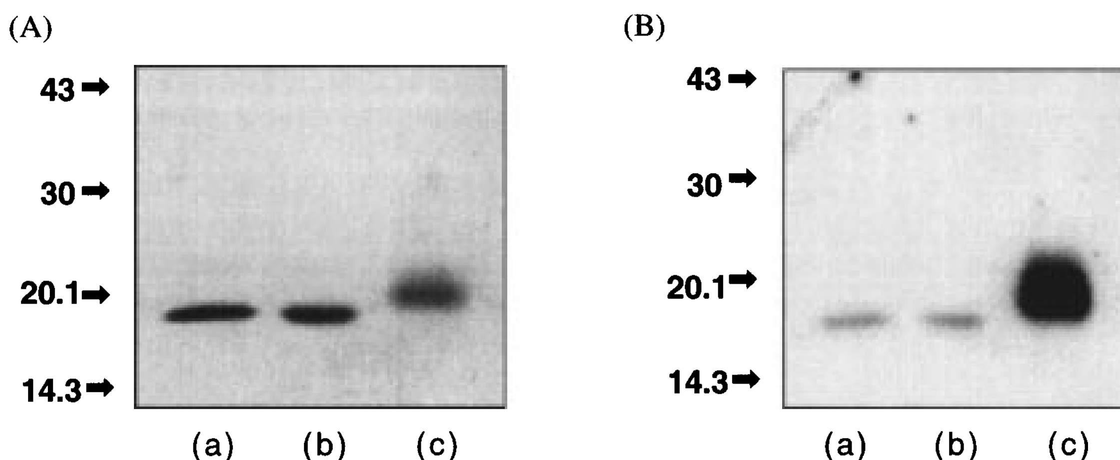


Figure 2. SDS-PAGE and detection of NANA with oxidation with $NaIO_4$. (A) 1 μg of IL-1αs were analysed on SDS-containing 15% polyacrylamide gel under reduced condition. IL-1αs were visualized with Coomassie Brilliant Blue staining. (B) 0.5 μg of IL-1αs were electrophoresed on SDS-containing 15% polyacrylamide gel under reduced condition. After electrophoresis, IL-1αs were transfered onto a PVDF membrane, oxidized with $NaIO_4$, biotinylated and reacted with HRP-avidin. Molecular weight standard electrophoresed in parallel are indicated to the left in kilodaltons. (a) untreated IL-1α (b) treated IL-1α (c) NANA-IL-1α.

spacer-mediated coupling, therefore, will be useful for the conjugation of NANA to other cytokines.

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