Biosci. Biotechnol. Biochem., **62** (6), 1171–1178, 1998

Synthesis of Artificial *N*-Glycopolypeptides Carrying *N*-Acetyllactosamine and Related Compounds and Their Specific Interactions with Lectins

Xiaoxiong ZENG, Takeomi MURATA, Hirokazu KAWAGISHI, Taichi USUI,† and Kazukiyo KOBAYASHI*

Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Ohya 836, Shizuoka 422-8529, Japan
**Graduate School of Engineering, Nagoya University, Chikusa, Nagoya 464-8603, Japan*

Received December 11, 1997

Artificial *N*-glycopolypeptides carrying *N*-acetyllactosamine (LacNAc) or related compounds were synthesized. First, sugars were converted into their corresponding β-glycosylamines with ammonium hydrogen carbonate. Then, the β-glycosylamines were condensated with the carboxyl groups of poly(L-glutamic acid). *N*-Glycopolypeptides with different degrees of substitution of sugars were isolated by passage through a column of Sephadex G-25. These synthetic polymers were used as model compounds in the analysis of oligosaccharide-lectin interactions. Interactions with some lectins were investigated by agar-gel double-diffusion tests and in terms of inhibition of hemagglutination. A glycopolypeptide substituted with LacNAc reacted with *Erythrina cristagalli* agglutinin (ECA), peanut (*Arachis hypogaea*) agglutinin (PNA), *Ricinus communis* agglutinin-120 (RCA_{120}), wheat germ (*Triticum vulgaris*) agglutinin (WGA) lectins, which recognize either galactosyl or *N*-acetylglucosamine (GlcNAc) residues. Other synthetic glycopolymers carrying *N*-acetylisolactosamine, GlcNAc, *N*,*N*′-diacetylchitobiose, or *N*,*N*′,*N*″-triacetylchitotriose also reacted with WGA, and these last two polymers inhibited hemagglutination most. Of these five glycopolypeptides, only the one substituted with LacNAc reacted with ECA. These sugar-substituted glycopolypeptides interacted specifically with the corresponding lectins, no matter how much shorter the sugar side chains of the glycopolymers were than those of natural glycoproteins.

Key words: *N*-glycopolypeptide; glycopolymer; lectin; molecular recognition; glycotechnology

Carbohydrate sequences of glycoproteins and glycolipids are information rich, initiating and regulating many biological recognition processes.[1,2] Glycoproteins usually can be found as several different glycoforms, and it can be difficult to prepare well-defined glycoproteins from natural sources in large amounts for the elucidation of structures and functions. More attention, therefore, has been paid to neo- or artificial glycoconjugate polymers, in which natural and synthetic polymers have pendant oligosaccharide moieties added.[3,4] A variety of naturally occurring and synthetic oligosaccharide derivatives have been attached to various kinds of macromolecules and used as vaccines or antigens, and in targeted drug delivery.[5-7] These polymers also can be used for the investigation of carbohydrate-protein interactions and biological recognition with lectins and anti-carbohydrate monoclonal antibodies.[8]

We have developed a simple synthetic method for glycopolypeptide conjugates in which lactose and $(GlcNAc)_2$ chains are attached to carboxyl groups of poly(L-glutamic acid) through β-*N*-linkages.[9] Here, we use the method on a bioactive oligosaccharide, LacNAc, and related mono- and oligosaccharides including iso-LacNAc, $(GlcNAc)_3$, $(GlcNAc)_2$, and GlcNAc. The LacNAc sequence is a major component of the core oligosaccharides of glycoproteins and glycolipids, which serve as differentiation antigens, tumor-associated antigens, and ligands of some receptors and enzymes.[10-12] A glycopolypeptide carrying LacNAc residues may have functions in various events of biological recognition. The interactions of such glycopolypeptides with lectins were investigated by double-diffusion tests in agar and by inhibition of hemagglutination.

Materials and Methods

Materials. GlcNAc was purchased from Yaizu Suisan Kagaku Co., Ltd. (Yaizu, Japan). $(GlcNAc)_2$, $(GlcNAc)_3$, LacNAc, iso-LacNAc, ECA, and poly(*p*-acryloyl-aminophenyl *N*-acetyl-β-lactosaminide) (PAP-LacNAc) were prepared as reported elsewhere.[13-17] Poly(L-glutamic acid) sodium salt (molecular weight 13,600, degree of polymerization 90) was obtained from Sigma (St. Louis, MO, USA), BOP and HOBt were obtained from Aldrich (Milwaukee, Wis., USA), and PNA, RCA_{120}, WGA were purchased from Seikagaku Corp. (Tokyo, Japan).

† To whom correspondence should be addressed.

Abbreviations: BOP, benzotriazol-1-yl-oxy-tri(dimethylamino)-phosphonium hexafluorophosphate; DS, degree of substitution; ECA, *Erythrina cristagalli* agglutinin; GlcNAc, 2-acetamido-2-deoxy-D-glucose; $(GlcNAc)_2$, 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose; $(GlcNAc)_3$, 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosy-(1→4)-2-acetamido-2-deoxy-D-glucose; HOBt, hydroxybenzotriazole; iso-LacNAc, β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucose; LacNAc, β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose; Me_2SO, dimethyl sulfoxide; PGA, poly(L-glutamic acid); PNA, peanut (*Arachis hypogaea*) agglutinin; RCA_{120}, *Ricinus communis* agglutinin-120; TPS, sodium 3-(trimethylsilyl)-propionate; WGA, wheat germ (*Triticum vulgaris*) agglutinin.

Analytical methods. HPLC was done with a liquid chromatograph Hitachi 6000 with a column of TSK gel 3000 PW_{XL} (ϕ7.8 × 300 mm, Tosoh Corp., Japan) and developed with 0.2 M NaCl at a flow rate of 0.5 ml/min with a UV detector at 210 nm. ^1H and ^{13}C NMR spectra were recorded with Fourier transform NMR spectrometer (Jeol JNM-EX 270 or JNM-EX 500). Chemical shifts are expressed in δ relative to TPS as an external standard.

General procedure for preparation of β-glycosylamines. The monosaccharide or oligosaccharide was dissolved to the concentration of 0.2 M in a 50 ml flask, and NH_4HCO_3 was added to saturation. Then the solution was stirred and incubated at 37°C in an uncovered flask. During the reaction, the solution was kept saturated by the addition of NH_4HCO_3 as needed. The reaction mixture was monitored by TLC with a 4:3:3:1 (by vol.) mixture of ethyl acetate, acetic acid, methanol, and water. The R_f was 0.60 for LacNAc and 0.35 for β-*N*-acetyllactosaminylamine, 0.60 for iso-LacNAc and 0.40 for β-*N*-acetylisolactosaminylamine, 0.71 for GlcNAc and 0.49 for β-*N*-acetylglucosaminylamine, 0.60 for $(GlcNAc)_2$ and 0.37 for β-*N*,*N*′-diacetylchitobiosylamine, and 0.54 for $(GlcNAc)_3$ and 0.34 for β-*N*,*N*′,*N*″-triacetylchitotriosylamine. When almost all of the sugar was converted to the 1-amino sugar, the reaction mixture was diluted with water, and NH_4HCO_3 was removed by concentration under reduced pressure with a bath temperature of 29–30°C. This procedure was repeated until there was no smell of NH_4HCO_3 in the concentrated solution. Finally the solution was freeze-dried, giving the desired glycosylamine in a yield of about 80% to 90%.

Preparation of PGA. The poly(L-glutamic acid) sodium salt obtained from Sigma was contained some unreacted methyl glutamate residues, seen in its NMR spectra. These residues all were converted to glutamic acid sodium salt as follows. One gram of the purchased preparation was dissolved in 20 ml of dichloroethane. Then 200 ml of a 0.12 N sodium hydroxide solution in a mixture of methanol and water (4:1 by vol.) was added dropwise to the solution, and the mixture was stirred at room temperature for 6 h. Then the precipitate was collected and washed with methanol. The precipitate was dissolved in 20 ml of cold water, and 5.2 ml of 1 N hydrochloric acid solution cooled in an ice bath was added dropwise to the solution with stirring for 2 h. The precipitate was collected by centrifugation (4°C, 24,100 × *g*) and washed with cold water several times. Finally the suspension of the precipitate in water was freeze-dried, affording PGA in a yield of 60%. ^1H NMR (D_2O, 30°C, 500 MHz) δ4.32 (peptide α-methine NHC*H*CO), 2.27 (peptide γ-methylene $CHCH_2CH_2$), 2.04 (peptide β-methylene $CHCH_2CH_2$). ^{13}C NMR (D_2O, 30°C, 500 MHz) δ176.23 (CO), 56.19 (peptide α-methine NHCHCO), 36.09 (peptide γ-methylene $CHCH_2CH_2$), 30.72 (peptide β-methylene $CHCH_2CH_2$).

Preparation of poly(N-acetyllactosaminyl-L-glutamine-co-glutamic acid) [*Poly(LacNAc/Gln-co-Glu)*, **1**]. PGA (20 mg, 0.0017 mmol) was dissolved in 0.3 ml of Me_2SO. Me_2SO solution (0.3 ml) containing BOP (170 mg, 0.385 mmol) and HOBt (18 mg, 0.133 mmol) was added and the resulting mixture was stirred magnetically for 15 min at room temperature. *N*-acetyllactosaminylamine (120 mg, 0.313 mmol) in Me_2SO (0.6 ml) was added and stirring was continued for 10 h under the same conditions. The solution was put immediately on a Sephadex G-25 column (ϕ 1.0 × 25 cm), and eluted with 0.02 M phosphate buffer (pH 7.4) containing 0.1 M NaCl at a flow rate of 1.0 ml/min. The carbohydrate component of the elution was monitored by the phenol-H_2SO_4 method at 485 nm. The eluate (1.0 ml/tube) had two peaks (F-1, tubes 16–31 and F-2, tubes 33–59). The first peak was collected, concentrated with an Amicon Diaflo ultrafiltration unit equipped with a YM-3 membrane operating at 2 kg/cm^2, and lyophilized, giving **1** (44 mg).

^1H NMR (D_2O, 30°C, 270 MHz) δ5.20 (d, J=8.1 Hz, H-1), 4.56 (d, J=7.6 Hz, H-1′), 4.38 (peptide α-methine NHC*H*CO), 3.62–3.98 (complex, other protons from LacNAc unit as side chain), 2.43 (peptide γ-methylene $CHCH_2CH_2$), 2.05 (peptide β-methylene $CHCH_2CH_2$ and $NHCOCH_3$). ^{13}C NMR (D_2O, 30°C, 270 MHz) δ184.02, 178.43, 177.44, 176.44, and 175.83 (CO), 105.84 (C-1′), 81.18 (C-4), 81.10 (C-1), 79.48 (C-5), 78.27 (C-5′), 75.58 (C-3), 75.47 (C-3′), 73.93 (C-2′), 71.53 (C-4′), 63.85 (C-6′), 62.89 (C-6), 56.99 (C-2), 55.88 (peptide α-methine NHCHCO), 36.49, and 34.73 (peptide γ-methylene $CHCH_2CH_2$), 30.83, and 29.37 (peptide β-methylene $CHCH_2CH_2$), 25.12 ($NHCOCH_3$).

Preparation of poly(N-acetylisolactosaminyl-L-glutamine-co-glutamic acid) [*Poly(iso-LacNAc/Gln-co-Glu)*, **2**]. Compound **2** was prepared in a similar manner as **1** (Table II). ^1H NMR (D_2O, 30°C, 270 MHz) δ5.21 (d, J=9.4 Hz, H-1), 4.50 (d, J=7.56 Hz, H-1′), 4.38 (peptide α-methine NHC*H*CO), 4.31 and 3.59–3.99 (complex, other protons from iso-LacNAc unit as side chain), 2.44 (peptide γ-methylene $CHCH_2CH_2$), 2.06 (peptide β-methylene $CHCH_2CH_2$ and $NHCOCH_3$). ^{13}C NMR (D_2O, 30°C, 500 MHz) δ183.71, 178.24, 177.36, 176.28, and 175.69 (CO), 106.08 (C-1′), 81.13 (C-1), 79.47 (C-5), 78.01 (C-5′), 76.91 (C-3), 75.54 (C-3′), 73.56 (C-2′), 72.38 (C-4), 71.54 (C-6), 71.47 (C-4′), 63.85 (C-6′), 57.20 (C-2), 56.38, and 55.77 (peptide α-methine NHCHCO), 36.17, 34.57, and 33.83 (peptide γ-methylene $CHCH_2CH_2$), 30.66, and 29.83 (peptide β-methylene $CHCH_2CH_2$), 25.01 ($NHCOCH_3$).

Preparation of poly(N,N′,N″-triacetylchitotriosyl-L-glutamine-co-glutamic acid) [*Poly((GlcNAc)$_3$/Gln-co-Glu)*, **3**]. Compound **3** was prepared in a similar manner as **1** (Table II). ^1H NMR (D_2O, 30°C, 270 MHz) δ5.16 (d, J=8.1 Hz, H-1), 4.67 (2d, $J_{1',2'}=J_{1'',2''}$=7.8 Hz, H-1′, H-1″), 4.38 (peptide α-methine NHC*H*CO), 3.52–4.00 (complex, other protons from $(GlcNAc)_3$ unit as side chain), 2.41 (peptide γ-methylene $CHCH_2CH_2$), 2.13 (peptide β-methylene $CHCH_2CH_2$), 2.13, and 2.04 ($NHCOCH_3$). ^{13}C NMR (D_2O, 30°C, 270 MHz) δ183.66, 178.36, 177.48, 176.31, and 175.76 (CO),

104.31 (C-1″), 104.08 (C-1′), 82.01 (C-4), 81.65 (C-4′), 81.13 (C-1), 79.24 (C-5″), 78.83 (C-5′), 77.43 (C-3″), 76.41 (C-3′), 75.69 (C-5), 75.09 (C-3), 72.67 (C-4″), 63.50 (C-6″, C-6′), 62.93 (C-6), 58.56 (C-2″), 58.02 (C-2′), 56.91 (C-2), 56.42 (peptide α-methine NHC*H*CHCO), 36.17, 34.72, and 33.91 (peptide γ-methylene $CHCH_2C*H_2$), 30.69, and 29.99 (peptide β-methylene $CHC*H_2CH_2$), 25.10 ($NHCOC*H_3$).

Preparation of poly(N,N′-diacetylchitobiosyl-L-glutamine-co-glutamic acid) [*Poly((GlcNAc)$_2$/Gln-co-Glu)*, **4**]. Compound **4** was prepared in a similar manner as **1** (Table II). ^{1}H NMR (D_2O, 30°C, 270 MHz) δ5.16 (d, J=9.5 Hz, H-1), 4.68 (d, J=7.9 Hz, H-1′), 4.38 (peptide α-methine NHC*H*CO), 3.54–4.02 (complex, other protons from (GlcNAc)$_2$ unit as side chain), 2.41 (peptide γ-methylene $CHCH_2C*H_2$), 2.14 (peptide β-methylene $CHC*H_2CH_2$), 2.14, and 2.05 ($NHCOC*H_3$). ^{13}C NMR (D_2O, 30°C, 270 MHz) δ183.89, 180.52, 178.31, 177.48, and 175.90 (CO), 104.38 (C-1′), 82.01 (C-4), 81.26 (C-1), 79.17 (C-5′), 78.87 (C-5), 76.46 (C-3′), 75.79 (C-3), 72.70 (C-4′), 63.56 (C-6′), 62.93 (C-6), 58.54 (C-2′), 56.84 (C-2), 55.90 (peptide α-methine NHCHCO), 36.53, 34.73, and 33.99 (peptide γ-methylene $CHCH_2CH_2$), 29.81 (peptide β-methylene $CHCH_2CH_2$), 25.12 ($NHCOCH_3$).

Preparation of poly(N-acetylglucosaminyl-L-glutamine-co-glutamic acid) [*Poly(GlcNAc/Gln-co-Glu)*, **5**]. Compound **5** was prepared in a similar manner as **1** (Table II). ^{1}H NMR (D_2O, 30°C, 270 MHz) δ5.18 (d, J=9.4 Hz, H-1), 4.37 (peptide α-methine NHC*H*CO), 3.55–3.97 (complex, other protons from GlcNAc), 2.44 (peptide γ-methylene $CHCH_2C*H_2$), 2.06 (peptide β-methylene $CHC*H_2CH_2$ and $NHCOC*H_3$). ^{13}C NMR (D_2O, 30°C, 270 MHz) δ183.99, 180.52, 178.31, 177.45, and 175.99 (CO), 81.33 (C-1), 80.59 (C-5), 77.18 (C-3), 75.54 (C-4), 63.56 (C-6), 57.41 (C-2), 56.12 (peptide α-methine NHCHCO), 36.62, 34.73, and 34.02 (peptide γ-methylene $CHCH_2CH_2$), 29.76 (peptide β-methylene $CHCH_2CH_2$), 25.10 ($NHCOCH_3$).

Double-diffusion test in agar. Four percent agar (3.0 g) was dissolved in 13.5 ml of 10 mM phosphate-buffered saline (PBS, pH 7.4) to give a concentration of 0.9%, and two drops of 1% sodium azide was added. The solution was poured into three glass dishes (42 mm, i.d.) to form a layer 3–4 mm thick. Wells were made with a steel puncher. Aqueous lectin solution (0.5 mg/ml PBS, 15 μl) and 15 μl of an *N*-glycopolypeptide solution (2 mg/ml, PBS) were added to the central and peripheral wells, respectively, with a syringe, and the plates were incubated at 30°C for 15 h. Gels were stained with 0.5% Amido Black 10B in 7.5% acetic acid and washed with 7.5% acetic acid.

Inhibition of hemagglutination by artificial N-glycopolypeptides and their corresponding sugars.[18] Fourfold minimum concentration lectin solution was prepared as follows. Portions (20 μl) of a 3% erythrocyte (Human blood type A) suspension was pipetted into each well of the twofold dilution series of lectin (20 μl) in 96-holes U-plate, then incubated for 1 h. The minimum concentration of lectin required for hemagglutination of erythrocyte was determined, and its fourfold concentration solution was used for the following assay.

Twofold serial dilution (20 μl) of *N*-glycopolypeptides and their sugars were prepared with PBS in a 96-holes microtiter U-plate. Then 20-μl portions of the lectin solution were pipetted into each hole and the plate was incubated at room temperature for 1 h. Then 40 μl of a 3% suspension of erythrocytes was added to each hole and the plate was incubated for 1 h. Agglutination of erythrocytes was looked for and the minimum concentration of *N*-glycopolypeptide or sugar that inhibited erythrocyte agglutination was identified.

Results and Discussion

Synthesis of N-glycopolypeptides

The scheme of *N*-glycopolypeptide synthesis is shown in Fig. 1.

(1) *Preparation of β-glycosylamines*

Firstly, the mono- and oligosaccharides were treated with a saturated solution of ammonium hydrogen carbonate according to Kochetkov's method.[19,20] Then, the reagent was removed by concentration of the solution and repeated dilution the powdery products with water. The product was 80–90% glycosylamine, and 10–20% unreacted sugar.

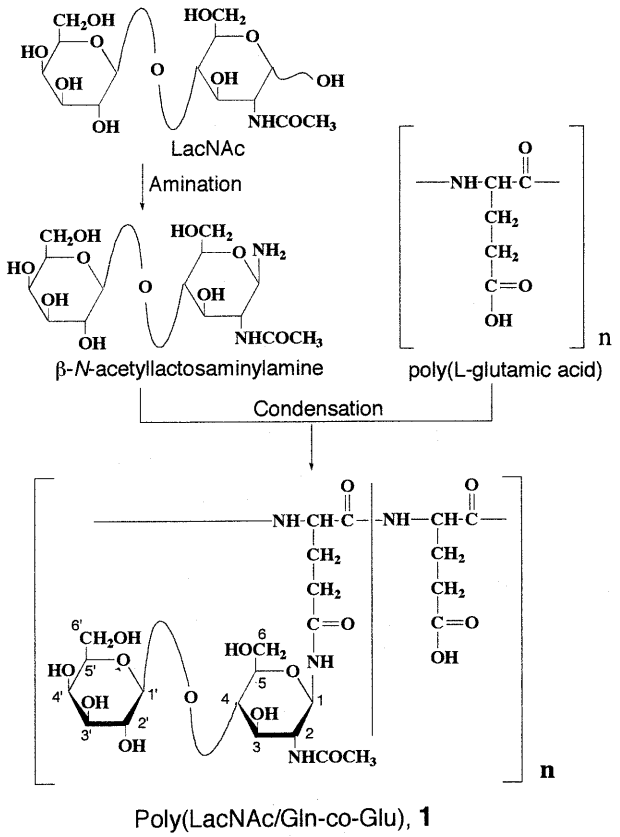


Fig. 1. Synthesis of Poly(*N*-Acetyllactosaminyl-L-glutamine-co-glutamic Acid) [Poly(LacNAc/Gln-co-Glu), **1**].

Table I. ^{13}C Chemical Shifts of Glycosylamines[a]

Glycosylamine		Chemical shift (δ), ppm							
		C-1	C-2	C-3	C-4	C-5	C-6	CO	$NHCOCH_3$
Galβ1→4GlcNAc-NH_2	I	87.10	58.83	75.47	81.74	78.29	63.20	177.51	25.23
II I	II	105.84	73.91	76.05	71.50	78.63	63.95	177.51	25.23
Galβ1→6GlcNAc-NH_2	I	87.35	59.21	77.41	72.87	78.11	71.77	177.63	25.21
II I	II	106.24	73.71	75.63	71.59	78.71	69.93	177.63	25.21
GlcNAcβ1→4GlcNAcβ1→4GlcNAc-NH_2	I	87.01	57.90	74.25	82.41	75.02	62.84	177.45	24.98
III II I	II	104.15	58.45	75.97	82.01	78.31	63.13	177.45	24.98
	III	104.33	58.65	76.30	72.58	78.78	64.41	177.45	24.98
GlcNAcβ1→4GlcNAc-NH_2	I	87.06	58.71	76.10	82.75	78.33	63.25	177.59	25.21
II I	II	104.42	58.53	76.41	72.45	78.83	63.49	177.52	25.05
GlcNAc-NH_2		85.61	56.77	74.81	71.11	77.60	61.37	169.79	22.88

[a] In D_2O, except for GlcNAc-NH_2, in Me_2SO.

Table I summarizes ^{13}C NMR spectral data of the glycosylamines. The configuration and glycosylamine structures were identified as follows with *N*-acetyllactosaminylamine, Galβ1→4GlcNAc-NH_2, used in the explanation as an example. The two anomeric proton signals at δ4.54 (J=8.1 Hz) and 4.25 (J=8.6 Hz) were attributed to H-1′ and H-1, both with the β-anomeric configuration. There was a characteristic signal at 87.10 ppm, which had shifted to a higher field than that of the reducing anomeric signals at 97.60 (C-1β) and 93.27 (C-1α) ppm of the starting sugar, LacNAc. This characteristic signal was assignable to the β-configuration, because the carbon signals arising from the β-anomeric structure of LacNAc closely corresponded to those of Galβ1→4GlcNAc-NH_2 except for C-1. No α-anomeric structure was detected. The amination described here was simple and protection of the hydroxyl groups of oligosaccharides was not needed. For the synthesis described below of *N*-glycopolypeptides, glycosylamines of 80–90% purity were used as is without chromatographic purification.

(2) *Transformation of β-glycosylamines into N-glycopolypeptides*

The 1-amino function of β-*N*-glycosylamine was coupled with the carboxyl group of PGA in the presence of BOP and HOBt as the condensation reagents as described elsewhere.[21] The solution was put on a column of Sephadex G-25 to separate the polymeric products from the low-molecular-weight reactants. Figure 2 shows a typical chromatogram after the reaction with *N*-acetyllactosaminylamine, and shows that two peaks had been separated by the chromatography. The F-1 fraction (tubes 16–31) was collected, ultrafiltered, and freeze-dried, affording *N*-glycosylated poly(L-glutamic acid) **1**. The degree of substitution (DS) in the mole fraction of the substituted residues in *N*-glycosylated poly(L-glutamic acid) as percentage was estimated from the ^1H NMR peak area of the *N*-glycosylated H-1 proton, peptide γ- and β-methylene protons, and acetyl protons. The large F-2 peak (tubes 33–59), which contained several reactants, was collected, concentrated, and chromatographed by a charcoal-Celite column. A mixture of unreacted *N*-acetyllactosaminylamine and LacNAc was recovered and recycled for amination. Typical data

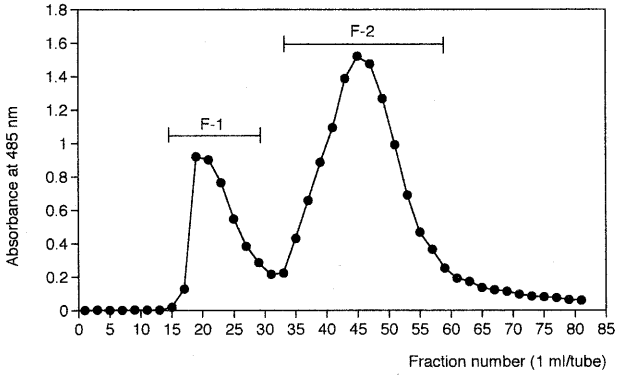


Fig. 2. Separation Chromatography of Condensation Products of *N*-Acetyl-lactosaminylamine and Poly(L-glutamic Acid) in the Presence of BOP and HOBt. Chromatography was done on a column (ϕ1.0×25 cm) of Sephadex G-25 and with 0.01 M phosphate sodium buffer (pH 7.4) containing 0.1 M NaCl at a flow rate of 1 ml/min.

about the preparations are summarized in Table II. Glycopolymers with different degree of substitution were prepared from different molar ratios of the reactants.

The structures of the synthetic glycopolypeptides (Fig. 3) were identified from their ^1H and ^{13}C NMR spectra. The linkage between the glycosyl residue and the γ-carboxamide of the glutamine residue was in the β-anomeric configuration. For example, *N*-β-linked anomeric proton signals appeared at δ5.20 ppm (J=8.1 Hz) for **1** (Fig. 4) and δ5.21 ppm (J=9.4 Hz) for **2**. The proton signals at δ4.56 ppm (J=7.6 Hz) for **1** and δ4.50 ppm (J=7.6 Hz) for **2** were assignable to the nonreducing terminal H-1′ of *O*-linked β-anomeric galactose involved in the LacNAc and iso-LacNAc units, respectively. In the ^{13}C NMR spectra, the *N*-β-linked C-1 signal shifted higher than the *O*-β-linked C-1′ signal. The *N*-β-linked C-1 signal was in a higher chemical shift at 81.10 ppm than the *O*-β-linked C-1′ at 105.84 ppm (Fig. 5). No other minor structure was detected.

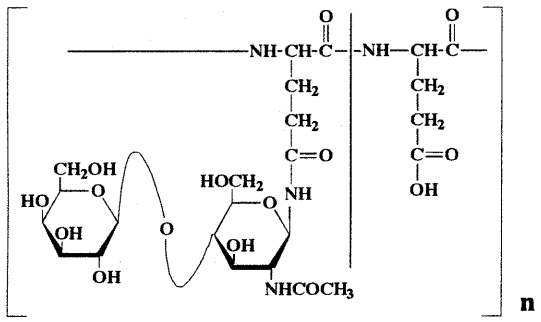
Interactions between N-glycopolypeptides and lectins

To evaluate molecular recognition by various synthetic *N*-glycopolypeptides, we investigated their interac-

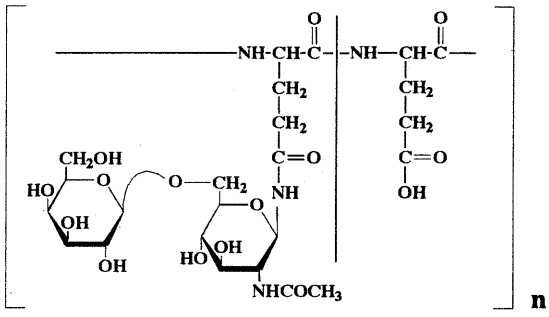
Table II. Synthesis of Glycopolypeptides **1**, **2**, **3**, **4**, and **5**[a]

Glycopolypeptide	PGA[b] (mg)	Glycosylamine		Coupling reagents		Yield (mg)	DS[e] (mol%)
		Glycosyl-NH_2	mg	BOP[c] (mg)	HOBt[d] (mg)		
1	20	LacNAc-NH_2	120	170	18	44	44
2	20	iso-LacNAc-NH_2	170	180	20	38	31
3	20	$(GlcNAc)_3$-NH_2	280	180	18	54	38
4	20	$(GlcNAc)_2$-NH_2	160	170	19	47	44
5	20	GlcNAc-NH_2	100	170	18	34	78

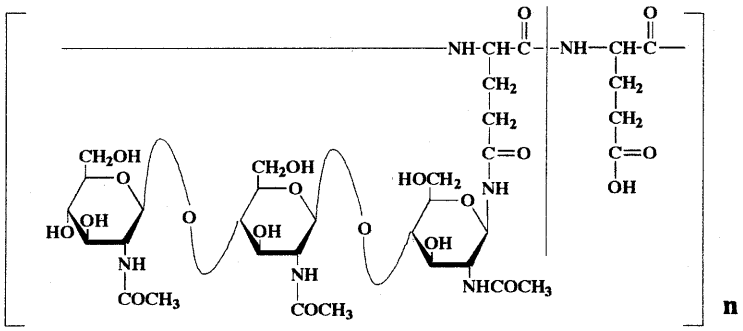
[a] See "Materials and Methods".
[b] Poly(L-glutamic acid).
[c] Benzotriazol-1-yl-oxy-tri(dimethylamino)-phosphonium hexafluorophosphate.
[d] Hydroxybenzotriazole.
[e] Degree of substitution.



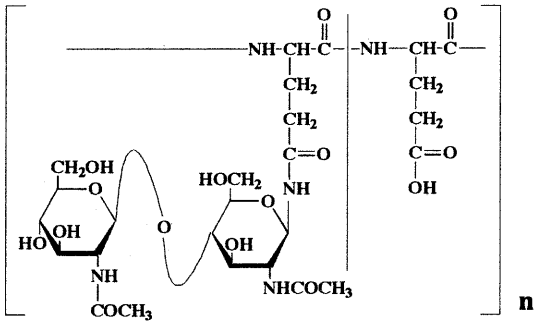
Poly(LacNAc/Gln-co-Glu), **1**



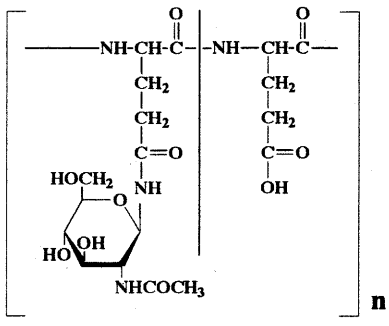
Poly(iso-LacNac/Gln-co-Glu), **2**



Poly($(GlcNAc)_3$/Gln-co-Glu), **3**



Poly($(GlcNAc)_2$/Gln-co-Glu), **4**



Poly(GlcNAc/Gln-co-Glu), **5**

Fig. 3. Structures of Synthesized *N*-Glycopolypeptides Carrying *N*-Acetyllactosamine and Related Sugars.

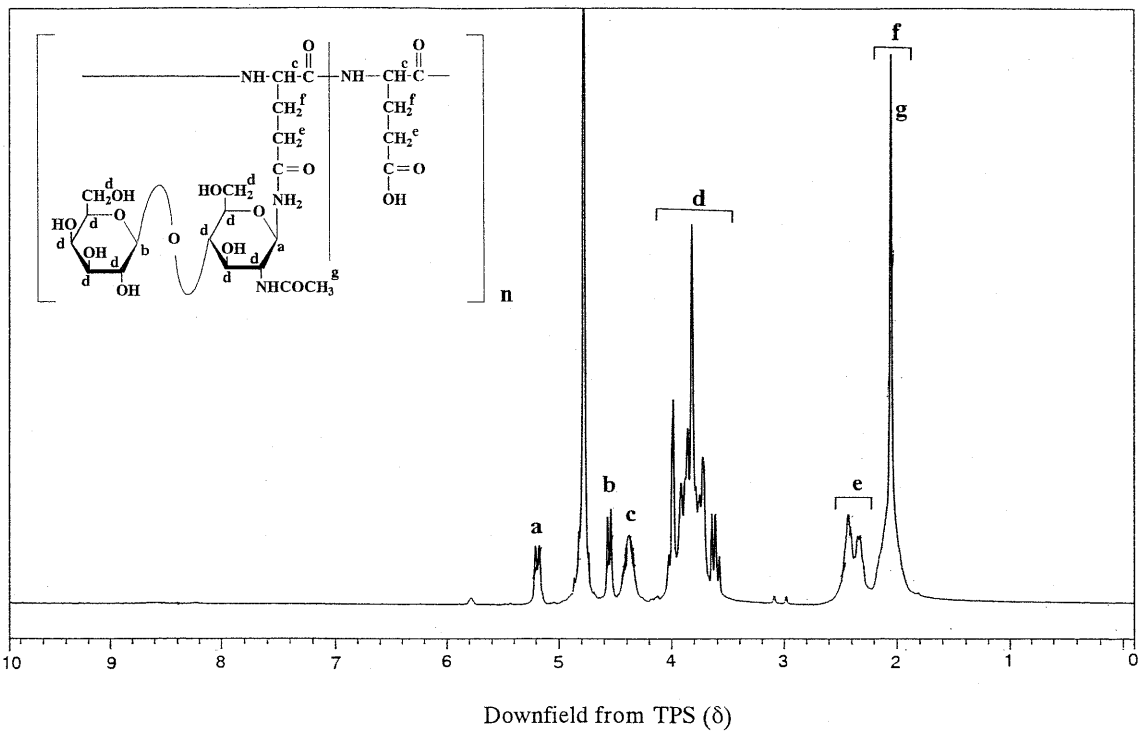


Fig. 4. ^{1}H NMR Spectrum of Poly(*N*-Acetyllactosaminyl-L-glutamine-co-glutamic Acid), **1** (D_2O, 30°C, 270 MHz).

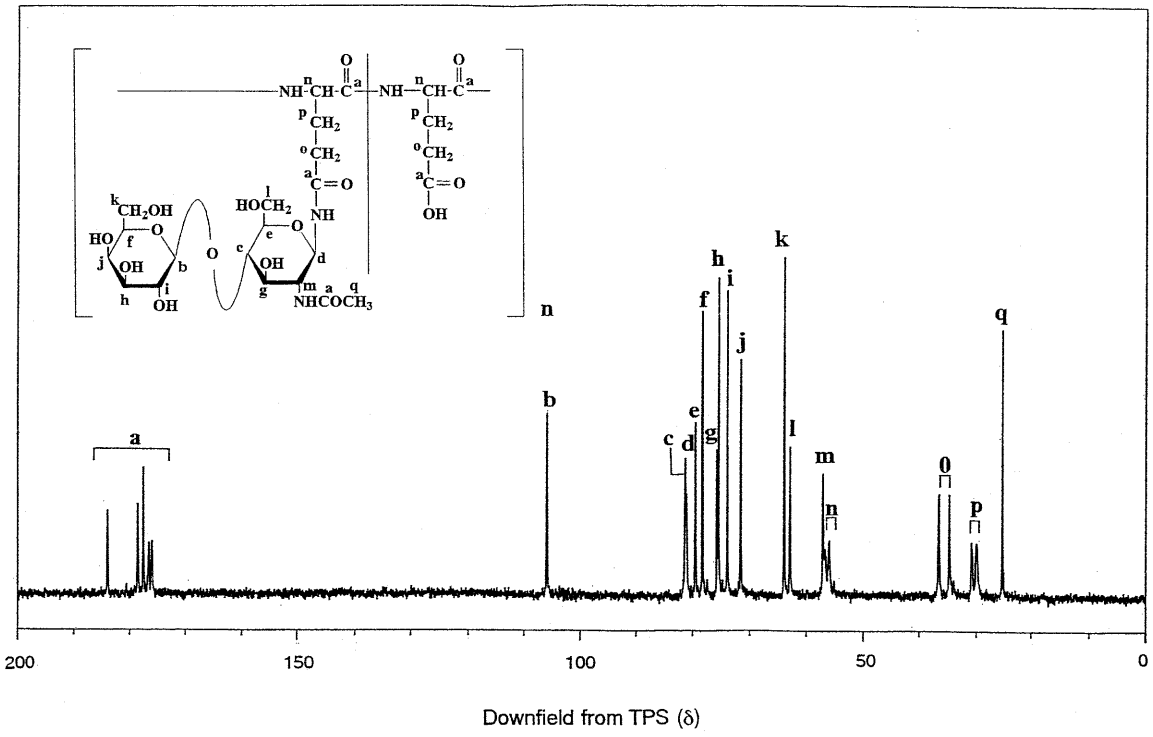


Fig. 5. ^{13}C NMR Spectrum of Poly(*N*-Acetyllactosaminyl-L-glutamine-co-glutamic Acid), **1** (D_2O, 30°C, 270 MHz).

tions with lectins by double-diffusion reactions in agar and by their inhibition of hemagglutination. The following lectins were used (their binding saccharides are given in brackets): ECA (Galβ1→4GlcNAc), PNA (Galβ1→3GalNAc), RCA_{120} (β-Gal), and WGA [$(GlcNAc)_n$].

Figure 6 shows results of a double diffusion test with WGA lectin. Sharp precipitin bands are seen between

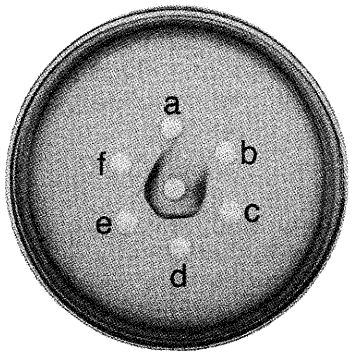


Fig. 6. Double-Diffusion Reaction of Synthetic *N*-Glycopolypeptides with WGA in Agar Gel. WGA was put in the center well. Portions of synthetic *N*-glycopolypeptides and poly(L-glutamic acid) sodium salt solutions were added to the peripheral wells. a, Poly (L-glutamic acid) sodium salt; b, **1**; c, **2**; d, **3**; e, **4**; f, **5**.

the central well with lectin and the surrounding wells containing polymers **2**, **3**, **4**, and **5**. There is a weak and dull band between **1** and ECA. The bands showed that the lectins had became insoluble after cross-linking with specific carbohydrate ligands along the *N*-glycopolypeptide chains. The double-diffusion test is an easy and rapid way to detect interactions between lectins and *N*-glycopolypeptides. The method, however, is merely qualitative, so we investigated inhibition of hemagglutination, as well.

The effects of *N*-glycopolypeptides and sugars on hemagglutination caused by lectins are summarized in Table III. The minimum inhibitory concentrations are expressed as the weight concentration (mg/ml) of the glycopolypeptides and also as the molar concentration of sugar units in the polymer. Hemagglutination caused by WGA was inhibited by all of the polymers (**1** to **5**). The effects of ECA were inhibited by polymer **1**, and the lectins binding galactosyl residue, RCA_{120} and PNA, were inhibited by both **1** and **2**. Therefore, ECA recognized the LacNAc units in polymer **1**, but it did not recognize polymer **2** with iso-LacNAc. Polymer **2** inhibited WGA, RCA_{120}, and PNA, but not ECA. This finding indicated that β-(1–4)-linked galactosyl residues were needed for binding with ECA. Little is known about the interaction of lectin with iso-LacNAc,[22,23] an artificial oligosaccharide. No inhibition was detected with other combinations. The qualitative results obtained by double-diffusion in agar and this test of inhibition by synthetic *N*-glycopolypeptides **1**, **3**, **4**, and **5** were consistent with the sugar specificities of these lectins reported for naturally occurring glycoproteins, glycolipids, and oligosaccharides.[16,22-24]

Inhibition by *N*-glycopolypeptides was compared with inhibition by corresponding oligosaccharides (Table III). The hemagglutination caused by ECA was inhibited by polymer **1** only, with a strength of close to that by LacNAc. Polymer **1** inhibited RCA_{120}, PNA and WGA weakly. Polymer **3**, which carried $(GlcNAc)_3$, inhibited WGA 10^3 times stronger than $(GlcNAc)_3$ itself did. Polymer **4**, carrying $(GlcNAc)_2$, inhibited WGA strongly. Polymer **5**, carried GlcNAc, inhibited strongly WGA, but its corresponding sugar did not. The interactions of glycopolymers **2**, **3**, **4**, and **5** with WGA were much stronger than those of the corresponding sugars. WGA has strong affinity for multi-antennary GlcNAc residues.[23,25] The $(GlcNAc)_n$ sequences along the PGA backbone have a cluster effect for WGA. Table III also shows the effects of the DS of the *N*-glycopolypeptide on the hemagglutination caused by WGA. The higher activities of polymers **2**, **3**, and **4** compared with those of their corresponding sugars were not affected by differences in the DS. The terminal galactosyl group of the

Table III. Inhibition by *N*-Glycopolypeptides and Sugars of Hemagglutination Caused by Lectins[a]

Inhibitor	Minimum inhibitory concentration, mg/ml (M[b])			
	WGA	ECA	RCA_{120}	PNA
1(15%)[c]	$1.25(1.0\times10^{-3})$	$0.16(1.3\times10^{-4})$	$2.50(2.0\times10^{-3})$	$1.25(1.0\times10^{-3})$
1(44%)	—[d]			
LacNAc	—	$0.15(3.9\times10^{-4})$	$0.62(1.6\times10^{-3})$	—
2(14%)	$0.0781(6.1\times10^{-5})$	—	$2.50(2.0\times10^{-3})$	$1.25(9.8\times10^{-4})$
2(31%)	$0.0781(9.9\times10^{-5})$			
iso-LacNAc	—	—	$1.20(3.1\times10^{-3})$	$2.39(6.2\times10^{-3})$
3(13%)	$2.0\times10^{-4}(1.1\times10^{-7})$	—	—	—
3(38%)	$2.0\times10^{-4}(2.1\times10^{-7})$			
$(GlcNAc)_3$	$0.16(2.6\times10^{-4})$	—	—	—
4(22%)	$2.0\times10^{-4}(2.0\times10^{-7})$	—	—	—
4(44%)	$3.9\times10^{-4}(5.6\times10^{-7})$			
$(GlcNAc)_2$	$1.25(2.9\times10^{-3})$	—	—	—
5(78%)	$0.0195(5.3\times10^{-5})$	—	—	—
GlcNAc	—	—	—	—

[a] The concentration of lectin used was fourfold the minimum to cause hemagglutination.
[b] Concentration of sugar unit.
[c] Degree of substitution of sugar in glycopolymer as percentage.
[d] —, no inhibition with 10 mg/ml sugar or 5 mg/ml *N*-glycopolypeptide.

Table IV. Inhibition by LacNAc-substituted Polymers and Their Corresponding Sugars of Hemagglutination Caused by Lectin

Inhibitor	Minimum inhibitory concentration, M		
	WGA	ECA	RCA_{120}
LacNAc	—[a]	3.9×10^{-4}	1.6×10^{-3}
1(15%)	1.0×10^{-3}	1.3×10^{-4}	2.0×10^{-3}
LacNAc-*p*NP[b]	—	4.1×10^{-6}	8.2×10^{-5}
PAP-LacNAc[c]	4.0×10^{-6}	4.1×10^{-6}	5.9×10^{-5}

[a] —, no inhibition with 10 mg/ml sugar or 5 mg/ml glycopolymer.
[b] *p*-Nitrophenyl *N*-acetyl-β-lactosaminide.
[c] Poly(*p*-acryloyl-aminophenyl-β-lactosaminide).

side chain in polymer **2** did not prevent binding with WGA, because polymer **5** had the same inhibitory effects. With **1**, even at the low DS of 15%, activity was weak, and with the DS of 44%, the polymer had no activity. The binding mode of *N*-glycopolypeptides **1** and **2** was much influenced by the position of the terminal galactosyl group in the side chain.

We earlier suggested a synthetic route for PAP-LacNAc, a homopolymer with a number-average molecular weight ($\bar{M}_n$) of 3.2×10^5.[17,22] Polymer **1** bound less strongly with the three lectins than this homopolymer did (Table IV). PAP-LacNAc inhibited WGA 10^3 times more than **1** did. Probably the strong interaction between PAP-LacNAc and WGA arises not only caused because of a cluster effect of the multiantennary oligosaccharide chains in the polymer, but also because of by the phenyl groups in the main chain. This suggestion is consistent with the earlier finding that WGA is inhibited more by (*p*-vinyl benzamido)-β-diacetylchitobiose because of the presence of hydrophobic aglycon than by $(GlcNAc)_2$.[20] Both of these polymers inhibited ECA and RCA_{120} in the same order in relative inhibition strength as that of the corresponding sugars LacNAc and *p*-nitrophenyl *N*-acetyl-β-lactosaminide. These results showed that the two lectins are much less subject to cluster effect by glycopolymer compared to WGA lectin.

In conclusion, five *N*-glycopolypeptides carrying LacNAc and related compounds were synthesized through two steps, amination and condensation. These glycopolymers reacted specifically with the corresponding lectins, no matter how much shorter the sugar side chains of the glycopolymers were than those of natural glycoproteins.

Acknowledgment

This work was supported by Grants-in-Aids from the Ministry of Education, Science, Sports, and Culture, Japan, by a Research Grant for Leading Research Utilizing Potential of Regional Science and Technology from the Science and Technology Agency, Japan, and by a Research Grant from the Ministry of Agriculture, Forestry, and Fisheries, Japan.

References

1) H. Kunz and W. K.-D. Brill, *Trends in Glycoscience and Glycotechnology*, **4**, 71–82 (1992).
2) C. Cambillau, In "*Glycoproteins*", ed. by J. Montreuil, J. F. G. Vliegenthart, and H. Schachter, Elsevier Science B. V., Amsterdam, 1995, p. 29.
3) R. Roy, F. D. Tropper, and A. Romanowaska, *Bioconjugate Chem.*, **3**, 256–261 (1992).
4) R. Roy, *Trends in Glycoscience and Glycotechnology*, **8**, 79–99 (1996).
5) N. K. Kochetkov, *Pure Appl. Chem.*, **56**, 923 (1984).
6) A. Rozalski, L. Brade, H.-M. Kuhn, J. Brade, P. Kosma, B. J. Appelmek, S. Kusumoto, and H. Paulsen, *Carbohydr. Res.*, **193**, 257 (1989).
7) K. Kobayashi, K. Akaike, and T. Usui, In "Methods in Enzymology", ed. by Y. C. Lee, and R. T. Lee, **242**, Academic Press, San Diego, 1994, pp. 226–235.
8) R. Roy and F. D. Tropper, *J. Chem. Soc. Chem. Commun.*, 1058–1061 (1988).
9) K. Kobayashi, E. Tawada, T. Akaike, and T. Usui, *Biochem. Biophy. Acta*, **1336**, 117–122 (1997).
10) J. C. Paulson, *Trends Biochem. Sci.*, **14**, 272 (1983).
11) K. Ando, K. Kikugawa, and M. Beppu, *J. Biol. Chem.*, **269**, 19394–19398 (1994).
12) I. Eggins, B. A. Fenderson, T. Toyokuni, B. Dean, M. Stround, and S.-I. Hakomori, *J. Biol. Chem.*, **264**, 9476 (1989).
13) K. Sakai, R. Katsumi, H. Ohi, T. Usui, and Y. Ishido, *J. Carbohydr. Chem.*, **11**, 553–565 (1992).
14) T. Usui, S. Morimoto, Y. Hayakawa, M. Kawagushi, T. Murata, Y. Matahira, and Y. Nishida, *Carbohydr. Res.*, **285**, 29–39 (1996).
15) J. A. Rupley, *Biochem. Biophy. Acta*, **83**, 245–255 (1964).
16) J. L. Iglesias, H. Lis, and N. Sharon, *Eur. J. Biochem.*, **123**, 247–252 (1992).
17) K. Kobayashi, N. Kakishita, M. Okada, T. Akaike, and T. Usui, *J. Carbohydr. Chem.*, **13**, 753–766 (1994).
18) H. Kawagishi, M. Yamawaki, S. Isobe, T. Usui, A. Kimura, and S. Chiba, *J. Biol. Chem.*, **269**, 1375–1379 (1994).
19) L. M. Likhosherstov, O. S. Novikova, V. A. Derevitsvitskaja, and N. K. Kochetkov, *Carbohydr. Res.*, **146**, C1–C5 (1986).
20) K. Kobayashi, A. Tsuchida, T. Usui, and T. Akaike, *Macoromolecules*, **30**, 2016–2020 (1997).
21) D. Hudson, *J. Org. Chem.*, **53**, 617–624 (1988).
22) T. Murata, and T. Usui, *Biosci. Biotech. Biochem.*, **61**, 1059–1066 (1997).
23) K. Matsuoka and S.-I. Nishimura, *Macromolecules*, **28**, 2961–2968 (1995).
24) I. J. Goldstein and R. D. Poretz, In "*The Lectins Properties, Functions and Applications in Biology and Medicine*", ed. by I. E. Liener, N. Sharon, and I. J. Goldstein, Academic Press, Orlando, 1986, pp. 35–244.
25) N. T. Shier, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 2078–2082 (1971).