Glycoconjugate J (1987) 4: 317-328

Preparation of Cluster Glycosides of *N*-Acetylgalactosamine That Have Subnanomolar Binding Constants Towards the Mammalian Hepatic Gal/GalNAc-specific Receptor

REIKO T LEE and YUAN C LEE*

Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, MD 21218 USA

Received June 30, 1987.

Key words: cluster N-acetylgalactosamine glycosides, mammalian hepatic Gal/GalNAc-lectin

Preparation of di- and tri-valent cluster glycosides containing *N*-acetyl-D-galactosamine (GalNAc) is described. Oligopeptides that contain a protected amino group and two or three free carboxyl groups are activated by methyl chloroformate and then coupled to 6-aminohexyl 2-acetamido-2-deoxy-β-D-galactopyranoside. The concentrations of the divalent GalNAc glycosides needed to produce 50% inhibition of the binding of asialoorosomucoid to the isolated, purified rat liver receptor specific for galactose and GalNAc and to the receptor on the hepatocyte surface were of the order of 10^{-8} M and 10^{-9} M, respectively. The binding affinity of the trivalent glycoside was 10- to 20-fold stronger than the divalent glycosides towards both the soluble receptor and intact hepatocyte.

The mammalian hepatic receptors specific for Gal/GalNAc have a strong affinity toward clustered galactosyl residues such as found in the complex-type oligosaccharide chains of desialylated serum glycoproteins [1]. In fact, the receptor on the hepatocyte surface can bind certain galactose-terminated, synthetic oligosaccharides of bi- and tri-antennary structure with affinity up to 10^3- and 10^6-fold higher, respectively, than the monovalent galactosides [2]. Synthetic ligands containing two to six galactosyl and lactosyl residues also exhibited varying degrees of this cluster effect, which not only depended on the number of galactosyl residues, but also on the overall structure of the ligand [3, 4]. If the galactosyl residues within a ligand are too close together, a strong cluster effect cannot be generated. One simple synthetic scheme which produced

---

Abbreviations: Z, benzyloxycarbonyl; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; AH, 6-aminohexyl; ASOR, asialoorosomucoid; DMF, *N*-dimethylformamide; DMSO, dimethylsulfoxide; Lac, lactosyl.

*Author for correspondence.

ligands of superior binding affinity involves the attachment of 6-aminohexyl lactoside *via* an amide bond to compounds that have two or three carboxyl groups [4].

In this paper, we report the preparation of GalNAc-containing, cluster glycosides using a similar, but slightly modified structural design. Since the receptor binds GalNAc much tighter than galactose (or lactose), these GalNAc cluster glycosides had, as expected, much stronger affinity than the corresponding lactosyl glycosides. Interestingly, however, the rat receptor bound the GalNAc glycosides 5- to 10-fold more avidly than the rabbit receptor, while the receptors from the two species showed a comparable affinity toward the lactosides. These powerful (K_d as low as 0.2 nM), low-molecular weight ligands will be useful reagents in both biochemical and cell biological studies of this receptor system as well as other GalNAc recognizing systems.

Materials and Methods

The following compounds were obtained as indicated: *N*-benzyloxycarbonyl L-aspartic acid (Z-Asp), *N*-acetyl-L-tyrosine, ethyl glycinate hydrochloride, γ-L-glutamyl-L-glutamic acid [Glu(Glu)], 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), methyl chloroformate, *N*-methyl-morpholine, and *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate all from Sigma Chemical Co. (St. Louis, MO, USA). Preparation of 6-aminohexyl β-lactopyranoside (LacAH), 6-(trifluoroacetamido)hexyl 2-acetamido-2-deoxy-β-D-galactopyranoside (GalNAcAH:TFA), and 6-aminohexyl 2-acetamido-2-deoxy-β-D-galactopyranoside (GalNAcAH) has been reported [5, 6]. Asialoorosomucoid (ASOR) was prepared as before [3] from orosomucoid which was kindly donated by Dr. M. Wickenhauser of the American Red Cross National Fractionation Center (Bethesda, MD, USA). The Triton-solubilized, purified receptors from rabbit and rat liver were prepared according to the method of Hudgin *et al.* [7] using an affinity column of asialofetuin-Sepharose. Isolated rat hepatocytes were prepared using the collagenase perfusion method based on Seglen [8].

TLC was performed on a precoated layer of silica gel 60, F_{254} (EM Industries, Cherry Hill, NJ, USA) using solvent A, ethyl acetate/isopropyl alcohol/water, 9/4/2 by vol, and solvent B, ethyl acetate/acetic acid/water, 3/2/1 by vol. For the detection of carbohydrate, TLC plates were sprayed with 15% sulfuric acid in 50% ethanol and heated at ~140°C. The amino group was detected by spraying with 0.2% ninhydrin in ethanol and heating the plates briefly. U.v. absorption spectra were obtained with a Perkin-Elmer 576 ST spectrophotometer. Concentration of the compounds that contain Z-groups was determined by using $\epsilon_{258} = 186$, and the compounds that contain tyrosyl groups, using $\epsilon_{277} = 1330$. Uncorrected melting points were determined using a Fisher-Johns apparatus. ^{1}H-NMR Spectra were obtained with a Varian 400 spectrometer and ^{125}I-labeled samples were counted with a Packard PRIAS PGD auto gamma counter. Elemental analyses were performed by the Galbraith Laboratories, Inc. (Knoxville, TN, USA). Lactose content in lactosides was determined with a phenol-sulfuric acid method [9] using lactose as standard. Galactosamine was determined using an automated amino sugar analyzer [10] with a slight modification as follows. Acid hydrolyzed, evaporated samples were fractionated on a column (1 × 11 cm) of BC-X8 (Benson Co., Reno, NV, USA) with 0.1 M sodium borate buffer, pH 7.4, containing 50 mM sodium sulfate as eluant, and the effluent was analyzed by the neocuproine colorimetric method. Amino acid analyses were done using the picotag method of Waters/Millipore (Milford, MA, USA) [11].

Hydrogenolysis was carried out in 10-60% acetic acid with 10% palladium on carbon (ca. 1/4 weight of the compound to be hydrogenated) as catalyst in a micro-Brown hydrogenator [12]. Activation of the carboxyl group with methyl chloroformate and subsequent coupling to an amino group were carried out as described [4]. In general, activation was carried out for 20 min in DMF in a dry ice-95% ethanol bath using a 0-15% excess of methyl chloroformate and *N*-methyl morpholine over the carboxyl compound. The I_{50} (the ligand concentration that inhibits 50% of the ^{125}I-ASOR binding) of the lactose- and GalNAc-containing compounds in the soluble receptor system and the isolated hepatocyte system was determined by inhibition assays [3]. Briefly, the binding of ^{125}I-ASOR by the receptor (10^{-9}-10^{-10} M) or hepatocytes ($\sim 2.5 \times 10^6$ cells/ml) was carried out at a fixed ^{125}I-ASOR concentration ($\sim 10^{-10}$ M) in the presence of an inhibitor at various concentrations ranging from approximately $0.01 \times I_{50}$ to $100 \times I_{50}$. Incubation was at 25°C for 30 min for the soluble assays, and at 2°C for 2 h with 2 rpm rotation for the hepatocyte surface-binding assay. The receptor-bound ^{125}I-ASOR was separated from the free ^{125}I-ASOR by an ammonium sulfate precipitation/filtration in the soluble assays, and by collecting the cell pellet after centrifugation of the suspension through a layer of silicon oil-mineral oil mixture in the cell-surface binding assay. For the determination of I_{50} of the ligands with very high affinity ($I_{50} < 10^{-9}$ M), the hepatocyte inhibition assay was modified as follows. Hepatocytes at 2×10^5 cells/ml were incubated in a total volume of 2.5 ml with $\sim 5 \times 10^{-11}$ M ^{125}I-ASOR and various concentrations of an inhibitor. After incubating at 2°C for 2 h, 1 ml aliquots of cell suspension were centrifuged through the oil mixture (0.5 ml) in 1.5 ml microfuge tubes. Tips containing the cell pellets were clipped and counted.

Results

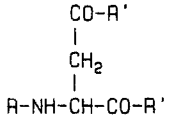
General Design for Preparing Di- and Tri-valent Cluster Glycosides

For divalent glycosides, the two carboxyl groups of aspartic acid were derivatized with glycine residues whose carboxyl groups were in turn derivatized with 6-aminohexyl glycosides. For trivalent glycosides, the three carboxyl groups of γ-L-glutamyl-L-glutamic acid were derivatized as described before for the lactoside derivative [4].

*Preparation of Z-DG$_2$ (**2**)*

To a suspension of ethyl glycinate hydrochloride (11.2 g, 80 mmol) in dichloromethane (300 ml) was added triethylamine (12 ml, 86 mmol), Z-Asp (5.35 g, 20 mmol) and EDAC (11.5 g, 60 mmol). The turbid mixture, after overnight stirring at room temperature, became a clear solution. It was extracted successively with 150 ml each of cold solutions of 0.3 M sulfuric acid, 1 M NaCl, saturated sodium hydrogen carbonate, and 1 M NaCl. The dichloromethane solution was dried with anhydrous sodium sulfate, filtered and evaporated to yield a crystalline product (**1**) in 85.5% yield (7.46 g, 17.1 mmol). The ^{1}H-NMR spectrum agreed well with the structure of **1**, having a correct ratio of aromatic H to methyl H. Recrystallized (from 100% ethanol) **1** gave m.p. 142-144°C and a correct elemental composition as shown below.

Analysis. Calculated for $C_{20}H_{27}N_3O_8$ (437.45): C, 54.91; H, 6.22; N, 9.61. Found: C, 54.75; H, 6.20; N, 9.68.



$$\mathrm{R{-}NH{-}\underset{}{\overset{\overset{\displaystyle CO{-}R'}{|}\ \overset{}{CH_2}\ |}{}}CH{-}CO{-}R'}$$

1	$R=C_6H_5CH_2OCO-$	$R'=-NHCH_2CO_2Et$
2	$R=C_6H_5CH_2OCO-$	$R'=-NHCH_2CO_2H$
3	$R=C_6H_5CH_2OCO-$	$R'=-NH(CH_2)_6O\text{-Lac}$
4	$R=C_6H_5CH_2OCO-$	$R'=-NHCH_2CONH(CH_2)_6O\text{-GalNAc}$
4	$R=C_6H_5CH_2OCO-$	$R'=-NHCH_2CONH(CH_2)_6O\text{-Lac}$
5	R=H	$R'=-NHCH_2CONH(CH_2)_6O\text{-GalNAc}$
6	R=Z-L-Tyr	$R'=-NHCH_2CONH(CH_2)_6O\text{-GalNAc}$
7	R=L-Tyr	$R'=-NHCH_2CONH(CH_2)_6O\text{-GalNAc}$
8	R=NAc-L-Tyr	$R'=-NHCH_2CONH(CH_2)_6O\text{-GalNAc}$

Saponfication of **1** was carried out at pH ~12 by adding NaOH to a solution of **1** (0.42 g, 1 mmol) in 70% ethanol (90 ml) until uptake of the alkali ceased. The solution was neutralized with Dowex 50 × 8 (100 mesh, H^+ form), filtered, the Dowex washed with 50% ethanol, and the filtrate evaporated. The residue containing **2** was purified by passing it through a column (5 × 217 cm) of Sephadex G-25 in 0.1 M acetic acid. Fractions containing **2** (R_F 0.04 in solvent A, u.v.-absorbing and charred slightly by sulfuric acid spray and heat) were combined and evaporated. The solid product (0.24 g, 0.6 mmol) was recrystallized from 100% ethanol to yield homogenous **2** having m.p. 198-199°C and a correct u.v. absorption spectrum and intensity.

Analysis. Calculated for $C_{16}H_{19}N_3O_8$ (381.33): C, 50.39; H, 5.02; N, 11.02. Found: C, 50.36; H, 5.33; N, 11.03.

*Coupling of Z-Asp and **2** with 6-Aminohexyl Lactoside*

In the previously described coupling of Z-Asp to LacAH [4], we noticed that the yield of the divalent product, Z-Asp(LacAH)$_2$, was unexpectedly low (~17%), and the yield did not increase much even when a 100% excess of LacAH was used. We surmised that this low yield resulted from the proximity of the two carboxyl groups (see the Discussion Section) and prepared an alternate dicarboxylic compound, **2**, having two carboxyl groups which are further apart. In this experiment, we coupled LacAH to Z-Asp and **2** under exactly the same conditions in order to compare the yield of the divalent product. Solutions containing 50 μmol of Z-Asp or **2** in 0.2 ml of DMF were treated with methyl chloroformate (115 μmol) and *N*-methyl-morpholine (118 μmol) and each mixture was added to a solution of 6-aminohexyl lactoside (65 mg, 145 μmol in 0.3 ml of DMSO). The reaction mixtures were processed as described [4] and the desired, disubstituted products were separated from other lactose-containing materials on a column (2.5 × 140 cm) of Sephadex G-15 in 1 mM acetic acid. Fig. 1 shows the elution profiles based on the lactose content of the effluent fractions determined by the phenol-sulfuric acid method. TLC examination in solvent B indicated that the disubstituted products were

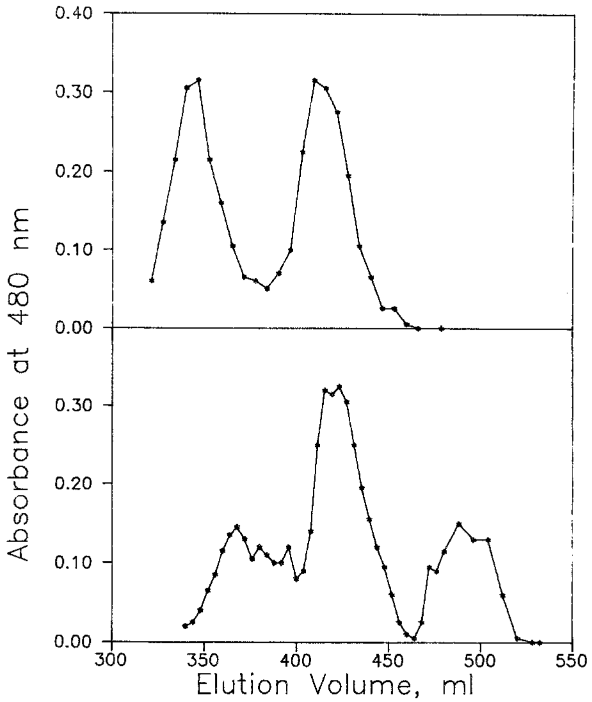


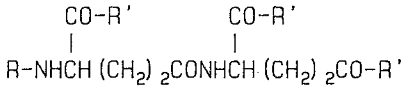
Figure 1. Sephadex G-15 (2.5 × 140 cm) gel filtration of the reaction mixtures resulting from coupling of LacAH to Z-Asp or Z-DG$_2$. The column was eluted with 1 mM acetic acid and the effluent was assayed by the phenol-sulfuric acid method. Upper panel, coupling to Z-DG$_2$; lower panel, coupling to Z-Asp.

located in the earliest peak. The peak at 400-450 ml of effluent contained the monosubstituted products as well as the unreacted LacAH. The yield of the disubstituted product, **3** [ZD(LacAH)$_2$], from Z-Asp was 16%, while that of **4′** [ZDG$_2$ (LacAH)$_2$] from **2** was 47%.

Preparation of Divalent GalNAc Glycosides

All the divalent GalNAc-glycosides were prepared utilizing **2**, since it gave a much higher yield of the disubstituted product than Z-Asp. Final products contained either an L-tyrosyl group or an *N*-acetyl-L-tyrosyl group, so that these glycosides could be radioiodinated for the direct binding studies.

Compound **2** (76 mg, 200 μmol) was activated with methyl chloroformate and coupled to GalNAcAH using a 25% excess of the glycoside over the carboxyl group. The disubstituted product ZDG$_2$(GalNAcAH)$_2$ (**4**) (R_f 0.21 in solvent B) was obtained in 30% yield (59 mg, 60 μmol) after two passages through the Sephadex G-15 column. Removal of the Z group from **4** by hydrogenolysis yielded DG$_2$(GalNAcAH)$_2$ (**5**) in 92% (47 mg, 55 μmol) yield. The product, which had an R_f = 0.07 upon TLC in solvent B, charred and also reacted with ninhydrin (orange). The product was purified on a column (1.8 × 64 cm) of Sephadex G-10 with water as eluant.



$$\mathrm{R{-}NH\overset{\overset{\displaystyle CO{-}R'}{|}}{C}H\,(CH_2)_2CONH\overset{\overset{\displaystyle CO{-}R'}{|}}{C}H\,(CH_2)_2CO{-}R'}$$

9	R=Z-L-Tyr	R'=-NH(CH$_2$)$_6$O-GalNAc
10	R=L-Tyr	R'=-NH(CH$_2$)$_6$O-GalNAc
11	R=L-Tyr	2R'=-NH(CH$_2$)$_6$O-GalNAc 1R'=-OH

The product **5** was derivatized either with a Z-tyrosyl group or with an *N*-acetyl-tyrosyl group. The Z-Tyr group was attached to **5** by adding *p*-nitrophenyl *N*-Z-tyrosinate (44 mg, 100 μmol) and triethylamine (6 μl, 43 μmol) to a solution of **5** (30 mg, 35 μmol) in DMSO (1 ml). After a few hours at room temperature, toluene (15 ml) was added and the mixture was left overnight. The precipitate was filtered to yield ~30 mg (~26 μmol) of solid, containing mainly ZYDG$_2$(GalNAcAH)$_2$ (**6**) (R_F 0.24 in solvent B). Because of its poor solubility, this solid was used in the next reaction without further purification. Hydrogenolysis of **6** as a suspension in 60% acetic acid (5 ml) with 10% Pd/C catalyst (8 mg) produced a major product which had an R_F of 0.05 in solvent B, and which charred as well as stained yellow with ninhydrin. This product, YDG$_2$(GalNAcAH)$_2$ (**7**) was purified by passing it through the Sephadex G-10 column. Peak fractions were evaporated to yield 10 mg (10 μmol) of **7**.

To attach an *N*-acetyl tyrosyl group to **5**, *N*-acetyl tyrosine (27 mg, 120 μmol) in 0.1 ml DMF was activated with methyl chloroformate (10 μl, 128 μmol) and *N*-methyl-morpholine (15 μl, 136 μmol) and the mixture was added to **5** (47 mg, 55 μmol) in 0.4 ml DMSO. Fractionation on the Sephadex G-15 column in 1 mM acetic acid yielded one major carbohydrate product of R_F 0.13 by TLC in solvent B. Relatively pure fractions were combined and chromatographed on the Sephadex G-10 column in water. Peak fractions were combined to yield homogeneous *N*-acetyl-YDG$_2$(GalNAcAH)$_2$ (**8**) in 55% yield (32 mg, 30 μmol).

*Preparation of Trivalent GalNAc Glycoside YEE(GalNAcAH)$_3$ (**10**)*

Preparation of a trivalent lactoside, YEE(LacAH)$_3$, has been reported [4]. The corresponding GalNAc glycoside was prepared similarly. Essentially it involves: 1) coupling Z-Tyr to Glu(Glu) to form Z-Tyr-Glu(Glu) (ZYEE); 2) coupling of GalNAcAH to ZYEE to form ZYEE(GalNAcAH)$_3$ (**9**); 3) removal of the Z-group to yield YEE(GalNAcAH)$_3$ (**10**). ZYEE (0.14 g, 0.25 mmol) was activated by the chloroformate method and reacted with GalNAcAH (0.33 g, 1.03 mmol), which was present in 37% excess over the carboxyl group. Carbohydrate-containing material was precipitated from the reaction mixture by adding a 20-fold volume of toluene. The precipitate was suspended in 5 ml of water, stirred for 2 h at room temperature, and filtered to yield 0.23 g of solid. TLC examination (in solvent B) of the solid showed that it contained a divalent product ZYEE(GalNAcAH)$_2$ (R_F 0.41) in addition to the desired trivalent product ZYEE(GalNAcAH)$_3$ (R_F 0.13). Because of the poor solubility of these compounds, the Z group was removed by hydrogenolysis before separation of the two compounds was attempted. Hydrogenolysis of the mixture produced two new carbohydrate components both of which were amino-positive (ninhydrin) and had lower R_F (0.12 and 0.04 in solvent B) than the starting compounds. The compound with R_F 0.04 is YEE(GalNAcAH)$_3$ (**10**), and the other with R_F 0.12 is YEE(GalNAcAH)$_2$ (**11**).

These two compounds were separated on a column (1.5 × 22.5 cm) of DEAE-Sephacel equilibrated in 10 mM Tris/HCl buffer, pH 7.6. YEE(GalNAcAH)$_3$ was eluted isocratically with two column volumes of the buffer, and YEE(GalNAc)$_2$ was eluted subsequently by a linear gradient of increasing NaCl concentration from 0-0.2 M. The fractions containing YEE(GalNAcAH)$_3$ and YEE(GalNAcAH)$_2$ were processed similarly by combining, concentrating to ~2 ml, and desalting on a column (2.2 × 144 cm) of Sephadex G-25 in 0.1 M acetic acid. YEE(GalNAcAH)$_3$ was obtained in 24% overall yield (~80 mg) and YEE(GalNAcAH)$_2$ in 13% overall yield (~35 mg). The by-product, YEE(GalNAcAH)$_2$, probably exists in isomeric forms. However, other than the determination of the galactosamine content (see below), no further characterization of the fine structure was made on this preparation.

*Preparation of 6-N-(N-Acetyl-L-tyrosyl)aminohexyl 2-Acetamido-2-deoxy-β-D-galactopyranoside (**12**)*

A monovalent GalNAc glycoside that can be iodinated was also prepared. *N*-Acetyl-L-tyrosine (123 mg, 0.55 mmol) in 0.5 ml DMF was activated with methyl chloroformate (43 μl, 0.55 mmol) and *N*-methyl-morpholine (61 μl, 0.56 mmol), and the mixture was added to a solution of GalNAcAH (156 mg, 0.49 mmol) in 0.4 ml DMSO. Toluene (30 ml) was added and the mixture was left overnight in the cold. TLC (solvent B) examination of the precipitate showed that it contained two spots that charred: one at R_F 0.59 was also u.v.-absorbing and was the desired product, **12**; the other with R_F 0.17, reacted with ninhydrin, and is the unreacted GalNAcAH. The solid was stirred with 2 ml of hot water and filtered to yield a solid product **12** (125 mg) which appeared homogeneous upon TLC in solvent B. A further 40 mg of solid was obtained upon concentration of the mother liquor, giving the total yield of 63% (165 mg, 0.31 mmol). The solid was crystallized from hot 95% ethanol; m.p. 203-206°C.

Analysis. Calculated for $C_{25}H_{39}N_3O_9$ (525.58): C, 57.13; H, 7.48; N, 7.99. Found: C, 55.01; H, 7.26; N, 7.66.

*Preparation of 6-N-(N-Acetyl-L-tyrosyl)aminohexyl Lactopyranoside (**12′**)*

The lactose analog of **12** was prepared in a similar way by substituting LacAH for GalNAcAH. The product (**12′**) was purified by passing the toluene-precipitated reaction mixture through the Sephadex G-15 column. TLC examination (solvent B) of the effluent fractions revealed that the only spot (R_F 0.56) which absorbed u.v. light and charred with sulfuric acid was present in the most-retarded peak (the major carbohydrate peak). The fractions were combined and evaporated to yield **12′** in 51% overall yield.

Characterization of the Products

Products **7**, **8**, **10**, **11**, **12** and **12′** were characterized by u.v. absorption spectrum, galactosamine and lactose analyses and for some products, by amino acid analysis. For products **10** and **12**, which were crystalline, the concentration determined from A_{277} was

Table 1. Carbohydrate content in mono-, di-, and tri-valent products.

Compound		GalN (or Lac) content mol/mol	
		Theoretical	Found
7	YDG$_2$(GalNAcAH)$_2$	2	1.77
8	NAcYDG$_2$(GalNAcAH)$_2$	2	1.94
10	YEE(GalNAcAH)$_3$	3	2.86
11	YEE(GalNAcAH)$_2$	2	1.85
12	NAcY(GalNAcAH)$_1$	1	0.99
12′	NAcY(LacAH)$_1$	1	0.99

95% (**10**) and 100.1% (**12**) of that from the weight. Amino acid analysis gave the correct ratios of amino acid for **8** and **10**. For **8**, the ratio of Y:D:G was 1.0 : 1.0 : 2.3 and for **10** the ratio of Y:E was 1.0 : 1.8. The yield of amino acids was in the range of 85 to 104%. As shown in Table 1, the galactosamine or lactose analyses of these compounds yielded the correct ratios of the sugar to tyrosine (A_{277}).

Affinity of the Cluster Glycosides Toward the Gal/GalNAc-Specific Receptors of Mammalian Liver

Affinity of the glycosides toward the Triton-solubilized, purified receptors from rat and rabbit liver and toward the isolated rat hepatocytes was determined using the inhibition assays. Results are shown in Table 2.

Discussion

The Gal/GalNAc-specific receptors of rat and rabbit hepatocytes avidly bind desialylated, complex-type oligosaccharides which have multiple non-reducing terminal galactose residues [2, 13]. Although the dissociation constant of monovalent galactose-containing ligands is in the range of 0.5-1 mM, interaction of multiple galactose residues simultaneously at the combining sites increases the affinity tremendously [1, 14]. A ligand containing three properly aligned, terminal galactose residues may express affinity close to a million-fold stronger than a simple galactoside [2]. Based on such observations, it has been postulated that on the mammalian hepatocyte surface, three galactose-combining sites of the receptor are arranged in a defined triangular configuration having inter-site distances of 150, 220, and 250 nm [15]. We have synthesized various cluster glycosides that contain up to six galactosyl residues and determined their binding affinity [3, 4]. The ligands that possess the strongest binding affinity per galactosyl residue are those which have connecting arms (from galactose residue to the branching point) that are sufficiently long and flexible so that the galactose residues can easily occupy the sites arranged in the above-mentioned triangular disposition. The divalent lactoside, **3**, represents one such ligand.

Since GalNAc is bound much tighter than galactose by this receptor [1], it was hoped that the proper clustering of two and three GalNAc residues would produce high affinity ligands that are simple in structure and are suitable for biochemical and cell

Table 2. Binding affinity of various synthetic ligands toward the Gal/GalNAc receptor.

	I_{50}, μM		
	Soluble receptor		Rat hepatocytes
	Rabbit	Rat	
Monovalent ligands			
Lac	500	600	300
LacAH	130	190	40
NAcY(LacAH)$_1$	440	260	670
GalNAc	190	14	4
GalNAcAH	23	5	4
GalNAcAH·TFA	11	4.6	15
NAcY(GalNAcAH)$_1$ **(12)**	14	2.4	10
Divalent ligands			
D(LacAH)$_2$	7	7	0.6
ZD(LacAH)$_2$	20	19	6
YD(LacAH)$_2$	10	8	4
DG$_2$(LacAH)$_2$	35	30	7
YDG$_2$(LacAH)$_2$	ND[a]	ND	8
DG$_2$(GalNAcAH)$_2$ **(5)**	1.0	0.08	0.004
YDG$_2$(GalNAcAH)$_2$ **(7)**	0.15	0.03	0.004
NAcYDG$_2$(GalNAcAH)$_2$ **(8)**	0.3	0.03	0.003
YEE(GalNAcAH)$_2$ **(11)**	0.5	0.05	0.003
Trivalent ligands			
YEE(LacAH)$_3$	1.7	3	0.05
YEE(GalNAcAH)$_3$ **(10)**	0.03	0.004	0.0002

[a] ND = not determined.

biological studies of this receptor system. We reported earlier the preparation of a divalent lactoside, **3**, by coupling 6-aminohexyl lactoside with Z-Asp whose carboxyl groups had been activated by methyl chloroformate [4]. It was noted that the yield of the divalent product in the coupling step seldom exceeded 20%. We speculated that this low yield resulted from the proximity of the two carboxyl groups which easily produced the anhydride of Asp in the chloroformate activation step, as depicted in equation 1 (Fig. 2). The anhydride would lead only to the monovalent product in the next step of the reaction with 6-aminohexyl lactoside. If such is the case, increasing the distance between the two carboxyl groups (as in **2**) should improve the yield of the disubstituted product. As described in the Results section, the divalent product was produced in 47% yield from **2** in comparison to 16% from Z-Asp. For this reason, we prepared all the divalent GalNAc glycosides based on the structure **2**. The activation of the carboxyl groups of **2**, their coupling to GalNAcAH and the subsequent purification of the products all proceeded similarly to the established, analogous processes for Z-Asp [4]. However, the initial coupled product, **4**, had to be kept under slightly acidic conditions at all times. When **4** (or **4'**) was exposed to a mild alkaline condition (0.1 M NH_4OH, room temperature), it converted quantitatively to a compound with a lower R_F (R_F of **4** was 0.21 and R_F of the new compound was 0.12 in solvent B). The new compound contained

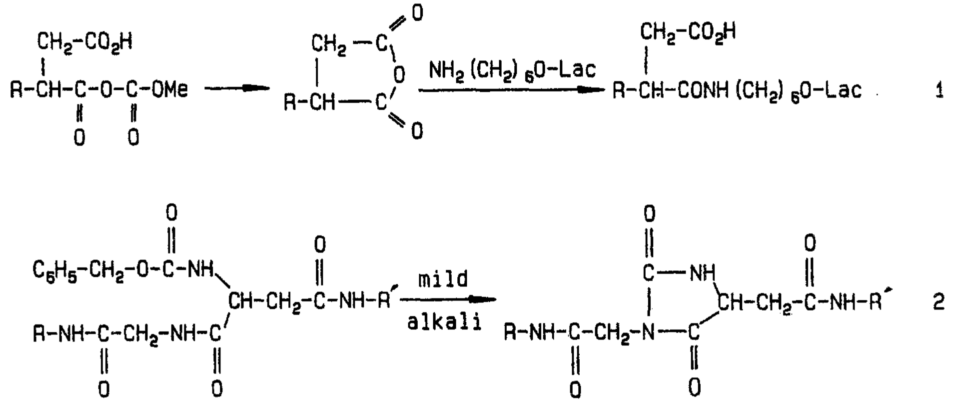


Figure 2. Side reactions during synthesis.

neither aromatic group (^{1}H-NMR and u.v. absorption) nor a primary amino group (ninhydrin). This side reaction required the presence of a Z-group, since compound **8** (which contains an NAcTyr group instead of a Z group) was unaffected by 0.1 M NH_4OH. The participation of a glycyl group was also implicated, for the reaction did not take place when compound **3** was treated with 0.1 M NH_4OH. The reaction probably occurs *via* a base-catalyzed cyclization involving the amido-group of one of the glycyl residues as shown in equation 2 (Fig. 2). Such cyclization leading to the formation of the hydantoins has been documented in peptides that contain Z-X-Gly structure [16].

As shown in Table 2, all the cluster ligands having two or three GalNAc residues per molecule were potent inhibitors. In the rat hepatocyte surface binding, I_{50} of the most potent ligand, **10**, was as low as 10^{-10} M. The I_{50} value of an inhibitor would approximate its true K_i value, if the assays are performed under conditions where both the concentrations of the reference ligand (^{125}I-ASOR) and receptor (or hepatocytes) are much lower than K_i of the inhibitor [17]. The original inhibitor assay of Connolly *et al.* [3] uses ^{125}I-ASOR and the receptor concentrations of $\sim 10^{-9}$-10^{-10} M, and is suitable for the estimation of the inhibition constant as low as 5×10^{-9} M. For those GalNAc glycosides whose I_{50} values are lower than 10^{-9} M, we carried out a modified assay using $\sim$10-fold lower concentrations of ^{125}I-ASOR and the receptor. A four-fold larger volume of the incubation mixture was sampled in order to increase the radioactive signal. The I_{50} of compound **8** determined in the modified assay was 3×10^{-9} M, while the value of 9×10^{-9} M was obtained under the previous assay condition.

The following observations and conclusions were made from the results presented in Table 2:-

1). The di- and tri-valent GalNAc glycosides exhibited strong cluster effects similar to the lactose series [4]. For both the lactose and GalNAc series, clustering of the first two sugar residues increased the affinity much more than the addition of the third residue.

2). As observed in the lactose cluster glycosides [4], affinity of the GalNAc cluster glycosides in the soluble receptor system was four- to 20-fold lower than in the hepatocyte system.

3). The presence of a positively charged group strategically located some distance from the terminal galactose (or GalNAc) may increase the affinity. For instance, compare LacAH and GalNAcAH with other lactose and GalNAc derivatives, and D(LacAH)$_2$ with other divalent LacAH glycosides. This effect is also manifested more strongly in the hepatocyte system than in the soluble system.
4). GalNAc glycosides are better ligands than the corresponding lactosides (or galactosides) in both the rat and rabbit systems. However, while the soluble rat and rabbit receptors have similar affinity toward the lactose derivatives, the rat receptor binds all the GalNAc ligands four- to 10-fold stronger than the rabbit receptor.

We are currently studying various aspects of interaction between the ligand and mammalian hepatic receptor using these high-affinity GalNAc cluster glycosides in the radioiodinated form. The strategy presented here for preparation of well-defined GalNAc-cluster ligands should also be useful for cluster ligands containing other sugars.

Acknowledgements

We wish to thank D. Reddar and N.L. Stults for the hepatocyte preparations, Dr. T.C. Wong for NMR spectroscopy and J. Lilly for amino acid analysis. This work was supported by National Institutes of Health Research Grant DK09970. Publication No. 1380 from the McCollum-Pratt Institute.

References

1 Ashwell G, Harford J (1982) Annu Rev Biochem 51:531-54.
2 Lee YC, Townsend RR, Hardy MR, Lönngren J, Arnarp J, Haraldsson J, Lönn H (1983) J Biol Chem 258:199-202.
3 Connolly DT, Townsend RR, Kawaguchi K, Bell WR, Lee YC (1982) J Biol Chem 257:939-45.
4 Lee RT, Lin P, Lee YC (1984) Biochemistry 23:4255-61.
5 Weigel PH, Naoi M, Roseman S, Lee YC (1979) Carbohydr Res 70:83-91.
6 Lee RT, Wong TC, Lee YC (1986) J Carbohydr Chem 5:343-57.
7 Hudgin RL, Pricer WE, Ashwell G, Stockert RJ, Morell AG (1974) J Biol Chem 249:5536-43.
8 Seglen PO (1976) Methods Cell Biol 13:29-83.
9 McKelvy JF, Lee YC (1969) Arch Biochem Biophys 132:99-110.
10 Lee YC (1972) Methods Enzymol 28:63-73.
11 Bidlingmeyer BA, Cohen SA, Tarvin TL (1984) J Chromatogr 336:93-104.
12 Brown CA, Brown HC (1966) J Org Chem 31:3989-95.
13 Baenzinger JU, Fiete D (1980) Cell 22:611-20.
14 Lee YC, Lee RT (1982) in Glycoconjugates, vol. 4, ed. Horowitz MI, Academic Press, New York, p 57-83.
15 Lee YC, Townsend RR, Hardy MR, Lönngren J, Bock K (1984) in Biochemical and Biophysical Studies of Proteins and Nucleic Acids, eds. Lo TB, Liu TY, Li CH, Elsevier, New York p 349-60.

16 Bodanszky M, Klausner YS, Ondetti MA (1976) in Peptide Synthesis, 2nd edition, Wiley, New York, p 22-23.

17 Chang KJ, Jacobs S, Cuatrecasas P (1975) Biochim Biophys Acta 406:294-303.