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New Synthetic Cluster Ligands for Galactose/*N*-Acetylgalactosamine-Specific Lectin of Mammalian Liver†

Reiko T. Lee, Paul Lin, and Yuan Chuan Lee*

ABSTRACT: Synthetic ligands containing up to six residues of nonreducing terminal galactose were prepared. The synthesis involved coupling of carboxyl groups of *N*-benzyloxycarbonylaspartic acid or of *N*-benzyloxycarbonyltyrosyl-γ-glutamylglutamic acid to the ω-amino group of the aglycon of a glycoside that contained up to three lactosyl residues. The benzyloxycarbonyl group was removed by hydrogenolysis before these ligands were tested as inhibitors to the binding of ^{125}I-asialoorosomucoid to the galactose/*N*-acetylgalactosamine lectin, both soluble and on the surface of freshly isolated mammalian hepatocytes. Each addition of a galactosyl residue to an existing ligand structure invariably increased the binding affinity of such a ligand. However, at each level of galactose valency, the binding constant varied as much as 1000-fold depending on the structure of the ligand. At a given level of valency, the binding strength of a cluster ligand depended mainly on two factors: (1) the maximum spatial inter-galactose distances and (2) the flexibility of the arm connecting galactosyl residues and the branch points. It has been postulated that the three galactose-combining sites of the lectin are arranged in space at the vertexes of a triangle whose sides are 15, 22, and 25 Å [Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., & Bock, K. (1984) in *Biochemical and Biophysical Studies of Proteins and Nucleic Acids* (Lo, T. B., Liu, T. Y., & Li, C. H., Eds.) pp 349–360, Elsevier, New York]. Ligands having inter-galactose distances shorter than these lengths were invariably poor ligands at their respective level of valency. Among the ligands having sufficiently long inter-galactose distances, those with the most flexible structure were the best inhibitors. The 50% inhibition of ^{125}I-asialoorosomucoid binding was achieved by 3×10^{-7} and 5×10^{-8} M, respectively, of the best synthetic bi- and trivalent ligands, and inhibitory power of these ligands was comparable to that of the most inhibitory bi- and triantennary oligosaccharide structures of natural origin [Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., Arnarp, J., Haraldsson, M., & Lönn, H. (1983) *J. Biol. Chem. 258*, 199–202].

The mammalian hepatic lectin specific for Gal[1] and GalNAc has a strong affinity (K_d = ca. 10^{-9} M) for desialylated serum glycoproteins such as ASOR[2] [e.g., Weigel (1980), Baenziger & Maynard (1980), and Connolly et al. (1983)] that bear multiple oligosaccharide chains of the complex type and thus can remove such glycoproteins rapidly from the circulation (Morell et al., 1971). The binding site of the lectin appears to be rather small, binding only the nonreducing terminal Gal (or GalNAc) and a portion of the penultimate sugar (Sarkar et al., 1979; Lee et al., 1982). Although galactosides will compete for the lectin site occupied by ASOR, the binding of monovalent galactosides to the lectin is much weaker, with K_d in the range of 10^{-4} M (Connolly et al., 1982). However, the binding strength is highly dependent on the number of galactosyl residues in a ligand, with K_d increasing exponentially with the increasing number of clustered Gal (Lee, 1982). The most spectacular cluster effect (an increase in the binding strength beyond that expected from the increase in Gal concentration) was observed (Lee et al., 1983) with a bivalent oligosaccharide, PENTA-2,4 with a K_d of 3×10^{-7} M, and a trivalent oligosaccharide, NONA I wtih a K_d of 8×10^{-9}

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[1] All sugars are of D configuration and in pyranose form. All amino acids are of L configuration.

[2] Abbreviations: ASOR, asialoorosomucoid; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; BSA, bovine serum albumin; AH, aminohexyl; LacAH, 6-aminohexyl β-lactopyranoside; AHT, (6-aminohexanamido)tris(hydroxymethyl)methane; Lac-BSA, BSA that has been modified with Lac-$SCH_2CONHCH_2CHO$ via reductive alkylation (Lee & Lee, 1980); Cbz, carbobenzyloxy; TLC, thin-layer chromatography.

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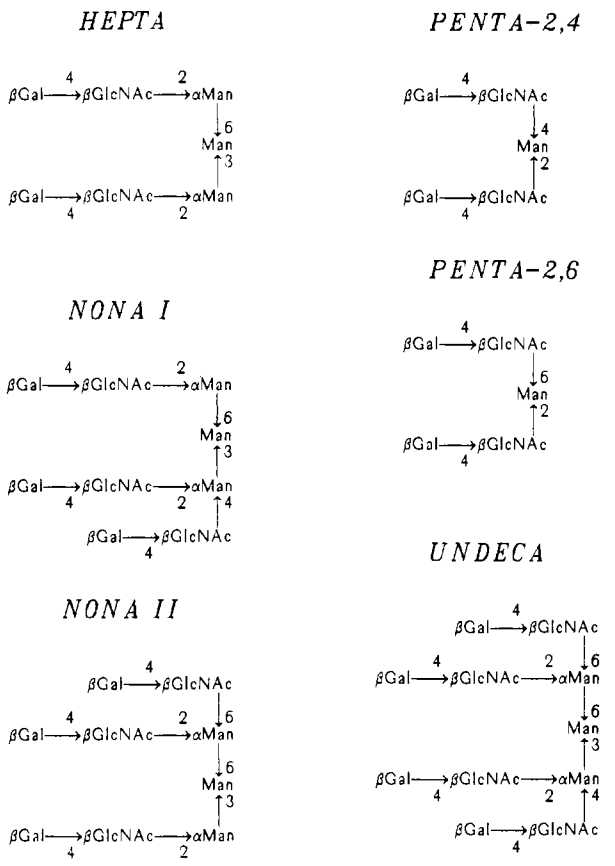
Chart I



(see Chart I for the structures). These binding constants indicate an increase in the binding strength over the monovalent galactosides of (2×10^3)- and 10^5-fold, while the Gal concentration was increasing only by 2- and 3-fold. From the binding studies of these and the other multivalent oligosaccharides, it was postulated that on the surface of the hepatocytes there exist three Gal-combining sites spatially arranged in such a way as to allow the most probable conformation (Bock et al., 1982) of NONA I (and presumably the triantennary glycopeptide) to bind very tightly (Lee et al., 1983).

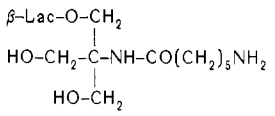
Earlier, we reported that synthetic cluster ligands of the type shown in Chart II, also exhibited a small but significant cluster effect, the decrease in K_d for each additional Gal in these ligands ranging from 3- to 50-fold (Connolly et al., 1982). In this paper, we describe a synthetic scheme to prepare cluster ligands by attaching ω-amino glycosides to carboxyl groups of aspartic acid and γ-glutamylglutamic acid. By incorporating a longer and flexible arm that connects the sugar moieties to the branching points, we were able to synthesize both bi- and trivalent cluster ligands containing only lactosyl groups as the sugar moiety with their binding strength approaching that of the best oligosaccharide structure of natural origin. The inhibition data of the newly synthesized cluster ligands were explained on the basis of molecular structure and the postulated spatial arrangement of the three Gal-combining sites of the lectin.

Materials and Methods

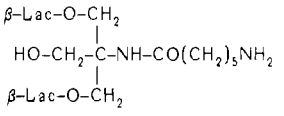
The following compounds were obtained from the indicated sources: *N*-carbobenzyloxyaspartic acid, γ-glutamylglutamic acid, *N*-carbobenzyloxytyrosine, and *N*-carbobenzyloxytyrosine *p*-nitrophenyl ester from Sigma Chemical Co.; EEDQ, methyl chloroformate, and *N*-methylmorpholine from Aldrich Chemical Co.; 1,2,3,4-tetra-*O*-acetyl-6-*O*-trityl-β-glucopyranose from Koch-Light Laboratories; carrier-free $Na^{125}I$ from Amersham Corp.

Chart II

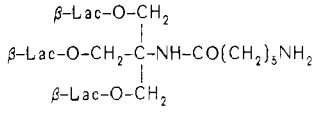
Mono-Lac-AHT



Bis-Lac-AHT



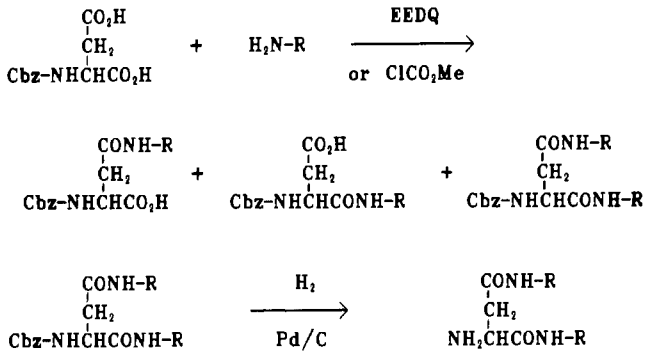
Tris-Lac-AHT



All the synthetic oligosaccharides shown in Chart I were kindly provided by Dr. J. Lönngren, and a diheteroglycan from *Streptococcus faecalis* was provided by Dr. J. H. Pazur. Orosomucoid was desialylated by a mild acid treatment as described before (Connolly et al., 1981) and radioiodinated by a modified (Kranz et al., 1976) chloramine T method of Greenwood et al. (1963). The detergent-solubilized lectin from rabbit liver was isolated according to the method of Hudgin et al. (1974). Methods for preparing 6-aminohexyl β-lactopyranoside (LacAH) (Weigel et al., 1979), mono-, bis- and tris-lactosylated derivatives of (6-aminohexanamido)tris(hydroxymethyl)methane (Chart II) (Lee, 1978), and lactose-containing BSA by reductive alkylation (Lee & Lee, 1980) have been described.

The sugar content of cluster ligands containing lactose was determined by a phenol–sulfuric acid method (McKelvy & Lee, 1969). For the compounds containing a trisaccharide, β-Gal(1–4) β-Glc(1–6)Glc, quantitation and identification of sugars were made by the sugar analyzer after acid hydrolysis of samples (Lee, 1972). Amino groups were quantitated by the fluorescamine method (Naoi & Lee, 1974) with asparagine as standard. UV-absorption spectra were obtained with a Perkin-Elmer spectrophotometer, Model 570. Standards used were *N*-Cbz-Asp and *N*-Cbz-Tyr, which gave maximum molar absorbance of 186 (258 nm) and 1360 (278 nm), respectively. Fast-atom bombardment (FAB) mass spectrometry for the determination of positive or negative molecular ions [$(M + H)^+$ or $(M - H)^-$] was performed on an MS-50 mass spectrometer (Kratos, Manchester, England) equipped with a 23-kG magnet. A sample consisted of 1 μL each of monothioglycerol and salt-free solution of a test glycoside (5–20 mM) and was bombarded with a xenon atom beam. The TLC examination of reaction mixtures and products were performed on silica gel layers (F_{254}) precoated on aluminum sheets (E. Merck). Solvents used were (solvent A) 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water, (solvent B) 3:2:1 (v/v) ethyl acetate–acetic acid–water, and (solvent C) 9:4:2 (v/v) ethyl acetate–2-propanol–water. Carbohydrate-containing compounds were visualized by spraying the plates with 15% sulfuric acid in 50% ethanol and heating on a hot plate at

Scheme I



~140 °C; amino-containing components were detected by spraying first with glacial acetic acid and then with 0.4% ninhydrin in 95% ethanol and heating the plates briefly; compounds containing aromatic groups were detected by exposing the plate to iodine vapor in an enclosed chamber for a few minutes. Radioactivity was counted with a Packard PRIAS automatic γ-counter, Model PGD.

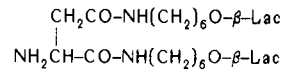
The binding affinity of the synthetic ligands to the solubilized binding protein was determined by a modified assay A of Hudgin et al. (1974) with various concentrations of a test compound (inhibitor). The concentration of the inhibitor causing 50% inhibition of ^{125}I-ASOR binding to the lectin (I_{50}) was obtained by plotting the percent inhibition vs. the logarithm of the inhibitor concentration (Connolly et al., 1982). Assays were performed with concentrations of both ^{125}I-ASOR and the lectin below 10^{-9} M so that the resulting I_{50} approximated the K_i (Chang et al., 1975). Rabbit and rat hepatocytes were prepared by the collagenase perfusion method of Seglen (1976) as described before (Connolly et al., 1982). The hepatocyte preparations were obtained routinely in 90–98% viability (Trypan blue exclusion), and 70–80% of the cells were as singles. The binding affinity of the synthetic ligands to the hepatocytes was determined by using an inhibition assay at 2 °C as described before (Connolly et al., 1982).

Per-*O*-acetylated β-Gal(1–4)β-Glc(1–6)Glc (mp 193–194 °C) was prepared from per-*O*-acetylated 1-bromo-α-lactopyranose and 1,2,3,4-tetra-*O*-acetyl-6-*O*-trityl-β-glucopyranose by a previously described procedure (Lee & Lee, 1982). The per-*O*-acetylated trisaccharide was then converted to the 1-bromo derivative (Weigel et al., 1979), which was in turn reacted with an amino-protected *N*-(6-aminohexanamido)-tris(hydroxymethyl)aminomethane (Lee, 1978) to give the amino-protected bis- and tris-glycosylated cluster ligands that, after de-*N*-protection, yielded the trisaccharide-containing ligands anlogous to those shown in Chart II.

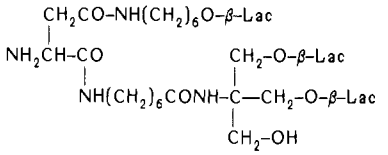
A new type of cluster ligand was prepared by attaching a number of different amino-containing glycosides (e.g., tris-LacAHT in Chart II) to the carboxyl groups of *N*-Cbz-Asp (Scheme I). Coupling of the amino and the carboxyl group was accomplished by using either EEDQ as a coupling agent or the mixed anhydride method (Barker et al., 1974). Some examples of the structures and the corresponding abbreviated nomenclature of these cluster ligands are shown in Chart III. A similar type of cluster ligand was prepared by using *N*-Cbz-Tyr-γ-Glu-Glu instead of *N*-Cbz-Asp. *N*-Cbz-Tyr-Glu-Glu was prepared by reacting γ-glutamylglutamic acid (0.5 mmol) and *N*-carbobenzyloxytyrosine *p*-nitrophenyl ester (0.6 mmol) in 15 mL of dry dimethyl sulfoxide and 0.11 mL of triethylamine for 4 h at room temperature. The reaction mixture was evaporated in vacuo, and the resulting oil was stirred with ethyl acetate (30 mL) overnight. TLC in solvent C showed that the resulting white solid contained the product

Chart III

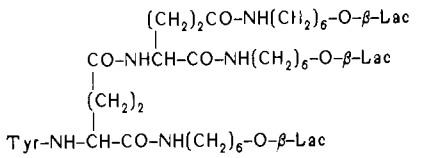
Asp(LacAH)$_2$



Asp(LacAH, bis-LacAHT)



Tyr-Glu-Glu-(LacAH)$_3$



Cbz-Tyr-Glu-Glu while the excess reagent and *p*-nitrophenol were extracted into ethyl acetate. The solid was dissolved in hot 95% ethanol and precipitated by addition of ether: yield, 49%; mp 133–137 °C. Examples of the EEDQ method and the mixed anhydride method are given below for the preparation of Cbz-Asp(tris-LacAHT)$_1$ and Cbz-Asp(tris-LacAHT)$_2$.

EEDQ Method. To a solution of *N*-Cbz-Asp (75 μmol) and tris-LacAHT (120 μmol) in 3.5 mL of water was added EEDQ (1 mmol), and the suspension was stirred overnight at room temperature. During the first several hours, 95% ethanol was added so as to result in the final ethanol concentration of ca. 50%. The suspension was evaporated to dryness, and the residue was suspended in 2 mL of water and centrifuged to remove the precipitate. The precipitate was washed with water (2 times 2 mL) by centrifugation, and the combined aqueous solution was washed once with toluene (2 mL). The water layer was evaporated to ca. 2 mL and then passed through a column (2 × 145 cm) of Sephadex G-25 with 0.1 M ammonium hydroxide as eluant (4-mL fractions). The elution profile obtained by the phenol–sulfuric acid assay revealed two peaks: the first peak contained Cbz-Asp(tris-LacAHT)$_2$ and the second contained Cbz-Asp(tris-LacAHT)$_1$ and a small amount of unreacted tris-LacAHT, as judged by TLC in solvent A. Products were obtained by evaporation of the appropriate fractions. To remove volatile salts in the residue, it was repeatedly dissolved in water and evaporated and then finally left in a vacuum desiccator containing concentrated sulfuric acid. Combined yield of the mono- and disubstituted products was 56%.

Mixed-Anhyride Method. *N*-Cbz-Asp (0.1 mmol) was dissolved in 0.6 mL of dry dimethylformamide and cooled in a dry ice–ethanol bath. Methyl chloroformate (20 μL, 0.23 mmol) and *N*-methylmorpholine (30 μL, 0.25 mmol) were added to the solution, and the mixture was kept in the cold bath for 20 min, after which time 100 μL of the mixture was withdrawn and added to a solution of tris-LacAHT (34 μmol) in 0.2 mL of dry dimethyl sulfoxide. After overnight at room temperature, 1 mL of water was added, and the mixture was fractionated on the column of Sephadex G-25 as described in the previous section. Combined yield of the two products was 48%.

Since a monosubstituted product such as Cbz-Asp(bis-LacAHT)$_1$ still contained a carboxyl group, it can be used in the

Table I: Some Properties of New Cluster Ligands

glycoside structure	nonglycoside structure: Cbz-Asp R_f[a]	R_f[b]	lactose/Cbz theory	lactose/Cbz found	Asp R_f[a]	lactose/NH_2 theory	lactose/NH_2 found
$(tris\text{-}LacAHT)_2$	0.12, 0.24[d]	0	6	7.0	0.02	6	5.6
$(tris\text{-}LacAHT)_1$	0.30	0.04	3	3.6	0.08	3	2.8
$(bis\text{-}LacAHT)_2$	0.21, 0.38[d]	0	4	4.0	0.12	4	3.8
$(bis\text{-}LacAHT)_1$	0.48	0.13	2	1.9	0.18	2	1.8
bis-LacAHT,mono-LacAHT	0.38	0.03	3	3.0	0.18	3	2.6
bis-LacAHT,LacAH	0.45	0.04	3	3.2	0.20	3	ND[e]
$(LacAH)_2$	0.72	0.20	2	2.0	0.42	2	ND
$(LacAH)_3$[c]	0.64	0.08	3	3.2	0.49	3	ND

[a] R_f in TLC with solvent A. [b] R_f in TLC with solvent B. [c] Derivatives of Cbz-Tyr-Glu-Glu and Tyr-Glu-Glu instead of Cbz-Asp and Asp. [d] Preliminary evidence indicates that two spots represent positional isomers (substitution at the α- or the β-carboxyl group of Asp). [e] ND, not determined.

second coupling reaction with another amino-containing lactoside, LacAH for instance, to yield asymmetrically substituted product Cbz-Asp(bis-LacAHT,LacAH). To obtain the product of this second coupling reaction in a homogeneous state, it was necessary to pass the mixture through a larger column (5 × 200 cm) of Sephadex G-25 in 0.1 M acetic acid. In all cases, elution position from the Sephadex columns and mobility on TLC aided in id ntifying the di- and the monosubstituted products. As shown in Table I, TLC in solvent A gave satisfactory results for most of the products. Solvent B, in which the mobility of all the products was markedly slower than in solvent A, was useful for the TLC of the compounds with high R_f values in solvent A, such as Cbz-Asp-$(LacAH)_2$. Also, R_f in solvent B depended mainly on the lactose content, R_f decreasing with increasing number of lactose residues per molecule. Identity of the products was established by compositional analysis on a weight basis as well as the ratio of lactose to Cbz (or Cbz-Tyr) group. Table I presents the experimentally determined ratios and the R_f values in TLC. In addition, the molecular weight of three of the products was determined by FAB mass spectrometry. Experimentally determined molecular masses were within 1 dalton of the theoretical values. The Cbz group of all the products was removed by hydrogenolysis in a micro-Brown hydrogenator (Brown & Brown, 1966) with 20% acetic acid as solvent and 10% palladium on carbon as catalyst. The hydrogenolysis product obtained by filtration and evaporation of the filtrate was ninhydrin positive (TLC), had an R_f value lower than the parent compound, and had the expected ratio of lactose to amino group (Table I).

Results

All the new synthetic cluster ligands were tested as inhibitors of ^{125}I-ASOR binding to rabbit (or rat) hepatocytes, and the resulting I_{50} values are presented in Table II. The I_{50} values of several inhibitors obtained in parallel inhibition experiments with rabbit and rat hepatocytes were identical within the experimental errors, suggesting that the binding characteristics of rabbit and rat hepatocytes under these conditions are very similar.

The first synthetic cluster ligands reported from this laboratory were galactosyl (Lee, 1978) and lactosyl (Kawaguchi et al., 1980) derivatives of (6-aminohexanamido)tris(hydroxymethyl)methane (Chart II). While these compounds showed clearly a demonstrable cluster effect (Connolly et al., 1982), the effect was far smaller than that manifested by the natural bi- and triantennary oligosaccharides and by the synthetic oligosaccharides (Chart I) representing the outer portions of the natural oligosaccharides (Lee et al., 1983). In view of the

Table II: Inhibition of ^{125}I-ASOR Binding by Small Cluster Ligands

inhibitor	Gal valency	I_{50} (μM) hepatocytes	I_{50} (μM) soluble lectin	ratio[a]
mono-GalAHT	1	420[b]	600[b]	1.4
mono-LacAHT		500[b]	520[b]	1.0
bis-GalAHT	2	87[b]	350[b]	4
bis-LacAHT		9[b]	210[b]	23
bis-Gal-Glc-GlcAHT		7	190	27
$Asp(bis\text{-}LacAHT)_1$		8	160	20
$Asp(LacAH)_2$		0.3	9.5	32
PENTA-2,4		0.3[c]	30	100
PENTA-2,6		4.5[c]	70	15
tris-GalAHT	3	8[b]	300[b]	38
tris-LacAHT		4[b]	180[b]	45
tris-Gal-Glc-GlcAHT		0.4	70	175
$Asp(tris\text{-}LacAHT)_1$		2	~100	50
Asp(bis-LacAHT,mono-LacAHT)		0.1	8	80
Asp(bis-LacAHT,LacAH)		0.1	5	50
$Tyr\text{-}Glu\text{-}Glu(LacAH)_3$		0.05	1	20
NONA I		0.007[c]	0.9	129
NONA II		0.15[c]	2.2	15
$Asp(bis\text{-}LacAHT)_2$	4	0.045	12	267
UNDECA		0.0034[c]	3	880
$Asp(tris\text{-}LacAHT)_2$	6	0.012	7	580

[a] The ratio of I_{50} obtained with soluble lectin to I_{50} obtained with hepatocytes. [b] Data from Connolly et al. (1982). [c] Data from Lee et al. (1983).

stereochemical restrictions imposed on the galactosyl residues of the tris-based glycosides, it was reasoned that further lengthening of the sugar moiety would improve the binding affinity. Consequently, we prepared analogous cluster ligands from a trisaccharide, β-Gal(1–4)β-Glc(1–6)Glc, and tested them in the inhibition assays. As shown in Table II, the binding affinity of bis- and tris-glycosylated ligands containing Gal, Lac, or Gal-Glc-Glc shows the following relationship: for the bis series, Gal $\ll$ Lac = Gal-Glc-Glc; for the tris series, Gal < Lac $\ll$ Gal-Glc-Glc.

While the binding affinity of the tris glycoside of Gal-Glc-Glc improved much over that of Gal, the I_{50} of the trisaccharide-containing ligand was still 50-fold higher than NONA I (Chart I), the best synthetic trivalent ligand tested so far. For further improvement of synthetic cluster ligands, we developed a more versatile synthetic scheme, so that a number of different structural features (such as the number of galactosyl residues per molecule and the length of the arm connecting the galactosyl residue to the joint) can be easily incorporated. The scheme is simply to attach various lactosides having an ω-amino group in the glycon [e.g., Weigel et al. (1979) and Lee (1978)] to the carboxyl groups of aspartic acid or γ-glutamylglutamic acid. All the cluster ligands shown in

Table III: Inhibition of ^{125}I-ASOR Binding by Some Macromolecular Cluster Ligands

inhibitor	I_{50} (nM) hepatocytes	I_{50} (nM) soluble lectin	ratio[a]
ASOR	0.5	5	10
Lac_6-BSA	220	2400	11
Lac_{20}-BSA	1.3	28	21
Lac_{40}-BSA	0.25	2.5	10
diheteroglycan (*S. faecalis*)	0.3	2.1	7

[a] The ratio of I_{50} obtained with soluble lectin to I_{50} obtained with hepatocytes.

Table I were prepared in this fashion. As shown in Table II, some bi- and trivalent ligands prepared by this scheme were better inhibitors than the tris glycoside containing Gal-Glc-Glc.

These synthetic ligands as well as the synthetic oligosaccharides shown in Chart I (Arnarp et al., 1981) were also tested as inhibitors with the Triton-solubilized binding protein from the rabbit liver, and the results are shown in Table II. Also, the inhibition potency of some macromolecular cluster ligands with both the hepatocytes and the soluble lectin is presented in Table III for comparison. Examination of Tables II and III indicates that the cluster effect is always greater for the hepatocytes than for the soluble lectin, in agreement with the previous report on the tris-galactosyl and -lactosyl ligands (Connolly et al., 1982). The ratio of the I_{50} obtained with the soluble lectin and the hepatocytes (the last column in Tables II and III) is a measure of difference in the clustering effect expressed by the two systems. The ratio appears to increase with increasing valency, reaching a ratio as high as 880 at four Gal residues per molecule (UNDECA, Table II). However, all the polyvalent, macromolecular ligands (such as ASOR) had a much smaller ratio of 7–20.

Discussion

Binding of a monovalent galactoside to the mammalian hepatocyte lectin is rather weak, the dissociation constant being in the range of (3–9) $\times 10^{-4}$ M (Lee et al., 1983; Connolly et al., 1982). However, polyvalent ligands, such as Gal-bearing neoglycoproteins, can attain a very strong binding ($K_d = 10^{-8}$ M or lower) by virtue of clustering of Gal residues (Lee, 1982). Interestingly, small synthetic polyvalent ligands (bis and tris glycosides shown in Chart II) also exhibited the cluster effect to some extent, but the effect was observed only with the isolated hepatocytes and not with the Triton-solubilized, purified lectin (Connolly et al., 1982). Recently, inhibition studies with a series of synthetic oligosaccharides, such as shown in Chart I, revealed that an oligosaccharide having only three terminal Gal residues could manifest a binding constant comparable to the neoglycoproteins containing 20–30 Gal residues (K_d = ca. 10^{-8} M) in the isolated rabbit hepatocytes system (Lee et al., 1983), indicating that a bulk of the cluster effect can be generated with only three Gal residues and that neither a large molecular size nor the protein backbone is necessary for the expression of the cluster effect. The most potent bivalent oligosaccharide in these studies was PENTA-2,4 ($K_d = 3 \times 10^{-7}$ M), which represents a portion of the desialylated complex-type triantennary oligosaccharide chain of α_1-protease inhibitor (Hodges et al., 1979). The most potent trivalent ligand was NONA I ($K_d = 7 \times 10^{-9}$ M), which contains structural elements of PENTA-2,4 and HEPTA and represents the outer portion of the above-mentioned triantennary oligosaccharide chain. This hugh increase in binding affinity (2000-fold from monovalent to the best bivalent and 50-fold from the best bivalent to the best trivalent) with each additional Gal residue, especially that from mono- to bivalent, is close to the maximum increase possible theoretically, when two Gal residues bind simultaneously with binding at each site contributing K_d of (3–9) $\times 10^{-4}$ M. It was suggested, therefore, that the spatial arrangement of the Gal residues of NONA I in its most probable conformation in solution (Bock et al., 1982) closely approximates the spatial arrangement of the three Gal-combining sites of the lectin (Lee et al., 1984). The three sites would then be situated on vertexes of a triangle whose sides are 15, 22, and 25 Å.

Table IV: Maximal Gal–Gal Distances in the Synthetic Cluster Ligands Determined with Fisher–Hirschfelder–Taylor Atomic Models

compound	maximum Gal–Gal spatial distance (Å)[a]
bis-GalAHT[b]	9
bis-LacAHT[b]	17
bis-Gal-Glc-GlcAHT[b]	30
$Asp(LacAH)_2$	28
$Tyr\text{-}Glu\text{-}Glu(LacAH)_3$	33, 41, 43
Asp(bis-LacAHT,LacAH)	17, 30, 30
Asp(bis-LacAHT,mono-LacAHT)	17, 36, 36

[a] Distance of 1 cm in the model closely approximates 1 Å in the real molecule. [b] Applies also to tris derivatives.

The synthetic cluster ligands reported here can be divided into three groups: (1) derivatives of tris(hydroxymethyl)-aminomethane (e.g., tris-LacAHT), (2) derivatives of Asp and Tyr-Glu-Glu with each carboxyl group bearing one terminal Gal [e.g., $Asp(LacAH)_2$], and (3) a hybrid type in which the tris-type derivative is attached to one or both of the carboxyl groups of Asp [e.g., Asp(bis-LacAHT,LacAH)]. We have determined, with Fischer–Hirschfelder–Taylor atomic models, the maximal Gal–Gal distances attainable in these cluster ligands and present them in Table IV. The molecular models also revealed that type 2 compounds, having linking arms between the lactose residues and the joints of at least six carbons or longer, are very flexible and allow the Gal residues to be separated spatially anywhere between zero and the maximum distance indicated in Table IV. On the contrary, the orientation of the Gal residues in space in the type 1 (tris-type) compounds is restricted, since only a single methylene group separates the sugars from the joint.

Of all the compounds presented in Table II, only bis-GalAHT, tris-GalAHT, tris-LacAHT, and $Asp(tris\text{-}LacAHT)_1$ possessed one or more inter-Gal distances that are shorter than those specified by the most probable NONA I conformation (i.e., 15, 22, and 25 Å) and manifested considerably inferior binding than others of equivalent valency (Table II). The rest of the compounds, though all were better inhibitors than those listed above, were still poorer inhibitors than PENTA-2,4 (bivalent) and NONA I (trivalent), despite the fact that they had adequate inter-Gal distances. Obviously, other factors play a role in determining the inhibitory potency. Flexibility seems to be one of the factors: i.e., the more flexible the aglycon of a ligand is, the more potent inhibitor this ligand will be [e.g., bivalent, $Asp(LacAH)_2$ > bis-LacAHT; trivalent, $Tyr\text{-}Glu\text{-}Glu(LacAH)_3$ > Asp(bis-LacAHT,LacAH) > tris-Gal-Glc-GlcAHT]. In fact, the most flexible bivalent ligand, $Asp(LacAH)_2$, has an inhibitory potency comparable to the most potent oligosaccharide, PENTA-2,4. It is likely that the Gal residues of $Asp(LacAH)_2$ can easily bind to any two of the three Gal-combining sites. The comparable binding affinity of $Asp(LacAH)_2$ and PENTA-2,4 suggests that a bulk of the binding strength in the lectin–ligand interaction is generated by the lactosyl moieties alone. However, the most flexible trivalent ligand, $Tyr\text{-}Glu\text{-}Glu(LacAH)_3$, has somewhat

weaker binding strength than NONA I, suggesting that the rest of the oligosaccharide in NONA I may have some energetical contribution either by stabilizing the most probable conformation or by actually interacting at a secondary site. A further increase in the Gal valency of a small ligand to four or six residues per molecule brought about a modest increase in the binding affinity [e.g., compare Asp(bis-LacAHT)$_2$ and Asp(bis-LacAHT, mono-LacAHT)]. This increase may be of a statistical nature, such as an increased local concentration of Gal near the combining sites, or due to the cross-interaction of such a ligand with another set of the Gal-combining sites that might be present in proximity.

Though the affinity of the Triton-solubilized lectin for monovalent ligands is very similar to that of the hepatocytes [Table II; also Connolly et al. (1982)], clustering of Gal residues caused only a modest increase in the binding affinity compared to the hepatocytes. Also, the lectin on the hepatocytes showed 25–20 times stronger binding of PENTA-2,4 than PENTA-2,6; this preference is lost when the solubilized lectin is used (compare PENTA-2,4 vs. PENTA-2,6 and NONA I vs. NONA II in Table II). These observations suggest that the lectin molecules on the hepatocyte surface may have a specific three-dimensional structure or orientation, which is responsible in presenting the Gal-combining sites in such a way as to achieve a very tight binding of the NONA I type structure. In the detergent-solubilized form, the lectin exists in the absence of calcium ion as a 250 000-dalton molecular species. Upon addition of calcium ion (which is needed for the binding of the ligand), the M_r becomes 600 000 (Anderson et al., 1982). It may be that, in the process of detergent solubilization, the original three-dimensional structure of the lectin molecule or a unique spatial arrangement of lectin molecules as maintained on the hepatocyte surface is lost or that the association of two 250 000-dalton units of the solubilized lectin does not attain the optimal binding structure of this lectin, thus resulting in the inferior binding of all the cluster ligands, especially those containing the PENTA-2,4 structure.

The ratio of the I_{50} values obtained in the soluble and the hepatocyte systems (the last column of Tables II and III) is a measure of the difference in the cluster effect of the two systems. The ratio of the I_{50} values reached the highest value at four to six residues of Gal per molecule of the ligand. Further increase in the Gal valency may eventually decrease the ratio, since a very potent inhibitor, a diheteroglycan of *Streptococcus faecalis* having a molecular weight of 15 000 and containing 18 nonreducing Gal residues per molecule (Pazur et al., 1973), had the smallest ratio of the I_{50} values of all the polyvalent ligands examined (Table III). Also, all the polyvalent macromolecules (ASOR and Lac-BSA's), regardless of the Gal valency, had the I_{50} ratio of 10–20, much smaller than those of small polyvalent ligands. ASOR, a desialylated glycoprotein with five oligosaccharide chains of the complex type, binds to the hepatocytes with roughly a 10-fold stronger affinity than the single chain (triantennary glycopeptide and NONA I). If one defines that a binding unit of the lectin consists of the three Gal-combining sites of the NONA I structure, the modest increase in binding of ASOR over NONA I can be explained on the basis of one molecule of ASOR binding to two (or more) binding units of the lectin. It may be that the polyvalent macromolecules in general can bind to multiple units of the lectin. Possibly, such a ligand can bind the multiple binding units of the solubilized lectin more readily than the lectin on the hepatocytes; this will tend to minimize the difference in I_{50} between the two systems.

The cluster ligands described here are much more readily synthesized than those used in our previous study (Lee et al., 1983). Ready availability of these ligands, which can be used to modify proteins or can be incorporated into insoluble matrices by simple means, should provide many new tools in the studies of carbohydrate-mediated endocytoses.

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Distance Measurements in Spin-Labeled Lysozyme†

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ABSTRACT: The single His-15 of hen egg lysozyme reacts with 2,2,6,6-tetramethyl-4-(bromoacetamido)piperidinyl-1-oxy or 2,2,5,5-tetramethyl-3-(bromoacetamido)pyrrolidinyl-1-oxy to give a spin-labeled enzyme [Wien, R. W., Morrisett, J. D., & McConnell, H. M. (1972) *Biochemistry 11*, 3707–3716]. High-field ^1H NMR spectra (300 and 500 MHz) of these species in ^2H_2O contain protein peaks selectively broadened by dipolar coupling to the unpaired electron spin. While usually difficult to discern in the spectrum itself, broadened resonances are revealed in difference spectra obtained by subtracting the original spectrum from one taken after reduction of the nitroxide radical with ascorbate. The heights of difference spectra peaks are related in a simple way to r^{-6}, where r is the label to proton distance. These distances were used to solve for the location of the electron spin by using algorithms from distance geometry. The spin was found to lie in a hydrophobic groove between Phe-3 and Asp-87. These results demonstrate the feasibility of spin-labeling for accurate distance measurements in proteins through the use of distance geometry.

The time-averaged structure of any molecule can be determined if one measures a sufficient number of interatomic distances. Distance geometry (Crippen, 1981; Havel et al., 1979; Kuntz et al., 1979) provides the mathematical framework to convert distance measurements, with the associated experimental errors, into actual three-dimensional structures. A great many distances are needed to solve a structure. One estimate (Havel et al., 1979) suggests an order of 1000–2000 measurements would be required. This is in agreement with low-resolution X-ray diffraction studies on small proteins. In analogy with the diffraction methods the more measurements, the higher the resolution of the final result. NMR methods have considerable potential for such measurements, especially with the new two-dimensional techniques using the nuclear Overhauser effect (NOE). Methods such as NOESY (Jeener et al., 1979; Kumar et al., 1981), coupled with the high fields now available, provide semiquantitative estimates for a large number of interproton distances in proteins (Wüthrich et al., 1982). But even if all possible NOE's were determined to better than ±1-Å accuracy, the structure of a small protein would be underdetermined and could not be solved without the use of idealized models. The major difficulty is that the distances measured with NOE techniques are all short (less than 4 Å in proteins) compared to the radius of the molecule. Longer distances are required to complete the structure (Havel et al., 1979).

Paramagnetic probes offer another avenue to intramolecular distances. Because of the great strength of the paramagnetic moment compared to the magnetic moment of the proton, line-broadening effects can be seen out to about 20 Å in molecules of ca. 10000 daltons (Wien et al., 1973; Krugh, 1976; Dwek et al., 1975). The two main types of probes are paramagnetic metal ions such as Mn^{2+} or Gd^{3+} and free radicals. Metal ions have been quite useful in special cases where well-defined binding sites exist (Campbell et al., 1975; Lee & Sykes, 1983). Nitroxide spin-labels appear to be more generally useful and offer the opportunity to put probes, one at a time, in several specific locations throughout a protein by taking advantage of the great variety of reactive groups available (Berliner, 1976; Likhtenshtein, 1976). The possibility of measuring many hundreds of 10–20-Å distances is well worth investigating.

As an initial test of this strategy, we have prepared a spin-labeled lysozyme derivative. Wien et al. (1972) showed that hen egg white lysozyme, labeled at His-15 with 2,2,5,5-tetramethyl-3-(bromoacetamido)pyrrolidinyl-1-oxy, induced broadening of proton resonances from inhibitors binding more than 15 Å away. They checked to see if resonances from the protein could be likewise measured, but the spin-labeled lysozyme spectrum was devoid of resolution. Addition of ascorbate reduced the nitroxide to the diamagnetic hydroxylamine, after which the NMR spectrum sharpened up.

The early work of Wien et al. (1972) was limited by the low magnetic field employed (23 kG, 100-MHz ^1H). Without spectral resolution of the labeled protein, changes in line widths due to dipolar broadening from the free electron could not be determined. We have now measured spin-labeled lysozyme at 300 and 500 MHz. Increased dispersion at these high fields provides much better resolution in spectra of the paramagnetic enzyme. Equally important was our ability to work at much lower protein concentration because of greatly increased sensitivity. High concentrations lead to substantial broadening even at 300 MHz due to intermolecular effects. Finally, we used difference spectroscopy to quantitate line-broadening effects (Campbell et al., 1975). We subtracted a spectrum of paramagnetic spin-labeled protein from a spectrum taken after reduction of the label to the diamagnetic hydroxylamine. This procedure results in a greatly simplified trace containing

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