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A New Class of Model Glycolipids: Synthesis, Characterization, and Interaction with Lectins

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A method is described for preparing model glycolipids by linking aldobionic acids to an alkylamine through an amide bond. These compounds may be rapidly prepared in large quantities. The glycolipids precipitate specifically with lectins. Precipitation occurs at glycolipid concentrations just above their critical micelle concentration.

Although lectin-reactive molecules in biomembranes are generally glycoproteins (1, 2), lectins also bind glycolipids (3–9). For a detailed study of lectin–glycolipid interaction we required substances of defined structure. Because isolation of naturally occurring glycolipids is time consuming and only small quantities are generally obtained pure, we synthesized these compounds. In this paper we report the synthesis, characterization, and some biological applications of a new class of model glycolipids which can be readily prepared in high yield as pure crystalline solids. A preliminary communication has already appeared (10).

MATERIALS AND METHODS

Materials. *N*-Phenyl-1-naphthylamine, ninhydrin, diphenylamine, *n*-octylamine, *n*-decylamine, *n*-undecylamine, *n*-dodecylamine, *n*-tetradecylamine, and *N*,*N*′-dicyclohexylcarbodiimide (DCC)[2] were obtained from the Aldrich Chemical Company (Milwaukee, Wis.). Methyl α-D-mannopyranoside, methyl α-D-galactopyranoside, melibiose, lactose, maltose, cellobiose, and gentiobiose were purchased from Pfanstiehl Laboratories, Inc. (Waukegan, Ill.). *n*-Hexadecylamine and *n*-tridecylamine were obtained from Eastman Kodak Company (Rochester, N. Y.). *n*-Octadecylamine was obtained from Koch-Light Laboratories, Ltd. (Colnbrook, Buck, England). Amberlite cation-exchange resin IR-120 C.P. was obtained from the Mallinkrodt Chemical Company (St. Louis, Mo.). Precoated silica gel G-60 tlc plates were obtained from Brinkmann Instruments, Inc. (Westbury, N. Y.). Concanavalin A was prepared by the method of Williams *et al.* (11). *Bandeiraea simplicifolia* (BS I) lectin was isolated by the method of Murphy and Goldstein (12). Castor bean lectin (RCA_I) was a generous gift of Dr. Marilyn Etzer, University of California, Davis.

N-Dodecylmelibionamide. Melibiose was oxidized to potassium melibionate (13) followed by conversion to the free acid by treatment with Amberlite IR-120 (H^+). The aqueous solution of the free acid was dried *in vacuo* at 40°C. Melibiono-1,5-lactone was formed by concentrating and drying melibionic acid by repeated evaporation from methanol and ethanol (14). Melibiono-1,5-lactone (1 g, 2.8 mmol) was dissolved in methanol (10 ml) by gentle heating, *n*-dodecylamine (0.6 g, 3.2 mmol) was added, and the reaction mixture was stirred overnight at room temperature. The precipitate which formed was filtered, washed with cold methanol, and air dried. Repeated recrystallization from methanol gave *N*-dodecylmelibionamide as a crystalline white

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[2] Abbreviations used: DCC, *N*,*N*′-dicyclohexylcarbodiimide; RCA_I, castor bean lectin; CMC, critical micelle concentration; salt/*P*; buffer, 0.01 M phosphate (pH 7.2), 0.5 M NaCl, 0.1 mM $MnCl_2$, and 0.1 mM $CaCl_2$; Con A, concanavalin A; PBS, phosphate buffered saline; Ac, acetyl; Man*p*, mannopyranoside; Gal*p*, galactopyranoside; Glc*p*, glucopyranoside.

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solid (yield, 70%; mp 155–156°C). It gave a single spot (R_f 0.30) on tlc (1-butanol:acetic acid:diethyl ether:water, 9:6:3:1) upon spraying with acidic diphenylamine (10). *Anal.* Calcd for $C_{24}H_{47}NO_{11}$: C, 54.80; H, 9.08; N, 2.66. Found: C, 54.86; H, 9.14; N, 2.57. Preparation of other model glycolipids followed the same reaction scheme. The products, melting points, and elemental analyses are listed in Table I. The crystallization solvent for all synthetic glycolipids except the *N*-alkylmaltobionamides was methanol; the maltobionamides were crystallized from ethanol.

Determination of critical micelle concentration (CMC). CMC determinations were carried out on an Aminco–Bowman fluorimeter at 23°C using a fluorescent probe (*N*-phenyl-1-naphthylamine). The *N*-phenyl-1-naphthylamine was excited at 360 nm and the emission recorded at 425 nm. The fluorescence change, plotted against glycolipid concentration, was extrapolated to the concentration at which fluorescence reached a minimum value, defined as the critical micelle concentration.

Precipitin reactions. A modification of the precipitin reaction described by So and Goldstein (15) was employed. Increasing quantities of glycolipid dissolved in salt/P_i buffer (0.01 M phosphate, pH 7.2; 0.5 M NaCl; 0.1 mM $MnCl_2$, and 0.1 mM $CaCl_2$) were added to tubes containing buffer and lectin (≈60 μg) in a total volume of 0.5 ml. Tubes were incubated at 25°C for 48 h, centrifuged, and washed with salt/P_i. The washed precipitates were dissolved in 0.05 M NaOH and protein was determined by a semimicro Lowry procedure (16). Sugar inhibition of the precipitin reaction was conducted by adding increasing amounts of carbohydrate hapten to tubes containing lectin. The

TABLE I

PROPERTIES OF SYNTHETIC GLYCOLIPIDS

Compound	mp (°C)	Analyses: % Calculated C	H	N	% Found C	H	N	Yield %	CMC in H_2O
N-Octyllactobionamide	130–132	51.16	8.37	2.98	51.24	8.38	3.05	86	
N-Decyllactobionamide	128–129	53.10	8.71	2.82	53.19	8.73	2.68	75	3.2 (1600)[a]
N-Undecyllactobionamide	149–150	54.00	8.87	2.74	53.94	8.82	2.70	86	
N-Dodecyllactobionamide	143–144	54.80	9.08	2.66	54.54	8.76	2.53	90	0.285 (150)
N-Tridecyllactobionamide	150–151	55.64	9.15	2.60	55.78	9.27	2.58	100	
N-Tetradecyllactobionamide	138–139	56.30	9.28	2.53	56.08	9.32	2.41	90	0.0312 (17.3)
N-Hexadecyllactobionamide	133–135	57.80	9.53	2.40	57.64	9.50	2.32	90	α[b]
N-Octylmaltobionamide	116–117	51.16	8.37	2.98	51.12	8.39	2.90	71	
N-Decylmaltobionamide	119–120	53.10	8.71	2.82	53.22	8.72	2.94	73	2.41 (1200)
N-Undecylmaltobionamide	122–123	54.00	8.87	2.74	53.91	8.76	2.68	74	0.88 (450)
N-Dodecylmaltobionamide	120–121	54.80	9.08	2.66	54.80	9.04	2.68	75	0.228 (119.9)
N-Tridecylmaltobionamide	122–123	55.64	9.15	2.60	55.53	9.19	2.57	74	0.074 (40)
N-Tetradecylmaltobionamide	119–120	56.30	9.28	2.53	56.46	9.35	2.52	80	0.032 (17.6)
N-Hexadecylmaltobionamide	119–120	57.80	9.53	2.40	57.64	9.45	2.40	89	α[b]
N-Octylmelibionamide	162–164	51.16	8.37	2.98	50.96	8.35	3.02	93	
N-Decylmelibionamide	160–162	53.10	8.71	2.82	52.98	8.74	2.82	89	3.01 (1500)
N-Undecylmelibionamide	157–158	54.00	8.87	2.74	53.89	8.01	2.64	38	0.989 (520)
N-Dodecylmelibionamide	155–156	54.80	9.08	2.66	54.86	9.14	2.57	69	0.209 (110)
N-Tridecylmelibionamide	152–154	55.64	9.15	2.60	55.52	9.04	2.52	50	0.074 (40)
N-Tetradecylmelibionamide	144–151	56.30	9.28	2.53	56.14	9.33	2.58	98	0.030 (16.6)
N-Hexadecylmelibionamide	148–150	57.80	9.53	2.40	57.76	9.55	2.49	94	α[b]
N-Tetradecylcellobionamide	112–114	56.30	9.28	2.53	56.28	9.27	2.47	100	
N-Hexadecylcellobionamide	105–107	57.80	9.53	2.40	57.53	9.42	2.36	100	
N-Decylgentiobionamide	146–148	53.10	8.71	2.82	52.60	8.64	2.76	100	
N-Dodecylgentiobionamide	141–143	54.80	9.08	2.66	54.63	8.91	2.64	95	
N-Tetradecylgentiobionamide	144–146	56.30	9.28	2.53	56.23	9.40	2.54	96	
N-Hexadecylgentiobionamide	132–134	57.80	9.53	2.40	57.94	9.65	2.48	95	

[a] Values represent mM concentration with μg/ml indicated in parentheses.

[b] CMC was too low (<2 μg/ml) to be determined by the fluorescence method.

precipitin reaction was initiated by addition of glycolipid at a final concentration which would precipitate maximal quantities of protein.

RESULTS AND DISCUSSION

Synthesis of the compounds in Table I follows a reaction scheme (Fig. 1) similar to that described for *N*-dodecylmelibionamide and *N*-dodecyllactobionamide (10). Initially, DCC was added to catalyze the formation of the aldobionamides. Subsequently it was discovered that amide formation proceeds via the aldobionolactone, formation of which is catalyzed by DCC. Lactone formation also may be effected by heating *in vacuo* and/or dissolution in organic solvents. In the present study, lactone formation was achieved by evaporating the lactobionic acid to dryness several times from ethanol or methanol *in vacuo* at 50°C. Comparison of the carbon-13 nmr spectra of free acid and lactone revealed the presence of carbonyl carbon atoms with a chemical shift at about 180.0 ppm with respect to trimethylsilane for the free aldobionic acid, and a resonance doubling and a shift toward trimethylsilane in the case of lactone carbonyl carbon atoms with peaks at 175.3 and 174.9 ppm. Addition of DCC is advisable when reaction mixtures contain mainly the free aldobionic acid and alkylamine. However, carbodimide may be omitted if aldobionolactone is used as reactant.

Occasionally, the aldobionamides appeared as a gel during recrystallization but repeated recrystallizations from methanol or ethanol provided an analytically pure, crystalline product. The *N*-alkyllactobionamides and *N*-alkylmaltobionamides generally formed crystalline products readily whereas other glycolipids often formed gels. Purity of the glycolipids was readily monitored by thin layer chromatography. When spray reagents were used, the glycolipids were ninhydrin and aldose positive only after hydrolysis (10). Further characterization by ir spectroscopy yielded the characteristic amide I (1655 cm^{-1}) and amide II (1550 cm^{-1}) bands. Elemental analysis also confirmed the assigned structures.

Preparation of aqueous solution of the glycolipids usually required heating and sonication. The solubility of the substances in aqueous solution was affected by both the alkyl chain length and the nature of the carbohydrate head groups. At 1 mg/ml, the alkyllactobionamides, cellobionamides, and gentobionamides (all containing β-D-glycosidic linkages) with alkyl chain lengths of 12 or more carbon atoms generally precipitated from aqueous solution on standing. In fact, the longer the alkyl chain, the more readily the glycolipids separated from solution. Chromatographically pure cellobionamides and gentiobionamides frequently formed gels and very viscous solutions.

In aqueous solution the synthetic glycolipids, like all surfactants, form micelles as detected by fluorescent enhancement of *N*-phenyl-1-naphthylamine which partitions very favorably into the detergent micelles. The quantum yield of the naphthylamine is increased in the presence of a hydrophobic substance (17). Figure 2 shows the enhancement of fluorescence observed in the presence of increasing quantities of glyco-

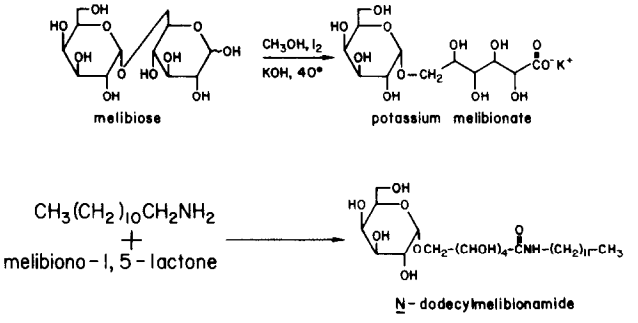

FIG. 1. Reaction scheme for preparation of *N*-dodecylmelibionamide.

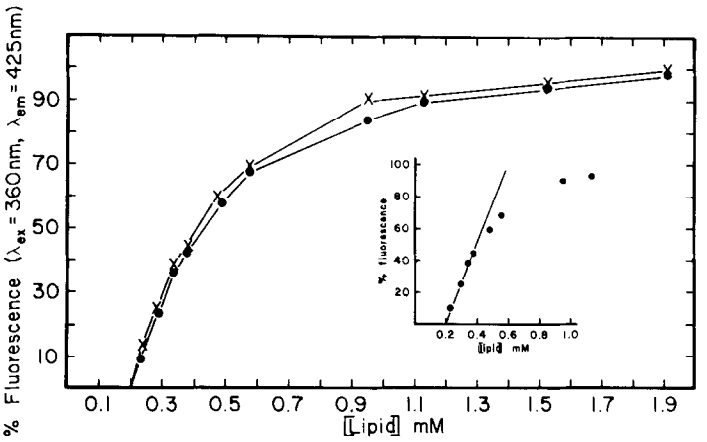

FIG. 2. Dependence of fluorescence of *N*-phenyl-1-naphthylamine on the concentration of glycolipid. (×) *N*-dodecylmelibionamide; (●) *N*-dodecylmaltobionamide. Inset represents extrapolation to 0% fluorescence.

lipid. Values of the CMC determined by this method (Table I) exhibited a logarithmic relationship with the number of carbon atoms in the alkyl chain. The least-squares fit of these data (Fig. 3) allows the formulation of a relationship which describes the free energy of micelle formation (18) in accordance with the equation $\Delta G = -RT \ln \text{CMC}$. The equations in Table II can be used to predict the ΔG of micelle formation for any glycolipid in a particular series by substituting the number of carbon atoms in the alkyl chain carbons for n_c. Values of 3300–3500 cal/mol (Table II) were obtained for the contribution of the polar head group and are indicative of a rather strong interaction with the solvent. The −680 cal/mol contribution to the free energy of micelle formation implies that from 6–7 carbons must be in the alkyl chain before ΔG becomes favorable for micelle formation. As detected by the similar curves in Fig. 3 and equations in Table II, CMC values are independent of the nature of the sugar moiety and of the nature and anomeric configuration of the glycosidic linkage.

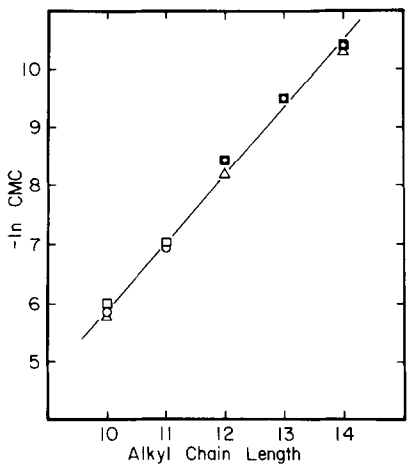

FIG. 3. Dependence of ln CMC on alkyl chain length. CMC values determined in distilled water. (○) *N*-alkylmelibionamides; (△) *N*-alkylactobionamides; (□) *N*-alkylmaltobionamides.

Micelle formation by the synthetic glycolipids results in structures containing multiple carbohydrate groups on the surface and alkyl chains concentrated in the interior region of the micelle. The synthetic glycolipids when present as micelles precipitate with lectins. Quantitative precipitin curves of concanavalin A (Con A) (Fig. 4), B_4 isolectin from *B. simplicifolia* seeds (BS I) (Fig. 5), and RCA_I (Fig. 6) were obtained with several glycolipids. In all three cases, precipitation occurs at the CMC (see Table III) and the lectin–glycolipid interactions were specific, in that a lectin precipitates only that glycolipid containing a carbohydrate with which it interacts. Since the CMC is governed by the number of carbon atoms in the alkyl chain, the quantity of glycolipid required for precipitation depends on the chain length. Comparisons of the point at which precipitation first occurred for the

TABLE II

ΔG OF MICELLE FORMATION[a]

N-Alkylmelibionamide

$\Delta G = 3516\ (\pm 394) - 698\ (\pm 30) n_c$ (cal/mol)

N-Alkylmaltobionamide

$\Delta G = 3000\ (\pm 259) - 661\ (\pm 21) n_c$ (cal/mol)

N-Alkyllactobionamide

$\Delta G = 3433\ (\pm 124) - 687\ (\pm 11) n_c$ (cal/mol)

[a] Equations are obtained by a least-squares fit to the data in Fig. 3 according to the equation $\Delta G = -RT$ ln CMC. The slope of the graph is equal to $-$ln CMC/alkyl carbon and the y-intercept ($n_c = 0$) is related to the ΔG of solubilization of the polar head group (18).

reactions of the *N*-alkylmaltobionamides with Con A, or reaction of *N*-alkylmelibionamides with the BS I lectin revealed that, as expected, 10-fold less glycolipid was required for each two-carbon increase in the alkyl chain length.

The incomplete precipitation of Con A by *N*-decylmaltobionamide and the failure of the *Bandeiraea simplicifolia* isolectins to precipitate *N*-decylmelibionamide are due to inhibition of precipitation by soluble, monomeric glycolipid molecules. At concentrations above the CMC a glycolipid monomer equal in concentration to the CMC is in solution. These monomeric glycolipids may act as competitive inhibitors in the same manner as mono- and oligosaccharides. The CMC values (in PBS buffer, Table III), for *N*-decylmaltobionamide and *N*-decylmelibionamide were determined to be 850 μg/ml, high enough to inhibit or abolish the precipitation with concanavalin A and the *B. simplicifolia*-B_4 isolectin, respectively. Figure 7 illustrates the manner in which *N*-decylmelibionamide inhibits precipitation of guaran and B_4 isolectin from *B. simplicifolia* seeds. On a molar basis, the glycolipid and methyl α-D-galactopyranoside are equally potent as inhibitors. Thus, the concentration of monomeric *N*-decylmelibionamide was sufficient

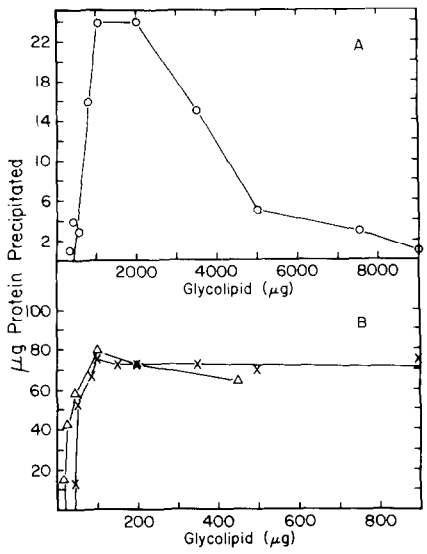

FIG. 4. Quantitative precipitin curves of *N*-alkylmaltobionamides with con A. Buffer consisted of PBS (Γ/2 0.5, pH 7.2). (A) (○) *N*-decylmaltobionamides; (B) (×) *N*-dodecylmaltobionamide; and (△) *N*-tetradecylmaltobionamide.

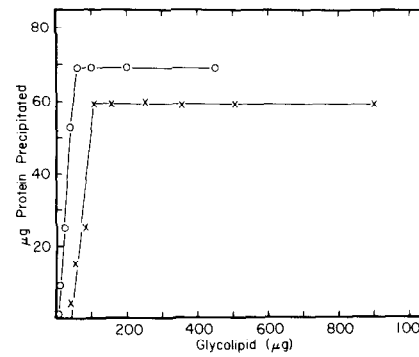

FIG. 5. Quantitative precipitin curves of *N*-alkylmelibionamides with BS I lectin. Buffer consisted of PBS (Γ/2 0.5, pH 7.2). (×) *N*-dodecylmelbionamide and (○) *N*-tetradecylmelibionamide.

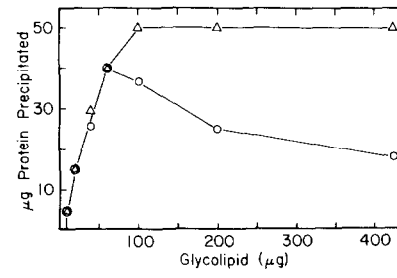

FIG. 6. Quantitative precipitin curves of *N*-tetradecylmelibionamide (○) and *N*-tetradecylactobionamide (△) with RCA_1. Buffer consisted of PBS (Γ/2 0.5, pH 7.2).

TABLE III

GLYCOLIPID–LECTIN INTERACTION

Glycolipid	Glycolipid concentration at point of precipitation (μg/ml)	Lectin (carbohydrate-binding specificity)	CMC[a] (μg/ml)
N-Decylmaltobionamide	900	Concanavalin A	850
N-Dodecylmaltobionamide	74	(α-D-Man*p* > α-D-Glc*p* ≫ α-D-GlcNAc*p*)	75
N-tetradecylmaltobionamide	12		5.5
N-Decylmelibionamide	—[b]	*Bandeiraea simplicifolia* B_4-isolectin	850
N-Dodecylmelibionamide	80	(α-D-Gal*p*)	75
N-Tetradecylmelibionamide	5		5.5
N-Tetradecylmelibionamide	12	RCA_1 (β-D-Gal*p* ≃ α-D-Gal*p*)	5.5
N-Tetradecyllactobionamide	12		5.5

[a] Critical micelle concentration in PBS buffer (Γ/2 0.5, pH 7.2).
[b] Precipitation did not occur due to high CMC.

to completely inhibit precipitate formation at the concentration at which micelles of this glycolipid first form.

Inhibition of lectin–glycolipid precipitation by monosaccharides further demonstrates the specificity of the precipitation reaction as well as the ability of glycolipids to compete with monosaccharides for the carbohydrate binding site. In Fig. 7, the competitive inhibitor, methyl-α-D-galactopyranoside, inhibits formation of the BS I lectin–*N*-dodecylmelibionamide complex at 0.5 mM whereas 10 mM methyl-α-D-mannopyranoside failed to inhibit. Methyl-α-D-mannopyranoside inhibits Con A–glycolipid complex formation of 0.5 mM while 10 mM methyl-α-D-galactopyranoside failed to inhibit (Fig. 8).

Finally, *N*-alkylaldobionamides in concentrations related to their CMC lyse erythrocytes. For example, *N*-decyl-, *N*-dodecyl-, and *N*-tetradecyl-aldobionamides cause lysis in the concentration ranges 800/1000, 30–40, and 3–4 μg/ml, respectively. This observation is consistent with the detergent property of these substances.

ACKNOWLEGMENTS

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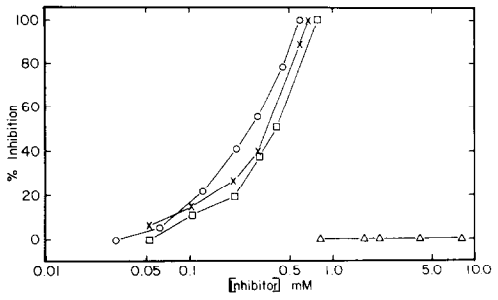

FIG. 7. Inhibition of BS I lectin–guaran and *N*-dodecylmelibionamide precipitation by low molecular weight ligands. Buffer consisted of PBS (Γ/2 0.5, pH 7.2); BS I (50 μg/tube), and guaran (7.5 μg/tube) inhibited by *N*-decylmelibionamide (○) or methyl-α-D-Gal*p* (×). BS I (50 μg/tube) and *N*-dodecylmelibionamide (150 μg/tube) inhibited by methyl-α-D-Gal*p* (□) or methyl-α-D-Man*p* (△).

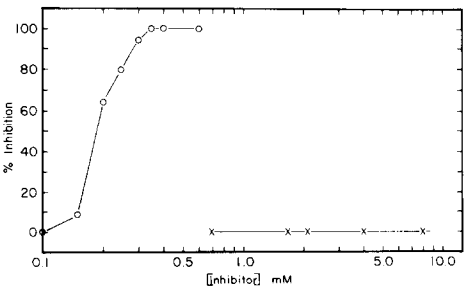

FIG. 8. Inhibition of Con A–*N*-dodecylmaltobionamide precipitation by low molecular weight ligands. Buffer consisted of PBS (Γ/2 0.5, pH 7.2); Con A (50 μg/tube) and *N*-dodecylmaltobionamide (100 μg/tube) inhibited by methyl-α-D-Man*p* (○) or methyl-α-D-Gal*p* (×).

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