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Synthesis of High-Affinity, Hydrophobic Monosaccharide Derivatives and Study of Their Interaction with Concanavalin A, the Pea, the Lentil, and Fava Bean Lectins[1]

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Concanavalin A (Con A) and agglutinins from the pea (PSA), lentil (LCH), and fava bean (VFA) constitute a group of D-mannose/D-glucose binding legume lectins. In addition to their sugar binding specificity, these lectins also contain sites that bind hydrophobic ligands. The present study explores a class of nonpolar binding sites reportedly present adjacent to the carbohydrate binding site in PSA, LCH, and VFA. A series of 2-*O*- and 3-*O*-substituted nitrobenzoyl and nitrobenzyl derivatives of methyl α-D-glucopyranoside and methyl α-D-mannopyranoside were synthesized. Evaluation of their binding to Con A, PSA, LCH, and VFA was carried out by the technique of hapten inhibition of precipitation reaction. The hapten inhibition assay results reveal that the presence of a methyl or methylene group at the O-2 or O-3 position of the sugar is essential for hydrophobic interaction with PSA, LCH, and VFA. The substitution of methyl by nitrobenzyl leads to enhanced binding (1.7–16.7 times for the 2-*O*-substituted compounds and 7.9–40.5 times for the 3-*O*-substituted compounds) with the *m*-nitrobenzyl group contributing to maximum binding. A hydrophobic interaction is also involved between Con A and 2-*O*-nitrobenzyl derivatives, resulting in enhanced binding, but the corresponding 3-*O*-isomers bind poorly due probably to steric reasons. These results may be rationalized on the basis of the recently published X-ray data of Con A and VFA. The nitrobenzyl derivatives, after transformation to their azido analogs, have potential applications in the photoaffinity labeling of these lectins.

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Concanavalin A (Con A)[3] from jack bean (*Canavalia ensiformis*) and agglutinins from the pea (*Pisum sativum*, PSA), the lentil (*Lens culinaris*, LCH), and the fava bean (*Vicia faba*, VFA) constitute a group of D-mannose/D-glucose binding legume lectins with high protein sequence homology and common carbohydrate binding properties. The molecular structure and carbohydrate binding specificity of these lectins have been extensively studied (1). Whereas Con A consists of four identical subunits, the other lectins are composed of two light (α) and two heavy (β) chains. With regard to their carbohydrate binding specificity, all four lectins require the pyranose forms of α-D-mannose/α-D-glucose. The hydroxyl groups most critical for Con A binding are those at positions C-3, C-4, and C-6 and it will tolerate considerable variations at the C-2 position. On the other hand, lectins from the pea, the lentil, and the fava bean require unmodified hydroxyl groups at C-4 and C-6.

The lectins PSA, LCH, and VFA, however, differ significantly from Con A in binding 3-*O*-substituted derivatives of D-mannose/D-glucose. Using the technique of hapten inhibition of hemagglutination, Allen and coworkers showed that the 3-*O*-methyl and 3-*O*-benzyl derivatives of glucose are 2–16 times more inhibitory than glucose for the pea, the lentil, and the fava bean lectins but are very poor or noninhibitors of Con A (2). Studies on the inhibition of the precipitation reaction of the pea and lentil lectins with a phosphomannan led to similar

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[3] Abbreviations used: Con A, the lectin from jack bean (*Canavalia ensiformis*); PSA, the lectin from the pea (*Pisum sativum*); LCH, the lectin from the lentil (*Lens culinaris*); VFA, the lectin from fava bean (*Vicia faba*); me, methyl; man*p*, mannopyranoside; glc*p*, glucopyranoside; PBS, phosphate-buffered saline, pH 7.2; TLC, thin-layer chromatography; ^{1}H NMR, proton magnetic resonance spectroscopy.

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results (2, 3). However, the 3-O-linked disaccharides of glucose, nigerose, and laminaribiose were found to be poor inhibitors of the pea, the lentil, and the fava bean lectins (2, 3). On the basis of these results, Allen and co-workers suggested the presence of a hydrophobic area in the sugar binding site of the lectin that interacts with the methyl group of the 3-*O*-methyl glucose and the methylene (or benzyl) group of 3-*O*-benzyl glucose. The 3-*O*-substituted D-mannose derivatives were not examined.

Sites which bind hydrophobic ligands are a common feature of many plant lectins, notably those present in leguminous plant seeds. Several classes of such sites have been described from non- to highly specific. The objective of the present study was to explore a class of nonpolar binding sites adjacent to the carbohydrate binding sites of PSA, LCH, and VFA. In designing the carbohydrate ligands as probes of this hydrophobic site, we kept in mind their potential application as intermediates in the preparation of photoaffinity labels for mapping the nonpolar binding site in these lectins. Thus the nitrobenzyl derivatives of methyl α-D-man*p* and methyl α-D-glc*p* were chosen because they could then be transformed to their photoactivable azido derivatives for affinity labeling. In this paper we report the synthesis of the monosaccharide derivatives **5**, **6**, and **19–30** and evaluation of their binding to Con A, PSA, LCH, and VFA by the technique of hapten inhibition of precipitation.

MATERIALS AND METHODS

Materials. Methyl α-D-mannopyranoside and methyl α-D-glucopyranoside were obtained from Pfansteil Laboratories, Inc. (Waukegan, IL). Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (1) and methyl 4,6-*O*-benzylidene-α-D-mannopyranoside (2) were prepared by following the literature procedures (4, 5). Methyl 2-*O*-methyl-α-D-mannopyranoside (31) and methyl 3-*O*-methyl-α-D-mannopyranoside (32) were synthesized according to the procedure of Nashed (6). Benzyl bromide, *m*-nitrobenzyl bromide, *p*-nitrobenzyl bromide, *p*-nitrobenzoyl chloride, imidazole, and 2,4,6-tri(pyridyl)-1,3,5-triazine were from Aldrich (Milwaukee, WI). Acetonitrile was distilled from calcium hydride under N_2. Amberlite IR-120 ion-exchange resin was purchased from Mallinckrodt Chemical Co. Silica gel 60 (70–220 mesh) was from EM Science (Gibbstown, NJ). Silica gel (32–63 mesh) for (flash) column chromatography was obtained from ICN Biochemicals (Cleveland, OH). Glass-backed silica gel 60 plates precoated with a 0.25-mm-thick layer of kieselgel 60F-254, for thin-layer chromatography, were obtained from E. Merck (Darmstadt, Germany). Components were visualized by uv quenching (254 nm) or by staining the plate in ceric ammonium molybdate (48.0 g ammonium molybdate and 2.0 g cerium(II)sulfate in 1 liter of 10% aqueous sulfuric acid). Con A was purified by the procedure of Agrawal and Goldstein (7). Lectins from the pea and the lentil were purchased from E. Y. Laboratories (San Mateo, CA). Fava bean lectin was kindly provided by Dr. W. J. Peumans of the Catholic University of Leuven, Belgium. Dextran B-1355 S, *Saccharomyces cerevisiae* mannan, and *Pichia* sp. and *Hansenula holstii* phosphomannans were available from other studies.

*Methyl 2-O-(p-nitrobenzoyl)-4,6-O-benzylidene-α-D-glucopyranoside (**3**) and methyl 3-O-(p-nitrobenzoyl)-4,6-O-benzylidene-α-D-glucopyranoside (**4**).* Imidazole (3.4 g, 50 mmol) was dissolved in chloroform (40 ml) by stirring and the mixture was cooled to ice-bath temperature. To this solution, *p*-nitrobenzoyl chloride (25 mmol in 22 ml chloroform) was added dropwise with stirring over 5 min. The reaction mixture was filtered to remove the solid material. Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (7.1 g, 25.2 mmol) was added to the filtrate, and the mixture was stirred at reflux for 18 h and concentrated under reduced pressure. The residue was chromatographed on silica gel using benzene/ethyl acetate (4/1) as the eluent. The first fraction crystallized from hexane/ethyl acetate to give **3** as needles (3.2 g, 30%), TLC R_f 0.67 (chloroform/methanol, 50/1), mp 196–198°C (Lit. (8) 195–197°C). The second fraction also crystallized from hexane/ethyl acetate to give **4** as needles (2.2 g, 20%), TLC R_f 0.61 (chloroform/methanol, 50/1), mp 233–235°C (Lit. (8) 233–235°C).

*Methyl 2-O-(p-nitrobenzoyl)-α-D-glucopyranoside (**5**).* Compound **3** (215 mg, 0.5 mmol) was suspended in 25 ml of 80% aqueous acetic acid, stirred at reflux for 3 h, and concentrated to dryness under reduced pressure. Column chromatography on silica gel (chloroform/methanol, 20/1) followed by crystallization from hexane/ethyl acetate furnished the title compound **5** as needles, TLC R_f 0.26 (chloroform/methanol, 15/1), mp 166–167°C. ^{1}H NMR (500 MHz, $CDCl_3$, and a drop of CD_3OD): δ 8.31 and 8.27 (ABq, 4H, p-$NO_2C_6H_4$), 5.02 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-1); 4.94 (dd, 1H, $J_{2,1}$ = 3.7 Hz, $J_{2,3}$ = 10 Hz, H-2); 4.10–3.20 (m, 8H, H-3, H-4, H-5, H-6, H-6′, and OCH_3); 2.21 (dd, 1H, J = 4.6 and 9.5 Hz, 6-OH).

*Methyl 3-O-(p-nitrobenzoyl)-α-D-glucopyranoside (**6**).* Compound **4** (215 mg, 0.5 mmol) was treated in the same manner as above to obtain the title product **6** as pale yellow needles, TLC R_f 0.33 (chloroform/methanol, 15/1), mp 94–95°C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.31 and 8.26 (ABq, 4H, p-$NO_2C_6H_4$); 5.36 (t, 1H, J = 9.5 Hz, H-3); 4.86 (d, J = 3.8 Hz, H-1); 3.93–3.74 (m, 5H, H-2, H-4, H-5, H-6, and H-6′); 3.50 (s, 3H, OCH_3); 2.51 (d, 1H, J = 5.3 Hz, 4-OH); 2.14 (d, 1H, J = 11.4 Hz, 2-OH); 1.19 (dd, 1H, J = 5.6 and 7.1 Hz, 6-OH).

*Typical procedure for the synthesis of methyl 2-O-(p-nitrobenzyl)-4,6-O-benzylidene-α-D-glucopyranoside (**9**) and methyl 3-O-(p-nitrobenzyl)-4,6-O-benzylidene-α-D-glucopyranoside (**12**).* The protected sugar (**1**) (400 mg, 1.4 mmol) and potassium carbonate (196 mg, 1.4 mmol, 1.0 eq) were partially dissolved in acetonitrile (5.7 ml) followed by the addition of *p*-nitrobenzyl bromide (307 mg, 1.4 mmol). The reaction mixture was stirred under N_2 overnight at reflux, diluted with ethyl acetate, and washed with 1 M citrate, saturated sodium bicarbonate, and saturated NaCl solution. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give a white solid. This residue was chromatographed on silica gel (eluting with a 2/1 mixture of petroleum ether/ethyl acetate) to give methyl 2-*O*-(*p*-nitrobenzyl)-4,6-*O*-benzylidene-α-D-glucopyranoside (**9**) (59 mg, 10% yield) and methyl 3-*O*-(*p*-nitrobenzyl)-4,6-*O*-benzylidene-α-D-glucopyranoside (**12**) (71 mg, 12% yield) as white solids. TLC (33% ethyl acetate in petroleum ether) R_f 0.33 (**9**), 0.20 (**12**), mp 106–107°C (**9**), 123–125°C (**12**). ^{1}H NMR data are listed in Table II.

*Methyl 2-O-(p-nitrobenzyl)-α-D-glucopyranoside (**21**).* Compound **9** (35 mg, 0.084 mmol) was dissolved in methanol (3 ml) and to this solution cation-exchange resin (IR-120-H^+, 0.3 g) was added. The reaction mixture was stirred overnight at 30°C. The methanolic solution was filtered through Whatman filter paper and the filtrate was concentrated under reduced pressure to a syrup. Column chromatography of this syrup on silica gel followed by lyophilization afforded the title compound (**21**) as a pale yellow powder (>80%). TLC (chloroform/methanol, 9/1) R_f 0.31, mp 115–116°C. ^{1}H NMR data are given in Table III.

*Methyl 3-O-(p-nitrobenzyl)-α-D-glucopyranoside (**24**).* Compound **12** (30 mg, 0.072 mmol) was treated in the same manner as above to obtain **24** as a pale yellow solid (90%). TLC (chloroform/methanol, 9/1) R_f 0.39, mp 109–111°C. ^{1}H NMR data are given in Table III.

Methods. Melting points were determined on a MELT-TEMP melting apparatus (Laboratory Devices, Holliston, MA) and are uncorrected. All ^{1}H NMR spectra were recorded on a Bruker AM 300 or AM 360 MHz spectrometer. Spectra were measured at ambient temperature in $CDCl_3$ or CD_3OD using tetramethylsilane as the internal standard.

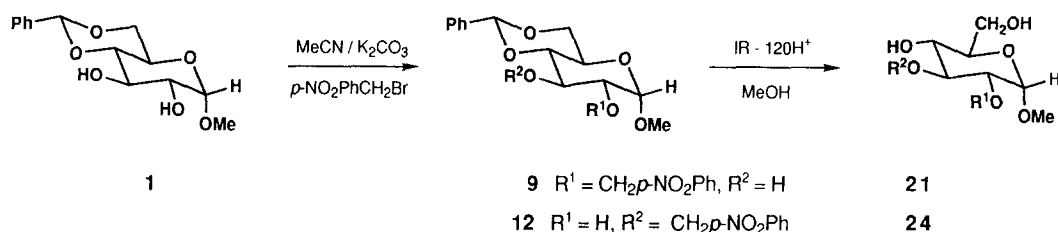

FIG. 1. Typical reaction scheme.

Mass spectra were recorded on a VG Analytical (70-S) mass spectrometer using ammonia as a reagent in the chemical ionization mode and *p*-nitrobenzyl alcohol as a matrix in the fast atom bombardment mode.

Quantitative precipitation and precipitation inhibition assay. A microprecipitation technique was employed for quantitative precipitation assay (9). Stock solutions of Con A (1 mg/ml), the lentil lectin (5 mg/ml), fava bean lectin (5 mg/ml), dextran B-1355 S (2 mg/ml), *S. cerevisiae* mannan (1 mg/ml), *Pichia* sp. phosphomannan (5 mg/ml), and *H. holstii* phosphomannan (5 mg/ml) were prepared in phosphate-buffered saline (PBS). In the case of the pea lectin, a stock solution (2 mg/ml) was prepared in PBS containing 0.1 mM $MnCl_2$. The precipitation reaction was conducted by adding Con A (15 μg/15 μl), pea lectin (60 μg/30 μl), lentil lectin (100 μg/20 μl), or fava bean lectin (50 μg/10 μl) to plastic vials (1.5 ml). Varying aliquots of polysaccharide solution were added and the mixtures were adjusted to a final volume of 100 μl using PBS containing 1 M NaCl solution. In the case of the pea lectin, a PBS solution containing 1 M NaCl and 0.1 mM $MnCl_2$ was used. The reaction mixtures were kept at room temperature for 2 days and centrifuged (11,500 rpm, 10 min) and the supernatant solutions removed. The precipitates were washed three times with 150 μl of PBS containing 1 M NaCl. The protein content of the precipitate was determined by Lowry's method (10) using bovine albumin as the standard.

Stock solutions of the hapten standards (methyl α-D-man*p* and methyl α-D-glc*p*) and monosaccharide derivatives **19–30** were prepared in double-distilled water. Hapten inhibition of the precipitation of lectin–polysaccharide complex was carried out by adding varying aliquots (0.5–50 μl) of the standard or the monosaccharide derivative (1–100 mM) to the plastic vial and adding an aliquot of lectin solution (same amount as used for the quantitative precipitation) and polysaccharide solution (15 μg/7.5 μl of dextran 1355 for Con A, 20 μg/20 μl of *S. cerevisiae* mannan for the pea lectin, 80 μg/16 μl of *Pichia* sp. or *H. holstii* phosphomannan for the lentil lectin, and 10 μg/5 μl of *S. cerevisiae* mannan for fava bean lectin). Incubation of the mixtures, processing, and protein determination of the precipitates were carried out as above for quantitative precipitation experiments.

TABLE I

Reaction Yield and Physical Properties of Compounds **7–30**

Compound number	Reaction yield[a] (%)	TLC R_f	mp (°C)	Compound number	TLC[b] R_f	mp (°C)
7	40	0.42[c]	110–112	**19**	0.34	119–120
8	12	0.27	102–103	**20**	0.32	Syrup
9	10	0.33	106–107	**21**	0.31	115–116
10	4	0.26[c]	118–120	**22**	0.42	90–91
11	12	0.17	149–150	**23**	0.40	Syrup
12	12	0.20	123–125	**24**	0.39	109–111
13	16	0.72	89–90	**25**	0.42	99–100
14	19	0.50	Syrup	**26**	0.40	95–96
15	23	0.50	Syrup	**27**	0.39	114–116
16	10	0.45	98–100	**28**	0.41	Syrup
17	20	0.29	Syrup	**29**	0.39	Syrup
18	19	0.24	Syrup	**30**	0.40	131–132

[a] Yield of isolated pure product.
[b] Chloroform/methanol (9/1).
[c] Petrolium ether/ethyl acetate (4/1) and petrolium ether/ethyl acetate (2/1) for **8**, **9**, and **11–18**.

RESULTS

Reaction of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**) with *N*-benzoyl imidazole in chloroform gave a mixture of methyl 2-*O*-(*p*-nitrobenzoyl)-4,6-*O*-benzylidene-α-D-glucopyranoside (**3**) and methyl 3-*O*-(*p*-nitrobenzoyl)-4,6-*O*-benzylidene-α-D-glucopyranoside (**4**) in 30 and 20% yields, respectively. The mp and ^{1}NMR data of **3** and **4** agree well with those reported by Ishido and coworkers (8). On stirring a suspension of **3** and **4** in 80% aqueous acetic acid at reflux for 3 h, complete debenzylidenation was effected. The crystalline products **5** and **6** showed a characteristic methine proton signal at δ 4.92 (dd, $J_{2,1}$ = 3.7 Hz and $J_{2,3}$ = 10 Hz, H-2) and at δ 5.36 (t, J = 9.5 Hz, H-3), respectively, confirming their structural assignment.

Monoalkylation of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**1**) or methyl 4,6-*O*-benzylidene-α-D-mannopyranoside (**2**) was carried out in dry acetonitrile at reflux with one equivalent of K_2CO_3 using benzyl bromide, *m*-nitrobenzyl bromide, or *p*-nitrobenzyl bromide as the alkylating agent (Fig. 1). Each of these reactions gave a mixture of monoalkylated products in moderate yields that were purified by flash chromatography on a silica gel column. The reaction yields, and mp data of the monoalkylated products **7–18** are reported in Table I and their ^{1}H NMR data are listed in Table II. Compound **8** was also analyzed by fast atom bombardment mass spectrometry in the positive ion mode and it showed the expected molecular ion peak at m/z 418 $[M + H^+]^+$.

Debenzylidenation was readily effected by stirring overnight a methanolic solution of each of the compounds **7–18** in the presence of IR-120 (H^+ form) cation-exchange resin. The deprotection in each case was nearly quantitative and the resultant product was purified on silica gel by column chromatography. Table I lists the physical properties of the products **19–30** and their ^{1}H NMR data are listed in Table III. Additional proof for

TABLE II

^{1}H NMR Data for **7–18**[a]

Compound number	H-1 $J_{1,2}$	H-2 $J_{2,3}$	H-3 $J_{3,4}$	H-4 $J_{4,5}$	H-5 $J_{5,6a}$	H-6a $J_{6,6a}$	H-6e $J_{6e,5}$	OH	O Me	ArCH$_2$O		H-2″ $J_{2'',3''}$	H-3″	H-4″ $J_{4'',5''}$	H-5″	H-6″ $J_{6'',5''}$	PhC<u>H</u>O$_2$	<u>Ph</u>CHO$_2$
7[b]	4.61	3.47	4.15	3.50	3.82	3.70	4.26	2.56	3.38	4.79	4.70			7.51–7.30			5.52	7.51–7.30
	3.6	9.3	9.3	9.6	10.0	10.1	4.7											
8	4.76				4.35–3.40				3.41	4.89	4.79	8.25	—	8.34	7.58–	7.70	5.52	7.58–7.30
	3.8													8.6	7.30	8.6		
9	4.78	3.52	4.20	3.48	3.83	3.71	4.28		3.42	4.91	4.83	7.56	8.21	—	8.21	7.56	5.52	7.50–7.33
	3.6	9.3	9.1	9.4	10.1	10.0	4.5					8.8	8.8		8.8	8.8		
10[b]	4.82				4.35–3.60			2.31	3.45	4.97	4.79			7.50–7.25			5.57	7.50–7.25
	3.8							7.3										
11	4.81				4.28–3.55				3.44	4.98	4.90	8.21	—	8.07	7.48–	7.67	5.54	7.48–7.30
	3.8													8.6	7.30	8.6		
12	4.81				4.29–3.55				3.45	4.99	4.92	7.51	8.11	—	8.11	7.51	5.52	7.45–7.30
	3.3											8.7	8.7		8.7	8.7		
13	4.75				4.30–3.70			2.48	3.36	4.75	4.70			7.53–7.30			5.57	7.53–7.30
	1.6							8.0										
14	4.78				4.30–3.70			2.58	3.37	4.86	4.79	8.26	—	8.14	7.52	7.72	5.58	7.55–7.30
	1.6							5.0						8.8	8.8	8.8		
15	4.78				4.30–3.70			2.43	3.38	4.90	4.82	7.55	8.22	—	8.22	7.55	5.59	7.52–7.30
	1.4							5.8				8.8	8.8		8.8	8.8		
16	4.74				4.35–3.70			2.76	3.37	4.84	4.70			7.55–7.20			5.62	7.55–7.20
17	4.77				4.32–3.73			2.66	3.37	4.89	4.82	8.18	—	8.10	7.50	7.66	5.58	7.50–7.30
	1.6													8.8	7.30	8.8		
18	4.77				4.30–3.70			2.55	3.39	4.90	4.83	7.48	8.14	—	8.14	7.48	5.58	7.48–7.30
												8.8	8.8		8.8	8.8		

[a] All spectra were measured at 300 MHz on $CDCl_3$ solutions unless otherwise mentioned.
[b] Measured at 360 MHz.

the structure was also obtained from mass spectrometry. The chemical ionization mass spectrum of **24**, methyl 3-*O*-(*p*-nitrobenzyl)-α-D-glucopyranoside showed the expected molecular ion peak at m/z 347 $[M + NH_4^+]^+$. Periodate oxidation of **20**, methyl 2-*O*-(*m*-nitrobenzyl)-α-D-glucopyranoside, and **23**, methyl 3-*O*-(*m*-nitrobenzyl)-α-D-glucopyranoside, was carried out according to the procedure of Avigad (11). Compound **20** consumed one equivalent of sodium periodate, whereas **23** did not react, thus providing unequivocal evidence that **20** and **23** are 2-*O*- and 3-*O*-isomers, respectively. Finally, methyl 2-*O*-methyl-α-D-mannopyranoside (**31**) and methyl 3-*O*-methyl-α-D-mannopyranoside (**32**) were prepared according to the procedure of Nashed (6).

Aqueous solutions of **5, 6,** and **19–32** (7.5–40 mM) for hapten inhibition study were prepared by gentle warming and or vortexing. Quantitative precipitation of Con A by dextran B-1355-S, PSA by *S. cerevisiae* mannan, LCH by *Pichia* sp. or *H. holstii* phosphomannan, and VFA by *S. cerevisiae* mannan was carried out by adding increasing amounts of the polysaccharide to a fixed concentration of the lectin in suitable buffer solution (see Materials and Methods). Each of the compounds **5, 6,** and **19–32** was assayed for its ability to inhibit the lectin precipitation reaction. Figures 2 and 3 show the typical inhibition curves obtained for LCH:phosphomannan system using **19–30** as haptens. The 50% inhibition concentration (IC_{50}) values obtained from these curves for **19–30** were normalized with respect to that of the standard monosaccharide haptens, methyl α-D-glc*p* and methyl α-D-man*p* to give relative inhibitory potencies (see Tables IV and V). For comparison, the relative inhibitory potencies of 2-*O*- and 3-*O*-methyl derivatives of methyl α-D-man*p* and 2-*O*- and 3-*O*-*p*-nitrobenzoyl derivatives of methyl α-D-glc*p* are also listed in Tables IV and V.

The *p*-nitrobenzoyl derivatives **5** and **6** were poor inhibitors for both Con A and PSA. The 2-*O*- and 3-*O*-methyl derivatives of methyl α-D-man*p*, **31** and **32,** were moderately more potent (2.4 and 6 times, respectively) than methyl α-D-man*p* for inhibiting the precipitation of PSA by *S. cerevisiae* mannan. All of the 2-*O*-benzyl and nitrobenzyl derivatives **19–21** and **25–27** were 1.3 to 16.7 times more inhibitory than the standards for all four lectins. The corresponding 3-*O*-isomers **22–24** and **28–30** were much more potent (2.8 to 40.5 times) than the standards for PSA, LCH, and VFA but were essentially noninhibitors of Con A.

DISCUSSION

Many legume lectins are known to possess at least three classes of nonpolar binding sites in addition to their car-

TABLE III

^{1}H NMR Data for **19–30**[a]

Compound number	H-1 $J_{1,2}$	H-2 $J_{2,3}$	H-3 $J_{3,4}$	H-4 $J_{4,5}$	H-5 $J_{5,6}$	H-6 $J_{6,6'}$	H-6' $J_{6',5}$	OH	OH	OH-6	OMe	ArCH2O		H-2'' $J_{2'',3''}$	H-3''	H-4'' $J_{4'',5''}$	H-5''	H-6'' $J_{6'',5''}$
19	4.62			3.94–3.32				2.81	2.72	2.03	3.35	4.70	4.60			7.38–7.29		
	3.4																	
20[b]	4.76			4.00–3.36							3.40	4.89	4.79	8.30	—	8.16	7.55	7.75
	3.6															8.2		7.7
21[b]	4.77			4.00–3.30							3.39	4.86	4.80	7.56	8.21	—	8.21	7.56
	3.5													8.7				8.7
22	4.76			3.87–3.50				2.35	2.16	1.96	3.44	5.03	4.73			7.38–7.28		
	3.8								9.0			11.5						
23[c]	4.67			3.85–3.40							3.42	4.97		8.34	—	8.11	7.56	7.81
	3.8															8.3		8.3
24	4.77			3.90–3.30				2.46	2.15	1.93	3.45	5.14	4.90	7.55	8.21	—	8.21	7.55
	3.8								9.4					8.1				8.1
25	4.79			0–3.37				2.47	2.24	2.07	3.36	4.74	4.55			7.40–7.26		
	0.8								9.8									
26[b]	4.78			3.95–3.50						2.17	3.37	4.84	4.79	8.27	—	8.16	7.54	7.69
	1.5															8.0		8.0
27[b]	4.79			3.90–3.40							3.38	4.81		7.53	8.22	—	8.22	7.53
	1.6													8.9				8.9
28[d]	4.75	3.94	3.67	3.98	3.54	3.83	3.76				3.34	4.69	4.57			7.40–7.30		
	1.7	3.2	9.4	9.8	3.4	12.1	2.9											
29[d]	4.76	4.01	3.70	4.08	3.55	2.89	3.78			3.13	3.34	4.82	4.71	8.29	—	8.15	7.52	7.69
	1.7	3.2	9.4	9.8	3.0	12.2	2.6									7.9		7.9
30[b,d]	4.75	4.03	3.69	4.02	3.56	3.89	3.82				3.37	4.86	4.81	7.58	8.22	—	8.22	7.58
	1.6	3.3	9.4	9.7	3.5	12.2	2.8							8.8				8.8

[a] All spectra were measured at 360 MHz on $CDCl_3$ solutions unless otherwise mentioned.
[b] Measured on $CDCl_3$ solution containing traces of CD_3OD.
[c] Measured at 300 MHz on CD_3OD solution.
[d] Spectral data of H-1 to H-6' measured on D_2O exchanged sample.

bohydrate and metal binding sites. Several legume lectins including lima bean lectin contain a specific, high-affinity binding site for adenine and its N-6 derivatives with association constants 10^5–10^6 M^{-1} (12). A low-affinity site that interacts with ligands such as tryptophan and indoleacetic acid (K_a 10^3–10^4 M^{-1}) is present in each subunit of Con A and many other lectins (13). The third class involves a hydrophobic site present adjacent to the carbohydrate binding site. In Con A, this site interacts with the phenyl groups of phenyl β-glucosides and mannosides

TABLE IV

Hapten Inhibition by Methyl α-D-Glucopyranoside and Derivatives

Compound number	Hapten (Me α-D-glucoside series)	Relative potency: Con A:dextran B-1355 S	PSA:mannan *S. cerevisiae*	LCH:phosphomannan *P. species*	VFA:mannan *S. cerevisiae*
	Me α-D-glucopyranoside	1.0	1.0	1.0	1.0
19	Me 2-*O*-benzyl-α-D-glc*p*	6.4	1.3	7.0	8.5
20	Me 2-*O*-(*m*-nitrobenzyl)-α-D-glc*p*	7.4	2.3	11.4	8.1
21	Me 2-*O*-(*p*-nitrobenzyl)-α-D-glc*p*	1.7	2.0	2.6	3.4
5	Me 2-*O*-(*p*-nitrobenzoyl)-α-D-glc*p*	<0.1	<0.07	—	—
22	Me 3-*O*-benzyl-α-D-glc*p*	<0.2	2.8	8.1	11.5
23	Me 3-*O*-(*m*-nitrobenzyl)-α-D-glc*p*	0.3	7.9	24.6	37.2
24	Me 3-*O*-(*p*-nitrobenzyl)-α-D-glc*p*	<0.3	20.0	16.4	26.8
6	Me 3-*O*-(*p*-nitrobenzoyl)-α-D-glc*p*	<0.1	<0.1	—	—

TABLE V

Hapten Inhibition by Methyl α-D-Mannopyranoside and Derivatives

Compound number	Hapten (Me α-D-mannoside series)	Relative potency			
		Con A:dextran B-1355 S	PSA:mannan *S. cerevisiae*	LCH:phosphomannan *H. holstii*	VFA:mannan *S. cerevisiae*
	Me α-D-mannopyranoside	1.0	1.0	1.0	1.0
25	Me 2-*O*-benzyl-α-D-man*p*	4.0	2.8	8.8	3.9
26	Me 2-*O*-(*m*-nitrobenzyl)-α-D-man*p*	5.9	5.0	16.7	5.4
27	Me 2-*O*-(*p*-nitrobenzyl)-α-D-man*p*	3.7	2.7	10.7	4.9
31	Me 2-*O*-methyl-α-D-man*p*		2.4	—	—
28	Me 3-*O*-benzyl-α-D-man*p*	0.1	14.7	34.9	16.2
29	Me 3-*O*-(*m*-nitrobenzyl)-α-D-man*p*	<0.1	13.2	40.5	17.2
30	Me 3-*O*-(*p*-nitrobenzyl)-α-D-man*p*	<0.1	13.6	32.0	15.2
32	Me 3-*O*-methyl-α-D-man*p*	—	6.0	—	—

(14). On the other hand, the methyl groups of 3-*O*-methyl-D-glucose and the methylene group of 3-*O*-benzyl-D-glucose have been reported to interact at a similar site in PSA, LCH, and VFA but not with Con A (2). Until now, D-mannose derivatives carrying hydrophobic substituents at C-3 position have not been examined.

In the present investigation we sought to obtain a better understanding of the site present in the legume lectins which interacts with hydrophobic ligands. A series of derivatives of methyl α-D-glc*p* and methyl α-D-man*p* containing 2-*O*- and 3-*O*-benzyl, *m*-nitrobenzyl, and *p*-nitrobenzyl substituents were synthesized according to the scheme shown in Fig. 1. The 2-*O*- and 3-*O*-methyl derivatives of methyl α-D-man*p* and 2-*O*- and 3-*O*-*p*-nitrobenzoyl derivatives (see Materials and Methods) of methyl α-D-glc*p* were also prepared as probes for elucidating the structural requirements for the hydrophobic ligands. Nitro substituents in the phenyl ring were chosen so that these compounds could serve as precursors for the preparation of azido derivatives for affinity labeling of these hydrophobic sites. The yield of the isolated intermediates **7–18** was moderate. ^{1}H NMR spectra of each of the intermediates **7–18** showed characteristic signals arising from aromatic ring protons, the methine proton of the benzylidene group (5.52–5.62 ppm), the anomeric proton (4.61–4.82), and the glycosidic methyl protons (3.36–3.45). Sugar ring protons of 2-*O*-substituted compounds **7** and **9** exhibited well-resolved resonances and hence were amenable to complete spectral assignment, whereas the remainder of the derivatives **10–18** showed overlapping signals in the sugar ring proton region (see Table II). ^{1}H NMR spectrum of **9** provided convincing spectral evidence for its identity as methyl 2-*O*-(*p*-nitrobenzyl)-4,6-*O*-benzylidene-α-D-glucopyranoside. Three resonances appearing at δ 4.78 (d, 1H, $J_{1,2}$ = 3.6 Hz), 4.20 (dt, 1H, $J_{3,4}$ = 9.1 Hz, $J_{3,2}$ = 9.3 Hz, $J_{3,OH}$ = 1.8 Hz), and 3.52 ppm (dd, 1H, $J_{2,1}$ = 3.6 Hz, $J_{2,3}$ = 9.3 Hz) were tentatively assigned to H-1, H-3, and H-2, respectively. On D_2O exchange, the signal at 4.20 ppm collapsed to a triplet and subsequent irradiation of the resonance at 4.78 ppm resulted in the simplification of the signal at 3.52 ppm into a doublet ($J_{2,3}$ = 9.3 Hz), thus confirming the structural assignment. In contrast to compounds **7** and **9**, the debenzylidenated 3-*O*-substituted derivatives **28–30** showed well-resolved sugar ring proton resonances. The identity of compounds **19–21**, **25–27** and **22–24**, **28–30** as the 2-*O*- and 3-*O*-isomers, respectively, was also established unambiguously by periodate oxidation (11).

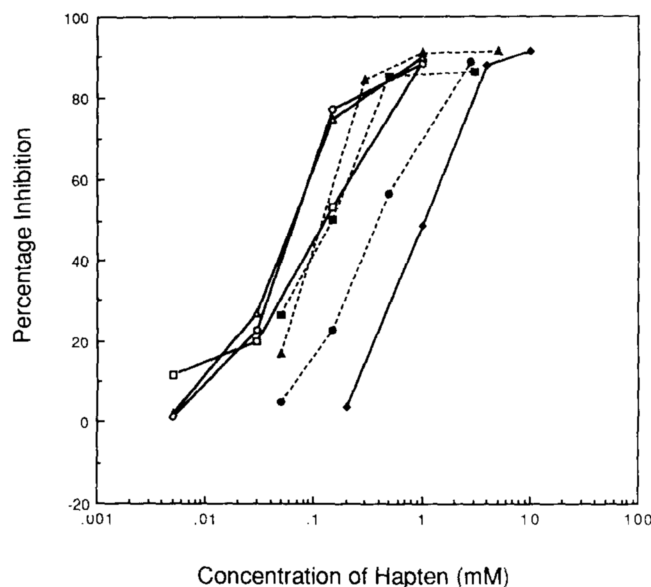

FIG. 2. Inhibition of lentil lectin (LCH)-*Pichia* sp. phosphomannan precipitation by haptens: (♦) Me α-D-glc*p*; (■) **19**; (▲) **20**; (●) **21**; (□) **22**; (△) **23**; and (○) **24**. Precipitation reaction vial contained 100 μg of lentil lectin and 80 μg of *Pichia* sp. phosphomannan. For details, see Materials and Methods.

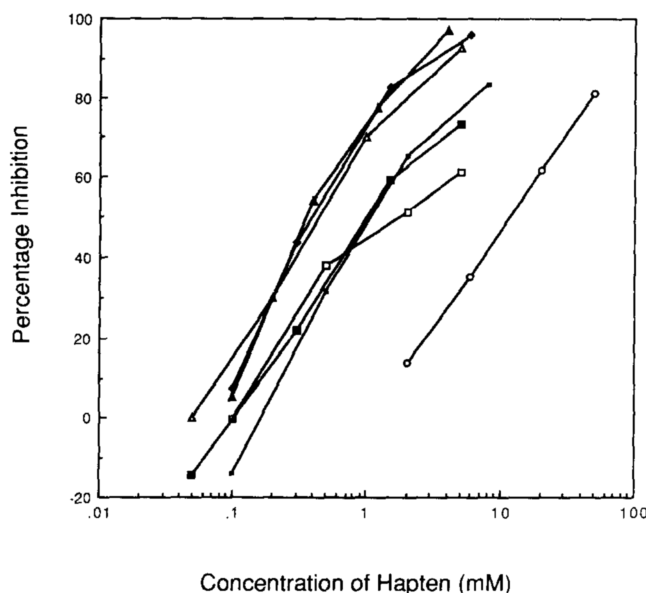

FIG. 3. Inhibition of lentil lectin (LCH)-*Hansenula holstii* phosphomannan precipitation by haptens: (○) Me α-D-man*p*; (□) **25**; (■) **26**; (■) **27**; (△) **28**; (▲) **29**; (♦) **30**. Precipitation reaction vial contained 100 μg of lentil lectin and 100 μg of *Hansenula holstii* phosphomannan. For details, see Materials and Methods.

Using a hapten inhibition of precipitation assay, we generated a complete set of inhibition curves and derived IC_{50} values for each derivative **5** and **6, 19–30,** and **31** and **32** against each of the lectins, Con A, PSA, LCH, and VFA. An example of such a study is shown in Figs. 2 and 3, which portray the effect of various 2-*O*- and 3-*O*-substituted derivatives **19–30** in the LCH:phosphomannan precipitation system. The most dramatic effect on this system is shown by substitution at the *O*-3 position of both methyl α-D-glc*p* and methyl α-D-man*p*. Methyl 3-*O*-(*m*-nitrobenzyl)-α-D-man*p* was found to be 40 times more potent than the parent compound, methyl α-D-man*p*!

The hapten inhibition assay results summarized in Table IV and Table V indicate the following: (i) the presence of a methyl or methylene group at the O-2 or O-3 position of methyl α-D-glc*p* and methyl α-D-man*p* is essential for hydrophobic interaction with PSA, LCH, and VFA; (ii) the substitution of methyl by benzyl leads to enhanced binding with the *m*-nitrobenzyl group contributing to maximum binding; and (iii) a ligand–protein hydrophobic interaction is also involved between Con A and 2-*O*-methyl, 2-*O*-benzyl, and 2-*O*-nitrobenzyl derivatives, resulting in greater binding, but the corresponding 3-*O*-isomers bind poorly due probably to steric hindrance.

These results may be rationalized on the basis of the recently published high-resolution X-ray data of Con A (15) and VFA (16). As shown by Reeke and Becker (16), the replacement of two large side chains, Leu-99 and Arg-228, found in Con A with smaller groups, Ala-212 and Gly-100, in VFA results in a region of significant structural difference between these two lectins. Thus the considerably open carbohydrate binding site in VFA allows the binding of α-D-glc*p*/α-D-man*p* derivatives with bulky substituents at the O-2 and O-3 positions unlike the situation with Con A. The two hydrophobic side chains Tyr-101 and Trp-129 in VFA could interact by Van der Waal contacts with the benzyl groups of **19–30,** leading to enhanced binding. Experiments are under way to prepare photoaffinity-labeled as well as spin-labeled derivatives based on the results of the present study in order to map the carbohydrate binding site in these legume lectins.

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