Protein–Carbohydrate Interaction. XX. The Interaction of Concanavalin A with Sophorose and Some of Its Derivatives*

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ABSTRACT: The interaction of concanavalin A, the phytohemagglutinin of the jack bean, with sophorose and several of its derivatives was studied by examining the extent to which these saccharides inhibited dextran–concanavalin A interaction. It is shown that the protein combining sites interact with the C-3, C-4, and C-6 hydroxyl groups of the *reducing* D-glucopyranose unit of sophorose in distinction to combination with similar hydroxyl groups of the *nonreducing* D-glucopyranosyl residue of α-linked glucose disaccharides.

The data which establish this point include the observations that: (1) 2-*O*-β-D-galactopyranosyl-D-glucose falls on the same line of inhibition as sophorose; D-galactose and its α- and β-glycosides are noninhibitors of dextran–concanavalin A interaction. (2) Sophoritol is a noninhibitor. (3) In analogy with the glycosides of D-glucose, α-methyl sophoroside is a better and β-methyl sophoroside a poorer inhibitor than sophorose. These modifications involve only the *reducing* moiety of sophorose. (4) Concanavalin A interacts to form a precipitate with bovine serum albumin containing multiple *p*-phenylazo-β-sophorosyl residues.

The phytohemagglutinin of the jack bean, concanavalin A (Sumner and Howell, 1935), has been shown to form a precipitate with a group of structurally related, branched polysaccharides which include glycogens, dextrans, yeast mannans, amylopectins, and certain levans (Sumner and Howell, 1936; Cifonelli *et al.*, 1956; Manners and Wright, 1962; Goldstein *et al.*, 1965a; Goldstein and So, 1965).

The stereochemical requirements of the combining sites of concanavalin A have been investigated in some detail by examining the extent to which a wide variety of low molecular weight carbohydrates inhibited concanavalin A–polysaccharide interaction (Goldstein *et al.*, 1965b; Smith and Goldstein, 1967; So and Goldstein, 1967b). On the basis of these studies it was suggested that unmodified hydroxyl groups at C-3, C-4, and C-6 of the α-D-glucopyranosyl or α-D-mannopyranosyl ring were necessary for binding to the protein. These particular hydroxyl groups appear to represent the minimum configurational features necessary for reaction with concanavalin A. We have postulated further that concanavalin A interacts with the terminal, nonreducing sugar residues of polysaccharide chain ends (Goldstein *et al.*, 1965a,b; Goldstein and Iyer, 1966).

It has been suggested by Hehre (1960, 1964) and Suzuki and Hehre (1964) that certain dextrans, classified as serotype A dextrans on the basis of their reactivity with type XII pneumococcus antiserum and characterized by a high content of α-D-(1→2)-glucosidic linkages, owe their substantial reactivity with concanavalin A to kojibiose residues (2-*O*-α-D-glucopyranosyl-D-glucose) situated within their molecular structure. The isolation of considerable quantities of kojibiose (Suzuki and Hehre, 1964; Torii *et al.*, 1963) from serotype A dextrans together with a characteristic shift in specific optical rotation which these dextrans display in cuprammonium solution (Scott *et al.*, 1957) appeared to support this view. Hehre (1964) has pointed out that D-glucopyranosyl residues linked α-(1→2) would still retain the configurational features necessary for interaction with concanavalin A, namely, unmodified hydroxyl groups at C-3, C-4, and C-6 of α-D-glucopyranosyl residues.

Recently published studies (Cifonelli *et al.*, 1966) on the chemical structure of S XII pneumococcal polysaccharide, which also forms a precipitate with concanavalin A, suggest the presence of α-D-(1→2)-glucosidic linkages, a possibility also commented on by others (Suzuki and Hehre, 1964; Goodman and Kabat, 1960).

In order to test this hypothesis, we have prepared a series of oligosaccharides containing α- and β-D-(1→2)-glucosidic linkages. This paper describes the extent to which sophorose (2-*O*-β-D-glucopyranosyl-D-glucose) and its derivatives interact with concanavalin A. A later communication will deal with the corresponding α isomer, kojibiose, and some of its derivatives.

* From the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan. *Received March 17, 1967.* Part of this work was done at the Department of Biochemistry, School of Medicine, State University of New York at Buffalo. This research was supported by National Institutes of Health Research Grants AI-05542, GM-09364, and AM-10171.

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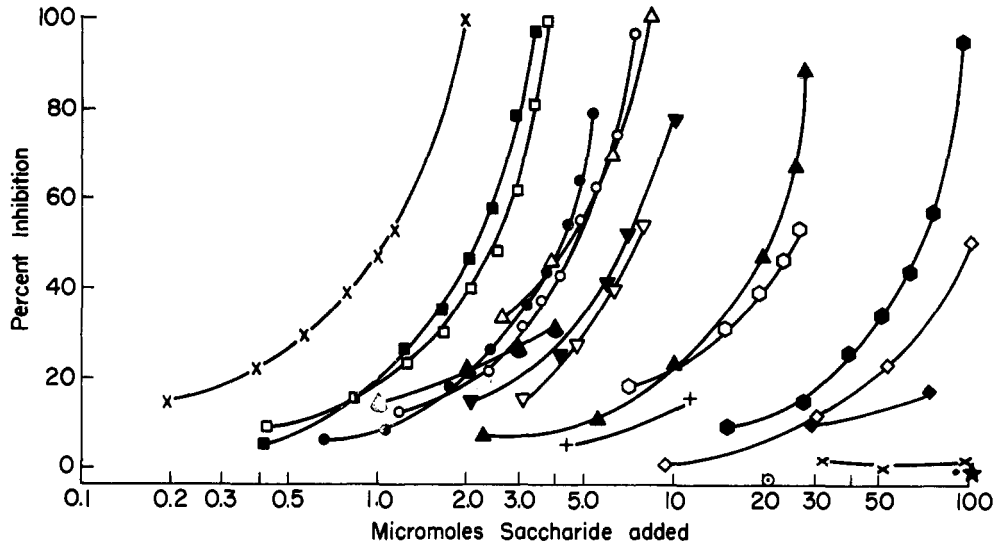

FIGURE 1: Inhibition by saccharides of dextran–concanavalin A precipitation. Each tube contained concanavalin A (300 μg), dextran NRRL B-1355-S (200 μg), and inhibitor as noted in a total volume of 1.0 ml. (×) α-Methyl sophoroside, (■) isomaltose, (□) methyl α-D-glucopyranoside, (●) maltose, (○) maltitol, (△) α-methyl sophoroside 2′,3′,4′,6′-tetraacetate, (▲) *p*-nitrophenyl β-sophoroside, (▼) sophorose, (▽) 2-*O*-β-D-galactopyranosyl-D-glucose, (▲) D-glucose, (○) β-methyl sophoroside, (+) sophorotriose, (◆) methyl β-D-glucopyranoside, (◇), gentiobiose, (◆) β-methyl sophoroside-2′,3′,4′,6-tetraacetate, (⊙) sophoritol, (X) cellobiose, (·) cellobiitol, and (★) laminaribiose.

Materials and Methods

Quantitative hapten inhibition analyses were conducted by the procedure developed in this laboratory and described in detail elsewhere (So and Goldstein, 1967a). Briefly it consisted of adding to a series of 3-ml centrifuge tubes (calibrated at 1.0, 1.5, and 2.0 ml, product of Bellco Glass, Inc., Vineland, N. J.) concanavalin A (300 μg) prepared according to the procedure of Agrawal and Goldstein (1965), dextran NRRL B-1355-S (200 μg, a gift of Dr. Allene Jeanes), and varying quantities of carbohydrate inhibitor. The volume was adjusted to 1.0 ml with buffered saline so that the final reaction mixture was 1 M in NaCl and 0.018 M with respect to phosphate buffer (pH 7.2). A control contained all components except inhibitor. After 24 hr at room temperature the precipitate was centrifuged, washed, and digested with 7 N H_2SO_4. Hydrogen peroxide (30%) was added to complete oxidation of carbohydrate and the $(NH_4)_2SO_4$ formed was determined by a micro ninhydrin procedure. Percentage inhibition was calculated from the expression $((A - B)/A) \times 100$, where A and B represent the quantity of nitrogen determined in the absence and presence of inhibitor, respectively.

Inhibitors. All carbohydrates employed as inhibitors were chromatographically homogeneous. Maltose was purified by conversion to its octaacetate followed by deacetylation. Maltitol was prepared from maltose by reduction with $NaBH_4$ followed by purification on paper. Sophorose was a gift of Dr. Hewitt G. Fletcher who also provided us with a generous sample of methyl 4,6-*O*-benzylidene-2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucosyl)-α-D-glucopyranoside. Sophoritol was prepared from sophorose by reduction with sodium borohydride followed by purification on paper. Gentiobiose was a gift of Dr. Milton Fisher. Sophorotriose was a gift of Dr. P. A. J. Goren. *p*-Nitrophenyl β-sophoroside was prepared by a procedure to be described elsewhere (R. N. Iyer and I. J. Goldstein, manuscript in preparation). It had mp 261–262° and $[\alpha]_D^{20}$ − 68° (*c* 1.0, water).

D-Glucose, cellobiose, and methyl α- and β-glucopyranoside were obtained commercially. Cellobiitol was a gift from Dr. Peter Allen and 2-*O*-β-D-galactopyranosyl-D-glucose was kindly provided by Professor K. Wallenfels.

Methyl 2′,3′,4′,6′-tetra-*O*-acetyl-α-sophoroside was prepared from methyl 4,6-*O*-benzylidene-2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucosyl)-α-D-glucopyranoside (0.5093 g) by refluxing in 50% aqueous acetic acid (v/v) for 10 min. Concentration on a rotatory evaporator *in vacuo* gave crystals (0.266 g) which upon recrystallization from ethanol had mp 164–165°, $[\alpha]_D^{21}$ +46.5 (*c* 1.12, water). *Anal.* Calcd for $C_{21}H_{32}O_{15}$: C, 48.2; H, 6.16. Found: C, 47.97; H, 5.97.

α-Methyl sophoroside (IV) was prepared from the above tetraacetate (151 mg) by dissolving in absolute methanol (5 ml) and adding a small piece of metallic sodium (*ca.* 5 mg). The reaction mixture was held at 50° for 30 min and allowed to stand overnight at 20° during which time crystallization ensued. The crystals were filtered (yield, 85 mg) and recrystallized from 80% ethanol (v/v), mp 260–262°, $[\alpha]_D^{21}$ +64° (*c* 1.06, water).

Anal. Calcd for $C_{13}H_{24}O_{11} \cdot 0.5H_2O$: C, 42.6; H, 6.9,

Found: C, 42.39; H, 7.00.

Methyl 4,6-*O*-benzylidene-2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucosyl)-β-D-glucopyranoside was synthesized by the procedure employed for the α anomer (Coxon and Fletcher, 1961). The product was crystallized from ethanol. Recrystallization from ethanol gave the pure substance, mp 193°, $[\alpha]_D^{20}$ −30° (*c* 1.0, chloroform).

Anal. Calcd for $C_{28}H_{36}O_{15}$: C, 54.89; H, 5.92. Found: C, 55.03; H, 5.99.

Methyl 2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucosyl)-β-D-glucopyranoside was prepared in an analogous fashion to the α isomer reported above. The benzylidene compound (504 mg) was dissolved in 50% acetic acid (25 ml) and refluxed for 10 min. The reaction mixture was concentrated *in vacuo* on a rotatory evaporator giving a crystalline mass. Recrystallization from absolute ethanol gave the substance (295 mg) as needles, mp 156°, $[\alpha]_D^{21}$ −18.5° (*c* 1.05, water).

Anal. Calcd for $C_{21}H_{32}O_{15}$: C, 48.2; H, 6.16. Found: C, 47.98; H, 5.99.

Methyl 2-*O*-(β-D-glucosyl)-β-D-glucopyranoside (β-methyl sophoroside) (V) was prepared by dissolving the above substance in methanol (25 ml) and adding a catalytic amount of sodium (*ca.* 5 mg). The reaction mixture was heated at 50° for 30 min and concentrated *in vacuo* on a rotatory evaporator to a syrup. The latter was dissolved in water and deionized (IR 120 H^+). Concentration gave crystals which were recrystallized from methanol to give an analytical sample (55 mg), mp 197°, $[\alpha]_D^{20}$ −37.3° (*c* 1.04, H_2O).

Anal. Calcd for $C_{13}H_{24}O_{11}\cdot 0.5H_2O$: C, 42.6; H, 6.9. Found: C, 42.58; H, 6.71.

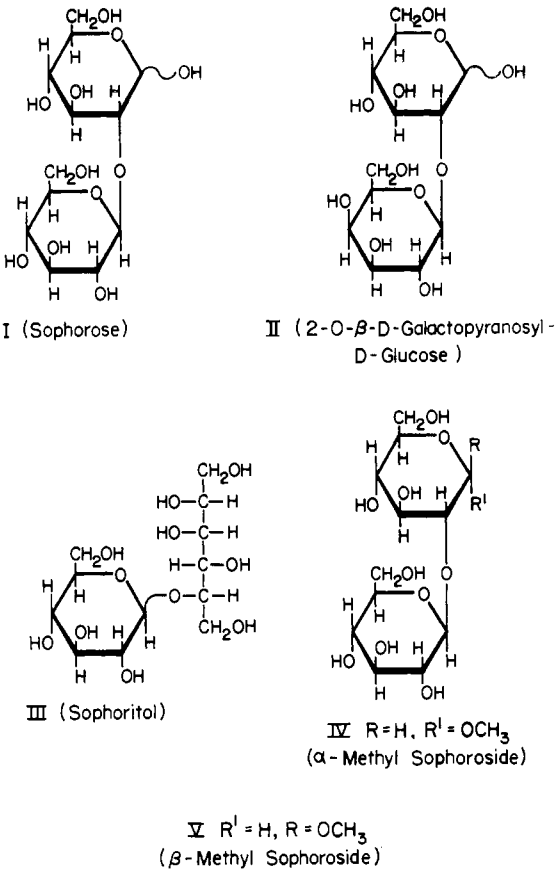

FIGURE 2: Structural formulas for sophorose and some of its derivatives.

Results

Inhibition curves for the carbohydrates examined as inhibitors of concanavalin A–dextran interaction are shown in Figure 1. In agreement with previous studies, methyl α-D-glucopyranoside is far more active as an inhibitor than D-glucose (Goldstein *et al.*, 1965b; Smith and Goldstein, 1967; So and Goldstein, 1967b). In this regard, all the α-linked glucobioses (*cf.* maltose and isomaltose) are superior to D-glucose.

In sharp contrast to the superior inhibiting power of the α-linked glucobioses are the disaccharides of D-glucose joined by β-glucosidic linkages. Methyl β-D-glucopyranoside, the prototype for β-linked disaccharides, required 70 μmoles for 50% inhibition, making it 3.5 times less potent than D-glucose and 30 times less active than the α-glycoside as an inhibitor of dextran–concanavalin A interaction. Laminaribiose and cellobiose which contain the β-D-(1→3)- and β-D-(1→4)-glucosidic linkage, respectively, did not inhibit concanavalin A–dextran interaction at concentrations of 100 μmoles whereas gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose) is a very poor inhibitor, requiring 100 μmoles for 50% inhibition (So and Goldstein, 1967b).

Sophorose (I) (Figure 2) which is characterized by a β-D-(1→2)-glucosidic linkage is unique in the β-linked glucobiose series, for it is three times more active as an inhibitor (7 μmoles for 50% inhibition) than D-glucose. In order to ascertain the basis for this behavior, several derivatives of sophorose were synthesized and tested for their activity as inhibitors.

2-*O*-β-D-galactopyranosyl-D-glucose (II) gave the same degree of inhibition as sophorose. On the other hand, sophoritol (III) exhibited no inhibition activity at a level of 20 μmoles. As controls for sophoritol, cellobiitol, and maltitol, the corresponding sugar alcohols of cellobiose and maltose, respectively, were tested. It was observed that cellobiitol was completely inactive at 100 μmoles, whereas maltitol exhibited approximately the same percentage inhibition as the parent sugar.

The α-methyl glycoside of sophorose (IV) showed approximately six times the inhibiting power of the parent disaccharide, whereas the corresponding β-methyl glycoside (V) is about 3.5 times poorer than sophorose. Thus, sophorose behaves in a fashion analogous to D-glucose in that both sugars become more potent inhibitors in the form of their corresponding α-methyl glycosides, whereas as their corresponding β-methyl glycosides they are poorer inhibitors than their parent sugars. It is also interesting to note the quantitative relationship that exists among these same four sugars, namely, that the methyl α- and β-sophorosides are approximately 2.5 times better

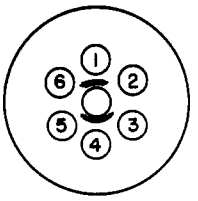

FIGURE 3: Diagram of agar gel diffusion patterns of the interaction of various carbohydrate–bovine serum albumin (BSA) conjugates with concanavalin A. All wells contain 0.1 ml. Central well contains concanavalin A (5 mg/ml); peripheral wells: (1) *p*-phenylazo-β-sophorose–BSA conjugate (5 mg/ml), (2) *p*-phenylazo-β-cellobiose–BSA conjugate (10 mg/ml), (3) *p*-phenylazo-β-lactose–BSA conjugate (10 mg/ml), (4) *p*-phenylazo-β-maltose–BSA conjugate (5 mg/ml), (5) *p*-phenylazo–BSA conjugate (20 mg/ml), and (6) *p*-phenylazogentiobiose–BSA conjugate (10 mg/ml).

inhibitors than the corresponding methyl α- and β-glucosides, respectively.

p-Nitrophenyl β-sophoroside is a more potent inhibitor than methyl β-sophoroside, 3.7 μmoles of the former giving 30% inhibition whereas the latter required about 15 μmoles for the same degree of inhibition. Precisely the same phenomenon was encountered when the inhibiting power of *p*-nitrophenyl β-D-glucopyranoside and methyl β-D-glucopyranoside were compared, the former being superior to the latter (Goldstein and Iyer, 1966). It was suggested that some type of nonspecific bonding of the hydrophobic phenyl group to a corresponding hydrophobic portion of the protein might account for this enhanced binding.

The effect of chemical modification of the nonreducing D-glucosyl moiety of the methyl glycosides of sophorose also was investigated. When the free hydroxyl groups of the terminal, nonreducing D-glucopyranosyl residue (positions C-2′, C-3′, C-4′, and C-6′) were blocked by *O*-acetyl groups, it was found that the percentage inhibition fell from a value of 1.1 μmoles for 50% inhibition of the free sugar to 4.3 μmoles for the tetra-*O*-acetyl derivative. A similar loss in inhibiting power was observed in the case of the β-methyl glycoside in that the corresponding quantities of sugar required for 16% inhibition fell from 6 μmoles for unsubstituted β-methyl sophoroside to 70 μmoles for the 2′,3′,4′,6′-tetra-*O*-acetyl derivative. Sophorotriose, the next higher homolog of sophorose (in which the three D-glucosyl residues are united by two β-(1→2)-glucosidic linkages), was found to be a much poorer inhibitor than sophorose, 12 μmoles being required to effect 16% inhibition compared to 2.4 μmoles for sophorose.

When *p*-nitrophenyl β-sophoroside was reduced catalytically to the corresponding *p*-aminophenyl derivative and coupled by diazotization to bovine serum albumin, the conjugate formed a precipitation band with concanavalin A (Figure 3). As reported previously, the maltose and glucose bovine serum albumin conjugates also reacted with concanavalin A, whereas the cellobiose and gentiobiose conjugates did not.

Discussion

The present study shows that specific terminal, nonreducing sugar residues (*e.g.*, the α-D-glucopyranosyl unit) are not, as we have previously suggested, the sole moieties capable of interacting with concanavalin A. Nevertheless, our results do confirm that it is the C-3, C-4, and C-6 hydroxyl groups of the reducing D-glucose unit of sophorose with which concanavalin A interacts, as we have previously suggested in the case of reactivity of α-D-glucopyranosyl residues. It already has been noted that certain modifications at the C-2 position of the D-glucopyranose ring are tolerated by the protein combining sites. Thus 2-deoxy-D-glucose, 2-*O*-methyl-D-glucose, and 2-acetamido-2-deoxy-D-glucose do not differ greatly from D-glucose in their capacity to inhibit dextran–concanavalin A interaction (Goldstein *et al.*, 1965b; Smith and Goldstein, 1967).

Consideration of the structure of sophorose I shows it to contain two potential receptor sites, namely, the C-3, C-4, and C-6 hydroxyl groups of the nonreducing β-D-glucopyranosyl unit and the C-3, C-4, and C-6 hydroxyl groups of the reducing moiety. The decision as to which of these two units (or indeed, the possibility that both of these units) is involved in binding to the active sites of the concanavalin A molecule has been investigated by studying the extent to which a number of sophorose derivatives inhibit concanavalin A–dextran interaction.

All evidence indicates that it is the *reducing* D-glucopyranose moiety of sophorose with which concanavalin A interacts. This is strongly supported by the following observations. (1) 2-*O*-β-D-Galactopyranosyl-D-glucose (II), which differs from sophorose only in the position of the C-4′ hydroxyl group of the nonreducing D-glycosyl residue, inhibits concanavalin A–dextran interaction to the same extent as sophorose. Inasmuch as we have shown repeatedly that neither D-galactose nor its α- or β-glycosides (lactose (4-*O*-β-D-galactopyranosyl-D-glucose) and melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) are noninhibitors) bind to the concanavalin A receptor sites, it must be concluded that the reducing moiety of II binds to concanavalin A.

(2) Reduction of sophorose with $NaBH_4$ converts it to sophoritol (III), in which the reducing D-glucopyranose moiety has been transformed into a D-sorbitol residue. Sophoritol is a noninhibitor of dextran–concanavalin A interaction. This indicates that the process of reduction has destroyed the carbohydrate binding sites, namely the spatial orientation of the C-3, C-4, and C-6 hydroxyl groups of the reducing moiety. It should be noted that maltitol, the alditol obtained by reduction of maltose, still inhibits concanavalin A–polysaccharide interaction to approximately the same extent as the parent sugar, indicating that maltose binds to concanavalin A by means of its nonreducing α-D-glucopyranosyl residue.

(3) In analogy with D-glucose and its methyl glycopyranosides, α-methyl sophoroside (IV) was six times more potent as an inhibitor than sophorose, whereas β-methyl sophoroside (V) was 3.5 times less potent an inhibitor than sophorose. These chemical modifications involve only the *reducing* moiety of sophorose (assuming of course that the conformation of the nonreducing residue remains unaltered). On the other hand, the conversion of maltose to β-methyl maltoside does not alter its inhibiting potency (Smith and Goldstein, 1967).

(4) Acetylation of the nonreducing D-glucosyl moiety of α- and β-methyl sophoroside lowered but did not abolish the capacity of these glycosides to inhibit dextran–concanavalin A interaction, demonstrating retention of the primary binding sites of these oligosaccharide glycosides.

Although these data are consistent with the interpretation that concanavalin A interacts with the reducing D-glucopyranose residue of sophorose several questions remain unanswered. Why is sophorose a better inhibitor than D-glucose (or α-methyl sophorose three times more potent than methyl α-D-glucopyranoside)? Does the bulky 2-*O*-glucopyranosyl substituent change the conformation of the reducing moiety, rendering it more complementary to the concanavalin A combining sites?

The explanation for the low inhibition activity of sophorotriose compared to that of sophorose may be the result of steric hindrance. Examination of Pauling–Corey–Koltun models of sophorotriose indicates that the nonreducing β-sophorosyl moiety offers considerable steric hindrance to the approach of the protein and probably obstructs to a large measure the interaction of the concanavalin A combining sites with the C-3, C-4, and C-6 hydroxyl groups of the reducing D-glucose moiety.

Recently it has been found that in common with other antibodies formed against disaccharide–protein antigens the antibodies elicited by bovine serum albumin *p*-phenylazo-β-sophoroside (P. Z. Allen, I. J. Goldstein, and R. N. Iyer, unpublished data) also are directed primarily against the *nonreducing* terminal glycosyl residue (Goebel *et al.*, 1934), in this case the β-D-(1→2)-glucopyranosyl unit. In this regard, immune antibodies differ markedly from the concanavalin A system.

This study does not provide a direct answer to the question: will concanavalin A combine with D-glucopyranosyl residues joined by α-D-(1→2)-glucosidic linkages in dextrans or S XII polysaccharide? Nevertheless, the study does indicate that concanavalin A has the capacity to interact with the C-3, C-4, and C-6 hydroxyl groups of a 2-*O*-substituted D-glucose unit. Further experiments are now in progress to provide answers to some of these interesting questions.

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