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Note

Synthesis and evaluation of chitosan-based, affinity-chromatography materials*

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Many methods have been reported for covalently coupling biomolecules to polysaccharides, with cellulose, agarose, dextran, and starch being the most commonly employed substrates[1–5]. The resultant conjugates have found application in enzyme and cell immobilization, immunology, drug delivery, and affinity chromatography. Chitosan, a linear polysaccharide containing mainly (1→4)-linked 2-amino-2-deoxy-β-D-glucopyranosyl residues, has attracted relatively little, albeit increasing, attention in this area, having found application in enzyme and cell immobilization, and drug delivery[6–8]. Indeed, the enzyme β-D-glucosidase has itself been immobilized covalently onto chitosan[6]. It has also been shown to be an ideal substrate for high-yielding, controllable, site-specific chemical modification *via* reductive amination[9] with its amine functionality[10], and the resultant derivatives have been reported to have interesting properties including viscosity modification[10,11], metal chelation[12,13], and gelation[14]. We describe herein a route to chitosan derivatives bearing pendant 1-thio-β-D-glucopyranosyl groups that have a potential utility as affinity chromatography materials for β-D-glucosidases.

The methods for preparing affinity-chromatography supports have been well reviewed[3,4]. Briefly, the ligand of choice must have (a) high affinity for the enzyme and (b) linkages resistant to chemical and enzymic hydrolysis, and furthermore (c) a "spacer-arm" between the ligand and support is usually desirable. For our application considered here, the 1-thio-β-D-glucopyranoside ligand fulfills criteria (a) and (b), as thioglycosides are relatively resistant to hydrolysis by glycosidases, and have been previously reported as ligands for affinity chromatography[15]. The bifunctional reagent, 1-allyloxy-2,3-epoxypropane, was incorporated to provide both

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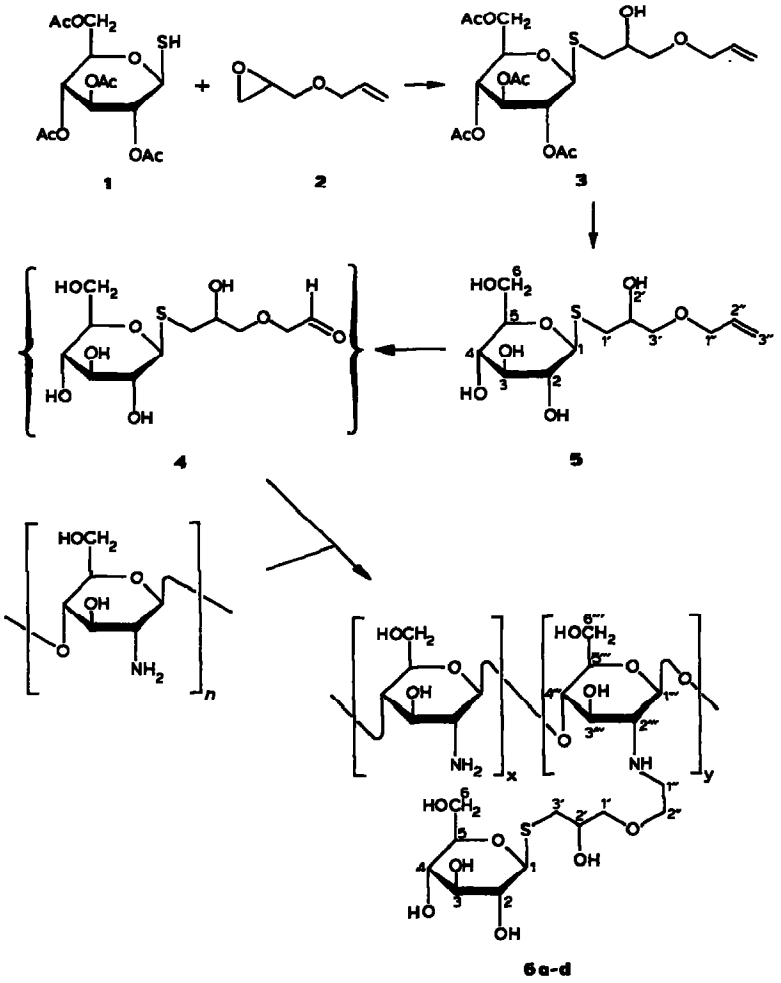

Scheme 1. Values of x and y in **6** indicate the fractional degree of substitution.

a spacer-arm and a convenient means for attachment to chitosan.

The synthesis of the desired 1-thio-β-D-glucopyranoside **5** was accomplished from 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (**1**) and 1-allyloxy-2,3-epoxypropane (**2**). Reductive ozonolysis of **5** provided the respective aldehyde **4**. Without further purification, this was bound to chitosan by reductive amination according to standard methods[10], to afford the respective derivatives **6a, b, c** and **d**, as listed in Table I. The ^{13}C-n.m.r. spectrum of **6a** showed the expected resonances indicating attachment of the thio-β-D-glucopyranosyl group. Derivatives **6b** and **6d** were crosslinked by reductive amination with glutaraldehyde, to provide products having a reduced solubility or hydration properties.

As a prelude to the testing of these materials as affinity supports, it was

TABLE I

SYNTHESIS AND CHARACTERISTICS OF CHITOSAN CONJUGATES

Derivative	*D.s.*[a]	*Reactant ratio*[b]	*Crosslinking (%)*	*Yield (%)*
6a	0.90	2.06		95
6b	0.90	2.06	5	70
6c	0.25	0.59		61
6d	0.25	0.59	15	60

[a]Degree of substitution, or y in **6**. [b]The ratio of aldehyde **4** to chitosan in the reaction mixture.

necessary to determine whether the thioglycosidic linkage is indeed resistant to enzymic hydrolysis. The monomer **5** was utilised for these tests, thus avoiding problems associated with the handling of polymeric materials, yet still providing a stringent test of lability. An assay for D-glucose based upon the coupling of hexokinase and D-glucose 6-phosphate dehydrogenase was utilized to detect enzyme-catalyzed turnover, since glycosidase-catalyzed hydrolysis of thioglycosides produces D-glucose and the free aglycon thiol. Incubation overnight of **5** (3.0mM) with β-D-glucosidase (~5 units) in the presence of coupling enzymes and cofactors resulted in no significant increase in absorbance; thus, no significant hydrolysis had occurred. The viability of the coupling enzymes at this stage was demonstrated by addition of a known amount of D-glucose and measurement of the expected response.

Binding of the thioglucoside **5** to the enzyme was investigated kinetically with β-glucosidases from almond and from *Alcaligenes faecalis*. Inhibition of hydrolysis of 4-nitrophenyl β-D-glucopyranoside by **5** was measured and K_i values of 35mM and 1.5mM determined for the β-glucosidases from almond and *Alcaligenes faecalis*, respectively. The K_m values for 4-nitrophenyl β-D-glucopyranoside for these two enzymes are 3mM (ref. 17) and 0.08mM (ref. 18), respectively. It therefore appears that both enzymes will bind the thioglucoside but, since the β-glucosidase from *Alcaligenes* has the greater affinity, it was used in testing the affinity support for binding of enzyme.

A preliminary test of the ability of the derivatized polymer to bind β-glucosidase was performed using the cross-linked preparation **6d**. No specific attempts were made to optimize the flow properties of the polymer. The effluent from the column was monitored by assaying the enzyme activity released with 4-nitrophenyl β-D-glucopyranoside, as shown in Fig. 1. Clearly β-glucosidase is bound to the column initially and eluted at high salt concentrations, thus indicating the potential of the material as an affinity support.

EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded with a 270-MHz

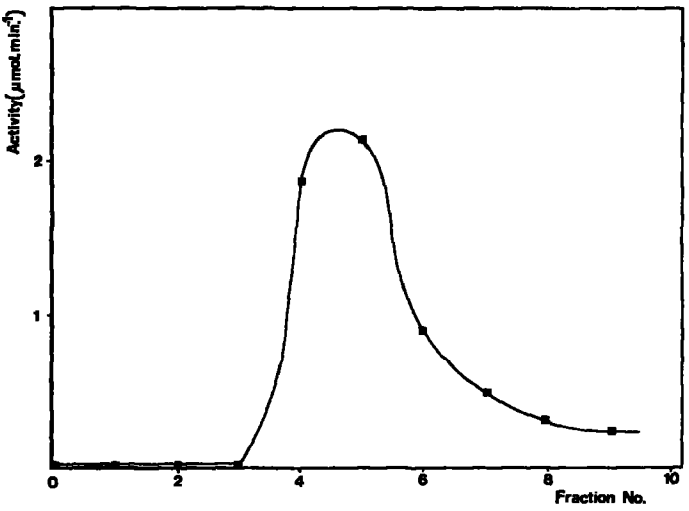

Fig. 1. Plot of β-glucosidase activity eluted from the affinity column as a function of fraction (0.13 mL), collected after application of the high-salt wash. See Experimental section.

spectrometer comprising an Oxford magnet and a Nicolet 1180 computer, and ^{13}C-n.m.r. spectra with a Bruker WH400 spectrometer at 100.6 MHz. Low-resolution mass spectra were recorded with a Varian/MAT CH4B or a Kratos/AEI MS50 mass spectrometer. Analytical thin-layer chromatography (t.l.c.) was performed with 0.20-mm precoated, aluminum-backed sheets of Silica Gel 60 F_{254} (E. Merck, Darmstadt, Germany). The solvent systems used for t.l.c. development were: (*a*) 4:1 ethyl acetate–hexane, and (*b*) 9:4:2 ethyl acetate–2-propanol–water. For detection of components, the t.l.c. sheets were sprayed with 30% H_2SO_4 in ethanol and heated on a hot plate to char. Liquid chromatography was performed on 230–400 mesh silica gel (Kieselgel 60, E. Merck, Darmstadt, Germany) according to the procedure of Still *et al.*[19]. All evaporations were performed under reduced pressure in a Büchi rotary evaporator.

Ozonolyses were performed at −78°, with a Welsbach Ozonator (90 V, 14 kPa input, O_2 pressure) O_3 source. O_3 was bubbled into the cooled solution *via* a sintered-glass bubbling tube until a pale-blue color persisted. The ozone source was turned off, and the solution purged with O_2 until colorless. Excess dimethyl sulfide was then added and the solution allowed to warm to room temperature, with stirring, for 2 h. Elemental microanalyses were performed by Mr. P. Borda, Microanalytical Laboratory, University of British Columbia. Degree of substitution (d.s.) values for **6a–d** were calculated from the microanalytical data, and were corrected for water content.

*3-Allyloxy-2-hydroxypropyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-*D*-glucopyranoside* (**3**). — 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose[20] **1**, (10.0 g, 27.5 mmol), 1-allyloxy-2,3-epoxypropane (**2**; 3.14 g, 27.5 mmol; Aldrich Chemical Co.),

and $NaHCO_3$ (2.31 g, 27.5 mmol) were stirred in ethanol (60 mL) for 4 h at reflux temperature. The solution was cooled, poured into chloroform (200 mL), washed with saturated $NaHCO_3$ solution (10 mL), saturated NaCl solution (2 × 100 mL), dried ($MgSO_4$), filtered, and evaporated; l.c. afforded diastereomeric **3**; ^{1}H-n.m.r. (270 MHz, $CDCl_3$): δ 5.87 (m, 1 H, H-2″), 5.32–4.96 (m, 5 H, H-2,3,4,3″a, 3″b), 4.55 (d, 1 H, $J_{1,2}$ 10.0 Hz, H-1), 4.30–4.05 (m, 2 H, H-6a,b), 4.00 (d, 2 H, $J_{2',3'}$ 5.0 Hz, H-3′a,b), 3.93 (m, 1 H, H-2′), 3.73 (m, 1 H, H-5), 3.51–3.45 (m, 2 H, H-1′a,b), 3.15–2.58 (m, 2 H, H-1″a,b), and 2.10, 2.06, 2.04, 2.00 (4 s, 12 H, 4 OAc); m.s.: *m/z* 478 (M^+), 418, and 258.

*3-Allyloxy-2-hydroxypropyl 1-thio-β-*D*-glucopyranoside* (**5**). — A solution of **3**, (7.3 g, 15.3 mmol) in anhydrous methanol (75 mL) was treated with 0.5M sodium methoxide in methanol (5.0 mL) until the reaction was complete (t.l.c., *b*). The solution was made neutral with Dowex 50-X8, (H^+, 100–200 mesh) ion-exchange resin, filtered, decolorized, and evaporated. Attempts to crystallize the syrupy residue were unsuccessful. Drying *in vacuo* (0.05 mm Hg) gave the diastereomeric mixture of **4** (4.5 g, 95%), foamy solid; ^{1}H-n.m.r. (270 MHz, D_2O): δ 5.89 (m, 1 H, H-2″), 5.38 (d, 1 H, $J_{2'',3a''}$ 17.0 Hz, H-3″a), 5.20 (d, 1 H, $J_{2'',3''b}$ 12.0 Hz, H-3″b), 4.49, 4.47 (2 d, 1 H, $J_{1,2}$ 7.0 Hz, H-1), 4.02 (d, 2 H, $J_{2',3'}$ 6.0 Hz, H-3′), 3.99 (m, 1 H, H-2′), 3.83 (dd, 1 H, $J_{6a,6b}$ 12.0, $J_{5,6a}$ 1.0 Hz, H-6a), 3.64 (dd, 1 H, $J_{6a,6b}$ 12.0, $J_{5,6b}$ 3.0 Hz, H-6b), 3.60–3.31 (m, 3 H, H-3,4,5), 3.26 (dd, 1 H, $J_{2,3}$ 9.0, $J_{1,2}$ 7.0 Hz, H-2), 2.92 (dd, 1 H, $J_{1''a,1''b}$ 14.0, $J_{1''a,2''}$ 5.0 Hz, H-1″a), 2.88–2.78 (m, 2 H, H-1′a,b), and 2.70 (dd, 1 H, $J_{1''a,1b''}$ 14.0, $J_{1a'',2''}$ 8.0 Hz, H-1″b); ^{13}C-n.m.r. (100.6 MHz, D_2O): δ 132.3 (C-2″), 84.1, 83.8 (C-1), 78.3, 78.2 (C-5), 75.6 (C-3), 70.7, 70.3, 68.0, 67.8 (C-2,3,4,5,2′,3′,1″), 59.3 (C-6), 31.7, and 31.6 (C-1′).

Ozonolysis of **5**. — The 1-thio-β-D-glucopyranoside **5** (3.42 g, 11.0 mmol) was dissolved in methanol (50 mL), cooled to −78°, and saturated with O_3. Dimethyl sulfide (3.43 g, 4.1 mL, 55.0 mmol) was added and the mixture was allowed to warm to ambient temperature for 2 h with stirring. Excess solvent was removed, and the syrupy residue dissolved in ethanol and precipitated by the addition of ether. After being precipitated twice and dried *in vacuo*, crude **4** was obtained (3.30 g, 96%).

Affinity ligand conjugates of chitosan. — (*a*). Chitosan (from crab shell, *N*-acetyl content <5%; Sigma Chemical Co.; 0.85 g, 5.3 mmol), dissolved in 5% aqueous acetic acid (50 mL), was treated with a solution of aldehyde **4**, (3.4 g, 10.9 mmol) in 5% aqueous acetic acid (10 mL), and sodium cyanoborohydride (Aldrich; 1.25 g, 20.0 mmol) for 24 h. The solution was dialyzed for six days against distilled water (6 × 1 L), filtered, and freeze dried to give **6a** (2.25 g, 95%); ^{13}C-n.m.r. (100.6 MHz, D_2O): δ 99.5 (C-1‴), 87.0 (C-1), 78.5 (C-5), 75.5 (C-3), 72.3, 71.8 (C-2,4), 67.6, 67.5, 66.5 (C-2″,2′,1′), 59.1 (C-6), 49.0 (C-3′), and 45.5 (C-1″).

Anal. Calc. for $[(C_6H_{11}NO_4)_{0.1}(C_{17}H_{31}NO_{11}S)_{0.9}]\cdot 1.9\,H_2O$: C, 41.21; H, 7.13; N, 3.02; S, 6.22. Found: C, 41.21; H, 7.00; N, 3.46; S, 6.83.

(*b*). Derivative **6a** (0.50 g, 1.3 mmol) was dispersed in 2.0% aqueous acetic acid with stirring, and treated with a 25% aqueous glutaraldehyde solution (0.10

mL, 0.26 mmol, 0.2 equiv.) and sodium cyanoborohydride (0.25 g, 4.0 mmol) for 24 h. The suspension was filtered and the precipitate washed with water. Drying *in vacuo* (7 Pa) provided **6b** (0.40 g, 69%).

Anal. Calc. for $[(C_{17}H_{31}NO_{11}S)_{0.9}(C_{8.5}H_{16}NO_4)_{0.1}]\cdot 0.65\ H_2O$: C, 45.68; H, 6.67; N, 3.16; S, 6.51. Found: C, 45.68; H, 6.92; N, 3.05; S, 6.40.

(*c*). Chitosan (0.70 g, 4.35 mmol) was dissolved in 5% aqueous acetic acid (50 mL) and treated with aldehyde **4** (0.8 g, 2.56 mmol, 0.59 equiv.) and sodium cyanoborohydride (1.0 g, 16.0 mmol), with stirring for 24 h. The solution was dialyzed for 6 days against distilled water (6 × 1 L), and freeze-dried to give **6c** (0.67 g, 61%).

Anal. Calc. for $[(C_6H_{11}NO_4)_{0.75}(C_{17}H_{31}NO_{11}S)_{0.25}]\cdot 0.88\ H_2O$: C, 42.06; H, 7.11; N, 5.61; S, 3.20. Found: C, 42.06; H, 6.85; N, 5.80; S, 3.29.

(*d*). A stirred solution of chitosan (0.70 g, 4.35 mmol) in 5% aqueous acetic acid (50 mL) was treated with aldehyde **4** (0.8 g, 2.56 mmol, 0.59 equiv.) and sodium cyanoborohydride (1.0 g, 16.0 mmol) for 24 h. Aqueous glutaraldehyde (25%) (0.40 mL, 1.0 mmol, 0.22 equiv.) and sodium cyanoborohydride (0.25 g, 4.0 mmol) were then added and allowed to stir for 24 h, resulting in a stiff, clear, and foamy gel. The gel was diluted with 5% aqueous acetic acid (100 mL), stirred, and dialyzed for 6 days against distilled water (6 × 2.0 L); freeze-drying gave **6d** (0.65 g, 60%).

Anal. Calc. for $[(C_6H_{11}NO_4)_{0.45}(C_{17}H_{31}NO_{11}S)_{0.25}(C_{8.5}H_{16}NO_4)_{0.3}]\cdot 0.43\ H_2O$: C, 45.13; H, 7.27; N, 5.54; S, 3.17. Found: C, 45.14; H, 7.22; N, 4.96; S, 3.04.

Enzyme studies. — All buffer chemicals, substrates, cofactors, and coupling enzymes were obtained from Sigma Chemical Co. Assays of D-glucose liberation were performed essentially as described previously[18,21] and standardised against a D-glucose standard solution. Quantities of coupling enzymes employed were sufficient that all glucose had been consumed within 5 min. Inhibition constants for the thioglucoside **4** with β-D-glucosidases from almond emulsin and *A. faecalis* were determined by use of a range of inhibitor concentrations (0.2–2.0 times K_i) at a fixed concentration (5mM and 0.1mM, respectively) of 4-nitrophenyl β-D-glucopyranoside in 50mM sodium phosphate buffer, pH 6.8 at 25°, as described previously[18]. Data were analyzed by means of a plot of v uninhibited/v inhibited *vs.* inhibitor concentration. The slope of such a plot equals $K_m/K_i\,(S + K_m)$ from which the K_i values could be determined.

The effectiveness of the affinity support was evaluated as follows: A small column (5 × 35 mm) was packed with the cross-linked polymer **6d**, equilibrated with buffer (5mM sodium phosphate, pH 6.8), and a small sample (2 mL) of *A. faecalis* β-D-glucosidase loaded on. After washing with the same buffer (5 mL), elution was effected with a buffer containing 0.5M NaCl and 5mM sodium phosphate, pH 6.8, and fractions (0.13 mL) collected.

β-Glucosidase activity, eluted from the affinity column, was estimated spectrophotometrically by measuring the rate of hydrolysis of 4-nitrophenyl β-D-glucopyranoside (0.25 mM) in 50mM sodium phosphate buffer, pH 6.8, upon addition of a fixed aliquot (10 μL) of column effluent.

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