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Note

Synthesis of 6′-*S*-α-D-glucopyranosyl-6′-thiomaltose (6^2-thiopanose) and its effect on the enzymic activity of glucoamylases from *Aspergillus niger**

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Most fungal glucoamylases possess a raw-starch binding area that is different from the active site[1-3] and located at a separate C- or N-terminal domain[4]. It is not yet known whether this binding site also plays a role in the hydrolysis or reversion reactions catalyzed by these enzymes. Several glucoamylases highly active in raw-starch hydrolysis are found to have, however, a strong debranching activity[5,6]. *Aspergilus niger* produces[2] two major forms of the enzyme, glucoamylase G1 and G2. As compared to glucoamylase G2, glucoamylase G1 contains a Ce-terminal extension of 104 amino acid residues that is required for the adsorption onto and the degradation of raw starch[2,7]. The enzyme releases both (1→4)- and (1→6)-linked α-D-glucose units from the non-reducing ends of starch and related poly- and oligo-saccharides; to obtain more information on the role of the (1→6)-linked unit in the enzymic process, a non-hydrolyzable substrate analog has been employed. We describe herein the synthesis of 6′-*S*-α-D-glucopyranosyl-6′-thiomaltose (6^2-thiopanose; **4**), in 80% yield, by displacement of the iodine atom of 1,2,3,6,2′,3′,4′-hepta-*O*-acetyl-6′-deoxy-6′-iodomaltose (**2**) by the activated form of 2,3,4,6-tetra-*O*-acetyl-1-*S*-acetyl-1-thio-α-D-glucopyranose (**1**), followed by *O*-deacetylation. The interaction of 6^2-thiopanose (**4**) with glucoamylases G1 and G2 from *Aspergillus niger* was tested by enzyme kinetics and u.v.-difference spectroscopy.

A previous paper[8] described the reaction of peracetylated 6-deoxy-6-iodomaltose with 2,3,4,6-tetra-*O*-acetyl-1-*S*-acetyl-1-thio-α-D-glucopyranose (**1**) to give peracetylated 6-*S*-α-D-glucopyranosyl-6-thiomaltose in high yield. The same procedure, applied to peracetylated 6′-deoxy-6′-iodo-maltose (**2**) afforded peracetylated 6^2-thiopanose (**3**) in 80% yield. The orientation of the newly formed thioglycosidic bond was expected to be

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* Dedicated to Professor Serge David on the occasion of his 70th birthday.

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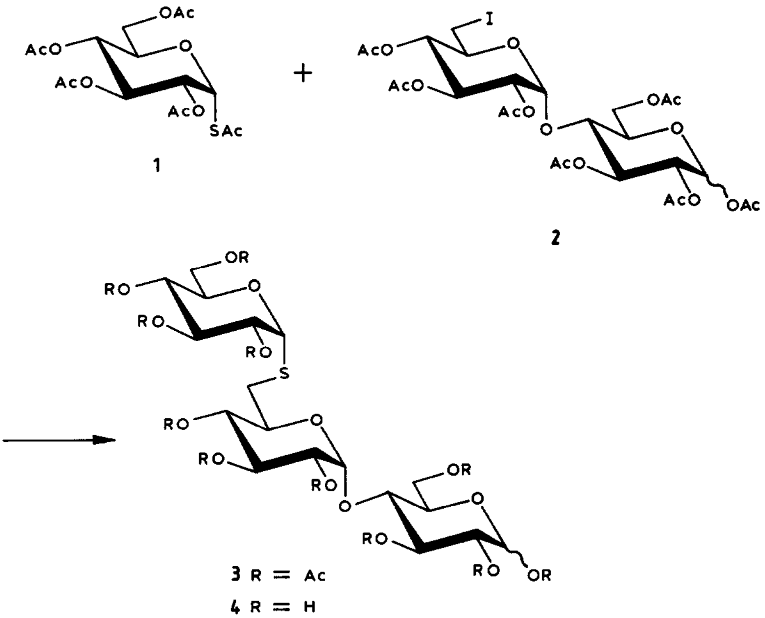

1,2-*cis* as isomerisation has never been observed under the conditions used. This finding was confirmed by the high optical rotation value of **3**, which was of the same magnitude as that found for the C-6 isomer[8]. A further confirmation of the 1″-thio-α-D-glucosyl-(1″→6′) bond was obtained by examination of the ^{1}H-n.m.r. spectrum. Thus, **3** gave a shielded doublet for H-6′a and H-6′b at δ 2.78 and 2.55, respectively, and a deshielded doublet for H-1″ at δ 5.82 with a splitting of 6 Hz.

TABLE I

Effect of 6^2-thiopanose (**4**) on glucoamylase kinetic parameters

		Substrate			
		Soluble starch		*Maltose*	
Enzyme form	*6^2-Thiopanose (mM)*	K_m (%)	k_{cat}[a] (s^{-1})	K_m (mM)	k_{cat}[a] (s^{-1})
GI	0	0.015 ± 0.0022	56 ± 7.2	0.74 ± 0.086	2.8 ± 0.22
	1	0.011 ± 0.0016	60 ± 6.4		
	2	0.006 ± 0.0010	72 ± 5.3	0.71 ± 0.09	2.3 ± 0.28
	4	0.005 ± 0.0007	81 ± 8.8		
G2	0	0.05 ± 0.007	57 ± 6.0	0.69 ± 0.10	3.0 ± 0.30
	2	0.06 ± 0.007	58 ± 6.8	0.83 ± 0.12	2.8 ± 0.28

[a] The K_{cat} value is expressed as the number of glucosyl bonds split per enzyme molecule per second. The parameters are averages of values of three experiments.

6^2-Thiopanose (**4**) at 2mM concentration exerted no significant effect on the k_{cat} and K_m values in the preliminary experiments on the hydrolysis of maltose catalyzed by glucoamylase G1 or G2, or the hydrolysis of soluble starch by glucoamylase G2 (Table I). The degradation of soluble starch by glucoamylase G1, however, was clearly enhanced by **4** (Table I). Thus, both apparent higher k_{cat} and lower K_m values, found in the presence of **4** at concentrations from 1 to 4mM, indicated that **4** acts as an activator. Plots of $1/v$ *vs.* $1/S$ at the various concentration of **4** (data not shown) were compatible with a nonessential activation mechanism[9] in which the reaction can occur in the absence of an activator. This has been found earlier for the effect of 4-thio-D-xylobiosyl residues on the hydrolysis of D-xylan, but not of oligomeric substrates, by a fungal D-xylanase[10]. An estimate of K_A, the dissociation constant for the activated glucoamylase complex[9,10], gave a value around 1mM.

In contrast, a weak inhibition by **4** of the catalytic activity of glucoamylase G1 was demonstrated by its influence on the progress of 3.8mM panose hydrolysis. The initial rate, V_0, of the α-D-(1→6)-linkage hydrolysis, as observed by ^{1}H-n.m.r. spectroscopy (Figl. 1), decreased from 0.068 to 0.030 min^{-1} and $T_{1/2}$ increased from 14.3 to 33.9 min in the presence of 3.7mM **4**. Therefore, the thio analog most likely interacts with glucoamylase in two different ways involving different areas of the enzyme surface: (a) the catalytic site that cleaves both α-(1→4)- and α-(1→6)-linked substrates[11] and (b) an additional binding site, possible the so-called raw-starch binding site which is present only in the G1 form of the enzyme and has been reported to possess high affinity for cyclodextrins[1,12,13]. Since such apparently counteracting effects of inhibition and activation result in a complex influence of **4** on the enzyme activity, a detailed and effector-consuming kinetic analysis of its mode of action has not been pursued.

U.v.-difference spectroscopy was employed to estimate the apparent dissociation constant, K_d, for **4** and glucoamylase G1 (Fig. 2). The calculated $\Delta\varepsilon_{max}$ value of 6300 M^{-1}·cm^{-1} at 292 nm (Fig. 3) is unusually large and 2–3-fold higher than that reported[14]

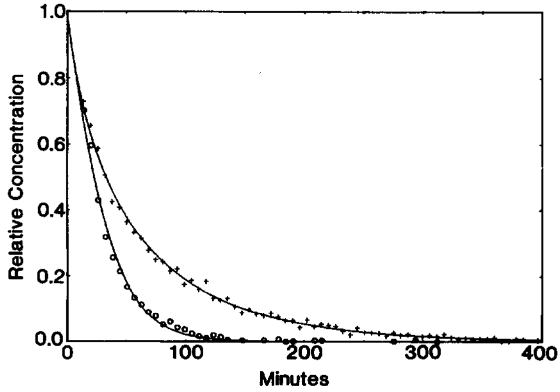

Fig. 1. Hydrolysis of 3.8mM panose with glucoamylase G1 from *Aspergillus niger* in the presence (+) and absence (○) of 3.7mM 6^2-thiopanose (**4**) as monitored by the loss of H-1″ signal.

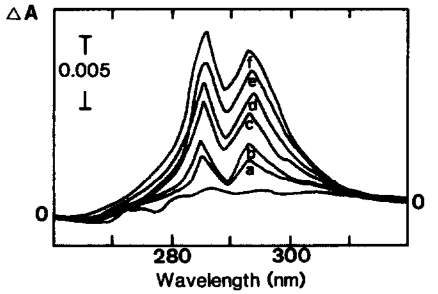

Fig. 2. Difference spectrum for glucoamylase G1 (14.7μM) induced by: (a) 0.31 (b) 0.61, (c) 0.90, (d) 1.19, (e) 1.91, and (f) 2.34mM 6^2-thiopanose (4).

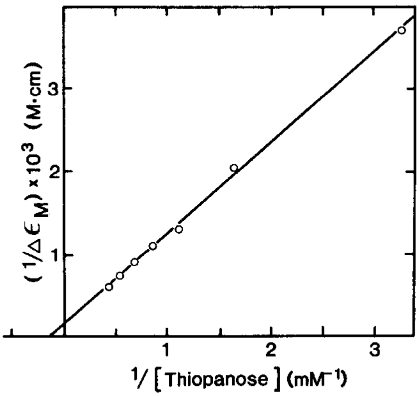

Fig. 3. Lineweaver-burk plot of the perturbation of the glucoamylase G1 spectrum at A_{292} ($\Delta\varepsilon$) by 6^2-thiopasose (4).

for other, ligand-induced difference spectra of an *Aspergillus niger* glucoamylase G1. Based on a $\Delta\varepsilon$ value between -1500 and -1600M^{-1}·cm^{-1}, characteristic of the denaturation blue shift for a tyrprophanyl residues[15], four tryptophanyl residues are assumed to be involved in the binding of **4** to glucoamylase G1. This is in agreement with two to four residues interacting with inhibitors or substrates in the active site, and another two from the C-terminal domain being critical for the raw-starch binding, as found in earlier chemical modification studies[14,16]. Although this might be an oversimplified interpretation, as the reported K_d value for beta-cyclodextrin association to the C-terminal domain[12,13] is two to three orders of magnitude smaller than the present apparent K_d value of 7.1mM, these observations indicate that **4** has a relatively low affinity for the site(s) probed by the spectral perturbation. Thus, **4** has an affinity comparable to that of isomaltotriose, which is hydrolyzed by the same enzyme with a K_m value of 11mM (ref. 17), and to that of panose, which has a K_m value of 6.3mM with the *Rhizopus delemar* glucoamylase[18]. Therefore, the major part of the spectral perturbation is likely not due to interaction with the raw-starch binding domain but with the active site area that comprises[19] also a third tryptophanyl residue, Trp 170, which is specifically protected by isomaltose, the smallest substrate containing the α-(1→6) bond. In addition to the large intensity, the shape of the difference spectrum induced by **4** for glucoamylase G1 displays features of both isomaltose as well as alpha- and beta-cyclodextrin-spectral perturbations (data not shown). Therefore, if binding of **4** to the raw-starch binding site contributes significantly to the perturbation of the u.v. spectrum, it has to occur with an affinity similar to that for the active site and, thus, much inferior to that of beta-cyclodextrin[12,13]. Owing to the relatively poor affinity for **4**, further studies using saturating concentrations for the establishment of the binding stoichiometry have not been performed.

In view of the complex formation and effects on glucoamylase activity observed at modest concentrations of **4**, it is proposed that **4** may be employed to further explore the binding areas, for example by differential labeling and identification of the amino acid residues involved in ligand binding.

EXPERIMENTAL

General. — The conditions used are indentical to those described in a previous communication[10].

S-*(2,3,4,6-Tetra-*O*-acetyl-α-*D*-glucopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-6-thio-α-*D*-glucopyranosyl)-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-*D*-glucopyranose* (**3**; *β anomer).* — Cysteamine (30 mg) was added to a stirred mixture of peracetylated 6′-deoxy-6′-iodomaltose[20] (**2**; 200 mg, 0.26 mmol) 2,3,4,6-tetra-*O*-acetyl-1-*S*-acetyl-1-thio-α-D-glucopyranose[21] (**1**; 160 mg, 0.39 mmol, 1.5 equiv.), and dithioerythritol (40 mg) in *N′,N″,N‴* hexamethylphosphoramide (4 mL). After 12 at room temperature, ice–water (20 mL) was added to the mixture. The precipitate was isolated by filtration through a bed of Celite and washed with water. Column chromatography using 1:1 (v/v) ethyl acetate–light pretroleum as eluent afforded **3** (200 mg, 80%), m.p. 89–90° (ethanol), $[\alpha]_D^{20}$ + 195° (*c* 1, chloroform); ^{1}H-n.m.r. (CDCl$_3$): δ 5.82(d, 1 H, $J_{1,2}$ 6 Hz, H-1″), 5.72(d, 1 H, $J_{1,2}$ 8 Hz, H-1), 5.30 (m, 4 H, H-1′,3,3′,3″), 5.0 (m, 4 H, H-2,2″,4,4″), 4.74 (dd, 1 H, $J_{1,2}$ 4, $J_{2,3}$ 10 Hz, H-2′), 4.41 (dd, 1 H, *J* 3 and 12 Hz, H-6,6′ or 6″), 4.35 (m, 1 H, H-5,6,6′ or 6″), 4.24 (dd, 1 H, *J* 4.5 and 12.5 Hz, H-6,6′ or 6″), 4.19 (dd, 1 H, *J* 4 and 12 Hz, H-6,6′ or 6″), 4.00 (m, 3 H, H-6,6′,5″ or 6″), 3.80 (o, 1 H, $J_{5,6}$ 2.5, $J_{5,6}$ 4.0, $J_{5,4}$ 10.0 Hz, H-5′), 2.78 (dd, 1 H, $J_{6a,5}$ 3.5,$J_{6a,6b}$ 14.5 Hz, H-6′a), and 2.55 (dd, 1 H, $J_{6b,5}$ 5.0 Hz, H-6b); ^{13}C-n.m.r. (CDCl$_3$) δ 95.4 (C-1′), 91.2 (C-1), 82.4 (C-1″), 62.8, 61.7 (C-6,6″), and 29.6 (C-6′).

Anal. Calc. for C$_{40}$H$_{54}$O$_{26}$S:C, 48.87; H, 5.53; S, 3.26. Found: C, 49.15; H, 5.29; S, 3.13.

S-*α-*D*-Glucopyranosyl-(1→6)-*O*-(6-thio-α-*D*-glucopyranosyl)-(1→4)-*D*-glucopyranose.* (**4**). — Compound **3** (200 mg, 0.2 mmol) was *O*-deacetylated by treatment with M sodium methoxide (200 μL) in methanol (20 mL). After neutralization and freeze-drying, **4** was quantitatively obtained. $[\alpha]_D^{20}$ + 140° (*c* 1, water); ^{13}C-n.m.r. (D$_2$O): δ 100.3 (C-1′), 96,6 (C-1β), 92.7 (C-1α), 85.9 (C-1″), 61.9 (C-6), 61-4 (C-6″), and 31.3 (C-6′).

Anal. Calc. for $C_{18}H_{32}O_{15}S{\cdot}2.5H_2O$:C, 38.22; H, 6.59; S, 5.67. Found: 37.94; H, 6.11; S, 5.96.

Effect of compound **4** *on glucoamylases G1 and G2 activity.* — Glucoamylases G1 and G2 were purified from a commercial product of *Aspergillus niger* glucoamylase, AMG 200 (Novo), as previously described[2]. For activity measurements, a stock solution of potato starch (Sigma, Chem. Co., S-2630) was prepared from a 1% slurry in 0.1M sodium acetate, pH 4.5, that was clarified by boiling, cooled on ice; and adjusted to a 0.5% concentration by addition of buffer. The k_{cat} and K_m values in the presence and absence of **4** were derived from Lineweaver–Burk plots of activities in the range of 0.01–0.45% of starch at 38° using 150–170nM of enzyme. The amount of D-glucose released was analyzed as reported earlier[2] on aliquots removed at appropriate time intervals. Kinetic parameters were likewise obtained from activities towards 0.3–10mM maltose at 25° using 1.4–1.5μM enzyme.

The progress of the hydolysis of the (1→6)-α-Dglucosyl bond in 3.8mM panose (Sigma) by 0.47μM of glucoamylase G1 at pH 4.3 and 27° was monitored by ^{1}H-n.m.r. spectroscopy (500 MHz)[22] (H-1″ signal at δ 4.93). The values of the initial rate V_0 and $T_{1/2}$ for the α-D-(1→6)-bond hydrolysis were calculated as described earlier[23].

The binding of **4** to glucoamylase G1 was monitored by u.v.-difference spectroscopy showing the perturbation of aromatic side-chains. The measurements were performed at concentrations of **4** between 0.3 and 2.4mM by use of double-chamber cuvettes (individual light path, 0.4375 cm) essentially as earlier reported[14]. The values of the dissociation constant, K_d, and the difference molar-absorption coefficient for the saturated enzyme, $\Delta\varepsilon_{max}$, were obtained from a double reciprocal plot[24] of $\Delta\varepsilon$, the difference absorbance at A_{292} divided by the protein concentration, as determined spectrophotometrically[14], and the concentration of **4**.

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