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Studies of the interaction of the maltose-binding protein of *Escherichia coli*, a closed-groove binder, with 4,6-*O*-ethylidenemalto-oligosaccharides (dp 2–5) and its regioselective labelling with 3-azibutyl 1-thio-α-(6-^{3}H)maltoside

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ABSTRACT

Four malto-oligosaccharides (dp 2–5), each with a 4,6-*O*-ethylidene group on the glucosyl unit at the non-reducing terminus, were synthesised and used to prove that the maltose-binding protein (MBP) of *E. coli* is a closed-groove binder. α-D-Glucosylation of 3-azibutyl 1-thio-α-D-(6-^{3}H)glucopyranoside yielded a ^{3}H-labelled, photolabile 1-thiomaltoside derivative that was used to chemically modify the binding site of MBP. The ^{3}H-labelled peptide containing 83% of the total radioactivity, which was isolated after tryptic cleavage of the modified MBP and sequenced, is part of the closed end of the MBP groove.

INTRODUCTION

Photoaffinity labelling is a useful method for investigating the binding area of a receptor protein of which the primary and possibly the tertiary structures are known. For many proteins, no suitable single crystals of a receptor–ligand complex are available that would allow determination of the three-dimensional structure and thereby the shape and functionality of the combining area.

The maltose-binding protein (MBP) of *Escherichia coli* is part of a system of at least five proteins which are responsible for the transport of malto-oligosaccharides through the outer lipid membrane, the periplasm, and the cytoplasmic membrane into the cell [1]. The MBP plays a key role because it mediates chemotaxis by malto-oligosaccharides and determines their rate of transport. It is specific

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for maltose and higher malto-oligosaccharides and the dissociation constants [2] (K_D) are $\sim\mu$M; D-glucose has no affinity for MBP [3]. Kinetic studies using a series of 3-azi-1-methoxybutyl α- and β-glycosides of malto-oligosaccharides (dp 2–8) indicated that the α-glycosides had lower K_D values and that the affinity increased with increasing dp up to 4–5 when plateaux of 40 and 350 μM were reached for the α- and β-series, respectively (Table I). These results were interpreted to indicate a close contact of the aglycon with the protein [4].

The 3-azi-1-methoxybutyl glycosides carried a photolabile diazirino group in the aglycon and were used [4] to label MBP. However, the susceptibility of the acetal group towards hydrolysis caused excessive loss of radioactivity when fragmentation of the labelled protein was attempted [5].

We now report on the binding to MBP of malto-oligosaccharide derivatives (dp 2–5), each of which carries a 4,6-*O*-ethylidene group at the glucosyl unit at the non-reducing terminus, and describe the labelling of MBP with photolabile 3-azibutyl 1-thio-α-(6-^{3}H)maltoside.

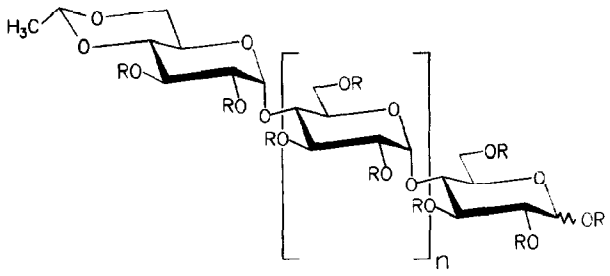
RESULTS AND DISCUSSION

Most carbohydrate-binding proteins recognise more-or-less extended structures of oligosaccharides. Judging from the specifity of MBP for malto-oligosaccharides, the protein could be a groove binder [6]. The fact that malto-oligosaccharides, modified at the reducing end, bind to MBP, but never reach the affinity of the parent malto-oligosaccharides, indicates non-binding contact of the "unnatural" structural element with the protein.

Four malto-oligosaccharide derivatives (**1**, **3**, **5**, and **6**) of dp 2–5, each with a 4,6-*O*-ethylidene group attached to the glucosyl unit at the non-reducing terminus, were synthesised and their K_D values with MBP were determined. Increase in the dp increased the affinity until, with **5** and **6**, binding constants of the same order as those for parent malto-oligosaccharides were reached [K_D values (μM): **1**, no affinity; **3**, 12; **5**, 1.8; **6**, 2.1]. These results prove MBP to be a groove binder, closed at the reducing end, but open at the non-reducing end. The minimal structural requirement for the chemically modified malto-oligosaccharides was two intact (1 → 4)-linked α-D-glucopyranose residues. Therefore, the overall binding area should correspond to approximately three such residues.

A comparison of the affinities of the 3-azi-1-methoxybutyl α-glycosides of maltose and maltotriose with those of the corresponding 3-azibutyl 1-thio-α-glycosides (**12** and **13**) [7] shows a significant increase of the affinities of the latter compounds (Table I). It is possible that the bulky and (for stereoelectronic reasons) more-rigid 3-azi-1-methoxybutyl group is accommodated less easily than the flexible 3-azibutyl group.

For the synthesis of the radioactively labelled ligands **12a–16a**, 3-azibutyl 1-thio-α-D-glucopyranoside (**7**) was 6-*O*-tritylated, then per-*O*-acetylated, and detritylated. The free hydroxyl group in compound **9** was then oxidised by methyl



1 n = 0, R = H
2 n = 0, R = Ac
3 n = 1, R = H
4 n = 1, R = Ac
5 n = 2, R = H
6 n = 3, R = H

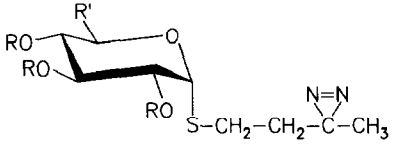
TABLE I

K_D Values of 3-azi-1methoxybutyl α- and β-maltosaccharides (dp 2–6, G_2–G_6, and **12** and **13**)

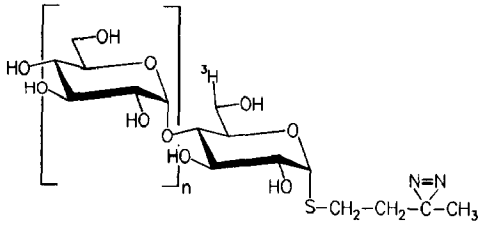
Compound	α-Series					β-Series					**12**	**13**
	G_2	G_3	G_4	G_5	G_6	G_2	G_3	G_4	G_5	G_6		
K_D value (μM)	350	100	80	40	40	500	450	350	350	350	5.7	3.1

sulfoxide–dicyclohexylcarbodiimide and the carbonyl group thus formed was reduced after *O*-deacetylation with sodium boro(^{3}H)hydride to give radiolabelled **7a**. The latter compound was glucosylated using CGT-ase and cyclomaltohexaose. The homologues **12a–16a** were isolated by HPLC.

For regioselective labelling of a binding area, it is essential that the receptor and the ligand should form only one stable complex in which the position of the



7 R = H, R' = CH_2OH
7a R = H, R' = CH^3HOH
8 R = Ac, R' = CH_2OTr
9 R = Ac, R' = CH_2OH
10 R = Ac, R' = CHO
11 R = H, R' = CHO



12a n = 1
13a n = 2
14a n = 3
15a n = 4
16a n = 5

ligand along the binding groove does not alter. With MBP, the two or three subsites towards the reducing end seem to provide most of the binding energy, because of the small difference in the K_D values of **12** and **13**. Thus, the α-glycoside of a malto-oligosaccharide will always be found with the aglycon in contact with the closed end of the groove. Therefore, covalent modification of this restricted part of the whole binding area should occur.

When **12a** was irradiated in the presence of MBP, 16% of the protein was chemically modified and radioactively labelled. The label was shown to be chemically stable and associated with the protein by SDS-PAGE (Fig. 1). Maltose protected MBP against photoaffinity labelling by **12a**. When the protein was fragmented by trypsin, HPLC (Fig. 2) of the peptides revealed that 83% of the total radioactivity was associated with one peptide. After determining the sequence of this peptide, it could be placed close to the *N*-terminus of the primary structure [8] comprising residues 7–11, where residue 12 (Asn) is likely to be the chemically modified position (Fig. 3).

At about the time these results were obtained, the structure of the MBP–maltose complex at 2.3 Å resolution was published [6]. The protein forms two domains with a groove to accommodate maltose with HO-1 touching Asp 14 and Lys 15 in the closed end of the groove. Judging from our results, the 3-azibutyl group of **12a** must lie along the backbone of Gly 13, Asn 12, and Ile 11 in order to react most probably with Asn 12.

The kinetic studies with chemically modified malto-oligosaccharides and the results of photoaffinity labelling are in accord with the three-dimensional structure of the MBP–maltose complex. These relatively simple means allow a crude description of a binding area and the localisation of peptide segments therein.

EXPERIMENTAL

General methods.—Melting points are uncorrected, optical rotations were measured with a Polartronic I instrument (Schmidt and Haensch). All reactions were monitored by TLC on Silica Gel 60 F_{254} (Merck) with the solvents indicated. Flash-column chromatography was performed on ICN silica gel (32-63, 60A). ^{1}H-NMR spectra (250 MHz) were recorded with a Bruker WM 250 spectrometer on solutions in $CDCl_3$ (internal Me_4Si). Photolabile compounds were irradiated with a Rayonet RPR reactor equipped with 16 lamps (RPR 3500 A). Kinetic data were determined with a Perkin–Elmer 650–10S fluorescence spectrometer. IR spectra were recorded with a Perkin–Elmer 1320 spectrophotometer. HPLC involved an LKB 2152 controller, three LKB 2150 pumps, a Rheodyne 7126 injector, an LKB variable wavelength monitor, and a Berthold HPLC radioactivity monitor LB 507 equipped with an 85:15 splitter and a Z-1000/4-cell scavenged with Quickszintflow 302. Radioactivity after TLC was detected using a Berthold Automatic TLC-Linear Analyzer and radioactivity in solutions was measured with

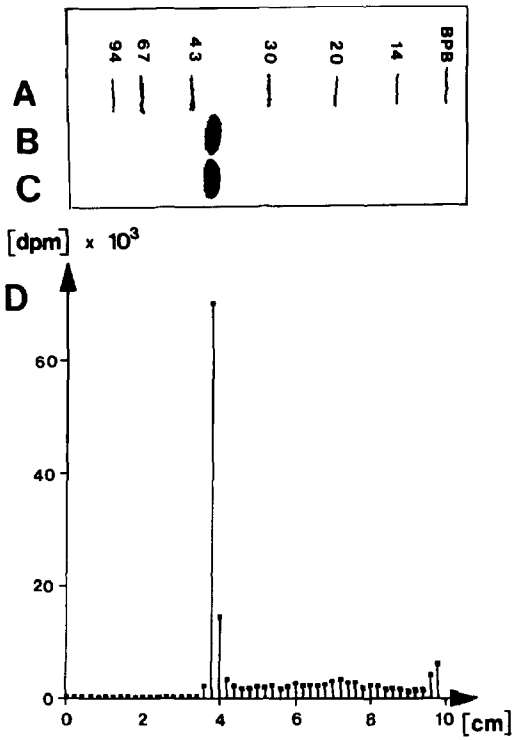


Fig. 1. SDS-PAGE of MBP: A, calibration proteins and kDa values; B, MBP; C, (^{3}H)MBP (see Experimental); D, lane C cut into strips and counted for radioactivity (see Experimental).

a Berthold BF 815 liquid scintillation counter using Quickszint 501 (Zinsser) for solutions in organic solvents and Quickszint 1 for aqueous solutions. NaB^3H_4 (100 mCi, 7 Ci/mmol) was purchased from Amersham–Buchler.

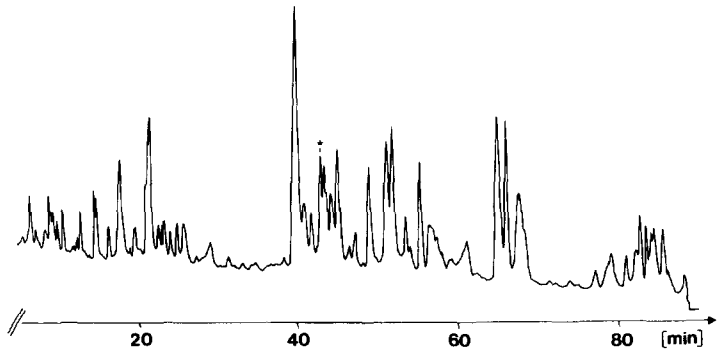


Fig. 2. HPLC (detection by UV absorption at 220 nm, see Experimental) of the mixture of peptides derived by digestion of (^{3}H)MBP with trypsin; (* designates the peptide which carried 83% of the total incorporated radioactivity).

Lys-Ile-Glu-Glu-Gly-Lys- Leu-Val-Ile-Trp-Ile-**Asn**-Gly-Asp-Lys - 15
Gly-Tyr-Asn-Gly-Leu-Ala-Glu-Val-Gly-Lys-Lys-Glu-Glu-Lys-Asp- 30
Thr-Gly-Ile-Lys-Val-Thr-Val-Glu-His-Pro-Asp-Lys-Leu-Glu-Glu- 45
Lys-Glu-Pro-Gln-Val-Ala-Ala-Thr-Gly-Asp-Gly-Pro-Asp-Ile-Ile- 60
Glu-Trp-Ala-His-Asp-Arg-Glu-Gly-Gly-Tyr-Ala-Gln-Ser-Gly-Leu- 75
Leu-Ala-Glu-Ile-Thr-Pro-Asp-Lys-Ala-Glu-Gln-Asp-Lys-Leu-Tyr- 90
Pro-Glu-Thr-Trp-Asp-Ala-Val-Arg-Tyr-Asn-Gly-Lys-Leu-Ile-Ala- 105

Fig. 3. The labelled peptide (7–15) with the potential residue for labelling (**Asn**) placed into the primary structure (first 105 amino acids from 370) of MBP.

Enzymes.—CGT-ase [(1 → 4)-α-D-glucan 4-α-D-glucanotransferase, cyclising, EC 2.4.1.19, 760 U/mL] from *Bacillus macerans* was a gift from Boehringer Mannheim; MBP, as a lyophilised powder, was a gift from Professor W. Boos; α-D-glucosidase (maltase, EC 3.2.1.20, 250 U/mL) from yeast was purchased from Boehringer Mannheim; and trypsin from bovine pancreas (TPCK-treated, 31 U/mg, research grade) was purchased from Serva.

Incubation of the 4,6-O-ethylidenemalto-oligosaccharides with α-D-glucosidase.—Separate solutions of maltopentaose and **1**, **3**, **5**, or **6** (1 mg each) in acetate buffer (250 μL, 0.1 M, pH 5.5) were incubated overnight with α-D-glucosidase (2 U, 30°). TLC (4:2:1 EtOAc–MeOH–H_2O) then showed complete degradation of maltopentaose into glucose and no degradation of the ethylidene derivatives.

Determination of the binding constants (K_D).—MBP (50 μg/mL) in Tris–HCl buffer (pH 7.4, 1 mL) was irradiated at 280 nm and the emission was recorded at 330 nm. Ligands were added as 10-μL aliquots and each concentration was measured with a newly prepared protein solution. The decrease of relative fluorescence (ΔF) was determined and each value was corrected with the ΔF for 10 μL of added buffer. The K_D values were determined by plotting ΔF (%)/log [I]. Ligands were used in the following concentrations (μM): 500, 200, 100, 50, 20, 10, 5, 2, 1, 0.5, and 0.1. The K_D values (μM), each the average of two measurements, were as follows: **1**, no affinity; **3**, 12; **5**, 1.8; **6**, 2.1; **12**, 5.7; **13**, 3.1.

Photoaffinity labelling of MBP.—A solution of MBP (2 mg, 49.4 nmol) and **12a** (185 μCi, 52.8 nmol/mL) in Tris–HCl (2 mL, 100 mM, pH 7.4) was flushed with N_2 (10 min), and the vessel was then closed and irradiated for 20 min. Dialysis against Tris–HCl (8°, 3 × 100 mL, 1 × 12 h, 2 × 2 h) and water (8°, 100 mL, 2 h), followed by freeze-drying, yielded [^{3}H]MBP-A (2 mg, 13.8 μCi, 16% of the protein labelled). MBP (1 mg) treated likewise was irradiated together with added maltose (10 mM). The [^{3}H]MBP-B obtained after dialysis contained 0.33 μCi (0.77% incorporation). Thus, maltose and **12a** occupy the same binding area in MBP.

Gel electrophoresis.—[^{3}H]MBP (~ 50 μg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%, SDS-PAGE; 0.1% SDS, 2% mercaptoethanol) [9]. The gel was stained with Serva Blue to show [^{3}H]MBP as a single band near the 43-kDa band of the calibration kit (Pharmacia). The gel was cut into slices (2 mm), Biolute-S (500 μL) was added, and after standing overnight at room

temperature, Quickszint 501 (4.5 mL) was added. After storage for 2 h at 8°, the radioactivity was determined: [^{3}H]MBP-A, 96000 dpm; [^{3}H]MBP-B, 7800 dpm (Fig. 1).

Tryptic degradation of [^{3}H]MBP-A.—A solution of [^{3}H]MBP-A (1 mg) in guanidinium HCl (1 mL, 6 M) was dialysed against water (2 × 100 mL, 2 × 1 h), the protein was centrifuged, and a suspension in NH_4HCO_3 (1 mL, 0.1 M) was treated with trypsin at 37° (15 μg, 12 h; additional 15 μg, 8 h). The peptides were subjected to HPLC (4 × 250 mm; Vydac protein and peptide C_{18}, 5 μm; MeCN–H_2O containing 5 mM trifluoroacetic acid, 750 μL/min), using the following MeCN gradient: 0–10 min, 0–10%; 10–80 min, 10–40%; 80–87 min, 40–80%; 87–90 min, 80–100%; 90–96 min, 100–0%; 80-μg samples were injected and the radiolabelled peptide was collected (42.9 min). Combined samples of the radiolabelled peptide were concentrated to yield 3.7 μCi of radiolabelled material (Fig. 2).

Edman degradation of the labelled tryptic peptide.—Automatic Edman degradation of the labelled peptide, which contained ~ 10% of impurity, was performed on an ABI 477A protein sequencer (20 degradation cycles) equipped online with an ABI 120A HPLC analyser for identification of the amino acid phenylthiohydantoins. The first three amino acids were shown to be Leu–Val–Ile. This tripeptide appears only once in the sequence of MBP and so could be placed into the primary structure of MBP as shown in Fig. 3. The next amino acid, Trp, could not be detected in cycle 4, since it was modified during the previous manipulations. The following amino acid (Ile) was detected in cycle 5, as the last amino acid of the labelled peptide. This makes residue 6 (Asn) a candidate as the labelled amino acid. This assumption is supported by the fact that no radioactive material was detectable in the extract of all cycles and that by extracting the glass-fibre support with water (250 μL) ~ 1 μCi of radioactive material was measured. TLC showed the radioactive material not to be a 1-thio-α-maltoside, and HPLC yielded a retention time for the radioactive material of 85 min under the conditions where that for the labelled peptide was 42.9 min. It appears that the modification prevented further Edman degradation.

4′,6′-O-Ethylidenemaltose (**1**).—A solution of maltose (1 g, 2.78 mmol) and *p*-toluenesulfonic acid · H_2O (330 mg, 1.73 mmol) in dry *N*,*N*-dimethylformamide (33 mL) was concentrated to dryness. To a solution of the syrupy residue in dry *N*,*N*-dimethylformamide (25 mL) was added acetaldehyde dimethyl acetal (0.6 mL, 5.5 mmol) [10], the mixture was stirred at 50° for 22 h in a closed vessel, then concentrated, and a solution of the residue in water (15 mL) was neutralised with LiOH, then concentrated. Flash-column (2 × 15 cm) chromatography (5:1 EtOAc–MeOH) of the residue gave amorphous **1** (695 mg, 68%), R_F 0.37 (7:2:1 EtOAc–MeOH–H_2O), $[\alpha]_D$ +113° (*c* 1, H_2O).

Treatment of **1** (100 mg, 0.27 mmol) with 1:2 Ac_2O–pyridine (3 mL) gave the amorphous 1,2,3,6,2′,3′-hexa-acetate **2** (155 mg, 91%), R_F 0.45 (1:2 cyclohexane–EtOAc, $[\alpha]_D$ +61° (*c* 1, $CHCl_3$). ^{1}H-NMR data: δ 6.23, 5.73 (2 d, 1 H, $J_{1\alpha\beta,2}$ 3.75

and 8.25 Hz, H-1α,β), 5.50, 5.3–5.4 (dd, m, 1 H, $J_{3\alpha,4}$ 8.4 Hz, H-3α,β), 5.25–5.4 (m, 1 H, H-3′), 5.33, 5.30 (2 d, 1 H, $J_{1'\alpha,\beta,2'}$ 4.2 and 3.9 Hz, H-1′α,β), 4.97, 4.96 (2 dd, 1 H, $J_{2\alpha,\beta,3}$ 10.2 and 9.45 Hz, H-2α,β), 4.83, 4.81 (2 dd, 1 H, $J_{2'\alpha,\beta,3'}$ 10.05 and 10.35 Hz, H-2′α,β), 4.65, 4.64 (2 q, 1 H, *J* 5.25 Hz, MeC*H*α,β), 4.50 (m, 1 H, H-6b), 4.25 (m, 1 H, $J_{6a,6b}$ 12.6 Hz, H-6a), 3.88–4.18 (m, 1 H, H-4′), 4.07, 4.0–4.15 (t, m, 1 H, $J_{4\alpha,5}$ 10.5 Hz, H-4α,β), 3.83 (m, 2 H, $J_{5,6a,b}$ 2.25 and 3.9, $J_{5',6'a,b}$ 3.0 and 3.75 Hz, H-5,5′), 3.48 (dt, 1 H, $J_{6'a,6'b}$ 9.75 Hz, H-6′b), 3.38 (dt, 1 H, H-6′a), 2.23, 2.11 (2 s, 3 H, AcO-1α,β), 1.96–2.14 (15 H, 5 OAc), 1.32 (2 d, 3 H, *Me*CH).

Anal. Calcd for $C_{26}H_{36}O_{17}$: C, 50.32; H, 5.85; Found: C, 51.09; H, 5.88.

4″,6″-O-Ethylidenemaltotriose (**3**).—A solution of maltotriose (1 g, 1.98 mmol) and *p*-toluenesulfonic acid · H_2O (277 mg, 1.45 mmol) and acetaldehyde dimethyl acetal (0.4 mL, 3.7 mmol) in dry *N,N*-dimethylformamide (17 mL) was treated, as described for maltose, to yield, after flash-column (2 × 15 cm) chromatography (7:2:1 EtOAc–MeOH–H_2O), amorphous hygroscopic **3** (523 mg, 50.5%), R_F 0.45 (4:2:1 EtOAc–MeOH–H_2O), $[\alpha]_D$ +98° (*c* 0.8, H_2O).

Treatment of **3** (80 mg, 0.16 mmol) with 1:2 Ac_2O–pyridine (2 mL) gave the 1,2,3,6,2′,3′,6′,2″,3″-nona-acetate **4** as a white solid (128 mg, 88%), R_F 0.34 (1:2 cyclohexane–EtOAc), $[\alpha]_D$ +59° (*c* 0.83, $CHCl_3$). ^{1}H-NMR data: δ 6.25, 5.75 (2 d, 1 H, $J_{1\alpha,\beta,2}$ 3.75 and 8.25 Hz, H-1α,β), 5.52 (dd, 0.5 H, $J_{3\alpha,4}$ 8.4 Hz, H-3α), 5.24–5.45 (m, 4.5 H, H-1′,1″,3β,3′,3″), 4.96, 4.95 (2 dd, 1 H, $J_{2,3\alpha,\beta}$) 10.05 and 8.25 Hz, H-2α,β), 4.7–4.83 (m, 2 H, H-2′,2″), 4.65 (q, 1 H, *J* 5.1 Hz, MeC*H*), 4.45–4.6 (m, 2 H, H-6b, 6′b), 3.83–4.34 (m, 7 H, H-4,4′,4″,5,5′,6a,6′a), 3.67 (dt, 1 H, $J_{5'',6''a,b}$ 10.2 Hz, H-5″), 3.48 (t, 1 H, H-6″b), 3.37 (t, 1 H, H-6″a), 1.94–2.25 (27 H, 9 Ac), 1.33 (d, 3 H, *Me*CH).

Anal. Calcd for $C_{38}H_{52}O_{25}$: C, 50.22; H, 5.77. Found: C, 50.92; H, 5.93.

4‴,6‴-O-Ethylidenemaltotetraose (**5**).—A solution of maltotetraose (250 mg, 0.38 mmol), *p*-toluenesulfonic acid · H_2O (43 mg, 0.22 mmol), and acetaldehyde dimethyl acetal (78 μL, 72 mmol) in dry *N,N*-dimethylformamide (3.5 mL) was treated, as described for maltose, to yield, after flash-column (1.5 × 15 cm) chromatography (5:2:1 EtOAc–MeOH–H_2O), amorphous hygroscopic **5** (134 mg, 51%), R_F 0.3 (4:2:1 EtOAc–MeOH–H_2O), $[\alpha]_D$ +135° (*c* 1, H_2O).

4⁗,6⁗-O-Ethylidenemaltopentaose (**6**).—A solution of maltopentaose (200 mg, containing cyclomaltohexaose), *p*-toluenesulfonic acid · H_2O (28 mg, 0.15 mmol), and acetaldehyde dimethyl acetal (50 μL, 46 mmol) in dry *N,N*-dimethylformamide (2 mL) was treated, as described for maltose, to yield, after flash-column (1 × 15 cm) chromatography (5:2:1 EtOAc–MeOH–H_2O), amorphous hygroscopic **6** (35 mg, 42 μmol), R_F 0.2 (4:2:1 EtOAc–MeOH–H_2O), $[\alpha]_D$ +60° (*c* 0.5, H_2O).

3-Azibutyl 2,3,4-tri-O-acetyl-1-thio-6-O-triphenylmethyl-α-D-glucopyranoside (**8**).—To a solution of **7** [7] (1 g, 3.6 mmol) in pyridine (10 mL) was added chlorotriphenylmethane (2.2 g, 7.2 mmol), and the solution was stirred overnight at room temperature. TLC then showed the formation of the 6-*O*-trityl derivative (R_F 0.63, 5:1 EtOAc–MeOH). Pyridine (10 mL) and acetic anhydride (5 mL) were added,

and the solution was stored at room temperature for 20 h, then poured onto crushed ice (200 mL), stirred vigorously for 1 h, and extracted with CH_2Cl_2 (3 × 200 mL). The combined extracts were washed with satd aq $NaHCO_3$ (5 × 100 mL) and water (100 mL), dried (Mg_2SO_4), and concentrated. Flash-column (2 × 15 cm) chromatography (4:1 cyclohexane–EtOAc) of the residue and crystallisation from EtOH yielded **8** (1.8 g, 77.8%), R_F 0.36 (2:1 cyclohexane–EtOAc), mp 115°, $[\alpha]_D$ +161° (*c* 0.93, $CHCl_3$). ^{1}H-NMR data: δ 7.42–7.2 (m, 15 H, 3 Ph), 5.73 (d, 1 H, $J_{1,2}$ 5.4 Hz, H-1), 5.33 (dd, 1 H, $J_{3,4}$ 10.05 Hz, H-3), 5.06 (dd, 1 H, $J_{4,5}$ 10.2 Hz, H-4), 5.05 (dd, 1 H, $J_{2,3}$ 10.05 Hz, H-2), 4.33 (ddd, 1 H, $J_{5,6a}$ 4.95, $J_{5,6b}$ 2.55 Hz, H-5), 3.20 (dd, 1 H, $J_{6a,6b}$ 10.2 Hz, H-6b), 3.13 (dd, 1 H, H-6a), 2.39–2.51 (m, 2 H, H-1′,1′), 2.08, 1.92 (2 s, each 3 H, 2 OAc), 1.70 (s, 3 H, AcO-4), 1.68 (t, 2 H, $J_{1',2'}$ 7.5 Hz, H-2′,2′), 1.0 (s, 3 H, H-4′,4′,4′).

Anal. Calcd for $C_{35}H_{38}N_2O_8S$: C, 65.00; H, 5.92. Found: C, 64.92; H, 5.84.

3-Azibutyl 2,3,4-tri-O-acetyl-1-thio-α-D-glucopyranoside (**9**).—To a well-stirred solution at 0° of **8** (1.3 g, 2 mmol) and NaI (1.1 g, 7.3 mmol) in MeCN (20 mL) was added chlorotrimethylsilane [11] (910 μL). When TLC indicated the absence of **8** (15 min), water (5 mL) was added to the ice-cooled mixture, which was stirred for 5 min, then poured into cold water (200 mL), and extracted with Et_2O (4 × 150 mL). The combined extracts were washed with aq satd $Na_2S_2O_3$ (2 × 150 mL), aq $NaHCO_3$ (150 mL), and then water (150 mL), dried (Mg_2SO_4), and concentrated. Flash-column (1.5 × 15 cm) chromatography (1:1 cyclohexane–EtOAc) of the residue and crystallisation from Et_2O–hexane yielded **9** (650 mg, 80%), R_F 0.53 (5:1 cyclohexane–EtOAc), mp 96°, $[\alpha]_D$ +176° (*c* 0.92, $CHCl_3$); ν_{max}^{KBr} 3520 cm^{-1} (OH). ^{1}H-NMR data ($CDCl_3$): δ 5.66 (d, 1 H, $J_{1,2}$ 6.0 Hz, H-1), 5.42 (t, 1 H, $J_{3,4}$ 10.05 Hz, H-3), 5.02 (t, 1 H, $J_{4,5}$ 10.05 Hz, H-4), 4.99 (dd, 1 H, $J_{2,3}$ 10.05 Hz, H-2), 4.18 (m, 1 H, H-5), 3.78 (m, 1 H, $J_{6a,6b}$ 10.2 Hz, H-6b), 3.57 (m, 1 H, H-6a), 2.31–2.52 (m, 2 H, H-1′,1′), 2.11, 2.08, 2.05 (3 s, each 3 H, 3 OAc), 1.66 (t, 2 H, $J_{1',2'}$ 7.5 Hz, H-2′,2′), 1.05 (s, 3 H, H-4′,4′,4′).

Anal. Calcd for $C_{16}H_{24}O_8N_2S$: C, 47.52; H, 5.98; Found: C, 47.91; H, 5.94.

3-Azibutyl 1-thio-α-D-(6-^{3}H)glucopyranoside (**7a**).—To a solution of **9** (500 mg, 1.24 mmol) and dicyclohexylcarbodiimide (800 mg, 3.9 mmol) in Me_2SO (15 mL) was added a trace of anhyd H_3PO_4. The mixture was stirred overnight at room temperature, then poured into satd aq NaCl (250 mL), and extracted with Et_2O (3 × 150 mL). The combined extracts were washed with satd aq $NaHCO_3$ (200 mL) and water (150 mL), then dried ($CaCl_2$), and concentrated. The residue was suspended in cyclohexane–EtOAc (1:1, 150 mL), filtered, and concentrated. Flash-column (1 × 12 cm) chromatography (1:1 cyclohexane–EtOAc) of the residue gave the aldehyde **10** (250 mg, 50%), R_F 0.2 (1:1 cyclohexane–EtOAc), ν_{max}^{KBr} 3600–3220 cm^{-1} (CHO). *O*-Deacetylation (10 mL of MeOH, 100 μL of methanolic M NaOMe) of **10** overnight gave, after flash-column (1 × 12 cm) chromatography (27:2:1 EtOAc–MeOH–H_2O), **11** (160 mg). To a solution of **11** (5 mg) in aq NaOH (1 mg/500 μL) was added $NaBH_4$ (5 mg), to yield 3-azibutyl 1-thio-α-D-glucopyranoside (**7**, characterised by comparison with an authentic sample [7]). To a

solution of **11** (155 mg, 0.56 mmol) in water (1 mL) was added aq 4% NaOH (20 μL). This solution was added to NaB^3H_4 (100 mCi, 7 Ci/mmol), and the mixture was stored overnight at room temperature, then evaporated. Excess of aldehyde was reacted overnight at room temperature and then for 1 h at 40° with Wanzlick's reagent [12] (1,2-dianilinoethane, 0.5 g, 2.36 mmol) in MeOH (10 mL) containing HOAc (50 μL) as catalyst. Column (1 × 15 cm) chromatography (27:2:1 EtOAc–MeOH–H_2O) of the residue twice yielded **7a** (55 mCi, 1.75 Ci/mmol, 55%), which was characterised by comparison with unlabelled **7** [7].

3-Azibutyl 1-thio-α-(6-^{3}H)maltoside (**12a**), *3-azibutyl 1-thio-α-(6-^{3}H)maltotrioside* (**13a**), *3-azibutyl 1-thio-α-(6-^{3}H)maltotetraoside* (**14a**), *3-azibutyl 1-thio-α-(6-^{3}H)maltopentaoside* (**15a**), and *3-azibutyl 1-thio-α-(6-^{3}H)maltohexaoside* (**16a**).—A solution of **7a** (50 mCi, 8 mg, 28.6 μmol) and cyclomaltohexaose (α-cyclodextrin, 10 mg, 10 μmol) in water (1 mL) was incubated with CGT-ase (10 μL, 7.6 U) at room temperature for 4 h, when TLC indicated that equilibrium had been reached. The mixture was freeze-dried, and column (1.5 × 10 cm) chromatography (5:2:1 EtOAc–MeOH–H_2O) of the residue yielded **7a** (30 mCi, 60%). Elution with 3:1 MeOH–H_2O gave the homologues (**12a–16a**).

Compound **7a** was treated as described above (1 mL of water, 10 mg of α-CD, 20 μL of CGT-ase, 4 h). The mixture was freeze-dried, and a solution of the residue in water (1 mL) was combined with the above homologues and kept frozen. The radiolabelled homologues were isolated by HPLC on a column (4.6 × 250 mm, 1 mL/min) of ODS hypersil (5 μm), using the following gradient of MeOH in H_2O: 0–5 min, 0–20%; 5–25 min, 20–30%; 25–30 min, 30–35%; 30–35 min, 35–100%; 35–45 min, 100–0%; and yielded (mCi): **7a** 11.9, **12a** 14.5, **13a** 6.1, **14a** 3.9, **15a** 1.8, **16a** 0.6. Retention times (min): **7a** 21.1, **12a** 22.6, **13a** 19.0, **14a** 15.2, **15a** 13.8, **16a** 13.4. R_F values: **7a** 0.63, **12a** 0.48, **13a** 0.34, **14a** 0.21, **15a** 0.09, **16a** 0.04 (5:2:1 EtOAc–MeOH–H_2O). All ^{3}H-labelled homologues were compared by autoradiography with the corresponding unlabelled compounds in order to prove their identity.

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