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Differential chemical modification of substrate binding areas in porcine-pancreatic alpha-amylase by three regioisomeric photolabile ligands

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Abstract

Three regioisomeric radiolabelled spacer-modified oligosaccharides: methyl 4′-*O*-[4-*S*-(3-azi-4-α-D-glucopyranosyloxy-1-[^{3}H]butyl)-6-deoxy-4-thio-α-D-*xylo*-hex-5-enopyranosyl]-α-maltoside (**12a**, G1-G3*), methyl 4-*O*-[4-*S*-(3-azi-4-α-maltosyloxy-1-[^{3}H]butyl)-6-deoxy-4-thio-α-D-*xylo*-hex-5-enopyranosyl]-α-D-glucopyranoside (**15a**, G2-G2*) and methyl 4-*S*-(3-azi-4-α-maltotriosyloxy-1-[^{3}H]butyl)-6-deoxy-4-thio-α-D-*xylo*-hex-5-enopyranoside (**16a**, G3-G1*) were synthesised and used as photoaffinity probes for the chemical modification of porcine-pancreatic alpha-amylase (PPA). Incorporation of covalently attached radioactivity amounted to 25–38% of the stoichiometric value. Tryptic digestion of the three labelled protein preparations PPA-G1-G3*, PPA-G2-G2*, and PPA-G3-G1* and the purification of the labelled peptides by fractional HPLC yielded altogether six pure components. On the basis of the published three-dimensional structure peptides G1-G3-II, G2-G2-II, and G2-G2-III were part of the catalytic site. G1-G3-I and G2-G2-I were part of the surface binding site. The major component derived from PPA, labelled by G3-G1*, corresponded to an area that is neither close to the active site nor to the surface starch-binding domain, which clearly indicates the presence of a third, hitherto undetected, substrate-binding site.

1. Introduction

Porcine-pancreatic alpha-amylase (PPA) is supposed to bind (1 → 4)-α-D-glucopyrano-oligosaccharides (maltodextrins) at its active center, which accommodates

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five D-glucosyl units. The hydrolytic cleavage of the glycosidic bond takes place between the second and the third unit, counting from the aglyconic end [1]. The amino acid sequence of two equally active isozymes, differing only insignificantly, are known [2], and the three-dimensional structure of isozyme 1 is known at 2.1 Å resolution [3]. A groove of 30-Å length corresponds well with the quoted five-subsite model of PPAs active site. A second, so-called starch-binding domain was also found, ~ 20 Å from the active site [4].

Although X-ray analysis is the optimal method for elucidating structural details of the receptor-binding area and locating a potential catalytic site, the relatively simple method of photoaffinity labelling may be adequate in such cases where only the three-dimensional protein structure and not the structure of the protein–ligand complex is known. The selectivity of chemical modification by photoaffinity labelling of carbohydrate-binding proteins has been demonstrated in two cases [5]; in one, the regiospecific labelling of the *E. coli* maltose-binding protein (MBP), was corroborated by a high resolution X-ray structural analysis of a MBP-maltose complex [6].

In a previous publication we described the preparation of three photolabile, isomeric, so-called spacer-modified tetrasaccharides G1-G3 (**12**), G2-G2 (**15**), and G3-G1 (**16**), which mimic the structure of maltopentaose, the supposedly optimal ligand to fully occupy the catalytic binding area of PPA. The three compounds are stable against enzymic degradation and competitively and effectively inhibit the hydrolysis of 4-nitrophenyl α-maltotrioside. Irradiating PPA in the presence of compound **12**, **15**, or **16** irreversibly deactivates the enzyme up to 98% without changing the K_M of the remaining active protein molecules [7]. Assuming for each protein–ligand complex the same binding mode AI, AII, and AIII [or other binding modes if a second, equally large binding-site is occupied, e.g., BI, BII, and BIII (Fig. 1)], the chemical modification by photoaffinity labelling ought to occur in differing areas because of the differently placed photolabile group in the ligand molecule. In this paper, we describe the chemical modification of the protein by radioactive ligands and the identification of the labelled peptide fragments.

2. Results

Syntheses of radioactive ligands — The assembly of the three radiolabelled ligands (**12a**, **15a**, and **16a**) is carried out by coupling 2-azi-4-benzoylthio-[^{3}H]butyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**5**) with three different, reactive aglyconic ends G3, G2, and G1 (**9**–**11**) [7] through thiolate alkylation to yield G1-G3* (**12a**), G1-G2* (**13**), and G1-G1* (**14**). Extending the glyconic end for the preparation of G2-G2* (**15a**) and G3-G1* (**16a**) is carried out by enzymic glucosylation of G1-G2* (**13**) and G1-G1* (**14**), respectively, using CGTase and α-cyclodextrin [8]. The radioactive label is conveniently introduced into the aglycon of **5** by oxidizing the suitably blocked glucoside **1** and subsequent reduction of the aldehyde (**2**) with sodium [^{3}H] borohydride. All further reactions take place in reasonable yields and are described in detail in the Experimental section.

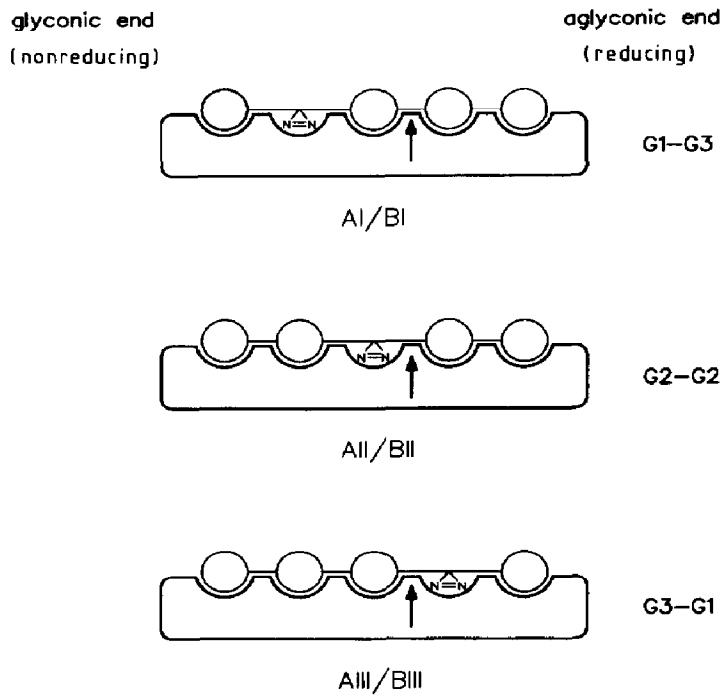
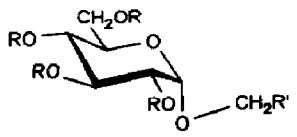


Fig. 1. Schematic presentation of PPA active site (A) and the starch-binding domain (B) with the three spacer-modified tetrasaccharides: ↑ indicates the catalytic site in A.

Chemical modification of PPA by radioactive ligands — Ligands G1-G3* (**12a**), G2-G2* (**15a**), and G3-G1* (**16a**), each with a specific radioactivity of 2.2 Ci/mmol, were incubated in a 5-fold molar excess for **12a**, a 73-fold molar excess for **15a**, and a 76-fold molar excess for **16a** with PPA. After irradiating the mixtures at 350 nm, the protein was denatured and carboxymethylated. The solution was exhaustively dialysed against TEA-buffer (50 mM, pH 7) and then against water. At regular intervals the dialysate was exchanged against fresh buffer until radioactivity in the former reached a plateau of ~ 22 000 cpm/mL. The radioactivity of the protein suspension after exhaustive dialysis slightly exceeds the amount expected according to former deactivation experiments [7] (Table 1). More radioactivity, associated with low-molecular-weight material, is eventually lost on rigorous purification of the protein. Loss is due to noncovalent association of radioactivity with the denatured protein and labile covalent bonds, such as ester links that hydrolyse during prolonged dialysis, and especially so when the sample is submitted to denaturing gel electrophoresis or to conditions of tryptic digestion. Stable, covalently bound radioactivity (Table 2) accounts in all cases for less than half the stoichiometric amount. For G1-G3* and G2-G2* the radioactive modification corresponds to ~ 38% of the irreversible deactivation found in previous experiments by determining enzymic activity kinetically after irradiation with nonradioactive ligands (Table 1) [7]. Using G3-G1*, the radioactive modification is ~ 62% of the irreversible deactivation found in previous experiments with nonradioactive ligand (Table 1) [7].



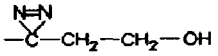
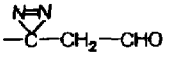
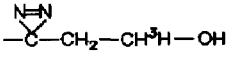
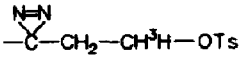
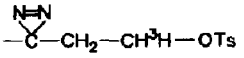
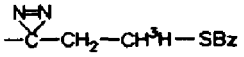
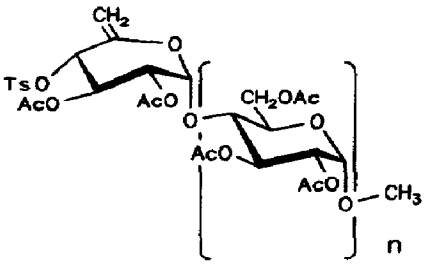
	R	R'
1	Bn	
2	Bn	
1a	Bn	
3	Bn	
4	Ac	
5	Ac	

Table 1
Irreversible inactivation of alpha-amylase with spacer-modified tetrasaccharides

Compound	**12**	**15**	**16**
Remaining activity (%)	3.6	1.9	60

Table 2
Stable, covalently bound radioactivity from the ligands **12a**, **15a**, and **16a**

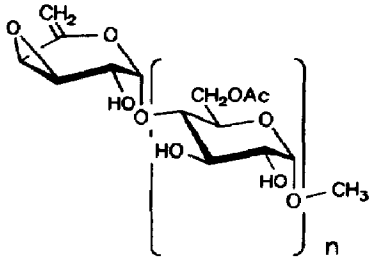
Compound	**12a**	**15a**	**16a**
Total radioactivity (%) covalently attached to protein in SDS-gel	35	38	25
μCi/mg protein	14	15.5	9.8
Yield of radioactivity (% of stoichiometric amount) of isolated, pure tryptic petides	G1-G3-I 20 G1-G3-II 6	G2-G2-I 12 G2-G2-II 3 G2-G2-III 50	G3-G1-I 30



6 n = 2

7 n = 1

8 n = 0

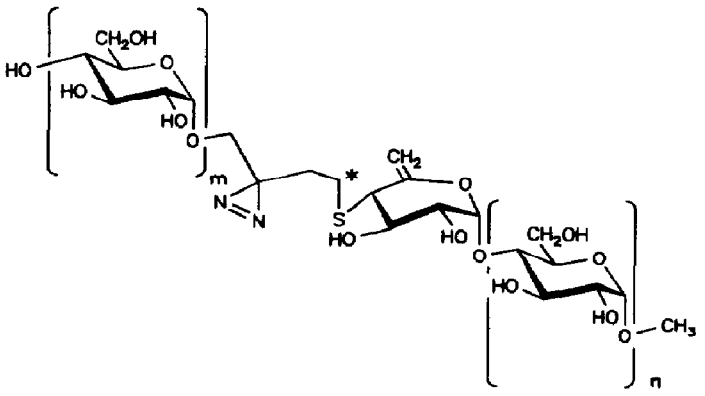


9 n = 2

10 n = 1

11 n = 0

Degradation of radiolabelled PPA — Each of the three radiolabelled protein samples PPA-G1-G3*, PPA-G2-G2*, and PPA-G3-G1* was digested with trypsin after denaturation and carboxymethylation with sodium iodoacetate. The conditions for total degradation were determined using an unlabelled protein sample. Repeated addition of trypsin was necessary for complete breakdown into a standard mixture of tryptic peptides. The HPLC elution pattern of a mixture of tryptic peptides from unlabelled PPA, monitored at 220 nm, is shown in Fig. 2. The profiles found for the tryptic mixture, containing the radiolabelled peptides, were almost indistinguishable. Splitting the eluate for parallel determination of radioactivity in a flow cell, reveals labelled compounds — not necessarily peptides — in certain areas where superposition with single peaks for peptides is not possible because of poor resolution of radioactivity. Separated compounds become again mixed in the 2-mL flow cell, and this is responsible for the very



12	m = 1	n = 2	* = H
12a	m = 1	n = 2	* = [^{3}H]
13	m = 1	n = 1	* = [^{3}H]
14	m = 1	n = 0	* = [^{3}H]
15	m = 2	n = 1	* = H
15a	m = 2	n = 1	* = [^{3}H]
16	m = 3	n = 0	* = H
16a	m = 3	n = 0	* = [^{3}H]

broad radioactivity signals. Whereas radioactivity from PPA-G1-G3* and PPA-G2-G2* is distributed in the earlier part of the elution profile (Fig. 2) showing a certain similarity, the radioactivity pattern of the eluate from PPA-G3-G1* appears significantly different.

Isolation and identification of radiolabelled peptides — The crude localisation of radioactivity in the elution profile (Fig. 2) allows the collection of peptide fractions that contain most of the radioactivity of a given sample. These radioactively enriched samples were rechromatographed and fractions collected, corresponding to observable UV-signals. In each fraction, radioactivity was determined by batchwise LSC-counting. After repeated fractionation under different HPLC conditions, solutions were obtained that contained only one radioactive peptide.

3. Discussion

Although photoaffinity labelling, using short-lived carbenes generated on irradiation from diazirines, causes indiscriminate chemical modification of the immediate chemical proximity, the extent of covalent-bond formation and the stability of the bond after it has

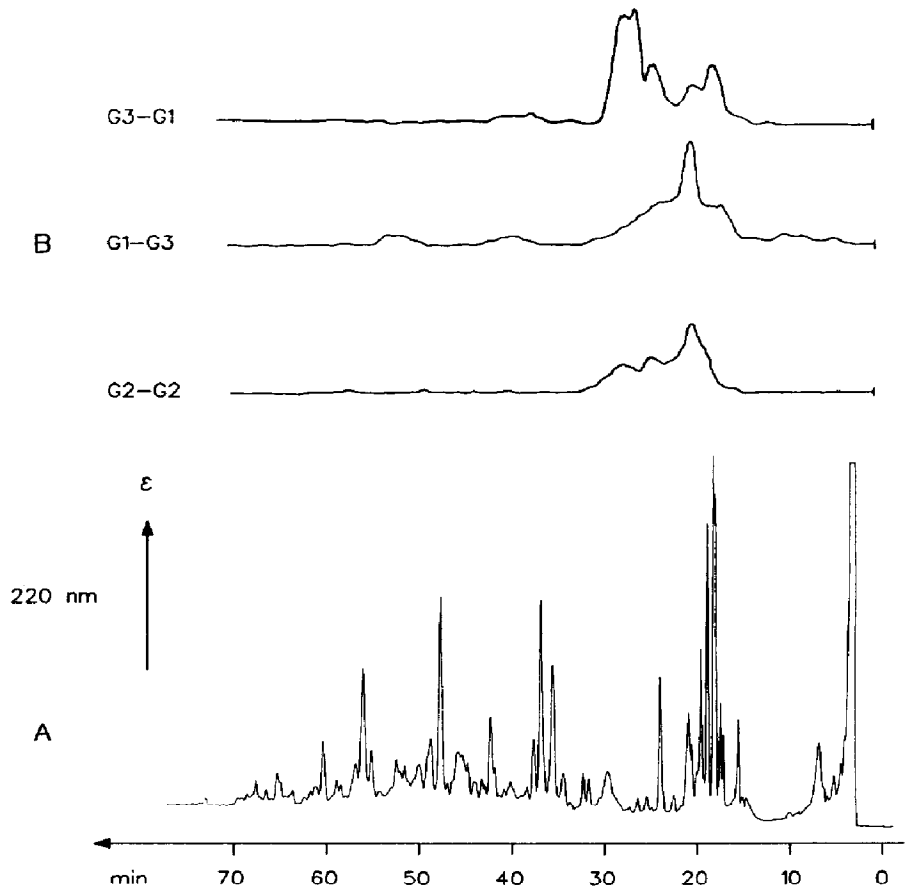


Fig. 2. Elution profile of HPLC separation of tryptic peptide mixtures derived from PPA labelled with G1-G3*, G2-G2*, and G3-G1*. Peptides are detected at 220 nm (A) and with a flow scintillation cell (B).

been formed depends on inherent factors of uncertainty such as distance and chemical reactivity of the neighbouring structures. Therefore, quantitative deductions based on the extent of chemical modification are not permissible. A low or even undetectable yield of a labelled peptide does not mean that the initial noncovalent interactions between the photolabile ligand and a certain binding area on the enzyme were insignificant. In our experience, nonspecific random labelling will not be detected as a single peptide with a radioactivity of 0.001%, or more, above the total. Isolated, radiolabelled peptides are therefore proof for a direct contact of the ligands reactive group with that section of the protein. Keeping these provisions in mind, the six isolated, uniform peptides could only be detected because of an intimate noncovalent contact of the corresponding reactive ligands with a specific binding area on the enzyme.

Qian et al. [3] have demonstrated that the catalytic groove area is made up of amino acids that appear as part of the structure of eight different tryptic peptides (Fig. 3, A), where Glu 233 and Asp 300 are supposed to be responsible for general base and acid catalysis [11]. According to the crude schematic presentation (Fig. 1), ligand G1-G3*

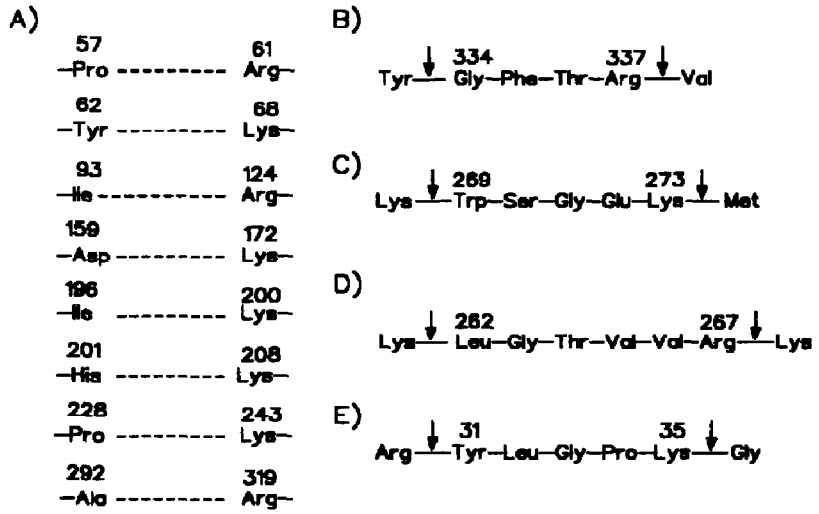


Fig. 3. (A) Amino acid sequence of tryptic peptides containing residues which appear to interact with the bound substrate [3]. (B) Amino acid sequence of G1-G3-I and G2-G2-I. (C) Amino acid sequence of G1-G3-II and G2-G2-II. (D) Amino acid sequence of G2-G2-III. (E) Amino acid sequence of G3-G1-I.

ought to label the part furthest from the glyconic [1] (nonreducing) end of the catalytically active area. This apparently involves the minor one of the two isolated peptides G1-G3-II, since that sequence belongs to one of the parts of the primary structure previously determined as forming the groove for the active site. As in that region only this one peptide could be detected, one can assume a direct, "on the spot" labelling by the spacer part of the ligand. The major peptide G1-G3-I is removed from the catalytic area and, according to the three-dimensional structure belongs to a second, noncatalytic binding site, the so-called starch-binding domain, for which no kinetic parameters and no structural details are known [3]. Therefore, the overall positioning of the ligand in the complex BI cannot be described. As G2-G2* labels the same peptide, although to a lesser extent, a certain similarity between the binding modes BI and BII (Fig. 1) may be assumed. The active-site groove is optimally labelled by G2-G2*, where the spacer, according to the schematic presentation AII, is situated close to the catalytic site. Two peptides, G2-G2-III, and to a much lesser degree G2-G2-II, situated in line, adjacent to each other, become labelled (Fig. 3). It may be speculated that the binding area to the glyconic end of the catalytic site is made up by the amino acids running from 267 to 273 in a direction towards the glyconic end of the bound ligand.

As a total surprise we found almost exclusive labelling of peptide G3-G1-I by the third spacer-modified oligosaccharide G3-G1* (Fig. 3). In the three-dimensional structure, this peptide is far removed from both the catalytic groove and from the second, so-called starch-binding domain. The result clearly indicates the presence of a third substrate-binding domain thus far undetected, and whose function is unknown. Equally

[1] The sequence of a glycan structure has been described by several terms, such as glyconic/aglyconic, west/east, nonreducing/reducing, or glycosidic/nonglycosidic end, of which we employ the first expression.

surprising is the fact that G3-G1* did not significantly label either the active site or the starch-binding domain (Fig. 3). This is difficult to explain if one considers "normal" competitive inhibition of enzyme activity by G3-G1 [7]. It may be significant, however, that G3-G1 by far is the least effective ligand for irreversibly deactivating the enzyme (Table 1).

4. Experimental

General methods.—Optical rotations were measured with a Polartronic I instrument (Schmidt und Haensch). All reactions were monitored by TLC on Silica Gel 60 F_{254} (E. Merck) with the solvents indicated. Flash-column chromatography was performed in ICN silica gel (32–63, 60A). ^1H NMR spectra (250 MHz) were recorded with a Bruker WM 250 spectrometer for solutions in $CDCl_3$ (internal Me_4Si). Photolabile compounds were irradiated with a Rayonet RPR reactor equipped with 16 lamps (RPR 3500 A). HPLC involved an LKB 2152 controller, two 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 Quickszint flow 302. Radioactivity after TLC was detected using a Berthold Automatic TLC-Linear Analyzer and radioactivity in solutions was measured with a Berthold BF 815 liquid scintillation counter using Quickszint 501 (Zinsser) for solutions in organic solvents and Quickszint 1 (Zinsser) for aqueous solutions. NaB^3H_4 (250 mCi, 8.8 Ci/mmol) was purchased from Amersham-Buchler.

Enzymes.—CGTase [(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; pancreatic alpha-amylase [(1 → 4)-α-D-glucan glucanohydrolase, EC 3.2.1.1, 6.5 mg/mL] Isoenzyme I from porcine pancreas was purified as described in Ref. [9]; beta-amylase [(1 → 4)-α-glucan maltohydrolase, EC 3.2.1.2, 2500 U/mL] from *Ipomoea batatas* was purchased from Boehringer Mannheim; and trypsin from bovine pancreas (TPCK-treated, 31 U/mg, research grade) was purchased from Serva.

Photoaffinity labelling of PPA.—A solution of PPA in 50 mM triethanolamine–triethanolamine · HCl buffer (pH 7.0, 10 mM $CaCl_2$) was flushed with N_2, and the vessel was then closed and irradiated for 12 min at 350 nm.

Gel electrophoresis.—PPA was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%; 0.1% SDS, 2% mercaptoethanol) [10]. The gel was stained with Serva Blue and a calibration kit (Pharmacia) was used. The gel was cut into slices (2 mm), Biolute-S (500 μL) was added, and after standing for 5 h at 30°C, Quickszint 501 (4.5 mL) was added. After storage for 2 h at 8°C, the radioactivity was determined by LSC.

Reduction and carboxymethylation of [^{3}H]PPA.—To the solution of [^{3}H]PPA was added 700 μL of 0.5 M Tris-HCl buffer (pH 7.5; 6 M guanidinium HCl, 5 mM EDTA, and 7 mM dithioerythritol) and then kept for 6 h at room temperature. Sodium iodoacetate (5 mg, 24 μmol) was added in the dark, followed, after 40 min, by 2-mercaptoethanol (100 μL). The protein was precipitated by dialysis against H_2O (4 × 400 mL) and collected by centrifugation.

Treatment of [^{3}H]PPA with trypsin.—A suspension of the reduced and carboxymethylated enzyme in 0.2 M $NaHCO_3$ buffer (1 mL) was treated with trypsin 1% for 6 h at 37°C. An additional amount of trypsin was then added and the digestion was continued for 10 h. A similar amount of unlabelled PPA was treated in the same way and the cleavage monitored by reversed-phase HPLC on a column (4.6 × 250 mm) of C-18 (VYDAC 218 TP) with MeCN–H_2O containing 10 mM CF_3CO_2H at 1 mL/min. The MeCN gradient was as follows: 0–10 min, 0–10%; 10–80 min, 10–40%; 80–90 min, 40–100%. The peptides were monitored at 220 nm.

Separation of the [^{3}H]-labelled peptides.—The [^{3}H]-labelled peptides were isolated from 90 μg samples by HPLC as described above. To prove homogeneity of the isolated peptides a different gradient and different eluents were used: eluent *A* (MeCN–phosphate buffer, pH 6, 5 mM, 80:20), eluent *B* (phosphate buffer, pH 6.5, 5 mM). The *A* gradient was as follows: 0–10 min, 0–12%; 10–80 min, 12–50%; 80–90 min, 50–100%. The [^{3}H]-labelled peptides were freeze-dried and stored at −20°C.

Edman degradation of the labelled tryptic peptides.—For sequence analysis, peptides were dissolved in 25 μL 30% MeCN, 0.1% CF_3CO_2H in water and applied to a pulsed-liquid gas-phase sequencer (Applied Biosystems Inc., model 477A, equipped with on-line HPLC analysis of the phenylthiohydantoin amino acids, model 120A).

*2-Azi-4-oxobutyl-2,3,4,6-tetra-*O*-benzyl-α-*D*-glucopyranoside* (**2**).—To a stirred solution of 2-Azi-4-hydroxybutyl-2,3,4,6-*O*-benzyl-α-D-glucopyranoside [7] (**1**) (300 mg, 0.48 mmol) in benzene (5.8 mL) was added pyridinium dichromate (PDC). After 18 h, PDC was filtered off and then the solvent was evaporated to yield a slightly red syrup. Flash column (2 × 30 cm) chromatography (1:3 EtOAc–cyclohexane) of the syrup yielded **2** (116 mg, 40%); R_f 0.37 (1:2 EtOAc–cyclohexane); $[\alpha]_D$ + 45° (*c* 1.09, EtOAc); $\lambda_{max}^{CH_2Cl_2}$ 320 nm. ^{1}H NMR data: δ 4.72 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 3.55 (dd, 1 H, $J_{2,3}$ 9.3 Hz, H-2), 3.95 (dd, 1 H, $J_{3,4}$ 9.7 Hz, H-3), 3.57–3.75 (m, 4 H, H-4, 5, 6, 6'), 3.32 (d, 1 H, $J_{1'a,1'b}$ 12 Hz, H-1'a), 3.37 (d, 1 H, H-1'b), 2.4 (dd, 1 H, $J_{3'a,3'b}$ 18 Hz, $J_{3'a,4}$ 1.5 Hz, H-3'a), 9.66 (dd, 1 H, H-4').

*2-Azi-4-hydroxy-4-[^{3}H]butyl 2,3,4,6-tetra-*O*-benzyl-α-*D*-glucopyranoside* (**1a**).—A solution of **2** (116 mg, 0.18 mmol) in freshly distilled 1,4-dioxane (700 μL, stirred for 4 h with $NaBH_4$) and 50 μL water was added to NaB^3H_4 (250 mCi, 8.8 Ci/mmol). The solution was stirred overnight and then evaporated. Flash-column (1.5 × 30 cm) chromatography (1:3 EtOAc–cyclohexane) of the residue yielded **1a** (168.3 mCi, 2.2 Ci/mmol, 67%), characterised by two-dimensional TLC with an authentic sample [7].

*2-Azi-4-(4-toluenesulfonyloxy)-4-[^{3}H]butyl 2,3,4,6-tetra-*O*-benzyl-α-*D*-glucopyranoside* (**3**).—To a solution of **1a** [168.3 mCi, 0.05 mmol in dry 20:1 CH_2Cl_2–pyridine (1 mL)] was added 4-toluenesulfonyl chloride (50 mg, 0.13 mmol), and the mixture was kept for 3.5 days at room temperature. To this 50 μL MeOH were added and the solution was kept for 2 h at room temperature. Then 10 mL of $CHCl_3$ were added, and the organic layer washed with satd aq $NaHCO_3$ (3 mL) and water (3 mL), dried ($MgSO_4$), and concentrated. Flash-column (1.5 × 28 cm) chromatography (1:4 EtOAc–cyclohexane) of the residue yielded syrupy **3** (134.6 mCi, 80%), characterised by two-dimensional TLC with an authentic sample [7].

*2-Azi-4-(4-toluenesulfonyloxy)-4-[^{3}H]butyl 2,3,4,6-tetra-*O*-acetyl-α-*D*-glucopyranoside* (**4**).—A solution of **3** (134.6 mCi) in dry CH_2Cl_2 (25 mL) containing 50 mg

NaOAc was ozonolysed (60 min, ~ 10 mmol/h, 0°C). NaOAc was filtered off and then the solution was evaporated. For *O*-debenzoylation the residue was dissolved in MeOH and NaOMe (1 M, 20 μL) was added. When TLC showed the end of the reaction (1.5 h), HOAc (30 μL) was added and then the solution was concentrated. The residue was dissolved in dry 20:1:1 CH_2Cl_2–Ac_2O–Et_3N (2 mL) and 4-dimethylaminopyridine (15 mg) was added. When the reaction was completed (3 h) MeOH was added to decompose the excess of Ac_2O. The solution was concentrated to yield a yellow syrup. Flash-column (1 × 26 cm) chromatography (2:3 EtOAc–cyclohexane) of the residue yielded syrupy **4** (56.7 mCi, 42%), characterised by two-dimensional TLC with an authentic sample [7].

2-Azi-4-benzoylthio-4-[^{3}H]butyl 2,3,4,6-tetra-O-*acetyl-α*-D-*glucopyranoside* (**5**).—To a solution of **4** (56.7 mCi) in acetone (3 mL) potassium thiobenzoate (15 mg) was added. The mixture was stirred for 5 h at room temperature. The solution was concentrated to yield a yellow syrup. Flash-column (1 × 30 cm) chromatography (2:3 EtOAc–cyclohexane) of the residue yielded syrupy **5** (45.4 mCi, 80%), characterised by two-dimensional TLC with an authentic sample [7].

Methyl 4'-O-*[4*-S-*(3-azi-4-α*-D-*glucopyranosyloxy-1-[^{3}H]butyl)-6-deoxy-4-thio-α*-D-xylo-*hex-5-enopyranosyl]-α-maltoside* (**12a**).—To a stirred solution of methyl 2,2',3,3',6,6'-hexa-*O*-acetyl-4'-*O*-[2,3-di-*O*-acetyl-6-deoxy-4-*O*-(4-toluenesulfonyl)-*α*-D-*xylo*-hex-5-enopyranosyl]-*α*-maltoside [12] (**6**) (24.8 mg, 0.025 mmol) in dry acetone (200 μL) was added dropwise methanolic NaOMe (33 μL, 1 M). Precipitated sodium 4-toluenesulfonate was removed by centrifugation and a solution of **5** (7 mCi) in dry acetone (130 μL) was added slowly to the supernatant solution of **9**. After 36 h, the mixture was washed through a column (0.5 × 2 cm) with MeOH and concentrated. HPLC (Hypersil ODS, 5 μm, column 250 × 8 mm, 35:65 MeOH–water, 2.5 mL/min, detection at 340 nm) of the residue gave **12a** (0.7 mCi, 10%). **12a** was characterised by two-dimensional TLC with an authentic sample [7].

Methyl 4-O-*[4*-S-*(3-azi-4-α-maltosyloxy-1-[^{3}H]butyl)-6-deoxy-4-thio-α*-D-xylo-*hex-5-enopyranosyl]-α*-D-*glucopyranoside* (**15a**).—To a stirred solution of methyl 2,3,6-tri-*O*-acetyl-4-*O*-[2,3-di-*O*-acetyl-6-deoxy-4-*O*-(4-toluenesulfonyl)-*α*-D-*xylo*-hex-5-enopyranosyl]-*α*-D-glucopyranoside [12] (**7**) (40 mg, 0.057 mmol) in dry acetone (500 μL) was added dropwise methanolic NaOMe (75 μL, 1 M). Precipitated sodium 4-toluenesulfonate was removed by centrifugation and a solution of **5** (20 mCi) in dry acetone (200 μL) was added slowly to the supernatant solution of **10**. After 24 h, the solution was diluted with water (500 μL), and concentrated to 300 μL. HPLC (Hypersil ODS, 5 μm, column 250 × 8 mm, 35:65 MeOH–water, 2.5 mL/min, detection at 340 nm) of the residue gave **13** (9 mCi, 45%).

To a solution of **13** (9 mCi) in water (500 μL) were added cyclomaltohexaose (13.9 mg, 0.014 mmol) and CGTase (3.5 μL, 2.7 U), and the solution was stored for 2 h at room temperature. The enzyme was inactivated by adding MeOH (500 μL) and freeze drying. The residue was dissolved in water (500 μL) and beta-amylase (1.4 μL, 3.5 U) was added, and the mixture was kept for 1.5 h at room temperature. The enzyme was then inactivated as described for CGTase, the solution centrifuged, and the product subjected to HPLC (MeOH gradient: 0–5 min, 0–20%; 5–20 min, 20–35%; 20–40 min, 35%; 40–50 min, 35–100%) to yield **15a** (1.7 mCi, 19%). Compound **15a** was characterised by two-dimensional TLC with an authentic sample [7].

*Methyl 4-S-(3-azi-4-α-maltotriosyloxy-1-[^{3}H]butyl)-6-deoxy-4-thio-α-*D*-xylo-hex-5-enopyranoside* (**16a**).—To a stirred solution of methyl 2,3-di-*O*-acetyl-6-deoxy-4-*O*-(4-toluenesulfonyl)-α-D-*xylo*-hex-5-enopyranoside [13] (**8**) (15.7 mg, 0.022 mmol) in dry acetone (350 μL) was added dropwise methanolic NaOMe (25 μL, 1 M). Precipitated sodium 4-toluenesulfonate was removed by centrifugation and a solution of **5** (15 mCi) in dry acetone (200 μL) was added slowly to the supernatant solution of **11**. The solution was stored for 8 h at room temperature, diluted with water (400 μL), and concentrated to 200 μL. HPLC (Hypersil ODS, 5 μm, column 250 × 8 mm, 35:65 MeOH–water, 2.5 mL/min, detection at 340 nm) of the residue gave **14** (9 mCi, 60%). To a solution of **14** (9 mCi) in water (500 μL) were added cyclomaltohexaose (13.9 mg, 0.014 mmol) and CGTase (3.5 μL, 2.7 U), and the solution was stored for 3 h at room temperature. The enzyme was inactivated by adding MeOH (500 μL) and freeze-drying. The residue was dissolved in water (500 μL) and beta-amylase (1.4 μL, 3.5 U) was added, and the mixture was kept for 1.5 h at room temperature. The enzyme was then inactivated as described for CGTase, the solution centrifuged, and the product subjected to HPLC (MeOH gradient: 0–5 min, 0–20%; 5–20 min, 20–35%; 20–40 min, 35%; 40–50 min, 35–100%) to yield **16a** (1.35 mCi, 15%). Compound **16a** was characterised by two-dimensional TLC with an authentic sample [7].

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