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Spacer-modified oligosaccharides with basic anchoring groups are inhibitors for endo-glycanases: porcine pancreatic alpha-amylase as model enzyme

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Abstract

By coupling methyl 2,3,6-tri-*O*-acetyl-4-*O*-(5-azido-6-*p*-tolylsulfonyloxyhexyl)-α-D-glucopyranoside with 2-benzoylthioethyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside, a spacer-modified disaccharide derivative, methyl 4-*O*-(5-azido-9-α-D-glucopyranosyloxy-7-thianonyl)-α-D-glucopyranoside, was obtained and then enzymatically glucosylated to yield the spacer-modified tri- and tetra-saccharide methyl 4-*O*-(5-azido-9-α-maltosyloxy-7-thianonyl)-α-D-glucopyranoside and methyl 4-*O*-(5-azido-9-α-maltotriosyloxy-7-thianonyl)-α-D-glucopyranoside, respectively, the extended spacer spanning the length of two (1 → 4)-linked pyranosyl units. The corresponding amines methyl 4-*O*-(5-amino-9-α-D-glucopyranosyloxy-7-thianonyl)-α-D-glucopyranoside, methyl 4-*O*-(5-amino-9-α-maltosyloxy-7-thianonyl)-α-D-glucopyranoside and methyl 4-*O*-(5-amino-9-α-maltotriosyloxy-7-thianonyl)-α-D-glucopyranoside, obtained by catalytic reduction, carry the basic functionality in a spacer position to allow ionic interaction with a catalytically active acidic group in porcine pancreatic alpha-amylase (PPA). Optimal inhibition of enzymic activity is by methyl 4-*O*-(5-amino-9-α-maltosyloxy-7-thianonyl)-α-D-glucopyranoside where three of the five subsites are occupied by glucosyl units and the spacer spanning the remaining subsites positions the amino group near the catalytic site.

Keywords: Spacer-modified oligosaccharide; Competitive inhibition; Alpha-amylase; Basic substrate analogues; Cationic anchoring group

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1. Introduction

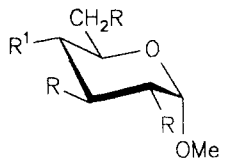
As far as structural investigations of glycosidic bond cleaving enzymes have explained the mechanisms of catalytic activity and corroborated the results of many kinetic studies, one can, without absenting oneself too far from the truth, generalise the so-called lysozyme model published by Phillips and his group [1]. Essentially, three elements of interaction can be encountered: specific binding of a more or less extended saccharide chain by mainly hydrogen bonding; special stabilisation of the conformationally distorted glycosyl moiety whose glycosidic bond becomes cleaved (stabilisation of the transition state); and the interaction of at least one proton donating group with the leaving glycosidic oxygen atom of the aglyconic end with concomitant proton transfer and formation of a salt bridge.

The importance of stable competitive inhibitors of glycoside hydrolase action is well established in glycobiology and major efforts are contributing to the search for natural products with inhibiting potential and the design and synthesis of new artificial inhibitors. Since general acid catalysis, partly initiating glycosidic bond cleavage, always requires the presence of an acidic group at the catalytic site, proton acceptors like amines, brought close to such a group, form salt bridges and thereby deactivate the catalytic centre. In addition, structural elements resembling the substrate or a hypothetic transition state, or both, in one molecule usually strengthen competitive inhibition. Many competitive inhibitors for glycosidases, whose substrates are glycosides or low molecular weight oligosaccharides are known, however only few such inhibitors directed against polysaccharide degrading endo-glycanases such as alpha-amylase have been described. Examples are certain acarboses and related compounds, where a carbocyclic centre unit has a distorted conformation, mimicking a hypothetical transition state, and in place of a glycosidic oxygen it carries a secondary, basic amino group. $(1 \rightarrow 4)$-α-Linked glucosyl units extending to the glyconic and the aglyconic end add the third important ingredient for optimal receptor ligand binding. It can be assumed that a molecule of five units occupies all five binding subsites [2] present in porcine pancreatic alpha-amylase (PPA) with a cationic charge on the especially well bound flattened carbocycle, neutralising one of the catalytically active groups. In such an optimal case, the inhibition constant can lie in the nM range [3].

Because of the relatively high costs of such natural inhibitors for endo-glycanases, like acarbose for amylases or allosamidine for chitinases etc., it is sensible to search for cheap and easily accessible synthetic analogues and test separately the inhibitory increments of basic alkylamino groups as potential cationic anchors and glycosyl units added to such cores. The investigations can be carried out with a readily available enzyme like PPA.

2. Results and discussion

Probably, the most simple and "flexible" competitive inhibitors of glycopyranosidases are the two diastereomeric 1,3-diamino-1,3-dideoxy-D-tetritols [4]. Flexibility of the acyclic compounds allows adaptation and "induced fitting" to the structure of active



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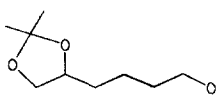
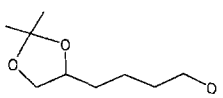
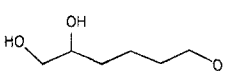
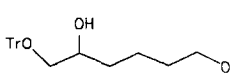
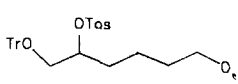
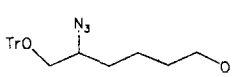
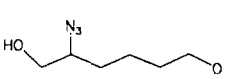
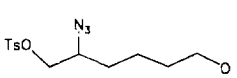
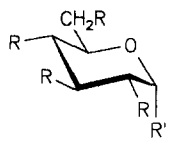
3	OBzl	
4	OAc	
5	OAc	
6	OAc	
7	OAc	
8	OAc	
9	OAc	
10	OAc	

sites and competitive inhibition is derived from both stereoselective hydrogen bonding as well as ionic interaction. For inhibiting an endo-glycanase, the effect of a basic functionality attached to a flexible, acyclic structure could be augmented by properly spaced glycosyl units occupying a maximal number of binding subsites. Such compounds would be stable against enzymic hydrolysis because one of the essential catalytic groups is neutralised by a salt bridge with the basic function of the inhibitor. In an earlier publication on photoaffinity labelling PPA, so-called spacer-modified oligosaccharides were used as ligands [5]. The compounds, where one glucosyl unit at a time is replaced by an acyclic spacer carrying a photoreactive group, mimic maltopentaose. Here, we describe syntheses of similar spacer-modified oligosaccharides (**15–20**) where the acyclic spacer carrying either a neutral azido group or an amino group replaces two subsequent glucosyl units in a maltodextrin chain.

Synthesis of the reducing moiety.—The reducing end must consist of an α-D-glucopyranoside carrying at C-4 a specially designed alkyl chain where the basic functionality can be regioselectively positioned and which allows facile coupling to the nonreducing moiety. The 4-*O*-alkyl chain is one part of the future spacer.

The starting material is methyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranoside (**2**) [6] which, on alkylation with 1,2-*O*-isopropylidene-6-iodohexane-1,2-diol (**1**), yielded methyl 2,3,6-tri-*O*-benzyl-4-*O*-(5,6-isopropylidenedioxyhexyl)-α-D-glucopyranoside (**3**). Catalytic debenzylation of **3** with Pd/H_2 and then acetylation yielded methyl 2,3,6-tri-*O*-



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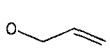
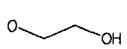
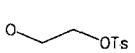
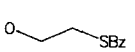
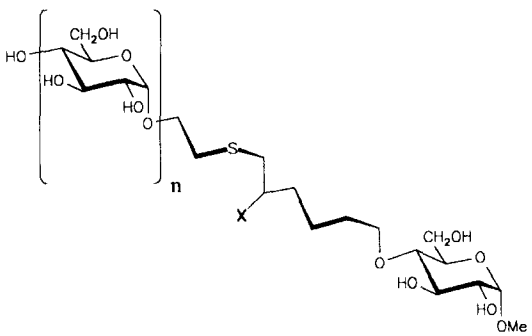
11	OBzl	
12	OBzl	
13	OAc	
14	OAc	

acetyl-4-*O*-(5,6-isopropylidenedioxyhexyl)-α-D-glucopyranoside (**4**). Partial deblocking of **4** with acetic acid [7] yielded compound **5** and, after tritylation, methyl 2,3,6-tri-*O*-acetyl-4-*O*-(5-hydroxy-6-triphenylmethoxyhexyl)-α-D-glucopyranoside (**6**), which was sulfonylated to **7**. Nucleophilic displacement of the tosyloxy group with azide (→ **8**) and then detritylation with $ZnBr_2$ [8] yielded **9**, from which methyl 2,3,6-tri-*O*-acetyl-4-*O*-(5-azido-6-*p*-tolylsulfonyloxyhexyl)-α-D-glucopyranoside (**10**) was obtained.

Synthesis of the nonreducing moiety.—A suitably blocked α-D-glucopyranosyl residue should carry the second part of the spacer as an aglycon.

Allyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**11**) [9], on ozonolysis and subsequent reduction yielded 2-hydroxyethyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**12**), from which 2-*p*-tolylsulfonyloxyethyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**13**) and, after nucleophilic displacement of the tosyloxy group with thiobenzoate, 2-benzoylthioethyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**14**) was obtained.



	n	X
15	1	N_3
16	2	N_3
17	3	N_3
18	1	NH_2
19	2	NH_2
20	3	NH_2

Coupling of the reducing and nonreducing moieties.—The free thiolate for the coupling reaction was generated in situ from **14**, with simultaneous *O*-deacetylation, by treatment with methanolic sodium methoxide. Addition of **10** in acetone resulted then in the formation of methyl 4-*O*-(5-azido-9-α-D-glucopyranosyloxy-7-thianonyl)-α-D-glucopyranoside (**15**).

Glucosylation of **15**.—D-Glucopyranosyl endgroups can be α-D-glucopyranosylated at C-4 through CGTase acting on cyclomaltohexaose (α-cyclodextrin) [10]. In such a way, a homologous series of maltooligosaccharides is obtained. Trimming with beta-amylase shortens maltodextrin chains to 1–3 glucose residues. This procedure was applied to **15**, and yielded diastereomeric mixtures of homologous spacer-modified tri- and tetra-saccharides, namely methyl 4-*O*-(5-azido-9-α-maltosyloxy-7-thianonyl)-α-D-glucopyranoside (**16**) and methyl 4-*O*-(5-azido-9-α-maltotriosyloxy-7-thianonyl)-α-D-glucopyranoside (**17**) which were separated by chromatography on Biogel P2. For identification by ^{1}H NMR, **15** was per-*O*-acetylated. With catalytic hydrogenation over palladium, each azide was converted into the corresponding amine. The thioether bond was not affected.

Enzymic assays.—All spacer-modified oligosaccharides, except the azide and the amine of G3–G1 (**17** and **20**), were essentially stable against enzyme catalysed hydrolysis. The rate of hydrolysis of the amine **20** was two times slower than for the azide **17** and as slow as the hydrolysis of maltotriose.

The inhibition assays were performed with 4-nitrophenyl α-maltotrioside as a chromogenic substrate. Determination of inhibition constants for the azides **15–17** and the amines **18–20** were carried out by Lineweaver–Burk, as well as Dixon plotting. The results are listed in Table 1.

Discussion.—Resistance against alpha-amylase catalysed hydrolysis clearly indicates a reduced formation of a productive complex **A**, because the glucopyranosyl unit at the reducing end carrying the flexible spacer at C-4 can occupy subsite V (complex **B**, Fig. 1). Affinity is augmented if the neutral azide is replaced by a basic amine capable of forming a salt bridge with an acidic group in the catalytic centre. It is noteworthy that the difference in binding between the azide **16** and the corresponding amine **19** is greatest in the case of the G2–G1 structure. This is apparently due to an optimal occupation of subsites by a minimal structure carrying a properly positioned basic anchoring group, which forms a salt bridge with an acidic group between subsite III and IV, where cleavage of the maltodextrin chain usually takes place [2] (Fig. 2). The simple principle of homologous spacer-modified oligosaccharides, carrying basic groups attached to the spacer, can be used for measuring the extension of a binding area. The difference in competitive inhibition between a neutral spacer-modified oligosaccharide and its basic analogue would be greatest in the case where maximal occupation of

Table 1
Inhibition of porcine pancreatic alpha-amylase by compounds 15–20

Compound	**15**	**16**	**17**	**18**	**19**	**20**
K_I [mmol]	36.6	8.1	2.4	11	0.53	0.42

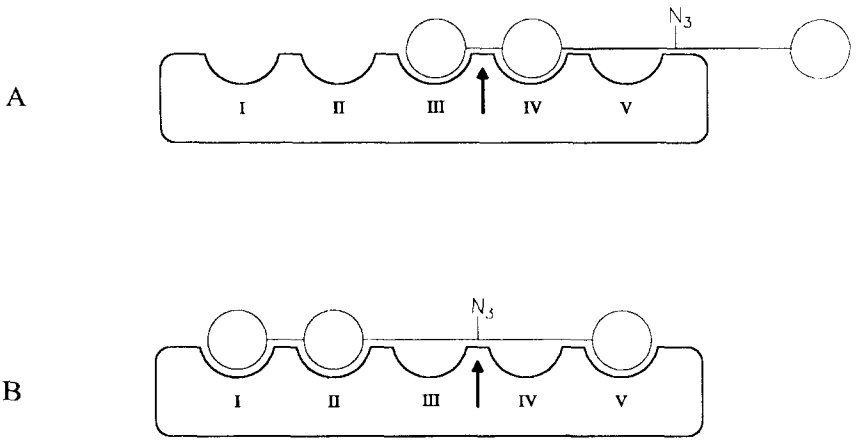


Fig. 1. Productive (**A**) and nonproductive complex (**B**) of compound **16** with porcine pancreatic alpha-amylase; (↑) indicates the catalytic site.

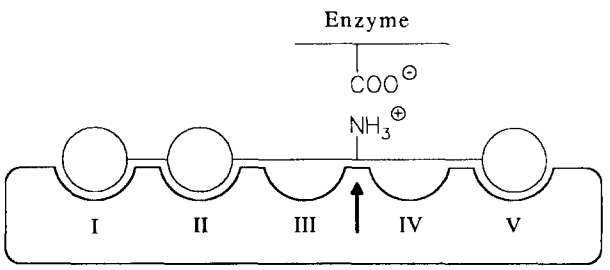


Fig. 2. Salt bridge formation between compound **19** and an acidic group at the catalytic site of PPA.

subsites is supplemented by the correct placing of the basic functionality. Lengthening the optimal structure **16** by adding one glucosyl unit leads to a further increase in affinity in contrast to the almost constant values between compounds **19** and **20**. The missing anchoring group in **17** may lead to a bending of the spacer, thus allowing the occupation of subsites I, II, III and V by glucosyl units (Fig. 3).

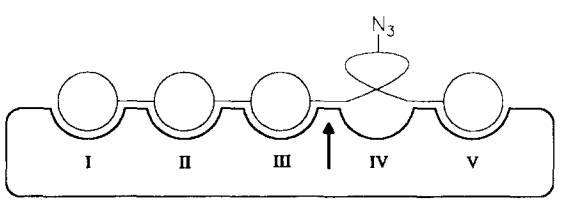


Fig. 3. Bending of the spacer in compound **17**.

3. Experimental

General methods.—All reactions were monitored by TLC on Silica Gel 60 F_{254} (E. Merck). Solutions were concentrated under reduced pressure. Column chromatography was carried out with Silica Gel 32–63, 60 A (ICN) or with Biogel P2 (400 mesh, Bio-Rad) at 40°C with distilled and degassed water. Optical rotations were measured with a Polartronic I (Schmidt and Haensch), IR spectra with a Perkin–Elmer 1320 spectrophotometer, and pH values with a WTW Type E 50 pH 0–14 pH meter. Kinetic data were obtained with an Eppendorf photometer (405 nm), with a transformation unit and a Siemens Kompensograph X–T C–1011. ^{1}H NMR spectra (250 MHz) were recorded with a Bruker WM 250 spectrometer for solutions in $CDCl_3$ (Internal Me_4Si).

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, alpha-amylase [(1 → 4)-α-D-glucan glucanohydrolase, EC 3.2.1.1, 1260 U/mg] from porcine pancreas and beta-amylase [(1 → 4)-α-D-glucan maltohydrolase, EC 3.2.1.2, 845 U/mg] from *Ipomoea batatas* were purchased from Boehringer Mannheim.

Enzymic investigations.—For the determination of the inhibition constants, commercial 4-nitrophenyl α-maltotrioside was used as substrate (0.17–5.3 mM, K_m 2.0 mM) at 30°C in 50 mM triethanolamine–triethanolamine HCl buffer (pH 7.0) containing 10 mM $CaCl_2$. Inhibitor concentrations [mmol] were **1**: 7.125–38; **2**: 2.375–14.25; **3**: 1.19–4.75 **4**: 4.75–14.25; **5**: 0.475–3.563; **6**: 0.356–2.375. The concentration of alpha-amylase in the assay was 15 U/mL.

1,2-O-Isopropylidene-6-iodohexane-1,2-diol (**1**).—To a solution of 1,2-*O*-isopropylidenehexane-1,2,6-triol 6-*p*-toluenesulfonate [11] (41 g, 125 mmol) in acetone, sodium iodide (56 g, 375 mmol) was added. The mixture was refluxed for 5 h. Precipitated potassium *p*-toluenesulfonate was filtered off and the solution concentrated to yield a yellow syrup. The syrup was dissolved in $CHCl_3$, washed with water, dried ($MgSO_4$), concentrated, and then purified by flash column (10 × 15 cm) chromatography (1:10 EtOAc–cyclohexane) to yield **1** (25 g, 70%); R_f 0.51 (1:2 EtOAc–cyclohexane); ^{1}H NMR: δ 4.08 (m, 2 H, H-1), 3.53 (m, 1 H, H-2), 1.36–1.72 (m, 4 H, H-3, 4), 1.75–1.90 (m, 2 H, $J_{5,6}$ 6.8 Hz, H-5), 3.20 (t, 2 H, H-6a, 6b), 1.35 (s, 1 H, CH_3), 1.40 (s, 1 H, CH_3).

Methyl 2,3,6-tri-O-benzyl-4-O-(5,6-isopropylidenedioxyhexyl)-α-D-glucopyranoside (**3**).—To a solution of methyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranoside [6] (**2**, 10 g, 21.5 mmol) in DMF (100 mL) at room temperature was added sodium hydride (3 g of a 60% suspension in oil washed with light petroleum) with stirring. 1,2-*O*-Isopropylidene-6-iodohexane-1,2-diol (**1**, 12.5 g, 44 mmol) was then added dropwise. After 3 h, the excess of sodium hydride was decomposed with MeOH, the clear solution was diluted with water (200 mL) and extracted with CH_2Cl_2 (3 × 150 mL), and the extract was dried ($MgSO_4$) and concentrated to dryness under reduced pressure. Flash column (9 × 15 cm) chromatography (1:5 EtOAc–cyclohexane) yielded **3** (8.5 g, 64%) as a colourless syrup; R_f 0.37 (1:2 EtOAc–cyclohexane); ^{1}H NMR: δ 4.61 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 3.50 (dd, 1 H, $J_{2,3}$ 9.8 Hz, H-2), 3.85 (t, 1 H, $J_{3,4}$ 9.8 Hz, H-3), 3.57–3.71 (m, 1 H, H-4), 3.64–3.78 (m, 1 H, H-5), 3.57–3.71 (m, 1 H, H-6a), 3.35–3.43 (m, 1 H, H-6b). Anal. Calcd for $C_{37}H_{48}O_8$: C, 71.61; H, 7.74. Found: C, 71.07; H, 7.72.

Methyl 2,3,6-tri-O-acetyl-4-O-(5,6-isopropylidenedioxyhexyl)-α-D-glucopyranoside (**4**).—A solution of **3** (8.5g, 13.7 mmol) in MeOH (200 mL) was hydrogenated in the presence of 10% Pd/C (500 mg). The resulting methyl 4-*O*-(5,6-isopropylidenedioxyhexyl)-α-D-glucopyranoside (R_f 0.54, 7:2:1 EtOAc–MeOH–H_2O) was treated with pyridine (50 mL) and acetic anhydride (50 mL) overnight. Acetic anhydride was decomposed, the solution diluted with water (250 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The extract was dried ($MgSO_4$) and concentrated. Flash column (4 × 20 cm) chromatography (1:2 EtOAc–cyclohexane) yielded **4** (5.44 g, 83%) as a colourless syrup; R_f 0.38 (1:1 EtOAc–cyclohexane); ^{1}H NMR: δ 4.86 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.80 (dd, 1 H, $J_{2,3}$ 9.8 Hz, H-2), 5.47 (t, 1 H, $J_{3,4}$ 9.9 Hz, H-3), 3.42 (m, 1 H, $J_{4,5}$ 10.1 Hz, H-4), 3.86 (ddd, 1 H, $J_{5,6a}$ 2.3 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 4.37 (dd, 1 H, $J_{6a,6b}$ 12.8 Hz, H-6a), 4.25 (dd, 1 H, H-6b), 3.46–3.57 (m, 2 H, H-1′), 1.25–1.70 (m, 2 H, H-2′), 1.25–1.70 (m, 2 H, H-3′), 1.25–1.70 (m, 2 H, H-4′), 3.95–4.10 (m, 1 H, H-5′), 3.46–3.57 (m, 2 H, H-6′), 3.40 (s, 3 H, OMe), 1.35 (s, 3 H, Me), 1.39 (s, 3 H, Me), 2.05 (s, 3 H, OAc), 2.15 (s, 3 H, OAc). Anal. Calcd for $C_{22}H_{36}O_{11}$: C, 55.45; H, 7.61. Found: C, 55.62; H, 7.71.

Methyl 2,3,6-tri-O-acetyl-4-O-(5,6-dihydroxyhexyl)-α-D-glucopyranoside (**5**).—A solution of **4** (14 g, 30 mmol) in $CHCl_3$ was treated with trifluoroacetic acid (10 mL) at room temperature for 12 h. The solution was diluted with water (200 mL) and extracted with CH_2Cl_2 (200 mL). The extract was dried ($MgSO_4$) and concentrated in vacuo. Flash column (5 × 20 cm) chromatography (3:1 EtOAc–cyclohexane) yielded **5** (9.8 g, 75%) as a colourless syrup; R_f 0.40 (7:1 EtOAc–cyclohexane); ^{1}H NMR: δ 4.87 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.82 (dd, 1 H, $J_{2,3}$ 9.8 Hz, H-2), 5.47 (dd,1 H, $J_{3,4}$ 9.0 Hz, H-3), 3.39 (t, 1 H, $J_{4,5}$ 10.2 Hz, H-4), 3.86 (ddd, 1 H, $J_{5,6a}$ 2.3 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 4.37 (dd, 1 H, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.25 (dd, 1 H, H-6b), 3.34–3.74 (m, 2 H, H-1′), 1.31–1.61 (m, 6 H, H-2′, H-3′, H-4′), 3.34–3.74 (m, 3 H, H-5′, H-6′), 3.39 (s, 3 H, OMe), 2.09 (s, 3 H, OAc), 2.14 (s, 3 H, OAc). Anal. Calcd for $C_{19}H_{32}O_{11}$: C, 52.33; H, 7.34. Found: C, 51.91; H, 7.15.

Methyl 2,3,6-tri-O-acetyl-4-O-(5-hydroxy-6-triphenylmethoxyhexyl)-α-D-glucopyranoside (**6**).—To a solution of **5** (9.8 g, 22.5 mmol) in pyridine was added chlorotriphenylmethane (12 g, 43 mmol) and the solution was stirred at room temperature. After 10 h the excess of chlorotriphenylmethane was decomposed with MeOH, the solution diluted with water (250 mL) and extracted with CH_2Cl_2 (3 × 150 mL). The extract was dried ($MgSO_4$) and concentrated. Flash column (5 × 20 cm) chromatography (1:2 EtOAc–cyclohexane) yielded **6** (12 g, 78.6%) as a colourless syrup; R_f 0.42 (1:1 EtOAc–cyclohexane). ^{1}H NMR: δ 4.86 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.82 (dd, 1 H, $J_{2,3}$ 9.3 Hz, H-2), 5.46 (t, 1 H, $J_{3,4}$ 9.5 Hz, H-3), 3.38 (t, 1 H,$J_{4,5}$ 10.2 Hz, H-4), 3.83 (ddd, 1 H, $J_{5,6a}$ 2.3 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 4.33 (d, 1 H, $J_{6a,6b}$ 12.0 Hz, H-6a), 4.22 (dd, 1 H, H-6b), 3.41–3.52 (m, 2 H, H-1′), 1.20–1.54 (m, 2 H, H-2′), 1.20–1.54 (m, 2 H, H-3′), 1.20–1.54 (m, 2 H, H-4′), 3.57 (m, 1 H, H-5′), 3.14 (dd, 1 H, $J_{6'a,6'b}$ 9.0 Hz, H-6′a), 3.02 (m 1 H, H-6′b), 3.38 (s, 3 H, OMe), 2.01 (s, 3 H, OAc), 2.03 (s, 3 H, OAc), 2.06 (s, 3 H, OAc), 2.10 (s, 3 H, OAc), 7.20–7.48 (m, 15 H, Ph). Anal. Calcd for $C_{38}H_{46}O_{11}$: C, 67.28; H, 6.85. Found: C, 67.42; H, 7.02.

Methyl 2,3,6-tri-O-acetyl-4-O-(5-p-tolylsulfonyloxy-6-triphenylmethoxyhexyl)-α-D-glucopyranoside (**7**).—A solution of **6** (12 g, 17.7 mmol) in CH_2Cl_2 (150 mL) was

treated with 4-dimethylaminopyridine (4.76 g, 40 mmol) and *p*-toluenesulfonyl chloride (6.75 g, 35 mmol) overnight. The excess of *p*-toluenesulfonyl chloride was decomposed with MeOH, the solution diluted with water (150 mL). The organic layer was separated, and the aqueous layer extracted with CH_2Cl_2 (3 × 100 mL). The extract was dried over $MgSO_4$ and concentrated. Flash column (5 × 20 cm) chromatography (1:5 EtOAc–cyclohexane) yielded **7** (9.45 g, 65%) as a colourless syrup; R_f 0.22 (1:5 EtOAc–cyclohexane); ^{1}H NMR: δ 4.86 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 4.80 (dd, 1 H, $J_{2,3}$ 9.8 Hz, H-2), 5.43–5.44 (t, 1 H, $J_{3,4}$ 9.8 Hz, H-3), 3.35 (t, 1 H, $J_{4,5}$ 10.2 Hz, H-4), 3.83 (ddd, 1 H, $J_{5,6a}$ 2.3 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 4.32 (d, 1 H, $J_{6a,6b}$ 12.0 Hz, H-6a), 4.21 (dd, 1 H, H-6b), 3.41–3.52 (m, 2 H, H-1′), 1.29–1.50 (m, 2 H, H-2′), 1.10–1.29 (m, 2 H, H-3′), 1.57–1.75 (m, 2 H, H-4′), 4.57 (m, 1 H, H-5′), 3.10 (t, 2 H, H-6′a, H-6′b), 3.36 (s, 3 H, OMe), 2.00 (s, 3 H, OAc), 2.04 (s, 3 H, OAc), 2.08 (s, 3 H, OAc), 2.10 (s, 3 H, OAc), 7.20–7.75 (m, 19 H, Ph), 2.42 (s, 3 H, Ph-Me). Anal. Calcd for $C_{45}H_{52}O_{13}S$: C, 64.95; H, 6.25; S, 3.85. Found: C, 65.49; H, 6.62; S, 3.70.

*Methyl 2,3,6-tri-*O*-acetyl-4-*O*-(5-azido-6-triphenylmethoxyhexyl)-α-*D*-glucopyranoside* (**8**).—To a solution of **7** (9.45 g, 11.4 mmol) in DMF (100 mL) was added sodium azide (3 g, 46 mmol), and the mixture was stirred at 60°C for 8 h. The solution was diluted with water (250 mL) and then extracted with CH_2Cl_2 (3 × 100 mL). The extract was dried over $MgSO_4$ and then concentrated. Flash column (5 × 20 cm) chromatography (1:5 EtOAc–cyclohexane) yielded **8** (7.54 g, 85%) as a colourless syrup; R_f 0.41 (1:2 EtOAc–cyclohexane); ν_{max}^{KBr} 2100 cm^{-1} (N_3); ^{1}H NMR: δ 4.86 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.82 (dd, 1 H, $J_{2,3}$ 9.8 Hz, H-2), 5.45–5.46 (t,1 H, $J_{3,4}$ 9.8 Hz, H-3), 3.39 (t, 1 H, $J_{4,5}$ 10.2 Hz, H-4), 3.82 (ddd, 1 H, $J_{5,6a}$ 2.2 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 4.33 (d, 1 H, $J_{6a,6b}$ 12.0 Hz, H-6a), 4.24 (dd, 1 H, H-6b), 3.31–3.54 (m, 2 H, H-1′), 1.15–1.50 (m, 6 H, H-2′, H-3′, H-4′), 3.31–3.54 (m, 1 H, H-5′), 3.10–3.24 (t, 2 H, H-6′a, H-6′b), 3.39 (s, 3 H, OMe), 2.01–2.04 (s, 3 H, OAc), 2.07–2.11 (s, 3 H, OAc), 2.12 (s, 3 H, OAc), 7.20–7.50 (m, 15 H, Ph). Anal. Calcd for $C_{45}H_{52}O_{13}N_3$: C, 64.79; H, 6.50; N, 5.97. Found: C, 64.08; H, 6.72; S, 5.44.

*Methyl 2,3,6-tri-*O*-acetyl-4-*O*-(5-azido-6-hydroxyhexyl)-α-*D*-glucopyranoside* (**9**).—To a solution of **8** (7.25 g, 10.3 mmol) in dry CH_2Cl_2 was added $ZnBr_2$ (20 g, anhyd) and then it was stirred for 0.5 h. Methanol (5 mL) was added and stirring was continued for 20 min. Then the solution was diluted with water (200 mL), the organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (3 × 50 mL), dried over $MgSO_4$ and then concentrated. Flash column (3 × 20 cm) chromatography (1:1 EtOAc–cyclohexane) yielded **9** (3.4 g, 70%) as a colourless syrup; R_f 0.43 (2:1 EtOAc–cyclohexane); ν_{max}^{KBr} 2100 cm^{-1} (N_3). ^{1}H NMR: δ 4.88 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.83 (dd, 1 H, $J_{2,3}$ 9.8 Hz, H-2), 5.48 (t, 1 H, $J_{3,4}$ 9.8 Hz, H-3), 3.41 (t, 1 H, $J_{4,5}$ 10.1 Hz, H-4), 3.86 (ddd, 1 H, $J_{5,6a}$ 2.3 Hz, $J_{5,6b}$ 4.4 Hz, H-5), 4.35 (d, 1 H, $J_{6a,6b}$ 12.0 Hz, H-6a), 4.26 (dd, 1 H, H-6b), 3.35 (t, 2 H, $J_{1',2'}$ 9.0 Hz, H-1′), 1.31–1.61 (m, 6 H, H-2′, H-3′, H-4′), 3.35–3.75 (m, 3 H, H-5′, H-6′a, H-6′b), 3.40 (s, 3 H, OMe), 2.07 (s, 6 H, OAc), 2.14 (s, 3 H, OAc). Anal. Calcd for $C_{19}H_{31}O_{10}N_3$: C, 49.45; H, 6.77; N, 9.11. Found: C, 49.73; H, 6.77; N, 9.65.

*Methyl 2,3,6-tri-*O*-acetyl-4-*O*-(5-azido-6-*p*-tolylsulfonyloxyhexyl)-α-*D*-glucopyranoside* (**10**).—A solution of **9** (3.4 g, 7.4 mmol) in CH_2Cl_2 (50 mL) was treated with 4-dimethylaminopyridine (1.5 g, 12.2 mmol) and *p*-toluenesulfonyl chloride (2.1 g, 11

mmol) overnight. The excess of *p*-toluenesulfonyl chloride was decomposed with MeOH, the solution diluted with water (100 mL). The organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (3 × 50 mL). The extract was dried over $MgSO_4$ and concentrated. Flash column (3 × 20 cm) chromatography (1:3 EtOAc–cyclohexane) yielded **10** (3.1 g, 67%) as a colourless syrup; R_f 0.41 (1:1 EtOAc–cyclohexane); ν_{max}^{KBr} 2090 cm^{-1} (N_3). ^1H NMR: δ 4.87 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.82 (dd, 1 H, $J_{2,3}$ 9.7 Hz, H-2), 5.46 (dd, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 3.40 (t, 1 H, $J_{4,5}$ 10.0 Hz, H-4), 3.85 (ddd, 1 H, $J_{5,6a}$ 2.2 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 4.35 (d, 1 H, $J_{6a,6b}$ 11.7 Hz, H-6a), 4.25 (dd, 1 H, H-6b), 3.32–3.60 (m, 2 H, H-1′), 1.25–1.59 (m, 2 H, H-2′, H-3′, H-4′), 3.32–3.60 (m, 1 H, $J_{5,6a}$ 4.5 Hz, $J_{5,6b}$ 6.8 Hz, H-5′) 4.08 (dd, 1 H, $J_{6'a,6'b}$ 9.7 Hz H-6′a), 3.35–3.75 (m, 1 H, H-6′b) 3.40 (s, 3 H, OMe), 2.05 (s, 3 H, OAc), 2.06 (s, 3 H, OAc), 2.14 (s, 3 H, OAc), 7.40–7.80 (m, 4 H, Ph), 2.46 (s, 3 H, Ph-Me); Anal. Calcd for $C_{26}H_{37}O_{13}N_3S$: C, 5.73; H, 6.02; N, 6.83. Found: C, 51.27; H, 6.26; N, 6.67.

2-Hydroxyethyl 2,3,4,6-tetra-O-*acetyl*-α-D-*glucopyranoside* (**12**).—Ozone (30 L per h, 10 mmol O_3 per h) was bubbled through a solution of allyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (**11**, 9 g, 24 mmol) in 1:1 CH_2Cl_2-MeOH (300 mL) at −75°C. After persistance of the blue colour of the solution for 10 min, the excess of O_3 was removed with a stream of O_2. Sodium borohydride (2 g, 53 mmol) was added, and the mixture was allowed to attain room temperature. The excess of $NaBH_4$ was decomposed with AcOH (15 mL) and then concentrated. Flash column (6 × 20 cm) chromatography (2:1 EtOAc–cyclohexane) yielded **12** (8.3 g, 90%) as a colourless syrup; R_f 0.35 (4:1 EtOAc–cyclohexane).

*2-*p*-Tolylsulfonyloxyethyl 2,3,4,6-tetra*-O-*acetyl*-α-D-*glucopyranoside* (**13**).—A solution of **12** (8.3 g, 22.3 mmol) in CH_2Cl_2 (100 mL) was treated with pyridine (10 mL) and *p*-toluenesulfonyl chloride. After 16 h at room temperature, the excess of *p*-toluenesulfonyl chloride was decomposed with MeOH and the solution diluted with water. The organic layer was separated and the aqueous layer extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried over $MgSO_4$ and then concentrated. Flash column (5 × 20 cm) chromatography (1:2 EtOAc–cyclohexane) yielded **13** (7.5 g, 69%) as a colourless syrup; R_f 0.38 (1:1 EtOAc–cyclohexane); $[\alpha]_D^{21}$ + 121°. ^1H NMR: δ 5.08 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.88 (dd, 1 H, $J_{2,3}$ 10.2 Hz, H-2), 5.43 (t, 1 H, $J_{3,4}$ 9.8 Hz, H-3), 5.06 (t, 1 H, $J_{4,5}$ 9.7 Hz, H-4), 4.02 (m, 1 H, H-5), 4.05–4.30 (m, 2 H, H-6a, H-6b), 3.88 (dt, 1 H, $J_{1'a,1'b}$ 12 Hz, $J_{1'a,2'}$ 5.3 Hz, H-1′a), 3.71 (dt, 1 H, $J_{1'b,2'}$ 5.1 Hz, H-1′b), 4.05–4.3 (m, 2 H, H-2′), 2.02 (s, 3 H, OAc), 2.04 (s, 3 H, Ph-Me). Anal. Calcd for $C_{45}H_{52}O_{13}S$: C, 50.55; H, 5.53; S, 5.87. Found: C, 50.69; H, 5.46; S, 5.63.

2-Benzoylthioethyl 2,3,4,6-tetra-O-*acetyl*-α-D-*glucopyranoside* (**14**).—To a solution of **13** (7.5 g, 13,7 mmol) in dry acetone (200 mL) was added potassium thiobenzoate (5 g, 21.8 mmol). After stirring for 2 h at room temperature, the solution was evaporated and the residue dissolved in water (200 mL). The suspension was extracted with $CHCl_3$ (3 × 100 mL), the combined organic layers were dried over $MgSO_4$ and then concentrated under reduced pressure. Flash column (2.5 × 20 cm) chromatography (1:3 EtOAc–cyclohexane) yielded **14** (6.1 g, 80%) as a colourless syrup; R_f 0.54 (1:1 EtOAc–cyclohexane); $[\alpha]_D^{21}$ + 80°. ^1H NMR: δ 5.15 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.85 (dd, 1 H $J_{2,3}$ 10.5 Hz, H-2), 5.50 (t, 1 H, $J_{3,4}$ 9.8 Hz, H-3), 5.05 (t, 1 H, $J_{4,5}$ 9.7 Hz, H-4), 4.02 (ddd, 1 H, $J_{5,6a}$ 4.8 Hz, $J_{5,6b}$ 2.3 Hz, H-5), 4.24 (dd, 1 H, $J_{6a,6b}$ 12.8 Hz,

H-6a), 4.97 (dd, 1 H, H-6b), 3.89 (dt, 1 H, $J_{1'a,1'b}$ 10.5 Hz, $J_{1'a,2'}$ 6.7 Hz, H-1'), 3.71 (dt, 1 H, $J_{1'b,2'}$ 6.7 Hz, H-1'b), 3.34 (t, 2 H, H-2'), 2.03 (s, 3 H, OAc), 2.05 (s, 3 H, OAc), 2.06 (s, 3 H, OAc) 2.08 (s, 3 H, OAc), 7.45–7.96 (m, 4 H, Ph). Anal. Calcd for $C_{23}H_{28}O_{11}S$: C, 53.93; H, 5.51; S, 6.26. Found: C, 53.36; H, 5.36; S, 6.03.

Methyl 4-O-(5-azido-9-α-D-glucopyranosyloxy-7-thianonyl)-α-D-glucopyranoside (**15**).—To a solution of **14** (4.1 g, 8 mmol) and **11** (3.1 g, 5 mmol) in dry MeOH (30 mL) was added methanolic MeONa (12 mL, 1 M) over 6 h. When the reaction was completed the solution was concentrated to dryness. Flash column (3 × 20 cm) chromatography (27:2:1 EtOAc–MeOH-water) yielded **15** (2.56 g, 92%) as a colourless solid; R_f 0.49 (7:2:1 EtOAc–MeOH-water); ν_{max}^{KBr} 2100 cm^{-1} (N_3). For ^1H NMR analysis compound **15** was acetylated; ^1H NMR: δ 4.86 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.81 (dd, 1 H, $J_{2,3}$ 10.5 Hz, H-2), 5.46 (t, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 3.42 (dt, 1 H, $J_{4,5}$ 9.8 Hz, H-4), 3.86 (m, 1 H, $J_{5,6a}$ 2.2 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 4.35 (dd, 1 H, $J_{6a,6b}$ 11.8 Hz, H-6a), 4.28 (dd, 1 H, H-6b), 3.43–3.62 (m, 2 H, H-1'), 1.30–1.70 (m, 2 H, H-2', H-3', H-4'), 4.11 (m, 1 H, H-5'), 2.73 (m, 2 H, H-6'), 2.82 (m, 2 H, H-8'), 3.43–3.62 (m, 2 H, H-9'), 5.12 (d, 1 H, $J_{1'',2''}$ 3.8 Hz, H-1''), 4.88 (dd, 1 H, $J_{2'',3''}$ 10.2 Hz, H-2''), 5.44 (t, 1 H, $J_{3'',4''}$ 9.8 Hz, H-3''), 5.08 (t, 1 H, $J_{4'',5''}$ 10 Hz, H-4''), 3.86 (m, 1 H, $J_{5'',6''a}$ 2.3 Hz, $J_{5'',6''b}$ 4.5 Hz, H-5''), 4.28 (m, 1 H, H-6''a), 4.11 (m, 1 H, H-6''b), 3.40 (s, 3 H, OMe), 2.0 (s, 3 H, OAc), 2.02 (s, 3 H, OAc), 2.07 (s, 6 H, OAc), 2.12 (s, 3 H, OAc). Anal. Calcd for $C_{21}H_{39}O_{12}N_3S$: C, 45.26; H, 7.00; N, 7.54; S, 5.75. Found: C, 45.02; H, 6.91; N, 7.36; S, 5.56.

Methyl 4-O-(5-azido-9-α-maltosyloxy-7-thianonyl)-α-glucopyranoside (**16**) *and methyl 4-O-(5-azido-9-α-maltotriosyloxy-7-thianonyl)-α-D-glucopyranoside* (**17**).—To a solution of **15** (1.8 g, 3.2 mmol) in water (10 mL) was added α-cyclodextrin (2 g, 2 mmol) and CGTase (75 μL, 58 U). The solution was kept for 4 h at room temperature. The enzyme was inactivated by heating to 95°C for 5 min, AcOH (50 μL) and beta-amylase (15 μL, 330 U) were added. After 1 h at room temperature, no homologues higher than **17** were detectable by TLC. Beta-amylase was then denatured as described for CGTase and the mixture concentrated under reduced pressure. Biogel chromatography of the residue gave colourless amorphous **16** (520 mg, 22.6%), R_f 0.36 (7:2:1 EtOAc–MeOH-water); ν_{max}^{KBr} 2100 cm^{-1} (N_3) and **17** (375 mg, 13.3%) R_f 0.24 (7:2:1 EtOAc–MeOH-water); ν_{max}^{KBr} 2100 cm^{-1} (N_3). Anal. Calcd for $C_{27}H_{49}O_{17}N_3S$ **16**: C, 45.09; H, 6.81; N, 5.84; S, 4.45. Found: C, 44.79; H, 6.76; N, 5.63; S, 4.21; Anal. Calcd for $C_{33}H_{59}O_{22}N_3S$ **17**: C, 44.98; H, 6.69; N, 4.76; S, 3.64. Found: C, 44.13; H, 6.33; N, 4.33; S, 3.52.

Methyl 4-O-(5-amino-9-α-D-glucopyranosyloxy-7-thianonyl)-α-D-glucopyranoside (**18**).—A solution of **15** (130.6 mg, 0.234 mmol) and methanolic HCl (234 μL, 0.234 mmol) in MeOH (5 mL) was hydrogenated in the presence of Pd/C (10%, 25 mg) for 1 h. The catalyst was filtered off and the filtrate was evaporated to yield **18** (131.1 mg, 99%), R_f 0.38 (30:20:15 $CHCl_3$–MeOH–NH_3). Compound **18** was acetylated for elemental and ^1H NMR analysis. ^1H NMR: δ 4.88 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.80–4.81 (dd, 1 H, $J_{2,3}$ 10.2 Hz, H-2), 5.46 (t, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 3.38 (d, 1 H, $J_{4,5}$ 9.8 Hz, H-4), 3.79–3.92 (m, 1 H, $J_{5,6a}$ 2.2 Hz, $J_{5,6b}$ 4.5 Hz, H-5), 4.35 (dd, 1 H, $J_{6a,6b}$ 12.0 Hz, H-6a), 4.26 (dd, 1 H, H-6b), 3.43–3.73 (m, 2 H, H-1'), 1.25–1.65 (m, 6 H, H-2', H-3', H-4'), 4.26 (m, 1 H, H-5'), 2.60–2.81 (m, 4 H, H-6', H-8'), 3.43–3.73 (m, 2 H, H-9'),

5.12 (d, 1 H, $J_{1'',2''}$ 3.8 Hz, H-1''), 4.09 (dd, 1 H, $J_{2'',3''}$ 10.3 Hz, H-2''), 5.44 (t, 1 H, $J_{3'',4''}$ 9.8 Hz, H-3''), 5.07 (t, 1 H, $J_{4'',5''}$ 9.8 Hz, H-4''), 3.79–3.92 (m, 1 H, H-5''), 4.06–4.18 (m, 2 H, H-6''a, H-6''), 5.93 (d, 1 H, NHAc), 3.40 (s, 3 H, OMe), 2.0 (s, 3 H, OAc), 2.02 (s, 3 H, OAc), 2.06 (s, 3 H, OAc), 2.07 (s, 6 H, OAc), 2.11 (s, 6 H, OAc), 2.15 (s, 3 H, OAc), 2.16 (s, 3 H, OAc). Anal. Calcd for $C_{37}H_{57}O_{20}NS$: C, 51.20; H, 6.62; N, 1.61. Found: C, 51.18; H, 6.38; N, 1.33.

Methyl 4-O-(5-amino-9-α-maltosyloxy-7-thianonyl)-α-D-glucopyranoside (**19**).—A solution of **16** (66.1 mg, 0.092 mmol) and methanolic HCl (92 μL) in MeOH (5 mL) was hydrogenated as described for compound **15**, to yield **19** (65.7 mg, 98%), R_f 0.28 (30:20:15 $CHCl_3$–MeOH–NH_3). Compound **19** was acetylated for elemental analysis. Anal. Calcd for $C_{49}H_{73}O_{28}NS$: C, 51.39; H, 6.32; N, 1.22; S, 2.78. Found: C, 51.77; H, 6.36; N, 1.55; S, 3.08.

Methyl 4-O-(5-amino-9-α-maltotriosyloxy-7-thianonyl)-α-D-glucopyranoside (**20**).—A solution of **17** (29.4 mg, 0.0334 mmol) and methanolic HCl (33.4 μL) in MeOH (5 mL) was hydrogenated as described for compound **15**, to yield **20** (29.2 mg, 98%), R_f 0.15 (30:20:15 $CHCl_3$–MeOH–NH_3). Compound **20** was acetylated for elemental analysis. Anal. Calcd for $C_{61}H_{89}O_{35}NS$: C, 50.76; H, 6.17; N, 0.97; S, 2.22. Found: C, 51.07; H, 6.27; N, 0.94; S, 2.37.

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