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The Substrate Specificity of the Enzyme Amyloglucosidase Towards a Pentadeoxy Derivative of Maltose

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2,6-Dideoxy-4-*O*-(2,3-dideoxy-α-D-*erythro*-hexopyranosyl)-1,5-anhydro-D-*arabino*-hexitol (2) has been synthesized and characterized by ^{1}H- and ^{13}C-NMR spectroscopy. The compound is a substrate for the enzyme amyloglucosidase, AMG (EC.3.2.1.3) and the reaction kinetics have been determined using ^{1}H-NMR spectroscopy. Compound 2 is hydrolysed by AMG with a half time of 139 min compared to 76 min for methyl maltoside (1) under identical reaction conditions.

Lemieux has recently proposed that key polar groups are essential for the recognition of oligosaccharides by antibodies [1-6]. We have been investigating this theory in a study of the substrate specificity of different deoxy-derivatives of maltose towards the enzyme amyloglucosidase (AMG) [7-9]. The enzyme is widely used technically in the processing of starch and in hydrolyzing maltose and higher oligomers into glucose.

This work [7-9] has shown, in agreement with Lemieux's proposal, that three hydroxyl groups (OH4', OH6' and OH3) are absolutely essential for the substrate activity, when maltose is hydrolyzed into glucose. The methyl 3-deoxy- and (4'deoxy)-β-maltosides have furthermore been shown not to be competitive inhibitors in the degradation of methyl β-maltoside, suggesting that the lack of one of these hydroxyl groups prevents the molecules from binding strongly to the active site of the enzyme. The other deoxy-derivatives (1, 2, 6, 2' or 3') of maltose are all substrates for the enzyme AMG, and generally hydrolyzed by the enzyme faster than the reference compound, methyl β-maltoside (**1**) [9].

This has raised the question, whether a molecule with only the three key-polar hydroxy-groups present is a substrate for the enzyme. We therefore wish to report on the syntheses of 1,2,6,2',3'-pentadeoxy-maltose (**2**) and its behaviour towards amyloglycosidase.

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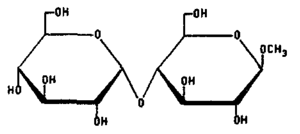

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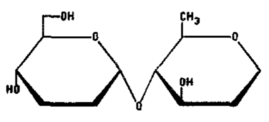

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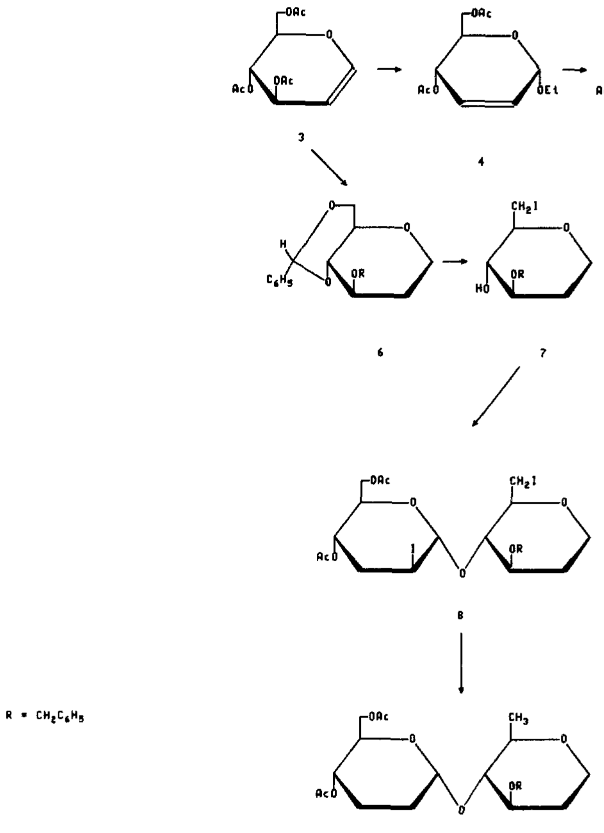


Materials and Methods

Melting points are uncorrected. Optical rotations were measured on a Perkin Elmer 241 polarimeter. NMR spectra were obtained on Bruker WH-90 and AM-500 NMR instruments. The spectra of protected compounds were measured in C^2HCl_3. Unprotected compounds were measured in ^2H_2O relative to the internal reference: acetone (δ 2.22) for ^{1}H-NMR spectra and dioxane (67.4 ppm) for ^{13}C-NMR spectra. Microanalyses were performed by Novo Microanalytical Laboratory, Copenhagen, Denmark. TLC was performed on silica gel-coated plates (Merck F-254, Darmstadt, W. Germany), the compounds being visualised by charring with sulfuric acid. Amyloglucosidase, AMG (EC 3.2.1.3) was a gift from Novo A/S, Denmark.

1,5-Anhydro-3-O-benzyl-2,6-dideoxy-6-iodo-D-arabino-hexitol (7)

A solution of 1,5-anhydro-4,6-*O*-benzylidene-2-deoxy-3-*O*-benzyl-D-glucitol (**6**) [10] (1.0 g, 3.07 mmol) in 0.1 M hydrochloric acid (10 ml) was kept for 20 h at 100°C with stirring and reflux. The mixture was diluted with water (10 ml), cooled in ice and filtered. The solution was purified by passing through a small column (5 × 2 cm) of mixed bed ion exchange resin. The column was washed with water (50 ml) and methanol (50 ml). Evaporation to dryness and drying in desiccator over P_2O_5 yielded 1,5-anhydro-3-*O*-benzyl-2-deoxy-D-glucitol as a syrup (582 mg, 2.45 mmol, 80%). The compound (582 mg) was dissolved in pyridine (12 ml) and the solution was cooled to 0°C, and stirred under N_2. Methanesulfonyl chloride (309 mg, 0.21 ml, 2.70 mol) was added from a syringe through a septum to the closed flask. The reaction mixture was left at 0°C for 16 h. The solution was evaporated and water (20 ml) was added. The aqueous phase was extracted with dichloromethane (2 × 50 ml) and the organic phase washed with 0.1 M hydrochloric acid (20 ml), a saturated solution of sodium hydrogen carbonate and water followed by drying ($MgSO_4$) and evaporation to dryness. The residue was purified by preparative TLC (ethyl acetate/hexane, 2/1 by vol). The main fraction was 1,5-anhydro-3-*O*-benzyl-2-deoxy-6-*O*-methanesulfonyl-D-glucitol (483 mg, 1.53 mmol, 62%) which was dissolved in *N,N*-di-methylformamide (5 ml) and treated with dry sodium iodide (573 mg, 3.82 mmol) at 100°C for 16 h. The mixture was evaporated and butanol (5 ml) was added and co-evaporated twice to remove the *N,N*-dimethylformamide. Water (10 ml) was added and the mixture was stirred for 1 h. The aqueous phase was extracted twice with dichlormethane (50 ml) and the organic phase washed with 10% sodium thiosulphate solution (10 ml) and three times with water (25 ml) followed by drying ($MgSO_4$) and evaporation to dryness. The residue was purified by preparative TLC (ethyl acetate/hexane, 3/5 by vol). This gave 1,5-anhydro-3-*O*-benzyl-2,6-dideoxy-6-iodo-D-*arabino*-hexitol (**7**) (364 mg, 115 mmol, 68%) as a syrup $[\alpha]_D$ -9.06 (*c* 0.9, chloroform). Analytical data calculated for $C_{13}H_{17}O_3I$: C, 44.85; H, 4.92. Found: C, 44.71; H, 4.93. NMR data (^1H and ^{13}C) are given in Table 1.

1,5-Anhydro-3-O-benzyl-2,6-dideoxy-6-iodo-4-O-(4,6-di-O-acetyl-2,3-dideoxy-2-iodo-D-arabino-hexopyranosyl)-D-arabino-hexitol (8)

A mixture of dry 1,5-anhydro-4,6-di-*O*-acetyl-2,3-dideoxy-D-*erythro*-hex-1-enitol (**5**) (390 mg, 1.82 mmol), dry 1,5-anhydro-3-*O*-benzyl-2,6-dideoxy-6-iodo-D-*arabino*-hexitol (**7**) (400 mg 1.115 mmol), dry *N*-iodo-succinimide (700 mg, 3.12 mmol) and 3Å molecular sieves in acetonitrile (8 ml) was stirred in the dark under dry nitrogen and cooling in ice-water for 1.5 h and then at room temperature for 2.5 h. The reaction mixture was diluted with dichloromethane (50 ml) and evaporated. The residue was diluted with dichloromethane (75 ml) and the dark red solution was washed with 4 N hydrochloric acid (3 × 10 ml) and three times with saturated sodium hydrogen carbonate solution and then water. Drying ($MgSO_4$) and evaporation to dryness yielded a brown syrup (953 mg), which was purified by preparative TLC using ethyl acetate/hexane, 1/1 by vol. This yielded **8** (423 mg, 0.61 mmol, 53%), which was characterized by its NMR (^1H and ^{13}C) parameters (Table 1).

Table 1. Nuclear magnetic resonance data for compounds **2-9**

^{1}H-NMR Data[b]	H1	H1′	H2	H2′	H3	H3′	H4	H5	H6	H6′
Compound.										
2, a-unit	5.44 1.5_{12} $4.0_{12'}$		1.98 3.9_{23} $12.5_{22'}$	1.85 $12.5_{2'3'}$	1.96 4.5_{34} $13.0_{33'}$	1.68 $10.5_{3'4}$	3.62 9.7_{45}	3.71	3.85 2.2_{56}	3.76 $5.5_{56'}$ 12.1_{66}
2, b-unit	3.93 1.8_{12} $5.0_{12'}$ $12.0_{11'}$	3.53 $2.0_{1'2'}$	2.03 5.0_{23} $13.0_{22'}$	1.71 $11.2_{2'3}$	3.78 8.8_{34}		3.32 9.2_{45}	3.42 6.1_{56}	1.36	
7	4.00 2.5_{12} $4.5_{12'}$ $12.0_{11'}$	3.41 $4.5_{1'2}$ $11.5_{1'2'}$	2.03 $4.5_{2'3}$ $14.0_{22'}$	1.65 $10.0_{2'3}$	3.42 9.5_{34}		3.32 9.5_{45}	2.89	3.49 2.2_{56}	3.29 $5.6_{56'}$ 11.5_{66}
8, a-unit	5.49 1.5_{12}		4.15 2.2_{23}		2.25 4.5_{34} $11.0_{33'}$	2.15 $10.0_{3'4}$	5.12 10.0_{45}	4.02	3.59 2.5_{56}	3.48 $6.5_{56'}$ 11.5_{66}
8, b-unit	4.03 2.0_{12} 14.0_{11}	3.48 $2.0_{1'2}$ $12.0_{1'2'}$	2.17 4.0_{23} $13.0_{22'}$	1.64 $12.0_{2'3}$	3.59 9.5_{34}		3.48 9.5_{45}	3.03	3.58 2.1_{56}	3.32 $6.0_{56'}$ 10.8_{66}
9, a-unit	5.36 1.5_{12} $1.5_{12'}$		1.76	1.67	1.90	1.71	4.60 9.0_{45}	3.95	4.12 2.5_{56}	4.05 $5.6_{56'}$ 12.0_{66}
9, b-unit	3.85 1.5_{12} $4.5_{12'}$ $12.0_{11'}$	3.30 $4.5_{1'2}$ $12.0_{1'2'}$	2.03 1.5_{23} $12.0_{22'}$	1.58 $4.5_{2'3}$	3.42 9.5_{34}		3.25 9.5_{45}	3.18 6.2_{56}	1.28	

^{13}C-NMR Data[c]	C1	C2	C3	C4	C5	C6	CH_2
Compound.							
2, a-unit	98.1	29.1	26.7	66.0	73.6	61.6	
b-unit	66.2	34.8	75.2	82.5	76.0	19.0	
7	65.4	30.3	79.9	74.4	78.1	7.80	70.7
8, a-unit	101.6	23.4	32.9	66.1	70.2	63.1	
b-unit	65.1	30.4	79.7	81.0	77.5	7.3	70.4
9, a-unit	97.7	28.9	23.9	68.1	69.3	63.5	
b-unit	65.4	31.2	81.5	81.3	75.5	19.2	70.7

[a] Measured at 500 MHz in ^2H_2O at 300 K as 0.1M samples using acetone (2.22 ppm) and dioxane (67.4 ppm) as internal references, respectively for ^{1}H-NMR and ^{13}C-NMR spectra.

[b] Chemical shifts (ppm) and coupling constants (Hz) given below.

[c] Carbon shifts assigned through carbon/proton correlation spectroscopy [13].

*1,5-Anhydro-3-O-benzyl-2,6-dideoxy-4-O-(4,6-di-O-acetyl-2,3-dideoxy-D-erythro-hexopyranosyl)-D-arabino-hexitol (**9**)*

To a dry solution of **8** (400 mg 0.58 mmol) in toluene (50 ml) was added tri-*n*-butyl tinhydride (1.00 ml, 3.75 mmol), and the mixture was stirred under nitrogen for 2.5 h. Evaporation gave a syrup which was purified by flash chromatography on a column packed with silica gel using ethyl acetate/hexane, 1/1 by vol, as eluant. The syrupy product (215 mg) was acetylated with acetic anhydride (1 ml) in pyridine (3 ml) for 2 h. Work up in the usual way gave a product (200 mg), which was purified by preparative TLC using ethyl acetate/hexane, 1/1 by vol, as eluant. This yielded **9** (180 mg 0.41 mmol 71%). $[\alpha]_D$ +60.06 (*c* 1.9, chloroform). Analytical data calculated for $C_{28}H_{32}O_8$; C, 63.29; H, 7.39. Found: C, 63.26; H, 7.41, NMR data (^1H and ^{13}C) are given in Table 1.

*1,5-Anhydro-2,6-dideoxy-4-O-(2,3-dideoxy-D-erythro-hexopyranosyl)-D-arabino-hexitol (**2**)*

The syrupy product **9** (86 mg, 0.20 mmol) was dissolved in methanol (20 ml) and acetic acid (4 ml) and 5% palladium on activated carbon (40 mg) was added. The mixture was stirred under 1 atm. hydrogen pressure for 24 h. The catalyst was filtered off, washed well with methanol, and the organic phase was evaporated to dryness yielding 1,5-anhydro-2,6-dideoxy-4-*O*-(4,6-di-*O*-acetyl-2,3-dideoxy-D-*erythro*-hexopyranosyl)-D-*arabino*-hexitol as a syrup (71 mg, 0.205 mmol ~100%). De-*O*-acetylation of this syrup in 0.1% sodium methoxide in methanol (5 ml) for 1 h gave after work up by addition of solid carbon dioxide, and evaporation to dryness a product (78 mg), which was purified by chromatography on Sephadex G-15 using methanol/water, 1/1 by vol, as eluant. This gave **2** as a pure syrup (51 mg, 0.19 mmol, 95%), which was characterized by its NMR data (Table 1).

Enzymatic Reaction

The substrate **1** or **2** (5 mg) was dissolved in acetate buffer (0.7 ml, 0.11 M, p^2H 4.3) prepared from anhydrous sodium acetate, acetic anhydride and deuterium oxide. AMG solution (20 μl, 4.8 mg dry enzyme/ml) in deuterated buffer solution was added and the sample transferred to a 5 mm NMR tube. ^1H-NMR spectra were recorded at 300 K with suitable time intervals at 500 MHz. The concentration of compound **1** was estimated from the signal from the anomeric protons where as the concentration of compound **3** was estimated from the signal from the deoxy signals, H-6 b.

Results and Discussion

Tri-*O*-acetyl-D-glucal (**3**) was rearranged into the 2,3-unsaturated compound **4** which by treatment with lithium aluminium hydride gave the 4,6-di-*O*-acetyl-3-deoxy-D-glucal (**5**) in good yield as described by Fraser-Reid *et al.*[11].

Tri-*O*-acetyl-D-glucal was furthermore converted into the known 1,5-anhydro-4,6-*O*-benzylidene-2-deoxy-D-*arabino*-hexitol, which was benzylated to afford **6** in high yield. Removal of the 4,6-*O*-benzylidene group by 0.1 M hydrochloric acid followed by mesylation of the primary hydroxy group and nucleophilic substitution with sodium iodide in *N,N*-dimethyl formamide gave the aglycone 1,5-anhydro-3-*O*-benzyl-2,6-dideoxy-6-iodo-D-*arabino*-hexitol (**7**) in 60% overall yield.

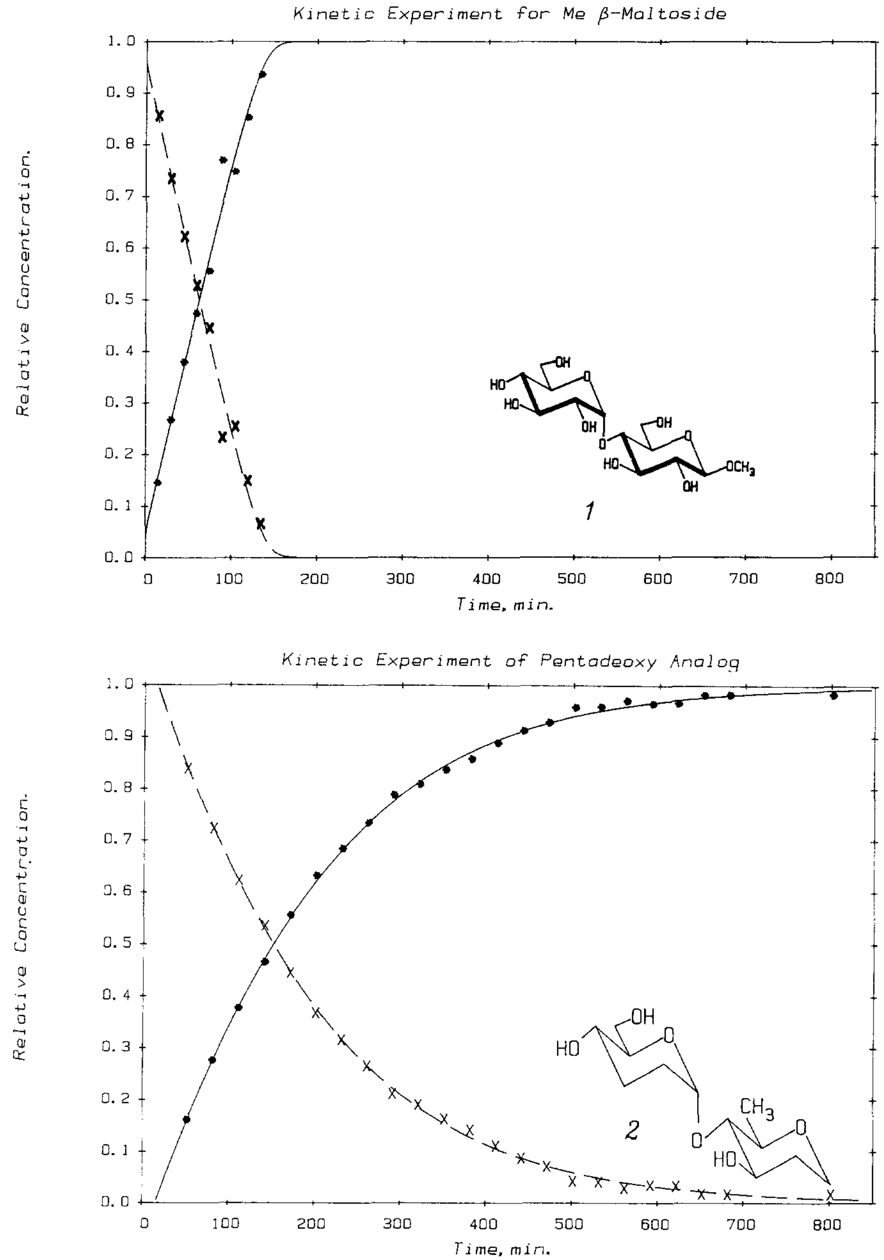
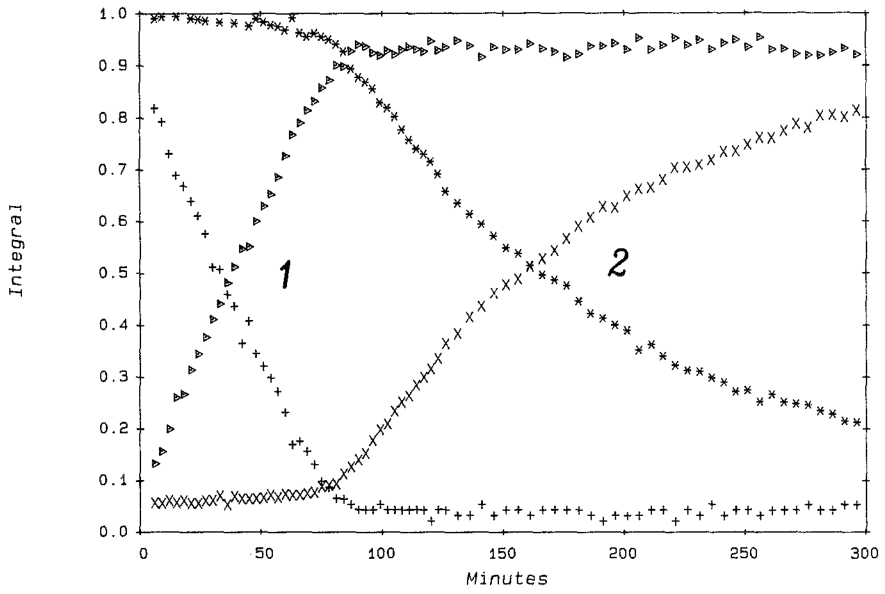


Figure 1. Enzymatic hydrolysis of substrates **1** and **2** with AMG using identical experimental conditions. A. Compound **1**, the data analysis gives a $V_0 = 7.4 \times 10^{-3}$ min and $t_{0.5} = 76$ min. B. Compound **2**, the data analysis gives a $V_0 = 4.9 \times 10^{-3}$/min and $t_{0.5} = 139$ min.

The 3-deoxy-D-glucal (**5**) and the aglycone **7** were treated with *N*-iodo-succinimide in acetonitrile as described by Thiem *et al.* [12] and the α-linked disaccharide **8** was isolated in 53% yield after chromatography. The iodine atoms were removed by reduction with tributyl tinhydride and **9** was isolated in 71% yield. Catalytical hydrogenolysis of the 3-*O*-benzyl group in methanol/acetic acid over Pd/C followed by removal of the acetyl



Substrates 1 and 2.

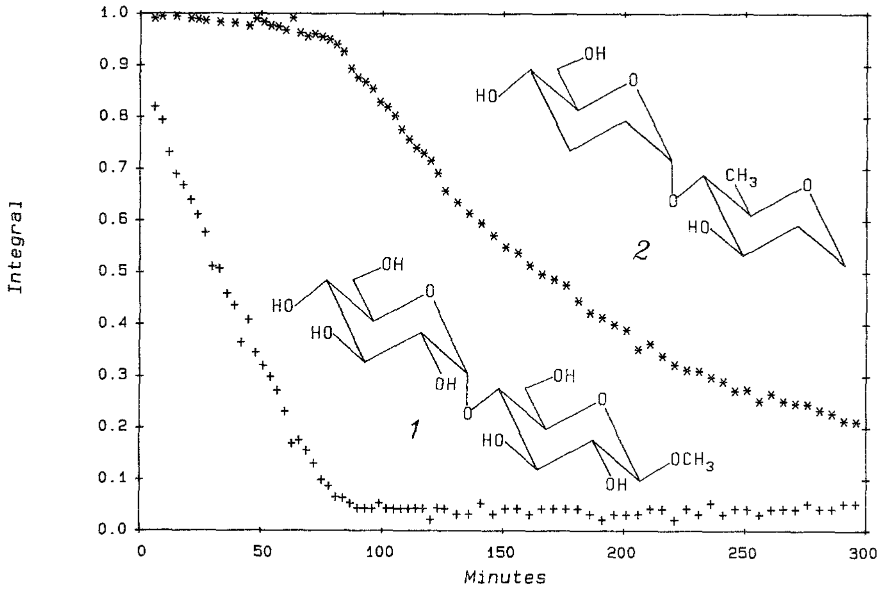


Figure 2. Competitive enzymatic hydrolysis of a mixture of substrates **1** and **2** (1:1). A. Substrate disappearance as a function of time for **1** and **2**, respectively. B. Simultaneous plot of substrate disappearance and product formation for **1** and **2** as a function of time.

groups with sodium methoxide in methanol gave the desired pentadeoxy compound **2** in 95% yield. The structure and purity of the product and the intermediates were all confirmed by their ^1H and ^{13}C NMR spectral data which are given in Table 1.

The compound proved to be a respectable substrate for the enzyme AMG as shown in Fig. 1. The half time for the hydrolysis of **2** is 139 min compared to 76 min for **1**, when the two compounds are hydrolyzed with AMG under identical experimental conditions. The results found when the two substrates are mixed and the progress of the hydrolysis followed by 500 MHz ^1H-NMR spectroscopy are shown in Fig. 2A and 2B. It is seen that the hydrolysis of the methyl maltoside (**1**) is completed before the enzyme reacts with the pentadeoxy compound (**2**), but that the half time of the reactions are not changed significantly for the two substrates compared to the values described above. The results are the same independent of the analysis of the substrates reacting (Fig. 2A) or the products formed (Fig. 2B). The affinity of the pentadeoxy derivative towards the enzyme is thus so much lower, due to the lack of the hydroxy groups, that the reaction first takes place when the normal substrate is fully hydrolyzed.

The results prove that the proposal by Lemieux, that key polar groups are absolutely essential for the recognition of oligosaccharides by antibodies, also is valid for the enzymatic hydrolysis of oligosaccharides by the enzyme amyloglucosidase, but that the overall strength of the binding in this case is much weaker than with the normal substrate.

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