Hydrolysis of Substrate Analogues Catalysed by β-D-Glucosidase from *Aspergillus niger*. Part II: Deoxy and Deoxyhalo Derivatives of Cellobiose†

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The hydrolysis of sixteen mainly deoxy and deoxyhalo derivatives of cellobiose catalysed by β-D-glucosidase from *Aspergillus niger* has been studied by means of ^{1}H NMR spectroscopy and progress-curve enzyme kinetics in both single-substrate and competition experiments. In the non-reducing ring of cellobiose it was found that the hydroxy groups at positions 2′, 3′, and 4′ are essential for the enzymatic hydrolysis. The primary hydroxy group on 6′ in this ring is, although important for the hydrolysis, not essential. The analogues modified at positions 3′ and 4′ and the 6′-bromo-6′-deoxy derivative were not inhibitors, whereas the 2′-deoxy derivative inhibited the enzymatic hydrolysis of methyl β-cellobioside to some extent. Of the analogues modified in the reducing ring, some were hydrolysed faster (e.g. the deoxy compounds) and some slower than methyl β-cellobioside in single-substrate experiments, but all derivatives were hydrolysed at a lower rate than this reference substrate in direct competition and displayed relatively weak inhibitory effects. The results are interpreted qualitatively with respect to changes in the free binding energies of the substrates and catalytic transition states based on the Michaelis–Menten mechanism, and some mechanistic implications of these findings are discussed.

Chemically modified substrates have, for many years, found application in the study of molecular recognition and enzyme mechanism and have proved to be one of the most fruitful approaches for the elucidation of specific interactions between macromolecular species and ligands, often accompanied by tremendous synthetic effort. We have recently reviewed this approach in the field of enzyme processing of carbohydrate substrates.[2]

Cellobiose [4-*O*-(β-D-glucopyranosyl)-D-glucose] occurs widely in plants, mainly as the prime hydrolysis product of cellulose catalysed by cellulases (EC 3.2.1.91). Hence, it constitutes the simplest cellulose model and the system cellobiose–cellobiase (β-D-glucosidase, EC 3.2.1.21) can be used to study the breakdown of β-linked D-glucose oligo- and poly-mers. We have previously described the use of ^{1}H NMR spectroscopy and progress-curve enzyme kinetics analysis to study of the β-D-glucosidase-catalysed hydrolysis of a number of methyl β-cellobioside analogous with deoxy functions in the reducing ring.[1] In this paper we describe the syntheses of some novel cellobiose derivatives and we report on further kinetic studies on the substrate specificity of a commercial β-D-glucosidase preparation from *Aspergillus niger*.

Edwards, in 1980, reviewed the chemistry of cellobiose and described the derivatives known at that time.[3] A number of cellobiose derivatives have since then been synthesized and described in the literature, most of which contain modifications of the two primary hydroxy groups. The most substantial of these works is that of Takeo *et al.*[4] We ourselves have placed much emphasis on the monodeoxy and monodeoxyhalo analogues of cellobiose in order to elucidate the carbohydrate–enzyme interactions of specific hydroxy groups.

β-D-Glucosidase from *Aspergillus niger* has previously been purified and characterized,[5–8] most recently by McCleary and Harrington[8] who used the commercial crude product from NOVO Industri A/S.[9] There are a number of disagreements between the results of these reports, especially in the molecular weight determinations and also in the Michaelis–Menten parameters. McCleary and Harrington[8] found K_m values of 1.89 and 0.8 mM for cellobiose and *p*-nitrophenyl β-D-glucopyranoside, respectively, so in the present study substrate concentrations well above K_m were used. Nothing is known about its amino acid sequence nor its amino acid composition, and hence the enzyme cannot be regarded as well characterized.

In this study, methyl β-cellobioside was chosen as the reference compound instead of cellobiose itself because mutarotation between α- and β-anomers is prevented. This

† Part I, see Ref. 1.

greatly simplifies the analysis of NMR spectra and the use of this compound enables one to distinguish between the hydrolysis products, glucose and methyl β-D-glucopyranoside, stemming from the two glucose units of the substrate. As pointed out previously[1] the conventional Michaelis–Menten parameters K_m and V_{max} are not well determined by simple progress-curve analysis – especially so when the reactions are severely product inhibited. Instead we use the accurately determinable parameters v_0 (initial rate) and $t_{\frac{1}{2}}$ (half-life) and compare these from experiments carried out under identical conditions.

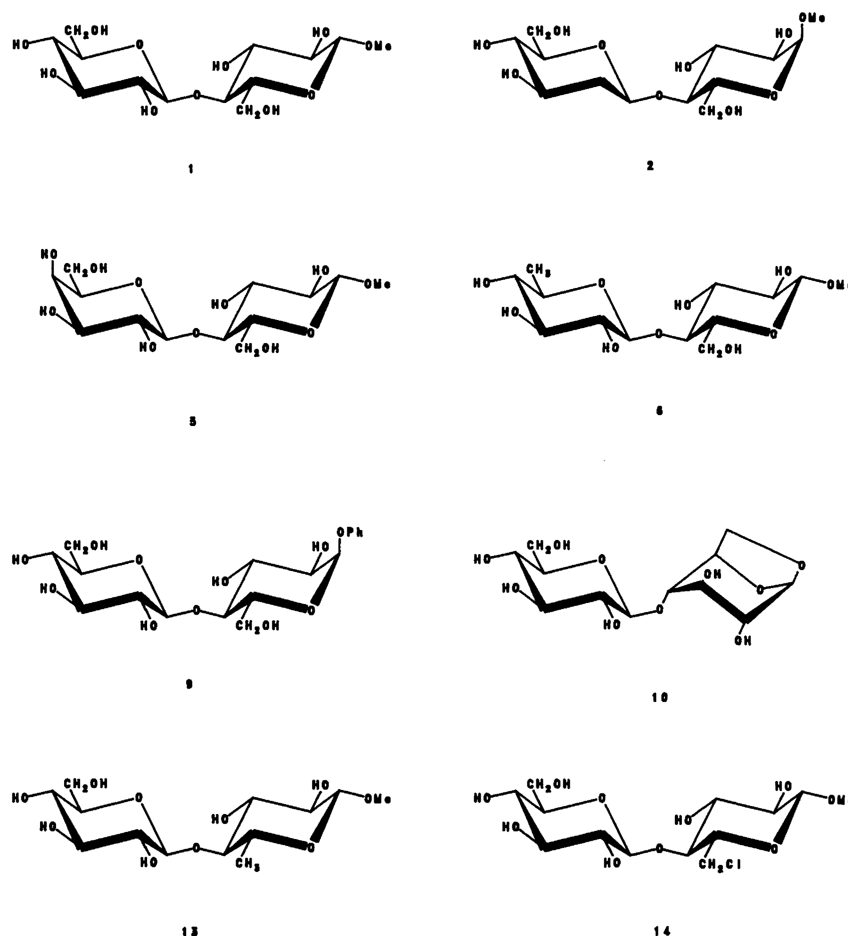
Results

Deoxy and deoxyhalo derivatives of cellobiose. Scheme 1 shows the structures of the cellobiose analogues used in this investigation. The syntheses of compounds not previously described are described below. The principal aim of the synthetic work was to obtain potentially interesting compounds and no effort has been made to optimize or to examine synthesis procedures thoroughly.

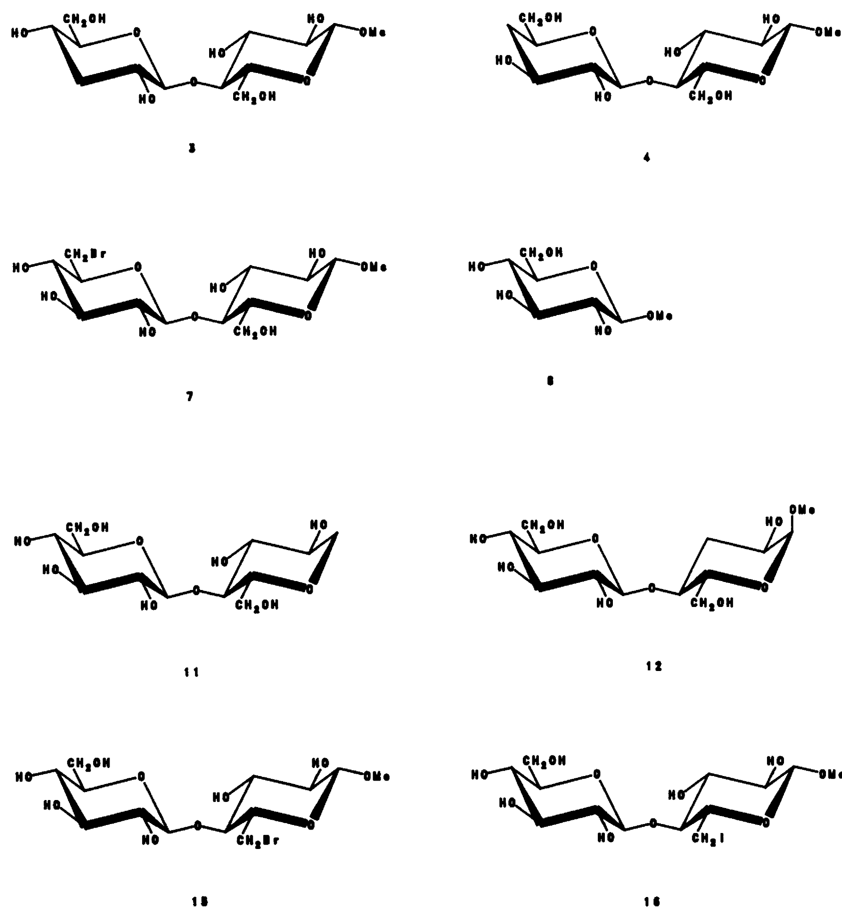
1,5-Anhydro-4-*O*-(β-D-glucopyranosyl)-D-glucitol **11** was prepared as shown in Scheme 2 from heptaacetyl-α-cellobiosyl bromide **24** via the heptaacetyl-1,5-anhydrocellobiitol **25** in 77 % yield. The use of hydrogen under pressure and triethylamine avoided the use of zinc for the reduction[10] and afforded a higher yield.

Methyl 3-deoxy-α-cellobioside **12** was synthesized analogously to the synthesis of the corresponding maltose derivative.[11] Heptaacetyl-α-cellobiosyl bromide **24** was first treated with sodium iodide in dry acetone followed by the addition of diethylamine which resulted in an elimination to produce heptaacetyl-2-hydroxycellobial **26** (Scheme 2). This was a more facile reaction than the method employed by Rao and Lerner who used 1,5-diazabicyclo[5.4.0]undec-5-ene for the dehydrohalogenation.[12] Compound **26** was then subjected to a Ferrier rearrangement by migration of the double bond to the 2,3-position catalysed by boron trifluoride–diethyl ether and in the presence of methanol the methyl α-glycoside **27** was obtained. Palladium-catalysed hydrogenation of **27** gave almost exclusively the desired *ribo* configuration and not the *arabino* of the methyl hexaacetyl-3-deoxy-α-cellobioside **28**. Deacylation by methoxide gave the unprotected methyl 3-deoxy-α-cellobioside **12**.

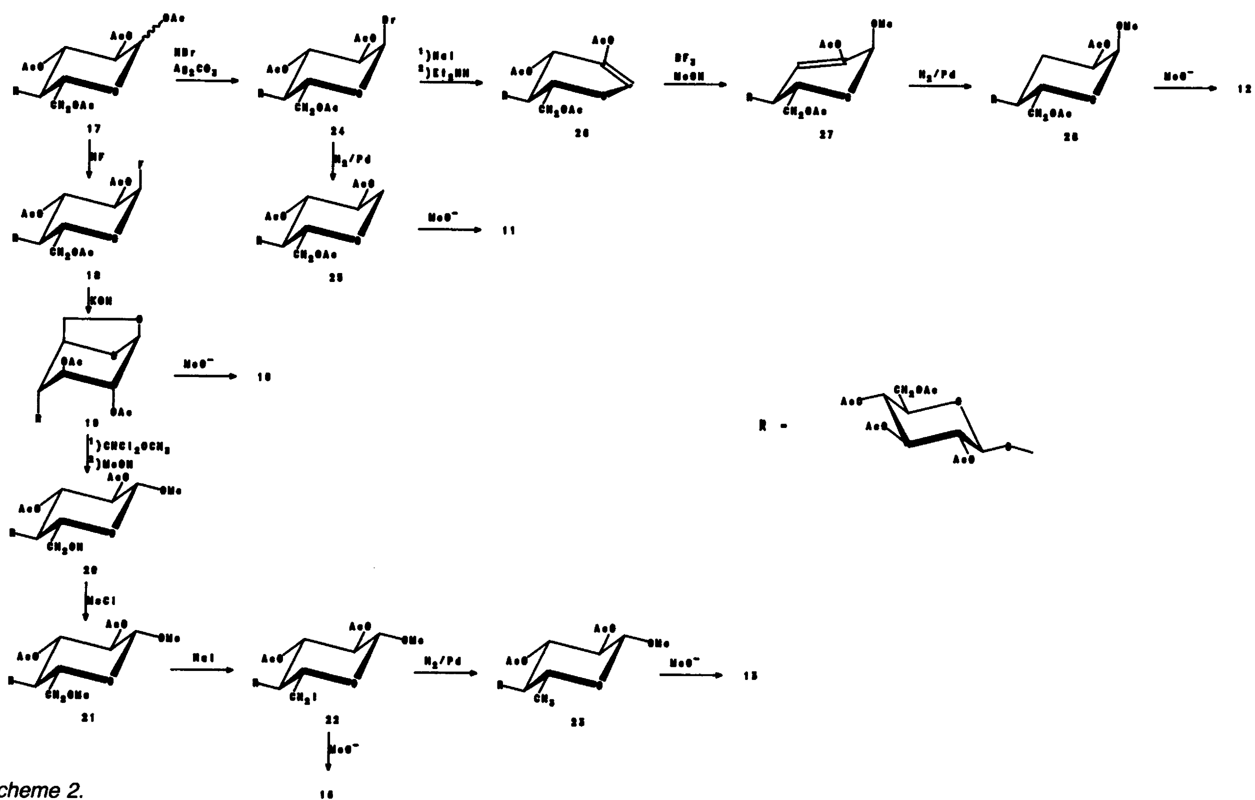
1,6-Anhydro-β-cellobiose **10**, methyl 6-deoxy-β-cellobio-



Scheme 1 (a).



Scheme 1 (b).



Scheme 2.

side **13**, and the 6-deoxy-6-iodo derivative **16** were also synthesized analogously to the corresponding maltose derivative[11,13] (Scheme 2).

Enzyme kinetics of derivatives modified in the non-reducing ring. The requirements for the enzyme to recognize specific hydroxy groups in the non-reducing ring were elucidated by preparing substrate analogues containing deoxy functions at the different positions. It is possible that some analogues which are not themselves substrates may be able to bind to the active site of the enzyme and thus express inhibitory effects.

Results for single-substrate experiments with analogues modified in the non-reducing ring are listed in Table 1. The results for the reference substrate methyl β-cellobioside were obtained from triplicate experiments. The relative error is smaller for the half-life than for the initial rate owing to the non-linearity of the least-squares regression analysis. Clearly, the hydroxy groups at positions 2′, 3′ and 4′ are essential for the analogue to be a substrate for the enzyme. The 4′-*epi* analogue (methyl β-lactoside) is not a substrate either. The 6′-deoxy derivative is slowly hydrolysed, whereas the 6′-bromo compound is not a substrate. These results are in very good accordance with the results obtained with β-D-glucosidase from *Aspergillus oryzae.*[14]

The results for the competition experiments with substrate analogues modified in the non-reducing ring are listed in Table 2. The 3′-deoxy, 4′-deoxy and 4′-*epi*, and 6′-bromo-6′-deoxy derivatives do not inhibit the hydrolysis of methyl β-cellobioside and these compounds appear not to be recognized by the enzyme at all at the concentrations studied.

The 6′-deoxy derivative is a poor inhibitor and its own hydrolysis rate is greatly reduced. Hence, this compound forms less stabilized enzyme–substrate and catalytic transition-state complexes than methyl β-cellobioside (see below).

Table 1. Single-substrate initial rates and half-lives of cellobiose analogues modified in the non-reducing ring relative to the single-substrate values for the reference substrate methyl β-cellobioside. The initial rates and half-lives were obtained by fitting of the progress curves of the enzymatic hydrolysis as described previously.[1] The reactions took place in D_2O at pH 4.75 and 27 °C.

Substrate	y_0/mM	Relative	
		v_0	$t_{\frac{1}{2}}$
1 (ref.)^a	28.1	1	1
2 (Me 2′-deoxy-α)	4.9	0	∞
3 (3′-deoxy)	29.4	0	∞
4 (4′-deoxy)	29.4	0	∞
5 (4′-*epi*)	28.1	0	∞
6 (6′-deoxy)	29.4	0.18	5.6
7 (6′-Br)	23.9	0	∞

$^a v_0 = 0.472 \pm 0.007$ mM min^{-1}, $t_{\frac{1}{2}} = 31.3 \pm 0.3$ min. Errors are standard deviations from triplicate experiments.

Table 2. Competition initial rates and half-lives of cellobiose analogues modified in the non-reducing ring relative to the single-substrate values for the reference substrate methyl β-cellobioside.

Substrate	y_0/mM	Relative	
		v_0	$t_{\frac{1}{2}}$
One-substrate experiment			
1 (ref.)^a	28.1	1	1
Two-substrate experiments			
1	28.1	0.73	1.4
2-deoxy-D-glucose	15.3		
1	28.1	0.02	84
2 (Me 2′-deoxy-α)	5.0	0	∞
1	28.1	1.0	0.99
3 (3′-deoxy)	29.4	0	∞
1	28.1	0.98	1.0
4 (4′-deoxy)	29.4	0	∞
1	28.1	0.99	1.0
5 (4′-*epi*)	28.1	0	∞
1	28.1	0.95	1.1
6 (6′-deoxy)	29.4	0.02	86
1	28.1	0.93	1.1
7 (6′-Br)	23.9	0	∞

$^a v_0$ and $t_{\frac{1}{2}}$ as in Table 1.

Methyl 2′-deoxy-α-cellobioside is not a substrate, although it is known that 2′-deoxy glycosides are very labile to acid hydrolysis.[14,15] In fact, a slow breakdown at a constant rate was observed for this compound, but since the rate was the same in single substrate as well as in competition experiments, it could be concluded that the observed breakdown was due to non-enzymatic acid-catalysed hydrolysis. This 2′-deoxy analogue is, however, the most potent inhibitor of the analogues tested. This suggests that the 2′-hydroxy group may be directly involved in the catalytic process.

Enzyme kinetics of derivatives modified in the reducing ring. Kinetic single-substrate experiments were carried out with a number of substrates modified in the reducing ring and the results are summarized in Table 3. In the one-substrate experiments some analogues are hydrolysed more rapidly than methyl β-cellobioside in the order 3-deoxy > 6-deoxy > 1-deoxy > 6-bromo-6-deoxy > Ph α-cell. > reference whereas the others are hydrolysed considerably slower. Thus it appears that increasing the hydrophobicity of the aglycone enhances the enzymatic hydrolysis, whereas inverting the aglycone ring from a $^4C_1$ to a $^1C_4$ chair

Table 3. Single-substrate initial rates and half-lives of cellobiose analogues modified in the reducing ring relative to the single-substrate values for the reference substrate methyl β-cellobioside.

Substrate	y_0/mM	Relative	
		v_0	$t_{\frac{1}{2}}$
1 (ref.)[a]	28.1	1	1
8 (Me β-D-glc*p*)[b]	25.7	0.43	9.1
9 (Ph α-cell.)	26.1	1.1	1.9
10 (1,6-anhydro)[b]	30.8	0.44	2.5
11 (1-deoxy)[b]	30.6	1.5	0.69
12 (3-deoxy-α)[b]	29.4	4.3	0.41
13 (6-deoxy)[b]	29.4	2.1	0.51
14 (6-Cl)	26.7	0.57	1.3
15 (6-Br)	23.9	1.3	0.65
16 (6-I)	21.5	0.53	1.4

[a] v_0 and $t_{\frac{1}{2}}$ as in Table 1. [b] Reported previously.[1]

Table 4. Competition initial rates and half-lives of cellobiose analogues modified in the reducing ring relative to the single-substrate values for the reference substrate methyl β-cellobioside.

Substrate	y_0/mM	Relative	
		v_0	$t_{\frac{1}{2}}$
One-substrate experiment			
1 (ref.)[a]	28.1	1	1
Two-substrate experiments			
1[b]	28.1	0.66	2.0
8 (Me β-D-glc*p*)	25.7		
1	28.1	0.54	1.9
9 (Ph α-cell.)	26.1	0.43	2.9
1[b]	28.1	0.91	1.1
10 (1,6-anhydro)[b]	30.8	0.07	14
1[b]	28.1	0.51	1.9
11 (1-deoxy)[b]	30.6	0.44	2.4
1[b]	28.1	0.93	1.1
12 (3-deoxy-α)[b]	29.4	0.52	1.9
1[b]	28.1	0.50	2.0
13 (6-deoxy)[b]	29.4	0.36	2.8
1	28.1	0.53	1.8
14 (6-Cl)	26.7	0.28	3.3
1	28.1	0.36	2.7
15 (6-Br)	23.9	0.15	2.8
1	28.1	0.21	4.2
16 (6-I)	21.5	0.09	4.1

[a] v_0 and $t_{\frac{1}{2}}$ as in Table 1. [b] Reported previously.[1]

conformation as in 1,6-anhydro-β-cellobiose decreases the hydrolysis rate.

For the 6-deoxy-6-halo series, the chloro and the iodo derivatives have almost the same kinetic parameters, whereas the bromo compound has an initial rate twice as large and a correspondingly shorter half-life. From a simple comparison of rates and half-lives, there is no clear trend as to the influence of steric factors of this group on the binding of the reducing ring.

Phenyl α-cellobioside has a greater initial rate than methyl β-cellobioside, but a longer half-life. This reaction is thus severely product inhibited and the enzyme thus forms a less firmly bound enzyme–substrate complex with phenyl α-cellobioside than with methyl β-cellobioside.[2]

Competition experiments were carried out with two simultaneous substrates (or substrate + inhibitor) with methyl β-cellobioside as the reference substrate together with an analogue modified in the reducing ring or methyl β-D-glucopyranoside. The results are shown in Table 4. Methyl β-cellobioside is hydrolysed faster than its competitor when in direct competition with the analogue, as is also the case when the analogues are hydrolysed faster in single-substrate experiments. When compared with the single-substrate experiment with methyl β-cellobioside, all analogues inhibit the hydrolysis of this somewhat, and the inhibitory effect on the reference substrate has the order 6-bromo-6-deoxy > 6-deoxy > 1-deoxy > Ph α-cell > 3-deoxy.

The reducing ring analogues are all inhibited by methyl β-cellobioside in such a way that the relative order of inhibition of the reference substrate on the analogues is 6-bromo-6-deoxy > 3-deoxy > 6-deoxy > 1-deoxy > Ph α-cell. In the order of decreasing inhibition which apart from the 6-bromo-analogue is the same order as the order of rates in the single-substrate experiments.

Discussion

The results for this β-D-glucosidase resemble those for β-D-galactosidase from *Escherichia coli*,[16,17] especially with regard to the requirements of the hydroxy groups in the non-reducing ring. There are, however, differences in product-inhibition properties and in the interactions with the reducing ring. Glucoamylase from *Aspergillus niger* showed a different pattern of specificity.[11,18–23] The essential hydroxy groups for this enzyme are those in positions 3, 4′ and 6′. Perhaps the hydroxy group in the 2′-position of cellobiose is engaged in neighbouring-group assistance and interacts with the catalytic carboxy group on the β-D-glucosidase (by hydrogen bonding), whereby the 2′-OH group could direct the acidic group of the active site into the right position for proton donation to the glycosidic oxygen atom. Similar observations and proposals have been made for β-D-galactosidase from *Escherichia coli*,[16,17] whereas the 2′-OH is non-essential for glucoamylase.[11,18–23] It must be pointed out that the 2′-deoxy derivative in this study was the methyl α-glycoside whereas the other analogues were

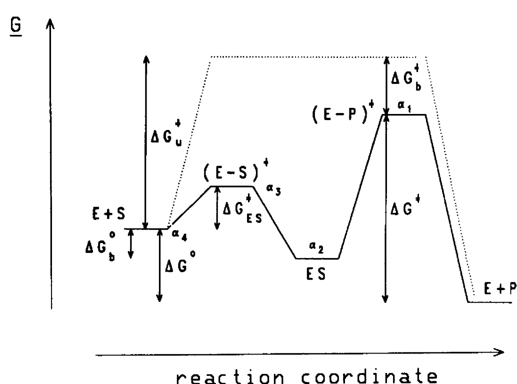
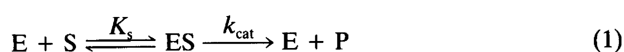


Fig. 1. Free-energy diagram of the β-D-glucosidase-catalysed hydrolysis of cellobiose analogues.

β-glycosides. It cannot be ruled out that the differences in behaviour could be due to this difference in anomeric configuration. The specificity for the 4′-position is mutual for all enzymes mentioned here (this is what distinguishes the substrates for β-D-glucosidase and β-D-galactosidase), and this specificity has also been found for D-xylose (D-glucose) isomerase.[24] It can be visualized that the substrate approaches the enzyme from the C-4′ end of the non-reducing ring, and then the group in the 4-position of this ring would be the first to encounter the active site. It is therefore likely that the specificity requirements are especially critical for this position. It can also be argued that any comformational changes in the substrate in going to the transition state can be recognized by movement in the position of this hydroxy group.

Fig. 1 shows a qualitative free-energy diagram referring to the simple Michaelis–Menten mechanism given in eqn. (1), where E is the free enzyme, S is the free sub-



$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (1)$$

strate, ES is the enzyme–substrate complex, and P is the product. K_s is the dissociation constant for the enzyme–substrate complex and is approximated (identified) with K_m, whereas k_{cat} is the catalytic rate constant ($= V_m/[E]_0$, where $[E]_0$ is the stoichiometric enzyme concentration). The ratio k_{cat}/K_m is the second-order rate constant (or the so-called binding specificity or catalytic power). The solid line corresponds to the enzyme-catalysed reaction, whereas the dotted line corresponds to the uncatalysed (or acid-catalysed) reaction. The scheme has been truncated. It is known that enzymatic reactions such as the ones in question consist of several intermediary steps with several bound ground-state as well as transition-state species. However, one of these steps is usually rate limiting, i.e. the transition state of this step and the preceding intermediate are kinetically the most important species. The free energies depicted in the figure are:

ΔG° free energy of the overall reaction,
ΔG°_b free binding (stabilization) energy of ES,
$\Delta G^\ddagger_{ES}$ free energy of activation for formation of ES,
$\Delta G^\ddagger_u$ free energy of activation for the uncatalysed reaction,
$\Delta G^\ddagger$ free energy of activation for the enzyme-catalysed reaction,
$\Delta G^\ddagger_b$ free binding (stabilization) energy of the catalytic transition state.

The absolute positions of the energy levels are arbitrary. The α factors are the factors by which the corresponding energy level is changed in the substrate analogues compared with the reference compound methyl β-cellobioside. An increase in a transition state will lead to a decrease in the rate constants, whereas an increase in a ground state will lead to an increase in the rate constants. A larger initial rate for an analogue compared with the reference substrate can result from either of two sources: the transition state of a rate-limiting step along the enzymatic pathway may be of lower energy and thereby give rise to a lower energy of activation. A lower-energy transition state can be formed either because of tighter binding to the enzyme or because the transition-state structure itself has an intrinsically lower energy. The other possibility for an increase in the initial rate may arise from looser binding of an intermediate preceding a rate-limiting step. This would also lead to a decrease in the energy of activation and thus a higher rate. In the first case the analogue would also be hydrolysed faster than the reference compound in direct competition with this and the mutual inhibitory effects are expected to be greater on the reference than on the analogue. The reverse is true for the second case, here the analogue would be hydrolysed more slowly than the reference in direct competition and the analogue would also be the most susceptible substrate for inhibition.

All compounds for which the energy of activation is reduced are hydrolysed faster than methyl β-cellobioside in single-substrate experiments, but are hydrolysed at a lower rate than the reference substrate in direct competition with this. So it seems for the deoxy compounds that the better substrates they are by themselves, the worse competitors they are to the reference substrate. A plausible explanation for this is that both a kinetically important enzyme–substrate (enzyme–intermediate) complex and a rate-limiting transition state in the Michaelis–Menten type reaction scheme (Fig. 1) are less stabilized for the fast analogues than for the reference compound, but in such a way that the destabilization of the enzyme–substrate complex is considerably larger than the destabilization of the transition state (i.e. $\Delta\Delta G^\circ_b > \Delta\Delta G^\ddagger_b$). This leads to lower energies of activation and will cause a rate increase in single-substrate experiments. But if, on the other hand, the substrate analogue has to compete directly with methyl β-cellobioside, the relative stabilizations of the catalytic transition states will determine the relative rates, and the relative order of rates will switch over in favour of the substrate which forms

the most stabilized catalytic transition state. In all cases tested, the most stabilized catalytic transition state was formed by the reference compound methyl β-cellobioside. According to this model 1,6-anhydro-β-cellobiose apparently forms a substantially less stable enzyme–substrate complex than methyl β-cellobioside, but it also forms a substantially higher-energy transition state, hence it exhibits a relatively low rate of hydrolysis and a weak inhibition. The qualitative energy diagram shown in Fig. 1 illustrates this feature. The 6-bromo-6-deoxy analogue is the only halo compound which has a higher energy of activation than the reference and is hydrolysed faster than this. This is because the destabilization of the enzyme–substrate complex is large enough to compensate for the relatively small destabilization of the catalytic transition state.

Methyl 6′-deoxy-β-cellobioside and 1,6-anhydro-β-cellobiose show the largest increases in the difference of binding energies of the transition states and also some of the largest increases in the difference of binding energies of the enzyme–substrate complex. However, the destabilization of the enzyme–substrate complex is not sufficiently large to compensate the rate reduction, and these compounds are therefore both relatively poor substrates and poor inhibitors.

Experimental

General procedures. ^{1}H NMR spectra were recorded on Bruker AM-500, Bruker AC-250, and Bruker HX-90 instruments and ^{13}C NMR spectra on Bruker AM-500, Bruker AC-250, and Bruker WH-90 instruments at 300 K. Tetramethylsilane was used as an internal reference (0 ppm) in deuteriochloroform solutions for both ^{1}H and ^{13}C spectra. For spectra recorded in deuterium oxide the HDO peak was used as internal reference (4.75 ppm) for ^{1}H spectra, whereas an external instrument reference was used for ^{13}C spectra in this solvent. NMR data are shown in Tables 5 and 6.

Glucose-free and lyophilised β-D-glucosidase from *Aspergillus niger* (EC 3.2.1.21, β-D-glucopyranoside glucohydrolase, cellobiase, Novozym 188) was a gift from NOVO Industri A/S. This enzyme is commercial and used in industry together with cellulases for the breakdown of cellulose to glucose. The enzyme is very stable, and no loss of activity could be detected for an enzyme batch after having been stored for 2 months at room temperature, in accordance with the manufacturer's specifications.[9] The commercial enzyme preparation from NOVO is very crude – only 1.5 % of the total protein is β-D-glucosidase,[8] but the pH- and temperature-profiles for stability and activity are the same for the crude and the purified preparations.[8,9] However, although activities towards maltose, lactose, trehalose, sucrose, and methyl α-D-glucopyranoside can be detected in the crude preparation, they were observed to be very low (<< 1 %) compared with the activities towards β-D-glucosides. Such secondary activities are common in a great number of enzyme preparations. In the time scales we used in our kinetic experiments, no secondary activities could be detected; these only become apparent upon prolonged standing. We primarily measured large catalytic effects and our interpretations are based only on such data. Most importantly, there was an intrinsic control in our experiments, in that all substrates were studied in both single substrate as well as in competition experiments, and since large mutual inhibitory effects are observed both of the reference substrate on the analogues and *vice versa,* it can, with reasonable confidence, be ruled out that any of the catalytic effects could be provided by other enzymes than the β-D-glucosidase in question.

The enzyme kinetics reactions took place in 0.1 M sodium acetate buffer in deuterium oxide, pH 4.75 (uncorrected meter reading) at 27 °C. The concentrations of substrates were 10 mg ml^{-1} (ca. 30 mM) and 15 μl of a solution of 20 mg ml^{-1} enzyme in the same buffer was added to 1 ml of the substrate solution to start the reaction. An aliquot of 600 μl in an NMR tube was then quickly degassed and placed in the spectrometer probe. Kinetic ^{1}H NMR spectroscopy and progress-curve analysis were then carried out as described previously.[1]

Optical rotations were measured on a Perkin Elmer 241 polarimeter, melting points are uncorrected and elemental analyses were carried out by Løven A/S's microanalytical laboratory. All reactions in organic solvents were carried out with the exclusion of moisture (calcium chloride).

Work-up from organic solvents was carried out by three washings with 4 M hydrochloric acid and three with saturated sodium hydrogen carbonate solution. The solutions were then dried with sodium or magnesium sulphate, filtered, and concentrated under diminished pressure at ca. 50 °C.

Deacylations took place in dry methanol containing ca. 1 mM sodium methoxide for approximately 0.5–1.0 h. Ions were removed by the addition of IRC-50 (H^{+}) ion-exchange resin equilibrated in methanol. After filtration of the resin, the solution was concentrated by vaporization under reduced pressure.

The palladium used as the catalyst for hydrogenations was a mixture containing 5 % palladium and activated carbon (Pd/C).

Where stated, unprotected sugars were separated on a Sephadex 15 column by elution with methanol–water (1:1). Fractions were collected by a fraction collector and examined for carbohydrate by charring with sulfuric acid. The relevant fractions were then pooled and concentrated. All reactions took place at room temperature unless otherwise stated.

Methyl 2′-deoxy-α-cellobioside **2** was a generous gift from Prof. Schmidt (Konstanz, Germany).[25] Methyl 3′-deoxy-β-cellobioside **3** was prepared by Bock and Pedersen[11] and was obtained as a by-product in equal amount from a glycoside synthesis of the corresponding maltose derivative, while methyl 4′-deoxy-β-cellobioside **4** was pre-

Table 5. ^{1}H NMR chemical shifts and observed first-order coupling constants of protected and unprotected disaccharides. Assignments marked with an asterisk may need to be reversed. The two first rows for each compound (designated by H′) contain data from the non-reducing ring and the rows below contain data from the reducing ring.

	Solvent		1	2	3	4	5	6	6′	OMe
1	D_2O	H′	4.52	3.32	3.53	3.43	3.50	3.94	3.75	
		J_3	7.7	8.0	9.2	9.2	2.0	6.3		
		J_2						12.1		
		H	4.42	3.32	3.65	3.65	3.64	4.01	3.83	3.59
		J_3	7.7	8.0			2.0	4.8		
		J_2						11.8		
2	D_2O	H′	4.77	1.87$_e$ 1.49$_a$	3.76	3.29	3.40	3.97	3.77	
		J_3	10.0 2.0	5.0$_e$ 12.0$_a$	9.5	9.9	2.2	6.0		
		J_2		13.0				12.3		
		H	4.85	3.64	3.82	3.71	3.78	3.89	3.81	3.44
		J_3	4.0	10.0	10.0	10.0	2.8	5.8		
		J_2						12.8		
3	D_2O	H′	4.51	3.53	2.22$_e$ 1.61$_a$	3.50	3.44	3.94	3.71	
		J_3	7.8	5.6 10.4	3.1$_e$ 8.6$_a$					
		J_2			13.9					
		H	4.42	3.33	3.66	3.76	3.65	4.01	3.83	3.60
		J_3	8.0	7.8			1.8	4.8		
6	D_2O	H′	4.56	3.32	3.48	3.20	3.52	1.34		
		J_3	7.7	9.2	9.6	9.6	6.0			
		H	4.38	3.32	3.64	3.43	3.50	3.93	3.74	3.57
		J_3	7.7	8.0			2.0	6.3		
		J_2						11.8		
7	D_2O	H′	4.59	3.35	3.55	3.50	3.66	3.86	3.70	
		J_3	7.9	9.4	9.2	9.1	1.8	5.4		
		J_2						11.4		
		H	4.42	3.34	3.66	3.66	3.66	4.01	3.83	3.59
		J_3	8.0	9.1				4.5		
		J_2						12.3		
9	D_2O	H′	4.58	3.38	3.55	3.48	3.55	3.96	3.77	
		J_3	7.8	8.0	9.2	9.2	2.0	6.4		
		J_2						12.0		
		H	5.66	3.79	4.09	3.81	3.86	4.01	3.93	
		J_3	4.0	8.4			2.4	5.5		
		J_2						12.0		
10	D_2O	H′	4.61	3.37	3.54	3.44	3.48	3.96	3.76	
		J_3	7.0	8.0	9.2	9.2	2.0	6.3		
		J_2						12.1		
		H	5.47	3.54	3.90	3.85	4.80	4.09	3.80	
		J_3	1.7			2.2		5.7		
		J_2						7.5		
11	D_2O	H′	4.51	3.33	3.58	3.43	3.49	3.93	3.75	
		J_3	8.0	8.0	9.0	9.4	2.1	5.8		
		J_2						12.3		
		H	3.49	3.29	3.54	3.64	3.64	3.98	3.78	
		J_3	1.9$_e$ 8.9$_a$	8.9	8.6		2.6	6.6		
		J_2						12.4		
12	D_2O	H′	4.51	3.23	3.46	3.36	3.46	3.87	3.70	
		J_3	8.1	9.1	9.1	9.5	2.5	4.2		
		J_2						12.3		
		H	4.69	3.80	2.34$_e$ 1.80$_a$	3.43	3.62	3.90	3.76	3.43
		J_3	3.5	3.9	4.9$_e$	9.8	2.5	5.6		
		J_2			11.9			12.6		

(cont.)

Table 5. (cont.).

	Solvent		1	2	3	4	5	6	6′	OMe
13	D_2O	H′	4.42	3.14	3.55	3.58	3.55	3.94	3.76	
		J_3	8.4	9.6	9.6	9.6	1.5	3.6		
		J_2						12.6		
		H	4.35	3.26	3.41	3.26	3.47	2.77		3.53
		J_3	8.4	9.1	9.6	9.6	6.0			
14	D_2O	H′	4.58	3.32	3.53	3.43	3.51	3.92	3.75	
		J_3	8.0	9.1	9.2	9.4	1.6	5.5		
		J_2						12.2		
		H	4.47	3.33	3.66	3.78	3.86	4.02	3.86	3.58
		J_3	8.0	9.1	9.2	9.0	2.2	9.6		
15	D_2O	H′	4.62	3.34	3.54	3.44	3.52	3.91	3.76	
		J_3	8.0	9.5	9.5	9.5	2.2	6.0		
		J_2						11.1		
		H	4.49	3.34	3.67	3.74	3.79	3.94	3.84	3.60
		J_3	8.0	8.9	8.9	9.0	2.2	4.2		
		J_2						12.1		
16	D_2O	H′	4.63	3.33	3.53	3.43	3.52	3.93	3.75	
		J_3	7.9	9.3	9.1	9.4	1.7	4.8		
		J_2						12.4		
		H	4.49	3.34	3.68	3.59	3.43	3.75	3.51	3.54
		J_3	8.1	9.4	9.0	8.9	1.3	6.6		
		J_2						11.5		
19	$CDCl_3$	H′	4.87	5.05	5.21	5.05	3.80	4.11	4.23	
		J_3	8.1	9.4	9.5	8.6	1.9	5.5		
		J_2						12.3		
		H	5.45	5.19	4.55	3.54		3.97	4.59	
		J_3						5.2		
		J_2						7.3		
20	$CDCl_3$	H′	4.64	4.93	5.17	5.06	3.69	4.04	4.36	
		J_3	8.1	9.5	9.6	9.7	2.0	4.4		
		J_2						12.4		
		H	4.41	4.87	5.17		3.39	3.93		3.49
		J_3	8.0	9.7		9.8	2.3			
		J_2						12.4		
21	$CDCl_3$	H′	4.63	4.93	5.18	5.07	3.70	4.05	4.35	
		J_3	7.9	9.3	9.4	9.6	2.3	4.4		
		J_2						12.5		
		H	4.42	4.88	5.18	3.84	3.63	4.56	4.38	3.49 3.11 (OMs)
		J_3	7.8	9.6	9.5	9.5	2.0	4.0		
		J_2						7.4		
22	$CDCl_3$	H′	4.66	4.94	5.16*	5.07	3.69	4.06	4.36	
		J_3	8.1	9.3	9.0	9.5	2.1	4.2		
		J_2						12.3		
		H	4.44	4.91	5.18*	3.64	3.27	3.62	3.29	3.52
		J_3	8.1	9.3	9.0	9.0	6.8			
		J_2						12.0		
26	$CDCl_3$	H′	4.68	4.98	5.18	5.10	3.69	4.25	4.29	
		J_3	7.9	9.2	9.4	9.7	1.8	4.4		
		J_2						12.4		
		H	6.59		5.69	4.43	4.08	4.29	4.25	
		J_3			4.7	9.2	2.0	4.5		
		J_2						12.4		
27	$CDCl_3$	H′	4.69	5.03	5.25	5.19	4.10	4.20	4.38	
		J_3	7.9	9.6	9.7	9.7	1.7	5.4		
		J_2						11.9		
		H	4.95		5.92	4.40	3.77	4.38	4.20	3.49
		J_3			2.0	10.0	2.8			
		J_2						10.7		

Table 6. ^{13}C NMR chemical shifts of protected and unprotected disaccharides. Assignments marked with an asterisk may need to be reversed. The first two rows for each compound (designated by C′) contain data from the non-reducing ring and the rows below contain data from the reducing ring.

	Solvent		1	2	3	4	5	6	OMe
1	D_2O	C′	103.3	74.0	76.4	70.3	76.8	61.4	
		C	103.9	73.7	75.2	79.6	75.6	60.9	58.1
2	D_2O	C′	104.0	68.7*	39.1	65.2*	79.6	61.7	
		C	105.6	73.8*	75.3	80.8	75.7	61.1	58.1
6	D_2O	C′	103.9	74.6	77.2	71.2	77.5	16.1	
		C	104.5	74.2	75.9	80.3	76.4	61.8	58.9
7	D_2O	C′	103.3	74.0	75.1*	72.0	75.8	33.3	
		C	103.9	73.7	75.0*	79.8	75.5	60.9	58.1
9	D_2O	C′	103.9	74.6	76.9	70.9	77.4	62.0	
		C	98.1	72.6*	72.3*	79.8	73.0*	61.0	
10	D_2O	C′	102.3	70.7	73.8	70.6	76.9*	65.9	
		C	102.3	74.8	78.4	79.6	76.3*	61.6	
11	D_2O	C′	103.4	74.0	76.3	70.3	76.8	61.4	
		C	79.9*	69.5	70.0	79.8*	76.8	61.1	
12	D_2O	C′	103.8	74.4	76.3	71.5	76.6	61.4	
		C	98.9	66.8	34.4	70.3	73.9	60.9	55.6
14	D_2O	C′	103.4	73.9	76.2	70.2	76.8	61.3	
		C	104.0	73.6*	73.9*	79.7	74.8	44.2	58.2
15	D_2O	C′	103.4	74.0	76.3	70.2	76.8	61.3	
		C	103.9	73.6	73.6	81.0	74.8	33.2	58.3
16	D_2O	C′	103.5	74.0	76.3	70.3	76.8	61.4	
		C	103.8	73.7	73.7	83.0	74.6	6.7	58.3
19	$CDCl_3$	C′	100.5	71.2	72.8	68.0	72.1	61.8	
		C	98.9	68.4	69.0	76.6	73.6	64.8	
20	$CDCl_3$	C′	100.5	71.7	73.0	67.9	71.8	60.2	
		C	101.7	71.5	72.6	74.9	74.9	61.6	57.4
21	$CDCl_3$	C′	100.3	71.7	72.9	67.8	72.0	61.5	
		C	101.6	71.3	72.3	76.8	72.7	67.0	57.2 38.0 (OMs)
22	$CDCl_3$	C′	100.6	71.7	72.9	67.6	72.0	61.8	
		C	101.1	71.5	72.0	79.8	73.7	4.2	57.1
23	$CDCl_3$	C′	100.8	71.7	72.7	67.7	72.5	61.4	
		C	101.1	75.4	72.7	76.8	70.7	17.0	56.7
25	$CDCl_3$	C′	100.5	71.4	72.7	67.6	71.7	61.4	
		C	77.2	66.6	67.8	76.3	73.1	61.6	
26	$CDCl_3$	C′	100.5	71.3	72.7	68.0	72.1	61.6	
		C	139.4	74.8	74.8	76.7	68.1	61.3	
27	$CDCl_3$	C′	101.5	71.3	72.7	67.6	71.9	62.5	
		C	117.8	145.5	94.7	73.4	68.3	62.0	56.1
28	$CDCl_3$	C′	101.8	71.3	72.7	68.2*	71.8	62.7	
		C	96.1	68.3*	31.3	75.0	68.4*	62.2	55.0

pared by Bock and Adelhorst[16,17] being identical with the corresponding lactose derivative.

Methyl β-cellobioside [methyl 4-*O*-(β-Dglucopyranosyl)-β-D-glucopyranoside] **1** was synthesized by the Koenigs–Knorr method[26] from commercial cellobiose octaacetate **17**. Methyl 6′-deoxy-β-cellobioside **6** and the methyl 6-deoxy-6-halo-β-cellobiosides **14** and **15** were obtained according to Takeo *et al.*[4] Phenyl α-cellobioside **9** was prepared from the peracetate obtained by the method of Helferich and Schmitz-Hillebrecht[27] by deacylation with methoxide.

*Methyl 2,6-di-O-acetyl-3-deoxy-4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-*erythro*-hex-2-enopyranoside* (**27**). Compound **26** (6.0 g, 9.7 mmol) was dissolved in dry dichloromethane (60 ml) and a mixture of dry methanol/boron trifluoride–diethyl ether/dichloromethane (1:3:11, 10.8 ml) was added and the solution was stirred (1.5 h). After the addition of 150 ml dichloromethane, the solution was washed with water and three times with saturated sodium hydrogen carbonate and worked up. Two recrystallizations from ethanol gave **27**. Yield 3.41 g, 60 %, m.p. 160–164 °C, $[\alpha]_D^{23}$ −16.5° (*c* 1.0, chloroform). Anal. $C_{25}H_{34}O_{16}$: C, H.

*Methyl 2,6-di-O-acetyl-3-deoxy-4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-α-D-*ribo*-hexopyranoside* (**28**). Compound **27** (2.5 g, 4.2 mmol) was dissolved in ethyl acetate (100 ml) and palladium on activated carbon was added (200 mg). The solution was stirred (20 h) under hydrogen. Filtration, evaporation of solvent, and two recrystallizations from ethanol gave compound **28**. Yield 2.27 g, 91 %, m.p. 155–158 °C, $[\alpha]_D^{23}$ −20.0° (*c* 1.0, chloroform). Anal. $C_{25}H_{36}O_{16}$: C, H.

*Methyl 3-deoxy-4-O-(β-D-glucopyranosyl)-α-D-*ribo*-hexopyranoside* (**12**). Compound **28** (1.1 g, 1.9 mmol) was deacylated with methoxide. After elution of the crude reaction mixture on Sephadex G-15 the product **12** was obtained. Yield 0.46 g, 74 % as a syrup. $[\alpha]_D^{23}$ − 49.9° (*c* 0.9, water). Anal. $C_{13}H_{24}O_{10}$: C, H.

Methyl 2,3-di-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranoside (**20**). To a solution of **19** (3.0 g, 5.2 mmol) in dry dichloromethane (21 ml) were added dry zinc bromide (0.36 g) and dichloromethyl methyl ether (6 ml) and the mixture was stirred (1.5 h). After evaporation to dryness, dry methanol (25 ml) and dry dichloromethane (5 ml) were added together with silver carbonate (3.6 g). This mixture was stirred overnight in the dark and then filtered through Celite and activated carbon. Work-up and recrystallization from ethanol gave **20**. Yield 2.0 g, 63 %, m.p. 180–182 °C, $[\alpha]_D^{23}$ −14.4° (*c* 1.0, chloroform).

Methyl 2,3-di-O-acetyl-6-O-methylsulfonyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranoside (**21**). To

a solution of **20** (1.5 g, 2.5 mmol) in dry pyridine (5.5 ml) was added methanesulfonyl chloride (1.5 ml, 19.4 mmol) and the mixture was stirred at 0 °C (2 h). After the addition of water (50 ml) the product crystallized within 1 h. This product was filtered off, dissolved in dichloromethane and worked up in the usual manner to give compound **21**. Yield 1.5 g, 88 %, m.p. 190–192 °C, $[\alpha]_D^{22}$ −12.3 (*c* 1.3, chloroform). Anal. $C_{26}H_{38}O_{19}S$: C, H.

Methyl 6-deoxy-6-iodo-4-O-(β-D-glucopyranosyl)-β-D-glucopyranoside (**16**). After deacylation of compound **22** (0.44 g, 0.61 mmol) with methoxide and recrystallization from ethanol–diethyl ether, compound **16** was obtained. Yield 0.23 g, 81 %, m.p. 147–153 °C. Column chromatography on Sephadex G-15 gave **16** with m.p. 161–163 °C, $[\alpha]_D^{23}$ −6.5° (*c* 1.1, water). Anal. $C_{13}H_{23}IO_{10}$: C, H.

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References

1. Bock, K. and Sigurskjold, B. W. *Eur. J. Biochem. 178* (1989) 711.
2. Bock, K. and Sigurskjold, B. W. In: Atta-ur-Rahman, Ed., *Studies in Natural Products Chemistry,* Elsevier, Amsterdam 1990, Vol. 7, pp. 29–86.
3. Edwards, R. G. In: Lee, C. K., Ed., *Developments in Food Carbohydrate,* Applied Science Publishers, Barking 1980, pp. 229–273.
4. Takeo, K.'i., Fukatsu, T. and Yasato, T. *Carbohydr. Res. 107* (1982) 79.
5. Vidmar, S., Turk, V. and Kregar, I. *Period. Biol. 87* (1985) 55.
6. Ge, S. G., Song, Y., Yang, S. J. and Zhang, S. Z. *Shengwu Huazue Zazhi 1* (1985) 67.
7. Dekker, R. F. H. *Biotechnol. Bioeng. 28* (1986) 1438.
8. McCleary, B. V. and Harrington, J. *Methods Enzymol. 160* (1988) 575.
9. NOVO Industri A/S, Bagsværd, Denmark, *Cellobiase. Novozym 188,* NOVO Enzyme Information, IB-number 198a-GB, 1979.
10. Fletcher, H. G., Jr. and Hudson, C. S. *J. Am. Chem. Soc. 70* (1948) 310.
11. Bock, K. and Pedersen, H. *Acta Chem. Scand., Ser. B 41* (1987) 617.
12. Rao, D. R. and Lerner, L. M. *Carbohydr. Res. 22* (1972) 345.
13. Bognár, R., Farkas, I., Menyhárt, M., Gross, H. and Paulsen, H. *Carbohydr. Res. 6* (1968) 404.
14. Mega, T. and Matsushima, Y. *J. Biochem. (Tokyo) 94* (1983) 1637.
15. Capon, B. *Chem. Rev. 69* (1969) 407.
16. Adelhorst, K. *Ph. D. Dissertation,* Technical University of Denmark, Lyngby, 1989.
17. Bock, K. and Adelhorst, K. *Carbohydr. Res. 202* (1990) 131.
18. Bock, K. and Pedersen, H. In: *Protein–Carbohydrate Interactions in Biological Systems,* Academic Press, London, 1986, pp. 173–182.
19. Bock, K. *Pure Appl. Chem. 59* (1987) 1447.
20. Bock, K. and Pedersen, H. *Acta Chem. Scand., Ser. B 42* (1988) 75.
21. Adelhorst, K., Bock, K., Pedersen, H. and Refn, S. *Acta Chem. Scand., Ser. B 42* (1988) 196.
22. Bock, K., Fernandez Bolanos Guzman, J. and Refn, S. *Glycoconjugate J. 4* (1987) 283.
23. Bock, K. In: Jaroszewski, J. W., Schaumburg, K. and Kofod, H., Eds., *NMR Spectroscopy in Drug Research,* Munksgaard, Copenhagen, 1988, pp. 119–134.
24. Bock, K., Meldal, M., Meyer, B. and Wiebe, L. *Acta Chem. Scand., Ser. B 37* (1983) 101.
25. Preuss, R. and Schmidt, R. R. *Synthesis* (1988) 694.
26. Newth, F. H., Nicholas, S. D., Smith, F. and Wiggins, L. F. *J. Chem. Soc.* (1949) 2550.
27. Helferich, B. and Schmitz-Hillebrecht, E. *Ber. Deutch. Chem. Ges. 66* (1933) 378.

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