Hydrolysis of Substrate Analogues Catalysed by β-D-Glucosidase from *Aspergillus niger*. Part III. Alkyl and Aryl β-D-Glucopyranosides*

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The hydrolysis of eighteen alkyl and aryl β-D-glucopyranosides and the disaccharides methyl β-cellobioside (reference substrate), cellobiitol, methyl β-gentiobioside, and methyl α-*C*-gentiobioside catalysed by β-D-glucosidase from *Aspergillus niger* has been studied using ^{1}H NMR spectroscopy and progress-curve enzyme kinetics in both single-substrate and competition experiments. The influence of chain length and stereochemistry of alkyl groups and substitutions of phenyl groups revealed that this enzyme has evolved preferentially to hydrolyse cellobiose despite low aglycone specificity. The implications of steric and polar or non-polar effects, which were shown to be important for the active site interactions on the energetics of the enzymatic activity as inferred from the kinetic experiments, are discussed.

One of the most fruitful approaches to the elucidation of specific interactions between macromolecular species and ligands has been the use of substrate analogues in the study of molecular recognition and enzyme mechanism. Most glycohydrolases possess low aglyconic specificity which enables these enzymes to catalyse the hydrolysis of alkyl and aryl glycosides, and disaccharides modified in the reducing ring. The hydrophilic–hydrophobic nature of the aglyconic site on the enzyme can thus be studied using such substrate analogues. We have recently reviewed the use of substrate analogues in the field of carbohydrate-degrading enzymes.[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). It thus 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 the β-D-glucosidase-catalysed hydrolysis of a number of methyl β-cellobioside analogues with deoxy functions in the reducing ring[3] as well as a number of other deoxy and deoxyhalo derivatives of cellobiose,[1] and we report in this paper on further kinetic studies on the substrate specificity of a commercial β-D-glucosidase from *Aspergillus niger* using alkyl and aryl β-D-glucopyranosides and the disaccharides cellobiitol, methyl β-gentiobioside, and methyl α-*C*-gentiobioside.

The enzyme β-D-glucosidase, the use of ^{1}H NMR spectroscopy as an analytical tool for monitoring enzyme kinetics and the use of methyl β-cellobioside as a reference substrate have been described previously.[1]

Results

The substrates used in this study are listed in Scheme 1 and have been characterized by their ^{1}H and ^{13}C NMR spectra which are tabulated in Tables 4 and 5.

β-D-Glc*p*-R

Compound	*R*
1	4-*O*-β-D-Glc*p*-OMe
2	OMe
3	OEt
4	O(*n*-Pr)
5	O(*n*-Bu)
6	O(*n*-C_5H_{11})
7	O(*n*-C_6H_{13})
8	O(*i*-Bu)
9	O(*i*-Pr)
10	O(*S*-2-Bu)
11	O(*S*-2-C_6H_{13})
12	O(*cyclo*-C_6H_{11})
13	4-*O*-D-Glc-1-ol
14	6-*O*-β-D-Glc*p*-OMe
15	7-*C*-α-D-6,7-dideoxy-*gluco*-heptopyranosyl-OMe
16	OPh
17	O(*o*-MeC_6H_4)
18	O(*p*-MeC_6H_4)
19	O(*o*-$NO_2C_6H_4$)
20	O(*p*-$NO_2C_6H_4$)
21	O(*R*-2-Bu)
22	O(*R*-2-C_6H_{13})

Scheme 1.

* Part II, see Ref. 1.

Single-substrate experiments. Single-substrate experiments with a number of alkyl and aryl β-D-glucopyranoside and the disaccharide analogues cellobiitol **13** (open-chain reducing end), methyl β-gentiobioside **14** [β-(1→6)-linkage], and methyl α-*C*-gentiobioside **15** (*C*-glycoside) were carried out, and the results are listed in Table 1.

For the alkyl β-D-glucopyranosides, all derivatives were hydrolysed more slowly than methyl β-cellobioside. The longer half-lives indicate that these substrates are influenced by substantial product inhibition. For the primary alkyl groups there is a decrease in rate from methyl to propyl and then an increase up to hexyl as depicted in Fig. 1. From a comparison of the initial rates and half-lives for the primary alkyl derivatives, it seems that the chain-length dependence arises from relatively destabilized enzyme–substrate complexes as well as destabilized catalytic transition states. The secondary alkyl derivatives are hydrolysed more slowly than the primary ones of corresponding size. The open-chain compound cellobiitol is slower than the reference, whereas the primary glycoside, methyl β-gentiobioside, is faster and the *C*-glycoside is not a substrate. The faster hydrolysis of methyl β-gentiobioside than of methyl β-cellobioside is consistent with the faster hydrolysis of primary alkyl glucosides compared with secondary ones.

Phenyl, *p*-tolyl and *o*-nitrophenyl β-D-glucopyranosides are hydrolysed at approximately the same rate as methyl β-cellobioside, whereas the *p*-nitrophenyl and especially the *o*-tolyl derivatives are considerably slower. The rate dependence on the substitution position for the methyl and nitro compounds may arise from conformational differences, or differences in the polar/non-polar interactions with the aglyconic binding site of the enzyme.

Competition experiments. Competition experiments with two substrates, one of which is the reference substrate methyl β-cellobioside, were performed corresponding to the single-substrate experiments summarized in Table 1. The results are listed in Table 2. Most alkyl and aryl β-D-glucopyranosides are heavily inhibited by methyl β-cellobioside in competition experiments and are hydrolysed only slowly, which makes their progress curves sigmoid in shape and prevents the application of a simple regression analysis. Hence the kinetic parameters in these cases are estimated directly from the progress curves and are not fitted.

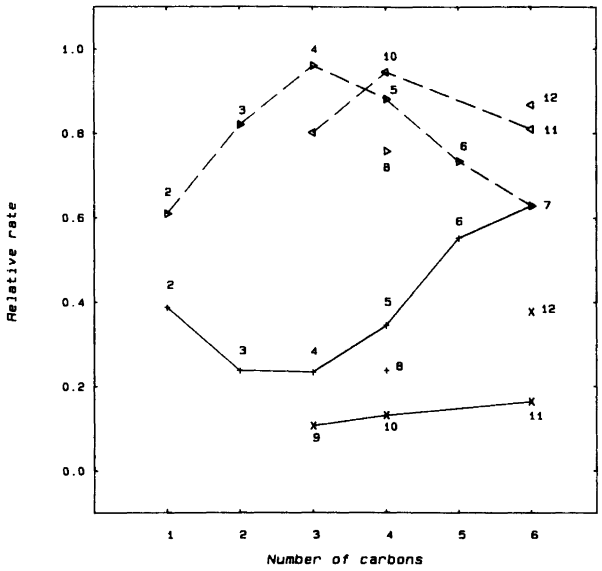

Fig. 1. Initial rate dependence on the chain length of primary and secondary alkyl β-D-glucopyranosides: +, relative initial rates for primary alkyl substrate analogues in single-substrate experiments; ▷, relative initial rates for methyl β-cellobioside in competition with the primary alkyl analogues; ×, relative initial rates for secondary alkyl substrate analogues in single-substrate experiments; ◁, designates relative initial rates for methyl β-cellobioside in competition with the secondary alkyl analogues. Numbers refer to compound numbers. Points not connected by lines represent the isobutyl and cyclohexyl derivatives, respectively.

Table 1. Relative initial rates and half-lives for the β-D-glucosidase-catalysed hydrolysis of alkyl and aryl β-D-glucopyranosides and the disaccharides cellobiitol, methyl β-gentiobioside, and methyl β-*C*-gentiobioside in single-substrate experiments. The initial rates and half-lives were obtained by fitting the progress curves of the enzymatic hydrolysis as described previously[3] and are tabulated relative to the results of the single-substrate experiments with the reference substrate methyl β-cellobioside. The reactions took place in D_2O at pH 4.75 and 300 K.

Substrate		Relative			
		Y_0/mM	v_0	$t_{1/2}$	$v_0 t_{1/2}$
1	(ref.)[a]	28.1	1	1	1
Alkyl β-D-glucopyranosides					
2	Methyl	22.2	0.39	3.6	1.4
3	Ethyl	22.3	0.24	10	2.4
4	Propyl	23.0	0.23	9.4	2.2
5	Butyl	22.4	0.34	5.7	1.9
6	Pentyl	22.5	0.55	3.7	2.0
7	Hexyl	22.2	0.63	2.1	1.3
8	Isobutyl	22.1	0.24	15	3.6
9	2-Propyl	22.9	0.11	19	2.1
10	(*S*)-2-Butyl	22.2	0.13	15	2.0
11	(*S*)-2-Hexyl	21.2	0.16	14	2.2
12	Cyclohexyl	38.2	0.38	7.4	2.8
Disaccharides					
13	Cellobiitol	22.1	0.72	1.7	1.2
14	Methyl β-gentiobioside	29.6	1.5	0.87	1.3
15	Methyl α-*C*-gentiobioside	10.3	0	∞	–
Aryl β-D-glucopyranosides					
16	Phenyl	22.3	0.98	1.4	1.4
17	*o*-Tolyl	22.1	0.03	102	3.1
18	*p*-Tolyl	22.1	0.99	1.1	1.1
19	*o*-Nitrophenyl	22.1	1.0	1.0	1.0
20	*p*-Nitrophenyl	22.1	0.40	2.5	1.0

[a] v_0 = 0.472 ± 0.007 mM min^{-1}, $t_{1/2}$ = 31.3 ± 0.3 min. Errors are standard deviations from triplicate experiments.

Table 2. Relative initial rates and half-lives for the β-D-glucosidase-catalysed hydrolysis of methyl β-cellobioside, alkyl and aryl β-D-glucopyranosides, and disaccharide analogues in competition experiments.

Substrate		Relative			
		Y_0/mM	v_0	$t_{1/2}$	$v_0 t_{1/2}$
Single-substrate experiment					
1	(ref.)[a]	28.1	1	1	1
Competition experiments					
Alkyl β-D-glucopyranosides					
1		22.4	0.61	1.6	1.0
2	Methyl	22.3	0.11	20	2.2
1		22.1	0.82	1.2	1.0
3	Ethyl	22.4	0.01	110	1.1
1		23.0	0.96	1.1	1.1
4	Propyl	22.4	0.03	35	1.1
1		22.4	0.88	1.2	1.1
5	Butyl	23.0	0.01	6.7	0.07
1		22.2	0.73	1.4	1.0
6	Pentyl	22.3	0.02	21	0.4
1		22.1	0.63	1.4	0.9
7	Hexyl	22.2	0.09	12	1.1
1		22.1	0.76	1.3	1.0
8	Isobutyl	22.2	0.03	35	1.1
1		23.0	0.80	1.2	1.0
9	2-Propyl	23.0	0.01	80	0.8
1		22.2	0.95	1.1	1.0
10	(*S*)-2-Butyl	23.0	0.02	66	1.3
1		22.4	0.81	1.3	1.1
11	(*S*)-2-Hexyl	21.0	0.08	11	0.9
1		28.1	0.87	1.2	1.0
12	Cyclohexyl	38.2	0.03	34	1.0
Disaccharides					
1		22.3	0.81	1.3	1.1
13	Cellobiitol	22.4	0.11	6.5	0.7
1		22.1	0.56	1.8	1.0
14	Methyl β-gentiobioside	22.1	0.15	3.8	0.6
1		22.1	1.0	1.0	1.0
15	Methyl α-*C*-gentiobioside	10.3	0	∞	–
Aryl β-D-glucopyranosides					
1		22.2	1.1	1.0	1.1
16	Phenyl	22.3	0.07	4.2	0.3
1		22.2	0.84	1.4	1.2
17	*o*-Tolyl	22.2	0.01	138	1.4
1		22.1	0.91	1.3	1.2
18	*p*-Tolyl	22.1	0.16	4.3	0.7
1		22.1	0.66	1.6	1.1
19	*o*-Nitrophenyl	22.1	0.16	5.0	0.8
1		22.2	0.30	3.6	1.1
20	*p*-Nitrophenyl	22.3	0.13	6.1	0.8

[a] v_0 and $t_{1/2}$ as in Table 1.

Table 3. Relative initial rates and half lives for the β-D-glucosidase-catalysed hydrolysis of *S*- and *R*-butyl- and hexyl β-D-glucopyranosides in competition experiments between the diastereomers and of methyl β-gentiobioside in competition with methyl α-*C*-gentiobioside.

Substrate		Relative			
		Y_0/mM	v_0	$t_{1/2}$	$v_0 t_{1/2}$
1	(ref.)[a]	28.1	1	1	1
Alkyl β-D-glucopyranosides					
10	(*S*)-2-Butyl	16.1	0.01	44	0.4
21	(*R*)-2-Butyl	16.1	0.01	90	0.9
11	(*S*)-2-Hexyl	10.0	0.02	15	0.3
22	(*R*)-2-Hexyl	10.0	0.01	34	0.3
14	Methyl β-gentiobioside	22.1	1.2	0.91	1.1
15	Methyl α-*C*-gentiobioside	10.3	0	∞	–

[a] v_0 and $t_{1/2}$ as in Table 1.

For the primary alkyl derivatives it is clear that the lower the rate they display in single-substrate experiments, the less the inhibitory effect in competition (Fig. 1). This tendency can also be seen in the secondary derivatives, although it is less pronounced (also shown in Fig. 1). This means that the reduction in rate for the alkyl derivatives is principally caused by an increase in the energy of the catalytic transition state (Fig. 1 in Ref. 1).

Cellobiitol is a relatively poor inhibitor, whereas methyl β-gentiobioside is somewhat stronger, and methyl α-*C*-gentiobioside does not inhibit at all. Methyl α-*C*-gentiobioside did not inhibit the reaction of methyl β-gentiobioside either (Table 3). The *C*-glycoside is not able to bind to the enzyme at all, whereas the open chain derivative binds less firmly to the enzyme both in the enzyme–substrate complex and in the catalytic transition state compared with the reference. These inhibition studies were only undertaken with inhibitor concentrations up to a certain value owing to limited quantities of the compounds. Possible inhibition at larger concentrations cannot be ruled out. Methyl β-gentiobioside is greatly inhibited by methyl β-cellobioside, so this compound forms a less stable enzyme–substrate complex than methyl β-cellobioside, but has a stabilization energy of the transition state of comparable size to that of the reference.

Only the nitro derivatives of the aromatic analogues inhibit the reference substrate to a significant extent. Phenyl and *p*-tolyl β-D-glucopyranosides possess substantial destabilizations of both the enzyme–substrate complex and the transition state of the same magnitude ($\Delta\Delta G^{\circ}_{b} \cong \Delta\Delta G^{\ddagger}_{b}$, Fig. 1 in Ref. 1). This represents a unique situation in which virtually the same kinetic parameters are observed for two different substrates in single-substrate experiments, but in which an almost complete absence of inhibition of one of the substrates on the other is observed in competition experiments.

In direct competition between the *R* and *S* diastereomers of 2-butyl and 2-hexyl β-D-glucopyranosides, it was found that the *S* diastereomers were hydrolysed faster than the *R* isomers (Table 3). This must be caused by a conformational preference due to the steric requirements of the aglyconic binding site. Theoretical calculations have confirmed that the conformations of the *S*-diastereomers are indeed the

ones that resemble the conformation of methyl β-cellobioside the most.[4]

Discussion

Nath and Rydon[5] have studied the mechanism of β-D-glucosidase from sweet almonds towards a number of aryl β-D-glucopyranosides and Mega and Matsushima[6] have studied the mechanism of β-D-glucosidase from *Aspergillus oryzae*. No obvious common trend is apparent for these three enzymes with this type of substrate, and though their overall mechanisms must be closely related, the free energies governing the interactions between the enzymes and different aromatic aglycones are somewhat different for the different enzymes.

It is evident that all the changes in the rates of hydrolysis stem from energy differences of relatively small magnitude. All binding energies of both the enzyme–substrate complex and the catalytic transition state are less favourable for the substrate analogues than for the reference compound, and except for the *p*-tolyl derivative **18** all activation energies are also less favourable. Thus it emerges that this enzyme, despite its low aglyconic specificity, has evolved towards cellobiose rather than β-D-glucopyranosides.

The observed inhibitory effects are directly reflected in the magnitudes of the changes in the stabilization of the enzyme–substrate complex. The transition state of the propyl glucoside is bound more firmly than both butyl and pentyl which gives rise to a relative low activation energy for the propyl compound. The reason for this phenomenon is not clear from these experiments.

The results for the alkyl derivatives suggest that when the carbon chain is long enough it may engage in hydrophobic interactions distant from the anomeric centre thereby becoming more tightly bound. This suggestion is supported when the results for the hexyl **7**, 2-hexyl **11**, and cyclohexyl **12** compounds are compared, inasmuch as the possibility of 'stretching out' to a distant hydrophobic region is suppressed in the last-mentioned substrate. In fact, **12** has very similar kinetic characteristics to the (*S*)-2-butyl derivative **10**.

A methyl group in the *ortho* position of the phenyl group gives a very unfavourable binding of the catalytic transition state whereas a nitro group in the same position does not. It thus seems that polar interactions take place at this position in the binding of the catalytic transition state, but not to the same extent in the binding of the enzyme–substrate complex (differential binding). The reverse seems to be the case for the *para* position where a nitro group gives rise to firmer binding of the enzyme–substrate complex than does a methyl group, but no difference can be seen for the binding of the catalytic transition state. Hence, it can be concluded that steric properties and polar–apolar interactions of the aglycone have significant effects on the catalytic hydrolysis. The results for the alkyl derivatives suggest that interactions between the enzyme and the aglycone are primarily hydrophobic and unspecific. However, the dramatic difference between the *ortho*- and *para*-tolyl derivatives indicates that specific interactions may also be of crucial importance.

Methyl β-gentiobioside is hydrolysed faster in the single-substrate experiments than methyl β-cellobioside. However, in the competition experiment methyl β-cellobioside is the fastest. This clearly demonstrates that enzymes have not only evolved with respect to the rate increases they cause, but also to be specific and this enzyme has evolved to hydrolyse β-1-4 linked D-glucopyranosyl units in preference to β-1-6 linkages in direct competition.

Tables 1–3 contain the products of the relative initial rates and half-lives. In Table 1 (single-substrate experiments) this product tends to be significantly greater than zero. This reflects a more pronounced product inhibition in these experiments compared with the hydrolysis of methyl β-cellobioside, since strong product inhibition will increase the half-life by a factor larger than the decrease in initial rate (which, of course, is not influenced by product inhibition). The fact that glucose is released in all cases means that $v_0 t_{1/2}$ is a good qualitative indicator of binding of the substrate analogues relative to glucose. It is clear from Table 1 that glucose binds more strongly to the enzyme than do any of the analogues, except for the *o*- and *p*-nitrophenyl derivatives. These two substrates also inhibit the hydrolysis of methyl β-cellobioside to the greatest extent.

In competition experiments (Tables 2 and 3) the reference compound has a $v_0 t_{1/2}$ value close to unity, as do many of the analogues. This means that in these experiments there is little change in the total inhibition during the course of the hydrolysis and that product inhibition 'takes over' from the inhibition that the two simultaneous substrates impose on each other. This in turn shows that glucose binds to the enzyme with a strength comparable to that of methyl β-cellobioside. The very moderate effect of the analogues on the hydrolysis of the reference confirms this conclusion. Competition experiments in which $v_0 t_{1/2} < 1$ represent cases where there is a net decrease in total inhibition during the reaction. The product inhibition for these analogues does not reach the same strength as the inhibition of methyl β-cellobioside on the substrate analogues and, consequently, these substrates bind much less firmly to the enzyme than methyl β-cellobioside. This can also be seen from their very low initial rates and their very weak inhibition of hydrolysis of the reference.

It can be concluded that estimations of initial rates and half lives in simple progress-curve kinetics of different substrate analogues under similar conditions, using both single-substrate and competition experiments, gives a good qualitative picture of the relative binding strength of the enzyme–substrate complexes as well as the catalytic transition states.

Table 4. The ^{1}H NMR chemical shifts and observed first-order coupling constants of the substrates.

		1	1′	2	3	3′	4	5	6	6′	Me
2	H′	4.39		3.27	3.50		3.39	3.47	3.93	3.73	
	J_3	8.0		9.5	9.1		9.8	2.2	6.0		
	J_2								12.2		
	H	3.58									
3	H′	4.46		3.24	3.48		3.36	3.43	3.91	3.90	
	J_3	8.0		9.0	8.8		9.6	2.1	5.6		
	J_2								12.6		
	H	3.72	3.96	1.22							
	J_3	7.1	7.1								
	J_2	9.8									
4	H′	4.38		3.18	3.41		3.29	3.37	3.84	3.64	
	J_3	8.0		9.0	9.0		9.1	2.0	5.6		
	J_2								12.4		
	H	3.55	3.80	1.55	0.84						
	J_3	6.8	6.8	7.5							
	J_2	9.8									
5	H′	4.43		3.23	3.46		3.37	3.4	3.89	3.69	
	J_3	7.9		8.4	9.0		9.2	2.3	5.6		
	J_2								12.2		
	H	3.65	3.90	1.58	1.35		0.89				
	J_3	7.3	7.4	7.1	7.3						
	J_2	9.5									
6	H′	4.36		3.16	3.39		3.27	3.4	3.82	3.62	
	J_3	8.0		8.9	9.0		9.3	1.6	5.5		
	J_2								12.3		
	H	3.58	3.83	1.54	1.24		1.24	0.79			
	J_3	6.8	6.6								
	J_2	9.9									
7	H′	4.36		3.15	3.39		3.37	3.33	3.82	3.62	
	J_3	8.0		8.8	9.3		8.6	1.8	5.3		
	J_2								11.8		
	H	3.57	3.83	1.53	1.2		1.2	1.2	0.78		
	J_3	6.7	6.9								
	J_2	9.7									
8	H′	4.46		3.28	3.49		3.39	3.45	3.92	3.73	
	J_3	8.0		9.3	9.1		9.7	2.2	5.7		
	J_2								12.3		
	H	3.45	3.71	1.91	0.93	0.92					
	J_3	7.0	6.8	6.7							
	J_2	9.7									
9	H′	4.55		3.23	3.50		3.38	3.46	3.92	3.72	
	J_3	8.0		9.0	9.1		9.4	2.1	6.1		
	J_2								12.4		
	H	1.26		4.12	1.22						
	J_3	6.3		6.3	6.1						
10	H′	4.55		3.24	3.49		3.38	3.46	3.92	3.72	
	J_3	8.0		9.3	9.2		9.7	2.3	6.0		
	J_2								12.4		
	H	1.24		3.89	1.5	1.62	0.91				
	J_3	6.3		6.2	7.4	7.4	7.5				
11	H′	4.53		3.23	3.48		3.37	3.2	3.95	6.71	
	J_3	8.0		8.6	9.0		9.0		5.4		
	J_2								12.5		
	H	1.24		3.92	1.48	1.61	1.34	1.34	0.88		
	J_3	7.1									
12	H′	4.58		3.23	3.49		3.38	3.44	3.90	3.72	
	J_3	8.1		8.7	9.1		9.4	1.9	5.8		
	J_2								12.3		

Contd.

Table 4. Contd.

		1	1′	2	3	3′	4	5	6	6′	Me
13	H′	4.57		3.34			3.46		3.64		
	J_3	8.0		9.4					5.7		
	J_2								12.2		
14	H′	4.29		3.22*	3.41		3.33	3.50	4.13	3.76	
	J_3	8.0		9.1	9.0		9.9	1.8	5.6		
	J_2								11.5		
	H	4.42		3.16*	3.41		3.33	3.35	3.83	3.73	3.48
	J_3	7.9		9.2	9.0		9.9	1.9	5.4		
	J_2								12.4		
16	H′	4.93		3.4	3.4		3.29	3.4	3.72	3.54	
	J_3							2.1	5.5		
	J_2								12.3		
	H			6.9	7.2		6.9	7.2	6.9		
17	H′	5.01		3.5	3.5		3.41	3.5	3.84	3.66	
	J_3							1.9	5.3		
	J_2								12.4		
	H										2.18
18	H′	4.95		3.44	3.50		3.38	3.5	3.82	3.64	
	J_3							2.1	5.4		
	J_2								12.4		
	H			6.94	7.11			7.11	6.94		2.19
19	H′	5.28		3.65	3.65		3.55	3.69	3.97	3.80	
	J_3							2.1	5.4		
	J_2								12.3		
	H				7.98		7.31	7.73	7.47		
20	H′	5.19		3.55	3.55		3.43	3.58	3.83	3.68	
	J_3								5.4		
	J_2								12.3		
	H			7.16	8.18			8.18	7.16		
21	H′	4.55								3.72	
	J_3	8.0									
	H	1.19			1.53	1.62	0.91				
	J_3	6.2									
22	H′	4.53									
	J_3	8.0									
	H	1.20			1.48	1.61	1.34	1.34	0.88		
	J_3	7.1									

Table 5. ^{13}C NMR chemical shifts of the substrates.

		1	2	3	4	5	6	Me
2	C′	104.2	74.0	76.9*	70.6	76.7*	61.7	
	C	58.1						
3	C′	102.7	74.0	76.8	70.5	76.8	61.7	
	C	67.0	15.1					
4	C′	103.1	74.1	76.8	70.6	76.8	61.7	
	C	73.2	23.1	10.6				
5	C′	103.1	74.1	76.8	70.6	76.8	61.7	
	C	71.3	31.8	19.4	14.0			
6	C′	103.1	74.1	76.8	70.6	76.8	61.7	
	C	71.7	29.4	28.3	22.7	14.3		
7	C′	103.1	74.1	76.8	70.6	76.8	61.7	
	C	72.9	31.8	29.7	25.7	22.9	14.3	
8	C′	103.4	74.1	76.8	70.6	76.8	61.7	
	C	78.1	28.7	19.3				
9	C′	101.2	74.0	76.7	70.6	76.7	61.7	
	C	21.8	73.9	23.2				
10	C′	102.3	74.3	76.6*	70.6	76.8*	61.7	
	C	20.8	79.7	29.2	9.6			
11	C′	102.4	74.2	76.7*	70.6	76.8*	61.7	
	C	22.9	78.6	36.2	27.6	21.5	14.3	
12	C′	101.0	74.0	76.7	70.5	76.7	61.6	
13	C′	103.7	74.5	77.0	70.6	77.0	61.7	
	C	63.3	73.4	70.7	80.4	72.4	63.8	
14	C′	104.4	74.1	76.7	70.4	76.6	61.7	
	C	103.8	74.1	76.9	70.6	75.9	69.6	58.4
16	C′	101.3	74.1	77.3*	70.6	76.7*	61.7	
	C	157.7	117.7	131.1	124.5	131.1	117.7	
17	C′	101.6	74.1	77.2*	70.6	76.8*	61.7	
	C	155.9	129.2	133.2	124.3	128.3	116.2	16.5
18	C′	101.6	74.1	77.2*	70.6	76.7*	61.7	
	C	155.6	117.8	131.4	134.4	131.4	117.8	20.8
19	C′	101.9	74.0	77.6	70.5	76.8	61.7	
	C	150.7		126.9*	124.5*	136.3	118.9	
20	C′	100.7	74.0	77.6	70.6	76.7	61.7	
	C		117.7	127.3		127.3	117.7	
21	C′	101.2	74.2	76.8*	70.5	76.7*	61.7	
	C	19.1	78.8	30.1	9.6			
22	C′	101.3	74.0	76.7*	70.6	76.8*	61.7	
	C	19.8	77.6	36.9	27.9	21.5	14.3	

* Assignments may be reversed.

Experimental

Methyl β-cellobioside **1** and methyl β-gentiobioside **14** were obtained by conventional Koenigs–Knorr synthesis from commercial cellobiose and gentiobiose octaacetates, respectively. Methyl α-*C*-gentiobioside **15** was a generous gift from Prof. Kishi.[7]

Cellobiitol (4-*O*-β-D-glucopyranosyl)-D-glucitol) **13** was synthesized from cellobiose by reduction with sodium borohydride in water.

Methyl β-D-glucopyranoside **2** was a commercial product, while **4**, **6**, **8**, **9**, **10**, **11**, **16**, **17**, and **18** were synthesized by Veibel and co-workers[8,9] in the 1930s. The other alkyl and aryl β-D-glucopyranosides were synthesized by conventional Koenigs–Knorr synthesis. The identification of compounds **10** and **11** as the *S*-diastereomers was made by ^{13}C NMR spectroscopy on the basis of the work by Seo *et al.*[10]

^{1}H NMR and ^{13}C NMR spectra (Tables 4 and 5) were recorded on Bruker AM-500 and Bruker AC-250 instruments at 300 K. Tetramethylsilane was used as an internal reference (0 ppm) for deuteriochloroform solutions for both ^{1}H and ^{13}C spectra. For spectra recorded in deuterium oxide the HDO peak was used as an internal reference (4.75 ppm) for ^{1}H spectra, whereas an external instrument reference was used for ^{13}C spectra in this solvent.

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 being kept for 2 months at room temperature, in accordance with the manufacturer's specifications.[11]

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 were 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.[1,3]

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