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Syntheses and testing of substrates and mechanism-based inactivators for xylanases

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Abstract

The syntheses of the 2,5- and 3,4-dinitrophenyl β-xylobiosides by two separate routes are described, as well as the syntheses of the 2,4-dinitrophenyl β-glycosides of 2-chloro-2-deoxy-xylobiose and 2-deoxy-2-fluoro-xylobiose. Both the 3,4- and 2,5-dinitrophenyl β-xylobiosides proved to be good substrates for the *Bacillus subtilis* xylanase, with k_{cat}/K_m values of 1.0 and 34.4 mM^{-1} s^{-1}, respectively. Excellent time-dependent inactivation of the exo-xylanase/glucanase from *Cellulomonas fimi* was provided by 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside, according to inactivation parameters of $k_i = 0.057$ min^{-1} and $K_i = 0.0035$ mM.

Keywords: Aryl β-xylobiosides; 2-Deoxy-2-halo-β-D-xylopyranosides; 2-Deoxy-2-halo-β-xylobiosides; Substrate; Inactivator; Glycosidase inhibitors; Endo-xylanase [β-(1 → 4)-Xylan xylanohydrolase, EC 3.2.1.8]

1. Introduction

Xylans are the most widely distributed polysaccharides in the biosphere, second only in mass to cellulose. They comprise a backbone of β-(1 → 4)-linked xylopyranose residues that are acetylated or glycosylated on the free hydroxyls with α-D-glucuronic acid, α-L-arabinose, or β-D-galactose [1]. As might therefore be expected, the degradation of xylan requires a number of different esterases and glycosidases, the most important amongst them being the endo-xylanases [β-(1 → 4)-xylan xylanohydrolases, EC 3.2.1.8], which cleave the backbone itself. These enzymes, particularly those devoid

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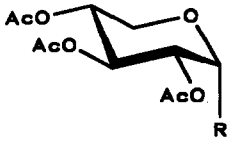
of cellulase activity, have significant commercial potential, particularly in the pulp and paper industry [2]. There has therefore been considerable interest in the structures and catalytic mechanisms of such enzymes.

Convenient assays for xylanases are therefore important for the more fundamental studies, as well as in their commercial applications. Unfortunately, the standard reducing-sugar assay using xylan as substrate can be quite irreproducible due to the changing nature of the substrate during the assay; hence, the varying K_m values generally obtained. Consequently this assay is not suitable for serious mechanistic studies, and is also problematic for more routine applications. Furthermore, since the more selective endo-xylanases such as that from *Bacillus subtilis* have no enzymatic activity on cellulose or on simple xylosides, these simpler substrates cannot be employed. The minimal substrate appears to be a xylobioside, and chromogenic glycosides of this sort whose hydrolysis can be monitored spectrophotometrically would appear to be the best. This has been recognised previously, and indeed aryl xylobiosides have been used as substrates in a very few cases [3]. However, those synthesised have not proven to be highly active as substrates, and these have very high K_m values, necessitating the use of large quantities for assays. One solution to this problem might be to synthesise xylobiosides with more active leaving groups. This should increase rates of reaction, hopefully resulting in higher k_{cat} values. In addition, since xylanases appear to follow a two-step mechanism (vide infra), the use of good leaving groups should increase the rate of formation of the intermediate relative to its decomposition, hopefully resulting in accumulation of the intermediate and therefore lowering the K_m values. The best phenolic leaving group generally employed with glycoside substrates is 2,4-dinitrophenol; hence, earlier attempts involved the synthesis of 2,4-dinitrophenyl β-xylobioside. Unfortunately, this derivative was too labile for general use as a substrate, but it did allow the monitoring of the stereochemical course of the reaction by ^{1}H NMR spectroscopy [4], the reaction being shown to occur with retention of anomeric configuration. Slightly less acidic phenols than 2,4-dinitrophenol (pK_a 3.96) would therefore be more desirable. Promising candidates would appear to be 2,5-dinitrophenol (pK_a 5.15) and 3,4-dinitrophenol (pK_a 5.36).

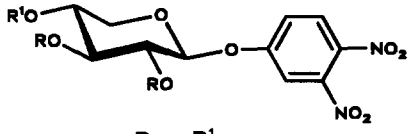
The design of suitable inactivators for xylanases that can be used to identify key catalytic residues requires prior knowledge of their mechanism. All the xylanases so studied to date have been shown to hydrolyse their glycosidic linkage with net retention of anomeric configuration [4], thus presumably following the double-displacement mechanism first proposed by Koshland [5]. This mechanism involves a two-step process in which a glycosyl–enzyme intermediate is formed and hydrolysed via oxocarbenium ion-like transition states [6]. 2-Deoxy-2-fluoro glycosides with good leaving groups have been shown previously [7] to function as good inactivators of this class of enzymes via the accumulation of a relatively stable glycosyl-enzyme intermediate. In this paper we describe the synthesis of 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside, as well as its 2-chloro analogue, as an inactivator for xylanases. The deoxyfluoro compound has already been used to inactivate and identify the catalytic nucleophile of the *B. subtilis* xylanase [8], and here we show it to also function as an effective inactivator of the exo-xylanase/glucanase from *Cellulomonas fimi*.

2. Results and discussion

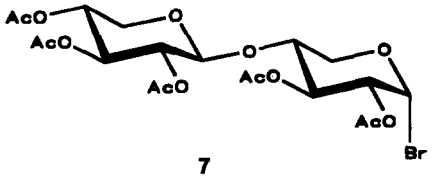
Synthesis of aryl β-xylobiosides.—The classical procedure for the synthesis of aryl glycosides involves reacting the protected glycosyl bromide in acetone with a solution of the phenol in aqueous NaOH [9]. However, the yields using this protocol are generally low (< 40%) and drop further when electron-deficient phenols are used, even when carried out under strictly anhydrous conditions [10]. Despite warnings that the use of 2,6-lutidine in Koenigs–Knorr reactions can cause the formation of orthoesters [11], this weak, slightly hindered base was found to provide dramatic improvement in chemical yields of these compound types. Quinoline has been used previously with the same result by Robertson and Waters [12]. Using this approach 2,5-dinitrophenyl 2,3,2′,3′,4′-penta-*O*-acetyl-β-xylobioside (**11**) was indeed synthesised in 64% yield by reaction of 2,3,2′,3′,4′-penta-*O*-acetyl-α-xylobiosyl bromide (**7**) with a slight excess of 2,5-di-



1 R = Br
2 R = O–C(=NH)–CCl$_3$



	R	R^1
3	Ac	Ac
4	OH	OH
5	Ac	$SiEt_3$
6	Ac	OH



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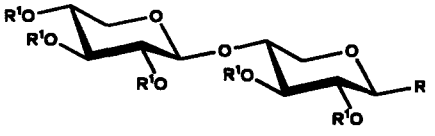
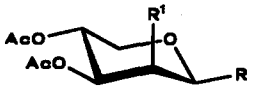
nitrophenol in the presence of 2,6-lutidine and silver carbonate in anhydrous acetonitrile. Unfortunately, this direct approach requires penta-*O*-acetylxylobiosyl bromide (**7**) as a starting material, which is a known compound [13] but is difficult to obtain in large quantities. The sample of xylobiose, from which **7** was synthesised in this study, was obtained after a laborious chromatographic workup by enzymatic degradation of birchwood xylan according to a published protocol [14].

In a recent publication [15] we described a general synthesis of aryl β-xylobiosides via coupling of a suitably partially protected β-D-xylopyranoside with α-D-xylopyranosyl trichloroacetimidate (**2**). This same strategy was employed for the synthesis of 3,4-dinitrophenyl β-xylobioside (**10**). Thus 3,4-dinitrophenol was glycosylated with tri-*O*-acetyl-α-D-xylopyranosyl bromide using the Koenigs–Knorr method under anhydrous conditions in the presence of 2,6-lutidine to give 3,4-dinitrophenyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (**3**) in 90% yield. Compound **3** was then deacetylated with ammonia in methanol [16] yielding 3,4-dinitrophenyl β-D-xylopyranoside (**4**) which was selectively 4-*O*-triethylsilylated, 2,3-di-*O*-acetylated, and desilylated to give 3,4-dinitrophenyl 2,3-di-*O*-acetyl-β-D-xylopyranoside (**6**) in 65% overall yield. Compound **6** was then xylosylated at its free 4-position using the trichloroacetimidate method to give crystalline 3,4-dinitrophenyl 2,3,2′,3′,4′-penta-*O*-acetyl-β-xylobioside (**9**).



	R	R^1
8	2-nitrophenyl	H
9	3,4-dinitrophenyl	Ac
10	3,4-dinitrophenyl	H
11	2,5-dinitrophenyl	Ac
12	2,5-dinitrophenyl	H

13



14 R = Cl R^1 = Cl
15 R = OAc R^1 = F

Synthesis of 2,4-dinitrophenyl 2-deoxy-2-halo-β-xylobiosides.—3,4-Di-*O*-acetyl-2-chloro-2-deoxy-β-D-lyxo- and -α-D-xylo-pyranosyl chloride (**14** and **16**) were obtained by chlorination of di-*O*-acetyl-D-xylal [17]. In contrast to the chlorination of tri-*O*-acetyl-D-glucal, where chlorine adds predominantly (5:1) from the α-face [18], addition of chlorine to di-*O*-acetyl-D-xylal occurs preferentially from the β-face giving the D-*lyxo* product in excess over the *xylo* product (1.9:1). The products were separated by column chromatography, crystallised, and characterised by ^{1}H NMR spectroscopy (Table 1). The xylosyl chloride **16** was then directly transformed into the 2,4-dinitrophenyl 3,4-di-*O*-acetyl-2-chloro-2-deoxy-α- and -β-D-xylopyranosides (**17** and **20**) in an 87% chemical yield using 2,4-dinitrophenol with silver carbonate and 2,6-lutidine in our modified Koenigs–Knorr reaction. This yield is remarkably high, given the low nucleophilicity of the phenol. The minor component formed was identified as the α-glycoside **17** from its ^{1}H NMR spectrum (Table 1), which shows a small coupling constant ($J_{1,2}$ 3.2 Hz) for its anomeric proton, while the other protons have large coupling constants consistent with their transdiaxial orientations. This formation of some α-xyloside is not surprising since the chlorine at C-2 of the sugar is a nonparticipating substituent in glycosylation reactions. In contrast, relatively small coupling constants were found for all the ring protons in the β-glycoside, indicating the adoption of a $^1C_4$ conformation. This is not surprising given the known strong preference for an axial orientation of electron-withdrawing substituents at the anomeric centre (the anomeric effect) and previous such observations on other xylopyranosyl halides [19].

Deacetylation of 2,4-dinitrophenyl glycosides with an *O*-acetyl group at C-2 by use of sodium methoxide in methanol results in decomposition of the glycoside. To avoid this problem an acidic deacetylation method using hydrogen chloride in methanol was developed for this type of activated glycoside [20], and indeed this method works well with 2-deoxy-2-halo-xylosides. However, such an approach was problematic for deprotection of xylobiosides due to the acid lability of the linkage between the two xylose units. Deacetylation under acidic conditions was found to result in significant solvolysis prior to removal of all the acetates. Fortunately, deprotection of the 2,4-dinitrophenyl 2-deoxy-2-halo-xylosides could be carried out using ammonia in methanol since the halogen stabilises the aryl glycoside linkage toward hydrolysis.

As a starting material for the synthesis of the 2-deoxy-2-fluoro-xylosides we used 1,3,4-tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-xylopyranose (**18**) [21], which was obtained by acetoxyfluorination of di-*O*-acetyl-D-xylal according to a published synthesis [22]. The compound was then activated as the glycosyl donor, 3,4-di-*O*-acetyl-2-deoxy-2-fluoro-α-D-xylopyranosyl bromide (**19**), using HBr in acetic acid and reacted with 2,4-dinitrophenol as described for the 2-chloro derivative to give 2,4-dinitrophenyl 3,4-di-*O*-acetyl-2-deoxy-2-fluoro-β-D-xylopyranoside (**25**) in 80% yield. No α-glycoside was isolated in this case. Compound **25** was then deacetylated with anhydrous ammonia in methanol to give 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-xylopyranoside (**26**).

The selective protection of the 2-deoxy-2-halo-xylosides **21** and **26** using tributyltin oxide–triethylsilyl chloride did not work satisfactorily. It is not clear whether or not the missing C-2 hydroxyl group and the consequent reduced coordination of the tin reagent is responsible for this failure, or whether it was due to the relatively small scale on which the reaction was carried out. However, these results required the formulation of

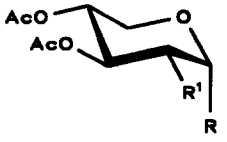
Table 1
^{1}H NMR data of the monosaccharides

Proton	Compound													
	3	**4**	**5**	**6** [a]	**14**	**16**	**17**	**20**	**21**	**22** [a]	**25**	**26**	**27** [b]	**28** [b]
H-1	5.37 d	5.22 d	5.06–5.24 m	5.33 d	6.00 dd	6.08 d	5.83 d	5.64 d	5.46 d	5.51 d	5.77 dd	5.54 dd	5.70 dd	5.72 dd
H-2	5.15 dd	3.44–3.68 m	5.06–5.24 m	5.17 dd	4.52 t	4.08 dd	4.11 dd	4.16 dd	3.77 t	4.12 dd	4.68 ddd	4.32 ddd	4.73 ddd	4.69 ddd
H-3	5.21 t	3.44–3.68 m	5.06–5.24 m	4.99 t	5.25 tt	5.51 dd	5.62 dd	5.25 t	3.47–3.70 m	5.08 t	5.21 dt	3.71 dt	5.12 dt	4.13 dt
H-4	4.97 dt	3.44–3.68 m	3.83–3.99 m	3.88 dq	4.89 dt	4.97 dt	5.05 ddd	4.90 dt	3.47–3.70 m	3.85 dq	4.86 q	3.64 dt	3.82 q	4.89 q
H-5a	3.65 dd	3.44–3.68 m	3.48 dd	3.62 dd	3.79 ddd	3.97–4.03 m	3.75 t	3.74 dd	3.47–3.70 m	3.66 dd	3.77 ddd	3.51 t	3.72dd	3.73 dd
H-5b	4.19 dd	4.04 d	3.39 dd	4.14 dd	4.35 dd		3.96 dd	4.24 dd	4.02 dd	4.21 dd	4.17 dd	3.98 dd	4.16 dd	4.21 dd
H-2′	7.37 d	7.62 d	7.33 d	7.37 d										
H-3′							8.79 d	8.71 d	8.73 d	8.72 d	8.70 d	8.74 d	8.72 d	8.81 d
H-4′														
H-5′	8.00 d	8.17 d	7.98 d	8.00 d			8.43 dd	8.43 dd	8.48 dd	8.43 dd	8.42 dd	8.48 dd	8.44 dd	8.45 dd
H-6′	7.25 dd	7.45 dd	7.22 dd	7.25 dd			7.46 d	7.44 d	7.60 d	7.43 d	7.48 d	7.61 d	7.48 d	7.47 d
CH_2CH_3			0.58 q											
CH_2CH_3			0.93 t											
O*H*				2.80 d						2.75 d				3.04 s
OAc	2.09 s		2.04 s	2.10 s	2.12 s	2.03 s	2.04 s	2.11 s		2.21 s	2.11 s		2.20 s	2.14 s
			2.07 s	2.13 s	2.17 s	2.09 s	2.10 s	2.17 s			2.17 s			
$J_{H,H(F)}$														
1,2	5.0	7.0		5.3	3.8	3.8	3.2	4.0	8.0	5.4	2.6	7.2	3.3	3.6
1,3					1.0									
1,F											9.8	4.3	9.3	8.7
2,3	6.5			7.2	3.8	10.5	10.8	5.6	8.0	7.2	4.0	8.2	5.0	5.4
2,5a											0.8			
2,F											44.4	50.8	45.3	46.4
3,4	6.5			7.2	3.9	10.0	9.5	5.6		6.8	3.5	8.2	4.5	4.5
3,5a					1.2									
3,F											10.9	14.8	11.6	12.2
4,5a	6.0		8.2	6.5	2.2	7.4	11.0	4.9		6.5	3.5	9.0	4.5	4.2
4,5b	4.1	5.4	4.8	4.0	1.7	10.0	6.0	3.5		4.0	2.8	4.2	3.0	3.5
4,O*H*				6.7						6.8				
5a,5b	12.4	11.4	11.2	12.0	13.2		11.2	12.5		12.0	13.0	10.8	12.2	12.8
2′,6′	2.6	2.4	2.8	2.6										
3′,5′							2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8
5′,6′	9.0	9.2	9.2	9.0			9.2	9.1	9.3	9.3	9.2	9.3	9.3	9.3

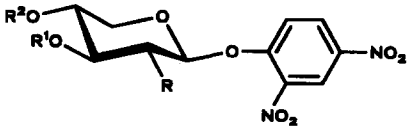
[a] In $CDCl_3$.
[b] In CD_3OD.

an alternative procedure. The most direct approach to the corresponding xylobiosides would be to xylosylate the unprotected monosaccharides, since there are only two hydroxyl groups which could be glycosylated. Unfortunately, however, reaction of **21** with xylosyl trichloroacetimidate (**2**) gave exclusively the 1,3-linked disaccharide (data not shown) not the desired 1,4-linked species. The strategy finally adopted involved acetylation of the unprotected xylosides with one equivalent of acetic anhydride in dioxane–pyridine, to yield the desired products **22** and **27** in a mixture with unreacted starting material, 4-*O*-acetyl derivatives, and diacetates. After purification of **22** and **27**, the other products were combined, deprotected and then recycled through the procedure.

The selectively 3-*O*-acetylated 2-deoxy-2-halo-β-D-xylosides were glycosylated with compound **2** to yield the peracetylated 2,4-dinitrophenyl 2-chloro-2-deoxy- and 2-deoxy-2-fluoro-β-xylobiosides (**23** and **29**). Both were deacetylated using ammonia in methanol



	R	R^1
16	Cl	Cl
17	2,4-dinitrophenyl	Cl
18	OAc	F
19	Br	F



	R	R^1	R^2
20	Cl	Ac	Ac
21	Cl	H	H
22	Cl	Ac	H
23	Cl	Ac	2,3,4-tri-*O*-acetyl-ß-D-xylopyranosyl
24	Cl	H	ß-D-xylopyranosyl
25	F	Ac	Ac
26	F	H	H
27	F	Ac	H
28	F	H	Ac
29	F	Ac	2,3,4-tri-*O*-acetyl-ß-D-xylopyranosyl
30	F	H	ß-D-xylopyranosyl

Table 2
^{1}H NMR data of the xylobiosides

Proton	Compound							
	9	**10**	**11**	**12**	**23**	**24**	**29**	**30**
H-1	5.28 d	5.14 d	5.43 d	5.27 d	5.45 d	5.48 d	5.66 dd	5.58 dd
H-2	5.09 dd	3.55 dd	5.03 dd	3.57 dd	4.02 dd	3.83 dd	4.62 ddd	4.39 ddd
H-3	5.20 t	3.60 t	5.18 t	3.63 t	5.24 t	3.72 dd	5.28 dt	3.88 q
H-4	3.89 dt	3.75 ddd	3.85 dt	3.77 dt	3.85 dt	3.82 ddd	3.83 m	3.84 dt
H-5a	3.57 dd	3.57 dd	3.64 dd	3.61 dd	3.62 dd	3.64 dd	3.70 dd	3.63 dd
H-5b	4.06 dd	4.09 dd	4.11 dd	4.12 dd	4.11 dd	4.18 dd	4.05 dd	4.14 dd
H-1′	4.58 d	4.35 d	4.59 d	4.36 d	4.61 d	4.39 d	4.67 d	4.36 d
H-2′	4.83 dd	3.23 dd	4.87 dd	3.23 dd	4.81 dd	3.23 dd	4.88 dd	3.23 dd
H-3′	5.11 t	3.32 t	5.13 t	3.32 t	5.08 t	3.32 t	5.10 t	3.32 t
H-4′	4.88 dt	3.50 ddd	4.91 dt	3.51 ddd	4.87 dt	3.50 ddd	4.89 dt	3.51 ddd
H-5′a	3.38 dd	3.24 dd	3.37 dd	3.24 dd	3.41 dd	3.24 dd	3.44 dd	3.24 dd
H-5′b	4.09 dd	3.90 dd	4.10 dd	3.90 dd	4.11 dd	3.91 dd	4.15 dd	3.90 dd
H-2″	7.34 d	7.59 d						
H-3″			7.87 d	8.01 d	8.70 d	8.72 d	8.70 d	8.73 d
H-4″			8.00 dd	8.03 dd				
H-5″	7.99 d	8.12 d			8.41 dd	8.47 dd	8.42 dd	8.48 dd
H-6″	7.23 dd	7.43 dd	8.13 d	8.19 d	7.38 d	7.59 d	7.44 d	7.61 d
OAc	2.02 s		2.02 s		2.01 s		2.03 s	
	2.03 s		2.03 s		2.02 s		2.04 s	
	2.04 s		2.05 s		2.04 s		2.16 s	
	2.06 s		2.11 s					
	2.07 s		2.12 s					
$J_{H,H(F)}$								
1,2	5.8	7.0	4.2	6.6	5.8	7.9	3.4	6.9
1,F							8.9	5.1
2,3	7.8	8.8	5.8	8.7	8.0	9.8	4.7	8.2
2,F							45.5	50.3
3,4	7.8	8.8	5.8	8.7	7.0	8.5	5.0	8.2
3,F							11.7	8.2
4,5a	7.2	9.5	5.6	8.7	7.0	10.0	4.2	9.0
4,5b	4.4	5.0	3.5	4.9	4.0	5.2	2.8	5.0
5a,5b	12.0	11.5	12.2	11.8	12.0	11.6	12.3	11.7
1′,2′	6.2	7.8	6.7	7.5	5.8	7.5	5.6	7.5
2′,3′	8.0	9.1	8.4	9.0	7.0	8.7	7.5	9.0
3′,4′	8.0	9.1	8.4	9.0	7.5	8.7	7.5	9.0
4′,5′a	8.0	10.2	8.6	10.3	7.3	10.2	7.2	10.0
4′,5′b	4.8	5.2	5.0	5.3	4.5	5.3	4.5	5.2
5′a,5′b	12.0	11.5	12.0	11.3	12.0	11.4	12.1	11.5
2″,6″	2.6	2.6						
3″,4″			8.8	8.8				
3″,5″					2.8	2.8	2.8	2.8
4″,6″			2.2	2.1				
5″,6″	9.2	9.1			9.4	9.3	9.2	9.5

to give the corresponding unprotected xylobiosides **24** and **30**, which were obtained as crystalline products and characterised by ^{1}H NMR spectroscopy (Table 2). Deoxyfluoro sugars were further characterized by ^{19}F NMR spectroscopy (Table 3).

Table 3
^{19}F NMR data of the fluorosugars

Compound	25	26	27	28	29	30
Chemical shift (ppm) [a]	−118.7 ddd	−123.4 ddd	−117.8 ddd	−119.5 ddd	−119.1 ddd	−122.8 ddd

[a] Relative to trifluoroacetic acid ($\delta = 0$).

Table 4
Kinetic parameters of xylobiosides with *B. subtilis* xylanase

Compound	Concentration range [mM]	$\Delta\epsilon$[a] (λ) ($M^{-1}\,cm^{-1}$)(nm)	pK_a Phenol	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
8	0.642–51.3	713 (400)	7.22	14.3	14.2±0.5	1.01
10	0.22–3.52	11709 (400)	5.36	8.3	3.4±0.3	2.44
10	3.52–17.6	11709 (400)	5.36	17.9	11.8±1.2	1.52
12	0.126–4.53	3571 (440)	5.15	75.6	2.2±0.1	34.36

[a] Under assay conditions.

Substrate assays with B subtilis *endo-xylanase.*—Michaelis–Menten parameters for the hydrolysis of **10** and **12**, as well as of the previously synthesised [15] substrate, 2-nitrophenyl β-xylobioside (**8**), were determined as described in the Experimental section and are presented in Table 4. Interesting kinetic behaviour was observed with **10** and **12** as shown by the biphasic Lineweaver–Burk plot for **10** in Fig. 1. Such behaviour is likely due to transglycosylation of the xylobiosyl enzyme intermediate to a second substrate molecule bound in the aglycone site at higher substrate concentrations. Such behaviour would only be observed kinetically if the second step, deglycosylation, were

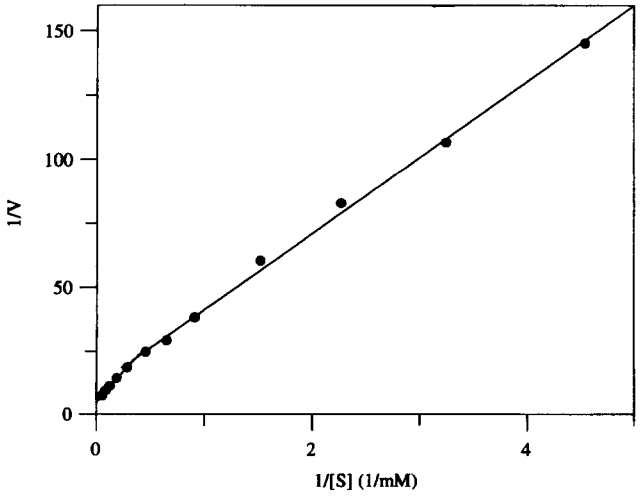


Fig. 1. Lineweaver–Burk plot for hydrolysis of 3,4-dinitrophenyl β-xylobioside by *B. subtilis* xylanase.

rate determining. Thus, the observation of such biphasic behaviour with the best leaving groups (thus presumably fastest glycosylation rates), but not with **8**, would suggest a change in the rate-determining step across the substrate series. TLC analysis of reaction mixtures confirmed that the reaction product at low substrate concentrations was xylobiose, while at high substrate concentrations longer aryl xylo-oligosaccharides were formed. Thus kinetic parameters determined at low substrate concentrations are those of the simple hydrolysis process, while those at high substrate concentration are for the transglycosylation process, the high K_m value reflecting the relatively poor binding in the aglycone site. A full analysis was performed for 3,4-dinitrophenyl β-xylobioside (**10**), as shown in Fig. 1, and parameters for both processes are provided in Table 4. However, due to the large quantities of substrate required for such a full analysis, a full evaluation was not performed for **12**, rates only being determined for the mechanistically more interesting hydrolysis process.

Once the deglycosylation step becomes rate-determining, K_m values should decrease with increasing substrate reactivity (leaving group ability), and this is apparent from the drop in K_m values across this (small) series. The considerably greater k_{cat} value for **12** than for **10** is somewhat surprising, given the similarity in phenol leaving group ability (pK_a). However, it is probably a consequence of the presence of an *ortho*-substituent in **12**, which has been shown in other glycosidases to result in higher k_{cat} values, possibly due to a ground-state destabilisation [23].

The best substrate for assaying this xylanase is **12**, since it has the highest k_{cat} and the lowest K_m values, thus allowing reactions to be performed with a minimum of substrate at high sensitivity. However, **10** has a larger extinction coefficient change upon cleavage under the assay conditions than **12**, thus making it a substrate of comparable utility. The poor extinction coefficient change of **8** under these conditions, due to its

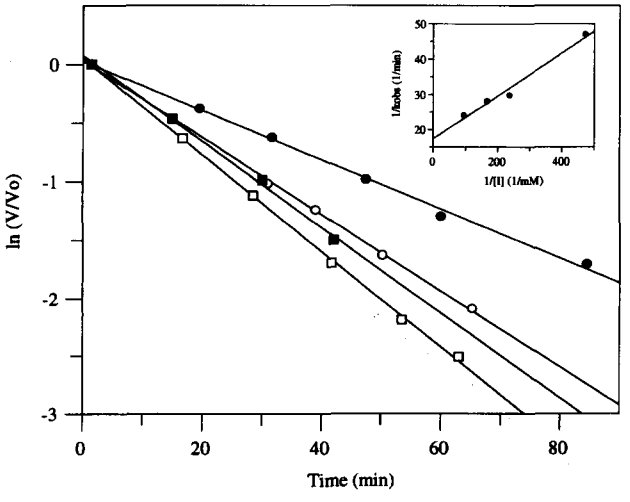


Fig. 2. Inactivation of *C. fimi* exo-xylanase/glucanase by 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside (**30**). First-order inactivation plots at the following concentrations of inactivator: (□) 10 uM; (■) 6 uM; (○) 4 uM; (●) 2 uM. Inset: Reciprocal replot of pseudo-first-order rate constants vs inactivator concentration.

high pK_a, makes it a poor choice for the preferred continuous assay, especially coupled with its high K_m value. However, it can be used successfully in stopped assays when phenol production is monitored at high pH values.

Inactivation studies with the C. fimi *exo-xylanase / glucanase (Cex).*—Incubation of Cex with 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside resulted in a time-dependent decrease in activity, as shown in logarithmic form in Fig. 2. Pseudo-first-order rate constants for inactivation were determined at each inactivator concentration, and these were plotted versus inactivator concentration in reciprocal form (Inset). This allows determination of the inactivation parameters, according to Scheme 1, of $k_i = 0.057$ min^{-1} and $K_i = 0.0035$ mM.

$$E + I \overset{K_i}{\rightleftharpoons} EI \overset{k_i}{\rightarrow} E.I$$

2-Deoxy-2-fluoro-xylobioside (**30**) is therefore an excellent time-dependent inactivator of the enzyme, presumably funtioning via the formation and accumulation of a relatively stable 2-deoxy-2-fluoro-xylobiosyl enzyme intermediate, analogous to that formed by Cex with the corresponding 2-deoxy-2-fluoro-glucoside [7] and cellobioside [24]. Interestingly **30** is the best inactivator yet found for this enzyme, on the basis of k_i/K_i values, being some 26-fold better than the cellobioside and 10^5-fold better than the glucoside. This is entirely consistent with the greater activity of this enzyme as a xylanase than a cellulase [25].

3. Experimental

General synthetic methods.—Melting points were determined with a Mel-Temp II apparatus (Laboratory devices, Holliston, MA. USA) and are not corrected. Reactions were monitored by TLC on DC-Alufolien Kieselgel 60 F_{254} 0.2 mm (E. Merck). Petroleum ether used had a boiling range of 30–50°C. ^1H NMR spectra were recorded with a Bruker AC 200 (200 MHz) or a Bruker WH 400 (400 MHz) and are referenced on the solvent peak. Unless stated otherwise, the spectra were recorded in $CDCl_3$ for fully acetylated, and in CD_3OD for unprotected sugars. ^1H NMR data are listed in Table 1 for monosaccharide and in Table 2 for disaccharide derivatives. Decoupling experiments were performed to obtain proton assignments. ^{19}F NMR spectra were recorded with a Bruker AC 200 (188.3 MHz) spectrometer using trifluoroacetic acid ($\delta = 0$ ppm) as an external standard. ^{19}F NMR data are listed in Table 3.

*2,5-Dinitrophenyl 2,3,2',3',4'-penta-*O*-acetyl-β-xylobioside* (**11**).—2,6-Lutidine (540 mg, 5.05 mmol) and Drierite (2 g) were added to a solution of anhydrous 2,5-dinitrophenol (0.9 g. 4.9 mmol) in anhydrous MeCN (20 mL). The mixture was stirred for 10 min under an atmosphere of dry nitrogen. Silver carbonate (1.5 g, 5.44 mmol) was added and stirring was continued in the dark while a solution of crude bromide **7** (1.4 g, ~ 2.5 mmol) in anhydrous MeCN (6 mL) was added dropwise. After complete addition the mixture was stirred for another 30 min and then filtered by suction. The residue was washed with EtOAc, the filtrate was concentrated, the residue was dissolved in CH_2Cl_2 (100 mL), and the solution was successively washed with 5% aq $NaHCO_3$ (3 × 100 mL), and water (100 mL), dried ($MgSO_4$), and concentrated. The residue was purified

by column chromatography (2:3 → 1:1 EtOAc–hexanes), and the product crystallised from the same solvents to give **11** (1.05 g, ~64%): mp 133–134°C: R_f 0.75 (2:1 EtOAc–hexanes). Anal. Calcd for $C_{26}H_{30}N_2O_{18}$: C, 47.42; H, 4.59; N, 4.25. Found: C, 47.55; H, 4.64; N, 4.19.

2,5-Dinitrophenyl β-xylobioside (**12**).—Dry ammonia was bubbled through a stirred suspension of **11** (686 mg. 1.04 mmol) in anhydrous MeOH (25 mL). When the reaction was complete as monitored by TLC (these reactions typically take 1–4 h), the solution was concentrated to dryness and the residue was purified by column chromatography (27:2:1 EtOAc–MeOH–H_2O). The product crystallised from MeOH–Et_2O to give **12** (451 mg, 96%): mp 175–176°C (dec.); R_f 0.27 (17:2:1 EtOAc–MeOH–H_2O). Anal. Calcd for $C_{16}H_{20}N_2O_{13}$: C, 42.86; H, 4.50; N, 6.25. Found: C, 42.62; H,4.63; N, 6.05.

*3,4-Dinitrophenyl 2,3,4-tri-*O*-acetyl-β-*D*-xylopyranoside* (**3**).—3,4-Dinitrophenol (0.9 g. 4.89 mmol) in anhydrous MeCN (20 mL) was glycosylated using 2,6-lutidine (576 mg, 5.38 mmol). Drierite (~ 3 g), silver carbonate (2.5 g, 9.07 mmol), and a solution of **1** (2.5 g, 7.37 mmol) in anhydrous MeCN (10 mL), as described for the synthesis of **11**. The reaction mixture was filtered by suction, the filtrate was concentrated to dryness, and the residue was purified by column chromatography (2:5 EtOAc–hexanes). The product was crystallised from the same solvents to give **3** (1.96 g, 90%): mp 180–181°C, R_f 0.46 (1:1 EtOAc–hexanes). Anal. Calcd for $C_{17}H_{18}N_2O_{12}$: C, 46.16; H, 4.10; N, 6.33. Found: C, 46.22; H, 4.13; N, 6.21.

*3,4-Dinitrophenyl β-*D*-xylopyranoside* (**4**).—Compound **3** (1.67 g, 3.78 mmol) was deacetylated in anhydrous MeOH (100 mL) as described for **12**. The product was purified by column chromatography (5:4:1 EtOAc–hexanes–MeOH) and then crystallised from EtOH–Et_2O–petroleum ether to give **4** (1.04 g, 87%): mp 149–150°C; R_f 0.44 (27:2:1 EtOAc–MeOH–H_2O). Anal. Calcd for $C_{11}H_{12}N_2O_9$: C, 41.78; H, 3.82; N, 8.86. Found: C, 41.62; H, 3.92; N, 8.69.

*3,4-Dinitrophenyl 2,3-di-*O*-acetyl-4-*O*-triethylsilyl-β-*D*-xylopyranoside* (**5**).—Compound **4** (1.38 g, 4.64 mmol) and dibutyltin oxide (1.27 g, 5.1 mmol) were refluxed in 1:2 benzene–toluene (150 mL) on a Dean Stark trap overnight. The solution was cooled in an ice bath under dry nitrogen, triethylsilyl chloride (1.17 mL, 6.96 mmol) was added, and the solution was stirred for 2 days. The mixture was concentrated to dryness, and the residue was dissolved in 1:1 MeCN–petroleum ether (300 mL). The petroleum ether layer was removed, and the MeCN layer was washed with fresh petroleum ether (2 × 100 mL). The acetonitrile was evaporated, and the residue was acetylated using 5:4 pyridine–Ac_2O (18 mL) overnight. The solution was transferred into water (300 mL), and the product was extracted with CH_2Cl_2 (5 × 50 mL). The combined extracts were neutralised with 5% aq $NaHCO_3$, washed with water (200 mL), dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (1:4 EtOAc–hexanes) and crystallised from the same solvents to give **5** (1.61 g, 71%): mp 128°; R_f 0.62 (1:2 EtOAc–hexanes). Anal. Calcd for $C_{21}H_{30}N_2O_{11}Si$: C, 49.02; H, 5.88; N, 5.44. Found: C, 48.92; H, 5.88; N, 5.38.

*3,4-Dinitrophenyl 2,3-di-*O*-acetyl-β-*D*-xylopyranoside* (**6**).—Water (6 mL) was added to a solution of **5** (415 mg, 0.807 mmol) in 1:2 Et_2O–AcOH (18 mL) and left at room temperature for 3 h. The solution was diluted with water (200 mL), and the product was extracted with CH_2Cl_2 (5 × 20 mL). The combined extracts were washed with water

(50 mL), neutralised with 5% aq $NaHCO_3$, washed with water (50 mL), dried, and concentrated. The residue was purified by column chromatography (3:2 EtOAc–hexanes), and the product crystallised from the same solvents to give **6** (294 mg, 91%): mp 138–139.5°C, R_f 0.18 (1:1 EtOAc–hexanes). Anal. Calcd for $C_{15}H_{16}N_2O_{11}$: C, 45.01; H, 4.03; N, 7.00. Found: C, 45.08; H, 4.11; N, 6.98.

*3,4-Dinitrophenyl 2,3,2',3',4'-penta-*O*-acetyl-β-xylobioside* (**9**).—A solution of borontrifluoride etherate (50 mg, 0.35 mmol) in anhydrous CH_2Cl_2 (5 mL) was added dropwise to a stirred mixture of **6** (632 mg, 1.58 mmol), **2** [26] (1.3 g, 3.09 mmol), and 3 Å molecular sieves (~ 0.5 g) in anhydrous CH_2Cl_2 (30 mL) at −25 to −20°C under dry nitrogen. After 30 min Et_3N (0.5 mL) was added, the solution was warmed to room temperature, filtered, and the filtrate was concentrated to dryness. The residue was purified by column chromatography (1:1 EtOAc–hexanes), and the product crystallised from the same solvents to give **9** (651 mg, 62%): mp 106–110°C; R_f 0.32 (1:1 EtOAc–hexanes). Anal. Calcd for $C_{26}H_{30}N_2O_{18}$: C, 47.42; H, 4.59; N, 4.27. Found: C, 47.51; H, 4.69; N, 4.19.

3,4-Dinitrophenyl-β-xylobioside (**10**).—Compound **9** (442 mg, 0.671 mmol) was deacetylated in anhydrous MeOH (30 mL) as described for **12**. The product crystallised from MeOH–Et_2O–petroleum ether to give **10** (263 mg, 87%): mp 204–205°C (dec); R_f 0.13 (27:2:1 EtOAc–MeOH–H_2O). Anal. Calcd for $C_{16}H_{20}N_2O_{13}$: C, 42.86; H, 4.50: N, 6.25. Found: C, 42.68: H, 4.50: N, 6.09.

*3,4-Di-*O*-acetyl-2-chloro-2-deoxy-β-*D*-lyxo- and -α-*D*-xylo-pyranosyl chloride* (**14** *and* **16**).—Dry chlorine was bubbled through a solution of **13** (1.29 g, 6.44 mmol) in CCl_4 (30 mL) at room temperature. The reaction mixture turned green when the starting material was used up. The solution was then purged with dry nitrogen to remove excess chlorine, concentrated, and the products isolated by column chromatography (1:3 Et_2O–petroleum ether).

The first-eluted compound **16** (512 mg, 29.3%) was crystallised from EtOAc–hexanes: mp 92–93°C; R_f 0.36 (1:3 Et_2O–petroleum ether).

The second-eluted compound **14** (952 mg, 54.5%) was crystallised from EtOAc–hexanes: mp 89–90°C; R_f 0.28 (1:3 Et_2O–petroleum ether).

*2,4-Dinitrophenyl 3,4-di-*O*-acetyl-2-chloro-2-deoxy-α- and -β-*D*-xylopyranoside* (**17** *and* **20**).—Anhydrous 2,4-dinitrophenol (1.4 g, 6.08 mmol) was glycosylated in anhydrous MeCN (50 mL) using 2,6-lutidine (0.60 g, 5.6 mmol), Drierite (~ 4 g), silver carbonate (2.0 g, 7.3 mmol), and a solution of **16** (1.1 g, 4.05 mmol) in anhydrous MeCN (5 mL), as described for **11**. The mixture was filtered by suction, and the filtrate was concentrated. The residue was dissolved in 1:1 CH_2Cl_2–water (100 mL). The organic layer was washed with 0.1 M HCl (100 mL), 5% aq $NaHCO_3$ (2 × 100 mL), and water (2 × 100 mL), dried, and concentrated. The anomeric mixture of the products was resolved by column chromatography (1:2 EtOAc–hexanes).

The first-eluted compound **20** was crystallised from EtOAc–hexanes (1.04 g, 61%): mp 139–140°C; R_f 0.30 (1:2 EtOAc–hexanes). Anal Calcd for $C_{15}H_{15}ClN_2O_{10}$: C, 43.03; H, 3.61; N, 6.69. Found: C, 42.91; H, 3.55; N, 6.50.

The second-eluted compound was **17** (0.45 g, 26%): R_f 0.23 (1:2 EtOAc–hexanes).

*2,4-Dinitrophenyl 2-chloro-2-deoxy-β-*D*-xylopyranoside* (**21**).—Compound **20** (650 mg, 1.55 mmol) was deacetylated in anhydrous MeOH (50 mL) as described for **12**. The

crude product was purified by column chromatography (10:9:1 EtOAc–hexanes–MeOH) and then crystallised from MeOH–Et_2O–petroleum ether to give **21** (423 mg, 81%): mp 159–160°C; R_f 0.19 (9:10:1 EtOAc–hexanes–MeOH). Anal. Calcd for $C_{11}H_{11}ClN_2O_8$: C, 39.48; H, 3.31; N, 8.37. Found: C, 39.55; H, 3.35; N, 8.18.

2,4-Dinitrophenyl 3-O-acetyl-2-chloro-2-deoxy-β-D-xylopyranoside (**22**).—A solution of **21** (322 mg, 0.962 mmol) in anhydrous dioxane (2 mL) was acetylated using Ac_2O (100 μL, 1.06 mmol) and pyridine (150 μL, 1.86 mmol) at room temperature overnight. The reaction mixture was diluted with toluene (20 mL) and concentrated to dryness. The products were separated by column chromatography (4:1 Et_2O–petroleum ether), and the slower moving monoacetate crystallised from EtOAc–hexanes to give **22** (123 mg, 34%): mp 118°C; R_f 0.46 (9:10:1 EtOAc–hexanes–MeOH). Anal. Calcd for $C_{13}H_{13}ClN_2O_9$: C, 41.45; H, 3.48; N, 7.44. Found: C, 41.54; H. 3.48; N, 7.44.

The second monoacetate **20**, as well as remaining starting material, were recovered and recycled.

2,4-Dinitrophenyl 3,2',3',4'-tetra-O-acetyl-2-chloro-2-deoxy-β-xylobioside (**23**).—Compound **22** (180 mg, 0.478 mmol) was glycosylated in anhydrous CH_2Cl_2 (10 mL) as described for **9**, using **2** (420 mg, 1.0 mmol) and borontrifluoride etherate (10 mg, 70 μmol). The crude product was purified by column chromatography (1:1 EtOAc–hexanes) and gave **23** (200 mg, 69%) as a colourless syrup: R_f 0.47 (1:1 EtOAc–hexanes).

2,4-Dinitrophenyl 2-chloro-2-deoxy-β-xylobioside (**24**).—Compound **23** (202 mg, 0.335 mmol) was deacetylated in anhydrous MeOH (20 mL) as described for **12**. The residue was purified by column chromatography (27:2:1 EtOAc–MeOH–H_2O), and the product was crystallised from MeOH–Et_2O–petroleum ether to give **24** (100 mg, 64%): mp 180–182°C; R_f 0.31 (17:2:1 EtOAc–MeOH–H_2O). Anal. Calcd for $C_{16}H_{19}ClN_2O_{12}$: C. 41.17; H, 4.10; N, 6.00. Found: C, 41.18; H, 4.20; N, 5.90.

3,4-Di-O-acetyl-2-deoxy-2-fluoro-α-D-xylopyranosyl bromide (**19**).—Hydrogen bromide in AcOH (45%, 10 mL) was added to a solution of **18** (159 mg. 0.569 mmol) in anhydrous CH_2Cl_2 (10 mL) at 0°C, and the temperature was maintained for 2 h. The mixture was then diluted with ice water (200 mL), and the product was extracted with CH_2Cl_2 (4 × 10 mL). The combined extracts were washed with water (100 mL), neutralised with 5% aq $NaHCO_3$ (100 mL), washed with water (100 mL), dried, and concentrated. The residue (170 mg) was used as crude product for further syntheses; R_f 0.53 (1:2 EtOAc–hexanes).

2,4-Dinitrophenyl 3,4-di-O-acetyl-2-deoxy-2-fluoro-β-D-xylopyranoside (**25**).—Anhydrous 2,4-dinitrophenol (185 mg, 1.0 mmol) was glycosylated in anhydrous MeCN (5 mL) using 2,6-lutidine (107 mg, 1.0 mmol), Drierite (~ 0.5 g), silver carbonate (276 mg, 1.0 mmol), and a solution of crude bromide **19** (170 mg, ~ 0.569 mmol) in anhydrous MeCN, as described for **3**. The residue was dissolved in CH_2Cl_2 (100 mL), washed with 0.1 M HCl (100 mL), 5% aq $NaHCO_3$ (100 mL), and water (100 mL), dried, and concentrated. The crude product was purified by column chromatography (1:2 EtOAc–hexanes) yielding **25** (185 mg, 80%) as a colourless syrup: R_f 0.35 (1:2 EtOAc–hexanes).

2,4-Dinitrophenyl 2-deoxy-2-fluoro-β-D-xylopyranoside (**26**).—Compound **25** (172 mg, 0.428 mmol) was deacetylated in anhydrous MeOH (10 mL) as described for **12**. The reaction mixture was concentrated, the residue was purified by column chromatog-

raphy (10:9:1 EtOAc–hexanes–MeOH), and the product was crystallised from MeOH–Et_2O–petroleum ether to give **26** (110 mg, 81%): mp 175°C; R_f 0.13 (9:10:1 EtOAc–hexanes–MeOH). Anal. Calcd for $C_{11}H_{11}FN_2O_8$: C, 41.52; H, 3.48; N, 8.80. Found: C, 41.74; H, 3.59; N, 8.61.

*2,4-Dinitrophenyl 3-*O*- and 4-*O*-acetyl-2-deoxy-2-fluoro-β-*D*-xylopyranoside* (**27** *and* **28**).—A solution of **26** (82 mg, 0.258 mmol) in anhydrous dioxane (1 mL) was acetylated using Ac_2O (25 μL, 0.265 mmol) and pyridine (40 μL, 0.495 mmol) as described for **22**. The resulting mixture was separated by column chromatography (4:1 Et_2O–petroleum ether). Compound **25** eluted first, followed by **28**; R_f 0.29 (4:1 Et_2O–petroleum ether). **27** (27 mg, 29%); R_f 0.20 (4:1 Et_2O–petroleum ether), and finally **26**. Products **25**, **26**, and **28** were pooled and recycled.

*2,4-Dinitrophenyl 3,2',3',4'-tetra-*O*-acetyl-2-deoxy-2-fluoro-β-xylobioside* (**29**).—Compound **27** (72 mg, 0.20 mmol) was glycosylated using **2** (420 mg, 1.0 mmol) and a solution of borontrifluoride etherate (10 mg, 80 μmol) in anhydrous CH_2Cl_2 (1 mL) as described for **9**. The crude product was purified by column chromatography (1:1 EtOAc–hexanes) yielding **29** (90 mg, 76%) as a colourless syrup: R_f 0.45 (1:1 EtOAc–hexanes).

2,4-Dinitrophenyl 2-deoxy-2-fluoro-β-xylobioside (**30**).—Compound **29** (90 mg, 0.153 mmol) was deacetylated in anhydrous MeOH (10 mL) as described for **12**. The concentrated reaction mixture was purified by column chromatography (27:2:1 EtOAc–MeOH–H_2O), and the product crystallised from MeOH–Et_2O–petroleum ether to give **30** (52 mg, 75%): mp 201°C; R_f 0.41 (17:2:1 EtOAc–MeOH–H_2O). Anal. Calcd for $C_{16}H_{19}FN_2O_{12}$: C, 42.67; H, 4.25; N, 6.22. Found: C, 42.83; H, 4.37; N. 6.07.

Enyzme kinetics.—The xylanase (EC 3.2.1.8) from *B. subtilis* was a generous gift of Dr Wakarchuk and was prepared as previously described [27]. All kinetic studies with this enzyme were performed in 20 mM MES buffer containing 50 mm NaCl (pH 6.0) at 40°C. BSA (0.1%) was added to the buffer at the lowest enzyme concentrations. Assays were run (3 min) in black quartz cells using 400 μL of substrate solution, and were started by addition of 10 μL of enzyme solution, corresponding to 2.2–62 μg of enzyme per reaction mixture. Data were analysed using the regular Michaelis–Menten equation and the GraFit program [28]. The spontaneous hydrolysis rates of the substrates were insignificant compared to the enzyme-catalysed rates. For the results see Table 4.

The exo-xylanase/glucanase Cex was provided by Dr Neil Gilkes, and its purification has been previously described [29]. Inactivation experiments with this enzyme were performed in 50 mm sodium phosphate buffer (pH 7.0) at 37°C. The enzyme was incubated with various concentrations of **30** and assayed at time intervals by removal of aliquots and assaying these as previously described [25]. Residual rates as a function of time at each inactivator concentration were fit to a first-order expression using the programme GraFit and pseudo-first-order rate constants (k_{obs}) were thus obtained. These were then directly fit to the expression below, again using GraFit to get values of k_i and K_i.

$$k_{obs} = \frac{k_i[I]}{K_i + [I]}$$

where $[I]$ is the inhibitor concentration.

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