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ARYL 4-THIOXYLOBIOSIDE AND 1,4-DITHIOXYLOBIOSIDES AS EFFECTORS OF THE ENZYMIC ACTIVITY FOR FUNGAL D-XYLANASES*

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ABSTRACT

Reaction of penta-*O*-acyl-4-thio-β-xylobiosyl bromide with either *o*-nitrophenol, *p*-nitrobenzenethiol, or *p*-aminobenzenethiol in acetone in the presence of potassium carbonate, followed by subsequent *O*-deacylation, led in excellent yield to *o*-nitrophenyl 4-thio-β-xylobioside (**8**), and *p*-nitrophenyl and *p*-aminophenyl 1,4-dithio-β-xylobiosides (**10**). Compound **8** behaved as a good chromogenic substrate for xylanases A and B from *Sporotrichum dimorphosporum* with respective K_m of 2.4 and 6mM. The *N*-acetyl derivative of **10**, as well as the free disaccharide 4-thioxylobiose, were competitive inhibitors for xylanase B (respective K_i of 2.5 and 0.72mM) but distinctly enhanced the rate of hydrolysis of D-xylan by xylanase A when incubated simultaneously with this substrate (respective K_A of 0.19 and 0.18mM); the rate of hydrolysis of the synthetic substrate **8** was not affected under similar conditions. These results suggest the possibility of a regulation mechanism for D-xylan hydrolysis by xylanase which may involve extracellular regulation by small oligosaccharides. The aminodithiodisaccharide **10** was coupled to CH-Sepharose 4B to give an affinity gel which retained xylanase activities.

INTRODUCTION

The characterization and the precise evaluation of the catalytic activity of glycanases are basic problems in the area of enzymic biomass conversion, since well defined substrates suitable for this purpose are usually not available. Heterogeneity of most of the macromolecular substrates usually used and variations in the action pattern of enzymes, or frequently multicomponent enzymic systems, are known impediments to reliable measurements. Evaluation of xylanases using purified xylan is a representative illustration of these difficulties.

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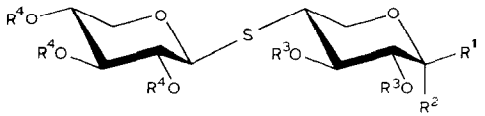
Nitrophenyl 4-thiomaltooligosaccharides, which are cleaved exclusively at the aglycon chromophoric site, were proposed previously to overcome this problem with α-amylase[5]. A similar approach is now undertaken with xylanases using aryl 4-thioxylobiosyl substrate analogs.

Affinity-chromatography purification of glycanases is another field of interest and aminophenyl 1-thioglycosides were already used as ligands for this purpose with glycosidases[6]. Thiooligosaccharides were suggested in a similar approach for glycanases[3] and aminophenyl 1,4-dithiocellobioside, as well as aminophenyl 1,4,4'-trithiocellotrioside, proved to be valuable ligands for the purification of cellobiohydrolases[7]. Following this approach, aryl 1,4-dithioxylobiosyl derivatives were selected for xylanases. Such substrate analogs may be readily prepared from previously described 4-thioxylobiose[1]. We report herein a synthesis in both the O and S series, and the enzymic behavior of these derivatives with well characterized, exocellular xylanase components from the lignolytic basidiomycete *Sporotrichum dimorphosporum*[8].

RESULTS AND DISCUSSION

4-Thioxylobiose (**1**) was previously prepared[1] as a gratuitous inducer of xylanases in *Trichoderma lignorum*. Preparation of derivatives of **1** was readily achieved from its precursor 1,2,3-tri-*O*-benzoyl-4-*S*-(2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl)-4-thio-D-xylopyranose[1] (**2**) by use of the bromide intermediate **3**. Reaction of *o*-nitrophenol, *p*-nitrobenzenethiol, or *p*-aminobenzenethiol with **3** in acetone in the presence of potassium carbonate led exclusively, and in excellent yield, to the corresponding 1,2-*trans* aryl glycosides **4–6**. The assignment of the anomeric configuration for compounds **5** and **6** is clearly inferred from the large



1 $R^1 = OH, R^2 = R^3 = R^4 = H$
2 $R^1 = H, R^2 = OBz, R^3 = Bz, R^4 = Ac$
3 $R^1 = H, R^2 = Br, R^3 = Bz, R^4 = Ac$
4 $R^1 = OC_6H_4NO_2\text{-}o, R^2 = H, R^3 = Bz, R^4 = Ac$
5 $R^1 = SC_6H_4NO_2\text{-}p, R^2 = H, R^3 = Bz, R^4 = Ac$
6 $R^1 = SC_6H_4NH_2\text{-}p, R^2 = H, R^3 = Bz, R^4 = Ac$
7 $R^1 = SC_6H_4NHAc\text{-}p, R^2 = H, R^3 = Bz, R^4 = Ac$
8 $R^1 = OC_6H_4NO_2\text{-}o, R^2 = R^3 = R^4 = H$
9 $R^1 = SC_6H_4NO_2\text{-}p, R^2 = R^3 = R^4 = H$
10 $R^1 = SC_6H_4NH_2\text{-}p, R^2 = R^3 = R^4 = H$
11 $R^1 = SC_6H_4NHAc\text{-}p, R^2 = R^3 = R^4 = H$

TABLE I

^{1}H-N M R DATA FOR RING PROTONS OF *O*-ACYLATED ARYL 4-THIO-β-XYLOBIOSIDE AND 1,4-DITHIO-β-XYLOBIOSIDES **4–7**[a]

Compound	*Chemical shifts (δ) and coupling constants (Hz)*											
	H-1 ($J_{1,2}$)	*H-2* ($J_{2,3}$)	*H-3* ($J_{3,4}$)	*H-4* ($J_{4,5a}$)	*H-5a* ($J_{a,b}$)	*H-5b* ($J_{4,5b}$)	*H-1'* ($J_{1,2}$)	*H-2'* ($J_{2,3}$)	*H-3'* ($J_{3,4}$)	*H-4'* ($J_{4,5a}$)	*H-5'a* ($J_{a,b}$)	*H-5'b* ($J_{4,5b}$)
4		5.56		3.48	3.79	4.51	4.79	4.98	5.17	4.86	3.37	4.10
		m		m	dd	dd	d	t	t	td	dd	dd
				(8.0)	(12.0)	(4.0)	(8.0)	(8.0)	(8.0)	(9.5)	(12.5)	
5	5.22	5.43	5.56	3.44	3.77	4.55	4.74	4.95	5.14	4.82	3.35	4.11
	d	t	dd	m	dd	dd	d	t	t	m	dd	dd
	(8.0)	(8.0)	(9.0)	(9.0)	(12.0)	(4.5)	(8.0)	(8.0)	(8.0)	(8.5)	(12.0)	(5.0)
6	4.83	5.38	5.54	3.33	3.69	4.48	4.79	5.31	5.18	5.31	3.38	4.12
	d	t	dd	m	dd	dd	d	t	t	m	m	dd
	(8.0)	(8.0)	(10.0)		(12.0)	(5.0)	(8.0)	(8.0)	(8.0)		(12.5)	(5.0)
7	4.87	5.32	5.48	3.32	3.64	4.43	4.71	4.88	5.11	4.83	3.30	4.0
	d	t	dd	dt	dd	dd	d	t	t	dt	dd	dd
	(8.5)	(8.5)	(10.0)	(10.0)	(12.0)	(4.5)	(8.5)	(8.5)	(8.5)		(12.0)	(9.0)

[a]For each compound: 1st line, chemical shift; 2nd line, multiplicity; and 3rd line, coupling constant (in parentheses).

coupling constants for H-1–H-3 in the ^{1}H-n.m.r. spectrum (Table I) and from correlations of the optical rotation within the series for the nitrophenyl derivative **4**. A further confirmation of the β-D-anomeric configuration for **4** was obtained from an examination of the ^{13}C-n.m.r. spectrum of the corresponding *O*-deacylated derivative **8**, which showed for C-1 the expected chemical displacement at lower field as compared to *o*-nitrophenyl α-D-glycosides[9]. *N*-Acylation of the aminophenyl derivative **6** with acetic anhydride–pyridine led to the *N*-acetyl derivative **7**, which is of interest as a model ligand since further immobilization of this substrate analog would involve an amide linkage[7]. Sodium methoxide deacylation afforded the aryl 4-thio- and 1,4-dithio-β-disaccharides **8–11** which were used for the interaction studies with xylanases.

Incubation of *o*-nitrophenyl 4-thio-β-xylobioside (**8**) in kinetic experiments with D-xylanases[8] A (pI 4.7) and B (pI 5.5), and monitoring of the reaction by the addition of sodium carbonate, led to the release of the characteristic yellow coloration for the nitrophenolate anion, indicative of the hydrolysis of the aglyconic linkage. Respective K_m values of 2.4 and 6mM were obtained for xylanases A and B, which may be compared with values in g · L^{-1} listed in Table II for these enzymes acting on D-xylan. This derivative may thus be considered as a valuable, well characterized chromogenic substrate for the evaluation of the enzymic activity of xylanases A and B of *S. dimorphosporum*, and hopefully extended to other D-xylanases.

In order to ascertain the use of *p*-aminophenyl 1,4-dithio-β-xylobioside (**10**) as a ligand for the purification of xylanases by affinity chromatography, this compound and the related precursor or derivative **1, 9** were tested in kinetic experiments with these enzymes. Owing to the low solubility in water of *p*-nitrophenyl 1,4-dithio-β-xylobioside (**9**) and, possibly, to an unspecific interaction of the aminophenyl derivative **10** with the enzymes, none of these compounds gave significant and reproducible values. The closely related *N*-acetyl-*p*-aminophenyl 1,4-

TABLE II

KINETIC PARAMETERS FOR THE HYDROLYSIS OF D-XYLAN AND *o*-NITROPHENYL 4-THIO-β-XYLOBIOSIDE (**8**) BY D-XYLANASES A AND B IN THE PRESENCE, OR NOT, OF 4-THIOXYLOBIOSE (**1**) AND *N*-ACETYL-AMINOPHENYL-1,4-DITHIO-β-XYLOBIOSIDE(**11**)

Enzyme	*Effector*	*Substrate*	
		D-*Xylan*	*Compound* **8**
Xylanase (pI 4.7)	None	K_m 2.6 g/L	K_m 2.4mM
	1	K_A 0.18mM	
	11	K_A 0.19 mM	
Xylanase B (pI 5.5)	None	K_m 7.8 g/L	K_m 6mM
	1	K_i 0.72mM	
	11	K_i 2.5mM	

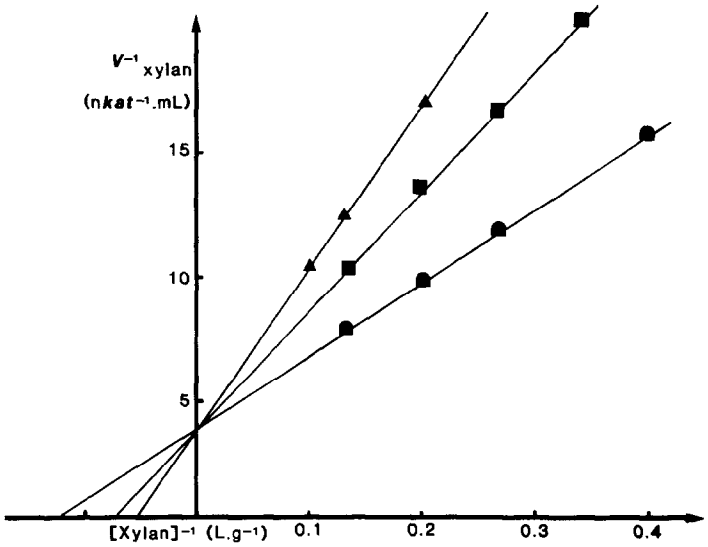


Fig. 1. Effect of various concentrations of *N*-acetyl-*p*-aminophenyl 1,4-dithio-β-xylobioside (**11**) on xylanase B catalytic activity (Lineweaver–Burk presentation): (—●—●—) no addition of **11** (—■—■—) mM **11** added, and (—▲—▲—) 2mM **11** added.

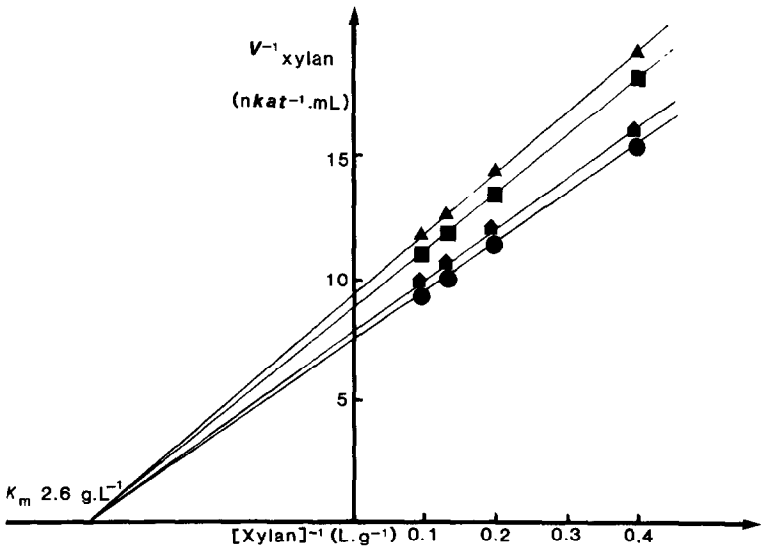


Fig. 2. Effect of various concentrations of *N*-acetyl-*p*-aminophenyl 1,4-dithio-β-xylobioside (**11**) on xylanase A catalytic activity (Lineweaver–Burk presentation): (—▲—▲—) no addition of **11**, (—■—■—) 0.25mM **11** added, (—●—●—) 0.5mM **11** added, and (—●—●—) mM **11** added.

dithio-β-xylobioside derivative **11** exhibited, however, the expected competitive inhibitory effect with xylanase B, when incubated in the presence of xylan. This behavior was also found for 4-thioxylobiose[1] (**1**). From the Lineweaver–Burk plots, respective K_i values of 0.72 and 2.5mM were determined for 4-thioxylobiose (**1**) and the aryl 1,4-dithio-β-xylobioside **11** (as shown on Fig. 1 for **11**).

A different and unexpected pattern was observed when each product analog (**1, 11**) was incubated with xylanase A and xylan. As shown in Fig. 2, an activation effect on the rate of hydrolysis of xylan was observed. This effect may be expressed on the basis that the total rate for an enzymic reaction is the sum of two rates involving (a) the breakdown of the enzyme–substrate complex into products with a rate constant k and (b) the breakdown of the activated enzyme–substrate complex into products with a rate constant k'. Thus the rate v for the present case of "non-essential activation" mechanism[10] may be expressed as shown in Eq. *1*,

$$v = \frac{k \cdot e \cdot (1 + ak'/K_A k)}{(1 + K_m/S) \cdot (1 + a/K_A)} \qquad (1)$$

where a is the activator concentration, K_A the dissociation constant of the activated enzyme–substrate complex, and e and S the respective enzyme and substrate concentrations. In the presence of an excess of substrate or of activator, the rate is supposed to fall within a defined limit which can be expressed respectively as shown in Eqs. *2* and *3*.

$$V_S = \frac{k \cdot e(1 + a/K_A \cdot k'/k)}{1 + a/K_A} \qquad (2) \qquad V_a = \frac{k' \cdot e}{1 + K_m/S} \qquad (3)$$

Equation *4*, which can be derived from Eq. *1*, shows that converging straight lines may be obtained, from experimental data in Fig. 2, by maintaining a constant substrate concentration and plotting $1/v$ *vs.* $1/a$ (Fig. 3).

$$\frac{1}{v} = \frac{1}{V_a} + \frac{K_A}{V_a} \cdot \left(1 - \frac{k}{k'}\right) \cdot \frac{1}{a} \qquad (4)$$

Equation *4* gives K_A at the intercept of the curves on the x-axis in Fig. 3 when the k/k' ratio is known. The k' value is obtained by plotting (Fig. 4) the reciprocal limiting rate against $1/a$ or $1/S$ according to Eqs. *2* or *3*, and the k value can be obtained according to Fig. 2, for an excess of xylan substrate alone, with the same xylanase concentration. A value of K_A may also be obtained from the slope of the reciprocal plot of limiting rate *vs.* concentration for compound **11** (Fig. 4), leading to respective values of 1.8 and 1.9mM for compounds **1** and **11** (Table II).

Xylan hydrolysis by xylanase A in the presence of 4-thioxylobiose (**1**) as well as the corresponding aryl 1-thio-β-glycoside **11** appears, thus, to be under control of an activation mechanism by these product analogs. Such activation is not seen in

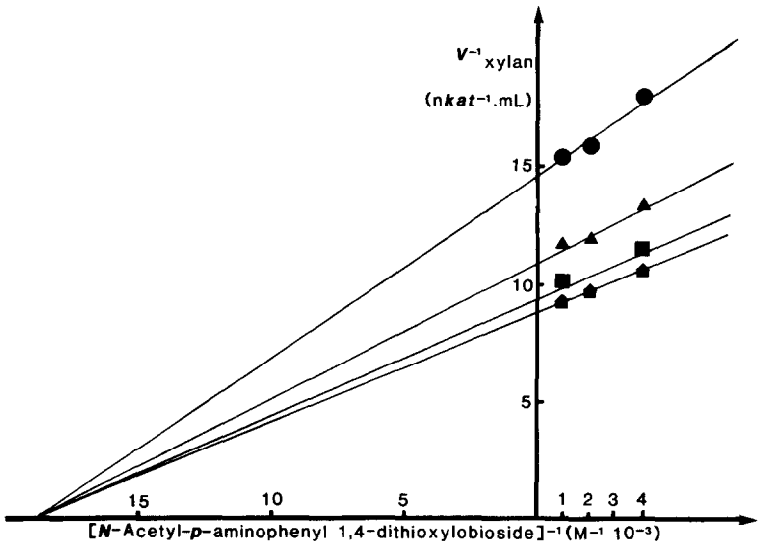


Fig. 3. Hydrolysis of xylan at respective concentrations of: (—●—●—) 2.5 g · L^{-1}, (—▲—▲—) 5 g · L^{-1}, (—■—■—), 7.5 g · L^{-1}, and (—☗—☗—) 10 g · L^{-1} by xylanase A in the presence of various concentrations of *N*-acetyl-*p*-aminophenyl 1,4-dithio-β-xylobioside (**11**) according to Eq. *4*.

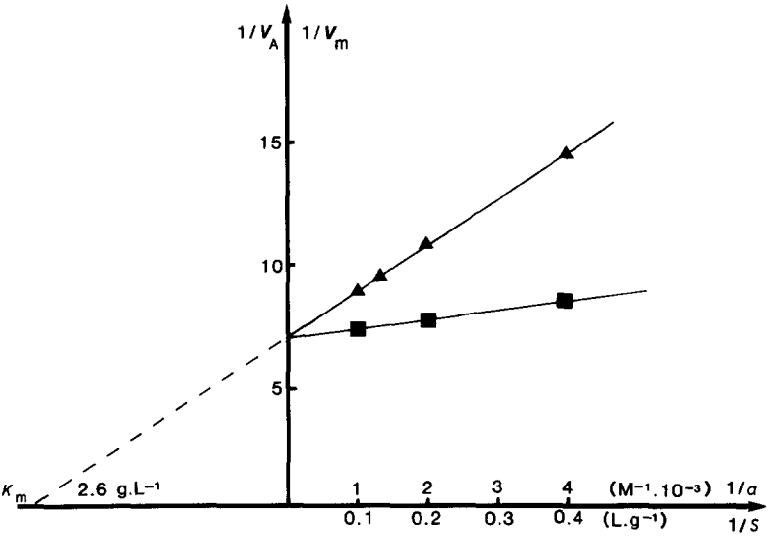


Fig. 4. Relationship between limiting rate hydrolysis of xylan by xylanase A and xylan concentration (—▲—▲—) xyland concentration, and (—■—■—) *N*-acetyl-*p*-aminophenyl 1,4-dithio-β-xylobioside concentration.

the hydrolysis of small oligomeric substrates like **8**. It was, however, previously observed in the presence of xylobiose[8].

As a working hypothesis, it may be assumed that binding of these small product analogs in a regulation site of the enzyme may induce some modification of the protein conformation resulting in an increase in the hydrolysis rate, with respect to the natural substrate, without modification of the affinity.

In view of the interactions exhibited by *N*-acetyl-*p*-aminophenyl 1,4-dithioxylobioside (**11**) with some components of the xylanase system of *S. dimorphosporum*, its precursor *p*-aminophenyl 1,4-dithioxylobioside (**10**) was tested as a ligand for the purification of xylanases by affinity chromatography. Compound **10** was coupled to a carboxylic-Sepharose derivative (CH-Sepharose 4B, Pharmacia) through an amide linkage by the usual activation with carbodiimide. From a partially purified enzyme extract containing amylase, cellulase, and xylanase activities[8], the xylanase component was retained and then eluted by increasing the ionic strength.

Regulation of the biosynthesis of cellulolytic and hemicellulolytic enzymes is known to be dependent on small, water-soluble fragments of the substrate, *i.e.*, oligosaccharides. Cellobiose, on one hand, is an inducer of cellulases in some strains at low concentration[11,12] but, on the other hand, may repress the biosynthesis of cellulases at higher concentrations. One can further infer, from the present results, that some components of the hydrolytic system may have their turnover increased by the presence of oligosaccharides, as was shown by the behavior of xylanase A with the 1,4-dithioxylobioside **11**.

EXPERIMENTAL

General methods. — Optical rotations were determined with a Quick polarimeter (Roussel et Jouan). The ^{1}H-n.m.r. spectra (Table I) were recorded at 250 MHz for solutions in (^{2}H)chloroform and the ^{13}C-n.m.r. spectra at 25.18 MHz for solutions in D_2O. Assignments were confirmed by double irradiation or the INDOR technique. The chemical shifts (δ) are reported relative to the signal of internal $SiMe_4$ and the coupling constants (in parentheses) are in Hz. Solutions were dried (Na_2SO_4) and evaporated under reduced pressure at temperatures below 45°. T.l.c. was performed on silica gel (Merck F_{254}, Merck, Darmstadt, Germany) with solvents indicated. Preparative chromatography used silica gel (Merck 60, 70–230 mesh).

General procedure for the preparation of o-*nitrophenyl 2,3-di*-O-*benzoyl-4*-S-*(2,3,4-tri*-O-*acetyl-β*-D-*xylopyranosyl)-4-thio-β*-D-*xylopyranoside* (**4**), *and* p-*nitrophenyl* (**5**) *and* p-*aminophenyl 2,3-di*-O-*benzoyl-4*-S-*(2,3,4-tri*-O-*acetyl-β*-D-*xylopyranosyl)-1,4-dithio-β*-D-*xylopyranoside* (**6**). — In a typical experiment, to 1,2,3-tri-*O*-benzoyl-4-*S*-(2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl)-4-thio-α-D-xylopyranose[1] (**2**, 1.08 g, 1.4 mmol) in dichloromethane (25 mL) at 0°, was added a solution of HBr in acetic acid (40% w/v, 10 mL). After 1 h at room temperature, the mixture

was washed with water (100 mL), then with a cold, saturated aqueous solution of $NaHCO_3$ (250 mL), and extracted with dichloromethane (150 mL). The resulting sirupy bromide **3** was used without characterization and purification in further steps. To its solution in acetone (25 mL) were added K_2CO_3 and *o*-nitrophenol, *p*-nitrobenzenethiol, or *p*-aminobenzenethiol (5 mmol), and the mixture was kept under N_2 atmosphere at room temperature. After 1 h, it was filtered on Celite and concentrated. The residue was dissolved in dichloromethane (200 mL), washed with ice-cold water (200 mL), ice-cold saturated Na_2CO_3 solution (200 mL), and again water. The resulting products, which all showed a major spot on t.l.c., were purified by column chromatography to yield the pure glycosides **4–6**. Yields, physical constants, eluents, and microanalytical data for these compounds are reported in Table III.

N-*Acetyl*-p-*aminophenyl 2,3-di*-O-*benzoyl-4*-S-*(2,3,4-tri*-O-*acetyl-β*-D-*xylopyranosyl)-1,4-dithio-β*-D-*xylopyranoside* (**7**). — The *p*-aminophenyl 1,4-dithiodisaccharide **6** (220 mg, 0.29 mmol), in 1:1 (v/v) pyridine–acetic anhydride (20 mL), was kept for 12 h at room temperature. The mixture was concentrated to a syrup which was dissolved in dichloromethane and washed with cold water. Concentration of the solution led to a syrup that crystallized from ether (see Table III for yield, physical constant, migration solvents, and microanalytical data).

o-*Nitrophenyl 4*-S-*β*-D-*xylopyranosyl-β*-D-*xylopyranoside (*o-*nitrophenyl β-4-thioxylobioside)* (**8**), *and* p-*nitrophenyl* (**9**), p-*aminophenyl* (**10**), *and* N-*acetyl*-p-*aminophenyl-4*-S-*β*-D-*xylopyranosyl-1,4-dithio-β*-D-*xylopyranoside* (**11**) (p-*nitrophenyl,* p-*aminophenyl, and* N-*acetyl*-p-*aminophenyl 1,4-dithio-β-xylobiosides).* — M Sodium methoxide in methanol (1 mL) was added to a solution of the peracylated aryl 4-thio- and 1,4-dithio-disaccharides (**4** and **5–7**, 1 g) in methanol (15 mL). After being stirred for 24 h, the solution was made neutral with Amberlite IR-120 (H^+) cation-exchange resin. Column chromatography of the oily residues afforded **8–11** in pure form, of which **10** and **11** were obtained as white solid foams after lyophilization of their aqueous solution. The yields, physical constants, eluents, and microanalytical data for **8–11** are reported in Table III; ^{13}C-n.m.r. data for *o*-nitrophenyl 4-thioxylobioside (**8**) in ($^2H_4$)methanol: δ 103.3 (C-1), 86.29 (C-1′), 79.09, 76.03, 75.26, 73.94, 71.03, 70.77, 67.76, and 45.92 (C-4).

Enzymes and enzymic assays. — *Sporotrichum dimorphosporum* D-xylanase A (pI 4.7) and B (pI 5.5) were prepared as described[8]. The enzyme homogeneity was checked by isoelectric focusing and electrophoresis on 15% poly(acrylamide) gel containing 0.1% sodium dodecylsulfate.

Xylanase activity was estimated from the measure of the increment of reducing groups, according to the method of Somogyi[13], on incubation with a borohydride-reduced D-xylan from birch wood[8]. An aqueous xylanase solution (0.1 mL) was mixed with various concentrations of suspended xylan in 0.1M acetate buffer (1.9 mL, pH 5, containing 5mM NaN_3). The mixture was incubated at 30° for periods of time ranging from 4 to 60 min, during which time the production of reducing sugars was linear. The absorbance was read at 500 or 660 nm on the supernatant solution following centrifugation at 27 000*g*.

TABLE III

CHROMATOGRAPHIC SOLVENTS, PHYSICOCHEMICAL, AND ANALYTICAL DATA FOR ARYL 4-THIO-β-XYLOBIOSIDE, 1,4-DITHIO-β-XYLOBIOSIDES (**8–11**) AND ACYLATED DERIVATIVES **4–7**

Compound	*Yield (%)*	*T.l.c. chromatogr. Solv. (v/v)*	*M.p. (degrees) (Solv. crys.)*	$[\alpha]_D^{20}$ *(degrees,c) (Solv.)*	*Anal.*							
					Calc.				*Found*			
					C	*H*	*N*	*S*	*C*	*H*	*N*	*S*
4	90	EtOAc–C_6H_{12} (1:1)		−3.3 (1.2) ($CHCl_3$)	57.37	4.65	1.86	4.25	57.29	4.67	1.90	4.41
5	60	Ether–C_6H_{12} (3:1)	161–164 (Ether)	−8.0 (0.9) ($CHCl_3$)	56.18	4.55	1.82	8.32	56.18	4 65	2.20	9.14
6	78	EtOAc–C_6H_{12} (1:1)	176.5–177.5 (EtOAc–C_6H_{12})	−16.1 (0.62) ($CHCl_3$)	58.45	5.00	1.89	8.66	58.42	5.11	2.29	9.15
7	90	EtOAc–C_6H_{12} (1:1)	217–219 (Ether)	−18.3 (1) ($CHCl_3$)	58.38	4.99	1 79	8.19	58.43	4.88	1.77	8.22
8	90	$CHCl_3$–MeOH–H_2O (65:25:4)	177–179 (MeOH)	−75.9 (0.18) (C_5H_5N)	45.82	5.01	3.34	7.63	46.74	5.11	3.31	7.71
9	90	ditto	159–162 (MeOH)	−8.2 (0.18) (C_5H_5N)	44.14	4.83	3.22	14.71	44 14	4.88	3.18	14.67
10	90	ditto		−6.7 (0.35) (MeOH)	45.39[a]	5.91[a]	3.30[a]	15.10[a]	45.33	5 90	3.18	14.31
11	90	ditto		−60.3 (0.82)	45.56[b]	5 91[b]	2.95[b]	13.50[b]	46.03	5.54	3.07	12.70

[a]For $C_{16}H_{23}O_7NS_2 \cdot H_2O$. [b]For $C_{18}H_{25}O_8NS_2 \cdot 1.5\ H_2O$.

The enzymic hydrolysis of *o*-nitrophenyl 4-thio-β-xylobioside (**8**) was monitored by the measure of the liberation of *o*-nitrophenol from the substrate analog, at different concentrations, in 0.1M acetate buffer (0.4 mL, pH 5, containing 5mM NaN_3), incubated at 30° with an aqueous solution of xylanase (0.1 mL). The reaction was stopped by adding M Na_2CO_3 (0.5 mL) and the absorbance was immediately read at 420 nm. K_m values were obtained from double reciprocal plots of Lineweaver–Burk curves. Enzymic activities in the presence of product analogs **1**, **11** (0.1 mL in 0.1M acetate buffer, pH 5, containing 5mM NaN_3), at final concentrations of 0.25, 0.5, 1.0, and 2.0mM, were determined as just described by use of either a xylan suspension (1.8 mL) or a solution of **8** (0.3 mL). All the given curves are ascribed to product analog **11**; however, the kinetic experiments with **1** gave curves having the same general aspect.

Affinity chromatography of D-*xylanases using Sepharose substituted by the ligand* p-*aminophenyl 1,4-dithioxylobioside* (**10**). — The affinity gel was prepared from compound **10** and CH-Sepharose 4B (Pharmacia, Sweden) following the recommendations of the manufacturer with slight modifications. The commercial, beaded agarose derivative (10 g) was swollen in an aqueous solution of NaCl (0.5M, 100 mL), washed with this solution (1 L), and then with water (1 L). The gel was poured into a solution of compound **10** (430 mg, 1.06 mmol) in water (40 mL), adjusted to pH 4.5 with 0.1M HCl. A solution of 3-(3-dimethylaminopropyl)-1-ethyl carbodiimide (1.2 g) in water (25 mL, pH 4.5) was added dropwise to the gel. The suspension was gently shaken in the dark for 24 h at room temperature, the pH being monitored intermittently and adjusted to 4.5. This was followed by addition of a solution of tris(hydroxymethyl)aminomethane (0.242 g, 2 mmol) in water (100 mL). After 3 h, the gel was poured on a column, and washed with water (1.5 L), and with a 0.1M carbonate buffer (pH 10.5, 300 mL) containing M NaCl. The column was stored in an acetate buffer (0.1M, pH 3.4, 300 mL) containing M NaCl. Prior to use, it was equilibrated with an 10mM acetate buffer (pH 5, 300 mL).

An enzymic extract from *Sporotrichum dimorphosporum*, prepared as described[8], was collected, after the preparative electrofocusing step, in the compartment having an isoelectric point around pI 5.5. It was deposed in water solution (0.5 mL) on the top of the affinity column. The elution was performed with 10mM acetate buffer (pH 5) by increasing stepwise the NaCl concentration (0.05 and 0.2M, 25 mL). Three fractions were obtained. Fraction I, eluted with the initial buffer, contained cellulase and amylase activities. Fractions II and III exhibited exclusively a xylanase activity (xylanase B, pI 5.5, in both fractions).

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