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Lipase-catalysed preparation of acetates of 4-nitrophenyl β-D-xylopyranoside and their use in kinetic studies of acetyl migration

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Abstract—Di-O-acetates and mono-O-acetates of 4-nitrophenyl β-D-xylopyranoside were prepared by use of lipase PS-30. Polarity of organic solvents and reaction time affected the regioselectivity of the di-O-acetylation as well as the yields of monoacetates. The kinetics of acetyl groups migration in these derivatives was studied in aqueous media using HPLC. Migration of the acetyl group strongly depended on pH. The highest rate of acetyl migration was observed from O-2 to O-3 in both 2,4-di-O-acetate and 2-O-acetate. On the contrary, acetyl exchange between O-3 and O-4 in both directions was slower than between O-2 and O-3. The 2,3-di-O-acetate and 4-O-acetate showed to be the most stable towards acetyl migration. The 3,4-di-O-acetate and 4-*O*-acetate were dominant in the corresponding equilibration mixtures.

Keywords: 4-Nitrophenyl β-D-xylopyranoside acetates; Lipase PS-30; Regioselectivity; Acetyl migration kinetics; Acetylesterase assay

1. Introduction

During evolution, nature has created many examples of carbohydrate esters with special distribution of acyl groups between hydroxyls at C-2 and C-3 of saccharide units. Partially O-acetylated plant polysaccharides (e.g., 4-*O*-methylglucuronoxylan,[1] glucomannan,[2] pectic polysaccharides[3]) or 2′(3′)-*O*-aminoacyl derivatives of D-ribose in t-RNA terminal adenosine, important in peptide synthesis,[4] can serve as suitable examples. The interest in systematic study of microbial esterases[5] involved in biodegradation of plant cell walls requires detailed knowledge of their catalytic properties. In this connection, there is a growing demand for a variety of modified[6] or regioselectively acetylated 4-nitrophenyl glycosides that could serve as precursors for chromogenic substrates important for investigation of catalytic properties of these enzymes[7] and for elaboration of simple enzyme assays.

Studies of acetylesterases operating on plant polysaccharides are complicated by the phenomenon of acetyl group migration (AcM) observed in partially acetylated carbohydrates in aqueous media. AcM has also been often observed in partially acetylated products in the process of preparative enzymatic hydrolysis of per-O-acetylated carbohydrates by lipases. Obviously, such phenomenon negatively affects the regioselectivity of the reaction.[8] This AcM can be avoided by application of controlled enzymatic hydrolysis at low pH (4–5),[9] by use of enzymatic alcoholysis in organic solvents[10] or by using bulkier acyl moieties. However, these conditions usually lead to a decrease in the rate of product formation.

The kinetics of irreversible consecutive acetyl migration from secondary to primary hydroxyl group of 2,3,4-tri-O-acetylated methyl α- and β-D-glucopyranoside ($4 \rightarrow 6$ AcM)[11] and 1,2-*O*-isopropylidene-α-D-hexofuranose acetates ($5 \rightarrow 6$ AcM)[12] in neutral condition is well documented. Although examples of reversible *trans* acyl migration were discussed in literature (e.g., $2 \rightarrow 3$ AcM on Bn-4-*O*-Me-β-D-Xyl*p*,[1a] on partially acetylated xylan[1b] or on xylo-oligosaccharides,[1e] acyl migration around the ring on cyclitols[13] or D-glucose derivatives[14])

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the positional, kinetic and thermodynamic aspects of AcM on a pyranoid ring have not been investigated in detail. It is known that AcM is significantly influenced by temperature and pH and that vicinal *trans*-migration in some cases occurs with nearly the same ease as vicinal *cis*-migration.[13–15]

The aim of this work is to present results on preparative transesterification in organic media catalysed by lipase PS-30 (from *Burkholderia cepacia*, Amano, further as LPS-30), leading to three possible di-O-acetates (compounds **1**–**3**) and three mono-O-acetates (compounds **4**–**6**) of 4-nitrophenyl β-D-xylopyranoside (NPh-β-D-Xyl*p*). The availability of acetates **1**–**6**, bearing the 4-nitrophenyl chromophore, led us to investigate the kinetics of AcM between equatorial oxygen atoms along the pyranoid ring in aqueous media. The results show that partially acetylated β-D-xylopyranoside compounds, depending on pH, are prone to vicinal *trans*-AcM in aqueous media. This fact may have an impact on acetylesterase enzymatic studies.

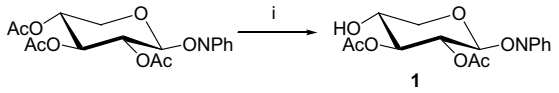
The choice to synthesise compounds **1–6** was supported by the following considerations:

(i) Microbial acetylxylan esterases deacetylate partially acetylated 4-*O*-methyl-D-glucuronoxylan, or its fragments generated upon the action of *endo*-β-(1→4)-xylanases at positions O-2 and O-3 of the β-D-xylopyranosyl residues.[5] Compounds **1**–**6** could serve as model substrates to investigate the specificity of acetylxylan esterases and the role of acetyl group migration in their mechanism of action; (ii) The 4-nitrophenyl aglycon in compounds **1**–**6** could mimic another xylopyranoside unit similarly as in 4-nitrophenyl aglycon-containing substrates of glycosidases and thus partly aid to proper orientation of the substrate in the enzyme binding site. Our recent study of the mode of action of *Streptomyces lividans* acetylxylan esterase indicated that acetylated methyl β-D-xylopyranosides could form productive complexes in two different orientations;[7] (iii) The compounds and their products of deacetylation could be easily monitored and quantified by HPLC using a spectrophotometric detector.

2. Results and discussion

2.1. Lipase-catalysed preparation of NPh-β-D-Xyl*p* acetates 1–6

According to published data for Me-β-D-Xyl*p*,[16] LPS-30 catalyses the deacetylation of per-O-acetylated NPh-β-D-Xyl*p* exclusively at O-4 to give the 2,3-diacetate **1**



Scheme 1. Reagents and conditions: (i) LPS-30, *n*-butanol, toluene, 40 °C, 68%.

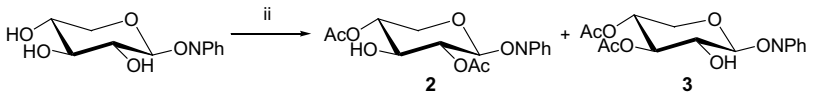
(Scheme 1). The transesterification condition (toluene and *n*-butanol) was used to eliminate migration of the acetyl group along the xylopyranoside ring. The reaction was slow but highly regioselective and **1** was isolated in 68% yield.

Some general trends regarding the regioselectivity of acetylation of D-glycopyranosides at O-2 and O-3 by LPS-30 have already been reported. The regioselectivity can be dependent, for example, on the anomeric configuration of the aglycon[17] or on the relationship between hydrophobicity and polarity of the solvent.[18,19]

The enzyme can also catalyse acetylation of NPh-β-D-Xyl*p*. Based on earlier studies of the acetylation of octyl-β-D-xylopyranoside[18] and phenyl 6-*O*-trityl-β-D-glucopyranoside,[19] we assumed that the hydrophobic character of the 4-nitrophenyl aglycon and the polarity of the solvent could affect the final proportion of diacetates **2** and **3**. Therefore, the effect of organic solvents with different polarity on the regioselectivity of the acetylation of NPh-β-D-Xyl*p* was evaluated with the aim to increase the proportion of the 2,4-diacetate **2**. The course of acetylation of NPh-β-D-Xyl*p* was monitored by HPLC.

In all solvents used, ranging from more to less hydrophobic character, a mixture of 2,4- and 3,4-diacetates (**2** and **3**) (Scheme 2) was produced in high yields but in different ratios. Table 1 shows a clear increase in the formation of the 2,4-diacetate in more hydrophobic solvents. A small amount of water in a hydrophobic solvent such as toluene (solvents 2 and 9) had a positive effect on 3,4-diacetate formation. We did not observe any appreciable production of per-O-acetylated NPh-β-D-Xyl*p*. This observation is in a good agreement with the results reported on Me-β-D-Xyl*p* conversion to the 3,4-diacetate in acetonitrile, and with the results with octyl-β-D-xylopyranoside affording the 2,4-diacetate as the major product of acetylation by LPS-30 in hexane.[18] Toluene was chosen as a solvent to obtain **2** and **3** in preparative scale and 62% of **3** and 25% of **2** were isolated by column chromatography after 72 h of shaking at 40 °C.

No AcM was observed during fractionation of acetylated products by column chromatography on silica



Scheme 2. Reagents and conditions: (ii) LPS-30, vinyl acetate, organic solvent (Table 1), 40 °C.

Table 1. Solvent effects on regioselectivity of the enzymatic acetylation of NPh-β-D-Xyl*p* by LPS-30 at 40 °C

No	Organic solvent	log P_{ow}[a]	Reaction time (h)	Conversion (%)	Ratio (**2**:**3**)
1	Cyclohexane	3.4	193	85	1:1.6
2	Toluene	2.69	75	94	1:2.1
3	Diisopropyl ether	1.9	50	100	1:2.8
4	Methyl isobutyl ketone	1.38	192	88	1:2.9
5	Ethyl acetate	0.73	195	90	1:3.1
6	Vinyl acetate	0.73	75	95	1:3.3
7	*t*-Butanol	0.4	193	89	1:3.8
8	Acetonitrile	−0.33	194	87	1:4.3
9	Toluene[b]		73	85	1:4.8

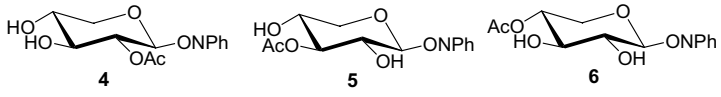
[a] Octanol/water partition coefficient.
[b] Without drying, without MS 4 Å (0.05% water).

gel. Acetyl migration due to contact with silica gel was also excluded by HPLC while monitoring the solutions of diacetates **1**–**3** shaken with silica gel in ethyl acetate (concentration 210 mg/mL) for several days.

Monoacetates **4**, **5** and **6** (Scheme 3) were obtained on acetylation of NPh-β-D-Xyl*p* by LPS-30 in several solvents. The reactions were monitored by HPLC. The composition of the reaction mixtures corresponding to maximum yields of monoacetates is shown in Table 2. The relative percentage of monoacetates showed the tendency of the 2-acetate **4** to be formed preferentially in more hydrophobic solvents whereas the 4-acetate **6** was formed in a greater proportion in more polar solvents (Table 2). Solvent effect was less pronounced in the case of the 3-acetate **5**.

2.2. Kinetic study of acetyl migration

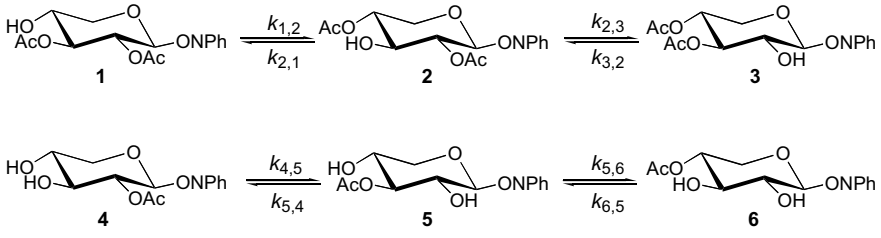
Solutions (1 mM) of diacetates **1**–**3** (pH 5, 6 and 7) and monoacetates **4**–**6** (pH 6) in phosphate buffer (0.1 M, containing 4% Me_2SO) were prepared and the time course of AcM in **1**–**6** was monitored by HPLC over a period of several hours at 40 °C until a thermodynamic equilibrium was reached. The proposed reaction sequence of AcM products is shown in Scheme 4. The 3,4-diacetate **3** was generated from the 2,3-diacetate **1**



Scheme 3.

Table 2. Monoacetylation of NPh-β-D-Xyl*p* by LPS-30 at 40 °C

Organic solvent	Reaction time (h)	Yield of monoacetates (%)	Relative proportion in the mixture (%)		
			4	**5**	**6**
Cyclohexane	153	57	46	54	0
Toluene	5	51	49	51	0
Diisopropyl ether	5.5	38	34	56	10
Methyl isobutyl ketone	50	67	53	38	9
Vinyl acetate	25.5	54	34	51	15
Ethyl acetate	75	54	32	50	18
t-Butanol	45.5	43	34	30	36
Acetonitrile	75	42	27	29	44



Scheme 4.

through the 2,4-diacetate **2** by first-order, reversible and consecutive reactions, and the same is true for the reverse reaction. On the other hand the 2,4-diacetate **2** produced **1** and **3** by two parallel, reversible and first-order AcM reactions. A similar reaction scheme occurred with monoacetates **4**, **5** and **6**. These processes could be described by eight rate constants indicated in Scheme 4.

The corresponding first-order rate constants were computed from exponential dependences between time and molar concentration of the starting compounds and migration products using equations applicable for the given kinetic systems.[20] Based on the average values of calculated four rate constants important in each case, the quantitative prediction of three migration products could be illustrated at any reaction time. The amounts of products of spontaneous hydrolysis (competitive reaction to AcM) close to thermodynamic equilibriums were small (about 10%) and therefore neglected. Figures 1 and 2 give examples of time course of AcM on diacetates **1**–**3** and monoacetates **4**–**6** at pH 6, respectively. The experimental points fit well the curves generated from average rate constants.

According to the rate constants calculated from measurements at pH 5, 6 and 7 at 40 °C (summarised in Table 3), AcM strongly depends on pH. The highest rate of AcM ($k_{2,3}$, $k_{4,5}$) at pH 6 was observed from O-2 to O-3 in diacetate **2** and in monoacetate **4**. Generally, the acetyl exchange between O-2 and O-3 in both directions is easier than between O-3 and O-4 (Table 3).

Tsuda and Yoshimoto[14] explained a similar phenomenon occurring in partially acylated methyl α-D-glucopyranoside by a smaller dihedral angle between O-2 and O-3 as compared to the angle between O-3 and O-4 of the glucopyranose ring in $^4C_1$ conformation and also by the smaller value of the torsion energy of a five-membered orthoacid intermediate between the neighbouring OH-groups. Based on crystallographic data for NPh-β-D-Xyl*p* in $^4C_1$ conformation,[21] it appears that the torsion angles between O-2 and O-3 or O-3 and O-4 are

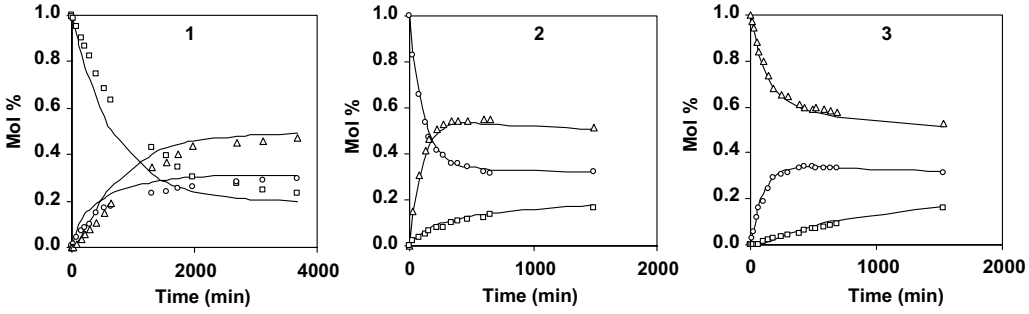


Figure 1. Interconversion of diacetates **1**, **2** and **3** at pH 6 as a function of time. The experimental points are fitted by curves generated using the rate constants $k_{1,2}$, $k_{2,1}$, $k_{2,3}$ and $k_{3,2}$ (Table 3). Symbols: □, **1**; ○, **2**; △, **3**.

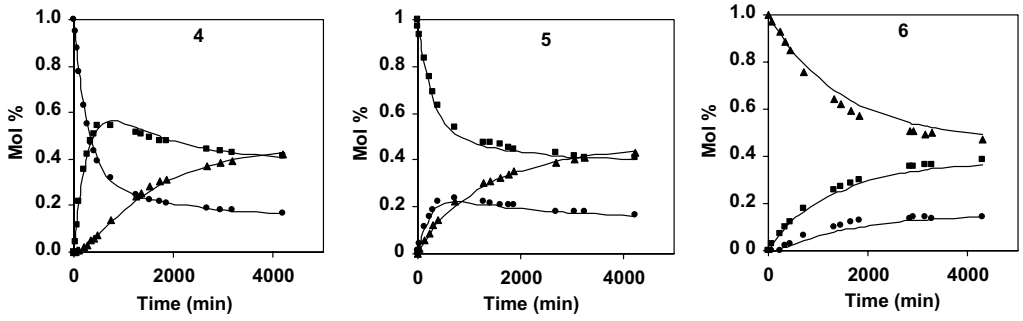


Figure 2. Interconversion of monoacetates **4**, **5** and **6** at pH 6 as a function of time. The experimental points are fitted by curves generated using the rate constants $k_{4,5}$, $k_{5,4}$, $k_{5,6}$ and $k_{6,5}$ (Table 3). Symbols: ●, **4**; ■, **5**; ▲, **6**.

Table 3. Average values of calculated rate constants (10^5 min^{-1}) shown in reaction in Scheme 4

pH	$k_{1,2}$	$k_{2,1}$	$k_{2,3}$	$k_{3,2}$	$k_{4,5}$	$k_{5,4}$	$k_{5,6}$	$k_{6,5}$
5	6.8	5.5	45.6	28.3				
6	122.4	75.8	529.2	336.1	266.5	105.9	45.4	37.8
7	1172.4	670.2	4657.9	2777.4				

similar (72.0° or 73.4°, respectively). Consequently, one may assume that the higher rate of AcM from O-2 to O-3 is due to a steric repulsive effect of the aglycon. On the other hand, a backward process could take place due to a higher reactivity of O-2 resulting from the inductive effect of the anomeric centre.

Derivatives **1** and **6** proved to be the most stable towards AcM. The corresponding rate constant $k_{2,1}$ and $k_{6,5}$ were depressed by one order of magnitude. As can be seen in Figures 1 and 2, the acetyl group at O-4 of NPh-β-D-Xyl*p* appears thermodynamically more stable because the 3,4-diacetate **3** and the 4-acetate **6** prevail in the equilibrium mixtures. On the other hand, the 2,3-diacetate **1** and 2-acetate **4** were found at equilibration in the lowest concentrations (Table 4). The final ratio of acetylated NPh-β-D-Xyl*p*-s was not significantly influenced by pH.

The values of rate constants determined at pH 6 (Table 3) also show that the AcM from between two selected position in diacetates is approximately 2–3 times faster than in monoacetates. This can be a consequence of steric and electronic factors associated with the presence of the second acetyl group on the β-xylopyranoside ring.

We are planning to use the prepared monoacetates and diacetates of NPh-β-D-Xyl*p* as new acetylxylan esterase substrates to establish the positional specificity of this extremely variable group of carbohydrate esterases and thus contribute to their classification. The information on the rate of AcM in individual compounds dissolved in buffers suitable for the enzyme assays is of considerable importance for the development of the assays. It is to be expected from our results that length in substrate solution preparation and incubation with enzymes may result in interconversion of the substrates due to AcM. Therefore, the ratios of all three mono- and diacetates of NPh-β-D-Xyl*p* formed from individual compounds during a relatively short time at three different pH values at 40 °C have been calculated and are presented in Tables 5 and 6.

Data in Tables 5 and 6 show clearly that, with regard to regioselectivity, the acetylxylan esterase assays should be as short as possible so the transformation of substrates due to AcM could be neglected. Stock substrate solutions will have to be prepared in solvents in which AcM does not proceed. Such solvent could be, for example, Me_2SO.

Table 4. Ratio of acetylated NPh-β-D-Xyl*p*-s at thermodynamic equilibrium at 40 °C

pH	Ratio of diacetates **1**:**2**:**3**	Ratio of monoacetates **4**:**5**:**6**
5	24:29:47	
6	20:31:49	15:39:46
7	18:31:51	

Table 5. Ratio of positional isomers of diacetates of NPh-β-D-Xyl*p* in 1 mM solutions of compounds **1**–**3** incubated in 0.1 M sodium phosphate buffer (pH 5.0–7.0) at 40 °C

Time of incubation (min)	pH	AcM products from **1**		AcM products from **2**		AcM products from **3**	
		2 (%)	**3** (%)	**1** (%)	**3** (%)	**1** (%)	**2** (%)
10		0.07	0.0002	0.06	0.45	0.00009	0.28
30	5	0.20	0.001	0.16	1.35	0.0007	0.84
60		0.40	0.006	0.32	2.67	0.003	1.66
120		0.79	0.02	0.64	5.22	0.01	3.24
10		1.18	0.03	0.73	5.05	0.01	3.21
30	6	3.30	0.26	2.05	13.83	0.10	8.78
60		5.99	0.95	3.71	24.24	0.37	15.39
120		10.04	3.14	6.22	37.92	1.23	24.08
10		8.72	2.03	4.99	31.86	0.69	19.00
30	7	16.81	10.99	9.61	51.72	3.75	30.84
60		22.02	23.81	12.59	54.89	8.12	32.73
120		26.88	39.07	15.37	53.32	13.32	31.79

Table 6. Ratio of positional isomers of monoacetates of NPh-β-D-Xyl*p* in 1 mM solutions of compounds **4**–**6** incubated in 0.1 M sodium phosphate buffer (pH 6.0) at 40 °C

Time of incubation (min)	AcM products from **4**		AcM products from **5**		AcM products from **6**	
	5 (%)	**6** (%)	**4** (%)	**6** (%)	**4** (%)	**5** (%)
10	2.61	0.006	1.04	0.45	0.002	0.38
30	7.51	0.05	2.99	1.33	0.02	1.10
60	14.14	0.20	5.62	2.58	0.07	2.15
120	25.09	0.73	9.97	4.90	0.24	4.08

3. Conclusion

All three possible diacetates **1**–**3** and monoacetates **4**–**6** were prepared from NPh-β-D-Xyl*p* by the catalytic action of LPS-30. Both mono- and diacetates of NPh-β-D-Xyl*p* will be used as substrates for classification of acetylxylan esterases on the basis of positional specificity. The diacetates will also serve as precursors for the synthesis of chromogenic substrates for hemicellulolytic hydrolases and esterases such as α-glucuronidase and feruloyl esterase. The reversible *trans*-migrations of the acetyl group along the flexible β-D-xylopyranoside ring in buffered aqueous media were characterised by rate constants. The results are important for studies of the substrate structure requirements, regioselectivity and mechanism of action of various carbohydrate esterases.

4. Experimental

4.1. General methods

LPS-30 was a kind gift from Amano (Japan). NPh-β-D-Xyl*p* was purchased from Lachema (Brno, Czech Republic). Per-O-acetylated 4-nitrophenyl β-D-xylopyranoside was prepared by conventional acetylation using Ac_2O in pyridine and after isolation crystallised from ethanol. Solvents were distilled from the appropriate drying agents before use. All reactions were monitored by TLC on Silica Gel 60 (0.25 mm, E. Merck, Darmstadt, Germany). Compounds were detected with 1% orcinol in 10% (v/v) EtOH soln of H_2SO_4 at ca. 200 °C. HPLC chromatographic analysis was carried out on a chromatograph equipped with a silica gel column (Biospher Si 100 5 μm). Each sample was injected through a 20 μL loop and the column was eluted with hexane–EtOAc at a rate of 1.0 mL/min. The elution of acetates of NPh-β-D-Xyl*p* was monitored with a calibrated UV detector at 305 nm. Column chromatography was performed on Silica Gel 60 (100–160 μm) in various solvent systems. Melting points were determined on a Kofler hot-stage and are uncorrected. Optical rotations were measured with a Perkin–Elmer 241 polarimeter at 20 °C. ^{1}H NMR spectra were recorded at 300 MHz with Bruker AM 300 (Me_4Si as internal standard). Homonuclear correlation with two-dimensional technique COSY-45 was used for complete assignment of protons. ^{13}C NMR spectra were recorded in $CDCl_3$ at 75 MHz and shifts are referenced to internal $CDCl_3$. Microanalyses were performed with a Fisons EA 1108 analyser.

4.2. 4-Nitrophenyl 2,3-di-*O*-acetyl-β-D-xylopyranoside (1)

4-Nitrophenyl 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside (1 g, 2.5 mmol) was dissolved in toluene (100 mL), then *n*-BuOH (0.8 mL) was added followed by addition of LPS-30 (5 g). The reaction mixture was shaken for 7 days at 40 °C and 200 rpm. The reaction was stopped by filtrating off the lipase. The solvent was eliminated under diminished pressure and the product was separated from the starting compound by column chromatography (1:1 toluene–EtOAc) to give **1** as a white solid (0.60 g, 68%): mp 135–136 °C (EtOAc–diisopropyl ether); $[\alpha]_D^{20}$ −49.8° (*c* 1.0, $CHCl_3$), lit.[22] 158–159 °C (EtOAc–hexane). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.22 (d, 2H, *J* 9.2 Hz, H-3′, H-5′), 7.09 (d, 2H, *J* 9.2 Hz, H-2′, H-6′), 5.29 (d, 1H, $J_{1,2}$ 5.7 Hz, H-1), 5.20 (dd, 1H, $J_{1,2}$ 5.7 and $J_{2,3}$ 7.5 Hz, H-2), 5.02 (t, 1H, $J_{3,4}$ 7.2 and $J_{2,3}$ 7.3 Hz, H-3), 4.18 (dd, 1H, $J_{4,5'}$ 4.3 and $J_{5,5'}$ 12.0 Hz, H-5′), 3.90 (dt, 1H, $J_{4,5'}$ 4.4, $J_{3,4}$ 7.0 and $J_{4,5}$ 7.1 Hz, H-4), 3.60 (dd, 1H, $J_{4,5}$ 7.2 and $J_{5,5'}$ 12.0 Hz, H-5), 2.16 (s, 3H, $COCH_3$), 2.11 (s, 3H, $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.0 ($COCH_3$), 169.2 ($COCH_3$), 161.1, 143.0, 2×125.8, 2×116.5 (C–Ar), 97.6 (C-1), 73.7 (C-3), 69.4 (C-2), 67.7 (C-4), 64.5 (C-5), 20.8 ($COCH_3$), 20.7 ($COCH_3$). Anal. Calcd for $C_{15}H_{17}NO_9$: C, 50.71; H, 4.82; N, 3.94. Found: C, 50.42; H, 5.16; N, 3.69.

4.3. The HPLC course monitoring of mono-O-acetylation and subsequent di-O-acetylation of NPh-β-D-Xyl*p* in different organic solvents

NPh-β-D-Xyl*p* (27.1 mg, 0.1 mmol) was dissolved in a selected organic solvent (954 μL), then MS 4 Å (250 mg), vinyl acetate (46 μL, 5 equiv) and 270 mg LPS-30 were added. The reaction mixtures were shaken at 40 °C at 200 rpm. Aliquots (20 μL) were withdrawn at different time intervals and filtered (Durapore filter, 0.45 μm). The filtered solution was analysed by HPLC. The elution times in 2:3 hexane–EtOAc, were 3.8 min for NPh-2,3,4-tri-*O*-Ac-β-D-Xyl*p*, 4.3 min for **3**, 5.6 min for **1**, 7.8 min for **2**, 10.2 min for **5**, 17.5 min for **6**, 20.5 min for **4** and 48.9 min for NPh-β-D-Xyl*p*. The amount of each acetate was calculated from the integration of chromatogram peak areas.

4.4. Preparative di-O-acetylation of 4-nitrophenyl β-D-xylopyranoside catalysed by LPS-30

NPh-β-D-Xyl*p* (1 g, 3.7 mmol) was suspended in toluene (300 mL). Molecular sieves (4 Å, 15 g), vinyl acetate (3.4 mL, 37 mmol, 10 equiv) and LPS-30 (9 g) were added. The reaction mixture was shaken at 40 °C and 200 rpm for 72 h. The reaction was stopped by filtrating off the lipase. The filtrate was concentrated and the residue fractionated by column chromatography (2:1 toluene–EtOAc) to give **3** as first eluted and **2** as next eluted compound.

4.4.1. 4-Nitrophenyl 2,4-di-*O*-acetyl-β-D-xylopyranoside (2). White solid (0.33 g, 25%), mp 153–155 °C (toluene), $[\alpha]_D^{20}$ −66.2° (*c* 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.21 (dt, 2H, *J* 2.1, 3.3 and 9.2 Hz, H-3′, H-5′), 7.10 (dt,

2H, *J* 2.1, 3.3 and 9.2 Hz, H-2′, H-6′), 5.30 (d, 1H, $J_{1,2}$ 5.7 Hz, H-1), 5.14 (dd, 1H, $J_{1,2}$ 5.7 and $J_{2,4}$ 7.8 Hz, H-2), 4.92 (ddt, 1H, $J_{4,5'}$ 4.4, $J_{4,5}$ 6.8 and $J_{3,4}$ 6.8 Hz, H-4), 4.23 (dd, 1H, $J_{4,5'}$ 4.4 and $J_{5,5'}$ 12.3 Hz, H-5′), 3.93 (m, 1H, H-3), 3.59 (dd, 1H, $J_{4,5}$ 6.6 and $J_{5,5'}$ 12.3 Hz, H-5), 2.82 (d, 1H, $J_{3,OH}$ 5.4 Hz, OH), 2.15 (s, 6H, 2×$COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.7 (*C*OCH_3), 170.1 (*C*OCH_3), 161.1, 143.1, 2×125.8, 2×116.6 (C–Ar), 97.9 (C-1), 71.9, 71.3, 70.9 (C-2, C-3, C-4), 64.6 (C-5), 20.9 (2 × CO*C*H_3). Anal. Calcd for $C_{15}H_{17}NO_9$: C, 50.71; H, 4.82, N, 3.94. Found: C, 50.61; H, 5.03; N, 3.83.

4.4.2. 4-Nitrophenyl 3,4-di-*O*-acetyl-β-D-xylopyranoside (3). White solid (0.81 g, 62%), mp 151–153 °C (EtOH), $[\alpha]_D^{20}$ −83.0° (*c* 1.0, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$): δ 8.22 (d, 2H, *J* 9.1 Hz, H-3′, H-5′), 7.11 (d, 2H, *J* 9.1 Hz, H-2′, H-6′), 5.23 (d, 1H, $J_{1,2}$ 5.8 Hz, H-1), 5.16 (t, 1H, $J_{2,3}$ 7.7 and $J_{3,4}$ 7.7 Hz, H-3), 5.01 (ddt, 1H, $J_{4,5'}$ 4.6, $J_{4,5}$ 7.3 and $J_{3,4}$ 7.4 Hz, H-4), 4.18 (dd, 1H, $J_{4,5'}$ 4.5 and $J_{5,5'}$ 12.1 Hz, H-5′), 3.90 (br dt, 1H, $J_{2,OH}$ 5.8, $J_{1,2}$ 5.9 and $J_{2,3}$ 7.3 Hz, H-2), 3.57 (dd, 1H, $J_{4,5}$ 7.4 and $J_{5,5'}$ 12.1 Hz, H-5), 2.79 (d, 1H, $J_{2,OH}$ 5.8 Hz, OH), 2.16 (s, 3H, $COCH_3$), 2.11 (s, 3 H, $COCH_3$). ^{13}C NMR (75 MHz $CDCl_3$): δ 170.6 (*C*OCH_3), 169.7 (*C*OCH_3), 161.2, 143.0, 2×125.8, 2×116.5 (C–Ar), 99.9 (C-1), 72.2 (C-3), 70.3 (C-2), 68.4 (C-4), 62.0 (C-5), 20.9 (CO*C*H_3), 20.8 (CO*C*H_3). Anal. Calcd for $C_{15}H_{17}NO_9$: C, 50.71; H, 4.82, N, 3.94. Found: C, 50.64; H, 4.89; N, 3.89.

4.5. Preparative mono-O-acetylation of 4-nitrophenyl β-D-xylopyranoside catalysed by LPS-30

Vinyl acetate (1.7 mL, 0.0185 mmol, 5 equiv), LPS-30 (5 g) and 4 Å molecular sieves (8 g) were added to a soln of NPh-β-D-Xyl*p* (1 g, 3.7 mmol) in *t*-BuOH (200 mL). The reaction mixture was shaken at 40 °C and 200 rpm for 40 h. The reaction was stopped by filtrating off lipase. The filtrate was concentrated and the residue was fractionated by column chromatography (2:1 → 1:1 toluene–EtOAc) to give first diacetates **2** and **3** (0.29 g, 22%), then monoacetate **5**. The second eluted monoacetate was **4** and the last eluted was **6**.

4.5.1. 4-Nitrophenyl 2-*O*-acetyl-β-D-xylopyranoside (4). White solid (0.22 g, 19%), mp 178–180 °C (EtOAc–toluene), $[\alpha]_D^{20}$ −7.9° (*c* 1.0, MeOH). ^1H NMR (300 MHz, $CDCl_3$): δ 8.21 (dt, 2H, *J* 2.1, 3.2 and 9.2 Hz, H-3′, H-5′), 7.10 (dt, 2H, *J* 2.1, 3.2 and 9.2 Hz, H-2′, H-6′), 5.21 (d, 1H, $J_{1,2}$ 6.2 Hz, H-1), 5.05 (dd, 1H, $J_{1,2}$ 6.3 and $J_{2,3}$ 7.9 Hz, H-2), 4.16 (dd, 1H, $J_{4,5'}$ 4.5 and $J_{5,5'}$ 11.8 Hz, H-5′), 3.85 (dt, $J_{4,5'}$ 4.5, $J_{3,4}$ 7.8 and $J_{4,5}$ 8.0 Hz, 1H, H-4), 3.75 (t, 1H, $J_{3,4}$ 7.8 and $J_{2,3}$ 7.7 Hz, H-3), 3.52 (dd, 1H, $J_{4,5}$ 8.2 and $J_{5,5'}$ 11.8 Hz, H-5), 2.16 (s, 3H, $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5 (*C*OCH_3), 161.1, 143.1, 2×125.8, 2×116.5 (C–Ar), 98.2 (C-1), 74.0 (C-2), 72.7 (C-3), 69.6 (C-4), 64.6 (C-5), 20.9 (CO*C*H_3), Anal. Calcd for $C_{13}H_{15}NO_8$: C, 49.84; H, 4.83, N, 4.47. Found: C, 49.65; H, 5.02; N, 4.29.

4.5.2. 4-Nitrophenyl 3-*O*-acetyl-β-D-xylopyranoside (5). White solid (0.19 g, 16%), mp 160–162 °C (CH_2Cl_2–cyclohexane), $[\alpha]_D^{20}$ −36.1° (*c* 1.0, MeOH). ^1H NMR (300 MHz, $CDCl_3$): δ 8.21 (dt, 2H, *J* 2.1, 3.3 and 9.3 Hz, H-3′, H-5′), 7.12 (dt, 2H, *J* 2.1, 3.3 and 9.3 Hz, H-2′, H-6′), 5.26 (d, 1H, $J_{1,2}$ 5.4 Hz, H-1), 4.94 (t, 1H, $J_{3,4}$ 7.1 and $J_{2,3}$ 7.1 Hz, H-3), 4.14 (dd, 1H, $J_{4,5'}$ 4.1 and $J_{5,5'}$ 12.1 Hz, H-5′), 3.86–3.93 (m, 2H, H-2, H-4), 3.58 (dd, 1H, $J_{4,5}$ 7.1 and $J_{5,5'}$ 12.1 Hz, H-5), 2.92 (d, 1H, *J* 5.5 Hz, OH), 2.80 (d, 1H, *J* 5.1 Hz, OH), 2.22 (s, 3H, $COCH_3$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.9 (*C*OCH_3), 161.3, 142.9, 2×125.9, 2×116.5 (C–Ar), 99.7 (C-1), 75.3 (C-3), 69.7, 67.9 (C-2, C-4), 64.4 (C-5), 21.0 (CO*C*H_3). Anal. Calcd for $C_{13}H_{15}NO_8$: C, 49.84; H, 4.83, N, 4.47. Found: C, 49.91; H, 4.98; N, 4.37.

4.5.3. 4-Nitrophenyl 4-*O*-acetyl-β-D-xylopyranoside (6). White solid (0.21 g, 18%), mp 142–143 °C (EtOAc–diisopropyl ether), $[\alpha]_D^{20}$ −83.7° (*c* 1.0, MeOH). ^1H NMR (300 MHz, $CDCl_3$): δ 8.21 (dt, 2H, *J* 2.1, 3.3 and 9.3 Hz, H-3′, H-5′), 7.13 (dt, 2H, *J* 2.1, 3.3 and 9.3 Hz, H-2′, H-6′), 5.16 (d, 1H, $J_{1,2}$ 6.8 Hz, H-4), 4.90 (dt, 1H, $J_{4,5'}$ 4.7, $J_{3,4}$ 7.7 and $J_{4,5}$ 7.5 Hz, H-4), 4.19 (dd, 1H, $J_{4,5'}$ 4.7 and $J_{5,5'}$ 12.2 Hz, H-5′), 3.82–3.88 (m, 2H, H-2, H-3), 3.54 (dd, 1H, $J_{4,5}$ 7.4 and $J_{5,5'}$ 12.2 Hz, H-5), 2.16 (s, 3H, $COCH_3$). ^{13}C NMR ($CDCl_3$): δ 170.7 (*C*OCH_3), 161.3, 143.0, 2×125.8, 2×116.6 (C–Ar), 100.1 (C-1), 72.8 (C-4), 72.5, 71.7 (C-2, C-3), 62.4 (C-5), 20.9 (CO*C*H_3). Anal. Calcd for $C_{13}H_{15}NO_8$: C, 49.84; H, 4.83, N, 4.47. Found: C, 49.69; H, 5.14; N, 4.31.

4.6. The HPLC kinetic study of acetyl migration on NPh-β-D-Xyl*p* acetates in phosphate buffer

Acetates **1**–**6** (0.002 mmol) were dissolved in Me_2SO (80 μL) and mixed with 0.1 M sodium phosphate buffer (1920 μL) of appropriate pH and preheated to 40 °C. The reaction mixtures were incubated at 40 °C. Aliquots (50 μL) were withdrawn at time intervals and directly injected to the HPLC system using a silica gel column eluted with 1:2 hexane–EtOAc. Elution times were 3.6 min for **3**, 4.3 min for **1**, 5.6 min for **2**, 6.6 min for **5**, 11.4 min for **6**, 14.6 min for **4** and 30.8 min for NPh-β-D-Xyl*p*. The data obtained by integration of the peak areas were used to calculate the rate constants of AcM.

4.7. Test for AcM in the presence of silica gel

Samples of diacetates **1**–**3** (0.7 mg) dissolved in EtOAc (2 mL) were shaken at 30 °C in the presence of 210 mg silica gel (Lachema, 100–160 μm). No traces of AcM products were observed in the filtrates of the mixtures within 3 days when monitored by HPLC.

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