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Regioselective enzymatic hydrolysis of acetylated pyranoses and pyranosides using immobilised lipases. An easy chemoenzymatic synthesis of α- and β-D-glucopyranose acetates bearing a free secondary C-4 hydroxyl group

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Abstract

Protected sugars with only one free hydroxyl group are useful building blocks for the synthesis of a large number of glycoderivatives. In order to avoid the problems of the classical chemical synthesis, we studied the regioselective enzymatic hydrolysis of different fully acetylated glycopyranoses and glycopyranosides. The main challenge was to obtain the hydrolysis of the substrates in only one position, with high regioselectivity, while avoiding any further hydrolysis towards partially acetylated sugars. *Candida rugosa* (CRL) and *Pseudomonas fluorescens* (PFL) lipases (EC 3.1.1.3) immobilised on octyl agarose afforded regioselective hydrolysis only in the 6- and 1-positions, respectively. Furthermore, a new one-pot chemoenzymatic approach has been developed in order to obtain α- and β-protected glucopyranoses bearing a free secondary C-4 hydroxyl group. For instance, 1,2,3,6-tetra-*O*-acetyl-α-D-glucopyranose was easily synthesised in good overall yield (70%) starting from 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose by regioselective enzymatic hydrolysis in the 6-position, catalysed by CRL, followed by a temperature- and pH-controlled acyl migration.

Keywords: Regioselective hydrolysis; Acetylated pyranosides; Lipase; Acetyl group migration

1. Introduction

Pure regioisomers of *O*-acetyl pyranosides bearing only one free hydroxyl group (AP) may be used as key intermediates for the synthesis of a large number of glycoderivatives (oligosaccharides, sugar esters, glycopeptides, etc.).[1,2] AP intermediates can be readily and selectively modified at their free hydroxyl group and are soluble in most organic solvents. Moreover, the protected final products can be easily deacetylated by very mild chemical or enzymatic processes. However, the preparation of APs with only one free hydroxyl group requires by classical chemical synthesis multistep reactions that may pose environmental problems for large-scale production, due to the use of organic solvents and toxic reagents.

For example, 1,2,3,6-tetra-*O*-acetyl-α-D-glucopyranose and related compounds, may be of particular interest due to the presence of (1→4)-glycosidic bonds in natural oligosaccharides and in those of pharmaceutical interest. However, only few papers describing suitable chemical syntheses of APs have been published.[3,4] The 1,2,3,4,6-penta-*O*-acetyl-α-D-pyranose (**1**) could economically be used in regioselective deacetylation for large-scale preparation of APs. Although a large number of procedures has been reported, suitable processes for the chemical hydrolysis of **1** only afford the deacetylation at the anomeric position.[5] An alternative approach for obtaining protected sugars with only one free secondary hydroxyl group, including the 4-position, employs dispiroketals for regioselective protection of vicinal diols.[6]

The complexity of the classical chemical approaches for regioselective protection/deprotection of sugars

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makes the use of enzymatic catalysts such as lipases or esterases an attractive and important tool for AP preparation. However, enzymatic deacylations of fully acylated pyranosides described in literature have some drawbacks.[2,7] In most cases the reactions are very slow or proceed with poor selectivity and yield. In addition these reactions often afford complex mixtures of tetra-, tri-, di-, and monoacetates, along with free monosaccharides such as glucose.

Some lipase-catalysed hydrolyses of fully acylated pyranosides have been reported to afford deacetylation at the anomeric or C-6 positions[8,9] in good yields. Regioselective hydrolysis at the primary position can be routinely obtained by protection of the C-1 hydroxyl group as the alkyl glycoside. The hydrolysis of sugar esters in positions other than the anomeric or the C-6 position with good regioselectivity and yield has never been reported. Furthermore, fully acetylated sugars are usually poor substrates when soluble lipases are used, whereas pentanoyl or longer chain esters result in improved activity.[8]

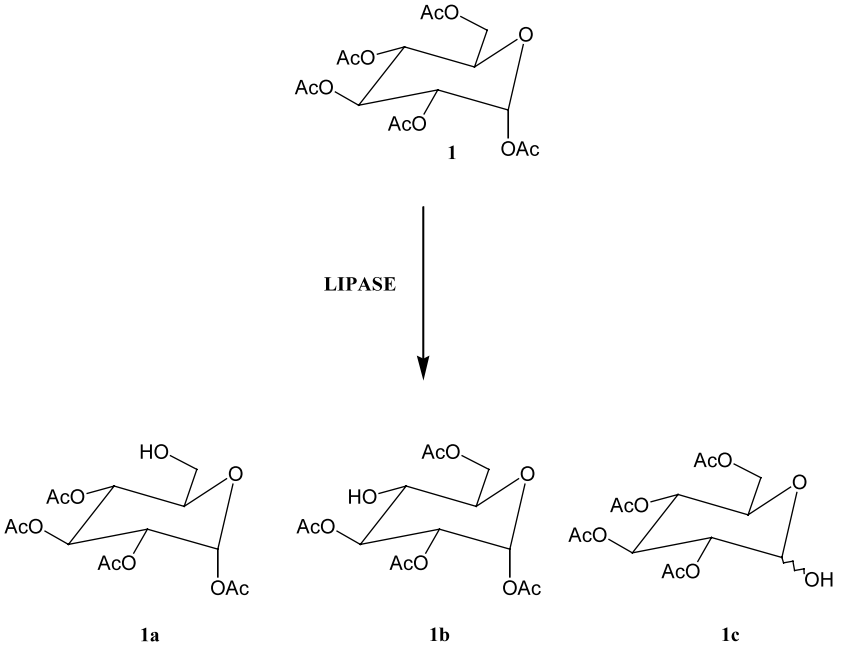
In a previous communication[10] we have reported that *Candida rugosa* (CRL) and *Pseudomonas fluorescens* (PFL) afford APs in good yield by hydrolysis of **1**. The main problem was to design a very active catalyst able to perform the selective hydrolysis of acetylated pyranoses in only one position. New lipase derivatives prepared by hydrophobic absorption on octyl agarose gel[11] have been used as catalysts. These immobilised lipases display good activity and regioselectivity in the hydrolyses performed in aqueous homogeneous media.[10]

In the present work we have extended the previous studies in order to obtain regioisomers of APs bearing only one free hydroxyl group in different position. We report the results obtained studying the regioselective enzymatic hydrolysis of different acetylated pyranosides, catalysed by CRL and PFL immobilised on octyl agarose. In particular, biocatalytic processes suitable for obtaining different regioisomers of AP in high yields that avoid any further hydrolyses towards partially acetylated or free sugars are described. Furthermore, a new, efficient chemoenzymatic process for the easy preparation of protected α- and β-D-glucopyranoses bearing a free secondary C-4 hydroxyl group is proposed.

2. Results and discussion

Enzymatic hydrolysis of 1,2,3,4,6-penta-O-*acetyl*-α-D-*glucopyranose (**1**).*—In the hydrolysis of **1** catalysed by lipases isolated from CRL and PFL, at acid pH, the regioselective hydrolysis only in the 6- and 1-positions, respectively, is obtained affording (Scheme 1) the corresponding tetra acetyl glucopyranoses (AP): 1,2,3,4-tetra-*O*-acetyl-α-D-glucopyranose (**1a**) and 2,3,4,6-tetra-*O*-acetyl-α,β-D-glucopyranose (**1c**).

In order to clarify the mechanism of the enzymatic hydrolysis of these substrates, we have considered the effect of different reaction conditions on the regioselectivity and yields. The influence of pH has been found to play a pivotal role in hydrolysis selectivity. In fact, as reported in Table 1, using CRL, as the pH increases,

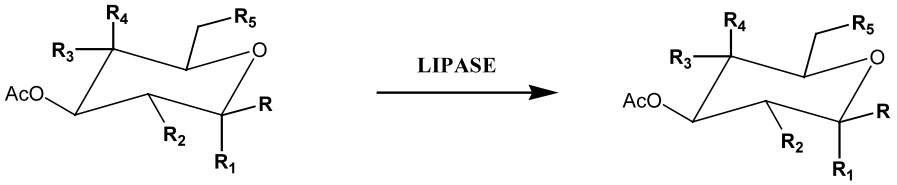


Scheme 1.

Table 1
Hydrolysis of α-D-glucose pentaacetate catalysed by CRL and PFL at different pH[a]

Lipase	pH	TAG % yield (*t*, h)	**1a**	**1b**	**1c**	Other TAG
CRL	4.00	90 (2 h)	100	–	–	–
CRL	5.00	75 (1.5 h)	98.3	1.7	–	–
CRL	6.00	60 (1.5 h)	94.9	2.8	–	2.3
CRL	7.00	57 (1 h)	28	70	–	2
CRL	8.00	46 (1 h)	21.7	61	–	17.3
CRL	9.00	34 (4 h)	40	28	–	32
PFL	5.00	86 (4.5 h)	–	–	100	–
PFL	7.00	>98 (3 h)	–	–	100	–

[a] Reaction conditions: phosphate buffer 25 mM, 20% acetonitrile; substrate concentration = 10 mM; 160 U of immobilised lipase in 20 mL of reaction mixture.



R = OAc; $\mathbf{R_1}$ = H; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OAc (**2**)
R = H; $\mathbf{R_1}$ = OMe; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OAc (**3**)
R = OMe; $\mathbf{R_1}$ = H; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OAc (**4**)
R = OBu; $\mathbf{R_1}$ = H; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OAc (**5**)
R = H; $\mathbf{R_1}$ = OAc; $\mathbf{R_2}$ = NHAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OAc (**6**)
R = OAc; $\mathbf{R_1}$ = H; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = H; $\mathbf{R_4}$ = OAc; $\mathbf{R_5}$ = OAc (**7**)
R = H; $\mathbf{R_1}$ = OAc; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = H; $\mathbf{R_4}$ = OAc; $\mathbf{R_5}$ = OAc (**8**)

R,$\mathbf{R_1}$ = H,OH; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OAc (**1c**)
R = H; $\mathbf{R_1}$ = OMe; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OH (**3a**)
R = OMe; $\mathbf{R_1}$ = H; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OH (**4a**)
R = OBu; $\mathbf{R_1}$ = H; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OH (**5a**)
R = H; $\mathbf{R_1}$ = OAc; $\mathbf{R_2}$ = NHAc; $\mathbf{R_3}$ = OAc; $\mathbf{R_4}$ = H; $\mathbf{R_5}$ = OH (**6a**)
R = OAc; $\mathbf{R_1}$ = H; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = H; $\mathbf{R_4}$ = OAc; $\mathbf{R_5}$ = OH (**7a**)
R = H; $\mathbf{R_1}$ = OAc; $\mathbf{R_2}$ = OAc; $\mathbf{R_3}$ = H; $\mathbf{R_4}$ = OAc; $\mathbf{R_5}$ = OH (**8a**)

Scheme 2.

regioselectivity and yield of APs markedly decrease. Performing the hydrolysis at pH 4, a 90% yield and complete regioselectivity toward the 6-position are obtained, whereas a low yield and a mixture of compounds **1a** and 1,2,3,6-tetra-*O*-acetyl-α-D-glucopyranose (**1b**) are observed under basic conditions. On the other hand PFL gives α/β **1c** (a 70:30 mixture of α and β anomers) in quantitative yields at all pH values tested.

The decrease of yield and the change in regioselectivity observed with CRL at basic pH may be assessed as a probable consequence of an acetyl group migration from the 4-position to the 6-position, which occurs in neutral or basic medium. The enzyme first hydrolyses the substrate in the 6-position, but because of acyl group migration, further hydrolysis can occur with a consequent reduction of the global AP's yield. At pH 7, for example, **1b** can be obtained as a major product (70:30 ratio with compound **1a**), but the global yield of AP is much lower than that obtained when the reaction is carried out at pH 4 (57% instead of 90% yield).

Enzymatic hydrolysis of different acetylated pyranosides.—We have studied the hydrolysis of different acetylated pyranosides (substrates **2**–**8**) catalysed by CRL and PFL (Scheme 2). As shown in Table 2, 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (**2**) was preferentially hydrolysed at the anomeric position with PFL to give α/β **1c** in good yield. CRL was poorly active towards this substrate and, surprisingly, afforded also product **1c**, although in very low yields (13%).

In order to also obtain the β anomers of APs bearing only one free hydroxyl group at the 6-position, the study of the regioselectivity of CRL and PFL was enlarged to include substrates in which the anomeric position was protected with an alkyl group such as methyl or butyl. The results obtained in the hydrolysis of the α and β anomers of methyl 2,3,4,6-tetra-*O*-acetyl-D-glucopyranoside (compounds **3** and **4**) are reported in Table 2. Using CRL as catalyst, the best activity was again obtained towards the α anomer **3**; however, the β anomer **4** was also hydrolysed at a positive rate (complete hydrolysis achieved in ~20 h).

Furthermore, CRL showed the same regioselectivity towards both substrates, allowing hydrolysis in the 6-position to give methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranoside (**3a**) and methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (**4a**) in ~90% yield. PFL was found to be completely inactive towards both substrates **3** and **4**.

In the hydrolysis of butyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**5**), butyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (**5a**) was obtained surprisingly with both lipases. With this substrate, PFL showed much higher activity in the hydrolysis than that shown towards substrate **4**. The activity of PFL towards **5** also resulted in much higher activity than that shown by CRL. The presence of a long aliphatic chain at the anomeric position probably enables PFL to hydrolyse this substrate. With this enzyme **5** was completely hydrolysed in only 7 h, and product **5a** was obtained in ~80% yield.

The regioselectivity of CRL has been further investigated considering other fully acetylated pyranoses (Scheme 2). In the hydrolysis of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucopyranose (**6**) CRL showed an activity and a regioselectivity close to that observed in the hydrolysis of compound **1**, allowing 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-α-glucopyranose (**6a**) to be obtained in good yield (78%).

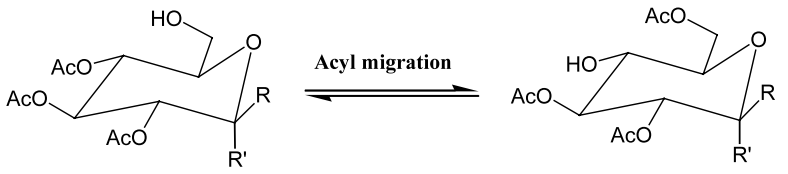
In the hydrolysis of 1,2,3,4,6-penta-*O*-acetyl-α- and β-D-galactopyranoses (compounds **7** and **8**), CRL confirmed the results obtained in the hydrolysis of substrates **1** and **2** concerning both activity and regioselectivity. Hydrolysis in the 6-position was in fact observed to obtain 1,2,3,4-penta-*O*-acetyl-α- and β-D-galactopyranoses (products **7a** and **8a**) in 94 and 43% yield, respectively (Table 2). The reaction rate in both examples was much higher for the α than for the β anomer.

One-pot chemoenzymatic synthesis of 1,2,3,6-tetra-O-acetyl-α-D-glucopyranose.—As previously shown in Scheme 1, CRL is able to hydrolyse in a regiospecific manner the penta-*O*-acetyl-α-D-glucopyranose (**1**) at the 6-position. However depending on the pH of the reaction, the 4-hydroxy derivative **1b** can also be isolated. In fact, both regioselectivity and yields decreased with increasing the pH of the reaction probably as a consequence of acetyl group migration from the 4-position to the 6-position, which occurs in neutral or basic medium. This migration has been better studied considering the effect of pH and temperature, in order to explore the possibility to control it to obtain the 4-hydroxy derivative **1b** in good yield (Scheme 3). The best results have been obtained at low temperature (4 °C) and moderately basic pH. At pH 8 a controlled acyl group migration

Table 2
Hydrolysis of acetylated glucopyranoses and glucopyranosides using CRL and PFL[a]

Substrate	Enzyme	Conversion (%)	*t* (h)	Yield (%)	Product
2	CRL	44	48	13	**1a**
2	PFL	98	20	90	**1a**
3	CRL	100	5	86	**3a**
3	PFL	<2	18	n.d.	n.d.
4	CRL	100	21	92	**4a**
4	PFL	<2	22	n.d.	n.d.
5	CRL	100	72	90	**5a**
5	PFL	100	7	81	**5a**
6	CRL	100	9	78	**6a**
7	CRL	100	19	94	**7a**
8	CRL	51	48	43	**8a**

[a] Reaction conditions: phosphate buffer 25 mM, 25% acetonitrile pH 5; substrate concentration = 20 mM; 40 U of immobilised lipase in 20 mL of reaction mixture; n.d. = not determined.



R = H R' = OAc **1a**
R = OMe R' = H **4a**

1b
4b

Scheme 3.

Table 3
Effect of pH on acetyl group migration from the 4-position to the 6-position at 4 °C

Compound	pH	Time (h)	Yield (%)[a]
1a	7	12	52
1a	7.5	12	75
1a	8	7	79
1a	8.5	4	48
1a	9	4	44
4a	7	4	44
4a	8	4	70

[a] Yield of reaction evaluated by HPLC analysis.

migration occurs allowing compound **1a** to be obtained in ~80% yield in 7 h (Table 3).

In order to evaluate the possibility to obtain α- and β-protected glucopyranoses bearing a free secondary C-4 hydroxyl group, we have extended the acyl migration studies to methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (**4a**). Also in this case, low temperature and basic pH allowed us to control the migration from the 4-position to the 6-position resulting in the isolation of methyl 2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**4b**). As reported in Table 3, by performing the reaction at pH 8, after 4 h we obtained the pure regioisomer **4b** in ~70% yield.

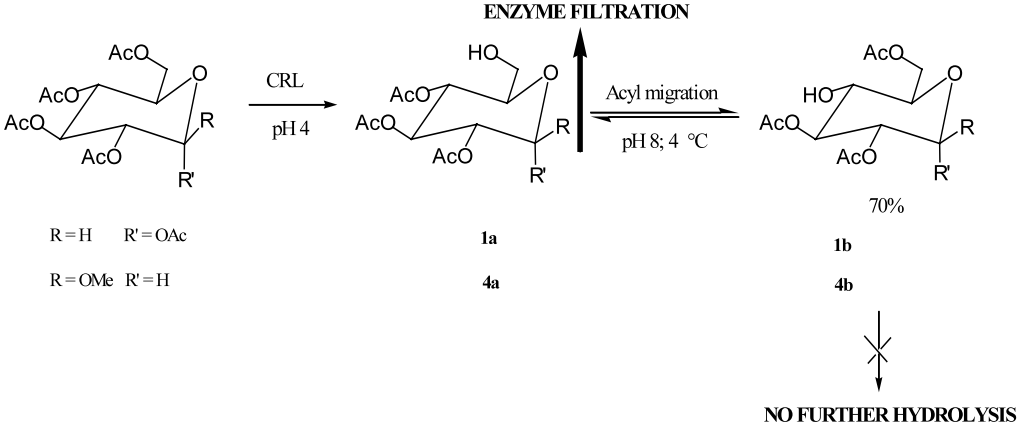
Further studies on 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose (**6a**) and 1,2,3,4-tetra-*O*-acetyl-α-D-galactopyranose (**7a**) are in progress.

Considering the good results obtained in the hydrolysis of α-D-glucose pentaacetate (**1**), as well as those obtained in the studies of acetyl group migration, we have investigated the possibility of the synthesis of **1b** by a new one-pot chemoenzymatic approach (Scheme 4). This synthesis has been developed by a correct combination of regioselective enzymatic hydrolysis of **1** and pH/temperature-controlled acyl group migration. In fact, starting from compound **1**, using CRL immobilised on octyl agarose, the hydrolysis performed at pH 4 and 25 °C allowed **1a** to be obtained in very good yield (90%). After enzyme derivative filtration, the reaction conditions were modified by lowering the temperature to 4 °C and increasing the pH to pH 8. Under these conditions a controlled acetyl group migration from the 4-position to the 6-position gave **1b** (70% overall yield after isolation). The same chemoenzymatic procedure has been applied to compound **4**. After a purification step, the pure regioisomer, methyl 2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**4b**), has been isolated in a yield of 68% (Scheme 4).

3. Conclusions

In this work we have described the regioselective hydrolysis of different acetylated pyranoses and pyranosides. The hydrolysis of α and β acetyl pyranoses catalysed by lipases isolated from *Candida rugosa* (CRL) and *Pseudomonas fluorescens* (PFL), afforded APs with only a free hydroxyl group in the 6-position or 1-position, respectively. By using CRL, the regioselective enzymatic hydrolysis of many acetylated pyranoses including α and β alkyl glucopyranosides was achieved Generally, this enzyme showed much more activity towards the α rather than the β acetylated pyranosides.

By a new one-pot chemoenzymatic approach, a free hydroxyl group in the 4-position was also obtained. This approach involved a temperature- and pH-controlled acyl migration performed on the product obtained by regioselective enzymatic hydrolysis in the 6-position catalysed by CRL of acetylated α- and β-D-glucose derivatives. According to this, 1,2,3,6-tetra-*O*-acetyl-α-D-glucopyranose (**1b**) can be easily synthesised in good overall yield, starting from a commercially available substrate and avoiding any intermediate purification. In the case of β anomers, glucopyranosides alkylated at the anomeric position must be used because of the low activity showed by CRL towards



Scheme 4.

penta-*O*-acetyl-β-D-glucose (**2**). As an example methyl 2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**4b**) has been prepared in good yield starting from methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**4**). The results reported in this work are of general interest for developing industrial processes for the preparation of sugar building blocks useful for the synthesis of oligosaccharides and other sugar derivatives. In fact, very easy-to-execute chemical procedures are used for the acetylation of sugars, while immobilised lipases are used to perform reactions that are difficult to develop using classical chemical reactions, i.e., the preparation of protected sugars having only one free hydroxyl group.

The results achieved in this work are strictly depending on a careful design and selection of the enzyme derivative used. The influence of the enzyme derivative preparation will be matter of forthcoming publications.

4. Experimental

General procedure.—CRL was obtained from Sigma Chemical Co., while PFL (Lipase AK Amano 20) was generously donated by Amano Enzyme Europe Ltd (Milton Keynes MK9; UK). Octyl-agarose (Sepharose CL-4B) was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All reagents were analytical grade.

The pH of the solutions during the enzymatic hydrolysis was kept constant using an automatic titrator 718 Stat Tritino from Metrohm (Herisau, Switzerland). HPLC analyses were performed using an HPLC Merck–Hitachi L-7100 (E. Merck, Darmstadt, Germany). The column was a Kromasil-C_{18} (250 × 4.6 and 5 μm) from Analisis Vinicos (Tomelloso, Spain). Analyses were run at 25 °C using an L-7300 column oven and UV detector L-7400 at 220 nm. The eluent was a mixture of 30% acetonitrile in phosphate buffer (10 mM) at pH 4; flow rate 1.0 mL/min.

Substrates that were not commercially available were synthesised using well-known chemical procedures, and their structures were confirmed by ^{1}H NMR spectroscopy. Columns for flash chromatography were made up with Silica Gel 60 (E. Merck) 60–200 μm or 40–63 μm. For 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-α-D-glucose (**6a**) elution of the flash chromatography column was performed with 30:70 acetone–dichloromethane; otherwise 40:60 hexane–ethyl acetate was used.

^{1}H NMR were recorded in $CDCl_3$ (δ = ppm) on a Bruker AMX 400 instrument. The different products obtained by enzymatic hydrolysis were characterised by COSY 2D NMR studies in order to assign the exact position of the hydrolysis.

Enzymatic hydrolyses.—All compounds were prepared following a general procedure: a suitable amount of immobilised lipase was added to a solution of substrate (20 mM) in phosphate buffer 25 mM (80%) and acetonitrile (20–30%) at the desired pH value. The hydrolytic reaction was carried out under mechanical stirring, and pH was controlled by automatic titration. Hydrolysis reactions were followed by HPLC. Finally, after filtration of the immobilised enzyme, the products were isolated by flash chromatography.

1,2,3,4-Tetra-O-*acetyl*-α-D-*glucopyranose* (***1a***). HPLC analysis: $t_R = 8.7$ min. ^{1}H NMR ($CDCl_3$): δ 1.93–2.1 (4 s, CH_3, 12H), 3.59–3.78 (2 m, ABX system, 2H-6), 3.61–3.75 (AB part of ABX system, $J_{1,3}$ 4.16 Hz, $J_{1,3}$ 2.36 Hz, $J_{1,2}$ 12.86 Hz, 2H-6), 3.95 (dq, *J* 2.36 Hz, *J* 4.16 Hz, *J* 10.31 Hz, 1H-5), 5.1 (dd, *J* 3.7 Hz, *J* 9.9 Hz, 1H-2), 5.14 (d, *J* 3.69 Hz, 1H-4), 5.56 (t, *J* 9.9 Hz, 1H-3), 6.38 (d, *J* 3.69 Hz, 1H-1). Anal. Calcd for $C_{14}H_{20}O_{10}$: C, 48.28; H, 5.79; O, 45.94. Found: C, 48.60; H, 5.40; O, 46.0.

2,3,4,6-Tetra-O-*acetyl*-α-D-*glucopyranose* (***1c***). HPLC analysis: $t_R = 7.1/8.2$ min. ^{1}H NMR ($CDCl_3$): δ 0.79 (t, *J* 7.3 Hz, CH_3, 3H), 1.29–1.43 (m, CH_2, 1H), 1.48–1.6 (m, CH_2, 1H), 2.01 (4 s, CH_3, 12H), 3.42–3.81 (2 m, ABX system, 2H), 3.44 (m, *J* 9.6 Hz, 1H-5), 3.48 and 3.64 (AB part of ABX system, $J_{1,3}$ 3.54 Hz, $J_{1,3}$ 2.4 Hz, $J_{1,2}$ 12.4 Hz, 2H-6), 4.42 (d, *J* 8 Hz, 1H-1), 4.76 (dd, *J* 7.9 Hz, *J* 9.7 Hz, 1H-2), 4.92 (t, *J* 9.8 Hz, 1H-4), 5.13 (t, *J* 9.6 Hz, 1H-3). Anal. Calcd for $C_{14}H_{20}O_{10}$: C, 48.28; H, 5.79; O, 45.94. Found: C, 48.50; H, 5.56; O, 45.94.

Methyl 2,3,4-tri-O-*acetyl*-α-D-*glucopyranose* (***3a***). HPLC analysis: $t_R = 6.8$ min. ^{1}H NMR ($CDCl_3$): δ 1.56 (s, CH_3, 3H), 1.99–2.08 (4 s, CH_3, 12H), 3.97 (m, 1H-5), 4.08 and 4.27 (2 m, ABX system, 2H-6), 4.1–4.25 (AB part of ABX system, $J_{1,3}$ 7.7 Hz, $J_{1,3}$ 4.59 Hz, $J_{1,2}$ 12.3 Hz, 2H-6), 4.89 (dd, *J* 10 Hz, *J* 6.6 Hz, 1H-2), 4.94 (d, *J* 3.51 Hz, 1H-1), 5.05 (t, *J* 9.8 Hz, 1H-4), 5.47 (t, *J* 9.9 Hz, 1H-3). Anal. Calcd for $C_{15}H_{22}O_{10}$: C, 49.72; H, 6.12; O, 44.16. Found: C, 49.32; H, 6.76; O, 43.92.

Methyl 2,3,4-tri-O-*acetyl*-β-D-*glucopyranoside* (***4a***). HPLC analysis: $t_R = 6.7$ min. ^{1}H NMR ($CDCl_3$): δ 1.9–2.01 (3 s, CH_3, 9H), 3.52 (s, CH_3, 3H), 3.53 (m, 1H-5), 3.6 and 3.8 (AB part of ABX system, $J_{1,3}$ 7.9 Hz, $J_{1,3}$ 2 Hz, $J_{1,2}$ 12.5 Hz, 2H-6), 4.46 (d, *J* 7.9 Hz, 1H-1), 4.94 (dd, *J* 9.7 Hz, *J* 8 Hz, 1H-2), 5.04 (t, *J* 9.5 Hz, 1H-3). Anal. Calcd for $C_{15}H_{22}O_{10}$: C, 49.72; H, 6.12; O, 44.16. Found: C, 48.60; H, 6.10; O, 45.30.

Butyl 2,3,4-tri-O-*acetyl*-β-D-*glucopyranoside* (***5a***). HPLC analysis: 40% acetonitrile in phosphate buffer (10 mM) at pH 4, flow rate 1.5 mL/min; $t_R = 8.3$ min. ^{1}H NMR ($CDCl_3$): δ 0.83 (t, *J* 7.4 Hz, CH_3, 3H), 1.22–1.32 (m, CH_2, 1H), 1.41–1.55 (m, CH_2, 2H), 1.93–2.01 (4 s, CH_3, 12H), 3.42–3.81 (2 m, ABX system, 2H), 3.64 (m, *J* 9.5 Hz, 1H-5), 4.05 and 4.20 (AB part of ABX system, $J_{1,3}$ 4.77 Hz, $J_{1,3}$ 2.44 Hz, $J_{1,2}$ 12.2 Hz, 2H-6), 4.43 (d, *J* 7.8 Hz, 1H-1), 4.90 (dd, *J* 7.9 Hz, *J* 9.6 Hz, 1H-2), 5 (t, *J* 9.8 Hz, 1H-4), 5.13 (t, *J* 9.5 Hz, 1H-3). Anal. Calcd for $C_{18}H_{28}O_{10}$: C, 53.46; H, 6.98; O, 39.56. Found: C, 53.60; H, 6.60; O, 39.80.

*2-Acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-α-*D*-glucopyranose (**6a**).* HPLC analysis: 20% acetonitrile in phosphate buffer (10 mM) at pH 4, flow rate 1.0 mL/min; $t_R = 6.8$ min. ^{1}H NMR ($CDCl_3$): δ 1.6 (s, CH_3, 3H), 1.93–2.07 (4 s, CH_3, 12H), 3.97 (m, 1H-5), 4.03 and 4.26 (2 m, ABX system, 2H-6), 4.05–4.24 (AB part of ABX system, $J_{1,3}$ 8.5 Hz, $J_{1,3}$ 3.92 Hz, $J_{1,2}$ 12.4 Hz, 2H-6), 4.47 (dd, *J* 9.9 Hz, *J* 6.75 Hz, 1H-2), 5.1 (t, *J* 9.7 Hz, 1H-4), 5.23 (t, *J* 9.9 Hz, 1H-3), 5.53 (d, *J* 9 Hz, 1H-NH), 6.1 (d, *J* 3.51 Hz, 1H-1). Anal. Calcd for $C_{14}H_{21}NO_9$: C, 48.41; H, 6.09; N. 4.03; O, 41.46. Found: C, 48.40; H, 6.10; N. 4.03; O, 41.47.

*1,2,3,4-Penta-*O*-acetyl-α-*D*-galactopyranose (**7a**).* HPLC analysis: $t_R = 8.6$ min. ^{1}H NMR ($CDCl_3$): δ 1.98–2.15 (4 s, CH_3, 12H), 4.04–4.16 (2 m, ABX system, 2H-6), 4.33 (t, *J* 6.45, 1H-5), 5.32 (m, 2H-2 + 3), 5.49 (t, *J* 8.7 Hz, 1H-4), 6.37 (d, *J* 2.8 Hz, 1H-1). Anal. Calcd for $C_{14}H_{20}O_{10}$: C, 48.28; H, 5.79; O, 45.94. Found: C, 48.50; H, 5.90; O, 45.60.

*1,2,3,4-Penta-*O*-acetyl-β-*D*-galactopyranose (**8a**).* HPLC analysis: $t_R = 8.4$ min. ^{1}H NMR ($CDCl_3$): δ 1.97–2.14 (4 s, CH_3, 12H), 3.51 and 3.8 (2 m, ABX system, 2H-6), 3.54–3.76 (AB part of ABX system, $J_{1,3}$ 7.64 Hz, $J_{1,3}$ 6.32 Hz, $J_{1,2}$ 11.7 Hz, 2H-6), 3.91 (dt, *J* 6.45 Hz, 1H-5), 5.13 (dd, *J* 3.42 Hz, *J* 10.4 Hz, 1H-2), 5.32 (t, *J* 8.37 Hz, 1H-3), 5.44 (d, *J* 3.39 Hz, 1H-4), 5.73 (d, *J* 8.26 Hz, 1H-1). Anal. Calcd for $C_{14}H_{20}O_{10}$: C, 48.28; H, 5.79; O, 45.94. Found: C, 48.40; H, 5.50; O, 46.10.

*Chemoenzymatic synthesis of AP bearing a free secondary C-4 hydroxyl group (compounds **1b** and **4b**).*— After the hydrolytic reaction catalysed by CRL at pH 4 were carried out following the procedure previously described under 'Enzymatic hydrolyses', the enzyme was filtered off. The solution was then cooled and step by step adjusted to pH 8, all the while controlling the temperature. The reaction was monitored by HPLC, and when the maximum concentration of **1b** (or **4b**) was achieved, the solution was extracted with Et_2O. After evaporation of the solvent under reduced pressure, the residue was purified by flash chromatography (hexane–ethyl acetate) to obtain the pure regioisomers **1b** (70% yield) or **4b** (68% yield).

*1,2,3,6-Tetra-*O*-acetyl-α-*D*-glucopyranose (**1b**).* HPLC analysis: $t_R = 8.5$ min. ^{1}H NMR ($CDCl_3$): δ 1.96–2.11 (4 s, CH_3, 12H), 3.59 (t, *J* 9.3 Hz, 1H-4), 3.92 (m, 1H-5), 4.18 and 4.42 (2 m, ABX system, 2H-6), 4.21–4.41 (AB part of ABX system, $J_{1,3}$ 8.6 Hz, $J_{1,3}$ 3.96 Hz, $J_{1,2}$ 12.6 Hz, 2H-6), 4.96 (dd, *J* 10.25 Hz, *J* 3.73 Hz, 1H-2), 5.28 (t, *J* 9.8 Hz, 1H-3), 6.23 (d, *J* 3.4 Hz, 1H-1). Anal. Calcd for $C_{14}H_{20}O_{10}$: C, 48.28; H, 5.79; O, 45.94. Found: C, 48.34; H, 5.54; O, 46.12.

*Methyl 2,3,6-tri-*O*-acetyl-β-*D*-glucopyranoside (**4b**).* HPLC analysis: $t_R = 7.12$ min. ^{1}H NMR ($CDCl_3$): δ 2.0–2.1 (3 s, CH_3, 9H), 3.495 (s, CH_3, 3H), 3.56 (m, 1H-4), 3.57 (m, 1H-5), 4.3 and 4.5 (2 dd, ABX system, 2H-6 $J_{1,3}$ 3.42 $J_{1,3}$ 3.9 $J_{1,2}$ 1.956), 4.45 (d, *J* 7.8 Hz, 1H-1), 4.9 (dd, *J* 9.7 Hz, *J* 8 Hz, 1H-2), 5.0 (t, *J* 9.5 Hz, 1H-3). Anal. Calcd for $C_{15}H_{22}O_{10}$: C, 49.72; H, 6.12; O, 44.16. Found: C, 48.50; H, 6.20; O, 45.30.

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