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Journal of Molecular Catalysis B: Enzymatic 3 (1997) 193–201

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Regioselective acylation of polyhydroxylated natural compounds catalyzed by *Candida Antarctica* lipase B (Novozym 435) in organic solvents

Bruno Danieli [a], Monica Luisetti [a], Giannantonio Sampognaro [a], Giacomo Carrea [b], Sergio Riva [b,*]

[a] *Dipartimento di Chimica Organica ed Industriale, Centro C.N.R. di Studio sulle Sostanze Organiche Naturali, Via Venezian 21, 20133 Milan, Italy*

[b] *Istituto di Chimica degli Ormoni, C.N.R., Via Mario Bianco 9, 20131 Milan, Italy*

Abstract

A systematic investigation on the transesterification activity and regioselectivity of the immobilized lipase B from *Candida antarctica* (Novozym 435) has been performed. This enzyme has been found to be quite active in such solvents as THF, acetone, dioxane, and in mixtures of these solvents with pyridine. Several glycopyranosides have been acetylated, showing that the regioselectivity displayed by Novozym 435 towards sugar secondary OH's is deeply influenced by the nature of the aglycone and by the stereochemistry of the glycosidic bond. This information on the regioselectivity of the lipase has been exploited for the preparation of acetyl derivatives of flavonoid glycosides.

Keywords: *Candida antarctica* lipase B; Novozym 435; Organic solvents; Regioselectivity; Sugar acylation; Flavonoid glycosides

1. Introduction

In recent years lipases have been used for the transformation of an innumerable number of organic compounds [1]. Stability, selectivity, and handyness are the properties that characterize these enzymes and that are responsible for their success among organic chemists. Several groups are trying to expand the scope of these hydrolases further on by searching for new lipases with interesting synthetic performances. A recent outcome of this effort is the so-called lipase B from *Candida antarctica* (CAB), enzyme that has been cloned and overexpressed into the host organism *Aspergillus oryzae* [2] and that, being now commercially available, has been used for the resolution of racemic compounds [3] and for the regioselective acylation of sugars with fatty acids in solvent-free reactions [4].

Being particularly interested in the regioselective enzymatic modification of natural compounds, we have studied CAB selectivity towards polyhydroxylated steroids [5] and natural glycosides [6]. In the latter case we have found that CAB is selective towards some of the primary OH's of complex glycosilated saponins, like ginsenoside Rg_1 [7], leaving all the secondary OH's of these molecules unaffected. To

* Corresponding author. Fax: +39-2-2841934; e-mail: ico@siam.mi.cnr.it.

PII S1381-1177(96)00055-0

get further data on CAB selectivity and to compare this information with the results obtained with another useful regioselective enzyme, the protease subtilisin Carlsberg [8], we have considered the alkaloids colchicoside (**1**) and thiocolchicoside (**2**) [9] as new targets. The unexpected results obtained in the enzymatic acylation of these two compounds (almost quantitative conversion to the corresponding 2′,6′-*O*-diacetate **1b** and **2b**) prompted us to systematically explore CAB selectivity towards sugars secondary OH's and in the following we will report the results of this study.

2. Experimental

2.1. Materials and methods

Cyclohexanol and the compounds **3**, **4**, **6**, **8–12**, **20**, were purchased from Aldrich. Quercitrin (**18**) and isoquercitrin (**19**) were from Extrasynthese. Colchicoside (**1**) and thiocolchicoside (**2**) were a generous gift from Indena SpA. The glycopyranosides **5**, **7**, **13–17** were prepared by us following standard procedures. *Candida antarctica* lipase B (Novozym 435) was a generous gift of Novo-Nordisk.

HPLC analyses were performed using a Lichrospher® 100 RP-18 5 μm column (from Merck) or a Partisil 5 silica column (from Whatman®) and a JASCO 880/PU instrument equipped with a JASCO 870 UV/VS detector. GC analyses were obtained using a capillary crosslinked methyl silicone gum column (HP-1, 25 m × 0.32 mm × 0.52 μm film thickness, Hewlett-Packard). Precoated silica gel 60 F_{254} plates from Merck were used for TLC. NMR spectra were obtained using either a Bruker AC-300 (300 MHz) or a Bruker AC-200 (200 MHz).

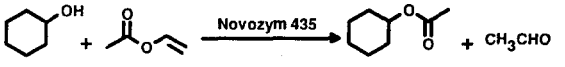
2.2. Influence of organic solvents on the activity of Novozym 435 (Table 1)

Cyclohexanol (15 μL, 0.15 mM) and vinyl acetate (40 μL, 0.45 mM) were dissolved in 1 mL of organic solvent (the list of the solvents used is reported in Table 1). Novozym 435 (4 mg) was added and the suspension shaken at 45°C. In order to evaluate the initial rate of acetylation, every 5 min a solution sample was withdrawn and subjected to GC analysis. Conditions: oven temperature at 50°C for 1 min, then from 50 to 130°C with a heating rate of 10°C/min. Retention times: cyclohexanol, 4.67 min; cyclohexanol acetate, 7.28 min. The degrees of conversion were similarly determined after 5 h and, if needed, after 24 and 48 h.

2.3. Sugars derivatization for GC analysis

A sample of the enzymatic reaction solution (30 μL) was diluted with pyridine (30 μL) and reacted with 1,1,1,3,3,3-hexamethyldisilazane (30 μL) and CF_3COOH (3 μL). After 5 min, 5 μL of this final solution was injected into the GC. Conditions: for **15** and **17**, oven temperature at 190°C for 30 min; for all the other sugar derivatives: oven temperature at 210°C for 1

Table 1
Influence of organic solvents on the activity of Novozym 435 in the acetylation of cyclohexanol



Solvent	Initial rate	Conversion (%)		
	(μmol/min)	5 h	24 h	48 h
Hexane	2.14	100		
Acetone	0.91	95	100	
Acetonitrile	0.76	85	100	
t-amyl alcohol	0.31	95	100	
t-butanol	0.18	75	100	
Dioxane	1.00	97	100	
DMF	0	—	—	
N-methyl-pyrrolidone	0	—	—	
3-pentanone	1.47	99	100	
Pyridine	0.06	10	19	23
THF	1.17	95	100	
THF-DMF 7:3	0.58	4	6	8
THF-pyridine 7:3	1.10	88	99	
THF-pyridine 5:5	1.04	78	99	
THF-pyridine 3:7	0.72	65	96	
Dioxane–pyridine 5:5	0.94	79	98	
Acetonitrile–pyridine 5:5	0.21	39	80	92

min, then from 210 to 250°C with a heating rate of 1°C/min.

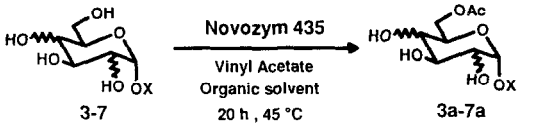
2.4. Acetylation of colchicoside (1) and thiocolchicoside (2)

The substrates **1** or **2** (100 mg) were dissolved in 7 mL of a 5:2 mixture of *t*-amyl alcohol and vinyl acetate (0.5 mL of pyridine was also added with **2**). CAB (150 mg) was added and the suspensions shaken at 45°C, following the conversion by TLC (eluent AcOEt–MeOH 9:1) and HPLC (RP-18 column, eluent: 10% CH_3CN and 90% H_2O (containing 0.1% CF_3COOH) for 20 min). After 40 h the enzyme was filtered, the solvent evaporated and the products **1b** (95%, as determined by HPLC) and **2b** (93%) purified by flash chromatography and characterized by ^{1}H-NMR. Selected ^{1}H-NMR data ($CDCl_3$) δ: **1b**: 7.43 (1H, s, H-8), 7.27 and 6.82 (each 1H, AB system, J = 11 Hz, H-11 and H-12), 6.73 (1H, s, H-4), 5.15 (1H, dd, $J_1 = 9.0$ Hz, $J_2 = 7.5$ Hz, H-2′), 4.88 (1H, d, 7.5 Hz, H-1′), 4.61 (1H, m, H-7), 4.48 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 5.1$ Hz, H-6′a), 4.38 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 1.5$ Hz, H-6′b), 3.98, 3.89 and 3.64 (each 3H, s, OCH_3), 2.17, 2.10, 1.98 (each 3H, s, CH_3CO). **2b**: 7.43 (1H, s, H-8), 7.23 and 7.02 (each 1H, AB system, $J = 11$ Hz, H-11 and H-12), 6.76 (1H, s, H-4), 5.18 (1H, t, $J = 8.0$ Hz, H-2′), 4.95 (1H, d, 8.0 Hz, H-1′), 4.60 (1H, m, H-7), 4.52 (1H, bd, $J = 12.0$ Hz, H-6′a), 4.32 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 6.1$ Hz, H-6′b), 3.88 and 3.64 (each 3H, s, OCH_3), 2.43 (3H, s, SCH_3), 2.16, 2.10, 2.00 (each 3H, s, CH_3CO). By selective irradiation of the signals at 5.15 (**1b**) and 5.18 (**2b**) the corresponding signals due to the anomeric H-1 became a singlet.

2.5. Acetylation of α-D-glycopyranosides (Table 2)

α-D-Glycopyranosides **3–7** (100 mg) were dissolved in 5 mL of a 4:1 mixture of THF-pyridine. Vinyl acetate (1 mL) and Novozym 435 were added and the suspensions shaken at 45°C, following the reactions by TLC (eluent AcOEt–MeOH 9:1) and GC. After 20 h the degrees of conversion were almost quantitative with all the five substrates. The enzyme was filtered, the solvents evaporated and the products purified by flash chromatography and characterized by ^{1}H-NMR. Selected ^{1}H-NMR data ($CDCl_3$) δ: **3a**: 4.77 (1H, d, $J = 3.5$ Hz, H-1), 4.49 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 4.6$ Hz, H-6a), 4.24 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 2.3$ Hz, H-6b), 3.42 (3H, s, OCH_3), 2.13 (3H, s, CH_3CO). **4a**: 5.55 (1H, d, $J = 3.3$ Hz, H-1), 4.46 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 4.5$ Hz, H-1), 4.18 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 2.7$ Hz, H-6b), 4.00 (1H, t, $J = 9.0$ Hz, H-3), 3.89 (1H, m, H-5), 3.71 (1H, dd, $J_1 = 9$ Hz, $J_2 = 3.3$ Hz, H-2), 3.46 (3H, s, OCH_3), 2.05 (3H, s, CH_3CO). **5a**: 4.98 (1H, d, $J = 3.8$ Hz, H-1), 4.73 and 4.55 (each 1H, d, $J = 12$ Hz, OCH_2Ph), 4.47 (1H, dd, $J_1 = 12.0$, $J_2 = 4.0$ Hz, H-6a), 4.14 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 2.0$ Hz, H-6b), 3.76 (2H, m, H-3 and H-5), 3.37 (1H, t, $J = 9$ Hz, H-4), 2.10 (3H, s, CH_3CO). **6a**: 4.83 (1H, d, $J = 3.0$ Hz, H-1), 4.42 (1H, dd, $J_1 = 11$ Hz, $J_2 = 5.8$ Hz, H-6a), 4.25 (1H, dd, $J_1 = 11$ Hz, $J_2 = 6.2$ Hz, H-6b), 3.43 (3H, s, OCH_3), 2.10 (3H, s, CH_3CO). **6b**: 5.10 (1H, dd, $J_1 = 9.8$ Hz, $J_2 = 3.0$ Hz, H-2), 4.87 (1H, d, $J = 3.0$ Hz, H-1), 4.39 (1H, dd, $J_1 = 11$ Hz, $J_2 = 5.8$ Hz, H-6a), 4.23 (1H, dd,

Table 2
Acetylation of α-D-glycopyranosides catalyzed by Novozym 435



Compound	2-OH	4-OH	X	6-OAc (product) [a] (%)
3	eq	eq	Me	98 (**3a**)
4	eq	eq	Ph	96 (**4a**)
5	eq	eq	CH_2Ph	99 (**5a**)
6	eq	ax	Me	74 (**6a**) [b]
7	ax	eq	CH_2Ph	97 (**7a**)

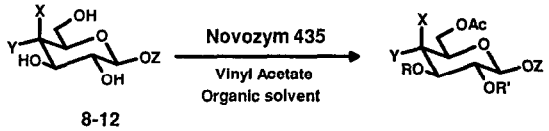
[a] Determined by GC and ^{1}H-NMR.
[b] 24% of 2,6-*O*-diacetate **6b**.

$J_1 = 11$ Hz, $J_2 = 6.2$ Hz, H-6b), 3.38 (3H, s, OCH_3), 2.17 and 2.10 (each 3H, s, CH_3CO). By selective irradiation of H-1, the signal at 5.10 ppm (H-2) became a doublet, $J = 9.8$ Hz). **7a**: 4.95 (1H, br s, H-1), 4.71 and 4.52 (each 2H, 2d, $J = 12$ Hz, OCH_2Ph), 4.56 (1H, dd, $J_1 = 12$ Hz, $J_2 = 4.0$ Hz, H-6a), 4.17 (1H, dd, $J_1 = 12$ Hz, $J_2 = 2.0$ Hz, H-6b), 4.01 (1H, br s, H-2), 3.90 (1H, br d, $J = 10$ Hz, H-3), 3.76 (1H, ddd, $J_1 = 10$ Hz, $J_2 = 4.0$ Hz, $J_3 = 2.0$ Hz, H-5), 3.64 (1H, t, $J = 10$ Hz, H-4), 2.15 (3H, s, CH_3CO).

2.6. Acetylation of β-D-glycopyranosides (Table 3)

Compounds **8** and **11** were reacted according to the previously described procedure. Compounds **9**, **10**, and **12** (100 mg) were dissolved in THF (4 mL) and, following the addition of vinyl acetate (1 mL) and CAB (50 mg), shaken at 45°C for the reaction times reported in Table 3 (TLC eluents AcOEt–MeOH in appropriate ratios). Usual work up gave the pure mono- and diacetates reported in Table 3. Selected ^{1}H-NMR data ($CDCl_3$) δ: **8a**: 4.91 (1H, t, $J = 8.7$ Hz, H-3), 4.47 (1H, dd, $J_1 = 12.2$ Hz, $J_2 = 3.8$ Hz, H-6a), 4.32 (1H, dd, $J_1 = 12.2$ Hz, $J_2 = 2.0$ Hz, H-6b), 4.25 (1H, d, $J = 8.7$ Hz, H-1), 3.57 (3H, s, OCH_3), 2.16 and 2.11 (each 3H, s, CH_3CO). **9a**: 5.01 (1H, t, $J = 8.7$ Hz, H-3), 4.95 (1H, d, $J = 7.5$ Hz, H-1), 4.46 (1H, dd, $J_1 = 11.3$ Hz, $J_2 = 4.5$ Hz, H-6a), 4.35 (1H, dd, $J_1 = 11.3$ Hz, $J_2 = 2.2$ Hz, H-6b), 3.80 (1H, dd, $J_1 = 8.7$ Hz, $J_2 = 7.5$ Hz, H-2), 2.20 and 2.12 (each 3H, s, CH_3CO). By selective irradiation of the signal at 3.80 ppm (H-2), the signal at 5.01 ppm (H-3) became a doublet and the signal at 4.95 ppm (H-1) became a singlet. **9b**: 5.06 (1H, t, $J = 7.6$ Hz, H-2), 5.00 (1H, d, $J = 7.6$ Hz, H-1), 4.50 (1H, dd, $J_1 = 11.3$ Hz, $J_2 = 4.5$ Hz, H-6a), 4.34 (1H, dd, $J_1 = 11.3$ Hz, $J_2 = 2.2$ Hz, H-6b), 3.71 (1H, t, $J = 7{,}6$ Hz, H-3), 2.15 and 2.11 (each 3H, s, CH_3CO). By selective irradiation of the signal at 5.06 ppm (H-2), the signal at 5.00 ppm (H-1) became a singlet and the signal at 3.71 ppm (H-3) became a doublet; by selective irradiation of the signal at 3.71 ppm (H-3), the signal at 5.06 ppm (H-2) became a doublet, while the signal at 5.00 ppm (H-1) remained a doublet. **10a**: 4.46 (1H, dd, $J_1 = 12.5$ Hz, $J_2 = 4.5$ Hz, H-6a), 4.29 (1H, dd, $J_1 = 12.5$ Hz, $J_2 = 2.0$ Hz, H-6b), 4.26 (1H, d, $J = 8.5$ Hz, H-1), 2.13 (3H, s, CH_3CO). **11a**: 4.42 (1H, dd, $J_1 = 11.3$ Hz, $J_2 = 6.2$ Hz, H-6a), 4.31 (1H, dd, $J_1 = 11.3$ Hz, $J_2 = 7.0$ Hz, H-6b), 4.17 (1H, d, $J = 7.5$ Hz, H-1), 3.93 (1H, br s, H-4), 3.58 (3H, s, OCH_3), 2.10 (3H, s, CH_3CO). **11b**: 4.83 (1H, dd, $J_1 = 9.8$ Hz, $J_2 = 2.8$ Hz, H-3), 4.23 (1H, d, $J = 7.8$ Hz, H-1), 3.56 (3H, s, OCH_3), 2.17 and 2.09 (each 3H, s, CH_3CO). **11c**: 4.92 (1H, dd, $J_1 = 9.8$ Hz, $J_2 = 7.8$ Hz, H-2), 4.23 (1H, d, $J = 7.8$ Hz, H-1), 3.49 (3H, s, OCH_3), 2.12 and 2.08 (each 3H, s, CH_3CO). **12a**: 4.93 (1H, dd, $J_1 = 8.0$ Hz, $J_2 = 3.4$ Hz, H-3), 4.91 (1H, d, $J = 7.6$ Hz, H-1), 4.32 (2H, s, H-6a and H-6b), 4.14 (1H, m, H-5), 4.08 (1H, t, $J = 3.4$ Hz, H-4), 3.86 (1H, t, H-2, $J = 7.5$ Hz, H-2), 2.20 and 2.08 (each 3H, s, CH_3CO). **12b**: 5.20 (1H, dd, $J_1 = 9.8$ Hz, $J_2 = 8.0$ Hz, H-2), 4.97 (1H, d, $J = 8.0$ Hz, H-1), 4.43 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 6.0$ Hz, H-6a), 4.33 (1H, dd, $J_1 = 12.0$ Hz, $J_2 = 6.3$ Hz, H-6b), 3.96 (1H, br

Table 3
Acetylation of β-D-glycopyranosides catalyzed by Novozym 435



8-12

Substrate	React. time	Product (%) [a]	X	Y	Z	R	R'
8	20 h	**8a** (97)	H	OH	Me	Ac	H
9	48 h	**9a** (77)	H	OH	Ph	Ac	H
		9b (23)	H	OH	Ph	H	Ac
10	8 h	**10a** (99)	H	OH	octyl	H	H
11	48 h	**11a** (24)	OH	H	Me	H	H
		11b (44)	OH	H	Me	Ac	H
		11c (31)	OH	H	Me	H	Ac
12	48 h	**12a** (92)	OH	H	Ph	Ac	H
		12b (6)	OH	H	Ph	H	Ac

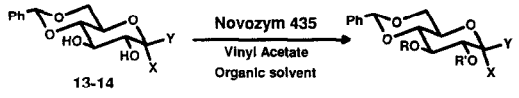
[a] Determined by GC and ^{1}H-NMR.

d, $J = 4.0$ Hz, H-4), 3.85 (1H, br t, $J = 6.1$ Hz H-5), 3.86 (1H, t, H-2, $J = 7.5$ Hz, H-2), 3.75 (1H, dd, $J_1 = 9.8$ Hz, $J_2 = 4.0$ Hz, H-3), 2.15 and 2.12 (each 3H, s, CH_3CO). By selective irradiation of the signal at 5.20 ppm (H-2), the signal at 4.97 ppm (H-1) became a singlet and the signal at 3.75 ppm (H-3) became a doublet.

2.7. Acetylation of methyl 4,6-O-benzyliden-D-α- and β-glucopyranosides (Table 4)

Compounds **13** and **14** (75 mg) were dissolved in 5 mL of vinyl acetate, CAB (50 mg) was added and the suspensions shaken at 45°C. Using **14** as a substrate, conversion was quantitative after 20 h, while with **13** approximately 40% of the starting material was present even after 9 days (determined by HPLC, silica column, eluent hexane–isopropanol from 90:10 to 75:25). Usual work up and purification by flash chromatography (eluent AcOEt–hexane 1:1) gave the pure products. Selected ^{1}H-NMR data ($CDCl_3$) δ: **13a**: 5.57 (1H, s, PhCH), 4.95 (1H, d, $J = 3.8$ Hz, H-1), 4.80 (1H, dd, $J_1 = 9.8$ Hz, $J_2 = 3.8$ Hz, H-2), 4.29 (1H, dd, $J_1 = 9.8$ Hz, $J_2 = 4.0$ Hz, H-6a), 4.17 (1H, t, 9.8 Hz, H-3), 3.84 (1H, dt, $J_1 = 9.8$ Hz, $J_2 = 4.0$ Hz, H-5), 3.75 (1H, t, 9.8 Hz, H-6b), 3.55 (1H, t, $J = 9.8$ Hz, H-4), 3.38 (3H, s, OCH_3), 2.18 (3H, s, CH_3CO). **14a**: 5.50 (1H, s, PhCH), 5.21 (1H, t, $J = 9.3$ Hz, H-3), 4.38 (1H, d, $J = 8.7$ Hz, H-1), 4.36 (1H, dd, $J_1 = 10.5$ Hz, $J_2 = 5.4$ Hz,

Table 4
Acetylation of methyl 4,6-*O*-benzyliden-D-α- and β-glucopyranosides catalyzed by Novozym 435

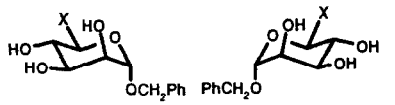


13-14

Substrate	X	Y	React. time	Product (%) [a]	R	R'
13	Me	H	9 days	**13a** (58)	H	Ac
14	H	Me	20 h	**14a** (79)	Ac	H
				14b (21)	H	Ac

[a] Determined by HPLC and ^{1}H-NMR.

Table 5
Acetylation of benzyl (D)- and (L)-α-mannopyranosides and α-rhamnopyranosides catalyzed by Novozym 435



(D) 7, 15 (L) 16, 17

Substrate	X	Main product (%) [a]
7	CH_2OH	6-*O*-Ac (97)
15	CH_3	4-*O*-Ac (20)
16	CH_2OH	6-*O*-Ac (64) [b]
17	CH_3	4-*O*-Ac (90)

[a] Determined by GC and ^{1}H-NMR.
[b] 29% of 4,6-*O*-diacetate (**16b**).

H-6a), 3.78 (1H, t, 10.5 Hz, H-6b), 3.63 (1H, t, $J = 9.8$ Hz, H-4), 3.58 (3H, s, OCH_3), 2.13 (3H, s, CH_3CO). **14b**: 5.56 (1H, s, PhCH), 4.92 (1H, t, $J = 9.3$ Hz, H-2), 4.45 (1H, d, $J = 8.7$ Hz, H-1), 4.37 (1H, dd, $J_1 = 10.5$ Hz, $J_2 = 5.4$ Hz, H-6a), 3.88 (1H, t, $J = 9.3$ Hz, H-3), 3.79 (1H, t, 10.5 Hz, H-6b), 3.58 (1H, t, $J = 9.8$ Hz, H-4), 3.50 (3H, s, OCH_3), 2.13 (3H, s, CH_3CO).

2.8. Acetylation of benzyl (D)- and (L)-α-mannopyranosides and α-rhamnopyranosides (Table 5)

Compounds **7** and **16** were dissolved in 4.4 mL of a mixture of acetone:pyridine 10:1 and compounds **15** and **17** in 4 mL of acetone. Vinyl acetate (1 mL) and CAB (50 mg) were added and the suspensions shaken at 45°C for 90 h (TLC eluent AcOEt–MeOH 95:5). Usual work up gave the pure products. Selected ^{1}H-NMR data ($CDCl_3$) δ: **7a** and **16a**: 4.95 (1H, br s, H-1), 4.71 and 4.52 (each 1H, d, $J = 12$ Hz, OCH_2Ph), 4.56 (1H, dd, $J_1 = 12$ Hz, $J_2 = 4.0$ Hz, H-6a), 4.17 (1H, dd, $J_1 = 12$ Hz, $J_2 = 2.0$ Hz, H-6b), 4.01 (1H, br s, H-2), 3.90 (1H, br d, $J = 10$ Hz, H-3), 3.76 (1H, ddd, $J_1 = 10$ Hz, $J_2 = 4.0$ Hz, $J_3 = 2.0$ Hz, H-5), 3.64 (1H, t, $J = 10$ Hz, H-4), 2.15 (3H, s, CH_3CO). **16b**: 5.07 (1H, t, $J = 10$ Hz, H-4), 4.98 (1H, br s, H-1), 4.72 and 4.53 (each 1H, d, $J = 12.2$ Hz, CH_2OPh), 4.31 (1H, dd, $J_1 = 12.5$ Hz, $J_2 = 5.2$

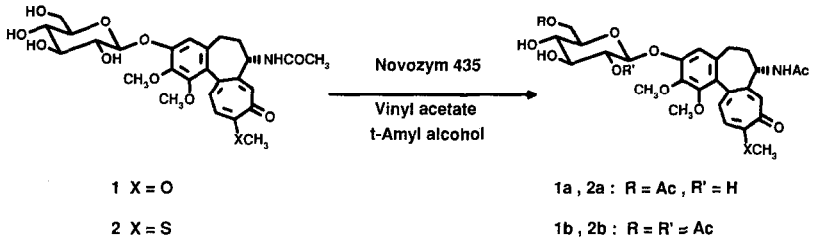


Fig. 1. Novozym 435 catalyzed acetylation of colchicoside (**1**) and thiocolchicoside (**2**).

Hz, H-6a), 4.07 (1H, dd, $J_1 = 12.5$ Hz, $J_2 = 1.8$ Hz, H-6b), 2.11 and 2.10 (3H, s, CH_3CO). **15a** and **17a**: 4.92 (1H, br s, H-1), 4.82 (1H, t, $J = 9.2$ Hz, H-4), 4.73 and 4.54 (each 1H, d, $J = 11.7$ Hz, CH_2OPh), 2.13 (3H, s, CH_3CO), 1.22 (3H, d, $J = 6.1$ Hz, CH_3-6).

2.9. Acetylation of isoquercitrin, quercitrin and rutin (Table 6 and Fig. 2)

Compounds **18–20** (0.2 mmol) were dissolved in 0.5 mL of pyridine. Acetone (4.4 mL), vinyl acetate (0.2 mL, ≈ 10 eq) and CAB (150 mg) were added and the suspensions shaken at 45°C for the time reported in Table 6, following the reactions by TLC (eluent $CHCl_3$–MeOH–H_2O 8:2:0.3) and HPLC (reverse phase C-18 column, eluent: linear gradient from 10% CH_3CN and 90% H_2O (containing 0.1% CF_3COOH) to 60% CH_3CN and 90% H_2O (containing 0.1% CF_3COOH) in 60 min). Usual work up and purification by flash-chromatography gave the pure products **18a–20a**. Selected ^{1}H-NMR data (d_6-DMSO, 80°C) δ: **18a**: 7.55 (2H, m, H-2′ and H-6′), 6.88 (1H, d, $J = 9$ Hz, H-5′), 6.45 and 6.24 (each 1H, d, $J = 1.8$ Hz, H-6 and H-8), 5.47 (1H, d, $J = 8.1$ Hz, H-1″), 4.88 (1H, t, $J = 8.5$ Hz, H-3″), 4.18 (1H, dd, $J_1 = 11$ Hz, $J_2 = 1.5$ Hz, H-6″a), 4.02 (1H, dd, $J_1 = 11$ Hz, $J_2 = 5.4$ Hz, H-6″b), 3.56-3.45 (2H, m, H-2″ and H-5″), 3.40 (1H, t, $J = 8.5$ Hz, H-4″), 2.09 and 1.80 (each 3H, s, CH_3CO). **19a**: 7.38–7.24 (2H, m, H-2′ and H-6′), 6.94 (1H, d, $J = 9$ Hz, H-5′), 6.42 and 6.25 (each 1H, d, $J = 1.8$ Hz, H-6 and H-8), 5.34 (1H, s, H-1″), 4.74 (1H, t, $J = 9.0$ Hz, H-4″), 4.08 (1H, m, H-2″), 3.78 (1H, dd, $J_1 = 9$ Hz, $J_2 = 2$ Hz, H-3″), 3.48 (1H, dq, $J_1 = 9$ Hz, $J_2 = 7$ Hz H-5″), 2.02 (3H, s, CH_3CO), 0.79 (3H, d, $J =$ 7.0 Hz, CH_3-6″). **20a**: 7.55 (2H, m, H-2′ and H-6′), 6.90 (1H, d, $J = 9$ Hz, H-5′), 6.41 and 6.23 (each 1H, d, $J = 1.9$ Hz, H-6 and H-8),

Table 6
Acetylation of the flavonoid glycosides **18–20** catalyzed by Novozym 435

Substrate		React. time	Main product (%)
Isoquercitrin	**18**	60 h	3″,6″-*O*-diacetate (79)
Quercitrin	**19**	150 h	4″-*O*-acetate (77)
Rutin	**20**	45 h	3″, 4‴-*O*-diacetate (91)

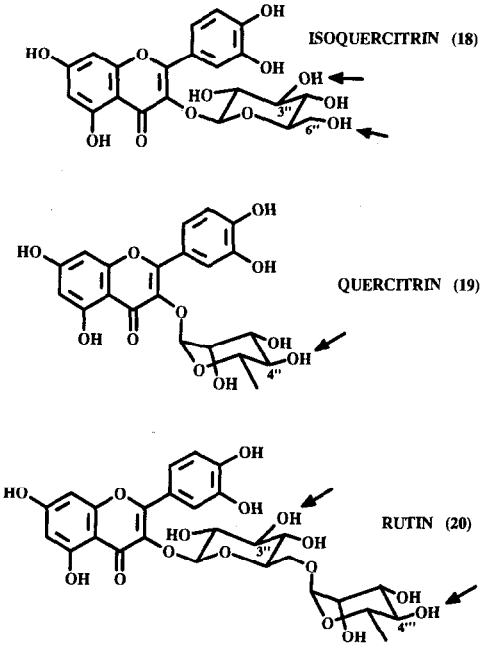


Fig. 2. Structures of isoquercitrin (**18**), quercitrin (**19**) and rutin (**20**).

5.53 (1H, d, $J = 8.0$ Hz, H-1″), 4.87 (1H, t, $J = 8.5$ Hz, H-3″), 4.69 (1H, t, $J = 9.0$ Hz, H-4‴), 4.53 (1H, s, H-1‴), 3.75 (1H, bd, $J =$ 11.5 Hz, H-6″a), 2.08 and 1.96 (3H, s, CH_3CO), 0.87 (3H, d, $J = 7.0$ Hz, CH_3-6″).

3. Results and discussion

In previous works [6,7] on the enzymatic acylation of terpene glycosides, we have shown that CAB directed its catalytic action on selected primary OH's. Therefore, by applying the usual protocol to the two alkaloids colchicoside (**1**) and thiocolchicoside (**2**), we expected to isolate the corresponding 6′-*O*-acetates **1a** and **2a**. Accordingly, CAB-catalyzed acetylation was very efficient but, to our surprise, the products quantitatively obtained were the 2′,6′-*O*-diacetate **1b** and **2b** (Fig. 1). In order to rationalize these results, we looked in the literature for further information on the selectivity of CAB towards sugars secondary OH's. We found only scant data [10,11] and therefore we decided to explore CAB selectivity systematically.

As sugars and their glycosides are usually quite polar compounds, we started this investigation by studying the performance of CAB in different organic solvents suitable for the solubilization of carbohydrates and of their derivatives. We chose cyclohexanol as a secondary alcohol model substrate and the results obtained in its enzymatic acetylation are reported in Table 1. We found that DMF and *N*-methyl–pyrrolidone were incompatible with CAB and that pure pyridine rapidly inactivated this lipase. On the other hand, compared with the 'good' solvent hexane, solvents like acetone, dioxane and THF were well accepted by the enzyme, both in terms of initial rates and final degree of conversion, even in mixture with pyridine (but, again, not with DMF).

Using mixtures of acetone or THF with up to 20% of pyridine as solvent, we studied the behavior of CAB with several simple glycopyranosides, differing each others for the steric series, the nature of the aglycone, and the stereochemistry of the glycosidic bond and/or of some of the secondary OH's (Tables 2–5). The degrees of conversion as well the ratios among the different acetylated isomers were determined by GC (after derivatization with 1,1,1,3,3,3-hexamethyldisilazane) or HPLC. The products were purified by flash chromatography and the structures determined by ^{1}H-NMR using, when it was necessary, selective decoupling or 2D-COSY experiments.

Table 2 shows the results obtained in the acetylation of different (D)-α-glycopyranosides. Acetylation was, in all cases, quantitative within 20 h and selectively directed to the primary OH's to give compounds **3a**–**7a**. Only with methyl α-galactopyranoside **6** the formation of 26% of the 2,6-*O*-diacetate **6b** was observed.

The behavior of the (D)-β-glycopyranosides **8**–**12** was completely different (Table 3). Considering the β-glucopyranosides first, we found that the nature of the aglycone deeply influenced the acetylation outcomes. When the aglycone was a long aliphatic chain, as in **10**, CAB directed its action only on the primary OH. On the other hand, acetylation of methyl β-glucopyranoside **8** gave the 3,6-*O*-diacetate **8a** almost quantitatively, while a 3:1 mixture of the 3,6-*O*- and 2,6-*O*-diacetate **9a** and **9b** was obtained from the phenyl β-glucopyranoside **9**. Finally, as already mentioned, when the aglycone was a large alkaloid moiety as in colchicoside **1** and in thiocolchicoside **2**, the corresponding 2′,6′-*O*-diacetates were obtained almost quantitatively. Moving to methyl and phenyl β-galactopyranoside **11** and **12**, the results were similarly puzzling. Contrary to the corresponding glucosides **8** and **9**, the selectivity of CAB with the methyl galactopyranoside **11** was quite poor (three products were obtained in similar amounts) while with the phenyl galactopyranoside **12** the 3,6-*O*-diacetate **12b** was formed in high yield (92%).

Table 4 reports the results obtained with two useful synthetic derivatives of glucose, methyl 4,6-*O*-benzyliden-D-α- and β-glucopyranoside

13 and **14**. These compounds have only two secondary OH's each, and the behavior of CAB with them was consistent with the previously described results: a very slow acetylation was observed with the α-methyl derivative **13**, while a fast but unselective transformation of the β-methyl isomer **14** was obtained. The results we have obtained on these two substrates with CAB were by far worse than those previously observed with *Pseudomonas cepacia* lipase [12].

Finally, as reported in Table 5, we compared the behavior of enantiomeric pairs of sugars, specifically the benzyl (D)- and (L)-α-mannopyranoside **7** and **16**, and the corresponding rhamnopyranoside **15** and **17**. Comparing the mannopyranosides first, again we found that CAB had a marked preference for the primary 6-OH. However, while with (D)-**7** the selectivity was almost absolute, a 36% of 4,6-*O*-diacetate was observed with the (L)-mannopyranoside **16**. As expected, the (D)-rhamnopyranosides **15** was quite refractary to the acylation by CAB and only 20% of the 4-*O*-acetate **15** was formed after 90 h. On the other hand, the (L)-rhamnopyranoside **17** furnished the 4-*O*-acetate **17a** in 90% yield. By comparing these results with the data published by Ronchetti and coworkers on the selectivities of three other lipases (from *Pseudomonas fluorescens*, *Candida cylindracea* and porcine pancreas) towards secondary OH's of enantiomeric pairs of sugars [13,14] we similarly observed that (L) rhamno- and mannopyranosides are better substrates than the corresponding enantiomers. However, in our case, both enantiomeric rhamnopyranosides have been acetylated at the 4-OH, while *Pseudomonas fluorescens* and porcine pancreatic lipases directed their actions to the 2-OH of D-rhamnopyranosides and to the 4-OH of their (L)-enantiomers [14].

To conclude this work, we decided to exploit some of these results for the selective acylation of flavonoid glycosides. In a previous paper [15] we have shown that the protease subtilisin was able to catalyze the acylation of the flavonoid D-glucopyranoside isoquercitrin (**18**) to afford the corresponding 6″-*O*-mono-, 3″-*O*-mono-, and 3″,6″-*O*-dibutanoyl derivatives, while the corresponding L-rhamnopyranoside quercitrin (**19**) was unaffected. On the light of the previous data, we thought that CAB could be able to acylate both **18** and **19**, and this actually was the case. As it is shown in Table 6, acetylation of quercitrin was very neat and the 4″-*O*-acetate **19a** was formed in 77% yield. As expected, acetylation of isoquercitrin gave a diacetate. 2D-COSY NMR unambiguously indicated that, in this case, the C-3″ OH was the secondary sugar hydroxyl that was acetylated to give the product **18a** in 79% yield. As a last example we considered the flavonoid disaccharide monoglycoside rutin (**20**), a flavonoid which possesses the same aglycone of **18** and **19** and whose sugar moiety is rutinose, that is 6-*O*-(L-α-rhamnopyranosyl)-D-glucose. This compound has only secondary sugar OH's and with subtilisin its acylation gave only the expected 3″-*O*-monoacyl derivative [16]. Acetylation of rutin with CAB gave a parallel result and the 3″, 4‴-*O*-diacetate **20a** was obtained as the only product in 91% yield after 45 h.

4. Conclusions

The results we have reported here, on the line of other systematic investigations on the regioselectivity displayed by lipases [17], clearly indicate the deep influence of the aglycone as well as of the stereochemistry of the glycosidic bond on the selectivity of CAB lipase towards sugars secondary OH's. Even though some of these data are quite puzzling, they offer useful suggestions on the outcomes that could be expected by using CAB with carbohydrates derivatives. All the information we have collected so far [5–7] clearly indicates that this lipase is particularly suitable for the selective acylation of complex glycosides possessing large aglycones and polysaccharides moieties. This interesting reactivity might be due to the absence of the lid usually observed in the 3D-structures of other

lipases [18]. As a consequence, the catalytic machinery of CAB is freely accessible even for large molecules, as it can be clearly deducted from the X-ray structure of this protein [19].

Molecular modelling simulation studies might likely offer useful suggestions to rationalize the results we have obtained. Further investigation on the possible influence of the nature of the organic solvent on the regioselectivity of this lipase is also in progress and the results will be reported in due course.

Acknowledgements

This work was partially supported by PIANO STRATEGICO 'Tecnologie Chimiche Innovative'.

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