

# Highly selective lipase-mediated discrimination of diastereomeric 5,6-epoxysteroids

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**Abstract**—Stereoisomerically pure 3 $\beta$ -hydroxy-5,6-epoxysteroids were obtained by combining selective chemical methods for  $\alpha$ - and  $\beta$ -epoxidation of  $\Delta^5$ -unsaturated steroids with enzymatic stereoselective esterification of the 3 $\beta$ -hydroxyl group. 5 $\beta$ ,6 $\beta$ -Epoxy-3 $\beta$ -hydroxysteroids were efficiently acylated by Novozym 435 and lipase AK, whereas 5 $\alpha$ ,6 $\alpha$ -epoxy-3 $\beta$ -hydroxysteroids were good substrates for *Candida rugosa* lipase. Mild enzymatic deacylation of the 3 $\beta$ -acetoxy group in the presence of the epoxy functionality was also accomplished by *C. rugosa* lipase-mediated hydrolysis.

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## 1. Introduction

Steroid epoxides are an important group of oxysterols, which regulate many biological functions, such as cell proliferation and cholesterol homeostasis.<sup>1</sup> For instance,  $\alpha$ - and  $\beta$ -epoxides of cholesterol are bioactive autooxidation products,<sup>1,2</sup> both being cytotoxic<sup>3</sup> and mutagenic,<sup>4</sup> while the 5 $\beta$ ,6 $\beta$ -epoxy functionality is found in a number of naturally occurring steroids possessing anti-tumour activities, such as the withanolides.<sup>5</sup> Furthermore, epoxides are synthetically valuable intermediates, since their facile ring opening allows the introduction of various functionalities in a stereospecific manner.<sup>6</sup> These properties have attracted considerable attention for the stereoselective synthesis of 5 $\alpha$ ,6 $\alpha$ -epoxides ( $\alpha$ -epoxides) and 5 $\beta$ ,6 $\beta$ -epoxides ( $\beta$ -epoxides) on the steroid nucleus.

Epoxidation of 3 $\beta$ -substituted  $\Delta^5$ -steroids with peracids,<sup>7</sup> such as 3-chloroperoxybenzoic acid (*m*-CPBA), is known to produce predominantly  $\alpha$ -epoxides due to the steric hindrance imposed by the C-10 and C-13 angular methyl groups. Current methods for the synthesis of  $\beta$ -epoxides include the use of halohydrins as intermediates,<sup>8</sup> the use of 3-halo substituents to block the entry of reagents from the alpha face,<sup>9</sup> chromyl diacetate,<sup>10</sup> potassium permanganate/inorganic salts,<sup>11</sup> chiral

ketones/oxone<sup>12</sup> and transition metal complexes in the presence of air or molecular oxygen as oxidant.<sup>13</sup>

However, the majority of these methods fail to give diastereomerically pure  $\alpha$ - and  $\beta$ -epoxides, thus more selective methods are still needed. Furthermore, the presence and stereochemistry of other substituents in the A- and B-rings can influence the stereoselectivity of the epoxidation method.<sup>11a,13b,14</sup>

Generally the methods used to prepare  $\beta$ -epoxides require the appropriate protection of other hydroxyl groups present on the molecule. Chemical deprotection, quite often of an ester, can be hampered by the sensitivity of the oxirane group towards the usual hydrolytic conditions. As a consequence, the synthesis of 5 $\beta$ ,6 $\beta$ -epoxy-hydroxysteroids remains a difficult task.

Recently, biocatalysis, often being complementary to conventional chemical reactions, has become an effective tool for achieving mild and selective transformations.<sup>15</sup> In particular, enzyme catalysis has played an important role for the selective interconversion of functional groups on the steroid structure.<sup>16,17</sup> For example, studies on the enzymatic regioselective esterification of hydroxyl groups located at different positions of the skeleton have shown a preference of lipases for OH's at the C-3 position and of the protease subtilisin Carlsberg for the hydroxyl groups on the D ring.<sup>18</sup> Moreover, the influence of the A/B rings junction (*cis* or *trans*) and of  $\Delta^4$ - or

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$\Delta^5$ -unsaturations on the lipases specifically towards 3-hydroxy steroids has also been demonstrated.<sup>18,19</sup>

In order to provide diastereomerically pure  $\alpha$ - or  $\beta$ -epoxysteroids by taking advantage of the known selectivity of enzymes towards steroidal substrates, we investigated the ability of lipases to discriminate between such epoxides through the acylation or deacylation of a hydroxyl moiety at the 3 $\beta$ -position. Additionally, the mild reaction conditions, provided by enzyme catalysis, were explored for the hydrolysis of 3 $\beta$ -acetoxy esters in the presence of the epoxy functionality.

## 2. Results and discussion

### 2.1. Chemical synthesis of epoxides

$\alpha$ -Selective epoxidations of both 3 $\beta$ -hydroxy- and 3 $\beta$ -acetoxy- $\Delta^5$ -unsaturated steroids were performed with magnesium monoperoxyphthalate hexahydrate (MMPP), which is safer and easier to remove than common peracids, like *m*-CPBA.<sup>20</sup> Furthermore,  $\beta$ -selective epoxidation of 3 $\beta$ -acetoxy- $\Delta^5$ -unsaturated steroids can be carried out through the low cost and eco-friendly<sup>11</sup> heterogeneous permanganate oxidation, following a procedure developed by our group.<sup>11b</sup> The  $\alpha/\beta$  ratio of epoxides was determined by the integration of the H-6 proton signal in the <sup>1</sup>H NMR spectra, which is known to appear as a doublet at around 2.9 ppm ( $J = 4$  Hz) in the  $\alpha$  isomer and as a doublet at 3.1 ppm ( $J = 2$  Hz) in the  $\beta$  isomer.<sup>21,13a</sup> As expected, the diastereomeric excesses ranged between 70% and 80%.

### 2.2. Stereoselective lipase-catalysed esterification of 3 $\beta$ -hydroxy-5,6-epoxysteroids to produce diastereomerically pure $\alpha$ -epoxides

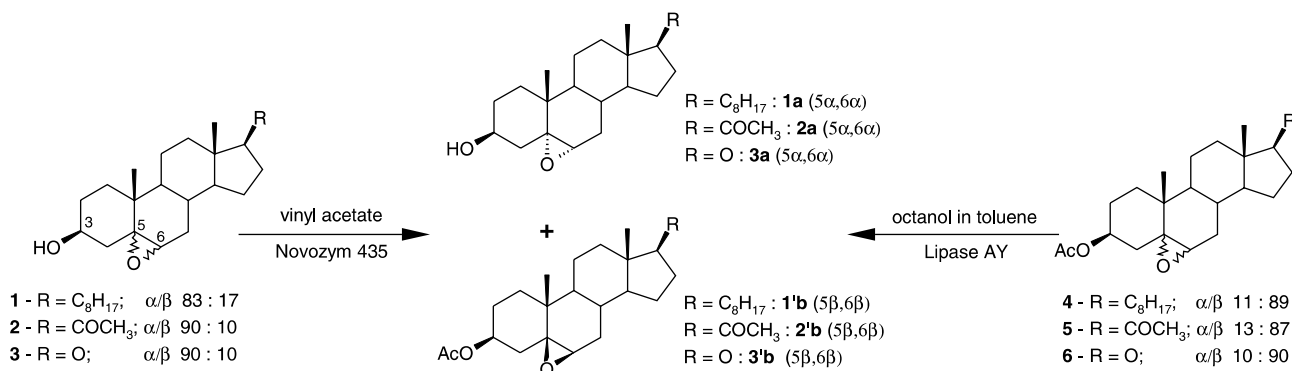
The lipase-catalysed esterification of a 3 $\beta$ -hydroxy group in the presence of a 5,6-epoxy functionality was studied by screening the performances of a range of 14 commercial hydrolases using diastereomerically enriched 5,6-epoxycholestan-3 $\beta$ -ol **1** ( $\alpha/\beta$  ratio 83:17) and 5,6-epoxy-3 $\beta$ -hydroxypregnan-20-one **2** ( $\alpha/\beta$  ratio

90:10) as substrates (Scheme 1, left side). Under irreversible transesterification<sup>22</sup> conditions (using vinyl acetate as the acyl donor and toluene as the solvent), TLC monitoring allowed the identification of a small group of lipases active on these substrates. Almost complete conversion of **1** was observed with *Candida rugosa* lipase, suggesting a good activity of this enzyme for the predominant  $\alpha$ -isomer, whereas minor conversion was noticed with Novozym 435, *Chromobacterium viscosum* lipase, lipase AK and lipase PS. Later on, the stereoselectivity of this group of lipases was more accurately measured by monitoring the acylation of the diastereomerically pure  $\alpha$ -epoxide **1a** and  $\beta$ -epoxide **1b** of cholesterol (obtained as described below) by GC. As shown in Table 1, the stereopreference of Novozym 435 for the acylation of the 3 $\beta$ -OH of the  $\beta$ -epoxide **1b** was very high, while two different commercial preparations of *C. rugosa* lipase confirmed a significant preference for the  $\alpha$ -epoxy isomer **1a** (first two entries of Table 1).

By exploiting these results, Novozym 435 was employed in preparative scale reactions to remove the minor  $\beta$ -isomer in  $\alpha$ -enriched mixtures of 3 $\beta$ -hydroxy-5,6-epoxysteroids (Scheme 1, left hand side). In all cases, the unreacted  $\alpha$ -epoxides, **1a**, **2a** and **3a**, were obtained in good isolated yields (67–72%) and 100% diastereomeric excesses, as confirmed by <sup>1</sup>H NMR analysis.

### 2.3. Stereoselective lipase-catalysed deacetylation of 3 $\beta$ -acetoxy-5,6-epoxysteroids to produce diastereomerically pure $\beta$ -epoxides

The next step was to find a suitable methodology for obtaining the complementary pure  $\beta$ -epoxides from the  $\beta$ -enriched mixtures of the corresponding acetates **4–6** prepared through permanganate epoxidation.<sup>11b</sup> Initially the stereoselective deacetylation of epimeric 3 $\beta$ -acetoxy-5,6-epoxysteroids was investigated using 1-octanol as an acyl acceptor in alcoholysis reactions (Scheme 1, right hand side). Exploiting the stereopreference of *C. rugosa* lipase for  $\alpha$ -epoxides (Table 1, lines 1–2), preparative scale reactions were run in the presence of lipase AY in order to remove the minor  $\alpha$ -epoxide in mixtures **4–6**. As expected, the unreacted 3-*O*-acetoxy  $\beta$ -epoxides (**1'b**, **2'b** and **3'b**) were obtained in good



**Scheme 1.** Novozym 435-catalysed stereoselective acetylation of 3 $\beta$ -hydroxy-5,6-epoxysteroids and lipase AY-catalysed stereoselective alcoholysis of 3 $\beta$ -acetoxy-5,6-epoxysteroids.

**Table 1.** Lipase-catalysed acetylation of **1a** and **1b**

	Substrate <sup>b</sup>	Conversion (%) <sup>a</sup>			
		90 min	3 h	6 h	24 h
<i>C. rugosa</i> lipase (Sigma) 3 mg	<b>1a</b>	62.3	84.8	86.5	96.4
	<b>1b</b>	3.9	6.5	9.8	12.7
Lipase AY <sup>c</sup> (Amano) 1.5 mg	<b>1a</b>	80.8	90.5	92	96.3
	<b>1b</b>	2.5	6.3	7.8	9.8
Novozym 435 <sup>d</sup> 10 mg	<b>1a</b>	0	0	0	1
	<b>1b</b>	14.3	26.4	47.9	82.3
Lipase PS <sup>e</sup> (Amano) 5 mg	<b>1a</b>	10.2	23.7	33	71.4
	<b>1b</b>	13.7	27.9	50	88.2
Lipase AK <sup>f</sup> (Amano) 3 mg	<b>1a</b>	1.7	2.4	4.3	11.9
	<b>1b</b>	15.2	29.6	53.1	90.6
<i>C. viscosum</i> lipase (Finnsugar) 30 mg	<b>1a</b>	0	0	0	4.6
	<b>1b</b>	4.5	9.6	16.7	41.7

<sup>a</sup> Evaluated by GC.<sup>b</sup> 10 mM 5 $\alpha$ ,6 $\alpha$ - or 5 $\beta$ ,6 $\beta$ -Epoxycholestan-3 $\beta$ -ol, **1a** or **1b**, (4 mg/ml) in toluene with 10% v/v vinyl acetate.<sup>c</sup> From *C. rugosa*.<sup>d</sup> From *C. antarctica*.<sup>e</sup> From *P. cepacia*.<sup>f</sup> From *P. fluorescens*.

isolated yields (62–75%) and 100% diastereomeric excess, as confirmed by <sup>1</sup>H NMR analysis. On the other hand, quite unexpectedly, neither Novozym 435 nor any other tested lipases were able to catalyse the selective alcoholysis of the 3 $\beta$ -acetoxy group of  $\beta$ -epoxides.

#### 2.4. Mild nonselective lipase-catalysed deacetylation of 3 $\beta$ -acetoxy-5,6-epoxysteroid mixtures

As a final step, we searched for reaction conditions able to promote a nonselective enzymatic alcoholysis, or hydrolysis, of the 3 $\beta$ -acetoxy group in epimeric 5,6-epoxysteroid mixtures (i.e., to transform **4–6** into **7–9**). In fact, a mild deacylation protocol is of synthetic value since most of the methods used to prepare 5 $\beta$ ,6 $\beta$ -epoxides are only applicable to 3-*O*-acylated substrates, and the subsequent chemical deacylation by standard procedures usually affect also the sensitive epoxy groups.

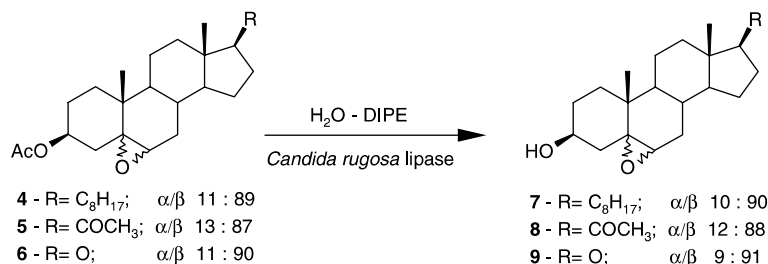
The lack of selectivity shown by lipase PS in the acylation of these substrates (Table 1) suggested that this lipase might be the enzyme of choice to accomplish the requested mild deacylation reaction. Unfortunately, this lipase did not accept 3 $\beta$ -acetoxy epoxysteroids as substrates in alcoholysis under hydrolysis reactions.

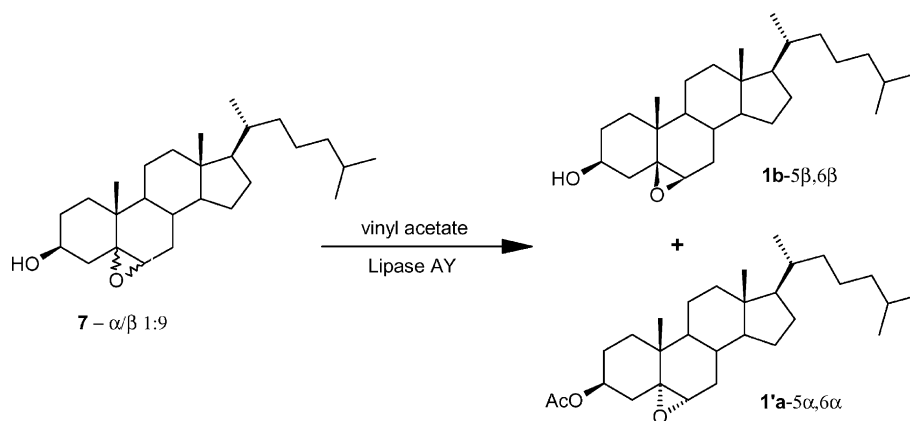
Therefore, we turned our attention again to *C. rugosa* lipase, as this enzyme showed a minor activity towards the  $\beta$ -epoxide epimers (Table 1). Careful optimisation of the reaction conditions indicated that water-saturated diisopropyl ether (DIPE) was the optimal system for performing the required deacylation in the presence of both  $\alpha$ - and  $\beta$ -5,6-epoxides. Accordingly, scale-up reactions allowed complete enzymatic hydrolysis of the 3 $\beta$ -acetate in the epimeric mixtures **4–6** without affecting the sensitive epoxides to give the corresponding alcohols **7–9** (Scheme 2).

The  $\beta$ -enriched mixture of 5,6-epoxides of cholesterol **7** thus obtained was submitted to AY-catalysed stereoselective acylation in order to remove the minor  $\alpha$ -epoxide, rendering diastereomerically pure 5 $\beta$ ,6 $\beta$ -epoxycholestan-3 $\beta$ -ol **1b** (Scheme 3), which was used to study the stereoselectivity of lipases to **1a** and **1b** by GC analysis (Table 1).

### 3. Conclusion

Herein we have reported the further usefulness of lipases for the selective modification of steroids. Specifically,

**Scheme 2.** *C. rugosa* lipase-catalysed mild deacylation of 3 $\beta$ -acetoxy-5,6-epoxysteroids.



**Scheme 3.** Lipase AY-catalysed stereoselective acetylation of 5,6-epoxycholestane-3β-ol 7.

diastereomerically pure  $\alpha$ -epoxides can be obtained by exploiting the strict selectivity of Novozym 435 for the minor  $\beta$ -epoxides components of epimeric mixtures. This biotransformation not only confirms the selectivity of this lipase, originally isolated from the yeast *Candida antarctica*, but is also a new noteworthy example of selectivity control by remote stereocentres.<sup>23</sup>

The screening of a library of commercially available lipases also indicated that the enzyme from *C. rugosa* lipase has an opposite selectivity compared to Novozym 435, thus allowing the isolation of diastereomerically pure  $\beta$ -epoxides via alcoholysis reactions.

Finally, a mild and clean deacylation reaction of the 3 $\beta$ -acetoxy group in the presence of the sensitive 5,6-epoxy moiety was attained using *C. rugosa* lipase as catalyst and water as nucleophile.

## 4. Experimental

### 4.1. General

All commercially available chemicals were used as supplied by the manufacturers. Steroids and crude *C. rugosa* lipase were from Sigma. Novozym 435 (immobilised *C. antarctica* lipase B) and Lypozyme IM 20 were from Novo Nordisk. Lipase PS (from *Pseudomonas cepacia*) was purchased from Amano and adsorbed on Celite as described elsewhere.<sup>24</sup> Lipase AK (from *Pseudomonas fluorescens*) and lipase AY (from *C. rugosa*) were from Amano. *C. viscosum* lipase was supplied by Finnsugar. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on a Bruker AC-300 at 300 and 75.47 MHz, respectively, using CDCl<sub>3</sub> as a solvent. Chemical shifts are reported on the  $\delta$  (ppm) scale and are relative to tetramethylsilane as the internal standard. Infrared spectra were recorded on a Jasco FT/IR-420 spectrometer. Optical rotations were determined on a Jasco P-1020 polarimeter. Melting points were measured in a Büchi B-540. TLC monitoring was done in toluene/diethyl ether (85:15) as eluent, and detected using Komarowsky's reagent.<sup>25</sup> The

degrees of conversion were determined by gas chromatography using a Hewlett Packard 5890 series II instrument, a HP-1 Crosslinked Methyl Silicone Gum (25 m, 0.32 mm ID, Hewlett Packard), oven temperatures ranging from 236 to 290 °C and H<sub>2</sub> as the carrier gas.

### 4.2. Chemical synthesis of 5 $\alpha$ ,6 $\alpha$ - and 5 $\beta$ ,6 $\beta$ -epoxysteroids

**4.2.1. Representative procedure for the epoxidation of 3 $\beta$ -hydroxy- $\Delta^5$  unsaturated steroids with MMPP.** To a solution of the 3 $\beta$ -hydroxy- $\Delta^5$ -unsaturated steroid (1 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 ml) and ethanol (2 ml), magnesium monoperoxyphthalate hexahydrate (MMPP, 990 mg, 2 mmol) was added and the suspension stirred at room temperature for 24 h. After this time the reaction was shown to have gone to completion (TLC control). Then, sodium chloride and diethyl ether were added and the suspension filtered through Celite. The filtrate was washed with 5% HCl, satd NaHCO<sub>3</sub>, water and brine, dried over MgSO<sub>4</sub> and evaporated to dryness, giving the  $\alpha$ -enriched epimeric mixtures of the epoxides as the only products.

**Compound 1:** <sup>1</sup>H NMR  $\delta$ : 0.61 (3H, s, CH<sub>3</sub>-18), 1.06 (3H, s, CH<sub>3</sub>-19), 2.91 and 3.07 (1H, d each,  $J = 4.362$  and 2.190 Hz, respectively, H-6), 3.90 (1H, m, H-3). The integration of the two signals (doublet peaks) due to the H-6 proton gave a 8.3:1.7  $\alpha/\beta$  ratio. <sup>13</sup>C NMR  $\delta$ : 59.33 (C-3), 65.77 (C-5), 68.70 (C-6). IR (ATR):  $\nu_{\max}$  1038.48, 1366.3, 1465.6, 2864.7, 2931.3, 3395 cm<sup>-1</sup>.

**Compound 2:** <sup>1</sup>H NMR  $\delta$ : 0.56 (3H, s, CH<sub>3</sub>-18), 1.00 (3H, s, CH<sub>3</sub>-19), 2.03 (3H, s, CH<sub>3</sub>-21), 2.92 and 3.08 (1H, d each,  $J = 4.359$  and 2.352 Hz, respectively, H-6), 3.90 (1H, m, H-3). The integration of the two signals (doublet peaks) due to the H-6 proton gave a 9:1  $\alpha/\beta$  ratio. <sup>13</sup>C NMR  $\delta$ : 59.06 (C-3), 63.33 (C-17), 65.67 (C-5), 68.58 (C-6), 209.52 (C-20). IR (ATR):  $\nu_{\max}$  1049.1, 1355, 1684, 1700.9, 2930.3, 3437.5 cm<sup>-1</sup>.

**Compound 3:** <sup>1</sup>H NMR  $\delta$ : 0.82 (3H, s, CH<sub>3</sub>-18), 1.09 (3H, s, CH<sub>3</sub>-19), 2.95 and 3.13 (1H, d each,  $J = 4.329$  and 2.532 Hz, respectively, H-6), 3.90 (1H, m, H-3). The

integration of the two signals (doublet peaks) due to the H-6 proton gave a 9:1  $\alpha/\beta$  ratio.  $^{13}\text{C}$  NMR  $\delta$ : 58.75 (C-3), 65.72 (C-5), 68.49 (C-6), 220.68 (C-17). IR (ATR):  $\nu_{\text{max}}$  1027.9, 1725.0, 2944.8, 3518  $\text{cm}^{-1}$ .

**4.2.2. Representative procedure for the epoxidation of 3 $\beta$ -acetoxy- $\Delta^5$ -unsaturated steroids with  $\text{KMnO}_4$ /inorganic salts.** To a solution of the 3 $\beta$ -acetoxy- $\Delta^5$ -unsaturated steroid (1 mmol) in  $\text{CH}_2\text{Cl}_2$  (5 ml), a mixture of finely powdered  $\text{KMnO}_4$  (350 mg) and  $\text{Fe}_2(\text{SO}_4)_3 \cdot (\text{H}_2\text{O})_n$  (175 mg) was added. Then, water (75  $\mu\text{l}$ ) and *t*-butyl alcohol (0.5 ml) were added and the reaction mixture left overnight at room temperature under magnetic stirring. After this time the reaction was shown to have gone to completion (TLC control). Sodium chloride and diethyl ether were added and the suspension was filtered through Celite. The filtrate was evaporated to dryness, giving the  $\beta$ -enriched epimeric mixtures of the epoxides as the only products.

Compound **4**:  $^1\text{H}$  NMR  $\delta$ : 0.64 (3H, s,  $\text{CH}_3$ -18), 1.00 (3H, s,  $\text{CH}_3$ -19), 2.03 (3H, s,  $\text{CH}_3\text{CO}$ ), 2.90 and 3.08 (1H, d each,  $J = 4.371$  and 1.941 Hz, respectively, H-6), 4.77 (1H, m, H-3). The integration of the two signals (doublet peaks) due to the H-6 proton gave a 1.1:8.9  $\alpha/\beta$  ratio.  $^{13}\text{C}$  NMR  $\delta$ : 59.14 (C-6), 65.15 (C-5), 71.36 (C-3), 170.21 ( $\text{CH}_3\text{CO}$ ). IR (ATR):  $\nu_{\text{max}}$  1030.8, 1243.9, 1363.4, 1465.6, 1731.8, 2866.7, 2933.2  $\text{cm}^{-1}$ .

Compound **5**:  $^1\text{H}$  NMR  $\delta$ : 0.59 (3H, s,  $\text{CH}_3$ -18), 1.01 (3H, s,  $\text{CH}_3$ -19), 2.03 (3H, s,  $\text{CH}_3\text{CO}$ ), 2.11 (3H, s,  $\text{CH}_3$ -21), 2.91 and 3.09 (1H, d each,  $J = 4.347$  and 2.163 Hz, respectively, H-6), 4.77 (1H, m, H-3). The integration of the two signals (doublet peaks) due to the H-6 proton gave a 1.3:8.7  $\alpha/\beta$  ratio.  $^{13}\text{C}$  NMR  $\delta$ : 62.42 (C-5), 63.35 (C-17), 63.60 (C-6), 71.19 (C-3), 170.55 ( $\text{CH}_3\text{CO}$ ), 209.37 (C-20). IR (ATR):  $\nu_{\text{max}}$  1029.8, 1243.9, 1356.7, 1698.9, 1735, 2930.0  $\text{cm}^{-1}$ .

Compound **6**:  $^1\text{H}$  NMR  $\delta$ : 0.85 (3H, s,  $\text{CH}_3$ -18), 1.04 (3H, s,  $\text{CH}_3$ -19), 2.04 (3H, s,  $\text{CH}_3\text{CO}$ ), 2.94 and 3.15 (1H, d each,  $J = 4.341$  and 2.493 Hz, respectively, H-6), 4.77 (1H, m, H-3). The integration of the two signals (doublet peaks) due to the H-6 proton gave a 0.9:9.1  $\alpha/\beta$  ratio.  $^{13}\text{C}$  NMR  $\delta$ : 62.56 (C-5), 63.17 (C-6), 71.10 (C-3), 170.53 ( $\text{CH}_3\text{CO}$ ), 220.77 (C-17). IR (ATR):  $\nu_{\text{max}}$  1029.8, 1240.0, 1727.9, 2946.7  $\text{cm}^{-1}$ .

### 4.3. Enzymatic acylation of 3 $\beta$ -hydroxy-5,6-epoxysteroids

In a typical screening assay, 3 $\beta$ -hydroxy-5 $\alpha$ ,6 $\alpha$ -epoxy- or 3 $\beta$ -hydroxy-5 $\beta$ ,6 $\beta$ -epoxy-steroid (10 mg) dissolved in toluene (0.9 ml) and vinyl acetate (0.1 ml) was added to the enzyme (50 mg of crude enzymes, 10 mg of Lypozyme IM 20 or 10 mg of Novozym 435) in 3 ml vials. The vials were stopped with a cap and shaken at 250 rpm at 45 °C. The reactions were monitored by TLC and GC (see Table 1).

**4.3.1. 5 $\alpha$ ,6 $\alpha$ -Epoxycholestan-3 $\beta$ -ol, 1a.** To a solution of 5,6-epoxycholestan-3 $\beta$ -ol **1** (150 mg,  $\alpha/\beta$  ratio 8.3:1.7) in

toluene (8.5 ml) and vinyl acetate (1.5 ml), Novozym 435 (75 mg) was added and the reaction shaken at 45 °C. After 24 h the enzyme was filtered off and the solvent evaporated. 5 $\alpha$ ,6 $\alpha$ -Epoxycholestan-3 $\beta$ -ol **1a** (100 mg, 67% yield) was isolated by flash chromatography (petroleum ether/ethyl acetate 1:1) as a single diastereomer  $[\alpha]_{\text{D}}^{21} = -44.5$ , (*c* 0.5,  $\text{CHCl}_3$ ) {lit.<sup>26</sup>  $[\alpha]_{\text{D}} = -47$ }, mp 141.5–142.7 °C (lit.<sup>26</sup> 141–142 °C) as indicated by  $^1\text{H}$  NMR.

**4.3.2. 5 $\alpha$ ,6 $\alpha$ -Epoxy-3 $\beta$ -hydroxypregnan-20-one, 2a.** To a solution of 5,6-epoxy-3 $\beta$ -hydroxypregnan-20-one **2** (150 mg,  $\alpha/\beta$  ratio 9.1:0.9) in toluene (8.5 ml) and vinyl acetate (1.5 ml), Novozym 435 (75 mg) was added and the reaction shaken at 45 °C. After 24 h the enzyme was filtered off and the solvent evaporated. 5 $\alpha$ , 6 $\alpha$ -epoxy-3 $\beta$ -hydroxypregnan-20-one **2a** (108 mg, 72% yield) was isolated by flash chromatography (petroleum ether, ethyl acetate 1:1) as a single diastereomer  $[\alpha]_{\text{D}}^{21} = +6.35$ , (*c* 0.84,  $\text{CHCl}_3$ ) {lit.<sup>27</sup>  $[\alpha]_{\text{D}} = +1$ }, mp 179.2–182.0 °C (lit.<sup>27</sup> 190–191 °C), as indicated by  $^1\text{H}$  NMR.

**4.3.3. 5 $\alpha$ ,6 $\alpha$ -Epoxy-3 $\beta$ -hydroxyandrostan-17-one, 3a.** To a solution of 5,6-epoxy-3 $\beta$ -hydroxyandrostan-17-one (**3**, 150 mg  $\alpha/\beta$  ratio 9:1) in toluene (8.5 ml) and vinyl acetate (1.5 ml), Novozym 435 (75 mg) was added and the reaction was shaken at 45 °C. After 24 h the enzyme was filtered off and the solvent was evaporated. 5 $\alpha$ ,6 $\alpha$ -Epoxy-3 $\beta$ -hydroxyandrostan-17-one (**3a**, 103 mg, 69% yield) was isolated by flash chromatography (petroleum ether/ethyl acetate 1:1.5) as a single diastereomer  $[\alpha]_{\text{D}}^{21} = -4.3$ , (*c* 0.5,  $\text{CHCl}_3$ , mp 225–228 °C (lit.<sup>9b</sup> 227–230 °C)}, as indicated by  $^1\text{H}$  NMR.

### 4.4. Enzymatic deacylation of 3 $\beta$ -acetoxy-5 $\alpha$ ,6 $\alpha$ - and 5 $\beta$ ,6 $\beta$ -epoxysteroids

In a typical screening assay, the 3 $\beta$ -acetoxy-5 $\alpha$ ,6 $\alpha$ -epoxy- or 3 $\beta$ -acetoxy-5 $\beta$ ,6 $\beta$ -epoxy-steroid (10 mg) having been dissolved in toluene (0.9 ml) and octanol (0.1 ml), were added to the enzyme (50 mg of crude enzymes, 10 mg of Lypozyme IM 20 or 10 mg of Novozym 435) in 3 ml vials. The vials were stopped with a cap and shaken at 250 rpm at 45 °C. The reactions were monitored by TLC over 5 days.

**4.4.1. 5 $\beta$ ,6 $\beta$ -Epoxycholestan-3 $\beta$ -yl acetate, 1'b.** To a solution of 5,6-epoxycholestan-3 $\beta$ -yl acetate **4** (180 mg,  $\alpha/\beta$  ratio 1.1:8.9) in toluene (8 ml) and octanol (2 ml), lipase AY (200 mg) was added and the reaction shaken at 45 °C. After 3 days, the enzyme was filtered off and solvent was evaporated. 5 $\beta$ ,6 $\beta$ -Epoxycholestan-3 $\beta$ -yl acetate **1'b** (112 mg, 62% yield) was isolated by flash chromatography (petroleum ether/ethyl acetate 2:1) as a single diastereomer  $[\alpha]_{\text{D}}^{21} = -0.57$ , (*c* 1.09,  $\text{CHCl}_3$ ) {lit.<sup>28</sup>  $[\alpha]_{\text{D}} = -0.2$ }, mp 109–111 °C (lit.<sup>28</sup> 112–113 °C), as indicated by  $^1\text{H}$  NMR.

**4.4.2. 5 $\beta$ ,6 $\beta$ -Epoxy-20-oxo-pregnan-3 $\beta$ -yl acetate, 2'b.** To a solution of 5,6-epoxy-20-oxo-pregnan-3 $\beta$ -yl acetate **5**, (180 mg,  $\alpha/\beta$  ratio 1.3:8.7) in toluene (8 ml) and octanol (2 ml), lipase AY (200 mg) was added and the reaction shaken at 45 °C. After 3 days, the enzyme was filtered off and the solvent was evaporated. 5 $\beta$ ,6 $\beta$ -Epoxy-20-oxo-pregnan-3 $\beta$ -yl acetate **2'b** (126 mg, 70% yield) was isolated by flash chromatography (petroleum ether/ethyl acetate 2:1) as a single diastereomer.  $[\alpha]_D^{21} = +50.6$ , (*c* 1.18, CHCl<sub>3</sub>) {lit.<sup>8a</sup>  $[\alpha]_D = +50$ }, mp 132–134 °C (lit.<sup>8a</sup> 132–136 °C), as indicated by <sup>1</sup>H NMR.

**4.4.3. 5 $\beta$ ,6 $\beta$ -Epoxy-17-oxo-androstan-3 $\beta$ -yl acetate, 3'b.** To a solution of 5,6-epoxy-17-oxo-androstan-3 $\beta$ -yl acetate **6** (180 mg,  $\alpha/\beta$  ratio 1:9) in toluene (8 ml) and octanol (2 ml), lipase AY (200 mg) was added and the reaction shaken at 45 °C. After 3 days, the enzyme was filtered off and the solvent evaporated. 5 $\beta$ ,6 $\beta$ -Epoxy-17-oxo-androstan-3 $\beta$ -yl acetate **3'b** (135 mg, 75% yield) was isolated by flash chromatography (petroleum ether/ethyl acetate 1:1) as a single diastereomer,  $[\alpha]_D^{21} = +43.0$ , (*c* 1.4, CHCl<sub>3</sub>) {lit.<sup>29</sup>  $[\alpha]_D = +40.5$ }, mp 186.7–188 °C (lit.<sup>29</sup> 185–187 °C), as indicated by <sup>1</sup>H NMR.

#### 4.5. Total hydrolysis of 3 $\beta$ -acetoxy-5 $\alpha$ ,6 $\alpha$ - and 5 $\beta$ ,6 $\beta$ -epoxysteroids

In a typical experiment, the 3 $\beta$ -acetoxy-5,6-epoxysteroid (150 mg), obtained by permanganate epoxidation, was dissolved in diisopropyl ether (35 ml) presaturated with water and then *C. rugosa* lipase (1.8 g) added. The mixture was shaken at 250 rpm, 45 °C and after 3 days the reactions were shown to have gone to completion. The products were isolated and the  $\alpha/\beta$  ratios quantified by <sup>1</sup>H NMR: 5,6-epoxycholestan-3 $\beta$ -ol **7** (136 mg,  $\alpha/\beta$  ratio 1:9), 5,6-epoxy-3 $\beta$ -hydroxypregnan-20-one **8** (130 mg,  $\alpha/\beta$  ratio 1.2:8.8) and 5,6-epoxy-3 $\beta$ -hydroxy-androstan-17-one **9** (135 mg,  $\alpha/\beta$  ratio 0.9:9.1).

#### 4.6. 5 $\beta$ ,6 $\beta$ -Epoxycholestan-3 $\beta$ -ol 1b

To a solution of 5,6-epoxycholestan-3 $\beta$ -ol **7** (136 mg,  $\alpha/\beta$  ratio 1:9) in toluene (6 ml) and vinyl acetate (1 ml), Lipase AY (100 mg) was added and the reaction shaken at 45 °C. After 3 days the enzyme was filtered off and the solvent evaporated. 5 $\beta$ ,6 $\beta$ -Epoxycholestan-3 $\beta$ -ol **1b** (100.5 mg, 74% yield) was isolated by flash chromatography (petroleum ether/ethyl acetate 1:1) as a single diastereomer,  $[\alpha]_D^{21} = +8.0$ , (*c* 0.5, CHCl<sub>3</sub>) {lit.<sup>29</sup>  $[\alpha]_D = +7.8$ }, mp 127–129 °C (lit.<sup>29</sup> 128–131 °C), as indicated by <sup>1</sup>H NMR.

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