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Regioselective enzymatic acylation of vicinal diols of steroids

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Abstract—Monoacylated derivatives of a complete set of 2,3- and 3,4-vicinal diols of steroids were prepared by regioselective lipase-catalysed transesterification reactions. The enzymes displayed different selectivities towards the vicinal diols depending on the configuration of the hydroxyl groups.

1. Introduction

Polyhydroxylated steroids bearing vicinal diols on the A-ring are frequently found in Nature and some of them have relevant biological activities. For instance, the *trans*-diaxial 2β,3α-di-hydroxy pattern is present in natural sulphated sterols with antiviral[1] or anti-angiogenic[2] action, while 2α,3α-diols isolated from marine sources hold cytotoxic activity.[3] Steroidal saponins displaying *trans*-diequatorial 2α,3β-vicinal diols are quite frequent as gitogenin derivatives with antitumor properties.[4] In turn, the 3β,4β-diol functionality is present in a variety of steroids like in the agosterols, which induce reversal of multidrug resistance[5] and proteasome inhibition,[6] as well as in formestane metabolites[7] and in volkendousins, which are potent antitumor agents.[8] Finally, the 3α,4β-vicinal diol pattern has been identified in contignasterol, a natural anti-inflammatory compound[9] and, recently, in a steroid possessing chemotaxis activity.[10]

The discovery that lipases and proteases are able to act in organic solvents opened the way to an intensive synthetic exploitation of these biocatalysts, which, as shown in hundreds of papers and several industrial applications, display remarkable chemo-, regio- and stereoselectivity.[11]

Specifically in the steroids field, enzyme catalysis can play an important role for the mild and selective interconversion of functional groups via regioselective transformations.[12–19] Studies on the transesterification of polyfunctionalyzed steroids have shown that hydrolases can have access to substituents either on the A-ring or on the D-ring and/or on the side-chain of steroids. Several lipases showed a preference for C-3 hydroxyl groups,[13,14] whereas the protease subtilisin Carlsberg catalysed the acylation of C-17 OH.[14] Moreover, stereoselective resolutions of epimeric alcohols located on the steroid side-chain have been carried out by lipase PS[15] and, more recently, by subtilisin.[16]

Concerning the modifications of A-ring substituents, the selectivity of lipases for 3-hydroxysteroids has been applied to the chemoenzymatic synthesis of pharmacologically relevant tibolone metabolites.[17] Quite recently, we have reported a highly selective lipase-catalysed preparation of epimerically pure 5α,6α- and 5β,6β-epoxysteroids through acylation or deacylation reactions at the C-3 OH.[18] Finally, the ability to discriminate among different hydroxyl groups on the A-ring has been demonstrated in the esterification of ecdysteroids catalysed by *Candida antarctica* lipase, which afforded the 2β-monoacyl derivatives in good yields.[19]

In this context, to further explore the enzymatic transformations of steroids, we endeavoured a systematic study on the selectivity of commercially available lipases towards a complete set of stereoisomeric 2,3- and 3,4-vicinal diols, to provide a new tool for the selective transformation of these molecules and of related natural compounds.

2. Results and discussion

Different synthetic strategies were used to afford the

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Table 1. Lipase-catalysed monoacylation of vicinal diols[a]

Substrate	Novozym 435	*C. rugosa* lipase	Lipase AY	Lipase PS	Lipase AK	*C. viscosum* lipase	Porcine pancreatic lipase	Lipozyme IM 20	Lipase CE
1	−	++	+	+	+	+	−	−	−
2	−	+	+	++	+	+	−	−	−
3	++	−	−	−	−	−	−	−	−
4	++	+[b]	+[b]	++	−	−	−	−	−
5	+	++	++	+	+	+	−	−	−
6	+	+	+	+	+	++	−	−	−
7	−	+	−	++	+	+	−	−	−
8	−	−	−	−	−	−		−	−

[a] Conversion and product(s) formation was evaluated by TLC.
[b] The formation of two products was observed.

requested stereoisomeric vicinal diols **1**–**8**. Woodward's *cis*-dihydroxylation method, which is known to be selective for the more hindered β-face of 5α-steroids,[20] was applied to Δ^2- or Δ^3-unsaturated precursors affording the *cis*-β diols **1** and **5** (Scheme 2). Complementarily, the *cis*-α diols **3** and **7** were accessed through osmium tetroxide-mediated dihydroxylation on the same olefins.[21] *Trans*-diequatorial diols **2** and **6** were obtained by two different approaches. Starting from cholestan-3-one, α-acetoxylation by lead tetracetate,[22] followed by stereoselective reduction by $NaBH_4/CeCl_3$ and deacetylation rendered the diol **2**. On the other hand, the 3β,4α-diol **6** was directly accessed by hydroboration of the Δ^4-3-one precursor.[23] Finally, *trans*-diaxial diols **4** and **8** were obtained by epoxide opening reactions.

As shown in the formula, the 2,3-diols were prepared by modifying the cholestane skeleton, whereas the difficulties found on separating the 5α- and 5β-epimers of Δ^3-cholestane by sequential crystallizations led us to prepare the set of 3,4-diols in the androstane series. The cholestane derivative **9** was also synthesised to confirm that lipases selectivity towards the A-ring OH was not affected by different substituents on the D-ring.

The performances of a panel of 9 commercial lipases were evaluated for the esterification of the vicinal diols **1**–**9**, using vinyl acetate as the acyl donor and toluene or acetone/THF as solvents for the cholestane or the androstane derivatives, respectively. TLC monitoring allowed the identification, for each substrate, of the lipase(s) able to promote the monoacylation of the substrates. As shown in Table 1, all the stereoisomeric vicinal diols were accepted as substrates by some of the enzymes tested with the exception of compound **8**.

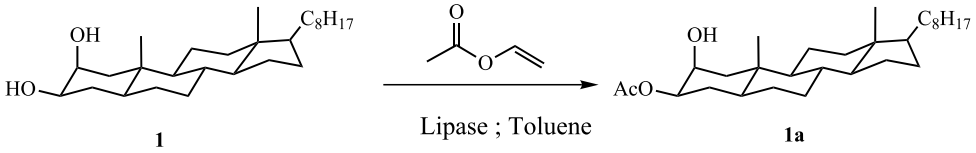
Lipases from different sources (*Candida antarctica*, column 2; *Candida rugosa*, columns 3 and 4; *Pseudomonas* strains, columns 5 and 6; *Chromobacterium viscosum*, column 7) were able to acylate the target compounds. Usually more than one enzyme was acting on the same substrate, with the notable exception of compound **3** (2α,3α-diol) acylated only by Novozym 435 (immobilized lipase B from *Candida antarctica*). Enzymatic acylations were highly regioselective, showing the formation of only one product by TLC, with the exception of *Candida rugosa* lipase (from Sigma or Amano) acting on compound **4**. Noteworthy, lipase PS and Novozym 435 showed complementary regioselectivity towards compound **4**.

For each substrate the best performing lipase (evaluated by TLC) was chosen for scale-up reactions, allowing the isolation of the corresponding monoester in good yields (Scheme 1). Products identification was easily done by NMR analysis (downfield shift of the signals due to the proton geminal to the acylated OH) and, when possible, by comparison with literature data.

The general preference of lipases for the C-3 OH[13,14] was observed with most of the substrates (Scheme 2). Specifically, the diequatorial vicinal diols **2** and **6** were converted into the corresponding 3β-acetate, showing a common preference of different lipases toward a 3β-equatorial OH in the presence of 2α-equatorial OH (substrate **2**) or of 4α-equatorial OH (substrate **6**).

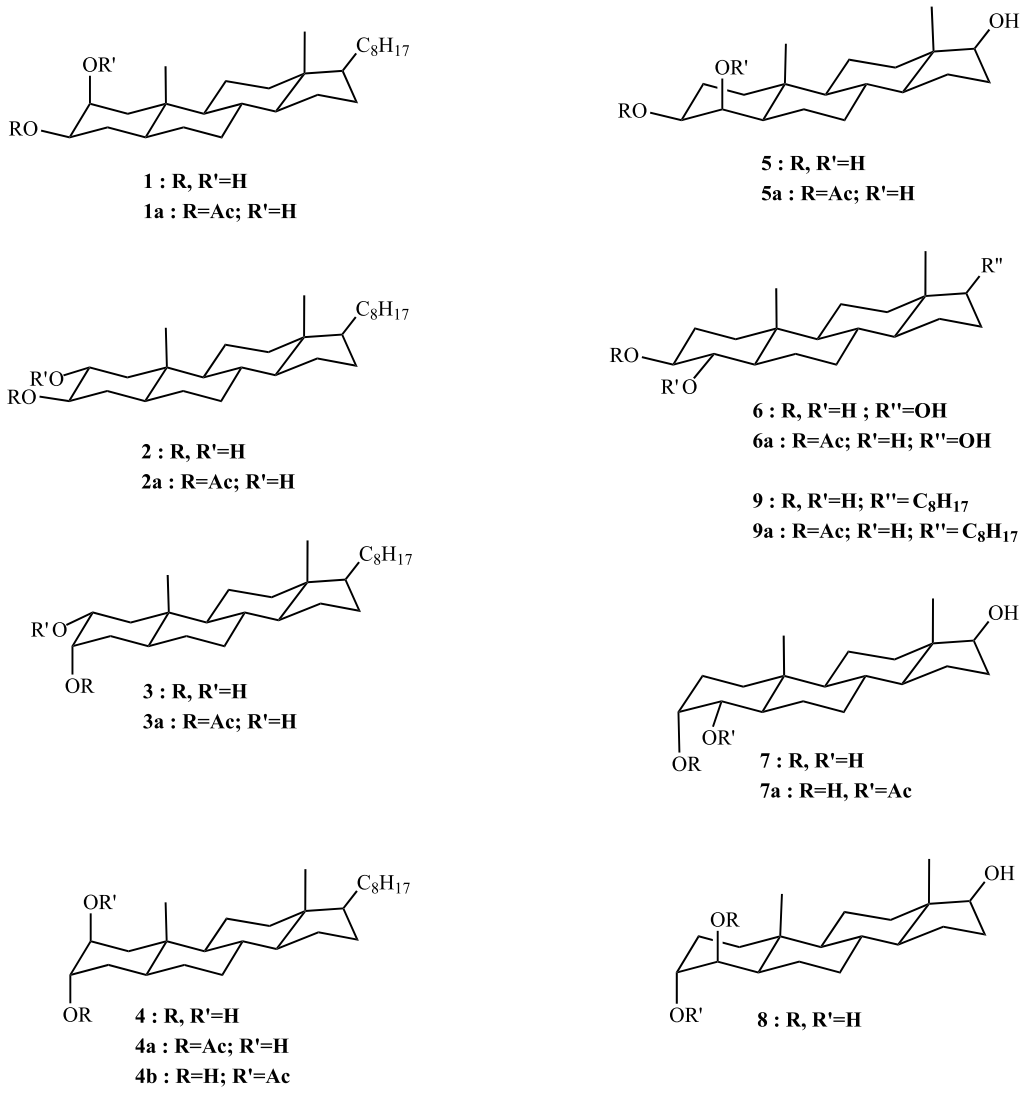
Concerning the diaxial 2β,3α- and 3α,4β-diols (substrates **4** and **8**), different outcomes were noticed. The diaxial 2β,3α-diol was differently accepted by the lipases tested. Whereas Novozym 435 converted this diol exclusively into the 3α-acetate **4a**, lipase PS showed opposite selectivity rendering the 2β-acyl derivative **4b** as the only product. Moreover, acylations catalysed by *Candida rugosa* lipases were not regioselective with this substrate. Conversely, the diaxial 3α,4β diol (**8**) was not accepted by any of the enzymes tested.

Finally, the equatorial/axial 2α,3α-diol (**3**), only accepted by Novozym 435, was acylated at the axial 3α-position, while, at variance, the 3α,4α-dihydroxy steroid (**7**) displaying axial/equatorial configuration, was acylated by different lipases at its equatorial 4α-OH.



1 **1a**

Scheme 1.



Scheme 2.

Moreover, in agreement with previous observations, the lipases tested did not catalyse any acylation of the 17β-OH (substrates **5**–**8**), that was found unaffected in each of the isolated products.

The ability of Novozym 435 to accept axial 3α-OH as a nucleophile has been reported previously.[13d] Herein, we noticed that this lipase catalyses the acylation of the 3α-OH even when a second hydroxyl is located at C-2 (substrates **3** and **4**), whereas the enzyme is inactive when the other OH is located at C-4 (substrates **7** and **8**).

Concerning lipase PS, the ability of this enzyme to esterify 3-hydroxy steroids has been previously observed with 3β-hydroxy-5,6-epoxy derivatives.[18] In the present work, lipase PS was found able to acylate most of the substrates (Table 1), catalysing their regioselective modification, not only at equatorial C-3 (product **2a**), but also at equatorial C-4 (product **7a**) and axial C-2 (product **4a**). Noteworthy, among the panel of tested enzymes, lipase PS was the only one able to acylate hydroxy groups located at the latter positions.

Candida rugosa lipase accepted most of the substrates. Specifically, this enzyme catalysed the acetylation of substrates **1**, **2**, **5** and **6** at their 3β-OH, as was expectable from previous studies.[13b,c] However, the 3α,4α-diol **7** was also acylated by *Candida rugosa* lipase at the C-4 OH, and the 2β,3α-diol **4** was acylated at both its hydroxy groups, showing that this enzyme has a quite variable affinity and selectivity pattern.

Chromobacterium viscosum lipase, which is known to have a strict selectivity for the 3β-hydroxyl of 5α-steroids,[14] showed the expected preference for diols carrying this OH (substrates **1**, **2**, **5** and **6**). In addition, acylation of the substrate **7** was also noticed, but at the 4α-OH.

Finally, the substrate **9**, a cholestane displaying 3β,4α-diequatorial configuration, was converted into the 3β-monoacetate **9a**, a result which confirmed that the differences in the steroid side chain do not influence the selectivity of lipases.

3. Conclusions

The reported results clearly show that lipases are able to

discriminate vicinal hydroxy groups located on the A-ring of steroids, being sensitive to the configuration of the different diols and affording the monoesters with high regioselectivity and good yields.

Considering the occurrence of steroidal vicinal diols in Nature, some of which being mono-acylated,[3,6] -glycosylated[4] or -sulphated,[1,10] these findings can offer useful synthetic tools for the preparation of these compounds and of related derivatives.

4. Experimental

4.1. General

All commercially available chemicals were used as supplied by the manufacturers. Steroids, porcine pancreatic lipase (13.3 U/mg) and crude *Candida rugosa* lipase (665 U/mg) were from Sigma. Novozym 435 (8600 PLU/g) and Lipozyme IM 20 (24 BIU/g) were from Novozymes. Lipase PS (30.8 U/mg, from *Pseudomonas cepacia*) was purchased from Amano and adsorbed on celite as described elsewhere.[16] Lipases AK (20 U/mg, from *Pseudomonas fluorescens*), CE (5.5 U/mg, from *Humicola lanuginosa*) and AY (30 U/mg, from *Candida rugosa*) were from Amano. *Chromobacterium viscosum* lipase (100 U/mg) was supplied by Finnsugar. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-300 at 300 M Hz and at 75.47 M Hz, respectively, using $CDCl_3$ and CD_3OD as solvents. Chemical shifts are reported on the δ (ppm) scale and are relative to tetramethylsilane as internal standard. Infrared spectra were recorder on a Jasco FT/IR-420 spectrometer. Mass spectra were recorded on a GCT Micromass spectrometer. Melting points were measured in a Büchi B-540. Flash chromatography was performed using silica gel (230–400 Mesh) from Merck and petroleum ether/ethyl acetate mixtures as eluents. TLC monitoring was done in petroleum ether/ethyl acetate as eluent and detected with the Komarowsky's reagent.[24]

4.2. Chemical synthesis of the diols

4.2.1. Cholestane-2β,3β-diol (1). According to a known procedure,[25] silica gel (70–230 Mesh, 100 g) was added to a solution of *p*-toluenesulfonic acid (3 g) in acetone (20 ml). The mixture was stirred, then evaporated under reduced pressure and left in a vacuum oven for 2 days. A mixture of cholestan-3β-ol (1.17 g, 3 mmol), *p*-toluenesulfonic acid/silica (15 g) and anhydrous toluene (200 ml) was stirred under reflux, until the consumption of the starting material (24 h). After cooling, diethyl ether was added and the silica was removed by filtration. The organic solution was washed with water, saturated bicarbonate solution and brine. Upon evaporation, cholest-2-ene (1.0 g, 90%) was recovered as a single product.

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.66 (3H, s, CH_3-18), 0.75 (3H, s, CH_3-19), 0.86 (6H, two d, $J=6.6$ Hz, CH_3-26 and CH_3-27), 0.90 (3H, d, $J=6.5$ Hz, CH_3-21), 5.59 (2H, m, H-2 and H-3). ^{13}C NMR δ: 125.96, 125.85, 56.49, 56.27, 54.07, 42.47, 41.45, 40.03, 39.78, 39.51, 36.17, 35.79, 35.61, 34.69, 31.82, 30.31, 28.77, 28.22, 28.00, 24.20, 23.82, 22.82, 22.56, 20.90, 18.68, 11.98, 11.67.

To a solution of cholest-2-ene (420 mg, 1.15 mmol) in glacial acetic acid (35 ml), I_2 (600 mg) and $Cu(OAc)_2$ (500 mg) were added and the reaction mixture was refluxed under magnetic stirring. After 6 h the reaction was complete (TLC control). Then, toluene and NaCl were added and the insoluble salts were filtered off. Diethyl ether was added to the filtrate and the organic phase was washed with water and saturated sodium bicarbonate solution. Evaporation of the solvents under reduced pressure rendered the crude product, which was dissolved in ethanol/chloroform (4:1) (20 ml), and treated with NaOH 16% aqueous solution (1 ml) for 2 h. Finally, upon addition of chloroform, the organic layers were washed with water, HCl 5%, and brine and evaporated to dryness. The residue was purified by flash chromatography (petroleum ether/ethyl acetate 4:1), affording pure cholestane-2β,3β-diol (**1**, 270 mg, 58%), which was crystallized from *n*-hexane, mp 173–175 °C (lit. 174–177[21a] and 175–176[26] °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.86 (6H, two d, $J=6.6$ Hz, CH_3-26 and CH_3-27), 0.89 (3H, d, $J=6.5$ Hz, CH_3-21), 1.00 (3H, s, CH_3-19), 3.63 (1H, dt, $J=4.6$, 10.8 Hz, H-3α), 4.02 (1H, brq, H-2α). ^{13}C NMR δ: 72.42, 70.22, 56.41, 56.26, 55.24, 45.33, 43.17, 42.62, 40.03, 39.50, 36.16, 35.78, 35.23, 34.83, 32.54, 31.96, 28.34, 28.22, 27.99, 24.17, 23.81, 22.80, 22.55, 21.29, 18.64, 14.56, 12.09. FTIR (ATR): ν_{max} 1049.1, 2850.3–2931.3, 3311.2 cm^{-1}.

4.2.2. Cholestane-2α,3β-diol (2). Glacial acetic acid (60 ml) was refluxed with acetic anhydride (5 ml) for 10 min, then cholestan-3-one (769 mg, 2 mmol) was added and, finally, lead tetracetate (1.5 g, 3.4 mmol) was slowly added. The reaction was heated under reflux, with magnetic stirring until the consumption of the starting material (24 h). After cooling, diethyl ether was added and the organic solution was washed with HCl 5%, saturated bicarbonate solution and brine. The crude product was purified by flash chromatography (petroleum ether/ethyl acetate 5:1) affording pure 3-oxocholestan-2α-yl acetate (710 mg, 80%), as a white amorphous powder, mp 120–122 °C (lit.[27] 124.7–125.2 °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.67 (3H, s, CH_3-18), 0.85 0.91 (9H, CH_3-21, CH_3-26 and CH_3-27), 1.12 (3H, s, CH_3-19), 2.15 (3H, s, CH_3CO), 5.29 (1H, dd, $J=6.6$, 12.9 Hz, H-2β). ^{13}C NMR δ: 204.3, 170.15, 74.46, 56.16, 56.06, 53.78, 47.84, 44.82, 43.57, 42.56, 39.71, 39.47, 37.18, 36.10, 35.74, 34.67, 31.56, 28.38, 28.20, 27.99, 24.16, 23.78, 22.80, 22.54, 21.60, 20.79, 18.63, 12.75, 12.04. FTIR (ATR): ν_{max} 1222.7, 1727.9, 1750.1 cm^{-1}.

To a solution of 3-oxocholestan-2α-yl acetate (700 mg, 1.6 mmol) in THF/methanol 2:1, $CeCl_3 \cdot 7H_2O$ (745 mg, 2 mmol) was added and the mixture was stirred for 10 min at room temperature before $NaBH_4$ (120 mg, 3.2 mmol, 8 equiv) was slowly added. After 30 min, the reaction was complete (TLC monitoring) and HCl 5% was added dropwise. The mixture was poured into water and extracted with diethyl ether. The organic solution was washed with

HCl 5%, saturated bicarbonate solution and brine, and evaporated to dryness, yielding 3β-hydroxycholestan-2α-yl acetate (641 mg, 91%), as a white powder, mp 73–75 °C.

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.64 (3H, s, CH_3-18), 0.84–0.90 (12H, CH_3-19, CH_3-21, CH_3-26, CH_3-27), 2.08 (3H, s, CH_3CO), 3.59 (1H, ddd, J=5.4, 9.5, 11.1 Hz, H-3α), 4.82 (1H, ddd, J=4.8, 9.4, 11.6 Hz, H-2β). ^{13}C NMR δ: 171.59, 76.49, 73.53, 56.28, 56.17, 54.17, 44.43, 42.52, 42.12, 39.81, 39.47, 37.27, 36.11, 35.89, 35.73, 34.70, 31.79, 28.19, 27.97, 27.77, 24.15, 23.77, 22.79, 22.53, 21.38, 18.63, 13.09, 12.02.

3β-Hydroxycholestan-2α-yl acetate (500 mg, 1.12 mmol) was treated with NaOH as described in Section 4.2.1. The crude product was purified by flash chromatography (petroleum ether/ethyl acetate 4:1), rendering cholestane-2α,3β-diol (**2**, 370 mg, 82%), which was crystallized from methanol, mp 196.0–197.5 °C (lit.[22b] 204 °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.64 (3H, s, CH_3-18), 0.84–0.91 (12H, CH_3-19, CH_3-21, CH_3-26, CH_3-27), 3.40 (1H, ddd, J=5.1, 8.9, 10.9 Hz, H-3α), 3.59 (1H, ddd, J=4.7, 9.0, 11.5 Hz, H-2β). ^{13}C NMR δ: 73.11, 72.3, 56.30, 56.20, 54.26, 45.03, 44.83, 39.89, 39.48, 38.14, 37.46, 36.13, 35.77, 35.57, 34.73, 31.88, 28.23, 28.00, 27.91, 24.17, 23.80, 22.81, 22.55, 21.36, 18.64, 13.50, 12.04. FTIR (ATR): ν_{max} 1051.0, 2850.1–2930.3, 3312.9 cm^{-1}.

4.2.3. Cholestane-2α,3α-diol (3). A solution of $K_3Fe(CN)_6$ (1 g), Et_3N (7 μl), K_2CO_3 (420 mg) and methanesulphonamide (96 mg) in 20 ml of *t*-BuOH/H_2O (3:2) was prepared. Cholest-2-ene (370 mg, 1 mmol), (Section 4.2.1) was dissolved in 30 ml THF/*t*-BuOH/H_2O (10:3:2) and added to the previous solution. Then, 30 μl of OsO_4 solution (0.1 mg/μl in CH_3CN) was added and the reaction was stirred at room temperature until the total consumption of the starting material (48 h). Sodium sulphite 5% was added and the mixture was stirred for 5 h. Upon addition of diethyl ether, the organic layer was washed with HCl 5%, saturated bicarbonate solution and brine, and then, evaporated. The residue was purified by flash chromatography (petroleum ether/ethyl acetate 4:1), rendering cholestane-2α,3α-diol (**3**, 242 mg), which was crystallized from *n*-hexane, mp 217–218 °C (lit.[22b] 216–219 °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.80 (3H, s, CH_3-19), 0.86 (6H, two d, J=6.6 Hz, CH_3-26 and CH_3-27), 0.90 (3H, d, J=6.6 Hz, CH_3-21), 3.77 (1H, br d, J=11.0 Hz, 2β-H), 3.96 (1H, br s, 3β-H). ^{13}C NMR δ: 69.27, 69.12, 56.35, 56.16, 54.16, 42.55, 40.93, 39.90, 39.49, 38.14, 36.90, 36.13, 35.78, 34.76, 34.22, 31.82, 28.22, 28.00, 27.65, 24.17, 23.80, 22.81, 22.55, 20.90, 18.65, 12.40, 12.06. FTIR (ATR): ν_{max} 1048.7, 2852.2–2930.0, 3315.0 cm^{-1}.

4.2.4. Cholestane-2β,3α-diol (4). A mixture of cholest-2-ene (400 mg, 1.1 mmol), $CHCl_3$ (15 ml) and 3-chloroperoxybenzoic acid (400 mg, 2.3 mmol) was stirred at room temperature. After the disappearance of the starting material (24 h), the reaction mixture was poured into water and extracted with CH_2Cl_2. The organic phase was washed with sodium sulphite 5%, water and brine, dried with $MgSO_4$ and evaporated at reduced pressure, affording 2α,3α-epoxycholestane (395 mg, 93%).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.64 (3H, s, CH_3-18), 0.75 (3H, s, CH_3-19), 0.86 (6H, two d, J=6.6 Hz, CH_3-26 and CH_3-27), 0.90 (3H, d, J=6.5 Hz, CH_3-21), 3.14 (2H, m, H-2β and H-3β).

According to a known procedure,[28] to a solution of 2α,3α-epoxycholestane (374 mg, 0.9 mmol) in acetone (30 ml), periodic acid (350 mg) in acetone/water (1:1.5 ml) was added and the mixture was heated to reflux for 5 min, then concentrated until 1/3 of the initial volume and kept at room temperature for 30 min. This mixture was refluxed again while water (2 ml) was added dropwise for 30 min. Finally, the mixture was cooled, concentrated under vacuum and purified by flash chromatography (petroleum ether/ethyl acetate 4:1), yielding cholestane-2β,3α-diol (**4**, 309 mg, 85%), which was crystallized from methanol, mp 183–185 °C (lit. 178–180[1b] and 197–200[21a] °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.86 (6H, two d, J=6.6 Hz, CH_3-26 and CH_3-27), 0.90 (3H, d, J=6.5 Hz, CH_3-21), 0.99 (3H, s, CH_3-19), 3.87 (2H, m, H-2α and H-3β). ^{13}C NMR δ: 71.82, 70.61, 56.41, 56.19, 55.10, 42.60, 40.54, 40.01, 39.49, 38.93, 36.14, 35.78, 35.73, 34.88, 31.90, 31.71, 28.20, 28.01, 24.13, 23.80, 22.82, 22.55, 20.86, 18.64, 14.59, 12.10. FTIR (ATR): ν_{max} 1047.9, 2843.2–2930.9, 3310.1 cm^{-1}.

4.2.5. Androstane-3β,4β,17β-triol (5). A mixture of testosterone acetate (661 mg, 2 mmol), glacial acetic acid (30 ml) and zinc dust (4 g) was stirred at room temperature. After disappearance of the starting material (5 h), diethyl ether was added and the suspension was filtered through a celite pad. The filtrate was evaporated under reduced pressure, then dissolved in diethyl ether and washed with water, saturated sodium bicarbonate solution and brine. After drying with $MgSO_4$, the organic phase was evaporated, affording an epimeric mixture of 5α- and 5β-androst-3-en-17β-yl acetate (595 mg, 94%).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.80 and 0.96 (3H, two s, 19-CH_3), 2.03 (3H, s, CH_3CO), 4.56 (1H, m, H-17α), 5.29 (1H, dq, H-4), 5.54 and 5.66 (1H, two m, H-3). The 5α- and 5β- epimeric ratio, evaluated by the integration of the H-3 multiplets,[29] was 0.8:1. Epimerically pure 5α-androst-3-en-17β-yl acetate was obtained after 3 sequential crystallizations in *n*-hexane.

5α-Androst-3-en-17β-yl acetate (200 mg, 0.63 mmol) reacted with I_2/$Cu(OAc)_2$, the product obtained was treated with NaOH (Section 4.2.1) and purified by flash chromatography (petroleum ether/ethyl acetate 1:2), rendering androstane-3β,4β,17β-triol (**5**, 95 mg), as a white amorphous powder, mp 257–260 °C (lit.[30] 263–265 °C).

Selected data: ^{1}H NMR ($CDCl_3$/CD_3OD) δ: 0.72 (3H, s, CH_3-18), 1.04 (3H, s, CH_3-19), 3.50 (1H, m, H-3α), 3.57 (1H, t, J=8.6 Hz, H-17α), 3.68 (1H, br t, J=2.30 Hz, H-4α). ^{13}C NMR δ: 81.89, 75.12, 72.67, 56.03, 51.64, 43.42, 37.66, 37.17, 36.12, 36.05, 32.54, 30.09, 26.34, 25.80,

23.79, 20.69, 14.98, 11.42. FTIR (ATR): ν_{max} 1064.6, 2845.5–2934.2, 3308.3 cm^{-1}.

4.2.6. Androstane-3β,4α,17β-triol (6). Testosterone (288.4 mg, 1 mmol) was dissolved in THF (10 ml) and cooled to 0 °C. Then, BH_3 in THF (1.0 M, 5 ml) was added slowly. The mixture was stirred for 3 h, then warmed to room temperature over 2.5 h and cautiously quenched with a 10 N NaOH aqueous solution (2 ml), followed by slow addition of a 30% H_2O_2 aqueous solution (2 ml). This mixture was stirred overnight, then poured into water and extracted with diethyl ether. The organic layers were washed with HCl 5%, saturated bicarbonate solution and brine. After drying with $MgSO_4$, the solvent was evaporated at reduced pressure and the residue purified by flash chromatography (petroleum ether/ethyl acetate 1:2), rendering androstane-3β,4α,17β-triol (**6**, 246 mg, 80%), which was crystallized from methanol, mp 253.3–255.8 °C (lit. 248–250[23a] and 258–259[30] °C).

Selected data: ^{1}H NMR ($CDCl_3/CD_3OD$) δ: 0.72 (3H, s, CH_3-18), 0.85 (3H, s, CH_3-19), 3.20 (1H, t, $J=8.7$ Hz, H-4β), 3.29 (1H, m, H-3α), 3.59 (1H, t, $J=8.5$ Hz, H-17α). ^{13}C NMR δ: 81.87, 76.52, 75.53, 55.24, 51.53, 51.48, 43.38, 37.75, 37.23, 36.85, 35.68, 31.76, 30.13, 28.78, 23.77, 23.10, 21.10, 13.89, 11.47. FTIR (ATR): ν_{max} 1066.0, 2847.5–2932.0, 3309.1 cm^{-1}.

4.2.7. Androstane-3α,4α,17β-triol (7). 5α-Androst-3-en-17β-yl acetate (200 mg, 0.63 mmol) (Section 4.2.5) was oxidized by OsO_4 (Section 4.2.3). The product was treated with NaOH (Section 4.2.1) and purified by flash chromatography (petroleum ether/ethyl acetate 1:2), affording androstane-3α,4α,17β-triol (**7**, 110.6 mg, 57%).

Selected data: ^{1}H NMR ($CDCl_3/CD_3OD$) δ: 0.72 (3H, s, CH_3-18), 0.85 (3H, s, CH_3-19), 3.45 (1H, d, $J=10.2$ Hz, H-4β), 3.6 (1H, t, $J=8.5$ Hz, H-17α), 3.97 (1H, s, H-3β). ^{13}C NMR δ: 82.40, 73.56, 70.14, 54.19, 50.76, 42.52, 37.50, 37.11, 36.89, 34.90, 31.76, 31.10, 23.47, 22.58, 21.20, 20.30, 12.70, 11.49. FTIR (ATR): ν_{max} 1065.0, 2846.1–2933.3, 3307.8 cm^{-1}.

4.2.8. Androstane-3α,4β,17β-triol (8). 5α-Androst-3-en-17β-yl acetate (290 mg, 0.92 mmol) obtained as described above (2.5) reacted with 3-chloroperoxybenzoic (Section 4.2.4). The product obtained was treated with periodic acid (Section 4.2.4), deacetylated with NaOH (Section 4.2.1) and purified by flash chromatography (petroleum ether/ethyl acetate 1:2), affording androstane-3α,4β,17β-triol (**8**, 142 mg), which was crystallized from methanol, mp 263–264.3 °C.

Selected data: ^{1}H NMR ($CDCl_3/CD_3OD$) δ: 0.71 (3H, s, CH_3-18), 1.03 (3H, s, CH_3-19), 3.49 (1H, br s, H-4α), 3.55 (1H, t, $J=8.6$ Hz, H-17α), 3.75 (1H, br q, $J=2.6$ Hz, H-3β). ^{13}C NMR δ: 82.54, 76.56, 71.24, 56.94, 52.51, 45.24, 44.08, 37.99, 37.09, 36.96, 33.32, 33.21, 30.61, 26.60, 25.11, 24.31, 20.97, 14.85, 11.67. FTIR (ATR): ν_{max} 1064.9, 2849.8–2935.1, 3309.5 cm^{-1}. FD-MS $m/z=308.2386$ (100%, M^+), 309.2424 (27%, M^++1), 290.2237 (17%, M^+-H_2O).

4.2.9. Cholestane-3β,4α-diol (9). Hydroboration of cholest-4-en-3-one (384 mg, 1 mmol) under the conditions described in Section 4.2.8 rendered cholestane-3β,4α-diol (**9**, 352 mg, 87%), which was crystallized from *n*-hexane, mp 235–237 °C (lit.[31] 237 °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.62 (3H, s, CH_3-18), 0.79–0.87 (12H, CH_3-19, CH_3-21, CH_3-26, CH_3-27), 3.18 (1H, t, $J=10.2$ Hz, H-4β), 3.29 (1H, m, H-3α). ^{13}C NMR δ: 76.24, 75.25, 56.54, 56.37, 54.6, 50.86, 42.63, 40.08, 39.62, 37.28, 36.36, 36.27, 35.92, 35.16, 31.74, 28.37, 28.26, 28.11, 24.28, 23.93, 22.83, 22.78, 22.58, 21.08, 18.70, 13.58, 12.11. FTIR (ATR): ν_{max} 1047.1, 2851.2–2930.2, 3315.0 cm^{-1}.

4.3. Enzymatic acylation of 2,3- and 3,4-vicinal diols

In a typical screening assay, a solution of the substrate (2 mg), in 0.9 ml of solvent (toluene for the cholestane diols or acetone/THF for the androstane diols) and vinyl acetate (0.1 ml) was prepared. This solution was added to the enzyme (30 mg of crude enzymes, 10 mg of Lipozyme IM 20 or 5 mg of Novozym 435) in 3 ml vials, which were stopped with a cap and shaken at 250 rpm at 45 °C. The reactions were monitored by TLC (see Table 1).

4.3.1. 2β-Hydroxycholestan-3β-yl acetate (1a). To a solution of cholestane-2β, 3β-diol (**1**, 25 mg, 0.062 mmol) in toluene (8 ml) and vinyl acetate (2 ml), *Candida rugosa* lipase (100 mg) was added and the reaction was shaken at 250 rpm, at 45 °C. After 24 h the reaction was complete. The enzyme was filtered off and the solvent was evaporated, the residue was purified by flash chromatography (petroleum ether/ethyl acetate 5:1) yielding 2β-hydroxycholestan-3β-yl acetate (**1a**, 22 mg, 80%), which was crystallized from methanol, mp 147–148 °C (lit.[26] 154 °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.86 (6H, two d, $J=6.5$ Hz, CH_3-26 and CH_3-27), 0.89 (3H, d, $J=6.5$ Hz, CH_3-21), 1.03 (3H, s, CH_3-19), 2.08 (3H, s, CH_3CO), 4.09 (1H, br q, $J=2.6$ Hz, H-2α), 4.78 (1H, ddd, $J=3.34$, 4.71, 11.75 Hz, H-3α). ^{13}C NMR δ: 170.13, 75.58, 68.72, 56.30, 56.19, 55.15, 45.40, 42.90, 42.59, 39.95, 39.47, 36.12, 35.76, 35.30, 34.77, 31.86, 28.58, 28.20, 28.13, 27.98, 24.13, 23.79, 22.80, 22.54, 21.34, 21.22, 18.62, 14.51, 12.07. FTIR (ATR): ν_{max} 1025.1, 1259.3, 1721.8, 2858.0–2950.2, 3510.8 cm^{-1}. FD-MS $m/z=$ 386.3462 (100%, M^+-CH_3COOH), 447.3828 (83%, M^++1), 404.3776 (67%, M^++1-CH_3CO), 448.3870 (25%, M^++2), 61.0324 (8%, $CH_3COOH+1$).

4.3.2. 2α-Hydroxycholestane-3β-yl acetate (2a). To a solution of cholestane-2α, 3β-diol (**2**, 50 mg, 0.124 mmol) in toluene (8 ml), vinyl acetate (1 ml) and lipase PS (100 mg) were added and the reaction mixture was shaken for 24 h, under the conditions described above. After usual work-up and purification by flash chromatography, 2α-hydroxycholestane-3β-yl acetate was recovered (**2a**, 48 mg, 87%) as a white powder, mp 157–159 °C. The TLC R_f of **2a** and of 3β-hydroxycholestan-2α-yl acetate (synthesised in Section 4.2.2) using petroleum ether/ethyl acetate (2:1) as eluent were 0.47, and 0.39, respectively.

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.84–0.90 (12H, CH_3-19, CH_3-21, CH_3-26, CH_3-27), 2.08 (3H, s, CH_3CO), 3.77 (1H, ddd, J=4.8, 9.4, 11.5 Hz, H-2β), 4.59 (1H, ddd, J=5.4, 9.5, 10.9 Hz, H-3α). ^{13}C NMR δ: 171.37, 78.99, 69.85, 56.13, 56.09, 54.03, 45.28, 44.34, 42.46, 39.75, 39.39, 36.81, 36.03, 35.68, 34.62, 32.53, 31.69, 30.82, 28.13, 27.90, 27.63, 24.08, 23.70, 22.70, 22.44, 21.25, 18.54, 13.17, 11.94. FTIR (ATR): ν_{max} 1025.9, 1259.8, 1718.8, 2857.0–2951.3, 3512.1 cm^{-1}. FI-MS m/z= 386.3553 (100%, M^+-CH_3COOH), 446.3842 (54%, M^+), 387.3591 (35%, M^++1-CH_3COOH), 447.3853 (33%, M^++1), 61.0251 (1%, $CH_3COOH+1$).

4.3.3. 2α-Hydroxycholestane-3α-yl acetate (3a). To a solution of cholestane-2α,3α-diol (**3**, 40 mg, 0.1 mmol) in toluene (8 ml), vinyl acetate (1 ml) and Novozym 435 (80 mg) were added and the reaction mixture was shaken for 2 days, under the conditions described above. After usual work-up and purification by flash chromatography, 2α-hydroxycholestane-3α-yl acetate was recovered (**3a**, 33 mg, 75%) and crystallized from methanol, mp 163–164 °C.

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.82 (3H, s, CH_3-19), 0.87 (6H, two d, J=6.5 Hz, CH_3-26 and CH_3-27), 0.90 (3H, d, J=6.5 Hz, CH_3-21), 2.12 (3H, s, CH_3CO) 3.84 (1H, dt, J=4.2, 11.4 Hz, H-2β), 5.12 (1H, br q, J=2.6 Hz, H-3β). ^{13}C NMR δ: 171.58, 73.03, 68.11, 56.33, 56.23, 54.14, 42.57, 41.70, 39.88, 39.47, 39.29, 36.74, 36.13, 35.78, 34.71, 32.21, 31.77, 28.23, 27.99, 27.45, 24.16, 23.83, 22.81, 22.54, 21.38, 20.89, 18.63, 12.55, 12.04. FTIR (ATR): ν_{max} 1027.4, 1259.8, 1714.7, 2859.0 2952.5, 3516.3 cm^{-1}. FI-MS m/z=446.3828 (100%, M^+), 386.3293 (91%, M^+-CH_3COOH), 447.3842 (29%, M^++1), 387.3349 (23%, M^++1-CH_3COOH).

4.3.4. 2β-Hydroxycholestane-3α-yl acetate (4a). To a solution of cholestane-2β,3α-diol (**4**, 50 mg, 0.124 mmol) in toluene (10 ml), vinyl acetate (1 ml) and Novozym 435 (80 mg) were added and the reaction mixture was shaken for 3 days, under the conditions described above. After usual work-up and purification by flash chromatography, 2β-hydroxycholestane-3α-yl acetate was recovered as a single product (**4a**, 47.6 mg, 86%), mp 110–111 °C (lit.[1b] 106–107 °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.86 (6H, two d, J=6.6 Hz, CH_3-26 and CH_3-27), 0.90 (3H, d, J=6.5 Hz, CH_3-21), 0.99 (3H, s, CH_3-19), 2.07 (3H, s, CH_3CO), 3.88 (1H, br s, H-2α), 4.82 (1H, br s, H-3β). ^{13}C NMR δ: 170.52, 72.92, 68.65, 56.43, 56.25, 54.97, 42.58, 40.46, 39.99, 39.89, 39.46, 36.13, 35.77, 35.34, 34.81, 31.85, 28.66, 28.21, 27.97, 24.11, 23.83, 22.80, 22.53, 21.40, 20.81, 18.62, 14.18, 12.06. FTIR (ATR): ν_{max} 1033.1, 1261.2, 1714.2, 2860.1–2953.8, 3500.3 cm^{-1}.

4.3.5. 3α-Hydroxycholestane-2β-yl acetate (4b). To a solution of cholestane-2β,3α-diol (**4**, 50 mg, 0.124 mmol) in toluene (9 ml), vinyl acetate (1 ml) and lipase PS (200 mg) were added and the reaction mixture was shaken for 3 days, under the conditions described above. After usual work-up and purification by flash chromatography, 3α-hydroxycholestane-2β-yl acetate was recovered as a single product (**4b**, 37 mg, 67%), mp 115.7–116.2 °C (lit. 85–86[1b] and 113[27] °C).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.64 (3H, s, CH_3-18), 0.86 (6H, two d, J=6.6 Hz, CH_3-26 and CH_3-27), 0.89 (3H, d, J=5.3 Hz, CH_3-21), 0.90 (3H, s , CH_3-19), 2.04 (3H, s, CH_3CO), 3.84 (1H, br q, J=2.3 Hz, H-3β), 4.87 (1H, br q, H-2α). ^{13}C NMR δ: 170.30, 73.15, 67.59, 56.35, 56.17, 54.85, 42.55, 39.92, 39.48, 38.52, 37.10, 36.12, 35.77, 35.56 34.94, 31.84, 31.74, 28.19, 28.11, 27.99, 24.12, 23.81, 22.80, 22.54, 21.44, 20.20, 18.63, 13.64, 12.06. FTIR (ATR): ν_{max} 1034.5, 1263.2, 1716.4, 2862.8–2928.4, 3481.8 cm^{-1}.

4.3.6. 4β,17β-Dihydroxyandrostane-3β-yl acetate (5a). To a solution of androstane-3β,4β,17β-triol (**5**, 35 mg, 0.114 mmol) in toluene (5 ml), THF (5 ml) and vinyl acetate (1 ml), *Candida rugosa* lipase (100 mg) was added and the reaction mixture was shaken for 3 days, under the conditions described above. After usual work-up and purification by flash chromatography (petroleum ether/ethyl acetate 2:1), 4β,17β-dihydroxyandrostan-3β-yl acetate was recovered (**5a**, 29.3 mg, 74%).

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.73 (3H, s, CH_3-18), 1.06 (3H, s, CH_3-19), 2.09 (3H, s, CH_3CO), 3.63 (1H, t, J= 8.7 Hz, H-17α), 3.83 (1H, br t, H-4α), 4.72 (1H, ddd, J= 3.2, 4.8, 8.0 Hz, H-3α). ^{13}C NMR δ: 170.27, 81.87, 75.53, 72.88, 55.29, 50.98, 48.71, 42.93, 36.85, 36.54, 35.61, 35.44, 31.82, 30.45, 25.55, 23.34, 22.13, 21.34, 20.11, 14.71, 11.11. FTIR (ATR): ν_{max} 1041.5, 1258.2, 1709.3, 2840.9–2943.0, 3442.0 and 3530.1 cm^{-1}. FD-MS m/z=351.2470 (100%, M^++1), 290.2104 (78%, M^+-CH_3COOH), 350.2468 (37%, M^+).

4.3.7. 4α,17β-Dihydroxyandrostane-3β-yl acetate (6a). To a solution of androstane-3β,4α,17β-triol (**6**, 30 mg, 0.1 mmol) in acetone (6 ml), THF (3 ml) and vinyl acetate (1 ml), *Chromobacterium viscosum* lipase (200 mg) was added and the reaction mixture was shaken for 3 days, under the conditions described above. After usual work-up and purification by flash chromatography, 4α,17β-dihydroxyandrostan-3β-yl acetate was recovered (**6a**, 20.2 mg, 60%), and crystallized from methanol, mp 182–184 °C.

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.73 (3H, s, CH_3-18), 0.86 (3H, s, CH_3-19), 2.09 (3H, s, CH_3CO), 3.46 (1H, t, J= 9.5 Hz, H-4β), 3.64 (1H, t, J=8.5 Hz, H-17α), 4.58 (1H, ddd, J=5.4, 9.2, 11.6 Hz, H-3α). ^{13}C NMR δ: 171.51, 81.85, 79.23, 72.48, 54.32, 51.29, 50.83, 42.88, 36.58, 36.01, 35.05, 31.00, 30.50, 25.62, 23.32, 22.48, 21.37, 20.54, 15.26, 13.51, 11.11. FTIR (ATR): ν_{max} 1035.6, 1262.2, 1707.7, 2841.6–2940.9, 3443.3 and 3522.3 cm^{-1}. FI-MS m/z=290.2061 (100%, M^+-CH_3COOH), 350.2541 (39%, M^+), 291.2042 (12%, M^++1-CH_3COOH).

4.3.8. 3α,17β-Dihydroxyandrostan-4α-yl acetate (7a). To a solution of 3α,4α,17β-trihydroxyandrostane (**7**, 118 mg, 0.38 mmol) in acetone (18 ml), THF (4 ml) and vinyl acetate (2 ml), lipase PS (500 mg) was added and the reaction mixture was shaken for 4 days, under the conditions described above. After usual work-up and purification by

flash chromatography, 3α,17β-dihydroxyandrostan-4α-yl acetate was recovered (**7a**, 102.3 mg, 76%) as a white powder.

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.72 (3H, s, CH_3-18), 0.87 (3H, s, CH_3-19), 2.09 (3H, s, CH_3CO), 3.64 (1H, t, J=8.5 Hz, H-17α), 4.01 (1H, q, J=2.9, 2.7 Hz, H-3β). 4.85 (1H, dd, J=11.8, 2.9 Hz, H-4β). ^{13}C NMR δ: 171.24, 82.76, 75.20, 67.58, 54.11, 50.67, 42.83, 42.48, 37.68, 36.80, 34.79, 31.14, 30.84, 27.48, 26.75, 23.41, 22.42, 21.13, 20.27, 12.82, 11.51. FTIR (ATR): ν_{max} 1039.2, 1265.1, 1710.7, 2841.6, 3445.7 and 3528.1 cm^{-1}. FI-MS m/z= 308.2328 (100%, $M^+ + 1 - CH_3CO$), 290.2052 (71%, $M^+ - CH_3COOH$), 350.2441 (62%, M^+), 332.2306 (26%, $M^+ - H_2O$), 351.2468 (12%, $M^+ + 1$).

4.3.9. 4α-Hydroxycholestane-3β-yl acetate (9a). To a solution of cholestane-3β,4α-diol (**9**, 50 mg, 0.124 mmol) in acetone (9 ml), vinyl acetate (1 ml) and *Chromobacterium viscosum* lipase (500 mg) were added and the reaction mixture was shaken under the conditions described above for 6 days. The usual work-up and flash chromatography yielded 4α-hydroxycholestane-3β-yl acetate (**9a**, 42.3 mg, 76%), mp 169–170 °C.

Selected data: ^{1}H NMR ($CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.84–0.91 (12H, CH_3-19, CH_3-21, CH_3-26, CH_3-27), 2.08 (3H, s, CH_3CO), 3.45 (1H, t, J=9.7 Hz, H-4β), 4.57 (1H, ddd, J=5.3, 9.1, 11.5 Hz, H-3α). ^{13}C NMR δ: 171.49, 79.24, 72.47, 56.30, 56.18, 54.21, 51.23, 42.45, 39.85, 39.46, 36.83, 36.10, 35.96, 35.76, 34.97, 31.44, 28.22, 27.97, 25.65, 24.12, 23.80, 22.79, 22.60, 21.53, 21.36, 20.94, 18.61, 13.46, 12.01. FTIR (ATR): ν_{max} 1036.6, 1263.2, 1715.4, 2860.9–2930.2, 3549.3 cm^{-1}. FI-MS m/z= 386.3400 (100%, $M^+ - CH_3COOH$), 446.3787 (33%, M^+), 387.3417 (27%, $M^+ + 1 - CH_3COOH$), 447.3893 (13%, $M^+ + 1$).

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