Regioselective Acylation of Bile Acid Derivatives with *Candida cylindracea* Lipase in Anhydrous Benzene

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Candida cylindracea lipase suspended in anhydrous benzene was utilized to regioselectively acylate the 3α-hydroxyl group on several bile acid derivatives. Systematic investigations of the specificity of this lipase toward different steroid skeletons and a study of enzyme stability under the reaction conditions were also carried out.

Introduction

Biologically active molecules containing different sensitive functional groups appear to be more and more suitable to exploit the potentialities of enzymes in organic synthesis.[1] Due to their polyfunctionalized rigid skeleton, steroids have always been a stimulating stereochemical exercise for organic chemists.[2] Moreover, their pharmaceutical properties and the high manufacturing cost of most of them make any achievement giving mild and efficient modifications of these natural compounds noteworthy.

In our previous studies we reported the use of hydroxysteroid dehydrogenases for the regioselective oxidoreduction of steroids in water[3] or in water–organic solvent two-phase systems.[4] These enzymes were used to synthesize steroid derivatives not easily achieved through conventional chemical reactions and to prepare regiospecifically deuterated bile acids useful for metabolic studies.[5]

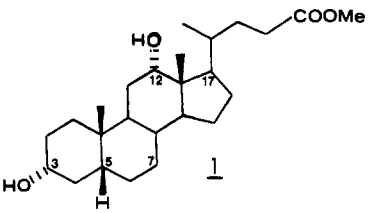
Recently another enzymatic reaction, transesterification catalyzed by lipase (from *Chromobacterium viscosum*) or protease (subtilisin) in dry acetone, has been used to obtain the regioselective acylation of polyhydroxylated steroids.[6] However, the substrate specificities of the enzymes employed made them unusable for the esterification of bile acids.

In the present study we found that lipase from *Candida cylindracea* suspended in an appropriate solvent can be successfully used for regioselective acylation of bile acids. Furthermore, systematic investigations of the specificity of this lipase toward different steroid skeletons and its stability under the reaction conditions were also carried out.

Results and Discussion

The substrate specifities of different lipases and proteases have been previously investigated for acylation of polyhydroxysteroids in dry acetone.[6] Interesting results were obtained with lipase from *Chromobacterium viscosum* (Ch.v) and with subtilisin Carlsberg. It was shown that the former enzyme selectively acylates the C-3 hydroxyl group when it is in the equatorial (β) position and the A/B ring fusion is in the trans configuration. On the other hand, subtilisin strongly prefers the other limb of the steroid skeleton, particularly C-17 or side-chain hydroxyl groups.

However, this same strict regioselectivity could prevent the two enzymes from acylating hydroxysteroids with different stereochemical characteristics, as, for instance, bile acid derivatives. In fact, no conversion was observed with either enzyme when deoxycholic acid, methyl ester (1) was used as substrate in dry acetone. This was probably due to the presence of the aliphatic chain, which obstructed the esterification of C-12α hydroxyl by subtilisin and to the cis fusion between rings A and B, which made 1 unsuitable to fit the Ch.v lipase active site.



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As solvent choice is one of the most important parameters in this kind of reaction,[1c] it occurred to us that perhaps some of the hydrolytic enzymes previously tested[6] might have been able to catalyze the esterification of 1 in less hydrophilic solvents. For instance, it has been reported[7] that lipase from *Candida cylindracea* (Cn.c) is practically inactive in acetone but has a good catalytic action in hexane, toluene, butyl ether, and other hydrophobic solvents. Following the general procedure,[6] compound 1 was dissolved in anhydrous benzene[8] containing trichloroethyl butanoate. Then one of the commercially available lipases was added to 1 mL of the reaction mixture and the suspensions were shaken at 45 °C and 250 rpm, with the degree of conversion monitored by HPLC. After 24 h, no products were detected for three of the four enzymes tested, but more than 80% conversion to a single new peak was observed in the presence of Cn.c lipase. The reaction was scaled up to 500 mg of 1 to characterize the new compound formed. After silica gel chromatography, the product was isolated and unambiguously identified by ^{1}H NMR as 3α-*O*-butanoyldeoxycholic acid, methyl ester. The esterification of C-3α OH was clearly indicated by a 0.5-ppm downfield shift of C-3βH, while C-12βH showed the same chemical shift as in 1.

To take full advantage of this interesting result, we first decided to investigate the substrate specificity of Cn.c

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(2) (a) Fieser, L. F.; Fieser, M. *Steroids*; Reinhold: New York, 1959. (b) Fried, J.; Edwards, J. A.; *Organic reactions in steroid chemistry*; Reinhold: New York, 1972.

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(8) This solvent was chosen because it afforded both good enzymatic activity and substrate solubility.

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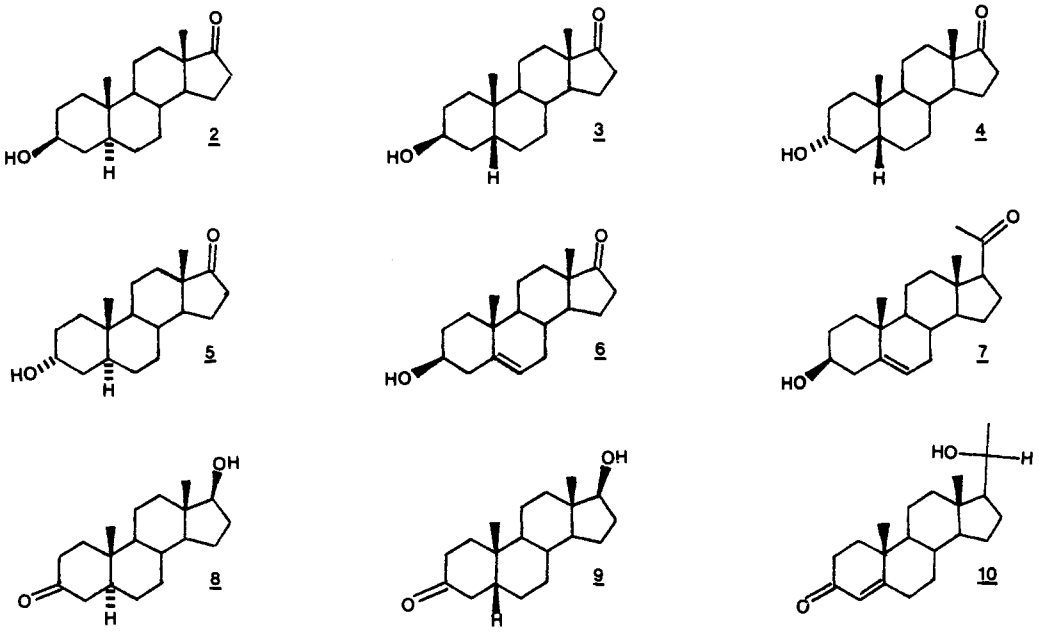
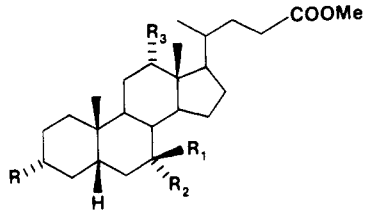
Chart I



lipase toward different steroid skeletons. Compounds **2–10** (Chart I) were chosen as model substrates. Using methyl butanoate[9] as acylating agent, we determined the initial reaction rates. The results, depicted in Table I, show that the stereochemical requirements of this enzyme are broader than those of Ch.v lipase.[6] The highest esterification rates at the C-3 hydroxyl were observed with **7** and, analogously to the results obtained with Ch.v lipase,[6] with **2** and **6**. However, product formation from the other compounds was also detected (only **5** was not recognized as a substrate). Moreover, the enzyme was able to catalyze the esterification of C-17β OH in **8**, on the other side of the steroid skeleton. Bile acid methyl esters **11–14** were



	R	R_1	R_2	R_3
11	OH	H	H	H
12	OH	H	OH	H
13	OH	OH	H	H
14	OH	H	OH	OH
16	OAc	H	H	H
17	OAc	OAc	H	H
18	OAc	H	H	OAc

also tested as substrates. They were all accepted by the enzyme's active site and their conversion rates are reported in Table II. In each case the formation of a single product was detected by HPLC and TLC, strongly suggesting that the enzyme is ineffective not only toward 12α-OH (as in **1**) but also toward 7α-OH and 7β-OH. In agreement with this hypothesis, no reaction was observed with 7α,12α-dihydroxy-3-oxo-5β-cholanoic acid, methyl ester (**15**).

Before scaling up the reactions with **12–14**, we carried out additional analytical tests to optimize the transesterification process. Using epiandrosterone (**2**) as substrate, we studied the effect on the reaction rate of the chain length of the acyl donor. As reported in Table III, the highest initial rates were observed with methyl butanoate and methyl dodecanoate; quite surprising was the marked drop in activity found with methyl hexanoate. We also investigated the stability of Cn.c lipase under reaction

Table I. Initial Rates of Acylation with Methyl Butanoate of Various Hydroxysteroids Catalyzed by Cn.c Lipase in Anhydrous Benzene

steroid	initial rate,[a] μmol/h
17-oxo-5α-androstan-3β-ol (**2**)	4.74
17-oxo-5β-androstan-3β-ol (**3**)	0.13
17-oxo-5β-androstan-3α-ol (**4**)	0.57
17-oxo-5α-androstan-3α-ol (**5**)	0
17-oxo-5-androsten-3β-ol (**6**)	4.15
20-oxo-5-pregnen-3β-ol (**7**)	8.08
3-oxo-5α-androstan-17β-ol (**8**)	0.75
3-oxo-5β-androstan-17β-ol (**9**)	0
3-oxo-4-pregnen-20β-ol (**10**)	0

[a] Conditions: 34 mM steroid (17 mM for **6** and **10**), 170 mM methyl butanoate (85 mM for **6** and **10**), 50 mg/mL of Cn.c lipase. Suspensions in 1 mL of anhydrous benzene were shaken at 250 rpm and at 45 °C. Every 10 min, 5-μL aliquots were withdrawn and assayed for the product by gas chromatography. At least three time points were taken for the initial rate determination. No product was detected in the absence of the enzyme.

Table II. Initial Rates of Acylation with Methyl Butanoate of Various Bile Acid Methyl Esters Catalyzed by Cn.c Lipase in Anhydrous Benzene

steroid	initial rate,[a] μmol/h
deoxycholic acid, methyl ester (**1**)	4.59
lithocholic acid, methyl ester (**11**)	1.58
chenodeoxycholic acid, methyl ester (**12**)	0.94
ursodeoxycholic acid, methyl ester (**13**)	0.67
cholic acid, methyl ester (**14**)	2.47
7α,12α-dihydroxy-3-oxo-5β-cholanoic acid, methyl ester (**15**)	0

[a] For conditions, see footnote to Table I. The products were assayed by HPLC (with **11** the product was assayed by gas chromatography).

(9) We used methyl esters to obtain reliable initial rates of acylation because the enzyme was much more stable with them than with trichloroethyl or trifluoroethyl esters (see text).

Table III. Effects of Chain Length of the Acyl Donor on the Transesterification Rate with Cn.c Lipase in Benzene

ester	initial rate,[a] μmol/h	rel rate
methyl acetate	1.38	20
methyl propionate	5.16	74
methyl butanoate	6.97	100
methyl hexanoate	1.44	21
methyl octanoate	4.02	58
methyl dodecanoate	5.70	82

[a] Conditions: 34 mM epiandrosterone (2), 170 mM methyl ester, 50 mg/mL of Cn.c lipase. Suspensions in 1 mL of anhydrous benzene were shaken at 250 rpm at 45 °C. Every 10 min, 5-μL aliquots were withdrawn and assayed by gas chromatography. At least three time points were taken for the initial rate determination. No product was detected in the absence of the enzyme.

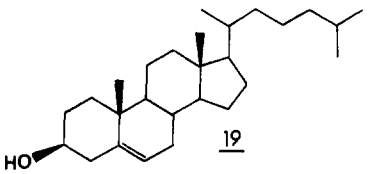
conditions. In the presence of 80 mM trichloroethyl butanoate (TCEB) or trifluoroethyl butanoate (TFEB) the half-lives of the enzyme shaken at 45 °C were 1.5 and 0.5 h. Instead, the enzyme suspended in 80 mM methyl butanoate or in pure benzene under the same conditions retained 75 or 88% of the original activity after 4 days. The poor stability of the enzyme in the presence of TCEB and TFEB might be due to the aspecific acylation of some amino acid residues essential for catalytic activity. Despite its inactivating effect, TCEB was chosen as acylating agent because of its much greater reactivity than that of methyl butanoate (20 times) and because it shifts the equilibrium toward the formation of the steroid ester.[1c] We also observed that the addition of water to the reaction mixture (up to 1% v/v) reduced the rate of esterification and caused the concomitant hydrolysis of the C-24 methyl ester. On the other hand, the addition of molecular sieves (Merck, 3 Å) to the reaction medium, which removed residual water from the enzyme, drastically reduced the reaction rates.

With bile acid methyl esters **12–14**, the reactions were scaled up and the products isolated in 70 to 85% yield. ^{1}H NMR analysis confirmed the selective monoesterification of C-3α hydroxyl.

In a recent paper,[10] Njar and Caspi utilized Cn.c lipase for the selective deprotection of polyacylated steroids. The reactions were performed in organic solvents, using 1-octanol instead of water as nucleophilic agent. Our attempts to use the same methodology to hydrolyze the acetyl esters in **16–18** were unsuccessful,[11] in a way confirming one of the reports in the literature,[10] which stated that the acetyl ester of **4** was not hydrolyzed and was recovered unchanged after 7 days. It should be mentioned that with **16–18** the steroids were the leaving groups, whereas in the esterification of **1–14** the leaving groups were MeOH, TCE, or TFE. This suggests that with **16–18** the formation of the acyl–enzyme intermediate is prevented or highly hindered by the bulky steroid molecule.

The sensitivity of Cn.c lipase toward modifications in the steroid side chain was also investigated briefly. The C-3β OH group of cholesterol (**19**) and of the more complex spirostan rockogenin (**20**) were both esterified by the enzyme. With the latter compound, a preparative scale experiment furnished the 3β-*O*-butanoyl ester in 89% yield.

In closing, we have demonstrated that, providing the right solvent is used, Cn.c lipase can be an efficient catalyst for regioselective esterification or polyhydroxylated steroids, extending what has previously been observed for Ch.v lipase and subtilisin. Exploiting Cn.c lipase's tolerance toward such bulky structures as **20**, we are currently investigating the catalytic properties of this enzyme with ecdyson derivatives and other more complex polyhydroxylated cyclic terpenoids.



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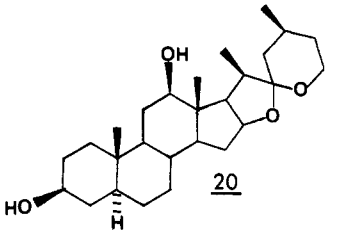

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Experimental Section

Materials. Lipases were obtained as follows: *Candida cylindracea* lipase Type VII (EC 3.1.1.3) and porcine pancreatic lipase Type II (EC 3.1.1.3) from Sigma, *Pseudomonas* sp. lipoprotein lipase (EC 3.1.1.34) from Toyobo, and *Pseudomonas fluorescens* lipoprotein lipase (EC 3.1.1.34) from Amano. In all experiments Cn.c lipase was used directly, as supplied by the manufacturer, without any pretreatment.

Steroids were obtained as follows: **3–9**, **12–14**, and **16–20** from Steraloids; **1**, **2**, **5**, **10**, and **11** from Sigma; **15** was prepared by enzymatic oxidation of **14** as we previously described.[4a]

Benzene (analytical grade) was used without further purification apart from drying by shaking with 3-Å molecular sieves (Merck). 2,2,2-Trichloroethyl butanoate and 2,2,2-trifluoroethyl butanoate were prepared as previously described.[6] Aliphatic acid methyl esters were from Aldrich. All other chemicals used in this work were purchased from commercial suppliers.

General Methods. Enzymatic transesterifications of compounds **2–10** were followed by gas chromatography (GC) with a 5-m HP1 capillary column coated with methylsilicone gum (Hewlett Packard) (N_2 as carrier gas, 30 mL/min, detector and injector port at 300 °C). Enzymatic transesterification of compounds **1** and **11–15** were followed by HPLC using a silica gel column (250 mm × 4.6 mm internal diameter, Whatman) eluted with the appropriate mixture of 2-propanol and hexane. The flow rate was 1 mL min^{-1} and readings were made at 220 nm.

Melting points are uncorrected and were determined in open-ended capillaries. ^{1}H NMR spectra were obtained on a Varian XL-200 (200 MHz) instrument in $CDCl_3$ with Me_4Si as internal standard. In addition to GC and HPLC, the purity of the products was also investigated by TLC with precoated silica gel 60 F_{254} plates (Merck) eluted with mixtures of chloroform and methanol. The spots were developed with Komarowsky's reagent.[12]

Stability of Cn.c Lipase. The enzyme (10–100-mg samples) was suspended in 1 mL of anhydrous benzene containing 900 mM methyl butanoate or 80 mM TCEB or 80 mM TFEB and shaken at 250 rpm and at 45 °C. At different incubation times, 1 mL of benzene containing 34 mM of **2** was added and the residual enzyme activity, i.e., product formation, measured by GC.

Preparative-Scale Acylations of 1, 12, 13, and 14. The following procedure for the acylation of **1** is representative. Cn.c lipase (2 g) was added to 50 mL of anhydrous benzene containing 1.23 mmol (500 mg) of **1** and 4 mmol of TCEB and the suspension was shaken at 250 rpm and at 45 °C. After 24 h a second addition of lipase (2 g) was made and the suspension shaken for another 24 h. HPLC showed that 96% of **1** was acylated. The enzyme was removed by filtration, the solvent evaporated, and the crude residue purified by flash chromatography using $CHCl_3$ as the

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(11) Compounds **16–18** (25 mM) were dissolved in benzene containing 300 mM 1-octanol and Cn.c lipase (50 mg/mL). The suspensions were shaken at 250 rpm and at 45 °C for 4 days. No hydrolysis was evidenced by HPLC and TLC.

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eluent. 3α-O-Butanoyldeoxycholic acid, methyl ester was obtained in 85% yield. The product was crystallized from diethyl ether–hexane: mp 110–2 °C. ^{1}H NMR: δ 0.60 (3 H, s, C-18 Me), 0.86 (3 H, s, C-19 Me), 3.94 (1 H, t, J = 2.8 Hz, H-12β), 4.67 (1 H, sept, J_1 = 10.6 Hz, J_2 = 4.9 Hz, H-3β). Anal. Calcd for $C_{29}H_{48}O_5$: C, 73.47; H, 10.20. Found: C, 73.75; H, 10.03.

The acylations of **12**, **13**, and **14** were carried out similarly. The products were recovered (yields between 70 and 78%) and characterized.

3α-O-Butanoylchenodeoxycholic acid, methyl ester: oil. ^{1}H NMR; δ 0.62 (3 H, s, C-18 Me), 0.88 (3 H, s, C-19 Me), 3.83 (1 H, q, J = 2.5 Hz, H-7β), 4.56 (1 H, sept, J_1 = 10.6 Hz, J_2 = 5 Hz, H-3β). Anal. Calcd for $C_{29}H_{48}O_5$: C, 73.47; H, 10.20. Found: C, 72.71; H, 9.80.

3α-O-Butanoylursodeoxycholic acid, methyl ester: oil. ^{1}H NMR: δ 0.65 (3 H, s, C-18 Me), 0.90 (3 H, s, C-19 Me), 4.10 (1 H, t, J = 3.0 Hz, H-7α), 4.65 (1 H, sept, J_1 = 10.6 Hz, J_2 = 4.9 Hz, H-3β). Anal. Calcd for $C_{29}H_{48}O_5$: C, 73.47; H, 10.20. Found: C, 72.84; H, 9.82.

3α-O-Butanoylcholic acid, methyl ester: mp 116–118 °C (from diethyl ether–hexane). ^{1}H NMR: δ 0.62 (3 H, s, C-18 Me), 0.86 (3 H, s, C-19 Me), 3.84 (1 H, q, J = 2.5 Hz, H-7β), 3.97 (1 H, t, J = 2.8 Hz, H-12β), 4.57 (1 H, sept, J_1 = 10.6 Hz, J_2 = 4.9 Hz, H-3β). Anal. Calcd for $C_{29}H_{48}O_6$: C, 71.15; H, 9.88. Found: C, 70.98; H, 9.93.

Preparation of 3β-O-Butanoylrockogenin. Rockogenin (**20**, 400 mg) was dissolved in 20 mL of anhydrous benzene containing 3 molar equiv of TCEB. Cn.c lipase (1 g) was added and the suspension was shaken at 250 rpm and at 45 °C for 24 h. The enzyme was filtered out, the solvent evaporated, and the crude residue purified by flash chromatography ($CHCl_3$–AcOEt, 9:0.6), yielding 413 mg (89%) of 3β-O-butanoylrockogenin: mp 195 °C (from MeOH). ^{1}H NMR: δ 4.68 (1 H, sept, J_1 = 11 Hz, J_2 = 5.7 Hz, H-3α). Anal. Calcd for $C_{31}H_{50}O_5$·MeOH: C, 71.91; H, 10.11. Found: C, 71.06, H, 9.83.

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Conformationally Constrained Peptides. Chirospecific Synthesis of 4-Alkyl-Substituted γ-Lactam-Bridged Dipeptides from L-Aspartic Acid

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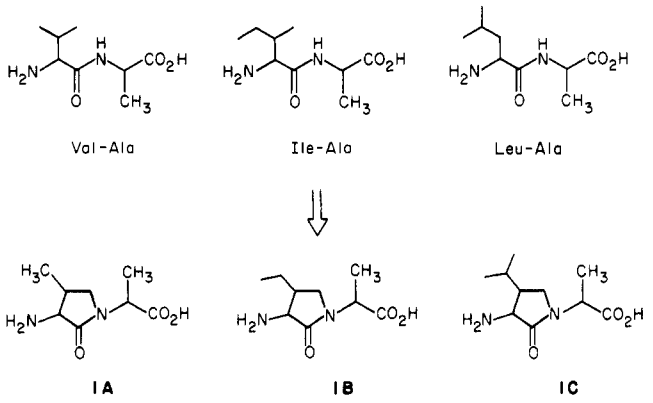
The synthesis of enantiomerically pure γ-lactam-bridged dipeptide analogues of Val-Ala, Ile-Ala, and β-MeLeu-Ala starting from L-aspartic acid is presented. *N*-(9-Phenylfluorenyl)-L-aspartic acid α-*tert*-butyl β-methyl diester and *N*-(9-Phenylfluorenyl)-L-aspartic acid dimethyl ester serve as the educts. They have been successfully alkylated at the β-carbon, C-3, with a variety of electrophiles and with total retention of asymmetric integrity at the α-carbon, followed by a regiospecific reduction of the β-methyl ester. Subsequent oxidation and reductive amination with alanine methyl ester affords the precursors of γ-lactam-bridged dipeptides which have been readily cyclized to the γ-lactams bearing the corresponding valine, isoleucine, and leucine side chains.

Introduction

Lactams as conformational constraints in peptide backbones are effective structural tools for probing the active conformations of bioactive peptides.[1-8] In a number of instances, locking bioactive peptides into active conformers by lactam backbone modification has led to increases in their potency.[1,9,10] Although several synthetic routes to lactam backbone modified peptides are known,[1,2,4,5-8,11-17] these methods commonly lack provision for retention of the amino acid side chain as a substituent on the lactam, place it at C-3 of the lactam, or do not permit continuing extension of the peptide chain. We now present a general synthetic methodology for preparation

Scheme I. Projections for γ-Lactam-Bridged Dipeptide Analogues



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