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Lipase-catalyzed regioselective preparation of fatty acid esters of hydrocortisone

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ABSTRACT

A series of fatty acid derivatives of hydrocortisone has been prepared by an enzymatic methodology. Nine 21-monoacyl products and one 3,11,17-triacetyl derivative, nine of them novel compounds, were obtained in a highly regioselective way through lipase-catalyzed esterification, transesterification and alcoholysis reactions. The influence of various reaction parameters such as acylating agent: substrate ratio, enzyme: substrate ratio, solvent, temperature and nature of acylating agent and alcohol was evaluated. Among the tested lipases, *Candida antarctica* lipase appeared to be the most appropriate and showed a high efficient behavior especially in a one-pot transesterification. The advantages presented by this methodology, such as mild reaction conditions and low environmental impact, make the biocatalysis a convenient way to prepare acyl derivatives of hydrocortisone. These lipophilic compounds are potential products in the pharmaceutical industry.

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1. Introduction

It is well known that glucocorticoids have a huge number of effects on physiologic systems. In addition to their physiologic importance, they are also among the most frequently used drugs, and often prescribed for their immunosuppressive and anti-inflammatory properties. [1]. As a consequence, glucocorticoids are widely used as drugs to treat inflammatory conditions such as arthritis or dermatitis, and as adjunction therapy for conditions such as autoimmune diseases [2].

Regarding their application in dermatology, topical glucocorticoids are the most frequently prescribed drugs [3]. Treatment with topical glucorticoids is effective, easy to administer, acceptable to patients and safe when used correctly [4]. The *in vivo* clinical effectiveness of a topical glucocorticoid depends on the bioavailability of the glucocorticoid within the skin at the site of action. For glucocorticoids the target cells are the keratinocytes and fibroblasts within the viable epidermis and dermis, where the gluocorticoid receptors are located [5].

The cellular uptake and residence time of the steroid as well as its affinity for the glucocorticoid receptor will determine the clinical effect [6]. The total uptake of steroid by fibroblasts and keratinocytes is related to drug lipophilicity [7].

The naturally occurring glucocorticoid is cortisol or hydrocortisone (**1**). It is produced by the adrenal gland and it is reversibly metabolized to biologically inactive cortisone by 11β-hydroxysteroid dehydrogenase [8]. Hydrocortisone has a relative low potency and a short duration of action. Although most of the glucocorticoids that are used therapeutically are synthetic derivatives of hydrocortisone with higher potency, hydrocortisone is preferred for long-term treatments and for use at sites of delicate skin such as facial and skin-fold areas and it is generally recommended for infants [9]. Taking into account these properties and considering that the lipophilicity and metabolic resistance of topic glucocorticoids may be increased by adding ester groups to their structure, it seems interesting to prepare acyl derivatives of hydrocortisone.

The use of enzymes and whole cells of microorganisms in synthesis of pharmaceuticals derivatives is increasing in the last years [10]. It is recognised that enzymes are capable of accepting a wide array of substrates, and catalyze enantio-, chemo- and regioselective reactions. As a result, biocatalysts allow carrying out different chemical transformations without the need for tedious protection and deprotection steps, especially in compounds with several functional groups.

Over the last years, biocatalysis in non-aqueous media has been widely used for several synthetic reactions such as esterification, transesterification, aminolysis, polymerization, etc. [11]. Enzymes are also well-known by its high enantioselective behavior and this property has formed the basis for the widespread use of enzymes for the synthesis of enantiomerically pure compounds [12]. Specifically in the steroid field, enzyme catalysis can play an important role in the mild and selective interconversion of functional groups via regio- and stereoselective transformations [13–16]. Studies carried out in our laboratory on the esterification

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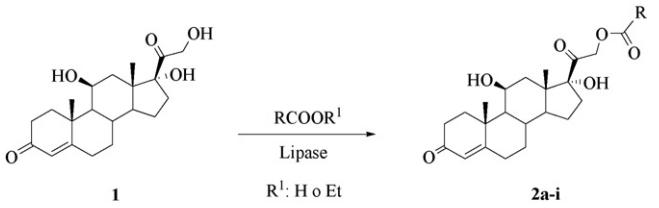


	R
2a	CH_3-
2b	$CH_3(CH_2)_4$-
2c	$CH_3(CH_2)_6$-
2d	$CH_3(CH_2)_8$-
2e	$CH_3(CH_2)_{16}$-
2f	$CH_3(CH_2)_7CH{=}CH(CH_2)_7$- *cis*
2g	$CH_3(CH_2)_7CH{=}CH(CH_2)_7$- *trans*
2h	$CH_3(CH_2)_3(CH_2CH{=}CH)_2(CH_2)_7$- *cis, cis*
2i	$CH_3(CH_2CH{=}CH)_3(CH_2)_7$- *cis, cis, cis*

Scheme 1.

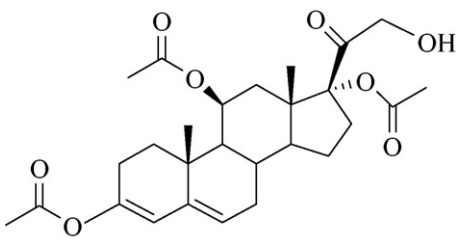
and transesterification of polyfunctionalyzed steroids, have shown that lipases can act on substituents either on A-ring or on the D-ring [17–19]. Taking into account these properties we have prepared fatty acid derivatives of dehydroepiandrosterone [20] and 3,17-β-estradiol [21] and a series of novel 20-succinates of pregnanes [22].

Hydrocortisone was converted into some pregnane and androstane derivatives by some strains of the fungi *Acremonium* [23] but no literature report has been found on enzymatic synthesis of lipophilic derivatives of hydrocortisone. In the present paper, we want to report the results obtained through lipase-catalyzed acylation and alcoholysis reactions in the preparation of acyl derivatives of hydrocortisone **2a**–**2i** (Scheme 1) and **4** (Formula 1).

2. Experimental

2.1. General

All solvents and reagents were of analytical grade. Fatty acids, fatty acid ethyl esters, Lipase from *Candida rugosa* (CRL) (905 U/mg solid) and type II crude from porcine pancreas (PPL) (190 U/mg protein) were purchased from Sigma Chemical Co.; *Candida antarctica* lipase B (CAL B): Novozym 435 (7400 PLU/g) and Lipozyme RM 1 M (LIP) (7800 U/g) were generous gifts of Novozymes Latinoamerica Ltd. and Novozymes A/S, both are commercially available from Novozymes North America Inc.; *Pseudomonas* lipase: Lipase PS Amano (PSL) (33,200 U/g) was purchased to Amano Pharmaceutical Co. All enzymes were used "straight from the bottle". Ethyl stearate and ethyl linolenate were prepared according a procedure previously described [24].



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Formula 1.

Enzymatic reactions were carried out on Innova 4000 digital incubator shaker, New Brunswick Scientific Co. at 200 rpm. Melting points were determined on a Fisher Johns apparatus and are uncorrected. Elemental analysis was carried out with a CE-440 Elemental Analyzer. Optical purities of products were determined by specific rotation with PerkinElmer 343 and Jasco P-1010 polarimeters. Solvents are indicated. All NMR spectra were recorded on a Bruker AM-500 (500 MHz for ^{1}H and 125.1 for ^{13}C). Chemical shifts (δ) are reported in ppm downfield from TMS as the internal standard. Coupling constant (J) values are given in Hz. Solvents are indicated. EI-MS were measured either in a VG TRIO-2 or in a Shimadzu QP-5000 mass spectrometer at 70 eV by direct inlet. Microwave reactions were carried in a SEM Discover monomode reactor using a closed vessel with magnetic stirring.

2.2. Microwave assisted peracetylation of hydrocortisone

3β,11β,17α,21-Tetraacetoxypregna-3,5-dien-20-one (***3***). A mixture of 180 mg (0.5 mmol) of **1**, 3 ml of acetic anhydride (32 mmol) and 1.5 ml of acetyl chloride (21 mmol) was heated under microwave irradiation (300 W) at 150 °C for 45 min. The reaction was then quenched with methanol and the solvent was evaporated. The crude residue purified by flash chromatography on silica gel using hexane/AcOEt (6:4) to yield 182 mg (88%) of 3; M.p.: 211–213 °C [25]; ^{1}H NMR: Table 3; ^{13}C NMR: Table 4.

2.3. Enzymatic monoacylation

2.3.1. General procedure

2.3.1.1. Ethyl carboxylate as acylating agent. To a solution of 0.4 mmol (145 mg) of hydrocortisone in 30 ml of toluene (or ethyl acetate in the preparation of **2a**), containing of the ethyl carboxylate (4 mmol), 300 mg of CAL B were added. The suspension was incubated at 110 °C with magnetic stirring (or shaken at 200 rpm and 55 °C for ethyl acetate) and the progress of reaction was monitored by TLC (hexane/ethyl acetate (6:4)). Once the reaction was finished, the enzyme was filtered off and the solvent evaporated under reduced pressure. The crude residue was purified by silica column chromatography (hexane/ethyl acetate (6:4)).

2.3.1.2. Carboxylic acid as acylating agent. To a solution of hydrocortisone (0.4 mmol) in toluene (50 ml), 4 mmol of the carboxylic acid and 300 mg of CAL B were added. The mixture was incubated at 110 °C with magnetic stirring, and the progress of the reaction was monitored by TLC (hexane/AcOEt (6:4)). Once the reaction was finished, the enzyme was filtered off and washed with dichloromethane (3 ml × 5 ml). The organic phases were combined and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude residue purified by flash chromatography on silica gel using hexane/AcOEt (6:4).

2.3.1.3. One-pot transesterification procedure. As described in Section 2.3.1.2 but in this case CAL B (360 mg) was added to a solution of hydrocortisone (0.4 mmol), the carboxylic acid (4 mmol) and 0.25 ml (4 mmol) of ethanol in 50 ml of toluene.

21-Acetoxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2a***). 160 mg (92%); M.p.: 217–218 °C, lit. [26] 221–223 °C); $[\alpha]_D$ +169 ($CHCl_3$), lit. [26] $[\alpha]_D$ +138° (acetone).^{1}H NMR and ^{13}C NMR are in accordance with reported data [27,28].

21-Hexanoyloxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2b***). As described in general procedure and using hexanoic acid (480 mg) or 577 mg of ethyl hexanoate as acylating agent: 168 mg (88%) with hexanoic acid, 172 mg (90%) with ethyl hexanoate and 178 mg (93%) by the one-pot procedure, of **2b**. M.p.: 124–125 °C. $[\alpha]_D$ (c = 2.88, $CHCl_3$): +99.4°. ^{1}H NMR: Table 3. ^{13}C NMR: Table 4. MS

(EI) *m/z* (rel. int.): 460 [M$^+$] (1), 344 (2), 285 (6), 43 (100). Analysis for $C_{27}H_{40}O_6$: calcd. C, 70.41; H, 8.75. Found: C, 70.25; H, 8.65.

21-Octanoyloxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2c***). As described in general procedure and using octanoic acid (596 mg) or 712 mg of ethyl octanoate as acylating agent: 177 mg (91%) with octanoic acid, 176 mg (90%) with ethyl octanoate and 179 mg (92%) by the one-pot procedure, of **2c**. M.p.: 104–105 °C. $[\alpha]_D$ (c = 1.00, $CHCl_3$): +109.3°. ^{1}H NMR: Table 3. ^{13}C NMR: Table 4. MS (EI): *m/z* (rel. int.): 488 [M$^+$] (12), 344 (30), 185 (11), 143 (23), 127 (72), 57 (100). Analysis for $C_{29}H_{44}O_6$: calcd. C, 71.28; H, 9.08. Found: C, 71.05; H, 9.05.

21-Decanoyloxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2d***). As described in general procedure, but using decanoic acid (712 mg) or 801 mg of ethyl decanoate as acylating agent: 149 mg (70%) with decanoic acid, 160 mg (75%) with ethyl decanoate and 166 mg (78%) by the one-pot procedure, of **2d**. M.p.: 117–118 °C. $[\alpha]_D$ (c = 1.00, $CHCl_3$): +69.5°. ^{1}H NMR: Table 3. ^{13}C NMR: Table 4. MS (EI) *m/z* (rel. int.): 516 [M$^+$] (4), 285 (38), 344 (16), 155 (46), 43 (100). Analysis for $C_{31}H_{48}O_6$: calcd. C, 72.06; H, 9.36. Found: C, 72.00; H, 9.25.

21-Octadecanoyloxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2e***). As described in general procedure, but using octadecanoic acid (1.18 g) or 1.25 g of ethyl octadecanoate as acylating agent: 159 mg (61%) with octanoic acid, 169 mg (65%) with ethyl octanoate and 185 mg (71%) by the one-pot procedure, of **2e**. M.p.: 73–75 °C. $[\alpha]_D$ (c = 0.62, $CHCl_3$): +46.5°. ^{1}H NMR: Table 3. ^{13}C NMR: Table 4. MS (EI)) *m/z* (rel. int.): 344 (3), 285 (2), 267 (2), 240 (5), 43 (100). Analysis for $C_{39}H_{64}O_6$: calcd. C, 74.48; H, 10.26. Found: C, 74.72; H, 10.35.

21-cis-9-Octadecenoyloxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2f***). As described in general procedure, but using *cis*-9-octadecenoic acid (1.17 g) or 1.24 g of ethyl *cis*-9-octadecenoate as acylating agent: 132 mg (51%) with the carboxylic acid, 148 mg (57%) with the ethyl carboxylate and 179 mg (69%) by the one-pot procedure, of **2f**. M.p.: 82–84 °C. $[\alpha]_D$ (c = 2.23, $CHCl_3$): +107.3°. ^{1}H NMR: Table 3. ^{13}C NMR: Table 4. MS (EI) *m/z* (rel. int.): 626 [M$^+$] (1), 265 (4), 237 (1), 121 (17), 41 (100). Analysis for $C_{39}H_{62}O_6$: calcd. C, 74.72; H, 9.97. Found: C, 74.85; H, 10.05.

21-trans-9-Octadecenoyloxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2g***). As described in general procedure, but using *trans*-9-octadecenoic acid (1.17 g) or 1.24 g of ethyl *trans*-9-octadecenoate as acylating agent: 135 mg (52%) with the carboxylic acid, 161 mg (62%) with the ethyl carboxylate and 177 mg (68%) by the one-pot procedure, of **2g**. M.p.: 75–78 °C. $[\alpha]_D$ (c = 0.71, $CHCl_3$): +97.2°. ^{1}H NMR: Table 3. ^{13}C NMR. Table 4. MS (EI): *m/z* (rel. int.): 626 [M$^+$] (12), 344 (13), 326 (7), 285 (18), 41 (100): Analysis for $C_{39}H_{62}O_6$: calcd. C, 74.72; H, 9.97. Found: C, 74.95; H, 10.15.

21-cis,cis-9,12-Octadecadienoyloxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2h***). As described in general procedure, but using *cis*,*cis*-9,12-octadecadienoic acid (1.16 g) or 1.23 g of ethyl *cis*,*cis*-9,12-octadecadienoate as acylating agent: 129 mg (50%) with the carboxylic acid, 157 mg (61%) with the ethyl carboxylate and 170 mg (66%) by the one-pot procedure, of **2h**. M.p.: 68–70 °C. $[\alpha]_D$ (c = 2.00, $CHCl_3$): +93.3°. ^{1}H NMR: Table 3. ^{13}C NMR: Table 4. MS (EI) *m/z* (rel. int.): 624 [M$^+$] (12), 344 (14), 329 (34), 301(10), 67 (100). Analysis for $C_{39}H_{60}O_6$: calcd. C, 74.96; H, 9.68. Found: C, 75.05; H, 9.86.

21-cis,cis,cis-9,12,15-Octadecatrienoyloxy-11β,17α-dihydroxypregn-4-en-3,20-dione (***2i***). As described in general procedure, but using *cis*,*cis*,*cis*-9,12,15-octadecatrienoic acid (1.15 g) or 1.22 g of ethyl *cis*,*cis*,*cis*-9,12,15-octadecatrienoate as acylating agent: 74 mg (30%) with the carboxylic acid, 121 mg (49%) with the ethyl carboxylate and 146 mg (59%) by the one-pot procedure, of **2i**. M.p.: 55–58 °C. $[\alpha]_D$ (c = 1.02, $CHCl_3$): +27.9°. ^{1}H NMR: Table 3. ^{13}C NMR: Table 4. MS (EI) *m/z* (rel. int.): 344 (4), 285 (3), 55 (63), 43 (100). Analysis for $C_{39}H_{58}O_6$: calcd. C, 75.20; H, 9.39. Found: C, 75.26; H, 9.48.

2.4. Enzymatic alcoholysis

3,11β,17α-Triacetoxy-21-hydroxypregna-3,5-dien-20-one (***4***). To a solution of **3** (136 mg (0.3 mmol)) in 15 ml of alcohol, 680 mg of CAL B were added. The suspension was shaken (200 rpm) at 55 °C and the progress of reaction was monitored by TLC. After 24 h, the enzyme was filtered off, the solvent was evaporated and the crude residue was purified by flash chromatography on silica gel using hexane/AcOEt (7:3) to yield 94 mg (75%) of **4**. M.p.: 55–57 °C. $[\alpha]_D$ (c = 1.02, $CHCl_3$): −15.6°. ^{1}H NMR: Table 3. ^{13}C NMR: Table 4. MS (EI) *m/z* (rel. int.): 488 [M$^+$] (0.5), 429 (2), 384 (3), 370 (1), 311 (9), 59 (4), 43 (100). Analysis for $C_{27}H_{36}O_8$: calcd. C, 66.38; H, 7.43. Found: C, 66.00; H, 7.16.

3. Results

The presence of the three hydroxyl groups in hydrocortisone (**1**) makes this compound an interesting model for enzymatic transformation. Therefore, we decided to study the behavior of lipases in acylation and alcoholysis reactions on this glucocorticoid.

3.1. Enzymatic acylation

The enzyme-catalyzed acylation of **1** allowed us to obtain, in a regioselective way, monoacylated derivatives with the acyl group exclusively suited in the 21 position of ring D of the steroidal skeleton (Scheme 1). In order to optimize the reaction conditions we have performed several experiments such as lipase screening and variation of the reaction parameters such as temperature, enzyme: substrate ratio (*E*/*S*) and acylating agent: substrate ratio (*A*/*S*).

3.1.1. Enzyme screening and solvent effect

Several commercial lipases in different solvents were evaluated in the acylation reaction of **1** with stearic acid: *C. rugosa* lipase (CRL), *C. antarctica* lipase B (CAL B); Lipozyme: lipase from the fungus *Rhizomucor miehei* (LIP), porcine pancreatic lipase (PPL) and lipase from *Pseudomonas* sp. (PS-C). The solvents selected for this screening were acetone, acetonitrile, hexane, diisopropyl ether and toluene. Reactions were carried out at 55 °C using an *E*/*S* ratio of 2 and an A: **1** ratio of 10. TLC monitoring allowed the identification of the lipase able to promote the acylation of **1**. In the absence of biocatalyst no product was obtained and, among the tested lipases, only CALB gave satisfactory results working in hexane or toluene.

It is well-known that hydrophobic water-immiscible solvents such toluene or hexane are a good medium for lipase-catalyzed reactions. The organic medium shows interesting advantages, such as the enhancement of solubility of reactants, activity and stability of the enzyme, the shift of the equilibrium towards product formation and easier separation of the enzyme from the reaction medium at the end of the reaction [11].

The spectroscopic analysis of the product from the CALB-catalyzed esterification in toluene showed that this lipase was completely regioselective because the stearyl derivative in the 21 position of hydrocortisone (**2e**) was obtained as the only product. Therefore CALB was the enzyme of choice for any further experiments on hydrocortisone esterification. The monoacylation of hydrocortisone on the hydroxyl of carbon 21 in compound **2e** was established by observing the downfield shifts in ^{1}H NMR, a double doublet of H-21 from δ 4.19 and 4.61 ppm in **1** to δ 4.85 and 5.03 ppm in the product **2e** (Table 3).

Many reports demonstrate that organic media not only influence the enzymatic activity but also the selectivity, and many examples have been observed about the correlation of enzyme regioselectivity with solvent parameters [29], especially with hydrophobicity described by log *P*. In this work, when polar solvents such as acetone or acetonitrile were used, no enzyme activity

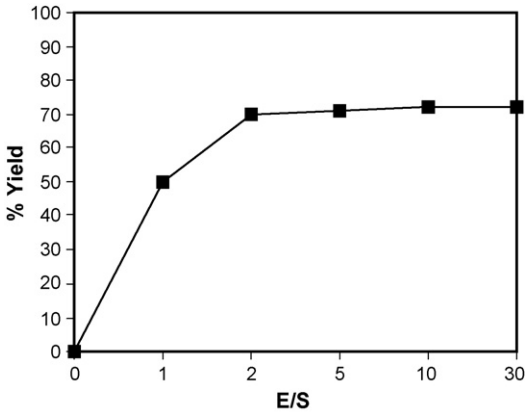


Fig. 1. Effect of enzyme:substrate ratio on the CALB-catalyzed synthesis of 21-hydrocortisone stearate (**2e**). Acylating agent: S 10, temperature 110 °C, time 24 h.

was observed. On the other hand, the enzymatic reactions using non polar solvents such as hexane and toluene, with low log *P* (log *P* hexane: 3.5 and log *P* toluene: 2.5) [30] gave very good results regarding lipase activity and regioselectivity.

3.1.2. Effect of enzyme: substrate ratio

The influence of the enzyme: substrate ratio in the enzymatic esterification was evaluated at 24 h, using *A*/*S*: 10, toluene as solvent at 110 °C and variable amounts of CAL B. From the obtained results (Fig. 1), it can be concluded that a ratio *E*/*S* of 2 is the most satisfactory.

3.1.3. Influence of temperature

With the aim of investigating the influence of temperature on the enzymatic esterification we performed it at 30 °C, 55 °C and 110 °C. The other reaction parameters were settled to their optimal values (CAL B, toluene, *E*/*S*: 2 and *A*/*S*: 10). The results in Table 1 (entries 1–3) show an increase in yield with the increase in temperature. Therefore we selected 110 °C as the reaction temperature. It is interesting to observe that the regioselectivity of the enzymatic acylation was kept unaltered with the increase of temperature reaction, being **2e** the only product obtained in every case.

3.1.4. Effect of acylating agent nature and acylating agent: substrate ratio

In view of our previous work on enzymatic acylation of steroids and other natural products, we decided to test the acylation reaction by using ethyl stearate and stearic acid as acylating agents [20,31,32]. The results obtained were both good and much the same (Table 2, entry 5), the hydrocortisone 21-stearate was obtained in a regioselective way, being the ethyl carboxylate slightly more efficient (65%) than the carboxylic acid (61%).

This behavior was kept in the preparation of the rest of products **2b**–**2i**. Accordingly, both the esterification and transesterification reaction of hydrocortisone with variable chain length saturated and unsaturated carboxylic acids and ethyl carboxylates from 2 to 18 carbon atoms gave the monoacyl derivative exclusively in position 21 in high yield.

As in previous work on catalyzed acylation of steroid compounds, it was also observed that the product yield was variable and decreased as the chain length of the acylating agent and unsaturation increased. Stereochemistry of the only double bond in the case of oleic and elaidic acid derivatives, had no significant effect on yield.

The influence of acylating agent: substrate ratio on reaction yield was evaluated in the esterification of hydrocortisone **1** with stearic acid in toluene using CALB. As expected, when we studied the influence of *A*/*S*, it was observed that a molar excess of fatty acid or ethyl carboxylate was advantageous for the reaction (Table 1, entries 3–6) with *A*/*S* 10 (entry 3) giving the best results (67% yield). A higher excess of fatty acid did not improve yields.

3.1.5. One-pot procedure

The previous results in enzymatic transesterification (ethyl carboxylate) and esterification (carboxyilic acid) of hydrocortisone following a biocatalytic way, prompted us to apply a one-pot procedure in which the acylating agent and the compounds of **2b**–**2i** were obtained in the same step. Therefore, we treated **1** with the carboxylic acid, ethanol and toluene as solvent in the presence of CAL B. The reaction was performed under reflux and with *E*/*S* 2, carboxylic acid/**1** 10 and carboxylic acid/alcohol 0.5.

It was observed that the products **2b**–**2i** were obtained in higher yield than in the enzymatic transesterification or esterification. The advantage in yield due to the application of the one-pot procedure was remarkable in the longer chain derivatives, for example it was observed a difference of 19% in **2i** yield employing this strategy (59% one-pot and 30% in the esterification) (Table 2, entry 9). Therefore, the one-pot procedure can be considered as the best enzymatic way to prepare the acyl derivatives of hydrocortisone.

Regarding the acetyl derivative, it was prepared by treatment of hydrocortisone with ethyl acetate as solvent and acylating agent and CAL B as biocatalyst. The product, obtained in excellent yield (92%) was identified as hydrocortisone-21 acetate (**2a**). Physical and ^{13}C spectral data were according to literature [26–28].

3.2. Enzymatic alcoholysis

The good results in yield and regioselectivity shown by CAL B in the acylation reactions of hydrocortisone in the hydroxyl group of 21 position, prompted us to study if the lipase showed the same behavior catalyzing the alcoholysis reaction on the peracetylated hydrocortisone (**3**). Scheme 2 shows the synthetic way from hydrocortisone.

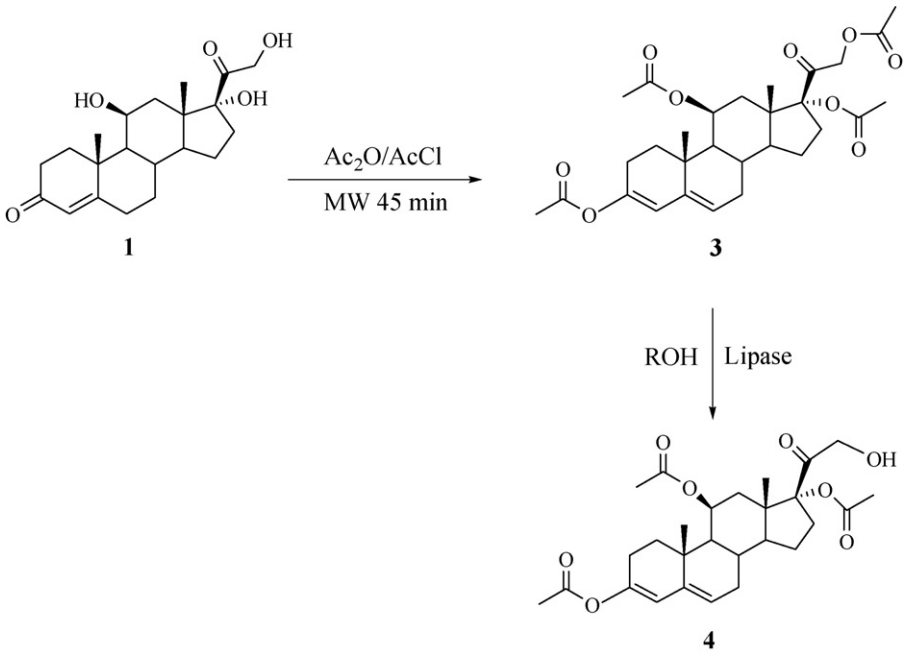
Table 1
Acylating agent/substrate ratio on the CALB-catalyzed synthesis of Hydrocortisone-21-stearate **2e**.

Entry	*A*/*S*	Temperature (°C)	Yield (%)
1	10	30	23
2	10	55	48
3	10	110	67
4	1	110	17
5	5	110	31
6	15	110	68

E/*S*: 2. Solvent: toluene.

Table 2
Lipase-catalyzed synthesis of 21-hydrocortisone esters from carboxylic acids, ethyl carboxylates and one-pot procedure (**2**).

Entry	Product	Acylating agent	Yield (%)		
			R		One-pot
			H	OEt	
1	**2a**	CH_3-COOR	–	92	–
2	**2b**	$CH_3(CH_2)_4COOR$	88	90	93
3	**2c**	$CH_3(CH_2)_6COOR$	91	90	92
4	**2d**	$CH_3(CH_2)_8COOR$	70	75	78
5	**2e**	$CH_3(CH_2)_{16}COOR$	61	65	71
6	**2f**	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOR$ *cis*	51	57	69
7	**2g**	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOR$ *trans*	52	62	68
8	**2h**	$CH_3(CH_2)_3(CH_2CH{=}CH)_2(CH_2)_7COOR$ *cis*,*cis*	50	61	66
9	**2i**	$CH_3(CH_2CH{=}CH)_3(CH_2)_7COOR$ *cis*,*cis*,*cis*	30	49	59



Scheme 2.

In order to obtain the substrate **3** we tested several procedures. We tried the peracetylation of hydrocortisone with acetic anhydride and several catalysts such as pyridine, p-toluensulfonic acid [26] and trimethylsilyl trifluoromethanesulfonate [33] at room temperature, reflux or microwave irradiation [34]. The microwave irradiation promoted the decomposition of the steroid and the other reaction conditions afforded only the 21-monoacetate in low yield.

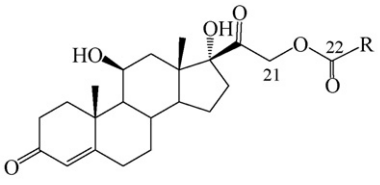
Finally, the substrate **3** was obtained in 88% yield by heating **1**, under microwave irradiation using a monomode reactor at 300 W, with a mixture of acetic anhydride: acetyl chloride 1.5.

At this point, we attempted to carry out the enzymatic alcoholysis of **3** using the same enzymes tested on the acylation reactions; ethanol, n-butanol and octanol as nucleophiles and acetonitrile, acetone, diisopropyl ether and ethanol as solvents. Only the reaction with CAL B and ethanol allowed us to obtain the triacetyl derivative **4** in very good yield (83%). Butanol gave the same product but in lower yield (11%) whereas octanol did not afford any product.

The product **4** was fully identified by spectroscopic methods. By comparison with the ^{1}H and ^{13}C spectra (Tables 3 and 4) of the tetraacetyl derivative **3**, run in the same solvent, we were able to assign the structure of compound 4. The ^{1}H NMR spectrum of **4** showed an upfield shift of H-21, double doublet at δ 4.64 and 4.82 ppm in **3** to a singlet at δ 4.27 ppm in the product **4**. Moreover only three singlets at δ 2.14, 2.08 and 2.01 corresponding to acetyl groups in positions 3, 11 and 17, were observed.

These results were obtained at 24 h, longer reaction times did not promote any change: neither the desired mono- and diacetylated derivatives of hydrocortisone or the product with the α,β-unsaturated ketone in carbon 3, were obtained.

Again the lipase showed a regioselective behavior acting only on carbon 21. Therefore, the enzymatic alcoholysis offers a good alternative to remove ester groups in carbon 21 keeping unaltered other esters in the molecule such as the enol-acetate protecting group in carbon 3. Although the remotion of acetyl group in C-21 of hydrocortisone, performed by chemical methods is not difficult, the enzymatic approach shows interesting advantages. The reaction is simple, it is performed at room temperature. Moreover, ethanol,



R

2a CH_3-
23

2b $CH_3(CH_2)_4$-
27 26-23

2c $CH_3(CH_2)_6$-
29 28-23

2d $CH_3(CH_2)_8$-
31 30-23

2e $CH_3(CH_2)_{16}$-
39 38-23

2f $CH_3(CH_2)_7CH{=}CH(CH_2)_7$- *cis*
39 38-32 31-30 29-23

2g $CH_3(CH_2)_7CH{=}CH(CH_2)_7$- *trans*
39 38-32 31-30 29-23

2h $CH_3(CH_2)_3(CH_2CH{=}CH)_2(CH_2)_7$- *cis, cis*
39 38-36 35-30 29-23

2i $CH_3(CH_2CH{=}CH)_3(CH_2)_7$- *cis, cis, cis*
39 38-30 29-23

Scheme 3.

Table 3
^{1}H NMR data of compounds **2a**–**2i**, **3**–**4**.

H no.	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**2i**	**3**	**4**
1α	1.85 m	1.88 m	1.89 m	1.87 m	1.86 m	1.86 m	1.86 m	1.87 m	1.48 m	1.50 m
1β	2.21 m	2.21 m	2.22 m	2.22 m	2.23 m	2.21 m	2.20 m	2.21 m	1.85 m	1.86 m
2α	2.34 dt (17.0, 4.5)	2.33 dt (17.0, 4.5)	2.33 dt (17.0, 4.5)	2.34 dt (17.0, 4.5)	2.35 dt (17.0, 4.5)	2.35 dt (17.0, 4.5)	2.35 dt (17.0, 4.5)	2.36 m	2.18 m	2.16 m
2β	2.50 m	2.51 m	2.51 m	2.51 m	2.50 m	2.51 m	2.50 m	2.50 m	2.44 m	2.45 m
3	–	–	–	–	–	–	–	–	2.16 s[a]	2.14 s[a]
4	5.70 d (1.2)	5.68 d (1.2)	5.71 d (1.2)	5.69 d (1.2)	5.69 d (1.2)	5.68 d (1.2)	5.68 d (1.2)	5.68 s	5.67 d (2.0)	5.69 d (2.0)
6α	2.25 m	2.25 m	2.22 m	2.23 m	2.22 m	2.23 m	2.23 m	2.23 m	5.33 t (3.5)	5.35 t
6β	2.51 m	2.50 m	2.53 m	2.52 m	2.51 m	2.51 m	2.51 m	2.51 m	1.88 m	1.90 m
7α	1.11 m	1.14 m	1.14 m	1.14 m	1.11 m	1.12 m	1.12 m	1.12 m	2.41 m	2.42 m
7β	2.03 m	2.04 m	2.03 m	2.05 m	2.01 m	2.06 m	2.06 m	2.06 m	2.18 m	2.21 m
8	2.07 m	2.08 m	2.08 m	2.09 m	2.09 m	2.09 m	2.09 m	2.08 m	1.37 dd (12.0, 3.2)	1.35dd (12.0, 3.2)
9	1.00 dd (11.0, 3.2)	1.04 dd (11.0, 3.2)	1.04 dd (11.0, 3.2)	1.02 dd (11.0, 3.2)	1.00 dd (11.0, 3.2)	1.01 dd (11.0, 3.2)	1.01 dd (11.0, 3.2)	1.01 dd (11.0, 3.2)	–	–
11	4.48 dd (6.2, 3.2)	4.47 dd (6.0, 3.2)	4.50 dd (6.2, 3.2)	4.48 dd (6.2, 3.2)	4.47 dd (6.2, 3.2)	4.47 dd (6.2, 3.2)	4.47 dd (6.2, 3.2)	4.47 dd (6.2, 3.2)	5.55 dd (6.5, 3.4) 2.14 s[a]	5.54 dd (6.5, 3.4) 2.08 s[a]
12α	2.06 m	2.06 m	2.06 m	2.06 m	2.06 m	2.06 d (3.7)	2.05 d (3.7)	2.05 d (3.7)	2.11 m	2.14 m
12β	1.75 dd (14.0, 2.6)	1.76 dd (14.0, 2.6)	1.76 dd (14.0, 2.6)	1.75 dd (14.0, 2.6)	1.75 dd (14.0, 2.6)	1.75 dd (14.0, 2.6)	1.75 dd (14.0, 2.6)	1.75 dd (14.0, 2.6)	1.97 m	2.02 m
14	1.72 m	1.73 m	1.73 m	1.70 m	1.71 m	1.70 m	1.70 m	1.70 m	1.77 m	1.80 m
15α	1.48m	1.48m	1.48 m	1.47 m	1.47 m	1.47 m	1.47 m	1.47 m	1.47 m	1.48 m
15β	1.80 m	1.84 m	1.84 m	1.83 m	1.81 m	1.83 m	1.84 m	1.84 m	1.86 m	1.86 m
16α	1.48 m	1.48 m	1.48 m	1.47m	1.47 m	1.47 m	1.47 m	1.47 m	1.88 m	1.90 m
16β	2.74 m	2.79 m	2.79 m	2.78 m	2.75 m	2.76 m	2.77 m	2.80 m	2.88 m	2.87 m
17	–	–	–	–	–	–	–	–	2.09 s[a]	2.01 s[a]
18	0.94 s	0.95 s	0.99 s	0.97 s	0.96 s	0.96 s	0.96 s	0.96 s	0.87 s	0.80 s
19	1.43 s	1.44 s	1.47 s	1.47 s	1.45 s	1.44 s	1.44 s	1.44 s	1.07 s	1.06 s
21	4.88 d (17.5) 5.04 d (17.5)	4.88 d (17.5) 5.03 d (17.5)	4.88 d (17.5) 5.04 d (17.5)	4.85 d (17.5) 5.03 d (17.5)	4.89 d (17.5) 5.03 d (17.5)	4.86 d (17.5) 5.02 d (17.5)	4.85 d (17.5) 5.02 d (17.5)	4.85 d (17.5) 5.02 d (17.5)	4.64d (16.5) 4.82d (16.5) 2.03 s[a]	4.27 s
23	2.43 td (7.6, 1.6)	2.43 td (7.5, 1.6)	2.45 td (7.5,1.6)	2.46 td (7.5, 1.6)	2.44 td (7.5, 1.6)	2.44 m	2.43 td (7.5, 1.6)	2.43 td (7.5, 1.6)	–	–
24	1.65 m	1.67 m	1.68 m	1.64 m	1.67 m	1.65 m	1.66 m	1.66 m	–	–
25	1.33 m	1.32 m	1.31 m	1.28 m	1.32 m	1.29 m	1.32 m	1.32 m	–	–
26									–	–
27	0.90 t (7.0)								–	–
28	–								–	–
29	–	0.89 t (7.0)			2.00 m	1.96 m	2.10 m	2.07 m	–	–
30	–	–			5.35 m	5.38 m	5.35 m	5.35 m	–	–
31	–	–	0.90 t (7.0)						–	–
32	–	–	–		2.00 m	1.96 m	2.77 m	2.83 m	–	–
33	–	–	–		1.32 m	1.29 m	5.35 m	5.35 m	–	–
34	–	–	–						–	–
35	–	–	–				2.10 m	2.83 m	–	–
36	–	–	–				1.32 m	5.35 m	–	–
37	–	–	–						–	–
38	–	–	–					2.07 m	–	–
39	–	–	–	0.90 t (7.0)	0.89 t (7.0)	0.88 t (7.0)	0.89 t (7.0)	0.89 t (7.0)	–	–

Solvent: $CDCl_3$. Carbon number: Scheme 3.
[a] Acetate signal. See formula in Scheme 3.

Table 4
^{13}C NMR of compounds **2a**–**2i**, **3**–**4**.

C No.	2b	2c	2d	2e	2f	2g	2h	2i	3	4
1	34.9	34.9	34.9	35.1	35.1	35.1	35.2	35.1	33.2	33.2
2	33.9	33.9	34.0	34.1	33.9	33.9	33.9	33.9	31.5	31.5
3	200.0	199.8	199.7	199.8	200.0	199.9	199.9	199.9	122.4 (20.5, 170.2)[a]	122.4 (21.0, 170.6)[a]
4	122.2	122.2	122.5	122.5	122.4	122.4	122.6	122.4	116.1	116.0
5	172.9	173.8	173.8	174.0	173.9	173.9	173.9	173.9	147.3	147.1
6	32.1	32.0	32.0	32.1	32.2	32.2	32.2	32.1	122.4	122.8
7	32.8	32.9	32.9	32.9	32.9	32.9	32.9	32.7	31.6	31.3
8	31.4	31.5	31.6	31.6	31.5	31.5	31.6	31.5	28.6	28.6
9	56.0	56.2	56.1	56.2	56.2	56.2	56.2	56.1	50.2	50.0
10	39.4	39.4	39.4	39.4	39.4	39.4	39.4	39.3	34.7	35.0
11	68.2	68.2	68.4	68.4	68.3	68.4	68.4	68.3	69.7 (21.1, 169.3)[a]	69.4 (21.2, 169.0)[a]
12	39.7	39.9	39.9	39.9	39.8	39.8	39.9	39.7	35.8	35.8
13	47.7	47.7	47.8	47.8	47.6	47.7	47.7	47.6	46.8	46.7
14	52.0	52.1	52.2	52.2	52.1	52.2	52.1	52.0	53.2	53.1
15	23.6	23.8	23.8	23.8	23.8	23.8	23.8	23.6	23.8	23.7
16	34.5	34.8	34.8	34.8	34.7	34.8	34.8	34.7	30.9	30.8
17	89.9	89.8	89.9	89.9	89.8	89.8	89.9	89.7	94.6 (20.9, 170.0)[a]	90.1 (21.0, 169.0)[a]
18	17.0	17.1	17.3	17.3	17.1	17.2	17.2	17.1	15.6	15.7
19	20.9	21.0	21.1	21.1	21.1	21.1	21.1	21.1	21.1	21.3
20	205.4	205.1	205.0	205.0	205.2	205.1	205.1	204.6	198.8	199.0
21	67.9	67.9	67.8	67.8	67.9	67.9	67.8	67.7	66.9 (20.9, 170.1)[a]	66.9
22	172.9	172.3	172.2	172.3	172.7	172.5	172.5	172.5	–	–
23	33.9	34.0	34.0	33.9	34.0	34.0	34.0	33.8	–	–
24	25.0	25.0	25.0	25.0	25.0	25.0	25.0	24.8	–	–
25	31.2	31.5	32.2	32.1	32.0	32.0	31.7	31.7	–	–
26	22.3	29.1	32.0	29.8	29.9	29.8	29.7	29.7	–	–
27	13.9	29.0	29.5	29.8	29.8	29.7	29.5	29.3	–	–
28	–	22.7	29.4	29.8	29.8	29.6	29.3	29.1	–	–
29	–	14.2	29.2	29.8	27.3	32.7	27.3	27.3	–	–
30	–	–	22.8	29.8	130.1	130.6	130.4	127.7	–	–
31	–	–	14.3	29.8	129.9	130.3	130.2	127.7	–	–
32	–	–	–	29.8	27.3	32.7	25.8	25.6	–	–
33	–	–	–	29.8	29.6	29.6	128.2	128.3	–	–
34	–	–	–	29.7	29.4	29.4	128.1	128.3	–	–
35	–	–	–	29.6	29.3	29.3	27.3	25.6	–	–
36	–	–	–	29.5	29.2	29.2	29.2	130.1	–	–
37	–	–	–	29.4	29.1	29.1	29.1	132.0	–	–
38	–	–	–	22.8	22.8	21.2	22.7	22.7	–	–
39	–	–	–	14.3	14.2	14.2	14.2	14.2	–	–

Solvent: $CDCl_3$. Carbon number: Scheme 3.

[a] Acetate signals.

used as nucleophile and solvent, is economic and has low toxicicty, and the lipase is recyclable and biodegradable.

4. Discussion

In our previous works we found that enzymes proved to be useful catalysts in steroid transformations. In this report, we use a lipase as biocatalyst in esterification and transesterification reactions of hydrocortisone with variable chain length saturated and unsaturated carboxylic acids and ethyl carboxylates from 2 to 18 carbon atoms. We have obtained nine acyl derivatives, eight of them novel compounds. All the products were identified by spectroscopic methods, ^{1}H and ^{13}C NMR spectra are reported. Signal assignments were made by comparison of the data with previous reports on hydrocortisone [26,27,34].

In order to get the best reaction conditions we studied the influence of various reaction parameters and procedures, affording the best results with *C. antarctica* lipase in the one-pot transesterification.

It is important to emphasize the completely regioselective behavior shown by the *C. antarctica* lipase. Only the 21 hydroxyl group acts as substrate of the enzymatic reaction and 21 acyl derivatives are exclusively obtained. It was also observed that the yield depends on the chain length and unsaturation grade of the acylating agent but not on the stereochemistry of the double bond.

Finally, this work also describes the application of the enzymatic approach to the preparation of 3,11β,17α-Triacetoxy-21-hydroxypregna-3,5-dien-20-one, novel compound obtained by enzymatic alcoholysis of a tetra-acyl derivative of hydrocortisone. Based on current results and the general behavior displayed by CAL B, the enzyme acts only at carbon 21 of hydrocortisone both in acylation and alcoholysis reactions.

This biotechnological procedure shows several advantages such as regioselectivity and low environmental impact. Through the regioselective behavior of the enzyme it is possible to obtain the desired product free of secondary by-products. Biocatalytic reactions offer a way for achieving green chemistry goals. The lipase is biodegradable and consequently more friendly to the environment than chemical catalysts. On the other hand, ethyl carboxylates and carboxylic acid are less toxic than most acylating agents commonly used in traditional synthetic procedures. Moreover, as the enzyme is insoluble in the reaction medium, it is easily removed by filtration at the end of the process and can be re-used. In the acetylation reaction of hydrocortisone, CAL B keeps 78% of its activity after ten reaction cycles.

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