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Immobilized Heterologous *Rhizopus Oryzae* Lipase as an Efficient Catalyst in the Acetylation of Cortexolone

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The enzymatic preparation of a monoacetyl derivative of the corticosteroid cortexolone, through a transesterification reaction, is described. The heterologous *Rhizopus oryzae* lipase, immobilized on three different supports, proved to be an efficient catalyst in the acylation reaction using a complex substrate such as cortexolone. Immobilization of the enzyme on Lewatit 1600 resin at pH = 7 and 25 °C was the best condition for catalysis of the acetylation reaction. The influence of various reaction parameters, such as the nature of the acetylating agent, the solvent, the temperature, and the ratios of acetylating agent to substrate, and enzyme to substrate, was evaluated. Using the response surface methodology and a central composite rotatable design, the specific yield of acetylated cortexolone was optimized by means of the study of the effect of the enzyme (E)/substrate (S) and the acylating agent (A)/substrate ratios. The ratios of 5 (E/S) and 31.6 (A/S) were predicted as the optimal values to reach the maximum specific yield of the product (P): 1.59 mmol P/mmol A·g E. The mild reaction conditions and low environmental impact make the biocatalytic procedure a convenient way to prepare the reported derivative of this biologically active steroid.

Introduction

Corticosteroids constitute the most frequently used drugs in the symptomatic treatment of inflammatory and proliferative skin diseases.[1] Among corticosteroids, cortexolone (**1**) and its 17α-propionate showed a potent antiandrogenic topical activity that is useful in the treatment of acne vulgaris.[2] Despite their beneficial effects, corticosteroids applied topically have also been associated with allergic contact dermatitis reactions.[3] This side effect results from the formation of reactive intermediates during skin metabolism, which are able to modify the side chain of nucleophilic amino acids, and, therefore, lead to the formation of modified proteins that are recognized as foreign by the skin immune system.[4] In this sense, Wilkinson and Jones suggested that corticosteroid molecules could be oxidized into their 21-dehydro derivatives and that the reaction of these α-oxo aldehydes with arginine could lead to the formation of modified proteins.[5] The hydroxy group at position 21 in corticosteroids is highly sensitive to oxidation by simple exposure to air of a methanolic solution in the presence of cupric acetate.[6] The preparation of an acyl derivative could avoid this oxidation and, consequently, the undesired side effects. It was observed that protection of the hydroxy group at position 17 does not inhibit the oxidation, because the same reaction carried out on corticosteroids with an acylated 17α-hydroxy group, such as hydrocortisone-17α-butyrate, also gave the expected α-oxo aldehydes.[7] Therefore, the preparation of cortexolone-21-acetate seems to be an interesting objective.

Cortexolone-21-acetate proved to be a good starting material in the biotransformation to hydrocortisone and hydrocortisone-21-acetate by parent and mutant strains of the filamentous fungus *Curvularia lunata*[8] and *Cunninghamella blakesleeana*.[9] It was observed that the position of the hydroxy group introduction, the selectivity of the bioconversion process, and its efficiency, were strongly dependent on the structure and substitution of the steroid molecule. Moreover, the conjugation of cortexolone derivatives to cytarabyme increased the stability of this nucleoside with anticancer activity.[10]

Regarding its synthesis, cortexolone-21-acetate was previously prepared from pregnenolone acetate in six steps.[11] However, to obtain the 21-monoester in a purity that was suitable for biological tests and pharmacological applications following this method, a complex and resource-intensive purification procedure is necessary.

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The application of enzymes in the synthesis of pharmaceuticals and natural product derivatives is well known.[12] Biocatalysis is a green alternative for the preparation of known and new compounds with physiological and therapeutic properties that are difficult to obtain by using conventional chemical methods.[13] Lipases have become attractive as biocatalysts for chemo-, regio-, and stereoselective reactions under mild conditions. They have been integrated into a variety of industrially interesting processes,[14] in particular, the preparation of enantiomerically pure pharmaceutical compounds.[15]

In the steroid field, enzyme catalysis can play an important role in the mild and selective interconversion of functional groups through regioselective transformations.[16–19] Studies carried out in our laboratory on the esterification and transesterification of polyfunctionalyzed steroids, have shown that lipases can act on substituents either on the A- or D-ring.[20,21] In addition, in previous papers, we observed that, in androstanes and pregnanes, *Candida rugosa* lipase showed a preference for C-3-hydroxy or -acyloxy groups, whereas *Candida antarctica* catalyzed the reactions at the D-ring.[22] Taking into account these properties, we have prepared fatty acid derivatives of dehydroepiandrosterone,[23] 3,17-β-estradiol,[24] and a series of novel 20-succinates of pregnanes.[25]

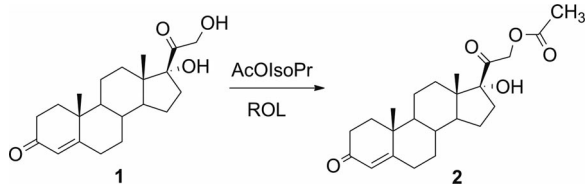
Regarding glucocorticoids, we carried out the lipase-catalyzed synthesis of acyl derivatives of hydrocortisone,[26] and, in the field of corticosteroids, Ferraboschi et al. reported the synthesis of cortexolone-17α-propionate catalyzed by *Candida rugosa* lipase.[27]

The extracellular sn-1,3 regioselective, native and recombinant, *R. oryzae* lipase (ROL) immobilized on different supports has been successfully used as a biocatalyst for the synthesis of human milk substitutes[28] and low caloric triacylglycerol,[29] flavor,[30,31] enzymatic resolution[32] and biodiesel production.[33]

The use of extracellular recombinant lipases is a well-referenced strategy that has been used to increase the productivity of the enzyme bioprocess production and to simplify processes downstream of the enzyme. *Pichia pastoris* is the preferred host yeast to produce recombinant lipases. The mature sequence of *R. oryzae* lipase has been produced in this cell factory under PAOX1 promoter using a strategy of mixed substrates.[34] The recombinant ROL has been characterized, and it showed a 40-fold higher specific activity compared with the commercial native ROL.[35]

When a lipase is selected as a biocatalyst for a given reaction, its application is often hampered by some characteristics: difficult recovery and reuse, low selectivity towards non-natural compounds and low stability under process conditions. Several strategies have been used to address these drawbacks. In this context, conformational engineering as been described as a very interesting approach that can be used to tune the lipase properties by using different immobilization protocols.[36,37] Thus, immobilization of a particular lipase in different orientations, with different rigidity, or in the presence of different microenvironments permits a range of biocatalysts to be generated from the same lipase with very different catalytic properties.[38,39]

In this work, we report the results obtained through lipase-catalyzed acetylation of cortexolone using an immobilized recombinant *Rhizopus oryzae* lipase (ROL) as catalyst (Scheme 1).



Scheme 1. Enzyme-catalyzed acetylation of cortexolone.

After fixing some operational parameters, the optimal conditions of enzyme/substrate ratio and acylating agent/substrate ratio have been determined by a central composite rotatable experiment design using the response surface methodology, to deliver the best immobilized derivative.

Results and Discussion

Preliminary experiments showed that the acetylation of cortexolone (**1**) using heterologous ROL as catalyst afforded cortexolone-21-acetate (21-acetoxy-17α-hydroxy-4-pregnen-3,20-dione) as the only product (Scheme 1) observed by HPLC analysis (see Figure S1 in the Supporting Information).

The identity of the product was confirmed by its melting point, which was in accordance with reported data,[11] and by spectroscopic methods. The acetylation on the hydroxy group of C-21 was confirmed by observing the downfield shift in the ^{1}H NMR signals of the 21-H resonance from δ = 4.28 and 4.56 ppm in substrate **1** to δ = 4.86 and 5.09 ppm in product **2**, respectively, and the appearance of a new signal corresponding to CH_3-23 of the acetyl group at δ = 2.17 ppm. There was no major variation in the methyl-18 chemical shift (moved from δ = 0.70 to 0.72 ppm), the methyl-19 chemical shift (from δ = 1.18 to 1.19 ppm) or the 4-H signal (from δ = 5.72 to 5.74 ppm). In the ^{13}C NMR spectrum, new signals of the carbonyl C-22 (δ = 171 ppm) and the methyl C-23 (δ = 20.6 ppm) from the new acetate group could be observed.

To optimize the reaction conditions, several experiments, using free and immobilized preparations of recombinant ROL, were performed to study the variation of the reaction parameters such as solvent, temperature, enzyme/substrate (E/S) ratio, acylating nature and acylating/substrate (A/S) ratio.

At first, we evaluated the behavior of free ROL (ROL0) and three immobilized preparations named: ROL1 (immobilized on Octadecyl Sepabeads), ROL2 (immobilized on Lewatit 1600) and ROL3 (immobilized on aldehyde-activated Lewatit CNP 105).

Previous experiments showed that the performance of enzymes in the acetylation reaction was improved when they were dried before use. The water percentage in the

three immobilized ROL was variable: 56% in ROL1, 46% in ROL2, and 70% in ROL3.

The four lipase preparations were evaluated in the acetylation of **1** with ethyl, vinyl, and isopropenyl acetate. Only the last substrate gave satisfactory results and was selected as acylating agent. In the absence of biocatalyst no product was obtained.

Among the tested enzymes, ROL2 gave the most satisfactory results. ROL1 and ROL3 showed a poor activity, and with ROL0 cortexolone and isopropopenyl acetate did not react at all (Table 1, Entries 1–12). In each case, only the C-21 hydroxy group of cortexolone was acetylated.

It was observed that the immobilized lipase ROL2 was much more reactive than the free lipase. Immobilization of the lipase fixes its open conformation, which can result in significant stabilization of the enzyme, especially for applications in organic solvent. Furthermore, the large differences in reactivity between the immobilized and the native heterologous enzyme could be explained by considering that the native enzyme is probably a mixture of different forms in organic solvent (aggregates, inactivated form for the solvent, lipase molecule in closed conformation, lipase molecules in open conformation, etc.) whereas in the immobilized form, homogeneous distributed monomeric lipase molecules predominate.

In the case of ROL2, immobilization of the lipase on Lewatit resin goes through a hydrophobic adsorption mechanism that differs from, for example, the simple adsorption on hydrophobic Sepabeads supports (ROL1) that was previously demonstrated.[40] Regarding ROL3 catalyst, the immobilization is performed though a multipoint covalent attachment on an aldehyde-activated support. The enzyme is immobilized through the rich area of lysines, and the open conformation is not fixed. Therefore, the "opening of the lid" and the accessibility of the active site seems to be blocked by this immobilization strategy.

Having established the optimal catalyst, a solvent screening was performed to establish the best solvent for this biocatalytic acetylation. With the aim that the enzyme activity and stability were affected as little as possible, three various-polarity solvents [hexane, diisopropyl ether (DIPE), and acetonitrile (MeCN)] were tested. The experiments (Table 1, Entries 2, 5, 8 and 11) clearly identified DIPE as the most efficient solvent for the reaction, and it was used in subsequent experiments for its ability to produce the highest conversion.

Regarding the optimum temperature (Table 1, Entries 13 and 14), we also performed the acetylation at 30 °C and, even after longer periods of time, ROL2 performance decreased to 20.2% conversion showing that the ROL2 activity increased with an increase in temperature. Therefore, 55 °C was chosen as the reaction temperature in subsequent experiments.

To determine the best acylating agent/substrate (A/S) ratio, reactions with various concentrations of the acylating agent were carried out (Figure 1).

An increase in conversion with an increase in A/S from 1 to 50 was observed. With A/S = 50 the conversion reached almost 100% under the present parameters.

To evaluate the enzyme/substrate (E/S) ratio, we performed the acetylation reaction using various ROL2 amounts (Figure 2). From these experiments we observed that the use of E/S ratios between 15 and 50 gave almost quantitative conversion.

Table 1. Optimization of reaction parameters for ROL-catalyzed acetylation of cortexolone (**1**).[a]

Entry	Enzyme	Solvent	Temperature [°C]	E/S ratio	A/S ratio	*t* [h]	Conversion [%]
			Solvent				
1	ROL0	hexane	55	30	25	48	n.d.
2	ROL0	DIPE	55	30	25	48	0.3
3	ROL0	MeCN	55	30	25	48	n.d.
4	ROL1	hexane	55	30	25	48	n.d.
5	ROL1	DIPE	55	30	25	48	4.9
6	ROL1	MeCN	55	30	25	48	n.d.
7	ROL2	hexane	55	30	25	48	23.6
8	**ROL2**	**DIPE**	**55**	**30**	25	48	52.1
9	ROL2	MeCN	55	30	25	48	n.d.
10	ROL3	hexane	55	30	25	48	n.d.
11	ROL3	DIPE	55	30	25	48	5.0
12	ROL3	MeCN	55	30	25	48	n.d.
			Temperature				
13	ROL2	hexane	30	30	25	72	9.8
14	ROL2	DIPE	30	30	25	72	20.2
			Time				
15	ROL 2	DIPE	55	30	50	1.5	6.7
16	ROL 2	DIPE	55	30	50	3	15.2
17	ROL 2	DIPE	55	30	50	8	27.7
18	ROL 2	DIPE	55	30	50	24	71.5
19	ROL 2	DIPE	55	30	50	**48**	99.4
20	ROL 2	DIPE	55	30	50	72	99.9

[a] Substrate concentration 1 mg/mL.

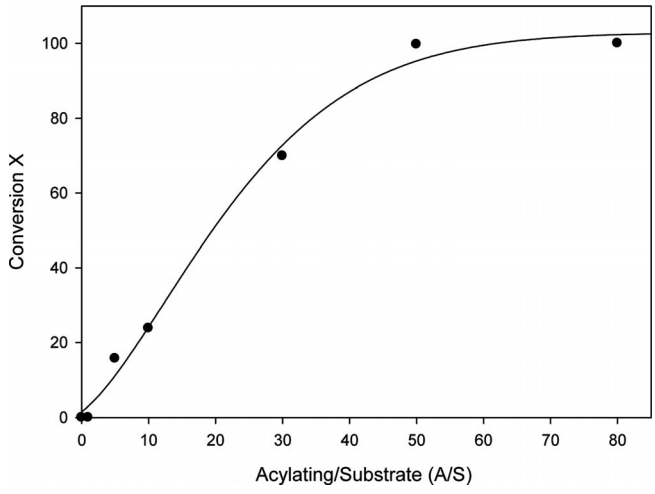


Figure 1. Influence of enzyme/substrate ratio on the conversion of cortexolone-21-acetate.

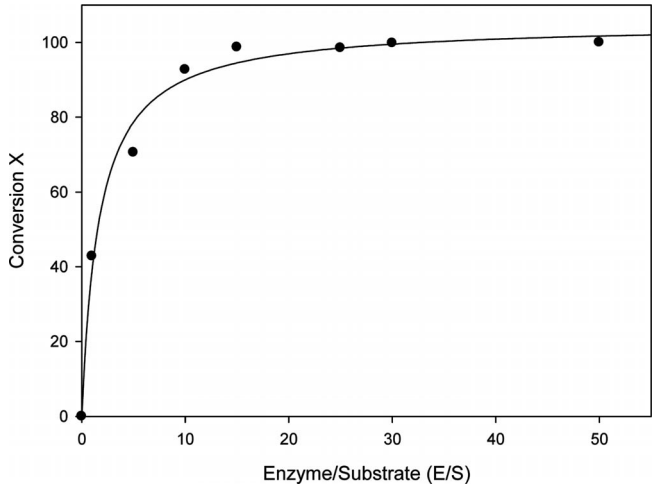


Figure 2. Influence of enzyme/substrate ratio on the conversion of cortexolone-21-acetate.

Taking into account the experiments mentioned above, we selected the following preliminary standard conditions for the biocatalytic acetylation of cortexolone with ROL2: E/S ratio 30, solvent DIPE, temperature 55 °C, and isopropenyl acetate/cortexolone ratio 50.

Finally, the optimum reaction time was also studied, and the results (Table 1, Entries 15–20) show that 48 h was optimal.

Using the response surface methodology and a central composite experimental design, the optimal values of E/S and A/S were determined with the aim of maximizing the specific yield defined as mmol of product (P) per mmol of acylating agent and gram of enzyme (mmol P/mmol A·g E) using ROL2. Taking into account the previous results, the selected enzyme/substrate ratio range was 5–50 and the acylating agent/substrate ratio was 30–70. The selected experimental design is described in Figure 3.

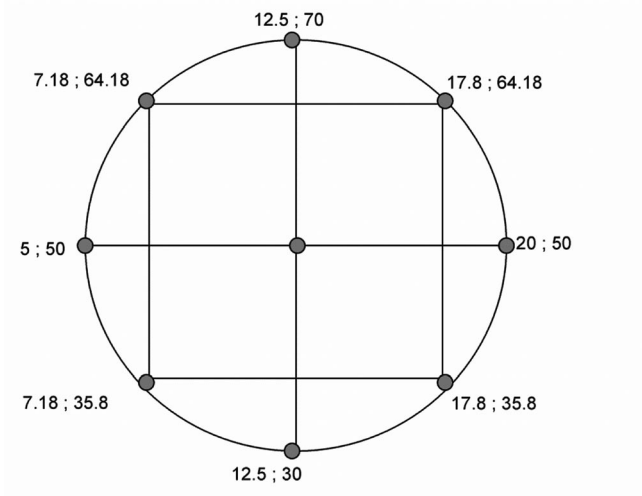


Figure 3. Diagram of the central composite rotatable design, using the nomenclature (E/S);(A/S).

The reaction conditions were also fixed following the results obtained in the acetylation reaction. Therefore, the biocatalyst was previously dried, temperature and shaking were fixed at 55 °C and 200 rpm, respectively, isopropenyl acetate was used as acetylating agent and DIPE as solvent. The productivities of each acetylation reaction are shown in Table 2.

Table 2. Specific yield of each acetylation reaction of the experimental design.[a]

E/S	A/S	Specific yield (mmol P/mmol A·g E)
5	50	1.56
7.18	35.8	1.34
12.5	30	0.92
17.8	35.8	0.72
20	50	0.51
17.8	64.18	0.33
12.5	70	0.46
7.18	64.18	0.84
12.5	50	0.81
12.5	50	0.81
12.5	50	0.81
12.5	50	0.81

[a] Reaction conditions: Substrate (1 mg/mL), DIPE, isopropenyl acetate, 55 °C, rotary shaking at 200 rpm, 48 h.

The obtained data was fitted to the mathematical model expressed in Equation (1) and the values of each coefficient, as well as the statistical parameter *p* value, are shown in Table 3.

Therefore, the equation obtained for the specific yield is expressed in Equation (1), and the corresponding response surface is plotted in Figure 4.

$$\text{Specific yield (mmol P/mmol A·g E)} = 0.8091 - 0.3275\cdot(E/S) - 0.1923\cdot(A/S) + 0.099\cdot(E/S)^2 - 0.0738\cdot(A/S)^2 \quad (1)$$

Firstly, the interaction effect of (E/S)·(A/S) had no significant effect on the specific yield, and the term E/S was the most significant one. Moreover, when the coefficients of E/S and A/S are compared, the former had higher values.

Table 3. Parameters of the empirical equations obtained for the stability response surfaces of specific yield (mmol P/mmol A·g E).

Coefficient	Value	*p* value
a	0.8091	<0.0001
b	–0.3275	<0.0001
c	–0.1923	0.0003
d	0.0990	0.0134
e	–0.0738	0.0414
f	0.0272[a]	0.4781

[a] Not considered a significant factor ($p > 0.05$).

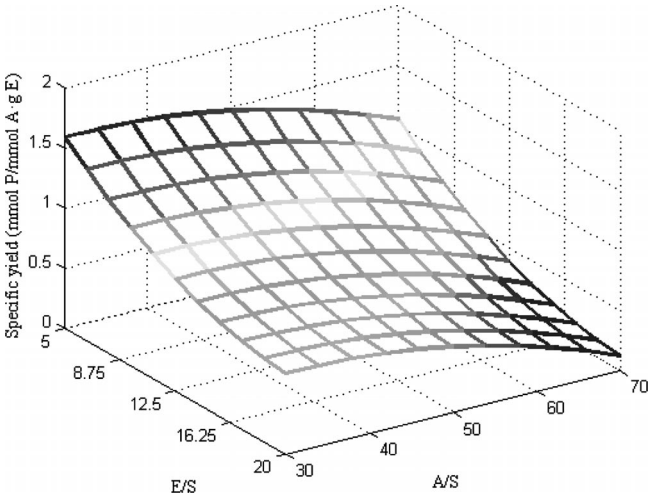


Figure 4. Response surface, according to the experimental design, corresponding to the specific yield of cortexolone-21-acetate under different ratios of E/S and A/S. Reactions were carried out at 55 °C and 200 rpm, using dry ROL2, DIPE as solvent and isopropenyl acetate as acetylating agent. Reaction time was 48 h.

Thus, the effect on the specific yield of the enzyme/substrate ratio was higher than the effect of the acylating agent/substrate ratio. The optimum values of E/S and A/S were 5 and 31.6, respectively, entailing a predicted maximum specific yield of 1.59 mmol P/mmol A·g E.

Because the acylating agent is in excess, some acetic acid could be produced by enzymatic hydrolysis of isopropenyl acetate. To test if acetic acid could act as a catalyst, we performed the acetylation of cortexolone without enzyme, using isopropenyl acetate and variable amounts of acetic acid (20, 40, 60, and 100 mM). In no case was any product detected after 48 h of reaction, indicating that acetic acid does not catalyze the acetylation of cortexolone.

Moreover, with the aim of studying the potential influence of acetic acid on the enzymatic activity, acetic acid (20 mM) was added to the enzymatic acetylation under the standard conditions. In this case, we observed that the presence of acetic acid did not affect the enzyme activity; cortexolone-21-acetate was obtained as the only product in 100% conversion after 48 h of reaction. In conclusion, under standard conditions the enzymatic acetylation of cortexolone is only catalyzed by the lipase, and the possible presence of acetic acid does not influence the enzyme behavior.

Finally, ROL2 is recyclable. Because the immobilized enzyme is insoluble in the organic reaction media, it is easily removed by filtration at the end of the process. It can be reused and, in this particular reaction, ROL2 kept 89% of its activity after four reaction cycles (Figure 5).

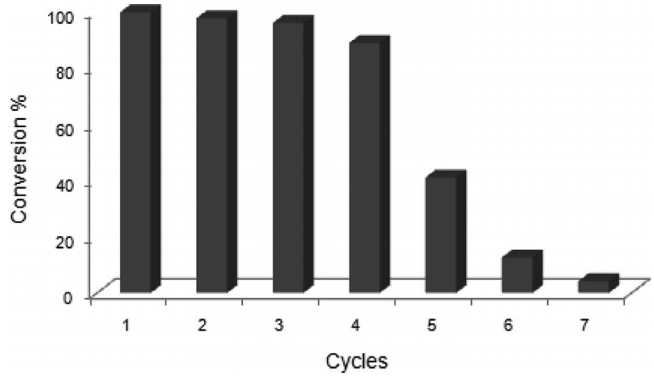


Figure 5. ROL2 reuse in the acetylation of cortexolone under standard conditions.

Conclusions

In this work we have studied the performance of three immobilized heterologous *Rhizopus oryzae* lipases as catalysts in the acetylation of cortexolone. The enzymatic reaction was regioselective, and only cortexolone-21-acetate was obtained. ROL immobilized on Lewatit 1600 (ROL2) proved to be the best biocatalyst in the preparation of the steroid derivative.

The reaction was performed at 55 °C, using isopropenyl acetate as acylating agent and diisopropyl ether as solvent. By means of a response surface methodology and a central composite rotatable design, the optimal values of enzyme/substrate ratio and acylating/substrate ratio were determined to maximize the specific yield. The optimal values of E/S and A/S were 5 and 31.6 respectively, lower than the values obtained from individual experiments, saving both enzyme and acylating substrate.

Finally, the enzymatic approach provides a simple and mild alternative method for the synthesis of this corticosteroid derivative. The application of ROL in an acetylation reaction on a steroid substrate such as cortexolone was not previously reported, and the results show that this enzyme can be an efficient catalyst on complex and rigid substrates such as steroids, which are difficult to transform in a regioselective way by using traditional synthetic procedures. Moreover, immobilized ROL kept 89% of its activity after four reaction cycles.

The broad application of steroid derivatives and, particularly, corticosteroids, as pharmaceuticals makes biocatalysis an appropriate way to prepare them in high purity, which is an essential requisite for a product designed for human consumption.

Experimental Section

General: All chemicals used in this work were of analytical grade and purchased from Sigma–Aldrich. The free and immobilized enzymes were dried in a vacuum drying oven (0.1 kPa, 30 °C) overnight before use. Enzyme/substrate ratio (E/S): enzyme amount (ROL0, 1, 2 and 3) in mg/substrate amount in mg. Acylating agent/substrate ratio (A/S): acylating agent amount in mg/substrate amount in mg. Enzyme activity: ROL1: 9.4 UI/mg, ROL2: 160 UI/mg, ROL3: 9.6 UI/mg. The enzymatic reaction was carried out with an Innova 4000 digital incubator shaker, New Brunswick Scientific Co., at 200 rpm. To monitor the reaction progress, aliquots were withdrawn and analyzed by HPLC with a Waters 1515 chromatograph equipped with a UV detector set a 254 nm, and Symmetry C18 (5 μm, 0.46 cm i.d. × 15 cm) eluted at 30 °C with a methanol/water mixture (80:20, v/v) at 0.5 mL min^{-1}. Retention times: cortexolone 4.4 min; cortexolone-21-acetate 5.7 min. Melting points were determined with a Fisher Johns apparatus. NMR spectra were recorded in $CDCl_3$ as solvent with a Bruker AC-200 NMR instrument operating at 200.1 and 50.2 MHz for ^1H and ^{13}C, respectively. Chemical shifts are reported in δ units (ppm) relative to tetramethylsilane (TMS) set at δ = 0 ppm; coupling constants are given in Hz.

Enzyme Production: ROL was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autònoma de Barcelona (UAB). This lipase is obtained by a mixed substrates-fed batch cultivation of a recombinant *P. pastoris* strain using methanol as inductor.[34] The culture broth was centrifuged and microfiltered to remove the biomass. The supernatant was concentrated by ultrafiltration with a Centrasette Pall Filtron system equipped with an Omega membrane of 10 kDa cut-off, and subsequently dialyzed against 10 mM Tris-HCl buffer (pH = 7.5) and thereafter lyophilized.[41]

Enzyme Immobilization

Standard Enzymatic Activity Assay Determination: To follow the immobilization process, the activities of ROL0 and its immobilized preparations (ROL1, ROL2 and ROL3) were analyzed spectrophotometrically measuring the increment in absorbance at 348 nm (ε = 5.150 M^{-1} cm^{-1}) produced by the release of *p*-nitrophenol (*p*NP) in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate buffer at pH = 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution (blank or supernatant) or suspension was added to 2.5 mL of substrate solution.

Adsorption of ROL on Octadecyl Sepabeads (ROL1): Sepabeads (EC-OD) (2 g) were added to sodium dihydrogen phosphate buffer (20 mL, 25 mM, pH = 7) containing lipase (25 mg; the amount of lipase was determined by Bradford's assay[42] of crude ROL). The mixture was then gently stirred at 25 °C and 250 rpm on a Coulter stirrer for 3 h. The solution was filtered through a sintered glass filter, and the supported lipase was washed several times with abundant distilled water. The percentage of immobilization was 80% determined by the Bradford's assay and the enzymatic activity assay described above.

Adsorption of ROL on Lewatit 1600 (ROL2): Lewatit VP OC1600 (2 g) was added to sodium dihydrogen phosphate buffer (20 mL, 25 mM, pH = 7) containing lipase (25 mg; the amount of lipase was determined by Bradford's assay[42] of crude ROL). The mixture was then gently stirred at 25 °C and 250 rpm on a Coulter stirrer for 3 h. The solution was filtered through a sintered glass filter, and the supported lipase was washed several times with abundant distilled water. The percentage of immobilization was 86% determined by Bradford's assay and the enzymatic activity assay described above.

Multipoint Covalent Immobilization of ROL on Aldehyde-Activated Lewatit CNP 105 (ROL3): The commercial resin Lewatit CNP 105 was previously activated with aldehyde groups as reported in the literature.[43] Aldehyde-activated Lewatit CNP 105 (2 g) was added to sodium hydrogen carbonate buffer (20 mL, 100 mM, pH = 10.1) containing lipase (25 mg; the amount of lipase was determined by Bradford's assay[41] of crude ROL). The mixture was then gently stirred at 25 °C and 250 rpm on a Coulter stirrer for 24 h. When the immobilization was finished (analyzed by enzymatic activity assay described above), $NaBH_4$ (20 mg) was added, and, after 30 min, the suspension was filtered through a sintered glass filter, and the covalently immobilized lipase was washed several times with abundant distilled water. The percentage of immobilization was 55% (determined by the Bradford's assay and the enzymatic activity assay described above).

Enzymatic Acetylation of Cortexolone: To a solution of cortexolone (0.1 g, 0.29 mmol) in diisopropyl ether (100 mL), isopropenyl acetate (0.94 g, 9.16 mmol) and ROL immobilized on Lewatit 1600 (0.5 g) were added. The reaction mixture was incubated at 55 °C and shaken at 200 rpm for 48 h. Once the reaction was finished, the enzyme was filtered off, washed with DIPE (3 × 10 mL), and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate, 1:1, v/v). After solvent evaporation, the product was isolated as a white solid (0.1 g, 0.26 mmol, 91% yield). M.p. 236–238 °C (ref.[11] 235–238 °C). ^1H NMR (200.1 MHz, $CDCl_3$): δ = 0.72 (s, 3 H, CH_3-18), 1.19 (s, 3 H, CH_3-19), 2.17 (s, 3 H, CH_3-23), 4.86 (d, J = 18 Hz, 1 H, CH_2-21), 5.09 (d, J = 18 Hz, 1 H, CH_2-21), 5.74 (s, 1 H, =CH-4) ppm. ^{13}C NMR (50.2 MHz, $CDCl_3$): δ = 14.5 (C-18), 17.4 (C-19), 20.6 (C-23), 68.0 (C-21), 123.9 (C-4), 171.0 (C-5), 171.2 (C-22), 199.7 (C-3), 205.3 (C-20) ppm. Reuse experiments: the filtered and washed enzyme was used in the next enzymatic acetylation under the same reaction conditions.

Effect of Enzyme/Substrate Ratio and Acylating Agent/Substrate Ratio on Specific Yield: The effect of enzyme/substrate (E/S) and acylating/substrate (A/S) ratio on the specific yield of cortexolone-21-acetate using ROL2 (ROL immobilized on Lewatit 1600) was studied by means of a Box-Hunter experiment design[44] and the response surface methodology. The applied central composite rotatable design (CCRD) was formed by 12 experiments, based in two variables having five levels each. The value of α was 1.41 and four center points for replication were carried out. The enzyme/substrate and acyl/substrate ratio ranges were selected by taking into account the results obtained in previous enzymatic acetylation reactions. The enzyme/substrate ratio range was 5–50 and the acylating/substrate ratio was 30–70. The values of both variables were codified from –1.41 to 1.41. The empirical response surfaces were built from the values of specific yield expressed as mmol P/mmol A·g E, after 48 h of acetylation reaction. The data results fit the empirical model expressed in Equation (2):

$$\text{Specific yield (mmol P/mmol A·g E)} = a + b\cdot(\text{E/S}) + c\cdot(\text{A/S}) + d\cdot(\text{E/S})^2 + e\cdot(\text{A/S})^2 + f\cdot(\text{E/S})\cdot(\text{A/S}) \quad (2)$$

The reaction conditions were fixed by taking into account the results obtained previously. Therefore, under standard conditions, the immobilized biocatalyst was used dry, and temperature and shaking were fixed at 55 °C and 200 rpm, respectively. DIPE was used as a solvent, and cortexolone (2 mg in 2 mL) was acetylated by using isopropenyl acetate.

The Sigma Plot 11.0 (Systat Software, Illinois, USA) was used to perform the statistical analysis and to fit the response surfaces.

Supporting Information (see footnote on the first page of this article): Spectral data for compound **2** and time course profiles of acetylation.

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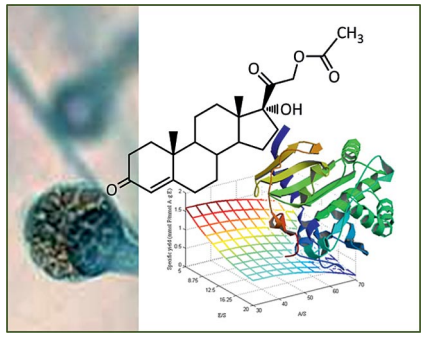
The regioselective acetylation of cortexolone was achieved by using an immobilized heterologous *Rhizopus oryzae* lipase. The mild reaction conditions and low environmental impact make the biocatalytic procedure a convenient way to prepare the monoacetyl derivative of this biologically active steroid.



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