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An efficient enzymatic preparation of 20-pregnane succinates: chemoenzymatic synthesis of 20β-hemisuccinyloxy-5α*H*-pregnan-3-one

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Abstract

Lipase-catalyzed transesterification of the 20 hydroxyl group in a series of pregnanes afforded novel 20-ethyl succinates that are not possible to prepare following the traditional synthetic methods. The reaction is stereoselective. The enzyme reacts selectively with the 20β epimers therefore only the 20β-succinyloxy derivatives are obtained. These compounds are obtained in variable yield, depending on the substitution in the ring A. The enzymatic approach allowed, for the first time, the synthesis of 20β-hemisuccinyloxy-5α*H*-pregnan-3-one, novel compound useful as a precursor of steroid–protein conjugates.

Keywords: *Candida antarctica* lipase B; 20-Pregnane succinates; Enzymatic stereoselective transesterification

1. Introduction

Steroid–protein conjugates show a wide range of applications. They are used in affinity chromatography to isolate proteins with specific functions such as steroid receptors,[1] and in new strategies of controlled drug release, for example, the selective intracellular delivery of the anti-inflammatory drug, dexamethasone, into endothelial cells.[2]

Steroid–protein conjugates are also used to obtain antihormonal agents. In the field of Endocrinology, the antihormonal agents capable of counteracting the physiological effects of endogenous hormones could serve as important biochemical and clinical tools.[3]

Several papers have previously described the efficacy of succinyl-substituted steroids as conjugates. For instance, Erlanger et al. have demonstrated, by using deoxycorticosterone 21-hemisuccinate and bovine serum albumin (BSA), that 20 NH_2 groups over 60 were substituted giving an efficacy of 33.33%.[4] In addition, when progesterone-11α-hemisuccinate-BSA was used as conjugate, 36 steroid molecules per protein molecule were found.[5] The conjugates can be prepared by linking the steroid hemisuccinate via an amide bond to the ε-amino groups of lysine in bovine serum albumin (BSA) by means of a mixed anhydride intermediate.[6]

Regarding the steroid, in the case of dexamethasone the preparation of 21-hemisuccinate is easy and the product is obtained in high yield. It is generally performed by reacting dexamethasone with excess of succinic anhydride and 4-dimethylaminopyridine for 24 h at room temperature.[7] Hemisuccinate derivatives of several steroids have been reported and some of them are commercial products. For example, there are several examples of application of hemiesters of succinic acid in different positions of the steroid skeleton. In the position 3: cholesterol hemisuccinate[8a] and pregnenolone hemisuccinate;[8b] 6: progesterone-6β-hydroxy-hemisuccinate;[8c]

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11α: progesterone-11α-hydroxy-hemisuccinate;[8d] 17: medroxy-progesterone-17-hemisuccinate[8e] and 17β-estradiol-17-hemi-succinate,[8f] and mostly in the position 21: the above mentioned dexamethasone-21-hemisuccinate,[6] 6α-methylprednisolone-21-hemisuccinate,[8g] hydrocortisone-21-hemisuccinate,[8h] 6β-hydroxycortisol-21-hemisuccinate,[8i] etc.

However, the approach used to prepare the above mentioned hemisuccinates involved the esterification of the hydroxyl group in position 20 of pregnanes but did not afford satisfactory results. Since pregnane-20-hemisuccinates are required as starting material in the preparation of steroid–protein conjugates that could be useful, *inter alia* in the study of the relationship between steroidogenesis and spermatogenesis in male amphibians in vivo,[9] it is desirable to have an efficient method for their preparation. With these guidelines in mind, we decided to apply an enzymatic approach.

The use of enzymes in the synthesis of pharmaceuticals and natural products' derivatives is increasing in the last few years.[10,11] Biotransformation is an area of interest for the preparation of new compounds with added value to the physiological and therapeutic properties, which are difficult to obtain by conventional chemical methods.[12]

In the last years, lipases have become attractive as biocatalysts for chemo-, regio-, and stereoselective reactions under mild conditions.[13] They can be used in a wide variety of organic solvents and do not require a coenzyme for activity.[14]

Specifically in the steroid field, enzyme catalysis can play an important role in the mild and selective interconversion of functional groups via regioselective transformations.[15–18] Studies carried out in our laboratory on the esterification and transesterification of polyfunctionalyzed steroids have shown that lipases can act on substituents either on A-ring or on the D-ring.[19,20] In addition, in previous papers we observed that, in androstanes and pregnanes, *Candida rugosa* lipase showed a preference for C-3 hydroxyl or acyloxy groups, whereas *Candida antarctica* catalyzed the reactions in D-ring.[21] Taking into account these properties we have prepared fatty acid derivatives of dehydroepiandrosterone[22] and 3,17-β-estradiol.[23]

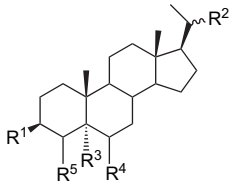
As a part of our ongoing project on enzymatic transformation of steroids, we report in the present paper that the results obtained by the application of a lipase-catalyzed reaction in the preparation of a series of novel 20-succinates of the pregnanes **1d**–**6d** (Fig. 1).

Encouraged by our success in lipase-catalyzed esterification in 20-hydroxypregnanes, we applied this methodology to the synthesis of 20β-hemisuccinyloxy-5α*H*-pregnan-3-one (**7**) (Fig. 1). In this paper we also report for the first time a chemoenzymatic way to prepare **7** that will allow determination of the level of circulating steroids during the reproductive season in male toads.[9]

2. Results and discussion

2.1. Enzymatic reactions

Our initial experiments to obtain the 20-succinates were performed using 3β-acetyloxy-20β-hydroxypregn-5-ene (**1b**)



1a: R^1 = AcO- ; R^2 = -α-OH ; R^3, R^4 = Δ^5
1b: R^1 = AcO- ; R^2 = -β-OH ; R^3, R^4 = Δ^5
1c: R^1 = AcO- ; R^2 = -α-OCO$(CH_2)_2$COOEt ; R^3, R^4 = Δ^5
1d: R^1 = AcO- ; R^2 = -β-OCO$(CH_2)_2$COOEt ; R^3, R^4 = Δ^5
1e: R^1 = HO- ; R^2 = -β-OH ; R^3, R^4 = Δ^5
1f: R^1 = HO- ; R^2 = -β-OCO$(CH_2)_2$COOEt ; R^3, R^4 = Δ^5
2a: R^1 = t-BuMe_3Si- ; R^2 = -α-OH ; R^3, R^4 = Δ^5
2b: R^1 = t-BuMe_3Si- ; R^2 = -β-OH ; R^3, R^4 = Δ^5
2c: R^1 = t-BuMe_3Si- ; R^2 = -α-OCO$(CH_2)_2$COOEt ; R^3, R^4 = Δ^5
2d: R^1 = t-BuMe_3Si- ; R^2 = -β-OCO$(CH_2)_2$COOEt ; R^3, R^4 = Δ^5
3a: R^1 = AcO- ; R^2 = -α-OH ; R^3 = R^4 = H
3b: R^1 = AcO- ; R^2 = -β-OH ; R^3 = R^4 = H
3c: R^1 = AcO- ; R^2 = -α-OCO$(CH_2)_2$COOEt ; R^3 = R^4 = H
3d: R^1 = AcO- ; R^2 = -β-OCO$(CH_2)_2$COOEt ; R^3 = R^4 = H
4a: R^1 = -O$(CH_2)_2$O- ; R^2 = -α-OH ; R^3, R^5 = Δ^5
4b: R^1 = -O$(CH_2)_2$O- ; R^2 = -β-OH ; R^3, R^5 = Δ^5
4c: R^1 = -O$(CH_2)_2$O- ; R^2 = -α-OCO$(CH_2)_2$COOEt ; R^3, R^5 = Δ^5
4d: R^1 = -O$(CH_2)_2$O- ; R^2 = -β-OCO$(CH_2)_2$COOEt ; R^3, R^5 = Δ^5
5a: R^1 = O= ; R^2 = -α-OH ; R^3, R^5 = Δ^4
5b: R^1 = O= ; R^2 = -β-OH ; R^3, R^5 = Δ^4
5c: R^1 = O= ; R^2 = -α-OCO$(CH_2)_2$COOEt ; R^3, R^5 = Δ^4
5d: R^1 = O= ; R^2 = -β-OCO$(CH_2)_2$COOEt ; R^3, R^5 = Δ^4
6b: R^1 = O= ; R^2 = -β-OH ; R^3 = R^4 = H
6d: R^1 = O= ; R^2 = -β-OCO$(CH_2)_2$COOEt ; R^3 = R^4 = H
7: R^1 = O= ; R^2 = -β-OCO$(CH_2)_2$COOH ; R^3 = R^4 = H

Figure 1. 20-Hydroxy-pregnane and its corresponding succinates.

as substrate and succinic anhydride as acylating agent, taking into account its good performance in the subtilisin-catalyzed esterification of pregnanes in the position 3 of ring A.[24] We studied the esterification catalyzed by several commercially available lipases: *C. antarctica* lipase B (CAL B), *Pseudomonas cepacia* lipase (PSL), *C. rugosa* lipase (CRL), porcine pancreatic lipase (PPL), and *Rhizomucor miehei* lipase (LIP), in a variety of solvents (*t*-amyl alcohol, acetonitrile, acetone, diisopropyl ether, chloroform, hexane, toluene, and tetrahydrofuran). None of the enzymes tested catalyzed the reaction with **1b**, and similar conditions applied to **1a**, **2a**, **2b**, **6a**, and **6b** gave the same unsatisfactory results. The only result obtained with succinic anhydride and **1b** was observed when we used CRL as biocatalyst and diisopropyl ether as solvent, but the product was the diol **1e** in 5% yield (Table 1, entry 1).

Using ethyl hemisuccinate instead of succinic anhydride and CAL B and CRL as biocatalysts (entries 2 and 3), it was possible to obtain the desired product **1d** but in very low amounts and accompanied by a mixture of steroid products such as **1e** and **1f**, together with diethyl succinate and succinic acid.

In summary, under these reaction conditions, neither succinic anhydride nor ethyl hemisuccinate proved to be good acylating agents.

In view of the previous satisfactory results that we had obtained in enzymatic transesterification reactions,[25,26] we screened diethyl succinate as acylating agent and **1b** as

Table 1
Lipase-catalyzed transesterification of pregnanes[a]

Entry	Substrate	Enzyme	Solvent	Acylating agent	Product (% yield)
1	**1b**	CRL	DIPE	Succinic anhydride	**1e** (5)
2	**1b**	CAL B	DIPE	Ethyl hemisuccinate	**1d** (6)
3	**1b**	CRL	DIPE	Ethyl hemisuccinate	**1d** (9)+**1e** (10)+**1f** (21)
4	**1b**	CAL B	Hexane	Diethyl succinate	**1d** (20)+**1e** (5)+**1f** (12)
5	**1b**	CAL B	Isooctane	Diethyl succinate	**1d** (43)
6	**1b**[b]	CAL B	Isooctane	Succinic anhydride	**1d** (39)
7	**1b**	CRL	Isooctane	Diethyl succinate	—
8	**1a**	CAL B	Isooctane	Diethyl succinate	—
9	**2a**	CAL B	Isooctane	Diethyl succinate	—
10	**2b**	CAL B	Isooctane	Diethyl succinate	**2d** (38)
11	**3a**	CAL B	Isooctane	Diethyl succinate	—
12	**3b**	CAL B	Isooctane	Diethyl succinate	**3d** (42)
13	**4a**	CAL B	Isooctane	Diethyl succinate	—
14	**4b**	CAL B	Isooctane	Diethyl succinate	**4d** (30)
15	**5a**	CAL B	Isooctane	Diethyl succinate	—
16	**5b**	CAL B	Isooctane	Diethyl succinate	**5d** (72)
17	**6b**	CAL B	Isooctane	Diethyl succinate	**6d** (77)

[a] Reaction conditions: E/S 5:1, A/S 3:1; temperature: DIPE and hexane: 55 °C, isooctane: 100 °C; reaction time: isooctane: 2 h, DIPE and hexane: 120 h.
[b] One-pot procedure.

substrate. The lipase from *C. antarctica* B (CAL B) with hexane as solvent gave the best results (**1d** in 20% yield) (entry 4), although not selective because 3β,20β-dihydroxypregn-5-ene (**1e**) and 20β-ethylsuccinoyloxy-3β-hydroxypregn-5-ene (**1f**) were also obtained in 5% and 12% yield, respectively. LIP showed a lower performance and the other lipases (PPL, PSL, and CRL) were completely inert.

In order to improve the activity of CAL B, we decided to perform the reactions at higher temperature and carried out the transesterification using three non-polar solvents: hexane (bp: 69 °C), toluene (bp: 110 °C), and isooctane (2,2,4-trimethylpentane) (bp: 99 °C) at their boiling temperatures. The best results were obtained with isooctane giving **1d** as the only product in 43% yield.

The succinoylation of **1b** on the hydroxyl of carbon 20 was confirmed by observing the shift in the ^{1}H NMR signal of H-20 resonance from 3.73 ppm in substrate **1b** to 4.86 ppm in product **1d**. There is also a significant difference in methyl-18 chemical shift, which moves from 0.77 ppm to 0.64 ppm.

In order to optimize the reaction conditions in isooctane, we performed several experiments varying other reaction parameters such as enzyme–substrate ratio (E/S) and acylating agent–substrate ratio (A/S). We have chosen as standard conditions diethyl succinate as acylating agent, isooctane as solvent, reflux temperature, an E/S ratio of 5 and A/S ratio of 3.

It is remarkable the variation in regioselective behavior displayed by the lipase (**1d**+**1e**+**1f**, Table 1, entry 4) in the transesterification reaction with diethyl succinate using hexane as solvent at 55 °C, in contrast with isooctane at 100 °C where **1d** was the only product obtained. Many reports demonstrated that organic media not only influence the enzymatic activity but also the selectivity,[27] and many examples have been observed about the correlation of enzyme regioselectivity with solvent parameters,[28] especially with hydrophobicity described by log P.[29] In this work, when polar solvents such as tetrahydrofurane or acetonitrile were used, no transacylation was observed. Considering non-polar solvents, if we compare hexane and isooctane the difference in polarity among them is low (log P hexane: 3.5 and log P isooctane: 4.5),[30] showing that the difference in solvent polarity alone does not explain the increase in reaction regioselectivity. Since in isooctane the reaction temperature is 45 °C higher than in hexane, we suggest that the increase in regioselectivity of enzymatic behavior could be associated with a kinetically controlled process.

In view of the promising results obtained in the enzymatic preparation of 20-ethyl succinate of **1b**, we decided to apply the same reaction conditions in the preparation of a series of pregnane-20-succinates. The results are summarized in Table 1 (entries 9–17). In every case the enzymatic transesterification was clean and the 20-ethyl succinates were the only derivatives obtained.

The yield was variable and seemed to be dependent more on the substituent at carbon 3 of ring A than on the presence of the double bond in ring A or B. The best yields were obtained with substrates **5b** (72%) and **6b** (77%) containing a carbonyl group in the position 3 of ring A. The carbonyl is a sterically smaller group in comparison with *tert*-butyldimethylsilyl group in **2b** and ethylendioxy group in **4b**, which afforded the lowest yields, 38% and 30%, respectively. The low influence of a double bond was observed in the similar yields obtained using the 3β-acetates **1b** (43%) with a double bond in position 5 and **3b** (42%) saturated in this position. Similar results can be observed for the substrates **5b** and **6b**, which differ in the double bond in 4 and afford **5d** and **6d** in almost the same yield.

The stereoselectivity of the enzymatic reaction, which only produces the 20β-succinyloxy derivative, deserves a special comment.

As can be observed in Table 1 (entries 9–18) from both stereoisomers in the 20 hydroxyl substrates, only the 20β isomer was a good substrate in the enzymatic transesterification with

CAL B and isooctane at reflux. Under these conditions, only the desired 20β-ethyl succinate was obtained, whereas the 20α one was inert to the enzymatic reaction in every case.

These results reveal a new type of stereoselective behavior of CAL B, which has been rationalized by molecular modeling.[31] We have also examined these enzymatic reactions using molecular modeling, in order to understand the structural relation between the enzymatic pocket and the characteristic steroidal framework, which accounts for the remarkable selectivity of the biological behavior of these compounds. In fact, only the 20β isomers have biological activity and would be useful in the preparation of steroid–protein conjugates.

By docking the substrates, we observed that the most favorable orientation for the C-20 hydroxyl respect to the acyl–Ser 105 complex resulted locating the A-ring of the steroidal skeleton deep inside the pocket, near the turn formed by residues 38–44 (Pro-Gly-Thr-Gly-Thr-Thr-Gly). Trp 104 locates close to steroidal skeleton, forcing methyl groups 18 and 19 to point to the opposite face. An appropriate trajectory for hydroxyl group attached to carbon 20 is needed in order to react with the acyl–enzyme complex, which is pointing to the plane formed by carbonyl group and oxygen of the ester moiety, opposite to His 224 in the triad. The difference in reactivity between 20α- and 20β-hydroxy epimers could be explained by the position of methyl 21 necessary to release the acyl–enzyme complex. The former shows steric interaction with Ile 189 (distance to residue is about 3 Å) whereas the latter seems to be less impeded to reach a reactive conformation (the substituent on carbon 20 closest to the residue is a hydrogen). Figure 2a and b shows the substrates **5a** and **5b**, together with acyl–Ser 105 and other highlighted residues that fix their position into the catalytic pocket.

The molecular modeling also explained the effect of bulky substituents attached to A-ring, such as 3-acetoxy, 3-*tert*-butyldimethylsilyl, and 3-ethylenedioxy, which afforded the corresponding products in lower yield. These groups increase the steric hindrance between residues 38–44 and substrate. However, it could be assumed that the steric hindrance is not the only cause of a decrease in enzymatic activity and also electronic effects from carbonyl group would be related to reaction yield. For 3-oxo compounds a favorable hydrogen bond interaction between the oxygen belonging to the carbonyl group and the hydroxyl of Ser 47 was observed, which would explain the increase in yield for these compounds (**5d** and **6d**).

On the other hand, the transesterification reaction of the 3β,20β-diol **1e** with CAL B transesterification in isooctane at reflux did not afford any product. As its solubility in the reaction system is the same as **6b** and the steric hindrance of hydroxyl group is not higher than a carbonyl, electronic effects must also be important in the enzymatic reaction.

At this point we could prepare both the acylating agent diethyl succinate and a series of 20β-pregnane ethyl succinates using a biocatalytic approach. This fact prompted us to apply a one-pot enzymatic procedure[32] in which the acylating agent and the ethyl succinate of **1b** were obtained in the same step. Therefore, we treated **1b** with succinic anhydride, ethanol, and isooctane as solvent in the presence of CAL B. The

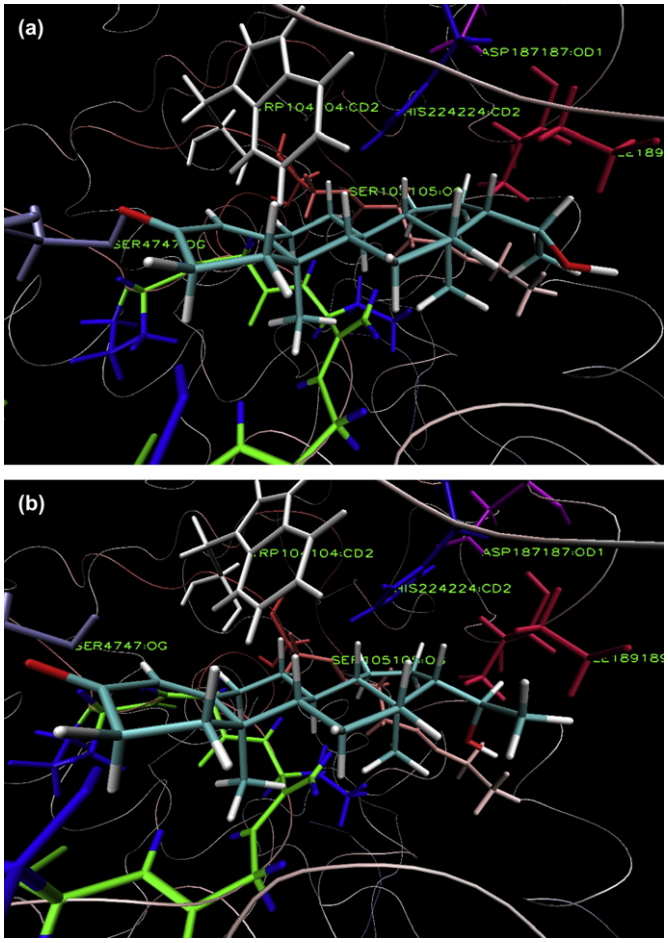


Figure 2. (a) Docking of compound **5a**. Trp 104 (white) and Ile 189 (red) make all methyl groups point below the steroidal rings. In this case, hydroxyl on carbon 20 is located opposite to Ser 105–acyl complex (brown) and the reaction cannot be achieved. Ser 47 (gray) shows a favorable interaction with carbonyl oxygen attached to carbon 3. Residues 38–44 (green backbone) are located near to the A-ring and interact sterically with bulky substituents at 3β position. (b) Docking of compound **5b**. For this configuration, hydroxyl on carbon 20 points toward the catalytic triad, which explains selectivity for 20-(*R*)-hydroxypregnanes transesterification.

reaction was performed under reflux and with an E/S ratio of 5, a succinic anhydride/**1b** ratio of 3, and a succinic anhydride/alcohol ratio of 1. It was observed that the ethyl derivative **1d** was obtained in similar yield (39%) as in the enzymatic transesterification with diethyl succinate as acylating agent (43%), so the one-pot procedure could be considered as an alternative method for the preparation of the succinyloxy derivatives.

Finally, the lipase is recyclable. As it is insoluble in the organic reaction media it is easily removed by filtration at the end of the process. It can be re-used and in this particular reaction, CAL kept 80% activity after eight reaction cycles.

2.2. Synthesis of enzyme substrates

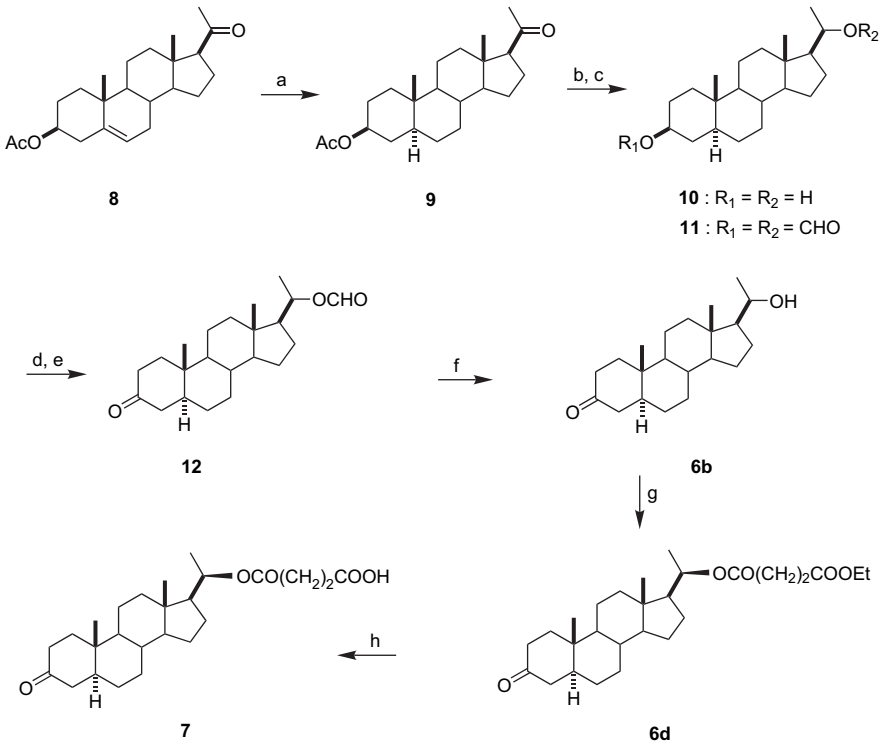
Pregnane derivatives **1**–**5** were synthesized from pregnenolone acetate using standard procedures previously reported by us.[33,34]

2.3. Chemoenzymatic synthesis of 20β-hemisuccinyloxy-5αH-pregnan-3-one

The enzymatic procedure was applied to the preparation of 20β-hemisuccinyloxy-5α-pregnan-3-one (**7**), not previously reported (Scheme 1). The substrate 20β-hydroxy-5α-pregnan-3-one (**6b**) was also prepared in our laboratory. In the synthetic route to **6b** we used as intermediate 3β,20β-dihydroxy-5α-pregnane **10**.[35] It was obtained from commercially available pregnenolone acetate (**8**) in three steps using standard procedures. Thus, catalytic hydrogenation of the Δ^5 double bond in **8**, following by simultaneous reduction of **10** of 20-carbonyl moiety and deprotection of 3-acetate **9** with LAH in THF rendered in 91% yield (three steps). The synthetic sequence is outlined in Scheme 1. Formylation of the two hydroxyl groups in **10** was accomplished using mixed formic acetic anhydride[36] rendering diformiate **11** in almost quantitative yield. Characteristic signals of the formyl protons and carbons in the NMR of **11** were especially diagnostic of the success of this reaction. Regioselective deformylation of **11** (KCO_3H/MeOH) and further oxidation with pyridinium chlorochromate (PCC) furnished the 3-keto-20-formyloxy pregnane **12** in 85% yield (from **11**). Spectroscopic evidence for this compound was the absence of a signal for a hydrogen at C-3 in the ^1H NMR and the signal at 204 ppm in the ^{13}C NMR characteristic of a 3-keto pregnane. Clean hydrolysis of the 20-formate in **12** was easily accomplished by treatment with KOH/MeOH, giving rise to 3-keto-20-hydroxy derivative **6b** in quantitative yield. The above mentioned CAL B-catalyzed transesterification of **6b** with ethyl succinate afforded the succinoylated derivative **6d** in 77% yield. Finally, compound **7** was obtained by regioselective cleavage of the ethyl group with bis(tributyltin)oxide following the procedure described by Mascaretti et al.[37]

3. Conclusions

Enzymatic catalysis allows the transesterification of the 20 hydroxyl group in a series of pregnanes affording novel 20-alkyl succinates (**1d**–**6d**) which are not possible to prepare following the traditional synthetic methods. Through a simple hydrolysis of these compounds **1d**–**6d**, it is possible to obtain various hemisuccinate derivatives. These derivatives can be useful as substrates in the synthesis of steroid–protein conjugates. It is important to emphasize the completely stereoselective behavior of the lipase. Only the 20β epimer acts as substrate of the enzymatic reaction. As a result the 20β-ethyl succinates are exclusively obtained. It was also observed that the yield depended on the substitution in carbon 3 of ring A. These experimental facts have been verified by docking of the substrates into the catalytic pocket of CAL B lipase. This biotechnological procedure shows several advantages such as mild reaction conditions and low environmental impact. Moreover, as 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 this particular reaction CAL B keeps 80% of its activity after eight reaction cycles. Finally, this work also describes the application of the enzymatic approach to the first synthesis of 5α-pregnane-3-one-20β-hemisuccinate **7**, a novel compound with potential application in the synthesis of antihormonal steroid–protein conjugates.



Scheme 1. Reagents and conditions: (a) H_2/Pd; (b) LAH, THF; (c) $HOCOCOCH_3$, Py; (d) KCO_3H, MeOH; (e) PCC, $BaCO_3$, Cl_2CH_2; (f) KOH, MeOH; (g) CAL B lipase, diethyl succinate, isooctane, reflux; (h) bis(tributyltin)oxide, toluene.

4. Experimental

4.1. General remarks

Lipase from *C. rugosa* (CRL) (905 U/mg solid) and type II crude from porcine pancreas (PPL) (190 U/mg protein) were purchased from Sigma Chemical Co.; lipase from *P. cepacia*: Lipase PS-C Amano II (804 U/g) was purchased from Amano Pharmaceutical Co. *C. antarctica* lipase B (CAL B): Novozym® 435 (7400 PLU/g) and *R. miehei* lipase: Lipozyme RM 1M (LIP) (7800 U/g) were generous gifts of Novozymes Latinoamerica Ltda. and Novozymes A/S. All enzymes were used 'straight from the bottle'. Chemical reagents and solvents were commercialized by Aldrich, Sigma, Fluka, or J.T. Baker. Enzymatic reactions were carried out on Innova 4000 digital incubator shaker, New Brunswick Scientific Co. at 200 rpm. Melting points were measured in a Fisher Johns apparatus and are uncorrected. Reactions were followed by TLC on Merck Silica gel 60F-254 aluminum sheets (0.2 mm thickness). For flash chromatography Merck Silica gel 60 (60–230 mesh) was used. Optical rotations were measured using chloroform as solvent with a Perkin Elmer 343 polarimeter. FTIR spectra were obtained on a Shimadzu FTIR-8300 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ as solvent using a Bruker AC-200 spectrometer operating at 200.13 MHz and 50.32 MHz for ^{1}H and ^{13}C, respectively, and a Bruker AM-500 NMR instrument operating at 500.14 MHz and 125.76 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts are reported in δ units relative to tetramethylsilane (TMS) set at 0 δ, and coupling constants are given in hertz.

4.2. Molecular modeling

CAL B structure was downloaded from RCSB Protein Data Bank (http://www.rcsb.org/pdb/).[38] Acyl moiety was added to Ser 105 and the geometry of the resulting complex was oriented according to data published by Pleiss et al.[39] and optimized using AMBER algorithm included on HyperChem version 7.5. All substrates were minimized using semiempirical AM1 method integrated on the same software mentioned above.

Docking experiments were run using GOLD version 2.0 (CCDC, Cambridge, UK). The active site radius was set to 10 Å and the GoldScore algorithm was used. Twenty solutions were obtained for each ligand. Structures in Figure 2 were generated using VMD 1.8.5.[40]

4.3. Synthesis of substrates (**1a–6a** and **1b–6b**) and acylating agents

4.3.1. 3β-Acetyloxy-20α-hydroxypregn-5-ene (**1a**) and 3β-acetyloxy-20β-hydroxypregnen-5-ene (**1b**)

Compounds **1a** and **1b** (identical with authentic standards)[35] were obtained from pregnenolone acetate (3β-acetyloxypregn-5-en-20-one) in 99% yield, by reduction with $NaBH_4$ in $MeOH/CH_2Cl_2$; the epimeric mixture (~1:9 taking into account the integration of the CH_3-13 signal in their ^{1}H NMR spectrum) was separated by chromatography on silica gel with hexane–ethyl acetate as eluent. ^{1}H NMR (200.13 MHz): **1a**: δ 0.68 (3H, s) (Me-18), 1.03 (3H, s) (Me-19), 1.23 (3H, d, J=6.0 Hz) (Me-21), 2.03 (3H, s), 3.73 (1H, m) (H-20), 4.62 (1H, br s) (H-3), 5.39 (1H, dd) (H-6); **1b**: δ 0.78 (3H, s) (Me-18), 1.04 (3H, s) (Me-19), 1.14 (3H, d, J=6.1 Hz) (Me-21), 3.72 (1H, m) (H-20), 4.58 (1H, br s) (H-3), 5.36 (1H, dd) (H-6).

4.3.2. 3β-tert-Butyldimethylsilyloxy-20α-hydroxypregn-5-ene (**2a**) and 3β-tert-butyldimethylsilyloxy-20β-hydroxypregn-5-ene (**2b**)

These compounds were obtained from pregnenolone acetate by reduction with LAH that rendered the 3,20-dihydroxypregne-5-ene as a mixture of 20α/20β epimers (1:9 by ^{1}H NMR) in almost quantitative yield. The mixture was dissolved in anhydrous DMF (20 mL) under nitrogen, imidazole (3 mmol) and *tert*-butyldimethylsilyl chloride (2 mmol) were added at 0 °C and the mixture was allowed to reach 25 °C. After 2 h the solution was poured into saturated aqueous NaCl (60 mL), extracted with diethyl ether (3×10 mL), dried with sodium sulfate, and evaporated under vacuum. The white solid obtained was purified by flash chromatography on silica gel using hexane/ethyl acetate (90:10), to give the title compound **2b** (Mp: 153–154 °C) in 78% yield. Continued elution with the same solvent afforded crystalline compound **2a** in 13% yield (Mp: 164–165 °C). **2a**: IR (film, cm^{-1}): 3406, 2962, 2847, 1454, 1245, 1120, 961, 687; ^{1}H NMR (500.14 MHz): δ 0.06 (6H, s) (Me–Si), 0.68 (3H, s) (Me-18), 0.89 (9H, s) (*t*-Bu–Si), 1.00 (3H, s) (Me-19), 1.23 (3H, d, J=6.2 Hz) (Me-21), 3.71 (1H, m) (H-20), 3.48 (1H, m) (H-3), 5.32 (1H, br d) (H-)6; ^{13}C NMR (50.32 MHz): δ −4.6, 12.5, 18.3, 19.4, 20.8, 23.5, 24.2, 25.9, 26.0, 31.6, 31.9, 32.1, 36.7, 37.5, 38.9, 41.6, 42.8, 50.2, 56.6, 56.0, 58.4, 70.4, 72.6, 121.1, 141.6. Anal. Calcd for $C_{27}H_{48}O_2Si$: C, 74.94%; H, 11.18%. Found: C, 75.06%; H, 11.04%. **2b**: IR (film, cm^{-1}): 3390, 2987, 2852, 1545, 1250, 1180, 964, 688; ^{1}H NMR (500.14 MHz): δ 0.06 (3H, s) (Me–Si), 0.77 (3H, s) (Me-18), 0.89 (9H, s) (*t*-Bu–Si), 1.01 (3H, s) (Me-19), 1.14 (3H, d, J=6.1 Hz) (Me-21), 3.74 (1H, m) (H-20), 3.48 (1H, m) (H-3), 5.31 (1H, br d) (H-6); ^{13}C NMR (50.32 MHz): δ −4.6, 12.4, 18.3, 19.5, 21.0, 23.7, 24.6, 25.7, 26.0, 26.0, 31.8, 32.0, 32.1, 36.7, 37.4, 40.0, 42.3, 42.9, 50.2, 56.3, 58.5, 70.6, 72.7, 121.0, 141.7. Anal. Calcd for $C_{27}H_{48}O_2Si$: C, 74.94%; H, 11.18%. Found: C, 75.10%; H, 10.97%.

4.3.3. 3β-Acetyloxy-20α- and 3β-acetyloxy-20β-hydroxy-5αH-pregnane (**3a** and **3b**)

3β-Acetyloxy-20-hydroxypregnane, α and β (**3a** and **3b**) (identical with authentic standards)[35] were obtained from 3β-acetoxypregnan-20-one, **2** in 97% yield, by reduction with $NaBH_4$ in $MeOH/CH_2Cl_2$; the epimeric mixture (~1:9 by ^{1}H NMR) was separated by chromatography on silica gel with hexane–ethyl acetate as eluent. ^{1}H NMR (500.14 MHz): **3a**: δ 0.67 (3H, s) (Me-18), 0.83 (3H, s) (Me-19), 1.13 (3H, d, J=6.0 Hz) (Me-21), 3.71 (1H, m) (H-20), 4.73 (1H, m) (H-3);

3b: δ 0.73 (3H, s) (Me-18), 0.82 (3H, s) (Me-19), 1.14 (3H, d, *J*=5.9 Hz) (Me-21), 3.72 (1H, m) (H-20), 4.72 (1H, m) (H-3).

*4.3.4. 3,3-Ethylenedioxy-20α- and 3,3-ethylenedioxy-20β-hydroxy-5-pregnene (**4a** and **4b**)*

Compounds **4a** and **4b** were obtained from 3-oxo-22,23-bisnor-4-cholenic acid in three steps.[41] Decarboxylation with iodobenzene diacetate and iodine in fresh CCl_4 under irradiation with a 300 W tungsten lamp rendered a mixture of 20α (*S*)/20β (*R*) epimers (3:2 by ^{1}H NMR), which was separated by chromatography in silica gel (hexane/ethyl acetate) giving rise to the mixture of two 20-iodo-4-pregnene-3-one 20*R* (38%) and 20*S* (56%). Protection of the 3-carbonyl group as an ethylene ketal and further replacement of iodine for hydroxide, via oxygenation of an intermediate 20-carbon radical, yielded the titled compounds, identical with an authentic standard[41] (**4a**: 40% yield, **4b**: 22% yield). ^{1}H NMR (200.13 MHz): **4a**: δ 0.68 (3H, s) (Me-18), 1.03 (3H, s) (Me-19), 1.23 (3H, d, *J*=6.2 Hz) (Me-21), 2.55 (1H, m) (Hβ-12), 3.71 (1H, m) (H-20), 3.94 (4H, m) (OCH_2CH_2O), 5.34 (1H, br d) (H-6); **4b**: δ 0.78 (3H, s) (Me-18), 1.04 (3H, s) (Me-19), 1.15 (3H, d, *J*=6.1 Hz) (Me-21), 2.55 (1H, m) (Hβ-12), 3.74 (1H, m) (H-20), 3.95 (4H, m) (OCH_2CH_2O), 5.35 (1H, br d) (H-6).

*4.3.5. 20α- and 20β-Hydroxypregn-4-en-3-one (**5a** and **5b**)*

Prepared from **4a** and **4b** by removal of the 3-ethylene ketal (acetone, *p*-toluensulfonic acid) giving the corresponding 20-hydroxy-4-pregnen-3-one identical to an authentic standard (**5a**: 83% yield, **5b**: 82% yield).[34] ^{1}H NMR (200.13 MHz): **5a**: δ 0.70 (3H, s) (Me-18), 1.18 (3H, s) (Me-19), 1.23 (3H, d, *J*=6.3 Hz) (Me-21), 3.72 (1H, m) (H-20), 5.72 (1H, br d) (H-4); **5b**: δ 0.78 (3H, s) (Me-18), 1.14 (3H, d, *J*=6.3 Hz) (Me-21), 1.23 (3H, s) (Me-19), 3.72 (1H, m) (H-20), 5.75 (1H, br d) (H-4).

4.3.6. Preparation of ethyl hemisuccinate

Ethanol (5.8 mL) was added to the solution of succinic anhydride (10 g, 100 mmol) in pyridine (20 mL). The reaction was allowed to proceed in a shaker at 55 °C overnight and then poured over a water/ice mixture. Hydrochloric acid (35%) was added dropwise to acidify and the product was extracted with ethyl acetate. Organic phase was washed with water, dried, and evaporated. Ethyl hemisuccinate: yield 90%, colorless liquid. ^{1}H NMR (200.13 MHz): δ 1.25 (3H, t), 2.65 (4H, m), 4.50 (2H, q); ^{13}C NMR (50.32 MHz): δ 13.9, 28.3, 28.5, 59.4, 171.1, 172.7. Anal. Calcd for $C_6H_{10}O_4$: C, 49.31%; H, 6.90%. Found: C, 48.89%; H, 6.98%.

*4.4. Synthesis of 20β-hydroxy-5αH-pregnan-3-one (**6b**)*

*4.4.1. 3β,20β-Pregnanediol (**10**)*

3β,20β-Pregnanediol (**10**) (Mp: 207–209 °C, identical with literature)[34] was obtained from pregnenolone acetate **8**, by catalytic hydrogenation of the double bond following by simultaneous reduction of the 20-carbonyl and 3β-acetate moieties with lithium aluminum hydride in THF (85% overall yield). ^{1}H NMR (200.13 MHz): δ 0.74 (3H, s) (Me-18), 0.81 (3H, s) (Me-19), 1.13 (3H, d, *J*=6.0 Hz) (Me-21), 3.59 (1H, m) (H-3), 3.72 (1H, m) (H-20).

*4.4.2. 3β,20β-Diformyloxy-5αH-pregnane (**11**)*

To a solution of the 3β,20β-pregnanediol (**10**) obtained above (390 mg, 1.2 mmol) in dry pyridine (10.0 mL) recently prepared formic acetic anhydride[41] (7 mL) was added at 25 °C under nitrogen atmosphere. After 2 h the solution was poured into cold 2 N HCl (50 mL), extracted with dichloromethane (3×15 mL), dried with sodium sulfate and the solvent evaporated, affording diformyloxypregnane **11** (438 mg, 97% yield). Mp: 143–145 °C. IR (film, cm^{-1}): 2928, 1707, 1449, 1177, 1041, 677; ^{1}H NMR (200.13 MHz): δ 0.63 (3H, s) (Me-18), 0.82 (3H, s) (Me-19), 1.20 (3H, d, *J*=6.6 Hz) (Me-21), 4.81 (1H, m) (H-3), 4.97 (1H, m) (H-20); ^{13}C NMR (50.32 MHz): δ 12.1, 12.3, 20.9, 23.4, 24.3, 25.6, 28.7, 29.7, 31.5, 33.2, 35.2, 36.2, 39.7, 39.9, 41.5, 44.7, 53.8, 55.6, 58.5, 70.6, 72.4. Anal. Calcd for $C_{23}H_{36}O_4$: C, 73.37%; H, 9.64%. Found: C, 73.13%; H, 9.71%.

*4.4.3. 20β-Formyloxyl-3β-hydroxy-5αH-pregnane (**12**)*

Compound **11** (438 mg) was dissolved in THF (10 mL) and methanol (50 mL) and potassium bicarbonate (3×35 mg) was added over 3 h. The reaction mixture was stirred for 4 h at 25 °C under nitrogen atmosphere, and the solution was neutralized with hydrochloric acid, concentrated and then diluted with water. Extractive workup with diethyl ether gave the 3β-hydroxy-20β-formyloxy steroid **12** (387 mg), which was purified by flash chromatography. Mp: 165–166 °C. IR (film, cm^{-1}): 3414, 2945, 1732, 1375, 1037, 963; ^{1}H NMR (200.13 MHz): δ 0.63 (3H, s) (Me-18), 0.79 (3H, s) (Me-19), 1.18 (3H, d, *J*=6.2 Hz) (Me-21), 3.59 (1H, m) (H-3), 4.96 (1H, m) (H-20); ^{13}C NMR (50.32 MHz): δ 12.4, 12.5, 21.3, 22.8, 24.4, 25.5, 28.6, 31.5, 32.1, 35.5, 36.2, 37.0, 38.2, 39.1, 44.9, 54.4, 55.9, 56.7, 63.9, 71.3, 72.9, 161.0. Anal. Calcd for $C_{22}H_{36}O_3$: C, 75.82%; H, 10.41%. Found: C, 75.63%; H, 10.51%.

*4.4.4. 20β-Formyloxy-5αH-pregnan-3-one (**13**)*

A suspension of pyridinium chlorochromate (862 mg, 4.0 mmol), barium carbonate (516 mg, 2.6 mmol), and 3 Å molecular sieves (700 mg) in anhydrous dichloromethane (15 mL) was stirred for 10 min under a nitrogen atmosphere at 0 °C. A solution of crude 3β-alcohol **12** (387 mg) in anhydrous dichloromethane (6 mL) was added and stirring continued at the same temperature for 2 h. The reaction mixture was diluted with ether and percolated through Florisil with dichloromethane/ether (1:1) and solvent was evaporated. Purification of the resulted residue by flash chromatography gave 20-formyloxypregnan-3-one **13** (309 mg, 77% yield, two steps). Mp: 153–154 °C. IR (film, cm^{-1}): 2932, 1718, 1690, 1434, 1168, 1142, 903; ^{1}H NMR (200.13 MHz): δ 0.63 (3H, s) (Me-18), 1.01 (3H, s) (Me-19), 1.21 (3H, d, *J*=6.0 Hz) (Me-21), 4.96 (1H, m) (H-20); ^{13}C NMR (50.32 MHz): δ 12.5, 12.9, 19.8, 21.4, 23.8, 24.6, 28.8, 31.6, 35.1, 35.7, 38.1, 38.4, 39.9, 42.5, 44.8, 46.4, 53.5, 54.8, 55.3, 71.3, 212.3. Anal. Calcd

for $C_{22}H_{34}O_3$: C, 76.26%; H, 9.89%. Found: C, 79.18%; H, 9.85%.

4.4.5. 20β-Hydroxy-5αH-pregnan-3-one (**6b**)

20β-Formyloxy-5α*H*-pregnan-3-one **13** (309 mg) was dissolved in THF (5 mL) and methanol (20 mL) and 5% aqueous potassium hydroxide (2 mL) was added. The resulting solution was stirred for 5 min at room temperature, neutralized with 2 N hydrochloric acid, and concentrated in vacuo to 1/10 of the original volume. Dilution with water and conventional workup with dichloromethane afforded 20β-hydroxy-pregnan-3-one **6b** (0.253 g, 91% yield). Mp: 185–187 °C. IR (film, cm^{-1}): 3433, 2926, 171, 1452, 1041, 883; ^{1}H NMR (500.14 MHz): δ 0.77 (3H, s) (Me-18), 1.02 (3H, s) (Me-19), 1.14 (3H, d, *J*=5.9 Hz) (Me-21), 3.73 (1H, m) (H-20); ^{13}C NMR (50.32 MHz): δ 11.6, 12.6, 21.3, 23.7, 24.5, 25.7, 28.9, 31.9, 35.3, 35.7, 38.2, 38.6, 40.0, 42.6, 44.7, 46.7, 53.9, 55.8, 58.6, 70.6, 212.2. Anal. Calcd for $C_{21}H_{34}O_2$: C, 79.19%; H, 10.76%. Found: C, 79.11%; H, 11.11%.

4.5. 20β-Hemisuccinyloxy-5αH-pregnan-3-one (**7**)

20β-Ethylsuccinyloxy-5α*H*-pregnan-3-one (**6d**) (112 mg, 0.25 mmol) was added to a solution of bis(tributyltin)oxide (0.31 mL, 0.6 mmol) in toluene (7 mL). The mixture was stirred at 80 °C for 15 h and then the solvent was evaporated in vacuo. The resulting oil was dissolved in EtOAc (10 mL) and extracted with 5% aqueous $NaHCO_3$ (3×5 mL). The aqueous phase was acidified to pH 4–5 with dilute HCl and extracted with EtOAc (3×5 mL). The organic phase was washed with brine (2×5 mL), dried (Na_2SO_4), and evaporated in vacuo to afford **7** (78 mg, 72% yield). Mp: 208 °C (dec). IR (film, cm^{-1}): 3445, 2915, 1727, 1689, 1387, 1036, 871; ^{1}H NMR (500.14 MHz) for **7**: δ 0.67 (3H, s) (Me-18), 1.03 (3H, s) (Me-19), 1.18 (3H, d, *J*=6.0 Hz) (Me-21), 1.28 (2H, m) (H-24), 2.62 (2H, m) (H-23), 4.91 (1H, m) (H-20); ^{13}C NMR (50.32 MHz): δ 11.5, 12.6, 19.9, 21.3, 24.3, 25.5, 28.9, 29.4, 29.7, 31.7, 35.3, 35.7, 38.2, 38.6, 39.3, 42.5, 44.7, 46.8, 53.9, 55.1, 55.7, 73.6, 171.4, 173.6, 212.2. Anal. Calcd for $C_{25}H_{40}O_5$: C, 71.39%; H, 9.59%. Found: C, 71.23%; H, 9.65%.

4.6. Enzymatic reactions

4.6.1. Enzymatic preparation of diethyl succinate

Diethyl succinate was prepared according to a known procedure and spectral data were in accordance with those reported in literature.[32] Colorless liquid, 88% yield. ^{1}H NMR (200.13): δ 1.24 (6H, t), 2.60 (4H, s), 4.13 (4H, q).

4.6.2. General procedure for the enzymatic succinoylation of pregnanes **1–6**

The lipase (500 mg) and the acylating agent (1 mmol) (succinic anhydride, ethyl hemisuccinate, or diethyl succinate) were added to a solution of the pregnane **1a/b–6a/b** (0.3 mmol) in the corresponding solvent (40 mL). The mixture was incubated in an orbital shaker at 55 °C and 200 rpm or at 100 °C with magnetic stirring, and the progress of the reaction was monitored by TLC (hexane/EtOAc 7:3). Once the reaction was finished, the enzyme was filtered off and washed with dichloromethane (3×5 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/EtOAc 95:5.

4.6.3. 3β-Acetyloxy-20β-ethylsuccinyloxypregn-5-ene (**1d**)

White solid (60 mg, 43% yield). Mp: 71–72 °C. $[\alpha]_D^{25}$ −25.0 (*c* 0.02, $CHCl_3$). IR (film, cm^{-1}): 2945, 1732, 1375, 1244, 1163, 1035, 963, 903; ^{1}H NMR (200.13 MHz): δ 0.64 (3H, s) (Me-18), 1.00 (3H, s) (Me-19), 1.13 (3H, d, *J*=6.1 Hz) (Me-21), 1.26 (3H, t, *J*=7.1 Hz) (CH_3, OEt), 2.03 (3H, s) (CH_3, OAc), 2.59 (4H, m) (CH_2, succinic), 4.14 (2H, q, *J*=7.1 Hz) (CH_2, OEt), 4.59 (1H, m, *J*=4.2 Hz) (H-3), 4.86 (1H, dq, *J*=6.1, 10.5 Hz) (H-20), 5.36 (1H, dd) (H-6); ^{13}C NMR (50.32 MHz): δ 12.3, 14.2, 19.3, 19.9, 20.9, 21.4, 24.3, 25.4, 27.7, 29.2, 29.6, 31.7, 31.8, 36.6, 36.9, 38.1, 39.1, 42.1, 50.0, 54.9, 56.0, 60.6, 73.3, 74.0, 122.4, 139.7, 170.6, 171.6, 172.3. Anal. Calcd for $C_{29}H_{44}O_6$: C, 71.28%; H, 9.08%. Found: C, 71.30%; H, 9.05%.

4.6.4. 3β,20β-Dihydroxypregn-5-ene (**1e**)

White solid (11 mg, 10% yield). Mp: 210–211 °C (lit.[34] 210–211 °C). $[\alpha]_D^{25}$ −65 (*c* 0.02, $CHCl_3$) (lit.[34] −64); ^{1}H NMR (200.13 MHz): δ 0.77 (3H, s) (Me-18), 1.01 (3H, s) (Me-19), 1.14 (3H, d, *J*=6.1 Hz) (Me-21), 3.50 (1H, m, *J*=4.2 Hz) (H-3), 3.72 (1H, dq, *J*=6.1, 10.5 Hz) (H-20), 5.34 (1H, dd) (H-6).

4.6.5. 20β-Ethylsuccinyloxy-3β-hydroxy-pregn-5-ene (**1f**)

White solid (27 mg, 21% yield). Mp: 112–113 °C. $[\alpha]_D^{25}$ −23.5 (*c* 0.02, $CHCl_3$). IR (film, cm^{-1}): 3264, 2936, 2360, 2342, 1732, 1449, 1377, 1163, 1054, 1022, 962; ^{1}H NMR (200.13 MHz): δ 0.64 (3H, s) (Me-18), 0.99 (3H, s) (Me-19), 1.14 (3H, d, *J*=6.1 Hz) (Me-21), 1.25 (3H, t, *J*=7.1 Hz) (CH_3, OEt), 2.59 (4H, m) (CH_2, succinic), 3.51 (1H, m, *J*=4.2 Hz) (H-3), 4.14 (2H, q, *J*=7.1 Hz) (CH_2, OEt), 4.86 (1H, dq, *J*=6.0, 10.5 Hz) (H-20), 5.34 (1H, dd) (H-6); ^{13}C NMR (50.32 MHz): δ 12.3, 14.2, 19.4, 19.9, 20.9, 24.3, 25.4, 29.2, 29.6, 31.6, 31.9, 36.5, 37.2, 39.2, 42.2, 42.3, 50.1, 55.0, 56.1, 60.6, 71.7, 73.3, 122.5, 140.8, 171.6, 172.3. Anal. Calcd for $C_{27}H_{42}O_5$: C, 72.61%; H, 9.48%. Found: C, 72.45%; H, 9.50%.

4.6.6. 3β-tert-Butyldimethylsilyloxy-20β-ethylsuccinyloxypregn-5-ene (**2d**)

White solid (51 mg, 38% yield). Mp: 75–77 °C. $[\alpha]_D^{25}$ −11.9 (*c* 0.02, $CHCl_3$). IR (film, cm^{-1}): 2930, 2901, 2857, 1732, 1462, 1379, 1252, 1223, 1161, 1088, 961, 887; ^{1}H NMR (200.13 MHz): δ 0.05 (6H, s) (Me–Si), 0.63 (3H, s) (H-18), 0.88 (9H, s) (*t*-Bu–Si), 0.98 (3H, s) (H-19), 1.14 (3H, d, *J*=6.1 Hz) (Me-21), 1.26 (3H, t, *J*=7.1 Hz), 2.58 (4H, m) (CH_2, succinic), 3.47 (1H, m, *J*=4.8 Hz) (H-3), 4.14 (2H, q, *J*=7.1 Hz) (CH_2, OEt), 4.85 (1H, dq, *J*=6.1, 10.5 Hz) (H-20), 5.30 (1H, dd) (H-6); ^{13}C NMR (50.32 MHz): δ −4.6, 12.3, 14.2, 18.2, 19.4, 19.9, 21.0, 24.3, 25.3, 26.0, 29.1, 29.6, 31.8, 31.9, 32.1, 36.6, 37.4, 39.2, 42.2, 42.8, 50.2, 55.0, 56.1, 60.7, 72.6, 73.3, 120.9, 141.6,

171.6, 172.3. Anal. Calcd for $C_{33}H_{56}O_5Si$: C, 70.67%; H, 10.06%. Found: C, 70.99%; H, 10.13%.

*4.6.7. 3β-Acetyloxy-20β-ethylsuccinyloxy-5αH-pregnane (**3d**)*

White solid (59 mg, 42% yield). Mp: 72–73 °C. $[\alpha]_D^{25}$ +14.1 (*c* 0.01, $CHCl_3$). IR (film, cm^{-1}): 2939, 1732, 1375, 1244, 1035, 963, 905, 886; ^1H NMR (200.13 MHz): δ 0.61 (3H, s) (Me-18), 0.81 (3H, s) (Me-19), 1.15 (3H, d, *J*=6.1 Hz) (Me-21), 1.26 (3H, t, *J*=7.1 Hz), 2.02 (3H, s) (CH_3, OAc), 2.60 (4H, m) (CH_2, succinic), 3.47 (1H, m, *J*=4.8 Hz) (H-3), 4.16 (2H, q, *J*=7.1 Hz) (CH_2, OEt), 4.86 (1H, dq, *J*=6.1, 10.5 Hz) (H-20); ^{13}C NMR (50.32 MHz): δ 12.2, 14.2, 19.9, 21.1, 21.5, 23.0, 24.2, 25.4, 27.5, 28.5, 32.0, 34.0, 35.3, 35.5, 36.7, 38.7, 39.3, 42.4, 54.2, 55.0, 55.8, 60.6, 73.3, 73.7, 170.7, 171.6, 172.3. Anal. Calcd for $C_{27}H_{42}O_5$: C, 70.99%; H, 9.45%. Found: C, 71.06%; H, 9.40%.

*4.6.8. 3,3-Ethylendioxy-20β-ethylsuccinyloxypregn-5-ene (**4d**)*

White solid (41 mg, 30% yield). Mp: 120–122 °C. $[\alpha]_D^{25}$ −8.1 (*c* 0.01, $CHCl_3$). IR (film, cm^{-1}): 2939, 2888, 2845, 1730, 1431, 1377, 1313, 1244, 1173, 1153, 1101, 1089, 1029, 961, 864; ^1H NMR (200.13 MHz): δ 0.64 (3H, s) (Me-18), 1.01 (3H, s) (Me-19), 1.14 (3H, d, *J*=6.1 Hz) (Me-21), 1.26 (3H, t, *J*=7.1 Hz), 1.96 (1H, m, *J*=2.8 Hz), 2.11 (1H, dd, *J*=2.8, 14.2 Hz), 2.59 (4H, m) (CH_2, succinic), 3.94 (4H, m), 4.14 (2H, q, *J*=7.1 Hz) (CH_2, OEt), 4.86 (1H, dq, *J*=6.1, 10.5 Hz) (H-20), 5.34 (1H, dd) (H-6); ^{13}C NMR (50.32 MHz): δ 2.3, 14.2, 18.9, 19.9, 20.9, 24.3, 25.4, 29.2, 29.6, 31.0, 31.7, 31.7, 36.3, 36.6, 39.2, 41.8, 42.2, 49.7, 54.9, 56.1, 60.6, 64.2, 64.4, 73.3, 109.4, 122.0, 140.2, 171.6, 172.3. Anal. Calcd for $C_{29}H_{44}O_6$: C, 71.28%; H, 9.08%. Found: C, 71.46%; H, 9.20%.

*4.6.9. 20β-Ethylsuccinyloxypregn-4-en-3-one (**5d**)*

White solid (105 mg, 72% yield). Mp: 70–71 °C. $[\alpha]_D^{25}$ +82.3 (*c* 0.08, $CHCl_3$). IR (film, cm^{-1}): 2942, 1732, 1664, 1376, 1230, 1159, 1073, 1027, 963, 864; ^1H NMR (200.13 MHz, $CDCl_3$): δ 0.66 (3H, s) (Me-18), 1.14 (3H, d, *J*=6.1 Hz) (Me-21), 1.16 (3H, s) (Me-19), 1.25 (3H, t, *J*=7.1 Hz), 2.58 (4H, m) (CH_2, succinic), 4.14 (2H, q, *J*=7.1 Hz) (CH_2, OEt), 4.86 (1H, dq, *J*=6.1, 10.5 Hz) (H-20), 5.71 (1H, s) (H-4); ^{13}C NMR (50.32 MHz): δ 12.4, 14.2, 17.3, 19.8, 20.9, 24.2, 25.3, 29.1, 29.5, 32.0, 32.8, 33.9, 35.4, 35.6, 38.6, 39.0, 42.2, 53.8, 54.8, 55.2, 60.6, 73.1, 123.8, 171.4, 171.6, 199.6. Anal. Calcd for $C_{27}H_{40}O_5$: C, 72.94%; H, 9.07%. Found: C, 72.87%; H, 9.05%.

*4.6.10. 20β-Ethylsuccinyloxy-5αH-pregnan-3-one (**6d**)*

White solid (112 mg, 77% yield). Mp: 68–69 °C. $[\alpha]_D^{25}$ +14.7 (*c* 0.01, $CHCl_3$). IR (film, cm^{-1}): 2945, 1732, 1670, 1375, 1244, 1035, 963, 903, 883; ^1H NMR (200.13 MHz): δ 0.63 (3H, s) (Me-18), 0.99 (3H, s) (Me-19), 1.13 (3H, d, *J*=6.1 Hz) (Me-21), 1.25 (3H, t, *J*=7.1 Hz), 2.58 (4H, m) (CH_2, succinic), 4.14 (2H, q, *J*=7.1 Hz) (CH_2, OEt), 4.85 (1H, dq, *J*=6.1, 10.5 Hz) (H-20); ^{13}C NMR (50.32 MHz): δ 11.4, 12.5, 14.2, 19.8, 21.3, 24.2, 25.4, 28.9, 29.1, 29.5, 31.7, 35.2, 35.6, 38.2, 38.5, 39.2, 42.4, 44.7, 46.7, 53.8, 55.0, 55.6, 56.4, 60.6, 73.2, 171.6, 172.3, 212.0. Anal. Calcd for $C_{27}H_{40}O_5$: C, 72.94%; H, 9.07%; O, 17.99%. Anal. Calcd for $C_{27}H_{42}O_5$: C, 72.61%; H, 9.48%. Found: C, 72.84%; H, 9.53%.

*4.6.11. Enzymatic one-pot preparation of 3β-ethylendioxy-Δ^5-pregnen-20β-ethyl succinate (**5d**)*

Succinic anhydride (100 mg, 1 mmol) and 500 mg of CAL B were added to a stirred solution of compound **5b** (108 mg, 0.3 mmol) in 100 μL of ethanol and 40 mL of isooctane. The mixture was heated at 100 °C and the progress of the reaction was monitored by TLC (hexane/EtOAc 7:3). Once the reaction was finished, the enzyme was filtered off and washed with dichloromethane (3×5 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 (hexane/EtOAc 95:5).

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