Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 1781–1788

BIOORGANIC & MEDICINAL CHEMISTRY

18-Vinyldeoxycorticosterone: a Potent Inhibitor of the Bovine Cytochrome *P*-450$_{11\beta}$

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Received 3 March 1998; accepted 11 May 1998

Abstract—18-Vinylprogesterone (18-VP) and 18-ethynylprogesterone (18-EP) have proved to be potent suicide inhibitors of *P*-450$_{11\beta}$, the last enzyme of aldosterone biosynthesis (Delorme, C.; Piffeteau, A.; Viger, A.; Marquet, A. *Eur. J. Biochem.* **1995**, *232*, 247; Delorme, C.; Piffeteau, A.; Sobrio, F.; Marquet, A. *Eur. J. Biochem.* **1997**, *248*, 252). This paper describes the synthesis of 18-vinyldeoxycorticosterone (18-VDOC), an analogue of deoxycorticosterone (DOC), the physiological substrate of the enzyme, and the evaluation of its reversible inhibiting properties for deoxycorticosterone and corticosterone oxidation by the bovine enzyme. 18-VDOC has been obtained by hydroxylation at C-21 of a 18-VP precursor. Its reversible K_i values are, respectively, 0.3 μM for the 11β-hydroxylation and 0.8 μM for the 18-hydroxylation. Hence, 18-VDOC is the strongest competitive inhibitor of bovine *P*-450$_{11\beta}$ described so far, but in contrast with 18-VP, it does not inhibit more efficiently the 18-hydroxylation than the 11-hydroxylation.

Introduction

P-450$_{11\beta}$, the last enzyme of aldosterone biosynthesis[1] (Scheme 1) plays a key role in the regulation of aldosterone production and the fundamental studies of this enzyme have a strong biological relevance with potential therapeutic applications. It was chosen as a target for the discovery of inhibitors which could decrease aldosterone overproduction occurring in pathological conditions.[2]

Several of these inhibitors, progesterone derivatives designed as suicide-substrates, have been synthesized and tested in our group.[3–6] Two of them, 18-vinyl progesterone (18-VP) and 18-ethynyl progesterone (18-EP) (Scheme 2) were found to be very efficient inhibitors of *P*-450$_{11\beta}$ either in rat adrenal cell-free extracts[4] or with the purified bovine enzyme.[7,8] They appeared as useful mechanistic tools to study the catalytic properties of this interesting *P*-450 which carries out three successive hydroxylations within the same active site.[7]

Furthermore, they have potential therapeutic interest. We have shown that they inhibit more efficiently the 18-hydroxylation steps (leading to aldosterone) than the 11β-hydroxylation step,[7] a property which was also observed in bovine adrenocortical cells.[9] In addition, they behave as potent antagonists of the mineralocorticoid receptor.[10]

This class of inhibitors has also been studied, in particular by the Merrell group, and their biological properties have been recently reviewed.[11] A comparison of the enzyme inhibitory activity of 18-EP and 18-EDOC (18-ethynyl deoxycorticosterone), hydroxylated at C-21 as is the physiological substrate of *P*-450$_{11\beta}$, has been carried out using rat adrenal glomerulosa homogenates.[11] The potency of 18-EDOC as inhibitor of the 18-hydroxylation has also been examined in calf adrenal cells by Yamakita et al.[12]

Very scarce data were given on 18-vinyl deoxycorticosterone (18-VDOC). We have previously found

Key words: 18-VDOC; *P*-450$_{11\beta}$; suicide-inhibitors; anti-aldosterone.

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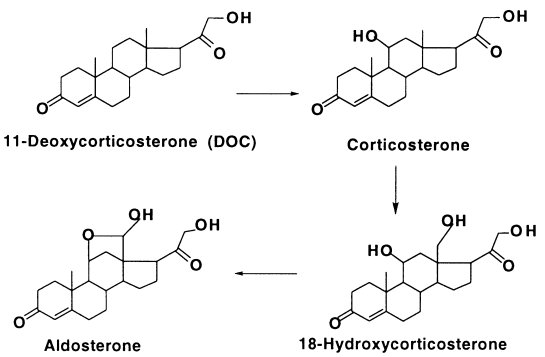
0968-0896/98/$—see front matter
PII: S0968-0896(98)00106-0



Scheme 1.

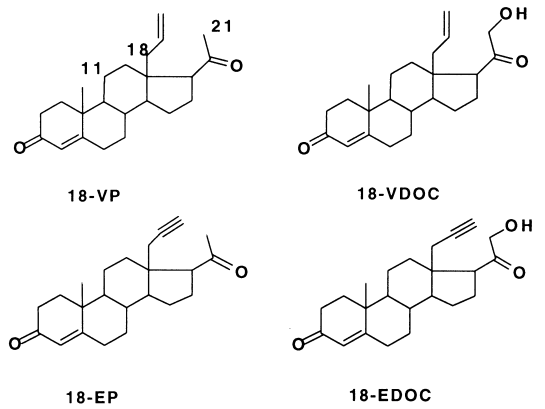
that 18-VP and 18-EP behave as suicide inactivators and that 18-VP was a fivefold better inhibitor than 18-EP, as reflected by the values of the apparent bimolecular rate constants k_i/K_i for time-dependent inactivation.[8] Hence we thought that it was useful to obtain more data on the properties of 18-VDOC.

In this paper we present a synthesis of 18-VDOC and kinetic results concerning its inhibition properties. In this work, only the reversible kinetic constants for corticosterone and aldosterone production catalyzed by the purified bovine P-$450_{11\beta}$ were measured.

Results

Chemistry: synthesis of 18-vinyl-deoxycorticosterone

The synthesis that we have achieved, described in Scheme 3, uses as starting material compound **2**, an intermediate in the synthesis of 18-vinyl-progesterone.[3] After removal of the dioxolane group, the alcohol function of **3** was protected with dihydropyrane in the presence of the pyridinium *p*-toluene sulfonate (PPTS) to give **4**, on which the key step, the hydroxylation at C-21 was attempted.



Scheme 2.

There are several well established procedures for this reaction, frequently reported on normal steroids with the 18-methyl group.[13] We have tested three of them, which failed when applied to **4**, bearing an allyl group at C-13.

Although the direct acetoxylation with lead tetracetate in acetic acid of 18-EP was described (with no characterization and no mention of the yield) in a patent,[14] we could obtain neither 18-VDOC nor 18-EDOC starting, respectively, from 18-VP or 18-EP. In both cases, the NMR spectrum of the crude product revealed, beside the expected singulets for the C-21 methylene and C-21 acetoxy groups, the disappearance of the vinyl and ethynyl protons, respectively. This suggests the addition of an acetate group on the terminal unsaturation, a reaction already described on other examples.[15,16]

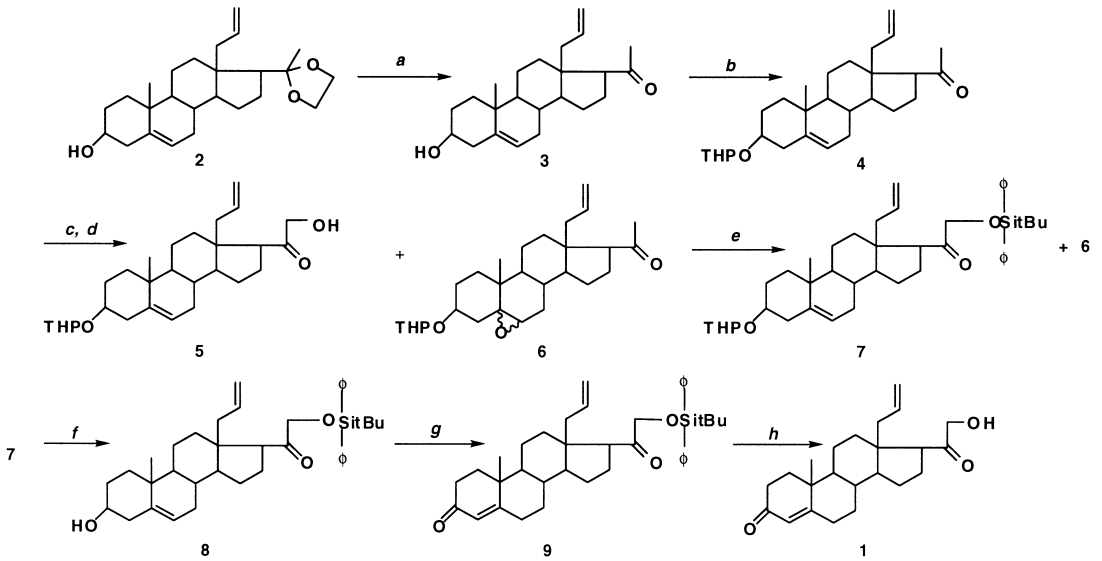
We then tried to prepare the 21-bromoketone, using phenyltrimethyl ammonium tribromide, a reagent which is unreactive towards double bonds.[17] This led also to a complex mixture resulting from the combination of different events, namely bromination at C-17 as well as C-21 and epimerisation of the side chain (the influence of the substituent at C-13 on the regioselectivity of the enolisation and the configurational stability of the C-17 side chain has been already reported).[18]

Another possible method using a diazoketone was also investigated. The β-ketoaldehyde **10** (hydroxymethylene form) obtained by treatment of the methyl ketone **3** with sodium hydride and ethyl formate, was converted into the diazoketone **11**, by reaction with sodium hydride and tosylazide (Scheme 4).[19,20]

Attempts to introduce an acetate group by treatment of the crude diazoketone with acetic acid were unsuccessful. Products resulting from the participation of the double bond of the 18-vinyl group were detected on the NMR spectrum of the crude product. To favor the substitution by the external nucleophile we performed the reaction with KBr in the presence of *p*-toluene sulfonic acid (TsOH). The bromoketone **12** was obtained, but as a mixture of 17α:17β isomers, that we were unable to separate efficiently.

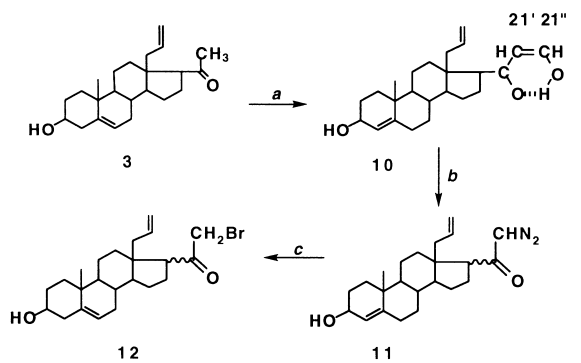
The microbial hydroxylation of 18-VP by the *Aspergillus niger* strain (ATCC 9142), which was reported to hydroxylate at C-21 several progesterone derivatives,[21] was also tried without success.

The method which allowed us to obtain **1**, although with a poor yield, relies on the peracid oxidation of a



Scheme 3. (a) *p*TosOH, acetone; (b) dihyropyrane, PPTS, CH_2Cl_2; (c) LDA, THF, Me_3SiCl; (d) MMPP, ethanol; (e) *t*BuPh$_2$SiCl, DMAP, pyridine, CH_2Cl_2; (f) AcOH, H_2O, THF; (g) N-Me piperidone, THF, Al (O*i*Pr)$_3$; (h) HCl, methanol.

silyl enol ether.[18] Treatment of methyl ketone **4** with LDA at −78 °C followed by quenching with trimethylchlorosilane afforded the 20(21) silyl enol ether. In our hands, the reaction was very capricious and the best yield of enol ether was 37% (determined on the NMR spectrum of the crude product). Epoxidation of this crude product with magnesium monoperoxyphthalate (MMPP) in ethanol[22] gave the hydroxy ketone **5** with a 81% corrected yield from silyl enol ether, according to the NMR spectrum of the crude product. The other identified compounds were the hydrolysis product of the tetrahydropyranyloxy group of **5** and the two epoxides **6**. Purification at this stage was difficult and only partially achieved. Silylation of the 21-hydroxyl group was performed in the presence of tertiobutyldiphenylsilyl chloride and DMAP. Pure **8** was obtained after deprotection of the 3-OH group. Oppenauer oxidation and final deprotection of the 21-OH group led to the targeted product **1**.



Scheme 4. (a) NaH, C_2H_5, HCOOEt, THF; (b) $TosN_3$, NaH, C_2H_5OH, THF; (c) KBr, TsOH, H_2O, CH_3CN.

Biochemical experiments

They were all carried out with the purified bovine reconstituted system that we have already described.[7]

In a preliminary assay, 18-VDOC was compared to 18-VP for the reversible inhibition of the 11β-hydroxylation step, using an initial concentration of substrate [^{14}C-DOC] close to the K_m value and different inhibitor concentrations. As shown in Figure 1, after 1 min in presence of 18-VDOC, a marked decrease in corticosterone production (only 30% of the total activity remaining) was observed at 1 μM of inhibitor. In contrast, under the same conditions, no inhibition was detectable in the presence of 1 μM 18-VP and a concentration of 10 μM of 18-VP was needed to obtain a significant decrease (23%) in corticosterone formation.

Inhibition kinetics

For kinetic determinations, 11β- and 18-hydroxylation reactions were studied by varying the concentration of substrates, respectively [^{14}C]11-deoxycorticosterone and [^{3}H]corticosterone, under initial velocity conditions as already described (see Experimental section). For the studies of reversible inhibition of the 11β-hydroxylation of DOC, the quantity of corticosterone formed during one to two minutes was examined with or without addition of 18-VDOC at three different concentrations (0.125–1 μM). According to the initial velocity

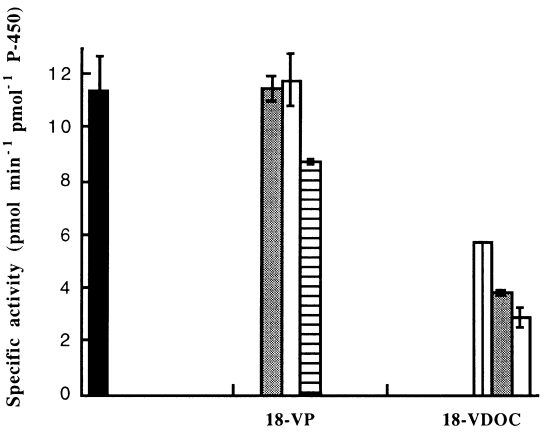


Figure 1. Inhibition of the transformation of DOC to corticosterone by 18-VP or 18-VDOC. 11β-Hydroxylase activity of bovine *P*-450$_{11\beta}$ (20 nM) was measured in a reaction mixture containing [^{14}C]11-deoxycorticosterone as substrate (1.25 μM) in the presence of inhibitors 18-VP or 18-VDOC at concentrations of: 0.25 μM (▥), 0.5 μM (▩), 1 μM (□), or 10 μM (▤). 100% is the production without inhibitor (■). Each activity is the average of duplicate measurements in the same experiment. Shown is a representative example of two independent experiments.

data, 18-VDOC bound competitively with respect to the substrate DOC (Fig. 2) and the dissociation constant K_i was of 0.3 ± 0.15 μM (SD). The enzyme kinetics for the reversible inhibition of 18-hydroxylation steps, that is, the production of 18-OH-corticosterone and aldosterone from corticosterone, were performed under identical conditions. The inhibition has been found to be also competitive with substrate (Fig. 3), with a K_i value of 0.8 ± 0.13 μM (SD). It will be noted that the K_i for both reactions are nearly identical. Under the same conditions, the K_m values for DOC and corticosterone were 1.97 ± 0.26 μM (SD) and 5.9 ± 0.18 μM (SD), respectively, consistent with the previously reported values.[7]

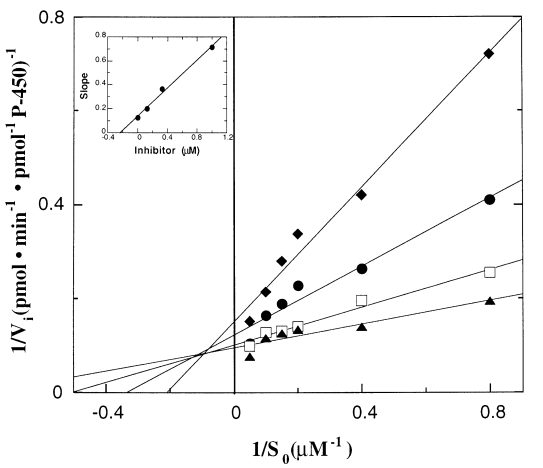


Figure 2. Inhibition of 11β-hydroxylase activity of bovine *P*-450$_{11\beta}$ by 18-VDOC. 11β-Hydroxylase activity of bovine *P*-450$_{11\beta}$ (40 nM) was studied with [^{14}C]11-deoxycorticosterone as substrate (1.25–20 μM), without (▲) or with 18-VDOC at 0.125 μM (□), 0.33 μM (●), or 1 μM (◆). Shown is a representative graph of at least two independent determinations. Duplicate measurements were run for each experiment.

Discussion

Among several 18-substituted progesterone derivatives synthesized and tested in our group, 18-EP and 18-VP, designed as suicide-substrates, proved to be potent inhibitors of aldosterone biosynthesis when tested with rat adrenal cell-free extracts,[4] with purified bovine *P*-450$_{11\beta}$[7] as well as with bovine adrenocortical cells.[9] Both compounds, which were found to inhibit more strongly the 18-hydroxylation step (aldosterone production) than the 11-hydroxylation have potential pharmacological interest as antimineralocorticoids. 18-VP is a better inhibitor than 18-EP in both reactions, as revealed by their respective reversible K_i values[7] (30 μM and 110 μM for 11β-hydroxylase activity and 5 μM and 30 μM for 18-hydroxylase activity, respectively), their k_i/K_i ratio for time-dependent inactivation[8] (470 $M^{-1}.s^{-1}$ for 18-VP and 89 $M^{-1}s^{-1}$ for 18-EP), and their effect on aldosterone production in adrenal cell-cultures.[9]

We have thus selected the 18-vinyl compound to examine the influence of an OH group at C-21 which is present in the substrate (DOC), on the inhibiting properties. Johnston et al. have considered the same point and compared the inhibitory potency of 18-EP and 18-EDOC for aldosterone production with rat adrenal

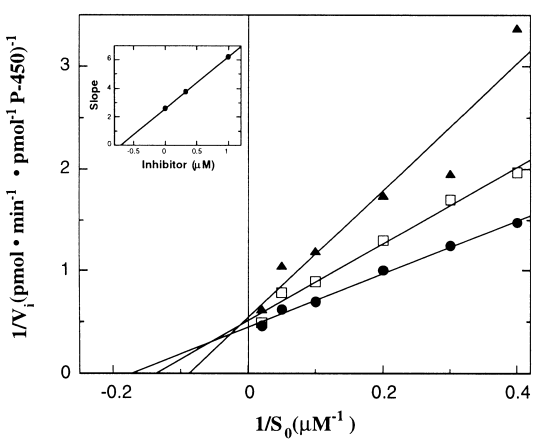


Figure 3. Inhibition of 18-hydroxylase activity of bovine *P*-450$_{11\beta}$ by 18-VDOC. 18-Hydroxylase activity of bovine *P*-450$_{11\beta}$ (450 nM) was studied with [^{3}H]corticosterone as substrate (2.5–50 μM), without (●) or with 18-VDOC at 0.33 μM (□), or 1 μM (▲). Shown is a representative graph of at least two independent determinations. Duplicate measurements were run for each experiment.

glomerulosa homogenates. With this nonpurified system, they found that 18-EP was more selective than 18-VP for inhibiting the C_{18}-hydroxylation activity. 18-EDOC was found slightly more potent than 18-EP (IC_{50} of 14 nM and 35 nM for aldosterone synthase assay, respectively).[11]

The reversible K_i values measured with our bovine purified reconstituted system revealed that 18-VDOC is 100-fold more efficient than 18-VP for inhibition of the 11β-hydroxylation step (0.3 μM and 30 μM, respectively), but only sixfold more efficient than 18-VP for the inhibition of the 18-hydroxylation step (0.8 μM and 5 μM, respectively). Thus, the presence of an OH group at C-21 strongly decreased the selectivity observed with 18-VP. We have interpreted the selectivity of 18-VP, on the basis of further experimental data, by postulating a conformational change of the enzyme after the 11-hydroxylation[7] allowing the proper orientation of the 18 methyl group with respect to the heme, the inhibitor showing a better affinity for the second conformation. Clearly, the hydroxyl group at C-21 influences the binding of the inhibitor.

Because of the absence of a three-dimensional structure of mammalian *P*-450 and despite their low sequence homologies, four bacterial *P*-450s are classically used as model for analysis of the substrate orientation in the heme pocket.[23] For instance, the modeling of corticosterone binding in the substrate pocket of mouse *P*-450coh[24] (coumarin 7α-hydroxylase) has been based on the three-dimensional structure of P450cam. It suggests a direct interaction of an arginine residue (−186 in *P*-450cam) with the 21-OH of the steroid by hydrogen bonding. It is plausible to assume that the interaction of the 21-hydroxyl group of 18-VDOC with a critical amino acid residue enhances its affinity for the native conformation of the enzyme, hence decreasing the selectivity for the inhibition of the 18-versus 11-hydroxylation.

Experimental

Chemical synthesis

All reactions using nonaqueous reagents were run under a dry argon atmosphere. Organic layers were dried on magnesium sulfate ($MgSO_4$). Column chromatography was performed on alumina 90 Merck (0.063–0.2 mm) or Kieselgel 60 Merck (70–230 mesh) and flash chromatography on Kieselgel 60 Merck (230–400 mesh). Reaction progress was monitored by analytical TLC on silica gel 60 F-254, alumina 150 F-254 or neutral alumina F-254 (type E) from Merck. Visualization of TLC was done by phosphomolybdic acid or by UV light. ^{1}H NMR spectra were recorded either on a Jeol GS X 400 or on a Bruker AC 200 or on a Bruker 300. Carbon magnetic resonance (^{13}C NMR) spectra were recorded on a Jeol GS X 400 or on a Bruker AC 200 at 100 MHz. Solutions in chloroform-*d* ($CDCl_3$) were used, with tetramethylsilane as an internal standard, coupling constants have been obtained by spin decoupling. Mass spectral data were obtained by chemical ionization (CI NH_3) or by electron impact (EI) on a Nermag R10-10C or R30-10 spectrometer. Infrared spectral data were recorded on a Perkin 1420 (values in cm^{-1}). Elemental analysis were performed by the Service Régional de Microanalyse (SIAR-Jussieu). All chemicals were purchased from Aldrich, Janssen, and Sigma.

3β-Hydroxy-18-vinyl-20,20-ethylenedioxypregn-5-ene (2). was prepared in nine steps from pregnenolone, as previously described.[3]

3β-Hydroxy-18-vinylpregn-5-ene-20-one (3). The dioxolane **2** (728 mg, 1.88 mmol) was dissolved in acetone (68 mL). *p*-Toluene sulfonic acid (36 mg, 0.19 mmol) was added. The mixture was stirred under argon, at room temperature, for 210 min. Then, the solvent was evaporated and the residue was extracted with CH_2Cl_2 (100 mL) and washed with water. Crystallization of the crude product in CH_2Cl_2-isopropyl ether gave 167 mg of white crystals. Flash chromatography of the residue in CH_2Cl_2/acetone 1% gave pure **3** (310 mg). The total yield was 74%: mp 156–157 °C; IR 3600, 1690, 1630 cm^{-1}; ^{1}H NMR (400 MHz) 1.00 (s, 3H, H-19), 2.15 (s, 3H, H-21), 3.51 (m, 1H, H-3), 4.96 (2dd, 2H, J = 17.4, 8.7 and 1.6 Hz, -CH = CH_2), 5.34 (m, 1H, H-6), 5.52 (m, 1H, -CH = CH_2). Anal. $C_{23}H_{34}O_2$ (C, H).

3β-[(Tetrahydro-2*H*-pyran-2yl)oxy]-18-vinylpregn-5-ene-20-one (4). The alcohol **3** (350 mg, 1.02 mmol) was dissolved in freshly distilled CH_2Cl_2 (10 mL) and kept under argon. Dihydropyrane (560 μL, 6.13 mmol, 6 equiv) and PPTS (51 mg, 0.2 equiv) were added. The mixture was stirred for 17 h at room temperature. Then diethylether (70 mL) was added and the organic solution was washed twice with water and evaporated. Purification of the crude product by flash chromatography (CH_2Cl_2-acetone, 1%) afforded pure **4** (399 mg, 92%): ^{1}H NMR (400 MHz) 0.96 (s, 3H, H-19), 2.10 (s, 3H, H-21), 3.44 (m, 2H, THP), 3.85 (m, 1H, H-3), 4.66 (brs, 1H-THP), 4.90 (2dd, 2H, J = 17.2, 8.8 and 2.1 Hz, -CH = CH_2), 5.29 (t, 1H, H-6), 5.47 (m, 1H, -CH = CH_2). Anal. $C_{28}H_{42}O_3$ (C, H).

3β-[(Tetrahydro-2*H*-pyran-2yl)oxy]-18-vinyl-21-hydroxypregn-5-ene-20-one (5). A solution of methylketone **4** (105 mg, 0.246 mmol) in dry THF (2 mL) was added dropwise at −78 °C to a solution of LDA (0.48 mL, 0.96 mmol, 4 equiv, 2 M in THF) in dry THF (4 mL) under argon. The mixture was stirred at −70 °C for 10 min. Then, trimethylsilylchloride (280 μL) was added

at −78 °C and the reaction was allowed to warm to room temperature. Extraction with ether containing 1% of Et_3N (100 mL) and washing with a saturated solution of $NaHCO_3$ and water afforded 156 mg of crude product, containing 37% of the silyl enol ether according to the NMR spectrum. ^{1}H NMR (400 MHz) 0.06, 0.14, and 0.20 (3s, Me_3Si), 1.02 (s, 3H, H-19), 3.49 (m, 2H, -THP), 3.91 (m, 1H, H-3), 4.58 (s, 2H, -O-C = CH_2), 4.71 (brs, 1H, -THP), 4.88 (m, 2H, -CH = CH_2), 5.35 (t, 1H, H-6), 5.87 (m, 1H, -CH = CH_2).

This crude product was dissolved in absolute ethanol (4 mL). MMPP (120 mg, 0.24 mmol) was added and the mixture was stirred overnight at room temperature under argon. Extraction with EtOAc and the usual workup afforded 154 mg of crude product which was purified by flash chromatography (cyclohexane/AcOEt, 5/1) to give starting material **4** (32 mg, 30%) and a mixture of α-hydroxyketone **5** and epoxides **6** (48 mg, 63% corrected yield). Both oxidation products **5** and **6** have the same R_f in cyclohexane/AcOEt (3/1 or 5/1). According the NMR spectrum, a 7:3 ratio was calculated for the mixture **5**:**6**, 81% corrected yield from silyl enol ether, 30% corrected yield from **4**.

5: ^{1}H NMR (200 MHz) δ 0.98 (s, 3H, H-19), 3.21 (t, 0.7 H, CH_2-OH, J = 4.7 Hz, exchanged with D_2O), 3.47 (m, 2H, -THP), 3.87 (m, 1H, H-3), 4.13 (ABX, 2H, H-21, J = 4.7 and 18.8 Hz, collapsed with D_2O), 4.68 (m, 1H, -THP), 4.92 (2bd, 2H, J = 16.5 and 7.7 Hz, -CH = CH_2), 5.31 (m, 1H, H-6), 5.36 (m, 1H, -CH = CH_2).

An analytical sample of pure **6** was obtained during the chromatography step. According to the NMR spectrum, **6** was a 8/2 mixture of α and β epoxides.

6: ^{1}H NMR (200 MHz) δ 0.96 (s, 0.6 H, H-19, β-epoxide) 1.06 (s, 2.4H, H-19, α-epoxide), 2.13 (s, 3H, H-21), 2.89 (dd, 0.8 H, J = 3.4 and 4.3 Hz, H-6, α-epoxide), 3.06 (dd, 0.2H, J = 4.6 and 6.8 Hz, H-6, β-epoxide), 3.45 (m, 1H, -THP), 3.69 (m, 0.2 H, H-3, β-epoxide), 3.84 (m, 1H + 0.8H, -THP + H-3 of α-epoxide), 4.65 (m, 1H, -THP), 4.93 (2dd, 2H, J = 18.7, 8.1 and 2.0 Hz, -CH = CH_2), 5.47 (m, 1H, -CH = CH_2).

3β-[(Tetrahydro-2*H*-pyran-2yl)oxy]-18-vinyl-21-tertiobutyldiphenylsilyloxypregn-5-ene-20-one (7). To a stirred solution of 73% pure **5** (38 mg), pyridine (15 μL) and DMAP (2.3 mg, 0.019 mmol) in dry CH_2Cl_2 (1 mL) (*t*-Bu)Ph_2SiCl (55 μL, 0.21 mmol, 3.3 equiv) was added. After the mixture was stirred for 5 days at room temperature, the reaction was quenched with H_2O and the aqueous layer was extracted with CH_2Cl_2. The crude product was partially purified by flash chromatography (cyclohexane/AcOEt, 5/1) to give **7** (74.5 mg) which was used without further purification for the next step. ^{1}H NMR (200 MHz) 1.12 and 1.20 (2s, 9H, *t*BuSi), 3.55 (m, 2H, -THP), 3.92 (m, 1H, H-3), 4.21 and 4.37 (2d, AB, J = 17.3 Hz, H-21), 4.77 (m, 1H, -THP), 5.0 (m, 2H, -CH = CH_2), 5.40 (m, 1H, H-6), 5.56 (m, 1H, -CH = CH_2), 7.35–7.84 (2m, 10H,-$SiPh_2$-).

3β-Hydroxy-18-vinyl-21-tertiobutyldiphenylsilyloxypregn-5-ene-20-one (8). Compound **7** (74.5 mg) was dissolved in the mixture AcOH/H_2O/THF (4/1/2, 1 mL) and the solution was stirred for 1 h at room temperature. Then, the solvent was evaporated in vacuum and the residue was extracted with CH_2Cl_2, washed with a solution of $NaHCO_3$ and water. After evaporation, the residue was purified by flash chromatography (cyclohexane/AcOEt, 5/1) to give pure **8** (10 mg, 71% from **5**). ^{1}H NMR (400 MHz) 1.00 (s, 3H, H-19), 1.09 and 1.42 (2s, 9H, *t*BuSi), 3.51 (m, 1H, H-3), 4.16 and 4.27 (2d, AB, J = 17.3 Hz, H-21), 4.91 (2bd, 2H, J = 18.1 and 8.2 Hz, -CH = CH_2), 5.34 (m, 1H, H-6), 5.40 (m, 1H, -CH = CH_2), 7.35–7.68 (2m, 10H, -$SiPh_2$-).

18-Vinyl-21-tertiobutyldiphenylsilyloxypregen-5-ene-3,20-dione (9). The alcohol **8** (10 mg, 0.017 mmol) was dissolved in dry toluene (3 mL) and *N*-methyl-4-piperidone (0.1 mL) was added. The mixture was heated to reflux under argon with a Dean-Stark apparatus. The first 1.5 mL of distillate were discarded. Aluminum isopropoxyde (14 mg, 0.07 mmol) was added and the mixture refluxed for 5 h. The toluene solution was diluted, neutralized with an aqueous solution of HCl 5% and washed with H_2O. Evaporation of the solvent afforded a residue (3.5 mg) which was used in the next step. ^{1}H NMR (200 MHz) 1.05 (s, 3H, H-19), 1.08 (s, 9H, *t*BuSi), 4.14 and 4.26 (2d, AB, J = 17.4 Hz, H-21), 4.92 (m, 2H, -CH = CH_2), 5.33 (m, 1H, -CH = CH_2), 5.71 (bs, 1H, H-4).

18-Vinyl-21-hydroxypregn-5-ene-3,20-dione 1 (18-VDOC). The crude silyl ether **9** (3.5 mg) was dissolved in a mixture MeOH–HCl 3% (1 mL) and stirred for 4 h at room temperature. The solution was then extracted with AcOEt, washed with a $NaHCO_3$ solution and water. Evaporation of the solvent gave 3 mg of residue which was purified by silica gel chromatography. Pure **1** (2 mg) was obtained as a glassy solid. ^{1}H NMR (200 MHz) 1.18 (s, 3H, H-19), 3.22 (dd, 1H, J = 4.9 and 4.4 Hz, 21-OH, collapsed with D_2O), 4.12 and 4.28 (2dd, 2H, ABX, J = 18.7 Hz, 4.9 and 3.8 Hz), 4.97 (2dd, 2H, J = 16.8, 9.9 and 2.2 Hz, -CH = CH_2), 5.40 (m, 1H, -CH = CH_2), 5.73 (bs, 1H, H-4); IR 3480, 1700, 1660, 1615 cm^{-1}; MS (EI) m/z 356, 325, 257, 206; HRMS calcd for $C_{23}H_{32}O_3$: 356.235142, found 356.23159.

3β-Hydroxy-18-vinyl-20-(hydroxy-2′-ethylene)-pregn-5-en-20-one (10). Methylketone **3** (122 mg, 0.35 mmol) was dissolved in 2.4 mL of dry THF and 1.2 mL of dry benzene was added. Sodium hydride (263 mg of a 95%

dispersion in mineral oil, 10.4 mmol) and absolute ethanol (75 μL, 58.8 mg, 1.27 mmol) were transferred to the steroid solution kept on an ice bath. The flask was placed under argon atmosphere and the reaction mixture was stirred vigorously at room temperature for 25 min. Ethyl formate (0.38 g, 5.1 mmol) was added dropwise with vigorous stirring. After 30 min, 1.8 N CH_3COOH (25 mL) was added cautiously (frothing) until pH 6.7. The usual workup (AcOEt, H_2O, $MgSO_4$) gave 163 mg of crude product as a mixture of 17α/17β isomers (ratio 40/60 calculated from the NMR spectrum). Purification by flash chromatography (CH_2Cl_2/acetone, 1%) afforded pure (17β)**10** (49 mg, 37%) as a yellow oil and a mixture of the 17β and 17α isomers (ratio 46/54, 80 mg, 62%). **10**(**17**β): ^{1}H NMR (400 MHz) δ 1.04 (s, 3H, H-19), 3.55 (m, 1H, H-3), 4.95 (2dd, 2H, J = 18.0, 11.2 and 1.3 Hz, -CH = CH_2), 5.38 (m, 1H, H-6), 5.53 (m, 1H, -CH = CH_2), 5.60 (d, 1H, J = 4.2 Hz, H-21′), 7.83 (d, 1H, J = 4.2 Hz, H-21″); MS(EI) m/z: 370, 311, 239, 159; HRMS calcd for $C_{24}H_{34}O_3$, 370.250792; found, 370.25084.

(**17**α): ^{1}H NMR (400 MHz) δ 1.03 (s, 3H, H-19), 2.85 (dd, 1H, J = 9.2 and 3.1 Hz, H-17), 3.54 (m, 1H, H-3), 5.14 (2bd, 2H, J = 18.0 and 11.2 Hz, -CH = CH_2), 5.38 (m, 1H, H-6), 5.46 (d, 1H, J = 4.5 Hz, H-21′), 5.87 (m, 1H, -CH = CH_2), 7.77 (d, 1H, J = 4.5 Hz, H-21″).

Extensive decomposition of **10** was observed during purification. The diazoketone **11** was thus prepared from the crude product.

3β-Hydroxy-18-vinyl-21-bromopregn-5-en-20-one 12 via 3β-hydroxy-18-vinyl-20-diazomethylpregn-5-en-20-one (11). Methylketone **3** (197 mg, 0.57 mmol) was transformed as described above into **10**. The usual work up (AcOEt, H_2O, $MgSO_4$) gave a crude product which was immediately dissolved in 2 mL of dry THF. The flask was placed on an ice bath and tosylazide (500 μL, 2.5 mmol, 0.49 g) was added under argon. Sodium hydride (60 mg of a 80% dispersion in mineral oil, 2 mmol) was added at 0 °C and vigorous stirring was continued for 15 min. The reaction was quenched with ice (frothing), washed with water, and dried ($MgSO_4$). The crude product was used without purification for the next step: IR N_{max} 2120 (C = N = N), 1620, 1590 cm^{-1}. The residue was dissolved in 2 mL of acetonitrile. An aqueous solution (500 μL) of KBr (300 mg) and APTS (40 mg) was added. The mixture was stirred for 17 h at room temperature. After evaporation of the solvent and usual workup, a purification by silica gel chromatography (cyclohexane/AcOEt, 3/1) gave **12** (38 mg, 16% from **3**) as a mixture of 17α/17β isomers (70/30 calculated from the NMR spectrum).

12: ^{1}H NMR (300 MHz) 1.03 (s, 2.1H, H-19, 17α isomer), 1.04 (s, 0.9H, H-19, 17β isomer), 2.78 (t, 0.3H, H-17, 17β isomer), 3.33 (dd, 0.7H, J = 8.3 and 2.7 Hz, H-17, 17α isomer), 3.53 (m, 1H, H-3), 3.91 (dd, 1.4H, J = 19.7 Hz, H-21, 17α isomer), 3.97 (dd, 0.6H, J = 18.6 Hz, H-21, 17β isomer), 5.02 (m, 0.6H, -CH = CH_2, 17β isomer), 5.15 (m, 1.4H, -CH = CH_2, 17α isomer), 5.34 (bs, 1H, H-6), 5.25 (m, 0.3H, -CH = CH_2, 17β isomer), 5.85 (m, 0.7H, -CH = CH_2, 17α isomer).

Biological Methods

Materials

11-Deoxy[4-^{14}C]corticosterone (1.85–2.22 GBq/mmol) and [1, 2, 6, 7-^{3}H] corticosterone (2.8–3.9 TBq/mmol) were purchased from NEN (Dupont de Nemours, France) and Amersham (France), respectively. 11-Deoxycorticosterone, corticosterone, and aldosterone were generous gifts from Roussel Uclaf (France). 18-Hydroxycorticosterone was obtained from Sigma (France). 18-Vinylprogesterone was synthesized as previously described.[3] NADPH was obtained from Boehringer (Mannheim). Sodium cholate was purchased from Sigma (France); soybean lecithin from Serva Feinbiochemica (Germany). All solvents and mineral salts were of the highest purity available from Prolabo (France).

Protein purification

Bovine fresh adrenal glands were obtained from a local slaughterhouse (Mantes-la-Jolie). Purification of the cytochrome P-$450_{11\beta}$ from bovine adrenocortical mitochondria (prepared from whole adrenal cortex) was performed as previously described.[7] The active heme content of the final enzymatic preparation (9 nmol/mg) was determined by the difference spectrum at 450 nm of the dithionite reduced CO-bound cytochrome versus the dithionite reduced cytochrome, assuming a molar absorption coefficient of 91 mM^{-1} cm^{-1}.[25]

Adrenodoxin and NADPH–adrenodoxin reductase were also purified from bovine adrenal mitochondria as already described.[7]

Enzyme kinetics

11β- and 18-hydroxylation enzymatic activities of cytochrome P-$450_{11\beta}$ were reconstituted as follows: all steroids were dissolved in ethanol (the final ethanol concentration in the incubation mixture should not exceed 2%). The incubation mixture contained P-$450_{11\beta}$ and the labeled substrate at concentrations described below, with or without inhibitor at different concentrations, adrenodoxin (10 μM), adrenodoxin reductase

(0.4 μM), 50 mM potassium phosphate buffer (pH = 7.4) and soybean lecithin sonicated in the same phosphate buffer for 3 min (2 mg/mL). The samples were pre-incubated at 30 °C for 30 s and the reaction, initiated by the addition of NADPH (400 μM) was allowed to proceed at 30 °C and was stopped by the addition of methanol. For 11β-hydroxylase activity, 11-deoxycorticosterone (including 462 Bq of 11-deoxy[4-^{14}C]corticosterone) was used as substrate (from 1.25 to 20 μM) with a *P*-450$_{11\beta}$ concentration of 40 nM. For 18-hydroxylase activity, corticosterone (including 7.4 kBq of [1, 2, 6, 7-^{3}H] corticosterone) was used as substrate (from 2.5 to 50 μM) with a *P*-450$_{11\beta}$ concentration of 450 nM.

After extraction of the solution with chloroform (2 vol), the organic phase was evaporated under a stream of nitrogen. The steroids were dissolved in 40 μL of ethanol and chromatographed on silica-coated TLC plates (eluent : acetone/toluene, 1/1). In this solvent system, the R_f values for DOC, corticosterone, aldosterone and 18-hydroxycorticosterone were, respectively, 0.72, 0.53, 0.32, and 0.23. The plates were analyzed using an automatic TLC linear analyzer (LB2821 Berthold). The amounts of steroid produced were calculated according to the formula A×B/C, where A is the initial amount of substrate (pmol), B is the total ^{3}H- or ^{14}C-radioactivity of all zones. Therefore, B/C represents the percentage of transformation of initial substrate to products during a fixed incubation time.

In the preliminary assay, the inhibition by 18-VDOC and 18-VP was assayed only for 11β-hydroxylase activity as described above with the following minor changes: *P*-450$_{11\beta}$ (20 nM) was incubated for 1 min with [^{14}C]-DOC 1.25 μM, in the absence or presence of inhibitor, either 18-VP (0.5, 1 or 10 μM) or 18-EP (0.25, 0.5 or 1 μM).

For K_i determinations, all experiments were run in duplicate for 30 s to 2 min for 11β-hydroxylation and for 1–3 min for the 18-hydroxylation, in the presence of varying concentrations of substrate, DOC or corticosterone, and at least three concentrations of inhibitor 18-VDOC (from 0.125 to 1 μM).

Kinetic constants were determined from linear regression analysis, after double reciprocal transformation of the experimental data. All the data are the average of at least two independent determinations. Values are the mean ± SD from two to four independent experiments.

Acknowledgements

The authors thank R. Azerad for providing the *Aspergillus niger* strain (ATCC 9142) and R. Lett for fruitful discussions.

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