Steroid substrate-induced epimerase mechanism in the active site of the human 11β-hydroxysteroid dehydrogenase type 1

Olivier Hennebert[a], Matthieu Montes[b], Alain Favre-Reguillon[c], Henry Chermette[d], Clotilde Ferroud[c] and Robert Morfin[a*]

Laboratoires de Biologie[a], Bioinformatique[b] EA-3199, and Chimie organique[c] UMR-7084 Conservatoire National des Arts et Métiers, 2 rue Conté, 75003 Paris, France, and CNRS[d] UMR-5180 Sciences Analytiques, Chimie Physique Théorique, Université de Lyon 1, Bât 210, 43 Boulevard du 11 novembre 1918, 69622 Villeurbanne Cedex, France

*Address correspondence to: R.M., (morfin@cnam.fr)

ABSTRACT

Cytochrome P4507B1 7α-hydroxylates dehydroepiandrosterone (DHEA), epiandrosterone (EpiA) and 5α-androstane-3β,17β-diol (Adiol). 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) interconverts 7α- and 7β- forms. Whether the inter-conversion proceeds through oxido-reductive steps or epimerase activity is investigated. Experiments using ^{3}H-labeled 7β-hydroxy-DHEA, 7β-hydroxy-EpiA and 7β-hydroxy-Adiol show the ^{3}H-label to accumulate in 7-oxo-DHEA trap but neither in 7-oxo-EpiA nor 7-oxo-Adiol traps. Computed models of 7-oxygenated steroids dock in the active site of 11β-HSD1 either in a flipped or turned form relative to cortisone and cortisol. 7-Oxo-steroid reduction in 7α- or 7β-hydroxylated derivatives results from either turned or flipped forms. 11β-HSD1 incubation in $H_2{}^{18}O$ medium with each 7-hydroxysteroid did not incorporate ^{18}O in 7-hydroxylated derivatives of EpiA and Adiol independently of the cofactor used. Thus oxido-reductive steps apply for the interconversion of 7α- and 7β-hydroxy-DHEA through 7-oxo-DHEA. Epimerisation may proceed on the 7-hydroxylated derivatives of EpiA and Adiol through a mechanism involving the cofactor and Ser_{170}.

INTRODUCTION

The 7α-hydroxylation of circulating dehydroepiandrosterone (DHEA), epiandrosterone (EpiA) and 5α-androstane-3β,17β-diol (Adiol) is catalyzed in animals and humans by the cytochrome P450 7B1 (CYP7B1)[1,2,3]. Thus, the 7α-hydroxylated derivatives of these steroids are found in human blood and urines[4,5]. Circulating 7α-hydroxy-DHEA is accompanied by almost equivalent quantities of 7β-hydroxy-DHEA[6,7]. Origin of 7β-hydroxy-DHEA and 7β-hydroxy-EpiA was investigated in several human models[8,9]. Use of a yeast-expressed recombinant human 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) helped to demonstrate that 7β-hydroxy-DHEA derived from 7α-hydroxy-DHEA through a 7-oxo-DHEA intermediate[10,11] (**Fig. 1**). Thus, the human 11β-HSD1 catalyzed the interconversion of 7α- and 7β-hydroxy-DHEA through its oxido-reductive activity on 7-hydroxylated DHEA substrates. Whether this occurred with the 7-hydroxylated derivatives of EpiA and Adiol was also investigated. Indeed inter-conversion of the 7α- and 7β-hydroxysteroids was obtained by use of human tissue preparations[8,9] and recombinant human 11β-HSD1[12,13]. Nevertheless, no production of a putative 7-oxo intermediate could be detected and it was hypothesized that the human 11β-HSD1 would act as an epimerase on the 7α- and 7β-hydroxylated derivatives of EpiA and Adiol. In contrast, when either 7-oxo-EpiA[12] or 7-oxo-Adiol[13] were used as substrates for the 11β-HSD1 in the presence of NADPH, reduction into 7α- and 7β-hydroxy derivatives was obtained (**Fig. 1**).

In this work, our aims were to assess the 11β-HSD1-mediated interconversion of 7α-hydroxy-EpiA and 7α-hydroxy-Adiol into their respective 7β-hydroxylated derivatives, and to ascertain whether traces of 7-oxo intermediaries were produced. To these ends, we used the recombinant human 11β-HSD1 and radio-labelled steroid substrates through experiments where the non radioactive 7-oxo-steroids were used as traps for their putative production by

the enzyme. In order to asses the mechanism taking place at the 11β-HSD1 active site, models of each 7-oxygenated steroid were computed and docked in the available crystal structure of human 11β-HSD1. This led to mechanistic assessment of the epimerisation mechanism.

RESULTS

Incubation of [^{3}H]-7β-hydroxy-DHEA, 7β-hydroxy-EpiA and 7β-hydroxy-Adiol in presence of respective 7-oxo-steroid traps

Absence of the enzyme or use of a boiled preparation resulted in no metabolism. No 11β-HSD1-mediated conversion of the substrates was found when cofactor was absent. $NADP^+$ supplementation led to the recovery of 38.8% of the radioactive label in the 7-oxo-DHEA fraction (**Table 1**). This proves that our experimental approach assesses the $NADP^+$-dependent oxidation of 7β-hydroxy-DHEA to 7-oxo-DHEA catalyzed by the recombinant human 11β-HSD1, and that NADPH is not generated in sufficient quantities from $NADP^+$ for catalysis of 7α-hydroxy-DHEA production. In contrast, no radioactivity deriving from the [^{3}H]-5α-reduced substrates was detected in 7-oxo-EpiA and 7-oxo-Adiol traps (**Table 1**). In [^{3}H]-7β-hydroxy-EpiA incubations, a portion of the radioactive label was recovered in the 7α-hydroxy-EpiA fraction after NADPH (1.3%) and $NADP^+$ (1.0%) supplementation. In [^{3}H]-7β-hydroxy-Adiol incubations, a portion of the radioactive label was recovered in the 7α-hydroxy-Adiol fraction after NADPH (7.0%) and $NADP^+$ (1.0%) supplementation. This indicates that either $NADP^+$ or NADPH was necessary for the recombinant human 11β-HSD1 to carry out the interconversion of both 5α-reduced 7β-hydroxysteroids to 7α-hydroxylated epimers. Absence of label in 7-oxo-EpiA and 7-oxo-Adiol suggests that the enzyme does not use this intermediary oxidation step.

Steroid structure examination and docking within 11β-HSD1 active site

Data computed within Kohn-Sham methodology (**supplementary mol.2 data**) helped modelling cortisone, cortisol and the six 7-hydroxysteroids and three 7-oxosteroids. Consideration of steroids bearing oxygen at the C_7 positions and comparison relative to cortisol and cortisone lead to the following: the steroid may be positioned as cortisol or either flipped or turned (**Fig. 2**). The 7-oxo groups of all flipped or turned 7-oxosteroids could match correctly with the 11-oxo group of cortisone. In 7α-hydroxysteroids, the axial 7α- position could possibly match with the axial 11β-hydroxyl of cortisol only after a flip of the molecule. This was not the case with the equatorial 7β- position which stood opposite to the axial 11β-hydroxyl when either flipped or flipped and turned. The turned molecule would be the only structure bringing the 7β-hydroxyl into the vicinity of the 11β-hydroxyl. It was also noticed that the formulae of Δ_5 steroids did not superpose exactly with those of 5α-reduced steroids. These comparisons led us to examine the fitting of each steroid relative to Tyr_{183} and Ser_{170} in the active site of the cofactor-supplemented enzyme. Cortisone positioned in the NADPH-fortified 11β-HSD1 active site proximal to the cofactor and to Tyr_{183} with minimum energy required (**Table 2**). No other cortisone position would dock properly within the site. This positioning results in cortisol production from cortisone reduction (**Fig. 3**). Once either turned or flipped, all 7-oxo-steroids were positioned in the site with the 7-oxo proximal to NADPH and to Tyr_{183}. Nevertheless, the distance between 7-oxo and Tyr_{183} was larger in 7-oxo-EpiA and 7-oxo-Adiol than in cortisone and 7-oxo-DHEA (**Table 2**). This structure-related displacement brought the Ser_{170} closer to the 7-oxo group. Thus NADPH-dependent reduction occurred on either turned or flipped 7-oxo-steroids, and led to the production of 7β- and 7α-reduced derivatives, respectively (**Fig. 4**). This positioning structural approach permits assessment of the events taking place in the active site. It indicates that production of 7α- and 7β-reduced forms may result from two different docking positions of the 7-oxo-steroid

substrates in the active site. In contrast, with 5α-reduced 7α-hydroxysteroids, the turned structures met with minimum energy requirements and the 7α-hydroxyl was positioned closer to Ser_{170} than Tyr_{183} (**Table 2**). With 5α-reduced 7β-hydroxysteroids, the flipped formulae met with minimum energy requirements and the 7β-hydroxyl positioned was in close vicinity to Ser_{170} while Tyr_{183} became more distal (**Table 2**). Differences in positioning of either 7α-hydroxy-EpiA and 7α-hydroxy-Adiol or 7β-hydroxy-Adiol and 7β-hydroxy-Adiol led us to question the absence of 7-oxo intermediate production and the interconversion process taking place in the presence of either NADPH or $NADP^+$. The possibility of an epimerisation process taking place led to two hypotheses: i) once docked and oxidized, the turned 7α-hydroxy-EpiA carbanion could react with H_2O and form an unstable ketone-hydrate leading to 7-oxo-EpiA immediately reduced to 7β-hydroxy-EpiA; ii) The turned 7α-hydroxy-EpiA carbanion could react with the proximal Ser_{170}, to form a stable hemi-ketal reduced then by NADPH in 7β-hydroxy-EpiA (**Fig. 5**). These mechanisms could apply to 7β-hydroxy-EpiA substrate after docking in the flipped position, and to 7α- and 7β-hydroxy-Adiol. Test of the first hypothesis required the use of $H_2{}^{18}O$.

Incubation of 7-oxygenated steroids in $H_2{}^{18}O$-reconstituted medium

We lyophilized 1 mg portions of the recombinant 11β-HSD1 in the presence of either $NADP^+$ or NADPH, and tested their activity before carrying out incubations in the presence of $H_2{}^{18}O$. As expected, no ^{18}O enrichment was detected in 7α-hydroxy-DHEA or 7β-hydroxy-DHEA after incubation of 7-oxo-DHEA in the presence of NADPH. Both 7α-hydroxy-DHEA and 7β-hydroxy-DHEA were oxidized into 7-oxo-DHEA in $NADP^+$-fortified incubations without ^{18}O incorporation. As expected from the mechanism proposed above, the reduction of 7-oxo-EpiA by NADPH produced both 7α-hydroxy-EpiA and 7β-hydroxy-EpiA containing no ^{18}O.

In the presence of $NADP^+$ as well as NADPH, 7α-hydroxy-EpiA and 7α-hydroxy-Adiol were converted to 7β-hydroxy-EpiA and 7β-hydroxy-Adiol containing no ^{18}O (**Table 3**). The ^{18}O-content of 7α-hydroxy-EpiA obtained from 7β-hydroxy-EpiA could not be measured due to the very low transformation yields. Thus, the 11β-HSD1 converts 7-oxo-steroids into both 7α- and 7β-epimers through a NADP(H)-dependent process which does not involve H_2O from the medium.

DISCUSSION

We have already shown that the recombinant human 11β-HSD1 is an active NADP(H)-dependent oxido-reductase interconverting cortisol and cortisone in addition to 7-oxo-DHEA and 7α-hydroxy-DHEA and 7β-hydroxy-DHEA[10]. This finding was extended to the native enzyme present in human skin tissue homogenates[11]. In the presence of NADPH, the recombinant human 11β-HSD1 reduced 7-oxo-DHEA into 7β-hydroxy-DHEA in preference to 7α-hydroxy-DHEA as indicated by a higher V_{max}/K_M ratio (**Table 2**)[10]. This preference for the 7β epimer was maintained in the $NADP^+$-dependent oxidation of 7β-hydroxy-DHEA and 7α-hydroxy-DHEA. Other assays with 7α- and 7β-hydroxylated derivatives of EpiA[12] and Adiol[13] gave results conflicting with those obtained with DHEA derivatives. Thus, the NADPH-dependent 7α-reduction of 7-oxo-epiA and 7-oxo-Adiol was preferred over the 7β-reduction by the enzyme through V_{max}/K_M values as shown in **Table 2**. In contrast to DHEA, the $NADP^+$-dependent oxidation of 7α- and 7β-hydroxy-EpiA and 7α- and 7β-hydroxy-Adiol did not result in 7-oxo derivative productions. Instead, inter-conversion of the 7α- and 7β-hydroxylated forms was observed, with a preference for the production of the 7β-hydroxylated epimers[12,13]. From these findings on 5α-reduced steroids, the mechanism driven by the recombinant human 11β-HSD1 remained open. Did the interconversion proceed

through oxido-reductive steps or through a direct epimeric transformation? Oxido-reduction should process through ketone formation. Our previous kinetic studies indicated through apparent K_Ms and V_{max} determinations that the conversion of 7α- into 7β- derivatives was preferred, as well as the reduction of 7-oxo into 7α- derivatives. On this basis, and because a very rapid oxido-reduction process could deplete the medium of a putative 7-oxo intermediate, we chose to use the 7β-hydroxysteroids as substrates for the recombinant human 11β-HSD1 with and without $NADP^+$ or NADPH supplementations. Search for a putative 7-oxo intermediate was assessed with use of the [^{3}H]-labelled 7β-hydroxysteroid together with the relevant non-radioactive 7-oxo-steroid for radioactivity trapping. In order to validate this model we used the known $NADP^+$-dependent 11β-HSD1-catalyzed oxidation of 7β-hydroxy-DHEA into 7-oxo-DHEA. Our evidence for [^{3}H]-label in the 7-oxo-DHEA originating from [^{3}H]-7β-hydroxy-DHEA indicated that the model was functional. No label occurred in the expected 7α-hydroxy-DHEA fraction with use of $NADP^+$ supplementation. Use of NADPH supplementation did not yield any [^{3}H]-7β-hydroxy-DHEA transformation product, thus indicating that the $NADP^+$-dependent oxidation step was a prerequisite for a further transformation. The validated model used with either [^{3}H]-7β-hydroxy-EpiA or [^{3}H]-7β-hydroxy-Adiol should yield [^{3}H]-label accumulation in the 7-oxo-EpiA and 7-oxo-Adiol traps, respectively. No label was found in the two 7-oxo-steroid traps, but small amounts of the radioactivity occurred at the level of 7α-hydroxylated derivatives. These formations did not occur in the absence of cofactor and were more dependent on NADPH than $NADP^+$. Two conclusions may be drawn from these findings. First, the recombinant human 11β-HSD1 does not carry out the $NADP^+$-dependent oxidation of 7β-hydroxy-EpiA and 7β-hydroxy-Adiol to 7-oxo-EpiA. Second, the enzyme carries out epimerisation of the 5α-reduced 7β-hydroxysteroids independently of the cofactor oxidation state. Nevertheless, presence of the

cofactor was necessary for such epimerisation. It should be noted that the NADPH dependent reduction of 7-oxo-EpiA and 7-oxo-Adiol was described with a preferred production of the 7α-hydroxylated derivatives[12,13]. It should also be noted that these findings obtained with the recombinant human 11β-HSD1 correlated well with other works using the native enzyme in human liver and intestinal preparations[8,9]. Interpretation of the present findings deserved assessment through close examination and comparison of the steroid structures relative to the enzyme activity. Cortisone and all 7-oxo steroids are substrates for the NADPH-dependent reduction by the recombinant 11β-HSD1. One may expect for the 11-oxo and 7-oxo groups an equivalent positioning proximal to NADPH within the enzyme active site. Indeed, either a flip or a turn of the 7-oxo-steroid structure does align the 7 and 11 sp_2 carbons of 7-oxo steroids and cortisone, respectively, as previously suggested[14,13] and as shown in **Fig. 2**. These model structures were computed within Kohn-Sham methodology (**see supplementary mol.2 data**) and their fitting into the 11β-HSD1 active site could be assessed. Thus, flipped formulae of 7-oxo and 7β-hydroxysteroids were docked in the site, while turned formulae of 7-oxo-steroids and 7α-hydroxysteroids docked as well in the site (**Fig. 3, Fig. 4**). When applied to 7-oxo-steroids, these two docking forms leads to the mechanism depicted in **Fig. 4** where 7-oxosteroids are reduced in the α or β position when docked in a turned or flipped position, respectively. This proposal is made attractive by the explanation it may provide for the stereospecific reduction of 7-oxo-cholesterol to 7β-hydroxy-cholesterol, exclusively[15,16,17]. Our model make it apparent that due to its large side chain, 7-oxo-cholesterol docking into the 11β-HSD1 active site may occur in the flipped position only, thereby leading to its reduction to 7β-hydroxy-cholesterol only.

Tyr_{183} is located proximal to the bound cofactor within the human 11β-HSD1 active site and is responsible for the reducing activity[18,19]. This is shown in **Fig. 4** and **Table 2** with measured distance between the C_7-borne oxygen and Tyr_{183}, Ser_{170} and the hydrogen donor C_4

of nicotinamide. The affinity of each 7-oxo-steroid for the active site (K_M) is not modified with the flipped or turned positions. Activity of the enzyme (V_{max}/K_M) may be related to short distances between the nicotinamide C_4-borne active hydrogen and the 7-oxo group, more so than distances from Tyr_{183} and Ser_{170}. Nevertheless, once produced, both 7α- and 7β-hydroxylated 5α-reduced steroids fit closer to Ser_{170} than to Tyr_{183}. In 5α-reduced derivatives (7α-hydroxy-EpiA, 7α-hydroxy-Adiol, 7β-hydroxy-EpiA, 7β-hydroxy-Adiol), absence of the 5-ene double bond and replacement of the sp_2 C_5 and C_6 by sp_3 structures induce major changes in the steroid structural configuration and alignment with cortisol. Our results indicate that occupation of the cofactor site was necessary for enzymatic action, but that neither $NADP^+$ nor NADPH modified the formation of 7α-and 7β-epimeric derivatives, and that no 7-oxo intermediate occurred in the trap. These observations led to question the oxido-reductive process. Since docking of 5α-reduced 7-hydroxysteroids brought the 7-hydroxyl in the close proximity of Ser_{170}, we hypothesize that cofactor-driven epimerisation could proceed either with use of a molecule of water or through a more stable hemi-ketal with Ser_{170} as depicted in **Fig. 5.** Validation of the first hypothesis required experimental test where $H_2{}^{18}O$ replaced H_2O contained in the enzyme site. Natural water was removed from cofactor loaded 11β-HSD1 by lyophilisation. Incubations with steroid substrates were carried out then in a medium reconstituted with $H_2{}^{18}O$ and no ^{18}O enrichment occurred in the "epimerized" products. These results imply that the second hypothesis involving Ser_{170} as responsible for the epimerisation process is held to be valid. This proposal relates well with the 7-oxygen distances from Ser_{170} and cofactor (**Table 2**) as well as with the measured K_M and V_{max}/K_M ratios (**Table 3**).

METHODS

Steroids. DHEA, EpiA and Adiol were obtained from Sigma-Aldrich. 7-Oxo-DHEA was from Steraloids. The 7-oxo-Adiol was donated by Sir E.R.H. Jones (Oxford, UK). Custom chemical synthesis by Roowin S.A. (Romainville, France) provided mg quantities of chemically pure 7α-hydroxy-DHEA, 7α-hydroxy-EpiA, 7β-hydroxy-DHEA, 7β-hydroxy-EpiA and 7-oxo-DHEA-17-ethylene-ketal. The latter steroid was reduced with hydrogen gas in the presence of Pd into 3β-hydroxy-5α-androstane-17-ethylene-ketal. After HCl treatment, the 3β-hydroxy-5α-androstane-7,17-dione (7-oxo-EpiA) was obtained. Both 7α-hydroxy-Adiol and 7β-hydroxy-Adiol were obtained after $NaBH_4$ (Sigma-Aldrich) reduction of 7α-hydroxy-EpiA and 7β-hydroxy-EpiA, respectively. The produced steroids were all purified by preparative HPLC and their identity and purity were assessed after GC/MS with evidence for a single peak containing the relevant molecular ion. [1,2,6,7-^{3}H] DHEA (74 Ci/mmol) and [1,2,4,5,6,7-^{3}H] dihydrotestosterone (110 Ci/mmol) were purchased from the New England Nuclear Corporation and were used for the production of [1,2,6-^{3}H] 7β-hydroxy-DHEA (61.4 Ci/mmol) and [1,2,4,5,6-^{3}H] 7β-hydroxy-EpiA (91.3 Ci/mmol) as previously described[20]. [1,2,4,5,6-^{3}H] 7β-Hydroxy-Adiol was obtained then after reduction of [1,2,4,5,6-^{3}H] 7β-hydroxy-EpiA by $AlLiH_4$. $H_2{}^{18}O$ (97% ^{18}O) was from Euriso-top (Saint Aubin, France). All solvents were of the reagent grade and obtained from Merck.

Steroid structure studies. The density functional theory within the Khon-Sham methodology[21] was used to model cortisol, cortisone and the 7-oxygenated steroids. The generalized gradient approximation was employed within Perdew-Burke-Ernzerhof exchange-correlation functional formulation[22]. Calculations were performed with use of the ADF06 program package[23,24]. The basis set was of triple-zeta quality + polarization with small frozen cores. Finally, the integration grid parameter, setting the numerical integration accuracy, was fixed to 5 (**supplementary mol.2 data**).

The recombinant human 11β-HSD1. The recombinant human 11β-HSD1 was obtained in microsomes of the *Sacharomyces cervisiae* strain W303-1B (Mat*a; leu2-3,112, his3-11,15; ade2-1; trp1-1; ura3-1; can*^{R}*; cyr*$^+$) transformed with the V60-HSD1 construct as previously described[10].

Bioinformatic analysis. The crystal structure of the human 11β-HSD1 with NADP was retrieved from the Protein Data Bank[25] (PDB code 1ILT). All heteroatoms were removed from the file and hydrogen atoms were added using the QuacPac program from Openeye Scientific Software. The docking experiments of the different steroids were performed using the flexible docking program Surflex[26]. The binding site for docking experiments was defined around the co-crystallized Adamantane Sulfone inhibitor. The 10 top scoring poses after docking were visually inspected in order to propose a binding mode for each compound. Selected images were generated by PyMol software.

Incubation protocols. Two different protocols were used, one for incubation with radiolabeled steroids, the second for incubation in 97% $H_2{}^{18}O$. i) Each 7β-hydroxysteroid (0.5 µmol) spiked with 20,000 dpm of the relevant [^{3}H]-steroid was mixed with 0.5 µmol of the relevant 7-oxo-steroid and dried under vacuum at the bottom of 10 mL glass tubes prior to addition of 66.7 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.4) containing 1 mM EDTA. The mixture was pre-incubated at 28°C for 5 min. The cofactor (either 1 µM NADP+ or NADPH in 500 µL buffer) was added to the steroid substrates. Incubations commenced at the addition of a suspension of yeast microsomes containing the 11β-HSD1 (63.8 µL, 1 mg protein) and addition of buffer to the 1 mL mark. The process continued in a shaking water bath at 28°C for 30 min. ii) Tubes containing aliquot portions of the buffered enzyme suspension (1 mL, 1

mg protein) were added with either NADP+ or NADPH and lyophilized. In order to avoid the putative reinsertion of environmental water, a $H_2{}^{18}O$ spray was produced at the moment of vacuum breaking. The medium was reconstituted with 1 mL $H_2{}^{18}O$ and the mixture was pre-incubated at 28°C for 5 min. Incubation commenced at the addition of the steroid substrate in 10µL ethanol. The process continued in a shaking water bath at 28°C for 30 min. All incubations ceased at the addition of 0.5 mL acetone followed by 2 mL ethyl acetate. Extraction of the steroids was carried out with 2 mL ethyl acetate and was repeated three times.

Steroid separation and analysis. The radio-steroids extracted were analyzed by TLC. The separation of 7α-hydroxy-DHEA (R_f 0.25) from 7β-hydroxy-DHEA (R_f 0.38) and 7-oxo-DHEA (R_f 0.55) on silica 60-coated glass plates (Merck, Darmstadt, Germany) required one development in ethyl acetate. Aluminium oxide-coated polyester plates from Machery-Nagel were developed twice in ethyl acetate for the separation of 7α-hydroxy-EpiA (R_f 0.47) from 7β-hydroxy-EpiA (R_f 0.55) and 7-oxo-EpiA (R_f 0.79). The same system and plates were used for the separation of 7α-hydroxy-Adiol (R_f 0.35) from 7β-hydroxy-Adiol (R_f 0.47) and 7-oxo-Adiol (R_f 0.55). The steroid containing zones were scrapped off the plate and counted. The counting of each sample was carried out for 30 min in order to accumulate sufficient counts for precise dpm measurements. Steroids recovered after incubations in the presence of $H_2{}^{18}O$ were transformed into TMS derivatives and analyzed by GC/MS as previously reported[8].

*Note: Supplementary data (**supplementary mol.2 data**) are freely available on*

http://bioinfo.cnam.fr/bioinfo/structuresteroidsinmol2/

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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Table 1: Incubation of 0.5 µM 7β-hydroxy-DHEA (**a**) 7β-hydroxy-EpiA (**b**) and 7β-hydroxy-Adiol (**c**) spiked with [1,2,6-^{3}H]-7β-hydroxy-DHEA (20,000 dpm), [1,2,4,5,6-^{3}H]-7β-hydroxy-EPIA (20,000 dpm) and [1,2,4,5,6-^{3}H]-7β-hydroxy-Adiol (20,000 dpm) in the presence of 0.5 µM 7-oxo-DHEA, 7-oxo-EPIA and 7-oxo-Adiol trap, respectively. Steroids from (**a**) were extracted after incubation and separated by TLC on silica before recovery (47-70 %) and counting. Steroids from (**b**) and (**c**) were extracted after incubation and separated by TLC on aluminium oxide before recovery (46-55 %) and counting. The background (24 dpm) was subtracted from each count. Data (dpm ± SEM) are means resulting from 3 separate experiments.

(a) Cofactor	**Incubation conditions**	**[^{3}H]-7α-hydroxy-DHEA fraction**	**[^{3}H]-7β-hydroxy-DHEA substrate**	**[^{3}H]-7-oxo-DHEA fraction**
NADPH (1 µM)	11β-HSD1	0	9,561 ± 1,587	0
	Boiled 11β-HSD1	0	9,458 ± 1,095	0
	No 11β-HSD1	0	11,446 ± 411	0
$NADP^+$ (1 µM)	11β-HSD1	0	9,050 ± 539	5,760 ± 527
	Boiled 11β-HSD1	0	11,085 ± 714	0
	No 11β-HSD1	0	10,633 ± 455	0
None	11β-HSD1	0	10,789 ± 269	0
	Boiled 11β-HSD1	0	12,533 ± 125	0
	No 11β-HSD1	0	10,790 ± 269	0
(b) Cofactor		**[^{3}H]-7α-hydroxy-EpiA fraction**	**[^{3}H]-7β-hydroxy-EpiA substrate**	**[^{3}H]-7-oxo-DHEA fraction**
NADPH (1 µM)	11β-HSD1	138 ± 8	10,119 ± 435	0
	Boiled 11β-HSD1	0	9,865 ± 172	0
	No 11β-HSD1	0	9,807 ± 246	0
$NADP^+$ (1 µM)	11β-HSD1	100 ± 14	10,048 ± 352	0
	Boiled 11β-HSD1	0	10,147 ± 192	0
	No 11β-HSD1	0	10,011 ± 285	0
None	11β-HSD1	0	10,803 ± 97	0
	Boiled 11β-HSD1	0	9,705 ± 212	0
	No 11β-HSD1	0	9,364 ± 198	0
(c) Cofactor		**[^{3}H]-7α-hydroxy-Adiol fraction**	**[^{3}H]-7β-hydroxy-Adiol substrate**	**[^{3}H]-7-oxo-Adiol fraction**
NADPH (1 µM)	11β-HSD1	746 ± 127	10,562 ± 163	0
	Boiled 11β-HSD1	0	9,699 ± 227	0
	No 11β-HSD1	0	9,664 ± 119	0
$NADP^+$ (1 µM)	11β-HSD1	164 ± 6	9,706 ± 138	0
	Boiled 11β-HSD1	0	9,810 ± 279	0
	No 11β-HSD1	0	9,650 ± 140	0
None	11β-HSD1	0	9,706 ± 366	0
	Boiled 11β-HSD1	0	9,987 ± 271	0
	No 11β-HSD1	0	9,864 ± 234	0

Table 2: 11β-HSD1-mediated reduction of cortisone and 7-oxo-steroids. Kinetic parameters were previously reported [10,12,13]. Distances between the oxygen borne at the steroid 7-position and Tyr_{183}, Ser_{170} hydroxyl groups and active hydrogen-bearing C_4 of nicotinamide (NA) in NADPH are given. The distances in brackets are those measured in each product deriving from the reduction process. Each steroid model was positioned and docked at minimum energy settings into the computerized crystal structure of the human 11β-HSD1. Distances were obtained through use of PyMol software.

Substrate (position)	**Product (position)**	**K_M (μM)**	**V_{max}/K_M**	**Tyr_{183} (Å)**	**Ser_{170} (Å)**	**NA (Å)**
Cortisone	*Cortisol*	2.8	0.4	2.85 *[1.81]*	2.10 *[2.68]*	1.90 *[2.30]*
7-Oxo-DHEA (turned)	*7α-Hydroxy-DHEA* (turned)	1.15	0.5	5.00 *[3.16]*	5.37 *[2.25]*	2.45 *[2.34]*
7-Oxo-DHEA (flipped)	*7β-Hydroxy-DHEA* (flipped)	1.13	7.4	2.31 *[3.81]*	1.91 *[5.81]*	2.29 *[4.97]*
7-Oxo-EpiA (turned)	*7α-Hydroxy-EpiA* (turned)	0.57	23.7	4.40 *[3.91]*	1.99 *[1.77]*	1.91 *[1.93]*
7-Oxo-EpiA (flipped)	*7β-Hydroxy-EpiA* (flipped)	0.52	5.8	2.34 *[2.06]*	3.42 *[1.98]*	2.29 *[2.57]*
7-Oxo-Adiol (turned)	*7α-Hydroxy-Adiol* (turned)	5.1	3.43	5.60 *[2.94]*	4.93 *[2.04]*	1.84 *[1.96]*
7-Oxo-Adiol (flipped)	*7β-Hydroxy-Adiol* (flipped)	6.8	0.22	3.11 *[1.81]*	3.20 *[1.96]*	2.11 *[2.42]*

Table 3: 11β-HSD1-mediated 7-hydroxysteroid epimerisation: ^{18}O incorporation from $H_2{}^{18}O$. Lyophilized 11β-HSD1 (1 mg) containing either NADPH or $NADP^+$ was reconstituted in 97% $H_2{}^{18}O$ prior to incubation with selected 7-oxygenated steroids. Substrates and metabolites were recovered, transformed into TMS derivatives and separated and analyzed by gas chromatography-mass spectrometry (GLC/MS). ^{18}O content of metabolites (M+2) was measured in M^+ and M^+-15 ions after comparison with M+2 levels in the same ions from authentic standards. * Conversion rate too low for measurement.

Conversion	Yield (%) of metabolite	^{18}O content in metabolite	K_M (μM)	V_{max}/K_M
7-oxo-DHEA to 7α-hydroxy-DHEA	49	0	1.15	0.5
7-oxo-DHEA to 7β-hydroxy-DHEA	30	0	1.13	7.4
7α-hydroxy-DHEA to 7-oxo-DHEA	66	0	70.0	0.2
7β-hydroxy-DHEA to 7-oxo-DHEA	90	0	9.5	1.9
7-oxo-EpiA to 7α-hydroxy-EpiA	70	0	0.57	23.7
7-oxo-EpiA to 7β-hydroxy-EpiA	30	0	0.52	5.8
7α-hydroxy-EpiA to 7β-hydroxy-EpiA	90	0	8.1	0.1
7β-hydroxy-EpiA to 7α-hydroxy-EpiA	0.1	0	*	*
7-oxo-Adiol to 7α-hydroxy-Adiol	41	0	5.1	3.43
7-oxo-Adiol to 7β-hydroxy-Adiol	43	0	6.8	0.22
7α-hydroxy-Adiol to 7β-hydroxy-Adiol	55	0	1.2	2
7β-hydroxy-Adiol to 7α-hydroxy-Adiol	40	0	21.0	0.5

Figure legends

Figure 1 11β-HSD1 mediated oxido-reduction and interconversion of the 7α- and 7β-hydroxylated derivatives of **a**: dehydroepiandrosterone (DHEA), **b**: epiandrosterone (EpiA) and **c**: 5α-androstane-3β,17β-diol (Adiol). Apparent V_{max}/K_M ratio values [10,12,13] are indicated for each NADP(H)-dependent reaction step. Absence of product formation is depicted by question marks and concerns the putative 11β-HSD1 mediated direct interconversion process.

Figure 2 Possible positioning relative to cortisone or cortisol for 7-oxygenated steroids in the 11β-HSD1 active site. Structure models were generated from computation within Kohn-Sham methodology (**supplementary mol.2 data**). The twelve 7-oxygenated DHEA derivative positions were selected for this tabular figure. Steroid backbone, oxygen and hydrogen are depicted in blue, red and white, respectively.

Figure 3 Docking of steroid structures within the 11β-HSD1 active site. The crystal structure of the human 11β-HSD1 retrieved from the Protein Data Bank (PDB code 1ILT) was used with QuacPac program from Openeye Scientific Software. Docking of the different steroids was performed using the flexible docking program Surflex. Cortisol (with $NADP^+$) and cortisone (with NADPH) native substrates were positioned in the site relative to Tyr_{183}, Ser_{170} (orange) and nicotinamide from NADP(H) (black) at minimum energy settings. Steroid backbone, oxygen and hydrogen are depicted in blue, red and white, respectively. 7oxo- and 7-hydroxysteroids fitted proximal to the cofactor and Tyr_{183} and Ser_{170} after either a turn or a flip of their structures relative to cortisone and cortisol, respectively. In this tabular figure the flip for 7-oxo-steroids and 7β-hydroxysteroids and the turn of 7α-hydroxysteroids exemplified in Fig. 2 were selected, respectively.

Figure 4 Proposed mechanism generating both 7α- and 7β-hydroxysteroids after 11β-HSD1-catalyzed NADPH-dependent reduction of 7-oxo-steroid precursors. The crystal structure of the human 11β-HSD1 retrieved from the Protein Data Bank (PDB code 1ILT) was used with QuacPac program from Openeye Scientific Software. Docking of the different steroids was performed using the flexible docking program Surflex. The steroid substrates (blue) and products were docked in the site relative to Tyr_{183}, Ser_{170} (orange) and nicotinamide from NADP(H) (black) at minimum energy settings for each flipped or turned structure. Enzymatic reduction of turned structures results in 7α-hydroxylated product while flipped structures result in 7β-hydroxylated products.

Figure 5 Hypotheses for 11β-HSD1-catalyzed epimerisation of 5α-reduced 7-hydroxysteroids. The steroid depicted is 7α-hydroxy-EpiA docked in the enzyme site with the 7α-hydroxyl proximal to Ser_{170} and distal to Tyr_{183}. The first hypothesis involves one molecule of H_2O which provides the –OH necessary for epimerisation to proceed through ketone-hydrate formation and a 7-ketone intermediate. Absence of ^{18}O enrichment after use of $H_2{}^{18}O$ eliminated such hypothesis. The second hypothesis implies reaction of Ser_{170} for hemi-ketal production prior to reduction. This model applies as well to 7α-hydroxy-Adiol and 7β-hydroxy-Adiol. Once produced, the 7β-hydroxy of 7β-hydroxy-EpiA becomes distal to Ser_{170} and this may justify the difficulty for the back reaction to proceed.

Fig. 1

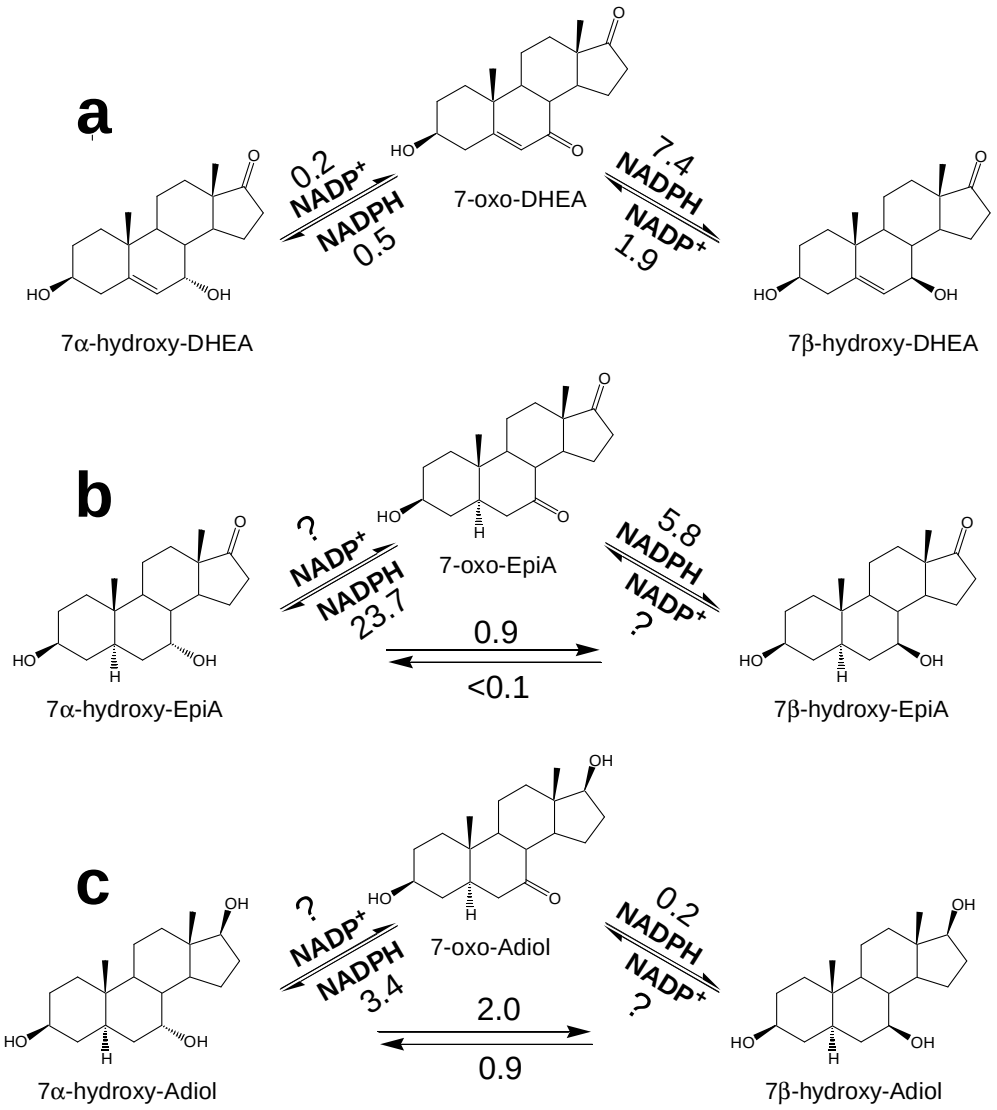
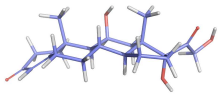
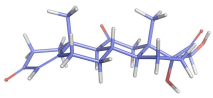
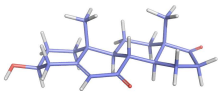
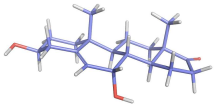


Fig. 2

Cortisol	**Cortisone**	**7-Oxo-DHEA**	**7α-Hydroxy-DHEA**	**7β-Hydroxy-DHEA**
				
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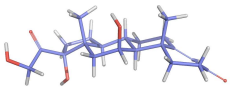	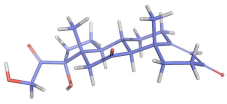	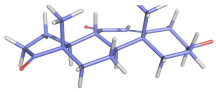	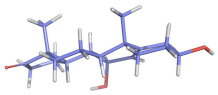	
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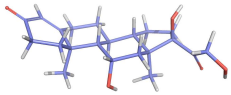	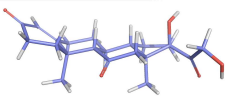	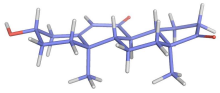	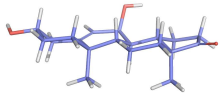	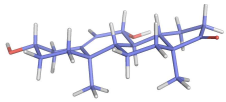
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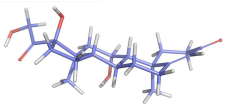
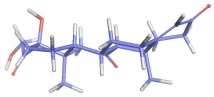
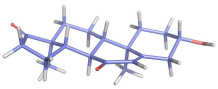
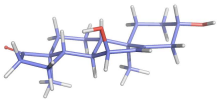
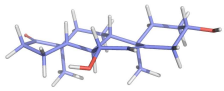
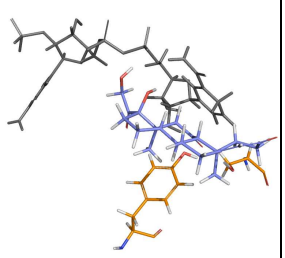
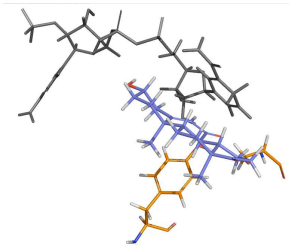
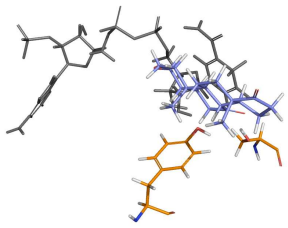
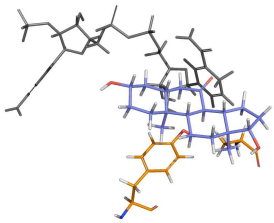
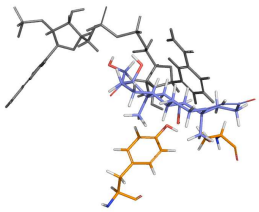
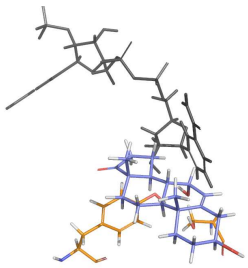
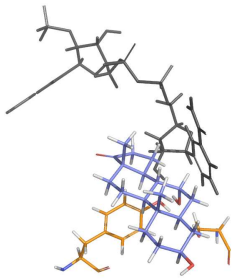
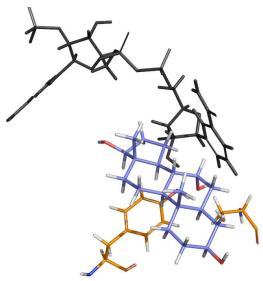
				

Fig. 3

Cortisone	**7-Oxo-DHEA**	**7-Oxo-EpiA**	**7-Oxo-Adiol**
			
Cortisol	**7α-Hydroxy-DHEA**	**7α-Hydroxy-EpiA**	**7α-Hydroxy-Adiol**
			
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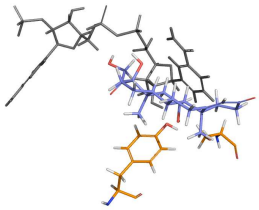	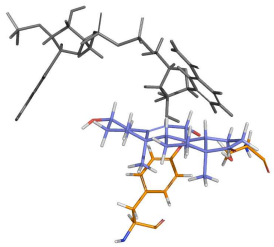	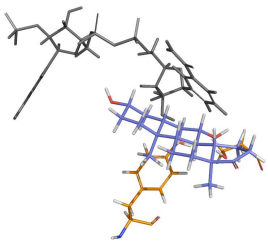	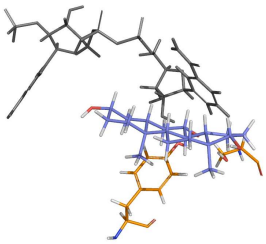

Fig. 4

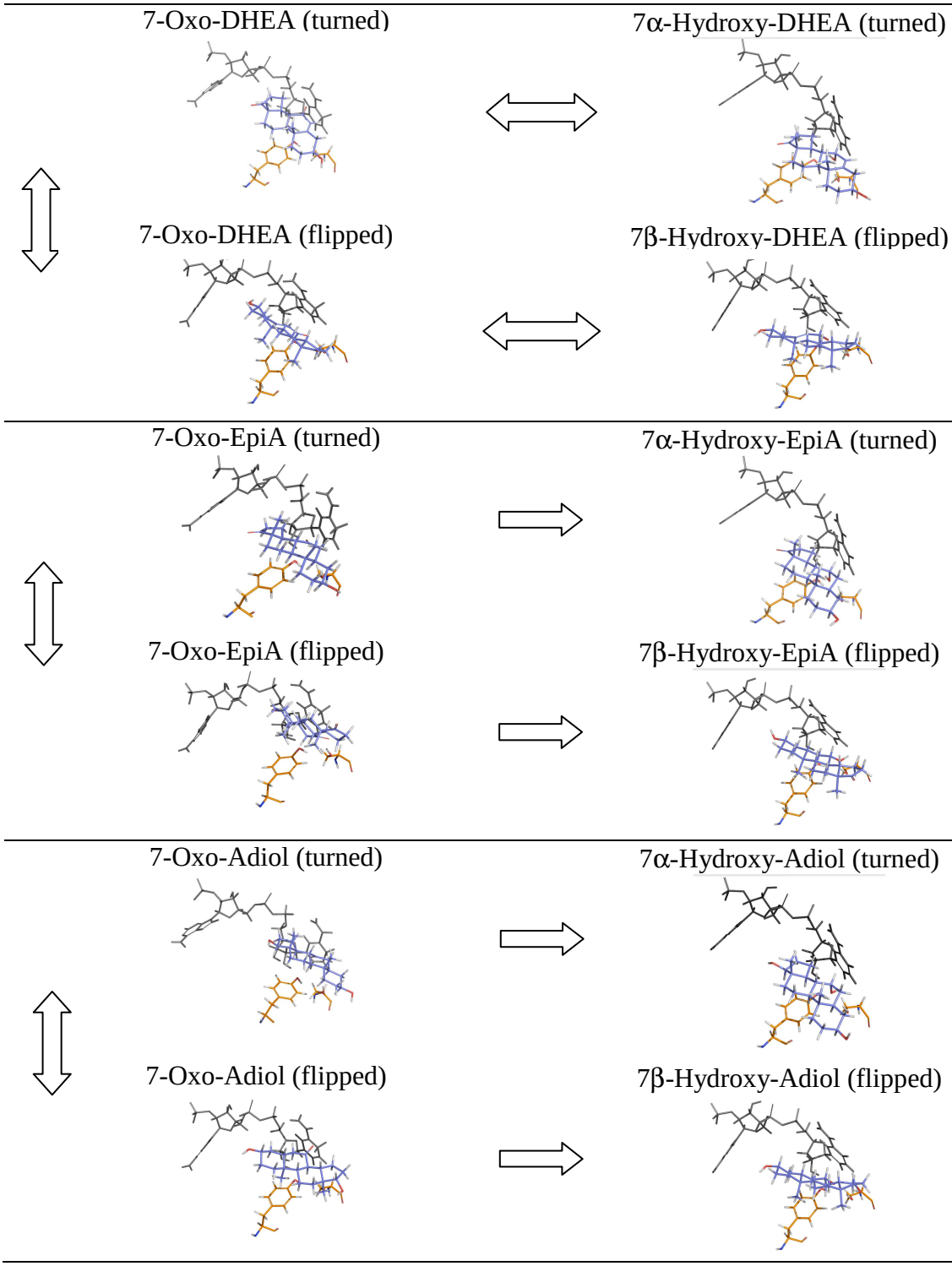

Fig. 5