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The catalytic mechanism of the 3-ketosteroid isomerase of *Digitalis lanata* involves an intramolecular proton transfer and the activity is not associated with the 3β-hydroxysteroid dehydrogenase activity

Nadine Meitinger [a,*], Jennifer Munkert [a], Rodrigo Maia de Pádua [b], José Dias de Souza Filho [c], Harald Maid [d], Walter Bauer [d], Fernão Castro Braga [b], Wolfgang Kreis [a]

[a] Friedrich-Alexander-Universität, Lehrstuhl für Pharmazeutische Biologie, Staudtstr. 5, D-91058 Erlangen, Germany
[b] Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6.627, 31270-901, Brazil
[c] Departmento de Quimica, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6.627, 31270-901, Brazil
[d] Friedrich-Alexander-Universität, Lehrstuhl für Organische Chemie, Henkestrasse 42, D-91054 Erlangen, Germany

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ABSTRACT

The isomerization of the Δ^5-3-ketosteroid isoprogesterone into the Δ^4-3-ketosteroid progesterone has been examined with recombinant 3β-hydroxysteroid dehydrogenase from *Digitalis lanata* (r*Dl*3βHSD), partially purified 3-ketosteroid isomerase from *Digitalis lanata* (*Dl*3KSI) and under non-enzymatic conditions in deuterium oxide (D_2O).

Studies indicate that the isomerization catalyzed by the *Dl*3KSI proceeds without significant isotope exchange between the medium and the steroid and thus involves an intramolecular proton transfer consistent with the mechanism of the bacterial 3-ketosteroid isomerase of *Pseudomonas testosteroni*. For the r*Dl*3βHSD as well as under non-enzymatic conditions deuterium was incorporated from the incubation buffer during isomerization. Together with a comparison of the rate of isomerization under the different conditions, it was demonstrated that r*Dl*3βHSD does not possess 3-ketosteroid isomerase activity.

Introduction

Many natural 3β-hydroxysteroids are Δ^5-olefins. The NAD(P) dependent enzymatic oxidation of these steroids into 3-ketosteroids is assigned to Δ^5-3β-hydroxysteroid dehydrogenases (EC 1.1.1.51) and strictly leads to the formation of Δ^4-3-ketosteroids. The latter reaction is attributed to 3-ketosteroid isomerase (EC 5.3.3.1). These reactions have been extensively investigated in bacteria as well as in mammals. In animals, both reactions are catalyzed by one bifunctional enzyme and several mammalian, membrane bound isoenzymes have been reported, all being members of the 3βHSD gene family.[1] On the other hand, in bacteria, oxidation and isomerization are typically catalyzed by two independent enzymes. For instance, in *Comamonas testosteroni* the soluble enzyme 3β/17β-hydroxysteroid dehydrogenase catalyzes the reversible dehydrogenation/reduction of the β-hydroxy/oxo groups at positions C-3 and C-17.[2] The bacterial 3KSI catalyzes the allylic isomerization of a variety of Δ^5-3-ketosteroids and is one of the most proficient enzymes known with k_{cat}/K_M approaching the diffusion limit.[3] The reaction mechanism of the bacterial 3KSI proceeds via a stereospecific intramolecular proton transfer which has already been demonstrated for the *Comamonas* enzyme using either unlabeled substrates in D_2O or labeled substrates in H_2O.[4–6]

The enzymatic conversion of the 3β-hydroxysteroid pregnenolone into the 3-ketosteroid isoprogesterone is probably involved in cardenolide biosynthesis. Cardenolides are a group of important pharmacologically active natural products.[7] However, compared with the mammalian and bacterial isoenzymes, there is not much information about 3βHSDs and 3KSIs in plants. Only a few 3βHSDs have been isolated from higher plants including the cardenolide producing plants *Digitalis lanata* and *Erysimum crepidifolium* along with the cardenolide-free plant *Arabidopsis thaliana*. In addition, some of these 3βHSD genes have been functionally expressed in *Escherichia coli*.[8–10] The question whether these plant-derived 3βHSDs possess 3KSI activity was addressed several times but it could not be answered unambiguously and

Abbreviations: 3KSI, Δ^5-3-ketosteroid isomerase; 3βHSD, Δ^5-3β-hydroxysteroid dehydrogenase.

* Corresponding author. Tel.: +49 9131 8528241; fax: +49 9131 8528243.
E-mail address: Nadine.meitinger@fau.de (N. Meitinger).

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some conflicting data have been reported.[8,10,11] For 3KSIs of plant origin no sequence information is provided in the public databases. We can only assume that plant 3KSI is not an ortholog of the bacterial 3KSI enzymes because homology searches using bacterial 3KSI sequences were not successful in the *Arabidopsis thaliana* genome or the *Digitalis purpurea* transcriptome.[12] Recently, the first plant 3KSI has, however, been purified from *Digitalis lanata* by our group and the characterization of the purified enzyme revealed that the *D. lanata* 3KSI resembles the bacterial enzymes. *Dl*3KSI was furthermore clearly separated from the *Dl*3βHSD by size exclusion chromatography.[12]

We here intended to demonstrate that the isomerization catalyzed by the *D. lanata* 3KSI involves an intramolecular proton shift to promote isoprogesterone isomerization as had been demonstrated previously for the *Comamonas* enzyme. Moreover, we want to add more evidence to support the assumption that 3KSI is not associated with 3βHSD in *D. lanata*. Therefore, we intended to show that in enzyme-free controls and in assays containing r*Dl*3βHSD isoprogesterone is only isomerized non-enzymatically and consequently, that the progesterone detected after incubation is only formed by chemical isomerization.

Results and discussion

The isomerization of isoprogesterone into progesterone was investigated in deuterium oxide under the following conditions: (a) enzyme catalysis with recombinant 3β-hydroxysteroid dehydrogenase from *Digitalis lanata* (r*Dl*3βHSD) using pregnenolone and NAD^+ as the substrate and cosubstrate, respectively; (b) enzyme catalysis with partially purified 3-ketosteroid isomerase from *D. lanata* (*Dl*3KSI) using isoprogesterone as the substrate; (c) enzyme catalysis with both r*Dl*3βHSD and partially purified *Dl*3KSI using pregnenolone and NAD^+ as the substrate and cosubstrate, respectively; (d) spontaneous isomerization of isoprogesterone in deuterium oxide without enzyme and (e) acid catalyzed isoprogesterone isomerization. Assays were then analyzed by HPLC/DAD/MS. This analysis allows us to quantify how much progesterone was formed in the different assays (UV/vis analysis) as well as to analyze if deuterium was incorporated into progesterone during the isomerization reaction (ESI-LC–MS analysis). The isoprogesterone used here was synthetized from pregnenolone (Sigma–Aldrich, Munich, Germany) according to Djerassi et al.[13] and purified by preparative TLC as specified in Ref. 12. The structure of isoprogesterone was verified by NMR analysis employing the NMR data reported for pregnenolone[14] as reference for obvious assignments of ^{13}C and ^{1}H signals. Because to our knowledge the NMR data of isoprogesterone have not been published yet the chemical shifts for all carbon atoms and protons of isoprogesterone are listed along with the ^{13}C, ^{1}H and ^{2}D NMR spectra in the Supplementary data.

It has already been demonstrated that the addition of a crude *Dl*3KSI preparation to a r*Dl*3βHSD activity test clearly shifted the ratio of isoprogesterone to progesterone toward progesterone.[12] UV/vis detection of isoprogesterone and progesterone present in the different assays described above confirmed these results (Fig. 1). The results of r*Dl*3βHSD activity tests which were performed at different temperatures showed that progesterone formation was significantly enhanced with increasing temperature. This was explained with an enhanced chemical isomerization at higher temperatures. When 3KSI alone was present in the assays, highest progesterone formation was seen at 37 °C which matches the temperature optimum of this enzyme.[12] Progesterone formation was slightly lower in the assays containing r*Dl*3βHSD and *Dl*3KSI than in the assays with *Dl*3KSI alone. This can be explained by the fact that both enzymes are active and consequently both, isoprogesterone and progesterone are formed until the assay is stopped. But still, the combination of both enzymes again illustrates that the isoprogesterone:progesterone ratio is clearly shifted toward progesterone after 3KSI addition. In comparison to the high isomerization rate promoted by 3KSI, the non-enzymatic production of progesterone was not significant when tested under the same conditions as the enzymatic (3KSI) assay. Bacterial 3KSIs belong to the most efficient enzymes.[3] *Dl*3KSI also has a high catalytic efficiency, but it could not be determined here because only partially purified *Dl*3KSI preparations were used. As soon as a recombinant form of *Dl*3KSI is available we will address this question in some detail.

ESI-LC–MS analysis was used to determine whether deuterium was incorporated from the medium into progesterone during the isomerization reactions. Hence, the ratio of the parent *m*/*z* 315 $[M+H]^+$ and *m*/*z* 316 $[M+1+H]^+$ ions detected in the positive ionization mode analysis was calculated for progesterone formed under the different assay conditions (Table 1). The mass spectrum of progesterone incubated for 30 min at 37 °C with 0.5 µL HCL in deuterium oxide as well as the mass spectra of progesterone formed in the 3KSI assay at 37 °C, the r*Dl*3βHSD assay at 50 °C and the assay containing both enzymes are shown in Figure 2 as examples.

The 315/316 ion ratio calculated for progesterone dissolved in water (1.82) corresponded well with the ion ratio calculated for

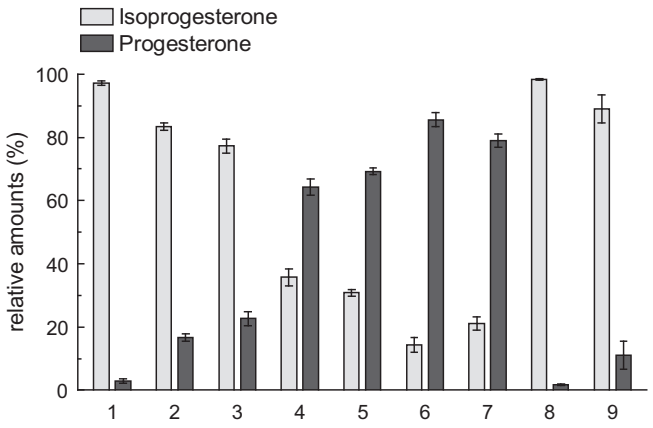

Figure 1. Conversation rate of isoprogesterone into progesterone determined for the different assay conditions by HPLC–UV/vis analysis. Mean ± SD of triplicates. **1**—r*Dl*3βHSD enzyme assay at 22 °C; **2**—r*Dl*3βHSD enzyme assay at 37 °C; **3**—r*Dl*3βHSD enzyme assay at 50 °C; **4**—r*Dl*3βHSD + *Dl*3KSI assay at 37 °C; **5**—*Dl*3KSI enzyme assay at 22 °C; **6**—*Dl*3KSI enzyme assay at 37 °C; **7**—*Dl*3KSI enzyme assay at 50 °C; **8**—non-enzymatic isomerization; **9**—acid catalyzed isomerization.

Table 1
Ratio of the parent *m*/*z* 315 $[m+H]^+$ and *m*/*z* 316 $[M+1+H]^+$ ions of progesterone formed under different assay conditions

Sample	315/316 ion ratio
Progesterone, RT[a]	1.82
Progesterone, 37 °C[b]	1.86 ± 0.045
Progesterone, 50 °C[b]	1.87 ± 0.037
3βHSD assay (d), 50 °C[b]	0.19 ± 0.016
3βHSD assay (d), 37 °C[b]	0.27 ± 0.011
3βHSD assay (d), 22 °C[b]	0.30 ± 0.010
3βHSD + 3-KSI assay (e)[b]	1.43 ± 0.019
3-KSI assay (a), 50 °C[b]	1.65 ± 0.073
3-KSI assay (a), 37 °C[b]	2.26 ± 0.051
3-KSI assay (a), 22 °C[b]	2.08 ± 0.040
Non-enzymatic isomerization (b)[b]	Not detectable
Acid catalyzed isomerization (c)[b]	0.44 ± 0.014

Samples were analyzed by ESI-LC–MS in the positive ion mode. Mean ± SD of triplicate.
[a] Sample in H_2O.
[b] Sample in deuterium oxide.

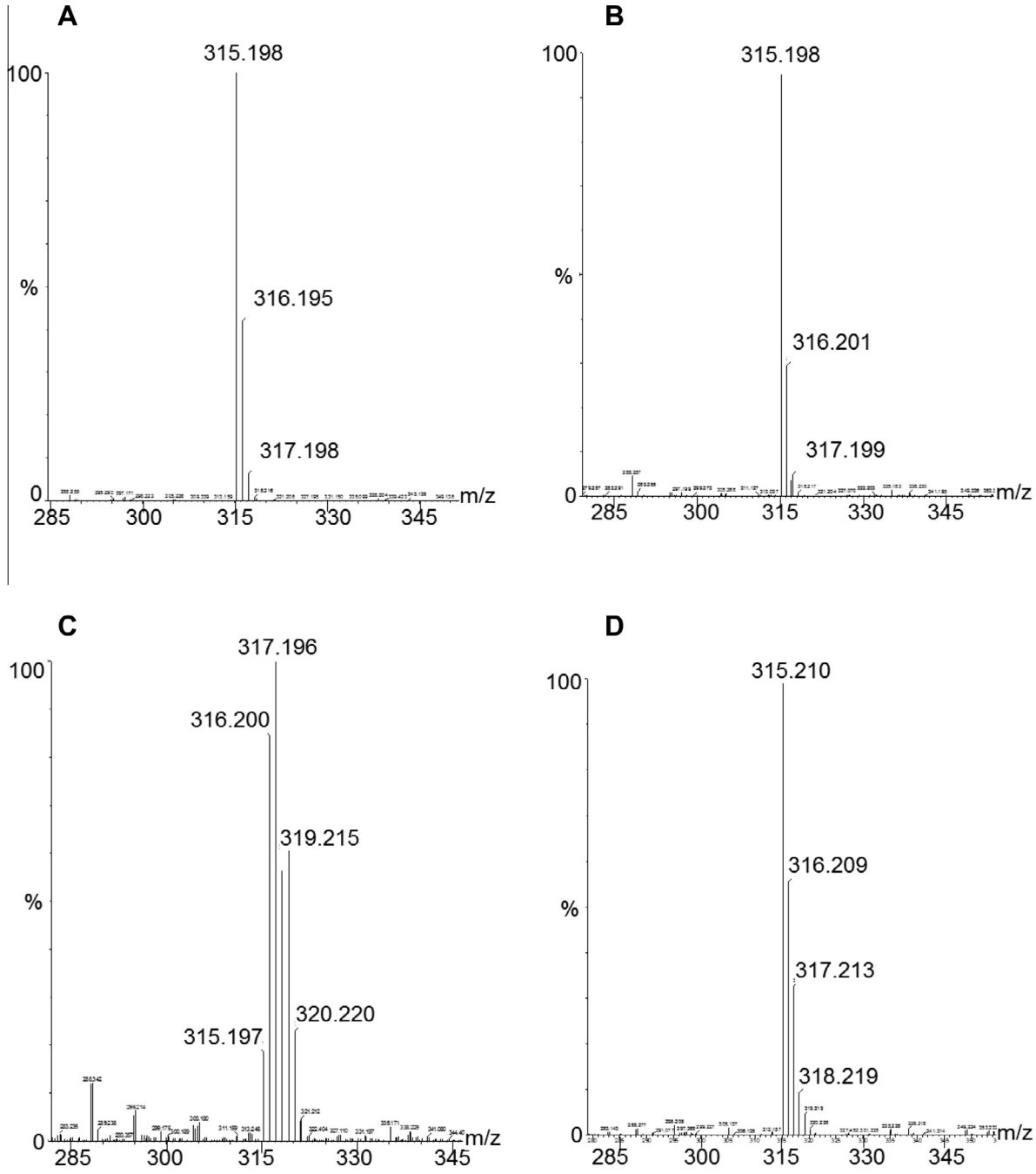

Figure 2. Mass spectra of progesterone detected by ESI-LC–MS analysis in the positive ionization mode. (A) Mass spectrum of progesterone incubated for 30 min at 37 °C with 0.5 μL HCl. (B) Mass spectrum of progesterone formed in the *Dl*3KSI assay at 37 °C. (C) Mass spectrum of progesterone formed in the r*Dl*3βHSD assay at 50 °C. (D) Mass spectrum of progesterone formed in the r*Dl*3βHSD + *Dl*3KSI assay at 37 °C.

progesterone which was incubated for 30 min with 0.5 μL 37% HCl in deuterium oxide at 37 °C and 50 °C (1.86 and 1.87, respectively). These controls prove that progesterone itself does not exchange protons with the incubation medium under the conditions applied here which was a prerequisite for the validity of our analyses and calculations.

The *m*/*z* 315/316 ion ratio obtained for progesterone formed in the r*Dl*3βHSD activity tests, as well as for the progesterone obtained by acid catalyzed isomerization, is considerably lower than the ion ratio of the progesterone controls due the high intensity of the ion at *m*/*z* 316. This substantiates that deuterium from the medium was incorporated into progesterone during the isomerization reaction. Mild reaction conditions (low acid concentration, low temperature and short incubation time) were chosen for the test for acid catalysis to reduce the chance that more than one deuterium was incorporated during progesterone formation, although this was not prevented completely. In contrast, a high ratio of the ions at *m*/*z* 315/316 was observed for progesterone formed in the 3KSI activity tests proving that no deuterium was incorporated from the medium during isomerization. This, in turn, demonstrates that the isomerization catalyzed by the *Dl*3KSI most probably proceeds via an intramolecular proton transfer. Deuterium incorporation into progesterone was thus attributed to a chemical isomerization only. The *m*/*z* 315/316 ion ratios calculated for progesterone formed in the assays containing both 3βHSD and 3KSI or 3KSI alone at 50 °C pointed out that both chemical and enzymatic isomerization

occurred under these conditions. When both enzymes were combined, progesterone was formed chemically during the first 4 h. Only when 3KSI was added to the reaction mixture after 4 h isoprogesterone was enzymatically isomerized into progesterone. In Figure 1 it was already shown that chemical isomerization increases with rising temperatures which explains the comparatively high *m*/*z* 315/316 ion ratio calculated for the progesterone formed in the 3KSI assay at 50 °C. Indeed 3KSI activity is lower at 50 °C than at 37 °C.[12]

In order to confirm the chemical and enzymatic isomerization of progesterone, disclosed by MS data, progesterone obtained by chemical and enzymatic isomerization was additionally analyzed by ^{2}H NMR (Fig. 3). We considered the direct NMR-analysis of the involved nucleus (^{2}H) to be the most promising and evident method in order to achieve proof for our suggested reaction mechanism. Other methods, e.g., detection of the lack of ^{1}H NMR signals, would be only indirect and thus prone to possible artifacts.

The spectrum of progesterone produced by chemical isomerization (Fig. 3A) revealed a broad singlet at δ 2.39 ppm, which was ascribed to H-6β in close agreement with literature data.[15] A further weak signal is found at δ 5.77 ppm which is the chemical shift of the proton/deuterium at the olefinic position H-4. It should be emphasized that the intensities of the signals at 2.39 and 5.77 ppm may not be compared directly. The inversion recovery method employed here is based on different T_1 between the solvent ($CDCl_3$) signal (set to zero crossing) and T_1 of the sample deuterium atoms. Since these T_1 numbers are unknown, differences in T_1 might manifest in errors which we estimate to ±50%. In contrast, the spectrum of progesterone which was obtained by enzymatic isoprogesterone isomerization does not exhibit the above mentioned relevant ^{2}H signals at δ 2.39 and 5.77 ppm (Fig. 3B). This finding confirms that an intramolecular proton transfer occurred during the enzymatic isomerization, thus corroborating our initial hypothesis.

To summarize, we demonstrated that *Digitalis lanata* 3KSI catalyzes the isomerization of isoprogesterone via an intramolecular proton transfer analogous to the bacterial enzyme using two different analytical methods. Moreover, we conclude that *Digitalis lanata* 3βHSD does not possess 3KSI activity.

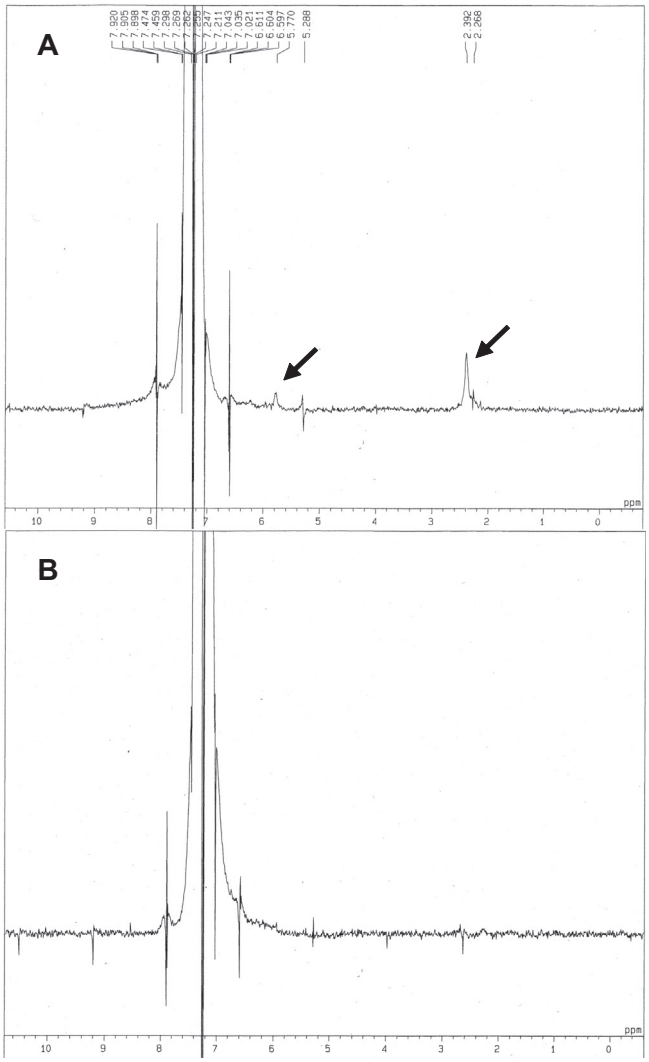

Figure 3. ^{2}H NMR spectra of progesterone. (A) ^{2}H NMR spectrum of progesterone formed by chemical isomerization. (B) ^{2}H NMR spectrum of progesterone formed by enzymatic isomerization. Comparable concentrations in both cases (A and B) are indicated by the approximately similar intensities of the ^{13}C-satellite signals of the solvent $CDCl_3$.

Enzyme preparations

For the production of r*Dl*3βHSD the *E. coli* strain M15 [pREP4] which was transformed with pQE30 plasmids (Qiagen, Hilden, Germany) containing the cDNA encoding the 3βHSD from *Digitalis lanata* was used.[8] The obtained His-tagged r*Dl*3βHSD was then purified employing Ni–NTA affinity chromatography according to the supplier's protocol (QIAexpressionist; Qiagen, Hilden, Germany).

*Dl*3KSI was partially purified from a crude protein extract made from 70 g of *D. lanata* leaves. After a fractionated ammonium sulfate precipitation in the concentration range of 25–75% ammonium sulfate the protein solution was passed through a Phenyl Sepharose 6 FF column (HiLoad 16/10) as specified in Ref. 12.

The obtained protein solutions were then lyophilized. For assaying the enzyme activity the lyophilized proteins were dissolved in deuterium oxide.

Isoprogesterone isomerization—composition of the assays

The r*Dl*3βHSD assay composed of 50 μL protein solution (final concentration 0.5 mg/mL), 25 μL pregnenolone solution and 30 μL NAD^{+} solution in a final volume of 250 μL. r*Dl*3βHSD was assayed at 22 °C, 37 °C and 50 °C for 4 h. The *Dl*3KSI activity test composed of 25 μL protein solution (final concentration 0.15 mg/mL) and 25 μL isoprogesterone solution in a final volume of 250 μL. *Dl*3KSI was assayed at 22 °C, 37 °C and 50 °C for 30 min. To combine both enzyme reactions (r*Dl*3βHSD and *Dl*3KSI) the r*Dl*3βHSD was assayed for 4 h at 37 °C as described, before 25 μL of the *Dl*3KSI solution were added and incubated for another 30 min at 37 °C. Non-enzymatic isomerization was analyzed by incubating 25 μL isoprogesterone solution in 250 μL deuterium oxide for 30 min at 37 °C. To study the acid catalyzed isomerization 25 μL of isoprogesterone solution was incubated for 30 min at 37 °C in deuterium oxide (final volume 250 μL) containing 0.5 μL HCl. Both steroid substrates (pregnenolone and isoprogesterone) were dissolved in deuterated methanol and added at a final concentration of 0.3 mM. The co-substrate NAD^{+} was dissolved in deuterium oxide and added at a final concentration of 0.6 mM. Digitoxigenin (1 mg/mL in methanol; 10 μL) was added to all assays as the internal standard. Reactions were terminated by adding 250 μL of dichloromethane and steroids were then extracted by vortexing for 30 s. Phase separation was facilitated by centrifugation at 13000×*g* for 5 min (Centrifuge Biofuge 13; Heraeus, Nuremberg, Germany) before the organic phase containing the steroids was removed and evaporated. Samples were then re-suspended in 100 μL methanol for HPLC/DAD/MS analysis.

HPLC/DAD/MS analysis

HPLC/DAD/MS analysis was conducted with a Waters Acquity UPLC (Binary Solvent Manager Waters Acquity) comprising Diodenarry (λ Detektor Acquity UPLC) and MS (TQ Detektor Acquity Ultra Performance LC) detection. 15 μL of sample was separated on a RP18 column (5 μm, LiChroCART 250-4, Merck, Darmstadt, Germany) with a flow rate of 1 mL/min employing a gradient of water (A) and acetonitrile containing 0.1% formic acid (B) as follows: 0 min 5% B; 10 min 95% B; 11 min 5% B; 13 min 5% B. Substances were analyzed at 220 nm (digitoxigenin), 210 nm (isoprogesterone) and 240 nm (progesterone). The mass chromatograms were recorded in the positive ionization mode. Afterward, extracted ion chromatograms were generated for m/z values 315 and 316 and the respective peaks were integrated to calculate the 315/316 ratio of the substances.

NMR-analysis of progesterone produced by enzymatic and acid catalyzed isoprogesterone isomerization

Progesterone samples produced by enzymatic and acid catalyzed isoprogesterone isomerization (ca. 1 mg each) were dissolved in 0.4 ml $CDCl_3$ for NMR measurements carried out on a JEOL Alpha500 spectrometer (^{1}H: 500 MHz). Isomerization conditions were used as described above with minor modification. The final assay volume was 1 mL and the incubation time of the acid catalyzed isomerization was extended to 6 h at 50 °C. Progesterone obtained from ca. 10 assays each was collected and purified by preparative TLC on silicagel 60 glass plates. Plates were developed with dichloromethane/ethyl acetate (9:1). Progesterone was visualized with anisaldehyde reagent[16] and eluted with 20 mL acetone. The solvent was evaporated to dryness in a rotary evaporator.

Sample shimming was done in the usual way on ^{2}H of the solvent. Deuterium spectra (^{2}H) were measured in unlocked mode by using the broadband transmitter channel with simultaneous ^{1}H decoupling. Prior to the measurements, T_1 of the $CDCl_3$ signals was determined by the inversion recovery method to 1.37. The spectra were recorded by the inversion recovery sequence (180°—delay—90°—Acq). According to the T_1 of ^{2}H in $CDCl_3$, the delay was set to 950 ms (= zero crossing of the $CDCl_3$ signal). Using this method, other deuterium atoms (from the purified progesterone samples) are expected to have different T_1 and should thus appear in the spectrum. However, signal intensities may not be compared directly using this method since the T_1 of the sample deuterium atoms are unknown. Further parameters of the spectra were 140 scans, 5 mm tube, relaxation delay 4.0 s, multinuclear probehead, non-spinning sample tube, measuring time per sample: 2.7 h.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.02.099.

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