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BIOSYNTHESIS OF PREGNANE DERIVATIVES IN SOMATIC EMBRYOS OF *DIGITALIS LANATA*

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Key Word Index—*Digitalis lanata*; Scrophulariaceae; foxglove; pregnanes; cardenolides; cholesterol monooxygenase; enzymes of pregnane metabolism; somatic embryos.

Abstract—Activities of the enzymes cholesterol monooxygenase (side chain cleaving) (SCCE), Δ^5-3β-hydroxysteroiddehydrogenase/Δ^5-Δ^4-ketosteroid isomerase, progesterone 5β-reductase, 3β-hydroxysteroid 5β-oxidoreductase, 3α-hydroxysteroid 5β-oxidoreductase, progesterone 5α-reductase and 3β-hydroxysteroid 5α-oxidoreductase which are involved in biosynthesis and transformation of pregnane derivatives were determined in different developmental stages of somatic embryos of *Digitalis lanata*. All enzymes were found to be present in proembryogenic masses (PEMs) as well as in globular and bipolar embryos. Most SCCE activity was found in the mitochondria fraction. Cholesterol, sitosterol and stigmasterol, precursors of the pregnanes, occurred in somatic embryos in amounts of about 1 μg mg^{-1} protein. Pregnenolone was found in traces only (about 20 ng mg^{-1} protein). Feeding of progesterone caused an increase of the contents of 5α- and 5β-pregnandione, progesterone, 5β-pregnane-3α-ol,20-one and 5α-pregnane-3β-ol,-20-one. In contrast, administration of cholesterol caused a small increase of pregnenolone only. These results indicate that the rate limiting step in pregnane (and probably in cardenolide biosynthesis) is compartmentation of CSSE in the mitochondria of the somatic embryos of *D. lanata*. © 1997 Elsevier Science Ltd

INTRODUCTION

Embryogenic cell strains of *D. lanata* are able to form large amounts of somatic embryos. In the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) these strains grow in the state of proembryogenic masses (PEMs). Somatic embryogenesis is elicited by substituting in the nutrient medium 2,4-D by naphthyl acetic acid (NAA) and lowering the auxin-cytokinin ratio. Starting from PEMs, globular and later bipolar embryos are formed. PEMs and early globular embryos (stage-I-globules) are free of cardenolides. Late globular embryos (stage-II-globules) and bipolar embryos synthesise cardenolides upon illumination [1]. The first main cardenolide built is glucodigifucoside [2]. It is still unknown why the late embryo structures synthesise cardenolides whereas the early stages do not. Several authors have speculated that the enzymes forming precursors and intermediates of cardenolide biosynthesis act as regulatory elements [3–5].

Cardenolides are derived from sterols via pregnane derivatives [6–8]. The first intermediate is pregnenolone (**5**). In mammals its formation is catalysed by cholesterol monooxygenase (side chain cleaving) (= side chain cleaving enzyme, SCCE; Table 1: enzyme *a*) [9]. SCCE is a membrane bound, mitochondrial P450 enzyme. It uses cholesterol (**2**) as substrate and catalyses its subsequent hydroxylation at the positions 20α and 22*R* followed by the final scission of isocapronal.

Cholesterol (**2**) [6, 7], 20α-hydroxycholesterol [6] and sitosterol (**3**) [8] are precursors of pregnanes and cardenolides in *D. lanata* plants. Radioactive labelled cholesterol was incorporated into pregnenolone in *D. purpurea* plants [10]. As yet, however, after cell disintegration formation of pregnenolone (**5**) by SCCE in plants could not be demonstrated, though with [26-^{14}C]cholesterol as substrate, cleavage of the side chain was shown to occur [3, 11, 12] (W. Kreis, and his coworkers, however, were not able to repeat these results [personal communication]).

In cell cultures and plants of different *Digitalis* sp. pregnenolone (**5**) was transformed to progesterone (**5**) [13, 14] and to cardenolides [14, 15]. Also progesterone (**6**) was incorporated into cardenolides [15]. The syn-

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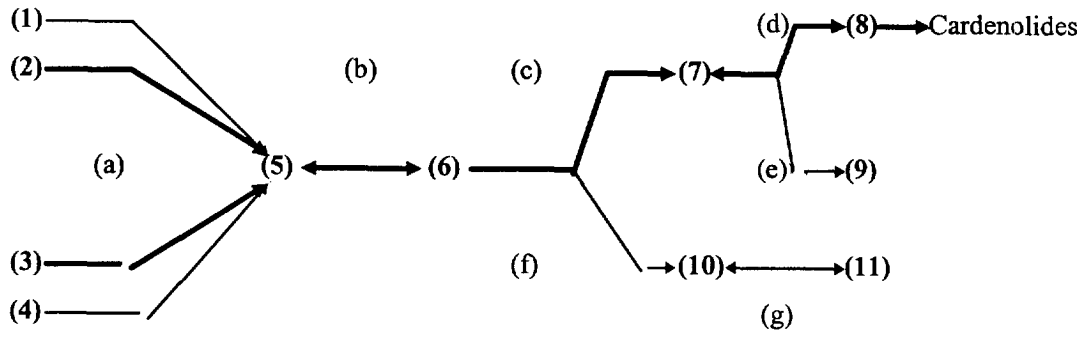
Table 1. Enzymes involved in pregnane biosynthesis and transformation in somatic embryos of *D. lanata*



1: Campesterol; **2**: cholesterol; **3**: sitosterol; **4**: stigmasterol; **5**: pregnenolone; **6**: progesterone; **7**: 5β-pregnane-3,20-dione; **8**: 5β-pregnane-3β-ol,20-one; **9**: 5β-pregnane-3α-ol,20-one; **10**: 5α-pregnane-3,20-dione; **11**: 5α-pregnane-3β-ol,20-one.

(*a*) Cholesterol monooxygenase (side chain cleaving) (= side chain cleaving enzyme, SCCE), EC 1.14.15.6 [9]
(*b*) Δ^5-3β-Hydroxysteroiddehydrogenase/Δ^5-Δ^4-ketosteroid isomerase (3β-HSD) [17]
(*c*) Progesterone 5β-reductase [18]
(*d*) 3β-Hydroxysteroid 5β-oxidoreductase [5]
(*e*) 3α-Hydroxysteroid 5β-oxidoreductase [4]
(*f*) Progesterone 5α-reductase [20]
(*g*) 3β-Hydroxysteroid 5α-oxidoreductase [21]

Cell material	Enzyme activities (μkat kg^{-1} protein)						
	(a)*	(b)	(c)†	(d)	(e)	(f)	(g)
PEMs	0.1–1	7.0	0.1	4900	70	0.1	42
Embryos	n.d.	1.5	n.d.	2000	60	0.2	30
Leaves	0.024	0.1	n.d.	1900	60	0.2	n.d.

* Cholesterol as substrate.
† Calculated values.
n.d.: not determined.

thesis of progesterone (**6**) is catalysed by the enzyme Δ^5-3β-hydoxysteroiddehydrogenase/Δ^5-Δ^4-ketosteroid isomerase (3β-HSD; Table 1: enzyme b). 3β-HSD was shown to occur in cell cultures and plants of *D. lanata* [17].

Progesterone (**6**) is reduced to 5β-pregnane-3,20-dione (**7**) by progesterone 5β-reductase (Table 1, enzyme c). Occurrence of this enzyme was demonstrated in leaves of *D. purpurea* [5, 18]. Progesterone 5β-reductase is of special importance in the biosynthesis of cardenolides, because the cardenolides occurring in *Digitalis* sp. are 5β-pregnane derivatives.

5β-Pregnane-3,20-dione (**7**) is converted in *D. purpurea* cell cultures to a series of different pregnane derivatives including those shown in Table 1 [19]. 5β-Pregnane-3β-ol,20-one (**8**) was built by 3β-hydroxysteroid 5β-oxidoreductase (Table 1, enzyme d), an enzyme found in preparations of cell cultures of *D. lanata* [5]. 5β-Pregnane-3,20-dione (**7**) is transformed to 5β-pregnane-3α-ol,20-one (**9**) by 3α-hydroxysteroid 5β-oxidoreductase (Table 1, enzyme e) [4].

Progesterone 5α-reductase (Table 1, enzyme f) forming 5α-pregnane-3,20-dione (**10**) competes for progesterone (**6**) with progesterone 5β-reductase (Table 1, enzyme c). Progesterone 5α-reductase was found in cell cultures of *D. lanata* [20]. 5α-pregnane-3,20-dione (**10**) is transformed to 5α-pregnane-3β-ol,20-one (**11**) by 3β-hydroxysteroid 5α-oxidoreductase (Table 1, enzyme g) in cell cultures of *D. purpurea* [21]. It has been suggested that progesterone 5α-reductase and 3α-hydroxysteroid 5β-oxidoreductase withdraw precursors from cardenolide biosynthesis [5]. In this paper we tried to find correlations between the activity of the pregnane forming and modifying enzymes mentioned above and the occurrence of the corresponding pregnane derivatives during somatic embryogenesis.

RESULTS AND DISCUSSION

Activities of the following enzymes involved in synthesis and metabolism of pregnane derivatives were determined in cell free extracts of PEMs, somatic embryos and for comparison also in leaves of *D. lanata*.

Cholesterol monooxygenase (side chain cleaving enzyme, SCCE) (Table 1, enzyme a)

SCCE activity was determined for the first time by quantification of the main product pregnenolone (**4**)

Table 2. SCCE activities in PEMs, somatic embryos and leaves of *D. lanata* using different substrates. In different experimental series deviating specific SCCE activities were found (0.1–1.0 μkat kg^{-1} protein). Therefore, data were normalized for leaf-extract with sitosterol as substrate = 100. Standard deviation within one series with three parallel experiments was ±20%

Substrates used in determination of SCCE activity	SCCE relative specific activity*		
	PEMs	Embryos	Leaves
Cholesterol (**2**)	n.d.	17	55
Sitosterol (**3**)	n.d.	39	100
20α-Hydroxycholesterol	72	19	28
22*S*-Hydroxycholesterol	94	22	28

* For calculation of enzyme activity the pregnenolone background (cf. Table 3) was subtracted.
n.d.: not determined in this series.

in cell free plant extracts. Enzyme activity was membrane bound and found in the mitochondria and microsomal fractions of PEMs, somatic embryos grown upon illumination and in the dark as well as in leaves (Table 1).

Formation of pregnenolone (**5**) was highest with cholesterol (**2**) and sitosterol (**3**) as substrates. However, the enzyme reacted also with 20α-hydroxycholesterol, 22*S*-hydroxycholesterol (Table 2), and at a lower rate with campesterol (**1**) and stigmasterol (**4**). The acception of 20α-hydroxycholesterol and 22*S*-hydroxycholesterol indicated that SCCE from *D. lanata* forms similar intermediates as mammalian SCCE [9]. The measurable enzyme activity was in the range of 0.1–1 μkat kg^{-1} protein in PEMs and somewhat lower in leaves (Table 1). The small amounts of pregnenolone (**5**) formed in the enzymatic tests become measurable by GC-MS using the single ion monitoring (SIM) mode by summing up 5 prominent masses.

Δ^5-3β-Hydroxysteroiddehydrogenase/Δ^5-Δ^4-ketosteroid isomerase (3β-HSD) (Table 1, enzyme b)

3β-HSD transforms pregnenolone (**5**) into progesterone (**6**). 3β-HSD activity was found in somatic embryos grown either in the light or in the dark (Table 1). PEMs showed the highest specific activity (up to 7 μkat kg^{-1} protein). In embryos the activity of the enzyme was lower (Table 1). In other *D. lanata* cell strains Seidel *et al.* [17] found 3β-HSD activities of 28–50 μkat kg^{-1} protein.

The activity of the enzyme was rather small in leaves (<0.1 μkat kg^{-1} protein) (Table 1, Fig. 1). This result was unexpected, because leaves are the main source of cardenolides in *D. lanata*. However, Stuhlemmer and Kreis [5] found no correlation between 3β-HSD activity and cardenolide formation.

Progesterone 5β-reductase (Table 1, enzyme c) and 3β-hydroxysteroid 5β-oxidoreductase (Table 1, enzyme d)

3β-Hydroxysteroid 5β-oxidoreductase transforms 5β-pregnane-3,20-dione (**7**) to 5β-pregnane-3β-ol,20-one (**8**). The specific activity of this enzyme was in the range of 2 mkat kg^{-1} protein (leaves and embryos) and 4 mkat kg^{-1} protein (PEMs) (Table 1). The activity of progesterone 5β-reductase, catalysing the conversion of progesterone (**6**) into 5β-pregnane-3,20-dione (**7**), was much smaller. It could only be measured indirectly in the crude extracts used. The product, 5β-pregnane-3,20-dione (**7**), was transformed by 3β-hydroxysteroid 5β-oxidoreductase directly to 5β-pregnane-3β-ol,20-one (**8**). Assuming that the rate limiting step in the formation of the latter compound was progesterone 5β-reductase a specific activity of above 0.1 μkat kg^{-1} protein was estimated. The expression of progesterone 5β-reductase in PEMs and early embryo stages, which do not synthesise cardenolides, is in contrast to results of Stuhlemmer *et al.* [4, 5] which showed progesterone 5β-reductase activity only in tissues which produced cardenolides.

Progesterone 5α-reductase (Table 1, enzyme f)

Progesterone 5α-reductase transforms progesterone (**6**) to 5α-pregnane-3,20-dione (**10**). The activity of this enzyme was in the range of 0.1 μkat in PEMs and somatic embryos (Table 1). Dark grown cultures exhibited slightly higher specific activities than cultures grown in the light. PEMs showed the lowest specific activity while in somatic embryos the specific activity was similar to that of young leaves. Progesterone 5α-reductase activity was also found by Stuhlemmer and Kreis [5] in various cell cultures and tissues of *D. lanata*.

3α-Hydroxysteroid 5β-oxidoreductase (Table 1, enzyme e)

3α-Hydroxysteroid 5β-oxidoreductase activity was found in PEMs, somatic embryos grown in the dark and upon illumination as well as in leaves. The activity of the enzyme was in the range of 60–70 μkat kg^{-1} protein (Table 1). Similar activities were reported by Stuhlemmer *et al.* [4] who examined tissue cultures of *D. lanata*.

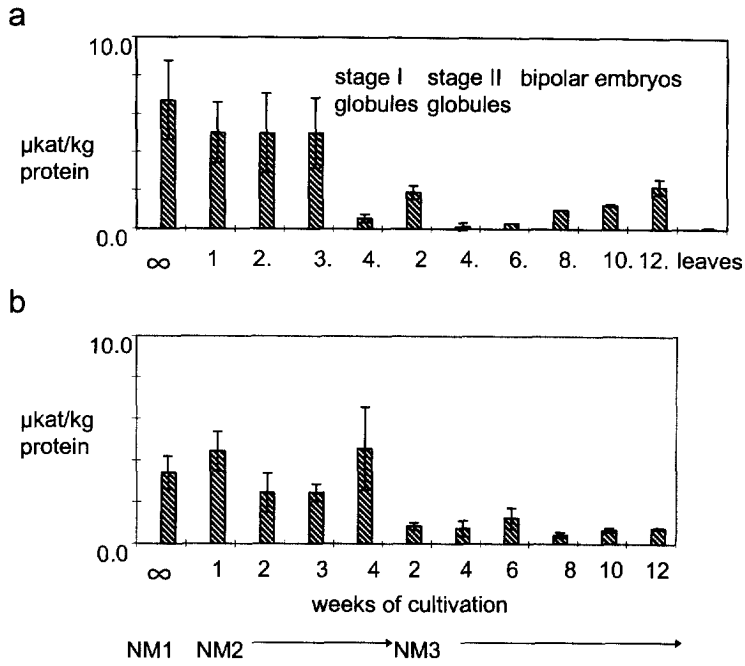


Fig. 1. Specific activity of 3β-HSD in somatic embryos. (a) Light grown cultures and leaves; (b) dark grown cultures. NM1–3 mark the change of culture media. Developmental stages are marked in the top of (a).

3β-Hydroxysteroid 5α-oxidoreductase (Table 1, enzyme g)

The enzyme catalyses the transformation of 5α-pregnane-3,20-dione (**10**) to 5α-pregnane-3β-ol,20-one (**11**) [21]. It was shown to occur in PEMs and somatic embryos (Table 1). Similar enzyme activities were found in somatic embryos grown in the light and in the dark.

In summary these results show that the enzymes (a) to (g) listed in Table 1 showed no increase in activity during embryo development and upon illumination of the embryos, i.e. conditions by which cardenolide formation is triggered. This indicates that the expression of these enzymes plays no key role in the regulation of cardenolide biosynthesis.

Sterols and pregnanes in PEMs and somatic embryos

Relatively high quantities of campesterol (**1**), sitosterol (**3**) and stigmasterol (**4**) were found in PEMs and somatic embryos (Fig. 2). Whereas the main sterol in PEMs was sitosterol in the somatic embryos it was stigmasterol. This shift may be a stress reaction, as it was recently reported for elicited parsley cell cultures [22]. The content of cholesterol (**2**) and fucosterol was relatively small. During embryo development the total sterol amount slightly decreased. In experiments with other *D. lanata* cell strains in addition to the sterols mentioned above cycloartenol and 24-methylenecycloartenol were detected [23].

In contrast to the sterols, pregnenolone (**5**) was present in traces only. It was detected by GC-MS using the SIM mode. The amounts found in mitochondria preparations of PEMs and somatic embryos was about 20 ng mg^{-1} protein (Table 3). The occurrence of pregnenolone agreed with the SCCE activity of these preparations. Progesterone (**6**) was neither found in PEMs nor in somatic embryos in spite of the relatively high 3β-HSD activities present.

*Feeding of cholesterol (**2**) and progesteron (**6**) to PEMs and somatic embryos*

Feeding of cholesterol (**2**) to PEMs (Table 3) and somatic embryos resulted in the formation of an increased, but still rather low level of pregnenolone (**5**). Other pregnane derivativess could not be detected. The low rate of pregnenolone formation indicates that SCCE has no free access to cholesterol (**2**).

After feeding of progesterone (**6**) in agreement with the presence of the enzymes discussed above, the pregnane derivative shown in Table 4 were detected. 5α-Pregnane derivatives represented the majority of the compounds, but 5β-pregnane-3β-ol,20-one (**8**) was shown also to occur in considerable quantities.

The rate limiting step in pregnane and cardenolide formation

In summary, the following results described in this paper indicate that compartimentation of the enzymes SCCE and its substrates restricts the synthesis of pregnenolone (**5**) and as consequence the formation of other pregnane derivatives and of cardenolides *in vivo*: (i) the occurrence of considerable amounts of the likely substrates of SCCE, i.e., cholesterol (**2**) and sitosterol (**3**), in PEMs and somatic embryos in contrast to very low amounts of the product pregnenolone

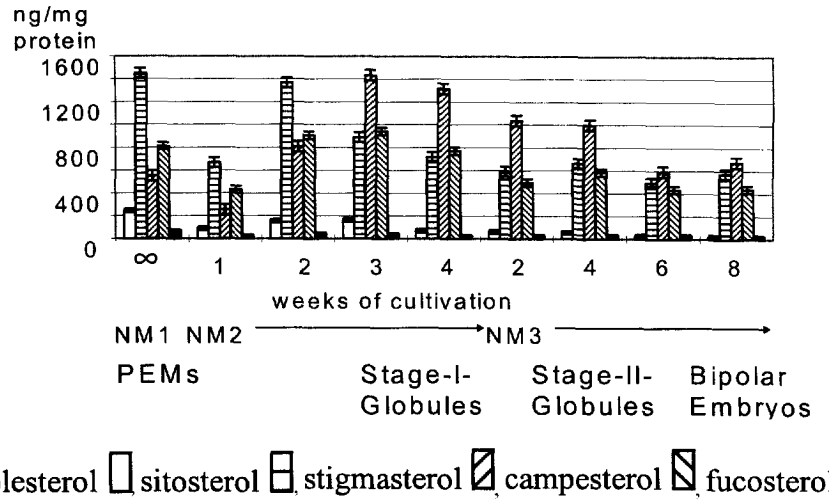


Fig. 2. Main sterols in PEMs and somatic embryos. NM1–3 indicate the nutrient media used for cultivation.

Table 3. Pregnenolone content in PEMs*

Treatment of PEMs	Pregnenolone content (ng mg^{-1} protein)
Control, grown in the light	20±10%
Control, grown in the dark	50±18%
Administration of:	
Cholesterol (**2**)	147±41%
Progesterone (**6**)	196±22%

* Lyophilized plant material was extracted with $CHCl_3$–MeOH–H_2O (5:10:4). After evaporation of the solvent steroids were separated on TLC (silica 60, 20 × 20 cm, $CHCl_3$–EtOAc 4:1). Steroid containing areas were scratched out and extracted with $CHCl_3$. After evaporation of $CHCl_3$ the residue was dissolved in MeOH and analysed by GC-MS SIM.

(**5**). (ii) The formation of different pregnane derivatives after feeding of progesterone (**6**) which indicates that these compounds are available for the enzymes of pregnane metabolism and (iii) the localization of SCCE in the mitochondria and the relatively small rise of pregnenolone (**5**) content after feeding of cholesterol (**2**) in spite of the fact that the cholesterol content of the mitochondria fraction was increased (data not shown). This result agrees with the fact that in mammals the biosynthesis of the pregnane-derived steroid hormones is mainly regulated by the transport of cholesterol into the mitochondria [24, 25].

EXPERIMENTAL

Cell cultures of D. lanata *EHRH. strain VIII.* PEMs and somatic embryos were grown as described by Scheibner *et al.* [26]. Basic nutrient medium (NM0, concs in mM): maltose 100, KNO_3 20, NH_4NO_3 20, KH_2PO_4 1.5, $CaCl_2$ 3, $MgSO_4$ 1.5, $FeSO_4$ 0.2, H_3BO_3 0.1, $MnSO_4$ 0.1, Na_2EDTA 0.1, $ZnSO_4$ 0.03, KI 5×10^{-3}, Na_2MoO_4 1×10^{-3}, myo-inositol 0.5, nicotinamide 10×10^{-3}, pyridoxin 2.5×10^{-3}, thiamine 1.5×10^{-3}. NM1 (for cultivation of PEMs): NM0 containing KH_2PO_4 6.5, 2,4-D 5×10^{-3} and kinetin 1×10^{-4}. NM2 (for start of embryo differentiation): NM0 containing NAA 5×10^{-3} and kinetin 1×10^{-4}. NM3 (formation of bipolar embryos): NM0 containing BA 5×10^{-3} and NAA 5×10^{-5}.

Feeding of cholesterol (**2**) *and progesterone* (**5**). Stock solns in EtOH (5 and 10 mg ml^{-1}) were added to the nutrient medium to a final concn of 50 and 100 μg ml^{-1}.

Table 4. Content of pregnane derivatives after feeding of progesterone

Pregnanes found	PEMs (ng mg^{-1} protein)	Stage-II-globules (ng mg^{-1} protein)
Progesterone (**5**)	818	864
5α-Pregnane-3,20-dione (**9**)	359	520
5α-Pregnane-3β-ol,20-one (**10**)	1796	1836
Pregnenolone (**4**)	196	146
5β-Pregnane-3β-ol,20-one (**7**)	117	152
5β-Pregnane-3α-ol,20-one (**10**)	540	136
Standard deviation	23%	20%

Enzyme assays. SCCE activity was determined in mitochondrial and microsomal frs obtained by freezing 15 g cells with liquid N_2 and stirring the material with 30 ml 50 mM sodium phosphate buffer containing 4 mM dithiothreitol pH 7.5 and 1.5 g H_2O insoluble polyvinyl pyrrolidone (Polyclar AT, Serva, Heidelberg). The extract was filtered through miracloth (Calbiochem, Bad Soden) and centrifuged 10 min 500 *g*. The supernatant was centrifuged 20 min 4800 *g*. The obtained pellet was dissolved in a small vol. of 75 mM sodium phosphate buffer pH 7.2. The enzyme assay (total vol. 0.5 ml) contained 150 mM NaCl, 5 mM glucose-6-phosphate, 1 mM $NADP^+$, 8.4 nkat glucose-6-phosphate dehydrogenase, 50 μg ml^{-1} substrate, 0.2% Tween 20 and 75 mM sodium phosphate buffer pH 7.2. The components were mixed in an ice-bath and preincubated for 30 min at 30°. After addition of the mitochondria prepn (1–3 mg protein per assay) the mixt. was incubated for 20 min at 30° on a shaker. After incubation etiocholan-17β-ol-3-one was added as int. standard (20 μg in 10 μl MeOH). Pregnenolone (**5**) was extracted with prechilled Et_2O (3 × 0.5 ml, 0°). After evapn of the Et_2O the residue was dissolved in 50 μl MeOH and quantified by GC-MS (single ion mode).

3β-HSD activity was measured according to Seidel *et al.* [17]. 50–100 mg Me_2CO-dry powder were extracted with 2 ml 20 mM sodium phosphate buffer pH 7.5 containing 0.25 M saccharose. After centrifugation (10 min at 10000 *g*) the supernatant was filtered through miracloth (Calbiochem, Bad Soden). 20 μl pregnenolone (**3**) (0.5 mg ml^{-1} DMSO) and 20 μl NAD^+ (10 mg ml^{-1} phosphate buffer) were added to 960 μl of the extract. The mixt. was incubated on a shaker (30 min at 37°). The reaction was stopped by addition of 50 μl testosterone (1 mg ml^{-1} EtOH) as int. standard. The steroids were extracted twice with 0.7 ml prechilled ether at 0°. The collected Et_2O phases were evapd and the remaining steroids resuspended in 100 μl MeOH. Progesterone (**6**) was quantified by GC-MS (total ion mode 50–500 amu).

Progesterone 5β-reductase and progesterone 5α-reductase were determined according to Seitz and coworkers [18, 20]. The products were quantified by GC-MS (total ion mode). 3α-Hydroxysteroid-5β-oxidoreductase and 3β-hydroxysteroid-5β-oxidoreductase were determined by the methods of Stuhlemmer *et al.* [4] and [5], respectively. The products were quantified by GC-MS (total ion mode).

Determination of steroids. Fresh plant material was frozen with liquid N_2 and lyophilized. 0.1 g dry plant material was extracted ×3 with 4 ml of a mixt. of $CHCl_3$–MeOH–H_2O (5:10:4) [27]. The collected extracts were evapd at 85° and sepd by TLC (Silica 60, 20 × 20 cm; $CHCl_3$–EtOAc, 4:1) [28]. A part of the plate was stained with anisaldehyde-reagent (0.5 ml anisaldehyde in 10 ml 98% HOAc, 85 ml MeOH and 5 ml 96% H_2SO_4) [29]. Steroid containing areas of the unsprayed part of the plate were scratched out and extracted with $CHCl_3$ (3 × 3 ml). After evapn of the $CHCl_3$ the residue was dissolved in 100 μl MeOH and examined by GC-MS (total ion mode) using testosterone and etiocholan-17β-ol,3-one as int. standards.

GC-MS. Performed using Hewlett-Packard 5890 equipped with 5972 Series MSD, column: HP-5 MS; 30 m × 0.25 mm; stationary phase: 0.25 μm crosslinked 5% phenylmethylsilicone, mobile phase: He, flow rate: 1 ml min^{-1}. Sepn of pregnane derivatives: temp. programme: 0 min: 150°, 10 min: 230°, 12 min: 270°, 13.2 min: 5β-pregnane-3α-ol,20-one (**8**), 13.5 min: 5β-pregnane-3β-ol,20-one (**7**), 13.7 min: pregnenolone (**4**), 14.0 min: 5α-pregnane-3β-ol,20-one (**10**), 14.4 min: 5α-pregnane-3,20-dione (**9**), 15.2 min: progesterone (**5**). Small amounts of pregnenolone were detected in single ion mode monitoring the characteristic *m/z* 231, 255, 283, 298, 316. Sepn of sterols: temp. programme: 0 min: 150°, 10 min: 230°, 22 min: 270°, 19.9 min: cholesterol; 22.3 min: campesterol; 23.1 min: stigmasterol; 24.9 min: sitosterol; 25.2 min: isofucosterol.

Protein concentrations. Determined by a modified Lowry procedure [30].

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