Phytochemistry 71 (2010) 1495–1505

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Highly conserved *progesterone 5β-reductase* genes (*P5βR*) from 5β-cardenolide-free and 5β-cardenolide-producing angiosperms

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ARTICLE INFO

Article history:
Received 20 April 2010
Received in revised form 2 June 2010
Available online 1 July 2010

Keywords:
Digitalis
Plantaginaceae
Progesterone 5β-reductase
Enone reductase
Substrate promiscuity
Cardenolide

ABSTRACT

Most cardenolides used in the therapy of cardiac insufficiency are 5β-configured and thus the stereo-specific reduction of the $\Delta^{4,5}$-double bond of a steroid precursor is a crucial step in their biosynthesis. This step is thought to be catalysed by progesterone 5β-reductases. We report here on the isolation of 11 *progesterone 5β-reductase* (*P5βR*) orthologues from 5β-cardenolide-free and 5β-cardenolide-producing plant species belonging to five different angiosperm orders (Brassicales, Gentianales, Lamiales, Malvales and Solanales). Amino acid sequences of the P5βR described here were highly conserved. They all contain certain motifs qualifying them as members of a class of stereo-selective enone reductases capable of reducing activated C=C double bonds by a 1,4-addition mechanism. Protein modeling revealed seven conserved amino acids in the substrate-binding/catalytic site of these enzymes which are all supposed to exhibit low substrate specificity. Eight *P5βR* genes isolated were expressed in *Escherichia coli*. Recombinant enzymes reduced progesterone stereo-specifically to 5β-pregane-3,20-dione. The progesterone 5β-reductases from *Digitalis canariensis* and *Arabidopsis thaliana* reduced activated C=C double bonds of molecules much smaller than progesterone. The specific role of progesterone 5β-reductases of P5βRs in cardenolide metabolism is challenged because this class of enone reductases is widespread in higher plants, and they accept a wide range of enone substrates.

1. Introduction

5β-Cardenolides are C_{23}-steroids containing a butenolide ring at C-17 and are derived from mevalonic acid via phytosterol and pregnane intermediates (Fig. 1). Further structural features are the *cis–trans–cis* configuration of the four annealed carbon rings and a hydroxyl function at C-14β. Several 5β-cardenolides, such as digoxin or digitoxin, are of special interest since they are used in the therapy of cardiac insufficiency in humans.

The reaction converting sterols into pregnenolone is thought to be catalysed by a mitochondrial cytochrome P450-dependent side chain cleaving enzyme (P450scc, CYP11A in animals) (Lindemann and Luckner, 1997) although no evidence of such a P450 has been found in plants as yet (Ohnishi et al., 2009). Subsequently, pregnenolone has to be modified in several steps and condensed with a C_2 unit to yield the 5β-cardenolide genin (Fig. 1). The preferred sequence of the individual biosynthetic steps leading to 5β-cardenolides is not yet clear and more than one pathway may be operative (Maier et al., 1986; Kreis et al., 1998) and it was suggested that a metabolic grid rather than a pathway should be used to display and describe cardenolide biosynthesis (Kreis and Müller-Uri, 2010).

The stereo-specific reduction of the $\Delta^{4,5}$-double bond of putative steroid precursors, such as progesterone, was investigated thoroughly. A progesterone 5β-reductase supposed to be involved in 5β-cardenolide metabolism (P5βR; EC 1.3.1.3) was isolated (Gärtner et al., 1994) and genes encoding this enzyme were cloned from several *Digitalis* species and functionally expressed in *Escherichia coli* (Herl et al., 2006a,b, 2008; Gavidia et al., 2007). The orthologues *Arabidopsis thaliana VEP1* gene encodes a protein which seems to be required for vascular strand development (Jun et al., 2002) but was also shown to be capable of reducing progesterone and other $\Delta^{4,5}$-steroids stereo-specifically *in vitro* (Herl et al., 2009). Only recently, Perez-Bermudez et al. (2010) reported on the isolation and functional expression of a novel progesterone 5β-reductase (termed P5βR2) from *Digitalis purpurea* that can be induced by stress.

The occurrence of genes coding for progesterone 5β-reductases in cardenolide-free species together with our observation that some P5βRs are highly substrate-promiscuous *in vitro* (Herl et al., 2006a,b; Burda et al., 2009; Schebitz et al., 2010) highlights the

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doi:10.1016/j.phytochem.2010.06.004

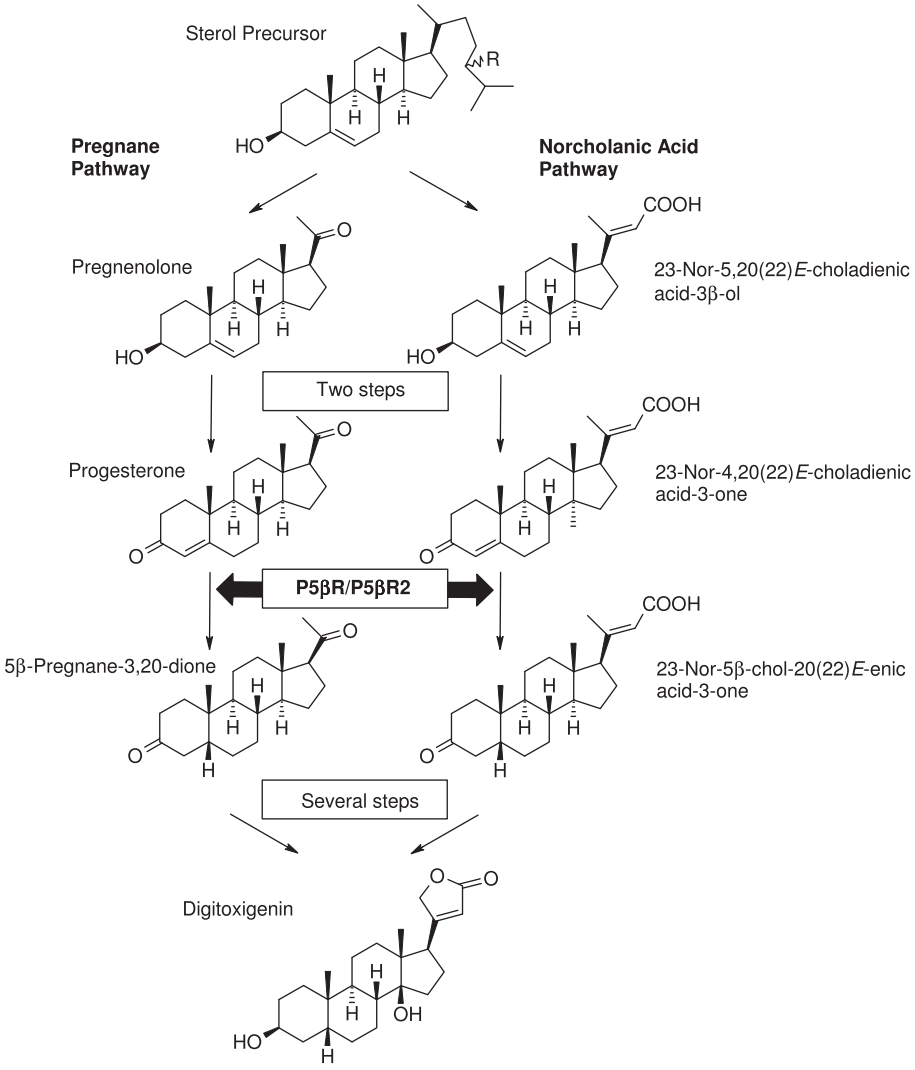

Fig. 1. Putative pathways leading from phytosterols to 5β-cardenolides such as digitoxigenin. P5βR/P5βR2 – progesterone 5β-reductases.

existence of a significant gap in our present understanding of the exact physiological function of P5βRs. We here isolated further *P5βR* genes, demonstrate that their substrate-binding site is highly conserved with regard to the amino acids flanking the two catalytic amino acids and assume that substrate promiscuity is a likely property of all P5βRs.

2. Results and discussion

2.1. Occurrence of 5β-cardenolides

5β-Cardenolides (Fig. 1) have been found in ca. 60 genera of the angiosperms (Singh and Rastogi, 1970; Melero et al., 2000; Kreis and Müller-Uri, 2010). So far they have been reported to occur in the monocots (Poales, Asparagales, Liliales), the basal eudicots (Ranunculales), the rosids (Crossosomatales, Myrtales, Celastrales, Malpighiales, Fabales, Rosales, Brassicales, Malvales) and the asterids (Gentianales, Lamiales, Solanales, Asterales). No records were found for the magnoliids (Fig. 2). About half of the genera investigated up to today belong to the Gentianales. It appears that 5β-cardenolides have a higher prevalence in phylogenetical younger angiosperms. Only recently, 5β-cardenolides were reported in the Asterales (Wang et al., 2007) and Crossosomatales (Klausmeyer et al., 2009). Therefore these compounds may have a wider distribution than previously assumed.

The unpredictable occurrence of 5β-cardenolides in the angiosperms raises the question whether this trait has evolved only once or several times during evolution. The analysis of genes suggested to be involved in 5β-cardenolide formation may help to address this question.

2.2. Occurrence of progesterone 5β-reductases

Progesterone 5β-reductases are thought to catalyse an important step in the 5β-cardenolide biosynthesis, namely the conversion of progesterone to 5β-pregnane-3,20-dione (Gärtner et al., 1990). Therefore P5βR was regarded to catalyse the first committed step in 5β-cardenolide biosynthesis (Gärtner and Seitz, 1993). Two different progesterone 5β-reductases (P5βR, P5βR2) have been shown to occur in the genus *Digitalis*. Following the nomenclature of the short-chain dehydrogenase/reductase (SDR) super-family of proteins (Persson et al., 2009) Perez-Bermudez et al. (2010) proposed that the family designation for progesterone 5β-reductases should be SDR75R and the protein designations should be SDR75R1 and SDR75R2 for P5βR and P5βR2, respectively. We here

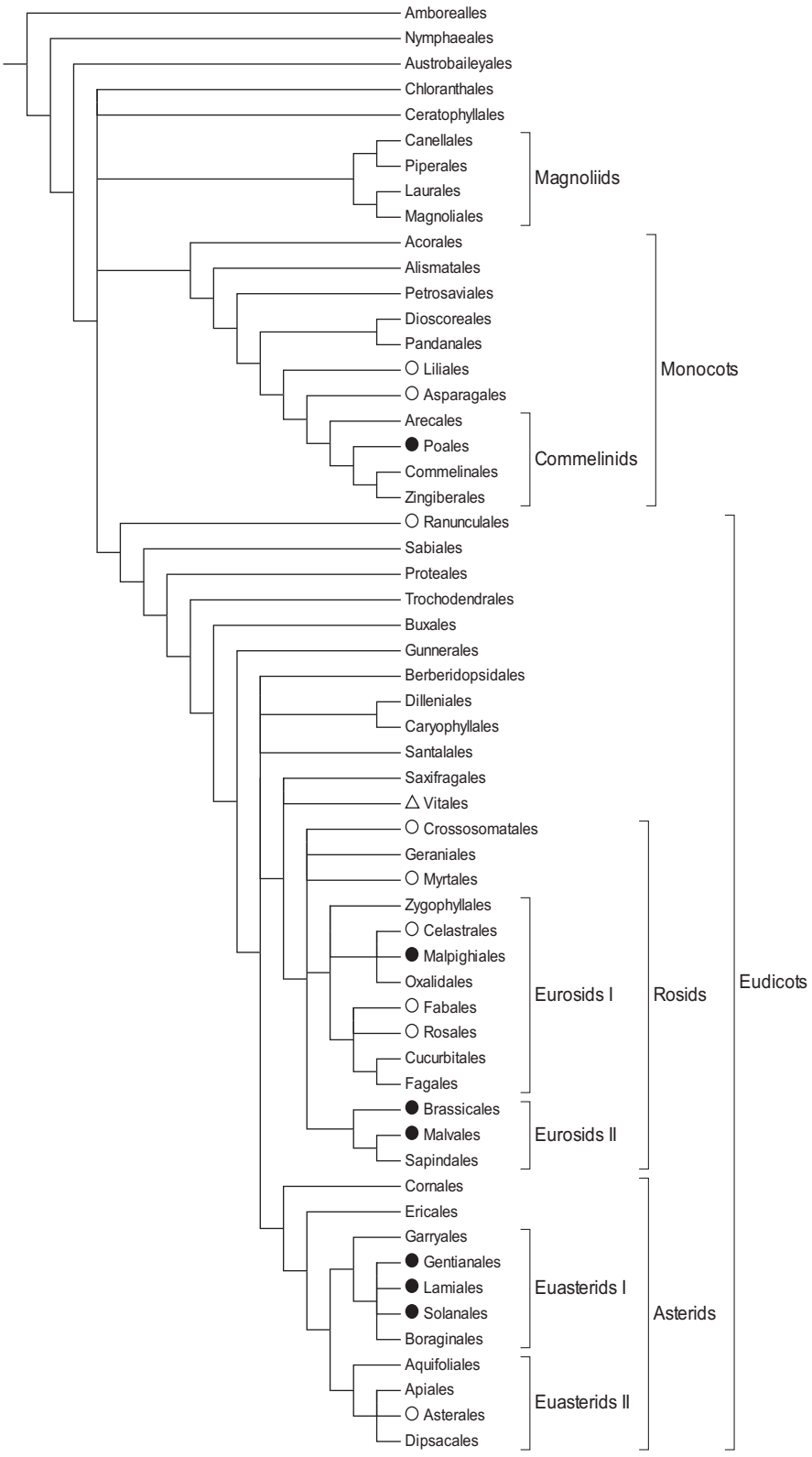

Fig. 2. Distribution of 5β-cardenolides in angiosperms. Orders reported to contain 5β-cardenolides are marked with circles (●,○). Filled circles (●) and open triangles (Δ) indicate that members of this order have been demonstrated previously or during this study to contain *P5βR* genes.

use the established terms P5βR and P5βR2 or speak of *P5βR-like* genes and P5βR-like enone reductases.

A high number of P5βR-like proteins in the public domain databases could be identified using published sequences of recombinant P5βRs from *Digitalis* species as a template (Gavidia et al., 2007 and own BLAST search). Bacterial *P5βR-like* genes form separate clusters (not shown) and among the angiosperms two individual clusters can be identified, one containing *Digitalis P5βR*, the

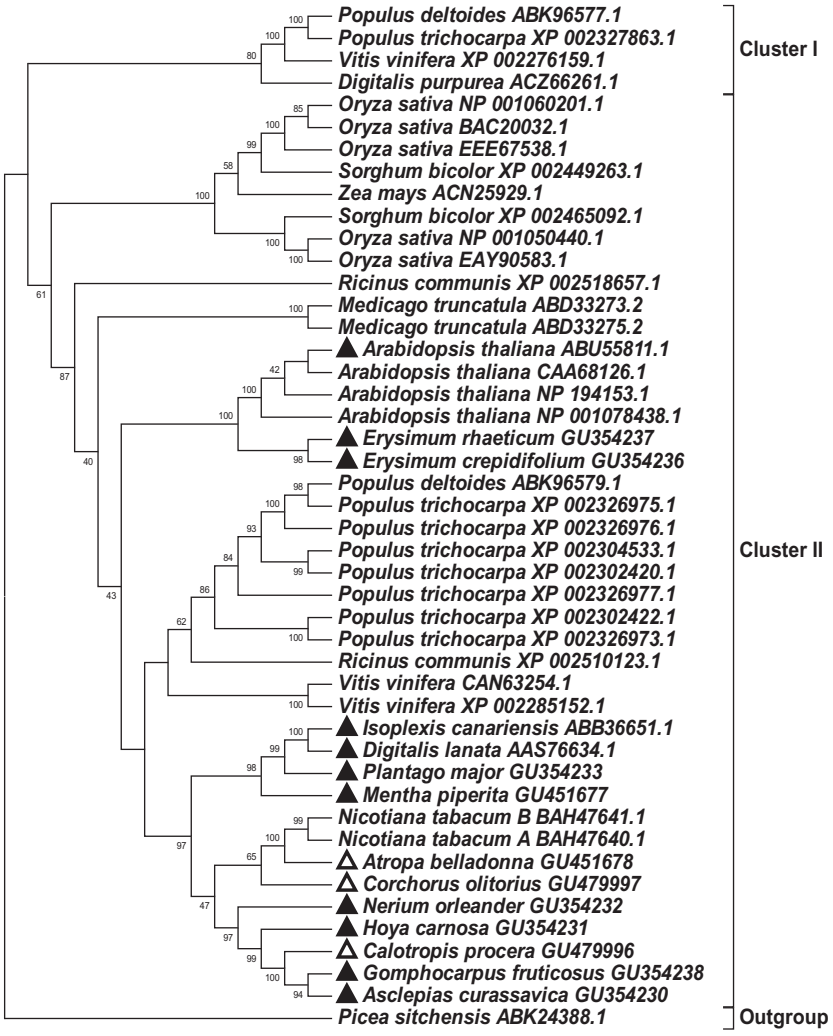

Fig. 3. Rooted neighbour-joining tree (Saitou and Nej, 1987) for P5βR-like enone reductases (strictly conserved motifs IV = NFYYxxED and V = TGxKxYxG). Data from protein blast search and the new sequences described here. The optimal tree with the sum of branch length = 5.13436521 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) greater than 40% are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 1050 positions in the final dataset. Cladistic analyses were conducted in MEGA4.1 (Tamura et al., 2007). Triangles (▲/△) indicate the species from which the new P5βR genes were cloned and the filled triangles (▲) indicate those species where the respective P5βR was functionally expressed. P5βR2 from *Digitalis purpurea* (ACZ66261) was included since it functionality as a progesterone 5β-reductase was shown although the motifs IV and V are slightly variant (Perez-Bermudez et al., 2010; Supplementary Fig. 2).

other one containing *Digitalis P5βR2* (Fig. 3). Although 5β-cardenolides are present in many angiosperms so far no entries (except for *Digitalis*) were found in the public databases indicating the presence of *P5βR* genes in these species. Therefore we here aimed at the isolation of *P5βR* genes from a set of 5β-cardenolide-producing species and close cardenolide-free relatives (Table 1) in order to address the question of relevance of progesterone 5β-reductases in 5β-cardenolide metabolism.

2.3. Isolation, cloning and sequence analysis of P5βR orthologues

The PCR amplification of putative *P5βR* genes was achieved with a set of primers deduced from GenBank™ entries for *Digitalis lanata P5βR* (Herl et al., 2006a), the *A. thaliana At5β-StR* (Herl et al., 2009) and the *enone reductase* gene of *Nicotiana tabacum* (Matsushima et al., 2008). We succeeded in the isolation of cDNAs from *Asclepias curassavica*, *Atropa belladonna*, *Calotropis procera*, *Corchorus olitorius*, *Erysimum crepidifolium* and *Erysimum rhaeticum*, *Gomphocarpus fruticosus*, *Hoya carnosa*, *Mentha piperita*, *Nerium oleander* and *Plantago major*. All new cDNA sequences reported here have been submitted to GenBank™ with the accession numbers listed in Table 1.

After sequencing of the cDNAs the corresponding protein sequences were deduced and aligned against the recombinant P5βR from *D. lanata* (Herl et al., 2006a). The deduced proteins share 68–96% sequence identity with the *D. lanata* P5βR and 50–54% identity with the *D. purpurea* P5βR2, respectively. A partial alignment presenting the SDR motifs I–VI of all proteins is shown in Table 2. Each of the genes isolated in the present study codes for enzymes containing all of these motifs. The full alignments (new P5βR gene products versus *D. lanata* P5βR and versus P5βR2, respectively) are provided as Supplementary Figs. 1 and 2.

Only 21 out of 85 deduced protein sequences obtained from a homology search (*e* values < e^{-70} compared to the *D. lanata* P5βR protein = ca. 50% identity) did not contain the conserved P5βR-specific variants of the motifs IV and V, namely NFYYxxED (containing the catalytic tyrosine residue) and TGxKxYxG (containing the cat-

Table 1
P5βR genes isolated here and those described previously with proof of function as progesterone 5β-reductase.

Species investigated	Order	Accession No.	ORF (nts)	5β-Cardenolides present	Functional P5βR
Arabidopsis thaliana[a]	Brassicales	EF579963	1167	–	+
Erysimum crepidifolium[b]	Brassicales	GU354236	1170	+	+
Erysimum rhaeticum[b]	Brassicales	GU354237	1170	+	+
Asclepias curassavica[b]	Gentianales	GU354230	1164	– (5α-Cardenolides)	+
Calotropis procera[b]	Gentianales	GU479996	1164	+	n.d.
Gomphocarpus fruticosus[b]	Gentianales	GU354238	1167	– (5α-Cardenolides)	+
Hoya carnosa[b]	Gentianales	GU354231	1164	–	+
Nerium oleander[b]	Gentianales	GU354232	1164	+	+
Digitalis lanata[c]	Lamiales	AY585867	1170	+	+
Digitalis purpurea[d]	Lamiales	AJ310673	1167	+	+
Digitalis purpurea[e]	Lamiales	GU062787	1182	+	+
Digitalis canariensis[f]	Lamiales	DQ218315	1170	+	+
Mentha piperita[b]	Lamiales	GU451677	1170	–	+
Plantago major[b]	Lamiales	GU354233	1199	–	+
Corchorus olitorius[b]	Malvales	GU479997	1164	+	n.d.
Atropa belladonna[b]	Solanales	GU451678	1164	–	n.d.
Nicotiana tabacum[g]	Solanales	AB488494	1170	–	+

n.d. – not determined.
[a] Herl et al., 2009.
[b] This paper.
[c] Herl et al., 2006a.
[d] Gavidia et al., 2007.
[e] Perez-Bermudez et al., 2010.
[f] Herl et al., 2006b.
[g] Matsushima et al., 2008.

Table 2
Alignment of the P5βR sequence motifs defined by Thorn et al. (2008) in the restricted short-chain dehydrogenase/reductases of the P5βR-like enone reductases. Conserved amino acids are highlighted. The numeration is given for orientation. The complete sequence alignments are shown in Supplementary Fig. 1.

Species	Motif I GxTGIxG	Motif II GxxRR	Motif III DxxD	Motif IV TgxKHYxGP	Motif V NFYYxxED	Motif VI WSVHRP
A. belladonna	31-GVTGIVG-39	58-GVARR-64	79-DISN-84	142-AGRKHYLGP-152	174-NFYYILED-183	195-WSVHRP-202
A. curassavica	32-GVTGIVG-40	59-GVARR-65	80-DIAN-85	143-TGGKHYCGP-153	175-NFYYSLED-184	196-WSVHRP-203
C. olitorius	32-GVTGIVG-40	59-GLARR-65	80-DISD-85	143-TGRKHYLGP-153	175-NFYYTLED-184	196-WSVHRP-203
C. procera	32-GVTGIVG-40	59-GVARR-65	80-DIAN-85	143-TGGKHYCGP-153	175-NFYYTLED-184	196-WSVHRP-204
D. canariensis	32-GVTGIIG-40	59-GVARR-65	80-DISD-85	143-TGRKHYMGP-153	176-NFYYDLED-185	197-WSVHRP-204
D. lanata	32-GVTGITG-40	59-GVARR-65	80-DISD-85	143-TGRKHYMGP-153	176-NFYYDLED-185	197-WSVHRP-204
E. crepidifolium	32-GVAGIVG-40	59-GVARR-65	80-DVSN-85	143-TGTKHYLGP-153	175-NFYYTQED-184	197-WSIHRP-204
E. rhaeticum	32-GVTGIVG-40	59-GVARR-65	80-DVSN-85	143-TGTKHYVGP-153	176-NFYYTQED-185	197-WSIHRP-204
G. fruticosus	33-GVTGIVG-41	60-GVARR-66	81-DIAN-86	144-TGGKHYCGP-154	176-NFYYTLED-185	197-WSVHRP-204
H. carnosa	32-GVTGIVG-40	59-GVARR-65	80-DIGN-85	143-TGGKHYAGP-153	175-NFYYTLED-184	196-WSVHRP-203
M. piperita	32-GVTGIVG-40	59-GVARR-65	80-DVSD-85	143-TGRKHYVGP-153	176-NFYYTLED-185	197-WSVHRP-204
N. oleander	32-GVTGIVG-40	59-GVARR-65	80-DISD-85	143-TGLKHYLGP-153	175-NFYYTLED-184	196-WSVHRP-203
P. major	32-GVTGIVG-40	59-GVARR-65	80-DISD-85	143-TGRKHYVGP-153	176-NFYYTQED-185	197-WSVHRP-204

alytic lysine residue). We also identified several *P5βR-like* genes by aligning *D. lanata* P5βR with proteins possessing higher *e* values but containing the conserved P5βR motifs IV and V.

The P5βRs deduced here and proteins possessing all P5βR motifs were used to investigate their relationship. It was tested whether structural similarity correlated with a phylogenetic relationship of the species included in this analysis. A possible evolutionary history was created using the neighbour-joining method (Saitou and Nej, 1987). The tree was rooted using *Picea sitchensis* as outgroup. This seemed appropriate because the respective *P. sitchensis* gene ABK 24338.1 also coded for a P5βR-like protein containing the conserved motifs IV (NFYYxxED) and V (TgxKxYxG) which we identified as characteristic for P5βR-like proteins (Fig. 3).

The cladogramme generated from all sequences showed that one cluster (Cluster II) of P5βRs correlated nicely with the assumed phylogenetic relationship of the species in the cluster. This implied that these genes have evolved from a common ancestral gene. A second cluster (Cluster I) contained *progesterone 5β-reductase* genes of *Populus*, *Vitis* and *D. purpurea*. The data set of Cluster I genes is too small to infer phylogenetic relationship. Interestingly, all three genera also contained paralogues in Cluster II. The occurrence of multiple forms of progesterone 5β-reductase genes in *Populus* and *Arabidopsis* with paralogues on different chromosomes is noteworthy. P5βRs (Cluster I) with a high degree of similarity and the motifs IV and V conserved existed in all 5β-cardenolide-containing genera investigated here (*Erysimum*, *Calotropis*, *Nerium*,

Corchorus) but also in plants containing 5α- but not 5β-cardenolides (*Gomphocarpus*, *Asclepias*) or even in cardenolide-free species (*Hoya*, *Mentha*, *Plantago*, *Atropa*).

Progesterone 5β-reductase genes coding for enzymes showing the conserved P5βR motifs IV and V, were up to now reported for 11 genera of angiosperms only. We here extended the list of genera shown to possess P5βR genes by another 10 genera. Most of the *P5βRs* isolated so far were from plant orders containing species capable of producing 5β-cardenolides (Fig. 3). However, the occurrence of *P5βR* genes seems not to be correlated with the occurrence of 5β-cardenolides. Especially the occurrence of *P5βR* homologues in gymnosperms is exciting since cardiac glycosides have so far not been reported from this class of seed plants. Although further experiments are required, our results and those of others (Perez-Bermudez et al., 2010) indicate that *P5βR* Cluster II orthologues may be present in many plant species.

2.4. Molecular modeling

Progesterone 5β-reductases are members of the large SDR super-family of enzymes (Jörnvall et al., 1995). All *P5βR* genes isolated here shared the characteristic features describing the new class of SDRs characterised by Thorn et al. (2008) on the basis of X-ray data. They can be classified as stereo-specific enone reductases capable of reducing activated C=C double bonds by a 1,4-addition mechanism. A hydride is transferred from NADPH to a carbonyl-activated C=C double bond and the carbonyl then protonated. In this way an enol intermediate is formed which tautomerises to the final product. Gavidia et al. (2007) erroneously suggested that the second tyrosine residue from the NFYYxxED motif acts as the catalytic base but only the experimentally determined crystal structure combined with site-directed mutagenesis (Thorn et al., 2008) revealed that it is the first tyrosine that is crucial for catalysis. Experiments in which the catalytic tyrosine residue was replaced by alanine or phenylalanine completely inactivated the protein (Thorn et al., 2008) whereas other mutations in the NFYYxxED motif did not (own unpublished results).

We here modeled the active sites and amino acid residues of the substrate-binding site using *D. lanata* P5βR (2V6G) as well as the progesterone-docked *D. lanata* structure as templates. The resulting complexes agree nicely with the docking experiments performed earlier (Herl et al., 2009) and are consistent with the expected reaction mechanism (Thorn et al., 2008). For each complex, a total of 16 amino acids located within 4.5 Å of either any progesterone atoms or atoms from the ribose and nicotine amide moiety of the co-substrate NADPH were considered as participating in the substrate-binding site and thereby contributing to substrate specificity (Table 2). As in the previously characterised P5βR-like proteins conserved Y179 and K147 residues were located in the active site. Interestingly, we found another five conserved amino acids within this pocket, namely W106, G145, F153, M215 and F353 (Fig. 4). G145, K147 and Y179 are amino acids displayed from the characteristic motifs IV and V (Table 3). On the other hand, W106, F153, M215 and F353 do not appear in any of the known sequence motifs but at the same time have a pronounced effect on the shape of the binding site. The conserved amino acids cluster around those parts of NADPH and progesterone that participate in the stereo-selective C=C double bond reduction in this way forming the conserved apex of an otherwise variable cone. No conserved amino acids could be found in the more remote parts of the catalytic pocket. To summarise, the enzymes under investigation not only are highly conserved with regard to certain motifs but also with regard to several individual amino acids in the catalytic/substrate-binding site.

When looking at the structure of the catalytic/substrate-binding site numerous indications can be found that progesterone is not the unique substrate for P5βRs *in vivo*, as was already deduced from the enzyme activity data (Herl et al., 2006a; Burda et al., 2009; Schebitz et al., 2010). For example, when docking progesterone into the substrate-binding site it becomes apparent that the bottom of this site is quite hydrophilic. This polarity is not mirrored in progesterone. In some of the P5βRs asparagine-205 is replaced by methionine and indeed a N205M mutant of a recombinant *D. lanata* P5βR shows an increased progesterone reductase activity when compared to wild type (data not shown). On the other hand, an asparagine residue is found at this position in many cardenolide-producing species. Hence, these P5βRs seem not to be optimised for the conversion of progesterone. These structural findings lead to the hypothesis that Cluster II P5βRs are substrate-promiscuous enone reductases. We suggest that these enzymes may be regarded as "pontoon" elements which can be utilised in various biochemical pathways or may contribute to

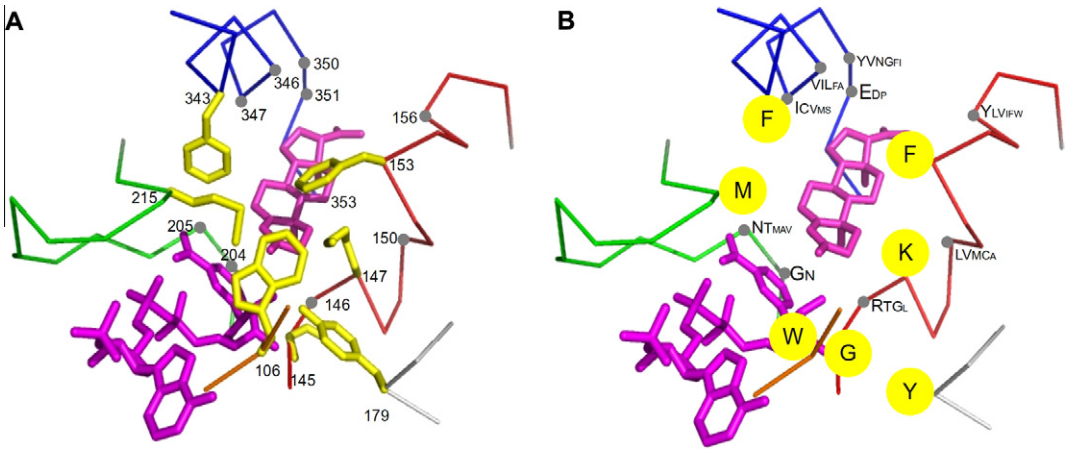

Fig. 4. (A) Catalytic pocket of P5βR-like enone reductases modeled on the crystal structure 2V6G (*D. lanata* P5βR). All amino acids located within 4.5 Å of either any progesterone atoms or atoms from the ribose and nicotine amide moiety of the cofactor NADPH were considered as participating in the substrate-binding site. In addition to the catalytic Y179 and K147 residues another five conserved amino acids could be identified within this pocket, namely W106, G145, F153, M215 and F353. The amino acids suggested to be part of the binding pocket are shown in Table 2. B. The frequency of occurrence of a certain amino acid in the variable parts of the pocket is symbolised by the font size used. Colours: conserved amino acids are shown (A) or highlighted (B) yellow. Green, red, blue, orange and grey lines indicate the different parts of the protein backbone contributing to the substrate-binding pocket. Substrate (progesterone) and co-substrate (NADPH) are shown in magenta.

Table 3
Amino acids suggested to be part of the binding pocket of P5βR-like enone reductases. All of the protein sequences deduced here are shown together with those of the P5βRs of *A. thaliana* (Herl et al., 2009), *D. lanata* (Herl et al., 2006a) and *D. canariensis* (Herl et al., 2006b). Structurally conserved amino acids are highlighted.

	Amino acid residue (numerations as for *D. lanata*)																
Species	106	145	146	147	150	153	156	179	204	205	215	343	346	347	350	351	353
A. belladonna	W	G	R	K	L	F	Y	Y	G	T	M	F	L	I	G	D	P
A. curassavica	W	G	G	K	C	F	Y	Y	G	N	M	F	I	C	Y	E	A
A. thaliana	W	G	T	K	L	F	V	Y	N	M	M	F	V	I	V	E	M
D. canariensis	W	G	R	K	M	F	Y	Y	G	N	M	F	V	I	N	E	F
D. lanata	W	G	R	K	M	F	Y	Y	G	N	M	F	V	I	N	E	F
E.crepidifolium	W	G	T	K	L	F	L	Y	N	T	M	F	V	I	V	E	M
E. rhaeticum	W	G	T	K	V	F	L	Y	N	T	M	F	V	I	V	E	M
G. fruticosus	W	G	G	K	C	F	Y	Y	G	N	M	F	I	C	Y	E	A
H. carnosa	W	G	G	K	A	F	W	Y	G	N	M	F	L	C	Y	E	A
M. piperita	W	G	R	K	V	F	V	Y	G	A	M	F	F	S	Y	E	P
N. oleander	W	G	L	K	L	F	F	Y	G	N	M	F	I	V	F	E	Q
P. major	W	G	R	K	V	F	I	Y	G	N	M	F	A	V	I	P	P

occult biosynthetic capacities recently defined as "silent metabolism" (Lewinsohn and Gijzen, 2009).

2.5. Heterologous expression of P5βR genes

The conditions described previously for a recombinant form of *D. lanata* P5βR (Herl et al., 2006a) were applied and optimised for expression of the new *P5βR* genes. The recombinant enzymes were Ni–NTA-purified and analysed for functionality applying the methods described earlier (Herl et al., 2006a,b, 2008, 2009). Showing data for r*Mp*P5βR, a recombinant form of *M. piperita* P5βR, Fig. 5 exemplifies the data sets collected for each expression experiment, including SDS PAGE, GC–MS and TLC analysis. TLC was used as a simple and fast screening tool whereas GC–MS analysis proved that only 5β-configured pregnanes were formed (Fig. 5).

Eight *P5βR* orthologues isolated from cardenolide-free plants as well as from cardenolide-containing (either 5β- or 5α-cardenolides) plants were successfully expressed in *E. coli* and demonstrated to reduce progesterone enantio-specifically to 5β-pregnane-3,20-dione. Some of the P5βR proteins obtained from cardenolide-free plants proved to be even more active towards progesterone than the respective proteins from cardenolide-containing plants (data not shown). *P5βR* homologues were expected to occur in 5β-cardenolide-free plants since the biochemical characterisation of the *VEP1* gene product of *A. thaliana* already indicated that P5βR enzymes from non-cardenolide plants can be more efficient than the *Digitalis* P5βR-like enone reductases investigated so far (Herl et al., 2009). The activity data presented here confirm these findings (Table 4).

When investigating substrate preferences and kinetic properties of r*Dl*P5βR, the recombinant, His-tagged form of a *D. lanata* P5βR, it was found that not only progesterone but also testosterone, 4-androstene-3,17-dione, cortisol, cortisone, canarigenone and 23-nor-4,20(22)*E*-choladienic acid-3-one (Herl et al., 2006a; Schebitz et al., 2010) were accepted as substrates. It has been demonstrated that the P5βR of *A. thaliana* is capable of reducing small molecules bearing an activated C=C double bond such as monocyclic enones and acyclic enoate esters. The essential structural element for substrates of P5βRs seems to be an activated C=C double bond. This structural element is present in several natural products, such as isopiperitenone and pulegone (menthol biosynthesis), which all have a biosynthetic counterpart with the C=C double bond eliminated. We therefore suggest that the P5βRs described so far should be designated "enone reductases" like the one isolated from tobacco cell cultures (Matsushima et al., 2008). This particular enone reductase contains all structural requirements to qualify it as a member of the P5βR-like SDRs of the P5βR Cluster II.

The substrate preferences of two of the recombinant P5βRs (those from *A. thaliana* and *Digitalis canariensis*) were investigated and compared. Both enzymes accepted progesterone and a broad variety of small cyclic and acyclic substrates bearing an activated C=C double bond (Table 4). The recombinant *A. thaliana* P5βR reduced all substrates ca. 10 times faster than the *D. canariensis* enzyme. This corroborates previous findings using steroid substrates only (Herl et al., 2009). Otherwise, the substrate preferences of the two P5βRs tested here were very similar. With both enzymes very high activities were measured for 2-cyclohexenone which represents a monocyclic substructure of progesterone. But-1-en-3-one, which may be regarded as the smallest possible mimic of progesterone, was reduced very efficiently, exceeding the relative activity measured for progesterone by a factor of 28. The high enantioselectivity of the reaction, as demonstrated here for progesterone, could also be seen with isophorone (ee > 99%), a very small prochiral progesterone mimic (Burda et al., 2009). It is assumed that all P5βRs of the Cluster II are substrate-promiscuous enzymes and that progesterone cannot be considered a unique substrate.

Kinetic constants of the new enzymes described here have not been collected as yet but have already been published for the P5βR-like enone reductases of, e.g., *D. lanata*, *D. purpurea*, *D. canariensis* and *A. thaliana* (Herl et al., 2006a,b, 2009; Gavidia et al., 2007). We are aware of the fact that even the detailed biochemical

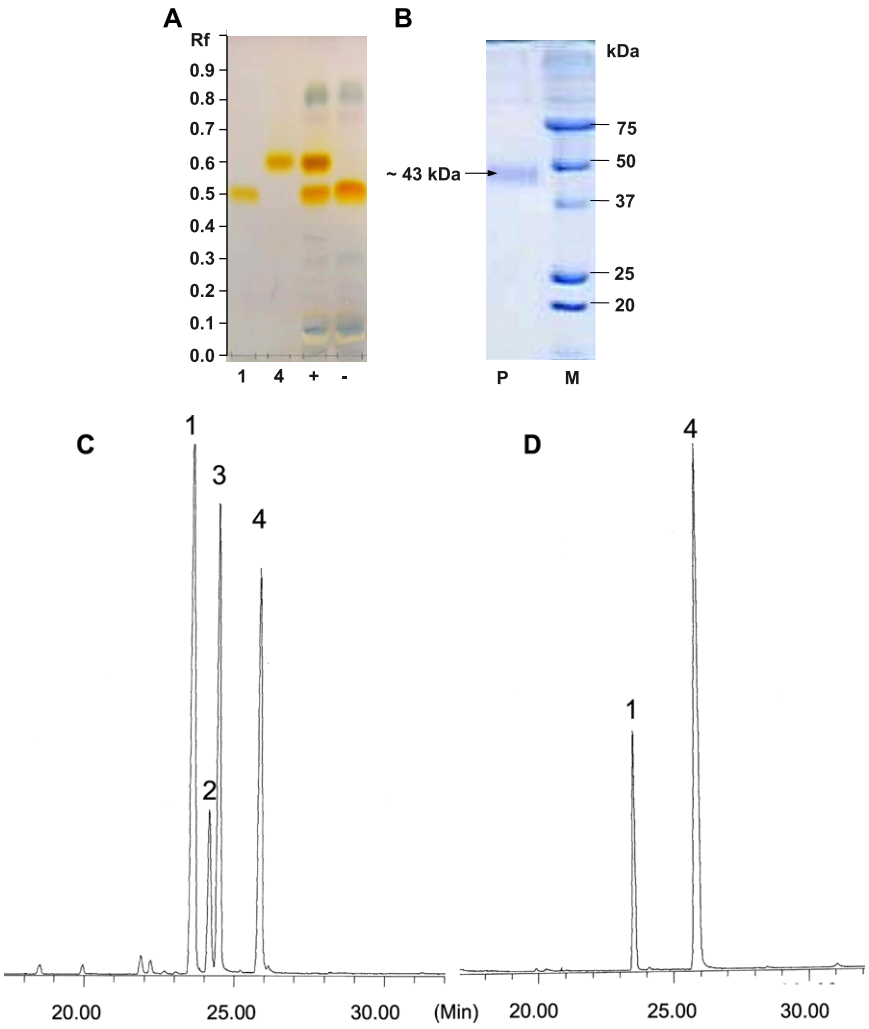

Fig. 5. Routine analysis exemplified with recombinant *Mentha piperita* P5βR (r*Mp*P5βR). (A) TLC analysis of P5βR activity: 1 – reference progesterone (1; *R*f = 0.5), 4 – reference 5β-pregnane-3,20-dione (2; *R*f = 0.6);+ – enzyme assay with the Ni–NTA-purified r*Mp*P5βR after 2 h; – – heat-inactivated control. (B) Expression of r*Mp*P5βR in *E. coli* analysed on SDS PAGE (12%). M – molecular weight marker. P – purified r*Mp*P5βR after Ni–NTA affinity chromatography. An arrow marks the recombinant protein at ~43 kDa. (C) GC–MS analysis of a standard mixture of 1 – 5β-pregnane-3,20-dione, *R*t = 23.70 min; 2 – isoprogesterone, *R*t = 24.19 min; 3–5α-pregnane-3,20-dione, *R*t = 24.55 min; 4–progesterone, *R*t = 25.93 min. (D) P5βR enzyme assay (2 h) – Enantio-selective reduction of progesterone using Ni–NTA-purified r*Mp*P5βR.

Table 4
Relative and specific activities of P5βRs from *D. canariensis* and *A. thaliana* towards various substrates.

	A. thaliana Enzyme activity		*D. canariensis* Enzyme activity	
	Relative (%)	Specific μkat/kg protein	Relative (%)	Specific μkat/kg protein
Progesterone	100	620	100	50.1
2-Cyclohexenone	1298	8048	1204	602.0
3-Methyl-2-cyclohexenone	19	118	52	26.1
But-1-en-3-one	2783	17255	Not determined	
3,5,5-Trimethyl-2-cyclohexenone	12	74	86	43.1
Ethyl acrylate	75	467	61	30.6
Ethylmethacrylate	64	397	64	32.1

characterisation of a given enzyme is not sufficient for deducing its metabolic role. However, the occurrence of closely related functional P5βRs in 5β-cardenolide-free as well as in 5β-cardenolide-containing plants lets us presume that Cluster II P5βRs are involved in more than just 5β-cardenolide biosynthesis. One may even challenge the role of P5βR in the cardenolide pathway(s) and the exact physiological role of P5βR still remains to be elucidated. Since substrate preferences of P5βR2s (Perez-Bermudez et al., 2010) have not been reported so far, only weak arguments for a more specific role of this particular enzyme in the formation of 5β-cardenolides have been provided as yet. Future knock-down or knock-out studies may help to solve this issue. Analysing *P5βR* genes of cardenolide-free mutants of cardenolide-producing species may also help to address this question. As far as the potential biotechnological application of enantio-selective enone reductases is concerned the structural data collected here will help to design P5βRs with improved or variant catalytic properties. The biochemical characterisation of some of the P5βRs is in progress.

3. Experimental

3.1. Plant material, chemicals, recombinant enzymes

Seeds, seedlings and plants were provided by the Botanic Garden of the University of Erlangen and identified by Walter Welß.

Specimens were deposited in the Erlangen University Herbarium. Plants were grown in the greenhouse until they produced sufficient leaf material for RNA extraction. They were watered every other day and supplemented once a week with nutrient solution (WUXAL® Universaldünger, Wilhelm Haug, Ammerbuch, Germany).

Steroids were purchased from Steraloids (Newport, USA). Isoprogesterone was synthesised according to Djerassi et al. (1956). All other chemicals were obtained from Sigma (Munich, Germany) or Merck (Darmstadt, Germany).

Recombinant forms of the P5βRs from *A. thaliana* and *D.* (*Isoplexis*) *canariensis* were prepared as described by Herl et al. (2006a,b) and Burda et al. (2009).

3.2. RNA extraction, cDNA synthesis and PCR

Only fresh, young leaves were used for RNA isolation. The leaves were frozen with liquid nitrogen and ground to a powder using mortar and pestle. Complete RNA isolation was carried out using a innuPREP Plant RNA Kit (Analytik Jena AG, Jena, Germany) following the manufacturer's instructions. We continued to use the PL buffer provided in the kit, because it yielded larger amounts of higher quality RNA. This fact was proven by photometric analysis with a NANODROP ND-1000 spectrophotometer.

The synthesis of P5βR cDNA was performed employing SuperScript™ III First-Strand Synthesis for reverse transcription PCR (RT-PCR) Kit (Invitrogen, Karlsruhe, Germany). RT-PCR was carried out in a Personal Cycler 20 (Biometra GmbH, Göttingen, Germany) according to the manufacturer's recommendation. Each reaction (50 μl total volume) contained 2.5 units peqGold *Taq* DNA Polymerase (Peqlab GmbH, Erlangen, Germany), 1× reaction buffer S, 0.5 mM of each dNTP, 2 mM of appropriate primers and 0.2 μg of the respective cDNA.

For standard PCR the following amplification programme was used: an initial denaturation at 94 °C for 5 min was followed by 30 cycles of a 20 s denaturation period at 94 °C, 1 min annealing at 50 °C and 2 min extension at 72 °C. Finally a 10 min extension at 72 °C was added to complete the amplification. PCR products were analysed by 1% agarose gel electrophoresis in a TAE buffer system. Gels were stained with ethidium bromide and visualised by illumination at UV_{365nm}. For size determination SmartLadder (Eurogentec GmbH, Köln, Germany) was used, producing a pattern of 14 regularly spaced bands ranging from 200 to 10.000 bp.

Degenerated as well as standard primers were deduced from the GenBank™ entries of the known P5βR-like genes from *A. thaliana* (AY062451.1), *D. lanata* (AAS93804.1) and *N. tabacum* (BAH47640.1). Different combinations of primers were tested in order to gain a specific PCR product for each gene isolation/cloning experiment. Finally, the full-length cDNA clones were amplified using the following primers: Soldir (ATG AGC TGG TGG GCT GG) and Solrev (CTA AGG AAC AAC TTT GTA GGC) for *A. belladonna*; Erydir (ATG AGT TGG TGG GGG) and Erysalrev (TAT AGT CGA CTG GAA ATC AAG GCA CGA TCT) for *E. crepidifolium* and *E. rhaeticum*; Gendir (ATG AGY TGG TGG TGG GCT) and Genrev (AGG AAC AAT CTT GTA AGC CTT) for *C. procera*, *G. fruticosus*, *C. olitorius*, *M. piperita*; Gengatedir (CAAC ATG AGY TGG TGG TGG GCT) and Gengaterev (CTA AGG AAC AAT CTT GTA AGC CTT) for *P. major*, *H. carnosa*, *A. curassavica*, *N. oleander*.

3.3. Subcloning and sequencing of PCR products using the pQE30UA vector system

For subcloning RT-PCR products were purified by 1% agarose gel electrophoresis using the "freeze and squeeze" technique (Thuring et al., 1975). The gel slice containing the desired DNA fragment was cut out under UV_{365nm} after straining with ethidium bromide and frozen at −80 °C for 5 min. The DNA was squeezed out of this gel fragment into a 1.5 ml Eppendorf tube and precipitated with 75% (v/v) isopropyl alcohol at 20 °C for 30 min. After centrifugation (12,000*g*, 15 min), the DNA pellet was washed with 70% ice-cold ethanol, again centrifuged and finally dried and re-dissolved in sterile double-distilled water.

The cloning of the purified DNA fragment into the pQE30UA vector system was performed following the instructions of the manufacturer (QIAexpressionist™, Hilden, Germany). A vector to insert ratio of 1:10 was chosen in the subsequent ligation step. Ligation was achieved over night at 16 °C. Plasmids were transferred to chemical competent *E. coli* M15 [pREP4] cells. Transformed cell colonies were selected on plates containing ampicillin and kanamycin.

The colonies were screened by colony PCR (Alshahni et al., 2009) using insert specific primers and the following PCR programme: Initial denaturation (95 °C/10 min), denaturation (95 °C/30 s), annealing (50 °C/1 min), elongation (72 °C/2 min) for 30 cycles and a final elongation step of 10 min. Plasmid DNA was isolated form positive clones using the peqGold Plasmid Miniprep Kit (Peqlab GmbH, Erlangen, Germany) and then sequenced by GATC Biotech AG (Konstanz, Germany).

3.4. Subcloning and sequencing of PCR products using the Gateway® Recombination Cloning system

In one set of experiments PCR products were cloned into an entry vector for the Gateway® recombination system following the manufacturer's instruction (Invitrogen, Karlsruhe, Germany). pENTR/D-TOPO, the directional TOPO entry vector was chosen for creating the entry clone. The sequences of the primers used for the RT-PCR contained the sites essential for the respective cloning procedure (Gengatediv: CTA AGG AAC AAT CTT GTA ACC TT and Gengaterer: CAA CAT GAG YTG GTG GTG GGC T). Destination cloning into the pDEST 17 vector was carried out following the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). All sequences obtained were analysed for correct orientation and reading frame. The appropriate constructs were transferred to chemically competent *E. coli* DH5α cells for maintenance, amplification and later on for expression in *E. coli* BL21(DE3).

3.5. Over-expression of P5βR cDNA cloned in pQE30UA or DESTINY vector in E. coli

Bacteria were incubated at 37 °C to an OD_{600} of 0.5–0.7. Isopropyl-β-D-thiogalactoside (IPTG) was added to induce expression of the recombinant His-tagged P5βR. Cultivation at 37 °C for up to 6 h (1 mM IPTG) as well as cultivation at 4 °C for up to 120 h (0.1 mM IPTG) was tested, before the His-tagged P5βR was isolated in its native form via batch purification using Ni–NTA (nickel–nitrilotriacetic acid) agarose following the procedure described in the manufacturer's instruction (QIAexpressionist™, Hilden, Germany). The Ni–NTA matrix was washed extensively with 20 mM imidazole buffer before affinity-bound proteins were eluted with 250 mM imidazole buffer. After purification of the recombinant P5βR the reaction buffer containing 100 mM HEPES-KOH (pH 8.0), 250 mM sucrose, 2 mM EDTA and 10 mM β-mercaptoethanol in PD-10 columns (GE Healthcare, Munich, Germany) replaced the elution buffer. Protein was quantified according to Bradford (1976). Protein analysis on SDS PAGE was performed as reported earlier (Müller-Uri and Reva, 2000).

3.6. Measuring progesterone 5β-reductase activity

To demonstrate progesterone 5β-reductase activity the method described by Stuhlemmer and Kreis (1996) and modified by Herl et al. (2006a) was used. In a final volume of 1000 μl the standard enzyme assay contained: 945 μl (200 μg/ml) purified protein,

6.4 mM $NADP^+$, 32.1 mM glucose 6-phosphate, 42 nkat glucose-6-phosphate dehydrogenase and either 0.3 mM (for TLC analysis) or 0.5 mM (for GC–MS analysis) progesterone. Heat-inactivated samples (10 min, 100 °C) served as controls. The mixture was incubated in 2 ml Eppendorf tubes at 40 °C for 2 h prior to extraction with 1000 µl dichloromethane. The organic phase was evaporated, the residue was dissolved in 50 µl methanol (for TLC) or 100 µl dichloromethane (for GC–MS) and then analysed. Activity tests were carried out three times or more.

Progesterone 5β-reductase activity was determined semiquantitatively using TLC which was carried out as described by Herl et al. (2006a). For product identification samples were analysed by GC Hewlett–Packard HP 6890 MSD Type 5890A in IE mode using helium as the carrier gas (constant flow 1 ml/min). A fused capillary column HP Optima five coated with 5% phenyl/95% dimethyl polysiloxane (30 m × 25 mm × 0.25 mm) was used. The GC column temperature was programmed from 200 °C (initial equilibrium time 4 min) to 300 °C at a ramp of 4 °C/min and then maintained at 300 °C for 6 min. The MSD was operated in full-scan mode from m/z = 50–600. The inlet and MS transfer line temperatures were maintained at 300 °C. Sample injection (2 µl) was in splitless mode. Pregnanes were identified by comparing their *R*t and fragmentation patterns with those of authentic compounds.

Spectrophotometric measurement of progesterone 5β-reductase activity was carried out as described by Burda et al. (2009). In contrast to the general procedure described above no NADPH-regenerating was added to the enzyme assay. The conversion of NADPH to $NADP^+$ was followed at 340 nm in the presence of the respective substrates (10 mM) and the co-substrate (12.5 mM). Relative activities towards the various substrates were determined by comparing the amount of $NADP^+$ released from NADPH with the activity measured for progesterone which was set to equal 100%.

3.7. In silico analysis

After sequencing the PCR products (GATC AG, Konstanz, Germany), the data were analysed using appropriate software packages (European Bioinformatics Institute). BLAST search of the GenBank™ database (<http://blast.ncbi.nlm.nih.gov/BLAST.cgi>) was used for searching and sequence analysis. The nucleotide sequences were translated into their corresponding amino acid sequence employing <http://www.expasy.ch/tools/dna.html>. Both of them were aligned using ClustalW (<http://www.ebi.ac.uk/clustalw/index.html>). Cladogrammes were constructed with the MEGA4.1 software package (Tamura et al., 2007). SWISS-MODEL (<http://swissmodel.expasy.org>) was used for protein structure homology modeling. The three-dimensional structural drawings were created with PyMOL (<http://www.pymol.org>).

Acknowledgements

We thank Gabriele Fischer for excellent technical assistance, Nadine Meitinger for a sample of isoprogesterone and Barbara White for linguistic advice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.06.004.

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