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Recombinant $\Delta^{4,5}$-Steroid 5β-Reductases as Biocatalysts for the Reduction of Activated C=C-Double Bonds in Monocyclic and Acyclic Molecules

Edyta Burda,[a] Marina Kraußer,[a] Gabriele Fischer,[b] Werner Hummel,[c] Frieder Müller-Uri,[b] Wolfgang Kreis,[b,*] and Harald Gröger[a,*]

[a] Department of Chemistry and Pharmacy, University of Erlangen-Nuremberg, Henkestr. 42, 91054 Erlangen, Germany
Fax: (+49)-9131-85-21165; phone: (+49)-9131-85-22554; e-mail: harald.groeger@chemie.uni-erlangen.de
[b] Department of Biology, University of Erlangen-Nuremberg, Staudtstr. 5, 91058 Erlangen, Germany
Fax: (+49)-9131-85-28243; phone: (+49)-9131-85-28241; e-mail: wkreis@biologie.uni-erlangen.de
[c] Institute of Molecular Enzyme Technology at the Heinrich-Heine-University of Düsseldorf, Research Centre Jülich
Stetternicher Forst, 52426 Jülich, Germany

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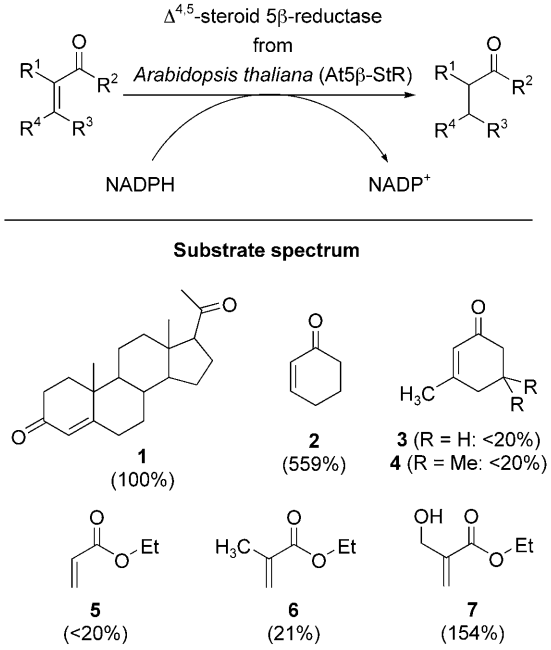
Abstract: It was found that $\Delta^{4,5}$-steroid 5β-reductases are capable of reducing also small molecules bearing an activated C=C double bond such as monocyclic enones and acyclic enoate esters. As preferred $\Delta^{4,5}$-steroid 5β-reductase (5β-StR) for this purpose, 5β-StR from *Arabidopsis thaliana* was used. In part, enzyme activities are even higher than that for progesterone. Successful preliminary biotransformations with enzymatic *in situ* cofactor recycling were also carried out. When using the prochiral compound isophorone as a substrate, a high enantioselective reaction course (>99% *ee*) was observed.

Keywords: alkenes; biotransformations; enoate reductases; enzyme catalysis; ketones; reduction

The biocatalytic reduction of activated C=C double bonds represents an enzymatic reaction with high application potential in organic synthesis.[1] By means of such a reaction type one can obtain access to chiral aldehydes and ketones,[2] carboxylates,[2c,3] and nitroalkanes.[2c,4] However, in contrast to the reduction of C=O double bonds of ketones,[5] aldehydes,[6] and keto esters[7] with alcohol dehydrogenases, the field of biocatalytic C=C-reduction is still poorly explored.[8] This is in particular due to the limited number of readily available and sufficiently stable recombinant enoate reductases. The workhorses in this field are enoate reductases from lower fungi and bacteria,[1] which show a broad substrate range, are available in recombinant form, and have been applied already in efficient reduction processes. This has been demonstrated, e.g., by impressive contributions from Shimizu et al.[2a,b] and Stewart et al.[2d,4a] who applied these enzymes in various C=C double bond reductions. In spite of the high suitability of these enoate reductases, there is an increasing demand for further enzymes being capable of reducing C=C double bonds, thus expanding the diversity of biocatalysts for this important reaction. An elegant step towards this direction has been recently reported, e.g., by Faber et al. demonstrating the suitability of a 12-oxophytodienoate reductase from tomato as an (air-stable) enoate reductase in organic syntheses.[2c,9,10] This example also shows that enzymes of plant origin can be used as efficient biocatalysts in organic synthesis. In this context we now communicate our preliminary results on the suitability of $\Delta^{4,5}$-steroid 5β-reductases of plant origin for the reduction of activated C=C double bonds in small monocyclic and acyclic molecules.

Recently, a full-length cDNA clone encoding a $\Delta^{4,5}$-steroid 5β-reductase (At5β-StR, EC 1.1.1.145/1.3.1.23), a member of the short-chain dehydrogenase/reductase (SDR) family, was isolated from *Arabidopsis thaliana* leaves and expressed in *Escherichia coli*.[11] A three-dimensional model of the enzyme highlights its close structural similarity to the previously described *Digitalis lanata* progesterone 5β-reductase for which the crystal structure was determined.[12] Referring to plant steroid reductases we were interested in investigating the suitability of these enzymes to accept smaller molecules bearing an activated C=C double bond. For our studies we used recombinant $\Delta^{4,5}$-steroid 5β-reductases from *Digitalis* (*Isoplexis*) *canariensis*[13] and *A. thaliana*.[11] The latter enzyme shows an activity of 0.4 U per mg of protein for progesterone,[11] thus making it interesting for organic synthetic purposes.

The investigation of the substrate spectrum has been carried out by means of a spectrophotometrical



Scheme 1. Substrate spectrum of At5β-StR (for this enzyme a specific activity of 0.4 U/mg was reported when using compound **1** as a substrate, see ref.[11]).

study, measuring the consumption of the cofactor depending on the type of substrate (according to Scheme 1). We found that (in contrast to the typically expected narrow substrate range of steroid-transforming enzymes) the recombinant $\Delta^{4,5}$-steroid 5β-reductase from *A. thaliana* (At5β-StR) shows an unexpectedly broad substrate spectrum (Scheme 1). Notably, small monocyclic and even acyclic substrates bearing an activated C=C double bond were accepted. A high relative enzyme activity of 559% was measured for 2-cyclohexenone (**2**), which exceeds significantly the one measured for progesterone (**1**, 100%).

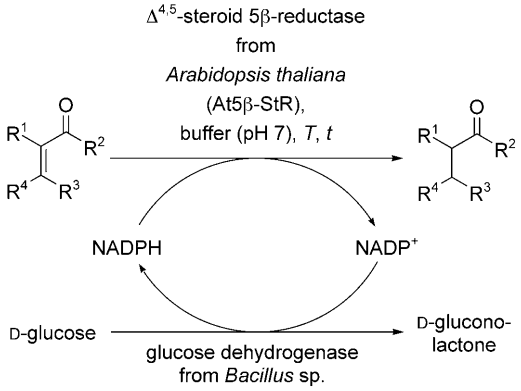
In contrast, for 3-methyl-substituted cyclohexenones as substrates low enzyme activities were observed with <20% for both 3-methyl-2-cyclohexenone (**3**) and 3,5,5-trimethyl-2-cyclohexenone (isophorone, **4**). The small monocyclic molecules **2** and **3** are of particular interest from a mechanistic perspective since both compounds can be considered as simple monocyclic mimics of progesterone (**1**) and represent substructures of **1**. Thus, the large deviation of the obtained enzyme activities for substrates **2** and **3** is surprising and will be subject to molecular modelling studies. Furthermore, we were interested if At5β-StR from *A. thaliana* also accepts acyclic compounds, in particular α,β-unsaturated enoate esters. However, using simple acrylate esters such as ethyl acrylate (**5**) and ethyl methacrylate (**6**) resulted in low enzyme activities of <20% and 21%, respectively. It is noteworthy that the presence of a hydroxy group led to an increase of enzymatic activity. When using hydroxylated methacrylate ester **7** (prepared *via* Baylis–Hillman reaction of ethyl acrylate and formaldehyde) as a substrate, an increased enzymatic activity of 154% was observed. This result is also interesting from a synthetic perspective since it shows the suitability of Baylis–Hillman products for enzymatic enoate reduction with At5β-StR. Furthermore, this study represents one of the rare examples demonstrating the capability of steroid reductases to accept small monocyclic and acyclic molecules bearing a C=C double bond in conjugation to an electron-withdrawing group as substrates.[14] Thus, it appears that steroid reductases, in particular progesterone reductases of plant origin, possess a broader substrate spectrum than widely expected.

Encouraged by the positive photometer experiments we subsequently studied the application of At5β-StR from *A. thaliana* as biocatalyst in preparative biotransformations with *in situ* cofactor regeneration. *In situ* regeneration[5] of the cofactor NADPH (which was used in a catalytic amount) was carried out using a glucose dehydrogenase under oxidation of D-glucose to D-gluconolactone, thus regenerating the required reduced form of the cofactor, NADPH. The reduction concept with At5β-StR from *A. thaliana* is shown in Table 1. When using 2-cyclohexenone (**2**) as substrate, reduction proceeded smoothly under formation of cyclohexanone with >95% conversion at 30–34 °C (Table 1, entry 1). This biotransformation underlines the suitability of this enzyme to reduce also non-steroid-type molecules on a preparative scale.

A reaction at a somewhat elevated reaction temperature of 40–41 °C gave similar results (entry 2). Besides At5β-StR from *A. thaliana* we identified a $\Delta^{4,5}$-steroid 5β-reductase from *Digitalis* (*Isoplexis*) *canariensis*[13] as a further steroid reductase from a plant to be suitable for the reduction of 2-cyclohexenone, leading to cyclohexanone with a high conversion of >95% as well (entry 3). It should be added that the $\Delta^{4,5}$-steroid 5β-reductase from *D. (I.) canariensis* was also successfully expressed in *E. coli*,[13] thus being available in the advantageous recombinant form, too.

As an example for substituted cyclohexenones, isophorone (**4**) was used as a substrate. The reduction also proceeded, but a low conversion of 14% was observed in the presence of At5β-StR from *A. thaliana* as a biocatalyst (entry 4). This result is in accordance with the lower enzyme activity for **4** (in comparison with **2**, see Scheme 1). This transformation of the prochiral compound **4** into chiral 3,3,5-trimethyl-2-cyclohexanone also enabled us to study the potential of At5β-StR from *A. thaliana* to catalyze reductions of activated C=C double bonds in an enantioselective fashion. We were pleased to find that the reduction of **4** proceeded highly enantioselectively in the presence of At5β-StR from *A. thaliana*, leading to 3,3,5-tri-

Table 1. Preparative biotransformations.



Entry[a]	R^1	R^2	R^3	R^4	T [°C]	Conversion [%]	*ee* [%]
1	H	-$(CH_2)_3$-		H	30–34	>95	–
2	H	-$(CH_2)_3$-		H	40–41	>95	–
3[b]	H	-$(CH_2)_3$-		H	30	>95	–
4	H	-$CH_2CMe_2CH_2$-		CH_3	30	14	>99
5	CH_2OH	OEt	H	H	r.t.[c]	15	n.d.[d]

[a] For general protocol, see Experimental Section; reaction time (t) was 24 h for entries 1 and 3, and 48 h for entries 2, 4 and 5.
[b] Steroid reductase from *Digitalis* (*Isoplexis*) *canariensis* was used in this experiment as enoate reductase.
[c] r.t. = room temperature.
[d] n.d. = not determined.

methyl-2-cyclohexanone with an enantiomeric excess of >99% *ee* (entry 4).

The acyclic Baylis–Hillman product **7** was also reduced by means of the At5β-StR from *A. thaliana*, although in this preliminary experiment conversion was low with 15% (Table 1, entry 5).

In conclusion, our studies revealed that, besides steroids as substrates, $\Delta^{4,5}$-steroid 5β-reductases are also capable of reducing small molecules bearing an activated C=C double bond such as monocyclic enones and acyclic enoate esters. As preferred steroid reductase for this purpose, At5β-StR from *A. thaliana* was used. In part, higher enzyme activities have been found than the one for the steroid substrate progesterone. Successful preliminary biotransformations with enzymatic *in situ* cofactor recycling confirmed the synthetic potential of steroid reductases for small molecules with an activated C=C-double bond. Currently, investigations of scope and limitations of these types of reduction processes with At5β-StR from *A. thaliana* are in progress with a focus on the development of processes with high volumetric productivities.

Experimental Section

Protocol for the Spectrophotometric Measurement of Enzyme Activity (According to Scheme 1)

The enzymatic activity was measured spectrophotometrically as the consumption of NADPH through oxidation to $NADP^+$ at a wavelength of 340 nm in the presence of the substrate ($\varepsilon_{340} = 6.3\ mmol^{-1}cm^{-1}$). A cuvette (1 mL) was filled with 960 μL of a buffered solution of a substrate (2 mM; phosphate buffer: pH 7.0, 50 mM), and 20 μL of a buffered solution of NADPH (NADPH: 12.5 mM; phosphate buffer: pH 7.0, 50 mM). A solution (20 μL) of At5β-StR from *A. thaliana* (purified; NADPH-dependent; 2.7 mg/mL) was then added. The relative activities were determined by comparison of measured enzyme activities for the substrates (in U/mL) with the one for progesterone (regarded as the reference experiment with a relative activity of 100%).

Protocol for Preparative Biotransformations (According to Table 1)

In a 100-mL round-bottom flask, a buffered solution of $\Delta^{4,5}$-steroid 5β-reductase from *A. thaliana* (At5β-StR; HEPES-KOH buffer, 5% glycerol; pH 8; prepared according to ref.[11]; entries 1 and 2: 0.945 mL, 3.29 mg/mL; entry 4: 1.15 mL, 4.20 mg/mL; entry 5: 1.12 mL, 2.7 mg/mL) or $\Delta^{4,5}$-steroid 5β-reductase from *D. (I.) canariensis* (HEPES-KOH buffer, 5% glycerol; pH 8; prepared according to ref.[13]; entry 3: 0.945 mL, 1.22 mg/mL) and a glucose dehydrogenase from *Bacillus* sp. (18 U; Amano Enzymes Inc.) were subsequently added to a solution of the enone or enoate ester (**2**: 0.41 mmol; **4**: 0.15 mmol; **7**: 0.25 mmol), D-glucose (2 mmol), and cofactor $NADP^+$ (0.04 mmol) in phosphate buffer (pH 7; 50 mM; entries 1–4: 70 mL; entry 5: 25 mL). The reaction mixture was stirred for 24–48 h (for details and reaction temperature, see Table 1), and subsequently the reaction mixture was extracted with dichloromethane (entries 1–3: three times 80 mL; entry 4: five times 80 mL; entry 5: five times 30 mL). The combined organic phases

were dried over magnesium sulfate, filtered and concentrated carefully under vacuum. The resulting crude product was then analyzed by means of proton NMR spectroscopy, and the conversion was determined from these data by comparison with data of reference samples (entries 1–4) or literature data (entry 5: for literature data, see ref.[15]). The determination of the enantiomeric excess (entry 4) was carried out by means of chiral gas chromatography (Shimadzu GC-17 A; column: CP-Chirasil-DEX; gas carrier: helium; flow: 1.3 mL/min; temperature program: 5 min at 60 °C, followed by an increase of 5 °C per min to 190 °C and 10 min at 190 °C).

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