Chapter 3

Molecular cloning and characterization of a broad substrate terpenoid oxidoreductase from *Artemisia annua*

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Abstract

From *Artemisia annua* L., a new oxidoreductase (Red 1) was cloned, sequenced and functionally characterized. Through bioinformatics, heterologous protein expression, and enzyme substrate conversion assays, the elucidation of the enzymatic capacities of Red1 was achieved. Red1 acts on monoterpenoids, and in particular functions as a menthone:neomenthol oxidoreductase. The kinetic parameter *k*cat/*K*m was determined to be 939 fold more efficient for the reduction of (-)-menthone to (+)-neomenthol, than results previously reported for the menthone:neomenthol reductase from *Mentha x piperita.* Based on its kinetic properties, the possible use of Red1 in biological crop protection is discussed.
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Introduction

Artemisia annua L. (*A. annua*) is primarily known for its production of the antimalarial drug artemisinin, which is extracted from the plant in small quantities (0.01-2% dry weight (Zhang et al., 2008)). Artemisinin belongs to the sesquiterpenoids, and this is also the class of compounds that has attracted most attention from researchers investigating this plant (Bertea et al., 2006; Bouwmeester et al., 1999; Mercke et al., 2000; Wallaart et al., 1999; Zhang et al., 2008). Less investigated is the biosynthesis of its monoterpenoid metabolic profile, even though studies of the composition of *A. annua* essential oil show that it contains many different monoterpenoids (Goelet al., 2007; Woerdenbag et al., 1993; Woerdenbag et al., 1994). Hence, in addition to artemisinin, *A. annua* contains many other terpenoids of commercial interest such as menthol (table 1). The enantiomer (-)-menthol is used in industry and pharmacy and is recognized as a high value natural compound. For biotechnological purposes, it would be desirable to isolate enzymes capable of producing menthol or other closely related compounds, such as (+)-neomenthol. In order to begin to understand the origin of oxygenated terpenoids in *A. annua*, we cloned and characterized an oxidoreductase, Red1 (Rydén et al., 2010). We showed that Red1 is very likely to be involved in the biosynthetic network of artemisinin. To investigate the potential use of Red1 in biotechnological applications, we here investigate the substrate specificity of the enzyme in more detail.

Previously, EST libraries from organs specialized in terpenoid biosynthesis have been used to clone and characterize enzymes involved in artemisinin biosynthesis (Covello et al., 2007; Mercke et al., 2000; Ro et al., 2006; Wallaart et al., 2001; Zhang et al., 2008). Here, we describe the heterologous over-expression and isolation of Red1, and investigate its enzymatic properties toward monoterpenoids.

Table 1. Phytochemical profile of *A. annua*.

Substrate	% of substrate in essential oil	Converted by Red1	References
Artemisinic alcohol	5.2-7.5	-	(Woerdenbag et al., 1993)
Artemisinic aldehyde	77-2450[1)]	-	(Lommen et al., 2006)
Borneol	0.6-20.0	-	(Woerdenbag et al., 1994), (Banthorpe et al., 1977)
Camphor	3.3-21.8	-	(Woerdenbag et al., 1993), (Bhakuni et al., 2001)
Carvone	Traces	-	(Goel et al., 2007)
Citral	n.d.	-	
Dihydroartemisinic alcohol	35-1984[1)]	-	(Lommen et al., 2006)

Table 1. Continuation.

Substrate	% of substrate in essential oil	Converted by Red1	References
Dihydroartemisinic aldehyde	23-781[1)]	+	(Lommen et al., 2006), (Rydén et al., 2010)
Dihydrocarveol	n.d.	-	
Dihydrocarvone	n.d.	+	
Germacrone	n.d.	-	
Humulene	0.2-0.7	-	(Woerdenbag et al., 1994)
Isopiperitenol	n.d.	-	
Limonene	0.23	-	(Ahmad et al., 1994)
Menthol and derivatives	Minor components	+	(Chalchat et al., 1991), (Lawrence, 1982)
Menthone	n.d.	+	
Neomenthol	n.d.	+	
Nootkatone	n.d.	-	
Perilla alcohol	n.d.	-	
Perilla aldehyde	n.d.	+	(Rydén et al., 2010)

Table 1. Continuation.

Substrate	% of substrate in essential oil	Converted by Red1	References
Terpineol	0.1-0.9	-	(H. J. Woerdenbag, et al., 1994)
Thujone	Traces-9.0	+	(Derek V. Banthorpe, et al., 1977), (D. V. Banthorpe, Baxendale, Gatford, & Williams, 1971), (Ahmad & Misra, 1994)

[1)] μg g^{-1} dry weight plant material.
n.d. – no data available.

Incubations of Red1 with a series of monoterpenoids such as menthone, carvone and limonene as well as sesquiterpenoids exemplified by nootkatone and germacrone, were performed to investigate whether Red1 is capable of reducing the ketone group to an alcohol in these systems, along with the reverse dehydrogenase reaction (figure 1). The contribution of Red1 to the metabolic profile of *A. annua* is discussed, as well as the prospects of Red1 in transgenic approaches.

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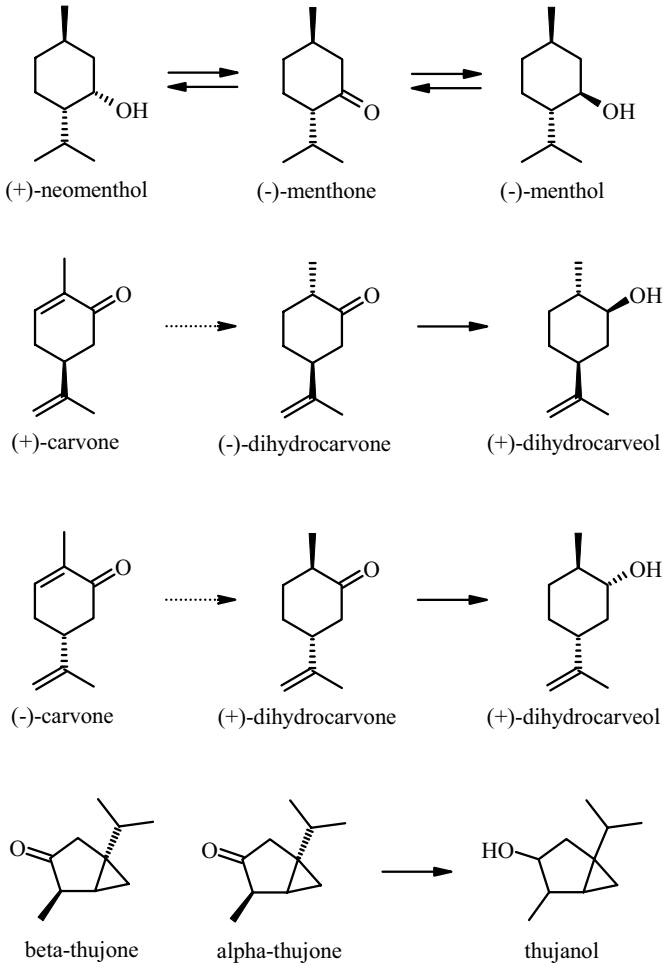
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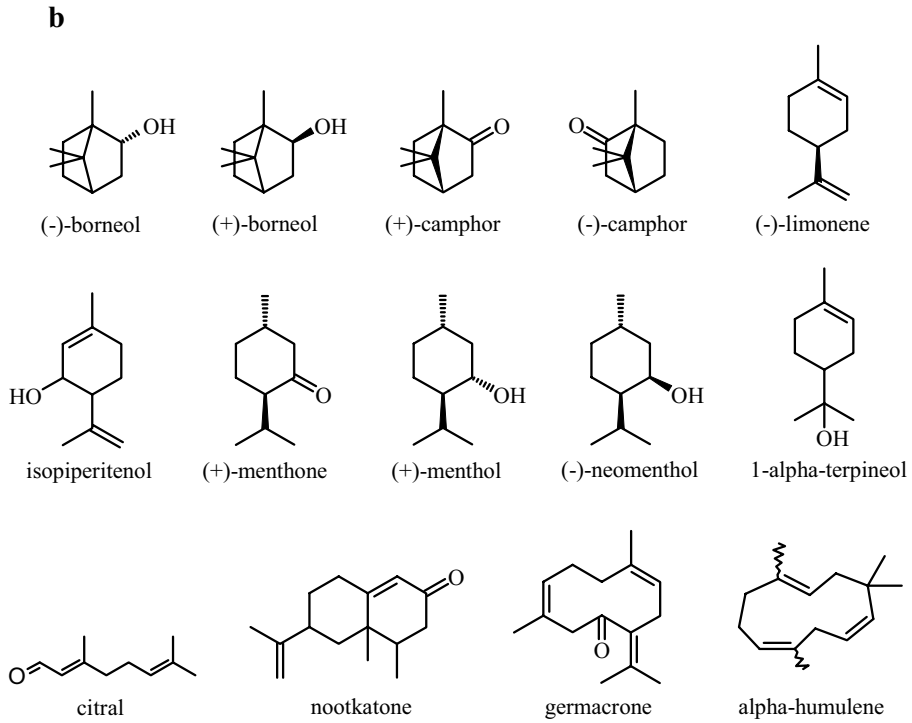

Figure 1. Substrates and conversion products for Red1. Solid arrows reprecent conversion by Red1.
a. Dashed arrow represents conversion via innate *E. coli* proteins.
b. Substrates not converted by Red1.

Results and Discussion

Sequence analysis

After sequencing 2180 ESTs and running a batch BlastX, 19 reductase candidates were identified and assigned putative functions. To further narrow down the number of candidates, a comparison with tomato, potato, rice, *Arabidopsis thaliana* (*A. thailana*), lettuce and sunflower EST libraries was done. To distinguish between

ubiquitous reductases and reductases likely to be relevant in sesquiterpene lactone biosynthesis, the following strategy was adopted: Reductases with a high similarity with lettuce and sunflower, but significantly lower similarity with the non-Asteraceae outgroups potato (*Solanaceae*), tomato (*Solanaceae*), rice (*Poaceae*) and *A. thaliana* (*Brassicaceae*), were considered to be promising candidates in the terpenoid biosynthesis. Based on the results from the BlastX search and the EST library comparisons, one candidate named *red1* was selected as the most promising candidate to relevant terpenoid biosynthesis. This was inferred from its similarity with the monoterpenoid reductase isopiperitenone reductase (Ringer et al., 2003) and its high similarity with a lettuce (74%) and sunflower EST (43%). The comparatively low similarity with rice (24%) and *A.thaliana* (33%) further solidified the hypothesis of Red1 to be a promising candidate. The full length sequence was therefore obtained using RACE-PCR.

Analysis of the full length sequence of *red1* with tBlastX revealed *Mentha x piperita* (*M. piperita*) (-)-isopiperitenone reductase, *M. piperita* (-)-menthone:(+)-neomenthol reductase, *M. piperita* menthol dehydrogenase and *Papaver bracteatum* (*P. bracteatum*) salutaridine reductase as top hits, with enzymatically confirmed functions. Alignment of these amino acid sequences including the deduced amino acid sequence of Red1 illustrate two conserved motifs, which are common for the short chain dehydrogenase/reductase (SDR) protein family (figure 2) (Kallberg et al., 2002): the N-terminal cofactor binding motif GxxxGxG (motif 1) and the catalytic domain YxxxK (motif 2). Common to the enzymes is their preference for NADP(H) over NAD(H), due to the basic lysine residue in motif 1 (Persson et al., 2003), which categorizes them as classical SDR enzymes (Kallberg et al., 2002). Two-by-two comparison of amino acid sequences produced similarity rankings of Red1 aligned with *P. bracteatum* salutaridine reductase (49% identity), *M. piperita* (-)-isopiperitenone reductase and *M. piperita* menthol dehydrogenase

(47% identity) and *M. piperita* (-)-menthone:(+)-neomenthol reductase (46% identity).

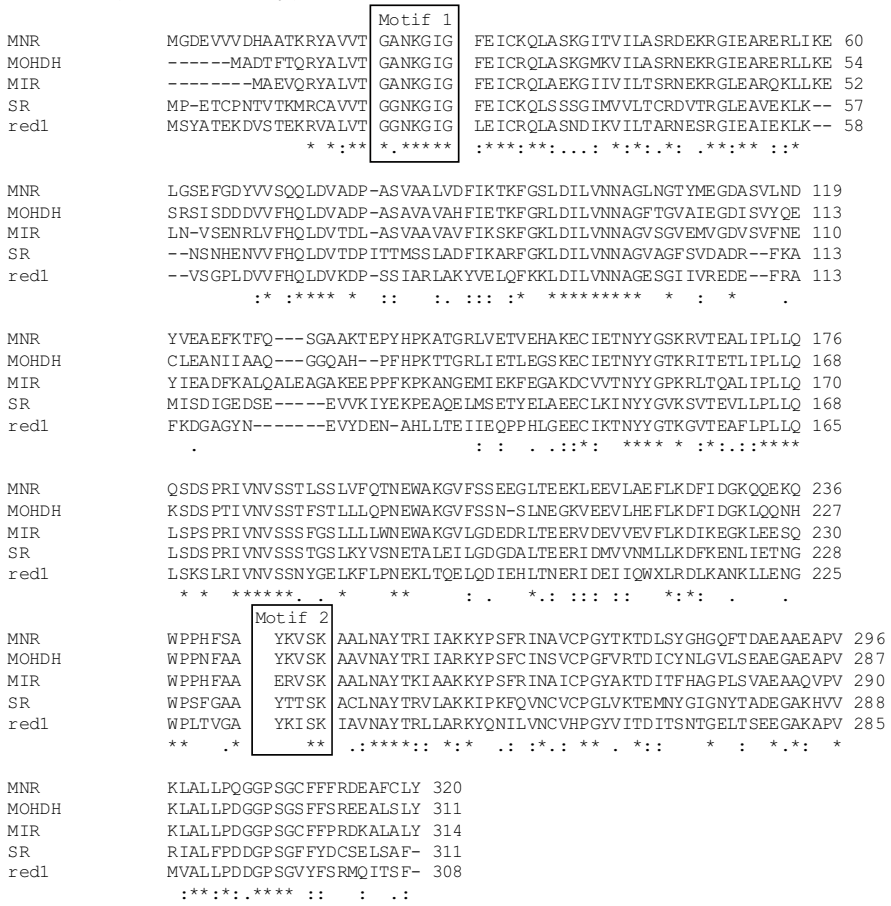

Figure 2. Alignment of homologous amino acid sequences against Red1. MIR = *Mentha x piperita* (-)-isopiperitenone reductase (accession number AY300162) (Ringer et al., 2003), MNR = *Mentha x piperita* (-)-menthone:(+)-neomenthol reductase (accession ABC88670) (Daviset al., 2005), MOHDH = *Mentha x piperita* menthol dehydrogenase (accession number AY288138) (Davis et al., 2005) and SR = *Papaver bracteatum* salutaridine reductase (accession number EF184229) (Geissler et al., 2007). Motif 1 GxxxGxG is a cofactor binding domain typical for SDR (short chain dehydrogenase/reductase

superfamily) and motif 2 YxxxK is a catalytic domain (Davis et al., 2005; Kallberg et al., 2002). (*): strictly conserved residues; (:): conserved substitutions; (.): semiconserved substitutions.

Reductase activities

Based on the bioinformatics analysis, it was hypothesized that Red1 would reduce exocyclic double bonds ketones. Initial experiments were performed with crude cytosolic extracts from *Escherichia coli* (*E. coli*) producing Red1 without purification. The crude protein extract was tested for conversion of (-)-camphor, (+)-camphor, (+)-carvone, (-)-carvone, citral, germacrone, α-humulene, (-)-limonene, (-)-menthone mixed with 10% (+)-menthone, (+)-nootkatone, 1-α-terpineol and β-thujone with NADPH as cofactor (figure 1). Conversion of the ketones into their corresponding alcohols was detected for the terpenes menthone, carvone and β-thujone, but not for the other monoterpenes and sesquiterpene ketones. Menthone was predominantly converted into (-)-menthol, with traces of neomenthol (figure 3a, b). Both enantiomers of carvone were converted into dihydrocarveol, but biotransformation of carvone to dihydrocarvone also occurred in the negative control (data not shown). β-Thujone was to a minor extent converted into its alcohol (figure 1, data not shown). This demonstrates that the reduction of the cyclic carbon double bond is due to native *E. coli* proteins. To discriminate between Red1 dependent conversion and conversion by *E. coli* enzymes, his-tagged Red1 protein was used in assays with selected monoterpenoids. With NADPH as cofactor, dihydrocarvone (figure 4a, b) and menthone (figure 3c, d, e) were efficiently converted to corresponding alcohols dihydrocarveol and menthol/neomenthol respectively (figure 1). The monoterpenes carvone and isopiperitenone were not converted (data not shown), suggesting that Red1 preferentially reduces saturated ketones. In a crude protein extract containing his-tagged Red1, (-)-menthone was converted predominantly to (-)-menthol with traces of

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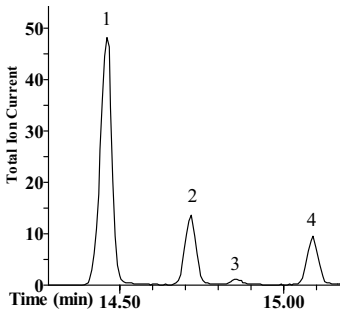
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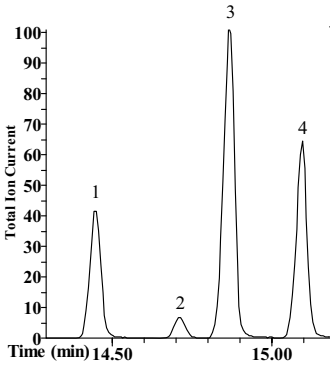
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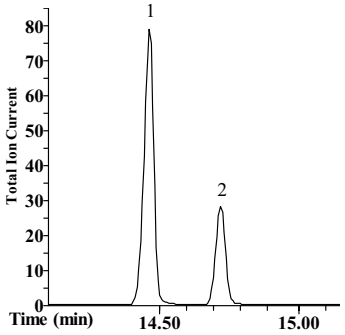
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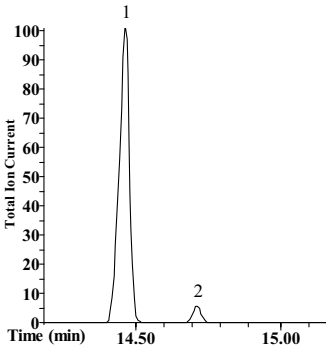
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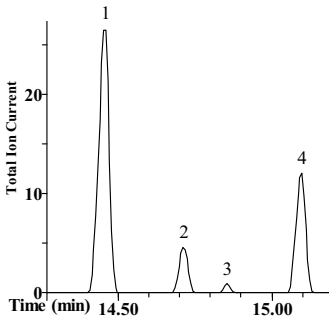
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Figure 3. GC chromatogram of conversion assays. 1 = (-)-menthone, 2 = (+)-menthone, 3 = neomenthol, 4 = (-)-menthol.

a. Crude preparation of Red1 with (-)-menthone and (+)-menthone as substrate.
b. Crude empty vector control with (-)-menthone and (+)-menthone as substrate.
c. Purified his-tagged Red1 with (-)-menthone and (+)-menthone as substrate.
d. Empty vector control with (-)-menthone and (+)-menthone as substrate.
e. Crude preparation of his-tagged Red1 with (-)-menthone and (+)-menthone as substrate.

neomenthol (figure 3e), whereas purified his-tagged Red1 converted (-)-menthone to (+)-neomenthol (main product) and (-)-menthol (figure 3c). The results with purified his-tagged Red1 confirmed the function of red1 to reduce ketone groups in saturated systems.

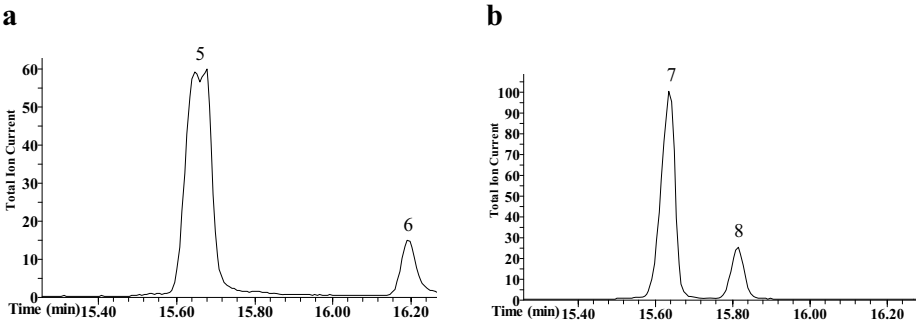

Figure 4. GC chromatogram of conversion assays. 5 = (-)-dihydrocarveol, 6 = (+)-dihydrocarveol, 7 = (-)-dihydrocarvone, 8 = (+)-dihydrocarvone.

a. Purified his-tagged Red1 with (-)-dihydrocarvone as substrate.
b. Empty vector control with (-)-dihydrocarvone as substrate.

Dehydrogenase activities

The capability of Red1 to perform the reverse reaction, oxidation of alcohol to ketone or aldehyde groups, was investigated using selected monoterpenes. Purified his-tagged Red1 was incubated with the monoterpenes (+)-borneol, (-)-borneol, (+)-menthol, (-)-menthol, (+)-neomenthol and (-)-neomenthol with NADP+ as cofactor (figure 1). The compounds (+)-borneol, (-)-borneol, (+)-menthol and (-)-neomenthol were not converted. (+)-Neomenthol proved to be well accepted and converted into (-)-menthone (figure 5a, b). Red1 showed interesting enantiomer specificity towards menthol, as it only accepted (-)-menthol as a substrate but not (+)-menthol (figure 6a, b). Another interesting property of Red1 is its ability to convert neomenthol to (-)-menthol via the intermediate (-)-menthone (figure 5a, b). These results show the capability of Red1 to reduce, as well as oxidize, monoterpenes. Of the monoterpenoid substrates tested, Red1 showed highest activity for the conversion of (-)-menthone to (+)-neomenthol.

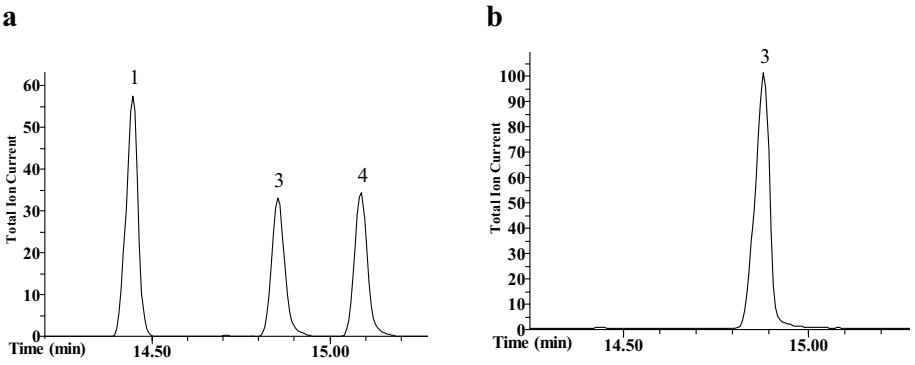

Figure 5. GC chromatogram of conversion assays. 1 = (-)-menthone, 3 = neomenthol, 4 = (-)-menthol.

a. Purified his-tagged Red1 with (+)-neomenthol as substrate.
b. Empty vector control with (+)-neomenthol as substrate.

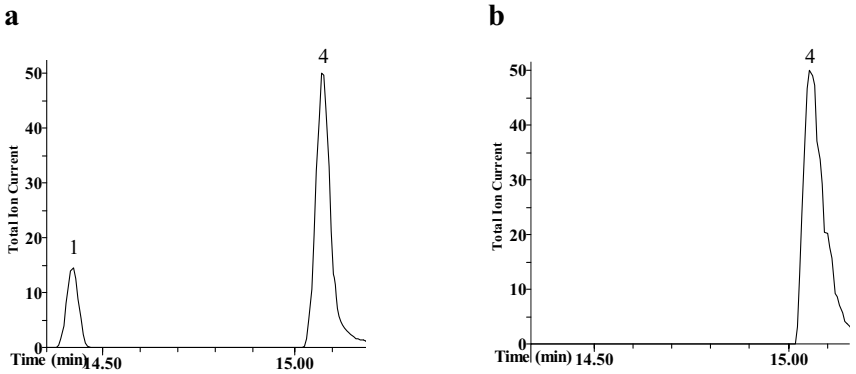

Figure 6. GC chromatogram of conversion assays. 1 = (-)-menthone, 4 = (-)-menthol.

a. Purified his-tagged Red1 with (-)-menthol as substrate.
b. Empty vector control with (-)-menthol as substrate.

Kinetic parameters

Activity of purified Red1 was studied in different buffers, by dialyzing against buffers A-F (table 2). The results show that Red1 is sensitive toward reducing agents, implying importance of sulphur bridges for activity, and further that the enzyme is stabilized by the addition of salt. The dependence of conversion activity on buffer pH was investigated, by comparing conversion assays performed in either potassium phosphate buffers, or Tris buffers, over a range of pH 6.0 to 9.0 with 0.5 unit increments. Red1 shows a broad pH optimum, ranging from 6.0 to 8.0 with maximum activity at pH 7.0. The optimal temperature for Red1 for menthone:neomenthol conversion was 30°C. The *K*m and *k*cat values for Red1 dependent reduction of (-)-menthone to (+)-neomenthol, were 7.1 µM and 0.60 s^{-1}, respectively. The kinetic parameters for the reverse reaction, oxidation of (+)-neomenthol to (-)-menthone, were *K*m 305 µM and *k*cat 0.91 s^{-1} (table 3, figure 7).

Table 2. Activity of purified Red1 in buffers A-F. Composition of buffers: A – 40 mM potassium phosphate buffer, 0.1% β-mercaptoethanol, pH 7.5, B – 40 mM potassium phosphate buffer, 1 mM ascorbic acid, pH 7.5, C – 40 mM potassium phosphate buffer pH 7.5, D – 20 mM potassium phosphate buffer pH 7.5, E – 40 mM potassium phosphate buffer, 100 mM KCl, pH 7.5, F – 20 mM potassium phosphate buffer, 100 mM KCl. Samples were performed in duplicates.

Buffer	Ratio (-)-menthone/(+)-neomenthol	Main differences in buffer composition
A	4.9	Reducing agent β-mercaptoethanol
B	6.1	Reducing agent ascorbic acid
C	4.3	High buffer salt concentration
D	4.3	Low buffer salt concentration
E	3.9	High buffer salt concentration and added KCl
F	3.3	Low buffer salt concentration and added KCl

Table 3. Kinetic parameters for conversion of (-)-menthone and (+)-neomenthol by Red1.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
(-)-menthone	7.1 ± 1.4	0.60	83620
(+)-neomenthol	305 ± 100	0.91	2990
Menthone:neomenthol reductase from *Mentha x piperitea* (Davis, Ringer et al. 2005)	674	0.06	89

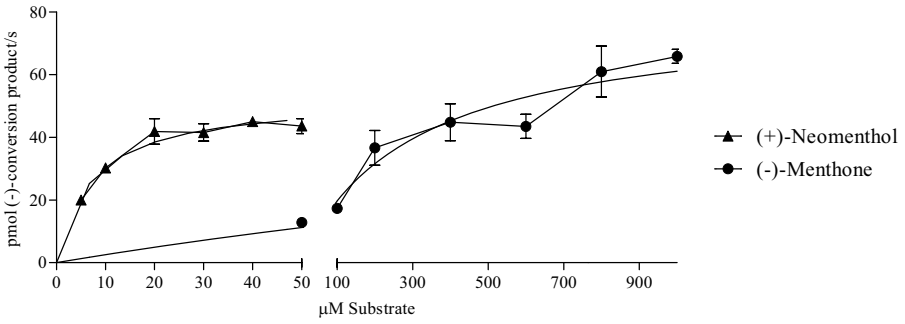

Figure 7. Michaelis-Menten curves for Red1 dependent conversion of (-)-menthone (●) and (+)-neomenthol (▲).

Concluding remarks

Studies of the biosynthesis of natural compounds in *Artemisia annua* has so far focused mostly on the biosynthesis of the antimalarial drug artemisinin. Previous studies involving Red1 pointed it out as an efficient and likely reducer of the ketone in the sesquiterpenoid artemisinin intermediate dihydroartemisinic aldehyde (Rydén et al., 2010). However, *Artemisia annua*, a member of the Asteraceae family well known for its sesquiterpenoid biosynthesis, also contains a range of other valuable and interesting terpenoids (table 1). The potential of Red1 to convert important monoterpenoids and sesquiterpenoids, was investigated while keeping in mind the natural metabolic profile of *A. annua* (table 1) (Bhakuni et al., 2001). To investigate a possible biotechnological use of Red1, monoterpenoids as well as sesquiterpenoids other than those found in *A. annua*, were selected and tested for suitability as substrates (table 1). Of all the tested monoterpenoid substrates, (-)-menthone proved to be the most efficiently converted. Hence, further kinetic characterization focused on (-)-menthone as substrate. Red1 shares sequence homology with the short chain dehydrogenase/reductase (SDR) protein family (Kallberg et al., 2002), and in particular reductases from mint (Davis et al., 2005) (figure 2). Common to SDRs is their action on molecules such as monoterpenoids (C_{10} compounds) rather than the sesquiterpeneoids (C_{15} compounds) (Kallberg et al., 2002). Conversion assays confirmed the ability of Red1 to reduce and oxidize short chain molecules. From the results summarized in table 1, it can be concluded that Red1 has broad substrate specificity (figure 1a).

(-)-Menthol is precent in *A. annua* in discrete amounts, and due to its industrial importance its precursor, (-)-menthone, was used as a substrate for Red1 (table 1). Crude protein extracts containing Red1 converted (-)-menthone to (-)-menthol (figure 3a, b). However, product specificity was shifted from (-)-menthol to (+)-neomenthol, when purified protein was used (figure 3c), indicating the

interference of *E.coli* proteins in the position of the chemical equilibrium between (-)-menthone and (-)-menthol. Another interesting feature of Red1 is its chemical equilibrium between (-)-menthone, (-)-menthol and (+)-neomenthol, which show a capability of producing (-)-menthol from (+)-neomenthol (figure 5a, b). This makes Red1 an interesting enzyme for biotechnology, as it is desirable to be able to convert (+)-neomenthol to (-)-menthol. The acceptance of (-)-menthol (figure 6a, b) and refusal of (+)-menthol as a substrate in a dehydrogenase reaction, further supports the enantioselective product formation and substrate specificity.

The capability of Red1 to efficiently convert (-)-menthone to (+)-neomenthol (table 3) may also be interesting for plant protection. The intrinsic value of (+)-neomenthol lies in its antimicrobial effect and it has been shown that the compound has a plant protective action (Choi et al., 2008). Albeit (+)-neomenthol has not been detected in *A. annua*, the usage of Red1 for biotechnological purposes may be warranted as deemed from the kinetic data (table 3). Production of (+)-neomenthol was accomplished in *A. thaliana* by overexpression of a (-)-menthone reductase from pepper (*Capsicum annuum*), which led to enhanced protection against *Pseudomonas syringae* pv *tomato* DC3000 and *Hyaloperonospora parasitica* isolate Noco2. Our results show that Red1 has a *k*cat/*K*m value that is 939 fold more efficient than that of (-)-menthone: (+)-neomenthol conversion by the corresponding *M. piperitea* enzyme (Davis et al., 2005), a plant species specialized in the biosynthesis of menthone and menthol related monoterpenoids. The oxidation of (+)-neomenthol to (-)-menthone by Red1 is unlikely to occur at any significant level, as the *k*cat/*K*m value of the reducing reaction is 28 fold higher than the *k*cat/*K*m value for the oxidative reaction (table 3). Furthermore, the *K*m value of the oxidative reaction is in the unphysiological range of 305 µM. Introduction of Red1 *in planta*, could hence contribute significantly to enhanced resistance against pathogens due to its catalytic efficiency, provided there is (-)-menthone available in the host.

Materials and Methods

Chemicals

(+)-Borneol, (-)-borneol, (-)-camphor, (+)-camphor, (-)-carvone , α-humulene, neomenthol and (-)-limonene were purchased from Extrasynthèse, Genay, France. (+)-Menthol was purchased from Tokyo Chemical Industry, (-)-menthol from Fluka, (-)-menthone and (+)-nootkatone were purchased from Fluka. (+)-Dihydrocarvone, germacrone, 1-α-terpineol and β-thujone were synthesized by the Department of Pharmaceutical Biology, Groningen, the Netherlands. A mixture of α-thujone and ß-thujone was purchased from Roth. (+)-Carvone, citral 95% cis-trans mixture were purchased from Sigma-Aldrich. Isopiperitenone was obtained as described by Lucker et al. (2004).

GC-MS

Analyses were performed on a HP5890 series II gas chromatograph coupled to an HP5972A mass selective detector (70eV) equipped with a ZB-5MS column (30 m x 0.25 mm i.d., film thickness 0.25 µm, Phenomenex, ordered at Bester, Amstelveen, The Netherlands). Inlet temperature was set to 250 °C and the detection temperature to 290 °C, with a constant helium flow of 1.0 ml/min. Samples were analyzed with the temperature program 55 °C for 4 min, followed by a ramp of 5 °C/min to 220 °C, followed by a ramp of 20 °C to 290 °C with a hold for 4.5 min. Peaks were identified using Wiley mass spectral library, NIST library, internal libraries or via authentic standards.

Sequencing

Constructs were sequenced by the commercial service of Macrogen using BigDye terminator cycling conditions.

Kinetic analysis

The kinetic parameters *K*m and Vmax were calculated with GraphPad Prism 5 for Windows (GraphPad Software Inc.), using standard settings for non-linear regression curve fitting in Michaelis-Menten mode.

Plant material

Plants were grown for collection of flowers using seeds (UG2006) obtained from University of Groningen (Wallaart et al., 1999). Seeds were sown the 3rd of August 2006 and grown under green house conditions using potting compost. Plants were watered as necessary without addition of fertilizer. When the plants started to flower displaying a range from buds to late flowering with pollen, the flowers were collected in four categories sorted after bud (F1), young flower (F2), old flower (F3) and old flower with pollen (F4). Flowers were frozen in liquid nitrogen and stored at -80 °C. Plant leaf material was picked the 28th of April 2003 and stored at -80 °C (Bertea et al., 2006).

Construction of cDNA and cloning of full length gene red1

A glandular trichome specific cDNA library previously constructed was further sequenced (Bertea et al., 2006). Obtained sequences were compared with the literature, using batch BlastX with standard settings. The EST fragments were additionally analyzed by alignment with EST libraries from tomato, potato, rice, *A. thaliana*, lettuce and sunflower. It was argued that fragments having high similarity with EST sequences from lettuce and sunflower, which both belong to the family Asteraceae, but comparatively low similarity with the non-Asteraceae family members potato, tomato and rice, would be specific for Asteraceae species and likely to be involved in terpenoid biosynthesis. Based on the combined information from the two alignment approaches, one gene fragment

named *red1* was selected for further analysis and 5' extension. Approximately 100 mg frozen flowers (stage F4) and mature leaves (O leaf) were ground in liquid nitrogen. Total RNA was isolated using TriPure (Roche Applied Science) and 1 µg of the isolated F4 and O leaf total RNA were used for 5' RACE cDNA amplification, using the Smart RACE cDNA amplification kit from Clontech. PCR was performed on an aliquot of the obtained F4 cDNA, using the internal primer Red1-RACE1 5' cgatttctctcattcgtgaggtgttcaatatcttggagc 3' and the universal primer mix from the Smart RACE cDNA amplification kit. The PCR product was purified from an agarose gel and ligated into the sequencing vector pGEMT-Easy (Promega) and sequenced. The full length gene was isolated form O leaf cDNA with the primers FL5-AseI-red1 5' accttggattaatatgtcatatgcaaccgagaaagatg 3' and FL3-red1-XhoI 5' accttggctcgagtcaaaatgaggttatttgcattcgac 3', using Phusion DNA polymerase (Finnzymes) under reaction conditions recommended by the manufacturer (Finnzymes) and 1 µl cDNA. The 953 bp PCR product was purified using the QIAquick PCR Purification Kit of Qiagen, and cloned into pGEMT for propagation and sequencing. Confirmed inserts were excised using AseI and XhoI and ligated into the expression vectors pET26b+ and pET28a+, previously restricted with NdeI and XhoI, forming the constructs pET26b+-red1 and pET28a+red1. Constructs were cloned into *E. coli* DH5α for propagation, where after plasmids were isolated and transformed into the expression host *E. coli* BL21 (DE3)-Rosetta2 following manufacturer's instructions (Novagen). The nucleotide sequence for *red1* has been deposited in the GenBank database under GenBankAccession Number GU167953.

Sequence analysis

The sequence of *red1* was analyzed via tBlastX using standard settings (Altschul et al., 1990). The amino acid sequences of selected top hits with confirmed enzymatic function, were extracted and aligned using ClustalW2 (Larkin et al., 2007). Sequences were

aligned using bl2seq for proteins with default settings to obtain identity percentages (Altschul et al., 1990).

Protein expression and purification

Seed cultures with 5 ml LB medium supplemented with 30 µg/ml kanamycine and 34 µg/ml chloramphenicol were initiated using one colony from fresh streak outs, and incubated at 37°C over night with shaking. Main cultures of 200 ml 2xTY (2.4% tryptone, 1.5% yeast extract, 0.75% NaCl) supplemented with antibiotics, as described above, were initiated in 1000 ml Erlenmayer-flasks at a calculated OD_{600} of 0.02 using aliquots from the seed cultures. Cultures were incubated at 37°C with shaking for 4 hours, and thereafter induced with 200 µl 1M IPTG, followed by incubation at 16°C with shaking for 14 hours. Cytosolic proteins were extracted using Bugbuster (Novagen) following manufacturer's instructions. Proteins emerging from the pET28a-red1 construct were purified on a nickel column using Ni-NTA resin (Qiagen), and dialyzed at 4°C against buffer G (20 mM KH_2PO_4, 150 mM KCl and 20% glycerol) using a 14 kDa filter (Viskase Companies, Inc.). Proteins were stored at -20°C. Purity was verified using SDS-PAGE.

Conversion assays

Initial conversion assays to determine substrate specificity were performed in 2 ml aliquots containing 20 µl purified protein or crude protein extract, 40 mM potassium phosphate buffer (pH 7.0), 1 mM DTT, 500 µM NADPH for reductase assays, or $NADP^+$ for dehydrogenase assays and 100 µM substrate. Mixtures were incubated at 30°C with gentle shaking over night. All reactions were performed in duplicates. Protein extracts from *E. coli* harboring the empty expression vectors pET26b+ and pET28a+ served as negative controls. Conversion assays to determine kinetic parameters contained 1.5 µg to 4.5 µg purified protein, 500 µM NADP+ or NADPH, 5-50 µM (-)-menthone or 50-1000 µM (+)-neomenthol in

buffer F (20 mM KH_2PO_4, 150 mM KCl, pH 7.0), amounting to a total reaction volume of 900 µl. Samples were incubated for 5 min. at 30°C and reactions were initiated by adding purified Red1. Kinetic assays were incubated for 10 min. at 30°C. The calculated molecular weight of 34,370 Da for Red1 was used to determine the molar amount of protein added in the assays. Enzyme reactions were stopped on ice and 45 nmol cis-nerolidol was added as an internal standard to samples. Samples were vortexed and 2 ml ethyl acetate was added, followed by vigorous mixing. Samples were centrifuged until complete phase separation was achieved. The organic phase was collected and dried with anhydrous Na_2SO_4 and analyzed by GC-MS as described above. Kinetic assay samples were prepared by adding 12 nmol cis-nerolidol as internal standard, extracting twice with 600 µl ethyl acetate, and concentrating samples under a gentle nitrogen flow. Samples were analyzed by GC-MS, as described above. Standard curves for quantitative analysis were constructed from authentic standards using (+)-neomenthol and (-)-menthone.

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