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NEPETALACTOL OXIDOREDUCTASE IN TRICHOMES OF THE CATMINT *NEPETA RACEMOSA*

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Key Word Index—*Nepeta racemosa*; Lamiaceae; catmint; trichome; biosynthesis; oxidoreductase; methylcyclopentane monoterpene; iridoid; nepetalactol; nepetalactone.

Abstract—Isomers of (7*S*)-nepetalactone are the principal constituents of the essential oil accumulated by plants of the genus *Nepeta* (catmints), with the *cis,cis*-isomer predominating in the catmint *N. racemosa*. An enzyme which catalyses the NAD^+-dependent oxidation of *cis,cis*-nepetalactol to *cis,cis*-nepetalactone has been identified in cell-free extracts of *N. racemosa* leaves. This enzyme has been partially purified and has been shown to be located within trichomes present on the surface of leaves of this species. Because glandular trichomes have been shown to be the site of accumulation of nepetalactones in *N. racemosa* and *N. cataria*, the presence of such activity within the trichomes suggests that nepetalactol may be an intermediate in the biosynthesis of nepetalactone.

INTRODUCTION

The methylcyclopentanoids, also referred to as iridoids, constitute the largest known monoterpenoid group [1]. Plants of the genus *Nepeta* (Lamiaceae = Labiatae), commonly known as catmints, accumulate an essential oil characterized by the preponderance of the methylcyclopentane monoterpene nepetalactones [2, 3]. In *N. racemosa*, the principal nepetalactone stereoisomer accumulated is *cis,cis*-nepetalactone (**1a**) in the majority of plants [2, 4]. Other stereoisomers (*cis,trans*-nepetalactone (**2a**) and *trans,cis*-nepetalactone (**3**)) are often present in trace amounts in these plants, but individual plants frequently occur which produce markedly different proportions of these nepetalactone isomers [4]. Nepetalactones are well-known as feline excitants [5, 6], and have been shown to be repellent to a number of insect species [1, 7]. Their production by insect and plant species most probably results from their effects on insect behaviour, and their role in these species is likely to be as defence chemicals [1, 7, 8]. The effect of this class of monoterpenoids on insect behaviour is not, however, exclusively one of repellance. Certain (7*S*)-nepetalactones and (7*S*)-nepetalactols have also been shown to act as aphid sex attractants (pheromones) [9–11], and more recently to act as attractants for aphid parasitoids (kairomones) [12, 13].

Early studies on the biosynthesis of *cis,trans*-nepetalactone (**2a**), the principal isomer present in *N. cataria*, established its origin from mevalonate, with demonstration of the incorporation of labelled mevalonate into nepetalactone in crude leaf homogenates [14]. The biosynthesis of methylcyclopentanoids in other plant species was shown to be derived from mevalonate via geraniol (**4**), or the (*Z*)-isomer nerol (**5**) [1]. However, further studies of nepetalactone biosynthesis in *N. cataria* indicated that the related mono-unsaturated compound, (3*S*)-citronellol (**6**) was a more efficient precursor in this species [15]. Bellesia *et al.* [15] have suggested (Scheme 1) that a 1,8-dioxygenated derivative ((3*S*)-8-oxocitronellal (**8**)) might directly be cyclized to the iridodial (**9**), known to be a key intermediate in the biosynthesis of methylcyclopentanoids in many plant species. It was further speculated that iridodial (**9**) is converted to nepetalactone **2a** by a Canizzaro-type reaction [15]. The literature is confusing in that the terms iridodial (e.g. **9**) and nepetalactol (e.g. (**1b**), (**2b**)) are sometimes used interchangeably. However, evidence that these compounds are not necessarily interconvertible structures dependent upon stereochemistry is discussed in detail elsewhere [11]. The pathway proposed by Bellesia *et al.* [15], and outlined in Scheme 1, prompted us to investigate the intermediacy of nepetalactol in the biosynthesis of nepetalactone.

In this paper, we confirm the presence of an enzyme which catalyses the oxidation of *cis,cis*-nepetalactol (**1b**) to *cis,cis*-nepetalactone (**1a**) in extracts of leaves

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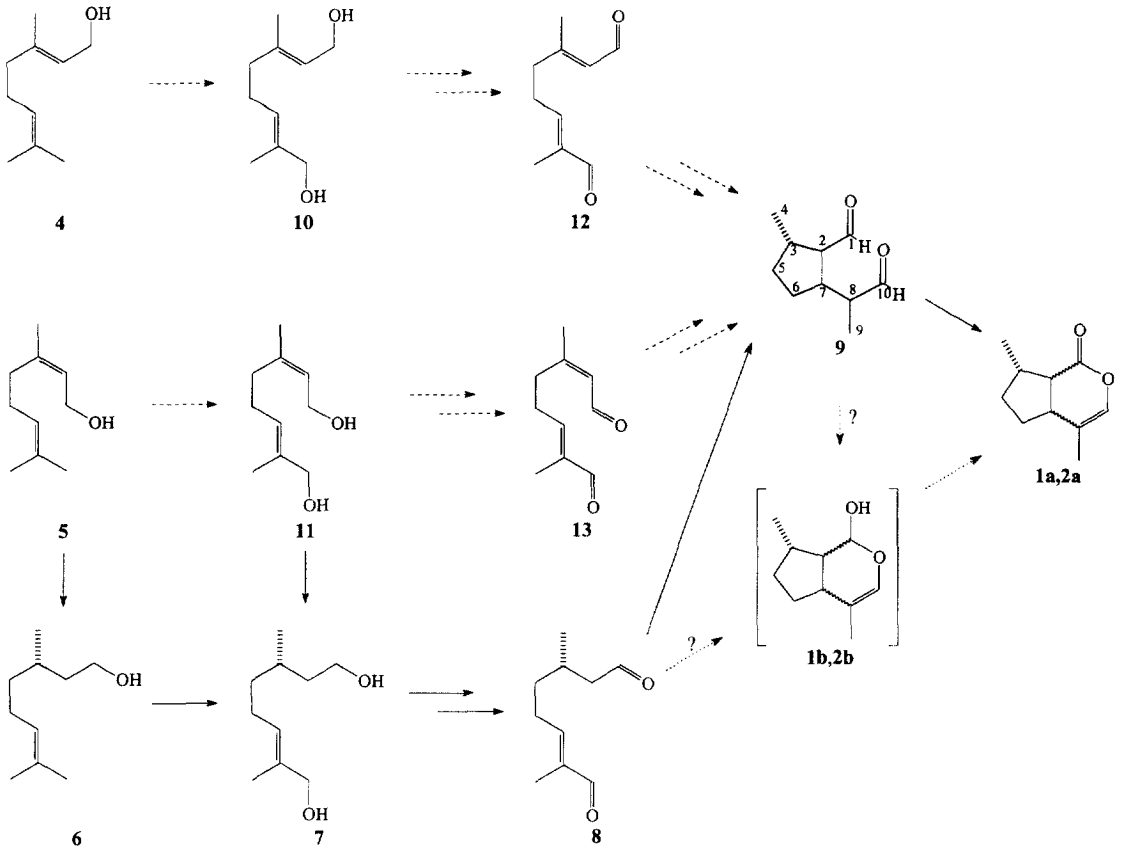


Scheme 1. Biosynthesis of nepetalactones from (3*S*)-citronellol (**6**) as proposed by Bellesia *et al.* [15] for *Nepeta* spp. (arrows). Included is the proposed pathway from geraniol/nerol (**4**/**5**) to iridodial (**9**) shown to occur in other plant species (dashed arrows), and the intermediacy of nepetalactols (**1b**, **2b**) as suggested by this work (dotted arrows).

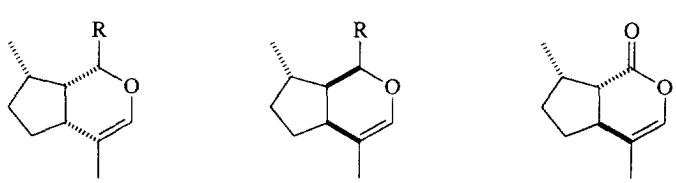
of the catmint *N. racemosa*. This activity, together with that of another oxidoreductase presumed to be involved in nepetalactone biosynthesis [16], is present in trichomes on *N. racemosa* leaves, known to be the site of accumulation of nepetalactones in this species [4].

RESULTS

Enzyme extraction and partial purification

Cell-free extracts prepared from young leaves of *N. racemosa* were found to catalyse the reduction of NAD^+, monitored by measuring absorbance changes at 340 nm, after the addition of nepetalactol (**1b**). This oxidoreductase activity remained in a 100,000 *g* supernatant, and thus could be attributed to a soluble protein. The enzyme could be concentrated by ammonium sulphate fractionation of the 100,000 *g* supernatant, and this fraction was used for preliminary experiments. Using such preparations, we found that activity was dependent on NAD^+, while $NADP^+$ was ineffective. Two different nepetalactol stereoisomers were assayed: in the *cis,cis*-nepetalactol (**1b**) assay, NAD^+ was reduced at 18.9 pkat mg^{-1}, and at 17.3 pkat mg^{-1} with *cis,trans*-nepetalactol (**2b**).

The NAD^+-dependent nepetalactol oxidoreductase could be precipitated between 40 and 60% saturated ammonium sulphate (Table 1). This allowed separation of this activity from the previously-characterised $NADP^+$-dependent acyclic monoterpenoid



1a R = =O
1b R = —OH
2a R = =O
2b R = —OH
3

Table 1. Partial purification of nepetalactol oxidoreductase from *N. racemosa*

Purification step	Volume (ml)	Protein (mg)	Activity (nkat)	Specific activity (nkat mg^{-1})	Purification (-fold)
60% $(NH_4)_2SO_4$ pellet	100	622	22.0	0.035	–
DEAE-Sephacel	100	396	17.1	0.043	1.22
5′-AMP Sepharose	25	0.70	1.9	2.71	63.0

oxidoreductase, which was precipitated between 60 and 80% saturated ammonium sulphate [16].

Subsequent ion-exchange chromatography did not greatly enhance the specific activity of the preparation (Table 1). A number of affinity media were therefore tested for purification of the enzyme, and of these the enzyme was found to reversibly bound to Matrex Orange A, Blue B and Green, as well as 5′-AMP. Experiments with Matrex Green indicated that activity could not be eluted with either NAD^+ or $NADP^+$ (up to 5 mM). With 5′-AMP, activity could be eluted biospecifically with NAD^+, and this medium gave maximum recovery of enzyme activity. Following ion-exchange chromatography, affinity chromatography on 5′-AMP Sepharose resulted in significant (60-fold) purification (Table 1). The enzyme could be eluted from this column with 0.3 mM NAD^+, yielding a preparation with specific activity 2.7 nkat mg^{-1}. Under the conditions employed for affinity chromatography, an appreciable proportion of the enzyme did not bind to the column, contributing to the high overall loss of activity during purification (Table 1).

The enzyme preparation after 5′-AMP Sepharose chromatography was analyzed by denaturing gel electrophoresis (SDS-PAGE). Silver staining of the resultant gel showed a principal band at 53 kD, together with a number of fainter bands (not shown). In an attempt to unambiguously determine the polypeptide composition of the enzyme, we took advantage of the fact that binding of the enzyme to Matrex Green was unaffected by the presence of nicotinamide cofactors. A preparation partially purified by ion-exchange chromatography was loaded onto a Matrex Green column in the presence of NAD^+, $NADP^+$ and NADH (each at 5 mM), conditions which might be expected to minimize the binding of contaminating oxidoreductases. After washing with loading buffer, the enzyme was eluted with a gradient of 0 to 1.5 M KCl, and a portion of each active fraction was concentrated by TCA precipitation and then subjected to SDS-PAGE. After silver-staining, all the fractions were found to contain a doublet at ca 50 kD, with a smaller band (ca 20 kD) present in the most active fractions. The Matrex Green purified preparation was also subjected to non-denaturing gel electrophoresis, and stained for activity in the presence of nepetalactol and nitroblue tetrazolium. Two stained bands were excised and analyzed by SDS-PAGE. After silver-staining, 3 principal bands (ca 53, 47 and 29 kD) were observed in each lane, although additional fainter bands were also present. In all, it would appear that nepetalactol oxidoreductase activity is associated with polypeptide(s) of ca 50 kD, but the data are by no means conclusive.

The purification protocol shown in Table 1, therefore, although yielding enzyme of sufficient purity for enzymological characterization, did not yield homogenous enzyme needed for reliable determination of its polypeptide composition. Further purification, e.g. inclusion of additional chromatographic steps, was not attempted for the purposes of this study because of the relatively low yields of enzyme obtained.

Characterization of activity

We had previously isolated an $NADP^+$-dependent oxidoreductase, active with acyclic monoterpenoid alcohols, from leaves of *N. racemosa* [16]. No detectable activity towards the substrates of this enzyme (the acyclic monoterpenoids geraniol (**4**), nerol (**5**) or (3*S*)-citronellol (**6**)) was detected with the partially-purified nepetalactol oxidoreductase. Thus the nepetalactol oxidoreductase is clearly distinct from the acyclic monoterpenoid oxidoreductase previously described.

In order to characterize the activity of the nepetalactol oxidoreductase, a series of mixtures containing partially-purified oxidoreductase, nepetalactol (**1b**) and NAD^+ were prepared, and incubated at 25° for up to 1 h. Incubations were extracted with organic solvent at 15 min intervals, and the extracts analyzed by gas chromatography. The chromatograms (not shown) showed a time-dependent decrease in the peak area of substrate nepetalactol (15.73 min), with a concomitant increase of a peak with a retention time of 14.67 min, which was identical to the retention time of authentic nepetalactone (**1a**). This peak was not detected in extracts from incubations with the enzyme in which the reaction had been stopped by addition of dichloromethane prior to substrate nepetalactol. Confirmation that nepetalactone was produced in these incubations was obtained by tandem gas chromatography-mass spectrometry (GC-MS) of the extract from a 1 h incubation (Fig. 1). In addition to the substrate nepetalactol (10.7 min), a peak with identical retention time (11.0 min) to authentic nepetalactone (**1a**) was observed. Mass spectrometric

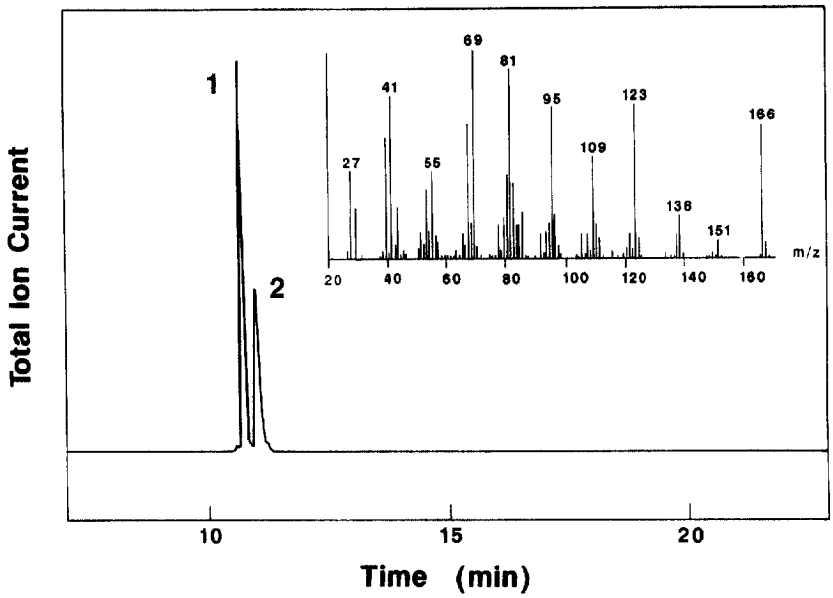


Fig. 1. Combined gas-chromatographic-mass spectrometric (GC-MS) analysis of the product of nepetalactol oxidation. Partially purified oxidoreductase (0.14 nkat) was incubated for 1 h with *cis,cis*-nepetalactol (1 mM) in 0.5 ml 20 mM tris-Cl, pH 8. After extraction into dichloromethane, the extract was analyzed by GC-MS. In the total ion chromatogram, peak 1 (10.7 min) had an identical retention time to authentic nepetalactol, and peak 2 (11 min) to authentic nepetalactone. The insert shows the mass spectrum of peak 2, identical to authentic nepetalactone.

analysis of this peak confirmed its identity as nepetalactone (see Experimental section). It was clear, therefore, that the partially purified oxidoreductase from leaves of *N. racemosa* specifically catalyzed the NAD^+-dependent conversion of *cis,cis*-nepetalactol (**1b**) to *cis,cis*-nepetalactone (**1a**).

Localization of activity

It has been suggested that glandular trichomes present on the leaves of *Nepeta* spp. might play some role in accumulation of the nepetalactone-rich essential oil of this genus [14, 17]. In labiate plants (= Lamiaceae) of the genus *Mentha*, peltate glands have been shown to contain *p*-menthane monoterpenoids, the principal constituents of the essential oil of these species [18]. Isolated *Mentha* glands have been shown to possess the ability to synthesize these monoterpenoids from mevalonic acid, and have been used as a starting material for purification of the enzymes involved [19, 20]. By analysis of samples obtained from the subcuticular cavity of *N. racemosa* and *N. cataria*, we have shown that peltate glandular trichomes of these species accumulate nepetalactones within the characteristic subcuticular space of these structures [4]. Northern analysis of the expression of a cytochrome P450 gene isolated from *N. racemosa*, which may encode a monoterpenoid hydroxylase within the nepetalactone biosynthetic pathway, has shown its expression to be confined to trichomes in this species [21]. It was of interest, therefore, to determine whether the nepetalactol oxidoreductase (and indeed the acyclic monoterpenoid oxidoreductase) of *N. racemosa* might also be located within such structures.

We employed the dry ice abrasion technique developed by Yerger *et al.* [22] to isolate trichomes from the leaf surfaces of *N. racemosa*. Microscopic examination showed that the resulting preparation consisted of a mixture of the three types of trichomes (simple, capitate and peltate) borne by this species [17]. Little or no leaf material or debris could be observed. After preparing a crude protein extract from this preparation (as well as from the trichome-denuded leaves which remain after abrasion), we assayed for the activity of both the $NADP^+$-dependent acyclic monoterpenoid oxidoreductase and the NAD^+-dependent nepetalactol oxidoreductase. The results (Table 2) show that nepetalactol oxidoreductase activity could only be detected in trichome preparations (specific activity: 0.28 nkat mg^{-1}) with no activity present in extracts from stripped leaves.

When the activity of the $NADP^+$-dependent acyclic monoterpenoid oxidoreductase [16] was measured in these preparations, although some activity was associated with trichomes, the majority was present in the stripped leaves. However, the specific activity of this enzyme was 3-fold greater in the trichome preparation than in the stripped leaves. This enzyme activity is, therefore, clearly present in trichomes, but also in cells of the leaf lamina. It was noticed that a significant proportion of acyclic monoterpenoid oxidoreductase activity was not retained using affinity chromatography during purification [16], which might

Table 2. Oxidoreductase activities in preparations from *N. racemosa* leaves

Preparation	Protein (mg)	Total activity (nkat)		Specific activity (nkat mg^{-1})	
		nepetalactol	nerol (10-OH-nerol)	nepetalactol	nerol (10-OH-nerol)
Stripped leaves	54.5	nd	9.8 (7.0)	nd	0.18 (0.13)
Trichomes	3.2	0.9	1.7 (1.2)	0.28	0.53 (0.37)

Leaves (5 g) were stripped of trichomes using dry ice abrasion, and a prepartion of soluble protein prepared from the resulting trichomes and trichome-denuded (stripped) leaves. These preparations were assayed for NAD^+-dependent nepetalactol oxidoreductase and $NADP^+$-dependent nerol and 10-hydroxynerol oxidoreductase activities. nd = not detected.

imply the presence of different isoforms of this enzyme in *N. racemosa*. If so, these isoforms may be expressed differentially in leaves, with one predominantly in trichomes, the other within the leaf lamina tissues. Microscopic examination showed that the stripped leaves did retain some trichomes in clefts presumably inaccessible to the dry ice particles, which may also account for the presence of acyclic oxidoreductase activity in this preparation. The total activity of the acyclic monoterpenoid oxidoreductase obtained in these extracts was significantly greater than that of the nepetalactol oxidoreductase; thus a low residual activity of nepetalactol oxidoreductase remaining in trichomes not removed from the stripped leaves might not have been detected in our assays. However, from the data it would appear that both oxidoreductase enzymes involved in nepetalactone biosynthesis are expressed in leaf trichomes of *N. racemosa*. From these experiments, the specific trichome type within which these enzymes are expressed cannot, however, be identified.

DISCUSSION

The results presented in this paper are consistent with a pathway involving nepetalactol **1b** as an intermediate in the biosynthesis of nepetalactone **1a** in *N. racemosa*. Methylcyclopentane monoterpenoid biosynthesis has been shown to be initiated by the hydroxylation at C-8 of either geraniol (**4**), nerol (**5**) or (3*S*)-citronellol (**6**) (Scheme 1), depending on the plant species (reviewed in [1, 23]). In *C. roseus*, 8-hydroxygeraniol (**10**) and 8-hydroxynerol (**11**) were shown to be incorporated into loganin [24, 25], with negligible incorporation of (3*S*)-citronellol (**6**) or 8-hydroxycitronellol (**7**). In *N. cataria*, however, 8-hydroxycitronellol (**7**) was more efficiently incorporated into nepetalactone than either nerol (**5**) or 8-hydroxynerol (**11**) [15]. We have shown that geraniol (**4**), nerol (**5**) and (3*S*)-citronellol (**6**) are hydroxylated by a membrane-bound enzyme, most likely a cytochrome P450, in both *N. racemosa* and *C. roseus* [23]. In both of these species, subsequent oxidation of the 8-hydroxylated compounds to dialdehydes is carried out by $NADP^+$-dependent oxidoreductase enzymes [16, 26]. The $NADP^+$-dependent oxidoreductase of *N. racemosa* is equally active with 8-hydroxygeraniol (**10**), 8-hydroxynerol (**11**), (3*R*)- and (3*S*)-8-hydroxycitronellol (**7**) [17]. Thus the hydroxylase of *N. racemosa* appears not to discriminate between (3*S*)-citronellol (**6**) and its unsaturated analogues, with the $NADP^+$-dependent oxidoreductase similarly unspecific with regard to the corresponding hydroxylated monoterpenoids. An enzyme responsible for cyclization of acyclic monoterpenoid aldehydes to yield the methylcyclopentane skeleton has yet to be characterised in either *C. roseus* or *Nepeta* spp. However, from the available evidence, it would seem likely that such an activity exists in these species. Cyclization of 8-oxogeranial (12) directly to iridodial (9) has been demonstrated in cell-free extracts of *Rauwolfia serpentina*, and the enzyme responsible partially purified [27]. In *N. cataria*, administration of [10-^{3}H]iridodial, already containing the cyclopentane ring system, has been shown to lead to significant incorporation of radiolabel into nepetalactone **2a** [15].

We have demonstrated here the existence of an enzyme catalyzing the oxidation of nepetalactol **1b** to nepetalactone **1a** in leaves of the catmint *N. racemosa*. The presence of such an enzyme implies that methylcyclopentanoid biosynthesis in this species may proceed from acyclic monoterpenoid dialdehydes to nepetalactones via a nepetalactol intermediate. If so, the presence of this enzyme in extracts of leaf trichomes from *N. racemosa* would support the hypothesis that trichomes (most likely peltate glandular trichomes) are the site of biosynthesis as well as accumulation of nepetalactones in *Nepeta* spp. However, we cannot exclude the possibility that the activity of this enzyme towards nepetalactol is adventitious, and that an alternative pathway may exist. Further analysis of this enzyme will be required for detailed characterization of its activity and its role in methylcyclopentane monoterpenoid biosynthesis. Confirmation of the actual pathway will require the isolation and characterization of the cyclase(s) responsible for formation of the methylcyclopentane carbon skeleton from acyclic precursors, and identification of their cyclised products.

EXPERIMENTAL

Plant material

Seed of *N. racemosa* L. (formerly *N. musinii* Spreng.) was obtained from Chiltern Seeds, Chiltern Stile, Cumbria, U.K. Plants were maintained in the glasshouse.

Chemicals

Authentic nepetalactone (**1a**) ((4a*R*,7*S*,7a*S*)-nepetalactone) was obtained by steam distillation of *N. racemosa* plants, purified by HPLC, and its structure verified by GC-MS (*m/z* 166 (M^+, 63%), 69 (100), 81 (90), 41 (77), 123 (74), 95 (73), 67 (65), 39 (58), 109 (48), 27 (42)) and NMR [28]. Nepetalactol (**1b**) ((4a*R*,7*S*,7a*S*)-nepetalactol) was prepared by DIBAL reduction of the corresponding nepetalactone (**1a**), and authenticated by NMR spectroscopy [28]. Nepetalactol (**2b**) ((4a*S*,7*S*,7a*R*)-nepetalactol) was prepared either by reduction of the corresponding nepetalactone (**2a**) ((4a*S*,7*S*,7a*R*)-nepetalactol) or by synthesis from (3*S*)-citronellol [11], and authenticated by NMR spectroscopy [28].

Enzyme isolation

Leaves of *N. racemosa* (200 g) were collected and homogenized using a mortar and pestle containing 10 g acid-washed sand, in 200 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 20% (*w/v*) glycerol, 50 mM Na metabisulphite, 10 mM Na ascorbate and 1 mM dithiothreitol (DTT). Only the first pair of expanded leaves were used. The homogenate was slurried (5 min) with 25 g polyvinylpolypyrrolidone and 12.5 g Amberlite XAD-4 before filtration through 4 layers of muslin. The filtrate was centrifuged at 20,000 *g* for 20 min, and the supernatant then centrifuged at 100,000 *g* for 60 min. Solid $(NH_4)_2SO_4$ was added gradually to the supernatant, with stirring, to 40% satn, and the soln stirred for a further 20 min at 4°. After centrifugation at 20,000 *g* for 30 min, the ppt was discarded and the supernatant was brought to 60% satn with $(NH_4)_2SO_4$, and re-centrifuged. The ppt was dissolved in 25 ml of 20 mM Tris-HCl (pH 8.0) containing 10% (v/v) glycerol and 1 mM DTT, and dialyzed overnight against 5 l of the same soln, at 4°.

Further purification of the enzyme was achieved by ion-exchange chromatography. The dialyzed preparation was applied to a 100 ml DEAE-Sephacel column, and after washing the column with 100 ml of 20 mM Tris-HCl (pH 8.0) containing 10% (v/v) glycerol and 1 mM DTT, eluted with a linear gradient of 0 to 0.5 M KCl (400 ml) in the same buffer, at 1.0 ml min^{-1}. Active fractions were pooled and dialyzed overnight against 5 l of buffer. This prepn was then applied to a 20 ml 5′-AMP-Sepharose column, and after washing with 50 ml of 20 mM Tris-HCl (pH 8.0) containing 10% (v/v) glycerol and 1 mM DTT, the enzyme was eluted with a linear gradient of 0 to 1.5 mM NAD^+ (100 ml) in the same buffer, at 0.5 ml min^{-1}. Active fractions were pooled and dialyzed as before, and stored prior to use at −70°.

Trichome preparation

Trichomes were removed from the surfaces of approximately 5 g of *N. racemosa* leaves, using the dry ice abrasion procedure described in Ref. [22]. Residual leaves were collected by sieving over 350 μm nylon mesh, and the trichomes (those passing through the sieve together with those adhering to the walls of the tubes used in the procedure) were suspended in 5 ml of 50 mM K-pi (pH 7.5) containing 10% (v/v) glycerol, 1 mM DTT, 10 mM Na metabisulphite and 10 mM Na ascorbate. The trichomes were sonicated on ice (12 × 15 s bursts, with 30 s intervals) using an MSE Soniprep, until microscopic examination indicated that no intact peltate trichomes remained. Polyvinylpolypyrrolidone (0.8 g) was added, and after 3 min of stirring, the preparation was centrifuged at 100,000 *g* for 1 h. The supernatant was used directly for assay of oxidoreductase activities. Residual (trichome-denuded) leaves were processed essentially as described previously (enzyme isolation section), to yield a 100,000 *g* supernatant fraction which was used directly for assay of oxidoreductase activities.

Enzyme assay

Nepetalactol oxidoreductase activity was routinely measured by following the reduction of NAD^+, essentially as described for the assay of the acyclic monoterpenoid oxidoreductase [16]. Each assay contained 2 mM nepetalactol and 0.4 mM NAD^+, in 1 ml 50 mM BisTrisPropane, pH 9. A Cary 3 spectrophotometer was used to follow reduction of the cofactor at 340 nm, with temperature maintained at 25°.

For analysis of the product of nepetalactol oxidation, partially-purified oxidoreductase (0.14 nkat) was incubated at 25° with 0.8 mM NAD^+ and 1 mM *cis,cis*-nepetalactol in 0.5 ml of 20 mM tris-Cl, pH 8. At 15 min intervals, these mixtures were extracted with 0.5 ml CH_2Cl_2. After concn to 100 μl, an aliquot (1.0 μl) was analyzed by gas chromatography (GC). A Hewlett-Packard HP5890 chromatograph equipped with a cool on-column inlet and flame ionization detector and employing a polar capillary column (25 m × 0.53 mm i.d. BP-20, SGE) was used, with He carrier gas. Combined gas chromatography-mass spectrometry employed the same chromatograph, using a non-polar capillary column (50 m × 0.32 mm i.d. HP-1, Hewlett Packard) coupled directly to a VG Analytical 70–250 mass spectrometer employing electron impact ionization (70 eV, 230°). Tentative identifications made by GC-MS were later confirmed by comparison of the mass spectral data with those of samples authenticated by NMR, and by peak

enhancement on GC when co-injected with authentic compounds.

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