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SUBSTRATE SPECIFICITY OF MONOTERPENOL DEHYDROGENASES FROM *FOENICULUM VULGARE* AND *TANACETUM VULGARE**

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Key Word Index—*Foeniculum vulgare*; Umbelliferae; fennel; *Tanacetum vulgare*; Compositae; tansy; monoterpenes; biosynthesis; dehydrogenases; fenchone; thujone.

Abstract—Soluble enzyme preparations from leaves of *Foeniculum vulgare* catalysed the pyridine nucleotide-dependent dehydrogenation of *l-endo*-fenchol to *d*-fenchone, while similar preparations from *Tanacetum vulgare* catalysed the pyridine nucleotide-dependent dehydrogenation of *d*-3-thujanol to *d*-3-thujone. The monoterpenol dehydrogenases were separated from alcohol (ethanol) dehydrogenase by gel-permeation chromatography, and the MW, pH optimum, cofactor preference and other general properties of these enzymes were examined. Specificity studies indicated that only a narrow range of monoterpenols, related to those structural classes produced *in vivo*, were oxidized by these dehydrogenases.

INTRODUCTION

While relatively few enzymes are thought to determine the basic skeletal class of the monoterpenes (e.g. pinanes, bornanes, thujanes, fenchanes, etc.) produced by a given plant, the complex mixture of structurally-related derivatives often encountered in the essential oils suggests that many enzymatic steps may be involved in the subsequent modification and interconversion of the monoterpenes. Whether these secondary transformations are catalysed by commonly-occurring enzymes of low specificity, or by unique, highly specific enzymes has been the subject of speculation [1–3], and some support for both possibilities is available. Thus, certain plant-derived alcohol (ethanol) dehydrogenases readily oxidize monoterpenols such as geraniol [4, 5] while, on the other hand, a rather specific geraniol dehydrogenase has been isolated from *Citrus* and resolved from ethanol dehydrogenase [6].

Recently, we described a partially-purified enzyme system from sage (*Salvia officinalis*, Labiatae) that catalysed the NAD-dependent oxidation of *d*-borneol to *d*-camphor and of *l*-thujol (now *l*-3-neoisothujanol)‡ to *l*-thujone (now *l*-3-isothujone) [7]. The activity, which was distinct from ethanol dehydrogenase, was rather specific for these particular monoterpenol substrates, and several types of evidence strongly suggested that the same dehydrogenase catalysed this key oxidation step in the biosynthesis of both *d*-camphor and *l*-3-isothujone, which are the major monoterpenes of sage [7]. On the basis of this evidence we speculated that monoterpenol oxidations might not be carried out by general alcohol dehydrogenases or by highly specific dehydrogenases, but rather by dehydrogenases which are specific for the particular structural groups of monoterpenes produced by the plant (i.e. 'group-specific' dehydrogenases). In this communication we describe the partial purification and characterization of monoterpenol dehydrogenases from fennel (*Foeniculum vulgare*, Umbelliferae), a plant which produces *d*-fenchone as the sole monoterpene ketone [23], and from tansy (*Tanacetum vulgare*, Compositae) which produces both *d*-3-thujone and *l*-camphor as major essential-oil components [9]. Substrate specificity studies with both of these enzyme preparations support the suggestion that monoterpenol oxidations are catalysed by group-specific dehydrogenases.

RESULTS

An NAD-dependent *l-endo*-fenchol dehydrogenase (FDH) activity could be demonstrated in crude

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‡Considerable confusion has resulted from the use of two systems of nomenclature for thujane monoterpenes. While much of the previous work on the thujane monoterpenes of *Salvia* [7, 8] and *Tanacetum* [9–15] has employed the traditional system of nomenclature [16–18], we have adopted here the more rational scheme proposed by Brown *et al.* [19], now coming into general use [20–22].

homogenates of fennel leaves, while an NAD-dependent *d*-3-thujanol dehydrogenase (TDH) activity was readily demonstrated in crude homogenates of tansy leaves. Differential centrifugation indicated that these dehydrogenases were soluble enzymes; essentially all the activity in both fennel and tansy preparations remained in the supernatant after centrifugation at 105 000 g for 90 min.

Resolution of monoterpenol dehydrogenases from alcohol (ethanol) dehydrogenases

Concentration of the soluble protein fraction by $(NH_4)_2SO_4$ precipitation followed by gel-permeation chromatography on Sephadex G-150 allowed the separation of FDH from alcohol (EtOH) dehydrogenase (ADH) in fennel-leaf preparations (Fig. 1a), and the separation of TDH from ADH in tansy preparations (Fig. 1b). ADH was located spectrophotometrically (Δ 340 nm), while the monoterpenol dehydrogenases were located by assay of NADH-[4-^{3}H] production from *l-endo*-fenchol-[2-^{3}H] (for FDH) and from both *d*-3-thujanol-[3-^{3}H] and *l*-3-neoisothujanol-[3-^{3}H] (for TDH). Each monoterpenol dehydrogenase was also located by *l*-camphor-[G-^{3}H] formation (TLC assay) from the alternate substrate *l*-borneol-[G-^{3}H]. The elution patterns of FDH and TDH were identical

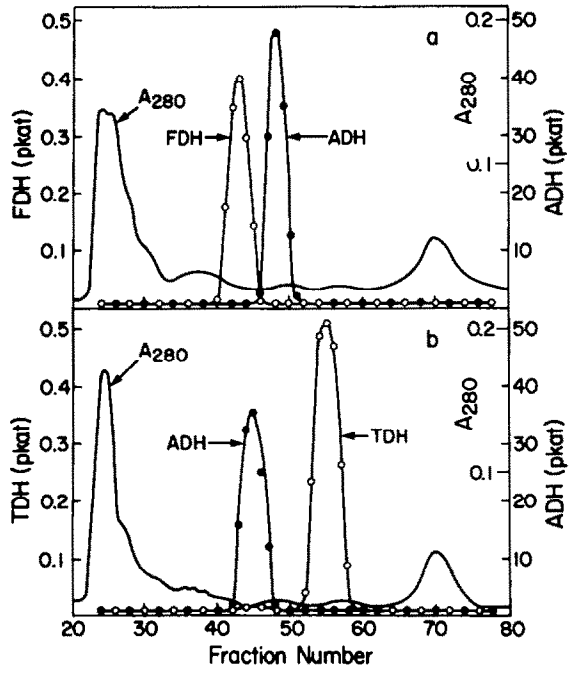


Fig. 1. (a) Sephadex G-150 column chromatography of the soluble-protein fraction obtained from a homogenate of fennel leaves. Absorbance at 280 nm (——), *l-endo*-fenchol dehydrogenase (FDH) activity (O—O), and ethanol dehydrogenase (ADH) activity (●—●) are plotted. (b) Gel-permeation chromatography of the soluble-protein fraction from a homogenate of tansy leaves on the same Sephadex G-150 column. Absorbance at 280 nm (——), ethanol dehydrogenase (ADH) activity (●—●), and *d*-3-thujanol dehydrogenase (TDH) activity (O—O) are plotted. Chromatography and assay procedures are described in the Experimental. V_0 was at fraction No. 24.

when assayed by NADH-[4-^{3}H] production or by *l*-camphor-[G-^{3}H] formation. Neither fennel nor tansy ADH possessed monoterpenol-dehydrogenase activity, nor did the monoterpenol dehydrogenases exhibit significant EtOH dehydrogenase activity (Fig. 1a, b). In the absence of pyridine nucleotide, no dehydrogenase activity was detected, and no activity was detected when boiled enzyme was incubated with the complete assay medium. For both FDH and TDH isolated by gel-permeation chromatography, product formation was proportional to added protein (up to the 150 μg protein level) and to the time of incubation (up to 90 min) under the standard assay conditions described.

General characteristics of monoterpenol dehydrogenases

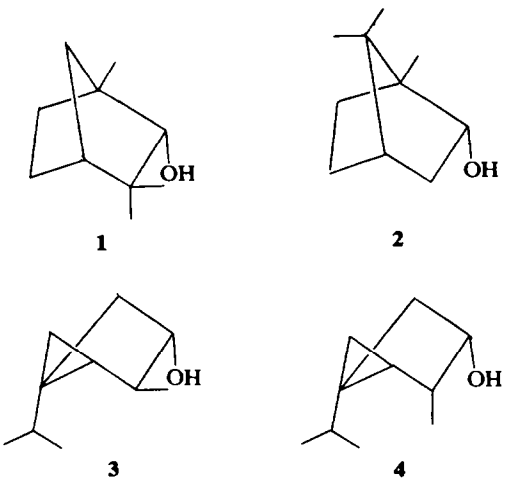
The MW of FDH from fennel was determined to be *ca* 89 000 by chromatography on a calibrated Sephadex G-150 column, while the MW of the ADH from this tissue was estimated to be 78 000 by similar means. The TDH isolated from tansy had a MW of roughly 66 500, while the ADH of this tissue exhibited a MW of 85 500 by calibrated gel-permeation chromatography. The monoterpenol dehydrogenase previously isolated from sage had a MW of 91 000 [7].

The FDH exhibited a pH optimum at 8.0 (half maximal activities at pH 7.0 and 9.2) when examined in sodium phosphate, barbital sodium-acetate, Tris–Cl, and glycine–NaOH buffers (all 50 mM). Maximum activity and stability of the enzyme were achieved in 50 mM sodium phosphate buffer (pH 8.0) which was used throughout. The TDH from tansy also exhibited maximum activity at pH 8.0 (half maximal activities at pH 6.7 and 9.3) when examined in the above buffers, and it was also most stable in 50 mM sodium phosphate buffer (pH 8.0).

FDH showed a slight preference for NAD ($K_m \sim 10^{-4}$ M) over NADP ($K_m \sim 2\times10^{-4}$ M) as cofactor, and at saturating concentrations of pyridine nucleotide (10^{-3} M), NADP yielded rates *ca* 85% of those observed with NAD. In the case of TDH, NAD ($K_m \sim 10^{-4}$ M) was also preferred to NADP ($K_m \sim 2\times 10^{-4}$ M), but at saturating concentrations of pyridine nucleotide (10^{-3} M) no significant difference in rates of monoterpenol oxidation was observed. Flavin nucleotides were ineffective as cofactors with both enzymes. The *d*-borneol/*l*-3-neoisothujanol dehydrogenase previously isolated from sage preferred NAD as cofactor ($K_m = 7\times10^{-5}$ M), and, like the dehydrogenases described here, it exhibited a pH optimum and stability optimum at pH 8.0 [7].

Substrate specificity of monoterpenol dehydrogenases

Estimated K_m for *l-endo*-fenchol (with FDH) and for *d*-3-thujanol (with TDH) was about 4×10^{-5} M, and specificity studies were carried out at the 2×10^{-4} M substrate level. FDH exhibited a strong preference for *l-endo*-fenchol (**1**) as substrate (Table 1). *l-endo*-Fenchol is the only fenchol stereoisomer formed from geranyl pyrophosphate in cell-free preparations from fennel leaf, and its role as the key intermediate in *d*-fenchone biosynthesis seems quite certain [23]. Of the other monoterpenols tested, only *l*-borneol (**2**), which closely resembles *l-endo*-fenchol (**1**), was oxidized at an appreciable rate. Thus, FDH was highly specific for the monoterpenol substrate,



1 2 3 4

and it oxidized at the highest rate that monoterpenol considered to be the natural precursor of *d*-fenchone *in vivo* [23]. In the case of TDH from tansy, both *d*-3-thujanol (**3**) and *l*-3-neoisothujanol (**4**) were oxidized at high rates (Table 1). Analysis of the volatile oil from immature tissue used in the present experiments (see Experimental) indicated the presence of the corresponding ketones *d*-3-thujone (53%) and *l*-3-isothujone (3%). While *l*-3-isothujone is a minor component of the oil in the present case, chemical races of tansy that produce isothujone as the major component are known [24, 25], and so this capacity to produce isothujone from the appropriate precursor is not surprising. While purification of the enzyme to homogenity would provide the only firm evidence that the diastereomeric thujanols (**3** and **4**) are oxidized by the same species, the fact the *d*-3-thujanol dehydrogenase and *l*-3-neoisothujanol dehydrogenase activities (as well as *l*-borneol dehydrogenase activity) were coincident on Sephadex G-150 chromatography strongly suggests this possibility. If the thujanol diastereomers are oxidized by the same enzyme, then the specificity studies suggest that an important feature of the substrate is the relative spatial orientation of the hydroxyl group and the isopropyl-substituted cyclopropane ring, and that the configuration of the C-4 methyl is less important (cf. **3** and **4**). In addition to the specific pair of 3-thujanols, the TDH oxidized *l*-borneol (**2**) to *l*-camphor at a respectable rate (Table 1). *l*-Borneol is the expected precursor of *l*-camphor [26, 27], a constituent comprising 19% of the volatile oil from the immature tansy leaves used here. Camphor is the major monoterpene produced by certain chemical races of tansy [24, 25].

Table 1. Monoterpenol specificity of the dehydrogenases isolated from *F. vulgare* and *T. vulgare*

Substrate	*F. vulgare* (Rel. rate)	*T. vulgare* (Rel. rate)
l-endo-Fenchol	100	3
d-endo-Fenchol	16	<2
l-3-Neoisothujanol	4	100
d-3-Thujanol	11	86
l-3-Isothujanol	<2	<2
d-3-Neothujanol	<2	<2
l-Borneol	28	34
d-Isoborneol	3	13
d-Borneol	7	<2
l-Isoborneol	11	5
l-Menthol	<2	<2
d-Neomenthol	<2	<2

Each reaction mixture, containing 100 μg protein (*F. vulgare*) or 120 μg protein (*T. vulgare*), 1 mM NAD, 0.2 mM monoterpenol, and 1 mM dithioerythritol in 1 ml 50 mM Na–phosphate buffer (pH 8.0), was incubated at 30° for 1 hr. For *F. vulgare* the rate of oxidation of *l-endo*-fenchol, which was taken as 100, was 3.9 pkat/mg. For *T. vulgare* the rate of oxidation of *l*-3-neoisothujanol, which was taken as 100, was 4.2 pkat/mg. For specificity studies, monoterpenols labeled at the carbinol carbon were used as substrates and the rate of formation of NADH-[4-^{3}H] was measured.

DISCUSSION

Recent studies on the cyclization of geranyl pyrophosphate in cell-free systems have indicated that *endo*-fenchol is the essential precursor of fenchone [23], and that borneol is the direct precursor of camphor [7, 26, 27]. The results reported here show that the corresponding dehydrogenases involved in fenchone and camphor biosynthesis are specific for those particular monoterpenol precursors synthesized by the 'cyclase' enzymes from geranyl pyrophosphate. Similarly, *l*-3-neoisothujanol (rather than the epimeric *l*-3-isothujanol) has been previously proposed as the key precursor of *l*-3-isothujone [7], a suggestion that finds further support in the present work. Banthorpe and associates [14] have suggested a concerted mechanism for the origin of *l*-3-neoisothujanol, which would yield *l*-3-isothujone on oxidation, and have proposed an 'X-group' mechanism for the origin of *d*-3-neothujanol, which would yield *d*-3-thujone on oxidation. While the present work, as well as studies with the dehydrogenase from sage [7], supports the intermediacy of *l*-3-neoisothujanol in *l*-3-isothujone biosynthesis, the specificity observed for *d*-3-thujanol suggests that this epimer (and not *d*-3-neothujanol) may be the actual precursor of *d*-3-thujone *in vivo*.

With the present work, our studies on monoterpenol dehydrogenases now encompass three species from three different families, and include a representative that produces but a single monoterpene ketone as well as those that produce significant quantities of two structurally distinct ketone types (i.e. bornanes and thujanes). The summation of the results indicates that monoterpenol oxidations are not catalysed by a general alcohol dehydrogenase, nor does a distinct, highly specific dehydrogenase appear to be required for each requisite oxidation. Rather, the results suggest that such oxidations may be catalysed by dehydrogenases which are specific (i.e. 'group specific') for the particular monoterpenols produced by that species.

EXPERIMENTAL

Plant material. F. vulgare was grown from seed under controlled conditions described previously [23]. Young filamentous leaves from the shoot apex of 21-day-old plants

were used in all experiments, and the monoterpene content of this tissue has been described [23]. Immature *T. vulgare* leaves (< 4 cm in length) from the shoot apex of young plants were obtained from a population growing on this campus. GC–MS analysis of the steam-distilled oil from this tissue indicated the presence of *d*-3-thujone (53%), *l*-3-isothjone (3%) and *l*-camphor (19%) as the only ketones.

Substrates and reagents. *d*-Borneol-[G-^{3}H], *l*-isoborneol-[G-^{3}H] (both 24.3 Ci/mol), *l*-borneol-[G-^{3}H], *d*-isoborneol-[G-^{3}H] (both 28.7 Ci/mol), and *l*-menthol-[G-^{3}H] (26.5 Ci/mol) were prepared by $^3H_2$ exposure, and purified by TLC as described previously [27, 28]. *d*-Borneol-[2-^{3}H] and *l*-isoborneol-[2-^{3}H] (60 Ci/mol), *l*-borneol-[2-^{3}H] and *d*-isoborneol-[2-^{3}H] (83.6 Ci/mol), *l*-3-neoisothujanol-[3-^{3}H] and *l*-3-isothujanol-[3-^{3}H] (83.6 Ci/mol), *l*-menthol-[3-^{3}H] and *d*-neomenthol-[3-^{3}H] (77.6 Ci/mol), *d*-3-thujanol-[3-^{3}H] and *d*-3-neothujanol-[3-^{3}H] (60 Ci/mol), and the *endo*-fenchols-[2-^{3}H] (67.5 Ci/mol) were prepared by reduction of the corresponding ketones with NaB^3H_4. Details of the prepn and TLC purification of labeled monoterpenols of the bornane [7], fenchane [23] and menthane [29] series are described elsewhere. Detailed procedures have also been reported for hydride reduction of thujone and isothujone [16], and TLC purification of the resulting diastereomeric thujanols [17]. Purities of the labeled monoterpenes were verified by GC–RC, and location of ^{3}H on the carbinol carbon of specifically labeled substrates was confirmed by CrO_3 oxidation, as described [7]. For use as substrates, the monoterpenols were suspended in H_2O with the aid of Tween-20 (10 μg/μmol) and sonication. It was independently verified that Tween-20 had no effect on the dehydrogenases when present at 20× the concn present in the substrate. The sources of monoterpene standards have been described previously [7, 8, 23]; other standards, reagents and biochemicals were obtained from Sigma Chemical Co. or Aldrich Chemical Co.

Enzyme preparation. All operations were carried out at 0–4°. Leaves (15 g) were homogenized in a Ten-Broeck homogenizer in 100 mM Na-Pi buffer (pH 6.5) containing 250 mM sucrose, 50 mM ascorbic acid, 50 mM $Na_2S_2O_5$, 5 mM $MgCl_2$ and 1 mM dithioerythritol. The homogenate was immediately slurried for 10 min with 15 g dry washed PVP (GAF Corp. Polyclar AT) [30] and 15 g washed and hydrated Amberlite XAD-4 resin (Rohm and Haas Corp.) [31] to remove phenolics and resinous material [30, 31]. The slurry was then filtered through several layers of cheesecloth, and the filtrate centrifuged at 27 000 g for 20 min. The resulting pellet was discarded and the supernatant centrifuged at 105 000 g for 90 min to yield the soluble protein fraction used as the source of the enzyme. For localization studies, the XAD-4 resin treatment was omitted and soluble PVP (GAF Corp. Plasdone K-90) [30] was substituted for the insoluble polymer. Particulate fractions were prepared directly by differential centrifugation.

Enzyme purification. Monoterpenol dehydrogenase activity was almost quantitatively precipitated from the soluble enzyme fractions with $(NH_4)_2SO_4$ [0–60% saturation (0.39 g/ml) in the case of FDH from fennel, and 0–80% saturation (0.56 g/ml) in the case of TDH from tansy]. The precipitates were collected by centrifugation at 27 000 g for 10 min, and then suspended in 5 ml of 50 mM Na-Pi buffer (pH 6.5) containing 2 mM ascorbic acid and 1 mM dithioerythritol. After centrifugation to remove denatured protein, the conc protein fraction was applied to a 2.5 × 100 cm column of Sephadex G-150, which was previously equilibrated and eluted with 50 mM Na-Pi buffer containing ascorbic acid and dithioerythritol as above. The column effluent was monitored at 280 nm, and 6.8 ml fractions were collected for assay of dehydrogenase activity. Fractions containing activity were pooled, concd by ultrafiltration (Amicon PM-10) and dialysed to the appropriate conditions.

Enzyme assays. ADH activity was assayed spectrophotometrically by the method of ref. [32]. For the assay of monoterpenol dehydrogenase activity, the standard reaction mixture contained 100–150 μg protein, 1 mM dithioerythritol, 1 mM of either NAD or NADP and 0.1 mM substrate alcohol (either [G-^{3}H]-labeled or specifically [^{3}H]-labeled at the carbinol carbon only), in a total vol. of 1 ml 50 mM Na–Pi buffer (pH 8.0). The reaction mixture was incubated in a sealed tube at 30° for up to 90 min. When the substrate was alcohol-[G-^{3}H], the reaction was stopped by chilling in ice and extracting the products with 2 ml Et_2O. The appropriate unlabeled ketone (15 mg) was added as an int. standard, and the labeled product was isolated by TLC (Si gel; hexane–EtOAc, 9:1) for the determination of ^{3}H content by liquid-scintillation spectrometry as described previously [7]. When the substrate was an alcohol specifically labeled with ^{3}H at the carbinol carbon, the reaction was stopped by chilling and repeated extraction of residual substrate with EtOAc. While the NADH-[4-^{3}H] or NADPH-[4-^{3}H] formed in the reaction could be isolated from the aqueous phase by ion exchange chromatography [33], measurement of the radioactivity remaining in the aq. phase after EtOAc extraction [scintillation spectrometry in ScintiVerse (Fisher Scientific); 34% efficiency for ^{3}H] was shown to provide a more convenient and reliable assay of product formation [7]. Appropriate boiled controls, and controls run without Py nucleotide, were included in each experiment, with individual controls in those experiments in which different control values were possible (e.g. pH optimum). All radiochemical assays were conducted with a s.d. of less than 3%. It was independently verified that dithioerythritol, which was always present in the assay medium, was not a substrate for the dehydrogenases and had no adverse effect on the reaction.

Protein determination. Protein in partially purified preparations was determined directly (after TCA precipitation) by the method of Lowry *et al.* [34], with bovine serum albumin as standard. Modification of the method to compensate for phenolic interference [35] was unnecessary.

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