Bull. Environ. Contam. Toxicol. (1988) 41:17–25

Archives of Environmental Contamination and Toxicology

Biotransformations of α-Terpineol in the Rat: Its Effects on the Liver Microsomal Cytochrome P-450 System

K. Madhava Madyastha* and V. Srivatsan

Bio-Organic Section, Department of Organic Chemistry, Indian Institute of Science, Bangalore 560 012, India

α-Terpineol (I), a monocyclic monoterpene tertiary alcohol, is widely used in the manufacture of perfumes, cosmetics, soaps and antiseptic agents. It was reported earlier (Horning et al. 1976) that this monoterpene alcohol when administered to humans is hydroxylated to p-menth-1,2,8-triol (II). It is not known whether α-terpineol also produces other metabolites during its metabolism in the mammalian system and if so, the nature of these metabolites.

It is known that some of the terpenoids when administered to rats or rabbits increase the activity of liver microsomal mixed-function oxidases (Parke and Rahman, 1969; Jori et al. 1969; Cinti et al. 1976; Chadha and Madyastha 1984; Madyastha and Chadha 1986), now called polysubstrate monoxygenases (PMSO). However, conflicting reports have appeared in literature regarding the effects of α-terpineol on liver microsomal cytochrome P-450 system. Parke and Rahman (1969) have reported that upon administration of some of the monoterpenes such as terpineol to rats once daily for three days results in the increase of liver microsomal cytochrome P-450 and drug-metabolizing activity. However, Cinti and "colleagues" (1976) showed a decrease in the levels of cytochromes P-450 and b_5 as well as aminopyrine N-demethylase activity upon injecting (i.p.) α-terpineol to rats once daily for three days. Hence, a reinvestigation was undertaken to establish the metabolic fate of α-terpineol and its effects on liver microsomal cytochrome P-450 system following oral administation of this monoterpene alcohol to rats.

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*To whom correspondence should be addressed.

MATERIALS AND METHODS

Cytochrome c, NADPH, NADH, dithiothreitol (DTT), methyl cellulose and α-terpineol were procured from Sigma Chemical Co., USA. α-Terpineol was purified by column chromatography over silica gel using hexane-ethylacetate (9:1) as the eluent and was found to be 99% pure as judged by g.l.c. analysis (Madyastha and Renganathan 1983).

UV and visible absorption spectra were recorded on a Hitachi 557 double wavelength double beam spectrophotometer. IR spectra were recorded on a Perkin Elmer model 397 spectrophotometer. NMR spectra were recorded with a Varian T-60 spectrometer. Gas chromatography-mass spectral analyses were performed on a Hewlett-Packard instrument. Gas liquid chromatography was performed on a Chemito model 3800 chromatograph fitted with a stainless steel column containing 5% QF_1 on chromosorb w. The column temperature was maintained at 160^{o}C and the detection was carried out using FID detector with N_2 as the carrier gas with a flow rate of 30 ml/min.

Thin layer chromatography (TLC) (silicagel G) of the metabolites were performed using hexane-ethylacetate (8:2, system I; 7:3, system II; 6:4, system III) as the solvent system. Metabolites were visualized either by exposing the plates to iodine or by spraying with 1% vanillin in 50% concn. H_2SO_4 and heating at 110^{o}C for 5 min.

Male albino rats (IISc., strain) weighing 185-225 g were used in the present study. For the biotransformation studies, α-terpineol was administered orally (600 mg/kg body wt.), once daily for 20 days as a suspension in 1% methyl cellulose solution (2 ml). Control rats were given only the vehicle. After dosing, control and experimental rats were housed separately in metabolism cages with free access to food and water. Urine was collected daily, adjusted to pH 3-4 and extracted twice with equal volume of diethyl ether. The aqueous portion remaining after ether extraction containing conjugated metabolites was then subjected to acid hydrolysis and then extracted with ether (Chadha and Madyastha 1984). Both the ether extracts were separated into neutral and acid fractions. The acidic fractions were esterified using diazomethane (Chadha and Madyastha 1984).

The rats used for studying the effects of α-terpineol on the hepatic microsomal cytochrome P-450 system were dosed as mentioned earlier. The contol and experimental rats were sacrificed after specified days of

dosing (Table 1) and the liver microsomes were prepared by the method of Lu and Levin (1972)

Protein concentrations were determined by the method of Lowry *et al.* (1951). The concentrations of cytochromes P-450 and b_5 in the liver microsomes were measured by the method of Omura and Sato (1964). The NAD(P)H-cytochrome *c* reductase activities were determined as reported earlier (Chadha and Madyastha 1982).

RESULTS AND DISCUSSION

The neutral fraction (1.1 g) on TLC analysis (system II) showed the presence of one major (R_f 0.69) and two minor (R_f 0.63 & 0.35) compounds. This fraction was subjected to column chromatography over silica gel (45 g) using hexane and hexane-ethylacetate mixtures as eluents. The compound with R_f 0.69 eluted with hexane-ethylacetate (9:1) was found to be α-terpineol on comparing the IR and NMR spectra with that of the authentic sample. The other two metabolites with R_f 0.63 and 0.35 were eluted with 17:3 and 15:5 hexane-ethylacetate, respectively. The compound with R_f 0.63 was found to be impure on the basis of g.l.c. analysis and could not be processed further for characterization due to paucity of material. The compound with R_f 0.35 was further purified by preparative TLC (system III) and the pure compound showed the following spectral characteristics. IR spectrum (liquid film) indicated the presence of hydroxyl function (3400 and 1360 cm^{-1}). NMR ($CDCl_3$) spectrum showed signals at 1.2 δ (6H,s), gem-dimethyl protons on a carbon carrying a hydroxyl group; 1.35 δ (3H,s), methyl protons attached to a carbon in a ring carrying a hydroxyl function, C-7; 1.5-1.95 δ (6H,m), the saturated methylene protons of the ring, C-3, C-5 & C-6; 2.0-2.4(2H,m), a methine proton (C-4) and a hydroxyl proton which disappeared on adding D_2O; 3.7(1H,m), a proton on a carbon carrying a secondary hydroxyl group (C-2) and 4.5 δ (2H, br peak), two hydroxyl protons which disappeared on adding D_2O. Based on these spectral characteristics, the compound was identified as p-methane-1,2,8-triol (II, Fig.1).

The neutral fraction (690 mg) from hydrolysed urine on TLC analysis (system II) showed a major compound (R_f 0.69) and a few minor compounds. The compound with R_f 0.69 was isolated by preparative TLC (system II) and was found to be α-terpineol on comparing the IR and NMR spectra with that of the authentic sample. The minor compounds could not be identified due to paucity of material.

TLC analysis (system I) of the esterified acid fraction (2.1 g) showed the presence of one major (R_f 0.44) and

two minor (R_f 0.33 and 0.23) metabolites. This fraction was subjected to column chromtography over silica gel (85 g) and the metabolites with R_f 0.44, 0.33 and 0.23 were eluted with hexane-ethylacetate 8:2, 7:3 and 6:4, respectively. The compound (880 mg) with R_f 0.44 was further purified by silica gel column (35 g) and eluted with hexane-ethylacetate (8:2). IR spectrum (liquid film) of the compound showed absorptions of a tertiary hydroxyl group (3450 and 1250 cm^{-1}) and an α, β-unsaturated carbonyl group (1705 cm^{-1}). NMR spectrum ($CDCl_3$) showed signals at 1.2 δ (d,gem-dimethyl protons on a carbon carrying hydroxyl group); a multiplet between 1.8-2.4 δ; a doubletat 3.7 δ (carbomethoxyl group); and a broad multiplet at 6.9 δ (β-proton of an α,β -unsaturated carbonyl system). The NMR data of the metabolite was similar to that of the authentic oleuropeic acid methyl ester and also to that reported earlier for the same compound (Shukla et al. 1968) except that the metabolite showed doublets at 1.2 and 3.7 δ instead of singlets as noticed in the case of authentic oleuropeic acid. This suggested that the metabolite isolated is a mixture containing oleuropeic acid methyl ester and a compound closely related to it. G.l.c. analysis (5% QF_1 on chromosorb w, column temp. 160°C), gave two peaks (R_t 7.2 and 9.0 min). The peak with R_t 7.2 min corresponded to that of authentic oleuropeic acid methyl ester and was enhanced when admixed with authentic compound. The mixture was hydrogenated at room temperature using Raney nickel catalyst and then subjected to g.l.c. analysis which gave only one peak with R_t 9.0 min. This observation suggests the presence of dihydrooleuropeic acid (IV) and oleuropeic acid (III) methyl esters in the mixture. The presence of these two compounds was confirmed by GC-mass spectral analysis (Fig.2).

Oleuropeic acid (III) methyl ester: m/z 180(M^+-H_2O), 140($C_8H_{12}O_2^+$), 121($C_9H_{13}^+$), 105($C_8H_9^+$), 80(C_6H_8) and base peak at 59($C_2H_3O_2$).

Dihydrooleuropeic acid (IV) methyl ester: m/z 182 (M^+-18), 142 ($C_8H_{14}O_2$), 122(C_9H_{14}, base peak), 107(C_8H_{11}), 81(C_6H_9) and 59($C_2H_3O_2$).

The methylated acid fraction from the hydrolysed urine upon TLC analysis (system II) showed a major (R_f 0.44) and a few minor metabolites. On g.l.c. analysis it gave three prominent peaks (R_t 5.7, 7.2 and 9.0 min). Peaks with R_t 9.0 and 7.2 min were enhanced on mixing with the mixture of oleuropeic (III) and dihydrooleuropeic acid (IV) methyl esters.

Biotransformation of α-terpineol to p-menthane 1,2,8-triol (II) was reported earlier (Horning et al. 1976).

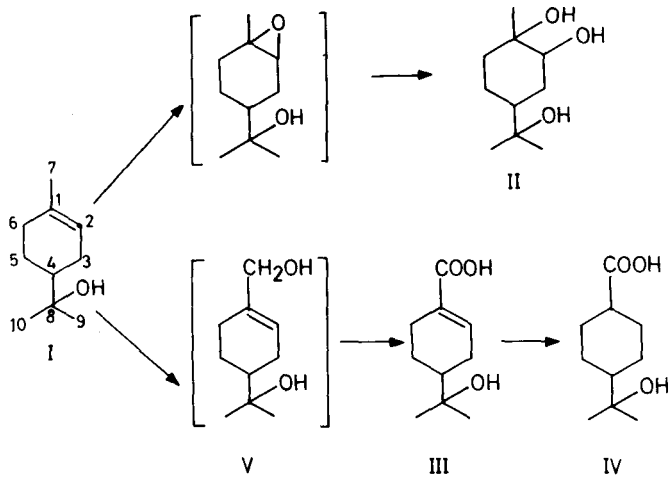

FIG.1 BIOTRANSFORMATIONS OF α- TERPINEOL (I) IN RAT

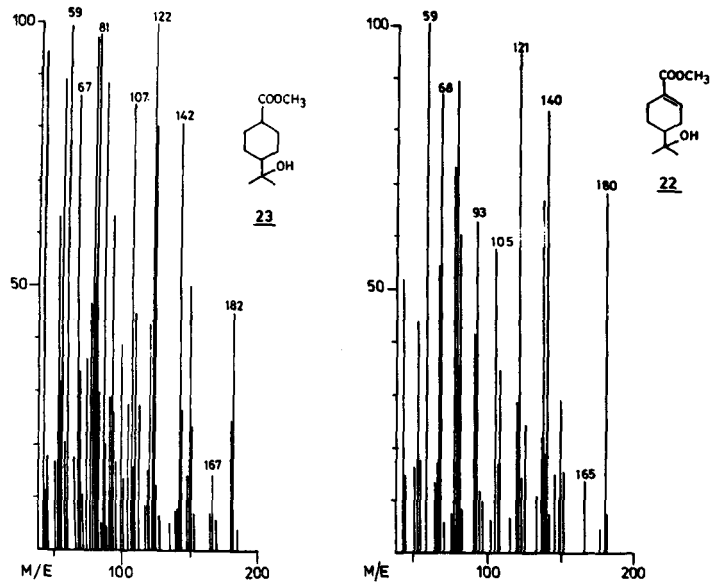

Fig.2 Mass spectra of the methyl esters of Dihydrooleuropeic(IV) and oleuropeic(III) acids.

TABLE - I EFFECT OF ORAL ADMINISTRATION OF α - TERPINEOL ON HEPATIC DRUG - METABOLISING ENZYMES

Days of treatment	Cyt P - 450 n mol mg^{-1} protein	% Change	Cyt b_5 n mol mg^{-1} protein	% Change	NADPH-Cyt c reductase n mol min^{-1} mg^{-1} protein	% Change	NADH-Cyt c reductase μ mol min^{-1} mg^{-1} protein	% Change
0 (control)	0.50 ± 0.10	–	0.51 ± 0.10	–	81 ± 20	–	0.73 ± 0.19	–
1	0.86 ± 0.14*	72 (+)	0.59 ± 0.09	16 (+)	112 ± 17*	38 (+)	0.63 ± 0.14	14 (-)
2	1.02 ± 0.21*	104 (+)	0.58 ± 0.10	14 (+)	117 ± 23*	43 (+)	0.73 ± 0.24	0
3	0.95 ± 0.13*	90 (+)	0.57 ± 0.03	12 (+)	99 ± 13	22 (+)	0.64 ± 0.19	12 (-)
6	0.77 ± 0.04*	54 (+)	0.52 ± 0.04	2 (+)	84 ± 11	3 (+)	0.62 ± 0.07	15 (-)
9	0.76 ± 0.10*	52 (+)	0.46 ± 0.01	10 (-)	87 ± 22	7 (+)	0.79 ± 0.13	8 (+)

All values represent mean ± S.D., N=3 sets, each set with 4 rats. (+) or (-) indicate increase or decrease with respect to control, respectively. * Values differing significantly from control for a $P < 0.05$. The values of the control rats for days one, two, three, six and nine did not differ appreciably from one another and hence they were combined and the mean value ± S.D. is presented in the table. Details of the experiment are given in the text.

This minor neutral metabolite is probably formed from an epoxide intermediate as hypothesized earlier. However, the present study clearly suggests that allylic methyl oxidation and the reduction of the 1,2-double bond are the major routes for the metabolism of α-terpineol in rat (Fig.1). Although allylic oxidation of C-1 methyl seems to be the major pathway, the alcohol p-menth-1-ene-7,8-diol (V) could not be isolated from the urine of rats. Probably this compound does not get accumulated and is readily oxidized further to oleuropeic acid (III). Similar observations have been made earlier in the case of Pseudomonas incognita which was shown to oxidize the allylic methyl of α-terpineol to oleuropeic acid (III) via the intermediacy of the corresponding alcohol (Madyastha and Renganathan 1984). The present studies have clearly demonstrated that the endocyclic double bond in oleuropeic acid (III) readily gets reduced to yield dihydrooleuropeic acid (IV). It is reasonable to assume that the allylic methyl group (C-7) gets oxidized prior to the reduction of the 1,2-double bond. Earlier investigators have noticed the resistance of isolated endocyclic double bond to reduction after oral administration of some of the terpenoids with endocyclic double bond to mammals (Kodama et al. 1974: Ishida et al. 1981; Ventura et al. 1985).

The effect of α-terpineol on the liver microsomal cytochrome P-450 system is summarized in Table 1. Oral administration of α-terpineol to rats increased the levels of liver microsomal cytochrome P-450 by 72, 104, 90, 54 and 52% after 1, 2, 3, 6 and 9 days of treatment, respectively. A moderate increase in the levels of liver microsomal NADPH-cytochrome c reductase was also observed during the first three days of repeated treatment (Table 1). The effect on cytochrome b_5 and NADH-cytochrome c reductase was not significant. Contrary to these observations, Cinti et al. (1976) reported that the levels of liver microsomal cytochromes P-450 and b_5 decreased considerably upon administration of α-terpineol to rats intraperitoneally or by the aerosol route. However, the present studies are in agreement with the observation made by Parke and Rahman (1969) regarding the ability of α-terpineol to induce liver microsomal cytochrome P-450 upon its oral administration to rats, although the extent of induction observed in the present studies was more than that reported by Parke and Rahman (1969). The difference in the mode of administration may be responsible for these incompatible observations. In fact earlier it was reported that bornylacetate, cineole, compounds closely related to α-terpineol, when administered to rats results in a significant increase in the levels of cytochromes P-450 and b_5, NADPH-cytochrome c reductase and N-demethylase activities (Cinti et al. 1976);

Madyastha and Chadha 1986). In the present studies maximal induction of cytochrome P-450 and NADPH-cytochrome c reductase was observed upon oral administration of α-terpineol once daily, for two days and further treatment decreased the levels considerably (Table 1). The initial induction of both these enzymes could result in the faster metabolism of α-terpineol resulting in the shortening of its half-life period. Hence, it is quite possible that the level of induction on further treatment may be less pronounced. Similar observations have been made earlier in the case of terpentine oil (Jarvisalo and Vainio, 1980).

In conclusion, the results of this study have demonstrated that allylic methyl oxidation of α-terpineol is the major route for its biotransformation in rat. The other interesting observation made was the reduction of the endocyclic double bond as seen in the formation of dihydrooleuropeic acid (IV) from oleuropeic acid (III). Like cineole, α-terpineol also when administered to rat increase the liver microsomal cytochrome P-450 system to a significant extent.

ACKNOWLEDGEMENT. Financial assistance from the Department of Science and Technology (DST), India is gratefully acknowledged.

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Received February 7, 1988; accepted April 28, 1988.