

Biosynthesis of β -Substituted Furan Skeleton in the Lower Furanoterpenoids: A Model Study

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Furanoterpenes are widely distributed in the plant kingdom. In this study we have carried out enzymatic synthesis of simple furan compounds from the molecules containing an α -isopropylidene ketone unit and the role of cytochrome P450 in this biotransformation has been conclusively established. Eight model compounds (acyclic, monocyclic, and bicyclic, 1–8), having an α -isopropylidene ketone unit, were synthesized and incubated with PB-induced rat liver microsomes in the presence of NADPH and O₂. GC-MS and NMR analyses of the product(s) indicated the formation of corresponding furano derivatives (11–18). Cytochrome P450 inhibitors, metyrapone, SKF-525, and carbon monoxide, inhibited the formation of furan (8) to 76, 62, and 97%, respectively. Incubation of dehydrofukinone (7), a naturally occurring terpene, with purified cytochrome P450, NADPH-cytochrome P450 reductase, and dilaurylphosphatidylcholine in the presence of NADPH and O₂ resulted in the formation of 10 and furanodehydrofukinone (19). Based on these observations, we propose one of the probable biosynthetic routes for lower furanoterpenoids in higher plants.

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Key Words: biosynthesis; biotransformation; furanoterpenes; furan; furanoeremophilane; P450; inhibition; reconstitution.

R-(+)-pulegone, the major constituent of pennyroyal oil from *mentha pulegium*, with an α -isopropylidene ketone unit, was demonstrated to convert to R-(+)-menthofuran by rat as well as human liver cytochrome P450 enzyme system (1, 2). It was suggested that R-(+)-pulegone regio-selectively undergoes hydroxylation at methyl group *syn* to the carbonyl unit, followed by spontaneous intramolecular cyclization to the hemi-

ketal and then dehydration to yield R-(+)-menthofuran (1). Earlier we have demonstrated similar conversions in case of S-(–)-pulegone as well as piperitenone to S-(–)-menthofuran and 6,7-dehydromenthofuran respectively, by rat liver microsomal cytochrome P450 (3, 4). It has been shown by *in vivo* feeding experiments with peppermint, that R-(+)-menthofuran is derived from R-(+)-pulegone (5). Recently it has been demonstrated that the microsomal preparations from the oil gland secretory cells of *Mentha pulegium*, transform R-(+)-pulegone to R-(+)-menthofuran (6). Considering above studies involving mammalian as well as plant cytochrome P450 enzyme systems, it is reasonable to assume that similar regiospecific oxidations of terpenes, containing an α -isopropylidene ketone unit, might be involved in the biosynthesis of molecules with β -substituted furan skeleton in plant. In fact several β -substituted furanoterpenes, viz. furanoeremophilanes, furanogermacranes, etc., have been isolated from natural sources (7–10) with the corresponding compounds having α -isopropylidene ketone unit (9, 10). Many of the furanoterpenes has been found to be toxic and used as a defense compounds by plants (11). Considering their abundance in the nature, we were interested to investigate the general mechanism of β -substituted furan ring formation.

We have synthesized several compounds which are either naturally occurring or very similar to naturally occurring with an α -isopropylidene ketone unit and these compounds were incubated with PB-induced rat liver microsomal cytochrome P450 system in presence of NADPH and O₂. In this paper we propose the model for probable biosynthetic route for lower furanoterpenoids.

MATERIALS AND METHODS

Chemicals. Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP⁺, and Tris-HCl were supplied by Sigma Chemical Co. (St. Louis, MO). Phenobarbital was a generous gift from IDPL (Hyderabad, India). Synthesis of mesityl oxide (1) (12), 2-isopropylidene cyclohexanone (2) (13), 4-methyl-2-isopropylidene cyclohexanone (3)

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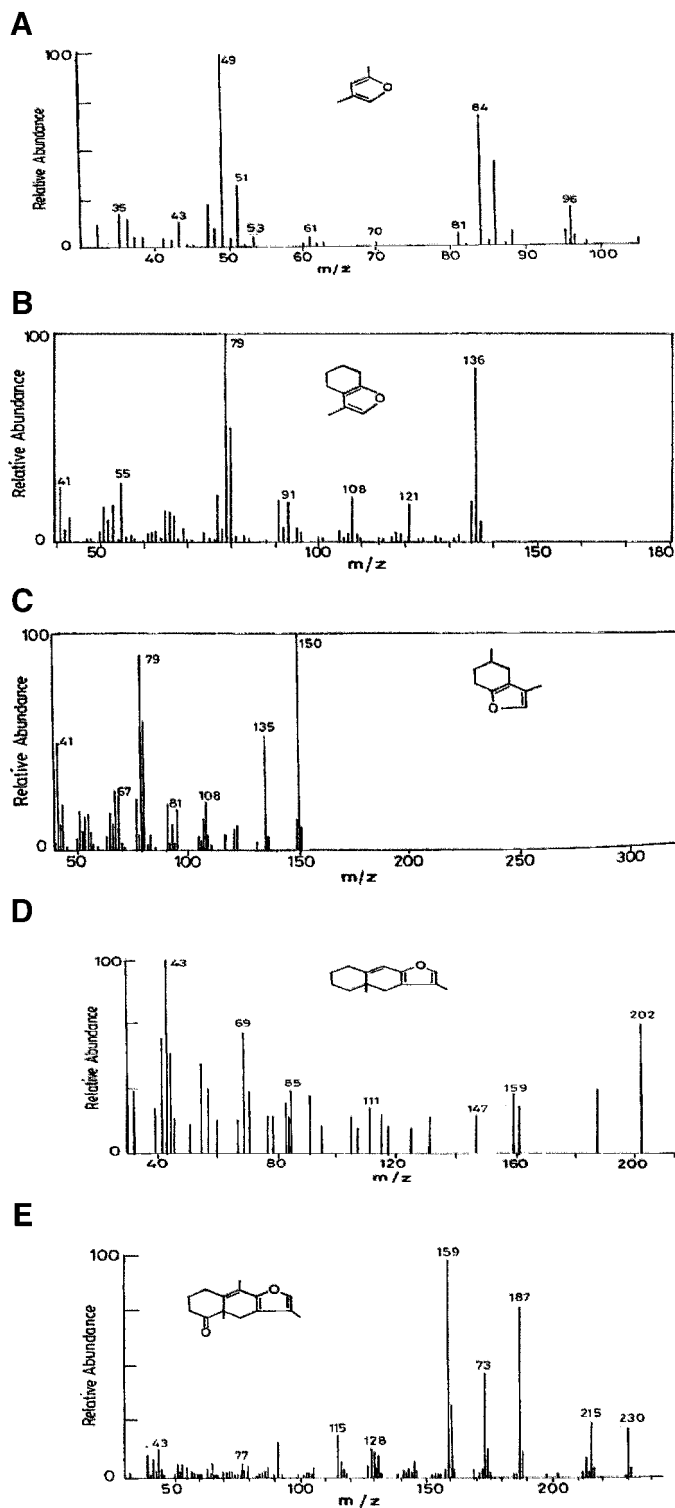


FIG. 1. Mass spectrum of 11 (A), 12 (B), 13 (C), 14 (D), and 15 (E). Substrates (1–5, 2 mM) were incubated separately with PB-induced rat liver microsomes in the presence of NADPH and O₂ and the extracted enzymatic product(s) were subjected to GC–MS analysis as described under Materials and Methods.

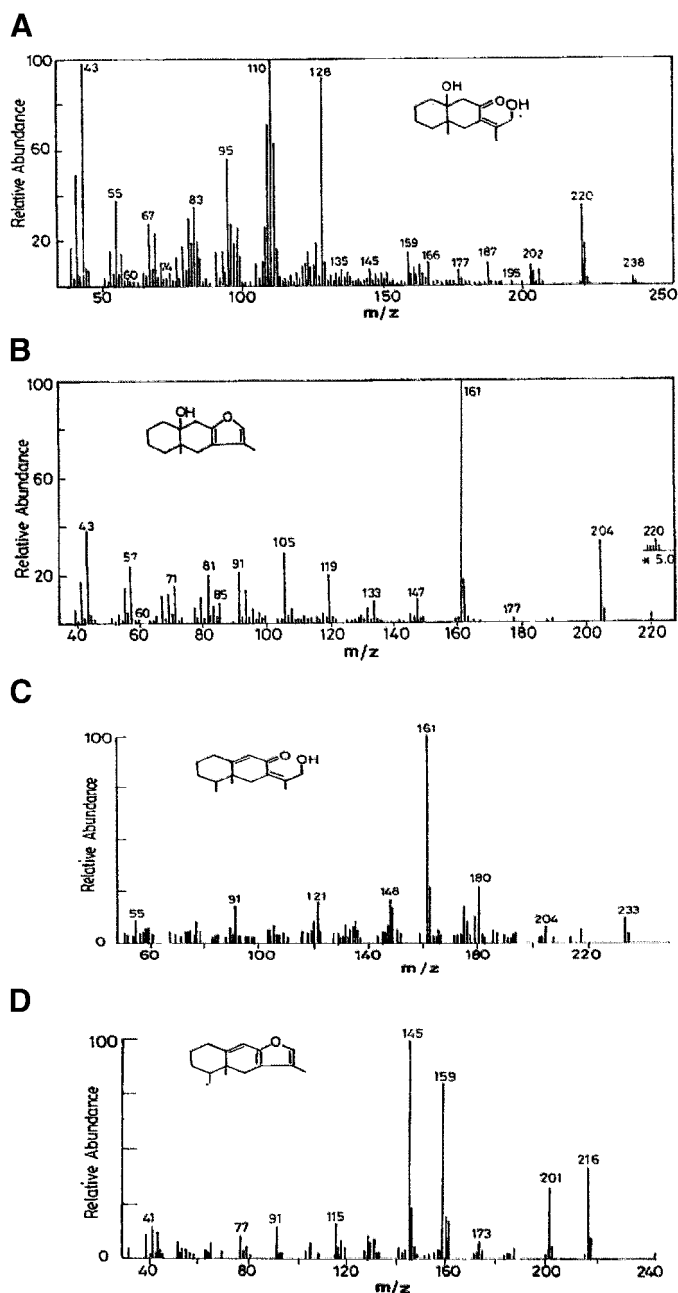


FIG. 2. Mass spectrum of 9 (A), 16 (B), 10 (C), and 17 (D). Substrates (6 and 7, 2 mM) were incubated separately with PB-induced rat liver microsomes in the presence of NADPH and O₂. In case of dehydrofukinone (7) the assay was also carried out with reconstituted cytochrome P450 system. The enzymatic product(s) formed were extracted and subjected to GC–MS analysis as described under Materials and Methods.

(13), 4,4a,5,6,7,8-hexahydro-3-isopropylidene-4a-methyl naphthalen-2-one (4) (14–16), 4,4a,5,6,7,8-hexahydro-3-isopropylidene-1,4a-dimethylnaphthalen-2,5-dione (5) (14–16), 4,4a,5,6,6,7,8,8a-octahydro-3-isopropylidene-4a-methyl-8a-hydroxy naphthalen-2-one (6) (14–16), dehydrofukinone (4,4a,5,6,7,8-hexahydro-3-isopropylidene-4a,5-dimethylnaphthalen-2-one) (7) (14–16) and ar-turmerone (8) (17), were carried out as reported in literature.

TABLE 1

Analysis of the Mass Spectra of Furan Derivatives (**11–18**) and Hydroxylation Products (**9, 10, 19, and 20**) Obtained in the Enzymatic Reaction

No.	Compound	Mass fragmentation (<i>m/z</i>)
9	4,4a,5,6,7,8-Hexahydro-3-isopropylidene-4a-methyl-8a,10-dihydroxy naphthalen-2-one	238 (M ⁺), 220 (M ⁺ -H ₂ O), 202 (M ⁺ -H ₄ O ₂), 187 (M ⁺ -CH ₇ O ₂), 128 (M ⁺ -C ₇ H ₁₀ O)
10	4,4a,5,6,7,8-Hexahydro-3-isopropylidene-4a,5-dimethyl-10-hydroxy-naphthalen-2-one	233 (M ⁺ -H), 204 (M ⁺ -CHO), 161 (M ⁺ -C ₄ H ₈ O)
11	2,4-Dimethyl furan	96 (M ⁺), 81 (M ⁺ -CH ₃), 53 (M ⁺ -C ₂ H ₃ O)
12	4,5,6,7-Tetrahydro-3-methylbenzofuran	136 (M ⁺), 121 (M ⁺ -CH ₃), 108 (M ⁺ -CO), 79 (M ⁺ -C ₄ H ₇ O)
13	4,5,6,7-Tetrahydro-3,5-dimethyl benzofuran	150 (M ⁺), 135 (M ⁺ -CH ₃), 108 (M ⁺ -C ₂ H ₂ O), 91 (M ⁺ -C ₃ H ₇ O)
14	4,4a,5,6,7,8-Hexahydro-3-4a-dimethyl naphtho(2,3-b)furan	202 (M ⁺), 187 (M ⁺ -CH ₃), 159 (M ⁺ -C ₂ H ₃ O), 131 (M ⁺ -C ₄ H ₇ O), 43 (M ⁺ -C ₁₂ H ₁₅)
15	4,4a,5,6,7,8-Hexahydro-3,4a,9-trimethylnaphtho(2,3-b)furan-5-one	230 (M ⁺), 215 (M ⁺ -CH ₃), 187 (M ⁺ -C ₂ H ₃ O), 173 (M ⁺ -C ₃ H ₅ O), 159 (M ⁺ -C ₄ H ₇ O)
16	4,4a,5,6,7,8,8a,9-Heptahydro-8a-hydroxy-3,4a-dimethylnaphtho(2,3-b) furan	220 (M ⁺), 204 (M ⁺ -CH ₄), 161 (M ⁺ -C ₃ H ₇ O), 147 (M ⁺ -C ₄ H ₉ O)
17	4,4a,5,6,7,8-Hexahydro-3,4a,5-trimethylnaphtho(2,3-b)furan	216 (M ⁺), 201 (M ⁺ -CH ₃), 173 (M ⁺ -C ₂ H ₃ O), 159 (M ⁺ -C ₃ H ₅ O), 145 (M ⁺ -C ₄ H ₇ O)
18	2[2-(4-Methyl phenyl)-propyl]-4-methyl furan	214 (M ⁺), 119 (M ⁺ -C ₆ H ₇ O), 91 (M ⁺ -C ₈ H ₉ O)
19	6-(4-Methyl phenyl)-6-hydroxy-2-methyl-hept-2-ene-4-one	232 (M ⁺), 217 (M ⁺ -CH ₃), 135 (M ⁺ -C ₆ H ₉ O), 83 (M ⁺ -C ₁₀ H ₁₃ O)
20	6-(4-Hydroxy methyl phenyl)-2-methyl-hept-2-ene-4-one	232 (M ⁺), 217 (M ⁺ -CH ₃), 201 (M ⁺ -CH ₃ O), 135 (M ⁺ -C ₆ H ₉ O), 83 (M ⁺ -C ₁₀ H ₁₃ O)

Treatment of animals and preparation of microsomes. Male albino rats (Wistar strain, 150–180 g) were administered phenobarbital (80 mg/kg, body wt) intraperitoneally in 0.9% saline solution for 4 days. Animals were sacrificed 24 h after the last injection. Microsomes were prepared from pretreated rats by differential centrifugation method (18). Microsomes were suspended in Tris-HCl buffer (0.05 M, pH 7.8) containing 0.25 M sucrose, and 20% glycerol (v/v) and were stored at -20°C.

Purification of liver microsomal cytochrome P450 system. Cytochrome P450 and NADPH-cytochrome P450 reductase were purified according to the method of Guengerich and Martin from phenobarbital induced rat liver microsomes (19).

Protein determinations were conducted following the method of Lowery *et al.* (20). Cytochrome P450 content of the liver microsomes was estimated according to method of Omura and Sato (21). NADPH-cytochrome P450 reductase was assayed according to method of Yasukochi and Masters (22).

General procedure for biotransformation in vitro. PB-induced rat liver microsomes (2 mg/ml) were incubated in the presence of glucose 6-phosphate (5 mM), NADP⁺ (0.5 mM), glucose-6-phosphate dehydrogenase (2 units), MgCl₂ (10 mM), substrate (**1–8**) (2 mM in 50 μl of acetone) and Tris-HCl (0.01 M, pH 7.4) in a total volume of 10 ml. The reaction was initiated by the addition of an NADPH-generating system, and the mixture was incubated aerobically in a rotary shaker for 1 h at 37°C. At the end of the incubation period, protein was precipitated by adding 4 ml each of saturated Ba(OH)₂ and 0.25 M ZnSO₄ solution. The precipitated protein was removed by centrifugation. The supernatant was extracted three times with 15–20 ml of distilled diethyl ether or pentane, dried over anhydrous Na₂SO₄, concentrated, and an aliquot was subjected to GC-MS analysis. In the case of compound **8** (ar-turmerone), the crude extract was subjected for chromatographic analysis.

Inhibitor studies. The microsomal protein was incubated with the inhibitor at 37°C for 5 min. before adding the substrate (ar-turmerone, **8**) and NADPH-generating system. The inhibitors, Metyrapone and SKF-525 were added to the assay mixture in acetone. Equal amount of the solvent used for suspending or dissolving the

inhibitor was added to the control assay. CO inhibitory experiments were carried out by bubbling CO into the enzyme solution for 2 min. Similarly N₂ was bubbled for 2 min into the enzyme solution used in the control assay. The total ether extracts after concentration were subjected to GC analysis.

Studies using reconstituted cytochrome P450 system. Reconstituted enzyme system contained cytochrome P450 (2 nmol/ml), NADPH-cytochrome P450 reductase (35 units), dilaurylphosphatidyl choline (30 μg/ml), Tris-HCl buffer (0.01 M, pH 7.4), KCl (0.15 M), EDTA (1 mM) and substrate 1 mM, dehydrofukinone (**7**), in a total volume of 2 ml. The reaction was initiated by the addition of NADPH (1 mM). The assay mixture was incubated at 37°C for 20 min. At the end of the assay, the reaction was stopped by immersing in ice and extracted with pentane (3 × 15 ml). The combined extract was dried over Na₂SO₄ and concentrated. An aliquot was used for analysis by GC-MS to identify the enzymatic products formed.

Chromatographic procedures. GC analyses were carried out on a Shimadzu Model 14A instrument equipped with a hydrogen flame ionization detector. The instrument was fitted with a Shimadzu HR-1 wide-bore capillary column (15 × 0.5 mm). Nitrogen was used as the carrier gas at 30 ml/min.

TLC was carried out on silica gel-G coated plates (0.25 mM) developed with hexane/ethyl acetate as solvent system (90:10, v/v). Compounds were visualized on the chromatograms by spraying the TLC plates with 1% vanillin in 50% H₂SO₄ followed by heating at 100°C for 5–10 min.

Spectra. Proton NMR spectra were recorded on a JEOL FT-90MHz spectrometer. Chemical shifts are reported in ppm, with respect to tetramethylsilane as the internal standard. Mass spectral analyses were performed on a JEOL-JMX-DX 303 instrument attached with a JMA-DA 5000 data system. A capillary column (50 m in length and 0.25 mm in diameter) containing either 3% SE-30 on Chromosorb-W or 10% QF on Chromosorb-W was used for the effective separation of metabolites. The column temperature was programmed between 60 and 100°C at a rate of 2°C/min and then to 220°C at 8°C/min. Helium was used as a carrier gas at a flow rate of 30 ml/min.

RESULTS AND DISCUSSION

Biotransformation by PB-Induced Rat Liver Microsomes

In this study we have used rat liver cytochrome P450 enzyme system to demonstrate the probable mechanism of biosynthesis of β -substituted furan skeleton in plants based on the recent demonstration that menthofuran is synthesized from R-(+)-pulegone in peppermint by the same means as in mammalian liver (6).

Test compounds **1–8** were incubated separately with phenobarbital induced rat liver microsomes in the presence of NADPH and O₂ and the incubation mixtures were subjected to GC–MS analysis (Figs. 1 and 2). All the compounds were biotransformed to corresponding furan derivatives, **11–18**, as judged by the GC–MS analysis, shown in Table 1. In case of **6** and **7**, we were able to isolate the respective hydroxyl intermediates **9** and **10**, which are involved in the formation of **18** and **19** (Fig. 4). The isolation and identification of compounds **9** and **10** along with **16** and **17**, demonstrates that the regioselective hydroxylation of the methyl group *syn* to the carbonyl is the primary step in the formation of furan skeleton. Conversion of mesityl oxide (**1**), to 2,4-dimethylfuran (**10**) indicates that it is the minimum carbon skeleton required for furan formation. Whereas transformation of compounds **1–8** to **11–18** shows that a wide variety of molecules, with an α -isopropylidene ketone unit, can be accepted as a substrates in this enzymatic reaction (Fig. 4).

Several attempts to obtain enough material for NMR spectra were unsuccessful, as furan compounds, **11–17**, obtained from **1** to **7** were either highly unstable or volatile in nature. While in case of ar-turmerone (**8**), the furan derivative was found to be fairly stable. The crude extract of the assay mixture, obtained in an enzymatic reaction using compound **8** as a substrate, was chromatographed on silica gel pencil column using different mixtures of hexane/ethyl acetate and three products were isolated. Based on the NMR [(CDCl₃, Fig. 3A) δ -7.1 (4H, s, phenyl aromatic protons), 6.05 (1H, bs, α -furan proton), 5.75 (1H, bs, β -furan proton), 2.30 (3H, s, CH₃ on phenyl ring), 1.97 (3H, s, CH₃ on furan ring) and 1.20 (3H, s, CH₃ on secondary carbon)] and the mass spectra (Fig. 3A, inset), most nonpolar metabolite (*R_f* 0.97 and *R_t* 2.5 min) was identified as a furan derivative, **18**, of ar-turmerone (**8**). Second compound (*R_f* 0.47 and *R_t* 5.3 min), based on its NMR [(CDCl₃, Fig. 3B) δ -7.4–7.0 (4H, aromatic protons), 5.92 (1H, bs, olefinic proton), 2.27 (3H, s, CH₃ on aromatic ring), 2.0 (3H, s, CH₃ *syn* to carbonyl), 1.8 (3H, s, CH₃ *anti* to carbonyl) and 1.43 (3H, s, CH₃ on tertiary carbon)] and mass spectra (Fig. 3B, inset), was identified as **12**. The most polar compound (*R_f* 0.12 and *R_t* 9.7 min) which showed the following spectroscopic characteristics: NMR (CDCl₃, Fig. 3C) δ -7.4–7.12 (4H, aromatic protons), 6.02 (1H, bs, olefinic proton), 4.65 (2H, s, hy-

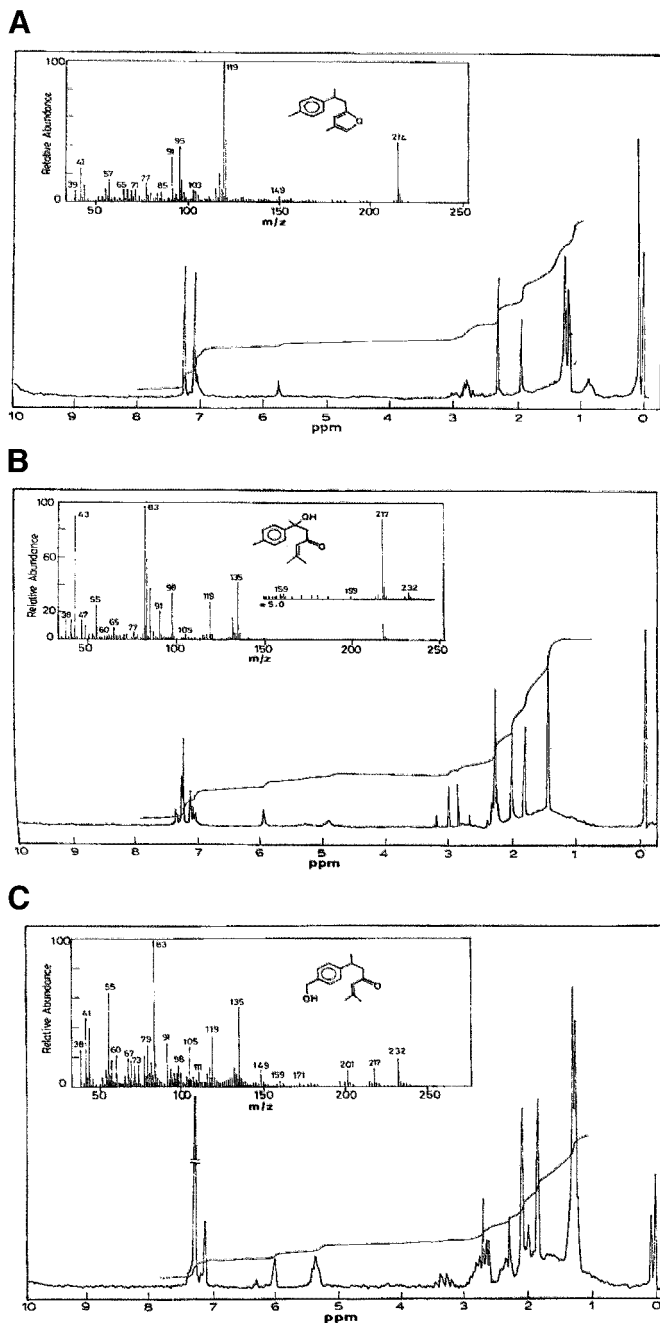


FIG. 3. NMR and mass spectrum (inset) of enzymatic products **18** (A), **19** (B), and **20** (C) formed from ar-turmerone (**1**). Substrate (ar-turmerone, 2 mM) was incubated with PB-induced rat liver microsomes in the presence of NADPH and O₂. The enzymatic products formed were extracted and purified by column chromatography as described under Materials and Methods.

droxymethyl protons), 2.1 (3H, s, CH₃ *syn* to carbonyl), 1.85 (3H, s, CH₃ *anti* to carbonyl) and 1.3 (3H, d, CH₃ on secondary carbon) and mass spectra (Fig. 3C, inset) was identified as **13**. Compounds **12** and **13** are formed through the benzylic hydroxylation by cytochrome P450 enzyme system, whereas furan (**11**) by allylic

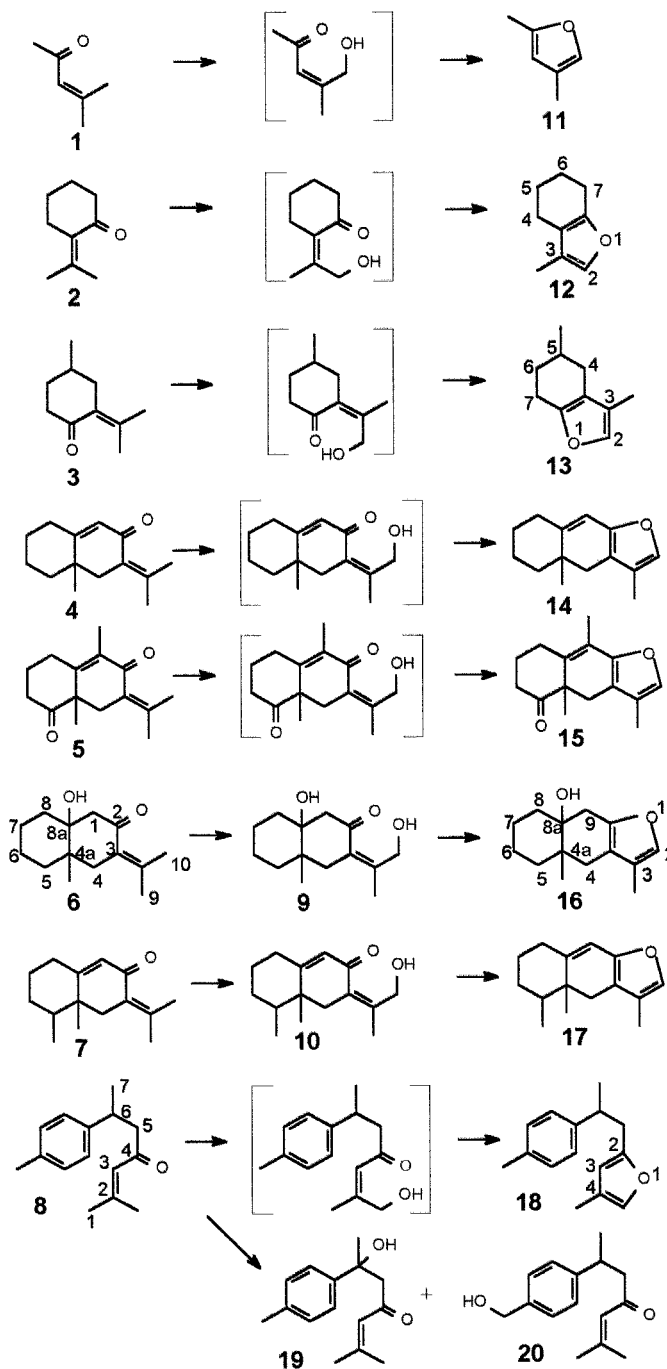


FIG. 4. Enzymatic synthesis of furans from the compounds having α -isopropylidene ketone units.

hydroxylation (Fig. 4). Identification of furan (**11**) by NMR and MS further corroborates the formation of β -substituted furan skeleton in the test compounds.

The assay carried out using ar-turmerone (**1**) as the substrate, indicated that the metyrapone, SKF-525 and CO, known inhibitors of cytochrome P450, inhibited the reaction by 76, 62, and 97%, respectively (Fig.

5). Inhibitor studies amply support the involvement of cytochrome P450 in the above transformation.

Reconstitution of Cytochrome P450 Hydroxylase Activity

We have chosen dehydrofukinone (**7**) for these studies because this compound cooccurs in nature with furanodehydrofukinone (**17**) and other related furanoremorephilanes (**9**, **10**). Reconstituted cytochrome P450 system (see Materials and Methods) was incubated with **7** in the presence of NADPH and O_2 . GC-MS analysis showed the presence of **10** and **17** in the assay mixture (Figs. 2C and 2D). The reconstitution of hydroxylation of dehydrofukinone (**7**) to **10** which leads to formation of furan derivative (**17**), clearly demonstrated that the reaction is cytochrome P450 mediated one. It further suggests that a similar mechanism might be operating in the biosynthesis of furanodehydrofukinone (**17**) in plants.

On the basis of our results, we propose the probable pathway for formation of β -substituted furan skeleton in furanoterpenoids. In the first step, it can be assumed that, due to hydrogen bonding with ketone (**21**), the molecule gets oriented in the active site in such a way that only methyl group *syn* to the carbonyl is exposed for the hydroxylation by cytochrome P450 enzyme. Thus the methyl group *syn* to the carbonyl gets hydroxylated regioselectively to give hydroxymethyl (**22**). In the second step, the close proximity of the hydroxyl

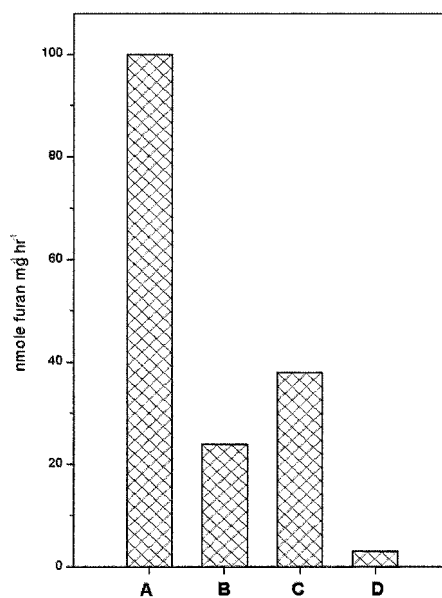


FIG. 5. Effect of inhibitors: A, control; B, metyrapone; C, SKF-525; and D, CO, on the formation of furan (**18**) by PB-induced rat liver microsomal cytochrome P450 system. The assay was carried out as described under Materials and Methods. One hundred percent activity (0.0% inhibition) represents 100 nmol $mg^{-1} h^{-1}$.

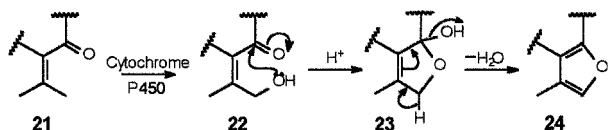


FIG. 6. Proposed pathway for biosynthesis of furanoterpenes.

to the ketone facilitate the intramolecular non enzymatic cyclization to give a hemiketal (23) which readily undergoes dehydration to yield the furan (24) in the third step (Fig. 6).

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