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IN VITRO BIOSYNTHESIS OF ISOPENTENYLACETOPHENONES IN *EUPATORIUM RUGOSUM*

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Key Word Index—*Eupatorium rugosum*; Compositae; snakeroot; isopentenylacetophenones; dehydrotremetone; cell free homogenate; biosynthesis.

Abstract—A cell free homogenate of *Eupatorium rugosum* leaves was prepared and utilized to study the biosynthesis of dehydrotremetone (**1**). Homogenates of young leaves were most efficient in the formation of **1**. The furan ring and its side chain were derived from an isoprenoid unit, which appeared to be isopentenyl pyrophosphate and not dimethylallyl pyrophosphate. Potential aromatic precursors such as 4-hydroxyacetophenone and 4-hydroxy-3[isopenten-(2)-yl]-acetophenone were poorly utilized. However, tremetone, especially in the presence of the coenzyme NADP, was very efficiently converted to **1**. An apparent intermediate in the pathway leading to **1** was isolated and appeared similar to 4-hydroxy-3[isopenten-(2)-yl]-acetophenone. It is proposed that the aromatic moiety of **1** is derived from acetate via a polyketide intermediate, which undergoes isoprenylation by isopentenyl pyrophosphate, followed by aromatization and furan ring closure.

INTRODUCTION‡

ALTHOUGH it has been previously demonstrated[1] that the aromatic ring of dehydrotremetone (**1**) is biosynthetically formed from acetate and the side-chain from an isoprene unit in *Eupatorium rugosum* leaves, the incorporation of possible intermediates in the pathway was difficult to demonstrate *in vivo*. Furthermore, the specific isoprene precursor, which is utilized to alkylate the polyacetate moiety, cannot be readily identified in such experiments, due to the impermeability of membranes by MVA[2] as well as by the isoprenoid pyrophosphates. It was of interest therefore to prepare a cell free system of *E. rugosum* tissue which could not only readily synthesize **1** *in vitro* but also accumulate and utilize any possible intermediates.

RESULTS

Preparation and properties of the cell-free system.

Because of the problems[3] associated with the extraction of enzymes from plant tissues a number of buffer systems and protecting agents[3,4] were initially added to the extraction

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‡ Abbreviations used: MVA-mevalonic acid, IPP-isopentenyl pyrophosphate, DMAPP-dimethylallyl pyrophosphate, EtSH-mercaptoethanol.

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TABLE 1. COFACTORS, INHIBITORS AND CONDITIONS AFFECTING THE INCORPORATION OF IPP-[1-^{14}C] INTO DEHYDROTREMETONE

Treatment*	Incorporation (nmol)	Per cent of control
Complete system (a)	0·78	[100]
−EDTA	0·11	14·0
−EtSH, −cysteine	0·18	23·0
−Sucrose	0·38	48·0
−$MgCl_2$	0	0
−Polyclar AT	0·18	23·0
pH 7·0	0·02	3·0
pH 8·0	0·06	8·0
Incubation in the dark	0·6	78·4
+Sonification	1·24	160·0
Complete system (b)	3·05	[100]
+$CaCl_2$ (4 mM)	3·29	108·0
+$FeSO_4$ (4 mM)	2·09	68·6
+$CuSO_4$ (4 mM)	1·66	54·3
+Iodoacetate (0·4 mM)	3·29	108·0
+Iodoacetate (2·0 mM)	1·10	36·0
+Iodoacetate (0·2 M)	0·27	8·8

* Each incubation in (a) contained 3·1 × 10^5 dpm of IPP-[1-^{14}C] as substrate and in (b) contained 3·7 × 10^6 dpm of IPP-[1-^{14}C].

medium with little recovery of activity. The extraction medium finally chosen was largely obtained through trial and error (Table 1) and consisted of 0·3 M sucrose, 0·01 M $MgCl_2$, 0·02 M EDTA, 0·02 M cysteine, 0·01 M EtSH, 0·01 M KF, 0·1 M Tris–HCl, pH 7·8, and polyclar AT (25% of the weight of the plant material). Different tissue disruption and extraction procedures were also tried, but grinding of the plant material in a mortar with dry ice and buffer, followed by sonication (Table 1) gave the best results. Rather unusual was the requirement for EDTA–$MgCl_2$ in a ratio of 2:1, and each appeared to be essential for activity (Table 1). In view of the inhibitory properties of $FeSO_4$ and $CuSO_4$ (Table 1) the high concentration of EDTA is most likely required to bind multivalent ions other than Mg^{2+} in the homogenate. Optimum activity was dependent on pH adjustment to 7·4–7·8 (Table 1). One or more of the enzymes involved appeared to contain sulfhydryl proteins since a requirement of EtSH and/or cysteine was found. Furthermore the sulfhydryl reagent iodoacetamide strongly inhibited the formation of dehydrotremetone (Table 1).

The selection of the proper plant material was also found to be important for the preparation of cell free homogenates capable of synthesizing dehydrotremetone (Table 2). Stem

TABLE 2. EFFECT OF AGE AND TREATMENT OF LEAVES ON THE FORMATION OF DEHYDROTREMETONE

Age and/or treatment*	Incorporation into dehydrotremetone (nmol)	(%)
Young leaves—fresh	1·86	0·13
Mature leaves—fresh	0·97	0·06
Old leaves—fresh	0·09	0·007
Young leaves—stored frozen	0	0
Young leaves—lyophilized—stored 4 months	3·37	0·21
Young leaves—lyophilized—stored 6 months	0	0
Young leaves—lyophilized—boiled homogenate	0	0

* 3·1 × 10^5 dpm of IPP-[1-^{14}C] were added as substrate to each incubation.

tissue showed little or no activity and root tissue, although highly active, did not synthesize dehydrotremetone but chromenes,[5] which are structurally related to the benzofurans.[6,7] Young, fresh leaves were a good source of enzymatic activity, which decreased with age of the leaves (Table 2). Young leaves could not be stored frozen because biosynthetic activity was lost. However, upon lyophilization, active homogenates could be obtained from young leaves up to 4 months after harvesting (Table 2) but thereafter activity rapidly declined.

Identification of the product of the biosynthetic reaction

The assay of the cell free formation of dehydrotremetone consisted of TLC and determination of the radioactivity of the eluted spot. Therefore, it was important to ensure that the recovered compound was not contaminated by substrate or by other radioactive compounds which were not derived via the same pathway. For this purpose the material eluted from the TLC zone was mixed with authentic dehydrotremetone and recrystallized. The spectific activity remained constant after the first recrystallization and the loss of radioactivity in the first crystallization was found to be due mainly to contamination by tremetone. However, since tremetone is also a benzofuran compound and derived via the same pathway, it was concluded that the TLC assay was sufficiently accurate for the present investigation. In experiments where new substrates were used the same procedure was employed to test the accuracy of the assay.

TABLE 3. THE UTILIZATION OF ISOPRENOID COMPOUNDS IN THE FORMATION OF DEHYDROTREMETONE

Substrate	Incorporation (nmol)
Experiment 1*	
IPP-[1-^{14}C]	2·54
IPP-[1-^{14}C] + ATP (2 mM)	2·60
DMAPP-[1-^{14}C]	0·59
DMAPP-[1-^{14}C] + ATP (2 mM)	0·71
MVA-[5-^{3}H]	0·
MVA-[5-^{3}H] + ATP (2 mM)	0·20
Experiment 2	
IPP-[1-^{14}C] (0·07 mM)	2·21
IPP-[1-^{14}C] + DMAPP (0·12 mM)	3·00
IPP-[1-^{14}C] + DMAPP (0·46 mM)	3·47
IPP-[1-^{14}C] + DMAPP (1·16 mM)	5·36
DMAPP-[1-^{14}C] (0·09 mM)	0·51
DMAPP-[1-^{14}C] + IPP (0·1 mM)	0·48
DMAPP-[1-^{14}C] + IPP (0·4 mM)	0·47
DMAPP-[1-^{14}C] + IPP (1·0 mM)	0·46

* In the Experiment 1 the amounts of radioactive substrate added to the incubations were as follows: IPP-[1-^{14}C] = $2{\cdot}02 \times 10^5$ dpm, DMAPP-[1-^{14}C] = $1{\cdot}64 \times 10^5$ dpm, MVA-[5-^{3}H] = $4{\cdot}4 \times 10^7$ dpm.

The utilization of isoprenoid substrates

Besides IPP-[1-^{14}C] other isoprene units were tested as substrates for the cell free formation of dehydrotremetone (Table 3). MVA-[^{3}H] was significantly better incorporated

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in the cell free system than in the intact plants.[1] However, both DMAPP-[1-^{14}C] and MVA-[5-^{3}H] were inferior to IPP-[1-^{14}C] as substrates. The incorporation of MVA-[5-^{3}H] was completely dependent on ATP, whereas the incorporation of DMAPP-[1-^{14}C] was only slightly increased by ATP (Table 3). ATP had no effect on the utilization of IPP-[1-^{14}C].

In a separate experiment (Table 3), the incorporation of IPP-[1-^{14}C] into dehydrotremetone was increased 2-fold by the addition of unlabeled DMAPP. However, where the concentration of DMAPP-[1-^{14}C] was kept constant and unlabeled IPP was added no increase in the utilization of DMAPP-[1-^{14}C] was observed.

Utilization of potential aromatic precursors

A number of possible precursors of dehydrotremetone were labeled with ^{3}H and incubated with the cell free homogenate. The results in Table 4 showed that only tremetone was utilized as much, upon the addition of NADP, as IPP-[1-^{14}C]. Of the expected aromatic precursors, 4-hydroxyacetophenone was virtually inactive and 4-hydroxy-3[isopenten-(2)-yl]acetophenone which appeared to be a reasonable precursor for the furan ring formation, was a very poor substrate compared to IPP-[1-^{14}C] and tremetone (Table 4).

TABLE 4. INCORPORATION OF POTENTIAL AROMATIC PRECURSORS INTO DEHYDROTREMETONE

Substrates*	Incorporation (%)
IPP-[1-^{14}C] ($4{\cdot}49 \times 10^{5}$ cpm)	0·1
4-Hydroxy-3[isopenten-(2)-yl]acetophenone-^{3}H	0·006
4-Hydroxyacetophenone-^{3}H	0·001
Tremetone-^{3}H	0·1†
Tremetone-^{3}H, + NADP	0·6†
Tremetone-^{3}H, + NADP, + boiled homogenate	0†

* The following amounts of radioactive substrates were added to the incubation mixtures: IPP-[1-^{14}C] = $6{\cdot}55 \times 10^{5}$ dpm, 4-hydroxy-3[isopenten-(2)-yl]acetophenone-^{3}H = $2{\cdot}26 \times 10^{7}$ dpm, 4-hydroxyacetophenone-^{3}H = $9{\cdot}2 \times 10^{6}$ dpm, II-^{3}H = $8{\cdot}08 \times 10^{6}$ dpm.

† Since tremetone and dehydrotremetone migrated close to each other in the TLC-assay system, cold dehydrotremetone was added upon termination of the incubation and the mixture recrystallized to constant sp. act.

Isolation of a possible intermediate in the formation of dehydrotremetone

During the TLC assay of the IPP-[1-^{14}C] incorporation experiments two other radioactive spots were obtained on the TLC-plates. One migrating close to the solvent front appeared to be carotenoid in nature and was therefore disregarded. Another area on the chromatogram, which was close to the origin on the TLC plate and therefore more polar than dehydrotremetone, was designated IPP-NO-3. IPP-[1-^{14}C] was incorporated into IPP-NO-3 15 times more efficiently than into dehydrotremetone. The ratio of incorporation of IPP-[1-^{14}C] into IPP-NO-3 and dehydrotremetone was constantly found to be $15{\cdot}5 \pm 2$ under a number of experimental conditions. It appeared therefore that IPP-NO-3 is biosynthetically related to dehydrotremetone and perhaps an intermediate in the pathway leading from IPP-[1-^{14}C]. Therefore IPP-NO-3-[^{14}C] was isolated and reincubated

with the cell free homogenate. As shown in Table 5 IPP-NO-3 was 8 times more efficiently incorporated into dehydrotremetone than IPP-[1-^{14}C].

TABLE 5. INCORPORATION OF IPP-NO-3-[^{14}C] INTO DEHYDROTREMETONE

Substrate*	Incorporation (%)	Relative to control (%)
IPP-[1-^{14}C]	0·14	[100]
IPP-NO-3-[^{14}C]	1·12	800
IPP-NO-3-[^{14}C], boiled homogenate	0	0

* The amounts of radioactive substrate added were as follows: IPP-[1-^{14}C] = 2·1 × 10^5 dpm, IPP-NO-3-[^{14}C] = 2·24 × 10^5 dpm.

From the positive reaction of IPP-NO-3 with the 2,4-dinitrophenol reagent and an identical TLC R_f value to 4-hydroxy-3[isopenten-(2)-yl]acetophenone it appeared at first that the two compounds were identical. Although chromatography of a mixture of ^{3}H-4-hydroxy-3[isopenten-(2)-yl]acetophenone and IPP-NO-3-[^{14}C] on TLC in three different solvent systems showed only one radioactive peak, the ^{3}H/^{14}C ratio was found to be lower than that of either of the compounds (Table 6, experiment 1).

TABLE 6. COMPARISON OF IPP-NO-3-[^{14}C] AND 4-HYDROXY-3[ISOPENTEN-(2)-YL]ACETOPHENONE-^{3}H

Experiment 1: ^{3}H/^{14}C ratio of IPP-NO-3-[^{14}C] and 4-hydroxy-3[isopenten-(2)-yl]acetophenone-^{3}H on TLC plates*

Solvent	^{3}H/^{14}C ratio
MeOH–C_6H_6 (2:98)	5·67
MeOH(C_6H_6 (5:95)	6·00
$CHCl_3$	5·50

Experiment 2: competitive utilization of IPP-NO-3-[^{14}C] and 4-hydroxy-3[isopenten-(2)-yl]acetophenone-^{3}H for the synthesis of dehydrotremetone

Sample	Ratioactivity ^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C ratio
Substrate mixture	80 727	9242	8·73
Isolated dehydrotremetone	1196	218	5·50
Reisolated IPP-NO-3	16 626	2208	7·52

* The original ^{3}H/^{14}C ratio of the mixture was 7·75.

These results indicated that the two compounds were probably similar in structure but not identical. This was further supported by experiment 2, Table 6 in which the two compounds with a known ^{3}H/^{14}C ratio were incubated with a cell free homogenate of the plant. The results showed that the ^{3}H/^{14}C ratio of the isolated dehydrotremetone was lower than that of the original substrate mixture, indicating that IPP-NO-3 was preferential utilized. Finally the IR spectra of the two compounds were compared. Although both compounds appeared to be ketones, and contained a hydroxyl function and an aromatic moiety, the spectra were not identical.

DISCUSSION

From the previous results[1] it appeared that the acetophenone moiety of dehydrotremetone is derived from acetate via a polyketide, which then would undergo cyclization to 4-hydroxyacetophenone, followed by isoprenylation. This sequence of reactions has already been postulated[8] and experimental evidence has been presented that in the formation of psoralen[9] and ergot alkaloids[10] the aromatic ring undergoes isoprenylation. However, in both these cases the aromatic precursor is not derived via a polyacetate intermediate. In the present work 4-hydroxyacetophenone, the most likely aromatic precursor of dehydrotremetone, was a very poor substrate and 4-hydroxy-3[isopenten-2-(yl)]acetophenone which has been postulated[8] to undergo cyclization to the benzofuran, was also a poor substrate. A number of explanations are possible to explain these results. Either (1) 4-hydroxy-3[isopenten-2-(yl)]acetophenone is not an intermediate in the pathway and isoprenylation occurs directly on the polyketide intermediate followed by aromatization or (2) the enzymes catalyzing the formation of dehydrotremetone are located in a subcellular particle into which entry of the aromatic precursors was difficult. In the latter case a poor utilization of the pyrophosphorylated isoprenoid compound and tremetone would have been expected, but in fact these substrates were found to be good precursors. A possible explanation therefore is isoprenylation before cyclization to an aromatic ring, a process which would bypass the destruction of the aromatic resonance energy in the transition state of the substitution of an aromatic ring.

The poor utilization of 4-hydroxy-3[isopenten-(2)-yl]acetophenone in conjunction with the isolation of IPP-NO-3 and the similarity of the two compounds, appeared to indicate that the former is not an intermediate in the formation of dehydrotremetone but that the true intermediate is IPP-NO-3. From preliminary results it appeared that IPP-NO-3 was an acetophenone-isoprenoid compound, but with the double bond in the side chain at a different position or with an epoxide function in the side chain.[8]

There appeared to be little doubt that the furan ring and its side chain was derived from an isoprenoid unit. In all cases reported in the literature, DMAPP appeared to be the preferred substrate for isoprenylation of aromatic compounds. However, in our results in all experiments IPP was preferentially incorporated over DMAPP by a factor of 5. Furthermore, the addition of unlabeled DMAPP to the radioactive IPP more than doubled the utilization of IPP-[1-^{14}C]. This would indicate that in *E. rugosum* IPP is the isoprenoid unit which participates in the isoprenylation reaction. Although unlikely, in view of the competition experiment performed, the possibility of the preferential destruction of DMAPP, or the rapid utilization of DMAPP for the formation of other isoprenoid compounds, in the crude homogenate, and consequently the preferential incorporation of IPP, cannot be excluded.

EXPERIMENTAL

Preparation of substrates. The chemical synthesis and purification of IPP-[1-^{14}C][11] (sp. act.: 0·1 μCi/μmol) and DMAPP-[1-^{14}C][10] (sp. act.: 0·14 μCi/μmol) has been reported.[10,11] MVA-[5-^{3}H] (sp. act. 200 μCi/μmol) was purchased (Schwarz/Mann). The synthesis of tremetone-[^{3}H], 4-hydroxy-3[isopenten-(2)-yl]acetophenone-[^{3}H] and 4-hydroxyacetophenone-[^{3}H] has been described.

Preparation of the cell free homogenate. Plant material was washed with H_2O, cut into small pieces and then ground in a mortar with dry ice, 3 vol of buffer (0·1 M Tris HCl, pH 7·8, 0·3 M sucrose, 0·01 M $MgCl_2$, 0·02 M

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EDTA, 0·02 M cysteine, 0·01 M EtSH, 0·01 M KF) and one part Polyclar AT to 4 parts plant material. After grinding for 15 min, the ratio of buffer to plant material was adjusted to 5:1 (v/w). For lyophilized leaves the ratio of buffer to plant material was adjusted to 20:1. The slurry was ground in an Omni-Mixer at maximum speed 4× for 10 sec at 0°. The homogenate was then filtered through 4 layers of cheesecloth and sonicated for 5 sec/ml. The homogenate (10 ml) was incubated with the appropriate substrate(s) and cofactor(s) for 14 hr at 30° with shaking. For all incubations IPP-[1-^{14}C] incorporation was used as a control. Background controls consisted of incubation of the substrate with 10 ml of homogenate previously immersed for 10 min in boiling H_2O.

Assay and isolation of intermediates. After the incubation the homogenate was shaken vigorously with 20 ml of $CHCl_3$, followed by centrifugation for 10 min at 15000 *g*. The $CHCl_3$ layer was removed and the extraction repeated once with 10 ml of $CHCl_3$. The combined $CHCl_3$ extracts were evaporated to dryness, the residue dissolved in 1 ml of C_6H_6 and passed through a silicic acid column (0·2 × 1 cm) and eluted with C_6H_6. 3 ml of eluate was collected, conc. under a stream of N_2 and examined by silica gel TLC using MeOH–C_6H_6 (1:49). The adsorbent containing the fluorescent band of dehydrotremetone (R_f 0·55) was removed and transferred into vials and counted in a liquid scintillation counter. Quenching and counting efficiency in the presence of silica gel was measured by the addition of an internal standard.

To ascertain the radioactive purity of dehydrotremetone, a number of incubations were extracted and several TLC plates used to obtain sufficient compound for recrystallization, which was then carried out as described before.[1] IPP-NO-3 was isolated from the C_6H_6 eluate of the silica gel columns by preparative TLC using the MeOH–C_6H_6 system. The radioactive band corresponding to IPP-NO-3 was removed, eluted with MeOH, concentrated and loaded on to another preparative TLC plates and run in MeOH–C_6H_6 (1:9). After location, removal and elution with MeOH, 1·5 mg of material was obtained with sufficient radioactivity to carry out the experiments described but not sufficient for PMR examination.