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BIOSYNTHESIS OF HALOGENATED MONOTERPENES IN *PLOCAMIUM CARTILAGINEUM*

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Key Word Index—*Plocamium cartilagineum*; Rhodophyta; alga; halogenated monoterpenes; stereospecifically labelled mevalonates; peroxidases.

Abstract—The biosynthesis of halogenated monoterpenes in this alga has been studied by feeding experiments using labelled bicarbonate, acetate and mevalonate. Seasonal effects have been observed in these studies and the incorporation of $H^{14}CO_3^-$ into primary metabolites is consistent with the expected reductive pentose pathway. The incorporations of [^3H, ^{14}C]mevalonates and limited chemical degradations are consistent with the expected cyclization to these rearranged monoterpenes. Attempts to obtain cell free systems to carry out all or part of the biosynthetic sequence or to halogenate various substrates have been unsuccessful, although a classical peroxidase has been isolated, characterized and partially purified.

INTRODUCTION

Halogenated monoterpenes are of limited occurrence in red alga and have been isolated from only four genera of Rhodophyta, namely *Chondrococcus*, *Microcladia*, *Ochtodes* and *Plocamium* [1]. Collections of *Plocamium cartilagineum* made in various parts of the world have yielded a considerable number of polyhalogenated monoterpenes, both polyhalogenated monoterpenes with a regular isoprenoid carbon skeleton and rearranged monocyclic compounds [2–6]. The incorporation of halogen atoms into these and related molecules has been the subject of considerable speculation [7]. Bromoperoxidases (EC 1.11.1.7) are enzymes that can oxidize bromide ions in the presence of hydrogen peroxide and subsequently brominate a variety of β-ketonic or phenolic substrates. Similar enzymes are almost certainly involved in the biosynthesis of polyhalogenated terpenes, but to date the only enzymes that have been characterized from algal sources are those involved with the biosynthesis of halogenated phenols or products derived from brominations of β-diketones [8–14]. Various addition reactions of halogens to olefinic and other unsaturated substrates have been shown to be catalysed by the chloroperoxidase enzyme from the fungus *Caldariomyces fumago* [15] but these products are achiral [16]. All naturally occurring halogenated terpenes isolated so far from algae which have asymmetric centres are chiral, although mixtures of diasteroisomers have been isolated in some cases [17]. The chirality is not restricted to halonium induced cyclization of terpenes (for which there are well documented chemical models) because many chiral acyclic halogenated terpenes are known. It would seem possible that there is a higher degree of substrate specificity in the haloperoxidase reactions involving terpenes than in those for phenolic and enolic halogenations. We have attempted both classical biosynthetic feeding experiments and some enzymological studies on local Australian *P. cartilagineum* to see if the techniques that have been successful in plant and microbial metabolism can be applied to macroscopic algae and to study the halogenation of terpenoid compounds. A very brief report on some of the general findings of this work has appeared in a recent review [18].

RESULTS

The analysis of *P. cartilagineum* extracts by GLC with electron capture detection and GLC/mass spectrometry with chemical ionization routinely showed the presence of the four alicyclic metabolites (**1–4**). The major metabolite (**1**) and the minor component (**2**) have been previously reported from *P. cartilagineum* collected in the Sydney region [3]. Compound **4** has been isolated from *P. violaceum* [1] and we find it makes up < 1% of the total monoterpene content. A very frustrating finding in this work was the repeated collection of samples of *P. cartilagineum* of both sterile plants and the tetrasporophyte [19] that were devoid of any halogenated monoterpenes. These algae were often located quite close, within a few metres, of algae containing upto 1% dry weight of monoterpenes and there are no obvious morphological differences. A locality metabolite variation in collections of *Plocamium* spp. has been described [20], but any alga totally lacking in metabolites would not be noticed in bulk collections for just isolation and characterization of metabolites. Small parts of all individual plants to be used for any biosynthetic experiment had therefore to be routinely checked for monoterpene content by GLC. We examined small sections of small and larger plants, new and old thalli of both the sterile plants and some tetrasporophytes and detected no difference in metabolite composition for samples collected over a 50 mile stretch of coastline. The metabolites were either present or absent. Large scale extractions showed the presence of trace amounts of other halogenated metabolites with shorter GLC retention times which have not yet been fully characterized but are probably less halogenated on acyclic metabolites. We have confirmed that the metabolites 3 and 4 are not artifacts of the isolation procedure by submitting purified 1 and 2 to the isolation procedures and showing that 3 and 4 are not produced, although an isomer of 4 has been made by heating an isomer of 2 to 150° in dimethylformamide [5].

Other terpenoid metabolites that would be expected to be labelled from [^{14}C]mevalonate feedings are carotenoids and sterols and so are of use in monitoring feeding experiments. We have identified α- and β-carotene, α-cryptoxanthin and lutein as the major carotenoids with trace amounts of zeaxanthin present. The only sterol isolated from the non-saponifiable lipid was cholesterol, but traces of 22-dehydrocholesterol was detected by GLC/MS.

The conditions finally established for the successful feedings to this alga were incubation in a minimum volume of vigorously aerated sea-water at 15–17° under constant illumination for periods of up to six days. The photosynthetic activities of *P. cartilagineum* kept under these conditions were checked by the rate of oxygen evolution and this showed a gradual decline over six days (Table 1). This decline in oxygen evolution could be lessened by using a much larger volume of sea-water, but this then led to even more dilution of any radioactively labelled compound fed. After several unsuccessful attempts to get incorporations of [^{14}C]acetate and [^{14}C]mevalonate into the monoterpene fraction it was established that $H^{14}CO_3^-$ could indeed label these metabolites and this showed that monoterpene synthesis was occurring to some extent under out laboratory conditions. Incorporations into monoterpenes were uniformly low and typical values for acetate, bicarbonate and mevalonate feedings are shown in Table 2. In all of these successful feeding experiments incorporations into caroteroids and cholesterol were usually of the order of 0.01 %, i.e. an order of magnitude higher than for the monoterpenes.

Significant incorporations of $H^{14}CO_3^-$ into water soluble metabolites, principally amino acids and sugars, was achieved under these conditions using algae collected at any time of the year. Figure 1 shows that the incorporation of $H^{14}CO_3^-$ into water soluble fractions was linear with time, and a dark control incubation of 4 days duration was comparable to the 1 min time sample. The distribution of label within the four fractions from the ion exchange separation is expressed as total activity in Table 3 and represented graphically as the percentage of total water soluble fraction in Fig. 2. There is an initial preferential incorporation of bicarbonate into the basic fraction containing the amino acids and into the acidic fractions containing the sugar phosphates and organic acids. Incorporation of label into sugars was gradual, increasing to levels equal to that of the amino acids (Fig. 2). Further separation of the amino acid and sugar fractions is shown in Figs 3 and 4 respectively. In the amino acid fraction, alanine was labelled initially with the greatest part of the radioactivity accumulating in serine over the time of the experiment, with a lesser rise for glycine. There was also preferential labelling within the neutral fraction, with galactose having 83 % of the activity of this class of compounds after 25 min. The acidic fractions 1 and 2, phosphate esters and organic acids respectively, were not fractionated because of the relatively low activities of these pools.

The incorporations of [^{14}C]mevalonate and [^{3}H, ^{14}C]mevalonates into the major metabolite (1) are shown in Table 4. These feedings were only successful in the period August–October, i.e. late winter and spring. The criteria of radiochemical purity is of paramount importance when working with such low levels of in-

Table 1. Photosynthetic activity of *P. cartilagineum*

Time from collection (days)	Rate of O_2 evolution (mg O_2/g/hr)
1	13.6
2	8.4
6	6.8

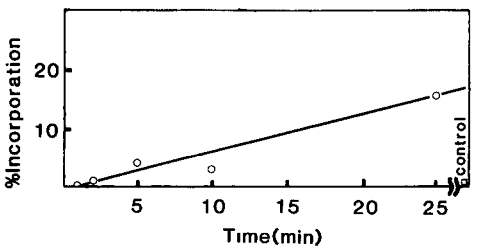

Fig. 1 The incorporation of activity from $H^{14}CO_3^-$ into water soluble metabolites in *P. cartilagineum*. The dark control was incubated for 4 days.

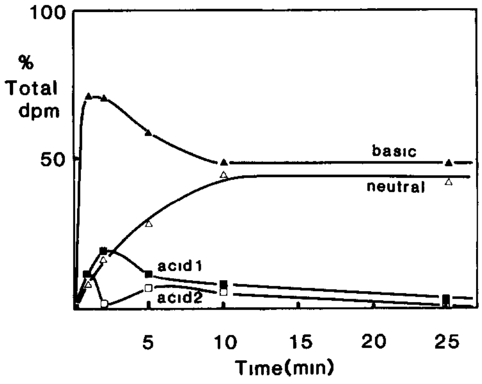

Fig. 2. The incorporation of activity from $H^{14}CO_3^-$ into various fractions of water soluble metabolites expressed as percentages of total incorporation.

Table 2. Incorporation of precursors into the monoterpene 1

Precursor	Time of successful feedings	Incorporation (%)
[1-^{14}C]Acetate	March–December	0.005–0.01
$H^{14}CO_3^-$	March–December	0.004–0.01
[2-^{14}C]Mevalonate	August–October	0.01

Table 3. Incorporation of $H^{14}CO_3^-$ into water soluble photosynthetic products

Time	Radioactivity (dpm × 10^{-3}) Neutral	Basic	Acid 1	Acid 2	Incorporation (%)
1	0.6	5.7	0.9	0.9	0.5
2	3.2	13.9	3.6	0.3	1.3
5	18.6	35.9	7.3	4.6	4.3
10	21.3	23.3	3.9	2.5	3.1
25	100.7	116.2	7.8	4.3	15.0
(dark control)	—	—	—	—	0.6

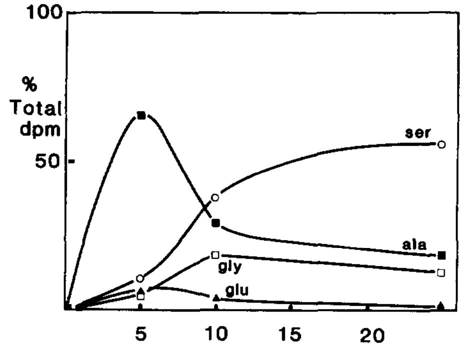

Fig. 3. The incorporation of activity from $H^{14}CO_3^-$ into the amino acids.

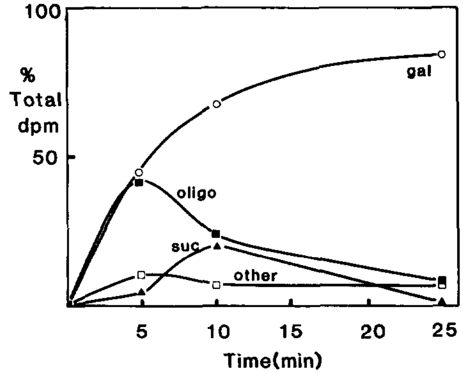

Fig. 4. The incorporation of activity from $H^{14}CO_3^-$ into sugars.

Table 4. Incorporation of labelled mevalonates into the monoterpene **1**

Precursor	Incorporation (%)	$^3H/^{14}C$ Substrate	1
[2-^{14}C]	0.01	—	—
[4*R*-^{3}H, 2-^{14}C]	0.003	1.75	1.82
[2*R*,*S*-^{3}H, 2-^{14}C]	0.009	4.74	3.63

corporations and consequently the results are only reported here for the major metabolite (**1**) which is highly crystalline and easily purified. Compound **1** was routinely crystallized five or six times until the same constant specific activity of both the sample and the material reisolated from the mother liquors was achieved. A complication in these experiments was sometimes the observation of a decline in the specific activity of **1** over several recrystallizations despite apparent zonations of radioactivity shown on autoradiograms which appeared to correspond to the monoterpene band. These problems were only sometimes observed in the mevalonic acid feedings: [^{14}C]acetate and $H^{14}CO_3^-$ incorporations, although low, did not show this effect. The metabolite **1**, obtained from feeding [2-^{14}C]mevalonate, was aromatized to the derivative **5** by heating with DBU and this derivative oxidized to acetic acid with chromic acid. Some 45% (±10%) of the activity was recovered in the acetic acid, the large errors being due to working with specific activities only several times above background. This means that there is no separate pool of dimethylallyl pyrophosphate which usually leads to unequal labelling of the two isoprenoid units of monoterpenes in higher plants [21]. The incorporations of [4*R*-^{3}H]- and [2*R*,*S*-^{3}H]-mevalonic acid are shown in Table 4. Both 4*R*-^{3}Hs are incorporated, while one quarter of the tritium is lost on incorporation of [2*R*,*S*-^{3}H]mevalonate, i.e. three out of four of these atoms are incorporated.

Attempts at conversion of [2-^{14}C]mevalonate into terpenes in cell free preparations were totally unsuccessful. Incubations of similar cell free systems with geraniol gave no products and incubations with geraniol pyrophosphate merely caused partial hydrolysis of the pyrophosphate, probably by a non-specific phosphatase. Also no bromo- or chloro-peroxidase activity, as measured by any decrease in absorbance at 278 nm of monochlorodimedone [15] could be detected over the pH range of 3–8.

However, a very labile classical peroxidase activity was detected in cell-free homogenates of *P. cartilagineum*. The enzymic activity is in the 105 000 *g* supernatant and was purified some 20-fold by precipitation with ammonium sulphate and gel filtration (Table 5). The M_r by gel filtration was 43 000 and when run on polyacrylamide gels and stained for peroxidase activity, three zones were apparent all migrating in front of the pink R-phycoerythrin contaminant. This preparation had a very broad pH profile for the oxidation of iodine and *o*-dianisidine in the presence of hydrogen peroxide extending from pH 3 to 8, with peaks of highest activity at pH 3.8

Table 5. Purification of peroxidase

Fraction	Total activity (kat × 10^{-5})	Total protein (mg)	Specific activity (kat/mg protein × 10^{-6})	Purification	Yield (%)
Crude homogenate	20.9	179	1.16	1	100
105 000 *g* supernat.	13.8	91	1.51	1.3	66
30–60% $(NH_4)_2SO_4$	4.38	7.2	6.06	5.2	21
Gel filtration	2.04	0.9	22.1	19	10

and 6.2 for both substrates, but not many conclusions can be drawn from this as the preparation containing at least three isoenzymes. This partially purified preparation also showed no activity with monochlorodimedone, bromide or chloride ions and peroxide over the range pH 3–8.

DISCUSSION

The difficulties that have been experienced in obtaining reliable incorporations of small precursors such as mevalonic acid into monoterpenes are well documented [21]. In macroscopic red algae an additional problem occurs, as the lack of any long distance transport or translocation mechanisms [22] means that the precursors must be introduced into the sea-water in which the alga is incubated. A fairly high ratio of sea-water to algae and vigorous aeration must be used or blooms of microorganisms occur under our conditions even if the plants or sections of the plants are treated with hypochlorite to attempt surface sterilization. The extensive dilution of the label may be a factor in only obtaining such low incorporations as we report here.

Only one report on the incorporation of small precursors into an algal secondary metabolite has so far been reported. This is the incorporation of ^{14}C-labelled acetate, palmitate and to a lesser extent malonate and butyrate into the metabolite tribromoheptene oxide in *Bonnemaisonia nootkama* [23]. No mention of seasonal effects was made in that work, although in the alga *Rhodomela larix* [12] bromoperoxidase activity could not be detected during the winter months. We do not know why we could only detect mevalonic acid incorporations in the period August–October, but it may be related to the age of the plant and the rate of growth and an increase in metabolite production at that time of the year. Thus seasonal variations in enzyme levels and therefore biosynthetic capacity may be common in algal secondary metabolites.

The oxygen electrode measurements provide a useful way of determining the 'state' of the alga before and during feeding experiments. We terminate the feeding experiments when this activity has dropped to half that of the freshly collected algae, as any longer incubation does not lead to higher incorporations. The incorporation of [^{14}C]bicarbonate into primary metabolites has been achieved throughout the whole year and the results are consistent with the normal operation of the reductive pentose phosphate cycle [24]. Dark carbon fixation under our conditions was negligible. The results shown in Figs 1–4 indicate that normal metabolic pathways are operating and that activity accumulates in both the monosaccharide and amino acid pools. Pulse feeding experiments by Kremer [24, 25] have shown that usually labelled amino acids are not accumulated, as the percentage of ^{14}C-labelling declines rapidly after a feeding pulse, suggesting rapid metabolic interconversions. Serine appears to show a different course of behaviour in our system, but we have not carried out any pulse labelling. Anyhow, the variety of primary metabolites labelled rapidly by [^{14}C]bicarbonate suggests that ample labelled precursors are present which may give rise to acetyl CoA and then mevalonic acid under the appropriate conditions. The incorporations of the order of 0.01% of the labelled acetate or mevalonate fed into the major carotenoids and cholesterol shows indeed that pathways involving mevalonic acid are active under our experimental conditions. The composition of the carotenoid and sterol fractions are unexceptional and are typically those of most Rhodophyta [26]. In higher plants it has also been noted that incorporations into higher terpenoids are usually much greater than into monoterpenes [21]. The problem of decreasing activity on repeated crystallizations after purification by GLC or TLC in plant monoterpenes biosynthesized from mevalonic acid has been described, and later explained by 'salvage pathways' which epoxidize and hydrate prenyl pyrophosphates to compounds which co-chromatograph with monoterpenes [27]. Very possibly similar process happen in algae as well, as this problem did not occur in $H^{14}CO_3^-$ feedings where the physiological substrate was used nor in [^{14}C]acetate feedings but only when [^{14}C] mevalonate was used. The magnitude of this diversion of supplied tracer to other products was very varied and affected about two-thirds of all our feeding experiments.

The very low incorporations (Table 2) observed into the metabolite **1** by all precursors must mean a very low rate of monoterpene synthesis under our conditions. We are confident that these results are meaningful as they are supported by the dual labelling experiments (Table 4) and limited chemical degradations. [4*R*-^3H, 2-^{14}C]Mevalonate is incorporated into **1** with an unchanged $^3H/^{14}C$ ratio, so two 4-pro-*R* hydrogens of mevalonic acid are present in **1**. On aromatization one of the ^3H labels is lost, shown as expected in Fig. 5. When [2*R*,*S*-^3H, 2-^{14}C]mevalonate was incorporated only three out of four tritiums were incorporated per two C-14s.

This is also as expected as shown in Fig. 5, and means that no tritium label is lost by isopentenyl pyrophosphate-dimethylallylpyrophosphate isomerase nor in the halogenation/cyclization reactions leading to the six membered ring. Oxidation with chromic acid of **1** obtained by feeding [2-^{14}C]mevalonate gave labelled acetic acid containing 45 ± 10% of the starting activity. It would appear, that in contrast to higher plants, in this alga both

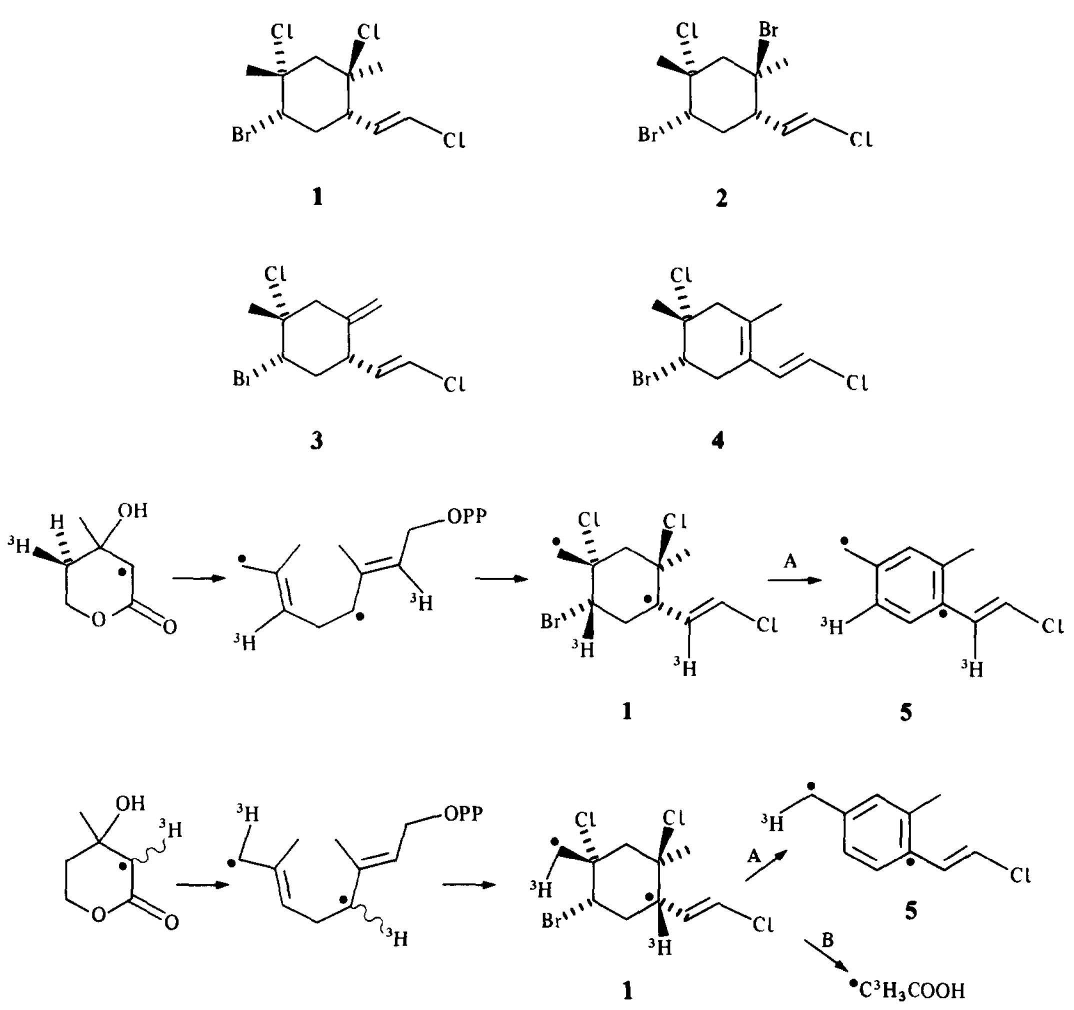

Fig. 5. Structures of the major halogenated metabolites of *P. cartilagineum* and the schemes for incorporations of [^{3}H, ^{14}C]mevalonates into 1 and chemical degradations.

isoprenoid units are equally labelled and this is supported by the ^{3}H incorporations. Some aspects of the suspected possible pathways to 1 and related metabolites have been suggested previously, but are given in more detail in Fig. 6. It has been proposed previously that an acyclic trihalo derivative (6) is a precursor of a halogen isomer (7) which can be rearranged, *in vivo*, to an isomer of 1 [29]. The problem then is how to get from geranyl pyrophosphate to 6 and how to cyclize 6 to 7. The terminal allylic chloride is presumably formed by a chloroperoxidase type enzyme acting on the trisubstituted olefin: attack by Cl^+ or a related enzyme bound species and elimination of a proton. In fact this type of reaction can be carried out by the action of hypochlorous acid on geraniol in non-aqueous solvents [30]. The doubly allylic chloride could be formed in several ways including (a) nucleophilic displacement of the allylic pyrophosphate by chloride ions and allylic substitution by Cl^+ without shift of the double bond, or (b) by 1,4-addition of Cl^+/Cl^- to a diene formed by elimination from geranyl pyrophosphate.

The terminal allylic chlorination must be stereospecific because the corresponding methyl group in 1 retains all of the label for [2-^{14}C]mevalonate in that isopene unit, i.e. the proton is eliminated from the *E*-methyl group. Diastereoisomers of 6 are known from the one plant, but it is not known if this reflects some lack of stereochemical control in synthesis or epimerization after synthesis [29]. It is however, worth recalling that the fungal chloroperoxidase from *Calariomyces fumago* shows no stereospecificity in a variety of reactions including 1,2- and 1,4-additions to appropriate olefins [16]. The cyclization of 6 to 7 could also involve a halonium ion as shown in Fig. 6, and the rearrangement of 7 to 1 is unexceptional.

All of our attempts to obtain a cell free system that would convert mevalonate, geraniol or geraniol pyrophosphate into halogenated monoterpenes were unsuccessful. We only observed some conversion of geraniol pyrophosphate to geraniol, probably by a non-specific phosphatase. Our attempts to detect chloro- or bromoperoxidase activity as measured by halogenation of monochlorodimedone were also unsuccessful. Possibly this substrate, which works so well for enzymes which halogenate β-diketones and related compounds, is not attacked by the halogenoperoxidases involved in terpene biosynthesis or alternatively, we have not chosen the correct conditions for preparation of our cell free extracts.

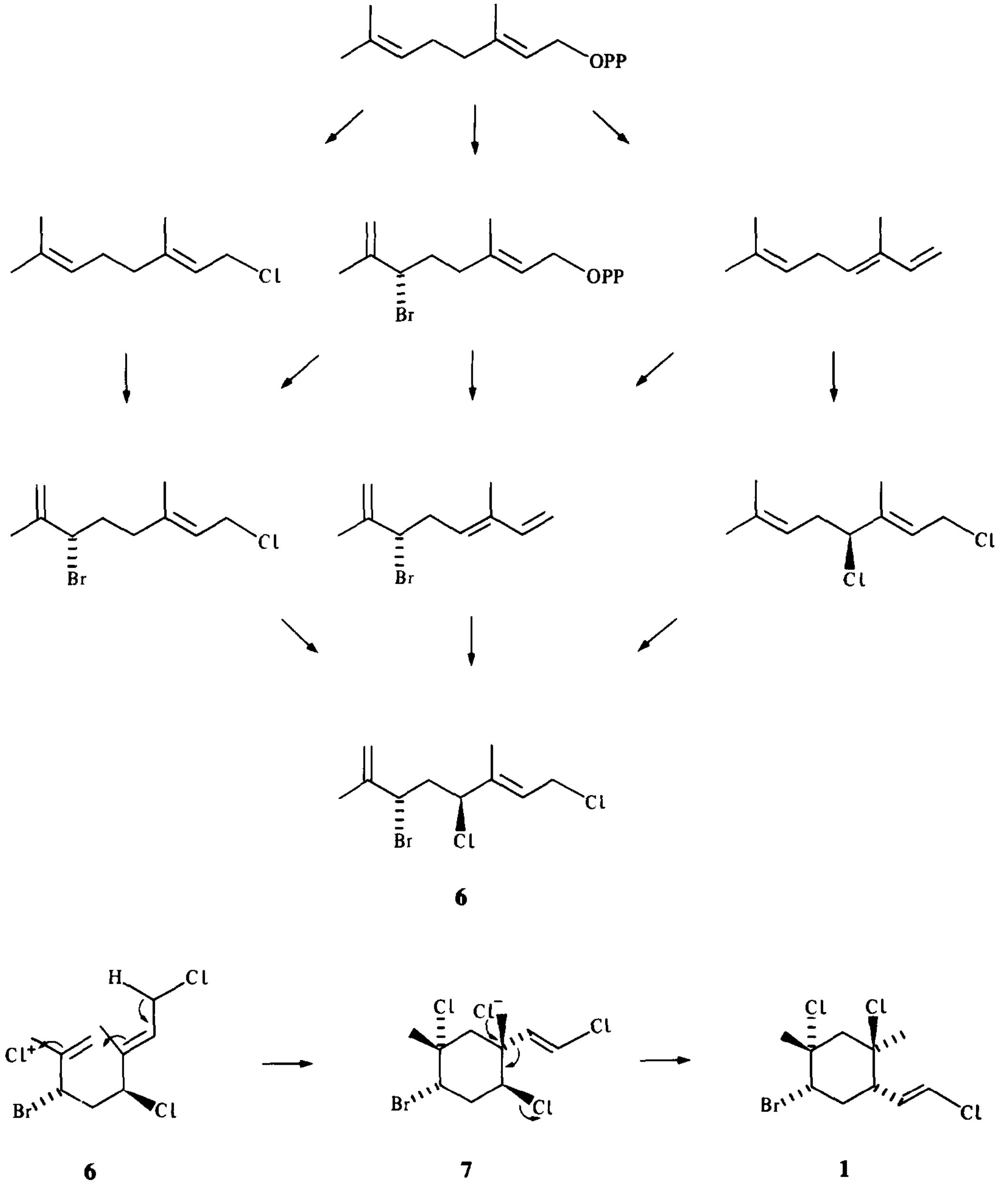

Fig. 6. Possible pathways from geranyl pyrophosphate to the trichlorobromo metabolite 1.

We did however, partially purify a classical plant peroxidase type of activity which is very labile, but is active over a very wide pH range. This enzyme preparation is very similar to others described previous from other alga [31] and does not appear to be related at all to enzymes involved in the biosynthesis of halogenated monoterpenes.

EXPERIMENTAL

Whole plants of *P. cartilagineum* [19] were collected intertidally from rock platforms between Wollongong and Sydney, N.S.W., transported to the laboratory in plastic bags on ice and maintained in vigorously aerated seawater at 15–16° under constant illumination. Metabolites 1–4 were isolated by extracting the algae with Me_2CO in a Waring blender and by chromatography: column and TLC on silica gel and HPLC on μ-Porasil with refractive index detection. In feeding experiments where only metabolite 1 was isolated the crude extract was separated by preparative TLC using petrol (40–60°)–CH_2Cl_2 (2:1), the zones visualized with I_2 and eluted with MeOH. The metabolite was then repeatedly crystallized from hexane. Sterols were isolated from the non-saponifiable lipid fraction by the method of Gibbons *et al.* [31]. Carotenoids were separated by TLC on alumina in EtOAc–CH_2Cl_2–petrol (5:2:7) and the three major zones eluted and rechromatographed on silica gel followed by elution with MeOH. Identifications were made by comparison of R_f values and visible spectra recorded in MeOH in a Cary 118 spectrophotometer.

Photosynthetic activities were obtained by measurement of O_2 evolution in an assimilation chamber saturated with O_2, at 20° and with a white light source of energy 214 kJ/m^2.

Fixation of $H^{14}CO_3^-$ was performed on 8 ml suspensions of finely cut algae in sea water at 20°. $H^{14}CO_3^-$ (0.7 μCi, 56.9 mCi/mMole) was added to the stirred suspension and a white light source (6.8 kJ/m^2) used to illuminate the samples for varying lengths of time. A H_2O soluble fraction was prepared and fractionated essentially by the method of Howard *et al.* [32], using Dowex-1 and -50 resins. The basic fraction was separated by high voltage electrophoresis in 6.3 % HCO_2H (pH 1.9) at 4.5 × 10^3V and visualization with ninhydrin. Amino acid analyses were also performed on a Beckman 121-M Amino Acid Analyser.

In feeding experiments where the monoterpenes were isolated, the whole plants were immersed in the minimum vol of vigorously aerated sea water at 14–16° under constant illumination, for periods of up to 6 days. Labelled precursors were added directly to the sea water.

Scintillation counting was performed on a Packard Tri-Carb counter using 38.5 % toluene, 38.5 % dioxane, 23 % MeOH 8 % (w/v) naphthalene, 0.5 % PPO and 0.01 % POPOP. Efficiencies were checked by addition of standard ^{14}C- and where necessary ^{3}H-hexadecane as well to all samples. GLC/MS was performed using a Finnigan 3200 GC-mass spectrometer coupled to a 6110 data system. A column (6 mm od × 1.5 mm) of 3 % OV-1 was used under a flow of 20 ml CH_4/min with temp programming from 100° at 6°/min. NMR spectra were recorded on a JEOL FX100Q NMR spectrometer operating in the FT mode using TMS as an internal reference in $CDCl_3$ soln. The metabolite **1** was aromatized by heating with DBU (10 molar excess) in dioxane at 70° until TLC indicated complete conversion. The aromatic derivative (**5**) was oxidized with chromic acid and H_2SO_4, and the HOAc collected by steam distillation. After neutralization and addition of cold carrier, the HOAc was converted to the *p*-bromophenacyl derivative in the usual way and purified by repeated crystallization.

[^{3}H]Geranyl pyrophosphate was prepared by reducing citral with NaB^3H_4 (100 mCi/mMole) in EtOH, adding cold geraniol, purifying via the $CaCl_2$ complex [33] to give geraniol (~ 90 μCi/mMole phosphorylating by the literature method [34] and separation of the pyrophosphate on silica gel with n-PrOH–H_2O–ammonia.

Cell free preparations were obtained by homogenization of the alga with a Polytron homogenizer for 5 min. In experiments using mevalonic acid or geranyl pyrophosphate the homogenizing medium contained 0.1 M Pi buffer, pH 7.4 (50 ml/10 g algal wet wt), 5 mM $MgCl_2$, 2 mM Cl_2, 5 mM β-ME, 1 mM dithiothreitol and 1 mM NaCl or NaBr and was clarified by centrifugation at 1000 *g* (10 min). For experiments using mevalonic acid supplements of ATP (to 3 mM), NADPH (0.5 mM) and glucose-6-phosphate (3 mM) were made after centrifugation.

For peroxidase preparations the algae were homogenized in 0.1 M Pi buffer, pH 7.4, containing 1 mM EDTA, and after centrifugation (1000 g, 10 min) the pellet was rehomogenized in fresh buffer The combined supernatants were then spun at 105 000 *g* for 1 hr. The assay mixture for peroxidase activity contained 0.5 mM *o*-dianisidine, 1.1 mM H_2O_2, 0.1 ml of enzyme soln and 2.7 ml buffer, pH 6.6. The oxidation was followed at 460 mM using the units of activity defined by Murphy and 'Oh Eocha [30]. Protein determinations were carried out by the biuret method or the microbiuret method for protein concentrations of less than 1 mg/ml. M_r determinations were run on ultragel AcA44 using standard proteins (Sigma) for calibration. Gel electrophoresis was performed on 7 % polyacrylamide prepared in Tris–HCl pH 8.9, and run in 52 mM Tris–65 mM glycine pH 8.9. Halogenoperoxidase assays were performed with monochlorodimedone as a substrate using chloride or bromide ions at various pHs essentially as described by Morris and Hager [15].

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