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IN VIVO BIOSYNTHESIS OF TRIBROMOHEPTENE OXIDE IN *BONNEMAISONIA NOOTKANA*

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Key Word Index—*Bonnemaisonia nootkana*; Bonnemaisoniaceae; Rhodophyta; algae biosynthesis; brominated C_7-compounds; tribromoheptene oxide; acetate; palmitate.

Abstract—Experimental evidence is presented supporting the involvement of acetate and palmitate in the biosynthesis of tribromoheptene oxide in this red alga.

Marine red algae of the Bonnemaisoniaceae (Florideophyceae) produce a variety of brominated secondary metabolites of unique structure and origin [1]. These compounds have strong antibiotic and antiseptic properties [2], seem to be contained in specialized vesiculate cells [3,4] and may function as chemical defenses against predators [1,2]. *Trans*-1,3,3-tribromo-1-heptene oxide, a major component of *Bonnemaisonia nootkana* (Esper) Silva, is probably derived biogenetically from 3-ketooctanoic acid via the methyl ketone, 2-heptanone [1], by base-catalysed, epoxide-forming reactions following halogenation and reduction. Methyl ketones occur in terrestrial plants and animals [5], but polybrominated methyl ketone derivatives seem to be restricted to members of the Bonnemaisoniaceae. Biosynthesis of naturally occurring methyl ketones can occur by catabolic decarboxylation of β-keto acids formed during the process of fatty acid β-oxidation [6] or during anabolic fatty acid biosynthesis [7]. We investigated these possibilities, and report here what we believe to be the first case of *in vivo* biosynthesis of marine macroalgal secondary metabolites from exogeneous substrates.

Freshly collected, healthy apices of *Bonnemaisonia nootkana* were incubated in limited volume, sterile sea water containing ^{14}C-labelled sodium acetate, sodium malonate, sodium butyrate, or sodium palmitate. Periodically, a portion of the plant from each medium was removed, washed and homogenized in hot chloroform–methanol. Chromatography of the extract on Florisil columns yielded an ether-soluble, 'organic-nonpolar' fraction and a methanol, 'organic-polar' fraction, both of which were assayed for radioactivity by liquid scintillation counting. The epoxide-containing, hexane/ether-soluble 'organic-nonpolar fraction' was labelled (0.5–4.0% total incorporated activity) from all substrates (Table 1). The majority of the label was present in the algal residue following chloroform–methanol extraction (30–50%), and the water-sololuble (38–60%) and methanol-soluble, 'organic-polar' fractions (20–38%). The organic-nonpolar fraction was fractionated by high pressure liquid chromatography on μ-Porasil to yield pure epoxide (>99.9% pure, confirmed by GC and 220 MHz NMR spectroscopy), which was dried, weighed, resuspended in scintillation fluor and assayed for presence of label. Incorporation of label from all sources into *trans*-1,3,3-tribromo-1-heptene oxide was confirmed (Table 1). Highest specific activity in the epoxide consistently resulted from [^{14}C]acetate and [^{14}C] palmitate (Table 1) in contrast to lower incorporation of label from butyrate and malonate. Specific activity in the epoxide increased 2.6- to 6.3-fold over a 12 hr period and was present in smaller amounts in dark-incubated plants. The test algae were not axenic, but uptake of supplied precursors was inferred. Microscopic analysis of the test algae confirmed the general absence of contaminating epimicroflora, which has been reported after scanning electron microscopy of various members of the Bonnemaisoniaceae [8] and which may be the result of the natural products themselves. Also, most of the non-epoxide associated ^{14}C was present as palmitic acid in plants incubated in sodium [^{14}C]palmitate, thus suggesting direct uptake and protonation of the exogenously supplied precursor.

The results support the suggested [1] involvement of acetate condensation or fatty acids in the biosynthesis of the brominated epoxide in *B. nootkana*. Low rates of synthesis from butyrate and malonate may reflect differential solubility or transport of these compounds rather than their lack of direct involvement in the biosynthetic pathway. Alternatively, pools of these acids may exist in the plant and effect uptake of exogenous stock. Unfortunately, the equal efficiency of labelling by acetate and palmitate, and ambiguous results of sequence analysis tests (unpublished), precluded determination of anabolic or catabolic formation of bromoheptanones in *B. nootkana*. Bromination probably occurs by activity of a peroxidase on 2-heptanone [9]. Epoxide biosynthesis in *B. nootkana* clearly involves dynamic, acetate-based fatty acid metabolism and thus is an example of the use of

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Table 1. Radioactivity from sodium acetate, sodium malonate, sodium butyrate and sodium palmitate contained in hexane–ether and methanol fractions of a chloroform extract from plants of *B. nootkana* following 6 hr incubation.

	Nonpolar-organic fraction*				Polar-organic fraction†
	Total		Epoxide‡		Total
^{14}C-labelled substrate	dpm ($\times 10^4$)	sp. act. (dpm/mg)	dpm	sp. act. (dpm/mg)	dpm ($\times 10^4$)
Acetate	2.90	1654	1323	1192	0.4
Malonate	0.29	48	157	112	1.8
Butyrate	0.14	26	73	23	2.0
Palmitate	4.60	1982	1680	1186	29.0

* Hexane–ether fraction from chloroform extract.

† Methanol fraction.

‡ Epoxide (*trans*-1,3,3-tribromo-1-heptene oxide) separated from nonpolar fraction by HPLC on μ-Porasil and assayed for activity.

existing pathways for metabolically efficient allelochemical elaboration [10].

In general, low rates of incorporation of label from organic precursors into secondary metabolites is not uncommon [11]. Similar patterns were not unexpected in *B. nootkana* because the 'gland cells', the probable location of bromoketone metabolism [3, 4] are a minor portion of total algal cytoplasmic biomass. However, the ability to incorporate label into the epoxide biosynthetically allows further studies of brominated natural products metabolism in this species.

Published data on incorporation of low molecular weight organics into macroalgal metabolites are few. Acetate uptake by *Ulva* [12] and ^{57}Co-cobalamin uptake by various tropical species [13] have been reported, but the molecular locations of the labels were not determined. Incorporation of label from amino acids into prokin and from uridine into RNA have been reported in a unicellular stage of the brown alga, *Fucus* [14], but our report of ^{14}C incorporation from acetate and palmitate into secondary metabolites of a red alga seems to be unique.

EXPERIMENTAL

Bonnemaisonia nootkana was collected from depths of 21–28 m in the submarine canyon near Carmel, California, by Scuba divers during the summer, a period of active growth. The algae were placed in chilled sea water in 2 quart polyethylene jars, and shipped in coolers with ice by air freight to San Diego. In the laboratory, the specimens were maintained in sterile, filtered (0.34 μm Millipore) sea water in a controlled environment chamber at 12–14°, 330 lx (cool white fluorescent light) continuous illumination. The experiments were performed on healthy plants within 24 hr of their collection.

Carrier-free radiolabel (New England Nuclear) solns containing 15 μCi sodium acetate (57.7 mCi/mM), 18 μCi sodium 2-[^{14}C]malonate (17 mCi/mM), 16 μCi sodium 1-[^{14}C]butyrate (24 mCi/mM), and 17 μCi sodium U-[^{14}C]palmitate (prepared by treating U-[^{14}C]palmitic acid, 928 mCi/mM, with sodium hydride in Et_2O stirred under N_2 for 30 min at room temp.) respectively were used as the test incubation media.

Healthy, *ca* 5 g fr. wt. apical portions were incubated under agitation (110 rpm) in beakers containing 350 or 500 ml sterile sea water with various radiolabels (see below) under the same conditions as the maintenance cultures. At the end of the incubation period, the algae were rinsed exhaustively in sterile, chilled sea water and quenched, homogenized and refluxed for 10 min in 50 ml hot $CHCl_3$–MeOH (1:1). The homogenized material was filtered through Whatman No. 4 paper, the excess $CHCl_3$–MeOH extract evapd *in vacuo*, and the residual aq. fraction removed. The non-aq. residue was resuspended in Et_2O and chromatographed on 0.5 × 7.0 cm Florisil columns by eluting with 7–8 ml 25% Et_2O–hexanes (to give the 'organic-nonpolar' fraction) followed by 5 ml 100% MeOH ('organic-polar' fraction). The fractions were weighed, placed in a toluene-based scintillation fluor 4.0% (w/w) PPO and 0.4% (w/w) POPOP, and assayed for radioactivity in a Beckman LS-100 scintillation counter, using an external standard. Following scintillation spectroscopy, the organic-nonpolar fraction was fractionated to give pure epoxide. Esterification was necessary to separate the epoxide from fatty acids by HPLC. The toluene fluor was removed by evapn *in vacuo*, and the free fatty acids in the residue esterified in diazomethane in Et_2O stirred at room temp. for 1 hr. Excess diazomethane was removed by heating the soln for 15 min at 40°, and excess Et_2O removed by evapn *in vacuo*. The residue was taken up in 10% Et_2O–hexanes and filtered through glass wool to remove ppt. (mainly scintillation fluor). Purification of the epoxide was achieved by HPLC using μ-Porasil (2 × 30 cm) support and 10% ether–hexanes as the solvent system (flow rate: 2 ml/min, 73–100 kg/cm^2). Chemical purity (>99.9%) of the epoxide after HPLC was confirmed by 220 MHz NMR and GLC with standards. The pure epoxide fraction was weighed and assayed by scintillation counting.

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