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2-CARBOMETHOXYOXEPIN: 1-CARBOMETHOXYBENZENE 1,2-OXIDE AND THE BIOSYNTHESIS OF METHYL SALICYLATE IN *PHELLINUS TREMULAE*

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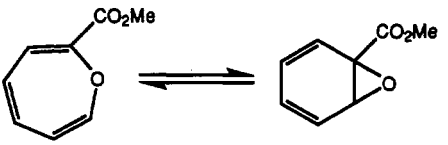
ABSTRACT.—Administration of labeled benzoic acid-α-^{13}C to liquid cultures of *Phellinus tremulae* afforded labeled methyl benzoate, methyl salicylate, and 2-carbomethoxyoxepin, which supports the intermediacy of an arene oxide in the biosynthesis of salicylic acid and its methyl ester.

The isolation of 2-carbomethoxyoxepin: 1-carbomethoxybenzene oxide [**1**] from liquid cultures of *Phellinus tremulae* (=*Fomes igniarius* var. *populus*) (Hymenochaetaceae) (1) provided the opportunity to investigate directly the hypothesis that arene oxides are intermediates in the biosynthesis of methyl salicylate and, consequently, salicyclic acid. This hypothesis is based on observations of substituent migration using labeled or strategically substituted aromatic compounds (2), as well as on the isolation of arene oxides from natural sources (3,4). Further evidence of arene oxide intermediacy was provided by the elegant study by Boyd and Berchtold (5) in which they prepared a number of 1-carboxybenzene and 1-carbomethoxybenzene oxides and showed that these undergo facile acid-catalyzed rearrangement to the corresponding phenols. In particular, they showed that 1-carbomethoxybenzene 1,2-oxide [**1**] is readily transformed to methyl salicylate via an acid-catalyzed NIH shift involving migration of the carbomethoxyl group.

The biosynthesis of salicylic acid and, similarly, methyl salicylate is generally regarded as proceeding via the shikimic acid pathway (6–8), although different intermediates seem to be involved as far as bacteria (6) and plants (7,8) are concerned. In bacteria, chorismic acid and isochorismic acid were shown to be the intermediates involved (6). In plants, it is believed that salicylic acid is derived from the phenylpropanoid pathway, in



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which phenylalanine is converted to *trans*-cinnamic acid by phenylalanine ammonia lyase (PAL) catalysis. Chain-shortening degradation leads to benzoic acid, which is oxidized to salicylic acid (7). The involvement of benzoic acid is supported by the results of the administration of [^{14}C]benzoic acid to plants with subsequent isolation of labeled salicylic acid (2,7), presumably through the 1,2-oxide of benzoic acid (2). Harper *et al.* (9,10) showed that *Phellinus* spp. are capable of converting aliphatic and aromatic carboxylic acids into their esters using chloromethane as methylating agent. Particularly, they have demonstrated that CD_3-labeled methyl benzoate is formed when *Phellinus pomaceus* is incubated in the presence of benzoic acid and deuterated chloromethane. Administration of salicylic acid does not yield methyl salicylate, which suggests that methyl salicylate is biosynthesized from methyl benzoate (9,10) and not by the methylation of salicylic acid. In order to test the in vivo participation of arene oxides as intermediates in the methyl salicylate biosynthesis (5), benzoic acid-α-^{13}C was administered to *P. tremulae*. The liquid culture was grown in the presence of Diaion HP-20 resin as reported previously (1) and was periodically supplemented with

injections of a sterile solution of sodium benzoate-α-^{13}C. After 16 days of growth, the resin and mycelium were separated from the culture medium and washed with CH_2Cl_2. The crude extract was chromatographed eluting with 0.5% MeOH in CH_2Cl_2. The incorporation of labeled benzoic acid was determined by ^{13}C-nmr spectroscopy of the isolated methyl benzoate, methyl salicylate, and 2-carbomethoxyoxepin [**1**]. The enrichment is shown in Table 1.

The level of incorporation of labeled benzoic acid in these three esters supports the hypothesis that methyl salicylate is biosynthesized by *P. tremulae* from benzoic acid via methyl benzoate and 2-carbomethoxyoxepin [**1**] (Scheme 1). Acid-catalyzed rearrangement affords methyl salicylate. In the study by Harper *et al.* on the methylation of benzoic acid by chloromethane in *P. pomaceus*, it was found that ester biosynthesis was maximal at 0.5 mM of benzoic acid and that it was inhibited when the benzoic acid concentration in the medium exceeded 3 mM (9). They reported that the decrease in the methyl benzoate pool was followed by a secondary peak in the chromatogram, which was not methyl salicylate, and proposed that the fungus was directing benzoic acid into an alternative metabolic pathway. On the basis of our results, it seems possible that 2-carbomethoxyoxepin [**1**] is the alternative metabolite.

Phellinus tremulae causes severe heartwood decay in aspen (*Populus tremuloides* Michx.) (11), an increasingly important timber resource in Canada (12). Salicyclic acid plays an important role in the induction of plant resistance to pathogens, the so-called systemic acquired resistance (SAR) (8). It has been suggested that salicyclic acid is the signal compound that induces SAR (13). Salicylic acid is produced by the monooxygenase catalyzed oxidation of benzoic acid (14), presumably via the arene oxide: oxepin. It appears that methyl salicylate does not induce SAR (8). It thus seems possible that the methylation of benzoic acid is the way in which *P. tremulae* is able to shut down induced resistance in aspen by diverting the salicylic acid to methyl salicylate, via 2-carbomethoxyoxepin. The odor of wintergreen (methyl salicylate) has long been associated with *Phellinus* heartwood rot (15).

TABLE 1. Incorporation of Benzoic Acid-α-^{13}C by *Phellinus tremulae*.

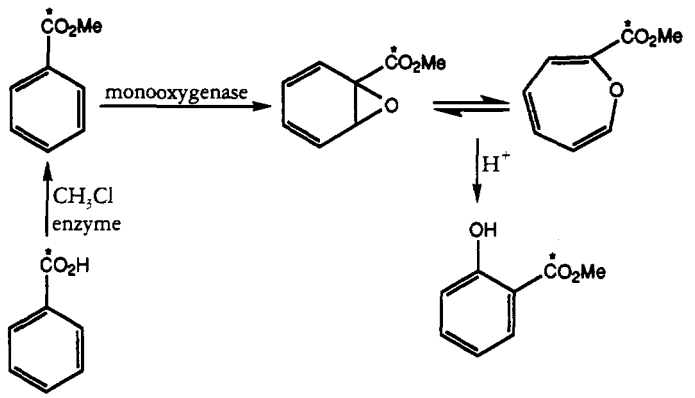
Compound	δ	Atom % enrichment[a]
Methyl benzoate.....	167.0	37
Methyl salicylate	170.6	38
2-Carbomethoxy-oxepin [**1**]	163.2	94

[a]Atom % enrichment = $\frac{\text{sodium benzoate labeled intensity}}{\text{natural abundance intensity}} - 1.1$

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra (^{1}H and ^{13}C) were obtained on a Bruker WH-400 spectrometer with an Aspect 3000 computer system. All nmr spectra were recorded in $CDCl_3$ solution using TMS as internal standard. Universal Scientific Incorporated Si gel 60 (40 microns) was used for flash chromatography. Analytical tlc was carried out on sections of E. Merck precoated aluminum sheets of Si gel 60 F-254. Ultraviolet-active materials were detected by visualization under a uv lamp (254 nm). Liquid media were prepared using Bacto™ malt extract broth (Difco Laboratories) and HP-20 Diaion resin (Mitsubishi Chemical Industries Ltd., supplied by TCI America, Portland, OR). Labeling experiments were performed with 99% benzoic acid-α-^{13}C purchased from MSD Isotopes, Montreal, Canada. All solvents were distilled prior to use.

GROWTH OF *PHELLINUS TREMULAE*: ADMINISTRATION OF BENZOIC ACID-α-^{13}C.—Cultures of *P. tremulae* (strain NOF 1464) were obtained from Dr. Y. Hiratsuka, Forestry Canada, Northern Forestry Centre, Edmonton, and are deposited at the University of Alberta Microfungus Herbarium (UAMH 7005). Agar plate cultures of *P. tremulae* were blended in a Waring blender with sterile H_2O and ca. 30 ml-aliquots were inoculated into 4-liter Erlenmeyer flasks charged with 2 liters of sterile malt extract medium (40 g of malt extract broth Difco, 40 g of Diaion HP 20 resin and 2 liters of redistilled H_2O). Benzoic acid-α-^{13}C (123 mg) was suspended in redistilled H_2O (40 ml) and solubilized by adding aqueous NaOH (1 M) dropwise with heating. The pH was kept slightly



SCHEME 1. Biosynthesis of methyl salicylate from benzoic acid in *Phellinus tremulae*.

acidic. This sterile solution was added periodically to the *P. tremulae* culture (2 liters) by injecting 10 ml after days 1, 3, 5, and 7 of growth. The culture was harvested at day 16.

HARVESTING AND SEPARATION.—The culture broth was separated from the resin and mycelium by vacuum filtration. Resin and wet mycelium were transferred to a sintered-glass funnel and extracted with CH_2Cl_2 (3×100 ml for each 2 liters culture). The organic extracts were combined and dried over anhydrous $MgSO_4$ or Na_2SO_4. Evaporation of the solvent under reduced pressure afforded 0.5 g/liter of a crude organic extract (dark yellow oil). Flash chromatography of the crude organic extract was carried out using 0.5% MeOH in CH_2Cl_2. The first three compounds eluted from the column were methyl salicylate, methyl benzoate, and 2-carbomethoxyoxepin.

Labeled methyl salicylate.—^{13}C nmr ($CDCl_3$, 100 MHz) enriched signal: δ 170.6 (C=O, 37% incorporation); natural abundance signals: δ 161.6 (C-2), 135.6 (C-4), 129.8 (C-6), 119.0 (C-5), 117.5 (C-3), 112.3 (C-1), 52.1 (OMe).

Labeled methyl benzoate.—^{13}C nmr ($CDCl_3$, 100 MHz) enriched signal: δ 167.0 (C=O, 38% incorporation); natural abundance signals: δ 132.8 (C-4), 130.1 (C-1), 129.5 (C-2, C-6), 128.2 (C-3, C-5), 51.9 (OMe).

Labeled 2-carbomethoxyoxepin [**1**].—^{13}C nmr ($CDCl_3$, 100 MHz) enriched signal: δ 163.2 (C=O, 94% incorporation); natural abundance signals: δ 137.0 (C-7), 133.6 (C-5), 132.9 (C-2), 128.6 (C-4), 123.9 (C-3), 118.1 (C-6), 52.4 (OMe).

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