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Biooxidation of Chrysanthemate Isobutenyl Methyl Groups Examined by Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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[1,3-^{13}C]Acetone (92.6 atom %) is used to label the isobutenyl methyl groups of methyl *trans*-chrysanthemate and *trans*-chrysanthemic acid. ^{13}C NMR chemical shifts are assigned for the analogous allylic carbon atoms of 12 alcohols, aldehydes, and acids prepared by chemical oxidation of methyl *trans*- and *cis*-chrysanthemates. The chemical shifts of these carbon atoms in pH 7.4 phosphate buffer are compared with those of metabolites formed on biooxidation of the ^{13}C-labeled compounds. Direct NMR examination of rat and mouse liver microsomal oxidase systems, the bile of treated rats, and the excreta of orally dosed lepidopterous larvae confirms the previously reported dependence of *E*/*Z* oxidase specificity on the system. Metabolites in rat liver enzyme systems and in the bile of treated rats indicate that the *E*-hydroxymethyl intermediate, but not its *Z* isomer, is rapidly oxidized to the corresponding acid. Studies with ^{14}C compounds establish the importance of isobutenyl methyl oxidation, glucuronide conjugation, and biliary excretion in eliminating the chrysanthemic acid isomers from the body of treated rats.

Chrysanthemate insecticides are detoxified in part by metabolic oxidation at the isobutenyl methyl substitutents (Casida and Ruzo, 1980). Previous studies using [^{14}C]-chrysanthemates and thin-layer chromatography (TLC) revealed some organismal differences in the stereoselectivity of the methyl hydroxylation reactions and in the extent of further oxidation at these sites or conjugation before excretion (Yamamoto and Casida, 1966; Yamamoto et al., 1969; Ueda et al., 1975a,b; Soderlund and Casida, 1977). These findings are not complete or definitive since many of the metabolites were unidentified or underwent some decomposition during workup and analysis. These difficulties might be overcome by in situ examination by ^{13}C nuclear magnetic resonance (NMR) spectroscopy of appropriate ^{13}C-labeled materials and comparison of the ^{13}C chemical shifts of the metabolites with those of suitable synthetic standards.

The present study utilizes ^{13}C NMR to determine the metabolic fate of the isobutenyl methyl groups of methyl (1*RS*,*trans*)-[^{13}C]chrysanthemate in rat and mouse liver enzyme systems and a lepidopterous larva. The in vivo relevance of these findings is further examined by comparing the in vitro metabolites with those present in bile from cannulated rats treated with (1*RS*,*trans*)-chrysanthemic acid bearing a ^{14}C label (for quantitation of urinary, fecal, and biliary metabolites) or a ^{13}C label (for identification of biliary metabolites by direct NMR examination).

MATERIALS AND METHODS

Designations. Figure 1 gives the structure of methyl chrysanthemate and some of its oxidation products along with their designations. Chrysanthemic acid is referred to as CA and its methyl ester as CA-CH_3. Isomers about the cyclcopropane ring are designated as cis or trans and of the side chain (from oxidation of the allylic methyl groups) as *E* or *Z* alcohols (CH_2OH), aldehydes (CHO), or carboxylic acids (COOH).

Chemicals. *trans*-CA and *trans*/*cis*-CA (7:3) were provided by Sumitomo Chemical Co. (Osaka, Japan). Sources of the ^{14}C-labeled cyclopropanecarboxylic acids used were as follows: (1*R*,*trans*)-[2,2-*dimethyl*-^{14}C]-CA (56 mCi/mmol) from Roussel-Uclaf-Procida (Paris, France); (1*R*,*trans*)-[*carbonyl*-^{14}C]-CA and (1*R*,*cis*)-[*carbonyl*-^{14}C]-CA (2.7 mCi/mmol) (Nishizawa and Casida, 1965; Ueda et al., 1974); (1*RS*,*trans*)-[*carbonyl*-^{14}C]-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (Cl_2CA) (58 mCi/mmol) from hydrolysis (0.5 N NaOH in methanol, 37 °C, 24 h) of (1*RS*,*trans*)-[*carbonyl*-^{14}C]permethrin (Gaughan et al., 1977).

Spectroscopy and Chromatography. NMR spectra were obtained with a Bruker WM-300 wide-bore spectrometer operating at 300.13 MHz (^{1}H) or 75.47 MHz (^{13}C)

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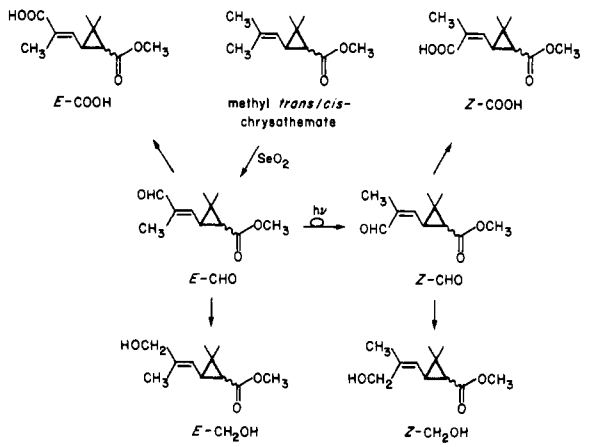

Figure 1. Conversion of unlabeled methyl chrysanthemate to isomeric alcohols, aldehydes, and carboxylic acids indicating designations used.

for samples in 5-mm tubes ($^1H/^{13}C$) or 10-mm tubes (^{13}C only). ^{13}C Metabolite analyses were carried out in 10-mm tubes on samples in 100 mM phosphate, pH 7.4, buffer containing acetone (1%) (internal standard and carrier solvent for substrate introduction) and D_2O (10%) (internal lock). Experiments were run for 4000–14 000 transients by using a 32° pulse width with a 3-s relaxation delay following data acquisition. Data were acquired and Fourier transformed over 16K data points with a simple experimental multiplication (line broadening 5 Hz) of the free induction decay.

TLC utilized silica gel chromatoplates (EM Laboratories, Inc., Elmsford, NY) of 0.25-mm thickness for analysis and 0.5-mm thickness for product isolation. ^{14}C compounds were detected by autoradiography and unlabeled products by ultraviolet quench or with an aqueous 0.05% $KMnO_4$ spray reagent.

Synthesis of Potential Metabolites of Methyl Chrysanthemate. The required products in unlabeled form were obtained as shown in Figure 1 by SeO_2 oxidation to the aldehydes (Crombie et al., 1970), photoisomerization, and reduction of the isomer mixture to the alcohols (Ruzo et al., 1982) or aerial oxidation to the carboxylic acids (Yamamoto et al., 1969). A mixture of *trans/cis*-CA-CH_3 (2.0 g) and SeO_2 (1.6 g) in absolute ethanol (20 mL) was refluxed for 1 h, concentrated under vacuum, and filtered. The filtrate was diluted with water and extracted with ether, and the ether was dried ($MgSO_4$) and evaporated. Distillation of the residue yielded *trans/cis*-[(*E*)-CHO]-CA-CH_3 (1.1 g, 85–95 °C/0.1 mmHg) and *trans/cis*-[(*E*)-CH_2OH]-CA-CH_3 (234 mg, 110–120 °C/0.1 mmHg) with an overall yield of 64%. The aldehyde mixture (100 mg) in degassed chloroform (50 mL) was photoisomerized by irradiation at 300 nm until equilibrium was reached (~4 h) either without sensitizer or by using isobutyrophenone (125 mg), in either case yielding equal amounts of the four isomeric aldehydes as determined by ^1H NMR. *trans*-[(*E*)-CHO]-CA-CH_3 and a mixture of the other three isomers of (CHO)-CA-CH_3 were separated from the reaction mixture by TLC (hexane-ether, 3:1). Treatment of any single aldehyde isomer or mixture of isomers (250 mg) with $NaBH_4$ (53 mg) in 2-propanol (20 mL) for 18 h at 20 °C and TLC (ether–hexane, 1:1) yielded the corresponding alcohol isomers. *trans*-[(*E*)-CH_2OH]-CA-CH_3, *trans/cis*-[(*E*)-CH_2OH]-CA-CH_3 and a mixture of all four isomers were prepared in this manner from the corresponding aldehydes. The acid standards were obtained by aerial oxidation of either *trans/cis*-[(*E*)-CHO]-CA-CH_3 or *trans/cis*[(*E/Z*)-CHO]-CA-CH_3 (prepared via photoisomerization). A solution of the aldehydes (40 mg) in 100 mM, pH 7.4, phosphate buffer (50 mL) containing $FeSO_4$ (~1 mg) was sonicated and oxygen was bubbled through the mixture for 48 h. Acidification (HCl) and extraction with ether followed by TLC (chloroform–methanol, 23:2) separated *trans*-[(*E*)-COOH]-CA-CH_3 from the other three isomers.

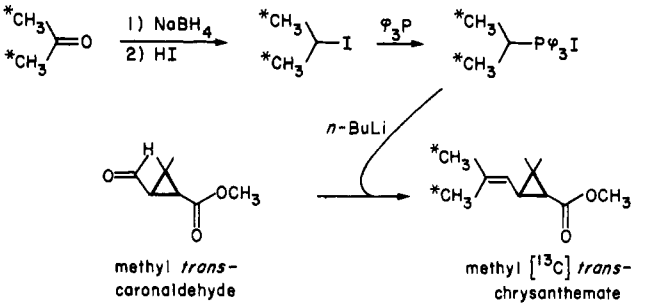

Figure 2. Synthesis of methyl *trans*-[^{13}C]chrysanthemate. Asterisks designate the positions of ^{13}C labels.

Synthesis of Methyl *trans*-[^{13}C]Chrysanthemate. Figure 2 gives the synthetic procedure based on Crombie et al. (1970). [1,3-^{13}C]Acetone (92.6 atom%, 500 mg, Prochem, London) was treated with $NaBH_4$ (200 mg) in triethylene glycol dimethyl ether (2 mL) and aqueous $NaHCO_3$ (5%, 0.2 mL) with stirring for 1 h at 20 °C. The mixture was dried ($MgSO_4$) and filtered, and the product was distilled out of the solvent at 100 °C. The product was treated with aqueous HI (57%, 2.5 mL) and distilled, and the distillate was extracted with ether (4 mL), which in turn was washed with $NaHCO_3$ (5%), dried ($MgSO_4$), and concentrated (Vigreux column). Triphenylphosphine (2.3 g) was added to the concentrate and the mixture heated at 130 °C for 12 h. Trituration of the residue with ether yielded the crude phosphonium salt (1.8 g, 48% from [^{13}C]acetone) [mp 189–195 °C; reported 192–194°C (Crombie et al., 1970)], which was used without further purification.

For Wittig coupling, the phosphonium salt (1.0 g) in dry ether (10 mL) at 0 °C, under positive N_2 pressure, was treated with *n*-butyl lithium (1.45 mL of 1.6 M solution in hexane) to form the deep red ylide. After stirring for 0.5 h at 20 °C, the mixture was again cooled to 0 °C, and methyl (1*RS*,*trans*)-caronaldehyde (434 mg) [prepared as described by Crombie et al. (1970)] in dry ether (5 mL) was added over 30 min. The mixture was stirred for 2 h at 20 °C, treated with water (10 mL), and extracted with ether. The organic layer was dried ($MgSO_4$), filtered, and evaporated. Microdistillation of the residue gave *trans*-[^{13}C]CA-CH_3 as a clear liquid (300 mg, 66%). A portion of the methyl ether was hydrolyzed by treatment with 5% NaOH in water–methanol (10:1) at 50 °C for 6 h. Acidification (HCl), extraction with chloroform, and drying ($MgSO_4$) yielded *trans*-[^{14}C]CA in nearly quantitative yield.

trans-[^{13}C]Phenothrin was prepared by adding 3-phenoxybenzyl bromide (60 mg) and tetra-*n*-butylammonium bromide (5 mg) to *trans*-[^{13}C]CA (20 mg) in methylene chloride (5 mL) and aqueous $NaHCO_3$ (5%, 2 mL). The mixture was stirred rapidly at 40 °C for 12 h, and the organosoluble portion was dried ($MgSO_4$) and purified by TLC (hexane–ether, 5:1), yielding the product (34 mg, 81%) as a clear liquid.

Metabolism in Rat and Mouse Liver Enzyme Systems. Microsomes (100000*g* pellet from 9000*g* supernatant) and the soluble fraction (100000*g* supernatant) were prepared (Bleeke and Casida, 1984) from male Swiss-Webster mice (20–25 g) or phenobarbital-induced male

Simonsen albino rats (180–200 g) (Simonsen Laboratories, Gilroy, CA). Reaction mixtures in 2.5-mL final volume of 100 mM phosphate, pH 7.4, buffer contained some or all of the following constituents: microsomes (1.5 mg of protein), soluble fraction (5 mg of protein), reduced nicotinamide adenine dinucleotide phosphate (NADPH) (2 mg), tetraethyl pyrophosphate (TEPP, a potent esterase inhibitor) (7×10^{-5} M), D_2O (0.25 mL), and the substrate ([^{13}C]CA-CH_3, [^{13}C]CA, or [^{13}C]phenothrin) (400 μg) added in acetone (25 μL). Incubations with the soluble fraction also contained $MgCl_2$ (1.8 mg) and KCl (6.2 mg). The mixtures were shaken in 25-mL Erlenmeyer flasks at 37 °C for 5 min (standard for all experiments). They were then stored for up to 8 h at 4 °C prior to examination by ^{13}C NMR spectroscopy.

Metabolism in Rats. Male rats (as above but without phenobarbital induction) were administered [^{14}C]CA (~0.25 μCi) at 1 mg/kg or [^{13}C]CA at 8 mg/kg either intraperitoneally (ip) or intraveneously (iv) into a femoral vein. The carrier vehicles were methoxy triglycol (150 μL) for the ip treatments and ethanol–Emulphur–0.9% aqueous NaCl (1:1:8) (200 μL) for the iv treatments. Urine, feces, and, in certain cases, [^{14}C]CO_2 were collected for 48 h from ip-treated rats housed individually in glass metabolism cages with food and water offered ad libitum. In biliary excretion studies, rats had their bile ducts cannulated with polyethylene tubing while under sodium pentobarbital (60 mg/kg ip) anesthesia (Marshall and Dorough, 1979). Rats were allowed to recover from surgery, with free access to food and water, for at least 16 h to confirm a consistent bile flow. Rats were then administered (1R,*trans*)[^{14}C]CA ip, and bile and urine were collected for 48 h. Alternatively, for iv treatments in short-term experiments, the rats were anesthetized, their bile ducts were cannulated, and they were then administered *trans*-[^{13}C]CA, (1*R*,*cis*)-[^{14}C]CA, or (1*RS*,*trans*)-[^{14}C]Cl_2CA. Bile was collected for 2 h following administration during which time the rats remained anesthetized.

To analyze the metabolites of (1*R*,*trans*)-[^{14}C]CA, the urine was acidified with dilute HCl to pH 1, saturated with $(NH_4)_2SO_4$, and extracted with ether–ethanol (3:1) (1 volume × 3), recovering 92–94% of the radiocarbon content. Application of this procedure to bile gave only 70% recovery. Feces were homogenized in methanol (Polytron) to recover 99% of their ^{14}C content. The ether–ethanol extract of urine (dried over $MgSO_4$), the methanol extract of feces, and the bile directly were quantitated by liquid scintillation counting and subjected to TLC (0.5-mm chromatoplates) in butanol–acetic acid–water (6:1:1) (BAW solvent system). Labeled products were recovered by gel extraction with methanol or acetone. Glucuronide conjugates of isolated urinary and biliary metabolites were cleaved with β-glucuronidase (333 units, Sigma Chemical Co., St. Louis, MO) in 3.3 mL of 40 mM phosphate, pH 6.8, buffer incubated for 4 h at 37 °C. Aglucons were further isolated by extraction as above and cochromatographed by TLC (toluene saturated with formic acid–ether, 10:3) or subjected to methylation (diazomethane) and two-dimensional TLC cochromatography [two developments in the first direction with hexane–ether (2:1) and one development in the second direction with toluene–ethyl acetate–methanol (15:5:1)] [based on Ueda et al. (1974, 1975a,b)].

The ^{13}C NMR spectrum was examined with the 0–1-h bile (2.0 mL) plus D_2O (0.5 mL) and acetone (20 μL).

Metabolism in *Pseudaletia unipuncta* (Noctuidae: Lepidoptera) Larvae. In a trial experiment, larvae were allowed to ingest [α-^{13}C]benzyl alcohol (91 atom%, MSD Isotopes, Montreal) (250 μg each) applied in acetone (5 μL) to a small piece of larval diet (~3 mm^2) that they ate in 3–6 h. They were then placed in NMR tubes along with glass wool to restrict their movement and a small glass capillary containing 25 μL D_2O and 25 μL of acetone for locking and referencing.

In a metabolism study, four last instar larvae (~250 mg each) were individually fed [^{13}C]CA-CH_3 (250 μg each) as above. They were maintained with untreated larval diet ad libitum and their excreta collected for 0–24 and 24–48 h after treatment. The excreta were extracted with cold 100 mM, pH 7.4, phosphate buffer with sonication. After ^{13}C NMR examination, the extract was acidified to pH 5.5 with H_3PO_4 and treated with β-glucosidase (30 units, Sigma) for 12 h at 37 °C and reexamined by ^{13}C NMR.

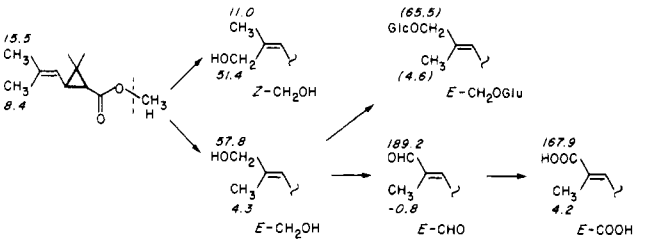

Figure 3. Metabolic oxidation of allylic carbon atoms of *trans*-chrysanthemic acid and methyl *trans*-chrysanthemate. Numbers are ^{13}C NMR chemical shifts (ppm) for the designated allylic carbon atoms of standard compounds (Table I). ^{13}C NMR chemical shifts for both allylic carbon atoms of the in vitro and in vivo metabolites of [^{13}C]CA and [^{13}C]CA-CH_3 (Table II) are in every case within 0.1 ppm of those for the relevant standards. The glucoside (Glc) is not available as a standard but cleaves with β-glucosidase to [(*E*)-CH_2OH]-CA-CH_3.

Table I. ^{13}C NMR Chemical Shifts of the *E* and *Z* Allylic Carbon Atoms of Potential Metabolites of Trans and Cis Isomers of Methyl Chrysanthemate

allylic carbon atoms		chemical shift, ppm[a]			
		E carbon		*Z* carbon	
E	*Z*	trans	cis	trans	cis
CH_3	CH_3	15.5[b]		8.4[b]	
CH_2OH[c]	CH_3	57.8	58.1	4.3[d]	
CH_3	CH_2OH[c]	11.0[d]		51.4[e]	51.4[e]
CHO	CH_3	189.2	184.1	−0.8[d]	
CH_3	CHO	*f*	*f*	185.9[g]	181.2[g]
COOH	CH_3	167.9[h]	161.7[h]	4.2[d]	
CH_3	COOH	*f*	*f*	171.2[h]	168.3[h]

[a] Relative to acetone (C=O = 206.0, CH_3 = 21.0 ppm) in 100 mM phosphate, pH 7.4, buffer. 2-Propanol (14.6 and 55.1 ppm) and acetol (15.3, 58.4, and 203.4 ppm) are not formed from acetone in any enzyme system examined. [b] Relative assignment based on Crombie et al. (1975). CA and CA-CH_3 give identical chemical shifts. [c] Chemical shifts (ppm) for the oxidized allylic carbons of the corresponding acetates are (*E*)-trans 59.7, (*Z*)-trans 60.0, (*E*)-cis 54.7, and (*Z*)-cis 53.7. [d] Assignment of the CH_3 group of the trans isomer by comparison with metabolite mixtures. [e] The cis and trans isomers are not resolved. [f] Not assigned. [g] Assignment assumes trans is downfield of cis as with the corresponding *E* isomers. [h] Chemical shifts (ppm) for the cyclopropanecarboxyl carbons are similar for *trans*- and *cis*-cyclopropanecarboxylates [see also Crombie et al. (1975) and Janes (1977)] and appear as follows (ppm): (*E*)-trans 165.9, (*Z*)-trans 164.9, (*E*)-cis 164.6, and (*Z*)-cis 165.2.

RESULTS

Assignment of ^{13}C NMR Resonances. Chemical shifts in aqueous medium are given in Table I and Figure 3 for the allylic carbon atoms of potential metabolites of methyl chrysanthemate. These shifts were identical at 2 and 25 °C. Interestingly, in the ^{1}H NMR spectra of the (*E*)-CH_2OH isomer the CH_2 protons resonate as a sharp singlet, but in the (*Z*)-CH_2OH isomer, they resonate as an AB

Table II. Stereoselectivity of in Vitro and in Vivo Metabolic Oxidation of Allylic Carbon Atoms of Methyl *trans*-Chrysanthemate and *trans*-Chrysanthemic Acid

allylic carbon atoms		relative intensity[a]	
E	*Z*	*E*	*Z*
Rat Microsomes, CA-CH_3			
CH_3	CH_3	31	26
CH_2OH	CH_3	13	12
CH_3	CH_2OH	9	9
Mouse Microsomes, CA-CH_3			
CH_3	CH_3	46	39
CH_2OH	CH_3	7	5
CH_3	CH_2OH	1.1	1.5
Rat Microsomes plus Soluble, CA-CH_3			
CH_3	CH_3	44	36
CH_3	CH_2OH	5	4
COOH	CH_3	3	8
Rat Bile, CA			
CH_3	CH_3	26	22
CH_2OH	CH_3	5	5
CH_3	CH_2OH	11	11
CHO	CH_3	5	5
COOH	CH_3	2	8
Lepidopterous Larval Excreta, CA-CH_3			
CH_2OGlc	CH_3	48	52

[a] Measured from relative peak areas (computer calculated).

quartet, indicating magnetic nonequivalence. This may be interpreted as being due to restricted rotation in the (*Z*)-CH_2OH isomer.

Metabolism in Rat and Mouse Liver Enzyme Systems. Reaction mixtures of [^{13}C]CA, rat liver microsomes and NADPH incubated for up to 20 min give ^{13}C NMR spectra identical with those of [^{13}C]CA alone in buffer, indicating that no oxidation occurs in the isobutenyl group of the free acid. Under the same conditions, [^{13}C]CA-CH_3 is appreciably oxidized after 5 min (the standard incubation time) to approximately equal amounts of two products with *trans*-[(*E*)-CH_2OH] and *trans*-[(*Z*)-CH_2OH] side chains. ^{13}C NMR is equivocal on whether these compounds are acids or esters, but the esterase inhibitor TEPP at 7×10^{-5} M does not significantly change the product distribution so they are probably esters. On longer incubation (20 min) with rat microsomes and NADPH, there is almost complete loss of starting CA-CH_3 and of most of the initial oxidation products, presumably via secondary reactions. The mouse liver microsome system shows greater specificity than the corresponding rat preparation for oxidation at the (*E*)-CH_3 group of the ester (Table II).

The soluble fraction with the microsomes selectively promotes further metabolism of one of the initial alcohol metabolites (Table II; Figure 4). *trans*-[(*Z*)-CH_2OH]-CA-CH_3 is still a major product but the *E* isomer is completely converted to *trans*-[(*E*)-COOH]-CA-CH_3. No peaks corresponding to the presumed aldehyde intermediates are observed.

trans-[^{13}C]Phenothrin is not a suitable substrate for NMR studies in the rat liver microsome–NADPH system under the above conditions in which *trans*-[^{13}C]CA-CH_3 is readily metabolized. The limiting factor appears to be the poor aqueous solubility of phenothrin (as noted also for other pyrethroids; Gammon et al., 1981) since even in buffer alone the ^{13}C signals are not visible after 4000 transients. Similar results are obtained with [^{13}C]phenothrin even at 8-fold higher dilution in 20-mm NMR tubes. Experiments with [^{13}C]CA-CH_3 and [^{13}C]phenothrin indicate that extraction of the acidified incubation mixtures with deuteriochloroform recovers the labeled materials but

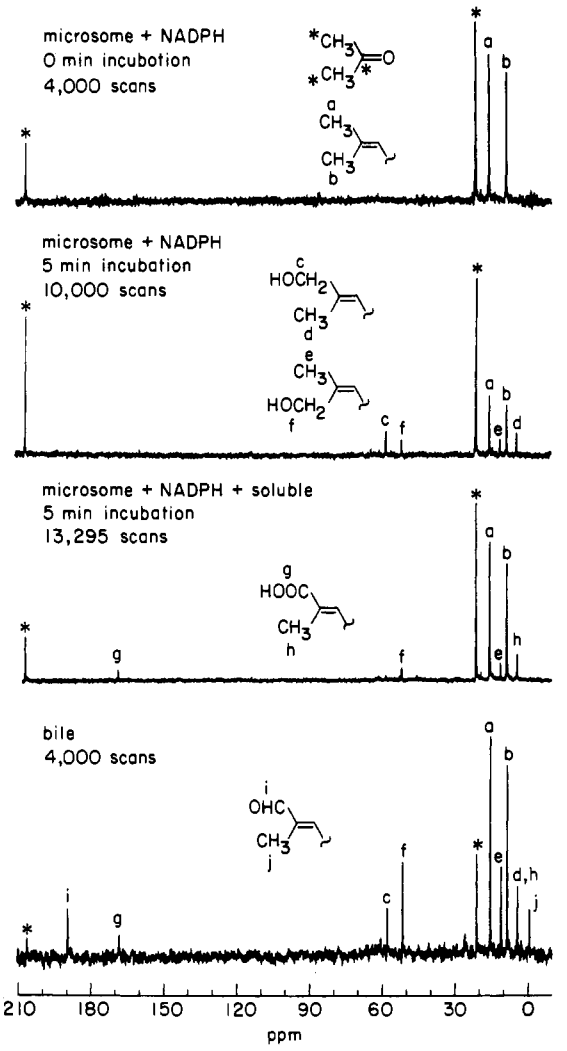

Figure 4. ^{13}C NMR spectra of metabolites of methyl *trans*-[^{13}C]chrysanthemate in rat liver microsomal oxidase systems and of *trans*-[^{13}C]chrysanthemic acid in the bile of intravenously treated rats. The ^{13}C signals of the acetone carrier solvent are indicated by asterisks. Spectra identical with the top figure are also obtained with [^{13}C]CA in the microsome plus NADPH system at both 0- and 5-min incubation.

with [^{13}C]CA-CH_3 this procedure is less favorable (because of increased background) than direct observation of the reaction mixture.

Metabolites Excreted in the Urine and Feces of Rats Treated Intraperitoneally with (1*R*,*trans*)-[^{14}C]Chrysanthemic Acid. Both (1*R*,*trans*)-[*carbonyl*-^{14}C]- and (1*R*,*trans*)-[2,2-*dimethyl*-^{14}C]CA are rapidly eliminated from ip-treated rats with ca. 80% of the radioactivity excreted within 48 h (Table III). There is little or no difference in the distribution of radioactivity in the excreta of animals treated with either of the two labeled compounds and there is very little decarboxylation, as evident by limited $^{14}CO_2$ evolution from rats treated with carbonyl-labeled material. Urine contains most of the radiocarbon, though fecal excretion is significant. The urinary metabolites are glucuronide conjugates of CA, (CH_2OH)-CA, (CHO)-CA, and (COOH)-CA plus small amounts of unconjugated (CH_2OH)-CA. The feces contain (COOH)-CA and three unidentified conjugates (8, 6, and 2% of the administered dose) (Table III). The latter conjugates are more polar than the glucuronides: they migrate only in the BAW system and extract poorly into ether–ethanol (2:1), even at pH 1; they are not efficiently methylated by diazomethane and bind tenaciously to silica gel.

Table III. Metabolites Excreted in the Urine and Feces of Rats Treated Intraperitoneally with (1*R*,*trans*)-[*2,2-dimethyl*-^{14}C]Chrysanthemic Acid at 1 mg/kg

metabolite[a]	recovery of administered radiocarbon, %[a]
Urine (54%)	
CA-Glc[b]	12
(CH_2OH)-CA[c]	14
(CH_2OH)-CA-Glc[b,c]	14
(CHO)-CA-Glc[b,c]	9
(COOH)-CA-Glc[b,c]	5
Feces (30%)	
(COOH)-CA	14
unidentified conjugates	16

[a] Average of results with two rats at 48 h after treatment with little further excretion after the first 24 h. Average percent excretion values for two rats receiving 1 mg/kg (1*R*,*trans*)-[*carbonyl*-^{14}C]CA are 56% in urine, 22% in feces, and 0.6% as $^{14}CO_2$. [b] Cleaved by β-glucuronidase to liberate the designated aglucon. [c] The TLC method could not distinguish *E* or *Z* oxidized products.

Table IV. Biliary Excretion in Rats Treated Intraperitoneally or Intravenously with ^{14}C Preparations of Chrysanthemic Acid or Its Dichlorovinyl Analogue at 1 mg/kg

^{14}C compound administered	recover of administered radiocarbon, %[a]
ip Treatment, 0–24- or 0–48-h Bile	
(1*R*,*trans*)-CA	72, 79[b]
iv Treatment, 0–2-h Bile	
(1*RS*,*trans*)-CA	73, 76, 95[c]
(1*R*,*cis*)-CA	82
(1*RS*,*trans*)-Cl_2CA	95

[a] Values are for individual rats. [b] One rat at 24 and one at 48 h. Biliary and urinary metabolites totaled 89–94% of the administered radiocarbon and less than 1% was excreted in the feces. [c] Urinary bladder contents contained 5–8% of the administered radiocarbon at 2 h.

Biliary Excretion and Biliary Metabolites of Chrysanthemic Acid Isomers and a Dichlorovinyl Analogue. Biliary excretion is the major route for elimination of ip- and iv-administered 1*R*,trans, 1*RS*,trans, and 1*R*,cis isomers of CA and of (1*RS*,*trans*)-Cl_2CA or their metabolites (Table IV). TLC examination of the bile both directly and following β-glucuronidase treatment revealed that all of the radioactivity is attributable to conjugates of CA and its oxidized derivatives. Most of the conjugates are cleaved by β-glucuronidase and the remainder chromatograph in a similar manner to the unidentified fecal metabolites.

^{13}C NMR examination of bile from rats receiving iv-administered (1*RS*,*trans*)-[^{13}C]CA revealed signals with chemical shifts identical with those of the parent compound and the four trans metabolites, i.e., [(*E*)-CH_2OH]- and [(*Z*)-CH_2OH]-CA, [(*E*)-CHO]-CA, and [(*E*)-COOH]-CA (Table II; Figure 4). These compounds were known to be glucuronide conjugates based on the ^{14}C studies. The site of conjugation must be the cyclopropanecarboxylate group since the chemical shifts of the allylic carbon atoms are identical with those of nonconjugated derivatives and these ^{13}C shifts are known (Table I) to be sensitive to derivatization in the side chain but not at the 1-carboxyl. Thus, methylation of the CA carboxyl group has no effect on the hydroxymethylene chemical shifts whereas modification of the alcohol function significantly changes these signals (Table I).

Metabolism of Methyl *trans*-[^{13}C]Chrysanthemate in a Lepidopterous Larva. Exploratory studies in which lepidopterous larvae were fed with [α-^{13}C]benzyl alcohol and examined directly 2 h later revealed ^{13}C NMR signals for alcohol and carboxylic acid products, but they were extremely broad and required long (~16-h) data acquisition times. An extract of the 24-h excreta showed similar products, but the signals were much sharper and the intensity indicated that most of the ^{13}C label was in the excreta. Further experiments were therefore limited to examination of material in more homogeneous media.

Excretion is an important route for elimination of metabolites of orally administered *trans*-CA-CH_3. ^{13}C NMR examination (20-h acquisition time) of buffer extracts of excreta collected within 24 h after treatment showed no starting material and only a single product that appeared to contain a $-CH_2-O-$ group in addition to a $-CH_3$ substituent but with different chemical shifts to any previously encountered (Figure 3). Treatment of this mixture with β-glucosidase caused a movement of the resonances to correspond with those of [(*E*)-CH_2OH]-CA-CH_3 (Table I; Figure 3). Excreta collected in the second 24-h period after treatment showed no metabolites.

DISCUSSION

^{13}C NMR examination has both advantages and disadvantages in studying metabolic processes (Scott and Baxter, 1981). This noninvasive approach avoids potential metabolite volatilization and alteration inherent in chromatographic workup and analysis by some other methods. However, sensitivity and solubility are limiting factors, and metabolite identifications are restricted to changes occurring at sites close to the labeled portion of the molecule. In the present study, the labeled allylic methyl groups are readily oxidized to various levels and each potential product considered gives a unique, readily assignable ^{13}C NMR chemical shift. Like all other metabolic probes, the method relies on the availability of synthetic standards. Caution is required in metabolite quantitation based on relative peak intensities from integration of ^{13}C resonances obtained using gated broad-banded decoupling because of varying nuclear Overhauser enhancements for carbons with or without attached hydrogens and differing T_1 (spin–lattice) relaxation times. However, with similar functionalities—in this case methyl groups—the enhancement is identical, and T_1 values are expected to be similar though not identical. Therefore, quantitation in the present metabolism experiments was by comparison of the relative integrals of the ^{13}C resonances in either the methyl or hydroxymethyl substituents. Support of this method came from the finding that the integration ratios of the vinyl proton resonances in an isomeric mixture of [(*E*/*Z*)-CH_2OH]-CA-CH_3 were identical with those for the methyl and hydroxymethyl ^{13}C resonances, indicating ^{13}C relaxation to be complete.

The metabolic fate of the isobutenyl group is shown in Figure 3 and is fully consistent with earlier studies. Hydroxylation of methyl groups by rat microsomes occurs with *trans*-CA-CH_3 but not with *trans*-CA, and in the former case the product ratios are not significantly changed by the esterase inhibitor TEPP, indicating that hydroxylation occurs more readily in the esters than in the free acid. Rat biliary metabolites of CA involve approximately equal amounts of oxidation at the (*E*)- and (*Z*)-methyl groups, showing that the specificity encountered with the in vitro oxidase system is the same as that seen in vivo. Mouse microsomes are much more specific than rat microsomes in selectively oxidizing the (*E*)-methyl substituent of CA-CH_3, a species specificity also recognized with

the chrysanthemate insecticide resmethrin (Ueda et al., 1975b; Soderlund and Casida, 1977). The present study with (1*RS,trans*)-chrysanthemates does not evaluate the possibility of selective *E* or *Z* hydroxylation for the 1*R*,-*trans* vs. the 1*S*,*trans* isomer (Ueda et al., 1975a,b; Soderlund and Casida, 1977). It is also inadequate to rule out small amounts of epimerization at the C-3 position as either a minor metabolic pathway (Ueda et al., 1975a,b) or an artifact of photodecomposition (Ruzo, 1982); due to the relative low signal/noise achieved, the presence of 5–10% isomerization would not be evident by the procedures used here.

A high degree of stereospecificity is evident in further oxidation of the initial hydroxymethyl compounds, both in vitro and in vivo in rats. The (*E*)-CH_2OH compound undergoes rapid oxidation relative to the (*Z*)-CH_2OH isomer. By analogy with the NMR observations on the standard compounds, this may be due to the CH_2 protons in the (*Z*)-CH_2OH isomer being less accessible to the oxidase than in the (*E*)-CH_2OH isomer.

Conjugation is a major factor determining the distribution and excretion of CA and CA-CH_3 metabolites. ^{13}C NMR readily differentiates whether the site of conjugation is the oxidized isobutenyl substituent or the cyclopropanecarboxylate function. The lepidopterous larvae conjugate the hydroxyl position of [(*E*)-CH_2OH]-CA-CH_3 as the glucoside for excretion. The findings with ^{14}C compounds establish that rats conjugate (1*RS,trans*)-CA and its oxidized derivatives at the cyclopropanecarboxylate group for efficient excretion via the bile. *cis*-CA and *trans*-Cl_2CA are also extensively eliminated in the bile, again presumably as glucuronides. The molecular weight range of these glucuronides is appropriate for biliary excretion (Smith, 1973). Thus, ^{13}C NMR is a useful supplement to other methods in studies on sequential oxidation and conjugation in xenobiotic metabolism.

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Registry No. (1*RS,trans*)-CA-CH_3, 15543-70-1; (1*RS,trans*)-CA, 705-16-8; (1*R,trans*)-CA, 4638-92-0; (1*S,cis*)-CA, 26771-11-9; (1*RS,trans*)-Cl_2CA, 55701-07-0; *trans*-[(*E*)-CH_2OH]-CA-CH_3, 93132-66-2; *cis*-[(*E*)-CH_2OH]-CA-CH_3, 93132-74-2; *trans*-[(*Z*)-CH_2OH]-CA-CH_3, 93132-67-3; *cis*-[(*Z*)-CH_2OH]-CA-CH_3, 93132-75-3; *cis*-[(*E*)-COOH]-CA-CH_3, 93132-68-4; *trans*-[(*E*)-COOH]-CA-CH_3, 93132-76-4; *trans*-[(*E*)-CH_2OH]-CA-CH_3-Glc, 93040-26-7; *cis*-[(*E*)-CH_2OH]-CA-CH_3-Glc, 93132-77-5; *cis*-[(*E*)-CH_2OH]-CA, 54984-66-6; *trans*-[(*E*)-CH_2OH]-CA, 54984-65-5; *cis*-[(*Z*)-CH_2OH]-CA, 54984-67-7; *trans*-[(*Z*)-CH_2OH]-CA, 54984-68-8; *cis*-[(*E*)-CHO]-CA, 93132-69-5; *trans*-[(*E*)-CHO]-CA, 93132-78-6; CA-Glc, 93132-70-8; (CH_2OH)-CA, 93132-71-9; (CHO)-CA-Glc, 93040-27-8; (COOH)-CA, 93132-73-1; (CH_2OH)-CA-Glc, 93132-72-0; (CHO)-CA-Glc, 93132-73-1.

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