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Registry No. 1, 104460-23-3; 2, 104459-75-8; (*E*)-3, 104474-16-0; (*NS*)-3, 104459-85-0; 4, 124482-57-1; 5, 124482-58-2; 3′-(2-chloro-4-(trifluoromethyl)phenoxy]-2-methoxyacetophenone, 104460-45-9; 3-chloro-4-fluorobenzotrifluoride, 78068-85-6; 3′-hydroxy-2-methoxyacetophenone, 54794-31-9.

Metabolism of the Insecticidally Active $GABA_A$ Receptor Antagonist 4-*sec*-[3,4-$^3H_2$]Butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane

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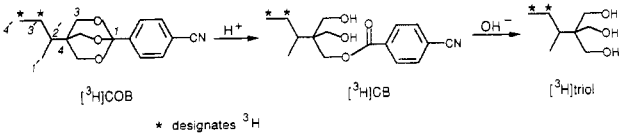
4-*sec*-[3,4-$^3H_2$]Butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane (referred to as [^{3}H]COB) was examined as an example of a new class of insecticidally active compounds that block the γ-aminobutyric acid gated chloride channel. Metabolites were identified by thin-layer cochromatography with standards from synthesis and by consideration of their hydrolytic and oxidative degradation products formed in situ on two-dimensional silica gel chromatoplates. Metabolism of [^{3}H]COB by mouse liver and housefly abdomen microsomes is dependent on fortification with NADPH. The *O*-methylene and *sec*-butyl sites are sensitive to oxidation. Each carbon of the *sec*-butyl group is individually functionalized with strong preference for the methylene site in the mouse but not the housefly microsomal system. *O*-Methylene hydroxylation initiates spontaneous cage opening to form an aldehyde that undergoes metabolic reduction, ultimately yielding the same cyanobenzoate ester of 2,2-bis(hydroxymethyl)-3-methylpentan-1-ol formed by direct hydrolysis. Houseflies injected with [^{3}H]COB form many if not all of the same metabolites, with major products being the aforementioned cyanobenzoate, the orthoester oxidized at the *sec*-butyl methylene site, and polar conjugates.

1,4-Disubstituted 2,6,7-trioxabicyclo[2.2.2]octanes are of interest as in vitro and in vivo probes for the γ-aminobutyric acid (GABA) gated chloride channel (Casida et al., 1985, 1988; Casida and Palmer, 1988) and as a new class of insecticidally active compounds (Palmer and Casida, 1985, 1987). 4-*tert*-Butyl-1-[^{3}H]phenyl-2,6,7-trioxabicyclo[2.2.2]octane (TBOB), used as a radioligand for the $GABA_A$ receptor–ionophore (Lawrence et al., 1985), is metabolized in houseflies, mice, and their microsomal oxidase systems to form cage-opened products and metabolites tentatively identified as involving modifications on both the 1- and 4-substituents (Scott et al., 1987). A newer radioligand probe is 4-*sec*-[3,4-$^3H_2$]-butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane (Nicholson et al., 1988) with enhanced biological activity relative to TBOB, i.e., housefly topical LD_{50} of 45 μg/g alone or 1.1 μg/g on pretreatment with the microsomal oxidase inhibitor piperonyl butoxide (PB), mouse intraperitoneal LD_{50} of 0.56 mg/kg, and 10-fold higher potency at the receptor target (Casida et al., 1988). This cyanoorthobenzoate (COB) undergoes cage opening in acid to form the cyanobenzoate (CB), which is hydrolyzed by base to the triol. This report considers the metabolic



fate of [^{3}H]COB in microsomal oxidase systems from mice and houseflies and in houseflies in vivo. Metabolites were identified by thin-layer chromatography (TLC) involving cochromatography with authentic standards or they were tentatively identified by comparisons of the hydrolytic and oxidative degradation products formed in situ on two-dimensional silica gel chromatoplates.

MATERIALS AND METHODS

Designations. Abbreviations are used as follows: 1′, 2′, 3′, and 4′ = sites undergoing metabolism in the *sec*-butyl group; AB = the aminobenzoate from hydrolysis of 1-(4-amidophenyl)-

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4-*sec*-butyl-2,6,7-trioxabicyclo[2.2.2]octane (amidoorthobenzoate or AOB); COB, CB, and triol as indicated above; hydroxy and keto derivatives defining the position of modification, e.g., 3′-CHOH-COB, 3′-CHOH-CB, and 3′-C=O-CB; MCPBA = *m*-chloroperoxybenzoic acid; MS = mass spectrometry; NMR = nuclear magnetic resonance; PCC = pyridinium chlorochromate; PSCP (used as a general esterase inhibitor) = phenylsaligenin cyclic phosphonate; TBOB = *tert*-butylbicycloorthobenzoate or 4-*tert*-butyl-1-phenyl-2,6,7-trioxabicyclo[2.2.2]octane; TEA = triethylamine.

Chromatography and Spectroscopy. TLC used precoated silica gel 60 F-254 chromatoplates (0.25-mm thickness) and the following solvent systems: HE = hexane–ethyl acetate (1:2) with 0.07% TEA; E = ether with 0.07% TEA; HD = hexane–dioxane (1:1) with 0.07% TEA; ECB = ether–carbon tetrachloride–1-butanol (10:12:1.2) with 0.07% TEA; TEM = toluene–ethyl acetate–methanol (15:5:1) with 0.07% TEA; BAW = 1-butanol–acetic acid–water (6:1:1). The number of developments are indicated, e.g., HE×3 for three developments with HE. Cochromatography involved detection of the authentic standards under UV light (254 nm) or with anisaldehyde–H_2SO_4 spray reagent (Stahl, 1969) and of the ^{3}H compounds by radioautography following spraying of the plates with En^{3}hance (NEN Research Products, Boston, MA). Proton NMR spectra were obtained at 300 MHz with a Bruker WM-300 spectrometer for samples dissolved in deuteriochloroform, unless otherwise specified. MS utilized the Hewlett-Packard 5985 system and chemical ionization at 230 eV with methane (0.8 Torr).

Radiochemicals. [^{3}H]COB was prepared at 27 Ci/mmol with one tritium in each of the 3′- and 4′-positions by reduction of 1-(4-cyanophenyl)-4-(1-methylprop-2-enyl)-2,6,7-trioxabicyclo[2.2.2]octane with tritium gas in ethyl acetate–TEA (25:1) containing 5% platinum on carbon (Ozoe et al., 1982) (NEN Research Products). [^{3}H]CB was obtained by hydrolysis of [^{3}H]COB in dichloromethane containing a trace of 6 N HCl at room temperature for 2.5 h. The desired product was isolated by TLC (HE×1) and characterized by cochromatography with authentic cold material.

Chemicals. COB was prepared from 3-methylpentan-1-ol by the general procedure described for the *tert*-butyl analogue (Casida et al., 1985). The product was obtained as white crystals, mp 133–135 °C. MS: $[M + 1]^+$ m/z 274. NMR: δ 7.71 and 7.64 [each 2 H, AA′BB′, J = 8 Hz, aromatic]; 4.13 [6 H, m, $(CH_2O)_3$]; 1.51 and 1.30 [each 1 H, m, CH_2CH]; 1.00 [1 H, m, CH_2CH]; 0.90 [3 H, d, J = 6 Hz, CH_3CH]; 0.88 [3 H, t, J = 7 Hz, CH_3CH_2]. The butenyl analogue, 1-(4-cyanophenyl)-4-(1-methylprop-2-enyl)-2,6,7-trioxabicyclo[2.2.2]octane, was prepared in a similar manner from 3-methyl-4-penten-1-ol and obtained after workup as white crystals, mp 127–128 °C. MS: $[M + 1]^+$ m/z 272. NMR: δ 7.70 and 7.62 [each 2 H, AA′BB′, J = 8 Hz, aromatic]; 5.60 [1 H, ddd, J = 7, 10, 17 Hz, CH_2=CH]; 5.09 [2 H, m, J = 10, 17 Hz, CH_2=CH]; 4.12 [6 H, s, $(CH_2O)_3$]; 2.15 [1 H, m, J = 7 Hz, CH_3CH]; 1.00 [3 H, d, J = 7 Hz, CH_3CH].

AOB was prepared by treating COB (205 mg) with MnO_2 (2 g) in dry dichloromethane (20 mL) with stirring for 4.5 days (Cook et al., 1966). Fractionation of the reaction mixture on Florisil with chloroform gave the starting material while elution with ethyl acetate yielded AOB (60 mg, 29%), mp 230–234 °C. MS: $[M + 1]^+$ m/z 292. NMR: δ 7.75 and 7.68 [each 2 H, AA′BB′, J = 8 Hz, aromatic]; 6.00 and 5.59 [each 1 H, br s, NH_2]; 4.14 [6 H, m, $(CH_2O)_3$]; 1.51 and 1.30 [each 1 H, m, CH_2CH]; 0.96 [1 H, m, CH_2CH]; 0.92 [3 H, d, J = 6 Hz, CH_3CH]; 0.89 [3 H, t, J = 7 Hz, CH_3CH_2]. The corresponding amidobenzoate (AB) was obtained by hydrolysis of AOB (20 mg) with 6 N HCl (12 μL) and alumina (1 g) in chloroform (5 mL) for 2 h. The alumina was filtered off and the product was isolated by preparative TLC (HE×1). Recrystallization from ether gave off-white crystals (>90% yield), mp 86–87 °C. MS $[M + 1]^+$ m/z 310. NMR (dimethyl-d_6-sulfoxide): δ 8.12 [1 H, br, NH], 8.05 and 8.00 [each 2 H, AA′BB′, aromatic]; 7.55 [1 H, br, NH]; 4.43 and 4.47 [2 H, AB, CH_2OCO]; 3.50 [4 H, AB, 2 × CH_2OH]; 3.00 [2 H, br s, OH]; 1.66 and 1.54 [each 1 H, m, CH_2CH]; 1.10 [1 H, m, CH_2CH]; 0.95 [3 H, d, J = 6 Hz, CH_3CH], 0.85 [3 H, t, J = 7 Hz, CH_3CH_2]. CB was prepared from COB (0.53 g) by hydrolysis in chloroform (60 mL) containing 12 N HCl (0.3 mL) and alumina (10 g) over 5 h. Chromatography of the crude reaction mixture over alumina with chloroform containing 4% methanol gave the product, which was subsequently crystallized from ether–hexane (0.53 g, 95%), mp 75–76 °C. MS: $[M + 1]^+$ m/z 292. NMR: δ 8.11 and 7.74 [each 2 H, AA′BB′, aromatic]; 4.57 and 4.52 [each 1 H, AB, CH_2OCO]; 3.88 and 3.73 [each 2 H, AB, CH_2OH]; 3.24 [br s, OH]; 1.62 [1 H, m, CH_3CH_2]; 1.54 [1 H, m, CH_3CH_2]; 1.05 [1 H, m, CH_2CH]; 0.93 [3 H, d, CH_3CH]; 0.91 [3 H, t, CH_3CH_2]. CB (0.53 g) in dichloromethane (80 mL) with PCC (0.39 g) under N_2 gave, after workup and Florisil chromatography, a mixture, which was fractionated by silica gel column chromatography, using ether–hexane (1:2) and ether–hexane (2:1) as eluting solvents. 3-CHO-CB, as an impure mixture containing the desired diastereomers in an approximately 1:1 molar ratio, was obtained as a white crystalline solid (0.08 g, 15%), mp 118–120 °C. MS: $[M + 1]^+$ m/z 290. NMR: δ 9.74 and 9.73 [each s, diastereomeric CHO]; 8.10 and 7.75 [each 2 H, AA′BB′, aromatic]; 4.80 and 4.65 [2 H, 2 × AB, diastereomeric CH_2OCO]; 4.05 and 3.90 [each 1 H, AB, CH_2OH]; 2.25 [1 H, br, OH]; 1.95 [1 H, m, CH_3CH_2]; 1.65 [1 H, m, CH_3CH_2]; 1.25 [1 H, m, CH_2CH]; 1.10–0.90 [6 H, m, diastereomeric CH_3CH_2 and CH_3CH].

The diastereomeric 3′-CHOH-COBs were synthesized from the butenyl analogue of COB via the epoxides. Thus MCPBA (52 mg) was added in small portions over a 5-min period at 0 °C to a stirred solution of the olefin (54 mg) in dichloromethane (10 mL) (Fieser and Fieser, 1967). The reaction mixture was maintained at room temperature with stirring, and further quantities of MCPBA (52 mg at each addition) were added in small amounts as above after 8, 24, 32, and 48 h. The reaction was maintained for a further 6 h after the last addition and then washed with saturated $NaHSO_3$ solution and water. After normal workup, the mixture of diastereomeric epoxides in an approximately 2:1 molar ratio (30 mg) was isolated by TLC (dichloromethane containing 4% acetone) in 56% yield. NMR: δ 7.65 and 7.60 [each 2 H, 2 × AA′BB′, J = 8 Hz, aromatic]; 4.20 [AB, J = 2 Hz, minor diastereomer $(CH_2O)_2$]; 4.17 [s, major diastereomer $(CH_2O)_3$]; 2.80–2.75 [2 H, m, epoxy protons]; 2.50 [d of d, J = 3, 6 Hz, major diastereomer, CHO], 2.35 [m, minor diastereomer, CHO]; 1.25 [1 H, m, CH_3CH]; 0.95 [3 H, 2 × d, diastereomeric CH_3CH]. Reduction of the epoxides utilized the general procedure of Brown et al. (1982). Thus $LiBH_4$ (~10 mg) was added to the epoxides (15 mg) in dry ether (15 mL) under N_2 with stirring. A further portion of $LiBH_4$ (~30 mg) was added and the mixture heated to reflux for 2.5 h. After cooling to room temperature, the reaction was quenched with dilute NaOH solution, with cooling as necessary followed by workup. Fractionation of the crude mixture by TLC (HE×1) gave the isomeric alcohols. Isomer A: (less polar) (5.0 mg, 33%) mp 172–175 °C. MS: $[M + 1]^+$ m/z 290. NMR: δ 7.70 and 7.61 [each 2 H, AA′BB′, J = 8 Hz, aromatic]; 4.25 [6 H, m, $(CH_2O)_3$]; 4.05 [1 H, br q, J = 7 Hz, $CHOH$]; 1.53 [br, OH]; 1.35 [1 H, m, CH_3CH]; 1.20 [3 H, d, J = 6 Hz, CH_3CHOH]; 0.91 [3 H, d, J = 7 Hz, CH_3CH]. Isomer B: (more polar) (3.3 mg, 22%) mp 163–166 °C. MS: $[M + 1]^+$ m/z 290. NMR δ 7.70 and 7.61 [each 2 H, AA′BB′, J = 8 Hz, aromatic]; 4.25 [6 H, m, $(CH_2O)_3$]; 3.75 [1 H, m, J = 6 Hz, $CHOH$]; 1.60 [br s, OH], 1.51 [1 H, m, J = 7 Hz, CH_3CH]; 1.21 [3 H, d, J = 6 Hz, CH_3CHOH]; 0.85 [3 H, d, J = 7 Hz, CH_3CH].

Microsomal Metabolism. Mouse liver and housefly abdomen microsomes were prepared by the procedure of Abernathy et al. (1973). Liver microsomes were stored at −70 °C and fly microsomes were used immediately after preparation. Mixtures containing [^{3}H]COB (0.04 nmol, ~1 μCi), microsomal protein (Bradford, 1976) (1 mg), and NADPH (0 or 2 μmol) in phosphate buffer (0.1 M, pH 7.4, 1.1 mL) were incubated at 37 (mouse) or 31 °C (housefly) for 1 or 2 h. Similar incubations with [^{3}H]CB (0.07 nmol, ~2 μCi) were carried out with and without added PSCP (10 nmol). The reactions were terminated by adding NaCl (0.2 g) and extraction with ether (4 mL × 2). The combined ether extracts were dried over anhydrous Na_2SO_4 and concentrated under N_2.

Metabolite mixtures were analyzed by TLC either directly or after chemical modification. Direct analysis involved one-dimensional development and cochromatography with authentic standards using three solvent systems, HE×3, E×2, and HD×2.

Table I. Metabolites of 4-*sec*-[3,4-$^3H_2$]Butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane in Mouse Liver and Housefly Abdomen Microsomal Oxidase Systems and in Houseflies

	metabolite recovery,[a] %		
	microsomes + NADPH		
compd or metabolite	mouse	housefly	housefly in vivo
COB	14.6 ± 2.1 (89.8)[b]	41.2 ± 0.4 (88.3)[b]	85.0 ± 0.2 (74.5)[c]
unknown A	1.0 ± 0.1	0.4 ± 0.1 (0.4)	<0.2 (<0.2)
3-CHO-CB	0.8 ± 0.0	0.4 ± 0.0 (0.4)	0.7 ± 0.1 (0.4)
3′-C=O-COB	23.4 ± 0.6 (3.2)	5.3 ± 0.1 (3.5)	3.9 ± 1.2 (2.5)
2′-COH-COB	6.7 ± 0.7	9.2 ± 0.1 (0.6)	0.6 ± 0.4 (0.3)
3′-CHOH-COB	12.5 ± 0.8	4.5 ± 0.1	0.6 ± 0.1 (0.5)
3′-C=O-CB	11.7 ± 0.4	4.4 ± 0.1	0.8 ± 0.1 (0.6)
CB	0.8 ± 0.0	1.8 ● 0.3 (1.3)	3.2 ● 1.9 (12.5)
1′-CH_2OH-COB	7.6 ± 0.4	9.9 ± 0.2 (1.0)	1.4 ± 0.1 (1.9)
4′-CH_2OH-COB	10.0 ● 1.1	14.7 ± 0.7 (1.5)	1.6 ± 1.0 (2.6)
unknown B	0.8 ± 0.2	<0.2	<0.2 (<0.2)
2′-COH-triol	<0.2	1.8 ± 0.1	0.5 ± 0.1 (0.7)
3′-CHOH-triol	3.4 ± 0.3	1.5 ± 0.1	<0.2 (<0.2)
4′-CH_2OH-CB	2.2 ± 0.3	1.1 ● 0.1	<0.2 (1.2)
1′-CH_2OH-CB	<0.2	1.1 ● 0.6	<0.2 (<0.2)
origin	2.7 ± 0.2 (3.2)	1.5 ● 0.6 (1.6)	0.8 ± 0.0 (1.0)
background	2.6 ± 0.3 (3.8)	1.2 ± 0.1 (1.4)	0.9 ± 0.0 (1.3)

[a] Yields are corrected for tritium loss from metabolic attack at the labeled positions as indicated in the text. The mean and standard deviation with microsomes are for six replicates involving half of the incubations in each case for 1 h and the other half for 2 h with slightly more metabolism at the longer time. The mean and standard deviation with houseflies are from four replicates. [b] Values in parentheses are for incubations without NADPH as mean of three replicates for mouse liver and two replicates for housefly abdomens. Values without NADPH are <0.2 except as shown. [c] Values are for 1 h after injection followed in parentheses by average of two replicates for 2 h after injection.

For two-dimensional analyses, the first solvent was HE×2 followed by either TEM×2 (for AOB and AB) or ECB×2 (for 3′-CHOH-COB). Modification by diazomethane treatment involved metabolite mixtures from mouse liver microsomes redissolved in methanol (0.5 mL) to which was added excess freshly generated diazomethane. After 12 h the solvent was evaporated and the products were analyzed by TLC (HE×3). The metabolites from mouse microsomes were also analyzed following in situ modification on the silica gel chromatoplates. Following development in the first dimension with HE×3, the plate was placed in a hood and after 4 days developed in the second dimension with the same solvent system. Alternatively, after development in the first dimension (HE×3), the plate was partially developed in the second dimension with toluene–acetic acid (4:1) to the point that the solvent covered the metabolite positions. The plate was dried for 10 min and the partial development procedure repeated twice. The plate was subsequently dried for 1 h and then developed fully (HE×3). In each case the chromatograms were analyzed by radioautography.

In Vivo Metabolism in Houseflies. Ten adult female houseflies (*Musca domestica* L., ~20 mg each) were individually injected (thorax) with dimethyl sulfoxide (0.2 μL) containing [^{3}H]COB (0.001 μg, ~0.1 μCi). One or two hours after injection, they were homogenized in acetone (3 mL) with a Polytron homogenizer. Following centrifugation, the supernatant was retained and the pellet reextracted with acetone. The supernatants were combined and cooled (dry ice–acetone), and the precipitates were filtered off. The clear solution was concentrated under N_2 and analyzed by TLC (HE×3). For analysis of excretion products, the same injection procedure was followed and the flies were held in a 25-mL beaker and supplied with water. The dose injected caused no discernible poisoning signs. After 48 h, the flies were homogenized in acetone, and the feces were washed from the beaker and extracted 3 times with methanol. After concentration under N_2, both extracts were analyzed by TLC (BAW×1).

Quantitation of Metabolites. Metabolite mixtures of COB were chromatographed (HE×3), the chromatoplates radioautographed, and the individual compounds quantitated by scraping the appropriate silica gel regions directly into scintillation vials for liquid scintillation counting. Metabolite yields were corrected for tritium loss from functionalization of the 3′- and 4′-positions of the 3′-CHOH, 3′-C=O, and 4′-CH_2OH derivatives assuming no isotope effect or further tritium exchange.

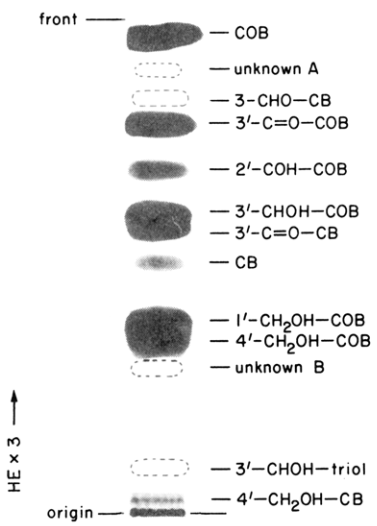

Figure 1. Metabolites of 4-*sec*-[3,4-$^3H_2$]butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane ([^{3}H]COB) formed on incubation with mouse liver microsomes and NADPH for 1 h and analyzed by extraction into ether, TLC (HE×3), and radioautography.

RESULTS

Metabolism of [^{3}H]COB and [^{3}H]CB in Mouse Liver and Housefly Abdomen Microsome Systems. [^{3}H]COB undergoes very little metabolism on incubation for 1 h with mouse liver or housefly abdomen microsomes in the absence of NADPH (Table I). In the presence of NADPH there was extensive metabolism by both sources of microsomes, and more than 10 metabolites are formed (Table I, Figure 1). Most of the metabolites of [^{3}H]COB therefore originate from oxidative metabolism.

[^{3}H]CB is metabolized by mouse liver microsomes, alone or with NADPH, almost exclusively to a single more polar metabolite, probably the corresponding triol (Figure 2). Addition of PSCP led primarily to recovery of starting material and a trace amount of triol, whereas PSCP plus NADPH gave small levels of triol and 3-CHO-CB. These

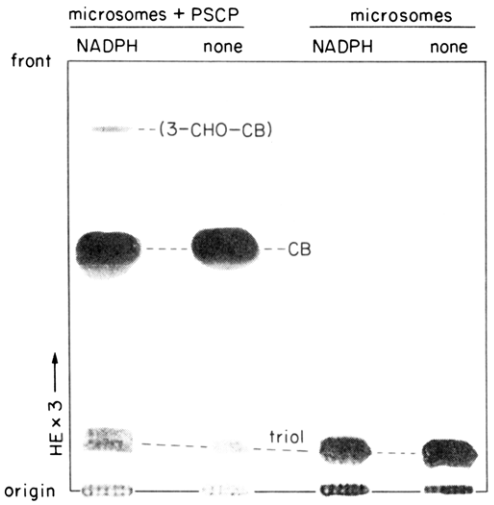

Figure 2. Metabolites of the cyanobenzoate [^{3}H]CB (from hydrolysis of [^{3}H]COB) formed on incubation with mouse liver microsomes alone and with NADPH and/or PSCP for 1 h and analyzed by extraction into ether, TLC (HE×3), and radioautography.

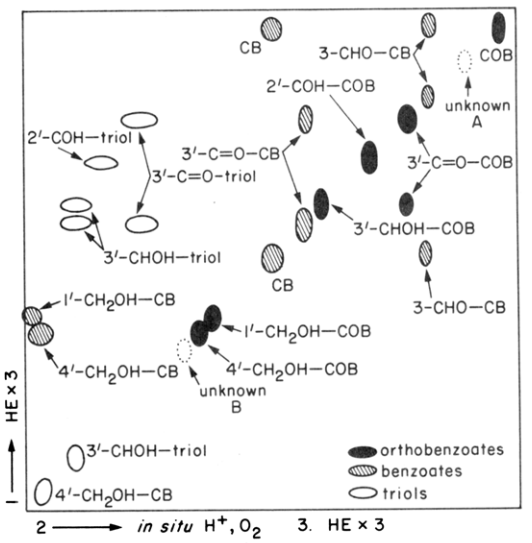

Figure 3. Metabolites of 4-*sec*-[3,4-$^3H_2$]butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane ([^{3}H]COB) formed on incubation with mouse liver microsomes and NADPH for 1 h and analyzed by extraction into ether, TLC in the first dimension (HE×3), holding the chromatoplates 4 days at 25 °C, TLC in the second dimension (HE×3), and radioautography.

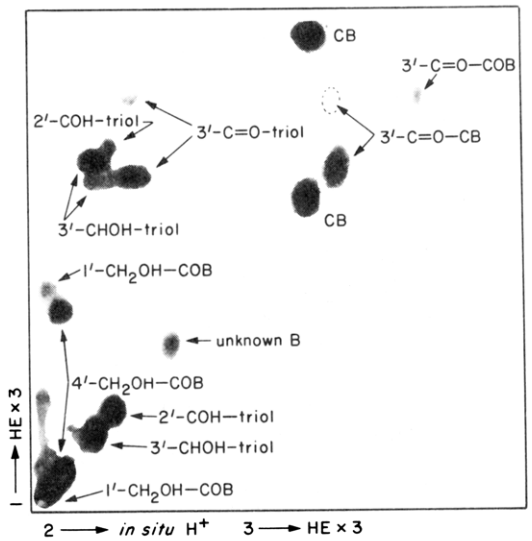

Figure 4. Metabolites of 4-*sec*-[3,4-$^3H_2$]butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane ([^{3}H]COB) formed on incubation with mouse liver microsomes and NADPH for 1 h and analyzed by extraction into ether, TLC in the first dimension (HE×3), TLC in the second dimension (three partial developments with toluene–acetic acid (4:1), drying, and then full development with HE×3), and radioautography.

results suggest a principally hydrolytic rather than oxidative mechanism for conversion of CB to triol. The small amount of triol formation with PSCP present may result from chemical hydrolysis or esterase-catalyzed hydrolysis not completely inhibited by PSCP.

Mouse Microsomal Oxidase Metabolites Identified by Cochromatography with Standards. In addition to starting material ([^{3}H]COB), three metabolites cochromatographed with authentic standards and were thus identified as 3-CHO-CB, 3′-CHOH-COB, and CB (Table II). No metabolite cochromatographed with AOB or AB in any of the solvent systems investigated. Treatment of the metabolite mixtures with diazomethane did not lead to any alteration in their TLC patterns, thereby ruling against the presence of carboxylic acid or phenol derivatives in the extracts.

Mouse Microsomal Oxidase Metabolites Tentatively Assigned by Their Hydrolysis and Oxidation Products. Several metabolites in incubation mixtures are tentatively assigned following their hydrolysis or oxidation effected in situ on silica gel chromatoplates (Figures 3 and 4). In these chromatograms, which were developed fully in both directions with the same solvent system (HE×3), any compound not falling along the diagonal axis represents a nonmetabolic alteration product. Under these conditions, COB decomposes to CB and 3-CHO-CB and CB itself oxidizes to 3-CHO-CB. These assignments are based on cochromatography with authentic standards. Similar experiments indicated below are used for tentative identification of other metabolites in the absence of synthetic standards.

3′-C=O-COB is tentatively assigned based on three observations. First, the cage structure is intact since it shows the acid lability characteristic of the orthobenzoates (Figure 4; complete hydrolysis is observed in other experiments over a longer period of time). Second, 3′-CHOH-COB, previously assigned by cochromatography with authentic material, slowly oxidizes to a less polar compound, with the same R_f as for 3′-C=O-COB, as anticipated under the mild oxidative conditions of the experiment. Finally, the metabolite is more polar than COB but less polar than 3′-CHOH-COB, as expected for the keto compound. 3′-C=O-COB is the major metabolite in mouse microsomes, i.e., 23% yield.

3′-C=O-CB, the hydrolysis product of 3′-C=O-COB, present in 12% yield, has the following properties consistent with its assignment: acid insensitivity indicating the absence of the orthobenzoate structure; acid treatment of 3′-C=O-COB generates a product with the same R_f value as this metabolite, and both of these compounds undergo further reaction to the same more polar derivative, presumably 3′-C=O-triol (Figures 3 and 4).

Three metabolites, designated 1′-CH_2OH-COB, 2′-COH-COB, and 4′-CH_2OH-COB, are assignable as orthobenzoates, based on their acid lability. The metabolite designated 2′-COH-COB is more polar than 3′-C=O-COB and less polar than 3′-CHOH-COB; it is further decomposed to a product that is more polar than 3′-C=O-triol and less polar than 3′-CHOH-triol (Figures 3 and 4). Metabolites referred to as 1′- and 4′-CH_2OH-COB are

Table II. TLC Properties of 4-*sec*-Butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane (COB) and Some of Its Derivatives and Metabolites

compd	R_f values in indicated solvent systems					
	HE×3	E×2	HD×2	HE×2[a]	ECB×2[b]	TEM×2[b]
COB[c]	0.97	0.83	0.84	0.78	0.85	0.83
unknown A	0.90	0.74	0.76	0.70	0.76	0.78
3-CHO-CB[c]	0.83	0.68	0.73	0.65	0.69	0.63
3′-C=O-COB[d]	0.79	0.60	0.71	0.60	0.63	0.61
2′-COH-COB[d]	0.70	0.57	0.66	0.51	0.59	0.57
3′-CHOH-COB[c]	0.61	0.49	0.64	0.45	0.52	0.52
3′-C=O-CB[d]	0.58	0.47	0.63	0.43	0.48	0.50
CB[c]	0.51	0.41	0.60	0.41	0.40	0.48
1′-CH_2OH-COB[d]	0.39	0.30	0.50	0.27	0.33	0.40
4′-CH_2OH-COB[d]	0.36	0.23	0.49	0.25	0.29	0.38
unknown B	0.34					
AOB[e]				0.24		0.28
3′-CHOH-triol[d]	0.10	0.11	0.30	0.09	0.12	0.18
4′-CH_2OH-CB[d]	0.03	0.03	0.18	0.05	0.06	0.12
AB[e]				0.03		0.07

[a] Solvent system used for the first dimension of all 2D cochromatography. [b] Alternative solvent systems used for the second dimension of 2D cochromatography. [c] [^{3}H]COB and its metabolites identified by cochromatography with authentic standards. [d] Metabolites tentatively identified by their oxidation or hydrolysis products generated on exposure of the chromatoplates to air or acid on silica gel. [e] Not detected as metabolites.

Table III. Effect of Site of *sec*-Butyl Oxidation on R_f Values of 4-*sec*-Butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo-[2.2.2]octane (COB), 3-*sec*-Butylphenol (BP), and 3-*sec*-Butylphenyl Methylcarbamate (BPMC)

substituent	R_f or relative R_f		
	COB[a]	BP[b]	BPMC[b]
none	0.97	0.97	0.97
3-C=O	0.79	0.74	0.56
2′-COH	0.70	0.54	0.37
3′-CHOH	0.61	0.43	0.22
1′-CH_2OH	0.39	0.40	0.17
4′-CH_2OH	0.36	0.29	0.17

[a] R_f with HE×3. [b] Relative R_f with benzene–ether (1:1), normalizing the R_f of BP (0.56) and BPMC (0.45) to 0.97 and the other products proportionately. The same R_f order is observed with ether–hexane (2:1), benzene–ether (10:3), benzene–ether–hexane (1:1:1), and hexane–ether (2:1) (Cheng and Casida, 1973).

assigned, on a tentative basis, by their relative R_f values, in comparison with authentic *sec*-butyl derivatives in two different series (Cheng and Casida, 1973; Table III). However, the possibility that either or both of these metabolites may be dihydroxy-COB derivatives cannot be unequivocally excluded.

1′- and 4′-CH_2OH-CB are designated as such based on their acid stability, relative polarity, and formation on decomposition of 1′- and 4′-CH_2OH-COB, respectively.

3′-CHOH-triol is tentatively assigned by comparison with the decomposition products of 3′-CHOH-COB and 3′-C=O-CB. 2′-COH-triol is so designated because it occurs as a metabolite of COB when incubation is prolonged to 2 h, it appears as the more polar of two products on decomposition of 2′-COH-COB (the trace intermediate although not shown in Figure 3 is presumably 2′-COH-CB), and its polarity falls between that of 3′-C=O-triol and 3′-COH-triol, a result consistent with 2′-COH-triol.

Housefly Microsomal Oxidase Metabolites. The metabolites detected with housefly microsomes are the same as those found with mouse microsomes although there are differences in relative proportions (Table I, Figure 5).

Housefly Metabolites. CB is the major metabolite of COB in houseflies. This shift in the pattern of metabolites represents the only noticeable difference with respect to the other studies (Table I, Figure 5). When the injected flies were held 48 h, most of the ^{3}H was excreted in feces

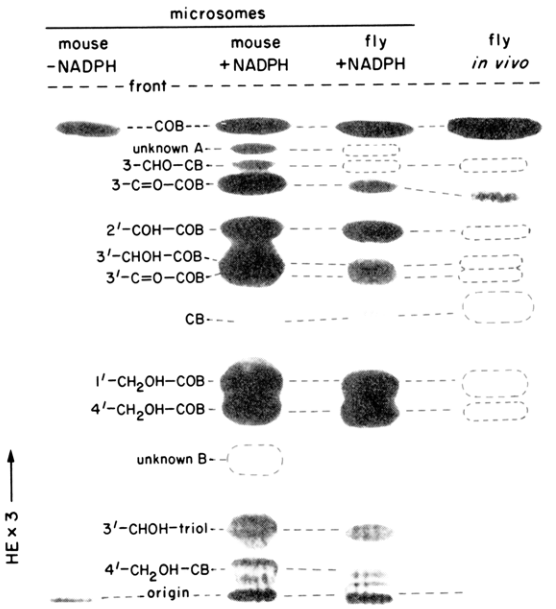

Figure 5. Metabolites of 4-*sec*-[3,4-$^3H_2$]butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane ([^{3}H]COB) formed on incubation with mouse liver microsomes alone, mouse liver microsomes with NADPH, and housefly microsomes with NADPH and on injection into living houseflies. Analyses involved extraction into ether, TLC (HE×3), and radioautography. The dotted lines represent minor metabolites not adequately represented in the figure but clearly evident by liquid scintillation counting of the appropriate gel regions.

(74% of the administered dose), and in the fecal extract 16 metabolites are discernible by TLC (BAW×1). These polar metabolites are not obtained in the in vitro studies and are considered to be conjugates. No attempts were made to further characterize these materials.

DISCUSSION

Figure 6 summarizes current knowledge on the metabolic pathways of COB. Although the scheme is based primarily on the organoextractable metabolites from the mouse liver microsomal oxidase system, it appears to also be applicable at least in part to the fly microsomal oxidase system and flies in vivo (compare MO, FO, and F in Figure 6). The major metabolic process is hydroxyla-

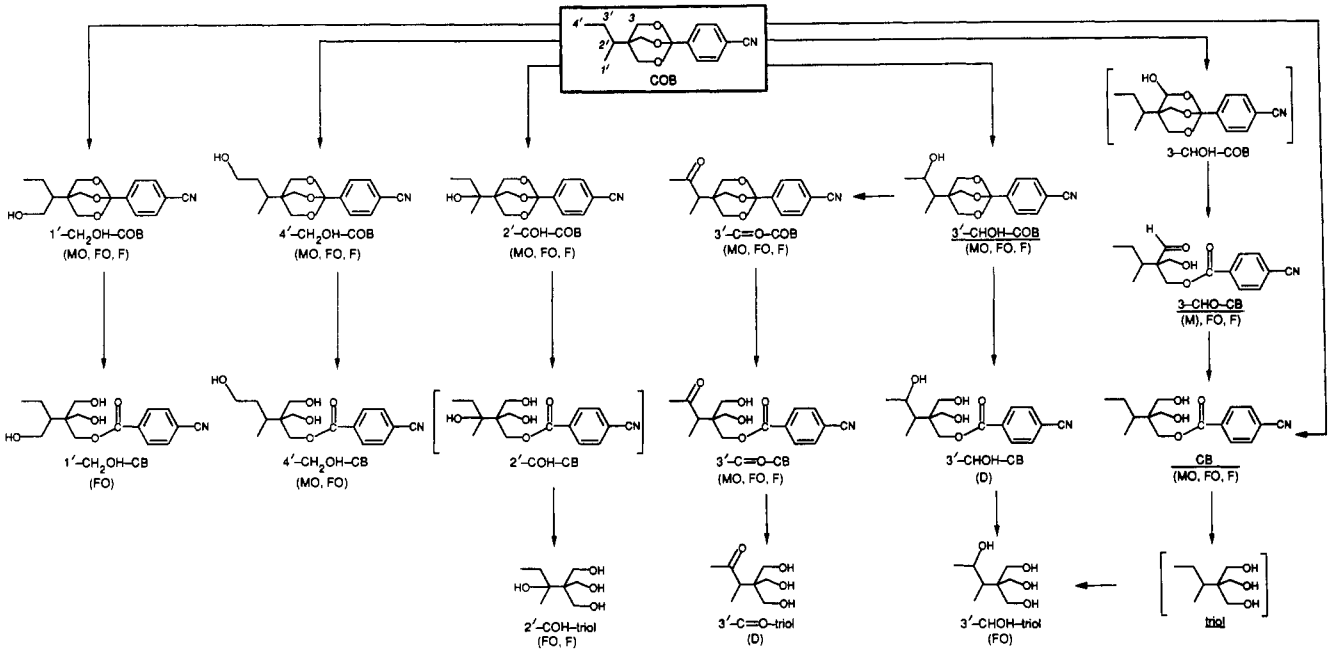

Figure 6. Metabolic pathways for 4-*sec*-butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane in the mouse liver and housefly abdomen microsome–NADPH systems indicating metabolites formed in the microsomal oxidase (MO) and housefly oxidase (FO) systems and in houseflies in vivo (F) and products formed on further decomposition (D). Compounds with brackets are expected intermediates. Compounds with designations underlined are identified by cochromatography.

Table IV. Site Specificity in Metabolism of 4-*sec*-[3,4-$^3H_2$]-Butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo[2.2.2]octane in Mouse Liver and Housefly Abdomen Microsomal Oxidase Systems and in Houseflies

	metabolism at indicated site,[a] %				
	sec-butyl				bicyclic
system	1′	2′	3′	4′	3
microsomes + NADPH					
mouse	8	7	51	12	19
housefly	11	11	16	16	12
housefly in vivo	1.4	1.1	5.3	1.6	5

[a] Summation of all metabolites involving indicated site of attack including COB, CB, and triol derivatives.

tion, which occurs at the 3-position of the bicyclic ring or at any position of the *sec*-butyl group.

O-Methylene hydroxylation initiates the ring-opening reaction (see also Scott et al., 1987) by forming 3-CHOH-COB, which decomposes spontaneously to 3-CHO-CB, a portion of which is reduced to CB. The end result is the same as hydrolysis of COB to CB but direct hydrolysis is not involved because NADPH is required.

The site specificity in metabolism of [^{3}H]COB is shown in Table IV. For mouse liver microsomes hydroxylation at the C-3′ position is favored, accounting for 51% of the recovered metabolites, with little preference for the other three positions. With fly abdomen microsomes, hydroxylation involves little specificity for various positions in the *sec*-butyl moiety. Since a number of polar metabolites are noted, it is plausible that some of these may represent multiple hydroxylation of the *sec*-butyl group.

3′-CHOH-COB undergoes further oxidation to 3′-C=O-COB, but in contrast, no evidence is available for oxidation of primary alcohol metabolites to the corresponding aldehydes or carboxylic acids. The initially formed hydroxy and keto derivatives of the orthobenzoates are labile. This leads to 3′-C=O-CB as a major metabolite.

The cyanobenzoates, once generated, are further hydrolyzed to give more polar compounds. It appears that esterases quickly hydrolyze CB to the triol, which in turn is rapidly converted to other metabolites. CB does not undergo hydroxylation reactions unless the esterases are inhibited and the incubation mixtures are fortified with NADPH. In the present studies there is no evidence to suggest any hydroxylation of the aromatic ring or hydrolysis of the nitrile to the amide (i.e., COB → AOB or AB). This is in contrast to TBOB where aromatic hydroxylation is a minor metabolic pathway (Scott et al., 1987).

PB synergizes the housefly toxicity of COB by 41-fold versus 21-fold for the corresponding *tert*-butyl compound (Casida et al., 1988; Palmer and Casida, 1985), a result consistent with the *sec*-butyl group offering several sites for metabolic detoxification. Enhanced insecticidal activity may be achieved therefore by introducing appropriate 4-substituents which are less sensitive to oxidative detoxification.

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Registry No. COB, 125172-14-7; 3-CHO-CB (isomer 1), 125172-15-8; 3-CHO-CB (isomer 2), 125196-92-1; 3′-C=O-COB, 125172-16-9; 2′-COH-COB, 125172-17-0; 3′-CHOH-COB (isomer 1), 125172-18-1; 3′-CHOH-COB (isomer 2), 125172-29-4; 3′-C=O-CB, 125172-19-2; CB, 125172-20-5; 1′-CH_2OH-COB, 125172-21-6; 4′-CH_2OH-COB, 125172-22-7; 2′-COH-triol, 125172-23-8; 3′-CHOH-triol, 125172-24-9; 4′-CH_2OH-CB, 125172-25-0; 1′-CH_2OH-CB, 125172-26-1; AOB, 125172-27-2; AB, 125172-28-3; NADPH, 53-57-6.

Evaluation of Volatile Compounds on the Germination of Seventeen Species of Weed Seeds

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Seventeen species of weed seeds were evaluated for their germination response to twelve naturally occurring, or related, volatile compounds. Germination of curly dock (*Rumex crispus*) was stimulated in darkness by 0.01 and 0.1 mL of methyl salicylate/10 L of air. Styrene, safrole, 2-cyanopyridine, and 5-methyl-2-hexanone also stimulated the dark germination of curly dock. Red sorrel (*Rumex acetosella*) dark germination was 67% (controls 3.4%) when exposed to styrene vapor. Methyl salicylate and benzyl acetate stimulated johnsongrass (*Sorghum halepense*) germination in darkness and light. Inhibition of germination was the usual response in a majority of tests. Vinylpyridines inhibited germination of most of the species tested.

Several naturally occurring volatile compounds stimulate the germination of some weed seeds (French and Leather, 1979) and also stimulate the germination of rust spores, including rusts of weeds (French et al., 1986). French (1985) reviewed the bioregulatory action of volatile flavor compounds on fungal spores and other propagules such as pine pollen and seeds, citing examples of germination stimulation and inhibition, induction of formative or morphological changes, and enzyme induction by this group of compounds. Recently Bradow and Connick (1988a,b) described the inhibitory activity of some volatile allelochemicals from the residues of *Amaranthus palmeri* on tomato, onion, and carrot seed germination. As part of our continuing effort to study mechanisms of controlling propagule germination, we investigated the effects of 12 volatile compounds on the germination of seeds of 17 weed species. These compounds were selected because of previously demonstrated biological activity in other propagules including morphological changes and stimulation of germination.