Structure–Biodegradability Relationships of Insecticidal 1,4-Disubstituted-2,6,7-trioxabicyclo[2.2.2]octanes†

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Selected 4-alkyl- and 4-aryl-1-(*p*-substituted-phenyl)-2,6,7-trioxabicyclo[2.2.2]octanes are among the most potent insecticides and $GABA_A$ receptor antagonists. The 4-substituents and para substituents determine not only the neurotoxicity but also the biodegradability in the mouse liver microsomal mixed-function oxidase system. Facile metabolism is conferred by 4-butyl (normal, secondary, and cyclo but not tertiary), 4-cyclohexyl, and 4-isopropyl substituents compared to nine other C_3–C_5-alkyl and aryl moieties. The *tert*-butyl substituent undergoes slow hydroxylation and the cyclohexyl moiety very rapid hydroxylation to several isomeric cyclohexanol derivatives. There are rapid conversions of *p*-methylthio to methyl sulfone, *p*-methoxyl to hydroxyl, and *p*-methyl to hydroxymethyl, with slower or no metabolism of 12 other para substituents. The *p*-ethynylphenyl moiety, which confers high insecticidal activity, is oxidatively metabolized not only to the corresponding phenylacetic acid (major) and acetophenone analogues, as expected, but also to the benzoic acid, benzaldehyde, and benzyl alcohol derivatives from oxidative cleavage of the ethynyl substituent.

2,6,7-Trioxabicyclo[2.2.2]octanes (TBOs) with suitable substituents in the 1- and 4-positions are potent insecticides and inhibitors of the γ-aminobutyric acid (GABA) gated chloride channel in mammalian brain (Casida et al., 1985, 1988, 1990; Obata et al., 1988; Palmer and Casida, 1985, 1989). The toxicity of many TBOs to houseflies is synergized by piperonyl butoxide (PB) (Table I), suggesting that they are detoxified by the microsomal mixed-function oxidase (MFO) system; dependent upon substitution there is enormous variation (2- to >263-fold) in the synergistic ratio (Palmer and Casida, 1985, 1987). 4-Cyclohexyl-1-(*p*-chlorophenyl)-2,6,7-trioxabicyclo[2.2.2]octane (4-*c*-Hex-TBO-Ph-Cl) but not its 4-*t*-Bu analogue is significantly synergized by PB in its toxicity to mice (Table I); as expected on this basis, the mouse liver MFO system readily detoxifies the *c*-Hex but not the *t*-Bu analogue relative to their action as inhibitors of the GABA-gated chloride channel (Casida et al., 1985). The MFO systems of mice and houseflies hydroxylate 4-*t*-Bu-TBO-Ph (TBOB) at the phenyl, methyl, and particularly an *O*-methylene substituent (Scott et al., 1987) and 4-*s*-Bu-TBO-Ph-CN at each carbon of the *s*-Bu group in addition to an *O*-methylene site (Deng et al., 1990) (Figure 1).

The toxicity of the TBOs is determined by both their affinity for the nerve binding site and their resistance to biodegradation. Systematic knowledge of structure–biodegradability relationships is therefore important in optimization of substituents for high insecticidal potency, selective toxicity, and suitable persistence. The present study helps to meet this need by examining a large series of TBOs and other $GABA_A$ receptor antagonists in mouse liver MFO systems. It attempts to provide chemical explanations for some of the detoxification mechanisms. Three specific aspects are examined as follows: the ease of metabolism of selected TBOs compared with other types of inhibitors of the GABA-gated chloride channel analyzed by GC-FID; the dependence of structure–biodegradability relationships on the 4-substituent of a series of *p*-iodophenyl-TBOs and on the para substituent of a series of TBOB analogues determined by GC-FID; identification of major metabolites of some of the most biodegradable TBOs by GC/MS techniques. Special attention is given to the metabolic fate of *p*-ethynylphenyl-TBOs because of their remarkably high insecticidal activity (Palmer and Casida, 1989).

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Table I. Mouse Intraperitoneal and Housefly Topical LD_{50} Values and Selectivity Ratios for 4-Cyclohexyl- and 4-*tert*-Butyl-1-(*p*-chlorophenyl)-2,6,7-trioxabicyclo[2.2.2]-octanes with No Synergist or with Piperonyl Butoxide

compd and synergist	LD_{50}, mg/kg mouse	LD_{50}, mg/kg housefly	LD_{50} ratio mouse/housefly
4-*c*-Hex-TBO-Ph-Cl[a]			
no synergist	52	10	5.2
PB	5	0.53	9.4
4-*t*-Bu-TBO-Ph-Cl[a]			
no synergist	1.1	10	0.11
PB	1.1	1.5	~0.7

[a] Data from Casida et al. (1985) and Palmer and Casida (1985).

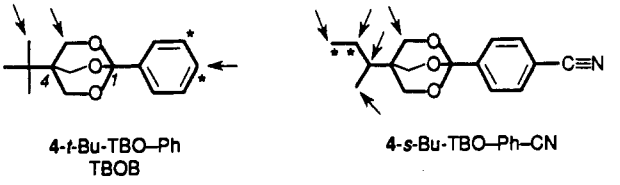

Figure 1. Sites of metabolic attack in mouse liver and housefly microsomal mixed-function oxidase systems for 4-*tert*-butyl-1-phenyl-2,6,7-trioxabicyclo[2.2.2]octane (TBOB) (Scott et al., 1987) and 4-*sec*-butyl-1-(*p*-cyanophenyl)-2,6,7-trioxabicyclo-[2.2.2]octane (Deng et al., 1990).

MATERIALS AND METHODS

Chemicals. Some of the TBOs used are described by Casida et al. (1985) and Palmer and Casida (1985). The *p*-iodophenyl-TBOs were prepared by the general procedure of Palmer and Casida (1989). The remainder either were previously reported (Palmer and Casida, 1989; Palmer et al., 1988) or were synthesized by the appropriate methods described therein.

Microsomal Preparations and Incubations. Microsomes were prepared from a 20% (w/v) homogenate of fresh livers of male albino Swiss-Webster mice in 100 mM, pH 7.4, sodium

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phosphate buffer using 12 complete strokes of a mechanically driven Teflon pestle in a glass mortar. The homogenates were centrifuged at 10000*g* for 20 min, and the supernatant was then centrifuged at 105000*g* for 60 min to obtain the microsomal fraction, which was subjected to one wash by resuspension in fresh buffer and resedimentation in the same manner. Following protein determination (Bradford, 1976), the microsomes were stored frozen at −80 °C for up to 60 days before use.

Standard incubation mixtures consisted of the microsomal protein (normally 0.5 mg but varied from 0.1 to 1.0 mg) and NADPH in 100 mM, pH 7.4, sodium phosphate buffer (1.2 mL) to which was added last the substrate in ethanol or acetone (5 μL) to give final concentrations of 1 mM NADPH and 17 μM substrate. Incubations in 2.5-cm-diameter flat-bottom vials were for 60 min in a water bath at 37 °C with shaking in air followed by cooling on ice. These standard conditions were not varied unless specifically stated otherwise. The same enzyme preparation was used throughout each compound series but different preparations were used in experiments presented in separate tables.

Analysis of Substrate Loss by GC-FID. For quantitation of substrate loss by GC-FID, the incubated mixtures were chilled, fortified with an internal standard (4-*t*-Bu-TBO-Ph-C≡CH or 4-*c*-Hex-TBO-Ph-Cl) added in acetone (5 μL), saturated with NaCl (~0.4 g), and extracted with diethyl ether (1 mL × 2) by vortexing for 10 s and centrifugation for 5 min each time to separate the water, ether, and protein interface. The ether was pipetted off into a conical centrifuge tube and evaporated to dryness under nitrogen, and acetone (200 μL) was added. An aliquot of 5.0 μL was analyzed as indicated below.

Analysis involved a Hewlett-Packard 5840A instrument with a flame ionization detector (FID) and a fused silica capillary column (0.25 mm × 15 m) with 0.25-μm film thickness of SE 54 (5% phenylmethylsiloxane) using helium as the carrier gas (9.2 mL/min, 40 cm/s), an injection port temperature of 150 °C, a detector temperature of 280 °C, and a temperature program of 100, 120, or 160 °C for 3 min and then 10 °C/min up to 300 °C with sample injection in the split mode. Acetone, tetrahydrofuran, and ethanol but not methanol are suitable GC injection solvents on the basis of studies with 4-*n*-Pr-TBO-Ph-I; apparently the 4-*n*-Pr derivative reacts completely with methanol in the injection port to give a product(s) not detected with standard GC-FID conditions. However, methanol is suitable for analysis of more stable compounds with other 4-substituents, e.g., *t*-Bu and Ph. Percentage metabolism was determined by comparing peak areas of the substrate before and after metabolism relative to those of the internal standard. This overall analytical procedure generally gives near quantitative recoveries from unincubated control.

Identification of Metabolites by GC/MS. The microsomal incubations were saturated with NaCl and extracted with ethyl acetate (1 mL × 2), which was evaporated to dryness under nitrogen. Formation of TMS derivatives involved treatment with acetonitrile (150 μL) followed by a 10% acetonitrile solution of *N,O*-bis(trimethylsilyl)acetamide (BSA) (30 μL) (Pierce, Rockford, IL) (0.5 h, 80 °C). Diazomethane and diazoethane were used in the normal manner to convert carboxylic acids to their methyl and ethyl esters, respectively. The derivatized samples were pooled in sets of six and evaporated under nitrogen to ~200 μL for analysis by GC/MS. Metabolites and their derivatives were identified by both GC/CI-MS and GC/EI-MS using the Hewlett-Packard 5985B instrument with conditions and temperature program defined later. Samples (1–2 μL) were injected splitless with an injection port temperature of 250 °C.

RESULTS

Metabolism of Variously Substituted 2,6,7-Trioxabicyclo[2.2.2]octanes, Polychlorocycloalkanes, and Tetramethylenedisulfotetramine by Mouse Liver Microsomal Mixed-Function Oxidases (Table II). Six TBOs were evaluated for ease of microsomal metabolism, which in each case is dependent on NADPH. Compounds lacking aryl moieties differ in their biodegradation with the 1-substituent involved, i.e., *c*-Hex > H. The ease of oxidation of 4-*t*-Bu-TBO-Ph-C≡CH is not altered by substitution of deuterium for the ethynyl hydrogen or by introduction of a cyano substituent in the 3-position. 4-*c*-Hex-TBO-Ph-Cl is the most rapidly metabolized compound in this group.

Table II. Metabolism of Variously Substituted 2,6,7-Trioxabicyclo[2.2.2]octanes, Polychlorocycloalkanes, and Tetramethylenedisulfotetramine by Mouse Liver Microsomal Mixed-Function Oxidases

compd	GC R_t,[a] min	NADPH-dependent metabolism,[b] %
trioxabicyclooctanes		
t-Bu-TBO-H	7.9	18 ± 3
t-Bu-TBO-*c*-Hex	12.2	61 ± 1
t-Bu-(3-CN-TBO)-Ph-C≡CH	11.2	28 ± 1
t-Bu-TBO-Ph-C≡CH[c]	9.5	27 ± 1
t-Bu-TBO-Ph-C≡CD	9.5	26 ± 1
c-Hex-TBO-Ph-Cl[c]	12.5	94 ± 3
polychlorocycloalkanes		
aldrin	7.0	39 ± 1
dieldrin	9.1	0
α-endosulfan	8.6	47 ■ 1
endosulfan sulfate	10.6	0
lindane	8.7	0
other		
tetramethylenedisulfotetramine	8.9	0

[a] SE 54 capillary column with a temperature program of 100 °C (for the first two TBOs) or 120 °C (for lindane and tetramethylenedisulfotetramine) or 160 °C (for the other compounds) for 3 min and then 10 °C/min to 300 °C. [b] Mean ± SE of four separate experiments with NADPH (each consisting of one or two analyses). Little or no substrate loss occurs on incubation with microsomes in the absence of NADPH as compared to unincubated controls. [c] Used as internal standards.

The TBOs considered above are more rapidly metabolized than three polychlorocycloalkanes, i.e., dieldrin, endosulfan sulfate, and lindane. Although extensively degraded, aldrin and α-endosulfan are converted to toxic metabolites (dieldrin and endosulfan sulfate in yields of 25 and 8%, respectively) that are resistant to further oxidation under these conditions. Tetramethylenedisulfotetramine under the standard protocol is very resistant to MFO metabolism.

Effect of 4-Substituent on the Metabolism of 1-(*p*-Iodophenyl)-2,6,7-trioxabicyclo[2.2.2]octanes by Mouse Liver Microsomal Mixed-Function Oxidases (Table III). The effect of the 4-substituent on biodegradation was examined in a series selected for no more than moderate metabolism elsewhere in the molecule, i.e., the TBO-Ph-I analogues as discussed later. The enzyme level used gave a range of 2–94% metabolism for compounds with different 4-substituents. In each case oxidation is implied by the absolute requirement for fortification of the microsomes with NADPH. Among the *n*-alkyl substituents, there appears to be a distinct chain link optimum with the biodegradability sequence of *n*-Bu > *n*-Pen > *n*-Pr. Similarly, in the cycloalkyl series the order of conferring biodegradability is *c*-Bu > *c*-Hex > *c*-Pen > *c*-Pr. Metabolism is more rapid for the *i*-Pr compound than for its *n*-Pr or *c*-Pr analogue. Three of the four butyl substituents examined confer high biodegradability, i.e., *n*-Bu, *s*-Bu, and particularly *c*-Bu but not *t*-Bu. A *trans*-2-methyl substituent enhances biodegradability in the cyclopropyl series as shown by *t*-2-Me-*c*-Pr > *c*-Pr or 1-Me-*c*-Pr. With the 4-phenyl moiety a *p*-F substituent does not substantially alter the metabolism, whereas the *m*-F substituent greatly retards biodegradation.

Effect of Para Substituent on the Metabolism of 4-*tert*-Butyl-1-(*p*-substituted-phenyl)-2,6,7-trioxabicyclo[2.2.2]octanes by Mouse Liver Microso-

Table III. Effect of 4-Substituent on the Metabolism of 4-Substituted-1-(*p*-iodophenyl)-2,6,7-trioxabicyclo[2.2.2]-octanes by Mouse Liver Microsomal Mixed-Function Oxidases

R-TBO-Ph-I R substituent	GC R_t,[a] min	NADPH-dependent metabolism,[b] %
c-Bu	12.5	94 ± 4
n-Bu	11.7	83 ± 4
s-Bu	12.0	76 ± 3
c-Hex	14.9	69 ± 2
i-Pr	10.9	63 ± 2
Ph	14.4	49 ± 2
p-F-Ph	14.2	43 ± 2
c-Pen	13.8	39 ± 2
n-Pen	12.7	33 ± 1
t-2-Me-*c*-Pr	11.5	32 ± 2
n-Pr	10.7	26 ± 1
t-Bu	11.9	12 ± 2
c-Pr	11.2	10 ± 3
1-Me-*c*-Pr	11.9	9 ± 2
m-F-Ph	14.2	2 ± 1

[a] SE 54 capillary column with a temperature program of 160 °C for 3 min and then 10 °C/min to 300 °C. R_t = 13.2 min for 4-*c*-Hex-TBO-Ph-Cl as an internal standard. [b] Mean ± SE of four separate experiments with NADPH (each consisting of one or two analyses). Little or no substrate loss occurs on incubation with microsomes in the absence of NADPH as compared to unincubated controls.

Table IV. Effect of Para Substituent on the Metabolism of 4-*tert*-Butyl-1-(*p*-substituted-phenyl)-2,6,7-trioxabicyclo-[2.2.2]octanes by Mouse Liver Microsomal Mixed-Function Oxidases

t-Bu-TBO-Ph-R R substituent	GC R_t,[a] min	NADPH-dependent metabolism,[b] %
SMe	12.4	98, 97[c]
OMe	10.7	95, 92
Me	9.1	74, 85
C≡CH	10.1	43, 48
H (TBOB)	7.7	16, 19
C≡N	10.8	17, 16
Cl	9.5	14, 14
t-Bu	11.5	10, 13
F	7.3	13, 10
SO_2Me	14.8	11, 11
OCF_3	7.4	8, 12
C≡CMe	12.2	8, 7
CF_3	7.3	4, 5
Br	10.6	2, 3
I	11.9	2, 2

[a] SE 54 capillary column with a temperature program of 160 °C for 3 min and then 10 °C/min to 300 °C. R_t = 13.2 min for 4-*c*-Hex-TBO-Ph-Cl as an internal standard. [b] Values are for two separate experiments with NADPH (each consisting of duplicate analyses). Little or no subtrate loss occurs on incubation with microsomes in the absence of NADPH as compared to unincubated controls. [c] Percentage NADPH-dependent metabolism at varying enzyme levels were 41, 64, and 100% at 0.1, 0.3, and 1.0 mg of protein, respectively; in each case only the SO_2Me metabolite was detected.

mal Mixed-Function Oxidases (Table IV). The nature of the para substituent has a marked influence on the biodegradability of compounds with no more than moderate metabolism at the 4-substituent, i.e., the 4-*t*-Bu-TBO-Ph or TBOB series. With the enzyme level used, the extent of metabolism varied from 98% for the *p*-SMe compound to 2% for the *p*-I analogue. In each case the metabolism is dependent on both microsomes and NADPH and is therefore considered to be mediated by cytochrome P-450. Substituents conferring rapid metabolism are SCH_3 > OCH_3 > CH_3 > C≡CH > H. The halogen-substituted compounds (F, Cl, Br, and I) are among the least biodegraded analogues with 2–14% metabolism.

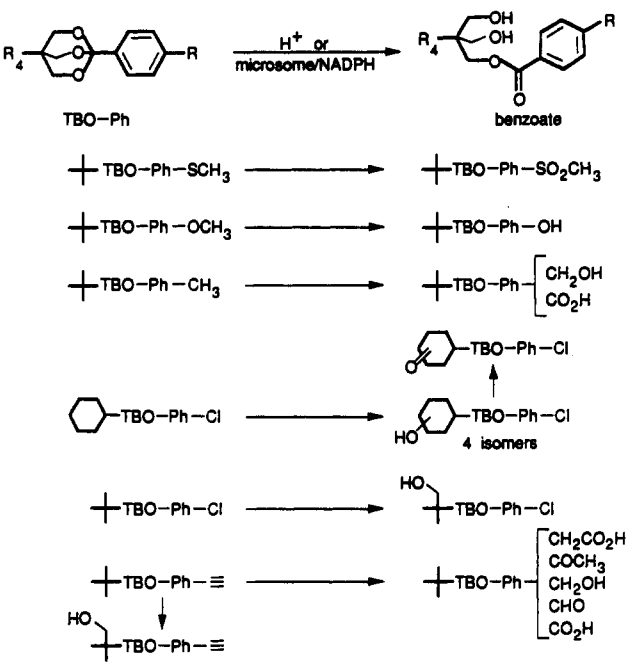


Figure 2. Metabolic reactions in mouse liver microsomal mixed-function oxidase systems for the *p*-methylthio, *p*-methoxy, and *p*-methyl analogues of 4-*tert*-butyl-1-phenyl-2,6,7-trioxabicyclo-[2.2.2]octane, for the 4-cyclohexyl and 4-*tert*-butyl analogues of 1-(*p*-chlorophenyl)-2,6,7-trioxabicyclo[2.2.2]octane, and for 4-*tert*-butyl-1-(*p*-ethynylphenyl)-2,6,7-trioxabicyclo[2.2.2]octane. The benzoates from ring-opening reactions are formed on both treatments with acid and NADPH-dependent microsomal systems.

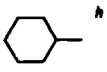
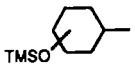
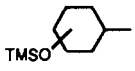
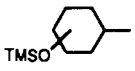
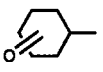
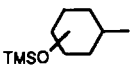
Other para substituents conferring similar or greater stability than H are C≡N, *t*-Bu, SO_2Me, OCF_3, C≡CMe, and CF_3.

Metabolites of 4-Alkyl-1-(*p*-substituted-phenyl)-2,6,7-trioxabicyclo[2.2.2]octanes in the Mouse Liver Microsomal Mixed-Function Oxidase System Analyzed by GC Cochromatography or GC/MS (Figure 2; Table V). Six compounds were selected for possible identification of the NADPH-dependent metabolites. GC cochromatography with a standard was used in one case and GC/CI-MS and GC/EI-MS for the other five compounds. With 4-*t*-Bu-TBO-Ph-SCH_3 there is rapid and total conversion of substrate and the appearance of only one metabolite detected by GC-FID in about 40% yield, which is identified on the basis of cochromatography (Table IV) with an authentic standard as the corresponding sulfone.

Under CI-MS analysis, the substrate TBOs and their metabolites all form $[M + H]^+$ (base peak ion) plus $[M + C_2H_5]^+$ and $[M + C_3H_5]^+$ adducts (not tabulated) with little fragmentation. The TMS-derivatized NADPH-dependent metabolites in addition show $[M + H - CH_4]^+$ and $[M + H - HOTMS]^+$ fragments (not tabulated) except for 4-*t*-Bu-TBO-Ph-OTMS, which lacks the $[M + H - HOTMS]^+$ fragment as expected. EI-MS gives more extensive fragmentation with two critical fragments providing the essential information for structural assignment: $[OCC_6H_4R]^+$ as the base peak ion and $[M - R_4 + H]^+$ as a minor fragment establish the nature of changes, if any, in the 1- and 4-substituents, respectively.

4-*t*-Bu-TBO-Ph-OCH_3 and 4-*t*-Bu-TBO-Ph-CH_3 each give one major NADPH-dependent product as a TMS derivative retaining the TBO system and modified in the para substituent. The major metabolite of the *p*-OCH_3 compound is the phenol assigned as 4-*t*-Bu-TBO-Ph-OTMS. 4-*t*-Bu-TBO-Ph-CH_3 is converted to 4-*t*-Bu-TBO-

Table V. GC/MS Data for Metabolites of Five 1,4-Disubstituted-2,6,7-trioxabicyclo[2.2.2]octanes in the Mouse Liver Microsomal Mixed-Function Oxidase System

$R_4C(CH_2O)_3CC_6H_4$-R[a]				EI-MS (rel intensity)[d]	
R_4	R	R_t,[b] min	CI-MS[c] $[M+H]^+$	base peak[e]	other
$(CH_3)_3C$	OCH_3[f]	13.4 (14.5)	279	135	278 (2), 248[g] (2), 222[h] (1), 191[i] (1), 153[j] (5), 152 (7), 107[k] (4), 92 (6), 77 (6)
$(CH_3)_3C$	OTMS[f]	15.6 (15.9)	337	193	336 (2), 321 (3), 306[g] (2), 279[h] (1), 249[i] (2), 211[j] (6), 177 (3), 149 (3), 135 (4), 121 (2), 96 (2), 91 (2), 79 (2), 75 (2), 73 (5)
$(CH_3)_3C$	CH_3[f]	10.5 (11.6)	263	119	262 (2), 232[g] (10), 206[h] (3), 175[i] (3), 137[j] (4), 91[k] (22), 81 (3), 79 (3)
$(CH_3)_3C$	CH_2OTMS[f]	18.1 (18.1)	351	207	350 (0.5), 335 (2), 320[g] (4), 305 (2), 294 (2), 275 (1), 261 (3), 225[j] (2), 179[k] (4), 135 (7), 118 (7), 90 (7), 89 (5), 75 (3), 73 (5)
$(CH_3)_3C$	CO_2TMS	20.6	365	221	v weak
$(CH_3)_3C$	C≡CH[f]	12.3 (12.8)	273	129	272 (2), 242[g] (5), 216[h] (1), 185[i] (1), 147[j] (4), 145 (5), 132 (8), 117 (14), 101[k] (15), 81 (3), 75 (13), 73 (11)
$(CH_3)_3C$	CHO	13.9	277	133	v weak
$(CH_3)_3C$	$COCH_3$	16.6	291	147	v weak
$(CH_3)_3C$	CH_2OTMS	18.1	351	207	225[j] (4), 179[k] (10), 160 (23), 147 (7), 129 (20), 118 (8), 117 (8), 90 (11), 89 (12), 75 (29), 73 (34)
$TMSOCH_2(CH_3)_2C$	C≡CH	18.4	361	129	227 (4), 219 (8), 216[h] (5), 203 (12), 184 (23), 169 (6), 147[j] (12), 144 (18), 124 (6), 116 (4), 111 (12), 103 (48), 101[k] (35), 75 (22), 73 (47)
$(CH_3)_3C$	CO_2TMS[l]	20.6	365	221	349 (7), 308[h] (2), 275 (5), 239 (3), 178 (5), 149 (19), 133 (4), 129 (4), 121 (4), 117 (4), 104 (12), 103 (11), 81 (10), 75 (12), 73 (16)
$(CH_3)_3C$	CH_2CO_2TMS[f,l,m]	21.1 (21.0)	379	235	378 (2), 363 (4), 348[g] (3), 322[h] (2), 289 (2), 261 (1), 253[j] (3), 221 (4), 207[k] (27), 192 (6), 177 (3), 163 (2), 147 (2), 145 (13), 135 (8), 118 (24), 107 (4), 90 (14), 75 (19), 73 (51)
$(CH_3)_3C$	Cl	11.8	283	139	282 (0.5), 254 (1.5), 252[g] (5), 228 (0.5), 226[h] (1.5), 195[i] (0.5), 159 (1), 157[j] (3), 141 (30), 129 (3), 117 (3), 113 (6), 111[k] (16), 96 (4), 81 (6), 79 (3), 75 (8), 73 (3)
$TMSOCH_2(CH_3)_2C$	Cl	17.8	371	139	237 (3), 229 (7), 226[h] (2), 215 (4), 213 (12), 201 (2), 184 (29), 171 (3), 169 (8), 159 (2), 157[j] (8), 144 (25), 141 (38), 129 (9), 113 (10), 111[k] (44), 103 (81), 75 (28), 73 (85)
	Cl[f]	19.5 (17.6)	309	139	308 (1), 278[g] (1), 247 (3), 228 (1), 226[h] (4), 191 (1), 159 (2), 157[j] (6), 147 (2), 141 (34), 122 (8), 113 (6), 111[k] (19), 107 (7), 93 (8), 81 (6), 79 (8), 75 (5), 73 (10)
	Cl[f]	24.4 (21.0) (21.3) (22.0) (23.0)[o]	397	139	228 (2), 226[h] (4), 215 (6), 213 (17), 195[i] (4), 169 (16), 167 (13), 157[j] (12), 141 (39), 129 (15), 120 (59), 111[k] (26), 105 (17), 103 (5), 101 (10), 91 (15), 75 (45), 73 (38)
	Cl	24.9	397	139	228 (2), 226[h] (8), 215 (7), 213 (21), 169 (5), 157[j] (7), 141 (36), 129 (37), 120 (14), 111[k] (14), 105 (7), 101 (9), 79 (15), 75 (28), 73 (28)
	Cl	25.1	397	139	228 (5), 226[h] (12), 215 (11), 213 (22), 195[i] (5), 169 (10), 167 (11), 157[j] (7), 141 (32), 129 (9), 120 (19), 111[k] (16), 91 (10), 79 (14), 75 (27), 73 (39)
	Cl[p]	25.3	323	139	204 (3), 185 (3), 157[j] (13), 149 (6), 141 (29), 135 (13), 129 (31), 111[k] (21), 93 (17), 79 (17), 75 (29), 73 (68)
	Cl[o]	26.1	397	139	396 (1), 381 (3), 228 (4), 226[h] (10), 215 (5), 213 (16), 169 (5), 159 (5), 157[j] (14), 141 (36), 129 (37), 120 (46), 111[k] (21), 101 (10), 92 (16), 91 (14), 79 (14), 75 (48), 73 (29)

[a] Substituents of the substrate are underlined and followed in subsequent lines by those of the metabolites. [b] Column: J&W DB5 30 m, 0.25 mm (i.d.), 0.25-μm film; helium carrier gas 15 psi. Temperature program: 140 °C for 1 min, 30 °C/min to 200 °C, 3 °C/min to 260 °C, 10 °C/min to 300 °C. [c] Methane reagent gas 0.9–1 Torr, source 130 °C. The base peak (100) is always $[M+H]^+$; $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$ are observed in all cases. [d] 70 eV, source 200 °C. [e] The base peak (100) is always $[OCC_6H_4R]^+$. [f] The corresponding benzoates from ring-opening reactions ($R_4C(CH_2OH)_2CH_2OCOC_6H_4R$) are observed as their bis-TMS derivatives (retention times given in parentheses); CI-MS gives $[M+H]^+$, $[M+H-CH_4]^+$, and sequential loss of HOTMS fragments from $[M+H]^+$. EI-MS major fragments are $[OCC_6H_4R]^+$ and 191 [TMSOCH=OTMS]$^+$, which is characteristic for 1,3-diols. [g] $[M-CH_2O]^+$. [h] $[M-R_4+H]^+$. [i] $[M-CH_2O-R_4]^+$. [j] $[H_2O_2CC_6H_4R]^+$. [k] $[C_6H_4R]^+$. [l] Methyl and ethyl esters are observed after derivatization with diazomethane and diazoethane, respectively. [m] The methyl ester ($R_4 = (CH_3)_3C$; $R = CH_2CO_2CH_3$) is also observed after extraction and BSA derivatization: R_t = 19.3 min; CI-MS $[M+H]^+$ = 321; EI-MS (rel intensity) 177 (100), 290[g] (2), 261 (2), 247 (2), 195[h] (4), 149[i] (4), 147 (3), 135 (4), 117 (3), 90 (5), 75 (5), 73 (17). [n] An unidentified metabolite is observed from this parent. R_t = 26.9 min; CI-MS $[M+H]^+$ = 367; EI-MS (rel intensity) 307 (1), 226 (5), 207 (1), 157 (7), 141 (36), 140 (7), 139 (100), 120 (19), 111 (17), 105 (15), 92 (16), 79 (11), 75 (6), 73 (5). [o] Major isomer. [p] TMS enol ether of ketone (or isomer) is also present: R_t = 26.4 min; CI-MS $[M+H]^+$ = 395; EI-MS base peak = 139.

Ph-CH_2OH as its major metabolite as indicated by the appropriate MS fragmentation of its TMS derivative; the corresponding benzoic acid is a minor metabolite.

4-*t*-Bu-TBO-Ph-C≡CH is converted to several metabolites of which six are identified by GC/EI-MS and GC/CI-MS (Figure 3). The major metabolite is 4-*t*-Bu-TBO-Ph-CH_2CO_2H, analyzed as its TMS ester with confirmation by conversion to the methyl and ethyl esters. The methyl ester 4-*t*-Bu-TBO-Ph-$CH_2CO_2CH_3$ surprisingly appears as a minor but consistent product in the metabolite mixture after derivatization with BSA (Figure 3). This carbomethoxy compound is an artifact of the extraction/analytical procedure since it is not present when derivatization is carried out with diazoethane and then BSA; it

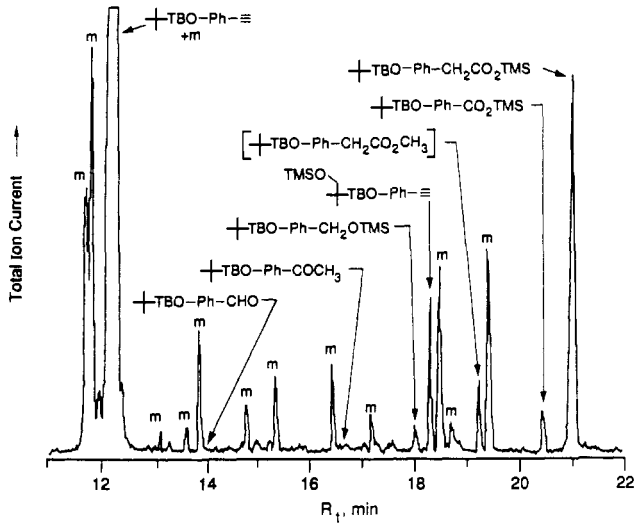


Figure 3. GC/CI-MS chromatogram of 4-*tert*-butyl-1-(*p*-ethynylphenyl)-2,6,7-trioxabicyclo[2.2.2]octane and some of its mouse liver microsomal mixed-function oxidase metabolites after treatment with *N,O*-bis(trimethylsilyl)acetamide. "m" designates components mostly from microsomes detected in both the presence and absence of substrate. The compound in brackets is considered to be an artifact formed during the analysis.

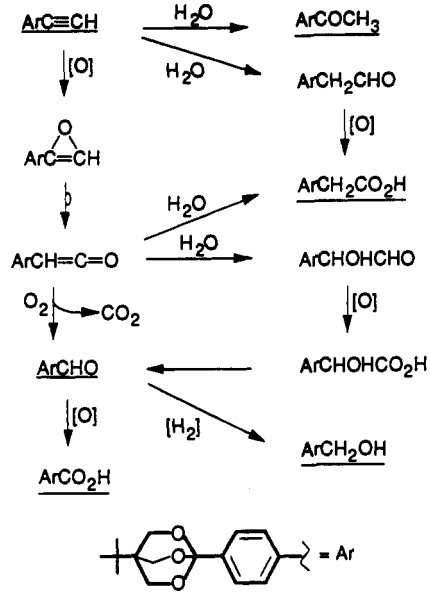


Figure 4. Possible mechanisms for mouse liver microsomal mixed-function oxidase metabolism of 4-*tert*-butyl-1-(*p*-ethynylphenyl)-2,6,7-trioxabicyclo[2.2.2]octane. The substrate and compounds observed as metabolites are underlined.

is probably formed by transesterification of the TMS ester with trace impurities of methanol in the solvents used. Another major metabolite is 4-$HOCH_2(CH_3)_2C$-TBO-Ph-C≡CH, with appropriate EI-MS and CI-MS characteristics as the TMS ether. A minor metabolite is 4-*t*-Bu-TBO-Ph-$COCH_3$, identified by cochromatography with an authentic standard, the $[OCC_6H_4COCH_3]^+$ ion in EI, and the appropriate $[M + H]^+$, $[M + C_2H_5]^+$, and $[M + C_3H_5]^+$ ions in CI. Three other metabolites are identified as products from oxidative cleavage of the ethynyl group. The benzoic acid derivative 4-*t*-Bu-TBO-Ph-CO_2H is assigned as the major ethynyl-cleavage product as determined by three separate experiments, each involving both EI-MS and CI-MS of the TMS, methyl, and ethyl esters. The corresponding aldehyde, a trace metabolite, is tentatively identified on the basis of the ions observed being $[M + H]^+$, $[M + C_2H_5]^+$, and $[M + C_3H_5]^+$ in CI and $[OCC_6H_4CHO]^+$ in EI. The benzylic alcohol is also observed, having the same R_t and CI and EI spectra as the major metabolite from 4-*t*-Bu-TBO-Ph-CH_3.

With 4-*t*-Bu-TBO-Ph-Cl, only one TBO metabolite is observed, for which MS data are consistent with hydroxylation at the *t*-Bu position. 4-*c*-Hex-TBO-Ph-Cl is readily hydroxylated at the cyclohexyl moiety to form one major and three minor isomers, plus a ketone from further oxidation of one or more of the cyclohexanol derivatives. An additional metabolite, TMS derivative $[M + H]^+$ = 367, is also modified on the cyclohexyl portion of the molecule but is not identified.

Several of the parent compounds and TBO-Ph metabolites are detected in part as their benzoate derivatives; i.e., bis-TMS ethers as derived from the parent TBO-Ph and tris-TMS ethers as derived from the hydroxylated metabolites. Although more extensive ring opening appears to be involved on fortification of microsomal incubations with NADPH, the quantitative aspects were not examined further in this phase of the present study.

DISCUSSION

Most of the TBOs are rapidly detoxified by MFO metabolism. This is evident by the effectiveness of PB as a synergist in houseflies (Palmer and Casida, 1985) and by the structure–biodegradability studies reported here with the mouse liver MFO system. In contrast, the polychlorocycloalkane insecticides, which are also inhibitors of the GABA-gated chloride channel, fall into two groups: poor substrates for MFO metabolism (i.e., lindane, dieldrin, and endosulfan sulfate) and precursors for toxic metabolites (i.e., α-endosulfan to endosulfan sulfate and aldrin to dieldrin).

Three portions of the molecule contribute to the structure–biodegradability relationships of the TBOs in the mouse liver MFO system, i.e., the *O*-methylene sites in the bicyclic ring, the 4-alkyl or 4-aryl moiety, and the 1-substituent including the para group of some of the orthobenzoates (Scott et al., 1987; Deng et al., 1990) (Figure 1). Hydroxylation at an *O*-methylene site leads to ring opening and formation of the diol benzoate (Figure 2), observed here after conversion to the bis-TMS derivative.

The 4-alkyl substituent markedly affects the biodegradability and is itself a site of metabolic alteration (Figure 2). The 4-butyl substituent confers extensive metabolism for three analogues (*n*, *s*, and *c*) versus the *t*-Bu moiety. This is consistent with the ease of hydroxylation at each carbon of the *s*-Bu substituent of 4-*s*-Bu-TBO-Ph-CN (Deng et al., 1990) compared to the slow metabolism of the *t*-Bu methyl groups (this study). The 4-cyclohexyl substituent is hydroxylated at multiple sites, contributing to the rapid detoxification of 4-*c*-Hex-TBOs in mice (e.g., Table I).

The para substituent of the TBO-Phs may also be the site of metabolic alteration (Figure 2). Although the cyanophenyl moiety is not detectably metabolized (Deng et al., 1990), the phenyl group appears to be hydroxylated (Scott et al., 1987). Facile MFO oxidations convert the SCH_3 substituent to the sulfone, the OCH_3 substituent to the corresponding phenol, and the CH_3 group to the benzyl alcohol derivative.

The *p*-ethynyl substituent is of particular interest because it is present in highly potent insecticides and chloride channel blockers (Figure 4). The extent of metabolism at 60 min is not affected by replacing the ethynyl proton of 4-*t*-Bu-TBO-Ph-C≡CH with deuterium. In contrast, kinetic rate studies on metabolite formation establish an isotope effect of 1.7 when the terminal proton

of phenylacetylene is replaced with deuterium (Kömives and Ortiz de Montellano, 1987). The major metabolite of 4-*t*-Bu-TBO-Ph-C≡CH is the phenylacetic acid derivative, probably formed by oxidation of the triple bond to give the oxirene, which rearranges to the more stable ketene followed by addition of water (Wade et al., 1979; Hammons et al., 1989). Although the acetophenone and mandelic acid derivatives are reported as metabolites of 4-ethynylbiphenyl (Wade et al., 1979), only the acetyl compound is observed with the analogous TBO substrate. The acetyl derivative is obtained by formal addition of H_2O to the triple bond. Oxidative cleavage of an aromatic ethynyl group is not previously reported in MFO systems and may be analogous in mechanism to that proposed for the autooxidation of phenylacetylene (Pritzkow and Rao, 1985). Thus, further oxidation of the ketene with loss of carbon dioxide leads to the aldehyde, which undergoes oxidation to the acid or reduction to the benzylic alcohol.

ABBREVIATIONS USED

BSA, *N,O*-bis(trimethylsilyl)acetamide; GABA, γ-aminobutyric acid; MFO, mixed-function oxidase; PB, piperonyl butoxide; TBO, 2,6,7-trioxabicyclo[2.2.2]octane (Figure 2); TMS, trimethylsilyl; CI, chemical ionization; EI, electron impact; FID, flame ionization detector; GC, gas chromatography; MS, mass spectrometry; TLC thin-layer chromatography; *n*, normal; *i*, iso; *s*, secondary; *t*, tertiary; *c*, cyclo; Me, methyl; Pr, propyl; Bu, butyl; Pen, pentyl; Hex, hexyl; Ph, phenyl.

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Registry No. *t*-Bu-TBO-H, 70636-87-2; *t*-Bu-TBO-*c*-Hex, 97720-37-1; *t*-Bu-(3-CN-TBO)-Ph-C≡CH, 107829-39-0; *t*-Bu-TBO-Ph-C≡CH, 108614-39-7; *t*-Bu-TBO-Ph-C≡CD, 130523-73-8; *c*-Hex-TBO-Ph-Cl, 97719-95-4; *c*-Bu-TBO-Ph-I, 130551-59-6; *n*-Bu-TBO-Ph-I, 130523-74-9; *s*-Bu-TBO-Ph-I, 130523-75-0; *c*-Hex-TBO-Ph-I, 130523-76-1; *i*-Pr-TBO-Ph-I, 130523-77-2; Ph-TBO-Ph-I, 130523-78-3; *p*-F-Ph-TBO-Ph-I, 130523-79-4; *c*-Pen-TBO-Ph-I, 130523-80-7; *n*-Pen-TBO-Ph-I, 130523-81-8; *t*-2-Me-*c*-Pr-TBO-Ph-I, 130523-82-9; *n*-Pr-TBO-Ph-I, 108614-59-1; *t*-Bu-TBO-Ph-I, 117439-67-5; *c*-Pr-TBO-Ph-I, 130523-83-0; 1-Me-*c*-Pr-TBO-Ph-I, 130523-84-1; *m*-F-Ph-TBO-Ph-I, 130523-85-2; 4-*t*-Bu-TBO-Ph-SMe, 130523-86-3; 4-*t*-Bu-TBO-Ph-OMe, 130523-87-4; 4-*t*-Bu-TBO-Ph-Me, 130523-88-5; 4-*t*-Bu-TBO-Ph-C≡CH, 108614-39-7; TBOB, 70637-05-7; 4-*t*-Bu-TBO-Ph-CN, 97720-14-4; 4-*t*-Bu-TBO-Ph-Cl, 97719-93-2; 4-*t*-Bu-TBO-Ph-*t*-Bu, 97720-21-3; 4-*t*-Bu-TBO-Ph-F, 97720-07-5; 4-*t*-Bu-TBO-Ph-SO_2Me, 130523-89-6; 4-*t*-Bu-TBO-Ph-O-CF_3, 130523-90-9; 4-*t*-Bu-TBO-Ph-C≡CCH_3, 130523-91-0; 4-*t*-Bu-TBO-Ph-CF_3, 97720-11-1; 4-*t*-Bu-TBO-Ph-Br, 97720-09-7; 4-*t*-Bu-TBO-Ph-I, 117439-67-5; aldrin, 309-00-2; dieldrin, 60-57-1; α-endosulfan, 959-98-8; endosulfan sulfate, 1031-07-8; lindane, 58-89-9; tetramethylenedisulfotetramine, 80-12-6; mixed function oxidase, 9040-60-2.