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Metabolism of [^{14}C]Crufomate (4-*t*-Butyl-2-chlorophenyl methyl methylphosphoramidate) by the Sheep†

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Abstract—Twenty-five metabolites were isolated from the urine, feces or plasma of sheep given single oral doses of [^{14}C]crufomate (4-*t*-butyl-2-chlorophenyl methyl methylphosphoramidate). These metabolites resulted from one or more of the following transformations: oxidation of a methyl group in the *t*-butyl moiety to yield either an alcohol or a carboxylic acid; hydrolysis of the phosphate and phosphoramidate bonds; oxidation of the *N*-methyl group to yield *N*-formyl phosphoramidates; methylation of the *N*-formyl group to yield *N*-methyl-*N*-formyl phosphoramidates; oxidative *N*-demethylation; conjugation with glucuronic acid. No ring-hydroxylation or dechlorination was observed, and no crufomate was isolated from the urine, feces or plasma.

Introduction

CRUFOMATE (4-*t*-butyl-2-chlorophenyl methyl methylphosphoramidate, A-1) is used as a systematic insecticide for the control of grubs, hornflies and cattle lice. The metabolic fate of ^{32}P labeled crufomate in sheep has been reported by Bauriedel and Swank.[1] Metabolites from crufomate were not identified in that study. The present study reports the metabolic fate of ^{14}C labeled crufomate given as single oral doses to sheep. The crufomate was labeled at the tertiary carbon of the tertiary butyl moiety.

Synthesis of some of the metabolites and of model compounds for characterization of metabolite structures is presented in a companion article.[2]

Experimental

RADIOLABELED COMPOUND

The [^{14}C]crufomate sample, 4-(1,1′-dimethyl[1′-^{14}C]ethyl)-2-chlorophenyl methyl methylphosphoramidate, was obtained from New England Nuclear Corporation. The radiopurity was greater than 99% (by thin-layer and gas-liquid chromatography, g.l.c.).

ANIMAL TREATMENT

Two wether sheep (sheep 1, 32 kg; sheep 2, 64 kg) were each given a single oral dose of 100 mg kg^{-1} of [^{14}C]crufomate. Each dose contained 0.5 mCi of ^{14}C. For isolation of plasma metabolites, a ewe (sheep 3, 82 kg) was given 77 mg kg^{-1} of [^{14}C]crufomate as a single oral dose, containing 0.977 mCi of ^{14}C. Expiration of $^{14}CO_2$ in the respiratory gases was measured from sheep 1 using methods reported previously.[3] Blood samples were collected from sheep 1 and 3 by means of a catheter in the jugular vein. Blood samples (50 ml, heparinized) were collected hourly from sheep 1 for the first 15 h after dosing, followed by samples 19, 24, 30 and 46 h after dosing. Blood was collected hourly from sheep 3 for the first 3 h after dosing and then continuously until 2 l were obtained. The plasma and blood cells from sheep 1 were separated and assayed separately for ^{14}C. The plasma from sheep 3 was assayed for ^{14}C and used for isolation of metabolites.

Urine from sheep 1 and 2 was collected 4, 8, 12, 20, 24, 32 and 48 h after dosing. The urine was kept at ice-bath temperature during collection and frozen for storage after ^{14}C assay. Feces were collected at 24 and 48 h after dosing. The feces were homogenized with water and freeze-dried.

INSTRUMENTATION AND QUANTITATION OF RADIOACTIVITY

All column eluates were monitored for radioactivity with a Packard Model 370E liquid scintillation flow system. An anthracene cell was used for aqueous eluents and cerium-activated glass beads for organic eluents. Radioactive components on chromatograms were located with a Packard Model 7200 chromatogram scanner. Radioactivity in urine and various extracts was quantitated by liquid scintillation in counting solution A;[4] a Nuclear-Chicago Mark I spectrometer was used. Tissue, fecal and red-blood cell radioactivity was quantitated by combustion of 100–200 mg of the freeze-dried samples in a Model 305 Packard Tri-Carb sample oxidizer. Infrared spectra

† Abbreviations: [^{14}C]crufomate = 4-*t*-butyl-2-chlorophenyl methyl methylphosphoramidate; BSTFA = bis-(trimethylsilyl)-trifluoroacetamide.

were obtained in micro-KBr pellets (10 mg KBr; 2 mm diameter pellets) with a Perkin-Elmer 337 infrared spectrometer equipped with a 4X beam condenser.

Before gas chromatography, all samples were treated with bis-(trimethylsilyl)trifluroroacetamide containing 1% trimethylchlorosilane (BSTFA) at room temperature for 18 h to form the trimethylsilyl derivatives (TMS derivatives). [$^2H_9$]TMS derivatives were prepared by the following procedure. The TMS derivatives were trapped from the g.l.c. and the TMS groups removed by hydrolysis with aqueous methanol. The samples were dried, reacted with bis-([$^2H_9$]trimethylsilyl)acetamide and gas-chromatographed.

Methyl derivatives of the metabolites were prepared with diazomethane or [$^2H_2$]diazomethane. The TMS derivatives were trapped from the gas chromatograph, and the TMS moieties hydrolyzed with aqueous methanol. These fractions were then reacted with diazomethane and again silylated before gas chromatography.

A Perkin-Elmer 801 gas chromatograph fitted with an effluent splitter was used for gas chromatography. Ten percent of the column effluent went to the flame-ionization detector and 90% was trapped in glass tubes for radioactivity detection and/or instrumental analysis. The column was a 6 ft, 1/8 in i.d. glass column packed with 3% SE-30 on 60/80 mesh Chromasorb W. The carrier gas was helium at a flow rate of 30 ml per min. The temperature was programmed at 10 °C per min from 100–250 °C. The injector was maintained at 200 °C. The detector was located in the column oven.

Mass spectra were obtained with solid sample inlet probes of either a Varian M-66 mass spectrometer equipped with a V-5500 control console or a Varian MAT CH-5 DF mass spectrometer capable of peak matching and determining decoupled metastable transitions.

COLUMN CHROMATOGRAPHY

Water-equilibrated Sephadex LH-20 (Pharmacia) was poured to form a 1.9 × 150 cm column [LH-20(H_2O)]. Samples (4 ml) were applied to the column and the radioactivity was eluted with water pumped at 1.5–2 ml per min.

The Sephadex LH-20 bicarbonate column [LH-20(HCO_3^-)] was prepared by first pouring water-equilibrated LH-20 to form a 0.9 × 110 cm column. The column was then equilibrated with two column volumes (approximately 50 ml) of 0.06 m $(NH_4)HCO_3$. Samples (1–2 ml) were applied to the column and the ^{14}C was eluted from the column with 0.06 M $(NH_4)HCO_3$ (0.5 ml per min).

Porapak Q (Waters Assoc.) was slurried with methanol and poured to form a 1 × 10 cm column. The column was washed with water, the sample applied in water and the column eluted with three column volumes of water. The radioactivity was eluted with methanol.

The cellulose ion exchange columns (Cellex GE and Cellex P, Bio-Rad) were prepared by pouring methanol slurries into 0.9 × 20 cm columns. The Cellex GE column was then conditioned with 1 M NH_4OH and the Cellex P column with 1 M HCl. The excess acid or base was displaced with water before the samples were added. The samples were applied to the columns and the ^{14}C eluted with water.

The cation exchange column (Ag-50 × 8[H^+], Bio-Rad) was poured in water (0.9 × 5 cm). The sample was applied in and eluted with water.

The DEAE Sephadex (A-25, Pharmaica) column was prepared by the method of Paulson *et al.*[5] DEAE Sephadex was swelled in 1 M KBr and then poured to form a 2.5 × 25 cm column; the column was washed with 1 l of water before use. The sample was applied in a small volume of water and the radioactivity was eluted with water (60 ml) followed by a KBr gradient (125 ml water in chamber 1 and 125 ml 1 M KBr in chamber 2).

PAPER CHROMATOGRAPHY

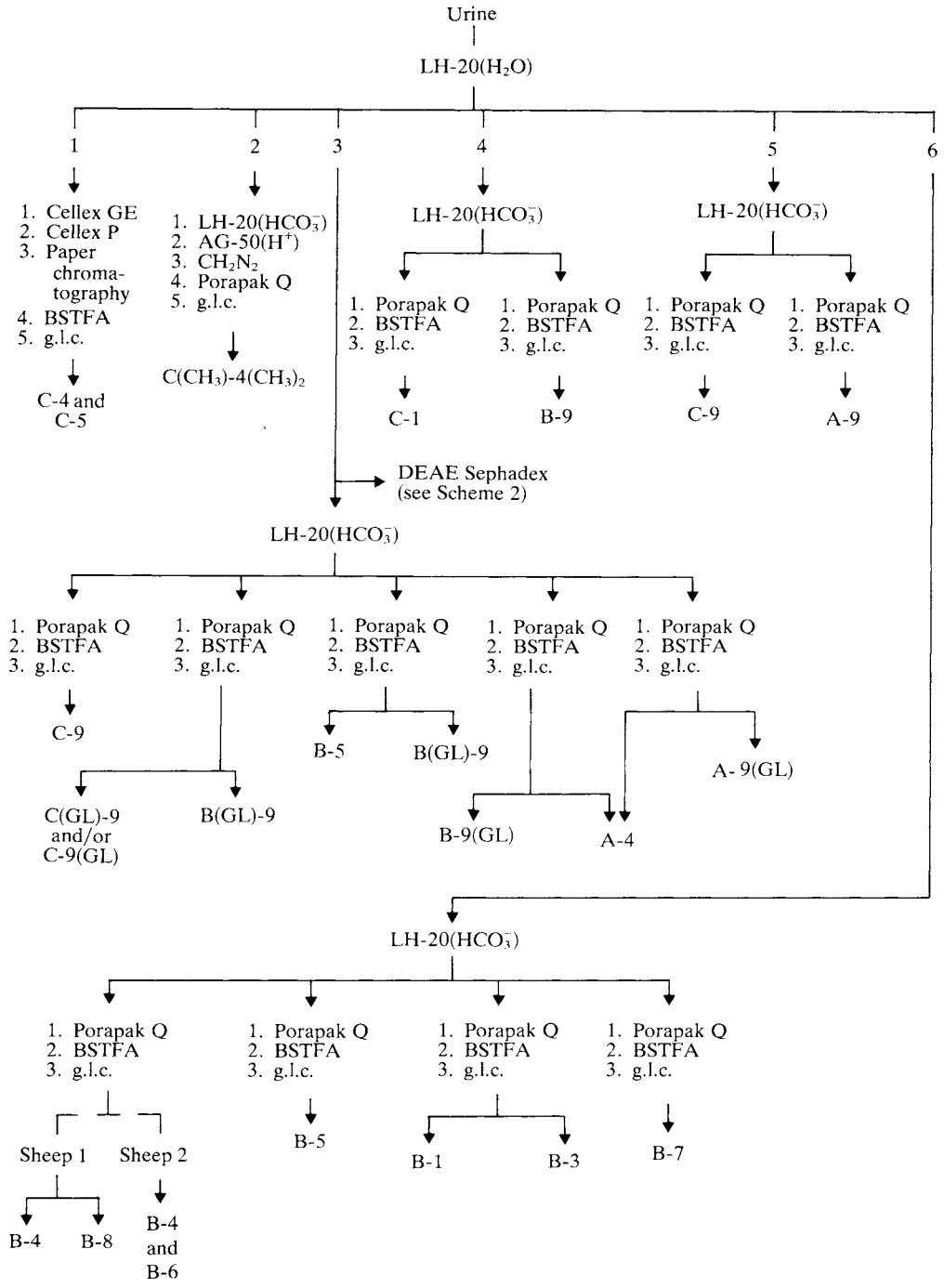
Samples were streaked across sheets of Whatman No. 1 paper and the chromatograms were developed with isopropanol + water + conc. NH_4OH (75:16:8, v/v/v).

ISOLATION OF METABOLITES

The flow diagrams given in Schemes 1 and 2 list the combinations of the above methods applied to the isolation of each urinary metabolite. The flow diagram in Scheme 3 lists these combinations as applied to the isolation of the plasma metabolites. Untreated urine was applied directly to the LH-20 (H_2O) column. The plasma metabolites were first adsorbed on the Porapak Q column from untreated plasma, the column was washed with water and the radioactivity eluted with methanol. The methanol eluate was then concentrated, diluted with water and applied to the LH-20 (H_2O) column.

All the metabolites, except those in LH-20 (H_2O) fractions 1 and 2 from the urine, bound to Porapak Q from aqueous solutions and were subsequently eluted with methanol. This procedure greatly facilitated the clean-up of the metabolites and the removal of eluent salts.

Freeze-dried feces were extracted three times with methanol and the methanol-soluble radioactive residue was dissolved in water. The fecal radioactivity was then adsorbed on Porapak Q from the aqueous solution, the column washed with water and the radioactivity eluted with methanol. The fecal radioactivity in the methanol eluate from Porapak Q was concentrated and applied to the LH-20 (HCO_3^-) column.

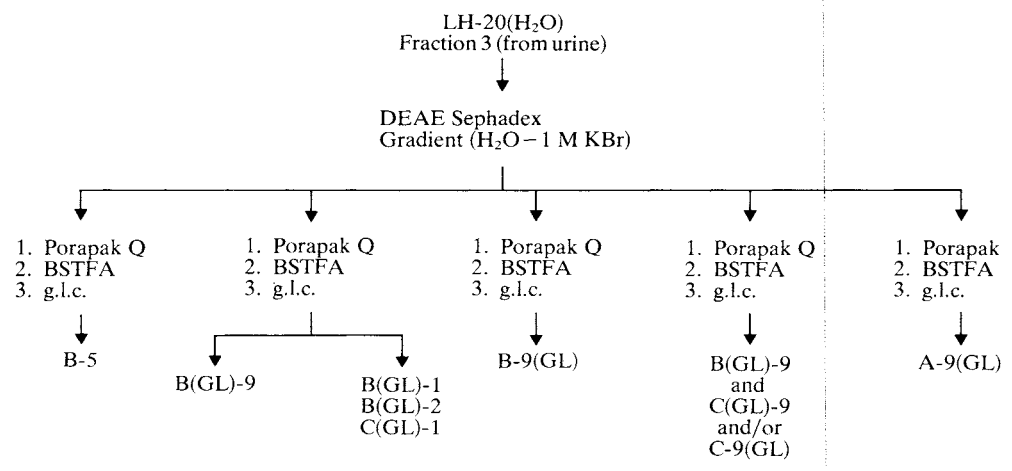


SCHEME 1. Separation of the radiolabeled metabolites in sheep urine after dosing with [^{14}C]crufomate.

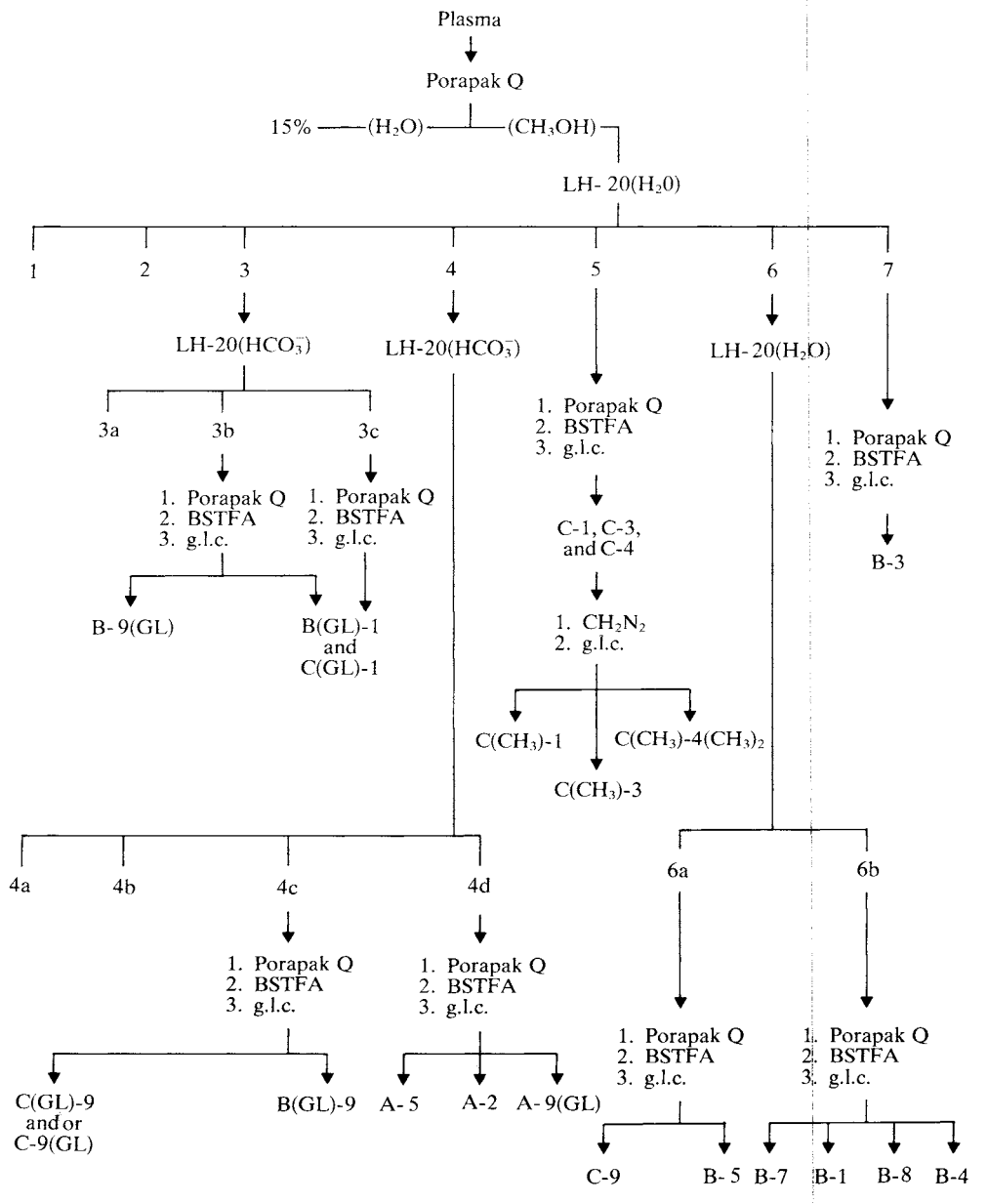
Results and discussion

Twenty-four metabolites of crufomate (A-1) were isolated from either the urine, feces or plasma from sheep given single oral doses of crufomate. Seven of these metabolites have been characterized as glucuronides. The assignments of structures to these glucuronides will be discussed separately in a companion paper.[6]

Because metabolism of crufomate involved changes in two moieties of the molecule, the unconjugated metabolites and the aglycones from the glucuronides have been coded with the letter-number designations given in the first column of Table 1. The latter designates the oxidation state of the tertiary butyl moiety (R_1, Table 1). The letter A designates the unchanged *t*-butyl group, B a hydroxylated *t*-butyl group and C a *t*-butyl group containing a carboxylic acid. The numbers 1–8 designate the structure of the phosphate or phosphoramidate attached to the phenolic oxygen. The number 9 designates that R_2 is a hydrogen. The nine structures represented by these numbers are given

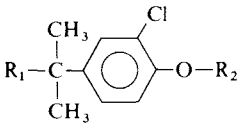


SCHEME 2. Separation of the 'glucuronide fraction' from urine [LH-20(H_2O) Fraction 3, Scheme 1] with DEAE Sephadex.



SCHEME 3. Separation of the radiolabeled metabolites in sheep plasma after dosing with [^{14}C]crufomate.

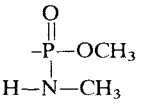
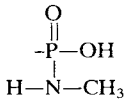
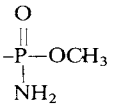
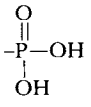
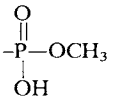
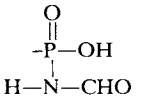
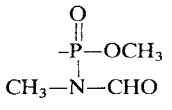
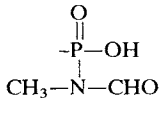
TABLE 1. Structures of the metabolites and compounds referred to in the text, illustrations and Tables 2–4.



R_1

Designation letter	Structure
A	CH_3
B	$HOCH_2$
C	HOOC

R_2

Structure	Designation number
	1
	2
	3
	4
	5
	6
	7
	8
-H	9

under R_2 in Table 1. By this system, crufomate is designated by A-1 and 2-chloro-4-*t*-butyl phenol by A-9. The locations of the substituents added to the metabolites by derivatization and the proposed bonding site of the glucuronic acid (whether on R_1 or R_2) are designated by parenthetical notations, i.e. B(TMS)-1(TMS) would designate B-1 with TMS groups replacing both the alcohol proton on R_1 and the amide proton on R_2.

The chemical names of the metabolites isolated from urine, feces and plasma are listed in Table 2. An estimate of the quantitation of each metabolite and a summary of the methods used to assign structures to each metabolite is given in Table 3.

Structures were assigned to the metabolites primarily on the basis of the mass spectral data obtained from the trimethylsilyl derivatives (TMS and [$^2H_9$]TMS, the TMS derivatives of metabolites methylated with diazomethane or [$^2H_2$]diazomethane (CH_2N_2-TMS) and the metabolites methylated with diazomethane (CH_2N_2). Mass spectra from the [$^2H_9$]TMS derivatives demonstrated the number of TMS moieties present in the metabolite derivatives and, along with determinations of metastable transitions, served to define some of the fragmentation pathways. Accurate mass determinations established the elemental compositions of selected fragment ions and molecular ions ($[M]^+$) that aided in structural assignments. The mass spectral data

TABLE 2. Chemical names of the nonconjugated metabolites from [C^{14}]crufomate

Code	Chemical name
A-1	4-*t*-Butyl-2-chlorophenyl methyl methylphosphoramidate
A-2	4-*t*-Butyl-2-chlorophenyl methylphosphoramidate
A-4	4-*t*-Butyl-2-chlorophenyl phosphate
A-5	4-*t*-Butyl-2-chlorophenyl methyl phosphate
A-9	4-*t*-Butyl-2-chlorophenol
B-1	2-Chloro-4(2-hydroxy-1,1-dimethylethyl)phenyl methyl methylphosphoramidate
B-3	2-Chloro-4(2-hydroxy-1,1-dimethylethyl)phenyl methyl phosphoramidate
B-4	2-Chloro-4(2-hydroxy-1,1-dimethylethyl)phenyl phosphate
B-5	2-Chloro-4(2-hydroxy-1,1-dimethylethyl)phenyl methyl phosphate
B-6	2-Chloro-4(2-hydroxy-1,1-dimethylethyl)phenyl formylphosphoramidate
B-7	2-Chloro-4(2-hydroxy-1,1-dimethylethyl)phenyl methyl formylmethylphosphoramidate
B-8	2-Chloro-4(2-hydroxy-1,1-dimethylethyl)phenyl formylmethylphosphoramidate
B-9	2-Chloro-4(2-hydroxy-1,1-dimethylethyl)phenol
C-1	2-[3-Chloro-4-[[(methoxy)(methylamino)phosphinyl]oxy]phenyl]-2-methylpropionic acid
C-3	2-[4-[[(Amino)(methoxy)phosphinyl]oxy]-3-chlorophenyl]-2-methylpropionic acid
C-4	2-[3-Chloro-4-[(dihydroxyphosphinyl)oxy]phenyl]-2-methylpropionic acid
C-5	2-[3-Chloro-4-[[(hydroxy)(methoxy)phosphinyl]oxy]phenyl]-2-methylpropionic acid
C-9	2-(3-Chloro-4-hydroxyphenyl)-2-methylpropionic acid

TABLE 3. Quantitation[a] of the metabolites present in plasma, urine and feces (% ^{14}C present) and summary of identification method

Metabolite code (Scheme 2)	Plasma %	Urine %	Feces %	Identification method[b]
A-1	ND[c]	ND	ND	
A-2	3	ND	ND	Conversion to A-1 with CH_2N_2
A-4	3	1	ND	S
A-5	11	ND	ND	S
A-9	3	5	ND	S
A-9(GL)[d]	9	6	ND	m.s.; glucuronidase
B-1	1	9	ND	S
B(GL)-1	1.5	<1	ND	m.s.
B(GL)-2	<1	<1	ND	m.s.; Ref. 6
B-3	2	3	ND	m.s.
B-4	<1	6	20	Synthesis of B-4 $(CH_3)_2$
B-5	<1	15	25	m.s.; conversion to B-4 $(CH_3)_2$
B-6	ND	4	ND	m.s.; i.r.; conversion to B-7 with CH_2N_2
B-7	2	5	ND	m.s.; i.r.; synthesis of A-7
B-8	2	5	ND	m.s.; i.r.; conversion to B-7 with CH_2N_2
B-9	<1	6	16	S
B(GL)-9	3	5	ND	m.s.; glucuronidase
B-9(GL)	11	17	ND	m.s.; glucuronidase
C-1	10	<1	ND	m.s.
C(GL)-1	<1	<1	ND	m.s.
C-3	5	ND	ND	m.s.
C-4	10	<1	ND	S
C-5	ND	<1	ND	Conversion to $C(CH_3)$-4$(CH_3)_2$
C-9	6	<1	ND	S
C(GL)-9 and/or C-9(GL)	1.5	2	ND	m.s.

[a] The quantitation of each metabolite is estimated from the amount of radioactivity trapped from the gas chromatograph. This assumes that mixtures of metabolites derivatized to the same extent.

[b] S indicates synthetic standards were available for comparison of mass spectra. m.s. and i.r.: metabolite characterized by mass spectral and/or infrared interpretation. For glucuronide characterization, see ref. 6.

[c] ND = none detected.

[d] GL = glucuronide.

obtained from the derivatives of the nonconjugated metabolites are given in Table 4.

The mass spectra from all derivatives of the nonconjugated metabolites contained molecular ions consistent with the structures assigned. $[M+2]^+$ ions were present with the appropriate relative intensity for the ^{37}Cl isotope.

MASS SPECTRAL DETERMINATIONS OF THE STRUCTURES OF R_1 MOIETIES

Assignment of the structures to the R_1 moieties in the phenolic portions of the metabolites were based on the following mass spectral data.

$R_1=CH_3$ (A series)

The mass spectra of the TMS, $[^2H_9]$TMS and CH_2N_2-TMS derivatives of the metabolites in which the *t*-butyl group remained unoxidized (A series, $R_1=CH_3$) were identical to those of the corresponding derivatives prepared from synthetic standards. These metabolites were assigned structures A-2, A-4, A-5 and A-9 (Table 1).

$R_1=CH_2OH$ (B series)

The mass spectra of all TMS derivatives of the metabolites in which the *t*-butyl group contained a primary alcohol (B series, $R_1=CH_2OH$) gave intense, chlorine-containing fragment ions at $[M-103]^+$ $[103=(CH_3)_3SiOCH_2]$ which shifted to $[M-112]^+$ for the $[^2H_9]$TMS derivatives. This fragmentation is characteristic of trimethylsilyl ethers of primary alcohols.[7] In addition, all the TMS and $[^2H_9]$TMS derivatives of the metabolites in the B series (except B-9) gave intense, chlorine-containing fragment ions at $[M-30]^+$. The presence of this $[M-30]^+$ ion in the mass spectra of $[^2H_9]$TMS derivatives showed that the methyl groups from the TMS moieties were not contained in the leaving group. Exact mass measurements of the $[M]^+$ and $[M-30]^+$ ions in the mass spectra from the TMS derivatives of B-4, B-7 and B-8 indicated that this loss resulted from the elimination of the elements of formaldehyde from the molecular ions. The measured masses of the $[M]^+$ and $[M-30]^+$ ions in all three spectra were within 2 ppm of the values calculated for the respective molecular ions and $[M-CH_2O]^+$ ions. Decoupled metastable determinations with B(TMS)-4(TMS)$_2$ showed that this $[M-30]^+$ ion was a metastable daughter from $[M]^+$. The metastable elimination of CH_2O from the molecular ion could not be attributed to phosphate methyl ester moieties[8] because none of the methyl esters of the known compounds A-1, -2, -4 or -5 gave this $[M-30]^+$ fragment ion, and methylation of B-4 with CD_2N_2 followed by trimethylsilylation [B(TMS)-4$(CD_2H_2]$ did not change the magnitude of the loss. These data indicated that the $[M-30]^+$ ions in the mass spectra of the TMS derivatives of the metabolites in the B series resulted from the elimination of CH_2O from the TMS ether moieties with rearrangement of the $(CH_3)_3Si$ group. The phosphorus-containing moieties were required for this metastable loss because this fragmentation mode was not observed in the mass spectrum of B(TMS)-9(TMS) ($R_2=H$). This fragmentation mode was assumed to involve the migration of the ethereal TMS group to the phosphate moiety[2] with subsequent loss of CH_2O.

These mass spectral data were the basis for assigning the structure of CH_2OH to R_1 in the metabolites of the B series. These mass spectral fragmentation modes of the TMS ethers of the metabolites of the B series were confirmed by synthesis of B(TMS)-1(TMS), B(TMS)-4$(CH_3)_2$ and B(TMS)-9(TMS).[2]

TABLE 4. Mass spectral data

m/e[a]	Rel. int.	Δ a.m.u. for [$^2H_9$]TMS	Ion description
A-1(TMS) from A-2	(1) CH_2N_2 → (2) BSTFA	A-1(TMS)	
363.1182 (Cl)	18	9	$[M]^+$
348 (Cl)	100	6 & 9	$[M-CH_3]^+$
328	38	9	$[M-Cl]^+$
318	1		
316	1		
312	2	6	$[348-HCl]^+$
292 (Cl)	3	6	$[348-C_4H_8]^+$
180	17	9	R_2 ion
166.5 (Cl; *m/2e*)	6	3	$[348-(CH_3)]^{2+}$(*hh*)[b]
152.5201 (Cl; *m/2e*)	20	3	$[348-(CH_3+C_2H_4)]^{2+}$(*ii*)[b]
A-2(TMS)			
349 (Cl)	7	9	$[M]^+$
334 (Cl)	22	6 & 9	$[M-CH_3]^+$
314	100	9	$[M-Cl]^+$
241 (Cl)	10	9	(*x*; Scheme 3)[b]
197 (Cl)	3	0	(*d*; Scheme 1)[b]
182 (Cl)	33	0	Scheme 5
169 (Cl)	11	0	(*g*; Scheme 1)[b]
168 (Cl)	3	0	(*i*; Scheme 1)[b]
166	22	9	R_2 ion
159.5 (Cl; *m/2e*)	1.5	3	$[343-CH_3]^{2+}$
145.5 (Cl; *m/2e*)	5.5	3	$[343-(CH_3+C_2H_4)]^{2+}$
A-4 (TMS)$_2$			
408 (Cl)	3.5	18	$[M]^+$
393 (Cl)	65	15 & 18	$[M-CH_3]^+$
373	100	18	$[M-Cl]^+$
337.0239 (Cl)	2	15	$[M-(CH_3+C_4H_8)]^+$
321 (Cl)	7.5	12	
305 (Cl)	3		
299	1.5		
285 (Cl)	1	9	
241.0813 (Cl)	3	9	(*x*; Scheme 3)[b]
225.0015	6	18	R_2 ion
211.0015	6.5	12	$[\{(CH_3)_2Si\}_2PO_4]^+$
209	1		
195	1.5	12	
189 (Cl; *m/2e*)	1	7.5	$[393-CH_3]^{2+}$
175 (Cl; *m/2e*)	0.3	7.5	$[393-(CH_3+C_2H_4)]^{2+}$
A-5 (TMS)			
350 (Cl)	9	9	$[M]^+$
335 (Cl)	100	9	$[M-CH_3]^+$
315	51	9	$[M-Cl]^+$
167	60	9	R_2 ion
A-4 (CH$_3$)$_2$			
292 (Cl)	15		$[M]^+$
277 (Cl)	100		$[M-CH_3]^+$
257	12		$[M-Cl]^+$
249 (Cl)	1		$[M-(CH_3+C_2H_4)]^+$
109	16		R_2 ion
A-9			
184 (Cl)	21		$[M]^+$
169 (Cl)	100		$[M-CH_3]^+$
141 (Cl)	16		$[169-C_2H_4]^+$ (*h*; Scheme 1)[b]
57	22		$[C_4H_9]^+$

TABLE 4 (*continued*)

m/e[a]	Rel. int.	Δ a.m.u. for [$^2H_9$]TMS	Ion description
A-9 (TMS)			
256 (Cl)	18		$[M]^+$
241 (Cl)	100		$[M-CH_3]^+$
169 (Cl)	3		(*g*; Scheme 1)[b]
141 (Cl)	<0.1		(*h*; Scheme 1)[b]
113 (Cl; *m/2e*)	2		$[241-CH_3]^{2+}$
99 (Cl; *m/2e*)	4.5		$[241-(CH_3+C_2H_4)]^{2+}$
B(TMS)-1			
379 (Cl)	10	9	$[M]^+$
364 (Cl)	11	6	$[M-CH_3]^+$
349 (Cl)	100	9	$[M-CH_2O]^+$
292 (Cl)	1.5		
276 (Cl)	49	0	$[M-(CH_3)_3SiOCH_2]^+$
182 (Cl)	80	0	Scheme 5
169 (Cl)	39	0	(*g*; Scheme 1)[b]
141(Cl)	3.5	0	(*h*; Scheme 1)[b]
108	38	0	R_2 ion
103	10	9	$[(CH_3)_3SiOCH_2]^+$
B(TMS)-1(TMS)			
451 (Cl)	9		$[M]^+$
436 (Cl)	20		$[M-CH_3]^+$
421 (Cl)	33		$[M-CH_2O]^+$
348 (Cl)	100		$[M-(CH_3)_3SiOCH_2]^+$
241 (Cl)	1.5		(*x*; Scheme 3)[b]
210.5 (Cl; *m/2e*)	<0.1		$[M-2CH_3]^{2+}$
180	15		R_2 ion
166.5 (Cl; *m/2e*)	<0.1		$[348-CH_3]^{2+}$ (*hh*)[b]
152.5 (Cl; *m/2e*)	0.3		$[348-(CH_3+C_2H_4)]^{2+}$ (*ii*)[b]
103	13		$[CH_3)_3SiOCH_2]^+$
B([$^2H_9$]TMS)-2([$^2H_9$]TMS) (see Scheme 8)			
455 (Cl)	13		$[M]^+$
437 (Cl)	18		$[M-C^2H_3]^+$
425 (Cl)	74		$[M-CH_2O]^+$
343 (Cl)	100		$[M-(C^2H_3)_3SiOCH_2]^+$
250 (Cl)	20		(*x*, Scheme 3)[b]
209.5 (Cl; *m/2e*)	<0.1		$[M-2C^2H_3]^{2+}$
182 (Cl)	16		Scheme 5
175	18		R_2 ion
169 (Cl)	10		(*g*; Scheme 1)[b]
148.5 (Cl; *m/2e*)	>0.1		$[343-(C^2H_3+C_2H_4)]^{2+}$
112	9		$[(C^2H_3)_3SiOCH_2]^+$
B(TMS)-3(TMS)			
437 (Cl)	19		$[M]^+$
422 (Cl)	35		$[M-CH_3]^+$
407 (Cl)	92		$[M-CH_2O]^+$
334 (Cl)	100		$[M-(CH_3)_3SiOCH_2]^+$
241 (Cl)	10		(*x*; Scheme 3)[b]
203.5 (Cl; *m/2e*)	3.5		$[M-2CH_3]^{2+}$
166	15		R_2 ion
159.5 (Cl; *m/2e*)	0.5		$[334-CH_3]^{2+}$
145.5 (Cl; *m/2e*)	4		$[334-(CH_3+C_2H_4)]^{2+}$
103	19		$[(CH_3)_3SiOCH_2]^+$
B(TMS)-4(TMS)$_2$			
496.1463 (Cl)	4	27	$[M]^+$
481 (Cl)	16	27 & 24	$[M-CH_3]^+$
466.1356 (Cl)	28	27	$[M-CH_2O]^+$
393 (Cl)	100	18	$[M-(CH_3)_3SiOCH_2]^+$
241 (Cl)	7	9	(*x*; Scheme 3)[b]
225	4	18	R_2 ion

TABLE 4 (*continued*)

m/e[a]	Rel. int.	Δ a.m.u. for [$^2H_9$]TMS	Ion description
B(TMS)-4(TMS)$_2$			
211	7.5	12	$[\{(CH_3)_2Si\}_2PO_4]^+$
189 (Cl; *m/2e*)	0.5	7.5	$[393-CH_3]^{2+}$
169 (Cl)	9	0	(*g*; Scheme 1)[b]
103	4	9	$[(CH_3)_3SiOCH_2]^+$
B(TMS)-5(TMS)			
438 (Cl)	3	18	$[M]^+$
423 (Cl)	22	15 & 18	$[M-CH_3]^+$
408 (Cl)	73	18	$[M-CH_2O]^+$
335 (Cl)	100	9	$[M-(CH_3)_3SiOCH_2]^+$
299	13	9	
241 (Cl)	10	9	(*x*; Scheme 3)[b]
169 (Cl)	15	0	(*g*; Scheme 1)[b]
168	1	0	(*i*; Scheme 1)[b]
167	2.5	9	R_2 ion
160 (Cl; *m/2e*)	0.3	3	$[135-CH_3]^{2+}$
146 M(Cl; *m/2e*)	1.8	3	$[335-(CH_3+C_2H_4)]^{2+}$
103	36	9	$[(CH_3)_3SiOCH_2]^+$
B(TMS)-4(CH$_3$*)*$_2$			
380 (Cl)	2		$[M]^+$
365 (Cl)	17		$[M-CH_3]^+$
350 (Cl)	100		$[M-CH_2O]^+$
335 (Cl)	4		$[350-CH_3]^+$
277 (Cl)	95		$[M-(CH_3)_3SiOCH_2]^+$
241 (Cl)	4		(*x*; Scheme 3)[b]
199	6		
169 (Cl)	5		(*g*; Scheme 1)[b]
109	14		R_2 ion
103	20		$[(CH_3)_3SiOCH_2]^+$
B(TMS)-7			
407.1079 (Cl)	5	9	$[M]^+$
392 (Cl)	1	6	$[M-CH_33]^+$
377.0972 (Cl)	26	9	$[M-CH_2O]^+$
351.0578 (Cl)	1.3	6	$[392-C_2H_3N]^+$
336.0714 (Cl)	6.5	9	$[377-C_2H_3N]^+$
335 (Cl?)	3	9	
305.0578 (Cl)	31	0	$[M-(CH_3)_3SiOCH]^+$
304.0502 (Cl)	8	0	$[M-(CH_3)_3SiOCH_2]^+$
277 (Cl?)	2.5	0	
276 (Cl?)	2.5	0	
264 (Cl?)	55	0	
263.0218 (Cl)	100	0	$[304-C_2H_3N]^+$
169 (Cl)	38	0	(*g*; Scheme 1)[b]
152.0388 (Cl)	12.5	0	$[C_9H_9Cl]^+$
141 (Cl)	10	0	(*h*; Scheme 1)[b]
137.5 (Cl; *m/2e*)	0.2	0	$[304-CHO]^{2+}$
136.0160	7.5	0	R_2 ion
123.5 (Cl; *m/2e*)	1.3	0	$[304-(CHO+C_2H_4)]^{2+}$
115	5	0	
113	5	0	
103	12	9	$[(CH_3)_3SiOCH_2]^+$
42	90	0	(*rr*; Scheme 6)[b]
B(TMS)-8(TMS)			
465.1324 (Cl)	0.1	18	$[M]^+$
450 (Cl)	0.1	15	$[M-CH_3]^+$
435.1210 (Cl)	0.5	18	$[M-CH_2O]^+$
409 (Cl)	0.3	15	$[450-C_2H_3N]^+$
394.0937 (Cl)	1.2	18	$[435-C_2H_3N]^+$
393 (Cl?)	1	18	
363.0795 (Cl)	5.5	9	$[M-(CH_3)_3SiOCH]^+$
362.0746 (Cl)	3.5	9	$[M-(CH_3)_3SiOCH_2]^+$
335 (Cl?)	2	9	

TABLE 4 (*continued*)

m/e[a]	Rel. int.	Δ a.m.u. for [$^2H_9$]TMS	Ion description
B(TMS)-8(TMS)			
334 (Cl?)	0.8	9	
322 (Cl?)	7	9	
321.0469 (Cl)	12	9	$[362-C_2H_3N]^+$
241 (Cl)	2	9	(*x*; Scheme 3)[b]
194.0404	9	9	R_2 ion
173.5 (Cl; *m/2e*)	0.2	3	$[362-CH_3]^{2+}$
169 (Cl)	15	0	(*g*; Scheme 1)[b]
159.5(Cl; *m/2e*)	0.7	3	$[362-(CH_3+C_2H_4)]^{2+}$
103	13	9	$[(CH_3)_3SiOCH_2]^+$
42	100	0	(*rr*; Scheme 6)[b]
Mixture of B(TMS)-6(TMS)/B(TMS)-4(TMS)$_2$			
496 (Cl)	14		
481 (Cl)	43		
466 (Cl)	73		
451 (Cl)	6		$[M]^+$ (B(TMS)-6(TMS)]
436 (Cl)	12		$[451-CH_3]^+$
421 (Cl)	98		$[451-CH_2O]^+$
411	2		
410	5		
409	7		
408	11		
407	2		
393 (Cl)	100		
377 (Cl)	2		
371	7		
357 (Cl)	4		
348 (Cl)	17		$[451-(CH_3)_3SiOCH_2]^+$
320 (Cl?)	16		$[348-CO]^+$
319 (Cl?)	10		
299	5		
241 (Cl)	10		
225	6		
221	4		
211	9		
169 (Cl)	18		
167	6		
103	10		
B(TMS)-9(TMS)			
344 (Cl)	2		$[M]^+$
329 (Cl)	2.5		$[M-CH_3]^+$
257 (Cl)	4		
241 (Cl)	100		$[M-(CH_3)_3SiOCH_2]$ (*x*; Scheme 3)[b]
157(Cl; *m/2e*)	1.4		$[M-2CH_3]^{2+}$
C(TMS)-1(TMS)			
465.1311 (Cl)	19	18	$[M]^+$
450 (Cl)	49	15	$[M-CH_3]^+$
430	8	18	$[M-Cl]^+$
421.1431 (Cl)	100	18	$[M-CO_2]^+$
420 (Cl?)	22	18	
406 (Cl)	4	15	$[421-CH_3]^+$
348 (Cl)	64	9	$[M-(CH_3)_3SiOOC]^+$
217.5 (Cl; *m/2e*)	4	6	$[M-2CH_3]^{2+}$
180	66	9	R_2 ion
166.5 (Cl; *m/2e*)	2	3	$[348-CH_3]^{2+}$
152.5 (Cl; *m/2e*)	8	3	$[348-(CH_3+C_2H_4)]^{2+}$
C(CH$_3$)-1			
335 (Cl)	18		$[M]^+$
320 (Cl)	0.5		$[M-CH_3]^+$

TABLE 4 (*continued*)

m/e[a]	Rel. int.	Δ a.m.u. for [$^2H_9$]TMS	Ion description
C(CH_3)-1			
304 (Cl)	1		$[M-CH_3O]^+$
300	32		$[M-Cl]^+$
276 (Cl)	100		$[M-CH_3OOC]^+$
182 (Cl)	83		Scheme 5
169 (Cl)	50		(*g*; Scheme 1)[b]
141 (Cl)	9		(*h*; Scheme 1)[b]
137.5 (Cl; *m/2e*)	0.5		$[276-H]^{2+}$
123.5 (Cl; *m/2e*)	1		$[276-(H+C_2H_4)]^{2+}$
108	100		R_2 ion
C(CH_3)-3(*TMS*)			
393 (Cl)	27		$[M]^+$
378 (Cl)	55		$[M-CH_3]^+$
358	100		$[M-Cl]^+$
334 (Cl)	61		$[M-CH_3OOC]^+$
318 (Cl)	13		$[378-CH_3OOCH]^+$
169 (Cl)	12		(*g*; Scheme 1)[b]
168 (Cl?)	5		(*i*; Scheme 1)[b]
166	10		R_2 ion
159.5 (Cl; *m/2e*)	1.5		$[334-CH_3]^{2+}$
145.5 (Cl; *m/2e*)	6		$[334-(CH_3+C_2H_4)]^{2+}$
Mixture of C(*TMS*)-4(*TMS*)$_2$[c]/*C*(*TMS*)-5(*TMS*)			
510 (Cl)	0.1	27	$[M]^+$[C(TMS)-4(TMS)$_2$]
495 (Cl)	3	24	$[510-CH_3]^+$
466 (Cl)	8	27	$[510-CO_2]^+$
465 (Cl?)	2	27	
452 (Cl)	1.5	18	$[M]^+$[C(TMS)-5(TMS)]
437 (Cl)	37	15	$[452-CH_3]^+$
408 (Cl)	100	18	$[452-CO_2]^+$
407 (Cl?)	39	18	
393 (Cl)	14	18	$[510-(CH_3)_3SiOOC]^+$
335 (Cl)	62	9	$[452-(CH_3)_3SiOOC]^+$
241 (Cl)	39	9	(*x*; Scheme 3)[b]
225	5	18	R_2 ion [C(TMS)-4(TMS)$_2$]
211	12	12	$[\{(CH_3)_2Si\}_2PO_4]^+$
169 (Cl)	30	0	(*g*; Scheme 1)[b]
167	8	9	R_2 ion [C(TMS)-5(TMS)]
117	30	9	$[(CH_3)_3SiOOC]^+$
C(CH_3)-4(CH_3)$_2$			
336 (Cl)	12		$[M]^+$
301 (Cl)	5		$[M-Cl]^+$
277 (Cl)	100		$[M-CH_3OOC]^+$
109	15		R_2 ion
C(*TMS*)-9(*TMS*)			
358 (Cl)	12		$[M]^+$
343 (Cl)	30		$[M-CH_3]^+$
241 (Cl)	100		$[M-(CH_3)SiOOC]^+$
225 (Cl)	2		
211 (Cl)	5		
191	30		
164 (Cl; *m/2e*)	1.5		$[M-2CH_3]^{2+}$
113 (Cl; *m/2e*)	1.5		$[241-CH_3]^{2+}$
99 (Cl; *m/2e*)	6		$[241-(CH_3+C_2H_4)]^{2+}$

[a] Exact masses determined for the underlined *m/e* values were compatible with the structures assigned. (Cl) denotes the presence of a chlorine isotope cluster. (Cl?) denotes the probable presence of chlorine.

[b] Parenthetical notations refer to ions described in Ref. 2.

[c] See Ref. 2 for spectrum from synthetic C(TMS)-4(TMS)$_2$.

R_1 = COOH (C series)

The mass spectra from the TMS derivatives of the metabolites characterized as carboxylic acids (R_1 = COOH, C series) gave intense fragment ions at $[M-117]^+$ [117 = $(CH_3)_3SiOOC$] which shifted to $[M-126]^+$ with [$^2H_9$]TMS derivatives. This fragmentation is characteristic for TMS-esters of carboxylic acids.[7] When a phosphorus-containing R_2 moiety was present, the C series TMS derivatives also gave intense, chlorine-containing fragment ions at $[M-44]^{\ddagger}$. The $[M-44]^{\ddagger}$ ions indicated a fragmentation mode analogous with the $[M-30]^{\ddagger}$ fragmentation mode observed from the TMS ether moieties of the B series metabolites. Exact mass measurements of the $[M]^{\ddagger}$ and $[M-44]^{\ddagger}$ ions in the mass spectrum of C(TMS)-1(TMS) indicated that the loss of 44 a.m.u. resulted from the elimination of CO_2 from the molecular ion. The measured masses of these ions were within 1.7 and 1.5 ppm, respectively, of the values calculated for $C_{18}H_{33}NO_5PClSi_2$ and $C_{17}H_{33}NO_3PClSi_2$. This evidence indicated that the $[M-44]^{\ddagger}$ fragment ions resulted from the elimination of CO_2 from the TMS carboxylic ester with rearrangement of the TMS moiety. As with the $[M-30]^{\ddagger}$ fragmentation mode observed with the B series metabolites, the presence of the phosphorus-containing moiety was required because this 44 a.m.u. loss was not observed in the mass spectrum of C(TMS)-9(TMS).

Two urinary metabolites of the C series (C-4 and C-5) were isolated from the LH-20(H_2O) fractions 1 and 2 (Scheme 1). These fractions eluted from the column as an unresolved doublet which we were unable to separate subsequently. The presence of C-4 and C-5 in both fractions was inferred from the following mass spectral data.

The mass spectrum of the TMS derivative of the metabolite(s) isolated from LH-20 (H_2O) fraction 1 [C(TMS)-4(TMS)$_2$/C(TMS)-5(TMS) Table 4] indicated the presence of two chlorine-containing components with molecular ions of masses 510 and 452. Associated with each molecular ion were chlorine-containing fragment ions at $[M-15]^+$, $[M-44]^{\ddagger}$ and $[M-117]^+$. The difference of 58 a.m.u. between these two sets of ions indicated either the presence of TMS derivatives of both structures C-4 and C-5 or a rearrangement of the 510 ion involving the elimination of 58 a.m.u. The [$^2H_9$]TMS derivatives of this fraction gave a mass spectrum in which the proposed molecular ions at masses 510 and 452 had shifted to 537 and 470, respectively. These changes in mass showed that the 510 ion contained three TMS groups and the 452 ion contained two TMS groups. Associated with the 537 ion and the 470 ion were chlorine-containing fragment ions at $[M-18]^+$ (18 = C^2H_3), $[M-44]^{\ddagger}$ (44 = CO_2) and $[M-126]^+$ [126 = $COOSi(C^2H_3)_3$]. If the ion with mass 470 was formed by a fragmentation mode of the 537 molecular ion, the leaving group would have had to contain nine deuterium atoms, presumably as C^2H_3 moieties, which would have given the leaving group the unlikely elemental composition of $C_4{}^2H_9H$. From these data we inferred that metabolites C-4 and C-5 were present in the first fraction eluted from the LH-20 (H_2O column).

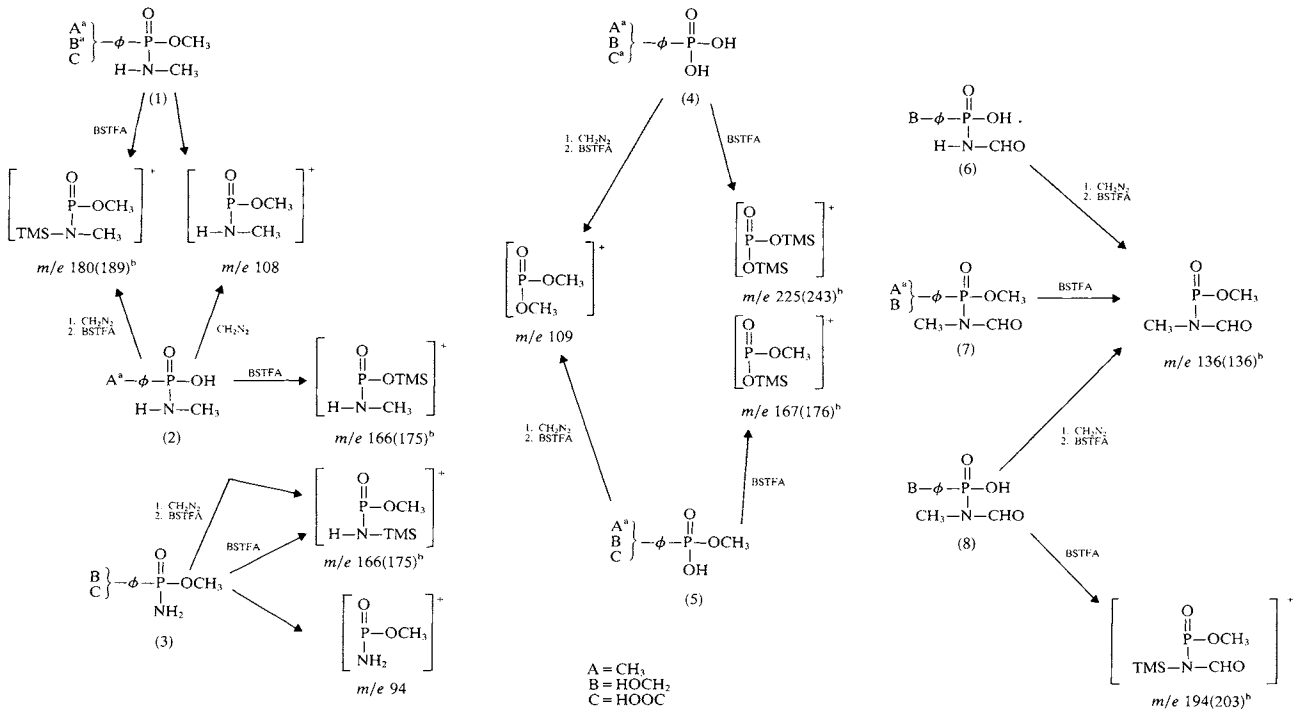
When LH-20 (H_2O) fraction 2 from the urine was treated with diazomethane only one radioactive component was present. This component gave a mass spectrum identical to that obtained from sythesized $C(CH_3)$-4$(CH_3)_2$.[2] We were unable to purify the radiolabeled metabolites in LH-20 (H_20) fraction 2 without treatment with diazomethane during the isolation procedure; therefore, this fraction may have contained a mixture of C-4 and C-5. The radioactivity in fractions 1 and 2 was assigned structures C-4 and C-5.

Metabolite C-3 was detected in the sheep plasma only. It co-chromatographed on the LH-20 columns with C-1 and C-4 and, as the TMS derivative, co-gas chromatographed with C(TMS)-1(TMS) and C(TMS)-4(TMS)$_2$ (deduced from the mass spectrum obtained from the mixture). This mixture of the TMS derivatives of C-1, C-3 and C-4 separated by g.l.c. after methylation. All were converted to the methyl esters as indicated by intense $[M-COOCH_3]^+$ ions. The mass spectra showed that both C-1 and C-3 added one methyl group, while C-4 added three. Methylated C-1 and C-3 gave mass spectral fragmentation patterns similar to that of $C(CH_3)$-4$(CH_3)_2$ (Table 4). In addition, methylated C-1 gave the rearrangement ion with a mass of 182. (For a discussion of the ion at *m/e* 182, see 'Mass spectral determination of structures of R_2 moieties' section and Scheme 5.) This ion was not present in the mass spectrum from methylated C-3. The absence of the *m/e* 182 ion and the evidence that the R_2 moiety from C-3 did not methylate (R_2 ion at 92) were the basis for assigning the structure to metabolite C-3.

It is of interest that C-4 from the plasma metabolites eluted from the LH-20 (H_2O) column much later than the C-4 isolated from untreated urine, and the C-4 in the plasma bound to Porapak Q from aqueous solution, but the C-4 in the urine would not bind. This anomolous behavior may have resulted from the existence of C-4 as an ionized salt in the basic sheep urine and in a different, partly unionized form in the plasma.

MASS SPECTRAL DETERMINATION OF STRUCTURES OF R_2 MOIETIES (TABLE 1 AND SCHEME 4)

Known compounds were available in the A series where R_2 = (1), (2), (4), (5), (7) and (9), which provided mass and infrared spectral correlations with metabolites in the B and C series that contained the same groups. All the derivatives of the compounds and metabolites known to contain phosphorus, except B(TMS)-6(TMS), gave fragment ions that resulted from cleavage between the phosphorus and the phenolic oxygen, with the charge remaining on the phosphorus-containing moiety (R_2 ion). The R_2 ions in the mass spectra of the various derivatives of the



SCHEME 4. Structures of ions in the mass spectra from the various derivatives of the compounds that were used to assign structures to the R_2 moieties. [a] Synethetic standards available. [b] Mass for [$^2H_9$]TMS derivative.

metabolites that were used to assign structures to these moieties are given in Scheme 4.

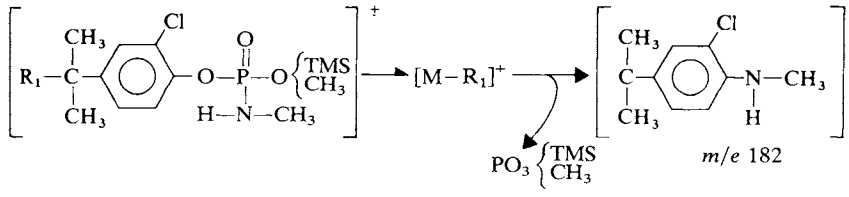
Synthetic compounds were not available where R_2 = (3), (6) and (8). The phosphoramidate methyl ester ($R_2 = 3$) was differentiated from the *N*-methylphosphoramidate ($R_2 = 2$) by reaction with diazomethane. The latter reacted with diazomethane to yield an $R_2 = 1$ fragment ion, whereas the former did not react with diazomethane.

R_2 ions were present in the mass spectra from the TMS derivatives of metabolites B-7 and B-8, but not B-6. The assignment of structures to the R_2 moieties of these proposed *N*-formyl phosphoramidates will be discussed below.

A chlorine-containing rearrangement fragment ion at *m/e* 182 (Scheme 5) inferred the presence of the *N*-methyl phosphoramidate group in the R_2 moieties of these molecules. In the mass spectrum of A-1 this ion was a metastable daughter from the $[M-CH_3]^+$ ion at *m/e* 276. This *m/e* 182 ion had an elemental composition of $C_{10}H_{13}NCl$ and can best be visualized as an *N*-methyl-2-chloro-4-isopropyl-anilinium ion. This *m/e* 182 ion shifted to *m/e* 185 in the mass spectrum from A-1 containing a synthetic trideutero *N*-methyl group[2] and this ion was observed only when the phosphoramide hydrogen was present. The *m/e* 182 ion was assumed to result from the metastable elimination of PO_3CH_3 [when $R_2 = (1)$] or $PO_3Si(CH_3)_3$ [when R_2 = the TMS ester of (2)] from the $[M-R_1]^+$ precursor.

STRUCTURAL CHARACTERIZATION OF THE PROPOSED *N*-FORMYL PHOSPHORAMIDATE METABOLITES (B-6, B-7 AND B-8)

Both B-7 and B-8 was isolated from the urine and plasma (Schemes 1 and 3). B-6 was isolated from the urine from sheep 2 only (Scheme 1). We were unable to separate B-6 from B-4.



SCHEME 5. Proposed fragmentation pathway leading to the production of the *m/e* 182 ion in mass spectra from the *N*-methyl phosphoramidate-containing metabolites.

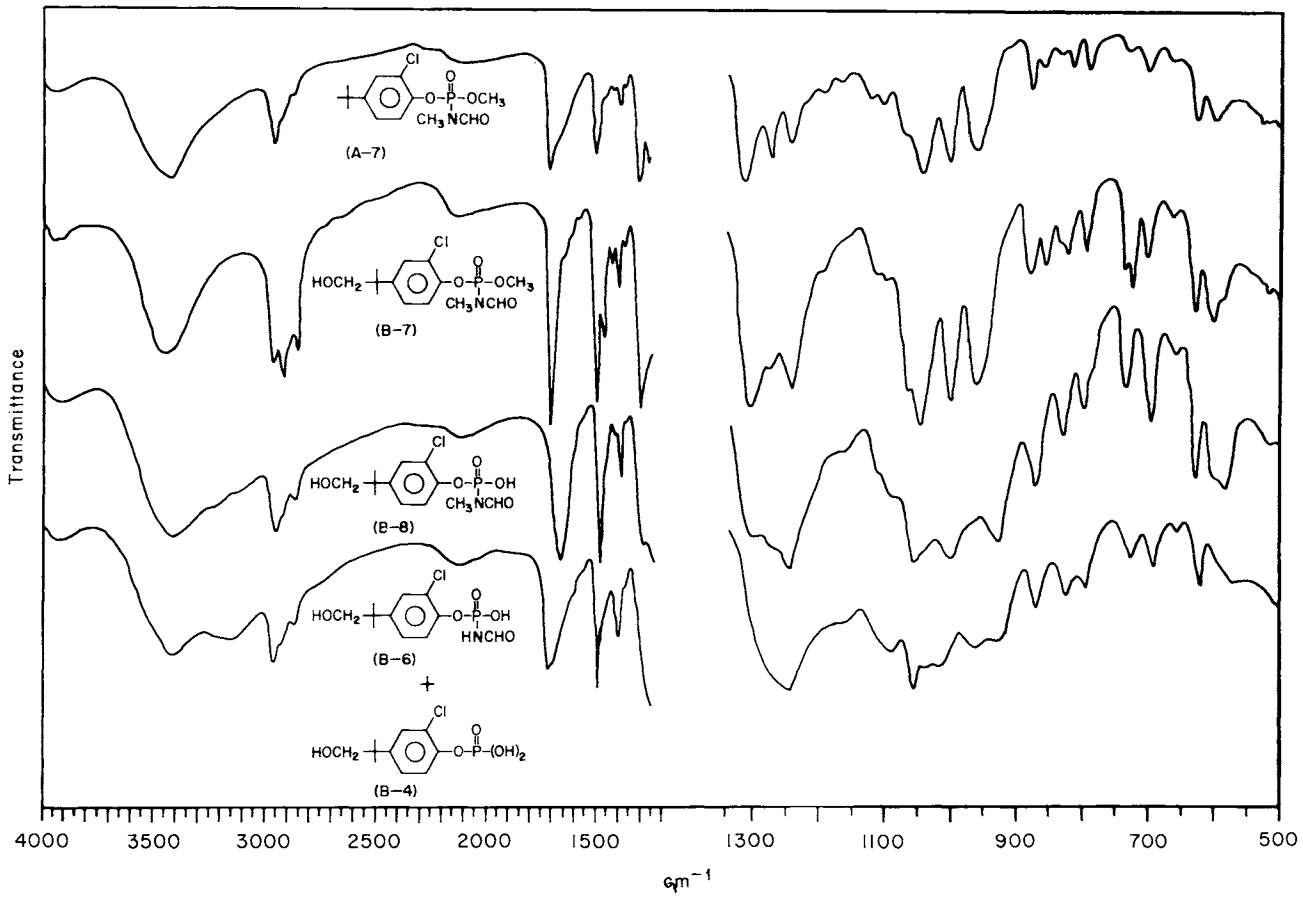


FIG. 1. Infrared spectra of A-7, B-7, B-8 and the mixture of B-6 with B-4.

The TMS derivatives of all three metabolites contained the fragment ions characteristic of the TMS derivatives of the B series compounds. The exact masses of these ions ($[M]^+$, $[M\text{-}CHO]^+$ and $[M-(CH_3)_3SiOCH_2]^+$) in the mass spectra of B(TMS)-7 and B(TMS)-8(TMS) agreed with the structures assigned.

Methylation of B-8 and B-6 (mixture of B-6 and B-4) with diazomethane partially converted B-6 and B-8 to B-7. This conversion indicated that B-6, B-7 and B-8 were analogs which differed only in the number of methyl groups in the phosphoramidate moieties. The evidence for the structures of the R_2 moieties of B-7, B-8 and B-6 will be presented below.

Metabolite B-7

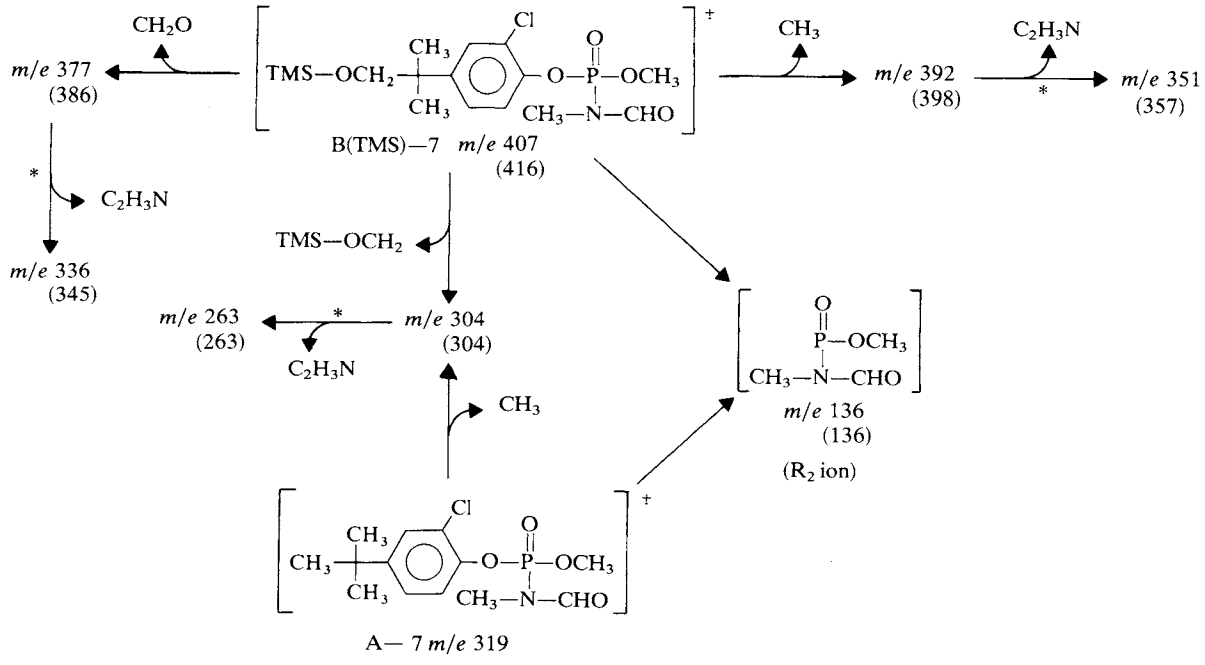
The A-7 analog of B-7 was synthesized and its structure confirmed by mass i.r. and n.m.r. spectrometry.[2] The i.r. spectra (KBr micropellet) from B-7 and A-7 (Fig. 1) were almost identical (A-7 also adsorbing strongly in the carbonyl region at 1705 cm^{-1}). The carbonyl group in B-7 was assumed to be present in the R_2 moiety because B-9 was the only radioactive compound present in the hydrolyzate of B-7 (10% NaOH, reflux, 18 h). This confirmed that B-7 was a metabolite of the B series.

Three mass spectral fragmentation modes were common to both A-7 and B(TMS)-7 (Scheme 6). These fragmentation modes resulted in ions at *m/e* 304, 263 and 136 (R_2 ion) in both spectra. The exact mass of the R_2 ion from B(TMS)-7 was within 3.5 ppm of that calculated for $C_3H_7NO_3P$. The R_2 ion from B($[^2H_9]$TMS)-7 remained at *m/e* 136 and thus demonstrated the absence of a TMS group in the $R_2 = 7$ fragment ion. The fragment ion at *m/e* 263 resulted from the metastable loss of C_2H_3N from the $[M-R_1]^+$ precursor at *m/e* 304. Two other metastable losses of C_2H_3N were present in the mass spectrum from B(TMS)-7 (Scheme 6). The mass spectra of A-7 synthesized with either a *N*-C^2H_3 or *O*-C^2H_3 group in the R_2 moiety demonstrated that only the *N*-methyl group was contained in the C_2H_3N leaving group.[2] The metastable elimination of C_2H_3N may be a characteristic fragmentation mode of the *N*-methyl-*N*-formyl phosphoramidate moiety because B(TMS)-8(TMS) also exhibited these fragmentation modes (Scheme 7). The similarities in the mass and i.r. spectra from A-7 and B(TMS)-7 and the elemental composition of the R_2 ion at *m/e* 136 were the basis for assigning the structure of the R_2 moiety of B-7.

In the purified form, B-7 and A-7[2] slowly eliminated carbon monoxide to form B-1 and A-1, respectively. This reaction was very slow requiring several months for complete conversion.

Metabolite B-8

Because B-8 was converted to B-7 when reacted with diazomethane and because B(TMS)-8(TMS) exhibited all the fragmentation modes (Scheme 7)



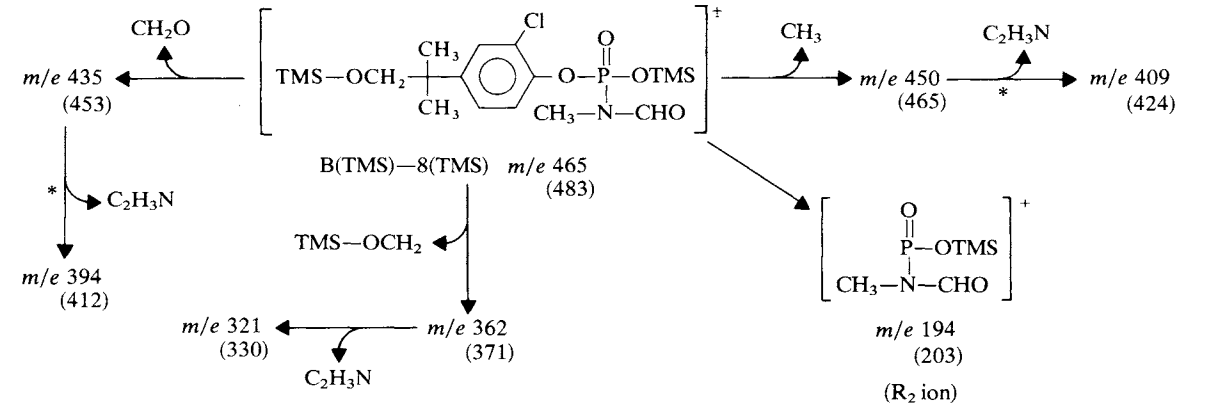
SCHEME 6. Mass spectral fragmentation modes of B(TMS)-7 and synthetic A-7.

involving the metastable elimination of C_2H_3N that were observed in the mass spectrum from B(TMS)-7, the R_2 moiety of B-8 was assumed to be a *N*-methyl-*N*-formyl phosphoramidate. The exact mass of the R_2 ion at *m/e* 194 in the mass spectrum of B(TMS)-8(TMS) agreed with the structure assigned. The R_2 ion shifted to *m/e* 203 for B([$^2H_9$]TMS)-8([$^2H_9$]TMS); this demonstrated the presence of one TMS moiety.

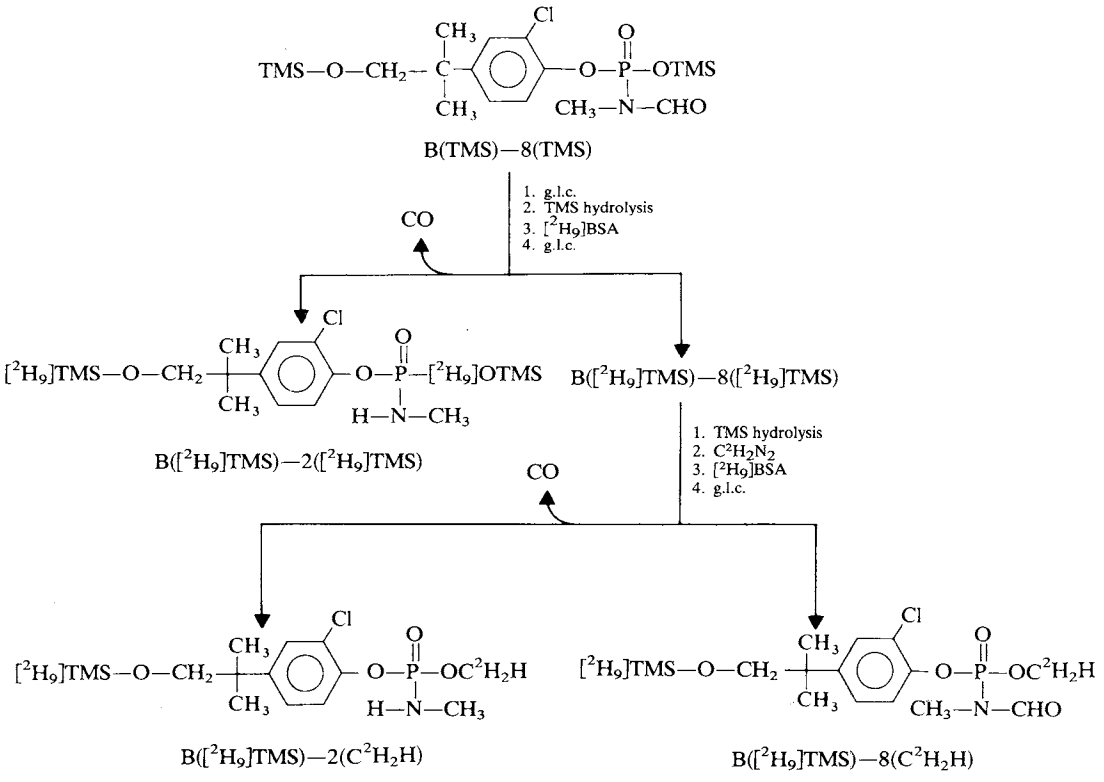
B-8 had a strong absorption band in the carbonyl region (1675 cm^{-1}, Fig. 1). The carbonyl group was assumed to be present in the R_2 moiety because B-9 was the only radioactive compound present in the hydrolyzate of B-8 (10% NaOH, reflux, 18 h).

B-8 in the purified form (B-8 regenerated from the diTMS derivative trapped from the g.l.c.) as the free acid eliminated carbon monoxide to form B-2. Experimental evidence for the elimination of carbon monoxide is summarized in Scheme 8. B([$^2H_9$]TMS)-2([$^2H_9$]TMS) and B([$^2H_9$]TMS)-2(C^2H_2H) were assigned as the structures for the two carbon monoxide elimination products because of the presence of the *m/e* 182 fragment ions (Scheme 5) in the mass spectra. The presence of the *m/e* 182 ion in the mass spectrum B([$^2H_9$]TMS)-2(C^2H_2H) indicated that the methyl group in B-8 was present as an *N*-methyl.

The elimination of carbon monoxide from B-8 was more rapid (2–3 days for complete conversion) than that observed with B-7 (possibly catalyzed by the free acid function). In the time required to carry out the reaction steps in Scheme 8 (2–3 h), approximately 10% of the B-8 had eliminated carbon monoxide to form B-2.



SCHEME 7. Mass spectral fragmentation of B(TMS)-8(TMS).



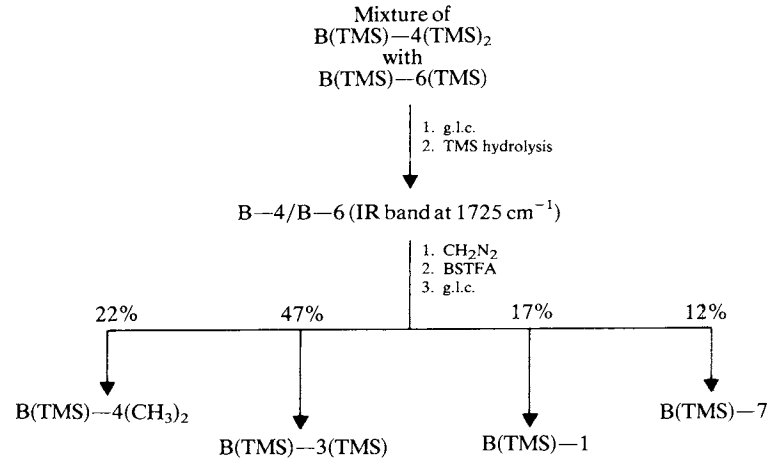
SCHEME 8. Evidence for the spontaneous elimination of carbon monoxide from B-8.

Metabolite B-6

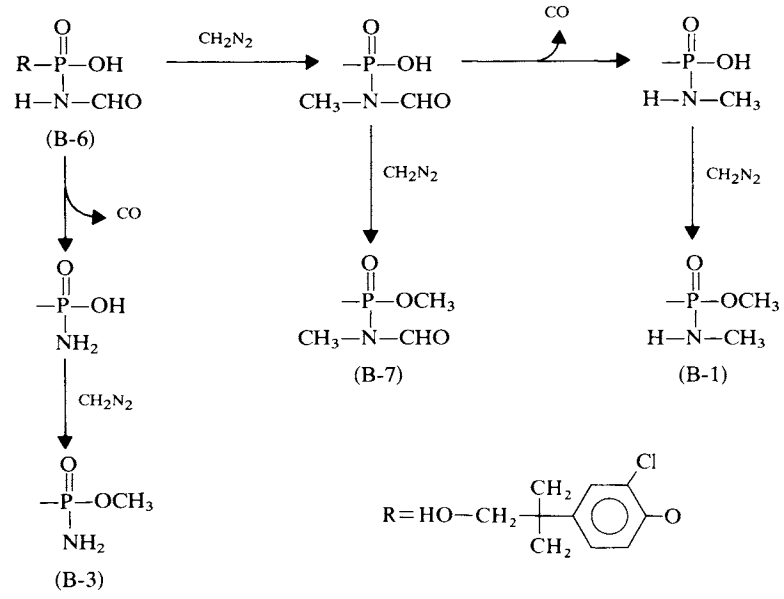
The TMS derivative of B-6 was observed as a metabolite that co-gas chromatographed with B(TMS)-4(TMS)$_2$. The mass spectrum from the mixture of B(TMS)-4(TMS)$_2$ and B(TMS)-6(TMS) is given in Table 4. The chlorine-containing ions at *m/e* 451 ($[M]^+$), 436 ($[M-CH_3]^+$), 421 ($[M-CH_2O]^+$) and 348 ($[M-CH_2OTMS]^+$) indicated that B(TMS)-6(TMS) was a metabolite of the B series in which R_2 was a phosphoramidate (from the odd mass [M$^+$]). B(TMS)-1(TMS), which yields these same fragment ions, was eliminated as the structure because no R_2 ion was present at 180.

When the mixture of B(TMS)-4(TMS)$_2$ and B(TMS)-6(TMS) was treated as outlined in Scheme 9, B-6 was converted to B-1, B-3 and B-7. Although the yields of B-7 were low (12% of the ^{14}C trapped from the g.l.c.), the conversion of B-6 to B-7 by treatment with diazomethane was evidence that B-6 was an *N*-formyl phosphoramidate. The absorption band at 1725 cm^{-1} in the i.r. spectrum from the mixture of B-4 and B-6 (Fig. 1) demonstrated the presence of a carbonyl group (B-4 had no absorption in the carbonyl region). Because the molecular ion for B(TMS)-6(TMS) was 14 a.m.u. less than that for B(TMS)-8(TMS), B-6 differed from B-8 only in that B-6 did not have the *N*-methyl group.

The modification in the R_2 moiety of B-6 which produced B-1, B-3 and B-7 was presumed to have resulted by the sequence outlined in Scheme 10. B-6 in



SCHEME 9. Flow scheme of the production of B-1, B-3, B-4 $(CH_3)_2$ and B-7 from the mixture of B-4 and B-6.



SCHEME 10. Proposed reaction sequence for the production of B-1, B-3 and B-7 from B-6.

the free acid form apparently eliminates carbon monoxide very readily because most of the radioactivity from B-6 was isolated, after methylation, as B-3 and B-1.

EXCRETION AND RESIDUES FROM [^{14}C]CRUFOMATE (SHEEP 1)

Eighty-two percent of the ^{14}C given to sheep 1 as a single oral dose was excreted in the urine within 48 h after dosing. No $^{14}CO_2$ was detected in the expired air and 4.7% of the ^{14}C was recovered in the feces. The carcass of the sheep, 48 h after dosing, contained 4.5% of the dose (eviscerated carcass, 2.8%; gastrointestinal tract and contents, 1%; assayed organs, 1.7%). Of the tissues assayed, liver, kidney and spleen contained the highest residues of ^{14}C from [^{14}C]crufomate. The residues in ppm of crufomate equivalents per gram of wet tissue were: kidney, 7.3; liver, 4.9; spleen, 1·3; lung, 0.9; heart and leg muscle, <0.3; visceral fat, none detected.

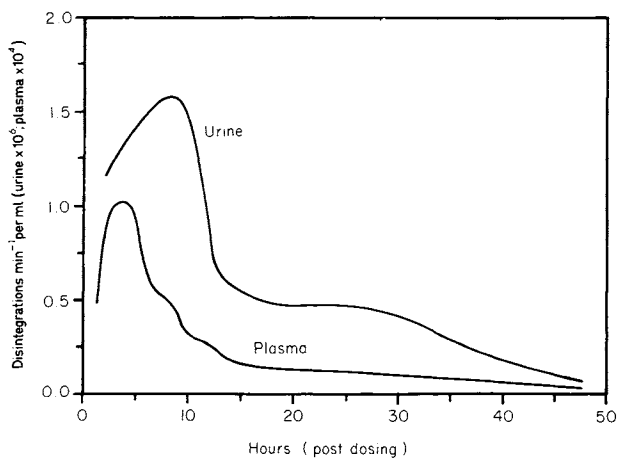


FIG. 2. Plasma and urine concentrations of ^{14}C with time after a single oral dose of [^{14}C]crufomate.

The period of maximum concentration of ^{14}C in the urine (4–8 h after dosing) paralleled the period of maximum blood concentration (3–5 h after dosing) of ^{14}C from [^{14}C]crufomate (Fig. 2). These data demonstrate the rapid absorption of the ^{14}C from [^{14}C]-crufomate into the circulatory system and, because no crufomate was detected in the blood or the urine by the separation methods used, also demonstrated the rapid metabolic transformation of crufomate.

The sheep blood levels of ^{14}C from [^{14}C]crufomate increased to a maximum of 25.4 μg equivalents of crufomate per ml within 3–5 h after dosing. This concentration decreased to 6 μ g per ml after 12 h and to less than 1μg per ml at 48 h. Ninety-eight percent of this blood ^{14}C was in the plasma fraction.

Three metabolites were isolated from sheep plasma that were not found in the urine. These were A-2, A-5 and C-3.

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