Metabolic Fate of *N,N*-Dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide in Rat, Dog, and Human

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Abstract □ The patterns of urinary excretion products of *N,N*-dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide (I) in the rat, dog, and human have been examined. After oral administration, the drug was extensively metabolized in all species and the amount of intact drug excreted in the urine was negligible (<1%). The metabolic pathways were *via* hydroxylation of the phenoxy ring, *N*-demethylation, *O*-methylation, and conjugation. The following metabolites have been identified in free and/or conjugated form: *N*-methyl-*N'*-(*p*-phenoxyphenyl)sulfamide (II), *N,N*-dimethyl-*N'*-*p*-(4-hydroxyphenoxy)phenylsulfamide (III), *N,N*-dimethyl-*N'*-*p*-(2-hydroxyphenoxy)phenylsulfamide (IV), *N*-methyl-*N'*-*p*-(4-hydroxyphenoxy)phenylsulfamide (V), *N*-methyl-*N'*-*p*-(2-hydroxyphenoxy)phenylsulfamide (VI), *N,N*-dimethyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VII), and *N*-methyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VIII). The major differences in the patterns of urinary metabolites were: (*a*) the monomethyl (II) was the major metabolite in the dog and human but a minor one in the rat, (*b*) the *para*-hydroxylated compound (III) was the major metabolite in the rat, (*c*) hydroxylation of the phenoxy ring in the *ortho*-position (IV and VI) was observed in the rat and dog but not in the human, and (*d*) the methoxylated compound (VII) was found only in rat urine.

Keyphrases □ *N,N*-Dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide—urinary excretion after oral administration, metabolic pathways, rat, dog, man □ Urinary excretion—metabolites of *N,N*-dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide, oral administration, rat, dog, man □ Metabolism, *N,N*-dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide—urinary products after oral administration, rat, dog, man

N,N-Dimethyl- *N'* -(*p*-phenoxyphenyl)sulfamide[1] (I) reduces cholesterol levels significantly in both tyloxapol[2]-treated and cholesterol–cholic acid-fed rats (1). It has no significant effect on the cholesterol levels in normal rats and dogs. TLC analyses indicated that the drug was extensively metabolized. The results from studies designed to characterize the metabolism of I in the rat, dog, and human are reported here.

EXPERIMENTAL

Dosage and Collection of Samples—*Rat*—Micronized I was suspended in 0.25% methylcellulose in sterile water[3] with a glass Teflon homogenizer. The suspension was administered *via* oral intubation to six Sprague–Dawley rats (three males and three females) at a dose level of 100 mg/kg. A second group of six rats (three males and three females) that received the vehicle only served as the control. The rats were dosed daily for 21 days. Urine samples were collected daily, pooled, and kept frozen until analyzed.

Dog—Four dogs, participating in chronic toxicity studies, had received daily oral doses of 100 mg/kg I for approximately 10 months. Urine samples were collected by catheterization at 4–8 hr after administration of the drug. Urine samples were also obtained from untreated animals.

Human—Four subjects each received a single 500-mg oral dose of I in hard-filled capsules. Urine samples were collected from −12 to 0 and from 0 to 24 hr after drug administration. The postadministration urine for each subject was pooled. Urine collected prior to drug administration was used as the control.

Extraction and Purification—Aliquots of the urine samples (150–200 ml) were acidified to pH 3.5 with acetic acid and extracted six times with an equal volume of chloroform. The chloroform layers were separated, pooled, and concentrated under reduced pressure. Residual chloroform in the extracted urine specimens was removed at 45° under reduced pressure (1 hr). The pH of the urine was adjusted to 5.0 with 1 *M* sodium acetate buffer (pH 5.0), and 1/100 volume of a concentrated β-glucuronidase aryl sulfatase solution[4] was added; the mixture was then incubated at 37° for 24 hr. The hydrolysis mixture was acidified to pH 3.5 and extracted three times with an equal volume of chloroform. The extracts were pooled and concentrated under reduced pressure. The control urine samples were processed in the same manner.

The concentrated extracts were streaked at the origin across three-fourths of the width of a 2-mm thick silica gel G-254 preparative TLC plate[5]. The corresponding control urine extract was streaked on the remaining one-fourth of the width of the plate. The plate was developed in a solvent system of chloroform–methanol (95:5). To obtain good resolution, the plate was developed, dried, and redeveloped three to five times. After development was completed, the chromatogram was visualized with a 254-nm UV lamp.

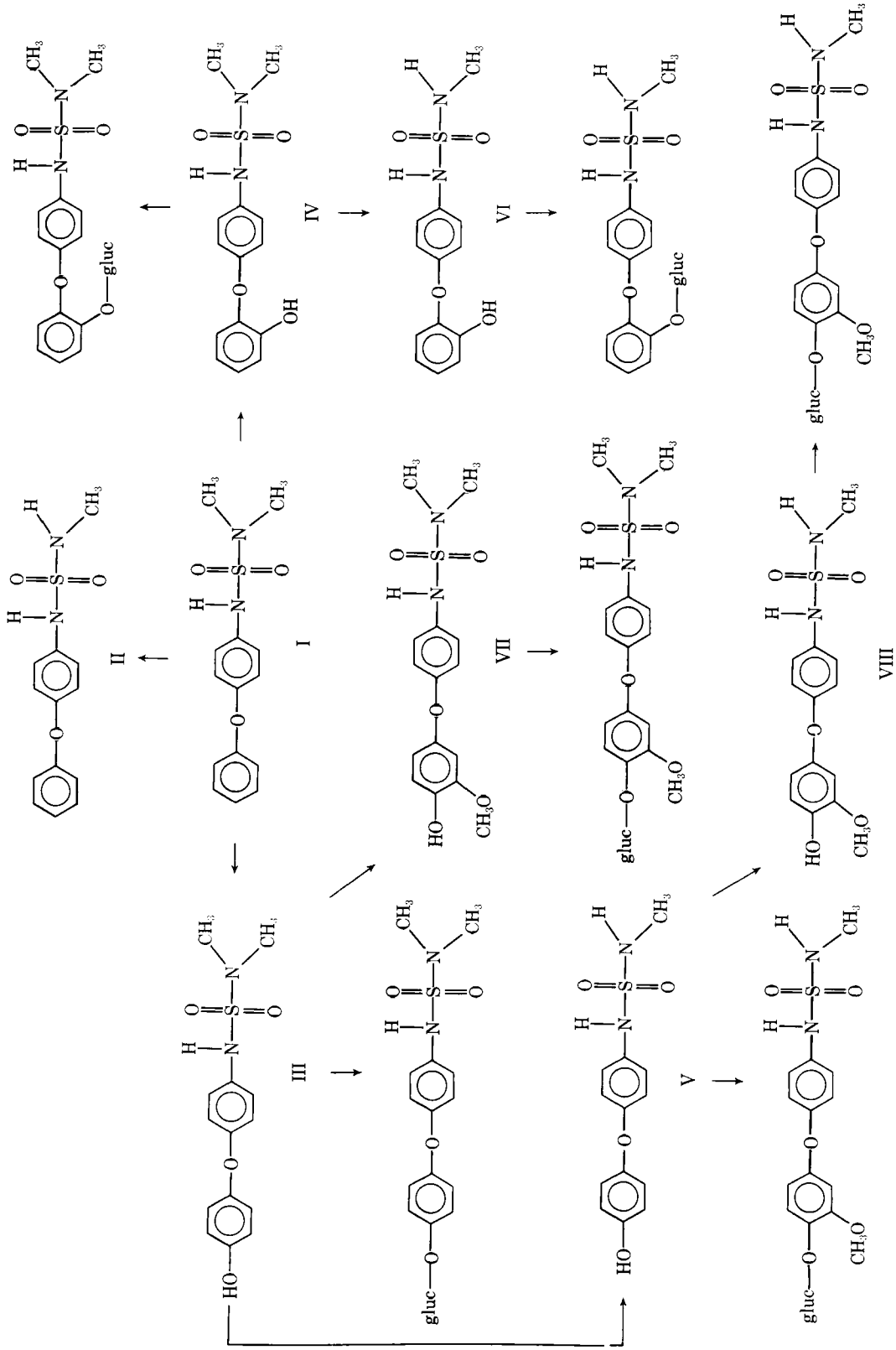
Zones that were present in the urine extracts from drug-treated subjects but not in the controls were scraped off the plate and eluted with chloroform–methanol (9:1). The purified extracts were evaporated to dryness. The residues were dissolved in a small amount of ethanol-free chloroform for introduction into the gas chromatograph[6]–mass spectrometer.

Identification of Metabolites—Identification of the urinary metabolites of I was carried out by using mass spectrometry, IR spectroscopy, and, when possible, NMR spectroscopy. Mass spectrometric analysis was routinely carried out as follows: 5 μl of a sample and 2 μl of bis(trimethylsilyl)acetamide were injected simultaneously into the gas chromatograph–mass spectrometer. A 1.21-m (4-ft) column of 1% SE-30 (methylpolysiloxy gum) on Gas Chrom Q was maintained at 210°. The mass spectrometer was operated at an ionization potential of 70 ev with an ion-source temperature of 290°. On-column silylation was selected for simplicity and conservation of material. Unless the metabolite being studied was contaminated with large amounts of urinary impurities, the on-column silylation technique generally provided a completely silylated derivative of the compound.

IR and NMR analyses were carried out without silylation. IR spectra were obtained with a spectrophotometer[7] and micro KBr pellets containing 50–100 μg of the sample. Since the NMR analysis[8] required 3–5 mg of sample, it was done only when that amount of material was available.

TLC Characterization of I Metabolites—Specific spray reagents were used to detect the phenolic and nonphenolic metabolites on the thin-layer chromatograms. Phenolic metabolites appeared immediately as a pink spot on the plate when sprayed with either of the following two reagents: (*a*) a freshly mixed 1:1 solution of 0.5% (w/v) aqueous ferric chloride and 0.5% (w/v) methanolic dipyridyl, and (*b*) a freshly mixed 1:1 solution of 0.5%

[1] V-25,030.
[2] Triton WR-1339.
[3] Upjohn Vehicle 122.
[4] Sigma type H-2 β-glucuronidase No. G-0876, 143,000 units/ml.
[5] Brinkmann.
[6] LKB-9000.
[7] Perkin-Elmer model 421.
[8] Varian XL-100.



Scheme I.—Proposed metabolic pathway of I in rat, dog, and human (gluc = glucuronide)

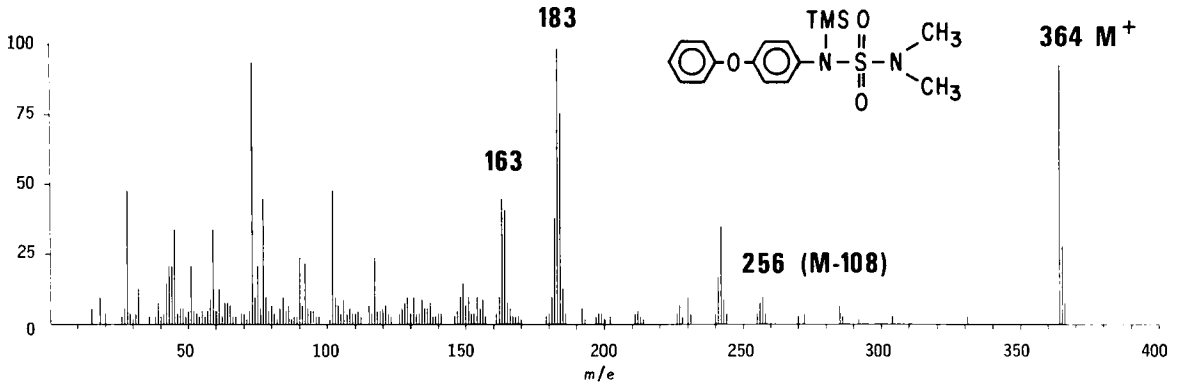


Figure 1—*Mass spectrum of trimethylsilyl derivative of I.*

benzidine in 0.17 *M* HCl and 10% aqueous sodium nitrite. These sprays were also used to detect any drug-related compounds obscured by other UV-absorbing materials present in the chromatographed urine extracts.

RESULTS AND DISCUSSION

Isolation and characterization studies of the urinary excretion products of I indicated extensive metabolism (Scheme I) of the drug in animals and humans. The metabolites were present in the urine mainly as glucuronide and/or sulfate conjugates. A summary of all detectable drug-related compounds found in the urine of I-treated rats, dogs, and humans is presented in Table I. A metabolic map, based on this information, is presented in Scheme I.

Combined GLC–mass spectrometry were used extensively to determine the structure of the metabolites. The mass spectrum of the trimethylsilyl derivative of I is presented in Fig. 1. The drug shows a strong molecular ion peak at *m/e* 364. Cleavage between the aniline and sulfamoyl group (M−108) gave another peak at 256. In the metabolism studies, the mass of these two fragments provided important information of *N*-demethylation pathways. The free phenoxyaniline ions appeared at *m/e* 183 and 184. Further fragmentation of the phenoxyaniline part of the molecule gave mass peaks at 163 and 164, which served as indicators of any metabolic modifications in the aromatic system.

Negligible amounts of unchanged drug were found in the urine specimens from animals and humans. In the rat and dog, the presence of trace amounts of the unchanged drug could be demonstrated only by GLC–mass spectrometric analysis. The relative amounts of each metabolite excreted were estimated from the size of the peaks on the gas chromatogram (Fig. 2). Evidence for the identification of the major metabolites is summarized below. The common mass peaks appearing in the mass spectra of I-related compounds are presented in Table II.

***N*-Methyl-*N'*-(*p*-phenoxyphenyl)sulfamide (II)**—This compound is the *N*-demethylation product of I found in the urine of the dog and human in unconjugated form. It was also found in the urine of the rat but only in trace amounts. The mass spectrum of the *N,N'*-bis(trimethylsilyl) derivative of II is shown in Fig. 3. The molecular ion was at *m/e* 422. Loss of the sulfamoyl group (M − 166) showed the material to be the desmethyl derivative of I. The fragmentation peaks at 163 and 164 indicated that the aromatic system was not modified. The most prominent peak in the spectrum was at *m/e* 198. Precise mass measurement gave the empirical formula of $C_{13}H_{12}NO$ (calc. 198.09189, found 198.09142). These results suggested that a methyl from one tri-

Table I—Summary of All Drug-Related Compounds Found in the Urine of Rat, Dog, and Human

Compound	Form of Excretion		Urine in which Compound Was Found		
	Free	Conjugated	Rat	Dog	Human
I	+	−	Trace	Trace	Trace
II	+	−	Trace	+	+
III	+	+	+	+	+
IV	+	+	+	+	−
V	+	+	+	+	+
VI	−	+	+	−	−
VII	−	+	+	−	−
VIII	−	+	+	+	+

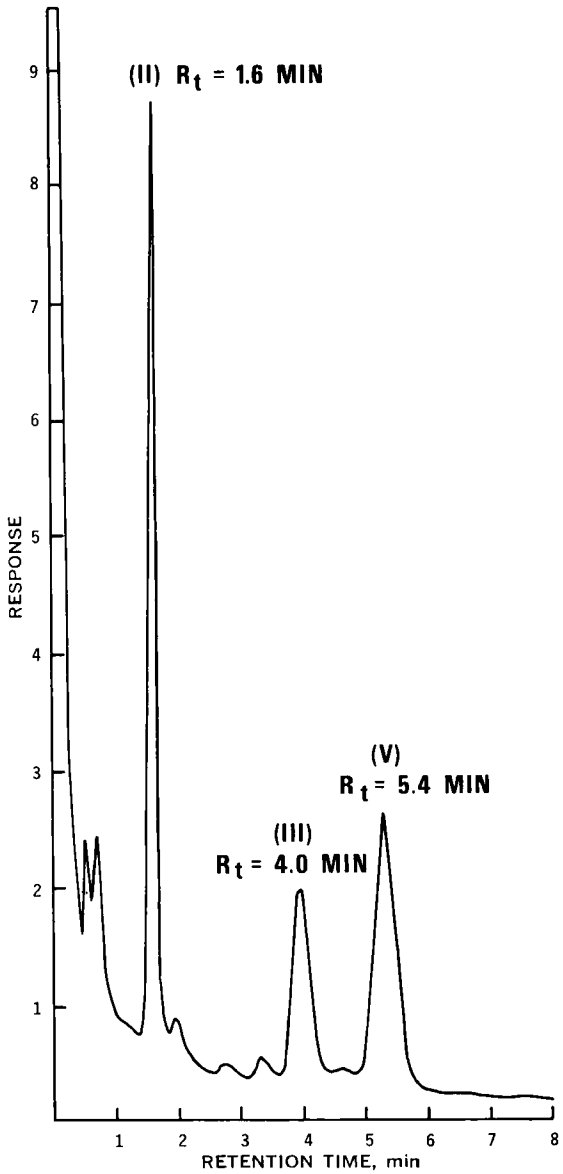


Figure 2—*Gas–liquid chromatogram of β-glucuronidase aryl sulfatase hydrolyzed (37° for 24 hr) human urine extract.*

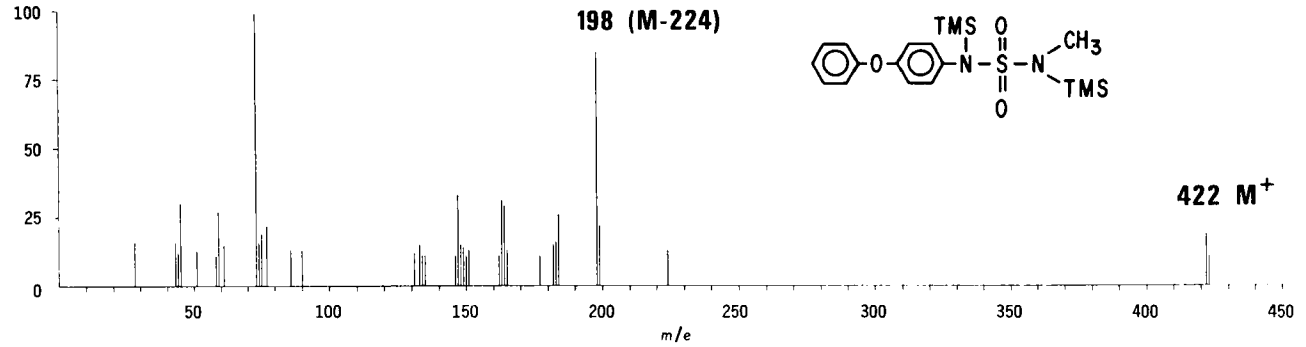


Figure 3—*Mass spectrum of bis(trimethylsilyl) derivative of II.*

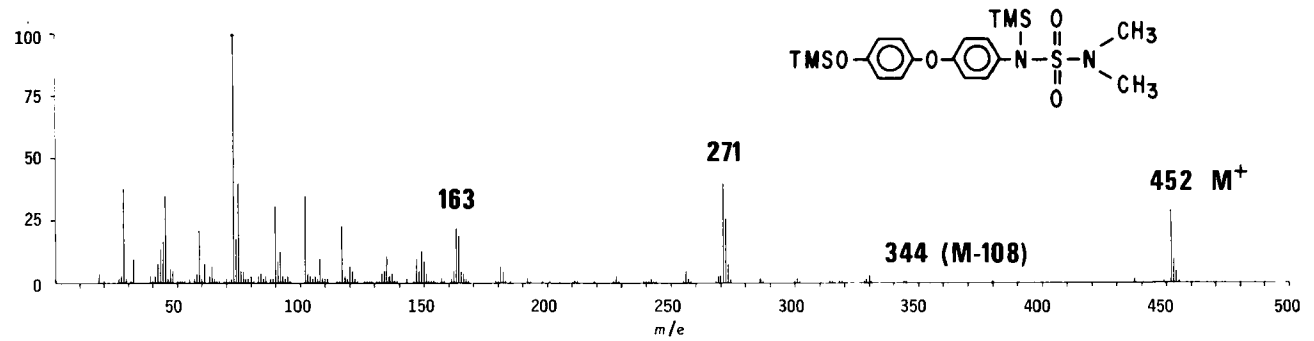


Figure 4—*Mass spectrum of bis(trimethylsilyl) derivative of III.*

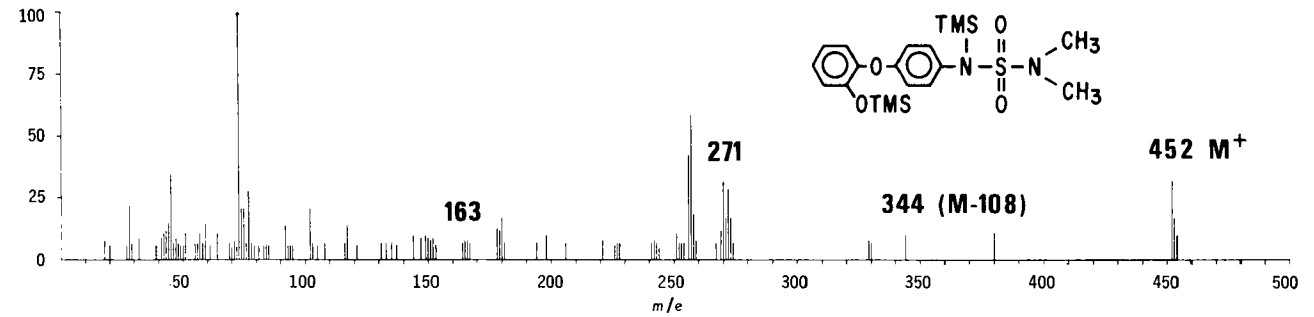


Figure 5—*Mass spectrum of bis(trimethylsilyl) derivative of IV.*

methylsilyl group migrated to the phenoxyaniline system upon electron impact. This unique property was shared by all desmethyl I-related compounds (Metabolites V, VI, and VIII).

Both IR and UV spectra of II were identical to unchanged I. The chromatographic and spectroscopic characteristics of II, synthesized independently, were identical with the isolated material. The hypolipidemic activity of II was equivalent to the parent, I.

***N,N*-Dimethyl-*N'*-*p*-(4-hydroxyphenoxy)phenylsulfamide (III)**—This metabolite was found both free and conjugated in the urine of all three species.

The mass spectrum of the bis(trimethylsilyl) derivative (Fig. 4) showed a molecular ion at *m/e* 452. Loss of 108 indicated that the sulfamoyl group was intact. The presence of the 163 and 164 pair indicated that the hydroxyl substitution did not affect the cleavage between the phenoxy oxygen and the aniline ring. Loss of the trimethylsilyl group from the aniline nitrogen and the loss of trimethylsilanol from the phenoxy ring gave strong mass peaks at *m/e* 271 and 181.

UV spectra of III were obtained in 95% ethanol and also with the addition of base (0.1 *N* KOH). In the absence of base, the spectrum showed a strong band at 238 nm and a weak band at 282 nm, which was similar to the parent compound (238, 270, and 278 nm). In the presence of 0.1 *N* KOH, the strong band was shifted to 252 nm and the weak band was shifted to 299 nm. The intensity of both bands was increased. The behavior was consistent with a monohydric phenol (4). The IR spectrum showed bands at 1295 and 1270 cm^{-1} which were phenol C—O vibrations. The features of the aromatic system and the sulfamoyl group of the parent compound were preserved.

The assignment of the hydroxyl group to the *para*-position of the phenoxy ring was confirmed by NMR. The spectrum showed two sets of *AA'BB'* multiplets, which indicated that all aromatic substitutions were *para*. On the thin-layer plate, III gave a strong positive response when sprayed with bipyridyl-ferric chloride reagent or benzidine reagent, which indicated the presence of the phenolic functional group.

Compound III, synthesized independently, had identical chromatographic and spectroscopic characteristics to the isolated material. The hypolipidemic activity of III was also equivalent to the parent, I.

***N,N*-Dimethyl-*N'*-*p*-(2-hydroxyphenoxy)phenylsulfamide (IV)**—This compound was found by its distinct chromatographic behavior both by TLC and GLC. Compound IV migrated more rapidly on silica gel G-254 than did III and had an R_f value very close to II. Compounds II and IV were distinguished from each other by spraying the plate with bipyridyl-ferric chloride reagent; IV appeared as a pink spot, whereas II remained colorless. Upon GLC, the trimethylsilyl derivative of IV (retention time 1.8 min) emerged much faster than the corresponding derivative of III (retention time 4.4 min). The mass spectrum (Fig. 5) of the bis(trimethylsilyl) derivative of IV showed the molecular ion at *m/e* 452, identical with its 4-isomer. The fragmentation pattern of IV

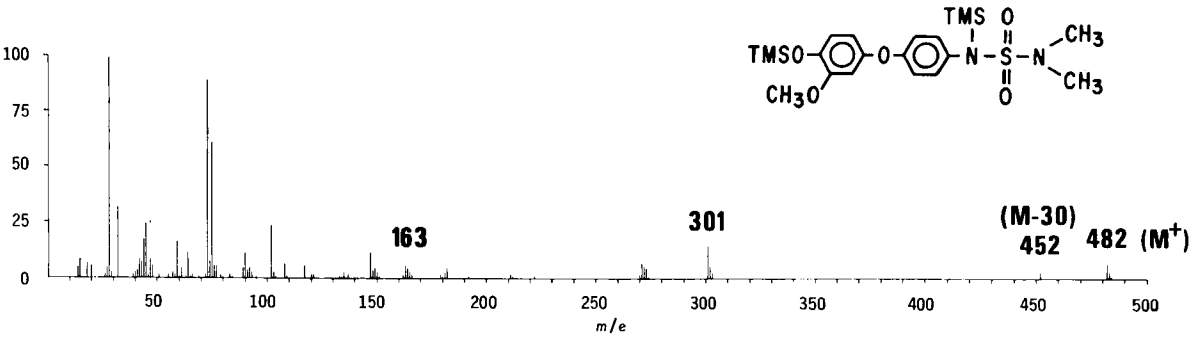


Figure 6—*Mass spectrum of bis(trimethylsilyl) derivative of VII.*

was similar to III except for the lack of the 163 and 164 pair, which indicated that a metabolic modification had occurred in the proximity of the oxygen between the two phenyl rings.

The NMR spectrum of IV could not be factored with certainty because the eight aromatic hydrogens form two highly coupled sets that were overlapping. One set of the multiplet was identified as an *AA'BB'* pattern, indicating that one phenyl ring had symmetrical *para-para* substitution. Thus, the possibility of hydroxylation on the aniline ring was ruled out. Hydroxylation in the *meta*-position relative to the phenoxy oxygen was unlikely because of the unfavorable inductive effect. Therefore, the hydroxyl group in IV was assigned to the *ortho*-position. The IR spectra of III and IV were similar.

Compound IV was found in both free and conjugated forms in the urine of the rat and dog *only*. It was not found in human urine during the 24 hr after drug administration.

***N*-Methyl-*N'*-*p*-(4-hydroxyphenoxy)phenylsulfamide (V)**—The *N*-desmethyl-4-hydroxy metabolite, both free and conjugated, was found in the urine of all three species. The mass spectrum of the trimethylsilyl derivative showed that the molecular ion was at *m/e* 510. Fragments at *m/e* 344 (M − 166) and 286 (M − 244) were characteristic of *N*-demethylated I. The presence of the 163 and 164 pair suggested that the hydroxyl substitution was not *ortho* to the phenoxy oxygen. Loss of trimethylsilanol was indicative of the relation of V with III.

The presence of a phenolic hydroxyl group in V was established by its UV spectra, reactivity with bipyridyl-ferric chloride reagent, and NMR spectrum. The UV spectrum taken in a neutral ethanol solution resembled that of the parent drug (238 and 282 nm). When the spectrum was redetermined at alkaline pH, a bathochromic shift of both bands to 252 and 298 nm, respectively, was observed, as was found for III, which suggested the presence of a monohydric phenol. NMR spectra showed two sets of *AA'BB'* multiplets, suggesting that all aromatic substitutions were *para*. The IR spectrum was also consistent with the structure assignment.

***N*-Methyl-*N'*-*p*-(2-hydroxyphenoxy)phenylsulfamide (VI)**—The metabolite was found in conjugated form in rat urine only. It was not isolated and its presence was established by GLC–mass spectrometric analysis of the partially purified extract containing III and VII. The mass spectrum of the trimethylsilyl derivative was identical to that of V except for the lack of the 163 and 164 pair. This suggested that VI was the 2-isomer of V (the *N*-demethylated analog of IV). On TLC, VI had a higher R_f than V and reacted with bipyridyl-ferric chloride reagent to give a pink-colored spot.

***N,N*-Dimethyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VII)**—Compound VII was a minor metabolite found only as a conjugate in rat urine. It had the same R_f value as III on TLC but was separated from III by repeated TLC. The mass spectrum (Fig. 6) of the trimethylsilyl derivative showed a molecular ion at *m/e* 482. Loss of 108 mass units indicated that the dimethyl sulfamoyl group was intact. Loss of the trimethylsilyl group from the aniline nitrogen (M − 72) followed by the loss of trimethylsilanol gave two prominent ions at 301 and 211. Presence of the 163 and 164 pair indicated that substitutions on the aromatic system were not in the close proximity of the diphenyl ether oxygen. Loss of 30 (CH_2O) and 31 (CH_3O) from all major peaks indicated the presence of a phenyl methyl ether linkage (5).

Since the amount of this compound in the urine was very

Table II—Common Mass Peaks Appearing in Mass Spectra of I-Related Compounds

m/e	Structure	Comments
73	$[Si(CH_3)_3]^+$	Appears in all spectra
75	$[HO{=}Si(CH_3)_2]^+$	Appears in all spectra of phenolic compounds
163	$[C_6H_5N{-}Si(CH_3)_2]^+$	Appears in all spectra except IV and VI
90	$[HO{-}Si(CH_3)_3]^+$	Appears in all phenolics
180	$[HO{-}C_6H_5N{-}Si(CH_3)_3]^+$	Appears in all phenolics
271	$[(CH_3)_3SiO{-}C_6H_5O{-}C_6H_5NH]^+$	Appears in all phenolics
256	$[C_6H_5OC_6H_5N{-}Si(CH_3)_3]^+$	Appears in I and II
185	$(C_6H_5OC_6H_5NH)^+$	Appears in I and II
344	$[(CH_3)_3SiO{-}C_6H_5OC_6H_5N{-}Si(CH_3)_3]^+$	Appears in all phenolics
198	$(C_6H_5OC_6H_5NCH_3)^+$	Appears in II
286	$[(CH_3)_3SiO{-}C_6H_5OC_6H_5NCH_3]^+$	Appears in all *N*-desmethyl hydroxylated metabolites
316	$[(CH_3)_3SiO{-}C_6H_5OC_6H_5NCH_3]^+$	Appears in VIII
M − 15	Loss of CH_3	Appears in all spectra
M − 30 or M − 31	Loss of HCHO or CH_3O	Appears in all metabolites with methoxyl group
M − 108	Loss of $SO_2N(CH_3)_2$	Appears in all compounds with sulfamide intact
M − 166	Loss of $SO_2N(CH_3)Si(CH_3)_3$	Appears in all desmethyl metabolites
M − 224	Loss of $SO_2N(CH_3)Si(CH_3)_3$ and trimethylsilyl from nitrogen	Appears in all desmethyl metabolites

small, it could not be isolated for further spectral analysis. Therefore, the positions of hydroxy and methoxy substitution on the aromatic system were assigned, based on the following information: (*a*) the presence of the 163 and 164 pair in the mass spectrum precluded the possibility of any substitution located *ortho* to the diphenyl ether oxygen; (*b*) the product of enzymatic hydroxylation of *para*-substituted monohydric phenols is catechol, which undergoes *O*-methylation yielding *para*-substituted *ortho*-methoxy phenol (6); and (*c*) catechol *O*-methyltransferase does not *O*-methylate resorcinol derivatives (7).

***N*-Methyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VIII)**—Compound VIII is the *N*-demethylation product of VII. It was found in the urine of all three species in conjugated form. The amount was very small but was separated from the crude urine extract by repeated TLC. The mass spectrum of VIII of the trimethylsilyl derivative showed that the molecular ion was at *m/e* 540. Loss of the *N*-trimethylsilyl-*N*-methylsulfamoyl group (M − 166) and the appearance of the 4-hydroxy-3-methoxy-*N*-methylphenoxyaniline ion at 316 (M − 224) indicated a monomethyl sulfamide analog. Loss of 30 or 31 mass units suggested it was a methoxy phenol. Presence of the 163 and 164 pair indicated that the hydroxyl and methoxy substitutions were not *ortho* to the phenoxy oxygen.

The NMR spectrum of VIII was difficult to interpret because of the overlapping of aromatic protons. However, a set of *AA'BB'* multiplets indicated one phenyl ring had *para-para* symmetric disubstitution. This ruled out the possibility that the aniline ring was modified. Based on the same argument as for VII, VIII was assigned the structure of the *N*-demethyl-4-hydroxy-3-methoxy analog of the parent drug.

In conclusion, the results of these experiments indicated that the metabolic fate of the parent drug was different in these three species. Rats can only demethylate the drug at reduced efficiency, as shown by the presence of only trace amounts of II (the *N*-demethylated product) and the presence of VII in rat urine. The lack of IV and VI (*ortho*-hydroxylated metabolites) in human urine may indicate that humans can only hydroxylate I in the *para*-position. However, since the human subjects in this study were only administered a single dose of the drug while the animals were administered multiple doses, this apparent species variation may be caused by enzyme induction in animals.

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Microbiological Synthesis of L-Dopa

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Abstract □ The ability of microorganisms to convert *N*-carbobenzoxyl-L-tyrosine, *N*-*tert*-butyloxycarbonyl-L-tyrosine, and *N*-formyl-L-tyrosine into their respective *N*-substituted L-dopa derivatives was studied. These tyrosine derivatives were examined because of the ease with which the blocking groups may be removed. L-Tyrosine derivatives were incubated with *Aspergillus ochraceous* or *Gliocladium deliquescens*, and the resulting L-dopa products were isolated and characterized. L-Dopa was metabolized by *G. deliquescens* to 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenethyl alcohol. L-Ascorbic acid and hydrocinnamic acid increased yields of L-dopa when added to fermentation media.

Keyphrases □ L-Dopa—microbiological synthesis, L-tyrosine derivatives incubated with *Aspergillus ochraceous* and *Gliocladium deliquescens*, isolation and identification of L-dopa products □ *Aspergillus ochraceous*—used for microbiological synthesis of L-dopa products from L-tyrosine derivatives □ *Gliocladium deliquescens*—used for microbiological synthesis of L-dopa products from L-tyrosine derivatives □ Microbiology—synthesis of L-dopa from L-tyrosine by microorganisms □ Levodopa—microbiological synthesis of L-dopa from L-tyrosine derivatives

Three different types of enzymes are known to catalyze the formation of L-dopa from L-tyrosine:

1. Tyrosine hydroxylase is an enzyme associated with the biosynthesis of norepinephrine. It catalyzes the tetrahydropteridine-dependent hydroxylation of L-tyrosine to L-dopa (1).

2. Tyrosinase catalyzes the oxidation of tyrosine to melanin. It is generally accepted that the reaction proceeds through the formation of L-dopa and hallochrome, with the eventual formation of the polymeric pigment, melanin (2).

3. β-Tyrosinase from *Escherichia intermedia* catalyzes the synthesis of L-dopa from L-tyrosine and pyrocatechol *via* β-replacement (3).

In view of the therapeutic importance of L-dopa (levodopa) in the treatment of Parkinsonism, the authors wish to record in detail the examination of reactions using microbial systems for the conversion