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Stereochemical aspects of the metabolism of the psychotomimetic amine 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane

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STEREOCHEMICAL ASPECTS OF THE METABOLISM OF THE PSYCHOTOMIMETIC AMINE 1-(2,5-DIMETHOXY-4-METHYLPHENYL)-2-AMINOPROPANE

by

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B.S., University of Utah, 1968

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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in the

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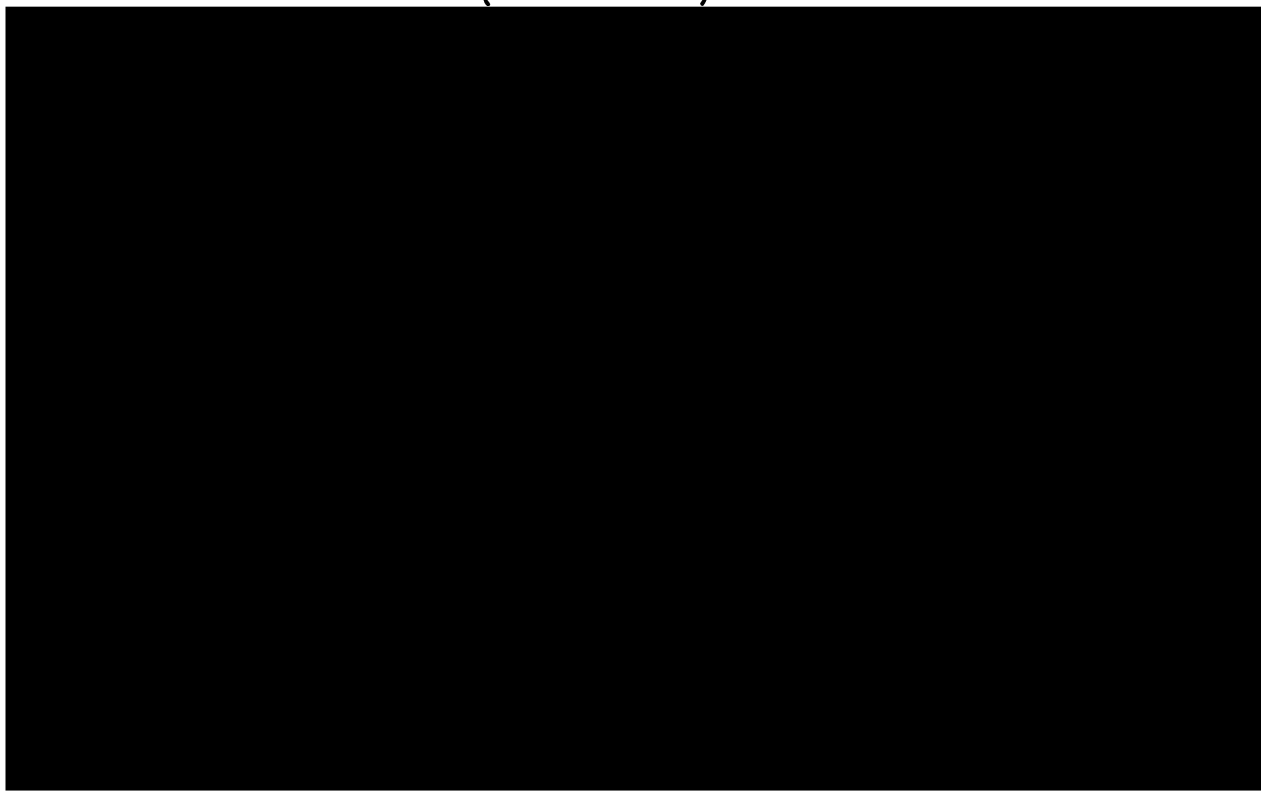
(San Francisco)



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The author wishes to gratefully acknowledge his patient wife and daughters and his understanding friends for their support and to express his sincere appreciation to his research advisor, Dr. Neal Castagnoli, Jr., for being an indefatigable optimist.

ABSTRACT

The possible role of the C-2 chiral center in the *in vivo* and *in vitro* metabolism of the psychotomimetic amine 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane was investigated. Preparation of 1-^{14}C labeled amine provided a means of following excretion patterns and obtaining quantitative estimations of unchanged compound and its metabolites by isotope dilution methods. When racemic ^{14}C-amine was administered intraperitoneally to rabbits, 54 - 75% of the administered radioactivity was excreted in the urine in 24 hours. The amine was extensively metabolized by rabbits. Less than 2% of the administered dose could be accounted for as unchanged amine in the 24 hour urine samples.

To serve as standards for comparison to metabolites, a number of compounds were synthesized. The potential metabolites prepared included 2,5-dimethoxy-4-(2-aminopropyl)benzoic acid, 1-(2,5-dimethoxy-4-hydroxymethylphenyl)-2-aminopropane, 1-(2,5-dimethoxy-4-methylphenyl)-2-propanone, 1-(2,5-dimethoxy-4-methylphenyl)-2-propanol, 1-(2,5-dimethoxy-4-methylphenyl)-2-propanone oxime and 2,5-dimethoxy-4-methylbenzoic acid.

The primary *in vivo* metabolic pathway was shown by isotope dilution analysis to involve C-4 oxidation to give 2,5-dimethoxy-4-(2-aminopropyl)benzoic acid. The 4-hydroxymethyl compound and the side chain oxidation products 1-(2,5-dimethoxy-4-methylphenyl)-2-propanone and 2,5-dimethoxy-4-methylbenzoic acid were not found as urinary metabolites.

Enantiomeric composition of the amine was determined by glpc analysis of the diastereomeric amides resulting from treatment with the chiral resolving agent S-(-)-N-pentafluorobenzoylprolyl 1-imidazolide. When racemic amine was administered to rabbits and the urine examined for unchanged compound, the ratio of R/S was found to be greater than 1.

Incubation of racemic amine in rabbit liver 10,000 x g supernatant fractions resulted in a marked preference for the S-enantiomer. However, when the individual isomers were incubated separately, the enantiomers were metabolized to approximately the same extent. Identification and quantification of the in vitro metabolites was accomplished with the aid of deuterium labeled amine and chemical ionization mass spectrometry. Stable isotope dilution analyses after incubation of hexadeuterio amine labeled in the methoxy positions indicated that a major in vitro pathway also involved C-4 methyl oxidation. The 4-hydroxymethyl compound accounted for 70% of the metabolites. The side chain oxidation products 1-(2,5-dimethoxy-4-methylphenyl)-2-propanone and 1-(2,5-dimethoxy-4-methylphenyl)-2-propanol were found to account for a minor metabolic pathway. The stereochemistry of metabolite formation was evaluated. Deuterium labeled amine was resolved. By incubating a pseudoracemic substrate composed of mixtures of labeled and unlabeled isomers, the enantiomeric source of the metabolites was determined. The S-enantiomer was found to be the main source of the propanone, propanol, and 4-hydroxymethyl metabolites.

DEDICATION

to

my father

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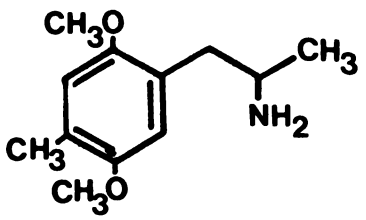
INTRODUCTION

The 1-phenyl-2-aminopropane system 2 is found in many pharmacologically active substances. Therapeutically useful central nervous stimulant and sympathomimetic 1-phenyl-2-aminopropanes[123] include amphetamine,[158] methoxamine[157] and ephedrine.[159] Minor structural modifications of substituents attached to the phenyl ring often dramatically alter the activity of central nervous stimulants. This class of drugs includes compounds which have variously been described as hallucinogens, psychotomimetics or psychedelics.[11] These substances are qualitatively similar in effect to the peyote alkaloid mescaline (3) but are generally more potent.[2] Similarities between the effects of these synthetic psychotogens and spontaneous mental aberrations observed in schizophrenics have been studied.[124,125]



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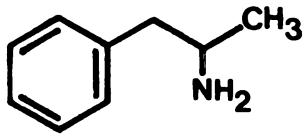

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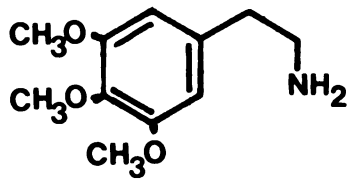

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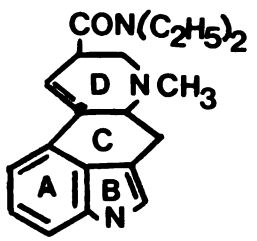
Extensive structure-activity relationship studies on the psychotomimetic 1-phenyl-2-aminopropanes have been carried out by Shulgin[4] and others[126-131] on more than fifty structural analogues. In general, increasing the chain length from 2-aminopropane to 2-aminobutane produces a less active compound while 2,4,5-trisubstitution on the phenyl ring provides optimal activity.[4] Alkylation on the nitrogen

lowers activity compared to the unsubstituted amine.[128]

Correlations other than substituent effects have been attempted on the psychotomimetic 1-phenyl-2-aminopropanes. Electron density relationships have been observed for some hallucinogenic 1-phenyl-2-aminopropane derivatives. Snyder and Merril[2] and later Kang and Green[1] have described a relationship between the highest occupied molecular orbital energies (a measure of the ability of these compounds to donate electrons) and hallucinogenic activity. Electron donation properties have been postulated to facilitate a reversible, low energy π-molecular complex which presumably forms with the brain receptor.[153] Antun et al.[132] have found a relationship between native fluorescence and hallucinogenic potency.

Steric requirements have also been correlated with activity. Psychotomimetic activity has been related to the propensity for the side chain of 1-phenyl-2-aminopropanes to reside in a conformation (4) related to the fixed ring system (ring C) of the hallucinogenic indole derivative, lysergic acid diethylamide (5).[2,3]



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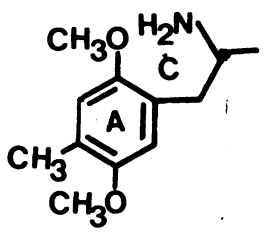

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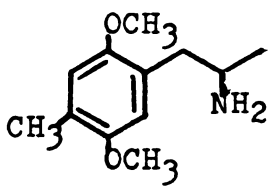
From these numerous structure-activity studies there are no definitive conclusions about activity as a function of substituents on the benzene ring. Several compounds with similar chemical structure and physical properties exhibit wide variations in activity. For example, 1-(2,3,4-trimethoxyphenyl)-2-aminopropane (6) is inactive in humans while the 2,4,5-trimethoxy analogue 7 is 17 times more potent than mescaline (3). Replacement of the 4-methoxy group of 7 with a methyl group produces compound 1 which is 80 to 100 times more potent than mescaline.[4] These pronounced



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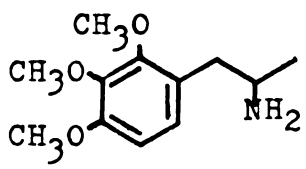

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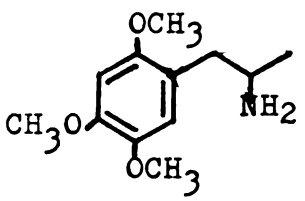

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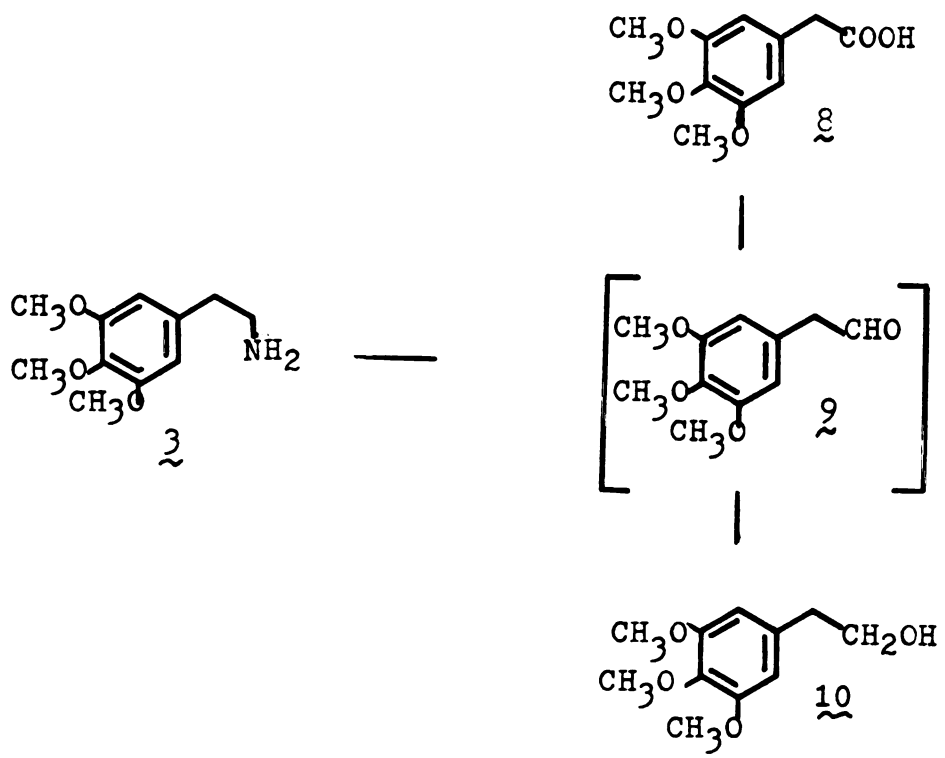
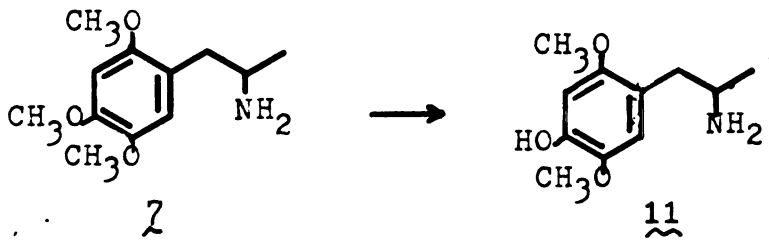
differences in activity have not been adequately explained by reported activity correlation studies. Knowledge of the metabolic fate of these amines may explain these differences in activity.[3,4,124,132,152,153] Harley-Mason et al.[152] studied the metabolism of mescaline with the rationale that the more information gained about the fate of known hallucinogens the easier it will be to search for endogenous psychotogens which may be responsible for schizophrenia. They suggested that mescaline itself is not hallucinogenic and proposed that a metabolite of the amine is the active

species. The 1 to 2 hour delayed onset of mescaline activity in humans was considered to be the lag time for the formation of an active metabolite. Further evidence was based on studies in mice which showed that the behavioral effects do not coincide with the concentration of unchanged amine in the brain.[162] The major metabolite of mescaline, 3,4,5-trimethoxyphenylacetic acid, was shown to be not active.[161] Friedhoff and Goldstein,[151] from aldehyde dehydrogenase inhibition studies, suggested that the active species is the aldehyde 9 or alcohol 10.



Antun et al.[132] have suggested that the pattern of aromatic substitution on the 1-phenyl-2-aminopropanes may offer

protection against metabolic inactivation. For example, they propose that 2,6- or tetra substitution protects against transformation by amine oxidase and that C-4 substitution blocks the formation of more readily excreted 4-hydroxyl compounds. Snyder et al.[153] reported that compound 1, first synthesized by A. Shulgin, is a more active substance because it is less susceptible to metabolic degradation than compound 7. A C-4 methyl group in place of the C-4



7 11

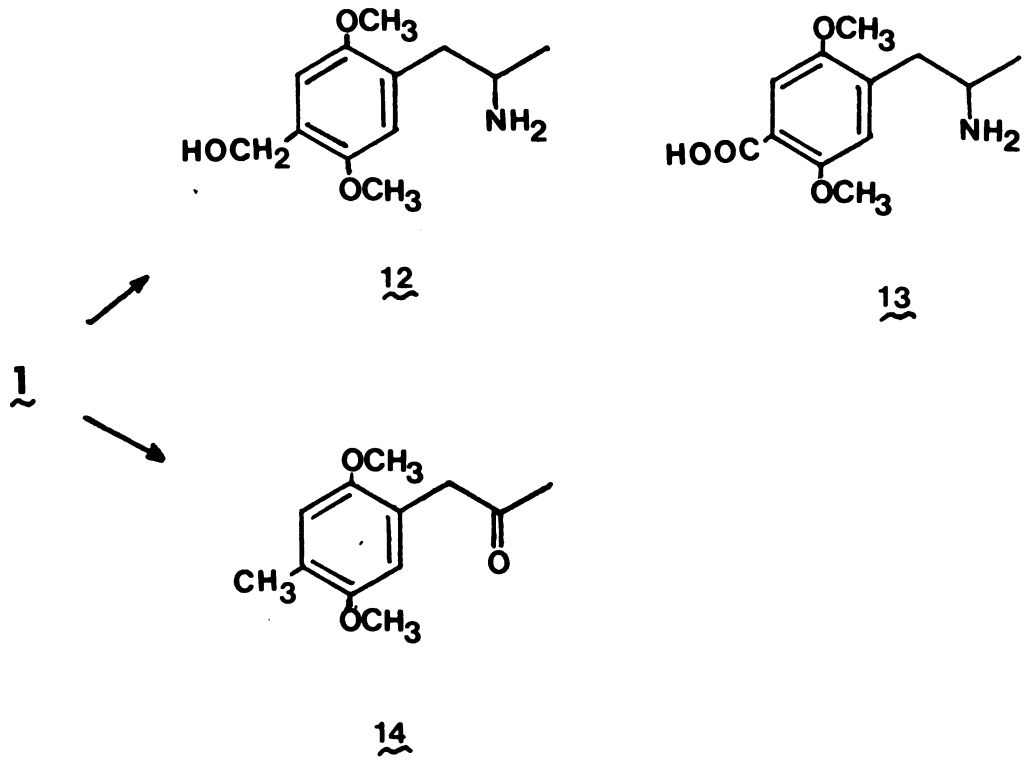
methoxy group of 7 was anticipated to block the metabolic pathway of demethylation and thus protect against excretion of the hydroxyl derivative 11 as a water soluble conjugate.

As part of an overall program to evaluate the role of metabolism in biological activity, an investigation of the comparative metabolism of structurally related but pharmacologically distinct 1-phenyl-2-aminopropane derivatives was undertaken. This thesis reports the results of studies on one of the most potent members of this series, 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (1, STP, DOM).

Shortly after 1 was identified by the U.S. Food and Drug Administration[137] as the active ingredient in the

hallucinogenic drug STP, several pharmacological and clinical evaluations of 1 in humans appeared in the literature.[133-136] At the oral doses tested, compound 1 did not produce marked changes in blood pressure, pulse rate, temperature, or pupillary diameter.[135] Visual and behavioral disturbances were noted at doses of the racemic compound above 5 mg which greatly resembled the effects of d-lysergic acid diethylamide (5, LSD) and mescaline.[134] Limited data suggest a difference in activity of the enantiomers of 1. Shulgin[89] has reported that the psychotomimetic activity of 1 in humans resides in the (-) isomer while the (+) isomer is nearly inactive. Benington and co-workers[63] have found the (-) isomer to be considerably more active than the (+) isomer in a rat behavior disruption assay. By measuring contractility of perfused isolated sheep intestine, Dyer et al.[140] concluded that the (-) isomer is six times more active than the (+) isomer. Consequently, any meaningful evaluation of metabolism vs activity must include a consideration of the stereochemistry of this system.

One study has appeared to date on the metabolic fate of 1. Ho et al.[15] reported that the major metabolic pathway of racemic 1 in rats involves the oxidation of the 4-CH_3 group to the alcohol 1-(2,5-dimethoxy-4-hydroxymethylphenyl)-2-aminopropane (12) and to the acid 2,5-dimethoxy-4-(2-aminopropyl)benzoic acid (13). A trace of the side chain oxidative deamination metabolite 1-(2,5-dimethoxy-4-methylphenyl)-2-propanone (14) was also observed.



Part I of this thesis is concerned with the net *in vivo* fate of 1 and its optical isomers in monkeys and rabbits. Part II describes *in vitro* studies on the stereochemically sensitive processes involved in the biotransformations of 1. Part III describes the synthetic work performed in association with the metabolic studies reported in Parts I and II, including the syntheses of compound 1 containing a ^{14}C label and a ^{2}H label, the synthesis of suspected metabolites, and the resolution of 1.

PART I. *In Vivo* Metabolism of 1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane (1)

Compound 1 is known to be a potent sympathomimetic in low doses in humans[134] and rats.[138] Because of the high potency of 1, only small doses can be administered to animals necessitating sensitive analytical procedures for the detection and estimation of the parent drug and its metabolites in biological fluids. To facilitate our studies we have employed mass spectrometric analyses coupled with stable isotopes and ^{14}C labeled 1 (see Part II of this thesis for mass spectral studies and Part III for syntheses of labeled compounds). The ^{14}C material allowed us to follow excretion patterns and to determine the fate of 1 in monkeys and rabbits.

Rhesus monkeys were selected as model animals for part of our metabolic work because they are phylogenetically close to man and have been shown in some cases to metabolize drugs similar to man.[48,54,160] Further studies were carried out on Dutch rabbits. Rabbits were chosen because of the extensive literature on rabbit metabolism of a model 1-phenyl-2-aminopropane, amphetamine (2)[54,61,62,65] and a model psychotomimetic, mescaline (3).[59,151]

A. Urinary Excretion Pattern of 1 in Rhesus Monkeys

In order to study the elimination of 1 and its metabolites in monkeys, ^{14}C-1 was administered at various dose levels. The elimination of administered radioactivity was found to be mostly via the kidneys and was essentially complete in 24 hours.

Rhesus monkeys (4-6 kg) confined to the sitting position in chairs were fitted with permanent arterial and venous catheters.[49] In one monkey, heart rate and systolic and diastolic blood pressure were monitored during intravenous infusion[49] of R:S-1. Administration of 0.2 mg/kg/hour resulted in an increase in both systolic and diastolic pressure accompanied by tachycardia. These effects are similar to those reported for rats.[138] At a dose of 2.5 mg given by intravenous infusion over a period of one hour, no discernible pharmacological effects other than cardiovascular effects were noted. Prolonged infusion of 2.3 mg/hour caused the monkey to become drowsy. Higher doses resulted in agitation, dyspnea and convulsions.

The data in Table 1 describe the ^{14}C urinary excretion pattern of 1 in monkeys. Between 80 and 90% of the administered dose was excreted in the urine in the first 24 hours.

Table 1

Urinary Excretion in Rhesus Monkeys After Intravenous Administration of ^{14}C-1

Total Dose (mg/kg)	Duration of Infusion (min)	% ^{14}C Dose Excreted in 24 Hours
1.0[a]	10	98
0.4[a]	40	86
0.2[a]	60	94
1.1[b]	150	80
3.3[b]	360	83

[a] Male Rhesus monkey (4.8 kg)
[b] Female Rhesus monkey (6.0 kg)

Percentages are of administered dose and were determined by liquid scintillation counting

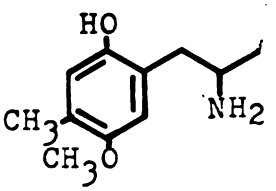
Partitioning of radioactive urinary metabolites by extraction of the urine samples adjusted to various pH values provided information about the polarity of the metabolites of 1. Less than 30% of the radioactivity excreted in 24 hours could be extracted in a separatory funnel with ether or ethyl acetate. About 20% was extractable at pH 12, very little at pH 8.5 and less than 5% at pH 1. The pH 12 extract would be expected to contain unchanged 1, basic and neutral metabolites, the pH 8.5 extract aminophenolic metabolites,

and the pH 1 extract, acidic metabolites. Continuous liquid-liquid extraction with ether yielded nearly the same extraction pattern.

It was apparent from the relatively small percentage of the urinary radioactivity that could be extracted into organic solvents that the parent amine had undergone conversion to highly polar metabolites.

An alternative approach to the initial isolation of polar metabolites by a heterogeneous extraction of a lyophilized urine sample with various solvents was also explored. The urine was extracted with ether at pH 12 to remove bases, the aqueous phase was lyophilized and the residue was digested with increasingly polar solvents (hexane, chloroform, ethyl acetate, and methanol). The radioactivity levels of the hexane, chloroform and ethyl acetate extracts were that of background while the methanol extract accounted for 98% of the total counts present. The final residue dissolved in water provided the remaining 2% of the radioactivity.

In order to investigate the possibility that glucuronide and/or sulfate conjugates of primary metabolites were among the polar metabolites formed, a study of the partitioning of urinary radioactivity before and after treatment with β-glucuronidase was undertaken. Of the possible metabolites predicted to be conjugated, the O-demethylated compounds 15 and 16 were considered likely. Charalampous _et al._[53] found that the O-demethylated aminophenolic metabolites of mescaline could be extracted with ethyl acetate at pH 8.5.



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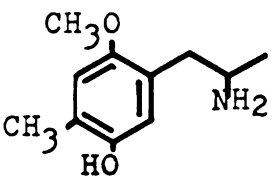

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One would expect the O-demthylated metabolites 15 and 16 also to be extractable at pH 8.5. However, treatment of radioactive urine samples with a β-glucuronidase solution with sulfatase activity at pH 5.4 or 5.0 produced a solution from which negligible counts could be extracted at pH 8.5 with ethyl acetate. This observation provides indirect evidence that 15 and 16 and glucuronide and sulfate conjugates of 15 and 16 represent at best minor urinary excretion productsin the biotransformation of 1 in the monkey. The enzyme-treated urine sample was also extracted at pH 1 and 12. The counts extracted were comparable to that of a radioactive urine sample which had not been treated with β-glucuronidase. This experiment does not conclusively prove that there are no glucuronide or sulfate conjugates present, however, since the activity of the enzyme preparation was not established. It is also possible that the conjugates were cleaved but the resulting aglycones were of high water solubility.

Further attempts to isolate possible water soluble metabolites were made using anion exchange chromatography. The monkey urine sample which had been lyophilized, extracted

with methanol and treated with β-glucuronidase was again lyophilized. The residue was placed on an anion exchange column at neutral pH and washed with water until neutral. The column was eluted with ammonium hydroxide, sodium hydroxide, and water until neutral pH, and finally by acetic acid. The elution pattern was followed by liquid scintillation counting.

Table 2

Purification of Water Soluble Metabolites by Ion Exchange Chromatography

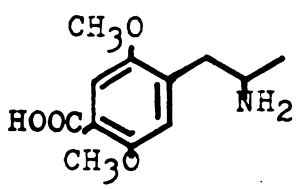
Fraction	Volume (ml)	Eluants	% ^{14}C Recovered
A	235	H_2O	nil
B	235	H_2O	nil
1-48	960	1.9N NH_4OH	nil
49-93	900	9.6N NH_4OH	nil
94-117	480	14.8N NH_4OH	nil
118-153	720	1.0N NaOH	nil
C	800	H_2O	nil
154-171	360	1.0N HOAc	nil
172-175	80	1.0N HOAc	84.3
176-182	140	1.0N HOAc	9.3
		Total	93.6

As can be seen from Table 2, nothing above background

was eluted by the water, ammonium hydroxide and sodium hydroxide fractions while acetic acid eluted 93.6% of the radioactivity. The fractions containing radioactivity were evaporated to give a yellow oil. Analysis by tlc (n-butanol: acetic acid:water, 4:1:1) showed a major radioactive band with R_f=0.5 and a minor band with R_f=0.4. Under a uv light, many nonradioactive spots were observed. The radioactivity from the major spot could be eluted from the tlc plate with methanol.

Purification of the major metabolite isolated by ion exchange was attempted by preparative thin layer chromatography on silica gel. The major radioactive area was scraped and then eluted with spectral grade methanol to give after evaporation about 3 mg (calculated from the specific activity of 1) of purified material. An nmr spectrum of the metabolite in formic acid in a micro nmr tube was attempted. The spectrum indicated that the sample was still impure and no structural assignments could be made. Analysis by uv also provided no structural evidence.

On the basis of the ion exchange results, it was clear that the major metabolite contained an acidic function and was probably an amino acid which would be consistent with its observed insolubility in organic solvents at any pH. The R_f value for the metabolite is consistent with the reported value[15] for amino acid 13. The identification and quantitative



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estimation of 13 and of other metabolites of 1 in rabbits by radiochemical isotope dilution analyses is reported later in this thesis.

B. Urinary Excretion Pattern of 1 in Dutch Rabbits

Summarized in Table 3 are the urinary excretion data obtained from rabbits after intraperitoneal (ip) injection of ^{14}C-1. Between 71 and 81% of the administered dose was excreted in the urine in 48 hours. Rabbits eliminate 1 and its metabolites mainly via the kidney as do monkeys but at a slower rate.

Table 3

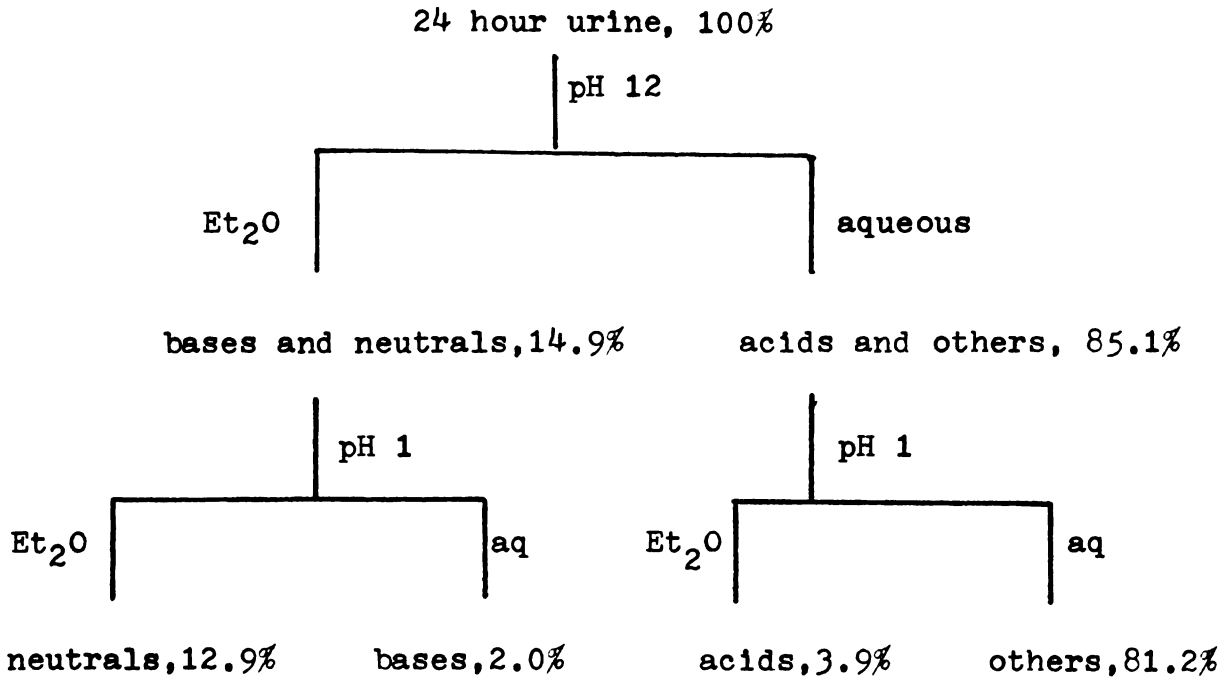
Excretion of ^{14}C in Urine After ip Dose of ^{14}C-1 in Three Rabbits

Dose (mg/kg)	% Dose Excreted in Urine	
	0-24 hr	24-48 hr
5.0	75	6
12.5	66	13
21.0	54	17

Percentages are from a different Dutch rabbit at each dose level and were determined by liquid scintillation counting

Extraction data of a typical rabbit urine sample after ip injection of ^{14}C-1 shows that 1 is extensively metabolized (see Figure 1). Of the total 19% of the ether extractable urinary radioactivity, the base fraction accounted for 2%, the neutral fraction 13% and the acid fraction, 4%. As was the case in the monkey, a major portion of the excreted radioactivity could not be extracted at any pH into ether. The attempts to separate and identify metabolites of 1 by direct isolation were of limited success. In order to be able to identify metabolites, isotope dilution studies were undertaken.

Figure 1. Flow sheet for the Partitioning of Urinary Radioactive Metabolites Between Ether and Water From A Typical Rabbit Experiment

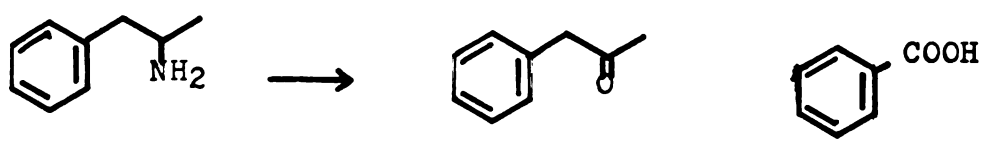


From a Dutch rabbit injected ip with ^{14}C-1 (7.4×10^6dpm). The 24 hour urine contained 75% of the administered dose. Percentages are of excreted dose and were determined by liquid scintillation counting.

C. Radiochemical Isotope Dilution Studies

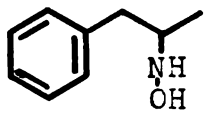
The identification of drug metabolites by classical methods of chemical analysis often fails because of limited amounts of compounds obtainable in pure form. This is particularly true because of the complex nature of biological mixtures.[163] Isotope dilution methods[144] offer a simplified approach to metabolite analysis[54] by removing the necessity of quantitative recovery, One in effect is allowed to increase the quantities involved by the addition of large amounts of unlabeled carrier. The major metabolic pathway of 1 in rabbits and monkeys was investigated by means of isotope dilution analysis.

From studies on compounds structurally related to 1 and of known metabolic fate, the possible major pathways of the metabolism of 1 can be anticipated. Amphetamine (2), the simplest 1-phenyl-2-aminopropane, undergoes metabolic oxidative deamination in rabbits to 1-phenyl-2-propanone (17)[165] and side chain cleavage to benzoic acid (18).[54] Labile



2 17 18

metabolites of amphetamine which under mild acidic or basic conditions decompose to 1-phenyl-2-propanone are also known. These metabolites, N-hydroxyamphetamine (19)[150]



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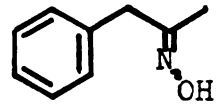

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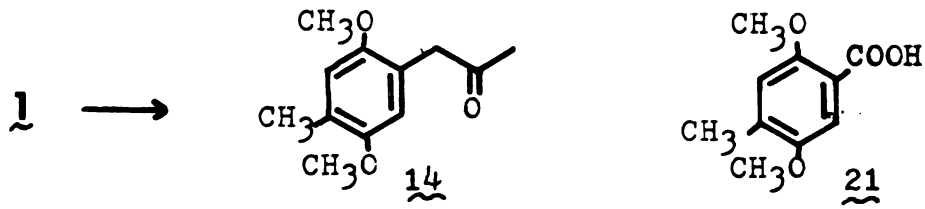
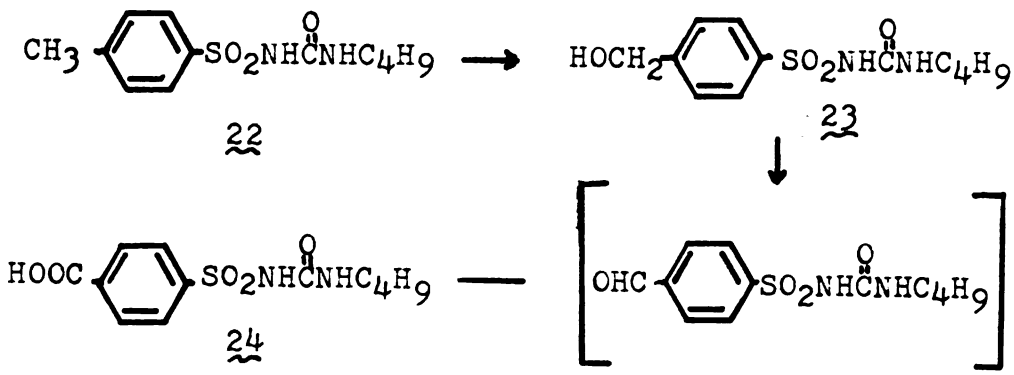
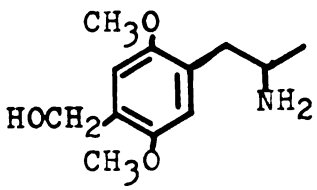
and 1-phenyl-2-propanone oxime (20),[62] represent various oxidation states of the amino group. From amphetamine metabolism studies, the pathway predicted for 1 would include the analogous side chain oxidation products 14 and 21.



Compound 1 contains a metabolically labile toluene moiety. In tolbutamide (22),[46,47] for example, the aromatic methyl group is metabolized to the hydroxymethyl derivative 23 and the carboxy derivative 24. Compound 1 would also be expected



to undergo aromatic methyl oxidation to yield 12 and 13.[15]



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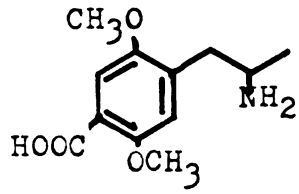

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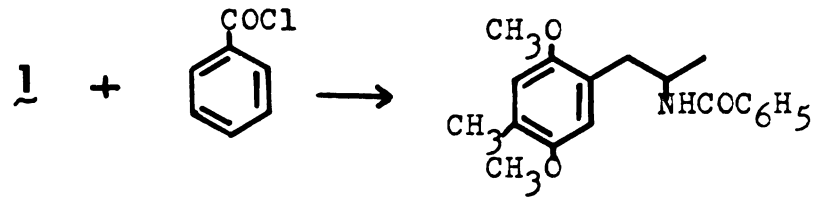
In order to determine if these pathways are important in the metabolism of 1, compounds 12, 13, 14 and 21 were synthesized to provide carrier materials for radiochemical isotope dilution studies. The discussion of these synthetic studies is included in Part III of this thesis.

By radiochemical isotope dilution analyses, the identity and concentration of these potential metabolites were determined in urine obtained from rabbits receiving ^{14}C-1 ip. The analyses involved intimate mixing of a known amount of unlabeled carrier with an aliquot of urine containing labeled metabolite. Dissolution of the carrier is necessary to ensure uniform dispersion of labeled metabolite in the carrier upon isolation and purification. The quantities of metabolites in the urine samples were calculated from the specific activity of the reisolated carrier. Amines 1 and 12 and amino acid 13 could not be crystallized when isolated from urine samples and were analyzed as benzoyl derivatives. Carrier ketone 14 and acid 21 were crystallized directly after isolation from urine samples.

1. Unchanged 1. Determination of the extent of excretion of 1 as unchanged amine was accomplished by addition of a known amount of unlabeled carrier 1 to an aliquot of urine. The ether soluble base fraction was treated with benzoyl chloride to form the crystalline benzamide derivative 25.



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Compound 25 is a well behaved, easily purified, high melting solid. In Table 4 are the data from rabbit experiments in which compound 1 was estimated by isotope dilution analysis. Only 0.5 to 2.0% of the administered dose (5-29 mg/kg) could be accounted for as unchanged amine. Thus, it is clear that the rabbit extensively and rapidly metabolizes compound 1. These findings should be compared to literature reports on the metabolism of mescaline in rabbits[164] and humans.[53,151] A large portion of administered mescaline is found in the urine as unchanged amine. The extensive metabolic degradation of 1 makes even more dramatic the differences in potency of these two amines. From a comparison of metabolic profiles in rabbits, the potency of 1 should be considered greater than the reported[4] 80-100 times that of mescaline.

Table 4

In Vivo Rabbit Metabolism of Amine 1

Dose (mg/kg)	Urine Collection (hours)	% Dose Excreted	% Unchanged Present
5	0-24	75	2.0
	24-48	6	0.5
12.5	0-24	66	0.9
	24-48	13	0.5
21	0-24	54	0.6
	24-48	17	-
22	0-20	*	*
	20-48	6	0.5
29	0-48	74	1.0

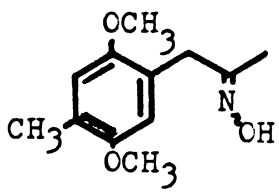
Male Dutch rabbits (1.5-2.0 kg) were injected ip with racemic ^{14}C-1 and the urine collected. Percentage dose excreted is in terms of total radioactivity administered. Isotope dilution analyses provided the percentage unchanged 1.

*Leakage in metabolic cage prevented quantitative estimation

2. Side Chain Metabolism. Since the side chain of 1 is likely to be sensitive to oxidative metabolism, the formation of ketoxime 26 and of ketone 14 was considered potentially important. It has been reported that treatment of 1-phenyl-2-propanone oxime with acid results in its rapid conversion to the corresponding ketone.[62] Therefore in our case, acid treatment of the urine aliquot and isotope dilution analysis for ketone 14 should provide an estimate of the combined amounts of 14 and 26. Ketone 14 was prepared by reduction of nitropropene 27 with iron powder.[154] In the analysis for ketone 14, less than 13% of the radioactivity excreted could be accounted for in the neutral fraction of the 24 hour urine and the recrystallized ketone was not radioactive. In a second rabbit experiment, 8% of the excreted ^{14}C could be accounted for as a combined base and neutral fraction. Isotope dilution analysis again showed ketone 14 not to be a urinary metabolite of 1.

It has been suggested in the literature that 1-phenyl-2-propanone, a major metabolite of amphetamine in the rabbit, is further metabolized to benzoic acid.[54] Consequently, 2,5-dimethoxy-4-methylbenzoic acid (21), prepared by oxidation of the readily available aldehyde 28 with potassium permanganate, was estimated by isotope dilution analysis in two rabbit experiments. The urine samples plus carrier were treated with HCl to hydrolyze potential conjugates. Once again no radioactivity was observed in the purified acid reisolated from the urine samples. The possibility that

the HCl treatment did not cleave a potential glycine conjugate (hippurate) of 21 was discarded after isotope dilution analysis with authentic hippurate 29 was negative.[155] Compound 29 was prepared from the acid chloride of 21 and glycine.[156]



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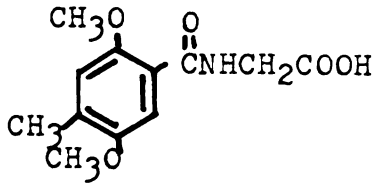

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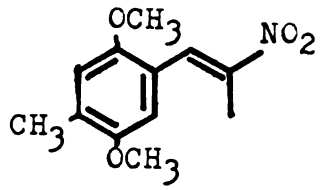

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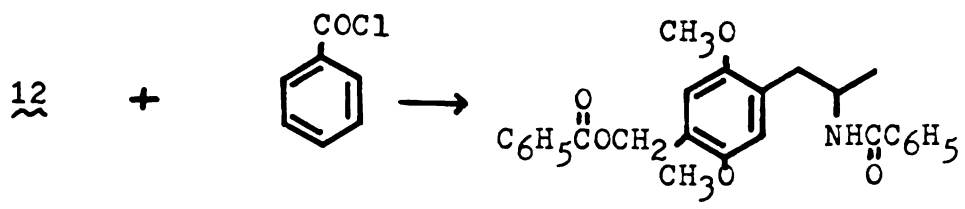
Thus, in terms of the metaboiltes investigated, oxidation of the side chain does not represent a major pathway in the in vivo metabolism of amine 1 in rabbits.

3. C-4 Metabolism. To test the possibility that oxidation at the C-4 aromatic methyl represents a major metabolic pathway of 1, the hydroxymethyl compound 12 and carboxy compound 13 were analyzed by isotope dilution. Compound 12, prepared by the reduction of phthaloyl aldehyde 30, was purified as its bis-benzoyl derivative 31 which was formed by treatment of the base fraction of the urine sample with benzoyl chloride in pyridine. Compound 31 may be easily



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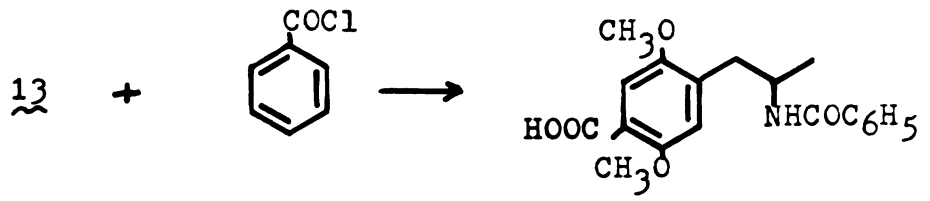
purified by recrystallization to constant specific activity. The hydroxymethyl compound 12 was found not to be a urinary metabolite in one rabbit although, as will be reported in Part II, compound 12 is a major metabolite of 1 in rabbit liver homogenates.

The carboxy compound 13 was prepared by oxidation of phthaloyl aldehyde 30. Carboxy metabolite 13 and carrier were purified as the benzoyl derivative 32 after treatment of the urine sample with NaOH and benzoyl chloride. Amino acid 13 was found to account for 38% of the administered dose and 51% of the 24 hour urine in one rabbit experiment. Aromatic methyl oxidation to the acid, therefore, represents a major pathway in the metabolism of 1 in rabbits.



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In Table 5 are the results from radiochemical isotope dilution analyses in a typical rabbit experiment. Only unchanged 1 and amino acid 13 were found to be urinary metabolites.

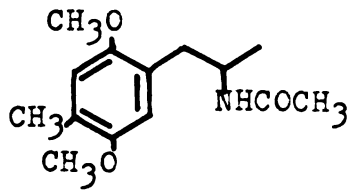
Table 5

Radiochemical Isotope Dilution Analysis of the *In Vivo* Metabolism of 1 in a Rabbit

Compound	Specific Activity (dpm/mg) (recrystallizations)	mp	% Dose
Unchanged 1	1. 210[a] 2. 188 3. 165	169-170[a] 169-170	1[a]
Amino Acid 13	1. 1443[c] 2. 1240 3. 1157	182-183[c] 182-183	47.2 40.6 37.9
Hydroxymethyl 12	1. <100[b] 2. <100	159-162[b]	<0.5
Propanone 14	1. <100 2. <100	50-52	<0.5
Acid 21	1. <100 2. <100 3. <100	120-122	<0.5

[a]As benzoyl derivative 25, see Table 4 for additional values
[b]As bis-benzoyl derivative 31
[c]As benzoyl derivative 32
A male Dutch rabbit was injected ^{14}C-1 (7.4×10^6 dpm) ip. Analyses were carried out on 10% aliquots (5 ml) of the 24 hour urine which contained 75% of the administered dose. Percentages are of administered dose.

While amino acid 13 is apparently the major urinary metabolite, less than half the administered dose was accounted for. Identification of additional metabolites was limited by the availability of unlabeled carriers. Other compounds considered to be possible metabolites but not available for analysis include the N-acetyl compound 33, propanolamine 34, propanol 35, and glucuronide, sulfate or other possible conjugates of amino acid 13 and amine 1.



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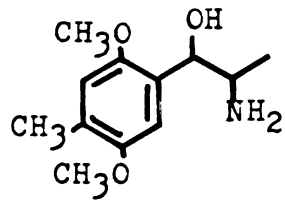

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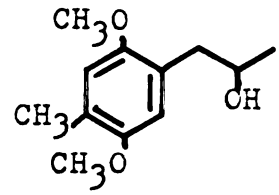

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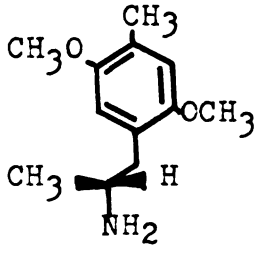
Further studies on the fate of amine 1 in rabbit liver are described in Part II of this thesis.

D. Stereoselective Excretion of Unchanged 1 in Rabbits

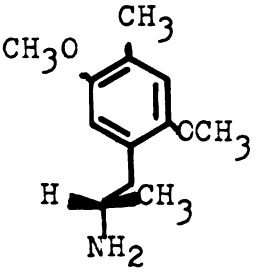
Knowledge of the metabolic fate of the individual optical isomers of 1 may also provide information about activity. Variations in pharmacological activity with the configuration about a chiral center in asymmetric molecules are well documented.[104] In general, the potency of a racemic mixture is found to be intermediate between the pharmacologically active and inactive enantiomers. Investigations concerned with stereochemical parameters associated with the metabolic fate of asymmetric drugs have attempted to explain in part such differences in activities.[90-95,105] The pharmacological[107-110] and biochemical[111] properties of R- and S-amphetamine and related 1-phenyl-2-aminopropanes[112] have been examined by a number of workers. In vivo metabolic studies have shown that S-amphetamine is excreted in the urine of man to a lesser extent than R-amphetamine.[97] Consistent with this excretion pattern is the reported substrate stereospecific hydroxylation of S-amphetamine to 1R:2S-norephedrine by dopamine-β-hydroxylase.[66] However, Axelrod[65] and more recently Hewick and Fouts[45] have reported that rabbit liver microsomal preparations which principally oxidatively deaminate amphetamine display a marked stereochemical preference for R-amphetamine. Additionally, Beckett has reported significant species-dependent stereoselective side-chain metabolic alterations in this system.[113] As has been observed with several 1-phenyl-2-aminopropanes,[84-88] the biological activities of the (+) and (-) forms of 1 differ, with the psychotomimetic activity residing in the

(-)-isomer.[63,89] In order to evaluate the potential importance of stereoselective processes in the metabolism of 1, we have examined the enantiomeric composition of unchanged amine excreted in the urine of rabbits treated intraperitoneally with racemic 1 was examined.

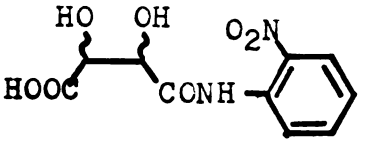
The absolute configurations of 1a and 1b were established by Matin et al.[6] Racemic 1 was resolved by recrystallization of salts obtained from (+)- and (-)-o-nitrotartranilic acid (36). A comparison of the cd curves of the N-salicylidine



1aR



1bS



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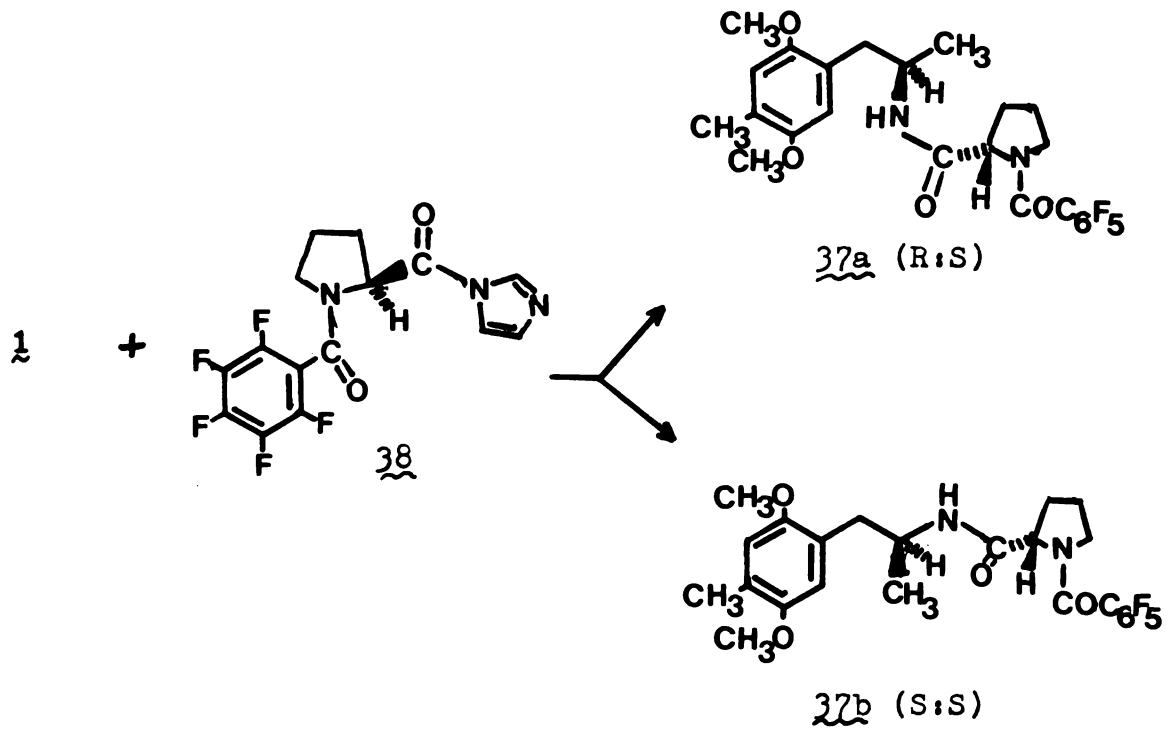
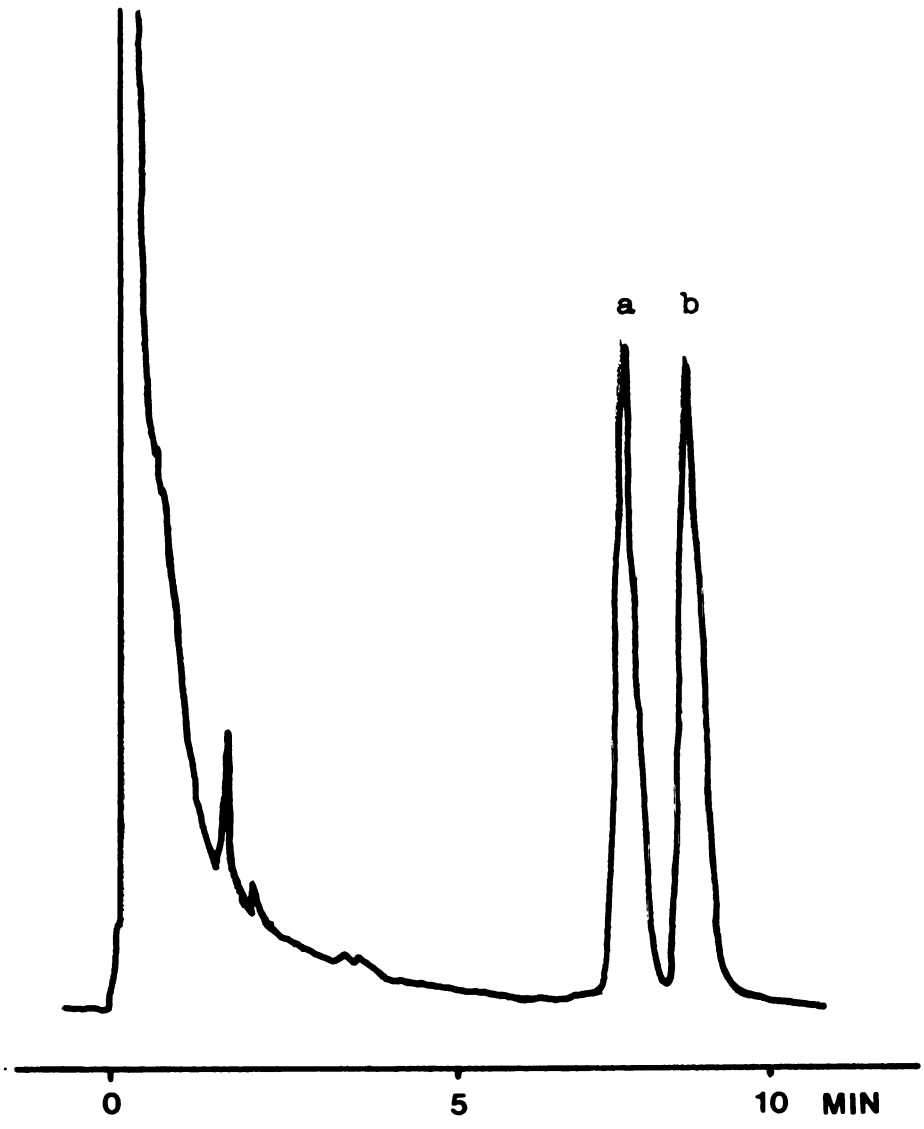
derivatives of the resolved amines with curves for the N-salicylidine derivatives of R(-)- and S(+)-amphetamine established the absolute configurations of 1a and 1b to be R(-) and S(+), respectively. Estimation of the relative concentrations of 1a and 1b was achieved by a glpc analysis of the diastereomeric amides 37a and 37b formed when a mixture of 1a and 1b is allowed to react with the chiral reagent S-(-)-N-pentafluorobenzoylprolyl 1-imidazolide (38).[99]



The peak with the shorter retention time was found to be associated with 37a and the peak with the longer retention time, 37b. Figure 2 shows that racemic 1, when derivatized, exhibits a clearly resolved glpc tracing with two equally intense peaks corresponding to 37a and 37b establishing that neither the reaction nor the detection processes show stereochemical preferences. Furthermore, with highly pure 1a and 1b, only one of the pair of peaks appears in the glpc tracing after derivatization, thus confirming the enantiomeric purity of 1a, 1b and 38 and indicating that the analytical procedure does not cause racemization.

Figure 2

Glpc Tracing of Racemic 1 After Treatment With Prolyl Reagent 38



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[a] R:S-37a, R_t=7.9 min
[b] S:S-37b, R_t=9.0 min

R:S-1 as free amine in benzene was treated with 38. Glpc on 6 ft 3% OV-17 column, oven temp 250°.

Five male Dutch rabbits (1.5-2.0 kg) were administered doses of amine 1 ranging from 5 to 29 mg/kg. When possible, the urine was collected and analyzed in fractions. However, in one experiment, the 0-24 hour urine collection volume was too small for analysis. In Tables 4 and 6 are summarized the salient data. By isotope dilution analysis, less than 2% of the excreted radioactivity could be accounted for as unchanged drug. Fortunately, the glpc tracings of control urine samples worked up according to the analytical procedure showed no interfering peaks. When low doses of 1 (5 and 12.5 mg/kg) were administered, no significant differences in enantiomeric composition were observed (R/S = 1). However, at the higher doses (21, 22, and 29 mg/kg) the analyses established that the R-enantiomer was present in the urine to a greater extent than the S-enantiomer.

Table 6

In Vivo Stereoselective Metabolism of 1 in Rabbits

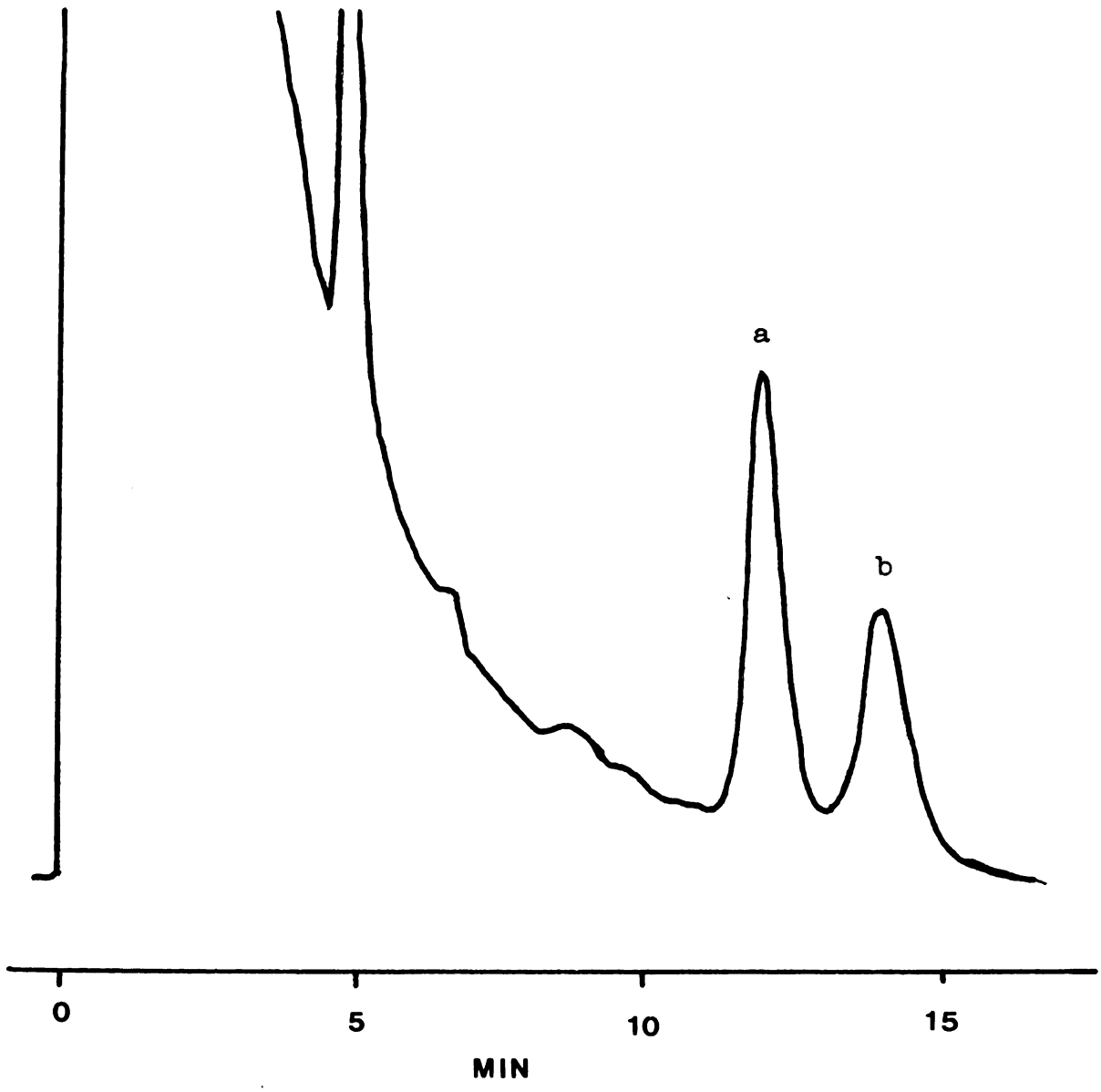
Dose (mg/kg)	Urine Collection (hours)	Enantiomeric Composition of Recovered Amine (R/S)
5	0-24	1.0
12.5	0-24	1.0
21	0-24	1.7
22	0-20	1.4
	20-48	1.4
29	0-48	1.1

See Table 4 for additional excretion data

Figure 3 shows a sample glpc tracing of the diastereomeric amides 37a and 37b obtained from urine after ip administration of R:S-1 (21 mg/kg). In this case nearly twice as much R isomer was excreted than S isomer (R/S=1.7).

Figure 3

Glpc Tracing Showing Stereoselective Metabolism of 1



[a]R:S-37a, R_t= 11.8 min
[b]S:S-37b, R_t= 13.8 min
The base fraction of a 24 hour urine sample after ip administration of racemic 1 (21 mg/kg) was treated with 38 followed by glpc analysis on a 6 ft 3% OV-17 column, oven temp 235°.

In order to determine if metabolic or chemical racemization of 1a or 1b was occurring, the pure enantiomers were administered ip in separate experiments.[13] Isolation of the base fraction and glpc analysis with the prolyl reagent established that in each case no racemization had occurred. While the present data do not permit a statistical analysis of dose vs enantiomeric composition of excreted parent drug, the results of the in vivo studies reported here suggest that the metabolic steps under stereochemical control may either represent minor pathways or that more than one stereoselective reaction is occurring. From the data presented later in this thesis describing the in vitro metabolism of racemic 1 as well as pure 1a and 1b by rabbit liver homogenates, it is apparent that stereoselective processes may be an important factor in the metabolism of 1. These in vitro studies also argue against stereoselective protein binding[100] as being solely responsible for the ratio of enantiomers observed in the urine after parenteral administration of 1 to the rabbit.

E. Experimental Section

Melting points were determined with a Thomas-Hoover Uni-Melt stirring oil capillary-tube melting point apparatus and are uncorrected. Gas-liquid partition chromatography was performed on a Varian Aerograph Model 2100-00 Life Sciences gas chromatograph. U-shaped 2 m x 2 mm id pyrex columns packed with 3% SE-30 or 3% OV-17 on acid washed, DMCS treated chromosorb W were used at column temperatures of 185° for SE-30 and 235-255° for OV-17. Glpc peak intensities were measured on a DuPont Curve Resolver Model 310 or by cutting and weighing. Specific activity measurements in isotope dilution studies were determined from 0.30 to 5.00 mg isolated carrier weighed on a Cahn balance. Liquid scintillation counting of urine or isotope dilution samples in 10 ml Aquasol (New England Nuclear) was performed on a Packard Tricarb Model 3375. All values were corrected for efficiency by ^{14}C-toluene internal standard (New England Nuclear) or by automatic external standard.

1. Heterogeneous solvent extraction of urinary radioactivity.- The combined 24 hour urine samples (1050 ml, 2.1×10^7 dpm, 23.9 mg calculated in terms of parent amine) from three monkey experiments which involved administration of ^{14}C-1 were adjusted to pH 10 with NH_4OH and extracted with Et_2O (3x500 ml). The aq layer (1.7×10^7 dpm) was evaporated to dryness on a rotary evaporator under high vacuum at room temp. The brown gummy residue was digested sequentially at room temp for 2 hours with 500 ml portions of isomeric C_6H_{14}, $CHCl_3$, EtOAc, and finally MeOH. The solid remaining after the extractions

was dissolved in H_2O (47 ml). The MeOH extraction accounted for 98% and the H_2O the remaining 2% of the radioactivity in the urine sample.

2. Attempted cleavage of possible glucuronide or sulfate conjugates.-The MeOH extract ($1.6x10^7$ dpm) from the heterogeneous solvent extraction described above was evaporated and the residue dissolved in H_2O (33 ml), adjusted to pH 5.4 with 0.1N acetate buffer (33 ml) and then incubated with β-glucuronidase solution (0.2 ml, approx 33,400 units, Sigma) at 37°for 48 hours. The solution was then made pH 8.4 with 10% NaOH and extracted with EtOAc (2x50 ml). The EtOAc layers contained only background counts. A second attempt to hydrolyze conjugates with β-glucuronidase was made on another a monkey urine sample. To 10 ml of the urine sample adjusted to pH 5.0 with 0.1N acetate buffer was added β-glucuronidase solution (0.1 ml). After stirring at 37°for 6 hours, the solution was extracted at pH 11.8 with Et_2O (3x20 ml) followed by EtOAc (3x20 ml) at pH 8.5 and Et_2O (3x20 ml) at pH 1. The counts extracted werethe same as from a urine sample not treated with β-glucuronidase. Ether at pH 11.8 extracted 20% and the combined extracts at pH 8.5 and 1 accounted for an additional 3% of the urinary radioactivity.

3. Attempted purification of water soluble metabolites.-The monkey urine sample which had been extracted with Et_2O and EtOAc at pH 12, 8.5 and 1 and which contained radioactive metabolites ($8.0x10^6$ dpm) was lyophilized. The residue was purified by an anion exchange resin (AG 1X4, 100-200 mesh,

chloride form, Biorad). The urine sample was conservatively estimated as having a molecular weight of 50 (200 meq total). A total of 140 g (200 ml) resin was converted to the hydroxide form by washing with H_2O (500 ml), 9 vol 1.0N NaOH (1600 ml), then H_2O until neutral by pH paper. The urine sample was placed on the column at neutral pH and washed with H_2O until neutral. The column was eluted in 20 ml fractions with 1.9N NH_4OH (960 ml), 9.6N NH_4OH (900 ml), 14.8N NH_4OH (480 ml), 1.0N NaOH (720 ml), H_2O (800 ml) until neutral by pH paper, followed by 1.0N AcOH (1000 ml). The elution was followed by liquid scintillation counting (see Table 2, page 13).

Purification of the major radioactive metabolite isolated by ion exchange was attempted by preparative tlc (silica gel, 0.5 mm glass plates, Merck, n-BuOH:AcOH:H_2O, 4:1:1). The major radioactive band (R_f=0.5) was scraped and eluted with MeOH (25 ml). The residue was still impure since no structural evidence could be gained from uv or nmr analysis.

4. Isotope dilution analyses for urinary metabolites.- The 24 hour urines containing 54 to 75% of the administered radioactivity of rabbits injected with ^{14}C-1 (5-29 mg/kg) were quick frozen in 10 equal aliquots. Each aliquot was then used for isotope dilution determinations according to the following details (see Tables 4 and 5).

(a) Unchanged Compound 1. To a solution of carrier 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (1, 15 to 25 mg weighed accurately) in 10% of the urine sample was added 5% NaOH (5 ml) and the resulting mixture was extracted with 3 x 15 ml Et_2O. The combined Et_2O layers were evaporated

and the residue treated with $CHCl_3$ (5 ml), 5% NaOH (5 ml) and benzoyl chloride (0.1 ml). The mixture was stirred at room temp overnight and then the $CHCl_3$ layer was separated and the aqueous layer extracted with $CHCl_3$ (10 ml). The combined $CHCl_3$ layers were dried ($MgSO_4$) and evaporated to leave the benzamide 25 which after 2 crystallizations from isopropanol reached a constant specific activity (see Tables 4 and 5).

(b) 2,5-Dimethoxy-4-(2-aminopropyl)benzoic acid (13).-To a solution of carrier 13 (195.3 mg) in 10% of the urine sample was added 10% NaOH (5 ml) and benzoyl chloride (0.5 ml) and the resulting mixture was stirred at room temp overnight. The mixture was filtered and the filtrate made pH 1 with 10% HCl. The ppt which formed was collected and washed with pet ether (30-60°, 100 ml). The remaining near colorless solid (benzamide 32) was then crystallized to constant specific activity from EtOH (see Table 5).

(c) 2,5-Dimethoxy-4-methylbenzoic acid (21).-To a mixture of carrier 21 (204.2 mg) and 10% of the urine sample was added an equal volume of conc HCl and the resulting mixture was then held at reflux for 1 hour. Upon cooling a ppt developed which was collected and recrystallized from water and was not radioactive.

(d) 1-(2,5-Dimethoxy-4-methylphenyl)-2-propanone (14).-A mixture of carrier 14 (123.9 mg) in 10% of the urine sample was treated with an equal volume of conc HCl and the resulting soln was stirred at room temp overnight. The mixture was then extracted with 3 x 20 ml

Et_2O. The combined Et_2O layers were dried ($MgSO_4$) and evaporated to leave an oil which could be recrystallized from hexane. The resulting pure propanone 14 (mp 50-52°) was not radioactive.

(e) 1-(2,5-Dimethoxy-4-hydroxymethylphenyl)-2-aminopropane (12).-A mixture of carrier 12 (32.9 mg) in 10% of the urine sample was made pH 13 with 10% NaOH the extracted with 5 x 25 ml Et_2O. The combined Et_2O layers were dried (K_2CO_3) then evaporated to leave a residue which was treated with benzoyl chloride (0.2 ml) and pyridine (5 ml). The mixture was heated in an oil bath at 75° for 25 min then cooled. The ppt which developed upon the addition of H_2O (12 ml) was collected, stirred with 5% Na_2CO_3 for 1 min then recollected. The resulting bis-benzoyl derivative 31 was recrystallized from EtOH and was not radioactive (see Table 5).

5. Stereoselective excretion of unchanged 1.-Male Dutch rabbits (1.5-2.0 kg) in metabolic cages were administered ip racemic ^{14}C-1 in normal saline in doses from 5 to 29 mg/kg. Urine was collected through a screen to separate urine and feces in 0-24 and 24-48 hr fractions and frozen until analyzed. Except when analyzing for metabolites each urine sample was divided into two parts, a 10% portion for isotope dilution assay of unchanged compound (see Table 4) and the remainder for glpc analysis after derivatization with the prolyl reagent 38 (see Table 6).

The pH of the fraction for glpc analysis was adjusted to 12 with 10% NaOH and then extracted with 3 x 40 ml portions

of hexane. The combined hexane layers were backwashed with 2 x 10 portions of 10% HCl. The combined acid layers were basified to pH 12 with 10% NaOH then extracted with 3 x 40 ml hexane. The carefully separated hexane layers were evaporated and dried by azeotropic distillation with 10 ml dry C_6H_6. Analysis by glpc of the residue dissolved in 0.1 ml C_6H_6 showed no peaks in the area of the diastereomeric amides 37a and 37b. Analysis of the residue after the addition of about 1 mg of 38 followed by gentle warming showed a chromatogram consisting of two peaks with retention times identical to those of amides 37a and 37b. The enantiomeric composition was determined by measuring the ratio of the peak areas of the diastereomers.

PART II. *In Vitro* Metabolism of 1

The *in vivo* experiments described in Part 1 of this thesis showed that the main metabolic pathway of amine 1 in rabbits involves aromatic methyl oxidation and that 1 is metabolized stereoselectively. In order to study in more depth the enzymatic processes responsible for the metabolism of amine 1, *in vitro* studies were undertaken.

A. The Liver Preparation

In a quantitative sense, the liver is by far the most important organ involved in the metabolism of foreign substances. Many of the drug metabolizing enzymes are located in the hepatic endoplasmic reticulum and can be partially purified as a microsomal fraction.[65,68] The microsomal enzyme system requires for activity a physiological pH, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), and molecular oxygen.[67] Amphetamine (2)[45,65] tolbutamide (22),[46,47] mescaline (3),[57,58] and other compounds[59,60] having structural similarities to 1 are known to be metabolized by microsomal enzymes. As a starting point therefore, the metabolic pattern of these compounds could be used to anticipate the pathway of 1.

In vitro liver studies are usually carried out with tissue slices, total tissue homogenates, 9,000 - 12,000 x g supernatant fraction of homogenates, or microsomes.

The supernatant fractions of homogenates used in this study have certain advantages over the other preparations mentioned. Compared to homogenates, 9,000 x g supernatant fractions provide a reproducible preparation for quantitative studies[50] and often possess greater activity even when expressed in terms of microsomal protein.[51] The 9,000 x g fraction contains most of the microsomes of the homogenate, few mitochondria and most of the soluble fraction enzymes,[50] and is easier to prepare than the microsomal fraction.

Two attempts to metabolize 1 by monkey liver 10,000 x g homogenates following a procedure for pentazocine metabolism[48] yielded only starting amine based on radiochemical isotope dilution analyses. This may be explained by the condition of the tissue. One liver was 4-12 hours old and the other was obtained from a monkey sacrificed by a lethal intravenous dose of pentobarbital. Rabbits were chosen for continuation of the in vitro studies of 1 for several reasons. Rabbits are readily available and relatively inexpensive and have been shown to be efficient metabolizers of 1-phenyl-2-aminopropanes.[54,69] Furthermore, studies on the fate of R and S-amphetamine using rabbit liver homogenates and microsomal preparations have been published.[29,45,65] In order to minimize variation from liver preparation to liver preparation due to age,[72] sex,[71] or species differences,[54,70] only male, six month old, Dutch rabbits were used.

Several attempts to demonstrate metabolism of ^{14}C-1 by

rabbit liver preparations following procedures for amphetamine outlined by Foreman *et al.*[29] and Hucker and co-workers,[62] were unsuccessful (100% recovery of 1 by isotope dilution analysis, see page 36). These systems consisted of a 10,000 x g supernatant fraction of a liver homogenate in phosphate buffer at pH 7.4 and contained substrate, Mg^{++}, nicotinamide, and an NADPH generating system composed of NADP and glucose-6-phosphate in varying proportions. After 2 hours at 37°, little metabolism of 1 by these systems could be detected by radiochemical isotope dilution analyses. One experiment using rat liver microsomes[26] in tris buffer fortified with NADPH also proved unsuccessful. The compositions of some model homogenate systems are summarized in Table 7.

The liver preparation found to be most effective was patterned after a system designed by McDaniel and co-workers[46] for the metabolism of tolbutamide. This mixture does not depend on and NADPH generating system. Instead, NADPH is added directly to the mixture immediatley before incubation. Although the utilization of NADPH by the enzyme mixture was not determined, the amount of NADPH per gram of liver used was equivalent to the reported[50] ratio required for maximal activity. This preparation metabolized 67% of compound 1 when the mixture consisted of amine 1 in 0.29 mM concentration (2.4 μmole/g liver).

Table 7

Compositions of model incubation mixtures for the *in vitro* metabolism of 1. All concentrations are expressed in terms of 1 g liver and in μmoles.

Substrate	amphetamine[29]	amphetamine[62]	compound 1[a]
Substrate conc	6.0	7.4	2.4-12.8
Buffer	Phos,pH 7.4	Phos,pH7.4	Tris/HCl,pH 7.4
NADP	4.0	0.5	—
NADPH	—	—	8.9
Nicotinamide	400	100	—
Glu-6-Phos	160	25	—
Mg^{++}	200	75	80
Total vol (ml)	40	6	8.3
Amt metab	2.6	4.8	1.1-1.9

[a]See Table 9, page 49, for additional data

B. Induction of Metabolism by Pretreatment with Phenobarbital

A number of compounds have been shown to increase the activity of liver drug metabolizing enzymes.[52] Among these are phenobarbital, benzo(a)pyrene, and 3-methylcholanthrene. These agents stimulate drug metabolism by increasing the synthesis of microsomal protein and are effective only after pretreatment of the animal.[52]

The data in Table 8 describe attempts stimulate the metabolism of 1 by rabbit liver preparations by pretreatment with phenobarbital. Two rabbits were injected with phenobarbital (15 mg/kg) intraperitoneally twice daily,[50] in one case for 3 days and in the other for 4 days. Twelve hours after the last dose the rabbits were sacrificed and the livers taken for metabolism studies. Little difference in the total disappearance of 1 in pretreated compared to control incubation mixtures was noted. It is possible that the lack of induction of metabolism was due either to the short period of pretreatment[50] or to the lack of additional NADPH cofactor needed for increased metabolism or to the type of inducer chosen. Further induction studies were not attempted.

Table 8

Effect of Pretreatment with Phenobarbital on the

In Vitro Metabolism of Amine 1

	Pretreatment with Phenobarbital		Control	
Experiment number	1[a]	2[b]	1	2
μmoles substrate	2.6	3.1	2.4	3.1
% substrate metab	61	56	67	56

[a]Pretreated twice daily for 4 days
[b]Pretreated twice daily for 3 days

Supernatant fractions (10,000 x g) of 1 g of liver from two male Dutch rabbits pretreated with phenobarbital (15 mg/kg) were compared to identical liver preparations from two rabbits not pretreated. Racemic ^{14}C-1 was incubated at 37° for 2 hours. Percentages were determined by radiochemical isotope dilution analyses.

C. *In Vitro* Stereoselective Metabolism of 1 in Liver

Further characterization of the parameters controlling the stereoselective metabolism observed in the *in vivo* experiments was pursued using rabbit liver preparations in an effort to more fully describe the nature of the processes under stereochemical control. Axelrod[65] in 1955 found a marked stereospecificity of the rabbit liver microsomal enzymes for the R-isomer of amphetamine. More recently, Hewick and Fouts[45] have shown that R-amphetamine is deaminated about 200% faster than S-amphetamine. In contrast to these findings, Debackere,[61] using the same spectrophotometric assay as Axelrod, found the S-isomer of amphetamine to be a better substrate than the R-isomer in sheep, cattle, swine, and horse liver. Goldstein and Anagnoste[66] reported that S-amphetamine but not R-amphetamine is an *in vitro* substrate for dopamine-beta-hydroxylase. The resulting formation of norephedrine from S-amphetamine has been suggested as a possible reason for the difference in activity of R- and S-amphetamine.[98]

As in the *in vivo* studies, the chiral acid derivative S-(-)-N-pentafluorobenzoylprolyl 1-imidazolide (38) was employed to determine the enantiomeric composition of unchanged 1 extracted from the incubates. The diastereomeric amides 37a (R:S) and 37b (S:S) formed with 38 and racemic 1 separate readily on several glpc columns including SE 30, OV 17 and OV 25; in each case 37a elutes before 37b.

The metabolism of racemic 1 was examined at several initial concentrations. In these studies the percent amine metabolized was established by radiochemical isotope dilution analysis starting with ^{14}C-1. In all experiments the livers were obtained from six month old male Dutch rabbits. When incubations were carried out with boiled liver homogenates, no evidence of metabolism was observed (100% recovery of unchanged amine by radiochemical isotope dilution analysis).

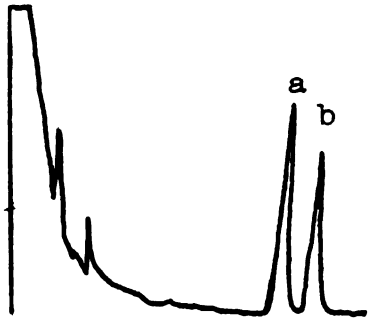
In Table 9 are given the results obtained from incubations of racemic 1 at various initial concentrations. In each experiment it is apparent that the S-enantiomer is more extensively metabolized than the R-enantiomer. In general the enantiomer ratio of unchanged amine (R/S) increases with decreasing initial concentration. On the basis of percent conversion and the R/S ratio of unchanged amine, it is possible to calculate the enantiomeric composition in each experiment. It is of interest to note that in these studies approximately the same number of mg of 1bS are consumed while with decreasing initial concentrations of racemic 1 there is a decrease in the metabolism of 1aR. The significance of this observation is at the present unclear.

In Figure 4 are examples of glpc tracings which show the enantiomeric composition of unchanged 1 after incubation of racemic 1 at various substrate concentrations. At an initial concentration of 0.5 mg/g liver, the metabolism of 1bS is nearly complete and only 1aR remains.

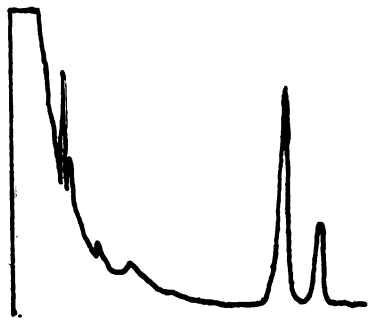
Figure 4

Glpc Tracings Showing the Enantiomeric Composition of Unchanged Amine After the Incubation of Racemic 1 in 10,000 x g Rabbit Liver Homogenates

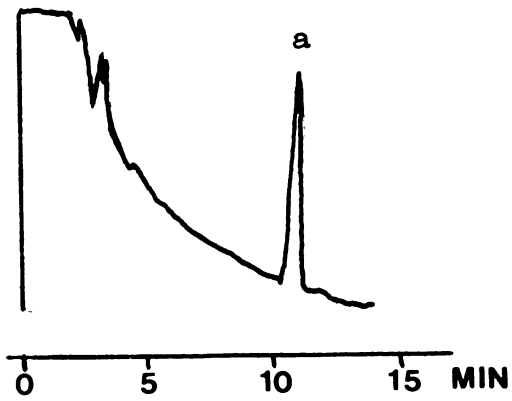
(a) 1.57 mg/g liver



(b) 0.75 mg/g liver



(c) 0.5 mg/g liver



[a] R:S-37a
[b] R:S-37b

Table 9

Stereoselectivity in the *In Vitro* Metabolism of Racemic **1** by 10,000 x g Rabbit Liver Homogenates

Exp. No.	Conc. of **1** mg/g liver (mM)	% Metab (R+S)	mg Metabolized per g liver R+S	S	R	R/S (metab)
1	1.57 (0.77)	30	.47	.29	.18	.64
2	0.75 (0.37)	55	.42	.29	.13	.44
3	0.75 (0.37)	56	.42	.30	.12	.38
4	0.64 (0.31)	61	.39	.29	.10	.35
5	0.58 (0.29)	67	.39	.28	.11	.39
6	0.50 (0.25)	63	.31	.25	.06	.24
7	0.50 (0.25)	57	.28	.22	.06	.27

Percentages are in terms of initial concentrations of racemic ^{14}C-**1** substrate and were determined by radiochemical isotope dilution analyses. Enantiomeric compositions were determined by glpc analysis of derivatives **37a** and **37b**.

D. An Apparent Enantiomeric Interaction in the *In Vitro* Metabolism of 1 in Liver

The dramatic stereochemical preference in the conversion of 1aR and 1bS required further attention, particularly in view of the opposite stereoselectivity reported by others for the metabolism of R- and S-amphetamine by rabbit liver microsomes. Other than methodology, the principal difference in our study and the amphetamine studies[45,65] was the fact that we were examining the metabolism of racemic 1 whereas in the case of amphetamine the pure enantiomers were involved. The possibility arises, therefore, that the stereochemical metabolic fate of amine 1 depends on the enantiomeric composition of the substrate.

To test this possibility, a comparison of the extent of metabolism of pure 1a and 1b by rabbit liver homogenates at three different concentrations was made (see Table 10). In each case the liver from a single animal was first homogenized and the homogenate was divided into three equal portions. Incubations were carried out with 1a, 1b and racemic 1. At the start of the incubation, aliquots of incubates containing 1a and 1b were combined. Glpc analysis of the mixture with the prolyl reagent established that the initial concentrations were equal. At the termination of the incubation period, the extents of conversion of 1a and 1b were determined by adding known quantities of the opposite enantiomer to the incubate and then analyzing for the unchanged amine with the prolyl

Table 10

Comparison of the Metabolism of 1a, 1b and Racemic 1 by 10,000 x g Rabbit Liver Homogenates

Exp.	Substrate	Initial Conc. mg/g liver (mM)	mg Metab.	Calculated R/S ratio unchanged 1
1	1aR	1.04 (.51)	0.45	1.0
	1bS	1.03 (.51)	0.45	
	1	1.57 (.77)	0.47	0.64
2	1aR	0.75 (.37)	0.49	1.1
	1bS	0.75 (.37)	0.44	
	1	0.75 (.37)	0.42	0.44
3	1aR	0.50 (.25)	0.28	0.90
	1bS	0.50 (.25)	0.32	
	1	0.50 (.25)	0.29	0.30

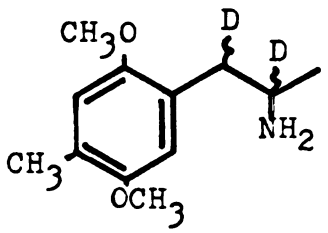
Initial concentrations are in terms of the equivalent of 1 g liver in 8.3 ml total volume. Total mg metabolized was determined by radiochemical or stable isotope dilution analysis or by glpc with the opposite enantiomer as internal standard. Enantiomeric composition was determined by glpc analysis of derivatives 37a and 37b.

reagent. Knowing the amount of the opposite enantiomer added, the glpc ratios permitted a direct calculation of the concentration of the amine metabolized. In one experiment (No. 3, Table 10), the extent of metabolism was determined by an alternate method. At the end of the incubation, equal aliquots of the incubates containing 1a and 1b were combined and the enantiomeric composition determined as before. To the remaining mixtures a known amount of the dideuterio derivative 1(d_2) of amine 1 was added. The base fractions were then submitted to direct insertion probe chemical ionization mass spectral (CIMS) analysis. Repeated scans over the MH^+ ions corresponding to amine 1 (d_0) and amine 1(d_2) provided a reasonably constant ratio throughout the evaporation time and was used to calculate the amount of 1 present in the mixture after incubation. This stable isotope dilution analysis is described in more detail later in this thesis. This technique was also employed to determine the percent metabolism of racemic 1 in experiment No. 3; in experiments No.1 and No. 2 the radiochemical isotope dilution analysis previously described was used.

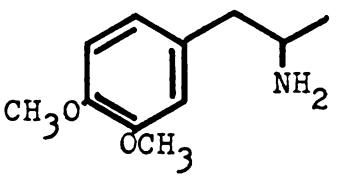
The data generated from these studies are summarized in Table 10. The most dramatic result is that at all three concentrations studied, the two enantiomers proved to be equally good substrates for the liver homogenate enzyme system. This is in marked contrast to the stereoselectivity observed in the metabolism of racemic 1. The simplest interpretation of these results is that one enantiomer is influencing the metabolism of its antipode, possibly via

substrate inhibition. Spectral shift studies on the binding of the S- vs R-amphetamine to cytochrome P-450 have shown that the S-enantiomer binds more strongly than the R-enantiomer.[45] Thus it is conceivable that the metabolism of 1aR is inhibited by 1bS. Such enantiomeric interactions have been reported.[142,143] For example, L-malate is a substrate for fumarate hydratase while D-malate is not although it acts as a competitive inhibitor of L-malate.[101]

Assuming these *in vitro* observations relate in some way to the *in vivo* metabolism of 1-phenyl-2-aminopropanes (and perhaps other asymmetric drugs), our studies suggest that the pharmacologic and toxicologic properties of racemic mixtures should receive independent attention. The recent report by Barfknecht and Nichols[102] that the psychotomimetic activity as measured in rat conditioned avoidance tests of racemic 1-(3,4-dimethoxyphenyl)-2-aminopropane (39) cannot be reproduced by either enantiomer may be explained by enantiomeric interactions in the metabolism of this amine.



1 (d_2)



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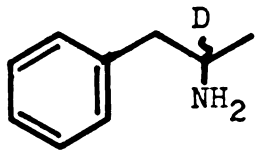
E. Identification of *In Vitro* Metabolites

The studies previously described in this thesis include the application of radiochemical techniques to the identification and quantification of suspected metabolites of amine 1. These techniques were limited, however, to the analysis of compounds anticipated to be metabolites for which authentic standards were available. Unequivocal identification of metabolites of unknown structure using radiochemical labeling can come from extensive purification of small quantities of metabolites from biological mixtures. In order to identify new metabolites of amine 1 while retaining sensitive quantitative capabilities, an alternative labeling technique was employed. This technique involved the application of stable isotope labeling and chemical ionization mass spectrometry (CIMS)[114] to our metabolism studies.

Any label which will make a metabolite easily recognizable among other substances will simplify the detection and identification of the metabolite. The detection of metabolites labeled with stableisotopes can be accomplished by mass spectrometric analysis. In this section are described studies with deuterium labeled amine 1.

One drawback of using deuterium labeled compounds is the possibility of isotope effects which may alter pharmacological activity[121] and rates of metabolism.[122] For example, placement of a deuterium in the alpha position of amphetamine (40) slows its metabolism by rat liver (k_H/k_D = 1.9)[29] and results

in a small isotope effect in its metabolism in man.[31] The effect of deuteration of the OCH_3 group on the *in vitro* demethylation of *o*-nitroanisole (41) shows a deuterium isotope effect k_H/k_D of 2.[33] The *in vitro* metabolism of trideuterio tolbutamide (42) labeled in the toluene position, however, produces hydroxymethyl- and carboxytolbutamide with no apparent isotope effect.[47] In using deuterium as a label



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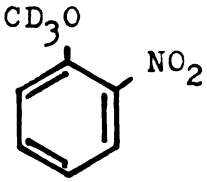

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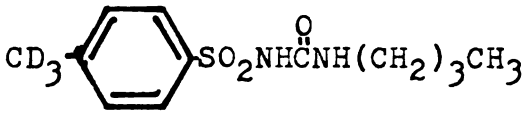


42

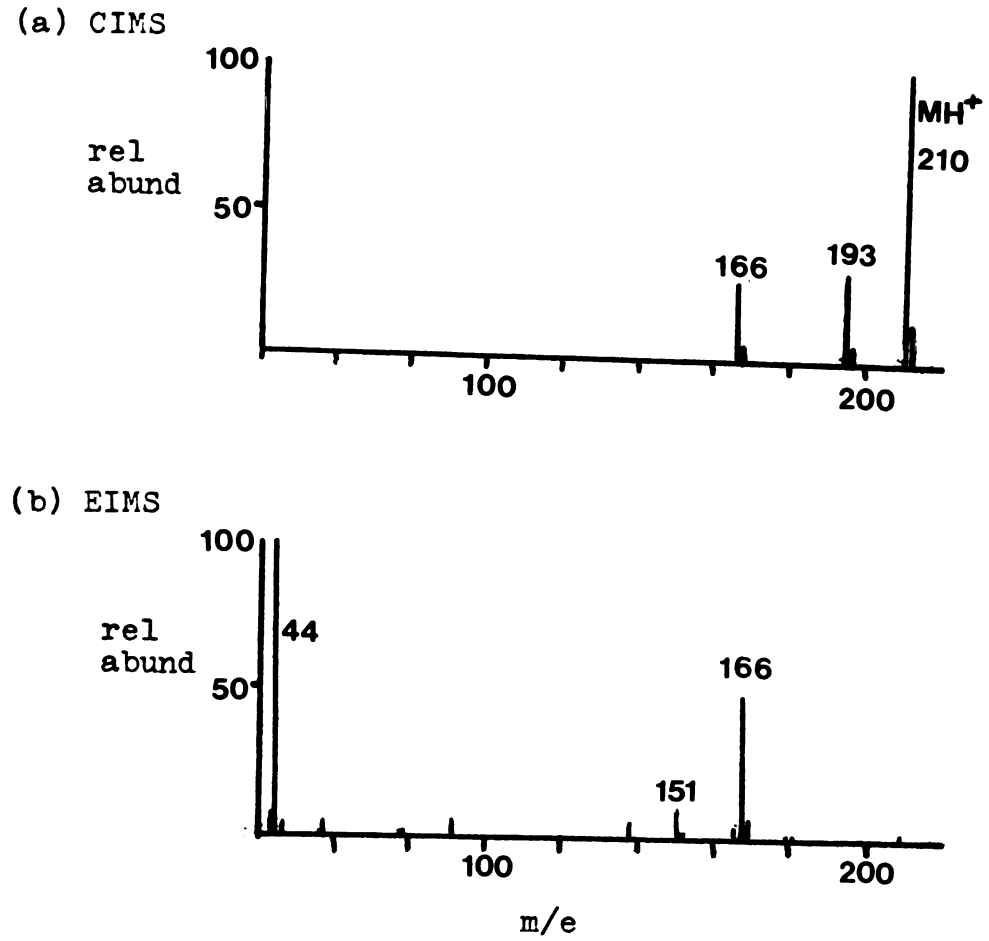
the approximation is made that the physical and chemical behavior of the deuterated molecule is the same as that of the unlabeled molecule, although the rates of metabolism may differ. Rates of metabolism will differ mainly when the rate limiting step in the process involves the cleavage of a C-D bond.[121] Secondary isotope effects must also be considered.[172]

Electron impact mass spectrometry (EIMS)[33,34] and gas chromatography EIMS combinations[118,119,150] have proved to be valuable tools in metabolism studies. These studies, however, are often hampered by the complexity of the EI spectra. CIMS is better suited for metabolism studies[167]

because CIMS produces spectra with relatively limited fragmentation patterns and usually an intense MH^+ ion. In general, CI mass spectra show fewer ions and more intense high mass ions than do EI mass spectra. The CI mass spectrum of compound 1 shows an abundant MH^+ while the EI mass spectrum of 1[117] consists of a base peak of m/e 44 and a parent peak with intensity less than 5% of the base (see Figure 5).

Figure 5

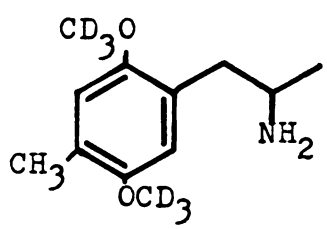
Comparison of Chemical Ionization and Electron Impact Mass Spectra of Amine 1



(a) CIMS, source temp 260°, reactant gas isobutane, 0.7 torr
(b) EIMS spectrum from reference 117

1. Identification of *In Vitro* Metabolites of 1 by Mass Spectrometric Doublet Analysis

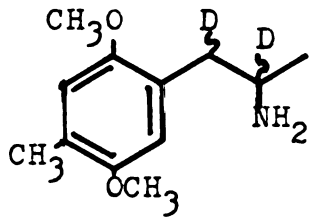
In order to identify the *in vitro* metabolites of amine 1 and to determine if isotope effects alter the fate of deuterated 1, a doublet analysis mass spectrometric technique was employed.[35] This technique involves the use of stable isotope mixtures which allow for identification of labeled metabolites by simple inspection of the mass spectrum for the presence of characteristic doublets. In our case, the doublets arose from incubation of an equal molar mixture of 1 (MH^+ = 210) and deuterated 1 (MH^+ = 216, 1-d_6) in a rabbit liver preparation. Recovered unchanged drug as well as the metabolites retaining the labeled site exhibited in the mass spectrum an MH,MH^+ + 6 doublet for the parent ion and any fragment ions containing the labeled site. The synthesis of hexadeuterio labeled amine 1(d_6) is described in Part III of this thesis. The labeled site was chosen in an attempt to minimize isotope effects. Amine 1 (d_6), labeled in both methoxy groups, was anticipated to avoid isotope effects in both C-4 methyl and side chain metabolism. The main limitation of 1(d_6) is in the analysis of O-demethylation reactions.



1 (d_6), MH^+ 216

In these reactions, isotope effects would be expected and the products formed would lose part or all of the stable isotope label.

The doublet analysis experiments were carried out with the liver homogenate preparations previously described. The mass spectrum (CIMS) of the initial substrate mixture, composed of an equal molar solution of $\underset{\sim}{1}$ (d_0) and $\underset{\sim}{1}$ (d_6), showed a doublet of equal height lines at m/e 210 and 216 for $\underset{\sim}{1}$ (d_0) and $\underset{\sim}{1}$ (d_6) respectively. To measure the amount of substrate consumed, a second deuterated amine $\underset{\sim}{1}$ (d_2) was prepared and used as an internal standard.[73,74] Dideuterio $\underset{\sim}{1}$($\underset{\sim}{1}$(d_2)MH^+ 212) was prepared by reduction of the corresponding phenylnitropropene with $LiAl^2H_4$ as described in Part III of this thesis. Quantification was achieved by comparing the ion



$\underset{\sim}{1}$ (d_2), MH^+ 212

current of the mass spectrometer at the MH^+ ion of the internal standard (m/e 212) to the MH^+ ion of the compound to be analyzed (m/e 210 or 216). Since in this case the internal standard differs from the compound to be measured only isotopically, the chemical and physical properties are the same in the work-up procedure.

After the two hour incubation period, the homogenate was separated into basic, neutral, acidic and amphoteric fractions by extraction with ether at varying pH. Each of the fractions was subjected to direct probe CIMS analysis.

In Figure 6 is a sample mass spectrum of the ether soluble base fraction. As can be seen from Table 11 and Figure 7, repeated scans over the MH^+ ions corresponding to $\underset{\sim}{1}(d_6)$ or $\underset{\sim}{1}(d_0)$ and the internal standard $\underset{\sim}{1}(d_2)$ provided a reasonably constant ratio throughout the evaporation curve. Thus, a ratio of peak heights of d_6/d_2 or d_2/d_0 can be taken at any point on the evaporation curve and used to calculate the amount of $\underset{\sim}{1}(d_6)$ or $\underset{\sim}{1}(d_0)$ present in the incubation mixture.

Figure 6

CIMS of Base Fraction

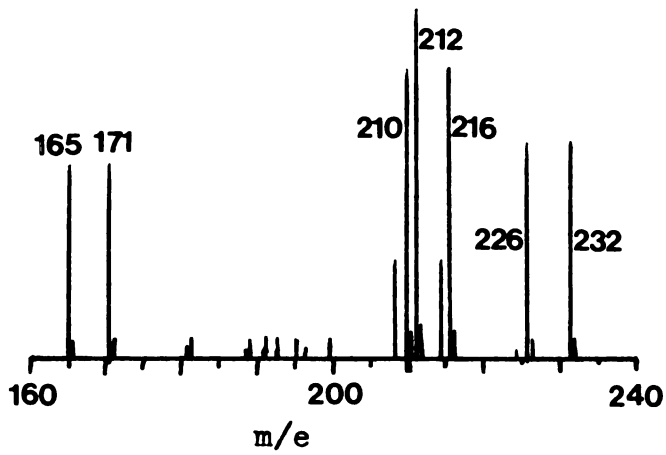


Table 11

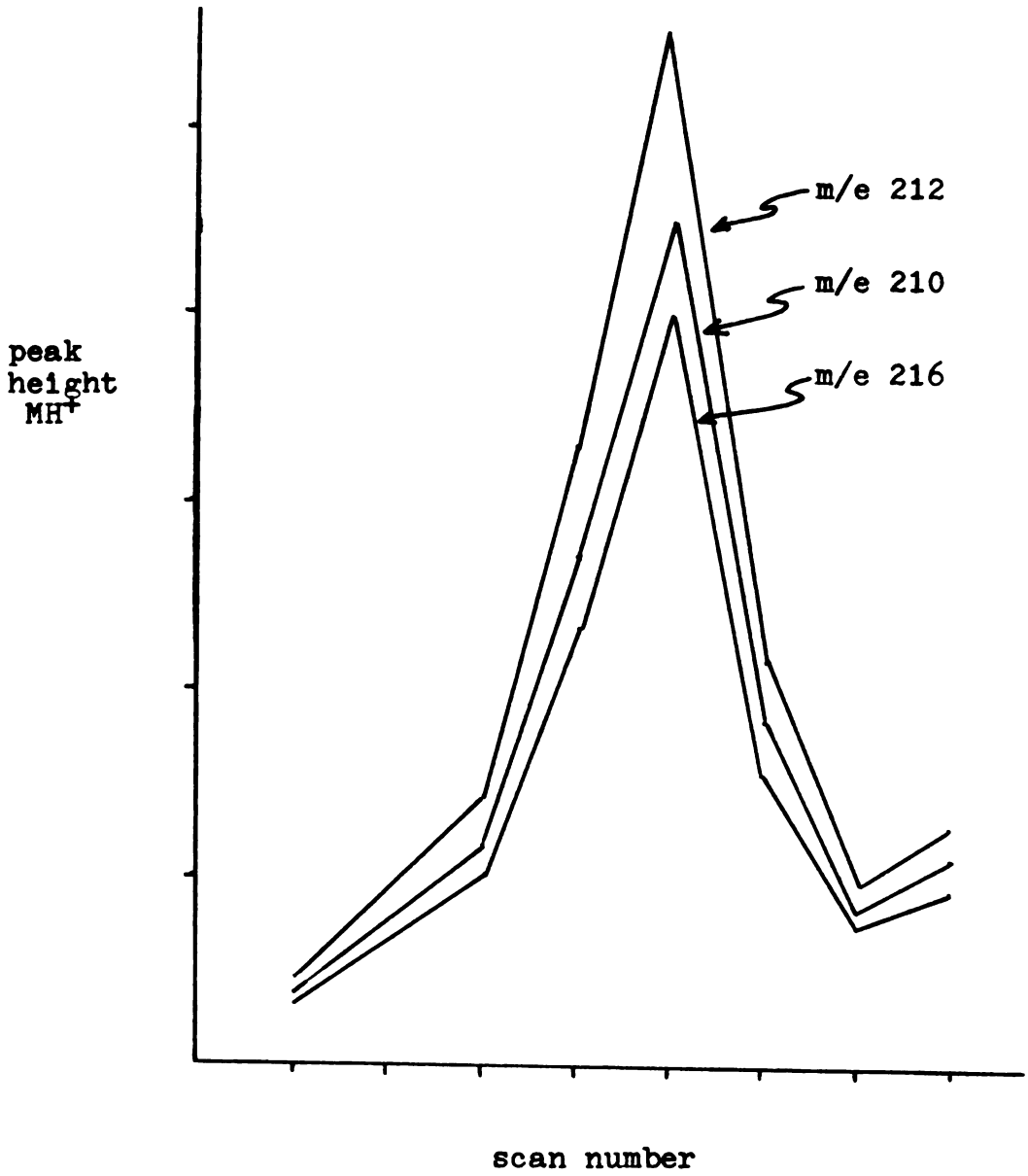
Reproducibility of Peak Height Ratios of d_2/d_o and d_6/d_2 MH^+ Ions of Amine 1 Along an Evaporation Curve

m/e 210 d_o (mm)	m/e 212 d_2 (mm)	m/e 216 d_6 (mm)	212/210 d_2/d_o ratio	216/212 d_6/d_2 ratio
3.6	4.2	3.0	1.17	0.72
6.3	7.6	5.4	1.20	0.71
11.5	14.0	10.0	1.22	0.72
27.0	33.0	23.0	1.22	0.70
45.0	55.0	40.0	1.22	0.73
18.0	21.5	15.0	1.19	0.70
8.0	9.5	7.0	1.19	0.74
10.5	12.5	9.0	1.19	0.72
		ave. ± S.E.M.	1.20±.02	0.72±.01

Peak height measurements are from direct insertion probe CIMS analysis at 0.7 torr, probe temperature 220°. Total time of evaporation curve about 1.5 min. The measurements are from the base fraction of a 10,000 x g liver homogenate with 1(d_o), 0.5 mg, and 1(d_6), 0.5 mg as subtrate. Internal standard 1(d_2), 0.5 mg was added at the end of the 2 hour incubation period.

Figure 7

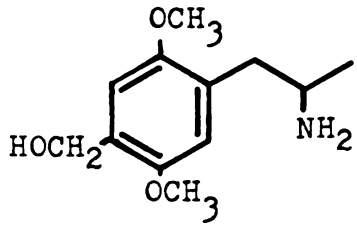
Mass Spectral Evaporation Curve of a Mixture of $\underset{\sim}{1}$ (d_0), $\underset{\sim}{1}$ (d_2) and $\underset{\sim}{1}$ (d_6)



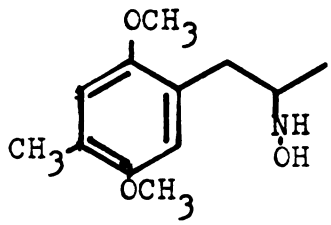
See Table 11 for experimental conditions

The finding that the m/e 216/210 ratio equaled 1 before and after incubation showed that 1 (d_6) is metabolized at the same rate as 1 (d_0). Therefore, at least in terms of disappearance of substrate, no measurable isotope effects were noted.

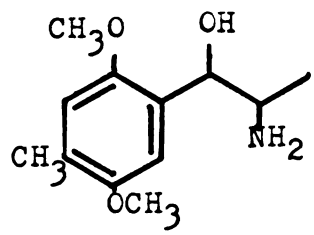
Also seen in the mass spectrum of the base fraction (Figure 6) is a doublet separated by six mass units (m/e 226 and 232). This doublet corresponds to a compound with a molecular weight 16 mass units greater than substrate 1 and is consistent with the incorporation of an oxygen atom into the molecule. High resolution mass spectrometric analysis showed the empirical formula to be $C_{12}H_{20}NO_3$. Of the possible structures for the metabolite, the hydroxymethyl compound 12 the N-hydroxy metabolite 43, and the beta-hydroxy compound 44 are the most likely. CIMS studies on model



12



43



44

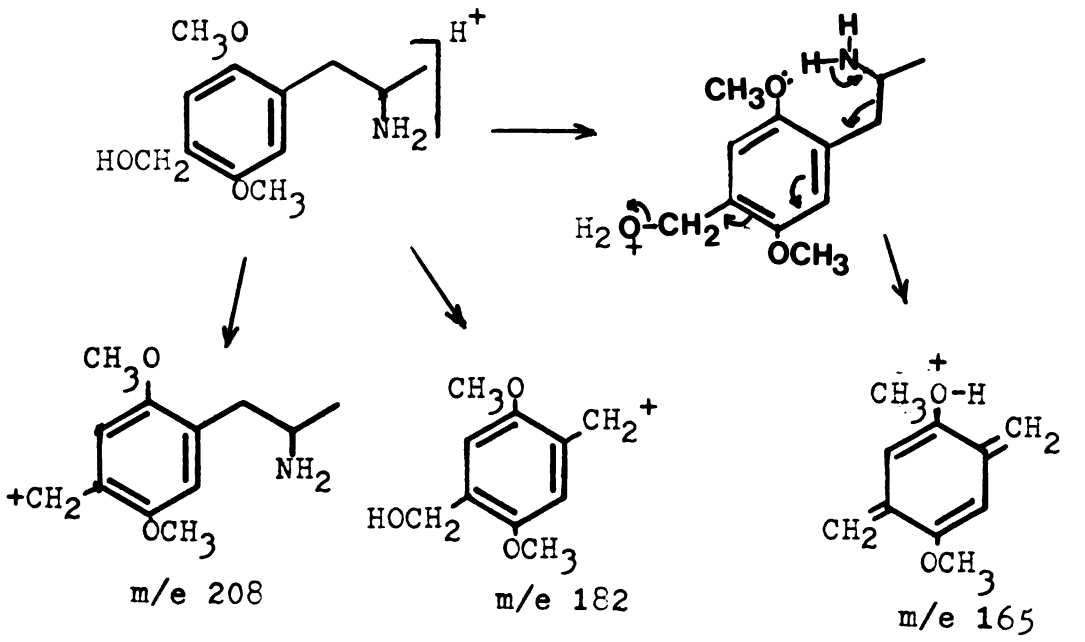
1-hydroxy-1-phenyl-2-aminopropanes such as ephedrine[116] show that these compounds are characterized by minor MH^+ ions

compared to the base peak, the protonated styrene ($MH^+ - H_2O$). The fact that the MH^+ ion is the base peak for the metabolite rules out structure 44. Attempts to synthesize 43 as a standard were unsuccessful. The hydroxymethyl metabolite 12 was synthesized in a pure form and was available for comparison to the metabolite. The data in Table 12 show that the fragmentation pattern of the metabolite and synthetic 12 is nearly identical. The observed stability of the metabolite in aqueous and ether solution also argues against 43 since it has been reported that N-hydroxy compounds are unstable.[150]

Table 12

Mass Spectral Data of Synthetic and Metabolic 4-Hydroxymethyl Compound 12

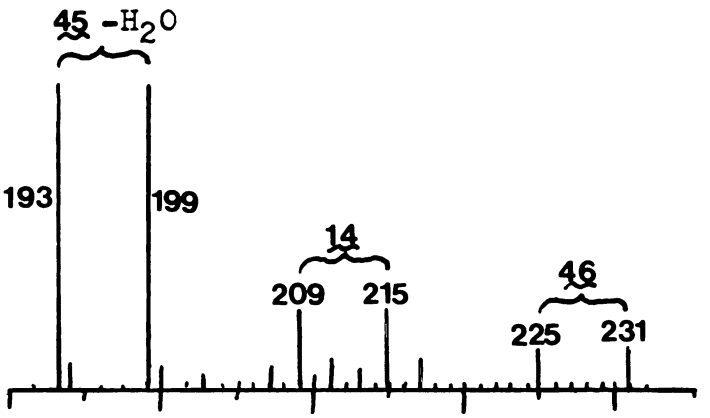
	% Relative Abundance	
m/e	Synthetic	Metabolic
226	100	100
208	50.0	48.2
182	10.4	11.8
165	91.7	91.0



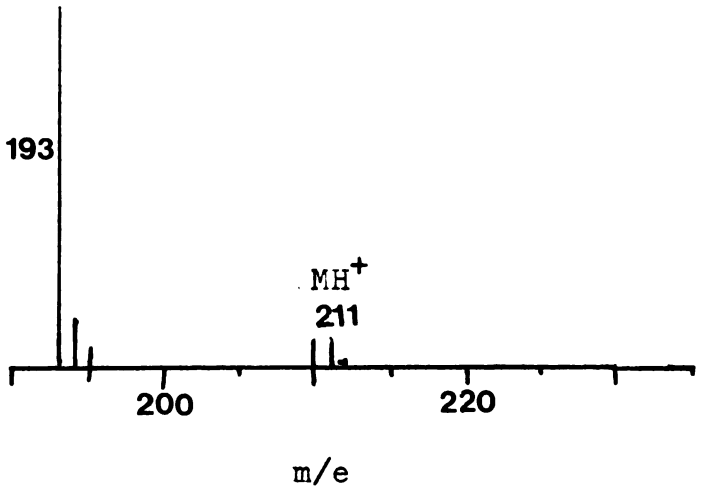
In Figure 8 is a CI mass spectrum of the neutral ether soluble fraction. Three doublets presumably from metabolites can readily be seen at m/e 231/225, 215/209 and 199/193.

Figure 8

(a) Neutral Fraction

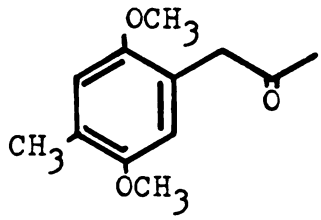


(b) Authentic propanol 45

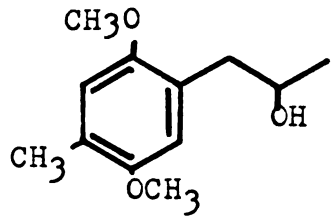


The 215/209 doublet can be accounted for by the oxidative deamination product, ketone 14. High resolution mass spectral analysis was consistent with the molecular formula for 14 (see Table 14). Peak enhancement glpc studies with authentic 14 on OV 17 and SE 30 columns provided additional proof of the identity of the metabolite.

The m/e 199/193 doublet arises from a fragment of neutral metabolite propanol 45. Examination of the mass spectrum 18 mass units above this doublet reveals the presence of the MH^+ doublet at m/e 217/211. Synthetic 45 was prepared (see Part III) for comparison with the metabolite.



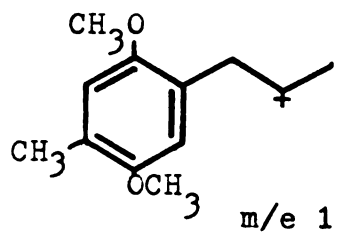
14



45

The CI mass spectrum even at low probe temperatures of synthetic propanol 45 shows a small MH^+ parent ion with the base peak in the spectrum resulting from a $MH^+ - H_2O$ fragment. High resolution mass spectral analysis of the $MH^+ - H_2O$ metabolite fragments verified the empirical formula as that of 45 (see Table 14) and glpc peak enhancement with authentic 45 on an OV 17 column added to the proof of identity of metabolite 45.

45 $\xrightarrow{-H_2O}$



m/e 193

The observed equal intensity of the doublet signals for the hydroxymethyl, propanone and propanol metabolites provides evidence that these metabolites are formed at equal rates from $\underset{\sim}{1}$ (d_0) and $\underset{\sim}{1}$ (d_6). Thus, no measurable isotope effects are involved in the <u>in vitro</u> formation of $\underset{\sim}{12}$, $\underset{\sim}{14}$, and $\underset{\sim}{45}$ from $\underset{\sim}{1}$ (d_6).

Analysis of the ether soluble acid fraction and the homogenate residue for characteristic doublets was unsuccessful. Failure to observe doublets in these fractions may be a result of metabolites being formed in quantities below the detectable limits. Mass spectral analysis may also have been hindered by the possible low volatility of the metabolites. Derivatization techniques to yield metabolite derivatives more volatile for mass spectral analysis were not attempted.

2. Stable Isotope Dilution Analysis of *In Vitro* Metabolites of Amine 1

Part I outlines the use of ^{14}C-labeled amine 1 in radiochemical isotope dilution analyses of metabolites. Radiolabeled 1 and its metabolites were analyzed after dilution with unlabeled synthetic carriers. In order to quantify the *in vitro* metabolites of 1, a modification of these techniques using stable isotope labeled 1 and CIMS was employed. There are several advantages with stable isotope labeling and CIMS for quantitative analyses. One advantage is the ease of sample preparation. In our case, only simple extraction procedures were needed to prepare the metabolite mixture for CIMS analysis. Recrystallization to constant specific activity is not necessary and analyses can be carried out on samples containing microgram quantities of metabolite. Although the usual precautions needed for radioactive substances do not apply, the disadvantage of isotope effects with stable labeled compounds must be considered.

Several pathways of the *in vitro* metabolism of 1 were determined by isotope dilution analyses of suspected metabolites after the incubation of racemic hexadeuterio-1 (1-d_6) in rabbit liver homogenates. To serve as internal standards, unlabeled synthetic metabolites were added to the homogenate at the end of the incubation period. The metabolites were, therefore, labeled with deuterium while the unlabeled materials served as carriers. Reisolation by simple extraction of

carriers and metabolites prepared the samples for CIMS analysis. A comparison of peak height ratios in the mass spectrum of metabolite to carrier provided a quantitative estimate of each suspected metabolite. The preparation of authentic standards is described in the synthesis section (Part III) of this thesis.

Scheme 1 contains the potential metabolites studied. In addition to compounds 1, 12, 13, 14, and 21 which were studied as potential *in vivo* metabolites by radiochemical isotope dilution techniques are the side chain oxidation products propanol 45 and the oxime 26.

Scheme 1

Potential *In Vitro* Metabolites of 1 in Rabbit Studied by Isotope Dilution Analysis

Base Fraction	Unchanged 1
	Hydroxymethyl Cpd 12
Neutral Fraction	Propanone Cpd 14
	Propanol Cpd 45
	Oxime Cpd 26
Acid Fraction	Benzoic Acid Cpd 21
Amphoteric Fraction	Amino Acid Cpd 13

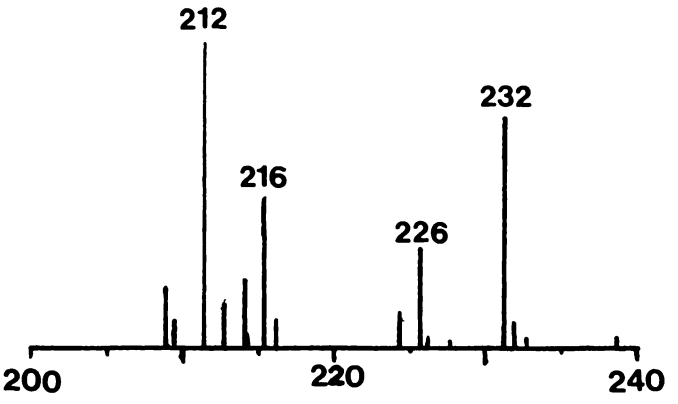
The separation of metabolites was based on the partitioning of authentic compounds into ether at pH 12 for the base fraction, 7.4 for the neutral fraction, and 1 for the acid fraction.

Racemic 1 (d_6) was incubated with the liver homogenate previously described for two hours. At the end of the incubation period, an ethanolic solution of 100-200 μg of each potential metabolite and 1 (d_2) were added to the homogenate and the incubation mixture was extracted with ether. A peak height ratio of 20:1 for carrier to metabolite is easily measured in the mass spectrum. From this ratio it is possible to quantitatively detect 5-10 μg of metabolite in the homogenate. High resolution mass spectrometric analyses were carried out on metabolites and carriers to verify the identity of the substances and to establish the purity of the m/e signals used to determine peak height ratios (see Table 14).

The amount of 1 (d_6) substrate present after incubation was calculated from the d_6/d_2 peak height ratio of MH^+ ions of the remaining 1 (d_6) compared to a known amount of 1 (d_2) internal standard. The amount metabolized was 37% of the substrate (0.38 mg total).

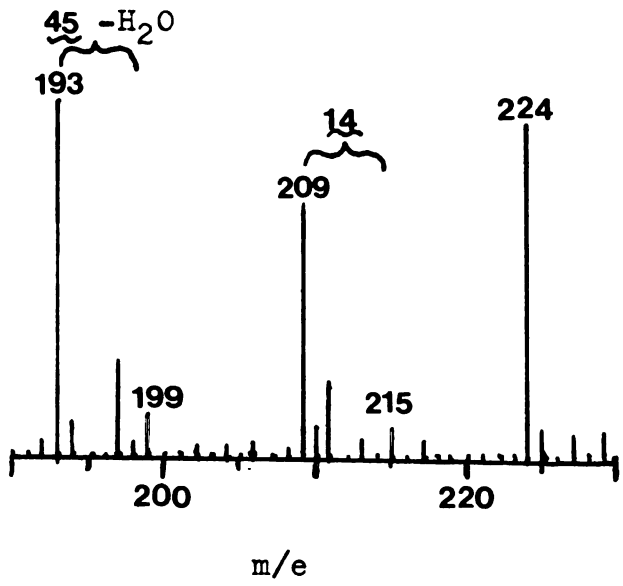
Figure 9

CIMS Analysis of Base Fraction



By a comparison of the m/e 232/226 ratio, the major *in vitro* metabolite was found to be the hydroxymethyl compound 12 (see Figure 9). This metabolite accounted for 70% of the total metabolites.

Figure 10



Stable Isotope Dilution Analysis of Neutral Fraction

Source temperature 130°, isobutane reactant gas, pressure 0.7 torr

Extraction at the pH of the incubation mixture (pH 7.4) yielded the ether soluble neutral metabolites along with contamination from the basic metabolite fraction. Backwashing with 10% HCl provided uncontaminated neutral metabolites. In this fraction were the propanone 14 the propanol 45, and the oxime 26 (see Figure10). The d_6/d_0 (m/e 215/209) peak height ratio was readily calculated from the MH^+ ions for 14. The d_6/d_0 ratio for 45 was calculated either from the MH^+ ions (m/e 217/211) or from the MH^+-H_2O fragment ions (m/e 199/193). The propanol 45 and the propanone 14 were found to be minor in vitro metabolites of amine 1. From the absence of an m/e 230 signal in the neutral fraction, it was determined that the oxime 26 is not a metabolite in this preparation.

CIMS analysis of the ether soluble acidic components provided a clean spectrum with the only major peak at m/e 197 corresponding to the MH^+ ion of internal standard benzoic acid 21 (d_0). Acid 21, therefore, is not an in vitro metabolite of 1 under the conditions tested.

Attempts to extract synthetic amino acid 13 into a variety of organic solvents at various pH values were unsuccessful. Attempts to analyze the homogenate directly for standard 13^{173} were hindered by the presence of numerous contaminating substances in the mass spectrum. Anion exchange chromatography was attempted on the homogenate to separate water soluble compounds not containing an acidic function from standard 13. The mass spectrum of the acid wash of the column was still complicated and did not show the presence

of 13. Attempts to analyze amino acid 13 as its more volatile benzoyl or pentafluoropropionyl derivative were made. The mass spectrum of the homogenate after treatment with benzoyl chloride or pentafluoropropionic anhydride did not contain signals for the respective derivatives of 13. From these results it is clear that stable isotope dilution analysis of the water soluble *in vitro* metabolite 13 requires extensive purification procedures. Although the radiochemical isotope dilution analysis described in Part I of this thesis could have been applied to the *in vitro* formation of 13, no further studies on the possible formation of 13 were undertaken.

The results of the stable isotope dilution analyses are summarized in Table 13. A total of 74% of the metabolites was accounted for by the three compounds 12, 14, and 45. As in the *in vivo* case, aromatic methyl oxidation proved to be the major pathway. The hydroxymethyl compound 12 accounted for most of the material metabolized. This metabolite was not found in rabbits *in vivo*. The major *in vivo* metabolite, amino acid 13, presumably arises from further oxidation of 12 This enzymatic oxidation is probably catalyzed in part by alcohol dehydrogenase (ADH).[165] The fact that hydroxymethyl compound 12 is the major *in vitro* metabolite suggests that the conditions might not have been optimal for ADH activity. ADH is located primarily in the soluble fraction of the cell[165] and would be expected to exist in the supernatant fraction of a 10,000 x g liver homogenate. The pH optimum for ADH, however, of 10-11 is significantly higher than pH 7.4 which

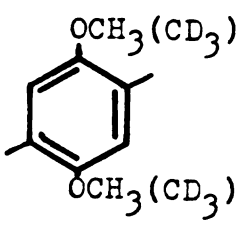
is optimal for microsomal activity. Furthermore, the cofactor used in our studies, NADPH, does not serve as a cofactor for ADH which requires NAD. The accumulation of the hydroxymethyl metabolite 12 in the homogenate is reasonable in terms of a possible lack of ADH activity in the liver preparation.

Table 13

In Vitro Metabolites of 1 (d_6) in 10,000 x g Rabbit Liver Homogenate

Compound	MH^+		% Total
	Metab.	Carrier	Metabolites
$HOCH_2ArCH_2\underset{NH_2}{C}HCH_3$	232	226	70
$CH_3ArCH_2\underset{OH}{C}HCH_3$	217[a]	211[a]	3
$CH_3ArCH_2\overset{O}{\overset{\|}{C}}CH_3$	215	109	1
$CH_3ArCH_2\overset{N}{\overset{\|}{C}}CH_3$ (N–OH)	230	224	0
$CH_3ArCOOH$	203	197	0

[a]Also calculated from m/e 199/193 MH^+ fragment

Ar.: 

The anticipated side chain oxidation was found to be only a minor pathway since the propanol 45 was found to account for 3% of the metabolites and the ketone 14 an additional 1%. The 3/1 ratio of formation of propanol to ketone may represent an equilibrium between 14 and 45. An equilibrium relationship in a liver preparation of phenylpropanone and phenylpropanol has been shown.[62]

Table 14

High Resolution CIMS of *In Vitro* Metabolites and Carriers Isolated From a 10,000 x g Liver Homogenate

Compound	MF (MH^+)	Mass	Found	Δ mMu error
1 (d_2)	$C_{12}H_{18}{}^2H_2NO_2$	212.1620	212.1623	0.3
1 (d_6)	$C_{12}H_{14}{}^2H_6NO_2$	216.1871	216.1879	0.8
12 (d_o)	$C_{12}H_{20}NO_3$	226.1443	226.1443	0.0
12 (d_6)	$C_{12}H_{14}{}^2H_6NO_3$	232.1819	232.1821	0.3
14 (d_o)	$C_{12}H_{17}O_3$	209.1178	209.1169	0.9
14 (d_6)	$C_{12}H_{11}{}^2H_6O_3$	215.1554	215.1559	0.5
45 (d_o)	$C_{12}H_{17}O_2$[a]	193.1228	193.1232	0.4
45 (d_6)	$C_{12}H_{11}{}^2H_6O_2$[a]	199.1605	199.1596	0.9
21 (d_o)	$C_{10}H_{13}O_4$	197.0814	197.0823	0.9
26 (d_o)	$C_{12}H_{18}NO_3$	224.1287	224.1283	0.4

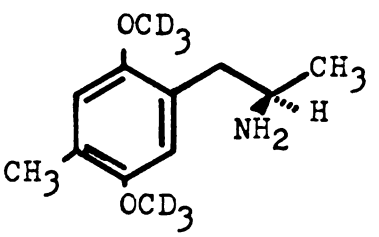
[a] MH^+-H_2O fragment

Deuterated metabolites were formed in a 10,000 x g rabbit liver homogenate from 1 (d_6) as substrate as described in the experimental section. At the end of the incubation period, compounds 1 (d_2), 12 (d_o), 14 (d_o), 45 (d_o), 21 (d_o), and 26 (d_o) were added as internal standards. The metabolites and carriers were isolated into ether fractions at various pH values. Source temp 250°, pressure 0.4 torr methane.

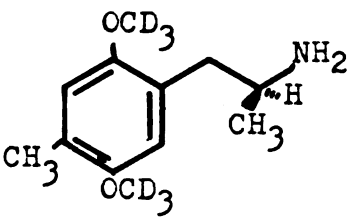
F. Stereoselective *In Vitro* Metabolite Formation

Compound 1 has been shown in previous sections of this thesis to be metabolized stereoselectively by rabbits. It was of interest to determine if the stereochemistry of the substrate was a controlling factor in the formation of the individual metabolites. In order to evaluate the stereochemical factors involved, the study of the formation of the metabolites of 1 was undertaken using the resolved R- and S-isomers of stable isotopically labeled 1 (d_6) and CIMS. From the stable isotope dilution analyses previously described it was found that deuterium atoms in the methoxy groups of 1 (d_6) did not interfere with either aromatic methyl oxidation or side chain metabolism.

The experiment involved the resolving of deuterium labeled 1. The stereospecifically labeled substrate was composed of an equal molar mixture of one enantiomer of deuterio 1 (d_6) and its opposite enantiomer protio 1 (d_0). The pseudoracemic composition of the substrate was confirmed by mass spectral analysis which displayed two equal height MH^+ signals at m/e 210 and 216 (see Table 15).

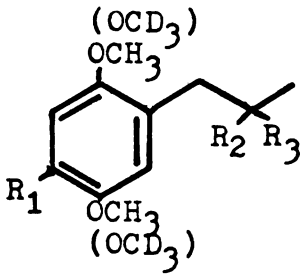


R-1 (d_6)



S-1 (d_6)

Glpc analysis of the substrate after treatment with prolyl reagent 38 also showed the mixture to be racemic. The resulting pseudoracemates (see Table 15) were then used to determine the enantiomeric source of the metabolites. Analysis of metabolites 12, 14 and 45 by CIMS indicated the presence or absence of label. This analysis of enantiomeric source of metabolite formation is independent of the chirality of the metabolite. For example, the propanone 14 no longer possesses an asymmetric center but the presence or absence of the deuterium label in the methoxy positions indicates from which enantiomer of 1 the ketone 14 is derived.



12: $R_1=CH_2OH$; $R_2=H$; $R_3=NH_2$

14: $R_1=CH_3$; $R_2R_3=O$

45: $R_1=CH_3$; $R_2=H$; $R_3=OH$

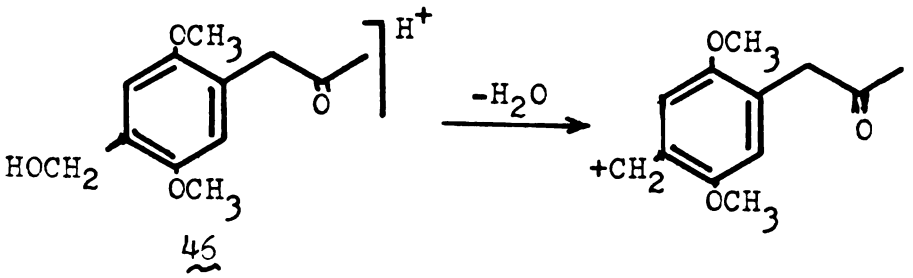
Three identical portions of a liver preparation from a single rabbit were used. The substrate incubated in one portion consisted of an equal molar mixture of racemic 1 (d_6) and racemic 1 (d_0). In the second portion was incubated an equal molar mixture of S-1 (d_6) and R-1 (d_0) and in the third portion an equal molar mixture of S-1 (d_0) and R-1 (d_6). Following incubation the percentage total amine metabolized was determined with the aid of 1 (d_2) as described previously.

A comparison of the results from these three combinations of substrate was also used to further evaluate potential deuterium isotope effects (see below).

The data generated in this experiment are summarized in Table 15). At the end of the two hour incubation period, the incubate containing racemic 1 (d_6) and racemic 1 (d_0) showed ions at m/e 210 and 216 of equal height corresponding to the MH^+ ions of 1 (d_0) and 1 (d_6) respectively. By comparison to the internal standard, about 0.23 mg of 1 (d_0) and 1 (d_6) (0.46 mg total, 46%) of the substrate (1.0 mg) was metabolized. Since the remaining amine gave a mass spectrum with the same d_6/d_0 ratio as the initial substrate, 1 (d_6) and 1 (d_0) were again seen to be nearly equal substrates for the enzymes in the liver preparation. Analysis of the d_6/d_0 ratios of unchanged amine in the other two mixtures showed the same stereoselective metabolic preference seen in the glpc studies described earlier (see Table 9). The R/S ratio of unchanged amine after incubation was 2.1 in one case and 1.8 in the other.

The stereochemistry of metabolite formation was evaluated. As before, neutral metabolites were extracted into ether at the pH of the incubation medium (pH 7.4). Basic metabolites were extracted at pH 12 with ether. The metabolites studied were the hydroxymethyl compound 12, the propanone 14, and the propanol 45. Also studied was a neutral metabolite with the structure tentatively assigned as the hydroxypropanone 46. The CIMS spectrum of the neutral fraction showed an MH^+ ion at m/e 225 and 231 for the labeled and unlabeled 46 plus a fragment consistent with the loss of water from the parent.

The structure of 46 is only tentative because the metabolite has never been isolated in a pure form, high resolution CIMS attempts were unsuccessful, and authentic 46 was not available for comparison.



46 m/e 207

In Table 15 are summarized the data which show the stereochemistry of the formation of some of the metabolites of amine 1. None of the metabolites studied was formed stereospecifically since no metabolite was formed exclusively from one of the isomers of 1. The formation of the metabolites was found, however, to be under stereochemical control. The R/S ratio of metabolites formed ranged from 0.2 in the case of the propanone 14 to 0.8 for the major metabolite, hydroxymethyl compound 12. While the data are from a single experiment and no statistical evaluation was possible, it is clear that each of the metabolites was formed to a greater extent from the S-isomer than from the R-isomer. The significance of this observation is at the present unclear though it does suggest that there is a stereoselective barrier acting before the substrate reaches the enzymatic site of metabolism. Compared to previous in vitro experiments (see

Table 15

Stereoselective Metabolite Formation From Amine 1

in a 10,000 x g Rabbit Liver Homogenate

Compound	Substrate		
	R:S-d_o, R:S-d_6	R-d_o, S-d_6	R-d_6, S-d_o
Amine 1			
before incubation	d_6/d_o=1.02	R/S=0.95	R/S=0.95
after incubation	d_6/d_o=1.00	R/S=2.1	R/S=1.8
% metabolized	46	42	48
% R metabolized	—	11	17
% S metabolized	—	31	31
$HOCH_2ArCH(NH_2)CHCH_3$	d_6/d_o=1.1	R/S=0.6	R/S=0.8
% of metabolites	70[a]		
$CH_3ArCH_2C(=O)CH_3$	d_6/d_o=1.2	R/S=0.2[c]	R/S=0.3[c]
% of metabolites	1[a]		
$CH_3ArCH_2CH(OH)CH_3$	d_6/d_o=1.1	R/S=0.5	R/S=0.4
% of metabolites	3[a]		
$HOCH_2ArCH_2C(=O)CH_3$[b]	d_6/d_o=1.1	R/S=0.5	R/S=0.3
% of metabolites	trace		

[a] See stable isotope dilution analyses of metabolites, Part II
[b] Structure tentatively assigned
[c] Calculated as if C=O were chiral
Initial substrate concentration in each mixture was 1.0 mg of 1

Ar:

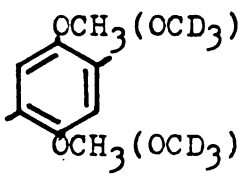


Table 9) the amount of substrate consumed in this experiment is at a maximum. The stereoselective process is possibly a function of enzyme saturation. The metabolic preference for S-1 may also be related to the stronger binding of S-amphetamine than R-amphetamine for microsomal P-450.[45,176] Kinetic studies on metabolite formation might provide more information on a possible metabolic interaction of R- and S-1 and their metabolites.

G. *In Vitro* Stereoselective Metabolism of 1 in Rabbit Brain

Amine 1 has been shown by autoradiographic studies to penetrate the blood brain barrier.[55,56] In view of this, *in vitro* brain metabolism studies of 1 in rabbits were initiated. The first attempt involved the application of the techniques described for liver homogenates to brain metabolism studies. The whole brain was removed immediately after decapitation, rinsed in ice cold 1.15% KCl then homogenized in tris/HCl buffer (pH 7.4) containing sucrose. Substrate ^{14}C-1 and NADPH were added and the mixture was incubated at 37°. Total time elapsed before incubation was one hour. Isotope dilution analysis of an aliquot of the mixture revealed, after one recrystallization of the benzamide 25, that 93.4% of the amine remained unchanged. Better success was achieved when steps were taken to maximize the viability of the tissue. The lag time between brain removal and incubation was reduced to less than five minutes by omitting the homogenization step. Since brain tissue requires more oxygen than other organs,[64] pure O_2 was bubbled through the incubation mixture. Two experiments were carried out using this revised technique. In the first experiment, isotope dilution analysis showed that almost 30% (0.19 mg) of the substrate had been consumed. Analysis of the incubate by treatment with prolyl reagent 38 followed by glpc on an OV 17 column showed a contaminating substance with a retention time identical to the amide 37b which hindered the enantiomeric composition determination. Analysis on an SE 30 column separated this contaminating

substance from the prolyl amides 37a and 37b. Analysis of the peak areas of 37a and 37b on SE 30 showed an R/S ratio of recovered 1 to be 1.1. Repetition of this experiment resulted in metabolism of 21% (0.12 mg) of the amine. The R/S ratio of unchanged substrate was again 1.1. No attempt was made to measure tissue viability and no energy source such as glucose was provided in the incubation medium.

The results summarized in Table 16 show that some metabolism did occur in the brain preparations and that 1b (S) is a slightly better substrate than 1a (R) when racemic 1 was substrate. The significance of these findings is unclear and no further brain studies were carried out.

Table 16

In Vitro Stereoselective Metabolism of Racemic ^{14}C-1 by Rabbit Brain

Exp.	Substrate Conc. mg ^{14}C-1 (mM)	% metab.	mg metab.	O_2	R/S unch. 1
1	0.62 (.10)	6	0.04	no	1.0
2	0.64 (.10)	30	0.19	yes	1.1
3	0.55 (.09)	21	0.12	yes	1.1
control	0.49 (.08)	0	0.0	yes	1.0

Incubations of racemic ^{14}C-1 were carried out with the whole brain (7-9 g) from three separate rabbits as described in the experimental section. The control experiment consisted of amine 1, buffer, and bubbling pure O_2 at 37° for 1 hour.

H. Experimental Section

1. Incubations. Male Dutch rabbits (6 mo) were stunned by a blow to the neck and their livers immediately removed and rinsed in ice cold 1.15% KCl. Five gram portions of the livers were homogenized in 6 volumes of 0.05 M Tris/HCl buffer (pH 7.4) containing 0.25 M sucrose with a Potter-Elvehjem type of homogenizer. The homogenate was centrifuged at 10,000 x g for 20 min in a Sorvall RCZ-B refrigerated centrifuge and the supernatant fraction used for incubation. The incubation mixture contained substrate (4.1-12.8 μmoles), homogenate (14 ml, equivalent to 2 g liver), NADPH (17.8 μmoles) and $MgCl_2$ (160 μmoles). The final total volume was 16.6 ml. The total time for preparation of the homogenate was one hour. The mixture was incubated open to the air for 1 or 2 hours at 37° in a metabolic shaker.

2. Determination of Enantiomeric Composition. The mixture to be analyzed was treated with 10% NaOH (1 ml) and was then extracted with 3 x 25 ml portions of hexane in a separatory funnel with gentle shaking. The combined hexane extracts were concentrated under vacuum and the residue in 20 ml anhydrous benzene azeotroped to dryness. Drying agents such as $MgSO_4$ adsorbed the amine and therefore could not be used. The dry residue was dissolved in 0.5 ml anhydrous benzene and treated with 1 mg of prolyl reagent 38. After about 15 min at room temp, the mixture was analyzed by glpc as described in the experimental section of Part I.

3. Quantitative Estimation of Amine 1. (a) Radiochemical isotope dilution analyses were carried out on the benzoyl derivative of 1 as previously described using 2 ml aliquots of the incubation mixture with 20 mg of added cold carrier 1. Two or three recrystallizations of the benzamide derivative 25 yielded material with constant specific activity.

(b) Glpc Determination. To the incubation mixture was added 0.75 mg of the opposite enantiomer. Work-up and derivatization with the prolyl reagent 38 proceeded as described above. Glpc analyses on OV 17 or SE 30 provided ratios of amides 37a and 37b from which the concentrations of the amine were calculated.[99]

(c) CIMS Analyses. In Experiment #3, Table 10, aliquots (2 ml) of the mixtures containing 1a, 1b, and racemic 1 were taken and to each was added 0.125 mg of internal standard 1 (d_2). After adjusting the pH to 12 with 10% NaOH, the mixtures were extracted with 3 x 5 ml Et_2O. The combined extracts were evaporated to dryness and the residues made anhydrous by azeotropic distillation with dry benzene. The resulting residues were transferred with EtOH to the direct insertion probe of the mass spectrometer. Repeated scans of the MH^+ ions, 210, 212, for 1 and 1 (d_2) were obtained during evaporation of the sample (source temperature 200-230°). Variation of the 210/212 ratio was less than 5%. The concentration of 1 was then calculated on the basis of this ratio.

4. Quantitative Estimation of the In Vitro Metabolites of Amine 1. Racemic 1 (d_6) (0.77 mg, 3.14 μmole) was incubated

in the liver homogenate described above. Stable isotope dilution analyses were carried out by the addition of carrier unlabeled metabolites at the end of the 2 hour incubation period. Each carrier metabolite served as an internal standard for the CIMS analysis of the deuterium labeled metabolite. The carrier metabolite mixture consisted of an ethanolic soln which contained in each 0.1 ml amine $\underset{\sim}{12}$ (103 μg), propanone $\underset{\sim}{14}$ (110 μg), propanol $\underset{\sim}{45}$ (209 μg), ketoxime $\underset{\sim}{26}$ (110 μg), acid $\underset{\sim}{21}$ (100 μg), and amino acid $\underset{\sim}{13}$ (100 μg). Internal standard $\underset{\sim}{1}$ (d_2) (0.76 mg) was also added to the homogenate. The resulting mixture was extracted at pH 7.4 with Et_2O (3 x 25 ml) to give the neutral fraction. The aq layer was adjusted to pH 12 with 10% NaOH and extracted with Et_2O (3 x 25 ml) to give the base fraction. After adjusting the pH of the aq layer to 1 with concd HCl, the mixture was again extracted with Et_2O (3 x 25 ml) to give the acid fraction. The Et_2O solutions containing the neutral, base and acid fractions were reduced in volume under vacuum to 0.1-1.0 ml and were then analyzed by CIMS for metabolites and carriers (see Table 13).

5. <u>In Vitro Brain Studies</u>. Male Dutch rabbits (6 mo) were guillotined and the whole brains (7-8 g) removed immediately and rinsed in ice cold 1.15% KCl. The preparation of the tissue is described in Experiments 1, 2 and 3. The incubation mixture contained substrate (^{14}C-$\underset{\sim}{1}$) and Tris/HCl pH 7.4 buffer (0.05 M, 20 ml) containing sucrose (0.25 M). The mixture was stirred with a magnetic stirrer for 1 hr at 37°. At the end of the incubation period a 2 ml aliquot was taken for isotope dilution analysis and the remainder was analyzed with the

prolyl reagent 38 for enantiomeric composition as described above.

(a) Experiment 1. The rabbit brain (7.8 g) was homogenized with a Potter-Elvehjem type of homogenizer before incubation of the substrate (0.62 mg ^{14}C-1) in the presence of NADPH (41.5 mg). The mixture was incubated open to the air at 37° for 1 hr. The total time elapsed before incubation was 1 hr. After incubation, carrier 1 was added to a 2 ml aliquot and isotope dilution analysis as the benzamide 25 was carried out. One recrystallization of 25 showed that 93.4% of the amine remained unchanged. Analysis of the remaining portion of the homogenate with the prolyl reagent 38 and glpc showed the R/S ratio of unchanged 1 to be 1.0.

(b) Experiment 2. The lag time between brain removal and incubation was shortened to 5 min by omitting the homogenization step and mincing the brain with scissors. The incubation mixture, which contained substrate (0.64 mg ^{14}C-1) and Tris/HCl buffer (pH 7.4), was treated by bubbling pure O_2 (about 120 ml/min) via a pasteur pipette during the 1 hr incubation period. At the end of the incubation period, a 2 ml aliquot of the 26 ml (total volume) incubation mixture was taken for isotope dilution analysis. Carrier 1 (22.8 mg) was added and the benzamide 25 was prepared. After 2 recrystallizations from EtOH, this reached a constant specific activity (1050 dpm/mg, 1030 dpm/mg). Almost 30% (0.19 mg) of the substrate had been consumed. The remaining 24 ml of the incubate was analyzed by treatment with prolyl reagent 38 followed by glpc on a 3% SE 30 column. The R/S

ratio of unchanged amine was 1.1.

(c) Experiment 3. This experiment followed the same procedure as Experiment 2. About 21% (0.12 mg) of the substrate (0.55 mg ^{14}C-1) was consumed as measured by isotope dilution with carrier 1 (19.6 mg). Two recrystallizations of benzamide 25 yielded a constant specific activity (1202 dpm/mg and 1180 dpm/mg). The R/S ratio of unchanged substrate was also 1.1.

(d) Control. To serve as a control, ^{14}C-1 (0.49 mg) in Tris/HCl buffer (0.05 M, pH 7.4, 25 ml) was stirred at 37° with pure O_2 bubbling through the solution for 1 hr and was then analyzed for disappearance of substrate and enantiomeric composition as above. Isotope dilution analysis showed 102% recovery of amine and glpc analysis with the prolyl reagent 38 showed an R/S ratio of remaining amine of 1.0.

PART III. Synthetic Studies

A major obstacle encountered in the detection of metabolites is the complexity of biological mixtures which conceals relatively small quantities of metabolites. Analyses involving biological fluids are greatly simplified by the use of isotopic markers.[139] Radioactive isotopes, usually ^{14}C or ^{3}H, can often be incorporated into drugs with simple chemical reactions. The fate of the labeled drug can then be followed by radiochemical techniques through biological systems with high sensitivity.[139] Stable isotopes of hydrogen and carbon have recently become popular for labeling in drug metabolism studies. Stable isotopes have been useful for the discovery of new metabolites[35,36] and in biotransformation mechanism studies.[30,34,37-39,42]

Another use for chemical synthesis in metabolism studies involves the preparation of authentic standards for comparison with biotransformation products as evidence for proof of structure. In most cases the isolation and purification of sufficient amounts of metabolite for structure determination by classical chemical methods is prohibited by the limited quantity of metabolite formed. Comparison of a partially purified metabolite to an authentic synthetic metabolite by such techniques as tlc R_f values, glpc retention times, color tests, reactivity and spectral properties provides evidence leading to the structure determination of the metabolite. Radioisotope dilution studies can also

provide proof of structure.[139] This technique can be used both qualitatively and quantitatively. Isotope dilution analyses with stable isotopes and mass spectrometry are also of considerable value in drug metabolism studies.

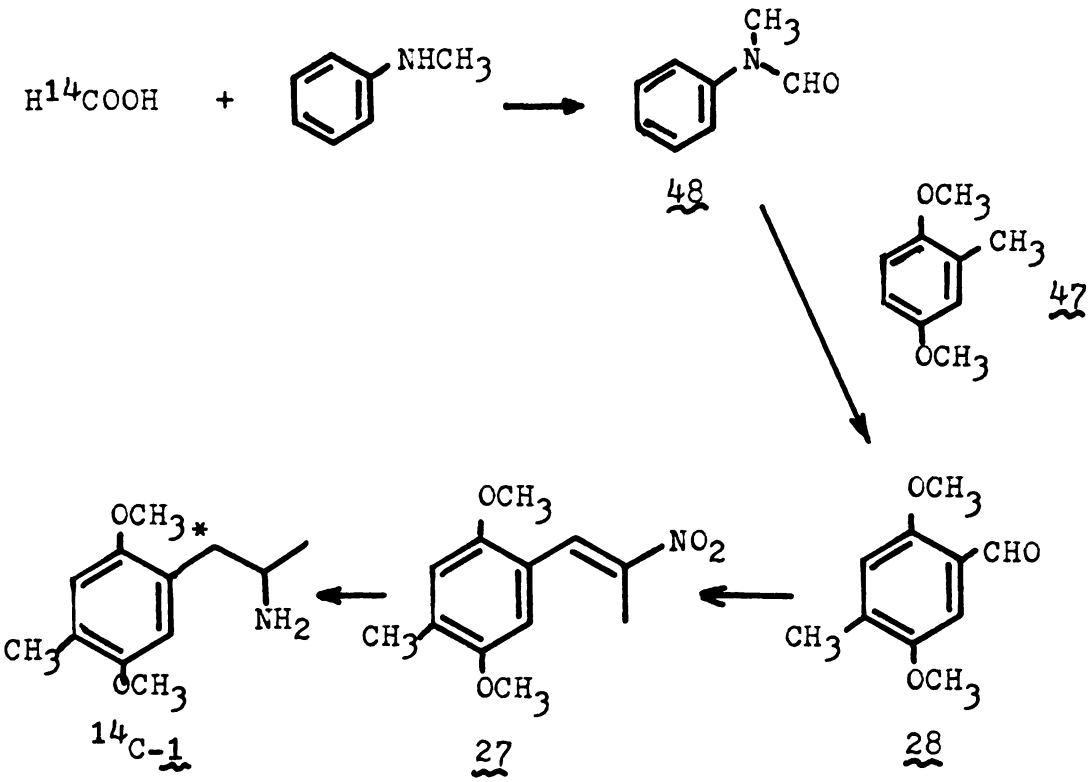
To more fully describe the metabolic fate of 1, ^{14}C-labeled amine was synthesized in order to follow excretion patterns and for quantitative estimation of metabolites by isotope dilution techniques. Deuterium labeled 1 was also prepared for metabolite identification and quantification utilizing chemical ionization mass spectrometry (CIMS). Also described in this chapter are the syntheses of several metabolites and suspected metabolites of amine 1.

A. Synthesis of ^{14}C-1

In order to follow the biotransformations of 1 and its metabolites, the synthesis of ^{14}C-labeled 1 was undertaken. The C-1 benzylic carbon atom of the side chain was considered both a convenient and strategic site for labeling. Amphetamine (2) shares the same side chain as 1 and serves as a model for metabolism studies. For amphetamine, metabolic formation of benzoic acid represents the most extensive reported enzymatic side chain cleavage.[54] A C(1) ^{14}C-label in amine 1 would still be present in a major fragment of the molecule if 1 were to undergo analogous side chain cleavage.

The well established phenylnitropropene route[80] for the synthesis of amines related to 1 from their corresponding benzaldehydes proved to be a convenient method for the production of ^{14}C-1. Of the numerous methods for the preparation of ^{14}C-benzaldehydes[77-79], the method chosen was by direct formylation of 2,5-dimethoxytoluene (47) under Vilsmeier-Haack conditions.[20] The synthetic pathway leading to ^{14}C-1 is shown in Scheme 2 . N-Methylformanilide-^{14}C=O,to be used for the formylation of 47 to give benzaldehyde 28, was prepared by the reaction of H^{14}COOH with N-methylaniline. The unique location of the newly introduced formyl group was established by glpc which showed only one product and by nmr analysis which displayed two aromatic singlets at 6.75 and 7.17 ppm consistent with their assigned p-orientation. Any other location of the formyl group would be

Scheme 2



expected to show o or m-coupling of the aromatic signals.

In Table 17 are summarized the data concerning our efforts to maximize the yield of N-methylformanilide (48). A molar ratio of N-methylaniline to formic acid of 2:1 gave optimal yields when the mixture was allowed to stand in the dark at room temperature for 3 days. The Vilsmeier-Haack formylation of 47 produced an oil which was purified as its bisulfite addition product in consistent yields of 75-80%. The conversion of aldehyde 28 to ^{14}C-labeled 1 followed the phenylnitropropene route except that AlH_3 instead of $LiAlH_4$ was employed to reduce the phenylnitropropene 27. By employing a 4:1 molar ratio of AlH_3 to 27, pure 1 was obtained in 80% yield.

Table 17

Formation of N-Methylformanilide from Formic Acid and N-Methylaniline

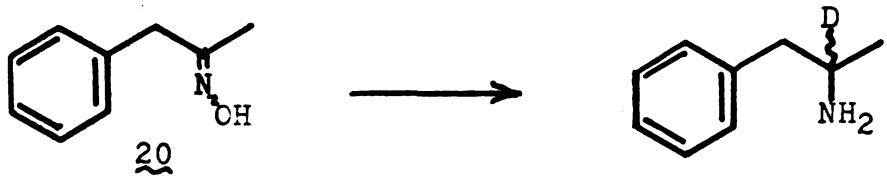
Runs	Time (days at room temp)	Molar ratio $PhNHCH_3$	Molar ratio HCOOH	Average % yield
2	0	1	1	51.5
1	3	1	1	67.0
1	12	1	1	50.0
8	3	2	1	67.8

Reaction details are described in experimental section

The overall yield of ^{14}C-1 in terms of $H^{14}COOH$ was 10.7% (550 mg) with 102% incorporation of label. The specific activity was determined by comparison to a ^{14}C-toluene standard and found to be 0.096 mCi/mmole (8.77×10^5 dpm/mg).

B. Synthesis of Deuterium Labeled 1

The application of stable isotopes to drug metabolism studies has already been discussed in detail in Part II of this thesis. Of the stable isotopes ^{2}H, ^{13}C, ^{15}N, and ^{18}O commercially available, deuterium was chosen to label amine 1. Deuterium precursors offer a combination of high isotopic purity with relatively low cost. Amphetamine (2) has been labeled by Foreman et al.[29] with deuterium at the C-2 carbon atom by the reduction of phenyl-2-propanone oxime (20) with $LiAl^{2}H_4$. Mescaline (3)[32] and amphetamine[16] labeled with deuterium in the C-1 and C-2 positions have been prepared by the reduction of the nitrostyrenes 49 and 50 with $LiAl^{2}H_4$.



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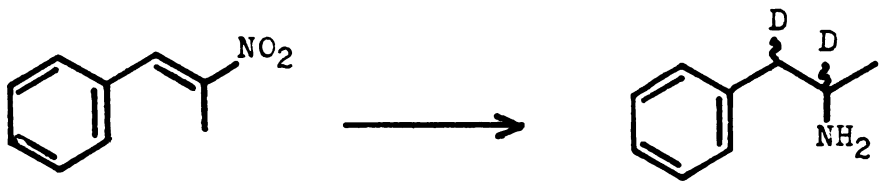

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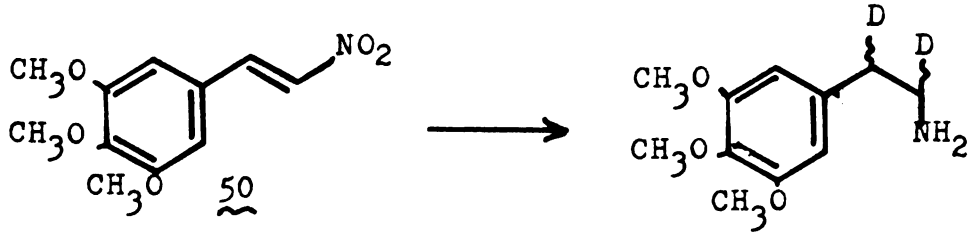

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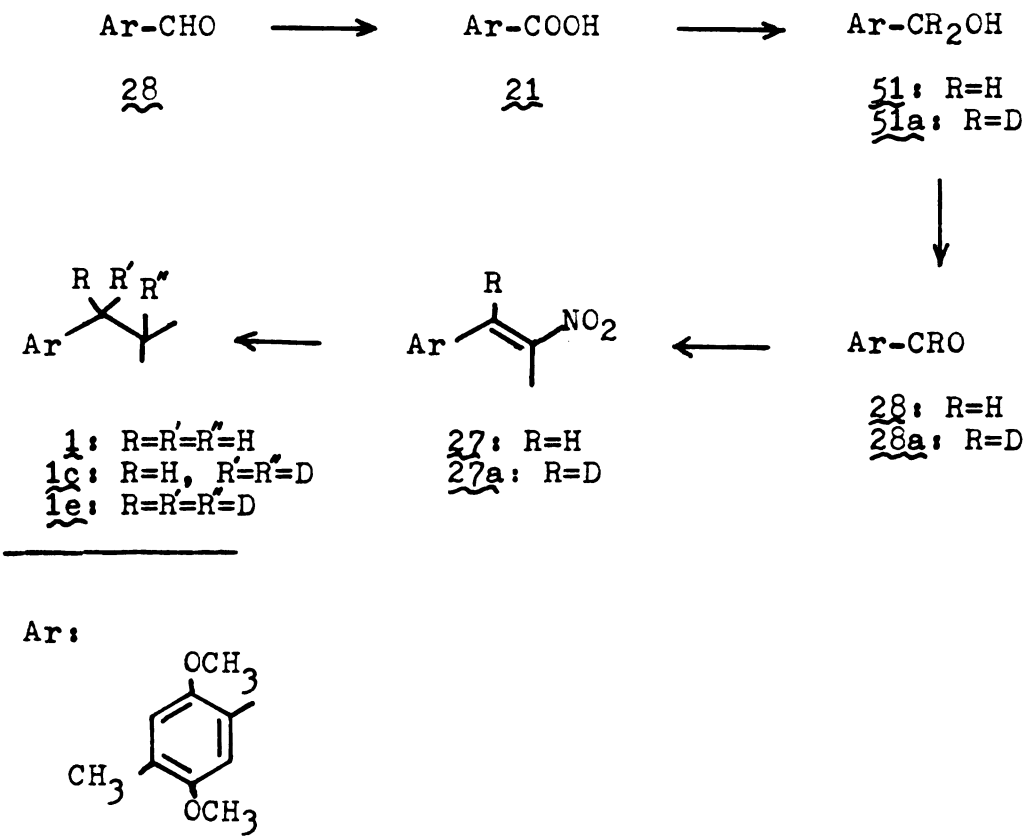
Several steps in the synthetic scheme for amine 1 described in detail for the production of ^{14}C-1 offer an opportunity for the replacement of a proton with deuterium. The most convenient method would be to use lithium aluminum deuteride in place of $LiAlH_4$ in the reduction of the phenylnitropropene 27. The resulting dideuterio product 1c, however, now has a second asymmetric center and the potential for existing as a diastereomeric mixture. The presence of these diastereomers might alter the metabolic processes sensitive to the configuration of both C-2 and the new asymmetric center, C-1. Enzymatic oxidation of the benzylic carbon of ethyl benzene, for example, has been shown to be sensitive to the configuration of C-1 deuterated ethyl benzene.[167] The possibility of replacing the remaining benzylic proton of 1c with deuterium to produce a deuterated compound without the creation of a new asymmetric center can be realized if aldehyde 28 can be deuterated on the aldehydic carbon to form 28a. The derivative of amine 1 which contains a deuterium label and no new asymmetric center is the trideuterio compound 1e. Scheme 3 shows the sequence for the production of 1e. All of the reactions are high yield. The critical step is in the formation of the deuterated aldehyde 28a.

There are numerous approaches to the synthesis of deuterated benzaldehydes including direct formylation of the aromatic ring,[20] Rosenmund reduction of benzoyl halides,[21] oxidation of benzyl alcohols[22] or the reduction of benzonitriles.[23] A newer and unique procedure for the production

Scheme 3



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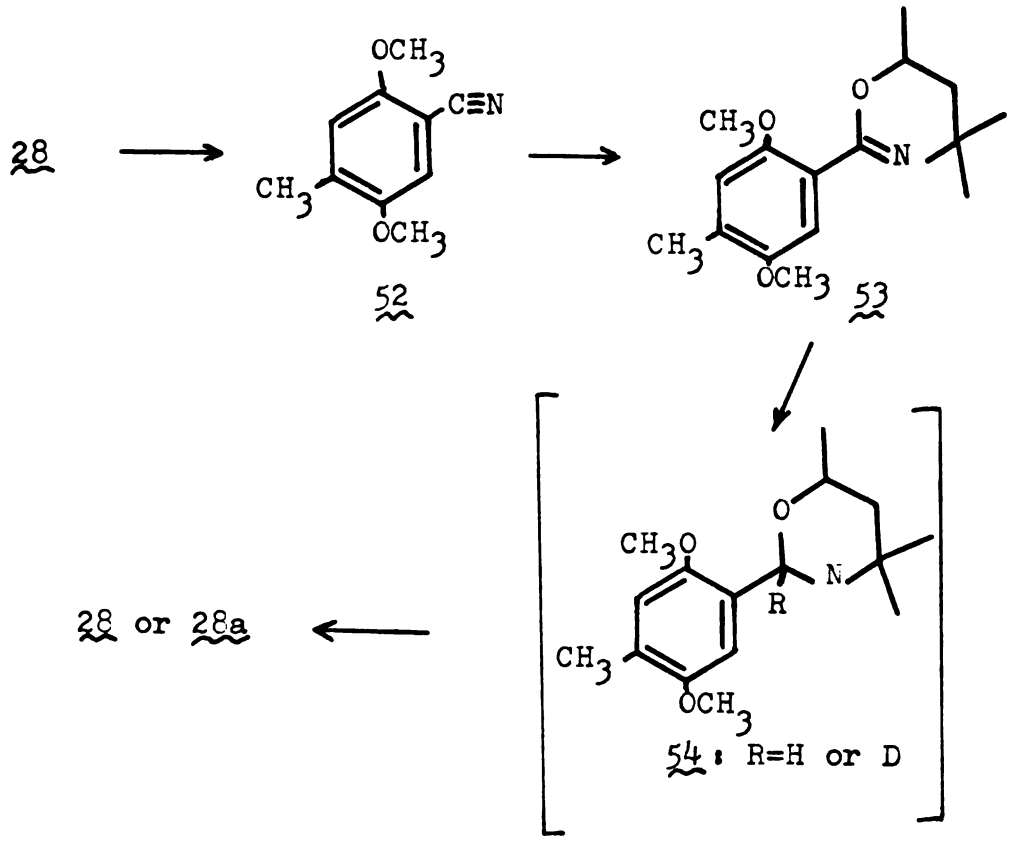
of C-1 deuterated aldehydes has been reported by Meyers and co-workers.[24] They reported high yield reductions with sodium borodeuteride of 5,6-dihydro-4H-1,3-oxazines to tetrahydro-1,3-oxazines which upon hydrolysis give C-1 deuterated aldehydes. Most of the compounds prepared by this method were aliphatic aldehydes. The only aromatic aldehyde reported was benzaldehyde which was obtained in 66% overall yield from the dihydro-1,3-oxazine.[25] The synthesis of the deuterated aldehyde 28a by this method was attempted. The nitrile 52 was prepared[17] in 62% yield from aldehyde 28. It is a well behaved colorless solid with a characteristic ir absorption at 2230 cm^{-1}. The cyclization of this nitrile

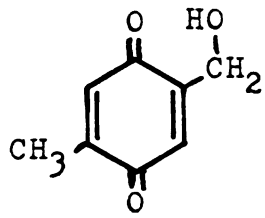


to the dihydrooxazine 53 was accomplished in no better than 26% yield. The reported yield for benzaldehyde dihydrooxazine is 45%.[25] The severity of the reaction conditions (concd H_2SO_4 is a reactant) probably accounts for the low yield. One attempt was made to reduce 53 with sodium borohydride and to hydrolyze the reduction product 54 to the aldehyde 28. However, no aldehyde proton could be detected in the nmr spectrum of the reaction mixture. During the reaction, the temperature was not held at -10° as the literature recommends[24] but was allowed to warm to +10°. Meyers reports[24] that at temperatures higher than -10° a significant amount undergoes ring opening rather than reduction to the aldehyde oxidation state. This process was abandoned

in favor of a higher overall yield sequence.

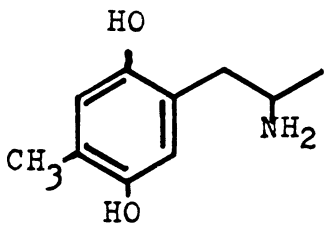
A second route to 28a involved as the critical step the oxidation of 2,5-dimethoxy-4-methylbenzyl alcohol (51) to the corresponding benzaldehyde (see Scheme 3). The benzoic acid 21 was prepared from the aldehyde 28 by oxidation with neutral permanganate in 50% yield.[27] The actual yield is greater since some starting material was recovered. Reduction of acid 21 with $LiAlH_4$ produced the alcohol 51 in 77% yield. The mp agreed with the literature value.[26] Of the various possible oxidizing agents[22] for the formation of aldehyde 28 from alcohol 51, ceric ammonium nitrate was tried first. Trahanovsky et al.[22] reported the oxidation of benzyl alcohol and related alcohols by ceric ammonium nitrate in water or 50% aqueous acetonitrile. Of the compounds studied, p-methoxybenzyl alcohol, 2,4,6-trimethylbenzyl alcohol and m-methoxybenzyl alcohol are most closely related to 51. They produced the corresponding aldehydes in 94, 89 and 25% yield respectively. Oxidation of the alcohol 51 was carried out with 0.5M ceric ammonium nitrate in 50% aqueous acetonitrile by the method described by Trahanovsky.[22] The nmr spectrum of an ether extract of the reaction mixture showed no starting material, no signals in either the methoxy or the aldehyde region. Crystallization of this material gave an orange solid the nmr spectrum of which was consistent with a 2,5-disubstituted benzoquinone.[168] An ir spectrum showed the presence of a 1,4-benzoquinone carbonyl[169] at 1655 cm^{-1}. From this evidence and a correct elemental analysis, the structure of the product was assigned that of the O-demethyl-

ated product, 2-hydroxymethyl-5-methyl-1,4-benzoquinone (55).



55

The relative ease with which the p-dimethoxy groups were cleaved under oxidative conditions may be related to the possible metabolic formation of quinone metabolites of 1. It has been suggested by Shulgin[4] that the enzymatic O-demethylation of the psychotomimetic 1-phenyl-2-aminopropanes along with quinone formation may lead to cyclization to form indoles similar in structure to known indole hallucinogens. The structural similarity between the O-demethylated product of 1, 56, and 6-hydroxydopamine (57), an agent which causes chemical sympathectomy, is also worth noting.



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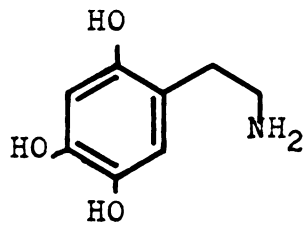

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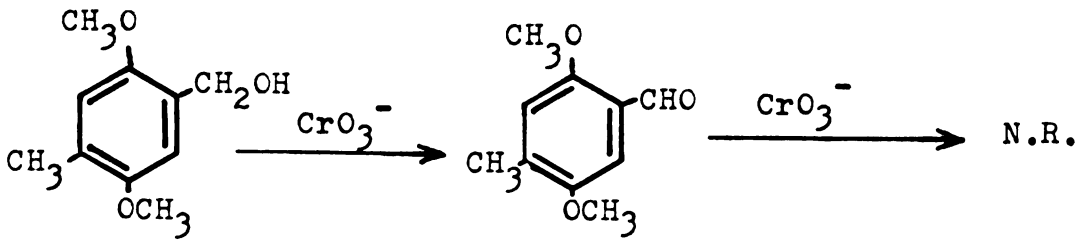
Attempts to oxidize aldehyde 28 with CrO_3^- (Jones reagent) gave only recovery of unreacted starting material. This somewhat unexpected resistance to oxidation suggested that the alcohol 51 might be selectively converted to aldehyde 28 with this reagent. This was found to be the case and provided a method for the synthesis of deuterated aldehyde 28a and the trideuterio compound 1e (see Scheme 3).



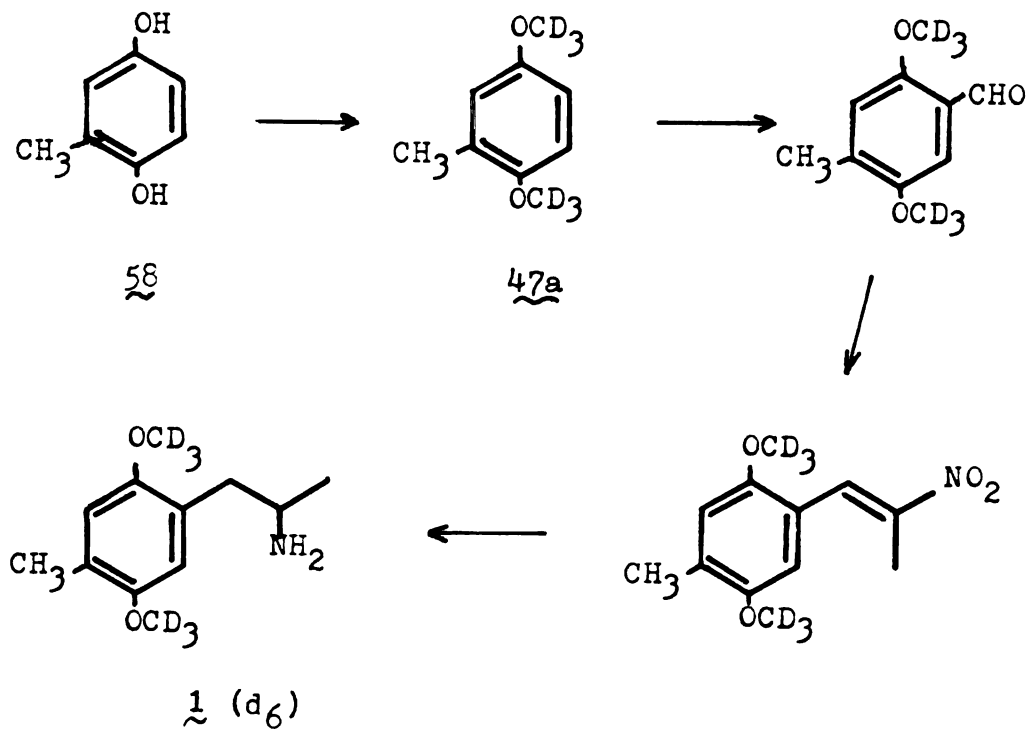
51 28

Although the scheme for the production of 1e was established, the actual synthesis was not carried out in favor of the production of more useful labeled compounds. The main uses of 1e in metabolism studies were considered to be as a substrate for the production of labeled metabolites and as an internal standard for the quantitative estimation of amine 1.

Metabolism studies with trideuterio compound 1e as substrate for the production of deuterated metabolites might be complicated by possible isotope effects that would arise from metabolism of the C-1 and C-2 deuterium atoms. Possible stereochemically sensitive biotransformations of the C-2

asymmetric center might be altered by a C-2 deuterium. In order to avoid these possible isotope effects, an alternate position for the label was chosen.

A position anticipated to have little effect on either C-4 methyl oxidation or side chain alterations is the methoxy groups. Hexadeuterio-1 (d_6) labeled in the methoxy groups was prepared starting with the methylation of methylhydroquinone (58) with methyl iodide-d_3 to give the deuterated toluene 47a. The nmr of 47a showed the absence of OCH_3 signals. Compound 47a was then converted to the aminopropane 1 (d_6) following the same aldehyde, phenylnitropropene route previously described for the synthesis of ^{14}C-1 (see Scheme 2). The overall yield in terms of methyliodide-d_3 was 23.8%. By CIMS analysis, the isotopic purity of 1(d_6) was found to be greater than 99%.



58 47a 1 (d_6)

As an internal standard for mass spectrometric quantitative estimation of 1 (d_0) or 1 (d_6), both dideuterio derivative 1c and trideuterio derivative 1e are equally useful, however, 1c is more readily synthesized. Dideuterio compound 1c was prepared by reduction of phenylnitropropene 27 with $LiAl^2H_4$ following the procedure of Lindeke and Cho.[16] The nmr spectrum was consistent with the incorporation of deuterium atoms at C-1 and C-2. The side chain CH_3 proton signal was observed as a singlet at 1.1 ppm. The C-1 proton signal appeared as two broad bands of equal areas at 2.5 and 2.7 ppm corresponding to the erythro and threo forms (see Figure 11).

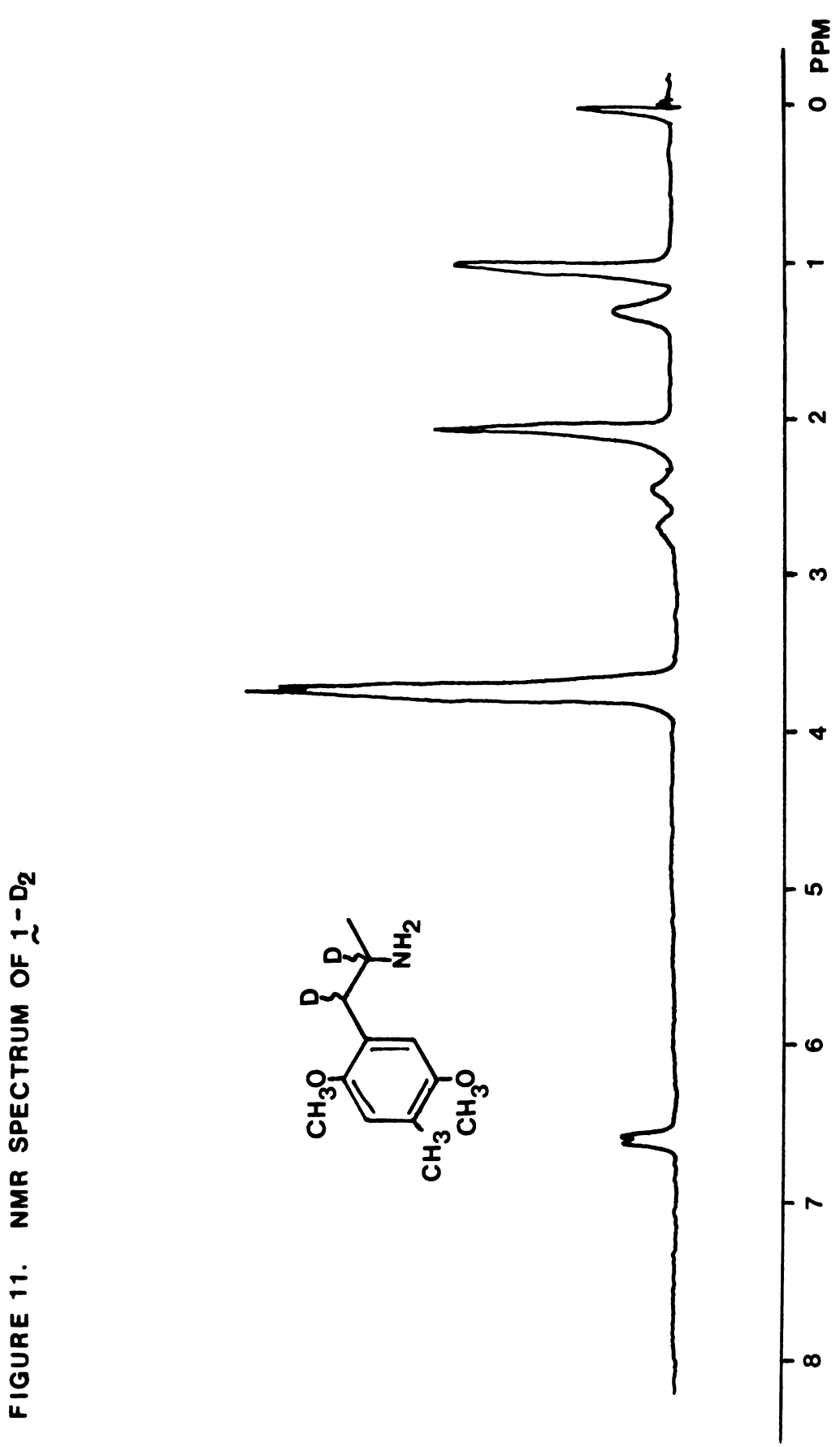
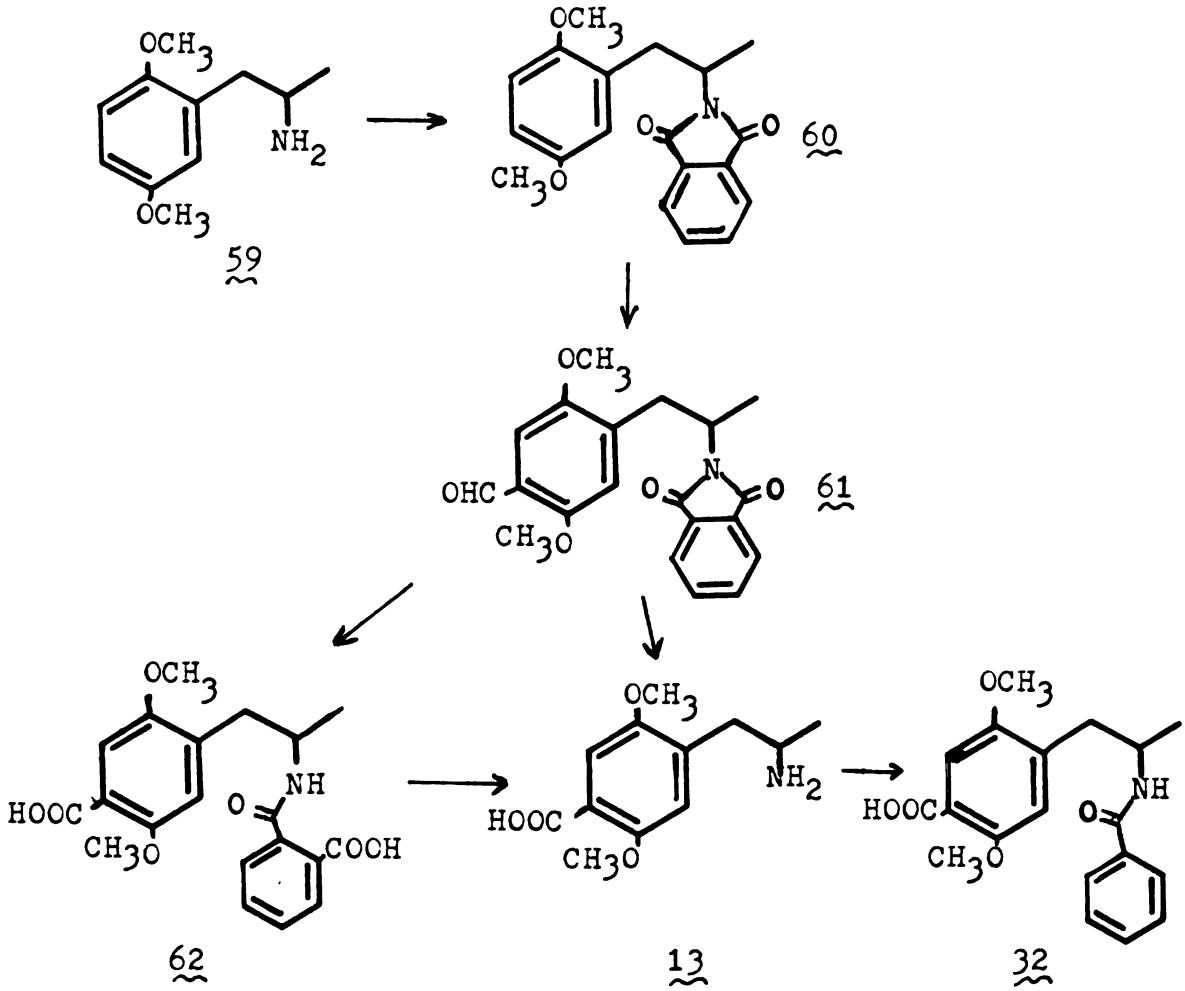


FIGURE 11. NMR SPECTRUM OF $\underset{\sim}{1}$-D_2

C. Synthesis of Potential Metabolites of 1

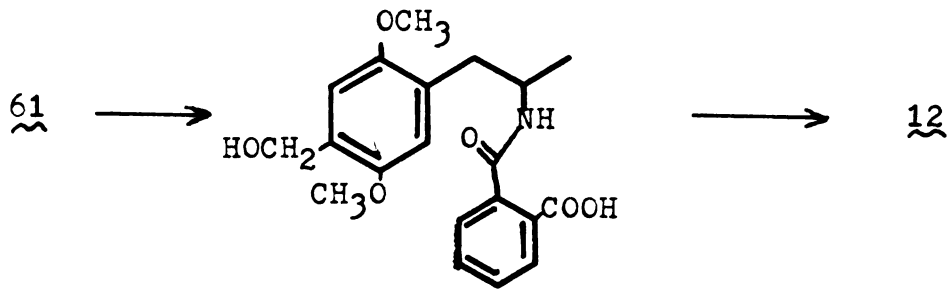
Ho and co-workers[14] reported in their work on the metabolic fate of 1 in rats the synthesis of the 4-hydroxymethyl metabolite 12 and the 4-carboxy metabolite 13. Protection of the amino group of 1-(2,5-dimethoxyphenyl)-2-aminopropane (59) with an N-phthaloyl group to give 60 followed by Vilsmeier-Haack formylation of the aromatic ring produced the phthalimidoaldehyde 61. Oxidation of 61 with Ag_2O under basic conditions simultaneously oxidized the aldehyde and hydrolyzed the phthaloyl to give a low yield of crude amino acid 13 which was characterized as its benzamide 32.[14] In our hands the oxidation of 61 did not form the amino acid 13 but produced instead a product which after purification by ion exchange chromatography proved to be an acid insoluble compound. Analysis by uv showed λ_{max} 280 nm which shifted to 317 nm with a drop of base indicating the presence of an aromatic carboxylic acid.[170] Integration of the aromatic region of the nmr spectrum indicated the presence of four phthaloyl protons. Titration showed the compound to contain a diacidic function. On the basis of the above evidence and microanalysis, the structure of the product was assigned that of the ring opened phthaloylbenzoic acid 62.

It has been observed that in the hydrolysis of the phthaloyl moiety the first amide linkage is broken more easily with base than acid, while the second amide linkage is cleaved more easily with acid than base.[145] This is



reasonable since the hydrolysis under basic conditions must lead to the charged carboxylate which would hinder attack by hydroxide on the remaining amide. Treatment of 62 with 10% HCl cleaved the phthaloyl group to give the desired amino acid 13 as its hydrochloride salt. The nmr spectrum of 13 in D_2O showed two singlets (7.10 and 7.28 ppm) consistent with p-orientation of two aromatic protons. Recrystallization from acetonitrile provided pure 13·HCl which was characterized by microanalysis.

The hydroxymethyl metabolite 12 was synthesized by the sodium borohydride reduction[146] of the phthalimidoaldehyde 61. Under aqueous basic conditions,[147] $NaBH_4$ reduction of 61 formed the hydroxymethylphthalamic acid 63. The nmr spectrum of the product collected upon acidification showed the disappearance of the aldehyde proton signal and the appearance of a methylene proton signal at 4.52 ppm.

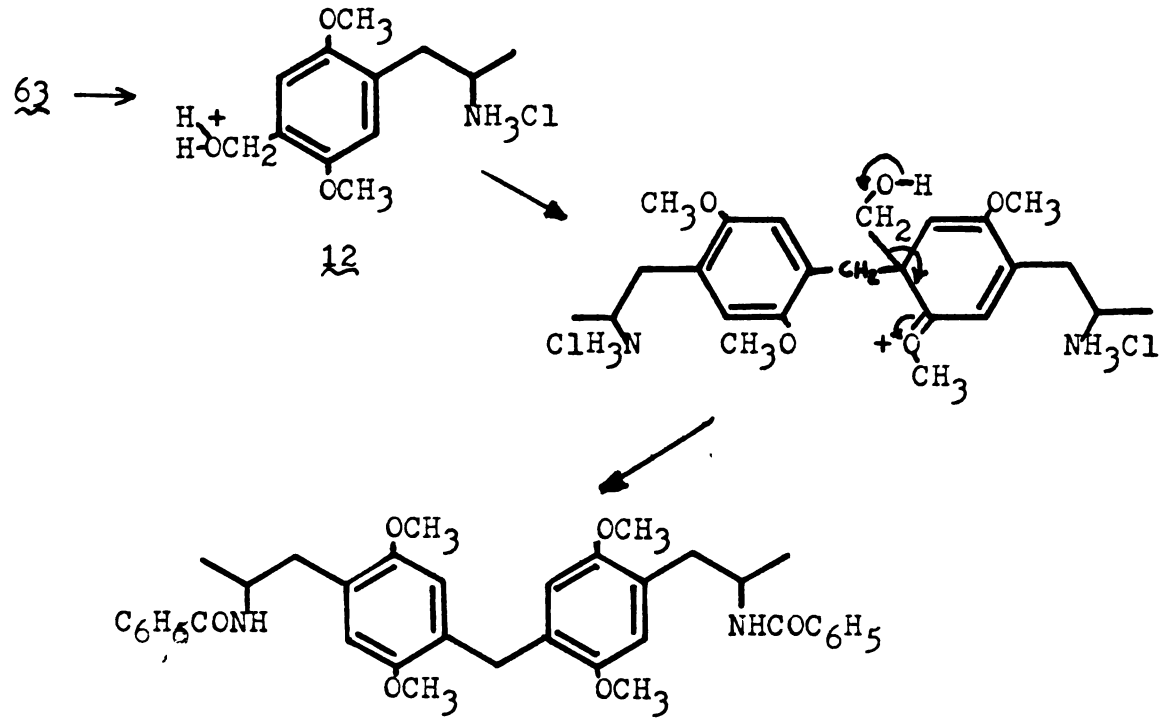


63

Several unsuccessful attempts to hydrolyze 63 with acid were made. Even the relatively mild conditions of 10% HCl at 60° destroyed the compound. The nmr spectrum showed the appearance of an NH_2 signal but the disappearance of the benzylic protons of the hydroxymethyl group. The benzyl alcohol portion of the molecule was apparently sensitive to acid. In order to isolate and characterize the major product from the reaction, a larger quantity of phthaloyl compound 63 was held at reflux in 10% HCl for two hours. The mixture was adjusted to pH 12 with 10% NaOH and extracted with ether followed by ethyl acetate. The organic layers were dried and evaporated to give an oil. Tlc (n-butanol:acetic acid: water, 4:1:1) revealed the presence of numerous compounds

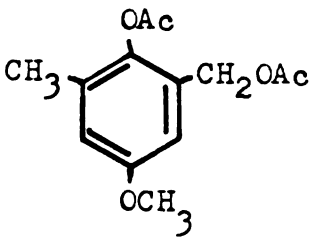
with the major spot at the origin. Treatment of an ether solution of the oil with HCl gas did not yield a solid. Purification of the major product was achieved by preparation of the benzoyl derivative. CIMS analysis of the recrystallized benzoyl derivative gave an MH^+ ion of 611 consistent with the bis-benzoyl derivative of the condensation product of two molecules of 12 with the loss of the elements of formaldehyde and water. The nmr spectrum supported the possibility of a self-condensation reaction by the presence of a singlet at 3.91 ppm suggesting a diphenylmethane structure. This evidence along with microanalysis led to the structure assignment as that of the diphenylmethane 64. A possible mechanism for the acid catalyzed self-condensation is suggested in Scheme 4.

Scheme 4

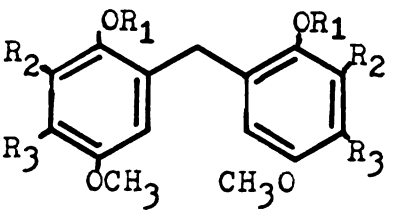


64

There are other examples of self-condensation reactions of this type. A side product from the base hydrolysis of 2-acetoxy-5-methoxy-3-methylbenzyl acetate (65) is bis-(2-hydroxy-5-methoxy-3-methylphenyl)methane (66).[12] Another example is in the chloromethylation of 2,5-dimethoxytoluene with formaldehyde and concentrated HCl.[28] A major product is the self-condensation product 67.



65



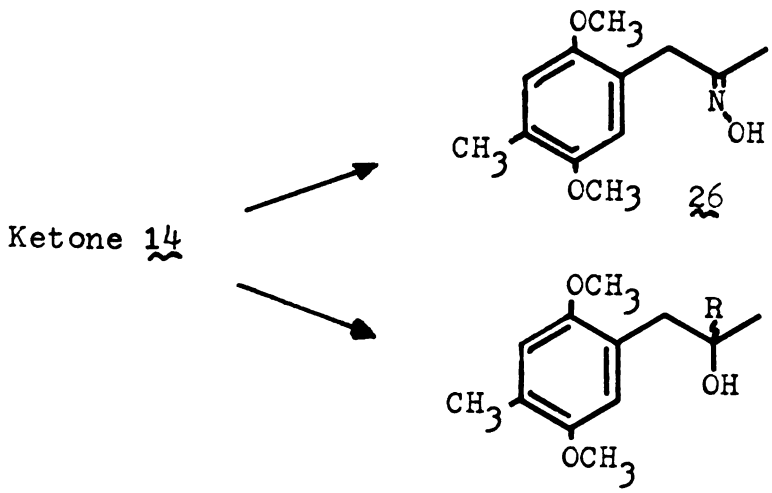
66: R_1=H, R_2=CH_3, R_3=H

67: R_1=CH_3, R_2=H, R_3=CH_3

Hydroxymethyl compound 12 was successfully prepared from phthalamic alcohol 63 by avoiding acidic conditions and hydrolyzing with 20% NaOH. The product had the same mp and nmr characteristics as the literature values.[14]

Synthesis of the ketoxime 26 from the ketone 14 and hydroxylamine[81,83] produced an oil which was crystallized from hexane. Nmr analysis of the oil showed two singlets for the side chain CH_3 at 1.78 and 1.85 ppm (1:2.5 ratio) consistent with the <u>syn</u> and <u>anti</u> forms[82] of 26. The nmr spectrum of the recrystallized product showed the presence of only one CH_3 singlet at 1.85 ppm indicating crystallization

of one isomer. This downfield singlet corresponds to the isomer with the CH_3 <u>syn</u> to the OH of the oxime.[171] Thus, the <u>anti</u> form of 26 is the isomer which crystallized.

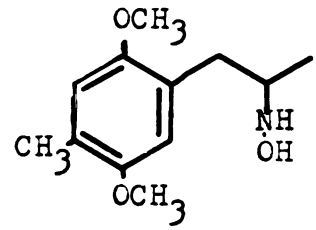


45: R=H

45a: R=D

The alcohol 45 was produced from the reduction of ketone 14 with sodium borohydride in ethanol. Analysis of the crude product by nmr showed the reaction was essentially quantitative. Recrystallization yielded an analytical sample. Reduction of 14 with lithium aluminum deuteride produced the monodeuterio alcohol 45a in high yield and high isotopic purity. Analysis of 45a by CIMS showed an MH^+ and an M^+ of about equal intensity even at low probe temperatures (150-180°). Examination of the MH^+-H_2O signal at m/e 194 indicated that the deuterium label in this fragment is stable. This deuterated material may be useful as a standard in the quantitative estimation of 45.

One attempt was made to synthesize the N-hydroxy compound 43. Feuer et al.[148] reported that ketoximes can be reduced with diborane to hydroxylamines. The reduction of ketoxime 26 with diborane in THF produced a viscous oil. Analysis by glpc on an OV-17 column showed three major peaks. Peak enhancement studies with authentic amine 1, ketone 14, and ketoxime 26 indicated the peaks in the mixture were 1, 14, and 26. The possibility that the hydroxylamine 43 was decomposing on the glpc column was discarded when a direct probe CIMS analysis at low probe temperature exhibited no m/e 226 corresponding to the MH^+ expected for 43. The nominal masses 224,210 and 209 were major lines in the mass spectrum and correspond to the MH^+ ions for 26, 1 and 14 respectively.



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D. Experimental Section

Melting points were determined with a Thomas-Hoover Uni-Melt stirring oil capillary tube melting point apparatus and are uncorrected. Gas-liquid partition chromatography was performed on a Varian Aerograph Model 2100-00 Life Sciences Gas Chromatograph. A U-shaped 2 m x 2 mm id pyrex column packed with 3% SE-30 or 3% OV-17 on acid washed, DMCS-treated chromosorb W was used at column temperature 185° for SE-30 and 170 –255° for OV-17. Purity of radioactive compounds was verified by tlc on alumina (Eastman Chromatogram 6063) with benzene or benzene-acetic acid (5:1) and by scanning the chromatogram with a Varian-Berthold Model LB2722-10 radio-scanner. Liquid scintillation counting of samples in 10 ml Aquasol (New England Nuclear) was performed on a Packard Tri-carb Model 3375. All values were corrected for efficiency by ^{14}C-toluene internal standard (New England Nuclear) or by automatic external standard. The infrared spectra were measured with a Perkin-Elmer Model 337 infrared spectrophotometer. Proton magnetic resonance spectra were determined at 60 MHz with a Varian Model A-60-A pmr spectrometer. The chemical shifts are expressed in values (ppm) relative to either TMS or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard. In the presentation of the pmr spectra, the following notations are used: s = singlet, d = doublet and comp m = complex multiplet. The chemical ionization mass spectra were obtained with a modified AEI Model MS902 double focus mass spectrometer equipped with a direct inlet system by Dr. R. Weinkam. Isobutane was used as the reactant gas

at 0.4-0.7 torr with a source temp of 140—240°. Microanalyses were performed by the Microanalytical Department, Berkeley.

N-Methylformanilide-^{14}C=O (48).-A mixture of formic acid-^{14}C (0.95 g, 19.9 mmole, 2 mCi) and N-methylaniline (4.3 g, 40.2 mmole) was allowed to react at room temp in the dark for 84 hr. The reaction mixture was transferred to a short path still and water and unreacted formic acid were removed at 100-110°/760 mm. The product distilled at 110-120°/5 mm. Further purification was achieved by partitioning the distillate between Et_2O (50 ml) and 10% HCl (10 ml). After extracting the aq layer a second time with Et_2O, the combined extracts were dried (Na_2SO_4) and removed under vacuum. Short path distillation provided pure 48 (1.39 g, 11.5 mmole, 52%): bp 110-120°/5 mm (lit.[175] bp 131°/22 mm.

2,5-Dimethoxy-4-methylbenzaldehyde-7-^{14}C (28).-To a stirred solution of 48 (1.39 g, 11.5 mmole) was added dropwise freshly distilled $POCl_3$ (1.5 g, 9.8 mmole) followed one hour later by 2,5-dimethoxytoluene (47, 3.0 g, 19.7 mmole). The stirred reaction mixture was maintained at 70° for 4 hours, cooled,then 10% NaOAc (15 ml) was added and the mixture was stirred at room temp overnight. The reddish-brown precipitate which separated was extracted into Et_2O (2 x 50 ml). The combined Et_2O layers were evaporated and to the residue was added $NaHSO_3$ reagent (32% $NaHSO_3$ in 20% aq EtOH). After 5 min of vigorous shaking, 250 ml H_2O were added and the resulting soln was washed with Et_2O (200 ml). The pH of the aq layer was adjusted to 12 with 10% NaOH and the soln was extracted with Et_2O (2 x 125 ml). The combined Et_2O

layers were dried (Na_2SO_4) and evaporated to yield 1.17 g of pure aldehyde 28 (6.5 mmole, 56.5%): mp 82-83° (lit.[12] mp 85-87°); pmr ($CDCl_3$) δ 2.26 ppm (s, 3H, $ArCH_3$), 3.80 and 3.87 (s, OCH_3), 6.80 and 7.23 (s, C_6H_2), 10.73 (s, CHO).

1-(2,5-Dimethoxy-4-methylphenyl)-2-nitropropene-1-^{14}C (27).-A soln of 28 (1.17 g, 6.5 mmole), NH_4OAc (0.40 g, 5.2 mmole), and nitroethane (0.7 ml, 9.9 mmole) in 10 ml glacial HOAc was held at reflux for 1.5 hr. After cooling, water (20 ml) was added to yield 1.15 g (4.9 mmole, 75%) crude product, mp 79-82°. Recrystallization from MeOH gave pure 27 (0.82 g, 3.5 mmole, 53.5%): mp 85-87° (lit.[174] 85.5-87.5°); pmr ($CDCl_3$) δ 2.29 ppm (s, $ArCH_3$), 2.43 (s, $NCCH_3$), 3.86 (s, 6H, OCH_3), 6.82 (s, C_6H_2), 8.30 (s, olefinic H).

Anal. Calcd for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.91; H, 6.26; N, 5.89.

(R:S)-1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane-1-^{14}C Hydrochloride (1·HCl).-Into a 250 ml 3-neck flask equipped with a magnetic stirrer, pressure equalizing dropping funnel, and dry N_2 system were placed $LiAlH_4$ (0.68 g, 18 mmole) and freshly distilled anhydrous THF (50 ml). The system was purged with dry N_2 and cooled to 3°. To this cooled, stirred suspension was slowly added 100% H_2SO_4 (0.93 g, 9 mmole). The resulting mixt was stirred for 30 min at 3° to complete reagent formation following which a 100 ml dry THF soln of nitropropene 27 (0.82 g, 4.5 mmole) was added over a 30 min period. After 2 additional hr at 3° followed by 4 hr at room temp, 1.6 ml of water was added carefully to destroy excess reagent followed by 1.6 ml of 15% NaOH and

4.8 ml water. To complete salt formation 9.0 ml of 15% NaOH was added and the mixture was stirred for 15 min and then filtered. The filter cake was digested 30 min with Et_2O and the organic solvents were combined and dried (K_2CO_3) and then concd to give the free amine. The crude product in 100 ml of anhyd Et_2O was saturated with dry HCl and after cooling, the Et_2O was decanted and the solid hydrochloride was crystallized from isopropanol to yield pure amine hydrochloride (0.55 g, 2.25 mmole, 64.3%): mp 185-188° (lit.[43] mp 184-185°); 0.096 mCi/mmole.

The benzamide 25 used in the isotope dilution analyses was obtained by stirring a mixture of 1 (unlabeled, 0.5 g, 2.04 mmole), benzoyl chloride (0.5 ml, 4.0 mmole) in $CHCl_3$ (5 ml) and 5% NaOH (20 ml) for 12 hr. The $CHCl_3$ was separated and the aq layer was extracted with 10 ml $CHCl_3$. The combined dried ($MgSO_4$) organic solvents were removed to give crude 25 (0.60 g, 1.9 mmole, 94%, mp 152-160°). Pure (R:S)-1-(2,5-dimethoxy-4-methylphenyl)-2-benzamidopropane (25), 0.38 g, 1.2 mmole, 61%) was obtained by crystallization from isopropanol: mp 169-170°; pmr ($CDCl_3$) δ 1.30 ppm (d, J = 6.5 Hz, CCH_3), 2.20 (s, $ArCH_3$), 2.77 - 3.05 (comp m, CH_2), 3.77 (s, OCH_3), 3.82 (s, OCH_3), 3.97 - 4.65 (comp m, NCH), and 6.50 - 7.90 (comp m, 7 aromatic protons); ir(KBr) ν_{max} 1645 cm^{-1} (C=O).

Anal. Calcd for $C_{19}H_{23}NO_3$: C, 72.82; H, 7.40; N, 4.47. Found: C, 72.66; H, 7.54; N, 4.45.

Resolution of (R:S)-1.-Following the method of Matin and co-workers,[6] free base 1 [(4.1 g, 20.0 mmole) obtained by extracting (3 x 50 ml Et_2O) a solution of 1·HCl (5.0 g, 20.4 mmole) in aq 10% NaOH (25 ml)] in ab EtOH (50 ml) was treated with a solution of (+)-o-nitrotartranilic acid (2.70 g, 10.0 mmole) in ab EtOH (75 ml) and the resulting mixture was allowed to stand overnight at room temp. The salt which formed (4.7 g) was shown to be 70% S (see under Experimental Section Part I for procedure to determine enantiomeric composition). After four recrystallizations from ab EtOH, the enantiomerically pure salt was obtained (1.4 g, 3.10 mmole, 31%). The free base was liberated by extraction of a pH 10 solution of the tartranilate salt with Et_2O (3 x 50 ml). After drying over K_2CO_3, dry HCl gas was passed through the Et_2O to ppt the HCl salt of 1b (0.56 g, 2.3 mmole, 11.5%): mp 198-200° (lit.[166] mp 204-205°). The combined mother liquors containing amine 1 enriched with 1a were worked-up to yield the pure base which was treated with (-)-o-nitrotartranilic acid and the resulting salt was recrystallized four times to yield the enantiomerically pure product in 30% yield. The hydrochloride salt of 1a (obtained in 14% yield) had a mp 200-202° (lit.[166] mp 204-205°). The enantiomerically pure hydrochloride salts of R- and S-1 (d_6) were also prepared following this procedure.

2,5-Dimethoxy-4-methylbenzonitrile (52).-A mixture of 2,5-dimethoxy-4-methylbenzaldehyde (28, 1.31 g, 7.3 mmoles), ammonium dibasic phosphate (7.0 g, 53.0 mmoles), 30 ml of nitroethane and 10 ml of glacial acetic acid was held at reflux for 20 hours. After removal of the volatile

components under reduced pressure, the residual oil was added to 100 ml of water with stirring and was then allowed to stand at room temp for 1 hr. The solid which formed was filtered (0.8 g, 62%). An analytical sample was obtained by recrystallization from EtOH: mp 127-129° (lit.[174] mp 127-129°); ir ($CHCl_3$) 2230 cm^{-1} (C≡N); pmr ($CDCl_3$) δ 2.25 ppm (s, CH_3), 3.79 (s, OCH_3), 3.85 (s, OCH_3), 6.80 and 6.91 (s, C_6H_2).

Anal. Calcd for $C_{10}H_{11}NO_2$: C, 67.78; H, 6.26; N, 7.90. Found: C, 67.91; H, 6.10; N, 8.02.

4,4,6-Trimethyl-2-(2,5-dimethoxy-4-methylphenyl)-5,6-dihydro-1,3-oxazine (53).-A solution of nitrile 52 (1.59 g, 9.0 mmole) in CH_2Cl_2 (15 ml) was added dropwise with stirring to 4.5 g of concd H_2SO_4 in an ice bath over a 20 min period followed by the dropwise addition of 2-methyl-2,4-pentanediol (1.06 g, 9.0 mmole). After an additional 2 hr of stirring in the cold, the mixture was poured with stirring on 50 g of cracked ice and was then half neutralized with 40% NaOH (1.1 ml). The CH_2Cl_2 layer was separated and the aq layer washed with 2 x 25 ml portions of chloroform. The aq layer was made alkaline with 40% NaOH and extracted with Et_2O (3 x 25 ml). The combined Et_2O layers were dried ($MgSO_4$) and evaporated to give 0.8 g (26%) of crude product. An analytical sample was prepared by short path distillation: 120-130°/0.2 mm; pmr ($CDCl_3$) δ 4.73 (s, OCH_3), 4.76 (s, OCH_3), 6.72 and 6.92 (s, C_6H_2).

Anal. Calcd for $C_{16}H_{23}NO_3$: C, 69.29; H, 8.36; N, 5.05. Found: C, 69.12; H, 8.15; N, 5.29.

2,5-Dimethoxy-4-methylbenzoic Acid (21).-To a stirred suspension of 2,5-dimethoxy-4-methylbenzaldehyde (28, 7.2 g, 40 mmole) in 150 ml H_2O maintained at 70-80° was added a soln of $KMnO_4$ (9.0 g, 56 mmole) in 180 ml H_2O over a 45 min period. After a additional hour, the pH was adjusted to 10 with aqueous 10% NaOH and the resulting soln filtered hot. The MnO_2 ppt was washed with 3 x 20 ml hot H_2O. Upon cooling of the combined filtrates, unreacted aldehyde separated and was collected. Acidification of this filtrate with 10% HCl gave the acid 21 (4.0 g, 21 mmole , 51% based on starting aldehyde). Crystallization from water provided the analytical sample: mp 122-124°; pmr ($CDCl_3$) δ 2.28 ppm (s, $ArCH_3$), 3.84 (s, OCH_3), 4.04 (s, OCH_3), 6.92 (s, arom H), 7.55 (s, arom H), 9.67 (s, COOH); ir ($CHCl_3$) ν_{max} 1740 cm^{-1} (C=O).

Anal. Calcd for $C_{10}H_{12}O_4$: C, 61.22; H, 6.16. Found: C, 60.97; H, 6.15.

2,5-Dimethoxy-4-methylbenzyl Alcohol (51).- To a suspension of $LiAlH_4$ (1.18 g, 31 mmole) and 50 ml of dry THF cooled in an ice bath and under N_2 was added dropwise acid 21 (3.2 g, 11.3 mmole) dissolved in dry THF (50 ml). The resulting suspension was stirred at room temp for 4 hr then the reaction was stopped by the careful addition of water (1 ml), followed by 10% NaOH (3 ml) and water (1 ml). The salts were filtered and washed with Et_2O (100 ml). The combined organic filtrates were dried ($MgSO_4$) and evaporated to give a white solid: 2.9 g, 96%, mp 68-78°. Crystallization from EtOH gave 2.4 g, 80%, mp 72-74° (lit.[44] mp 78°).

2-Hydroxymethyl-5-methyl-1,4-benzoquinone (55).-To a solution of alcohol 51 (173 mg, 0.93 mmole) in 3 ml acetonitrile was added with stirring 1M $Ce(NH_4)_2(NO_3)_6$ (2 ml, 2 mmole). The mixture immediately turned dark brown then within a few seconds turned yellow. After 2 additional min of stirring at room temp, the soln was extracted with Et_2O (2 x 40 ml). The combined Et_2O layers were washed with 20 ml sat $NaHCO_3$, dried ($MgSO_4$) and evaporated to give an orange solid (104 mg, 73%, mp 72-74°). An analytical sample was obtained by sublimation: 50°/0.5 mm; mp 90-93°; ir ($CHCl_3$) ν_{max} 1655 cm^{-1} (C=O); pmr ($CDCl_3$) δ 2.05 (d, J = 1.5 Hz, CH_3), 4.55 (bd s, CH_2), 6.60 (q, J = 1.5 Hz, 1H), 6.80 (t, J = 1.5 Hz, 1H).

Anal. Calcd for $C_8H_8O_3$: C,63.15; H, 5.30. Found: C, 62.93; H, 5.32.

1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane-1,2-^{2}H Hydrochloride 1(d_2).-Following the procedure of Lindeke and Cho,[16] a solution of nitropropene 27 (4.74 g, 20 mmole) in dry THF (100 ml) was added dropwise to a stirred suspension of $LiAl^2H_4$ (99%, 2.2 g, 52 mmole) in dry THF (100 ml). The mixture was stirred at room temp overnight then worked up following the procedure for the synthesis of 1. The free amine was distilled: bp 98-120°/0.25 mm, 3.0 g, 71.0%, HCl salt mp 185-188° (lit.[43] protio-1·HCl mp 184-185°); pmr ($CDCl_3$) δ 1.12 (s, $CDCH_3$), 1.35 (bd s, NH_2), 2.23 (s, $ArCH_3$), 2.50 and 2.72 (bd s, ArCHD), 3.80 (s, OCH_3), 6.68 (s, arom H), 6.72 (s, arom H); CIMS (MH^+) 99+% isotope incorporation.

2,5-Dimethoxytoluene-d_6 (47a).-Into a 3-neck 500 ml flask cooled in an ice bath and equipped with a magnetic stirrer, reflux condenser and N_2 system was added 200 ml anhyd MeOH and KOH (19.7 g, 352 mmole). After the system was flushed with N_2 and cooled for 10 min, methylhydroquinone (22.0 g, 176 mmole) dissolved in 120 ml MeOH was added slowly followed by methyl iodide-d_3 (99.5%, 50.0 g, 352 mmole) added dropwise. The mixture was heated to reflux for 24 hr and was then cooled and the salts were filtered and washed with Et_2O (100 ml). The filtrate was evaporated under vacuum and the residue, dissolved in Et_2O (100 ml), was extracted with 5% NaOH (4 x 50 ml). The combined aq layers were washed with 50 ml Et_2O. The Et_2O layers were combined, dried ($MgSO_4$), then evaporated to give 13.7 g (51%) of crude product. Distillation (43-45°/0.25 mm) yielded 12.6 g (47%) pure 47a: pmr ($CDCl_3$) δ 2.23 (s, $ArCH_3$), 6.77 (s, C_6H_3).

2,5-Dimethoxy-4-methylbenzaldehyde-d_6 (28a).-Following the procedure for the preparation of 28, compound 47a (12.5 g, 79.1 mmole) was formylated with N-methylformanilide (11.8 g, 87 mmole) and $POCl_3$ (13.3 g, 87 mmole) in 74.4% yield (10.9 g, mp 82-84°, lit.[12] mp 85-87°): pmr ($CDCl_3$) δ 2.27 (s, $ArCH_3$), 6.83 (s, arom H), 7.27 (s, arom H), 10.43 (s, CHO).

1-(2,5-Dimethoxy-4-methylphenyl)-2-nitropropene-d_6 (27).- Following the procedure for the preparation of 27, the aldehyde 28a (10.9 g, 59 mmole) was condensed with nitroethane to yield the nitropropene 27a (11.5 g, 81.1%, mp 83-90°): pmr ($CDCl_3$) δ 2.30 (s, $ArCH_3$), 2.42 (s, $CNCH_3$), 6.81 (s, arom H), 8.33 (s, ArCHC).

(R:S)-1-(2,5-Dimethoxy-4-methylphenyl)-2-aminopropane-d_6 Hydrochloride 1(d_6).-Following the procedure for the preparation of 1, nitropropene 27a (11.5 g, 47.5 mmole) was reduced with $LiAlH_4$ (7.2 g, 190 mmole) in dry THF to yield crude 1 (d_6)(12.2 g). Distillation (85-105°/0.25 mm) gave 8.1 g (79.5%) of pure amine: 1·HCl pmr (D_2O) δ 1.41 (d, J = 6.5 Hz, $CHCH_3$), 2.30 (s, $ArCH_3$), 2.83 - 3.20 (comp m, CH_2), 3.45 - 4.15 (comp m, CHN), 7.01 (bd s, C_6H_2); CIMS (MH^+) 99+% incorporation of isotope.

N-(2-Carboxybenzoyl)-2,5-dimethoxy-4-(2-aminopropyl)-benzoic Acid (62).-A mixture of the phthalimidoaldehyde 61 (5.0 g, 14.2 mmole), prepared by the method of Ho *et al.*,[14] Ag_2O (6.38 g, 27.5 mmole) and NaOH (5.07 g, 127 mmole) in 85 ml water was stirred overnight at room temp. After heating for 1.5 hr on a steam cone, the reaction mixture was allowed to cool and was filtered. The filtrate was acidified with 10% HCl to pH 1 and the resulting white ppt (4.3 g, 11.1 mmole, 78%, mp 178-181°) was crystallized from EtOH to yield pure 62 (2.9 g, 7.5 mmole, 53.5%): mp 186-188°; pmr (DMSO-d_6) δ 1.13 ppm (d, J = 6.5 Hz, CCH_3), 2.65 - 3.05

(comp m, CH_2), 3.77 (s, OCH_3), 3.95 - 4.65 (comp m,NCH), 7.0 - 7.96 (comp m, 6 aromatic protons); ir (KBr) ν_{max} 1720 cm^{-1} (acid C=O), 1650 (amide C=O).

Anal. Calcd for $C_{20}H_{21}NO_7$: C, 62.01; H, 5.46; N, 3.62. Found: C, 61.87; H, 5.39; N, 3.96.

2,5-Dimethoxy-4-(2-aminopropyl)benzoic Acid (13).-A suspension of the amidoacid 62 (1.2 g, 3.1 mmole) in 25 ml 10% HCl was heated at reflux for 3 hr and the mixture was cooled and washed with 3 x 40 ml EtOAc. Removal of the water under vacuum yielded a brown oil which upon trituration with Et_2O gave crude amino acid 13·HCl (0.83 g, 3.0 mmole, 98%, mp 80-135°). Crystallization from CH_3CN provided pure 13 as its HCl salt (0.2 g, 0.73 mmole, 23%): mp 196-198°; pmr (D_2O) δ 1.47 ppm (d, J = 6.5 Hz, CCH_3), 2.85 - 3.30 (comp m, CH_2), 3.65 - 4.1 (comp m, NCH), 4.03 (s, OCH_3), 7.10 (s, arom H), and 7.28 (s, arom H); ir (KBr) ν_{max} 1730 cm^{-1} (C=O).

Anal. Calcd for $C_{12}H_{18}NO_4Cl$: C, 52.27; H, 6.58; N, 5.08; Cl, 12.9. Found: C, 52.19; H, 6.69; N, 5.03; Cl, 13.1.

The benzamide 32 of the amino acid 13 was prepared by the method of Ho and co-workers[14] by the treatment of 13 (0.2 g, 0.7 mmole) with benzoyl chloride (0.24 g, 1.7 mmole). The pure benzamide (0.16 g, 64%) had a mp of 183-184° (lit.[14] mp 185-186°; EtOH); pmr (DMSO-d_6) δ 1.30 ppm (d, J = 6.5 Hz, CCH_3), 2.85 - 3.10 (comp m, CH_2), 3.83 (s, OCH_3), 4.10 - 4.65 (comp m, NCH), and 6.98 - 8.00 (comp m, 7 aromatic protons).

N-(2-Carboxybenzoyl)-1-(2,5-dimethoxy-4-hydroxymethylphenyl)-2-aminopropane (63).-A soln of $NaBH_4$ (0.36 g, 9.5 mmole) in 1N NaOH (45 ml) was stirred with the N-phthalimidoaldehyde 61[14] (3.0 g, 8.4 mmole) at room temp for 3 hr followed by heating on a steam cone for 2 hr. After cooling, the suspension was filtered and the filtrate was acidified to pH 1 with 10% HCl. The white ppt which formed was collected: 2.6 g, 85%, mp 156-159°. Recrystallization from ab EtOH/Et_2O gave 1.4 g (3.8 mmole, 46%) of compound 63: mp 171-172°; pmr (DMSO-d_6) δ 1.14 ppm (d, J = 6.5 Hz, CH_3), 2.62 - 2.98 (comp m, $ArCH_2C$), 3.73 (s, OCH_3), 3.78 (s, OCH_3), 4.52 (s, $ArCH_2O$), 6.80 - 7.95 (comp m, 6 aromatic protons).

Anal. Calcd for $C_{20}H_{23}NO_6$: C, 64.33; H, 6.21; N, 3.75. Found: C, 63.86; H, 6.30; N, 3.82.

Bis-2,5-dimethoxy-4-(2-aminopropyl)phenylmethane (68).-A suspension of the phthaloylalcohol 63 (0.86 g, 2.2 mmole) in 10% HCl (25 ml) was held at reflux for 2 hr. After cooling, the mixture was extracted with EtOAc (2 x 50 ml). The combined EtOAc layers were dried (Na_2SO_4) and evaporated to give an oil (0.5 g, 1.3 mmole, 57%) the nmr of which showed the presence of mainly one compound: pmr ($CDCl_3$) δ 1.10 ppm (d, J = 6.0, CH_3), 1.72 (s, NH_2), 2.35 - 2.82 (comp m, CH_2), 2.89 - 3.38 (comp m, CHN), 3.68 (s, OCH_3), 3.79 (s, OCH_3), 3.91 (s, $ArCH_2Ar$), 6.69 (s, C_6H_2). Compound 68 was further characterized as its bis-benzoyl derivative 64 prepared as described above for amine 1 and crystallized from EtOH: mp 196-198°; pmr ($CDCl_3$) δ 1.30 ppm (d, J = 6.5 Hz, CH_3),

2.65 - 3.03 (comp m, CH_2), 3.65 (s, OCH_3), 3.74 (s, OCH_3), 3.90 (s, $ArCH_2Ar$), 4.01 - 4.66 (comp m, CHN), 6.51 - 7.83 (comp m, 14 aromatic protons); CIMS (MH^+) 611 (100%).

Anal. Calcd for $C_{37}H_{42}N_2O_6$: C, 72.76; H, 6.93; N, 4.59. Found: C, 72.93; H, 6.83; N, 4.61.

1-(2,5-Dimethoxy-4-hydroxymethylphenyl)-2-aminopropane(12)- Method 1. A solution of carboxyphthaloylalcohol 63 (2.5 g, 6.7 mmole) in aqueous 20% NaOH (50 ml) was held at reflux for 20 hr. The resulting suspension was cooled then extracted with Et_2O (50 ml) and EtOAc (3 x 50 ml). The combined organic layers were dried ($MgSO_4$) and evaporated to give 0.79 g (53%) of an oil. Attempts to crystallize the product from heptanes, hexane or EtOH/Et_2O failed. Sublimation, or preparative tlc did not provide a solid. Treatment of an Et_2O solution of the oil with HCl gas gave an oil which, by nmr analysis, was more impure.

Method 2. A suspension of phthaloylaldehyde 61 (1.68 g, 4.8 mmole) in an aqueous solution (30 ml) of $NaBH_4$ (200 mg) was stirred at room temp for 2 hr. Solid NaOH (3.0 g) was then added and the mixture heated to reflux for 20 hr. After cooling, the suspension was extracted with EtOAc (2 x 50 ml). The combined EtOAc layers were dried ($MgSO_4$) and evaporated to give a low melting white solid (0.54 g, 50.1%). Crystallization from heptanes gave pure 12: mp 90-92° (lit.[14] mp 92-95°, heptanes); pmr ($CDCl_3$) δ 1.13 ppm (d, J = 7 Hz, CH_3), 2.05 (s, NH_2), 2.66 (comp m, CH_2), 3.15 (comp m, CHN), 3.80 (s, OCH_3), 3.82 (s, OCH_3), 4.68 (s, $ArCH_2$), 6.71 (s, arom H),

6.93 (s, arom H), lit.[14] 6.74 ppm (s, arom H), 6.95 (s, arom H).

The bis-benzoyl derivative 31 of 12 was produced by heating a mixture of amine 12 (0.13 g, 0.6 mmole), benzoyl chloride (0.3 ml) and pyridine (3 ml) on a steam cone for 5 min. The ppt which developed upon addition of water (10 ml) was collected and shaken in a test tube with 5% Na_2CO_3 (5 ml). A gum was collected (0.23 g, 92%) and crystallized from EtOH: mp 162-163°; pmr ($CDCl_3$) δ 1.33 ppm (d, J = 6 Hz, CCH_3), 2.82 - 3.10 (comp m, CCH_2), 3.80 (s, OCH_3), 3.86 (s, OCH_3), 4.05 - 4.62 (comp m, CHN), 5.41 (s, CH_2O), and 6.63 - 8.20 (comp m, 12 aromatic protons).

Anal. Calcd for $C_{26}H_{27}NO_5$: C, 72.04; H, 6.28; N, 3.23. Found: C, 72.30; H, 6.12; N, 3.30.

1-(2,5-Dimethoxy-4-methylphenyl)-2-propanone oxime (26).- Powdered NaOH (1.1 g, 27.5 mmole) was added with stirring to a soln of ketone 14[154] (1.15 g, 5.5 mmole) and $NH_2OH \cdot HCl$ (0.6 g, 8.6 mmole) in 75% aq EtOH. The mixture was heated and held at reflux for 10 min. After cooling, the contents were poured into 10% HCl (20 ml) and a light yellow oil separated which was extracted into Et_2O (2 x 30 ml). Evaporation of the Et_2O layers gave 1.1 g (90%) of an oil: pmr ($CDCl_3$) δ 1.78 and 1.85 (s, $CNCH_3$). Crystallization from hexane gave 1.01 g (82%, mp 72-74°). Recrystallization from hexane gave pure 26 (0.46 g, 37%, mp 73-75°), a second crop gave an additional 0.13 g (mp 64-72°, total yield 48%): pmr ($CDCl_3$) δ 1.85 (s, $CNCH_3$), 2.24 (s, $ArCH_3$), 3.54 (s, CH_2),

3.78 (s, OCH_3), and 3.81 (s, OCH_3), 6.70 (s, arom H), and 6.75 (s, arom H).

Anal. Calcd for $C_{12}H_{17}NO_3$: C, 64.55; H, 7.67; N, 6.27. Found: C, 64.69; H, 7.67; N, 6.41.

(R:S)-1-(2,5-Dimethoxy-4-methylphenyl)-2-propanol (45).- A mixture of ketone 14[154] (0.2 g, 0.96 mmole), $NaBH_4$ (0.06 g, 1.6 mmole) and ab EtOH (10 ml) was stirred at room temp for 1 hr then evaporated to dryness. The residue was stirred with water (10 ml) then extracted with Et_2O (2 x 20 ml). The Et_2O layers were dried ($MgSO_4$) and evaporated to give a white solid the nmr of which showed the reaction to be essentially quantitative. Recrystallization from hexane provided the analytical sample: mp 80-82°; pmr ($CDCl_3$) δ 1.20 ppm (d, J = 6.0 Hz, CH_3), 2.22 (s, Ar CH_3), 2.63 - 2.92 (comp m, CH_2), 3.76 (s, OCH_3), 3.8 - 4.40 (comp m, CHN), 6.99 (s, arom H), and 7.02 (s, arom H).

Anal. Calcd for $C_{12}H_{18}O_3$: C, 68.55; H, 8.63. Found: C, 68.69; H, 8.53.

(R:S)-1-(2,5-Dimethoxy-4-methylphenyl)-2-propanol-2-^{2}H (45a).-A soln of ketone 14[154] (1.91 g, 9.2 mmole) in dry THF (50 ml) was added dropwise to a stirred suspension of $LiAl^2H_4$ (99%, 1.0 g, 24 mmole) in dry THF (50 ml). The mixture was stirred at room temp for 7 hr and the reaction was stopped by the careful addition of water (1.0 ml) followed by 10% NaOH (1.5 ml) and water (3 ml). The salts were filtered and the filter cake digested for 30 min with Et_2O. The

organic layers were combined, dried ($MgSO_4$) and concd to give the alcohol 45a (1.90 g, 98.5%, mp 80-81°). Recrystallization from hexane gave 1.76 g (8.4 mmole, 91%): mp 80-82°; pmr ($CDCl_3$) δ 1.20 ppm (s, $CDCH_3$), 2.22 (s, $ArCH_3$), 2.76 (s, CH_2), 3.76 (s, OCH_3), 7.70 (s, C_6H_2); CIMS (MH^+) 99+% isotope incorporation.

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