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IDENTIFICATION OF TERTIARY AMINOMETHYLENEDIOXYPROPIOPHENONES AS URINARY METABOLITES OF SAFROLE IN THE RAT AND GUINEA PIG

E. O. OSWALD, L. FISHBEIN, B. J. CORBETT AND M. P. WALKER

National Institute of Environmental Health Sciences, National Institutes of Health, Public Health Service and Department of Health, Education and Welfare, P.O. Box 12233, Research Triangle Park, N.C. 27709 (U.S.A.)

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SUMMARY

Three nitrogen-containing metabolites of safrole (1-allyl-3,4-methylenedioxybenzene) are excreted in the urine of rats and/or guinea pigs following oral or intraperitoneal administration. The major safrole basic ninhydrin-positive metabolites of the guinea pig and rat are 3-*N*,*N*-dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone and 3-piperidyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone, respectively. In addition, the rat also excretes the above *N*,*N*-dimethylaminoketone and trace amounts of 3-pyrrolidinyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone. All three of these aminoketones decompose to form 1-(3′,4′-methylenedioxyphenyl)-3-propen-1-one.

INTRODUCTION

An earlier report from this laboratory[1] indicated the formation of urinary basic ninhydrin-positive metabolites following oral or intraperitoneal administration of a number of components of essential oils, *e.g.* myristicin, safrole, isosafrole, asarone (*trans*) or β-asarone (*cis*) to male rats.

In order to account for the various observed physiological responses[1] after administration of myristicin and some of the above propenylbenzene derivatives, it was suggested[2] that the substituted benzene derivative may be converted biologically to amphetamines.

The present report describes the identification and verification of structure of the basic nitrogen-containing metabolites of safrole.

EXPERIMENTAL

Commercial-grade safrole (J. T. Baker), was further purified by silicic acid column chromatography to a purity of 99% or greater as determined by thin-layer and gas–liquid chromatography, infrared spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy before administration. All organic solvents were Baker Analyzed reagent grade.

Preparation of biological samples

Male Sprague–Dawley rats (200–500 g) from Charles River Breeding Labs, Wilmington, Mass., and male Hartley strain guinea pigs (200–500 g) from Hazleton Research Animals, Inc., Burtonsville, Md., were given oral and/or intraperitoneal injection of safrole (75–300 mg/kg) for the desired time period. In experiments when the concentration of the metabolite was critical, the animals were injected intraperitoneally with safrole (75–200 mg/kg) daily for periods up to 15 days. Urine was collected, stored, and extracted as described earlier[1]. The safrole basic urine fraction was then concentrated 300–1200-fold and the ninhydrin-positive components were separated by thin-layer chromatography using the methanol system[1] and located by ultraviolet light[1]. The silica gel containing the ninhydrin-positive component was quickly collected, and this material eluted with methanol, thence concentrated *in vacuo* under N_2, and the final metabolite quantitatively transferred with spectro-grade chloroform to an appropriate conical vial. The solvent was removed under N_2, an appropriate volume of spectro-grade chloroform added to the sample, and the material stored under nitrogen at $-20°$ until further use.

Prolonged exposure of these metabolites to alkaline conditions increased the breakdown to the ninhydrin-negative "ketone". Because of this lability, the ninhydrin-positive metabolite after initial chromatographic purification was reduced immediately with an excess of sodium borohydride (10 mg) or sodium borodeuteride in ethanol (10 ml). The mixture was stirred for about 15 min, the excess sodium borohydride or deuteride removed by addition of excess acetic acid, and the reduction mixture concentrated to dryness *in vacuo* in order to remove the alcohol and acetic acid. The reduction mixture was then made basic (pH 14) with NaOH, the reduced metabolite of safrole extracted repeatedly with chloroform, and the combined chloroform solutions taken to dryness and transferred to a conical vial. In order to remove any breakdown products formed during purification and reduction, the reduction mixture was then purified using the methanol system[1] and eluted as described above for the unreduced metabolites.

All spectral data were collected as soon as possible after the final thin-layer purification. In some instances the reduced metabolites were also monoacetylated with acetyl chloride and pyridine or acetic anhydride and pyridine.

All of the metabolites and their reduced and acetylated derivatives were characterized chemically on thin-layer chromatography with previously described ninhydrin, chromatropic acid and 2,4-dinitrophenylhydrazine[1] reagents.

Collection of qualitative spectra

Infrared spectra. Infrared spectra of all the isolated urinary safrole basic metabolites and their authentic synthetic analogs were obtained using a Perkin–Elmer 621 infrared spectrophotometer. The desired material (usually 200–1000 μg) in spectro-grade chloroform was applied as a film to a NaCl disc, all traces of the solvent removed, and the spectra determined. This material could then be removed from the salt disc with spectro-grade chloroform and used for other characterization studies.

Ultraviolet spectra. Safrole basic metabolites were dissolved in absolute ethanol and the absorption spectrum obtained using a Beckman DK-2 spectrophotometer.

Nuclear magnetic resonance spectra. Usually 500–2000 μg of the purified safrole basic metabolite was dissolved in about 100 μl of $\geq 99.9\%$ deuterated chloroform

(Diaprep, Inc., Atlanta, Ga.) containing 3% tetramethyl silane (≥99.9% Aldrich Chemical Co., NMR grade). The solution was then transferred to an elongated cylindrical micro-NMR tube (6 mm × 10 mm, Wilmad Glass Co., Buena, N.J.). The NMR spectra were obtained using a Varian HA-100 NMR spectrometer with tetramethylsilane as the reference at room temperature (28°).

Mass spectra. Low-resolution mass spectrometry was performed on 0.5–10-μg samples of metabolite using a Perkin–Elmer 270 GC mass spectrometer. Since major decomposition occurred during introduction of the metabolites by way of the gas chromatograph, most of the mass spectra were collected by introducing the sample by direct probe with the routine voltage of 70 eV and bombardment of 100 μA.

Preparation of tertiary aminomethylenedioxypropiophenones

3-*N*,*N*-Dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone·HCl (35% yield, m.p. 195–196°) (lit., m.p. 192°)[5]; 3-piperidyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone·HCl (21% yield, m.p. 212–213°), (lit., m.p. 211–212°)[5]; and 3-pyrrolidinyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone·HCl (40% yield, m.p. 202–203°), (lit., m.p. 199°)[5] were synthesized by the Mannich reaction[3] *via* the reaction of 3,4-methylenedioxyacetophenone (K and K Labs, Plainview, N.J.) with paraformaldehyde and the appropriate secondary amine hydrochloride in ethanol. The Mannich salt was recrystallized from ethanol–ether, neutralized with NaOH and the free base extracted into chloroform. The chloroform was removed *in vacuo* to yield the free base. These free bases of the above tertiary aminomethylenedioxypropiophenones underwent analogous reactions (including reduction, acetylation and decomposition upon heating to the respective unsaturated ketone[4]) as those described for the isolated metabolites.

RESULTS AND DISCUSSION

Metabolite I

There appears to be only one basic ninhydrin-positive metabolite of safrole excreted in the urine of guinea pigs, (R_F of 1.0, relative to the standard 3,4-methylenedioxyamphetamine using the methanol system[1]). This metabolite reacts with ninhydrin upon heating to yield a pink material. The ultraviolet spectrum of Metabolite I has a maximum absorption at 310 and 274 mμ indicating that some grouping is conjugated with the aromatic ring. As is shown in Fig. 1, safrole, a non-conjugated system, has maximum absorption at 286 mμ. The Metabolite I from the guinea pig, upon reduction has a maximum ultraviolet absorption at 286 mμ, similar to that of safrole. Infrared spectra of Metabolite I indicate a carbonyl absorption at 1670 cm^{-1} (Fig. 2) with a very sharp band at 1605 cm^{-1}. Metabolite I upon reduction with sodium borodeuteride yields a very broad hydroxy absorption band at 3400–3300 cm^{-1} (Fig. 3). The monoacetylated derivative of reduced Metabolite I has a carbonyl absorption at 1730 cm^{-1} and no hydroxy absorption in the 3400-cm^{-1} region (Fig. 4).

Attempts to gas chromatograph Metabolite I yielded a nonpolar ninhydrin-negative material which will be described later. However, direct probe introduction of these metabolites into the mass spectrometer was very useful. The Metabolite I has a parent mass of 221 (see Fig. 5), with a base peak of 58, and with very abundant m/e = 149 and less abundant m/e = 121. Upon reduction with sodium borohydride,

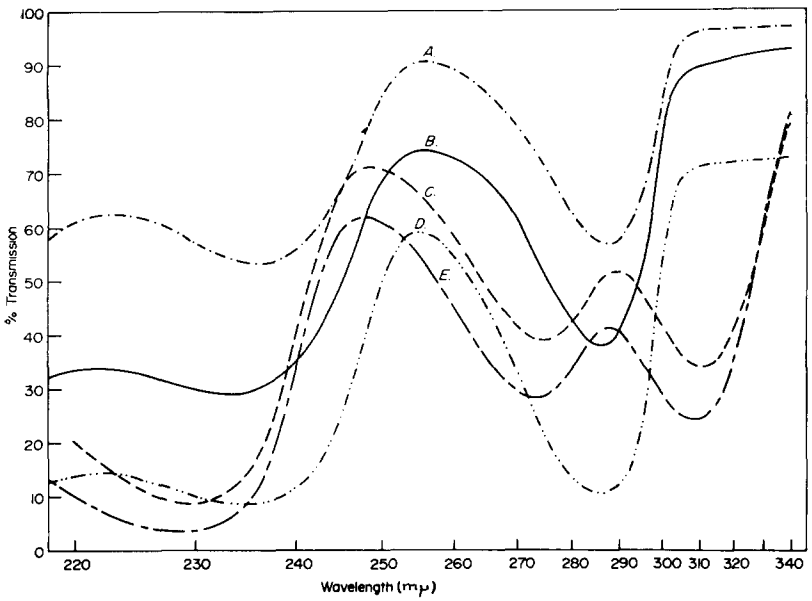

Fig. 1. Ultraviolet spectra. A, safrole; B, sodium borohydride-reduced rat Metabolite II; C, synthetic 3-piperidyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone; D, sodium borohydride-reduced synthetic 3-piperidyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone; E, rat Metabolite II.

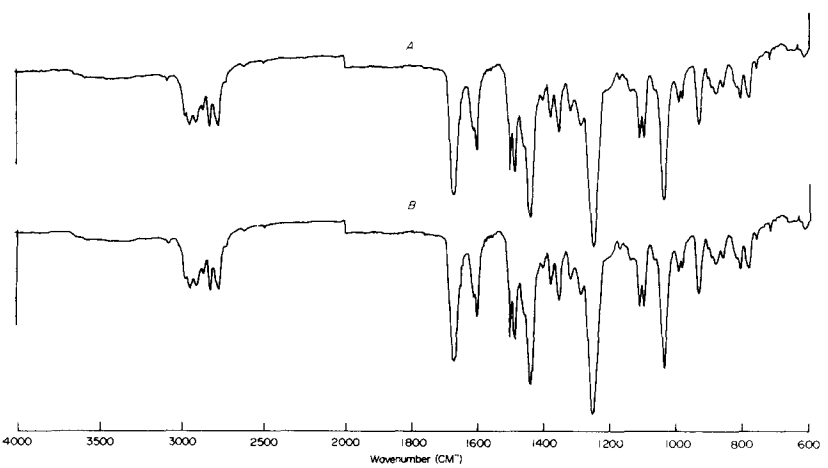

Fig. 2. Infrared spectra. A, guinea pig Metabolite I; B, synthetic 3-*N*,*N*-dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone

the reduced Metabolite I has a parent mass of 223 (Fig. 6), with a base peak of $m/e = 58$ with abundant 148 and 121 fragments. A significant (P-18) fragment (Fig. 6) with a mass of 205 is also present for the reduced Metabolite I. The guinea pig Metabolite I which was reduced with sodium borodeuteride has a parent mass of 224, indicating that only one of the deuterium from the reduction is non-exchangeable. The other deuterium on the oxygen of the hydroxy group is exchangeable. Upon

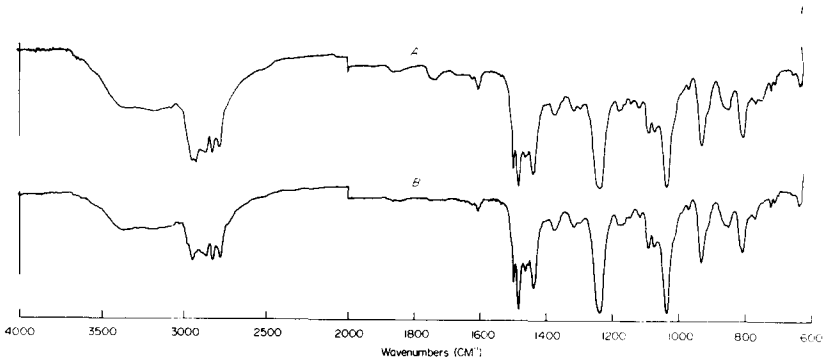

Fig. 3. Infrared spectra. A, sodium borohydride-reduced guinea pig Metabolite I; B, sodium borohydride-reduced synthetic 3-*N*,*N*-dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone.

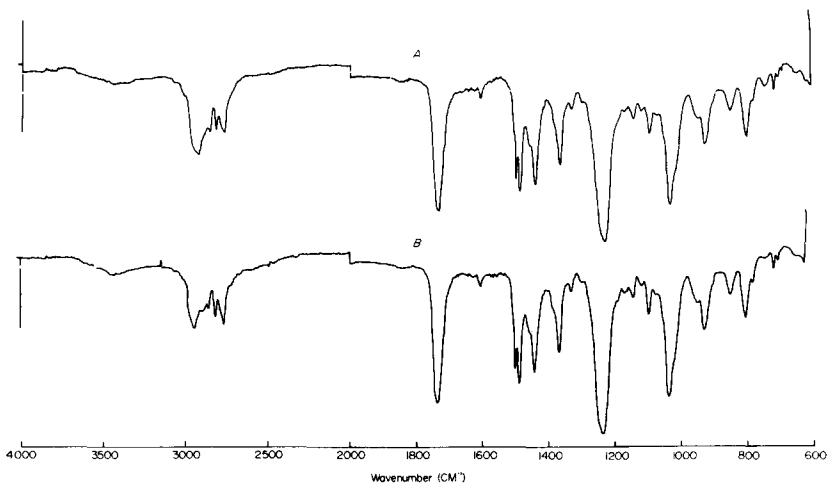

Fig. 4. Infrared spectra. A, acetylated sodium borohydride-reduced guinea pig Metabolite I; B, acetylated sodium borohydride-reduced synthetic 3-*N*,*N*-dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone.

acetylation of the sodium borohydride reduced Metabolite I, the abundance of the original reduced parent mass of 223 is decreased and the 222 mass fragment is very abundant with the parent mass of the acetylated derivative being 265.

The methylenedioxy ring feature of Metabolite I was indicated *via* its NMR spectra (Fig. 7, sharp singlet-two protons 5.98–6.04 ppm). Reduction of Metabolite I with sodium borodeuteride results in the removal of the triplet (4.78–4.88 ppm), indicative of the methine proton on the carbon atom adjacent to the ring, further localizing the non-exchangeable deuterium from the reduction of Metabolite I (Fig. 8).

The foregoing information indicates that guinea pig Metabolite I has a methylenedioxyphenyl ring which is conjugated with a carbonyl group linked to a methylene *N*,*N*-dimethylamino unit. The entire molecule has a mass of 221 prior to reduction and acetylation.

The ultraviolet, infrared, nuclear magnetic resonance and mass spectra of Metabolite I are identical to those obtained from an authentic sample of 3-*N*,*N*-dimethylamino-1-(3',4'-methylenedioxyphenyl)-1-propanone (see Figs. 1, 2, 5 and 7).

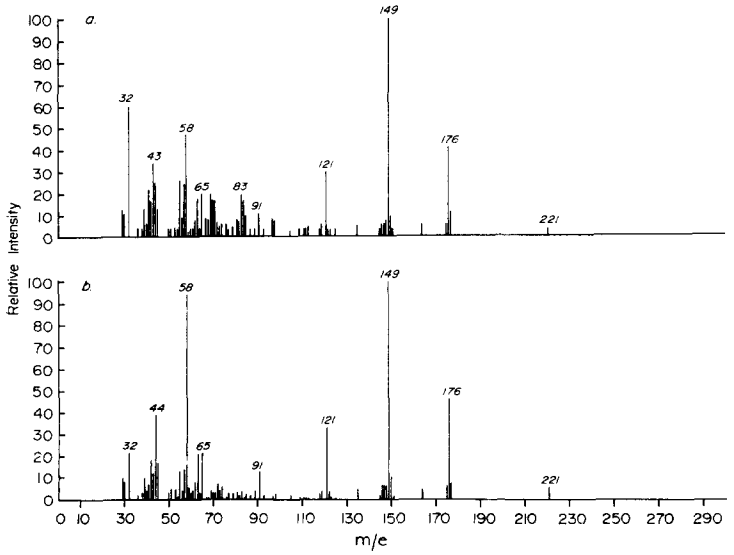

Fig. 5. Mass spectra. a, Guinea pig Metabolite I. b, synthetic 3-*N*,*N*-dimethylamino-1-(3',4'-methylenedioxyphenyl)-1-propanone.

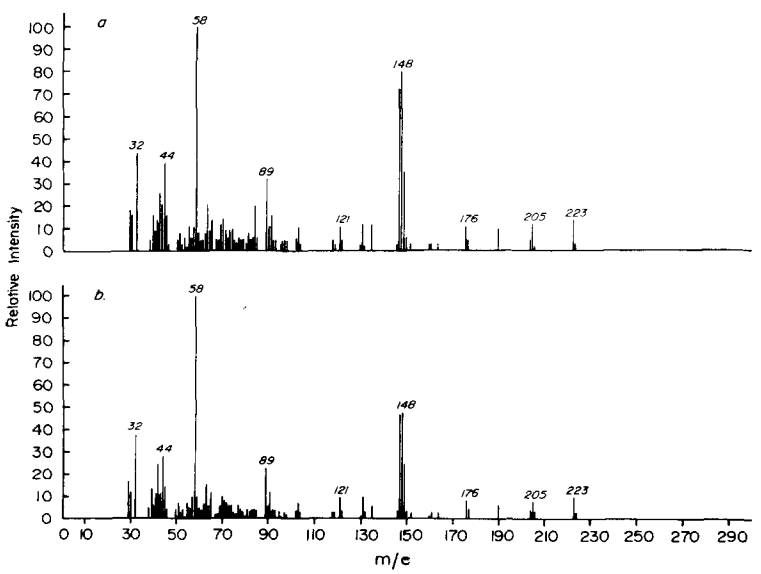

Fig. 6. Mass spectra. a, Sodium borohydride-reduced guinea pig Metabolite I. b, Sodium borohydride-reduced synthetic 3-*N*,*N*-dimethylamino-1-(3',4'-methylenedioxyphenyl)-1-propanone.

The reduction and acetylation products of the synthetic product with the analogous derivatives of guinea pig Metabolite I were also identical (see Figs. 3, 4, 6 and 8), thus providing further verification of the structure (Fig. 9).

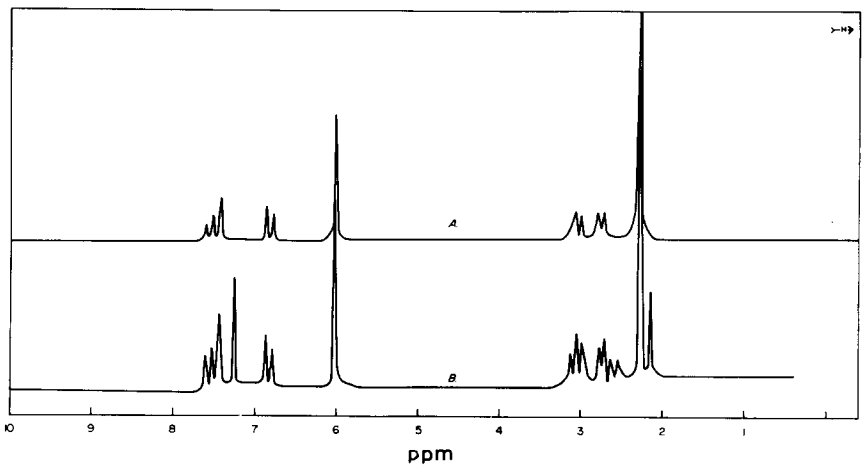

Fig. 7. NMR spectra. A, synthetic 3-*N*,*N*-dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone; B, guinea pig Metabolite I.

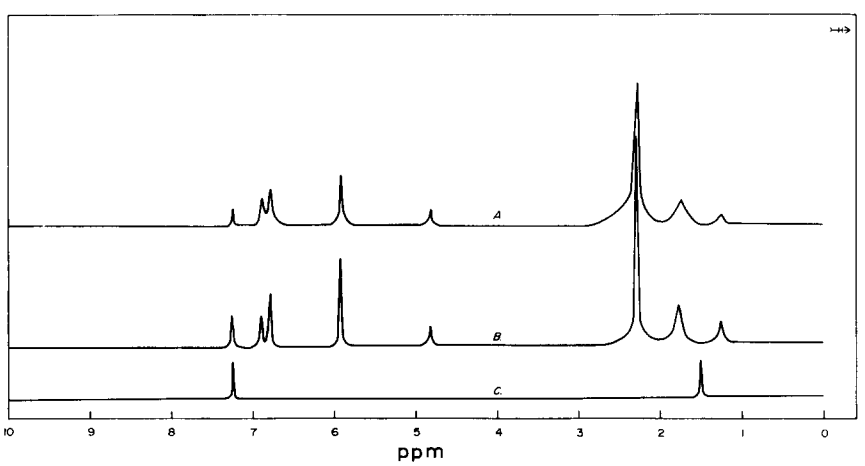

Fig. 8. NMR spectra. A, sodium borohydride-reduced synthetic 3-*N*,*N*-dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone; B, sodium borohydride-reduced guinea pig Metabolite I; C, solvent system: deuterated chloroform–tetramethyl silane.

Metabolite II

The rat excretes the same type of safrole basic metabolites as does the guinea pig. 3-*N*,*N*-dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone (Metabolite I) is, however, a minor metabolite of the rat. The Metabolite I of the rat is contaminated with a trace component which will be discussed later.

The major metabolite of the rat (Metabolite II) has a R_F of 2.2 relative to the standard amphetamine using the methanol system[1], and upon heating reacts with ninhydrin to yield a dark purple material. The ultraviolet spectrum of Metabolite II is similar to that described for Metabolite I (Fig. 1) with maximum absorption at 310 and 274 mμ, indicating the conjugated system. The 1670- and 1605-cm^{-1} conjugated carbonyl absorption is absent upon reduction with sodium borohydride or deuteride. The reduced Metabolite II with the hydroxyl absorption at 3400–3300 cm^{-1} was acetylated to yield the monoacetyl derivative with a 1730-cm^{-1} carbonyl absorption and no hydroxy group.

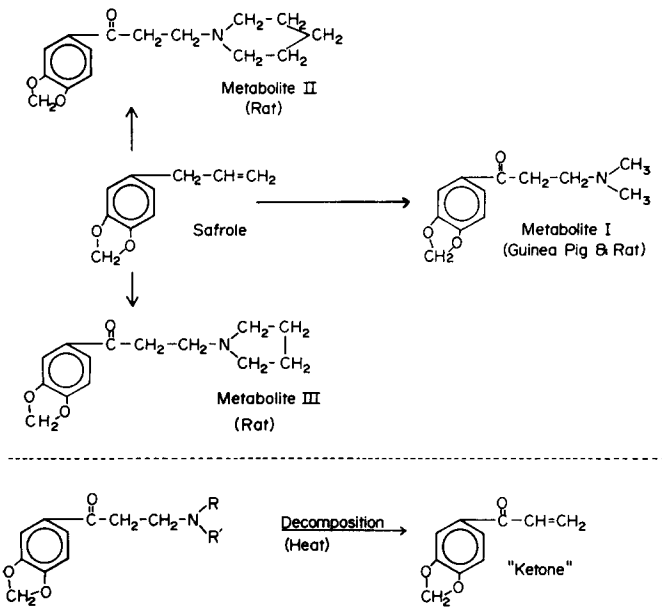

Fig. 9. Chemical structures of rat and guinea pig safrole basic metabolites*.

Metabolite II has a parent mass of 261 with a base peak of 98, less abundant 176, 149, and 121 mass fragments, and no significant mass fragments in the region between 176 and 261. Upon reduction with sodium borohydride, this derivative has a parent mass of 263 with a very significant (P-18) fragment of mass 245, including the 148 and 131 fragments. Reduction of Metabolite II with sodium borodeuteride yields a material with a parent mass of 264, indicative of one non-exchangeable deuterium present in the molecule from the reduction. Acetylation of the reduced Metabolite II (m/e = 263) produced the monoacetylated derivative with a parent mass of 305 and a very prominent 262 fragment.

As exemplified by NMR spectra, the methylenedioxy ring is intact (sharp singlet 5.98–6.04 ppm) and the aromatic protons (multiplet two protons, 7.44–7.60 ppm; and multiplet one proton, 6.80–6.88 ppm) are the same as in Metabolite I. Upon reduction there is a shift in the aromatic region, (two protons 6.80 ppm and one proton

* Additional spectra of Metabolites II and III may be obtained from the authors upon request.

6.90 ppm), as seen for the dimethylamino-substituted ketone and the triplet (4.78–4.88 ppm), indicating the methine proton is absent upon reduction with sodium borodeuteride.

The major basic urinary safrole metabolite of the rat (Metabolite II) was thus found to be 3-piperidyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone as shown by its identical ultraviolet, infrared, mass and NMR spectra with that of an authentic synthesized sample (Fig. 9).

Metabolite III

As indicated above, both the rat and guinea pig produce the 3-*N*,*N*-dimethylamino-1-(3′,4′-methylenedioxyphenyl)-1-propanone from safrole. However, there is a trace contaminate in the rat Metabolite I. From the direct probe mass spectra it is clear that the contaminate has a parent mass of 247. Upon reduction with sodium borohydride, the parent mass is 249 with an abundant (P-18) mass fragment of 231. The very abundant 84 mass fragment for this Metabolite III seems to be the base peak. Reduction of the trace component with sodium borodeuteride gives a material with a mass of 250, again indicating only one non-exchangeable deuterium is present. Acetylation of the reduced mixture gave a material with a mass of 291.

All of the mass fragments for this trace metabolite are the same as those for Metabolites I and II, except for the parent mass of 247 and the 84 base peak suggesting a different amine component from the previously described tertiary aminoketones.

Attempts to gas chromatograph these materials using the Perkin–Elmer GC mass spectrometer with its particular designed separator were unsuccessful. The ninhydrin-positive metabolites decompose to a less polar material which is ninhydrin negative, chromatropic positive[1], and which contains a carbonyl group.

However, subsequent investigation of the reduced rat Metabolite I mixture utilizing an LKB GC mass spectrometer revealed the presence of a pyrrolidinyl ketone with minor decomposition on OV-17 at 175°. The LKB GC mass spectrum of this Metabolite III has a parent mass of 249 with a base of 84, with less abundant 231, 230, 149, 148 and 147 fragments and was shown to be identical with that of an authentic sample of 3-pyrrolidinyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone (Fig. 9).

"Ketone"

The pH and thermal lability of the three ninhydrin-positive tertiary aminoketones produced from safrole has been cited earlier. The nonpolar decomposition product reacts with 2,4-dinitrophenylhydrazine. The synthetic unsaturated ketone produced by heating of the aminoketone[4] retains the methylenedioxy moiety, but is no longer basic. The mass spectrum of this ketone indicates a parent mass of 176 with a base peak of 149 and less abundant 121, 91 and 63 mass fragments. Reduction of the unsaturated ketone yields the respective alcohol with a 151 base peak. The synthetic ketone produced by heating of the above dimethylaminoketone has the same structure, 1-(3′,4′-methylenedioxyphenyl)-3-propen-1-one as the decomposition product from the basic safrole Metabolites I, II and III. Fig. 9 illustrates the formation of the "ketone" from tertiary aminomethylenedioxypropiophenones as well as the structures of Metabolites I, II and III formed from safrole.

The reactivity of the basic aminoketones has been shown earlier. A tertiary

amine would not be expected to react with ninhydrin. However, the process of heating the thin-layer chromatographic plate following spraying with ninhydrin results in the decomposition of the tertiary aminoketone to the amine and unsaturated ketone. The free secondary amines, *e.g.* dimethylamine, piperidine and pyrrolidine, subsequently react with ninhydrin to yield red, dark purple and bright yellow colored complexes, respectively.

Biological significance

Earlier reports[2] have suggested that substituted benzene derivatives may be converted biologically to amphetamines. A large series of substituted amphetamines[2] were synthesized and their psychoactive properties were examined in order to explain the psychotropic responses produced by various non-nitrogen-containing propenylbenzene derivative of essential oils.

The presently reported investigations were undertaken to firmly document the biological formation of amphetamines from the compounds of essential oils. No amphetamine production was detected. Instead, more chemically active tertiary aminoketones were isolated and structurally identified.

We have described the identification and structure of three nitrogen-containing metabolites of safrole which are excreted in the urine of rats and guinea pigs following both oral and intraperitoneal administration. As discussed earlier, the production of these ninhydrin-positive basic metabolites of safrole require the presence of a double bond in the side chain. A possible mechanism to account for the formation of the three tertiary aminomethylenedioxypropiophenones suggests that safrole may undergo a biological allylic oxidation to first form the allylic ketone which could then condense with a secondary amine in the presence of the appropriate enzyme system to yield the final excreted tertiary aminoketones.

These tertiary aminopropiophenones may very likely be the active metabolites which elicit the psychotropic activity of the propenylbenzene derivatives of the essential oils. The pharmaceutical industry has produced many psychotropic drugs[6] which have very similar chemical structures to those of the above reported metabolites and which produce many of the psychoactive responses[1] that are reported for the components of the essential oil. In addition, the tertiary aminoketones upon oxidation to the N-oxide also may be the pathological tumor producing agents derived from some of the essential oils.

Until more is known about these particular tertiary aminopropriophenones and their potential biological properties, we can conclude only that safrole is converted biologically to the tertiary aminomethylenedioxypropiophenones, not to the amphetamines. We have verified that non-nitrogen-containing components of essential oils are converted biologically to very basic nitrogen-containing metabolites *in vivo*.

The elucidation of the basic urinary ninhydrin-positive metabolites of isosafrole, myristicin and the *cis*- and *trans*-asarones is presently in progress. Additional investigations are also underway to determine the potential biological hazards of these nitrogen-containing metabolites. It is hoped that these studies involving the tertiary aminoketones will contribute to the better understanding of the physiological as well as the pathological action of the allylic and propenylic benzene derivatives in natural food products[1] and essential oils.

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