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Metabolism of amphetamines. Identification of *N*-oxygenated products by gas chromatography and mass spectrometry

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A sensitive method for the isolation and identification of *N*-oxygenated metabolites and metabonates of medicinal amines is described. A combination of gas chromatography and mass spectrometry is effective in separating and identifying secondary hydroxylamines, oximes and nitrones, all of which are *N*-oxygenated products of secondary amines. These products give mass spectra containing diagnostic fragment ions which are of great value in identification. Primary hydroxylamines are oxidized on column to oximes and can be separated and identified as such. This oxidation is avoided if the primary hydroxylamine is introduced by direct inlet into the mass spectrometer. Secondary hydroxylamines are more stable during gas chromatographic examination although some decomposition to nitrone and secondary amine does occur. In contrast, oximes and nitrones show no tendency to decompose when gas chromatographed.

In vitro, and in some instances *in vivo* metabolic *N*-oxidation of amphetamine and its derivatives produces *N*-hydroxy compounds as initial metabolites. These hydroxylamines are subsequently converted by physical (e.g. heat), chemical and perhaps metabolic processes to oximes (Beckett, Van Dyk & others, 1971) or to nitrones (Beckett, Coutts & Ogunbona, 1973a) depending on whether the hydroxylamine is primary [RNHOH] or secondary [RN(OH)R']. The isolation of a hydroxylamine, an oxime or a nitrone in a metabolic study, therefore, is indicative of metabolic *N*-oxidation. *N*-Oxygenated metabolites and metabonates are not readily available. Also, because of their instability, it is imperative that metabolically produced hydroxylamines and nitrones are isolated and identified as rapidly as possible. The only feasible method of accomplishing this seemed to be by means of gas chromatography (g.c.), mass spectrometry (m.s.) and a combination of these, provided it was established that hydroxylamines, oximes and nitrones could be separated by g.c. and would give rise to mass spectra which were diagnostic of these groups of compounds. A few isolated examples of the use of g.c. and m.s. in the identification of the oxime derived from amphetamine (Hucker, Michniewicz & Rhodes, 1971; Beckett & Al-Sarraj, 1973) and the *N*-hydroxylated metabolite of phentermine (Cho, Lindeke & Hodshon, 1972) have been reported. We have established that primary and secondary hydroxylamines, oximes and nitrones derived from amphetamines can be separated without prior derivatization (which may result in undesired decomposition) by means of g.c. and give rise to mass spectra which are diagnostic of these compounds.

MATERIALS AND METHODS

Hydroxylamines. The primary hydroxylamines, Ia, Ib, and Ic were supplied as succinate salts by Smith, Kline and French Corporation, Philadelphia. Compound Id

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hydrochloride, m.p. 121–123°, was obtained by catalytic hydrogenation of 1-(2,5-dimethoxy-4-methylphenyl)-2-nitropropene (Coutts & Malicky, unpublished). The six secondary hydroxylamines (IIa-IIe, IV) were products of *m*-chloroperbenzoic acid oxidation of the corresponding amphetamines (IIIa-IIIe and *N*-benzylamphetamine) and were isolated and characterized as oxalates. Their preparation will be described elsewhere (Beckett, Coutts & Ogunbona, 1973b). *N*-Hydroxynorfenfluramine (Ie) acid oxalate, m.p. 160–161°, was similarly synthesized from norfenfluramine.

Oximes. The oximes Va, m.p. 72–73°; Vb, m.p. 71–72°; Vd, m.p. 75–76°; Ve, m.p. 90–91°; VI, b.p. 106–108° (10 mm); and VII, m.p. 67–68° were prepared as colourless or pale yellow products by K. K. Midha and A. Saunders from the corresponding ketones by the action of hydroxylamine. Oximes Vc and Vf were the products of catalytic (10% palladium-charcoal) hydrogenation of 1-(2-methoxyphenyl)-2-nitropropene and 2-nitro-1-(3,4,5-trimethoxyphenyl)propene respectively, in ethanolic hydrochloric acid (Coutts & Malicky, unpublished) and were characterized as their hydrochlorides, Vc HCl, m.p. 122–124° and Vf HCl, m.p. 121–123°.

All synthetic hydroxylamines and oximes gave C, H and N analyses consistent with their structures and gave single spots when examined by t.l.c.

Qualitative conversion of secondary hydroxylamines to nitrones. To an aqueous solution of the secondary hydroxylamine (IIb-IIe) (10 mg) was added yellow mercuric oxide (25 mg) and the suspension shaken for 30 min during which time a black colour (Hg) developed. The solution was filtered and the filtrate extracted with ether. The ether extract was concentrated to about 100 μl on a water-bath at 43° and then examined by g.c. and g.c./m.s. Three peaks were observed, i.e. nitrone (VIII or IX) (main product) and smaller peaks of oxime (Va or Vd) and the corresponding ketone as indicated by m.s.

Gas chromatography. All compounds were chromatographed on a Perkin-Elmer Model F-11 instrument which incorporated a flame-ionization detector. A glass column, length 1·0 m, o.d. 0·25 inch, containing 7½% Carbowax on Chromosorb W, acid-washed and DCMS-treated, was used with nitrogen (40 lb inch^2) as carrier gas. Pertinent retention times (Rt) of compounds used (in min) are listed in Tables 1–4 at an oven temperature of 158° except where otherwise stated.

Combined gas chromatography/mass spectrometry. This was performed on a Perkin-Elmer model 270 instrument using a 7½% Carbowax column (length 1 m) identical to that described above. Helium (10 lb inch^2) was the carrier gas. The following temperatures were used: oven 160–180°, injection port 180–200°, manifold 215°, ion source 185°, and an ionizing potential of 70 eV was employed.

Mass spectra. Direct inlet mass spectra were recorded on an A.E.I. MS-9 or MS-12 mass spectrometer at an ionizing potential of 70 eV. Source temperature varied between 145 and 200° depending on the sample. Accurate mass measurements were determined on the MS-9 instrument by the peak matching method.

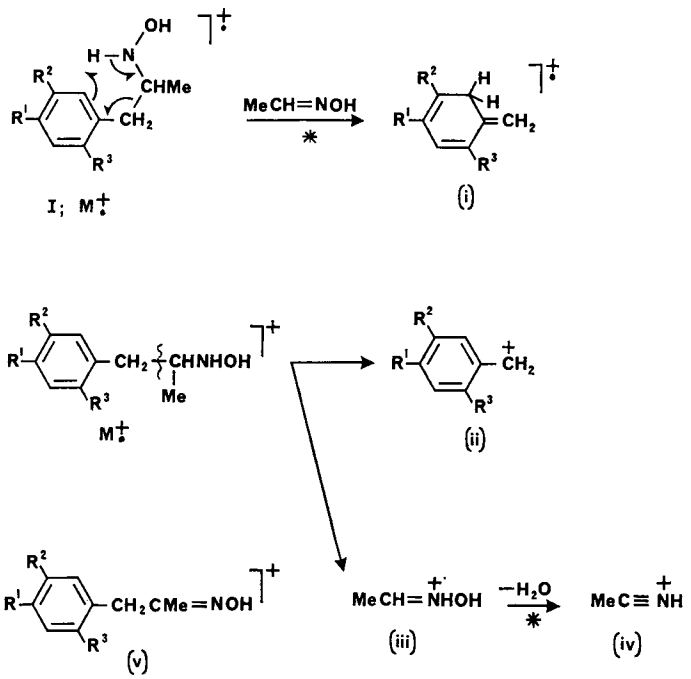
RESULTS AND DISCUSSION

Primary hydroxylamines

The mass spectra of five primary hydroxylamines (Ia-e) were recorded. Compounds Ia, c, e are metabolic products of amphetamine (Beckett & Al-Sarraj, 1972), *p*-methoxyamphetamine (Beckett & Midha, unpublished) and norfenfluramine (Beckett

& Ogunbona, unpublished) respectively. All were examined by direct inlet into the mass spectrometer and each gave a simple spectrum which differed appreciably from the amphetamine from which it was derived (Fig. 1). Ions considered of diagnostic value in identifying these primary hydroxylamines are listed in Table 1 and identified in Scheme 1.

The modes of fragmentation are greatly influenced by the nature of the substituents on the aromatic ring. The ion of greatest diagnostic value is of m/e 60 which is an abundant ion in all spectra and is identified as ion (iii) in the scheme. A metastable ion



Scheme 1.

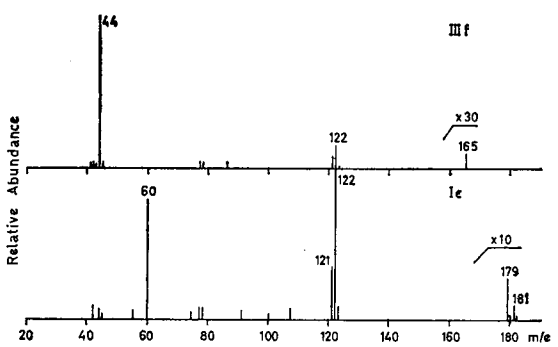
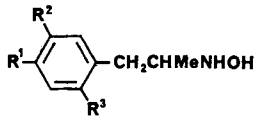


FIG. 1. Mass spectra of *p*-methoxyamphetamine (IIIf) and *N*-hydroxy-*p*-methoxyamphetamine (Ic).

Table 1. *Primary hydroxylamines—gas-chromatographic retention times, and relative abundances of diagnostic ions in their direct inlet mass spectra.*



I

						% relative abundance				
	R^1	R^2	R^3	Rt	M^+	(i)	(ii)	(iii)	(iv)	(v)
(a)	H	H	H	4·1	1·0	11	23	100	19	0·4
(b)	OH	H	H	10·0[a]	0·6	37	22	100	11	3·3
(c)	OMe	H	H	16·3	1·1	100	35	80	9	2·8
(d)	Me	OMe	OMe	13·1[a]	8·2	100	13	45	8	0·8
(e)	H	CF_3	H	3·4	—	2	10	100	4	—

(a) = Column temp. 196°.

of m/e 29·4 in each spectrum supports the expulsion of a molecule of water from (iii) to yield ion (iv) m/e 42. A third ion (ii) present in all spectra is, like (iii), formed by benzylic cleavage of the molecular ion. No other ions are observed in the spectrum of *N*-hydroxynorfenfluramine (Ie) which has the strong electron-withdrawing trifluoromethyl group as a ring substituent. In contrast, the spectra of the remaining four *N*-hydroxyamphetamines (Ia-d) contain an ion identified as (i) which is particularly abundant in those compounds with strong electron-donating groups in the aromatic ring (Ic, d). These four hydroxylamines also give a very weak molecular ion and an $[M-2]^+$ ion, also of low abundance, which is identified as ion (v). Aromatic hydroxylamines have previously been shown to give rise to $[M-2]^+$ ions (Coutts & Mukherjee, 1970). The elemental compositions of the ions i-iv in the spectrum of Id were determined by accurate mass measurements and were consistent with the structures shown in Scheme 1.

When primary *N*-hydroxyamphetamines are analysed by g.c., they decompose at the injection port to the corresponding oxime although decomposition is minimized by suitably positioning the column (Beckett & Al-Sarraj, 1973). Thus g.c. identification of a metabolite as an oxime can indicate that the metabolite is either an oxime or a primary hydroxylamine. A preliminary thin-layer chromatographic (t.l.c.) examination of metabolites will distinguish between these possibilities (Beckett & Al-Sarraj, 1973).

Since column position at the injection port could not be adjusted, primary hydroxylamines could not be directly identified by combined g.c./m.s. Complete conversion to oxime occurred. Sufficient quantities of the primary hydroxylamine metabolite must be obtained and introduced directly into the mass spectrometer to give meaningful spectra characteristic of the hydroxylamine.

Secondary hydroxylamines

Secondary hydroxylamines are more stable than primary hydroxylamines. Synthetic samples of *N*-hydroxy derivatives of *N*-methyl-, *N*-ethyl-, *N*-propyl-, and *N*-butylamphetamine (IIa-d) and *N*-hydroxyfenfluramine (IIe) were examined by g.c. Provided the column fitted snugly at the injection port, each gave a single peak, the retention time of which was approximately six to eight times that of the corresponding

N-alkylamphetamine (III). Under other circumstances, decomposition occurred. The two decomposition products derived from each of the compounds IIb-e were identified, by comparison of their mass spectra with those of authentic samples, as the corresponding amine(III) and the nitrone (VIII or IX). However, thermal decomposition of *N*-hydroxy-*N*-methylamphetamine (IIa) gave the amine IIIa and the oxime (Va).

When the major peak in each of the g.c./m.s.-treated compounds (IIa-IIe) was scanned, the mass spectra obtained were consistent with the hydroxylamine structures (II) and appreciably different from that of the corresponding amphetamines (III) (Fig. 2). None of the mass spectra of compounds of general structure (II) showed a molecular ion, but fragment ions were present which enabled these compounds to be identified. Of particular diagnostic value were the ions (ix) and (xi) (Table 2).

Hydroxylamines IIb-e gave spectra which contained an abundant m/e 60 ion (iii) ($MeCH{=}\overset{+}{N}HOH$) which was absent from *N*-hydroxy-*N*-methylamphetamine (IIa) as expected since this ion (iii) arose from ion (ix) by the well-known hydrogen rearrangement which occurs in secondary amines only when the *N*-alkyl group contains more than one C atom.

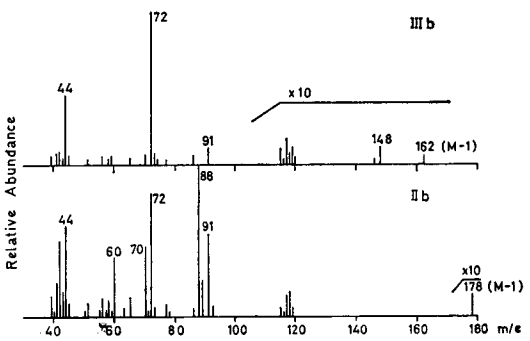
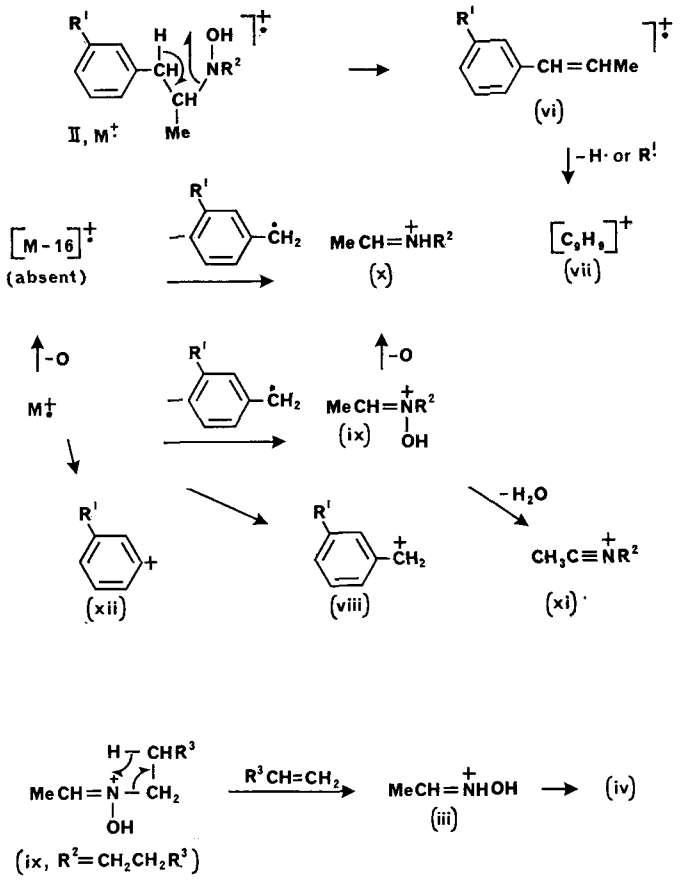


FIG. 2. Mass spectra of *N*-ethylamphetamine (IIIb) and *N*-ethyl-*N*-hydroxyamphetamine (IIb).

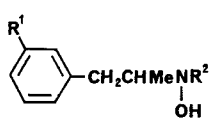
Mass spectra of secondary hydroxylamines (IIa-e) were also recorded by the direct inlet technique. The resulting spectra were similar to those obtained by the g.c./m.s. method, except that the diagnostic ion (ix) became appreciably more abundant. Accurate mass measurements were made of all the fragment ions derived from hydroxylamine IIe (Table 2) and were consistent with the structures depicted in Scheme 2.

A direct inlet mass spectrum of *N*-benzyl-*N*-hydroxyamphetamine (IV), a metabolite of benzylamphetamine (Beckett, Coutts & Gibson, unpublished), was also recorded. It contained only three ions of relative abundance greater than 10%, *viz*, m/e 150 (27%), 91 (100%) and 65 (11%), the formation of which can be readily interpreted (Scheme 3). The diagnostic ion, m/e 150, was absent from the spectrum of *N*-benzylamphetamine, being replaced by one of m/e 134 ($MeCH{=}\overset{+}{N}HCH_2Ph$). A g.c./m.s. could not be recorded in this instance because the retention time on a 7½% Carbowax column at 200° was in excess of 2 h and the compound underwent decomposition on an OV-17 (2%) column to the corresponding nitrone and amine.



Scheme 2.

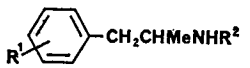
Table 2. *Secondary hydroxylamines—gas-chromatographic retention times, and relative abundances of diagnostic ions in their g.c.-mass spectra.*



II

	R^1	R^2	Rt	% relative abundances								
				(M-1)	(iii)	(vi)	(vii)	(viii)	(ix)	(x)	(xi)	(xii)
(a)	H	Me	3·3	—	—	8	14	40	91	100	42	12
(b)	H	Et	3·5	1·5	39	16	14	55	100	81	46	12
(c)	H	nPr	4·6	2·0	60	29	20	85	100	63	38	16
(d)	H	nBu	6·5	1·2	90	32	28	95	100	92	34	16
(e)	CF_3	Et	2·7	3·5	16	3	5	28	100	30	14	0·4

Table 2.—*continued*



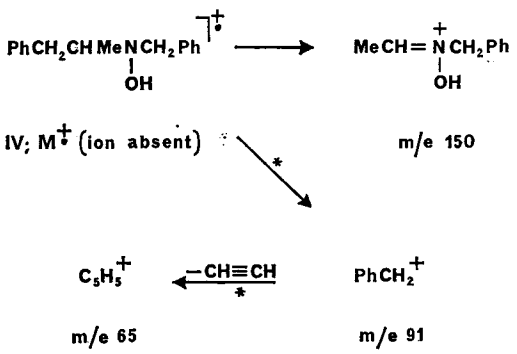
III

	R^1	R^2		R^1	R^2
(a)	H	Me	(d)	H	nBu
(b)	H	Et	(e)	*m*-CF_3	Et
(c)	H	nPr	(f)	*p*-OMe	H

Oximes

The mass spectra of six oximes (Va-f) derived from amphetamine structures were also examined by mass spectrometry. Compound Va (Hucker & others 1971) Vb and Vc (Beckett, Coutts & Midha, unpublished) and Vd (Beckett, & others, 1973a) are *in vitro* metabolic products of the corresponding amphetamine. Two additional oximes (VI and VII), which were not derived from amphetamines, were also examined to see whether they differed appreciably in mass spectral behaviour from compounds of general structure V. One of these reference compounds (VI) is an *in vitro* metabolic product of propylhexedrine (Beckett, Saunders & Midha, unpublished).

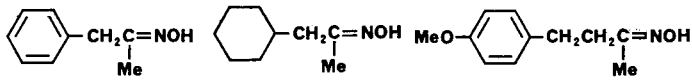
Direct inlet mass spectra of compounds Va-f gave spectra with abundant (17–100%) molecular ions, which assisted in the identification of these compounds. Fragmenta-



Scheme 3.

tion of these ions however, varied greatly according to the nature of the ring substitution pattern (Table 3). Fragmentations common to all were the formation of the ions M-17, M-32 and M-33. The last of these can be considered the most important diagnostic ion and its formation is concluded to be the result of the sequential loss of H_2O and $\cdot CH_3$ from the molecular ion. Appropriate metastable ions (m/e 182·5, 170·1) in support of this mechanism are found in the spectrum of Vd. The profound effect of the ring substituent on fragmentation is illustrated by the differences in relative abundance of the fragment ions m/e 58 and the absence of an [M-31] ion from all but one of the spectra. 1-Phenyl-2-propanone oxime (Va) gave an ion m/e 58 of appreciable abundance. This ion was a minor fragment in the spectra of compounds Vb, c, e and f, all of which possess electron-donating substituents. In contrast, the ion m/e 58

Table 3. *Oximes—gas-chromatographic retention times, and relative abundances of diagnostic ions in their direct inlet mass spectra.*

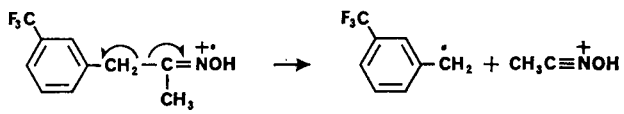


V VI VII

Cmpd	Substituents 2	3	4	5	6	Rt	Base peak m/e	% relative abundances M+	M-17	M-18	M-19	M-31	M-32	M-33	m/e58
Va	H	H	H	H	H	5·5	91	70	19	25	12	—	25	40	23
b	H	H	MeO	H	H	4·3[a]	121	27	13	—	—	—	9	13	2
c	MeO	H	H	H	H	3·1[a]	91	17	6	3	—	74	10	13	4
d	H	CF_3	H	H	H	4·4	58	45	12	42	19	—	6	32	100
e	H	O-CH_2-O		H	H	9·7[a]	135	95	5	1	2	—	4	15	2
f	H	MeO	MeO	MeO	H	23·1[a]	239	100	17	—	—	—	7	27	9
VI						2·1	73	0·5	—	—	—	—	—	—	3
VII						7·4[a]	121	9	3	—	—	—	—	—	—

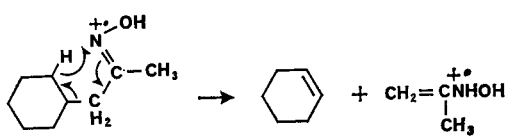
(a) = Oven temperature 196°

was the base peak in the spectrum of Vd which contained the strong electron-withdrawing trifluoromethyl ring substituent. Its presence obviously influences the ease of benzylic cleavage.



Vd

The mass spectra of the isomeric metabolites, Vb and c differ appreciably. The reason for this is that in the latter, a methoxyl radical can be expelled from the molecular ion as a result of an *ortho-effect*, thus producing a strong [M-31] i.e. m/e 148 fragment (Coutts & Malicky, 1973).

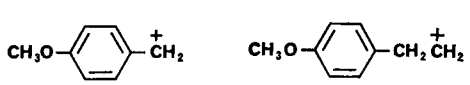


VI

In contrast with the behaviour of oximes of general structure V, the oximes VI and VII gave spectra with molecular ions of low abundance and lacking the diagnostic fragments discussed above. Only three prominent ions were observed in the spectrum of VI, viz. m/e 73 (100%), 55 (22%) and 41 (13%). The base peak was formed in a manner typical of aliphatic oximes (Goldsmith, Becher & others, 1966).

Two abundant ions were present in the spectrum of oxime VII, m/e 121 (100%) and 135 (20%), and are identified as (xiii) and (xiv) respectively.

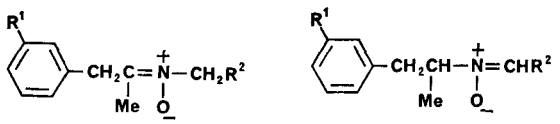
The eight oximes described above were stable when examined by combined g.c./m.s. and gave spectra which were almost superimposable on those obtained by the direct inlet method.



xiii xiv

This study on oximes illustrates that a variety of structural features can influence the pattern of fragmentation of these compounds and shows that the structures of oxime metabolites can be deduced from the fragmentation pattern displayed in their mass spectra when they are examined by g.c./m.s. techniques.

Table 4. *Nitrones—gas-chromatographic retention times, and relative abundances of diagnostic ions in their g.c.—mass spectra.*



VIII IX

				Ions, % relative abundances						
	R^1	R^2	Rt	M^+	$(M-17)^+$	(vi)	(vii)	(viii)	(xv)	(xvi)
(a)	CF_3	Me	5·5	1·1	0·9	89	61	100	52	2·1
(b)	H	Me	7·4	1·1	—	100	49	98	55	3·6
(c)	H	Et	8·4	0·5	0·5	100	48	82	31	3·2
(d)	H	nPr	11·5	0·5	2·6	100	34	86	28	2·8

Nitrones

The major *in vitro* metabolic product of fenfluramine (IIIe) was deduced to be *N*-ethyl-α-methyl-α-(*m*-trifluoromethylbenzyl)nitrone (VIIIa) by its g.c./m.s. behaviour and its facile reduction to, or preparation from *N*-hydroxyfenfluramine (IIe). A preliminary account of this research has been published (Beckett & others, 1973a). Three other metabolites of general structure II, i.e. compounds IIb, c and d, are readily oxidized to nitrones by shaking with yellow mercuric oxide and are reformed when the solution of the nitrone is reduced with lithium aluminium hydride. *N*-Hydroxy-*N*-methylamphetamine (IIa) behaved atypically and did not give a nitrone. The nitrones which were produced had longer g.c. retention times than the *N*-hydroxy-amphetamines from which they were derived (Table 4). Their method of preparation indicates that they possess structures VIII or IX. Each nitrone could be separated as an isolated peak by g.c. and when the peak was scanned in the mass spectrometer, each gave a spectrum which was useful for identification purposes (Fig. 3). All spectra contained a molecular ion of low abundance and a characteristic M-R^2 ion together with four additional abundant ions which are identified in Scheme 4. It is now recognized that this m.s. evidence is insufficient to define which structure of the two possibilities (VIII and IX) is the correct one since fragmentation mechanisms can be devised to produce all diagnostic fragment ions (Table 4) from either structure (Scheme 4). Attempts to prepare a pure sample of nitrone VIIIc for an (nmr) study to distinguish between the two possibilities have so far proved unsuccessful. The action of *m*-chloroperbenzoic acid on *N*-propylamphetamine produced a mixture of nitrone, oxime (Va) and other

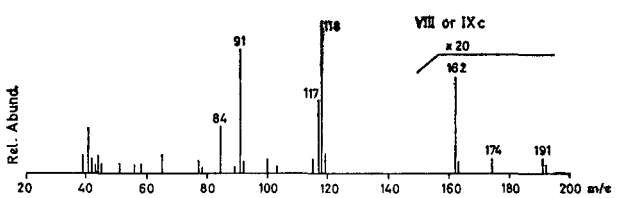
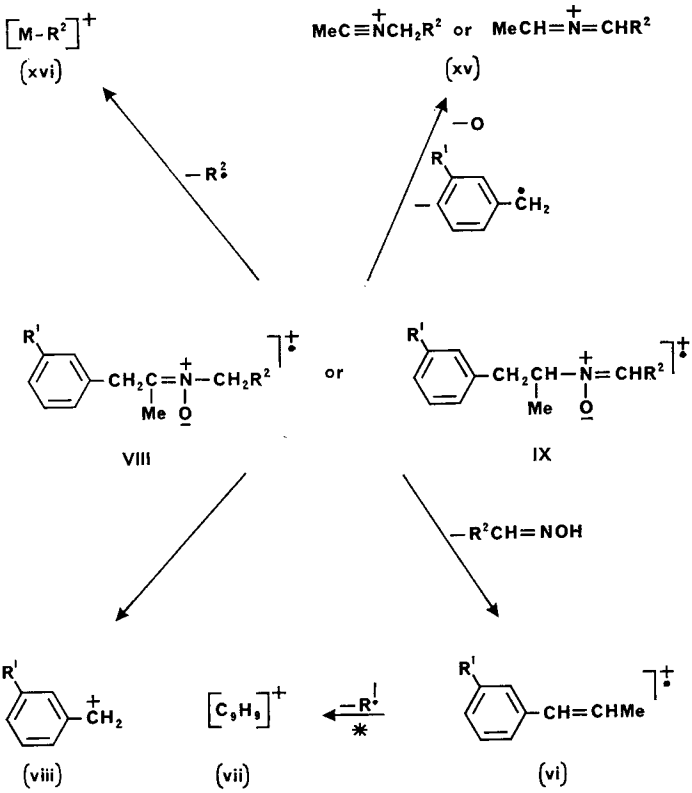


FIG. 3. Mass spectra of nitrone derived from *N*-hydroxy-*N*-propylamphetamine (i.e. compound VIII or IXc).



Scheme 4.

minor products which could be separated on a microscale by g.c. methods but not by chemical or physical means in quantities sufficient for nmr. The mass spectrum of this g.c.-separated nitrone, however, was identical to that of the major *in vitro* metabolite of *N*-propylamphetamine.

Acknowledgements

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