METABOLIC AND CHEMICAL STUDIES ON α,*N*-DIARYLNITRONES

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SUMMARY

The metabolism of a number of α,*N*-diarylnitrones has been studied *in vitro* using male hamster microsomes. All substrates produced benzaldehyde and an uncharacterised substance as metabolites. If the metabolic reaction was carried out in the light, or if the metabolic extracts were exposed to light, the chemical formation of two new compounds was observed. One compound had chromatographic and spectroscopic properties identical to the corresponding amide; the other compound had properties which suggested it was an oxaziridine.

The results are discussed in relation to the formation of amides as metabolites of *N*-benzyl anilines. It is concluded that nitrones are not on the above metabolic pathway, but that they may form amides by chemical rearrangement.

KEY WORDS

metabonates, diarylnitrones, oxaziridines, *N*-benzoylanilines, microsomes, *in vitro* metabolism, hamster

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INTRODUCTION

During previous studies on the metabolism of *N*-benzylanilines, the corresponding *N*-benzoyl compounds were recognised as minor metabolites /1-3/. This led us to investigate the mechanism of amide formation, during which we studied the metabolism of possible intermediates in the processes. It was found, in some cases, that *N*-benzylanilines gave rise to both the amide and the corresponding nitrone. This indicated that *N*-hydroxylation (Figure 1, route c) leading to [II] followed by dehydrogenation (route d) to the nitrone [III] and rearrangement to the amide [VI] via an oxaziridine [IV] may be a possible route (pathways c,d,e,f) to the formation of these carbonyl compounds as suggested by Gooderham and Gorrod /1/. The results from studies using enzyme activators, inducers and inhibitors also supported this concept /1/. In order to determine whether the mechanism proposed above is involved in the metabolic formation of amides, authentic α,*N*-diphenylnitrones, i.e., α,*N*-diphenylnitrone (DPN), α-phenyl-*N*-(4-chlorophenyl)nitrone (PCPN), α-phenyl-*N*-(4-methylphenyl)nitrone (PMPN) and α-phenyl-*N*-(2,4,6-trimethyl)-phenylnitrone (PTMPN), themselves being metabolic products of *N*-benzylaniline (NBA), *N*-benzyl-4-chloroaniline (NBCA), *N*-benzyl-4-methylaniline (NBMA) and *N*-benzyl-2,4,6-trimethylaniline (NBTMA), respectively, were used as substrates (Figure 2) in *in vitro* microsomal oxidation systems.

Although the chemistry of nitrones has been extensively investigated /4,5/, no report has been published regarding the metabolic fate of diaromatic nitrones. The chemical formation of the corresponding amides and oxaziridines by the action of light on nitrones has been a subject of a number of investigations. Splitter and Calvin /6/ have shown that diaromatic nitrones give unstable oxaziridine intermediates which react further to produce amides with protic solvents by C-*N* transoxygenation (Figure 1, pathways e and f). These authors postulated that oxaziridines were unstable transient intermediates.

This communication reports experiments designed to investigate the possibility that amides, reported as minor metabolites of secondary benzylic amines, may arise via metabolic rearrangement of nitrones as proposed by Gooderham and Gorrod /1/.

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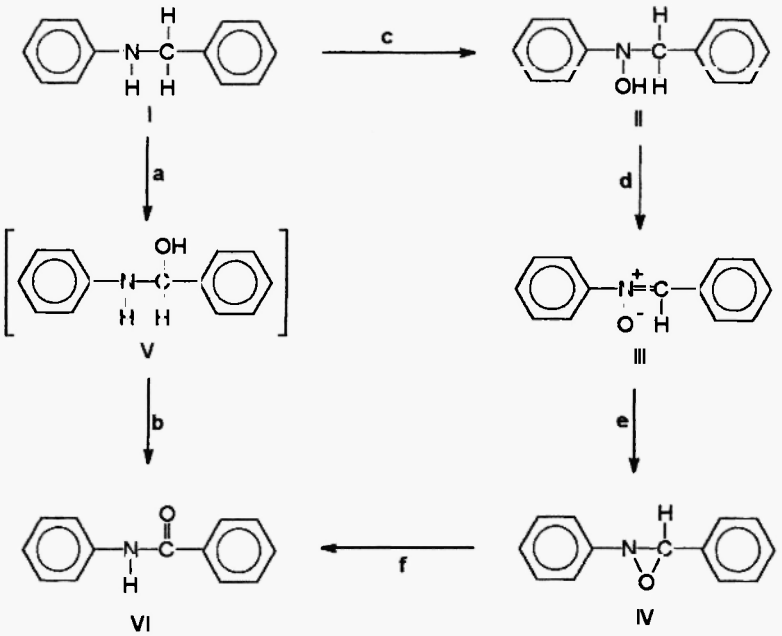
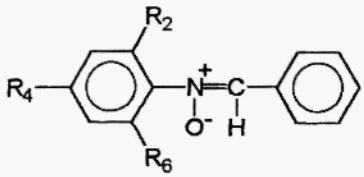


Fig. 1: Proposed mechanisms for the metabolic formation of amides from *N*-benzylanilines.



Abbreviation	R_2	R_4	R_6
DPN	-	-	-
PMPN	-	CH_3	-
PCPN	-	Cl	-
PTMPN	CH_3	CH_3	CH_3

Fig. 2: Structures of α,*N*-diphenylnitrones studied.

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MATERIALS AND METHODS

Materials

The nitrones used as substrates, i.e., DPN, PCPN, PMPN and PTMPN, were prepared by direct oxidation of the corresponding *N*-benzylanilines with metachlorperbenzoic acid /7,8/ and recrystallised from petroleum ether. The potential amide metabolites NBZA, NBZCA, NBZMA and NBZTMA were prepared by the Schotten-Baumann reaction using the corresponding acyl halide /9/ and recrystallised from ethyl alcohol. All the compounds prepared were characterised by UV, IR, NMR, mass spectrometry and elemental analysis. All primary anilines, corresponding aldehydes, organic solvents, and chromatographic materials were obtained from either Aldrich Chemical Company, or British Drug Houses, UK.

High performance liquid chromatography

The HPLC isocratic system used comprised an LCD Analytical constaMetric 3200 solvent delivery system, a Rheodyne syringe loading sample injector valve (model 7125) fitted with a 20 μl sample loop, a Milton Roy spectroMonitor 3100 variable wavelength UV detector, and a Milton Roy model 4000 computing integrator. The HPLC column was a Spherisorb μ-Bondapak CI8 5 μm (25 cm x 4.6 mm I.D.) purchased from Phase Separations Limited, Deeside, UK, in series with a guard column of pellicular ODS purchased from Whatman International Ltd., Maidstone, Kent. HPLC analyses were also carried out using the above system in conjunction with a high speed, multiple wavelength UV/VIS detector (Rapiscan SA6508, Severn Analytical, UK), scanning from 190 to 364 nm. The detector was coupled to a PC (Tandon AT) allowing data capture and manipulation via the Rapiscan programme version 2.0. The reaction products were eluted under isocratic conditions with a mobile phase of acetonitrile: 0.02M phosphate buffer, pH 7.0 (S1=30:70 v/v); (S2=40:60 v/v), at a flow rate of 2 ml/min. The metabolic products were initially detected by their absorbance at 254 nm and the retention times of compounds under theses conditions are given in Table 1. The Rapiscan UV detector connected to the standard HPLC system was used to obtain UV spectra of both authentic and metabolic or chemical

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products of nitrone transformation. The UV absorption maxima data for authentic compounds are presented in Table 1.

TABLE 1

HPLC retention times and absorption maxima for the separation and characterisation of related nitrones and some of their potential metabolites

Compound	Abbreviation	Retention time (min.) S1	S2	λ max. (nm)
Benzaldehyde	B	4.6	3.5	254
N-benzoylaniline	NBZA	10.4	ND	263
α,N-diphenylnitrone	DPN	7.9	ND	315
α,N-diphenyloxaziridine	DPO	14.6	ND	238
N-benzoyl-4-chloraniline	NBZCA	26.0	ND	265
α-phenyl-N-(4-chlorophenyl)nitrone	PCPN	15.5	ND	318
α-phenyl-N-(4-chlorophenyl)oxaziridine	PCPO	32.9	ND	238
N-benzoyl-4-methylaniline	NBZMA	20.2	ND	265
α-phenyl-N-(4-methylphenyl)nitrone	PMPN	16.7	ND	316
α-phenyl-N-(4-methylphenyl)oxazindine	PMPO	27.1	ND	240
N-benzoyl-2,4,6-trimethylaniline	NBZTMA	ND	8.0	210
α-phenyl-N-(2,4,6-trimethylphenyl)nitrone	PTMPN	ND	10.5	300

Incubation and extraction procedures

Glucose-6-phosphate dehydrogenase was purchased from the Boehringer Mannheim Corporation (London) Ltd. Nicotinamide adenine dinucleotide phosphate (NADP) was obtained from Sigma Ltd. Glucose-6-phosphate was purchased from British Drug House, Poole, Dorset (UK). All other chemicals were obtained as mentioned previously. Male hamsters were used as enzyme source. Hepatic microsomes were prepared at 0°C using the calcium chloride precipitation method /10/. Incubations were carried out in a shaking water bath at 37°C using a standard cofactor solution at pH 7.4. Cofactors consisting of NADP (2 μmol), glucose-6-phosphate (10 μmol), glucose-6-phosphate dehydrogenase (1 unit), $MgCl_2$ (20 μmol) prepared in phosphate buffer (2 ml, 0.2 M, pH 7.4) were preincubated for 5 minutes before the addition of microsomes and substrate (5 μmol).

The incubation time was 30 minutes after which the reaction was terminated and the reaction mixture extracted by the addition of

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dichloromethane (5 ml). The incubates were extracted with a further 5 ml of dichloromethane and the combined organic extracts were evaporated to dryness using a stream of N_2 at 25°C. Dichloromethane has been shown to be the solvent of choice for the extraction of nitrones, amines, amides, arylhydroxylamines and related compounds /3/. Because of the known chemical ability of nitrone and oxaziridine functional groups /11/, incubations were carried out in the dark and light was avoided during subsequent extraction and concentration procedures. These conditions were used to prevent the chemical conversion of any oxaziridine present to the isomeric amide during analysis. The dried organic residues were reconstituted in 200 µl of methanol for HPLC analysis.

In additional studies authentic nitrones or products from metabolic experiments were reconstituted in methanol and allowed to stand at room temperature (20°C) in artificial light for various periods prior to analysis. These reaction mixtures were also analysed by HPLC as described above.

RESULTS AND DISCUSSION

Analysis of extracts from the metabolism of the various nitrones failed to show the presence of the corresponding amide, if the analysis was carried out immediately after the termination of incubations carried out under dark conditions and all subsequent manipulations precluded light, although in all cases benzaldehyde was observed as a metabolite. However if the metabolic extracts from nitrone metabolism were stored at room temperature and exposed to light for more than six hours, chemical formation of two new compounds was observed, one having chromatographic properties identical to the corresponding amide and the other an unknown compound, with longer retention times than either amides or nitrones. These latter were thought to be oxaziridines. In addition from each substrate a further compound was observed running between benzaldehyde and the substrate (Figure 3b;5). Figure 3 shows the HPLC chromatogram obtained with authentic compounds (a) or following metabolism and exposure to light for 6 hours (b) using α-phenyl-4-methylphenylnitrone (PMPN) as a substrate. Similar results were obtained following incubations of other nitrones. If the analysis was carried out immediately following incubation, the compounds designated [3] and [4] were absent in all experiments. The minimum time required for detection of these two

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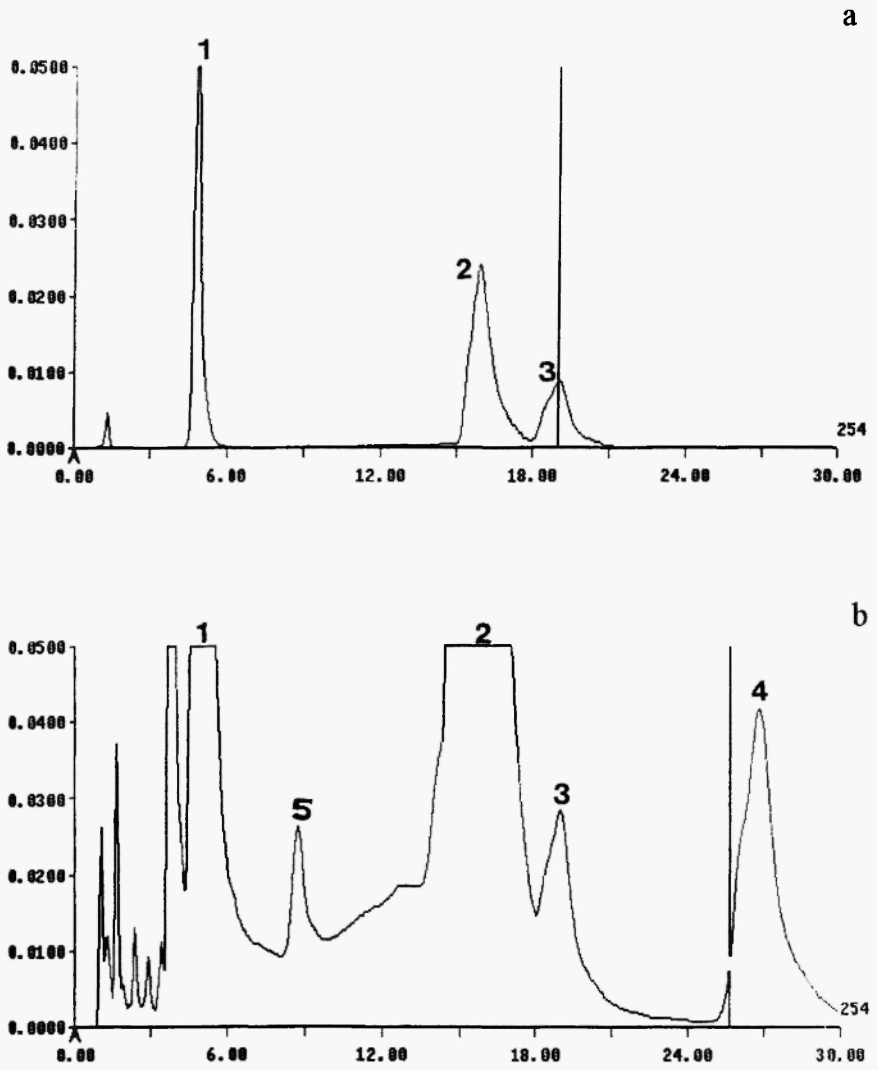

Fig. 3: a. HPLC separation of PMPN and its potential metabolites. **b**. Typical HPLC chromatogram obtained following extraction from male hamster microsomal incubation mixture with PMPN as substrate (after six hours irradiation) (1=B, 2=PMPN, 3=NBZMA, 4=PMPO). For abbreviations see Table 1; see text for incubation and analytical conditions.

products [3 & 4], thought to be corresponding amide and an unknown product, was six hours under our analytical conditions. No further change in the photochemical formation of amide and the other product

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was observed with further increases of time. The availability of a Rapiscan UV detector directly connected to the HPLC system enabled the UV spectra of these photochemical products to be recorded. In each case the compound corresponding to [3], i.e. running directly after the nitrone, had a UV spectrum identical to that of the corresponding authentic amide. Compound [4] (derived from PMPN) had a UV spectrum different from that of the parent nitrone (PMPN) and the corresponding amide (NBZMA), having a maximum absorption at 240 nm. Analogous observations were made with the other nitrones (Figure 4). It has previously been shown by Splitter and Calvin /6/ that DPN photo-rearranged to a compound with an absorption maximum at about 230 nm, which they proposed to be the corresponding oxaziridine. In our experiments (Figure 4a) a compound having an absorption maximum at this value was also detected when DPN was used as a substrate and the metabolic extracts exposed to light. This suggests that the "unknown" compounds formed by photo-rearrangement in our experiments were the corresponding oxaziridines.

Although no reference compounds were available for comparison with the unknown products, which we suggest are oxaziridines, their UV spectra were quite different from both the corresponding nitrone and amide and their absorption maxima were shifted to a shorter wavelength. The absorption spectra of these products were similar to those of oxaziridines in earlier studies /6/. The mass spectrum of the unknown compound from irradiated PMPN (details not given) also showed a molecular ion peak, suggesting an isomer of the nitrone, and consistent with the compound being an oxaziridine. In order to confirm the proposed oxaziridine structure, the synthesis of α-phenyl-*N*-(4-methylphenyl)oxaziridine (PMPO) was attempted by the reaction of metachlorperbenzoic acid with the corresponding imine /12,13/. The corresponding imine, *N*-benzylidene-p-toluidine (NBDMA) was prepared by reaction of benzaldehyde with toluidine /14/. Whilst the full details are not given it can be reported that evaporation of the reaction mixture under reduced pressure yielded multiple products as shown by TLC and HPLC. HPLC examination of the mixture showed the presence of the corresponding amide. Mass spectral analysis of the reaction mixture showed a molecular ion peak indicating the presence of an oxygenated product. However, no oxaziridine could be isolated. These results indicate that oxaziridines formed from the corresponding imines are probably not sufficiently stable to be isolated and are

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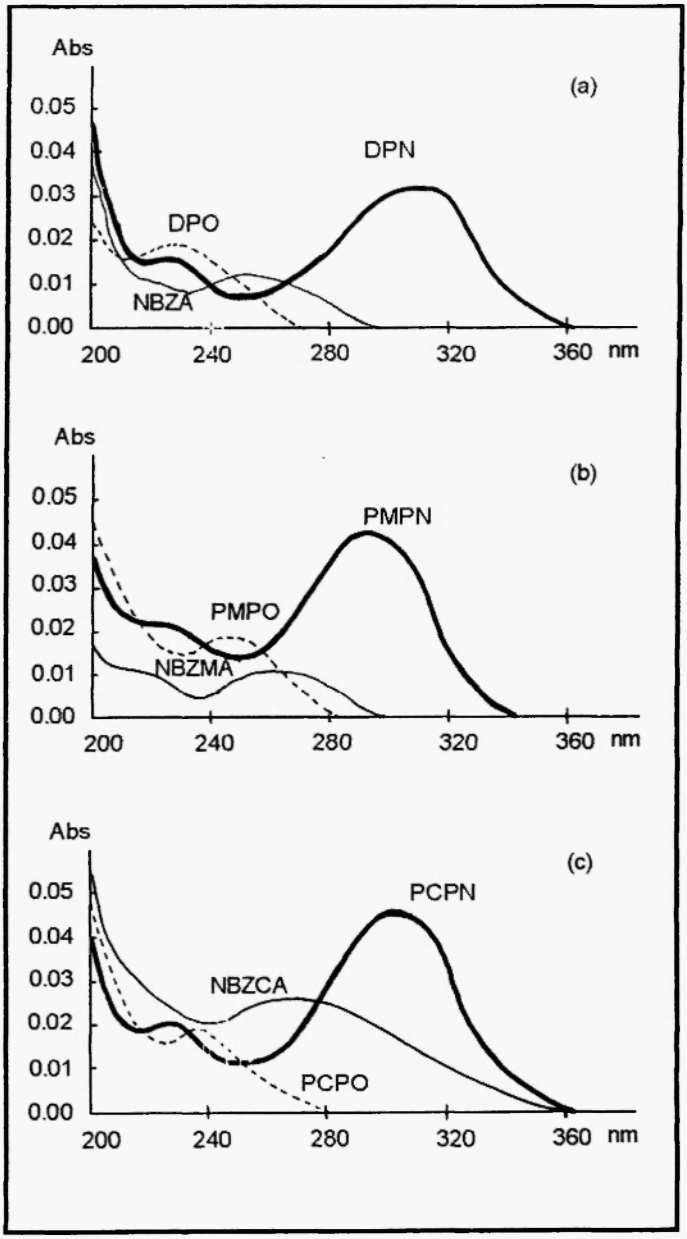

Fig. 4: A curve overlay from absorption spectra of the α,*N*-diphenylnitrones: (a) DPN, (b) PMPN, (c) PCPN, and their chemical rearrangement products following microsomal metabolism by hepatic male hamster microsomes after six hours irradiation.
▬ nitrones; — amides; ---- oxaziridines.

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spontaneously converted to the corresponding isomeric amides. This finding is in line with previous studies, with similar results /6/. The preparation of a wide variety of aromatic-aliphatic oxaziridines by oxidation of the appropriate imines with peracetic acid has been reported /15,16/. However, 2-phenyl or 2-benzyloxaziridines could not be prepared by this method /15/.

Following microsomal metabolism of PTMPN, no amide metabolite was detected. On irradiation of PTMPN (following metabolism or direct irradiation of a methanolic solution [2 mg/ml]), no compound having properties similar to an oxaziridine was observed although the corresponding amide was detected by HPLC. The chemical formation of the corresponding amide was further confirmed using a Rapiscan UV detector.

Regarding substance [5] (Figure 3): if the metabolism of nitrones occurred by simple hydrolysis, then these compounds would be expected to be the corresponding arylhydroxylamines. However, these compounds did not have chromatographic or spectroscopic properties corresponding to either arylhydroxylamines or the nitroso analogues. It may be that these compounds are imines produced chemically by condensation of benzaldehyde and aromatic amine as recently observed during the metabolism of certain *N*-benzylanilines /17/.

The present findings show that nitrones are not metabolic intermediates in the formation of amide metabolites from *N*-benzylanilines. These experiments indicate that the chemical generation of the amide may depend upon the rearrangement of a nitrone precursor and may involve an oxaziridine intermediate. This is one source of the amides observed by HPLC in microsomal extracts using secondary *N*-benzylanilines as substrates. The work presented indicates that amides detected as "metabolites" of *N*-benzyl aromatic amines may in part be formed by chemical breakdown of the metabolically formed nitrone. This is a further example of "metabonate" formation. A similar mechanism for the formation of the corresponding formamide metabolite from a tertiary arylaliphatic amine, methadone, has been proposed /12/. This present study clearly shows that amides can be produced from aromatic nitrones during work-up procedures, probably via unstable diaromatic-oxaziridine intermediates.

The metabolic experiments carried out in this work do not support the involvement of a metabolic *N*-oxidative pathway in the formation of these carbonyl functions. However, it should also be considered that the polarity of preformed nitrones may prevent their easy transport

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across lipoprotein membranes and hence prevent their further metabolism, in contrast to nitrones formed on the luminal side of the membrane.

Despite the failure of the above to support the role of nitrones as intermediates in amide formation from secondary anilines, it is clear that an alternative route to amides has to be functioning in order to account for the early observation of Gooderham and Gorrod /1/ who clearly indicated the enzymic nature of the process by the use of enzyme inducers and inhibitors. Additionally it should be remembered that amide formation from *N*-benzyl anilines does not require either irradiation or prolonged storage prior to analysis. Recently, a novel observation has been made indicating the enzymic formation of amides and/or nitrones as a minor metabolic pathway following *N*-oxygenation of the corresponding imines /3,18/ (Figure 5). This process may also involve oxaziridines as intermediates. This possible metabolic route to amides from secondary anilines via these novel intermediates would

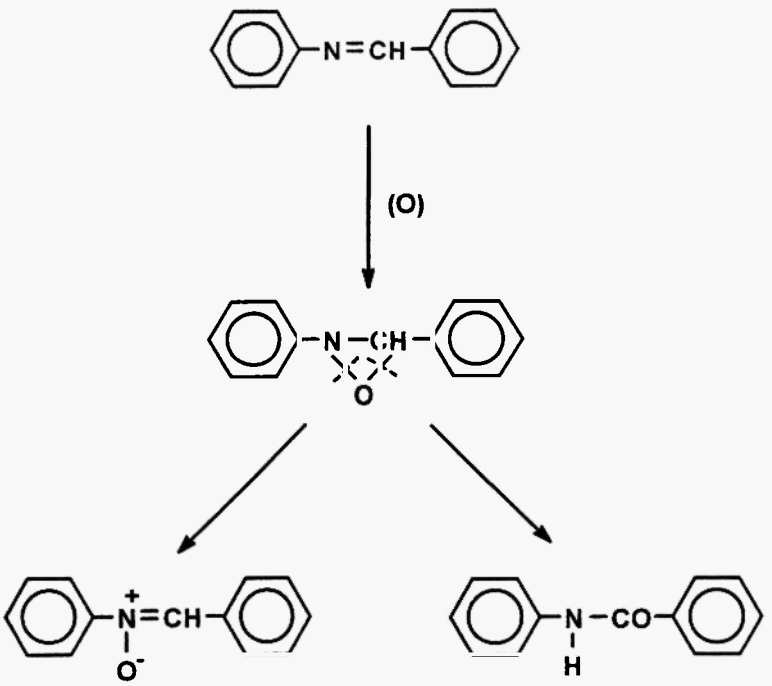

Fig. 5: The possible metabolic route to amides via *N*-oxygenation of imines.

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require that imines are formed as metabolites. Preliminary experiments /17/ indicate that this pathway does occur by a chemical mechanism as discussed earlier. The formation and metabolism of imines from *N*-benzylanilines and the role of the stability of the corresponding oxaziridines in amide formation are currently under investigation in our laboratories.

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REFERENCES

1. Gooderham NJ, Gorrod JW. Microsomal formation of amides from secondary aromatic amines. In: Gorrod J W, et al., Development of Drugs and Modern Medicines. Chichester: Ellis Horwood Ltd., 1986; 318-323.
2. Ulgen M, Gorrod JW. Microsomal formation of N-benzyl-4-hydroxymethylaniline from N-benzyl-4-methylaniline. J Pharm Pharmacol 1992; 44: 865-866.
3. Ulgen M. Metabolic and chemical determinants of amide formation from benzylic amines. Ph.D. thesis, Kings College, University of London; 1992.
4. Hamer J, Macaluso A. Nitrones. Chem Rev 1964; 64: 473-495.
5. Delpierre GR, Lamchen M. Nitrones. Quarterly Reviews 1964; 329-348.
6. Splitter JS, Calvin M. The irradiation products of several nitrones. J Org Chem 1965; 30: 3427-3436.
7. Gorrod JW, Gooderham NJ. Improved preparation of α,N-diphenylnitrones and N-benzyl-N-phenylhydroxylamines by direct oxidation of secondary anilines. Arch Pharm 1986; 319: 261-265.
8. Ulgen M, Gorrod JW. Synthesis, characterisation and chromatographic studies on some new substituted α,N-diarylnitrones. J Pharm Univ Mar 1992; 8: 135-143.
9. Vogel AI. Practical Organic Chemistry. 3rd ed. London: Longmans, 1961.
10. Schenkman JB, Cinti DL. Preparation of microsomes with calcium. Methods in Enzymology 1978; 52: 83-89.
11. Spence GG, Taylor EC, Buchardt O. The photochemical reactions of azoxy compounds, nitrones and aromatic amine N-oxides. Chem Rev 1970; 70: 231-239.
12. Abott FS, Slatter JG, Burton R, Kang GI. Methadone metabolism in the rat in vivo: Identification of a novel formamide metabolite. Xenobiotica 1985; 15: 129-140.

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13. Boyd DR, Neill DC, Watson CG. Dynamic stereochemistry of imines and derivatives. Part VI. Stereochemistry of the peroxyacid-imine route to oxaziridines. J Chem Soc Perkin Trans II 1975; 1813-1821.
14. Law HD. Electrolytic reduction. Part V. Benzylidene bases. J Chem Soc 1912; 101: 154-166.
15. Emmons WD. The synthesis of oxaziranes. J Am Chem Soc 1956; 78: 6208-6209.
16. Emmons WD. The preparation and properties of oxaziranes. J Am Chem Soc 1957; 79: 5739-5774.
17. Low CM, Ulgen M, Gorrod JW. The recognition of a diarylimine as a metabonate produced during incubation of N-benzyl-4-chloroaniline with hepatic microsomal preparations. J Pharm Pharmacol 1994; 46: in press.
18. Gorrod JW, Ulgen M. Metabolic N-oxygenation of diarylimines: A new metabolic reaction of potential toxicological significance. Drug Metab Rev 1993; 26: 241-252.

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