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In vitro hepatic microsomal metabolism of *N*-benzyl-*N*-methylaniline

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Abstract

In the present study, the in vitro microsomal metabolism of a tertiary aniline, *N*-benzyl-*N*-methylaniline (NBNMA) was studied to determine whether this compound produces an amide derivative (benzoyl) together with N-dealkylation and C- and N-oxidation products as metabolites. The preparations of the corresponding potential metabolites were undertaken and were separated using TLC and HPLC. Incubations were performed using rat microsomal preparations fortified with NADPH. The substrate and its potential metabolites were extracted into dichloromethane in the presence of NaCl and examined by TLC and HPLC–UV. The results indicated that NBNMA did not produce the corresponding amide (benzoyl derivative) or N-oxide metabolite but was dealkylated to the corresponding secondary amine. Two *p*-hydroxylated phenolic metabolites were also observed. These findings support the concept that nitrones are essential intermediate metabolites for the formation of amides from secondary aromatic amines (chemical rearrangement to amide via an oxaziridine intermediate). The carbinolamine produced from NBNMA does not seem stable enough to allow further oxidation to the amide and therefore this intermediate is broken down to the dealkylation products. N-Dealkylations and *p*-hydroxylations are major metabolic reactions following in vitro hepatic microsomal metabolism of the benzylic tertiary aniline, NBNMA.

Keywords: Microsomes; Metabolism; Tertiary anilines; Amide formation

1. Introduction

Studies on the in vitro metabolism of substituted *N*-benzylanilines by hepatic microsomal preparations from various species have previously been performed to establish the formation of the corresponding amides [1–5]. Two mechanisms have been proposed for the metabolic formation of amides [5]. One mechanism suggests that initial oxygen attack results in hydroxylation of the benzylic carbon atom to form a carbinolamine intermediate, which is further oxidised to give the amide (Fig. 1, R = H). If amide formation utilises this route, then metabolism of *N*-benzyl-*N*-methylaniline (NBNMA) may give a N-benzoyl metabolite by further oxidation of the carbinolamine (Fig. 1, R = CH_3).

Another route proposed for the formation of an amide from a secondary aniline requires an initial oxygenation step on the constituent nitrogen giving an N-hydroxy compound which could be further oxidised to a nitrone, followed by conversion of the nitrone to the corresponding amide via an oxaziridine intermediate (Fig. 2, R = H).

Previous studies on the in vitro hepatic metabolism of *N*-benzylaniline (NBA) and *N*-benzyl-4-methylaniline (NBPT) showed the formation of the corresponding amide and nitrone [1–6] in a number of species. Therefore, an in vitro metabolic study on NBNMA (Fig. 3), the N-methyl derivative of NBA, was carried out. NBA itself was previously shown to be one of the best substrates for amide formation [1–4]. The aim was to establish whether the corresponding amide (the N-benzoyl derivative) is produced. This study with NBNMA was planned to inhibit initial nitrone formation by introducing the methyl moiety into the substrate molecule which would prevent oxygenation of the constituent nitrogen to hydroxylamine and hence nitrone. If the corresponding amide is detected, this

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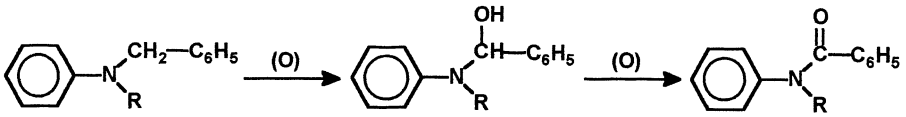


Fig. 1. Possible formation of amides from benzylic secondary (R = H) andtertiary (R = CH_3) amines via a carbinolamine intermediate.

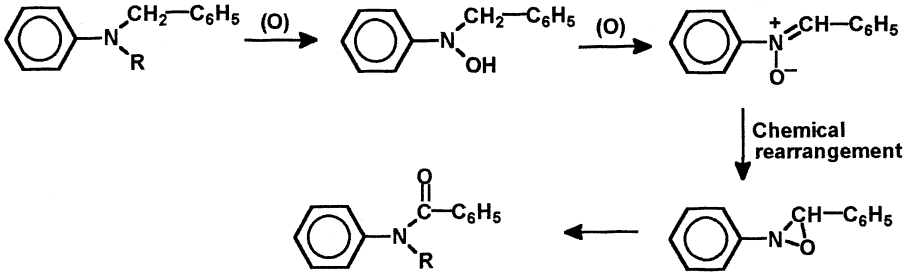


Fig. 2. Possible formation of amides from benzylic secondary (R = H) amines via nitrones and oxaziridines (if R = CH_3, this reaction is not possible).

would mean that the N-oxidative route proposed above is probably not involved in the formation of amides and benzylic carbon oxidation may be involved (Fig. 1, if R = CH_3). In contrast, a negative result for amide formation from NBNMA will support the concept that metabolic amide formation requires initial N-oxidation to nitrone. From our previous experiments on the metabolism of other benzylic anilines, it is predicted that a number of other metabolic products, i.e. the phenolic metabolites, debenzylated amines and the corresponding aldehydes, would be produced from this substrate. The results of the above experiments are the subject of this communication.

2. Chemistry

The metabolic study on NBNMA required the synthesis of three potential metabolites, namely, *N*-benzoyl-*N*-methylaniline (NBZNMA), *N*-benzyl-*N*-methylaniline-*N*-oxide (NBNMAO) and *N*-benzyl-*N*-methyl-4-hydroxyaniline (NBNM4HA). NBZNMA was prepared by the reaction of NMA and benzoyl chloride in aqueous alkali [7,8]. Synthesis of the corresponding N-oxide (NBNMAO) was achieved by the action of H_2O_2 on NBNMA in acetic acid [9–11]. The 4-hydroxylated derivative of NBNMA (NBNM4HA) was obtained by direct benzylation of 4-(methylamino)phenol in acetone [12–14]. Alkylation of the phenol function to yield an ether was avoided by the use of $NaHCO_3$ instead of strong alkali.

NBZNMA [8] and NBNMAO·HCl [10,11] had identical melting points to those reported previously. The structure of NBNMAO was further confirmed by the use of EI-MS which displayed the correct M^+ peak (m/z 213). In addition, the expected fragmentation pathway [15], such as the loss of oxygen (m/z 197), formaldehyde (m/z 183), benzaldehyde (m/z 107), $CH_3^\bullet$ (m/z 198) and Ar–$CH_2^\bullet$ (m/z 106) from M^+ and the subsequent loss of $CH_3^\bullet$ (m/z 182) or Ar–$CH_2^\bullet$ (m/z 106) from the parent amine (m/z 197), was observed.

The structure of NBNM4HA was determined on the basis of ^{1}H NMR and EI-MS data besides elemental analysis. Chemical shifts observed in the ^{1}H NMR spectrum were consistent with the structure (see text). The fragmentation pathway for this compound was similar to those reported for EI-MS of *N*-benzyl-*N*-methyl-4-aminophenol [16] displaying the fragment ions arising from the loss of $CH_3^\bullet$ (m/z 198), $C_6H_5^\bullet$ (m/z 136) and Ar–$CH_2^\bullet$ (m/z 122).

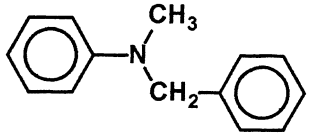
3. Chemical experimental section

N-Methylaniline (NMA), *N*-methyl-*N*-benzylaniline (NBNMA), benzyl and benzoyl chlorides were purchased from Aldrich. 4-(Methylamino)phenol sulphate was purchased from Fisons, UK. All solvents, glacial acetic acid and hydrogen peroxide were obtained from Merck.

3.1. Preparation of potential metabolites of NBNMA

3.1.1. N-Benzoyl-N-methylaniline (NBZNMA) [8]

N-Benzoyl-*N*-methylaniline was prepared by the benzoylation of *N*-methylaniline (1.07 g, 0.01 mol) in the



NBNMA

Fig. 3. Structure of *N*-benzylic tertiary aniline, NBNMA.

Table 1
Chromatographic properties of NBNMA and its potential metabolites [a]

Compound	Abbreviation	TLC ($R_f \times 100$)	HPLC, R_t (min)
N-Benzyl-*N*-methylaniline	NBNMA	74	19.67
N-Benzoyl-*N*-methylaniline	NBZNMA	49	4.84
N-Benzyl-*N*-methylaniline-*N*-oxide	NBNMAO	0	16.59
N-Methylaniline	NMA	61	3.57
N-Benzylaniline	NBA	68	10.29
Benzaldehyde	B		2.97
N-Benzyl-4-hydroxyaniline	NB4HA	33	4.47
N-Benzyl-*N*-methyl-4-hydroxyaniline	NBNM4HA	44	5.88

[a] HPLC solvent system: acetonitrile–0.02 M phosphate buffer, 40:60, v/v (pH 7) (flow rate: 2 ml/min; λ_{max}: 254 nm). TLC solvent system: petroleum ether–acetone (70:30, v/v) (b.p. of the petroleum ether is 40–60°C).

presence of NaOH solution (10 ml, 2N). The resulting mixture was kept overnight and then extracted with diethyl ether (3×20 ml). The ethereal solution was subsequently washed with HCl solution (5%, 3×20 ml) and distilled water in order to eliminate any unreacted *N*-methylaniline. Evaporation of the solvent afforded the amide (yield 62%). M.p. 64°C [8]. UV (ethanol), λ_{max} (nm): 276.0

3.1.2. *N*-Benzyl-*N*-methylaniline-*N*-oxide (NBNMAO) [10,11]

N-Benzyl-*N*-methylaniline (1.97 g, 0.01 mol) was dissolved in acetic acid (10 ml). To this stirred solution, portions of H_2O_2 (35%, 1.7 ml, 0.02 mol) were added dropwise. The mixture was kept for 3 days without stirring. The reaction mixture was cooled to -10°C (in ice–salt mixture) and made alkaline by adding chilled NaOH (20%) dropwise. The separated brownish oil was then extracted with diethyl ether in order to eliminate any unreacted amine. The aqueous phase was saturated with NaCl and extracted with DCM (3×20 ml). Evaporation of the solvent gave crude N-oxide. Recrystallisation from diethyl ether–methanol mixture afforded NBNMAO in a pure state (yield 45%), m.p. 73–77°C. After passing dry hydrogen chloride through a solution of the N-oxide (in chloroform) the HCl salt formed, m.p 128°C, as previously reported [10,11]. UV (ethanol), λ_{max} (nm): 252.4, 269.2 (shoulder). EI-MS, m/z (% relative intensity): 213 (M^+, 100), 197 (28), 182 (4), 122 (52), 106 (16), 91 (40), 77 (16), 65 (6), 51 (5).

3.1.3. *N*-Benzyl-*N*-methyl-4-hydroxyaniline (NBNM4HA)

4-Methylaminophenol sulphate was treated with $NaHCO_3$ solution (5%), followed by extraction with diethyl ether. The solvent was then evaporated and the remaining solid, 4-methylaminophenol (0.62 g, 0.005 mol) was dissolved in acetone (30 ml) and $NaHCO_3$ (0.5 g, 0.006 mol) was added. This mixture was refluxed and benzyl chloride (0.005 mol, 0.63 g) was dropped through the condenser. The reaction was monitored by TLC and stopped when the level of a new substance formed was maximal. Any remaining acetone was evaporated and the solid remaining was dissolved in diethyl ether, dried over anhydrous sodium sulphate and dry hydrogen chloride was passed through the solution. The precipitated hydrochloride salt was washed with diethyl ether and recrystallised from diethyl ether (yield 20%). M.p. 173–175°C. UV (ethanol), λ_{max} (nm): 270.0, 315.5. EI-MS, m/z (% relative intensity): 214 (23), 213 (M^+, 100), 196 (1.8), 161 (2.6), 136 (13), 122 (23), 91 (11), 65 (2) [16].

4. Analytical separation section

N-Benzylaniline (NBA) was obtained from British Drug Houses (BDH), Poole, UK. 4-(Benzylamino)phenol (NB4HA) was purchased from Eastman, Rochester, NY, USA. *N*-Benzoyl-*N*-methylaniline (NBZNMA), *N*-benzyl-*N*-methyl-4-aminophenol (NBNM4HA) and *N*-benzyl-*N*-methylaniline-*N*-oxide (NBNMAO) were synthesised as reported in the text. The separation techniques used were based on TLC and an isocratic HPLC system. TLC was carried out using precoated silica gel GF254 0.25 mm on glass (Merck) using petroleum ether–acetone (70:30, v/v) as the solvent system. The plates, after development, were examined under UV light (254 nm) and then sprayed with diazotised sulphanilic acid reagent (specific for phenolic compounds), followed by sodium carbonate. Metabolic standards were eluted with a mobile phase composition of acetonitrile–0.2 M phosphate buffer (40:60, v/v) (final pH 7), at a flow rate of 2 ml/min. The metabolic products were detected by their absorbance at 254 nm. Table 1 shows TLC R_f and HPLC R_t values of the substrate and its potential metabolites.

All chromatographic solvents were obtained from Merck. The HPLC column (Spherisorb C18 5 μm, 25 cm length × 4.6 mm i.d.) was purchased from Phase Separations Ltd., Deeside, UK. The guard column packing material (Whatman Pellicular ODS) was pur-

chased from Whatman International Ltd., Maidstone, UK. The HPLC chromatograph consisted of an isocratic system comprising one 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 integrator. A rapiscan SA6508 UV detector was connected to the HPLC to directly obtain UV spectra of eluting metabolites from NBNMA.

5. Biological section

Glucose-6-phosphate dehydrogenase was purchased from the Boehringer Mannheim Corporation (London) Ltd. Nicotinamide adenine dinucleotide phosphate mono sodium salt (NADP) and glucose-6-phosphate disodium salt were obtained from Sigma. Hepatic washed rat microsomal preparations were prepared at 0°C using the calcium chloride precipitation method [17]. Incubations were carried out in a shaking water bath at 37°C for 30 min using a standard co-factor solution at pH 7.4. Co-factor generating solutions 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 pre-incubated for 5 min before addition of microsomes (1 ml), equivalent to 0.5 g original liver and substrate (2 μmol in 50 μl methanol per flask). Metabolic reactions were stopped by extraction with dichloromethane (DCM) (2 × 5 ml) after adding NaCl (0.8 g). The DCM extracts were evaporated to dryness using a stream of N_2 at 20°C. Dry

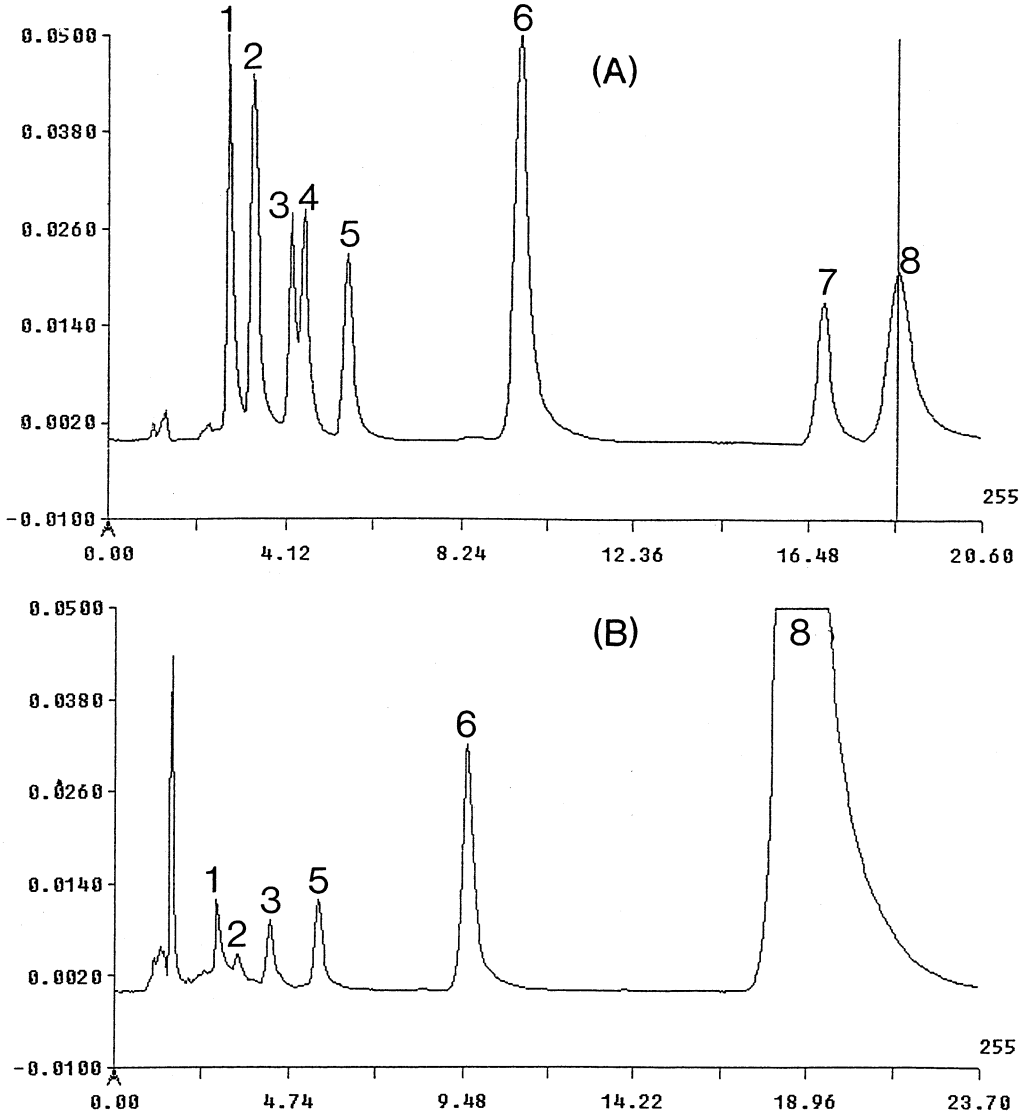


Fig. 4. HPLC chromatogram obtained (A) from standards, (B) following extraction from male rat microsomal incubation mixture with NBNMA* as substrate (1 = B, 2 = NMA, 3 = NB4HA, 4 = NBZNMA, 5 = NBNM4HA, 6 = NBA, 7 = NMNBAO, 8 = NBNMA (substrate)). * See Table 1 for abbreviations.

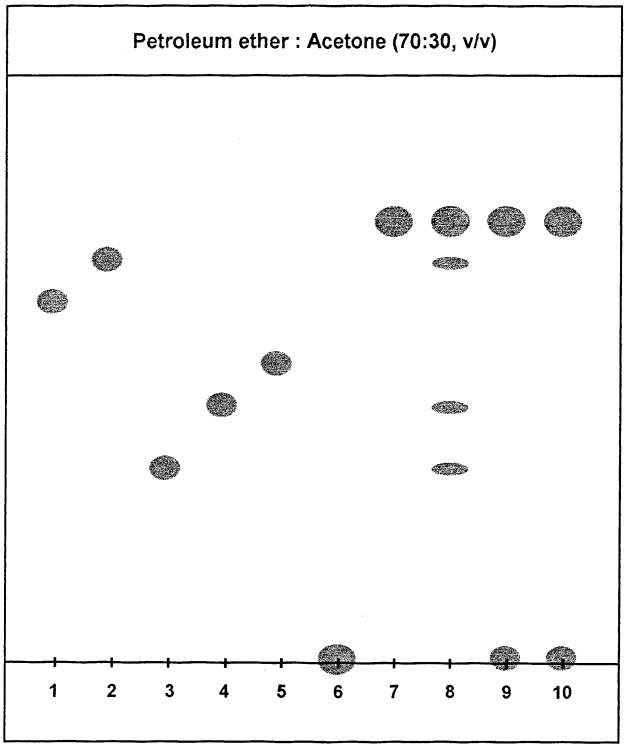


Fig. 5. TLC chromatogram obtained following extraction from male rat microsomal incubation mixture with NBNMA* as substrate. 1, NMA; 2, NBA; 3, NB4HA; 4, NBNM4HA; 5, NBZNMA; 6, NBNMAO; 7, NBNMA; 8, test; 9, control (denatured microsomes); 10, control (without co-factors). * See Table 1 for abbreviations.

organic residues were reconstituted in 200 μl of methanol for HPLC and 100 μl of methanol for TLC analysis.

6. Results and discussion

Examination of extracts obtained following the in vitro metabolism of NBNMA failed to show the formation of the N-benzoyl derivative as a metabolite. However, benzaldehyde and both secondary amines, i.e. NMA and NBA, were observed by HPLC (Fig. 4) and TLC (Fig. 5). The formation of the N-oxide could not be demonstrated. The two phenolic metabolites, i.e. NBNM4HA and NB4HA, were also detected, having identical HPLC and TLC chromatographic properties to those of authentic standards (Figs. 4 and 5). These metabolites also gave a positive response to diazotised sulphanilic acid reagent (with an orange colour) which supports our proposal. Further confirmation of the formation of all metabolites from NBNMA was achieved using a rapiscan UV detector (Fig. 6). From N-dealkylated metabolites, only NBA was detected by TLC (Fig. 5). Fig. 7 shows established metabolic pathways for NBNMA.

This present work, using the benzylic tertiary amine NBNMA as a substrate, clearly supports the proposal that the formation of amides requires initial nitrone

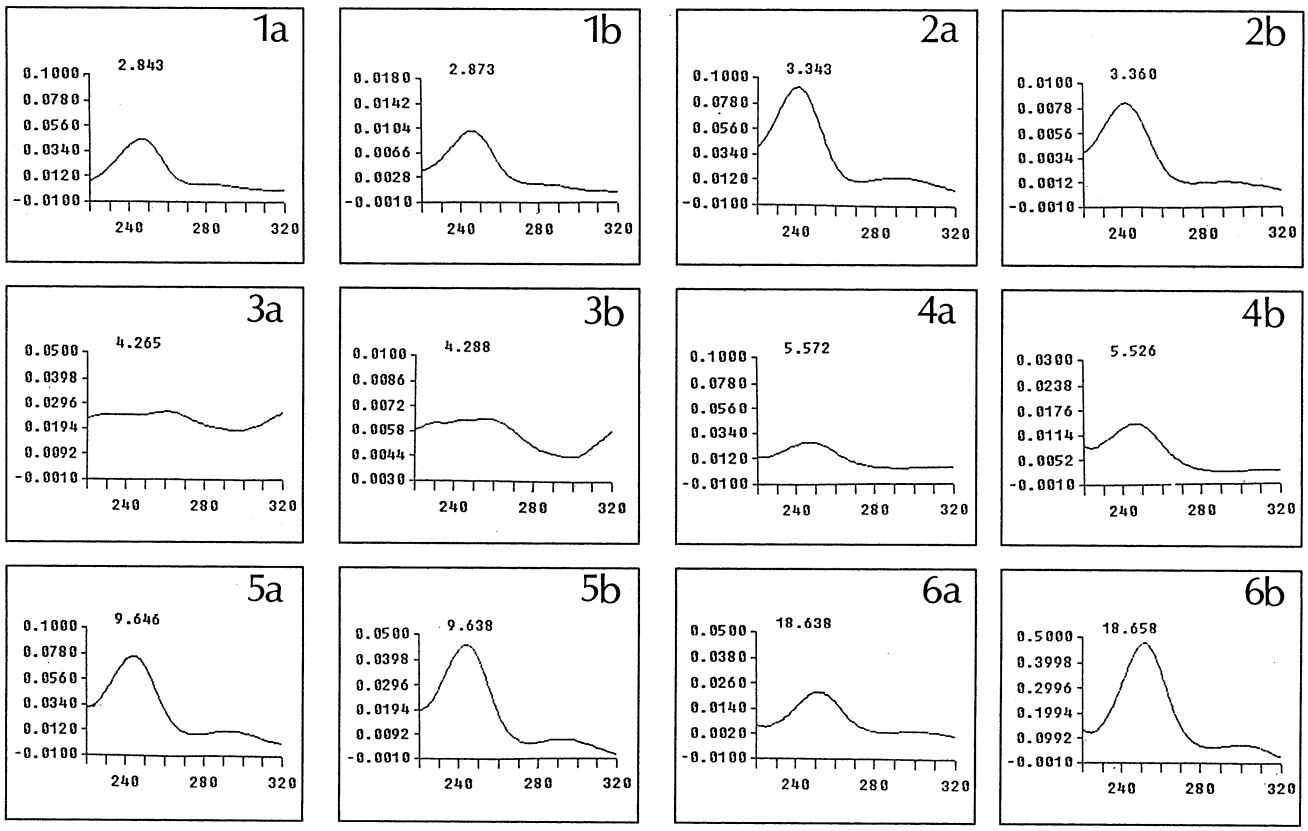


Fig. 6. UV spectra of NBNMA* metabolites. Authentics: 1a, B; 2a, NMA; 3a, NB4HA; 4a, NBNM4HA; 5a, NBA; 6a, NBNMA. Metabolites: 1b, B; 2b, NMA; 3b, NB4HA; 4b, NBNM4HA; 5b, NBA; 6b, NBNMA. * See Table 1 for abbreviations.

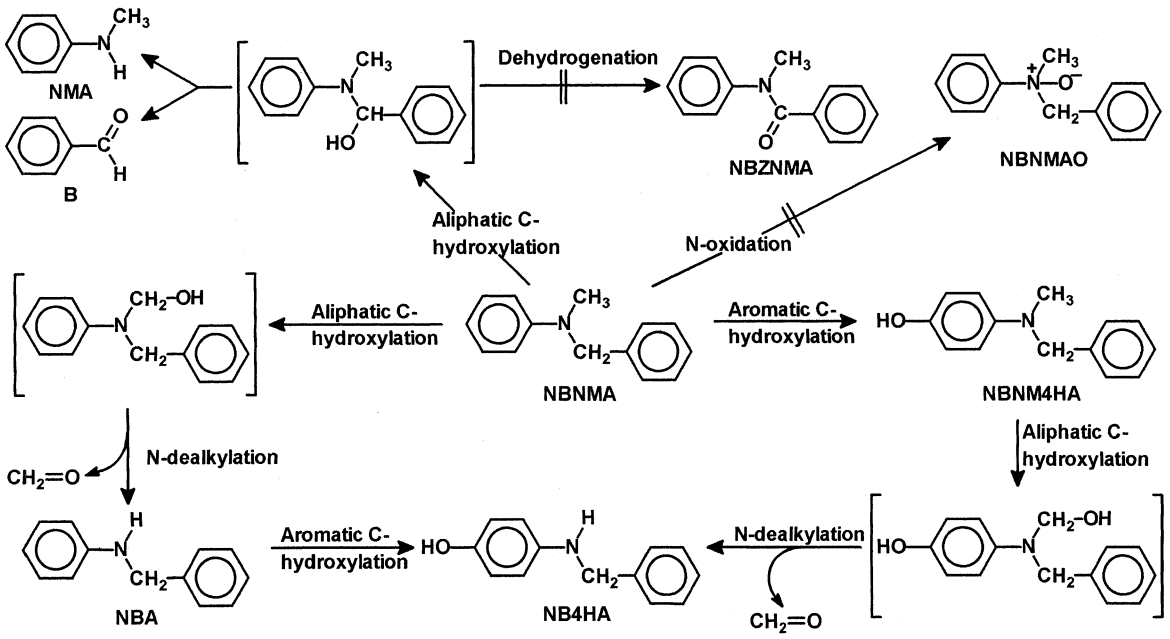


Fig. 7. Possible metabolic intermediates involved in the biotransformation of NBNMA.

formation which can only be formed from secondary amines (Fig. 2), as it did not indicate the formation of the corresponding amide. The methyl moiety introduced into the substrate prevented nitrone formation which supports our previous proposal that α-C benzylic oxidation leads to debenzylation, whereas nitrone formation leads to amide formation [4–6,18]. It has previously been shown that nitrones can chemically rearrange to oxaziridines that yield amides following ring cleavage [18].

The two phenolic metabolites observed during the metabolism of NBNMA are the first examples from a tertiary benzylic amine substrate. However, secondary benzylic anilines are known to form *p*-hydroxylated metabolites in vitro as a major metabolic pathway [2,6]. In a previous study, *N*,*N*-dialkyl- and *N*-alkyl-4-aminophenols derived from *N*-ethyl-*N*-methylaniline have also been detected from incubation mixtures of rabbit hepatic microsomes [19].

In the present study, the N-oxidation of NBNMA to the corresponding N-oxide (NBNMAO) was prevented by electronic and/or steric influences. In addition, failure to detect this N-oxide metabolite in our experimental conditions may be due to low extractability of this metabolite from the aqueous phase or it may have been formed in a very low amount. Alternatively, as benzylic N-oxides often undergo the 'Meishenheimer rearrangement' to produce the corresponding alkoxyamines [8,9,14], this well-known reaction may account for the failure to detect the N-oxide in the present study. Experiments on the metabolism of NBNMAO are in progress to examine these proposals further.

Acknowledgements

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