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IN VITRO METABOLIC FATE OF N-SUBSTITUTED DIBENZYLAMINES : INVESTIGATION OF BIOLOGICAL OXIDATION SITE ON NITROGEN COMPOUNDS WITH DIFFERENT pK_a

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SUMMARY

The *in vitro* microsomal metabolism of four model tertiary amines ie. N-ethyldibenzylamine (EDBA), tribenzylamine (TBA), N,N-dibenzylaniline (DBAN) and N,N-dibenzylbenzamide (BZDBA) was studied to establish whether the corresponding N- and C-oxidation products such as the corresponding amides, dealkylation products, N-oxides and phenols formed. These compounds were selected as substrates to establish the effect of the varying pK_a and logP characteristics on their *in vitro* metabolism. The substrates and their potential metabolites were synthesized and then separated using HPLC. Incubations were performed using rat microsomal preparations fortified with NADPH. The substrates and their potential metabolites were extracted into dichloromethane and examined by HPLC. Results from HPLC analysis indicated that all these substrates failed to produce the corresponding amides as metabolites. However, the dealkylation metabolites were observed with all the substrates used as major metabolites. The substrates EDBA and TBA produced the corresponding N-oxide metabolites. Three phenolic metabolites of DBAN were also demonstrated. These findings support the concept that nitrones are essential metabolic intermediates for the formation of amides and this reaction requires a secondary amine as substrate. In addition, the metabolic N-oxidation of these substrates depends on the pK_a of the constituent nitrogen of the molecule.

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ÖZET

N-Etildibenzilamin (EDBA), tribenzilamin (TBA), N,N-dibenzilanilin (DBAN) ve N,N-dibenzilbenzamid (BZDBA) bileşiklerinin *in vitro* hepatik mikrozomal metabolizması, karşılık gelen N-oksit, dealkilasyon, amid ve fenol gibi N- ve C- oksidasyon metabolitlerinin oluşup oluşmayacağını tespit etmek için çalışıldı. Bu substratlar, değişen pK_a ve logP özelliklerinin *in vitro* metabolizmalarına etkisini anlamak amacıyla seçildi. Substratlar ve olası metabolitleri sentez edilip spektral tekniklerle yapıları ve saflıkları aydınlatıldı. Daha sonra HPLC ile ayırımları sağlandı. İnkübasyonlar NADPH varlığında sıçan mikrozomal preparatları kullanılarak yapıldı. Substratlar ve olası metabolitleri diklorometana çekilerek HPLC ile analizleri yapıldı. Deneylerden alınan sonuçlar dört substratın da karşılık gelen amid metabolitlerini vermediğini, ancak temel metabolit olarak dealkilasyon ürünlerini verdiğini gösterdi. EDBA ve TBA ile karşılık gelen N-oksit metabolitleri tespit edildi. Substrat olarak DBAN kullanıldığında, üç fenolik metabolit tespit edildi. Elde edilen bulgular, nitronların amid oluşumunda sekonder aminlerin metabolizması ile oluşan esas ara ürünler olduğu ve bu reaksiyonun substrat olarak sekonder amin gerektirdiği hipotezlerini desteklemektedir. Ayrıca, bu substratlarda metabolik N-oksidasyon azot atomunun pKa değerine bağlı olarak meydana gelmektedir.

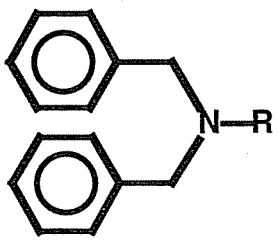
Key words : Tertiary amines, *in vitro* metabolism, the effect of pK_a.

INTRODUCTION

Tertiary amines constitute the structure of many drugs and xenobiotics such as industrial and environmental chemicals. Earlier reports showed that N-oxidation and N-dealkylation were common metabolic pathways for this function. In the case of aromatic tertiary amines, aromatic hydroxylation may also occur (1,2,3,4). Previous studies have shown that metabolic N-oxidation is possible if the nitrogen center is basic. In the case of non-basic compounds, α-carbon oxidation is more favourable. Such a compound, N-methylcarbazole, has been reported to produce N-hydroxymethyl intermediate (5).

Alicyclic amines were also reported to be metabolised by initial N-oxidation or α-carbon oxidation, which could then result in formation of lactam metabolites (6, 7). N,N-Dialkylamides are another group of non-basic nitrogen compounds giving dealkylation products as a result of α-carbon oxidation (8, 9).

In the present study, rat microsomal metabolism of four dibenzylamine derivatives, namely, N-ethyldibenzylamine (EDBA), tribenzylamine (TBA), N,N-dibenzylaniline (DBAN) and N,N-dibenzylbenzamide (BZDBA) were carried out (Figure 1). The aim was to establish whether the corresponding N- and C-oxidation products such as the corresponding amides (the mono benzoyl derivative), dealkylation products, N-oxides and phenols produced. It was of interest to see if the varying pK_a and logP characteristics of these substrates affect their *in vitro* metabolism.



Compound	R
EDBA	$-CH_2-CH_3$
TBA	$-CH_2-C_6H_5$
DBAN	$-C_6H_5$
BZDBA	$-CO-C_6H_5$

Figure 1: Structures of substrates used in this study.

RESULTS AND DISCUSSION

The substrates and their potential metabolites were synthesized by previously reported methods (Table 1) and their structures and purities were confirmed by spectral and chromatographic techniques together with their melting point analyses. The synthesis and some spectral data of EDBAO, DB4AP and DBZBA are presented in the Experimental.

In vitro hepatic microsomal metabolism of EDBA and TBA both produced the corresponding dealkylation product DBA by deethylation and debenzylation, respectively (Figures 2 and 3). However, EDBA did not yield EBA by debenzylation (Figure 2). This finding supports the previous concept that the smaller alkyl group is preferentially dealkylated when two different alkyl groups are present in the molecule (10). These two substrates with basic nitrogens (for

Table 1: Chromatographic properties of N-substituted dibenzylamines and their potential metabolites

Compound	Abbreviation	HPL Solvent	HPLC Rt (min)	TLC Solvent (Rf x 100)
N-Ethyldibenzylamine . HCl (14)	EDBA.HCl	S_1	31.99	A_1(82) (base)
Dibenzylamine	DBA	S_1	18.04	A_1(52)
N-Ethylbenzylamine	EBA	S_1	8.05	A_1(18)
N-Acetyldibenzylamine (15)	ADBA	S_1	7.64	A_1(55)
N-Benzoyl-N-ethylbenzylamine (16, 17)	BZEBA	S_1	7.77	A_1(65)
N-Ethyldibenzylamine-N-oxide	EDBAO	S_1	14.91	A_i (0)
Benzaldehyde	B	S_1	2.97	A_1(70)
Tribenzylamine (18, 19)	TBA	S_2	12.76	A_1(81) , A_2(77)
Dibenzylamine	DBA	S_2	5.07	A_1(52) , A_2(65)
Tribenzylamine-N-oxide (20)	TBAO	S_2	9.77	A_1 (1) , A_2(57)
Benzaldehyde	B	S_2	2.11	A_1(70) , A_2(ND)
N,N-Dibenzylaniline (21)	DBAN	S_3	32.02	A_3(85)
N-Benzylaniline	NBA	S_3	10.27	A_3(68)
Aniline	AN	S_3	4.52	A_3(49)
4-(N,N-Dibenzylamino)phenol (22)	DB4AP	S_3	13.75	A_3(47)
N-Benzyl-4-aminophenol	NB4AP	S_3	6.23	A_3(33)
4-Aminophenol	4AP	S_3	3.78	A_3(13)
Benzaldehyde	B	S_3	5.12	A_3(65)
N,N-Dibenzylbenzamide (23)	BZDBA	S_1	18.31	A_1(59) , A_4(39)
N-Benzylbenzamide (24)	BZBA	S_1	4.05	A_1(46) , A_4(37)
N,N-Dibenzoylbenzylamine (25)	DBZBA	S_1	15.37	A_1(61) , A_4(63)
Dibenzylamine	DBA	S_1	18.19	A_1(52) , A_4(31)
Benzaldehyde	B	S_1	2.96	A_1(70) , A_4(67)

S_1 = Acetonitrile - Phosphate buffer , pH 7 , (40:60, v/v) , flow rate 2 ml/min , max= 225 nm
S_2 = Acetonitrile - Phosphate buffer , pH 6 , (60:40, v/v) , flow rate 2 ml/min , max= 230 nm
S_3 = Acetonitrile - Phosphate buffer , pH 7 , (60:40, v/v) , flow rate 2 ml/min , max= 230 nm

A_1 = Petroleum ether (b.p. 40-60 C) - Acetone , (60:40, v/v)
A_2 = Chloroform - MeOH , (80:20, v/v)
A_3 = Petroleum ether (b.p. 40-60 C) - Acetone , (70:30, v/v)
A_4 = Petroleum ether (b.p. 40-60 C) - DCM , (25:75, v/v)
ND : Not Determined

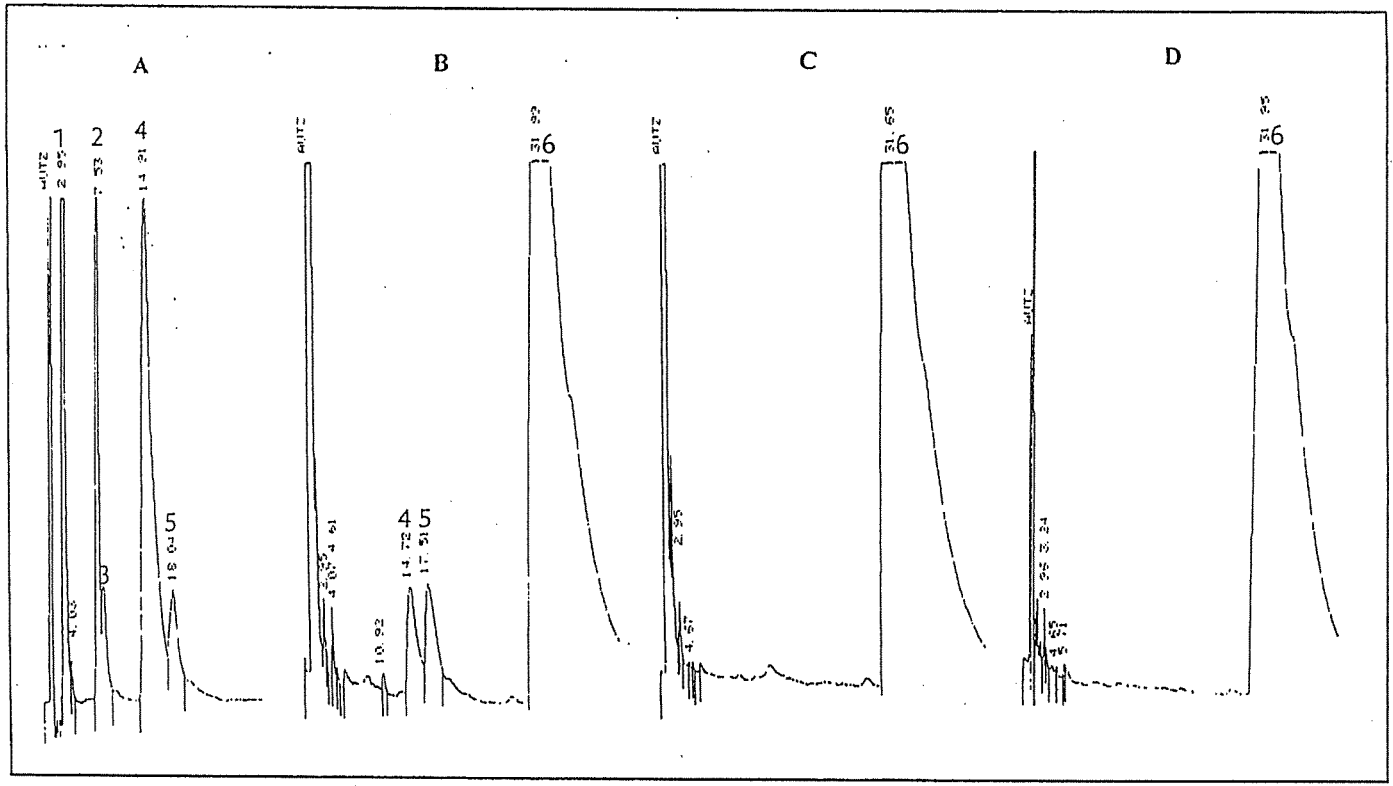


Figure 2: HPLC Chromatogram obtained **[A]** from standards, **[B]** following extraction from male rat microsomal incubation mixture with EDBA as substrate, **[C]** from control incubation with denatured microsomes and **[D]** from control incubation without Co-factors.
(1 = B , 2 = ADBA + BZEBA , 3 = EBA , 4 = EDBAO , 5 = DBA , 6 = EDBA. HCl)
(see text or table 1 for the abbreviations)

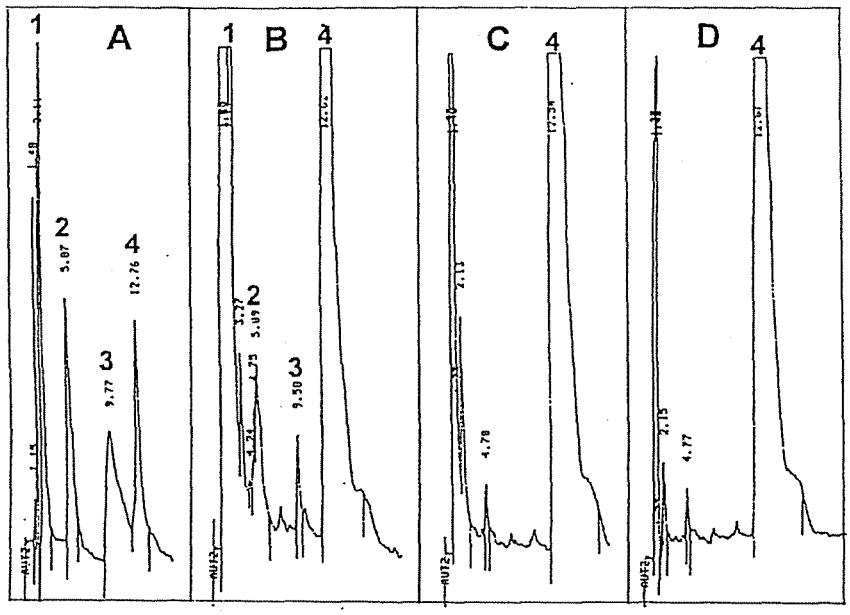


Figure 3: HPLC Chromatogram obtained [A] from standards, [B] following extraction from male rat microsomal incubation mixture with TBA as substrate, [C] from control incubation with denatured microsomes and [D] from control incubation without Co-factors (1 = B, 2 = DBA, 3 = TBAO, 4 = TBA).
(see text or table 1 for the abbreviations)

pK_a values see Table 2) gave the N-oxide metabolites (Figures 2 and 3). Figures 4 and 5 show the established metabolites of EDBA and TBA.

With remaining two substrates, DBAN and BZDBA, metabolic N-oxidations did not seem possible because of their low pK_a values (1.03 and -1.80, respectively). DBAN produced mono- and di-debenzylation metabolites and three phenolic products (Figure 6). The established metabolites of DBAN are given in Figure 7.

BZDBA, the least basic substrate, only gave mono-debenzylation product. HPLC and TLC examination supported the lack of di-dealkylation and hydrolysis (Figure 8). These findings are in line with previous studies on this class of compounds (N,N-dialkyl benzamides) (8,9). An unknown metabolite was also detected by HPLC. Figure 9 shows the total metabolic picture for this substrate.

Table 2: The results obtained from the rat microsomal metabolism of N-substituted dibenzylamines and their pK_a and logP values

Compd.	Log P	pK_a	N.Dealkylation	Amide formation	Aromatic hydroxylation	N-Oxidation	Hydrolysis
EDBA	3.31	8.16	**DBA**^{HT}	*NO*	*ND*	**EDBAO**^{H}	*NA*
TBA	4.33	6.94	**DBA**^{HT}	*NO*	*ND*	**TBAO**^{H}	*NA*
DBAN	4.92	1.03	**BAN**^{HT} , **B4AP**^{H}, **AN**^{H}, **4AP**^{H}	*NO*	**DB4AP**^{HT} , **B4AP**^{H}, **4AP**^{H}	*ND*	*NA*
BZDBA	4.53	-1.80	*NO*	*NO*	*ND*	*ND*	*NO*

NA : Not Applicable , *ND* : Not Determined, *NO* : Not Observed, *H* : Detected by HPLC, *HT* : Detected by TLC & HPLC.
see figure 1 for the structures ; table 1 for the abbreviations

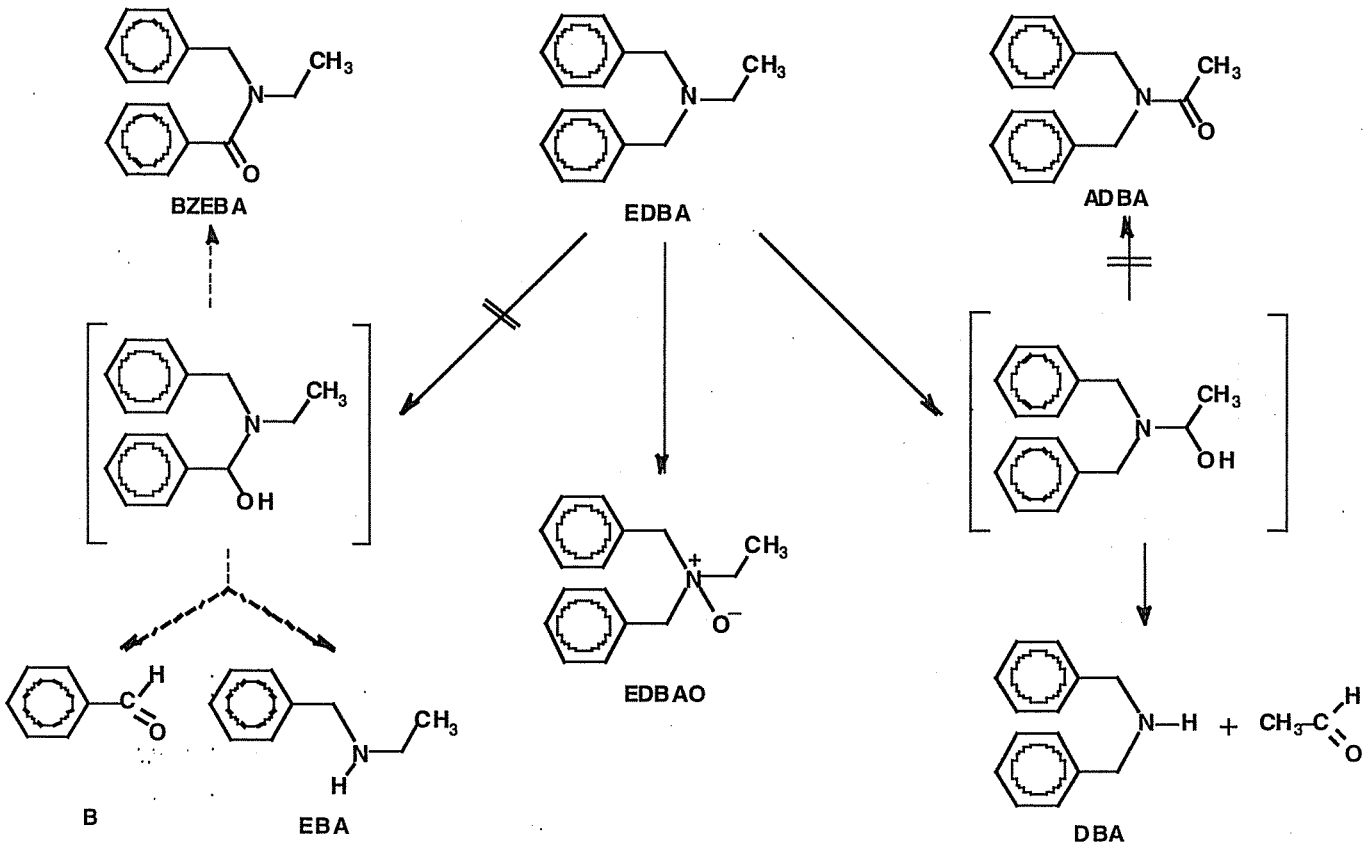


Figure 4: Established metabolic pathways for EDBA.

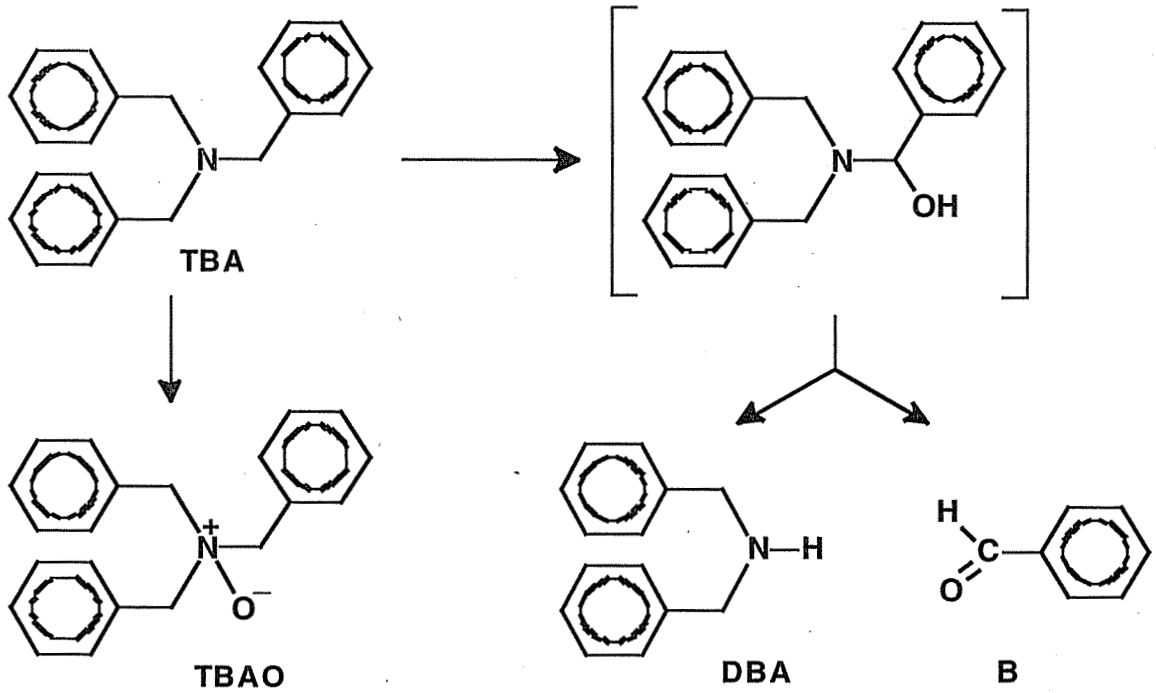


Figure 5: Established metabolic pathways for TBA.

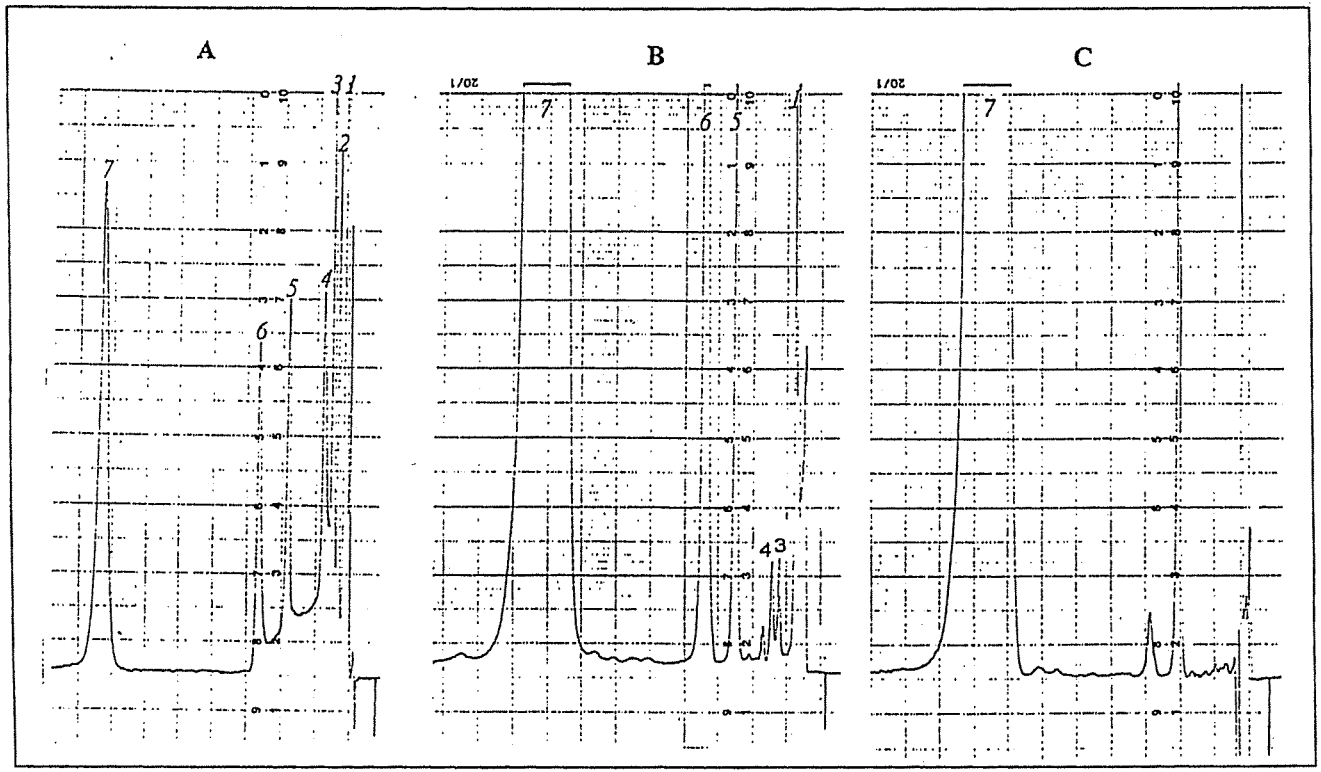


Figure 6: HPLC Chromatogram obtained **[A]** from standards, **[B]** following extraction from male rat microsomal incubation mixture with DBAN as substrate **[C]** from control incubation with denatured microsomes.
(1 = 4AP, 2 = AN, 3 = B, 4 = B4AP, 5 = BAN, 6 = DB4AP , 7 = DBAN)
(see text or table 1 for the abbreviations)

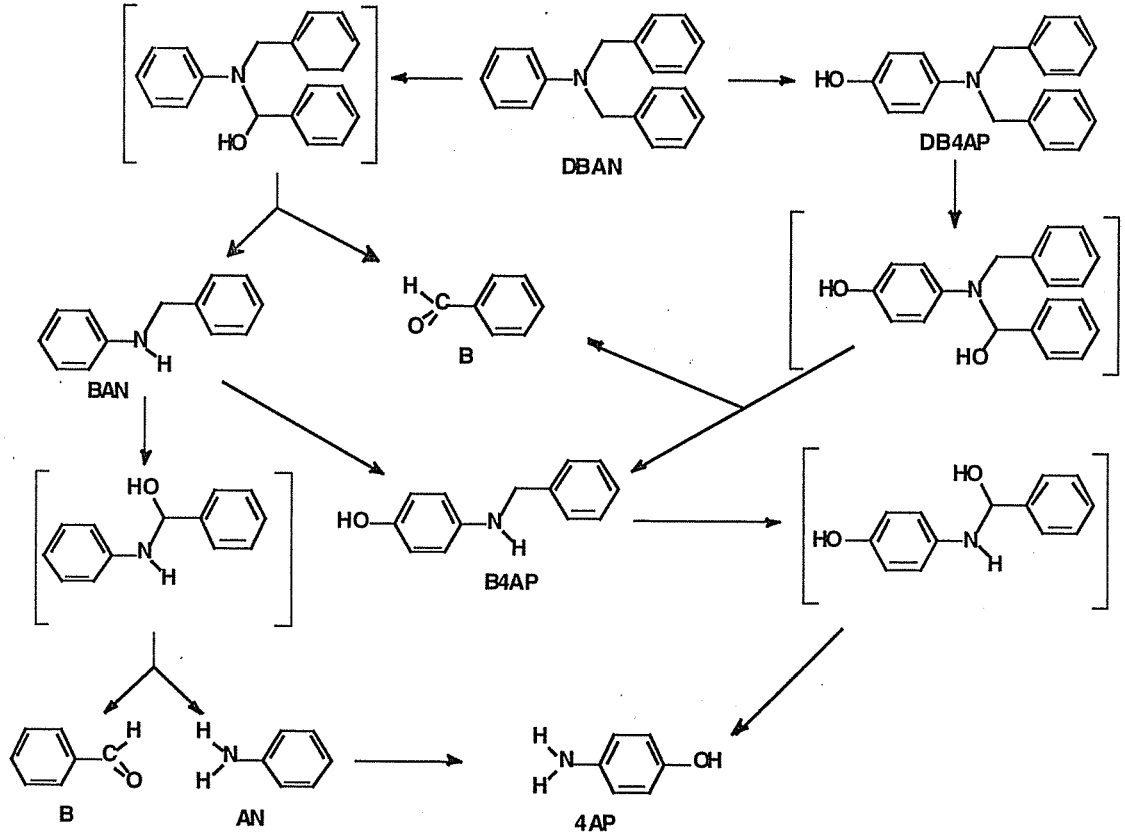


Figure 7: Established metabolic pathways for DBAN.

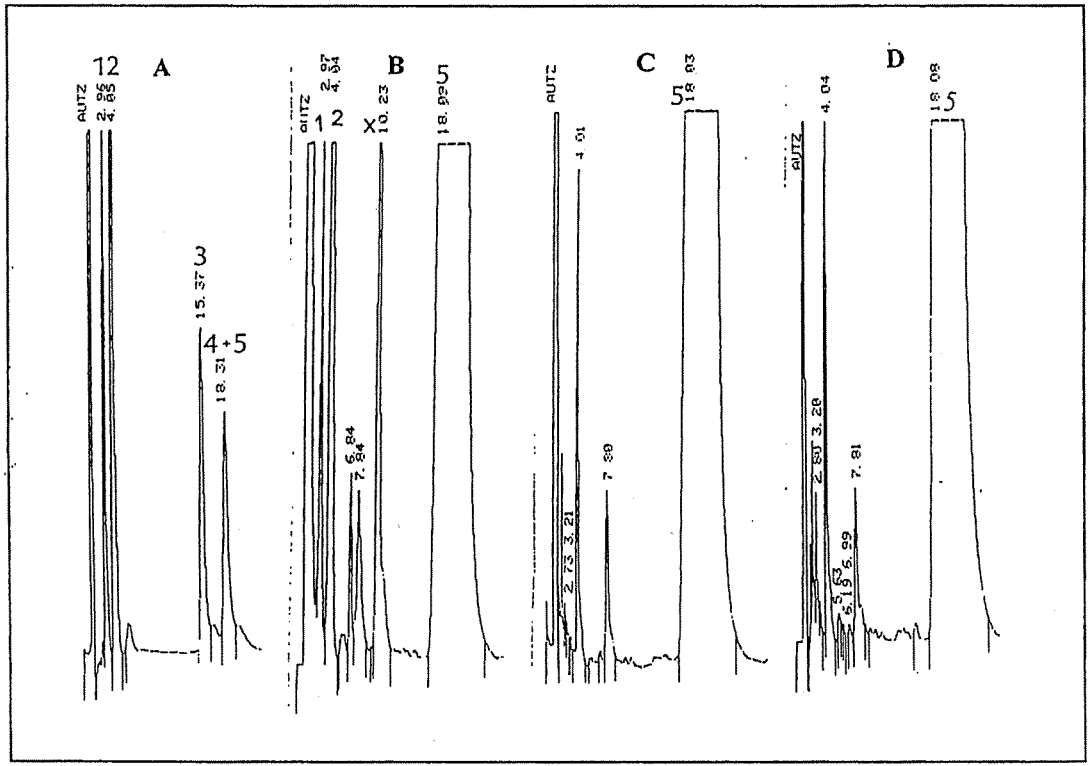


Figure 8: HPLC Chromatogram obtained **[A]** from standards, **[B]** following extraction from male rat microsomal incubation mixture with NBZDBA as substrate, **[C]** from control incubation with denatured microsomes and **D** from control incubation without co-factors. (1 = B, 2 = BZBA, 3 = DBZBA, 4 = DBA, 5 = BZDBA , X = Unknown metabolite)
(see text or table 1 for the abbreviations)

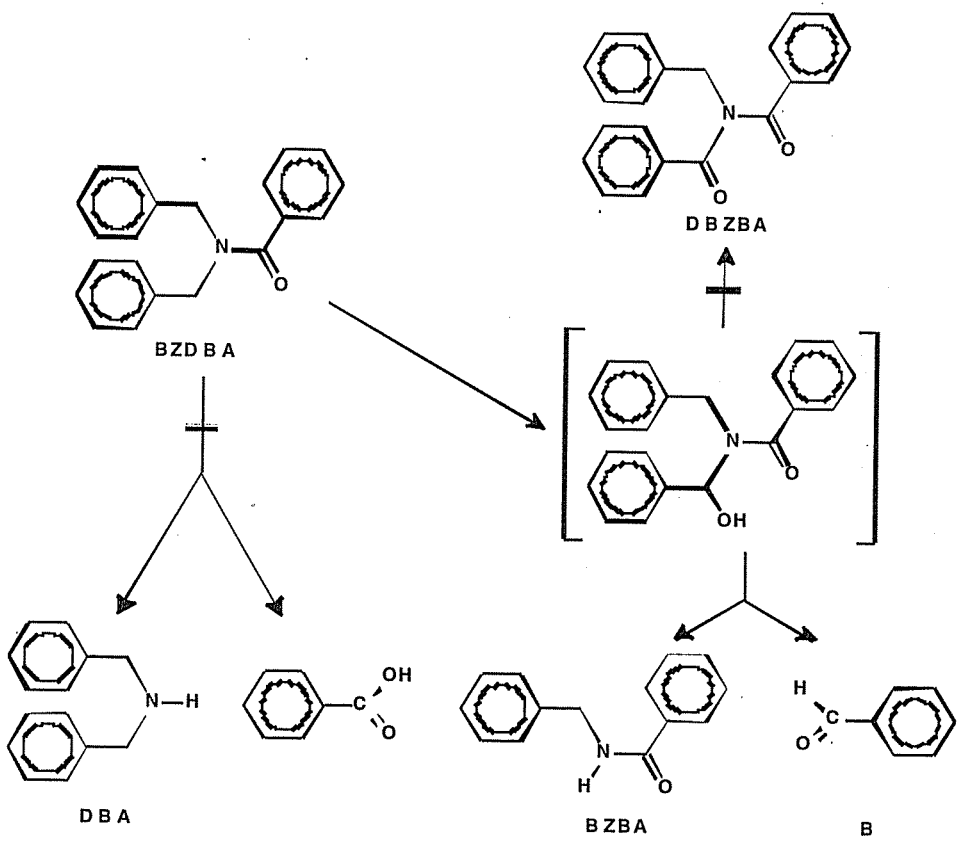


Figure 9: Established metabolic pathways for BZDBA.

All the substrates failed to produce the corresponding amide metabolites (α-carbon oxidation). This observation supports the proposal that the formation of amides is only possible by the initial formation of nitrone intermediate which can only arise from secondary amines (11-13).

EXPERIMENTAL

Materials and Methods

Acetic anhydride (AA), 4-aminophenol (4AP), aniline (AN), benzaldehyde (B), benzoyl chloride (BZC), benzyl chloride (BC), dichloromethane (DCM), diethylether, glacial acetic acid, hydrogen peroxide (35%) sodium borohydride (Merck); dibenzylamine (DBA) (Aldrich), N-ethylbenzylamine (EBA) (British Drug Houses), N-benzylaniline (BAN), N-benzyl-4-aminophenol (B4AP), glucose-6-phosphate dehydrogenase (Boehringer Mannheim), nicotinamide adenine dinucleotide phosphate mono sodium salt (NADP), glucose-6-phosphate disodium salt (Sigma). All chromatographic solvents were obtained from Merck Chemical Company. The HPLC column (Spherisorb C18 5µm (25cm length x 4.6mm i.d.) was purchased from Phase Separations Limited, Deeside, UK. The guard column packing material (Whatman Pellicular ODS) was purchased from Whatman International Ltd., Maidstone, Kent, UK. The HPLC chromatograph consisted of an isocratic system comprising a LCD analytical constaMetric 3200 solvent delivery system, a Rheodyne syringe loading sample injector valve (model 7125) fitted with a 20µL 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 N-substituted dibenzylamines. Computer-generated log P and pK_a estimations for the substrates were carried out using the programs ProLog P version 4.2D, and pKalc version 2.0C (Compudrug Chemistry Ltd, Budapest, Hungary).

Synthesis of Substrates and Their Potential Metabolites

This work required the synthesis of the substrates and most of their potential metabolites (Table 1) and their structures and purities were confirmed by spectral and chromatographic analysis. The compounds were prepared by previously reported methods (Table 1).

N-Ethyldibenzylamine-N-oxide (EDBAO) : Hydrogen peroxide (30% w/w, 0.0155 mol) was added to a solution of EDBA (0.0052 mol) whilst stirring this mixture vigorously at ambient temperature. The reaction mixture was kept in dark for a further 5 days without stirring. At the end of this period, the flask content was cooled to -10°C and chilled solution of NaOH (20% w/v) was added to provide an alkaline solution followed by extraction with diethylether to remove the unreacted amine. The remaining aqueous phase was then saturated with NaCl and then extracted with DCM. Evaporation of the organic solvent under reduced pressure followed by recrystallization of crude product from diethylether-methanol afforded the N-oxide in a pure state. M.p. 124-125.5 °C, yield 42%. **UV** (EtOH) λ_{max} (ε): 262(1569), 218(16338), 207(20586). **IR** (KBr) ν (cm^{-1}): 3413 (H_2O); 3092, 3064, 3037, 3010 (=CH); 2983 (CH_3); 2939 (CH_2); 1584, 1495, 1457 (C=C) ; 1381 (CH_3 groups,C-H sym bend, N-oxide N-O str); 891,723 (monosubs. benzene). **^{1}H-NMR** (400 MHz) (CD_3OD/TMS), δ (ppm): 1.39 (t,3H,- $CH_2\underline{CH_3}$); 2.99 (q,2H,-$C\underline{H_2}CH_3$); 3.30 (CH_3OH/CD_3OH); 4.39 (q,4H,-CH_2-Ar); 4.88 (H_2O/CD_3OD); 7.39-7.88 (m,9H,Ar-H). **EI-Mass** (70 eV): 241(M^+), 225, 210, 197, 196, 181, 149, 135, 134, 120, 107, 106, 105, 91 (100%), 77, 65, 63, 58, 57, 56, 51. **Anal.** for $C_{16}H_{19}NO$. H_2O (calc./found): C 74.09/74.72, H 8.16/8.34, N 5.40/5.16.

4-(N,N-Dibenzylamino)phenol hydrochloride (DB4AP.HCl) (22)**:** A mixture of 4-aminophenol (0.01 mol), benzyl chloride (0.03 mol) and $NaHCO_3$ (0.03 mol) was refluxed in acetone for 24 hrs, then cooled and filtered. The solution thus obtained was evaporated to dryness. The remaining oil was dissolved in diethylether and this solution was dried over anhydrous Na_2SO_4. Ethereal solution was treated with dry hydrogen chloride to yield DBAP as hydrochloride salt. Recrystallization of the crude product from diethylether-methanol afforded DB4AP.HCl in a pure state. M.p.215-218 °C, yield 67%. **UV** (EtOH) λ_{max} (ε): 321(1115), 250(6259), 207(25535). **IR** (cm^{-1}) ν (KBr): 3160 (Ar-OH), 3070, 3040, 3000 (=CH); 2920, 2860 (CH_2); 2750, 2690, 2580 (N-H); 1610, 1515, 1480, 1405 (C=C); 1285, 1215 (O-H bend. and C-O str.); 895 (disubs. benzene); 745, 690 (mono subs. benzene). **EI-Mass** (70 eV): 289(M^+), 213, 212, 199, 198, 197, 121, 120, 106, 94, 93, 91 (100%), 80, 78, 77, 65, 63.

N,N-Dibenzoylbenzylamine (DBZBA) (25)**:** A mixture of N-benzylbenzamide (0.002 mol), benzoyl chloride (0.004 mol) and NaH (0.002 mol) was refluxed in benzene for 15 hrs, then cooled and filtered. The solution thus obtained was evaporated to dryness. The crude product was washed with MeOH to re-

move unreacted benzoyl chloride, dried and recrystallized from petroleum ether (b.p. 40-60 °C)-ether. M.p. 87-90 °C, yield 48%. **UV** (EtOH) λ_{max} (ε): 253(26334). **IR** (cm^{-1}) ν (KBr): 3454 (H_2O), 3026 (=CH), 2929, 2860 (CH_2), 1697, 1649 (C=O), 1550, 1512, 1442 (C=C), 710, 694 (mono subs. benzene). **EI-Mass** (70 eV): 315 (M+), 224, 211, 210 (100%), 194, 193, 105, 91, 77, 51. **Anal.** for $C_{21}H_{17}NO_2$. $^1/_2H_2O$ (calc./found): C 77.75/78.63 , H 5.59/5.59, N 4.32/4.12.

Analytical procedures for the detection and identification of substrates and their potential metabolites

The separation techniques used were based on TLC and an isocratic HPLC system. TLC was carried out using prepared silica gel GF254 0.25 mm on glass plates (E.Merck, Darmstadt) with suitable solvent systems. Table 1 shows TLC R_f x 100 and HPLC R_t values of the substrates and their potential metabolites, using these solvent systems. In the HPLC analysis, the reaction products were eluted with a number of mobile phases consisting of acetonitrile : 0.2 M phosphate buffer at different ratios. The metabolic products were detected by their absorbance at 225 or 230 nm. Retention times of compounds under these conditions are given in Table 1.

Incubation and Extraction Procedures

Hepatic washed rat microsomal preparations were prepared at 0°C using the calcium chloride precipitation method of Schenkman & Cinti (26). Incubations were carried out in a shaking water bath at 37°C for 30 minutes using a standard co-factor solution at pH 7.4. Co-factor generating solutions consisting of NADP (2 mol), glucose-6-phosphate (10 mol), glucose-6-phosphate dehydrogenase (1 unit), $MgCl_2$ (20 mol) prepared in phosphate buffer (2mL, 0.2M, pH7.4) were pre-incubated for 5 minutes before addition of microsomes (1 mL) equivalent to 0.5 g original liver and substrate (2 mole in 50 mole methanol per flask). Metabolic reactions were stopped by extraction with DCM (2x5mL) including 0.8 g of NaCl. The DCM extracts were evaporated to dryness using a stream of N_2 at 20°C. Dry organic residues were reconstituted in 200 L of MeOH for HPLC and 100 L of MeOH for TLC analysis.

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