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IN VITRO MICROSOMAL METABOLISM OF SOME N-BENZYL SUBSTITUTED TERTIARY AMINES IN THE RAT

FAREDE BAZI N-BENZİL SÜBSTİTÜENTİ TERTİARİ AMİNLERİN İN VİTRO MİKROZOMAL METABOLİZMASI

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The in vitro hepatic microsomal metabolism of three different substituted benzylic tertiary amines ie. N-isopropyl-N,N-dibenzylamine (PDBA), N-(2,4,6-trimethylbenzyl)-N,N-dibenzylamine (MDBA) and N-(2,6-dichlorobenzyl)-N,N-dibenzylamine (CDBA) was studied in the rat to establish whether N-oxidation and N-dealkylation reactions occured. Substrates and their potential metabolites ie. the corresponding N-oxides and secondary amines were synthesised. The structures of these compounds were confirmed by UV, IR, ^{1}H-NMR and MASS spectroscopic techniques and their elemental analyses. Separation was achieved by thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC). Substrates were incubated with the microsomes isolated from rat liver in the presence of co-factors including NADPH. Substrates and metabolites were then extracted from biological systems with dichlromethane. Metabolites occured were compared with their authentic standards and the metabolic pathways were clarified. Oxidative dealkylation leading to the formation of the corresponding secondary amines and aldehydes were detected as major routes of metabolism for these there substrates. However, no N-oxide metabolites were detected.

Üç farklı sübstitüe benzilik tersiyer aminin (N-isopropil-N,N-dibenzilamin) (PDBA), N-(2,4,6-trimetilbenzil)-N,N-dibenzilamin (MDBA) ve N-(2,6-diklorobenzil)-N,N-dibenzilamin (CDBA) in vitro hepatik mikrozomal metabolizması, sıçanda N-oksidasyon ve N-dealkilasyon reaksiyonlarının oluşup oluşmadığını tayin etmek için çalışıldı. Substratlar ve bunlara karşı gelen N-oksit ve sekonder amin gibi olası metbolitler sentezlendi. Bu bileşiklerin yapıları UV, IR, ^{1}H-NMR, kütle spektroskopik yöntemleri ve elementel analiz ile aydınlatıldı. Ayırım, ince tabaka kromatografisi (İTK) ve yüksek basınçlı sıvı kromatografisi (HPLC) ile sağlandı. Substratlar, NADPH içeren ko-faktörlerin varlığında sıçan karaciğerinden izole edilen mikrozomal preparatlarla inkübasyona tabi tutuldu. Substrat ve oluşan metabolitler biyolojik sistemden diklorometana çekildi. Oluşan metabolitler standartlarla karşılaştırıldı ve metabolik yollar açığa çıkartıldı. Bu üç substrat için metabolizmanın birincil yolu olarak bunlara karşı gelen sekonder amin ve aldehitlerin oluşumuna yol açan oksidatif dealkilasyon tespit edildi. Ancak, N-oksit metabolitleri tespit edilmedi.

Keywords: *Benzylic tertiary amines, in vitro metabolism, microsomes*

Anahtar kelimeler: *Benzil tersiyer aminler, in vitro metabolizma, mikrozomlar*

Introduction

The majority of drugs, toxicants and environmental chemicals have tertiary amine function. N-Oxidation and N-dealkylation are the most important routes in the metabolism of certain tertiary amines (1,2,3) although no report is found on the *in vitro* tertiary benzylic amine metabolism. In previous studies, metabolism of certain tertiary amines by hepatic microsomal preparations from different species has been examined using chromatographic techniques. These studies showed that these compounds produced N-oxygenated (4,5,6,7) and N-dealkylated (4,7,8,9) metabolites. It has been demonstrated that the high electron density occurs in the basic amines and this situation results in spontaneous breakdown of the carbinolamine to give the corresponding dealkylated amine and aldehyde. When the electron donating groups are present adjacent to the methylene, increased rates of dealkylation are observed. The basicity of the nitrogen atom in tribenzylamine is increased by electron donating substituents on the ring (10). In the present study, methyl or chloro substituents were connected to the aromatic ring or; an isopropyl moiety was introduced to the nitrogen atom to modify the metabolic N-oxidation and N-dealkylation reactions in the tertiary

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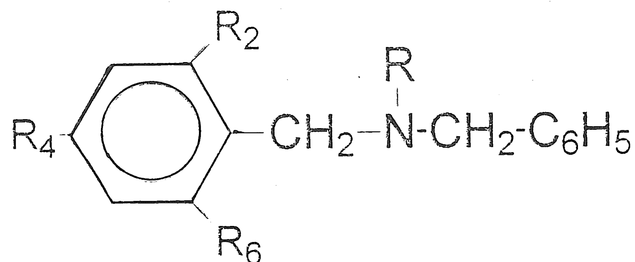
Table 1. Structures of substrates used in this study. (see table 2 for abbreviations)



Compound	R	R2	R4	R6
MDBA	C_6H_5-CH_2-	CH_3-	CH_3-	CH_3-
CDBA	C_6H_5-CH_2-	Cl-	H-	Cl-
PDBA	$(CH_3)_2CH$-	H-	H-	H-

amines (Table 1).

The aim for this study was to investigate whether these benzylic tertiary amines were metabolized into the N-oxygenated and/or N-dealkylated products (Table 1, Fig. 1). The proposed substrates and their potential metabolites were synthesised and characterised using spectroscopic techniques and were separated using TLC and HPLC. The results from the *in vitro* oxidative microsomal metabolism of the proposed substrates with hepatic washed rat microsomes fortified with NADPH are presented.

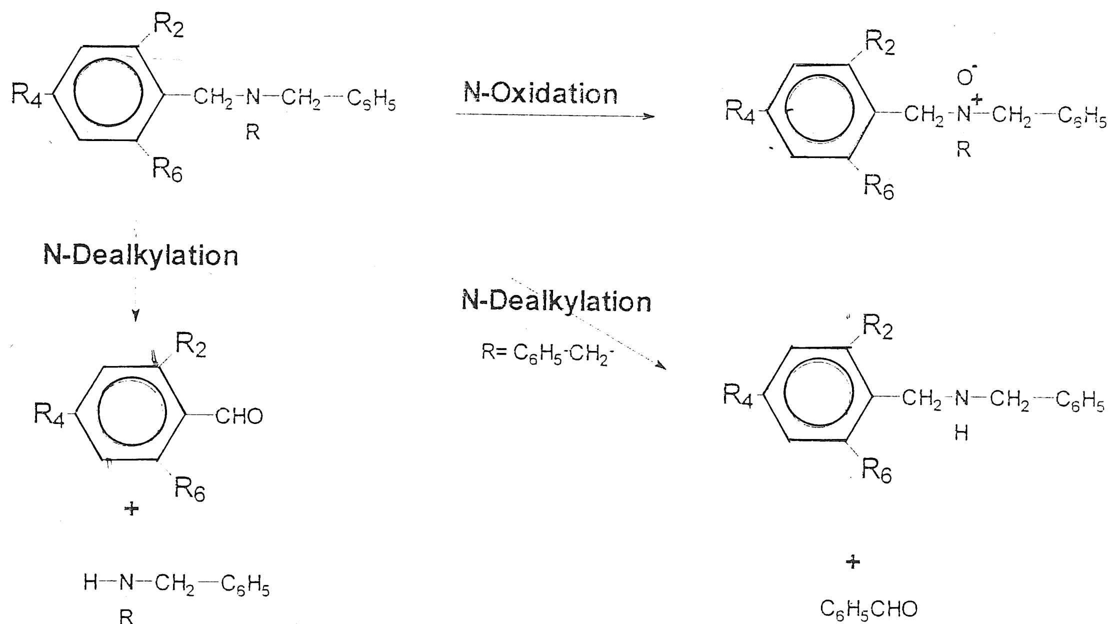


Fig.1. Proposed metabolic pathways for N-substituted benzyl/isopropyl-N,N-dibenzyl tertiary amines used as substrates in this study (see table 1 for the structures)

Materials and Methods

Chemicals

Dibenzylamine, benzyl chloride, 2,4,6-trimethylbenzyl chloride, α,2,6-trichlorotoluene, 2,6-dichlorobenzaldehyde and 2,4,6- trimethylbenzaldehyde were all purchased from Aldrich Chemical Company, UK. Calcium, sodium and potassium chlorides were purchased from British Drug Houses (B.D.H.) (Poole, Dorset, UK). Benzaldehyde, glacial acetic acid, hydrogen peroxyde (30%), sodium hydroxyde and potassium iodide were obtained from E. Merck (Darmstadt, Germany). m-Chloroperoxybenzoic acid (m-CPBA) and N-benzylisopropylamine were purchased from Sigma Chemical Company. Acetonitrile (HPLC grade) and all other chromatography solvents were purchased from Lab-Scan (Germany). Plastic-backed TLC plates precoated with silica-gel $60F_{254}$, potassium dihydrogenorthophosphate hydrate, disodium hydrogenphosphate and sucrose were obtained from E. Merck (Darmstadt, Germany). 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 Ltd. Magnesium chloride ($MgCl_2.6H_2O$) was obtained from FSA Laboratory (UK).

Albino Wistar rats (200-250 g) were used in this study. The animals were deprived of food overnight prior to sacrifice, but were allowed water ad libitum. They were previously fed on a balanced diet. Hepatic washed microsomes were prepared by calcium chloride precipitation method as described by Schenkman and Cinti (11).

Instrumentation

Melting points were determined with a Buchi (B-530) apparatus and were uncorrected. Spectroscopic data were recorded with the following instruments: UV spectra were recorded on a Shimadzu-260 UV spectrophotometer, IR spectra (in KBr) on a ATI Unicom Mattson 1000 FT-IR spectrophotometer, ^{1}H-NMR spectra on a Perkin Elmer NMR $COCCl_3$ as a solvent and Mass spectra on a Mass spectrometer with an ionisation potential of 70eV. C,H,N analyses were carried out on a model 240 XY Control and 1106 Carlo Erba Equipments, TUBITAK Instrumental Analysis Lab, Gebze Turkey.

High Performance Liquid Chromatography (HPLC)

HPLC column Spherisorb C_{18} 5μm(25 cm length x 4.6 mm i.d.) was purchased from Phase Separations Limited, Deeside, UK. The guard column packing material (Whatman Pellicular ODS) was purchased from Whatman Internationl Ltd., Maidstone, Kent, 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.

The "acetonitrile : phosphate buffer" mobile-phase compositions and flow rates were as follows:
A. (70:30,v/v pH:6), flow rate 1.5 ml/min
B. (60:40,v/v pH:6), flow rate 2 ml/min
C. (50:50,v/v pH:6), flow rate 1 ml/min

HPLC retention times of the substrates and their potential metabolites are shown in table 2.

Thin Layer Chromatographic Analysis

TLC was carried out using plastic-backed TLC plates precoated with silicagel $60F_{254}$ with the following solvent systems: 1. benzene:methanol (80:20,v/v); 2. chloroform: acetone:ammonia (90:10:0.5,v/v); 3. petroleum ether (b.p.40-60°C):acetone (70:30,v/v); 4. benzene:petroleum ether: acetone (10:10:80,v/v); 5. petroleum ether: acetone (10:90,v/v); 6. dichloromethane: ethyl acetate: acetone (10:10:80,v/v). The plates, after development, were examined under UV light (254 nm), Rfx100 values were recorded (Table 2) and the plates were sprayed with Ehrlichs reagent (to detect for primary and secondary amines), Dragendorff reagent (for N-oxides and tertiary amines) and 2,4-Dinitrophenylhydrazine reagent (for benzaldehyde).

Incubation and Extraction Procedures

Incubations were carried out in a shaking water-bath at 37°C using a standard co-factor solution consisting of NADP (2μmol), G-6-P (10 μmol), G-6-P dehydrogenase suspension (1 unit) and aqueous MgCl2 (50%,w/w) (20 μmol) in phosphate buffer (0.2M, 2ml) at pH 7.4. Co-factors were pre-incubated for 5 min to generate NADPH, before the addition of microsomes (1 ml equivalent to 0.5 g original liver) and substrate (5 μmol) in methanol (50 μl). Control experiments with denaturated microsomes (98°C, 10 min) or without co-factors were also carried out. The incubation was continued for 30 min, terminated and extracted with dichloromethane (2x5 ml). The organic extracts were evaporated. The residues were reconstituted in 200 μl of methanol for HPLC and 50 μl methanol for TLC. A typical HPLC chromatogram of MDBA and its potential metabolites is shown in figure 2a. An HPLC chromatogram following MDBA metabolism by rat liver microsomes is shown in figure 2b.

Synthesis of substrates ie. N-isopropyl-N,N-dibenzylamine (PDBA), N-(2,4,6-trimethylbenzyl)-N,N-dibenzylamine (MDBA) and N-(2,6-dichlorobenzyl)-N,N-benzylamine (CDBA)

PDBA was synthesized as follows: Equimolar amonuts ($25x10^{-3}$ mol) of N-benzylisopropylamine, potassium iodide and aqueous sodium hydroxide solution (10%) were refluxed while stirring vigorously. When the temperature increased to 90°C, equimolar amount of benzyl chloride was added to the mixture dropwise over half an hour. During the reaction, a solid was formed. The reaction was stopped when the level of tertiary amine was maximal. This was monitored by TLC. The mixture was cooled, the yellowish orange solid formed was recrystallized from ethanol-water

Table 2. Chromatographic properties of substrates and their potential metabolites

Compound (Abbreviation)	HPLC Rt (Solvent system)*	TLC Rfx100 value (Solvent system)*
N-(2,4,6-trimethylbenzyl)-N,N-dibenzylamine (MDBA)	14.2 (A)	79 (1) ; 74 (2)
N-(2,4,6-trimethylbenzyl)-N,N-dibenzylamine-N-oxide (MDBAO)	11.9 (A)	37 (1) ; 6 (2)
N-(2,4,6-trimethylbenzyl)-N-benzylamine (MBA)	7.3 (A)	57 (1) ; 49 (2)
2,4,6-Trimethylbenzaldehyde (TMB)	3.3 (A)	68 (1) ; 66 (2)
Dibenzylamine (DBA)	4.8 (A) ; 4.6 (B) ; 8.0 (C)	49 (1) ; 38 (2) ; 51 (3) ; 34 (4) ; 49 (5) ; 55 (6)
Benzaldehyde (B)	2.5 (A) ; 2.1 (B) ; 2.9 (C)	65 (1) ; 71 (2) ; 54 (3); 71 (4) ; 69 (5) ; 72 (6)
N-(2,6-dichlorobenzyl)-N,N-dibenzylamine (CDBA)	20.7 (B)	75 (3) ; 77 (4)
N-(2,6-dichlorobenzyl)-N,N-dibenzylamine-N- oxide (CDBAO)	14.8 (B)	6 (3) ; 18 (4)
N-(2,6-dichlorobenzyl)-N-benzylamine (CBA)	6.9 (B)	66 (3) ; 65 (4)
2,6-Dichlorobenzaldehyde (CB)	2.7 (B)	59 (3) ; 68 (4)
N-Isopropyl-N,N-dibenzylamine (PDBA)	14.7 (C)	72 (5) ; 68 (6)
N-Isopropyl-N,N-dibenzylamine-N-oxide (PDBAO)	14.7 (C)	7 (5) ; 9 (6)
N-Isopropylbenzylamine (PBA)	5.1 (C)	24 (5) ; 26 (6)

*see text

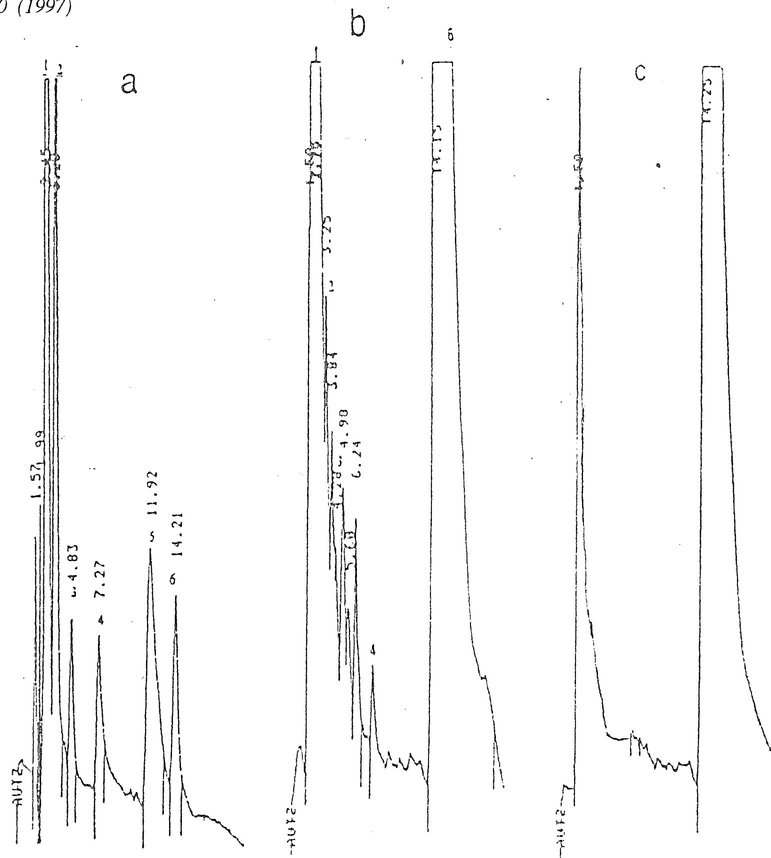


Fig.2. a. HPLC chromatogram of MDBA and its potential metabolites
b. HPLC chromatogram of *in vitro* MDBA metabolites formed by hepatic rat microsomes
c. Control experiment with denaturated microsomes
(1=B, 2=TMB, 3=DBA, 4=MBA, 5= MDBAO, 6 MDBA) (see table 2 for abbreviations)

to give the bright white crystalls of PDBA (Table3). MDBA and CDBA were prepared by the alkylation of dibenzylamine with the corresponding alkyl halides (12). Equimolar amounts of 2,4,6-trimethylbenzyl chloride ($7.5x10^{-3}$ mol) or α ,2,6-trichlorotoluene (10^{-2} mol) was added to the equimolar amount of dibenzylamine in aqueouns sodium hydroxide solution (10%) gradually in small portions over thirty minutes while stirring and heating under reflux. The reaction was monitored by TLC. Cooled residues were washed with water. The crude products were recrystallized from ethanol-water to give the white crystalls of MDBA and white powder of pure CDBA (Table 3). ^{1}H-NMR for CDBA (MeOD, δ ppm) 3.52 (s,4H, 2xCH_2), 3.85 (s,2H,CH_2), 7.11-7.33 (m,13H, Ar protons). Dragendorff reagent formed a pale orange colour with all of the tertiary animes synthesized.

Synthesis of secondary amines ie. N-(2,4,6-trimethylbenzyl)-N-benzylamine (MBA) and N-(2,6-dichlorobenzyl)-N-benzylamine (CBA)

These aliphatic secondary amines were synthesized by the alkylation reaction. Equimolar amounts of benzylamine ($5x10^{-3}$ mol) in methanol and potassium hydroxide were refluxed while constantly stirring. When the temperature increased to 90°C, equimolar amount of alkyl halides in methanol was added to the mixture dropwise. Reactions were stopped when TLC monitoring showed that the level of benzylamine was minimal. The mixture was evaporated and the residue was washed with water. Pure secondary amines, MBA and CBA were obtained by preparative TLC as liquids using the solvents system of petroleum ether (b.p. 40-60°C): dichloromethane (25:75, v/v). Both MBA and CBA gave orange colour with Dragendorff reagent ^{1}H-NMR for CBA (MeOD,δ ppm): 3.84 (s,2H,Ar-CH_2), 4.02 (s,2H,NH-CH_2), 4.91 (s,1H,NH), 7.20-7.41 (m,8H,Ar protons).

Synthesis of N-oxides ie. N-isopropyl-N,N-dibenzylamine-N-oxide (PDBAO), N-(2,4,6-trimethylbenzyl)-N,N-dibenzylamine-N-oxide (MDBAO) and N-(2,6-dichlorobenzyl)-N,N-dibenzylamine-N-oxide (CDBAO)

PDBAO and CDBAO were synthesized from corresponding tertiary amines by m-chloroperoxybenzoic acid (m-CPBA) oxidation (13,14). The solution of m-CPBA in dichloromethane was added to the equimolar

Table 3. Analytical data for substrates and their potential metabolites

Compound as abbreviated	m.p. (°C) and description	Yield (%)	UV λmax (nm)	IR ν (cm^{-1})	C,H,N analysis Calc. (Found) C	H	N
MDBA	71-72 bright white crystal	56	204	3100,1500, 760-755	87.49 (87.44)	8.26 (8.42)	4.25 (4.14)
MDBAO $.H_2O$	89-90 white powder	18	206	3050, 1500, 980	79.30 (80.01)	8.04 (7.95)	3.85 (3.83)
MBA	- dark yellow liquid	5	204	3350-3300, 1190-1130, 1500, 900	85.30 (88.42)	8.84 (7.28)	5.85 (5.25)
CDBA	66-67 white powder	39	204	3100-3000, 1500, 860-800	70.79 (70.88)	5.38 (5.33)	3.93 (3.90)
CDBAO $.H_2O$	77-80 white crystal	14	205	3100-3000, 1500-1400, 900	64.61 (63.72)	5.42 (5.12)	3.59 (3.42)
CBA	- yellow liquid	17	203	3350,3100-3000,1500, 900, 660	63.18 (63.68)	4.92 (5.12)	5.26 (5.33)
PDBA	32-34 white crystal	59	207	3100-3000 1600-1400,	85.30 (85.18)	8.84 (8.90)	5.85 (5.90)
PDBAO $.H_2O$	104-105 white crystal	34	205	3100-3000, 1500, 980	74.69 (73.99)	8.48 (8.38)	5.12 (4.93)

amount of tertiary amine ($2.6x10^{-3}$ mol) (in dichloromethane) dropwise over thirty minutes at 0-4°C while stirring. The reaction was monitored by TLC and stopped when the level of N-oxides appeared to be maximal. The reaction mixture was washed with dilute sodium sulphite (2x15 ml), dilute sodium carbonate (2X15 ml), and saturated sodium chloride solution (2x15 ml) respectively to remove the excess of m-CPBA. DCM phases were bulked, dried with anhydrous sodium sulphate and evaporeted. PDBAO and CDBAO mixtures were obtained as white powders followed by washing with petroleum ether: TLC analyses, even after repeated washing, showed the presence of m-CPBA and the corresponding tertiary amines as impurities. Column chromatography was carried out with the solvent system of chloroform:methanol (80:20,v/v). CDBAO and PDBAO were obtained as white crystalls (Talbe 3). ^{1}H-NMR for CDBAO (MeOH, μ ppm): 4.51 (s,4H,$2xCH_2$), 4.79 (s,2H,CH2), 7.15-7.70 (m, 13H,Ar protons). MDBAO was synthesized from corresponding tertiary amine by action with hydrogene peroxide (15). MDBA ($0.91x10^{-3}$ mol) in glacial acetic acid (10 ml) was stirred for 48 hours at room temparature with hydrogen peroxyde (30%, $6x10^{-3}$ mol). Chilled sodium hydroxide solution (40%) was added to the reaction mixture dropwise at 0-4°C. The precipitate formed was filtered and washed with cold water. Pure MDBAO was obtained as a white powder by washing with cooled petroleum ether: acetone (95:5,v/v): several times (Table 3). N-Oxides gave a dark orange colour with Dragendorff reagent (16).

Characterisation of compounds

All the substrates and their potential metabolites synthesized were characterized by UV, IR, MASS data and elemental analysis. The elemental analysis showed that all N-oxydes synthesised absorbed one mol of water in their molecule. CDBA and its potential metabolites were also characterized by ^{1}H-NMR spectral spectral data using TMS as the internal standart. Analytical data are presented in Table 3.

Results and Discussion

The present method employed for the benzylation of aliphatic secondary amines gave rise to the desired products (12,17). In order to prepare secondary amines, either the corresponding amide or imine derivatives could be prepared and these intermediates could be reduced by $NaBH_4$ or $LiAlH_4$. But, when the secondary amines were prepared by this method, the yields of products were very low. Therefore, secondary amines were also obtained by benzylation of primary amines.

To synthesize the corresponding N-oxides, two methods were employed ie. PDBAO and CDBAO were prepared by the action of m-CPBA on the corresponding tertiary amines and MDBAO was prepared by the action of H_2O_2 (30%) in glacial acetic acid on the MDBA (15).

The El-Mass spectral analysis of substrates and their potential metabolites showed the expected molecular ion peaks and fragmentation patterns (Table 4). In each spectrum, the base peak corresponded to the probable structure. A proposed fragmentation pathway for PDBAO is shown in Fig.3.

IR analyses of tertiary amines and their potential metabolites showed aromatic C-H and C=C stretching bands at the 3100-3000 and 1600-1400 cm^{-1} regions respectively. The presence of N-oxides was confirmed by a band observed at the 980-970 cm^{-1}. Secondary amines were characterized by a weak N-H stretching band at the 3350-3250 cm^{-1} region.

Elemental analysis was consistent with the required structures (Table 3). ^{1}H-NMR spectroscopy confirmed the structre of CDBA and its potential metabolites.

Incubation of substrates with hepatic rat microsomal preparations produced a variety of metabolites. All substrates showed metabolic debenzylation as expected. The results obtained from HPLC analysis indicate that two different metabolic pathways are present in debenzylation reactions. Ring cleavage via carbinolamine as intermediate occur in both non-substituted benzyl and substituted benzyl rings. The HPLC chromatogram of MDBA and its potential metabolites *in vitro* MDBA metabolites formed by hepatic rat microsomes are shown in figures 2a and 2b. The formation of dealkylated products was observed by using only HPLC (but not TLC). No metabolites were observed in control experiments using denaturated microsomes or in the absence of confactors. This demonstrated that the products were enzymically formed and metabolic reaction were required NADPH as co-factor. No N-oxides were detected in any case. The electronic effect of benzyl groups may prevent N-oxidation of the tertiary amine substrates used in this study. Alternatively, these N-oxides may decompose in the incubation conditions or may be intermediates of metabolic dealkylation (18). In order to clarify which mechanism is operative,

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Fig.3. Proposed electron impact mass fragmentation pathyways for PDBAO

$(C_6H_5-CH_2)_2\overset{+}{N}(O^-)-CH(CH_3)_2$ $\rceil^{+\cdot}$

$[M]^{+\cdot}$; m/z : 255

M-16

$(C_6H_5-CH_2)_2\overset{+\cdot}{N}-CH(CH_3)_2$

m/z : 239

$- CH_3^{\cdot}$

$(C_6H_5-CH_2)_2\overset{+}{N}=CH-CH_3$

m/z : 224

$- C_6H_5-CH_2^{\cdot}$

$C_6H_5-CH_2=\overset{+}{N}(O^-)-CH(CH_3)_2$ $\rceil^{+}$

m/z : 164

$C_6H_5-CH_2^{+}$

m/z : 91

$-CH_2^{\cdot}$

$C_6H_5^{+}$

m/z : 77

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Table 4. Major ion peaks in the direct electron impact (70eV) mass spectra of substrates and their potential metabolites (see table 2 for abbreviations)

Compound	Molecular formula (M.W.)	m/z (% Relative intensity)
MDBA	$C_{24}H_{27}N$ (329.487)	65 (7.2), 91 (62.1), 106 (22.4), 132 (100), 196 (59.1), 238 (8.3), 329 (24.6)
MDBAO .H_2O	$C_{24}H_{29}NO_2$ (363.503)	105 (6.1), 119 (23.5), 133 (100), 212 (3.7), 254 (2.5), 329 (0.16), 345 (4.5)
MBA	$C_{17}H_{21}N$ (239.362)	65 (4.2), 77 (4.9), 91 (29.6), 106 (17.1), 132 (100), 148 (1.6), 239 (27.5)
CDBA	$C_{21}H_{19}Cl_2N$ (356.29)	65 (13.3), 91 (100), 159 (24.9), 210 (11), 278 (43.4), 281 (5.7), 354 (7.3), 355 (40.3)
CDBAO .H_2O	$C_{21}H_{21}Cl_2NO_2$ (390.406)	91 (61.5), 159 (51.5), 182 (7), 194 (5), 212 (100), 280 (5.5), 371 (20)
CBA	$C_{14}H_{13}Cl_2N$ (266.157)	91 (100), 106 (32.5), 160 (10.2), 174 (59.2), 188 (26.2), 266 (56.5)
PDBA	$C_{17}H_{21}N$ (239.362)	65 (46.5), 83 (15), 91 (83), 105 (7.50), 132 (20), 162 (17), 181 (43), 224 (100), 239 (47.90)
PDBAO .H_2O	$C_{17}H_{23}NO_2$ (273.378)	77 (9), 91 (100), 105 (6.5), 134 (8), 164 (23.5), 224 (28.5), 239 (12), 255 (15)

it is neccessary to investigate the *in vitro* metabolic fate of these N-oxides as substrates. This is currently being investigated in our laboratory.

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