Metabolism of Oxybutynin:† Establishment of Desethyloxybutynin and Oxybutynin *N*-Oxide Formation in Rat Liver Preparations Using Deuterium Substitution and Gas Chromatographic Mass Spectrometric Analysis

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Oxybutynin is rapidly metabolized in rat liver microsomes. Two major primary oxidation products were identified as *N*-desethyl oxybutynin and oxybutynin *N*-oxide. Deuterium substituted substrate was used to aid the identification. *N*-Desethyl oxybutynin was characterized by gas chromatography electron impact mass spectrometry as its trifluoroacetamide derivative and oxybutynin *N*-oxide was indicated by the presence of a decomposition product, 2-oxo-3-butenyl-2 cyclohexyl-2-phenylglycolate, as elucidated from the gas chromatographic mass spectrometric analysis. The formation of this product from synthetic oxybutynin *N*-oxide was verified and occurs by two consecutive rearrangements upon thermolysis of the unstable *N*-oxide. Attempted titanous chloride reduction of oxybutynin *N*-oxide resulted in the formation of the hydrolytic products 2-cyclohexyl-2-phenylglycolic acid and 4-diethylamino-2-butynol.

INTRODUCTION

Oxybutynin (OB), an α-acetylenic amine with anticholinergic properties which was previously used in the treatment of gastrointestinal hypermotility,[1,2] has recently regained interest as a drug for treatment of urinary bladder disorders.[3,4] Although it was observed as early as 1965 that in the rat[1] oxybutynin is almost entirely metabolized, no reports have so far been published concerning the identification of the metabolites. In the course of studies on mechanistic details of the metabolic transformation of α-acetylenic amines[5–7] and as part of a pharmacological re-evaluation of oxybutynin, the metabolism of this amine is presently being investigated.

This paper reports on the formation, isolation and characterization of desethyloxybutynin (DOB) and oxybutynin *N*-oxide (OBNO) which appear to be the major primary oxidation products in rat liver microsomes. In the chemical characterization, which is based on comparative mass spectrometric and gas chromatographic mass spectrometric studies, including the use of deuterium substituted substrate, emphasis is placed on the discovered chemical instability of OBNO. This is one of two simultaneously presented reports describing the gas chromatographic mass spectrometric properties of unstable, drug-related *N*-oxides of tertiary α-acetylenic amines. For the other report, see *Biomed. Mass Spectrom.* **8**, 514 (1981).

† 4-Diethylamino-2-butynyl 2-cyclohexyl-2-phenylglycolate.

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Abbreviations: OB = oxybutynin; DOB = *N*-desethyl oxybutynin; OBNO = oxybutynin *N*-oxide; OBCPG = 2-oxo-3-butenyl 2-cyclohexyl-2-phenylglycolate; CPGA = 2-cyclohexyl-2-phenylglycolic acid; DEBO = 4-diethylamino-2-butynol; FNBT = 4-fluoro-3-nitrobenzotrifluoride; PCPG = 4-piperidino-2-butynyl 2-cyclohexyl-2-phenylglycolate; TFA = trifluoracetyl.

EXPERIMENTAL

Instrumentation

Infrared (IR) and nuclear magnetic resonance (NMR) spectra were routinely recorded for the whole synthetic sequence and were in full accordance with the proposed structures. The apparatus used was a Perkin–Elmer 599B spectrophotometer and a Perkin–Elmer R-12 spectrometer, respectively. NMR spectra were obtained in C^2HCl_3 solutions unless otherwise stated. Chemical shifts are expressed in ppm. (δ) with tetramethylsilane as the internal marker.

Gas–liquid chromatography (GC) was performed on a Pye–Unicam 104 chromatograph with flame ionization detection (FID). A 1.8 m × 2 mm glass column containing 3% OV 17 on Gas Chrom Q (Supelco^R) was used. The temperatures of the inlet and detector were 270 and 300 °C respectively. The column temperature was 230 °C and the N_2 carrier gas flow 40 ml min^{-1}.

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Mass spectral identification of the metabolites was performed on a Varian MAT 311A gas chromatograph mass spectrometer connected to a PDP 11/34 computer system. Spectra were obtained under electron impact (EI) conditions at an ionizing voltage of 70 eV unless otherwise stated. The trap current was 1000 μA, the accelerating voltage 3.0 kV and the temperature of the ion source around 250 °C. The gas chromatograph was equipped with a 0.3 mm × 2.5 m capillary column coated with SE 30 and connected to the mass spectrometer by open split coupling. The column was operated at temperatures around 220 °C with He as the carrier gas. Computerized background subtractions were made when analysing the metabolic extracts.

In one set of experiments in which OBNO and its decomposition products were analysed, gas chromatography mass spectrometry (GCMS) was performed on a Varian 1400 gas chromatograph combined with an LKB 9000 mass spectrometer. In this case the gas chromatograph mass spectrometer was connected to a Raytheon 704 computer system.[8] The gas chromatograph was equipped with a 2 mm × 1.5 m silanized glass column containing Chromosorb G coated with 3% OV 101. The injector and column temperatures were 230 °C and 200 °C, respectively. The ionizing potential was 18 eV, the trap current 60 μA and the accelerating voltage 3.5 kV. The temperature of the ion source was 290 °C. Direct inlet (DI) analysis was performed on the LKB 9000 under conditions similar to those used for GCMS.

Melting points were determined with a Leitz 350 hot stage microscope and are uncorrected. The microanalyses were carried out at Kemicentrum, University of Lund, Box 740, S-222 07 Lund, Sweden, and were correct within 0.4% of the theoretical value.

Analytical thin-layer chromatography (TLC) was done on precoated silica gel (60 PF, Merck) plates. Preparative TLC was performed with Merck's silica PF gel, spread on glass plates to a thickness of 1 mm.

Chemicals and synthesis

Oxybutynin-HCl was obtained from Bristol-Myers, New York. Technical grade methyl 2-cyclohexyl-2-phenylglycolate was kindly provided by Dr Stanley Dykstra at Mead Johnson, Evansville, USA. Perdeuteroparaformaldehyde with a minimum isotopic purity of 99% was obtained from E. Merck, Darmstadt; glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH from Sigma Co., St Louis, USA. 1-Fluoro-2-nitro-4-trifluoromethylbenzene was obtained under the name of 4-fluoro-3-nitrobenzotrifluoride (FNBT) from Pierce, Rockford, USA. Other chemicals were obtained from various commercial sources and were, where necessary, of analytical grade.

Stock phosphate buffer solutions were prepared by dissolving KH_2PO_4 (81.7 g) in water (800 ml), adjusting the pH to 7.5 by titration with KOH (5 M) and diluting with water to 1000 ml. The water used was doubly distilled.

2-Cyclohexyl-2-phenylglycolic acid (CPGA).[9] Technical grade methyl 2-cyclohexyl-2-phenylglycolate was purified by repeated vacuum distillation and the fraction boiling at 125–130 °C/0.4–0.5 mm Hg (53–67 Pa) was collected and used in the hydrolysis.

Methyl 2-cyclohexyl-2-phenylglycolate (5.0 g; 0.02 mol), 20% sodium hydroxide (15 ml) and methanol (40 ml) were stirred at 50 °C for 3 h. The mixture was cooled in ice, made acidic (pH about 1) with conc. HCl and the free acid was extracted into ether. The solution was dried ($MgSO_4$) and concentrated *in vacuo*. Treatment of the residue with light petroleum yielded the acid as white crystals. Yield, 4.3 g (91%); m.p., 159–161 °C. IR (KBr): ν_{max} (cm^{-1}): 3400 (O—H), 3400–2300 (COOH) and 1730 (C=O). NMR (C^2HCl_3 + 10% $C^2H_3O^2H$)(δ): 7.78–7.10 (m, 5H, aromatic), 4.28 (s, 2H, OH, COOH) and 2.45–0.90 (m, 11H cyclohexyl). Mass spectrometry: *m/z* (%): 234 (2), 189 (79), 171 (4), 153 (100), 129 (6), 107 (35), 106 (30), 105 (27), 91 (11), 83 (11), 79 (10), 77 (10), 55 (21) and 41 (5).

2-Propynyl 2-cyclohexyl-2-phenylglycolate. A mixture of CPGA (4.2 g; 0.018 mol), lithium carbonate (1.4 g; 0.019 mol) and propargyl bromide (2.3 g; 0.019 mol) in dimethylformamide (50 ml) was stirred at 40 °C for 12 h. Water (25 ml) was then added and the ester was extracted into ether. The solution was washed with brine, dried ($MgSO_4$), concentrated and distilled *in vacuo*. Yield, 4.2 g (86%); b.p., 135 °C/0.01 mm Hg (1.3 Pa). Anal.: ($C_{17}H_{20}O_3$): C,H. IR (NaCl): ν_{max} (cm^{-1}): 3520 (O—H), 3300 (≡C—H), 2140 (C≡C) and 1730 (C=O), NMR(δ): 7.80–7.14 (m, 5H, aromatic), 4.71 (t, 2H, O—CH_2—C≡C), 3.57 (*s*, 1H, OH), 2.45 (t, 1H, C≡CH) and 2.45–0.75 (m, 11H, cyclohexyl). Mass spectrometry: *m/z* (%): 189 (100), 170 (3), 151 (23), 129 (5), 107 (34), 105 (59), 91 (13), 79 (11), 77 (13), 55 (15) and 41 (6).

4-Diethylamino-2-but[4,4-$^2H_2$]ynyl 2-cyclohexyl-2-phenylglycolate. 2-Propynyl 2-cyclohexyl-2-phenylglycolate (400 mg; 1.47 mmol), perdeuteroparaformaldehyde (61 mg; 1.91 mmol), diethylamine (118 mg; 1.62 mmol), glacial acetic acid (97 mg; 1.62 mmol) and a trace of CuCl in dioxane (10 ml) were stirred at 70 °C for 3 h. Water (5 ml) was added and the mixture was acidified to pH 1 (6 M HCl) and washed with ether (2 × 5 ml). The aqueous phase was made alkaline with K_2CO_3 and extracted with ether (4 × 10 ml). The etheral extract was dried (K_2CO_3) and the solvent was removed *in vacuo*. The residue was dissolved in ether and the product was precipitated with HCl in ether. Recrystallization from ethanol–ether, yielded 470 mg (80%) of deuterated oxybutynin hydrochloride, m.p. 116–118 °C. IR (KBr): ν_{max} (cm^{-1}): 3600–3200 (O—H, N^+—H), 2750–2430 (N^+—H), 1730 (C=O). NMR(δ): 7.80–7.10 (m, 5H, aromatic), 4.77 (s, 2H, O—CH_2—C≡C), 3.49 (s, 1H, OH), 2.91 (q, 4H, N—CH_2) and 2.50–0.90 (m) + 1.32 (t) (17 H, cyclohexyl + CH_3). Mass spectrometry: *m/z* (%): 360 (2), 359 (8), 358 (2), 344 (100), 343 (2), 342 (5), 276 (6), 190 (6), 189 (41), 128 (8), 127 (6), 126 (4), 107 (23), 105 (27), 99 (17), 91 (10), 79 (7), 77 (5), 56 (9) and 55 (10). The minimum isotopic purity was 98% (GCMS).

4-Ethylamino-2-butynyl 2-cyclohexyl-2-phenylglycolate (DOB). Oxybutynin (2.0 g; 5.6 mmol) dissolved in diethyl ether (10 ml) was cleaved by adding cyanogen

bromide (0.6 g; 5.6 mmol) in diethyl ether (10 ml) and stirring the mixture at room temperature for 18 h.[10] Most of the ether was then evaporated and the residue was fractionated on a silica gel column using a mixture of diethyl ether and light petroleum as the eluent. Evaporation of the solvent *in vacuo* gave 4-bromo-2-butynyl 2-cyclohexyl-2-phenylglycolate as an oil, which was used without further purification. Yield, 1.5 g (75%). IR (NaCl), ν_{max} (cm^{-1}): 3520 (O—H), 1730 (C=O) and 615 (C—Br). NMR(δ): 7.77–7.12 (m, 5H, aromatic), 4.75 (m, 2H, O—CH_2—C≡C), 3.81 (t, 2H, Br—CH_2—C≡C), 3.52 (s, 1H, OH) and 2.50–0.90 (m, 11H, cyclohexyl). Mass spectrometry: *m/z* (%): 349 (0.4), 347 (0.4), 285 (0.3), 284 (2), 283 (2), 282 (2), 281 (2), 233 (1), 191 (3), 189 (100), 171 (4), 152 (4), 151 (45), 133 (7), 131 (6), 129 (7), 108 (4), 107 (51), 106 (9), 105 (76), 91 (20), 82 (10), 79 (16), 69 (4), 67 (5), 55 (20), 52 (12), 51 (7) and 41 (8).

The bromide (0.5 g; 1.4 mmol) was stirred with an excess of ethylamine (0.3 g; 6.7 mmol) in toluene (5 ml) at room temperature for 15 h. The reaction mixture was washed with a 10% K_2CO_3 solution and the organic solvent was removed *in vacuo*. Purification on preparative silica gel plates (60 F-254, E. Merck) with diethyl ether as the mobile phase yielded 4-ethylamino-2-butynyl 2-cyclohexyl-2-phenylglycolate. IR (NaCl), ν_{max} (cm^{-1}): 3500–3400 (O—H, N—H) and 1730 (C=O). NMR(δ): 7.77–7.10 (m, 5H, aromatic), 4.70 (m, 2H, O—CH_2—C≡C), 3.37 (t, 2H, N—CH_2—C≡C), 2.62 (q, 2H, N—CH_2—C), 2.45 (s, 2H, NH and OH) and 2.50–0.90 (m) + 1.07 (t) (14 H, cyclohexyl + CH_3). Mass spectrometry: *m/z* (%): 329 (1), 296 (3), 247 (21), 246 (10), 190 (17), 189 (100), 171 (7), 129 (7), 114 (5), 107 (41), 105 (18), 97 (81), 95 (13), 94 (5), 91 (16), 83 (5), 82 (4), 81 (9), 80 (7), 79 (14), 77 (9), 69 (8), 68 (4), 67 (7), 57 (5), 56 (5), 55 (14), 44 (11), 42 (5), 41 (13). Treatment of the base with HCl in ether gave the HCl salt which was recrystallized from ethanol + ether. Yield, 150 mg (30%); m.p. 142–144 °C. The purity of the product was verified by TLC using $CHCl_3 + CH_3OH + NH_3$ (90:10:1) as the developing system. After drying, the plate was visualized by ultraviolet (UV) irradiation (254 nm) and by spraying with iodine (2% in methanol); only one spot with R_f 0.68 could be detected. The purity was also tested by gas chromatography. The gas chromatographic analysis showed a purity of ≥99%.

4-Diethylamino-2-butynol (DEBO). The amino alcohol was prepared according to Cologne and Poilane.[11] Yield, 56%. IR (NaCl), ν_{max} (cm^{-1}): 3500–3000 (O—H) and 3000–2800 (C—H). NMR(δ): 4.22 (t, 2H, O—CH_2—C≡C), 4.07 (s, 1H, -OH), 3.41 (t, 2H, C≡C—CH_2—N), 2.47 (q, 4H, N—CH_2) and 1.07 (t, 6H, $-CH_3$). Mass spectrometry *m/z* (%): 141 (18), 127 (9), 126 (100), 86 (6), 58 (15), 56 (8) and 41 (11).

4-Diethylamino-2-butynyl 2-cyclohexyl-2-phenylglycolate *N*-oxide (OBNO). To a solution of OB (1.5 g, 4 mmol) in EtOH (5 ml) was added a solution of H_2O_2 (1.2 g, 35%, 12 mmol). After completed oxidation (24 h) at room temperature, excess H_2O_2 was destroyed by addition of a Pt foil and subsequent stirring for 2 h. The solvent was then evaporated *in vacuo* without heating. To remove solvent residues, the colourless, viscous residue was repeatedly redissolved in EtOH and then ether, with evaporation in between, and finally dried *in vacuo* (1 mm Hg, 133 Pa). The whole procedure was performed at the lowest possible temperature, as the product decomposes under colouration at ambient temperatures. The crude product 1.2 g (80%) was stored under N_2 at −20 °C. The NMR spectrum of the crude product was compatible with an *N*-oxide but showed the presence of impurities. Thus, as compared with the spectrum of OB, the changes in shifts 3.37 → 4.10 (N—CH_2—C≡C), 2.62 → 3.33 (N—CH_2—C) and 4.70 → 4.76 (O—CH_2—C≡C) indicated the presence of an oxidized nitrogen. However, a peak at 4.55 constituting about 15% of that at 4.10 (N—CH_2—C≡C) suggested the presence of allenic protons (>C=C=CH_2), which indicated a rearrangement product present as an impurity. Analytical TLC (MeOH + EtAc + TEA, 85:15:12) revealed two significant components with R_f values of 0.50 and 0.80. Similarly, preparative TLC ($CHCl_3$ + MeOH, 3:1) revealed two bands as visualized by UV irradiation. These were scraped off and the products were eluted with MeOH. After evaporation of the extract, from the lower band crystalline material was obtained. Direct inlet mass spectrometric analysis (Fig. 6) gave results in accordance with the *N*-oxide structure. Attempts to recrystallize the material from ether were unsuccessful, with a rapid colouration of the solution.

4-Piperidino-2-butynyl 2-cyclohexyl-2-phenylglycolate (PCPG). The synthesis of this amine, used as internal standard for the quantitation of OB, has recently been described.[12]

Microsomal incubations

Microsomes were prepared as described earlier[13] from livers of male Sprague-Dawley rats (200–250 g). Incubations were conducted aerobically for up to 20 min at 37 °C in 50 ml Erlenmeyer flasks and were run in duplicate. Microsomal suspension equivalent to 0.5 g wet liver (~10 mg protein) was incubated in a total volume of 5 ml of 0.15 M potassium phosphate buffer at pH 7.5 containing 1.5 μmol NADP, 18 μmol glucose-6-phosphate, 20 μmol of $MgCl_2$, 5 IU glucose-6-phosphate dehydrogenase and OB or [$^2H_2$]OB as stated. The incubation was started by the addition of the microsomes.

Analytical procedures

The metabolic reaction was terminated by transferring the entire contents of the flasks to 50 ml extraction tubes containing 0.5 ml 20% $ZnSO_4$ solution. Precipitated protein was removed by centrifugation and the pellets were washed with 1.15% KCl (1 ml). After centrifugation and adjustment of the pH to 9.0 (2 M Na_2CO_3) the combined aqueous solution and washings were extracted with CH_2Cl_2 (1 × 5 ml). The CH_2Cl_2 extracts were filtered through silanized glass wool, transferred into 12 ml conical centrifuge tubes and evaporated to near dryness under a stream of dry N_2. The residues were dissolved in 0.5 ml CH_2Cl_2 and treated with trifluoroacetic anhydride (50 μl) at room temperature overnight. The samples were then washed

with saturated $NaHCO_3$ (1 ml), centrifuged and subjected to gas chromatographic and gas chromatographic mass spectrometric analysis. Reference samples were prepared by adding OB and DOB (see chemicals and synthesis), each to a final concentration of 100 μM, to microsomal suspensions (5 ml), omitting the cofactors, and then immediately carrying them through the assay.

To estimate the disappearance of OB a modification of the previously described gas chromatographic mass spectrometric method[12] was used, substituting a flame ionization detector for the mass spectrometer. In these cases an appropriate amount of internal standard (PCPG) was added to the samples prior to extraction. Standard curves were prepared from incubation mixtures containing OB and PCPG but without cofactors.

Microsomal protein[14] and cytochrome P-450[15] were determined according to standard methods.

Stability studies of oxybutynin *N*-oxide

The behaviour of OBNO when exposed to elevated temperatures in a gas chromatograph injection block was investigated by injecting dichloromethane solutions (1 mM) of synthetic OBNO and monitoring the gas chromatographic effluent by FID and mass spectrometric analysis.

To investigate the behaviour of OBNO under the experimental conditions for $TiCl_3$ reduction,[16] 0.5 mmol of synthetic OBNO was dissolved in 1 ml EtOH to which was added 5 M HCl (2 ml) and 15% $TiCl_3$ (5 M, 1 ml). The mixture was left overnight at ambient temperature and the pH was then brought to above 10. The alkaline aqueous solution was extracted with CH_2Cl_2 (2 × 5 ml), filtered through silanized glass wool, evaporated under dry N_2 to a small volume and analysed by GCMS.

In one set of experiments the CH_2Cl_2 extract was evaporated to dryness and the residue was dissolved in 0.1 ml of saturated $Na_2B_4O_7$ to which was added 1 ml of a FNBT solution (0.3 ml in 100 ml DMSO).[17] The mixture was left at room temperature for 2 h, after which H_2O (3 ml) was added and the mixture was extracted with CH_2Cl_2 (5 ml). The organic extract was filtered through silanized glass wool, evaporated to a small volume and analysed by GCMS. Reference samples were prepared by dissolving DEBO (see chemicals and synthesis) (0.1 mmol) in saturated $Na_2B_4O_7$ and processing the samples as above.

To investigate for the presence of CPGA (see chemicals and synthesis) formed as a result of ester cleavage, the acidic aqueous phase after $TiCl_3$ reduction was extracted with CH_2Cl_2 (1 × 10 ml). After filtration through silanized glass wool, the solvent was evaporated at low temperature, the residue treated with diazomethane in ether[18] and analysed by GCMS. Reference samples were prepared by dissolving synthetic CPGA (0.1 mmol) in the 5M HCl–$TiCl_3$ mixture and then carrying it through the assay.

RESULTS AND DISCUSSION

The oxidative functionalization of OB is associated with the microsomal fraction of the liver, requires oxygen and is NADPH-dependent. At a 100 μM initial substrate concentration a rapid decrease in the OB level was seen (Fig. 1), indicating a very rapid metabolism. Gas chromatographic analysis of the trifluoracetyl (TFA)-derivatized methylene chloride extracts exhibited six clearly separated peaks (Fig. 2), three of which could be attributed to OB (peak 3) and related structures (peaks 2 and 4). Thus, peaks 2 and 4 were absent from extracts from zero time incubates, from incubations carried out with heat-inactivated microsomes and when cofactors had been omitted. The intensity of both these gas chromatographic peaks increased with incubation time relative to that of OB (peak 3).

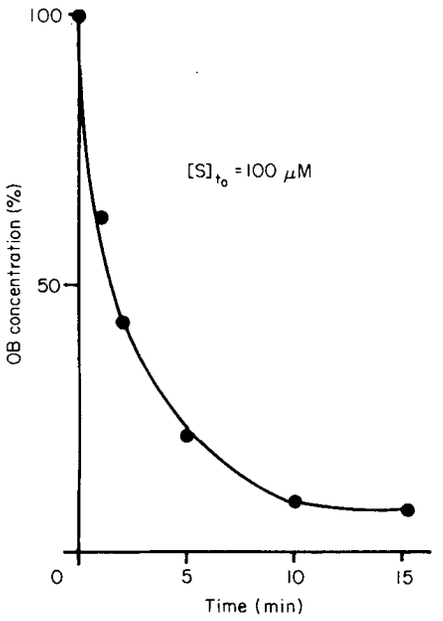


Figure 1. Disappearance of OB during NADPH-dependent metabolism in rat liver microsomes. Each point represents the mean of four incubations using two different liver preparations. SEM ≤ ±2%. For the experimental particulars see Experimental section.

The mass spectrum of OB present in peak 3 is shown in Fig. 3. The spectrum contains a molecular ion at m/z 357 and an $[M-15]^+$ base peak ion at m/z 342, but is dominated by the formation of the m/z 189 ion and its further decomposition. Further details on the fragmentation pattern have been published elsewhere[12] and data on the mass spectrum of $[^2H_2]$OB are given in the Experimental section.

After incubation of OB and $[^2H_2]$OB, the mass spectra of gas chromatographic peak 4 contained fragment ions m/z 342, 193, 192, 189 and 344, 195, 194, 189 (Fig. 4(a)), respectively, suggesting a TFA derivative of DOB, which was confirmed by absolute identity between the spectra of the TFA derivatives of the non-deuterated metabolite and reference sample (Fig. 4(b)). The molecular ion is missing from both spectra. The fragmentation pattern is dominated by the base peak ion m/z 189 and its subsequent decomposition, indicating an intact 2-cyclohexyl-2-phenylglycolate moiety (cf. Fig. 3). The uneven fragment ion m/z 193 arises through C—O cleavage in the acetylenic chain concomitant to hydrogen migration, a fragmentation analogous to that seen in the mass spectrum of 4-piperidino-2-butynyl 2-cyclohexyl-2-phenylglycolate.[12]

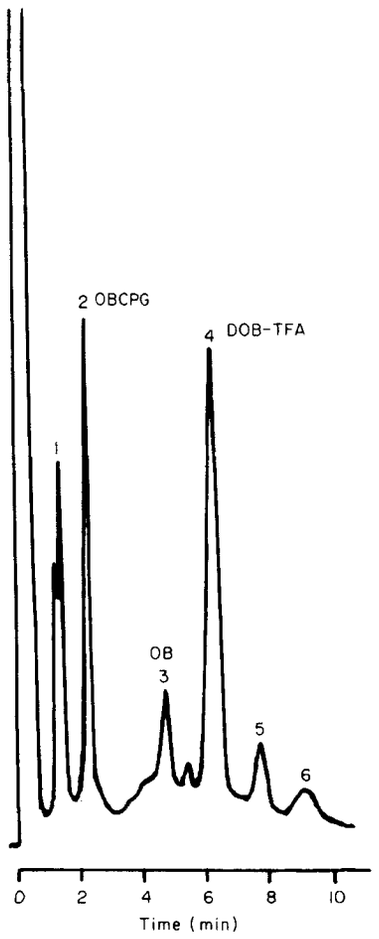


Figure 2. Gas chromatogram of an extract after a 10 min incubation of OB with a rat liver microsomal preparation. Peaks 2, 3 and 4 represent OB and identified products. Peak 1 was present in the blank and t_0 extracts. Peaks 5 and 6 comprise as yet unidentified products. For the gas chromatographic conditions see Experimental section.

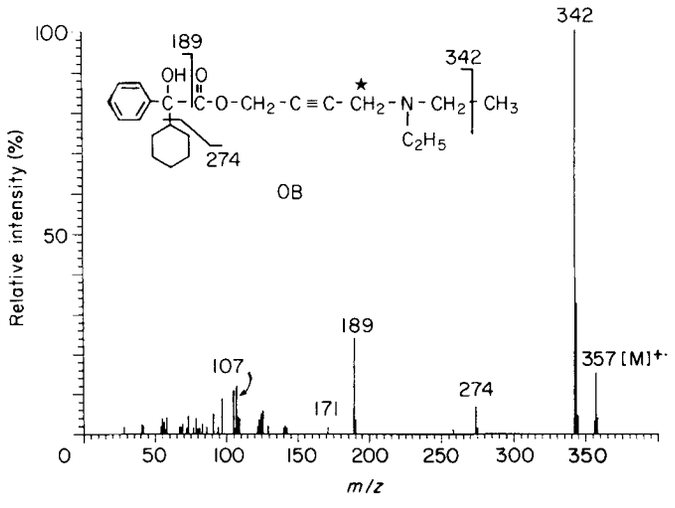


Figure 3. EI mass spectrum of OB, present in peak 3 in Fig. 2, obtained with the Varian Mat 311 A gas chromatographic mass spectrometric system. For details, see Experimental section. The asterisk denotes the C-4 position carrying the label in $[^2H_2]$OB.

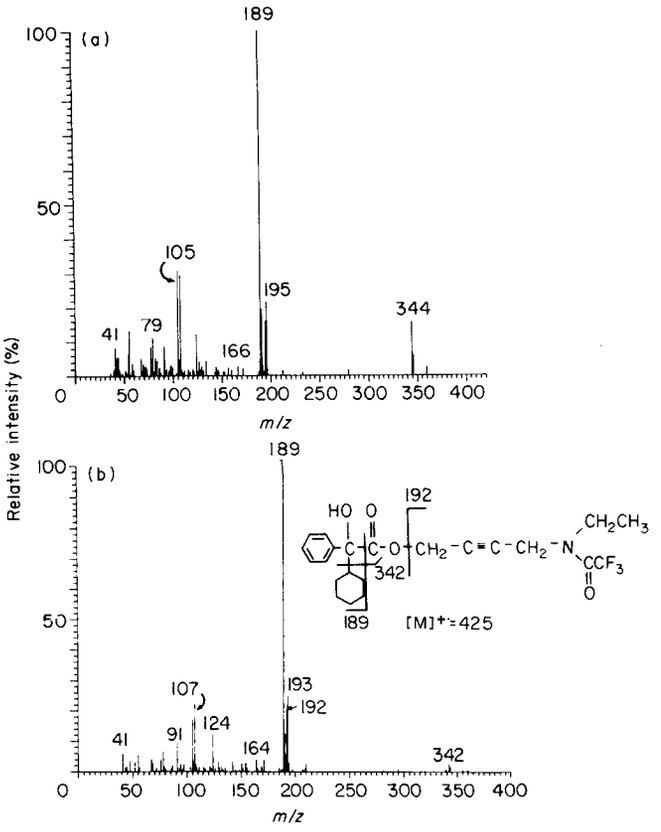


Figure 4. EI mass spectrum of peak 4 in Fig. 2 after incubation of $[^2H_2]$OB (a) and of a TFA derivatized reference sample of DOB (b). The spectra were obtained with the Varian Mat 311 A gas chromatographic mass spectrometric system. Details are given in the Experimental section.

Gas chromatographic peak 2 in Fig. 1 showed a mass spectrum with ions present at *m/z* 189, 171, 151, 107, 105, 91 and 77, indicating the presence of an intact 2-cyclohexyl-2-phenylglycolate structure (cf. Fig. 3) but only a few other ions of diagnostic importance were observed, i.e. *m/z* 70, 202 and 220. The highest mass detected was found at *m/z* 220, 31 u above *m/z* 189, which is inconceivable as a molecular ion. However, when after incubation of $[^2H_2]$OB the corresponding mass spectrum was investigated (Fig. 5(a)), an upward shift of 2 u in masses *m/z* 70, 202 and 220 indicated the presence also of the 4-carbon residue of the 4-amino-2-butynyl chain. As none of the fragments could be attributed to ions with the charge residing on a basic nitrogen or to nitrogen containing a TFA residue, two options were considered—either the nitrogen was lost or it had undergone oxidation to an *N*-oxide. However, the loss of diethylamine, due to metabolic α-carbon oxidation at the C-4 propynylic carbon, could be excluded, as in that case at least one of its deuterium atoms would be lost. This left the *N*-oxide as a remaining possibility, and when a sample of synthetic OBNO was subjected to gas chromatographic mass spectrometric analysis (Fig. 5(b)), the mass spectrum of the most prominent gas chromatographic peak (Fig. 5(c)) was indeed identical to that of gas chromatographic peak 2 in the incubation extract.

Aliphatic *N*-oxides are characterized by a thermal instability,[19–21] which is pronounced in those of α-acetylenic amines.[22] Accordingly, it was conceivable

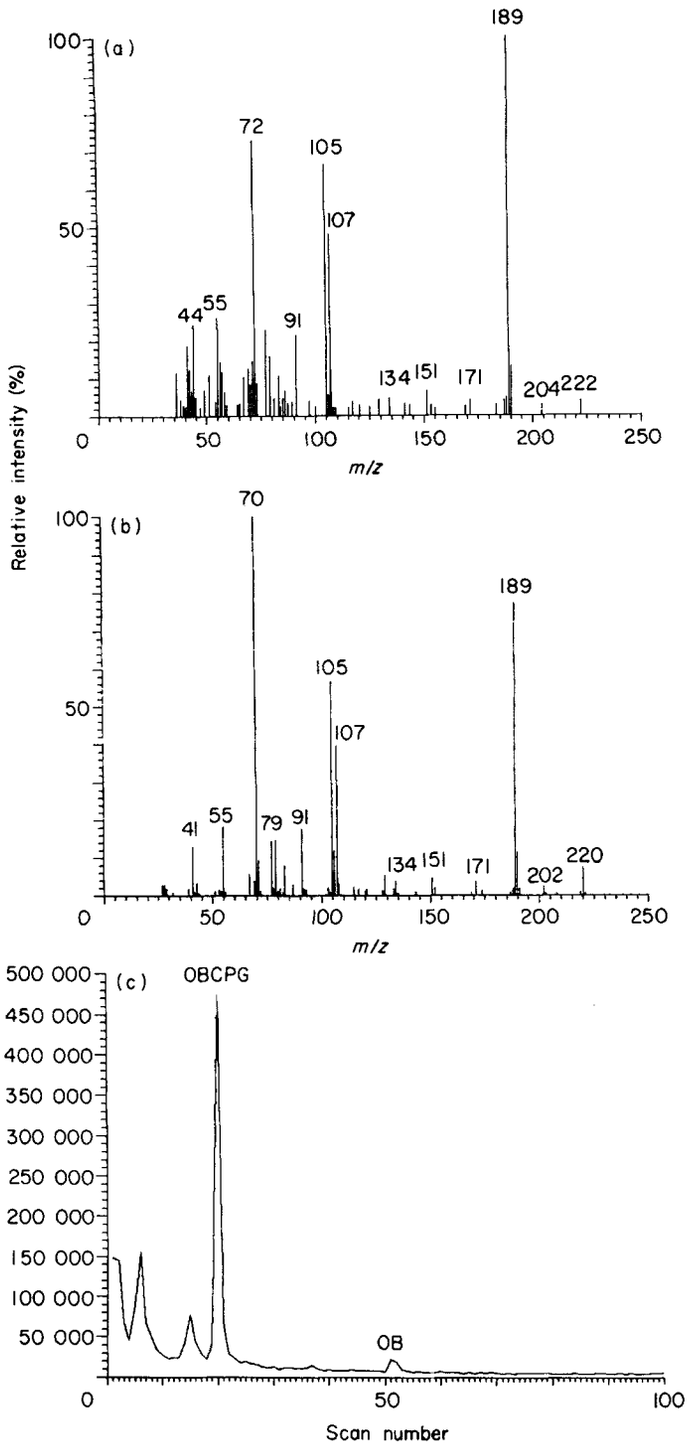
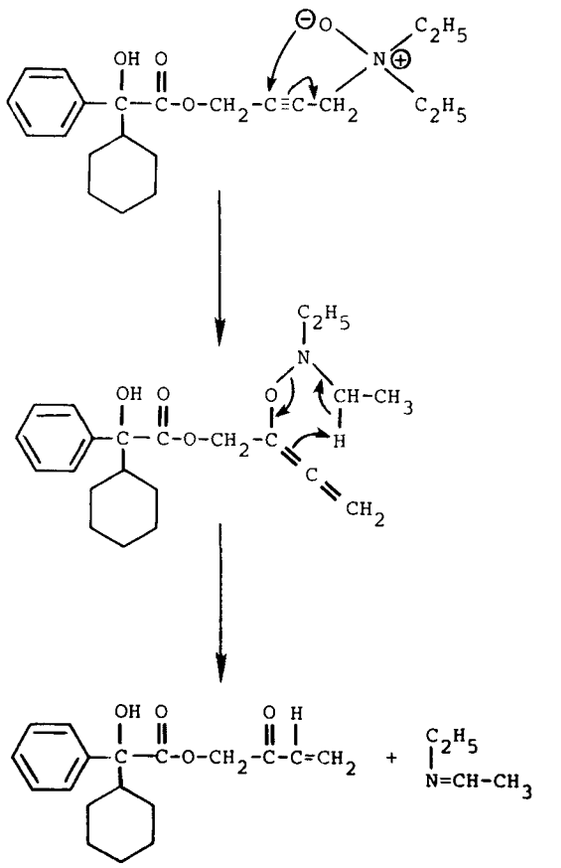


Figure 5. EI mass spectrum of peak 2 in Fig. 2 after incubation of $[^2H_2]$OB (a) and of the most prominent peak (spectrum 20) in the gas chromatographic pyrolysis of OBNO (b). (c) Total ion current profile after pyrolysis of OBNO. Data were obtained with the Varian Mat 311 A gas chromatographic mass spectrometric system. Details are given in Experimental section.

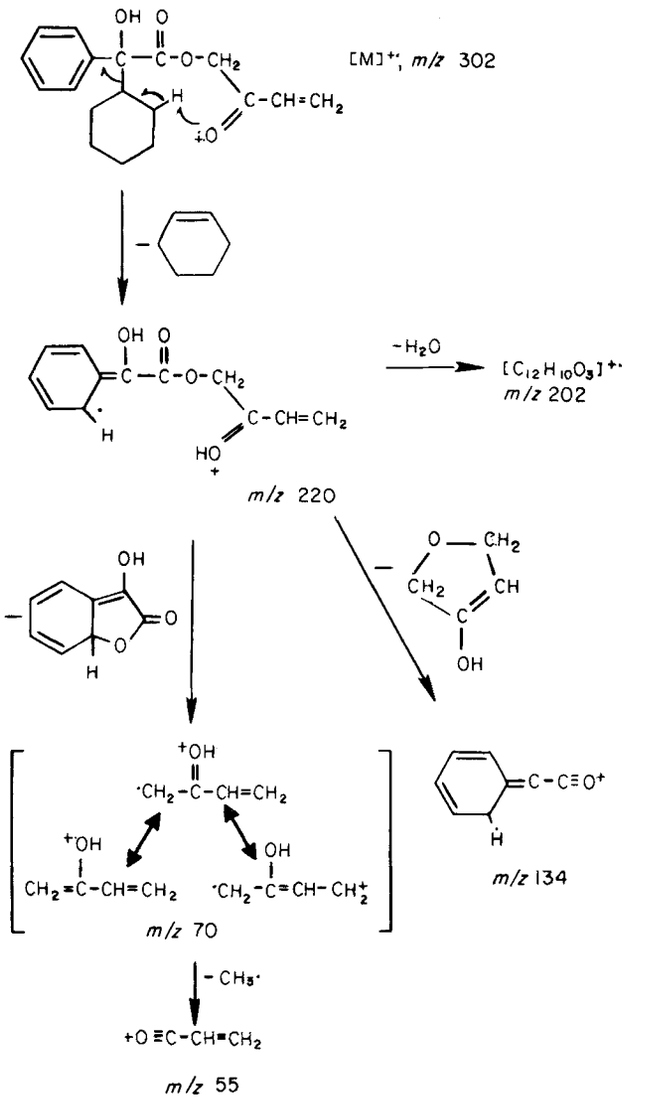


Scheme 1. Mechanism for the generation of OBCPG from the chemical decomposition of OBNO as concluded from the analogous decomposition of pargyline *N*-oxide.[7]

that the compound contained in the gas chromatographic peak 2 originated from OBNO formed by metabolism. Information on the structure and route of formation of the decomposition product was gained from our previous studies on pargyline, another α-acetylenic amine.[23] When subjected to gas chromatographic mass spectrometric analysis, pargyline *N*-oxide decomposes by two consecutive rearrangements, yielding propenal and Schiff bases.[23] With the same mechanism applied on OBNO (Scheme 1), one of the decomposition products should constitute an α,β-unsaturated ketone (2-oxo-3-butenyl 2-cyclohexyl-2-phenylglycolate, OBCPG) with a molecular weight of 302.

Although the mass spectrum in Fig. 5(b) is devoid of an $[M]^{+\cdot}$ ion at m/z 302, its fragmentation pattern (Scheme 2) is compatible with the structure of OBCPG in Scheme 1. Thus the loss of a cyclohexene entity from m/z 302 will give rise to a radical ion at m/z 220, which by loss of H_2O generates m/z 202. Cleavage of the CH_2—O bond results in the formation of the m/z 70 base peak ion, which in the case of the $[^2H_2]$-substituted compound retains both labels (m/z 72). Ejection of a methyl radical from m/z 70 after hydrogen scrambling then generates m/z 55 (in the $[^2H_2]$-substituted variant m/z 55, 56, 57).

Contrary to the spectrum obtained when synthetic OBNO was subjected to GCMS (Fig. 5), its DI mass spectrum (Fig. 6) was indicative of an *N*-oxide with a small but distinct $[M]^{+\cdot}$ ion present at m/z 373. Another ion of low intensity at m/z 342 indicates that the $[M]^{+\cdot}$ ion to some extent loses $CH_3O\cdot$, generating an immonium ion. Analogous losses of ·OH have been shown in the fragmentation of *N*-oxides of cyclic aliphatic amines.[21,24] Substantiated by a base peak ion at m/z 126, the DI spectrum of OBNO contributes additional evidence for the rearrangement as depicted in Scheme 1. Thus the formation of m/z 126 by direct



Scheme 2. Suggested fragmentation pattern compatible with the OBCPG structure in Scheme 1, cf. the mass spectrum in Fig. 5(b).

fission of the C-1–C-2 σ-bond in the acetylenic chain is unlikely, as this bond is rather adamant in most other compounds related to OB (cf. Figs 3, 4 and Ref. 24) and a rearranged, isomeric molecular ion (Fig. 6, cf. Scheme 1) can better explain the ease of formation of the base peak ion and also account for the presence of ions at *m/z* 220, 70 and 55, the latter previously encountered in the mass spectrum in Fig. 5(b). Moreover, an analogous, rearranged molecular ion was implicated in the fragmentation of the *N*-oxide of another α-acetylenic amine, BL 14 (*N*-(5-pyrrolidino-3-pentynyl)succinimide).[24]

Titanous chloride reduction followed by analysis of the amine formed is a method sometimes used to quantitate *N*-oxides in metabolic extracts.[25] This method, although successfully used for determination of pargyline and BL 14 *N*-oxides[6,7] in *in vitro* studies, did not work for OBNO. Thus, when a 15-min incubate, which was low in OB (cf. Fig. 1) and was expected to contain substantial amounts of OBNO, was reduced, subsequent gas chromatographic or gas chromatographic mass spectrometric analysis showed no increase in the OB level. Control experiments performed with synthetic OBNO gave the same results, however, and the acidic conditions used for the reduction, were shown to cause hydrolysis of the ester function in OB. The hydrolytic products, DEBO and CPGA, were both identified by GCMS as their FNBT (Fig. 7) and methyl ester[26] derivatives, respectively. As the formation and characterization of hydrolytic products in OB metabolism is extraneous to this report, further details of this matter will be presented elsewhere.

The metabolic studies of OB conducted so far indicate a compound which is extensively metabolized. The present work provides evidence that DOB and OBNO—both to be expected as a result of functionalization of a lipophilic tertiary amine—are distinct metabolites in rat liver preparations. The problems concerning the identification of tertiary *N*-oxides in metabolic mixtures are accentuated by the inherent chemical instability of OBNO. Knowledge of the chemical properties of *N*-oxides can explain the presence of decomposition products during the course of the analysis. The detection of the decomposition product was in this case a serendipity finding due to the overall lipophilic character of the *N*-oxide which made extraction into CH_2Cl_2 possible. That the *N*-oxide of OB undergoes a

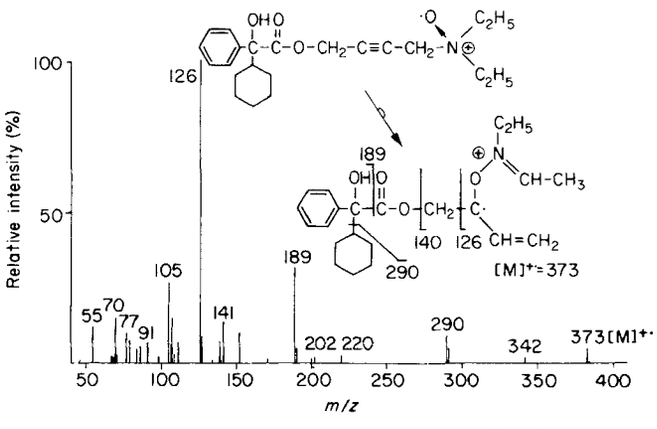


Figure 6. Direct insertion, EI mass spectrum of OBNO obtained on the LKB 9000 mass spectrometer. For further details, see Experimental section.

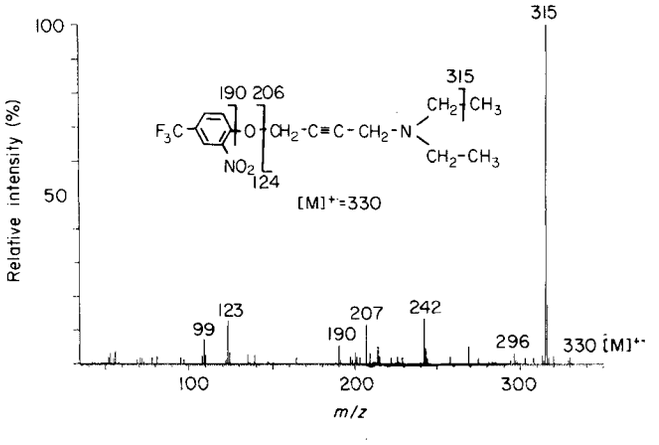


Figure 7. EI mass spectrum of FNBT-derivatized DEBO formed during attempted Ti^{3+} reduction of OBNO. The spectrum was obtained with the LKB gas chromatographic mass spectrometric system (see Experimental section).

rearrangement analogous to those of pargyline and BL 14 *N*-oxides suggests that this is a general feature of α-acetylenic *N*-oxides, a property that will undoubtedly render metabolic *N*-oxidation of α-acetylenic amines *in vivo* difficult to survey.

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REFERENCES

1. P. M. Lish, J. A. La Budde, E. L. Peters and S. I. Robbins, *Arch. Int. Pharmacodyn.* **156**, 467 (1965).
2. C. W. Hock, *Curr. Ther. Res.* **9**, 437 (1967).
3. A. C. Diokmo and J. Lapides, *J. Urol.* **108**, 307 (1972).
4. C. M. Fredericks, C. F. Anderson and D. L. Kreulen, *Invest. Urol.* **12**, 317 (1975).
5. B. Lindeke, G. Hallström, E. Anderson and B. Karlén, *Xenobiotica* **8**, 341 (1978).
6. G. Hallström, B. Lindeke and E. Anderson, *Xenobiotica* **11**, in press.
7. G. Hallström, B. Lindeke, A. H. Khuthier and M. A. Al-Iraqi, *Chem.-Biol. Interactions* **34**, 185 (1981).
8. C. G. Hammar, G. Pettersson and P. T. Carpenter, *Biomed. Mass Spectrom.* **1**, 397 (1974).
9. K. Hoffmann and H. Schellenberg, *Helv. Chim. Acta* **30**, 292, (1947).
10. J. B. Bicking, C. M. Robb, R. L. Smith, E. J. Cragoe Jr, F. A. Kuehl Jr and L. R. Mandel, *J. Med. Chem.* **20**, 35 (1977).
11. J. Colonge and G. Poilane, *Bull. Soc. Chim. Fr.* 502 (1955).
12. B. Lindeke, H. Brötell, B. Karlén, G. Rietz and A. Vietorisz, *Acta Pharm. Suec.* **18**, 25 (1981).
13. A. K. Cho, B. Lindeke and C. Y. Sum, *Drug Metab. Dispos.* **2**, 1 (1974).
14. P. J. Geiger and S. P. Bessman, *Anal. Biochem.* **49**, 467 (1967).
15. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2379 (1964).
16. R. T. Brooks and P. D. Sternglanz, *Anal. Chem.* **31**, 561 (1959).
17. J. Vessman and S. Strömberg, *Anal. Chem.* **49**, 369 (1977).
18. T. J. De Boer and H. J. Backer, in *Organic Synthesis*, Coll. Vol. IV, p. 250. J. Wiley & Sons, New York (1963).
19. N. Castagnoli Jr, J. C. Craig, A. P. Melikian and S. K. Roy, *Tetrahedron* **26**, 4319 (1970).
20. D. Schüller and H. P. Harke, *Org. Mass Spectrom.* **7**, 839 (1973).
21. J. K. Faulkner and K. J. A. Smith, *J. Pharm. Pharmacol.* **26**, 473 (1974).
22. A. H. Khuthier, M. A. Al-Iraqi, G. Hallström and B. Lindeke, *J. Chem. Soc. Chem. Commun.* 9 (1979).
23. G. Hallström, B. Lindeke, A. H. Khuthier and M. A. Al-Iraqi, *Tetrahedron Lett.* **21**, 667 (1980).
24. G. Hallström, B. Lindeke and E. Anderson, *Acta Pharm. Suec.* **17**, 31 (1980).
25. A. H. Beckett, J. W. Gorrod and P. Jenner, *J. Pharm. Pharmacol.* **23**, 55 S (1971).
26. H. R. Sullivan, J. G. Page and S. L. Due, *Biomed. Mass Spectrom.* **2**, 53 (1975).

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