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ASPECTS OF METABOLISM OF TAMOXIFEN BY RAT LIVER MICROSOMES

IDENTIFICATION OF A NEW METABOLITE: *E*-1-[4-(2-DIMETHYLAMINOETHOXY)-PHENYL]-1, 2-DIPHENYL-1-BUTEN-3-OL *N*-OXIDE

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Abstract—Metabolism of tamoxifen *N*-oxide by phenobarbitone-induced rat liver microsomes gave a major metabolite which was identified as *E*-1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenyl-1-buten-3-ol *N*-oxide (α-hydroxytamoxifen *N*-oxide) by comparison of mass spectral properties with synthetic material. This new metabolite was also formed from tamoxifen. Tamoxifen epoxide was synthesised; its microsomal metabolism gave the corresponding *N*-oxide. Neither tamoxifen epoxide nor its *N*-oxide was detected as a product of tamoxifen metabolism.

The synthetic antiestrogen tamoxifen {*Z*-(1-[4-(2-dimethyl - aminoethoxy)phenyl] - 1,2 - diphenyl - 1 - butene} (**1a**) is currently in use in the clinic for the treatment of estrogen-dependent breast cancer [1, 2]. Over recent years, much evidence has emerged that metabolism may play an important role in modulating the activity of this compound [3]. Originally a phenolic derivative (4-hydroxytamoxifen) was the only serum metabolite detected [4], but later Adam *et al.* [5] showed the major serum metabolite to be the *N*-desmethyl derivative. Hepatic microsomal metabolism studies showed a similar metabolic profile [6] with the identification of tamoxifen *N*-oxide (**1b**) [7] as a further metabolite. Subsequent metabolism studies of tamoxifen using hepatocytes (unpublished results, Dr I. B. Parr) revealed the presence of a minor metabolite which we were unable to identify at that time.

Comparison of the estrogen receptor (ER) binding of tamoxifen and its metabolites [8–10] showed that tamoxifen, *N*-desmethyltamoxifen and tamoxifen *N*-oxide have similar affinity to ER but that 4-hydroxytamoxifen has a much higher affinity. We were interested in elucidating the nature of the unidentified metabolite to determine whether it had significant biological activity. From mass spectral data it was thought that this metabolite could be an *N*-oxide and thereby possibly formed via tamoxifen *N*-oxide. For this reason we have studied the metabolism of tamoxifen *N*-oxide. The results are also of interest as part of a study in determining how a change in the basic nature of the side chain affects the metabolic profile and might be of interest in evaluating the possible use of tamoxifen *N*-oxide therapeutically.

We now report that using a phenobarbitone-induced rat liver microsomal system, a hitherto unreported metabolite is formed when either tamoxifen *N*-oxide or tamoxifen is the substrate. Tamoxifen epoxide (**2a**), which had been tentatively identified as a minor metabolite of tamoxifen using a phenobarbitone-induced rat liver microsomal system [11], was synthesised. Its properties and microsomal metabolism are reported. The results of our experiments are discussed in relation to how the alteration of the functionality of tamoxifen can influence the site of biotransformation within the molecule.

MATERIALS AND METHODS

Materials. Tamoxifen (**1a**) and other starting triphenylbutene derivatives (**1c**), (**3a**), (**4a**) were synthesised by reported procedures [12, 13]. All chemical reagents and biochemicals used were commercially available.

Chromatography. HPLC apparatus and running conditions have been described previously [7]. Samples for HPLC analysis were concentrated to dryness and dissolved in 500 μl HPLC grade methanol.

Reverse-phase TLC was carried out using 20 × 20 cm Whatman $KC_{18}F$ plates with methanol: water: diethylamine (80:20:1) as the running solvent (System I). Normal phase TLC employed 5 × 20 cm (0.25 mm) silica gel 60 F-254 (Merck) plates with chloroform: methanol: 28% aqueous ammonia (90:10:0.5) as the solvent system (System II). The R_f values for metabolites and synthetic compounds are listed in Table 1.

In vitro *metabolism*. Microsomes were prepared from the livers of male Wistar rats (200–300 g) dosed with sodium phenobarbital in their drinking water at a concentration of 0.5 g/l for at least 10 days before use. Animals were sacrificed by cervical dislocation, the livers were excised immediately, washed with ice-cold aqueous 1.15% KCl, minced and homogenised (Potter Elvehjem homogenizer) in 4 vol. of aqueous

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Table 1. Chromatographic mobilities of compounds described herein

	R_f I	R_f II
Z-Tamoxifen (**1a**)	0.08	—
Tamoxifen epoxide (**2a**)	0.14	0.61
Z-Tamoxifen *N*-oxide (**1b**)	0.16	0.12
4-Hydroxytamoxifen	0.20	0.37
α-Hydroxytamoxifen (**3a**)	0.26	—
β-Hydroxytamoxifen (**4a**)	0.26	—
Deoxygenated Metabolite X	0.26	—
Tamoxifen *N*-oxide epoxide (**2b**)	0.27	—
4-Hydroxytamoxifen *N*-oxide	0.33	0.07
Tamoxifen *N*-oxide, Metabolite Y	0.34	—
Tamoxifen *N*-oxide, Metabolite X	0.44	—
α-Hydroxytamoxifen *N*-oxide (**3b**)	0.45	—
β-Hydroxytamoxifen *N*-oxide (**4b**)	0.45	—

I = RP-TLC, MeOH:H_2O:Diethylamine, 80:20:1.
II = Silica TLC, $CHCl_3$:MeOH:28% aq. ammonia, 90:10:0.5.
Compounds visualised under u.v. at 254 nm.

1.15% KCl. The homogenate was centrifuged (1000 *g*, 15 min, 4°), the supernatant was centrifuged at 10,000 *g* (20 min) then at 120,000 *g* (1 hr). The resulting microsomal pellet was washed by suspension in aqueous 1.15% KCl and centrifugation as described above. The final pellet was suspended in aqueous 1.15% KCl (1 ml/g of fresh liver), frozen in liquid nitrogen and stored at −20°. Samples were assayed for protein content [14]. Cytochrome P-450 was assayed by its carbon monoxide difference spectrum [15] after reduction with sodium dithionite. An extinction coefficient of 91/mM/cm between 450 and 490 nm was assumed. Incubations were carried out in stoppered 25 ml Erlenmeyer flasks using a standard incubation mixture containing 1 ml microsomes (equivalent to 15–20 mg protein, 1.5–2.0 nmoles P450/mg protein), 0.1 M phosphate buffer (3 ml; pH 7.4) and substrate (0.5 mg in 50 μl methanol). After pre-incubation at 37° for 2 min the NADPH regenerating system was added, in 1 ml of buffer [NADP, 2.5 mg; D-glucose-6-phosphate, 18 mg; $MgCl_2$, 10 mg; D-glucose-6-phosphate dehydrogenase (Boehringer, yeast, grade II, 5 μl of a solution containing 5 mg protein/ml, specific activity 140 units/mg)]. Incubations were carried out in a shaking waterbath at 37° for 90 min, flushing with oxygen for 30 sec at 30-min intervals. For the controls, substrate was added at the end of the incubation. For samples analysed by HPLC, internal standard was added at the end of the incubation.

Substrate and metabolites were extracted with 3 × 7 ml ethyl acetate over 1 g sodium chloride in 15 ml Sovirel tubes, after adjusting the pH to 9.0 with 0.13 M sodium hydroxide. The extracts were concentrated and analysed by HPLC or by TLC, isolated metabolites were subjected to electron impact (E.I.) mass spectrometry.

*Deoxygenation of metabolite X.** (see Results) A sample of the metabolite X was deoxygenated by dissolution in methanol (0.5 ml) and treatment with a 15% aqueous solution of titanium (III) chloride (50 μl). After 1 hr at room temperature, the solution was partitioned between ether (1 ml) and aqueous sodium hydroxide (0.5 M; 1.2 ml). The ether solution was concentrated and the residue applied to a 5 × 20 cm reverse-phase TLC plate. Elution with System I and collection of the material of R_f 0.26 gave deoxygenated material.

Instrumentation. NMR spectra of solutions in $CDCl_3$ were recorded using a 60 MHz Perkin Elmer R12B spectrometer or of solutions in DMSO-d_6 using a 250 MHz Bruker WM250 spectrometer, the latter by courtesy of the University of London Intercollegiate Research Service. U.v. spectra were recorded using a Pye Unicam SP8-150 spectrophotometer and E.I. mass spectra (70 eV) using a VG 7070H spectrometer with a direct insertion probe and VG 2235 data system.

SYNTHESIS

Reaction of 1,1,2-triphenyl-1-butene with peracid has been shown to give the corresponding epoxide [16]. Treatment of tamoxifen (**1a**) with excess 3-chloroperbenzoic acid not only epoxidised the central double bond but also oxidised the nitrogen; the product was identified as the *N*-oxide (**2b**) (Scheme I). The mass spectrum of (**2b**) thus showed an abundant fragment at *m/z* 342 formed by Cope elimination of the side chain characteristic of an *N*-oxide [7] and sixteen mass units higher than the corresponding fragment of tamoxifen *N*-oxide (**1b**). The *N*-oxide (**2b**) was *N*-deoxygenated by reduction with triphenylphosphine, but tamoxifen epoxide (**2a**) was better prepared from the readily available *Z*-chloroethoxy compound (**1c**) [12], epoxidation of which gave (**2c**). Treatment of epoxide (**2c**) with dimethylamine gave tamoxifen epoxide (**2a**) in good yield as a crystalline solid. The epoxide structure was apparent from ^1H NMR in which the ethyl methylene protons now appear as separate multiplets owing to the adjacent chiral centre that has been introduced.

Epoxidation of tamoxifen

A solution of *Z*-tamoxifen (100.5 mg, 0.27 mmol) [12] in benzene (2 ml) was treated with 3-chloroperoxybenzoic acid (mCPBA; 200 mg, 1.16 mmol) and the mixture set aside. After 16 hr the mixture was filtered, the filtrate concentrated and the resulting residue triturated under aqueous sodium hydroxide (1.5 M; 10 ml). The product was extracted with dichloromethane (3 × 10 ml), the combined organic layers dried with anhydrous sodium sulphate, filtered through Celite and concentrated to give a white foam of *tamoxifen* N-*oxide epoxide* (**2b**), yield 99 mg; NMR (DMSO-d_6): δ0.70 (*t*, *J* 7.3 Hz, 3H, CH_3CH_2), 1.21 and 2.14 (2 × *dq*, *J* 14.2, 7.3 Hz, 2H, CH_3CH_2), 2.99 (*s*, 6H, NMe_2), 3.38 (*br*, $OCH_2CH_2N+H_2O$), 4.32 (*m*, 2H, OCH_2CH_2N), 6.65 (*d*, *J* 8.7 Hz, 2H, H-3,5 of C—C_6H_4—O), 7.05–7.45 (*m*, 9H, ArH), 7.59 (*d*, *J* 8.3 Hz, 2H, ArH), 7.80 (*m*, 1H, ArH); mass spectrum: *m/z* 387 (M^+·—O, 1%), 342 (M^+—Me_2NOH, 5%), 315 (M^+—$Me_2NOCH_2CH_2$, 11%), 208 (M^+—Me_2NOH—PhCOEt, 34%), 182 (18%),

* The use of X and Y herein has no connection with previous use of this notation to define tamoxifen metabolites.

181 (13%), 165 (21%), 72 ($Me_2NCH_2CH_2^+$, 100%), 58 ($Me_2^+N{=}CH_2$, 71%); see Fig. 3.

cis- 2- *Ethyl*- 2,3-*diphenyl*-3-[4-(2-*chloroethoxy)phenyl)]oxirane* (**2c**)

Z-1-[4-(2-Chloroethoxy)phenyl]-1,2-diphenyl-1-butene (**1c**; 2.0 g, 5.5 mmol) [12] with mCPBA (1.45 g, 7.2 mmol) in benzene (20 ml) for 3 hr and work up as above, extracting with ether (2 × 15 ml) instead of dichloromethane, and recrystallisation of the product from light petroleum (b.p. 80–100°) gave the *title compound* as colourless needles, yield 1.70 g (81%), m.p. 83–84° NMR ($CDCl_3$):δ0.80 (*t*,*J* 7 Hz, 3H, CH_3), 1.40 and 2.18 (2 × *dq*, *J* 15 Hz, 7 Hz, 2H, CH_3CH_2), 3.59 (*m*, 2H, OCH_2CH_2Cl), 3.97 (*m*, 2H, OCH_2CH_2Cl), 6.52 (*d*, *J* 10 Hz, 2H, H-3,5 of C—C_6H_4—O), 6.9–7.7 (*m*, 12H, ArH); mass spectrum: *m/z* 378 ($M^{+\cdot}$, 20%), 321 (M^+—EtCO, 8%), 244 (M^+—PHCOEt, 100%), 181 (M^+—PhCOEt—CH_2CH_2Cl, 38%) Anal. Calc. for $C_{24}H_{23}ClO_2$: C, 76.1; H, 6.1; Cl, 9.4. Found: C, 76.1; H, 6.2; Cl, 9.3%.

cis-2-*Ethyl*-2,3-*diphenyl*-3-[4-(2-*dimethylaminoethoxy)phenyl]oxirane, tamoxifen epoxide* (**2a**)

cis-2-Ethyl-2,3-diphenyl-3-[4-(2-chloroethoxy) phenyl]oxirane (**2c**; 433 mg) and a solution of dimethylamine in ethanol (33% w/v; 10 ml) was heated in a sealed vessel at 80° for 18 hr. The resulting solution was partitioned between ether (100 ml) and aqueous sodium hydroxide (0.5 M; 50 ml). The ether solution was dried with anhydrous sodium sulphate and concentrated to give an oil which crystallised from light petroleum (b.p. 40–60°) giving the *title compound*, yield 319 mg (72%), m.p. 78–80°. U.v. (EtOH): λ_{max} 208 ($\log_{10}\varepsilon$ 4.45), 231 (4.26), 246 sh. (3.07), 271 (3.09), 279 (3.09), 286 (3.02); NMR ($CDCl_3$): δ 0.82 (*t*, *J* 7.4 Hz, 3H, CH_3CH_2), 1.40 and 2.10 (2 × *dq*, *J* 15 Hz, 7.4 Hz, 2H, CH_2CH_2), 2.25 (*s*, 6H, NMe_2), 2.60 (*t*, *J* 5.8 Hz, 2H, OCH_2CH_2N), 3.87 (*t*, *J* 5.8 Hz, 2H, OCH_2CH_2N), 6.58 (*d*, *J* 8.8 Hz, 2H, H-3,5 of C—C_6H_4—O), 7.0–7.6 (*m*, 12H, Ar*H*); mass spectrum: *m/z* 387 (M^+, 22%), 315 (M^+—$Me_2NCH_2CH_2$, 32%), 253 (M^+—PhCOEt, 20%), 181 (M^+—PhCOEt—$Me_2NCH_2CH_2$, 12%), 165 (14%), 72 ($Me_2NCH_2CH_2^+$, 100%), 58 ($Me_2N{=}CH_2^+$, 100%). Anal. Calc. for $C_{26}H_{29}NO_2$: C, 80.6; H, 7.5; N, 3.6. Found: C, 80.3; H, 7.5; N, 3.5%.

An identical sample of tamoxifen epoxide was obtained by treatment of the *N*-oxide (**2b**) (101 mg, 0.24 mmol) with triphenylphosphine (138 mg, 0.53 mmol) in toluene (2 ml) at reflux for 30 min and column chromatography of the product on silica gel (type H; 8 g) with elution by 4:4:1 light petroleum (b.p. 40–60°)–ether–triethylamine, yield 22 mg (24%).

N-*oxidations*

N-Oxides of the tamoxifen derivatives were prepared by oxidation of the amines with hydrogen peroxide. Compound (**3b**)* gave analysis figures corresponding to a monohydrate and those of (**4b**) to a solvate in which was present 0.5 equiv. each of water and hydrogen peroxide. Tamoxifen *N*-oxide has previously been shown to crystallise with water and hydrogen peroxide [7].

E-1-[4-(2-*Dimethylaminoethoxy) phenyl*]-1,2-*diphenyl*-1-*buten*-3-*ol* N-*oxide* (**3b**)

E-1-4-(2-Dimethylaminoethoxy)phenyl]-1,2-diphenyl-1-buten-3-ol (**3a**) [13] (102 mg) was taken up in a mixture of methanol (2 ml) and tetrahydrofuran (2 ml) and treated with aqueous hydrogen peroxide (30% w/v; 2 ml). After 45 hr the mixture was partitioned between water (20 ml) and dichloromethane (20 ml). The dichloromethane solution was dried with anhydrous sodium sulphate and concentrated. The residue was taken up in 4:1 light petroleum (b.p. 40–60°)–dichloromethane (18 ml) and the *title compound* crystallised as a monohydrate, yield 111 mg (100%), m.p. 152–155° after recrystallisation from 1:1 benzene–cyclohexane; NMR (DMSO-d_6) δ 0.98 [*d*, *J* 6.4 Hz, 3H, $CH_3CH(OH)$], 3.04 (*s*, 6H, NMe_2), 3.3 (*br*, OCH_2CH_2N and H_2O), 4.32 (*m*, 2H, OCH_2CH_2N), 4.58 [*m*, 1H, $CH_3CH(OH)$], 6.62 (*d*, *J* 8.7 Hz, 2H, H—3,5 of C—C_6H_4—O), 6.78 (*d*, *J* 8.7 Hz, 2H, H—2,6 of C—C_6H_4—O), 7.1–7.45 (m, 1OH, 2 × Ph); mass spectrum: *m/z* 387 ($M^{+\cdot}$—O, 2%), 373 (0.8%), 369 (M^+—O—H_2O, 0.3%), 342 (M^+—Me_2NOH, 1.5%), 327 (0.6%), 324 (M^+—Me_2NOH—H_2O, 0.4%), 252 (2%), 209 (PhCH—C_6H_4—$OCH{=}CH_2^+$, 28%), 183 (7%), 165 (4%), 72 ($Me_2NCH_2CH_2^+$, 17%), 58 ($Me_2NCH_2^+$, 100%); see Fig. 1. Anal Calc. for $C_{26}H_{29}NO_3$. H_2O: C, 74.1; H, 7.4; N, 3.3. Found: C, 74.5; H, 7.4; N, 3.2%.

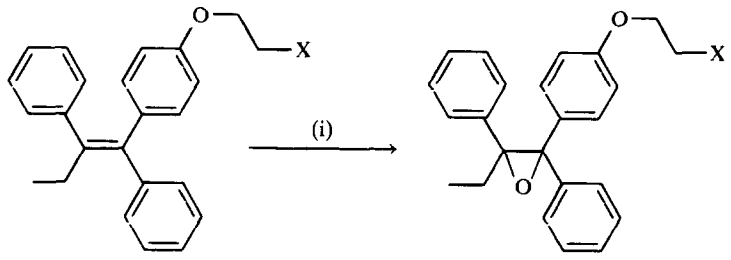
Z-1-[4-(2-*Dimethylaminoethoxy)phenyl*]-1,2-*diphenyl*-1-*buten*-4-*ol* N-*oxide* (**4b**)

Z-1-[4-(2-Dimethylaminoethoxy)phenyl-1,2-diphenyl-1-buten-4-ol (**4a**) [13] (84 mg) was oxidised by the method used above for (**3b**) to give the *title compound* as a solvate with both water and hydrogen peroxide, yield 70 mg (75%), m.p. 167–170° after recrystallisation from 1:1 benzene–cyclohexane; NMR (DMSO-d_6): δ 2.56 (*t*, *J* 7.3 Hz, 2H, $HOCH_2CH_2$), 3.03 (*s*, 6H, NMe_2), 3.30 (*t*, *J* 7.3 Hz, 2H, $HOCH_2CH_2$), 3.46 (*br*, OCH_2CH_2N and H_2O), 4.33 (*m*, 2H, OCH_2CH_2N), 4.69 (*br*, 1H, OH), 6.63 (*d*, *J* 8.8 Hz, 2H, H—3,5 of C—C_6H_4—O), 6.74 (*d*, *J* 8.8 Hz, 2H, H—2,6 of C—C_6H_4—O), 7.1–7.41 (*m*, 1OH, 2 × Ph); mass spectrum: *m/z* 387 ($M^{+\cdot}$—O, 5%), 342 (M^+—Me_2NOH, 61%), 311 (M^+—Me_2NOH—CH_2OH, 32%), 268 (M^+—$Me_2NOCH_2CH_2O$—CH_2OH, 60%), 191 (15%), 91 (21%), 72 (25%), 58 ($Me_2NCH_2^+$, 100%). Anal. Calc. for $C_{26}H_{29}NO_3 \cdot \frac{1}{2}H_2O$, $\frac{1}{2}H_2O_2$: C, 72.7, H, 7.3; N, 3.3. Found: C, 72.8; H 7.1; N, 3.3%.

RESULTS

HPLC analysis of tamoxifen microsomal incubation extracts exhibited metabolism similar to that observed previously [7]. The extent of metabolism

* Although the metabolite (**3b**) has *E*-nomenclature whereas tamoxifen (**1a**) and the β-hydroxy compound (**4b**) have *Z*, all these compounds have the same configuration of the groups about the central double bond, having the disubstituted ring *trans* to the ethyl group.



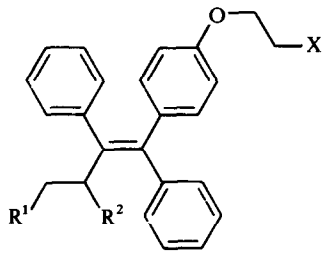
(1) (2) (a) X = NMe_2

(b) X = $N^+Me_2O^-$

(c) X = Cl

Reagents: (i), 3-Cl.C_6H_4.CO_3H, C_6H_6

Scheme 1

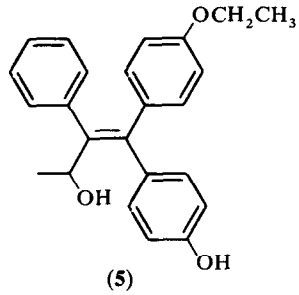


(a) X = NMe_2

(b) X = $N^+Me_2O^-$

(c) X = Cl

(3) R^1 = H, R^2 = OH
(4) R^1 = OH, R^2 = H



(5)

was in the range 35–42% (over 3 experiments), the metabolites then identified were 4-hydroxytamoxifen (R.T. = 314 s) 3.0–5.0%, tamoxifen *N*-oxide (R.T. = 369 s) 13.3–17.2%, and *N*-desmethyltamoxifen (R.T. = 662 s) 14.1–17.5% (calculated as percentage area under curve). Incubation of tamoxifen *N*-oxide with an identical microsomal system gave 17.0% metabolism. Metabolites detected were tamoxifen (R.T. = 552 s) 3.5%, *N*-desmethyltamoxifen 1.3%, and the major metabolite, designated metabolite X (R.T. = 240 s), 12.3%. Further investigation into the metabolism of tamoxifen *N*-oxide, using reverse-phase TLC separation (System I) gave two bands of higher polarity than the substrate. One of these was the major product, metabolite X (R_f 0.44) having R.T. = 240 s on HPLC examination. The other band (R_f 0.34) was designated Y which on reverse-phase TLC (System I) had chromatographic properties similar to those of synthetic 4-hydroxytamoxifen *N*-oxide [17]. Elution of this band and re-chromatography on silica TLC (System II) revealed the presence of more than one component, one band co-chromatographing with the synthetic compound. Insufficient material was available in these bands for mass identification and confirmation of structure.

Identification of metabolite X from tamoxifen N-*oxide*

In the mass spectrum of metabolite X the highest mass peak was at *m/z* 387 and there was a fragment at *m/z* 342 (see Fig. 1). This loss of 45 mass units is characteristic of an *N*-oxidised side chain and is seen in the spectrum of tamoxifen *N*-oxide (**1b**) itself. From the peak of highest *m/z* it was deduced that the molecular weight of the metabolite is 403; the molecular ion itself was not seen owing to facile loss of the *N*-oxide oxygen, or Cope elimination giving the fragment at *m/z* 342. Therefore, metabolism resulted in an extra oxygen atom being incorporated into the molecule. The abundant fragment in the

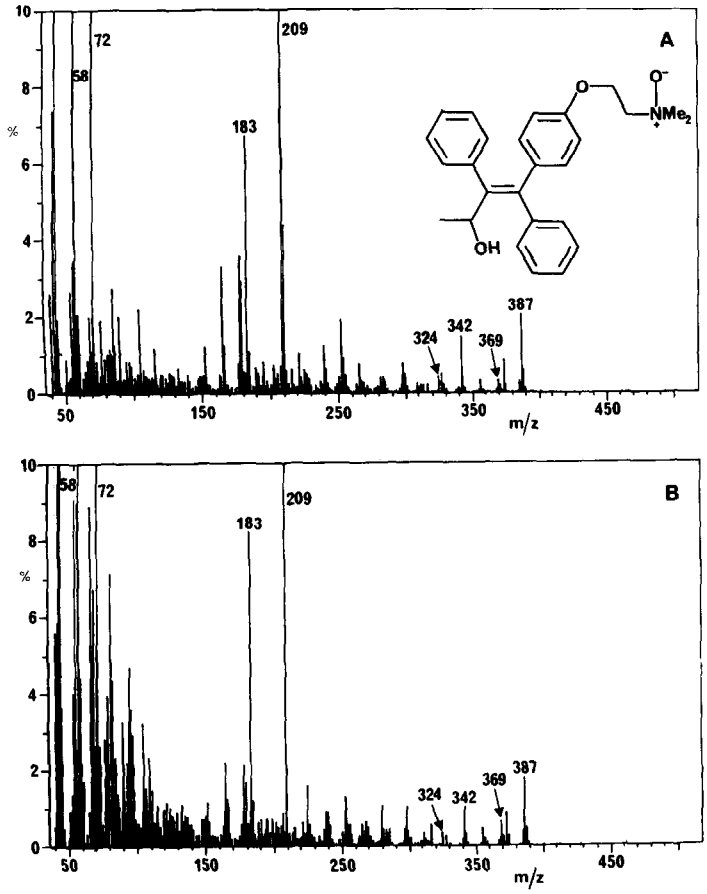


Fig. 1. Electron impact mass spectra of *E*-1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenyl-1-buten-3-ol *N*-oxide; A = synthesised compound, B = isolated metabolite.

spectrum at m/z 209 is thought to be a result of cleavage of the central ethylene linkage and led us initially to believe that this bond was saturated. Such is the case for the epoxide (**2a**). However, in the mass spectrum of the epoxide (Fig. 2) there was no signal at m/z 209. Closer examination of the mass spectrum of the metabolite revealed ions at m/z 369 and 324 attributable to loss of water from the ions at m/z 387 and 342 respectively. An ion of m/z 369 was also seen in the mass spectrum of the hydroxylated tamoxifen (**3a**) and on this basis, the metabolite was suspected to be the *N*-oxide (**3b**). Analysis of the metabolite by reverse-phase TLC revealed that it was significantly more polar than tamoxifen *N*-

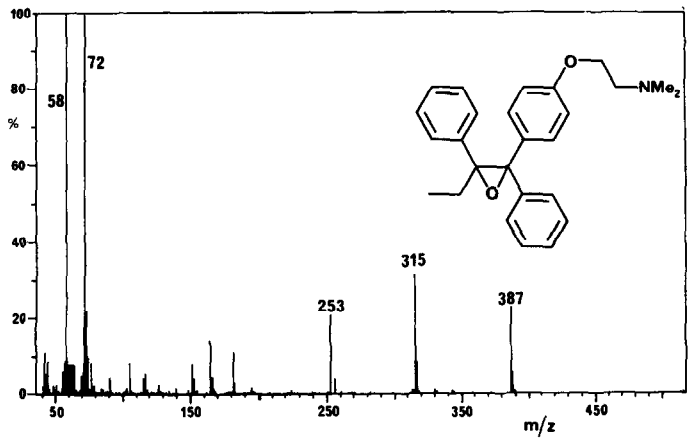
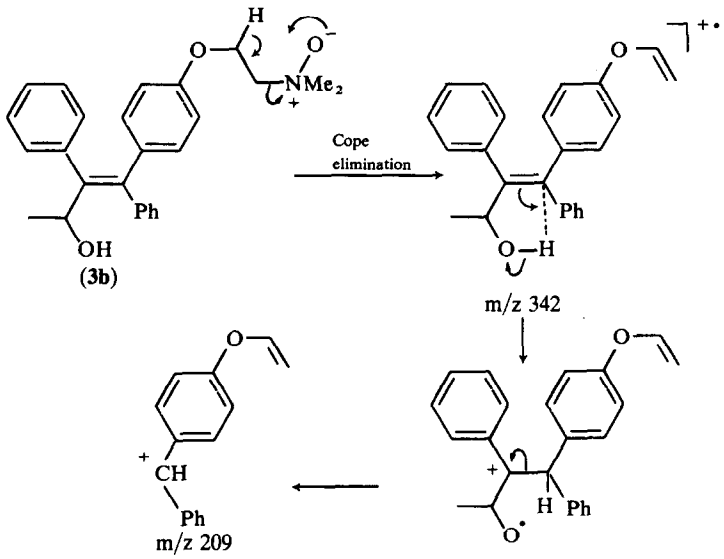


Fig. 2. Electron impact mass spectrum of tamoxifen epoxide.



Scheme 2. Proposed mass spectral fragmentation of the metabolite (**3b**).

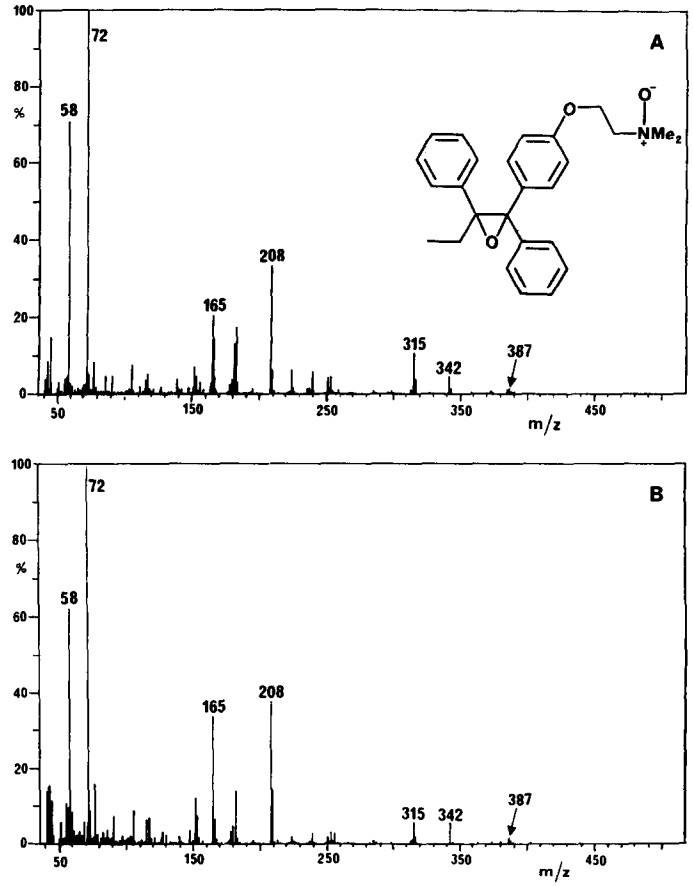


Fig. 3. Electron impact mass spectra of tamoxifen *N*-oxide epoxide; A = synthesised compound, B = isolated metabolite.

oxide. Comparison was then made with synthetic samples of the *N*-oxides (**3b**) and (**4b**). Both synthetic compounds had identical R_f values to that of the metabolite. Further evidence was sought by deoxygenation of the metabolite by titanium (III) chloride. The deoxygenated material gave an identical R_f value to both of the alcohols (**3a**) and (**4a**), hence the chromatographic determinations did not exclude the primary alcohol (**4b**) as a possible structure for the metabolite. However, the identification of metabolite X as the secondary alcohol (**3b**) was confirmed by examination of the mass spectra of the synthetic *N*-oxides (**3b**) and (**4b**). The spectrum of synthetic (**3b**) showed good correlation with that of metabolite X (Fig. 1) but the spectrum of the primary alcohol (**4b**) showed a completely different fragmentation pattern; there was an abundant loss of $HOCH_2$ giving rise to a strong fragment at *m/z* 311 not seen with metabolite X and no fragments due to loss of water. The hitherto unexplained fragment at *m/z* 209 for the metabolite may be the result of fission of the central double bond promoted by the presence of the hydroxyl group, possibly as shown in Scheme 2.

Attempted detection of microsomal epoxidation

In view of our identification of a new metabolite from tamoxifen *N*-oxide and in an attempt to determine whether the proposed metabolite tamoxifen epoxide [11] was produced using our microsomal system, the metabolism of tamoxifen itself was reinvestigated using reverse-phase TLC (System I). This showed both bands X and Y (isolated from tamoxifen *N*-oxide) to be formed in small amounts when tamoxifen was the substrate, by comparison of R_f values and mass spectrometry. Using reverse-phase TLC, tamoxifen epoxide would have an R_f similar to those of tamoxifen *N*-oxide and 4-hydroxytamoxifen (Table 1). Therefore, this area of the chromatogram was removed, eluted with methanol and re-run on normal phase silica (System II) against the synthesised standards. As expected, the major constituent was tamoxifen *N*-oxide together with a small amount of 4-hydroxytamoxifen; tamoxifen epoxide could not be detected. However, the synthetic tamoxifen epoxide had properties similar to those reported by Ruenitz *et al.* [11] for the metabolite. The mass spectrum of synthetic tamoxifen epoxide (Fig. 2) showed an abundant fragment due to loss of propiophenone not reported by Ruenitz *et al.*, and the u.v. spectrum had a maximum absorbance at 231 nm whereas Ruenitz quoted a figure of 220 nm. However, the chromatographic mobility was consistent with the reported observations [11], tamoxifen epoxide being slightly more polar than tamoxifen.

The microsomal metabolism of tamoxifen epoxide (**2a**) was also investigated to determine its stability in the microsomal system. This led to the identification of a single more polar metabolite, shown by E.I. mass spectrometry and comparison with synthetic compound, to be tamoxifen *N*-oxide epoxide (**2b**) (Fig. 3). This was not detected as a metabolite of tamoxifen itself, therefore it is likely that tamoxifen epoxide is not formed as a metabolite of tamoxifen, using the microsomal system herein described.

DISCUSSION

In this study, we have conclusively identified a previously unreported metabolite of tamoxifen as *E*-1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenyl-1-buten-3-ol *N*-oxide (α-hydroxytamoxifen *N*-oxide) (**3b**). By comparison of the new metabolite with authentic material obtained by synthesis, the possibility of overlooking alternative structures has been avoided.

In some early studies on the metabolism of tamoxifen, Fromsen *et al.* [6] identified a metabolite in rat bile (metabolite F) (**5**) indicating that tamoxifen had undergone metabolism at three positions. This would at first seem unlikely and certainly metabolite F could not be formed initially. However, from our findings there is a possibility that it is formed by way of the metabolite, *alpha*-hydroxytamoxifen *N*-oxide (**3b**), by reductive cleavage of the oxidised dimethylamino group and *para* (4-) hydroxylation. A second minor product of metabolism appeared to be comprised of more than one compound of which one may be 4-hydroxytamoxifen *N*-oxide but insufficient material was available for mass spectral identification. Recently, tamoxifen epoxide was tentatively identified as a microsomal metabolite of tamoxifen [11]. However, using a similar microsomal system we were unable to detect this metabolite by comparison with synthesised tamoxifen epoxide. Incubation of synthetic tamoxifen epoxide with the microsomal system led to further metabolism, tamoxifen *N*-oxide epoxide being the only metabolite detected. Since none of this product was observed with tamoxifen as the substrate, epoxidation is probably not a significant metabolic route for tamoxifen in our microsomal system. It is interesting, however, that the epoxide group in tamoxifen epoxide is stable to metabolism in contrast to that in diethylstilbestrol epoxide where further metabolism occurs giving 4′-hydroxypropiophenone [18].

We have shown that modification of the basic side-chain in tamoxifen can promote a pathway of oxidative metabolism at a point which is different from that occurring in tamoxifen. The hepatic microsomal preparation contains an array of metabolising enzymes with differing functions and a change in nature of the tamoxifen side-chain may affect transport to, affinity for, and orientation of binding of a substrate for a specific enzyme system. In this study the pathway of allylic hydroxylation was promoted, possibly due to the relatively high polarity of the *N*-oxide. *In vivo* an *N*-oxide may be excreted rapidly, or may be reduced metabolically to the original amine so that the microsomal system used here may not reflect the human *in vivo* situation. However, if the concentration of tamoxifen *N*-oxide *in vivo* becomes sufficient for further metabolites to be formed, α-hydroxylation may be of significance. Results obtained from *in vivo* studies should therefore be of value.

The effect that is reported here is comparable to the switching of metabolism by deuterium where, by substitution of protons by deuterium, metabolism of one pathway can be suppressed and/or another accentuated [19]. For tamoxifen, *N*-oxidation of the amine nitrogen to give its *N*-oxide has suppressed

further metabolism of the basic side-chain (e.g. demethylation), presumably since the nitrogen atom is no longer basic. It is also worth noting that the *alpha*-hydroxylation pathway is one which is not normally seen. It is unfortunate that, in this particular situation, the metabolite obtained (**3b**) would have a low binding affinity for the estrogen receptor. *alpha*-Hydroxylation of tamoxifen reduces the binding affinity by a factor of 10 [13] and it would not be improved by *N*-oxidation. However, in other cases it is possible that more active metabolites might be formed. *N*-Oxidation is a major pathway of tamoxifen metabolism, at least *in vitro*; it appears that by completely blocking *N*-oxidation, formation of a more active antiestrogenic metabolite might be favoured. We are undergoing studies with other synthesised tamoxifen analogues to determine whether this can be achieved.

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