Biochemical Pharmacology, Vol. 36, No. 4, pp. 573–577, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

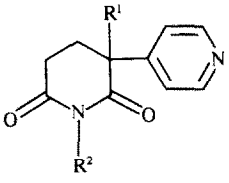
Metabolism and pharmacokinetics of the N- and C-*n*-octyl analogues of pyridoglutethimide [3-ethyl-3-(4-pyridyl)piperidine-2,6-dione]: novel inhibitors of aromatase

(*Received* 1 *July* 1986; *accepted* 18 *September* 1986)

Pyridoglutethimide [PyG, 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione] is the parent member of a homologous series of compounds possessing selective inhibitory activity towards the enzyme complex aromatase. It has been developed as a potential successor to aminoglutethimide [AG, 3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] which is now a well established treatment for hormone-dependent metastatic breast carcinoma in postmenopausal women [1, 2]. Potential advantages of PyG over AG have been discussed elsewhere [3, 4].

AG and PyG have similar aromatase inhibitory activities and, since *in vivo* the duration of response to AG is limited, it would be of interest to determine whether an analogue of PyG which is more potent *in vitro* might prove more effective *in vivo*. We have identified higher N-alkyl analogues (1-substituted series) of PyG as well as homologues of PyG in which the ethyl residue is replaced by higher alkyl groups (3-substituted series) as more potent inhibitors of aromatase [5]. The optimum chain length in either the 1- or 3-substituted series is C_8 [respectively, 3-ethyl-1-*n*-octyl-3-(4-pyridyl)piperidine-2,6-dione (N-octylPyG) and 3-*n*-octyl-3-(4-pyridyl) piperidine-2,6-dione (C-octylPyG)]. The inhibitory activity towards aromatase for N-octylPyG ($K_i = 0.12\ \mu M$, testosterone as substrate) and C-octylPyG ($K_i = 0.09\ \mu M$) was higher than for PyG ($K_i = 1.1\ \mu M$) (M. G. Rowlands, unpublished results). Previously we have reported on the metabolism and pharmacokinetics of PyG in the rat and the rabbit. Plasma levels were high and well maintained [3] with a plasma half-life of 6 hr in rat and 16.4 hr in rabbit. The sole metabolite detected to date is the *N*-oxide [3] which does not inhibit aromatase [4].

We now report comparable studies in these species of these new inhibitors.



	R^1	R^2
N - Octyl PyG	$C_2H_5^-$	$C_8H_{17}^-$
C - Octyl PyG	$C_8H_{17}^-$	H−

METHODS

Synthesis

3-Ethyl-1-*n*-octyl-3-(4-pyridyl)piperidine-2,6-dione [N-octylpyridoglutethimide (N-octylPyG)] and 3-*n*-octyl-3-(4-pyridyl)piperidine-2,6-dione [C-octylpyridoglutethimide (C-octylPyG)] were synthesised as previously described [5]. The corresponding d_{17}-analogues were respectively made from PyG (0.45 mmol) and from 4-pyridylacetonitrile (2.54 mmol) using d_{17}-octyl bromide at the appropriate stage in these syntheses.

Chromatography

High pressure liquid chromatography (HPLC) apparatus consisted of a Waters dual pump system as described previously [3] with u.v. detectors at 254 nm and 229 nm. The following mobile phases were used: *system I*, acetonitrile–0.01 M phosphate buffer, pH 7.0, 50:50 v/v; *system II*, isocratic 30% acetonitrile in phosphate buffer for 3 min then a gradient, increasing to 60% acetonitrile until 4.5 min; after 9 min, the solvent was returned to the original compositions (for 3 min); there was a 6 min re-equilibration period between samples. *System III*, acetonitrile–0.01 M phosphate buffer, pH 7.0, 70:30 v/v. The flow rate was 1.5 ml/min. Sample extracts, after concentration to dryness, were reconstituted in HPLC grade acetonitrile (500 μl for microsomal extracts; 200 μl/ml for plasma extracts), aliquots of which (4–10 μl) were injected onto the column.

For normal phase TLC separations, glass plates (5 × 20 cm or 20 × 20 cm) coated with silica gel 60 F_{254} (Merck, 0.25 mm layer) were developed with chloroform–methanol, 19:1.

Mass spectrometry

Direct insertion mass spectra were determined in the electron impact (EI) mode (ionising voltage 70 eV, trap current 100 μA) at an ion-source temperature of 160° or in the chemical ionisation (CI: ammonia or methane) mode (50 eV, 200 μA) using a VG 7070H spectrometer and 2235 data system.

Pharmacokinetic studies

Wistar rats (150–250 g) and New Zealand White rabbits (3–3.5 kg) were obtained from Olac., Oxon, U.K. and maintained on standard laboratory diets. N-OctylPyG was administered to rats at a dose of 0.121 mmol/kg as the HCl salt in water (0.1 ml; i.v., i.p. and p.o.). C-OctylPyG was administered in dimethylsulphoxide (DMSO); rats received 0.132 mmole/kg (0.1 ml, i.v.) and the rabbit received 0.039 mmole/kg (0.3 ml, i.v.). Blood samples were taken at 5 (i.v.), 15, 30 min, 1, 2, 4 and 6 hr by cardiac puncture for rats (5–6 ml), using one animal per time point, and by bleeding from the ear vein for rabbits (3 ml). Plasmas were stored at −20° prior to extraction, using the method previously described [3]. Aliquots (4 μl, in triplicate) of the 200 μl samples were subjected to HPLC and the plasma concentration determined from the calibration curves after correction for recoveries which were determined using pretreatment plasma (1 ml) to which either N-octylPyG or C-octylPyG (5 μg) was added.

Calibration curves were constructed for N-octylPyG and C-octylPyG using standard solutions (0.01 mg/ml, 0.1 mg/ml, 1.0 mg/ml) plotting area under the peak against concentration.

In vitro *metabolism studies*

Microsomes were prepared from the livers of male Wistar rats (200–300 g) pre-treated with sodium phenobarbital, and incubations carried out as previously described [6] using 0.5 mg substrate. After addition of sodium chloride (1 g), substrate and metabolites were extracted with ethyl acetate (3 × 7 ml). The combined extracts were concentrated on a rotary film evaporator, the residues taken

up in HPLC grade acetonitrile (500 μl) and analysed by HPLC (C-octylPyG, System II) or TLC; isolated metabolites were subjected to EI and CI mass spectrometry.

RESULTS

Pharmacokinetic studies

The calibration curves obtained with the prepared standards for N-octylPyG and C-octylPyG showed a linear relationship between peak area and concentration over the range under consideration. The limit of detection (254 nm) for both N-octylPyG and C-octylPyG was 20 ng per injection. Recovery of N-octylPyG was 70% from rat plasma whereas C-octylPyG gave 100% recovery from both rat and rabbit plasma. The HPLC retention time for C-octylPyG in System I was 230 sec and in System II was 650 sec. The retention time for N-octylPyG in System III was 225 sec. No u.v. absorbing interfering components were observed when untreated plasma extracts were developed in the above TLC and HPLC systems.

Following i.v. administration of N-octylPyG the peak plasma concentration at 15 min was 7.6 μg/ml. This value fell rapidly with no detectable N-octylPyG in the 2, 4 and 6 hr plasma extracts (Fig. 1A). Following i.p. and p.o. administration, plasma levels at 15 min were 1.0 μg/ml and 1.4 μg/ml respectively with no detectable N-octylPyG at later times.

C-OctylPyG (0.132 mmole/kg) was administered i.v. to rats as the free base; a peak plasma concentration of 7.3 μg/ml was obtained at 15 min and the calculated plasma half-life was 50 min. There was no detectable C-octylPyG in the 6 hr plasma extract (Fig. 1B). Similarly in the rabbit, C-octylPyG (0.039 mmole/kg) gave a peak plasma concentration of 3.2 μg/ml at 5 min and a plasma half-life of 42 min. At 6 hr, C-octylPyG was undetectable in the plasma extract (Fig. 1C).

In vitro *metabolism studies*

Metabolism of N-octylPyG. TLC examination of the organic extracts following incubation of N-octylPyG with microsomes revealed the presence of u.v. absorbing bands not observed in control extracts (Fig. 2). A comparison of the microsomal metabolism of N-octylPyG with that of N-octylPyG-d_{17} revealed some quantitative but no qualitative differences. The metabolites separated on TLC were removed and subjected to EI and CI mass spectrometry.

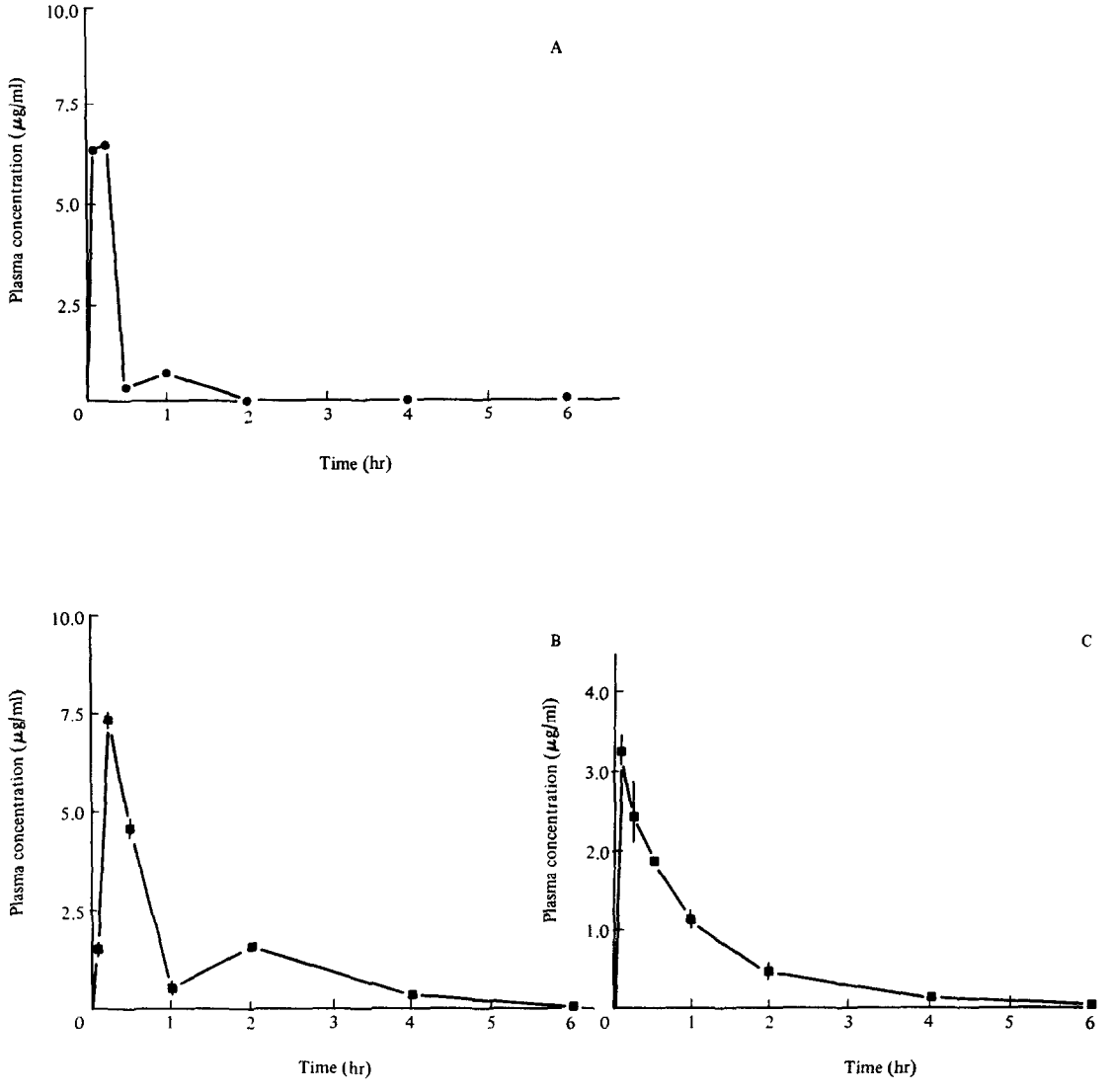


Fig. 1. Plasma concentration–time profile after i.v. administration: (A)N-octylPyG to rat; (B) C-octylPyG to rat. (C) C-octylPyG to rabbit. Substrates quantified by HPLC as described in the text.

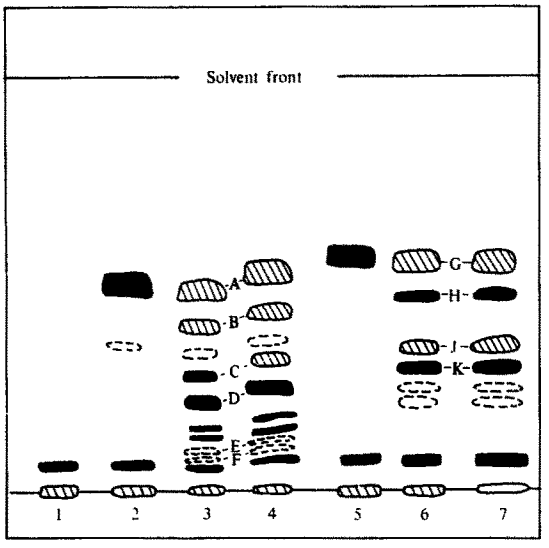


Fig. 2. Diagramatic representation of a thin-layer chromatogram in chloroform:methanol (19:1) of extracts of microsomal incubations. (1) Control—microsomes and cofactors; (2) microsomes and cofactors with N-octylPyG added immediately prior to extraction; (3) microsomes and cofactors incubated with N-octylPyG at 37° for 60 min; (4) As (3) with N-octylPyG-d_{17} as substrate; (5–7) As (2–4) with C-octylPyG as substrate.

Quantification of C-octylPyG microsomal metabolism. The microsomal metabolites of C-octylPyG were fewer, permitting separation and quantification by HPLC of some of them. The microsomal half-life for C-octylPyG was 40 min and for the deuterated analogue, 46 min (calculated from results up to 45 min). The metabolites were also quantified by HPLC using System II (see Fig. 3A and B). By supplementing the microsomal incubates with additional cofactors at intervals of 20 min [7], a further metabolite was detected (R.T. = 104 sec) and the product at R.T. 220 sec increased, whereas that at R.T. 170 sec decreased. Metabolites from the 100 min incubate were isolated by TLC and eluted with methanol. The resulting methanolic solutions (100 μl) were examined by mass spectrometry (EI and CI), and also subjected to HPLC (System II) allowing the mass spectral findings to be equated with HPLC quantification. Components **H** (R.T. = 220 sec), and **J** (R.T. = 104 sec) were each unique but **K** comprised four components of R.T. 142, 170, 190 and 205 sec, separation of which was insufficient for recovery and individual MS analysis. However, their identity as mono-hydroxy derivatives was clear from the CI spectrum as was the presence among them of the ω-1 hydroxy derivative, from the α-cleavage ions in the EI spectra (Table 1). Ions corresponding to $[M\text{-alkyl}]^+$ for ω-2 and ω-3 hydroxy derivatives were also present in these spectra.

Some general comments can be made which apply to metabolites of both N- and C-octylPyG following mass spectrometry. Monohydroxylation in the octyl residue increased the molecular weight by 16 daltons in the non-deuterated compounds and by 15 daltons in the deuterated

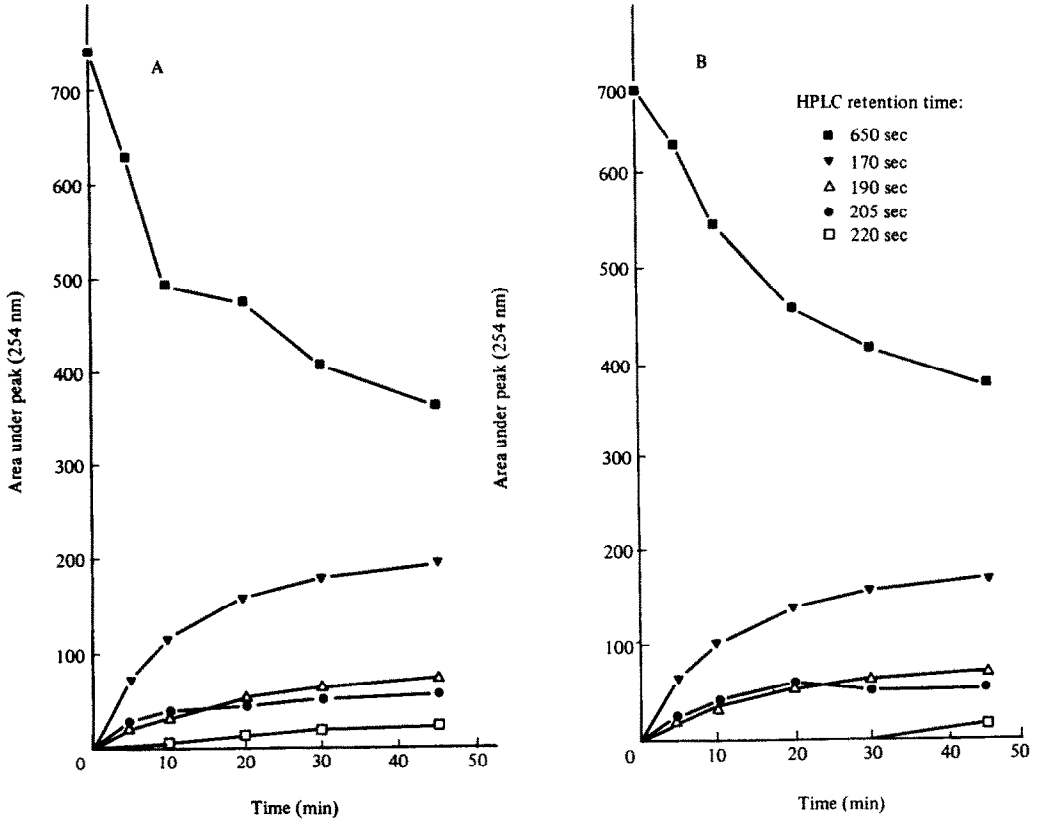


Fig. 3. Concentration–time profile for (A) C-octylPyG and (B) C-octylPyG-d_{17} and their metabolites detected after incubation of the substrate with phenobarbital-induced rat liver microsomes at 37°. Substrates and metabolites were quantified by HPLC at 254 nm using System II, as described in the text.

Table 1. Major ions in the mass spectra of N-octylPyG (A–F), C-octylPyG (G–K), their deuterated analogues and their metabolites

Compound	$[M + H]^+$ (CI spectrum)	Structurally significant peaks in the EI spectrum. Relative abundance (%) and structural assignments are given in parentheses () followed by m/z values, where available, for corresponding ions in the spectra of deuterated counterparts []
N-OctylPyG (**A**)	331 [348]	330 (40, $M^{\cdot+}$) [347], 302 (50, $[M - C_2H_4]^{\cdot+}$) [319], 133 (100)
7-Oxooctyl (**B**)	345 [360]	344 (19, $M^{\cdot+}$) [359], 329 (6, $[M - CH_3]^+$) [341], 315 (15, $[M - C_2H_5]^+$) [330], 301 (23, $[M - CH_3CO]^+$) [313], 287 (100, $[M - CH_2COCH_3]^+$) [297], 43 (72, CH_3CO^+) [46]
Hydroxyoxooctyl (**C**)	361	extensive fragmentation, no unambiguously assigned high mass ions
7-Hydroxyoctyl (**D**)	347 [363]	331 (18, $[M - CH_3]^+$) [344], 328 (12, $[M - H_2O]^{\cdot+}$) [343], 80 (100), 45 (29, CH_3CHOH^+) [49]
Dihydroxyoctyl (**E, F**)	363 [378]	extensive fragmentation, no unambiguously assigned high mass ions.
C-OctylPyG (**G**)	303	302 (5, $M^{\cdot+}$) [319], 190 (100, $[M - C_8H_{17}]^{\cdot+}$) [191]
7-Oxooctyl (**H**)	317	316 (8, $M^{\cdot+}$), 301 (4, $[M - CH_3]^+$), 273 (5, $[M - CH_3CO]^+$), 259 (68), 190 (100), 43 (69, CH_3CO^+)
Hydroxyoxooctyl (**J**)	333	extensive fragmentation, no unambiguously assigned high mass ions
Hydroxyoctyl (**K**)	319	303 (7, $[M - CH_3]^+$) [316], 289 (8, $[M - C_2H_5]^+$) [300], 275 (11, $[M - C_3H_7]^+$) [284], 190 (100) [191], 45 (25, CH_3CHOH^+) [49]

counterparts. The introduction of one keto-function raised the molecular weight by 14 and 12 daltons respectively. The CI spectra in particular unambiguously defined molecular weight since $[M + H]^+$ ions were the base peak (in both methane and ammonia spectra) whereas a molecular ion was sometimes weak or absent in the EI spectra. Products of ω-1 oxidation were characterised by α-cleavage ions [8] $[M - CH_3]^+$ and $[M - CD_3]^+$. Other positions of oxidation were not so clearly discriminated by characteristic fragmentation in the present study and are not specified in Table 1, which give total or partial structural assignments to the metabolites based on the foregoing arguments.

The metabolites of both analogues were most conveniently separated by TLC (Fig. 2) prior to analysis by mass spectrometry.

DISCUSSION

Both octyl derivatives have plasma half-lives in the rat and the rabbit (C-octylPyG) substantially shorter than the values (6 hr and 16.4 hr respectively) for the parent drug PyG [3], implying a much more rapid rate of clearance as a result of the multiple possibilities for oxidative metabolism in the alkyl chains.

To assess their susceptibility to oxidative metabolism the *in vitro* metabolism of the octyl derivatives was determined using rat liver microsomes. TLC analysis showed that many more metabolites were formed from N-octylPyG than from C-octylPyG and because of this complexity together with the more rapid disappearance of N-octylPyG from rat plasma *in vivo* we confined our investigations on N-octylPyG to attempts to characterise metabolites and selected C-octylPyG for a more detailed quantitative study.

An HPLC system was developed (System II) with which the more polar metabolites of C-octylPyG could be quantified. This revealed several products, the formation of which was both time- and NADPH-dependent (see Fig. 3). In order to obtain sufficient quantities of these various metabolites for MS identification the microsomal system was supplemented with additional cofactors at 20-min intervals [7] producing an increase in the concentration of certain metabolites with time coupled with a decline in others implying secondary metabolism. This was confirmed by MS analysis of these metabolites separated on TLC, an aliquot of each being subjected to HPLC to determine the R.T. of these products and thus correlation with the quantitative studies. As the proportion of metabolite **K** decreased (a major component being the ω-1 hydroxyl product—see Table 1) so metabolites **H** and **J** increased in concentration. Analysis by MS (Table 1) showed **H** to be the ω-1 keto metabolite and **J** to be an hydroxy-keto metabolite.

Deuterium labelling, used to confirm the nature and location of functional groups introduced by metabolism, can also introduce isotope effects in the formation of metabolites [9]. This aspect was studied for C-octylPyG. Deuteration increased the half-life for microsomal metabolism from 40 to 46 min, corresponding to a small isotope effect (IE, $k_H/k_D = 1.15$). The principal hydroxylation pathway (170 sec; Fig. 3) was also slightly retarded in the deuterated analogue, but the minor pathways were not significantly affected (Fig. 3). Keto derivatives are formed via two successive cleavages of C–H (or C–D) linkages. The resulting isotope effects delayed the appearance of this derivative of deuterated C-octylPyG (220 sec; Fig. 3). From these metabolic investigations it is apparent that microsomal metabolism of both C- and N-octylPyG occurs primarily in the alkyl side-chain. We detected no pyridine *N*-oxidized products (contrast pyridoglutethimide [3]) and no metabolites formed as a result of oxidation in the glutarimide ring (contrast aminoglutethimide [10, 11]). Both such oxidation products would have resulted in molecular weight increases which were equal for both deuterated and non-deuterated compounds but this was not observed.

The *in vitro* metabolism studies reported here indicate extensive oxidation of both substrates. Hence the short plasma half-lives may be attributed to oxidative metabolism, facilitating rapid excretion. For C-octylPyG, the microsomal half-life is of the same order as that observed *in vivo* in both rat and rabbit, confirming extensive metabolism. However, HPLC examination (System II) of plasma obtained from animals treated with C-octylPyG revealed no peaks corresponding to the metabolites identified in extracts of the microsomal incubates within the detection limits of the system.

Acknowledgements—This investigation was supported by grants to the Institute of Cancer Research from the Cancer Research Campaign, the Medical Research Council and the British Technology Group. The authors thank Miss Linda Lovell for assistance with *in vivo* animal work and Professor A. B. Foster for his interest in this project.

Drug Development Section
CRC Laboratory
Institute of Cancer Research
Clifton Avenue
Sutton
Surrey, SM2 5PX, U.K.

ALISON SEAGO*
MICHAEL H. BAKER
JOHN HOUGHTON
CHUI-SHEUNG LEUNG
MICHAEL JARMAN

REFERENCES

1. R. J. Santen, E. Badder, S. Lerman, H. Harvey, A. Lipton, A. E. Boucher, A. Manni, H. Rosen and S. A. Wells, *Breast Cancer Res. Treat.* **2**, 375 (1982).
2. A. L. Harris, T. J. Powles, I. E. Smith, R. C. Coombes, H. T. Ford, J. C. Gazet, C. L. Harmer, M. Morgan, H. White, C. A. Parsons and J. A. McKinna, *Eur. J. Cancer clin. Oncol.* **19**, 11 (1983).
3. A. Seago, P. E. Goss, L. J. Griggs and M. Jarman, *Biochem. Pharmac.* **35**, 2911 (1986).
4. A. B. Foster, M. Jarman, C.-S. Leung, M. G. Rowlands, G. N. Taylor, R. G. Plevey and P. Sampson, *J. Med. Chem.* **28**, 200 (1985).
5. A. B. Foster, M. Jarman and C.-S. Kwan (nee Leung), British Patent Application No. 8517999. Publication No. 2162177A (1986).
6. R. McCague and A. Seago, *Biochem. Pharmac.* **35**, 827 (1986).
7. A. Seago and J. W. Gorrod, in *Biological Oxidation of Nitrogen in Organic Molecules* (Eds. J. W. Gorrod and L. A. Damani), p. 187. Ellis Horwood, Chichester (1985).
8. I. Howe, D. H. Williams and R. D. Bowen, *Mass Spectrometry: Principles and Applications*, 2nd Edn, p. 134. McGraw-Hill, New York (1981).
9. A. B. Foster, *Adv. Drug. Res.* **14**, 1 (1985).
10. H. Egger, F. Bartlett, W. Itterley, R. Rodebaugh and C. Shimanskas, *Drug Metab. Dispos.* **10**, 405 (1982).
11. A. B. Foster, L. J. Griggs, I. Howe, M. Jarman, C.-S. Leung, D. Manson and M. G. Rowlands, *Drug Metab. Dispos.* **12**, 511 (1984).

* To whom correspondence should be sent.