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Technical Note

Microbial Transformation of Tamoxifen

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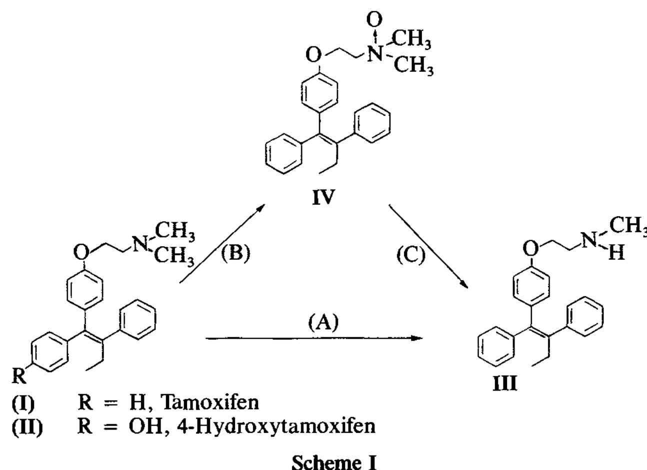
KEY WORDS: tamoxifen; microbial metabolism; tamoxifen *N*-oxide; *N*-desmethyltamoxifen; *Gliocladium roseum.*

INTRODUCTION

Tamoxifen (I), a nonsteroidal antiestrogen used in the treatment of breast cancer (1,2), is a pure estrogen antagonist in the chicken oviduct (3), a partial estrogen agonist/antagonist in the rat uterus (4), and a complete estrogen agonist in the mouse uterus (5). One possible explanation for the disparate action of tamoxifen in these species is the differential formation in mice, rats, and chickens of tamoxifen metabolites that have different estrogenic or antiestrogenic properties. Studies on the metabolism of tamoxifen (6–10) by mammalian species showed the formation of 4-OH tamoxifen (II), *N*-desmethyltamoxifen (III), and tamoxifen *N*-oxide (IV). Thus, interest in studying the metabolism of tamoxifen using mammalian systems has been sparked mainly because of the ubiquitous use of this drug in breast cancer therapy.



Scheme I

In view of the fact that some microbes display the full range of drug metabolism observed in mammals (11–13), we carried out this study to assess the metabolic activity of a large number of fungi for their ability to transform tamoxifen. The results obtained from this study showed that tamoxifen was resistant to metabolic transformation by most fungi tested. Only *Gliocladium roseum* showed appreciable transformation of tamoxifen (I) to *N*-desmethyltamoxifen (III) and tamoxifen *N*-oxide (IV), in 28 and 57% overall yields.

MATERIALS AND METHODS

General

Melting points were determined on a Fisher–Jones hot-plate apparatus and are uncorrected. Infrared spectra were taken with a Perkin–Elmer 281 spectrophotometer using Nujol disks. High-resolution mass spectra were determined on an LKB 9000 GC mass spectrometer. ^{1}H NMR were obtained on a 300-MHz Nicolet NT-300-W3 spectrometer using tetramethylsilane (TMS) as the internal standard and $CDCl_3$ as the solvent. Tamoxifen and *N*-desmethyltamoxifen were provided by ICI, Stewart Pharmaceuticals, Delaware.

Cultures and Fermentation Screening Procedures

Biotransformation experiments were performed using shake culture techniques by a two-stage fermentation procedure in a medium consisting of 2% glucose, 0.5% yeast extract, 0.5% K_2HPO_4, 0.5% neopeptone, and 0.5% NaCl. The medium was adjusted to pH 7 before sterilization by autoclaving for 15 min. After 72 hr of incubation in the above medium, 5 ml of Stage I culture was used as the inoculum for fresh Stage II cultures (50 ml/250-ml flask). After 24 hr of incubation of Stage II cultures, tamoxifen was added (0.25 mg/ml medium) as a suspension in dimethylformamide (180 mg/ml DMF). Culture controls consisted of fermentation blanks in which the organism was grown under identical conditions without substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and incubated under the same conditions.

Fermentation Sampling and Thin-Layer Chromatography (TLC)

The fermentations were sampled by extraction of 5 ml of culture broth with 5 ml of chloroform. After evaporation of the solvent, the residue was spotted on precoated silica gel TLC plates (0.25 mm). The plates were developed in tol-

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uene–methanol–acetone (5:2:2) and visualized by spraying with 1% ceric sulfate in 3 N H_2SO_4.

Transformation of Tamoxifen by *Gliocladium roseum* NRRL1829

A total of 360 mg of tamoxifen dissolved in 2 ml of DMF was evenly distributed among twenty-eight 250-ml Erlenmeyer flasks, each containing 50 ml of Stage II culture. The cultures were incubated (250 rpm, 27°C) for 3 days (no detectable improvement of the yield was observed after 3 days) and extracted three times with 1 liter of $CHCl_3$. The combined extracts were washed with water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* (40°C) to give 420 mg residue. The residue was applied to preparative TLC plates (1-mm-thick silica gel P/UV-254, E. Merck, Darmstadt) and developed first in $CHCl_3$–CH_3OH (9:1), followed by a second development in toluene–methanol–acetone (5:2:2). The bands were visualized under UV light and the areas corresponding to the metabolites were scraped from the plates, eluted with acetone–methanol, and evaporated to dryness. The yellowish residue (205 mg) with a 0.20 R_f value was crystallized from $CHCl_3$/ethyl acetate to afford 195 mg of pure IV. m.p. 65–66°C; IR (KBr) 2950, 2875, 1610, 1580, 1450, 965, 700, cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.91 (t, 3H, J = 8Hz, CH_3 CH_2), 2.45 (q, 2H, CH_3 CH_2); 3.25 [s, 6H, $N(CH_3)_2$], 3.6 (t, 2H, J = 6Hz, NCH_2 CH_2O), 4.46 (t, 2H, NCH_2 CH_2O), 6.53 and 6.77 (two d, 2H, J = 9Hz, aromatic AB system), 7.13 and 7.26 (two s each 5H, two phenyl); MS m/z (% R.A.) 387 [M^+, 0.2%], 371 [$(M-O)^+$, 2.7%], 357 [$(M-O)^+-CH_3$, 15.8%], 326 [$(M-O)^+-N(CH_3)_2$, 100%], 311 [$(M-O)^+-CH_2N(CH_3)_2$, 6.6%], 300 [$(M-O)^+-CH_2CH_2-\overset{+}{N}=CH_2$, 27.5%], 72 [$CH_2CH_2N(CH_3)_2^+$, 7.2%], 58 [$CH_2$ $-CH_3$ $N(CH_3)_2^+$, 21.1%], 18 [H_2O, 20.8%]; mass spectrum, calcd. for $C_{26}H_{29}NO_2$ m/e 387.5206, found m/e 387.5184.

The residue (99 mg) with an R_f value of 0.48 was crystallized from $CHCl_3$/acetone to yield 81 mg of pure III. m.p. 221–222°C; ^1H NMR ($CDCl_3$) δ 0.89 (t, 3H, J = 5Hz, CH_3 CH_2), 2.1 (q, 2H, CH_3CH_2), 2.54 (s, 3H, $N-CH_3$), 3.10 (m, 2H, $N-CH_2CH_2$), 4.01 (t, 2H, OCH_2CH_2), 6.55 and 6.76 (2 d, each 2H, J = 8Hz, aromatic), 7.08 and 7.29 (2s, each 5H, aromatic), 9.3 (s, 1H, NH); MS m/z (% R.A.) 358 ($M^+ + 1$, 18.1%), 357 (M^+, 62%), 301 ($M^+ + 1 - CH_2CH_2NHCH_3$, 24.9%), 300 ($M^+ - CH_2CH_2NHCH_3$, 100%), 58 ($CH_2CH_2NHCH_3^+$, 16.2%), 44 ($CH_2NHCH^+_3$, 47.3%); mass spectrum, calcd. for $C_{25}H_{27}NO$ m/z 357.4944, found m/z 357.4883.

RESULTS AND DISCUSSION

Screening with 96 known fungal species showed that tamoxifen was extremely resistant to metabolic transformation as evidenced by the fact that only one fungus, namely, *Gliocladium roseum* NRRL 1829, showed significant transformation. Large-scale fermentations of tamoxifen using *G. roseum* resulted in the formation of two compounds that showed the same chromatographic and spectral characteristics (IR, NMR, and MS) as the synthetic reference standards of *N*-desmethyltamoxifen (III) and tamoxifen *N*-oxide (IV).

It is quite interesting to note that *G. roseum* resulted in the formation of both the *N*-oxide and the *N*-desmethyl metabolite. The questions regarding the role of *N*-oxides as intermediates in the oxidative *N*-dealkylation of tertiary amines have been the subject of controversy (14–17). Indeed, several workers have shown that tertiary amines can be dealkylated by a one-step process involving cytochrome P-450 mixed-function oxidase as shown in Pathway A (16–18), while others presented evidence to indicate a two-step mechanism involving *N*-oxide synthetase requiring reduced pyridine nucleotides followed by *N*-demethylase as shown in Pathways B and C (14,18). Preliminary studies on the metabolism of tamoxifen *N*-oxide by *G. roseum* showed the formation of *N*-desmethyltamoxifen. Future studies using partially purified enzymes will be carried out to explore further the mechanism of *N*-dealkylation by *G. roseum*.

The results obtained from this study clearly show that tamoxifen is very resistant to biotransformation by microorganisms. Although we cannot explain the reasons for these results, it is worthy to note that during many of these transformations, a considerable amount of tamoxifen was retained in the cells, and any attempts at exhaustively extracting these cells using different solvents resulted in only 35–70% recovery of tamoxifen. In a preliminary experiment using whole-cell suspensions of active and heat-inactivated cell cultures, recovery of tamoxifen ranged from 82 to 94% from the heat-inactivated cell cultures, as opposed to 35 to 70% from the active cultures. These initial studies suggest metabolic activation of tamoxifen followed by possibly irreversible binding to cell constituents. Current studies using ^3H-tamoxifen are under way.

REFERENCES

1. G. Leclercq and J. C. Heuson. *Anticancer Res.* 1:217–222 (1981).
2. V. C. Jordan. *Cancer Treat. Rep.* **60**:1409–1419 (1976).
3. R. L. Sutherland, J. Mester, and E. E. Baulieu. *Nature* (*Lond.*) 267:434–435 (1977).
4. M. J. K. Harper and A. L. Walpole. *J. Reprod. Fert.* 13:101–119 (1967).
5. L. Terenius. *Acta Endocrinol. Copenh.* **66**:431–447 (1971).
6. J. L. Borgna and H. Rochefort. *Mol. Cell. Endocrinol.* 20:71–88 (1980).
7. H. K. Adam, E. J. Douglas, and J. V. Kemp. *Biochem. Pharmacol.* 27:145–147 (1979).
8. V. C. Jordan. *Breast Cancer Res. Treat.* 2:123–138 (1982).
9. V. C. Jordan, M. M. Collins, L. Rowsby, and G. Prestwich. *J. Endocrinol.* 75:305–316 (1977).
10. A. B. Foster, L. J. Griggs, M. Jarman, J. M. S. Van Maanen, and H. R. Schulter. *Biochem. Pharmacol.* 29:1977–1979 (1980).
11. R. V. Smith and J. P. Rosazza. *J. Natl. Prod.* 46:79–91 (1983).
12. P. J. Davis, S. K. Yang, and R. V. Smith. *Appl. Environ. Microbiol.* 48:327–331 (1984).
13. A. M. Clark, J. D. McChesney, and C. D. Hufford. *Med. Res. Rev.* 5:231–253 (1985).
14. D. M. Ziegler and F. Pettit. *Biochem. Biophys. Res. Commun.* 15:188–193 (1964).
15. P. Willi and M. H. Bickel. *Arch. Biochem. Biophys.* 156:772–779 (1973).
16. B. B. Brodie, J. R. Gillette, and B. N. LaDu. *Annu. Rev. Biochem.* 27:427–443 (1958).
17. R. E. McMahon and H. R. Sullivan. *Xenobiotica* 7:377–382 (1977).
18. S. Hamill and D. Y. Cooper. *Xenobiotica* 14:139–149 (1984).