# Biphenyl Oxide Hydroxylation by *Cunninghamella echinulata*

Françoise M. Seigle-Murandi,*,† Serge M. A. Krivobok,† Régine L. Steiman,† Jean-Louis A. Benoit-Guyod,‡ and Georges-André Thiault§

Laboratoire de Botanique, Cryptogamie, Biologie Cellulaire et Génétique and Laboratoire de Toxicologie et Ecotoxicologie, Groupe pour l'Etude du Devenir des Xénobiotiques dans l'Environnement (GEDEXE), UFR de Pharmacie, Université J. Fourier (Grenoble I), B.P. 138, 38243 Meylan Cédex, France, and Produits Chimiques Auxiliaires et de Synthèse (PCAS), 23 Rue Bossuet, 91160 Longjumeau, France

Screening of selected fungi proved *Cunninghamella echinulata* most suitable for inquiry into hydroxylation of biphenyl oxide. The process depended on cultivation conditions and yielded up to 76% 4-hydroxybiphenyl oxide and about 6% 4,4'-dihydroxybiphenyl oxide.

4-Hydroxybiphenyl oxide is an important ingredient of Fenoxicarb (Zurflüh et al., 1986), an insecticide that prevents normal insect development. Presently, it is synthesized from anisole (Thiault, unpublished results). Yet several fungi catalyze hydroxylation of aromatic compounds without undergoing ring cleavage (Clark et al., 1985; Kieslich, 1984; Smith and Rosazza, 1975) including *Cunninghamella elegans*, *Aspergillus niger*, *Rhizopus stolonifer*, *Cunninghamella bainieri*, *Penicillium chrysogenum* (Smith and Rosazza, 1975), *Neurospora crassa*, and other members of the Mucorales (Cerniglia et al., 1978), thus providing a natural alternative.

We have previously described conditions required by *Absidia spinosa* for the formation of 5-hydroxybenzimidazole from benzimidazole (Chapelle et al., 1986; Seigle-Murandi et al., 1986). The mechanism of aromatic hydroxylation by some organisms has been elucidated recently. The use of microorganisms in the simulation of mammalian metabolic processes is classical and provides information on the mechanisms of action, toxicity, pharmacological activity (Davis, 1988; Smith and Rosazza, 1982; Smith et al., 1977), and metabolic pathways of xenobiotics (Reighard and Knapp, 1986), though with some substrates (PAH) there might be significant differences (Cerniglia, 1984).

Although extensive work has been done on biphenyl (Dodge et al., 1979; Wiseman et al., 1975; Cox and Golbeck, 1985), no study was concerned with microbial hydroxylation of biphenyl oxide. Studies suggested that *Cunninghamella echinulata* effectively parahydroxylated some synthetic substrates (Pasutto et al., 1987). The possible toxicity of biphenyl oxide toward previously selected fungal strains (Chapelle et al., 1986) has been checked and the bioconversion of biphenyl oxide investigated under various culture conditions. The purpose of this work is to obtain a specific hydroxylation of biphenyl oxide at position 4.

## MATERIALS AND METHODS

**Fungal Strains and Culture Conditions.** Microorganisms were taken from the collection of our Institute (CMPG, Collection Mycology Pharmacy Grenoble). Most of them were Fungi Imperfecti isolated from different sources (Seigle-Murandi et al., 1980a; Seigle-Murandi et al., 1981). They were stored at 4 °C on solid malt extract medium (1.5%). Each strain was cultivated both on malt extract medium (15 g/L) and on Galzy and Slonimski synthetic medium (Galzy and Slonimski, 1957) without glucose and with agar (1.5%). Various amounts of biphenyl oxide were added as a solution in ethanol, and the media were sterilized at 121 °C for 20 min. The final concentrations of biphenyl oxide (BPO) were 0.01, 0.1, 0.5, 1.5 and 10 g/L. Malt extract medium was used to examine the toxicity of BPO upon the microorganisms. Toxicity is expressed by the diameter of inhibition (millimeters). Cultivation on synthetic medium with biphenyl oxide as the only carbon source was a test of consumption or biotransformation. Synthetic medium was also used without BPO to estimate the nutritive supply of agarose that is not an inert matrix (Seigle-Murandi et al., 1980b). Cultivation was at 24 °C for 8 days.

*C. echinulata* selected after cultivation on solid media was cultivated in liquid media. Mycelial inocula were used throughout the investigation. They were prepared by homogenization of 5-day-old cultures grown in 250-mL Erlenmeyer flasks; each contained 50 mL of growth media with biphenyl oxide (0.01 g/L) for induction. Two milliliters of homogenized culture was inoculated in 100 mL of liquid media. Four different liquid media (pH 6) were used: Galzy and Slonimski medium (Galzy and Slonimski, 1957) with glucose (5 g/L); soy medium (Smith and Rosazza, 1974); cornstarch medium (Golbeck et al., 1983); and tartaric acid medium (modification of dimethylsuccinic acid medium) (Ander et al., 1984) with glucose (5 g/L). Cultivation was done in 500-mL Erlenmeyer flasks containing 100 mL of medium under shaking conditions (180 rpm) at 24 °C. Biphenyl oxide (0.5 g/L, final concentration) was added to a 2-day-old culture according to the method of Smith et al. (1980). The cultivation was carried out for 3 more days. According to the results obtained, two kinetic studies on the production of 4-hydroxybiphenyl oxide (4-OH-BPO) and 4,4'-dihydroxybiphenyl oxide (4,4'-di-OH-BPO) and on the depletion of biphenyl oxide by *C. echinulata* were carried out in tartaric acid medium (glucose 5 g/L, pH 6). The fungus was grown for 7 days at 24 °C in a 1-L batch fermentor (Biolafitte). The agitation speed was 250 rpm. The aeration rate was 2 L/min. Biphenyl oxide was added on day 2 (0.5 g/L). The only difference between the two kinetic studies was the amount of inoculum (i.e., 20 and 40 mL, respectively). Culture controls consisted of fermentation blanks in which the fungus was grown under identical conditions but without substrate. Substrate controls consisted of sterile media containing the same amount of substrate and incubated under the same conditions. Each series of experiments was made in triplicate.

**Extraction of Metabolites.** Each culture medium was filtered off; the aqueous phase was acidified to pH 2.0 with 6 M HCl and extracted with bidistillate ethyl acetate (3 volumes). The combined extracts were dried over anhydrous $Na_2SO_4$ and evaporated to dryness at 30 °C under vacuum. The residue was dissolved in ethyl acetate for thin-layer chromatography (TLC) or in methanol for high-performance liquid chromatography (HPLC).

**Analyses.** *TLC.* TLC was carried out on silica gel 60 $F_{254}$ plates (Merck). Different solvent systems were used: 1, benzene/acetic acid (50:10); 2, chloroform/acetone (40:10); 3, cyclohexane/ethyl formate/formic acid (30:20:2). The $R_f$ values were as

* Author to whom correspondence should be addressed.

† Laboratoire de Botanique, Cryptogamie, Biologie Cellulaire et Genetique.

‡ Laboratoire de Toxicologie et Ecotoxicologie.

§ Produits Chimiques Auxiliaires et de Synthèse.

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**Table I. Biotransformation of Biphenyl Oxide by *C. echinulata* Grown in Four Various Liquid Media (3 Days after the Addition of Biphenyl Oxide, 0.5 g/L)**

| media | mycelial dry wt, g/L | 4-OH-BPO produced, g/L | product yield, % |
|---|---|---|---|
| soy | 4.4 | 0.101 | 20.2 |
| cornstarch | 5.1 | 0.021 | 4.2 |
| Galzy and Slonimski | 3.9 | 0.104 | 20.8 |
| tartaric acid | 3.3 | 0.192 | 38.4 |

follows: biphenyl oxide (BPO), 0.92 (system 1), 0.89 (system 2), 0.81 (system 3); 4-hydroxybiphenyl oxide (4-OH-BPO), 0.65 (system 1), 0.76 (system 2), 0.30 (system 3); 4,4'-dihydroxybiphenyl oxide (4,4'-di-OH-BPO), 0.26 (system 3). The detection of 4-OH-BPO and of 4,4'-di-OH-BPO was made at 254 nm and confirmed by spraying chromatograms with diazotized parasulfanilic acid. Their concentration and the depletion of biphenyl oxide were calculated by scanning chromatograms with a high-performance thin-layer chromatographic scanning apparatus (Shimadzu CS 930).

*HPLC.* HPLC was performed with a liquid chromatograph (Waters Associates) equipped with a pump (Model 510), an injector (Model U6K), and a UV detector (Lambda-Max Model 481). The semipreparative column was 7.5 mm inside diameter × 300 mm long, filled with Spherisorb ODS 2-$C_{18}$, 5 $\mu$m (Société Française Chromato Colonne, Neuilly-Plaisance, France). The mobile phase was methanol/water (70:30 v/v), pH 5.0, and flow rate 1.7 mL/min. Detection was at 254 nm.

*GC-MS.* Gas chromatography (GC) was carried out with a Girdel instrument (Model 32) equipped with a capillary column (SE-30, 25 m × 0.32 mm) programmed from 130 to 270 °C at 6 °C/min. The carrier gas was He (1.5 mL/min). Mass spectrometry (MS) was performed with a Nermag (Model R 10-10 C) mass spectrometer. The compounds, dissolved in dry pyridine, were silylated with bis(trimethylsilyl)trifluoroacetamide for 2 h at room temperature. Identification of products was based on comparison with authentic reference compounds (from PCAS, Longjumeau, France).

## RESULTS AND DISCUSSION

**Preliminary Experiments.** Previous studies on the ability of 610 strains of micromycetes to introduce a hydroxy group into a benzimidazole substrate (Chapelle et al., 1986) showed that an efficient hydroxylation was obtained with *A. spinosa* (CMPG 428), *A. niger* (CMPG 401), *Aspergillus parasiticus* (CMPG 349), *Penicillium verrucosum* var. corymbiferum (CMPG 208), and *Ulocladium botrytis* (CMPG 148). *C. echinulata* (CMPG 668) has been widely used to introduce a hydroxy group into aromatic substrates (Pasutto et al., 1987). Biological oxidation of aromatic structures may be a detoxification process or it may represent a step in the partial or total consumption of a substrate. Thus, the possible toxicity of biphenyl oxide had to be determined, followed by its eventual biotransformation by selected fungi.

Preliminary studies were made with several concentrations of BPO from 0.01 to 10 g/L. Toxicity assay on solid malt extract medium showed that all the concentrations exerted a toxic effect: growth was diminished or inhibited. Cultivation on the synthetic Galzy and Slonimski medium without glucose showed that *A. spinosa* and especially *C. echinulata* were the more performant strains. A final concentration of BPO of 0.5 g/L was chosen for the experiments in liquid media.

*C. echinulata* was cultivated in four different liquid media. Mycelial dry weights and amounts of 4-OH-BPO produced are given in Table I. The production of 4-OH-BPO was inversely correlated with the growth of the fungus since the best results (38%) were obtained with the tartaric acid medium in which growth was the poorest. On the contrary, the lowest yield was obtained in cornstarch medium (4%), where the growth of the fungus was the best. Complex media such as cornstarch medium and soy

**Table II. Kinetic Studies of Biotransformation of Biphenyl Oxide (BPO) by *C. echinulata* Grown in a Batch Fermentor (Tartaric Acid Medium, BPO 0.5 g/L)**

| | sampling time,[a] days | BPO depletion, % | 4-OH-BPO g/L | 4-OH-BPO yield, % | 4,4'-di-OH-BPO g/L | 4,4'-di-OH-BPO yield, % |
|---|---|---|---|---|---|---|
| A[b] | 2 | 44.2 | 0.095 | 19.0 | 0.006 | 1.2 |
| | 3 | 63.8 | 0.240 | 48.0 | 0.007 | 1.4 |
| | 4 | 92.5 | 0.098 | 19.6 | 0.008 | 1.6 |
| | 5 | 95.6 | 0.015 | 3.0 | 0.010 | 2.0 |
| B[b] | 2 | 82.6 | 0.380 | 76.0 | 0.013 | 2.6 |
| | 3 | 84.8 | 0.285 | 57.0 | 0.018 | 3.6 |
| | 4 | 95.2 | 0.280 | 56.0 | 0.025 | 5.0 |
| | 5 | 98.3 | 0.265 | 53.0 | 0.032 | 6.4 |

[a] Sampling time (days) after addition of biphenyl oxide. [b] A and B are studies differing by the quantity of inoculum (A, 20 mL; B, 40 mL; see Material and Methods).

medium allow good growth of most microorganisms and have been used for the hydroxylation of naphthalene (Cerniglia et al., 1978) and biphenyl (Golbeck et al., 1983). Soy medium gave a yield of 20% for 4-OH-BPO, but extraction and purification of the metabolites were difficult. Cultivation in synthetic medium avoided these difficulties and allowed better control of the bioconversion and its further optimization. The 4-OH-BPO yield was better with tartaric acid medium (38%) than with Galzy and Slonimski medium (20%). This difference might be related to nitrogen concentration as tartaric acid medium is a nutrient nitrogen limited medium (2.6 mM), while Galzy and Slonimski medium is not (82.5 mM). The nitrogen limitation of the medium has been shown to be a very important factor in the metabolization of some substrates, particularly lignocellulosic compounds (Ander et al., 1984). Nitrogen starvation might also possibly be an important factor to be considered for hydroxylation. This hypothesis must be checked by using different substrates and other strains of fungi. Furthermore, tartaric acid medium is buffered (pH 6), while Galzy and Slonimski medium is not. The relationship between the degree of hydroxylation and culture medium aeration was also shown: the transformation yield in tartaric acid medium was 18% with static cultures compared to 38% with agitated cultures.

**Production of 4-Hydroxybiphenyl Oxide by *C. echinulata* in Batch Fermentor.** The kinetic studies in batch fermentors differed only in the quantity of the inoculum, which was more important in the second experiment. In the first experiment, oxidation of BPO (Table II, A) was apparent 2 days after its addition. The bioconversion yield was higher (48%) on day 3 than on days 4 and 5 and indicated that 4-OH-BPO was itself metabolized. In the second experiment (Table II, B), the highest transformation yield was obtained on day 2 (76%) and then decreased but remained higher (53%) than in experiment A (3%). The BPO depletion was comparable in experiments A and B (95% and 98%, respectively).

We proposed above that nitrogen starvation is important but not the only factor to be considered. The agitation speed and the aeration rate were higher in batch fermentors compared with shaker flasks. In shaker flasks (Table I), the bioconversion yield was only 38% after 3 days, while in batch fermentors it reached 48% after 3 days (experiment A) and 76% after 2 days (experiment B) (Table II). When aeration in batch fermentors was 0.5 L/min instead of 2 L/min, the yield of 4-OH-BPO was reduced by 62%. The biomass of inoculum and the physiological status of the fungus must also be considered. The inoculum of experiment A was half that of experiment B in the batch fermentor. The recovery of 4-OH-BPO was higher and more rapidly obtained in experiment B, the lag phase being

shorter. Each of these different parameters intervenes in the biotransformation process.

**Metabolite Related to 4-Hydroxybiphenyl Oxide.** 4-OH-BPO production was accompanied by the appearance of another metabolite detected when chromatograms were sprayed with diazotized parasulfanilic acid. After extraction and purification on a semipreparative HPLC column, mass spectra led to the identification of 4,4′-di-OH-BPO. The yield of formation of this compound is shown in Table II as a function of time: after 5 days of cultivation, it amounted to 2% in experiment A and 6% in experiment B. The synthesis of this compound increased but in relation to neither the depletion of BPO nor the formation of 4-hydroxybiphenyl, which decreased rapidly after days 3 or 4. 4-Hydroxybiphenyl is probably not only hydroxylated, giving 4,4′-di-OH-BPO, but also metabolized to sulfonic acid conjugates that were not detected by use of diazotized parasulfanilic acid. Such an observation has been made during the hydroxylation of biphenyl by *A. parasiticus* (Golbeck et al. 1983). Conjugation reactions of xenobiotics either as sulfates or as glucuronides is a main metabolic pathway in mammals and is known to be important for detoxification and excretion (Hackett et al., 1983; Hackett and Griffiths, 1985). It also occurs in plant systems (Harborne and King, 1976; Imperato, 1980). Sulfatation by microbial systems is extremely rare, and glycosylation is not a common pathway (El-Sharkawy and Abul-Hajj, 1987). However, it was shown that *C. elegans* could form glucuronide and sulfate conjugates of phenolic compounds (Cerniglia et al., 1982) and *Aspergillus toxicarius* of biphenyl (Golbeck and Cox, 1984). More recently, results obtained with *Streptomyces fulvissimus* demonstrated that it has the ability to carry out sulfatation reactions (Ibrahim and Abul-Hajj, 1989). But the importance of conjugation in microbial systems is far from being clearly understood and needs additional studies.

## CONCLUSION

After preliminary studies with six fungi, *C. echinulata* was chosen to hydroxylate BPO to 4-OH-BPO. Using four different media, we have demonstrated that higher bioconversion was obtained in nutrient nitrogen limited medium. The intensity of the metabolic process is related to the density of inoculum, agitation, and aeration of the medium. Our results show that the optimal incubation lasted 2–3 days since longer periods resulted in the appearance of 4,4′-di-OH-BPO and other metabolites, probably sulfate conjugates, while the quantity of 4-OH-BPO decreased rapidly.

The classical synthesis of 4-OH-BPO passes through many steps, and the raw materials are expensive. The optimization of the microbial transformation process might give an alternative route that is faster and cheaper.

## LITERATURE CITED

Ander, P.; Eriksson, K. E.; Yu, H. S. Metabolism of lignin-derived aromatic acids by wood-rotting fungi. *J. Gen. Microbiol.* **1984**, *130*, 63–68.

Cerniglia, C. E. Microbial metabolism of Polycyclic Aromatic Hydrocarbons. *Adv. Appl. Microbiol.* **1984**, *30*, 31–71.

Cerniglia, C. E.; Hebert, R. L.; Szaniszlo, P. S.; Gibson, D. T. Fungal transformation of naphthalene. *Arch. Microbiol.* **1978**, *117*, 135–143.

Cerniglia, C. E.; Freeman, J. P.; Mitchum, R. K. Glucuronide and sulfate conjugation in the fungal metabolism of aromatic hydrocarbons. *Appl. Environ. Microbiol.* **1982**, *43*, 1070–1075.

Chapelle, F.; Steiman, R.; Seigle-Murandi, F.; Luu Duc, C. 5-Hydroxylation of benzimidazole by Micromycetes. I. Strains selection. *Appl. Microbiol. Biotechnol.* **1986**, *23*, 430–433.

Clark, A. M.; McChesney, J. D.; Hufford, C. D. The use of microorganisms for the study of drug metabolism. *Med. Res. Rev.* **1985**, *5*, 231–253.

Cox, J. C.; Golbeck, J. H. Hydroxylation of biphenyl by *Aspergillus parasiticus*: approaches to yield improvement in fermentor cultures. *Biotechnol. Bioeng.* **1985**, *27*, 1395–1402.

Davis, P. J. Microbial models of mammalian drug metabolism. *Dev. Ind. Microbiol.* **1988**, *29*, 197–219.

Dodge, R. H.; Cerniglia, C. E.; Gibson, D. T. Fungal metabolism of biphenyl. *Biochem. J.* **1979**, *178*, 223–230.

El-Sharkawy, S.; Abul-Hajj, Y. Microbial transformation of zearalenone. I. Formation of zearalenone-4-O-beta-glycoside. *J. Nat. Prod.* **1987**, *50*, 520–521.

Galzy, P.; Slonimski, P. Variations physiologiques de la levure au cours de la croissance sur l'acide lactique comme seule source de carbone. *C. R. Acad. Sci.* **1957**, *245D*, 2423–2426.

Golbeck, J. H.; Cox, J. C. The hydroxylation of biphenyl by *Aspergillus toxicarius*: conditions for a bench scale fermentation process. *Biotechnol. Bioeng.* **1984**, *26*, 434–441.

Golbeck, J. H.; Albaugh, S. A.; Radmer, R. Metabolism of biphenyl by *Aspergillus toxicarius*: induction of hydroxylating activity and accumulation of water-soluble conjugates. *J. Bacteriol.* **1983**, *156*, 49–57.

Hackett, A. M.; Griffiths, L. A. The quantitative disposition of 3-O-methyl-(+)-[$^{14}$C]catechin in man following oral administration. *Xenobiotica* **1985**, *15*, 907–914.

Hackett, A. M.; Griffiths, L. A.; Broillet, A.; Wermeille, M. The metabolism and excretion of (+)-[$^{14}$C]cyanidanol-3 in man following oral administration. *Xenobiotica* **1983**, *13*, 279–286.

Harborne, J. B.; King, L. Flavonoid sulfates in Umbelliferae. *Biochem. Syst. Ecol.* **1976**, *4*, 111–115.

Ibrahim, A. R.; Abul-Hajj, Y. J. Aromatic hydroxylation and sulfatation of 5-hydroxyflavone by *Streptomyces fulvissimus*. *Appl. Environ. Microbiol.* **1989**, *55*, 3140–3142.

Imperato, F. A new sulfated flavonol glycoside in the fern *Asplenium fontanum* Bernh. *Chem. Ind.* **1980**, *1980*, 540–541.

Kieslich, K. In *Biotechnology*: *Biotransformations*; Kieslich, K., Ed.; Verlag Chemie: Weinheim, FRG, 1984; Vol. 6a.

Pasutto, F. M.; Singh, N. N.; Jamali, F.; Coutts, R.; Abuzar, S. Microbiological systems in organic synthesis: preparation of racemic Prenalterol utilizing *Cunninghamella echinulata*. *J. Pharm. Sci.* **1987**, *76*, 177–179.

Reighard, J. B.; Knapp, J. E. Microbial models of mammalian metabolism. *Pharm. Int.* **1986**, *7*, 92–94.

Seigle-Murandi, F.; Nicot, J.; Sorin, L.; Genest, L. C. *Rev. Ecol. Biol. Sol.* **1980a**, *17*, 149–157.

Seigle-Murandi, F.; Steiman, R.; Lacharme, J. *Bull. Trav. Pharm. Lyon* **1980b**, *24*, 7–19.

Seigle-Murandi, F.; Nicot, J.; Sorin, L.; Lacharme, J. *Cryptogam. Mycol.* **1981**, *2*, 217–237.

Seigle-Murandi, F.; Steiman, R.; Chapelle, F.; Luu Duc, C. 5-Hydroxylation of benzimidazole by Micromycetes. II. Optimization of production with *Absidia spinosa*. *Appl. Microbiol. Biotechnol.* **1986**, *25*, 8–13.

Smith, R. V.; Rosazza, J. P. Microbial models of mammalian metabolism. *J. Pharm. Sci.* **1975**, *64*, 1737–1759.

Smith, R. V.; Rosazza, J. P. Microbial transformations as a means of preparing mammalian drug metabolites. In *Microbial transformations of bioactive compounds*; Rosazza, J. P., Ed.; CRC Press: Boca Raton, FL, 1982; Vol. 2, pp 1–42.

Smith, R. V.; Acosta, D.; Rosazza, J. P. Cellular and microbial models of investigation of mammalian metabolism of xenobiotics. *Adv. Biochem. Eng.* **1977**, *5*, 70–100.

Smith, R. V.; Davis, P. J.; Clark, A. M.; Glover-Milton, S. Hydroxylations of biphenyl by fungi. *J. Appl. Bacteriol.* **1980**, *49*, 65–73.

Wiseman, A.; Gondal, J. A.; Sims, P. 4′-hydroxylation of biphenyl by yeast containing cytochrome P-450: radiation and thermal stability, comparisons with liver enzyme (oxidized and reduced forms). *Biochem. Soc. Trans.* **1975**, *3*, 278–281.

Zurflüh, R.; Pfiffner, A.; Broger, E.; Fischer, U.; Lohri, B.; Schlageter, M.; Schneider, F. Fenoxycarb. A novel insect controlling agent; synthetic approaches. *6th Int. Congr. Pestic. Chem., 6th* **1986**.

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