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Microbial hydroxylation. I. Hydroxylation of aniline by *Aspergillus alliaceus*, *A. albertensis* and *A. terreus*

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SUMMARY

Six hundred and seventy microorganisms were screened for the ability to perform stereoselective aromatic hydroxylation reactions of industrial significance, using aniline as a model substrate. TLC and HPLC analyses with diode array detection were used to identify and characterize hydroxylase activities. Of 79 cultures belonging to the species *Aspergillus alliaceus*, *A. albertensis*, and *A. terreus*, 26 strains produced 2-aminophenol. Thirty strains were able to hydroxylate aniline in the *para* position. Five strains of *A. terreus* produced an unidentified phenolic compound in high yield.

INTRODUCTION

Screening for microbial enzymes is useful for several reasons. Fungal monooxygenase systems often perform reactions identical to mammalian liver P-450 enzymes [9,23]. Since microbial enzymes are easier to work with and scale up, they have been used to study metabolism of antitumor compounds [21], carcinogenic compounds [5,19], and toxic fungal metabolites [8]. They also have been used to study the metabolic fate of insecticides [22] and herbicides [13] in the environment. Microbial transformations of therapeutically useful compounds provide an important source of new active derivatives [16].

The types of microorganisms known to carry out such useful enzymatic reactions are often *Pseudomonas* species [15], *Streptomyces* species, or filamentous fungi of genera such as *Cunninghamella*, *Mucor* [15], or *Beauveria* [24]. The genus *Aspergillus* also is a major source of species capable of many useful biotransformation reactions. For example, *A. niger* is known for the ability to hydroxylate acyclic and cyclic terpenes [1,10,14,17,20]. These biotransformations are useful in the production of fragrances, flavors, and anticancer compounds. *A. ochraceus* has been studied and used for its ability to regio- and stereoselectively hydroxylate progesterone in the 11-α position, a biotransformation useful in steroid synthesis [4]. *A. ochraceus* also has been studied for the stereoselective hydroxylation of β-thujone [11]. *A. alliaceus* has been studied for its aromatic hydroxylation of the antitumor compound ellipticine [6]. *A. parasiticus* performs *para*-hydroxylation of biphenyl [7], a reaction of use in the plastics and dye industries.

One of the reasons so many applications have been found for microbial enzymes is that they often can achieve regioselective and stereoselective hydroxylations that are difficult to achieve chemically [18]. There are chemical methods to achieve aromatic hydroxylation such as the use of superacids [12]. However, the range of products formed by these methods is limited primarily to *meta*-hydroxylated compounds [2], and the range of compounds that would be suitable starting materials is limited by their stability under the reaction conditions.

In this project we screened a large number of microbial isolates, mostly filamentous fungi, for regioselective aryl hydroxylases of potential industrial significance. Specific biotransformations of the model substrate, aniline, to monohydroxylated products were identified by comparisons with reference chemical standards using thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). Six hundred microorganisms were initially screened. A total of 120 strains belonging to 17 genera and 77 species were from the Agricultural Research Service Culture Collection (NRRL), Peoria, IL, USA and 480 environmental strains were isolated from bark, soil, and sludge samples collected from several sites in Illinois, Wisconsin, North Carolina, and Montana, USA. Although some instances of hydroxylase activity were detected in other genera in this initial broad screen (unpublished data), striking results with five strains of two *Aspergillus* species led us to perform a

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focused screen to investigate closely related strains. The second screen resulted in identification of additional biocatalysts with aryl hydroxylase activities. We describe here the hydroxylation capabilities detected from strains of *Aspergillus alliaceus*, *A. albertensis*, and *A. terreus* in the two screens.

MATERIALS AND METHODS

Reference chemicals

Aniline (99.5 +%, 2-aminophenol (99%), 3-aminophenol (98%) and 4-aminophenol (98%) were obtained from Aldrich Chemical Co., Milwaukee, WI, USA. Chemicals were used as received after purity was checked by TLC.

Strains and media

In an initial broad screen of 600 microorganisms, five of nine cultures of *A. alliaceus* or *A. terreus* were able to hydroxylate aniline. A second screen focusing on 70 strains of *A. albertensis*, *A. alliaceus*, and *A. terreus* identified additional hydroxylating cultures. All strains of *A. alliaceus, A. albertensis*, and *A. terreus* were from the Agricultural Research Service Culture Collection (NRRL), Peoria, IL, USA. Table 1 lists the culture numbers and names of strains in which aniline hydroxylase activity was detected.

All cultures were stored in 25% glycerol at −80 °C, and grown as slant cultures for one to two weeks on one of the following: Sabouraud maltose agar (Difco) containing 2% agar, YPD agar (1.0% yeast extract, 2.0% Bacto-peptone, 2.0% dextrose, 2.0% agar), or modified Czapek's agar (containing 0.3% $NaNO_2$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 20% sucrose, and 2% agar). Soy flour–glucose medium, adapted from Betts et al. [3], consisting of 2% dextrose, 0.5% soy flour, 0.5% yeast extract, 0.5% K_2HPO_4, and 0.5% NaCl, adjusted to pH 7.0 with 5 *N* HCl, was used in the screening of cultures. Media were sterilized at 121 °C for 15 min.

Fermentation protocol

A two-stage fermentation was used. Initial cultures were grown in 25 ml soy flour–glucose medium held in 125-ml Erlenmeyer flasks using foam plugs (Jaece, N. Tonawanda, NY, USA). Inoculum was prepared by agitation of the surface of each slant culture with a few milliliters of sterile medium, forming a suspension which was used to inoculate medium in a flask. Incubations were carried out at 250 r.p.m., 28 °C in a New Brunswick Scientific (Edison, NJ, USA) Model G-27 PsycroTherm Incubator Shaker (1″ diameter circular orbit). After three days, 2.5 ml of each culture was transferred to a fresh flask of the same medium. Twenty-four hours later 10 mg of substrate (aniline) was added to each flask as a solution in 100 μl of dimethylformamide. For control cultures, no substrate or dimethylformamide was added. Additional control flasks contained only sterilized medium or sterilized medium plus dimethylformamide. Several samples were taken from each flask over a six-day period.

TABLE 1

Aspergillus cultures able to hydroxylate aniline[a]

Culture name	NRRL culture no.	Metabolites produced[b]
A. alliaceus	315	A4
A. terreus	1921	A2, A4, U
A. terreus	6300	A2
A. terreus	13030	A2
A. alliaceus	13076	A4
A. terreus	255	A4
A. terreus	256	A2, A4
A. terreus	259	A4
A. terreus	260	A4
A. terreus	265	A4
A. terreus	267	A2, A4
A. terreus	268	A4
A. terreus	269	A4
A. terreus	270	A2
A. terreus	272	A2
A. terreus	273	A2, A4
A. terreus	535	A2
A. terreus	539	A2
A. terreus	571	A2
A. terreus	640	A2
A. terreus	648	A4
A. terreus	649	A4
A. terreus	655	A4
A. terreus	675	A4
A. terreus	676	A4
A. terreus	680	A2, A4
A. terreus	1216	A2
A. terreus	1217	A4
A. terreus	1218	A2, A4
A. terreus	1221	A2, A4
A. alliaceus	1236	A4
A. terreus	1245	A4
A. terreus	1246	A2
A. terreus	1789	A4
A. terreus	1920	A2
A. terreus	1965	A2, A4
A. terreus	2281	A2, A4
A. terreus	4609	A2
A. albertensis	20602	A4
A. terreus	A-200	A4
A. terreus	A-201	A2
A. terreus	A-211	A2
A. terreus	A-212	A2
A. terreus	A-311	U
A. terreus	A-315	U
A. terreus	A-202	A4, U
A. terreus	A-210	A2
A. alliaceus	A-2360B	A4
A. terreus	A-5787	A2, A4, U

[a]The first five cultures are from an initial broad screen. The rest of the cultures are from an in-depth screen of three species.
[b]A2 = 2-aminophenol, A3 = 3-aminophenol, A4 = 4-aminophenol, U = unidentified phenol.

Assay procedure

Samples from each flask (2.5 ml) were poured or pipetted into 12 × 75 mm Pyrex tubes with push-in stoppers (Sarstedt Laboratory Wares, Newton, NC, USA). Sampling times were 12, 24, 48, 72, and 144 h after addition of substrate. Cultures that grew in clumps too large to pipet were first homogenized (Omni 1000 2-speed homogenizer with autoclave-sterilized 10 × 95 mm generator, Omni International, Waterbury, CT, USA). Each 2.5-ml sample was extracted with 0.75 ml ethyl acetate by agitation for 5 s using a Thermolyne Maxi-mix (Pierce Chemical Co., Rockford, IL, USA) and centrifugation at 2793 × *g* for 5 min. The ethyl acetate extract layers were transferred into Titertube MicroTubes in 8 × 12 racks (Bio-Rad Laboratories, Richmond, CA, USA) to be used for TLC. For HPLC analysis, the ethyl acetate extracts were evaporated to dryness under a stream of nitrogen and the residue was dissolved in methanol.

Thin-layer chromatography (TLC)

Extracts (75 μl) were spotted along with standard solutions onto 0.25-mm thick Silica Gel 60 F-254 Pre-Coated TLC Plates (E. Merck, Darmstadt, Germany) using a Titertek Digital Multichannel Pipette (EFLAB, Finland). For the focused screen of 70 strains of three *Aspergillus* species, 150 μl were spotted. In general, TLC solvents and spray reagents used were those of Smith and Rosazza [23]. Plates of samples from aniline-fed culture and standards were developed in benzene–methanol–acetic acid (90:16:8). A second solvent system, toluene–piperidine (75:30), was used to confirm results. Spots were visualized first under UV_{254} light and again after spraying sequentially with 5% aqueous ferric nitrate, 0.5% sulfanilic acid in 2% HCl and 0.5% aqueous sodium nitrite (1:1), then 1% ferric chloride in methanol–water (1:1).

High performance liquid chromatography (HPLC)

Methanolic solutions of samples from aniline-fed cultures or standards were chromatographed using a Spectra-Physics SP8800 pump (Spectra-Physics, St Charles, MO, USA), a Waters WISP 710B autoinjector (Waters Associates, Milford, MA, USA), an ABI 30 × 4.6 mm Spheri-5RP-18 guard column (Applied Biosystems, Ramsey, NJ, USA), a Waters 300 × 3.9 mm μBondapak C-18 pre-column, a DuPont 250 × 4.6 mm Zorbax RX-C8 column (DuPont Co., Wilmington, DE, USA), and an ABI 1000S Diode Array detector. The solvent used was a linear gradient over 15 min from 100% A to 100% B, where A consisted of 85 mM $(NH_4)_2PO_4$ buffer, pH 7.0, 15% methanol, and 2.5% isopropanol; and B consisted of 60 mM $(NH_4)_2PO_4$ buffer, pH 7.0, 37.5% methanol, and 5% isopropanol. The flow rate used was 1.0 ml min^{-1} and eluted peaks were detected at 230 nm and recorded.

RESULTS

Hydroxylase activities identified in a broad screen

From the initial TLC investigation of 600 microorganisms from diverse genera and geographic locations, we identified

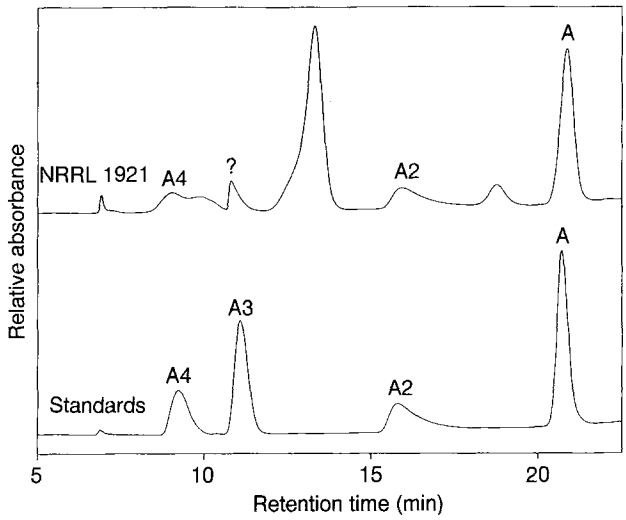

Fig. 1. HPLC chromatogram at 230 nm of 2- and 4-aminophenol production by culture NRRL 1921 after 24-h incubation. Top trace = sample extract from culture NRRL 1921. Bottom trace = reference standards. A4 = 4-aminophenol (elution vol. 9.24 ml); A3 = 3-aminophenol (elution vol. 11.09 ml); A2 = 2-aminophenol (elution vol. 15.8 ml); A = aniline (elution vol. 20.71 ml).

five cultures belonging to two species of *Aspergillus* that were able to hydroxylate the model substrate aniline in the *ortho* and/or the *para* positions. These organisms are listed in the first five lines of Table 1. Production of 2-aminophenol (A2) and/or 4-aminophenol (A4) was confirmed by chromatography in a second TLC solvent system showing the presumed metabolites at the same R_F as standards.

HPLC analysis using a diode array detector (Figs 1–3) allowed unambiguous identification of peaks from culture

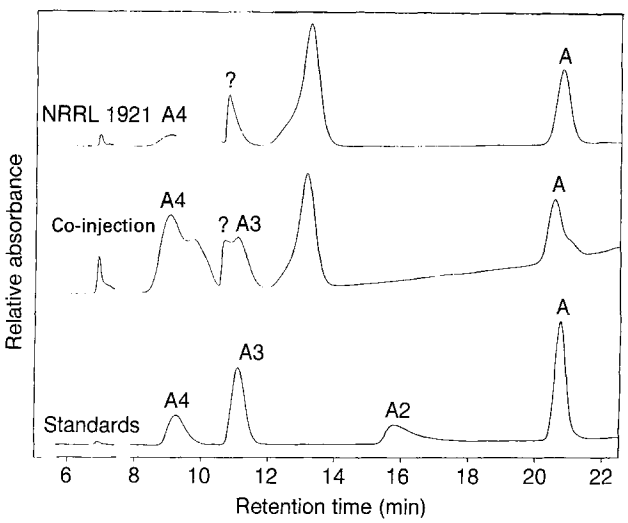

Fig. 2. HPLC chromatogram at 230 nm of co-injection of NRRL 1921 48-h sample extract and reference standards (peak identification as in Fig. 1). Top = culture NRRL 1921. Middle = co-injection of reference standards A4 and A3 only, and culture NRRL 1921. Bottom = reference standards.

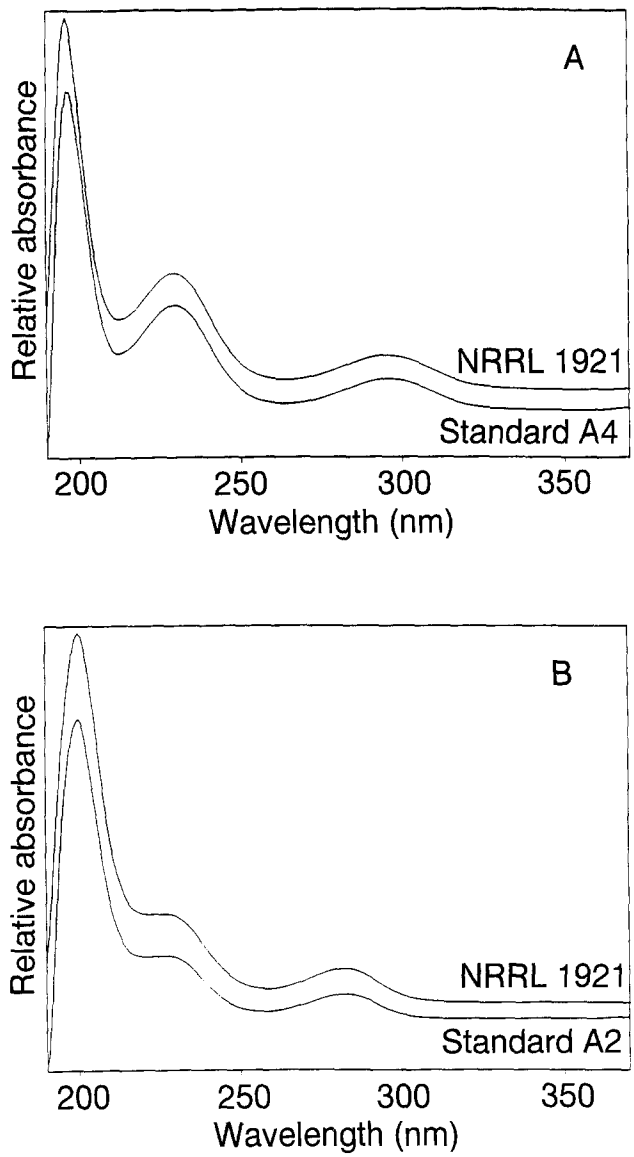

Fig. 3. HPLC/diode array detection absorbance spectra (190–370 nm) from selected NRRL 1921 and standard peaks. (A) Spectra of peak A4 from culture NRRL 1921 24-h extract (upper trace) and of A4 standard (lower trace); (B) Spectra of peak A2 from culture NRRL 1921 24-h extract (upper trace) and of A2 standard (lower trace). (Peak identification as in Fig. 1.)

samples having the same elution volume and absorbance spectra as reference standards. Figure 1 shows that at 24 h after addition of aniline, culture NRRL 1921 contained peaks A4 and A2 which have elution volumes corresponding to those of 4-aminophenol (A4) and 2-aminophenol (A2), respectively. After 48 h, the 2-aminophenol (A2) was no longer seen (Fig. 2). Co-injection was performed to clarify whether peaks seen in the sample were identical with reference compounds. The middle trace of Fig. 2 shows the HPLC elution profile at 230 nm resulting from co-injection of the NRRL 1921 48-h sample and reference standards 4-aminophenol (A4) and 3-aminophenol (A3). Of two overlapping peaks in the sample, the first peak co-eluted with 4-aminophenol (peak A4), resulting in an enlarged peak. However, an unidentified peak in the sample was shown to have a slightly different elution volume from peak A3, and thus was not 3-aminophenol.

Further confirmation of the identity of peaks was achieved by comparison of absorbance spectra (190–370 nm) from a diode array detector. For example, Fig. 3 shows that the spectrum of 4-aminophenol (A4) matches well with the spectrum of peak A4 from culture NRRL 1921, and the spectrum from peak A2 agrees with the 2-aminophenol (A2) standard spectrum. The spectrum from the unidentified peak did not match that of standard 3-aminophenol (data not shown).

Control fermentations of cultures to which no aniline had been added showed that the compounds detected in aniline-fed cultures were not normal fungal products. Using the same assay procedure as with the other samples, no 2-, 3-, or 4-aminophenol was detected in any of the controls. In addition to the monohydroxyaminophenols, culture NRRL 1921 produced an unidentified phenol in high yield (data not shown).

Focused screen of Aspergillus alliaceus, A. albertensis, *and* A. terreus

Subsequent to the initial broad screen of 600 microorganisms, further screening of 70 additional NRRL strains belonging to the species *Aspergillus alliaceus*, *A. albertensis*, and *A. terreus* identified 42 more hydroxylating strains. TLC analysis using 150 μl of each culture extract increased the sensitivity of the screen. Of the 70 additional cultures screened, 27 strains were found to hydroxylate aniline in the *para* position, 23 in the *ortho* position, and four cultures produced the same unidentified phenol as strain NRRL 1921 (Table 1). No cultures were identified which hydroxylated aniline in the *meta* position.

DISCUSSION

Extensive screening was carried out using TLC and HPLC analyses. From an initial screen of 600 cultures of various genera, we identified two strains of *A. alliaceus* and three strains of *A. terreus* able to hydroxylate aniline in either the *ortho* or *para* positions. Aniline hydroxylase activity was also detected from other genera in the initial screen; some of these were environmental isolates identified as belonging to the genus *Fusarium* (data not shown).

From the focused screen of 70 strains belonging to the species *A. alliaceus*, *A. albertensis*, and *A. terreus*, we were able to identify 42 additional strains which possessed aniline hydroxylase activity. More than half of our isolates from these three species gave positive results in our aniline hydroxylase assay procedure. These results point to the widespread occurrence of such enzymes in these species. However, to our knowledge, strain NRRL 315 is the only one of these strains which has been previously reported to have hydroxylase activity [6].

In further work we have investigated other substrates and other types of oxidations of commercial importance,

using these microbes and others obtained based on literature reports of oxidative capabilities. In our screening studies the microbial world has proved to be a rich source of biocatalysts able to perform enzymatic oxidations of industrial importance. In the future, the economic viability of such useful microbial transformations may be improved by genetic studies, strain selection, and optimization of fermentation conditions.

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REFERENCES

1 Arfmann, H.A., W.R. Abraham and K. Kieslich. 1988. Microbial ω-hydroxylation of *trans*-nerolidol and structurally related sesquiterpenoids. Biocatalysis 2: 59–67.

2 Berrier, C., H. Carreyre, J.C. Jacquesy and M.P. Jouannetaud. 1990. Hydroxylation régiosélective en méta de dérivés chlorés de l'aniline et du phénol. New J. Chem. 14: 283–287.

3 Betts, R.E., D.E. Walters and J.P. Rosazza. 1974. Microbial transformations of antitumor compounds. I. Conversion of acronycine to 9-hydroxyacronycine. J. Med. Chem. 17: 599–602.

4 Bihari, V., A.K. Joshi, A.W. Khan and S.K. Basu. 1988. Biochemical engineering studies for steroid transformations: 11-α-hydroxylation of progesterone using *Aspergillus ochraceus*. J. Microb. Biotechnol. 3: 45–50.

5 Cerniglia, C.E., W. Mahaffey and D.T. Gibson. 1980. Fungal oxidation of benzo[*a*]pyrene by *Cunninghamella elegans*. Biochem. Biophys. Res. Commun. 94: 226–232.

6 Chien, M.M. and J.P. Rosazza. 1979. Microbial transformations of natural antitumor agents. VII. Formation of 8- and 9-hydroxyellipticines. Drug Metab. Disposit. 7: 211–214.

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

8 El-Sharkawy, S.H. and Y.J. Abul-Hajj. 1988. Microbial transformation of zearalenone. 2. Reduction, hydroxylation, and methylation products. J. Org. Chem. 53: 515–519.

9 Ferris, J.P., M.J. Fasco, F.L. Stylianopoulou, D.M. Jerina, J.W. Daly and A.M. Jeffrey. 1973. Monooxygenase activity in *Cunninghamella bainieri*: evidence for a fungal system similar to liver microsomes. Arch. Biochem. Biophys. 156: 97–103.

10 Iida, M., A. Mikami, K. Yamakawa and K. Nishitani. 1988. Microbial hydroxylation of (−)-α-santonin by *Aspergillus niger*. J. Ferment. Technol. 66: 51–55.

11 Ilidrissi, A., M. Berrada and M. Holeman. 1990. Bioconversions de cétones cycliques monoterpeniques. Fitoterapia 41: 23–29.

12 Jacquesy, J.-C., M.-P. Jouannetaud, Y.V. Morellet and Y. Vidal. 1986. Hydroxylation directe d'anilines en aminophénols. Bull. Soc. Chim. Fr., No. 4: 625–629.

13 Kaufman, D.D. and J. Blake. 1973. Microbial degradation of several acetamide, acylanilide, carbamate, toluidine and urea pesticides. Soil Biol. Biochem. 5: 297–308.

14 Lamare, V., A. Archelas, R. Faure, M. Cesario, C. Pascard and R. Furstoss. 1989. Microbial transformations. 14. Regioselective hydroxylation of (1R)-caryolanol by *Aspergillus niger*. A reexamination of the ^{13}C nmr spectrum of caryolanol. Tetrahedron 45: 3761–3768.

15 Latorre, J., W. Reineke and H.-J. Knackmuss. 1984. Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange. Arch. Microbiol. 140: 159–165.

16 Lesma G., G. Palmisano and S. Tollari. 1983. Microbial hydroxylation of heteroyohimbine alkalids. J. Org. Chem. 48: 3825–3828.

17 Madyastha, K.M. and N.S.R.K. Murthy. 1988. Transformations of acetates of citronellol, geraniol, and linalool by *Aspergillus niger*: regiospecific hydroxylation of citronellol by a cell-free system. Appl. Microbiol. Biotechnol. 28: 324–329.

18 March, J. 1985. Advanced Organic Chemistry: Reactions, Mechanisms, and Structure, 3rd edn, p. 498. Wiley, New York.

19 McMillan, D.C., P.P. Fu and C.E. Cerniglia. 1987. Stereoselective fungal metabolism of 7,12-dimethylbenz[*a*]anthracene: identification and enantiomeric resolution of a K-region dihydrodiol. Appl. Environ. Microbiol. 53: 2560–2566.

20 Miyazawa, M., K. Yamamoto, Y. Noma and H. Kameoka. 1990. Bioconversion of (+)-fenchone to (+)-6-*endo*-hydroxyfenchone by *Aspergillus niger*. Chem. Express 5: 237–240.

21 Petroski, R.J., R.B. Bates, G.S. Linz and J.P. Rosazza. 1983. Microbial transformations of natural antitumor agents XXII: conversion of bouvardin to *O*-desmethylbouvardin and bouvardin catechol. J. Pharm. Sci. 72: 1291–1294.

22 Sariaslani, F.S., L.R. McGee and D.W. Ovenall. 1987. Microbial transformation of precocene II: oxidative reactions by *Streptomyces griseus*. Appl. Environ. Microbiol. 53: 1780–1784.

23 Smith, R.V. and J.P. Rosazza. 1974. Microbial models of mammalian metabolism. Aromatic hydroxylation. Arch. Biochem. Biophys. 161: 551–558.

24 Vigne, B., A. Archelas and R. Furstoss. 1991. Microbial transformations. 18. Regiospecific *para*-hydroxylation of aromatic carbamates mediated by the fungus *Beauveria sulfurescens*. Tetrahedron 47: 1447–1458.