

## Microbiological Hydroxylation of Estradiol: Formation of 2- and 4-Hydroxyestradiol by *Aspergillus alliaceus*

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Microorganisms known to hydroxylate alkaloids, amino acids, and aromatic substrates were examined for their potential to hydroxylate 17 $\beta$ -estradiol and estrone. Thin-layer chromatography of fermentation extracts revealed a wide range of steroid products. *Aspergillus alliaceus* (UI 315) was the only culture capable of producing good yields of catechol estrogens with 17 $\beta$ -estradiol. The organism also transformed estrone but not to catechol products. Analytical experiments with high-performance liquid chromatography revealed that *A. alliaceus* formed 4- and 2-hydroxyestradiol with yields of 45 and 16%, respectively. A preparative-scale incubation was conducted in 2 liters of medium containing 1 g of 17 $\beta$ -estradiol as substrate. 4-Hydroxyestradiol was isolated and identified by proton nuclear magnetic resonance and high-resolution mass spectrometry. Ascorbic acid was added to microbial reaction mixtures as an antioxidant to prevent the decomposition of unstable catechol estrogen metabolites. The microbial transformation of 17 $\beta$ -estradiol by *A. alliaceus* provides an efficient one-step method for the preparation of catechol estrogens.

Microbial transformations have long been valued for the production of nearly every class of steroid hormone product (4, 11, 18). Important reactions catalyzed by microbial enzymes include  $\Delta^1$ -dehydrogenation, side-chain cleavage with various sterols, and hydroxylations at the 11 $\beta$  and 16 $\alpha$  positions. The successful application of microbial transformations as an early form of biotechnology found wide acceptance in the industrial preparation of steroids. The features of microbial transformation reactions which render them useful for the synthesis of hormone products include the high regio- and stereochemical specificities displayed, the mild reaction conditions, and the ability to introduce molecular oxygen into nonactivated carbon positions about the steroid ring.

The catechol estrogens are a major group of natural estrogenic compounds formed by aromatic hydroxylation in mammalian tissues. These compounds are implicated in a wide variety of biological actions, and they are considered to play still-undefined roles in the mediation of estrogen action in mammals (2, 6, 7, 9, 12, 13). Recent interest in catechol estrogens prompted an investigation of the potential for microorganisms to prepare catechol estrogens from suitable precursor steroids. An examination of the literature revealed only one study in which microbial transformations had been used to prepare catechol estrogen derivatives of 17 $\alpha$ -methyleneestradiol (17). We describe the production of 2- and 4-hydroxyestradiols as microbial transformation products formed when 17 $\beta$ -estradiol undergoes aromatic hydroxylation by the fungus *Aspergillus alliaceus* (UI 315).

### MATERIALS AND METHODS

Melting points were determined in open-ended capillary tubes and are uncorrected. UV spectra were measured in ethanol solutions with a model SP1800 Pye Unicam spectrophotometer. High-resolution mass spectra were obtained through the services of the Midwest Center for Mass Spectrometry, Department of Chemistry, University of Ne-

braska, Lincoln, with a Kratos AEI MS-50 spectrometer by using the direct inlet mode. Proton nuclear magnetic resonance (NMR) spectra were obtained on samples dissolved in deuteriochloroform containing tetramethylsilane as an internal standard. Spectra were determined with a Bruker WM360 360.13-MHz instrument operating in the Fourier transforming mode.

**Chromatography.** Thin-layer chromatography (TLC) was performed on 0.25-mm silica gel GF-254 plates freshly activated at 110°C before use. Plates were developed in chloroform-ethyl acetate-acetone (30:10:5, vol/vol), and the developed chromatograms were visualized by fluorescence quenching at 254-nm UV light and by spraying with a solution of 0.2% ceric ammonium sulfate in 50% phosphoric acid. Sprayed plates were warmed with a heat gun to develop characteristic colors for the various steroidal phenols and catechols. In this chromatographic system, various compounds displayed the following  $R_f$  values and colors: 17 $\beta$ -estradiol, 0.55 (yellow); 4-hydroxyestradiol, 0.45 (pink); and 2-hydroxyestradiol, 0.33 (pink).

High-pressure liquid chromatography (HPLC) was performed with a Waters ALC/GPC 202 instrument equipped with a U6K Universal injector and a Beckman model 153 UV detector with a 280-nm UV filter. Analytical determinations were made with a Merck Lichrosorb Diol column (10- $\mu$ m packing, 0.46 [inside diameter] by 25 cm) with a mixture of chloroform and methanol (99:1) as the eluting solvent. Lichrosorb Diol columns have found wide application in the determination of catechol estrogens (1). Flow rates of 2 ml/min were achieved at an operating pressure of 1,000 lb/in<sup>2</sup>. Retention volumes for compounds involved in this work were as follows: estrone, 6.2 ml; 17 $\beta$ -estradiol, 8.1 ml; 4-hydroxyestradiol, 14.3 ml; and 2-hydroxyestradiol, 18.4 ml. Linear standard curves for estrone, 17 $\beta$ -estradiol, and 4-hydroxyestradiol were obtained by injecting 10- $\mu$ l volumes of solutions of each compound in ethyl acetate into the column and by correlating peak heights with concentrations of compounds. Curves were linear over the concentration range of 0.2 to 10  $\mu$ g. Estrone was used as an internal

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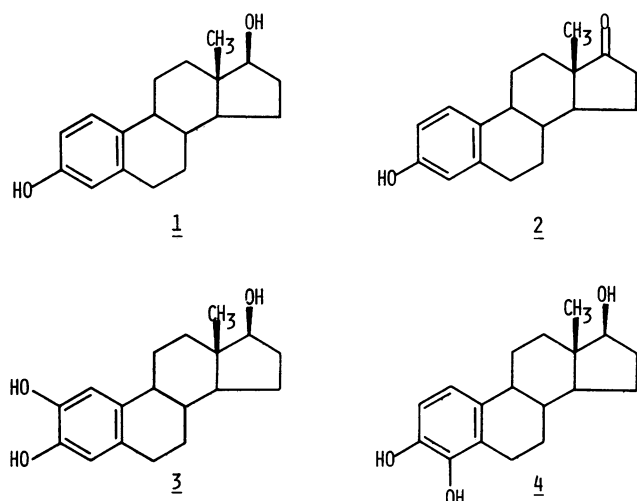


FIG. 1. Structures of 17 $\beta$ -estradiol (1), estrone (2), 2-hydroxyestradiol (3), and 4-hydroxyestradiol (4).

standard to measure extraction efficiency and for quantitative analyses of steroid-containing incubations by the internal standard ratios method (20). Linear ratio curves of 17 $\beta$ -estradiol/estrone ( $r = 0.9996$ ) and 4-hydroxyestradiol/estrone ( $r = 0.9926$ ) were observed in this work.

Preparative HPLC was performed on an Alltech Lichrosorb Diol column (10- $\mu$ m packing, 0.94 [inside diameter] by 50 cm) with the same eluting solvent. Nominal operating conditions with the preparative column were a flow rate of 3.5 ml/min at 1,100 lb/in<sup>2</sup>. Under these conditions, 4-hydroxyestradiol (Fig. 1, compound 4) possessed an elution volume of 101 ml.

**Chemicals.** 17 $\beta$ -Estradiol (1) and estrone (2) were obtained from Sigma Chemical Co., St. Louis, Mo. The identities of these compounds were confirmed by melting point, TLC, HPLC, and proton NMR measurements before they were used in experiments. 2-Hydroxyestradiol (3) and 4-hydroxyestradiol (4) were obtained from Steraloids Co., Wilton, N.H., and their purities were verified by HPLC before use.

**Fermentation procedure.** All microorganisms mentioned are maintained in the culture collection of the College of Pharmacy, University of Iowa, Iowa City. Cultures were grown according to our usual two-stage fermentation protocol (3) in soybean meal-glucose medium of the following composition: soybean meal, 5 g; glucose, 20 g; yeast extract, 5 g; sodium chloride, 5 g; dibasic potassium phosphate, 5 g; and distilled water, 1,000 ml. The complete medium was adjusted to pH 7.0 with 6 N HCl and sterilized in an autoclave at 121°C for 15 min before use.

Fermentations were conducted at 27°C on New Brunswick model G25 Gyrotory shakers operating at 250 rpm and describing a 2.5-cm orbital stroke. Cultures were grown in steel-capped Delong culture flasks containing one-fifth of their volumes in culture medium. Screening- and analytical-scale experiments were conducted in 125-ml Delong flasks, and preparative-scale incubations were conducted in 1,000-ml Delong flasks. Fermentations were initiated by suspending surface growth from slants in 5 ml of sterile medium and by using the resulting suspension to inoculate stage 1 cultures. Thick 72-h-old stage 1 cultures were used to inoculate stage 2 fermentations. The steroid substrate was dissolved in *N,N*-dimethylformamide (10% [wt/vol] solutions) and added to 24-h-old stage 2 cultures to a final

concentration of 0.4 mg/ml. The progress of microbial transformation reactions was monitored by TLC or HPLC by the following sampling procedure.

Samples (4 ml) were withdrawn from substrate-containing cultures at 0, 6, 12, 24, 36, and 48 h. These were extracted with 1 ml of ethyl acetate, and 30- $\mu$ l portions of the extracts were spotted onto TLC plates for analysis. Quantitative analytical experiments were performed as described below.

**Preparation of 4-hydroxyestradiol from 17 $\beta$ -estradiol by *A. alliaceus*.** A stage 2 *A. alliaceus* culture was grown in 2 liters of medium held in 10 1-liter Delong flasks. A total of 1.0 g of 17 $\beta$ -estradiol in 7.5 ml of *N,N*-dimethylformamide was evenly distributed among the 24-h-old cultures, and 1 g of ascorbic acid was added to each flask 12 h later. Substrate-containing cultures were incubated for 72 h; the fermentation beers were then combined, and the cells and other solids were removed by filtration through cheesecloth. Solids were discarded, and the filtrates were exhaustively extracted with six 300-ml portions of ethyl acetate. The extract was dried over anhydrous sodium sulfate and evaporated to a brown, nondrying oil (880 mg). HPLC analysis of the extract revealed a ratio of 4- and 2-hydroxyestradiol similar to that observed in analytical experiments and showed that the extract contained 396 mg of 4-hydroxyestradiol (38% yield).

The crude extract was dissolved in 1 ml of chloroform-ethyl acetate (3:1) and applied to a Baker 3405 silica gel column (2 by 13 cm) in the same solvent mixture. The column was developed with the same solvent mixture containing 2% glacial acetic acid while the solvent reservoir was constantly purged with a slow stream of argon gas. Fractions (0.5 ml) were collected and kept under argon until analyzed by TLC. Similar fractions (50 to 80 fractions) were combined to give a total of 276 mg of crude 4-hydroxyestradiol. The sample was dissolved in ethyl acetate (150 mg/ml), and 50- $\mu$ l volumes of this mixture were injected into the preparative HPLC Lichrosorb Diol column to prepare the analytical sample for spectral analysis. Peaks corresponding to 4-hydroxyestradiol (retention volume, 101 ml) were shaved and recycled to collect the pure product. The samples were collected, evaporated to dryness, and stored in argon to prevent decomposition. The preparative analytical sample of 4-hydroxyestradiol was used to obtain physical data.

**Kinetics of catechol formation from estradiol by *A. alliaceus*.** Qualitative analytical experiments revealed that *A. alliaceus* formed high yields of catechol estrogen metabolites when incubated with 17 $\beta$ -estradiol. The kinetics of the production of catechols and the mass balance values were determined by growing 50 cultures of *A. alliaceus* in 125-ml Delong flasks. Stage 2 cultures each received exactly 10 mg of 17 $\beta$ -estradiol in 0.1 ml of *N,N*-dimethylformamide and were again placed on the Gyrotory shaker. Control cultures received no 17 $\beta$ -estradiol. At 3 and 36 h after steroid addition, 100 mg of ascorbic acid in 2 ml of distilled water was added to each flask as an antioxidant (10, 14). Three estradiol-containing flasks and one control culture were harvested at each of several time intervals and analyzed for the presence of 17 $\beta$ -estradiol and catechol estrogens as follows. The internal standard, estrone (2.0 mg), was added in 1 ml of methanol, and the flasks were shaken for 1 min before 10 ml of ethyl acetate was added. Solvent-containing flasks were shaken at 250 rpm for exactly 5 min, a portion of the ethyl acetate phase was collected and filtered through a bed of ethyl acetate-wetted sodium sulfate to remove water, and 10 to 50  $\mu$ l of the clear yellow ethyl acetate extract was injected for HPLC analysis. The results of this experiment can be seen in Fig. 3.

TABLE 1. Microbial transformation products formed from estradiol (1) and estrone (2)

| Strain (no.) <sup>a</sup>                       | Products from estradiol <sup>b</sup> |   |     |         | Products from estrone |   |   |         |
|---|--------------------------------------|---|-----|---------|-----------------------|---|---|---------|
|   | 2                                    | 3 | 4   | Unknown | 1                     | 3 | 4 | Unknown |
| <i>Aspergillus alliaceus</i> (UI 315)           | -                                    | + | +++ | -       | -                     | - | - | +++     |
| <i>Aspergillus niger</i> (UI 172)               | -                                    | - | +   | +++     | -                     | - | - | +++     |
| <i>Aspergillus ochraceus</i> (ATCC 1008)        | +                                    | - | -   | ++      | -                     | - | - | -       |
| <i>Caldariomyces fumago</i> (ATCC 16373)        | +                                    | + | +   | ++      | +                     | + | + | ++      |
| <i>Cunninghamella echinulata</i> (NRRL 3655)    | -                                    | - | -   | +++     | -                     | - | - | +++     |
| <i>Cunninghamella blakesleeana</i> (ATCC 8688A) | -                                    | - | -   | -       | -                     | + | + | +++     |
| <i>Cunninghamella elegans</i> (ATCC 9245)       | -                                    | - | +   | +++     | -                     | + | + | +++     |
| <i>Curvularia lunata</i> (NRRL 2178)            | -                                    | - | -   | +++     | ++                    | - | - | -       |
| <i>Gliocladium deliquescens</i> (UI 1086)       | +                                    | - | -   | ++      | -                     | - | - | +       |
| <i>Helicostylum piriforme</i> (QM 6945)         | -                                    | - | -   | -       | -                     | - | - | -       |
| <i>Mucor mucedo</i> (UI 4605)                   | -                                    | - | -   | -       | -                     | - | - | -       |
| <i>Penicillium brevi-compactum</i> (ATCC 10418) | -                                    | - | +   | ++      | -                     | - | - | -       |
| <i>Phanerochaete chrysosporium</i> (ME 446)     | +                                    | - | -   | ++      | -                     | - | - | -       |
| <i>Polyporus versicolor</i> (ATCC 12674)        | -                                    | - | +   | -       | ++                    | - | - | ++      |
| <i>Rhizopus</i> sp. (UI 224)                    | +                                    | + | +   | +++     | -                     | - | - | +++     |
| <i>Sepedonium chrysospermum</i> (ATCC 13378)    | -                                    | - | -   | -       | -                     | - | - | -       |
| <i>Streptomyces griseus</i> (ATCC 13273)        | -                                    | + | +   | -       | -                     | - | - | +       |
| <i>Streptomyces griseus</i> (ATCC 10137)        | -                                    | + | +   | +++     | -                     | + | - | +++     |
| <i>Streptomyces griseus</i> (NRRL 8090)         | -                                    | - | -   | -       | -                     | - | - | -       |

<sup>a</sup> Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; UI, College of Pharmacy Culture Collection, University of Iowa, Iowa City; NRRL, National Regional Research Laboratories, Agricultural Research Service, Department of Agriculture, Peoria, Ill.; QM, Quartermaster Culture Collection, Natick, Mass.; ME, Forest Products Laboratory, Madison, Wis.

<sup>b</sup> Relative amounts of metabolites formed indicated as: -, none; +, 5%; ++, 10%; +++, 25% or greater yield (TLC estimates only).

## RESULTS

Microorganisms screened for their potential to hydroxylate 1 and 2 are listed in Table 1 along with the transformation products observed in screening-scale experiments. Of those strains examined, two of the three *Cunninghamella* strains, one of the two *Streptomyces* strains, and the only *Caldariomyces* strain formed catechol estrone products with a yield of no more than 10% as estimated by TLC. These cultures demonstrated the presence of a reductase system capable of converting estrone into estradiol. As with estrone, estradiol was converted into a variety of polar, unknown metabolites by most of the microorganisms tested. Five cultures oxidized the 17 $\beta$ -hydroxyl group of 1 to form estrone (2) but not in high yield. Two of the three *Aspergillus* and *Streptomyces* spp. tested formed 4-hydroxyestradiol.

The preparative-scale incubation with *A. alliaceus* provided an extract which gave the HPLC elution pattern shown in Fig. 2. By HPLC analysis, the overall yield of 4-hydroxyestradiol in this reaction was determined to be 38%. The major microbial transformation product was obtained by preparative HPLC, and the analytical sample was compared with authentic 4-hydroxyestradiol by mass and NMR spectral analyses and by TLC and HPLC. High-resolution electron impact mass spectrometry gave *m/e* 288.17320 (100%, calculated for C<sub>18</sub>H<sub>24</sub>O<sub>3</sub> 288.17288). The proton NMR spectrum gave signals at ppm values of 0.78 (s, 3H, 18-CH<sub>3</sub>), 1.25 (s, 1H, 17 $\alpha$ -H), 6.83 (d, 1H, *J* = 8.5 Hz, 2-H), 7.16 (d, 1H, *J* = 8.5 Hz, 1-H).

Results of an experiment designed to determine both the kinetics of formation of catechol estrogens from estradiol and the mass balance obtained in the biotransformation process are shown in Fig. 3. Estradiol was immediately taken up by *A. alliaceus* cells. Nearly 50% of the substrate was gone within 6 h of addition. Significant levels of 4-hydroxyestradiol were measured at 24 h, and maximum yields of 45% for 4 and 16% for 3 were measured at 48 and 60 h, respectively.

## DISCUSSION

Microorganisms and their enzymes have been widely exploited for their abilities to chemically transform the structures of steroids, alkaloids (4, 11, 18), and other groups

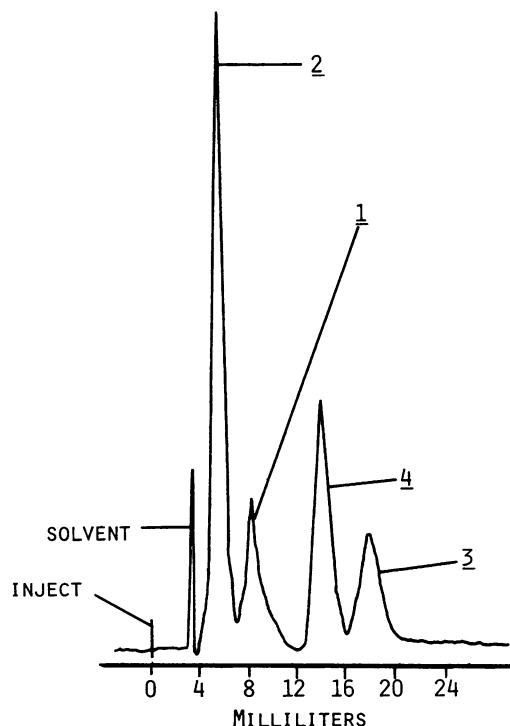


FIG. 2. Typical high-pressure liquid chromatogram of a 48-h fermentation extract. Extract (20  $\mu$ l) was injected under the conditions described in the text.

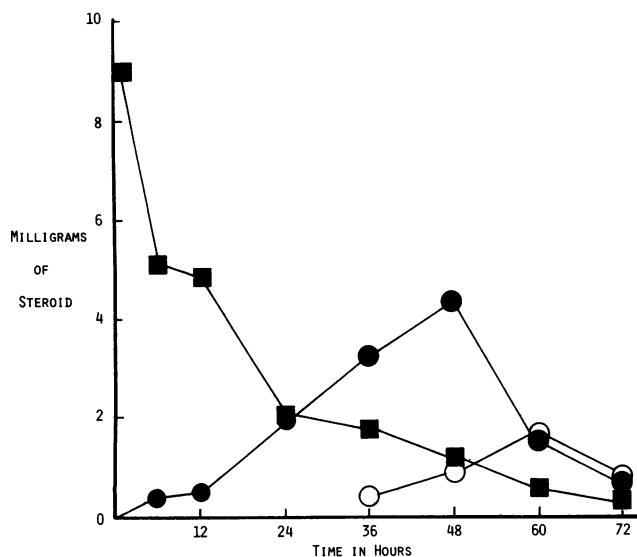


FIG. 3. Kinetics of the hydroxylation of 17 $\beta$ -estradiol (■) by *A. alliaceus* in the formation of 2-hydroxyestradiol (○) and 4-hydroxyestradiol (●).

of biologically active compounds (15). These biocatalysts accomplish highly regio- and enantiospecific transformations of even complex molecules under very mild reaction conditions without the need for protecting groups commonly used in synthetic organic chemistry. These features rendered microbial transformations attractive for possible use in the preparation of catechol estrogens.

Twenty microorganisms from our culture collection which had previously demonstrated their capacities to introduce molecular oxygen into the structures of aromatic amino acid and alkaloid substrates (3, 5, 14, 16, 19) were examined for their potential to hydroxylate 17 $\beta$ -estradiol and estrone. None of the cultures formed large quantities of catechol products with estrone as the substrate. Estradiol (1) proved to be a better substrate for the production of catechol products by the same 20 microorganisms. Most of the cultures used in screening-scale experiments produced much larger amounts of more polar products which were presumed to be metabolites hydroxylated at other positions. In general, no genus specificity could be observed in the formation of catechol metabolites.

Of all the cultures examined, *A. alliaceus* reproducibly converted 17 $\beta$ -estradiol into a more polar product with TLC mobility and color identical to that of 4-hydroxyestradiol (4) in good yield. Qualitative HPLC analysis of fermentations of *A. alliaceus* confirmed the identity of the metabolite as 4-hydroxyestradiol (4), and preparative fermentations were conducted to isolate sufficient quantities of the metabolite for mass and NMR spectral analyses. Ascorbic acid was added to substrate-containing flasks to prevent oxidative decomposition of the catechol products. Extracts obtained from the 72-h-old preparative-scale culture were stored in an argon atmosphere to prevent decomposition of the catechol. All HPLC solvents were degassed and continuously purged with a slow stream of argon for the same purpose. Analytically pure 4-hydroxyestradiol was obtained by collecting the peak eluting at 101 ml and by recycling the most enriched fractions through the preparative Lichrosorb Diol column.

The high-resolution mass spectrum gave a molecular ion of 288 for C<sub>18</sub>H<sub>24</sub>O<sub>3</sub> or one more oxygen atom than the starting material (1). The position of the oxygen atom

introduced by the microorganism was confirmed by proton NMR spectral analysis. The introduction of oxygen into position 4 results in a steroid molecule in which two A-ring aromatic protons occur *ortho* to one another. This arrangement of aromatic protons results in a characteristic AB spectral pattern in which each proton signal is equally split into a doublet (8, 17). Aromatic proton signals in the isolated estradiol metabolite occurred as an AB doublet pattern centered at 6.83 and 7.16 ppm ( $J = 8.5$  Hz). These spectral properties were identical to those previously reported for 4-hydroxyestradiol, and they confirm the identity of the *A. alliaceus* metabolite as 4 (8, 17). Insufficient 2-hydroxyestradiol was isolated from these experiments for full spectral identification.

An analytical experiment was conducted to determine both the kinetics of catechol estrogen formation and the mass balance between the 17 $\beta$ -estradiol consumed and the 2- and 4-hydroxyestradiol products formed. An analysis of substrate-containing cultures by the internal standard ratios method provided the results shown in Fig. 3. 4-Hydroxyestradiol (4) was the major product formed (44% yield), while 2-hydroxyestradiol was formed with a yield of 16%. At 48 h, the total mass balance accounted for approximately 65% of all substrate added and consumed. The fate of the remaining 35% of substrate is unknown. However, it is possible that the catechol products may be undergoing further metabolism or decomposition in the incubation mixtures.

Schubert et al. (17) described microbial transformations of 17 $\alpha$ -methyl-estradiol with *Aspergillus flavus*. The major compounds characterized in this study were the 2-, 4-, and 6-monohydroxylation products. The major reaction, accounting for approximately 30% of the hydroxylated steroid, was 6 $\alpha$ -hydroxylation. Catechol products were formed with a combined yield of approximately 20%, while more polar product(s), most probably representing dihydroxylated metabolites, were obtained with an overall yield of 70%.

Our study clearly demonstrates the potential for microorganisms to produce catechol estrogens from 17 $\beta$ -estradiol as substrate. It was surprising to observe that estrone would not serve as a substrate with this strain of *A. alliaceus*, and the reasons for this are unclear. Investigations on the enzymatic mechanisms of catechol estrogen formation by this culture of *A. alliaceus* are in progress.

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