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ORIGINAL PAPER

Modification of small molecules by using cytochrome P450 expressed in *Escherichia coli*

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Abstract We developed a system for bioconverting diverse compounds using P450s produced in *Escherichia coli*. Vectors for the expressing various P450 cDNAs quickly and easily in *E. coli* were developed by using several restriction enzyme sites. Three types of P450 (2C2, 2C29, and 2D22) were produced using these plasmids. Substrates were directly added to the incubation medium and metabolized. To obtain pure product from the medium, we first tried production of P450 in synthetic medium. The amount of another P450 2C43 produced in the synthetic medium was similar to the amount produced in Luria broth (LB) medium. Next, estradiol, a steroid, was added as a substrate, incubated, and the metabolite was extracted and analyzed by high-performance liquid chromatography. The metabolite extracted from synthetic medium was purer than that obtained from LB medium. Three P450s (2C29, 2C2, and 2A4) metabolized testosterone at different positions. P450 2C29 metabolized 7-ethoxycoumarin, androstendione, and dehydroepiandrosterone in this medium. P450s produced in the synthetic medium may be useful for producing various modified compounds for high-throughput screening.

Keywords P450 · Bioconversion · Hydroxylation

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Introduction

The cytochrome P450 superfamily comprises of a number of heme-thiolate monooxygenases that are subdivided into families and subfamilies on the basis of their amino acid sequence homologies [1, 14, 17, 21]. These enzymes play critical roles in the oxidation of xenobiotics, including drugs and environmental pollutants, and therefore represent a primary focus of toxicological and drug metabolism research. During the catalytic cycle, the enzyme functions as a terminal oxidase that utilizes molecular oxygen. The reducing electrons necessary for substrate metabolism by a cytochrome P450 are supplied by the oxidation of NADPH, catalyzed by NADPH-P450 reductase [20]. NADPH-P450 reductase is a 78-kDa membrane-bound flavoprotein containing a discrete FMN, FAD, and NADPH binding domain.

In addition to the hydroxylation, P450 catalyzes a wide range of other reactions, including dealkylation, epoxidation, desaturation, and oxidative ester and ether cleavage [6]. Furthermore, various P450s are known to hydroxylate some molecules, such as steroids, at different positions, and one P450 has been reported to metabolize a variety of compounds [10, 24, 25]. For example, P450 2A4 shows testosterone 15-alpha-hydroxylase, dehydroepiandrosterone 7-alpha-hydroxylase, and coumarin 7-hydroxylase activities, whereas P450 2C14 metabolizes testosterone to 16-OH testosterone [24, 25]. P450s in general show overlapping

but different substrate specificities. Recently, the cDNAs encoding various P450s have been co-expressed in *Escherichia coli* with NADPH-P450 reductase, and the metabolites measured [3, 7, 11, 26]. The membranes isolated from P450-producing cells and the purified enzymes catalyzed hydroxylation and demethylation of small molecules.

Because of the above-mentioned properties, P450s have the potential to be excellent enzymes for modifying lead compounds for drug development. Combinatorial chemistry, which came into use in the early 1990s, has dramatically changed the process of drug discovery. Combinatorial chemistry has contributed to a large increase in the number of compounds that can be subjected to high-throughput screening (HTS). In fact, many small molecules, such as inhibitors of anthrax lethal factor, an estrogen-related receptor agonist, and inhibitors of cathepsin, have been developed by this procedure [8, 13, 29]. The diversity of compound libraries is one of the keys to increasing the number of hits in HTS. A variety of methods of synthesizing numerous compounds have been developed [2, 4, 18]. For example, methods using phage libraries have been used for HTS [6, 22]. However, there has been little work on the modification of small molecules or the production of new compounds by the direct use of enzymes. We therefore developed a system for bioconverting small molecules such as steroids in *E. coli* by the action of P450, with the goal of developing a method of synthesizing a wide variety of compounds easily and quickly.

The vectors were constructed using the common restriction enzyme recognition sites to express various P450 genes. The substrates were added directly to the synthetic medium and metabolized. Several steroids were metabolized in the medium, which was similar to Luria broth medium.

Materials and methods

Materials

Isopropyl-beta-D-thiogalactopyranoside, methanol, delta-amino levulinic acid, and ethyl acetate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Testosterone, estradiol, androstenedione, dehydroepiandrosterone, 7-ethoxycoumarin, and oligonucleotides were obtained from Sigma (St. Louis, MO). pT7 vector was obtained from Novagen (Madison, WI), and pCR 2.1 was obtained from Invitrogen (Carlsbad, CA). Taq polymerase and reverse transcriptase were obtained from Takara Shuzo (Ohtu, Japan). The other chemicals were of the purest grade commercially available.

Construction of P450 gene expression vectors

Vectors for expression of P450 genes in *E. coli* were constructed as shown in Fig. 1. RT-PCR for the gene encoding 2C29 was done by using mRNA from a mouse liver (Table 1; primer 1 and primer 2). The *N*-terminals of the amplified fragments were modified by PCR (Table 1; primers 3 and 4). To create the *Sma*I site, PCR was done using primers 5 to 7 sequentially three times. Primers 8 and 9 and 10 and 11 were respectively used to create the *Eco*RI and *Xho*I sites. Primer 4 was added as the 3′primer for all PCRs. The amplified fragments were cloned into pCR 2.1 and sequenced.

The plasmid was cut and subcloned into pT7 or pBluescript (vectors containing *Sma*I, *Xho*I or *Eco*RI were named pT7Sma, pT7Xho or pBlueEco, respectively). pCW vector containing cDNA of human NADPH-cytochrome P450 reductase was constructed as described by Iwata et al. [11]. Co-expression of P450 genes with the reductase gene was achieved by using a biscitronic expression vector with a double tac promoter and selection for ampicillin resistance. The fragments encoding the cDNAs of various P450s were amplified by RT-PCR. The amplified DNAs for various P450s were subcloned into pCR 2.1 and sequenced. The plasmid containing cDNA of P450 was cut with *Sal*I and *Sma*I and the fragments obtained were subcloned into the *Sal*I site and *Sma*I site of pT7Sma. The plasmid containing cDNA of P450 2D22 was cut with *Sal*I and *Eco*RI, and the fragments obtained were subcloned into the *Sal*I site and *Eco*RI sites of pBlueEco. Next, the obtained plasmid was cut with *Nde* I and *Sal* I, and the fragment was subcloned into the *Nde* I and *Sal* I sites on pCW vector containing cDNA of reductase. cDNAs for 2C43 and 2D22 were obtained by RT-PCR of mRNA of monkey liver and mouse liver, respectively [21]. Expression vector for P450 2A4 and cDNA for P450 2C2 were obtained as described previously [24, 25].

P450 production and culture growth conditions

Escherichia coli DH5*a* was transformed with pCW vector containing cDNA of P450. After preincubation in the synthetic medium (called IU medium) at 37°C for 3 days, 3 ml of the inoculate was added to 300 ml of IU medium and incubated at 37°C and 400 *g* to an OD600 of between 0.2 and 0.3. Following the addition of isopropyl-1-thio-beta-D-galactopyranoside (1 μM) and

Fig. 1 Construction of expression vectors using restriction enzyme sites. The oligonucleotides for *N*-terminal modification and the corresponding amino acids are indicated, and the restriction sites are *underlined*

Table 1 Primers for the construction of P450-expression vectors

Primers	Oligonucleotides
Primer 1 and 2 for RT-PCR	5′-CAGAGC TCTGGGAGAGGGAAGCTCC-3′ 5′-TTAGAGAGGAATGAAGCAGAGCTG-3′
Primer 3 and 4 for the modification of of 2C29	5′-GGAATTCCATATGGCAACGCAATCTTCTGGACGAGGAAACTCC-3′ 5′GGGTCGACTTAGAGAGGAATGAA GCAGAGCTG-3′
Primer 5, 6 and 7 for *Sma* I of 2C29	5′-CCATATGGCAACGCAATCTTCTGGACGAGG-3′ 5′AATCTTCTGGACGAGGAAAACTCCCTCC-3′ 5′GCTCGAGGAGGAAAACTCCCTCCCGGGCCCACG-3′
Primer 8 and 9 for *Eco* RI of 2C29	5′-CCATATGGCAACGCAATCTTCTGCTCGAGG-3′ 5′-AATCTTCT GGA CGA G GAATTCTCCCTCCTG-3′
Primer 10 and 11 for *Xho* I of 2C29	5′-CCATATGGCAACGCAATCTTCTGCTCGAGG-3′ 5′-GCAATCTTCTGCTCGAGGAAAACTCCCTCC-3′
Primer 12 and 13 for 2D22	5′GGAATTCTCCCTCCTGGCCCTATGCCGTGG-3′ 5′-GGGTCGACTTAGCGGGGCAAAGCACAGAGC-3′
Primer 14 and 15 for 2C2	5′AGAATTCTCCCTCCCGGGCCCACTCCTTTG-3′ 5′AGTCGACTCAGGCAGGAATGAAGCTGAGCT-3′

The under lines indicate the restriction enzyme sites.

substrate, cultures were grown at 25°C and 400 *g* for 40 to 48 h. In the case of expression in Luria broth (LB) medium, after preincubation at 37°C for 1 day, 3 ml of the inoculate was added to 300 mL of IU medium and treated as described above.

Bioconversion of steroids

The inoculate was centrifuged at 2,000*g* for 10 min. A 7-ml aliquot of the supernatant was added to 3 ml of ethyl acetate and vortexed for 5 min. After centrifugation at 1,000*g* for 8 min the upper phase was removed. The extraction was done three times, and the resulting solution was air-dried. Fifty microliters of 50% methanol was added. The metabolites were separated at 40°C on a TSK-gel Super ODS column (Tosoh, Tokyo, Japan) using 40% aqueous methanol at a flow rate of 1.2 ml/min (LaChrom, Hitachi, Tokyo, Japan). Metabolite peaks were monitored at a wavelength of 210 nm.

Analytical methods

The P450 content of cells was determined by CO-difference spectroscopy analysis according to the method of Omura and Sato [19] using a dual-beam spectrophotometer. Protein contents were determined according to the methods of Bradford [5].

Fig. 2 *N*-terminal of amino acid sequences of five P450s, aligned on the proline–glycine-rich region, which is conserved in all P450s (indicated by *asterisks*)

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2C29    MDLVVFLATLSCLILLSLWRQSSGRGKLPPGPTPL
2C2     MDPVVVLVLCLSCLLLLSLWKQSHGGGKLPPGPTP
2C43    MDSLVVLVLCLCLSCLLLLSLWRQRSGRGKLPPGPTPL
2A4     MLTSGLLLVAAVAFLSVLVLMSVWKQRKLSGKLPPGPTP
2D22    MRLPTGAELWPIAIFTVIFLILVNLMHWRQRWTAHYPPGPMPWP
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Results and Discussion

Construction of P450 expression vectors

The *N*-terminal region of P450 must be truncated and modified if it is to be expressed in *E. coli* [11]. Therefore, PCR must be repeated several times to construct each expression vector. This construction procedure is complex and expensive. In addition, to bioconvert various compounds in *E. coli*, vectors that express many kinds of P450 genes must be constructed. Therefore, we produced new plasmids to construct expression vectors quickly and easily.

At the primary structural level, P450s have several conserved regions, including a heme-binding site, a substrate-recognition site, and a membrane-insertion site [9, 28]. The proline–glycine-rich site between the hydrophobic amino-terminal membrane-anchoring segment and the globular part of the protein is conserved in nearly all microsomal P450s [30] and is thought to be important in the proper folding of microsomal proteins (Fig. 2). Therefore,this site was used to construct a common vector for expressing P450 genes in *E. coli*. cDNA for P450 2C29 was isolated from mRNA of mouse liver using RT-PCR (Fig. 1), and the *N*-terminal region of the amplified fragment was modified by using PCR. The modified fragment was inserted into pCW vector (a P450 expression vector) and we determined whether or not it was expressed. The transformed cells showed a typical CO-difference spectrum (data not shown). The genetic code for the proline–glycine-rich region was CCN-GGN. We transferred this sequence to the sequence CCCGGG, which was cut with the restriction enzyme *Sma* I. We constructed expression vectors using two other sites (for *Eco*RI and *Xho*I) near the *Sma* I site, as shown in Fig. 1. We produced two different P450s (P450 2C2 using the *Sma*I site, 2D22 using the *Eco*RI site) using these constructed vectors. These plasmids were cut with *Nde* I and *Sal* I, and the obtained fragments were inserted into pCW vector.

The recombinant *E. coli* DH5*a* cells transformed with these constructed vectors were cultured. After incubation, the cells were collected, and the amount of P450 was measured using a spectrophotometer. Three types of P450 (2C2, 2C29, and 2D22) showed Soret peaks (450 nm) typical of P450s in the reduced CO-difference spectrum (Fig. 3). Cells transformed with pCW vector containing the *Xho*I site did not show a peak at 450 nm, but cells transformed with vector containing the *Eco*RI site did show a peak (data not shown). As a result, the two vectors containing the *Eco*RI or *Sma*I sites were useful for expressing many P450s quickly and easily in *E. coli*. The P450 superfamily contains more than 50 types in vertebrate, plant or insect. And the overall stereostructure among P450s has been found to be almost completely conserved in crystal structure studies [15, 23, 27]. So it suggests that these vectors containing *Sma*I site or *Eco*RI site can be applied to expression of the genes of various P450s in *E. coli*.

Production of P450 using synthetic medium

We added the substrates directly to the synthetic medium and analyzed the metabolites by high-performance liquid chromatography (HPLC) to obtain the products hydroxylated by P450. First we tried to produce P450 2C43 in synthetic medium to get a hydroxylated product of higher purity. 2C43 metabolized the steroid hormone estradiol. The concentration of P450

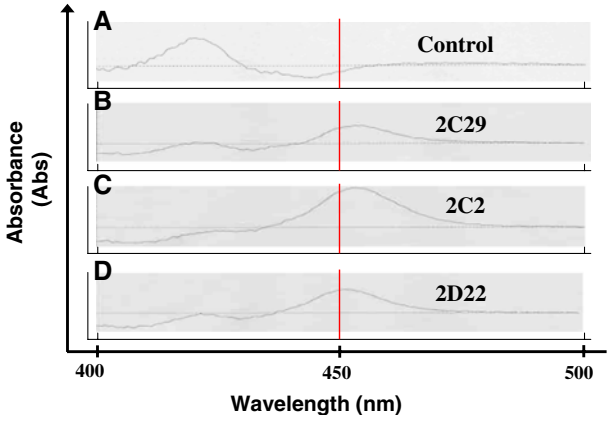


Fig. 3 Reduced CO-difference spectra of cells expressing P450 genes: **a** control, cells were transformed with only pCW vector, **b** P450 2C29 *Sma* I, **c** P450 2C2, and **d** P450 2D22

produced in *E. coli* was measured using CO-difference spectroscopy. The amount of P450 produced in the synthetic medium was the same as in LB medium (Fig. 4).

To determine the purity of the incubation medium, we compared the P450-hydroxylated products by HPLC. The inoculates prepared using the LB and synthetic media were extracted with ethyl acetate, dried, and analyzed by HPLC. The HPLC spectra showed hydroxylated peaks in both media at 210 nm (Fig. 5, retention time 4.8 min). However, the level of contaminants with retention times lower than 5 min was drastically reduced when the synthetic medium was used. This result indicates that the synthetic medium is useful for obtaining pure hydroxylated product. Mass spectrometric analysis indicated that the product at 4.8 min was hydroxylated estradiol.

Next,the androgen testosterone was metabolized by using P450 2C43 and the product was determined by HPLC (Fig. 6). No product was observed in the inoculate without P450 or testosterone (Fig. 6a, b), but one product was detected in the medium containing both (Fig. 6c). Unlike estradiol, testosterone (2.8 μg/ml) in 7 ml of synthetic medium was almost completely converted to product. This result indicates that P450 2C43 completely metabolized testosterone under these conditions.

Purified P450 2C43 has been reported to hydroxylate testosterone at the 17-position [16]. Therefore, we compared the HPLC retention time of our metabolite with that of androstenedione, which is the product of oxidation of testosterone at the 17-position. The metabolite and androstenedione were detected at the same retention time (Fig. 7b, 18 min). The fact that, like purified P450, P450 in the synthetic medium metabolized the steroid suggests that this system may be suitable for bioconversion of compounds for drug development.

After initial testing, drug candidates are tested on animals, and many drugs do not make it through this in vivo testing. Metabolism by P450 yields products that are more water-soluble and thus changes the efficiency of the drugs in vivo. Bioconversion of failed drug candidates using various P450s and then retesting may open the way to the development of new drugs. Furthermore, this technique may be applicable for producing various materials, such as fiber and platform.

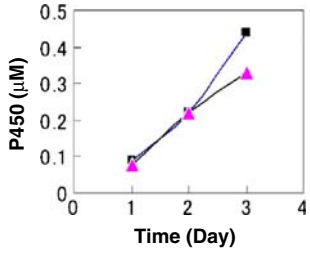


Fig. 4 Time course of P450 production in synthetic medium (*filled triangle*) and in LB medium (*filled square*) after the addition of Isopropyl b-D-thiogalactopyranoside in *E. coli* co-expressing the P450 2C43 gene and reductase. Bacterial cells were harvested from 50 ml of culture

Bioconversion of steroids using P450

Different P450s are known to hydroxylate the same steroid at different positions [24, 25]. Therefore, we tested our system with three P450s to determine whether a steroid would be hydroxylated at different positions by

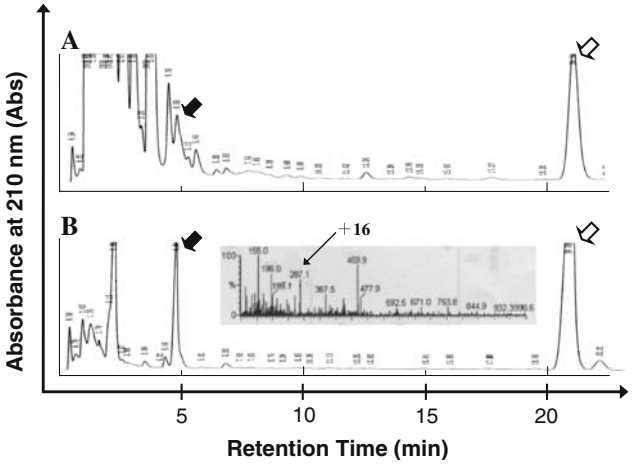


Fig. 5 HPLC analysis of estradiol metabolites produced by the action of P450 2C43 in vivo. P450 2C43 was incubated with estradiol in synthetic medium (**a**) and LB medium (**b**) for 48 h. *Open* and *solid arrows* indicate substrate and hydroxylated product, respectively. *Inset* shows the mass spectrum, with an *arrow* indicating the peak representing hydroxylated estradiol

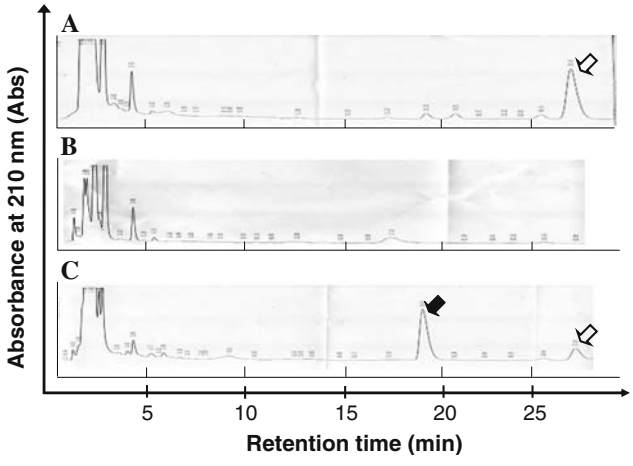


Fig. 6 HPLC analysis of testosterone metabolites produced by the action of P450 2C43 in vivo: **a** inoculate without P450, **b** inoculate without testosterone, and **c** testosterone incubated with P450. *Open* and *solid arrows* indicate substrate and product, respectively

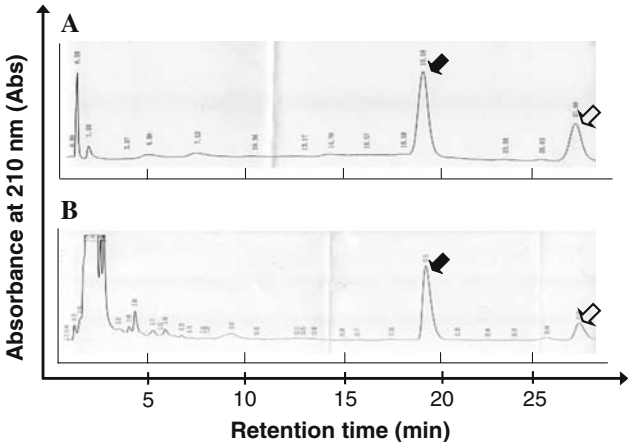


Fig. 7 Comparison of hydroxylated testosterone product and androstenedione by HPLC: **a** androstenedione and testosterone and **b** testosterone incubated with P450 2C43

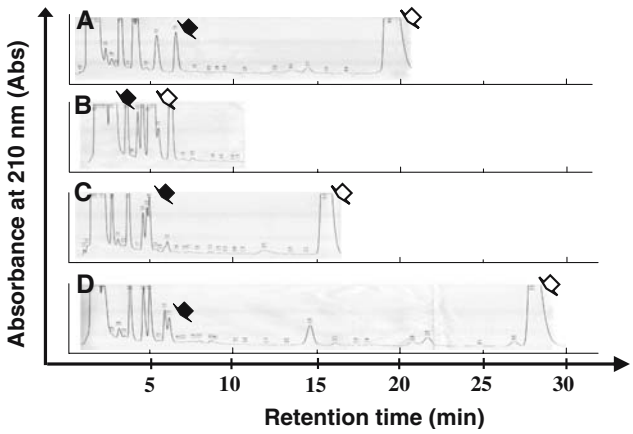


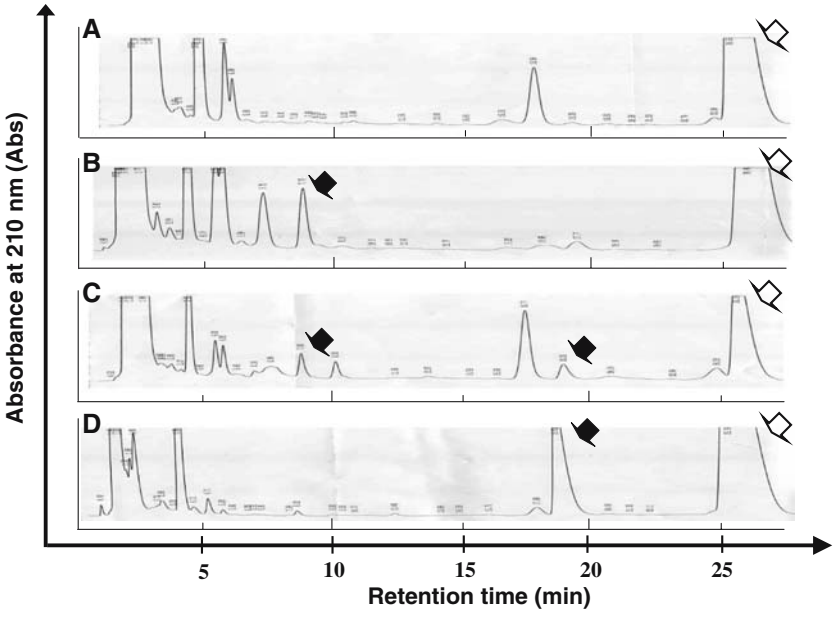
Fig. 9 Bioconversion by P450 2C29 incubated with **a** testosterone, **b** 7-ethoxycoumarin, **c** androstenedione, or **d** dehydroepiandrosterone

the three P450s. Testosterone was incubated with P450 2C2, P450 2C29, and P450 2A4, and the metabolites were analyzed by HPLC (Fig. 8). HPLC analysis indicated that the products of metabolism by the three P450s had different retention times (P450 2C29, 8 min; P450 2C2, 8 min and 18 min; P450 2A4 18 min). These results suggest that, with our system, a variety of modified compounds could be obtained from one molecule, depending on which P450 was chosen.

Microsomal P450s have been reported to metabolize many substrates, and its substrate specificity is overlapping but different [24, 25]. Therefore, we decided to determine whether P450 would hydroxylate testosterone, 7-ethoxycoumarin, androstenedione, and dehydroepiandrosterone in the synthetic medium. We used P450 2C29 as the P450. Hydroxylated products were obtained from all the compounds, at retention times of 7.2, 3.0, 5.1, and 7.0 min, respectively (Fig. 9). This result suggests that many compounds can be modified by one type of P450 and thus that this system may be useful for the bioconversion of many compounds to modified forms for high-throughput screening.

Recently, HTS has been used to screen potential new drugs from among millions of small molecules [8, 13, 29]. When HTS was first developed, compounds were subjected to it without preliminary design or screening; but recently, owing to the low success rate in finding hits and the high cost, target molecules are now designed to some extent, so that modified molecules are subjected to HTS. P450s have the potential to

Fig. 8 Bioconversion of testosterone by P450s after incubation in synthetic medium for 48 h: **a** control, cells were transformed with only pCW vector, **b** P450 2C29, **c** P450 2C2, or **d** P450 2A4



permit the synthesis of many modified forms from various types of compounds. Furthermore, mutant P450s produced using recombinant technologies undergo changes in substrate specificity [24]. This report raises the possibility of developing mutated P450s with different substrate specificities and using the mutants to increase the number of compounds available that have been modified by P450s. Our results suggest that our system may be important and versatile for modifying small molecules for HTS.

Recently, a number of P450 crystal structures have been determined [15, 23, 27]. Molecular models for the binding and metabolism of drugs have been developed on the basis of crystal structures (P450 2D6, for example) [12]. In the future, researchers may be able to select in advance a specific P450 for modifying a particular molecule for drug discovery, and thus produce a new drug.

It is also possible that combining and incubating many types of P450s together in our system and using them to metabolize compounds may open the way for the synthesis of many new modified products. We have found that new products are detected when two different P450s are incubated together (data not shown). Much more research is needed on the incubation of many P450s together and on the use of such combinations to metabolize a variety of small molecules for drug screening.

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