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# Oxygenation Reactions of Various Tricyclic Fused Aromatic Compounds Using Escherichia coli and Streptomyces lividans Transformants Carrying Several Arene Dioxygenase Genes

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# Oxygenation Reactions of Various Tricyclic Fused Aromatic Compounds Using *Escherichia coli* and *Streptomyces lividans* Transformants Carrying Several Arene Dioxygenase Genes

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**Bioconversion (biotransformation) experiments on arenes (aromatic compounds), including various tricyclic fused aromatic compounds such as fluorene, dibenzofuran, dibenzothiophene, carbazole, acridene, and phenanthridine, were done using the cells of *Escherichia coli* transformants expressing several arene dioxygenase genes. *E. coli* carrying the phenanthrene dioxygenase (*phdABCD*) genes derived from the marine bacterium *Nocardioides* sp. strain KP7 converted all of these tricyclic aromatic compounds, while *E. coli* carrying the *Pseudomonas putida* F1 toluene dioxygenase (*todC1C2BA*) genes or the *P. pseudoalcaligenes* KF707 biphenyl dioxygenase (*bphA1A2A3A4*) genes was not able to convert these substrates. Surprisingly, *E. coli* carrying hybrid dioxygenase (*todC1::bphA2A3A4*) genes with a subunit substitution between the toluene and biphenyl dioxygenases was able to convert fluorene, dibenzofuran, and dibenzothiophene. The cells of a *Streptomyces lividans* transformant carrying the phenanthrene dioxygenase genes were also evaluated for bioconversion of various tricyclic fused aromatic compounds. The ability of this actinomycete in their conversion was similar to that of *E. coli* carrying the corresponding genes. Products converted from the aromatic compounds with these recombinant bacterial cells were purified by column chromatography on silica gel, and identified by their MS and $^{1}H$ and $^{13}C$ NMR analyses. Several products, e.g., 4-hydroxyfluorene converted from fluorene, and *cis*-1,2-dihydroxy-1,2-dihydrophenanthridine, *cis*-9,10-dihydroxy-9,10-dihydrophenanthridine, and 10-hydroxyphenanthridine, which were converted from phenanthridine, were novel compounds.**

**Key words:** tricyclic aromatic compounds; phenanthridine; arene dioxygenase; *Escherichia coli*; *Streptomyces lividans*

Polycyclic aromatic hydrocarbons and polycyclic aromatic heterocycles are widespread environmental pollutants, which are derived from coal and petroleum.[1)] Studies on their microbial degradation are important for the developments of bioremediation technologies against these mutagenic or carcinogenic compounds.[2)]

Bacterial species that are able to metabolize small aromatic hydrocarbons, such as benzene,[3)] toluene,[3)] xylene,[4)] and naphthalene,[5)] are well known, and the genes and enzymes mediating their metabolism have been extensively analyzed.[6–9)] On the other hand, only a few reports describe bacterial species that degrade polycyclic aromatic hydrocarbons and polycyclic aromatic heterocycles, which are compounds with more than two rings, such as anthracene,[10)] phenanthrene,[10,11)] dibenzofuran,[12)] and carbazole.[13)] It is generally considered that these polycyclic aromatic compounds (PACs) are refractory to biodegradation.[14)] One of the rate-limiting steps in the degrada-

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tion of the pollutants seems to be the first oxygenation reaction that is frequently catalyzed by PAC dioxygenase. This is a multicomponent enzyme, which consists of ferredoxin, ferredoxin reductase, and an iron-sulfur protein, and introduces both atoms of molecular oxygen into an aromatic nucleus.[15] Genes coding for PAC dioxygenase have been isolated from several bacteria, i.e., *Pseudomonas* sp. strains NCIB 9816-4,[15] *Pseudomonas* sp. strain C18,[16] *Pseudomanas* sp. strain CA10,[17] *Burkhorderia* sp. strain RP007,[18] *Comamonas testosteroni* GZ39,[19] and *Nocardioides* sp. strain KP7,[20] and the functions of the encoded enzymes have been elucidated.

Bacterial species that are able to metabolize the industrial wastes, polychlorinated biphenyls (PCBs), which are refractory to biodegradation, are also known, and the genes and enzymes mediating their metabolism have been analyzed.[21] Several genes encoding biphenyl dioxygenase, which catalyzes the first oxygenation reaction, have been isolated from soil bacteria such as *Pseudomonas pseudoalcaligenes* KF707,[22] *Pseudomonas putida* KF715,[23] *Burkholderia cepacia* LB400,[24] and *Rhodococcus globerulus* P6,[25] and the functions of the encoded enzymes have been analyzed.

In the bacterial degradation not only of PACs but also of PCBs and small aromatic hydrocarbons such as toluene, the first reaction is ordinarily catalyzed by a multicomponent dioxygenation enzyme consisting of ferredoxin, ferredoxin reductase, and an iron-sulfur protein (Here, we call these arene dioxygenases, comprehensively). Many genes coding for arene dioxygenase have been cloned separately and the encoded enzymes have been characterized as their examples in part are described above, while a few reports display comparative studies on the characteristics among the dioxygenation enzymes.[26] Here, we describe comparative evaluation in the oxygenation reactions of various tricyclic fused aromatic compounds as well as other aromatic compounds, using *Escherichia coli* transformants expressing several arene dioxygenase genes, i.e., the phenanthrene dioxygenase genes of the marine bacterium *Nocardioides* sp. strain KP7,[20] the toluene dioxygenase genes of the soil bacterium *Pseudomonas putida*,[27] the *P. pseudoalcaligenes* KF707 biphenyl dioxygenase genes,[28] and hybrid dioxygenase genes with the subunit substitution between the toluene and biphenyl dioxygenases, which include large ($\alpha$) subunit from the former and small ($\beta$) subunit from the latter dioxygenase.[29] Oxygenation reactions of various tricyclic fused aromatic compounds by the *Streptomyces lividans* transformant expressing the phenanthrene dioxygenase genes[30] are also described. Furthermore, we have newly found that the recombinant cells carrying the phenanthrene dioxygenase genes are capable of converting phenanthridine into its two *cis*-diol forms and the monohydroxylated product.

## Materials and Methods

*Plasmids, bacterial strains, and growth conditions.* Plasmids for *Escherichia coli* used in this study are listed in Table 1. Vector pUC118 was purchased from Takara Shuzo Co. *E. coli* BL21 (DE3)[31] and *E. coli* JM109[32] were used as hosts for plasmid pHA171 and plasmids pJHF3051, pKF6622, and pKF6256, respectively, and cultured in LB medium[32] or M9 medium[32] at 30°C or 37°C. Ampicillin (Ap) (50 to 150 $\mu$g/ml) was added when needed.

Plasmid pIJ6021-phdABCD, in which the *Nocardioides* sp. KP7 *phdABCD* genes were inserted into the expression vector pIJ6021 for *Streptomyces* species carrying the kanamycin (Km) resistance gene and the thiostrepton-inducible promoter *PtipA*,[33] has been described.[30] *Streptomyces lividans* TK21[34] was used as a host for plasmid pIJ6021-phdABCD, and cultured in YEME medium or minimal medium[34] at 30°C. Minimal medium contained glucose (1%), L-asparagine (0.05%), $K_2HPO_4$ (0.05%), $MgSO_4 \cdot 7H_2O$ (0.02%), and $FeSO_4 \cdot 7H_2O$ (0.001%). Km was used at a final concentration of 5 $\mu$g/ml when necessary.

*General recombinant DNA techniques.* Restriction

**Table 1.** Plasmids for *E. coli* Used in This Study

| Plasmids (Vectors) | Genes[a] | Source | Enzyme common Names | References |
|---|---|---|---|---|
| PHA171 (pT7-7) | *phdABCD* | *Nocardioides* sp. KP7 | Phenanthrene dioxygenase | 20) |
| pJHF3051 (pUC119) | *todC1C2BA* | *Pseudomonas putida* F1 | Toluene dioxygenase | 27, 29) |
| pKF6622 (pUC118) | *bphA1A2A3A4* | *Pseudomonas pseudoalcaligenes* KF707 | Biphenyl dioxygenase | this study |
| pKF6256 (pUC118) | *todC1::bphA2A3A4* | *P. putida* F1 and *P. pseudoalcaligenes* KF707 | Toluene/biphenyl dioxygenase | this study |

[a] *phdA*, *todC1*, and *bphA1* code for an iron-sulfur protein large subunit $\alpha$, and *phdB*, *todC2*, and *bphA2* encode its small subunit $\beta$. *phdC*, *todB*, and *bphA3* encode ferredoxin, and *phdD*, *todA*, and *bphA4* code for ferredoxin reductase.

enzymes and T4 DNA ligase were purchased from Takara Shuzo. DNA manipulation was done in *E. coli*[32] and in *S. lividans*,[34] as described.

*Construction of plasmids pKF6622 and pKF6256.* A 6.8-kb *Xho*I fragment carrying the *bphA1A2A3A4-bphB-bphC* genes from *P. pseudoalcaligenes* KF707 (GenBank accession no. M83673)[28] was isolated and inserted into the *Xho*I site of vector pUC118, and then the *bphB-bphC* genes were eliminated by deleting a 1.4-kb *Ppu*MI fragment from this plasmid, creating pKF6622. An 1.4-kb *Sac*I-*Bgl*II fragment carrying the *bphA1* gene was eliminated from the plasmid pKF6622, instead, an 1.4-kb *Sac*I-*Bgl*II fragment carrying the *todC1* gene from *P. putida* F1 (GenBank J04996) was isolated and inserted into the the *Sac*I/*Bgl*II site of the resultant plasmid, creating pKF6256. In plasmids pKF6622 and pKF6256, the inserted arene dioxygenase genes were positioned to have the transcriptional read-through from the *lac* promoter of pUC118.

*Conversion experiments by cocultivation with cells and substrates. E. coli* BL21 (DE3) harboring pHA171 or *E. coli* JM109 harboring pJHF3051, pKF6622, or pKF6256 was grown in LB medium containing 150 μg/ml of Ap at 30°C with reciprocal shaking (175 rpm) for 8 h. Five milliliters of this culture was inoculated into 100 ml of M9 medium with 150 μg/ml of Ap, 10 μg/ml of thiamine, and 0.4% (w/v) glucose in Erlenmeyer flask at 30°C with reciprocal shaking (175 rpm) for 16–17 h, of which the absorbance in OD 600 nm reaches approximately 1. One mM (the final concentration) of isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture, and further cultivated for 4 h. The cells were collected by centrifugation, washed once with M9 medium, and then resuspended in 100 ml of fresh M9 medium with 150 μg/ml of Ap, 10 μg/ml of thiamine, 0.4% (w/v) glucose, and 1 mM (the final concentration) of IPTG, along with 10 mg or 1 mM (the final concentration) of each substrate, and cultivated in Erlenmeyer flask at 30°C with reciprocal shaking (175 rpm) for 2–3 days.

*S. lividans* TK21 harboring pIJ6021-phdABCD was grown in 100 ml of YEME medium containing 5 μg/ml of Km in a shaking (Sakaguchi) flask at 30°C with reciprocal shaking (200 rpm) for 2 days. One milliliter of this culture was inoculated into the same medium, and cultivated under the same conditions. After 24 h, thiostrepton was added to the culture at a final concentration of 5 μg/ml to induce transcription from *PtipA*, and cultivated further for 24 h. The mycelium was collected by centrifugation and washed once with minimal medium. Then, 1 g (wet wt) of mycelium was resuspended in 100 ml of fresh minimal medium, along with 1 mM (final concentration) of each substrate, and cultivated in Sakaguchi flasks at 30°C with reciprocal shaking (200 rpm) for 2–3 days.

Substrates, e.g., anthracene, phenanthrene, 5,10-dihydroanthracene, 9,10-dihydrophenanthrene, fluorene, dibenzofuran, dibenzothiophene, carbazole, phenoxathiin, acridine, and phenanthridine, were purchased from Aldrich Chemical Co., Wako Pure Chemical Co., or Kanto Chemical Co. The respective substrates were dissolved in small amounts of ethanol and added to the culture.

*Extraction and HPLC analysis of converted products.* To extract the converted products as well as the substrates, methanol (MeOH) with the same volume as the cultured medium was added to the coculture of the transformed cells of *E. coli* or *S. lividans*, and mixed for 30 min. After centrifugation to remove cells, the liquid phase was used for high-pressure liquid chromatography (HPLC) analysis or for further purification steps of the converted products.

The liquid phase (80 μl) was put through HPLC on a Puresil $C_{18}$ column (4.6 × 250 mm, Waters) with a photodiode array detector (model 996, Waters). It was developed at a flow rate of 1 ml/min with solvent A ($H_2O$-MeOH, 1:1) for 5 min, followed by a 凹-shaped gradient (No.3; Waters) from solvent A to solvent B (MeOH-2-propanol, 6:4) for 15 min, and with solvent B for 13 min, and monitored with max absorbance between 230–350 nm

*Purification and identification of converted products.* The liquid phase (1,000 ml), which was obtained by the procedure described above, was concentrated *in vacuo*, and extracted with ethyl acetate (EtOAc) (500 ml × 2). The organic layer was concentrated *in vacuo*, and analyzed by thin-layer chromatography (TLC) on silica gel [0.25-mm Silica Gel 60 (Merck)]. This was developed with solvent systems as follows: fluorene, hexane–EtOAc (10:1); 5,10-dihydroanthracene, hexane–EtOAc (10:1); 9,10-dihydrophenanthrene, hexane–EtOAc (10:1); dibenzofuran, hexane–EtOAc (10:1); dibenzothiophene, hexane–EtOAc (1:1); carbazole, hexane–EtOAc (3:1); phenoxathiin, hexane–EtOAc (1:1); acridine, dichloromethane ($CH_2Cl_2$)–MeOH (20:1); phenanthridine, $CH_2Cl_2$–MeOH (10:1). The converted products as well as the substrates, which were contained in the organic phase, were put through column chromatography on silica gel [20 by 250 mm, Silica Gel 60 (Merck)]. This was developed with solvent systems as follows: fluorene, hexane–EtOAc (10:1); 5,10-dihydroanthracene, hexane–EtOAc (20:1); 9,10-dihydrophenanthrene, hexane–EtOAc (20:1); dibenzofuran, hexane–EtOAc (10:1); dibenzothiophene, hexane–EtOAc (4:1→1:1) (stepwise), carbazole, hexane–EtOAc (5:1); phenoxathiin, hexane–EtOAc (3:1); acridine, $CH_2Cl_2$–MeOH (30:1); phenanthri-

dine, hexane-EtOAc (1:1→EtOAc) (stepwise).

The structures of the converted products were analyzed by mass (MS) (EI-MS, JEOL DX-303) and nuclear magnetic resonance (NMR) (500 MHz, JEOL GX-500) spectra. TMS was used for the internal standard.

(*R*) and (*S*)-methoxy-(2-naphtyl)acetic acid (2NMA) esters were prepared in a manner reported by Kusumi *et al.*[35]

## Results

*Biotransformation of various aromatic compounds with recombinant E. coli cells*

In the beginning, we did biotransformation (bioconversion) experiments of various aromatic compound-substrates listed in Table 2, using the recombinant *E. coli* cells expressing four species of the arene dioxygenase genes shown in Table 1. After the respective substrates and cells were co-cultured for 2–3 days, the ratio of the products converted from the substrates was measured through HPLC. Results are shown in Table 2.

*E. coli* (pHA171) harboring the phenanthrene dioxygenase (*phdABCD*) genes derived from the marine bacterium *Nocardioides* sp. strain KP7, was not able to transform the monocyclic aromatic hydrocarbons *n*-butylbenzene, *t*-butylbenzene, biphenyl, and diphenylmethane, while the other three recombinant *E. coli* strains carrying the arene dioxygenase genes derived from *Pseudomonas* species were able to convert all of these substrates. On the other hand, *E. coli* (pHA171) converted the tricyclic fused aromatic heterocycles, i.e., dibenzofuran, dibenzothiophene, carbazole, phenoxathiin, acridine, and phenanthridine, in addition to tricyclic fused aromatic hydrocarbons such as anthracene, phenanthrene, and fluorene. On the contrary, both transformants of *E. coli* (pJHF3051), carrying the *P. putida* F1 toluene dioxygenase (*todC1C2BA*) genes, and *E. coli* (pKF6622), carrying the *P. pseudoalcaligenes* KF707 biphenyl dioxygenase (*bphA1A2A3A4*) genes, were capable of transforming none of these tricyclic aromatic compounds with one exception; *E. coli* (pKF6622) converted anthracene with low efficiency. It was also surprising that *E. coli* (pKF6256) carrying the hybrid dioxygenase (*todC1::bphA2A3A4*) genes with the subunit substitution between the toluene and biphenyl dioxygenases was able to convert fluorene, dibenzofuran, and dibenzothiophene. Especially, this *E. coli* transformant converted dibenzofuran more efficiently than *E. coli* (pHA171).

*Biotransformation of tricyclic fused aromatic compounds with recombinant S. lividans cells*

The cells of *S. lividans* (pIJ6021-phdABCD) were also used to do biotransformation experiments with various tricyclic fused aromatic compounds listed in Table 2. This actinomycete transformant converted the substrates in the same way as those converted with the *E. coli* (pHA171). The converted products were identical to those converted with the *E. coli* transformants. The rates in the bioconversion of these tricyclic aromatic compounds with the recombinant *S. lividans* cells seemed to be more efficient than or similar to those with the cells of *E. coli*

**Table 2.** Bioconversion of Various Aromatic Compounds by Recombinant *E. coli* Cells

| Substrates \ Plasmids | pHA171 | pJHF3051 | pKF6622 | pKF6256 |
|---|---|---|---|---|
| *n*-Butylbenzene | — | + + | + + + | + |
| *t*-Butylbenzene | — | + + + | + | + + + |
| Biphenyl | — | + + + | + + + | + |
| 4-Methoxybiphenyl | — | — | + + + | — |
| Diphenylmethane | — | + + | + + + | + + + |
| 1-Methoxynaphthalene | + + + | — | — | — |
| Anthracene | + + + | — | + | + |
| Phenanthrene | + + + | — | — | — |
| 5,10-Dihydroanthracene | + + | NT | NT | NT |
| 9,10-Dihydrophenanthrene | + + + | NT | NT | NT |
| Fluorene | + + | — | — | + |
| Dibenzofuran | + | — | — | + + |
| Dibenzothiophene | + + | — | — | + |
| Carbazole | + + | — | — | — |
| 9-Methylcarbazole | — | — | — | — |
| Xanthene | — | — | — | — |
| Phenoxathiin | + | — | — | — |
| Phenoxazine | — | — | — | — |
| Phenothiazine | — | — | — | — |
| Acridine | + | — | — | — |
| Phenanthridine | + + + | — | — | — |

+, 1–20%; + +, 21–60%; + + +, 61–100%; NT, not tested.

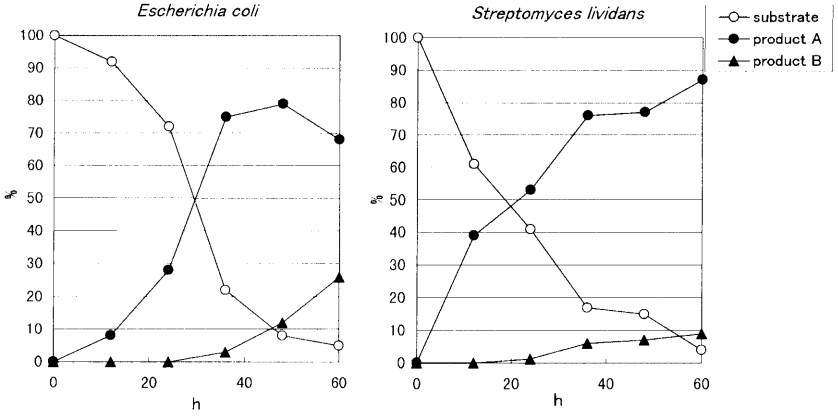



**Fig. 1.** Bioconversion of Phenanthridine by Co-culture with the Cells of *E. coli* (pHA171) and *S. lividans* (pIJ6021-phdABCD).
1 mM of the substrate and 10 mg (wet wt) of the cells per ml, which were fully induced with IPTG or thiostrepton, were used.
substrate, phenanthridine; product A, mixture of diols; product B, monohydroxylated product.
Diols were identified as *cis*-1,2-dihydroxy-1,2-dihydrophenanthridine and *cis*-9,10-dihydroxy-9,10-dihydrophenanthridine as shown later. The monohydroxylated product was identified to be 10-hydroxyphenanthridine as shown later.



(pHA171) (data not shown). As one example, Fig. 1 shows rates of the biotransformation of phenanthridine using the cells of *S. lividans* (pIJ6021-phdABCD) as well as those of *E. coli* (pHA171). In this case, the conversion rates of the substrate with the *S. lividans* and *E. coli* transformants were similar.

*Structural analysis of biotransformed products*
*Products converted from fluorene*

TLC analysis of the crude products (105 mg) converted from fluorene with *E. coli* (pHA171) revealed the presence of two metabolites with Rf values of 0.2 (compound 1) and 0.5 (compound 2). Both compounds were purified by silica gel column chromatography to yield 1 (10 mg) and 2 (3 mg) as colorless oils. Compound 2 proved to be 9-fluorenol (Fig. 2) by comparison with the previously reported spectral data (MS and NMR)[36].

The molecular fomula of compound 1 was determined to be $C_{13}H_{10}O$ by MS [*m/z* 182 ($M^+$)] and $^1H$ and $^{13}C$ NMR spectra. Analysis of DQF COSY, and C-H COSY spectra of 1 proposed that one phenolic OH function was attached to the aromatic ring of fluorene. The position of the phenolic OH proved to be C-4 by the observation of long range couplings from H-9 ($\delta$3.91) to C-1 ($\delta$117.6) and vicinal $sp^2$ $^1H$-$^1H$ spin network from H-1 to H-3. Thus, compound 1 was identified to be 4-hydroxyfluorene (Fig. 2). This was a novel compound. Its NMR data are shown in Table 3.

*A product converted from 5,10-dihydroanthracene*

TLC analysis of the crude products (100 mg) converted from 5,10-dihydroanthracene with *E. coli* (pHA171) or *S. lividans* (pIJ6021-phdABCD) found one metabolite with Rf 0.2 (compound 3). This metabolite was purified by silica gel column chromatography to yield 3 (15 mg) as a colorless oil. This compound (3) was identified as *cis*-1,2-dihydroxy-1,2-dihydro-anthracene (Fig. 2) by its MS [*m/z* 212 ($M^+$)] and NMR spectral data.[17] We observed that the conversion of 5,10-dihydroanthracene to anthracene occurred in cells of both non-transformed *E. coli* and *S. lividans* (data not shown). It is thus likely that the endogenous synthesis of anthracene from 5,10-dihydroanthracene first occurred in the transformed *E. coli* or *S. lividans*, followed by the dioxygenation reaction to produce compound 3.

*A product converted from 9,10-dihydrophenanthrene*

TLC analysis of the crude products (50 mg) converted from 9,10-dihydrophenanthrene with *E. coli* (pHA171) or *S. lividans* (pIJ6021-phdABCD) found one metabolite with Rf 0.3 (compound 4). This metabolite was purified by silica gel column chromatography to yield 4 (10 mg) as a colorless oil. The molecular formula of this compound (4) was determined to be $C_{14}H_{12}O$ by its MS [*m/z* 196 ($M^+$)] and $^1H$ and $^{13}C$ NMR spectra. Analysis of DQF COSY, and C-H COSY spectra of 4 proposed that one phenolic OH function was attached to the aromatic ring of 9,10-dihydrophenanthrene. The position of the phenolic OH proved to be C-3 by the observation of long range couplings from H-1 ($\delta$7.11) and H-10 ($\delta$2.80) to C-4a ($\delta$135.7) and from H-1 to C-3 ($\delta$154.5). Thus, compound 4 was identified to be 3-hydroxy-9,10-dihydrophenanthrene (Fig. 2). This was a novel compound. Its NMR data are shown in Table 4.

*Products converted from dibenzofuran*

TLC analysis of the crude products (32 mg) converted from dibenzofuran with *E. coli* (pKF6256) found two metabolites with Rf values of 0.2 (com-

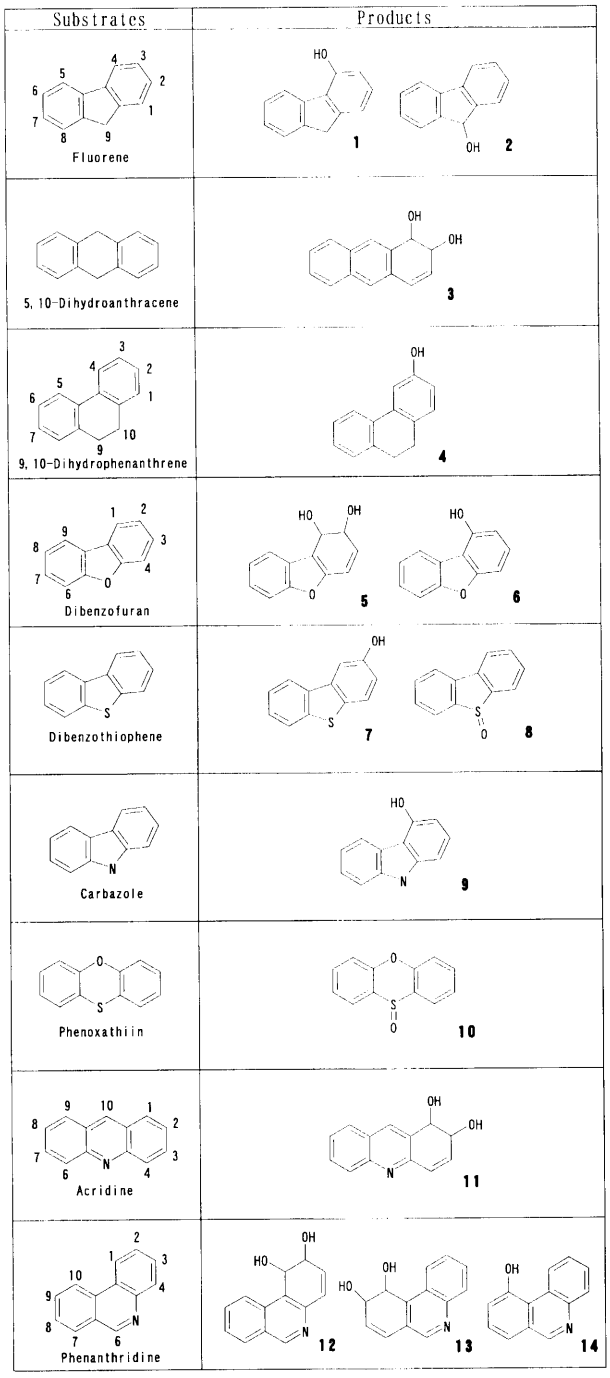



**Fig. 2.** Structures of Products Bioconverted from Respective Tricyclic Fused Aromatic Compounds.

pound 5) and 0.3 (compound 6). Both metabolites were purified by silica gel column chromatography to yield 5 (10 mg) and 6 (3 mg) as colorless oils. Compounds 5 and 6 were identified to be *cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran and 1-hydroxydibenzofuran (Fig. 2), respectively, by comparison with the previously reported spectral data (MS and NMR).[37]

**Table 3.** 500 MHz $^1$H NMR and 125 MHz $^{13}$C NMR Spectral Data of 4-Hydroxyfluorene (1) in $CDCl_3$

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1 | 7.13[a] | 117.6 |
| 2 | 7.13[a] | 127.5 |
| 3 | 6.72 (dd, J = 1.8, 6.7) | 113.7 |
| 4 | 5.17 (4-OH) | 151.6 |
| 4a | | 128.6 |
| 4b | | 140.6 |
| 5 | 8.09 (d, J = 7.3) | 123.5 |
| 6 | 7.36 (dd, J = 7.3, 7.3) | 126.8 |
| 7 | 7.25 (dd, J = 7.3, 7.3) | 125.9 |
| 8 | 7.51 (d, J = 7.3) | 124.4 |
| 8a | | 142.5 |
| 9 | 3.91 (s) | 37.3 |
| 9a | | 145.7 |

[a] Resonances in one-dimentional spectra obscured by overlapping signals.

**Table 4.** 500 MHz $^1$H NMR and 125 MHz $^{13}$C NMR Spectral Data of 3-Hydroxy-9,10-dihydrophenanthrene (4) in $CDCl_3$

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1 | 7.11 (d, J = 7.9) | 129.1 |
| 2 | 6.72 (dd, J = 3.0, 7.9) | 114.2 |
| 3 | | 154.5 |
| 4 | 7.23[a] | 110.6 |
| 4a | | 135.7 |
| 4b | | 134.2 |
| 5 | 7.67 (d, J = 7.9) | 123.7 |
| 6 | 7.27 (ddd, J = 3.0, 7.9, 7.9) | 126.9 |
| 7 | 7.23[a] | 127.6 |
| 8 | 7.23[a] | 128.2 |
| 8a | | 137.6 |
| 9 | 2.86 (m) | 29.3 |
| 10 | 2.80 (m) | 28.1 |
| 10a | | 129.8 |

[a] Resonances in one-dimentional spectra obscured by overlapping signals.

*Products converted from dibenzothiophene*

TLC analysis of the crude products (23 mg) converted from dibenzothiophene with *E. coli* (pHA171) found two metabolites with Rf values of 0.4 (compound 7) and 0.2 (compound 8). Both compounds were purified by silica gel column chromatography to yield 7 (3.6 mg) and 8 (1.2 mg) as colorless powders. Compounds 7 and 8 were identified to be 2-hydroxydibenzothiophene and dibenzothiophene sulfoxide (Fig. 2) by their MS and NMR spectral data, respectively.[38]

*A product converted from carbazole*

TLC analysis of the crude products (54 mg) converted from carbazole with *E. coli* (pHA171) found one spot with Rf 0.3 (compound 9). This compound was purified by silica gel column chromatography, yielding 9 (11.8 mg) as a colorless oil. Compound 9 was identified as 1-hydroxycarbazole (Fig. 2) by comparison with the previously reported spectral data (MS and NMR).[39]

*A product converted from phenoxathiin*

TLC analysis of the crude products (30 mg) converted from phenoxathiin with *E. coli* (pHA171) or *S. lividans* (pIJ6021-phdABCD) found one metabolite with Rf 0.4 (compound 10). This compound was purified by silica gel column chromatography to yield 10 (3.6 mg) as a pale yellow oil. Compound 10 was identified as phenoxathione sulfoxide (Fig. 2) by its MS [*m/z* 216 ($M^+$)] and NMR spectral data.[40]

*A product converted from acridine*

TLC analysis of the crude products (20 mg) converted from acridine with *E. coli* (pHA171) or *S. lividans* (pIJ6021-phdABCD) found one metabolite with Rf 0.2 (compound 11). This compound was purified by silica gel column chromatography to yield 11 (5.0 mg) as a yellow oil. Compound 11 was identified as *cis*-1,2-dihydroxy 1,2-dihydro-acridine (Fig. 2) by comparison with the previously reported spectral data (MS and NMR).[41]

*Products converted from phenanthridine*

TLC analysis of the crude products (150 mg) converted from phenanthridine with *E. coli* (pHA171) or *S. lividans* (pIJ6021-phdABCD) revealed the presence of three metabolites with Rf values of 0.3 (compound 12), 0.4 (compound 13), and 0.7 (compound 14). These compounds were purified by silica gel column chromatography to yield 12 (10 mg), 13 (8 mg), and 14 (3 mg) as yellow powder or oil.

The molecular formula of compound 12 was determined to be $C_{13}H_{11}NO_2$ by its MS [*m/z* 213 ($M^+$)] and $^1H$ and $^{13}C$ NMR spectra. Analyses of $^1H$-$^{13}C$ COSY and DQF COSY spectra showed that this compound was a dihydrodiol derivative of phenanthridine. The regiochemical assignment of the 1,2-diol was confirmed by the long range carbon-proton connectivities observed between H-6 ($\delta$9.20) and C-7 ($\delta$127.5) in HMBC spectrum and $^1H$-$^1H$ spin networks from H-7 ($\delta$8.0) to H-10 ($\delta$8.15) in DQF COSY spectrum. *cis* Cofiguration of the 1,2-diol was shown by the coupling constant ($J_{1,2}=4.5$ Hz). From these data, compound 12 was identified as *cis*-1,2-dihydroxy-1,2-dihydrophenanthridine (Fig. 2).

The molecular formula of compound 13 was determined to be $C_{13}H_{11}NO_2$ by its MS [*m/z* 213 ($M^+$)] and $^1H$ and $^{13}C$ NMR spectra. Analyses of $^1H$-$^{13}C$ COSY and DQF COSY spectra showed that this compound was also a dihydrodiol derivative of phenanthridine. 9,10-Diol regiochemical assignment was confirmed by the long range $^1H$-$^{13}C$ connectivities observed from H-6 ($\delta$8.75) and H-3 ($\delta$7.70) to C-4a ($\delta$147.4) in HMBC spectrum and vicinal $^1H$-$^1H$ spin networks from H-1 ($\delta$8.20) to H-4 ($\delta$7.97) in DQF COSY spectrum. *cis* Configuration of the 9,10-diol was proved by the coupling constant ($J_{1,2}=4.5$ Hz). Compound 13 was thus identified as *cis*-9,10-dihydroxy-9,10-dihydrophenanthridine (Fig. 2).

The molecular formula of compound 14 was determined to be $C_{13}H_9NO$ by its MS [*m/z* 195 ($M^+$)], $^1H$ and $^{13}C$ NMR spectra. DQF COSY analysis found two vicinal $^1H$-$^1H$ $sp^2$ spin networks from H-1 ($\delta$9.55) to H-4 ($\delta$8.10) and from H-7 ($\delta$7.69) to H-9 ($\delta$7.40). HMBC experiment proved the long range couplings from H-6 ($\delta$9.25) to C-7 ($\delta$119.8) and H-9 ($\delta$7.40) to C-10 ($\delta$155.6). From these data, compound 14 was identified as 10-hydroxyphenanthridine (Fig. 2).

Compounds 12, 13, and 14 were all novel compounds. Their $^1H$ and $^{13}C$ NMR data are shown in Table 5.

The absolute stereochemistry of compounds 12 and 13 were determined by $^1H$ NMR analysis of diastereomeric esters formed with (*R*)- and (*S*)-2-NMA[42] (Modified Mosher's method[43]). Their $\Delta(\delta R-\delta S)$ values are summarized in Fig. 3. The sign of $\Delta\delta$ are systematically arranged to the right and left sides to the 2NMA planes in 12 and 13. From these data, the absolute configurations of C-1 and C-2 in

**Table 5.** 500 MHz $^1H$ and 125 MHz $^{13}C$ NMR Spectral Data of *cis*-1,2-Dihydroxy-1,2-dihydrophenanthridine (12), *cis*-9,10-Dihydroxy-9,10-dihydrophenanthridine (13), and 10-Hydroxyphenanthridine (14) in DMSO-$d_6$

| | 12 | | 13 | | 14 | |
|---|---|---|---|---|---|---|
| Position | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | 5.12(m) | 64.5 | 8.20 (d, J=7.9 ) | 123.8 | 9.55 (d, J=7.9) | 127.6 |
| 2 | 4.55(m) | 70.0 | 7.63 (dd, J=7.9, 7.9) | 127.0 | 7.63 (dd, J=7.9, 7.9) | 126.7 |
| 3 | 6.10(d, J=9.8) | 136.5 | 7.70 (dd, J=7.9, 7.9) | 128.5 | 7.70 (dd, J=7.9, 7.9) | 128.3 |
| 4 | 6.57 (dd, J=3.0, 9.8) | 127.5 | 7.97 (d, J=7.9) | 129.5 | 8.10 (d, J=7.9) | 129.2 |
| 4a | | 145.5 | | 147.4 | | 144.2 |
| 6 | 9.20 (s) | 152.1 | 8.75 (s) | 149.7 | 9.25 (s) | 153.7 |
| 6a | | 127.5 | | 124.8 | | 120.1 |
| 7 | 8.06 (d, J=7.9) | 127.5 | 6.61 (dd, J=3.0, 9.8) | 122.5 | 7.69 (d, J=7.9) | 119.8 |
| 8 | 7.60 (dd, J=7.9, 7.9) | 126.6 | 6.05 (ddd, J=1.8, 1.8, 9.89) | 135.7 | 7.62 (dd, J=7.9, 7.9) | 128.2 |
| 9 | 7.80 (dd, J=7.9, 7.9) | 130.9 | 4.45 (m) | 69.5 | 7.40 (dd, J=7.9, 7.9) | 116.9 |
| 10 | 8.15 (d, J=7.9) | 122.6 | 5.11 (m) | 63.8 | | 155.6 |
| 10a | | 134.0 | | 138.9 | | 128.4 |
| 10b | | 124.0 | | 126.0 | | 123.9 |

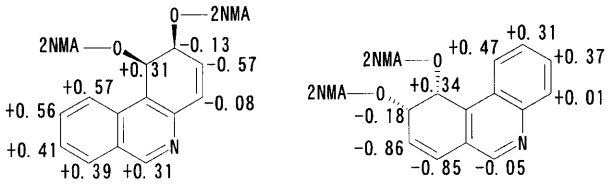
compound 12 were determined to be *R* and *S*, respectively, and those of C-10 and C-9 in compound 13 were also *R* and *S*, respectively, as shown in Fig. 3.

## Discussion

The cells of *E. coli* carrying the toluene dioxygenase (*todC1C2BA*) and biphenyl dioxygenase (*bphA1A2A3A4*) genes seem to have similar substrate ranges judging from the results shown in Table 2. These *E. coli* transformants were able to use as the substrates monocyclic aromatic hydrocarbons with one-larger substituents such as *n*-butyl benzene, *t*-butyl benzene, biphenyl, and diphenylmethane, while these transformants were not able to convert the tricyclic fused aromatic hydrocarbons and heterocycles shown in Table 2, except for the case of anthracene. However, the cells of *E. coli* carrying the hybrid dioxygenase (*todC1::bphA2A3A4*) genes with the subunit substitution between the toluene and biphenyl dioxygenases were able to convert the tricyclic fused aromatic compounds with a fluorene skeleton, i.e., fluorene, dibenzofuran, and dibenzothiophene. This result demonstrates the feasibility of extending the substrate specificity of aromatic compounds in an arene dioxygenase enzyme through subunit substitution. Similar results are observed in the bioconversion of trichloroethylene.[29]

The phenanthrene dioxygenase (*phdABCD*) genes are likely to have the characteristics in contrast to those of the above *Pseudomonas* arene dioxygenase genes as shown in Table 2. Especially it is notable that the bacterial cells carrying the *phdABCD* genes were able to efficiently convert the substrates with a phenanthrene skeleton, such as phenanthrene and phenanthridine. *E. coli* as well as *S. lividans* carrying the genes converted phenanthridine into the three oxygenated products, i.e., *cis*-1,2-dihydroxy-1,2-dihydrophenanthridine (12), *cis*-9,10-dihydroxy-9,10-dihydrophenanthridine (13), and 10-hydroxyphenanthridine (14). In Fig. 1, 10-hydroxyphenanthridine appeared behind the occurrence the two *cis*-dios. It is considered that *cis*-9,10-dihydroxy-9,10-dihydrophenanthridine was converted to the monohydroxylated compound non-enzymatically, due to the structural instability of the *cis*-diol. In Fig. 2, several further monohydroxylated products in a benzene ring, which are compounds 1, 4, 6, 7, and 9, were obtained with the bacterial cells carrying the *phdABCD* or *todC1::bphA2A3A4* genes. Similarly, we speculate that the *cis*-diols are generated with arene dioxygenase in the beginning, and then converted to these monohydroxylated products non-enzymatically, due to the structural instability of the *cis*-diols.

In this study, we have shown that the *S. lividans* transformant that expresses the phenanthrene dioxygenase genes is able to convert the various tricyclic




*cis*-1,2-Dihydroxy-1,2-dihydrophenanthridine (12)

*cis*-9,10-Dihydroxy-9,10-dihydrophenanthridine (13)

**Fig. 3.** NMR Data of the NMA-esterified Derivatives from Phenanthridine.
Numbers are $\Delta(\delta S\text{-}\delta R)$ values assigned.

fused aromatic compounds to their oxygenized forms, which seems to be more feasible for further degradation reactions. Therefore, this recombinant strain may function as a trigger for biodegradation by microbial consortia in the soil environment.

In this study, we have also displayed several novel compounds, i.e., 4-hydroxyfluorene (1) and 3-hydroxy-9,10-dihydrophenanthrene (4) in addition to the three phenanthridine derivatives (12,13,14), which were produced through the bioconversion with the recombinant cells carrying the arene dioxygenase genes. Many of the products obtained in this study (Fig. 2) seem to be difficult to synthesize with the methods of organic chemistry. It would be possible to complement the methods of combinatorial chemistry through extending the applicable category of the biotechnological methods shown in this paper. In the near future, we would like to aim at the establishment of the biological technologies that we call "BioCombiChem" (Biology-based Combinatorial Chemistry).

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