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Biocatalytic synthesis of monocyclic arene-dihydrodiols and -diols by *Escherichia coli* cells expressing hybrid toluene/biphenyl dioxygenase and dihydrodiol dehydrogenase genes

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Abstract

The hybrid toluene/biphenyl dioxygenase, which is encoded by the *todC1* gene of *Pseudomonas putida* F1 and the *bphA2A3A4* genes of *Pseudomonas pseudoalcaligenes* KF707, has substrate ranges wider than toluene dioxygenase endoced by the *todC1C2BA* genes of *P. putida* F1. We carried out growing cell reactions by *Escherichia coli* expressing the *todC1-bphA2A3A4* genes for the comprehensive production of monocyclic arene-dihydrodiols. As a result, we successfully biotranformed acetophenone-related compounds (acetophenone, propiophenone, and butyrophenone) to the corresponding *cis*-dihydrodiols. Furthermore, we performed the bioconversion experiments by *E. coli* cells expressing the *bphB* (dihydrodiol dehydrogenase) gene in addition to *todC1-bphA2A3A4* to produce a series of monocyclic arene-diols. Consequently, toluene, benzene, stylene, *p*-xylene, acetophenone, propiophenone, butyrophenone, and trifluoroacetophenone were converted to the corresponding vicinal diols. The antioxidative activity of these generated diol compounds was markedly higher than that of the substrate used.

Keywords: Hybrid toluene/biphenyl dioxygemase; Dihydrodiol dehydrogenase; Monocyclic arenes; Antioxidant; Biotransformation; *Escherichia coli*

1. Introduction

Toluene dioxygenase that is encoded by the *todC1C2BA* genes from *Pseudomonas putida* F1 [1] has been used to generate cyclohexadiene-*cis*-diols (*cis*-dihyrodiols) from many structurally diverse monocyclic aromatic compounds [2]. Biphenyl dioxygenase, which is composed of BphA1, BphA2 [large (α) and small (β) subunits of an iron–sulfur protein, respectively], BphA3 (ferredoxin), and BphA4 (ferredoxin reductase), from *Pseudomonas pseudoalcaligenes* KF707 [3], seems to have substrate ranges similar to those of toluene dioxygenase, but to have substrate preference for monocyclic aromatic compounds with one-larger substituents [4,5]. The gene (*todC1*) coding for the large subunit of iron–sulfur protein of toluene dioxygenase was replaced to *bphA1* that was followed by *bphA2A3A4* of strain KF707, which was inserted into the *Escherichia coli* vector pUC118, to create plasmid pKF6256 [4]. The function of this hybrid toluene/biphenyl dioxygenase (TodC1BphA2A3A4) is shown in Fig. 1. *E. coli* cells possessing pKF6256 were shown to have substrate ranges wider than those expressing the *todC1C2BA* genes of *P. putida* F1 and different from those expressing the *bphA1A2A3A4* genes of *P. pseudoalcaligenes* KF707, e.g., *E. coli* (pKF6256) was able to biotransform dibenzofuran and dibenzothiophnene that were not the substrates for TodC1C2BA or BphA1A2A3A4 [4]. It was also reported that *E. coli* cells expressing *todC1-bphA2A3A4* degraded

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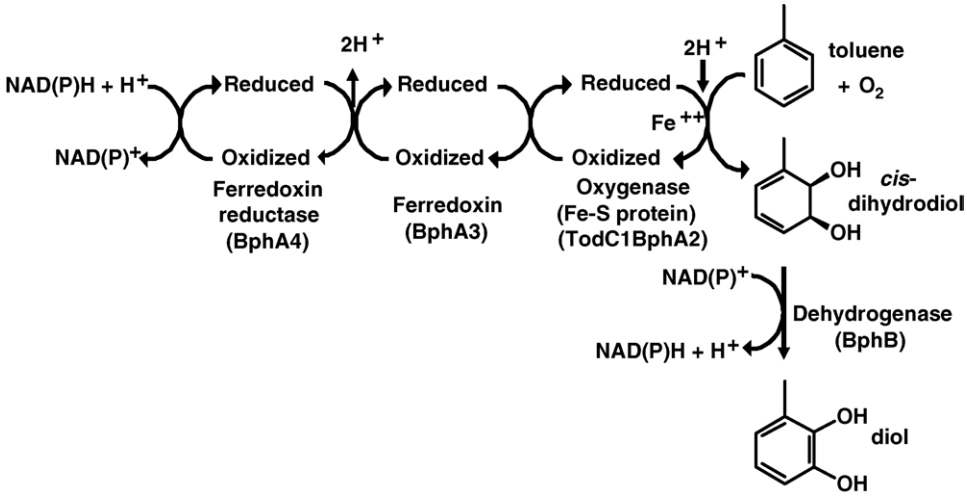

Fig. 1. Catabolic pathway from toluene to diol (3-methyl-benzene-1,2-diol) via *cis*-dihydrodiol (3-methyl-cyclohexa-3,5-diene-1,2-diol).

trichloroethylene much faster than *E. coli* cells expressing *todC1C2BA* or *bphA1A2A3A4* [6]. Maeda et al. [7] showed that the iron–sulfur proteins, TodC1BphA2, and BphA1A2, were present as heterohexamers, whereas the iron–sulfur protein of TodC1C2 was present as a heterotetramer. These results suggest that it may be feasible to synthesize industrially interesting tough-to-make organic compounds derived from a variety of monocyclic aromatic compounds by *E. coli* cells expressing the *todC1-bphA2A3A4* genes.

Recently, we have developed comprehensive and simple biocoversion procedures by growing cell reactions using *E. coli* that expressed sequential biphenyl metabolic genes [living cells-based combinatorial chemistry (CellCombiChem)] [8,9]. In this report, we show CellCombiChem using *E. coli* expressing the *todC1-bphA2A3A4* genes for the production of the *cis*-dihydrodiols from various monocyclic aromatic compounds. We also show CellCombiChem using *E. coli* expressing the subsequent *bphB* gene encoding dihydrodiol dehydrogenase (Fig. 1) in addition to the above hybrid genes for the production of the vicinal diols from various monocyclic aromatic compounds. Such monocyclic derivatives of *cis*-dihydrodiols and vicinal diols are attractive as starting materials for the chemical synthesis of pharmaceuticals, agrochemicals and other industrially useful compounds. Our previous studies have shown that compounds including a monocyclic arene-vicinal diol (catechol) in the molecular structures possess potent antioxidative activities [8,10]. Thus, the antioxidative activity of the diol compounds generated in the present study is also examined.

2. Experimental

2.1. Plasmids and bacterial strains

The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pKF6256 carrying the *todC1-bphA2A3A4* genes for their expression in *E. coli* vector pUC118 has previously been described [4].

2.2. Construction of the plasmid

The 1.1 kb *Cla*I–*Hin*dIII region of plasmid pJHF101 was deleted after the digestion with *Cla*I/*Hin*dIII to disrupt *bphC*, and religated after the treatment with KOD polymerase (Toyobo) to create plasmid pUC6256B for the expression of *todC1-bphA2A3A4* as well as *bphB*.

2.3. Growing cell reactions

E. coli JM101 harboring pKF6256 or pUC6256B was grown in an LB medium [11] containing Ampicillin (Ap; 150 μg/ml) at 30 °C with reciprocal shaking for 7–8 h until the absorbance at OD 600 nm had reached approximately 1. Eight milliliters of this culture was inoculated into 100 ml of an M9 medium [11] containing 150 μg/ml of Ap, 0.4%

Table 1
Bacterial strains and plasmids used in this study

Strains or plasmid	Relevant characteristic(s)	References
Strains		
Escherichia coli JM109	Host strain for DNA manipulation	[11]
Escherichia coli JM101	Host strain for bioconversion	[11]
Plasmids[a]		
pKF6256	Ap^r, plasmid carrying the *todC1-bphA2A3A4*	[4]
pJHF101	Ap^r, plasmid carrying the *todC1-bphA2A3A4BC*	[6]
pUC6256B	Ap^r, plasmid carrying the *todC1-bphA2A3A4B*	This study

[a] Plasmids pKF6256 and pUC6256B are respectively available from Kyushu University (Kensuke Furukawa; kfurukaw@agr.kyushu-u.ac.jp) and Marine Biotechnology Institute (Norihiko Misawa; norihiko.misawa@mbio.jp).

(w/v) glucose, 1mM (final concentration) of isopropyl β-D-thiogalactopyranoside (IPTG), and 10 mg of each substrate in a Sakaguchi flask, and co-cultivated at 30 °C with reciprocal shaking for 2 days.

The substrates used in this study were purchased from Sigma–Aldrich Co. or Maybridge Chemical Co. The respective substrates were dissolved in small volume of dimethyl sulfoxide (DMSO) and added to the culture.

2.4. Extraction and HPLC analysis of the converted products

To extract the converted products as well as the substrates, 100 ml of methanol (MeOH) was added to the co-culture, and mixed for 30 min. After centrifuging to remove the cells, the liquid phase was analyzed by high-pressure liquid chromatography (HPLC) or used for further purification of the converted products. The liquid phase (80 μl) was applied to HPLC in an XTerra C_{18} column (4.6 mm × 150 mm, Waters) with a photodiode array detector (model L-7455, Hitachi). Development was at a flow rate of 1 ml/min with solvent A [5% acetonitrile (CH_3CN) and 20 mM phosphoric acid] for 3 min, then by a linear gradient from solvent A to solvent B (95% CH_3CN and 20 mM phosphoric acid) for 15 min, and finally with solvent B for 10 min, the maximum absorbance being monitored in the range of 200–500 nm.

2.5. Purification and identification of the converted products

The liquid phase (2000 ml), which had been obtained by the procedure just described, was concentrated in vacuo and extracted with ethyl acetate (EtOAc; 500 ml × 2). The resulting organic layer was concentrated in vacuo and analyzed by thin-layer chromatography (TLC) on silica gel (E. Merck 60F-254 0.25-mm silica gel plates). The formed products were purified by column chromatography on Silica Gel 60 (20 mm × 250 mm, Merck). Their structures were analyzed by mass spectral data [MS (EI) and HRMS (EI); Jeol DX505W], nuclear magnetic resonance (NMR) spectral data (400 MHz, Bruker AMX400), and $[\alpha]_D$ data (Horiba SEPA-300).

*2.5.1. 3-Methyl-cyclohexa-3,5-diene-1,2-diol (**1**: product converted from toluene by TodC1bphA2A3A4)*

The crude EtOAc extract (74.9 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 60:1) to yield 9.0 mg of **1** (yield 6.6%).

*2.5.2. 3,5-Cyclohexadiene-1,2-diol (**2**: product converted from benzene by TodC1bphA2A3A4)*

The crude EtOAc extract (52.1 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 20:1) to yield 7.1 mg of **2** (yield 4.9%).

*2.5.3. 3-Vinyl-cyclohexa-3,5-diene-1,2-diol (**3**: product converted from styrene by TodC1bphA2A3A4)*

The crude EtOAc extract (91.8 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 100: 1) to yield 33.1 mg of **3** (yield 24.9%).

*2.5.4. 2,5-Dimethyl-phenol (**4**: product converted from p-xylene by TodC1bphA2A3A4)*

The crude EtOAc extract (66.4 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 100:1) to yield 24.9 mg of **4** (yield 21.6%).

4: HRMS (EI) calcd. for $C_8H_{10}O$ (M^+), 122.0732; found 122.0729. ^{1}H NMR ($CDCl_3$) δ: 2.17 (s), 2.23 (s), 6.55 (s), 6.63 (d, J = 7.4 Hz), 6.96 (d, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 15.3 (C-7), 20.9 (C-8), 115.7 (C-3), 120.5 (C-1), 121.4 (C-5), 130.7 (C-6), 137.0 (C-4), 153.5 (C-2).

*2.5.5. 1-(5,6-Dihydroxy-cyclohexa-1,3-dienyl)-ethanone (**5**) and 1-(3-hydroxy-phenyl)-ethanone (**6**) (products converted from acetophenone by TodC1bphA2A3A4)*

The crude EtOAc extract (64.7 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 20:1) to yield 13.9 mg of **5** (yield 10.8%) and 6.9 mg of **6** (yield 6.1%).

6: HRMS (EI) calcd. for $C_8H_8O_2$ (M^+), 136.0524; found 136.0521. ^{1}H NMR ($CDCl_3$) δ: 2.53 (s), 7.02 (dd, J = 2.3, 7.9 Hz), 7.27 (dd, J = 7.9, 8.6 Hz), 7.43 (d, J = 2.3 Hz), 7.45 (d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 26.7 (C-8), 114.6 (C-2), 120.6 (C-4), 121.1 (C-6), 129.9 (C-5), 138.5 (C-1), 156.2 (C-3), 198.7 (C-7).

*2.5.6. 1-(5,6-Dihydroxy-cyclohexa-1,3-dienyl)-propan-1-one (**7**) and 1-(3-hydroxy-phenyl)-propan-1-one (**8**) (products converted from propiophenone by TodC1bphA2A3A4)*

The crude EtOAc extract (68.2 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 20:1) to yield 26.1 mg of **7** (yield 20.8%) and 4.1 mg of **8** (yield 3.7%).

7: HRMS (EI) calcd. for $C_9H_{12}O_3$ (M^+), 168.0787; found 168.0789. $[\alpha]_D^{25}$ +83.7 (c 1.00, MeOH). ^{1}H NMR ($CDCl_3$) δ: 1.02 (t, J = 7.7 Hz), 2.65 (q, J = 7.7 Hz), 4.34 (m), 4.56 (d, J = 6.2 Hz), 6.03 (m), 6.18 (dd, J = 1.7, 9.2 Hz), 6.88 (d, J = 5.9 Hz). ^{13}C NMR ($CDCl_3$) δ: 8.3 (C-9), 30.4 (C-8), 64.4 (C-6), 69.2 (C-5), 122.7 (C-3), 133.3 (C-2), 135.7 (C-1), 139.1 (C-4), 202.3 (C-7).

8: HRMS (EI) calcd for $C_9H_{10}O_2$ (M^+), 150.0681; found 150.0681. ^{1}H NMR ($CDCl_3$) δ: 1.15 (t, J = 6.8 Hz), 2.91 (q, J = 6.8 Hz), 6.98 (dd, J = 2.3, 7.5 Hz), 7.26 (dd, J = 7.5, 8.6 Hz), 7.38 (d, J = 2.3 Hz), 7.45 (d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 8.2 (C-9), 31.9 (C-8), 114.4 (C-2), 120.1 (C-6), 120.6 (C-4), 129.8 (C-5), 138.4 (C-1), 156.0 (C-3), 200.5 (C-7).

*2.5.7. 1-(5,6-Dihydroxy-cyclohexa-1,3-dienyl)-butan-1-one (**9**: product converted from butyrophenone by TodC1bphA2A3A4)*

The crude EtOAc extract (100.4 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 30:1) to yield 28.0 mg of **9** (yield 22.8%).

9: HRMS (EI) calcd. for $C_{10}H_{14}O_3$ (M^+), 182.0943; found 182.0940. $[\alpha]_D^{25}$ +98.6 (*c* 1.69, MeOH). ^{1}H NMR ($CDCl_3$) δ: 0.85 (t, J = 7.2 Hz), 1.56 (tq, J = 7.2, 7.9 Hz), 2.58 (7, J = 7.9 Hz), 4.33 (m), 4.56 (d, J = 6.6 Hz), 6.03 (m), 6.17 (dd, J = 1.8, 9.3 Hz), 6.87 (d, J = 5.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 13.8 (C-10), 17.9 (C-9), 39.1 (C-8), 64.2 (C-6), 69.3 (C-5), 122.7 (C-3), 133.5 (C-2), 135.9 (C-1), 139.2 (C-4), 201.9 (C-7).

*2.5.8. 1-(2,3-Dihydroxy-phenyl)-2,2,2-trifluoro-ethane (**10**: product converted from 2,2,2-trifluoroacetophenone by TodC1bphA2A3A4)*

The crude EtOAc extract (65.1 mg) was subjected to column chromatography (hexane–EtOAc = 2:1) to yield 2.2 mg of **10** (yield 1.9%).

10: HRMS (EI) calcd. for $C_8H_5F_3O_3$ (M^+), 197.0356; found 197.0351. ^{1}H NMR ($CDCl_3$) δ: 6.64 (d, J = 7.5 Hz), 6.73 (dd, J = 7.5, 8.2 Hz), 6.86 (d, J = 8.2 Hz). ^{13}C NMR ($CDCl_3$) δ: 113.5 (C-8), 116.2 (C-4), 118.5 (C-1), 120.4 (C-5), 120.6 (C-6), 143.8 (C-2), 145.2 (C-3), 195.5 (C-7).

*2.5.9. 3-Methyl-benzene-1,2-diol (**11**) and 2,3-dihydroxy-benzoic acid (**12**) (products converted from toluene by TodC1bphA2A3A4 and BphB)*

The crude EtOAc extract (118.6 mg) was subjected to column chromatography (hexane–EtOAc = 8:1 → CH_2Cl_2–MeOH = 10: 1 (stepwise)) to yield 63.4 mg of **11** (yield 47.0%) and 5.4 mg of **12** (yield 3.2%).

*2.5.10. Cathecol (**13**: product converted from benzene by TodC1bphA2A3A4 and BphB)*

The crude EtOAc extract (58.5 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 20:1) to yield 29.1 mg of **13** (yield 20.6%).

*2.5.11. 3-Vinyl-benzene-1,2-diol (**14**: product converted from stylene by TodC1bphA2A3A4 and BphB)*

The crude EtOAc extract (100.3 mg) was subjected to column chromatography (hexane–EtOAc = 4:1) to yield 49.8 mg of **14** (yield 38.1%).

*2.5.12. 3,6 Dimethyl-benzene-1,2-diol (**15**: product converted from p-xylene by TodC1bphA2A3A4 and BphB)*

The crude EtOAc extract (81.3 mg) was subjected to column chromatography (hexane–EtOAc = 8:1) to yield 8.7 mg of **15** (yield 6.7%).

15: HRMS (EI) calcd. for $C_8H_{10}O_2$ (M^+), 138.0681; found 138.0677. ^{1}H NMR ($CDCl_3$) δ: 2.16 (s), 5.05 (brs), 6.55 (s). ^{13}C NMR ($CDCl_3$) δ: 15.4 (C-7, 8), 121.6 (C-1, 4), 121.8 (C-5, 6), 141.6 (C-2, 3).

*2.5.13. 1-(2,3-Dihydroxy-phenyl)-ethanone (**16**: product converted from acetophenone by TodC1bphA2A3A4 and BphB)*

The crude EtOAc extract (189.3 mg) was subjected to column chromatography (hexane–EtOAc = 20:1) to yield 49.8 mg of **16** (yield 39.2%).

16: HRMS (EI) calcd. for $C_8H_8O_3$ (M^+), 152.0473; found 152.0472. ^{1}H NMR ($CDCl_3$) δ: 2.55 (s), 5.85 (s), 6.74 (dd, J = 8.0, 8.0 Hz), 7.15 (dd, J = 1.4, 8.0 Hz), 7.20 (dd, J = 1.4, 8.0 Hz), 12.39 (s). ^{13}C NMR ($CDCl_3$) δ: 26.6.5 (C-8), 118.9 (C-1), 118.9 (C-5), 120.4 (C-4), 121.4 (C-6), 145.3 (C-3), 149.5 (C-2), 205.0 (C-7).

*2.5.14. 1-(2,3-Dihydroxy-phenyl)-propan-1-one (**17**: product converted from propiophenone by TodC1bphA2A3A4 and BphB)*

The crude EtOAc extract (136.9 mg) was subjected to column chromatography (hexane–EtOAc = 30:1) to yield 84.0 mg of **17** (yield 67.7%).

17: HRMS (EI) calcd. for $C_9H_{10}O_3$ (M^+), 166.0630; found 166.0633. ^{1}H NMR ($CDCl_3$) δ: 1.16 (t, J = 7.3 Hz), 2.95 (q, J = 7.3 Hz), 5.82 (s), 6.73 (dd, J = 7.8, 8.1 Hz), 7.04 (dd, J = 1.3, 7.8 Hz), 7.22 (dd, J = 1.3, 8.1 Hz), 12.50 (s). ^{13}C NMR ($CDCl_3$) δ: 8.1 (C-9), 31.6 (C-8), 118.8 (C-5), 119.0 (C-1), 120.0 (C-4), 120.5 (C-6), 145.5 (C-3), 149.5 (C-2), 207.6 (C-7).

*2.5.15. 1-(2,3-Dihydroxy-phenyl)-butan-1-one (**18**: product converted from butyrophenone by TodC1bphA2A3A4 and BphB)*

The crude EtOAc extract (69.0 mg) was subjected to column chromatography (hexane–EtOAc = 30:1) to yield 29.3 mg of **18** (yield 24.1%).

18: HRMS (EI) calcd. for $C_9H_{10}O_3$ (M^+), 180.0787; found 180.0786. ^{1}H NMR ($CDCl_3$) δ: 0.95 (t, J = 7.4 Hz), 1.70 (tq, J = 7.4, 7.4 Hz), 2.89 (t, J = 7.4 Hz), 6.74 (dd, J = 7.9, 8.1 Hz), 7.04 (dd, J = 1.3, 7.9 Hz), 7.20 (dd, J = 1.3, 8.1 Hz), 12.55 (s). ^{13}C NMR ($CDCl_3$) δ: 13.7 (C-10), 17.8 (C-9), 40.3 (C-8), 118.8 (C-5), 119.1 (C-1), 120.1 (C-4), 120.7 (C-6), 145.4(C-3), 149.6 (C-2), 207.3 (C-7).

*2.5.16. 1-(2,3-Dihydroxy-phenyl)-2,2,2-trifluoro-ethane (**10**: product converted from 2,2,2-trifluoroacetophenone by TodC1bphA2A3A4 and BphB)*

The crude EtOAc extract (82.3 mg) was subjected to column chromatography (hexane–EtOAc = 2:1) to yield 5.2 mg of **10** (yield 4.4%).

2.6. In vitro inhibitory activity against lipid peroxidation

A rat brain homogenate was prepared according to the method of Kubo et al. [12] with some modifications, as described [10].

3. Results

3.1. Biotransformation by E. coli expressing todC1-bphA2A3A4

Respective compounds were biotransformed through the co-cultivation with the cells of *E. coli* JM101 carrying plas-

mid pKF6256, which expressed the hybrid toluene/biphenyl dioxygenase genes (*todC1-bphA2A3A4*). The converted products were analyzed by chromatographic and spectroscopic methods.

3.1.1. Toluene

The product converted from toluene (**1**) was identified to be *cis*-(1*S*,2*R*)-3-methyl-cyclohexa-3,5-diene-1,2-diol by direct comparison with an authentic sample [purchased from Chemical Synthesis Services (CSS)] by their TLC, ^{1}H NMR spectra, and $[\alpha]_D$ value.

Considering the characteristics of enzymatic reactions, the stereochemistry of all the prepared 1,2-dihydrodiols shown below can be conformed to be identical to that of **1**.

3.1.2. Benzene

The product converted from benzene (**2**) was identified to be *cis*-(1*S*,2*R*)-cyclohexadiene-1,2-diol by comparison with the previously reported spectral data (MS and NMR) [13].

3.1.3. Styrene

The product converted from styrene (**3**) was identified to be *cis*-(1*S*,2*R*)-3-vinyl-cyclohexa-3,5-diene-1,2-diol by comparison with the previously reported spectral data (MS and NMR) [14].

3.1.4. p-Xylene

The molecular formula of the product converted from *p*-xylene (**4**) was determined to be $C_8H_{10}O$ by HRMS (EI). The analyses of ^{1}H and ^{13}C NMR spectra revealed that one phenolic OH function was attached at benzene ring. Thus, **4** was identified to be 2,5-dimethyl-phenol.

3.1.5. Acetophenone

Acetophenone was converted into two products (**5** and **6**). The product **5** was identified to be 1-((5*S*,6*R*)-5,6-dihydroxy-cyclohexa-1,3-dienyl)-ethanone [15] by comparison with its previously reported spectral data (MS and NMR).

The molecular formula of **6** was determined to be $C_8H_8O_2$ by HRMS (EI), and ^{1}H and ^{13}C NMR spectra. Analysis of DQF COSY, and HMQC spectra of **6** proposed that one phenolic OH function was attached to the benzene ring. The position of the phenolic OH proved to be C-3 by the observation of vicinal sp^2 ^{1}H–^{1}H network from H-4 (δ_H 7.02) to H-6 (δ_H 7.45) and meta sp^2 ^{1}H–^{1}H coupling between H-2 (δ_H 7.43) and H-4. Thus, **6** was identified to be 1-(3-hydroxy-phenyl)-ethanone.

3.1.6. Propiophenone

Propiophenone was converted into two products (**7** and **8**). The molecular formula of **7** was determined to be $C_9H_{12}O_3$ by HRMS (EI). Analyses of DQF COSY, and HMQC spectra of **7** proposed that **7** was a dihydrodiol derivative of propiophenone. The regiochemical assignment of the 2,3-dihydrodiol was conformed by the observation of long range ^{1}H–^{13}C coupling from H-2 (δ_H 4.59) to C-7 (δ_C 199.9). Thus, **7** was identified to be 1-((5*S*,6*R*)-5,6-dihydroxy-cyclohexa-1,3-dienyl)-propan-1-one. This was a novel compound.

The molecular formula of **8** was determined to be $C_9H_{10}O_2$ by HRMS (EI). Analysis of DQF COSY, and HMQC spectra of **8** proposed that one phenolic OH function was attached to the benzene ring of propiophenone. The position of the phenolic OH proved to be C-3 by the observation of vicinal sp^2 ^{1}H–^{1}H network from H-4 (δ_H 6.98) to H-6 (δ_H 7.45) and meta sp^2 ^{1}H–^{1}H coupling between H-2 (δ_H 7.38) and H-4. Thus, **8** was identified to be 1-(3-hydroxy-phenyl)-propan-1-one.

3.1.7. Butyrophenone

The molecular formula of the product from butyrophenone (**9**) was determined to be $C_{10}H_{14}O_3$ by HRMS (EI). Analyses of DQF COSY, and HMQC spectra of **9** proposed that **9** was a dihydrodiol derivative of butyrophenone. The regiochemical assignment of the 2,3-dihydrodiol was conformed by the observation of long range ^{1}H–^{13}C coupling from H-6 (δ_H 6.87) to C-7 (δ_C 201.9) and vicinal sp^2 ^{1}H–^{1}H spin network from H-4 (δ_H 6.17) to H-6. Thus, **9** was identified to be 1-((5*S*,6*R*)-5,6-dihydroxy-cyclohexa-1,3-dienyl)-butan-1-one. This was a novel compound.

3.1.8. Trifluoroacetophenone

The molecular formula of the product from trifluoroacetophenone (**10**) was determined to be $C_8H_5F_3O_3$ by HRMS (EI). Analyses of DQF COSY, and HMQC spectra of **10** suggested the attachment of two phenolic OH functions at benzene ring. The positions of the phenolic OHs were determined to be C-2 and C-3, since vicinal sp^2 ^{1}H–^{1}H spin network from H-4 (δ_H 6.86) to H-6 (δ_H 6.64) was observed. These findings enabled **10** to be 1-(2,3-dihydroxy-phenyl)-2,2,2-trifluoro-ethane. This was a novel compound.

3.2. Biotransformation by E. coli expressing todC1-bphA2A3A4 and bphB

Respective compounds were biotranformed through the co-cultivation with the cells of *E. coli* JM101 carrying plasmid pUC6256B, which expressed the dihydrodiol dehydrogenase (desaturase) gene (*bphB*) in addition to the hybrid toluene/biphenyl dioxygenase genes (*todC1-bphA2A3A4*). The converted products were analyzed by chromatographic and spectroscopic methods.

3.2.1. Toluene

The products that were converted from toluene were identified to be 3-methyl-benzene-1,2-diol (**11**) [16] and 2,3-dihydroxy-benzoic acid (**12**) [17] by comparison with the previously reported spectral data (MS and NMR).

3.2.2. Benzene

The product converted from benzene was identified to be catechol (**14**) by the direct comparison with an authentic sample [purchased from Aldrich] by TLC and HPLC.

3.2.3. Styrene

The product converted from styrene was identified to be 3-vinyl-benzene-1,2-diol (**14**) [18] by comparison with the previously reported spectral data (MS and NMR).

3.2.4. p-Xylene

The molecular formula of the product converted from *p*-xylene (**15**) was determined to be $C_8H_{10}O_2$ by HRMS (EI). ^{1}H NMR spectra of **15** showed only 4H signals meaning that **15** was a symmetrical compound. Analyses of DQF COSY, and HMQC spectra of **15** suggested the attachment of two phenolic OH functions at benzene ring. The ^{1}H–^{13}C long range couplings observed from H-7 (8) (δ_H 2.16) to C-1 (4)(δ_C 121.6), C-2 (3) (δ_C 141.6), and C-6 (5) (δ_C 121.8) in HMBC experiment confirmed the positions of the phenolic OHs to be C-2 and C-3. These findings enabled **15** to be 3,6 dimethyl-benzene-1,2-diol.

3.2.5. Acetophenone

The molecular formula of the product from acetophenone (**16**) was determined to be $C_8H_8O_3$ by HRMS (EI). Analyses of DQF COSY, and HMQC spectra of **16** suggested the attachment of two phenolic OH functions at benzene ring. The positions of the phenolic OHs were determined to be C-2 and C-3, since vicinal sp^2 ^{1}H–^{1}H spin network from H-4 (δ_H 7.15) to H-6 (δ_H 7.20) was observed. These findings enabled **16** to be 1-(2,3-dihydroxy-phenyl)-ethanone.

3.2.6. Propiophenone

The molecular formula of the product from propiophenone (**17**) was determined to be $C_9H_{10}O_3$ by HRMS (EI). Analyses of DQF COSY, and HMQC spectra of **17** suggested the attachment of two phenolic OH functions at benzene ring. The positions of the phenolic OHs were determined to be C-2 and C-3, since vicinal sp^2 ^{1}H–^{1}H spin network from H-4 (δ_H 7.04) to H-6 (δ_H 7.22) was observed. These findings enabled **17** to be 1-(2,3-dihydroxy-phenyl)-propan-1-one.

3.2.7. Butyrophenone

The molecular formula of the product from butyrophenone (**18**) was determined to be $C_{10}H_{12}O_3$ by HRMS (EI). Analyses of DQF COSY, and HMQC spectra of **18** suggested the attachment of two phenolic OH functions at benzene ring. The positions of the phenolic OHs were determined to be C-2 and C-3, since vicinal sp^2 ^{1}H–^{1}H spin network from H-4 (δ_H 7.04) to H-6 (δ_H 7.20) was observed. These findings enabled **18** to be 1-(2,3-dihydroxy-phenyl)-butan-1-one.

3.2.8. Trifluoroacetophenone

The product converted from trifluoroacetophenone (**10**) was identified to be 1-(2,3-dihydroxy-phenyl)-2,2,2-trifluoro-ethane by the direct comparison with authentic sample on TLC and HPLC. Three milligrams of **10** was obtained in this experiment.

Table 2
Inhibitory effects of converted products on lipid peroxidation in a rat brain homogenate

Compound	IC_{50} (μM)
Toluene	>1000
11	10
12	>100
Benzene	>100
13	12
Styrene	>100
14	7.3
Xylene	>100
15	4.4
Acetophenone	>100
16	3.3
Propiophenone	>100
17	3.3
Butyrophenone	>100
18	9.0
Trifluoroacetophenone	>100
10	3.0
Catechin (positive control)	20

3.3. Antioxidative activity of the converted products

The converted products by the *todC1-bphA2A3A4* and *bphB* genes contain catecholic OH function in their structures. Therefore, they were expected to possess antioxidative activity. We examined their in vitro inhibitory effects towards lipid peroxidation (Table 2). As a result, all of the products except for **12** showed much stronger antioxidative activity than to the substrates. Judging from the results shown in Table 2, all of the substrates had no antioxidative activity.

4. Discussion

The hybrid toluene/biphenyl dioxygenase enzyme, TodC1BphA2A3A4, was able to biotransform a variety of monocyclic aromatic compounds. Toluene, benzene, styrene, acetophenone, propiophenone, and butyrophenone were converted to their *cis*-dihydridiol forms (Fig. 2), this being the typical reaction mediated by this enzyme, while the monohydroxy products were generated from *p*-xylene, acetophenone, and propiophenone through the *E. coli* cells expressing the hybrid dioxygenase. These substrates initially seem to have been converted to their corresponding dihydrodiols, and then to the more stable monohydroxylated forms in a non-enzymatic manner. It is surprising that in the case of trifluoroacetophenone, not the dihydrodiol but diol forms were obtained as the converted product (Fig. 2). *E. coli* may have endogenous dehydrogenation activity for this substrate, while it is not strong. The same phenomenon has been observed in the bioconversions of flavonoids when using *E. coli* expressing the modified biphenyl dioxygenase genes [*bphA1*(2072)*A2A3A4*] [10].

Dihydrodiol dehydrogenase, BphB, derived from *P. pseudoalcaligenes* KF707 seems to be capable of efficiently

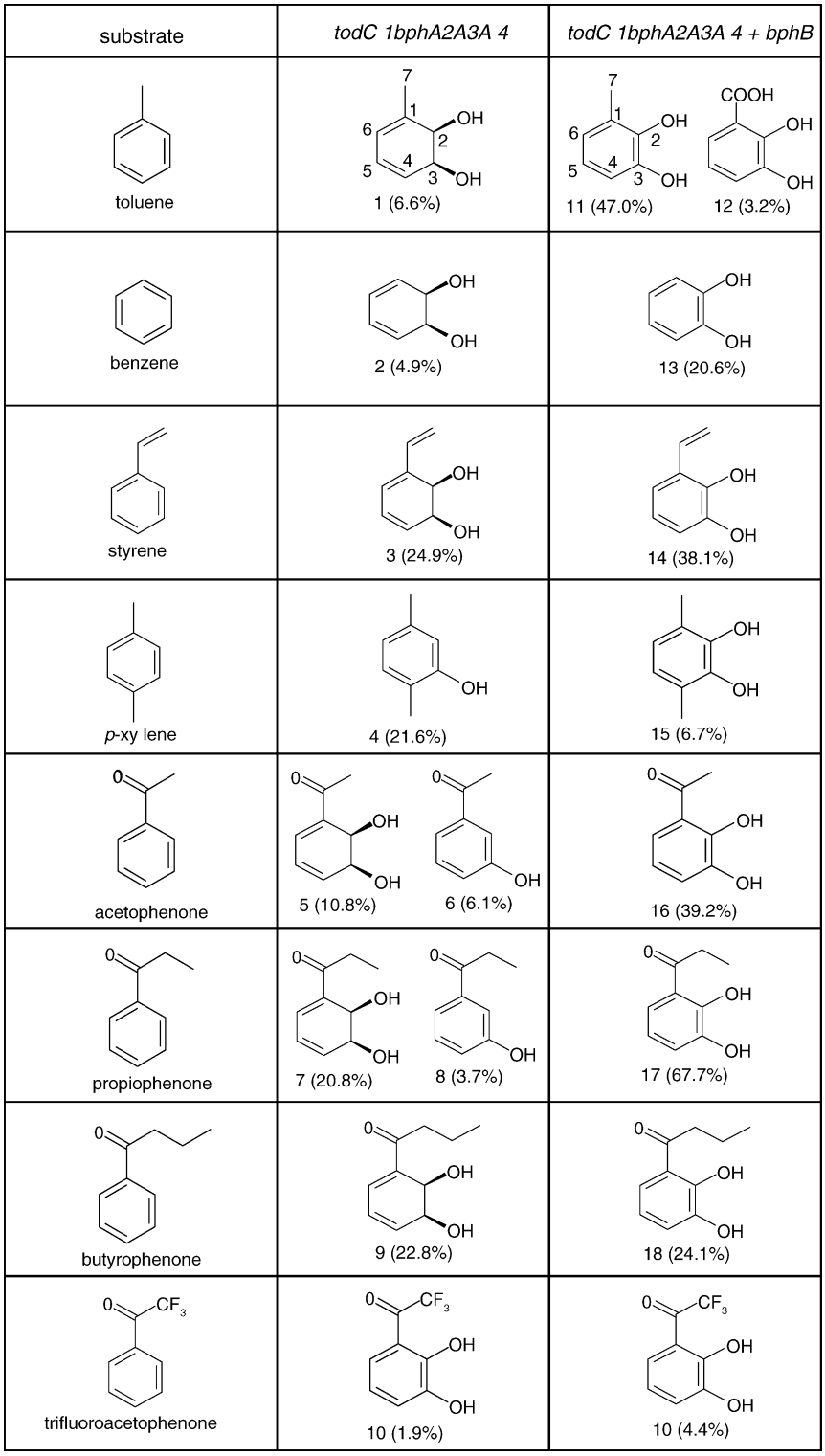

Fig. 2. Bioconversion of various monocyclic aromatic compounds through the living cells of *E. coli* carrying plasmid pKF6256 expressing *todC1-bphA2A3A4* or plasmid pUC6256B expressing *todC1-bphA2A3A4* and *bphB*. Percent value in the parentheses represents the yield of the products purified.

converting the monocyclic arene-dihydrodiol forms generated through the hybrid dioxygenase, to their corresponding monocyclic diol forms, indicating that the native BphB has broad substrate specificity. We have also shown in this study, the comprehensive biocatalytic synthesis of monocyclic arene-diols with the structure of catechol type. In the case of toluene, the generated diol was further oxidized at the allylic methyl position to yield a carboxylic derivative.

The products (catechol compounds) converted by TodC1BphA2A3A4 and BphB showed potent anitioxidative activity on lipid peroxidation test (Table 1). Therefore, they could be useful for the prevention of pathological disturbance such as myocardial and cerebral ischemia [19,20], and atheroscleorosis [21]. This result suggest that our CellCombiChem system is effective for producing biologically active organic chemicals, which are difficult or are not feasible to be synthesized by methods with chemical synthesis.

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