Available online at www.sciencedirect.com

Journal of Molecular Catalysis B: Enzymatic 23 (2003) 9–16

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Enzymatic synthesis of novel antioxidant flavonoids by *Escherichia coli* cells expressing modified metabolic genes involved in biphenyl catabolism

Kazutoshi Shindo [a,*], Yukiko Kagiyama [a], Ryoko Nakamura [a], Akihiro Hara [b], Hiroshi Ikenaga [b], Kensuke Furukawa [c], Norihiko Misawa [b]

[a] *Department of Food and Nutrition, Japan Women's University, 2-8-1, Mejirodai, Bunkyo-ku, Tokyo 112-8681, Japan*
[b] *Marine Biotechnology Institute, Heita, Kamaishi 026-0001, Japan*
[c] *Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan*

Received 7 February 2003; received in revised form 4 March 2003; accepted 4 March 2003

Abstract

The *bpha1(2072)A2A3A4* gene cluster codes for a modified biphenyl dioxygenase holoenzyme with broad substrate specificity. In the previous report, we have shown the biotransformation of flavone, flavanone, 6-hydroxyflavone and 6-hydroxyflavanone by *Streptomyces lividans* cells carrying *bphA1(2072)A2A3A4*. In the present study, we successfully biotransformed chalcone and 7-hydroxyisoflavone, which are categorized with other groups of flavonoids, by using recombinant *Escherichia coli* cells expressing the same genes. We also biotransformed various flavonoids by *E. coli* cells expressing the *bphB* (dihydrodiol dehydrogenase) gene in addition to the *bphA1(2072)A2A3A4* genes. Flavone, flavanone, 6-hydroxyflavone, 6-hydroxyflavanone, chalcone, and 7-hydroxyisoflavone, which were used as substrates, were converted with high efficiency to their corresponding diols, whose structures were determined by HREI-MS, ^{1}H NMR and ^{13}C NMR data. The antioxidative activity of these generated diol compounds was markedly higher than that of the substrates used.

Keywords: Biphenyl dioxygemase; Dihydrodiol dehydrogenase; Flavonoid; Antioxidant; Biotransformation; *Escherichia coli*

---

1. Introduction

The degradation of polychlorinated biphenyls (PCBs), which are serious environmental pollutants, is enzymatically initiated by the action of biphenyl dioxygenase, as has been elucidated for the biphenyl-degrading bacteria, *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia cepacia* LB400 [1,2]. Biphenyl dioxygenase is a multi-component enzyme consisting of BphA1, BphA2 (large and small subunits of iron–sulfur protein, respectively), BphA3 (ferredoxin), and BphA4 (ferredoxin reductase). This enzyme (BphA) is responsible for the conversion of a biphenyl to its dihydrodiol, which is further converted to 2,3-dihydroxybiphenyl by the subsequent dehydrogenation (desaturation) catalyzed by BphB (Fig. 1).

Modified *bphA1* genes have recently been generated by DNA shuffling, using the *bphA1* genes derived from *P. pseudoalcaligenes* KF707 and *B. cepacia* LB400 [3]. One of the shuffled genes, *bphA1(2072)*, has been shown to mediate with broad substrate specificity, when expressed in combination with

---

[*] Corresponding author. Tel.: +81-3-5981-3433; fax: +81-3-5981-3426.
E-mail address: kshindo@fc.jwu.ac.jp (K. Shindo).

1381-1177/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1381-1177(03)00038-9

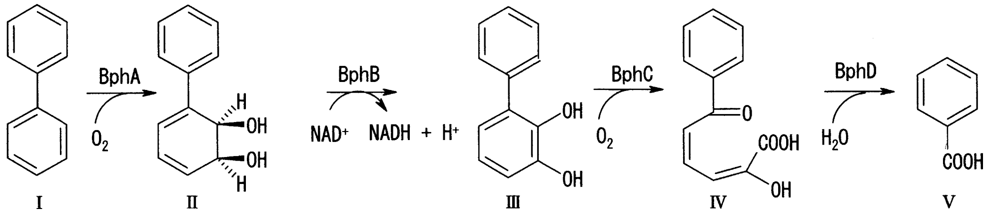

Fig. 1. Biphenyl catabolic pathway for *P. pseudoalcaligenes* KF707 and *B. cepacia* LB400. Key to compounds: I, biphenyl; II, 3-phenylcyclohexa-3,5-diene-1,2-diol (dihydrodiol compound); III, 2,3-dihydroxybiphenyl (3-phenylbenzene-1,2-diol); IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta*-cleavage compound); V, benzoic acid. Key to enzymes: BphA (*BphA1A2A3A4*), biphenyl dioxygenase; BphB, dihydrodiol dehydrogenase (desaturase); BphC, 2,3-dihydroxybiphenyl dioxygenase; BphD, *meta*-cleavage compound hydrolase.

bphA2A3A4 from *P. pseudoalcaligene* [4]. Various molecular species, in which heterocyclic aromatics are linked with phenyl or benzyl groups, could be converted with high efficiency to the corresponding dihydrodiols and/or monohydroxy derivatives by recombinant *Escherichia coli* or *Streptomyces lividans* strains carrying the modified biphenyl dioxygenase genes [*bphA1(2072)A2A3A4*] [4,5]. However, it is not clear whether the dihydrodiols generated would be capable of being used as substrates for BphB to synthesize the corresponding diols.

Flavonoid pigments are universally distributed in higher plants and are the source of the yellow and orange to blue colors in petals, fruits, leaves and roots [6]. Flavonoids have recently attracted considerable attention due to their beneficial effect on health, e.g. their antioxidative activity is considered to be of medical and nutritional importance. Although 6467 flavonoids in nature are listed in *The Handbook of Natural Flavonoids* [7], the biological transformation of these flavonoids has not been well studied. It may be a feasible approach to produce “unnatural” natural flavonoids by enzymatic conversion. This conversion is likely to be mediated by modified biphenyl dioxygenase, since flavonoids also have the molecular structure of a heteroaromatic ring (such as a chromone ring) linked with a phenyl group. In the previous study [8], we successfully transformed flavone, flavanone, 6-hydroxyflavone and 6-hydroxyflavanone by *S. lividans* cells expressing *bphA1(2072)A2A3A4* genes. In this case, the products generated were not the corresponding dihydrodiols but diols, and/or the monohydroxy derivatives, as shown in Fig. 2.

We report in this present paper the biotransformation of chalcone and 7-hydroxyisoflavone by *E. coli* cells expressing the same genes. We further show the efficient bioconversion of flavone, flavanone, 6-hydroxyflavone, 6-hydroxyflavanone, chalcone and 7-hydroxyisoflavone to their corresponding diols by *E. coli* cells expressing the *bphB* gene in addition to *bphA1(2072)A2A3A4*. The antioxidative activity of the generated diol compounds is also examined.

2. Experimental

2.1. Plasmids, bacterial strains, and growth conditions

Plasmid pKF2072 carrying the *bphA1(2072)A2A3-A4* genes for their expression in *E. coli* has previously been described [4]. *E. coli* JM109 [9] was used as a host for plasmid pKF2072 and the other plasmid (pBS2072B), and was cultured in an LB medium or M9 medium [9] at 30 or 37 °C. Ampicillin (Ap, 150 μg/ml) was added when needed.

2.2. Construction of the plasmid

The 1.9 kb *Xba*I-*Bgl*II fragment, which contains *bphA1* (2072), was isolated from plasmid pKF2072. The 3.78 kb *Bgl*II-*Cla*I fragment carrying the *bphA2A3A4* and *bphB* genes of *P. pseudoalcaligenes* KF707 was isolated from plasmid pJHF18 [10]. Both of these fragments were inserted together into the *Xba*I-*Cla*I site of pBluescript II

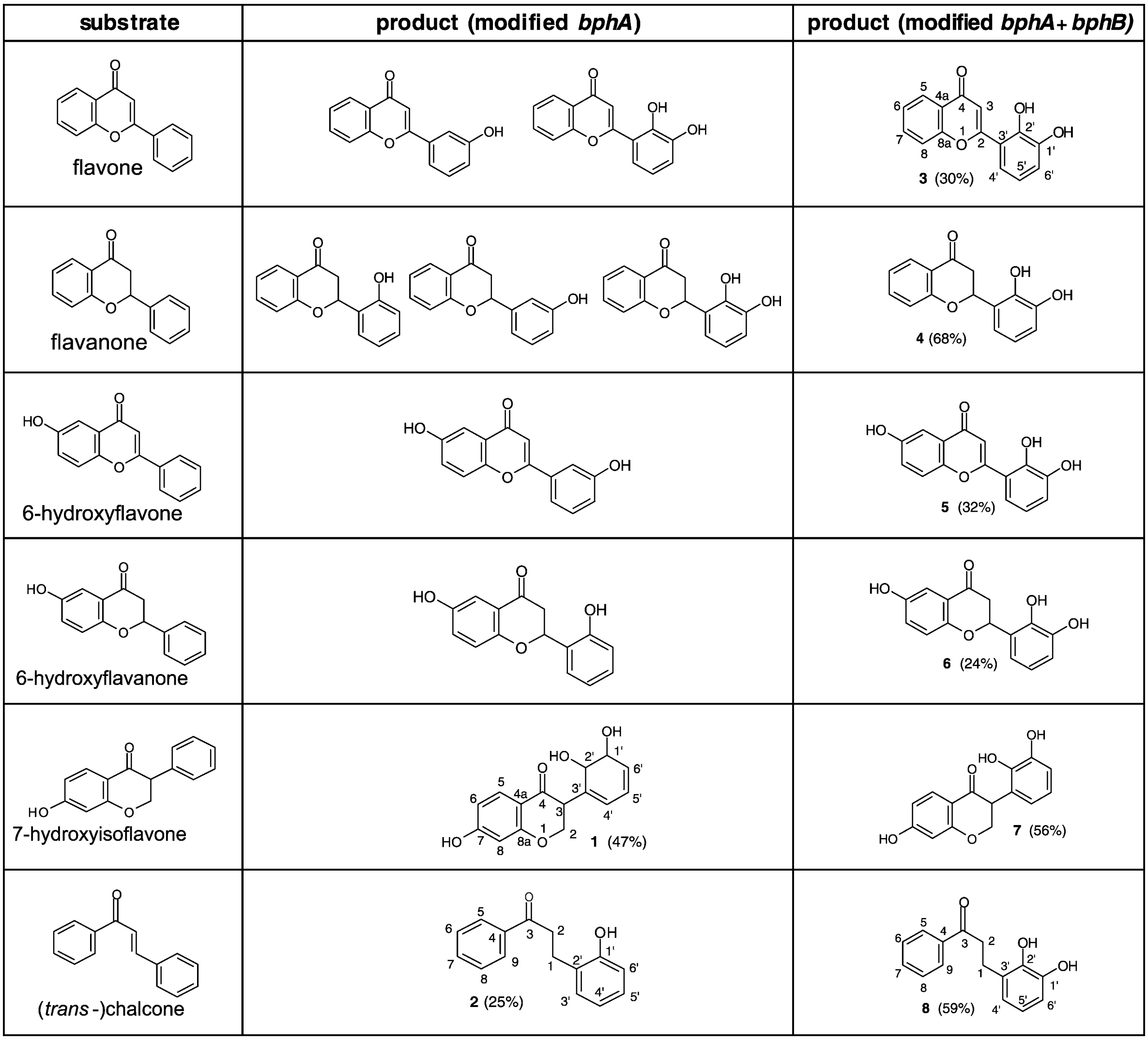

Fig. 2. Bioconversion of various flavonoids by using the cells of *E. coli* carrying modified biphenyl dioxygenase genes [*bphA1(2072)A2A3A4*] and by using the dihydrodiol dehyrogenase gene (*bphB*) in addition to these dioxygenase genes. The percentage values in parentheses represent the yield of each purified product.

SK, creating plasmid pBS2072B. In this plasmid, the *bphA1(2072)*::*bphA2A3A4bphB* genes were inserted in an orientation to give the transcriptional read-through from the *lac* promoter of the vector. The respective DNA sequences of the *bphA1* (2072) gene and the *bphA2A3A4bphB* genes are available from DDBJ/Genbank under accession numbers AB085748 and M83673.

2.3. Conversion experiments

E. coli JM109 harboring pKF2072 or pBS2072B was grown in an LB medium containing Ap at 30 °C while reciprocally shaking at 175 rpm (rotation per minute) for 8 h. Five milliliters of this culture was inoculated into 100 ml of an M9 medium containing 150 μg/ml of Ap, 10 μg/ml of thiamine, and 0.4%

(w/v) glucose in a Sakaguchi flask at 30 °C while reciprocally shaking at 175 rpm for 16–17 h until the absorbance at OD 600 nm had reached approximately 1. One millimolar (final concentration) of isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture, and cultivation was continued for a further 4 h. The cells were collected by centrifugation, washed once with the M9 medium, and then resuspended in 100 ml of a fresh M9 medium containing 150 μg/ml of Ap, 10 μg/ml of thiamine, 0.4% (w/v) glucose, 1 mM (final concentration) of IPTG, and 10 mg of each substrate, before being cultivated in a Sakaguchi flask at 30 °C while reciprocally shaking at 175 rpm for 2 days.

The substrates used in this study were purchased from Aldrich Chemical Co., Sigma Chemical Co. or Maybridge Chemical Co. The respective substrates were dissolved in small volume of 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, a volume of methanol (MeOH) equal to that of the culture medium was added to the co-culture of the transformed cells of *E. coli*, 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 (1400 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) and nuclear magnetic resonance (NMR) spectral data (400 MHz, Bruker AMX400).

2.5.1. 3-(2,3-Dihydroxycyclohexa-1(6),4-dienyl)-7-hydroxychromen-4-one

The compound **1** is a product converted from 7-hydroxyisoflavone by modified bphA. The crude EtOAc extract (55.9 mg) was subjected to column chromatography (dichloromethane (CH_2Cl_2)–EtOAc = 10:1) to yield 47.0 mg of **1**. MS (EI) *m/z* 272 (M^+). HRMS (EI) calcd. for C15H12O5 (M^+), 272.0683; found 272.0685. ^{1}H NMR (DMSO-d_6) δ: 4.19 (1H, m), 4.31 (1H, m), 5.81 (dd, 1H, $J = 2.0, 9.6$ Hz), 5.93 (ddd, 1H, $J = 2.4, 5.7, 9.6$ Hz), 6.42 (d, 1H, $J = 5.3$ Hz), 6.84 (d, 1H, $J = 2.6$ Hz), 6.91 (dd, 1H, $J = 2.6, 8.8$ Hz), 7.92 (d, 1H, J=8.8 Hz). ^{13}C NMR (DMSO-d_6) δ: 67.0 (C-2′), 69.4 (C-1′), 102.0 (C-8), 115.3 (C-6), 116.3 (C-4), 122.7 (C-5′), 122.9 (C-3), 123.9 (C-4′), 127.2 (C-5), 132.5 (C-3′), 133.0 (C-6′), 153.0 (C-2), 157.2 (C-8a), 162.6 (C-7), 175.4 (C-4).

2.5.2. 3-(2-Hydroxyphenyl)-1-phenylpropan-1-one (**2**: product converted from (trans)-chalcone by modified bphA)

The crude EtOAc extract (187.0 mg) was subjected to column chromatography (hexane–EtOAc = 4:1) to yield 24.9 mg of **2**. MS (EI) *m/z* 226 (M^+). HRMS (EI) calcd. for $C_{15}H_{14}O_2$ (M^+), 226.0994; found 226.0992. ^{1}H NMR ($CDCl_3$) δ: 2.98 (t, 2H, $J = 6.2$ Hz), 3.38 (t, 2H, $J = 6.2$ Hz), 6.78 (ddd, 1H, $J = 1.1, 7.1, 7.4$ Hz), 6.86 (dd, 1H, $J = 1.1, 7.0$ Hz), 7.05 (dd, 1H, $J = 7.0, 7.4$ Hz), 7.06 (d, 1H, $J = 7.1$ Hz), 7.38 (dd, 1H, $J = 7.4, 7.4$ Hz), 7.50 (dd, 1H, $J = 7.4, 7.4$ Hz), 7.91 (d, 1H, $J = 7.4$ Hz). ^{13}C NMR ($CDCl_3$) δ: 23.5 (C-1), 29.7 (C-2), 117.4 (C-6′), 120.7 (C-4′), 127.7 (C-2′), 127.9 (C-5′), 128.3 (C-5), 128.3 (C-9), 128.6 (C-6), 128.6 (C-8), 130.5 (C-3′), 133.7 (C-7), 136.0 (C-4), 154.5 (C-1′), 202.0 (C-3).

2.5.3. 2-(2,3-Dihydroxyphenyl)chromen-4-one (3: product converted from flavone by modified bphA + bphB)

The crude EtOAc extract (189.9 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 30:1) to yield 30.0 mg of **3**. MS (EI) *m/z* 254 (M^+). HRMS (EI) calcd. for $C_{15}H_{10}O_4$ (M^+), 226.09940; found 226.09942. ^{1}H NMR (DMSO-d_6) δ: 6.82 (dd, 1H, J = 7.9, 7.9 Hz), 6.98 (dd, 1H, J = 1.8, 7.9 Hz), 7.11 (s, 1H), 7.35 (dd, 1H, J = 1.8, 7.9 Hz), 7.47 (dd, 1H, J = 7.9, 7.9 Hz), 7.71 (d, 1H, J = 8.0 Hz), 7.80 (ddd, 1H, J = 1.8, 7.9, 7.9 Hz), 8.03 (dd, J = 1.8, 7.9, 7.9 Hz). ^{13}C NMR (DMSO-d_6) δ: 111.0 (C-3), 117.6 (C-6′), 118.3 (C-3′), 118.4 (C-8), 118.5 (C-4′), 119.2 (C-5′), 123.1 (C-4a), 124.7 (C-5), 125.2 (C-6), 134.1 (C-7), 145.3 (C-2′), 145.9 (C-1′), 155.8 (C-8a), 161.1 (C-2), 177.2 (C-4).

2.5.4. 2-(2,3-Dihydroxyphenyl)chroman-4-one (4: product converted from flavanone by modified bphA + bphB)

The crude EtOAc extract (103.2 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 20:1) to yield 68.0 mg of **4**. MS (EI) *m/z* 256 (M^+). HRMS (EI) calcd. for $C_{15}H_{12}O_4$ (M^+), 226.0994; found 226.0994. ^{1}H NMR (DMSO-d_6) δ: 2.76 (dd, 1H, J = 13.4, 16.5 Hz), 3.16 (dd, 1H, J = 3.0, 16.5 Hz), 5.78 (dd, 1H, J = 3.0, 13.4 Hz), 6.70 (dd, 1H, J = 7.9, 7.9 Hz), 6.80 (dd, 1H, J = 1.2, 7.9 Hz), 6.93 (dd, 1H, J = 1.2, 7.9 Hz), 7.07 (d, 1H, J = 7.9 Hz), 7.08 (dd, 1H, J = 7.9, 7.9 Hz), 7.57 (ddd, 1H, J = 1.8, 7.9, 7.9 Hz), 7.79 (dd, 1H, J = 1.8, 7.9 Hz). ^{13}C NMR (DMSO-d_6) δ: 42.5 (C-3), 74.4 (C-2), 115.1 (C-4′), 117.0 (C-6′), 118.0 (C-8), 119.1 (C-5′), 121.3 (C-6), 125.8 (C-4a), 126.1 (C-3′), 126.4 (C-5), 136.2 (C-7), 140.7 (C-2′), 145.2 (C-1′), 161.5 (C-8a), 192.0 (C-4).

2.5.5. 2-(2,3-Dihydroxyphenyl)-6-hydroxychromen-4-one (5: product converted from 6-hydroxyflavone by modified bphA + bphB)

The crude EtOAc extract (309.5 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 5:1) to yield 31.5 mg of **5**. MS (EI) *m/z* 270 (M^+). HRMS (EI) calcd. for $C_{15}H_{10}O_5$ (M^+), 270.0528; found 270.0522. ^{1}H NMR (DMSO-d_6) δ: 6.78 (dd, 1H, J = 7.4, 7.4 Hz), 6.95 (dd, 1H, J = 1.8, 7.4 Hz), 7.05 (s, 1H), 7.22 (dd, 1H, J = 3.5, 8.6 Hz), 7.29 (d, 1H, J = 3.5 Hz), 7.30 (dd, 1H, J = 1.8, 7.4 Hz), 7.57 (d, 1H, J = 8.6 Hz). ^{13}C NMR (DMSO-d_6) δ: 107.4 (C-5), 110.0 (C-3), 117.3 (C-6′), 118.4 (C-3′), 118.4 (C-4′), 118.4 (C-5′), 119.7 (C-8), 122.9 (C-7), 124.0 (C-4a), 145.5 (C-2′), 146.0 (C-1′), 149.5 (C-8a), 154.7 (C-6), 160.9 (C-2), 177.1 (C-4).

2.5.6. 2-(2,3-Dihydroxyphenyl)-6-hydroxychroman-4-one (6: product converted from 6-hydroxyflavanone by modified bphA + bphB)

The crude EtOAc extract (170.7 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 20:1) to yield 23.9 mg of **6**. MS (EI) *m/z* 272 (M^+). HRMS (EI) calcd. for $C_{15}H_{12}O_5$ (M^+), 272.0685; found 272.0685. ^{1}H NMR (DMSO-d_6) δ: 2.68 (dd, 1H, J = 2.4, 16.4 Hz), 3.06 (dd, 1H, J = 13.4, 16.4 Hz), 5.65 (dd, 1H, J = 2.4, 13.4 Hz), 6.68 (dd, 1H, J = 7.3, 7.3 Hz), 6.77 (d, 1H, J = 7.3 Hz), 6.91 (d, 1H, J = 7.3 Hz), 6.92 (d, 1H, J = 8.8 Hz), 7.02 (dd, 1H, J = 3.2, 8.8 Hz), 7.10 (d, 1H, J = 3.2 Hz). ^{13}C NMR (DMSO-d_6) δ: 42.7 (C-3), 74.4 (C-1), 110.0 (C-5), 115.0 (C-8), 115.0 (C-6′), 117.0 (C-4′), 119.0 (C-5’), 120.7 (C-4a), 124.5 (C-7), 126.1 (C-3′), 142.4 (C-2′), 145.2 (C-1′), 151.5 (C-6), 154.9 (C-8a), 192.3 (C-4).

2.5.7. 3-(2,3-Dihydroxyphenyl)-7-hydroxychromen-4-one (7: product converted from 7-hydroxyisoflavone by modified bphA + bphB)

The crude EtOAc extract (118.0 mg) was subjected to column chromatography (hexane–EtOAc = 2:1) to yield 56.4 mg of **7**. MS (EI) *m/z* 270 (M^+). HRMS (EI) calcd. for $C_{15}H_{10}O_5$ (M^+), 270.0528; found 270.0522. ^{1}H NMR (DMSO-d_6) δ: 6.63 (dd, 1H, J = 2.4, 7.7 Hz), 6.66 (dd, 1H, J = 7.1, 7.7 Hz), 6.79 (dd, 1H, J = 2.4, 7.1 Hz), 6.87 (d, 1H, J = 2.1 Hz), 6.93 (dd, 1H, J = 2.1, 8.8 Hz), 7.94 (d, 1H, J = 8.8 Hz). ^{13}C NMR (DMSO-d_6) δ: 102.1 (C-8), 115.2 (C-6), 115.2 (C-6′), 116.5 (C-4a), 118.7 (C-5′), 120.1 (C-3′), 121.7 (C-4′), 122.1 (C-3), 127.2 (C-5), 143.8 (C-2′), 145.6 (C-1′), 154.6 (C-2), 157.5 (C-8a), 162.1 (C-7), 174.9 (C-4).

2.5.8. 3-(2,3-Dihydroxyphenyl)-1-phenylpropan-1-one (8: product converted from (trans)-chalcone by modified bphA + bphB)

The crude EtOAc extract (187.0 mg) was subjected to column chromatography (hexane–EtOAc = 4:1) to yield 58.5 mg of **8**. MS (EI) *m/z* 242 (M^+). HRMS (EI) calcd. for $C_{15}H_{14}O_3$ (M^+), 242.0943; found 242.0944.

^{1}H NMR (DMSO-d_6) δ: 2.85 (t, 2H, J = 7.3 Hz), 3.25 (t, 2H, J = 7.3 Hz), 6.52 (dd, 1H, J = 7.5, 7.5 Hz), 6.57 (d, 1H, J = 7.5 Hz), 6.63 (d, 1H, J = 7.5 Hz), 7.50 (dd, 2H, J = 7.6, 8.6 Hz), 7.61 (dd, 1H, J = 7.6, 7.6 Hz), 7.96 (d, 2H, J = 8.6 Hz). ^{13}C NMR (DMSO-d_6) δ: 24.8 (C-1), 38.2 (C-2), 113.3 (C-6′), 118.7 (C-5′), 120.2 (C-4′), 127.8 (C-5), 127.8 (C-9), 128.0 (C-2′), 128.7 (C-6), 128.7 (C-8), 133.0 (C-7), 136.6 (C-4), 143.1 (C-2′), 144.9 (C-1′), 199.6 (C-3).

2.6. In vitro inhibitory activity against lipid peroxidation

A rat brain homogenate was prepared according to the method of Kubo et al. [11] with some modifications. In brief, a male Wistar rat weighting about 300 g was intraperitoneally anaesthetized with sodium pentobarbital (50 mg/kg), and the brain was transcardially perfused with 10 mM phosphate-buffered saline (pH 7.4) to completely remove blood from the brain. After killing by decapitation, the whole brain, except for the cerebellum, was immediately homogenized with a Teflon homogenizer for 30 s in 15 ml of an ice-cold 100 mM phosphate buffer (pH 7.4). The reaction mixture for the assay consisted of 0.5% (w/v) of the homogenate, 100 μM sodium ascorbate as an initiator for the generation of oxygen radicals, and a sample dissolved in MeOH. The mixture was incubated at 37 °C for 1 h while reciprocally agitating. Malondialdehyde (MDA) was stoichiometrically formed in the reaction mixture according to the concentration of lipid peroxides. MDA thus formed was allowed to react with thiobarbituric acid for spectrophotomeric quantification at 532 nm. The percentage inhibition was calculated as follows: $(1-(T-B)/(C-B)) \times 100$ (%), in which T, C and B are respectively the A_{532} readings of the treatment drug, the control (peroxidation without a drug) and the zero time control (no peroxidation).

3. Results

3.1. Biotransformation of the flavonoids with modified bphA

Respective flavonoids were biotransformed through the co-cultivation with the cells of *E. coli* carrying plasmid pKF2072, which expresses the modified biphenyl dioxygenase genes [*bphA1(2072)A2A3A4*]. The products converted were analysed by chromatographic and spectroscopic methods.

3.1.1. Flavone, flavanone, 6-hydroxyflavone and 6-hydroxyflavanone

The products, which were converted from flavone, flavanone, 6-hydroxyflavone and 6-hydroxyflavanone, were compared by TLC and HPLC with the authentic samples [8] to confirm their identities as shown in Fig. 2.

3.1.2. 7-Hydroxyisoflavone

The molecular formula of the product **1** was determined to be $C_{15}H_{12}O_5$ by HRMS (EI) and ^{1}H NMR and ^{13}C NMR spectral data. An analysis of the DQF COSY and HMQC spectra of **1** indicated **1** to be the dihydrodiol derivative of the phenyl side chain. The 1′,2′-diol regiochemical assignment was confirmed by the long-range ^{1}H-^{13}C coupling from H-4′ (δ, 6.42) to C-3 (δ, 122.9) and C-2′ (δ, 67.0). These findings indicated **1** to be 3-(2,3-dihydroxycyclohexa-1(6),4-dienyl)-7-hydroxychromen-4-one (Fig. 2). Product **1** was a novel compound, the yield of the purified product being shown in Fig. 1.

3.1.3. trans-Chalcone

The molecular formula of the product **2** was determined to be $C_{15}H_{14}O_2$ by HRMS (EI) and ^{1}H NMR and ^{13}C NMR spectral data. In the ^{1}H NMR and ^{13}C NMR spectra of 2, the signals due to $\Delta^{1,2}$ of the substrate had disappeared, while two coupled methylene signals (δ_H 3.00 (δ_C 30.1) and δ_H 3.23 (δ_C 40.4)) were observed. This confirmed the reduction of $\Delta^{1,2}$ in **2**. Furthermore, *vicinal* spin networks due to one-substituted and *ortho*-substituted benzene were observed in the DQF COSY spectrum of **2**. Consistent with the molecular formula of 1, the replacement of one phenolic OH function in either benzene ring was proved. The position of the phenolic OH function was determined to be C-1′ by the observation of ^{1}H-^{13}C long-range coupling from H-1 (δ 2.85) to C-1′ (δ 154.5) and to C-3′ (δ 130.5). Thus, **2** was identified as 3-(2-hydroxyphenyl)-1-phenylpropane-1-one (Fig. 2), a novel compound. The reduction of $\Delta^{1,2}$ was observed in the co-culture of (*trans*)-chalcone with host *E. coli* JM109. Therefore, this reduction was independent of *bphA*.

3.2. Biotransformation of the flavonoids with modified bphA and bphB

Respective flavonoids were biotransformed through the co-cultivation with the cells of *E. coli* carrying plasmid pBS2072B, which expresses the dihydrodiol dehydrogenase (desaturase) gene (*bphB*) in addition to the modified biphenyl dioxygenase genes. The products converted were analyzed by chromatographic and spectroscopic methods.

3.2.1. Flavone

Converted product **3** was compared by TLC and HPLC with an authentic sample of 2-(2,3-dihydroxyphenyl)chromen-4-one to confirm its identity as shown in Fig. 2.

3.2.2. Flavanone

Converted product **4** was compared by TLC and HPLC with an authentic sample of 2-(2,3-dihydroxyphenyl)chroman-4-one to confirm its identity as shown in Fig. 2.

3.2.3. 6-Hydroxyflavone

The molecular formula of product **5** was determined to be $C_{15}H_{10}O_5$ by HRMS (EI) and ^1H NMR and ^{13}C NMR spectral data. An analysis of the DQF COSY and HMQC spectra of **5** suggested the attachment of two phenolic OH functions at C-1′ and C-2′. The positions of the phenolic OHs were confirmed by the observation of ^1H-^{13}C long-range coupling from H-4’ (δ 7.30) to C-2 (δ 160.9) and to C-2′ (δ 145.5), and a *vicinal* spin network from H-4′ to H-6′ (δ 6.95). These findings enabled **5** to be determined as 2-(2,3-dihydroxyphenyl)-6-hydroxychromen-4-one (Fig. 2), this being a novel compound.

3.2.4. 6-Hydroxyflavanone

The molecular formula of product **6** was determined to be $C_{15}H_{12}O_5$ by HRMS (EI) and ^1H NMR and ^{13}C NMR spectra data. An analysis of DQF COSY and MHQC spectra of **6** suggested the attachment of two phenolic OH functions at C-1′ and C-2′. The positions of the phenolic OHs were confirmed by the observations of ^1H–^{13}C long-range coupling from H-4′(δ 6.91) to C-2 (δ 74.4) and to C-2′ (δ 145.5), and from H-5′ (δ 6.68) to C-1′ (δ 145.2) and to C-3′ (δ 126.1), and a *vicinal* spin network from H-4′ to H-6′ (δ 6.77). These findings enabled **6** to be determined as 2-(2,3-dihydroxyphenyl)-6-hydroxychroman-4-one (Fig. 2), this being a novel compound.

3.2.5. 7-Hydroxyisoflavone

The molecular formula of product **7** was determined to be $C_{15}H_{10}O_5$ by HRMS (EI) and ^1H NMR and ^{13}C NMR spectral data. An analysis of the DQF COSY and HMQC spectra of **7** suggested the attachment of two phenolic OH functions at C-1′ and C-2′. The positions of the phenolic OHs were confirmed by the observation of ^1H-^{13}C long-range coupling from H-6′ (δ 6.79) to C-1′ (δ 145.6) and to C-2′ (δ 143.8), and a *vicinal* spin network from H-4′ (δ 6.66) to H-6′. These findings enabled **7** to be determined as 3-(2,3-dihydroxyphenyl)-7-hydroxychromen-4-one (Fig. 2), this being a novel compound.

3.2.6. trans-Chalcone

The molecular formula of product **8** was determined to be $C_{15}H_{14}O_3$ by HRMS (EI) and ^1H NMR and ^{13}C NMR spectral data. The $\Delta^{1,2}$ of the substrate in **8** was also reduced, as with **2**. An analysis of the DQF COSY and HMQC spectra of **8** suggested the attachment of two phenolic OH functions at C-1′ and C-2′. The positions of these phenolic OHs were confirmed by the observation of ^1H-^{13}C long-range coupling from H-1 (δ 2.85) to C-2′ (δ 143.1), C-3′ (δ 128.0) and C-4′ (δ 120.2), and a *vicinal* spin network from H-4′ (δ 6.57) to H-6′ (δ 6.63). These findings enabled **8** to be determined as 3-(2,3-dihydroxyphenyl)-1-phenylpropan-1-one (Fig. 2), this being a novel compound.

3.3. Antioxidative activity of the converted products

The antioxidative activity of flavonoids is important for their medicinal and nutritional values. The converted products reported in this paper contained some phenolic OH functions in their structures, so the in vitro inhibitory activity toward lipid peroxidation was examined. The results are summarized in Table 1. All the products showed stronger antioxidative activity than to the substrate. In particular, compounds 3–8 possessing the catechol structure showed sufficient antioxidative activity.

Table 1
Inhibitory effects of flavonoids on lipid peroxidation in a rat brain homogenate

Compound	IC_{50} (μM)
Flavone	>200
3	25
Flavanone	140
4	73
6-Hydroxyflavone	>200
5	2.7
6-Hydroxyflavanone	33
6	15
7-Hydroxyisoflavone	>200
1	190
7	25
(*trans*)-Chalcone	>200
2	62
8	7.0
Catechin (positive control)	20

4. Discussion

The biphenyl dioxygenase *bphA1(2072)A2A3A4*, which was modified by directed protein evolution (DNA shuffling) [4], was able to transform various flavonoids. 7-Hydroxyisoflavone was converted to its dihydrodiol form (Fig. 2), this being the typical reaction mediated by this enzyme, while the monohydroxy products were generated from flavone, flavanone, 6-hydroxyflavone, 6-hydroxyflavanone and (*trans*-)chalcone through the *E. coli* cells expressing the modified dioxygenase (Fig. 2). These substrates 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 flavone and flavanone, not the dihydrodiol but the diol forms were observed from the products converted by the recombinant *E. coli* cells carrying only *bphA1(2072)A2A3A4* (Fig. 2). *E. coli* is likely to have endogenous dehydrogenation activity, although it is not strong. The same result has also been obtained when using *S. lividans* as the host [8].

Dihydrodiol dehydrogenase (desaturase) BphB that is derived from *P. pseudoalcaligenes* KF707 was capable of efficiently converting the flavonoid dihydrodiol forms, which would have been generated through the above-mentioned dioxygenase, to the corresponding flavonoid-diol forms. This result demonstrates that the native BphB had broad substrate specificity. We have shown for the first time in this study efficient enzymatic syntheses of flavonoid-diols with a structure of the catechol type. The diol products generated were “unnatural” natural flavonoids, which have not previously been detected in nature, with strong antioxidant activity.

Acknowledgements

The authors thank Drs. Sueharu Horinouchi and Yasuo Ohnishi of The University of Tokyo for their valuable discussions.

References

[1] K. Furukawa, Curr. Opin. Biotechnol. 11 (2000) 244–249.
[2] N. Kimura, A. Nishi, M. Goto, K. Furukawa, J. Bacteriol. 179 (1997) 3936–3943.
[3] T. Kumamaru, H. Suenaga, M. Mitsuoka, T. Watanabe, K. Furukawa, Nat. Biotechnol. 16 (1998) 663–666.
[4] N. Misawa, K. Shindo, H. Takahashi, H. Suenaga, K. Iguchi, H. Okazaki, S. Harayama, K. Furukawa, Tetrahedron 58 (2002) 9605–9612.
[5] K. Shindo, R. Nakamura, I. Chinda, Y. Ohnishi, S. Horinouchi, H. Takahashi, K. Iguchi, S. Harayama, K. Furukawa, N. Misawa, Tetrahedron 59 (2003) 1895–1900.
[6] J.B. Harbone, in: T.W. Goodwin (Ed.), Plant Pigments, Academic Press, New York, 1988, pp. 299–343.
[7] J.B. Harborne, H. Baxter, Handbook of Natural Flavonoids, vols. 1–2, Wiley, Chichester, 1999.
[8] H.-K. Chun, Y. Ohnishi, K. Shindo, N. Misawa, K. Furukawa, S. Horinouchi, J. Mol. Catal. B: Enzym. 21 (2003) 113–121.
[9] J. Sambrook, D. W. Russell, Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.
[10] J. Hirose, A. Suyama, S. Hayashida, K. Furukawa, Gene 138 (1994) 27–33.
[11] K. Kubo, I. Yoshitake, Y. Kumada, H. Shuto, N. Nakamizo, Arch. Int. Pharmacodyn. Ther. 272 (1984) 283–295.