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Biotransformation of flavone and flavanone by *Streptomyces lividans* cells carrying shuffled biphenyl dioxygenase genes

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Abstract

The *bphA1*(*2072*)*A2A3A4* gene cluster codes for a shuffled biphenyl dioxygenase holoenzyme with broad substrate specificity. The *bphA1*(*2072*) encoding the large subunit of an iron–sulfur protein is a hybrid gene generated by DNA shuffling using *bphA1* of *Pseudomonas pseudoalcaligenes* KF707 and *bphA1* of *Burkholderia cepacia* LB400. The *bphA2* encoding the small subunit of an iron–sulfur protein, *bphA3* encoding a ferredoxin and *bphA4* encoding a ferredoxin reductase are from *P. pseudoalcaligenes* KF707. The *bphA1*(*2072*)*A2A3A4* gene cluster was positioned downstream of a thiostrepton-inducible promoter P_{tipA} on a high-copy-number vector pIJ6021, and introduced into the Gram-positive, soil-inhabiting, filamentous bacterium *Streptomyces lividans*. Biotransformation of some flavonoids by the recombinant *S. lividans* cells was examined and the products were determined by EI-MS, ^{1}H and ^{13}C NMR. Flavone was converted into two products, 2′,3′-dihydroxyflavone (a major product) and 3′-hydroxyflavone (a minor product). Flavanone was converted into three products, 2′,3′-dihydroxyflavanone (a major product), 2′-hydroxyflavanone and 3′-hydroxyflavanone (minor products). 2′,3′-Dihydroxyflavanone was a novel compound. The biotransformation of flavone and flavanone proceeded very efficiently; 1 mM of each of the substrate was almost completely converted to the corresponding di- or mono-hydroxy form in 24 h. Furthermore, 6-hydroxyflavone and 6-hydroxyflavanone were also converted into 2′,6-dihydroxyflavone and 3′,6-dihydroxyflavanone, respectively. Among these products, 2′,3′-dihydroxyflavone and 2′,3′-dihydroxyflavanone showed free radical-scavenging activity.

Keywords: Biphenyl dioxygenase; Flavanone; Flavone; Biotransformation; *Streptomyces lividans*

1. Introduction

Flavonoids are a diverse group of plant natural products synthesized from phenylpropanoid and acetate-derived precursors [1–3]. In plants, they play important roles in growth and development, and in defense against microorganisms. Moreover, the

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biological properties of flavonoids, e.g. their antioxidant activity, are considered to be of medicinal and nutritional importance of these compounds. Although 6467 falavonoids in nature are listed in "*The Handbook of Natural Flavonoids*" [4], the artificial modification of flavonoids has not been well studied. Therefore, it is promising to produce "unnatural–natural flavonoids" by genetic engineering or biotransformation.

Biphenyl dioxygenases are known as initial key enzymes to degrade environmental pollutants, mainly polycyclic aromatic compounds, which are derived from coal and petroleum. The enzyme is a multi-component enzyme that consists of BphA1, BphA2 (large and small subunits of the iron–sulfur enzyme, respectively), BphA3 (a ferredoxin), and BphA4 (a ferredoxin reductase). Modified *bphA1* genes were generated by DNA shuffling using *bphA1* of *P. pseudoalcaligenes* KF707 and *bphA1* of *B. cepacia* LB400 [5]. One of the shuffled genes, *bphA1*(*2072*), was found to encode a modified large subunit of the iron–sulfur enzyme with broad substrate specificity. Many heterocyclic aromatic compounds could be converted at high efficiency to the corresponding 1,2-dihydrodiol and/or monohydroxy derivatives by the *Escherichia coli* transformant in which *bphA1*(*2072*) was expressed together with *bphA2A3A4* from *P. pseudoalcaligenes* [6]. We also constructed a plasmid for expression of the modified biphenyl dioxygenase in the Gram-positive, soil-inhabiting, filamentous bacterium *Streptomyces lividans*. The *Streptomyces* transformant could catalyze the same conversions as those by the *E. coli* transformant at the same or a better efficiency (our unpublished results). Therefore, we tried to biotransform some flavonoids, which are regarded as heterocyclic aromatic compounds, using *S. lividans* cells carrying shuffled biphenyl dioxygenase genes. In this paper, we describe di- and/or mono-hydroxylation of the B-ring of flavone and flavanone by the modified biphenyl dioxygenase. The major product from flavanone, 2′,3′-dihydroxyflavanone, was a novel compound. The mechanism of formation of 2′,3′-dihydroxy derivatives, instead of 2′,3′-dihydrodiol derivatives, was not clear. However, production of 2′,3′-dihydroxy derivatives is interesting because the *o*-dihydroxy structure in the B-ring is essential for the effective antioxidant activity of flavonoids [7].

2. Experimental

2.1. Plasmids, bacterial strains, and general recombinant DNA techniques

Plasmid pKF2072 carrying the *bphA1(2072)A2A3A4* gene cluster was described [6]. The expression vector pIJ6021 for *Streptomyces* species, which carries the kanamycin resistance gene and thiostrepton-inducible promoter P_{tipA} [8], was a gift from E. Takano and M. J. Bibb, John Innes Centre, Norwich, UK. *S. lividans* TK21 [9] was used as a host for expression of *bphA1*(*2072*)*A2A3A4*, and cultured in YEME medium [9] or minimal medium [10] at 30 °C. Kanamycin was used at a final concentration of 5 μg/ml when necessary. Plasmid pUC18 and *E. coli* JM109 for DNA manipulation were purchased from Takara Shuzo. Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were purchased from Takara Shuzo. DNA was manipulated in *S. lividans* [9] and *E. coli* [11], as described earlier. Nucleotide sequences were determined by the dideoxy chain termination method with the Thermo sequenase fluorescence-labeled primer cycle sequencing kit (Amersham) on an automated DNA sequencer.

2.2. Construction of plasmids

bphA1(*2072*) (1.4 kb) was amplified by PCR using pKF2072 as a template and the following two primers; A1F (CCCGGTACCGGAGGTATA*CAT**ATG***AGCTCAGCAATCAAAGAAGTG; underlined, italic and bold letters indicate a *Kpn*I site, an *Nde*I site and a start codon of *bphA1*(*2072*), respectively) and A1R (CCCGGATCC*AGATC T*AACGAT TGCGTCT TGA-**TCA**GGGCTTGAGCGT; underlined, italic and bold letters indicate a *Bam*HI site, a *Bgl*II site and a termination codon of *bphA1*(*2072*), respectively). The amplified fragment was digested with *Kpn*I plus *Bam*HI and cloned into pUC18 between the corresponding sites, resulting in pUC18-*bphA1*(*2072*). Similarly, *bphA2* (0.4 kb) was amplified using the following two primers; A2F (CCCGGTACC*AGATCT*AGAAGG-GAGTTCAAC**ATG**GTGGGCTGGACG; underlined, italic and bold letters indicate a *Kpn*I site, a *Bgl*II site and a start codon of *bphA2*, respectively) and A2R (CCCAAGCTTACCTCC**TCA**GAAGAACATGCTC-

AGGTTGTT; underlined and bold letters indicate a *Hin*dIII site and a termination codon of *bphA2*, respectively). The amplified fragment was digested with *Kpn*I plus *Hin*dIII and cloned into pUC18 between the corresponding sites, resulting in pUC18-*bphA2*. *bphA3* and a 5′-portion of *bphA4* (0.9 kb) were amplified using the following two primers; A3F (CCCAAGCTT**ATG**AAATTTACCAGAGTTTGTG-ATCGAAGA; underlined and bold letters indicate a *Hin*dIII site and a start codon of *bphA3*, respectively) and A3R (CCCGGATCC*GGTCACC*CAGCACGCG-CACCAGCAACTCGT; underlined and italic letters indicate a *Bam*HI and *Bst*PI site, respectively). The amplified fragment was digested with *Hin*dIII plus *Bam*HI and cloned into pUC18 between the corresponding sites, resulting in pUC18-*bphA3A4′*. A 3′-portion of *bphA4* (0.7 kb) was amplified using the following two primers; A4F (CCCAAGCTT*GGTCA-CC*GGACCGGGGCATGG; underlined and italic letters indicate a *Hin*dIII and *Bst*PI site, respectively) and A4R (CCCGGATCCTCC**TCA**ATTCGGTTTGGC-TTTGAG; underlined and bold letters indicate a *Bam*HI site and a termination codon of *bphA4*, respectively). The amplified fragment was digested with *Hin*dIII plus *Bam*HI and cloned into pUC18 between the corresponding sites, resulting in pUC18-*bph′A4*. All the amplified fragments were sequenced to confirm no PCR errors. Both the 1.4 kb *Kpn*I–*Bgl*II fragment of pUC18-*bphA1* (*2072*) and the 0.4 kb *Bgl*II–*Hin*dIII fragment of pUC18-*bphA2* were cloned together into pUC18 between the *Kpn*I and *Hin*dIII sites, resulting in pUC18-*bphA1*(*2072*)*A2*. Both the 0.9 kb *Hin*dIII–*Bst*PI fragment of pUC18-*bphA3A4′* and the 0.7 kb *Bst*PI–*Bam*HI fragment of pUC18-*bph′A4* were cloned together into pUC18 between the *Hin*dIII and *Bam*HI sites, resulting in pUC18-*bphA3A4*. Both the 1.8 kb *Kpn*I–*Hin*dIII fragment of pUC18-*bphA1*(*2072*)*A2* and the 1.6 kb *Hin*dIII–*Bam*HI fragment of pUC18-*bphA3A4* were cloned together into pUC18 between the *Kpn*I and *Bam*HI sites, resulting in pUC18-*bphA1*(*2072*)*A2A3A4*. Finally, the 3.4 kb *Nde*I–*Bam*HI fragment of pUC18-*bphA1*(*2072*)*A2A-3A4* was cloned into pIJ6021 between the corresponding sites, resulting in pIJ6021-*bphA1*(*2072*)*A2A3A4*. On pIJ6021-*bphA1*(*2072*)*A2A3A4*, the four genes were under the control of the thiostrepton-inducible promoter P_{tipA} and they would be co-transcribed in *S. lividans* containing pIJ6021-*bphA1*(*2072*)*A2A3A4*. *bphA1*(*2072*) was supposedly translated by using the Shine-Dalgarno (SD) sequence of the expression vector. Just upstream from the start codons of *bphA2* and *bphA3*, artificial SD sequences were designed. The translational coupling of *bphA3* and *bphA4* would occur because the start codon of *bphA4* was originally overlapped with the termination codon of *bphA3*.

2.3. Conversion experiments

S. lividans TK21 harboring pIJ6021-*bphA1*(*2072*) *A2A3A4* was grown in 100 ml of YEME medium containing 5 μg/ml of kanamycin in a shaking (Sakaguchi) flask at 30 °C with reciprocal shaking (120 rpm) for 2 days. One milliliter of this culture was inoculated into the same medium, and cultivated under the same conditions. After 24 h, thiostreptone was added to the culture at a final concentration of 5 μg/ml to induce transcription from P_{tipA}. After an additional 24 h of incubation the mycelium was collected by centrifugation and washed once with minimal medium. Then 100 mg (wet weight) mycelium was resuspended in 100 ml of fresh minimal medium, and the substrate, flavone, flavanone, 6-hydroxyflavone, or 6-hydroxyflavanone, was added to the mycelium suspension at a final concentration of 1 mM. The mycelium and substrates were incubated on a reciprocal shaker (120 rpm) at 30 °C for 24 h, followed by HPLC analysis of the culture supernatant. All the substrates were purchased from Aldrich or Sigma.

2.4. Purification and identification of converted products

The culture supernatant (about 100 ml) was extracted with 100 ml of ethyl acetate (EtOAc). The organic layer was concentrated in vacuo, and analyzed by thin-layer chromatography (TLC) on silica gel (0.25 mm Silica Gel 60 (Merck)). The solvent systems were as follows: flavone, hexane:EtOAc (1:1); flavanone, hexane:EtOAc (3:1); 6-hydroxyflavone, hexane:EtOAc (1:1); 6-hydroxyflavanone, CH_2Cl_2:EtOAc (1:1). 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 the following

solvent systems: flavone, hexane:EtOAc (3:1 → 1:1) (stepwise); flavanone, hexane: EtOAc (3:1); 6-hydroxyflavone, CH_2Cl_2:EtOAc (1:1); 6-hydroxyflavanone, CH_2Cl_2:EtOAc (1:1).

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

- Compound **1**: ^{1}H NMR (DMSO-d_6): 6.82 (dd, J = 7.9, 7.9), 6.98 (dd, J = 1.8, 7.9), 7.11 (s), 7.35 (dd, J = 1.8, 7.9), 7.47 (dd, J = 7.9, 7.9), 7.71 (d, J = 8.0), 7.80 (ddd, J = 1.8, 7.9, 7.9), 8.03 (dd, J = 1.8, 7.9, 7.9). ^{13}C NMR (DMSO-d_6): 111.0, 117.6, 118.3, 118.4, 118.5, 119.2, 123.1, 124.7, 125.2, 134.1, 145.3, 145.9, 155.8, 161.1, 177.2.
- Compound **2**: ^{1}H NMR (DMSO-d_6): 6.92 (s), 7.00 (dd, J = 1.8, 7.9), 7.37 (dd, J = 7.9, 7.9), 7.44 (dd, J = 1.8, 1.8), 7.48 (dd, J = 7.9, 7.9), 7.52 (dd, J = 1.8, 7.9), 7.76 (d, J = 7.9), 7.83 (ddd, J = 1.8, 7.9, 7.9), 8.05 (dd, J = 1.8, 7.9), 9.88 (s). ^{13}C NMR (DMSO-d_6): 106.9, 112.8, 117.2, 118.5, 118.8, 123.3, 124.8, 125.5, 130.2, 132.4, 134.3, 155.6, 157.9, 162.7, 177.0.
- Compound **3**: See Table 1.
- Compound **4**: ^{1}H NMR (DMSO-d_6): 2.80 (dd, J = 3.1, 16.5), 3.18 (dd, J = 12.8, 16.5), 5.58 (dd, J = 3.1, 12.8), 6.75 (ddd, J = 1.8, 1.8, 7.3), 6.92 (d, J = 1.8), 6.93 (d, J = 7.3), 7.08 (dd, J = 7.9, 7.9), 7.09 (d, J = 7.9), 7.20 (dd, J = 7.3, 7.3), 7.58 (ddd, J = 1.8, 7.9, 7.9), 7.78 (dd, J = 1.8, 7.9), 9.52 (s). ^{13}C NMR (DMSO-d_6): 43.5, 78.7, 113.4, 115.3, 117.0, 118.0, 120.7, 121.4, 126.3, 129.6, 136.2, 140.3, 157.4, 161.0, 191.5.
- Compound **5**: ^{1}H NMR (DMSO-d_6): 2.77 (dd, J = 3.1, 16.5), 3.20 (dd, J = 13.4, 16.5), 5.78 (dd, J = 3.1, 13.4), 6.87 (dd, J = 7.9, 7.9), 6.88 (d, J = 7.9), 7.09 (dd, J = 7.9, 7.9), 7.09 (d, J = 7.9), 7.19 (dd, J = 7.9, 7.9), 7.47 (d, J = 7.9), 7.59 (ddd, J = 1.8, 7.9, 7.9), 7.79 (dd, J = 1.8, 7.9), 9.81 (s). ^{13}C NMR (DMSO-d_6): 42.4, 74.3, 115.4, 118.0, 119.1, 121.3, 121.3, 124.9, 126.3, 126.9, 129.3, 136.2, 154.2, 161.5, 191.9.
- Compound **6**: ^{1}H NMR (DMSO-d_6): 6.81 (s), 6.98 (dd, J = 2.4, 7.9), 7.25 (dd, J = 3.1, 8.6), 7.31 (d, J = 3.1), 7.34 (dd, J = 7.9, 7.9), 7.41 (dd, J = 2.4, 2.4), 7.47 (dd, J = 2.4, 7.9), 7.61 (d, J = 8.6). ^{13}C NMR (DMSO-d_6): 105.8, 107.5, 112.7, 116.9, 118.7, 119.7, 123.1, 124.2, 130.2, 132.6, 149.2, 155.0, 158.0, 162.3, 176.9.
- Compound **7**: ^{1}H NMR (DMSO-d_6): 2.71 (dd, J = 2.4, 16.5), 3.10 (dd, J = 13.4, 16.5), 5.65 (dd, J = 2.4, 13.4), 6.86 (dd, J = 7.9, 7.9), 6.87 (d, J = 7.9), 6.94 (d, J = 8.5), 7.02 (dd, J = 3.1, 8.5), 7.11 (d, J = 3.1), 7.17 (dd, J = 7.9, 7.9), 7.46 (d, J = 7.9), 9.41 (s), 9.76 (s). ^{13}C NMR (DMSO-d_6): 42.6, 74.2, 109.9, 115.4, 118.9, 119.1, 120.7, 124.5, 125.3, 126.9, 129.2, 151.5, 154.1, 154.8, 192.1.

2.5. Free radical-scavenging activity

Free radical-scavenging activity was measured essentially by the method described by Yamaguchi et al. [12] using 1,1-diphenyl-2-picrylhydrazyl (DPPH). A portion of the sample solution (100 μl) was mixed with 100 mM MES buffer (pH 6.0, 50 μl) and 1 mM DPPH in ethanol (50 μl). The mixture was shaken vigorously and left to stand for 30 min at room

Table 1
The physico-chemical properties and spectral data of 2′,3′-dihydroxyflavanone (**3**)

Appearance	Pale yellow powder
Molecular formula	$C_{15}H_{12}O_4$
EI-MS	256 (M^+)
Melting point	36–38 °C
UV λ_{max} nm (ε)	214.4 (55700), 253.2 (23500), 320 (7000)
IRν (KBr) (cm^{-1})	3400, 1685, 1605, 1465, 1307, 1227, 767
^{1}H NMR (DMSO-d_6)	2.76 (dd, J = 13.4, 16.5), 3.16 (dd, J = 3.0, 16.5), 5.78 (dd, J = 3.0, 13.4), 6.70 (dd, J = 7.9, 7.9), 6.80 (dd, J = 1.2, 7.9), 6.93 (dd, J = 1.2, 7.9), 7.07 (d, J = 7.9), 7.08 (dd, J = 7.9, 7.9), 7.57 (ddd, J = 1.8, 7.9, 7.9), 7.79 (dd, J = 1.8, 7.9)
^{13}C NMR (DMSO-d_6)	42.5, 74.4, 115.1, 117.0, 118.0, 119.1, 121.3, 121.3, 125.8, 126.4, 136.2, 140.7, 145.2, 161.5, 192.0

temperature in the dark. The absorbance at 515 nm (A_{515}) by DPPH was measured by UV-Vis spectrophotometry. The concentration of the sample, which showed 50% radical-scavenging activity, was determined.

3. Results and discussion

3.1. Biotransformation of flavone and flavanone by recombinant S. lividans cells

A number of heterocyclic aromatic compounds could be converted to the corresponding 1,2-dihydrodiol and/or monohydroxy derivatives by the *S. lividans* transformant carrying the shuffled biphenyl dioxygenase genes (*bphA1*(*2072*)*A2A3A4*) at the same or higher efficiency, compared to those by the *E. coli* transformant (our unpublished results). Therefore, we tried biotransformation of some flavonoids, which are regarded as heterocyclic aromatic compounds, using *S. lividans* cells carrying the shuffled biphenyl dioxygenase genes. We used 1 mM of flavone and flavanone and confirmed their conversion by HPLC analyses (data not shown). The biotransformation of flavone and flavanone proceeded very efficiently; 1 mM of each substrate was almost completely converted in 24 h. Next, we used 100 mg (4.5 mM) of the substrates to prepare the converted products for determination of their structures.

3.1.1. Products converted from flavone (Fig. 1)

TLC analysis of the crude products (180 mg) converted from flavone by the *S. lividans* transformant revealed the presence of two compounds with Rf values of 0.1 (compound **1**) and 0.3 (compound **2**). Both compounds were purified by silica gel column chromatography to yield **1** (13 mg) and **2** (2.4 mg) as pale yellow powders.

The molecular formula of compound **1** was determined to be $C_{15}H_{10}O_4$ by MS [*m/z* 254 (M^+)] and ^1H and ^{13}C NMR spectra. Analysis of DQF COSY and C–H COSY spectra of **1** proposed that two phenolic OH functions were attached to the phenyl side chain. The positions of the phenolic OH were proved to be 2′ and 3′ by the observation of long range couplings from H-5′ (δ 6.82) to C-1′ (δ 118.3) and C-3′ (δ 145.9) and from H-4′ (δ 6.98) to C-2′ (δ 145.3) and vicinal sp^2 ^1H–^1H spin network from H-4′ to H-6′. Thus, compound **1** was identified to be 2′,3′-dihydroxyflavone [13].

The molecular formula of compound **2** was determined to be $C_{15}H_{10}O_3$ by MS [*m/z* 238 (M^+)] and ^1H and ^{13}C NMR spectra. Analysis of DQF COSY and C–H COSY spectra of **2** proposed that one phenolic OH function was attached to the phenyl side chain. The position of the phenolic OH was proved to be 3′ by the analysis of the sp^2 ^1H–^1H spin network from H-2′ to H-6′. Thus, compound **2** was identified to be 3′-hydroxyflavone [14].

3.1.2. Products converted from flavanone (Fig. 1)

TLC analysis of the crude products (258 mg) converted from flavanone by the *S. lividans* transformant revealed the presence of three compounds with Rf values of 0.1 (compound **3**), 0.3 (compound **4**) and 0.4 (compound **5**). Each compound was purified by silica gel column chromatography to yield **3** (16 mg), **4** (4.6 mg) and **5** (4.4 mg) as pale yellow powders.

The molecular formula of compound **3** was determined to be $C_{15}H_{12}O_4$ by MS [*m/z* 256 (M^+)] and ^1H and ^{13}C NMR spectra. Analysis of DQF COSY and C–H COSY spectra of **3** proposed that two phenolic OH functions were attached to the phenyl side chain. The positions of the phenolic OH were proved to be 2′ and 3′ by the observation of long range couplings from H-5′ (δ 6.70) to C-1′ (δ 125.8) and C-3′ (δ 145.2) and from H-4′ (δ 6.80) to C-2′ (δ 140.7) and vicinal sp^2 ^1H–^1H spin network from H-4′ to H-6′. Thus, compound **3** was identified to be 2′,3′-dihydroxyflavanone. This was a novel compound. The physico-chemical properties of **3** were shown in Table 1.

The molecular formula of compound **4** was determined to be $C_{15}H_{12}O_3$ by MS [*m/z* 240 (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 phenyl side chain. The position of the phenolic OH was proved to be 3′ by long range couplings from H-5′ (δ 7.20) to C-1′ (δ 140.3) and C-3′ (δ 157.4) and the analysis of the sp^2 ^1H–^1H spin network from H-2′ to H-6′. Thus, compound **4** was identified to be 3′-hydroxyflavanone [15].

The molecular formula of compound **5** was determined to be $C_{15}H_{12}O_3$ by MS [*m/z* 240 (M^+)] and ^1H and ^{13}C NMR spectra. Analysis of DQF COSY and C–H COSY spectra of **5** proposed that one phenolic

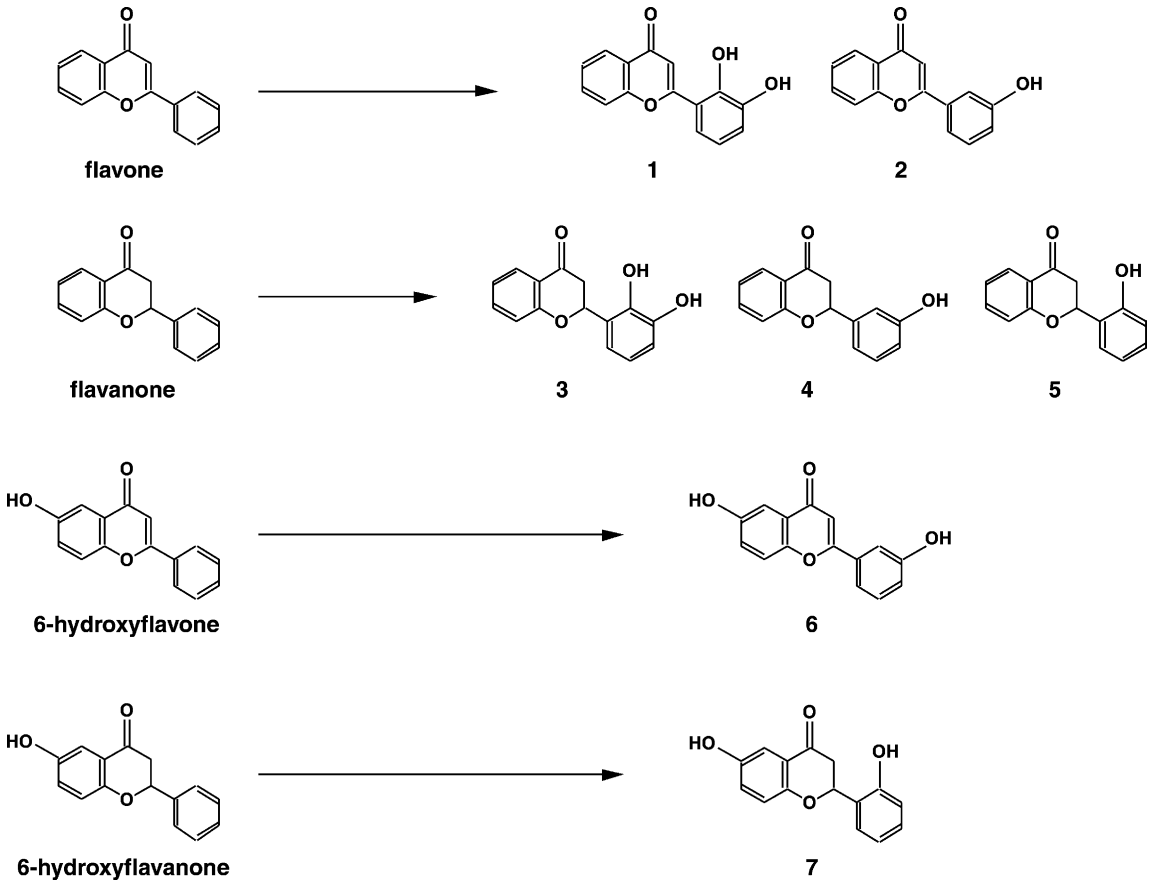

Fig. 1. Biotransformation of flavonoids by the *S. lividans* transformant expressing the modified biphenyl dioxygenase. The structures of converted products were determined by MS and ^{1}H and ^{13}C NMR.

OH function was attached to the phenyl side chain. The position of the phenolic OH was proved to be 2′ by the observation of the vicinal sp^2 ^{1}H–^{1}H spin network from H-3′ to H-6′. Thus, compound **5** was identified to be 2′-hydroxyflavanone [16].

The major products converted from flavone and flavanone were 2′,3′-dihydroxy derivatives, but not dihydrodiol derivatives. These results were very surprising because the modified biphenol dioxygenase converted heterocyclic aromatic compounds into the corresponding dihydrodiol and/or monohydroxy derivatives. For example, 2-phenylquinoline was converted to 3-(2-quinolyl)-3,5-cyclohexadiene-1,2-diol by the transformant (Fig. 2A). We speculate that flavone and flavanone were converted to their dihydrodiol derivatives in the first step and then the dihydrodiol derivatives were converted to the dihydroxy derivatives by dehydrogenation and to the monohydroxy derivatives by dehydration (Fig. 2B). However, it is unclear why the dehydrogenation of the dihydrodiol derivatives occurs only for the flavonoids. As described below, 2′,3′-dihydroxy derivatives were not produced when 6-hydroxyflavone and 6-hydroxyflavanone were treated by the transformants.

3.2. Biotransformation of some derivatives of flavone and flavanone by the S. lividans transformant

We examined biotransformation of some derivatives of flavone and flavanone, including 3-hydroxyflavone, 6-hydroxyflavone, 7-hydroxyflavone, chrysin (5,7-dihydroxyflavone), apigenin (4′,5,7,-trihydroxyflav-

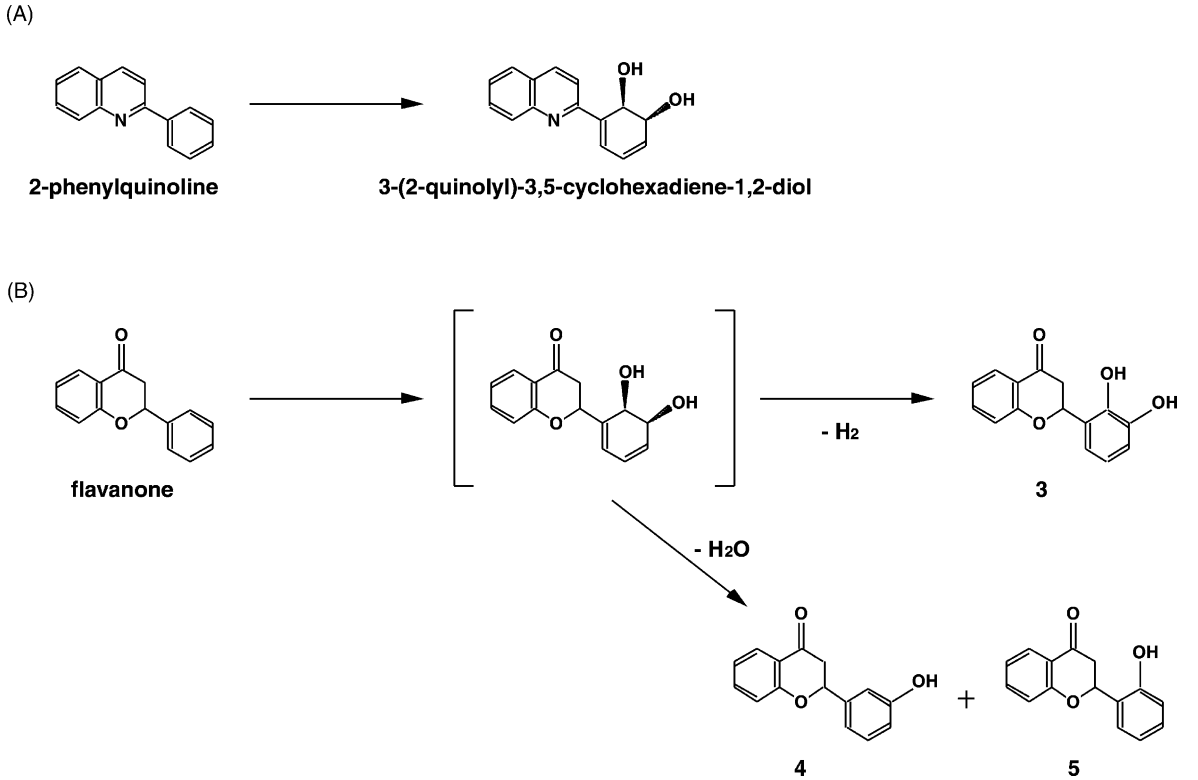

Fig. 2. Conversion of 2-phenylquinoline into 3-(2-quinolyl)-3,5-cyclohexadiene-1,2-diol (A) and probable pathways of conversion of flavanone into the di- and mono-hydroxy derivatives (B) by the *S. lividans* transformant expressing the modified biphenyl dioxygenase. When flavanone was used as a substrate, the dihydrodiol derivative is probably produced in the first step and then the dihydrodiol derivatives are converted to the dihydroxy derivative by dehydrogenation and to the monohydroxy derivatives by dehydration.

one), α-naphtoflavone, β-naphtoflavone, 6-hydroxyflavanone, and naringenin (4′,5,7-trihydroxyflavanone), by the modified biphenyl dioxygenase. One millimoler of each substrate was added to the recombinant *S. lividans* cells and incubated for 24 h at 30 °C, followed by HPLC analysis of the culture supernatant. The conversion of these compounds, except for 6-hydroxyflavone and 6-hydroxyflavanone, was not detected (data not shown). The conversion efficiency of 6-hydroxyflavone and 6-hydroxyflavanone was lower than those of flavone and flavanone; 40% of flavone and 10% of flavanone remained in 24 h reactions. We speculate that the substitutional group(s) causes the structural hindrance for the access of the enzyme. We used 100 mg (4.2 mM) of 6-hydroxyflavone and 6-hydroxyflavanone to prepare the converted products for determination of their structures.

3.2.1. A product converted from 6-hydroxyflavone (Fig. 1)

TLC analysis of the crude products (260 mg) converted from 6-hydroxyflavone by the *S. lividans* transformant found one compound with an Rf of 0.2 (compound **6**). This compound was purified by silica gel column chromatography to yield **6** (8.5 mg) as a pale yellow powder. The molecular formula of compound **6** was determined to be $C_{15}H_{10}O_4$ by MS [*m/z* 254 (M^+)] and ^1H and ^{13}C NMR spectra. Analysis of DQF COSY and C–H COSY spectra of **6** proposed that one phenolic OH function was attached to the phenyl side chain. The position of the phenolic OH was proved to be 3′ by long range couplings from H-5′ (δ 7.34) to C-1′ (δ 132.6) and C-3′ (δ 158.0) and the analysis of the

sp^2 ^1H–^1H spin network from H-2′ to H-6′. Thus, compound **6** was identified to be 3′,6-dihydroxyflavone [17].

3.2.2. A product converted from 6-hydroxyflavanone (Fig. 1)

TLC analysis of the crude products (250 mg) converted from 6-hydroxyflavanone by the *S. lividans* transformant found one compound with an Rf of 0.1 (compound **7**). This compound was purified by silica gel column chromatography to yield **7** (8.5 mg) as a pale yellow powder. The molecular formula of compound **7** was determined to be $C_{15}H_{12}O_4$ by MS [*m/z* 256 (M^+)] and ^1H and ^{13}C NMR spectra. Analysis of DQF COSY and C–H COSY spectra of **7** proposed that one phenolic OH function was attached to the phenyl side chain. The position of the phenolic OH was proved to be 2′ by the observation of the vicinal sp^2 ^1H–^1H spin network from H-3′ to H-6′. Thus, compound **7** was identified to be 2′,6-dihydroxyflavanone [18].

Both 6-hydroxyflavone and 6-hydroxyflavanone were converted to their monohydroxy derivatives. However, the positions of the phenolic OH function were different; the 3′ position for 6-hydroxyflavone and the 2′ position for 6-hydroxyflavanone. The monohydroxy product converted from flavone also contained a hydroxy group at the 3′ position. Both the 2′- and 3′-hydroxy derivatives were produced when flavanone was converted by the transformants. We have no explanation for the difference of the positions of the phenolic OH function that was introduced by the reaction. While the dihydroxy derivatives were the major products from flavone and flavanone, not dihydroxylation but monohydroxylation of the B-ring occurred when 6-hydroxyflavone and 6-hydroxyflavanone were used as substrates. We also have no explanation for this difference.

3.3. Free radical-scavenging activity of the dihydroxy products from flavone and flavanone

Antioxidant activity of flavonoids is important for their medicinal and nutritional values. We therefore, analyzed free radical-scavenging activity of flavone, flavanone and their converted products by using DPPH (Table 2). Among these compounds, 2′,3′-dihydroxyflavone and 2′,3′-dihydroxyflavanone showed free radical-scavenging activity. Flavone, flavanone and their monohydroxy products did not show any activity. These results are reasonable, because the *o*-dihydroxy structure in the B-ring was reported to be essential for the effective antioxidant activity of flavonoids [7].

Table 2
Fifty percent radical-scavenging activity concentration of some flavonoids by the DPPH method

Flavonoids	50% radical-scavenging activity concentration (μg/ml)
Flavone	>200
2′,3′-Dihydroxyflavone	39
3′-Hydroxyflavone	>200
Flavanone	>200
2′,3′-Dihydroxyflavanone	16
2′-Hydroxyflavanone	>200
3′-Hydroxyflavanone	>200

4. Conclusions

Flavone and flavanone were converted mainly to the 2′,3′-dihydroxy derivatives by *S. lividans* cells in which the modified biphenyl dioxygenase was expressed. 2′,3′-dihydroxyflavanone, a major product from flavanone, was a novel compound. The 2′,3′-dihydroxy derivatives showed free radical-scavenging activity.

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