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Synthesis of vicinal diols from various arenes with a heterocyclic, amino or carboxyl group by using recombinant *Escherichia coli* cells expressing evolved biphenyl dioxygenase and dihydrodiol dehydrogenase genes

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Abstract—Various aromatic molecules, in which heterocycles are linked with a phenyl or benzyl group, were converted to their respective 2,3-diols (catechols) in the benzene ring by growing cell reactions using recombinant *Escherichia coli*, which expressed the evolved biphenyl dioxygenase [*bphA* (2072)] genes and the subsequent bacterial dihydrodiol dehydrogenase (*bphB*) gene. These vicinal diol products showed strong in vitro inhibitory activity against the lipid peroxidation induced by free radicals and strong scavenging activity towards DPPH radicals. The vicinal diols were also synthesized from ionized monocyclic aromatics incorporating an amino or carboxyl group.

1. Introduction

We intend to develop a system for the comprehensive bioconversion of a series of organic chemicals by growing cell reactions using recombinant microbes which express different combinations of a sequence of biocatalytic genes. This intention attempts to perform comprehensive 'chemical synthesis' by using the multiple biocatalytic functions of the cells to link the diversity of genes (DNA) to that of chemicals. Figure 1 suggests the concept of such living cells-based combinatorial chemistry (CellCombiChem). CellCombiChem could significantly make it practical to synthesize chemicals, which are difficult or impractical to synthesize by chemical methods. It is also important to use an enzyme with broad substrate specificity or preference to achieve comprehensive bioconversion. Directed protein evolution could be one of the most powerful methods for generating such enzymes.[1]

It is usually difficult to introduce hydroxy group(s) into an aromatic ring *regio* or *stereo*-specifically by a chemical synthesis, although the industrial need for this is strong. For attempting such a hydroxylation reaction we have started our study on CellCombiChem with the biocatalytic genes mediating biphenyl catabolism, since carrying out the first dioxygenation reaction would be difficult by the extracted enzyme, which includes ferredoxin and ferredoxin reductase and needs $NAD(P)H^+$ that must be regenerated. The selection is also based on the following reasons: during the past 35 years, the *stereo* and *regio*-specific syntheses of *cis*-dihydrodiols (*cis*-dihydrocatechols, *cis*-cyclohexadiene-diols) have mainly been performed by toluene dioxygenase-mediated microbial conversion, so as to generate two hundred structurally diverse *cis*-dihydrodiols,[2] several of which have been applied as chiral intermediates for versatile synthetic applications by synthetic chemists worldwide.[2,3] These results show that toluene dioxygenase, which is an enzyme structurally similar to biphenyl dioxygenase, desirably has very broad substrate specificity; it may therefore be feasible to introduce hydroxy group(s) into a wide range of aromatic compounds *stereo*- and *regio*-specifically with biphenyl dioxygenase, and *regio*-specifically with the subsequent dehydrogenation enzyme. Many studies on the degradation of environmental pollutants such

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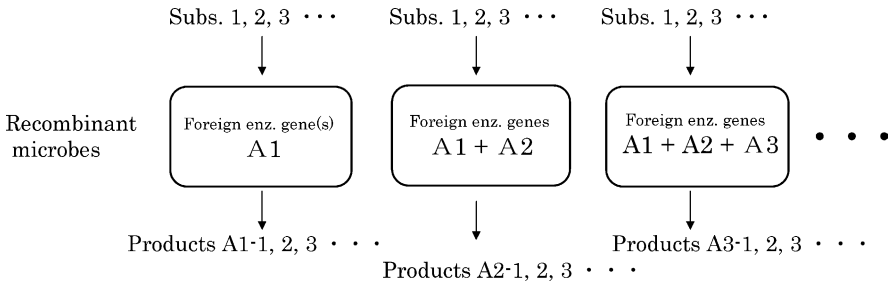


Figure 1. Concept of living cells-based combinatorial chemistry (CellCombiChem). Subs. 1, 2, 3… means a series of organic chemicals used as substrates. Foreign enz. gene(s) A1 represents foreign gene(s) coding for metabolic enzyme A1. Products A1-1, 2, 3… are generated from Subs. 1, 2, 3… through the living cells of recombinant microbes expressing the A1 gene(s). A2, which is usually a metabolic enzyme subsequent to A1, can convert A1-1, 2, 3… to A2-1, 2, 3… , which are further converted to A3-1, 2, 3… by enzyme A3.

as polychlorinated biphenyls (PCBs) have been performed by using biphenyl dioxygenases, but little work has been done on the synthesis of useful compounds by using this enzyme.[2,4]

The degradation of biphenyl and PCBs is enzymatically initiated by the action of biphenyl dioxygenase, as has been elucidated for the biphenyl-degrading bacteria, *Pseudomonas pseudoalcaligenes* KF707,[4,5] *Burkholderia* sp. strain LB400,[6] and *Sphingomonas yanoikuyae* strain B1.[2] Biphenyl dioxygenase is a multi-component enzyme consisting of BphA1, BphA2 [large α and small β subunits of iron–sulfur protein, respectively], BphA3 (ferredoxin), and BphA4 (ferredoxin reductase), as shown in Figure 2.[2,4,7–9] This enzyme (BphA) is responsible for the conversion of a biphenyl to its *cis*-dihydrodiol [(1*S*,2*R*)-dihydroxy-3-phenylcyclohexa-3,5-diene].[2,8] This compound is further converted to 3-phenylbenzene-1,2-diol by the subsequent desaturation reaction, which is catalyzed by BphB (biphenyl *cis*-dihydrodiol dehydrogenase).[7,9] The substrate-binding sites of biphenyl dioxygenase are considered to be present in its α-subunit (BphA1). This consideration is supported by the results of an X-ray structural analysis of naphthalene dioxygenase,[10] which is an enzyme with a topology slightly different from that of biphenyl dioxygenase, and by data obtained from DNA shuffling and in vitro mutagenesis experiments.[4,11] Modified *bphA1* genes were generated by DNA shuffling, using the *bphA1* genes derived from *P. pseudoalcaligenes* KF707 and *Burkholderia* sp. LB400.[11a] One of the shuffled genes, *bphA1* (2072), has been shown to mediate with broad substrate specificity, when expressed in combination with *bphA2A3A4* from *P. pseudoalcaligenes*.[8] We have shown for the first time that various molecular species, in which heterocyclic aromatics are linked with phenyl or benzyl groups, can be converted with high efficiency to the corresponding *cis*-dihydrodiols (*cis*-dihydrocatechols) by recombinant *Escherichia coli* or *Streptomyces lividans* strains carrying the evolved biphenyl dioxygenase genes [*bphA1*(2072)*A2A3A4*: *bphA* (2072)] (Fig. 3).[8,12] The *cis*-dihydrodiols generated may be capable of being used as substrates for BphB to synthesize their respective diols (catechols).

We report here the formation of vicinal diols (catechols) from various aromatic molecules, in which heterocycles including heteroaromatics are linked with phenyl or benzyl groups, through the growing cells of *E. coli* that express the *P. pseudoalcaligenes bphB* gene in addition to the *bphA* (2072) genes (Fig. 3). The antioxidative activity of these vicinal diols is examined. The vicinal diol formation from ionized monocyclic aromatics incorporating an amino or carboxyl group is also described.

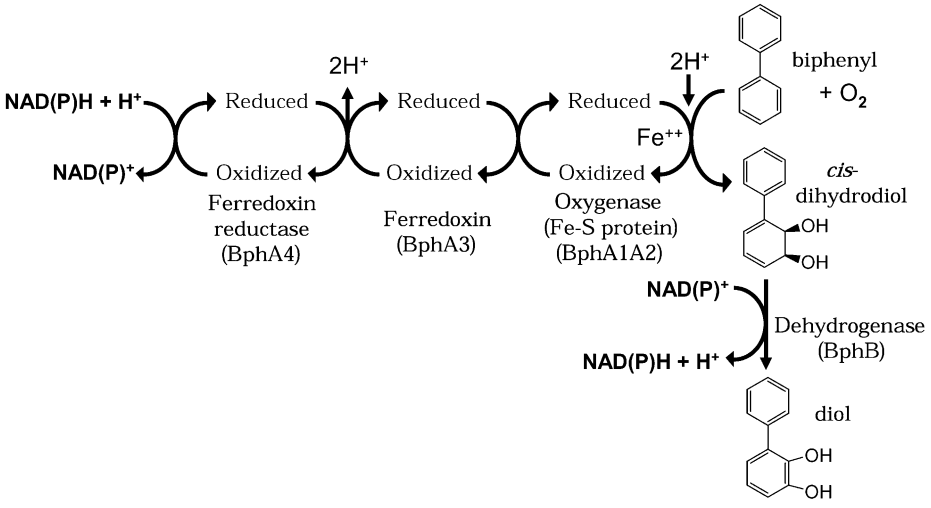


Figure 2. Catabolic pathway from biphenyl to diol (3-phenylbenzene-1,2-diol) via *cis*-dihydrodiol [(1*S*,2*R*)-dihydroxy-3-phenylcyclohexa-3,5-diene] for the biphenyl-degrading bacterium, *Pseudomonas pseudoalcaligenes* KF707. *Sphingomonas yanoikuyae* strain B1 seems to have the same pathway.[2]

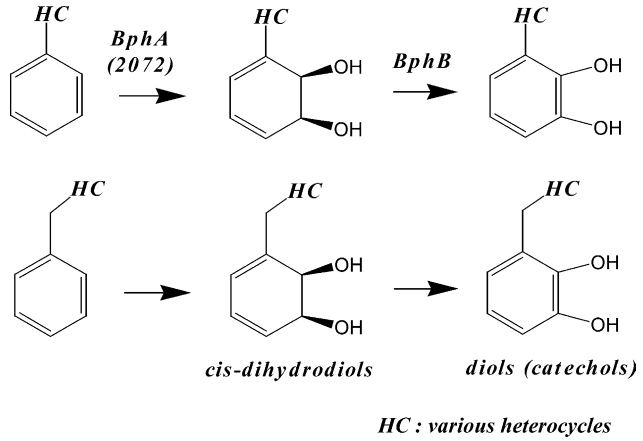


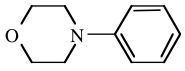
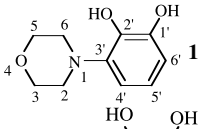
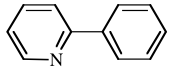
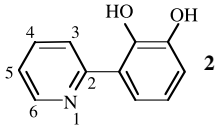
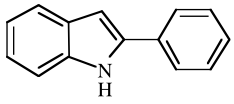
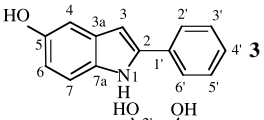
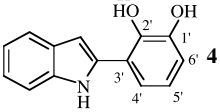
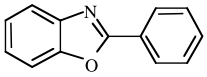
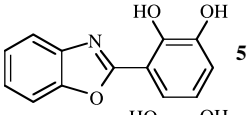
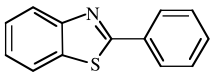
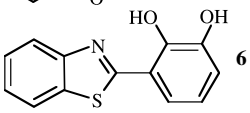
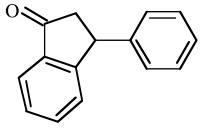
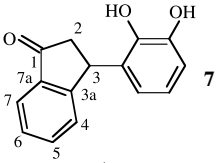
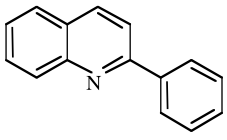
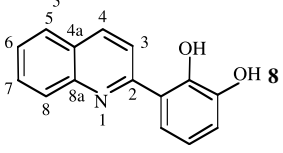
Figure 3. Bioconversion of a series of organic chemicals, in which various heterocycles are linked with phenyl or benzyl groups. The results of our study show that such compounds can be converted to the corresponding *cis*-dihydrodiols (*cis*-dihydrocatechols) by BphA (2072), these then being further converted to diols (catechols) by BphB.

2. Results and discussion

2.1. Biotransformation of heterocyclic compounds with phenyl moieties

4-Phenylmorpholine, 2-phenylpyridine, 2-phenylindole, 2-phenylbenzoxazole, 2-phenylbenzothiazole, and 2-phenylquinoline, whose structures are shown in Table 1, were used as examples of such substrates for bioconversion experiments. 3-Phenyl-1-indanone, in which not a heterocycle but a cyclic hydrocarbon substituted with oxygen is linked with a phenyl group, was also included in the experiments (Table 1). All of the substrates were converted by co-cultivation with recombinant *E. coli* cells possessing plasmid pBS2072B which carries the *bphA1*(2072)*A2A3A4* [*bphA* (2072)] and *bphB* genes. The structures of the biotransformed products were determined by HRMS (EI) and ^{1}H and ^{13}C NMR analyses, including a 2D spectral analysis. All the products were vicinal diols (catechols), except for one product converted from 2-phenylindole (Table 1). The yields of the respective purified products were 6.8–77% (Table 1). Products 2, 7 and 8 were found to be novel compounds according to the CAS database. In all the bioconversion experiments of this present study, we used 10 mg of each substrate per 100 ml culture (0.01%; w/v).

Table 1. Bioconversion of various aromatics, in which heterocycles are linked with phenyl groups, through the living cells of *E. coli* expressing the *bphA* (2072) and *bphB* genes

Substrate	Product	Yield (%)
 4-Phenylmorpholine	 **1**	30
 2-Phenylpyridine	 **2**	26
 2-Phenylindole	 **3**  **4**	9.0, 6.8
 2-Phenylbenzoxazole	 **5**	77
 2-Phenylbenzothiazole	 **6**	74
 3-Phenyl-1-indanone	 **7**	70
 2-phenylquinoline	 **8**	31

It may be thought that the substrate content is in a low level. However, the scale-up experiments in the substrates are easy to do up to 10-fold (0.1%; w/v), as needed (our unpublished results).[12]

When co-cultured with 2-phenylindole, a product with one hydroxy group in the indole ring (**3**) was obtained, in addition to the typical diol product (**4**). *E. coli* (pKF2072), which carries the *bphA1*(2072)*A2A3A4* genes, also generated the monohydroxylated product (**3**) in addition to the corresponding *cis*-dihydrodilol form (data not shown). We therefore consider that the indole ring has affinity for the active site facilitating an oxygenation reaction in the BphA (2072) enzyme and that the monohydroxy form is non-enzymatically generated from a *cis*-dihydrodiol due to the structural instability of the vicinal dihydrodiol in the indole ring. Such non-enzymatic dehydration from *cis*-dihydrodiols has often been observed.[8,9,12,13]

The results of our previous study show that heteroaromatic compounds with phenyl moieties were converted to their respective *cis*-dihydrodiols (*cis*-dihydrocatechols) when co-cultured with *E. coli* (pKF2072) carrying the *bphA* (2072) genes.[8] Examples of such substrates include 1-phenylpyrrole, 3-methyl-1-phenylpyrazole, and 4-phenylpyrimidine, in addition to the substrates shown in Table 1. 1-Phenylpyrrole, 3-methyl-1-phenylpyrazole, and 4-phenylpyrimidine are also very likely to be converted to the corresponding vicinal diols by *E. coli* (pBS2072B). Flavonoid pigments such as flavone and 6-hydroxyflavone can also be regarded as having a structure in which the heteroaromatic ring, the chromen-4-one ring, is linked to a phenyl group. We have shown that flavone and 6-hydroxyflavone were converted to their respective vicinal diols via *E. coli* or *S. lividans* carrying only the *bphA* (2072) genes[9,14] and via *E. coli* carrying the *bphA* (2072) and *bphB* genes more efficiently.[9] Seeger et al. have also shown that the isoflavonoids, 7-hydroxyisoflavone and 7-hydroxy-8-methyl-isoflavone, were biotransformed to the vicinal diol forms by *E. coli* harboring the *bphA* and *bphB* genes derived from *Burkholderia* sp. LB400.[15]

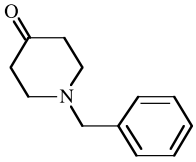
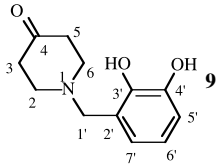
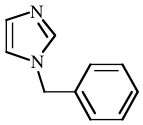
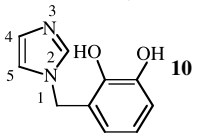
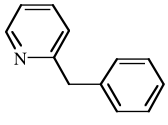
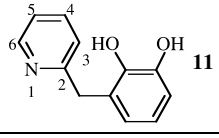
2.1.1. 4-Phenylmorpholine. The molecular formula of product **1** converted from 4-phenylmorpholine was determined to be $C_{10}H_{13}NO_3$ by HRMS (EI). In the ^{1}H NMR spectrum, signals derived from the morpholine moiety of 4-phenylmorpholine were completely preserved, while only the 3H sp^2 methine signals of the benzene ring were observed. Consistent with its molecular formula, the replacement of two phenolic OH functions in the benzene ring is proposed. The positions of these two phenolic OH functions were determined to be at C-1′ and C-2′ by the observation of a vicinal sp^2 spin network from H-4′ (δ 6.38) to H-6′ (δ 6.49) in the DQF COSY spectrum. Thus, **1** was identified to be 3-morpholin-4-ylbenzene-1,2-diol (Table 1).

2.1.2. 2-Phenylindole. The molecular formula of product **3** converted from 2-phenylindole was determined to be $C_{14}H_{11}NO$ by HRMS (EI). In the ^{1}H NMR spectrum, signals due to the phenyl moiety were completely conserved, while only 4H signals derived from the indole ring were observed. Consistent with its molecular formula, the replacement of a phenolic OH function in the indole ring is proposed. The position of this phenolic OH function was determined to be at C-5 by the observation of ^{1}H–^{13}C long-range coupling from H-4 (δ 6.82) to C-3 (δ 98.0) and the small coupling constant of H-4 (J=2.0 Hz, *meta* coupling between H-4 and H-6 (δ 6.61)). From these findings, **3** was determined to be 2-phenyl-1H-indole-5-ol (Table 1).

2.2. Biotransformation of heterocyclic compounds with benzyl moieties

Several heterocyclic compounds with benzyl moieties (1-benzylpiperidone, 1-benzylimidazole, and 2-benzylpyridine), whose structures are shown in Table 2, were also examined in the same manner and found to have been converted. The biotransformed products were identified as the vicinal diols shown in Table 2. The yields of the respective purified products were 20–68% (Table 2). Each of these three products was found to be a novel compound according to the CAS database. We have shown for the first time in the present study that heterocycles not having aromaticity and linked to a phenyl or benzyl moiety (4-phenylmorpholine and 1-benzylpiperidone) could be

Table 2. Bioconversion of several aromatics, in which heterocycles are linked with benzyl groups, through the living cells of *E. coli* expressing the *bphA* (2072) and *bphB* genes

Substrate	Product	Yield (%)
 1-Benzylpiperidone	 **9**	23
 1-Benzylimidazole	 **10**	68
 1-Benzylpyridine	 **11**	20

converted to the corresponding vicinal diols through the *E. coli* (pBS2072B) cells.

Several heteroaromatic compounds with benzyl moieties (1-benzylimidazole, 4-benzylisothiazole, and 2-benzylpyridine) have been shown to be converted to the corresponding *cis*-1,2-dihydrodiols when co-cultured with *E. coli* (pKF2072) carrying the *bphA* (2072) genes.[8] 4-Benzylisothiazole should also be converted to its vicinal diol by *E. coli* (pBS2072B) incorporating the *bphA* (2072) and *bphB* genes.

2.2.1. 1-Benzylpiperidone. The molecular formula of product **9** converted from 1-benzylpiperidone was determined to be $C_{12}H_{15}NO_3$ by HRMS (EI). In the ^{1}H NMR spectrum, signals due to the piperidone moiety of 1-benzylpiperidone were completely preserved, while only the 3H sp^2 methine signals of the benzene ring were observed. Consistent with its molecular formula, the replacement of two phenolic OH functions in benzene ring is proposed. The positions of these two phenolic OH functions were determined to be at C-3′ and C-4′ by the observation of a vicinal sp^2 spin network from H-5′ (δ 6.80) to H-7′ (δ 6.47) in the DQF COSY spectrum. From these findings, **9** was determined to be 1-(2,3-dihydroxy-benzyl)-piperidin-4-one (Table 2).

2.3. Biotransformation of a hetrocyclic aromatic compound

When heteroaromatic compounds with phenyl moieties, in which methyl or hydroxy groups are substituted, were used as the substrates for BphA (2072), these substrates were often converted to the hydroxylated forms in their heteroaromatic rings, e.g. 2-(2-hydroxyphenyl)benzoxazole was biotransformed to its *cis*-dihydrodiol in the benzoxazole ring [(4*R*,5*S*)-2-(2-hydroxyphenyl)-4,5-dihydro-1,3-benzoxazole-4,5-diol].[8] We show here that BphB was functional to produce the corresponding diol (**12**) from the *cis*-dihydrodiol (Table 3). The yield of the purified product was 20% (Table 3). This product was a novel compound.

2.3.1. 2-(2-Hydroxyphenyl)benzoxazole. The molecular formula of product **12** was determined to be $C_{13}H_9NO_4$ by HRMS (EI). In the ^{1}H and ^{13}C NMR spectra, signals due to C-1′–C-6′ were preserved, while only 2H signals derived from the benzoxazole ring were observed. Consistent with its molecular formula, the replacement of two phenolic OH functions in the benzoxazole ring is proposed. The positions of these two phenolic OH functions were determined to be at C-4 and C-5 by the coupling constant between H-4 and H-5 (J=8.5 Hz) and the ^{1}H–^{13}C long-range couplings from H-6 (δ 6.88) to C-3a (δ 129.2), C-5 (δ 142.2) and C-7a (δ 143.2). From these findings, **12** was determined to be 2-(2-hydroxyphenyl)benzoxazole-4,5-diol (Table 3).

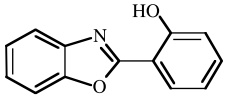
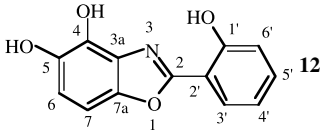
2.4. Biotransformation of aromatic compounds with an amino group

Ionized aromatic molecules incorporating carboxylic acid or primary amine moieties in their structures have frequently been used as building blocks for the chemical synthesis of pharmaceuticals, agrochemicals, and other industrially useful organic molecules. We further examined the ability of the evolved biphenyl dioxygenase [BphA (2072)] and dihydrodiol dehydrogenase (BphB) enzymes by bioconversion experiments on such ionized compounds. Primary amines such as aniline and benzylamine that incorporated an amino group in their molecules were not converted by the recombinant *E. coli* and *S. lividans* cells.[12] This could be due to their lack of permeability into the cell and/or to the stronger affinity of the amino group to the iron-incorporating active site of the enzyme. Therefore, the amino groups of aniline, benzylamine, and phenylethylamine were protected as a *tert*-butyl-carbamate by stirring with di-*tert*-butyl dicarbonate [(*t*-BOC)$_2$O] and NaOH in aqueous dioxane (Fig. 4(A)). Their protected groups should then be easy to remove by an acidic treatment. The *t*-BOC derivatives synthesized were successfully converted to the corresponding vicinal diols with high efficiency (75–85%) by the recombinant *E. coli* (pBS2072B) cells (Fig. 4(A)). All of the diol primary amines that were synthesized by the combination of biological and chemical methods are difficult to synthesize by purely chemical means. Indeed, the 3-(1-amino-ethyl)-benzene-1,2-diol was a novel compound according to the CAS database.

We used the racemic mixture of phenylethylamine as the substrates. The products (**15**) converted from their *t*-BOC derivatives were analyzed by HPLC in a Chiralcel OD-H column (10×250 mm, Daicel), which was developed with hexane-2-propanol (9:1). As a result, both the chiral products were found to generate from the *t*-BOC derivatives of racemic phenylethylamine, showing no enantio-selectivity of the *E. coli* cells expressing *bphA* (2072) and *bphB* towards bioconversion of such substrates.

2.4.1. Phenyl-carbamic acid *tert*-butyl ester. The molecular formula of product **13** was determined to be $C_{11}H_{13}NO_4$ by HRMS (EI). In the ^{1}H and ^{13}C NMR spectra, signals due to the *t*-BOC moiety (C-1′–C-6′) were preserved, while only 3H signals derived from the benzene ring were observed.

Table 3. Bioconversion of an aromatic, in which a heteroaromatic is linked with a phenyl group substituted with a hydroxyl group, through the living cells of *E. coli* expressing the *bphA* (2072) and *bphB* genes

Substrate	Product	Yield (%)
 2-(2-Hydroxyphenyl)benzoxazole	 **12**	20

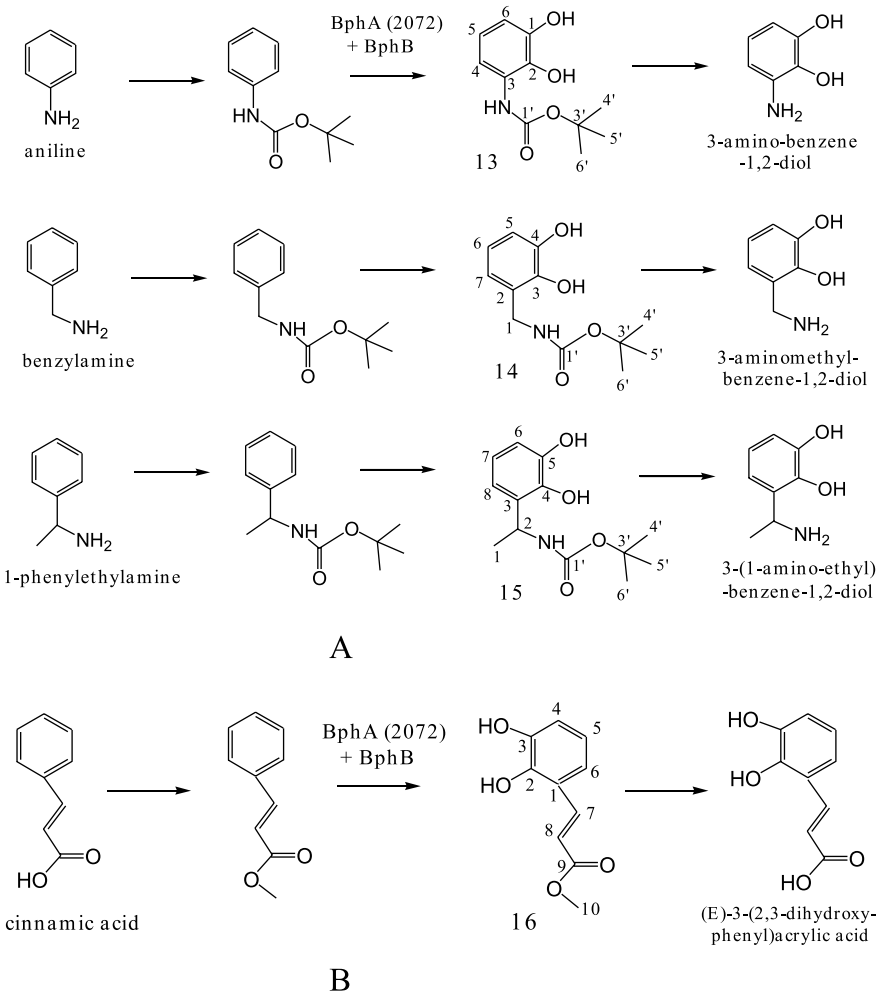


Figure 4. Bioconversion of several monocyclic aromatics incorporating an amino group (A) and a monocyclic aromatic incorporating a carboxyl group (B), by using the living cells of *E. coli* expressing the *bphA* (2072) and *bphB* genes, on the combination of the simple chemical methods.

Consistent with the molecular formula, the replacement of two phenolic OH functions in the benzene ring is proposed. The positions of these two phenolic OH functions were determined to be at C-1 and C-2 by observation of the vicinal sp^2 network of C-4 (δ 6.33)–C-5 (δ 6.66)–C-6 (δ 6.68). From these findings, **13** was determined to be (2,3-dihydroxy-phenyl)-carbamic acid *tert*-butyl ester (Fig. 4(A)).

2.5. Biotransformation of an aromatic compound with a carboxyl group

Cinnamic acid, which includes a carboxyl group in the molecule, was not converted by the recombinant *E. coli* cells. This could be due to its lack of permeability into the cells. Therefore, the carboxyl group of cinnamic acid was protected as a methyl ester by stirring in a 5% HCl–MeOH solution. The methyl ester should then be easy to hydrolyze by an alkaline treatment. The synthesized cinnamic acid methyl ester was converted to the corresponding vicinal diol with high efficiency (86%) by the recombinant *E. coli* (pBS2072B) cells (Fig. 4(B)).

2.5.1. Cinnamic acid methyl ester. The molecular formula of product **16** was determined to be $C_{10}H_{10}O_4$ by HRMS (EI). In the ^1H and ^{13}C NMR spectra, signals due to the side chain (C-7–C-10) were preserved, while only 3H signals derived from the benzene ring were observed. Consistent with its molecular formula, the replacement of two phenolic OH functions in the benzene ring is proposed. The positions of these two phenolic OH functions were determined to be at C-2 and C-3 by observation of the vicinal sp^2 network of C-4 (δ 6.83)–C-5 (δ 6.65)–C-6 (δ 7.05). From these findings, **16** was determined to be (*E*)-3-(2,3-dihydroxy-phenyl)-acrylic acid methyl ester (Fig. 4(B)).

2.6. Antioxidative activity of the converted products

The converted products described in 2.1–2.3 contained phenolic OH functions in their structures. We examined their in vitro inhibitory effects towards the lipid peroxidation induced by free radicals in a rat brain homogenate (Table 4A) and their scavenging activity towards DPPH (1,1-diphenyl-2-picrylhydrazyl) radicalsB). Each of the products showed much stronger antioxidative activity than to the corresponding substrate. Judging from the results shown in B, none of the substrates had antioxidative activity. The results suggest that our system of CellCombiChem would be effective for producing biologically active organic chemicals

Table 4

(A) Inhibitory effects of the respective compounds on lipid peroxidation in a rat brain homogenate

Substrate	IC_{50} (μg/ml)	Product	IC_{50} (μg/ml)
4-Phenylmorpholine	63	1	1.8
2-Phenylpyridine	140	2	3.5
2-Phenylindole	4.9	3	0.19
		4	0.038
2-Phenylbenzoxazole	>200	5	0.26
2-Phenylbenzothiazole	70	6	0.11
3-Phenyl-1-indanone	28	7	1.1
2-Phenylquinoline	>200	8	0.98
1-Benzylpiperidone	53	9	1.8
2-Benzylimidazole	>200	10	3.5
2-Benzylpyridine	78	11	1.6
2-(2-Hydroxyphenyl)benzoxazole	>200	12	0.18

(B) DPPH radical-scavenging activity of the respective compounds

Substrate	IC_{50} (μg/ml)	Product	IC_{50} (μg/ml)
4-Phenylmorpholine	>200	1	2.3
2-Phenylpyridine	>200	2	51
2-Phenylindole	>200	3	45
		4	30
2-Phenylbenzoxazole	>200	5	21
2-Phenylbenzothiazole	>200	6	13
3-Phenyl-1-indanone	>200	7	18
2-Phenylquinoline	>200	8	17
1-Benzylpiperidone	>200	9	33
2-Benzylimidazole	>200	10	24
2-Benzylpyridine	>200	11	51
2-(2-Hydroxyphenyl)benzoxazole	>200	12	78

which are difficult or impractical to synthesize by chemical methods.

3. Conclusions

The majority of pharmaceuticals and agrochemicals include a heterocycle not having aromaticity, an aromatic heterocycle (heteroaromatic), or a benzene ring in their molecular structures. We have shown that a wide array of aromatic molecules, in which a heterocycle with or without aromaticity is linked with a phenyl or benzyl group, can be biotransformed to the respective *cis*-2,3-dihydrodiols in the benzene ring by the growing cells of *E. coli* expressing the evolved *bphA* genes [*bphA* (2072)],[8] and further biotransformed to the respective 2,3-diols by those expressing the *bphB* genes in addition to the *bphA* (2072) genes (Fig. 3). It has also been shown that ionized monocyclic aromatics incorporating an amino group or a carboxyl group in their molecules can be converted to the respective 2,3-diols in the benzene ring, by using the *E. coli* cells that expresses the *bphA* (2072) and *bphB* genes, on the combination of the simple chemical methods. The results of this present and past study should enable CellCombiChem to be achieved when using the biphenyl-catabolic genes in the initial two steps (Fig. 1). CellCombiChem using the *bphC* gene, which mediates the third enzymatic step, in addition to the *bphA* (2072) and *bphB* genes, is already found to be successful to produce a series of aromatic compounds with a picolinic acid (pyridine-2-carboxylic acid) in their molecules, whose results will be presented elsewhere. The comprehensive production of such organic chemicals, which are difficult or impractical to chemically synthesize, could intend to serve as new starting materials for the chemical synthesis of pharmaceuticals, agrochemicals and other industrially useful compounds, or as a unique screening source for 'lead'-discoveries or ligand-hunting by pharmaceutical companies.

4. Experimental

4.1. Plasmids, bacterial strains, and chemicals

Plasmid pBS2072B, which contained the modified biphenyl dioxygenase [*bphA* (2072)] genes and the subsequent biphenyl-2,3-dihydrodiol 2,3-dehydrogenase (*bphB*) gene derived from *P. pseudoalcaligenes* KF707, has been described.[9] *E. coli* JM109 was used as the host for the plasmid. The chemicals used as substrates were purchased from Aldrich Chemical Co., Wako Pure Chemical Co., or Kanto Chemical Co. The respective substrates were each dissolved in a small volume of dimethyl sulfoxide (DMSO) and added to the culture.

4.2. Growing cell reactions

E. coli JM109 harboring pBS2072B was grown in an LB medium[16] containing Ampicillin (Ap; 150 μg/ml) at 30 °C with reciprocal shaking for 7–8 h. Five milliliters of this culture was inoculated into 100 ml of an M9 medium[16] with Ap (150 μg/ml), thiamine (10 μg/ml), and glucose (0.4%; w/v) in an Erlenmeyer flask at 30 °C with reciprocal shaking and left for 16–17 h, until the absorbance at OD 600 nm reached approximately 1. The cells were collected by centrifugation, resuspended in a fresh M9 medium (100 ml) with Ap (150 μg/ml), thiamine (10 μg/ml), 0.4% (w/v) glucose, and 1 mM (the final concentration) of isopropyl β-D-thiogalactopyranoside (IPTG) as well as each substrate (10 mg), and cultivated in the Erlenmeyer flask at 30 °C with reciprocal shaking for 2–3 days.

4.3. Extractions and HPLC analysis of the converted products

To extract the converted products and the substrates, an equal volume of methanol (MeOH) to that of the culture medium was added to the co-culture of the transformed cells of *E. coli*, and the mixture 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 (4.6×150 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.

4.4. Purification and identification of the products

The liquid phase (2,000 ml) which had been obtained by the procedure just described was concentrated in vacuo and then 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 E. Merck silica gel plates (60F-254)]. The formed products were purified by column chromatography on silica gel [20× 250 mm^2, Silica Gel 60 (Merck)]. Their structures were analyzed by mass spectrometry (MS) [MS (EI) and HRMS (EI), Jeol AX-505W instrument] and nuclear magnetic resonance (NMR) (400 MHz, Bruker AMX400 instrument).

4.4.1. 3-Morpholin-4-ylbenzene-1,2-diol (1; the product converted from 1-phenylmorpholine). The crude extract (86.5 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=30:1) to yield 30.0 mg of **1**. MS (EI) *m/z* 195 (M^+). HRMS (EI) calcd for $C_{10}H_{13}NO_3$ (M^+), 195.0896; found, 195.0899. ^{1}H NMR (DMSO-d_6) δ: 2.87 (m, 4H), 3.71 (m, 4H), 6.38 (dd, 1H, J=1.4, 7.8 Hz), 6.49 (dd, 1H, J=1.4, 8.1 Hz), 6.55 (dd, 1H, J=7.8, 8.1 Hz). ^{13}C NMR (DMSO-d_6) δ: 50.8 (C-2, C-6), 66.4 (C-3, C-5), 109.4 (C-4′), 110.5 (C-6′), 118.7 (C-5′), 138.0 (C-2′), 140.8 (C-3′), 145.5 (C-1′).

4.4.2. 3-(2-Pyridyl)benzene-1,2-diol (2; the product converted from 2-phenylpyridine). The crude extract (130.0 mg) was subjected to column chromatography (hexane–EtOAc=10:1) to yield 26.0 mg of **2**. MS (EI) *m/z* 187 (M^+). HRMS (EI) calcd for $C_{11}H_9NO_2$ (M^+), 187.0633; found, 187.0633. ^{1}H NMR ($CDCl_3$) δ: 6.92 (dd, 1H, J=1.2, 7.9 Hz), 6.75 (dd, 1H, J=7.9, 7.9 Hz), 7.18 (dd, 1H, J=5.3, 5.6 Hz), 7.27 (dd, 1H, J=1.2, 7.9 Hz), 7.76 (ddd, 1H, J=1.6, 5.3, 7.9 Hz), 7.83 (d, 1H, J=7.9 Hz), 8.42 (dd, 1H, J=1.6, 5.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 115.5 (C-6′), 116.8 (C-4′), 118.1 (C-3′), 118.6 (C-5′), 119.1 (C-3), 121.6 (C-5), 137.9 (C-4), 145.6 (C-6), 146.0 (C-1′), 147.4 (C-2′), 157.6 (C-2).

4.4.3. 2-Phenylindol-5-ol (3) and 2-indol-2-ylbenzene-1,2-diol (4) (the products converted from 2-phenylindole). The crude extract (191.0 mg) was subjected to column chromatography (hexane–EtOAc=3:1) to yield 6.8 mg of **3** and 9.0 mg of **4**.

3. MS (EI) *m/z* 209 (M^+). HRMS (EI) calcd for $C_{14}H_{11}NO$ (M^+), 209.0841; found, 209.0857. ^{1}H NMR (DMSO-d_6) δ: 6.61 (dd, 1H, J=2.0, 8.8 Hz), 6.70 (s, 1H), 6.82 (d, 1H, J=2.0 Hz), 7.17 (d, 1H, J=8.8 Hz), 7.27 (dd, 1H, J=7.7, 7.7 Hz), 7.42 (dd, 2H, J=7.7, 7.7 Hz), 7.79 (d, 2H, J=7.7 Hz), 8.70 (brs, 1H), 11.20 (brs, 1H). ^{13}C NMR (DMSO-d_6) δ: 98.0 (C-3), 103.7 (C-4), 111.7 (C-7), 112.0 (C-6), 124.8 (C-2′, C-6′), 127.2 (C-4′), 128.9 (C-3′, C-5′), 129.4 (C-3a), 131.7 (C-7a), 132.5 (C-1′), 137.9 (C-2), 150.9 (C-5).

4. MS (EI) *m/z* 225 (M^+). HRMS (EI) calcd for $C_{14}H_{11}NO_2$ (M^+) 225.0790; found, 225.0786. ^{1}H NMR ($CDCl_3$) δ: 6.74 (dd, 1H, J=1.6, 7.8 Hz), 6.80 (s, 1H), 6.80 (dd, 1H, J=7.8, 7.9 Hz), 7.06 (dd, 1H, J=7.8, 7.8 Hz), 7.13 (dd, 1H, J=7.8, 7.8 Hz), 7.25 (dd, 1H, J=1.6, 7.8 Hz), 7.36 (d, 1H, J=7.8 Hz), 7.58 (d, 1H, J=7.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 100.0 (C-3), 111.0 (C-7), 114.1 (C-6′), 119.1 (C-3′), 120.0 (C-5), 120.0 (C-4′), 120.4 (C-4), 120.8 (C-5′), 122.1 (C-6), 128.2 (C-3a), 136.3 (C-7a), 141.0 (C-2′), 143.4 (C-1′).

4.4.4. 3-Benzoxazol-2-ylbenzene-1,2-diol (5; the product converted from 2-phenylbenzoxazole). The crude extract (155.0 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=30:1) to yield 76.8 mg of **5**. MS (EI) *m/z* 227 (M^+). HRMS (EI) calcd for $C_{13}H_9NO_3$ (M^+), 227.0582; found, 227.0583. ^{1}H NMR (DMSO-d_6) δ: 6.88 (dd, 1H, J=7.8, 7.8 Hz), 7.03 (dd, 1H, J=1.8, 7.8 Hz), 7.45 (2H), 7.47 (dd, 1H, J=1.8, 7.8 Hz), 7.82 (dd, 1H, J=1.4, 8.0 Hz), 7.84 (1H). ^{13}C NMR (DMSO-d_6) δ: 110.1 (C-7), 110.5 (C-3′), 117.3 (C-4′), 119.3 (C-4), 119.5 (C-6′), 119.9 (C-5′), 125.4 (C-5), 125.8 (C-6), 139.4 (C-3a), 146.2 (C-1′), 146.9 (C-2′), 148.7 (C-7a), 162.8 (C-2).

4.4.5. 3-Benzothiazol-2-ylbenzene-1,2-diol (6; the product converted from 2-phenylbenzothiazole). The crude extract (165.0 mg) was subjected to column chromatography (hexane–EtOAc=20:1) to yield 73.5 mg of **6**. MS (EI) *m/z* 243 (M^+). HRMS (EI) calcd for $C_{13}H_9NO_2S$ (M^+), 243.0355; found, 243.0350. ^{1}H NMR (DMSO-d_6) δ: 6.82 (dd, 1H, J=7.2, 7.2 Hz), 6.96 (dd, 1H, J=1.2, 7.2 Hz), 7.44 (dd, 1H, J=7.3, 7.3 Hz), 7.53 (dd, 1H, J=7.3, 8.0 Hz), 7.54 (dd, 1H, J=1.2, 7.2 Hz), 8.05 (d, 1H, J=8.0 Hz). ^{13}C NMR (DMSO-d_6) δ: 117.7 (C-6′), 118.4 (C-3′), 118.5 (C-4′), 119.5 (C-5′), 122.0 (C-4), 122.1 (C-7), 125.2 (C-6), 126.6 (C-5), 133.9 (C-7a), 145.7 (C-2′), 146.3 (C-1′), 151.4 (C-3a), 166.4 (C-2).

4.4.6. 3-(2,3-Dihydroxyphenyl)indan-1-one (7; the product converted from 3-phenyl-1-indanone). The crude extract (148.0 mg) was subjected to column chromatography (hexane–EtOAc=2:1) to yield 70.3 mg of **7**. MS (EI) *m/z* 240 (M^+). HRMS (EI) calcd for $C_{15}H_{12}O_3$ (M^+), 240.0786; found, 240.0786. ^{1}H NMR (DMSO-d_6) δ: 2.60 (dd, 1H, J=3.4, 18.9 Hz), 3.09 (dd, 1H, J=8.2, 18.9 Hz), 4.80 (dd, 1H, J=3.4, 8.2 Hz), 6.34 (dd, 1H, J=1.9, 7.7 Hz), 6.52 (dd, 1H, J=7.5, 7.7 Hz), 6.66 (dd, 1H, J=1.9, 7.5 Hz), 7.28 (dd, 1H, J=0.8, 7.6 Hz), 7.41 (ddd, 1H, J=0.8, 7.6, 7.6 Hz), 7.60 (dd, 1H, J=7.6, 7.6 Hz), 7.65 (d, 1H, J=7.6 Hz). ^{13}C NMR (DMSO-d_6) δ: 39.5 (C-3), 44.3 (C-2), 113.8 (C-6′), 118.7 (C-4′), 118.9 (C-5′), 122.6 (C-7), 126.5 (C-4), 127.4 (C-6), 130.1 (C-3′), 136.3 (C-7a), 143.2 (C-2′), 145.2 (C-1′), 158.2 (C-3a), 205.8 (C-1).

4.4.7. 3-(2-Quinolyl)benzene-1,2-diol (8; the product converted from 2-phenylquinoline). The crude extract (210.0 mg) was subjected to column chromatography (hexane–EtOAc=10:1) to yield 31.3 mg of **8**. MS (EI) *m/z* 237 (M^+). HRMS (EI) calcd for $C_{15}H_{11}NO_2$ (M^+), 237.0790; found, 237.0788. ^{1}H NMR (DMSO-d_6) δ: 6.78 (dd, 1H, J=7.9, 7.9 Hz), 7.64 (d, 1H, J=7.9 Hz), 7.65 (dd, 1H, J=7.2, 7.2 Hz), 7.84 (dd, 1H, J=7.2, 8.0 Hz), 8.03 (d, 1H, J=8.0 Hz), 8.04 (d, 1H, J=7.2 Hz), 8.33 (d, 1H, J=8.7 Hz), 8.56 (d, 1H, J=8.7 Hz). ^{13}C NMR (DMSO-d_6) δ: 117.5 (C-6′), 117.9 (C-4′), 118.2 (C-3), 118.3 (C-5′), 118.7 (C-3′), 126.3 (C-4a), 126.8 (C-8), 127.0 (C-6), 128.0 (C-5), 131.0 (C-7), 138.4 (C-4), 143.9 (C-8a), 146.6 (C-1′), 149.1 (C-2′), 158.2 (C-2).

4.4.8. 1-[(2,3-Dihydroxyphenyl)methyl]piperidin-4-one (9; the product converted from 1-benzylpiperidone).

The crude extract (104.0 mg) was subjected to column chromatography (hexane–EtOAc = 1:1) to yield 22.5 mg of **9**. MS (EI) *m/z* 221 (M^+). HRMS (EI) calcd for $C_{12}H_{15}NO_3$ (M^+), 221.1053; found, 221.1055. ^{1}H NMR ($CDCl_3$) δ: 2.46 (t, 2H, J = 5.8 Hz), 2.83 (t, 2H, J = 5.8 Hz), 3.76 (s, 2H), 6.47 (dd, 1H, J = 0.9, 7.5 Hz), 6.65 (dd, 1H, J = 7.5, 7.5 Hz), 6.80 (dd, 1H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$) δ: 40.7 (C-3, C-5), 52.5 (C-2, C-6), 60.3 (C-1′), 114.4 (C-5′), 119.7 (C-7′), 119.9 (C-6′), 120.8 (C-2′), 144.2 (C-3′), 144.7 (C-4′), 206.8 (C-4).

4.4.9. 3-(Imidazolylmethyl)benzene-1,2-diol (10; the product converted from 1-benzylimidazole). The crude extract (140.0 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 10:1) to yield 67.7 mg of **10**. MS (EI) *m/z* 190 (M^+). HRMS (EI) calcd for $C_{10}H_{10}N2O_2$ (M^+), 190.0742; found, 190.0748. ^{1}H NMR (DMSO-d_6) δ: 5.05 (s, 1H), 6.47 (d, 1H, J = 7.5 Hz), 6.57 (dd, 1H, J = 7.3, 7.3 Hz), 6.72 (d, 1H, J = 7.3 Hz), 6.85 (brs. 1H), 7.11 (brs, 1H), 7.65 (brs, 1H). ^{13}C NMR (DMSO-d_6) δ: 45.0 (C-1′), 115.1 (C-5′), 119.0 (C-6′), 119.5 (C-7′), 119.7 (C-5), 124.6 (C-2′), 128.1 (C-4), 137.4 (C-2), 143.2 (C-3′), 145.2 (C-4′).

4.4.10. 3-(2-Pyridylmethyl)benzene-1,2-diol (11; the product converted from 2-benzylpyridine). The crude extract (109.7 mg) was subjected to column chromatography (hexane–EtOAc = 10:1) to yield 20.0 mg of **11**. MS (EI) *m/z* 201 (M^+). HRMS (EI) calcd for $C_{12}H_{11}NO_2$ (M^+), 201.0790; found, 201.0789. ^{1}H NMR (DMSO-d_6) δ: 4.00 (s, 2H), 6.54 (d, 1H, J = 7.2 Hz), 6.54 (d, 1H, J = 7.2 Hz), 6.64 (dd, 1H, J = 7.2, 7.2 Hz), 7.21 (dd, 1H, J = 4.8, 7.6 Hz), 7.22 (d, 1H, J = 7.6 Hz), 7.70 (ddd, 1H, J = 1.6, 7.6, 7.6 Hz), 8.45 (dd, 1H, J = 1.6, 4.8 Hz). ^{13}C NMR (DMSO-d_6) δ: 38.6 (C-1′), 113.9 (C-6′), 118.9 (C-5′), 120.9 (C-7′), 121.4 (C-5), 122.8 (C-3), 126.6 (C-2′), 137.0 (C-4), 143.4 (C-3′), 145.6 (C-4′), 148.4 (C-6), 160.8 (C-2).

4.4.11. 2-(2-Hydroxyphenyl)benzoxazole-4,5-diol (12; the product converted from 2′-hydroxy-2-phenylbenzoxazole). The crude extract (185.0 mg) was subjected to column chromatography (hexane–EtOAc = 4:1) to yield 19.5 mg of **12**. MS (EI) *m/z* 243 (M^+). HRMS (EI) calcd for $C_{13}H_9NO_4$ (M^+), 243.0532; found, 243.0531. ^{1}H NMR (DMSO-d_6) δ: 6.88 (d, 1H, J = 8.5 Hz), 7.05 (dd, 1H, J = 7.8, 8.5 Hz), 7.06 (d, 1H, J = 8.5 Hz), 7.10 (d, 1H, J = 8.5 Hz), 7.48 (ddd, 1H, J = 1.6, 8.5, 8.5 Hz), 7.93 (dd, 1H, J = 1.6, 7.8 Hz). ^{13}C NMR (DMSO-d_6) δ: 100.1 (C-6), 110.4 (C-2′), 114.3 (C-7), 117.2 (C-6′), 119.8 (C-4′), 126.9 (C-3′), 129.2 (C-3a), 133.5 (C-5′), 135.8 (C-4), 142.2 (C-5), 143.2 (C-7a), 157.6 (C-1′), 160.9 (C-2).

4.4.12. (2,3-Dihydroxy-phenyl)-carbamic acid *tert*-butyl ester (13; the product converted from phenyl-carbamic acid *tert*-butyl ester). The crude extract (132.5 mg) was subjected to column chromatography (hexane–EtOAc = 5:1) to yield 30.0 mg of **13**. MS (EI) *m/z* 225 (M^+). HRMS (EI) calcd for $C_{11}H_{13}NO_4$ (M^+), 225.1002; found, 225.1001. ^{1}H NMR ($CDCl_3$) δ: 1.46 (s, 9H), 6.33 (dd, 1H, J = 1.9, 7.0 Hz), 6.66 (dd, 1H, J = 7.0, 7.7 Hz), 6.68 (dd, 1H, J = 1.9, 7.7 Hz). ^{13}C NMR (CDCl3) δ: 28.2 (C-4′, C-5′, C-6′), 82.8 (C-3′), 111.2 (C-4), 112.6 (C-6), 120.8 (C-5), 125.1 (C-3), 135.2 (C-2), 147.6 (C-1), 155.6 (C-1′).

4.4.13. (2,3-Dihydroxy-benzyl)-carbamic acid *tert*-butyl ester (14; the product converted from benzyl-carbamic acid *tert*-butyl ester). The crude extract (86.5 mg) was subjected to column chromatography (hexane–EtOAc = 4:1) to yield 8.6 mg of **14**. MS (EI) *m/z* 239 (M^+). HRMS (EI) calcd for $C_{12}H_{17}NO_4$ (M^+), 239.1158; found, 239.1156. ^{1}H NMR ($CDCl_3$) δ: 1.37 (s, 9H), 4.12 (d, 2H, J = 6.9 Hz), 5.23 (brs, 1H), 5.79 (s, 1H), 6.52 (d, 1H, J = 7.5 Hz), 6.67 (dd, 1H, J = 7.5, 7.8 Hz), 6.81 (d, 1H, J = 7.8 Hz), 9.53 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 28.3 (C-4′, C-5′, C-6′), 50.0 (C-1), 81.6 (C-3′), 114.6 (C-5), 120.3 (C-6), 121.3 (C-7), 125.1 (C-2), 142.7 (C-3), 146.4 (C-4), 158.8 (C-1′).

4.4.14. [1-(2,3-Dihydroxy-phenyl)-ethyl]-carbamic acid *tert*-butyl ester [15; the product converted from (1-phenyl-ethyl)-carbamic acid *tert*-butyl ester]. The crude extract (183 mg) was subjected to column chromatography (CH_2Cl_2–EtOAc = 5:1) to yield 7.6 mg of **15**. MS (EI) *m/z* 253 (M^+). HRMS (EI) calcd for $C_{13}H_{19}NO_4$ (M^+), 253.1314; found, 253.1312. ^{1}H NMR ($CDCl_3$) δ: 1.37 (s, 9H), 1.49 (d, 3H, J = 6.9 Hz), 4.86 (d, 1H, J = 8.0 Hz), 4.92 (dq, 1H, J = 6.9, 8.0 Hz), 5.88 (s, 1H), 6.66 (dd, 1H, J = 1.3, 7.7 Hz), 6.75 (dd, 1H, J = 6.9, 7.7 Hz), 6.79 (dd, J = 1.3, 6.9 Hz), 9.37 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 19.4 (C-1), 28.3 (C-4′, C-5′, C-6′), 43.6 (C-2), 81.4 (C-3′), 113.8 (C-6), 116.1 (C-8), 120.7 (C-7), 129.7 (C-3), 142.0 (C-4), 146.5 (C-5), 157.9 (C-1′).

4.4.15. (*E*)-3-(2,3-Dihydroxy-phenyl)-acrylic acid methyl ester (16; the product converted from cinnamic acid methyl ester). The crude extract (130 mg) was subjected to column chromatography (CH_2Cl_2–MeOH = 40:1) to yield 80.0 mg of **16**. MS (EI) *m/z* 194 (M^+). HRMS (EI) calcd for $C_{10}H_{10}O_4$ (M^+), 194.0579; found, 194.0581. ^{1}H NMR ($CDCl_3$) δ: 3.69 (s, 3H), 6.55 (d, 1H, J = 15.8 Hz), 6.65 (dd, 1H, J = 7.8, 8.0 Hz), 6.83 (d, 1H, J = 7.8 Hz), 7.05 (d, 1H, J = 8.0 Hz), 7.90 (d, 1H, J = 15.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 51.3 (C-10), 116.7 (C-8), 116.9 (C-4), 118.8 (C-6), 119.2 (C-5), 121.3 (C-1), 140.4 (C-7), 145.5 (C-2), 145.7 (C-3), 167.2 (C-9).

4.5. In vitro inhibitory activity against lipid peroxidation

4.5.1. Inhibitory effect on lipid peroxidation. In vitro inhibitory activity of the respective diols against lipid peroxidation were measured using a rat brain homogenate as described.[9]

4.5.2. DPPH radical-scavenging activity. The scavenging activity towards DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals was measured essentially according to the method described by Kubo et al.[17] A portion of the sample solution (100 μl) was mixed with a 100 mM MES buffer (pH 6.0, 50 μl) and 1 mM DPPH in ethanol (50 μl). The mixture was shaken vigorously and then left to stand for 30 min at room temperature in the dark. The absorbance at 515 nm (A_{515}) by DPPH was measured by UV–vis spectrophotometry. The concentration of the sample resulting in 50% radical-scavenging activity was determined.

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