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Hydroxylation of various molecules including heterocyclic aromatics using recombinant *Escherichia coli* cells expressing modified biphenyl dioxygenase genes

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Abstract—Various molecules, in which heterocyclic aromatics are linked with phenyl or benzyl groups, were converted to their corresponding *cis*-dihydrodiols by recombinant *Escherichia coli* cells expressing modified biphenyl dioxygenase genes. Heterocyclic aromatic compounds with substituted phenyl or aliphatic moieties were also biotransformed to the hydroxylated products by the cells. Many of the converted products were novel compounds. These compounds are potentially useful as versatile starting materials for the chemical synthesis of pharmaceuticals and biologically active organic molecules.

1. Introduction

Since the first report by Gibson's group in 1968 that cyclohexadiene-*cis*-diols were generated through microbial oxidation of benzene and toluene,[1] numerous *cis*-dihydrodiols (*cis*-diols) have been produced. To date over two hundred structurally diverse arene-*cis*-diols have been generated by bacterial dioxygenation enzymes.[2,3] Since the late 1980's these *cis*-diols have been shown to be used as chiral synthons for the enantiospecific synthesis of versatile natural products and therapeutic agents.[2–7]

The enzymes vital to dihydrodiol formation consist of ferredoxin, ferredoxin reductase, and an iron–sulfur protein composed of large (α) and small (β) subunits. Introduction of both molecular oxygen atoms into an aromatic nucleus occurs in an NAD(P)H-dependent manner.[2,8] The genes coding for the four components of the enzyme were isolated first from *Pseudomonas pseudoalcaligenes* KF707 (biphenyl dioxygenase),[9] and then from many other bacterial species including *P. putida* F1 (toluene dioxygenase),[10] *Pseudomonas* sp. strain 9816-4 (naphthalene dioxygenase),[11] *Nocardioides* sp. strain KP7 (phenanthrene dioxygenase),[12] and *Burkholderia cepacia* LB400 (biphenyl dioxygenase).[13] These genes have frequently been expressed in *Escherichia coli*, and used to oxidize a variety of arenes to their corresponding *cis*-diols in significant yields.[5,6,10] The substrates for the toluene-dioxygenase-mediated *cis*-dihydroxylation include a great number of monocyclic aromatics with benzene skeleton, whose hydrogen(s) is (are) substituted preferentially with smaller group(s) such as methyl, ethyl, vinyl, carboxyl, and halogenated groups.[2,3] Polycyclic-fused-aromatic hydrocarbons including naphthalene and phenanthrene have also been transformed to their corresponding *cis*-diols by naphthalene and phenanthrene dioxygenases.[2,3,8,14] Linked aromatics such as biphenyl and its chlorinated substituents have been converted to the *cis*-diols by biphenyl dioxygenase.[3,9,13,15] *cis*-Diol formation from dicyclic (bicyclic)- or tricyclic-fused heterocyclic aromatics (heteroaromatics) such as quinoline, dibenzofuran and phenanthridine by arene dioxygenase has also been reported.[2,3,16] These bioconversion experiments have been performed by worldwide research groups for over three decades. However, no reports exist that describe *cis*-diol formation from a heterocyclic aromatic molecule, in which a heteroaromatic ring is linked with a bulky group such as phenyl group.

In this article we describe the bioconversion of a variety of such heterocyclic aromatic molecules using the cells of an *E. coli* transformant that carries biphenyl dioxygenase genes modified with DNA shuffling. The resulting products from the *cis*-dihydroxylation described should be important as

Keywords: heterocyclic aromatics; *cis*-dihydrodiol; biphenyl dioxygenase; *Escherichia coli*.

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Table 1. Bioconversion of various aromatic compounds by several recombinant *E. coli* cells

Substrates	Plasmids			
	pHA171 (PDO)	pJHF3051 (TDO)	pKF6622 (BDO)	pKF2072 (modified BDO)
n-Butylbenzene	–	48	72	49
t-Butylbenzene	–	89	14	87
Biphenyl	–	63	75	100
4-Methoxybiphenyl	–	–	64	68
Diphenylmethane	–	35	72	94
1-Methoxynaphthalene	77	–	–	62
Anthracene	65	–	13	13
Phenanthrene	100	–	–	–
Fluorene	58	–	–	–
Dibenzofuran	16	–	–	13
Dibenzothiophene	9	–	–	8
Carbazole	42	–	–	–
Xanthene	–	–	–	11
Phenothiazine	–	–	–	–
Acridine	6	–	–	–
Phenanthridine	90	–	–	–
1-Phenylpyrrole	–	75	100	100
1-Phenylpyrazole	–	–	–	47
3-Methyl-1-phenylpyrazole	–	–	63	100
2-Phenylpyridine	–	–	–	14
4-Phenylpyrimidine	–	23	–	100
2-Phenylindole	–	–	23	71
2-Phenylbenzoxazole	–	–	45	100
2-Phenylbenzothiazole	–	–	81	36
3-Phenyl-1-indanone	–	–	93	97
2-Phenylquinoline	–	–	53	89

PDO, phenanthrene dioxygenase; TDO, toluene dioxygenase; BDO, biphenyl dioxygenase; the number denotes the conversion ratio (%).

chiral intermediates for the chemical synthesis of various therapeutic agents and agrochemicals, since many of them include heterocycles in their molecular structure.

2. Results and discussion

2.1. Construction of evolved biphenyl dioxygenase genes

The *bphA1* gene encodes the large subunit (ion–sulfur protein) of biphenyl dioxygenase from *P. pseudoalcaligenes* KF707 and *B. cepacia* LB400. Shuffled *bphA1* gene variants were constructed by DNA shuffling of both the corresponding genes, *bphA1* (KF707) and *bphA1* (LB400).[17,18] Shuffled variants were digested with *Sac*I and *Bgl*II, and inserted into plasmid pJHF18Δ*Mul*I just upstream of the *bphA2A3A4BC* genes of *P. pseudoalcaligenes* KF707,[17,19] and then introduced into *E. coli*. The resultant transformants were assayed for the production of ring *meta*-cleavage products (yellow) from polychlorinated biphenyls (PCBs).[17] One was found that has the double-intensified *meta*-cleavage activity compared with the *E. coli* strains carrying *bphA1* (KF707) and *bphA1* (LB400) solely. This evolved *bphA1*-carrying *E. coli* strain was also found to convert benzene, toluene, and ethylbenzene, compared to the *E. coli* strains expressing the *bphA1* parent genes, which showed negligible conversion.[20] Plasmid DNA was prepared from the cells including the evolved *bphA1* gene, and then the 1.43 kb *Ppu*MI fragment was eliminated to disrupt the *bphB* and *bphC* genes. The resultant plasmid carrying the shuffled (evolved) *bphA1*::*bphA2A3A4* genes, which code for holoenzyme of biphenyl dioxygenase, was designated pKF2072. The amino acid sequences of the modified BphA1 [BphA1 (2072)] was different by four and fifteen amino acids compared with those of BphA1 (KF707) and BphA1 (LB400), respectively. In this BphA1 (2072), His255, Val258, Gly268, and Phe277 of BphA1 (KF707) were changed to Gln255, Ile258, Ala268, and Tyr277 of BphA1 (LB400), respectively.[21]

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

Three distinctive genes encoding arene dioxygenases exist. These are the *phdABCD* genes encoding phenanthrene dioxygenase (plasmid pHA171) derived from the marine bacterium *Nocardioides* sp. strain KP7,[12] the *todC1C2BA* genes coding for toluene dioxygenase (plasmid pJHF3051) derived from *P. putida* F1,[22,23] and the *bphA1A2A3A4* genes encoding biphenyl dioxygenase (plasmid pKF6622) from *P. pseudoalcaligenes* KF707.[9,16] Bioconversion (biotransformation) experiments were performed with various aromatic compounds as substrates, using the *E. coli* cells expressing the three arene dioxygenase-encoding genes as well as the *E. coli* cells expressing the shuffled biphenyl dioxygenase genes (plasmid pKF2072). The substrates and cells were co-cultured for 2–3 days, after which the ratio of the products converted from the substrates was measured by high performance liquid chromatography (HPLC). Results are shown in Table 1. *E. coli* (pHA171) preferentially converted tricyclic-fused aromatics such as anthracene, phenanthrene, and phenanthridine. The structures of the converted products have been described previously.[16] Both strains of *E. coli* (pJHF3051) and *E. coli* (pKF6622) were incapable of transforming tricyclic-fused aromatics except for anthracene in the latter strain, but capable of converting monocyclic aromatic hydrocarbons with one large substituent such as *n*-butyl benzene, *t*-butyl benzene, biphenyl,

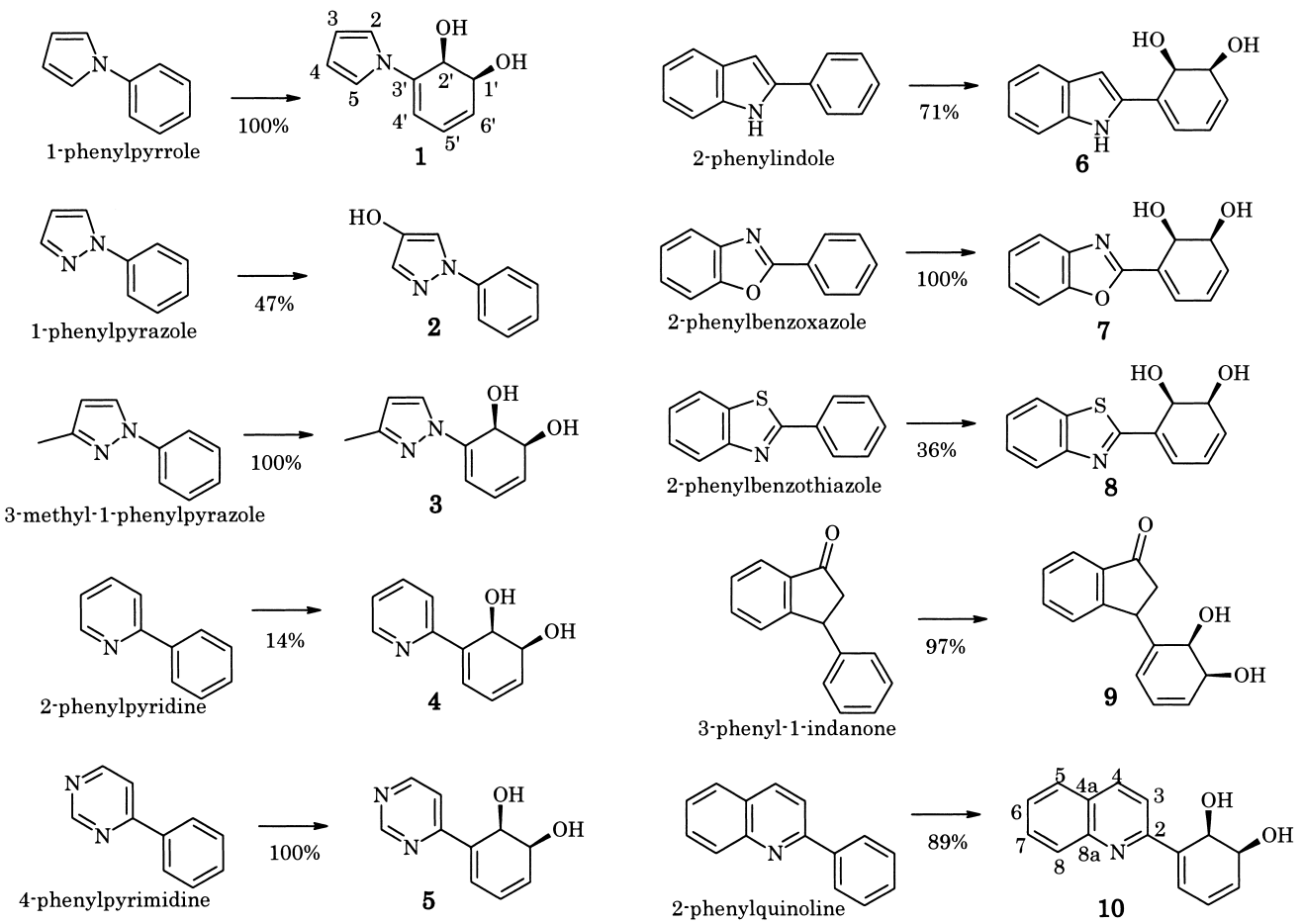

Figure 1. Bioconversion of various heteroaromatic compounds, in which heterocyclic molecules are linked with phenyl group, using the cells of *E. coli* carrying the modified biphenyl dioxygenase genes. The number below arrows shows the conversion ratio. All the converted products were novel compounds except for **2**.

and diphenylmethane. *E. coli* (pHA171) did not perform these conversions. It is notable that a difference existed between *E. coli* (pJHF3051) and *E. coli* (pKF6622) in their ability to convert heterocyclic forms, in which heteroaromatic molecules are linked with phenyl group (below 1-phenylpyrrole in Table 1), the latter being able to convert many of these compounds. *E. coli* (pKF2072) was found to have an extended substrate specificity, in comparison with the parent strain *E. coli* (pKF6622). Specifically, this *E. coli* strain harboring pKF2072 transformed all of the heterocyclic compounds (below 1-phenylpyrrole in Table 1) significantly, including 1-phenylpyrazole, 2-phenylpyridine, and 4-phenylpyrimidine that *E. coli* (pKF6622) did not convert. These cells of *E. coli*(pKF2072) were used for further experiments.

2.3. Biotransformation of heteroaromatic compounds including phenyl or benzyl moieties

Biphenyl is a linked form of two phenyl groups. Although not reported to date it could be considered that one phenyl group linked with a heterocyclic-aromatic ring could also act as a substrate for biphenyl dioxygenase. 1-Phenylpyrrole, 1-phenylpyrazole, 3-methyl-1-phenylpyrazole, 2-phenylpyridine, 4-phenylpyrimidine, 2-phenylindole, 2-phenylbenzoxazole, 2-phenylbenzothiazole, 3-phenyl-1-indanone, and 2-phenylquinoline, the structures of which are shown in Fig. 1, were used as examples of such substrates for bioconversion experiments. The structures of products converted through the cells of *E. coli* (pKF2072) were determined through HRMS (EI) and ^{1}H and ^{13}C NMR

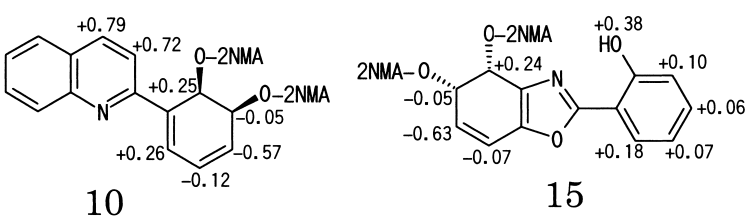

Figure 2. Application of modified Mosher's method in determination of the absolute configuration of (1*S*,2*R*)-3-(2-quinolyl)-3,5-cyclohexadiene-1,2-diol (**10**) and (4*R*,5*S*)-2-(2-hydroxyphenyl)-4,5-dihydro-1,3-benzoxazole-4,5-diol (**15**). The assignments of H-5–H-8 in **10** were obscured by the overlappings of naphthalene signals of 2NMA.

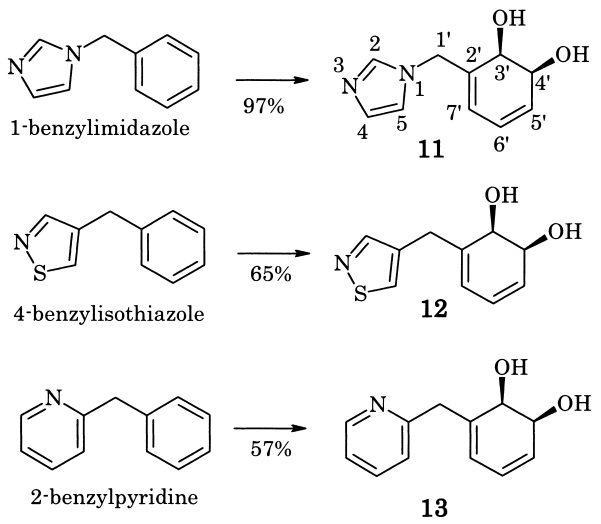

Figure 3. Bioconversion of several heteroaromatic compounds, in which heterocyclic molecules are linked with benzyl group, using the cells of *E. coli* carrying the modified biphenyl dioxygenase genes. The number below arrows shows the conversion ratio. These converted products were novel compounds.

analysis including 2D spectral analysis. All the products except for the product derived from 1-phenylpyrazole were the corresponding *cis*-1,2-dihydrodiol forms (Fig. 1). Taking into account the characteristics of the dioxygenase enzymes,[3] the absolute diastereomeric forms of the products are also illustrated in Fig. 1. The absolute stereochemistry of 3-(2-quinolyl)-3,5-cyclohexadiene-1,2-diol (**10**) (a conversion product of 2-phenylquinoline) was determined as a representative by ^{1}H NMR analysis of diastereomeric esters formed with (*R*) and (*S*)-methoxy-(2-naphthyl)acetic acid (2NMA). $\Delta(\delta R-\delta S)$ Values are summarized in Fig. 2. The sign of $\Delta\delta$ are systematically arranged right and left sides to the 2NMA planes. From these results, the absolute configurations of C-1′ and C-2′ in **10** were demonstrated to be *S* and *R*, respectively, as expected. It was also surprising that heteroaromatic compounds, in which phenyl group are linked with more bulky dicyclic-fused heteroaromatics, were efficiently converted (**6**–**10**) (Fig. 1). Several heteroaromatic compounds including benzyl moieties (1-benzylimidazole, 4-benzylisothiazole, and 2-benzylpyridine), instead of phenyl moieties, were also examined in the same manner. The converted products were identified as the corresponding *cis*-1,2-dihydrodiol forms as shown in Fig. 3. These results show that the recombinant *E. coli* (pKF2072) possessing the modified biphenyl dioxygenase enzyme is able to convert a broad range of heteroaromatic compounds with phenol and benzyl moieties into *cis*-diols.

The molecular formula of product (**2**) converted from 1-phenylpyrazole was determined to be $C_9H_8N_2O$ by HRMS (EI) as well as ^{1}H and ^{13}C NMR spectral data. In the ^{1}H NMR spectrum, signals derived from the phenyl moiety of 1-phenylpyrazole were completely preserved, while only 2H signals of pyrazole ring was observed. Consistent with its molecular formula, the replacement of a phenolic OH function in pyrazole ring was proposed. The position of the phenolic OH was determined to be C-4 by the comparison of ^{13}C NMR data of **2** with that of pyrazole (Fig. 1). The structure of **2** was confirmed by the comparison with previously reported NMR data.[24] Only this compound, among many of the examined heterocyclic compounds with phenol or benzyl moiety, was converted not to *cis*-diol but to a compound with hydroxyl group in a heterocycle ring by the *E. coli* transformant. We consider that the pyrazole ring may have stronger affinity for the active site facilitating an oxygenation reaction in this enzyme compared with a phenyl ring. It is likely that substitution of one methyl group in pyrazole ring reduces its affinity as shown in an example of **3**.

2.4. Biotransformations of other heteroaromatic compounds

We further examined the ability of the modified biphenyl dioxygenase enzyme via bioconversion experiments. Heteroaromatic compounds with phenyl moieties, in which methyl or hydroxyl groups are substituted, and heteroaromatic compounds with one aliphatic chain instead of phenyl or benzyl moieties were used as substrates. These substrates were converted to the hydroxylated forms, as shown in Fig. 4.

2.4.1. 2-*p*-Tolylpyridine. The molecular formula of the product (**14**) was determined to be $C_{12}H_{11}NO$ through its HRMS (EI) and ^{1}H and ^{13}C NMR spectral data. In DQF COSY spectrum of **14**, signals due to *p*-tolyl moiety and vicinal spin network H-4 (δ 7.29)–H-5 (δ 7.15)–H-6 (δ 8.11) in the pyridine ring were observed. From these

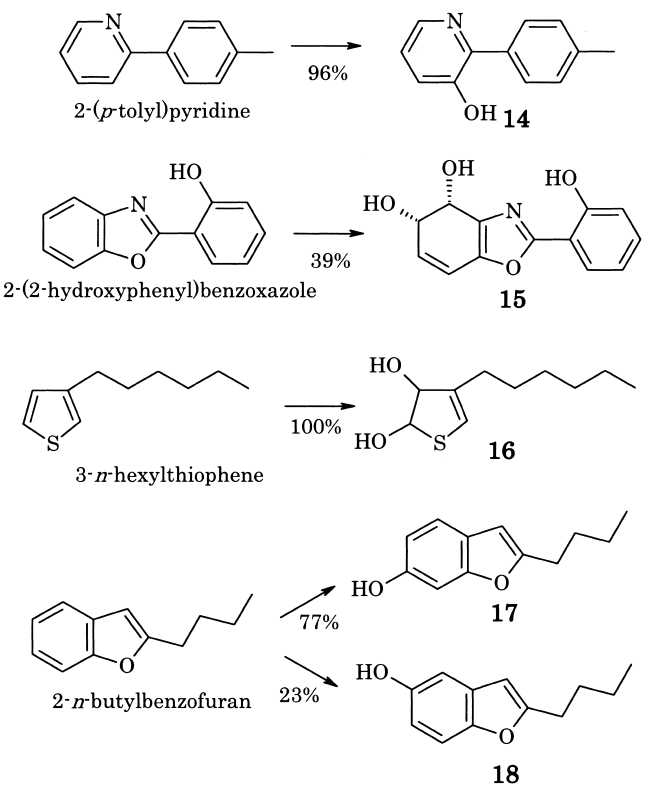

Figure 4. Bioconversion of several heteroaromatic compounds, in which heterocyclic molecules are linked with substituted phenyl or aliphatic groups, using the cells of *E. coli* carrying the modified biphenyl dioxygenase genes. The number below arrows shows the conversion ratio. **15**, **17**, and **18** were novel compounds.

findings, **14** was identified as 2-(4-methylphenyl)-3-pyridiol (Fig. 4).

2.4.2. 2-(2-Hydroxyphenyl)benzoxazole. The molecular formula of the product (**15**) was determined to be $C_{13}H_{11}NO_4$ through HRMS (EI) and ^1H and ^{13}C NMR spectral data. Analysis by $^1H-^{13}C$ COSY and DQF COSY spectra showed that **15** was dihydrodiol derivative of benzoxazole ring. The 4,5-diol regiochemical assignment was confirmed by the long range $^1H-^{13}C$ connectivities observed between H-6 (δ 5.95) and C-7a (δ 145.5) and H-7 (δ 6.57) and C-3a (δ 135.8) in HMBC spectrum. Thus, compound **15** was identified as 2-(2-hydroxyphenyl)-4,5-dihydro-1,3-benzoxazole-*cis*-4,5-diol as shown in Fig. 4. The absolute stereochemistry of **15** was determined by ^1H NMR analysis of diastereomeric esters formed with (*R*) and (*S*)-2NMA. $\Delta(\delta R-\delta S)$ values are summarized in Fig. 2. The sign of $\Delta\delta$ are systematically arranged right and left sides to the 2NMA planes. From these results, the absolute configurations of C-4 and C-5 in **15** were determined to be *R* and *S*, respectively.

2.4.3. 3-*n*-Hexylthiophene. The molecular formula of the product (**16**) was determined to be $C_{10}H_{18}O_2S$ through HRMS (EI) and ^1H and ^{13}C NMR spectral data. Analysis by ^1H, ^{13}C and DQF COSY spectra determined that **16** was 4-hexyl-2,3-dihydro-2,3-thiophenediol as shown in Fig. 4.

2.4.4. 2-*n*-Butylbenzofuran. The molecular formulas of both products (**17**, **18**) were determined to be $C_{12}H_{14}O_2$ through HRMS (EI) and ^1H and ^{13}C NMR spectral data. Analyses by ^1H, ^{13}C and DQF COSY spectra revealed that a phenolic OH function was replaced in benzofuran ring of 2-butylbenzofuran in **17** and **18**. In the HMBC spectrum of **17**, $^1H-^{13}C$ long range couplings from H-4 (δ 7.24) to C-6 (δ 152.4) and C-7a (δ 155.4) were observed. Therefore, **17** was identified to be 2-butybenzo[*b*]furan-6-ol (Fig. 4). In the HMBC spectrum of **18**, $^1H-^{13}C$ long range couplings from H-7 (δ 7.21) to C-5 (δ 152.1) and C-3a (δ 129.7) were observed. Therefore, **18** was determined to be 2-butybenzo[*b*]furan-5-ol (Fig. 4).

3. Conclusions

Enzyme-mediated formation of cyclohexadiene-*cis*-diols from benzene rings in a wide range of heterocyclic-aromatic compounds including phenyl and benzyl moieties has been shown in this study for the first time. The enantiomerically pure metabolites are easily prepared using the recombinant bacterial cells that carry the modified biphenyl dioxygenase genes, and purified by simple column chromatography, as shown in Section 4. Such *cis*-diols including hetero-aromatics seem to be very important as versatile starting materials for the enantioselective chemical synthesis of biologically active organic molecules, such as therapeutic agents that include heterocycles in their molecular structure. We have further shown the introduction of (a) hydroxyl group(s) into heteroaromatic rings in several heteroaromatic compounds including methyl- or hydroxyl-substituted phenyl and aliphatic moieties. It has also been shown here that the use of biphenyl dioxygenase is effective for the synthesis of organic molecules of industrial profit. So far, the biphenyl dioxygenase-mediated transformation of aromatics to *cis*-diols has mainly been studied for bioremediation of environmental pollutants, although numerous conversion experiments have been performed using toluene dioxygenases with an industrial purpose as described in Section 1. This paper also describes in vitro evolution of biphenyl dioxygenases through DNA shuffling, which has extended their substrate specificity to hetero-aromatic compounds. Similar approaches have been utilized in the environmental field with PCB bioconversions.[17,25–27]

4. Experimental

4.1. General

4.1.1. Plasmids, bacterial strains, and growth conditions. Plasmids pHA171,[12] pJHF3051,[22,23] and pKF6622[9,16] were described. *E. coli* BL21 (DE3)[28] and *E. coli* JM109[29] were used as hosts for plasmid pHA171 and plasmids pJHF3051, pKF6622, and pKF2072, respectively, and cultured in LB medium[29] or M9 medium[29] at 30°C or 37°C. Ampicillin (Ap) (50–150 μg/ml) was added when needed.

4.1.2. General recombinant DNA techniques. Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo. DNA manipulation was done in *E. coli* as described.[29]

4.1.3. DNA shuffling. The *bphA1* genes were shuffled between the corresponding genes of *P. pseudoalcaligenes* KF707 and *B. cepacia* LB400 as described.[17]

4.1.4. Chemicals and conversion experiments. *E. coli* BL21 (DE3) harboring pHA171 or *E. coli* JM109 harboring pJHF3051, pKF6622, or pKF2072 was grown in LB medium containing 150 μg/ml of Ap at 30°C with reciprocal shaking (175 rpm) for 8 h. Five milliliters of this culture was inoculated into 100 ml of M9 medium with 150 μg/ml of Ap, 10 μg/ml of thiamine, and 0.4% (w/v) glucose in Erlenmeyer flask at 30°C with reciprocal shaking (175 rpm) for 16–17 h, of which the absorbance in OD 600 nm reaches approximately 1.1 mM (the final concentration) of isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture, and further cultivated for 4 h. The cells were collected by centrifugation, washed once with M9 medium, and then resuspended in 100 ml of fresh M9 medium with 150 μg/ml of Ap, 10 μg/ml of thiamine, 0.4% (w/v) glucose, and 1 mM (the final concentration) of IPTG, along with 10 mg or 1 mM (the final concentration) of each substrate, and cultivated in Erlenmeyer flask at 30°C with reciprocal shaking (175 rpm) for 2–3 days.

Substrates used in this study were purchased from Aldrich Chemical Co., Wako Pure Chemical Co., or Kanto Chemical Co. The respective substrates were dissolved in small volume of ethanol and added to the culture.

4.1.5. Extractions and HPLC analysis of converted products. To extract the converted products as well as the substrates, an equal volume of methanol (MeOH) to the cultured medium was added to the co-culture of the transformed cells of *E. coli*, and mixed for 30 min. After

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

4.2. Purification and identification of products

The liquid phase (1400 ml), which was obtained by the procedure described above, was concentrated in vacuo, and extracted with ethyl acetate (EtOAc) (500 ml×2). The organic layer was concentrated in vacuo, and analyzed by thin-layer chromatography (TLC) on silica gel (0.25 mm E. Merck silica gel plates (60F-254)). The formed products were purified through column chromatography on silica gel (20×250 mm^2, Silica Gel 60 (Merck)). Their structures were analyzed by mass (MS) (MS (EI) and HRMS (EI), JEOL DX303) and nuclear magnetic resonance (NMR) (500 MHz, JEOL GX500) spectra. (*R*) and (*S*)-2NMA esters were prepared in a manner reported by Kusumi et al.[30]

4.2.1. 3-(1*H*-1-Pyrrolyl)-3,5-cyclohexadiene-*cis*-1,2-diol (1) (product converted from 1-phenylpyrrole). The crude EtOAc extract (25 mg) was subjected to column chromatography (hexane–EtOAc=10:1) to yield 5.0 mg of **1**. MS (EI) *m/z* 177 (M^+). HRMS (EI) calcd for $C_{10}H_{11}NO_2$ (M^+), 177.0790; found 177.0791. ^{1}H NMR ($CDCl_3$) δ: 4.44 (d, 1H, *J*=6.1 Hz), 4.62 (ddd, 1H, *J*=3.0, 3.0, 6.1 Hz), 5.71 (dd, 1H, *J*=2.4, 9.8 Hz), 5.91 (d, 1H, *J*=6.1 Hz), 5.97 (ddd, 1H, *J*=2.4, 6.1, 9.8 Hz), 6.26 (dd, 2H, *J*=2.4, 2.4 Hz), 6.99 (dd, 2H, *J*=2.4, 2.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 68.3 (C-2′), 70.7 (C-1′), 109.6 (C-4′), 110.6 (C-3,4), 118.5 (C-2,5), 122.8 (C-5′), 127.9 (C-6′).

4.2.2. 1-Phenyl-4-hydroxy-pyrazole (2) (product converted from 1-phenylpyrazole). The crude EtOAc extract (55 mg) was subjected to column chromatography (hexane–EtOAc=2:1) to yield 7.0 mg of **2**. MS (EI) *m/z* 160 (M^+). HRMS (EI) calcd for $C_9H_8N_2O$ (M^+), 160.0637; found 160.0637. ^{1}H NMR ($CDCl_3$) δ: 7.17 (m, 1H), 7.34–7.38 (3H), 7.50–7.55 (3H). ^{13}C NMR ($CDCl_3$) δ: 113.8 (C-5), 118.5 (C-2′,6′), 126.1 (C-4′), 129.3 (C-3′,5′), 131.2 (C-3), 140.2 (C-1′), 142.6 (C-4).

4.2.3. 3-(3-Methyl-1*H*-1-pyrazolyl)-3,5-cyclohexadiene-*cis*-1,2-diol (3) (product converted from 3-methyl-1-phenylpyrazole). The crude EtOAc extract (68 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=20:1) to yield 18 mg of **3**. MS (EI) *m/z* 192 (M^+). HRMS (EI) calcd for $C_{10}H_{12}N_2O_2$ (M^+), 192.0900; found 192.0898. ^{1}H NMR ($CDCl_3$) δ: 2.28 (s, 3H), 4.54 (m, 1H), 4.81 (d, 1H, *J*=6.0 Hz), 5.86 (dd, 1H, *J*=3.0, 5.0 Hz), 6.00–6.08 (2H), 6.15 (d, 1H, *J*=2.5 Hz), 7.65 (d, 1H, *J*=2.5 Hz). ^{13}C NMR ($CDCl_3$) δ: 13.7 (5-CH3), 66.9 (C-1′), 68.3 (C-2′), 107.5 (C-4), 108.4 (C-4′), 123.2 (C-5′), 127.7 (C-6′), 128.1 (C-3), 137.2 (C-3′), 150.6 (C-5).

4.2.4. 3-(2-Pyridyl)-3,5-cyclohexadiene-*cis*-1,2-diol (4) (product converted from 2-phenylpyridine). The crude EtOAc extract (50 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=40:1) to yield 10 mg of **4**. MS (EI) *m/z* 189 (M^+). HRMS (EI) calcd for $C_{11}H_{11}NO_2$ (M^+), 189.0790; found 189.0793. ^{1}H NMR (DMSO-d_6) δ: 4.34 (dd, 1H, *J*=2.5, 5.5 Hz), 4.56 (d, 1H, *J*=5.5 Hz), 5.89 (d, 1H, *J*=10.2 Hz), 6.04 (ddd, 1H, *J*=3.0, 5.5, 9.8 Hz), 6.92 (d, 1H, *J*=5.5 Hz), 7.21 (dd, 1H, *J*=4.9, 8.0 Hz), 7.63 (d, 1H, *J*=8.0 Hz), 7.75 (dd, 1H, *J*=8.0, 8.0 Hz), 8.54 (d, 1H, *J*=4.9 Hz). ^{13}C NMR (DMSO-d_6) δ: 65.2 (C-2′), 71.0 (C-1′), 119.7 (C-3), 122.1 (C-5), 123.4 (C-5′), 124.0 (C-4′), 135.5 (C-6′), 137.7 (C-4), 138.1 (C-3′), 149.3 (C-6), 156.7 (C-2).

4.2.5. 3-(4-Pyrimidinyl)-3,5-cyclohexadiene-*cis*-1,2-diol (5) (product converted from 4-phenylpyrimidine). The crude EtOAc extract (23.5 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=30:1) to yield 6.6 mg of **5**. MS (EI) *m/z* 190 (M^+). HRMS (EI) calcd for $C_{10}H_{10}N_2O_2$ (M^+), 190.0743; found 190.0751. ^{1}H NMR ($CDCl_3$) δ: 4.54 (d, 1H, *J*=6.0 Hz), 4.84 (d, 1H, *J*=6.0 Hz), 6.16–6.24 (2H), 6.91 (d, 1H, *J*=4.9 Hz), 7.52 (dd, 1H, *J*=1.8, 5.5 Hz), 8.66 (d, 1H, *J*=5.5 Hz), 9.11 (d, 1H, *J*=1.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 67.3 (C-2′), 69.3 (C-1′), 116.3 (C-5), 123.7 (C-5′), 128.0 (C-4′), 135.4 (C-3′), 135.4 (C-6′), 157.2 (C-4), 158.3 (C-2).

4.2.6. 3-(1*H*-2-Indolyl)-3,5-cyclohexadiene-*cis*-1,2-diol (6) (product converted from 2-phenylindole). The crude EtOAc extract (86 mg) was subjected to column chromatography (hexane–EtOAc=10:1) to yield 5 mg of **6**. MS (EI) *m/z* 227 (M^+). HRMS (EI) calcd for $C_{14}H_{13}NO_2$ (M^+), 227.0947; found 227.0948. ^{1}H NMR (DMSO-d_6) δ: 4.34 (2H), 4.70 (d, 1H, *J*=5.5 Hz), 4.97 (d, 1H, *J*=6.0 Hz), 5.78 (d, 1H, *J*=9.2 Hz), 6.01 (ddd, 1H, *J*=2.4, 5.5, 9.2 Hz), 6.50 (d, 1H, *J*=5.5 Hz), 6.61 (s, 1H), 6.94 (dd, *J*=7.9, 7.9 Hz), 7.06 (dd, 1H, *J*=7.9, 7.9 Hz). ^{13}C NMR (DMSO-d_6) δ: 66.8 (C-2′), 70.5 (C-1′), 100.4 (C-3), 110.8 (C-7), 118.4 (C-4′), 119.1 (C-5), 120.0 (C-4), 121.8 (C-6), 122.6 (C-5′), 128.3 (C-3a), 131.0 (C-3′), 133.0 (C-6′), 137.3 (C-2), 137.7 (C-7a).

4.2.7. 3-(1,3-Benzoxazol-2-yl)-3,5-cyclohexadiene-*cis*-1,2-diol (7) (product converted from 2-phenylbenzoxazole). The crude EtOAc extract (65.4 mg) was subjected to column chromatography (CH_2Cl_2–EtOAc=1:1) to yield 26.1 mg of **7**. MS (EI) *m/z* 229 (M^+). HRMS (EI) calcd for $C_{13}H_{11}NO_3$ (M^+), 229.0739; found 229.0735. ^{1}H NMR (DMSO-d_6) δ:4.41 (m, 1H), 4.62 (dd, 1H, *J*=5.5, 5.5 Hz), 4.97 (d, 1H, *J*=5.5 Hz), 5.18 (d, 1H, *J*=7.1 Hz), 6.08–6.15 (2H), 7.10 (d, 1H, *J*=4.9 Hz), 7.33–7.40 (2H), 7.68 (dd, 1H, *J*=2.0, 6.7 Hz), 7.72 (dd, 1H, *J*=2.0, 6.7 Hz). ^{13}C NMR (DMSO-d_6) δ: 64.2 (C-2′), 70.4 (C-1′), 110.5 (C-7), 119.6 (C-4), 121.9 (C-5′), 125.4 (C-5), 126.5 (C-6), 126.5 (C-3′), 129.1 (C-4′), 139.4 (C-6′), 141.6 (C-3a), 149.9 (C-7a), 162.7 (C-2).

4.2.8. 3-(1,3-Benzothiazol-2-yl)-3,5-cyclohexadiene-*cis*-1,2-diol (8) (product converted from 2-phenylbenzothiazole). The crude EtOAc extract (68 mg) was subjected to column chromatography (CH_2Cl_2–EtOAc=5:1) to yield 32.5 mg of **8**. MS (EI) *m/z* 245 (M^+). HRMS (EI) calcd for

$C_{13}H_{11}NO_2S$ (M^+), 245.0511; found 245.0508. ^1H NMR ($CDCl_3$) δ: 4.51 (m, 1H), 5.00 (d, 1H, J=6.1 Hz), 6.21 (dd, 1H, J=4.9, 9.2 Hz), 6.26 (dd, 1H, J=4.3, 9.2 Hz), 6.78 (d, 1H, J=4.9 Hz), 7.34 (dd, 1H, J=7.3, 7.3 Hz), 7.44 (dd, 1H, J=7.3, 7.3 Hz), 7.81 (d, 1H, J=7.3 Hz), 7.94 (d, 1H, J=7.3 Hz). ^{13}C NMR ($CDCl_3$) δ: 66.4 (C-1′), 68.6 (C-2′), 121.4 (C-7), 123.0 (C-4), 124.6 (C-6′), 125.8 (C-6), 126.5 (C-5), 127.8 (C-4′), 132.4 (C-3′), 133.3 (C-5′), 133.7 (C-7a), 153.0 (C-3a), 168.0 (C-2).

4.2.9. 3-(*cis*-5,6-Dihydroxy-1,3-cyclohexadienyl)-1-indanone (9) (product converted from 3-phenyl-1-indanone). The crude EtOAc extract (57 mg) was subjected to column chromatography (CH_2Cl_2–EtOAc=2:1) to yield 10 mg of **9**. MS (EI) *m/z* 242 (M^+). HRMS (EI) calcd for $C_{14}H_{14}O_3$ (M^+), 242.0943; found 242.0943. ^1H NMR ($CDCl_3$) δ: 2.68 (dd, 1H, J=3.1, 18.9 Hz), 3.01 (dd, 1H, J=7.9, 18.9 Hz), 4.17–4.28 (3H), 5.70 (d, 1H, J=4.9 Hz), 5.91 (m, 1H), 5.95 (m, 1H), 7.38 (dd, 1H, J=7.3, 7.3 Hz), 7.45 (d, 1H, J=7.3 Hz), 7.58 (dd, 1H, J=7.3, 7.3 Hz), 7.74 (d, 1H, J=7.3 Hz). ^{13}C NMR ($CDCl_3$) δ:43.0 (C-3), 43.6 (C-2), 67.8 (C-1′), 71.6 (C-2′), 120.4 (C-4′), 123.8 (C-4), 125.3 (C-5′), 126.5 (C-7), 127.9 (C-5), 128.0 (C-6′), 134.7 (C-6), 138.1 (C-3a), 142.7 (C-3′), 156.1 (C-7a), 206.9 (C-1).

4.2.10. 3-(2-Quinolyl)-3,5-cyclohexadiene-*cis*-1,2-diol (10) (product converted from 2-phenylquinoline). The crude EtOAc extract (55 mg) was subjected to column chromatography (hexane–EtOAc=10:1) to yield 12 mg of **10**. MS (EI) *m/z* 239 (M^+). HRMS (EI) calcd for $C_{15}H_{13}NO_2$ (M^+), 239.0947; found 239.0947. ^1H NMR ($CDCl_3$) δ: 4.50 (dd, 1H, J=3.0, 6.7 Hz), 5.08 (d, 1H, J=6.7 Hz), 6.23 (m, 1H), 6.78 (m 1H), 7.46 (dd, 1H, J=6.7, 6.7 Hz), 7.49 (dd, 1H, 6.7, 6.7 Hz), 7.69 (d, 1H, J=6.7 Hz), 7.73 (d, 1H, J=6.7 Hz), 7.96 (d, 1H, J=8.5 Hz), 8.07 (d, 1H, J=9.2 Hz). ^{13}C NMR ($CDCl_3$) δ: 66.7 (C-1′), 69.6 (C-2′), 117.8 (C-3), 125.1 (C-4′), 125.1 (C-5′), 126.6 (C-7), 126.8 (C-8a), 127.4 (C-8), 128.9 (C-5), 130.0 (C-6), 131.7 (C-6′), 136.4 (C-3′), 136.7 (C-4), 146.5 (C-4a), 157.1 (C-2).

4.2.11. 3-(1*H*-1-Imidazolylmethyl)-3,5-cyclohexadiene-*cis*-1,2-diol (11) (product converted from 1-benzylimidazole). The crude EtOAc extract (65 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=7:1) to yield 2.0 mg of **11**. MS (EI) *m/z* 192 (M^+). HRMS (EI) calcd for $C_{10}H_{12}N_2O_2$ (M^+), 192.0900; found 192.0889. ^1H NMR (DMSO-d_6) δ: 3.72 (m, 1H), 4.00 (m, 1H), 4.62 (d, 1H, J=15.9 Hz), 4.77 (d, 1H, J=15.9 Hz), 5.58 (d, 1H, J=5.5 Hz), 5.75 (dd, 1H, J=3.0, 9.1 Hz), 5.82 (m, 1H), 6.89 (s, 1H), 7.10 (s, 1H), 7.61 (s, 1H). ^{13}C NMR (DMSO-d_6) δ: 48.4 (C-1′), 66.6 (C-3′), 68.2 (C-4′), 120.5 (C-7′), 122.3 (C-6′), 131.4 (C-5′), 137.8 (C-2′).

4.2.12. 3-(4-Isothiazolylmethyl)-3,5-cyclohexadiene-*cis*-1,2-diol (12) (product converted from 4-benzylisothiazole). The crude EtOAc extract (34 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=40:1) to yield 8.0 mg of **12**. MS (EI) *m/z* 209 (M^+). HRMS (EI) calcd for $C_{10}H_{11}NO_2S$ (M^+), 209.0511; found 209.0505. ^1H NMR (DMSO-d_6) δ: 3.52 (d, 1H, J=16.5 Hz), 3.62 (d, 1H, J=16.5 Hz), 3.78 (dd, 1H, J=6.0, 6.0 Hz), 4.03 (m, 1H), 4.61 (d, 1H, J=6.7 Hz), 4.66 (d, 1H, J=6.0 Hz), 5.55 (d, 1H, J=5.5 Hz), 5.68 (dd, 1H, J=3.0, 9.8 Hz), 5.80 (dd, 1H, J=5.5, 9.8 Hz), 8.42 (s, 1H), 8.70 (s, 1H). ^{13}C NMR (DMSO-d_6) δ: 30.8 (C-1′), 67.8 (C-4′), 68.5 (C-3′), 120.0 (C-7′), 123.0 (C-6′), 129.5 (C-5′), 137.3 (C-4), 140.7 (C-2′), 145.6 (C-5), 158.8 (C-3).

4.2.13. 3-(2-Pyridylmethyl)-3,5-cyclohexadiene-*cis*-1,2-diol (13) (product converted from 2-benzylpyridine). The crude EtOAc extract (55 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=50:1) to yield 5.6 mg of **13**. MS (EI) *m/z* 203 (M^+). HRMS (EI) calcd for $C_{12}H_{13}NO_2$ (M^+), 203.0947; found 203.0947. ^1H NMR (DMSO-d_6) δ: 3.55–3.62 (2H), 3.82 (m, 1H), 4.03 (m, 1H), 5.57 (d, 1H, J=4.9 Hz), 5.66 (dd, 1H, J=3.0, 9.7 Hz), 5.80 (m, 1H), 7.21 (dd, 1H, J=5.5, 6.0 Hz), 7.27 (d, 1H, J=7.6 Hz), 7.70 (ddd, 1H, J=4.9, 6.0, 7.6), 8.46 (d, 1H, J=5.5 Hz). ^{13}C NMR (DMSO-d_6) δ: 42.0 (C-1′), 68.3 (C-4′), 68.7 (C-3′), 120.6 (C-7′), 121.4 (C-5), 123.2 (C-3), 123.2 (C-6′), 129.6 (C-5′), 136.6 (C-4′), 140.4 (C-2′), 148.9 (C-6), 159.9 (C-2).

4.2.14. 2-(4-Methylphenyl)-3-pyridiol (14) (product converted from 2-*p*-tolylpyridine). The crude EtOAc extract (65 mg) was subjected to column chromatography (CH_2Cl_2) to yield 9.0 mg of **14**. MS (EI) *m/z* 185 (M^+). HRMS (EI) calcd for $C_{12}H_{11}NO$ (M^+), 185.0841; found 185.0838. ^1H NMR (DMSO-d_6) δ: 2.33 (s, 3H), 7.15 (dd, 1H, J=4.3, 7.9 Hz), 7.21 (d, 2H, J=7.9 Hz), 7.29 (d, 1H, J=7.9 Hz), 7.91 (d, 2H, J=7.9), 8.11 (d, 1H, J=4.4 Hz), 10.06 (s, 1H). ^{13}C NMR (DMSO-d_6) δ: 20.8 (1′-CH3), 123.0 (C-5), 123.3 (C-4), 128.2 (C-2′,6′), 128.7 (C-3′,4′), 135.2 (C-4′), 137.0 (C-1′), 140.1 (C-6), 144.4 (C-2), 152.2 (C-3).

4.2.15. 2-(2-Hydroxyphenyl)-4,5-dihydro-1,3-benzoxazole-*cis*-4,5-diol (15) (product converted from of 2-(2-hydroxyphenyl)benzoxazole). The crude EtOAc extract (40 mg) was subjected to column chromatography (CH_2Cl_2–EtOAc=10:1) to yield 7.8 mg of **15**. MS (EI) *m/z* 245 (M^+). HRMS (EI) calcd for $C_{13}H_{11}NO_4$ (M^+), 245.0688; found 245.0691. ^1H NMR (DMSO-d_6) δ: 4.50 (2H), 5.22 (d, 1H, J=5.5 Hz), 5.33 (d, 1H, J=6.7 Hz), 5.95 (d, 1H, J=10.0 Hz), 6.57 (dd, 1H, J=2.4, 10.0 Hz), 7.00 (dd, 1H, J=7.3, 7.3 Hz), 7.04 (d, 1H, J=8.6 Hz), 7.39 (dd, 1H, J=7.3, 8.6), 7.79 (d, 1H, J=7.3 Hz), 10.92 (s, 1H). ^{13}C NMR (DMSO-d_6) δ: 64.4 (C-4), 71.3 (C-5), 111.0 (C-2′), 112.5 (C-7), 116.9 (C-6′), 120.0 (C-4′), 126.0 (C-3′), 132.3 (C-5′), 135.8 (C-3a), 136.0 (C-6), 145.5 (C-7a), 156.2 (C-1′), 158.8 (C-2).

4.2.16. 4-Hexyl-2,3-dihydro-2,3-thiophenediol (16) (product converted from 3-*n*-hexylthiophene). The crude EtOAc extract (37.5 mg) was subjected to column chromatography (hexane–EtOAc=5:1) to yield 10.0 mg of **16**. MS (EI) *m/z* 202 (M^+). HRMS (EI) calcd for $C_{10}H_{18}O_2S$ (M^+), 202.1028; found 202.1023. ^1H NMR ($CDCl_3$) δ: 0.88 (t, 3H, J=7.3 Hz), 1.20–1.28 (6H), 1.45 (m, 2H), 2.16 (t, 2H, J=7.3 Hz), 4.53 (s, 1H), 5.54 (s, 1H), 5.82 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 13.8 (C-6′), 22.6 (C-5′), 27.8 (C-2′), 28.8 (C-3′), 29.2 (C-1′), 31.7 (C-4′), 79.5 (C-3), 81.7 (C-2), 117.8 (C-5), 137.3 (C-4).

4.2.17. 2-Butylbenzo[*b*]furan-6-ol (17) and 2-butylbenzo-[*b*]furan-5-ol (18) (products converted from 2-*n*-butylbenzofuran). The crude EtOAc extract (45 mg) was

subjected to column chromatography (hexane–EtOAc=10:1) to yield 17 mg of **17** and 5 mg of **18**.

17 MS (EI) *m/z* 190 (M^+). HRMS (EI) calcd for $C_{12}H_{14}O_2$ (M^+), 190.0994; found 190.0991. ^{1}H NMR ($CDCl_3$) δ: 0.91 (t, 3H, *J*=7.3 Hz), 1.37 (m, 2H), 1.67 (m, 2H), 2.67 (m, 2H), 4.81 (s, 1H), 6.24 (s, 1H), 6.67 (dd, 1H, *J*=2.4, 7.9 Hz), 6.88 (s, 1H), 7.24 (d, 1H, *J*=7.9 Hz). ^{13}C NMR ($CDCl_3$) δ: 13.8 (C-4′), 22.1 (C-3′), 28.1 (C-1′), 29.8 (C-2′), 98.1 (C-7), 101.5 (C-3), 111.4 (C-5), 120.2 (C-4), 122.6 (C-3a), 152.4 (C-6), 155.4 (C-7a), 158.9 (C-2).

18 MS (EI) *m/z* 190 (M^+). HRMS (EI) calcd for $C_{12}H_{14}O_2$ (M^+), 190.0994; found 190.0994. ^{1}H NMR $CDCl_3$) δ:0.91 (t, 3H, *J*=7.3 Hz), 1.37 (m, 2H), 1.67 (m, 2H), 2.67 (m, 2H), 4.58 (s, 1H), 6.23 (s, 1H), 6.76 (dd, 1H, *J*=2.0, 8.0 Hz), 6.84 (d, 1H, *J*=2.0 Hz), 7.21 (d, 1H, *J*=8.0 Hz). ^{13}C NMR ($CDCl_3$) δ: 13.8 (C-4′), 22.1 (C-3′), 28.2 (C-1′), 29.8 (C-2′), 101.7 (C-3), 101.7 (C-4), 111.0 (C-7), 111.5 (C-6), 129.7 (C-3a), 149.8 (C-7a), 152.1 (C-5), 161.6 (C-2).

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