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Hydroxylation of ionized aromatics including carboxylic acid or amine using recombinant *Streptomyces lividans* cells expressing modified 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. These *bphA1*(2072)*A2A3A4* genes were expressed in the actinomycetes *Streptomyces lividans* using a thiostrepton-inducible promoter P_{tipA}. Biotransformation experiments of various aromatics including carboxylic acid or amine in their molecular structure, such as 1-naphthoic acid, 2-(1-naphthyl)acetic acid, diphenylamine, and 1-benzyl-4-piperidone, were performed using the recombinant *S. lividans* cells. These ionized aromatics were converted to the corresponding 1,2-dihydrodiol, mono- or tri-hydroxy forms in 48 h. The structure of the converted products was determined by their EI-MS, ^{1}H- and ^{13}C NMR analysis, and several products were found to be novel compounds.

1. Introduction

Degradation of the environmental pollutants, polychlorinated biphenyls (PCBs) is initiated enzymatically by the action of biphenyl dioxygenase.[1] This enzyme is a multicomponent enzyme that consists of BphA1, BphA2 (large and small subunits of iron-sulfur protein, respectively), BphA3 (ferredoxin), and BphA4 (ferredoxin reductase).

Recently, modified *bphA1* genes have been generated by DNA shuffling using the *bphA1* genes derived from the gram-negative bacteria *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia cepacia* LB400.[2] One of the shuffled genes, *bphA1*(2072), has been shown to mediate a broad substrate specificity, when expressed in combination with *bphA2A3A4* from *P. pseudoalcaligenes*.[3] Various molecular species, in which heterocyclic aromatics are linked with phenyl or benzyl groups, were shown to be converted to their corresponding 1,2-dihydrodiols successfully by this recombinant *Escherichia coli* strain carrying the modified biphenyl dioxygenase genes [*bphA1*(2072)*A2A3A4*].[3] The 1,2-dihydrodiols produced including heteroaromatics have important industrial potential 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. In addition this study also illustrated that the use of biphenyl dioxygenase could mediate the effective synthesis of high value organic molecules.

So far, the biphenyl dioxygenase-mediated transformation of aromatics to *cis*-diols has mainly been studied for bioremediation of environmental pollutants. Plasmid, pIJ6021-*bphA1*(2072)*A2A3A4*, for the expression of the modified biphenyl dioxygenase genes in the gram-positive, soil-inhabiting, filamentous bacterium *Streptomyces lividans* has also been constructed using a thiostrepton-inducible promoter P_{tipA} on a high-copy-number vector pIJ6021.[4] The *S. lividans* transformant carrying this plasmid was able to catalyze the same conversions as those by the *E. coli* transformant at the same or improved efficiency (Y. Ohnishi and S. Horinouchi, unpublished results).

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Ionized aromatic molecules including carboxylic acid or amine moieties in their molecular structure have frequently been used as building blocks for the chemical synthesis of various therapeutic agents and agrochemicals.[5] Biological modification of these molecules through hydroxylation reactions is a promising way to extend a variety of biologically active chemicals. In this paper we describe hydroxylation of ionized aromatic molecules, such as 1-naphthoic acid, 2-(1-naphthyl)acetic acid, diphenylamine, 1-benzyl-4-piperidone, benzyl-carbamic acid *tert*-butyl ester and phenyl-carbamic acid *tert*-butyl ester, through the modified biphenyl dioxygenase-carrying *S. lividans* cells.

2. Results

2.1. Biotransformation of 1-naphthoic acid and 2-(1-naphthyl)acetic acid

Aromatics including the carboxylic acids (1-naphthoic acid or 2-(1-naphthyl)acetic acid) were converted to the mono-hydroxy derivatives. A 40–60% conversion was achieved from each substrate using the *S. lividans* cells expressing the modified biphenyl dioxygenase genes (plasmid, pIJ6021-*bphA1*(2072)*A2A3A4*). In contrast, the recombinant *E. coli* cells expressing the same modified biphenyl dioxygenase genes (plasmid, pKF2072) were not able to convert these aromatic molecules.

2.1.1. 1-Naphthoic acid. 1-Naphthoic acid was converted to two products by the *S. lividans* transformant. The molecular formulas of both the products (**1**, **2**) were determined to be $C_{11}H_8O_3$ by HR-EIMS. Analysis by $^1H-^{13}C$ COSY and DQF COSY spectra revealed one phenolic OH was replaced in the naphthalene ring of products **1** and **2**. In the HMBC spectrum of **1**, $^1H-^{13}C$ long range couplings from H-2 (δ 8.12) to carbonyl carbon (δ 168.2) and C-4 (δ 157.8) were observed. Therefore, product **1** was identified to be 4-hydroxy-1-naphthoic acid[6] (Fig. 1). *Visinal* sp^2 spin network of H-2 (δ 8.07)–H-3 (δ 7.48)–H-4 (δ 8.37) in the DQF COSY spectrum and $^1H-^{13}C$ long range couplings from H-2 to carbonyl carbon (δ 168.8) and H-4 to C-5 (δ 153.6) in the HMBC spectrum showed that product **2** was 5-hydroxy-1-naphthoic acid[7] (Fig. 1). The yield of the products purified is also shown in Figure 1.

2.1.2. 2-(1-Naphthyl)acetic acid. 2-(1-Naphthyl)acetic acid was converted to two products by the *S. lividans* transformant. The molecular formulas of both the products (**3**, **4**) were determined to be $C_{12}H_{10}O_3$ by HR-EIMS. Analysis by $^1H-^{13}C$ COSY and DQF COSY spectra revealed one phenolic OH was replaced in the naphthalene ring of products **3** and **4**. In the HMBC spectrum of **3**, $^1H-^{13}C$ long range couplings from H-1′ (δ 3.96) to C-2 (δ 128.2) and H-2 (δ 7.18) to C-4 (δ 152.3) were observed. Therefore, product **3** was identified to be 2-(4-hydroxy-1-naphthyl)acetic acid[8] (Fig. 1). *Visinal* sp^2 spin network of H-2 (δ 7.39)–H-3 (δ 7.39)–H-4 (δ 8.16) in the DQF COSY spectrum and $^1H-^{13}C$ long range couplings from H-2 to C-1′ (δ 39.2) and H-4 to C-5 (δ 153.0) in the HMBC spectrum showed that product **4** was 2-(5-hydroxy-1-naphthyl)acetic acid[9] (Fig. 1).

2.2. Biotransformation of diphenylamine and 1-benzyl-4-piperidone

Aromatics including secondary and tertiary amine (diphenyl amine and 1-benzyl-4-piperidone, respectively) were used as substrate for biotransformation experiments. Both the compounds were converted to the corresponding mono- or tri-hydroxy forms, or 1,2-dihydrodiol form with high efficiency (60–80%) by the recombinant *S. lividans* cells. The recombinant *E. coli* was also able to convert these aromatic molecules.

2.2.1. Diphenylamine. Diphenylamine was converted to three products by the *S. lividans* transformant. The molecular formulas of the two products (**5**, **6**) were determined to be $C_{12}H_{11}NO$ by HR-EIMS. Analysis by $^1H-^{13}C$ COSY and DQF COSY spectra revealed one phenolic OH was replaced in the benzene ring of products **5** and **6**. Observation of *visinal* spin networks from H-3 (δ 6.88) to H-5 (δ 7.08) and H-2′ (δ 6.68) to H-6′ (δ 6.68) confirmed that **5** was 2-anilonophenol[10] (Fig. 1). The symmetrical ^1H NMR spectra revealed that **6** was 4-anilonophenol[11] (Fig. 1).

The molecular formula of the remaining product (**7**) was determined to be $C_6H_{11}NO_3$ by HR-EIMS. Analysis by $^1H-^{13}C$ COSY and DQF COSY spectra revealed three phenolic OHs were replaced in the benzene rings. The positions of the OH moieties were determined to be C-2, C-3′ and C-4′ by the observation of *visinal* spin networks from H-3 (δ 7.32) to H-6 (δ 7.77), between H-5 (δ 7.42) and H-6 (δ 6.84) as well as *meta* coupling between H-2′ (δ 6.31) and H-6′ (δ 6.84). From these findings, product **7** was determined as 4-(2-hydroxyanilino)-1,2-benzenediol (Fig. 1). **7** was a novel compound.

2.2.2. 1-Benzyl-4-piperidone. 1-Benzyl-4-piperidone was converted to one product by the *S. lividans* transformant. The molecular formula of the product (**8**) was determined to be $C_{11}H_{17}NO_3$ by HR-EIMS. Analysis by $^1H-^{13}C$ COSY and DQF COSY spectra showed that **8** was a dihydrodiol derivative of the benzene ring. The 2,3-diol regiochemical assignment was confirmed by the observation of sp^2 *visinal* spin network of H-4 (δ 5.70)–H-5 (δ 5.90)–H-6 (δ 5.90). Thus, product **8** was determined as 4-(2-hydroxyanilino)-1,2-benzenediol (Fig. 1). The absolute stereochemistry of **8** was determined by ^1H 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 Figure 2. The sign of $\Delta\delta$ are systematically arranged right and left sides to the 2NMA planes. From these results, the absolute configuration of C-2 and C-3 in **8** were determined to be *R* and *S*, respectively. **8** was also a novel compound.

2.3. Biotransformation of benzyl-carbamic acid *tert*-butylester and phenyl-carbamic acid *tert*-butylester

Benzylamine and aniline, which include an amino group (primary amine) in their molecular structure, were not converted by the recombinant *S. lividans* cells or by the recombinant *E. coli* cells. This could be due to the luck of their permeability into the cell or perhaps their affinity to the iron-including active site of the enzyme. Therefore, the amino groups of benzylamine and aniline were protected as

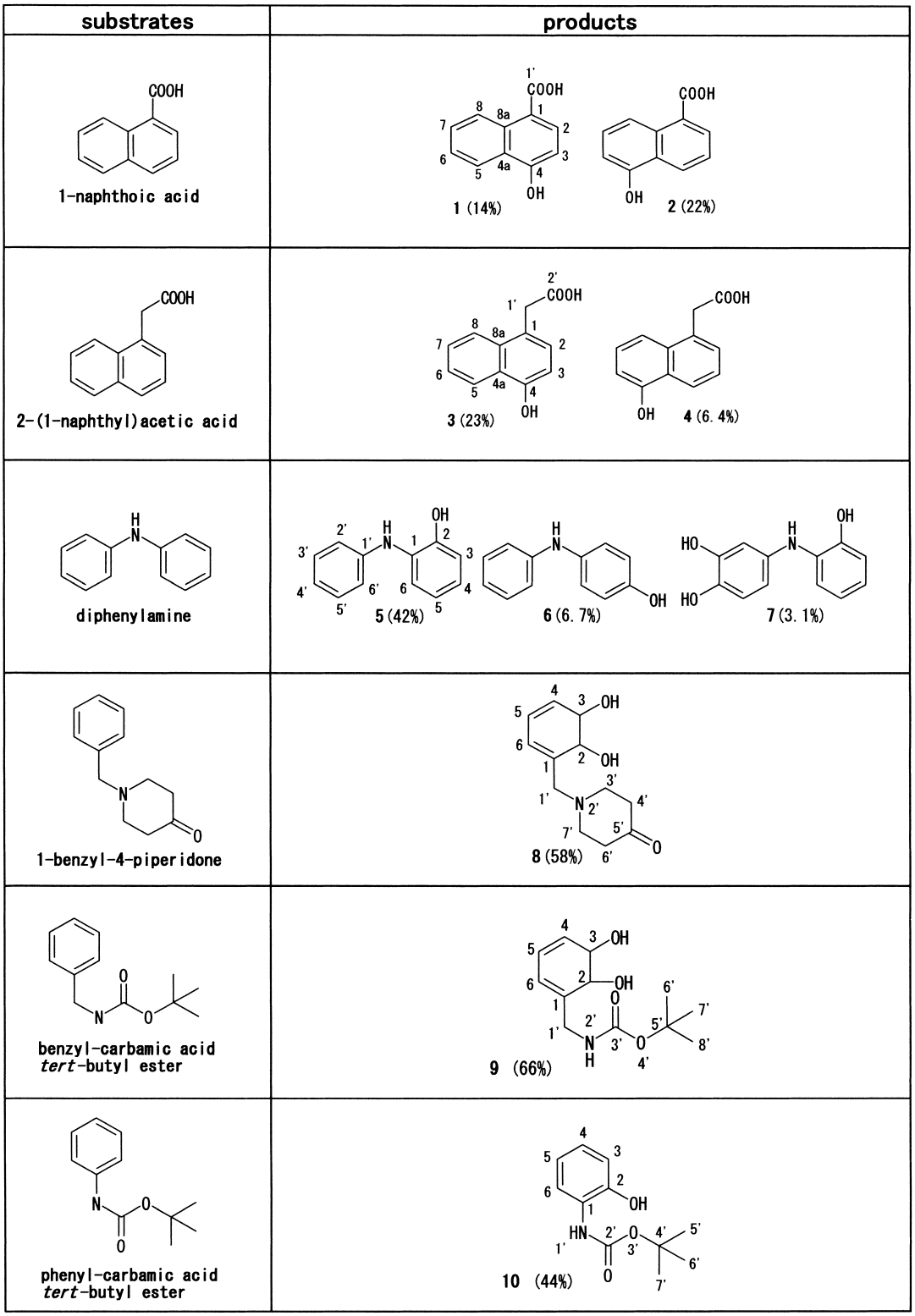

Figure 1. Structures of products bioconverted from respective aromatic compounds. Percent value in the parentheses represents the yield of the products purified.

tert-butyl carbamate by stirring with di-*tert*-butyl dicarbonate [$(t\text{-BOC})_2O$] and NaOH in aqueous dioxane. Their protected groups should easily be removed by acidic treatment. The *t*-BOC derivatives synthesized were successfully converted to the corresponding 1,2-dihydrodiol or monohydroxy forms with high efficiency (75–85%) by the recombinant *S. lividans* cells. The recombinant *E. coli* was also able to convert these aromatic molecules.

2.3.1. Benzyl-carbamic acid *tert*-butyl ester. Benzyl-carbamic acid *tert*-butyl ester was converted to one product by the *S. lividans* transformant. The molecular formula of

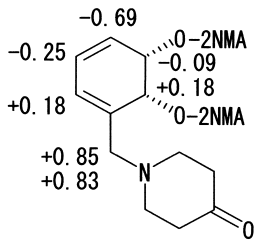

Figure 2. Application of modified Mosher's method in determination of the absolute configuration of 1-[((5*S*,6*R*)-5,6-dihydroxycyclohexa-1,3-dienyl)-methyl]piperidin-4-one (**8**).

the product (**9**) was determined to be $C_{12}H_{19}NO_4$ by HR-EIMS. Analysis by HMQC and DQF COSY spectra showed that **9** was dihydrodiol derivative. The 2,3-diol regiochemical assignment was confirmed by the long range $^1H-^{13}C$ connectivities from H-1′ (δ 3.75) to C-1 (δ 138.1), C-2 (δ 68.6) and C-6 (δ 120.4). Thus, **9** was determined to be (5,6-dihydroxy-cyclohexa-1,2-dienylmethyl)-carbamic acid *tert*-butyl ester (Fig. 1).

2.3.2. Phenyl-carbamic acid *tert*-butyl ester. Phenyl-carbamic acid *tert*-butyl ester was converted to one product by the *S. lividans* transformant. The molecular formula of the product (**10**) was determined to be $C_{11}H_{15}NO_3$ by HR-EIMS. Analysis by HMQC and DQF COSY spectra revealed that one phenolic OH was attached at benzene ring. C-4 (δ 68.2). Observation of *visinal* sp^2 spin networks from H-3 (δ 6.89) to H-6 (δ 7.03) confirmed that the OH function was attached at C-2. Thus, **10** was determined to be (2-hydroxy-phenyl)-carbamic acid *tert*-butyl ester (Fig. 1).

3. Discussion

Traditionally, biphenyl dioxygenase catalyzes the formation of *cis*-1,2-dihydrodiol from a benzene ring.[1] In this sense, compounds **8** and **9** were typical products converted by this enzyme. However, the other products formed were not *cis*-1,2-dihydrodiol, but monohydroxylated or trihydroxylated products. The monohydoxylated forms are considered to be generated non-enzymatically by dehydration from the corresponding 1,2-dihydrodiols, as a consequence of their structural instability. The reason is not clear why in the case of product **7** a catechol was generated instead of *cis*-dihydrodiol from a benzene ring. But, the similar catechol-type products have also shown to be produced by the modified biphenyl dioxygenase-mediated transformation of flavone and flavanone.[4] Endogenous desaturation enzymes, which are responsible for the conversion from *cis*-dihydrodiol to catechol, may exist in the *Streptomyces* species used.

This study has shown an advantage of using *Streptomyces* species instead of *E. coli* as hosts for bioconversion experiments. It is significant that the *E. coli* transformant expressing the modified dioxygenase genes was found not to convert 1-naphthoic acid and 2-(1-naphthyl)acetic acid, which were converted by the *Streptomyces* transformant. It is possible that the difference would depend principally on better permeability of ionized substrates through the cell membrane in *Streptomyces* species (gram positive bacteria) compared with *E. coli* (gram negative bacterium).

Enzyme-mediated introduction of (a) hydroxyl group(s) into the benzene rings of various aromatic compounds including carboxylic acid or amine moieties has been described in this study. The hydroxylated metabolites are easily prepared with high yields using the recombinant bacterial cells that carry the modified biphenyl dioxygenase genes. The scale-up experiments should be easy to do. For example, in the case of 1-benzyl-4-piperidone as the substrate, the addition of one gram to 1-liter culture gave 326 mg of the converted compound (data not shown). Purification can be achieved by classical chromatographic techniques, as described in Experimental. Such ionized aromatics including hydroxyl groups seem to be very important as starting or condensing materials for the chemical synthesis of biologically active organic molecules such as therapeutic agents.

In conclusion, the present study and our previous report[3] have shown that the use of evolved biphenyl dioxygenases is effective for the synthesis of high value organic molecules used in the industrial sector. Many of the products generated by this study are difficult to be synthesized with existing methods of organic chemistry. It would be feasible to complement the methods of combinatorial chemistry with the biotechnological methods described in our papers. We aim to establish such biological technologies alongside combinatorial chemistry, in an approach we term 'Bio-CombiChem' (Biology-based Combinatorial Chemistry).

4. Experimental

4.1. Plasmids, bacterial strains, and growth conditions

Plasmid pKF2072 carrying the *bphA1*(2072)*A2A3A4* genes for their expression in *E. coli* has been described.[3] *E. coli* JM109[12] was used as a host for plasmid pKF2072, and cultured in LB medium[12] or M9 medium[12] at 30 or 37°C. Ampicillin (Ap) (50–150 μg/ml) was added when needed.

The expression vector pIJ6021 for *Streptomyces* species, which carries the kanamycin resistance gene and thiostrepton-inducible promoter P_{tipA} has been described.[13] A plasmid, pIJ6021-*bphA1*(2072)*A2A3A4*, for the expression of the modified biphenyl dioxygenase genes in *S. lividans* has been constructed using vector pIJ6021.[4] On plasmid pIJ6021-*bphA1*(2072)*A2A3A4*, the four genes are positioned under the control of the thiostrepton-inducible promoter P_{tipA} enabling co-transcription in *S. lividans*. *S. lividans* TK21[14] was used as a host for this plasmid, and cultured in YEME medium[15] or minimal medium[15] at 30°C. Kanamycin was used at a final concentration of 5 μg/ml when necessary.

4.2. Conversion experiments

E. coli JM109 harboring 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. One millimolar (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 100 μg/ml (the final concentration) of each substrate, and cultivated in Erlenmeyer flask at 30°C with reciprocal shaking (175 rpm) for 2–3 days.

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 10 mg or 1 mM (the final concentration) of each substrate was added to the mycelium suspension. 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.

4.3. Purification and identification of converted products

The culture supernatant (about 1 l) was extracted with 1 l 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: 1-naphthoic acid, CH_2Cl_2–MeOH (10:1); 2-(1-naphthyl)acetic acid, CH_2Cl_2–MeOH (10:1); diphenylamine, CH_2Cl_2; 1-benzyl-4-piperidone, CH_2Cl_2–MeOH (10:1); benzyl-carbamic acid *tert*-butyl ester, CH_2Cl_2–MeOH (10:1); phenyl-carbamic acid *tert*-butyl ester, hexane–EtOAc (3:1). The converted products as well as the substrates, present in the organic phase, were put through column chromatography on silica gel (20 by 250 mm, Silica Gel 60 (Merck)).

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. (*R*)- and (*S*)-2NMA esters were prepared in a manner reported by Kusumi et al.[16]

4.3.1. 4-Hydroxy-1-naphthoic acid (1) and 5-hydroxy-1-naphthoic acid (2) (product converted from 1-naphthoic acid). The crude EtOAc extract (90 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=15:1) to yield 15.6 mg of **1** and 24.0 mg of **2**.

Compound **1**. ^{1}H NMR (DMSO-d_6) δ: 6.90 (d, 1H, J=7.9 Hz), 7.49 (dd, 1H, J=7.3, 7.3 Hz), 7.58 (dd, 1H, J=7.3, 9.1 Hz), 8.12 (d, 1H, J=7.9 Hz), 8.22 (d, 1H, J=7.3 Hz), 9.02 (s, 1H, J=9.1 Hz); ^{13}C NMR (DMSO-d_6) δ: 106.9 (C-3), 117.0 (C-1), 124.6 (C-4a), 124.7 (C-6), 125.5 (C-8), 127.8 (C-7), 132.8 (C-5), 132.8 (C-2), 132.9 (C-8a), 157.8 (C-4), 168.2 (C-1′). (Found: M$^+$, 188.0470. Calcd for, 188.0473 ($C_{11}H_8O_3$)).

Compound **2**. ^{1}H NMR (DMSO-d_6): 6.92 (d, 1H, J=7.3 Hz), 7.40 (dd, 1H, J=7.3, 7.3 Hz), 7.48 (dd, 1H, J=7.3, 7.3 Hz), 8.07 (dd, 1H, J=2.0, 7.3 Hz), 8.24 (d, 1H, J=7.3 Hz), 8.37 (dd, 1H, J=2.0, 7.3 Hz); ^{13}C NMR (DMSO-d_6) δ: 108.2 (C-6), 116.2 (C-8), 123.5 (C-3), 125.1 (C-4a), 126.7 (C-4), 127.7 (C-1), 128.1 (C-7), 130.0 (C-2), 132.0 (C-8a), 153.6 (C-5), 168.8 (C-1′). (Found: M$^+$, 188.0466. Calcd for, 188.0473 ($C_{11}H_8O_3$)).

4.3.2. 2-(4-Hydroxyl-1-naphthyl)acetic acid (3) and 2-(5-hydroxyl-1-naphthyl)acetic acid (4) (product converted from 2-(1-naphthyl)acetic acid). The crude EtOAc extract (80 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=15:1) to yield 24.6 mg of **3** and 6.9 mg of **4**.

Compound **3**. ^{1}H NMR ($CDCl_3$) δ: 3.96 (s, 2H), 6.72 (d, 1H, J=7.9 Hz), 7.18 (d, 1H, J=7.9 Hz), 7.45 (dd, 1H, J=7.9, 7.9 Hz), 7.51 (dd, 1H, J=7.9, 8.5 Hz), 7.88 (d, 1H, J=8.5 Hz), 8.22 (d, 1H, J=7.9 Hz); ^{13}C NMR ($CDCl_3$) δ: 38.0 (C-1′), 113.5 (C-3), 122.0 (C-5), 122.1 (C-1), 123.7 (C-8), 125.4 (C-7), 125.7 (C-4a), 126.5 (C-6), 128.2 (C-2), 133.7 (C-8a), 152.3 (C-4), 175.5 (C-2′) (Found: M$^+$ 202.0633. Calcd for 202.0630 ($C_{12}H_{10}O_3$)).

Compound **4**. ^{1}H NMR ($CDCl_3$) δ: 4.03 (s, 2H), 6.80 (d, 1H, J=7.3 Hz), 7.30 (dd, 1H, J=7.3, 7.3 Hz), 7.39 (2H), 7.51 (d, 1H, J=7.3 Hz), 8.16 (dd, 1H, J=2.5, 7.9 Hz); ^{13}C NMR ($CDCl_3$) δ: 39.2 (C-1′), 108.5 (C-6), 116.0 (C-8), 122.3 (C-4), 124.5 (C-3), 124.9 (C-4a), 126.6 (C-7), 128.5 (C-2), 130.2 (C-1), 133.4 (C-8a), 153.0 (C-5), 174.1 (C-2′). (Found: M$^+$202.0631. Calcd for 202.0630 ($C_{12}H_{10}O_3$)).

4.3.3. 2-Anilinophenol (5), 4-anilinophenol (6), and 4-(2-hydroxyanilino)-1,2-benzenediol (7) (products converted from diphenylamine). The crude EtOAc extract (234 mg) was subjected to column chromatography (Hexane–CH_2Cl_2=1:1 to CH_2Cl_2–MeOH=100:1, stepwise) to yield 46.3 mg of **5**, 8.4 mg of **6** and 3.4 mg of **7**.

Compound **5**. ^{1}H NMR ($CDCl_3$) δ: 5.14 (drs, 1H), 5.72 (s, 1H), 6.68 (d, 2H, J=7.9 Hz), 6.79 (dd, 1H, J=7.9, 7.9 Hz), 6.80 (dd, 1H, J=7.9, 7.9 Hz), 6.88 (d, 1H, J=7.9 Hz), 6.99 (dd, 1H, J=7.9, 7.9 Hz), 7.08 (d, 1H, J=7.9 Hz), 7.13 (dd, 2H, J=7.9, 7.9 Hz); ^{13}C NMR ($CDCl_3$) δ: 115.3 (C-3), 115.8 (C-2′, C-6′), 120.3 (C-4′), 121.0 (C-5), 124.5 (C-6), 126.0 (C-4), 129.4 (C-3′, C-5′), 129.1 (C-1), 145.3 (C-1′), 150.9 (C-2). (Found: M$^+$ 185.0845. Calcd for 185.0841 ($C_{12}H_{11}NO$)).

Compound **6**. ^{1}H NMR ($CDCl_3$) δ: 4.64 (brs, 1H), 5.42 (brs, 1H), 6.76 (d, 2H, J=8.6 Hz), 6.80 (dd, 1H, J=7.3, 7.3 Hz), 6.87 (d, 2H, J=7.3 Hz), 6.99 (d, 2H, J=8.6 Hz), 7.18 (dd, 2H, J=7.3, 7.3 Hz); ^{13}C NMR ($CDCl_3$) δ: 115.7 (C-2′, C-6′), 116.1 (C-3, C-5), 119.6 (C-4′), 122.4 (C-2, C-6), 129.3 (C-3′, C-4′), 135.8 (C-1), 145.1 (C-1′), 151.1 (C-4). (Found: M$^+$ 185.0847. Calcd for 185.0841 ($C_{12}H_{11}NO$)).

Compound **7**. ^{1}H NMR ($CDCl_3$) δ: 6.31 (d, 1H, J=1.8 Hz), 6.84 (dd, 1H, J=1.8, 9.8 Hz), 7.32 (d, 1H, J=8.6 Hz), 7.35

(dd, 1H, *J*=7.9, 8.6 Hz), 7.42 (d, 1H, *J*=9.8 Hz), 7.52 (ddd, 1H, *J*=1.2, 8.6, 8.6 Hz), 7.77 (dd, 1H, *J*=1.2, 7.9 Hz); ^{13}C NMR ($CDCl_3$) δ: 107.0 (C-2), 116.2 (C-3′), 125.5 (C-5′), 130.5 (C-6′), 132.9 (C-4′), 133.4 (C-1′), 134.8 (C-5), 135.3 (C-6), 144.1 (C-2), 148.5 (C-4), 149.6 (C-3), 155.0 (C-1). (Found: M^+ 217.0733. Calcd for 217.0739 ($C_{12}H_{11}O_3$)).

4.3.4. 1-[(5,6-Dihydroxycyclohexa-1,3-dienyl)methyl]-piperidin-4-one (8) (product converted from 1-benzyl-4-piperidone). The crude EtOAc extract (536 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=30:1) to yield 68.0 mg of **8**.

Compound **8**. ^{1}H NMR ($CDCl_3$) δ: 2.36 (dd, 2H, *J*=6.1, 6.1 Hz), 2.64 (ddd, 1H, *J*=6.1, 12.2, 12.2 Hz), 2.81 (ddd, 1H, *J*=6.1, 12.2, 12.2 Hz), 3.05 (d, 1H, *J*=12.8 Hz), 3.40 (d, 1H, *J*=12.8 Hz), 4.02 (m, 1H), 4.38 (d, 1H, *J*=5.5 Hz), 5.70 (m, 1H), 5.90 (2H); ^{13}C NMR ($CDCl_3$) δ: 40.7 (C-4′, C-6′), 52.4 (C-3′, C-7′), 61.0 (C-1′), 65.9 (C-3), 72.0 (C-2), 122.2 (C-6), 125.3 (C-4), 127.8 (C-5), 135.4 (C-1), 207.7 (C-5′). (Found: M^+ 223.1212. Calcd for 223.1209 ($C_{12}H_{17}NO_3$)).

4.3.5. (5,6-Dihydroxy-cyclohexa-1,3-dienylmethyl)-carbamic acid *tert*-butyl ester (9) (product converted from benzyl-carbamic acid *tert*-butyl ester). The crude EtOAc extract (320 mg) was subjected to column chromatography (CH_2Cl_2–MeOH=30:1) to yield 75.6 mg of **9**.

Compound **9**. ^{1}H NMR ($CDCl_3$) δ: 1.37 (s, 9H), 3.75 (dd, 1H, *J*=5.8, 16.1 Hz), 3.88 (m, 1H), 4.97 (brs, 1H), 4.08 (m, 1H), 4.21 (m, 1H), 5.77 (m, 1H), 5.80 (m, 1H), 5.87 (m, 1H); ^{13}C NMR ($CDCl_3$) δ: 28.3 (C-6′, 7′, 8′), 68.0 (C-3), 68.6 (C-2), 79.9 (C-5′), 120.4 (C-6), 124.4 (C-5), 128.7 (C-4), 138.1 (C-1), 156.4 (C-3′). (Found: M^+ 241.1312. Calcd for 241.1315 ($C_{12}H_{19}NO_4$)).

4.3.6. (2-Hydroxy-phenyl)-carbamic acid *tert*-butyl ester (10) (product converted from benzyl-carbamic acid *tert*-butyl ester). The crude EtOAc extract (160.9 mg) was subjected to column chromatography (hexane–EtOAc=10:1) to yield 44.3 mg of **10**.

Compound **10**. ^{1}H NMR ($CDCl_3$) δ: 1.46 (s, 9H), 6.63 (brs, 1H), 6.78 (ddd, 1H, *J*=1,2, 7.3, 7.9 Hz), 6.89 (dd, 1H, *J*=1.2, 7.8 Hz), 6.95 (ddd, 1H, *J*=1.7, 7.3, 7.8 Hz), 7.03 (dd, 1H, *J*=1.7, 7.9 Hz), 8.10 (brs, 1H); ^{13}C NMR ($CDCl_3$) δ: 28.2 (C-5′, C-6′, C-7′), 82.1 (C-4′), 118.8 (C-3), 120.7 (C-5), 121.4 (C-6), 125.6 (C-1), 125.6 (C-4), 147.4 (C-2), 155.0 (C-2′). (Found: M^+ 209.1053. Calcd for 209.1053 ($C_{11}H_{15}NO_3$)).

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