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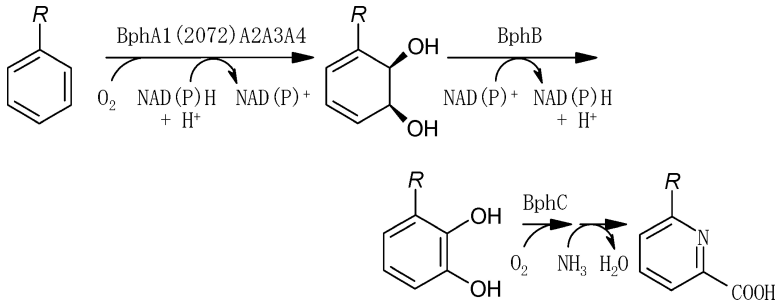

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Conversion from Arenes Having a Benzene Ring to Those Having a Picolinic Acid by Simple Growing Cell Reactions Using *Escherichia coli* that Expressed the Six Bacterial Genes Involved in Biphenyl Catabolism

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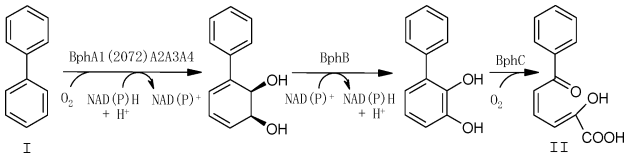
Aromatic compounds having a picolinic acid (pyridine-2-carboxylic acid) in the molecules are rare in nature and usually difficult to chemically synthesize. Using the multiple biocatalytic functions of cells may, however, enable us to achieve "chemical synthesis" of a series of organic compounds that are difficult or impractical to synthesize chemically. We report here a system for the comprehensive bioconversion of a series of aromatic compounds with a benzene ring to those with a picolinic acid, which was performed by simple growing cell reactions using recombinant microbes. We constructed a recombinant *Escherichia coli* strain expressing the six genes involved in biphenyl catabolism, these being the *bphA1*(2072)*A2A3A4* genes encoding the evolved biphenyl dioxygenase, the *bphB* gene encoding dihydrodiol dehydrogenase, and the *bphC* genes encoding catechol 2,3-dioxygenase. Their enzymatic functions are shown in Scheme 1.[1]

The *bphA1* gene encoded the large (α) subunit of the iron−sulfur protein of biphenyl dioxygenase which contains the substrate-binding site.[2] The evolved *bphA1* (2072) gene,[3] which had been constructed by DNA shuffling between *bphA1* from the biphenyl-degrading soil bacteria, *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia* sp. LB400, encoded a large subunit with a broad substrate range.[4] This gene was inserted into the *Sac*I/*Bam*HI site of plasmid pJHF18 [5] carrying the *bphA2A3A4BC* genes derived from *P. pseudoalcaligenes* KF707 [6] to construct plasmid pSHF1072. All the six genes in this plasmid were oriented to have transcriptional read-through starting from *bphA1* (2072) by the *lac* promoter of vector pUC118 upon induction by IPTG.[7]

We selected biphenyl, 2-phenyl naphthalene, 3-phenylindanone, 2-phenylquinoline, and 2-phenylbenzoxazole, and the six flavonoids, flavanone, flavone, 6-hydroxyflavanone, 6-hydroxyflavone, 7-hydroxyflavanone, and (*trans*)-chalcone, as substrates for the biotransformation experiments.[8] *E. coli* JM109 harboring pSHF1072 was grown in an LB medium[7] containing 150 μg/mL of ampicillin (Ap) at 30 °C with reciprocal shaking (175 rpm) for 6−7 h until the absorbance at OD 600 nm had reached approximately 1. Eight milliliters of this culture was inoculated into 100 mL of an M9 medium[7] with 150 μg/mL of Ap, 1 mM of IPTG, 0.4% (w/v) glucose, and 10 mg of each substrate in a Sakaguchi flask, and co-cultivated at 30 °C with reciprocal shaking (175 rpm) for 2 days. To extract the converted products as well as the substrates, a volume of methanol equal to that of the culture medium was added to the co-culture and mixed for 30 min. After centrifuging to remove cells, the liquid phase was applied to a high-performance liquid chromatographic (HPLC) analysis for further purification of the converted products. The structure of each purified product was determined by HRFAB-MS and by ^{1}H and ^{13}C NMR data.[9]

Scheme 1. Catabolic Pathway from Biphenyl (I) to the Meta-Cleavage Compound (II)



The products converted from the respective substrates were surprisingly found to be aromatic compounds, in which a benzene ring had been replaced with a picolinic acid, as shown in Table 1. Product **11** from (*trans*)-chalcone showed a reduction of $\Delta^{1,2}$ due to endogenous activity in the *E. coli* host.[10] Except for the product from biphenyl, 6-phenylpyridine-2-carboxylic acid (**1**), all of the remaining products were novel compounds according to the CAS database. Each substrate-to-product conversion ratio was in the range of 13−84% as shown in Table 1.

As an example of the structural identification, the molecular formula of product **6** from flavanone was determined to be $C_{15}H_{11}O_4N$ by HRFAB-MS. The presence of a carboxylic acid function in **6** was demonstrated by treating **6** with phenasylbromide and triethylamine to give the phenasyl ester of **6**. An analysis of the DQF COSY and HMQC spectra of **6** showed the A and C rings in flavanone to have been completely preserved, while the signals due to the B ring had disappeared. Except for the signals due to the A and C rings, vicinal coupled sp^2 methine signals (C-2′ (δ_H 8.02, δ_C 124.3) − C-3′ (δ_H 8.07, δ_C 138.7) − C-4′ (δ_H 7.87, δ_C 124.3)) and three sp^2 quarternary carbons (δ_C 148.8, δ_C 157.3, δ_C 166.1 (carboxylic acid)) were observed in the ^{1}H and ^{13}C NMR spectra of **6.** The ^{1}H−^{13}C long-range coupling from H-3′ to δ_C 148.8 (C-5′) and δ_C 157.3 (C-2′) in the HMBC spectrum and the vicinal spin couplings of $J_{2',3'}$ (7.7 Hz) and $J_{3',4'}$ (7.6 Hz) confirmed the presence of a pyridine ring composed of the carbons of C-2′ to C-5′. The linkage of C-2′ to the C ring and of C-5′ to carboxylic acid was determined from the ^{13}C chemical shift data. These findings collectively indicated **6** to be 6-oxo-6-(4-oxo-chroman-2-yl)-pyridine-2-carboxylic acid.

The products by the catabolic enzymes, BphA1(2072)A2A3A4BC, would have been aromatic compounds with a meta-cleavage group, i.e., ones with 2-hydroxymuconic ϵ-semialdehyde (Scheme 1). Since we were unable to detect such meta-cleavage products, these compounds are considered to have been unstable in the co-culture,

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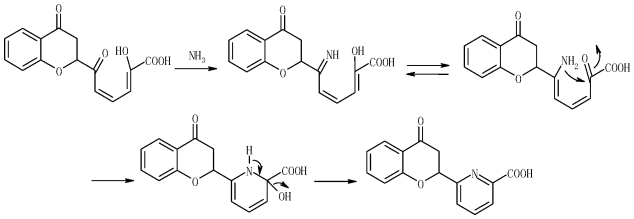
Table 1. Bioconversion of Various Aromatic Compounds with Phenyl Groups by Co-culture with the Cells of *E. coli* Carrying the *bphA1* (2072), *bphA2*, *bphA3*, *bphA4*, *bphB*, and *bphC* Genes[a]

substrate	product	substrate	product
biphenyl	**1** (75%)	flavanone	**6** (65%)
2-phenyl naphthalene	**2** (25%)	flavone	**7** (37%)
3-phenylindanone	**3** (13%)	6-hydroxyflavanone	**8** (28%)
2-phenylquinoline	**4** (29%)	6-hydroxyflavone	**9** (46%)
2-phenyl benzoxazole	**5** (23%)	7-hydroxyflavanone	**10** (84%)
		(*trans*)-chalcone	**11** (41%)

[a] The percentage values in parentheses represent the conversion ratio of each substrate to the corresponding product, which was measured by HPLC.

Scheme 2. Proposed Routes for Generating the Aromatic Compound with a Picolinic Acid from a Compound with a 2-Hydroxymuconic ϵ-Semialdehyde



and rapidly converted to the picolinic acid compounds in a nonenzymatic manner, the speculated routes being shown in Scheme 2. Ammonia is very likely to have been derived from NH_4Cl (1 g/L; 30−40-fold equivalent amounts of the substrates) in the M9 medium. Asano et al. have also found that the meta-cleavage compound, 2-hydroxymuconic ϵ-semialdehyde, was biotransformed from the monocyclic aromatic molecule, catechol, through the cell suspension of *E. coli* which expressed a bacterial catechol 2,3-dioxygenase gene (similar to *bphC*), and then was chemically converted to picolinic acid by such severe conditions as stirring in 25% ammonia water or autoclaving with ammonium phosphate.[11] We also carried out the biotransformation experiments of flavanone with different concentrations of NH_4Cl in the co-culture to determine its required concentration.

Consequently, the picolinic acid product started to be generated with 0.35 g/L of NH_4Cl and reached a plateau between 0.75 and 10 g/L of NH_4Cl.

We used 10 mg of each substrate per 100 mL of culture (0.01% w/v) in all of the foregoing bioconversion experiments. To

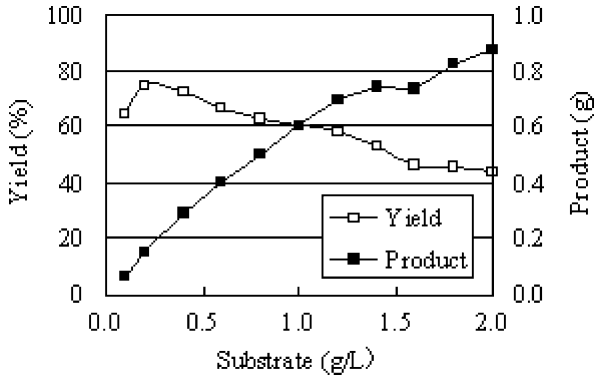


Figure 1. Scaled-up test on the substrate by using flavanone. Product (g) represents wt of the purified picolinic acid compound. Yield (%) shows the ratio, purified product/substrate added.

substantiate that the substrate content was not at too low a level, we conducted scaled-up experiments on the substrate using flavanone as the example, the results being shown in Figure 1. It was found to easily scale-up the substrate by 20-fold (0.2% w/v) with more than a moderate level of yield (40−80%). We have reported in the present study a simple method for producing various picolinic acid-incorporating compounds (which are substituents at the C-6 position) from aromatic compounds with a benzene ring by ordinary co-culture with recombinant *E. coli* and each substrate. The comprehensive production of such organic chemicals, which are very 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.

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Supporting Information Available: Methods for HPLC analysis and purification of the converted products, and HRFAB-MS, ^{1}H- and ^{13}C NMR data of **1**−**11.** This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) The microbial biodegradation of biphenyl and polychlorinated biphenyls (PCBs) has been reviewed: (a) Furukawa, K. *Curr. Opin. Biotechnol.* **2000**, *11*, 244−249. (b) Furukawa, K.; Suenaga, H.; Goto, M. *J. Bacteriol.* **2004**, *186*, 5189−5196.
(2) Carredano, E.; Karlsson, A.; Kauppi, B.; Choudhury, D.; Parales, R. E.; Parales, J. V.; Lee, K.; Gibson, D. T.; Eklund, H.; Ramaswamy, S. *J. Mol. Biol.* **2000**, *296*, 701−712.
(3) DDBJ/EMBL/GenBank accession no. AB085748.
(4) Misawa, N.; Shindo, K.; Takahashi, H.; Suenaga, H.; Iguchi, K.; Okazaki, H.; Harayama, S.; Furukawa, K. *Tetrahedron* **2002**, *58*, 9605−9612.
(5) Hirose, J.; Suyama, A.; Hayashida, S.; Furukawa, K. *Gene* **1994**, *138*, 27−33.
(6) DDBJ/EMBL/GenBank accession no. M83673.
(7) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1989
(8) The chemicals used as substrates were purchased from Aldrich Chemical or Tokyo Kasei Kogyo. The respective substrates were each dissolved in a small volume of dimethyl sulfoxide (DMSO) and added to the culture.
(9) The structures were analyzed by their mass spectral data [MS (FAB) and HRMS (FAB); JEOL JMS-700T] and nuclear magnetic resonance (NMR) spectral data (400 MHz, Bruker AMX400).
(10) Shindo, K.; Kagiyama, Y.; Nakamura, R.; Hara, A.; Ikenaga, H.; Furukawa, K.; Misawa, N. *J. Mol. Catal. B: Enzym.* **2003**, *23*, 9−16.
(11) Asano, Y.; Yamamoto, Y.; Yamada, H. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 2054−2056.

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