Oxidation of Naphthenoaromatic and Methyl-Substituted Aromatic Compounds by Naphthalene 1,2-Dioxygenase†

SERGEY A. SELIFONOV, 1,2,* MAGDALENA GRIFOLL, 3 RICHARD W. EATON, 4 AND PETER J. CHAPMAN 4

Center for Environmental Diagnostics and Bioremediation, University of West Florida, 1 and Gulf Ecology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Gulf Breeze, Florida 32561; Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108; 2 and Department of Microbiology, University of Barcelona, 08028 Barcelona, Spain

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Oxidation ofacenaphthene,acenaphthylene,andfluorenewasexaminedwithrecombinantstrainPseudomonas aeruginosa PAO1(pRE695)expressingnaphthalenedioxygenagenesclonedfromplasmidNAI7. Acenaphtheneunderwentmonoxygenationto1-acenaphthenewithsubsequentconversionto1-acenaphthenoneandcis- and trans-acenaphthene-1,2-diols,whileacenaphthylene wastdioxygenatedtogivecis-acenaphthene-1,2-diol. Nonspecificdehydrogenaseactivitiespresentinthehoststrainledtotheconversionofbothoftheacenaphthene-1,2-diolsito1,2-acenaphthoquinone. The latter was oxidized spontaneously to naphthalene-1,8-dicarboxylic acid. No aromatic ring dioxygenation products were detected fromacenaphtheneandacenaphthylene. Mixedmonoxygenasedioxygenagenactions of naphthalenedioxygenasenonfluorenewieldedproducts ofbenzyclic9-monooxygenation,aromaticringdioxygenation,orboth. Theactionof naphthalenedioxygenasenon a variety ofmethyl-substituted aromaticcompounds,including1,2,4-trimethylbenzenearndimersofdimethylnaphthalene, resultedinin theformation ofbenzyclicalcohols, i.e., methyl group monoxygenation products, which were subsequently converted to the corresponding carboxylicacidsbydehydrogenase(s)inthehoststrain. Benzyclicmonoxygenationofmethyl groupswasstronglypredominantover aromaticringdioxygenationandessentiallynonspecificwithrespected to the substitution pattern of the aromatic substrates. Inadditiontomonoxygenatingbenzylicmethyl andmethylenegroups, naphthalenedioxygenasbehavedasasulfoxygenase, catalyzing monoxygenation of the sulfur heteroatom of 3-methylbenzothiophene.

Naphthenoaromaticcompoundsconstituteagroup of aromaticchemicals withbenzyclic methylenic or methylenegroups. Theyareabundant constituents of polycyclic aromatic compound (PAC)mixtures,oneofthelargest groups of priority pollutants (45). Compounds withbenzyclicmethylenegroups (e.g.,acenaphtheneandfluorene) occuras major constituents ofcoal-derived products suchascreosote andcoal tar (47). Methyl-substituted aromaticcompoundsare alsofound in coal-derived products butin small quantities relative to those ofthe corresponding unsaturated nuclei. Crude oils and petroleum-derived materials also contain an extensive series of naphthenoaromatic chemicals in which unsubstituted parent hydrocarbons are relatively less abundant than in coal-derived materials (1, 33).

Biodegradation of materials containing PACs may have value in the development of remediative technologies that address environmental pollution by these chemicals (31, 32). From the standpoint of the structural complexity of PACs found in such complex mixtures as fossil fuels, microbial mechanisms contributing to the degradation of naphthenoaromatic compounds have been insufficiently investigated. An extensive body of knowledge on the bacterial degradation of PACs has been built over yearsof biochemical and genetic studies, yet these have addressed primarily the degradation of unsubstituted aromatic nuclei (8). The bacterial catabolic pathways for chemicals of the latter group (e.g., naphthalene or phenanthrene) principally involve sequences of reactions initiated by reductive dioxygenation of the aromatic ring (19). Broad substrate specificity is now a well-recognized property of bacterial enzymes catalyzing early reactions of catabolic pathways for PAC utilization since Evans et al. (16) first showed oxidation of phenanthrene and anthracene by naphthalene-grown bacteria. However, little is known of how bacterial arenedioxygenases act on naphthenoaromatic compounds and, therefore, influence their fate during biodegradation of PAC mixtures.

In recent years, various monoxygenase-type reactions catalyzed by toluene dioxygenase (TDO) acting on indan and indene (6, 46) or by naphthalene dioxygenase (NDO) acting on indan, indene (18), and indanones (35) have emerged as characteristic of oxygenases normally performing dioxygenation of aromatic rings. Earlier studies of the bacterial oxidation ofacenaphtheneandacenaphthylenebyamutant strain of Beijerinckia sp. (37) showed that several oxygenated products are formed from these compounds as a result of the oxidative activity normally responsible for initial reactions in the catabolism of aromatic hydrocarbons, such as biphenyl. Product formation was explained as resulting from monoxygenation ofacenaphtheneanddioxygenationofacenaphthylenealongwithnonspecific conversion of formed sec-alcohols to the corresponding ketones. However, the observed monoxygenase reactions were not unambiguously attributed to the activity of a single mono- or dioxygenase.

Although a number of reports describe monoxygenation of the methyl groups of substituted aromatic hydrocarbons, such as xylenes or methylbenzothiophenes (3, 4, 10, 11, 29, 34, 44), these reactions have not been attributed to actions of arene dioxygenases. Only TDO has been rigorously implicated in mo-
noxygcnation of the methyl groups of 2- and 3-nitrotoluenes (36).

A possible role of arene dioxygenases in the oxidation of sulfur heteroatoms in S-heterocyclic aromatic compounds is of particular interest because that reaction is often catalyzed by bacteria that possess dioxygenation-initiated catabolic pathways for degradation of PACs, such as naphthalene, biphenyl, and fluorene (17, 21, 22, 25, 28, 30). Aromatic sulfur heterocycles, such as benzo[ghi]perylene and dibenzo[ghi]perylene, also occur in coal-tar derived and petroleum materials, and a number of reports demonstrate that they can be metabolized via a sequence of reactions akin to those of naphthalene or alkylbenzene catabolism (14, 22, 27, 28). However, there is no direct experimental evidence available at present to implicate arene dioxygenases in the reactions of sulfoxidation. Recently, a dihydrodiol-accumulating mutant derivative, UV4, of the toluene-degrading Pseudomonas putida NCIMB 11767 was shown to form chiral sulfoxides from a number of aryl-substituted S-heterocycles. However, few experimental details were reported to provide unequivocal evidence implicating TDO, or another oxygenase, in the sulfoxidation reactions (2).

The present study focuses on nondioxygenase oxidative reactions catalyzed by NDO encoded by the plasmid NAH7. This NDO, like that from Pseudomonas sp. strain 9816-4, initiates naphthalene catabolism by NAD(P)H-dependent incorporation of dioxygen into naphthalene to form cis-1,2-dihydroxy-1,2-dihydronaphthalene (15, 26, 43). The use of a recombinant strain, Pseudomonas aeruginosaa PAO1(pRE695) (13), expressing NDO genes cloned in the absence of genes encoding subsequent enzymes of the naphthalene catabolic pathway, allows firm attribution of the formation of oxygenated products other than arene-cis-dihydriodols to NDO. We report here the action of NDO of the NAH7 plasmid on a variety of naphthoaromatic hydrocarbons, on methyl-substituted aromatic hydrocarbons, and on aromatic S-heterocycles.

(A preliminary account of this work has appeared elsewhere [39].)

MATERIALS AND METHODS

Organisms, growth, biotransformations, and product recovery. P. aeruginosaa PAO1 harboring plasmid pRE695, which carries NDO genes cloned from plasmid NAH7, was used in this study (13). The strain was grown in 500-m1 volumes in mixed LB-mineral medium (1:1, vol/vol) (23), supplemented with glucose (1 g/liter) in 2-liter Erlemeyer flasks on a rotary shaker (180 rpm) or in 8-liter volumes (13-liter flasks) aerated by magnetic stirring (300 rpm) at 30°C. Naphthalene 1,2-dioxygenase was induced by the addition of isopropl-β-D-thioglucopyranoside (0.5 mM, 2 h) in the late exponential phase of growth. Cells were harvested by centrifugation (8,000 × g), washed twice with 50 mM potassium phosphate buffer (pH 7.5), and resuspended in the same buffer to give a suspension with an optical density at 600 nm of 1.8 before incubation with different test compounds. Hydrocarbons were supplied at concentrations of 1 g/liter, while naphthoaromatic sec-alcohols, ketones, and S-heterocycles were supplied at 200 mg/liter. Incubations with the host strain, P. aeruginosaa PAO1 lacking plasmid pRE695, and incubations without cells were used as control experiments. Incubations were performed at 30°C for 30 h with a rotary shaker and Erlemeyer flasks for 24 h. Subsequently, cells (and undissolved substrates, where present) were removed by centrifugation, and supernatants were extracted with ethyl acetate (2 × 1 volume) and then acidified (to pH 2 to 3) before extraction again with ethyl acetate. Extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure at 30°C.

Derivatization methods. Materials recovered by extraction under acidic conditions were methylated with an ethereal solution of diazomethane prior to gas chromatography–mass spectrometry (GC-MS) analysis. Acetylation with acetic anhydride in the presence of pyridine was used for derivatization of fluorine-related products.

Analytical methods. GC-MS and GC-Fourier transform infrared spectroscopy (GC-FTIR) were performed using a Hewlett-Packard 5890 series II gas chromatograph equipped with HP5971 mass-selective and HP 5965B infrared detectors. Compounds were separated on an HP-5 capillary column (25 m by 0.32 mm, 0.25-μm film thickness) with helium as the carrier gas (at 23 cm/s). The column temperature was held isothermally at 50°C for 1 min and then increased to 290°C at a rate of 5°C/min (conditions A) or 10°C/min (conditions B). The mass spectrometer was operated at an electron ionization energy of 70 eV. Injector, transfer line, and analyzer temperatures were set at 150, 300, and 300°C, respectively. Instrumental library searches and comparisons with available authentic compounds were used for identification of metabolites. The relative amounts of metabolites were calculated for duplicate samples from the peak areas of GC-MS total ion current chromatograms. Total ion current integrated responses (excluding peak of residual starting material) were used as 100% internal standard values for each sample analyzed.

Thin-layer chromatography (TLC) was performed on 0.25-mm-thick silica gel plates (Merck Silica Gel 60 F254) with methylene chloride or ethyl acetate as the developing solvent.

Chemicals. All chemicals were of the highest purity commercially available.

Commercial preparations of acenaphthylene from a number of sources contained various amounts of acenaphthenes (1 to 15%), as determined by GC and GC-MS. To avoid artifacts due to insufficient purity of this chemical for the biotransformation studies, acenaphthylene with a content of acenaphthenes not exceeding 0.1% (by GC) was prepared by dehydration of 1-acenaphthenol. 1-Acenaphthenol (2 g, recrystallized from hexane) was mixed with 50 ml of 30% phosphoric acid and heated at 80°C with occasional stirring until it could no longer be detected by TLC of small samples removed over the course of dehydration (1.5 h). Aacenaphthenol was recovered by extraction with pentane, further purified by sublimation, and stored in the dark at room temperature until further use.

1-Bromoacetophenone was prepared at 10-mmol scale by reduction of a 10% solution of potassium salt of 1,8-naphthaldehydic acid in 0.1 M potassium phosphate buffer (pH 7.5) with excess sodium borohydride to give 8-hydroxyethyl-1-naphthalene-1-carboxylic acid. Acidification of the reaction mixture to pH 2, followed by extraction with ethyl acetate, yielded chromatographically pure 1,8-naphthalenesulfonic acid (98%).

Authentic acenaphthene (37), cis- and trans-acenaphthene-1,2-diols (24), and 1-hydroxy-9-fluorenone (40) were prepared by published methods. Identities of all authentic materials were confirmed by GC-MS and 1H nuclear magnetic resonance spectroscopy.

RESULTS AND DISCUSSION

The biotransformation of acenaphthene, acenaphthylene, fluorene, and indane symmetrically substituted dimethylnaphthalenes (DMNs), 1,2,4-tetramethylbenzene, and three different benzo[b]thiophenes was examined with P. aeruginosaa PAO1(pRE695), expressing cloned NDO genes, and with P. aeruginosaa PAO1 lacking pRE695. This allowed all observed initial oxygen incorporation reactions to be attributed to the action of NDO.

Oxidation of acenaphthene by cells of PAO1(pRE695) yielded a number of products (Table 1; Fig. 1), with 1-acenaphthene (compound 2) and 1-acenaphthenone (compound 1) accounting for up to 65% (by GC) of all recovered products. Biotransformations of acenaphthene, 1-acenaphthene, and 1-acenaphthenone also yielded a considerable amount of both cis- and trans-2-acenaphthene-1,2-diols (24), with each isomer accounting for up to 15% of recovered products. In contrast to acenaphthene, acenaphthylene was converted by cells of P. aeruginosaa PAO1(pRE695) to acenaphthene-1,2-diol (compound 4, up to 76%) with virtually exclusive cis configuration of its hydroxyl groups (more than 99% of the cis isomer). Small amounts (1 to 3%) of acenaphtheno-1,2-quinone (compound 6) and traces of compound 3 (tentatively identified as acenaphthene-1,2-diol or its ketol tautomer) were also observed in experiments with acenaphthene and acenaphthylene. Aacenaphtho-1,2-quinone was also formed as a major product from either cis- or trans-2-acenaphthene-1,2-diol when incubated with the NDO-containing strain.

The presence of small amounts of 1-acenaphthenone (compound 1) detected by GC-MS in experiments with acenaphthene (free of acenaphthene) and with either cis-acenaphthene-1,2-diol or trans-acenaphthene-1,2-diol was attributed to thermal dehydration of diols in the GC injector. 1-Acenaphthene was not detected by TLC analysis of the biotransformation products where traces of authentic ketone could easily be detected as a result of its intense blue fluorescence upon illumination of TLC plates with 254-nm UV light.

A combination of several mechanisms may be proposed to

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explain the formation of comparable amounts of both cis- and 
trans-acenaphthene-1,2-diols from acenaphthene by P. aerugi-
osa PAO1(pRE695) (Fig. 1). The first involves sequential 
monooxygenation of each methylenic group of acenaphthene 
by NDO. An alternative mechanism may be suggested in view 
of recent data indicating that NDO from 
other reactions. Absolute stereochemistry is not in-
tended.

A similar desaturation of acenaphthene to acenaphthylene cat-
ylized by NDO of the NAH7 plasmid would furnish the latter 
compound to undergo dioxygenation to cis-acenaphthene-1,2-
diol. Finally, reversible dehydrogenase reductase activity of 
the host strain might also contribute to the distribution of cis and 
trans isomers. In fact, (±)-trans-acenaphthene-1,2-diol was 
partially converted to its cis-isomer when incubated with cells 
of either PAO1(pRE695) or the host PAO1 lacking pRE695. 
Evidence for such dehydrogenase reductase activity in the host 
strain was also obtained when incubations with (+)-acenap-
thenol and fluorenol yielded the corresponding ketones and 
cis- and (±)-trans-acenaphthene-1,2-diols gave acenaphtho-
1,2-quinone; the ketones were partially reduced to the corre-
sponding sec-alcohols by this strain. Although establishing the 
absolute configurations of the chiral products, 1-acenaphthenol 
and trans-acenaphthene-1,2-diol, formed by strain 
PAO1(pRE695) was beyond the scope of this work, it is im-
portant to note that enantioselective action of NDO on (1R) 
or (1S)-acenaphthenol along with enantioselective dehydroge-
nase reductase activity of the host strain might also affect the 
distribution of cis- and trans-diols.

Comparison of the products of acenaphthene and acenaph-
thylene oxidation by P. aeruginosa PAO1(pRE695) (Fig. 1) 
with those formed by a biphenyl-oxidizing strain, Beijerinckia 
sp. strain B8/36 (37), indicates the similarity of the oxidation 
pathways in these two organisms. Although it was not possible 
to rigorously implicate a particular oxygenase in the observed 
oxidations of these compounds by strain B8/36 with the recombi-
nant strain PAO1(pRE695), both the initial 1-monooxygena-
tion of acenaphthene and 1,2-dioxygenation of acenaphthy-
lene can now be unambiguously attributed to NDO.

It is interesting to note that considerable amounts (3 to 5%) 
of naphthalene-1,8-dicarboxylic acid (1,8-NDA; compound 7), 
recovered as its anhydride (compound 8) under the acidic 
conditions of extraction, were found among the products of 
biotransformations of acenaphthene, acenaphthylene, and 
their oxidation products (Table 1). Incubations of acenaphth-
quinone without cells also resulted in the accumulation of 
1,8-NDA, indicating spontaneous oxidation of the quinone 
rather than a biological reaction. Neither PAO1(pRE695) nor 
PAO1 metabolizes 1,8-NDA further. In an earlier study of the 
oxidation of acenaphthene and acenaphthylene by Beijerinckia
sp. strain B8/36 (37), 1,8-NDA was not reported among the oxidation products. However, a wild-type strain of *Beijerinckia* sp., strain B1, accumulated this compound as a final product of acenaphthene and acenaphthylene oxidation (38). 1,8-NDA (or its anhydride) has also been reported as a dead-end product in oxidations of acenaphthene and acenaphthylene by a bacterial strain that utilizes naphthalene, fluorene, or phenanthrene (22). Although there are no published data available on the further bacterial metabolism of 1,8-NDA, this compound has been established as an intermediate in acenaphthene catabolism by *Alcaligenes* sp. strains (41), and a number of bacterial strains capable of utilizing eitheracenaphthene or ace napthylene as a sole carbon and energy source also grow with 1,8-NDA.

NDO in strain *P. aeruginosa* PAO1(pRE695) exhibited both monoxygenase and dioxygenase activities toward fluorene (Table 2; Fig. 2). Benzylic 9-monoxygenation was responsible for the formation of 9-fluorenone (compound 10) and 9-fluorenol (compound 9), while aromatic ring dioxygenation products were detected as phenolic compounds (compounds 11 to 14) apparently derived from dehydration of putative 1,2- and 3,4-cis-dihydrodiols. GC-MS analysis of the acetylated bio transformation products from fluorene, 9-fluorenone, or 9-fluorenol also revealed a number of minor metabolites (compounds 15 to 24) with oxygen incorporated at both the benzylic position C-9 and in the aromatic ring. Detection of a series of four phenolic isomers of hydroxyfluorene (compounds 11 to 14), of 9-hydroxyfluorene (compounds 17 to 20), and of four compounds (compounds 21 to 24) with MS fragmentations

![FIG. 2. Transformation of fluorene by *P. aeruginosa* PAO1(pRE695). Bold arrows indicate reactions catalyzed by NDO.](http://aem.asm.org/)

### Table 2. GC-MS data of the products formed from starting substrates of fluorene (H), 9-fluorenone (J), and 9-fluorenol (I) by *P. aeruginosa* PAO1(pRE695)

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Starting substrate</th>
<th>GC R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>m/z of major ion peaks (abundance [%])</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>H, J</td>
<td>27.03</td>
<td>180(M&lt;sup&gt;+&lt;/sup&gt; , 100), 152(32), 126(4), 76(13), 63(6)</td>
<td>9-Fluorenone&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>H, I</td>
<td>27.55</td>
<td>182(M&lt;sup&gt;+&lt;/sup&gt; , 81), 181(100), 165(14), 152(36), 139(2), 126(3), 91(5), 76(16)</td>
<td>9-Fluorenol&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>H</td>
<td>30.15</td>
<td>182(M&lt;sup&gt;+&lt;/sup&gt; , 100), 181(70), 165(26), 139(4), 128(32), 76(20)</td>
<td>(?)-Hydroxyfluorene&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>H</td>
<td>30.46</td>
<td>182(M&lt;sup&gt;+&lt;/sup&gt; , 100), 181(46), 166(13), 165(14), 152(27), 139(1), 126(3), 82(5), 76(12)</td>
<td>1-Hydroxyfluorene&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>H</td>
<td>30.65</td>
<td>182(M&lt;sup&gt;+&lt;/sup&gt; , 100), 181(76), 165(26), 152(31), 127(3), 91(6), 76(11)</td>
<td>(?)-Hydroxyfluorene&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>H</td>
<td>30.80</td>
<td>182(M&lt;sup&gt;+&lt;/sup&gt; , 100), 181(57), 165(12), 152(26), 126(3), 91(6), 76(10)</td>
<td>2-Hydroxyfluorene&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

### Notes:
- Only those substrates from which a particular biotransformation product was formed are indicated.
- Identification is based on match of mass spectra (fragmentation and peak intensity) and capillary GC retention times with those of authentic samples.
- Tentative assignment of structure.
- Compounds detected under conditions B after acetylation (excluding derivatives of compounds listed under conditions A).
- Four isomers possible.
### TABLE 3. GC-MS data of products formed from methyl-substituted aromatic compounds by *P. aeruginosa* PAO1(pRE695)

<table>
<thead>
<tr>
<th>Product no.</th>
<th>Relative amt (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GC&lt;sup&gt;c&lt;/sup&gt; Rt (min)</th>
<th>m/z of major ion peaks (abundance [%])</th>
<th>Suggested structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>From 1,5-dimethyl-naphthalene:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>3</td>
<td>15.86</td>
<td>186(M&lt;sup&gt;+&lt;/sup&gt;, 6), 171(14), 170(68), 169(19), 155(52), 141(100), 127(7), 115(43)</td>
<td>1-Methoxy-4,8-dimethylnaphthalene&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>26</td>
<td>75</td>
<td>16.68</td>
<td>172(M&lt;sup&gt;+&lt;/sup&gt;, 92), 157(27), 155(19), 153(17), 143(100), 128(72), 115(31)</td>
<td>1-Hydroxymethyl-5-methyl naphthalene&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>27</td>
<td>12</td>
<td>17.21</td>
<td>200(M&lt;sup&gt;+&lt;/sup&gt;, 84), 185(4), 169(100), 157(5), 141(58), 139(21), 128(6), 115(33)</td>
<td>5-Methyl-1-naphthoic acid methyl ester&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>28</td>
<td>9</td>
<td>19.86</td>
<td>188(M&lt;sup&gt;+&lt;/sup&gt;, 81), 170(19), 157(18), 141(100), 129(85), 128(65), 115(21)</td>
<td>1,5-bis(Hydroxymethyl) naphthalene&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>From 2,6-dimethyl-naphthalene:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>17</td>
<td>16.42</td>
<td>172(M&lt;sup&gt;+&lt;/sup&gt;, 100), 157(30), 155(26), 153(11), 143(97), 128(72), 115(31)</td>
<td>2-Hydroxymethyl-6-methyl naphthalene&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>16.64</td>
<td>172(M&lt;sup&gt;+&lt;/sup&gt;, 100), 157(22), 143(9), 129(29), 128(28), 115(10)</td>
<td>3,7-Dimethyl-1-naphthol&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>31</td>
<td>43</td>
<td>17.29</td>
<td>200(M&lt;sup&gt;+&lt;/sup&gt;, 75), 185(1), 169(100), 155(2), 141(51), 115(23)</td>
<td>6-Methyl-2-naphthoic acid methyl ester&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>32</td>
<td>13</td>
<td>19.84</td>
<td>188(M&lt;sup&gt;+&lt;/sup&gt;, 55), 171(7), 143(22), 129(100), 115(10)</td>
<td>2,6-bis(Hydroxymethyl) naphthalene&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>From 2,7-dimethyl-naphthalene:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>74</td>
<td>16.43</td>
<td>172(M&lt;sup&gt;+&lt;/sup&gt;, 100), 157(26), 155(20), 153(11), 143(99), 141(33), 128(73), 115(31)</td>
<td>2-Hydroxymethyl-7-methyl naphthalene&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>From 1,8-dimethyl-naphthalene:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>14</td>
<td>15.57</td>
<td>170(M&lt;sup&gt;+&lt;/sup&gt;, 74), 155(4), 141(100), 139(24), 127(1), 115(23)</td>
<td>8-Methyl-1-naphthaldehyde&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>35</td>
<td>9</td>
<td>15.83</td>
<td>170(M&lt;sup&gt;+&lt;/sup&gt;, 95), 169(100), 153(36), 141(59), 139(25), 127(12), 115(41)</td>
<td>Unidentified</td>
</tr>
<tr>
<td>From 1,2,4-trimethylbenzene:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>20</td>
<td>10.28</td>
<td>136(M&lt;sup&gt;+&lt;/sup&gt;, 51), 121(21), 118(100), 105(18), 93(34), 91(54), 77(33)</td>
<td>2,5-Dimethylbenzyl alcohol&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>43</td>
<td>28</td>
<td>10.38</td>
<td>136(M&lt;sup&gt;+&lt;/sup&gt;, 60), 121(20), 118(100), 105(16), 93(30), 91(50), 77(30)</td>
<td>2,4-Dimethylbenzyl alcohol&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>44</td>
<td>24</td>
<td>10.51</td>
<td>136(M&lt;sup&gt;+&lt;/sup&gt;, 97), 121(100), 119(11), 107(41), 105(40), 93(66), 91(75), 77(40)</td>
<td>3,4-Dimethylbenzyl alcohol&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>45</td>
<td>3</td>
<td>10.87</td>
<td>164(M&lt;sup&gt;+&lt;/sup&gt;, 63), 149(16), 133(100), 132(94), 105(64), 91(10), 77(34)</td>
<td>2,5-Dimethylbenzonic acid methyl ester&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>46</td>
<td>4</td>
<td>10.99</td>
<td>164(M&lt;sup&gt;+&lt;/sup&gt;, 42), 149(6), 133(100), 132(55), 105(36), 91(6), 77(21)</td>
<td>2,4-Dimethylbenzonic acid methyl ester&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>47</td>
<td>9</td>
<td>11.82</td>
<td>164(M&lt;sup&gt;+&lt;/sup&gt;, 36), 149(3), 133(100), 105(27), 91(11), 77(8)</td>
<td>3,4-Dimethylbenzonic acid methyl ester&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>20</td>
<td>12.37</td>
<td>152(M&lt;sup&gt;+&lt;/sup&gt;, 2), 134(100), 133(97), 119(5), 105(43), 91(41), 77(31)</td>
<td>1,2-bis(Hydroxymethyl)-4-methylbenzene&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>49</td>
<td>2</td>
<td>14.18</td>
<td>152(M&lt;sup&gt;+&lt;/sup&gt;, 65), 134(97), 121(57), 105(66), 93(76), 91(100), 77(71)</td>
<td>1,4(3)-bis(Hydroxymethyl)-3(4)-methylbenzene&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Analysis after methylation with diazomethane.

<sup>b</sup> Based on total ion current response of mass-selective detector.

<sup>c</sup> Under conditions A.

<sup>d</sup> Identification is based on instrumental library match.

<sup>e</sup> Identification is based on match of mass spectra (fragmentation and peak intensity) and capillary GC retention times with those of authentic samples.
consistent with 9-hydroxyfluorene-dihydrodiols indicated that dioxygenation of the aromatic ring can occur at both the 1,2 and 3,4 positions. Detection of compounds 21 to 24 also pointed out that incorporation of dioxygen into the aromatic ring can occur with both syn and anti configurations with respect to the stereochemistry of the hydroxyl at C-9. The concentrations of phenolic and dihydrodiol products accounted for approximately 30% of that of 9-fluorenone and 9-fluorenol in fluorene biotransformation experiments. In contrast to the oxidation ofacenaphthene, NDO acted on fluorene both in a normal dioxygenating mode and in a monooxygenating mode.

Indan was also oxidized by P. aeruginosa PAO1(pRE695), yielding 1-indanol (65%), 1,3-dihydroxyindan (20%), 1-indanone (6%), and 1-indenol (5%) as the principal products (data not shown). In this respect, the action of NDO of the plasmid NAH7 was similar to that described for the nearly identical NDO of Pseudomonas sp. strain 9816-4 (18) and TDO of P. putida F1 (46).

Methyl-substituted aromatic compounds, such as symmetrically substituted dimethylnaphthalenes and 1,2,4-trimethylbenzene, were all oxidized by strain PAO1(pRE695), as evidenced by the formation of a variety of oxygenated products (Table 3). The major route of oxidation for all tested methyl-substituted aromatic compounds involved incorporation of one atom of oxygen into a methyl substituent of the substrate molecule, resulting in formation of the corresponding benzyl alcohols (compounds 26, 29, 34, and 38) from the DMNs (Fig. 3). Both the α- and β-methyl groups of DMNs were monooxygenated by NDO. NDO also showed no preferential oxidation of any of the three nonequivalent methyl substituents of 1,2,4-trimethylbenzene, as indicated by formation of comparable amounts of the three dimethylnaphtalcohol compounds 42, 43, and 44. In the cases of 1,5-DMN, 2,6-DMN, 1,8-DMN, and 1,2,4-trimethylbenzene, the formation of bis-hydroxymethyl-substituted compounds was also detected, indicating subsequent monooxygenation of a second methyl group. Dehydrogenase activity of the host strain is apparently responsible for further conversion of the primary alcohols to carboxylic acids (e.g., compounds 27, 31, 35, 39, and 41) via the corresponding aldehydes. Such transformations of aromatic alcohols and aldehydes to acids have been observed in this strain (12). The lactone (compound 40) detected only in experiments with 1,8-DMN is evidently formed from 8-hydroxymethyl-1-naphthoic acid as a result of its spontaneous ring closure under acidic extraction conditions.

Biotransformation of 1,8-DMN also yielded an unidentified product (compound 37) whose MS fragmentation indicates incorporation of one atom of oxygen and the loss of two atoms of hydrogen with respect to the molecular mass of the starting substrate. Its molecular ion (m/z 170) matches those of 8-methyl-1-naphthaldehyde (compound 36) and acenaphthenol (compound 2), but the fragmentation patterns are different for all of these compounds. On the basis of its mass spectrum, a cyclic ether structure of the 1,8-naphtho(b)pyran type is suggested for compound 37. However, it remains to be established whether the formation of such a cyclic ether linkage between the methyl groups of 1,8-DMN should be attributed to the activity of NDO or to an intramolecular dehydration of 1,8-bis(hydroxymethyl)naphthalene during GC analysis.

Small amounts of naphthols (compound 25 from 1,5-DMN and compound 30 from 2,6-DMN) were apparently formed by dehydration of the cis-dihydrodiols, minor products of dioxygenation of the aromatic ring, indicating an alternative mechanism of oxidation of these hydrocarbons.

To investigate possible sulfoxidation reactions catalyzed by NDO, P. aeruginosa PAO1(pRE695) was incubated with dibenzothiophene, benzothiophene, and 3-methylbenzothio- phene. All S-heterocyclic compounds were oxidized by this strain, as indicated by the accumulation of a number of metabolites. No appreciable amounts of the corresponding sulfoxides or sulfones were observed in experiments with diben-
zothiophene or benzothiophene. GC-MS analysis of methylated transformation products from 3-methylbenzothiophene revealed three major compounds: compound 50 (GC Rf = 15.10, 35%), compound 51 (Rf = 15.51, 20%), and compound 52 (Rf = 16.96, 45%). Compounds 50 and 51 were identified as 3-hydroxymethylbenzothiophene [MS, m/z(%): 164(92), 147(88), 135(100), 131(12), 103(8), 91(35), 89(17)] and benzothiophene-3-carboxylic acid methyl ester [MS, m/z(%): 192(54), 177(2), 161(100), 133(22), 89(27)], respectively. Compound 52 had a mass spectrum [m/z(%): 164(18), 148(37), 147(70), 136(65), 135(100), 131(9), 121(22), 115(12), 103(19), 91(58), 89(10), 77(20)] indicative of incorporation of one atom of oxygen into 3-methylbenzothiophene, with a very strong infrared absorbance band at 1,081 cm−1 (GC-FTIR) characteristic of sulfoxide S=O stretching. On the basis of GC-MS-FTIR analysis and comparison of spectral data with those in the literature (17), this metabolite was identified as 3-methylbenzothiophene sulfoxide. Only a trace of compound 53 (Rf = 17.09, <0.2%) with a mass spectrum matching that of 3-methylbenzothiophene sulfone (17) was detected. Formation of these products indicates that NDO is capable of two different monoxygenase reactions with 3-methylbenzothiophene, namely, (i) oxidation of the methyl group or (ii) sulfoxidation (Fig. 4).

The absence of S-oxygenation product accumulation from dibenzothiophene may be explained by preferential dioxygenation of the aromatic ring and by insufficient accessibility of the S-heteroatom for activated oxygen species at the catalytic center of NDO. In the case of the benzothiophene biotransformation, a number of products consistent with 2,3-dioxygenation were detected. Such attack on benzothiophene by isopropylbenzene dioxygenase results in an unstable cis,2,3-dioxygenation product readily undergoing both spontaneous opening of the S-heterocyclic ring and isomerization (14). It would appear that the extent to which NDO, and similar enzymes, can play a role in the sulfoxidation processes depends largely on the structures of S-heterocyclic aromatic compounds, their substitution patterns, and the degree of hindrance of the S-heteroatom.

It has been shown previously that TDO of P. putida F1 monoxygenates benzylic methylene groups of indan (46) and methyl groups of nitrotoluenes (36) and that NDO of Pseudomonas sp. strain 9816-4 monoxygenates indan (18) and indanes (35). Evidently, many, but not all, known arene dioxygenases can catalyze monoxygenase reactions of naphthenoaromatic or methyl-substituted aromatic substrates. For example, the purified dibenzofuran-4,4a-dioxygenase, which performs an angular attack on dibenzofuran and fluorenone, does not oxidize the methylene group of fluorene (7). As demonstrated in this study, NDO of the plasmid NAH7 preferentially acts as a monoxygenase on a number of naphthenoaromatic compounds possessing benzylic methylene groups and on methyl-substituted aromatic compounds. With the exception of the fluorene biotransformation, products indicative of dioxygenation of the aromatic ring of test hydrocarbons did not account for more than 3% of the recovered material. Oxidation of benzylic methyl groups by NDO was essentially non-specific with respect to their positions in the aromatic ring, as indicated by formation of monoxygenation products from various dimethylnaphthalenes and from 1,2,4-trimethylbenzene. Significant formation of aromatic ring oxidation products from fluorene (~30%) may indicate that the extent to which monoxygenation of benzylic methylene groups prevails over dioxygenation of aromatic rings is probably determined in part by their accessibility to the activated oxygen species at the enzyme active site. In addition to these benzylic monoxygenase reactions, NDO can catalyze monoxygenation of thiocellulose sulfur, as demonstrated by the formation of 3-methylbenzothiophene sulfoxide from 3-methylbenzothiophene. Understanding the extent to which monoxygenase activity of arene dioxygenases is dictated by the relative chemical reactivity of benzylic functional groups and S-heteroatoms or is determined by structural differences at their catalytic sites awaits more detailed comparative studies of these enzymes from bacteria that use alternative catabolic strategies (20, 22, 40, 42) for the utilization of naphthenoaromatic compounds.

From an environmental standpoint, the accumulating body of experimental evidence indicating fortuitous monoxygenative attack by arene dioxygenases, such as NDO, is of special interest. For example, in view of the abundance of naphthalene and phenanthrene in coal-derived materials and the ready availability of arene dioxygenases for bacterial catalolism of such unsubstituted aromatic substrates, these enzymes are inevitably implicated in the monoxygenation of a number of co-occurring naphthenoaromatic, methyl-substituted aromatic, and S-heterocyclic aromatic constituents. Although the actions of arene dioxygenases will be accompanied by the loss of the latter compounds, formation of oxygenated products, such as naphthenoaromatic ketones and aromatic carboxylic acids, can be expected. These products are not usually accommodated by pathways providing complete degradation of unsubstituted arenes. Catabolism of products formed as a result of monooxygenation of the naphthenoaromatic, methyl-substituted aromatic, and S-heterocyclic aromatic compounds demands alternative biochemical strategies and, therefore, a more diverse microbial flora. Furthermore, formation of oxygenated polycyclic aromatic products with polar keto, hydroxyl, and carboxyl functional groups is likely to enhance their solubility and bioavailability and to facilitate their migration into ecosystems adjacent to sites affected by PAC pollution. Emerging evidence indicating the toxicity of the products formed during the biotransformation of PACs (5, 9) emphasizes the need for systematic studies of biochemical mechanisms that determine the extent and the limitation of microbial degradation of chemical mixtures derived from fossil fuels.

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