Specificity of Substrate Recognition by *Pseudomonas fluorescens* N3 Dioxygenase

THE ROLE OF THE OXIDATION POTENTIAL AND MOLECULAR GEOMETRY*

(Received for publication, May 19, 1997, and in revised form, September 12, 1997)

Patrizia Di Gennaro‡, Guido Sello§§, Daniele Bianchi, and Paolo D’Amico

From the ‡Dipartimento di Genetica e Biologia dei Microorganismi, Università degli Studi, via Celoria 26, 20133 Milan, Italy, §§Dipartimento di Chimica Organica e Industriale, Università degli Studi, via Venezian 21, 20133 Milan, Italy, and Istituto G. Donegani, via Fauser 4, 28100 Novara, Italy

*Pseudomonas fluorescens* N3 is able to grow on naphthalene as the sole carbon and energy source. The mutant TTC1, blocked at the dihydrodiol dehydrogenase level, which can transform the hydrocarbon into the corresponding dihydrodiol, has been used to produce bioconversion products. To rationalize the different grades of conversion obtained with different substrates, a study was performed using non-naphthalene derivatives, including benzenes, conjugated benzenes, and polycyclic aromatic hydrocarbons. The corresponding diols obtained by bioconversion have been isolated and characterized. A theoretical model that considers both energy and geometry factors has been proposed to rationalize the experimental data. Good agreement has been found between the calculated values and the experimental results.

*Pseudomonas* dioxygenases are a family of closely related enzymes that can add an oxygen molecule to a substrate double bond (1). Their ability to make such a transformation on aromatic compounds, often the first step in the biodegradation of such compounds (2), is particularly interesting. The high stability of aromatic compounds requires an unusually high redox potential that, in most cases, is made available by two or three component enzymes (3). The active site is believed to belong to the class of Rieske-type iron-sulfur proteins, in which the iron, coordinated by two histidine nitrates and two cysteine sulfurs, is, at the same time, the end point of a long redox chain transporting the necessary electrons from NADH (or NADPH) and the coordination site of molecular oxygen (3). In the general framework of this type of dioxygenase, the specificity of recognition could be related to the geometric and functional characteristics of the active site. As a consequence, there are well-known oxygenases that transform monocyclic compounds (benzene (4) or toluene (5)), naphthalenes (6), or polycyclic aromatic hydrocarbons (7).

Since 1992, our research group has been interested in exploiting the power of the dioxygenase of *P. fluorescens* N3 that we isolated from the activated sludge of a wastewater treatment plant (8). The wild type is able to completely degrade naphthalene and some of its derivatives and to transform many other naphthalenes into the corresponding salicylic acids (8).

We later isolated a mutant strain (TTC1) blocked at the dihydrodiol dehydrogenase level (9), and then we cloned the naphthalene dioxygenase gene in *Escherichia coli* JM109 (10). Thus, we could efficiently produce dihydrodiols from many naphthalenes carrying substituents in both position 1 and position 2. The yield seemed to be correlated to two aspects, the electronic characteristics of the substituents and their position, and was higher for electron releasing groups in position 2. However, the yield trend was not readily understandable, so we decided to extend our investigation to assess the specificity of N3 dioxygenase.

Two strongly correlated actions were considered: the experimental verification of substrate specificity (by testing diverse aromatic substrates) and the theoretical design of a model that could be used to understand enzyme substrate recognition and, possibly, to quantify the yield of the transformation. In this paper, we describe the experimental results together with the model and its application.

**EXPERIMENTAL PROCEDURES**

**General Procedures**—Chemical characterization of the products was made by 1H NMR spectroscopy using Bruker AC200 and AC300 instruments. Chemical shifts are reported as δ (ppm) relative to tetramethylsilane as the internal standard; coupling constants are given in Hz. Mass spectra were recorded at 70 eV on a 7070EQ instrument updated by VG Instruments. Values of [α]D were obtained with a Perkin-Elmer 554 polarimeter.

**Culture Conditions**—*P. fluorescens* TTC1 (NCIMB 40605) cells were routinely grown in M9 medium (11) with succinate and salicylic acid as an inducer (0.1 mM) according to a previously described procedure (9). At the end of the fermentation period (optical density 1.2, overnight incubation), the cells were harvested by centrifugation and immediately used in the biotransformation.

**Naphthalene Dioxygenase Assay**—The naphthalene dioxygenase activity was determined by a photometric method as described previously (12). One unit of dioxygenase activity was defined as the amount of cells (dry cell weight) producing 1 μmol of 1,2-dihydro-1,2-dihydronaphthalene in 1 min under the test conditions.

**Biotransformation Conditions**—In a general procedure, biotransformations using *P. fluorescens* TTC1 were carried out as described below. The cells, corresponding to 400 units of naphthalene dioxygenase activity, were resuspended in 600 ml of M9 minimal medium (11) with 5 mM succinate. The substrate for bioconversion was supplied directly to the culture at the concentration of 1 g/liter. The reactions were carried out at 30 °C for 5 h, and the diol accumulation was monitored by high performance liquid chromatography analysis.1 At the end of the reaction, the cells and the unreacted substrate were removed by centrifugation for 10 min. The supernatant of the culture was extracted four times with 150 ml of ethyl acetate. The organic phase was dried over *Na2SO4* and the solvent was removed under reduced pressure at 30 °C in the presence of 10 mg of Amberlite IRA900 basic resin. The 1 The reaction time used as a standard for the yield comparisons (5 h) was selected on the basis of the kinetic data that have been measured for many substrates and show linear oxidation profiles thus supporting the absence of enzyme inactivation at this stage.
residue was purified by flash chromatography on silica gel using a 9:1 mixture of hexane and ethyl acetate as eluent.

The dihydrodiol 9b (Table I) is highly unstable under the recovery conditions and could be characterized only after spontaneous dehydration to give, as the sole product, the phenol. The dehydration prone compounds 26 and 30b (Table I) were characterized after transformation in the corresponding stable diacetates by reaction with acetic anhydride in pyridine.

The unstable compounds 10b, 27a, and 27b (Table I) could not be isolated in a pure enough form for mass and [α]_D determination.

The characteristics of the obtained compounds are reported below, and their structures are reported in Table I.

**cis-3,4-Dihydro-3,4-dihydroxystilbene (9a)**
\[\text{[α]_D} 118.1 \text{ (c 1 MeOH); MS, m/z } 214, 196, 176, 158, 130; \] 1H NMR (d_6DMSO/D_2O) δ 4.10 (1H, dd, J_4,5 4.3, J_4,3 4.6), 4.40 (1H, d, J_3,4 4.6), 5.90 (1H, dd, J_5,6 9.8, J_5,4 4.3), 6.41 (1H, d, J_5,6 9.8), 7.07–6.91 (3H, m), 7.29–7.17 (3H, m). Analyzed: C_{14}H_{14}O_{2} requires H, 6.59; C, 78.48; found, H, 6.38; C, 77.17.

**cis-2,3-Dihydroxy-1-phenylcyclohexa-4,6-diene (10a)**
\[\text{[α]_D} 190.4 \text{ (c 1 MeOH); MS, m/z } 188, 187, 173, 169, 158, 140, 120; \] 1H NMR (CDCl_3) δ 4.45 (1H, d, J_2,3 6.0), 4.60 (1H, ddd, J_3,2 6.0, J_3,4 1.8, J_3,5 1.8), 5.90 (1H, dd, J_5,4 4.5, J_5,6 8.8), 6.20 (1H, ddd, J_5,6 5.5, J_5,4 9.2, J_5,3 1.8), 6.35 (1H, d, J_5,6 5.5), 7.30–7.60 (3H, m). Analyzed: C_{12}H_{12}O_{2} requires H, 6.43; C, 76.57; found, H, 6.28; C, 75.12.

**cis-3,4-Dihydroxy-1-phenylcyclohexa-2,6-diene (10b)**
1H NMR (CDCl_3) δ 4.30 (2H, m), 6.20 (1H, dd, J_5,4 4.5, J_5,6 8.8), 6.40 (1H, d, J_5,6 8.8), 6.90 (1H, d, J_5,6 8.5), 7.30–7.60 (3H, m). The abbreviation used is: MS, mass spectrum.

**cis-1,2-Dihydroxyacenaphthylene (25)**
MS: m/z (relative intensity) 186 (M+, 84), 168 (100), 155 (14), 140 (64); 1H NMR (d_6DMSO/D_2O) δ 5.30 (2H, s), 7.62–7.42 (2H, m), 7.78 (1H, d, J_5,4 7.2). Analyzed: C_{12}H_{10}O_{2} requires H, 5.41; C, 77.40; found, H, 5.45; C, 78.50.

**cis-2,3-Dihydro-2,3-diacetoxybiphenylene (26)**
MS: m/z (relative intensity) 270 (M+, 2.5); 228 (13); 210 (25); 186 (45); 168 (100); 157 (35); 1H NMR (CDCl_3) δ 2.1 (3H, s), 5.75 (2H, s), 5.90 (2H, s), 7.30–7.42 (3H, m). Analysis: C_{16}H_{14}O_{4} requires H, 5.22; C, 71.10; found, H, 5.12; C, 70.96.

**cis-1,2-Dihydro-1,2-dihydroxyfluorene (27a)**
1H NMR (CDCl_3) δ 4.40–4.55 (2H, m), 5.95 (1H, d, J = 9.2), 6.10 (1H, ddd, J = 2.0, J = 2.5, J = 9.2), 7.15–7.60 (4H, m). The abbreviation used is: MS, mass spectrum.

**cis-3,4-Dihydro-3,4-dihydroxyfluorene (27b)**
1H NMR (CDCl_3) δ 3.4 (2H, s), 4.50 (1H, m), 5.75 (1H, d, J = 6.5), 5.9 (1H, dd, J = 9.0, J = 1.5), 6.35 (1H, dd, J = 9.0, J < 1), 7.15 (4H, m).
cis-(1R,2S)-1,2-Dihydro-1,2-dihydroxyanthracene (28) \[\text{[a]}_{D}^{25} +235.0 \text{ (c 1 MeOH)}; \text{MS: } m/z \text{ (relative intensity)} 212 (M+2,82) 194 (53); 181 (33); 166 (100); \] 1H NMR (d6-DMsol) \[\delta 4.20 (1H, \text{dd, } J_{3,4} = 2.4, J_{4,3} = 4.3), 6.64 (1H, d, J_{1,2} = 4.35, J_{2,3} = 4.3), 6.46 (1H, d, J_{1,2} = 4.35), 6.08 (1H, dd, J_{3,4} = 8.7, J_{4,3} = 4.3), 6.66 (1H, d, J_{1,2} = 8.7), 7.49–7.40 (2H, m), 7.63 (1H, s), 7.92–7.77 (3H, m). \] Analyzed: C_{14}H_{12}O_{2} requires H, 5.70; C, 79.23; found, H, 5.65; C, 78.05. 

5.70; C, 79.23; found, H, 5.45; C, 77.64. 

RESULTS 

Experimental Results—To gain more experimental data concerning the active site requirements, we enlarged the number of test compounds, adding to the naphthalene derivatives some substrates that could provide significant data to the description of the active site. In agreement with the lines previously reported, we were interested in selecting substrates with either different energy requirements or a particular geometric shape. Consequently, we moved toward aromatic compounds of other classes. Three sets of compounds were chosen: benzene derivatives (benzene, isobutyl benzene, bifenyl, and diphenylmethane) (set 1); conjugated benzene derivatives (phenylacetylene, diphenylacetylene; cis- and trans-stilbenes, 1,1-diphenylethylene, coumarine, and biphenyl) (set 2); and polycyclic hydrocarbons (biphenylene, fluorene, acenaphthene, anthracene, phenanthrene, and anthraquinone) (set 3). These compounds are representative of increasing electron delocalization with a consequent decreasing of oxidation potentials, different geometry, and different conformational freedom.

To have a consistent set of experimental data, all of the bioconversions were made using standardized procedures, without any attempt at getting the best possible yields. Therefore, we chose the mutant strain TTC1 in a 3-liter flask. To ensure the standardization of the culture conditions, we also used the enzyme activity test previously reported (12). When the yield was very low, we used either the E. coli recombinant pVL1343 + pMS13 (10) or the reactor procedure (13) to get enough product to characterize. The bioconversion products are reported in Table I.

None of the benzene derivatives (set 1) showed recognition by the enzyme activity test previously reported (12). When the yield was very low, we used either the E. coli recombinant pVL1343 + pMS13 (10) or the reactor procedure (13) to get enough product to characterize. The bioconversion products are reported in Table I.

### Table II

| No. | Compound | Product | Yield (mmol/liter) | Energy (eV) | Yield (E) (mmol/liter) | Geometry | Class | E0 | Yield (E,G) (mmol/liter) |
|-----|----------|---------|-------------------|-------------|------------------------|----------|------|---|-------------------------|
| 1   | Benzene  | 0       | -9.65             | 0           | 0                      |          |      |   |                         |
| 2   | i-Butylbenzene | 0       | -9.34             | 0           | 0                      |          |      |   |                         |
| 3   | Diphenylmethane | 0       | -9.32             | 0           | 0                      |          |      |   |                         |
| 4   | Benzyl | 0       | -9.34             | 0           | 0                      |          |      |   |                         |
| 5   | Phenylacetylene | 0       | -9.28             | 0           | 0                      |          |      |   |                         |
| 6   | 1,1-Diphenylethylene | 0       | -8.86             | 3.20        | Out*                   | 0        | 0    |   |                         |
| 7   | Diphenylethylene | 0       | -8.75             | 4.25        | Out*                   | 0        | 0    |   |                         |
| 8   | trans-Stilbene | 9A     | -8.49             | 6.73        | Out*                   | 0        | 0    |   |                         |
| 9   | cis-Stilbene | 9B     | 0.37              | -8.90       | 2.81                   | 1.2 A    | 0.196| 0.54 |
| 10  | Biphenyl | 10A    | 0.64              | -8.95       | 2.34                   | 1.2 B    | 0.043| 0.16 |
| 11  | Naphthalene | 11     | 4.63              | -8.71       | 4.63                   | 0        | 0.990| 4.58 |
| 12  | 2-Methoxy | 12     | 4.01              | -8.48       | 6.83                   | 2        | 0.564| 3.75 |
| 13  | 2-Carbomethoxy | 13     | 1.14              | -9.01       | 1.76                   | 2        | 0.564| 0.89 |
| 14  | 2-Ethyl | 14     | 2.89              | -8.63       | 5.39                   | 2        | 0.564| 2.94 |
| 15  | 2-Methyl | 15     | 2.84              | -8.62       | 5.49                   | 2        | 0.564| 2.99 |
| 16  | 2-Chloro | 16     | 1.83              | -8.86       | 3.20                   | 2        | 0.564| 1.70 |
| 17  | 2-Bromo | 17     | 0.93              | -8.90       | 2.81                   | 2        | 0.564| 0.98 |
| 18  | 1-Methoxy | 18A    | 1.56              | -8.40       | 7.59                   | 1 A      | 0.196| 0.56 |
| 19  | 1-Ethyl | 18B    | 0.16              | -8.58       | 5.87                   | 1 A      | 0.196| 1.14 |
| 20  | 1-Methyl | 19     | 1.42              | -8.58       | 5.87                   | 1 A      | 0.196| 1.14 |
| 21  | 1-Carbomethoxy | 20     | 0.90              | -8.54       | 6.26                   | 1 A      | 0.196| 1.22 |
| 22  | 1-Chloro | 21A    | 1.06              | -8.99       | 1.95                   | 1 A      | 0.196| 0.38 |
| 23  | 1-Bromo | 21B    | 0.29              | -8.99       | 1.95                   | 1 A      | 0.196| 0.38 |
| 24  | Coumarin | 22A    | 4.02              | -8.77       | 4.06                   | 1 A      | 0.196| 0.21 |
| 25  | Aconophethylene | 22B    | 0.86              | -8.77       | 4.06                   | 1 A      | 0.196| 0.21 |
| 26  | Biphenylene | 23A    | 1.19              | -8.84       | 3.39                   | 1 A      | 0.196| 0.66 |
| 27  | Fluorene | 23B    | 0.95              | -8.84       | 3.39                   | 1 A      | 0.196| 0.21 |
| 28  | Anthracene | 24A    | 0.14              | -8.12       | 10.27                  | 2 A      | 0.043| 0.48 |
| 29  | Anthraquinone | 24B    | 0.14              | -8.12       | 10.27                  | 2 A      | 0.043| 0.48 |
| 30  | Phenanthrene | 25A    | 0.99              | -8.61       | 5.59                   | 1.2 A    | 0.196| 1.09 |
| 31  | Phenanthrene | 25B    | 0.19              | -8.61       | 5.59                   | 1.2 B    | 0.043| 0.28 |

*a Calculated yield using Eq. 2.

*b Compound class determined using the substituent position.

*c Correction factor due to the substituent position and calculated as reported in the text.

*d Calculated yield corrected by the geometric factor (Eq. 3).

*e Compound not fitting into the accepted volume.
the enzyme; even the presence of a highly lipophilic substructure (isobutyl or CH₂CH₂Ph) did not lead to oxidation.

As soon as the conjugation was extended we observed an interesting change: of all the conjugated benzene derivatives (set 2), only cis-stilbene and biphenyl were recognized and gave two isomeric products each. It is clear that conjugation sufficiently lowered the oxidation potential, but geometric requirements dictated the conversion chances.

For the set 3 compounds, we were confident of the magnitude of the oxidation potentials; nevertheless, we still needed to verify both the bond involved in the transformation and the shape requirements. Of the six compounds, five were recognized and gave different products; the remaining one (anthraquinone) was found to be inactive. This result is a clear sign of the importance of the oxidation potential, which, as soon as some basic geometric requirements are satisfied, functions as the discriminating factor. In set 3 compounds, it is also very interesting to look at the product structures because of their peculiarity. Fluorene gives products in full agreement with expectations (two regioisomers coming from the attack at different bonds of the same ring); phenanthrene and anthracene also apparently give the expected products, but they do not come from the attack at the most oxidizable bond (bond 9–10). In any case, the products exactly reproduce the regioisomers of substituted naphthalenes. Finally, biphenylene andacenaphthyene give products that show a geometry that is peculiar but in agreement with the most oxidizable bond.

**Theoretical Studies**—Enzyme active site modelling is still a developing activity (14) that presents two fundamental approaches. The first approach utilizes a good knowledge of the active site obtained by different methodologies (15, 16, 17). The second approach deduces the specificity of recognition using the structure characteristics of accepted substrates (18).

Our problem is, to a certain degree, unique. In fact, we do not attempt to predict a complex biological activity, as is the case in medical chemistry, but model a biological transformation that is well defined in its outcome; i.e., we are interested in the rationalization of a known chemical reaction. We do not know two facts: the redox potential of the enzyme and its shape recognition. Thus, we need to identify the descriptor that directs the energy need and a volumetric representation of the allowed size of the molecules.

**Oxidation Potential**—The conversion mechanism of aromatic compounds to the corresponding dihydrodiols has been deeply discussed, and a two-step mechanism has been agreed on: the preliminary addition of an oxygen molecule, mediated by the Rieske-type iron-sulfur protein, and a subsequent reduction by NADH electrons through electron chain transfer (3) (see Scheme I).

We can consider the first step irreversible and, consequently, the second step not important when calculating the yield of the transformation. In this respect, the discriminating factor is the facility to oxidize of the naphthalene double bond, a facility that can, in turn, be related to the energy of the corresponding molecular orbital, i.e., of the molecular orbital located at the bond.

To determine these energies, we ran several calculations using a semiempirical quantomechanics program, AM1 (19), that has been found to be consistently reliable for our molecules. The energies obtained are reported in Table II. Most of the time, the energies correspond to the highest occupied molecular orbitals. Because the compounds can be considered
conformationally rigid, we could almost always avoid searching for the most stable conformation, and even in the few cases in which a partial rotational freedom was present (e.g. ethyl or methoxy substituents), the search was very easy. The calculations were also made for the naphthalene derivatives to compare a more comprehensive set of compounds. The corresponding conversion yields are reported in Table II.

**Shape Analysis**—The molecular shape can be well represented by the corresponding Van der Waals’ volume, which is easily calculated by many commercial programs (20). If we superimpose all of the accepted compounds (9-23, 25-28, and 30), taking care of exactly matching the double bonds that are transformed, and then we calculate the Van der Waals’ volume corresponding to such superimposed molecules, we obtain a comprehensive volume (TotVol) (Fig. 1A). TotVol is the volume inside the enzyme active site that is certainly free; consequently, all of the molecules that fit inside TotVol fulfill the shape requirements for acceptance.

Performing the same operation on the set of compounds (6-8) that, even possessing a molecular orbital of the correct energy, are not transformed into the corresponding diols, we obtain their combined volume (NotVol) (Fig. 1B). Subtracting TotVol from NotVol, we get a portion of space (ExtVol) (Fig. 1C) that is characteristic of NotVol only. Thus, we know that any compound that occupies ExtVol cannot be accepted by the enzyme. Two examples of molecules that do not fit into TotVol are shown in Fig. 2. The orientation of 1,1-diphenylethylene puts the nonaromatic double bond in the supposed reactive position. Even if the orbital energy is above the calculated threshold, the molecule does not react because one of its benzene rings is outside TotVol. Similarly, trans-stilbene oriented as required for the reaction of one of its benzene rings puts the other benzene ring outside TotVol; consequently, trans-stilbene is not recognized by the enzyme.

**Quantitative Calculations**—Looking at the experimental yields, it is possible to note their dependence on both the substituent nature and its position. The substituent nature is, of course, represented by the orbital energy, and consequently, quantification can easily be made inside a homogeneous set. On the other hand, the role of the substituent position is not automatically considered by the calculation. We took into consideration different possibilities (e.g. calculating the lipophilicity/lipophobicity of the substituents (Ref. 21 and references therein) or their Van der Waals’ radii), but we did not find any efficient descriptor. To overcome this difficulty, we tried a different approach (see below).

Still to be quantified is the correction needed for the orbital energies. The calculated energy of the attacked orbital of naphthalene is −8.71 eV. It was selected as the reference energy, and a corresponding correction has been assigned to any change equal to 0.1 eV. To calculate the conversion yields (μ) we correlated the experimental yields of 2-substituted naphthalenes with the calculated energies; we obtained the following line.

$$\Delta \mu = 5.035 \times \Delta E + 0.453 \quad (r = 0.955) \quad (Eq. 1)$$

This equation gives a yield correction factor $F_E$ equal to 0.958 for each 0.1 eV energy unit. The use of this correction factor in the following equation (Eq. 2) allows the calculation of the theoretical yields correlated to the orbital energy difference only (Table II),

$$\mu_i = \mu_{naph} + [E_i - E_{naph}]0.1 \times F_E \quad (Eq. 2)$$

where $\mu_i$ is the calculated yield of compound i, $\mu_{naph}$ is the naphthalene conversion yield (i.e. 4.63 mmol/liter), $E_{naph}$ is the orbital energy of naphthalene (i.e. −8.71 eV), and $F_E$ is the energy correction factor. From Eq. 2, we can also guess the energy threshold that would exclude a compound ($\mu = 0$); it is equal to −9.2 eV (Fig. 3).

**DISCUSSION**

If we consider the experimental results together, they indicate that a minimal extension of the aromatic substructure is required for both energy and geometric recognition, that molecular shapes are restricted to a well defined area, and that the products depend on both the energy of the attacked bond and its juxtaposition inside the active site. The data gathered by the experiments proved essential in the design of the theoretical model.

Let us now make some general considerations. All of the compounds are nearly insoluble in water, although this characteristic does not seem to be important. For example, operating with the E. coli recombinant, we can completely convert 1 g liter⁻¹ of naphthalene to its corresponding dihydrodiol.

Diol products are known to be toxic at high concentrations (12), but our operative concentrations were far from these levels.

The bioconversion products always showed some common characteristics: the two hydroxyl groups are cis-oriented; the absolute stereochemistry, when checked, was always the same (1R,2S referred to naphthalene dihydrodiol); most of the dihydrodiols showed no tendency to lose water and rearomatize, although some of them are highly unstable (e.g. one of the cis-stilbene derivatives, the fluorene derivatives, and the biphenyl derivatives).

The reported yields were calculated using high performance liquid chromatography analysis before isolation and characterization of the products to minimize the noise that could derive from the isolation procedure.

To test for acceptance we can operate thus: calculate the energy of the orbitals that can in principle react and exclude all
portions of the solution that the wrong energy (lower than $-9.2$ eV); fit the molecule into the accepted volume in the correct orientation and accept all the solutions falling inside it; or fit the molecule into the excluded volume in the correct orientation and eliminate all the solutions falling inside it.

The compounds that have the correct energy but are neither inside the accepted volume nor inside the excluded volume must be experimentally tested.

To quantitatively assess the effect of the substituent nature and position in naphthalenes, we then reconsidered the results by separating all the compounds into subclasses. We defined each subclass by considering the position of the substituent (1, or 2, or 1,2, or 2,3) and the type of product obtained (type A if the hydroxyl groups were on the same half space of the substituent; type B if not). We can highlight four different classes: unsubstituted naphthalene (class 0); 2-substituted naphthalenes (class 2); 1- and 1,2-substituted naphthalenes of subclass A (class 1A/1,2A); 1- and 1,2-substituted naphthalenes of subclass B, and 2,3-substituted naphthalenes (class 1B/1,2B/2,3).

When we correlated the calculated yields with the experimental yields inside each class, we obtained four lines (see Fig. 4, diagram 2) with the following slopes and correlation coefficients: (class 0) $m = 0.99$, $r = 0.999$; (class 2) $m = 0.564$, $r = 0.979$; (class 1A/1,2A) $m = 0.196$, $r = 0.971$; (class 1B/1,2B/2,3) $m = 0.043$, $r = 0.665$ ($m = 0.035$, $r = 0.843$, excluding compound 18B). Thus we can modify Eq. 2 to take into account also the substitution factor, obtaining the following equation,

$$\mu_{s} = (\mu_{naph} + ([E_{i} - E_{naph}] / 0.1) \times F_{g}) \times F_{s}$$

(Eq. 3)

where $F_{s}$ depends on the substitution pattern of the compound, and it is equivalent to the slope $m$ calculated from the correlations reported above. Using the yields calculated by Equation 3 we can obtain the correlation line reported in Fig. 5, diagram 3.

As can be noted from the diagram shown in Fig. 5, the
calculated values are in surprisingly good agreement with the experimental results (r = 0.967; m = 0.91). There are a few exceptions that we will discuss, but we would like to stress the nice behavior of our model, considering that the compounds of the set are different and include different classes of aromatics.

Let us now go back to the exceptions. Looking at Table II, we can find four compounds that correlate poorly. For 1-substituted compounds, we can locate three exceptions: the chlorine, bromine, and carbomethoxy derivatives. It is clear that the experimental yields are greater than those calculated. We have no explanation for this anomalous behavior, although it is worth noting that all these compounds carry a substituent that is electron withdrawing by field effect.\(^3\)

The last exception is anthracene. Its experimental conversion is lower than predicted. It is possible that the length of anthracene represents the limit of acceptance of the active site, a limit that is in agreement with the fact that 2-substituted naphthalenes do not give products of subclass B.

Conclusion—In this paper, we have reported the experimental results of the bioconversion of aromatic compounds by a \textit{P. fluorescens} mutant strain blocked at the dehydrogenase level. Then, using these results and the data obtained in previous experiences, we described a theoretical model that can rationalize the different behavior of the diverse substrates. The model proposed indicates the two main requirements that must be satisfied to have active site recognition: the minimum oxidation potential and the correct shape. The compounds used in this study demonstrated that the recognition is not limited to naphthalene derivatives and that the scope of the conversion can be greatly expanded. In addition, the amount of products is acceptable and, mainly by using the recombinant strains in a reactor procedure, can assure the availability of a number of optically pure dihydrodiols that constitute a chiral pool for synthetic use.

REFERENCES
1. Harayama, S., and Timmis, K. N. (1989) in \textit{Genetics of Bacterial Diversity} (Hopwood, D. A., and Chater, K. F., eds) pp. 151–174, Academic Press, London
2. Dagley, S. (1986) in \textit{The Bacteria} (Sokatch, J. R., ed) Vol. 10, pp. 527–555, Academic Press, London
3. Mason, J. R., and Joannou, C. L. (1990) Methods Enzymol. \textbf{188}, 52–60
4. Gibson, D. T., Yeh, W.-K., Liu, T.-N., and Subramanian, V. (1982) in \textit{Oxgenases and Oxygen Metabolism} (Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, M. J., Ernst, L., and Estabrook, R. W., eds) pp. 51–62, Academic Press, London
5. Ensley, B. D., Gibson, D. T., and Laborde, A. L. (1982) \textit{J. Bacteriol.} \textbf{149}, 945–954
6. Takizawa, N., Kaida, N., Torigoe, S., Moritani, T., Sawada, T., Sato, S., and Kiyochara, H. (1994) \textit{J. Bacteriol.} \textbf{176}, 2444–2449
7. Bestetti, G., Di Gennaro, P., Galli, E., Leoni, B., Pelizzoni, F., Sello, G., and Bianchi, D. (1994) \textit{Appl. Microbiol. Biotechnol.} \textbf{40}, 791–793
8. Bestetti, G., Bianchi, D., Bosetti, A., Di Gennaro, P., Galli, E., Leoni, B., Pelizzoni, F., and Sello, G. (1995) \textit{Appl. Microbiol. Biotechnol.} \textbf{44}, 306–313
9. Di Gennaro, P. (1996) \textit{Produzione di cis-diidrodioli di naftaleni sostituiti mediante la naftalene 1,2-diossigenasi di Pseudomonas fluorescens N3 clonata in E. coli}. Ph.D. Thesis, Università degli Studi di Milano, Milano
10. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) \textit{Molecular Cloning: A Laboratory Manual}, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Cidaria, D., Deidda, F., and Bosetti, A. (1994) \textit{Appl. Microbiol. Biotechnol.} \textbf{41}, 689–692
12. Bosetti, A., Bianchi, D., Andrisiolo, N., Cidaria, D., Cesti, P., Sello, G., and Di Gennaro, P. (1996) \textit{J. Chem. Technol. Biotechnol.} \textbf{66}, 375–381
13. Kuntz, I. D., Meng, E. C., and Shoichet, B. K. (1994) \textit{Acc. Chem. Res.} \textbf{27}, 117–123
14. Swaminathan, P., Hariharan, M., Murali, R., and Singh, C. U. (1996) \textit{J. Med. Chem.} \textbf{39}, 2141–2155
15. Nugiel, D. A., Jacobs, K., Kaltenbach, R. F., III, Worley, T., Patel, M., Meyer, D. T., Judhay, P. K., De Lucza, G. V., Snyrew, T. E., Klabe, R. M., Bachelor, L. T., Rayner, M. M., and Seitz, S. P. (1996) \textit{J. Med. Chem.} \textbf{39}, 2156–2169
16. Snyder, J. P., Rao, S. N., Koehler, K. F., and Vedani, A. (1993) \textit{3D QSAR Drug Design} (Kohiny, H., ed) pp. 536–554, ESCOM, Leiden, The Netherlands
17. Silverman, B. D., and Platt, D. E. (1996) \textit{J. Med. Chem.} \textbf{39}, 2129–2140
18. Stewart, J. J. P. (1990) \textit{MOPAC}, version 6.0, Quantum Chemistry Program Exchange n.527, Indiana University, Bloomington, IN
19. CHEMX (1995) version Jul95, Chemical Design Ltd., Chipping Norton, Great Britain
20. Provencher, L., and Jones, J. B. (1994) \textit{J. Org. Chem.} \textbf{59}, 2729–2732

\(^3\) However, the same effect is not shown by substituents in position 2.