

## Oxidative Pathway After Reduction of p-Aminoazobenzene by *Pseudomonas cepacia*

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The wastewaters of dyestuff industries in Gifu district are applied for mixed treatment with municipal sewage by activated sludge. The rates of elimination in these cases are not always high. Toxicities of the dye against microorganisms cause degradation of purifying functions in treatment plants and the standards for elimination of biochemical oxygen demand (BOD) are difficult to be attained. Therefore, when there is a great deal of wastewater from dyestuff industries, primary treatment is required with dye-assimilating bacteria in dyestuff industries. In the soil obtained from the drain trench of dyestuff industries in the Hashima district, a few kinds of dye-assimilating bacteria were isolated and identified (Ogawa *et al.* 1978). To investigate the possibility of primary treatment with these strains, the metabolic pathways of dyestuffs with such bacteria were studied.

In the previous study, p-aminoazobenzene was found to be subject to reductive fission of the azo bond and acetylation of the amino group (Idaka *et al.* 1987). In a continuation of the study of the previous metabolites, the authors isolated and identified other metabolites, o-aminophenol, m- and p-acetamidophenol, and 3,4-dihydroxyacetanilide, from the culture broth of *Pseudomonas cepacia* 13NA. The isolation and identification of these metabolites are reported in the present study.

### MATERIALS AND METHODS

3,4-Dihydroxyacetanilide was synthesized by the hydroxylation of 4-nitrocatechol followed by acetylation (Szent-Gyorgyi *et al.* 1976). Crystallized yield was 50 %: mp 171-173 °C; MASS, m/z 167 ( $M^+$ ); NMR, 2.1 ppm (s, 3H). Technical grade o-aminophenol and m- and p-acetamidophenol were purified by recrystallization with ethanol followed by sublimation.

The strain of *Pseudomonas cepacia* used in this study was isolated from the draining trenches of the dyestuff works in the Hashima district (Ogawa *et al.* 1978).

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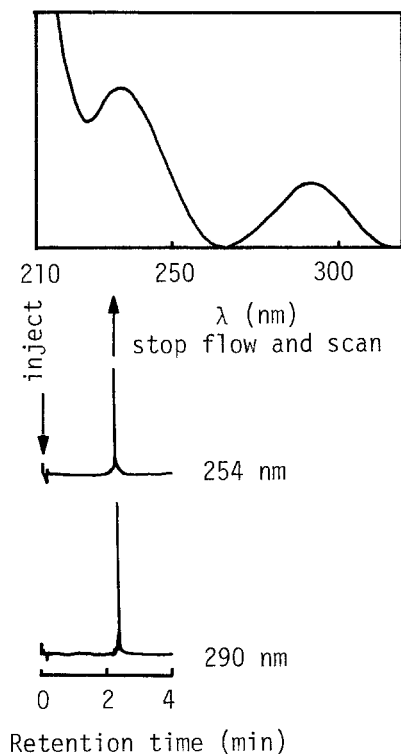


Figure 1. HPLC and rapid scan UV spectrum with stopped-flow method of the *o*-aminophenol corresponding fraction.

Conditions: column, Permaphase ODS,  $\Phi$  2.1 mm ID x 500 mm, column temp., 40 °C; elution solvent system, 40 % MeOH/H<sub>2</sub>O; pressure, 60 kg/cm<sup>2</sup>; flow rate, 2.5 ml/min; and detection, UV, 254 nm and 290 nm. UV spectrum was measured at the apex of the peak with stopping the flow for 20 sec (scanning rate between 210 nm and 350 nm).

The culture fluid was concentrated *in vacuo* by a rotary evaporator and extracted continuously with EtOH. The ethanol phase was concentrated *in vacuo*, passed through a short silica gel (Wako Gel W-200, Wako Chemicals Co., Osaka, Japan) column, separated and identified with TLC, HPLC and GC-MS analysis.

## RESULTS AND DISCUSSION

When *p*-aminoazobenzene was added as a substrate for *P. cepacia* 13NA, aniline, *p*-phenylenediamine, acetanilide, *p*-aminoacetanilide and *p*-phenylenediacetamide were separated and identified as major metabolites from the CH<sub>2</sub>Cl<sub>2</sub> extractable fraction. On the other hand, *o*-aminophenol, *m*- and *p*-acetamidophenol and 3,4-dihydroxyacetanilide were isolated and identified as minor metabolites from the EtOH extractable fraction.

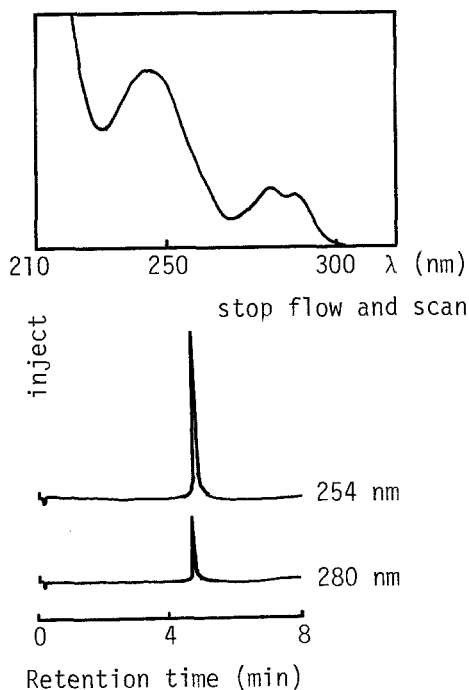


Figure 2. HPLC and rapid scan UV spectrum with stopped-flow method of the m-acetamidophenol corresponding fraction. Conditions: column, Permaphase ETH,  $\phi$  2.1 mm ID x 500 mm; column temp., 40 °C; elution solvent system, n-hexane/CH<sub>2</sub>Cl<sub>2</sub>/EtOH (94 : 3 : 3); pressure, 30 kg/cm<sup>2</sup>; flow rate, 1.5 ml/min; and detection, UV, 254 nm and 280 nm.

As to the metabolites, the EtOH extractable fraction was concentrated and passed through a short silica gel column. The o-aminophenol corresponding fraction was collected and applied to a silica gel TLC.  $R_f$  (0.35, developing solvent, 5 % MeOH/CH<sub>2</sub>Cl<sub>2</sub>) of the o-aminophenol corresponding fraction which coincided with the  $R_f$  of the authentic one. The fraction was then applied to HPLC. The conditions of the elution and the elution pattern of the fraction are shown in Figure 1. It shows that one of the trace metabolites from p-aminoazobenzene coincided with o-aminophenol. Moreover, the eluate from the Permaphase ODS column was analyzed by GC-MS using 2 % OV-1 column (1 m x  $\phi$  3 mm ID) at 100 C. The o-aminophenol corresponding peak by total ion monitor appeared at  $R_t$  2.3 min and m/z 109 as shown by its molecular peak. From these results the o-aminophenol corresponding peak was identified as an authentic one.

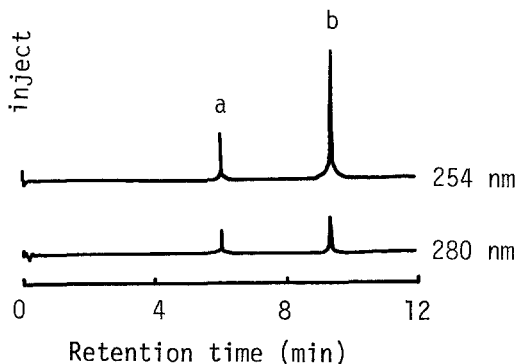


Figure 3. HPLC of the 3,4-dihydroxyacetanilide and p-acetamidophenol corresponding fraction. Conditions: column, Permaphase ETH,  $\phi$  2.1 mm x 500 mm; column temp., 40°C; elution solvent system, n-hexane/CH<sub>2</sub>Cl<sub>2</sub>/EtOH (94 : 3 : 3); pressure, 30 kg/cm<sup>2</sup>; flow rate, 1.5 ml/min; and detection, UV, 254 nm and 280 nm.

The previous study demonstrated that the free amino group of p-aminoazobenzene was acetylated during degradation by *P. cepacia* 13NA. Similarly, acetamidophenols were analyzed as aminophenol metabolites. The m-acetamidophenol corresponding fraction, which was more polar than the p-phenylenediamine corresponding fraction, was collected and applied to silica gel TLC ( $R_f$  0.17; developing solvent, 5 % MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The m-acetamidophenol corresponding fraction coincided with the  $R_t$  of the authentic one.

This fraction was later applied to HPLC. The conditions of the elution and the elution pattern of this fraction are shown in Figure 2. The figure indicates that one of the trace metabolite amounts from p-aminoazobenzene coincided with m-acetamidophenol. The GC-MS analysis showed an  $R_t$  of 3.6 min (column temperature, 140 °C) and m/z 151 which coincided with an authentic m-acetamidophenol.

Since the third fraction was more polar than m-acetamidophenol, the corresponding fraction was collected under UV illumination. The fraction applied to the silica gel TLC showed two spots at  $R_f$ : 0.15 (compound b) and 0.12 (compound a). The HPLC pattern of the fraction is shown in Figure 3. As the result of GC-MS analysis, peaks by the total ion monitor were shown at  $R_t$ , 2.2 min (a) and 3.6 min (b) and the molecular ion peak was respectively shown at m/z 167 (a) and 151 (b). Since the results of each analysis coincided with authentic p-acetamidophenol, compound (b) was identified as p-acetamidophenol. In the case of GC-MS analysis of compound (a), fragment ion peaks of m/z 168, 125 and 96 occurred besides the molecular ion peak. In the case of authentic 3,4-dihydroxyacetanilide, fragment ion peaks of m/z 168, 125 and 96

occurred besides the molecular ion peak; however, in the case of 2,4-dihydroxyacetanilide, a fragment ion peak of  $m/z$  149 occurred in addition to the molecular and fragment ion peaks of  $m/z$  168, 167, 125 and 96. As a result of comparing compound (a) with authentic 3,4-dihydroxyacetanilide, TLC, HPLC, GC-MS and UV spectrum, all analyses coincided perfectly and compound (a) was identified as 3,4-dihydroxyacetanilide.

Aniline was added as a substrate for *P. cepacia* 13NA in the medium, and metabolic compounds from aniline were examined. o-Aminophenol, m- and p-acetamidophenol and 3,4-dihydroxyacetanilide were identified by TLC, HPLC and GC-MS as minor metabolites from the EtOH extractable fraction.

Like aniline, p-phenylenediamine is also one of the major metabolic compounds from p-aminoazobenzene, so p-phenylenediamine was added in the medium as a substrate and trace metabolites were identified. As a result, p-acetamidophenol and 3,4-dihydroxyacetanilide from the EtOH extractable fraction were identified by TLC, HPLC and GC-MS.

During strain 13NA's metabolic processes of p-aminoazobenzene, reduction of the azo bond acetylation of the resulting amines and the continued hydroxylation of the aromatic ring were acknowledged to have occurred. When aniline was employed as a substrate (like p-aminoazobenzene), o-aminophenol, m- and p-acetamidophenol and 3,4-dihydroxyacetanilide were obtained as metabolites. Aniline was recognized to be a metabolic intermediate from these results. Aniline was known to be hydroxylated mainly at the para position as determined by measuring the urinary metabolites of the rat (Piotrowski 1961).

Likewise, when using p-phenylenediamine as a substrate, p-acetamidophenol and 3,4-dihydroxyacetanilide were identified as metabolites. Ryan (1972) proposed the hypothetical mechanism that p-aminophenol was formed by oxidative deamination from p-phenylenediamine in the rat and was subsequently reduced.

Since 3,4-dihydroxyacetanilide was isolated by using either aniline or p-phenylenediamine as a substrate, dihydroxylation was presumed to be caused by p-aminophenol as a direct intermediate.

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#### REFERENCES

- Idaka E, Ogawa T, Horitsu H (1987) Reductive metabolism of aminoazobenzenes by *Pseudomonas cepacia*, Bull Environ Contam Toxicol  
Ogawa T, Idaka E, Yatome C (1978) Studies on the treatment of the waste water containing dyestuffs by microorganisms. In: Arima K (ed) Microbiology for Environment Cleaning. Published under the support of the Ministry of Education, grant No. 212204-1977. pp 426-437

Piotrowski J (1961) Metabolism of small doses of aniline in rabbits. Med. Pracy 12:309-317  
Ryan AJ (1972) Factors involved in the metabolism of xenobiotics in mammals. Aust J Pharm Sci NS1:30-34  
Szent-Gyorgyi A, Chung RH, Boyajian MJ, Tishier M, Arison BH, Schoenewaldt EF, Wittick JJ (1976) Agaridoxin, a mushroom metabolite. Isolation, structure, and synthesis. J Org Chem 41:1603-1606

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