

# The Enzymic Reductions of Aryl-aldehydes and Aryl-alcohols in *Penicillium baarnense*

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The ability of whole cells of *P. baarnense* to reduce aryl-aldehydes and aryl-alcohols to the corresponding methyl derivatives has been demonstrated. Cell free experiments show that two separate enzymes, an aryl-alcohol dehydrogenase and an aryl-alcohol reductase, both requiring NADPH are involved in the reductions. The two enzyme activities have been resolved by gel chromatography.

## Preparation of aromatic substrates and products

*2-Hydroxy-4-methoxy-5,6-dimethylbenzaldehyde* (1). 5-Methylorcyaldehyde (12 mmol) was methylated with  $\text{CH}_3\text{I}$  (6 mmol) in acetone (50 ml) in the presence of  $\text{K}_2\text{CO}_3$  (12 mmol). Work-up of the product gave after recrystallizations from ethanol/water and sublimation at  $100^\circ\text{C}/1\text{ mmHg}$  1 as slightly yellow needles (11 mmol, 92% yield), m.p.  $122-123^\circ\text{C}$ ,  $\text{M}^+$  180. Anal.  $\text{C}_{10}\text{H}_{12}\text{O}_3$ : C, H.  $^1\text{H NMR}$  (60 MHz,  $\text{CDCl}_3$ ): 2.03 (3 H, s), 2.40 (3 H, s), 3.88 (3 H, s), 6.25 (H, s) 10.23 (H, s), 12.66 (H, s). IR (KBr)  $1620\text{ (s) cm}^{-1}$ .

*2-Hydroxy-4-methoxy-5,6-dimethylbenzylalcohol* (2). To 1 (0.5 mmol) in benzene (20 ml) sodium bis(2-methoxyethoxy)aluminium dihydride (1 mmol) dissolved in benzene (10 ml) was added dropwise. The reaction mixture was kept in  $\text{N}_2$  atmosphere below  $40^\circ\text{C}$ . The benzene was removed after 20 min and water and dry ice added to the residue. The product was extracted with ether. After drying over anhydrous  $\text{Na}_2\text{SO}_4$ , evaporation and recrystallization from cyclohexane 2 was obtained as colorless prisms (0.3 mmol, yield 59%). M.p.  $110-112^\circ\text{C}$ ,  $\text{M}^+$  182. Anal.  $\text{C}_{10}\text{H}_{14}\text{O}_3$ : C, H.  $^1\text{H NMR}$  (60 MHz):  $\text{DMSO}-d_6 + \text{CD}_3\text{OD}$ : 1.90 (3H, s), 2.10 (3H, s), 3.63 (3H, s), 4.43 (3H, s), 6.33 (H, s), 9.07 (H, s) IR (KBr)  $1600\text{ (s) } 3520\text{ (s) cm}^{-1}$ .

*4-Hydroxy-2-methoxy-5,6-dimethylbenz( $^{14}\text{C}$ )-aldehyde* (3). A Gattermann reaction on 3-methoxy-5,6-dimethyl resorcinol using  $\text{Zn}(\text{CN})_2$  mixed with 1 mCi  $\text{Na}^{14}\text{CN}$  gave  $^{14}\text{C}$ -labelled 3. Recrystallization from ethanol/water yielded colorless needles (yield 94%, specific radioactivity  $9.3 \times 10^5$  dpm/mg). M.p.  $240-242^\circ\text{C}$ ,  $\text{M}^+$  180. Anal.  $\text{C}_{10}\text{H}_{12}\text{O}_3$ : C, H, IR (KBr)  $1600\text{ (s), } 1640\text{ (s), } 3000\text{ (s, b) cm}^{-1}$ .

*1-Hydroxy-3-methoxy-4,5,6-trimethylbenzene* (4). Hydrogenation of 3 at atmospheric pressure using PdO as catalyst gave 4 (yield 96%). M.p.  $120-121.5^\circ\text{C}$  after recrystallization from hexane.  $\text{M}^+$  166. Anal.  $\text{C}_{10}\text{H}_{14}\text{O}_2$ : C, H.  $^1\text{H NMR}$

The main secondary product produced by *Penicillium baarnense* when grown on Raulin-Thom medium is barnol (4,6-dimethyl-5-ethylpyrogallol). In a recent publication a detailed pathway for the formation of barnol from acetate and methionine was elaborated.<sup>1</sup> A crucial step in this pathway is the reduction of the carboxyl function of the aromatic compound to a fully reduced methyl group. It was shown that some phenolic aldehydes were reduced to methyl derivatives when added to whole cells of the organism. The aldehydes were all homologues of the intermediate aldehyde appearing in barnol formation. In the present paper the reducing system in the organism is further studied by using various O-methylated aromatic aldehydes and their corresponding alcohols as substrates for reactions in whole cells as well as in cell-free extracts.

## EXPERIMENTAL

*Culture conditions.* *Penicillium baarnense* v. Beyma, CBS 315.19 was grown as previously described<sup>1</sup> on Raulin-Thom medium at  $28^\circ\text{C}$  in conical flasks on a rotary shaker.

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(60 MHz,  $\text{CDCl}_3$ ): 2.16 (9 H, d), 3.75 (3 H, s), 4.90 (H, s), 6.26 (H, s).

*2-Hydroxy-4-methoxy-6-methylbenzaldehyde* (evernin aldehyde) (5). Orcylaldehyde was O-methylated as described above.  $^{14}\text{C}$ -Labelled 4 was obtained by using orcy( $^{14}\text{C}$ )aldehyde. M.p. 63 °C (lit.<sup>3</sup> 65 °C), yield 91 %.

*2-Hydroxy-4-methoxy-6-methylbenzylalcohol* (evernin alcohol) (6). The title product was obtained after reduction of 5 evernin aldehyde as described for 2. M.p. 112–113 °C, yield 71 %, spec. radioactivity  $7.5 \times 10^5$  dpm/mg,  $M^+$  168. IR (KBr) 1595 (s), 1620 (s), 3160 (s), 3540 (s)  $\text{cm}^{-1}$ .

*1-Hydroxy-3-methoxy-5,6-dimethylbenzene* (7). Hydrogenation of evernin aldehyde as described for 4 gave 7. Purification was performed by sublimation at 90 °C, 0.1 mmHg, and recrystallization from ethanol/water. M.p. 95 °C, yield 92 %. Anal.  $\text{C}_9\text{H}_{12}\text{O}_2$ : C, H.

#### Feeding experiments with whole cells

$^{14}\text{C}$ -Labelled evernin aldehyde (3 mg, specific radioactivity  $6.9 \times 10^5$  dpm/mg) was added to a 3 days old culture (350 ml of medium). After 20 h the culture filtrate was extracted with ether. The ether solution was passed through Florisil and evaporated.

Non-labelled 7 1-hydroxy-3-methoxy-5,6-dimethylbenzene (100 mg) was added to the residue. Sublimation at 80–90 °C, 0.1 mmHg, followed by recrystallization from  $\text{CCl}_4$ /cyclohexane to constant specific radioactivity gave labelled compound.

In the same way evernin alcohol (4 mg,  $7.5 \times 10^5$  dpm/mg) yielded radioactive 7, 1-hydroxy-3-methoxy-5,6-dimethylbenzene.

*2-Hydroxy-4-methoxy-5,6-dimethylbenzaldehyde* (1) was fed to the cells in two separate experiments. Radioactive 1 gave after incubation as described a single radioactive product chromatographically identical with 4. In the other experiment nonlabelled 1 (45 mg) was distributed in 3 cultures. After 48 h 4 (10 mg) was isolated.

#### Cell-free experiments

The mycelium (37 g wet weight) from 3 days old cultures was homogenized in a cooled mill with glass beads (70 g) in a 0.1 M phosphate buffer (40 ml) pH 7.5 containing  $10^{-3}$  M mercaptoethanol,  $10^{-4}$  M EDTA and  $10^{-4}$  M phenylmethylsulfonyl fluoride. The supernatant obtained after centrifugation at 45 000 *g* for 60 min was precipitated with  $(\text{NH}_4)_2\text{SO}_4$  to 80 % saturation. The precipitate was dissolved in the phosphate buffer (6 ml) and the solution passed through a column of Sephadex G-25 equilibrated with the same buffer. The eluate was used as enzyme solution in most of the

experiments. In spite of gel filtration the eluate contained traces of mannitol which together with the mannitol:NADP<sup>+</sup> oxidoreductase present in the eluate regenerated NADPH which presented stoichiometric evaluation of the reactions. The enzyme solution was incubated with evernin aldehyde (18  $\mu\text{mol}$ ) dissolved in a minimal volume of ethanol and NADPH (7  $\mu\text{mol}$ ) for 5 h at room temperature. The reaction was terminated by the addition of  $(\text{NH}_4)_2\text{SO}_4$  and extraction of the products with ether.

The products were separated by thin layer chromatography on silica gel (benzene–dioxane–acetic acid, 95:24:4). Three radioactive compounds were obtained and identified with the radioactivity distributed as indicated with the percentage figures: 5 (18 %), 6 (10 %) and 7 (72 %).

In a similar experiment but with the addition of D-mannitol (360  $\mu\text{mol}$ ) the only detectable radioactive compound after incubation was 7.

Also when radioactive evernin alcohol was used as substrate but without addition of D-mannitol 7 was the only radioactive substance on the chromatogram.

Incubation with 5-methylorcy( $^{14}\text{C}$ )aldehyde (8) as substrate gave nonreacted aldehyde (37 %), the corresponding alcohol (9) (18 %) and fully reduced compound<sup>3</sup> (10) (42 %).

#### Separation of enzymes

The crude cell-free extract was treated with protamin sulfate. The precipitate (48 000 *g*, 20 min) was discarded and the proteins in the solution were fractionated by  $(\text{NH}_4)_2\text{SO}_4$  precipitation.

The protein fraction obtained from the interval 35–80 % of saturation with  $(\text{NH}_4)_2\text{SO}_4$  was redissolved in the buffer and applied to an Ultrogel Aca 34 column (2.5  $\times$  50 cm) previously equilibrated with the phosphate buffer, pH 7.5. The enzyme activities of the fractions (3.5 ml each) were tested with evernin alcohol and evernin aldehyde respectively as  $^{14}\text{C}$ -labelled substrates together with NADPH.

The fractions between 129–147 ml all catalyzed the reduction of evernin alcohol to the methyl derivative. These fractions did not show any activity against evernin aldehyde. However, the fractions between 172–192 ml used the aldehyde as substrate with the formation of the alcohol, that was not utilized as substrate for a further reduction.

#### RESULTS AND DISCUSSION

In the formation of barnol an intermediate carboxyl function is reduced to a methyl group. The initial attack on the carboxyl group is

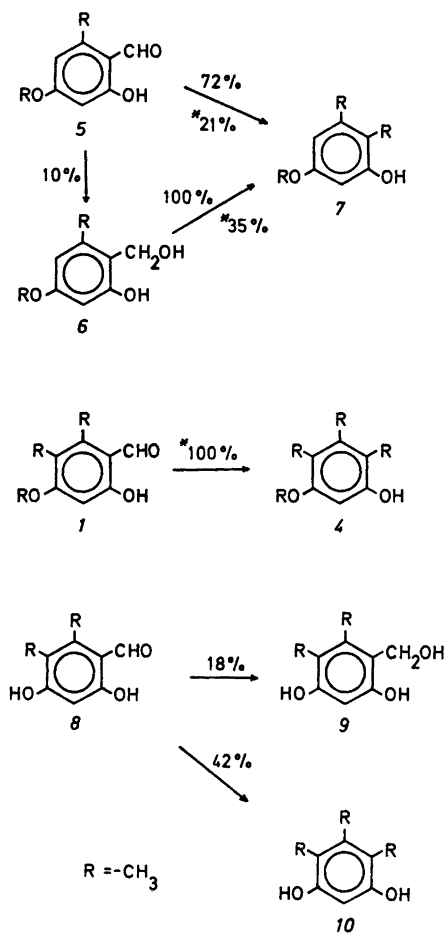


Fig. 1. Reductions of aryl derivatives *in vitro* and *in vivo* (marked with asterisks).

probably on the level of a thioester, which is believed to be an early enzyme-bound intermediate. Neither the aldehyde nor the alcohol have been reported as free metabolites in *P. baarnense*. In a previous publication it was shown that whole cells of *P. baarnense* are able to reduce certain diphenolic aldehydes to the corresponding methyl derivatives. As the benzylalcohols are difficult to prepare from the used diphenolic aldehydes it could not be established if the alcohols could be used as substrates for the formation of the methyl compounds. However, insertion of an *O*-methyl group *para* to the aldehyde group did not prevent reduction of the aldehyde function by

whole cells (Fig. 1). From an *O*-methyl aldehyde the corresponding alcohol (evernin alcohol) was prepared. When this alcohol was added to a whole cell system it was converted to the methyl derivative indicating that the reduction of the carboxyl function passes the oxidation level of an alcohol.

A protein fraction obtained by  $(NH_4)_2SO_4$  precipitation of a crude cell-free extract and freed from low molecular weight compounds by gel chromatography catalyzed in an NADPH dependent process the reduction of evernin aldehyde to a mixture of evernin alcohol and the methyl derivative. With an excess of NADPH the methyl derivative was the sole product (Fig. 1). When evernin alcohol was used as substrate a quantitative reduction to the methyl derivative was obtained. This protein fraction also reduced orcyaldehyde to the corresponding alcohol and the methyl derivative.

The enzyme activities were resolved in two separate protein fractions by gel chromatography on Ultrogel AcA 34. The one with the higher molecular weight catalyzed the reduction of vernin alcohol to the methyl derivative showing no activity towards the aldehyde. The other protein fraction reduced the vernin aldehyde to evernin alcohol but not further.

The crude protein solution contains thus an aryl-alcohol dehydrogenase and an aryl-alcohol reductase both requiring NADPH as hydrogen carrier.

The overall process of the reduction of the intermediate carboxyl function should involve

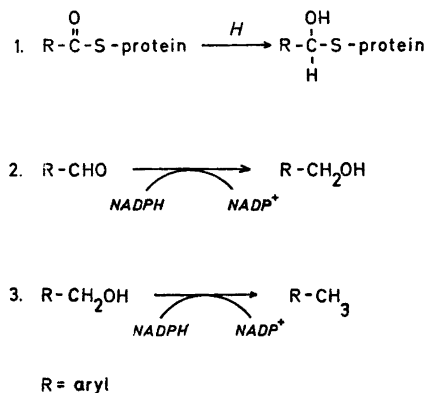


Fig. 2. Enzyme reactions participating in the conversion of a carboxyl function to a methyl group.

an aryl-aldehyde dehydrogenase as a third enzyme, and the reaction sequence can thus be visualized as in Fig. 2.

The enzymic reduction of an alcohol to the corresponding hydrocarbon compound has to our knowledge not been reported before and interesting mechanistic studies await. Further studies on the individual enzymes are in progress.

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