Fig. 2. *Radial growth (as a measure of fungicide resistance) of test strains on plates containing different concentrations of three inhibitors of eburicol 14α-demethylase (etaconazole, imazalil, fenarimol) and one fungicide that does not inhibit this specific enzyme (benomyl; control).* For each strain also the microsomal cytochrome P450 reductase activity is shown; m.c.: multiple copy.

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[2] T. Sakaki, M. Akiyoshi-Shibata, Y. Yabusaki, K. Manabe, H. Urakami, H. Ohkawa, *Pharmacogen.* **1991**, *1*, 86.
[3] J.M. van den Brink, R.F.M. van Gorcom, PCT Int. Pat. Application PCT/WO/94/29453.
[4] J.M. van den Brink, C.M.J. van Zeijl, C.A.M.J.J. van den Hondel, R.F.M. van Gorcom, *DNA Cell Biol.* **1995**, *14*, 719.
[5] J.G.M. van Nistelrooy, J.M. van den Brink, J.A.L. van Kan, R.F.M. van Gorcom, M.A. de Waard, *Mol. Gen. Genet.* **1996**, *250*, 725.

Stereochemistry of Enzymatic Hydrolysis of Nitriles

Hiromichi Ohta*

Nitriles constitute a unique class of compounds in organic chemistry. However, one of the drawbacks of conversion of nitriles is that they are generally resistant to hydrolysis, and hence, rather drastic reaction conditions are required. In this way, enzyme-catalyzed hydrolysis of nitriles is one of the most important biotransformations of synthetic substrates, as it can be carried out under mild reaction conditions [1]. Hydrolysis of a cyano group is achieved without affecting ester and

acetal functionalities, which is very difficult *via* ordinary chemical reactions.

It is already established that there are three kinds of enzymes which concern in hydrolysis of nitriles, as shown in *Scheme 1*. In most cases, reaction proceeds in two steps *via* intermediary formation of an amide. These two steps are catalyzed by two enzymes. Thus, in some cases, hydrolysis reaction can be stopped at the stage of an amide, without forming any trace of a carboxylic acid [2]. Another

interesting feature of nitrile-hydrolyzing enzymes is their stereoselectivity. Enzymes attack the positive carbon of a nitrile and an amide with recognition of the chirality or prochirality of the same carbon skeleton. Thus studies on the stereoselectivities of a nitrile hydratase and an amidase would be a good model for understanding how enzymes are controlling the stereochemical course of the reactions.

We have screened a nitrile-hydrolyzing microorganisms using benzonitrile and/or 3-hydroxypropionitrile as the sole source of nitrogen, and found that an actinomycetes, *Rhodococcus rhodochrous*, was the most active strain, which is now deposited

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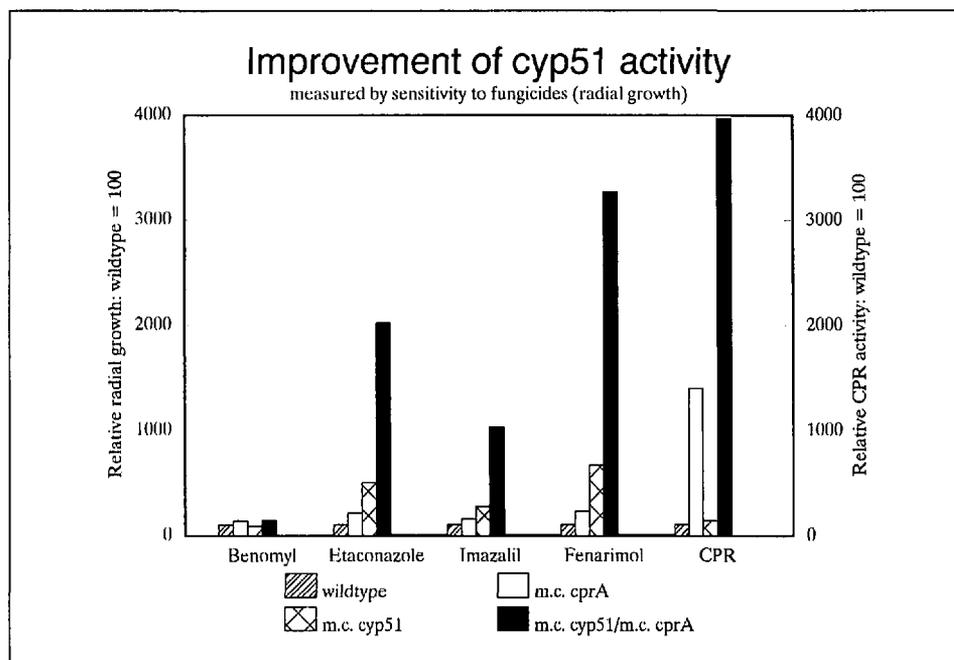3-14-1 Hiyoshi
Yokohama 223, Japan

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to Institute of Fermentation Osaka, Japan, with a registry number IFO 15564.

Hydrolysis of (±)-α-Arylpropionitriles

Through the studies on the hydrolysis of substituted benzonitriles, we noticed that amidase is sensitive to steric hindrance. This fact was expected to be effective to distinguish between (R)- and (S)-configuration of (±)-α-arylpropionitriles, which was proved to be true. When (±)-α-(p-methoxyphenyl)propionitrile (1) was incubated with the grown cells of R. rhodochrous, corresponding (R)-amide 2 and (S)-carboxylic acid 3 was obtained in high yields. The e.e. of both products was also excellent (Scheme 2). The stereochemical outcome of this reaction indicates that nitrile hydratase catalyzes hydrolysis of both enantiomers to give racemic amide. On the other hand, amidase accelerates the hydrolysis of only one enantiomer. Thus, the reaction profile as a whole shows that amidase has a fairly high enantioselectivity to (S)-amide.
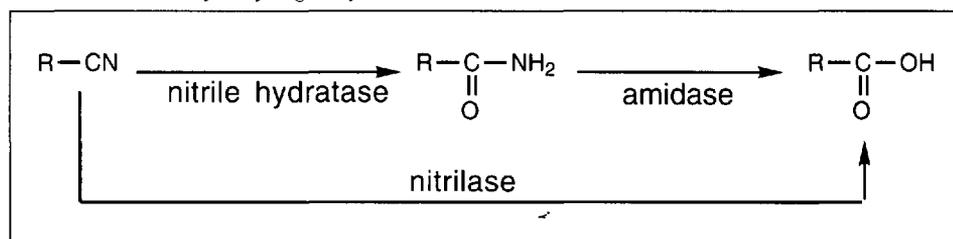
Hydrolysis of (±)-3-Benzoyloxypentanenitrile

The hydrolysis of an aliphatic nitrile proceeded in the same manner as described above. When (±)-3-benzoyloxypentanenitrile was subjected to the microbial hydrolysis, two products were obtained according to the same stereochemical selectivity of two enzymes, i.e., nitrile hydratase showed no enantioselectivity, while amidase preferentially hydrolyzed (S)-amide. Thus, in this case, the reaction was carried out using racemic amide 4 as shown in Scheme 3. Although some esterase activity (hydrolysis of benzoyl-ester group) was observed, expected products were obtained in good to excellent yields by controlling the reaction time [3].

The absolute configuration of resulting carboxylic acid was (S), similarly to the case of α-arylpropionitriles. However, when the spatial arrangement of the ligands around the asymmetric carbons are compared, it is noteworthy that the aryl groups of two compounds occupy the opposite direction with each other, as shown below.
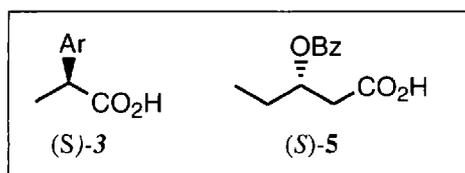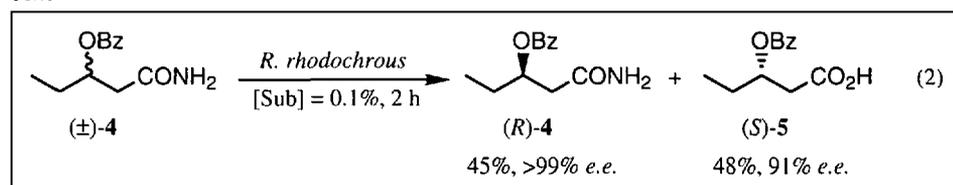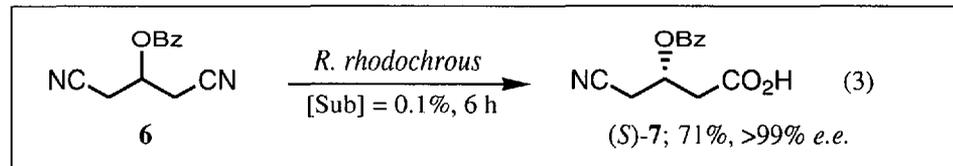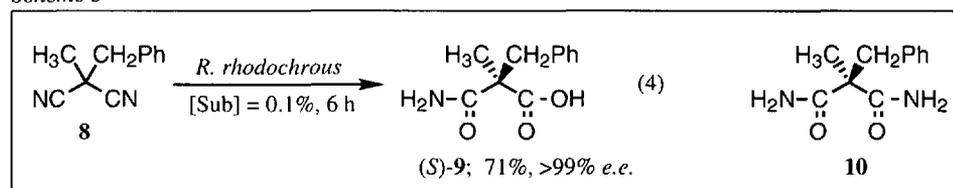
Scheme 1. *Nitrile-Hydrolyzing Enzymes*

Scheme 2
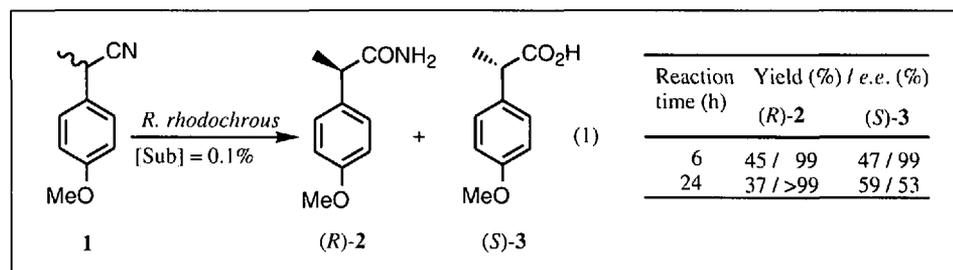
Scheme 3

Scheme 4

Scheme 5

Hydrolysis of β-Benzoyloxyglutarodinitrile

Then, it is an interesting problem to see what will happen with prochiral dinitriles. Thus, we prepared prochiral dinitriles structurally resembling to 1 and 4. First, a compound in which the terminal methyl group of β-benzoyloxypentanenitrile was replaced by a cyano group was designed, i.e., β-benzoyloxyglutarodinitrile (6). In this case, if the nitrile hydratase has no enantioselectivity, racemic cyano amide will be formed first. It will be transformed to an optically active cyano carboxylic acid via an enantioselective reaction catalyzed by amidase, leaving the other enantiomer of cyano amide, or nitrile hydratase will further attack the racemic cyano amide to afford prochiral diamide. To our surprise, what happened was entirely different, the sole product was (S)-cyano carboxylic acid 7, as shown in Scheme 4 [4]. This result clearly indicates that the first step hydrolysis promoted by the nitrile hydratase was enantioselective, unlike the reactions of racemic mononitriles. The configuration of obtained monocarboxylic acid is the same as that of 5, including its spatial arrangement. Comparing Reaction 2 and 3, it is suggested that the presence of a polar group (CN) at the terminal position

will be the key to determine the enantioselectivity of the nitrile hydratase, although the precise mechanism is not clear at present.

Hydrolysis of Malononitrile

Is the nitrile hydratase always enantioselective if there are two cyano groups in a molecule? To answer this question, malononitriles were used as the substrate, only one representative example is shown below [5]. Benzylmethylmalononitrile (8) gave amide carboxylic acid 9, in an entirely different manner compared with the

reaction of 6 (*Scheme 5*). First, both of two cyano groups were hydrolyzed, one to a carboxy and the other to an amide group. Control experiments revealed that the key intermediate was diamide 10, and this was proved to be also obtained starting from racemic cyano amide. Thus, nitrile hydratase showed no enantioselectivity. The chirality of the product was determined by recognition of the prochirality of 10 by the amidase.

In conclusion, while nitrile hydratase exhibits enantioselectivity only to a limited number of substrates, amidase catalyzes highly enantioselective reactions of a wide range of amides.

[1] 'Enzyme Catalysis in Organic Synthesis,' Ed. by K. Drauz and H. Waldmann, VCH, Weinheim, 1994, pp. 365–375.
[2] Y. Asano, T. Yasuda, Y. Tani, H. Yamada, *Agric. Biol. Chem.* 1982, 46, 1183.
[3] H. Kakeya, N. Sakai, T. Sugai, H. Ohta, *Tetrahedron Lett.* 1991, 32, 1343.
[4] M. Yokoyama, N. Imai, T. Sugai, H. Ohta, *J. Mol. Catal., B Enzymatic* 1996, 1, 135.
[5] H. Kakeya, N. Sakai, A. Sano, M. Yokoyama, T. Sugai, H. Ohta, *Chem. Lett.* 1991, 1823.
[6] M. Yokoyama, T. Sugai, H. Ohta, *Tetrahedron Asymmetry* 1993, 4, 1081.

Enantioselective Oxidation by Non-Heme Iron Monooxygenases from *Pseudomonas*

Marcel G. Wubbolts*, Sven Panke, Jan B. van Beilen, and Bernard Witholt

Introduction

Pseudomonas oleovorans alkane hydroxylase (EC 1.14.13.5) and xylene oxygenase (EC 1.14.13.-) from *P. putida* mt-2 are industrially relevant monooxygenases used for the production of optically active epoxides and (hetero)aromatic alcohols and acids [1]. Oxidation of aryl allyl ethers to (+)-aryl glycidyl ethers by alkane hydroxylase has thus provided a synthesis route towards the optically active β-blockers (–)-(S)-metoprolol and (–)-(S)-atenolol, developed by *Shell* and *Gist-Brocades* (Pat. EP 256586, US 49562843, and [2]). Heterocyclic aromatic acids produced by *Lonza* using these monooxygenases and subsequent enzymes (Pat. US 5242816, US 5236832, and [1]) are of use for the synthesis of pharmaceuticals such as the anti-hyperglycemia drug Glipizide.

The industrial application of these monooxygenases from *P. oleovorans* and *P. putida* mt-2 is limited to the use of the

wild-type organisms, which fortuitously accumulate the desired products as non-metabolizable intermediates. Synthesis of 'up-stream' intermediates, such as hetero-
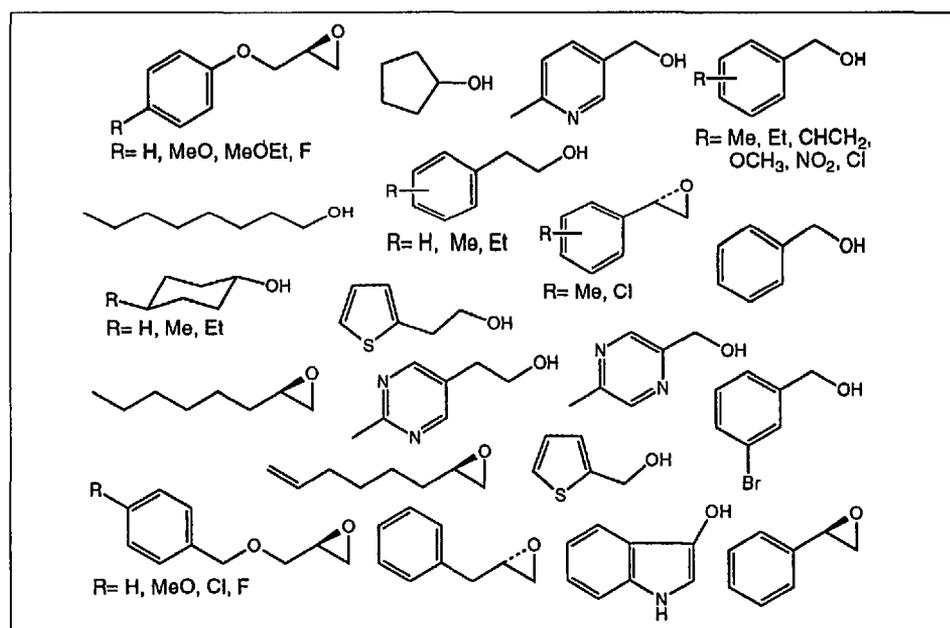
cyclic aromatic alcohols, or of metabolizable intermediates (*e.g.* derivatives of benzyl alcohol or benzoic acid) is not feasible with these strains. Furthermore, the wild-type biocatalysts is grown on alkanes (*P. oleovorans*) or xylenes (*P. putida* mt-2), which are substrates that can compete with the desired starting compounds, thus reducing productivity.

We have constructed, by genetic engineering, biocatalysts that contain alkane hydroxylase or xylene oxygenase, regulated by inducers that do not cause competitive inhibition, that are devoid of undesired 'down-stream' metabolic activities. Furthermore, we have introduced *xyl*- and *alk*-based 'biotransformation cassettes' into the chromosome of *E. coli* and *Pseudomonas* strains in order to obtain

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Figure. *Products of alkane-hydroxylase or xylene-oxygenase-mediated oxidation of aliphatic and (heterocyclic) aromatic compounds* [1][2][7–9]. Optically active epoxides without an assigned absolute configuration are represented with a dashed line. Substituents (R) that are accepted by the enzymes are indicated below each molecule.