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Diastereoselective Reduction of Ethyl α-Methyl-β-Oxobutanoate by Immobilized *Geotrichum candidum* in an Organic Solvent

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Abstract: Diastereoselectivity in microbial reduction of ethyl α-methyl-β-oxobutanoate is controlled by using immobilized microbe in an organic solvent in the presence of cyclopentanol as an additive to enhance the syn-selectivity.

Asymmetric reduction of ketones with microbes has widely been employed for the preparation of chiral alcohols.[1,2] However, serious disadvantages have still existed in microbial reduction; an alcohol of only one specific configuration is obtained by a microbe and usually the enantioselectivity of the reduction is unsatisfactory. To control stereochemistry of microbial reduction and to enhance enantioselectivity, several methods have been developed including the modification of a substrate,[3,4] the use of selective inhibitor,[5] the immobilization of a microbe[6] and the use of organic solvent.[7] Namely, the use of an inhibitor is effective to control the stereochemistry of microbial reduction where plural enzymes are responsible. If the added material plays a role of an accelerator, instead of an inhibitor, the situation will be more desirable. Indeed, we report here a novel method for controlling the diastereoselectivity of microbial reduction by means of an added accelerator. Cyclopentanol acts as an effective additive to increase the syn-selectivity of microbial reduction of an α-alkyl-β-keto ester.

Reduction of ethyl 2-methyl-3-oxobutanoate (**1**) with *Geotrichum candidum* IFO 4597[8] in water (substrate concentration = 33 mM) for 3 h gave the corresponding hydroxy ester (**2**) in the *syn/anti* ratio of *35/65*. The anti-selectivity of the reduction increased with the increase of reaction time: the *syn/anti* ratio after 70 h was *11/89*; *i.e.*, the *anti*-**2** was produced predominantly. Similar phenomenon was already observed by Buisson *et al*[9] and was claimed to be one of the methods for controlling diastereoselectivity toward the anti-product. When the microbe was immobilized with a water-adsorbent polymer[10] and the reaction was conducted in hexane, the microbial reduction gave the product with *syn/anti* ratio of *46/54*; the selectivity shifts slightly toward the syn side. To our surprise, the *syn/anti* ratio did not change drastically after prolonged reaction in hexane contrary to that observed in an aqueous system; the value was *35/65* even after 70 h. Thus, it seems that the reduction under an organic atmosphere prefers to produce the syn-product more than that in an aqueous solution. However, the syn-selectivity observed herein is still unsatisfactory. On the other hand, it has been found that the addition of cyclopentanol to the reduction system increases the syn-selectivity. Thus, the *syn/anti* ratio is enhanced up to *95/5* in the presence of 0.1 M of cyclopentanol. Cyclopentanol is able to shift the diastereoselectivity toward the *syn*-hydroxy ester. The results are summarized in Table 1.

Cyclopentanol exerts similar effect in an aqueous system, although the efficiency is small compared to that in the organic system and the anti-preference becomes dominant after prolonged reaction time. Naked microbe is

also effective to the reaction in hexane, but the immobilization enhances the reduction rate[10] as well as the formation of the syn-product.

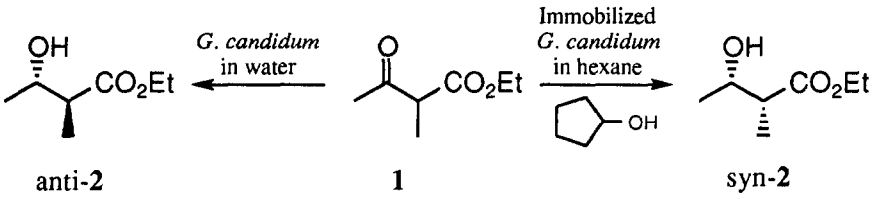

Table 1. Diasteroselectivities under Various Reaction Conditions

State of the Microbe	Solvent	Cyclopentanol (M)	*Syn/Anti* Ratio After 3 h	After 70 h
Naked	Water	0	35/65	11/89
Naked	Water	0.1	39/61	7/93
Immobilized	Hexane	0	46/54	35/65
Immobilized	Hexane	0.1	95/5	95/5

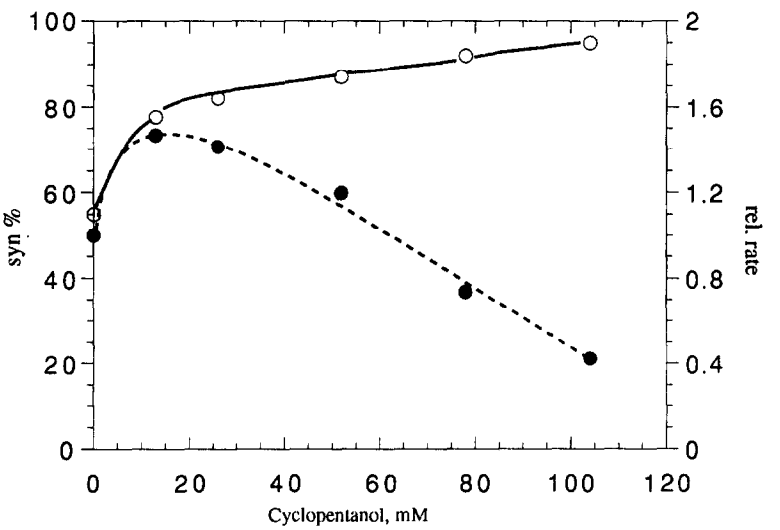

Fig. 1. Effect of cyclopentanol on diastereoselectivity and relative rate.

—○— syn % - -●- - rel. rate

Although the syn-diastereoselectivity increases with the increase in concentration of cyclopentanol as was described above, the reduction rate is not a monotonous function of the concentration of cyclopentanol: when the concentration of cyclopentanol increases, the reduction rate increases up to 13 mM of the alcohol and then the rate

decreases gradually. Thus, diastereoselectivity and reaction rate are controlled independently by cyclopentanol.

We confirmed that *Geotrichum candidum* oxidizes cyclopentanol into cyclopentanone. Therefore, we suggest that cyclopentanol in the present reaction system plays to keep the coenzyme in the reduced form ready to participate in the reduction. Dehydrogenases that afford the syn-isomer selectively may have larger Km for the coenzyme than the others.

The effects of the concentration of cyclopentanol on the reaction rate and diastereoselectivity are explainable with the idea that certain enzymes are inhibited by cyclopentanol and, at the same time, accelerated by NAD(P)H produced through the oxidation of cyclopentanol. That is, the additive, cyclopentanol, competes with **1** to be trapped by dehydrogenases responsible to the reduction. Consequently, the increase in the concentration of cyclopentanol reduces apparent activity of the enzymes. On the other hand, a high concentration of cyclopentanol contributes to promote the formation of NAD(P)H form $NAD(P)^+$ through a coupled oxidation of cyclopentanol. If a group of dehydrogenases that afford the syn-product selectively is more activated by the increase in the concentration of NAD(P)H than the group of dehydrogenases that afford the anti-product because of their difference in Km, the increase in the concentration of cyclopentanol will result in the increase in the syn-selectivity.

We reported that NADH-dependent glycerol dehydrogenase from *Geotrichum candidum* (GGDH) oxidizes cyclopentanol and an α-keto ester is reduced smoothly under the catalysis of GGDH in the presence of cyclopentanol.[10] GGDH is thus a candidate to keep the coenzyme in the reduced form. However, it is obvious that GGDH is not the enzyme that reduces **1** with a high syn-selectivity, because the reduction of **1** with GGDH is very slow and anti-preferential (*syn/anti* = *45/55*). The enzymes that reduce the coenzyme and those that reduce the substrate are not necessarily the same.

The preferential formation of the anti-product after prolonged reaction time as originally reported by Buisson *et al*[9] may be accounted for by the reverse oxidation of *syn*-**2** initially produced. The reaction is run under aerobic conditions, which may inevitably tend to keep the coenzyme in the oxidized form. Consequently, there are good chances for the microbe to oxidize the product through the reversed process of the reaction. If the dehydrogenases that interact with the syn-product are more active than the others under certain concentrations of $NAD(P)^+$, the final product after prolonged reaction period may be exceeded by the anti-isomer over the syn-isomer. The phenomenon is similar to preferential formation of *cis*-stilbene after prolonged photochemical *cis-trans* isomerization of this olefin. Cyclopentanol increases the amount of the reduced form of the coenzyme at the sacrifice of the amount of its oxidized form preventing the equilibrium from the oxidation process and, consequently, from the production of the anti-product.

Since the enzymes that participate in the present reduction are not yet isolated, the detailed role of cyclopentanol as an effective additive to control the stereochemistry of microbial reduction is ambiguous. The present method is worth to be tested for controlling the diastereoselectivity of other microbial reductions.

In a typical experiment, a solution of **1** (0.1 mmol) and cyclopentanol (1 mmol) in 6 ml of hexane was added to the immobilized microbe (0.5 g of the polymer (BL-100)[10] to 3 ml of microbial suspension) and the resulting suspension was shaken (100 stroke/min) at 30 °C for 17 h. Tridecane (10 mg, internal standard for GC) was added and the reaction mixture was filtered. The biocatalyst was washed with ether (5 ml) and the filtrates were combined. The chemical yield (56 %) and *syn-anti* ratio in the product (*95/5*) was measured using a GC-column (PEG, 0.25 mm x 20 m, 100 °C, He, 2 ml/min). The *anti*-hydroxy ester eluted faster (2.9 min) than the syn-isomer (3.2 min).[11] The product was subjected to a column chromatography on silica gel (eluent; hexane:CH_2Cl_2 = 2:1)[12] and the absolute configuration and e.e. of **2** (syn: 98 % (3*S*), anti: 95 % (3*S*)) were determined on a chiral capillary GC-column (Chiraldex G-TA, 0.25 mm x 30 m, 70 °C, He, 2 ml/min).

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12. The unchanged starting material (**1**) should be removed from the product prior to subject to the chiral capillary GC, otherwise, the presence of **1** interferes the complete separation of four isomers of **2**.

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