Asymmetric Reduction of Synthetic Ketones by Marine Microorganisms

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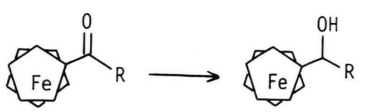
Three strains of bacteria reducing (trifluoroacetyl)ferrocene (**3**) to optically pure (*R*)-2,2,2-trifluoro-1-hydroxyethylferrocene (**4**) and one bacterial strain reducing **3** to (*S*)-**4** of moderate optical purity were isolated from sea-water collected at the coastal areas in Ibaraki prefecture of Japan. The former three strains were identified as *Micrococcus lylae, Micrococcus luteus,* and *Deleya marina* and the latter as *Bacillus licheniformis.* These strains also asymmetrically reduced some other synthetic ketones, *e.g.,* 2,2,2-trifluoroacetophenone (**7**) and phenyl trimethylsilyl ketone (**9**). Further screening of microorganisms capable of reducing **3** was done with bacteria isolated from sea-water of the deep sea (Okinawa trough, Japan trench, and Mariana trough) and of the pelagic areas (Indian Ocean and South China Sea). Most of these marine strains preferentially reduced **3** to (*R*)-**4** similar to the coastal strains, but the frequency of finding very highly enantioselective strains (*i.e.,* those forming **4** of >90% e.e.) was remarkably high in several sites of the deep sea and pelagic areas as compared with the coast and terrestrial environment.

Introduction

Asymmetric reduction of ketones is frequently encountered in organic synthesis, and enzymic or microbial procedures are successfully used for many species of usual ketones [1]. However, unnatural ketones such as organometallic ones are often poor substrates for the presently available enzymes or microorganisms. Acetylferrocene (**1**) was reduced neither with horse liver alcohol dehydrogenase nor with baker's yeast [2, 3]. Because the desired product, (+)- or (−)-1-hydroxyethylferrocene (**2**), is very useful in the production of asymmetric reagents or catalysts [4], we made much effort to find microorganisms capable of reducing **1.** The first screening was done with terrestrial microorganisms, but no prominent reduction was found. We then turned our attention to marine microorganisms, because few reports have appeared on the reductive activity of marine strains with synthetic ketones so that we expected unknown possibilities in this biosphere.



1 R = CH_3 **2** R = CH_3
3 R = CF_3 **4** R = CF_3

Fig. 1. Reaction scheme 1.

Here, we report the stereoselectivity of some species of marine microorganisms in the reduction of synthetic ketones. (Trifluoroacetyl)ferrocene (**3**) was used as the ketone substrate for screening because of the rapid reduction as compared with **1** [5].

Materials and Methods

Chemicals

The ketones **3, 9, 11,** and **13** were prepared by the methods described previously [2, 5–7]. Other ketones, **1, 5,** and **7,** were obtained from Tokyo Chemical Industries Ltd. (Tokyo). The racemic alcohols, (±)-**4,** (±)-**8,** (±)-**10,** and (±)-**12,** were prepared by $NaBH_4$ reduction of the corresponding ketones. The optically active specimens for HPLC standards, (*R*)-(−)-**4** [5], (*S*)-(+)-**8,** and (*S*)-(−)-**10,** were prepared by microbial reduction: (+)-**8** with *Xanthomonas campestris* NIAS 1076 in 13% yield, $[\alpha]_D^{23}$ + 20° (*c* = 0.8, CH_2Cl_2) (lit. $[\alpha]_D^{22}$ − 1.9° (*c* = 3, CH_2Cl_2) for (*R*)-(−)-**8** of 6.6% e.e. [8]), MS *m/z:* 176.0440 (M^+); calcd. $C_8H_7F_3O$ = 176.0449; (−)-**10** with *Torulopsis magnoliae* IFO 661 in 80%

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yield, $[\alpha]_D^{23}$ − 74° (*c* = 3.7, toluene) (lit. $[\alpha]_D$ − 48.4° (*c* = 1.8, toluene) for (*S*)-(−)-**10** of 46% e.e. [9]), MS *m/z:* 180.0968 (M^+); calcd. $C_{10}H_{16}OSi$ = 180.0970. Silica gel TLC plates (0.2 mm thick) were obtained from Merck. All other reagents were of the best grade available commercially.

Screening for reduction of **3** *with coastal microorganisms*

Samples of surface sea-water were collected at the coasts of Kashima and Oarai in Ibaraki prefecture and Ito in Shizuoka prefecture of Japan. About 250 strains of microorganisms were isolated from these samples by plate culture method with marine agar (type 2216, Difco).

Each strain of microorganism was cultured in a test tube containing 8 ml marine broth (type 2216, Difco) with shaking at 30 °C for 24 h. The cells were collected by centrifugation, suspended in a mixture of 0.18 ml phosphate buffer (0.1 M, pH 7.0) containing 1% glucose and 3% Tween 80 and 20 µl EtOH containing 1 µl of **3.** The mixtures were shaken at 30 °C for 48 h, and then extracted with ether. After concentration by vaporization, the extracts were analyzed by TLC (silica gel F_{254} developed with benzene; R_f 0.42 for **4** and R_f 0.78 for **3**). The conversion rate was determined with a TLC scanner. The spots of **4** were extracted with ether for optical purity determination by HPLC. The chromatographic conditions are given in the legend to Fig. 2.

Reduction of ketones **1, 5, 7, 9, 11,** *and* **13**

Reduction of these ketones with 4 selected strains was carried out in the same way as described above, but the produced alcohols **6, 8,** and **10** were analyzed by GC: column, silica capillary with a chemically bonded OV-1701 phase (0.25 mm i.d. × 25 m); carrier (He) flow rate, 0.7 ml/min; column temperature, 110 °C for **5**～**8** and 130 °C for **9** and **10;** retention time, 4.1, 1.7, 4.4, 5.6, 4.7, and 5.1 min for **5, 7, 6, 8, 9,** and **10,** respectively. Alcohols **2** and **12** were analyzed by TLC. R_f values for **1, 2, 11,** and **12** were 0.40, 0.26, 0.51, and 0.24, respectively, on silica gel F_{254} plates developed with 10% EtOAc in benzene. Optical purity of alcohols **8** and **10** were determined by HPLC in the same condition as described in the legend to Fig. 2 (retention time: 20.6 min for (*R*)-**8,** 21.7 min for (*S*)-**8,** 11.2 min for (*S*)-**10,** and 20.1 min for (*R*)-**10**). Optical purity of alcohols **2, 6,** and **12** were determined by the method reported before [2].

Biological taxonomy

Taxonomic studies were carried out according to Bergey's Manual of Systematic Bacteriology [10] or to the related publications [11−13].

Screening with microorganisms isolated from sea-water of the deep sea and the pelagic areas

Samples of sea-water were collected at the sites marked on the map in Fig. 4. The depth is given in the Figure. 15, 37, and 5 strains of bacteria used in the present study were isolated from sea-water collected at Okinawa trough [14], Japan trench (33°11.9′N, 141°43.0′E), and Mariana trough (20°26.19′−21°05.95′N, 143°53.18′−143°18.08′E) [15], respectively. The medium and temperature for isolation were 1/2 TZ medium [14] and 20 °C for Okinawa strains, marine agar and 4 °C for Japan trench strains, and 1/2 TZ medium and 4 °C for Mariana strains. One hundred and sixty-eight strains of bacteria were isolated with PPES-II medium at 20 °C from sea-water of Indian ocean, the bay of Bengal, the strait of Malacca, and South China sea [16]. The screening for the reduction of **3** with these strains was done in the same way as described for the coastal strains, but the temperature for cultivation and bioreduction was 20 °C and the culture medium (marine broth, type 2216) was supplemented with 1% glucose.

Results and Discussion

Ketone reduction by coastal microorganisms

Approximately one half of the coastal strains showed strong or weak reductive activity for the ketone **3.** Most of the strains yielded preferentially the *R*-configurated alcohol (**4**) as is seen from Fig. 2b and 2c. However, a few strains (*e.g.,* the strain for Fig. 2a) could also give the alcohol rich in the *S* enantiomer.

Four strains (M1-14, M2-7, M2-10, and M8-10) were selected for their high stereoselectivity and conversion rate, and their ability to reduce various synthetic ketones was investigated. The manganese-containing ketone (**13**) was not

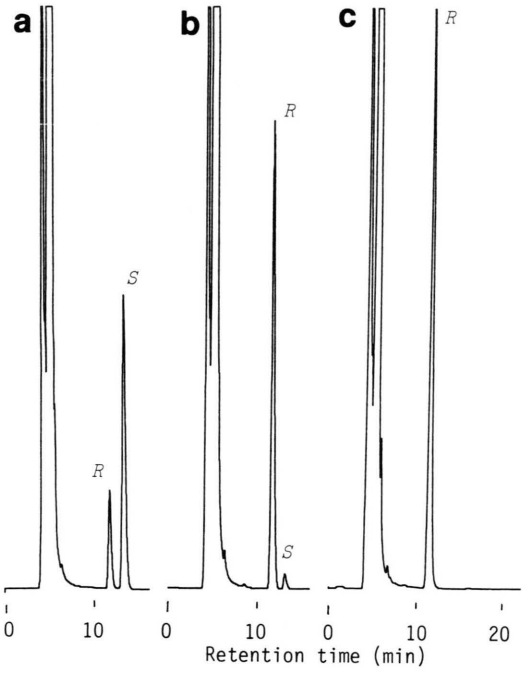

Fig. 2. HPLC chromatograms for **4** produced by strains M8-13 (a), M8-18 (b), and M8-10 (c). Conditions: column, Chiralcel OD, 4.6 × 250 mm (Daicel); solvent, 7.5% 2-propanol in hexane; flow rate, 0.5 ml/min; detection, UV at 215 nm; retention time, 11.9 min for (*R*)-**4** and 13.5 min for (*S*)-**4**.

reduced by any strain, but other ketones were reduced by all or some of the present strains. The results are summarized in Table I.

Acetylferrocene (**1**) was reduced by the strains M1-14 and M2-7. The conversion rate as well as the optical purity of the product (**2**) were not high, but they showed an important phenomena, that is, the *R* enantiomer of **2** was preferentially produced. All strains so far studied reduce **1** preferentially to (*S*)-**2** [2]. The microbial reduction of **1** to (*R*)-**2** is reported here for the first time. Another important fact was that strain M2-10, though less stereoselective for the screened substrate (**3**), showed a remarkably high stereoselectivity in the reductin of acetophenone (**5**) and its tricarbonylchromium complex (**11**). The produced alcohols were rich in the *S* enantiomer except for the trifluorinated alcohol (**8**). However, the configuration of (*R*)-**8** is substantially the same as those of (*S*)-**4,** (*S*)-**6,** and (*S*)-**12,** and hence the stereoselectivity of strain M2-10 was constant for all reducible ketones. On the other hand, the stereoselectivity of strains M1-14 and M2-7 in the reduction of **3** was reversed for acetophenone (**5**) and trimethylsilyl ketone (**9**). The stereoselectivity of strain M8-10 decreased by the exchange of the ferrocenyl group (in **3**) for the phenyl group (in **7**), while it was recovered by another exchange of the trifluoromethyl group (in **7**) for the trimethylsilyl group (in **9**). Thus, the present strains have an unique stereoselectivity in the reduction of some synthetic ketones.

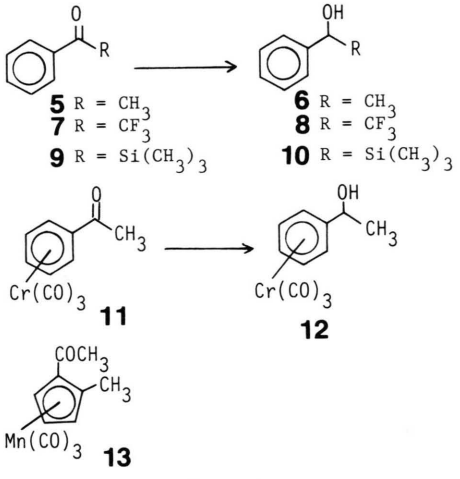

Fig. 3. Reaction scheme 2.

Table I. Absolute configuration, optical purity (% e.e.), and conversion rate (in parentheses, %) for the alcohols produced by four marine microorganisms.

Strain	Reaction **3→4**	**1→2**	**5→6**	**7→8**	**9→10**	**11→12**
M1-14	*R* > 99 (98)	*R* 79 (6)	*S* 93 (1)	*S* 65 (18)	*S* 42 (50)	*R* 28 (37)
M2-7	*R* > 99 (97)	*R* 68 (15)	*S* 91 (4)	*S* 87 (60)	*S* 13 (91)	*R* 42 (10)
M2-10	*S* 58 (40)	n.t. (<1)	*S* 99 (28)	*R* 89 (74)	*S* 2 (10)	*S* 96 (8)
M8-10	*R* > 99 (37)	(0)	(0)	*S* 43 (8)	*S* 80 (14)	(0)

n.t. = not tested.

Taxonomic characteristics

Strain M 1-14 as well as strain M 2-7 are Gram-positive nonmotile cocci, occurring predominantly in tetrads, forming no spores, and slightly degrading glucose in anaerobic conditions. These characteristics correspond with those of the genus *Micrococcus* [10a]. With other characteristics, the presence of Asp residue in the cell wall peptidoglycan for strain M 1-14 well fits with the description for *M. lylae* [10a]. On the other hand, strain M 2-7 was grown on inorganic nitrogen agar. This is characteristic of *M. luteus* according to the literature [10a]. The amino acid composition of the cell wall peptidoglycan for strain M 2-7 (Lys : Gly : Ala : Glu : Asp = 1 : 1 : 2 : 1 : 0) is also consistent with that found with a reference strain, *M. luteus* IFO 3333. Although some atypical characteristics are found with both strains, we identify strain M 1-14 as *M. lylae* and strain M 2-7 as *M. luteus*.

Strain M 2-10 is Gram-positive motile rods and endospore-forming facultative anaerobes. These characteristics suggest that strain M 2-10 belongs to the genus *Bacillus* [10b]. From the shape of spores and sporandiums and other characteristics, this strain is identified as *B. licheniformis*. The above three strains did not require Na^+ ion for growth.

Strain M 8-10 is Gram-negative motile rods with a polar flagellum. It showed an oxidative behavior in O-F test and required Na^+ ion for growth. These characteristics indicated that this strain belongs to *Pseudomonas* [10c] or its very near relative, the genus *Deleya* [11]. The quinone type (Q-9) is consistent with that of *Deleya* [11]. The species in *Deleya* with a polar flagellum is *D. marina* [12]. The characteristics for strain M 8-10 are, though with a minor exception (mannose utilization), all consistent with those reported for *D. marina* [11–13], leading to the identification of strain M 8-10 as *D. marina*.

Reduction with microorganisms from the deep sea and pelagic areas

To find more excellent microorganisms for the asymmetric ketone reduction, the screening for the reduction of **3** was continued with expanding the object from the coastal microorganisms to those living in the deep sea and pelagic areas. The distribution of microorganisms concerning the stereoselectivity in the reduction is shown in histograms (Fig. 4).

The stereoselectivity of terrestrial microorganisms in the reduction of **3** widely distributes from low to high degree, but most of the strains preferentially produce the (*R*)-alcohol ((*R*)-**4**). The strains belonging to the highest class (*i.e.*, those producing **4** of 90 ~ 100% e.e.) are, however, only 8% of the terrestrial strains tested (Fig. 4a).

The stereoselectivity of microorganisms obtained at the coasts of Kashima and Oarai (Fig. 4b) shows a similar feature with that of the terrestrial ones. For the coastal microorganisms of Izu, their stereoselectivity is not high (Fig. 4c).

A noteworthy difference from the above results appears for the microorganisms obtained from Okinawa trough (1200 ~ 1600 m deep, Fig. 4d). Ten of the fifteen strains from this site produced (*R*)-**4** of the highest class, while only one strain was poorly stereoselective. Some of the strains were derived from sea-water collected near hydrothermal vents in the trough. Microorganisms (16 strains) obtained from the very deep sea, Japan trench (5000 ~ 6000 m), were also capable of reducing the synthetic ketone (**3**). Although they included strains of moderate stereoselectivity, all they showed the preference for (*R*)-**4** (Fig. 4e). Microorganisms obtained from another point of Japan trench (21 strains, 6000 m deep) and Mariana trough (5 strains, 4200 ~ 5100 m deep) were almost inactive in the reduction of **3.**

For the microorganisms in the pelagic areas, most of the isolates from sea-water of Indian ocean and the bay of Bengal produced (*R*)-**4** of high optical purity (Fig. 4f ~ 4i). For instance, about 70% of the sixteen strains obtained at the sampling site h belongs to the highest class (Fig. 4h). The inclination that less stereoselective strains are hardly found is also seen for other sampling sites in the pelagic areas (Fig. 4k and 4l). In summary, most of the microorganisms living in the deep sea and pelagic areas reduce the ketone (**3**) preferentially to the *R* enantiomer of **4** similarly to the terrestrial and coastal strains, but the percent of highly stereoselective strains in the several points of the former (average more than 59%) is about 10 times large as compared with that of the latter (6%).

The marine strains were grown in glucose-supplemented marine broth. The terrestrial microor-

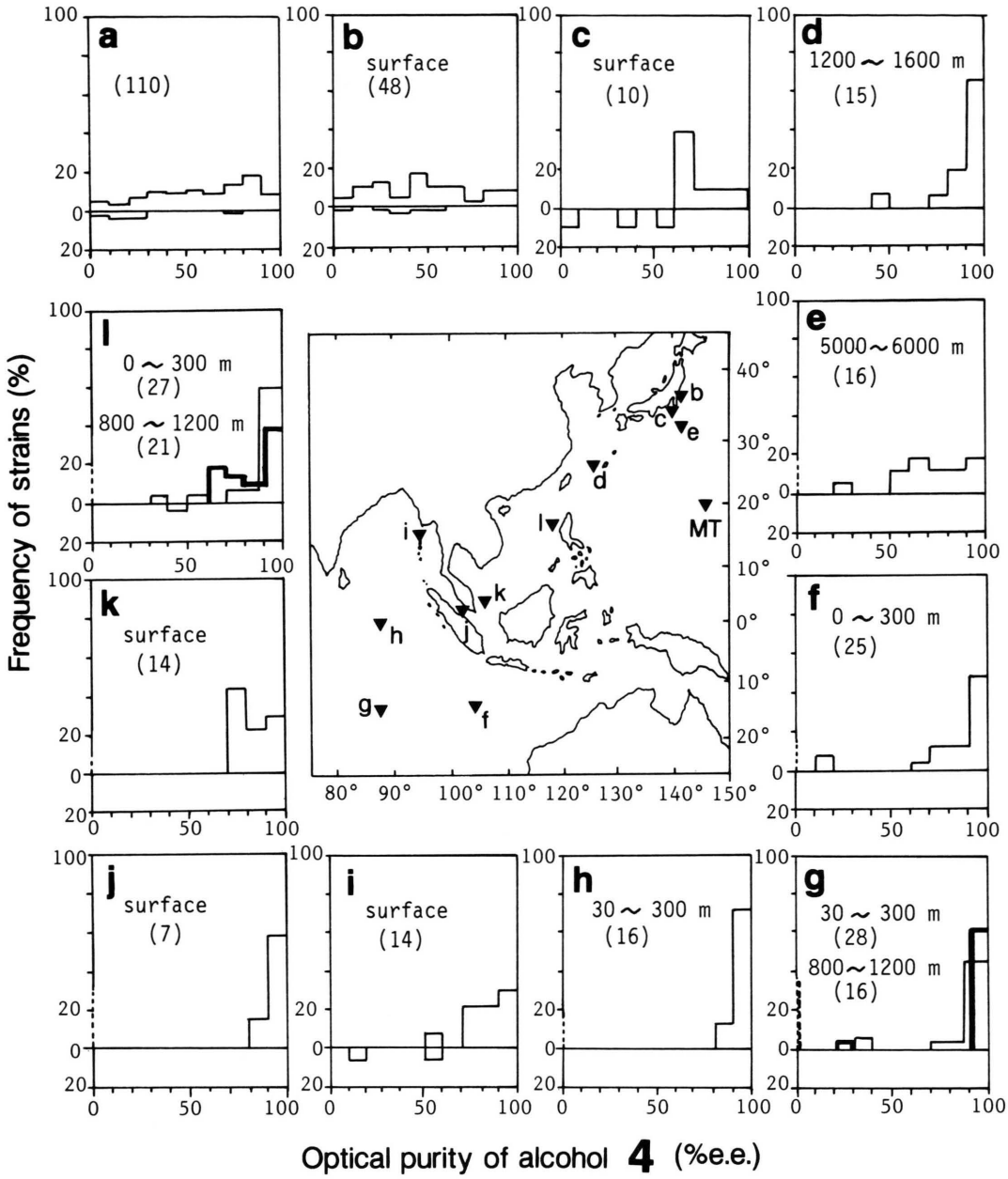

Fig. 4. Stereoselectivity in the reduction of **3** by microorganisms isolated from sea-water of the deep sea and pelagic areas. The strains are classified to 10 classes according to the optical purity of the produced alcohol (**4**), and the percentage of strains belonging to the class is given as a bar. Upward bar represents preferential production of (*R*)-**4** and downward bar that of (*S*)-**4.** Dotted line on the vertical axis represents percentage of strains inactive in the reduction. The data for terrestrial microorganisms (a) were obtained in the previous study [5]. Marine microorganisms were isolated from sea-water samples collected at the coast of Kashima-Oarai (b) and Izu (c), and at Okinawa trough (d), Japan trench (e), sites in Indian ocean (f ~ h), the bay of Bengal (i), the strait of Malacca (j), and sites in South China sea (k, l). Alphabetic letters in the map correspond to the above sampling sites. MT indicates Mariana trough. The sampling depth and the number of test strains are given in the Figure. The latter is in parentheses. At the sites for g and l, sea-water was collected from two depth. Thin line represents shallow point.

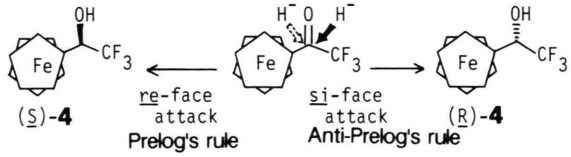

Fig. 5. Stereoselectivity in the reduction of (trifluoroacetyl)ferrocene (**3**).

ganisms were grown in the modified YM medium [5]. A comparative study was done, in which the Kashima-Oarai strains were grown in the latter medium and tested for their reductive activity. The result was substantially the same as Fig. 4b (data not shown), suggesting that the difference in culture medium did not have a great effect on the stereoselectivity. Taxonomy for the microorganisms of the deep sea and pelagic areas should be studied in future.

The preferential production of the (*R*)-alcohol in the reduction of **3** [5] is a very interesting fact, because this stereoselectivity is contrary to Prelog's rule [17], which expects the microbial ketone reduction being usually conducted on the *re*-face of the carbonyl group. The present study shows that the "anti-Prelog's rule" (Fig. 5) is functioning in the wide range of marine microorganisms and moreover that there is an inclination for the microorganisms living in the deep sea and pelagic areas to hold this rule more strictly than the coastal ones. It can be concluded that the probability to find unique and superior microorganisms for asymmetric ketone reduction is considerably high in the marine environment.

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