[Chem. Pharm. Bull.
32(4)1333—1341(1984)]

# The Use of Microorganisms in Organic Synthesis. III.[1] Microbiological Asymmetric Reduction of Methyl 3-(2-Furyl)-2-methyl-3-oxopropionate

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(Received July 21, 1983)

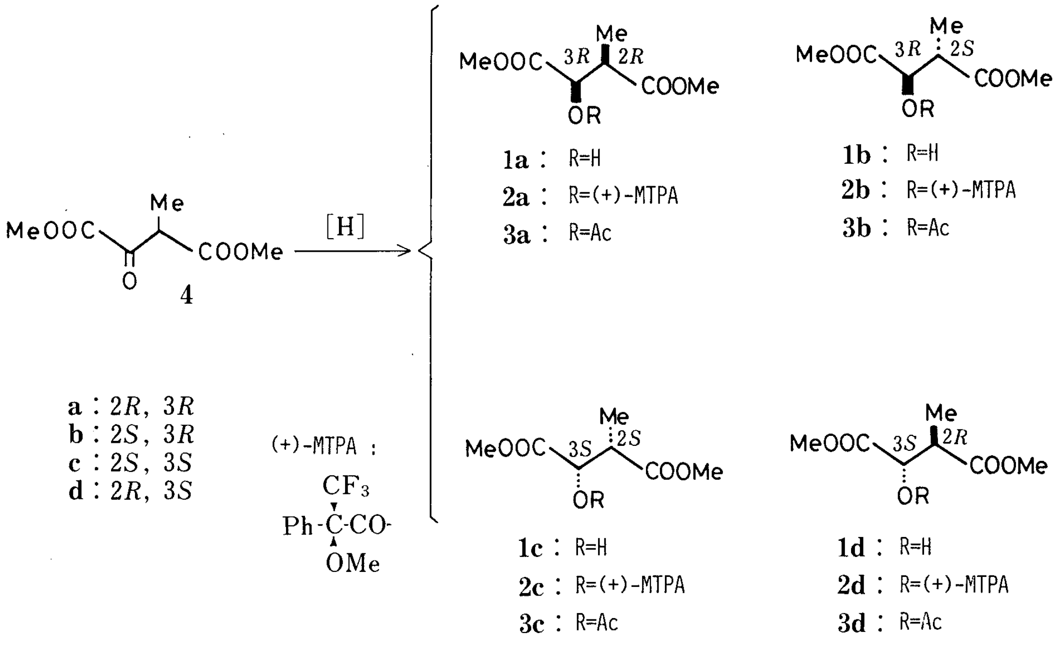
Microbiological asymmetric reduction of methyl 3-(2-furyl)-2-methyl-3-oxopropionate (**5**) by various yeasts was carried out. Four kinds of methyl 3-(2-furyl)-3-hydroxy-2-methyl propionates (**6a**—**6d**) could be obtained separately from the prochiral $\beta$-keto ester **5** by reduction with properly selected microorganisms. In particular, the desired *syn*-isomer **6a** was obtained with high optical purity (>99% e.e.). Both the chemical yield and the optical purity of the reduction products (**6a**—**6d**) were significantly improved when fermentation was carried out on a large scale using a 30 l jar fermentor or a 200 l tank.

**Keywords**——$\alpha$-methyl $\beta$-keto ester; $\alpha$-methyl $\beta$-hydroxy ester; asymmetric reduction; microbioloical reduction; yeast

Optically active 2-methylmalates **1a**—**1d** are considered to be useful building blocks for the synthesis of polyoxo- and polyenemacrolide antibiotics and insect pheromones, because they can be regarded as a functionalized 2-methyl-3-hydroxy propionic acid unit, which frequently appears in the above natural products.[2] Excellent synthetic methods have been developed for the *anti*[3]-isomers **1b** and **1d**,[2b] but for the *syn*[3]-isomers **1a** and **1c**, only multistep syntheses involving rather tedious optical resolution have been reported.[2a] Therefore, an effective method for the synthesis of the *syn*-isomers, particularly **1a**, is still required.

In the previous paper,[4] we reported that when dimethyl 2-methyl-3-oxosuccinate **4** was reduced with *Candida albicans*, the desired optically active *syn*-dimethyl 2-methylmalate **1a** (2*R*, 3*R*) was obtained in high optical purity (95% e.e.) along with the *anti*-isomer **1b** (2*S*, 3*R*; 58% e.e.). However, chromatographic separation of *syn*-**1a** and *anti*-**1b**, which are produced in a ratio of *ca.* 2:1, was found to be quite difficult. In order to overcome this difficulty, methyl 3-(2-furyl)-2-methyl-3-oxopropionate **5**[5] was chosen as a substrate, because a furan ring is readily convertible to carboxylic acid afterwards, and it was hoped that through this structural modification separation of the diastereoisomers obtained after reduction would become possible. Fortunately, separation was found to proceed quite smoothly by simple column chromatography. The present report deals with the asymmetric reduction of **5** with various yeasts, as well as separation of the products and determination of the stereostructure and optical purity of each product.

Reduction of **5** with actively fermenting baker's yeast (*Saccharomyces cerevisiae*) did not proceed at all. However, preliminary screening experiments using thirty-nine kinds of yeasts[6] showed that some microorganisms can reduce this substrate **5**. In these screening experiments, about 5 mg of **5** was used and the progress of the reduction was monitored by chromatography. Various yeasts such as *Kloeckera saturnus, Lipomyces starkeyi, Saccharomyces*



a : 2*R*, 3*R*
b : 2*S*, 3*R*
c : 2*S*, 3*S*
d : 2*R*, 3*S*

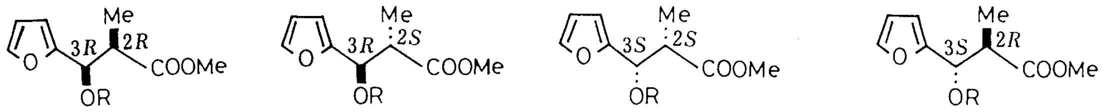
**1a** : R=H
**2a** : R=(+)-MTPA
**3a** : R=Ac

**1b** : R=H
**2b** : R=(+)-MTPA
**3b** : R=Ac

**1c** : R=H
**2c** : R=(+)-MTPA
**3c** : R=Ac

**1d** : R=H
**2d** : R=(+)-MTPA
**3d** : R=Ac

Chart 1



**6a** : R=H
**7a** : R=(+)-MTPA
**8a** : R=Ac

**6b** : R=H
**7b** : R=(+)-MTPA
**8b** : R=Ac

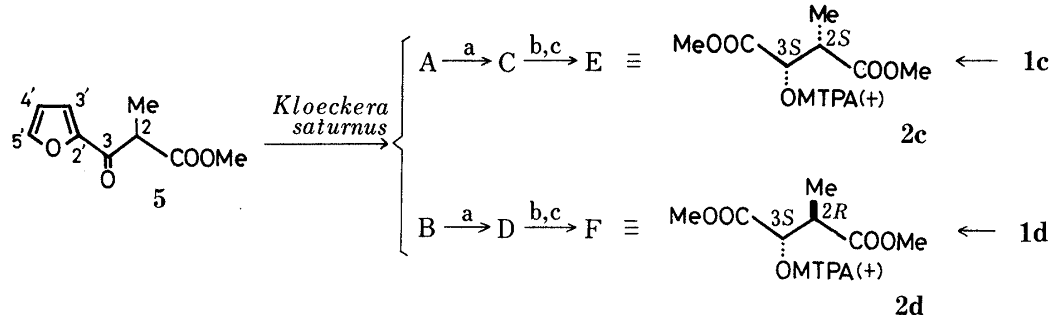
**6c** : R=H (=A)
**7c** : R=(+)-MTPA (=C)
**8c** : R=Ac

**6d** : R=H (=B)
**7d** : R=(+)-MTPA (=D)
**8d** : R=Ac

Chart 2

*delbrueckii, Saccharomyces fermentati, Candida humicola, Candida guilliermondii,* and *Candida albicans* were found to be effective for the asymmetric reduction of the $\alpha$-methyl $\beta$-keto ester **5**. At this stage, before proceeding further, it was necessary to determine the stereostructures of the four possible isomers **6a**—**6d**.

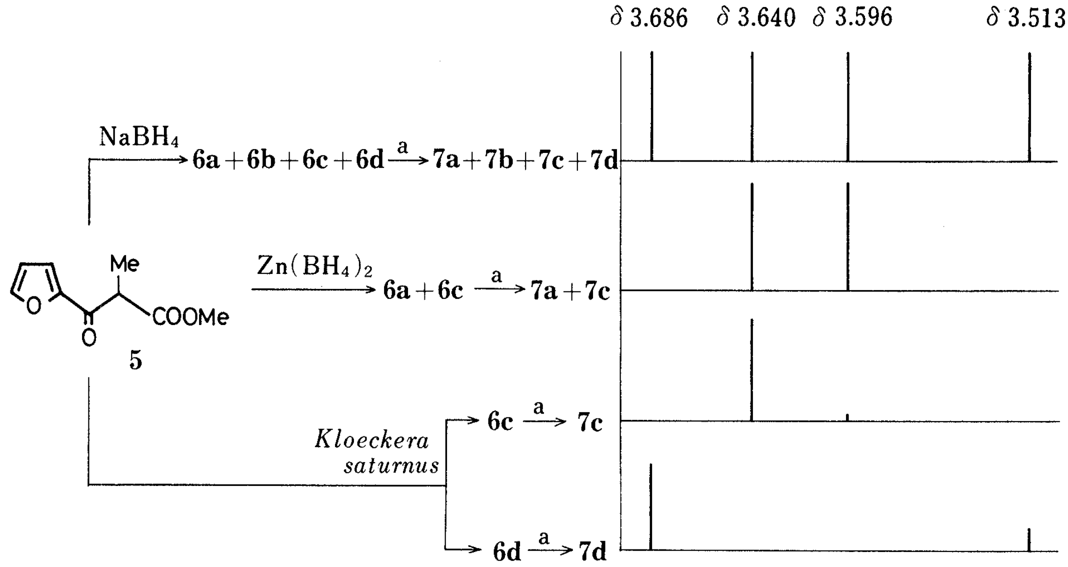
Initially, a substantial amount of **5** was subjected to reduction with *Kloeckera saturnus*, which was arbitrarily chosen from the above effective yeasts: namely, according to the reported cultivation method,[6] a total of 4.02 g (*ca.* 800 mg in each experiment) of **5** in a total of 4 l (800 ml in each experiment) of liquid medium was incubated with *Kloeckera saturnus* at 30 °C for 3 d. In this case, the resulting diastereomeric mixture could be separated by simple chromatography into two optically active alcohols A (682 mg; 17% yield, $[\alpha]_D^{18}$ $-10.73°$ ($c=4.8$, $CHCl_3$)) and B (455 mg; 11% yield, $[\alpha]_D^{18}$ $-27.29°$ ($c=5.5$, $CHCl_3$)) as the main products. For the purpose of determining the stereostructure and optical purity, A and B were separately condensed with (+)-$\alpha$-methoxy-$\alpha$-trifluoromethylphenylacetic acid chloride ((+)-MTPACl[7]) in pyridine and the resulting esters C and D were subjected to ozonolysis in $CH_2Cl_2$ under dry ice-acetone cooling and then esterified with $CH_2N_2$ to yield the corresponding esters E and F, respectively. The 400 MHz nuclear magnetic resonance (NMR) spectra of E and F were found to be identical with those of authentic (+)-MTPA esters **2c** (*syn*; 2*S*, 3*S*)[4] and **2d** (*anti*; 2*R*, 3*S*),[4] so that the absolute configuration of E and thence those of A and C are 2*S*, 3*S* (therefore A = **6c**, C = **7c**) and those of F and thence B and D are 2*R*, 3*S* (therefore B = **6d**, D = **7d**).



a : (+)-MTPACl/pyridine
b : 1) $O_3/CH_2Cl_2$, 2) 30% $H_2O_2$
c : $CH_2N_2$

Chart 3

Next, in order to prepare the four possible stereoisomers **6a**—**6d**, the $\alpha$-methyl $\beta$-keto ester **5** was reduced with $NaBH_4$ and the products were directly converted to the corresponding (+)-MTPA esters **7a**, **7b**, **7c**, and **7d**. The signals due to the four types of ester methyl protons appeared in distinctly different fields ($\delta$ 3.513, 3.596, 3.640, and 3.686) in the 400 MHz NMR spectrum without any shift reagent. The signals at $\delta$ 3.640 and 3.686 could be ascribed to **7c** (2*S*, 3*S*) and **7d** (2*R*, 3*S*) by direct comparison with those of the authentic specimens obtained above. Then, the $\beta$-keto ester **5** was reduced with $Zn(BH_4)_2$, which is known to give predominantly *syn*-isomers[8] **6a** and **6c**. The reduction products were converted to the (+)-MTPA esters as usual. Only two peaks appeared in the ester methyl region, as expected. One of the peaks was identified as that of **7c** and consequently the other peak ($\delta$ 3.596) could be ascribed to the other *syn*-isomer **7a**. Thus, the remaining unassigned peak at $\delta$ 3.513 should be due to the other *anti*-isomer **7b**. The relation between the signals and the structures was thus established to be as shown in Chart 4. From the small peaks due to the isomers present in **7c** and **7d**, the optical yields of **7c** and **7d** were calculated as 83 and 59% e.e., respectively.



a : (+)-MTPACl/pyridine

Chart 4

Now that we had a means to identify the reduction products, further screening experiments were undertaken using about 50 mg of **5** and the yeasts which had been found to be effective in the preliminary screening experiments. The chemical yield (as (+)-MTPA ester),

TABLE I

| Entry | Microorganism | Substrate **5** (mg) | Chemical yield (%) as (+)-MTPA ester | *syn*/*anti* | Optical yield (% e.e.) |
|---|---|---|---|---|---|
| 1 | *Kloeckera saturnus* | 4020 (48)[b] | 28[a] (12)[b] | 60/40 (54/46)[b] | *syn* **6c**: 83 (87)[b]<br>*anti* **6d**: 59 (53)[b] |
| 2 | *Saccharomyces delbrueckii* | 57 (46)[b] | 43 (50)[b] | 39/61 (41/59)[b] | *syn* **6a**: 90 (>99)[b]<br>*anti* **6b**: 91 (94)[b] |
| 3 | *Saccharomyces fermentati* | 45 (48)[b] | 21 (36)[b] | 40/60 (44/56)[b] | *syn* **6a**: 84 (>99)[b]<br>*anti* **6b**: 86 (98)[b] |
| 4 | *Candida humicola* | 58 (51)[b] | 28 (4)[b] | 36/64 (28/72)[b] | *syn* **6a**: 70 (>99)[b]<br>*anti* **6b**: 65 (90)[b] |
| 5 | *Candida guilliermondi* | 50 (47)[b] | 53 (24)[b] | 38/62 (13/87)[b] | *syn* **6a**: 89 (85)[b]<br>*anti* **6b**: 67 (98)[b] |
| 6 | *Candida albicans* | 46 (49)[b] | 23 (7)[b] | 0/99 (0/99)[b] | *anti* **6b**: >99 (97)[b] |

*a*) Isolation yield.
*b*) Data obtained in cases where yeast cells were used.

optical purity (% e.e.) and the ratio of *syn*-/*anti*-isomers are summarized in Table I.

The main features of the present results are as follows. 1) When *Kloeckera saturnus* was used, $C_3$-(*S*)-alcohols **6c** and **6d** were obtained, whereas other yeasts produced isomeric $C_3$-(*R*)-alcohols **6a** and **6b**. 2) High optical purity was obtained in some cases (*Saccharomyces delbrueckii*; 90—91% e.e., *Candida albicans*; >99% e.e.); however, when *Kloeckera saturnus, Saccharomyces fermentati, Candida humicola* and *Candida guilliermondii* were used, the optical yields were rather unsatisfactory. 3) Usually, no *syn*-/*anti*-selectivity was observed, but when *Candida albicans* was used, only the *anti*-isomer was obtained although the yield was poor. 4) In cases where yeast cells were used, the optical yield was significantly better in every case. 5) Products obtained as a mixture of diastereoisomers could be separated by simple silica gel column chromatography. Therefore, it become possible to prepare and isolate all four isomers by combining the above features.

Next we carried out large-scale reduction of **5** using a 30 l jar fermentor (working volume; 15 l) and a 200 l tank (working volume; 100 l) under the same cultivation conditions as used in the small-scale experiments. The following four yeast strains, *Kloeckera saturnus, Saccharomyces delbrueckii, Saccharomyces fermentati*, and *Candida albicans* were used for large-scale cultivation.[9] Chemical yield and optical purity (% e.e.) were calculated by the same method as described for the small-scale experiment and the results are summarized in Table II. When *Kloeckera saturnus* and *Saccharomyces fermentati* were used, both the chemical yield and the optical purities of the reduction products were significantly improved.

An effective conversion of optically active **6a**—**6d** obtained on a large scale into the desired optically active methylmalates (**1a**—**1d**) was then examined and it was found that the secondary hydroxyl group of the reduction products should be masked by conversion into the acetate before subjecting them to ozonolysis. Selective hydrolysis of acetate masking group in the presence of methyl esters was effected by treating the triesters **3a**—**3d** with 30% HBr–AcOH.[2b] The optical purities of methylmalates (**1a**—**1d**) thus obtained were individually determined by the previously described (+)-MTPA ester method and it was found that no decrease in optical yield occurred in the conversion process.

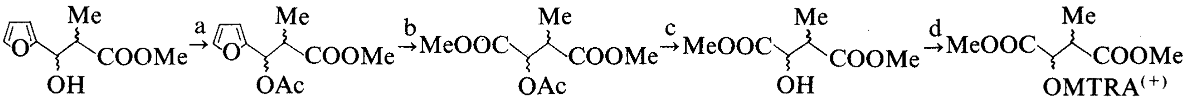
In conclusion, four kinds of methyl 3-(2-furyl)-3-hydroxy-2-methyl propionates (**6a**—**6d**) were obtained from the prochiral *β*-keto ester **5** by reduction with properly selected microorganisms. In particular, the desired *syn*-isomer **6a** was obtained with high optical purity.

TABLE II

| Entry | Microorganism | Substrate **5** (g) | Cultivation time (after addition of the substrate) | Products Chemical yield (%) | Optical yield (% e.e.) |
|---|---|---|---|---|---|
| 1[a] | *Kloeckera saturnus* | 7.522 | 96 h<br>(24 h) | **6c**: 2.936 g (39%)<br>**6d**: 2.534 g (33%) | **6c**: 83<br>**6d**: 73 |
| 2[b] | *Kloeckera saturnus* | 37.039 | 92 h<br>(20 h) | **6c**: 9.509 g<br>**6c**+**6d**: 8.153 g<br>**6d**: 4.699 g<br>Total: 22.361 g (60%)[c] | **6c**: 80<br>**6d**: 67 |
| 3[a] | *Saccharomyces delbrueckii* | 5.274 | 120 h<br>(48 h) | **6a**: 0.223 g (4%)<br>**6b**: 0.441 g (8%) | **6a**: 98<br>**6b**: 97 |
| 4[a] | *Saccharomyces fermentati* | 5.131 | 164 h<br>(90 h) | **6a**: 1.436 g (28%)<br>**6b**: 1.485 g (29%) | **6a**: >99<br>**6b**: >99 |
| 5[b] | *Saccharomyces fermentati* | 36.345 | 164 h<br>(92 h) | **6a**: 9.635 g<br>**6a**+**6b**: 7.681 g<br>**6b**: 11.084 g<br>Total: 28.400 g (77%)[c] | **6a**: >99<br>**6b**: 98 |
| 6[a] | *Candida albicans* | 4.759 | 96 h<br>(24 h) | **6b**: 0.671 g (14%) | **6b**: 96 |

*a*) Data obtained in 30 l jar fermentor.
*b*) Data obtained in 200 l tank.
*c*) Yields after single silica gel column chromatography.

TABLE III



| | | | | |
|---|---|---|---|---|
| (2*R*, 3*R*)-**6a**: >99% e.e. | **8a** (89%) | **3a** (60%) | **1a** (61%) | **2a**: >99% e.e. |
| (2*S*, 3*R*)-**6b**: 98% e.e. | **8b** (82%) | **3b** (42%) | **1b** (44%) | **2b**: 98% e.e. |
| (2*S*, 3*S*)-**6c**: 83% e.e. | **8c** (78%) | **3c** (54%) | **1c** (52%) | **2c**: 84% e.e. |
| (2*R*, 3*S*)-**6d**: 73% e.e. | **8d** (72%) | **3d** (37%) | **1d** (51%) | **2d**: 72% e.e. |

a: $Ac_2O$/DMAP in pyridine. b: 1) $O_3/CH_2Cl$, −78 °C, 2) 30% $H_2O_2$, 3) $CH_2N_2$.
c: 30% HBr/AcOH in MeOH. d: (+)-MTPACl in pyridine.

Interestingly, both the chemical yield and the optical purity of the reduction products (**6a**—**6d**) were significantly improved when fermentation was carried out on a large-scale using a 30 l jar fermentor or a 200 l tank.

Synthesis of biologically active natural products is being undertaken using mainly the *syn*-isomer **6a**.

## Experimental

All melting points were measured with a Kofler micro melting point apparatus and are uncorrected. IR spectra ($CCl_4$) were measured on a JASCO A-3 spectrophotometer. NMR spectra were measured either on a JEOL FX-60 spectrometer or a JEOL FX-400 instrument. Spectra were taken as 5—10% w/v solutions in $CDCl_3$ with $Me_4Si$ as an internal reference. Gas chromatography-mass spectroscopy (GC-MS) was carried out on a Hitachi RMU-6M mass spectrometer and high-resolution mass spectra were taken with a Hitachi M-80 GC-MS spectrometer. Values of $[\alpha]_D$ were measured on a Perkin-Elmer model 241 MC polarimeter.

**Synthesis of Methyl 3-(2-Furyl)-2-Methyl-3-oxopropionate 5**——*tert*-Butyl alcohol (14.8 g, 0.2 mol) was added to a suspension of 60% NaH (8 g, 0.2 mol) in absolute toluene (100 ml) under reflux, and the reaction mixture was stirred for 1 h, then a solution of methyl 2-furoate (12.6 g, 0.1 mol) and methyl propionate (17.6 g, 0.2 mol) in absolute toluene (20 ml) was added. The whole was stirred for 2 h under reflux. After cooling, the reaction mixture was diluted with aqueous AcOH and extracted with ether. The ether extract was washed with sat. $NaHCO_3$ aq. and sat. NaCl aq., then dried over $MgSO_4$. Removal of the solvent gave an oily product, which was chromatographed on silica gel (300 g) to give the $\beta$-keto ester **5** (13.321 g, 73% yield) as a homogeneous oil from the *n*-hexane–ethyl acetate (19 : 1—10 : 1) eluent. *Anal.* High-resolution MS. Calcd for $C_9H_{10}O_4$ ($M^+$; *m/e*): 182.057. Found: 182.058. IR $\nu_{max}$ $cm^{-1}$: 1682, 1745. NMR $\delta$: 1.483 (3H, d, $J=7.1$ Hz, 2-Me), 3.706 (3H, s, COOMe), 4.191 (1H, q, $J=7.1$ Hz, 2-H), 6.564 (1H, dd, $J_{4',5'}=1.7$ Hz, $J_{3',4'}=3.6$ Hz; 4'-H), 7.286 (1H, dd, $J_{3',5'}=0.8$ Hz, $J_{3',4'}=3.6$ Hz, 3'-H), 7.612 (1H, dd, $J_{3',5'}=0.8$ Hz, $J_{4',5'}=1.7$ Hz, 5'-H).

**Asymmetric Reduction of 5 with *Kloeckera saturnus***——i) Asymmetric reduction of **5** with *Kloeckera saturnus* was carried out according to our reported fermentation procedure.[6)] Test tubes (25 mm × 200 mm) containing 10 ml of culture medium comprising 5% glucose, 0.1% $KH_2PO_4$, 0.1% $(NH_4)_2SO_4$, 0.05% urea, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, 0.1% yeast extract, a trace of mineral solution (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnCl_2 \cdot 4H_2O$, 0.1% $ZnSO_4 \cdot 7H_2O$; 0.2 ml per 100 ml of culture medium) and tap water (pH 7.0) were inoculated with *Kloeckera saturnus* and cultured at 30 °C for 2 d with continuous shaking. Then 1 ml of the above seed culture was transferred to 800 ml of the same medium as described above. After 3 d of cultivation, *ca.* 800 mg of the substrate **5** was added to the 800 ml of seed culture then the cultivation was continued for a further 3 d under the same conditions. Five asymmetric reductions of **5** on this scale (finally 4.02 g of **5**) were carried out.

ii) The reaction mixture was filtered with the aid of celite and the filtrate was extracted with ether. The ether extract was washed with sat. NaCl aq., then dried over $MgSO_4$. Removal of the solvent gave an oily product, which was chromatographed on silica gel (150 g) with *n*-hexane–ethyl acetate (9 : 1) to provide three fractions. The first fraction (2.310 g, 57% recovery) was confirmed by direct comparison to be the starting material **5**. The second fraction A (682 mg, 17% yield) and the third fraction B (455 mg, 11% yield) were homogeneous oils. A: *Anal.* High-resolution MS. Calcd for $C_9H_{12}O_4$ ($M^+$; *m/e*): 184.073. Found: 184.074. $[\alpha]_D^{18}$ $-10.73°$ ($c=4.8$, $CHCl_3$). IR $\nu_{max}$ $cm^{-1}$: 1718, 1735 (sh), 3530. 400 MHz NMR $\delta$: 1.211 (3H, d, $J=7.2$ Hz, 2-Me), 2.980 (1H, qq, $J=7.2$ Hz, $J_{2,3}=4.1$ Hz, 2-H), 3.706 (3H, s, COOMe), 5.026 (1H, t, $J_{2,3}=4.1$ Hz, 3-H), 6.282 (1H, dd, $J_{3',5'}=0.8$ Hz, $J_{3',4'}=3.4$ Hz, 3'-H), 6.334 (1H, dd, $J_{4',5'}=1.8$ Hz, $J_{3',4'}=3.4$ Hz, 4'-H), 7.360 (1H, dd, $J_{4',5'}=1.8$ Hz, $J_{3',5'}=0.8$ Hz, 5'-H). B: *Anal.* High-resolution MS. Calcd for $C_9H_{12}O_4$ ($M^+$; *m/e*): 184.073. Found: 184.072. $[\alpha]_D^{18}$ $-27.29°$ ($c=5.5$, $CHCl_3$). IR $\nu_{max}$ $cm^{-1}$: 1720, 1740 (sh), 3500, 3600 (sh). 400 MHz NMR $\delta$: 1.092 (3H, d, $J=7.2$ Hz, 2-Me), 3.029 (1H, qq, $J=7.2$ Hz, $J_{2,3}=7.4$ Hz, 2-H), 3.160 (1H, br s, 3-OH), 3.732 (3H, s, COOMe), 4.784 (1H, dd, $J_{3,OH}=4.6$ Hz, $J_{2,3}=7.4$ Hz, 3-H), 6.294 (1H, dd, $J_{3',4'}=3.2$ Hz, $J_{3',5'}=0.8$ Hz, 3'-H), 6.335 (1H, dd, $J_{4',5'}=1.7$ Hz, $J_{3',4'}=3.2$ Hz, 4'-H), 7.384 (1H, dd, $J_{4',5'}=1.7$ Hz, $J_{3',5'}=0.8$ Hz, 5'-H).

**Preparation of (2*S*, 3*S*)-(+)-MTPA Ester (C) and Conversion of C into E by Ozonolysis**——i) Pyridine (0.5 ml) was added to a mixture of A (45 mg) and (+)-MTPACl (93 mg), and the reaction mixture was stirred for 48 h at room temperature. After the addition of $H_2O$, the reaction mixture was extracted with ether. The ether extract was washed with sat. NaCl aq., then dried over $MgSO_4$. Removal of the solvent gave an oily product, which was subjected to preparative thin layer chromatography [prep. TLC; silica gel (20 cm × 20 cm); solvent, *n*-hexane–ethyl acetate (3 : 1)] to provide the (2*S*, 3*S*)-(+)-MTPA ester (C; 86 mg, 88% yield) as a homogeneous oil. *Anal.* High-resolution MS. Calcd for $C_{19}H_{19}F_3O_6$ ($M^+$; *m/e*): 400.113. Found: 400.112. 400 MHz NMR $\delta$: 1.299 (3H, d, $J=7.1$ Hz, 2-Me), 3.505 (3H, d, $J=1.1$ Hz, OMe), 3.640 (3H, s, COOMe), 6.350 (1H, d, $J_{2,3}=6.4$ Hz, 3-H). The optical purity of C and thence A was found to be 83% e.e.

ii) Ozone was passed through a solution of C (58 mg) in $CH_2Cl_2$ (4 ml) under dry ice-acetone cooling for 20 min, then 30% $H_2O_2$ aq. (4 ml) was added to the ozonolyzed product and the reaction mixture was stirred for 20 min at room temperature. The reaction mixture was then extracted with ether after addition of $H_2O$, and the ether extract was washed with sat. NaCl aq., then dried over $MgSO_4$. Removal of the solvent gave a residue, which was treated with $CH_2N_2$ in ether solution to provide an oily product. It was subjected to preparative silica gel TLC [solvent; *n*-hexane–ethyl acetate (3 : 1)] to afford the dimethyl ester E (8.2 mg). The 400 MHz NMR spectrum of E was identical with that of authentic (2*S*, 3*S*)-(+)-MTPA ester **2c**.[4)]

**Preparation of (2*R*, 3*S*)-(+)-MTPA Ester (D) and Conversion of D into F by Ozonolysis**——i) Pyridine (0.5 ml) was added to a mixture of B (46 mg) and (+)-MTPACl (95 mg), and the reaction mixture was stirred for 48 h at room temperature. After the addition of $H_2O$, the reaction mixture was worked up and purified in the same way as described for C to provide the (2*R*, 3*S*)-(+)-MTPA ester (D; 91 mg, 91% yield) as a homogeneous oil. *Anal.* High-resolution MS. Calcd for $C_{19}H_{19}F_3O_6$ ($M^+$; *m/e*): 400.113. Found: 400.114. 400 MHz NMR $\delta$: 1.045 (3H, d, $J=7.1$ Hz, 2-Me), 3.442 (3H, d, $J=1.1$ Hz, OMe), 3.686 (3H, s, COOMe), 6.158 (1H, d, $J_{2,3}=10.7$ Hz, 3-H). The optical purity of D and thence B was found to be 59% e.e.

ii) Ozone was passed through a solution of D (57 mg) in $CH_2Cl_2$ (4 ml) under dry ice-acetone cooling for 20 min, then 30% $H_2O_2$ aq. (4 ml) was added to the ozonolyzed product and the reaction mixture was stirred for 20 min at room temperature. The reaction mixture was worked up, esterified with $CH_2N_2$ in ether solution and purified in the same way as described for E to afford the dimethyl ester F (11 mg). The 400 MHz NMR spectrum of F was identical with that of authentic (2*R*, 3*S*)-(+)-MTPA ester **2d**.[4)]

**Preparation of Four Alcohols (6a, 6b, 6c and 6d) and Their (+)-MTPA Esters (7a, 7b, 7c, and 7d)**——i) A mixture of **5** (505 mg) and $NaBH_4$ (100 mg) in EtOH (2 ml) was stirred for 70 min at 0 °C, and the reaction mixture was extracted with ether after the addition of $H_2O$. The ether extract was washed with sat. NaCl aq., dried over $MgSO_4$ and concentrated to give a mixture of four alcohols (**6a**+**6b**+**6c**+**6d**; 460 mg, 90% yield). **6a**+**6c**; NMR $\delta$: 3.704 (3H, s, COOMe), **6b**+**6d**; NMR $\delta$: 3.735 (3H, s, COOMe). The ratio of *syn* (**6a**+**6c**)/*anti* (**6b**+**6d**) was found to be 1.24 : 1 by NMR analysis.

ii) Pyridine (0.5 ml) was added to a mixture of the four alcohols (52 mg) and (+)-MTPACl (107 mg), and the reaction mixture was stirred for 42 h at room temperature. After the addition of $H_2O$, the reaction mixture was extracted with ether. The ether extract was washed with sat. NaCl aq., dried over $MgSO_4$ and concentrated to afford an oily product, which was chromatographed on silica gel (30 g) to provide the four (+)-MTPA esters (**7a**+**7b**+**7c**+**7d**; 98 mg, 87% yield) as a homogeneous oil from the *n*-hexane–ethyl acetate (19 : 1) fraction. **7a**+**7c**: 400 MHz NMR $\delta$: 1.188, 1.299 (each 3H, d, $J$=7.1 Hz, 2-Me), 3.453, 3.505 (each 3H, d, $J$=1.1 Hz, OMe), 3.596, 3.640 (each 3H, s, COOMe). **7b**+**7d**: 400 MHz NMR $\delta$: 1.027, 1.045 (each 3H, d, $J$=7.1 Hz, 2-Me), 3.397, 3.442 (each 3H, d, $J$=1.1 Hz, OMe), 3.513, 3.686 (each 3H, s, COOMe).

**Preparation of Racemic *syn*-Alcohols (6a+6c) and Their (+)-MTPA Esters (7a+7c)**——i) A solution of $Zn(BH_4)_2$ in dry ether (5 ml) [prepared from a 0.69 M soln of $ZnCl_2$ in ether (80 ml) and $NaBH_4$ (4 g) in ether (300 ml)] was added to a solution of **5** (138 mg) in dry ether (5 ml) under an argon atmosphere at 0 °C, and the reaction mixture was stirred for 1 h. After the addition of 10% HCl aq., the whole was extracted with ether. The ether extract was washed with sat. $NaHCO_3$ aq. and sat. NaCl aq., then dried over $MgSO_4$. Removal of the solvent gave 132 mg of a homogeneous oil. *syn* (**6a**+**6c**): 400 MHz NMR $\delta$: 3.706 (3H, s, COOMe), *anti* (**6b**+**6d**): 400 MHz NMR $\delta$: 3.732 (3H, s, COOMe). The ratio of *syn*/*anti* was found to be 17.7 : 1 by NMR analysis.

ii) Pyridine (0.5 ml) was added to a mixture of the above $Zn(BH_4)_2$ reduction products (32 mg) and (+)-MTPACl (54 mg), and the reaction mixture was stirred for 48 h at room temperature. After the addition of $H_2O$, the reaction mixture was worked up and purified in the same way as described for C to provide the *syn*-(+)-MTPA esters (**7a**+**7c**; 58 mg, 83% yield) as a homogeneous oil. 400 MHz NMR $\delta$: 1.188, 1.299 (each 3H, d, $J$=7.1 Hz, 2-Me), 3.453, 3.505 (each 3H, d, $J$=1.1 Hz, OMe), 3.596, 3.640 (each 3H, s, COOMe).

**Screening Experiment with Various Yeasts**——Microorganisms mentioned in the previous report[6] were used to reduce the starting material **5**. Erlenmeyer flasks (500 ml) containing 100 ml of the same culture medium as in the case of *Kloeckera saturnus* cultivation were inoculated with microorganisms and cultured at 30 °C for 3 d with continuous shaking. Then the substrate (*ca.* 50 mg of compound **5**) was added to the 100 ml of seed culture and the mixture was incubated further for 3 d under the same conditions. The reaction mixtures were separately worked up in the same way as in the case of *Kloeckera saturnus* reduction to give crude reduction products. Pyridine (0.5 ml) was added to a mixture of each reduction product and (+)-MTPACl (*ca.* 80 mg), and the reaction mixture was stirred for 48 h at room temperature. The reaction mixtures were worked up and purified in the same way as in the case of C to afford the (+)-MTPA esters as shown in Table I. The results are summarized in Table I.

**Screening Experiment with Yeast Cells**——Various yeast cells were obtained by centrifugation of each yeast after cultivation on the same scale (100 ml) as described above. Then, a suspension of yeast cells, sucrose (10 g) and substrate **5** (*ca.* 50 mg) in $H_2O$ (50 ml) was shaken at 30 °C for 5 h. The reaction mixture was worked up and purified in the same way as in the case of *Kloeckera saturnus* reduction to give crude reduction products. Pyridine (0.5 ml) was added to a mixture of each reduction product and (+)-MTPACl (*ca.* 60 mg), and the reaction mixture was stirred for 24 h at room temperature. The reaction mixture was worked up and purified in the same way as described for C to provide (+)-MTPA esters as shown in Table I. The data are summarized in Table I.

**Large-Scale Cultivation in a 30 l Jar Fermentor or 200 l Tank**——The procedure for large-scale cultivation in a 30 l jar fermentor or 200 l tank will be reported elsewhere.[9]

i) With *Kloeckera saturnus* (entries 1 and 2): After cultivation, the crude reaction mixture was chromatographed on silica gel (500 g—1.5 kg) to give the results shown in Table II. NMR spectra of reduction products (**6c** and **6d**) were identical with those of the authentic specimens described above. **6c**; $[\alpha]_D^{18}$ −10.73° ($c$=4.8, $CHCl_3$), **6d**; $[\alpha]_D^{23.5}$ −28.89° ($c$=5.4, $CHCl_3$). A part of **6c** (56 mg) and **6d** (54 mg) was separately converted to the corresponding 3,5-dinitrobenzoates, which were recrystallized from MeOH to give colorless needles. **6c**-3,5-dinitrobenzoate, mp 132.5—133 °C. *Anal.* Calcd for $C_{16}H_{14}N_2O_9$: C, 50.80; H, 3.73; N, 7.41. Found: C, 50.80; H, 3.78; N, 7.37. **6d**-3,5-dinitrobenzoate, mp 81—82 °C. *Anal.* Calcd for $C_{16}H_{14}N_2O_9$: C, 50.80; H, 3.73; N, 7.41. Found: C, 50.58; H, 3.80; N, 7.35. The optical purities of **6c** and **6d** were determined by the (+)-MTPA ester method described above and the results are shown in Table II.

ii) With *Saccharomyces delbrueckii* (entry 3): After cultivation, the crude reaction mixture was chromatographed on silica gel (200 g) to afford the reduction products (**6a** and **6b**) along with the starting material (**5**; 3.616 g, 69% recovery) as shown in Table II. The NMR spectra of the reduction products were identical with those of the authentic enantiomers (**6c** and **6d**) described above. **6a**; $[\alpha]_D^{23}$ +9.20° ($c$=5.47, $CHCl_3$), **6b**; $[\alpha]_D^{23}$ +36.79° ($c$=5.8, $CHCl_3$). The optical purities of **6a** and **6b** were determined by the (+)-MTPA ester method described above and the results are shown in Table II.

iii) With *Saccharomyces fermentati* (entries 4 and 5): After cultivation, the crude reaction mixture was chromatographed on silica gel (300 g—1.5 kg) to give the results shown in Table II. The NMR spectra of the reduction products (**6a** and **6b**) were identical with those of the authentic enantiomers (**6c** and **6d**) described above. **6a**; $[\alpha]_D^{24.5}$ +12.7° ($c$=5.0, $CHCl_3$), **6b**; $[\alpha]_D^{24.5}$ +38.76° ($c$=5.0, $CHCl_3$). A part of **6a** (55 mg) and **6b** (54 mg) was converted to the corresponding 3,5-dinitrobenzoates, which were recrystallized from MeOH to give colorless needles. **6a**-3,5-dinitrobenzoate, mp 132.5—133.5 °C. *Anal.* Calcd for $C_{16}H_{14}N_2O_9$: C, 50.80; H, 3.73; N, 7.41. Found: C, 50.67; H, 3.81; N, 7.31. **6b**-3,5-dinitrobenzoate, mp 82.5—83 °C. *Anal.* $C_{16}H_{14}N_2O_9$: C, 50.80; H, 3.73; N, 7.41. Found: C, 50.83; H, 3.82; N, 7.33. The optical purities of **6a** and **6b** were determined by the (+)-MTPA ester method described above, and the results are shown in Table II.

iv) With *Candida albicans* (entry 6): After cultivation, the crude reaction mixture was chromatographed on silica gel (200 g) to afford **6b** (671 mg, 14% yield). The NMR spectrum of **6b** was identical with that of the authentic specimen **6b** described above. **6b**; $[\alpha]_D^{23}$ +34.42° ($c$=5.47, $CHCl_3$). The optical purity of **6b** was found to be 96% e.e. by the (+)-MTPA ester method described above.

**Conversion of Reduction Products (6a—6d) into Dimethyl 2-Methylmalates (1a—1d)**——i) A mixture of a reduction product [**6a** (520 mg), **6b** (566 mg), **6c** (510 mg) or **6d** (546 mg)] obtained by a large-scale cultivation, pyridine (2.5 ml) and $p$-dimethylaminopyridine (20 mg) in $Ac_2O$ (2.5 ml) was stirred for 16—45 h at room temperature. Each reaction mixture was extracted with ether after the addition of $H_2O$. Each ether extract was washed with sat. NaCl aq., then dried over $MgSO_4$. Removal of the solvent gave an oily product which was separately chromatographed on silica gel (25 g) to give the corresponding acetate [**8a** (567 mg, 89% yield), **8b** (569 mg, 82% yield), **8c** (489 mg, 78% yield), or **8d** (482 mg, 72% yield)] as a homogeneous oil from the $n$-hexane–ethyl acetate (40 : 1—19 : 1) eluate. **8a**, **8c**: IR $\nu_{max}$ cm$^{-1}$: 1740. NMR $\delta$: 1.264 (3H, d, $J$=7.2 Hz, 2-Me), 2.086 (3H, s, 3-OAc), 3.150 (1H, qq, $J$=7.2, 7.2 Hz, 2-H), 3.624 (3H, s, COOMe), 6.127 (1H, d, $J$=7.2 Hz, 3-H). **8b**, **8d**; IR $\nu_{max}$ cm$^{-1}$: 1745. NMR $\delta$: 1.026 (3H, d, $J$=7.1 Hz, 2-Me), 2.004 (3H, s, 3-OAc), 3.301 (1H, qq, $J$=7.1, 10.2 Hz, 2-H), 3.714 (3H, s, COOMe), 5.984 (1H, d, $J$=10.2 Hz, 3-H).

ii) Ozone was passed through a solution of an acetate [**8a** (567 mg), **8b** (569 mg), **8c** (489 mg), or **8d** (482 mg)] in $CH_2Cl_2$ (10 ml) under dry ice-acetone cooling for 30 min, then 30% $H_2O_2$ aq. (2 ml) was added to the ozonolyzed product and the reaction mixture was stirred for 20 min at room temperature, then extracted with ether after the addition of $H_2O$. The extract was washed with sat. NaCl aq., then dried over $MgSO_4$. Removal of the solvent gave a residue, which was treated with a solution of $CH_2N_2$ in ether to provide an oily product. Each oily compound was chromatographed on silica gel (25 g) to afford the corresponding acetoxy diester [**3a** (327 mg, 60% yield), **3b** (233 mg, 42% yield), **3c** (255 mg, 54% yeild), or **3d** (173 mg, 37% yield)] as a homogeneous oil from the $n$-hexane–ethyl acetate (9 : 1) eluate. **3a**, **3c**: IR $\nu_{max}$ cm$^{-1}$: 1750. NMR $\delta$: 1.236 (3H, d, $J$=7.1 Hz, 2-Me), 2.135 (3H, s, 3-OAc), 3.089 (1H, qq, $J$=7.1, 4.2 Hz, 2-H), 3.716, 3.767 (each 3H, s, COOMe), 5.525 (1H, d, $J$=4.2 Hz, 3-H). **3b**, **3d**; IR $\nu_{max}$ cm$^{-1}$: 1750. NMR $\delta$: 1.233 (3H, d, $J$=7.1 Hz, 2-Me), 2.144 (3H, s, 3-OAc), 3.122 (1H, qq, $J$=7.1, 5.3 Hz, 2-H), 3.718, 3.765 (each 3H, s, COOMe), 5.283 (1H, d, $J$=5.3 Hz, 3-H).

iii) A mixture of an acetoxy diester [**3a** (327 mg), **3b** (233 mg), **3c** (255 mg), or **3d** (173 mg)] and 30% HBr–AcOH (0.2 ml) in MeOH (2 ml) was stirred for 15 min at 0 °C and then stirred for 16—18 h at room temperature. The reaction mixture was extracted with ether after the addition of $H_2O$. The ether extract was washed with sat. NaCl aq., then dried over $MgSO_4$. Removal of the solvent gave an oily product, which was chromatographed on silica gel (25 g) to provide the dimethyl 2-methylmalate [**1a** (160 mg, 61% yield), **1b** (82 mg, 42% yield), **1c** (107 mg, 52% yield), or **1d** (72 mg, 51% yield)] as a homogeneous oil from the $n$-hexane–ethyl acetate (8 : 1) eluate. Each dimethyl 2-methylmalate (**1a**—**1d**) was identical (IR and NMR) with the corresponding authentic specimen (**1a**—**1d**).[2a,4] **1a**: $[\alpha]_D^{26}$ −15.34° ($c$=5, $CHCl_3$), $[\alpha]_D^{26}$ −4.94° ($c$=5, $Et_2O$). **1b**: $[\alpha]_D^{22}$ −7.16° ($c$=4.4, $CHCl_3$). **1c**: $[\alpha]_D^{25}$ +12.64° ($c$=5, $CHCl_3$), $[\alpha]_D^{28}$ +5.20 ($c$=5, $Et_2O$). **1d**: $[\alpha]_D^{28}$ +5.62 ($c$=5, $CHCl_3$).

iv) Pyridine (0.3 ml) was added to a mixture of a dimethyl 2-methylmalate [**1a** (20 mg), **1b** (18 mg), **1c** (20 mg), or **1d** (19 mg)] and (+)-MTPACl (32—35 mg), and the reaction mixture was stirred for 42 h at room temperature, then worked up and purified in the same way as described for C to give the corresponding (+)-MTPA ester [**2a** (40 mg, 89% yield), **2b** (33 mg, 82% yield), **2c** (38 mg, 84% yield), or **2d** (37 mg, 85% yield)] as a homogeneous oil. The optical purities of the (+)-MTPA esters are shown in Table III.

**Acknowledgement** The authors are grateful to Dr. A. Seino of Kaken Pharmaceutical Co., Ltd. for supplying microorganisms and for valuable advice. We also wish to thank the Second laboratory, Kaken Pharmaceutical Co., Ltd. for their assistance in the large-scale cultivation. This work was supported by a grant for "Life Science" to this Institute from the Science and Technology Agency of Japan.

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