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A Synthesis of (–)-Sitophilate by Utilizing Yeast-mediated Reduction of an Enol Ester†

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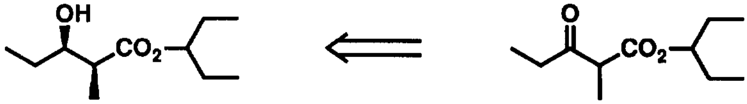
Abstract The microbial reduction of 1'-ethylpropyl 2-methyl-3-oxopentanoate, a β-keto ester possessing bulky substituent, as well as the corresponding enol ester was examined Epimeric mixture of hydroxy ester, containing (2*S*,3*S*)-isomer as the major product (92%*e e* , 43%*d e*) was obtained *via* the reduction of enol ester with growing cells of *Pichia farinosa* IAM 4682 in 63% yield The resulting β-hydroxy ester was converted to (2*S*,3*R*)-isomer (88%*e e* , 96%*d e*), of which *e e* was further enhanced by the lipase-catalyzed partial hydrolysis of the corresponding chloroacetate to give (-)-sitophilate, (99%*e e* , 98%*d e*) an aggregation pheromone of *Sitophilus granarius* L

Introduction

Sitophilate (1'-ethylpropyl 3-hydroxy-2-methylpentanoate, **1a**) is an aggregation pheromone isolated from *Sitophilus granarius* L [1] The absolute configuration was determined[2,3] by the comparison of spectral, chromatographic and biological properties between the natural product and both enantiomers which were chemically synthesized [4,5] Three reports on the syntheses of this pheromone as well as its antipode have been published so far Chong reported the first synthesis by utilizing a selective epoxide opening reaction with an organocopper reagent [4] The synthesis of both enantiomers was also reported by Mori and Ishikura, starting from methyl 3-hydroxypentanoate of microbial origin [5] Miyazawa and Yoshida demonstrated lipase-catalyzed enantioselective esterification as the key-step for the synthesis of **1a** [6] Since sitophilate itself is an α-substituted β-hydroxy ester, it seems to be prepared by microorganism-mediated reduction of α-substituted β-keto ester [7,8] Our recent study on the yeast-mediated reduction of this type of compounds[9] prompted us the examination of such approach toward this target molecule



(2*S*,3*R*)-**1a** Sitophilate

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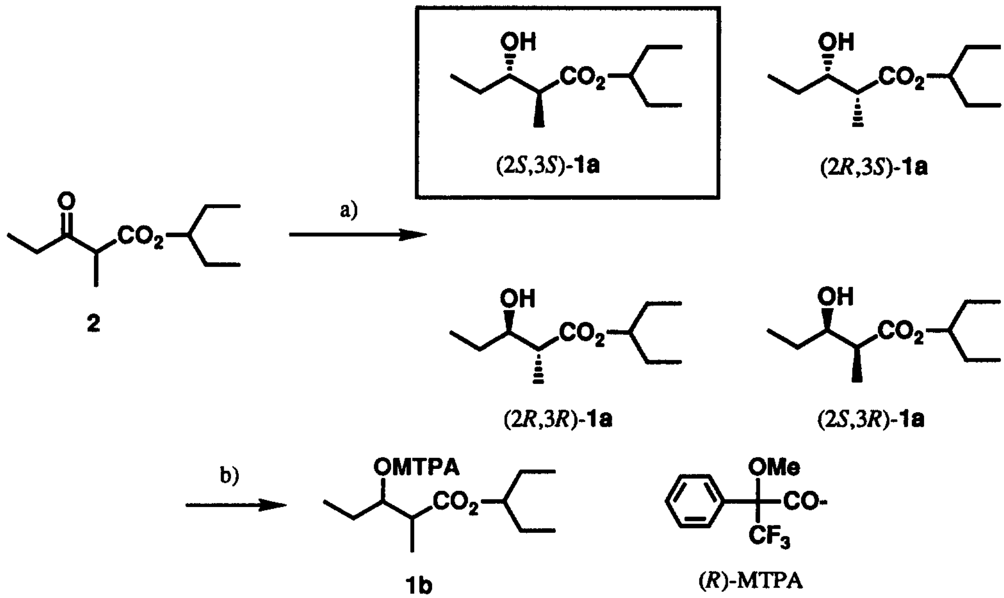
Fig. 1.

† Preparation of Enantiomerically Enriched Compounds by Using Enzymes, Part 14 For Part 13, see ref 11b The experimental part was mainly taken from the B Sc thesis of D S (March, 1990)

Reduction of β-keto ester with the resting cells of Pichia farinosa IAM 4682

At first, bakers' yeast was subjected to the reduction of β-keto ester **2**, however, almost no reduction occurred. We therefore turned our attention to *Pichia farinosa* IAM 4682, a kind of yeast whose unusual aptitude, so-called *anti*-Prelog's rule selectivity[10] for the reduction of simple methyl alkenyl ketones was observed recently.[11] Expecting to provide (3*R*)-**1a**, the cultivated cells of *P. farinosa* were incubated with **2** to result in giving a diastereomeric mixture of **1a** (70%).



a) *Pichia farinosa* IAM 4682 (resting cell), 70%, b) MTPA-Cl/C_5H_5N, quant.

Scheme 1. Reduction of β-keto ester with resting cells of *P. farinosa* IAM 4682

Table 1. Results on the yeast reduction using resting cells

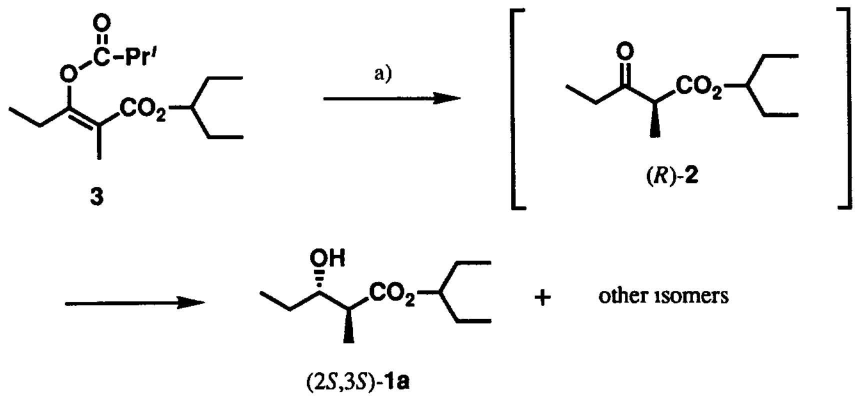
Substrate	Yd of 1a (%)	Isomeric ratio[a)] (%) (2*S*,3*S*)	(2*R*,3*S*)	(2*R*,3*R*)	*e.e.* (%) of major product	*d.e.* (%)
2	70	65.5	15.5	19.0	55	69
3	68	68.9	10.5	20.6	54	79

a) (2*S*,3*R*)-isomer was obtained only in a trace amount.

To our disappointment, the major product was (2*S*,3*S*)-isomer of **1a** [55% enantiomeric excess (*e.e.*), 69% diastereomeric excess (*d.e.*), Scheme 1]. Table 1 summarizes the isomeric ratios which were determined by the 400 MHz ^{1}H NMR analysis[4] of the corresponding (*R*)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) esters.[12] Although the desired (2*S*,3*R*)-**1a** was produced in only a trace amount, we decided to continue our efforts to secure (2*S*,3*S*)-**1a** with higher *e e* and *d e* because the major (2*S*,3*S*)-**1a** had been able to convert to the desired isomer by Mitsunobu inversion [5]

Reduction of enol ester with the resting cells of Pichia farinosa

Expecting the improvement of *e e* by means of "low concentration feeding" of the substrate,[13] the corresponding enol ester **3** was designed[9] as the next candidate for substrate Another reason for this selection was that the microorganism showed enantioface-selective protonation in the case of hydrolysis of α-substituted ketone enol ester [14] Our previous results suggest that hydrolysis should afford (*R*)-**2** which will be subsequently reduced If the rate of reduction is substantially faster than the racemization of this intermediate, the *d e* of the product is expected to be enhanced A smooth conversion of **3** to **1a** (68%) actually took place and the *d e* of the product was higher to some extent than that of the product from the keto ester, as expected Unfortunately, the *e e* was almost as low as in the case of the keto ester (Table 1) GLC analysis immediately after the addition of **3** (15 min) showed that most of **3** was hydrolyzed to **2**, indicating that the rate of hydrolysis is very fast This fact explains the reason why there was not observed much difference concerning *e e* between keto ester and enol ester as the substrate



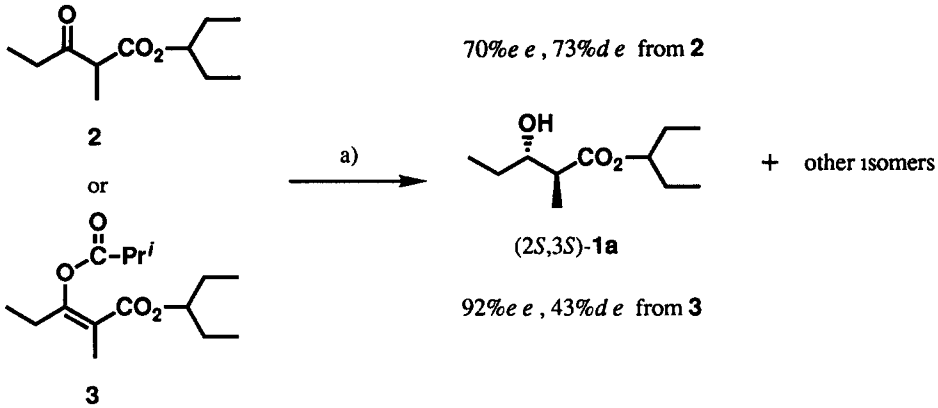
a) *Pichia farinosa* IAM 4682 (resting cell), 68%

Scheme 2. Reduction of enol ester with resting cells of *P. farinosa* IAM 4682

Again, the reaction conditions of affording higher *e e* s were extensively examined Addition of many kinds of additives,[8,11] changes of reaction conditions such as rate of aeration (under anaerobic or aerobic), pH and the kind of buffer solution, other kinds of carbon and energy source (ethanol, glycerol) resulted in no enhancement of the *e e* of (2*S*,3*S*)-**1a**

Reduction with the growing cells of Pichia farinosa

The solution to this problem was obtained by an unexpected observation shown below In the case that freeze-dried cells were incubated with **2**, no reduction was observed during several days Prolonged reaction period over one week, however, brought about a slow production of **1a** (5-10% per day) The newly grown microorganism after 2 weeks was confirmed to be identical with the original strain This result indicates that the microorganism in growing stage can also reduce the substrate **2**, as well as the resting cells which are normally used for biochemical reduction In the experiment using growing cells, the substrate was added 4 h after inoculation The reduction completed for both substrates (**2** and **3**) within 14 h Although the product from **2** was a mixture with moderate *e e* (**1a** 70%*e e* , 73%*d e*), an enhanced enantioselectivity was realized (**1a** 92%*e e* , 43%*d e*) for the reduction of **3** The results are listed in Table 2 A higher activity of the reductive enzyme as well as a lower one of the hydrolytic enzyme brought about a well-balanced successive two enzymatic reactions in growing cells A unique feature of multiple enzyme system of this microorganism was thus revealed



a) *Pichia farinosa* IAM 4682 (growing cell)

Scheme 3. Reduction with growing cells of *P farionosa* IAM 4682

Table 2. Results on the yeast reduction using growing cells

Substrate	Yd of **1a** (%)	Isomeric ratio (%) (2*S*,3*S*)	(2*R*,3*S*)	(2*R*,3*R*)	(2*S*,3*R*)	*e e* (%) of major product	*d e* (%)
2	65	73 6	13 6	12 8	0 0	70	73
3	63	68 7	26 3	2 8	2 2	92	43

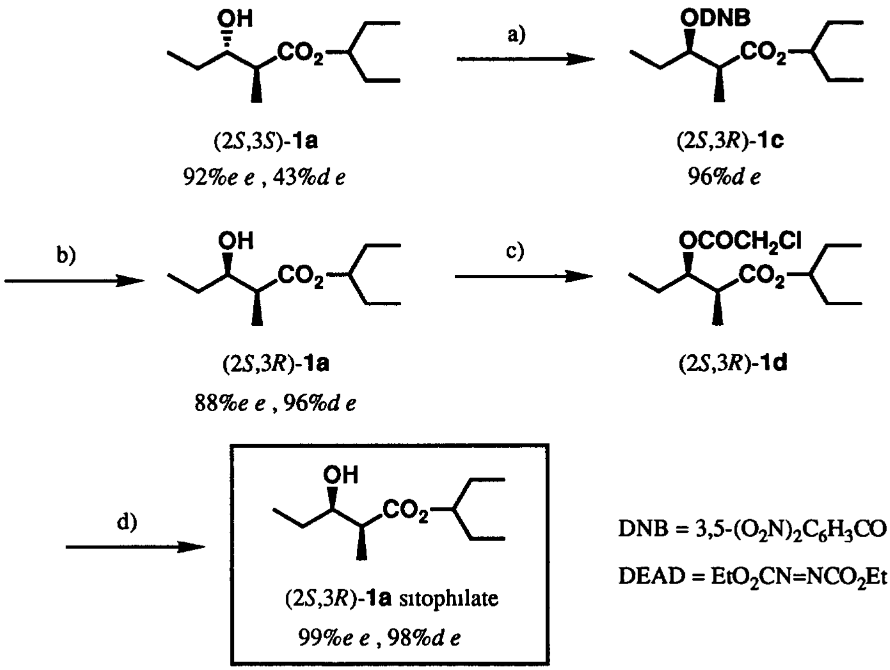
Conversion to natural product

Encouraged by the fact that (2*S*,3*S*)-**1a** with a high *e e* and a moderate *d e* could be obtained, the product was converted to the corresponding 3,5-dinitrobenzoate **1c** according to Mori's procedure [5] A modified Mitsunobu inversion[15] worked well to give (2*S*,3*R*)-**1c** in 49% yield The *d e* of **1c** reached 96%, because of the removal of the undesired isomer through the decomposition of the intermediate, as well as the recrystallization of the crude product The dinitrobenzoate **1c** was hydrolyzed to (2*S*,3*R*)-**1a** in 91%, $[\alpha]_D^{22}$ -3 71° (chloroform), whose *e e* was determined to be 88% by the ^{1}H NMR analysis of the corresponding MTPA ester **1b**

Final enantiomeric purification of **1a** was successfully accomplished by an enzyme-catalyzed reaction The corresponding chloroacetate **1d** (93%) was partially hydrolyzed with lipase PS (Amano, from

Pseudomonas) to afford **1a** (93% conversion, 83%yield), $[\alpha]_D^{26}$ -4 06° (chloroform) Thus the natural isomer with both high *e e* and *d e* (99%*e e* , 98%*d e*) was obtained

In conclusion, (-)-sitophilate **1a** was synthesized by a yeast-mediated reduction of corresponding enol ester as the key-step



a) DNBOH, Ph_3P, DEAD/THF, 49%, b) K_2CO_3/MeOH-THF, 91%,
c) $(ClCH_2CO)_2O/C_5H_5N$, DMAP 93%, d) *Pseudomonas* lipase, 83%

Scheme 4. Synthesis of natural sitophilate

EXPERIMENTAL

All b ps and m ps were uncorrected IR spectra were measured as films for oils and KBr discs for solids on a Jasco IRA-202 spectrometer ^{1}H NMR spectra were measured in $CDCl_3$ with TMS as the internal standard at 400 MHz on a JEOL JNM GX-400 spectrometer Optical rotations were recorded on a Jasco DIP 360 polarimeter Hitachi 163 and Shimadzu GC-9A gas chromatograph were used for GLC analysis Freshly distilled tetrahydrofuran (THF) from sodium-benzophenone ketyl was employed for anhydrous reaction Silica gel 60 K070-WH (70-230 mesh) of Katayama Chemical Co was used for column chromatography

1'-Ethylpropyl 2-methyl-3-oxopentanoate **2** A diastereomeric mixture of **1a**[1] (16 40 g, 86 3 mmol) was oxidized with neutral chromic acid[16] to give **2** (13 2 g, 81%), b p 89°C/1 5 Torr, IR νmax 2960, 2930, 2875, 1750, 1720, 1460, 1380, 1260, 1200, 1105, 920, 880 cm^{-1}, ^{1}H NMR δ 0 87 (3H, t, *J*=7 5Hz), 0 88 (3H, t, *J*=7 5Hz), 1 08 (3H, t, *J*=7 3 Hz), 1 34 (3H, d, *J*=6 8 Hz), 1 51-1 75 (4H, m), 2 51 (1H, dq, *J*=7 3, 18 1 Hz), 2 63 (1H, dq, *J*=7 3, 18 1 Hz), 3 53 (1H, q, *J*=7 3 Hz), 4 79 (1H, tt, *J*=5 4, 6 8 Hz), GLC (column, 15% BDS, 2 m, 80°C + 5°C/min, N_2, 0 8 kg/cm^2) rt 7 5 min (Found C, 65 73, H, 9 74 Calc for $C_{11}H_{20}O_3$ C, 65 97, H, 10 07%)

1'-Ethylpropyl (Z)-2-methyl-3-(2-methylpropionyloxy)pent-2-enoate **3** To a suspension of NaH (0 8 g, 60% in mineral oil) in Et_2O (15 ml), a soln of **2** (1 99 g, 10 5 mmol) in Et_2O (10 ml) was added dropwise at 0°C under Ar After stirring for a while at room temp, isobutyric anhydride (3 31 g, 21 0 mmol) was added dropwise at 0°C After stirring overnight, the mixture was poured into sat NH_4Cl aq and extracted with Et_2O A small portion of aq pyridine was added to the extract, and the mixture was stirred for 30 min at room temp to decompose excess amout of anhydride Then the mixture was washed with 1N HCl, water, sat $NaHCO_3$ aq and brine, dried (Na_2SO_4) and concentrated *in vacuo* The residue was purified by SiO_2 flash column chromatography (100 g) Elution with hexane/EtOAc (10/1) followed by bulb-to-bulb distillation afforded **3** (2 26 g, 85%) b p 115°C/2 5 Torr; IR vmax 2980, 2950, 2880, 1740, 1720, 1460, 1380, 1210, 1100, 925, 885 cm^{-1}, ^{1}H NMR δ 0 88 (6H, t, J=7 4 Hz), 1 07 (3H, t, J=7 5 Hz), 1 24 (6H, d, J=6 8 Hz), 1 50-1 62 (4H, m), 1 92 (3H, s), 2 36 (2H, q, J=7 5 Hz), 2 63-2 76 (1H, septet, J=6 8 Hz), 4 78 (1H, quint, J=6 2 Hz) By the comparison of its ^{1}H NMR spectrum with a related compound reported previously,[9] (Z)-geometry was deduced GLC (same condition for **2**) rt 13 7 min (Found C, 66 94, H, 9 47 Calc for $C_{15}H_{26}O_4$ C, 66 64, H, 9 69%)

Authentic sample of 1'-ethylpropyl 3-hydroxy-2-methylpentanoate **1a** B p 100-120°C/1 6 Torr, IR vmax 3500, 2970, 2940, 2890, 1730, 1710, 1460, 1380, 1250, 1180, 1100, 970, 915 cm^{-1}, ^{1}H NMR δ (C_6D_6) 0 76 (1 5H, t, J=7 3 Hz), 0 77 (1 5H, t, J=7 3 Hz), 0 77 (1 5H, t, J=7 3 Hz), 0 78 (1 5H, t, J=7 3 Hz), 0 89 (1 5H, t, J=7 3 Hz), 0 94 (1 5H, t, J=7 3 Hz), 1 10 (1 5H, d, J=7 3 Hz), 1 16 (1 5H, d, J=7 3 Hz), 1 20-1 55 (6H, m), 2 53 (0 5H, d, J=6 3 Hz), 2 36-2 45 (1H, m), 2 56 (0 5H, d, J=6 3 Hz), 3 47-3 54 (0 5H, m), 3 78 (0 5H, ddd, J=6 3, 6 3, 12 6 Hz), 4 80-4 87 (1H, m) GLC (same condition for **2**) rt 8 7 min [(2R*,3R*)-**1a**], 9 5 min [(2R*,3S*)-**1a**], Capillary GLC (column, PEG-20M, 50 m, 170°C/min, N_2, 2 3 kg/cm^2) rt 6 6 min [(2R*,3R*)-**1a**], 7 1 min [(2R*,3S*)-**1a**] A small portion was converted to (R)-MTPA ester **1b**[4] ^{1}H NMR of C_2-C$\underline{H}_3$ δ 1 07 [0 75H, d, J=6 8 Hz, (2S,3S)], 1 11 [0 75H, d, J=6 8 Hz, (2R,3S)], 1 17 [0 75H, d, J=6 8 Hz, (2R,3R)], 1 18 [0 75H, d, J=6 8 Hz, (2S,3R)]

Reduction by the resting cells of Pichia farinosa IAM 4682 reduction of 2 *Pichia farinosa* IAM 4682[††] was cultivated according to the reported procedure [11] The washed wet cells (250 g) was suspended in buffer solution (0 1M, 840 ml) To this was added glucose (84 g) and the mixture was shaken for 30 min (150 cpm) at 30°C Then an emulsion of **2** (500 mg, 2 50 mmol) in Triton X-100 soln (0 2%, 10 ml, sonicated for 5 min) was added and the mixture was further shaken for 1 day at 30°C The mixture was centrifuged (3000 rpm) and the supernatant was extracted with Et_2O after saturating with NaCl The precipitated cells were sonicated in acetone and filtered The filtrate was concentrated *in vacuo* and the residue was extracted with Et_2O Solid material on the filter was further extracted with Et_2O by applying sonication The organic extracts were combined and washed with water and brine, dried (Na_2SO_4) and the solvent was evaporated at at nospheric pressure through a Vigreux column The residue was purified by SiO_2 flash column chromatography (40 g) Elution with hexane/EtOAc (10/1) followed by bulb-to-bulb distillation afforded **1a** (354 mg, 70%) Its IR spectrum was identical with that of an authentic **1a** Capillary GLC (same condition for authentic **1a**) rt 6 6 min [(2R,3R)- + (2S,3S)-**1a**, 84 5%], 7 1 min [(2R,3S)-**1a**, 15 5%] A small portion was converted to (R)-MTPA ester **1b** ^{1}H NMR of C_2-C$\underline{H}_3$ δ 1 07 [1 97H, d, J=6 8 Hz, (2S,3S)], 1 11 [0 57H, d, J=6 8 Hz, (2R,3S)], 1 17 [0 46H, d, J=6 8 Hz, (2R,3R)], the diastereomeric ratio was calculated by the comparison of area of these signals and the result was listed in Table 1

Reduction of 3 In the same manner as described above, enol ester **3** (680 mg, 2 50 mmol) was reduced to give **1a** (344 mg, 68%) The diastereomeric ratio was determined by the NMR analysis of **1b** (see Table 1)

[††] In our current works, the name of *Pichia miso* IAM 4682 has been used for this yeast Recently the Institute of Applied Microbiology, University of Tokyo, changed the name of this strain to *Pichia farinosa* IAM 4682

Reduction by the growing cells of Pichia farinosa IAM 4682 reduction of 2 The ingredients of the medium are as follows glucose (1%), yeast extract (0 1%), peptone (0 7%), KH_2PO_4 (0 25%), K_2HPO_4 (0 25%), at pH 6 5 A loopful of *P farinosa* was inoculated to the sterilized medium (100 ml) in 500 ml-shaking flask and the flask was shaken (120 cpm) for 2 days at 30°C The first seed culture (5 ml) thus obtained was inoculated to the same medium and shaken for 12 h at 30°C The second seed culture (each 52 5 ml) and antifoam (Nacalai Tesque, Antifoam-AF emulsion, 10% in water, each 2 ml) was then added to two batches of the medium (1000 ml) in a 5000 ml-Erlenmeyer cultivating flask with two internal projections The flasks were shaken on a gyrorotator (180 rpm) After 4 h the substrate **2** (each 250 mg, total 500 mg, 2 50 mmol) was added and the cultivation was continued for further 14 h The workup was caried out in the same manner as described for the reduction with resting cells **1a** 328 mg (65%)

Reduction of 3 In the same manner as described above, enol ester **3** (680 mg, 2 50 mmol) was reduced to give **1a** (318 mg, 63%) The diastereomeric ratio of the products was determined by the NMR analysis of **1b** (see Table 2)

(2S,3R)-1'-ethylpropyl 3-(3,5-dinitro)benzoyloxy-2-methylpentanoate **1c** According to the reported procedure,[5] **1a** (566 mg, 2 75 mmol, obtained by the reduction of **3** with growing cells, 92%*e e*, 43%*d e*) was converted to 3,5-dinitrobenzoate **1c** (539 mg, 49%), m p 42 5-43 5°C (lit [5] m p 33-33 5°C), $[\alpha]_D^{20}$ -5 94° (c=1 06, $CHCl_3$) [lit [5] $[\alpha]_D^{23}$ -6 52° (c=0 97, $CHCl_3$)] Its IR and NMR spectra were in good accord with those reported previously [5] The *d e* of the present sample was estimated to be 96%, by the existence of a small C_2-$C\underline{H}_3$ δ 1 27 [d, *J*=7 3 Hz, (for 2*S*,3*S*)] signal in its NMR spectrum (Found C, 54 57, H, 5 93, N, 7 16 Calc for $C_{18}H_{24}N_2O_8$ C, 54 54, H, 6 10, N 7 07%)

(2S,3R)-(-)-1'-ethylpropyl 3-hydroxy-2-methylpentanoate **1a** According to the reported procedure,[5] **1c** (539 mg, 1 39 mmol) was converted to **1a** (251 mg, 91%) $[\alpha]_D^{22}$ -3 71° (c=1 05, $CHCl_3$) [lit.[5] $[\alpha]_D^{24}$ -3 9° (c=1 74, $CHCl_3$)] Its IR and NMR spectra were in good accord with those reported previously [5] In addition, a small C_2-$C\underline{H}_3$ δ 1 11 [d, *J*=7 3 Hz, (for 2*S*,3*S*)] signal was observed in its NMR spectrum Capillary GLC (same condition for authentic **1a**) rt 6 6 min (1 9%), 7 1 min (98 1%), its *d e* was confirmed to be 96% The *e e* was estimated to be 88% by the NMR analysis of **1b**

(2S,3R)-1'-ethylpropyl 3-chloroacetoxy-2-methylpentanoate **1d** To a soln of **1a** (220 mg, 1 09 mmol) in pyridine (2 2 ml) was added chloroacetic anhydride (380 mg, 2 eq) and 4-(*N,N*-dimethylamino)pyridine (10 mg) with ice-cooling The mixture was stirred for 1 h at room temp Then ice-water was added and the resulting mixture was further stirred for 30 min The mixture was extracted three times with Et_2O The extract was washed with water, 1N HCl, water, sat $NaHCO_3$ aq and brine, dried (Na_2SO_4) and concentrated *in vacuo* The residue was purified by SiO_2 flash column chromatography (15 g) Elution with hexane/Et_2O (19/1-9/1) afforded **1d** (282 mg, 93%), IR vmax 1760, 1730, 1460, 1380, 1285, 1255, 1180, 1090, 970 cm^{-1} GLC (same condition for **2**) rt 17 2 min (single peak) This sample was employed for next step without further purification

(2S,3R)-(-)-1'-ethylpropyl 3-hydroxy-2-methylpentanoate (sitophilate) **1a** To a mixture of **1a** (282 mg, 1 01 mmol) in phosphate buffer (0 1M, 28 ml, pH 7) was added lipase PS (140 mg) and the resulting mixture was stirred for 16 h at 30°C A small portion was extracted with Et_2O and analyzed by GLC to indicate 92 7% conversion of the reaction Then the mixture was extracted with Et_2O The extract was washed with brine, dried (Na_2SO_4) and concentrated *in vacuo* The residue was purified by SiO_2 flash column chromatography (20 g) Elution with pentane/Et_2O (4/1-3/1) afforded **1a** (170 mg, 83%), b p 90-95°C/3 Torr (bulb-to-bulb distillation), $[\alpha]_D^{26}$ -4 06° (c=0 91, $CHCl_3$) [lit [5] $[\alpha]_D^{24}$ -3 9° (c=1 74, $CHCl_3$)], IR vmax 3480, 2970, 2950, 1730, 1455, 1250, 1190, 1100, 1030, 980, 920 cm^{-1}, ^1H NMR (C_6D_6) δ 0 76 (3H, t, *J*=7 5 Hz), 0 77 (3H, t, *J*=7 5 Hz), 0 89 (3H, t, *J*=7 5 Hz), 1 16 (3H, d, *J*=7 3 Hz), 1 24-1 50 (6H, m), 2 30 (1H, d, *J*=4 4 Hz), 2 39 (1H, dq, *J*=4 4, 7 4 Hz), 3 77 (1H, ddt, *J*=4 4, 8 8, 4 4 Hz), 4 83 (1H, tt, *J*=5 4, 7 4 Hz) Its IR and NMR spectra were in good accord with those reported previously [5] Capillary GLC (same condition for authentic **1a**) rt 6 6 min (0 9%), 7 1 min (99 1%), its *d e* was 98% (Found C, 65 29, H, 10 30 Calc for $C_{11}H_{22}O_3$ C, 65 31, H, 10 96%) The *e e* was estimated to be 99% by the NMR analysis of **1b**

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