41. Production of (+)-(*S*)-Ethyl 3-Hydroxybutyrate and (−)-(*R*)-Ethyl 3-Hydroxybutyrate by Microbial Reduction of Ethyl Acetoacetate

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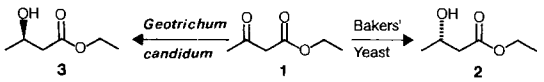
Summary

The production of (*S*)-ethyl 3-hydroxybutyrate (**2**) from ethyl acetoacetate (**1**) in higher yields and with higher optical purity than with the usual procedures was achieved by continuous addition of the substrate **1** and sucrose to an aerated suspension of bakers' yeast. The microbial reduction of **1** by the fungus *Geotrichum candidum* LINK yields – for the first time – the antipode **3**.

In the last years a growing interest in chiral building blocks arose in synthetic organic chemistry. Besides chiral natural products such as D-glucose, L-amino acids, tartaric acid, lactic acid, *etc.* also synthetic compounds prepared by microbial or enzymatic transformation of prochiral precursors became available. (*S*)- and (*R*)-Ethyl 3-hydroxybutyrate (**2** and **3**) proved to be versatile intermediates in the synthesis of various natural compounds [1–3].

Procedures for the reduction of ethyl acetoacetate **1** with bakers' yeast to (*S*)-ethyl 3-hydroxybutyrate (**2**) were reported by *Deol et al.* [4] and also by *Seuring & Seebach* [3] as well as by *Seebach & Zueger* [5]. However, these laboratory procedures though easy and simple for small scale work yield **2** of moderate optical purity, usually in the range of 85–90% (e.e.)[1]). The addition of 10–15% sucrose to the yeast suspension without aeration forces the yeast to uneconomical glucose metabolism. Furthermore, the addition of **1** in doses of 10 ml/l is in our experiments detrimental to the enantioselectivity of the reduction (see the *Table*).



Major improvements in the production of **2** from **1** with bakers' yeast were achieved by using a fermentor equipped with a flat-blade stirrer, temperature control and pH-control and two pumps suitable for the continuous addition of

[1]) Recently, *Meyers & Amos* [2] reported an optical purity in excess of 95%, but no experimental details were given.

Table. *Optical purity of (S)-ethyl 3-hydroxybutyrate* (**2**) *with respect to the ethyl acetoacetate* (**1**) *concentration*

Substrate concentration	(**1**)	g/l	0–1	5	10	15	20
Optical purity (e.e.)	(**2**)	%	95–97	72	70	70	58

The data of the first column (0–1 g/l) were drawn from the 50-l-batch experiment described in the *Figure* at 70 h. Total substrate fed at this time was 36 g/l. The data of the other concentrations were obtained from shake-flask experiments (100 ml). The reduction was complete after 24 h for 5 and 10 g/l substrate concentration, 90 and 70%, respectively, of the substrate were reduced at 15 and 20 g/l. Reduction did not proceed further until 72 h. The optical purity remained constant (±2%) during the whole experiment, the data shown in the *Table* were obtained after 72 h.

sucrose and ethyl acetoacetate solutions, independently[2]). The course of the production of **2** in a 50-l-batch fermentation is outlined in the *Figure.*

In a typical experiment 2.25 kg of **1** yielded 1.29 kg of **2** [56%; chem. purity: 99%; $[\alpha]_D^{20} = +41.3°$ ($c = 1$, $CHCl_3$)]. The observed optical purity was 95–97% (e.e.)[3]) and compares favourably to the 85–90% usually obtained by the procedure published before [3–6]. By continuous feeding of the ketoester **1** the actual concentration in the fermentor could be kept below 0.5 g/l until the end of the reaction, where a rise in the concentration indicates a lower reduction rate attributable to the beginning of cell death. In order to produce 1 kg of **2**, a total amount of 3.9 kg of yeast, 1.8 kg of **1** and 3.5 kg of sucrose is required. The process is not yet thoroughly optimized.

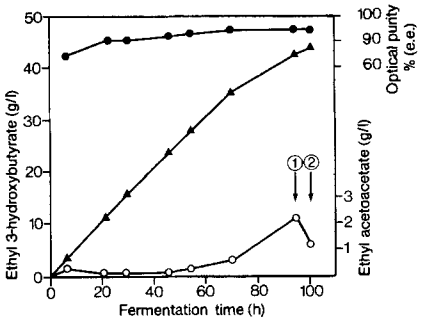


Figure. *Course of the reduction of ethyl acetoacetate with bakers' yeast.* The reaction was carried out in a 50-l-fermentor (*Giovanola Frères SA,* Monthey) as described in the experimental section. Feeding of sucrose (30% in dist. water) and of ethyl acetoacetate (25% in ethanol) were 100 ml/h each. The optical purity of the product was determined by esterification with (*S*)-1-phenylethyl isocyanate and GC. analysis. The arrows indicate the stop of the ethyl acetoacetate feeding ① and the stop of the fermentation and start of extraction ②. (▲—▲) (*S*)-Ethyl 3-hydroxybutyrate; ○—○ ethyl acetoacetate; ●—● Optical purity).

[2]) As the transformation reaction is carried out under non-sterile conditions using commercially available bakers' yeast, any conventional stirred tank reactor equipped with an aeration system could also be used.

[3]) No (*R*)-enantiomer could be detected by NMR. with Eu(hfc) as a shift reagent (1.5% detection limit); 1.5–2.5% of (*R*)-enantiomer have been observed with GC. analysis after derivatization with (−)-camphanic acid chloride or (−)-(*S*)-1-phenylethyl isocyanate.

Reduction of **1** with the fungus *Geotrichum candidum* leads to (*R*)-ethyl 3-hydroxybutyrate (**3**). To our knowledge, this is the first report of the reduction of **1** to the (*R*)-hydroxyester **3** with living cells. In 1977, *Hochuli et al.* [7] reported the enzymatic reduction of **1** to **3** with pure dihydroxyacetone reductase from *Mucor javanicus*. So far however, this process, needing regeneration of costly coenzymes, is not feasible for the production of larger quantities of **3**. The reduction with whole mycelium of two different strains of *Mucor javanicus* leads to **2** with low to moderate optical purity (4–82% **2**).

The reduction with *Geotrichum candidum* depends sensitively on the metabolic state of the cells. Batches of high optical yield were poorly reproducible so far. In addition, the overall yield (36% *vs.* 56%) and the total substrate input (13 g/l *vs.* 45 g/l) were considerably lower than in the yeast process. As the (*R*)-enantiomer **3** is available by depolymerization of polyhydroxybutyrate [5], this method may be preferred to the microbial reduction.

Experimental Part

Analytical procedures. Samples (1 to 5 ml) were withdrawn from the fermentors and extracted 2× with 50 ml portions of CH_2Cl_2. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The residual was dissolved in CH_2Cl_2 to give approx. a 1% solution and the ratio of substrate to product was determined by GC. (2 m × 2 mm, 5% *Carbowax 20 M* on 100/120 supelcoport, 80–120°, 4°/min). For the determination of the enantiomeric purity approx. 10 mg of the dried extract were dissolved in 1 ml of toluene and 50 µl of (−)-(*S*)-1-phenylethyl isocyanate were added (alternatively, the extracts were dissolved in 1 ml of pyridine and 50 mg of (−)-camphanic acid chloride were added). After standing at r.t. overnight, the samples were analyzed by GC. (20 m capillary column *SE-54*, 50–330°, 5°/min).

Preparation of (S)-ethyl 3-hydroxybutyrate (**2**). In a 50-l-fermentor (*Giovanola Frères SA*, Monthey, Switzerland) 5 kg of compressed bakers' yeast (*Klipfel AG*, Rheinfelden, Switzerland) and 1 kg of sucrose (commercial grade) were dissolved in 40 l of deionized water. Temperature was controlled at 30°, agitation (flat-blade stirrer with baffles) was 600 rpm and aeration was set to 10 l of air per min. The pH was kept between 3–4 by controlled addition of 4N NaOH or 4N HCl. Polypropyleneglycol was added as an antifoam agent, if necessary. After 30 min ethyl acetoacetate (**1**) (25% *v/v* in ethanol abs.) and sucrose (30% *w/v* in deionized water) were added continuously at a rate of 100 ml per h each. Samples were withdrawn twice a day and analyzed as described above and the feeding rate of **1** lowered if necessary. After 100 h of fermentation time (9 l acetoacetate solution added, 2.25 kg) the cells were centrifuged and the liquid (55 l) extracted three times with an equal volume of AcOEt. The aqueous phase was discarded and the organic phase concentrated *in vacuo*. The product **2** was purified by distillation (b.p. 70°/13 Torr), 1291.3 g (56.5% yield) were obtained. Chemical purity: 99% (GC.); $[\alpha]_D^{20} = +41.3°$ ($c = 1$, $CHCl_3$); optical purity: 97% (e.e.) (determined as camphanic ester).

$C_6H_{12}O_3$ (132.159) Calc. C 54.53 H 9.15% Found C 54.29 H 9.00%

Preparation of (R)-ethyl 3-hydroxybutyrate (**3**). In a 10-l-fermentor *(New Brunswick Scientific Co.)* 7 l of a sterile solution of 5% *(w/v)* glucose (commercial grade), 1% *(w/v)* yeast extract *(Difco Laboratories)* and 1% *(w/v)* bactopeptone *(Difco Laboratories)* were inoculated with 400 ml of a 1-day old culture of *Geotrichum candidum*, CBS 233.76, in the same medium. The fermentor was run for 96 h at 30°, 600 rpm and 7 l of air/min and then 350 g of glucose and 100 ml of ethyl acetoacetate were added. After 44 h incubation the cells were separated by centrifugation and the aqueous phase was extracted as described before. From 61.8 g of the crude extract, 36.7 g (36% yield) of **3** were obtained after distillation. Chemical purity: 98.8% (GC.); $[\alpha]_D^{20} = -39.1°$ ($c = 1$, $CHCl_3$); optical purity 89.6% (e.e.) (determined as camphanic ester).

$C_6H_{12}O_3$ (132.159) Calc. C 54.53 H 9.15 Found C 54.42 H 9.30

REFERENCES

[1] *G. Fráter,* Helv. Chim. Acta *62*, 2825 (1979).
[2] *A. I. Meyers & R. A. Amos,* J. Am. Chem. Soc. *102*, 870 (1980).
[3] *B. Seuring & D. Seebach,* Helv. Chim. Acta *60*, 1175 (1977).
[4] *B. S. Deol, D. D. Ridley & G. W. Simpson,* Austr. J. Chem. *29*, 2459 (1976).
[5] *D. Seebach & M. Zueger,* Helv. Chim. Acta *65*, 495 (1982).
[6] *D. D. Ridley & M. Stralow,* J. Chem. Soc., Chem. Commun. *1975*, 400.
[7] *E. Hochuli, K. E. Taylor & H. Dutler,* Europ. J. Biochem. *75*, 433 (1977).