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Asymmetric reductions of ethyl 2-(benzamidomethyl)-3-oxobutanoate by yeasts

Raffaella Gandolfi [a,*], Edoardo Cesarotti [b], Francesco Molinari [c], Diego Romano [c], Isabella Rimoldi [b]

[a] *Università degli Studi di Milano, Institute of Organic Chemistry 'A. Marchesini', Via Venezian 21, 20133 Milan, Italy*
[b] *Department of Inorganic, Metallorganic and Analytical Chemistry 'L. Malatesta', Via Venezian 21, 20133 Milan, Italy*
[c] *Department of Food Science and Microbiology, Via Mangiagalli 25, 20133 Milan, Italy*

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ABSTRACT

The stereoselective reduction of ethyl 2-(benzamidomethyl)-3-oxobutanoate **1** using yeasts was investigated among a restricted number (12) of yeasts. *Kluyveromyces marxianus var. lactis* CL69 diastereoselectively produced (2*R*,3*S*)-ethyl 2-(benzamidomethyl)-3-hydroxybutanoate **2**, whereas *Pichia glucozyma* CBS 5766 gave (2*S*,3*S*)-**2** as the major stereoisomer. The biotransformations were independently optimized for minimizing by-product formation and maximizing the diastereoselectivity. Under optimized conditions, *K. marxianus var. lactis* CL 69 gave the (2*R*,3*S*)-ethyl 2-(benzamidomethyl)-3-hydroxybutanoate **2** with ee > 99% and de = 98%, while *P. glucozyma* CBS 5766 allowed for the production of (2*S*,3*S*)-**2** with ee > 99% and de = 86%.

1. Introduction

The use of microbial cells as catalysts in organic chemistry is attractive since they provide a number of stereoselective enzymes, such as oxidoreductases and hydrolases.[1] The simultaneous occurrence of different enzymes or isoenzymes in whole cells may affect the overall selectivity; isolation of the desired enzyme or optimization of culture and biotransformation conditions can reduce side-reactions allowing for excellent yields and (stereo)-selectivity.[2,3] Yeasts are commonly used for catalyzing different biotransformations, most often carbonyl reductions, since this microbial group is known to produce stereoselective membrane-bound dehydrogenases; decarboxylases and esterases are also produced as cell-bound enzymes.[4–6]

The yeast-mediated reduction of racemic α-substituted β-keto-esters has been thoroughly studied since in a few cases the carbonyl reduction occurs with concomitant dynamic kinetic resolution of the C-2 stereocentre furnishing high yields of the corresponding enantiomerically pure α-substituted β-hydroxyester stereoisomers.[7–9] This can be due to the action of dehydrogenases on the enol form or occurrence of an enantioselective keto-reductase acting only on one enantiomer, leaving the opposite enantiomer unreacted and prone to enolization.[8] It must be observed that the processes of enolization might be also enzymatically catalyzed.[10]

A drawback of the use of whole cells is the possible occurrence of esterase activities catalyzing ester hydrolysis and furnishing β-ketoacids which are spontaneously decarboxylated to the corresponding ketones; the ketones thus produced can be further reduced. Several strategies have been employed to limit the formation of these by-products, including the use of esters with different alkyl groups,[11,12] medium engineering[13,14] or the addition of specific inhibitors.[15,16]

Enantiomerically pure β-amino acids are useful building blocks in the synthesis of many natural products and/or biologically active compounds and a few reviews deal with their production by chemical[17–19] and enzymatic[20] means. β-Hydroxy-β-amino acids are valuable intermediates for drug syntheses such as in the preparation of β-lactams;[21,22] these molecules can be obtained by hydrogenation of the corresponding β-keto-β-amido esters with organometallic catalysts[23–25] or with reductases/hydrogenases.[26–28]

In this work, we have focused our attention on the biotransformations of ethyl 2-(benzamidomethyl)-3-oxobutanoate **1** by yeasts. This biotransformation has been previously carried out using plant cells furnishing (2*R*,3*S*)-ethyl 2-(benzamidomethyl)-3-hydroxybutanoate **2** using cultures of *Parthenocissus tricuspidata* and (2*S*,3*S*)-**2** with *Gossypium hirsutum*;[26] the reactions occurred with excellent stereoselectivity, but the substrate concentrations used were quite low (0.12 mg/mL) and reaction times were 3 days. Biotransformations of ethyl 2-phtalimidomethyl-3-oxobutanoate catalyzed by whole cells and partially purified enzymes from different *Kluyveromyces marxianus* strains gave low diastereomeric and enantiomeric excesses.[27,28]

Herein, we report the stereoselective reduction of **1** with different yeasts. The best results were obtained with *K. marxianus var. lactis* CL69 which gave (2*R*,3*S*)-**2**, while *Pichia glucozyma* CBS 5766 gave (2*S*,3*S*)-**2** as the predominant stereoisomer.

* Corresponding author. Tel.: +39 02 503 14609; fax: +39 02 503 14615.
E-mail address: raffaella.gandolfi@unimi.it (R. Gandolfi).

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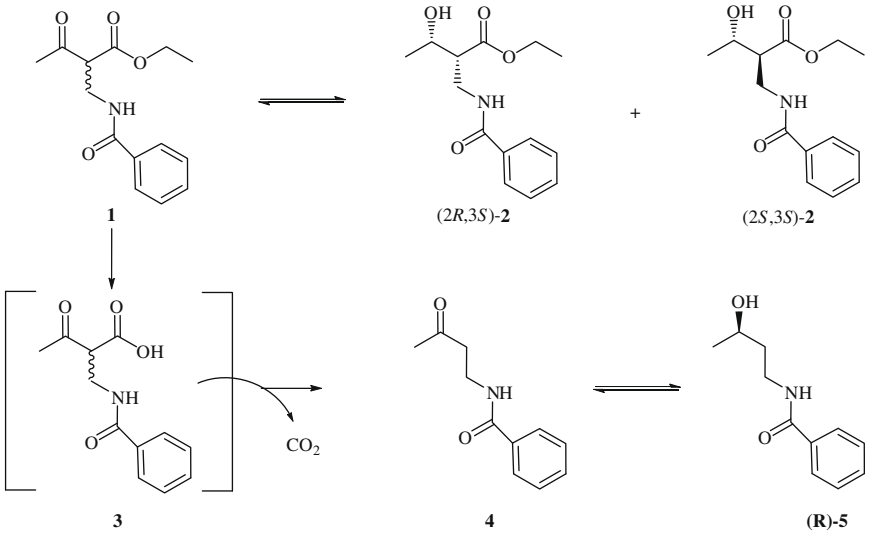

Scheme 1. Biotransformations of ethyl 2-(benzamidomethyl)-3-oxobutanoate **1**.

2. Results and discussion

A preliminary screening using conventional and non-conventional yeasts for the modification of ethyl 2-(benzamidomethyl)-3-oxobutanoate **1** was performed; the biotransformations of **1** catalyzed by yeasts are summarized in Scheme 1.

Two products were observed in all cases: ethyl 2-(benzamidomethyl)-3- hydroxybutanoate **2** and *N*-(3-oxobutyl)benzamide **4**, the latter product was further reduced to (*R*)-**5** (ee > 99%) only by *Pichia etchellsii* (Table 1). Carbonyl reduction of **1** occurred with high enantioselectivity furnishing the (*S*)-stereocentre at C3 (Prelog's rule) with all the yeasts tested. *N*-(3-Oxobutyl)benzamide **4** is formed through the action of cell-bound esterase(s) which produced the carboxylic acid **3** and subsequent spontaneous decarboxylation.

The (2*R*,3*S*)/(2*S*,3*S*) ratio of **2** was variable. Among the microorganisms which preferentially produce (2*S*,3*S*)-**2** (*anti*-configuration), *P. glucozyma* CBS 5766 gave the best diastereomeric excess, although with low yields. *K. marxianus var. lactis* CL 69 furnished the highest de of the *syn*-diastereoisomer (2*R*,3*S*).

In the light of these preliminary results, we studied different parameters (glucose and cell concentration, temperature, pH, substrate concentration) of the biotransformations with *K. marxianus var. lactis* CL 69 and *P. glucozyma* CBS 5766 with the aim of obtaining high yields of **2** with high stereoselectivity. The best results with *K. marxianus var. lactis* CL 69 were obtained with 30 g L^{-1} of cells (dry weight), employed at pH 7.0, 30 °C and substrate concentration of 0.5 g L^{-1} in the presence of 50 g L^{-1} of glucose; *P. glucozyma* CBS 5766 furnished the best results with 20 g L^{-1} of cells (dry weight), employed at pH 6.0, 27 °C and substrate concentration of 4.5 g L^{-1} in the presence of 30 g L^{-1} of glucose. The reduction of ethyl 2-(benzamidomethyl)-3-oxobutanoate **1** under these conditions is reported in Table 2.

Finally, preparative biotransformations aimed at producing (2*R*,3*S*)-**2** and (2*S*,3*S*)-**2** were independently carried out on a 1 L scale under optimized conditions. The reduction with *K .marxianus var. lactis* CL69 was carried out with recycling of the biocatalyst: the whole cells were re-used after centrifugation and a decrease in activity was observed after 6 cycles, when 3 g of substrate was totally converted and 2.67 g of (2*R*,3*S*)-**2** was obtained after

Table 1
Reduction of ethyl 2-(benzamidomethyl)-3-oxobutanoate **1** by different yeasts

Microorganisms	Time (h)	**2** (%)	ee **2** (%)	(2*R*,3*S*)/(2*S*,3*S*) **2** (%)	**4** (%)	**5** (%)
C. boidini CBS6056	24	23	>99	30/70	50	—
K. lactis CBS2359	96	49	>99	86/14	26	—
K. marxianus CBS1553	96	44	14	32/68	52	—
K. marxianus var. lactis CL69	72	70	>99	89/11	26	—
P. anomala CBS110	96	62	>99	58/42	20	—
P. etchellsii MIM	120	45	>99	71/29	6	37
P. glucozyma CBS5766	72	21	> 99	19/81	32	—
S. cerevisiae CBS1782	24	14	>99	36/64	15	—
S. cerevisiae CBS3093	24	34	57	41/59	8	—
S. cerevisiae CBS3081	24	40	85	33/67	11	—
S. cerevisiae NCYC73	24	11	>99	36/64	12	—
S. cerevisiae Zeus	96	15	>99	38/62	67	—

Table 2
Reduction of ethyl 2-(benzamidomethyl)-3-oxobutanoate **1** under the optimized conditions

Microorganisms	Time (h)	**2** (%)	ee **2** (%)	(2*R*,3*S*)/(2*S*,3*S*) **2** (%)	**4** (%)
P. glucozyma CBS5766	24	72	>99	7/93	15
K. marxianus var. lactis CL69	24	100	>99	99/1	—

purification. The biotransformation with *P. glucozyma* CBS 5766 was performed in a batch mode: 4.5 g L^{-1} of **1** was converted into (2*S*,3*S*)-**2** (2.75 g after purification) having a de = 86% and ee > 99%.

3. Conclusion

We have shown that biotransformations of ethyl 2-(benzamidomethyl)-3-oxobutanoate **1** with different yeasts lead to different products with satisfactory yields and high stereoselectivity. Under optimized conditions, *K. marxianus var. lactis* CL 69 gave the (2*R*,3*S*)-ethyl 2-(benzamidomethyl)-3-hydroxybutanoate **2** with ee > 99% and de = 98%, while *P. glucozyma* CBS 5766 allowed for the production of (2*S*,3*S*)-**2** with ee > 99% and de = 86%. These biotransformations are easier to perform and have better yields and reaction times compared with the ones catalyzed by cell plants[26] and have much higher stereoselectivities than the ones catalyzed by dehydrogenases from *K. marxianus* CBS 6556 and KCTC 7155 using ethyl 2-phthalimidomethyl-3-oxobutanoate as substrate.[27,28]

4. Experimental

4.1. Ethyl 2-(benzamidomethyl)-3- hydroxybutanoate 2

The standard was obtained by reduction with $NaBH_4$ (1:5) in ethanol. The absolute configuration was assigned in accordance with the literature.[29,30]

4.2. Preparation of *N*-(3-oxobutyl)benzamide 4

N-(3-Oxobutyl)benzamide **4** was obtained by biotransformation. Therefore, 2 g L^{-1} of ethyl 2-(benzamidomethyl)-3- oxobutanoate **1** and 10 g L^{-1} of lipase from *Candida cylindracea* were added in 0.1 M phosphate buffer, pH 7. The biotransformation was carried out at 30 °C under magnetic stirring. After 72 h, the reaction was extracted three times with ethyl acetate. The collected organic phases were dried over Na_2SO_4 and reduced under vacuo. The crude extract was purified with preparative TLC on silica gel (Kiesel 60 with fluorescent indicator) and was characterized by mass spectroscopy (Thermo Finnigan LCQ Advantage system) and ^{1}H NMR (Varian 200 MHz). ^{1}H NMR($CDCl_3$): δ = 2.20 (s, CH_3); 2.81–2.85 (t, CH_2); 3.68–3.74 (q, CH_2); 6.89 (br, NH); 7.41–7.77 (m, C_6H_5). $C_{11}H_{13}NO_2$ calculated: 191.23, found 214.08 (M^++Na(23)).

4.3. Preparation of *N*-(3-hydroxybutyl)benzamide 5

The *N*-(3-oxobutyl)benzamide was reduced by $NaBH_4$ (1:5) in ethanol. The reaction was maintained at room temperature under magnetic stirring. After 12 h, were added water and CH_2Cl_2 to recover the product **5**. The organic extract was dried over Na_2SO_4 and concentrated in vacuo. The product was purified via preparative TLC and was analyzed. ^{1}H NMR($CDCl_3$): δ = 1.27 (d, CH_3); 1.65–1.77 (m, CH_2); 3.27–3.42 (m, CH); 3.91–3.95 (m, CH_2); 6.80 (br, NH); 7.41–7.80 (m, C_6H_5), $C_{11}H_{15}NO_2$ calculated: 193.25, found 216.0 (M^++Na(23)).

The absolute configuration was assigned by catalytic asymmetric hydrogenation with Ru(II) complexes prepared with (−)-tetraMe BITIOP[31] as a chiral ligand.

In a Schlenk tube sealed under argon the substrate was added to the precatalyst followed by 20 mL of a solvent, the solution was stirred for 30 min and then transferred to an autoclave with a cannula.

The stainless steel autoclave (200 mL), equipped with temperature control (60 °C) and magnetic stirrer, was purged five times with hydrogen, after the transfer of the reaction mixture, the autoclave was pressurized (50 atm). At the end the autoclave was vented and the mixture was analyzed by NMR spectra and HPLC.

By asymmetric hydrogenation of substrate **4** using (−)-tetraMe BITIOP–Ru(II) complex we have obtained the product **5** with an (*R*)-configuration.[32]

4.4. Microorganisms: culture conditions

Strains from official collections or from our collection (Microbiologia Industriale Milano) were routinely maintained on malt extract (8 g L^{-1}, agar 15 g L^{-1}, pH 5.5). To obtain cells for biocatalytic activity tests, the microorganisms were cultured in 500 mL Erlenmeyer flasks containing 100 mL of medium and were incubated for 48 h at 28 °C on a reciprocal shaker (100 spm). The yeasts were grown on malt extract with 5 g L^{-1} Difco yeast extract, except *P. glucozyma* CBS 5766 grown on malt extract with 50 g L^{-1} Difco yeast extract. Fresh cells from submerged cultures were centrifuged (5000 rpm per 10′) and washed with 0.1 M phosphate buffer, pH 7, prior to use.

4.5. Bioreduction conditions

General procedure for the screening: reductions were carried out in 10 mL screw-capped test tubes with a reaction volume of 3 mL with cells (20 g L^{-1}, dry weight) suspended in 0.1 M phosphate buffer, pH 7, containing 5% of glucose and 4 g L^{-1} of ethyl 2-(benzamidomethyl)-3-oxobutanoate as substrate **1**. The reactions were carried out at 28 °C with magnetic stirring.

Optimization studies were carried out with *K. marxianus var. lactis* CL69 and *P. glucozyma* CBS 5766. The desired amount of cells was suspended in different 0.1 M phosphate buffers containing glucose and neat substrate was added to reach the desired concentration; the suspensions obtained were magnetically stirred at different temperatures.

The biotransformation was stopped by centrifugation of the cellular suspension; the liquid fraction was extracted with ethyl acetate. The organic extracts were dried over Na_2SO_4, and the solvent was removed under reduced pressure. After flash chromatography on silica gel eluted with CH_2Cl_2–di-*iso*-propyl ether (2:1) the pure products were obtained.

4.6. Analytical methods

Five hundred microliters of the crude reaction mixture were extracted with ethyl acetate. The organic phases were dried on Na_2SO_4 and were analyzed. The course of the reaction was monitored by TLC on silica gel Kiesel 60 with fluorescent indicator (CH_2Cl_2:diethyl ether = 2:1) and the molar conversion and diastereoisomeric and enantiomeric excess were determined by chiral HPLC analysis (Merck-Hitachi L-7100 equipped with Detector UV6000LP) on CHIRALPACK AD column (hexane:*iso*-propanol = 90:10, flow:0.6 mL min^{-1}, detector: diode array).

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