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Chemoenzymatic synthesis of 2-oxabicyclo[3.3.1]nonan-3-one enantiomers *via* microbial reduction by *Absidia coerulea* AM 93

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ABSTRACT

Microbial enantioselective reduction of (±)-diethyl 2-(3-oxocyclohexyl)malonate (**1**) has been described. A screening test on twenty-four fungi strains was carried out. Most of the microorganisms preferred bioreduction of (+)-isomer of δ-ketoester (**1**) to (+)-*trans* δ-hydroxy ester (**2**) with the anti-Prelog selectivity. Biotransformation conditions using *Absidia coerulea* AM 93 were optimized with respect to the growth medium, temperature and pH. An effect of 48 chemical additives on the course of biotransformation was checked. (−)-Diethyl 2-((*S*)-3-oxocyclohexyl)malonate ((−)-**1**) (ee = 98%) and (+)-diethyl 2-((1*R*, 3*R*)-3-hydroxycyclohexyl)malonate ((+)-**2**) (ee = 99%) were isolated and subjected to chemical lactonization, leading to (+)-(1*R*, 5*S*)-2-oxabicyclo[3.3.1]nonan-3-one ((+)-**3**) and (−)-(1*S*, 5*R*)-2-oxabicyclo[3.3.1]nonan-3-one ((−)-**3**). The absolute configuration of product (−)-**1** was confirmed by comparison of its optical rotation with the literature data. The absolute configuration the carbon atom bearing hydroxyl group in product (+)-**2** was determined using the Mosher's ester.

1. Introduction

δ-Lactone ring is a structural fragment of many natural products. Lactones play important role in food industry as flavour components [1–4], in agriculture as sex attractant pheromones [5–8] and also they are used as chiral building blocks for synthesis of pharmaceuticals [9]. Their physiological activity depends on configuration of stereogenic centres and on enantiomeric purity of the compounds [1–8].

Under our project we are interested in synthesis of optically active lactones with [3.3.1] carbon skeleton as potential antifungal agents. Naturally occurring, biologically active lactones of such a structure are presented in Fig. 1. They are isolated from stem bark of *Goniothalamus giganteus* (Annonaceae) [10–12] and *Illicium floridanum* [13,14], and from fruit kernels of *Otoba parvifolia* [15,16].

Styryl lactones, shown in Fig. 1, exhibit antitumor activity against several human tumor cells [10–12], whereas sesquiterpene lactones isolated from *Illicium* species are known as GABA antagonists in central nervous system [14]. The third group—lactones from *Otoba parvifolia* show antibiotic activity against such important microorganisms as *Staphylococcus aureus*, *Staphylococcus epidemis* and *Peptococus sp.* [15,16].

In recent years, syntheses of lactones using chemoenzymatic methods have been extensively investigated. These strategies bond the most efficient biocatalysis with chemical synthesis. Quick development of biocatalysis results from the fact, that enzyme-catalyzed reactions are highly enantioselective and regioselective, usually more than chemical synthesis using chiral catalysts. Several review articles have been published on the use of enzymes in organic synthesis [17–20]. Particularly two groups of enzymes: lipases and dehydrogenases are very useful [18–21]. Biological reductions catalyzed either by isolated enzymes or by whole microbial cells provide an attractive method that may be applied to a broad range of ketones [22–24] and ketoesters [25,26]. The reports comprise search for efficient and enentioselective biocatalysts and optimization of the process with respect to yields and enantiomeric excess of products [22–26]. Many biotransformations are performed in organic solvents [19] or in hydrophobic ionic liquids [20]. One of the ways to improve the yield of process is to add either inhibitors or activators to the cultivation mixture [27–33].

Herein we report on the resolution of racemic (±)-diethyl 2-(3-oxocyclohexyl)malonate (**1**) by means of microbial reduction, which could be a good alternative to organocatalytic conjugate 1,4-additions [34,35] of a variety of malonates to α,β-unsaturated enones. Optimization of the transformation conditions occupies a large part of this work. The aim of this part of the research was to increase enantiomeric excess of diethyl 2-(3-oxocyclohexyl)malonate (**1**). Effects of the additives on activities of the dehydrogenases were assessed.

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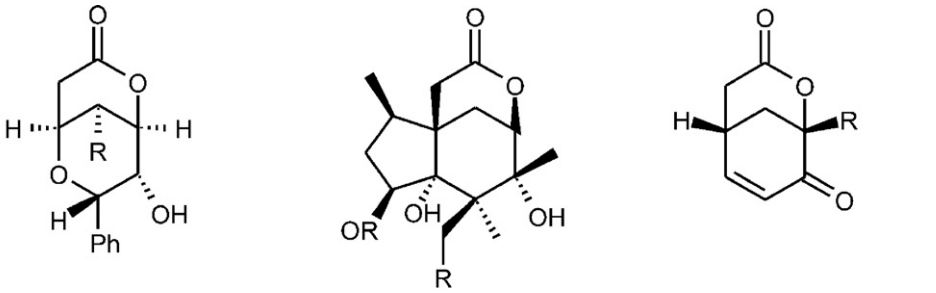


Fig. 1. Natural lactones with [3.3.1] carbon skeleton.

2. Materials and methods

2.1. Analysis

The structures of compounds **1**–**3** were determined based on ^{1}H NMR, ^{13}C NMR, COSY, DEPT, HMQC, IR and GC-MS. NMR spectra were recorded on Bruker DRX-500 spectrometer. The residual protic solvent $CHCl_3$ (δ_H = 7.26 ppm) in $CDCl_3$ was used as internal standard. ^{13}C NMR (δ_C = 77.0 ppm). Infrared spectra were measured on a Mattson FTIR-300 Thermo Nicolet spectrometer. Molecular mass was confirmed on a Varian Chrompack GC-MS CP-3800 Saturn 2000 GC-MS with an ionizing energy of 70 eV, using HP-1 column (crosslinked methyl silicone gum, 25 m × 0.32 mm, 0.25 μm film thickness). Optical rotation was measured on an Autopol IV automatic polarimeter (Rudolph).

Compositions of crude biotransformation extracts as well as purity of isolated products were determined by TLC and GC. TLC was carried out on silica gel Kieselgel 60 F_{254} glass plates (Merck) with petroleum ether:acetone:*iso*-propanol:ethyl acetate (40:1:3:1, v/v/v/v) or petroleum ether:acetone (2:1, v/v) as developing systems. The compounds were visualized by spraying the plates with 1% $Ce(SO_4)_2$, 2% $H_3[P(Mo_3O_{10})_4]$ in 10% H_2SO_4, followed by heating. The same eluents were applied to preparative column chromatography (silica gel: 60 230–400 mesh) for separation and purification of final products. Gas chromatography analyses were performed on a Hewlett Packard 5890A series II instrument, fitted with a flame ionization detector (FID), using column: Carbowax (30 m, 0.53 mm, 0.88 μm film thickness). H_2 at a flow rate of 2 cm^3/min was used as a carrier gas.

Conversion degree and enantiomeric excess were determinated by capillary GC using a chiral column–CP-cyclodextrin (25 m × 0.25 mm, 0.25 μm film thickness).

2.2. Synthesis of diethyl 2-(3-oxocyclohexyl)malonate ((±)-**1**)

The solution of cyclohex-2-en-1-one (3.5 g, 36.4 mmol) in 10 cm^3 of dry ethyl ether was added dropwise to a stirred solution of diethyl malonate (17.9 g, 112.0 mmol) with NaH (0.95 g, 39.6 mmol) and the stirring was continued at room temperature. When the reaction was complete (TLC, GC, 1 h) the mixture was diluted with ethyl ether (150 cm^3) and washed successively with aqueous solution of 10% HCl (50 cm^3) and water (until neutral). After solvent evaporation, diethyl malonate was distilled off under reduced pressure. The crude product was purified by column chromatography (eluent:petroleum ether:acetone, 2:1, v/v) to give 8.57 g of (±)-**1** in 92% yield. Physical and spectral data of (±)-**1** are as follows: Colourless oil; R_f = 0.61 (PE:acetone, 2:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 1.27 and 1.28 (two t, J = 7.2 Hz, 6H, 2× $CO_2CH_2CH_3$), 1.51 (qd, J = 12.6 Hz, J = 3.6 Hz, 1H, one of $^{6}CH_2$), 1.69 (qt, J = 12.6 Hz, J = 3.6 Hz, 1H, one of $^{6}CH_2$),), 1.90–1.94 and 1.92–1.97 (two m, 1H, one of $^{5}CH_2$), 2.05–2.09 (m, 1H, one of $^{5}CH_2$), 2.26 (ddd, J = 8.1 Hz, J = 8.7 Hz, J = 12.6 Hz 1H, one of $^{4}CH_2$), 2.25 (t, J = 12.6 Hz 1H, one of $^{4}CH_2$), 2.39–2.40 and 2.40–2.41 (two m, 1H, one of $^{2}CH_2$), 2.43–2.44 and 2.45–2.46 (two m, 1H, one of $^{2}CH_2$), 2.50–2.56 (m, 1H, $HCCH_2$) 3.29 (d, J = 8.1 Hz, 1H, $CH(CO_2C_2H_5)_2$) 4.20 and 4.21 (two q, J = 7.2 Hz, 4H, 2× $CO_2CH_2CH_3$); ^{13}C NMR (151 MHz), δ (ppm): 14.05 (2× $CO_2CH_2CH_3$), 24.51 (CH_2), 28.77 (CH_2), 38.00 (CH), 41.00 (CH_2), 45.09 (CH_2), 56.90 ($CH(CO_2C_2H_5)_2$), 61.52 and 61.53 (2× $CO_2CH_2CH_3$), 167.73 and 167.82 (C=O, ester), 209.62 (C=O); IR (film, cm^{-1}): 1731.13 (s), 1733.47 (s), 1738.58 (s); GC-EIMS: 257 (M+1).

2.3. Synthesis of trans diethyl 2-(3-hydroxycyclohexyl)malonate ((±)-**2**)

Diethyl 2-(3-oxocyclohexyl)malonate ((±)-**1**) (1.250 g, 4.88 mmol) was dissolved in ethanol (25 cm^3) and sodium borohydride (195.5 mg, 4.90 mmol) was added. The reaction mixture was stirred at room temperature. When the reaction was complete (TLC, GC, 3 h), ethanol was evaporated off in vacuo. The reaction mixture was diluted with ethyl ether (75 cm^3) and washed successively with 10% aqueous solution of HCl (5 cm^3) and water (until neutral). Ethereal extract was dried over $MgSO_4$. After solvent evaporation, the crude product was purified by column chromatography (eluent:petroleum ether:acetone, 2:1, v/v) to give 1.03 g pure (±)-**2** in 82% yield. The physical and spectral data of (±)-**2** are as follows: Colourless oil; R_f = 0.45 (PE:acetone, 2:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm): 1.22 and 1.23 (two t, J = 7.2 Hz, 6H, 2× $CO_2CH_2CH_3$), 1.61–1.63 and 1.64–1.66 (two m, 2H, $^{6}CH_2$), 1.73 (quintet, J = 3.5 Hz, 2H, $^{5}CH_2$), 1.90–1.99 (m, 2H, $^{4}CH_2$), 2.05–2.09 and 2.07–2.10 (two m, 2H, $^{2}CH_2$) 2.11–2.14 (m, 1H, $HCCH_2$) 3.15 (d J = 8.1 Hz, 1H, $CH(CO_2C_2H_5)_2$), 3.55–3.61 (m, 1H, HC(OH)), 4.14 and 4.17 (two q, J = 7.2 Hz, 4H, 2× $CO_2CH_2CH_3$); ^{13}C NMR (151 MHz), δ (ppm): 14.06 (2× $CO_2CH_2CH_3$), 23.43 (CH_2), 29.41 (CH_2), 35.13 (CH), 36.28 (CH_2), 39.65 (CH_2), 57.75 ($CH(CO_2C_2H_5)_2$), 61.23 and 61.27 (2× $CO_2CH_2CH_3$), 70.12 (HCOH), 168.40 and 168.46 (C=O, ester); IR (film, cm^{-1}): 1733.22 (s), 1738.51 (s), 3393.23 (s); GC-EIMS: 259 (241) (M+1).

2.4. Synthesis of 2-oxabicyclo[3.3.1]nonan-3-one ((±)-**3**)

Trans diethyl 2-(3-hydroxycyclohexyl)malonate ((±)-**2**) (0.453 g, 1.76 mmol) was refluxed with 10% ethanolic KOH solution (30 cm^3). When the reaction was complete (TLC, 5 h), ethanol was evaporated off in vacuo. The reaction mixture was diluted with ethyl ether (50 cm^3) and washed successively with 10% aqueous solution of HCl (10 cm^3) and water to pH = 6. Ethereal extract was dried over $MgSO_4$. After solvent evaporation, the crude

product was diluted with benzene (25 cm^3) and p-toluenosulfonic acid ($TsOH \times H_2O$) (0.35 g, 1.78 mmol) was added. The reaction mixture was refluxed for 5 h. Next, benzene was evaporated off and the product was diluted with ethyl ether (50 cm^3) and washed with 10% aqueous solution of NaOH (5 cm^3) and water (until neutral). The crude product was purified by column chromatography (eluent:petroleum ether:acetone:*iso*-propanol:ethyl acetate, 40:1:3:1, v/v/v/v) to give 150 mg of pure (±)-**3** in 61% yield.

*2.4.1. Synthesis of (−)-(1S, 5R)-2-oxabicyclo[3.3.1]nonan-3-one ((-)-**3**)*

Prepared from 120 mg (0.47 mmol) of (+)-diethyl 2-((1*R*, 3*R*)-3-hydroxycyclohexyl)malonate ((+)-**2**) according to the procedure described for preparation of (±)-**3**, obtained 42 mg. Yield: 64%, $[\alpha]_{589}^{20} = -18.6$ ($c = 2.05$, $CHCl_3$).

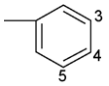
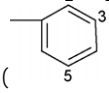
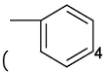
*2.4.2. Synthesis of (+)-(1R, 5S)-2-oxabicyclo[3.3.1]nonan-3-one ((+)-**3**)*

Prepared from 143 mg (0.56 mmol) of (−)-diethyl 2-((*S*)-3-oxycyclohexyl)malonate ((−)-**1**), which was dissolved in 5 cm^3 of ethanol and reduced with 25 mg (0.55 mmol) of $NaBH_4$ according to the procedure given for racemic (±)-**1**. After flash column purification 115 mg of product (−)-**2** ($[\alpha]_{589}^{20} = -1.04$ ($c = 3.11$, $CHCl_3$)) was obtained with 80% yield.

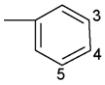
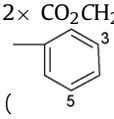
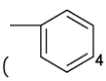
(−)-Isomer of 2-oxabicyclo[3.3.1]nonan-3-one ((−)-**3**) was prepared from 97 mg (0.38 mmol) of (−)-diethyl 2-((1*S*, 3*S*)-3-hydroxycyclohexyl)malonate ((−)-**2**) according to the procedure described for preparation of (±)-**3**. Yield: 59%, $[\alpha]_{589}^{20} = +17.9$ ($c = 1.5$, $CHCl_3$) (ee = 93%).

The physical and spectral data of 2-oxabicyclo[3.3.1]nonan-3-one (3) are as follows: $R_f = 0.20$ (PE:acetone:*iso*-propanol:ethyl acetate, 40:1:3:1) ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 1.74–1.54 (m, 6H, $^7CH_2$, $^8CH_2$, $^9CH_2$), 1.92–2.02 and 2.03–2.08 (two m, 2H, $^6CH_2$), 2.24–2.26 (m, 1H, ^{5}CH), 2.45 (d, $J = 18.56$ Hz, 1H, one of $^4CH_2$), 2.70 (dd, $J = 18.56$ Hz, $J = 6.64$ Hz, 1H one of $^4CH_2$), 4.70–4.72 (m, 1H, H^1CO); ^{13}C NMR (151 MHz), δ (ppm): 15.93 ($^8CH_2$), 25.98 ($^7CH_2$), 30.31 ($^9CH_2$), 30.96 (^{5}CH), 35.99 ($^6CH_2$, $^2CH_2$), 75.43 (H^1CO), 171.96 (C=O); IR (film, cm^{-1}): 1218.14 (s), 1725.55 (s); GC-EIMS: 141 (M+1).

2.4.3. Synthesis of diethyl 2-((1R, 3R)-3-((2′S)-3′,3′,3′-trifluoro-2′-methoxy-2′-phenyl-propionyloxy)cyclohexyl)malonate (2-S-MTPA)

(+)-*Trans* diethyl 2-((1*R*, 3*R*)-3-hydroxycyclohexyl)malonate ((+)-**2**) (70 mg, 0.271 mmol) was dissolved in dry pyridine (1 cm^3), then (*R*)-2-metoxy-2-trifluoromethylphenylacetic acid chloride (57 mg, 0.273 mmol) was slowly added and reaction mixture was stirred at room temperature. When reaction was completed (TLC, 10 h), the mixture was diluted with ethyl ether (30 cm^3) and washed successively with aqueous solution of 10% HCl (5 cm^3) and water (until neutral). Ethereal extract was dried over $MgSO_4$. After solvent evaporation, the crude product was purified by column chromatography (eluent:petroleum ether:acetone, 4:1, v/v) to give 93 mg of the product (yield 81%). Physical and spectral data of **2-*S*-MTPA** obtained are as follows: ^{1}H NMR (δ, ppm): 1.25 and 1.26 (two t, $J = 7.0$ Hz, 6H, $CO_2CH_2CH_3$), 1.28–1.42 m 2H, $^6CH_2$), 1.68–1.72 and 1.70–1.74 (two m, 1H, one of $^5CH_2$), 1.83 (dt, 1H, $J = 13.5$ Hz, $J = 3.5$ Hz, one of 1H $^5CH_2$), 2.01–2.09 (m, 2H, $^4CH_2$), 2.02–2.16 (m, 2H, $^2CH_2$), 2.13–2.17 (m, 1H, $HCCH_2$), 3.20 (d, $J = 8.5$ Hz, 1H, $HC(CO_2Et)_2$), 3.53 (s, 13H, OCH_3), 4.19 (q, $J = 7.0$ Hz, 4H, $CO_2CH_2CH_3$), 4.95–5.01 (m, 1H, H^3COOC(CF_3), 7.49–7.51 (m, 2H ), 7.36–7.38 (m, 3H ). ^{13}C NMR (δ, ppm): 14.04; 14.06 ($2\times$ $CO_2CH_2CH_3$), 23.14 ($^6CH_2$), 29.09 ($^5CH_2$), 30.84 ($^2CH_2$), 35.30 ($^4CH_2$), 35.99 ($HCCH_2$), 55.33 (OCH_3), 57.34 ($HC(CO_2Et)_2$), 61.38 ($2\times$ $CO_2CH_2CH_3$), 75.01 (H^3COOC(CF_3)), 127.24 (), 128.32 (), 129.50 (), 132.36 (), 165.65 ($COOC(CF_3)$). 168.11 and 168.17 ($2\times$ ($CO_2C_2H_5$)).

2.4.4. Synthesis of diethyl 2-((1R, 3R)-3-((2′R)-3′,3′,3′trifluoro-2′-methoxy-2′-phenyl-propionyloxy)cyclohexyl)malonate (2-R-MTPA)

2-*R*-MTPA: was prepared using 75 mg (0.291 mmol) of (+)-*trans* diethyl 2-((1*R*, 3*R*)-3-hydroxycyclohexyl)malonate ((+)-**2**) and 59 mg (0.283 mmol) of (*S*)-2-metoxy-2-trifluoromethylphenylacetic acid chloride according to the same procedure as for **2-*S*-MTPA**. 89 mg of the product was obtained (yield 73%). Physical and spectral data of **2-*R*-MTPA** obtained are as follows: ^{1}H NMR (δ, ppm): 1.24 and 1.25 (two t, $J = 7.0$ Hz, 6H, $CO_2CH_2CH_3$), 1.30–1.44 (m 2H, $^6CH_2$), 1.70–1.74 and 1.72–1.76 (two m, 1H, one of $^5CH_2$), 1.86 (dt $J = 3.5$ Hz, $J = 13.5$ Hz, one of $^5CH_2$), 1.98–2.05 (m, 1H, one of $^4CH_2$), 2.09–2.11 (m, 1H one of $^4CH_2$), 2.12–2.26 (m, 2H, $^2CH_2$), 2.29–2.32 ($HCCH_2$), 3.18 (d, $J = 8.5$ Hz, 1H, $HC(CO_2Et)_2$), 3.53 (s, 1H, OCH_3), 4.16 and 4.20 (two q, $J = 7.0$ Hz 4H, $2\times$ $CO_2CH_2CH_3$), 4.96–5.2 (m, 1H, H^3COOC(CF_3)), 7.50–5.52 (m, 2H ), 7.37–7.39 (m, 3H ), ^{13}C NMR (δ, ppm): 13.99, 14.01 ($2\times$ $CO_2CH_2CH_3$), 23.20 ($^6CH_2$), 29.06 ($^5CH_2$), 31.13 ($^2CH_2$), 35.09 ($^4CH_2$), 36.00 ($HCCH_2$), 55.25 (OCH_3), 57.33 ($HC(CO_2Et)_2$), 61.33 ($2\times$ $CO_2CH_2CH_3$), 74.99 (H^3COOC(CF_3), 127.24 (), 128.30 (), 129.48 (), 132.35 (),165.62 ($COOC(CF_3)$, 168.06 and 168.12 ($2\times$ ($CO_2C_2H_5$)).

2.5. Biocatalysts

The following microorganisms were obtained from the collection of Institute of Biology and Botany, Medical University in Wrocław: *Absidia coerulea* AM 93, *A. cylindrospora* AM 336, *Aphanocladium album* AM 417, *Ascosphaera aspis* AM 496, *Aspergillus ochraceus* AM 456, *A. wenthi* AM 413, *Beauveria bassiana* AM 278, *B. bassiana* AM 446, *Circinella muscaes* AM 302, *Dothiorella ribis* AM 273, *Fusarium oxysporum* AM 13, *F. solani* AM 203, *Fusicoccum amygdali* AM 258, *Laetiporus sulphurens* AM 524, *Mortiella vinaceae* AM 149, *Mucor circinelloides* AM 385, *Penicillium camemberti* AM 83, *P. notatum* AM 904, *Rhizopus nigricans* AM 394, *Rhodotorula glutinis* AM 242, *R. rubra* AM 4, *Saccharomyces cerevisiae* AM 464, *Trichoderma viride* AM 523, and *Yarrowia lipolytica* AM 71. Whereas the strains of *Hanseniaspora vinaea* 912, *Papularia rosea* 17, and *Penicillium vermiculatum* 30 came from the Collection of the University of Environmental and Life Sciences in Wrocław.

2.5.1. Initial screening

Screening procedure: The microorganisms were cultivated at 25 °C in 300 cm^3 Erlenmeyer flasks containing 75 cm^3 of the following nutrients: 1% solution of peptone and glucose (3%). After 3–5 days of growth, 20 mg of a substrate in 0.5 cm^3 of acetone was added to the shaken cultures. The transformation was being continued for 3, 6, 9, 12, 21 or 81 h. The products were extracted with diethyl ether and analysed by TLC and GC. Enantiomeric excesses were determined by GC (CP-cyclodextrin, 25 m × 0.25 mm, 0.25 μm film

thickness) under the following conditions: injector 200 °C; detector 250 °C; flow 2 ml/min (carrier gas–H_2); temperature program: 120 °C for 1 min, 0.4 °C/min to 160 °C.

2.5.2. Effect of growth medium

Portions of 0.5 cm^3 of the inoculum (spore concentration of 10^7 cm^{-3}) were transferred to 300 cm^3 Erlenmeyer flasks, each containing 75 cm^3 of one of six sterile growth media. The following media were used: **C**: (3 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g KCl, 0.01 g $FeSO_4$, 0.5 g $MgSO_4 \times 7H_2O$, 30 g saccharose/1000 ml H_2O);) **E**: (4 g yeast extract, 10 g starch, 0.1 g K_2HPO_4, 0.05 g $MgSO_4 \times 7H_2O$/1000 ml H_2O). **G**: (10 g glucose, 0.5 g K_2HPO_4, 0.5 g, asparagine/1000 ml H_2O); **M**: (40 g glucose, 2 g asparagine, 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \times 7H_2O$, 0.5 g tiamine/1000 ml H_2O); **P**: 30 g glucose, 10 g peptone/1000 ml H_2O S: (2.5 mg geistageine, 2.5 g NaCl, 2.5 g K_2HPO_4, 10 g glucose/1000 ml H_2O). The cultures were incubated at 26 °C with shaking at 150 rpm. After 5 days of cultivation, 20 mg of diethyl 2-(3-oxocyclohexyl)malonate (**1**) in 0.5 cm^3 of acetone was added to each flask. The conversion and enantiomeric excesses were measured after 9 h of the transformation, followed by the extraction with diethyl ether (25 cm^3). Dry mass of *A. coerulea* AM 93 in different media was established after washing and lyophilization.

2.5.3. Effect of temperature

Portions of 0.5 cm^3 of the inoculum (spore concentration of 10^7 cm^{-3}) were transferred to 300 cm^3 Erlenmeyer flasks, each containing 75 cm^3 of growth medium **P**. The cultures were incubated at one of the following temperatures: 21, 25, 28, 30, or 32 °C, with shaking at 150 rpm. After 5 days of cultivation, 20 mg of diethyl 2-(3-oxocyclohexyl)malonate (**1**) in 0.5 cm^3 of acetone was added to each flask. The conversion and enantiomeric excesses were measured after 9 h of the transformation followed by the extraction with diethyl ether (25 cm^3). Dry mass of *A. coerulea* AM 93 in different media was established after washing and lyophilization.

2.5.4. Effect of pH

Portions of 0.5 cm^3 of the inoculum (spore concentration of 10^7 cm^{-3}) were transferred to 300 cm^3 Erlenmeyer flasks, each containing 75 cm^3 of growth medium **P**. The cultures were incubated at 28 °C with shaking at 150 rpm. After 5 days of cultivation, the biomass was washed under sterile conditions and transferred to Erlenmeyer flasks, each containing 75 cm^3 of one of four phosphate buffer solutions of different pH values (4.5; 6.2; 7.2; 8.5). Next 20 mg of diethyl 2-(3-oxocyclohexyl)malonate (**1**) in 0.5 cm^3 of acetone was added to each flask. The conversion and enantiomeric excesses were measured after 9 h of the transformation followed by the extraction with diethyl ether (25 cm^3). Dry mass of *A. coerulea* AM 93 in different media was established after washing and lyophilization.

2.5.5. Effect of substrate concentration

Portions of 0.5 cm^3 of the inoculum (spore concentration of 10^7 cm^{-3}) were transferred to 300 cm^3 Erlenmeyer flasks, each containing 75 cm^3 of growth medium **P**. The cultures were incubated at 28 °C with shaking at 150 rpm. After 5 days of cultivation, one of the following amounts of 2-(3-oxocyclohexyl)malonate (**1**) were added to each flask: 20 mg (1 mmol/dm^3), 40 mg (2 mmol/dm^3), 60 mg (3 mmol/dm^3), 80 mg (4 mmol/dm^3), 100 mg (5 mmol/dm^3) or 150 mg (7 mmol/dm^3). The conversion and enantiomeric excesses were measured after 9 and 12 h of the transformation followed by the extraction with diethyl ether (25 cm^3). Dry mass of *A. coerulea* AM 93 was established after washing and lyophilization.

2.5.6. Effect of inhibitors/activators addition

Portions of 0.5 cm^3 of the inoculum (spore concentration of 10^7 cm^{-3}) were transferred to 300 cm^3 Erlenmeyer flasks, each containing 75 cm^3 of growth medium **P**. The cultures were incubated at 28 °C with shaking at 150 rpm. After 5 days of growth, specified amounts of either an inhibitor or an activator (10, 30 or 50 mg) were added to each flask and the cultures were incubated for the next 1 h. Then, 20 mg of 2-(3-oxocyclohexyl)malonate (**1**) in 0.5 cm^3 of acetone was added. The biotransformation was stopped after 1.5 h. The conversion and enantiomeric excesses were measured. Dry mass of *A. coerulea* AM 93 was established after washing and lyophilization. Simultaneously, under the same conditions, transformations without the additives were run as a control.

*2.5.7. Preparative resolution of racemic diethyl 2-(3-oxocyclohexyl)malonate-(±)-**1***

Absidia coerulea AM 93 was cultivated at 28 °C in ten 300 cm^3 Erlenmeyer flasks, each containing 75 cm^3 of solution of peptone (1%) and glucose (3%) in deionized water. After 5 days of growth, the cultures of microorganism were incubated with 15 mg of the Eschenmoser salt for 1 h. Next, racemic diethyl 2-(3-oxocyclohexyl) malonate (**1**), 75 mg in 0.5 cm^3 of acetone for each flask (initial concentration of 1 mg/cm^3), was added to the shaken cultures. After 12 h the products were extracted with diethyl ether (750 cm^3) and dried ($MgSO_4$). The solvent was evaporated off and the crude product mixture (720 mg; 49% of molar conversion of **1** by GC) was separated by column chromatography (silica gel, petroleum ether:acetone, 2:1) to give 310 mg of (−)-diethyl 2-((*S*)-3-oxocyclohexyl)malonate ((−)-1) (ee = 98%),{$[\alpha]_{589}^{20} = -3.4$ (c = 4.25, $CHCl_3$)}; and 290 mg of (+)-diethyl 2-((1*R*, 3*R*)-3-hydroxycyclohexyl)malonate ((+)-2) (ee = 99%) {$[\alpha]_{589}^{20} = +1.2$ (c = 3.7, $CHCl_3$)}. Overall yield of isolated products was 80%.

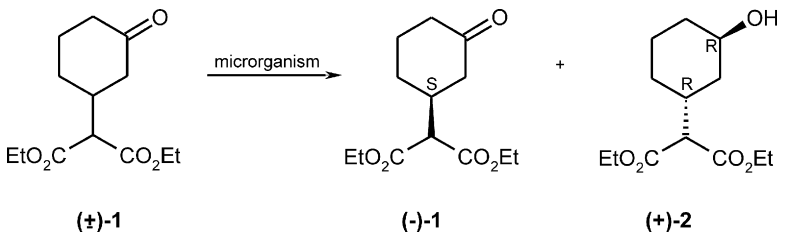
3. Results and discussion

3.1. Initial screening

In the initial screening we searched for microorganisms capable of resolution of racemic diethyl 2-(3-oxocyclohexyl)malonate (**1**) by enantioselective reduction to the corresponding *cis* or *trans* alcohols. We examined twenty-four fungi strains belonging to twenty different species. In general, the tested microorganisms, except for *Laetiporus sulphurens* AM 524, preferred reduction of (+)-*R*-enantiomer of the racemic δ-ketoester (**1**) to (+)-*trans* diethyl 2-(3-hydroxycyclohexyl)malonate with moderate both conversion and enantiomeric excess (Scheme 1).

The *trans*-diastereoisomer obtained is a product of the anti-Prelog's reduction. The *cis*-isomer was the major product in biotransformations with *Ascosphaera aspis* AM 496 (37.7%), *Aspergillus ochraceus* AM 456 (58.0%), *Fusicoccum amygdale* AM 258 (70.1%), and *Rhodotorula glutinis* AM 242 (45.1%)—unfortunately, it has never been isolated in a pure form.

We planned to obtain (+)- and (−)-2-oxabicyclo[3.3.1]nonan-3-one (**3**) from two enantiomers of 2-(3-hydroxycyclohexyl)malonate (**2**). One enantiomer of **2** can be obtained by biotransformation and the second one may be obtained by chemical reduction of the unreacted substrate (**1**). Unfortunately, there is no chemical method for reduction of 1,3-substituted ketones to *cis* diastereoisomers as the major products. This is the reason why we concentrated on microorganisms performing reduction to *trans* products with a high diastereoselectivity. The selected results of this transformation (leading preferentially to the *trans* alcohol) are presented in Table 1.



Scheme 1.

Table 1
Microbial reduction of racemic diethyl 2-(3-oxocyclohexyl)malonate (±)-**1**—composition of product mixtures (by GC).

Entry	Microorganism	Time [h]	Conversion of **1** [%]	Ee of **1** [%]	Ee of trans-**2** [%]	De of trans-**2** [%]
1	*Absidia coerulea* AM 93	3	47	(−) 82	(+) 99	95
		12	58	(−) 99	(+) 89	92
2	*Aphanocladium album* AM 417	21	24	(−) 14	(+) 22	94
		81	79	(−) 42	(+) 62	95
3	*Beauveria* bassiana AM 278	3	42	(−) 72	(+) 89	76
		6	65	(−) 62	(+) 57	73
4	*Beauveria bassiana* AM 446	6	29	(−) 9	(+) 5	71
		9	47	(−) 8	(+) 10	71
5	*Fusarium solani* AM 203	6	37	(−) 29	(+) 99	55
		12	53	(−) 27	(+) 76	63
6	*Penicillium camemberti* AM 83	6	44	(−) 39	(+) 33	77
		12	74	(−) 19	(+) 44	69
7	*Penicillium notatum* 904	3	35	(−) 42	(+) 20	81
		6	51	(−) 82	(+) 32	83
8	*Rhizopus nigricans* AM 394	9	22	(−) 21	(−) 63	62
		81	80	(−) 51	(−) 35	79
9	*Saccharomyces cerevisiae* AM 464	6	35	(−) 29	(−) 47	86
		21	75	(−) 45	(−) 12	83
10	*Rhodotorula rubra* AM 4	3	67	(−) 62	(+) 69	61
		6	72	(−) 79	(+) 67	61
11	*Yarrowia lipolytica* AM 71	9	36	(−) 22	(+) 19	58
		21	83	(−) 32	(+) 31	75

Four of the tested microorganisms: *Absidia coerulea* AM 93 (ee = 99%), *Beauveria bassiana* AM 278 (ee = 72%), *Penicillium notatum* AM 904 (ee = 99%), and *Rhodotorula rubra* AM 4 (ee = 79%) transformed substrate **1** with a high enantioselectivity.

In the initial screening *A. coerulea* AM 93 [36–38] transformed racemic diethyl 2-(3-oxocyclohexyl)malonate ((±)-**1**) to (+) isomer of *trans* diethyl 2-(3-hydroxycyclohexyl)malonate ((+)-**2**) in 3 h with 99% of enantiomeric excess and 44% of conversion. During the first 3 h the process of reduction was fast (44%) and highly enantioselective (ee = 99%), whereas later on it proceeded much slower (8.9% of progress in 9 h). Therefore, not only did we optimized biotransformation conditions with respect to the growth medium, temperature and pH, but also we have noticed that the added compound changed activity of dehydrogenases present in the microorganism.

3.2. Optimization of biotransformation conditions

3.2.1. Effect of growth medium

The cultures of *Absidia coerulea* AM 93 were cultivated in the six growth media of different composition (see Section 2.5.2). A degree of conversion of diethyl 2-(3-oxocyclohexyl)malonate (**1**) as well as enantioselectivity of the process and dry mass of the culture were measured. The results obtained are presented in Table 2.

Such big differences in enantioselectivity of the process run in different growth media suggest that the ingredients of the cultivation media may affect the dehydrogenases activity. The best growth medium with respect to the conversion degree and enantiomeric excess of the substrate was the mixture of peptone and glucose (growth medium P).

Table 2
Effect of growth medium on dry mass, enzyme activity, degree of conversion and enantiomeric excesses of (−)-**1** and (+)-**2**.

Entry	Growth medium	Dry mass [g]	Enzymatic activity[a]	Conversion of **1** [%]	Ee of **1** [%]	Ee of trans-**2** [%]
1	C	0.49	7.9	45	(−) 62	(+) 93
2	E	0.44	5.4	15	(−) 8	(+) 99
3	G	0.46	6.8	36	(−) 40	(+) 99
4	M	0.49	7.8	45	(−) 52	(+) 95
5	S	0.47	10.4	57	(−) 13	(+) 92
6	P	0.60	7.5	52	(−) 92	(+) 95

[a] Enzymatic activity $A = a\,b\,h^{-1}\,g^{-1}$ (a, amount of substrate [μmol]; b, percent of conversion/100; h, time [h]; g, mass of lyophilized mycelium [g]) A_A, enzymatic activity of the microorganisms with additives; A_C, enzymatic activity of the microorganism-control.

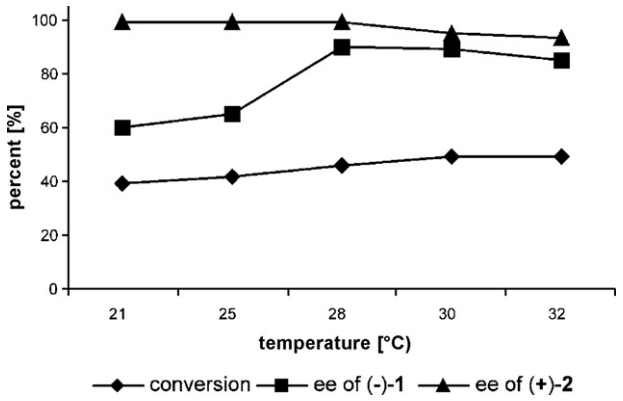


Fig. 2. Effect of temperature on substrate conversion and enantiomeric excesses of (−)-1 and (+)-2.

We have also examined growth process of *Absidia coerulea* AM 93 in the cultivation medium P. We observed that the initial pH of the medium (6.5) gradually changed with the progress of growth.

3.2.2. Effect of temperature

In the next experiment we determined optimal temperature for the microbial activity. In general, conversion of diethyl 2-(3-oxocyclohexyl)malonate (**1**) is directly proportional to temperature within the range of 21–32 °C (about 1 percent of conversion increase for 1 °C) (Fig. 2).

Within the range of 25–28 °C the ee of 2-(3-hydroxycyclohexyl)malonate (**2**) is high and the ee of (−)-**1** grows. When the temperature reached 30–32 °C, we observed a decrease of the enantioselectivity of dehydrogenases. Therefore, 28 °C was chosen as the optimal temperature of the process.

3.2.3. Effect of pH

A degree of conversion of diethyl 2-(3-oxocyclohexyl)malonate (**1**) and an enantioselectivity of the biotransformation process were evaluated in different pH values. The results are presented in Fig. 3.

We observed, that bioreduction of the substrate is more efficient and more enantioselective under acidic conditions than under neutral or alkaline ones.

3.2.4. Effect of substrate concentration

The aim of this experiment was to find out what is the maximal amount of the substrate that may be successfully transformed by a 5-day *Absidia coerulea* AM 93 culture, cultivated in 75 cm^3 of the growth medium P, without the loss of enantioselectivity compared to the screening test. An impact of the substrate amount on the

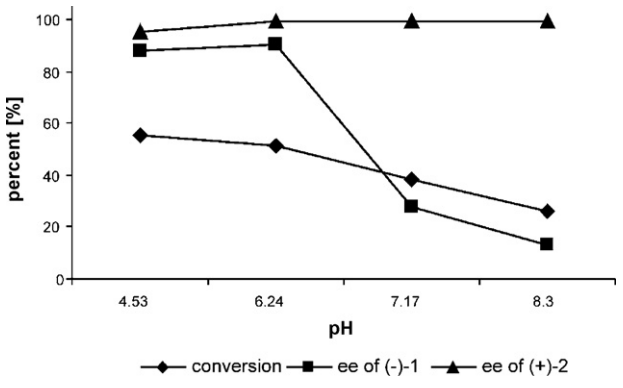


Fig. 3. Effect of pH on conversion and enantiomeric excesses of (−)-1 and (+)-2.

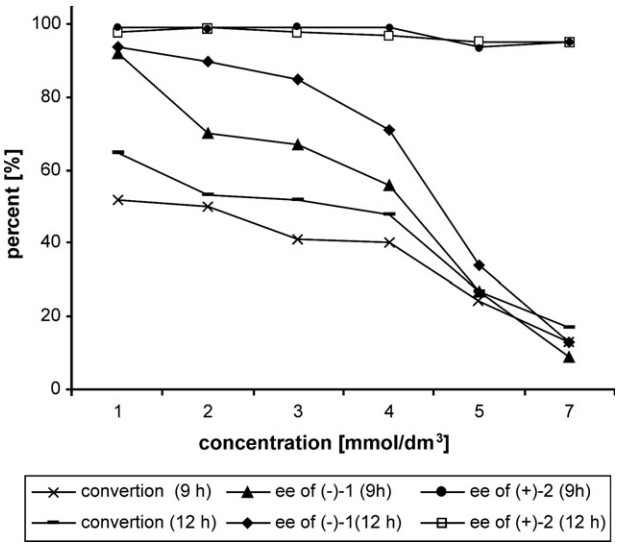


Fig. 4. Effect of substrate concentration on conversion and enantiomeric excesses of (−)-1 and (+)-2.

conversion degree and on enantiomeric excess of the products were determined. The results are presented in Fig. 4.

Degree of conversion of (±)-**1** depends on substrate concentration. For substrate concentration of from 1 to 4 mmol/dm^3 the conversion degree decreases along with an increase of concentration by about 3% per 1 mmol/dm^3. Whereas, for concentration of (±)-**1** of above 4 mmol/dm^3 it drops as much as 10% per 1 mmol/dm^3.

If the degree of conversion is less than 50% then the enantiomeric excess of product 2 is above 93%. These data suggest that it is a single dehydrogenase that is responsible for reduction (+)-diethyl 2-((*R*)-3-oxocyclohexyl)malonate ((+)-**1**) to (+)-diethyl 2-((1*R*, 3*R*)-3-hydroxycyclohexyl)malonate.

An activity of the dehydrogenase is proportional to the amount of (+)-**2**, calculated from concentration of the substrate and the degree of conversion. Concentration of the racemic ketone **1** affects the enzyme activity. Initially, an increase in substrate concentration activates the dehydrogenase, but when the concentration of ketone **1** is above 4 mmol/dm^3, it begins to inhibit it.

In summary, at this stage of the study it is only (+)-diethyl 2-(3-hydroxycyclohexyl)malonate (**2**) that is obtained as a pure enantiomer, whereas the (−) isomer of diethyl 2-(3-oxocyclohexyl)malonate (**1**) is obtained only with some enantiomeric excess. Longer reaction time (12 h instead of 9 h) does not improve the results.

3.2.5. Effect of additives on activity of dehydrogenases

Incubation of the microorganism culture with some additives is a known method of changing of activity and enantioselectivity of dehydrogenases [27–33]. For the experiments we employed 48 compounds, the structures of which are presented in Fig. 5.

Activity of dehydrogenases (calculated with respect to dry lyophilized biomass of the microorganism [27]) and differences between enantiomeric excesses of biotransformation carried out with additives and a control sample are presented in a graph (Fig. 6).

We divided the tested compounds into the following groups: **A**: acryl, propionic, and crotonic acid or their derivatives and 5 or 6-member ring compounds with oxygen or nitrogen as heteroatoms; **B**: compounds containing bromine atom; **C**: compounds with a carbonyl group; **E**: inorganic salts. Most of the compounds are of low molecular weight and contain a double bond along with a functional group in the allylic position. In the last group of compounds, marked as **D**, we included the compounds of different structures—it com-

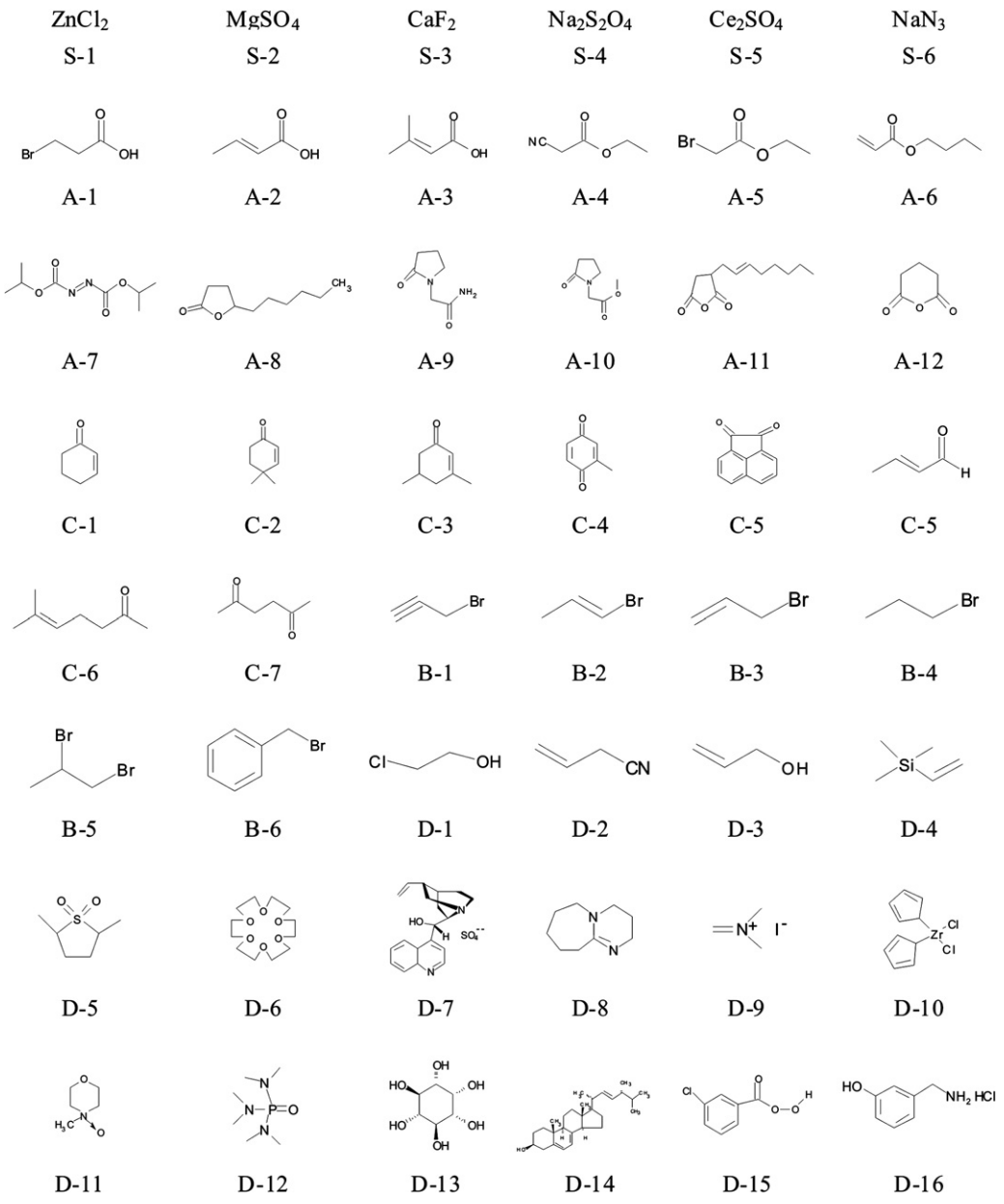


Fig. 5. Chemical structures and symbols of the additives used. $\Delta A = A_A - A_C \times 100\%/\mathrm{Ac}$, $A = a\,b\,h^{-1}\,g^{-1}$ (a, amount of substrate [μmol]; b, percent of conversion/100; h, time [h]; g, mass of lyophilized mycelium [g]) A_A, enzymatic activity of the microorganisms with additives; A_C, enzymatic activity of the microorganism-control. $\Delta ee = ee_A - ee_C$; ee_A, enantiomeric excesses of the microorganisms with additives; ee_C, enantiomeric excesses of the microorganism-control.

prises natural compounds like ergosterol (D-14) or myo-inozytol (D-13), and organic salts like Eschenmoser salt (D-3), cinchonine sulphate (D-7) or zircocene dichloride (D-10).

All the examinated inorganic salts inhibited activity and enantioselectivity of dehydrogenases, whereas the salts with organic ligands, like the Eschenmoser salt (D-3), cinchonine sulphate (D-7) or zircocene dichloride (D-10) expressed a high activating and enantioselectivity-improving effect. The Eschenmoser salt – N,N-dimethylmethylene-ammonium iodide – is a small molecule, but such compounds as cinchonine sulphate or zircocene dichloride have large, spatial structures. Similarly high activity was observed for HMPA (D-12) (hexamethylphosphoramide).

As we expected, the compounds with a carbonyl group act as activators for dehydrogenases. Four cyclic α,β-unsaturated ketones were checked (C-1, C-2, C-3 and C-4). The first three compounds increased the dehydrogenases activity by 40–50% and the enentiomeric excesses by 17–43%, whereas the fourth one—toluchinon, occurred to be an inhibitor. Compound C-2 considerably affects the diastereoselectivity of the reaction. It activates the *cis*-reduction dehydrogenases, but unfortunately, it does not inhibit the dehydrogenase responsible for the reduction to *trans* alcohol.

Crotonaldehyde (C-6), an aliphatic aldehyde with an α,β-unsaturated bond, inhibits the bioreduction, whereas the similar compound – 6-methylhex-5-en-2-one (C-7) – has the opposite effect. The shift in a double bond position in relation to the carbonyl group changes ΔA from −83% for C-6 to +103% for C-7, whereas Δee changes from −32% for C-6 to +42% for C-7. The similar results were observed for bromine derivatives: the compounds B-1 and B-3 have β-unsaturated bonds and both show inhibiting activity, which is lost for compound B-2 with a double bond in the α-position and for B-4 with no double bond at all. Interestingly, an introduction of the second bromine atom to 1-bromopropane (B-4) increases the activity of the dehydrogenase by +60%, and the enantioselectivity by 59% (compound B-5).

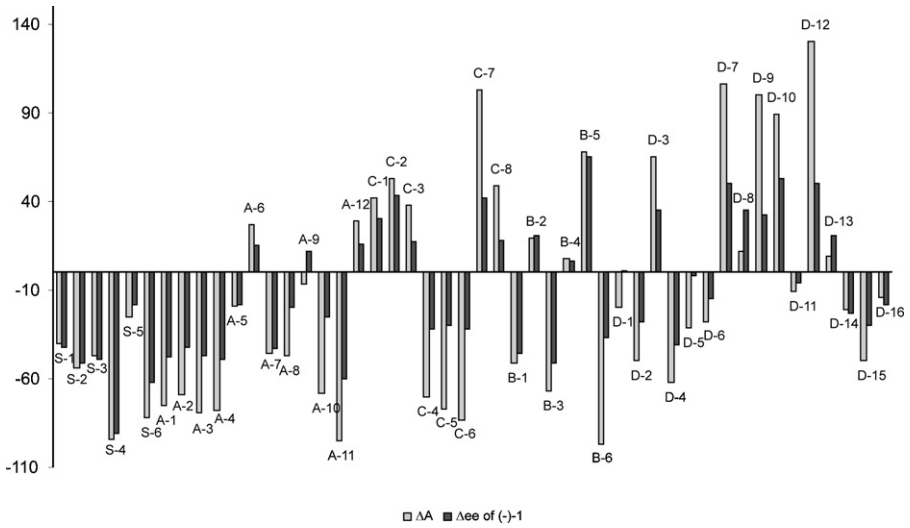


Fig. 6. Effect of additives on differences in enzymatic activity and enantiomeric excesses calculated for samples with additives and for control samples. $\Delta A = A_A - A_C \times 100\%/Ac$, $A = a\,b\,h^{-1}\,g^{-1}$ (a, amount of substrate [μmol]; b, percent of conversion/100; h, time [h]; g, mass of lyophilized mycelium [g]) A_A, enzymatic activity of the microorganisms with additives; A_C, enzymatic activity of the microorganism-control. $\Delta ee = ee_A - ee_C$; ee_A, enantiomeric excesses of the microorganisms with additives; ee_C, enantiomeric excesses of the microorganism-control.

In general, we have observed, that the tested organic acids and their derivatives, except for n-butyl acrylate (A-6; $\Delta A = 27\%$; $\Delta ee = 15\%$) and glutaric anhydride (A-12; $\Delta A = 29\%$; $\Delta ee = 16\%$), inhibit the activity of the dehydrogenases.

Allylic alcohol (D-3; $\Delta A = 38\%$; $\Delta ee = 17\%$) is an activator for oxidoreductases, whereas the replacement of the hydroxyl group with CN (D-2) or Br (B-3) inhibits the bioreduction. The aromatic compounds: acenaphthenequinone (C-5), benzyl bromide (B-6), 3-chloroperbenzoic acid (D-15) act as inhibitors.

To sum up, 19 out of the 48 tested additives proved to be strong dehydrogenase inhibitors. We have selected 5 compounds which increase the activity and the enantioselectivity of oxidoreductases in the examined bioreduction process using *Absidia coerulea* AM 93: C-7 ($\Delta A = 103\%$; $\Delta ee = 42\%$), D-7 ($\Delta A = 106\%$; $\Delta ee = 50\%$), D-9 ($\Delta A = 100\%$; $\Delta ee = 32\%$), D-10 ($\Delta A = 89\%$; $\Delta ee = 53\%$), and D-12 ($\Delta A = 130\%$; $\Delta ee = 50\%$).

The observed strongly activating effect of the molecules D-7, D-9, D-10, and D-12 on the dehydrogenases comes from the fact that these compounds can be easily attachable by microbial enzymes. Probably, the studied dehydrogenase is an allosteric enzyme, because it is activated by small compounds (C-7, D-9), and also by large, spatial molecules (D-7, D-9) which force change of conformation. Compounds D-7 and D-9 have ionic bond with an organic ligand. Zyrcone as a transition metal may form coordination bonds, as well as the highly polarized HMPA molecule. However, the most important may be the presence of a nitrogen atom that could be coordinated to metal in the active site.

For further research we used the Eschenmoser salt as an activator, due to its small molecule which is not troublesome in the product mixture.

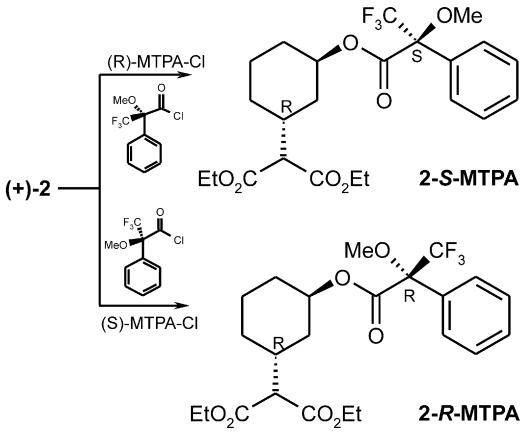
The final optimized biotransformation conditions are as follows: temperature: 28 °C; growth medium: 1% peptone and 3% glucose in deionized water; pH: 3.4–3.8. The microorganism was incubated with the Eschenmoser salt for 1 h before adding the substrate in concentration of 1 g/dm^3. After 12 h of the reaction we observed almost ideal resolution (GC analysis of the crude reaction mixture). After chromatographic separation we obtained (−)-diethyl 2-((1*S*)-3-oxocyclohexyl)malonate ((-)-**1**) {$[\alpha]_{589}^{20} = -3.4$ ($c = 4.25$, $CHCl_3$) (ee = 98%)} and (+)-diethyl 2-((1*R*, 3*R*)-3-hydroxycyclohexyl)malonate ((+)-**2**) {$[\alpha]_{589}^{20} = +1.2$ ($c = 3.7$, $CHCl_3$) (ee = 99%)}.

3.3. Determination of absolute configuration

Absolute configuration of diethyl 2-(3-oxocyclohexyl)malonate (**1**) was confirmed by a comparison of the sign of optical rotation with the literature data [38–40]. Absolute configuration of the carbon bearing hydroxyl group in (+)-**2** was determined by the Mosher ester method [41]. Single enantiomers of the alcohol were treated separately with two enantiomers of the Mosher's acid chloride (Scheme 2).

The resulting pair of diastereomeric esters (**2-*S*-MTPA**, **2-*R*-MTPA**) was analyzed by high field ^{1}H MNR, ^{13}C NMR, COSY, DEPT and HMQC, in $CDCl_3$ as a solvent.

The absolute configuration of alcohol (+)-**2** was determined by interpretation of the sign of $\Delta\delta^{SR}$ value, using empirical models [41]. This method is based on the anisotropic effect between the phenyl group of the chiral auxiliary MTPA with the substituents L1 and L2 of the alcohol. Mosher assumed that the ***R***- and ***S*-MTPA** esters of secondary alcohols exist in a conformation in which CH(OR), the carbonyl, and the CF_3 groups are situated in the same plane. When ligand L1 ($^2CH_2$) is more bulky than L2 ($^4CH_2$), it results in a negative value of $\Delta\delta^{SR}$ (in both ^{1}H NMR and ^{13}C NMR). In the case of MTPA-derivative of (+)-**2** we observed $\Delta\delta^{SR} < 0$, which means that the absolute configuration of the carbon bearing hydroxyl group in (+)-**2** alcohol is *R* (Fig. 7).



Scheme 2.

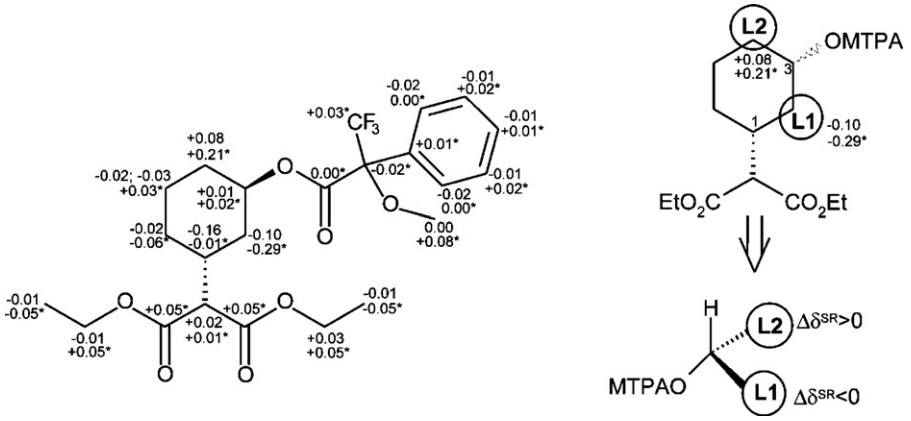
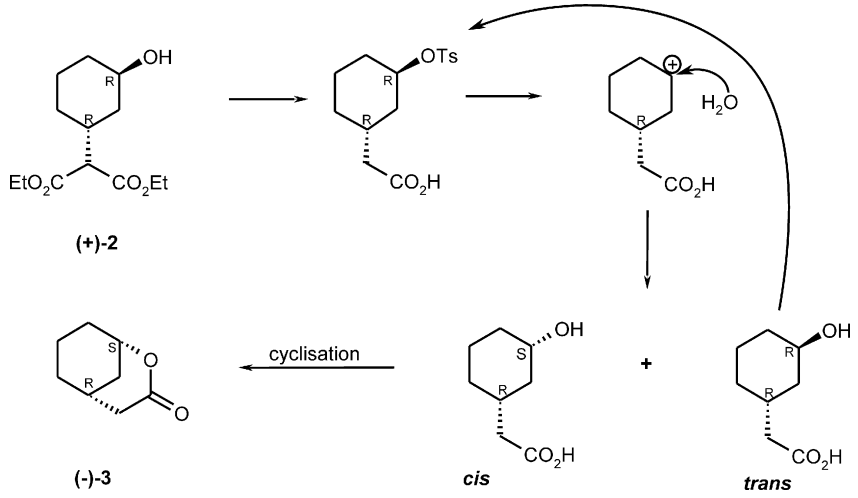


Fig. 7. $\Delta\delta$ values for **2-*S*-MTPA** and **2-*R*-MTPA** calculated from ^{1}H NMR and ^{13}C NMR spectra. *$-\Delta\delta$ for ^{13}C NMR.



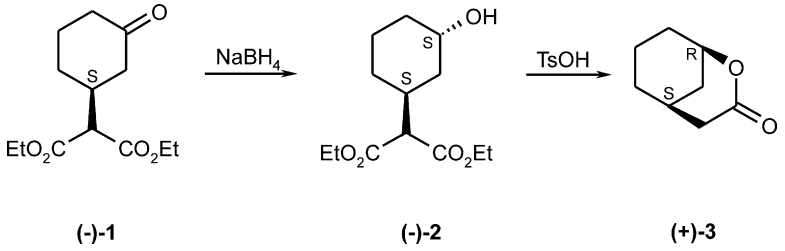
Scheme 3.

3.4. Lactonization of (+)- and (−)-isomer of diethyl 2-(3-hydroxycyclohexyl)malonate (*2*)

Chemical reduction of diethyl 2-(3-oxocyclohexyl)malonate (**1**) with borohydride derivatives led to a diastereoisomeric mixture of *trans* and *cis* alcohols (ratio 9:1). Under acidic conditions [15% BF_3 in diethyl eter or THF:H_2O:$HClO_4$ (pH = 1), or TsOH × H_2O in benzene or toluene] racemic *cis* diethyl 2-(3-hydroxycyclohexyl)malonate cyclized to the corresponding bicyclic lactone: 2-oxabicyclo[3.3.1]nonan-3-one (±)-**3** [42,43]. Under the same conditions the *trans*-diastereoisomer of **2** did not undergo this process. Refluxing of *trans* diethyl 2-(3-hydroxycyclohexyl)malonate (*trans*-(±)-**2**) with p-toluenosulfonic acid in benzene led to the unsaturated diester as the main product (80%). The product of dehydratation was more stabile than lactone **3**. Therefore, at first we hydrolyzed *trans* diethyl 2-(3-hydrocyclohexyl)malonate (**1**) to the corresponding mono acid, and in next step, we performed lactonization by refluxing the acid with TsOH × H_2O in benzene [44]. As a result, the desired *cis* lactones were obtained.

The inversion of configuration is the result of formation of a tosylate, which is an easy-leaving group. The carbocation formed is then attacked by a water molecule, which leads to a mixture of *cis* and *trans*-diastereoisomers of 2-(3-hydroxycyclohexyl)propionic acid. The *cis* isomer closes to *cis* lactone (−)-(1*S*, 5*R*)-2-oxabicyclo[3.3.1]nonan-3-one ((−)-**3**) $\{[\alpha]_{589}^{20} = -18.6$ ($c = 2.05$, $CHCl_3$), ee = 95%$\}$, whereas the *trans* one is tosylated again (Scheme 3).

The unreacted diethyl (−)-2-(3-oxocyclohexyl)malonate ((−)-**1**) was isolated from the biotransformation mixture and reduced



Scheme 4.

with sodium borohydride to the corresponding *trans* hydroxy diester (−)-**2** {$[\alpha]_{589}^{20} = -1.04$ (c = 3.11, $CHCl_3$)} with 95% ee.

(+)-(1*R*, 5*S*)-2-oxabicyclo[3.3.1]nonan-3-one ((+)-**3**) was prepared from (−)-diethyl 2-((1*R*, 3*S*)-3-hydroxycyclohexyl)malonate (−)-**2** in the same way as the opposite isomer {$[\alpha]_{589}^{20} = +17.9$ (c = 1.5, $CHCl_3$), ee = 93%} (Scheme 4).

4. Conclusions

To sum up, we obtained (+) and (−) isomers of 2-oxabicyclo[3.3.1]nonan-3-one (**3**) from the corresponding two enantiomers of *trans* diethyl 2-(3-hydroxycyclohexyl)malonate (**2**).

Four fungal strains: *Absidia coerulea* AM 93, *Beauveria bassiana* AM 278, *Penicillium notatum* AM 904, *Rhodotorula rubra* AM 4 have ability to resolve racemic diethyl 2-(3-oxocyclohexyl)malonate (**1**) by enantioselective reduction.

Highly enantioselective biotransformation by *Absidia coerulea* AM 93 leading to formation of (−)-diethyl 2-((*S*)-3-oxocyclohexyl)malonate (**1**) (ee = 98%) and (+)-diethyl 2-((1*R*, 3*R*)-3-hydroxycyclohex-1-yl)malonate (**2**) (ee = 99%) can be an interesting alternative for the enantioselective Michael addition.

The biotransformation conditions were optimized. We have selected 5 compounds which considerably increase both the activity and enantioselectivity of the oxidoreductases of *Absidia coerulea* AM 93. These are: acetonylacetone C-7 (ΔA = 103%; Δee = 42%), cinchonine sulphate D-7 (ΔA = 106%; Δee = 50%), Eschenmoser salt D-9 (ΔA = 100%; Δee = 32%) (ΔA = 100%; Δee = 32%), zircocene dichloride D-10 (89%), and HMPA D-12 (ΔA = 130%; Δee = 50%).

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