Chemoenzymatic Synthesis of (*S*)-8-*O*-Methylmellein by *Candida parapsilosis* Carbonyl Reductase

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Abstract: A screening of various *ortho*substituted 2-oxopropylbenzene derivatives with carbonyl reductases and alcohol dehydrogenases, respectively, revealed that compounds containing *ortho*-substituents with only little steric demand are feasible substrates. (*S*)-8-*O*-Methylmellein (**1**) was synthesized by stereoselective enzymatic reduction of 2-methoxy-6-(2-oxopropyl)benzonitrile (**5i**) with *Candida parapsilosis* carbonyl reductase as the key step, and completed by a one-step hydrolysis and intramolecular cyclization.

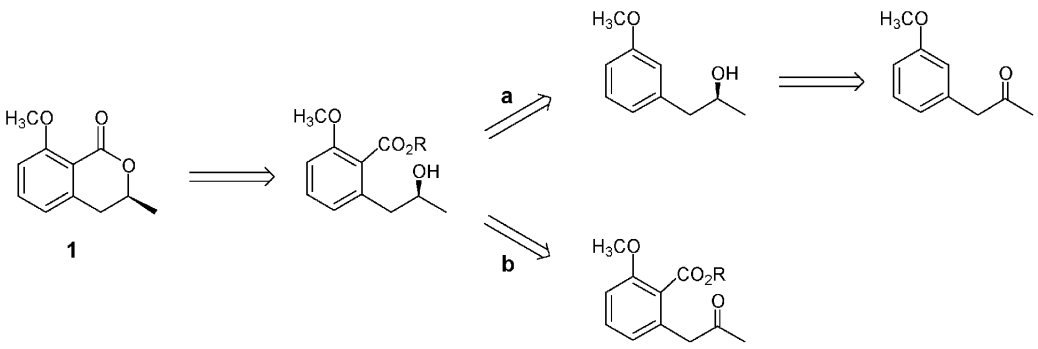
Key words: stereoselective synthesis, dihydroisocoumarin, alcohol dehydrogenase, oxidoreductase, Weinreb reaction

In the past 40 years more than 160 dihydroisocoumarins have been isolated and identified from microbial, plant and insect sources.[1,2] Members of this class of natural products exhibit a wide range of biological activities like antitumour,[3] antibiotic[4] and antiviral[5] properties, making them interesting as pharmaceutical agents. The key step in most chemical syntheses is the stereoselective preparation of a 2-(2-hydroxypropyl)benzoic acid ester derivative to obtain the desired product after cyclization. Therefore, a number of chemical syntheses applying chiral auxiliaries have been published using e.g. the Sharpless epoxidation,[6,7] the Sharpless asymmetric dihydroxylation[8] or the chiral pool.[9] The scope of introducing the stereocenter by an enzymatic reduction of the corresponding prochiral benzyl methyl ketone, however, is limited, partially because of the bulky *ortho* ester group that hinders the molecular recognition in an enzyme/substrate complex. Therefore, the reduction of 4,6-dimethoxy-2-(2-oxopropyl)benzoic acid methyl ester with baker´s yeast (*Saccharomyces cerevisiae*) gave only 25% isolated yield after 42 days, yet 99% ee.[10] Reduction of the same benzyl methyl ketone by chemical methods resulted in only moderate stereochemical control (< 62% ee).[10] To circumvent this problem, Napolitano et al. applied the *ortho*-metallation[11] to introduce the carboxyl moiety after generating the stereocenter (Scheme 1, pathway a),[12] which affords a multistep protecting-group strategy. Our aim was the most straight forward approach, utilizing isolated enzymes for reduction of the entirely substituted benzyl methyl ketone (Scheme 1, pathway b).

To our knowledge, there have been no reports so far, of any dihydroisocoumarin synthesis utilizing an isolated enzyme to introduce chirality. The preferred enantiomer obtained by this enzymatic synthesis exhibits the (*S*)-configuration as most of the naturally occurring dihydroisocoumarins do.[1]

We effected on an enzyme screening with oxidoreductases accepting (2-oxopropyl)benzene **(5a)** as a substrate for reduction. Unfortunately, the carbonyl reductase from *Candida parapsilosis* (*CP*CR) [EC 1.1.1.184], and the alcohol dehydrogenases from *Lactobacillus brevis* (*LB*-ADH), *Rhodococcus erythropolis* (*RE*-ADH), *Thermoanaerobium brockii* (*TB*-ADH), horse liver (HL-ADH) and *Saccharomyces cerevisiae* (Y-ADH) [all EC 1.1.1.1], all showed no reduction activities on 2-(2-oxopropyl)benzoic acid methyl ester (**5b**), the ethyl ester **5c**, or on the free acid.

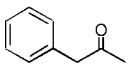
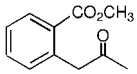
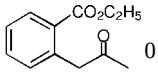
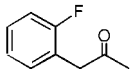
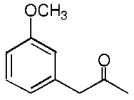
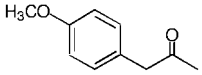
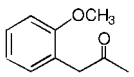
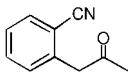
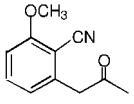
In a next step, we carried out a substrate screening to determine whether (2-oxopropyl)benzene derivatives with less bulky *ortho* substituents are accepted for reduction.



Scheme 1

The enzymes *CP*CR and *LB*-ADH were chosen because of their already known substrate ranges, and also since they reduce (2-oxopropyl)benzene **(5a)** itself in excellent yields and in high ee values.[13] Both enzymes were found to reduce 2-fluoro-(2-oxopropyl)benzene **(5d)**, the compound with the least bulky functional group attached to the *ortho*-position. Also, *CP*CR tolerated the methoxy group **(5g)**, however, the three possible methoxy regioisomers **(5e-g)** pointed out once more the hindering influence of the *ortho* substitution (Table). Nevertheless, the remaining enzymatic activity is sufficient for synthetic applications. Because the methoxy- and the cyano-group show similar steric demand,[14] we also tested nitriles **(5h,i)**, which should be hydrolyzed and cyclized to the corresponding dihydroisocoumarin after enzymatic reduction of the keto group. As expected, *CP*CR tolerated the substrates containing a nitrile-functionality in the *ortho*-position.

Table Substrate Screening

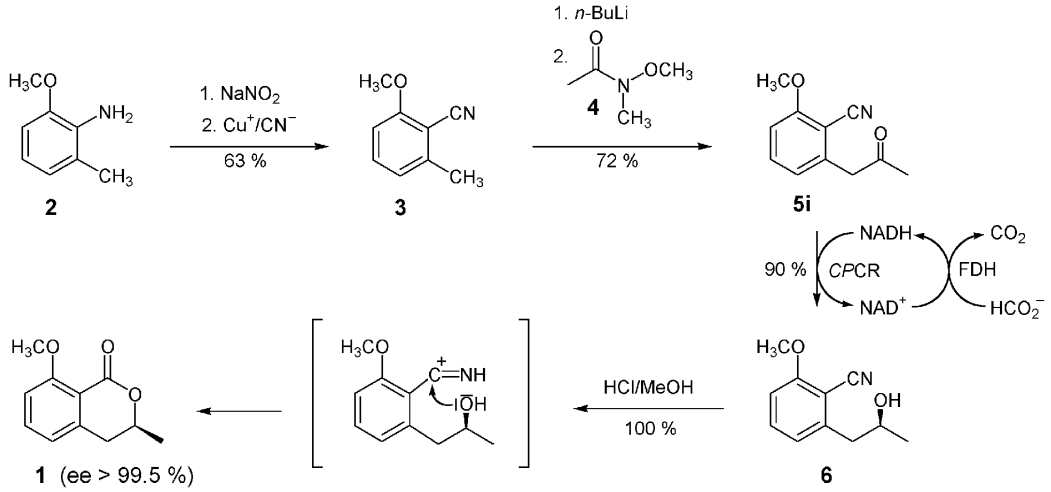
Compound	Substrate[a]	Activity *CP*CR (%)[b]	Activity *LB*-ADH (%)[b,c]
5a		100	100
5b		0	0
5c		0	0
5d		47	100
5e		64	58
5f		61	25
5g		30	0
5h		30	0
5i		21	0

[a]Assay conditions were: 5 mM substrate, 0.25 mM NAD(P)H, 100 mM TEA/NaOH buffer, pH 7.
[b]Enzyme activity was determined by the decrease of the NAD(P)H extinction at 340 nm relative to 2-oxopropylbenzene.
[c]The additionally tested enzymes *RE*-ADH, *TB*-ADH, HL-ADH and Y-ADH showed no superior reduction potency.

2-Methoxy-6-(2-oxopropyl)benzonitrile (**5i**) was obtained by a simple two-step synthesis starting from **2** via a Sandmeyer reaction using Cu(I)CN to give 2-methoxy-6-methylbenzonitrile (**3**).[15] In the second step, a Weinreb acylation[16] using *N*-methoxy-*N*-methylacetamide (**4**) in the presence of a stoichiometric amount of lithium diisopropylamide (LDA) was carried out. The enzymatic reduction of **5i** with *CP*CR (U_S = 14.2 U/mL) was done on a 200 mg scale, using the formate dehydrogenase (FDH), from *Candida boidini*; EC 1.2.1.2/formate system[17] for regeneration of NADH. The crude product **6** was used without further purification in the following hydrolyzation step. It was refluxed in a solution of concentrated hydrochloric acid/methanol (1:1, v:v)[18] for 12 h (Scheme 2). This led to the desired (*S*)-8-*O*-methylmellein (**1**) in quantitative yield and high optical purity (ee > 99.5%). The ee % was determined with GC, by comparison with racemic **1**, which was obtained through methylation of commercially available (±)-mellein (Sigma). The absolute configuration (*S*) was deduced by comparison of the optical rotation with literature data.[6] The driving force of the hydrolysis must be the attack of the hydroxyl group to the iminium cation (Scheme 2), since doubly *ortho*-substituted nitriles usually sustain these reaction conditions.[15] Accordingly, nitrile **5i** remained in the reaction mixture after the enzymatic reduction. It was not affected by the acidic hydrolysis and could be separated by column chromatography.

In summary we have developed a short and convenient chemoenzymatic synthesis of (*S*)-8-*O*-methylmellein (**1**) that should further enable easy access to a great variety of dihydroisocoumarins. The reduction of nitrile **5i** by *CP*CR is followed by an easy one-pot procedure to obtain (*S*)-8-*O*-methylmellein (**1**) in an overall yield of 90% and excellent enantiomeric excess of > 99.5%. We also showed that the nitrile functionality can be used advantageously as a mimic for the steric demanding carboxylic moiety in a chemoenzymatic synthesis.

All solvents were used in p.a. quality and if necessary dried by standard methods. Chemicals were purchased from Aldrich, Lancaster and Fluka. *TB*-ADH, HL-ADH and Y-ADH were purchased from Sigma. *CP*CR, *LB*-ADH and *RE*-ADH were isolated at the Institute of Enzymetechnology, University of Düsseldorf. 2-Methoxy-6-methylbenzonitrile (**3**) was prepared by a Sandmeyer-reaction from 2-methoxy-6-methylbenzeneamine (**2**).[15] Benzoic acid esters **(5b,c)** were prepared by decarboxylation and acylation of homophthalic acid,[19] completed by esterification according to published procedures.[20] TLC was performed using Merck silica gel $60F_{254}$. Flash column chromatography was done using silica gel (Merck, 60 mesh). All mps (B-540, Büchi) are uncorrected. ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C (75.5 MHz, $CDCl_3$) spectra were recorded on an AMX-300 (Bruker Physik AG) spectrometer. IR spectra (KBr pellets) were measured with a Pye Unicam SP 1000 spectrophotometer. Chrompack CP9002 using a *heptakis* (2,3,6-*tri*-*O*-methyl)-β-cyclodextrin/polysiloxane-column (50.0 m x 320 µm) was used for GC. Optical rotations were measured on a Perkin Elmer 241 polarimeter. GCMS: Hewlett Packard (HP 6890 series) GC-system fit-



Scheme 2

ted with a HP 5973 mass selective detector (EI, 70 eV) and a HP-5MS column. HRMS and microanalyses were performed at the analytical department, " Kekulé-Institut für Organische Chemie und Biochemie" (University of Bonn, Germany).

2-(2-Oxopropyl)benzonitrile (5h);[21] Typical Procedure

A solution of $(i\text{-Pr})_2NH$ (0.7 mL, 5.0 mmol) and BuLi (3.1 mL, 5.0 mmol; 1.6 M in hexane) in THF (15 mL) was stirred under N_2 at r.t. for 5 min and then cooled to –78°C. 2-Methylbenzonitrile (586 mg, 5.0 mmol) dissolved in THF (15 mL) was added and the red solution was stirred for 10 min. Amide **4** (0.55 mL, 5.5 mmol) was then added dropwise, stirring was continued for 1 h at –78°C before adding 2 M HCl (15 mL). The solution was warmed up to r.t., additional 2 M HCl (50 mL) was added and the mixture extracted with CH_2Cl_2 (3 x 50 mL). The organic layer was dried (Na_2SO_4) and evaporated. Flash column chromatography (EtOAc) yielded the nitrile **5h** (580 mg, 73%) as a yellow oil, R_f = 0.61.

^{1}H NMR: δ = 2.23 (s, 3H, CH_3), 3.95 (s, 2H, CH_2), 7.4-7.6 (m, 4H, ArH).

^{13}C NMR: δ = 30.2 (CH_3), 48.7 (CH_2), 113.3 (ArC_q), 117.9 (CN), 127.8, 131.0, 132.8, 133.0 (ArCH), 138.2 (ArC_q), 203.7 (CO).

GCMS: t_R = 8.59 min, *m/z* (%) = 159 (17) [M^+], 117 (100), 89 (50), 63 (34), 51 (12).

2-Methoxy-6-(2-oxopropyl)benzonitrile (5i)

Compound **5i** was prepared using **3** (740 mg, 5.0 mmol). Purification by flash column chromatography (PE:EtOAc, 1:1) afforded **5i** (684 mg, 72%) as a yellow oil, R_f = 0.36.

IR : ν = 2220, 1725, 1588, 1485, 1445, 1330, 1290, 1165, 1085, 770, 738 cm^{-1}.

^{1}H NMR: δ = 2.20 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2), 6.80 (m, 2H, ArH), 7.40 (m, 1H, ArH).

^{13}C NMR: δ = 30.1 (CH_3), 48.7 (CH_2), 56.2 (OCH_3), 102.8 (ArC_q), 109.9 (ArCH), 115.5 (CN), 122.6, 134.0 (ArCH), 140.0, 161.8 (ArC_q), 203.7 (CO).

GCMS: t_R = 10.41 min, *m/z* (%) = 189 (16) [M^+], 147 (100), 118 (31), 104 (11), 89 (14), 76 (15), 63 (8.6), 51 (7.5).

HRMS : *m/z* Calcd. for $C_{11}H_{11}NO_2$:189.0784. Found:189.0787.

Anal.Calcd for $C_{11}H_{11}NO_2$: C, 69.8; H, 5.9; N, 7.4. Found: C, 69.4; H, 5.9; N, 7.9.

(*S*)-2-(2-Hydroxypropyl)-6-methoxybenzonitrile (6)

Nitrile **5i** (200 mg, 1.1 mmol), NAD^+ (35 mg, 50 µmol) and sodium formate (17 g, 250 mmol) were dissolved in 100 mM triethanolamine buffer (250 mL, pH 7). The solution was adjusted to pH 7 and FDH- (200 µL, 57 U/ml), and *CP*CR-soln (200 µL, U_S = 14.2 U/mL) were added. The progress of the reaction was controlled by GCMS while additional *CP*CR (200 µL) was added every 12 h (because of denaturing and inhibiting effects to the enzyme) up to a total of 1 mL (14.2 U). After a total reaction time of 96 h, the solution was acidified with 2M HCl (50 mL) and extracted with CH_2Cl_2 (3 x 50 mL). The organic layer was dried (Na_2SO_4) and evaporated to give **6** (180 mg, 90%), 92% purity (GCMS). This crude product was used without further purification in the hydrolysis step.

IR : ν = 3370, 2985, 2220, 1725, 1600, 1585, 1480, 1440, 1285, 1215, 1080, 775 cm^{-1}.

^{1}H NMR : δ = 1.21 (d, 3H, $J_{H,H}$ = 6.2Hz, CH_3), 2.80-2.95 (m, 2H, CH_2), 3.85 (s, 3H, OCH_3), 4.05 (m, 1H, CH), 6.75-6.95 (m, 2H, ArH), 7.45 (m, 1H, ArH).

^{13}C NMR : δ = 23.3 (CH_3), 44.1 (CH_2), 56.2 (OCH_3), 68.3 (CHOH), 102.6 (ArC_q), 109.1 (CN), 116.0 (ArC_q), 122.7, 133.8, 144.7 (ArCH), 162.0 (ArC_q).

GCMS: t_R = 10.56 min, *m/z* (%) = 191 (M^+, 1.6%), 147 (100), 118 (43), 104 (15), 89 (12), 77 (14), 63 (4.6), 51 (5.5).

(*S*)-8-*O*-Methylmellein (1)[6]

Compound **6** (166 mg, 0.86 mmol) was dissolved in 32% HCl/MeOH (1:1, v/v; 40 mL). The solution was heated for 12 h under reflux, while the progression was controlled by GCMS. After complete conversion the product was extracted with CH_2Cl_2 (3 x 50 mL). The organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by flash column chromatography (PE:EtOAc, 1:1) to afford **1** (156 mg, 94%) as a colorless solid (ee > 99.5%); mp = 87°C (Lit.[6] mp = 86.5-87.5°C); $[\alpha]^{26}_D$ = +257 (c =0.5, $CHCl_3$) [Lit.[6] $[\alpha]^{26}_D$ = +261 (c = 0.52, $CHCl_3$)].

^{1}H NMR : δ = 1.39 (d, 3H $J_{H,H}$ = 6.4 Hz, CH_3), 3.80 (d, 2H $J_{H,H}$ = 4.7 Hz, CH_2), 3.86 (s, 3H, OCH_3), 4.47 (m, 1H, CH), 6.75 (m, 2H, ArH), 7.39 (m, 1H, ArH).

^{13}C NMR : δ = 21.0 (CH_3), 36.4 (CH_2), 56.6 (OCH_3), 74.5 (CH), 111.2 (ArCH), 114.0 (ArC_q), 119.6, 134.9 (ArCH), 142.3, 161.5 (ArC_q), 163.1 (CO).

GC (180°C): t_R = 83.68 min.

GCMS: = 10.98 min, *m/z* (%) = 192 (M^+, 82), 177 (6.8), 148 (100), 118 (34), 105 (56), 90 (93), 77 (43), 63 (16), 51 (30).

HRMS (M^+): *m/z* Calcd. for $C_{11}H_{12}O_3$: 192.0786. Found: 192.0779.

***rac*-8-*O*-Methylmellein**

Racemic mellein was methylated with methyl iodide in a suspension of potassium hydroxide in dimethyl sulfoxide, according to literature methods.[22]

GC (180 C): t_R = 82.15 min (*R*-enantiomer), 83.61 min (*S*-enantiomer).

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