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Microbial alcohol dehydrogenase screening for enantiopure lactone synthesis: Down-stream process from microtiter plate to bench bioreactor

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ABSTRACT

One-pot conversion with whole cells of bacteria was performed for biooxidation of *meso* monocyclic (**3a–b**) and bicyclic diols (**3c–e**) into corresponding chiral lactones of bicyclo[4.3.0]nonane structure (**2a–b**) as well as *exo*- and *endo*-bridged lactones with the structure of [2.2.1] (**3c–d**) and [2.2.2] (**3e**). *Micrococcus* sp. DSM 30771 was selected as biocatalyst with significant alcohol dehydrogenase activity. Among tested strains, microbial oxidation of *meso* diols **3a–e** catalyzed by *Micrococcus* sp. afforded enantiomerically pure ((+)-(2*S*,3*R*)-**2c** (ee = 99%), (+)-(2*S*,3*R*)-**2e** (ee = 99%)) or enriched ((+)-(1*S*,5*R*)-**2a** (ee = 90%), (−)-(1*S*,5*R*)-**2b** (ee = 86%), (+)-(2*S*,3*R*)-**2d** (ee = 80%)) lactone moieties. Comparative study with respect to microbial cultivation as well as biooxidation was undertaken to verify agreement of secondary metabolite biosynthesis in different scales: from MTP (4 mL), across shake flask (100 mL) till bioreactor (4 L). The results from biotransformations showed quite similar dependence in oxidation of all substrates **3a–e** in MTP and flasks as well, thereby confirmed the validity and reasonable approach of using MTP for preliminary studies.

1. Introduction

A large number of redox reactions in organic chemistry are still based on heavy metals, many of which are expensive, toxic, environmentally unacceptable and difficult to remove from the products mixture. As a consequence, any metal-independent method to perform oxidation reactions would present a viable alternative. Whole cells or pure enzymes biotransformations are definitively attractive approach due to a number of benefits.

Among different biocatalytic strategies of synthesis of optically active lactones, based on kinetic resolutions [1,2] and stereoselective reactions [3–7], we are especially interested in biooxidation of diols. One-pot oxidation catalyzed by native alcohol dehydrogenase from horse liver (HLADH) of nonsymmetric aliphatic 1,4- and 1,5-; 1,6-diols to corresponding γ- [6], δ- and ε-lactones [3] have been evaluated by our research group so far. It is worth to mention that HLADH isolated from horse liver was extensively used in the 80s [8–12]. The extremely rare function of HLADH was due to chemoselective oxidation of only one of the enantiotopic hydroxy group of *meso* diols possessing acyclic, monocyclic and bicyclic structures. Unfortunately, native HLADH is not available anymore, but a recombinant HLADH *Escherichia coli* was recently prepared and commercially distributed. A comparative study was performed by using both enzymes, unfortunately rec-HLADH was not as stereoselective as the native HLADH [6,13]. Therefore, we have recently extended screening of effective redox enzymes, by using other several commercially available alcohol dehydrogenases, however none of them proved to be promising enzyme capable to play a crucial role in synthesis of enantiomerically pure lactones. Hence, it is purposeful to look for new biocatalysts able to conduct one-step conversion of racemic diols to optically pure lactones.

Microtiter plates have recently become an attractive alternative to shake flasks mainly because of the possibility of automation and miniaturization. Application of MTPs ensure among others high aeration rates and mixing intensity, no splashing, low media

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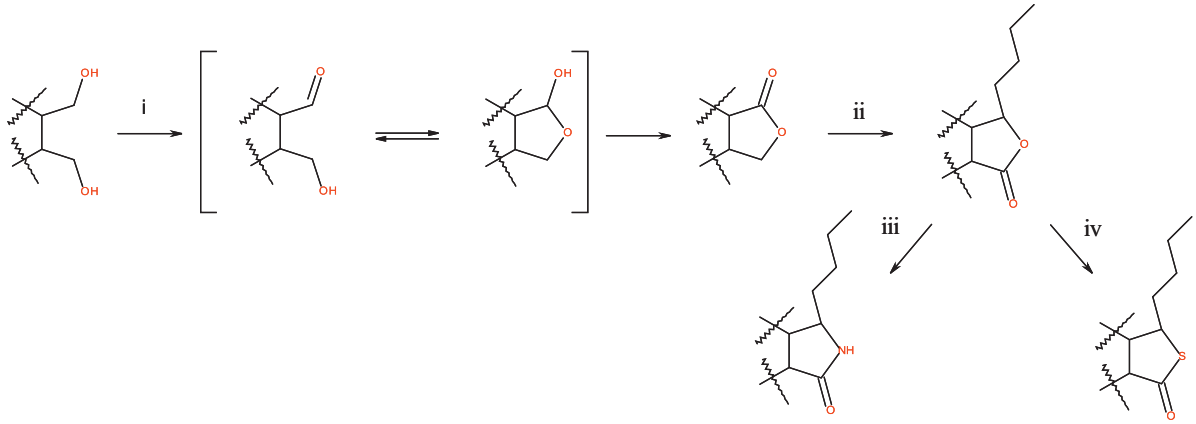

i- bacteria; ii- (1) n-C_4H_9MgBr (2 equiv.), $Zn(BH_4)_2$ (0.25 equiv.), THF, rt, (2) HCl_{aq}, (3) TPAP(cat.), NMO, CH_2Cl_2, 0°C;

iii- NH_3 H_2O, p, T (in Berghof reactor); iv- Lawesson's reagent, BF_3 Et_2O.

Fig. 1. Application of highly stereoselective biooxidation step in biosynthesis of optically active phthalide derivatives.

evaporation and the last but not least impressive workload associated with time-savings as well as less risk of cross-contamination regarding their design of unique cover system, called *sandwich cover* [14,15]. A mature, easy-to-use, cost-effective and rapid handling system for the cultivation of large numbers of microbial strains was designed by *Duetz* [16]. The smaller requirements for biomass levels increase the role of MTPs both for research and industrial applications. Nowadays, a large choice in compatible side equipment for handling MTP (dispensers, microplate readers, centrifuges, multichannel pipettes, pipetting robots, etc.) are applied for biocatalyst identification, enzymatic assays and immunoassays, evaluation of kinetic data, elucidation of metabolic pathways, media development, establishment of analytical protocols and cell cultivation [15,17–21].

In order to better exploit the results of MTP experiments, it was important to precisely ascertain the scalability of MTP to standard laboratory fermenter. Therefore, comparative study was undertaken to verify agreement of strain physiological data and secondary metabolite biosynthesis in different scales, from MTP (4 mL) across shake flask (100 mL) till bioreactor (4 L). In this paper we have performed microbial transformations of selected *meso* monocyclic (**3a–b**) and bicyclic diols (**3c–e**) affording enantiomerically pure and enriched corresponding bicyclic (**2a–b**) and tricyclic lactones (**2c–e**) with previously defined fungistatic activity.[13] Elaboration of highly stereoselective biooxidation step will be crucial in multi-step synthesis of enantiomerically pure lactones of bicyclo[4.3.0]nonane structure comprising a large group of phthalide derivatives. Such lactones with C4 butyl chain in the structure will be obtained in two step synthesis on the basis of literature data [22]. Besides, this process will open the door to biosynthesis of other optically active phthalide derivatives among them lactames and thiolactones, and their corresponding biological activity will be tested (Fig. 1).

2. Materials and methods

2.1. Analysis

^{1}H NMR and ^{13}C NMR spectra were recorded for $CDCl_3$ solutions on a Bruker Avance™ 600 (600 MHz) spectrometer. IR spectra were determined on a Thermo-Nicolet IR300 FT-IR spectrometer. Optical rotation was measured on an Autopol IV automatic polarimeter (Rudolph) in $CHCl_3$ solutions, concentrations denoted in g/100 mL. Analytical TLC technique (SiO_2, DC-Alufolien Kieselgel 60 F_{254}, Merck) were performed with solvent system: methylene chloride–methanol, 95:5. Visualization was made using a solution of 1% $Ce(SO_4)_2$ and 2% phosphoromolybdenic acid in 10% H_2SO_4, followed by heating. Preparative column chromatography (SiO_2, Kieselgel 60, 230–400 mesh, 40–63 μm, Merck) was performed with application of methylene chloride–methanol (95:5) or hexane–acetone (3:1) as eluent. Gas chromatography analysis (GC, FID, carrier gas H_2) was carried out on Agilent Technologies 7890N (GC System) with HP-5 column (cross-linked methyl silicone, 30 m × 0.32 mm × 0.25 μm). Enantiomeric excesses of the products were determined on chiral columns: Cyclosil-B (30 m × 0.25 mm × 0.25 μm) for lactones **2a**, **2b**, **2c**, **2e** and Astec Chiral-DEX B-PM (30 m × 0.25 mm × 0.12 μm) for lactone **2d**. Lactones compound were separated and identified by GC–MS according the next temperature programs: **2a**, **2b** and **2c** 120 °C, 220 °C (10 °C/min), 250 °C (20 °C/min) (1 min). The total run time was 12.5 min; **2e** 80 °C, 220 °C (7 °C/min), 250 °C (20 °C/min) (1 min). The total run time was 22.5 min; **2d** 80 °C, 120 °C (1 °C/min), 200 °C (20 °C/min) (5 min). The total run time was 49.0 min.

Retention times were established for lactones as follow:

t_R (−)-(1*R*,5*S*)-**2a** = 6.17 min, t_R (+)-(1*S*,5*R*)-**2a** = 6.23 min.
t_R (+)-(1*R*,5*S*)-**2b** = 6.62 min, t_R (−)-(1*S*,5*R*)-**2b** = 6.70 min.
t_R (−)-(2*R*,3*S*)-**2c** = 7.69 min, t_R (+)-(2*S*,3*R*)-**2c** = 7.79 min.
t_R (+)-(2*S*,3*R*)-**2d** = 42.51 min, t_R (−)-(2*R*,3*S*)-**2d** = 42.65 min.
t_R (+)-(2*S*,3*R*)-**2e** = 16.16 min, t_R (−)-(2*R*,3*S*)-**2e** = 16.65 min.

2.2. Chemicals

cis-4-Cyclohexene-1,2-dicarboxylic anhydride (**1b**), *cis*-5-norbornene-*endo*-2,3-dicarboxylic anhydride (**1c**), *cis*-5-norbornene-*exo*-2,3-dicarboxylic anhydride (**1d**), *endo*-bicyclo[2.2.2]oct-5-ene-2,3-dicarboxylic anhydride (**1e**) and $LiAlH_4$ were purchased from Sigma–Aldrich Chemical Co., while *cis*-cyclohexane-1,2-dicarboxylic anhydride (**1a**) was purchased from Fluka BioChemika.

2.3. Reduction of anhydrides *1a–e*

A solution of anhydride **1a–e** (6 mmol) in a mixture of diethyl ether (20 mL) and tetrahydrofuran (10 mL) was added dropwise to $LiAlH_4$ (8 mmol) in diethyl ether (20 mL). The mixture was stirred for 16 h under reflux. When the reaction was completed (controlled by GC, TLC), water was added to decompose the excess