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Effective fungal catalyzed synthesis of P-chiral organophosphorus compounds

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Abstract—An easy and efficient method for the microbial kinetic resolution of a substrate ethyl hydroxy(phenyl)methane(P-phenyl)phosphinate, which contains two stereogenic centers, is reported. To obtain the desired purpose biocatalyst, living fungal cells of *Geotrichum candidum* were cultivated with or without the supplementation of chemical additives influencing its activity. The biotransformation products were isolated with good yield and in enantiomerically pure forms.

1. Introduction

Microorganisms have been used for many years for the biological transformation of organic compounds. Recently, the use of both whole microorganisms and their enzymatic systems to carry out stereospecific and stereoselective reactions has taken on greater significance, as these reactions have demonstrated their usefulness in the asymmetric synthesis of the molecules of biological importance.[1,2] There are numerous methods, which have allowed us to improve the native enzyme nature and adapt them for particular reactions.[3–6] The processes based upon biotransformations belong to the field of 'Green' chemistry, which is an increasingly developed field in bioorganic synthesis.

Phosphonic acids, compounds containing the Lewis acid moiety R–CP(O)$(OH)_2$, are characterized by a stable, covalent carbon to phosphorus bond. Asymmetric synthesis of chiral phosphonates has gathered considerable attention, due to their application in the synthesis of other phosphorus compounds of various uses[7,8] and because of their potential biological activity.[9] Among this group, α-heteroatom substituted phosphinic acids are of particular interest because of their usefulness in the development of catalytic antibodies[10] and pharmacologically active chemicals.[11] It has been established that the biological activity of phosphonic acids is largely determined by the absolute configuration of the stereogenic α-carbon atom.[8] Therefore, there is a growing interest in the preparation of such compounds in enantiomerically pure forms. Microbial transformations of hydroxyphosphonates (hydroxyphosphinates) containing one asymmetric centre have been investigated for several years,[12] and so it seemed obvious that the biocatalysis of compounds with two stereogenic centers was only a matter of time. The application of a microbial culture for the biocatalysis of such hydroxyphosphinate compounds has recently been reported.[13–15] The first, but limited, application of biocatalysis in the deracemization of ethyl hydroxy(phenyl)methane(P-phenyl)phosphinate has also been reported.[15] Microbes preferentially hydrolyzed compound with an (*S*)-configuration at the α-carbon, whereas a lack of stereoselectivity was observed toward the phosphorus atom.

Previously reported studies allowed the resolution of only one pair of diastereoisomers of the starting material to enantiomerically pure forms.

Herein we have extended the scope of this approach by using fungal strains of different origins as biocatalysts for the enantioselective biotransformation of racemic ethyl hydroxy(phenyl)methane(P-phenyl)phosphinate.

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2. Results and discussion

Racemic ethyl hydroxy(phenyl)methane(P-phenyl)phosphinate was used as a substrate in the biocatalytic process catalyzed by different types of fungal strains. The racemic mixture consisted of four stereoisomers: a left pair (R_pR),(S_pS) and a right one (R_pS),(S_pR).[15]

Isolated enantiomers of the above-mentioned compounds are very attractive for further application as chiral building blocks in chemoenzymatic synthesis. Some effort has already been undertaken to separate all the enantiomeric forms of ethyl hydroxy(phenyl)methane(P-phenyl)phosphinate[15] but the results, based on the lipase activity, showed it to be unsuccessful.

In the method reported here, the way of biocatalyst preparation allowed us to achieve the desired direction of microbial transformation of the substrate. Results were strongly dependent upon the sort of chemical additive used in the cultivation process. The mixture of products was analyzed by ^{31}P NMR with the addition of quinine as chiral discriminator. This allowed us to determine each stereoisomer of the examined compound.[16] As shown in ^{31}P NMR spectra in Figure 1, by means of the preincubation of biocatalyst's cells in the presence of methyl-*iso*-propyl ketone, only one stereoisomer of the racemic mixture of the substrate was transformed (Fig. 1B). Remarkable results were obtained in the case of preincubation of the biocatalysts in the presence of oxalacetic acid, which turned out to be crucial for the effectiveness of the stereoselective biotransformation of the diastereoisomers of ethyl hydroxy(phenyl)methane(P-phenyl)phosphinate, because both pairs of diastereoisomers were resolved (Fig. 1A). These results allowed us to separate the obtained diastereoisomeric mixture using common column chromatography techniques.[15] The next step included the determination of the absolute configuration of the unreacted stereoisomers of ethyl hydroxy(phenyl)methane(P-phenyl)phosphinate. Usually NMR spectra are used as a tool for the determination of the absolute configuration of hydroxyphosphonates and hydroxyphosphinates,[8,17] mainly because of the simple method of sample preparation and because small amounts of the examined compound are required. In the case reported here, Mosher's approach turned out to be effective as well. For the (*R*)-MTPA conformer, the signal observed in the NMR spectra, derived from L2 nuclei is moved upfield, because of anisotropic effect exerted by the phenyl

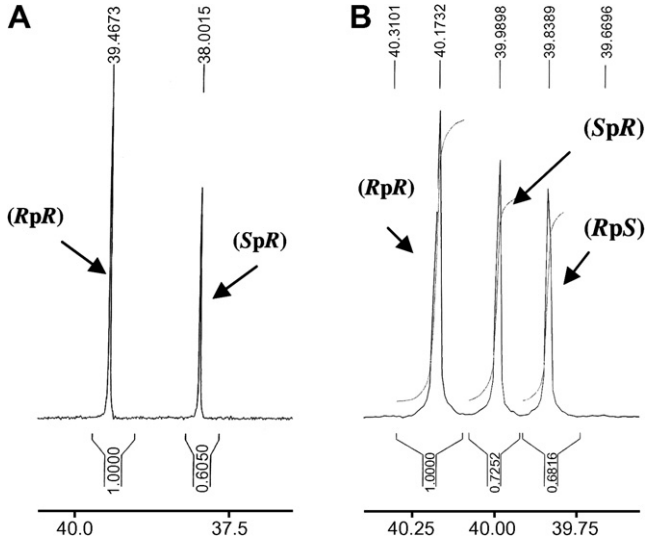
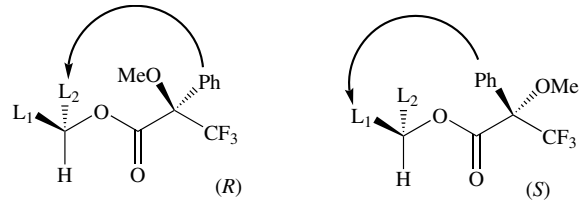
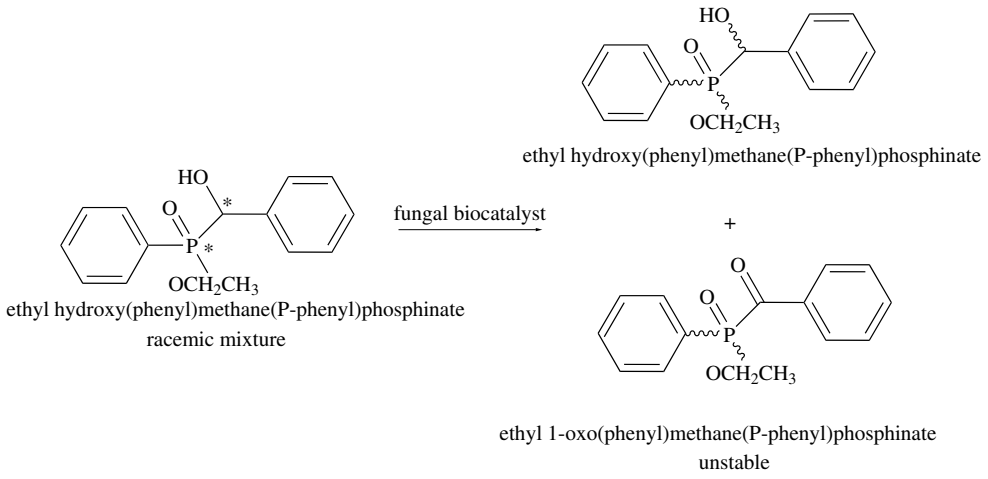


Figure 1. ^{31}P NMR spectra of biotransformation products recorded with quinine. (A) Oxalacetic acid as the additive, (B) methyl-*iso*-propyl ketone as the additive.



Scheme 1. The anisotropic effect of a phenyl ring on chemical shifts in MTPA esters.[17]



Scheme 2. Possible enantioselective biooxidation.

group. In the case of (*S*)-MTPA, the L1 group is obscured (Scheme 1).[17]

According to the literature data[15] and analysis of the ^{31}P NMR spectra of biotransformation products recorded with quinine (shift reagent), the absolute configuration of the stereoisomers of ethyl hydroxy(phenyl)methane(P-phenyl)-phosphinate has been determined. The results obtained, when methyl-*iso*-propyl ketone was used as an additive, show that only the (S_pS)-stereoisomer was transformed (Fig. 1B, the yield was up to 20%). The application of oxalacetic acid gave (R_pR) and (S_pR) diastereoisomers with excellent enantiomeric excess (>99%) and chemical yield up to 48% (Fig. 1A). The examined biotransformations were stereoselective and isomers bearing α-carbon atom with (*S*)-configuration were preferentially transformed with a lack of stereoselectivity toward the phosphorus atom. These results are in good agreement with those observed previously.[13–15]

There are at least two possible mechanisms of the presented biotransformation. First, it could be assumed that with enantioselective oxidation of one isomer, the oxidized form is unstable in water and splits (Scheme 2). The second possibility is the selective O-dealkylation of the phosphinate.[18] However, the ^{31}P NMR spectra suggests the biooxidation route of the microbial conversion, because there are no other signals characteristic to organophosphorus compounds except the one coming from the substrate.

In fact, in the reported case, the most important things are the satisfactory results and further applicability of biotransformation method.

3. Experimental

3.1. Materials

3.1.1. Microorganisms. Fresh baker's yeast was purchased from Wołczyn, Poland (strain PW-A-79002); *Penicillium oxalicum* (IT 17), *Aspergillus flavus* (IT 89) were a generous gift from Professor Anna M. Picco (Department of Land Ecology, Section of Mycology, University of Pavia, Italy); *Geotrichum candidum* (DSM 6593) and *Rhodotorula glutinis* (DSM 10134) were purchased from DSMZ—Deutsche Sammlung von Microorganismen und Zellkulturen GmbH.

3.1.2. Chemicals. All materials were obtained from commercial suppliers: Sigma, Aldrich, Fluka, POCh, Serva and used without purification.

3.2. Methods

3.2.1. Synthesis of ethyl hydroxy(phenyl)methane(P-phenyl)phosphinate. Synthesis according to the literature.[15] Mixture of (R_pR) and (S_pS) isomers—^{31}P NMR δ (ppm): 39.56; ^1H NMR: δ (ppm) 1.32 (t, $J = 7.0$ Hz, 3H, OCH_3), 3.93–4.18 (m, 2H, OCH_2), 5.16 (d, $J = 10.0$ Hz, 1H, CHP), 7.20–7.64 (m, 10H, aromatic protons); ^{13}C NMR δ (ppm): 16.71 (d, $J = 6.1$, CH_3), 62.09 (OCH_2), 73.41 (d, $J = 111.3$, CHP), 127.18, 127.22, 127,92 (d, $J = 3.1$), 128.08, 128.10, 128.25, 132.68 (d, $J = 2.2$), 132.77, 133.06, 136.58 (aromatic carbons).

Mixture of (R_pS) and (S_pR) isomers—^{31}P NMR δ (ppm): 38.00; ^1H NMR: δ (ppm) 1.27 (t, $J = 7.0$ Hz, 3H, OCH_3), 3.93–4.18 (m, 2H, OCH_2), 5.10 (d, $J = 7.4$ Hz, 1H, CHP), 7.20–7.64 (m, 10H, aromatic protons); ^{13}C NMR δ (ppm): 16.69 (d, $J = 5.5$, CH_3), 62.05 (OCH_2), 73.79 (d, $J = 110.3$ Hz, CHP), 127.47, 127.50, 128,17, 128.24 (d, $J = 2.4$), 128.38, 128.47, 132.77, 132.83 (d, $J = 2.2$), 132.12, 136.28 (aromatic carbons).

3.2.2. Cultivation of the microorganisms. Fungal strains (*P. oxalicum*, *A. flavus*, *G. candidum* and *Rhodotorula gracillis*) were routinely maintained on potato dextrose agar, which provided profuse sporulation suitable for inoculum collection. All fungal strains were cultured in a modified Czapek liquid medium, which consisted of 30 g/L sucrose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 2.64 g/L $(NH_4)_2SO_4$, 0.01 g/L $FeSO_4$, and 0.5 g/L KH_2PO_4, pH 7.2. Cultures were grown at 100 rpm in 250-mL Erlenmeyer flasks containing 100 mL medium, which were inoculated with a spore suspension in 0.05% Triton X-100 and incubated at 28 °C. After 3 days of cultivation, a fungal biomass was separated by centrifugation (4500 rpm/10 min). The cell pellet was washed with water and centrifuged again. Then the biocatalyst biomass was transferred into the 250-mL Erlenmeyer flask containing 100 mL of cultivation medium lacking sucrose and incubated under the condition of culturing. After a 24 h starvation period, the wet cells were used directly as a biocatalyst or cultivated again for 48 h with one of the chemicals: sodium pyruvate, methyl-*iso*-propyl ketone, and oxalacetic acid were applied in a final concentration of 8 mM. The biomass was then separated by centrifugation, washed twice with distilled water, and centrifuged again.

3.2.3. Biotransformation procedure. Wet fungal biomass (30–50 g) and 50 mg of substrate were added to a 250-mL Erlenmeyer flask containing 30 mL of water. The reaction mixtures were incubated at room temperature and 100 rpm for 3 days. The biocatalyst was separated by centrifugation and the supernatant was extracted twice using ethyl acetate and dried over anhydrous magnesium sulfate. After filtration, the organic phase was evaporated and the product analyzed by means of ^{31}P NMR spectroscopy using quinine as a chiral discriminator.[16] After the addition of methyl-*iso*-propyl ketone, the (S_pS)-stereoisomer was obtained with a yield of up to 20%, while the application of oxalacetic acid allowed us to gain the pair of the (R_pR) and (S_pR) diastereoisomers with chemical yields of up to 48%. In every case, the enantiomeric excess was >99%.

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