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First biological conversion of chiral heterophosphonate derivative – Scaling and paths of conversion discussion

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ABSTRACT

Presented work describes the first approach for the biocatalytic resolution of racemic mixtures of heterophosphonate derivative. *Penicillium funiculosum* and *Rhodotorula mucilaginosa* were successfully applied for the biological conversion of racemic mixture of 1-amino-1-(3′-pyridyl)methylphosphonic acid **3**. Both microorganisms carried out the kinetically driven process leading to conversion of one from the substrate enantiomers, leaving the second one unreacted. Application of *R. mucilaginosa* allowed obtaining pure enantiomer of the substrate (yield 100%, *e.e* 100% - unreacted isomer) after 24 h of biotransformation of **3** in the laboratory scale process (Method E), applying biocatalyst pre-treatment step − 24 h of starvation. In case of other biocatalyst, application of whole cells of *P. funiculosum* in laboratory scale process, also resulted in conversion of the racemic mixture of substrate **3** *via* oxidative deamination into ketone derivative, which was then bioreduced (second step of the process) into 1-hydroxy-1-(3′-pyridyl)methylphosphonic acid **4.** This time two products were isolated: unreacted substrate and hydroxy compound **4**. Conversion degree ranged from 30% (standard procedure, method A) to even 70% (with extra addition of sodium pyruvate - method B2). However, in this case, bioconversion was not enantioselective – products: amino- and hydroxyderivative were obtained as racemic mixtures. Both biocatalysts were also tested towards the scaling so other biocatalytic procedures were introduced - with immobilized fungal mycelium. In case of *Rhodotorula mucilaginosa* this approach failed (data not shown) but *Penicillium funiculosum* turned out to be active and also selective. Thus, application of this biocatalyst in the half-preparative scale, continuous-flow bioprocess (Method *C*2) resulted in the obtaining of pure *S*-**3** (100% *e.e.*) isomer with the 100% of conversion degree, without any side products. Recorded NMR spectra allowed confirming the reaction progress and its selectivity and also postulating possible mechanism of conversion.

1. Introduction

Phosphonic acids are organophosphorus compounds with stable P—C bond. Important group of phosphonates are *α*-aminophosphonic acids, analogues of the natural amino acids in which the carboxylic moiety is replaced by phosphonic functionality. They have inhibitory activity, act as antibiotics, crop protection agents, herbicides or peptide mimetics. These wide range of possible applications are, in the most cases, characteristic for chiral compounds of defined absolute configurations [1–5]. Considering the importance and the applicability of organophosphorus compounds, the next derivatives of such structures have focused the attention. Among others, phosphonates molecules with the hetero atom incorporated into the phenyl ring. Current literature data confirm, that different P—C compounds (as enantiomers of defined absolute configuration or as motifs in the drug structures) are applied in medicine [6,7], and also as building blocks for the chemical synthesis of phosphonic pseudopeptides, which act as enzymes inhibitors [8–11] (Fig. 1). Thus, this is the not fully explored source of molecules of great pharmaceutical potential.

Presented work describes the first biocatalytic attempt resulted in the obtaining of pure enantiomer of phosphonate derivative with the heteroatom incorporated in its structure. Thus, the aim of this study was to set the biocatalytic method of resolution of racemic mixture of 1-amino-1-(3′-pyridyl)methylphosphonic acid **3** (Fig. 2) using the fungal biocatalyst of confirmed previously, dual activity towards aminophosphonic acid, oxidative and reductive one (e.g. *P. funiculosum*) [14,15]. Such approach was meant to lead to the optically pure products: unreacted aminophosphonic acid **3** and if possible to hydroxyphosphonate

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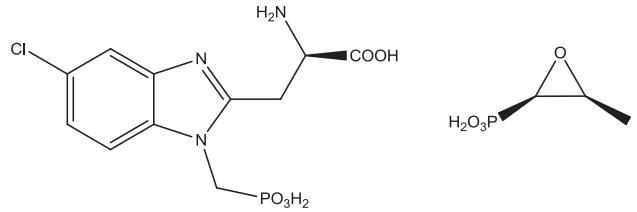

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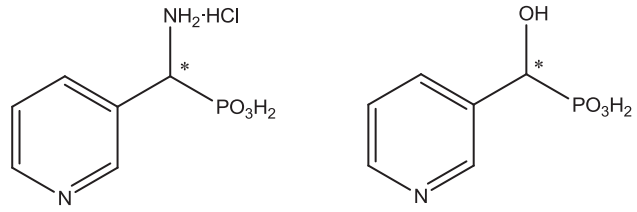
Fig. 1. Examples of phosphonates of pharmaceutical meaning: **1-** antagonist of NMDA receptor [12], **2-** phosphomycin, antibiotic activity [13].



3 4

Fig. 2. Chemical structures of 1-amino-1-(3′-pyridyl)methylphosphonic acid **3** and 1-hydroxy-1-(3′-pyridyl)methylphosphonic acid **4**.

4. These assumptions about the mechanism of the bioconversion are based on the previous discoveries, described for the other aminophosphonic acids [14,15].

2. Materials and methods

All chemicals were commercially available – purchased from: Fluka, Avantor Performance Materials Poland S.A., Sigma Aldrich.

NMR (Nucleic Magnetic Resonance) spectra were measured on a Bruker Avance™ 600 at 600.58 MHz for ^{1}H and 243.12 MHz for ^{31}P in D_2O (99.9% of atom D). Chemical shifts (δ) were reported in ppm and coupling constants (*J*) are given in Hz. ^{1}H NMR spectra are referenced to the central line from solvent ($\delta = 4.78$ for water). The biotransformation products were analyzed by ^{31}P NMR and ^{1}H NMR.

MS spectrum was prepared using high resolution mass spectrometer with analyzer time-of-flight (TOFMS) from LCT Premier XE.

2.1. Synthesis of substrate 3

1-amino-1-(3′-pyridyl)methylphosphonic acid **3** was synthesized according to the method described in literature [16,17]. The aminophosphonic acid was obtained in a three-step synthesis based on addition of diethyl H-phosphonate to the previously prepared imine and followed by acidic hydrolysis of the resulting aminophosphonate ester.

Neat benzhydrylamine (8.1 mL, 0.047 mol) was injected at room temperature to a solution of 3-pyridinecarboxaldehyde (4.38 mL, 0.047 mol) in CH_2Cl_2 (50 mL) and the reaction was stirred overnight. After that time, anhydrous Na_2SO_4 (5.0 g) was added and the mixture was stirred for additional 0.5 h. After removal of the drying agent the reaction was concentrated under reduced pressure affording crude imine as white solid that was used in the next step.

The imine (10 g, 0.037 mol) was dissolved in toluene (50 mL) and diethyl H-phosphonate [HP(O)(OEt)$_2$] (4.76 mL, 0.037 mol) followed by triethylamine (5.1 mL, 0.037 mol) were added. The resulting reaction mixture was heated at 120 °C for 8 h and then cooled down to room temperature and concentrated under reduced pressure. The resulting yellowish solid was essentially pure aminophosphonate ester that was used directly in the next step.

The crude aminophosphonate ester (10.0 g, 0.024 mol) was dissolved in toluene (50 mL) and aq. 6 M HCl (15 mL) was added. The resulting biphasic reaction mixture was vigorously stirred at 120 °C for 4 h. After that time, the reaction was cooled to room temperature, transferred to a separatory funnel and the layers were separated. The organic layer was discarded and the aqueous phase was evaporated to dryness under reduced pressure affording crude 1-amino-1-(3′-pyridyl) methylphosphonic acid that was further purified by recrystallization from mixture H_2O/EtOH affording the desired aminophosphonic acid hydrochloride as white non-hygroscopic powder (4.7 g, yield 85%).

^{31}P NMR (D_2O, δ, ppm): 8.42
^{1}H NMR (D_2O, δ, ppm): 8.81(s, l H, py-2), 8.73(d, l H, py-6), 8.61(d, 1H, py-4), 8.04 (t, 1H, py-5), 4.69 (d, 1H, CHP, $J = 16.3$ Hz)

2.2. Synthesis of product 4 (racemic mixture)

1-hydroxy-1-(3′-pyridyl)methylphosphonic acid **4** was obtained according to the literature data [16,17].

The 3-pyridinecarboxaldehyde (4.38 mL, 0.047 mol) was dissolved in toluene (50 mL) and diethyl H-phosphonate [HP(O)(OEt)$_2$] (6.10 mL, 0.047 mol) followed by triethylamine (6.5 mL, 0.047 mol) were added. The resulting reaction mixture was heated at 120 °C for 8 h and then cooled down to room temperature and concentrated under reduced pressure. The resulting crude hydroxyphosphonate was purified as oxalate salt. For that purpose, the crude hydroxyphosphonate (8 g, 0.036 mol) was dissolved in acetone (50 mL), and a solution of oxalic acid (9.1 g, 0.072 mol) in acetone (50 mL) was added. The resulting mixture was refrigerated and the precipitated pure oxalate of hydroxyphosphonate as orange solid was filtered and dried on air. Subsequently, the free ester was liberated from the oxalate salt by treatment with excess of 5% aqueous sodium carbonate and extraction with dichloromethane (4 × 30 mL). The organic extract was dried oved Na_2SO_4 and evaporated to dryness under reduced pressure affording pure hydroxyphosphonate as light orange solid that was used in the next step.

The hydroxyphosphonate (7 g, 0.031 mol) was dissolved in dichloromethane (50 mL) and fresh bromotrimethylsilane (BrTMS) (14.6 mL, 0.111 mol) was added. The resulting reaction mixture was stirred at room temperature overnight. After that time, solvent was evaporated under reduced pressure and the resulting oily residue was treated with methanol (50 mL) and stirred at room temperature overnight. After evaporation of solvent the crude 1-hydroxy-1-(3′-pyridyl) methylphosphonic acid was purified by recrystallization from methanol yielding the desired product as white solid (5.0 g, yield 84%).

^{31}P NMR (D_2O, δ, ppm): 14.68
^{1}H NMR (D_2O, δ, ppm): 8.71 (s, 1H, py-2), 8.60 (d, 1H, py-6, $J = 5.7$ Hz), 8.55 (d, 1H, py-4, $J = 8.0$ Hz), 7.96 (t, 1H, py-5, $J = 6.0$ Hz), 5.05 (d, 1H, CH-P, $J = 14.6$ Hz)

2.3. Microorganism and culturing conditions

Rhodotorula mucilaginosa (DSM 70403) was purchased from German Collection of Microorganisms and Cell Cultures (Germany). *Penicillium funiculosum* (Thom) S3 was isolated from soil sample [18]. Tested strains were cultivated on the standard, commercially available Potato Dextrose Broth (PDB). *P. funiculosum* mycelium grow 4 days in 250 mL cultivation flask with shaking 135 rpm at 25 °C until the mid-log phase was achieved. After that, biomass of *P. funiculosum* (5 g) was separated by filtration and washed twice with distilled water while *R. mucilaginosa* (4 g) was cultivated for 5 days and after that time biomass was separated by centrifugation (20 °C, 5000 rpm, 10 min).

2.4. Biotransformation procedure

2.4.1. Biotransformation catalyzed by P. funiculosum

Method A: Wet fungal biomass (5 g) and 3 mM of substrate **3** (50 mL of distilled water solution) were incubated in 250 mL Erlenmeyer flasks

Table 1
Parameters of polyurethane foams.

Type	Classification number	Size of pores [μm]
BULPREN	S 28280	2300–3300
FILTREN	TM 25133	1060–1600

at 135 rpm, 25 °C. The bioconversion of **3** was carried out for 5 days. Then, the biocatalyst was separated by filtration and the supernatant was evaporated.

Method B: The cells of *P. funiculosum* (5 g) were separated after mid-log phase and biotransformation was carried out as follow:

Method B1: Bioconversion was carried out according to Method A with extra addition (8 mM) of appropriate additive (sodium pyruvate, oxaloacetic acid, methyl isopropyl ketone, methyl vinyl ketone).

Method B2: Bioconversion was carried out according to Method B1 with the previous starvation period – preincubation for 24–48 h under starvation conditions with addition of 8 mM of chemical additive (sodium pyruvate, oxaloacetic acid, methyl isopropyl ketone, methyl vinyl ketone). After this time, 3 mM of substrate **3** was added and incubated for the next 5 days.

Method C:

P. funiculosum was immobilized on polyurethane foams (1 cm × 1 cm cube, Bogmar Łódź, Poland, Table 1.). Filtren TM are polyether based polyurethane foams and Bulpren® S types are reticulated polyester based polyurethane foams.

Polyurethane foams (30 pieces − 1 cm × 1 cm cube) were added to flask with appropriate nutrition medium PDB, sterilized and then inoculated. The immobilized mycelium (overgrown foams) of *P. funiculosum* was separated after 4 days.

Method C1: After immobilization, bioconversion was carried out in 250 mL Erlenmeyer flasks containing 100 mL of 3 mM of substrate **3** under shaking conditions (135 rpm) for 5 days at 25 °C.

Method C2: For continuous flow column experiments, a semi-preparative scale plastic tube-bioreactor of 13.5 cm length and 2.5 cm internal diameter was used. The bioreactor was packed with immobilized cells of *P. funiculosum* and 11.2 mM solution of substrate **3** (200 mL) was pumped (5 mL/min) from a reservoir into the reactor. The bioprocess was conducted for 5 days (I cycle) and later, fresh water solution of substrate **3** was pumped (II cycle) for 4 days.

2.4.2. Biotransformation catalyzed by R. mucilaginosa

Method D: Wet biomass of *R. mucilaginosa* (4 g) was used for biotransformation of substrate **3** – 30 mg (1.34 mM), process was carried out for 48 h (100 mL of distilled water at 135 rpm, 25 °C).

Method E: Bioconversion according to Method D with the previous starvation step (in 100 mL of distilled water for 24 h at 135 rpm, 25 °C). Afterward, 30 mg (1.34 mM) of substrate **3** was added to biotransformation media and bioprocess was carried out for 24 h.

Method F: Biomass cultivated on 1000 mL of PDB medium was used in scaling up process in batch – bioreactor (BioFlo Model C32, New Brunswick Scientific). Biomass (40 g) and 1000 mL of distilled water was placed in bioreactor and preincubated for 24 h under starvation conditions in 23 °C with aeration 0.5 L/min and agitation 200 rpm. After that time 300 mg (1.34 mM) of substrate **3** was added and incubated for next 24 h.

For every biotransformation variation appropriate control experiments were performed, what allowed confirming the stability of the biocatalyst and the substrate and also the biocatalytic origin of the arising product. Experiments were performed in triplicate.

2.5. Enantiomeric excess assignment

^{31}P NMR samples were prepared as follows: after separation of biomass, biotransformation mixture was evaporated and dissolved in deuterium oxide (600 μL). Then α-cyclodextrin (100 mM) as a chiral solvating agent [19] was added. The pH value of compound **3** solution was adjusted with NaOD or DCl solutions to the pD ≈ 10–11.

2.6. Absolute configuration assignment

Absolute configuration was established tentatively, using the model - similar compound: 1-aminophenylmethanephosphonic acid and was defined as *S*-1-amino-1-(3′-pyridyl)methylphosphonic acid **3** by the optical rotation measurement and according to the literature data (optical rotation $[\alpha]_D = -2$ (2 M NaOH)) [20].

3. Results and discussion

P. funiculosum and *R. mucilaginosa* were successfully applied for the resolution of racemic mixture of 1-amino-1-(3′-pyridyl)methylphosphonic acid **3**. Experiments began with the setting of the method of evaluation of the progress and the enantioselectivity of the reaction. To analyze these data, *α*-cyclodextrin as the chiral solvating agent was applied. Measurements *via* ^{31}P nuclear magnetic resonance (NMR) spectroscopy recorded with addition of *α*-cyclodextrin are simple and efficient method for determination of enantiomeric purity of phosphonates derivatives [19]. The solution pD have significant impact on the ability to form inclusion (guest–host) complexes with the chiral solvating agent and consequently on enantiodiscrimination efficiency so method should be optimized for each compound. [19,21–24]. The NMR samples were prepared in acidic, neutral and basic pH values at the particular concentrations of α-cyclodextrin for substrate **3** (Table 2). Such preliminary individual experiments allowed establishing the optimum conditions for NMR analysis.

As it is seen above (Table 2) the best shifts difference (Δδ) for enantiomers of tested substrate **3** was set as pD 10 and such value was applied for further experiments.

Among other tested fungal strains (data not shown) *P. funiculosum* and *R. mucilaginosa* were selected as active biocatalysts towards the aminophosphonic substrate **3**. As it was experimentally proven both biocatalysts were active but under different process conditions (medium, form of biocatalyst cells). Application of *R. mucilaginosa* allowed resolving the racemic mixtures of the substrate, but only in the process with low concentration of starting compound (1.34 mM) and with the free cells systems in the simplified model of batch process whereas *P. funiculosum* was effective only as immobilized mycelium in the model flow reactor process and also after scaling to 11.2 mM substrate solution. In both cases the path of bioconversion started from the oxidative deamination and then, in case of molds, ketone reduction was observed, whereas in case of yeasts the only noted step was the starting oxidation. These differences were crucial for the setting of the optimal bioconversion protocols. *P. funiculosum* required the conditions supporting the coenzyme regeneration systems whereas for *R. mucilaginosa* the preincubation under starvation conditions was the most important step influencing the effectiveness and selectivity of the process, because this approach force the yeasts to utilize the substrate as source of elements. Thus, after 5 days of bioconversion by *P. funiculosum*, according to method A, the conversion degree reached 34% (calculated according to NMR Fig. 3A) but ^{31}P NMR spectrum recorded with the addition of *α*-cyclodextrin showed that the course of the bioconversion is non-stereospecific (Fig. 3B). However from the recorded spectra and previously

Table 2
^{31}P NMR chemical shifts differences (Δδ) observed for racemic mixture of analyzed, chiral hetero organophosphorous compound **3** (10 mM) at the presence of *α*-cyclodextrin (100 mM).

pD	2	7	10
Δδ	0.00	0.06	0.08

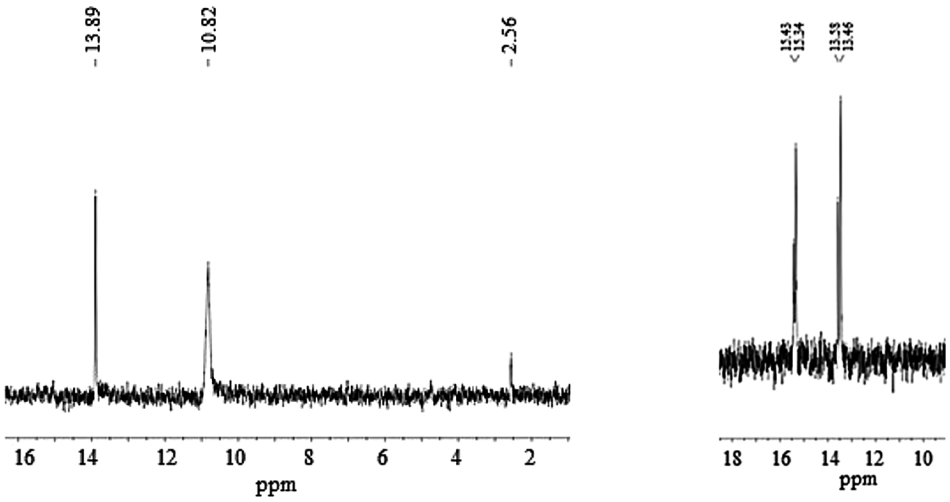


Fig. 3. (A) ^{31}P NMR spectrum of biotransformation of **3** using *P. funiculosum* as biocatalyst (Method A): substrate **3** (10.82 ppm), 1-hydroxy-1-(3′-pyridyl)methylphosphonic acid **4** (13.89 ppm), side product (2.56 ppm). (B) ^{31}P NMR spectrum recorded after standard bioconversion (Method A) of 1-amino-1-(3′-pyridyl) methylphosphonic acid **3** using *P. funiculosum* (with α-CD at pD ≈ 10).

performed and published work [14] it can be assumpted that aminophosphonate (**3**) is transformed to ketophosphonate and then to hydroxyphosphonate (**4**) (Fig. 3) but both the product and the unreacted substrate were racemic.

The path of bioconversion of a novel substrate 1-amino-1-(3′-pyridyl)methylphosphonic acid **3** into the final 1-hydroxy-1-(3′-pyridyl) methylphosphonic acid **4** was confirmed by the addition of 5 mg of chemically synthetized α-hydroxyphosphonate **4** (stable under the experimental conditions) to the NMR samples. This allowed to identify particular signals on the ^{31}P NMR spectrum and proved the predicted bioconversion step (Fig. 4).

Literature data [16,17] allowed to confirm that the signal of hydroxyphosphonate **4** on ^{31}P NMR spectrum is located at δ ≈ 13.89 ppm (Fig. 3). Also, mass spectroscopy confirmed the presence of substrate **3**: MS (TOF MS ES-): 189.0436 and product **4**: MS (TOF MS ES-): 236.0659. Mass spectrum suggested that 1-hydroxy-1-(3′-pyridyl)methylphosphonic acid **4** had two ions of sodium and protonated nitrogen in phenyl ring.

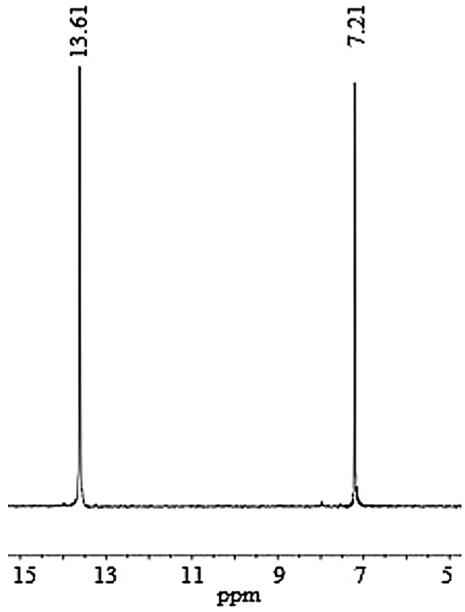


Fig. 4. ^{31}P NMR spectrum recorded after extra addition of chemically synthetized hydroxyphosphonate **4** to examined sample: substrate **3** (7.21 ppm) and product **4** (13.61 ppm).

P. funiculosum bioreaction path was running according to the following pattern: applied xenobiotic **3** (10.82 ppm) (Fig. 3) was converted *via* one pot cascade reactions (proton borrowing processes) oxidation, which resulted in the aminophosphonate **3** bioconversion into corresponding ketophosphonate, which is then reduced to hydroxyphosphonate **4** (13.89 ppm) (Fig. 3, Fig. 5) [14,15]. Aminophosphonic substrate **3** – exogenous source of hydrogen is oxidized, then reduced and biological coenzymes are regenerated into the oxidized form with the ketophosphonate proton acceptor and the hydrogen - borrowing cycle is completed. Similar examples of using the xenobiotics as elements of the cells redox cycle in fungal cells were described previously [25–27].

Regarding discussed results, efforts to improve the stereoselectivity and effectiveness of the reaction were undertaken. Modifications of biotransformation procedure, including the addition of the starvation step were provided. Deficiency of the nutrients induced the stress conditions for fungal mycelium, what in many cases, triggers on the secondary metabolic pathways, which are often crucial for bioconversion of non-physiological substrates, such as 1-amino-1-(3′-pyridyl) methylphosphonic acid **3**.

It is proven that the addition of catalytic amount of some chemicals of low weight to the reaction media can affect dehydrogenases activity *via* the impact on the balance between the oxidized and reduced forms of coenzymes, which are essential for redox reactions. This can be achieved directly – additives are proton acceptors or donors or indirectly – additives influence the redox metabolic paths (as substrates, intermediates, products or their analogues), increasing the demands for exogenous source of protons – e.g. xenobiotic. Thus, mycelium of *P. funiculosum* was incubated under standard condition at the presence of co-substrate (Method B1) or the additive was used after the 24–48 h starvation period (Method B2). In both cases, only two co-substrates (Table 3): methyl isopropyl ketone and sodium pyruvate influence the bioconversion of substrate **3** by *P. funiculosum* and were stable under experimental conditions. These modifications allowed to obtain higher conversion degree of 1-amino-1-(3′-pyridyl)methylphosphonic acid **3**, over 60% (Table 3).

Despite the good conversion degree, discussed approach failed considering the enantioselectivity. Such results could be explained by the nature of the enzymes involved in the substrate conversion. Possible presence of enzymes isoforms of opposite stereoselectivity and similar activity towards studied compound or what is also possible, the low substrate specificity of single enzyme can be the reason of the lack of the selectivity [28]. However, regarding the good effectiveness of studied bioconversion and its novelty it was worth to perform the next

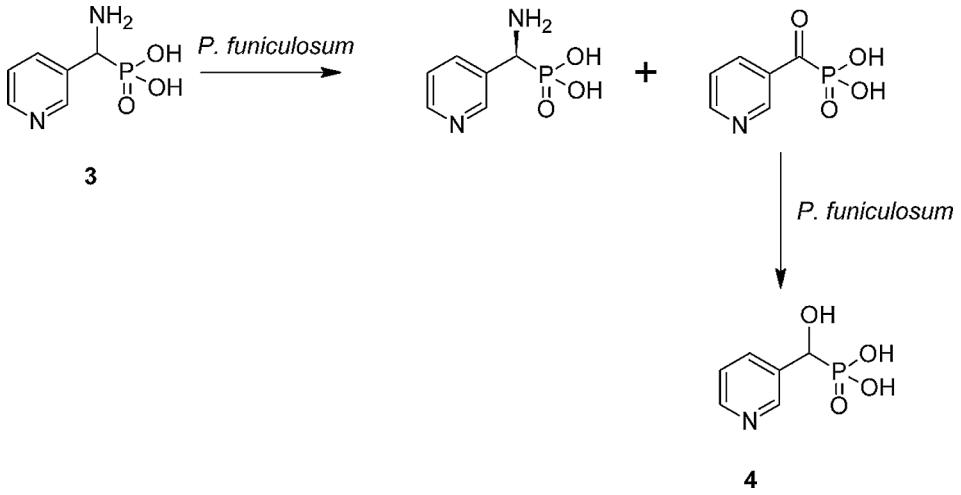


Fig. 5. Scheme of two step biotransformation of compound **3** to corresponding ketophosphonate and then to hydroxyphosphonate **4** catalyzed by *P. funiculosum*.

Table 3
The influence of the co-substrates on the effectiveness of biotransformation of substrate **3** with *P. funiculosum* under standard conditions (Method B1)[a] and with starvation step (Method B2).[b]

Cofactor	$t_{preincubation\ under\ starvation\ conditions}$ [h]	Conversion [%]
Sodium pyruvate	0[a]	32
	24[b]	35
	48[b]	69
Methyl isopropylketone	0[a]	44
	24[b]	60
	48[b]	64

[a] Standard procedure without starvation process.
[b] Mycelium preincubated under starvation conditions.

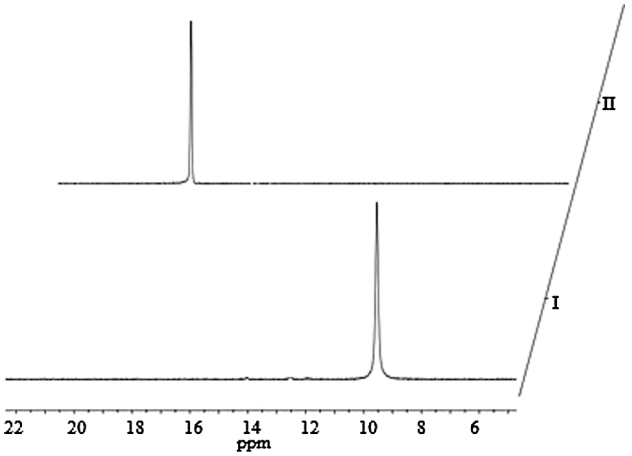


Fig. 6. ^{31}P NMR spectra after bioconversion of 1-amino-1-(3′-pyridyl)methylphosphonic acid **3** in the continuous-flow system: spectrum I: after 5 days (I cycle) of bioconversion of **3**, spectrum II: recorded after the addition of α-CD at pD ≈ 10 (100% *e.e.*).

experiments trying to set the conditions allowing to obtain optically pure products. Among others, biocatalysts engineering such as immobilization is the method of choice [29]. This can affect the selectivity of the bioconversion depending for example on the enzymes localization in the cell. If the proteins involved in the process are attached to the cells envelopes, immobilization can be critical for their activity. Such effect was observed as a consequence of the next experiments performed with immobilized fungal mycelium. Thus, *P. funiculosum* immobilized on polyurethane foams TM 25133 or S 28280 (Method C) was applied as biocatalyst in the two simplified systems of: batch and flow reactors.

Considering the model of the batch reactor (Method C1), experiments were carried out under shaking conditions, for 5 days and also this time, reaction was non-stereoselective, but the conversion degree of substrate **3** - was again improved - up to 67%. The breakthrough was occurred in the last experiment with the model of the continuous - flow reactor (Method C2) with higher concentration of the substrate 11 mM. Results were very good and really surprising. The only product single unreacted enantiomer of 1-amino-1-(3′-pyridyl)methylphosphonic acid **3** was obtained with *e.e.* of 100% (Fig. 6). Second step of this conversion is the reduction of prochiral intermediate – ketophosphonate to chiral product – hydroxyphosphonate. These conversion is a sequence of cascade reactions.

Absolute configuration was established using the model - similar compound: 1-aminophenylmethanephosphonic acid and was defined as *S*-1-amino-1-(3′-pyridyl)methylphosphonic acid **3** by the optical rotation measurement and according to the literature data (optical rotation $[\alpha]_D = -2$ (2 M NaOH)) [20].

Application of immobilized cells of *P. funiculosum* in the continuous - flow process has advantages over the simplified batch reactor because of few reasons: better biomass stability, removing xenobiotics of possible toxicity from the cells surface, decreasing the concentration of the substrate around the cells. This phenomenon: lack of the selectivity in the one process and high selectivity in the other one, applying the same biocatalyst and immobilization procedure but different model of the reactors can be explain in few ways. For example by the presence of at least two isoenzymes of different enantioselectivity "*E*" towards substrate enantiomers - they can differ in at least two ways. Thus, one isoenzyme can be further more active towards particular enantiomer (in described process towards "*R*", which is converted, "*S*" remain unreacted) while the second one - enzyme of opposite enantioselectivity is of low activity or belongs to the inducible proteins and appeared in the reaction medium after some time, so that, the products of its activity are not formed under applied conditions (taking into account the force flow and substrate removal from the mycelium environment). The second explanation is that the enzymes differs in the strength of the substrate binding and the force flow removes the substrate from the enzyme of one particular enantioselectivity, such findings were reported previously [30,31]. Different mode of action was observed, despite of the final result of bioconversion of substrate **3**, for other tested biocatalyst – *R. mucilaginosa*. Also this time, preliminary experiments were rather of low effectiveness - after 2 days of biotransformation (method D) enantiomeric excess of obtained product reach only about 20%, so the starvation period before bioconversion was applied (method E). This

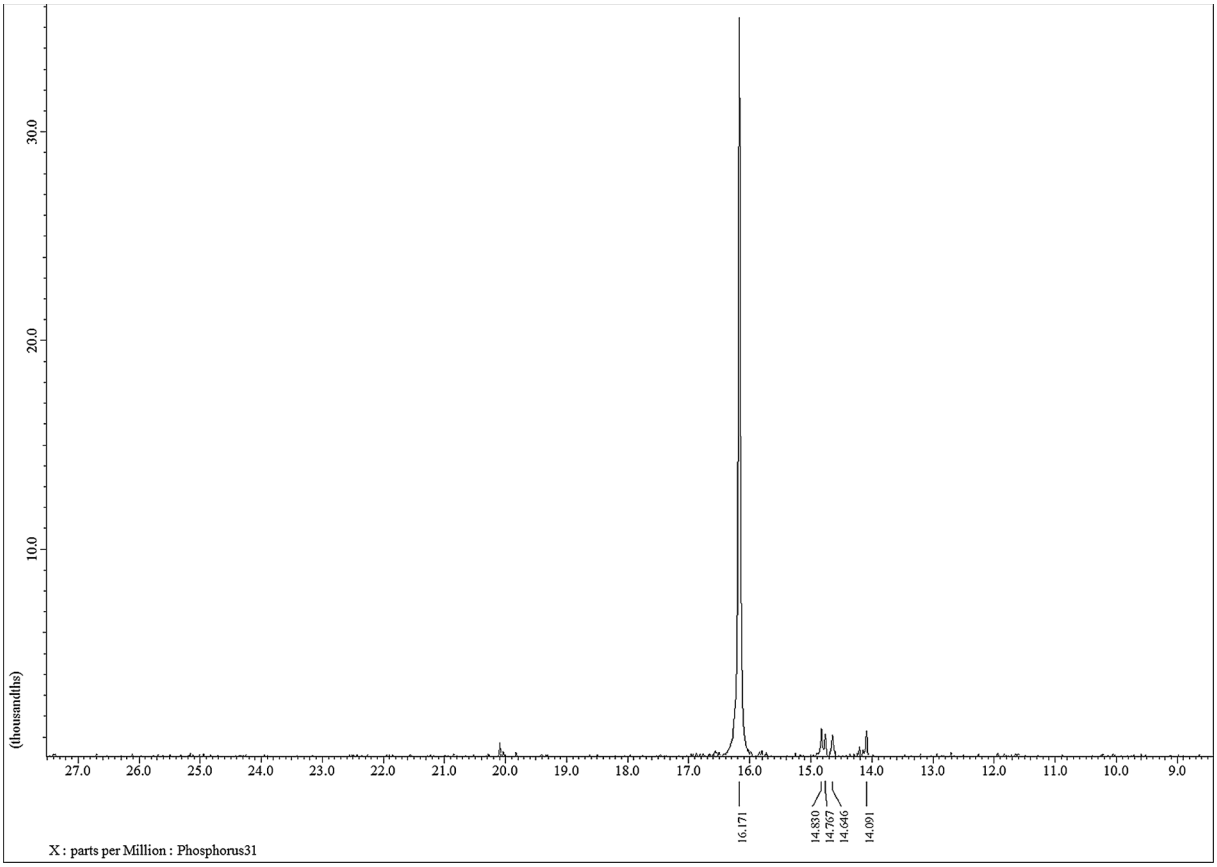


Fig. 7. ^{31}P NMR spectrum of products after biotransformation catalyzed by *Rhodotorula mucilaginosa* (Method E); ^{31}P NMR spectrum was recorded with addition of α-CD as chiral solvating agent at pD ≈ 10 (100% *e.e.*)

simple change allow to obtain pure enantiomer of compound **3** (Fig. 7) after only 24 h of biotransformation.

In case of tested yeasts, the preincubation of biocatalyst under starvation conditions was crucial to force the viable cells to utilize the substrate as carbon, phosphorus and nitrogen sources, considering the short duration of the process and the lack of the intermediates and side products. For effective proliferation in a heterogeneous environment, microorganisms have to harmonize the cell growth and division with nutrient availability. Fungi are constantly exposed to changes in the availability of nitrogen, so the efficient regulation of nitrogen metabolism is necessary for their growth [32]. Fungi preferentially use NH_4^+ and glutamine as a nitrogen source, but in the absence of these sources, they are able to use less easily assimilated compounds such as nitrate, urea, uric acid, amines, amides, purines, and pyrimidines. [33,34]. In studies of *V. dahlia* metabolism under nitrogen starvation, proteomic analysis show increase in production of enzymes for protein metabolism such as exopeptidases, metallopeptidases, endopeptidases and amino acid transporters. These results suggest that during nitrogen starvation fungi search for source of nitrogen inside and outside of the cell. [32] Nitrogen metabolite repression is the regulatory mechanism that enables adjustment of metabolism to the available nitrogen sources [35].

Tested compound could also serve as source of phosphorus atom for living cells as a consequence any trace of phosphorus, except unreacted enantiomer of compound **3**, was recorded on ^{31}P NMR spectrum. Some fungi are known for their ability to degrade aminophosphonates [36,37]. Due to structural diversity of phosphonates different enzymatic systems for their degradation are required. Enzymes that take part in the phosphonate utilization were identified as phosphonatase, phosphonoacetate hydrolase, phosphonopyruvate hydrolase and C—P lyase [36].

The assumptions that the substrate is converted as a source of elements were indirectly confirmed by the scaling experiments (Method F) - after 24 h of biotransformation both enantiomers of the substrate were degraded. This can be explained by too high oxygen concentration in bioreactor, which increase the metabolism level and influence the amino acid oxidases (FAD-dependent) activity, and as a consequence – facilitated substrate mineralization. Yeasts amino acid oxidases (AAO) are involved in amino acids utilization in two ways: without amino acid uptake substrate can be use as nitrogen source or with a transport of amino acid into cell as a nitrogen and carbon source. [38,39] Cell-surface, extra- and endocellular amino acid oxidases were found in fungal and bacterial cells [40,41]. Extracellular oxidases are characterized by broad substrate specificity so number of amino acid transporters can be limited. Ammonium ions NH_4^+, obtained from amino acids, are transported across the plasma membrane and serve as a source of nitrogen. [39,42] In yeast cells D-amino acids oxidase (DAAO) is involved in catabolic utilization of exogenous amino acids This highly stereoselective flavoenzyme catalyze oxidative deamination of D-amino acids to corresponding imino acids (Fig. 8). In the presence of molecular oxygen $FADH_2$ is oxidized spontaneously to FAD and oxygen is reduced to hydrogen peroxide. In the next step imino acids are non-enzymatically hydrolyzed to α-keto acids and ammonia [43].

4. Conclusions

Kinetically driven, effective, enantioselective bioconversion of novel phosphonic derivative − 1-amino-1-(3′-pyridyl)methylphosphonic acid **3** on laboratory and half-preparative scale was performed. Process with *P. funiculosum* begins with the oxidative deamination to ketophosphonic acid, which was then bioreduced in one pot reaction to the corresponding hydroxy- compound. So, the reaction cascade constitutes

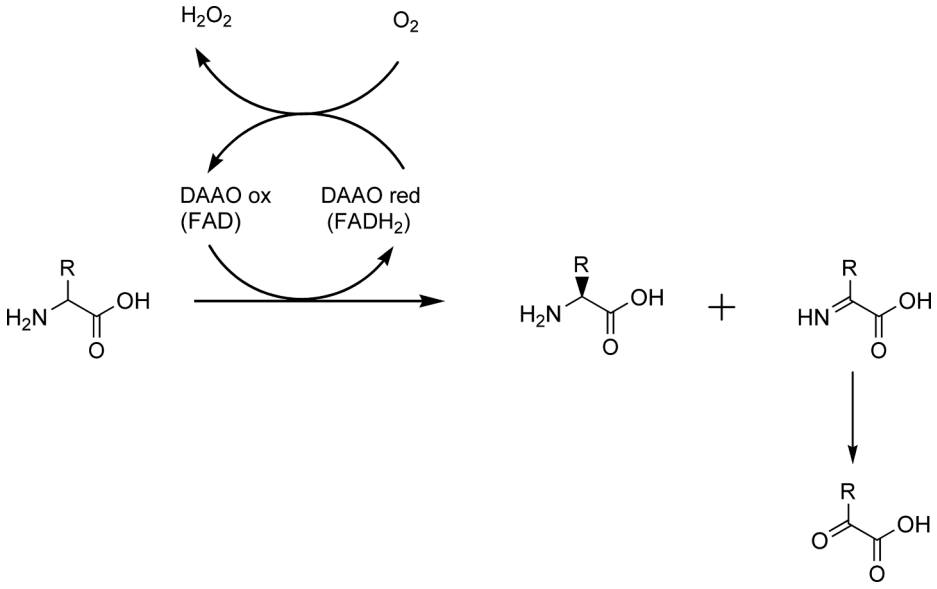


Fig. 8. Oxidative deamination of amino acids by D-amino acids oxidase (DAAO).

the redox cycle (laboratory scale experiments, shake flask system). Another mechanism was observed in process catalyzed by *R. mucilaginosa* (also laboratory scale experiments, shake flask system), where one from the enantiomers of aminophosphonic acid was mineralized in a very short time (serving as source of elements) and the second one remain unreacted. Scaling protocols were successful - pure isomer of compound (*S*)-**3** was obtained during continuous-flow bioprocess with immobilized *P. funiculosum* – (solution of substrate was increased from 3 mM to 11 mM).

These findings are significant, because this is the first report about the stereoselective bioconversion of chiral phosphonates with the heteroatom incorporated into their structure and because of the scaling.

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