Microbial biotransformation of two phosphonoacetic acid derivatives bearing two stereomeric centres

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Abstract
Three strains of microorganisms: Bacillus subtilis, Serratia liquefaciens and Escherichia coli were tested as whole-cell biocatalysts for the kinetic resolution of isomers of two new phosphonoacetic acid derivatives. Used compounds possess two chiral centres – one at the carbon adjacent to both functional groups and the other at the phosphorus. Biocatalytic hydrolysis of 2-butyryloxy-2-(butoxyethoxyphosphinyl)acetic acid and 2-butyryloxy-2-(isobutoxyethoxyphosphinyl)acetic acid with whole cells of Bacillus subtilis produced corresponding hydroxyphosphonates with diastereoselectivity ranging from 50 to 60%.

Key words: hydroxyphosphonates, lipolytic activity, biotransformations

Introduction
In today’s chemistry, biological means such as whole microbial cells or purified enzymes are increasingly being used for completing complex synthetic processes. One of the challenging applications of biocatalysis is the separation of enantiomers. Due to the fact that biological activity of many compounds is often inseparably connected with their stereochemistry, it becomes essential that pure enantiomers can be obtained at a certain point of the synthesis. Kinetic resolution of enantiomers with the use of microorganisms or enzymes is one of the routes to make that separation relatively easy and cost effective.

Hydroxyphosphonates possessing stereogenic center(s) constitute a group of substrates used for synthesis of a vast number of structurally variable organophosphonic compounds (Kolodiazhnyi, 2005; Plazuk et al., 2006; Pawar et al., 2006; Nesterov and Kolodiazhnyi, 2006; Kolodiazhnyi, 2012). Even though their properties and potential uses are yet to be discovered, they have been demonstrated to be efficient antiviral (Kolodiazhnyi, 2005; Magee and Evans, 2012), antibacterial and antifungal agents (Kolodiazhnyi, 2005). In addition, α-hydroxyphosphonate derivatives of tetrato[1,5-a]quinoline were found to possess antibacterial and antifungal properties (Kategaonkar et al., 2010). Our earlier studies, aimed at obtaining pure enantiomers of organophosphonic compounds, demonstrated that some hydroxyphosphonates might be delivered with good or excellent enantioselectivity by means of biotransformations (Skwarczyński et al., 1999; Malinowska et al., 2011). It was also found that analogous compounds like hydroxyphosphinates, may be produced in a similar way (Majewska et al., 2006; Majewska et al., 2009).

The purpose of this study was to extend the scope of our previous research aimed at the resolution of racemic mixtures of organophosphonic compounds that may possess some potential biological activity. We focused on the hydrolysis of 2-butyryloxy-2-(butoxyethoxyphosphinyl)acetic and 2-butyryloxy-2-(isobutoxyethoxyphosphinyl)acetic acids (Fig. 1; compounds 3a and 3b) using whole-cell biocatalysis in order to obtain enantiomerically pure forms of 2-hydroxy-2-(butoxyethoxyphosphinyl)acetic acid and 2-hydroxy-2-(isobutoxyethoxyphosphinyl)acetic acid (Fig. 2; compounds 2a and 2b). Three species of bacteria, Bacillus subtilis, Escherichia coli and Serratia liquefaciens, were tested. These microorganisms were selected from several bacterial strains stored in the Department of Bioorganic Chemistry, Wroclaw University of Technology collection. The selection was made on the basis of best lipolytic activity measured by Spirit blue agar test and previously observed results for the hydrolysis of butyryloxyphosphonates (Majewska et al., 2006; Malinowska et al., 2011). Compounds 2a and 2b were also chemically synthesized as a racemic mixture of all isomers, to confirm the identity of the hydrolysis products.
There is no literature data suggesting that these compounds have been synthesized and explored previously. Several papers report microbial transformation of similar compounds. Kinetic resolution of enantiomers of ethyl hydroxy(phenyl)methane(\(P\)-phenyl) phosphinate (a compound with two stereogenic centres but without the carboxylic group) has been tested earlier, using several bacterial strains (Majewska et al., 2006). Enantioselective biooxidation by fungi of ethyl hydroxy(phenyl)methane(\(P\)-phenyl) phosphinate has also been previously investigated (Klimek-Ochab et al., 2008).

**Materials and methods**

All materials were purchased from Sigma Aldrich (St. Louis, Missouri, United Staes), POCh (Gliwice, Poland) or BIOCORP (Warszawa, Poland) and were used without further purification.
B. subtilis and S. liquefaciens were obtained from our own collection and were identified by the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. E. coli was purchased from DSMZ.

NMR (Nucleic Magnetic Resonance) spectra were measured on a Bruker Avance™ 600 at 600.58 MHz for 1H; 243.12 MHz for 31P and 75.45 MHz for 13C in CDCl3 (99.8% of atom D, contains 0.03% v/v TMS) or on a Bruker Avance™ DRX 300 instrument operating at 300.13 MHz for 1H; 243.12 MHz for 31P and 151.02 MHz for 13C in CDCl3 (99.8% of atom D, contains 0.03% v/v TMS). Chemical shifts (δ) are given in ppm. 1H NMR are referenced to the internal standard TMS (δ = 0.00) and 13C NMR spectra to the central line of CHCl₃ (δ = 77.23).

The synthesized compounds were purified by gradient column chromatography using Merck Silica Gel 60 (63-230 mesh).

**Synthesis of compounds 1**

Compounds 1a and 1b were synthesized according to the method described below (Kosolapoff, 1951).

A mixture of 82.8 g (0.6 mol) of diethyl phosphate and 44.4 g (0.6 mol) of n-butanol or iso-butanol was slowly heated in a distillation apparatus (under normal pressure) up to oil bath temperature of 150°C, when a brisk reaction is initiated and ethanol distillation began. The bath was kept at 150-155°C until 16 ml of ethanol was collected. The mixture was then cooled to room temperature (RT). Next, the mixture was distilled under reduced pressure, and three fractions were collected (first at 90-100°C, second at 100-120°C and third at 125-140°C). The pressure of distillation was maintained at 17 hPa. Redistillation of the middle fraction repeated twice, gave pure ethyl butyl phosphate 1a (18.7 g, 18.8%), or pure ethyl iso-butyl phosphate 1b (8.5 g, 8.5%). The purity of compounds 1a and 1b was monitored by TLC (Thin Layer Chromatography) using ethyl acetate/hexane/isopropanol (4:7:1 v/v) as eluent.

**1a:**

1H NMR (CDCl₃, δ, ppm): 0.93 (t, J = 7.4 Hz, 3H, POCH₂CH₂CH₂CH₃), 1.37 (t, J = 7.1 Hz, 3H, POCH₂CH₂CH₂CH₃), 1.38-1.48 (m, 2H, POCH₂CH₂CH₂CH₂CH₃), 1.64-1.73 (m, 2H, POCH₂CH₂CH₂CH₂CH₃), 4.05-4.20 (m, 4H, POCH₂CH₂CH₂CH₂CH₃), 6.81 (d, J = 692.4 Hz, 1H, PCH₂).

31P NMR (CDCl₃, δ, ppm): 8.23.

**1b:**

1H NMR (CDCl₃, δ, ppm): 0.96 (d, J = 6.7 Hz, 6H, POCH₂CH(CH₃)₂), 1.37 (t, J = 7.1 Hz, 3H, POCH₂CH₂CH₃), 1.87-2.06 (m, 2H, POCH₂CH(CH₃)₂), 3.77-3.91 (m, 2H, POCH₂CH₃), 4.06-4.21 (m, 2H, POCH₂CH₃), 6.82 (d, J = 695.5 Hz, 1H, PCH₂).

31P NMR (CDCl₃, δ, ppm): 8.61.

**Synthesis of compounds 2**

Compounds 1a and 1b were converted to 2a and 2b according to a modified method (Caplan, 2000).

3.32 g (20 mmol) of ethyl butyl phosphate (or ethyl iso-butyl phosphate) was mixed with 1.84 g (20 mmol) glyoxylic acid monohydrate and 2.79 ml (20 mmol) triethylamine. The resulting solution was stirred for 2 h at RT. Thereafter, the reaction mixture was dissolved in 10 ml of distilled water and triethylamine was removed from the solution by ion exchange column (Dowex® 50W X8 50-100 mesh). After purification, 2.93 g (61% yield) of pure 2-butyryloxy-2-[butoxyethoxyphosphinyl]acetic acid 2a, or 3.59 g (75% yield) of pure 2-butyryloxy-2-[iso-butoxyethoxyphosphinyl]acetic acid 2b were obtained.

**2a:**

1H NMR (CDCl₃, δ, ppm): 0.94 (t, J = 7.4 Hz, 3H, POCH₂CH₂CH₂CH₃), 1.37 (t, J = 7.1 Hz, 3H, POCH₂CH₂CH₃ the second pair of enantiomers), 1.38 (t, J = 7.1 Hz, 3H, POCH₂CH₂CH₃ the second pair of enantiomers), 1.35-1.49 (m, 2H, POCH₂CH₂CH₂CH₃), 1.64-1.75 (m, 2H, POCH₂CH₂CH₂CH₃), 4.18-4.34 (m, 4H, POCH₂CH₂CH₂CH₃), 4.57 (d, J = 16.1 Hz, 1H, PCH₂).

31P NMR (CDCl₃, δ, ppm): 16.80.

13C NMR (CDCl₃, δ, ppm): 13.74 (POCH₂CH₂CH₃), 16.51 (d, J = 6.2 Hz, POCH₂CH₃), 18.78 (POCH₂CH₂CH₂CH₃), 32.62 (d, J = 4.3 Hz, POCH₂CH₂CH₂CH₃), 64.62 (d, J = 7.0 Hz, POCH₂CH₂CH₃ one pair of enantiomers), 65.06 (d, J = 7.2 Hz, POCH₂CH₂CH₃ a second pair of enantiomers), 68.62 (d, J = 7.4 Hz, POCH₂CH₂CH₂CH₃ CH₃ one pair of enantiomers), 68.68 (d, J = 7.4 Hz, 1H, PCH₂CH₃ a second pair of enantiomers), 68.65 (d, J = 7.5 Hz, POCH₂CH₂CH₂CH₃ a second pair of enantiomers), 68.66 (d, J = 156.9 Hz, PCH a second pair of enantiomers), 170.55 (d, J = 10.3 Hz, _COOH).

**2b:**

1H NMR (CDCl₃, δ, ppm): 0.96 (d, J = 6.6 Hz, 6H, POCH₂CH(CH₃)₂), 1.37 (t, J = 7.4 Hz, 3H, POCH₂CH₂CH₃), 1.87-2.06 (m, 2H, POCH₂CH(CH₃)₂), 3.77-3.91 (m, 2H, POCH₂CH₃), 4.06-4.21 (m, 2H, POCH₂CH₃), 6.82 (d, J = 695.5 Hz, 1H, PCH₂).
J. Szyszkowiak, P. Majewska

428

CH3), 1.84-2.08 (m, 2H, POCH2CH2(CH3)2), 3.90-4.03 (m, 2H, POCH2CH2CH3), 4.58 (d, J = 16.0 Hz, 1H, PCH).

31P NMR (CDCl3, δ, ppm): 17.10 (one pair of enantiomers), 17.12 (a second pair of enantiomers).

13C NMR (CDCl3, δ, ppm): 16.17 (d, J = 4.9 Hz, POCH2CH2H3), 18.67 (2C, POCH2CH(CH3)2), 29.31 (d, J = 6.0 Hz, POCH2CH(CH3)2), 64.42 (d, J = 6.8 Hz, POCH2CH3, one second pair of enantiomers), 64.62 (d, J = 6.8 Hz, POCH2CH3, a second pair of enantiomers), 68.83 (d, J = 161.2 Hz, PCH, one pair of enantiomers), 67.99 (d, J = 161.2 Hz, PCH, a second pair of enantiomers), 171.31 (d, J = 10.3 Hz, COOH).

Synthesis of compounds 3

Compounds 1a and 1b were converted to 3a and 3b without the isolation of hydroxyphosphonates 2a and 2b according to a modified method described in the literature (Caplan, 2000; Majewska, 2006).

3.32 g (20 mmol) of ethyl butyl phosphite (or ethyl iso-butyl phosphite) was mixed with 1.84 g (20 mmol) glyoxylic acid monohydrate and 2.79 ml (20 mmol) triethylamine. The resulting solution was stirred for 2 h at RT. Thereafter, the reaction mixture was dissolved in 100 ml of chloroform, placed in an ice bath and 2.07 ml (20 mmol) of butyryl chloride was slowly added drop-wise. After completion of the reaction, which lasts for two days as monitored by TLC, the resulting solution was extracted with 100 ml of distilled water, the organic phase was evaporated and the product was purified by column chromatography using dichloromethane:isopropanol in a ratio of 100:5 as eluent. After purification, 3.46 g (59% yield) of pure 2-butyryloxy-2-[butoxyethoxyphosphinyl]acetic acid 2a, or 3.94 g (67% yield) of pure 2-butyryloxy-2-[isobutoxyethoxyphosphinyl]acetic acid 2b were obtained.

3a:

1H NMR (CDCl3, δ, ppm): 0.93 (t, J = 7.4 Hz, 3H, POCH2CH2CH2H), 0.98 (t, J = 7.4 Hz, 3H, CH2CH2CH2 CH3), 1.36 (t, J = 7.0 Hz, 3H, POCH2CH2CH3), 1.61-1.77 (m, 4H, CH2CH2CH2CH3), 1.90-2.17 (m, 2H, CH2CH2CH3), 2.37-2.54 (m, 2H, CH2CH2CH3), 4.13-4.40 (m, 4H, POCH2CH2CH3), 4.13-4.40 (m, 4H, POCH2CH2CH3), 5.54 (d, J = 17.8 Hz, 1H, POCH2CH2CH3).

31P NMR (CDCl3, δ, ppm): 14.77.

13C NMR (CDCl3, δ, ppm): 13.53 (2C, CH2CH2CH2H), 16.28 (d, J = 5.9 Hz, POCH2CH2H), 18.67 (2C, CH2CH2CH2CH3), 29.31 (d, J = 6.0 Hz, POCH2CH2CH3), 64.42 (d, J = 6.8 Hz, POCH2CH3, one second pair of enantiomers), 64.62 (d, J = 6.8 Hz, POCH2CH3, a second pair of enantiomers), 68.83 (d, J = 161.2 Hz, PCH, one pair of enantiomers), 67.99 (d, J = 161.2 Hz, PCH, a second pair of enantiomers), 171.31 (d, J = 10.3 Hz, COOH).

3b:

1H NMR (CDCl3, δ, ppm): 0.94 (d, J = 6.5 Hz, 6H, POCH2CH2CH2CH3), 0.98 (t, J = 7.5 Hz, 3H, CH2CH2 CH3), 1.36 (t, J = 7.1 Hz, 3H, POCH2CH2H), 1.64-1.77 (m, 2H, CH2CH2CH3), 1.90-2.03 (m, 1H, POCH2 CH2CH2CH3), 2.37-2.54 (m, 2H, CH2CH2CH3), 3.92-3.99 (m, 2H, POCH2CH2CH2CH3), 4.24-4.31 (m, 2H, POCH2CH2CH3), 5.55 (d, J = 17.7 Hz, 1H, PCH).

31P NMR (CDCl3, δ, ppm): 14.67 (one pair of enantiomers), 14.70 (a second pair of enantiomers).

13C NMR (CDCl3, δ, ppm): 13.68 (CH2CH2CH3), 16.45 (d, J = 6.0 Hz, POCH2CH2H), 18.41 (CH2CH2 CH3), 18.66 (2C, POCH2CH2CH3), 29.28 (d, J = 6.0 Hz, POCH2CH2CH3), 35.66 (CH2CH2CH3), 64.83 (d, J = 5.6 Hz, POCH2CH3, one pair of enantiomers), 64.90 (d, J = 5.6 Hz, POCH2CH3, a second pair of enantiomers), 67.95 (d, J = 161.2 Hz, PCH, one pair of enantiomers), 67.99 (d, J = 161.2 Hz, PCH, a second pair of enantiomers), 74.10 (d, J = 6.2 Hz, POCH2CH2CH3, one pair of enantiomers), 74.18 (d, J = 6.2 Hz, POCH2CH2CH3, a second pair of enantiomers), 166.49 (COCH2CH2CH3), 172.17 (d, J = 10.3 Hz, COOH).

Microorganisms, growth and whole cell biotransformation conditions

Microorganisms were cultivated in a medium tested previously for stimulating vigorous growth and the lipolytic activity (Majewska et al., 2006).

One liter of the medium contained 10 g soluble starch, 1 g yeast extract, 5 g (NH4)2SO4, 2 g K2HPO, and 100 µl of tributyrin and 11 mixed in distilled water. The microorganisms were incubated at 26°C with shaking at 150 rpm
Microbial biotransformation of two phosphonoacetic acid derivatives bearing two stereomeric centres

A - pair of enantiomers

B - pair of enantiomers

Fig. 3. $^{31}$P NMR spectra with quinine as a chiral solvating agent of biotransformation reaction of 3a with Bacillus subtilis after 6 days. On the left, there is product – hydroxyphosphonate 2a, on the right – unreacted substrate 3a

for 24 h. Subsequently, the cells were centrifuged at $1449 \times g$ for 10 min. Biotransformations were performed in 100 ml solution of 0.017 M phosphate buffer, pH 7.0, and 50 μl of substrate with (150 rpm shaking; RT). Then the biomass was centrifuged at $1449 \times g$, the supernatant evaporated and the products of biotransformation were extracted with acetonitrile three times. Thereafter, the organic solvent was evaporated and the products were analyzed by means of $^{31}$P NMR spectroscopy using quinine as a chiral discriminator.

Results and discussion

Firstly it must be noted that the chemical shift of each isomer on $^{31}$P NMR spectra is only tentative at this stage of research and the correlation of the signals on $^{31}$P NMR spectra (with chiral solvating agent – quinine) and absolute configuration of isomers is unknown (Fig. 3). However, previous experiments on structurally similar compounds with the use of analogical method (Majewska et al., 2006; Majewska et al., 2009) and deductive analysis of the obtained data allowed us to cautiously assign NMR signals to enantiomers and diastereomers. The starting substrate is a mixture of four stereoisomers – two enantiomeric mixtures of diastereomers (denoted as A and B). Therefore, the conversion of each pair of enantiomers was analyzed separately.

In the case of compounds with one center of chirality, the process of ester hydrolysis should be terminated at conversion equal to 50% when the enzyme works with 100% enantioselectivity. In such a case, only one of the enantiomers is converted to hydroxyphosphonate. In the case of four stereoisomers however, this simple picture becomes far more complex. The reaction of hydrolysis can proceed enantioselectively or diastereoselectively, where both selectivities can be observed at the same time.

Stereochemistry of compounds 2a, 2b, 3a and 3b is unknown and there are no data in the literature about it. Attempts to separate enantiomeric mixtures A and B by non-chiral methods such as chromatography proved unsuccessful. Some enantioselectivity was observed when S. liquefaciens was used as a biocatalyst, but it did not exceed 25% ($ee$ – enantiomeric excess, see Table 3). In other cases, only diastereoselectivity was observed ($de$ – diastereomeric excess, see Table 1 and Table 2). B. subtilis appeared to carry out hydrolysis most effectively (see Table 1 and Fig. 2) compared with two other organisms (see Table 2 and Table 3) with conversion up to 80% after 6 days for compound 3a. B. subtilis hydrolyzed both compounds without enantioselectivity but with diastereoselectivity reaching 60%. In addition, B. subtilis hydrolyzed the enantiomeric mixture denoted as A (one mixture of two enantiomers) less efficiently than
Table 1. Biotransformations with *Bacillus subtilis*

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<th>Compound</th>
<th>Time [days]</th>
<th>Conversion [%]</th>
<th>$de$ of product [%]</th>
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Table 2. Biotransformations with *Escherichia coli*

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Table 3. Biotransformations with *Serratia liquefaciens*

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<th>Conversion [%]</th>
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the mixture denoted as B (the second mixture of two enantiomers) for both compounds: 3a and 3b. The highest de was obtained for compound 3a after five days of reaction (52%) with reaction yield reaching 47%. When the yield of reaction exceeded 50%, stereospecificity dropped significantly (to 33%). In the case of compound 3b, better stereospecificities were obtained, albeit with lower yields of reaction.

Conclusions

Among the three studied microorganisms, only B. subtilis appeared to be a moderate biocatalyst in terms of stereopreference of hydroxyphosphonate production. The interesting finding of this work is that this biocatalyst hydrolyzed isomers of tested compounds with both (R) and (S) configurations located at carbon atom. Usually, this kind of reaction is more or less enantioselective rather than diastereoselective.

Acknowledgements

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