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ORIGINAL ARTICLE

Kinetic resolution of (±)-diethyl- and dibenzyl hydroxy(phenyl) methanephosphonates and their acyl derivatives with lipases

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Abstract

A wide variety of commercially available lipases and microbial whole cells were tested for biotransformations of (±)-diethyl and dibenzyl hydroxyl(phenyl)methanephosphonates. Biocatalytic hydrolysis of acylated hydroxyphosphonates by whole cells of *Bacillus subtilis* gave optically active compounds with 95%ee $_S$. Enantioselectivities obtained when using commercially available enzymatic preparations were less satisfactory, leading to both compounds with an enantiomeric excess in the range 15–35%. Screening lipases for their ability to acylate these phosphonates or to hydrolyze their acylated derivatives enabled selection of enzymes and organisms suitable for use in both processes.

Keywords: *kinetic resolution, hydroxyphosphonates, microbial hydrolysis, lipase*

Introduction

The exploitation of whole cells of microorganisms and isolated enzymes in organic synthesis has significantly increased in recent decades, because stereoselective biocatalytic processes constitute a useful alternative to asymmetric synthesis of chiral compounds. Among several classes of enzymes involved in enantioselective biotransformations lipases are the most commonly used, because of their wide tolerance towards synthetic substrates and substantial stereoselectivity of catalyzed reactions.

Hydroxyphosphonates are used in many industrial applications. Some of them are used as additives to domestic detergents or as antiscaling agents in the water treatment industry. Many of them exhibit interesting biological activities (antibacterial, antiviral or anticancer) and are considered as inhibitors of various classes of enzymes (Collinsova and Jiracek 2000; Kolodiazhnyi 2005). Their biological activity strongly depends on their structural, so preparation of this class of compound in their chiral forms is the focus of many research groups (Kolodiazhnyi 2006).

There are a significant number of publications concerning preparation of enantiomerically enriched diethyl and dibenzyl hydroxy(phenyl)methanephosphonates (Figure 1) by asymmetric chemical synthesis (Kolodiazhnyi 2005) but only a few examples of kinetic resolution of enantiomers by biocatalytic procedures, with the most developed methods relying on application of lipases.

Skwarczyński et al (1999) described enantioselective hydrolysis of diethyl *n*-butyryloxy(phenyl) methanephosphonate (**2a**) by whole cells of *Pseudomonas fluorescens*. The reaction resulted in an enantiomeric excess of 80% at 39% substrate conversion. Several publications have described the biotransformation of dimethyl hydroxy(phenyl) methanephosphonate (Rove and Spilling 2001; Davies at all 1998; Pamies and Backvall 2003) but enzymatic kinetic resolution of **1a** or **1b** and their derivatives has not been described so far. Rove and Spilling (2001) used *Pseudomonas* sp. lipase in the second step of chemoenzymatic synthesis of non racemic (70% ee $_R$) dimethyl acetoxy(phenyl)methanephosphonate obtained by asymmetric synthesis with titanium alkoxide. Enzymatic hydrolysis led to further enrichment of one enantiomer and resulted in pure (>99% ee $_R$) hydroxyphosphonate. Davies

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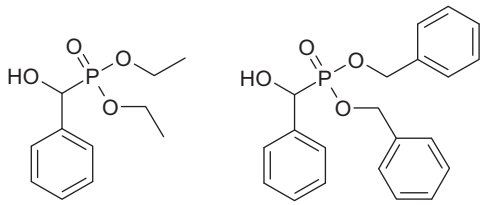

1a **1b**

Figure 1. Structures of diethyl hydroxyl(phenyl)methanephosphonate (**1a**) and dibenzyl hydroxyl(phenyl)methanephosphonate (**1b**).

at all (1998) used lipase F–AP 15 *(Rhizopus oryzae)* for enzymatic hydrolysis of the same acyloxyphosphonate and obtained 81%ee_S of alcohol and 81% ee_R ester. In contrast, preliminary studies on acylation of dimethyl hydroxy(phenyl)methanephosphonate with *Pseudomonas cepacia* lipase did not give satisfactory results (only 2% of yield) (Pamies and Backvall 2003).

Preliminary results on the enzymatic hydrolysis of acyloxy(phenyl)methanephosphonates encouraged us to investigate this reaction further. Thus the reactions were carried out using a wider set of microorganisms and a wide variety of commercially available enzymes (Table I). We also decided to use two substrates - **1a** and **1b -** as they differ in size, shape and hydrophobicity. The enzymatic acylation of **1a** and **1b** in an anhydrous environment was also investigated for comparison (Figure 2).

Methods

All materials were purchased from commercial suppliers: Sigma, Aldrich, Fluka, POCh, Serva,

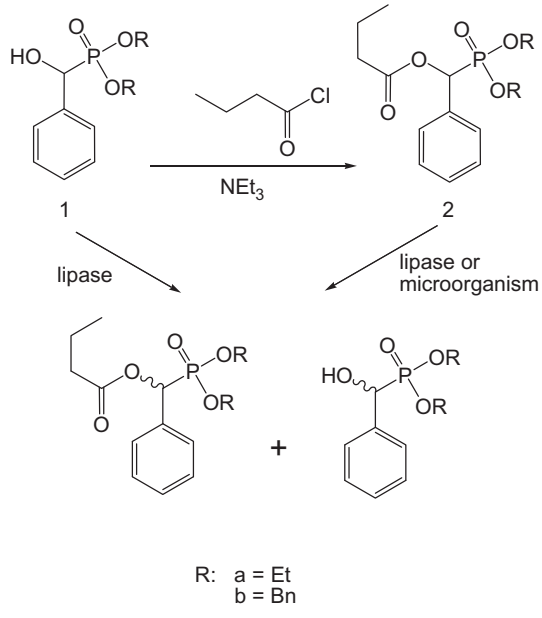

Figure 2. Scheme of biotransformation reactions.

and were used without further purification. The sources of lipases were: *Pseudomonas cepacia* (Sigma-Aldrich), *Pseudomonas cepacia* on Sol-gel-AK (Fluka), *Pseudomonas fluorescens* (Sigma-Aldrich), *Candida cylindracea* (Sigma-Aldrich), *Candida antarctica* A – CLEA (Fluka), *Rhizopus niveus* (Fluka), *Rhizopus oryzae* (Fluka), *Rhizopus species* (Serva), *Penicillium roqueforti* (Sigma-Aldrich), *Penicillium camemberti* (Sigma-Aldrich), *Aspergillus niger* (Sigma-Aldrich), *Mucor javanicus* (Sigma-Aldrich), *Rhizomucor meihei* (Sigma-Aldrich), porcine pancreas (Fluka), hog pancreas (Sigma-Aldrich), *Lipozyme®* (Fluka) and *Novozyme* 435 (generous gift *from* Novozymes). NMR spectra were measured on a Bruker Avance TM 600 at 600.58 MHz for ^1H; 243.12 MHz for ^{31}P and 151.02 MHz for ^{13}C in $CDCl_3$ or on a Bruker Avance DRX 300 instrument operating at 300.13 MHz for ^1H and 121.50 MHz for ^{31}P in $CDCl_3$. Chemical shifts (δ) are reported in ppm and coupling constants ($\mathcal{J}$) are given in Hz. ^1H NMR are referenced to the internal standard TMS (δ = 0.00), ^{13}C NMR spectra to the center line of $CHCl_3$ (δ = 77.23) and 85% phosphoric acid in H_2O for ^{31}P NMR spectra was used as external reference. Synthesized compounds were purified by gradient column chromatography using Merck Silica Gel 60 (63–230 mesh).

Synthesis of 1

Compounds **1a** and **1b** were synthesized according to Texier-Boullet and Lequitte (1986): 5 g of aluminum oxide was mixed with 5 g of potassium fluoride and powdered in a grinder. A mixture of 20 mmol of benzaldehyde 20 mmol and of diethyl or dibenzyl phosphite was added into this mixture and left at room temperature for 12 h. The reaction progress was monitored by TLC using dichloromethane/ethyl acetate (5:3 v/v for compound **1a** and 4:1 v/v for compound **1b**) as eluent. After this time the mixture was suspended in dichloromethane, insoluble components were filtered and solvent was evaporated. The desired compounds **1a** and **2a** were purified by recrystallization from a hexane - dichloromethane mixture.

1a:
Yield 82%, ^{31}P NMR (121.50 MHz, $CDCl_3$) δ 22.09 ppm; ^1H NMR (300.13 MHz, $CDCl_3$) δ 1.19, 1.25 ppm, 2xt, 6H, ($P(O)CH_2C\underline{H}_3$, $\mathcal{J}$ = 7.1Hz); 4.07-3.88 ppm, m, 4H ($P(O)C\underline{H}_2CH_3$); 5.00 ppm, d, 1H ($\mathcal{J}_{HP}$ = 10.9Hz); 7.48-7.24 ppm, m, 5H (H_{Ar}), mp. 73–75°C (lit. 64–65°C (Belciug et al. 1995), 75–77°C (Sardarian, Kaboudin 1997), 78–80°C (Goldeman, Soroka 2006), 82–84°C (M.M. Kabachnik et al. 2009).

Table I. Screening of hydrolysis (**2a** and **2b**) and acylation (**1a** and **1b**) reactions.

	Compound			
	2a	**2b**	**1a**	**1b**
	Conversion [%]			
Lipase	**Hydrolysis**		**Acylation**	
Candida cylindracea	100	100	8	–
Candida antarctica A	100	91	28	–
Rhizopus species	–	–	–	–
Rhizopus niveus	–	–	–	–
Rhizopus oryzae	5	8	6	–
Rhizomucor miehei	9	–	–	–
Mucor miehei	–	–	–	–
Penicillium camembertii	–	–	–	–
Penicillium roquefortii	–	–	–	–
Aspergillus niger	85	26	–	–
Pseudomonas cepacia	13	11	4	5
Pseudomonas fluorescens	18	15	5	4
Porcine pancreas	93	16	–	–
Hog pancreas	53	28	–	–
Immobilized *Pseudomonas cepacia*	–	–	–	–
Lipozyme	–	–	–	–
Novozyme 435	–	–	7	8
Microorganism	**Hydrolysis**			
Serrratia liquefaciens	100	–		
Escherichia coli	100	16		
Bacillus subtilis	100	20		
Pseudomonas fluorescens	98	10		
Pseudomonas aeruginosa	100	17		

"-" – Conversion 0-5% in hydrolysis reaction.

1b:
Yield 77%, ^{31}P NMR (121.50 MHz, $CDCl_3$) δ 22.41 ppm; ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 4.97–4.83 ppm, m, 4H (P(O)C$\underline{H}_2$C$_6$H$_5$); 5.06 ppm, d, 1H ($\mathcal{J}_{HP}$ = 10.4 Hz); 7.45-7.20 ppm, m, 15H (H_{Ar}), mp. 100–101°C, (lit. 103–104°C (Hoffmann 1988), 117–118°C (Pawar et al. 2006).

Synthesis of 2

Compounds **1a** and **1b** were converted into **2a** and **2b** with *n*-butyryl chloride by the following procedure: 10 mmol of compound **1** was added to 100 ml of the reaction medium composed of chloroform and triethylamine (10:1 v/v), followed by addition of 11 mmol of *n*-butyryl chloride. The resulting solution was stirred for 2 h at room temperature and then refluxed for 6 h. After this time the mixture was washed successively with: 100 ml of 5% hydrochloric acid, 100 ml of distilled water and dried over anhydrous magnesium sulphate. The product was purified by silica gel column chromatography using dichloromethane/ethyl acetate (5:3 v/v for compound **2a** and 4:1 v/v for compound **2b**) as eluent.

2a:
R_f = 0.68, Yield 93%. ^{31}P NMR (121.50 MHz, $CDCl_3$) δ 18.55 ppm; ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 0.95 ppm, t, 3H, $\mathcal{J}$ = 7.4Hz, (C(O)$CH_2CH_2C\underline{H}_3$); 1.21, 1.24 ppm, 2xt, $\mathcal{J}$ = 7.1Hz , 6H, (P(O)$CH_2C\underline{H}_3$,); 1.69 ppm, sextet, 2H, $\mathcal{J}$ = 7.4Hz, (C(O)$CH_2C\underline{H}_2CH_3$); 2.43 ppm, t, 2H, $\mathcal{J}$ = 7.4Hz, (C(O)$C\underline{H}_2CH_2CH_3$); 4.11-3.94 ppm, m, 4H (P(O)$C\underline{H}_2CH_3$); 6.17 ppm, d, 1H ($\mathcal{J}_{HP}$ = 13.5Hz); 7.50-7.27 ppm, m, 5H (H_{Ar}).

2b:
R_f = 0.75, Yield 78%. ^{31}P NMR (121.50 MHz, $CDCl_3$) δ 19.55 ppm; ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 0.87 ppm, t, 3H, $\mathcal{J}$ = 7,4Hz, (C(O)$CH_2CH_2C\underline{H}_3$); 1.59 ppm, sextet, 2H, $\mathcal{J}$ = 7.4Hz, (C(O)$CH_2CH_2CH_3$); 2.43 ppm, t, 2H, $\mathcal{J}$ = 7.7Hz, (C(O)$C\underline{H}_2CH_2CH_3$); 4.99-4.77 ppm, m, 4H (P(O)$C\underline{H}_2C_6H_5$); 6.21 ppm, d, 1H ($\mathcal{J}_{HP}$ = 13.5Hz); 7.44-7.19 ppm, m, 15H (H_{Ar}).

Enzymatic hydrolysis of 2

Enzymatic hydrolysis reactions were carried out in a biphasic system (3.8 ml) consisting of 0.05 M phosphate buffer, pH 7.0 (3.0 ml) and a mixture of di*iso*propyl ether (0.2 ml) with *n*-hexane (0.6 ml). After addition of 0.2 mmol of substrate and 50 mg of a suitable lipase (for other amounts see Table II) reactions were carried out at room temperature with shaking (150 rpm). The reeaction was stopped after certain periods of time and the mixture extracted twice with 15 ml of ethyl acetate, then organic phase was dried over anhydrous magnesium sulphate. After filtration, the organic solvent was removed by evaporation. The resulting alcohol and ester were separated by preparative HPLC. The hydroxyphosphonate obtained was analyzed by ^{31}P NMR using quinine as a chiral discriminator.

Enzymatic esterification of 1

Enzymatic acylations were carried out in di*iso*propyl ether (2ml) with the addition of 20 mg of powdered molecular sieves (3Å mesh). 0.02 mmol of the substrate, and 50 mg of a suitable lipase (for other amounts see Table III) and 0.165 mmol of vinyl *n*-butyrate were added. Reactions were carried out at 36 °C in a shaker (150 rpm). Reactions were stopped after certain periods of time by filtration followed by evaporation of the organic layer. The conversion and enantiomeric excess of the remaining substrate were analyzed by ^{31}P NMR using quinine as a chiral discriminator.

All compounds recovered from enzymatic or microbial hydrolyses were separated and purified by

Table II. Enzymatic hydrolysis of **2a** and **2b**.

Compound	Lipase	Amount of enzyme [mg]	Time [h]	conversion [%]	ee of alcohol **1a** or **1b**[%] (configuration)	ee of ester **2a** or **2b**[%] (configuration)[a]	E[b]
2a	*Candida cylindracea*	50	2	48	35 (*R*)	32 (*S*)	2,79
	Candida antarctica A	50	2.5	48	17 (*S*)	16 (*R*)	1,62
	Pseudomonas cepacia	150	120	11	6 (*R*)	1 (*S*)	1,14
	Pseudomonas fluorescens	150	120	12	6 (*R*)	1 (*S*)	1,14
	Aspergillus niger	50	48	56	40 (*S*)	51 (*R*)	3,74
	Porcine pancreas	50	2	49	16 (*R*)	15 (*S*)	1,58
	Hog pancreas	100	72	53	17 (*R*)	19 (*S*)	1,67
2b	*Candida cylindracea*	50	12	53	11 (*R*)	12 (*S*)	1,39
	Candida antarctica A	50	43	47	5 (*R*)	4 (*S*)	1,15
	Pseudomonas cepacia	200	168	17	8 (*S*)	2 (*R*)	1,19
	Pseudomonas fluorescens	200	168	14	21 (*S*)	3 (*R*)	1,58
	Aspergillus niger	100	120	43	16 (*R*)	12 (*S*)	1,54
	Porcine pancreas	150	168	25	12 (*S*)	4 (*R*)	1,32
	Hog pancreas	150	120	20	17 (*S*)	4 (*R*)	1,47

a – The ee_s values were then obtained from the equation $C = ee_s/(ee_s + ee_p)$.
b- $E = \ln[(1 - C(1 + ee_p)]/\ln[(1 - C(1 - ee_p)]$.

HPLC (Varian, Dynamax HPLC Column 250 × 21.4 mm; MICROSORB 300-10 C18) in a gradient of water : acetonitrile (10 minutes from 30% of acetonitrile in water to 60% of acetonitrile, followed by a 2 minute increasing gradient to 95% of acetonitrile, then maintained for 5 min). Retention times: 9,0 min – **1a**, 12,7 min – **2a**; 13,1 min – **1b**, 15,8 min – **2b**.

Microorganisms, growth and whole cell biotransformation conditions

Three microorganisms were from our own collection. They were *Pseudomonas aeruginosa*, *Bacillus subtilis and Serratia liquefaciens* and were identified by Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany. *Escherichia coli* and *Pseudomonas fluorescens* were purchased from DSMZ.

Microorganisms were grown in a lipolytic medium tested previously (Majewska et al. 2006). This medium consisted of 10 g of soluble starch, 1 g of yeast extract, 5 g of $(NH_4)_2SO_4$, 2 g of K_2HPO_4, 100 µl of tributyrin and 1000 ml of distilled water. Microorganisms were incubated at 26°C with shaking at 150 rpm for 1 day after which the cells were centrifuged at 3500 rpm for 15 min and washed twice in 0.017 M phosphate buffer, pH 7.0. Biotransformations were performed in 100 ml of 0.017 M phosphate buffer, pH 7.0, after addition of 50 µl of substrate, with shaking at 150 rpm at room temperature. Then the biomass was centrifuged, the supernatant extracted twice with ethyl acetate and dried over anhydrous magnesium sulfate. After filtration, the organic solvent was evaporated and the resulting alcohol and ester separated by preparative HPLC. The product was analyzed by ^{31}P NMR spectroscopy using quinine as a chiral discriminator.

Table III. Enzymatic acylation of **1a** and **1b**.

Compound	Lipase	Amount of enzyme [mg]	Time [h]	conversion [%]	ee of alcohol **1a** or **1b**[%] (configuration)	ee of ester **2a** or **2b**[%] (configuration)[a]	E[b]
1a	*Candida cylindracea*	300	168	12	13 (S)	95 (R)	47,56
	Candida antarctica A	50	96	52	17 (R)	16 (S)	1,60
	Pseudomonas cepacia	300	168	8	3 (R)	35 (S)	2,12
	Pseudomonas fluorescens	300	168	14	10 (R)	61 (S)	4,61
	Rhizopus oryzae	300	168	15	0	0	–
	Novozyme 435	300	168	13	7 (R)	47 (S)	2,96
1b	*Candida antarctica A*	300	168	9	15 (S)	52 (R)	5,65
	Pseudomonas fluorescens	300	168	11	6 (S)	49 (R)	3,06
	Novozyme 435	300	168	15	16 (S)	91 (R)	23,89

a – The ee_s values were then obtained from the equation $C = ee_s/(ee_s + ee_p)$.
b- $E = \ln[(1 - C)(1 - ee_s)]/\ln[(1 - C)(1 + ee_s)]$.

Spirit blue agar plates

The lipolytic activity of microorganisms was tested on Spirit blue agar plates. Several media were tested and the best one was chosen in order to achieve the most vigorous growth and the best lipolytic activity visualization. 5 g of peptone from meat, 5 g of peptone from casein, 5 g of yeast extract, 20 g of agar-agar and 0.15 g of Methyl Blue (Spirit Blue) were suspended in 1 l of distilled water and autoclaved (15 min at 121°C). In a separate flask lipase reagent was prepared by suspending of 6 ml tributyrin and 0.6 g gum arabic in 24 ml of distilled water. Lipase reagent was also autoclaved (15 min at 121°C). Both mixtures were cooled to at least 50°C (stabilization of the emulsion), poured into sterile plates and allowed to solidify. Inoculated plates were incubatad at 30 ± 2°C for 72 hours and after this time, clear zones around colonies of lipolytic microorganisms were observed.

Results and Discussion

Screening

Screening was carried out using a wide variety of available enzymatic preparations and microorganisms. Reactions were carried out using both types of biocatalyst for a standard time (6 days for hydrolysis by whole cells and 7 days for hydrolysis and acylation by lipases) at room temperature (Table I). Enzymatic hydrolyses were carried out in a biphasic system (phosphate buffer and the mixture of *n*-hexane and di*iso*propyl ether) using 50 mg of biocatalyst. The acylation reaction was carried out under anhydrous conditions (di*iso*propyl ether) and therefore only isolated enzymes were tested.

In all the cases conversion of compound **2a** was significantly higher than that of **2b**. Only biocatalysts giving more than 10% substrate conversion in the screening reaction were considered for further studies. Whereas hydrolysis of compounds **2** gave satisfactory results the synthesis of these compounds by acylation of hydroxyphosphonates **1** failed (only one reaction occurred with a conversion higher than 10%). Therefore, in the hope of achieving better substrate/product ratio the enzymes showing any activity were used in further studies.

Enzymatic hydrolysis

In order to optimize the reaction, the time of reaction and amount of enzyme were adjusted to achieve approximately 50% of substrate conversion (Table II). This is typical when trying to set up conditions for kinetic resolution of enantiomers. Hydrolysis of compounds **2a** and **2b** provided products enriched in *R* or *S*- stereoisomers depending on the lipase applied.

Higher values of enantiomeric excess were achieved for hydrolysis of **2a**. Unfortunately, reactions exhibited low to moderate stereoselectivities. In most cases enzymatic hydrolysis of **2a** provided products enriched in the *R*-enantiomer of the alcohol. The only exceptions were lipases from *Candida antarctica* and *Aspergillus niger*, which more selectively hydrolyzed the substrate of opposite configuration, thus providing the S-product. The reverse stereoselectivity of most of lipases was observer in case of hydrolysis of **2b**.

Enzymatic acylation

Despite the high excess of enzyme used and prolonged reaction time, we were not able to obtain conversions higher than 8–15% (Table III). The only enzyme which exhibited satisfactory activity was lipase from *Candida antarctica* A, which yielded 52% conversion of substrate **1a** within 4 days. However, even in this case the reaction had poor enantioselectivity (17%ee).

Microbial hydrolysis

The esters **2a** and **2b** were also hydrolyzed using whole bacterial cells. These microorganisms were previously tested for their lipolytic activity using Spirit Blue agar plates. The kinetic resolution was carried out in a buffered medium and reactions were stopped after reaching the conversion range of ~50% or after 6 days (Table IV).

Microbial hydrolysis gave the most satisfactory results. All the reactions carried out when **2a** was used as substrate provided *S*-**1a** with different ee values, depending on the strain used. The best results were obtained using *B. subtilis* – after 8 hours a product of 95% ee was obtained with satisfactory yield. *Escherichia coli* might also be considered as a potentially useful catalyst; however, high ee % values were only achieved at low conversions. In contrast, hydrolysis of **2b** did not give satisfactory results, with both conversions and enantioselectivities being exceptionally low.

We were unable to calculate ee% values for the unreacted **2**. Neither diethyl- or dibenzyl acetoxyphenylmethanephosphonates showed separation of signals in their ^{31}P NMR spectra in the presence of α- cyclodextrin. A better method for determination of ee values of these esters may be their chemical conversion into hydroxyphosphonates and using methods suitable for alcohols – ^{31}P NMR spectra

Table IV. Microbial hydrolysis of **2a** and **2b**.

Compound	Lipase	Time [h]	conversion [%]	ee of alcohol **1a** or **1b**[%] (configuration)	ee of ester **2a** or **2b**[%] (configuration)[a]	E[b]
2a	control	144	12	26 (S)	4 (*R*)	1,76
	Serrratia liquefaciens	96	61	16 (S)	25 (*R*)	1,71
	Escherichia coli	18	36	64 (S)	36 (*R*)	6,43
		24	48	41 (S)	38 (*R*)	3,39
	Bacillus subtilis	8	44	95 (S)	75 (*R*)	87,74
		10	47	92 (S)	82 (*R*)	60,71
		12	51	74 (S)	77 (*R*)	15,35
	Pseudomonas fluorescens	96	43	20 (S)	15 (*R*)	1,72
	Pseudomonas aeruginosa	4	27	14 (S)	5 (*R*)	1,39
		8	32	6 (S)	3 (*R*)	1,16
2b	control	144	3	2 (S)	<0,5 (*R*)	1,04
	Serrratia liquefaciens	144	4	8 (S)	0 (*R*)	1,18
	Escherichia coli	144	16	15 (S)	3 (*R*)	1,39
	Bacillus subtilis	144	20	6 (S)	2 (*R*)	1,14
	Pseudomonas fluorescens	144	10	6 (S)	1 (*R*)	1,14
	Pseudomonas aeruginosa	144	17	8 (S)	2 (*R*)	1,19

a – The ee_s values were then obtained from the equation $C = ee_s/(ee_s + ee_p)$.
b- $E = \ln[(1 - C(1 + ee_p)]/\ln[(1 - C(1 - ee_p)]$.

with quinine (presented in this paper). Various attempts to chemically hydrolyse **2** were undertaken. However, both acidic (TFA, H_2SO_4 in water: methanol mixture) and basic hydrolysis (K_2CO_3 or triethylamine in water, or NaOH in methanol solution) led to the decomposition of **2b** as confirmed by TLC and ^{31}P NMR spectra. Thus, these values were calculated from the NMR data obtained for hydroxyphosphonates (ee) and substrate conversion depending on the type of reaction (hydrolysis or acylation).

Absolute configuration assignment

The absolute configuration of both compounds was correlated with the literature data (Zhou et al. 2008; Hammerschmidt, Hanbauer 2000). For compounds **1a** and **1b**, the ratio of enantiomers was calculated on the basis of ^{31}P NMR spectra when quinine was used as chiral discriminator. For example, optically active dibenzyl hydroxyl(phenyl)methanephosphonate obtained by hydrolysis of **2b** with lipase from *Aspergillus niger* yielded dextrorotatory product composed of a non-equimolar mixture of enantiomers, which gave two signals in the ^{31}P NMR spectrum taken in the presence of quinine, with the predominant signal at 23.19 ppm.

Conclusions

A systematic study on the action of wide variety of lipases and some lypolitic microorganisms towards preparation of optically active diethyl and dibenzyl hydroxy(phenyl)methanephosphonates has been undertaken. Hydrolysis of *n*-butyryloxyphenylmethanephosphonates appeared to be a far better method than acylation of hydroxyphosphonate. However, only microbial hydrolysis of compound **2a** by whole cells of *B. subtilis* gave the major enantiomer (*S*) of compound **1a** with high enantiomeric excess 95% and satisfactory yield. The remaining biocatalysts gave unsatisfactory results.

It is noteworthy that a significant change in the size of the phosphonate moiety, namely the use of dibenzyl esters instead of diethyl ones, resulted in a significant decrease of reaction yield and enantioselectivity. Moreover, major products of the opposite configuration were typically obtained. This stereoselective outcome is probably caused by renumbered order of size of substituents according to the Kazlauskas rule. Thus, the increase of size of the phosphoryl substituent might be a method to reverse the stereoselectivity of lipases.

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