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Batch and in-flow kinetic resolution of racemic 1-(*N*-acylamino) alkylphosphonic and 1-(*N*-acylamino)alkylphosphinic acids and their esters using immobilized penicillin G acylase

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ABSTRACT

The kinetic resolution of racemic 1-(*N*-acylamino)alkylphosphonic acids **3** (R^3 = OH) and their dimethyl esters **1**, as well as 1-(*N*-acylamino)alkylphosphinic acids **4** (R^3 = H or Ph) using penicillin G acylase (PGA) immobilized on three types of mesoporous silicas in both a batch slurry system and in a continuous-flow reactor was studied. The initial hydrolytic deacylation rates in the presence of those catalysts were measured and the relationships between the substrate structure and the enzyme efficiency are discussed. The stereospecific hydrolysis of the *N*-acyl group of both racemic *N*-acylated phosphorus analogues of amino acids and their esters catalyzed by the immobilized PGA proved to be a highly effective method for the kinetic resolution of all the investigated compounds, with the stereochemical preference of PGA for (*R*)-substrates.

1. Introduction

Chiral, non-racemic α-aminoalkylphosphonic and α-aminoalkylphosphinic acids have attracted significant attention from chemists and biochemists for many years due to their diverse biological activity and wide range of applications, mainly as pharmaceuticals and agrochemicals.[1–6]

Recently we reported the stereospecific hydrolysis of the *N*-acyl group of both racemic 1-(*N*-acylamino)alkylphosphonic acids **3** (R^3 = OH) and their dimethyl esters **1** catalyzed by the native penicillin G acylase (PGA) as a highly effective method for the kinetic resolution of these compounds.[7] Looking at this problem from an application perspective, we deemed it important to undertake a study of the same process, but employing immobilized PGA. Immobilization of the enzyme provides a number of important advantages, including: (i) the ease of enzyme separation from the reaction mixture, which in turn simplifies reaction mixture processing; (ii) better handling and re-use of the enzyme; and (iii) the possibility of employing an immobilized enzyme in continuous-flow systems, with positive effects on the performance and process economy. Other possible improvements include increased operational and storage enzyme stability, and increased enzyme resistance against extremes in pH, temperature and ionic strength.[8]

PGA has tremendous industrial application in the hydrolytic deacylation of penicillin G to 6-aminopenicillanic acid (about 20,000 t a^{-1}) and its reacylation to semi-synthetic β-lactam antibiotics (ampicillin, amoxicillin, cephalexin, cefaclor, cefadroxil and others),[9] with immobilized PGA accounts for 88% of worldwide 6-aminopenicillanic acid production.[10–14]

PGA has been immobilized on various matrices, ranging from natural and synthetic organic polymers to inorganic carriers.[10,14–16] Inorganic mesoporous silicas such as SBA-15 or MCF offer some special advantages, such as uniform, tunable pore size and structure, large surface area, opened pore structure, openness to a wide variety of chemical modifications, great potential for high enzyme loading and stability towards organic solvents.[10–12,14] Diverse methods for PGA immobilization were evaluated and compared in a comprehensive review by Sheldon et al.[10] Covalent immobilization has generally been favored in the case of this enzyme.[10,17,18]

Herein, we report our studies on the immobilization of PGA from *Escherichia coli* (EC 3.5.1.11) on powder mesoporous silica of SBA-15 and MCF type and on the application of immobilized PGA for the kinetic resolution of racemic 1-(*N*-acylamino) alkylphosphonic and 1-(*N*-acylamino)alkylphosphinic acids and their esters, including a comparative study on the effectiveness of immobilized and native PGA in these processes (Scheme 1). A successful kinetic resolution of an α-aminophosphonic acid derivative in a continuous-flow microfluidic system containing the PGA

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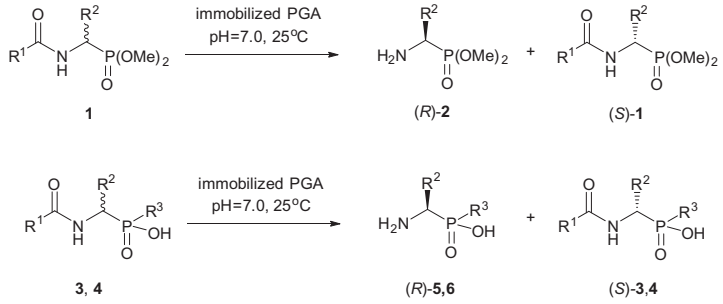


Scheme 1. Kinetic resolution of 1-(*N*-acylamino)alkylphosphonic, 1-(*N*-acylamino)alkylphosphinic acids and their esters using immobilized forms of penicillin G acylase.

immobilized inside the multichannel siliceous monolith with bi-modal pore structure is also reported.

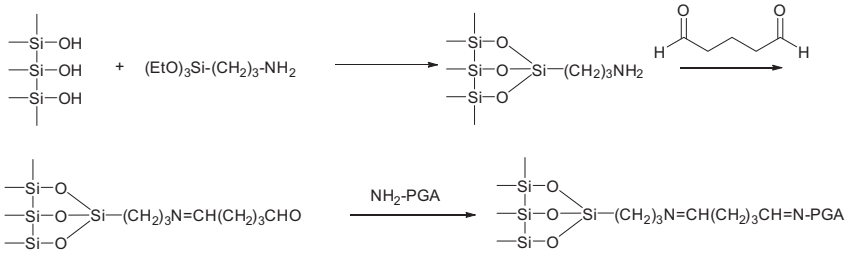
2. Results and discussion

2.1. PGA immobilization

PGA was immobilized on two types of mesoporous silicas (MPSs): SBA-15 (Santa Barbara Amorphous) and MCF (mesoporous cellular foams). MPSs have a rigid, open-pore structure and a large surface area that can be densely covered with various anchor groups. They can be synthesized to obtain pore sizes ranging from 2 to 40 nm. Moreover they are environmentally acceptable, structurally stable and resistant to microbial attacks. The mesoporous silicas were prepared by the templating method reported in our previous papers.[20–22] The silicas were next activated by exposure to water vapour at room temperature for 5 h, then dried at 200 °C and treated with (3-aminopropyl)triethoxysilane in toluene to obtain mesoporous silicas functionalized with 3-aminopropyl groups with amino groups density of ca. 1.5 mmol per 1 g of silica (Scheme 2).[24] Further activation of the functionalized silicas with a solution of glutaraldehyde in phosphate buffer bonded the glutaraldehyde by the covalent azomethine bond. Treatment of these silicas with a solution of PGA in phosphate buffer covalently bonded the enzyme via its amino groups with the free formyl groups of the glutaraldehyde moiety.

The immobilized PGA was finally obtained in the form of a suspension of silica powders in a phosphate buffer (pH 7.5), and it was stored at 4 °C in this form. The measured specific activity of the enzyme immobilized on SBA-15 and MCF was 273 and 2066 U/g, respectively, whereas the amounts of the protein bonded were equal to 20.9 mg/g for SBA-15 (immobilization yield 38%) and 49.4 mg/g for MCF (immobilization yield 95%). Thus the measured activity was more than seven times larger for the MCF-based enzyme than for SBA-15, despite there being only about two-fold larger the amount of protein bonded on to MCF. This can be explained by the specific structural features of the applied supports. Very open cellular pores (20–50 nm in diameter and ca. 2 cm^3/g mesopore volume) connected by the wide windows (14–18 nm) typical for MCFs allow for the unhindered diffusion of substrates and products. The smaller hexagonally arranged cylindrical pores of SBA-15 (6–8 nm, 0.8 cm^3/g mesopore volume) may be big enough to contain molecules of the attached enzyme, but may restrict substrate or product diffusion.

PGA was also immobilized in siliceous monolithic rods (MH) with a hierarchical pore structure. The monoliths (45 mm in length and 6 mm in diameter) were prepared by using the Nakanishi method[25] with minor modification, as recently described in our papers (Fig. 1a).[23,24] Their functionalization with aminopropyl



Scheme 2. Immobilization of penicillin G acylase on mesoporous silicas.

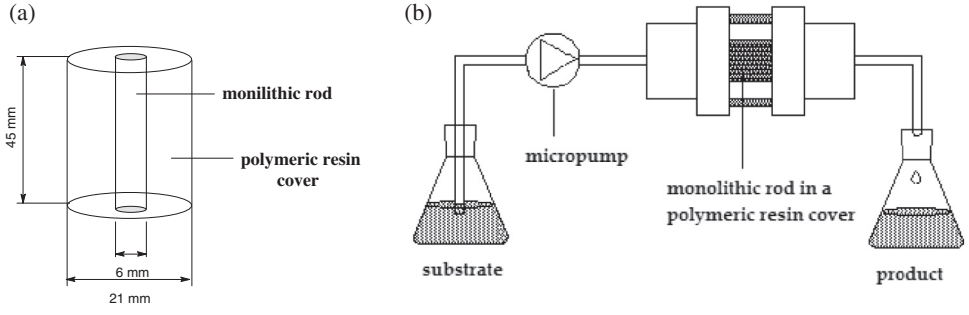


Figure 1. (a) PGA immobilized in a monolithic rod clad with polymeric resin. (b) A single-rod continuous-flow microreactor system.

groups was achieved by immersing the rods in a solution of 3-(aminopropyl)triethoxysilane in toluene for 72 h at 80 °C with intensive stirring. Each monolith was then clad with an epoxide polymer resin (L285MGS-H285MGS type) to obtain a single-rod multichannel microreactor. Each microreactor was rinsed with a series of solvents under flow, after which a solution of glutaraldehyde in phosphate buffer was pumped through the microreactor and, after rinsing with water and phosphate buffer, a solution of PGE in the phosphate buffer was pumped through the microreactor in a circular system for 3 h. Finally, the microreactor was rinsed again with water and a series of buffers.

The calculated amount of protein bounded inside the monolith (14.5 mg, or 44.6 mg/g of monolith, for a monolith weight of 0.325 g) was very close to that obtained for MCF (49.4 mg/g). This can also be explained by its very open pore structure (ca. 1 cm^3/g of mesopore volume, and over 2.5 cm^3/g of macropore volume) as determined by the nitrogen adsorption and mercury porosimetry experiments.[23,24]

2.2. Initial hydrolysis rate measurements

The initial rates of enzymatic hydrolytic deacylation of *N*-acylated 1-aminoalkylphosphonic and 1-aminoalkylphosphinic acids, as well as their esters, were measured for each specific substrate in a series of preliminary experiments in order to assure optimal substrate conversion of approximately 50% in the further kinetic resolution experiments (Scheme 1). The measurements were carried out in a water solution of phosphate buffer (pH 7.0) at 25 °C, with intensive stirring, using various amounts of the immobilized enzyme depending on the specific reactivity of substrate (Table 1). Approximately 2 mL of aliquots of the reaction mixture were extracted at various reaction times through Minisart® SRP 4 syringe filters to stop the reaction. Concentrations of the reaction product and the unconverted substrate in the reaction mixtures were determined by means of ^{1}H NMR spectroscopy using pivalic acid as the internal standard. The determined initial hydrolysis rates for immobilized PGA were compared in Table 1 with those reported in our previous paper for native PGA.[7]

Most of the values for the initial hydrolysis rates for the reactions catalyzed with PGA immobilized on MCF or SBA-15 were similar or slightly lower in comparison with those obtained for the reactions of the same compounds catalyzed by native PGA.[7] However, the initial hydrolysis rates in the presence of MCF-immobilized PGA were higher by a factor of 6.6 and 1.8, respectively, in the case of the phosphonic analogue of asparagine **3c** and the *H*-phosphinic analogue of alanine **4b**. Overall, the results obtained with the immobilized PGA confirm the relationship between the substrate structure and the enzyme efficiency observed for the native PGA and reported in our previous paper.[7] Thus the initial hydrolysis rate of the *N*-phenylacetylamino group is higher in comparison with the *N*-benzyloxycarbonylamino group. cf. compounds **4a** and **4b**; the hydrolysis rates of the free acids are distinctly higher when compared to the corresponding methyl esters, cf. **1c** and **3b**, as well as **1d** and **3c**, while the hydrolysis rate rapidly decreases when increasing the steric effect of the substituent at the α-position, cf. **1a** versus **1b**–**d**. The presence of a bulky substituent at the phosphorus atom in the phenylphosphinic acid derivative **4c** only slightly reduces its reactivity in comparison with the corresponding *H*-phosphinic analogue **4b**.

2.3. Kinetic resolution of racemic 1-(*N*-acylamino)alkylphosphonic and 1-(*N*-acylamino)alkylphosphinic acids and their esters in a batch system

The kinetic resolutions of *N*-acylated 1-aminoalkylphosphonic and 1-aminoalkylphosphinic acids as well as their esters were carried out in a solution of phosphate buffer (pH 7.0) at 25 °C with intensive stirring using various reaction times and amounts of immobilized enzyme depending on the specific reactivity of the substrate to achieve substrate conversion close to 50% (Scheme 1, Table 1). The reaction was stopped by filtering off the immobilizate by using folded paper filters. The unconverted substrates and hydrolysis products were separated by column chromatography on silica gel (for the esters) or by ion-exchange chromatography on DOWEX 50W X8 (H^+ form) for the free acids. The enantiomeric excesses in the unreacted substrates were determined as described in our previous paper[7] based on the integration of ^{31}P NMR or ^{1}H NMR signals of both enantiomers and using quinine as the chiral discriminating agent (Table 1). The enzyme enantioselectivities *E*, as the most intrinsic expression of enzyme stereoselectivity, were calculated as a function of the enantiomeric excess in the unchanged substrate and conversion of the substrate, as previously described.[7]

As can be seen from Table 1, highly effective kinetic resolutions with *E*-values exceeding 100 were obtained for all of the investigated *N*-acylated substrates, i.e. for the phosphonic analogues of valine, leucine, and asparagine **3a**–**c**, the dimethyl esters of the phosphonic analogues of alanine, phenylalanine leucine and asparagine **1a**–**d**, *H*-phosphinic analogues of alanine **4a**–**b** and the phenylphosphinic analogue of alanine **4c**. It is noteworthy that for two of the investigated substrates, i.e. the phosphonic analogue of valine **3a** and the dimethyl ester of leucine **1c**, the kinetic resolution catalyzed with native PGA gave only a moderate enzyme enantioselectivity (22 and 66, respectively).[7] The specific rotations of the unchanged substrates and hydrolysis products were close to those obtained for the same compounds in the kinetic resolution experiment using native PGA[7], which confirms the stereochemical preference of both native and immobilized PGA for the (*R*)-substrate.

2.4. Kinetic resolution of racemic 1-(*N*-phenylacetylamino)-2-carbamoylethylphosphonic acid dimethyl ester 1d in a flow system in a single-rod microfluidic microreactor

In a preliminary experiment, a solution of substrate **1d** (3 mmol/L) in phosphate buffer (pH 7) was pumped through the microreactor (Fig. 1b) with a gradually diminishing flow rate, starting with a value of 0.5 mL/min, to determine the dependency between the flow rate and substrate conversion (Table 2). Assuming that the free space inside the monolith is equal to 0.82 mL, which corresponds to an estimated monolith porosity of 65% at its geometrical volume of 1.27 mL, and that the activity of PGA in the monolith is equal to 333.8 U, the average hydrolysis rate *V* of the substrate was calculated at each specific flow rate (Table 2). A good linear relationship between log(*V*) and substrate conversion *C* was observed (Fig. 2). Extrapolation of this relationship to $C = 0$ allowed us to determinate the initial hydrolysis rate in the microreactor equal to 6.07×10^{-4} μmol/U min (which corresponds to $\log(V) = -3.2169$). The latter value is very close to the initial hydrolysis rate of the same substrate catalyzed by the native PGA, as reported in our previous paper (6.83×10^{-4} μmol/U min).[7]

The experiment described above revealed that a substrate conversion close to 50% can be achieved at a flow rate of 0.01 mL/min. Therefore, in the final kinetic resolution experiment, a solution of substrate (3 mmol/L, 100 mL) in phosphate buffer was pumped through the microreactor at a flow rate of 0.01 mL/min. The unconverted substrate (*S*)-**1d** and the hydrolysis product (*R*)-**2d** were isolated from the outflow (87 mL) by column chromatography on silica gel as described above in yields of 47% and 44%, respectively. Their specific rotations, +5.4 and −2.4, respectively, were close to those obtained for the same compounds obtained in the kinetic resolution experiment using native PGA.[7] The enantiomeric excess in the unchanged substrate was determined to be 99% (^{1}H NMR

Table 1
Enzymatic hydrolysis of *N*-acylated 1-aminoalkylphosphonic acids **3**, their esters **1** and 1-aminoalkylphosphinic acids **4** catalyzed by immobilized PGA

Substrate				Activity of PGA [U][a]/matrice	Initial hydrolysis [μmol/U min]		Kinetic resolution results									
No.	R^1	R^2	R^3		Immobilized PGA	Native PGA[7]	Prod. No.	Time [h]	Conversion	Yield [%][b]		$[\alpha]_D^{20}$		Product config.	$ee_s \times 100$ [%]	E
										Substrate	Product	Substrate	Product			
1a	$PhCH_2$	Me	—	1.44/MCF	$1.41 \pm 0.16 \times 10^{1}$	1.46×10^{1}	**2a**	0.67	0.48	43	49	+43.9[c]	−6.4[d]	(*R*)	99	>100
1b	$PhCH_2$	$PhCH_2$	—	306/MCF	$5.13 \pm 0.64 \times 10^{-4}$	5.21×10^{-4}	**2b**	144	0.49	45	43	+28.7[e]	−25.2[f]	(*R*)	99	>100
1c	$PhCH_2$	*i*-$PrCH_2$	—	270/MCF	$4.59 \pm 0.34 \times 10^{-4}$	6.89×10^{-4}	**2c**	96	0.48	48	47	+31.7[g]	−17.2[h]		99	>100
1d	$PhCH_2$	H_2NCOCH_2	—	198/MCF	$2.26 \pm 0.19 \times 10^{-4}$	6.83×10^{-4}	**2d**	48	0.47	42	32	+5.4[i]	−2.4[j]		87	300
3c	$PhCH_2$	H_2NCOCH_2	OH	134/MCF	$2.78 \pm 0.17 \times 10^{-2}$	4.19×10^{-3}	**5c**	24	0.49	46	41	+7.6[k]	−32.8[l]	(*R*)	99	>100
4a	$PhCH_2O$	Me	H	2.45/MCF	$0.78 \pm 0.16 \times 10^{-1}$	1.70×10^{-1}	**6a**	72	0.48	45	44	+47.6[m]	−6.7[n]	(*R*)	92	1990
4b	$PhCH_2$	Me	H	3.96/MCF	$1.10 \pm 0.06 \times 10^{-1}$	6.20	**6a**	1	0.48	46	49	+67.8[p]	−6.8[r]	(*R*)	91	450
4c	$PhCH_2$	Me	Ph	2.31/MCF	4.80 ± 0.49	4.28	**6b**	2	0.49	48	47	+81.1[s]	−38.4[t]		99	>100
3a	$PhCH_2$	*i*-Pr	OH	345/SBA	$2.15 \pm 0.08 \times 10^{-4}$	7.16×10^{-4}	**5a**	144	0.49	46	45	−0.7[u]	+1.0[v]	(*R*)	99	>100
3b	$PhCH_2$	*i*-$PrCH_2$	OH	259/SBA	$2.91 \pm 0.13 \times 10^{-3}$	7.44×10^{-3}	**5b**	48	0.49	49	43	+31.6[w]	−24.1[z]	(*R*)	99	>100

[a] Activity of the immobilized PGA used.
[b] Isolated yield after chromatography.
[c] (*c* 2.4 MeOH), Lit.[4] $[\alpha]_D^{20}$ = +44.5 (*S*) (*c* 1, MeOH).
[d] (*c* 1.5, MeOH).
[e] (*c* 0.9, $CHCl_3$).
[f] (*c* 1.8, MeOH), Lit.[26] $[\alpha]_D^{20}$ = +25.9 (*S*) (*c* 0.9, $CHCl_3$).
[g] (*c* 2, $CHCl_3$).
[h] (*c* 2, $CHCl_3$).
[i] (*c* 0.1, CH_3CN).
[j] (*c* 1.6, CH_3CN).
[k] (*c* 1, 1 M NaOH).
[l] (*c* 1, H_2O), Lit.[27] $[\alpha]_D^{20}$ = −33.0 (*R*) (*c* 1, H_2O).
[m] (*c* 0.25, 1 M NaOH), Lit.[28] $[\alpha]_D^{20}$ = +48.3 (*R*) (*c* 1, acetic acid).
[n] (*c* 1, H_2O), Lit.[29] $[\alpha]_D^{20}$ = −6.8 (*R*) (*c* 1, H_2O).
[p] (*c* 0.1, H_2O), Lit.[29] $[\alpha]_D^{20}$ = +74.0 (*S*) (*c* 0.5, H_2O).
[r] (*c* 1, H_2O), Lit.[29] $[\alpha]_D^{20}$ = −6.8 (*R*) (*c* 0.5, H_2O).
[s] (*c* 1, 1 M NaOH).
[t] (*c* 0.4, 1 M NaOH).
[u] (*c* 1, 1 M NaOH).
[v] (*c* 2, 1 M NaOH).
[w] (*c* 1, 1 M NaOH).
[z] (*c* 0.5, 1 M NaOH), Lit.[30] $[\alpha]_D^{20}$ = −25.0 (*R*) (*c* 1, 1 M NaOH).

Table 2
The dependence of the conversion of 1-(*N*-phenylacetylamino)- 2-carbamoylethylphosphonic acid dimethyl ester (*R*,*S*)-**1d** versus the flow rate

Flow rate [mL/min]	Contact time [min]	Conversion *C*	Initial hydrolysis *V* [μmol/U·min]
0.5	1.65	0.085	2.31×10^{-4}
0.4	2.07	0.095	1.65×10^{-4}
0.3	2.75	0.118	1.16×10^{-4}
0.2	4.13	0.162	7.05×10^{-5}
0.1	8.25	0.242	2.63×10^{-5}
0.05	16.5	0.312	8.50×10^{-6}
0.01	82.6	0.490	5.33×10^{-7}

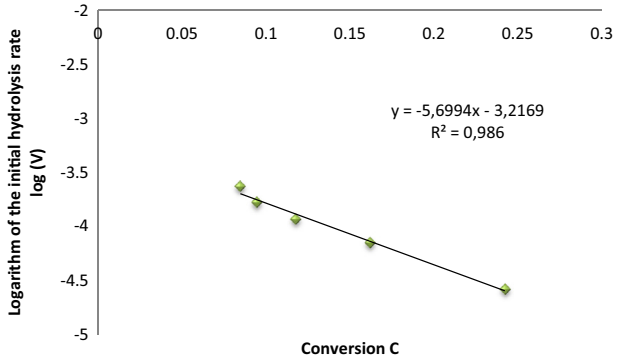


Figure 2. Variation of the initial hydrolysis rate *V* of (*R*,*S*)-**1d** versus the substrate conversion *C* in a semi-logarithmic system.

method). Thus the performed experiment proved that kinetic resolution of racemic *N*-acylated phosphorus analogues of amino acids in a flow system using siliceous monolithic rods as PGA carriers is potentially one of the most convenient and effective methods for kinetic resolution of this class of compounds.

3. Conclusions

Two kinds of mesoporous silicas, i.e. MCF and SBA-15 as well as multichannel siliceous monoliths (MH) were successfully functionalized with penicillin G acylase (PGA) to obtain biocatalysts with the PGA specific activity of 2066, 273 and 1027 U/g, respectively, at the immobilization yields of 95%, 38% and 75%, respectively.

The immobilization of PGA on MCF or SBA-15 has been proven to give effective biocatalysts for the kinetic resolution of racemic 1-(*N*-acylamino)alkylphosphonic and 1-(*N*-acylamino)alkylphosphinic acids and their esters carried out in a batch system. In most of the investigated cases, the initial hydrolytic deacylation rates in the presence of those catalysts were similar or slightly lower in comparison with reactions of the same compounds catalyzed by native PGA.[7] However, the initial hydrolysis rate in the presence of MCF-attached PGA was higher by factors of 6.6 and 1.8, respectively, for the phosphonic analogue of asparagine **3c** and the *H*-phosphinic analogue of alanine **4b**. The results obtained for the immobilized PGA confirm the relationships between the substrate structure and the enzyme efficiency as observed for native PGA and reported in our previous paper.[7]

The stereospecific hydrolysis of the *N*-acyl group of both racemic *N*-acylated phosphorus analogues of amino acids and their esters catalyzed by PGA immobilized on MCF or SBA-15 proved to be a highly effective method for kinetic resolution of all the investigated compounds. Highly effective kinetic resolutions with *E*-values exceeding 100 were obtained for the phosphonic analogues of leucine and asparagine **3b**–**c**, dimethyl esters of the phosphonic analogues of alanine, phenylalanine and asparagine **1a**–**b** and **1d**, *H*-phosphinic analogues of alanine **4a**–**b** and the phenylphosphinic analogue of alanine **4c**, as well as for the phosphonic analogue of valine **3a** and the dimethyl ester of leucine **1c**. In the case of the last two compounds, the kinetic resolution catalyzed with the native PGA only gave a moderate value of enzyme enantioselectivity (22 and 66, respectively).[7] As in the case of the native PGA,[7] the stereochemical preference of the immobilized PGA for the (*R*)-substrates was found.

The initial hydrolytic deacylation rate of racemic 1-(*N*-phenylacetylamino)-2-carbamoylethylphosphonic acid dimethyl ester **1d** in a flow system with PGA immobilized in the siliceous monolith (6.07×10^{-4} μmol/U min) was proven to be very close to those measured for the native PGA and the same substrate (6.83×10^{-4} μmol/U min).[7] High yields of isolation of the hydrolysis product (*R*)-**2d** and the unconverted (*S*)-**1d** with high enantiomeric purity of the latter compound (99%) demonstrate that the kinetic resolution of *N*-acylated phosphorus analogues of amino acids in a flow system using siliceous monolithic rods with a hierarchical pore structure as PGA carriers is potentially one of the most convenient and effective methods for the kinetic resolution of this class of compounds.

4. Experimental

4.1. General

Melting points were determined using capillary tubes in a Stirling SMP 3 apparatus and are uncorrected. IR-spectra were measured on a Nicolett 6700 FT-IR spectrophotometer (ATR method). ^{1}H and ^{13}C NMR spectra were recorded on Varian instruments at operating frequencies of 400 or 600 MHz and 100 or 150 MHz, respectively, using TMS as a resonance shift standard. ^{31}P NMR spectra were recorded on a Varian instrument at an operating frequency of 161.8 MHz with 80% orthophosphoric acid as an external resonance shift standard. Column chromatography was conducted using Merck 60 silica gel (70–230 mesh). Ion-exchange chromatography was carried out using DOWEX 50W X8 in the H^+ form. Routine monitoring of reactions was done with the use of thin layer chromatography, using TLC plates coated with silica gel (Merck 60 F_{254}). Optical rotations were measured on a JASCO V-650 polarimeter. Buffers used: phosphate buffer (Na_2HPO_4/KH_2PO_4) pH 7.5, phosphate buffer (Na_2HPO_4/KH_2PO_4) pH 7.0, acetate buffer (0.2 M CH_3COOH/CH_3COONa) pH 4.5, Tris–HCl buffer (tris(hydroxymethyl)aminomethane/0.2 M HCl) pH 7.8.

4.2. Chemicals and enzyme

The syntheses of racemic 1-(*N*-acylamino)alkylphosphonic and 1-(*N*-acylamino)alkylphosphinic acids and their esters are described in our previous paper.[7] Penicillin G acylase from *Escherichia coli* (20.34 U/mg) was purchased from CBC Biotech S.r.l. (Italy). The specific activity of native penicillin G acylase and its immobilized forms was determined with penicillin G as a substrate according to the PDAB method.[19]

4.3. Immobilization of PGA

4.3.1. Immobilization on MCF and SBA-15

Mesoporous silica foams MCF and SBA-15 were prepared as described in our previous papers.[20–22] The supports (2 g) were then treated with water vapour at room temperature for 5 h, after which they were dried at 200 °C for 2 h and then treated with a solution of (3-aminopropyl)triethoxysilane (0.54 g, 3 mmol) in toluene (60 mL) at 80 °C for 24 h to obtain mesoporous silicas functionalized with 3-aminopropyl groups with a density of amino groups

of ca. 1.5 mmol per 1 g of silica. The functionalized silica carriers (1.5 g of each) were washed with ethanol (2 × 35 mL), water (2 × 35 mL) and a phosphate buffer pH 7 (2 × 35 mL). Centrifugation (20 min, 9000 rpm) was applied for the separation of the silica carriers. The carriers were then activated with a solution of glutaraldehyde in phosphate buffer pH 7 (2.5%, 30 mL) at room temperature for 45 min. After activation, the carriers were washed with water (3 × 35 mL) and phosphate buffer pH 7.5 (2 × 35 mL) and, after separation, treated with a solution of PGA in phosphate buffer pH 7.5 (25 mL, protein concentration 3.1 mg/mL) and gently shaken at room temperature for 2.5 h, then left at 4 °C for 12 h. After separation of the enzyme solution, the carriers were rinsed with phosphate buffer pH 7.5 (2 × 35 mL), 0.5 M NaCl in phosphate buffer pH 7.5 (35 mL), acetate buffer pH 4.5 (35 mL) and water (2 × 35 mL), left in Tris–HCl buffer pH 7.8 (35 mL) at 4 °C for 12 h, rinsed once again with water (35 mL) and suspended in phosphate buffer pH 7.5 (35 mL).

The specific activity of the immobilizates was determined by adopting the procedure of Shewale et al.,[19] using the hydrolysis of penicillin G as the standard reaction. The activity was measured by adding 1 mL of suspension of immobilized enzyme in 0.1 M phosphate buffer pH 7.5 to a solution of penicillin G in the same buffer (9 mL, 10 mg/mL). After exactly 5, 10, 15 and 20 min of incubation with stirring at 37 °C, a 0.5 mL sample was taken off and the released product was determined by conventional tests.[19]

4.3.2. Immobilization of PGA on silica monoliths

The preparation of silica monolith rods (45 mm in length and 6 mm in diameter) is described in our recent paper.[23] The rods (12 pieces, 3.5 g) were immersed in a solution of (3-aminopropyl)triethoxysilane (3.14 g, 17.5 mmol) in toluene (140 mL) for 72 h at 80 °C under intensive stirring. The monoliths were then clad with epoxide polymer resin (L285MGS-H285MGS type) to obtain a single-rod microfluidic microreactors.[23] Each microreactor was rinsed in a flow with ethanol (20 mL), water (20 mL), and phosphate buffer pH 7 (20 mL) at a flow rate of 0.5 mL/min. A solution of glutaraldehyde (2.5%) in phosphate buffer (0.1 M, pH 7.0, 25 mL) was then pumped through the microreactor, and after rinsing with water (20 mL) and phosphate buffer pH 7.5 (20 mL), a solution of PGE in phosphate buffer pH 7 (6 mL, protein concentration 3.26 mg/mL) was pumped through the microreactor in a circular system for 3 h. Finally, the microreactor was rinsed with phosphate buffer pH 7.5 (20 mL), a solution of NaCl (0.5 M) in phosphate buffer pH 7.5 (10 mL), acetate buffer pH 4.5 (10 mL), water (15 mL), Tris–HCl buffer pH 7.8 (5 mL), water (15 mL) and phosphate buffer pH 7.5 (5 mL), and stored in the last buffer.

4.4. Kinetic resolution of racemic 1-(*N*-acylamino)alkylphosphonic and 1-(*N*-acylamino)alkylphosphinic acids and their esters in a batch system

4.4.1. Initial hydrolysis rate measurement

Racemic substrates **1, 3**, or **4** (0.2 mmol) were dissolved in phosphate buffer (0.2 M, pH 7.0, 5–15 mL) and the temperature of the mixture was adjusted to 25 °C. The pH was then adjusted to 7.0 by the addition of 1 M aqueous NaOH as necessary. A suspension of the appropriate amount of immobilized penicillin G acylase (see Table 1) in phosphate buffer (0.45 mL) was then added, after which the reaction mixture volume was adjusted to 20 mL and the resulting mixture was stirred at 25 °C. The substrate conversion was determined by extracting 2 mL of the aliquot of the reaction mixture through Minisart® SRP 4 syringe filters to stop the reaction. After evaporation of water under reduced pressure, a precisely measured amount of a solution of pivalic acid in D_2O was added as the internal standard (approximately 0.5 mg of the pure compound), the residue was dissolved in D_2O, and the amount of substrate and hydrolysis product was determined by ^1H NMR.

4.4.2. Kinetic resolution experiments

4.4.2.1. Kinetic resolution of racemic 1-(*N*-acylamino)alkylphosphonic and 1-(*N*-acylamino)alkylphosphinic acids. Racemic free acids **3** or **4** (1 mmol) were dissolved in phosphate buffer (0.2 M, pH 7.0, 75 mL) and the temperature of the mixture was adjusted to 25 °C. The pH was adjusted to 7.0 by the addition of 1 M aqueous NaOH as necessary. A suspension of the appropriate amount of immobilized penicillin G acylase (see Table 1) in phosphate buffer (5–15 mL) was then added and the resulting mixture was stirred at 25 °C for the time given in Table 1. The reaction was stopped by filtering off the immobilizate by using folded paper filters. After evaporation of water under reduced pressure, the residue was dissolved in water (5 mL) and passed through a DOWEX 50W X8 (H^+ form) column (50 mL) which was rinsed with water. The first fractions contained the remaining free acids **3a–c** or **4a–c**, whereas the further ninhydrin-positive fractions contained deacylation products **5a–c** or **6a–b**.

4.4.2.2. Kinetic resolution of racemic 1-(*N*-acylamino)alkylphosphonic acid esters. Racemic esters **1** (1 mmol) were dissolved in phosphate buffer (0.2 M, pH 7.0, 75 mL) and the temperature of the mixture was adjusted to 25 °C. The reactions were carried out as described above. After evaporation of the water from the reaction mixture, the residue was extracted with CH_2Cl_2 (4 × 3 mL), the extract was dried over $MgSO_4$, the solvent was evaporated under reduced pressure, and the residue was separated by column chromatography (silica gel, CH_2Cl_2/MeOH 20:1).

4.5. Kinetic resolution of racemic 1-(*N*-phenylacetylamino)-2-carbamoylethylphosphonic acid dimethyl ester 1d in a single-rod microfluidic microreactor in a flow system

4.5.1. Dependency of the conversion of 1-(*N*-phenylacetylamino)-2-carbamoylethylphosphonic acid dimethyl ester versus the flow rate

A solution of substrate **1d** (94.2 mg, 0.3 mmol, 3 mmol/L) in phosphate buffer (100 mL, pH 7), was pumped through a microreactor (Fig. 1) at 25 °C with a gradually diminishing flow rate, starting from the value of 0.5 mL/min. In order to determine the conversion of the substrate 2 mL of the outflow was evaporated under reduced pressure, a precisely measured amount of a solution of pivalic acid in D_2O was added as the internal standard (approximately 0.5 mg of the pure compound), the residue was dissolved in D_2O and the amount of unconverted substrate and hydrolysis product was determined by ^1H NMR.

4.5.2. Kinetic resolution experiment in a flow system

A solution of substrate **1d** (94.2 mg, 0.3 mmol, 3 mmol/L) in phosphate buffer (100 mL, pH 7), was pumped through the microreactor (Fig. 1) at 25 °C with a flow rate of 0.01 mL/min. The outflow was collected (87 mL) and worked up as described in Section 4.4.2.1. The unreacted substrate **1d** and the hydrolysis product **2d** were isolated by column chromatography on silica gel (CH_2Cl_2/MeOH 10:1, v/v) in yields of 47% (18 mg) and 44% (11 mg), respectively, with specific rotations of +5.4 and −2.4, respectively. The determination of the enantiomeric excess in the substrate gave a value of 99% (^1H NMR method).

4.6. The structure and the enantiomeric excesses determination

The structure of the hydrolysis products and the unchanged substrates was determined by comparing their spectroscopic

(^{1}H-, ^{13}C-, ^{31}P-, IR) and HRMS data with the data reported in our previous paper for the same compounds. [7] The determination of the enantiomeric excesses was performed by ^{1}H or ^{31}P NMR methods using quinine as the chiral discrimination agent, as previously described for the same compounds.[7]

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