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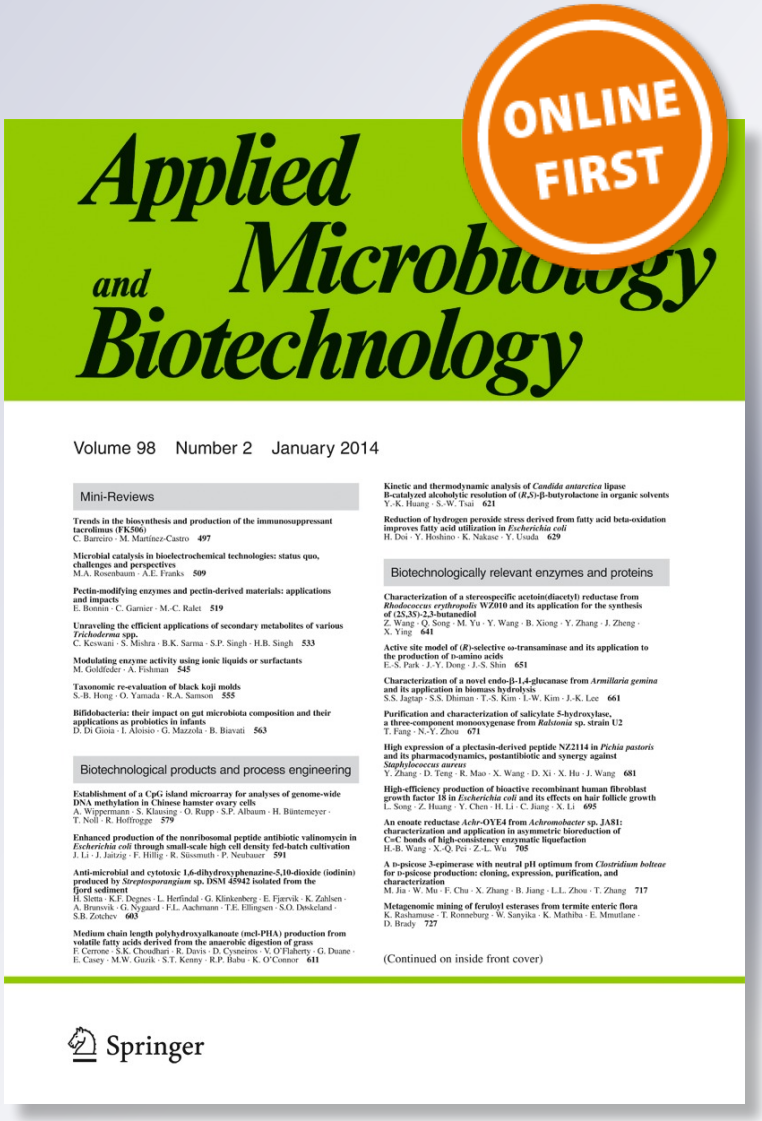

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MINI-REVIEW

Current state and perspectives of penicillin G acylase-based biocatalyses

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Abstract In the course of more than 60-year history, penicillin G acylase (PGA) gained a unique position among enzymes used by pharmaceutical industry for production of β-lactam antibiotics. Kinetically controlled enzymatic syntheses of cephalosporins of novel generations in which PGA catalyzes coupling of activated acyl donor with nucleophile belong among the latest large-scale applications. Contrary to rather specific roles of other enzymes involved in β-lactam biocatalyses, the PGA seems to have the greatest potential. On the laboratory scale, other applications with industrial potential were described, e.g., directed evolution of the enzyme to meet specific demands of industrial processes or its modification into the enzyme catalyzing reactions with novel substrates. The fact that β-lactams represent the most important group of antibiotics comprising 65 % of the world antibiotic market explains such a tremendous and continuous interest in this enzyme. Indeed, the annual consumption of PGA has recently been estimated to range from 10 to 30 million tons. The application potential of the enzyme goes beyond the β-lactam biocatalysis due to its enantioselectivity and promiscuity: the PGA can be used for the production of achiral and chiral compounds convenient for the preparation of synthons and active pharmaceutical ingrediences, respectively. These biocatalyses, however, still wait for large-scale application.

Keywords Penicillin G acylase · β-lactam biocatalysis · Enantioselectivity · Kinetically controlled syntheses

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Introduction

The role and benefits of β-lactams for human society were aptly summarized in the review of Srirangan et al. (2013): in the course of a nearly centenarian history of the β-lactams, natural penicillins and cephalosporins have been firstly used as therapeuticals and secondly as templates for production of semisynthetic β-lactam antibiotics (SSBAs) of novel generations. The research into their development was driven by the increasing resistance of microorganisms to natural β-lactam antibiotics, penicillins, or low antimicrobial activity of cephalosporins. The story of a penicillin G acylase (PGA, EC 3.5.1.11; penicillin amidohydrolase, penicillin acylase) started in the 1950s when the enzyme was described by Sakaguchi and Murao (1950). The enzyme structure, physicochemical characterization, synthesis, and maturation in production strain can be found elsewhere (e.g., Rajendhran and Gunasekaran 2004; Sio and Quax 2004; Chandel et al. 2008; Srirangan et al. 2013).

The PGA from *Escherichia coli* (PGA^{Ec}) is a robust enzyme and in the 1960s was very quickly recognized by several pharmaceutical companies as the enzyme with high industrial potential: activity optimum up to 60 °C (Erarslan et al. 1991), denaturation temperature T_m of 64.5 °C (Grinberg et al. 2008), and long-term operational stability after immobilization (Ospina et al. 1992). In the 1980s, the enzyme served as an industrial catalysts for the large-scale production of β-lactam nuclei 6-aminopenicillanic acid (6-APA) and 7-aminodeacetoxy cephalosporanic acid (7-ADCA) that were

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used for chemical production of SSBAs. Such a hydrolytic usage of the PGA was the first step in overtaking chemical production of SSBAs by biocatalysis.

PGAs belong to the structural superfamily of N-terminal nucleophile hydrolases and, so far, 21 PGA producers were described among bacteria, fungi, and yeasts (Tishkov et al. 2010; Grulich et al. 2013). Other classes of enzymes belonging to the same enzyme superfamily are penicillin V acylases (PVA, EC 3.5.1.11.) and cephalosporin acylases (CA, EC 3.5.1.93). Glutaryl-7-ACA acylase (GA, EC 3.5.1.93) is a member of the latter class.

Increasing demand for SSBAs with novel traits resulted in extensive search for further enzymes capable of biotransformation of β-lactams. Among them, the enzymes such as α-amino acid ester hydrolase (AEH, EC 3.1.1.43), cephalosporin C acetylhydrolase (CAH, EC 3.1.1.41), D-amino acid oxidase (DAO, EC 1.4.3.3), hydantoinase (EC 3.5.2.2), carbamoylase (EC 3.5.1.77), and expandase (EC 1.14.20.1) should be highlighted. Currently, the total number of all enzymes studied for their implementation into bioprocesses for syntheses of SSBAs exceeds 40 (Barber et al. 2004).

Large-scale syntheses of SSBAs catalyzed by PGAs from corresponding β-lactam nuclei and acyl donors began to dominate over chemical ones in this century, when these processes were found to be economically viable. This was namely supported by the fact that it was possible to run the enzymatic processes in existing equipment, with the enzymes being a part of organic chemist's toolbox (Bruggink et al. 1998).

Penicillin G (penG), the first β-lactam antibiotic discovered in the 1930s of the last century, is currently produced in amount exceeding 60 kT/year (Thykaer and Nielsen 2003). Majority of penG is further processed and biotransformed into semisynthetic penicillins and cephalosporins (based on nucleophiles 6-APA and 7-ADCA) that exhibit improved pharmacological traits. The fact that more than 50 cephalosporins are currently marketed (Ozcengiz and Demain 2013) just emphasizes the importance of a biocatalysis of β-lactams.

Several excellent reviews were written in the last decade on the PGA. The reviews usually stress or combine certain aspects of the enzyme catalysis such as the mechanism of catalysis and enzyme specificity, the enzyme improvement or modification, the way of an enzyme immobilization and production of different biocatalysts, the usage of PGAs in water or organic solvents, production of enantiopure active pharmaceutical ingredients, industrial applications, etc. The scope of this review is to present a survey of the "total" biocatalytic potential of PGA and give a complex view on the enzyme versatility and application potential. The enzyme industrial potential is compared with that of other enzymes that may compete with PGA in biotransformation of β-lactams.

Reaction mechanism of PGA

The use of the PGA in bioprocesses is of great industrial interest as an environmentally friendly and economic alternative to chemical processes. The enzyme may act as a transferase or a hydrolase and has as a catalytic residue the N-terminal serine on β-subunit (Serβ1) that is activated by a bridging water molecule. If dehydrated, the PGA becomes inactive, and therefore, some water must be present. The enzyme recognizes the side chains of substrates composed of hydrophobic moiety (phenyl-, pyridyl-, thienyl-, tetrazolyl-, CN-, etc.), connected with a short spacer (–CH2–, –OCH2–, –SCH2–, –CHOH–, –CHNH2–) to carboxyl group or its derivative (–COOH, –COOR, –CONHR).

The acyl donor first associates with the enzyme at acyl subsite of the active site that is highly specific for hydrophobic groups (Duggleby et al. 1995) and acylates the Serβ1 to give the acyl-enzyme intermediate. This complex is, in turn, attacked by a water molecule (hydrolysis) or β-lactam nucleus (synthesis) interacting with the aminic subsite preferring hydrophylic, negatively charged groups. The reaction product, a nucleus or SSBA, is finally released. The reaction mechanisms for hydrolysis and synthesis catalyzed by PGAs are identical but reaction conditions are different (see recent review of Srirangan et al. 2013).

Syntheses of β-lactam antibiotics catalyzed by PGA

Two different strategies for the enzymatic synthesis of β-lactam antibiotics were described and reviewed elsewhere (e.g., Giordano et al. 2006; Volpato et al. 2010): a direct condensation reaction known as a thermodynamically or equilibrium controlled synthesis and kinetically controlled approach in which the PGA catalyzes coupling of the activated acyl donor (ester or amide) with the nucleophile. It should be noted that starting with racemic mixture of activated acyl donor, for example in the case of ampicillin the PGA^{Ec} gave both enantiomers (*R*)- and (*S*)-ampicillin, as expected from the low enantioselectivity of this enzyme. Since (*R*)-ampicillin is more biologically active enantiomer (Morimoto et al. 1972), coupling reactions are performed with (*R*)-enantiomers of acyl donors in the case of chiral acyl side chains of the antibiotic.

The yields of the thermodynamically controlled syntheses are determined by the thermodynamic equilibrium and are independent of the enzyme properties. In the kinetically controlled reaction, the yield is higher and kinetic parameters of the enzyme play a crucial role (e.g., Youshko et al. 2002 Ribeiro et al. 2005). From the economic viewpoint, it is important to keep the molar ratio acyl donor/nucleophile as low as possible. However, hydrolysis of the activated acyl donor becomes the parameter of critical importance when approaching stoichiometric ratio of the reactants.

Kinetically controlled synthetic reaction has been systematically studied and parameters relevant for evaluation of enzyme synthetic efficiency were introduced (e.g., Gabor et al. 2005 Jager et al. 2007).

Synthetic applications of PGAs were reported with penam (6-APA) as well as cephem nuclei (7-ACA, 7-ADCA, or 3′-functionalized nucleophile).

The effect of chemical modification of cephalosporin nuclei derived from 7-ACA (3′-functionalization) was studied by Terreni et al. (2005). 3′-modified, cephalosporanic nuclei are more stable at more acidic pH which keeps the amino group at position 7′ in its undissociated form (a prerequisite for PGA-catalyzed syntheses; Barber et al. 2004) and minimizes the hydrolysis of synthesized β-lactam antibiotics. Simultaneous 7′-enzymatic acylation/3′-chemical derivatizations of 7-ACA/7-ADCA or 6′-/3′-derivatizations of 6-APA yield β-lactam antibiotics with improved traits; cefamandole (the nucleophile 7-TACA), cefazolin (7-ZACA), cefonicid (7-SACA), cefaclor (7-ACCA), cefprozil (7-APRA) are examples of 7′-/3′-processed cephalosporins, and pivampicillin represents only penicillin processed simultaneously at position 6′ and 3′ (Table 1).

So far, the wild-type enzymes of ten microorganisms (*E. coli*—PGA^{Ec}, *Xanthomonas citri*—PGA^{Xc}, *Alcaligenes faecalis*—PGA^{Af}, *Kluyvera cryocrescens*—PGA^{Kcr}, *Kluyvera citrophila*—PGA^{Kc}, *Providencia rettgerri*—PGA^{Pr}, *Kluyveromyces citrophila*—PGA^{Kci}, *Achromobacter* sp.—PGA^{Asp}, *Arthrobacter viscosus*—PGA^{Av}, and *Bacillus megaterium*—PGA^{Bm}), recombinant enzymes, such as evolved PGA^{Ec} (Alkema et al. 2002a, b, 2004; Jager et al. 2008; Deaguero et al. 2012; Cecchini et al. 2012) or PGA^{Bm} (Wang et al. 2007), chimeric and metagenomic PGAs (Zhou et al. 2003; Gabor and Janssen 2004; Gabor et al. 2005; Jager

Table 1 Recent examples of β-lactam antibiotics synthesized by PGA

Antibiotic	β-lactam nucleus	Acyl donor	Used by
Ampicillin	6-APA	PGA	Alkema et al. 2002a, b; Youshko et al. 2002, 2004; Gabor and Janssen 2004; Gabor et al. 2005; Jager et al. 2007, 2008; Marešová et al. 2010
		PGME	Illanes and Fajardo 2001; Alkema et al. 2002b; Gabor and Janssen 2004; Ribeiro et al. 2005; Jager et al. 2007; Deaguero et al. 2012; Bečka et al. 2013
Amoxicillin	6-APA	HPGME	Alkema et al. 2002b; Goncalves et al. 2005; Bergeron et al. 2009; Wu et al. 2010; Marešová et al. 2010; Bečka et al. 2013
		HPGA	Alkema et al. 2002b; Youshko et al. 2002, 2004; Gabor et al. 2005; Jager et al. 2007
Cefamandole	7-TACA	MAME	Terreni et al. 2007; Estruch et al. 2008
Cefazolin	7-ZACA	TzAA-OMe	Park et al. 2000; Terreni et al. 2007; Estruch et al. 2008
Cefonicid	7-SACA	MAME	Terreni et al. 2007; Estruch et al. 2008
Cefaclor	7-ACCA	PGME	Wei et al. 2003; Yang and Wei 2003; Zhang et al. 2008; Estruch et al. 2008; Cecchiny et al. 2012
Cefadroxil	7-ADCA	HPGME	Alkema et al. 2002b
		HPGA	Alkema et al. 2002b; Gabor et al. 2005; Jager et al. 2007
Cephalexin	7-ADCA	PGME	Hernández-Justiz et al. 1998; Alkema et al. 2002b; Wei et al. 2003; Gabor and Janssen 2004; Estruch et al. 2008; Li et al. 2008b; Illanes et al. 2009; Bernardino et al. 2009; Aguirre et al. 2010; Bahamondes et al. 2012; Cecchiny et al. 2012
		PGA	Schroën et al. 1999, 2001; Alkema et al. 2002b; Youshko et al. 2002, 2004; Zhou et al. 2003; Gabor and Janssen 2004; Gabor et al. 2005; Cheng et al. 2006; Wang et al. 2007; Jager et al. 2007; Estruch et al. 2008; Marešová et al. 2010; Cechiny et al. 2012
Cephalothin	7-ACA	2-TAA	Shaw et al. 2000; Estruch et al. 2008
Cefprozil	7-APRA	HPGME	Cecchiny et al. 2012
	7-GPRA→ 7-APRA	HPGHE	Feng et al. 2008
Cephaloglycin	7-ACA	PGME	Terreni et al. 2007; Estruch et al. 2008
Penicillin G	6-APA	PAA	Alkema et al. 2002a; Gabor et al. 2005
Pivampicillin	POM-6-APA	PGME	Kim and Lee 1996

6-APA 6-aminopenicillanic acid, *7-ACA* 7-aminocephalosporanic acid, *7-ADCA* 7-aminodeacetoxycephalosporanic acid, *7-ACCA* 7-amino-3-chloro cephalosporanic acid, *7-TACA* 7-amino-3-(1-methyl-1,2,3,4-tetrazol-5-yl)thiomethylcephalosporanic acid, *7-ZACA* 7-amino-3-(5-methyl-1,2,3,4-thiazol-2-yl)thiomethylcephalosporanic acid, *7-SACA* 7-amino-3-(1-sulphomethyl-1,2,3,4-tetrazol-5-yl)thiomethylcephalosporanic acid, *7-GPRA* 7-phenylacetamido-3-propenylcephalosporanic acid, *7-APRA* 7-amino-3-propenylcephalosporanic acid, *POM-6-APA* pivaloyloxymethyl 6-aminopenicillanic acid, *PAA* phenylacetamid, *PGA* phenylglycine amide, *HPGHE* 4-hydroxy-D-phenylglycine hydroxyethyl ester, *HPGA* D-*p*-hydroxyphenylglycine amide, *PGME* phenylglycine methyl ester, *HPGME* hydroxyphenylglycine methyl ester, *TzAA-OMe* tetrazolylacetic acid methyl ester, *MAME* mandelic acid methyl ester, *2-TAA* 2-thienylacetamide

Except 6-APA, 7-ADCA and 7-ACA, other nucleophiles are 3′-functionalized

et al. 2007), were studied in syntheses of SSBAs. Recently published syntheses are listed in Table 1.

Nowadays, catalysts based on the immobilized PGA^{Ec}, PGA^{Af}, and PGA^{Asp} are routinely used for large-scale production of SSBAs in kinetically controlled syntheses.

Current status of PGA-based synthetic applications

PGA-based catalysts are used in industry under conditions far from the physiological conditions inside the cell. Moreover, PGA should catalyze reactions also with unnatural substrates. To meet these requirements, PGA has to be improved in terms of pH and temperature stability, activity temperature optimum, tolerance to organic solvents, and sensitivity to inhibition by reactants. Also, engineered PGA was expected to recognize further β-lactams as substrates or acquire better catalytic traits (e.g., improved ratio of the initial rate of the product synthesis to the initial rate of the hydrolysis of activated acyl donor) rendering syntheses of SSBAs more competitive with chemical processes.

Overproduction and the way of stabilization of PGA represent further aspects that are essential for any industrial application of the enzyme.

Much effort has already been spent to improve the production of PGA by the recombinant strains used in industry because downstream processing represents significant expenses in catalyst manufacturing costs of which they may account for 40–65 % (Forciniti 2008).

The following approaches should be considered to construct microbial production platform: (1) maximization of the enzyme overproduction by fine tuning of *pac* gene expression, protein translation, processing, and folding (extensively reviewed for bacterial and yeast expression systems by Srirangan et al. 2013); (2) engineering host cell physiology so that the intracellular stress response solicited by metabolic burden is reduced, e.g., the extracellular expression described by Orr et al. (2012; *E. coli*) and Westers et al. (2004; *B. subtilis*); (3) evaluation of different expression systems in terms of stability of the state of enzyme overproduction, e.g., segregational and structural instability of the recombinant plasmid, gene dosage effect, constitutive or inducible expression, minimizing of the lysis of the recombinant cell in the course of the production stage (e.g., lyses of bacterial cultures caused by phages or metabolic burden of the heterologous enzyme); and (4) the way of cultivation in continuously stirred bioreactor (high cell density, fed-batch cultures of bacteria and yeasts, e.g., cultures of recombinant strain based on *E. coli* (Liu et al. 1999; Lin et al. 2002), *bacilli* (Yang et al. 2006; Zhang et al. 2006), or *Pichia pastoris* (Senerovic et al. 2006; Marešová et al. 2010).

Anyway, it can be concluded that high-expression systems based on *E. coli* host bearing different *pac* genes are almost exclusively used for the large-scale production of PGAs. Consequent downstream processing also exhibits industrial feasibility. So far, there is no report available on the application of any yeast expression system on industrial scale.

A lot of effort has been already invested into PGA immobilization. Established technologies of the enzyme stabilization vary from the enzyme immobilization on solid matrices (predominantly for production of nucleophiles) to entrapment or encapsulation of cross-linked enzyme aggregates (CLEA). Immobilization techniques were reviewed, e.g., by Kallenberg et al. (2005).

Syntheses of semisynthetic β-lactams with improved PGAs

The knowledge of the crystal structure of PGA^{Ec} (McVey et al. 2001) was used to increase the protein performance in kinetically controlled syntheses of SSBAs by replacement of amino acid residues of the active site (e.g., Alkema et al. 2002a, b, 2004; Gabor and Janssen 2004; Jager et al. 2008; Deaugero et al. 2012). Engineered PGA constructed by shuffling the *pac* genes of PGA^{Ec}, PGA^{Kcr}, and PGA^{Pr} improved the synthetic activity by 40 % when compared to the PGA^{Pr}. The chimeric enzymes of PGA^{Ec} and PGA^{Kcr} with additional point mutations displayed a 40–90 % increase in the relative rate of acyl transfer to the β-lactam nucleus during ampicillin synthesis and were also improved in the production of amoxicillin, cephalexin, and cefadroxil (Jager et al. 2007). Yang and Wei (2003) engineered the PGA^{Bm} and reported that the mutation of the surface of the β-subunit increased stability of the enzyme at acidic pH or to organic solvents.

Low stereoselectivity of the wild-type PGA^{Ec} toward (*R*)-phenylglycine methyl ester (PGME) excludes the usage of the racemate of acyl donor for large-scale syntheses of SSBAs. Deaguero et al. (2012) improved the stereoselectivity of the PGA by protein engineering. The enzyme with AA substitution Phe24ßAla exhibited stereoselectivity more than 98 % toward (*R*)-PGME in comparison to the wild-type enzyme. The improved enzyme could be exploited in ampicillin synthesis from the racemate of activated acyl donor.

Directed evolution of PGA into cephalosporin acylase

The PGA and GA are members of the enzyme superfamily of N-terminal (Ntn) hydrolases. Although AA sequences are rather different (identity of 13.3 %) and the enzymes hydrolyze very different substrates, the structural homologies of their active sites are very high. Therefore, Oh et al. (2004) tried to modify the PGA into CA and thus produce 7-ACA from cephalosporin C (cephC). They introduced simultaneously up to seven replacements of amino acid residues in the active site which improved the activity of PGA toward

glutaryl-7-ACA as much as 7.6 times. The improvement was accompanied with simultaneous reduction of activity toward penG. This modification clearly suggests the way how to enlarge the portfolio of hydrophobic aromatic side chains accepted by the PGA by hydrophilic linear side chains. However, the modification cannot be used for large-scale production of 7-ACA: the mutant has only 2.1 % of deacylation activity when compared with that of GA from *Pseudomonas diminuta* deacylating GL-7-ACA. Contrary to PGA, GA was changed to cephalosporin C-acylase that is used on large scale for "single-step" production of 7-ACA from cephC (this review; see "Cephalosporin acylases" section).

Application of PGA in "one-pot" cascade conversions

Cascade conversions, which combine multiple reactions without intermediate recovery steps, are intensively studied at laboratory scale. These one-pot processes render syntheses more environmentally friendly, economically viable, because they can be performed in smaller reactor volumes, at shorter reaction times with higher volumetric and space time yields, and further reduce production of wastes (Sheldon 2008; Blum et al. 2010).

Schroën et al. (2002) used the cascade reactions for the production of cephalexin from adipoyl-7-ADCA and (*R*)-PGME by concerted biocatalysis with two enzymes GA (hydrolytic production of 7-ADCA) and PGA (coupling reaction of 7-ADCA with (*R*)-PGME).

Another excellent example of concerted application of PGA and AEH has been suggested recently. The enzymes were used for the production of ampicillin from penG. The process does not require crystallization of 6-APA after PGA-catalyzed hydrolysis of penG, because the activity of AEH is not inhibited by phenylacetic acid, a byproduct of the hydrolysis. Thus, AEH catalyst can be used directly in a solution of reactants after the hydrolysis. In this process, PGA^{Ec} and AEH from *X. citri* were used, and the reaction times were significantly reduced in comparison with the ones previously observed in the one-pot production of amoxicillin from penG that used the PGA for both steps (Du et al. 2009; Wu et al. 2010).

Enantioselectivity of PGA

PGA exhibits enantioselectivity and diastereoselectivity, traits that increase the industrial potential of the enzyme. The research into this topic was started in the 1960s of the last century and has been reviewed recently (Grulich et al. 2013). It should be stressed that none of these features were so far exploited on industrial scale, but application potential of these reactions has already been envisaged (Solano et al. 2012).

Two types of enantioselective reactions are catalyzed by PGA frequently with a high degree of enantiomeric excess (*ee*): enantioselective reverse hydrolysis and enantioselective hydrolysis. In both reactions, one enantiomer undergoes the acylation (reverse hydrolysis) or its acylated derivative is hydrolyzed (hydrolysis). Products of the reactions could be of pharmaceutical interest because exogenously supplied enantiomerically pure drugs may exhibit diverse interactions with chiral targets such as enzymes, receptors, or ion channels (Aboul-Enein and Abou-Basha 1997), and optically pure enantiomers can serve as chiral building blocks for APIs.

A growing interest into discovering application potential of the enantioselectivity of PGA can be documented by examples that have been published recently and are listed below.

Enantioselective reverse hydrolysis

The PGA^{Ec} catalyzes enantioselective resolution of racemate of esters of aromatic α-amino acids (Basso et al. 2000), (*RS*)-phenylglycinonitrile (Chilov et al. 2003), racemic mixtures of α-amino acids and glutamine (Carboni et al. 2006) with high degree of *ee* for (*S*)-enantiomers. The racemates of β-amino acids (Li et al. 2008a) and β-aminoesters (e.g., Roche et al. 1999) are resoluted with high degree of *ee* for (*R*)-enantiomers. The products of resolutions serve as synthons or APIs. The PGA^{Af} analogously resoluted racemic mixtures of aromatic α-amino acids and their *p*-substituted derivatives (Gong et al. 2011) and β-amino acids and their *p*-substituted derivatives (Li et al. 2013).

Enantioselective hydrolysis

The PGA^{Ec} was used to prepare enantiopure (2*S*,3*S*)-(+)-3-hydroxyleucine (Fadnavis et al. 1997), or (*S*)-enantiomer of 2-chlorophenylglycine (Fadnavis et al. 2008), (*R*)-mandelic acid derivatives which are chiral side chains for preparations of 3′-functionalized cephalosporins (Rocchietti et al. 2002), and azasugars from racemic mixtures (Venkataiah et al. 2011). The PGA^{Kc} was used for preparation of (*R*)-*tert*-leucine by enantioselective hydrolysis of N-phenylacetyl-*tert*-leucine racemic mixture (Liu et al. 2006).

Application of PGA in protection/deprotection of reactive amino groups

To give a brief insight into this type of PGA application, three processes may be mentioned. The protection/deprotection was used for production of enantiopure (*R*) and (*S*)-1-phenylethylamine from the racemic mixture of 1° amine by PGA^{Af} that are of pharmaceutical importance (Guranda et al. 2004). The PGA^{Ec} was used for the protection/deprotection of amino groups of L-amino acid esters during chemoenzymatic synthesis of biologically active dipeptides by Didžiapetris et al.

(1991) and Švedas and Beltser (1998). Carboni et al. (2004) performed PGA^{Ec}-catalyzed protection/deprotection of esters of nonpolar D-amino acids, components of some biologically important peptides. The use of biocatalysis in protection/deprotection represents "green" alternative to commonly used chemical processes using toxic and expensive chemicals (Kadereit and Waldmann 2001).

Application of PGA in prodrug activation

It is known that a prodrug may offer many advantages for handling over the drug itself into which it is converted. In specific cases, this activation process consists in a simple removal of an N-acyl protecting group. The PGA fused with monoclonal antibody L6 mAb activates chemically prepared prodrug N-acyl palytoxin derivative into palytoxin derivative by the deacylation reaction. The drug is cytotoxic against cells expressing the Na^+–K^+ ATPase associated with the toxin receptor. The same activating effect of the PGA was observed also with prodrugs N-(phenylacetamido) doxorubicin and N-(phenylacetyl) melphalan (Yang et al. 2011).

PGA-catalyzed promiscuous reactions

The PGA is capable of catalyzing several reactions with completely different reaction mechanisms to those described above (Wu et al. 2005), and therefore, it is classed among the enzymes designated as promiscuous enzymes. The research into the promiscuity of PGA^{Ec} has started recently.

The enzyme catalyzes: (1) Markovnikov addition reaction (Wu et al. 2005, 2006) which is an electrophile addition onto a double bond of a nucleophile; (2) transesterification reactions where an ester is transformed into another one through an interchange of the alkoxy moieties (Lindsay et al. 2002; Liu et al. 2011); and (3) Henry reaction known in organic chemistry to form carbon–carbon bond in the reaction of nucleophilic nitroalkane with an electrophilic aldehyde or ketone (Wang et al. 2010). From the viewpoint of the large-scale processes, the yields of the reaction products are very low and reaction times are counted in days.

Enzymes competing with PGA for syntheses of SSBAs

As regards industrial potential of biotransformations of β-lactams, the following enzymes deserve a brief description of their structure and biocatalysis: α-amino acid ester hydrolase (AEH), PVA, and cephalosporin acylases (CA and GA). Except AEH, these enzymes have already been used in large-scale processes for the production of SSBAs.

The primary nucleophiles (6-APA and 7-ACA/7-ADCA) and acyl side chains (position R) recognized by the enzymes PGA, PVA, AEH, CA, and GA are shown in Fig. 1.

α-Amino acid ester hydrolases

AEHs (formally β-lactam acylases, EC 3.1.1.43) are able to catalyze the transfer of the acyl group from α-amino acid esters to β-lactam nucleophiles such as cephems and penams in kinetically controlled syntheses or to a water molecule (hydrolyses; Polderman-Tijmes et al. 2002a) analogously to PGA.

AEHs are composed of four identical subunits each having 614 amino acid residues that form a dimer of dimers with 81 residues that make up the subunit interfaces. The catalytic triad of Ser205, Asp338, and His370 conserved among the AEHs indicates that these enzymes are members of enzyme family of serine hydrolases (Polderman-Tijmes et al. 2002b; Barends et al. 2003).

It has been concluded that the AEHs catalyze their reactions through a common acyl-enzyme intermediate (Blinkovsky and Markaryan 1993) which corresponds to the mechanism described for PGAs and for peptide hydrolysis by serine hydrolases.

Since AEHs require substrate with α-amino group, they are not inhibited by phenylacetic acid (Blinkovsky and Markaryan 1993). Together with their ability to accept various β-lactam nuclei, they were used for the syntheses of β-lactam antibiotics listed in Table 2. The slightly acidic pH optimum of the AEH, which is beneficial for β-lactams stability, is another advantage of AEHs for biocatalytic applications, as is their stereospecificity toward the acyl donor (Fernandez-Lafuente et al. 2001). The syntheses listed in Table 2 were catalyzed by AEHs from *Xanthomonas rubrillineans*, *X. citri*, *Acetobacter turbidans*, or *Xanthomonas* sp. Except cefatrizine, all other β-lactams were also synthesized by PGA.

Penicillin V acylases

PVAs (EC 3.5.1.11) catalyze the hydrolysis and synthesis of phenoxyacetyl-substitued β-lactam antibiotics. It is capable of hydrolyzing substrates with the benzyl side chain, but still the preferred substrate remains penicillin V (Kumar et al. 2008). Its biotechnological potential lies in the production of 6-APA from penicillin V. Only one structural gene was studied in details: the PVA from *Bacillus sphaericus* (Olsson et al. 1985). Sequence homology with PGAs is very low. However, the crystal structure of the PVA showed N-terminal cysteine and fold typical for PGAs (Suresh et al. 1999).

pH optimum (5.6–8.5) is lower (Margolin et al. 1980) than the optimum of the PGA (6.5–8.5; Schumacher et al. 1986) which can be advantageous for industrial processes, since the

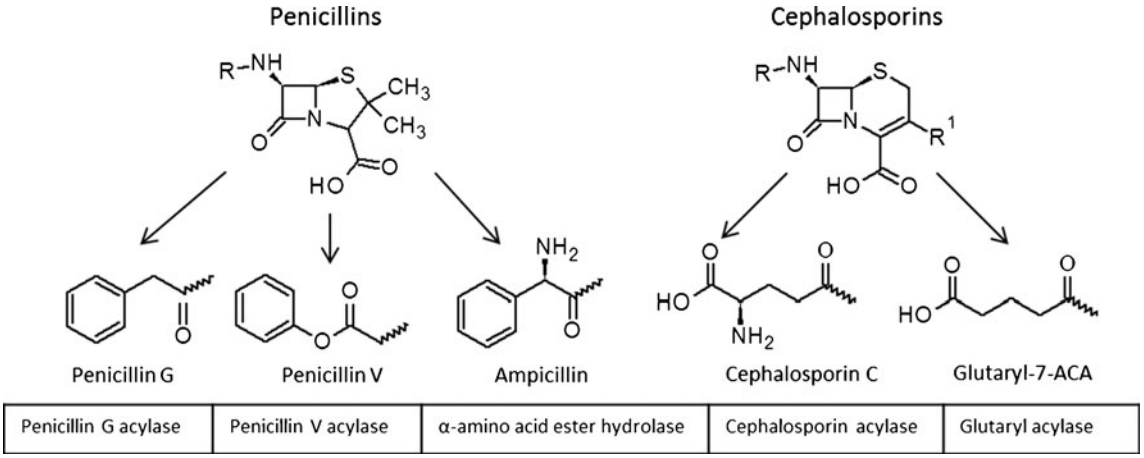

Fig. 1 Examples of enzymes used in biotransformations of β-lactams and their primary substrates

chemical degradation of 6-APA is less at lower pH values (Shewale and Sudhakaran 1997; Kumar et al. 2008).

The PVA from *Streptomyces mobaraensis* showed high acyl-transfer activity in reactions for the kinetically controlled synthesis of β-lactam antibiotics. It was able to catalyze synthesis of deacetoxycephalosporin V from 7-ADCA and phenoxyacetic acid methyl ester, dihydropenicillin F (6-APA and hexanoic acid methyl ester), or penicillin K (6-APA and octanoic acid methyl ester; Koreishi et al. 2007).

Cephalosporin acylases

Cephalosporin acylases (EC 3.5.1.93, CA/CCA/GA) recognize β-lactam antibiotics with cephalosporanic acid nucleus such as cephC or glutaryl-7-ACA, that have side chains with charged groups. Therefore, these enzymes, just as AEHs but unlike PGAs and PVAs, accept β-lactam antibiotics with hydrophilic side chains as their substrates (Fritz-Wolf et al. 2002). Some researchers classify CAs into two groups depending on their substrate specificity: the GAs also known as cephalosporanic acid acylases and cephalosporin-C acylases (CCA; Aramori et al. 1991).

The crystal structure of the GA resembles the crystal structure of the PGA and structural homologies of their active sites are very high (Duggleby et al. 1995; this review, see "Directed evolution of PGA into cephalosporin acylase" section).

The GA is an industrially relevant enzyme since it is employed in the two-step enzymatic deacylation of cephC to 7-ACA. The enzyme is responsible for the hydrolysis of glutaryl-7-ACA, which results from the conversion of cephC to 2-ketoadipoyl-7-ACA catalyzed by DAO with the consequent formation of H_2O_2 that causes spontaneous decarboxylation to glutaryl-7-ACA (Monti et al. 2000). The GA has also very low specificity for cephC and the enzyme cannot be exploited for the one-step production of 7-ACA without further improvements (Pollegioni et al. 2005).

Since all clinically important cephalosporins are produced semisynthetically from 7-ADCA, 7-ACA, and their derivatives, much effort has been spent to redesign metabolic fluxes in production fungal strains and develop multipurpose host platforms for production of nonnative β-lactams (Ward 2012).

While *Penicillium chrysogenum* cannot produce cephalosporins and cephamycins, it is an attractive fungus because of its capacity to overproduce penG. The fungus was successfully

Table 2 SSBAs synthesized with AEH

Antibiotic	β-lactam nucleus	Acyl donor	Used by
Ampicillin	6-APA	PGME	Fernandez-Lafuente et al. 2001; Blum and Bommarius 2010
Amoxicillin	6-APA	HPGME	Kato et al. 1980
Cefaclor	7-ACCA	PGME	Wang et al. 2012
Cafadroxil	7-ADCA	HPGME	Ye et al. 2012
Cephalexin	7-ADCA	PGME	Takahashi et al. 1972, 1974; Blinkovsky and Markaryan 1993; Polderman-Tijmes et al. 2002a,b; Barends et al. 2006; Blum and Bommarius 2010
Cefprozil	7-APRA	HPGME	Ye et al. 2012
Cephaloglycin	7-ACA	PGME	Takahashi et al. 1972
Cefatrizine	7-ATTC	HPGME	Pan et al. 2013; Ye et al. 2012

7-ATTC 7-amino-3-[(1*H*/1,2,3-triazol-4-ylthio)methyl]-cephalosporanic acid

engineered to synthesize cephalosporins adipoyl-7-ACA, adipoyl-7-ADCA (Crawford et al. 1995; van de Sandt and de Vroom 2000; Robin et al. 2001) and novel cephem β-lactam adipoyl-7-amino carbamoyl cephalosporanic acid (adipoyl 7-ACCCA) if cultures are fed with adipate. From these novel β-lactams, semisynthetic cephalosporins, such as ceftriaxone, cefazolin and ceftazidine (van den Berg et al. 2007; Harris et al. 2009), may be prepared. When the culture of the recombinant *P. chrysogenum* was fed with phenylacetic acid, phenylacetyl-7-ADCA was produced.

The GA of *Pseudomonas* SY-77 (Sio et al. 2002) was genetically engineered to hydrolyze adipoyl derivatives of the abovementioned cephalosporins into the nuclei for syntheses of SSBAs.

Recently, GA was successfully changed into cephalosporin C acylase (CCA) by directed enzyme evolution. The enzyme of *Pseudomonas* sp. SE83 was evolved by six point mutations into CCA that splits cephC directly into 7-ACA and D-α-amino-adipic acid (Shin et al. 2005). It was reported (Zahel et al. 2013) that the enzyme is used at industrial scale for a single-step production of 7-ACA, thus replacing two enzymatic process exploiting the enzymes GA and DAO (Barber et al. 2004).

Two examples of the GA engineering together with construction of the recombinant strain of *P. chrysogenum* abovementioned class GA among competitors with the PGA for production of cephalosporin nucleophiles.

Future trends for PGA applications

The current demands of industries on enzyme-based biocatalyses are: (1) enhancement of existing catalyst productivity and (2) development of novel catalysts. These requirements are inevitable to facilitate development of economical, large-scale processes. It has already been proved by laboratory experiments that the genetic engineering of the enzymes and natural diversity of microorganisms play an important role in achievement of these requirements. Fast progress in bioinformatics, molecular modeling and docking experiments with substrates of industrial importance represent a promising approach for predicting improvements of the PGA as regards the substrate specificity, kinetic parameters, and stereoselectivity (e.g., Braiuca et al. 2003, 2006).

Moreover, the productivity of PGA-catalyzed reactions can be improved by the way of enzyme immobilization and application of water-miscible organic solvents or nonaqueous solutions (Datta et al. 2013). Mass transfer limitations in the catalyst are now a major barrier for further improvement of the biocatalyst performance (Kallenberg et al. 2005). The way of catalyst preparation should therefore be case-specific. Particularly, the specific activity and operational stability of the catalyst have to be considered for a given process, and diffusion limitations that reduce the product yield, selectivity to xenobiotics, etc., have to be reduced as much as possible (van Roon et al. 2007). PGA-activated membranes used in a membrane bioreactor or combination of immobilization procedure and mutagenesis of the PGA surface (Abian et al. 2004; Cecchini et al. 2007, 2012) are promising approaches in a catalyst preparation. Replacement of nonconserved surface residues with lysine or assembling eight new glutamic residues distributed throughout the enzyme (Montes et al. 2007) did not affected the enzyme synthetic activity for cephalexin, cefprozil, or cefaclor and improved the way of the enzyme immobilization. Such a strategy appears to be generally applicable for enhancing enzyme stability in organic solvents.

Potentials of PGA and AEH for synthetic applications

PGAs and AEHs are enzymes capable of catalyzing related reactions and both enzymes can be used in the process of SSBAs preparation.

Unlike PGA, AEH is not able to hydrolyze penG and phenylacetyl-7-ADCA into 6-APA and 7-ADCA (Polderman-Tijmes et al. 2002a); only antibiotics with α-amino acid acyl side chains could be hydrolyzed or synthesized by AEHs (Barends et al. 2003). Moreover, in comparison to the PGA, soluble AEH has low thermostability with T_{opt} of 25 °C and observed half-life of 5 min at 30 °C (Blum and Bommarius 2010). On the other hand, low product hydrolysis due to the low affinity of the AEH for amides (Blinkovsky and Markaryan 1993), slightly acidic pH optimum (AEH=pH 6, PGA=pH 7.5–8; Schroën et al. 1999) and stereospecificity toward the acyl donor (Fernandez-Lafuente et al. 2001) make AEH a promising alternative to PGA for the synthesis of α-amino acid-containing cephalosporins. Seven of 13 β-lactam antibiotics listed in Table 1 can be synthesized by both enzymes. So far, not a single piece of information is available in literature on the large-scale applications of AEH.

Concerted application of PGA and AEH is an excellent example of the process for the production of ampicillin from penG that was laboratory developed and meets most of the demands of the modern bioprocess.

Potential of PGA promiscuity and enantioselectivity for large-scale processes

Application of water-miscible organic solvents or a nonaqueous solution promoted the syntheses of optically active compounds such as ketones (Kumaraguru and Fadnavis 2012), amino acids (Fadnavis et al. 2008), chiral alcohols, acids, and their esters (Roche et al. 1999; Basso et al. 2000). The organic solvent even alters the catalytic traits of the PGA into promiscuity such as Henry and esterification reactions (Liu et al. 2011; Wang et al. 2010). Nevertheless, reaction yields were

very poor and reaction times very long. Thus, further organic cosolvents should be evaluated in order to understand their effects on the PGA catalysis and improvements of the reaction yields. Reported adverse effects of nonaqueous media could be overcome by the use of ionic liquids as an alternative to solvents (Lee 2012).

Because it was shown that temperature and pH can affect the PGA enantioselectivity and the catalyst productivity (Topgi et al. 1999; Yao and Lalonde 2003; Chilov et al. 2003; Gong et al. 2011), the site-directed mutagenesis based on docking experiments could increase the application potential of enantioselectivity of the PGA (Calleri et al. 2002; Massolini et al. 2003).

Immobilization techniques have already been successfully applied to the production of catalysts convenient for the syntheses of SSBAs. Among others, the following examples of immobilization have not yet been taken into account as regards an improvement or scale-up of the enantioselectivity-based bioprocesses: CLEAs that have many economical and environmental benefits in the context of the industrial biocatalysis (Sheldon 2011) or immobilization of the PGA onto iron oxide nanoparticles coated with polyethyleneimine followed by cross-linking with glutaraldehyde (Bahman et al. 2013).

Conclusions

Based on the knowledge of the broad enzyme specificity, PGA was successfully improved by engineering to meet the demands of industry (existing biocatalyses) or changed into the enzyme catalyzing reactions with novel substrates (novel biocatalyses). A high demand for production of SSBAs continuously drives development of the biotechnologies involving the PGA as a catalyst, and therefore, there is a consensus of the research and industrial community on the future potential of the PGA as an industrial enzyme. Current research is aimed at the reduction of costs of the process, production of β-lactams of novel generations belonging to the category of "biopures", and development of further applications exploiting sophisticated catalysts based on immobilized PGA for the production of APIs and chiral synthons.

As to other enzymes currently involved in β-lactams biocatalyses, although indispensable in a given process, they usually play a specific role, and their potential for further improvements is limited.

Although a great number of PGAs of different origin has been described so far, a limited number of the recombinant production strains are currently used on industrial scale with recombinant *E. coli* being the major host. The dominance of the recombinant *E. coli* among bacterial expression systems has been reviewed recently by Chen (2012).

The future of β-lactam biocatalyses definitely consists in concerted application of enzymes in cascade reactions. These one-pot processes meet most of the demands of the modern bioprocess.

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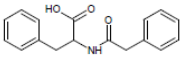
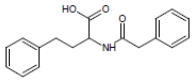
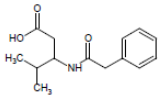
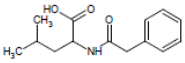
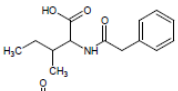
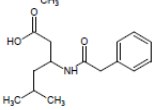
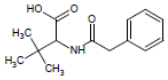
Opravný list

Str. 52.: V textu má být „22,5 x 22,5 x 22,5 Å“ místo „60 x 60 x 60 Å“.

Tab. 9, str. 70.:

Substráty	Struktura	Využití produktu	Literární odkazy
N-PhAc-α-fenylalanin		α-(*S*)-fenylalanin - přímý prekurzor neuromodulátoru fenyletylaminu	158
N-PhAc-α-homofenylalanin		(*S*)-homofenylalanin - stavební blok léčivé látky pro léčbu hypertenze a kardiovasculárních onemocnění	159
N-PhAc-β-leucin		Stavební blok pro syntézu terpenoidů nebo proteinů	160
N-PhAc-α-leucin		(*S*)-enantiomer - stavební blok pro syntézu proteinů	161
N-PhAc-α-isoleucin		(*S*)-isoleucin - doplněk potravy	
N-PhAc-β-homoleucin		Modulátor aktivity myeloperoxidasy, stavební blok biologicky aktivních tripeptidů	162, 163
N-PhAc-α-*terc*-leucin		(*S*)-*terc*-leucin - stavební blok pro syntézu léčivých látek proti AIDS a chemoterapeutik	96, 164

Tab. 10, str. 71.:

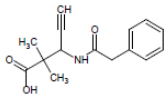
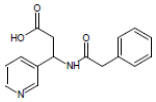
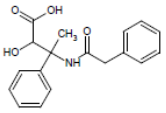
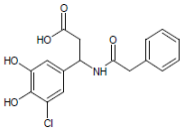
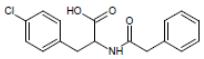
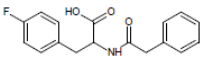
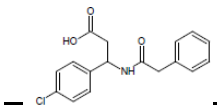
Substráty	E-hodnoty	
	PGA^{A}	**PGA^{Ec}**
N-PhAc-α-fenylalanin	90 ± 5	90 ± 5
N-PhAc-α-homofenylalanin	80 ± 5	80 ± 5
N-PhAc-β-leucin	68 ± 3	60 ± 5
N-PhAc-α-leucin	80 ± 5	80 ± 5
N-PhAc-α-isoleucin	80 ± 5	80 ± 5
N-PhAc-β-homoleucin	85 ± 4	55 ± 5
N-PhAc-α-*terc*-leucin	105 ± 5	75 ± 5

Str. 71.: V textu má být „N-PhAc-α-homofenylalaninu“ místo „N-PhAc-α-DL-homofenylalaninu“.

Tab. 13, str. 78.:

Substrát	Enantiomer	Vzdálenost (Å)				Predikován jako reaktivní	Predikovaná preference
		$O^{Ser1\beta} \rightarrow C$	$N^{Ala69\beta} \rightarrow O$	$N^{Asn241\beta} \rightarrow O$	$O^{Gln23\beta} \rightarrow H$		
N-PhAc-α-fenylalanin	(*S*)	3,4	4,0	4,0	3,9	Ano	(*S*)
	(*R*)	**5,2**	**6,3**	**7,9**	**5,4**	Ne	
N-PhAc-α-homofenylalanin	(*S*)	3,3	3,7	3,9	3,3	Ano	(*S*)
	(*R*)	**4,6**	**6,2**	**7,4**	**5,2**	Ne	
N-PhAc-β-leucin	(*R*)	3,2	3,7	4,0	3,5	Ano	(*R*)
	(*S*)	3,4	3,9	4,0	**4,4**	Ne	
N-PhAc-α-leucin	(*S*)	3,4	3,9	3,8	3,0	Ano	(*S*)
	(*R*)	3,4	4,0	4,1	**4,6**	Ne	
N-PhAc-α-isoleucin	(*S*)	3,6	4,1	3,9	2,8	Ano	(*S*)
	(*R*)	**4,5**	**6,2**	**7,4**	**5,1**	Ne	
N-PhAc-β-homoleucin	(*S*)	3,2	3,8	3,7	2,9	Ano	(*S*)
	(*R*)	3,4	3,8	4,0	**4,4**	Ne	
N-PhAc-α-*terc*-leucin	(*S*)	3,3	4,0	4,1	3,3	Ano	(*S*)
	(*R*)	**4,7**	**6,3**	**7,5**	**4,8**	Ne	

Tab. 14, str. 80.:

Substrát	Struktura	Využití produktu	Literární odkazy
N-PhAc-3-aminopent-4-ynová kyselina		(*S*)-enantiomer - stavební blok pro syntézu léčiva Elarofiban	165
N-PhAc-3-amino-3-pyridin-propanová kyselina		(*S*)-enantiomer - stavební blok pro syntézu léčiva Xemilofiban	166
N-PhAc-3-amino-3-benzyl-2-hydroxybutanová kyselina		(*S*)-enantiomer - stavební blok pro syntézu léčiva Paclitaxel	167
N-PhAc-3-amino-3-(3-chloro-4,5-dihydroxyfenyl) propanová kyselina		(*S*)-enantiomer - stavební blok pro syntézu léčiva Lidamycin	168
N-PhAc-*p*-*Cl*-α-fenylalanin		(*R*)-enantiomer - prekurzor pro syntézu analgetika Zolmitriptanu	92
N-PhAc-*p*-*F*-α-fenylalanin		(*R*)-enantiomer - stavební blok léčiva Abarelix	92
N-PhAc-*p*-*Cl*-β-fenylalanin		(*S*)-enantiomer - látka využívaná pro nejrůznější laboratorní účely	Sigma-Aldrich

Tab. 15, str. 81.:

Substrát	Enantiomer	Vzdálenost (Å)				Predikovaný jako reaktivní	Predikovaná preference
		$O^{Ser1\beta} \rightarrow C$	$N^{Ala69\beta} \rightarrow O$	$N^{Asn241\beta} \rightarrow O$	$O^{Gln23\beta} \rightarrow H$		
N-PhAc-3-aminopent-4-ynová kyselina	(*R*)	**3,9**	**4,5**	**4,7**	**4,7**	Ne	-
	(*S*)	3,5	**4,4**	**4,3**	**5,3**	Ne	
N-PhAc-3-amino-3-pyridin-propanová kyselina	(*R*)	3,2	3,7	3,9	3,1	Ano	(*R*)
	(*S*)	**4,2**	**4,2**	**5,0**	**5,0**	Ne	
N-PhAc-3-amino-3-benzyl-2-hydroxybutanová kyselina	(*S*)	3,3	3,8	4,0	3,4	Ano	(*S*)
	(*R*)	**4,2**	**6,1**	**7,8**	3,8	Ne	
N-PhAc-3-amino-3-(3-chloro-4,5-dihydroxyphenyl) propanová kyselina	(*R*)	3,1	3,6	4,0	2,9	Ano	(*R*)
	(*S*)	3,6	4,1	**4,3**	4,2	Ne	
N-PhAc-*p*-*Cl*-α-fenylalanin	(*S*)	3,2	3,8	3,8	2,0	Ano	(*S*)
	(*R*)	**4,8**	**6,6**	**8,0**	4,2	Ne	
N-PhAc-*p*-*F*-α-fenylalanin	(*R*)	3,6	4,1	4,0	2,6	Ano	-
	(*S*)	3,4	4,0	4,1	3,7	Ano	
N-PhAc-*p*-*Cl*-β-fenylalanin	(*R*)	3,2	3,6	4,0	3,2	Ano	(*R*)
	(*S*)	**4,4**	**6,7**	**7,9**	3,4	Ne	

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Penicillin G acylase from *Achromobacter* sp. CCM 4824

An efficient biocatalyst for syntheses of beta-lactam antibiotics under conditions employed in large-scale processes

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Abstract Penicillin G acylase from *Achromobacter* sp. (NPGA) was studied in the enzymatic synthesis of β-lactam antibiotics by kinetically controlled *N*-acylation. When compared with penicillin acylase of *Escherichia coli* (PGA), the NPGA was significantly more efficient at syntheses of ampicillin and amoxicillin (higher S/H ratio and product accumulation) in the whole range of substrate concentrations. The degree of conversion of 6-aminopenicillanic acid to amoxicillin and ampicillin (160 mM 6-APA, 350 mM acyl donor methylester·HCl, pH 6.3, 25 °C, reaction time of 200 min) with immobilized NPGA equaled 96.9 % and 91.1 %, respectively. The enzyme was highly thermostable with maximum activity at 60 °C (pH 8.0) and 65 °C (pH 6.0). Activity half-life at 60 °C (pH 8.0) and at 60 °C (pH 6.0) was 24 min and 6.9 h, respectively. Immobilized NPGA exhibited long operational stability with half-life of about 2,000 cycles for synthesis of amoxicillin at conversion conditions used in large-scale processes (230 mM 6-APA, 340 mM D-4-hydroxyphenylglycine methylester·HCl, 27.5 °C, pH 6.25). We discuss our results with literature data available for related penicillin acylases in terms of their industrial potential.

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Keywords *Achromobacter* sp. · Penicillin G acylase · β-Lactam antibiotics · Kinetically controlled synthesis · Immobilized enzyme

Introduction

Penicillin acylases (EC 3.5.1.11; penicillin amidohydrolases) of prokaryotic origin are important industrial catalysts, routinely used for large-scale production of β-lactam nuclei (N), a 6-aminopenicillanic acid (6-APA) or 7-aminodeacetoxycephalosporanic acid (7-ADCA) by hydrolysis of penicillin G (PEN G) or deacetoxycephalosporin G. The enzymes belong to the family of N-terminal nucleophilic hydrolases with catalytically active N-terminal serine of β-subunit. Active enzyme is formed from an inactive precursor (pre-pro-enzyme) by a complex process as described for penicillin acylases of *Alcaligenes faecalis* (Kasche et al. 2003) and *Escherichia coli* (Ignatova et al. 2005). The mature enzyme accumulates in the periplasmic space of Gram-negative bacteria as a heterodimer composed of a small α and large β subunits. Both subunits participate in the formation of active site (Duggleby et al. 1995; Alkema et al. 2002).

The enzymes are currently applied also to industrial syntheses of β-lactam antibiotics from a nucleophile (β-lactam nucleus, N) and corresponding acyl donor (AD, D-phenylglycine or D-4-hydroxyphenylglycine) (Chandel et al. 2008).

Thermodynamically controlled coupling of AD with N catalyzed by PGA in water yields low amounts of an antibiotic due to established reaction equilibrium (Diender et al. 1998; Schroën et al. 1999). Significantly higher yields of the

products were achieved with activated AD in the form of amide or methylester in kinetically controlled syntheses (Bruggink et al. 1998). It has been reported that the conversion of N into β-lactam antibiotic depends on parameters such as kinetic parameters of the enzyme, chemical properties of reactants, their concentrations in the reaction mixture, as well as the reaction conditions (Diender et al. 2000; Youshko et al. 2000, 2001; Ribeiro et al. 2005; Illanes et al. 2007).

At high molar ratio of activated AD to N, the synthesis yields higher amount of an antibiotic (Illanes et al. 2007). From the economical viewpoint, it is important to keep the molar ratio AD/N as low as possible. However, hydrolysis of the activated AD becomes the parameter of critical importance when approaching stoichiometric ratio of the reactants. The hydrolysis of the activated AD is either spontaneous namely at pH above 6.0 or catalyzed by the enzyme (Ospina et al. 1996; Diender et al. 1998; Youshko et al. 2000; Ribeiro et al. 2005).

The kinetics of enzyme-catalyzed synthetic reaction has been systematically studied and parameters relevant for evaluation of enzyme synthetic efficiency were introduced (Youshko et al. 2001; Youshko et al. 2002). The ratio of initial rate of the product synthesis $(V_{Ps})_{init}$ to initial rate $(V_{Ph})_{init}$ of free acid formation (hydrolytic product of activated AD) is known as synthesis/hydrolysis (*S*/*H*) ratio. Thus, the *S*/*H* ratio designated also as selectivity coefficient (Ribeiro et al. 2005) is an important parameter determining the preference of the interaction of the acyl-enzyme intermediate with β-lactam nucleus instead of molecule of water (Youshko et al. 2002). The *S*/*H* ratio increases with nucleophile concentration in the reaction mixture according to equation $S/H=(V_{Ps}/V_{Ph})_{init}=\beta_0[N]/(1+\beta_0\gamma[N])$ (Youshko et al. 2002; Gabor et al. 2005), where [*N*] stands for β-lactam nucleophile concentration. For low [*N*], the equation becomes $(V_{Ps}/V_{Ph})_{init}=\beta_0[N]$, and there is a linear relationship between *S*/*H* and [*N*] with the slope β_0, a parameter describing the nucleophilic reactivity. The enzyme selectivity between hydrolysis of the product and that of activated acyl donor is quantified by the parameter α, which equals the ratio of enzyme specificity constants for antibiotic hydrolysis to that of acyl donor: $\alpha=(k_{cat}/K_m)_{Ps}/(k_{cat}/K_m)_{AD}$ (Youshko et al. 2002; Gabor et al. 2005). The parameters *S*/*H*, α and maximum of antibiotic amount formed during synthesis (P_{Smax}) are used in evaluation of synthetic properties of novel penicillin acylases (Hernández-Justiz et al. 1999; Alkema et al. 2002; Gabor et al. 2005; Cheng et al. 2006; Jager et al. 2007).

We described a novel penicillin G acylase (NPGA) from soil isolate *Achromobacter* sp. CCM 4824 (Plháčková et al. 2003; Škrob et al. 2003). It was shown that NPGA efficiently hydrolyzes semi-synthetic β-lactam antibiotics like ampicillin, amoxicillin and cephalexin, besides PEN G and deacetoxycephalosporin G. The *npga* gene was sequenced (GenBank NCBI Accession number AY919310) and cloned in the host *E. coli* BL21 on a multicopy plasmid pKX1P1 (Kyslík et al. 2011).

Cai et al. (2004) studied penicillin acylase from the related microorganism *Achromobacter xylosoxidans* that was designated as PGA650. The authors expressed the gene in *E. coli* BL21 and reported a high thermal stability of the enzyme. However, they did not evaluate the enzyme as to synthetic properties. Gabor et al. (2005) identified novel penicillin acylase PAS2 by cloning and functional screening of the gene bank prepared from environmental DNA of enriched culture of microorganisms obtained from sandy soil. The amino acid (AA) sequence of the enzyme showed the high identity with PGA650 and the authors found increased synthetic activity (higher β_0 and P_{Smax}) in comparison with PGA from *E. coli*, to amoxicillin and ampicillin at low concentrations of reactants. These authors used kinetic model of Youshko et al. (2002) and also calculated P_s values for high concentrations of reactants.

Based on AA sequences of penicillin acylases deposited in databases, the identity of amino acid sequences of α and β subunits of our NPGA and PGA650 is 90.8 % and 96.6 %, respectively. If NPGA and PAS2 are compared, the identity of AA sequence of α and β subunit is 75.1 % and 87.2 %, respectively.

The aim of the present work is to determine synthetic properties of NPGA from *Achromobacter* sp. CCM 4824 and compare them with data on related enzymes PGA650 and PAS2 reported in literature. The NPGA was purified and properties in kinetically controlled syntheses of β-lactam antibiotics were determined. Analogous study was performed with purified PGA from *E. coli* RE3(pKA18), another enzyme available in the laboratory (Sobotková et al. 1996).

To test synthetic properties at concentrations of reactants employed in industry, immobilized NPGA and PGA were tested in syntheses using high substrate concentrations. Moreover, performance of immobilized NPGA for amoxicillin synthesis was determined in repeated conversion cycles.

Materials and methods

Microorganisms

Recombinant strain *E. coli* BL21(pKX1P1) (Kyslík et al. 2011) and *E. coli* RE3(pKA18) (Sobotková et al. 1996) were used to prepare biomass for purification of NPGA and PGA, respectively.

Enzyme purification and immobilization

NPGA (specific activity of 50 U/mg protein) was purified as described by Škrob et al. (2003). Soluble PGA of *E. coli*

(specific activity of 60 U/mg protein) was purified according to Kutzbach and Rauenbusch (1974). The activity of 1 unit (U) was defined as the amount of NPGA or PGA producing 1 μmol of phenylacetic acid per min in 0.05 M sodium phosphate buffer (pH 8.0) containing 1 % (w/v) PEN G at 37 °C.

Immobilized enzyme catalysts were supplied by Fermenta Biotech Ltd., India: Fermase PA® 1500 (immobilized PGA), specific activity of 81 U/g_{ww} (g of wet weight) or 516 U/g_{dw} (g of dry weight) (PEN G, pH 8, 37 °C) and Fermase NA® 150 (immobilized NPGA), specific activity of 58 U/g_{ww} or 432 U/g_{dw} (PEN G, pH 8, 37 °C). The immobilization procedure was based on polyacrylamide gel entrapment of the enzyme, combined with cross-linking with glutaraldehyde (Datla et al. 2011).

Media and culture conditions

The strain *E. coli* BL21(pKX1P1) was grown in LB medium (shaken flask cultures) or in a stirred bioreactor in mineral medium (0.4 % $(NH_4)_2SO_4$, 1.36 % KH_2PO_4, 0.3 % NaOH, 0.2 % $MgSO_4 \cdot 7H_2O$, 0.02 % $CaCl_2 \cdot 6H_2O$, 0.01 % $FeSO_4 \cdot 7H_2O$, pH 7.2) that was supplemented with casein hydrolyzate (10 g/l) and glycerol (10 g/l) (medium MCHGly) as described earlier (Kyslík et al. 2011). Culture conditions in a stirred bioreactor for *E. coli* RE3(pKA18) were described elsewhere (Sobotková et al. 1996).

Assay of activity and titration of NPGA with phenylmethanesulfonyl fluoride

The biomass harvested from the culture of the strain *E. coli* BL21(pKX1P1) was washed with 0.05 M phosphate buffer (pH 7.5) and the hydrolytic activity of NPGA was assayed with whole cells (0.04–0.1 mg_{dw} per ml of assay solution) by titration (titration system Radiometer) in 0.05 M sodium phosphate buffer supplemented with 1 % (w/v) PEN G at 37 °C. pH of 8.0 was maintained with 0.1 M NaOH. To assay activity of purified enzyme, the solution with 0.1–0.2 μg protein/ml was used. Titration of NPGA active sites with phenylmethanesulfonyl fluoride (PMSF) (Švedas et al. 1997) was used to estimate the enzyme concentration in crude enzyme preparation. Protein concentrations were assayed with BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

Kinetic parameters for substrate hydrolysis (K_m, V_{max}, k_{cat})

The parameters were determined with purified enzymes NPGA and PGA in 0.05 M potassium phosphate buffer (pH 7.0) at 30 °C. Concentrations of reactants were monitored by HPLC. The relationship between the initial reaction rate and a substrate concentration (ranging from 1 to 1,000 μM) was determined for each substrate in three independent experiments. The kinetic parameters K_m and V_{max} were calculated using the Hanes–Wolf plot and ANOVA calculator. The catalytic constant k_{cat} for PEN G was derived from V_{max} and molar concentration of the enzyme in reaction (calculated from protein amount and NPGA molecular weight of 89 kDa).

Kinetic parameters for β-lactam antibiotic synthesis (S/H, β_0, P_{Smax})

A temperature of the reaction solution containing an activated AD (15 mM, D-phenylglycine amide or D-4-hydroxyphenylglycine amide) and N (1–200 mM 6-APA or 7-ADCA for determination of β_0; 25 mM 6-APA or 7-ADCA for S/H and P_{Smax}) in 0.05 M potassium phosphate buffer (pH 7.0) was adjusted to 25 °C. The reaction was started by addition of purified enzyme (5–40 μl per ml of the reaction mixture) and the course of the reaction was monitored by HPLC. Initial rates of formation of an antibiotic and free amino acid from hydrolyzed acyl donor were calculated.

HPLC assay of reaction products

The concentrations of reaction components were determined by HPLC (Dionex-Summit) using Tessek SGX C18 5 μm, column (4×250 mm; Tessek, Czech Republic). The mobile phase (0.01 M sodium phosphate buffer and methanol) differed for substrates as follows: pH 3.0 and 10 % MetOH for amoxicillin and cefadroxil; pH 5.6 and 30 % MetOH for ampicillin and cephalexin, pH 6.5 and 40 % MetOH for penicillin G and deacetoxycephalosporin G.

Syntheses of antibiotics at high concentrations of substrates using immobilized NPGA and PGA

Kinetically controlled syntheses were performed in a stirred reactor with working volume of 100 ml (400 rpm, temperature of 25 °C) and pH was maintained with 12.5 % (w/v) ammonia water. The nucleophile (6-APA or 7-ADCA) was added to water and dissolved by addition of ammonia water (pH was maintained at pH 7.0), activated AD (D-phenylglycine methylester·HCl or D-4-hydroxyphenylglycine methylester·HCl) was added and required pH was maintained with ammonia water. The reaction was started by catalyst addition and the pH was maintained. The course of the reaction was monitored by HPLC.

Determination of catalyst operational half-life for amoxicillin synthesis

Repeated batch cycles were performed in microprocessor-controlled automatic device (Assortis Electric s.r.o., Czech

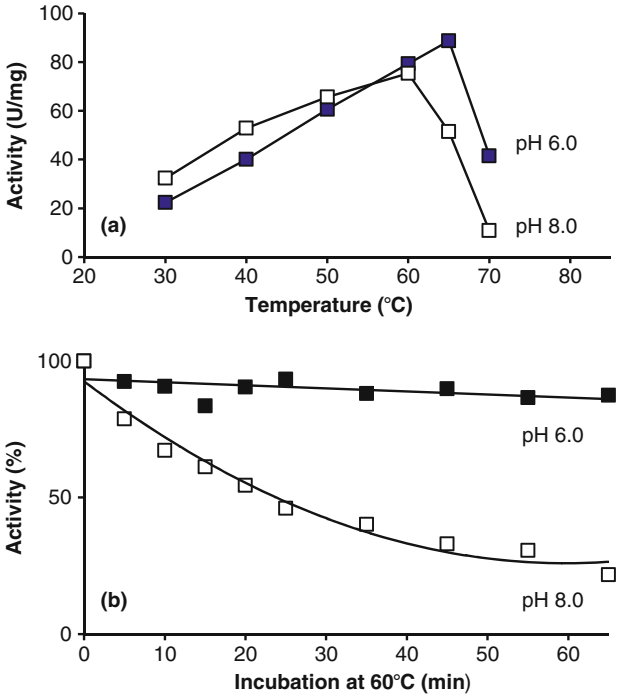

Fig. 1 **a** The effect of temperature on the activity of purified NPGA with 1 % penicillin G dissolved in 0.15 M NaCl; pH 6.0 (*filled square*) or 8.0 (*empty square*) was maintained by titration with 0.1 M NaOH. **b** Activity of purified NPGA in the course of incubation in 0.05 M sodium phosphate at pH 6.0 (*filled square*) or 8.0 (*empty square*) and temperature of 60 °C

Republic) with reactor of working volume up to 150 ml. Conversions were performed at 27.5 °C, frequency of stirring was 400 rpm and pH was maintained at 6.28 with 12.5 % ammonia water. Stock solution for ten cycles (900 ml) was composed of 6-APA (50 g) and D-4-hydroxyphenylglycine methylester·HCl (75 g) dissolved in water (final pH of 5.8), filtered and kept at 4 °C. Reaction was started by addition of 90 ml of stock solution into a reaction vessel containing 11 g_{ww} (1.75 g_{dw}) of the catalyst Fermase NA-150. After 130 min of conversion, the pH-stat was switched off, the reaction mixture was pumped out, and the reactor was filled with 150 ml of washing solution (0.16 M NaOH) to rinse the catalyst and remove residual amoxicillin. After 5 min of stirring, the solution (pH 7.9) was removed from the reactor and the next cycle was started by addition of 90 ml of stock solution. The time course of selected conversion cycles was monitored by HPLC. The initial conversion rates were calculated from decrease of 6-APA in time period of 0–60 min and expressed in % of 6-APA conversion into amoxicillin per minute. The final degree of conversion was determined after 130 min of the reaction. Hydrolytic activity of the catalyst (U/g_{ww}) for PEN G was assayed by titration using about 0.2 g_{ww} of the catalyst, which was withdrawn from the reactor and washed with water. The catalyst was returned to the reaction after assay.

Results

Purification of the NPGA

Fed-batch culture of the strain *E. coli* BL21(pKX1P1) was performed in a stirred bioreactor as described previously (Kyslík et al. 2011) in medium MCHGly. A volumetric activity of NPGA reached 33,000 U/l of fermentation broth (specific activity of 1,400 U/g_{dw}) after 22 h of cultivation. The biomass was disintegrated and the enzyme amount was assayed by titration of enzyme catalytic sites with inhibitor PMSF in cell-free protein extract. NPGA concentration corresponded to 0.9 g of the enzyme per liter of fermentation broth. This amount corresponds to constitutive production of NPGA that represents about 20 % of the cell soluble protein. The enzyme was purified 25-fold: the specific hydrolytic activity (PEN G, pH 8.0, 37 °C) was 50.4 U/mg of protein, which corresponded to the k_{cat} of 72 s^{-1}. This enzyme preparation was used in further experiments.

Effect of temperature on activity of NPGA

The temperature optimum for activity was determined in the range of temperatures from 30 °C to 70 °C in 0.05 M phosphate buffer at pH 6.0 or 8.0. At pH of 8.0, the activity

Table 1 Steady-state (hydrolytic) kinetic parameters of NPGA and PGA for antibiotics and activated acyl donors

Reaction conditions: 0.05 M potassium phosphate buffer (pH 7.0), 30 °C; ± stands for standard deviation for 95 % confidence interval

D-PGA D-phenylglycine amide, *D-HPGA* D-4-hydroxyphenylglycine amide

Substrate	NPGA (*Achromobacter* sp. CCM 4824)			PGA (*Escherichia coli* ATCC 9637)		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
D-PGA	26±1.5	19.1±1.6	1.36±0.14	29±1.7	31.6±2.6	0.93±0.09
D-HPGA	34±3.2	23.9±2.1	1.43±0.18	21±2.0	18.8±1.7	1.12±0.14
Ampicillin	22±0.8	0.45±0.02	49.1±2.6	21±0.8	3.57±0.14	6.03±0.32
Amoxicillin	27±1.1	0.22±0.01	124.2±8.0	14±0.6	1.54±0.08	9.03±0.58
Cephalexin	19±0.3	0.80±0.06	23.6±1.8	36±0.6	2.82±0.21	12.7±1.0
Cefadroxil	39±1.2	0.55±0.02	71.1±3.8	25±0.8	0.84±0.04	29.7±1.6

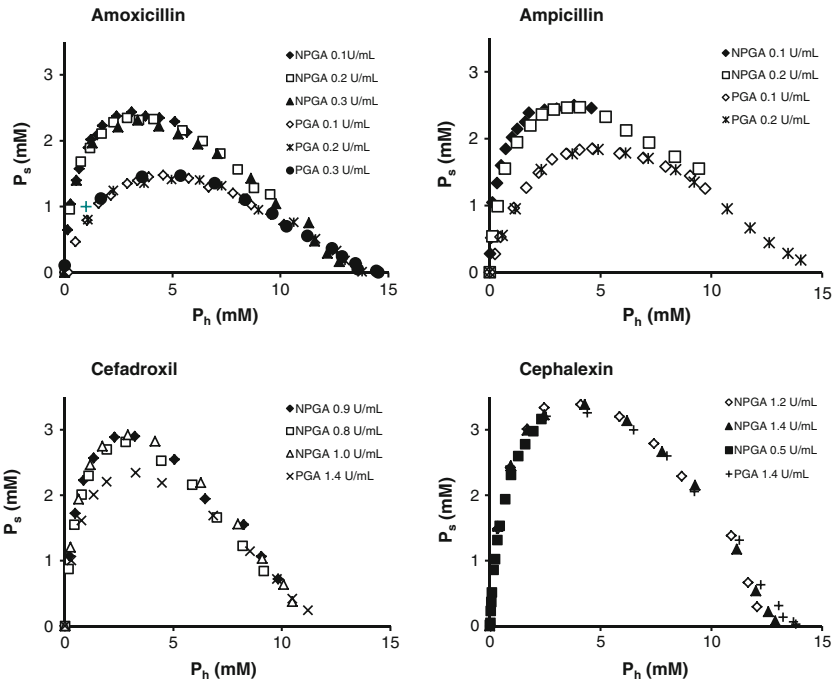

Fig. 2 Relationship between antibiotic synthesis (P_s) and formation of free amino acid (P_h) in the course of synthetic reaction catalyzed by purified NPGA and PGA. Reaction conditions: 15 mM acyl donors (D-4-hydroxyphenylglycine amide, D-phenylglycine amide), 25 mM nucleophiles (6-APA, 7-ADCA), 0.05 M phosphate K^+, pH 7.0, 30 °C. Different quantities of acylases were used

maximum was achieved at 60 °C while at pH of 6.0 the maximum was shifted to 65 °C (Fig. 1a). The stability of the enzyme activity at the temperature of 60 °C was measured in 0.05 M phosphate buffer and pH 6.0 or 8.0. The half-life of NPGA activity at pH of 6.0 and 8.0 was 6.9 h and 24 min, respectively (Fig. 1b).

Kinetic parameters of NPGA for hydrolyses

NPGA-catalyzed hydrolyses of β-lactam antibiotics and acyl donor amides were measured in phosphate buffer at pH of 7.0 and temperature of 30 °C (Table 1). The values of kinetic constants k_{cat}, K_m and substrate specificity constant k_{cat}/K_m confirmed the high catalytic activity to substrates with D-4-hydroxyphenylalanyl- (amoxicillin or cefadroxil) or D-phenylalanyl- (ampicillin and cephalexin) side chain. Values of k_{cat}/K_m of NPGA descend in the following order: amoxicillin > cefadroxil > ampicillin > cephalexin. This order corresponds to descending affinity to acyl-residue of a substrate: D-4-hydroxyphenylalanyl > D-phenylalanyl.

Kinetically controlled syntheses of β-lactam antibiotics at low substrate concentrations

The performance of purified enzymes NPGA and PGA in kinetically controlled syntheses of antibiotics amoxicillin, ampicillin, cefadroxil and cephalexin were compared in reaction mixtures containing low concentrations of substrates: 15 mM activated AD (D-phenylglycine amide or D-4-hydroxyphenylglycine amide) and 25 mM N (6-APA or 7-

Table 2 Kinetic parameters α, β_0, *S*/*H* ratio and P_{Smax} for NPGA and PGA

Acyl donor (AD)	β-Lactam nucleophile	Antibiotic (Ab)	α		β_0 (M^{-1})		*S*/*H*		P_{Smax} (mM)	
			NPGA	PGA	NPGA	PGA	NPGA	PGA	NPGA	PGA
D-PGA	6-APA	Ampicillin	36.1	6.5	153	30	3.8	0.8	2.5	1.9
D-HPGA	6-APA	Amoxicillin	87.1	8.1	129	31	3.2	0.8	2.4	1.5
D-PGA	7-ADCA	Cephalexin	17.4	13.7	149	162	3.7	3.9	3.4	3.3
D-HPGA	7-ADCA	Cefadroxil	49.8	12.3	98	96	2.5	2.4	2.9	2.3

Syntheses of antibiotics in solution of 15 mM acyldonor with 25 mM β-lactam nucleophile in 0.05 M potassium phosphate, pH 7.0, at 30 °C, catalysed with NPGA or PGA. Acyl donors: *D-PGA* D-phenylgycine amide, *D-HPGA* D-4-hydroxyphenylglycine amide; nucleophiles: *6-APA* 6-aminopenicillanic acid, *7-ADCA* 7-aminodeacetoxycephalosporanic acid. $\alpha=(k_{cat}/K_m)_{Ab}/(k_{cat}/K_m)_{AD}$ is the ratio of hydrolytic specificity constants k_{cat}/K_m of antibiotic and corresponding acyldonor determined in 0.05 M potassium phosphate, pH 7.0, at 30 °C; $\beta_0=[(V_{Ps}/V_{Ph})_{init,N}]/[N]$ is the coefficient of nucleophile reactivity calculated from linear part of relationship of initial synthesis/hydrolysis ratio (*S*/*H*) vs. nucleophile concentration; P_{Smax} is the maximum concentration of antibiotic accumulated in the course of the reaction

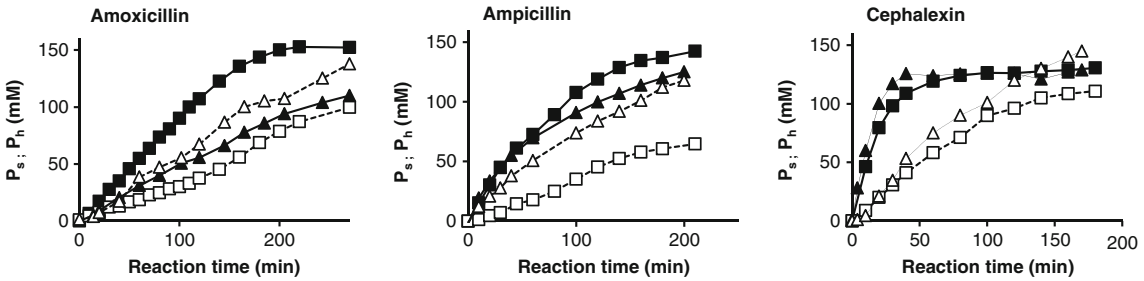

Fig. 3 Time courses of kinetically controlled syntheses of amoxicillin, ampicillin and cephalexin. Syntheses of amoxicillin and ampicillin were carried out with 160 mM 6-APA and 350 mM acyl donor D-4-hydroxyphenylglycine methylester·HCl (amoxicillin) and D-phenylglycine methylester·HCl (ampicillin). Synthesis of cephalexin was performed with 140 mM 7-ADCA and 340 mM D-phenylglycine methylester·HCl. Antibiotic (P_s, *filled square*, *filled triangle*) and free amino acid (P_h, *empty square*, *empty triangle*) productions catalyzed by NPGA (*filled square*, *empty square*) and PGA (*filled triangle*, *empty triangle*) in the course of the syntheses were monitored by HPLC. Syntheses were performed at pH 6.3 and temperature of 25 °C

ADCA). Concentrations of a product (antibiotic, P_s) and free amino acid (product of hydrolysis of activated acyl donor and antibiotic, P_h) were determined by HPLC in the course of syntheses catalyzed by various amounts of enzymes. The relationships of these parameters are shown in Fig. 2. It is evident that the P_s/P_h ratio is independent on enzyme concentration used in reaction (15 mM AD and 25 mM N). The enzyme concentration affects only the reaction rate, i.e., the time in which P_s maximum (P_{Smax}) is achieved. The values of P_{Smax} and P_{Smax}/P_h ratio for given initial concentrations of the reactants and reaction conditions are affected solely by kinetic parameters of individual enzymes. Figure 2 illustrates that NPGA is significantly more effective in syntheses of antibiotics amoxicillin and ampicillin than PGA. Both enzymes are comparable as regards syntheses of cefadroxil and cephalexin. In the latter case, almost no difference in P_{Smax} between NPGA and PGA was observed. The maximum amount of each product formed by these enzymes is shown in Table 2. For both enzymes, the values of P_{Smax} descend in the following order: cephalexin, cefadroxil, ampicillin and amoxicillin.

The parameters *S*/*H* ratio, α and β_0 are shown in Table 2. We found that the *S*/*H* ratio increased linearly in concentration of N ranging from 0 to 50 mM. The parameter β_0 for 6-APA was five times higher for NPGA in comparison with PGA while β_0 for 7-ADCA was almost identical for both enzymes. This results in similar product accumulation for syntheses of cephalexin and cefadroxil. The maximum P_{Smax} for cefadroxil and cephalexin synthesized by NPGA is 2.9 and 3.4 mM, respectively, and the maxima synthesized by PGA are 2.3 and 3.3 mM, respectively. These values are higher than those for ampicillin and amoxicillin, which is connected with higher values of β_0 and lower α for 7-ADCA in comparison with 6-APA.

Syntheses of β-lactam antibiotics at high substrate concentrations catalyzed by immobilized enzymes

We compared synthetic performances of NPGA and PGA at high substrate concentrations (140–160 mM N and 340–350 mM acyl donor methylester) employed in industrial processes. The experiments were done with immobilized enzymes Fermase NA® 150 (NPGA) and Fermase PA® 1500 (PGA). NPGA synthesized more efficiently both the amoxicillin and the ampicillin which is shown in time courses of parameters P_s and P_h (Fig. 3). The plot P_s vs. P_h under these experimental conditions is in Fig. 4. In the case of cephalexin synthesis, much smaller differences

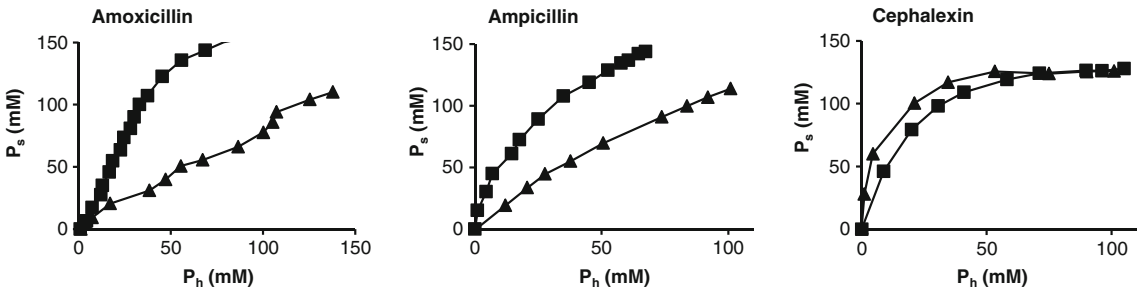

Fig. 4 Relationship between antibiotic synthesis (P_s) and formation of free amino acid (P_h) produced in the course of syntheses of amoxicillin (160 mM 6-APA and 350 mM D-4-hydroxyphenylglycine methylester·HCl), ampicillin (160 mM 6-APA and 350 mM D-phenylglycine methylester·HCl) and cephalexin (140 mM 7-ADCA and 340 mM D- phenylglycine methylester·HCl) at high substrate concentrations, using NPGA (*filled square*) or PGA (*filled triangle*)

Table 3 Syntheses of amoxicillin, ampicillin, and cephalexin by immobilized NPGA at high substrate concentrations

Antibiotic	N: 6-APA (mM)	N: 7-ADCA (mM)	AD: D-HPGMe (mM)	AD: D-PGMe (mM)	AD/N	$(V_{Ps})_{init.}$ (U/$g_{cat.dw}$)	$(V_{Ps})_{init.}$ ($kg_{antibiotic}$/$kg_{cat.dw}$/h)	$(V_{Ph})_{init.}$ (U/$g_{cat.dw}$)	*S/H*	Conversion of N (%)	Reaction time (min)	pH
Amoxicillin	130		320		2.46	167	4.2	56	2.98	96.8	140	6.3
Amoxicillin	160		350		2.19	85	2.1	27	3.15	96.9	200	6.3
Amoxicillin[a]	240		360		1.50	76	1.9	18	4.25	91.3	240	6.3
Ampicillin	160			350	2.19	171	4.1	60	2.85	91.1	200	6.3
Ampicillin	400			600	1.50	351	8.5	57	6.17	92.0	300	5.9
Ampicillin[a]	400			540	1.35	272	6.6	26	10.55	94.0	270	5.9
Cephalexin		160		360	2.25	185	3.9	26	7.01	92.0	210	6.3
Cephalexin[b]		240		360	1.50	139	2.9	22	6.47	91.1	210	6.3
Cephalexin		300		400	1.33	208	4.3	19	11.24	88.5	300	6.3

Reaction conditions: volume of reaction mixture 100 ml, temperature 25 °C, pH 6.3 or 5.9 (maintained by addition of 12.5 % (w/v) NH_4OH), 6 g_{ww} of the catalyst

Activated acyl donors (AD): *D-HPGMe* (D-4-hydroxyphenylglycine methylester·HCl) and *D-PGMe* (D-phenylglycine methylester.HC); β-lactam nucleophiles (N): 6-APA and 7-ADCA; *AD/N* molar ratio of acyldonor to nucleophile, $(V_{Ps})_{init.}$ is initial rate of product synthesis − synthetic activity; $(V_{Ph})_{init.}$ is the initial rate of formation of free aminoacids − hydrolytic activity; *S/H* ratio=$(V_{Ps}/V_{Ph})_{init.}$, $kg_{antibiotic}$ denotes kg of synthetized antibiotic, $g_{cat.dw}$ or $kg_{cat.dw}$ denotes g or kg of dry weight of catalyst

[a] 10 g_{ww} of the catalyst

[b] 9 g_{ww} of the catalyst

between both enzymes were found. PGA-catalyzed synthesis was somewhat faster (Fig. 3), but product accumulation at the end of the reaction was identical for both the enzymes.

The efficiency of immobilized NPGA was further tested in syntheses of these antibiotics at concentrations of nucleophile up to 400 mM and AD/N ratio ranging from 1.33 to 2.46 (Table 3). The maximum conversion degrees were 96.9 % (amoxicillin, reaction time 200 min), 94 % (ampicillin, 270 min) and 92 % (cephalexin, 210 min). The molar AD/N ratio for synthesis of amoxicillin, ampicillin and cephalexin was 2.19, 1.35 and 2.25, respectively. *S/H* ratio of NPGA determined at synthesis of amoxicillin, ampicillin and cephalexin was 3.15, 10.55 and 7.0, respectively.

Synthetic activities calculated from initial rate of product synthesis with 160 mM N were 2.14 kg (amoxicillin), 4.14 kg (ampicillin) and 3.86 kg (cephalexin) per kg_{dw} of catalyst per hour. These activities increase with nucleophile concentration and AD/N ratio (Table 3).

Repeated conversions and operational stability of the catalyst

Immobilized NPGA (Fermase NA® -150) was used for synthesis of amoxicillin from 6-APA (230 mM) and D-4-hydroxyphenylglycine methylester·HCl (340 mM). Reaction temperature and pH was maintained at 27.5 °C and 6.25, respectively. Reaction time was set to 130 min and the catalyst was used in 308 repeated conversion cycles performed by automated bioreactor. Conversion degree varied between 90 % and 92 %, and the activity of catalyst after 308 conversion cycles (Fig. 5) was 93.4 % of initial value. This finding corresponds to operational half-life of more than 2,000 conversion cycles.

Discussion

High-expression prokaryotic system (Kyslík et al. 2011) for production of NPGA from *Achromobacter* sp. CCM 4824 (Plháčková et al. 2003) and the commercial catalysts for

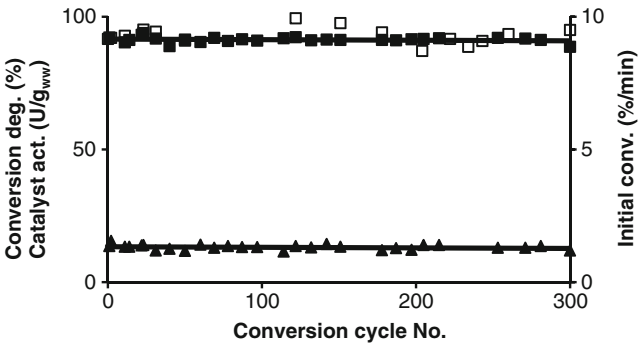

Fig. 5 Stability of immobilized NPGA in consequent conversion cycles of 6-APA into amoxicillin (230 mM nucleophile, 340 mM D-4-hydroxyphenylglycine methylester·HCl, pH 6.25 and 27.5 °C). Final conversion degree (*filled square*, %) and hydrolytic activity (*empty square*, U/g_{ww}) were assayed. Initial conversion rates (*filled triangle*, %/min) were calculated from initial 60 min of conversion cycle and expressed in percentage of nucleophile converted into antibiotic. The catalyst sampled for hydrolytic activity assay was returned into reaction mixture

kinetically controlled syntheses of β-lactam antibiotics (Datla et al. 2011) were developed previously. Two closely related *pac* genes have been cloned and characterized by two groups: Cai et al. (2004) studied PGA650 encoded by the *pac* gene from *A. xylosoxidans* and Gabor et al. (2005) PAS2 encoded by environmental gene. Therefore, we have focused on characterization of our NPGA to be able to compare the enzyme with literature data on PGA650 and PAS2 as to their stability traits and efficiency for kinetically controlled syntheses of β-lactam antibiotics under conditions employed in industrial processes. In this respect, unfortunately, some important properties of enzymes PGA650 or PAS2 were not reported. To have a corresponding set of experimental data obtained under comparable conditions, we decided to perform analogous study with PGA of *E. coli* cloned in our laboratory previously.

We have found that the temperature stability of NPGA from *Achromobacter* sp. CCM 4824 was higher than that published for PGA650 from *A. xylosoxidans*, an enzyme considered as the most stable penicillin acylase reported so far (Cai et al. 2004). At pH 8.0 and 60 °C, NPGA exhibited the half-life of 24 min in contrast to 8 min determined for PGA650. Guranda et al. (2004) measured rate constant of inactivation kinetics (k_{inact}) for PGA of *E. coli* at lower temperature of 50 °C and at pH ranging from 3.5 to 9.5. The minimum inactivation constant corresponding to the half-life of 9 h was found at pH 6.0. At pH 8.0 and 50 °C, the half-life was 7–10 min, which corresponds to the data obtained for PGA by Cai et al. (2004).

The stability of NPGA at pH 6.0 and temperature of 60 °C determined by our group (half-life up to 6.9 h) is noteworthy because it is advantageous for the application of NPGA in β-lactam antibiotic syntheses usually performed at a pH value of around 6.0.

The study of kinetically controlled syntheses catalyzed with soluble enzymes NPGA and PGA at lower substrate concentrations showed that NPGA is a significantly better catalyst for ampicillin and amoxicillin syntheses than PGA. The same conclusion was reported by Gabor et al. (2005) who compared PAS2 and penicillin acylase from *E. coli*.

Using a kinetic model of Youshko et al. (2002) for syntheses of antibiotics, Gabor et al. (2005) simulated synthesis of ampicillin with PAS2 at higher substrate concentrations (above 100 mM 6-APA) and stated that the P_{Smax} value should be lower than that for PGA. We found experimentally that NPGA had higher productivity than PGA of *E. coli* also at high concentrations of substrates. This might be the most important difference in synthetic properties of NPGA and PAS2.

The operational stability of immobilized NPGA at high concentrations of reactants (half-life of 2,000 cycles of repeated syntheses of amoxicillin) based on the extreme temperature stability of soluble enzyme (half-life 6.9 h, at 60 °C and pH 6.0) together with the high efficiency in synthesis of ampicillin make the NPGA a better class of catalyst for 6-APA *N*-acylations. We suppose that stability of NPGA could predetermine the enzyme also for catalytic use in organic solvents.

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Research review paper

Perspectives and industrial potential of PGA selectivity and promiscuity

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ABSTRACT

Penicillin G acylases (PGAs) are robust industrial catalysts used for biotransformation of β-lactams into key intermediates for chemical production of semi-synthetic β-lactam antibiotics by hydrolysis of natural penicillins. They are used also in reverse, kinetically controlled synthetic reactions for large-scale productions of these antibiotics from corresponding beta-lactam nuclei and activated acyl donors. Further biocatalytic applications of PGAs have recently been described: catalysis of peptide syntheses and the resolutions of racemic mixtures for the production of enantiopure active pharmaceutical ingredients that are based on enantioselective acylation or chiral hydrolysis. Moreover, PGAs rank among promiscuous enzymes because they also catalyze reactions such as trans-esterification, Markovnikov addition or Henry reaction. This particular biocatalytic versatility represents a driving force for the discovery of novel members of this enzyme family and further research into the catalytic potential of PGAs. This review deals with biocatalytic applications exploiting enantioselectivity and promiscuity of prokaryotic PGAs that have been recently reported. Biocatalytic applications are discussed and presented with reaction substrates converted into active compounds useful for the pharmaceutical industry.

Contents

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1. Introduction

Penicillin acylases belong to the N-terminal nucleophile (Ntn) serine hydrolase superfamily of proteins where they represent three distinct families: penicillin G acylases (PGA), penicillin V acylases and cephalosporin acylases (or ampicillin acylases) (Oh et al., 2004). Although their native role in microorganisms (i.e. bacteria and fungi) has not been so far unambiguously elucidated, they are thought to be involved in the catabolism of natural compounds bearing phenylacetyl moiety (Galan et al., 2004).

Penicillin G acylases (EC 3.5.1.11; PGA, penicillin amidohydrolase) are robust industrial catalysts that have been routinely used for several decades for production of 6-aminopenicillanic acid (6-APA) by hydrolysis of natural penicillin G (Arroyo et al., 2003; Chandel et al., 2007; Elander, 2003; Sio and Quax, 2004). In addition to the large-scale production of the key intermediates 6-APA or 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) (Chandel et al., 2007) for chemical syntheses of semi-synthetic β-lactam antibiotics (Chandel et al., 2007), PGAs have been recently applied by pharmaceutical companies (DSM Fine Chemicals, Fermenta Biotech Ltd) also in reverse, synthetic reactions for the large-scale production of β-lactam antibiotics from corresponding β-lactam nuclei and activated acyl donors. While PGAs generally catalyze the hydrolytic reactions at alkaline pH, at acidic or neutral pH they promote acylations. An excellent review (Volpato et al., 2010) deals with the hydrolytic production of β-lactam antibiotic nuclei and kinetically controlled syntheses of β-lactam antibiotics, topics that are beyond the scope of this review.

Thorough research into the structure of PGA and understanding of substrate–enzyme interactions revealed that PGAs are versatile enzymes catalyzing reactions over a wide range of compounds and exhibiting stereoselectivity.

Enzyme enantioselectivity, in general, is a phenomenon with great potential for the pharmaceutical industry. Chiral molecular entities called enantiomers exist always in pairs of which usually only one enantiomer is biologically active or can serve as an intermediate of such compound. Research into enantioselectivity of PGA started in the 60's of the last century and dealt with resolutions of racemic mixtures of α-amino acids and their derivatives (Cole, 1969 Lucente et al., 1965), α-amino alcohols and α-amino nitriles (Romeo et al., 1971). Resolution experiments were later extended to α-substituted α-amino acids (Rossi and Calcagni, 1985), racemic mixtures of β, γ, δ, ε-amino acids (Margolin, 1993; Rossi et al., 1977), aliphatic amines (Rossi et al., 1978), β-aryl-β-amino acids (Soloshonok et al., 1995), β-alkyl-β-amino acids (Soloshonok et al., 1994), phosphonic and phosphonous analogs of alanine (Solodenko et al., 1993), primary or substituted carbinols (Fuganti et al., 1988; Waldmann, 1989), β-amino ketones (Cainelli et al., 1997) and the dipeptide aspartame (Fuganti and Grasselli, 1986).

In this review, we will focus on biocatalytic applications exploiting enantioselectivity and promiscuity of prokaryotic PGAs, two properties that were discovered or profoundly studied during the last decade. We have focused on biocatalyses providing active compounds useful for the pharmaceutical industry (APIs), or their precursors.

1.1. Sources of penicillin G acylases

Bacterial PGAs belong to the best characterized industrial enzymes. Their traits and biocatalytic potential have been discussed in detail elsewhere (Arroyo et al., 2003; Chandel et al., 2007; Sio and Quax, 2004; van Langen et al., 2000). PAs were reported in bacteria belonging to genera *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Escherichia*, *Kluyvera*, *Providencia*, *Shigella*, *Xanthomonas*, and *Xylella* (Parmar et al., 2000; Shewale and Sivaraman, 1989; Škrob et al., 2003; Sudhakaran et al., 1992). Nature biodiversity seems to be always a major source of novel PGAs with interesting characteristics. The novel bacterial producers that were isolated and added on the list during the last decade are shown in Table 1.

The knowledge of nucleotide sequences of the *pga* (or *pac*) structural genes encoding PGAs and their regulatory regions was exploited for improvement of the enzyme parameters, construction of recombinant strains expressing wild-type or genetically-engineered genes or mining environmental genes encoding PGAs (Gabor et al., 2004). Some of the recombinant strains are used in industry as "tailor-made" strains for overproduction of PGAs (Kallenberg et al., 2005) and consequent production of robust biocatalysts.

Examples of expression systems for PGAs based on different bacterial or yeast hosts are listed in Table 2. Although about 20 different *pga* genes were cloned and at least 14 structural genes were sequenced, only four PGAs were studied as regards enantioselectivity and promiscuity (see further chapters). Unlike bacterial hosts, the yeast expression systems have so far not been reported as industrial production strains for PGAs. Obviously, extended fermentation time (up to 120 h) might be one of the reasons for preferential use of bacterial systems although the yeast host *Pichia pastoris* offers the choice for cytoplasmic or extracellular expression.

1.2. Penicillin G acylase-based catalyst

Enzyme stabilization is required for industrial success of the biocatalyst because enzymes are naturally evolved proteins catalyzing reactions under physiological conditions. Once stabilized, the enzymes retain their capabilities to catalyze reactions under mild conditions and, furthermore, may acquire traits that render them exploitable in organic syntheses. In the latter case, enzyme immobilization eliminates the problem of protein unfolding caused by organic solvents that are frequently present in reaction mixtures. The effect of solvents on enzyme activity and stereoselectivity was reported elsewhere (Illanes et al., 2012). Expected outcomes of enzyme immobilization are: increased enzyme activity in organic solvents, increased temperature stability (Polizzi et al., 2006), long-term operational stability, and catalyst recovery for its repeated usage (Miletic et al., 2012).

Table 1
Recently isolated microorganisms producing PGA.

Microorganism	Enzyme localization	Reference
Bacillus subtilis BAC4	Extracellular	Supartono et al. (2008)
Achromobacter sp. CCM 4824	Periplasmic	Škrob et al. (2003)
Achromobacter xylosoxidans strain 650	Periplasmic	Cai et al. (2004)
Bacillus badius PGS10 (*Bacillus* sp. PGS10)	Intracellular	Rajendhran et al. (2002) (2003)
Shigella boydii (clinical isolate)	Periplasmic	Montazam et al. (2009)
Bacillus sp. MARC-0103	Extracellular	Tahir et al. (2009)
Thermus thermophilus HB27	Membrane fraction	Torres et al. (2012)

Table 2
The *pga* structural genes cloned in microbial hosts.

Host strain	Donor strain of *pga* gene	Reference
Bacillus subtilis	*Alcaligenes faecalis* CICC AS1.767	Zhou et al. (2003b)
	Arthrobacter viscosus 8895GU	Ohashi et al. (1989)
	Bacillus megaterium	Kang et al. (1991)
Escherichia coli	*Shigella boydii* clinical isolate	Montazam et al. (2009)
	Bacillus badius PGS10	Rajendran and Gunasekaran (2007)
	Bacillus megaterium UN1	Meevootisom and Saunders (1987)
	Escherichia coli ATCC 11105	Robas et al. (1993); Zhang et al. (1990)
	Escherichia coli RE3	Sobotková et al. (1996)
	Alcaligenes faecalis ATCC 19018	Deak et al. (2003); Verhaert et al. (1997)
	Arthrobacter viscosus ATCC 15294	Konstantinovic et al. (1994)
	Arthrobacter viscosus 8895GU	Ohashi et al. (1988)
		Ohashi et al. (1989)
	Achromobacter sp. CCM 4824	Kyslík et al. (2011)
	Achromobacter xylosoxidans strain 650 (enzyme PGA650)	Cai et al. (2004)
	Escherichia coli 194	Meevootisom and Saunders (1987)
	Kluyvera cryocrescens (*Kluyvera citrophila* ATCC 21285)	García and Buesa (1986)
	Kluyvera cryocrescens (*K. citrophila*)	Wen et al. (2005)
	Environmental DNA (enzyme PAS2)	Gabor et al. (2005)
	Providencia rettgeri (*Proteus rettgeri* ATCC 31052)	Daumy et al. (1986)
	Providencia rettgeri (*Proteus rettgeri* Cyc1)	Daumy et al. (1986)
	Shuffled structural gene (*E. coli* × *K. cryocrescens* × *P. rettgeri*)	Jager et al. (2007); Zhou et al. (2003a)
Proteus mirabilis	*Escherichia coli* ATCC 11105	Gumpert et al. (1996)
Pseudomonas aeruginosa	*Escherichia coli* ATCC 9637	Krzeslak et al. (2009)
Pichia pastoris X-33	*Escherichia coli* RE3	Marešová et al. (2010)
	Providencia rettgeri	Ševo et al. (2002)
Saccharomyces cerevisiae CBL1-30	*Escherichia coli*	Ljubijankić et al. (1999)
	Providencia rettgeri	Ljubijankić et al. (1999)

Maturation of enzymes takes place in natural compartments of the cell (as regards PGA, see Section 2) and this process yields a mature form (e.g., tertiary and quarternary structures) with hydrophobic core, an active site, and hydrophilic surface. The aspects of enzyme interactions with material surfaces and nature of the interface between the enzyme and material surfaces were reviewed by Talbert and Goddart (2012).

As a general rule, it is important that the functional groups used for immobilization are not involved in the catalytic activity of the enzyme. The properties of a support material are also important. For example, in the case of porous supports, the pores should be large enough for an enzyme molecule because an average size of a folded, mature enzyme ranks 3–6 nm. From this viewpoint, pores are classified into micropores (less than 2 nm), mesopores (2–50 nm), and macropores (above 50 nm) according to the IUPAC recommendations.

After immobilization, a substrate should have an easy access to the catalytic site of the bound enzyme and the site should not be distorted by immobilization. These aspects are addressed in reviews such as Sheldon (2007), Mateo et al. (2007) and Fernandez-Lafuente (2009).

Immobilization on insoluble carrier beads is still the conventional way of PGA stabilization for applications in bioprocesses dealing with hydrolytic production of β-lactam antibiotic nuclei.

Cross-linked enzyme aggregates (CLEAs) are a novel type of carrierless catalysts. These aggregates appear to have considerable industrial potential due to their high activity retention and stability, ease of preparation from technical, crude enzyme preparations and carrierless basis (Sheldon, 2007). The technique was applied also on PGA (Cao et al., 2001) to produce particles having the size of about 45 um (Van Langen et al., 2002). Embodiment of CLEAs into gel matrix represents another method of generation of enzyme biocatalysts (Wilson et al., 2009) suitable for applications under both mild (water phase) and unnatural, harsh conditions (organic solvents). High specific activities make these catalysts a matter of choice for kinetically controlled syntheses of semi-synthetic β-lactam antibiotics at large-scale processes.

Stabilization of PGA on glyoxyl agarose with stabilization factor of 8000 (Alvaro et al., 1990) and 7000 (Guisan et al., 1993) was reported for the enzymes from *Escherichia coli* and *Kluyvera citrophila*, respectively. An outstanding review on this topic was written by Kallenberg et al. (2005).

The latest developments in PGA immobilization include i) chemical and genetic modification of the protein surfaces (Lopez-Gallego et al., 2005; Montes et al., 2006, 2007) in combination with immobilization on hydrophilic supports activated with glyoxyl groups (Mateo et al., 2006), ii) preparation of a catalyst based on CLEAs stabilized with polyionic polymers (Pchelintsev et al., 2009; Wilson et al., 2009), and iii) stabilization of PGA on immobilized metal ion affinity membrane (Chen et al., 2012).

The PGA undergoes a relatively large conformational change during catalysis, an acyl-induced exposure of a reactive serine (McVey et al., 2001). An oriented immobilization conferring a different rigidity to the enzyme and distorting its structure has been studied and its effect on synthesis of β-lactam antibiotics (Fernandez-Lafuente et al., 1998; Tereni et al., 2001) or enantioselectivity (Rocchietti et al., 2002) was described. Mateo et al. (2007) concluded that the development of enzyme immobilization in a fully controlled fashion (i.e., via a particular area and conferring a desired rigidity) is a prerequisite for preparation of convenient catalyst.

2. Structure of bacterial PGA, enzyme maturation and mechanism of catalysis

2.1. Structure and enzyme maturation

The data on the expression of the structural *pga* gene and post-translational processing of PGA precursor were obtained predominantly from studies on the PGAs of enterobacteria: the enzymes share an αββα sandwich structural motif and accumulate in the periplasmic space as heterodimers. To reach this stage of a mature, properly folded enzyme, the inactive prepropeptide consisting of a signal peptide, small α-subunit, propeptide and large β-subunit has to undergo a rather complex processing (Brannigan et al., 1995). Processing of the enzyme precursor in *E. coli* begins in cytoplasm where the nascent peptide is stabilized with Ca^{2+}. The signal sequence of the precursor is cleaved off during crossing the cytoplasmic membrane

and the β-subunit is then released by intra-molecular, autocatalytic cleavage in periplasmic space (Ignatova et al., 2005). Intermolecular, sequential removal of the propeptide from proPGA generates the C-terminus of the α-subunit. The PGA from Gram-positive bacteria such as *Bacillus megaterium* or *Arthrobacter viscosus* is secreted to the growth medium on crossing the cytoplasmic membrane (Chandel et al., 2007). Mature enzyme may be found in cytosol (Xu et al., 2005), in the periplasmic space of Gram negative bacteria (Daumy et al., 1986) or secreted into the medium by Gram positive bacteria (Zhou et al., 2003b).

2.2. Diversity of prepropeptides and α- and β-subunits of PGAs

The number of AA residues in the majority of bacterial PGA prepropeptides exceeds 800 residues. The prepropeptide of metagenomic PGA PAS2, is the largest one and consists of 874 AA. The identity of the prepropeptide from *E. coli* with those of other representatives of the family *Enterobacteriaceae* is very high: over 99% and about 83% with *Shigella* sp. and *Kluyvera cryocrescens*, respectively, and about 61% with *Providencia rettgeri*. It shows only about 50% identity with the prepropeptide identified in the genus *Achromobacter* and less than 26% identity with those from representatives of Gram positive genera *Arthrobacter* and *Bacillus*.

Similar results regarding the level of protein identity were found for α-subunits (the length slightly above 200 AA residues) from different bacteria. The β-subunit (about 550 AA residues) seems to be rather conserved in length and exhibits high homology.

Table 3 shows the list of sequenced prepropeptides of bacterial PGAs and comparison of their sizes with those of mature heterodimers in terms of the number of AA residues.

2.3. Catalytic site of PGA

An active site of PGA^{Ec} consists of two regions which are involved in enzyme catalysis (Basso et al., 2002b): 1. aminic subsite preferring hydrophilic groups including catalytic AA residuum ser1β, and 2. acyl binding subsite which accepts hydrophobic groups (Duggleby et al., 1995). A detailed mechanism of hydrolysis of penicillin G or synthesis of β-lactam antibiotics involving formation of tetrahedral intermediates was reviewed by Giordano et al. (2006). The same tetrahedral intermediates are also formed between the nucleophile, the acyl donor and catalytic ser1β in enantioselective resolutions.

Amino acid residues ser1β, gln23β, ala69β and asn241β (Oh et al., 2004) are recognized as essential amino acid residues of aminic subsite in the eleven PGAs so far characterized (Table 3). It is proposed that the general topology and the quarternary structure of the active site might be conserved throughout the PGA family (Barbero et al., 1986).

Slight variations in amino acid residues are evident in the acyl binding subsite that is formed in *E. coli* by met142α, arg145α, phe146α, phe24β, thr32β, pro49β, val56β, trp154β, and ile177β (Oh et al., 2004; van der Laan et al., 1999). The acyl binding site is responsible for the substrate specificity of the enzyme and the AA residues located on α-subunits of PGAs are conserved among all representatives of families *Enterobacteriaceae* (including PAS2 (Gabor et al., 2004) from uncultured γ-*Proteobacterium*) and *Alcaligenaceae*. PGAs from the representatives of Gram positive genera *Arthrobacter* and *Bacillus* lack the residue arg145α and PGA of *A. viscosus* also shows the replacement phe146α → leu.

As for the amino acid residues of β-subunit, PGAs from the representatives of Gram positive genera *Arthrobacter* and *Bacillus* bear the replacements in all AA positions (phe24β → VAL, thr32β → leu, pro49β → phe, val56β → met, trp154β → tyr and ile177β → leu), while AA residues in Gram negative bacteria are unchanged except for the replacement val56β → leu in genera *Achromobacter*, *Alcaligenes*, *Providencia* and γ-*Proteobacterium*.

The AA residues involved in interaction with Ca^{2+} ion are rather conserved among the PGAs. Usually six AA residues are complexing a Ca^{2+} ion which results in a stable conformation of the mature enzyme (glu152α; asp/asn336β; val/thr338β; asp339β; pro/ile/glu/asp468β and asp/glu515β) (Duggleby et al., 1995; Kasche et al., 2003; McDonough et al., 1999).

So far, the crystal structures of three PGAs have been determined: PGA from *E. coli* (24 structure variants) (Alkema et al., 2000, 2004; Done et al., 1998; Hewitt et al., 2000; Kasche et al., 2003; McVey et al., 2001), *Alcaligenes faecalis* (2 variants) (Varshney et al., 2012) and *P. rettgeri* (McDonough et al., 1999).

Detailed knowledge of the crystal structure of PGA^{Ec} and positions of AA residues involved in substrate binding and catalysis revealed the aminic subsite of PGA with a wide tolerance to substrates while the pocket for the acylic moiety is highly selective (Massolini et al., 2008; Fig. 1).

3. Enantioselectivity of PGA

According to the IUPAC recommendations, the preferential formation of one stereoisomer over another is an enzyme characteristic called stereoselectivity. When the stereoisomers are enantiomers, the phenomenon is called enantioselectivity (Moss, 1996). PGA was found to catalyze two principal reactions: enantioselective reverse hydrolysis and enantioselective hydrolysis.

Table 3
Diversity of prepropeptides of PGA and subunits α and β of enzyme mature form.

Source	Family	Accession no.	Size[a] (AA)	MW[a] (Da)	α (AA)	β (AA)	References
Achromobacter sp. CCM 4824	*Alcaligenaceae*	NCBI ID: AAY25991	863	95036.5	230	557	Plháčková et al. (2003), Škrob et al. (2003)
Achromobacter xylosoxidans	*Alcaligenaceae*	NCBI ID: AAP20806	843	92911	229	557	Cai et al. (2004)
Alcaligenes faecalis	*Alcaligenaceae*	NCBI ID: AAB71221	816	92233	202	551	Verhaert et al. (1997)
Arthrobacter viscosus	*Micrococcaceae*	NCBI ID: AAA22077	802	92112	208	537	Konstantinovic et al. (1994)
Bacillus badius	*Bacillaceae*	NCBI ID: AAZ20308	805	92519	212	538	Rajendran and Gunasekaran (2007)
Bacillus megaterium	*Bacillaceae*	NCBI ID: AAB41343	802	91987	208	537	Kang et al. (1991), (1994)
Escherichia coli	*Enterobacteriaceae*	NCBI ID: AAA24324	846	94670	209	557	Oh et al. (1987)
γ-*Proteobacterium* (uncultured, eDNA)	Unclassified (environmental sample)	NCBI ID: AAS87335	874	96849	229	555	Gabor et al. (2004), (2005)
Kluyvera cryocrescens	*Enterobacteriaceae*	UniProtKB/Swiss-Prot: ID: P07941	844	93571	209	555	Barbero et al. (1986)
Providencia rettgeri	*Enterobacteriaceae*	PIR ID: A56681	837	94372.5	205	553	Ljubijankic' et al. (1992)
Shigella sp. D9	*Enterobacteriaceae*	NCBI ID:ZP_08393531	846	94494	209	557	Ward et al. (2009)
Thermus thermophilus HB27	*Thermaceae*	NCBI ID: YP_005941	776	87683	234	521	Torres et al. (2012)
Shuffled construct 6G8 *K. cryocrescens* × *E. coli*	*Enterobacteriaceae* derived	Not deposited	846	94379	209	557	Jager et al. (2007)
Synthetic construct	*Enterobacteriaceae* derived	NCBI ID: AAP13564	842	94730	207	553	Zhou et al. (2003a)

[a] Prepropeptide.

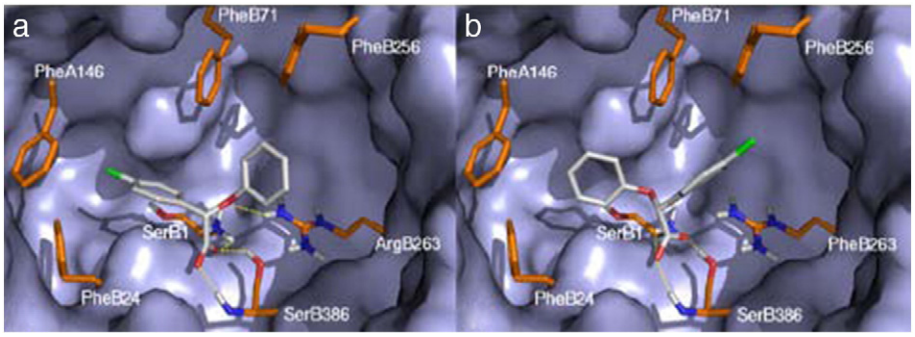

Fig. 1. Binding mode of *(S)*-phenyloxy-2-chlorphenylacetic acid (a) and *(R)*- phenyloxy-2-chlorphenylacetic acid (b) in active site of PGA (Massolini et al., 2008).

Molecular modeling of "enzyme–ligand" interactions (consisting of identification of energy-favorable binding sites on the protein molecule for a given ligand by the computational method GRID (Goodford, 1985), "in silico" docking experiments and the data processing by the multivariate chemometric method PCA) that occur in the active site of an enzyme, was used to understand molecular mechanisms of enzyme enantioselectivity. This computational method is a matter of choice for 1) understanding experimentally obtained results in enantioselective studies, 2) anticipation of the changes in enzyme selectivity after modification of the enzyme structure and 3) prediction of enzyme selectivity for further substrates and novel xenobiotics (Braiuca et al., 2006; Kazlaukas, 2000).

Pioneering molecular modeling experiments with PGA were performed by Basso et al. (2002b) who modeled unstable transient states of binding of the substrate in the active site of the enzyme. The resulting tetrahedral intermediate consists of the reactive carbonyl carbon of the phenyl acetic residue (acyl donor), the O^{γ} of the catalytic AA residue ser1β and the amino group of the L-phenylglycine methyl ester (nucleophile) (Fig. 2).

The method of molecular modeling was used to explain different selectivities of PGA^{Ec} and PGA^{Pr} (Basso et al., 2002a; Braiuca et al., 2004) or PGA^{Af} and PGA^{Ec} (Braiuca et al., 2003). In the latter case, a 3D-model of the crystal structure of PGA^{Af} was constructed by means of homologous modeling based on crystal structures of PGA^{Ec} and PGA^{Pr}. The model crystal structure revealed the narrower cleft of PGA^{Af} which may enhance the enantioselectivity towards aminoesters (Braiuca et al., 2003). The 3-D crystal structure of PGA^{Af} was determined by Varshney et al. (2012) but its comparison with the model crystal of Braiuca et al. has not yet been done.

3.1. Enantioselective reverse hydrolysis (also known as asymmetric synthesis or enantioselective acylation)

In the presence of activated acyl donor and a racemic mixture of a nucleophile, PGA^{Ec} was highly (above 90%) enantioselective towards aromatic amino acids while with aliphatic amino acids the enantioselectivity was low. Models of transition-states of binding of D- or L- analogs of aromatic amino acid methyl esters in acylation reactions revealed the possibility of formation of an additional hydrogen bond between the carbonyl group of the ester of the L-enantiomer and arg263β. Therefore, complex energy of tetrahedral intermediate $E_{L\text{-tet}}$ is lower than $E_{D\text{-tet}}$. Moreover, molecular dynamic simulations showed that polar (hydrophilic) rather than hydrophobic interactions in the active site are mainly responsible for both the ligand affinity and PGA enantioselectivity in reverse hydrolyses of racemic mixtures of these aromatic compounds (Basso et al., 2002b). Complex energies of tetrahedral intermediates of D- and

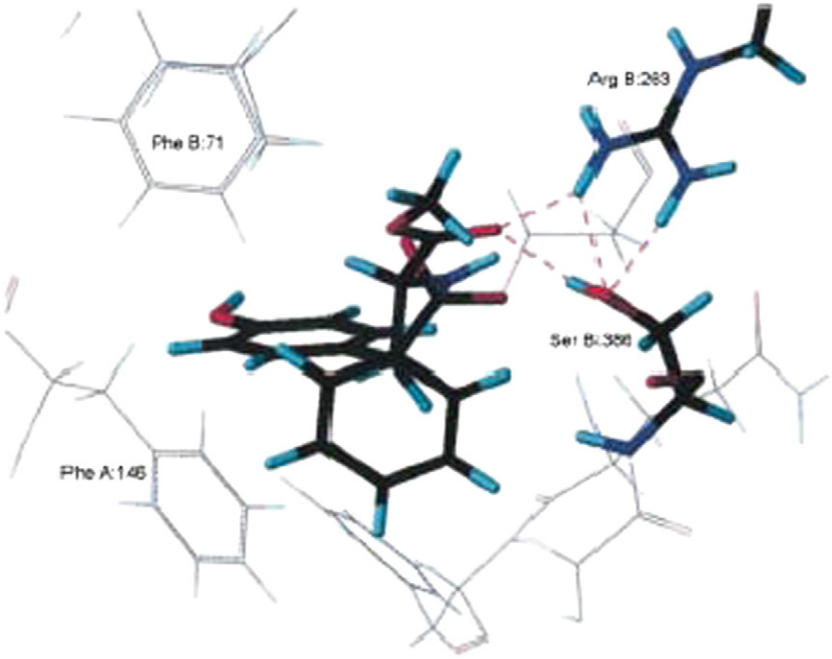

Fig. 2. Structure of the tetrahedral intermediate with 4-hydroxyphenylacetic derivative and L-phenylglycyl methyl ester (PhGlyOMe) focused on AA residues in the aminic subsite. The red dotted lines represent the net of H-bonds net among the carbonyl oxygen of PhGlyOMe, argβ263 and serβ386 that stabilize the intermediate (Basso et al., 2002a).

L- esters of aliphatic amino acids are similar. Usually, a difference of 5 kcal/mol in the complex energies is considered as the basis of enantioselectivity (Ke et al., 1998).

3.2. Enantioselective hydrolysis

Molecular basis of enantioselective hydrolysis catalyzed by PGA has been studied by combining enantiomeric separation on chiral stationary phase and molecular modeling (Massolini et al., 2003).

A stationary chiral phase based on immobilized PGA was used for the resolution of a mixture of 35 racemates (2-aryloxyalkanoic acids, their isosteric analogs and 2-arylpropionic acids). It was concluded that enzyme enantioselectivity decreases with increasing dimension of substituents both on benzene ring (A) and stereogenic center (R′). On the other hand, enantioselectivity increases if additional aromatic ring (R′) is a substituent of the stereogenic center (Fig. 3). This study confirmed the importance of hydrophobic interactions for chiral recognition of compounds by PGA (Calleri et al., 2002).

The experimental results of chiral separations were also elucidated by molecular modeling. Superimposing and analysis of geometry of *(R)*- and *(S)*- p-chloro-phenoxyalkanoic acid methyl esters showed that tetrahedral intermediate of *(R)*-enantiomer has the methyl group localized in a shallow pocket formed by residues phe24β, gln23β and pro22β. On the other hand, the methyl group of *(S)*-enantiomer is easily accessible by hydrophobic pocket created by side chains of AA residues phe71β, phe146β and ala69β, which results in better access of the molecule to the active center. Because of the spatial arrangement of the active site, the methyl group in this configuration does not disrupt catalysis. Therefore, Π-electron planar systems (e.g., aromatic rings) are preferred in this position as substituents. This enables the "stacking" of substituent planar ring to side chains of phe71β and phe146β on the basis of hydrophobic interactions (Massolini et al., 2008; Fig. 4).

Calleri et al. (2004) investigated enantioselective hydrolysis of nine racemic mixtures of methyl esters of 2-aryloxyalkanoic acids and isosteric analogs in reactions catalyzed by immobilized PGA of *E. coli*. They confirmed their experimental findings by molecular modeling of transition states of enzyme–substrate complexes and showed that the formation or collapse of the tetrahedral intermediate formed by Ser1β attack on carbonyl group is the ground of PGA selectivity towards the substrates.

Molecular modeling experiments of Lavecchia et al. (2007) confirmed in the active site of PGA^{Ec} the electrostatic interactions of residues ser1β, ser386β and arg263β with *(S)*-enantiomers of 2-aryloxyalkanoic acids. The interactions among arg263β and *(R)*-enantiomers are missing, which leads to the enzyme preference towards *(S)*-enantiomers at enantioselective hydrolyses.

Guncheva et al. (2004) carried out kinetic studies and molecular modeling concerning PGA enantioselectivity with a series of phenylacetylarylamides. A higher PGA hydrolytic activity was demonstrated towards these substrates substituted simultaneously with NH_2 and COOH groups. If only one functional group is present, the substrate binding to the enzyme becomes weaker. Furthermore, the carboxyl group must be located in the substrate molecule in the same way as in the case of nitrophenyl-*p*-amino benzoic acid (NIPAB), a substrate used for chromogenic assay of PGA activity.

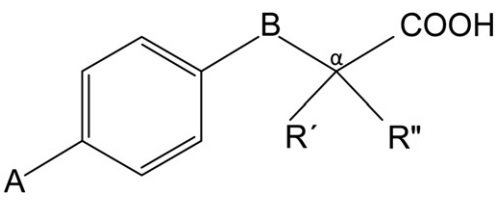

Fig. 3. General scheme of 2-arylalkanoic acids.

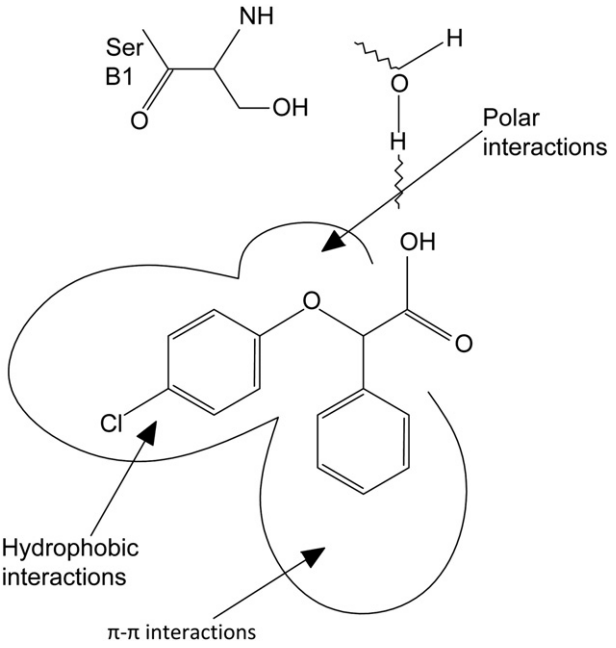

Fig. 4. Ligand–enzyme interactions involved in active site of PGA for acidic compounds (Massolini et al., 2008).

PGA^{Ec}-catalyzed hydrolyses of 2-aryloxy-2-arylacetic acids and their thioester derivates were studied by Massollini et al. (2006). They investigated the effect of different electron-donating or -withdrawing substituents of phenoxyl and phenyl rings and bulkiness of substituents on stereogenic center on enantioselectivity. It was expected that electrostatic interactions among polar groups of the enzyme molecule and carboxyl group or ethereal oxygen of the substrate contribute to stabilization of the enzyme–substrate complex and, consequently, improve enzyme enantioselectivity. This was observed with electron-withdrawing substituents while electron-donating substituents reduced the enantioselectivity. Temporini et al. (2007) studied the enantioselectivity of PGA with the same substrates but differing in the distance of phenyl ring from the stereogenic center owing to the introduction of alkyl residue. PGA exhibited the highest enantioselectivity towards *(S)*-enantiomers of the substrates with alkyl residue formed by two carbon atoms. PGA enantioselectivity towards aryl-substituted substrates was reviewed by Massolini et al. (2008).

While the enantioselective acylations and enantioselective hydrolyses catalyzed by PGA^{Ec} were extensively studied, the stereospecificity of the enzyme towards newly described xenobiotics, precursors of important chiral synthons, still has to be elucidated (Massolini et al., 2003). Studies on enantioselectivity of other bacterial PGAs are scarce although different stereospecificities can be predicted from the diversity of PGAs (Table 3).

4. Application of PGA enantioselectivity for production of pure chiral compounds

Many synthons or active pharmaceutical ingredients (APIs) are chiral compounds. Although enantiomers have identical chemical and physical properties in achiral environments (except for the rotation of polarized light plane) they behave differently in a chiral, biological environment. It was found that critical physiological processes show 100% stereoselectivity. In these terms, exogenously supplied enantiomerically-pure drugs may exhibit diverse interactions with chiral targets such as enzymes, receptors and ion channels (Aboul and Aboul, 1997).

76% of new active substances launched on the market in 2000 was enantiomerically pure substances and in 2001 enantiomerically pure

substances represented 36% of the total amount of APIs available on the market (Massollini et al., 2006). Around 2007, the percentage of enantiomerically pure drugs introduced to the market represented 75% (Lavecchia et al., 2007). Biocatalysis leading to production of building blocks for enantiopure drugs on industrial scale has been reviewed by Solano et al. (2012).

Enantioselective reverse hydrolysis and enantioselective hydrolysis may be used for preparation of pure chiral compounds with industrial potential from racemic mixtures. Both reactions may be performed in water phase alone, supplemented with co-solvents and ionic liquids (ILs), or in organic solvents alone.

The following chapters are focused on applications of PGA enantioselectivity and promiscuity that were described in the last decade and have an industrial potential. The overwhelming majority of applications were described with immobilized PGA of *E. coli*, a robust catalyst convenient for biocatalyses under harsh conditions in organic solvents. In a few cases, immobilized PGAs from the bacteria *A. faecalis*, *A. viscosus*, and *K. citrophila* were also used in enantioselective resolutions.

Different groups of chiral compounds that were the subjects of research into enantioselectivity of PGAs are shown in Fig. 5.

4.1. Enantioselective reverse hydrolysis

4.1.1. Enantioselective reverse hydrolysis (enantioselective acylation) in water phase

Since Zmijewski et al. (1991) reported that PGA is a suitable catalyst for carrying out kinetically controlled acylation in resolution of racemates, the PGAs of two bacterial species, i.e. *E. coli* and *A. faecalis*, were used in these reactions. Only the PGA^{Ec} was used by three research groups as a free enzyme (Chilov et al., 2003; Deaguero et al., 2010, 2012; Topgi et al., 1999).

PGA was applied in the resolution of a racemic mixture of (*RS*)-phenylglycinonitrile to *(R)*-phenylglycinonitrile, an important intermediate for the synthesis of antibiotics (Chilov et al., 2003) and on racemic mixtures of α-amino acids, intermediates for the production of semi-synthetic β-lactam antibiotics, agricultural chemicals and other new drugs (Kim and Lee, 1996). Recently, aqueous solutions of racemic mixtures of glutamate and glutamine were also resolved by PGA with a high degree of enantiomeric excess (ee) while the racemic mixtures of aspartate, asparagine and serine were resolved with a lower degree of ee. However, these asymmetric syntheses of enantiomerically pure amino acids have never led to an economically viable process (Carboni et al., 2006).

Topgi et al. (1999) used PGA^{Ec} to synthesise *(S)*-enantiomer of ethyl 3-amino-4-pentynoate. The enantiomer was obtained after resolution of trimethylsilylated racemic mixture of ethyl 3-amino-4-pentynoate by immobilized PGA^{Ec}. *(S)*-enantiomer, an important chiral synthon for syntheses of bioactive pseudopeptides (xemilofiban, an anti-platelet agent), was prepared with high reaction yield and $ee_{(s)}$. Landis et al. (2002) optimized this enantioselective resolution with different acyl donors and racemic mixtures of nucleophiles. When they used an unprotected racemic mixture of ethyl 3-amino-4-pentynoate and phenyl acetic acid, the initial rate of reaction was higher. The same reaction yield and $ee_{(s)}$ were obtained.

Gong et al. (2011) studied enantioselectivity of PGA^{Af} with racemic mixtures of phenylalanine and its *p*-substituted derivatives. Pure D-enantiomers of these compounds are important building blocks used for syntheses of antiviral, antidiabetic, analgesic and other drugs. D-enantiomers of *p*-substituted phenylalanine derivatives were obtained with high ee.

Deaguero et al. (2012) sought to improve the diastereoselectivity of PGA^{Ec} towards *(R)*-phenylglycine methyl ester, an acyl donor for kinetically controlled synthesis of ampicillin, via saturation mutagenesis based on the spatial arrangement of *(R)*-enantiomer in active site of PGA. The enzyme with AA substitution Phe24βAla exhibited improved diastereoselectivity (*de*) of 98% towards *(R)*-phenylglycine methyl ester in comparison to the wild type PGA^{Ec}.

4.1.2. Enantioselective reverse hydrolysis in two-phase water systems or organic phase

In the presence of organic solvents PGA accepts various L-amino acids as substrates. In the mixture of toluene and equimolar amounts of acyl donor and both enantiomers of the nucleophile, PGA^{Ec} catalyzed the resolution of aromatic amino acids with high ee while this

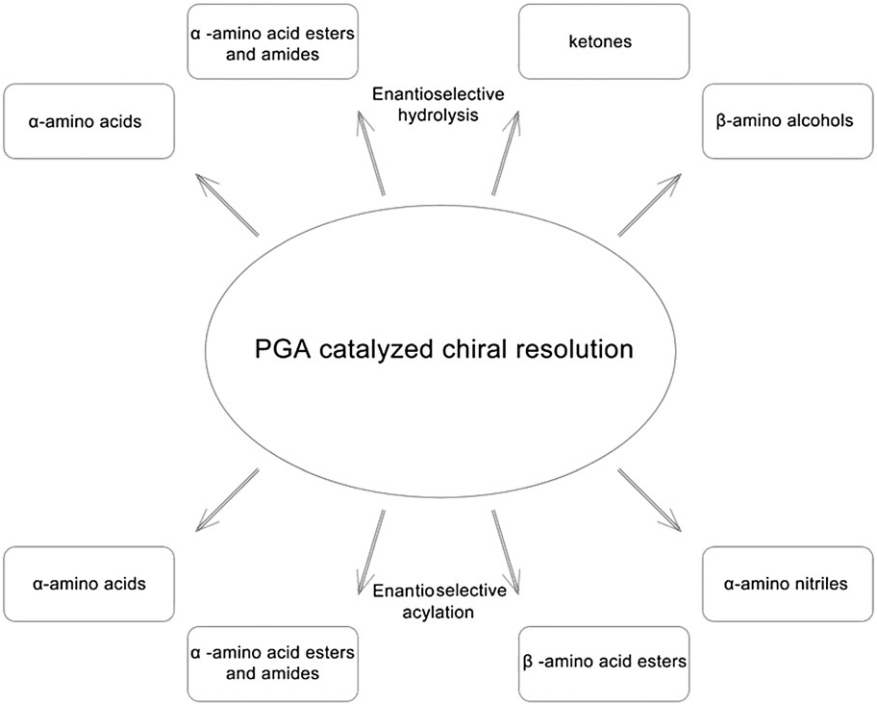

Fig. 5. Scheme of PGA-catalyzed chiral resolutions.

parameter for aliphatic amino acids was significantly lower (Basso et al., 2002b).

Basso et al. (2000) showed that PGA^{Ec} catalyzed enantioselective acylation of L-enantiomers of 4-phenylglycine methyl ester and 4-hydroxyphenylglycine methyl ester in organic solvents such as toluene and dichloromethane. Pure D-enantiomers, used as acyl donors in syntheses of β-lactam antibiotics, may be obtained with $ee_{(s)}$ of 98%.

PGA^{Ec} also catalyzes resolutions of racemic mixtures of β-aminoesters to pure *(R)*-β-aminoesters, while the *(S)*-enantiomers undergo the acylation. (*R*/*S*)-methyl 3-amino-3-(4-methoxyphenyl)propionate seems to be the best substrate among the tested β-aminoesters. The reaction mixture contained toluene and water in the ratio of 98:2 and the reaction was totally enantioselective (Roche et al., 1999).

4.2. Enantioselective hydrolysis

These reactions were catalyzed by PGAs from *E. coli*, *A. faecalis*, *A. viscosus*, and *K. citrophila* and the publications dealing with enantioselective hydrolysis are listed in Table 4. Four groups of chiral compounds subjected to enantioselective hydrolyses are shown in Fig. 5.

4.2.1. Enantioselective hydrolysis in water phase with co-solvents

4.2.1.1. Alpha-amino acids. Immobilized PGA^{Ec} was used to obtain enantiopure (*2S,3S*)-(+)-3-hydroxyleucine via enantioselective hydrolysis of N-phenylacetyl-3-hydroxyleucine racemic mixture. The product is an important substance for preparation of naturally occurring compounds cyclodepsipeptides, components of antibiotics containing peptide lactones as APIs. Reaction mixture consisted of acetonitrile and water in a 4:6 ratio and $ee_{(p)}$ equaled 99% (Fadnavis et al., 1997). Liu et al. (2006) used PGA^{Kc} for the preparation of L-*tert*-leucine by enantioselective hydrolysis of N-phenylacetyl-*tert*-leucine racemic mixture. D-*tert*-Leucine was prepared via acid-catalyzed hydrolysis of remaining D-enantiomer. Overall reaction yield of L-*tert*-leucine and D-*tert*-leucine was 80.6% and 83.1%, respectively, and ee of L-*tert*-leucine was 99%. Preparation of optically pure leucine derivatives is of great importance because these compounds can serve as chiral building blocks for pharmacologically active peptide components of anti-tumor or anti-AIDS drugs (Bomamrius et al., 1995). Fadnavis et al. (2008) used PGA^{Ec} to resolve a racemic mixture of 2-chlorophenylglycine and obtained *(S)*-enantiomer of 2-chlorophenylglycine, an important intermediate in the synthesis of antiplatelet agent Clopidogrel, with high ee.

4.2.1.2. Ketones. Kumaraguru and Fadnavis (2012) exploited PGA^{Ec} for resolution of a racemic mixture of 4-oxocyclopent-2-en-1-yl 2-phenylacetate in diisopropyl ether. Enantiomerically pure *(R)*-and *(S)*-4-hydroxy-2-cyclopentenones are versatile intermediates for the synthesis of a large number of complex natural products such as prostaglandins, prostacyclins, thromboxane and nucleosides. Chemoenzymatic synthesis of azasugars 1,4-dideoxyallonojirimycine and 1,4-dideoxymannojirimycine involves enantioselective hydrolysis of a racemic mixture of N-phenylacetyl derivative of homoserine lactone performed by PGA^{Ec} (Venkataiah et al., 2011). These compounds have potential use in a wide range of therapeutic strategies, including the treatment of viral infection (HIV), cancer, diabetes, tuberculosis, and lysosomal storage diseases, and as inhibitors of the growth of parasitic protozoa.

4.2.1.3. Amides and esters of acids. Rocchietti et al (2002) used PGA^{Ec} for preparation of *(R)*-mandelic acid derivatives which are chiral side-chains for preparations of 3′-functionalized cephalosporins. They immobilized the enzyme to agarose beads by glyoxyl multipoint or limited linkage. Moreover, they used agarose activated by glutaraldehyde and Eupergit C. Except for the latter support where the enantioselectivity

Table 4
Chiral resolutions catalyzed by PGAs: enantioselective reverse hydrolysis and enantioselective hydrolysis.

Author	Type of reaction	Racemic mixture	Enzyme form	Type of carrier	Product of industrial interest	Y (%), ee (%)
Chilov et al. (2003)	ERH	Phenylglycinonitrile	SE		N-PhAc-(*S*)-phenylglycinonitrile	E > 500 (towards *(S)*-enantiomer
Carboni et al. (2006)	ERH	Glutamic acid methyl ester Glutamine		DSM	D-Glutamate D-Glutamine	ee = 90% ee = 97%
Topgi et al. (1999)	EH	N-PhAc-3-Amino-(5-trimethylsilyl)-4-pentynoate ethyl ester	IE	(Rohm, Boehringer-Mannheim)	N-PhAc-(*S*)-3-amino-4-(5-trimethylsilyl)-pentynoate ethyl ester	
Gong et al. (2011)	ERH	α-Phenylalanine and its derivatives	IE	Oxirane acrylic beads	D-α-Phenylalanine and its derivatives	ee = 99%
Landis et al. (2002)	ERH	3-Amino-4-pentynoate ethyl ester	IE	Recordati	*(S)*-3-Amino-4-pentynoate ethyl ester	ee = 95%
Deaguero et al. (2012)	ERH	Phenylglycine methyl ester	SE		*(R)*-Ampicillin	de = 98%
Basso et al. (2002a,b)	ERH	α-AA methyl ester	IE	Roche	N-PhAc-L-Amino acid methyl ester	ee > 90% (aromatic AA)
Basso et al. (2000)	ERH	Phenylglycine methyl ester 4-Hydroxyphenylglycine methyl ester	IE	Celite R-640 Rods	N-PhAc-L-Phenylglycine methyl ester N-PhAc-L-4-Hydroxyphenylglycine methyl ester	ees = 98%
Roche et al. (1999)	ERH	β-aminoesters	IE	Chiro-CLEC	*(S)*-β-Amino esters	ee > 90%
Fadnavis et al. (1997)	EH	N-PhAc-3-Hydroxyleucine	IE		(2*S*, 3*S*)-3-Hydroxyleucine	ee = 99%
Liu et al. (2006)	EH	N-PhAc-α-*tert* -Leucine	IE	Amberzyme	L-*tert*-Leucine, N-PhAc-D-*tert* -leucine	ee(*s*) = 98.5%, ee(p) = 99%
Fadnavis et al. (2008)	EH	N-PhAc-2-Chlorophenylglycine	IE	Roche	S-α-ACAA[a]	ee > 99%
Cainelli et al. (1997)	EH	N-PhAc-3-Amino-azetidin-2-one	IE	Recordati	*(S)*-3-amino-azetidin-2-one	ee(*s*) = 94
Kumaraguru and Fadnavis (2012)	EH	N-PhAc-4-Oxocyclopent-2-en-1-yl 2-phenylacetate	IE	Epoxy activated carrier	*(S)*-4-hydroxycyclopent-2-en-1-yl 2-phenylacetate	E > 100
Venkataiah et al. (2011)	EH	N-PhAc-Homoserine lactone	IE	Epoxy activated carrier	*(S)*-(−)-α-N-phenylacetylamido-γ-butyrolactone	ee = 99%
Rocchietti et al. (2002)	EH	N-PhAc-Mandelic acid iso-propylamide derivatives	IE	Eupergit C, agarose gel beads different binding techniques	*(R)*-Mandelic acid	ee = 73%, Y = 47%
Yao and Lalonde (2003)	EH	Methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate		Roche, Bioscience	(4R)-Methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate	E > 50%, ee = 98%
Fadnavis et al. (2006)	EH	N-PhAc-3-amino-3-ethyl-1-propanol	IE	Roche	*(R)*-3-Amino-3-ethyl-1-propanol	ee > 99%, Y > 45%

[a] *(S)*-α-Amino-(2-chlorophenyl)acetic acid.

was reduced, the way of immobilization did not change the enantioselectivity when compared with free PGA. This is the one and only report dealing with the influence of the way of immobilization on enantioselectivity of PGA.

PGA^{Ec}-catalyzed enantioselective hydrolysis was used to prepare intermediates for the production of dioxolane nucleotides or synthons for the synthesis of natural compounds in high enantiomeric purity (Yao and Lalonde, 2003).

4.2.1.4. Alcohols. PGA^{Ec} immobilized on epoxy support also catalyzed enantioselective hydrolysis of a racemic mixture of N-phenylacetylated derivative of 3-amino-3-phenyl-1-propanol. *(S)*-enantiomer was hydrolyzed into *(S)*-amino alcohol of high enantiomeric purity (Fadnavis et al., 2006). Enantiomerically pure *(S)*-3-amino-3-phenyl-1-propanol is used as an important intermediate for the preparation of the drug Fluoxetine (Gao and Sharpless, 1988).

4.3. Application of PGA in protection and deprotection of reactive amino groups

Both enantioselective hydrolysis and enantioselective acylation can be used for the resolution of a racemic mixture of 1° amines. A racemic mixture of 1-phenylethylamine was resolved by soluble PGA^{Af} into *(S)*-enantiomer and acylated *(R)*-enantiomer. The latter was isolated and hydrolyzed by PGA^{Af} (a deprotection step) to get enantiopure *(R)*-1-phenylethylamine. Both enantiomers are used in the pharmaceutical industry (Guranda et al., 2004). These experiments confirmed the results of Švedas et al. (2002a,b) who compared enantioselectivities of PGA^{Ec} and PGA^{Af} with racemic mixtures of 1° amines and their N-PhAcetylated or N-PhGlycylated derivatives. They found much better enantioselectivity of PGA^{Af}.

PGA^{Ec} was used for the protection/deprotection of reactive groups of L-amino acid esters during the chemoenzymatic synthesis of biologically active dipeptides by Didžiapetris et al. (1991) and Švedas and Beltser (1998).

Biologically important peptides also contain D-amino acids. Carboni et al. (2004) performed for the first time PGA^{Ec}-catalyzed protection/deprotection of a series of esters of non-polar D-amino acids in an organic solvent. The ability of PGA to accept a D-enantiomer of a nucleophile was ascribed to modification of enantioselectivity of the PGA in the presence of non-aqueous medium.

The use of biocatalysis in protection/deprotection represents a "green" alternative to commonly used chemical processes using toxic and expensive chemicals (Kadereit and Waldmann, 2001).

4.4. Asymmetric, partial hydrolysis of a prochiral compound

Cabrera et al. (2007) used PGA^{Ec} to catalyze enantioselective hydrolysis of the prochiral compound diethyl or dimethyl phenylmalonate (diester of phenyl-malonic acid). Prochiral diester is asymmetrically transformed into just one of the two possible isomers, a chiral intermediate monoester (+)-methyl phenylmalonate. The final product, a prochiral diacid, has not been formed. The reaction proceeds up to 100% conversion (instead of the maximum of 50% in resolution of racemic mixtures) obviously for two reasons: the enzyme prefers the substrate over the product and the reaction is enantioconvergent. Although it is an interesting example of biocatalysis, the chiral monoester does not have any industrial application.

5. Exploitation of PGA in analytical devices

A high share of chiral compounds in the API market is the reason why pharmaceutical and biotechnological companies spent a considerable effort in developing a universal method for direct separation of pure enantiomers from racemic mixtures of chiral compounds. Chiral stationary phases based on immobilized enantioselective enzymes represent an efficient and rapid method for the separation of enantiomers. HPLC column bearing immobilized PGA^{Ec} as a chiral selector was developed and used as an enzyme reactor for the resolution of mixtures of racemates (Massolini et al., 2001).

The technique combines the advantages of capillary electrophoresis (e.g., low consumption of sample and solvent) with the possibility to test stationary phases of various chemistry for improving process selectivity. Moreover, linkage with mass spectrometry allows immediate identification of reactants. The review of Calleri et al. (2004) deals with the development and analytical applications of a PGA-based stationary phase.

Chiral resolution for 27 of the 35 racemates of acids by chiral stationary phase based on immobilized PGA was reported by Calleri et al. (2002). The most important parameter affecting enantioselectivity was the mobile phase because its composition controlled the retention order of the enantiomers. The same research group extended the applicability of PGA as a chiral selector towards 2-aryloxyalkyl- and 2-arylalkyl-2-aryloxyacetic acids, compounds having pharmacological importance (Temporini et al., 2007).

The way of PGA immobilization used in the development of a chiral stationary phase was studied by Gotti et al. (2006). They found that the enantioselective properties of a chiral selector did not change with the method of immobilization.

At present, the technique based on PGA covalently immobilized on a monolithic epoxy silica column represents a highly sensitive microseparation method convenient for quantitative analysis of pharmaceutical preparations (e.g., profens) and detection of impurities (Burns and May, 2003; Gotti et al., 2012).

The microseparation techniques discussed above are of great value for the evaluation of PGA enantioselectivity determination of the most preferred chiral compound among related compounds under given separation conditions. Using these techniques with PGAs of different bacterial origins as chiral selectors for the same set of racemates could be a convenient way to match the enzyme with the chiral process.

Another analytical application of PGA concerns hydrolytic activity of the enzyme. Rojanarata et al. (2010) developed a UV spectrophotometric method for the determination of amoxicillin in oral formulations. The method is fast, sensitive and inexpensive. Two enzymes are employed in the method: the D-4-hydroxyphenyl glycine side chain of amoxicillin cleaved off by PGA reacts with 2-oxoglutarate formed in a reaction catalyzed by D-phenylglycine aminotransferase. The product of the reaction, 4-hydroxybenzoylformate, was assayed spectrophotometrically. The method belongs among green analytical methods and could be useful for multi-assays performed in an autoanalyzer.

6. Synthesis of dipeptides

Conventional chemical synthesis of peptides requires donor and acceptor activation accompanied by protection of the reactive group under harsh conditions. Biocatalysis allows simple introduction of protecting, environment-friendly agents to reactive groups under mild conditions while the activation is still the matter of chemistry. Moreover, the number of protecting groups in biocatalysis can be significantly reduced and, in the case of very selective biocatalysts, no protecting groups are required. Proteases were reported as efficient catalysts for kinetically controlled syntheses of dipeptides with C^{α}-esterified amino acid as an activated acyl donor and N^{α},C^{α}-free amino acids as nucleophiles (Yokozeki and Hara, 2005). The examples of dipeptide syntheses discussed below (Table 5) were catalyzed by soluble or immobilized PGA^{Ec}.

Khimiuk et al. (2003) presented a chemoenzymatic synthesis of various dipeptides containing D-(−)-phenylglycine moiety from activated acyl donor D-(−)-phenylglycine amide and specific L-amino acid in reactions catalyzed by PGA in aqueous solution. This is the first step towards the synthesis of enantiomerically pure diketopiperazine, which can have potent cytotoxic (Boger and Zhou, 1993) and antitumor

Table 5
Non-enantioselective applications of PGA.

Reference	Type of reaction	Reaction substrate	Enzyme form	Type of carrier	Product of industrial interest	Y (%), ee (%)
Guranda et al. (2004)	Protection	(+/−)-1-Phenylethylamine	SE		*(S)*-1-Phenylethylamine	eep = 99.1%
	Deprotection	*(R)*-PhGly-*(R)*-1-Phenylethylamine[a]	SE		*(R)*-1-Phenylethylamine	eep = 99.1%
Carboni et al. (2004)	Protection	D-Amino acid esters	IE	Boehringer-Mannheim	N-PhAc-D-Amino acid esters	Y = 75–95%
	Deprotection	N-PhAc-D-Amino acid esters	IE	Boehringer-Mannheim	D-Amino acid esters	
Khimiuk et al. (2003)	PS	D-PhGlycine Amide + L-amino acid	IE	Assemblase	D-(−)-PhGly-L-amino acid	Y = 72–94%
van Langen et al. (2000)	PS	D-PhGlycine Amide + L-PhGlycine	IE	Assemblase	D-PhGly-L-phenylglycine	Y = 69%
	PS	L-PhGlycine methyl ester (2×)			L-PhGly-L-PhGlycine methyl ester	Y = 63%
Didžiapetris et al. (1991)	Protection	Amino acid esters + phenylacetic acid			N-PhAc-amino acid esters	
	Deprotection	N-PhAc-Peptide peptide				
Švedas and Beltser (1998)	Protection	Esters of various amino acids	SE		N-PhAc-amino acid esters	
Wu et al. (2005)	MAR	4-Nitroimidazole + vinylacetate	IE	Acrylic beads	4-Nitroimidazole derivative	Y = 74%
Wu et al. (2006)	MAR	Allopurinul + vinylester	IE (SE		Allopurinol derivative	
Liu et al. (2011)	TE	Guaifenesin + vinylacetate	IE	Acrylic beads	1-O-Acetylguaiphenesin	Y = 84%
Wang et al. (2010)	HR	4-Nitrobenzaldehyde + nitroethane	IE	Acrylic beads	1-(4-Nitrophenyl)-2-nitropropanol	

[a] *(R)*-Phenylglycyl-substituted *(R)*-1-phenylethylamine.

(Gomez-Monterey et al., 2008) properties. In this case, the application of PGA does not use the trait of enantioselectivity.

Van Langen et al. (2000) synthesised stereochemically pure phenylglycine dipeptides without any need for protecting groups using immobilized PGA^{Ec}. They used L-phenylglycine (nucleophile) and D-phenylglycine amide (acyl donor) or L-phenylglycine methyl ester as both nucleophile and acyl donor. In the former case, D-phenylglycyl-L-phenylglycine was prepared. When using the second pair of reactants, L-phenylglycyl-L-phenylglycine methyl ester was obtained. Both dipeptides in activated forms (as esters) undergo a ring closure to corresponding diketopiperazine (Purdie and Benoiton, 1973) that can be used as a synthon for fungicidal and antiviral agents (Svokos et al., 1971).

Unfortunately, both groups did not use racemate of the nucleophile and, therefore, the results do not testify to the enantioselectivity of the enzyme. The reactions represent a formation of the peptide bond between activated acyl donor and L-nucleophile.

Nucleophiles for the synthesis of dipeptides by biocatalysis are restricted to hydrophobic amino acids and processes for their productions still do not meet industrial requirements.

7. PGA as a promiscuous enzyme

The enzyme capable of catalyzing several chemical reactions with completely different reaction mechanisms (Wu et al., 2005) is designated as a promiscuous enzyme. Research into the promiscuity of PGA of *E. coli* has been carried out recently in connection with the production of nitroalkanols, modified APIs and N-heterocycle derivatives (Table 5).

Three very recent reactions differing in the mechanism of catalysis have so far been ascribed to PGA^{Ec}.

7.1. Markovnikov addition reaction

The reaction mechanism of Markovnikov addition consists of electrophile addition onto a double bond of a nucleophile.

Wu et al. (2006) studied the ability of PGA to catalyze Markovnikov addition with different vinylesters as substrates. It was found that the enzyme activity decreased with increasing length of vinyl ester chain and lower reaction yields of products were achieved with more sterically hindered vinylesters as reaction substrates.

The effect of different substituents on imidazole ring in the reactions with vinylesters was analogously studied. 4-Nitroimidazol used as the electrophile gave in these additions the highest yield. With pyrole, pyrazole and 1,2,4 triazole, the reaction yield was lower and the amount of the product formed in the reaction was decreasing in a descending order with 1,2,4 triazole, imidazole, pyrazole, and pyrole as electrophiles. Markovnikov additions were also studied with the electrophile uracil; however, in this case the reaction gave a very low yield even after prolonged reaction time (8 days).

PGA-catalyzed Markovnikov addition reaction with allopurinol (electrophile) was also tested (Wu et al., 2005). The product of the reaction was N-heterocycle derivative, which indicated a potential synthetic route for bioactive N-derivatives of heterocyclic compounds. Wu et al. (2005) suggested a hypothetical mechanism of Markovnikov addition reaction catalyzed by PGA, where serβ1 of active site of PGA plays a crucial role (Fig. 6).

7.2. Transesterification

Transesterification is a term used for reactions where an ester is transformed into another one through an interchange of the alkoxy moieties.

Transesterification activity of penicillin G acylase of *E. coli* was demonstrated by Lindsay et al. (2002) who studied the activation effect of nonbuffer salts on PGA catalyzing transesterification reaction of phenoxyacetic acid methyl ester with 1-propanol in hexane. The enzyme was found to be over 750-fold more reactive upon lyophilizing in the presence of KCl. The same group (Lindsay et al., 2003) later focused its effort on identification of the salt mixture used at lyophilisation of PGA and having the highest activation effect. The study revealed that the formulation consisting of 98% (w/w) of a salt mixture KAc:CsCl = 1:1, 1% (w/w) enzyme, and 1% (w/w) potassium phosphate buffer was 35 000-fold more active in hexane than the salt-free formulation. Thus, a remarkably active biocatalyst in hydrophobic media was obtained by lyophilizing an enzyme in the presence of nonbuffer salts.

Liu et al. (2011) reported the transesterification reaction of vinyl acetate with guaifenesin as a promiscous reaction of PGA. Guaifenesin, an API for the preparation of anxiolytics and central myorelaxants, and its structurally similar derivatives were used to study substrate binding into the active site of the PGA during transesterification reactions.

Although PGA has a broad substrate specificity (including substrates with phenyl acetyl or phenoxy acetyl moieties), it has no specificity for vinyl acetate. As 10% water solution, N-methylimidazole was found to modulate the enzyme activity and alter the catalytic ability of PGA from amidation to esterification. Liu et al. (2011) proposed that N-methylimidazole assisted the enzyme in ester bond formation. The presence of N-methylimidazole in active site of the enzyme may activate the substrate by enhancing the nucleophilicity of the hydroxyl group. FTIR spectra revealed enhanced B-sheet structure of PGA in solution containing N-methylimidazole instead of D_2O. In addition, docking simulations confirmed the influence of N-methylimidazole on the active site: Glnβ23 and Asnβ242 displayed minor rearrangements, whereas Serβ1 showed evident movement towards the center (Liu et al., 2011; Fig. 7).

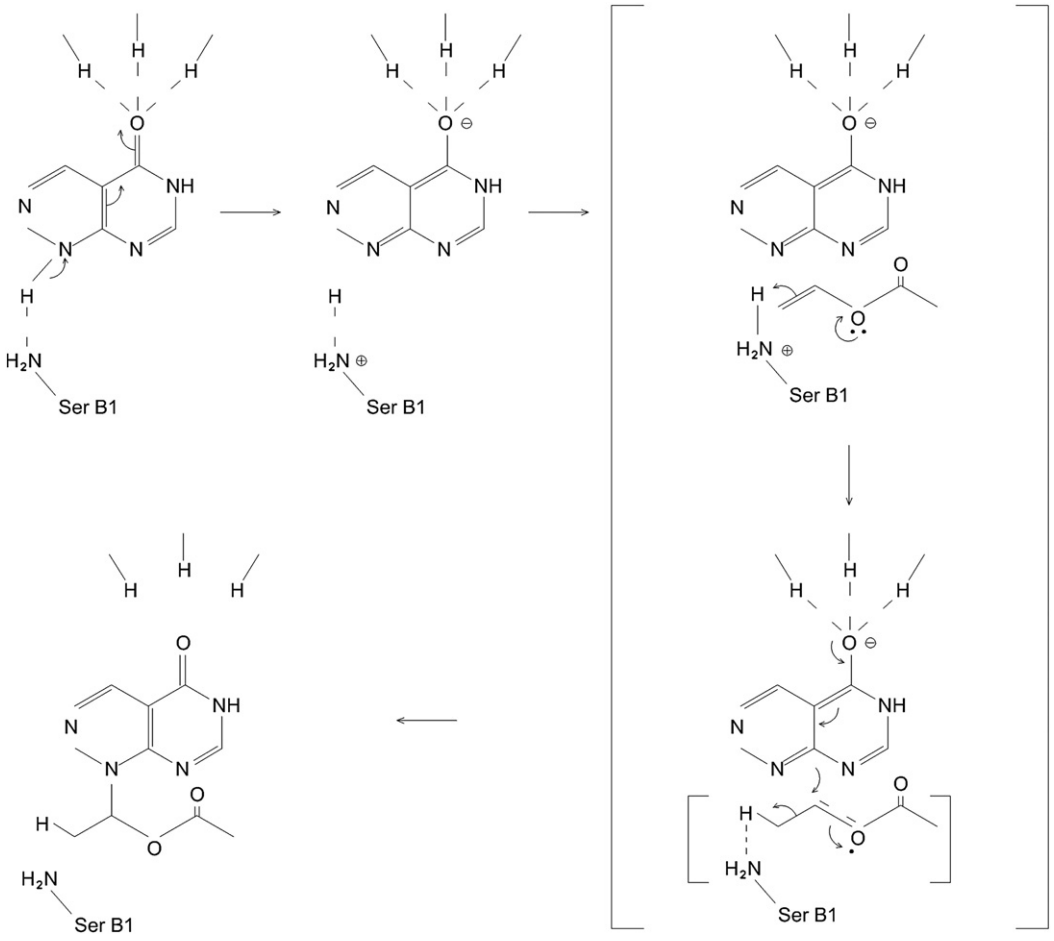

Fig. 6. Proposed mechanism of PGA-catalyzed Markovnikov addition reaction.

Guaifenesin was found to be the best substrate for PGA-catalyzed transesterification among the group of related APIs. Anyway, the productivity of a biocatalyst is very low. The pharmacological potential of 1-O-acetylguaifenesin as an API has yet to be evaluated.

7.3. Henry reaction

The reaction is known in organic chemistry and represents a formation of carbon–carbon bond in a reaction of nucleophilic nitroalkane with an electrophilic aldehyde or ketone. Products of this coupling are β-nitroalkanols that are useful for syntheses of intermediates for the preparation of many biologically active compounds, e.g. fungicides (Mikite et al., 1982). Biocatalytic approaches to Henry (Nitroaldol) reaction were reviewed by Milner et al. (2012). There are two biocatalytic approaches to enantio-enriched products of the Henry reaction reported in the literature: direct enzyme-catalyzed Henry reaction or initial chemical formation of the β-nitroalkanols followed by enzymatic kinetic resolution of the stereoisomers. Lipases, transglutaminases, hydroxynitril lyases and proteases are known to catalyze Henry reaction.

Wang et al. (2010) used three acylases to catalyze the synthesis of β-nitroalkanols in the presence of DMSO: PGA^{Ec}, Amano acylase from *Aspergillus oryzae* and D-aminoacylase from *E. coli*. The yield obtained with immobilized PGA was very low but still detectable. Among these enzymes, D-aminoacylase was the best catalyst of the direct enzyme-catalyzed Henry reaction.

Since the reactions were carried out under harsh denaturing conditions (DMSO, 50 °C), the role of DMSO itself in Henry reaction should be considered. In general, the effect of DMSO on the enzyme structure and its function differs according to physico-chemical traits of a protein (Johannesson et al., 1997) and co-solvent concentration (Roy et al., 2012). Wang et al. (2010) did not exclude that DMSO itself may affect a catalytic promiscuity of enzymes. Busto et al. (2011) observed that the Henry reaction can also occur through non-specific catalysis since BSA itself efficiently catalyzes this reaction.

As in promiscuous reactions discussed above, the productivity of PGA catalyst is very low and the reaction has not been evaluated in terms of enantioselectivity.

8. Exploitation of PGA-catalyzed N-deacylation in biosensors

Biosensor is defined as an analytical tool consisting of a biological component, a biosensing layer, and physical signal transducer. According to the mode of signal transduction, sensors are classified as electrochemical, optical, thermal and piezoelectric ones. Electrochemical sensors, in their turn, are divided into four types: potentiometric, amperometric, conductometric and ion-selective field-effect transistor sensors (Dzyadevich et al., 2006). Immobilized enzymes are especially used as biosensing components of electrochemical sensors that have a wide application in clinical diagnostics, food quality control, monitoring of in-process variables of fermentations in bioreactors or environmental sensing. A new role of biosensors is envisaged in screening processes aimed at discovering new drugs (Keusgen, 2002).

This review deals with electrochemical, optical, thermal and piezoelectric sensors.

More than half of the enzyme sensors reported are electrochemical biosensors (Hasanzadeh et al., 2012) that work on the following principle: a target analyte interacts with the biological part of the sensor (e.g., an enzyme immobilized on the surface of a pH-sensitive

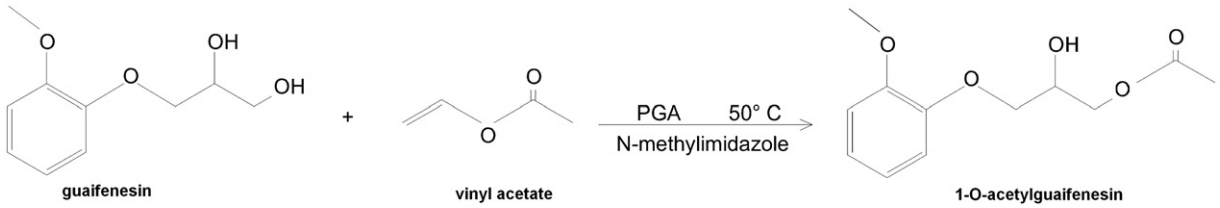

Fig. 7. Scheme of PGA-catalyzed transesterification reaction.

device, amperometric electrode or semiconductor structure) and the resulting biological signal (a charged product of enzymatic reaction) is converted into a physical signal by a transducer. The most frequently used immobilization techniques are enzyme entrapment and cross-linking methods (Poghossian et al., 2001). Potentiometric types of biosensors and those based on ion-selective field-effect transistors have attracted the attention of researches due to their small size, fast response time, the possibility of bulk production (Dzyadevich et al., 2006; Keusgen, 2002), and their development goes ahead very fast.

Liu et al. (1998) developed a biosensor for fermentation process control based on ion-selective transducer with field effect, the biological component of which was PGA. The response time of the sensor to different concentrations of penicillin G was 30 s. The biosensor showed high sensitivity and long-term operational stability when employed for off-line monitoring penicillin G in culture broth during fermentation (Liu and Li, 2000).

Analytical devices called enzyme thermistors (Mosbach et al., 1975) are used as sensors in flow meters for gases and liquids. The evolution of heat is a general property accompanying biochemical transformations and immobilized enzymes (as a thin film) serve as biorecognition elements for thermometric measurements. Yakovleva et al. (2013) developed sensitive enzyme thermistors based on immobilized PGA that were applied to determine penicillin G concentration ranging from 0.02 to 200 mM.

The development of an integral thermal biosensor array for the simultaneous determination of several analytes is currently of great interest for bioprocess monitoring: a single flow micro-channel is serially partitioned into several distinct reaction zones each containing one immobilized enzyme. It has been concluded (Yakovleva et al., 2013) that one of the attractive directions in future developments of biosensors is a hybrid sensor that combines different detection principles.

9. Concluding remarks

Prokaryotic PGAs exhibit the traits required of enzymes with high industrial potential: robustness in terms of temperature and pH stability, wide substrate specificity, and stereoselectivity. Some of these traits have already been exploited on industrial scale in bioprocesses for production of semisynthetic β-lactam antibiotics, others still wait for their biotechnological applications. No matter how much the structure of the mature enzyme is conserved among prokaryotic microorganisms, producers of novel PGAs have been isolated and described recently and further findings could be envisaged, especially if metagenome mining is employed to a larger extent. So far, about 14 structural genes encoding PGAs from prokaryotes have been sequenced so that the primary structures of enzymes are known.

Many synthons or active pharmaceutical ingredients (APIs) are chiral compounds. Although enantiomers have identical chemical and physical properties in achiral environments, they behave differently in chiral, biological environment. Therefore, the phenomenon of stereoselectivity has undoubtedly a high industrial potential.

The knowledge of crystal structure and interactions of AA residues involved in enzyme catalysis with the molecule of substrate is of paramount importance. Unfortunately, our detailed knowledge of enzyme–ligand interactions is limited only to three representatives of PGAs the crystals of which were prepared. Exact knowledge of the quartenary structure is important also for 1) modeling-based elucidation of the stereospecificity of the enzyme towards newly developed and described xenobiotics, precursors of important chiral synthons, and 2) development of methodologies permitting enzyme immobilization in a fully controlled fashion (i.e., via a particular area) and conferring a desired rigidity on the catalyst.

Crystal structure-based modeling of interactions of substrates with PGA will allow the prediction of the structures of substrates for PGA of industrial interest and, vice versa, selection of the optimal enzyme structure among different PGAs for a given stereoselective reaction. In this respect, microseparation techniques may be of great value for fast comparison of enantioselectivity of different PGAs. Using this technique with PGAs of different bacterial origins as chiral selectors for the same set of carefully selected racemates could be a convenient way to match the enzyme with the chiral process.

At present, experimental research into enantioselectivity of PGAs (i.e., enantioselective acylations and enantioselective hydrolyses) has been limited only to PGAs from four different microorganisms but the results confirmed that different stereospecificities can be anticipated for PGAs that differ in quarternary structures.

Asymmetric, partial hydrolysis of a prochiral compound is an interesting PGA-catalyzed reaction because it is enantioconvergent and conversion of substrate into chiral product equals 100% instead of the maximum of 50% in resolutions of racemic mixtures.

Modulation of enantioselectivity could be a useful tool for preparation of novel biologically active compounds. It should be stressed that there are only three reports on modulation of stereoselectivity of PGA: i) diastereoselectivity of PGA^{Ec} towards *(R)*-phenyl glycine methyl ester, an acyl donor for kinetically controlled synthesis of ampicillin, via saturation mutagenesis based on the spatial arrangement of *(R)*-enantiomer in active site of PGA (Deaguero et al., 2010, 2012). Modified enzyme exhibited improved diastereoselectivity towards *(R)*-phenyl glycine methyl ester and was used in kinetically controlled synthesis of β-lactam antibiotics using racemic mixture of activated acyl donor, ii) the way of immobilization of PGA on Eupergit C (Rocchietti et al., 2002) reduced the enantioselectivity in comparison with free enzyme, and iii) PGA-catalyzed amidation in water phase was changed into esterification in organic solvent (Liu et al., 2011). This type of stereoselectivity modulation requires a detailed explanation because esterification catalyzed by PGA is not enantioselective and could be considered as promiscuous reaction. Carboni et al. (2004) hypothesized that a non-aqueous medium may increase PGA enantioselectivity towards D-α-amino acids. However, they did not perform resolution of racemate in non-aqueous medium.

Promiscuity of PGA has been discovered only recently. All promiscuous reactions are slow, they require long reaction time to obtain small quantities of the products. They might become interesting for pharmaceutical industry when the enzyme is subject to direct enzyme evolution or reaction conditions are optimized. Since the crystal structure of PGA is known, molecular modeling could be a tool of choice to increase the efficiency of PGA in catalysis of these reactions. Instead of directed enzyme evolution, PGAs from other bacteria could be evaluated for their promiscuity. So far, the phenomenon of promiscuity has been studied solely with PGA^{Ec}.

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Resolution of α/β-amino acids by enantioselective penicillin G acylase from *Achromobacter* sp.

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Abstract:
Penicillin G acylases (PGAs) are enantioselective enzymes catalyzing a hydrolysis of stable amide bond in a broad spectrum of substrates. Among them, derivatives of α– and β-amino acids represent a class of compounds with high application potential. PGA^{Ec} from *Escherichia coli* and PGA^{A} from *Achromobacter* sp. CCM 4824 were used to catalyze enantioselective hydrolyses of seven selected N-phenylacetylated α/β-amino acid racemates. The PGA^{A} showed higher stereoselectivity for three enantiomers of N-PhAc-β-homoleucine, N-PhAc-α-*tert*-leucine and N-PhAc-β-leucine. To study the mechanism of enantiodiscrimination on molecular level, we have constructed a homology model of PGA^{A} that was used in molecular docking experiments with the same substrates. *In-silico* experiments successfully reproduced the data from experimental enzymatic resolutions confirming validity of employed modeling protocol. We employed this protocol to evaluate enantiopreference of PGA^{A} towards seven new substrates with application potential. For five of them, high enantioselectivity of PGA^{A} was predicted for.

Keywords: Penicillin G acylase, enantioselectivity, homologous model, docking experiments, α/β-amino acid

Abbreviations: N-phenylacetyl amino acid (N-PhAc-amino acid)

1. Introduction

Penicillin G acylases (EC 3.5.1.11; PGA, penicillin amidohydrolase) are robust industrial catalysts routinely used for decades for biotransformation of penicillins and cephalosporins [1-4]. Their biocatalytic potential expanded tremendeously once PGAs were found to be enantioselective and promiscuous [5]. Syntheses of semisynthetic β-lactam antibiotics, peptide syntheses and resolutions of racemic mixtures *via* enantioselective acylation or hydrolysis of broad range of substrates (e.g., amino acids, ketones, amino acid esters or amides, amino nitriles or amino alcohols) catalyzed by PGAs represent biocatalyses with high potential especially for pharmaceutical industry. The PGAs are heterodimers composed of α- and β-subunit and belong to the structural superfamily of N-terminal nucleophilic hydrolases (Ntn hydrolases). Recent resolutions of three dimensional PGA crystal structures provided detailed insight into the active site of this enzyme and allowed better understanding of a catalytic mechanism. An active site of PGA^{Ec}

consists of two regions [6]: 1. aminic subsite preferring hydrophilic groups including catalytic aa residues Ser1ß, and other essential amino acid residues Gln23ß, Ala69ß and Asn241ß and 2. acyl binding subsite which accepts hydrophobic groups [7] consisting of Met142α, Arg145α, Phe146α, Phe24ß, Thr32ß, Pro49ß, Val56ß, Trp154ß and Ile177ß. Catalytic mechanism of hydrolysis of penicillin G, synthesis of ß-lactam antibiotics or enantioselective resolution involves the nucleophilic attack of the O^{γ} hydroxyl group of Ser1ß on the carbonyl carbon of the amide bond [8-9]. As the consequence of the attack, a covalent intermediate of an acyl-enzyme complex *via* a tetrahedral transition state is formed. Asn241ß and Ala69ß form the "oxyanion hole" that balances the negative charge and thus lowers the energy of the reactive tetrahedral intermediate. Gln23ß is also shown to interact with the nucleophilic part of a substrate and contribute to the stabilization of the tetrahedral intermediate.

The PGA^{A} from the mutant strain *Achromobacter* sp. CCM 4824 [10] has been firstly characterized by Škrob et al. [11]. Molecular mass of α-subunit and β-subunit is 27.0 and 62.5 kDa, respectively. The results showed that the enzyme is more efficient biocatalyst in comparison with PGA^{Ec} as regards syntheses of semi-synthetic β-lactam antibiotics. Its potential for kinetically controlled syntheses of semisynthetic β-lactam antibiotics has been shown recently [12] and has already been used at industrial scale by Fermenta Biotech Ltd.

Here we present results of the research into enantioselectivity of PGA^{A}. First, the enantioselective resolutions of racemic mixtures of α/β-amino acids having considerable potential for pharmaceutical applications were determined experimentally. Then we describe a construction of a homology model of PGA^{A} and consequent molecular docking experiments employed to understand molecular basis of PGA^{A} enantioselectivity. Experimentally validated model of the enzyme was used to predict PGA^{A} enantioselectivity towards new, hitherto non-investigated substrates.

2. Experimental

2.1. Microorganisms and culture conditions

Recombinant strain *E. coli* BL21(pKX1P1) and *E. coli* RE3(pKA18) was used to prepare biomass for purification of PGA^{A} and PGA^{Ec}, respectively. Fed batch cultures of the strains in a stirred bioreactor were described earlier [13-14].

2.2. Enzyme purification and hydrolytic activity assay

PGA^{A} was purified as described by Škrob et al. [11] and PGA^{Ec} according to Kutzbach and Rauenbusch [15]. Purified PGA^{Ec} and PGA^{A} has the specific activity of 60 and 50 U/mg protein, respectively. The activity of 1 unit (U) was defined as the amount of an enzyme producing 1 µmol of phenyl acetic acid per minute from PenG (2% w/v) in 0.05 M sodium phosphate buffer (pH 8.0) at 37 °C.

2.3. Chemical synthesis of N-phenylacetyl-amino acid racemic mixtures

Phenylacetyl chloride (0.044mol) was added dropwise to 100 mL of NaOH solution (10 %, w/v) of racemic mixture of an amino acid (0.04mol) kept on ice bath. The reaction mixture was acidified to pH 2 with 6M HCl, and N-phenylacetyl amino acid (N-PhAc amino acid) was extracted three times with ethyl acetate and recrystallized from the ethyl acetate solution. Purity of the product was determined by HPLC using C-8 reverse phase column. Eluent consisted of H_2O (containing 0.1 % TFA) : acetonitrile in ratio 7 : 3. For more details on products yields, chemical shifts in the ^{1}H-NMR and ^{13}C-NMR spectra and data from MS analyses see Supplementary data (Fig. S1-S7).

2.4. Enantioselective hydrolysis of N-PhAc-amino acid racemic mixtures

The reactions were carried out in a pH-stat at 30 °C under continuous stirring. A water solution (25 mL) containing 0.025 M N-PhAc-amino acid racemic mixture was incubated at pH 7.5 for 30 min. 50U of PGA^{Ec} or PGA^{A} were added to the reaction mixture and the pH was maintained at 7.5 by titration (2M NH_4OH).

Concentrations of reactants were analyzed by HPLC using Dionex P580 Pump, C-8 column and a Dionex PDA-100 detector set at 215 nm. The mobile phase consisted of a mixture of acetonitrile : water (containing 0.1 % TFA) = 3 : 7.

The enantiomeric excess of the products (ee_p) was determined by HPLC using Dionex P580 Pump, Sumichiral OA 5000 column and UV detector set at 215 nm or Daicel Crownpak CR(+) column using the Dionex PDA-100 detector set at 200 nm. Composition of eluent solution was as follows: for Sumichiral OA 5000 column (flow rate of 0.4 mL/min) - 2 mM $CuSO_4$ containing 5% or 2% isopropanol; for Daicel Crownpak CR(+) column (flow rate of 1 mL/min) - aqueous solution of $HClO_4$ (pH = 1) containing methanol (10% v/v). Retention times of enantiomers of reaction products are summarized in Supplementary data (Table S1).

E values describing enantioselectivities of PGA^{Ec} or PGA^{A} were determined using Eq. (1):

$$E = \frac{\ln(A/A_0)}{\ln(B/B_0)} \qquad \text{(Eq. 1)}$$

where A_0 and A represent the concentrations of the faster reacting enantiomer at the reaction times 0 and t, resp. B_0 and B denote the concentrations of the slower reacting enantiomer at the reaction times 0 and t, resp. [16]. The enantiomeric ratio E was also determined by non-linear regression using Eq. (2) which is derived from Sih´s equation describing a relationship between E, degree of conversion (c) and the enantiomeric excess ee_p [16].

$$c = 1 - ((1 + ee_p) \times (1 - ee_p)^{-E})^{1/(E-1)} \qquad \text{(Eq. 2)}$$

2.5 Molecular modeling

A homology model of PGA^{A} was prepared using the sequence of amino acids from GenBank (AAY25991). The most suitable template for modeling was identified by PSI-BLAST search using the ExPaSy server against sequences in the Protein Data Bank. The PGA^{A} query sequence was then aligned with the best template – PGA^{Ec} enzyme (PDB-ID 1GM7, sequence identity 50%) using ClustalOmega [17]. Identity of amino acid residues forming the active site between the template and the query was 88%. Only two substitutions occurred within the seventeen amino acid residues forming the active site (Val56ß→Leu56ß and Ser149α→Ala149α), and no insertions or deletions were observed for these positions. Alignments were prepared with a constraint between the Ser1β residue of the query sequence and the corresponding serine residue of the template. The homology modeling based on alignment of PGA^{A} and PGA^{Ec} was performed using the Swiss-model webserver [18]. The α and β chains of penicillin acylases were modelled separately by calculating a number of minimized intermediate models which were ranked by the structure quality Z score. One model of the α chain and one of the β chain had to be chosen from this ensemble of structures, not only on the basis on the Z score, but also by taking into account the reciprocal positions of the two chains. The final model was evaluated using a program Verify 3D [19] with the template validation data used as the baseline to assess the respective models. To facilitate reproducibility of the work, the model was deposited to Protein Model DataBase [20] under the following access code PM0080082. The analysis of Ramachandran plot generated by RAMPAGE server [21] suggested a high quality of the homology model with more than 95 % of residues located in the favored region, for detailed report from the analysis see Supplementary data (Fig. S8). This finding was further confirmed by low Z-score of -0.29 reported by QMEAN server [22-23] indicating that the quality of the model is comparable to the high-resolution crystal structures of proteins of similar size. Next, the PGA^{A} model was protonated *via* H++ server at pH 7.5 using default settings. Protonation of catalytically important amino acid residues was modified manually so that they conform to specific reaction mechanism, i.e., enantioselective hydrolysis of N-PhAc-α/β-amino acids [24].

Molecular docking calculations were performed through AutoDock Vina plug-in for PyMol [25]. Structures of substrates were prepared using Avogadro molecular editor [26] and energetically minimized in four steps of the steepest descent using MMFF94 force field [27]. Substrate and protein structures were converted to AutoDock Vina compliant format by AutoDock Vina plug-in for PyMol. For docking, the grid box (22.5 x 22.5 x 22.5 Å) covering the PGA^{A} active site was defined and centered on the center of mass of Ser1β, Ala69β and Asn241β residues. The docking calculations were performed using Autodock Vina software [28] with an exhaustiveness of 200, maximum of 9 generated binding modes and maximum energy difference between the best and the worst binding modes of 3 kcal/mol. Visualization of docked binding modes and analysis of its geometries were performed in PyMol [29]

software. Mechanism-based geometric criteria for prediction of substrate reactivity [30] were derived accordingly to the catalytic mechanism of PGAs. To evaluate reactivity of docked substrate conformations, the binding modes were assessed by using following criteria: (i) distance between nucleophilic oxygen atom O^{γ} of Ser1β and attacked carbon from carbonyl group of the substrate reflecting the probability of nucleophilic attack, (ii) lengths of hydrogen bonds between nitrogen atoms from amino groups of Ala69β and Asn241β and the oxygen atom from the carbonyl group of substrate that stabilize the negative charge of the reactive tetrahedral intermediate, and (iii) length of hydrogen bond between oxygen atom from carbonyl group of Gln23β and leaving nitrogen group of substrate that also contributes to the stabilization of the tetrahedral intermediate (Fig. 1). To derive particular cutoff distances for these criteria, high-quality structures of PGA complexed with substrates and their analogs were analyzed (Supplementary data, Table S2). Since such evaluation provides only qualitative two-state prediction of substrates reactivity – reactive and non-reactive, we can accurately predict only potential for highly enantioselective discrimination. In such a case, one enantiomer is predicted as poorly active, while the other one as well active. It is important note that minor differences in enantioselectivity cannot be predicted using employed approach. Additionally, the interactions present in predicted binding modes were analyzed by PoseView [31].

3. Results and discussion

Research into enantioselectivity of PGAs started in 60s of the last century and dealt mainly with resolutions of racemic mixtures of α-amino acids and their derivatives [32-33]. So far, main concern on PGA^{A} dealt with syntheses of several important semi-synthetic β-lactam antibiotics. In order to asses a broader potential of PGA^{A}, we have focused on seven α/β-amino acids (Table 1) that have application potential as building blocks of active pharmaceutical ingredients. These substrates were used to compare enantioselectivities of PGA^{A} with PGA^{Ec}, an enzyme exhibiting 50 % sequence identity with PGA^{A} [34].

Racemates of substrates listed in Table 1 were used in enantioselective hydrolytic reactions catalyzed by purified PGA^{Ec} and PGA^{A} and their enantioselectivity values (E) were determined using HPLC analysis (Table 2). A high degree of enantioselectivity (E = 90 ± 5) for both PGA^{A} and PGA^{Ec} was observed in enzyme-catalyzed hydrolyses of N-PhAc-α-phenylalanine. Both PGA^{A} and PGA^{Ec} exhibited similar degree of enantioselectivity towards substrate N-PhAc-α-leucine and N-PhAc-α-isoleucine. On the other hand, PGA^{A} exhibited markedly higher enantioselectivity (in descending order) with: N-PhAc-β-homoleucine, N-PhAc-α-*tert*-leucine and N-PhAc-β-leucine (Table 2).

A lot of effort has been invested into the structural analysis of PGAs and understanding of its substrate-enzyme interactions. Amino acid residues Ser1β, Gln23β, Ala69β and Asn241β [42] are recognized as essential amino acid residues of aminic subsite in the eleven PGAs so far characterized [5]: Ala69β and Asn241β participate in formation of "oxyanion - hole", Gln23β stabilizes E-S tetrahedral intermediate and the Ser1β residue serves as a catalytic residue. It is proposed that the general topology and the quarternary structure of the active site cavity might be conserved throughout the PGA family [43]. However, slight variations in amino acid residues are evident in the acyl binding subsite that is formed in *E. coli* by Met142α, Arg145α, Phe146α, Phe24β, Thr32β, Pro49β, Val56β, Trp154β, and Ile177β [42,44]. Due to so far unsuccessful effort to crystallize the PGA^A (data not shown), we decided to prepare homology model of PGA^A to study molecular mechanism beyond enantioselectivities of this enzyme.

The model of PGA^A was used as a receptor in molecular docking experiments with both enantiomeric forms of seven substrates studied experimentally (Table 1). The binding energies predicted by molecular docking strongly correlated (R= -0.76) with the molecular weight of investigated substrates. However, the largest difference in the predicted binding energies between enantiomers of a given substrate was 0.7 kcal/mol only (Table S3). Since such difference is within the error margin of the scoring function, the observed difference between binding affinities of individual enantiomers should be considered as negligible without any significant influence on the enantioselectivity of PGA^A. The predicted structures of complexes were further analyzed in respect to the interactions of substrates with amino acid residues involved in the catalysis (i.e. Ala69β, Asn241β, Gln23β and Ser1β). For all N-PhAc-α-amino acids and N-PhAc-β-homoleucine, the distances from functional groups of important amino acid were markedly shorter for *(S)*-enantiomer which predicted this enantiomer as the preferred form (Table 3). On the contrary, *(R)*-N-PhAc-β-leucine was preferred enantiomer. Acyl moieties of all enantiomer pairs adopted binding modes that allowed the moiety to fit in the acyl-binding site of PGA^A, in which their phenyl rings formed π-π interactions with Phe24β and most frequently also hydrophobic interactions with Phe24β, Thr68β and Ala69β, see Supplementary data (Fig. S9 and S10). Based on our docking experiments we recognized, in addition to acyl binding subsite, also the “secondary hydrophobic” subsite formed mainly by the following amino acid residues: Ala69β, Phe71β, Ala149α and Phe146α.

Two mechanisms of PGA^A’s enantioselectivity may be recognized accordingly to the bulkiness of substrate’s substituent.

Substrates with bulky substituents: N-PhAc-α-phenylalanine, N-PhAc-α-homophenylalanine, N-PhAc-α-isoleucine, and N-PhAc-α-*tert*-leucine. They all adopted similar binding mode in the aminic subsite without exception (Fig. 2a). The bulky, hydrophobic nucleophilic substituents were all oriented to the secondary hydrophobic subsite. In the case of

N-PhAc-α-phenylalanine and N-PhAc-α-homophenylalanine containing the second aromatic ring in the structure of their nucleophilic substituents, additional π-π and hydrophobic interactions were formed between this ring and either with Phe71β or Phe146α residues within this site see Supplementary data (Fig. S9). The carboxyl moieties of all four substrates were oriented towards part of the active site with positively charged and hydrophilic amino acid residues (e.g. Arg263β, Ser390β, Asn214β and N-terminal part of Ser1β). Oxygens from the carbonyl group of (*S*)-enantiomers were always properly stabilized by amino acid residues of the "oxyanion-hole" in the orientation enabling nucleophilic attack upon the carbon atom of the carbonyl by Ser1β. Also hydrogens from amino groups of substrates were properly stabilized by Gln23β. On the contrary, binding modes of (*R*)-enantiomers had their peptide bonds oriented in the opposite direction (Fig. 2a). This orientation resulted into larger spatial separation of substrates' carbonyl oxygen and hydrogen of stabilizing functional amino group of the enzyme. Such binding modes also effectively shielded the attacked carbon from the nucleophilic attack by Ser1β. All this facts combined suggest that (*R*)-enantiomers of this group of substrates were poorly reactive at best, while (*S*)-enantiomers of these compounds were better stabilized for reaction as is illustrated by all four measured distances being much shorter contributing to its predicted preference (Table 3).

Substrates with less bulky substituents: N-PhAc-β-homoleucine, N-PhAc-α-leucine, N-PhAc-β-leucine. The binding modes of (*S*)-enantiomers of N-PhAc-β-homoleucine and N-PhAc-α-leucine followed the scheme for substrates with the bulky substituents (Fig. 2b), with the exception that these substituents were not oriented towards the secondary hydrophobic subsite of the binding cavity. The predicted binding modes of these two (*S*)-enantiomers fulfilled all criteria for their reactivity (Table 3).

In the case of (*R*)-enantiomers of N-PhAc-β-homoleucine and N-PhAc-α-leucine, the carboxyl groups pointed towards the secondary hydrophobic subsite and simultaneously their non-polar substituents pointed towards the positively charged and hydrophilic residues (Fig. 2b). These binding modes allowed the peptide bonds of (*R*)-enantiomers of N-PhAc-β-homoleucine and N-PhAc-α-leucine to adopt similar orientation as (*S*)-enantiomers of N-PhAc-β-homoleucine and N-PhAc-α-leucine and therefore to partially restore necessary stabilization of their carbonyl oxygens by the residues of the "oxyanion-hole" and orientation of attacked carbon towards catalytic nucleophile (Table 3). However, the amino groups of substrates were not stabilized by Gln23β, and the obviously non-complementary interactions required by these binding modes should further disfavor their formation. In the case of N-PhAc-β-leucine racemic mixture, binding modes of both enantiomers followed the same pattern as described above but (*R*)-enantiomer was the preferred form (Table 3).

Since the molecular docking of seven substrates into the homology model of PGA^A confirmed experimental results obtained with purified PGA^A (Table 2), we employed this approach for *in-silico* prediction of enantioselective hydrolyses of non-conventional α/β-amino acid enantiomers of which have an application potential (Table 4). We should stress here that enantioselective hydrolyses of majority of racemates of these substrates catalyzed by so far characterized PGAs have not yet been reported. A reference to stereoselectivity of PGA with N-PhAc-3-aminopent-4-ynoic acid derivative and N-PhAc-*p*-Cl-β-phenylalanine can be found in research studies [45-46].

Results of docking experiments with pure enantiomers of racemates of seven N-PhAc-α/β-amino acids into active site cavity of homology model of PGA^A are shown in Table 5.

Similarly to previous analysis, both enantiomers exhibited comparable binding energies ruling out a differential binding as a source of potential enantiopreference of PGA^A (Table S3). Therefore we further focused on evaluation of differences in potential reactivity of these novel substrates using validated geometric criteria. As shown for α/β-amino acids, such analysis was able to successfully predict high enantioselectivity in cases, when reactivity of one enantiomer was seriously disfavored. Based on this analysis, we predicted high enantiopreference of PGA^A for three (*R*)-enantiomers and two (*S)*-enantiomers of novel substrates (Table 5). However, we could not entirely exclude possibility of moderate-to-low enantioselectivity for remaining two substrates. In the case of N-PhAc-3-aminopent-4-ynoic acid, binding of its rigid and extended ethynyl group into the secondary hydrophobic subsite of the PGA^A prevented stabilization of carbonyl oxygen of both enantiomers by the residues of "oxyanion hole" as well as proper stabilization by interactions with Gln23β, predicting both enantiomers as inactive or poorly active. The binding modes of (*R*)-enantiomers of both N-PhAc-3-amino-3-pyridin-propanoic acid and N-PhAc-3-amino-3-(3-chloro-4,5-dihydroxyphenyl) propanoic acid had their aromatic nucleophilic substituents located in the secondary hydrophobic subsite as was the case of the binding modes for the group of substrates containing bulkier substituents. Interestingly, the (*S*)-enantiomers of these compounds were bound with their carboxyl groups in the secondary hydrophobic subsite, while their aromatic nucleophilic substituents pointed towards hydrophilic part of the active site in the same fashion as the group of substrates containing less bulky substituents. Since the aromatic substituents of these two substrates carry also some polar groups, their binding modes seemed to be relatively easy to accommodate into the PGA^A's binding site. In any case observed binding modes of (*S*)-enantiomers provided poor interactions with all four functionally important residues (Table 5). This analysis suggested high enantiopreference towards (*R*)-enantiomers of these two

substrates. In the case of N-PhAc-3-amino-3-benzyl-2-hydroxybutanoic acid, both enantiomers complied to the binding modes observed for group of substrates having the bulky substituent, with the exception that hydroxyl group of (*S*)-enantiomer took over the interaction of carboxyl group with hydrophilic part of the active site cavity. Nevertheless, a difference in measured distances indicated strong (*S)*-preference (Table 5). Substrates N-PhAc-*p*-Cl-α-DL-phenylalanine and N-PhAc-*p*-Cl-β-DL-phenylalanine fully followed behavior of substrates containing bulkier substituents including predicted enantiopreference towards their (*S*)- and (*R*)-enantiomers, respectively. Enantiomers of N-PhAc-*p*-F-α-DL-phenylalanine adopted extended conformations because their nucleophilic substituents could fit better into aminic subsite being possibly stabilized by π-π interactions with Phe71β. Although the carboxyl group of (*R*)-enantiomer was being oriented into the secondary hydrophobic subsite, the distances to interacting groups were favorable for both enantiomers predicting their good reactivity (Table 5). Therefore, no conclusion on possible enantioselectivity of PGA^{A} towards this substrate could be drawn.

4. Conclusions

In this study, we present results of the research into enantioselectivity potential of a novel industrial catalyst PGA^{A} towards racemic mixtures of α/β-amino acids, pure enantiomers of which have an application in pharmaceutical industry. We have demonstrated that PGA^{A} exhibited markedly higher enantioselectivity than PGA^{Ec} (in descending order) with: N-PhAc-β-homoleucine, N-PhAc-α-*tert*-leucine, and N-PhAc-β-leucine. We have constructed a homology model of PGA^{A} using availability of a crystal structure of PGA^{Ec}. We were able to evaluate plausibility of the model by using molecular docking calculations: the results of molecular modeling were identical with the enantiopreference of PGA^{A} found for all experimentally studied substrates. Follow-up docking experiments with seven non-conventional N-PhAc-α/β-amino acids enabled us to predict the enzyme enantiopreference for most of these substrates. Predictions indicated high PGA^{A}'s enantiopreference for (*R*)-enantiomers of three compounds and (*S)*-enantiomers of two substrates. *In-silico* prediction of enantiopreference revealed PGA^{A} convenience towards production of (*S*)-enantiomer of N-PhAc-3-amino-3-benzyl-2-hydroxybutanoic acid, a potential building block for paclitaxel. *In-silico* prediction of PGA^{A}'s enantiomer preference could be powerful tool for selection of compounds that are not available on the market but can serve as a source of chiral compounds for pharmaceutical industry.

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Ethical statement

The authors are aware of ethical standards and declare that they have no conflicts of interests. The research does not involve human being or animals.

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Figure captions

Fig. 1. Mechanism-based geometric criteria developed for evaluation of reactivity of docked substrates in active site of PGA^{A}. The essential amino acid residues of *PGA^{A}*'s active site are shown as green sticks. Binding mode of (*S*)-N-PhAc-*tert*-leucine is shown as cyan sticks. The analyzed distances D_1 ($O^{Gln23\beta}\rightarrow$ H), D_2 ($O^{Ser1\beta}\rightarrow$ C), D_3 ($N^{Asn241\beta}\rightarrow$O) and D_4 ($N^{Ala69\beta}\rightarrow$O) are displayed as yellow dashed lines.

Fig. 2. Predicted binding modes of seven experimentally tested substrates within the active site cavity of PGA^{A}. A) Binding modes of enantiomers with bulky substituents (moieties: α-phenylalanine, α-homophenylalanine, α-isoleucine, and α-tert-leucine). B) Binding modes of enantiomers with less bulky substituents. Grey surface - the active site cavity; red surface - the acyl-binding subsite; orange surface - the secondary hydrophobic subsite. Crucial catalytic amino acid residues of the active site are green; preferred and non-preferred enantiomers are cyan and yellow, respectively.

Supplementary data
Resolution of α/β-amino acids by enantioselective penicillin G acylase from *Achromobacter* sp.

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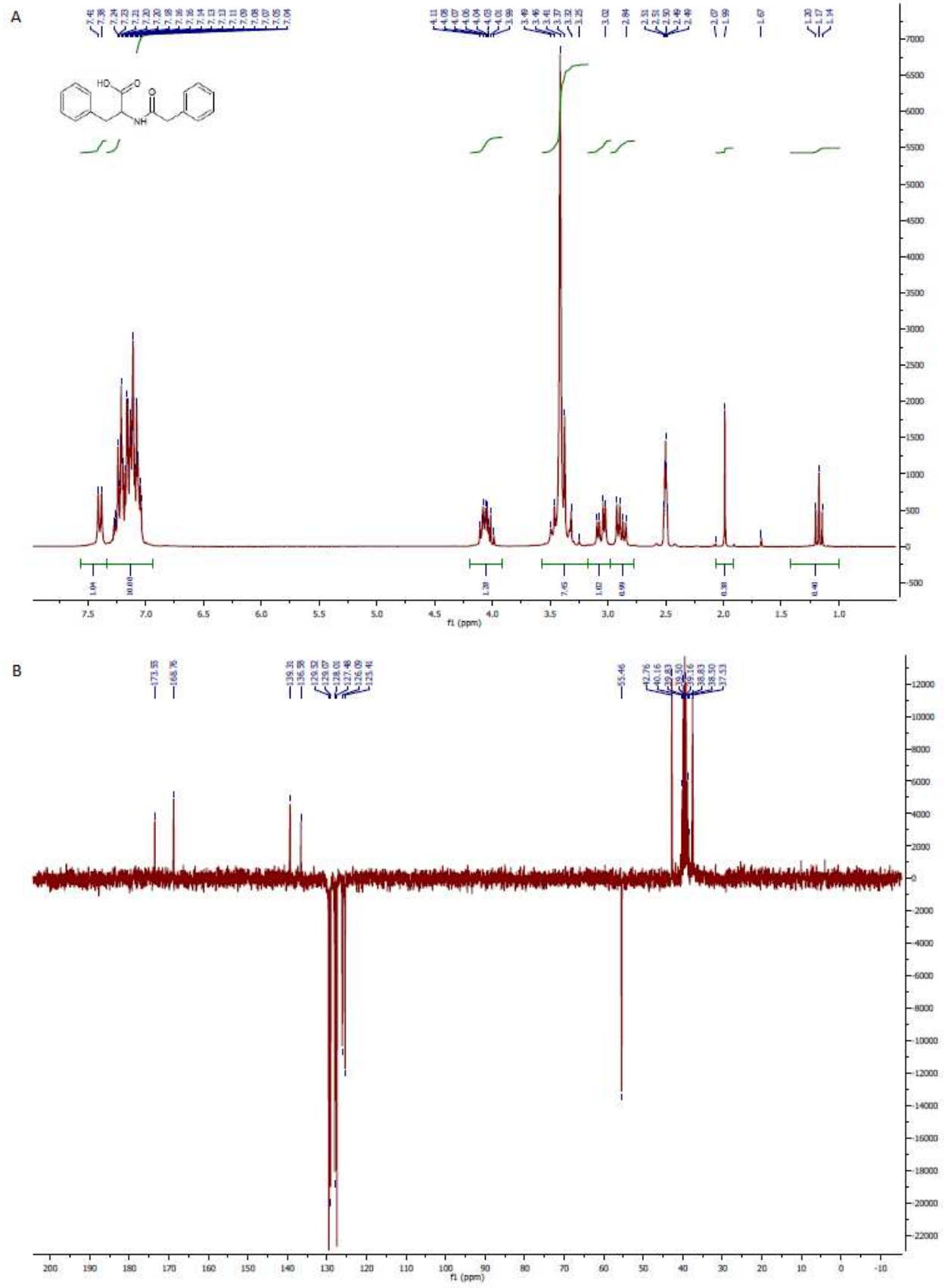


Fig. S1. ^{1}H (A) and ^{13}C (B) NMR spectra of N-PhAc-α-phenylalanine

A) ^{1}H NMR: (DMSO) δ 7.41 (d, J = 7.5, 1H, NH), 7.00-7.30 (m, 10H, ArH), 3.95-4.15 (m, 1H, CH), 3.3-3.5 (m, 2H,CH_2CO), 3.0-3.10 (m, 1H, $ArCH_2$), 2.8-2.9 (m, 1H, $ArCH_2$)

B) ^{13}C NMR: (DMSO) δ 173.55, 168.76, 139.31, 136.58, 129.52, 129.07, 128.01, 127.48, 126.09, 125.41, 55.46, 42.76, 38.50

MS (ES^+) m/z 306.1091 $(M + Na)^+$ - M = 283 g/mol

(1g, 72 %).

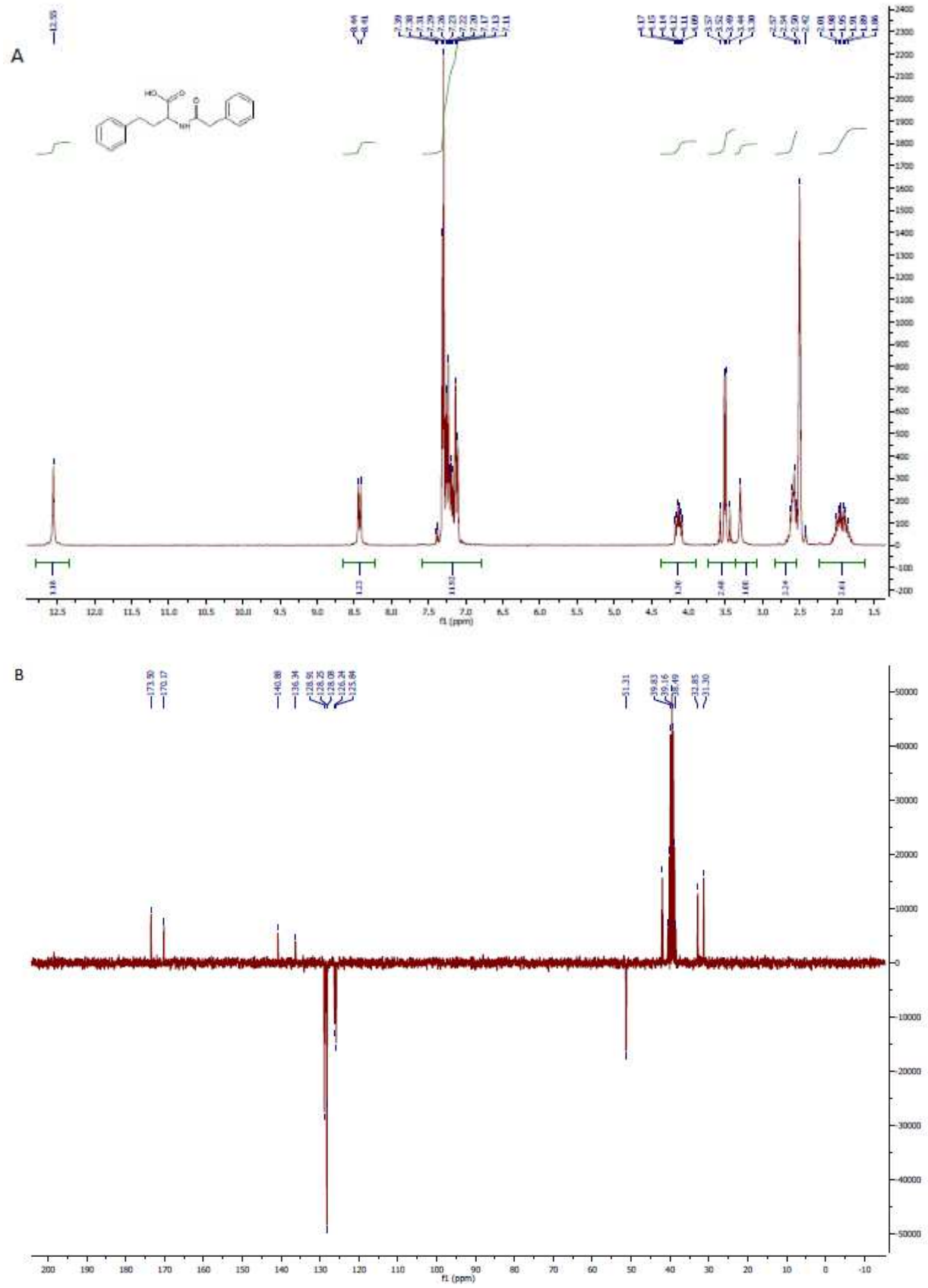


Fig. S2. ^{1}H (A) and ^{13}C (B) NMR spectra of N-PhAc-α-homophenylalanine

A) ^{1}H NMR: (DMSO) δ 12.55 (s, 1H, COOH), 8.43 (d, J = 7.5, 1H, NH), 7.0-7.4 (m, 10H, ArH), 4.0-4.2 (m, 1H, CHN), 3.51 (d, 2H, CH_2CO), 2.5-2.7 (m, 2H, $ArCH_2$), 1.75-2.1 (m, 2H, CH_2); 3.3 (1H)

B) ^{13}C NMR: (DMSO) δ 173.5, 170.17, 140.88, 136.34, 128.92, 128.25, 128.08, 126.24, 125.84, 51.31, 41.97, 32.86, 31.31

MS (ES^+) m/z 320.1256 $(M + Na)^+$ - M = 297 g/mol

(1g, 79 %).

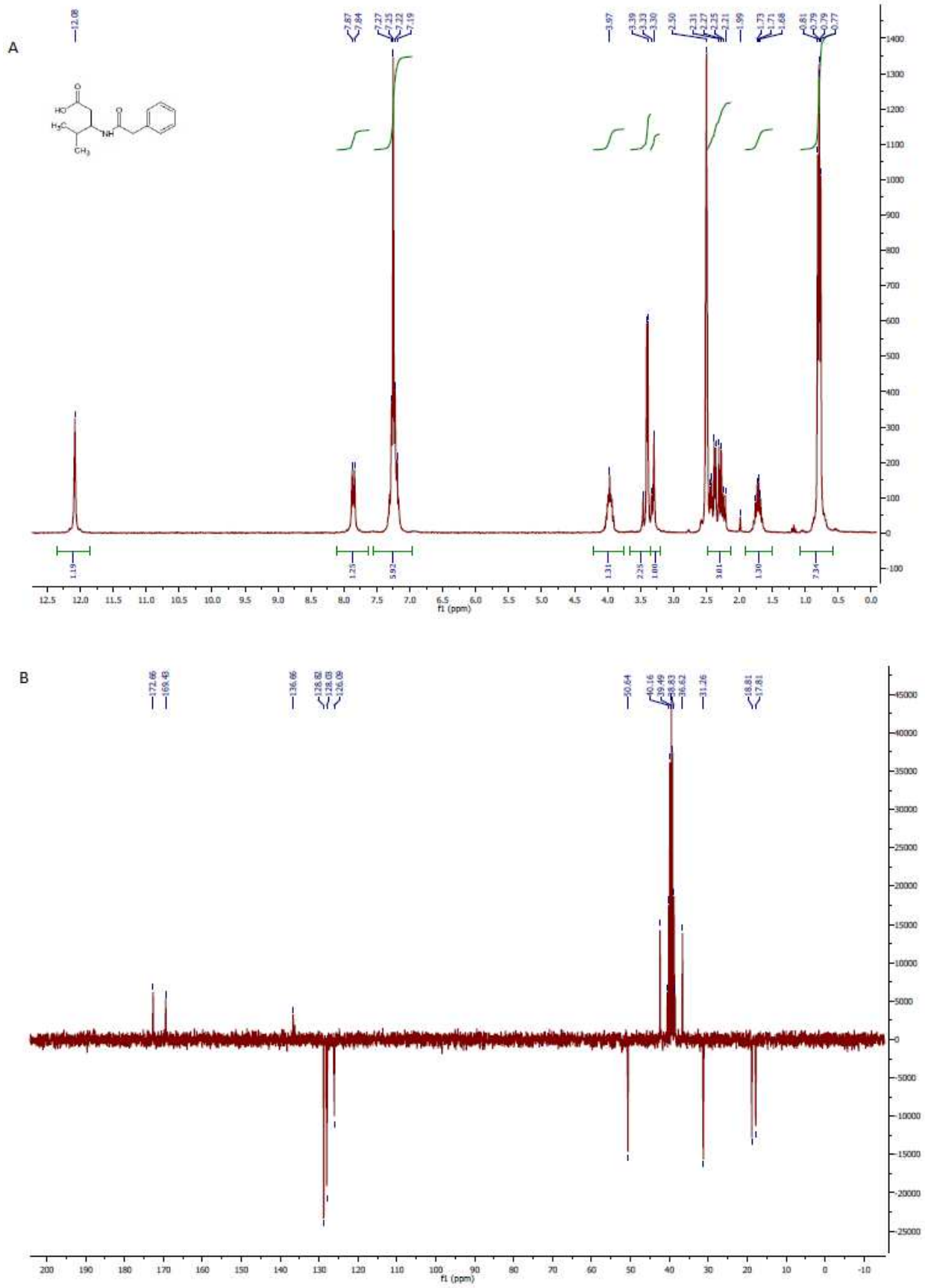


Fig. S3. ^{1}H (A) and ^{13}C (B) NMR spectra of N-PhAc-β-leucine

A) ^{1}H NMR: (DMSO) δ 12.10 (s, 1H, COOH), 7.89 (d, J=10, 1H, NH), 7.1-7.4 (m, 5H, ArH), 3.8-4.1 (m, 1H, CHN), 3.36 (d, 2H, CH_2CO), 3.25-3.35 (m, 2H), 2.2-2.45 (m, 2H, CH_2), 1.6-1.8 (m, 1H, CH), 0.78 (d, J = 50 Hz, 3H, CH_3), 0.8 (d, J= 50 Hz, 3H, CH_3)

B) ^{13}C NMR: (DMSO) δ 172.66, 169.42, 136.23, 128.82, 128.03, 126.09, 50.63, 42.39, 36.62, 31.27, 18.82, 17.82

MS (ES^+) m/z 250.1433 $(M + H)^+$ - M = 249 g/mol

(1g, 69 %).

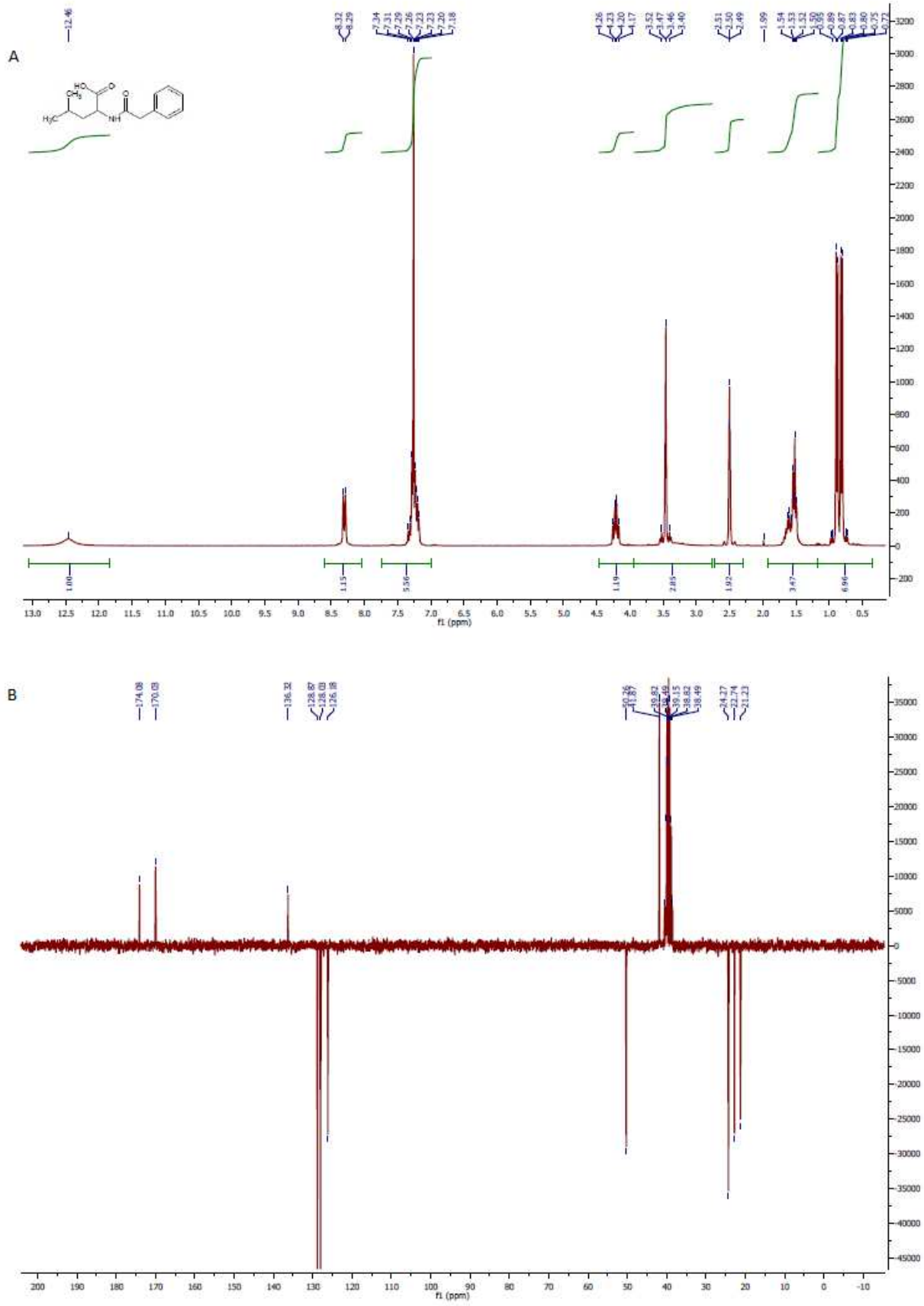


Fig. S4. ^{1}H (A) and ^{13}C (B) NMR spectra of N-PhAc-α-leucine

A) ^{1}H NMR: (DMSO) δ 12.45 (bs, 1H, COOH), 8.30 (d, J=7.5, 1H, NH), 7.15-7.3 (m, 5H, ArH), 4.26 (ddd, J = 7.5; 7.5; 15 Hz, 1H, CHN), 3.52 (s, 2H, CH_2CO), 1.49-1.65 (m, 3H, CH_2, CH), 0.86 (d, J = 15 Hz, 3H, CH_3), 0.84 (d, J = 17.5 Hz, 3H, CH_3)

B) ^{13}C NMR: (DMSO) δ 174.08, 170.03, 136.32, 128.03, 127.22, 126.19, 50.27, 41.88, 24.28, 22.75, 21.24

MS (ES^+) m/z 250.1436 $(M + H)^+$ - M = 249 g/mol

(1g, 76 %).

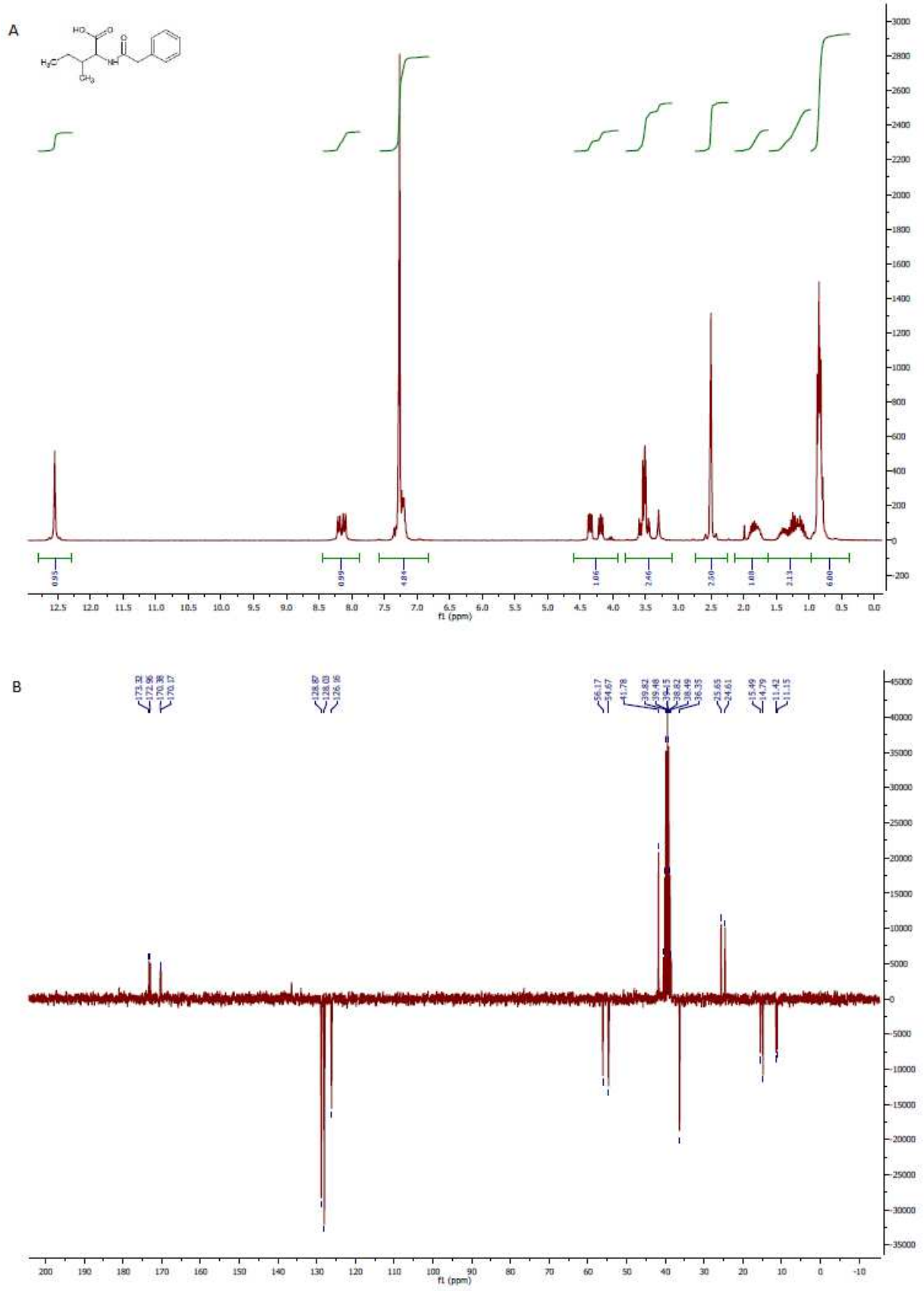


Fig. S5. ^{1}H (A) and ^{13}C (B) NMR spectra of N-PhAc-α-isoleucine

A) ^{1}H NMR: (DMSO) δ 12.55 (s, 1H, COOH), 8.0-8.25 (m 1H, NH), 7.1-7.3 (m, 5H, ArH), 4.1-4.4 (m, 1H, CHN), 3.4-3.6 (m, 2H, CH_2CO), 1.7-1.9 (m,1H, CH), 1.0-1.5 (m, 2H, CH_2), 0.75-1.0 (m, 6H, 2xCH_3)

B) ^{13}C NMR: (DMSO) δ 173.32, 172.96, 170.38, 170.17, 137, 128.87, 128.03, 126.16, 56.17, 54.68, 41.78, 36.35, 25.66, 24.62, 15.49, 14.79, 11.42, 11.15

MS (ES^+) m/z 250.1434 $(M + H)^+$ - M = 249 g/mol

(1g, 77 %).

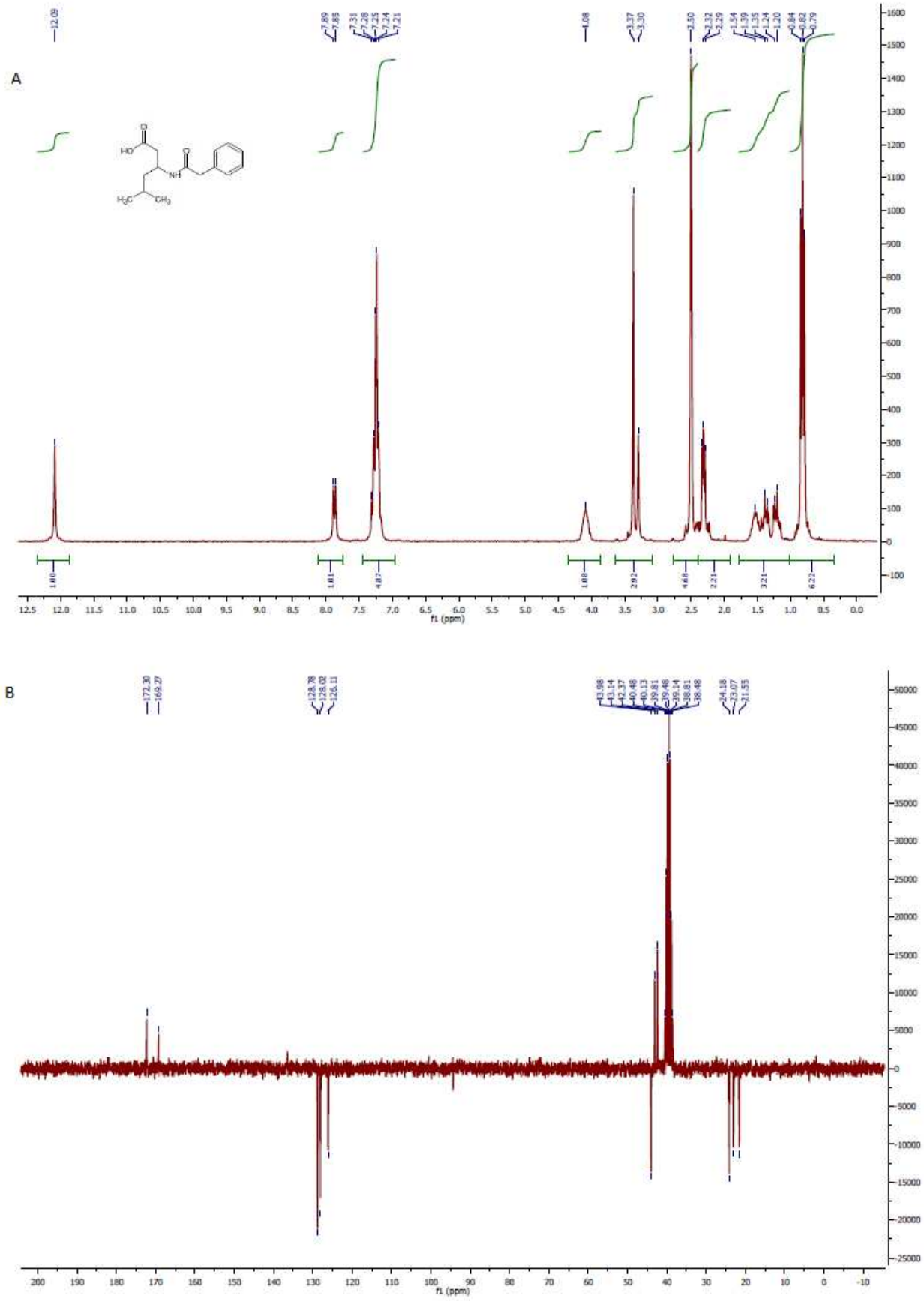


Fig. S6. ^{1}H (A) and ^{13}C (B) NMR spectra of N-PhAc-β-homoleucine

A) ^{1}H NMR: (DMSO) δ 12.10 (s, 1H, COOH), 7.87 (d, J=7.5, 1H, NH), 7.1-7.3 (m, 5H, ArH), 4.0-4.2 (m, 1H, CHN), 3.37 (s, 2H, CH_2CO), 3.3 (s, 1H,), 2.25-2.4 (m, 2H, CH_2), 1.18-1.61 (m, 3H, $CH+CH_2$), 0.83 (d, J= 5 Hz, 3H, CH_3), 0.80 (d, J = 7.5 Hz, 3H, CH_3)

B) ^{13}C NMR: (DMSO) δ 172.29, 169.27, 136.49, 128.89, 128.02, 126.12, 43.99, 43.14, 42.37, 40.49, 24.19, 23.08, 21.56

MS (ES^+) m/z 264.16 $(M + H)^+$ - M = 263 g/mol

(1g, 73 %).

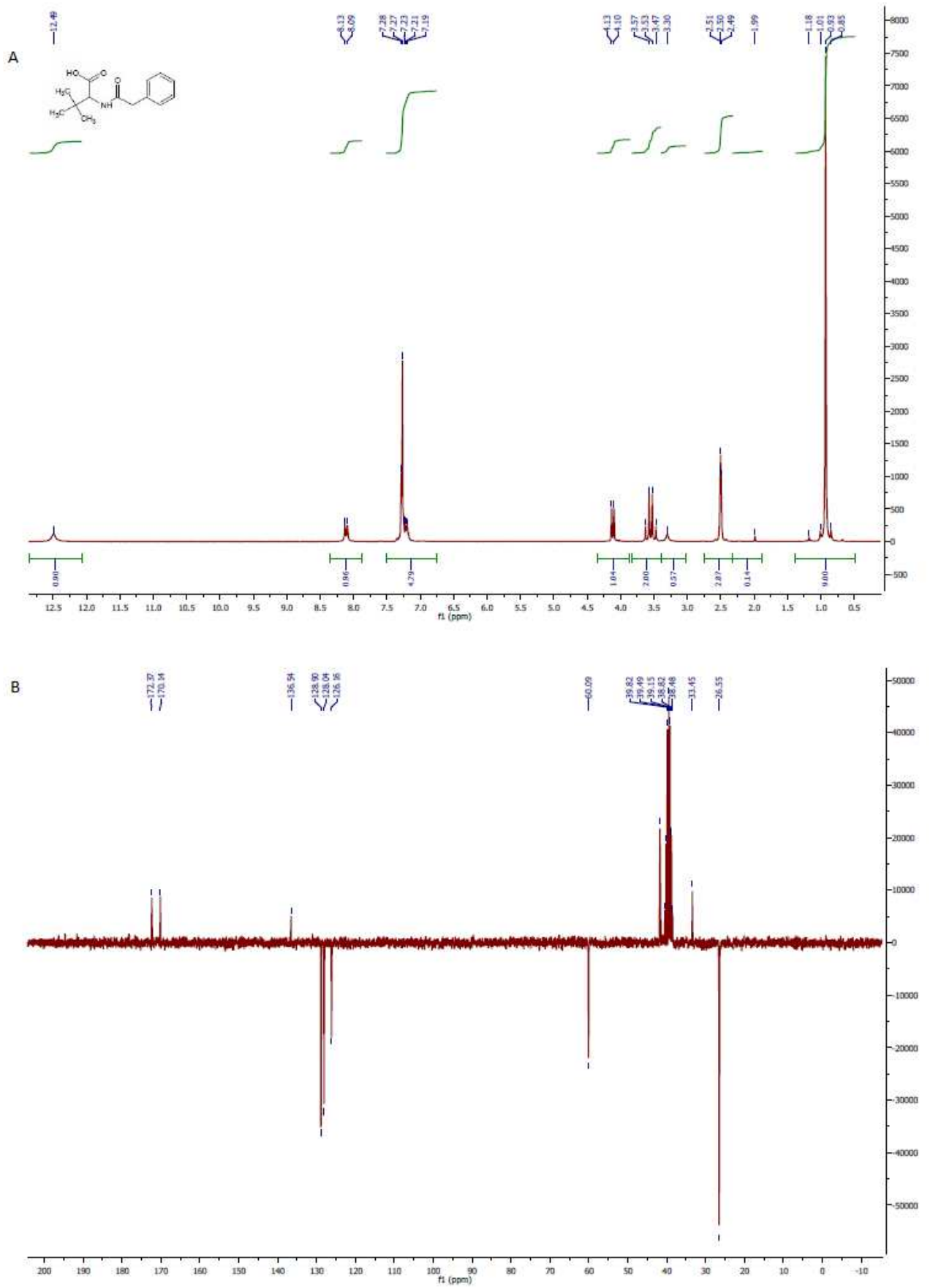


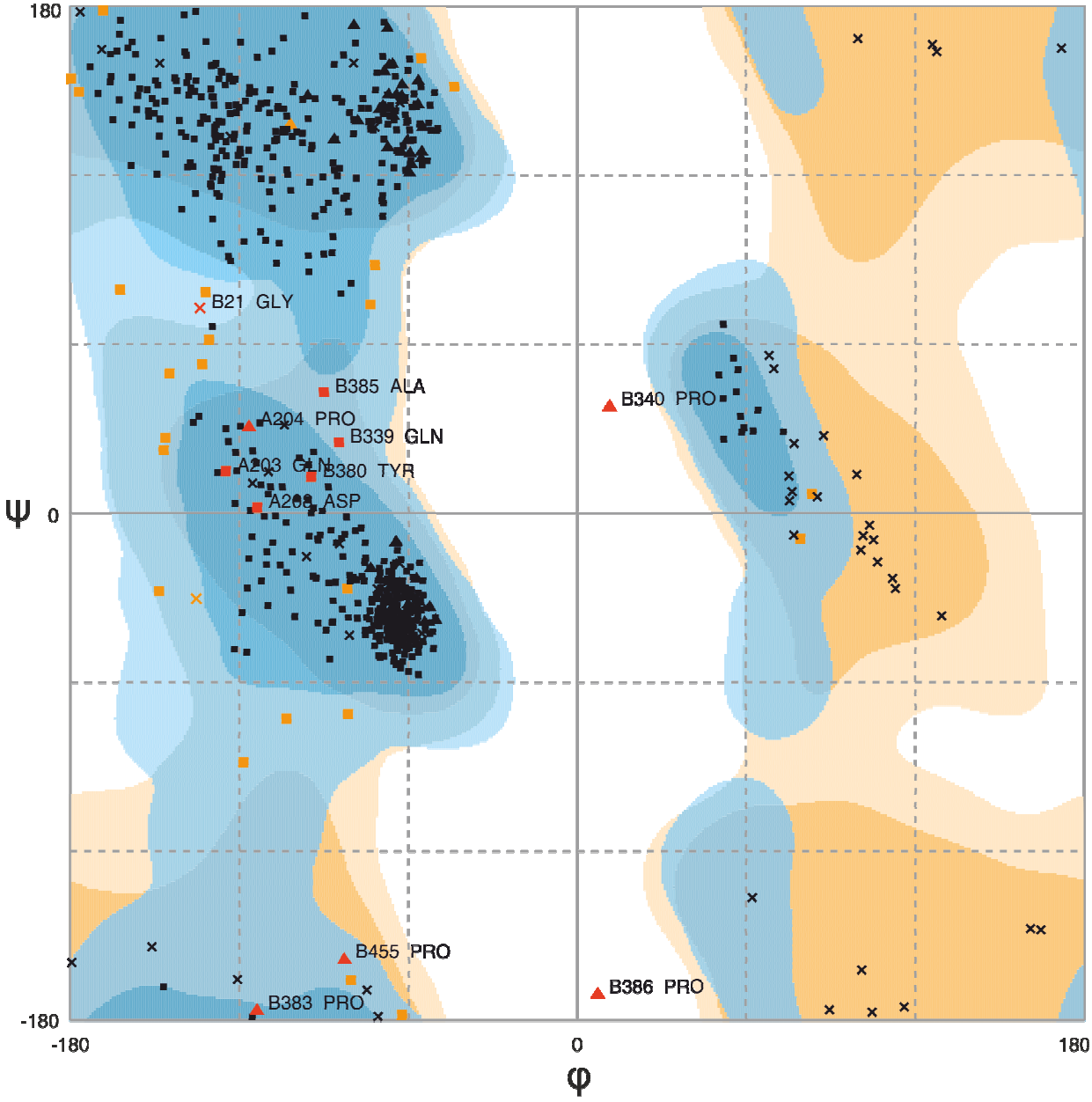
Fig. S7. ^{1}H (A) and ^{13}C (B) NMR spectra of N-PhAc-*α*-*tert*-leucine

A) ^{1}H NMR: (DMSO) δ 12.49 (s, 1H, COOH), 8.11 (d, J = 10 Hz, 1H, NH), 7.2-7.3 (m, 5H, ArH), 4.11 (d, J= 7.5 Hz, 1H, CHN), 3.4-3.6 (m, 2H, CH_2CO), 0.93 (s, 9H, CH_3)

B) ^{13}C NMR: (DMSO) δ 172.37, 170.14, 136.54, 128.90, 128.04, 126.16, 60.09, 41.74, 33.46, 26.56

MS (ES^+) m/z 250.14346 $(M + H)^+$ - M = 249 g/mol

(1g, 80 %).



Number of residues in favoured region (~98.0% expected) : 724 (95.3%)
Number of residues in allowed region (~2.0% expected) : 25 (3.3%)
Number of residues in outlier region : 11 (1.4%)

Fig. S8. Assessment of Ramachandran plot for homology model of PGA^{A} generated by RAMPAGE server.

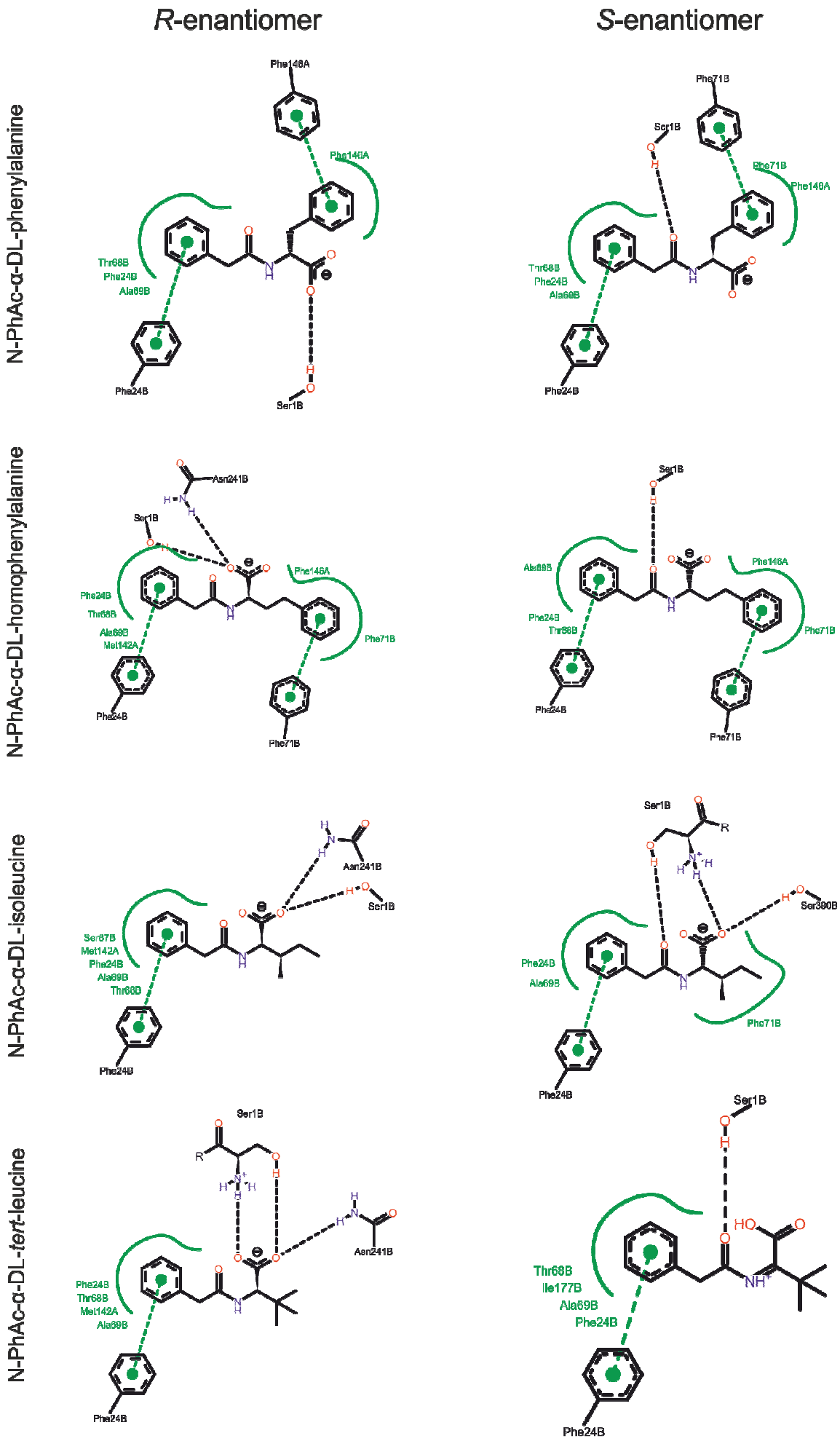


Fig. S9. Interactions of PGA^{A} with substrates carrying bulky substituents reported by PoseView.

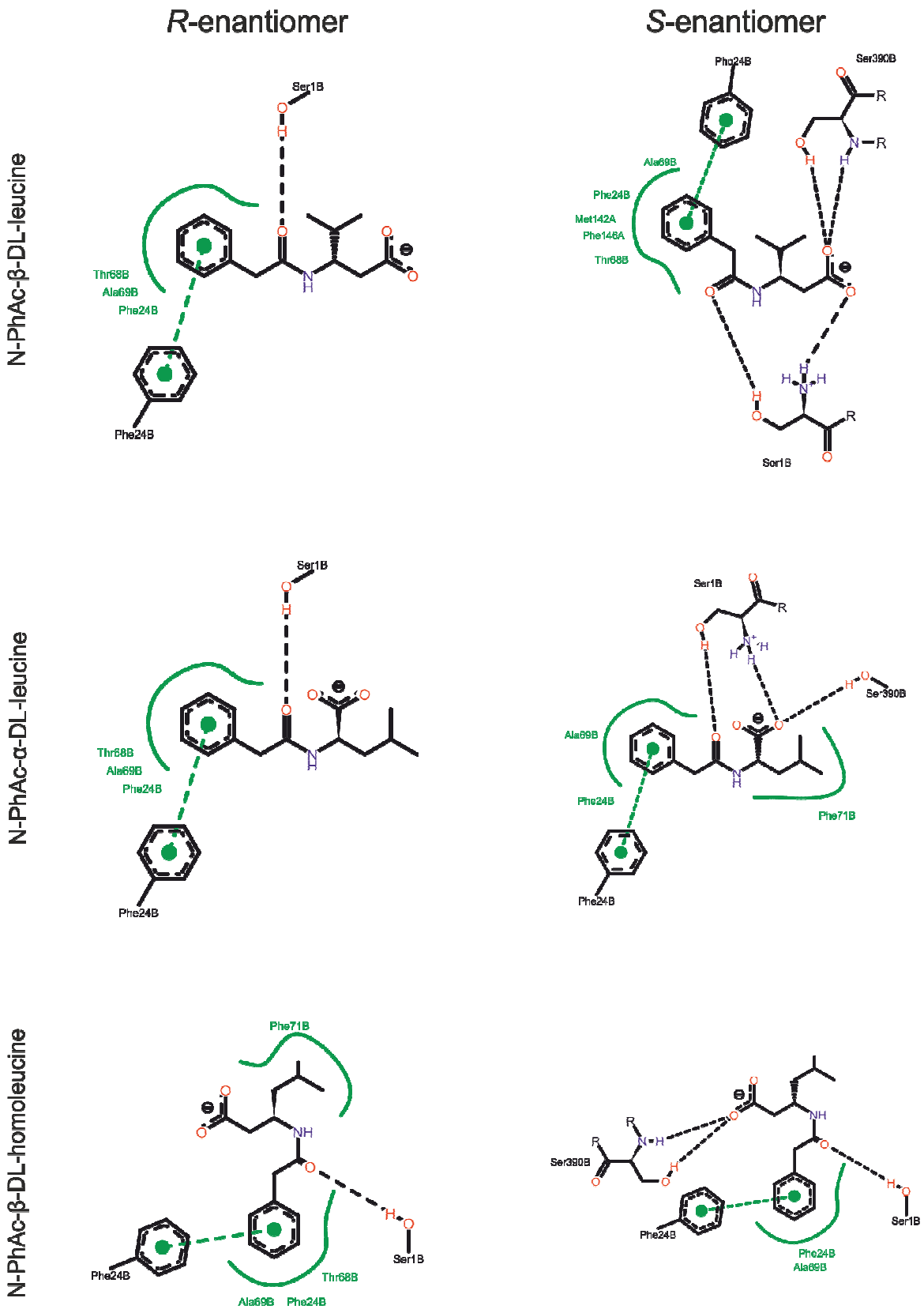


Fig. S10. Interactions of PGA^{A} with substrates carrying less bulky substituents reported by PoseView.

Table S1 Retention times of enantiomers of reaction products.

Product	Retention time (min)	
	(*S*)-enantiomer	(*R*)-enantiomer
α-phenylalanine[a]	7.5	6.7
α-homophenylalanine[a]	11.5	9.1
β-leucine[b]	15.6	17.4
α-leucine[b]	57.1	30.3
α-isoleucine[a]	7.4	4.7
β-homoleucine[a]	13.6	12.0
α-*tert*-leucine[c]	9.2	8.4

[a] Chiral stationary phase: column Daicel Chrompak R (+), 5μm - (250 x 4.0 mm I.D.), Daicel, France
Mobile phase: $HClO_4$ water solution, pH=1, flow rate 0.4 mL/min, 25°C, detection at 200 nm

[b] Chiral stationary phase: column Sumichiral OA-5000, 5 μm - (250 x 4.0 mm I.D.), SCAS, Japan
Mobile phase: 2mM $CuSO_4$-isopropanol in ratio of 95:5, flow rate 1mL/min, 25°C, detection at 215 nm

[c] Chiral stationary phase: column Sumichiral OA-5000, 5 μm - (250 x 4.0 mm I.D.), SCAS, Japan
Mobile phase: 2mM $CuSO_4$-isopropanol in ratio of 98:2, flow rate 1mL/min, 25°C, detection at 215 nm

Table S2 Derivation of mechanism-based geometric criteria for PGA reactions.

PDB-ID of complex	Ligand	Distance (Å)			
		$O^{Ser1\beta} \rightarrow C$	$N^{Ala69\beta} \rightarrow O$	$N^{Asn241\beta} \rightarrow O$	$O^{Gln23\beta} \rightarrow H$
1GM7	penicillin G	3.3	3.6	3.7	3.6
1GM8	penicillin sulfoxide	3.3	3.7	3.7	3.9
1GM9	penicillin sulfoxide	2.9	2.8	3.0	3.3
Maximum cutoff distances		**3.6**	**4.1**	**4.1**	**4.3**

For the analysis, polar hydrogen atoms were added to ligands and protein structures were aligned to 1GM7 using PyMOL program. Distances were measured also in PyMOL program. In case of structures 1GM7 and 1GM8 carrying mutation Asn241β→Ala241β, the distance $N^{Asn241\beta} \rightarrow O$ was measured to corresponding amino acid residue form structure 1GM9. The cutoff distances were derived as maximal observed distance increased by 10% to partially compensate errors from molecular modeling.

Table S3 Predicted binding energies of substrates docked into the active site of PGA[A].

Substrate	Binding energy [kcal/mol]		
	(*R*)-enantiomer	(*S*)-enantiomer	difference (*R*-*S*)
N-PhAc-α-DL-phenylalanine	-8.0	-8.3	0.3
N-PhAc-α-DL-homophenylalanine	-8.6	-7.9	-0.7
N-PhAc-β-DL-leucine	-6.8	-7.0	0.2
N-PhAc-α-DL-leucine	-6.6	-6.8	0.2
N-PhAc-α-DL-isoleucine	-7.4	-6.7	-0.7
N-PhAc-β-DL-homoleucine	-7.7	-7.3	-0.4
N-PhAc-α-DL-*tert*-leucine	-7.7	-7.1	-0.6
N-PhAc-3-aminopent-4-ynoic acid	-7.7	-7.9	0.2
N-PhAc-3-amino-3-pyridin-propanoic acid	-7.5	-7.2	-0.3
N-PhAc-3-amino-3-benzyl-2-hydroxybutanoic acid	-8.2	-8.4	0.2
N-PhAc-3-amino-3-(3-chloro-4,5-dihydroxyphenyl) propanoic acid	-8.1	-8.2	0.1
N-PhAc-*p*-Cl-α-D-phenylalanine	-8.5	-8.1	-0.4
N-PhAc-*p*-F-α-DL-phenylalanine	-8.1	-7.7	-0.4
N-PhAc-*p*-Cl-β-DL-phenylalanine	-8.1	-8.1	0.0