Enantioselective Penicillin Acylase–catalyzed Reactions

Factors Governing Substrate and Stereospecificity of the Enzyme

V. K. ŠVEDAS, M. V. SAVCHENKO, A. I. BELTSER, AND D. F. GURANDA

A. N. Belozersky Institute of Physicochemical Biology
Moscow State University
000958 Moscow, Russia

For many years, penicillin acylase from *Escherichia coli* has been considered first of all as a practically important enzyme of industrial use for modification of β-lactam antibiotics.[1–3] During the last years, penicillin acylase has become a subject of new attentive interest from both fundamental and practical points of view.[4] The crystal structure of penicillin acylase at 1.9-Å resolution has been reported and, principally, a new mechanism to endow the enzyme with its catalytic properties has been suggested.[5] Penicillin acylase has been used for deprotection of the amino groups in amino acid derivatives,[6,7] for enzymatic resolution of enantiomers of fluoro-substituted α-amino acids,[8] secondary alcohols, and α-substituted phenylacetic acids,[9–11] and for enantioselective acylation during synthesis of loracarbef.[12] In contrast to all other acylases, penicillin acylase has been shown to be an effective biocatalyst for resolution of enantiomers of aminoalkylphosphonic acids,[13] aminoalkylphosphonous acids,[14] and β-amino acids[15,16] (FIGURE 1). One can thus see that penicillin acylase is an enzyme of wide substrate specificity. Nevertheless, the reported studies on the substrate specificity of penicillin acylase have been done so far mainly on a semiquantitative level or only for a very limited number of substrates.[17–20] Benzylpenicillin, which is often claimed as the preferred substrate of penicillin acylase from *E. coli*,[1–3] is not, for example, one of the five most reactive substrates characterized so far (TABLE 1). Stereospecificity of this enzyme in practice was not studied.

Let us consider the specificity of penicillin acylase to the substrate of the general formula (FIGURE 2). The following problems have been studied: capacity of acyl group binding pocket ρ1; specificity of ρ2 and ρ3 subsites; and chiral discrimination of the substrate in the penicillin acylase active center.

ACYL GROUP BINDING POCKET

Topography of the acyl group binding pocket (subsite ρ1) was studied by the bifunctional inhibitors, alkylboronic acids, with a changing length of the alkyl chain;[21] penicillin acylase specificity to the acyl group of the substrate was also investigated for the series of *N*-acylated glycines[22] (FIGURE 3). There is a remarkable increase of inhibition with a growing alkyl radical, but the capacity of the binding pocket is

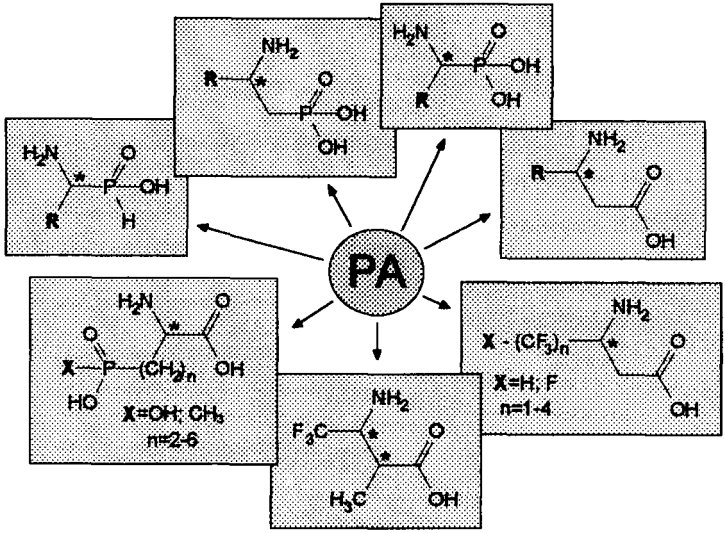


FIGURE 1. Structures of the organoelement analogues of natural amino acids and nonconventional amino acids prepared in optically pure form using penicillin acylase (PA).

limited by the amyl radical (the length of the amyl radical most closely corresponds to the length of the benzyl radical, as in the best substrates of penicillin acylase). Further increase of the alkyl chain leads to the distinguished decrease of the inhibition. Practically, the same kind of dependence is observed in the case of penicillin acylase–catalyzed hydrolysis of *N*-acylglycines. The important similarity between these two dependencies is that the slope of the dependencies is close to a value of two; thus, the binding of the alkyl chain is not just a simple extraction of the alkyl chain from water to the hydrophobic pocket of the enzyme, but also an elimination of some thermodynamically unfavorable contacts with water in the enzyme active center—or, in other words, an expulsion of some water molecules from the binding pocket. As an illustration, a model "knife-scabbard" can be used.

TABLE 1. The Most Reactive Substrates of Penicillin Acylase

Substrate	k_{cat}/K_M ($M^{-1} \cdot s^{-1}$)	%
Phenylacetyl-Abu[a]	$4.6 \cdot 10^7$	100
Phenylacetyl-Phe	$3.7 \cdot 10^7$	80
Phenylacetyl-Ala	$2.1 \cdot 10^7$	46
Phenylacetyl-Ala^{PH}	$2.0 \cdot 10^7$	43
Phenylacetyl-Gly	$2.0 \cdot 10^7$	43
Benzylpenicillin	$1.7 \cdot 10^7$	37
Phenylacetyl-Gly-Val	$1.5 \cdot 10^7$	33
Phenylacetyl-Gly-Leu	$1.1 \cdot 10^7$	24
Phenylacetyl-Gly-Phe	$0.9 \cdot 10^7$	20
Phenylacetyl-Ala^{P}	$0.64 \cdot 10^7$	14

[a]Abu = alpha-aminobutyric acid.

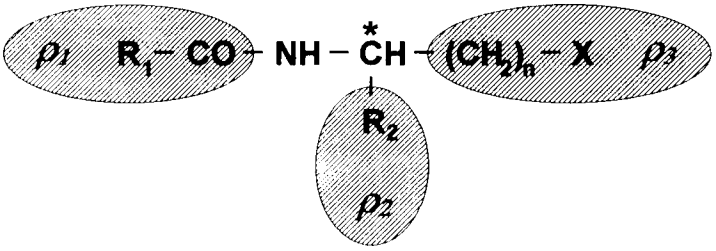


FIGURE 2. Schematic representation of the penicillin acylase substrate and location of its structural fragments in the corresponding subsites of the enzyme active center.

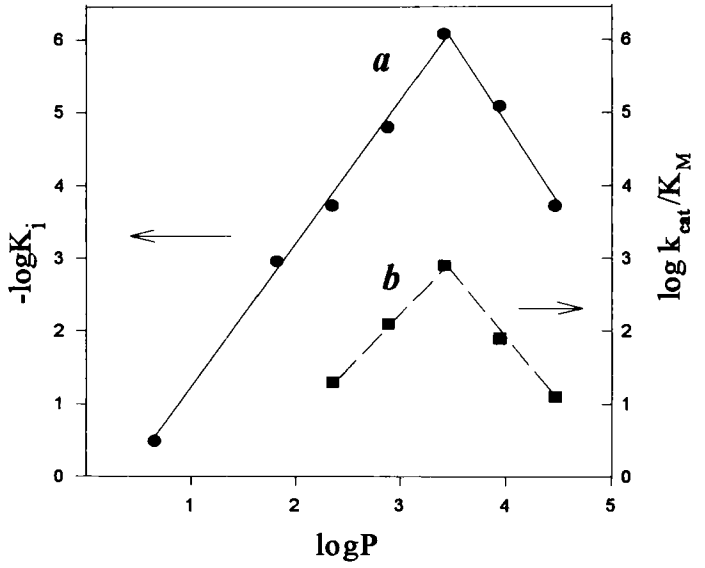
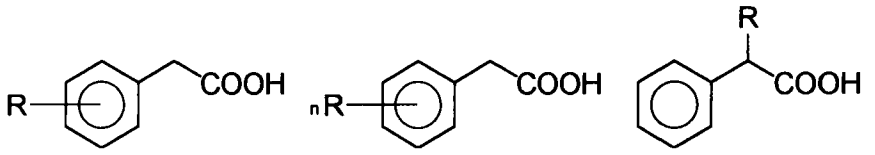


FIGURE 3. Penicillin acylase inhibition by alkylboronic acids (*a*) and penicillin acylase–catalyzed hydrolysis of *N*-acylated glycines (*b*): dependencies of the inhibition constants (*a*) and of the second-order rate constants of the hydrolysis (*b*) on the hydrophobicity of the alkyl radical.



where R= H, OH, F, Cl, Br, NH_2, NO_2, OCH_3

FIGURE 4. Structures of penicillin acylase inhibitors—phenylacetic acid derivatives.

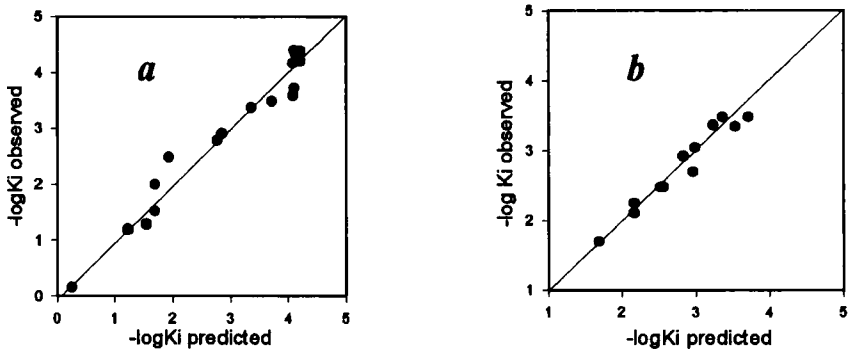


FIGURE 5. Penicillin acylase inhibition by phenylacetic acid derivatives: two-parameter correlations of the inhibition constants with the hydrophobicity and molecular volume of the inhibitor—(*a*) for *p*- and *m*-substituted PAA derivatives (equation 1); (*b*) for *o*- and α-substituted PAA derivatives (equation 2).

TABLE 2. Specificity of Penicillin Acylase to *N*-Phenylacetyl-α-Amino Acids

R[a]	k_{cat}/K_M ($M^{-1} \cdot s^{-1}$)
H	$1.9 \cdot 10^7$
CH_3	$2.1 \cdot 10^7$
C_2H_5	$4.6 \cdot 10^7$
i-C_3H_7	$5 \cdot 10^5$
i-C_4H_9	$3.8 \cdot 10^5$
C_4H_9	$2 \cdot 10^6$
Ph	$4.7 \cdot 10^6$
Ph-CH_2	$3.7 \cdot 10^7$
NH_2-$(CH_2)_4$	$2 \cdot 10^6$
PO(OH)(CH_3)-$(CH_2)_3$	$3.1 \cdot 10^6$
HOOC-CH_2	$1.7 \cdot 10^6$
H_2N-CO-CH_2	$1.2 \cdot 10^4$

[a]R in RCH(NHCOCH$_2$Ph)COOH.

TABLE 3. Specificity of Penicillin Acylase to *N*-Phenylacetyl-β-Amino Acids

R[a]	k_{cat}/K_M ($M^{-1} \cdot s^{-1}$)
CF_3	$3.1 \cdot 10^5$
CH_3	$2.8 \cdot 10^6$
C_3H_7	$1.3 \cdot 10^5$
i-C_3H_7	740
i-C_4H_9	$3 \cdot 10^4$
Ph	$1.1 \cdot 10^6$
4-F-Ph	$9 \cdot 10^5$
3,4,5-$(MeO)_3$-Ph	$1 \cdot 10^6$

[a]R in RCH(NHCOCH$_2$Ph)CH$_2$COOH.

TABLE 4. Stereospecificity of Penicillin Acylase to α-Amino Acid Derivatives

Substrate	$\frac{(k_{cat}/K_M)^L}{(k_{cat}/K_M)^D}$
Phenylacetyl-Ala^P	58,000
Phenylacetyl-Ala^{PH}	1400
Phenylacetyl-Ala	1000
Phenylacetyl-AlaOMe	280
Phenylacetyl-Ala-D-Ala	65

X-ray structures of the penicillin acylase modified by phenylmethylsulfonylfluoride and of the enzyme complex with phenylacetic acid, published recently, confirmed this observation.[5]

For a more detailed characterization of the acyl group binding subsite ρ1, penicillin acylase inhibition by 30 substituted phenylacetic acid (PAA) derivatives was studied (FIGURE 4). The competitive inhibition was found in all cases.

The best inhibitors were PAA and their derivatives with hydrophobic substituents (Cl-, Br-, etc.) in the *m*-position. The specificity was as follows: *m*-PAA > *p*-PAA > *o*-PAA. The derivatives of PAA with the polar substituents (-OH, -NH_2) in the α-position showed the lowest inhibition effect. No significant stereospecificity to the α-substituted derivatives (with an asymmetric α-carbon) was detected. A two-parameter correlation of the inhibition constants with the hydrophobicity (log P) and molecular volume (MolVol) of the inhibitor was shown. Two correlation series with different roles of the hydrophobicity were obtained: (i) for *p*- and *m*-substituted derivatives (FIGURE 5a),

$$-\log K_i = 1.2(\pm 0.1) \cdot \log P - 5.4(\pm 0.9) \cdot 10^4 \cdot \text{MolVol} + 6.5(\pm 0.8); \quad (1)$$

(ii) for *o*- and α-substituted inhibitors (FIGURE 5b),

$$-\log K_i = -0.4(\pm 0.04) \cdot \log P - 3.3(\pm 0.2) \cdot 10^4 \cdot \text{MolVol} + 8.0(\pm 0.3). \quad (2)$$

The *p*-OH and *m*-NO_2 derivatives did not fit the correlation. The following conclusions can thus be made based on the correlations obtained: despite the strongly limited capacity of the hydrophobic pocket ρ1 in the penicillin acylase active center (FIGURE 2), there is a possibility of additional hydrophobic interactions at the *m*-position of the benzyl radical and of H-bond formation at the *p*-position (≈6 kJ/mol); also, the hydrophobic pocket is covered by the polar narrow crack, which is not wider than 1.4–1.8 Å.

TABLE 5. Stereospecificity of Penicillin Acylase to Lysine Derivatives

Substrate	$(k_{cat}/K_M)^L$	$(k_{cat}/K_M)^D$	$\frac{(k_{cat}/K_M)^L}{(k_{cat}/K_M)^D}$
α-*N*-Phac-Lys	$2.0 \cdot 10^6$	13	150,000
di-α,ε-*N*,*N*-Phac-Lys	$5.9 \cdot 10^6$	$2.4 \cdot 10^3$	25
ε-*N*-Phac-Lys	$6.7 \cdot 10^6$	$4.6 \cdot 10^4$	1.5

TABLE 6. Stereospecificity of Penicillin Acylase for Different Substrate Series: $RCH(NHCOCH_2Ph)X$

R	X	$(k_{cat}/K_M)^L/(k_{cat}/K_M)^D$	X	R	$(k_{cat}/K_M)^L/(k_{cat}/K_M)^D$	X	R	$(k_{cat}/K_M)^L/(k_{cat}/K_M)^D$
CH_3	CH_2COOH	220	COOH	C_4H_9	400	CH_2COOH	CH_3	220
	$COOCH_3$	280		i-C_3H_7	780		CF_3	500
	$PO(OH)(OCH_3)$	520		i-C_4H_9	950		i-C_4H_9	940
	COOH	1000		CH_3	1000		p-Br-Ph	6600
	PO_2H_2	1400		p-F-Ph	2600		p-CH_3O-Ph	7900
	$PO(O\text{-}i\text{-}C_3H_7)_2$	1800		o-F-Ph	2800		p-Cl-Ph	9300
	$PO(OCH_3)_2$	2300		Ph	4300		3,4,5-$(CH_3O)_3$-Ph	12,500
	$PO(OH)_2$	58,000		$PO(OH)(CH_3)(CH_2)_3$	5300		p-F-Ph	13,000
				Ph-CH_2	5700		Ph	14,000
Ph	$PO(OH)_2$	300		NH_2-$(CH_2)_4$	150,000			
	COOH	4300						
	CH_2COOH	14,000						

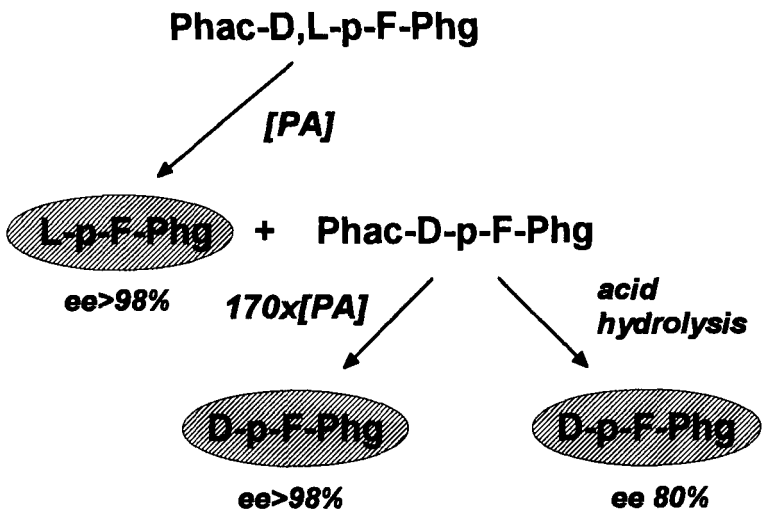


FIGURE 6. Biocatalytic preparation of D-*p*-fluorophenylglycine.

SPECIFICITY OF THE SUBSITE ρ2

Penicillin acylase has a wide substrate specificity to the α-amino acid side chain (TABLE 2). *N*-Phenylacetyl-β-amino acid derivatives (TABLE 3) are much less reactive substrates in comparison with the corresponding α-amino acid derivatives. The most remarkable decrease in the penicillin acylase catalytic activity is observed in the case of α- and β-amino acids with a branched alkyl side chain. Thus, sterically

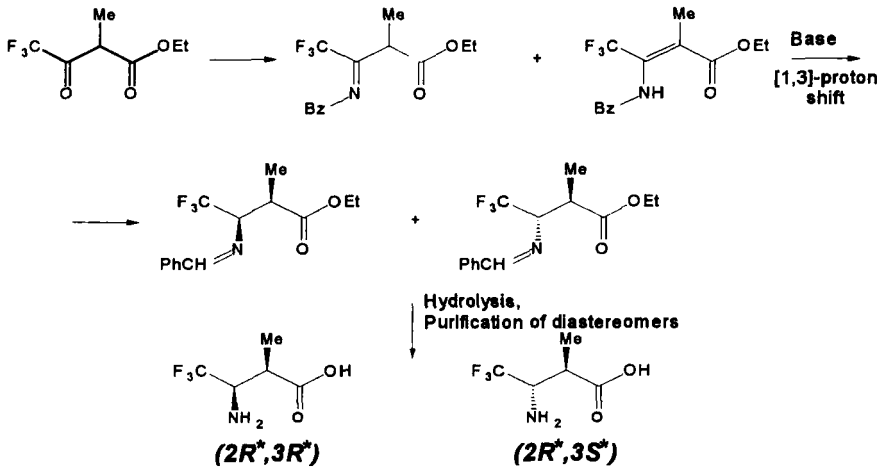


FIGURE 7. Scheme of the stereoselective synthesis of diastereomers of α-methyl-β-trifluoromethyl-β-alanine.

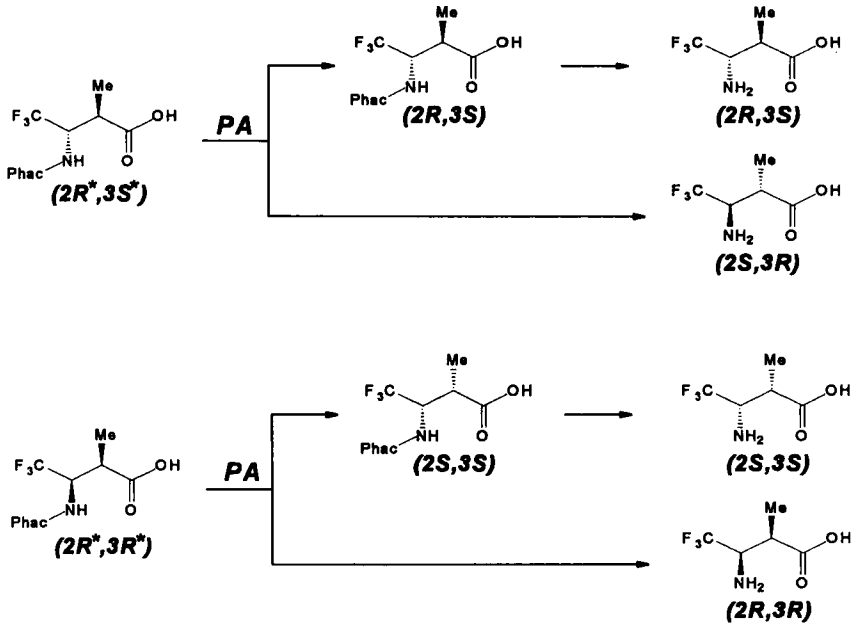


FIGURE 8. Scheme of the penicillin acylase–catalyzed preparation of the four isomers of α-methyl-β-trifluoromethyl-β-alanine.

controlled interactions are the most important factor governing substrate specificity at subsite ρ2.

INTERACTIONS AT SUBSITE ρ3

Interaction of the negative charge of the substrate at subsite ρ3 is the most important factor for chiral discrimination in the penicillin acylase active center (TABLE 4). The highest stereospecificity, equal to 58,000, is observed in the case of 1-*N*-phenylacetylaminoethylphosphonic acid (phosphonic analogue of alanine); hydrolysis of 1-*N*-phenylacetylaminophosphonous acid (phosphonous analogue of alanine) is characterized by about the same stereospecificity as for *N*-phenylacetylalanine hydrolysis. However, when the negatively charged group of the substrate is blocked, the penicillin acylase stereospecificity is remarkably decreased. Thus, it is reasonable to suggest the presence of a positively charged group in the subsite ρ3 that is responsible for a rather effective electrostatic interaction with the negatively charged group of the substrate. Experimental data on the hydrolysis of the different *N*-phenylacetylated derivatives of lysine strongly support this suggestion (TABLE 5). Penicillin acylase–catalyzed hydrolysis of α-*N*-phenylacetyl-L-lysine (the positively charged side chain group is located in subsite ρ2 and the carboxy group is in subsite ρ3) proceeds with the same second-order rate constant, $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, as *N*-phenylacetyl-L-norleucine hydrolysis. Therefore, the positive charge in this case does

not have any significant influence on the catalytic properties of the enzyme. In the case of the D-enantiomers (where the side chain should be located at subsite ρ3 and the carboxy group at subsite ρ2), hydrolysis of α-*N*-phenylacetyl-D-lysine proceeds with a second-order rate constant of 13 $M^{-1} \cdot s^{-1}$, which is 380 times lower than the second-order rate constant for hydrolysis of *N*-phenylacetyl-D-norleucine. When the amino group of the side chain radical is acylated and the positive charge is extinguished, the second-order rate constant for di-α,ε-*N*,*N*-phenylacetyl-D-lysine hydrolysis is increased by more than 18,000-fold.

Hence, the chiral discrimination of the substrate in the penicillin acylase active center is based on the balance of the electrostatic interactions of the substrate with the positive charge in subsite ρ3 and sterically controlled hydrophobic interactions at subsite ρ2.

PENICILLIN ACYLASE–CATALYZED PREPARATION OF ENANTIOMERS

In general, penicillin acylase–catalyzed hydrolysis of *N*-phenylacetylated α- and β-amino acids is characterized by a rather high stereospecificity (TABLE 6). It should be mentioned that the stereospecificity in enzymatic hydrolysis of *N*-phenylacetyl-β-amino acids is lower, with the exception of *N*-phenylacetyl-β-phenylalanine and its derivatives. Because of the high stereospecificity, penicillin acylase can be used for preparative synthesis of the enantiomers of different amino acids.

In some cases, penicillin acylase possesses high stereospecificity and good catalytic activity in the hydrolysis of both enantiomers of the substrate. It is especially

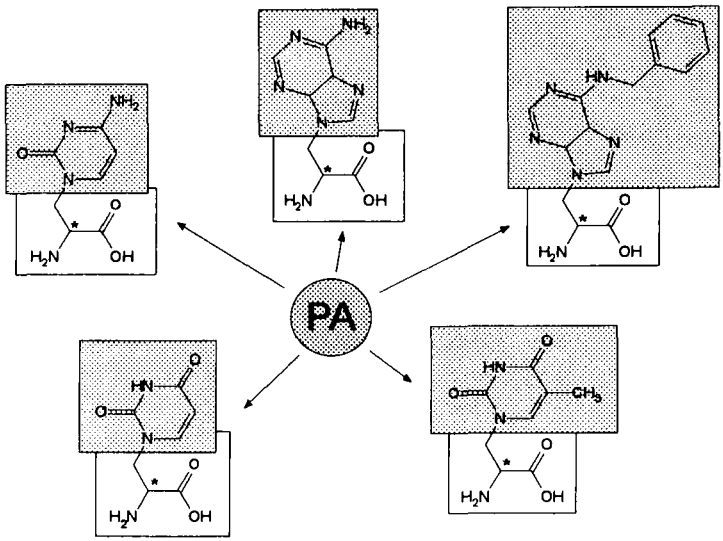


FIGURE 9. Structures of nucleo-amino acids prepared in optically pure form using penicillin acylase.

important when acidic hydrolysis of the D-enantiomer of the substrate proceeds with remarkable racemization (FIGURE 6).

The combination of the stereoselective synthesis of diastereomers and the consequent biocatalytic resolution of pure diastereomers into the pair of enantiomers provided a basis for the synthesis of each of the four isomers of the compounds with two chiral centers. α-Alkyl-β-fluoroalkyl-substituted β-amino acids represented a visual example for such a chemoenzymatic approach.[23] Diastereomers of free α-methyl-β-trifluoromethyl-β-alanine were synthesized and isolated in diastereomerically pure form by recrystallization (FIGURE 7). Phenylacetyl derivatives were synthesized with a good yield and the preparative enzymatic resolution of the four isomers was performed (FIGURE 8).

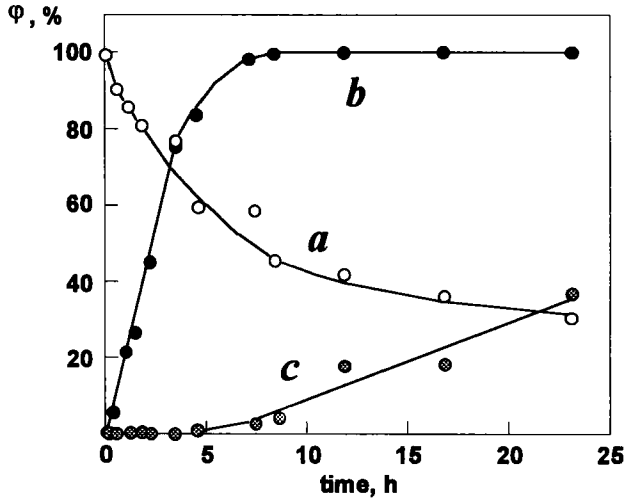


FIGURE 10. Progress curves of penicillin acylase–catalyzed hydrolysis of D,L-*N*-phenylacetyl-β-(Cytozinyl-N^1)-α-alanine (*a*) and accumulation of L-enantiomer (*b*) and D-enantiomer (*c*) of the nucleo-amino acid. Conditions: pH 7.5, 0.1 M phosphate, 25 °C.

Nucleo-amino acids represent a new field for application of penicillin acylase in preparation of enantiomers (FIGURE 9). Enzymatic hydrolysis of *N*-phenylacetylated nucleo-amino acids is quite a stereospecific reaction, leading to the consequent hydrolysis of both enantiomers (FIGURE 10). Enantiomers of four synthetic nucleo-amino acids and the natural nucleo-amino acid, 3-(6-benzylaminopurin-9-yl)alanine, were prepared with high optical purity.[24]

REFERENCES

1. VANDAMME, E. J. 1980. Microbial enzymes and bioconversions. *In* Economic Microbiology. Volume 5. A. A. Rose, Ed.: 467–522. Academic Press. New York.
2. ABBOTT, B. J. 1976. Adv. Appl. Microbiol. **20:** 203–257.
3. SHEWALE, J. G. & H. SIVARAMAN. 1989. Proc. Biochem. **24:** 146–154.
4. VIRDEN, R. 1990. Biotechnol. Genet. Eng. Rev. **8:** 189–218.

5. DUGGLEBY, H. J., S. P. TOLLEY, C. P. HILL, E. J. DODSON, G. DODSON & P. C. E. MOODY. 1995. Nature **373:** 264–268.
6. ŠVEDAS, V. K., I. YU. GALAEV, YU. A. SEMILETOV & G. A. KORSHUNOVA. 1983. Bioorg. Chem. **9:** 1139–1141.
7. WALDMANN, H. & D. SEBASTIAN. 1994. Chem. Rev. **94:** 911–937.
8. SOLOSHONOK, V. A., V. K. ŠVEDAS, V. P. KUKHAR, I. YU. GALAEV, E. V. KOZLOVA & N. YU. SVISTUNOVA. 1993. Bioorg. Khim. **19(4):** 478–483.
9. WALDMANN, H. 1989. Tetrahedron Lett. **30(23):** 3057–3058.
10. FUGANTI, C., C. M. ROSELL, S. SERVI, A. TAGLIANI & M. TERRENI. 1992. Tetrahedron Asymmetry **3(3):** 383–386.
11. BALDARO, E., P. D'ARRIGO, G. PEDROCCHI-FANTONI, C. M. ROSELL, S. SERVI, A. TAGLIANI & M. TERRENI. 1993. Tetrahedron Asymmetry **4(5):** 1031–1034.
12. ZMIJEWSKI, M. J., B. S. BRIGGS, A. R. THOMPSON & I. G. WRIGHT. 1991. Tetrahedron Lett. **32(13):** 1621–1622.
13. SOLODENKO, V. A., T. N. KASHEVA, V. P. KUKHAR, E. V. KOZLOVA, D. A. MIRONENKO & V. K. ŠVEDAS. 1991. Tetrahedron **47(24):** 3989–3998.
14. SOLODENKO, V. A., M. Y. BELIK, S. V. GALUSHKO, V. P. KUKHAR, E. V. KOZLOVA, D. A. MIRONENKO & V. K. ŠVEDAS. 1993. Tetrahedron Asymmetry **4(9):** 1965–1968.
15. SOLOSHONOK, V. A., A. G. KIRILENKO, N. A. FOKINA, I. P. SHISHKINA, S. V. GALUSHKO, V. P. KUKHAR, V. K. ŠVEDAS & E. V. KOZLOVA. 1994. Tetrahedron Asymmetry **5(6):** 1119–1126.
16. SOLOSHONOK, V. A., N. A. FOKINA, A. V. RYBAKOVA, I. P. SHISHKINA, S. V. GALUSHKO, A. E. SOROCHINSKY, V. P. KUKHAR, V. K. ŠVEDAS & M. V. SAVCHENKO. 1995. Tetrahedron Asymmetry **6(7):** 1601–1610.
17. COLE, M. 1969. Biochem. J. **115:** 733–739.
18. KUTZBACH, C. & E. RAUENBUSCH. 1974. Hoppe-Seyler's Z. Physiol. Chem. **354:** 45–53.
19. FUGANTI, C., P. GRASSELLI, S. SERVI, A. LAZZARINI & P. CASATI. 1987. J. Chem. Soc. Chem. Commun., p. 538–539.
20. MARGOLIN, A. I., V. K. ŠVEDAS & I. V. BEREZIN. 1980. Biochim. Biophys. Acta **616:** 283–289.
21. ŠVEDAS, V. K. 1976. Ph.D. thesis. Department of Chemistry, Moscow State University.
22. BELTSER, A. I. 1995. Diploma work. Department of Chemistry, Moscow State University.
23. SOLOSHONOK, V. A., A. G. KIRILENKO, N. A. FOKINA, V. P. KUKHAR, S. V. GALUSHKO, V. K. ŠVEDAS & G. RESNATI. 1994. Tetrahedron Asymmetry **5(7):** 1225–1228.
24. BAGERZADEH, G., A. A. KOZLOVSKY, N. V. SUMBATIAN, G. A. KORSHUNOVA, M. V. SAVCHENKO & V. K. ŠVEDAS. 1995. In preparation.