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# Enzymatic Preparation of Both L- and D-Enantiomers of Phosphonic and Phosphonous Analogues of Alanine Using Penicillin Acylase

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**ABSTRACT:** D-Enantiomers of N-acylated 1-aminoethylphosphonic and 1-aminoethylphosphonous acids were able to be hydrolyzed with high concentrations of penicillin acylase in a reasonable time period. This finding was used to prepare both L- and D-enantiomers of these phosphorus analogues of alanine by stepwise enzymatic hydrolysis of their racemic N-phenylacetyl derivatives using the same enzyme - penicillin acylase - by simply changing the enzyme/substrate ratio.

Phosphorus analogues of α-amino acids have received considerable attention in the past because of their potential or proven bioactivity[1]. It is well recognized that the bioactivity of these compounds is dependent on their stereoconfiguration[1], which makes obtaining them in enantioenriched (up to enantiopure) forms of practical significance. The enzymatic resolution of racemates was recently shown to be a useful approach for preparing enantiomeric α–aminophosphonic[2] and α-aminophosphonous[3] acids (APA). In this approach, racemic N-acylated APA are hydrolyzed stereoselectively by penicillin acylase (EC 3.5.1.11) to give L-APA and intact D-stereoisomers of the substrates which are then transformed into D-APA by prolonged refluxing in acidic medium. The latter procedure is not optimal because of possible racemization[4]. We demonstrate here that this transformation can be accomplished under mild conditions (neutral medium, room temperature) using high concentrations of penicillin acylase. The utility of such an approach is exemplified by the preparation of both L- and D-enantiomers of the phosphorus analogues of alanine, namely 1-aminoethylphosphonic ($Ala^P$) and 1-aminoethylphosphonous ($Ala^{P\text{-}H}$) acids which are used in the biological researches[5].

Penicillin acylase has been shown to reveal high enantioselectivity in the hydrolysis of L- and D-forms of 1-(N-phenylacetylamino)ethylphosphonic acid (PhAc-$Ala^P$), the rate of D-form hydrolysis being low[6]. As a consequence, under optimal conditions (initial racemic substrate concentration about 0.1 M, enzyme concentration about $10^{-7}$ M), only the L-substrate is hydrolyzed by the enzyme in 1-2 h; the hydrolysis of the D-form is negligible even after prolonged incubation times[2] However, computer simulation of the enzymatic hydrolysis of PhAc-$Ala^P$ indicates that an increase in enzyme concentration can result in a significant increase in the rate of D-substrate hydrolysis[2]. Indeed, when D-PhAc-$Ala^P$ was incubated with a high enzyme concentration (50-100 times as large as that used for L-$Ala^P$ preparation) over 3-4 days, D-$Ala^P$ was isolated

in good yield and excellent optical purity (ee >99%[7]) Thus, owing to the high enantioselectivity of the process, the enzymatic hydrolysis of racemic PhAc-Ala$^{P}$ can be carried out in two separate stages without any special precautions at low enzyme concentration only the L-form of the substrate is hydrolyzed, the D-form can be hydrolyzed with noticeable velocity only by enhanced amounts of the enzyme

In comparison with N-acylated Ala$^{P}$, the hydrolysis of 1-(N-phenylacetylamino)ethylphosphonous acid (PhAc-Ala$^{P\text{-}H}$) by penicillin acylase proceeds with moderate enantioselectivity ($E$ = 1440), the rate of D-substrate hydrolysis being relatively high ($K_D$ = 1 37·10$^{4}$ M$^{-1}$s$^{-1}$). In this case, the hydrolysis of the L-substrate is accompanied with the appreciable hydrolysis of the D-form even at low enzyme concentration Nevertheless, the computer simulation of the process using the kinetic parameters found (fig 1) shows that L-Ala$^{P\text{-}H}$ can be obtained in satisfactory yield and with high optical purity under appropriate conditions.[2] In practice, the controlled enzymatic hydrolysis of racemic PhAc-Ala$^{P\text{-}H}$ at low enzyme concentration (see below) resulted in L-Ala$^{P\text{-}H}$ in 80% yield with 94% ee[7]. The incubation of the remaining D-substrate with an increased amount of penicillin acylase for 36 h led to D-Ala$^{P\text{-}H}$ with a low ee, 76% Due to the relatively high $K_D$ value, the 5-10-fold increase in the enzyme amount is enough to produce the effective hydrolysis of D-PhAc-Ala$^{P\text{-}H}$ To obtain enantiopure D-PhAc-Ala$^{P\text{-}H}$, the racemic substrate was incubated with low enzyme concentration for twice the time period (compared with that used for L-Ala$^{P\text{-}H}$ preparation under the same conditions), or the D-substrate remaining after L-Ala$^{P\text{-}H}$ preparation was further incubated with low enzyme concentration for one more time period. It is to be noted that prolonged incubation under these conditions does not lead to a significant decrease in the yield of enantiopure D-PhAc-Ala$^{P\text{-}H}$ The enzymatic hydrolysis of this compound results in D-Ala$^{P\text{-}H}$ of excellent optical purity (ee >99%[7]). Thus, because of the moderate enantioselectivity of penicillin acylase in regard to PhAc-Ala$^{P\text{-}H}$, the course of enzymatic hydrolysis of this substrate has to be carefully controlled to achieve the high optical purity of the desired products, especially during the L-Ala$^{P\text{-}H}$ preparation

In summary, both L- and D-enantiomers of 1-aminoethylphosphonic and 1-aminoethylphosphonous acids are obtained in high chemical and optical yields through the step by step enzymatic hydrolysis of their racemic N-phenylacetyl derivatives using the same enzyme - penicillin acylase - by simply changing the enzyme/substrate ratio

*L-1-Aminoethylphosphonic acid* (L-Ala$^{P}$ ) was obtained as described[2] after 2-h stirring of 0 12 M D,L-PhAc-Ala$^{P}$ and 0.8·10$^{-7}$ M penicillin acylase solution in 0 01 M phosphate buffer, pH 6 85 Yield 82% (after crystallization from water-ethanol), mp 274-277 °C dec, ee >99%[7] , $[\alpha]_D^{20}$ -17.0 (c 0 5, 1N NaOH). Lit[8], $[\alpha]_D^{20}$ -16.9 (c 2, 1N NaOH) The second product of the process, D-PhAc-Ala$^{P}$ , was isolated in 78% yield, mp 149-152 °C (water), $[\alpha]_D^{20}$ +38 0 (c 0 5, $H_2O$), and used in D-Ala$^{P}$ preparation (see below).

*D-1-Aminoethylphosphonic acid* (D-Ala$^{P}$). D-PhAc-Ala$^{P}$ (243 mg, 1 mmol) was stirred with 100 ml of 1·10$^{-6}$ M penicillin acylase solution in 0 01 M phosphate buffer, pH 7.0, at r t for 3 days, the pH being maintained at 6 9-7 1 by addition of 1N KOH The mixture was treated as described[2], and, after cation-exchange and crystallization from water-ethanol, 94 mg (75%) of D-Ala$^{P}$ was obtained, mp 277-279 °C dec, ee >99%[7] , $[\alpha]_D^{20}$ +16.7 (c 0.5, 1N NaOH). Lit[8] , $[\alpha]_D^{20}$ +16 8 (c 2, 1N NaOH)

*L-1-Aminoethylphosphonous acid* (L-Ala$^{P\text{-}H}$). D,L-PhAc-Ala$^{P\text{-}H}$ (700 mg, 3 1 mmol) was dissolved in 40 ml water, and pH was adjusted to 7.95 with 1N KOH. Immobilized penicillin acylase[2] (0.5 g, specific activity 2 10$^{3}$ U/g) was added, and the mixture was stirred at r.t , the reaction progress being controlled by HPLC After stirring for 1.5 h the enzyme was filtered off, the filtrate was acidified to pH 5 with 20% HCl and washed

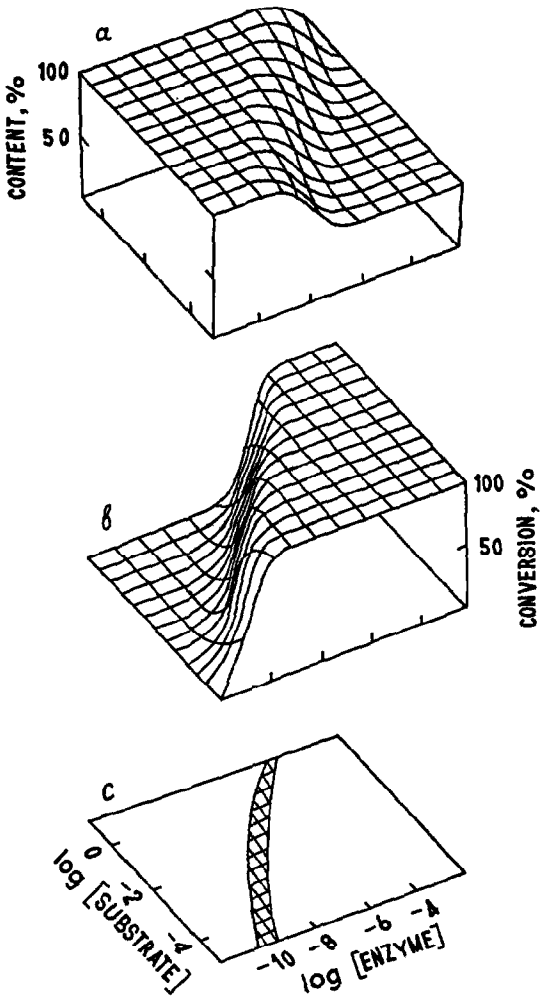


Fig. 1. Computer simulation of D,L-PhAc-Ala$^{P-H}$ enzymatic hydrolysis.

The conversion of L-PhAc-Ala$^{P-H}$ to L-Ala$^{P-H}$ (b) and the content of the L-Ala$^{P-H}$ in the total Ala$^{P-H}$ (a) in dependence on the initial concentrations of penicillin acylase and racemic substrate after 1 h of the enzymatic reaction are presented. The cross-hatched area (c) shows the concentration range where the conversion of L-form of the substrate and the content of L-Ala$^{P-H}$ exceed 90% and 98%, respectively.

with ether. The water solution was concentrated *in vacuo* to 15 ml volume and passed through a Dowex 50Wx8 ($H^+$-form) using water as an eluent. Weakly acidic ninhydrin-positive fractions were evaporated, the waxy residue was dissolved in 20% HCl (4 ml) and evaporated to dryness. The glassy material was dissolved in absolute ethanol (4 ml), and propylene oxide (3 ml) was added. The solid precipitate was filtered off, washed with absolute ethanol and ether, and dried *in vacuo* at 80 °C to give 134 mg (80%) L-$Ala^{P-H}$, mp 220-223 °C dec, ee 94%[7], $[\alpha]_D^{20}$ -6.0 (c 0.5, $H_2O$). Lit[9]., $[\alpha]_D^{20}$ -6.4 (c 2, $H_2O$).

*D-1-Aminoethylphosphonous acid* (D-$Ala^{P-H}$). D,L-PhAc-$Ala^{P-H}$ was stirred for 3.5 h with immobilized penicillin acylase under conditions described above for L-$Ala^{P-H}$ preparation. The products of the reaction were separated on Dowex 50Wx8 ($H^+$-form, eluent - water). D-PhAc-$Ala^{P-H}$ was isolated from more acidic ninhydrin-negative and chlorine-tolidine-positive fractions and crystallized from dioxane. Yield 73%, mp 138-140 °C, $[\alpha]_D^{20}$ +74.0 (c 0.5 $H_2O$). This compound (227 mg, 1 mmol) was dissolved in 25 ml water, and pH was adjusted to 7.8 with 1N KOH. Immobilized penicillin acylase[2] (2 g, $2 \cdot 10^3$ U/g) was added, and the mixture was stirred at r.t. for 36 h. The mixture was treated as described above for L-$Ala^{P-H}$ preparation to give 90 mg (83%) D-$Ala^{P-H}$, mp 222-224 °C dec, ee >99%[7], $[\alpha]_D^{20}$ +6.8 (c 0.5, $H_2O$). Lit[9]., $[\alpha]_D^{20}$ +7.0 (c 2, $H_2O$).

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6. Mironenko D.A., Kozlova E.V., Svedas V.K., Solodenko V A., Kasheva T.N., Kukhar V.P. *Biokhimiya*, **1990**, *55*, 1124. Enantioselectivity $E$ is expressed as a ratio of bimolecular rate constants, $K_L$ and $K_D$, for enzymatic hydrolysis of L- and D-forms of the substrate. For PhAc-$Ala^P$, $E$ was found to be 58000, the $K_D$ being 111 $M^{-1}s^{-1}$
7. Enantiomeric excesses (ee) of the optically active aminophosphonic (-phosphonous) acids obtained were determined by HPLC after pre-column derivatization with *o*-phthalaldehyde and N-acetyl-L-cysteine (Galushko S.V., Belik M.Y., Solodenko V.A. *J. Chromatogr.*, submitted for publication).
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