Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 2221–2229

BIOORGANIC & MEDICINAL CHEMISTRY

Use of Enzyme Penicillin Acylase in Selective Amidation/Amide Hydrolysis to Resolve Ethyl 3-amino-4-pentynoate Isomers

Ravindra S. Topgi,[a] John S. Ng,[a] Bryan Landis,[b] Ping Wang[b] and James R. Behling[a,*]

[a]*R&D, Chemical Sciences, SEARLE, 4901 Searle Parkway, Skokie, IL 60077, USA*
[b]*Bioprocess Group, MONSANTO, Chesterfield, St. Louis, MO 63198, USA*

Received 10 May 1999

Abstract—The β-amino acid, (*S*)-ethyl-3-amino-4-pentynoate, is a chiral synthon used in the synthesis of xemilofiban hydrochloride, an anti-platelet agent. A biocatalytic approach was developed to resolve (*R*)- and (*S*)-enantiomers of ethyl 3-amino-4-pentynoate in enantiomerically pure form employing the enzyme Penicillin acylase. In the acylation, phenylacetic acid was used as an acylating agent. We have shown that both the acylation and deacylation can be employed and that the activity of the enzyme Penicillin acylase can be controlled by maintaining an appropriate pH of the reaction medium.

Introduction

In recent years there has been a surge of research activity in the area of β-amino acids. Besides exhibiting powerful antibacterial properties themselves, β-amino acids are key constituents of many naturally occurring and synthetic compounds of pharmaceutical and medicinal interests, such as peptides, terpenes, alkaloids, macrolides and β-lactam antibiotics. Most recently, the emerging importance of the Taxol-related family of antitumor agents and the antiplatlet aggregation agent xemilofiban has intensified research to prepare these compounds effectively on a large scale. A monograph and a number of reviews dealing with synthesis of β-amino acids have been published recently.[1–11] The search for novel asymmetric synthetic methods for preparing β-amino acids has received much attention (e.g. catalytic,[12–14] stiochiometric,[15–20] manipulation of chiral pool,[21–25] and enzymatic syntheses[26]). In comparison with non-biological based approaches, enzymatic syntheses have shown reaction specificity, enantioselectivity, rate enhancement and favorable environmental impacts.[27]

Although the biocatalytic approach has been successfully applied in the production of α-amino acids, its application for the preparation of enantiomerically pure β-amino acids is gaining much more interest.[28–34] Amino acylases[35] and amino peptidases,[36] which are commonly used for the resolution of α-amino acids, do not resolve β-amino acids, or show low affinity and stereoselectivity in the hydrolytic processes. Lipases catalyze the enantioselective hydrolysis of the esters with modest stereoselectivity.[26] The remarkable selectivity (both regio and enantioselectivity) of amidases and mild reaction conditions have been successfully utilized in several commercialized biocatalytic processes.[37]

Penicillin G. amidohydrolase (PGA) (E.C. 3.5.1.11) from *Escherichia coli* ATCC 9637 is a unique enzyme that exhibits exceptionally high affinity to phenylacetic acid derivatives.[38,39] This capability has been advantageously used to achieve moderate to excellent stereochemical discrimination between corresponding enantiomers in the hydrolytic cleavage of the phenylacetyl group from α-aminoalkylphosphonic acids,[40,41] α-, β- and γ-amino carboxylic acids,[42–44] sugars,[45] amines,[46] peptides,[47] and esters of phenylacetic acid.[48,49] The chemoselectivity of PGA for the phenylacetyl moiety has led to the development of new protecting groups for amino, hydroxy and carboxy functions, which have been used in peptide, carbohydrate and β-lactam chemistry.[50–54]

In this paper, we report a selective amidation/amide hydrolysis using the enzyme Penicillin G amidohydrolase from *E. coli* to synthesize (*R*)- and (*S*)-enantiomers of ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate in optically pure form.

Key words: Penicillin acylase; resolve; β-amino acid; acylation; deacylation.
* Corresponding author.

0968-0896/99/$ - see front matter
PII: S0968-0896(99)00155-8

Results and Discussion

As a part of ongoing efforts to synthesize a potent, orally active anti-platelet agent, xemilofiban hydrochloride **(1)**,[55–58] we proposed to develop an efficient chemoenzymatic process for **(2)**, the chiral β-amino acid ester synthon for xemilofiban hydrochloride **(1)** (Fig. 1).

Enzymatic approach

The catalytic activity of the enzyme PGA is pH dependent. In the pH range 7–8.5 it exhibits deacylation activity, whereas at acidic pH 5–6 acylation is observed.[59] This well pronounced pH dependent enzyme activity offers the possibility of exploring selective amidation/amide hydrolysis to synthesize (*R*)- and (*S*)-enantiomers of ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate in optically pure form (Fig. 2).

Deacylation

The racemic ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate (**3**) needs to be acylated prior to the deacylation by the enzyme PGA. In the presence of triethylamine, phenylacetyl chloride reacts with a racemic amine to yield a phenylacetyl derivative **4**. Heptane or ethyl acetate can be used as the reaction medium. The reaction at room temperature for two hours gave the desired product **4** (Fig. 3). The racemic ethyl 3-(*N*-phenylacetyl)-5-(trimethylsilyl)-4-pentynoate **(4)** was placed in the phosphate buffer (pH 7.4). The pH of the medium was adjusted to ~7.5. At room temperature immobilized enzyme Penicillin G amidohydrolase was added to the reaction medium and the pH of the reaction medium was maintained at approximately 7.4 using dilute potassium bicarbonate solution. The HPLC was used to monitor the reaction. The reaction was usually complete in approximately 20 h. After completion of the reaction, the reaction mixture was extracted with ethyl acetate. The ethyl acetate extract was then extracted with dilute hydrochloric acid. The organic phase contained the (*S*)-amide (**6a**) (96% yield). The pH of the aqueous phase was raised to ~8 using potassium carbonate and extracted with ethyl acetate to obtained (*R*)-amine (**7a**) (91% yield). Similarly, starting with the racemic ethyl 3-(*N*-phenylacetyl)-4-pentynoate (**5**) (*S*)-amide (**6b**) (95% yield) and (*R*)-amine (**7b**) (92% yield) were obtained.

The results of the PGA catalyzed hydrolysis of **4** and **5** were similar, but because the final product xemilofiban hydrochloride **(1)** does not contain the trimethylsilyl group at the alkyne carbon, it was preferable to perform

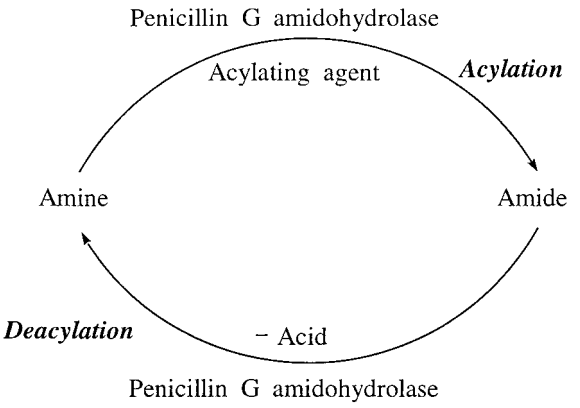


Figure 2. Acylation and deacylation by enzyme Penicillin G amidohydrolase.

desilylation prior to the enzymatic deacylation. Desilylation of the phenylacetyl derivative **4** was achieved by treatment with 0.2 equivalents of sodium ethoxide in ethanol at room temperature. Desilylation was complete in one hour. This racemic amide **5** was then subjected to the PGA catalyzed deacylation reaction. Because of the selective preference of PGA towards the (*R*)-enantiomers, the rate of deacylation of both (*R*)-enantiomers is significantly higher than the (*S*)-enantiomers. The result is the selective deacylation of the (*R*)-enantiomers (Fig. 4).

The *R*-amine **(7)** is a liquid, whereas the (*S*)-amide **(6)** is a solid. The difference in this physical property enabled easy separation of antipodes by filtration.

The desired *S*-amide **(6)** needs to be deacylated prior to the synthesis of the final compound, xemilofiban hydrochloride **(1)**. The slow rate of deacylation of the (*S*)-amide by the enzyme PGA can be enhanced significantly by using a high concentration of the enzyme. The temperature can also be increased to increase the rate of enzymatic hydrolysis. The (*S*)-amide obtained from the above reaction can be deacylated to liberate the free (*S*)-amine **(9)** using excess PGA (Fig. 5).

Acylation

Racemic ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate (**3**) is an insoluble liquid that forms an emulsion in water, whereas the (*R*)-phenylacetamide (**10**) is an insoluble solid. Based on the difference in this physical property, the equilibrium of the enzyme acylation reaction can be shifted towards the synthesis of the amide by

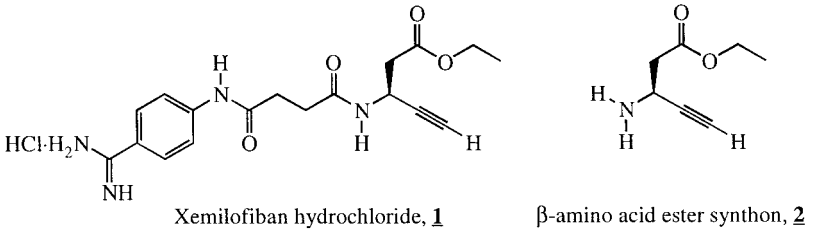


Figure 1. Anti-platelet agent xemilofiban hydrochloride and β-aminoacid ester synthon.

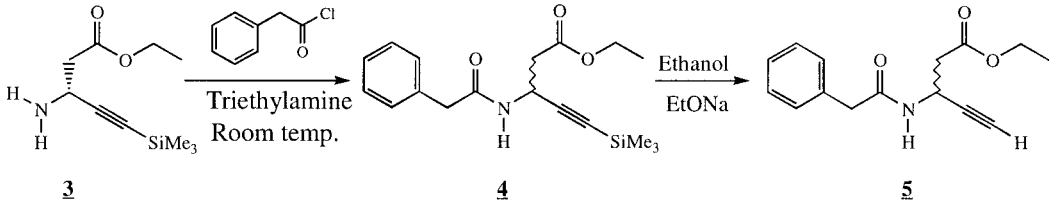


Figure 3. Synthesis of desilylated phenylacetamide.

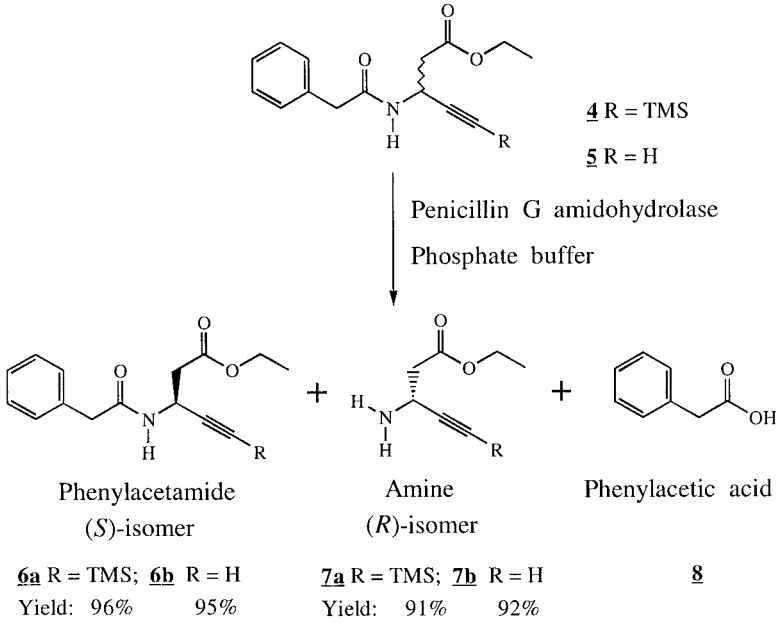


Figure 4. Hydrolysis of phenylacetamides using Penicillin G amidohydrolase.

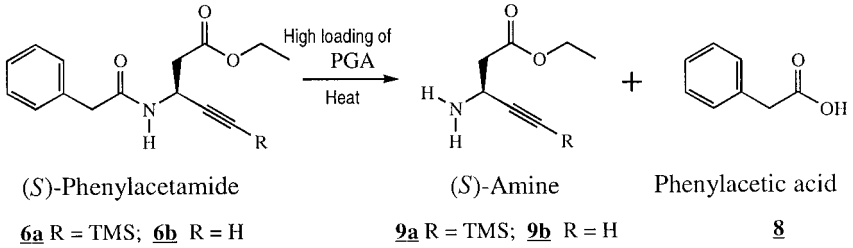


Figure 5. Hydrolysis of (*S*)-phenylacetamide using Penicillin G amidohydrolase.

precipitating the formed acylated product[60] (Fig. 6). The racemic ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate (**3**) was added to dilute hydrochloric acid. The pH of the reaction medium was then adjusted to 6. Phenylacetic acid (2 equiv) was added and the pH of the medium was readjusted to 6. Soluble Penicillin G amidohydrolase (50 units/100 mg of racemic amine) was added and the reaction was allowed to stir at room temperature. After completion of the reaction, the pH of the reaction mixture was adjusted to 4. The filtration of the reaction mixture gave (*R*)-amide **(10)** in a quantitative yield. Chiral HPLC analysis of this isolated amide showed absence of (*S*)-amide. The pH of the filtrate was raised to ~8 and extracted with ethyl acetate to obtain (*S*)-amine **(11)** (yield: 90%). The chiral HPLC analysis indicated *R:S* ratio of 2:98.

Compared to the deacylation approach, the acylation methodology does not require prior chemical phenylacylation and also eliminates the final deacylation of the *S*-amide using excess PGA. The isomerization of the undesired (*R*)-enantiomer allows for maximum conversion of racemic amine to the desired (*S*)-amine.

The Enzyme Penicillin G Amidase

The commercial supply is primarily derived from expressing the enzyme Penicillin G. amidohydrolase (E.C. 3.5.1.11) ATCC 9637 in *E. Coli.* We obtained soluble enzyme from Calbiochem and Boehringer-Mannheim. The immobilized enzyme was received from Sigma, Rohm-Pharma and Boehringer-Mannheim.

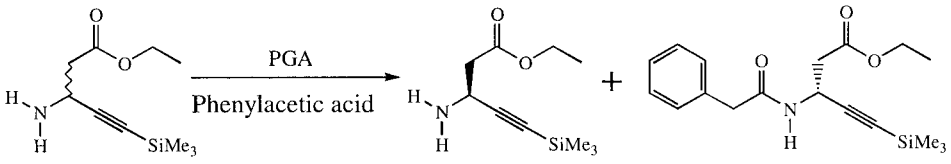


Figure 6. Acylation using Penicillin G amidohydrolase.

Under our experimental conditions, recovery of immobilized enzyme made it preferable to the soluble enzyme.

Acyl Group Specificities

The enzyme active site has a well defined structural requirement for the acyl moiety. PGA shows a rather broad substrate specificity.[53] In the case of substituted phenyl acetamides, PGA displays higher specificity to the phenylacetyl group. Instead of p-hydroxyphenyl acetyl and phenoxyacetyl groups, the phenylacetyl group was considered for its easier bulk availability. It has been reported that the esters of phenylacetic acid have enhanced reactivity in PGA enzyme reactions.[61,62] Commercially available acylating agents such as methyl phenoxyacetate, methyl phenylacetate, ethyl phenylacetate and phenylacetic acid were used. All of these reagents were compatible with the conditions used in the enzyme reaction. Methyl phenoxyacetate gave higher yields of amide than methyl phenylacetate. Phenylacetic acid proved quite reactive under our enzymatic reaction conditions. However, based on ease of handling and the possibility of recyclization without derivatization, we chose phenylacetic acid as the preferred acylating agent.

Organic Cosolvent

The interaction between the enzyme reaction center and acyl moiety of the substrate is a prerequisite for the product formation. Therefore, solubility of the substrate in the reaction medium is important. Although the enzyme PGA prefers an aqueous environment, reactions can be run in the presence of a low percentage of organic cosolvent.[63] In order to solubilize the substrates in water, a certain amount of organic cosolvent was required. Experiments were performed using acetone, tetrahydrofuran, acetonitrile, isopropanol, methanol, and toluene. Methanol and acetonitrile showed compatibility in the PGA catalyzed deacylation, whereas acetone was a more acceptable solvent in acylation reactions. Even though the reaction was a heterogeneous mixture without an organic cosolvent, the enzyme performance was equally good.

In general, the presence of an organic cosolvent leads to a more complicated chemical environment in the enzymatic reaction medium. The enzymatic reaction carried out in a medium without organic cosolvent gave one enantiomer in the solid form and the other in the liquid form. This allowed easy separation of the enantiomers.

Phosphate Buffer

Phosphate buffer is known to enhance the activity of the enzyme Penicillin G amidase.[64] Our studies showed that the enzyme displayed satisfactory activity in water without phosphate buffer. Use of water as a reaction medium would be advantageous for industrial applications.

pH of the Reaction Medium

The pH of the reaction medium dictates the activity as well as the function of the enzyme PGA. Therefore, the pH plays an important role. Enzyme PGA from different sources might show different activity as well as selectivity toward (*R*)- and (*S*)-enantiomers depending on the pH of the reaction medium. The acylation reaction was studied at different pH values ranging from 4.5 to 7.5. The enzyme showed maximum acylation activity around pH 5.3. However, at pH values below 5.2 acylation activity sharply declined. A pH lower than 5 might have a detrimental effect on enzyme stability and activity. The pH range 5.25–6.25 (Fig. 7) seems to be optimum for phenylacetamide formation.

Reaction Monitoring

The difference in reaction rates for the PGA catalyzed enzymatic deacylation of (*R*)- and (*S*)-enantiomers permits the deacylation of one of the enantiomers selectively. Cleavage of 100% of the (*R*)-amide corresponds to 50% conversion of the racemic amide. The ideal process would consume 100% of the (*R*)-amide. However, high temperature, longer reaction time and high concentration of enzyme promote the deacylation of the (*S*)-amide. In order to limit the cleavage of the phenylacyl group of the (*S*)-enantiomer, the reaction has to be stopped at 50% consumption of the starting racemic amide. Progress of the enzymatic deacylation reaction and consumption of 50% of the racemic amide can be determined by two methods: base addition and HPLC.

Base addition method

As the enzyme cleaves the phenylacyl group, phenylacetic acid is formed, which lowers the pH of the reaction medium. Base was added to maintain the starting pH (Note: use of ammonium hydroxide led to the formation of desilylated byproducts. Desilylation was eliminated when bicarbonates were used). Consumption of the base equivalent of 50% of the starting material indicated 50% conversion point. In the acylation reaction this approach was not used as there was no phenylacetic acid liberation.

HPLC method

Reverse phase HPLC analysis on a Vydac-C18 analytical column used a gradient of acetonitrile (0.1% triethylamine) in water (0.05% phosphoric acid) to quantify the total amide in the reaction mixture. Chiral HPLC analysis on (S,S) Whelk-O Chiral column used isopropanol:hexane (30:70) as a solvent system to separate and quantify the (*R*)- and (*S*)-enantiomers. Monitoring by HPLC proved very convenient for both the deacylation and acylation reactions.

Cleavage of the (*S*)-Amide

The rate of deacylation of (*S*)-amide by PGA is significantly lower than the corresponding (*R*)-amide. A large excess of enzyme and longer reaction times were used to achieve the cleavage of (*S*)-amide. The (*S*)-amide was suspended in phosphate buffer (0.01 M, pH ~7.5) at room temperature, followed by the addition of enzyme Penicillin G amidohydrolase (2.5 times the weight of (*S*)-amide). The (*S*)-amide was cleaved in ~21 h and was monitored by Chiral HPLC.

Quantification of Free Amines

At pH above 7.5 the (*R*)-and (*S*)-amines are practically insoluble in water. Organic solvents were used to extract the free amines from the aqueous reaction medium at pH ~8. As the free amines are devoid of a strong chromophore, their quantification on HPLC using UV detection required prior derivatization. *p*-Fluorobenzoyl, 1-naphthoyl and phenylacetyl derivatives of the racemic amine were prepared and their behavior on the chiral HPLC column was studied. Based on ease of preparation and HPLC analysis, the 1-naphthoyl derivatives (Fig. 8) were preferred.

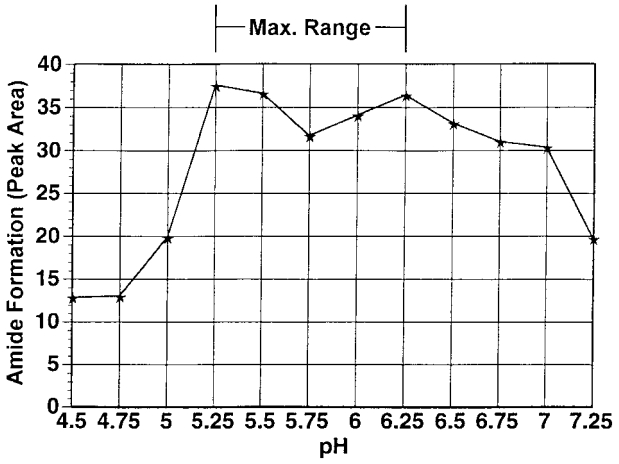


Figure 7. pH Profile for PGA catalysed phenylacetamide formation.

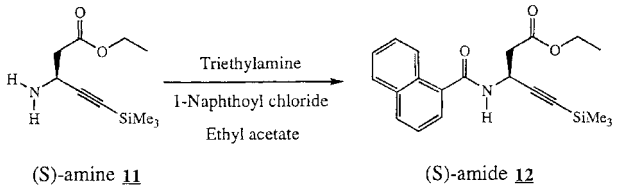
Scale-up

The enzymatic acylation reaction was scaled-up at Boehringer-Mannheim facility in Germany. Three piloting runs were performed (Table 1). The details will be reported in a subsequent paper.

Conclusion

We have demonstrated that PGA can be an effective and efficient catalyst for enantioselective acylation as

a) Synthesis of 1-naphthoyl derivative:



(S)-amine 11 (S)-amide 12

b) Chiral analysis of 1-naphthoyl derivative:

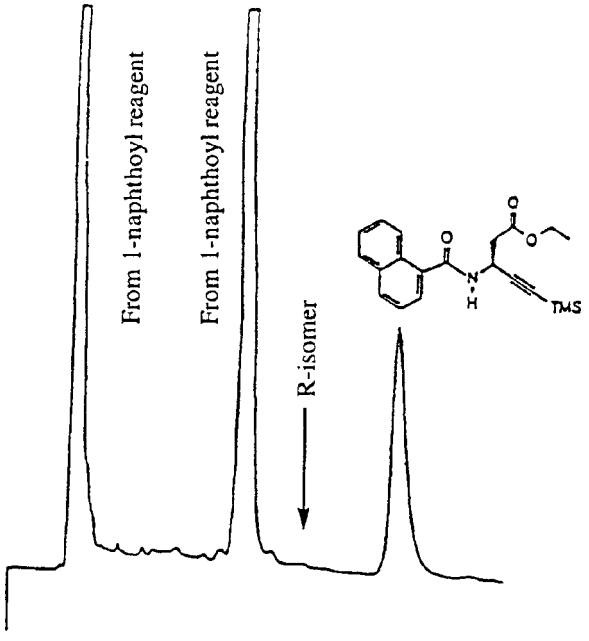


Figure 8. Synthesis of 1-naphthoyl derivative and chiral analyis of enzymatic reaction.

Table 1. Summary of piloting. Analysis of (*S*)-amine isolated

	Piloting- 1	Piloting- 2	Piloting- 3
Purity%	100.0	100.0	99.8
ee%	91.1	98.4	97.7
Yield%	42.4	42.2	43.4

well as deacylation of ethyl 3-amino-4-pentynoate. Synthesis of the desired antipode (*R* or *S* enantiomer) can be achieved by selecting either amidation or amide hydrolysis, as demonstrated in the synthesis of (*R*)- and (*S*)-enantiomers of ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate. Optimization of enzymatic reaction conditions to achieve higher ee involves the identification of the reaction conditions that expand the energy difference between the transition states of competing diastereomeric pathways. The present biocatalytic approach, which offers the possibility of synthesizing separately both enantiomers of β-amino acids/esters, could serve as a method of choice, especially from the points of view of generality, economy and simplicity of experimental procedure.

Experimental

Materials. High purity solvents obtained from commercial suppliers were used as received. Racemic amine[56] was obtained from our pilot plant and was used as such. Phenylacetyl chloride, 1-naphthoyl chloride, and triethylamine were obtained from Aldrich. Phenylacetic acid and phosphate buffer were obtained from Sigma. Soluble as well as immobilized Penicillin G amidohydrolase were obtained from Boehringer-Mannheim. Chiral HPLC column (S,S) Whelk-O was obtained from Regis chemical company. Melting points were determined on a Thomas–Hoover melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 300 MHz with TMS as an internal reference. IR spectra were recorded on a Perkin–Elmer 685 spectrophotometer. MIR refers to multiple internal infrared spectroscopy. High resolution mass spectra were recorded on a Finnigan MAT8430 instrument. Elemental analyses were conducted on a Control Equipment CEC240-XA instrument.

Synthesis of ethyl 3-(*N*-phenylacetyl)-5-trimethylsilyl-4-pentynoate, (4). The crude sample of racemic ethyl-3-amino-5-trimethylsilyl-4-pentynoate, **(3)**, (225 g, 77% amine purity, SC-56988) was dissolved in ethyl acetate (2 L). Triethylamine (1.5 mol) was added under nitrogen atmosphere. The reaction flask was cooled in an ice-salt bath. Under vigorous stirring, phenylacetyl chloride (1 mol) was added drop by drop. Ethyl acetate (250 mL) was added to facilitate the stirring. After completing the addition of phenylacetyl chloride, the reaction was stirred at the same temperature for about 30 min, then warmed to room temperature and stirred for about 2 h. TLC (solvent system: 96:3.5:0.5; CH_2Cl_2:EtOH:NH_4OH) of the reaction mixture indicated completion of the reaction. The precipitated triethylamine hydrochloride was filtered, and the precipitate was washed with ethyl acetate (2×50 mL). Washes were combined with the filtrate. Silica gel (400 g) was added to the filtrate, and the solvent was removed under reduced pressure (bath temp.: 50°C, vacuum: 27'') to obtain a cake. The cake was loaded on a silica pad (250 g), and the system was washed with a gradient of dichloromethane in hexane followed by mixtures: CH_2Cl_2:EtOH:NH_4OH, 96:3.5:0.5, and 92:7:1. All the fractions containing the required material were combined and evaporated to dryness under reduced pressure. Crystallization of the residue in hexane gave 107 g of ethyl 3-(*N*-phenylacetyl)-5-trimethylsilyl-4-pentynoate, **(4)**, as a white crystalline solid. Mp: 74–75°C. IR (MIR): 3270, 2175, 1729, 1658, 1545, 1377, 1356, 1248, 1178, 1097, 1073, 1031, 947, 843, 760, 707 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.12 (s, 9H, TMS); 1.15–1.24 (t, 3H, CH_3), 2.54–2.72 (m, 2H, CH_2), 3.57 (m, 2H, CH_2), 4.05–4.15 (m, 2H, CH_2), 5.07–5.14 (m, H, C-H), 6.20–6.27 (d broad, H, N-H), 7.24–7.39 (m, 5H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$): δ −0.26, 14.14, 38.59, 39.95, 43.54, 60.72, 88.02, 102.90, 127.26, 128.86, 129.29, 134.54, 169.68, 170.04; MS *m*/*z*: 168, 190, 198, 200, 260, 291, 315, 332 (M^{+1}, 100); anal.: $C_{18}H_{25}NO_3Si$ requires C: 65.22, H: 7.60, N: 4.23. Found C: 65.18, H: 7.33, N: 3.99%. The mother liquor contained polar products along with the desired phenylacetyl derivative. The evaporation of the mother liquor under reduced pressure gave a thick oil (15 g).

Desilylation of ethyl 3-(*N*-phenylacetyl)-5-trimethylsilyl-4-pentynoate, 4. The above crude material (15 g) obtained from the evaporation of the mother liquor, which contained ethyl 3-(*N*-phenylacetyl)-5-trimethylsilyl-4-pentynoate, **(4)**, was dissolved in ethanol (200 proof dehydrated, 250 mL) under nitrogen atmosphere. Under vigorous stirring, sodium methoxide (25 wt% in methanol, 8 mL) was added drop by drop. The reaction was stirred at room temperature for 4 h. TLC (Solvent system: 92:7:1; CH_2Cl_2:EtOH:NH_4OH) indicated the completion of the reaction. The reaction mixture was neutralized with dilute sulfuric acid and filtered. The filtrate was evaporated to dryness, and the residue was dissolved in methanol and impregnated on reverse phase silica gel (40 g). The impregnated silica gel was loaded on a small pad of reverse phase silica gel and eluted with a gradient of methanol in water. The fractions containing ethyl 3-(*N*-phenylacetyl)-4-pentynoate were combined and concentrated under reduced pressure. The residue was recrystallized to give 6.7 g of ethyl 3-(*N*-phenylacetyl)-4-pentynoate, **(5)**, as a white crystalline solid. IR (MIR): 3271, 1734, 1634, 1538, 1493, 1454, 1407, 1374, 1340, 1312, 1286, 1209, 1177, 1143, 1091, 1069, 1034, 653 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.18–1.25 (t, 3H, CH_3), 2.22–2.25 (d, H, acetylenic), 2.58–2.75 (m, 2H, CH_2), 3.8 (s, 2H, CH_2), 4.08–4.17 (q, 2H, CH_2), 5.05–5.14 (m, H, C-H), 6.27–6.37 (d broad, H, N-H), 7.23–7.40 (m, 5H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$): δ 14.10, 37.70, 39.45, 43.50, 60.88, 71.39, 81.39, 127, 33, 128.92, 129.31, 134.40, 169.88, 170.08; MS *m*/*z*: 168, 190, 198, 219, 234, 241, 260 (M^{+1}, 100); anal.: $C_{15}H_{17}NO_3$ requires C: 69.48, H: 6.61, N: 5.40. Found C: 69.38, H: 6.48, N: 5.32%.

Deacylation of ethyl 3-(*N*-phenylacetyl)-5-trimethylsilyl-4-pentynoate, 4, using Enzyme Penicillin G amidohydrolase. Phosphate buffer (500 mL, 0.01 M prepared from 0.1 M phosphate buffer, pH 7.4) was added to racemic ethyl 3-(*N*-phenylacetyl)-5-trimethylsilyl-4-pentynoate, **(4)**, (5 g, 15 mmol). The pH was 7.4. Immobilized Penicillin G amidohydrolase (2 g, 345 units/g) was added. The reaction mixture was stirred at room temperature. During the reaction the pH of the reaction medium was maintained around 7.4 using dilute $KHCO_3$ solution (1:1, saturated $KHCO_3$:water) with an auto titrator. After 24 h stirring, the reaction mixture was extracted with ethyl acetate (3×75 mL). The combined organic extract was extracted with dilute hydrochloric acid (pH 2, 3×75 mL). The hydrochloric acid extracts were combined, and preserved. The organic extract was washed with dilute potassium bicarbonate (pH 8, 2×25 mL), dried over sodium sulfate and evaporated to dryness under reduced pressure to obtain (2.4 g, 96%) unreacted (*S*)-amide, **6a**, as a white crystalline compound. M.p.: 74–76°C. IR (MIR): 3243, 3059, 2963, 2174, 1742, 1641, 1550, 1495, 1312, 1275, 1250, 1152, 1041, 964, 898, 845, 648 cm^{-1}; Sp. Rot. (c = 2 cm, methanol): $[\alpha] = D = -71.9°$ ^1H NMR (400 MHz, $CDCl_3$): δ 0.12 (s, 9H, TMS); 1.18–1.24 (t, 3H, CH_3), 2.54–2.72 (m, 2H, CH_2), 3.58 (m, 2H, CH_2), 4.07–4.15 (m, 2H, CH_2), 5.08–5.15 (m, H, C-H), 6.21–6.28 (d, broad, H, N-H), 7.26–7.39 (m, 5H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$): δ −0.25, 14.15, 38.54, 39.90, 43.59, 60.75, 88.07, 102.79, 127.30, 128.89, 129.31, 134.46, 169.65, 170.10; anal.: $C_{18}H_{25}NO_3Si$ requires C: 65.22, H: 7.60, N: 4.23. Found C: 64.87, H: 7.80, N: 4.25%.

The acidic aqueous phase containing the amine was basified using dilute potassium bicarbonate solution to attain pH ~7.8 and extracted with ethyl acetate (3×75 mL). The ethyl acetate extracts were combined, dried over sodium sulfate and evaporated to dryness under reduced pressure to obtain (1.45 g, 91%) (*R*)-amine, **7a**. The small quantity of (*R*)-amine was derivatized using 1-naphthoyl chloride. 1-Naphthoyl (*R*)-amide is a white crystalline compound. IR (MIR): 3273, 2958, 2175, 1734, 1642, 1513, 1371, 1293, 1248, 1093, 1026, 839, 778, 759, 699 cm^{-1}; Sp. Rot. (c = 2 cm, methanol): $[\alpha]_D = +43.3°$ ^1H NMR (400 MHz, $CDCl_3$): δ 0.19 (s, 9H, TMS), 1.26–1.33 (t, 3H, CH_3), 2.80–2.95 (m, 2H, CH_2), 4.16–4.28 (m, 2H, CH_2), 5.40–5.48 (m, H, C-H), 6.81–6.88 (d broad, H, N-H), 7.44–8.38 (m, 7H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$): δ −0.22, 14.23, 39.06, 40.07, 60.91, 88.51, 102.81, 124.69, 125.22, 125.32, 126.43, 127, 15, 128.30, 130.16, 130.93, 133.56, 133.70, 168.15, 170.37; MS *m*/*z*: 190, 204, 224, 268, 282, 296, 340, 368 (M^+1, 100). Anal.: $C_{21}H_{25}NO_3Si$ requires C: 68.63, H: 6.86, N: 3.81. Found C: 68.38, H: 6.82, N: 3.71%.

Deacylation of ethyl 3-(*N*-phenylacetyl)-4-pentynoate, 5, using Enzyme Penicillin G amidohydrolase. Phosphate buffer (250 mL, 0.01 M prepared from 0.1 M phosphate buffer, pH 7.4) was added to racemic ethyl 3-(*N*-phenylacetyl)-4-pentynoate (1.94 g, 7.5 mmol). The pH was 7.57. Immobilized Penicillin G amidohydrolase (2 g, 345 units/g, Boehringer–Mannheim) was added. The reaction mixture was stirred at room temperature. During the reaction the pH of the reaction medium was maintained around 7.5 using dilute $KHCO_3$ solution (1:1, saturated $KHCO_3$:water) with an auto titrator. After 24 h stirring, the reaction mixture was extracted with ethyl acetate (3×75 mL). The organic extracts were combined and extracted with dilute hydrochloric acid (pH 2.0, 3×75 mL). The hydrochloric acid extracts were combined and preserved. The organic extract was washed with dilute potassium bicarbonate (pH 8, 2×25 mL), dried over sodium sulfate and evaporated to dryness under reduced pressure to obtain unreacted (*S*) amide, **(6b)**, (0.92 g, 95%) as a white crystalline solid. IR (MIR): 3279, 3205, 1733, 1641, 1530, 1413, 1351, 1317, 1180, 1114, 1073, 1030, 954, 763, 699 cm^{-1}; Sp. Rot. (c = 2 cm, methanol): $[\alpha]_D = -41.4°$ ^1H NMR (400 MHz, $CDCl_3$): δ 1.20–1.25 (t, 3H, CH_3), 2.22–2.25 (d, H, acetylenic), 2.58–2.75 (m, 2H, CH_2), 3.8 (s, 2H, CH_2), 4.08–4.17 (q, 2H, CH_2), 5.05–5.13 (m, H, C-H), 6.26–6.36 (d broad, H, N-H), 7.22–7.39 (m, 5H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$): δ 14.10, 37.68, 39.42, 43.56, 60.89, 71.36, 81.34, 127, 37, 128.96, 129.33, 134.35, 169.83, 170.11; MS *m*/*z*: 168, 190, 198, 200, 214, 221, 234, 242, 246, 261 (M^+1, 100); anal.: $C_{15}H_{17}NO_3$ requires C: 69.48, H: 6.61, N: 5.40. Found C: 69.30, H: 6.52, N: 5.34%.

The acidic aqueous phase containing the amine was basified using dilute potassium bicarbonate solution to attain pH ~7.8 and extracted with ethyl acetate (3×75 mL). The ethyl acetate extracts were combined, dried over sodium sulfate, and evaporated to dryness under reduced pressure to obtain (*R*)-amine, **(7b)**, (0.49 g, 92%). The small quantity of (*R*)-amine was derivatized using 1-naphthoyl chloride. 1-Naphthoyl (*R*)-amide is a white crystalline compound. IR (MIR): 3288, 1734, 1636, 1522, 1430, 1376, 1288, 1187, 1025, 783 cm^{-1}; Sp. Rot. (c = 2 cm, methanol): $[\alpha]_D = -5.6°$ ^1H NMR (400 MHz, $CDCl_3$): δ 1.26–1.32 (t, 3H, CH_3), 2.37 (d, H, acetylenic), 2.84–2.97 (m, 2H, CH_2), 4.17–4.26 (m, 2H, CH_2), 5.40–5.47 (m, H, C-H), 6.92–6.98 (d, broad, H, N-H), 7.44–8.36 (m, 7H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$): δ 14.16, 38.04, 39.53, 61.06, 71.69, 124.65, 125.26, 126.45, 127, 22, 128.31, 131.05, 133.29, 133.67, 168.25, 170.44: Anal.: $C_{18}H_{17}NO_3$ requires C: 73.20, H: 5.80, N: 4.74. Found C: 69.97, H: 5.73, N: 4.84%.

Acylation of ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate, 3, using Enzyme Penicillin G amidohydrolase. Crude free amine, **3**, (racemic, 500 mg) was added drop by drop to dilute HCl acid (50 mL, pH 1.9) with stirring. After 10 min, the insoluble material was extracted using *t*-butyl methyl ether (25 mL). The layers were separated, and the pH of the aqueous phase was adjusted to 6 using ammonium hydroxide (2.5 N). Phenylacetic acid (2 equiv) was added and the mixture was stirred for about 10 min. The pH was adjusted to 6 and the mixture was stirred for about 10 min. Soluble enzyme (250 units, Boehringer–Mannheim) was added, and the mixture was stirred at room temperature for 42 h. The pH of the reaction mixture was adjusted to 4 and stirring was continued for 10 min. The product was collected by

filtration and washed with water (pH 4, 10 mL). The washes were combined with the main filtrate. The filtrate was preserved for the isolation of amine. The product collected was dissolved in ethyl acetate (25 mL) and batch extracted with water (pH 8.3, 2×15 mL). The ethyl acetate phase was dried over sodium sulphate and evaporated to dryness under reduced pressure gave 256 mg of (*R*)-amide, **(10)**, as a white solid. The chiral HPLC analysis of this isolated amide indicated absence of (*S*)-amide and very insignificant amount of desilylated amide. Mp: 76–78°C. IR (MIR): 3247, 2174, 1742, 1641, 1551, 1495, 1312, 1275, 1250, 1155, 1041, 964, 898, 845 cm^{-1}; Sp. Rot. (c = 2 cm, methanol): $[\alpha]_D = +70.4°$ ^1H NMR (400 MHz, $CDCl_3$): δ 0.11 (s, 9H, TMS); 1.18–1.23 (t, 3H, CH_3), 2.53–2.70 (m, 2H, CH_2), 3.57 (m, 2H, CH_2), 4.07–4.14 (m, 2H, CH_2), 5.07–5.13 (m, H, C-H), 6.22–6.27 (d, broad, H, N-H), 7.23–7.38 (m, 5H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$): δ−0.25, 14.15, 38.58, 39.93, 43.60, 60.73, 88.06, 102.82, 127.26, 128.89, 129.28, 134.47, 169.60, 170.02; anal.: $C_{18}H_{25}NO_3Si$ requires C: 65.22, H: 7.60, N: 4.23. Found C: 65.33, H: 7.66, N: 4.22%.

The washes were combined with the preserved filtrate, treated with ammonium hydroxide (2.5 N) to obtain pH 8.2 and extracted with ethyl acetate (2×25 mL). The organic phase was dried over sodium sulphate and evaporated to dryness under reduced pressure to give 145 mg of (*S*)-amine, **(11)**. This material (145 mg) was dissolved in ethyl acetate (50 mL). In presence of triethylamine, an aliquot was treated with 1-naphthoyl chloride at room temperature. Chiral HPLC analysis of the 1-naphthoylated material indicated the desired silylated amine ratio 2:98 (*R:S*). 1-Naphthoyl silyl (*S*)-amide is a white crystalline solid. IR (MIR): 3285, 2958, 2174, 1734, 1642, 1513, 1371, 1292, 1247, 1026, 839 cm^{-1}; Sp. Rot. (c = 2 cm, methanol): $[\alpha]_D = -51.6°$ ^1H NMR (400 MHz, $CDCl_3$): δ 0.19 (s, 9H, TMS), 1.27–1.33 (t, 3H, CH_3), 2.81–2.96 (m, 2H, CH_2), 4.15–4.25 (m, 2H, CH_2), 5.40–5.47 (m, H, C-H), 6.82–6.88 (d broad, H, N-H), 7.45–8.38 (m, 7H, aromatic); ^{13}C NMR (500 MHz, $CDCl_3$): δ−0.21, 14.24, 39.08, 40.08, 60.92, 76.77, 77.03, 77.28, 88.52, 102.83, 124.70, 125.24, 125.33, 126.44, 127.16, 128.31, 130.17, 130.94, 133.57, 133.71, 168.17, 170.38; MS *m*/*z*: 190, 204, 224, 239, 272, 296, 304, 322, 343, 368 (M^{+1}, 100); anal.: $C_{21}H_{25}NO_3Si$ requires C: 68.63, H: 6.86, N: 3.81. Found C: 68.25, H: 6.88, N: 3.57%.

Acknowledgements

We gratefully acknowledge our Bioprocess group at St. Louis for discussions, Larry Miller for providing analytical methodology, Kathy McLaughlin and Dr. John Dygos for valuable discussions and manuscript preparation.

References

1. Ng, J.; Topgi, R. S. *Current Opinion In Drug Discovery and Development* **1998**, *1*, 314 and references cited therein.
2. Spatola, A. F. In *Chemistry and Biochemistry of the Acids, Peptides and Proteins*; Weinstein, B., Ed.; Marcel Dekker: New York, 1983; Vol. 7, pp. 331.
3. Huang, H.; Iwasawa, N.; Mukaiyama, T. *Chem. Lett*. **1984**, 1465.
4. Drey, C. N. C. In *Chemistry and Biochemistry of the Amino Acids*; Barret, G. C., Ed.; Chapman and Hall: New York, 1985; Chapter 3.
5. Griffith, O. W. *Ann. Rev. Biochem*. **1986**, *55*, 855.
6. Hecht, S. M. *Acc. Chem. Res*. **1986**, *19*, 383.
7. Kim, S.; Chang, S. B.; Lee, P. H. *Tetrahedron Lett*. **1987**, *28*, 2735.
8. Helms, G. L.; Moore, R. E.; Niemczura, W. P.; Patterson, G. M. L.; Tomer, K. B.; Gross, M. L. *J. Org. Chem*. **1988**, *53*, 1298.
9. Tanner, D.; Somfai, P. *Tetrahedron* **1988**, *44*, 613.
10. Kim, S.; Lee, P. H.; Lee, T. A. *J. Chem. Soc., Chem. Commun*. **1988**, 1242.
11. Kunieda, T.; Nagamatsu, T.; Higuchi, T.; Hirobe, M. *Tetrahedron Lett*. **1988**, *29*, 2203.
12. Estermann, H.; Seebach, D. *Helv. Chim. Acta* **1988**, *71*, 1824.
13. Takagi, M.; Yamamoto, K. *Tetrahedron* **1991**, *47*, 8869.
14. Lubell, W. D.; Kitamura, M.; Noyori, R. *Tetrahedron Asymmetry* **1991**, *2*, 543.
15. Rico, J. G.; Lindmark, R. J.; Rogers, T. E.; Bovy, R. *J. Org. Chem*. **1993**, *58*, 7948.
16. Amoroso, R.; Cardillo, G.; Sabatino, P.; Tomasini, C.; Trere, A. *J. Org. Chem*. **1993**, *58*, 5615.
17. Mukai, C.; Kim, I. J.; Furu, E.; Hanaoka, M. *Tetrahedron* **1993**, *49*, 8323.
18. Swindell, C. S.; Tao, M. *J. Org. Chem*. **1993**, *58*, 5889.
19. Masters, J. J.; Hegedus, L. S. *J. Org. Chem*. **1993**, *58*, 4547.
20. Bunnage, M. E.; Burke, A. J.; Davies, S. G.; Goodwin, C. J.; Ichihara, O. *Tetrahedron* **1994**, *50*, 3975.
21. Gmeiner, P. *Tetrahedron Lett*. **1990**, *40*, 5717.
22. Norman, B. H.; Morris, M. L. *Tetrahedron Lett*. **1992**, *33*, 6803.
23. Juaristi, E.; Quaintana, D. *Tetrahedron Asymmetry* **1992**, *3*, 723.
24. Burgess, K.; Liu, L. T.; Pal, B. *J. Org. Chem*. **1993**, *58*, 4758.
25. Jefford, C. W.; Wang, J. B.; Lu, Z.-H. *Tetrahedron Lett*. **1993**, *34*, 7557.
26. Patel, R. N.; Banerjee, A.; Howell, J. M.; McNamee, C. G.; Brozozowski, D.; Mirfakhrae, D.; Nanduri, V.; Thottathil, J. K.; Szarka, L. *Tetrahedron Asymmetry* **1993**, *4*, 2069.
27. Jones, J. B. *Tetrahedron* **1986**, *42*, 3351.
28. Rossi, D.; Lucente, G.; Romeo, A. *Experientia* **1977**, *33*, 1557.
29. Lu, Y.; Miet, C.; Kunesch, N.; Poisson, J. E. *Tetrahedron Asymmetry* **1991**, *2*, 871.
30. Soloshonok, V. A.; Fokina, N. A.; Rybakova, A. V.; Shishkina, I. P.; Galushko, S. V.; Sorochinsky, A. E.; Kukhar, V. P.; Savchenko, M. V.; Svedas, V. K. *Tetrahedron Asymmetry* **1995**, *6*, 1601.
31. Cardillo, G.; Tolomelli, A.; Tomasini, C. *J. Org. Chem*. **1996**, *61*, 8651.
32. Soloshonok, V. A.; Soloshonok, I. V.; Kukhar, V. P.; Svedas, V. K. *J. Org. Chem*. **1998**, *63*, 1878.
33. Ebert, C.; Gardossi, L.; Linda, P. *J. Mol. Catal. B*. **1998**, *5*, 241.
34. Cardillo, G.; Tolomelli, A.; Tomasini, C. *Eur. J. Org. Chem*. **1999**, *1*, 155.
35. Chenault, H. K.; Dahmer, J.; Whitesides, G. *J. Am. Chem. Soc*. **1989**, *111*, 6354.
36. Meijer, E. M.; Boesten; W. H. Y.; Schoemaker; H. E.; van Balken; J. A. M. In *Biocatalysis in Organic Synthesis;* Tramper, J.; van der Plas, H. C.; Links, P. Eds.; Elsevier: Amsterdam, 1985; p. 135.

37. Drauz, K.; Waldmann, H., Eds. *Enzyme Catalysis in Organic Synthesis*. VCH: New York, 1995, pp. 386.
38. Didziapetris, R.; Drabnig, B.; Sehellenberger, V.; Jakubke, H.-D.; Svedas, V. *FEBS Lett*. **1991**, *287*, 31.
39. Stoineva, I. B.; Galunsky, B. P.; Lazanov, V. S.; Ivanov, I. P.; Petkov, D. D. *Tetrahedron* **1992**, *48*, 1115.
40. Solodenko, V. A.; Kasheva, T. N.; Kukhar, V. P.; Kozlova, E. V.; Mironenko, D. A.; Svedas, V. K. *Tetrahedron* **1991**, *47*, 3989.
41. Solodenko, V. A.; Belik, M. Y.; Hgalushko, S. V.; Kukhar, V. P.; Kozlova, E. V.; Mironenko, D. A.; Svedas, V. K. *Tetrahedron Asymmetry* **1993**, *4*, 1965.
42. Margolin, A. L.; Svedas, V. K.; Berezin, I. V. *Biochim. Biophys. Acta* **1980**, *616*, 283.
43. Anderson, E.; Mattiasson, B.; Hahn-Hagerdal, B. *Enz. Microb. Technol*. **1984**, *6*, 301.
44. Margolin, A. L. *Tetrahedron Lett*. **1993**, *34*, 1239.
45. Waldmann, H. *Kontakte* **1991**, *2*, 33.
46. Romeo, A.; Lucente, G.; Rossi, D.; Zanotti, G. *Tetrahedron Lett*. **1971**, *21*, 1799.
47. Svedas, V. K.; Galaev, I. Yu.; Semiletov, Yu. A.; Korshunova, G. A. *Bioorgan. Khim*. **1983**, *9*, 1139.
48. Fuganti, C.; Rosell, C. M.; Servi, S.; Tagliani, A.; Terreni, M. *Tetrahedron Asymmetry* **1992**, *3*, 383.
49. Baldaro, E.; D'Arrigo, P.; Pedrocchi-Fantoni, G.; Rosell, C. M.; Servi, S.; Tagliani, A.; Terreni, M. *Tetrahedron Asymmetry* **1993**, *4*, 1031.
50. Fuganti, C.; Grasselli, P.; Casati, P. *Tetrahedron Lett*. **1986**, *27*, 3191.
51. Baldaro, E.; Faiardi, D.; Fuganti, C.; Grasselli, P.; Lazzarini, A. *Tetrahedron Lett*. **1988**, *29*, 4623.
52. Didziapetris, R.; Drabnig, B.; Schellenberger, V.; Jakubke, H.-D.; Svedas, V. *FEBS Lett*. **1991**, *287*, 31.
53. Hermann, P. *Biomed. Biochim. Acta* **1991**, *50*, 19.
54. Stoineva, I. B.; Galunsky, B. P.; Lozanov, S. V.; Ivanov, I. P.; Petkov, D. D. *Tetrahedron* **1992**, *48*, 115.
55. Zablocki, J. A.; Rico, J. G.; Garland, R. B.; Rogers, T. E.; Williams, K.; Schretzman, L. A.; Rao, S. A.; Bovy, P. R.; Tjoeng, F. S.; Lindmark, R. J.; Toth, M.; Zupec, M.; McMackins, D. E.; Adams, S. P.; Miyano, M.; Markos, C. S.; Milton, M. N.; Paulson, S.; Herin, M.; Jacquin, P.; Nicholson, N.; Panzer-Knodle, S. G.; Haas, N. F.; Page, G. D.; Szalony, J. A.; Taite, B. B.; Salyers, A. K.; King, L. W.; Campion, J. G.; Feigen, L. P. *J. Med. Chem*. **1995**, *38*, 2378.
56. Babiak, K.; Babu, S.; Behling, J. R.; Boys, M. L.; Cain-Janicki, K. J.; Doubleday, W. W.; Farid, P.; Hagen, T. J.; Hallinan, E. A.; Hansen Jr., D. W.; Korte, D. E.; McLaughlin, K. T.; Medich, J. R.; Nugent, S. T.; Orlovski, V.; Park, J. M.; Peterson, K. B.; Pilipauskas, D. R.; Pitzele, B. S.; Tsymbalov, S.; Stahl, G. L. *Preparation of ethyl 3(*S*)-[4-[4-(aminoiminomethyl) phenyl] amino-1,4-dioxobutyl]amino]-4-pentynoate*. US Patent: 5536869. July 16, 1996.
57. Cossy, J.; Schmitt, A.; Cinquin, C.; Buisson, D.; Belotti, D. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1699.
58. Boys, M. L. *Tetrahedron Lett*. **1998**, *39*, 3449.
59. Shewale, J. G.; Deshpande, B. S.; Sudhakaran, V. K.; Ambedkar, S. S. *Process Biochem. Intl*. **1990**, 97.
60. Zmijewski, M. J.; Briggs, B. S.; Thompson, A. R.; Wright, I. G. *Tetrahedron Lett*. **1991**, *32*, 1621.
61. Fuganti, C.; Grasselli, P.; Servi, S.; Lazzarini, A.; Casati, P. *Tetrahedron* **1988**, *44*, 2575.
62. Fuganti, C.; Rosell, C. M.; Rigoni, R; Servi, S.; Tagliani, A.; Terreni, M. *Biotech. Lett*. **1992**, *14*, 543.
63. Andersson, E.; Mattiasson, B.; Hahn-Hagerdal, B. *Enzyme Microb. Technol*. **1984**, *6*, 301.
64. Ebert, C.; Gardossi, L.; Linda, P. *Tetrahedron Lett*. **1996**, *37*, 9377.