ENANTIOPURE *R*(-)-3-AMINOISOBUTYRIC ACID SYNTHESIS USING *Pseudomonas aeruginosa* AS ENANTIOSPECIFIC BIOCATALYST

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Abstract - The main goal of this research was the synthesis of enantiopure *R*(-)-3-aminoisobutyric acid from dihydrothymine with good yield, high stereospecificity and relative simplicity. Seventy two percent yield of the product was obtained in three steps. Step one consisted of dihydrothymine racemization. Step two was a dihydropyrimidinase reaction involving the *Pseudomonas aeruginosa* 10145 bacterial strain as the biocatalyst. Step three was performed with a diazotization reaction. The bacteria's enzymes determined the stereochemistry of the process since the diazotization reaction did not interfere at this point. The results of this work provide an interesting method for the production of commercial β-amino acids from other substituted-dihydrothymines.
Keywords: β-Amino acid; *R*(-)-3-aminoisobutyric acid; Biocatalysis; *Pseudomonas aeruginosa*.

INTRODUCTION

In recent years, β-amino acids have attracted attention. These molecules have the ability to form unusual peptidelike chains when they are linked together, or with other compounds, with the linkage being hydrolytically stable. This property presents a potential applicability for pharmaceuticals, exhibiting a better bioavailability than usual protein drugs, for example (Borman, 1997; Liljeblad and Kanerva, 2006).

The molecule *R*(-)-3-aminoisobutyric acid is a β-amino acid that shows promising applications when linked to another active one, being applied in the synthesis of new drugs (Daines and Pendrak, 2002; Ferrer *et al*., 2002; Frick *et al*., 2002; Zhao *et al*., 2008). The first report about enantiomerically pure *R*(-)-3-aminoisobutyric acid production referred to the chemical synthesis of racemic 3-aminoisobutyric acid with sequential chemical resolution (Pollack,

1943). Unfortunately, this method was an expensive procedure. Some years later, the process utilizing *Saccharomyces cerevisiae* providing a biological resolution became an alternative method. In this case, the yeast metabolized the total amount of the "*S*" isomer form, leaving only the "*R*" isomer form in the reactor. However, the maximum process yield was 50%, because the microorganism used the other 50% for nourishment (Kakymoto and Armstrong, 1961; Pollock, 1973). Due to its potential application in the medical industry, the development of a new approach to produce enantiomerically pure *R*(-)-3-aminoisobutyric acid with high yield, stereospecificity, relative simplicity and low coast is of great value.

Mei *et al*. (2009) described about fifty bacteria species (Example: *Pseudomonas aeruginosa*, *Bacillus* sp., *Clostridium glycolicum*) with hydantoinasic-dihydropyrimidinasic activity, including the characteristics of their catalytic stereochemistry. Based on this information, an option to be considered is to test

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the efficiency of some of these described bacteria for producing enantiomerically pure β-amino acids or intermediates. The aim of this study was to describe a preparative production of enantiopure R(-)-3-aminoisobutyric acid from the main substrate dihydrothymine utilizing the bacterium *Pseudomonas aeruginosa*, which presents enantioselective dihydropyrimidinase hydrolyzing activity, as the biocatalyst and a sequential diazotization reaction.

EXPERIMENTAL

Production of Enantiomerically Pure R(-)-3-Aminoisobutyric Acid

The culture of *Pseudomonas aeruginosa* 10145, provided by The Culture Collection of the INCQS – FIOCRUZ, Rio de Janeiro, Brazil, was maintained in 20% glycerol and stored at -15 °C. Cells were transferred to nutrient agar media and incubated at 30 °C during 24 h. For pre-culture, the strain from a culture on nutrient agar was transferred into tubes containing 10 mL of growing broth consisting of 1.0% glycerol, 0.5% yeast extract, 1.0% sodium chloride. The pH of the medium was initially adjusted to 7.0. After 24 h, 10 mL of cell suspension was inoculated into a 500 mL flask containing 100 mL of the same medium used in pre-culture with 0.01% added dihydrothymine as dihydropyrimidinase inducer. The culture temperature and agitation rate were 30 °C and 150 rpm, respectively. Cell growth was monitored by turbidity at 600 nm up to the stationary phase. Biomass was harvested by centrifugation at 4 °C and 12000 g for 30 minutes and washed three times with 100 mM borate buffer, pH 9.0. *Pseudomonas aeruginosa* 10145 biomass, 250 mg wet weight of cells, was suspended in 20 mL of 100 mM borate buffer, pH 9.0. Dihydrothymine was added to a final concentration of 10 mM and was incubated under 150 rpm orbital agitation at 35 °C. Dihydropyrimidinase activity of the bacterial culture was estimated colorimetrically by quantification of *N*-carbamoyl-R(-)-3-aminoisobutyrate and by quantification of the remaining dihydrothymine, as described below. After reaction completion, cells were eliminated by centrifugation at 4 °C and 12000 g for 30 minutes. The supernatant was concentrated *in vacuo* to about fifty percent of the original volume. *N*-carbamoyl-R(-)-3-aminoisobutyrate was precipitated by acidification with concentrated hydrochloric acid to pH 2.0, under about 0 °C. The precipitate was recovered by centrifugation at 10 °C and 5000 g for 10 minutes, washed with distilled water and dried *in vacuo*. *N*-carbamoyl-R(-)-

3-aminoisobutyrate and sodium nitrite were dissolved in 3.5 M hydrochloric acid up to a concentration of 0.5 mM each. The reactor flask was incubated under 150 rpm orbital agitation at about 0 °C. The extent of the diazotization reaction was followed by colorimetric ninhydrin assay (Machado *et al.*, 2005) to detect the R(-)-3-aminoisobutyric acid produced and *N*-carbamoyl-R(-)-3-aminoisobutyrate consumption, as described below. After completion of the reaction, the reaction mixture was diluted in deionized water and deposited on a Dowex 50 cation exchange column. The column was washed three times with deionized water and eluted with 0.2 M ammonium formate, pH 4.0, solution. R(-)-3-Aminoisobutyric acid was recovered by evaporation *in vacuo*.

Assay of Dihydropyrimidinase Activity of Bacterial Culture and *N*-Carbamoyl-R(-)-3-Aminoisobutyrate Characterization

N-carbamoyl-R(-)-3-aminoisobutyrate produced was quantified as follows: 150 μL of 10% *p*-dimethylaminobenzaldehyde in 6 N hydrochloric acid was added to 400 μL of reaction sample pre-treated with 700 μL of 12% trichloroacetic acid. The precipitated material was eliminated by centrifugation. Absorbance at 450 nm of the supernatant was measured and compared with a standard curve of *N*-carbamoyl-2-aminoisobutyrate (Morin, 1993). *N*-carbamoyl-R(-)-3-aminoisobutyrate produced was dried *in vacuo* and polarimetry was conducted in a Jasco DIP-370 Digital polarimeter. FTIR of this intermediate was performed on a Perkim-Elmer Spectrum BXII 60508 Pike Miracle in order to verify and confirm the identity of the produced molecule. The remaining dihydrothymine was determined in the supernatant of the reaction mixture by HPLC using a reverse phase C_{18} Spherisorb ODS-2 column (4.6 x 250 mm, ISCO). The mobile phase used was 10% acetonitrile, 0.1% trichloroacetic acid and water at a flow rate of 0.8 mL.min^{-1}. The column eluent was detected at 230 nm. A standard curve of dihydrothymine was constructed in order to determine its concentration by integration of the corresponding peak. FTIR of dihydrothymine was performed with a Perkim-Elmer Spectrum BXII 60508 Pike Miracle in order to verify the identity of the molecule and compare with the intermediate and final product.

Characterization of the Target Product

R(-)-3-aminoisobutyric acid was analyzed to confirm its enantiomeric purity/enantiomeric excess and molecular identity. Chiral HPLC was performed by

the protocol described by Alonso *et al*. (2008), slightly modified. It was conducted at room temperature with a Nucleosil Chiral-1 column (4.6 x 250 mm, Macherey-Nagel) using 1mM copper sulfate as the mobile phase at a flow rate of 0.7 mL.min⁻¹. *R*(-)-3-aminoisobutyric acid was submitted to racemization with 1M NaOH and two peaks of the same area were obtained with retention times of 3.80 minutes (*R*) and 4.97 minutes (*S*), respectively. The column eluent was detected at 225 nm. Elementary analysis was performed on a Perkim-Elmer 2400 CHN analyzer. The melting point was obtained on a capillary apparatus. Polarimetry was conducted with a Jasco DIP-370 Digital polarimeter. FTIR was performed with a Perkim-Elmer Spectrum BXII 60508 Pike Miracle. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were performed on a Brucker Spectros 300.

RESULTS AND DISCUSSION

The enantiopure *R*(-)-3-aminoisobutyric acid synthesis strategy applied in this study involved three steps (Figure 1): 1- dihydrothymine racemization, 2- dihydropyrimidinase hydrolysis and 3- a diazotization reaction. The first two were performed by using the selected stereospecific bacterial bioconversor, *P. aeruginosa* strain 10145, coupled with an alkaline medium. The last step consisted of a diazotization reaction. Steps one and two defined the process stereochemistry since the diazotization reaction (step three) does not interfere at this point (Garcia and Azerad, 1997; Keil *et al*., 1995).

Pseudomonas aeruginosa strain 10145 was previously identified to be the most efficient microorganism tested for production of *N*-carbamoyl-*R*(-)-3-aminoisobutyrate compared to some other species and strains (Côrtes, 2009). This specific bacterial strain was able to convert dihydrothymine into enantiomerically pure *N*-carbamoyl-*R*(-)-3-aminoisobutyrate with more than 76% yield in five hours. Progress of the biocatalytic process was measured by quantification

of the remaining dihydrothymine and *N*-carbamoyl-*R*(-)-3-aminoisobutyrate produced (Figure 2) and by FTIR. After eight hours, FTIR showed the presence of bands of axial deformation: 1,703 cm⁻¹ (intense signal, C=O amide I in acyclic molecule), 1,568 cm⁻¹ (intense signal, asymmetric, C=O carboxylic acid), 3,495 cm⁻¹ (medium signal, N-H amine) and 3,249 cm⁻¹ (medium signal, N-H amide). Bands of axial deformation were absent at: 1,717 cm⁻¹ (intense signal, C=O amide I in cyclic molecule) and 3,248 – 2,843 cm⁻¹ (weak signal, symmetric and asymmetric, N-H amide) and bands of angular deformation absent at: 1,646 cm⁻¹ (medium signal, N-H amide II) and 830 cm⁻¹ (medium and large signal, symmetric, N-H amide), all characteristic of the substrate dihydrothymine. The product absolute configuration was confirmed by polarimetry: $[\alpha]^{25}_D$ -11.7° (c = 0.1, 3M HCl). These data suggested dihydrothymine molecule ring opening, and, consequently, the production of *N*-carbamoyl-*R*(-)-3-aminoisobutyrate. As a control, dihydrothymine solution was also observed to be stable under the process conditions for ten hours.

A subsequent diazotization reaction converted the enantiopure *N*-carbamoyl-*R*(-)-3-aminoisobutyrate into enantiopure *R*(-)-3-aminoisobutyric acid, the target product, with 96.5% yield, resulting in an approximately 72% total yield. The product characterization is described below. Its absolute configuration was confirmed by polarimetry, corresponding to the value found in the literature (Crumpler *et al*., 1951). The enantiomeric excess was determined to be higher than 98%.

The molecular characterization *of R*(-)-3-aminoisobutyric acid provided: M.p. 177-179°C. Microanalytical data (calculated) C 47.05% (46.60%), H 8.52% (8.74%) N 13.71% (13.59%). FTIR (KBr) 1651, 1589, 1501 and 1398 cm⁻¹. ^{1}H NMR (D$_2$O, 300 MHz) δ (ppm) 2.56-2.75 (m, 2H, β-CH$_2$) 2.13-2.24 (m, 1H, α-CH) 0.78 (d, *J* = 6.9 Hz, 3H, CH$_3$). ^{13}C NMR (D$_2$O, 75MHz) δ (ppm) 179.50 (COOH) 40.24 (β-CH$_2$) 37.10 (α-CH) 13.00 (α-CH$_3$). $[\alpha]^{25}_D$ -10.2° (c = 0.5, methanol). Chiral HPLC (Nucleosil Chiral-1, CuSO$_4$ 1 mM) retention time 3.80 minutes (*R*).
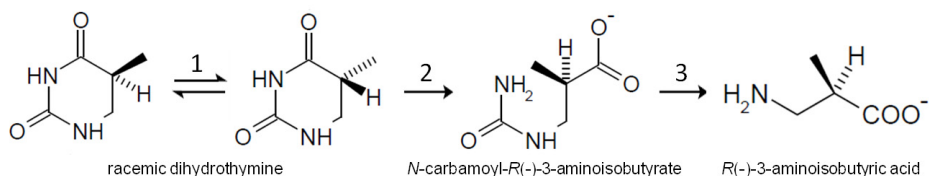

Figure 1: Scheme of the *R*(-)-3-aminoisobutyric acid production sequence. (1) racemization, (2) *R*-dihydropyrimidinase and (3) diazotization reaction.
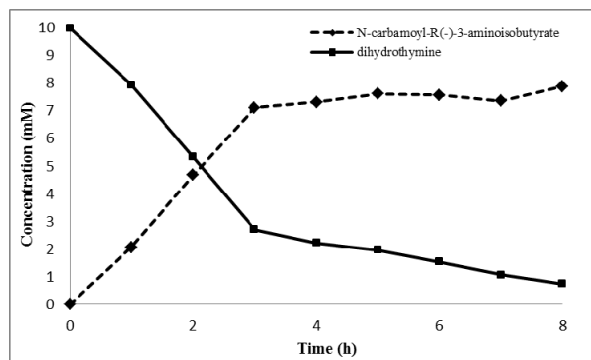
Figure 2: Progress curve of: (•) *N*-carbamoyl-*R*(-)-3-aminoisobutyrate synthesis and (▪) dihydrothymine ring opening.

On the basis of our results and available information in the literature (Arcuri *et al.*, 2000; Ishikawa *et al.*, 1997) it is possible to propose the probable mechanism of this stereospecific process: racemic dihydrothymine was converted exclusively to the *R*-isomer of *N*-carbamoyl-*R*(-)-3-aminoisobutyrate by the *R*-preferential dihydropyrimidinase in combination with a racemization in the alkaline medium (pH 9.0). The *N*-carbamoyl-*R*(-)-3-aminoisobutyrate was then converted to *R*(-)-3-aminoisobutyric acid by a diazotization reaction.

Our results show that the method described is an interesting route for low weight β-amino acid production, complementing the other described effective methods: 1) the Arndt-Eister homologation method, which consists of adding an additional carbon atom between the carboxy and amino groups of α-amino acids. The advantage of this specific method is obtaining the enantiomerically pure β-amino acids when the appropriate α-amino acids are utilized. However, a disubstituition may occur, forming α, β-amino acids, and it also requires diazomethane, making the reaction unsuitable for large-scale use (Matthews and Seebach, 1997); 2) A second most utilized method is the one based on kinetic resolution with hydrolytic enzymes, which are sensitive to the substrate contents of amino and ester functionalities and make this resolution procedure non-general for developing many different forms of β-amino acids. On the other hand, it was the first successful approach, using the enzyme penicillin acylase to hydrolysis the phenyl-acetyl group enantioselectively (Liljeblad and Kanerva, 2006). Soloshonok *et al.* (1995) synthesized some β-aryl-β-amino acids in enantiomerically pure form in a good yield by penicillin acylase catalyzed resolution of their racemic *N*-phenylacetyl derivatives, employing a simple set of reactions and separations of enzymatically resolved species.

CONCLUSION

According to our results, the process biocatalyzed by *Pseudomonas aeruginosa* strain 10145 plus a diazotization reaction is a competitive route to produce enantiopure *R*(-)-3-aminoisobutyric acid and possibly other commercially interesting β-amino acids by converting substituted dihydrothymines into the respective enantiopure β-amino acids. The great advantage of the present method was the possibility of obtaining good yields and higher selectivity, combined with relative simplicity of the synthesis, including low cost of chemical supplies, resulting in a low-cost process. However, additional study is necessary to evaluate the suitability to scale up the process, define the possible dihydrothymine substituent groups and determine the process drawbacks.

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