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Enzymatic resolution of *N*-acetyl-homophenylalanine with mammalian kidney acetone powders

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Abstract—Kidney acetone powders (KAP) from beef, dog, hog, rat, and sheep have been evaluated for resolving *N*-acetyl-DL-homophenylalanine. We also propose a simple protocol for the preparation of both enantiomers of homophenylalanine by enzymatic resolution using mammalian KAP. The beef kidney afforded the best results, rendering the highest isolated yields, 37.5% and 41% and enantiomeric excesses of 94% and >99% for both D-*N*-Ac-homophenylalanine and L-homophenylalanine, respectively.

1. Introduction

Most of angiotensin converting enzyme (ACE) inhibitors, such as Benazepril **1**, Temocapril **2**, Cilazapril **3**, and Enalapril **4** (Fig. 1), contain an L-homophenylalanine ethyl ester moiety in their structure, which can be efficiently introduced through nucleophilic displacement of the corresponding chiral amine over (*R*)-2-nosyloxy-4-phenylbutyric acid ethyl ester **5**, as shown in Scheme 1 for the synthesis of Benazepril **1**,[1] with a concomitant inversion of configuration. The homochiral α-substituted ester **5** can be obtained from D-homophenylalanine (D-HPA) **6** (Scheme 2).

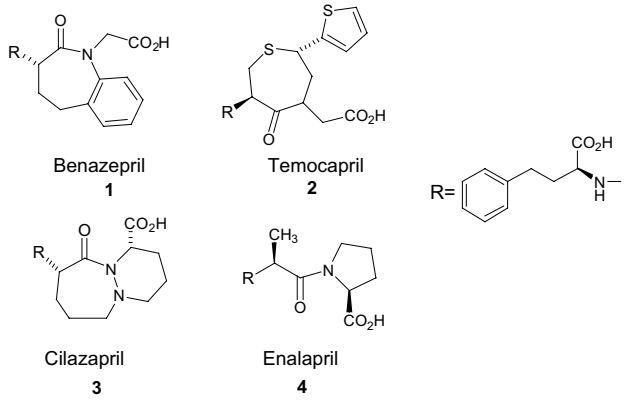
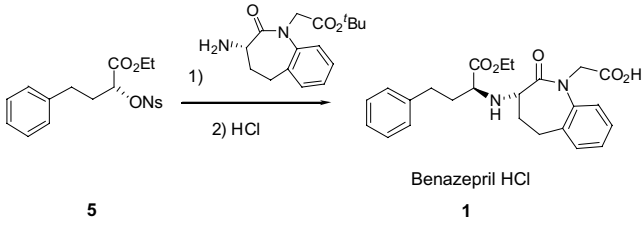
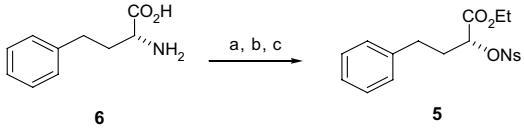


Figure 1. Some ACE inhibitors holding the L-HPA moiety.



Scheme 1. Synthesis of Benazepril from (*R*)-2-nosyloxy-4-phenylbutyric acid ethyl ester.



Scheme 2. Reagents: (a) $NaNO_2$, H_2SO_4, H_2O (89%); (b) $SOCl_2$, ethanol, (65%); (c) NsCl, Et_3N (74%).

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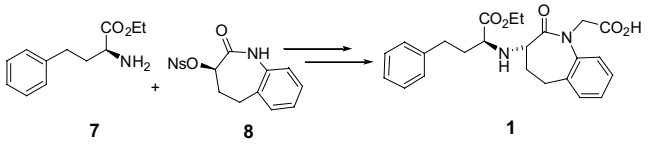
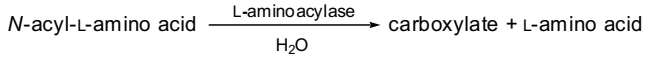


Scheme 3. Synthesis of Benazepril from L-HPA-ethyl ester.

Recently, Chang and Yang[2] reported a new synthetic strategy for the synthesis of Benazepril **1**, one of the most potent ACE inhibitors, which makes use of L-homophenylalanine ethyl ester **7** for the nucleophilic attack of 4-nitrobenzenesulfonic acid (3*R*)-2-oxo-2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepin-3-yl ester **8**, as shown in Scheme 3. Both enantiomers of homophenylalanine (HPA) have a potential application in the synthesis of ACE inhibitors.

While a great variety of methods for the preparation of both enantiomers of HPA have been reported,[3a–g] many of them are impractical, mainly due to their cost. Although the enzymatic resolution of HPA ethyl ester by lipases has been described,[3c] to the best of our knowledge, no report has been made on an enzymatic resolution strategy using aminoacylases.

Enzymatic resolution of amino acid derivatives is usually a simple and efficient method for the preparation of enantiopure amino acids. One of the most widely used enzymes for carrying out the resolution of α-acylamido acids is the pig kidney L-aminoacylase (*N*-acyl-L-amino-acid amidohydrolase, EC 3.5.1.14), which is commercially available.[4] This enzyme catalyzes the following reaction:

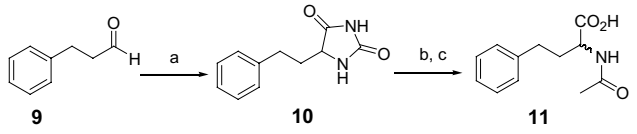


Despite the convenience of chemo-enzymatic methods in synthetic organic chemistry, there still exists some uneasiness amongst some synthetic chemists regarding the handling of enzymes and other biochemical techniques. Furthermore, most commercially available enzymes are expensive and can be unstable, unless kept under specific storage conditions. Most of these drawbacks could be avoided by using crude enzymatic preparations; however, the use of these raw preparations is much less explored than the use of pure enzymes.[5a–e] Herein, we report results on the evaluation of the kidney acetone powders (KAPs) from different mammalian species such as beef, dog, hog, rat, and sheep for resolving *N*-acetyl-DL-HPA **11**. We also propose a practical and reliable protocol for the preparation of both enantiomers of HPA by enzymatic resolution using mammalian KAP.

2. Results and discussion

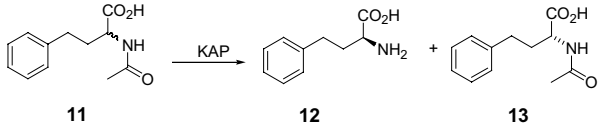
As part of our studies toward the synthetic development of precursors for the manufacture of enantiopure ACE inhibitors, we explored a practical procedure for the synthesis of D-HPA. As a result, a new synthetic approach for the synthesis of Benazepril **1**, involving the use of L-HPA was described;[2] this procedure is potentially suitable for the synthesis of other ACE inhibitors. As both enantiomers of HPA can be used as precursors to ACE inhibitors,[2,3a–j] we believe a resolution strategy is an appropriate choice.

Racemic *N*-acetyl-HPA **11** was obtained in a typical α-amino acid synthetic pathway involving hydrolysis of 5-phenetylhydantoin **10**, which was prepared by a Bucherer–Bergs protocol,[6] with an overall yield of 50% from 3-phenylpropanaldehyde **9** (Scheme 4).



Scheme 4. Reagents and conditions: (a) KCN, $(NH_4)_2CO_3$ (75%); (b) NaOH, H_2O, 130 °C (74%); (c) Ac_2O, NaOH, 0–5 °C (90%).

The *N*-acyl amino acid **11** was subjected to enzymatic resolution with KAP from different mammalian species with their specific activities being calculated (Scheme 5).



Scheme 5. Enzymatic resolution of *N*-acetyl HPA with mammalian KAP.

In a preliminary test, kidneys from five species were explored: beef, dog, hog, rat, and sheep. Dog and rat KPAs did not produce significant bioconversion after 90 h of reaction, so, they were discarded from this study.

As shown in Tables 1 and 2, beef kidney acetone powder was the most efficient in this biotransformation, giving the highest isolated yields and enantiomeric excesses (ee) for both L-HPA **12** and D-*N*-Ac-HPA **13**.

In order to determinate the ees of **12** and **13** by chiral HPLC, it was necessary to derivatize both compounds into their corresponding methyl esters, using a method reported by Lin et al.[9]

Table 1. L-HPA **12** from enzymatic resolution of **11**

Mammalian	Isolated yield (%)	$[\alpha]^a$ (*c* 1, 3 M HCl)	Ee[b] (%)
Sheep	34	+44.8	99.4
Beef	41	+45.2	99.9
Hog	40	+45.1	99.9

[a] Lit.[7] $[\alpha] = +47.5$ (*c* 1.0, 1 M HCl).
[b] Determined as the methyl ester on a CHIRALCEL OD column.

Table 2. D-*N*-Ac-HPA **13** from enzymatic resolution of **11**

Mammalian	Isolated yield (%)	[α][a] (*c* 1, CH_3OH)	Ee[b] (%)
Sheep	26%	−18.9	94
Beef	37.5%	−19.5	>99
Hog	33%	−19.4	96

[a] Lit.[8] [α] = −19.2 (*c* 1.0, CH_3OH).
[b] Determined as the methyl ester on a CHIRALCEL OD column.

A series of tubes containing varying substrate (*N*-acetyl-DL-homophenylalanine) concentration were prepared and the reaction started by the addition of the KAP extract. L-HPA was measured colorimetrically by the ninhydrin method modified by Doi et al.[10] Commercial DL-HPA was used as standard.

Samples were taken at different times to follow the time course of the hydrolysis. Linear product concentration versus time was obtained for 360 min under our experimental conditions. From these data, initial reaction rates were calculated for each substrate concentration and the data fitted to hyperbolic kinetics by nonlinear regression analysis with the program Prism 3.0 cx for Macintosh. The fitted curve and parameters are shown in Figure 2.

From the obtained V_{max} value and the measured protein concentrations in the sample, a specific activity of 3.9 μmole min^{-1} mg^{-1} was calculated for beef material.

A simplified estimation of the specific activities of sheep and hog extracts were performed by measuring the HPA produced over a fixed incubation time, at variable *N*-acetyl-DL-HPA concentration. Measured rates were lower than those in Figure 2, so the linearity of the reaction rate at each substrate concentration can be assumed with confidence. The data for all the KAP extracts are shown in Table 3.

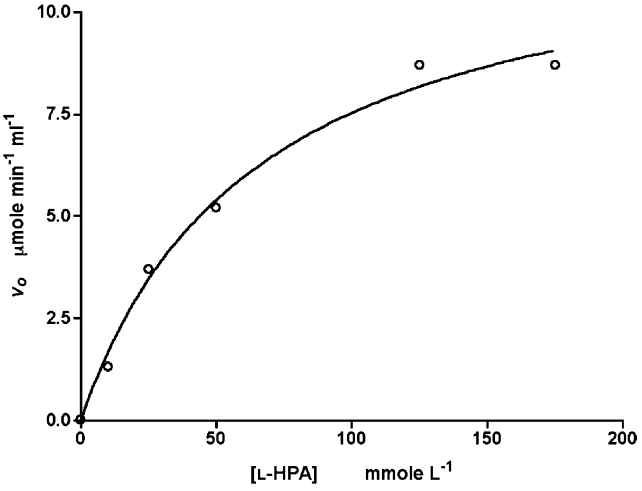


Figure 2. Curve of initial velocity against substrate concentration. Data fitted to Michaelis–Henri kinetics, as detailed in the text. Extract concentration, 3.165 mg mL^{-1}. V_{max}, 12.4±0.98 μmole min^{-1} mL^{-1}; $K_m = 65.0 \pm 12.7$ mmole L^{-1}.

Table 3. Specific activities for the different KAPs

KAP	Specific activity (μmole min^{-1} mg^{-1})
Beef	3.91
Hog	2.51
Sheep	2.54

3. Experimental

3.1. Preparation of kidney acetone powders

Beef, sheep, and hog kidneys were obtained from recently slaughtered animals from a nearby slaughterhouse. Dog kidneys were supplied by the local dog control center and rat kidneys from the animal breeding facility of our university. Fresh sheep kidneys (142 g) were blended in 420 mL of chilled acetone using a household blender and filtered through a Büchner funnel. The cake was treated twice in the same manner, until the filtrate was colorless. The solid residue was air dried under the hood and sifted on a 20-mesh sieve. The procedure yielded 22.4 g of dry powder, which was kept at −20 °C. A similar procedure was used to obtain beef, dog, hog, and rat KAP. A typical KAP preparation kept its specific activity after six months.

3.2. Chemical synthesis

3.2.1. DL-Homophenylalanine 14. A 1 L stainless steel reactor was charged with 81.73 g (0.402 mol) of DL-5-phenethylhydantoin **10**, 400 mL of water and 48.31 g (1.207 mol) of NaOH. After closing the vessel, the mixture was heated for four hours at 130–132 °C. The reaction mixture was treated with activated charcoal for 5 min, filtered over diatomaceous earth and washed with water. After acidifying to pH 6.3 with 10 M HCl and cooling to 0–5 °C for 1 h, a beige powder crystallized out, which was filtered, washed with water, and dried at 60 °C until a constant weight was seen. The yield of **14** was 56.63 g (74.3%), mp 279–280 °C [lit.[11] m.p. 282 °C (dec)].

3.2.2. DL-*N*-Acetyl-homophenylalanine 11. To a cold solution of 12.5 g (69.7 mmol) of **14** in 115 mL of water, and 2.79 g (69.7 mmol) of NaOH (0–5 °C), were added 9.26 g (90.6 mmol) of acetic anhydride, while the pH was kept in the range 11–11.5 by adding 50% NaOH, at the same temperature. Temperature and pH were maintained for one hour; after acidifying to pH 2.3 with 10 M HCl, the mixture was kept at 0–5 °C for 1 h, filtered and washed with the minimum amount of water. The yield of compound **11** was 13.8 g (89.5%), m.p. 145–147 °C, [lit.[12] m.p. 147–148 °C].

3.3. Enzymatic hydrolysis

A mixture of 2.4 g of **11** and 60 mL of 0.2 M potassium phosphate buffer pH 7.5, containing 10 mg L^{-1} of $CoCl_2$ was adjusted to pH 7.5 with 6 M NaOH. The solution

was divided into three 20 mL fractions; to each one, 200 mg of kidney acetone powders of beef, hog, and sheep, were added, respectively, and then incubated at 37 °C for 24 h in an orbital shaker at 250 rpm. After cooling to 5 °C, the pH was adjusted to 11.0 with 1 M NaOH under vigorous stirring and the reaction mixtures was centrifuged separately at 3200*g* and 5 °C for 15 min. Each supernatant was acidified to pH 6.3 with 6 M HCl and cooled to 0–5 °C for half an hour. The L-HPA **12** precipitate was filtered and the mother liquors saved. The solid was dissolved in 1 M HCl, treated with activated charcoal, filtered over diatomaceous earth and its pH again adjusted to 6.3, then filtered, washed with water and dried at 60 °C until constant weight. The yields of L-HPA **12** obtained for different KAPs are shown in Table 1. The filtrate from each of the above samples, containing unreacted D-*N*-Ac-HPA **13**, was adjusted to pH 2.3 with 10 M HCl, cooled to 0 °C for 2 h, vacuum filtered, washed with chilled water until the pH of the washing was between 3 and 4 and dried at 60 °C until constant weight. The yields of D-*N*-Ac-HPA **13** are shown in Table 2.

3.4. Determination of ee of 12 and 13

3.4.1. L-Homophenylalanine methyl ester. L-HPA **12** (300 mg, 1.67 mmol) was suspended in anhydrous methanol (6 mL) and cooled to −10 °C in an ice-salt bath. Then, 156 μL (260 mg, 2.18 mmol) of thionyl chloride were added dropwise and the reaction mixture kept for 1 h at −5 to −10 °C and then at 40–45 °C until TLC monitoring (7:3 isopropanol–ammonium hydroxide; developed with 0.2% ninhydrine reagent spray) indicated the L-HPA had disappeared. The solution was then evaporated in a rotatory evaporator. The solid was dissolved in the minimum amount of cold water, made alkaline with sodium bicarbonate and extracted with ethyl acetate (3 × 5 mL), dried, and evaporated to dryness. The L-HPA methyl ester was submitted to chiral HPLC analysis, on a CHIRALCEL OD column; hexane–isopropanol (90:10). *N*-Acetyl-D-HPA **13** was treated as above to give the corresponding methyl ester, which was submitted to chiral HPLC analysis under the same conditions.

3.5. Enzyme assays and specific activity of L-aminoacylase in beef KAP

To measure L-aminoacylase activity in KAP, a soluble preparation was made by suspending 200 mg of each KAP in 10 mL of 0.2 M potassium phosphate buffer pH 7.5, at 5 °C. The suspension was centrifuged and the supernatant kept in an ice bath. Protein concentration was determined with the bicinchoninate method.[13]

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