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Oxidation of Nα-protected-L-lysine by *Rhodotorula graminis* to produce novel chiral compounds

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Abstract

The chiral intermediates (*S*)-3,4-dihydro-1,2(2*H*)-pyridinedicarboxylic acid, 1-(phenylmethyl)ester [BMS 202665-01] and (*S*)-3,4-dihydro-1,2(2*H*)-pyridinedicarboxylic acid, 1,1-dimethylethyl ester [BMS 264406-01] were prepared by oxidation of Nα-carbobenzoxy-L-lysine (Nα-CBZ-L-lysine) and Nα-*t*-butoxycarbonyl-L-lysine (Nα-*t*-BOC-L-lysine), respectively, by cell suspensions of *Rhodotorula graminis* SC 16005. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

The current interest in enzymatic production of chiral amino acids and amino acid derivatives lies in the preparation of intermediates for pharmaceutical synthesis.[1–11] Oxidation of L-lysine by L-lysine-α-ketoglutarate aminotransferase from *Flavobacterium flavescens*,[12] *Rhodotorula glutinis*,[13] *Candida utilis*,[14] *Streptomyces lactamdurans*,[15] and *Saccharomyces cerevisiae*,[16] has been demonstrated. L-Lysine-α-ketoglutarate aminotransferase catalyzes ε-transamination of L-lysine with α-ketoglutarate. The terminal amino group of L-lysine is transferred to α-ketoglutarate to yield L-glutamate and α-aminoadipate-δ-semialdehyde, which is rapidly converted into the intramolecularly dehydrated form 1-piperideine-6-carboxylic acid.[17–19]

L-Lysine-α-ketoglutarate aminotransferase contains bound pyridoxal 5′-phosphate as a prosthetic group.[12,17–19] An NAD^+-dependent L-lysine ε-dehydrogenase from *Agrobacterium tumefaciens* was also shown to catalyze the oxidation of L-lysine to α-aminoadipate-δ-semialdehyde, which is rapidly converted into 1-piperideine-6-carboxylic acid.[20]

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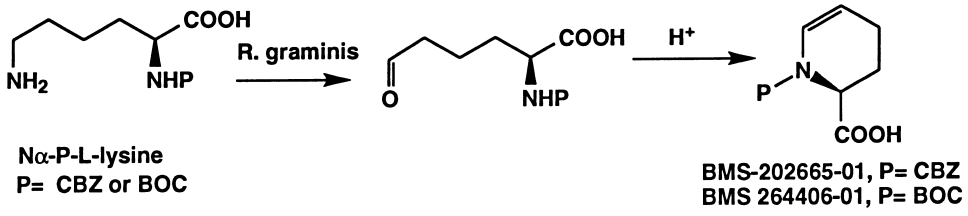
Table 1
Oxidation of Nα-*t*-BOC-L-lysine by cell suspensions of *Rhodotorula graminis* SC16005

Reaction Time (Hours)	Nα-t-BOC-L-lysine (g/L)	Conversion (%)
0	10.0	0
24	7.7	23
48	5.1	49
72	3.0	70
96	2.0	80

Reaction was carried out as described in the Experimental section.

In this report we describe the oxidation of Nα-*t*-BOC-L-lysine and Nα-CBZ-L-lysine by cell suspensions of *Rhodotorula graminis* SC 16005 to prepare novel chiral intermediates (Scheme 1). Products (BMS 264406-01 and BMS 202665-01) of Nα-*t*-BOC-L-lysine and Nα-CBZ-L-lysine are chiral intermediates potentially useful in the synthesis of some ACE (angiotensin converting enzyme) inhibitors effective as antihypertensive drugs.[7,8]



2. Results and discussion

2.1. Oxidation of Nα-t-BOC-L-lysine

Cells of *Rhodotorula graminis* SC 16005 were grown in a 75 L fermentor as described in the Experimental section. About 3 kg of wet cell paste were obtained from each fermentation run. Cell suspensions (20%, w/v, wet cells) were used to conduct the oxidation of Nα-*t*-BOC-L-lysine. Results are as shown in Table 1. A conversion of 80% was obtained on the basis of substrate utilized after a 96 h reaction period. From a portion of the reaction mixture (2.5 L), 8.2 g of product (*S*)-3,4-dihydro-1,2(2*H*)-pyridinedicarboxylic acid, 1,1-dimethylethyl ester [BMS 264406-01] was isolated in overall 41% yield.

2.2. Oxidation of Nα-CBZ-L-lysine

Cells of *Rhodotorula graminis* SC 16005 (20% cell suspension, w/v, wet cells) were used to conduct the oxidation of Nα-CBZ-L-lysine as described in the Experimental section. Results are as shown in Table 2. A conversion of 72% was obtained on the basis of substrate utilized. From the reaction mix-

Table 2
Oxidation of Nα-CBZ-L-lysine by cell suspensions of *Rhodotorula graminis* SC16005

Reaction Time (Hours)	Nα-CBZ-L-lysine (g/L)	Conversion (%)
0	10.0	0
24	8.1	19
48	6.5	35
72	4.3	57
96	2.8	72

Reaction was carried out as described in the Experimental section.

ture (0.5 L), 1.5 g of product (*S*)-3,4-dihydro-1,2(2*H*)-pyridinedicarboxylic acid, 1-(phenylmethyl)ester [BMS 202665-01] was isolated in overall 40% yield.

An NAD^+-dependent oxidation of L-lysine by L-lysine ε-dehydrogenase from *Agrobacterium tumefaciens* has been demonstrated.[20] L-Lysine is oxidatively deaminated to yield α-aminoadipate δ-semialdehyde, which is spontaneously converted to 1-piperideine-6-carboxylate. We have evaluated the oxidation of L-lysine, Nα-CBZ-L-lysine and Nα-*t*-BOC-L-lysine by cell suspensions of *Agrobacterium tumefaciens*. Only L-lysine was oxidized by *Agrobacterium tumefaciens* (unpublished results).

A different enzyme (L-lysine ε-aminotransferase) was demonstrated from *Streptomyces lactamdurans*.[15] In this organism, the precursor of the α-aminoadipoyl side-chain of cephamycin C is L-lysine. L-Lysine ε-aminotransferase required α-ketoglutarate for the reaction. The terminal amino group of L-lysine is transferred to α-ketoglutarate to yield L-glutamate and α-aminoadipate-δ-semialdehyde, which is converted into the dehydrated form 1-piperideine-6-carboxylic acid. L-Lysine ε-aminotransferase was also demonstrated in *Rhodotorula graminis*, which grew on a medium containing L-lysine as the sole nitrogen source and accumulated α-aminoadipic-δ-semialdehyde. Aminotransferases have been used in the preparation of amino acids and amines.[21]

In this report we have demonstrated the biocatalytic preparation of novel chiral intermediates (*S*)-3,4-dihydro-1,2(2*H*)-pyridinedicarboxylic acid, 1-(phenylmethyl)ester [BMS 202665-01] and (*S*)-3,4-dihydro-1,2(2*H*)-pyridinedicarboxylic acid, 1,1-dimethylethyl ester [BMS 264406-01] by oxidation of Nα-CBZ-L-lysine and Nα-*t*-BOC-L-lysine, respectively, by cell suspensions of *Rhodotorula graminis* SC 16005. BMS-202665-01 and BMS-264406-01 are potential chiral intermediates required for the chemical synthesis of antihypertensive drugs.[7,8]

3. Experimental

3.1. Materials and methods

Nα-*t*-BOC-L-Lysine and Nα-CBZ-L-lysine were purchased from Sigma Chemicals (St. Louis, MO).

The proton magnetic resonance (^{1}H-NMR) and carbon magnetic resonance (^{13}C-NMR) spectra were recorded on a Brucker AM-300 spectrometer.

3.2. Microorganism

Rhodotorula graminis SC 16005 was obtained from the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute. Microbial cultures were stored at −90°C in vials.

3.3. Growth of microorganism

One vial of culture was used to inoculate 100 mL of medium A containing 1% malt extract, 1% yeast extract, 2% glucose and 0.3% peptone. The medium was adjusted to pH 6.8 before sterilization. Cultures were grown at 28°C and 280 RPM for 48 h. Cultures were harvested by centrifugation at 18 000×g for 15 min, washed with 0.1 M potassium phosphate buffer pH 8.0, and used for oxidation studies.

3.4. Oxidation by cell suspensions

Cells of *Rhodotorula graminis* SC 16005 were suspended in 100 mM potassium phosphate buffer (pH 8.0) at 20% (w/v, wet cells) cell concentration. Cell suspensions were supplemented with 10 mg/mL of Nα-CBZ-L-lysine and 10 mg/mL of α-ketoglutarate. Oxidation was conducted at 28°C and 150 RPM. Periodically, samples of 0.1 mL were taken and diluted with 0.9 mL of acetonitrile and 0.1 mL of trichloroacetic acid were added. The sample was then incubated at room temperature for 2 h, centrifuged, and filtered through a 0.2 μm LID/X filter and analyzed by HPLC. Similarly, the oxidation of Nα-*t*-BOC-L-lysine was carried out by cells of *Rhodotorula graminis* SC 16005.

3.5. Growth of organisms in a fermentor

Rhodotorula graminis SC 16005 cultures were grown in a 75 L fermentor containing 50 L of medium A containing 0.025% UCON antifoam. Growth consisted of several inoculum development stages and a fermentation stage. Inoculum development consisted of F1 and F2 stages. In the F1 stage, a frozen vial of each culture was inoculated into 100 mL of medium A contained in a 500 mL flask. Growth was carried out at 28°C and 280 RPM for 48 h on a rotary shaker. In the F2 stage, 10 mL of F1 stage culture was inoculated into 1 L of medium A and incubated at 28°C and 280 RPM for 24 h. Fermentors containing 50 L of medium A were inoculated with 3 L of inoculum of each culture from an F2 stage. Fermentation was conducted at 25°C and 500 RPM with 15 LPM (liter per min) aeration for 48 h and then cells were collected and stored at −90°C until further use. About 3 kg of wet cell paste were obtained from each fermentation batch.

3.6. Preparative scale biotransformation of Nα-CBZ- and Nα-t-BOC-L-lysine

Frozen cells from the above batches were used to conduct the oxidation reaction in a 5 L reactor. Cells (600 g wet cells) were first washed with 20 mM potassium phosphate buffer (pH 8.0) and washed cells were suspended in 2.75 L of 0.1 M potassium phosphate buffer (pH 8.0). Cell suspensions were supplemented with 30 g of Nα-CBZ-L-lysine and 21 g of disodium α-ketoglutarate. The oxidation reaction was carried out at 28°C and 300 RPM with 0.5 LPM aeration for 42 h. After a 24 h biotransformation period, an additional 10 g of disodium α-ketoglutarate were added. The pH was maintained between 7.6 and 8.0. Periodically, samples were prepared as described above and analyzed by HPLC to determine the % conversion of Nα-CBZ-L-lysine to Nα-CBZ-6-hydroxy-2-piperidine carboxylic acid.

Biotransformation of Nα-*t*-BOC-L-lysine was carried out in a 20 L Braun bioreactor. Frozen cells (1.87 kg) of *Rhodotorula graminis* SC 16005 were washed with 20 mM potassium phosphate buffer (pH 8.0) and washed cells were suspended in 12.5 L of 0.1 M potassium phosphate buffer (pH 8.0). Cell suspensions were supplemented with 125 g of Nα-*t*-BOC-L-lysine and 76 g of disodium α-ketoglutarate. The oxidation reaction was carried out at 28°C and 300 RPM with 0.5 LPM aeration for 96 h. After 24 and 72 h, an additional 38 g of disodium α-ketoglutarate were added. The pH was maintained between 7.6 and 8.0. Periodically, samples were prepared as described above and analyzed by HPLC to determine the % conversion of Nα-*t*-BOC-L-lysine to Nα-*t*-BOC-6-hydroxy-2-piperidine carboxylic acid.

*3.7. Isolation of (*S*)-3,4-dihydro-1,2(2*H*)-pyridine dicarboxylic acid, 1-(phenylmethyl) ester*

The enzymatic reaction mixture (0.5 L) was centrifuged at 20 000×g for 15 min to remove cells and the supernatant solution was lyophilized to obtained 2.5 g of material. The crude lyophilized material was dissolved in 23 mL of ethyl acetate and 23 mL of water were added. The pH of the mixture was adjusted to 2.95 with concentrated phosphoric acid. The aqueous phase was separated and extracted with 12 mL of ethyl acetate.

The combined organic extract was stirred with 23 mL of 0.1 M sodium phosphate buffer (pH 8.0) and the pH was adjusted to 9.4 with 1 M NaOH. The lower phase was separated and the upper phase was extracted with 12 mL of water. The combined aqueous extract was stirred with 23 mL of ethyl acetate and adjusted to pH 2.8 with concentrated phosphoric acid. The organic phase was separated, washed with three 2 mL portions of water and concentrated under reduced pressure to produce a colorless oily residue (1.65 g). The oily residue was dissolved in 10 mL of CH_2Cl_2 and chromatographed on a silica gel 60 column (2.5×25 cm), eluted with 400 mL of CH_2Cl_2:CH_3OH (9:1) and subsequently with 600 mL of the same solvent (4:1). Fractions (20 mL) were collected and assayed by TLC on silica gel (developed with CH_2Cl_2:CH_3OH, 3:1) and detected by Ehrlich reagent (*p*-dimethylaminobenzaldehyde, 20 g/L in conc. CH_3COOH:HCl, 85:15, v/v), then heated to develop an intense cherry-pink color). Fractions 13–26 which gave Ehrlich positive reaction (R_f 0.44) were combined and concentrated under reduced pressure to produce a colorless viscous oil (1.5 g). $[\alpha]_D=-14.4$ (c=0.6, CH_3CN). MS: m/z 262 $(M+H^+)$ and 218 $(M+H-CO_2)^+$; ^{1}H-NMR ($CDCl_3$): 7.3 (m, 5H); 6.86 (d, 0.5H); 4.85 (m, 1H); 2.35 (m equatorial 1H); 2.02 (m, 2H); 1.89 (m, axial 1H). ^{13}C-NMR: 174.69, 155.10, 155.01, 137.55, 125–130, 125.33, 125.06, 107.24, 106.79, 68.89, 68.71, 55.59, 55.25, 24.48, 24.44, 19.46, 19.26.

The NMR spectra indicate that the compound exists as a mixture of rotamers that are stable on the NMR time scale.

*3.8. Isolation of (*S*)-3,4-dihydro-1,2(2*H*)-pyridine dicarboxylic acid, 1,1-dimethyl ester*

The enzymatic reaction mixture (2.5 L) was centrifuged at 20 000×g for 15 min to remove cells, the supernatant solution was stirred with 1.25 L of ethyl acetate and the pH of the mixture was adjusted to 3.0 with concentrated phosphoric acid. The upper phase was separated and mixed with 10 g of acid-washed Celite and filtered. The filtrate was stirred with 600 mL of H_2O and adjusted to pH 7.75 with 1 M NaOH. The lower phase was separated and stirred with 300 mL of ethyl acetate and the pH of the mixture was adjusted to 3.0 with concentrated phosphoric acid. The upper phase was separated and the lower phase was extracted with a second 300 mL portion of ethyl acetate. The combined upper phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to produce a residue (11 g), which was then dissolved in 100 mL of ethyl acetate and refluxed for 4 h. The ethyl acetate was removed under reduced pressure to obtain a pale yellow viscous oil (8.2 g). $[\alpha]_D=-15.3$ (c=0.6, CH_3CN). MS: m/z 228 $(M+H)^+$;

^{1}H-NMR ($CDCl_3$): 11.2 (s, 1H); 6.87 (dd, 1H); 5.01 (m, 1H); 4.9 (m, 1H); 2.5 (m, 2H); 1.8 (m, 2H); 1.55 (m, 9H). ^{13}C-NMR: 177.4, 177.1, 152.5, 152.2, 124.6, 124.2, 104.7, 104.4, 81.7, 81.5, 54.0, 52.9, 28.1, 23.4, 23.2, 18.4, 18.2.

3.9. Analytical methods

Analysis of Nα-CBZ-L-lysine and Nα-*t*-BOC lysine was carried out using a Hewlett–Packard HPLC. A Vydac C-18 protein and peptide column (100×4.5 mm, ID 5 m) was used under the following conditions. The mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 70% methanol:30% water:0.1% trifluoroacetic acid (solvent B) used in a linear gradient of 0 to 100% B from 0 to 25 min and 100% A from 26 to 30 min. The flow rate was 1 mL/min and the detection wavelength was 211 nm. The retention times for Nα-CBZ-L-lysine, Nα-CBZ-6-hydroxy-2-piperidine carboxylic acid, Nα-*t*-BOC-L-lysine and Nα-*t*-BOC-6-hydroxy-2-piperidine carboxylic acid are 10.92 min, 17.9 min, 9.27 min and 16.7 min, respectively.

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