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Enzymatic preparation of 5-hydroxy-L-proline, *N*-Cbz-5-hydroxy-L-proline, and *N*-boc-5-hydroxy-L-proline from (α-*N*-protected)-L-ornithine using a transaminase or an amine oxidase

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ABSTRACT

N-Cbz-4,5-dehydro-L-prolineamide or *N*-Boc-4,5-dehydro-L-prolineamide are alternative key intermediates for the synthesis of saxagliptin, a dipeptidyl peptidase IV (DPP4) inhibitor recently approved for treatment of type 2 diabetes mellitus. An efficient biocatalytic method was developed for conversion of L-ornithine, *N*-α-benzyloxycarbonyl (Cbz)-L-ornthine, and *N*-α-*tert*-butoxycarbonyl (Boc)-L-ornithine to 5-hydroxy-L-proline, *N*-Cbz-5-hydroxy-L-proline, and *N*-Boc-5-hydroxy-L-proline, respectively. Rec. *Escherichia coli* expressing lysine-ε-aminotransferase and rec *Pichia pastoris* expressing L-ornithine oxidase were used for these conversions. *N*-Cbz-5-hydroxy-L-proline, and *N*-Boc-5-hydroxy-L-proline were chemically converted to key intermediates *N*-Cbz-4,5-dehydro-L-prolineamide and *N*-Boc-4,5-dehydro-L-prolineamide, respectively.

1. Introduction

The enzyme DPP4, which regulates plasma levels of the insulinotropic glucagon-like peptide-1(7–36) (GLP-1(7–36)) hormone, has emerged as a novel therapeutic target for the treatment of type 2 diabetes [1,2]. Inhibitors of DPP4 have been shown to elevate levels of GLP-1(7-36) which increases glucose-stimulated insulin secretion, decreases glucagon secretion, delays gastric emptying, and increases the β-cell mass of pancreatic islets, and thereby improves regulation of blood glucose levels in diabetic patients [3–5]. Recently, pyrrolidine-based small molecule inhibitors of DPP4, such as (2*S*)-{[(3-hydroxyadamantan-1-yl)amino]acetyl}-pyrrolidin-2-carbonitrile and (2*S*)-1-{2-[(5-cyanopyridin-2-yl)amino]ethylamino}acetyl-2-pyrrolidinecarbonitrile have emerged as potent and selective, orally bioavailable therapeutics for type 2 diabetes [6–10].

During the course of a program to develop DPP4 inhibitors, an efficient process for the preparation of synthetic intermediates *N*-Cbz-4,5-dehydro-L-prolineamide (**3**) or *N*-Boc-4,5-dehydro-L-prolineamide (**4**) was required [11]. We have previously reported the conversion of *N*-α-Cbz-L-lysine and *N*-α-Boc-L-lysine to (*S*)-3,4-dihydro-1,2(2*H*)-pyridinedicarboxylic acid, 1-(phenylmethyl)ester and (*S*)-3,4-dihydro-1,2(2*H*)-pyridinedicarboxylic acid, 1,1-dimethylethyl ester, respectively, by cell suspensions of *Rhodotorula graminis* SC 16005 [12]. We have also reported the transfer of the ε-amino group of lysine in the dipeptide *N*-Cbz-L-homocysteinyl L-lysine to α-ketoglutarate to produce [4*S*-(4a,7a,10ab)]1-octahydro-5-oxo-4-[(Cbz)amino]-7H-pyrido-[2,1-b] [1,3]thiazepine-7-carboxylic acid using a novel L-lysine ε-aminotransferase (LAT) from *Sphingomonas paucimobilis* SC 16113 cloned and overexpressed in *Escherichia coli* [13]. In this report we have explored a similar approach to the conversion of L-ornithine derivatives (*N*-α-Cbz-L-ornithine and *N*-α-Boc-L-ornithine) to intermediates required for the synthesis of saxagliptin. A biocatalytic procedure was developed, based upon the enzymatic conversion of L-ornithine, *N*-α-Cbz-L-ornthine, and *N*-α-Boc-L-ornithine to 5-hydroxy-L-proline, *N*-Cbz-5-hydroxy-L-proline (**1**), and *N*-Boc-5-hydroxy-L-proline (**2**), respectively.

2. Materials and methods

2.1. Growth of *E. coli* BL21-DE3 (pBMS2000-LAT)

E. coli BL21-DE3 (pBMS2000-LAT) containing cloned lysine ε-amino transferase from *S. paucimobilis* SC 16113 [13] was grown as described below: F1 stage: A 1 mL aliquot from a vial of *E. coli* BL21-DE3 (pBMS2000-LAT) was inoculated into each 500-mL flask containing 100 mL of MT5 medium [2.0% yeastamin, 4.0% glycerol, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.125% $(NH_4)_2SO_4$, 0.0246% $MgSO_4 \cdot 7H_2O$ (added post-autoclaving from a sterile 1 M solution) and 0.005% kanamycin (added post-autoclaving from a sterile 5% solution)]. The medium had been batched with deionized water, adjusted to pH 7.2, and autoclaved for 30 min at 121 °C. Each flask was incubated at 30 °C for 24 h at 250 rpm. At the time of transfer to the next stage, OD_{600} was ca. 5.5–8.0 when measured against a medium MT5 blank.

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F2 stage: A 100 mL portion of F1 stage culture was inoculated into each 4-L flask containing 1-L of MT5-M1 medium [2.0% quest Hy-Pea, 1.85% tastone-154, 4.0% glycerol, 0.6% Na_2HPO_4, 0.125% $(NH_4)_2SO_4$, 0.0246% $MgSO_4{\bullet}7H_2O$ (added post-autoclaving from a sterile 1 M solution) and 0.005% kanamycin (added post-autoclaving from a sterile solution)]. The medium was batched with deionized water, adjusted to pH 7.4, and autoclaved for 30 min at 121 °C. Flasks were then incubated on a shaker at 30 °C and 250 rpm. At an OD_{600} of ca. 0.8, 10 mL of a filter-sterilized 3.5 mM β-isopropyl thiogalactoside (IPTG) solution was added aseptically to a final concentration of 35 μM in the 1 L of broth. All filter sterilization of medium components was conducted with a 0.2 μm cellulose nitrate filter. After 24 h growth, the flasks were harvested by centrifugation at 11,325 × *g* for 10 min at 4 °C. The final recovery was 26.8 g wet cells from each flask which were stored at −70 °C until assayed for activity.

2.2. Growth of *E. coli* BL21-DE3 (pBMS2000-LAT) in a 15-L fermentor

E. coli BL21-DE3 (pBMS2000-LAT) was grown in flasks (F1-stage and F-2 stage) as described above. The fermentor contained 13-L of medium MT5-M1 with 0.04% antifoam UCON to control foaming. The pH after batching was adjusted to 7.4. Sterilization was conducted at 121 °C for 20 min. After sterilization, the temperature was reduced to 30 °C and the volume was about 13 L. Prior to inoculation, a filter-sterilized solution containing $MgSO_4{\bullet}7H_2O$ and kanamycin (combined in 500 mL of H_2O) was added aseptically to the tank to a final concentration of 1 mM (0.0246%) and 50 mg/L, respectively. The fermentor was inoculated with broth from one F2 flask (1 L inoculum) to yield an optical density (600_{nm}) in the tank of ca. 0.35. At a CO_2 off-gas level of 0.15–0.25, filter-sterilized IPTG (125 mg dissolved in 500 mL H_2O) was added aseptically to yield a final concentration of 35 μM. This off-gas level was achieved after 4 h. If necessary, an $OD_{600} \sim 0.8$ may also be used as the criterion for inducer (IPTG) addition. Because antifoam and other solids in MT5-M1 affect medium clarity, the following procedure was used to measure optical density. 1 mL of broth was centrifuged at 5000 × *g* for 10 min and the supernatant was discarded. The pellet was re-suspended in water to the same 1 mL volume. This suspension was then further diluted 1:10 with water (200 μl suspension plus 1.8 mL water) for OD measurement. The value obtained was multiplied by 10 to get the final reading. The fermentation was conducted at 30 °C, 1 vvm aeration, 500 rpm agitation, and 690 mbar pressure. The pH was controlled at 7.0 with NH_4OH and the foaming was controlled by addition of antifoam UCON on demand. Samples were taken during the course of the fermentation for determination of wet cell weights, enzyme activity, pH, dry cell weight, glycerol, and nitrogen content. Off-gas CO_2 from the fermentor was monitored continuously with a gas analyzer. A significant drop in CO_2 off-gas was used to end the run. When CO_2 decreased from its peak to a value of ca. 0.6, the fermentor was cooled to 10 °C and the cells were recovered by centrifugation. The cell paste was rinsed with 1 L of 10 mM pH 7.0 sodium phosphate. The cell paste was removed and stored at −70 °C.

2.3. Enzymatic conversion of *N*-α-Cbz-L-ornithine to *N*-Cbz-5-hydroxy-L-proline (**1**) using lysine-ε-aminotransferase

To a 250-mL Erlenmeyer flask were added 1.33 g of *N*-α-Cbz-L-ornithine (final concentration 0.1 M), 1.9 g of α-ketoglutarate disodium salt (final concentration 0.2 M) and 50 mL 0.1 M potassium phosphate buffer, pH 8. After the solids were dissolved, 0.5 g frozen *E. coli* cells BL21-DE3 (pBMS2000-LAT) expressing lysine-ε-aminotransferase was added and mixed. The flask was shaken at 28 °C, 200 rpm. 1 h after the start of the incubation the pH was adjusted to 8 by addition of 1 M K_2HPO_4. After 15 h, the concentration of α-N -Cbz-L-ornithine had decreased to 0.87 mg/mL and a new HPLC product peak appeared. Incubation was continued for a total of 39 h, and then the cells were removed by centrifugation. The reaction mixture was extracted at pH 3.5 (adjusted with 6 M H_2SO_4) with three 40-mL portions of ethyl acetate. Concentration of the extract gave 1.22 g (4.61 mmole, 92% yield) of *N*-Cbz-5-hydroxy-L-proline (**1**) as a non-crystalline residue.

2.4. Conversion of *N*-Cbz-5-hydroxy-L-proline (**1**) to *N*-Cbz-4,5-dehydro-L-prolineamide (**3**) [14,15]

A 99-mg portion of **1** (0.374 mmole) was stirred with 1.5 mL of anhydrous methanol and 30 mg of Amberlyst 15 resin (H+) under nitrogen for 1 h. The mixture was filtered, rinsing with 2 mL of 1,2-dichloroethane. The filtrate was concentrated in a nitrogen stream, and the residue was redissolved in 1,2-dichloroethane and reconcentrated to remove any remaining methanol. The residue was dissolved in 1 mL of 1,2-dichloroethane, stirred under nitrogen with 0.233 mL (1.34 mmole) of *N*,*N*-diisopropylethylamine (DIPEA), cooled in an ice bath, and 0.186 mL of trimethylsilyl trifluoromethanesulfonate (TMSOTf, 1.03 mmole) was added dropwise with stirring. After 0.5 h 1 mL of concentrated ammonium hydroxide was added. The lower (organic) phase was separated and the rich aqueous phase concentrated to remove excess ammonia. The aqueous solution, 2 mL, was stirred with 115 mg (0.870 mmole) of ammonium sulfate, 2 mL of ethyl acetate and 158 mg (0.570 mmole) of 4-(4,6-dimethoxy-1,3,5-triazen-2-yl)-4-methylmorpholinium chloride (DMT-MM). After 3 h, the upper phase was separated and washed with 1 M KH_2PO_4 and water. Concentration of the organic extract gave 96 mg of residue that was 59% (w/w) *N*-Cbz-4,5-dehydro-L-prolineamide by HPLC assay. Purification by preparative TLC on Whatman 20 cm × 20 cm PLK5F silica gel plate, eluting with dichloromethane-methanol, 9:1, and collecting a band with Rf 0.65–0.77 gave 46.5 mg (0.189 mmole) of crystalline *N*-Cbz-4,5-dehydro-L-prolineamide (**3**) (50% yield from **1**), indistinguishable by HPLC from authentic material prepared by an alternative route. ^{1}H NMR (300 MHz, DMSO-d6, 333 K) δ = 7.35 (m, 5H, Ph–), 6.9 (br, 2H, –$CONH_2$), 6.57 (*J* = 4.4, 2.2, 2.2, 1H, H-5), 5.11 (s, 2H, –OCH_2–), 5.03 (*J* = 4.3, 2.5, 2.5, 1H, H-4), 4.51 (*J* = 11.7, 5.1, 1H, H-2), 2.99 (*J* = 16.6, 11.7, 2.4, 2.4, 1H, H-3a), 2.53 (*J* = 16.8, 4.8, 2.5, 2.3, 1H, H-3b).

2.5. Enzymatic conversion of *N*-α-Boc-L-ornithine to *N*-Boc-5-hydroxy-L-proline (**2**) using lysine-ε-amino transferase

To a 50-mL Erlenmeyer flask were added 0.93 g of *N*-α-Boc-L-ornithine (final concentration 0.4 M), 1.52 g of α-ketoglutarate disodium salt (final concentration 0.8 M) and 10 mL 0.1 M potassium phosphate buffer, pH 8. After the solids dissolved, 1.0 g of frozen *E. coli* BL21-DE3 (pBMS2000-LAT) cells expressing lysine-ε-amino transferase was added and mixed. The flask was shaken at 28 °C, 200 rpm for 20 h. After the cells were removed by centrifugation there was no detectable *N*-α-Boc-L-ornithine remaining in the supernatant. A 4 mL portion of the supernate was extracted at pH 3.5 (H_2SO_4) with several 5-mL portions of ethyl acetate and additional portions after saturation with sodium chloride. Concentration of the combined extract gave 334 mg (1.44 mmole, 91% yield) of *N*-(Boc)-5-hydroxy-L-proline (**2**) as a slightly amber oil.

2.6. Preparation of *N*-Boc-4,5-dehydro-L-prolineamide (**4**)[14,15]

A 100-mg portion of **2** (0.432 mmole) was converted to the methoxy aminal and treated with TMSoTf as described above to prepare the protected dehydroproline. Water (1 mL) was added to the mixture. After warming to room temperature, 57.0 mg (0.431 mmole) of ammonium sulfate and 161.8 mg (0.585 mmole) of 4-(4,6-dimethoxy-1,3,5-triazen-2-yl)-4-methylmorpholinium chloride (DMT-MM) were added to the stirred mixture. After 30 min the rich lower (organic) phase was separated and washed with 15 mM ammonia. The organic extract was concentrated and the residue purified by preparative TLC as described above, eluting with dichloromethane-methanol, 19:1. Collection of a band with Rf 0.46–0.56 gave 43.3 mg (0.204 mmole, 47% yield from **2**) of *N*-(Boc)-4,5-dehydro-L-prolineamide (**4**) as a colorless oil that was indistinguishable by HPLC from authentic material prepared by an alternative route [11]. The product was stored at −20 °C as a solution in 1,2-dichloroethane. ^{1}H NMR (300 MHz, DMSO-d6, 333 K) δ = 6.5–7.5 (br, 2H, –$CONH_2$), 6.48 (*J* = 4.4, 2.1, 2.1, 1H, H-5), 4.93 (*J* = 4.3, 2.5, 2.5, 1H, H-4), 4.39 (*J* = 11.6, 5.4, 1H, H-2), 2.94 (*J* = 16.6, 11.6, 2.4, 2.4, 1H, H-3a), 2.50 (*J* = 16.6, 5.4, 2.5, 2.1, 1H, H-3b), 1.40 (s, 9H, –CMe3).

2.7. Isolation of microbial cultures with ornithine oxidase activity

Soil samples were collected from the Bristol–Myers Squibb New Brunswick, New Jersey, site. About a gram of soil sample was suspended in 5 mL of water, mixed thoroughly and samples were allowed to settle. The supernatant solutions from various samples were inoculated in 25 mL of medium A ($NaNH_4HPO_4$ 1 g, K_2HPO_4 0.5 g, KH_2PO_4 0.5 g, $MgSO_4{\bullet}7H_2O$ 200 mg, NaCl 10 mg, $FeSO_4{\bullet}7H_2O$ 10 mg, $MnSO_4{\bullet}4H_2O$ 10 mg, glycerol 20 g and *N*-α-Boc-L-ornithine 5 g in 1 L of water). After 96 h of growth when the medium became turbid, cultures were transferred to medium A containing 1.5% agar in petri dishes. From these enrichment cultures, three organisms were isolated that used *N*-α-Boc-L-ornithine as a sole nitrogen source. One faster growing culture, later identified as a *Pichia* strain (designated as SC 16539), was grown in 500-mL flasks containing 100 mL of medium A. The culture broth after growth gave an HPLC peak at 4.6 min (residual *N*-α-Boc-L-ornithine) and a new peak at 5.53 min (MS consistent with the *N*-Boc-5-hydroxy-L-proline **2**).

2.8. Conversion of *N*-α-Boc-L-ornithine to *N*-Boc-5-hydroxy-L-proline **2** by *Pichia* sp. SC16539

Pichia sp. SC16539 was inoculated from a slant into 10 mL of medium B (0.1 M pH 7 sodium phosphate buffer containing per liter 20 mL glycerol, 1 g yeast extract, 0.2 g $MgSO_4{\bullet}7H_2O$, 10 mg NaCl, 10 mg $FeSO_4{\bullet}7H_2O$, 10 mg $MnSO_4{\bullet}4H_2O$, and 5 g filter-sterilized *N*-α-Boc-L-ornithine (dissolved in water added after autoclaving)) in a 50-mL flask, and shaken for three days at 28 °C, 250 rpm. Inoculum (2.5 mL) was used to inoculate 50 mL of the same medium in a 250-mL flask. This flask was also shaken for three days at 28 °C, 250 rpm. Sterile glycerol was added to 10%, and the suspension was transferred to culture vials which were stored at −70 °C. These vials were used to inoculate subsequent flasks. Two 500-mL flasks containing 100 mL of medium B were inoculated with an entire vial (1.5 mL) and incubated on a rotary shaker at 28 °C, 250 rpm. A 4-L flask containg 1-L of medium B was inoculated with 25 mL of 24 h inoculum from a 500-mL flask and incubated on a rotary shaker shaken at 28 °C, 225 rpm for 4 days. A 100-mL portion of broth was centrifuged at 12,000 × *g* for 15 min and the supernatant was retained. The supernatant was extracted at pH 3.5 (H_2SO_4) with several portions of ethyl acetate. The aqueous phase, still containing significant product, was saturated with sodium chloride and extracted with an additional portion of ethyl acetate. Concentration of the combined extract gave 450 mg of *N*-Boc-5-hydroxy-L-proline **2** as an oil.