Enantioselective Enzymatic Cleavage of *N*-Benzyloxycarbonyl Groups

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Abstract: A new enzymatic process for the enantioselective cleavage of *N*-benzyloxycarbonyl (Cbz) groups from protected amino acids and related compounds has been developed. The Cbz-deprotecting enzyme was isolated from cell extracts of *Sphingomonas paucimobilis* SC 16113 and purified to homogeneity. The purified protein has a molecular weight of 155,000 daltons and a subunit size of 44,000 daltons.

Keywords: biocatalysis; cleavage of *N*-benzyloxycarbonyl groups, enantioselectivity, enzymes, protecting groups, *Sphingomonas paucimobilis*

Introduction

Amino groups often require protection during synthetic transformations elsewhere in the molecule; at some point, the protecting group must be removed. Enzymatic protection and deprotection under mild conditions have been demonstrated previously. Penicillin G amidase and phthalyl amidase have been used for the enzymatic deprotection of the phenylacetyl and phthaloyl groups from the corresponding amido or imido compounds.[1–5] Acylases have been used widely in the enantioselective deprotection of *N*-acetyl-DL-amino acids.[6] Enzymatic deprotection of *N*-carbamoyl-L-amino acids and *N*-carbamoyl-D-amino acids has been demonstrated by microbial L-carbamoylases and D-carbamoylases, respectively.[7–10]

The Cbz group is commonly used to protect amino and hydroxy groups during organic synthesis. Chemical deprotection is usually achieved by hydrogenation with a palladium catalyst.[11–15] However, during chemical deprotection some groups are reactive under (e.g., carbon-carbon double bonds) or may interfere with (e.g., thiols or sulfides) the hydrogenolysis conditions. In addition, the process is not enantioselective. In this paper, we report an alternative enantioselective enzymatic deprotection process that can be performed under mild conditions without damaging any otherwise susceptible groups in the molecule.

Results and Discussion

Various Cbz-protected amino acids were evaluated as a substrates for enzymatic deprotection using cell extracts of *S. paucimobilic* SC 16113. Results are as shown in Table 1. Only Cbz-L-amino acids were deprotected, giving complete conversion to the corresponding L-amino acid. Cbz-D-amino acids gave $<2\%$ reaction yield. These results demonstrated that the enzyme is enantioselective and can be used for the resolution of racemic Cbz-amino acids. *N*-α, ε-Cbz-L-Lysine and *N*-ε-L-lysine gave a lower yield (33% and 48%) of L-lysine at 1 mg/mL substrate input. Upon decreasing the substrate input to 0.5 mg/mL the yield rose to 75% and 98%. *O*-Cbz-L-Tyrosine was completely converted to L-tyrosine.

The dipeptides *N*-α-Cbz-L-phenylalanine-L-alanine and *N*-α-Cbz-L-phenylalanine-L-leucine were also completely deprotected; however, the tripeptide Cbz-glycine-glycine-glycine gave a lower reaction yield due to poor substrate specificity for the enzyme. No other products, except deprotected tripeptide were observed.

Racemic Cbz-amino acids were also evaluated as substrates for hydrolysis by cell extracts of *S. paucimobilic* SC 16113. As anticipated only the L-enantiomer was hydrolyzed, giving the L-amino acids in >48% yield and >99% ee. The unreacted Cbz-D-amino acids were recovered in >48% yield and >98% e.e. (Table 2).

Cell extracts of *S. paucimobilic* SC 16113 also catalyzed the deprotection of [4*S*-(4a,7a,10ab)]-octahydro-5-oxo-4-{[(phenylmethoxy)carbonyl]amino}-

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Table 1. Enzymatic deprotection Cbz-L-amino acids.[a]

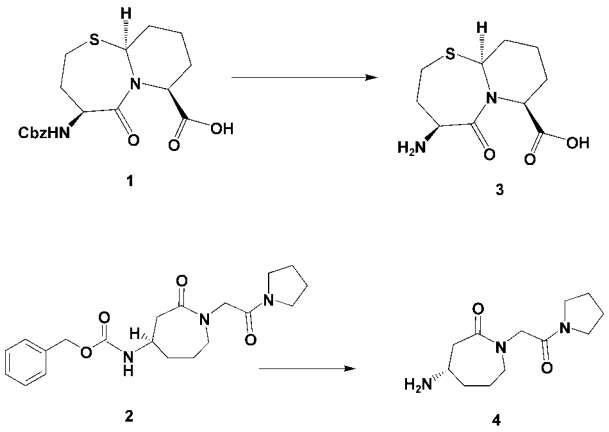
Substrate (input mg/mL)	Product	Conversion [%]
N-α-Cbz-L-tyrosine (1)	L-tyrosine	100
O-Cbz-L-tyrosine (1)	L-tyrosine	100
N-α-Cbz-L-leucine (1)	L-leucine	100
N-α-Cbz-L-phenylalanine (1)	L-phenylalanine	100
N-α-Cbz-L-proline (1)	L-proline	100
N-α-Cbz-L-lysine (1)	L-lysine	98
N-α-Cbz-D-tyrosine (1)	D-tyrosine	1.6
N-α-Cbz-D-leucine (1)	D-leucine	1.2
N-α-Cbz-D-phenylalanine (1)	D-phenylalanine	0
N-α-Cbz-D-lysine (1)	D-lysine	0.7
N-α-Cbz-D-proline (1)	D-proline	0
N-α, ε-(Cbz)-L-lysine (1)	L-lysine	33
N-α, ε-(Cbz)-L-lysine (0.5)	L-lysine	72
N-ε-Cbz-L-lysine (1)	L-lysine	48
N-ε-Cbz-L-lysine (0.5)	L-lysine	98
N-α-Cbz-L-phenylalanine-L-leucine (1)	L-phenylalanine-L-leucine	85
N-α-Cbz-L-phenylalanine-L-alanine (1)	L-phenylalanine-L-alanine	100
N-α-Cbz-glycine-glygine (1)	L-glycine-glycine	53
N-α-Cbz-glycine-glygine (0.5)	L-glycine-glycine	75
N-α-Cbz-glycine-glygine-glycine (1)	L-glycine-glycine-glycine	5
N-α-Cbz-glycine-glygine-glycine (0.5)	L-glycine-glycine-glycine	23

[a] The Cbz-amino acids (5–10 mg) were incubated with 10 mL of cell extract of *S. paucimobilis* at 42 °C for 18–20 h at 150 rpm on a shaker. The reactions were terminated by addition of 2 volumes of 50% acetonitrile containing 0.4% trifluoroacetic acid. The reaction mixtures were analyzed for substrate and product concentration by HPLC.

Table 2. Enzymatic resolution of Cbz-amino acids.

Substrate	Product	HPLC yield [%]	L-Amino acids ee [%]	Cbz-D-Amino acids	
				HPLC yield [%]	ee [%]
N-α-Cbz-DL-tyrosine	L-tyrosine	48	99.4	49.5	99.4
N-α-Cbz-DL-proline	L-Proline	49.8	99.8	49	98
N-α-Cbz-DL-phenylalanine	L-phenylalanine	48	99.4	49.5	99.3
N-α-Cbz-DL-lysine	L-Lysine	49	99.9	48.8	99.4

7*H*-pyrido[2,1-*b*][1,3]thiazepine-7-carboxylic acid (**1**) and {(3*S*)-hexahydro-2-oxo-1-[2-oxo-2-(1-pyrrolidinyl)-ethyl]-1*H*-azepin-3-yl}carbamic acid phenylmethyl ester (**2**), resulting in the formation of 6-amino hexahydro-2,2-dimethyl-7-oxo-1*H*-azepine-1-acetic acid, ethyl ester (**3**) and (*S*)-1-[(3-aminohexahydro-2-oxo-1*H*-azepine-1-yl) acetyl]pyrrolidine (**4**), respectively. In each reaction, quantitative HPLC yields of **3** and **4** were obtained. Compound **3** is an intermediate in the synthesis of an antihypertensive drug.[16–19]



The Cbz-deprotecting enzyme was purified 178-fold from cell extracts of *S. paucimobilic* SC 16113 (Table 3). The purified protein gave a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis corresponding to a subunit molecular weight of 45,000 daltons. The molecular weight of purified protein was determined to be 154,000 by gel filtration column chromatography. This indicates that the enzyme is a homotrimer. The L-carbamoylase from *Alcaligenes xylosoxidans* [7] is a homodimeric enzyme with a subunit molecular weight of 65,000 daltons and D-carbamoylase from *Comamonas* sp. E222c [10] is a homotrimeric

Table 3. Purification of Cbz-deprotecting enzyme from extracts of *S. paucimobilis* SC 16113.

Step	Volume [mL]	Total protein [mg]	Activity [units]	Specific activity [units/mg]	Purification [fold]
Cell extracts	700	1845	170	0.092	1
DE-52 column	500	380	165	0.43	4.6
Ammonium sulfate	60	300	149	0.49	5.3
Phenyl sepharose	28	3.6	3.28	0.91	9.9
Sephacryl S-200	14	0.14	2.3	16.4	178

enzyme with a subunit molecular weight of 40,000 daltons. N-Terminal and internal peptide sequences of the purified protein have been determined for cloning of this enzyme in a suitable host; this work will be described in a separate paper.

Conclusion

In this report, we have described a novel enantioselective enzymatic process for the deprotection of benzyloxycarbonylamino acids to prepare L-amino acids and benzyloxycarbonyl-D-amino acids in high yield and enantiomeric excess.

Experimental Section

Materials

Substrates **1** and **2** and authentic product standards 3 and 4 were synthesized by the Process Research and Development Department, Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ). The physicochemical properties including spectral characteristics (^{1}H NMR, ^{13}C NMR, mass spectra) were in full accord for all these compounds.[16–20] *N*-Benzyloxycarbonyl(N-α-Cbz)-amino acids were purchased from Aldrich Chemical Company, Milwaukee, WI.

Selective Techniques for Isolation of Microorganisms

A selective culture technique was used to isolate microorganisms that were able to utilize *N*-α-Cbz-L-lysine as the sole source of nitrogen. Soil samples were collected from various sites in New Jersey. About a gram of the soil sample was suspended in 5 mL of water and mixed thoroughly and then allowed to settle. The supernatant solutions from various samples were inoculated into medium A (2% glucose, 1% N-α-Cbz-L-lysine, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4$, 0.001% $FeSO_4$, 0.001% $ZnSO_4$, pH 7.0). After 96 h of growth the medium became turbid and cultures were transferred to medium A containing 1.5% agar contained in petri plates. From this enrichment culture technique eight different types of colonies were isolated. One culture was sent out to American Type Culture Collection (Rockville, MD) for identification. The culture was identified as *Sphingomonas paucimobilis* strain SC 16113.

Growth of Sphingomonas paucimobilis

S. paucimobilis SC 16113 was grown on medium A containing 1% *N*-α-Cbz-L-phenylalanine or *N*-α-Cbz-L-lysine as the sole source of nitrogen. After 48 h growth, cultures were transferred to the above medium containing 1.5% agar contained in petri plates. Cultures from the petri plates were inoculated into 100 mL medium B (0.015% yeast extract, 2% glucose, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4$ and 0.2% NaCl, pH 7) containing 1% *N*-α-Cbz-L-phenylalanine and/or *N*-α-Cbz-L-lysine for the preparation of vials, which were stored at $-70\,^{\circ}C$. One vial (containing 1 mL of *Sphingomonas paucimobilis* in medium B) was used to inoculate 100 mL of medium B. Cultures were grown at 28 °C and 280 rpm for 48 h on a rotary shaker. Cells were harvested by centrifugation at 18,000 × g for 15 minutes and stored at $-70\,^{\circ}C$.

S. paucimobilis SC 16113 was grown in 25-L fermentors containing 15 L of medium B containing 0.025% SAG and 0.025% Dow Corning antifoam. Growth consisted of a inoculum development stage and a fermentation stage. Inoculum development consisted of F1 and F2 stages. In the F1 stage, 1 mL culture of S. *paucimobilis* SC 16113 was inoculated into 100 mL of medium B. The growth was carried out in 500-mL flasks at 28 °C and 250 rpm for 24 hours. In the F2 stage, 100 mL of F1 stage culture were inoculated into 1 L of medium B in a 4-L flask and incubated at 28 °C and 100 rpm for 24 h. A fermentor containing medium B was inoculated with 4 L of F2 stage inoculum and grown at 28 °C and 220 rpm agitation with 250 LPM (liters per minute) aeration. During fermentation, cells were periodically harvested by centrifugation from 200 mL of culture broth.

Preparation of Cell Extracts of *S. paucimobilis* SC 16113

Cell extracts were prepared at 4 – 7 °C. Cells (2 g) were washed with 25 mM potassium phosphate buffer pH 8.0 (buffer A) and washed cells were suspended in 10 mL of buffer A containing 10 mM Na-ethylenediaminetetraacetic acid (EDTA). To the cell suspensions, 0.1 mL of 100 mM phenylmethylsulfonyl fluoride (PMSF) solution in isopropanol and 0.1 mL of 0.5 M dithiothreitol (DTT) were added. Cell suspensions (20% w/v, wet cells) were passed through a French press at 15,000 psi pressure and disintegrated cells were centrifuged at 25,000 × g for 30 min at 4 °C. The supernatent solution obtained after centrifugation was referred to as the cell extract. Cell suspensions of more than 100-mL volumes were disintegrated with a Microfluidizer (Microfluidics, Inc) at 12,000 psi (two passages) and disintegrated cells were centrifuged at 25,000 × g

for 30 min to obtain a cell extract. Protein in cell extracts was estimated by Bio-Rad protein reagent using bovine serum albumin as a standard. The assay mixture contained 1 – 10 μL of enzyme fraction, 0.8 mL water and 0.2 mL Bio-Rad reagent. After mixing, the absorbance of the solution was measured at 595 nm.

Biotransformation using Cell Extract

Various Cbz-protected amino acids (10 mg) and other substrates were incubated with the cell extracts (10 mL) at 42 °C for 18 – 20 h. The reactions were terminated by addition of 2 volumes of 50% acetonitrile containing 0.4% trifluoroacetic acid (TFA). The reaction mixtures were analyzed for substrate and product concentration by HPLC.

HPLC Analysis

Analysis was performed using a Hewlett-Packard (HP) 1090 instrument with a Vydac C-18 reverse phase column. The mobile phase consisted of solvent A containing 0.1% trifluoroacetic acid (TFA) in water and solvent B containing 0.1% TFA in 70% acetonitrile:30% water. The following gradient of solvent A and B was used for the separation of substrates and products. 0 min: A 100%, 0 – 15 min: A 50% and B 50%, 15 – 25 min: B 100%, 25 – 26 min: A 100%, and 26 30 min: A 100%. The flow rate was 1 mL/min. The column temperature was ambient, and the detection wavelength was 215 nm. Under these conditions, the retention times for [4*S*-(4a,7a,10ab)]-octahydro-5-oxo-4-{[(phenylmethoxy)carbonyl]-amino}-7*H*-pyrido[2,1-*b*][1,3]thiazepine-7-carboxylic acid **1** and its deprotected analogue **3** were 15.48 min and 28 min, respectively. The retention times for *N*-α-Cbz-L-phenylalanine and L-phenylalanine were 16.99 min and 7.35 min, respectively. All other Cbz-containing compounds were analyzed using the above HPLC method.

Analyses of the [(3*S*)-hexahydro-2-oxo-1-[2-oxo-2-(1-pyrrolidinyl) ethyl]-1H-azepin-3-yl]carbamic acid phenylmethyl ester **2** and its deprotected analogue **4** were carried out on a YMC basic column (5 μm; 4.6 × 150 mm). The mobile phase consisted of solvent A containing 0.1% H_3PO_4 in water and solvent B containing 90% acetonitrile in water. The linear gradient of solvent A and B over 20 min was used for the separation of substrates and products. The flow rate was 1 mL/min. The column temperature was ambient, and the detection wavelength was 220 nm. The retention times for **2** and **4** were 14.8 min and 6.7 min, respectively.

Resolution of enantiomers of Cbz-amino acids was carried out by using a Chiralcel OJ-RH column (5 μm; 4.6 × 150 mm). The column was equilibrated with 0.05% TFA in methanol: water (20:80). A gradient in 30 min with solvent A [0.05% TFA in methanol:water (20:80)] running from 100 – 0% and solvent B [0.05% TFA in methanol: acetonitrile (20:80)] running from 0 – 100% was applied at 0.8 mL/min flow rate. The detector was set up at 220 nm. Retention times were: Cbz-D-phenylalanine, 21.06 min; Cbz-L-phenylalanine, 21.69 min; Cbz-D-tyrosine, 19.15 min; Cbz-L-tyrosine 19.68 min; Cbz-D-proline, 14.85 min, Cbz-L-proline 15.41 min.

Resolution of enantiomers of amino acids was carried out by using a Regis Ligand Exchange Column (5 μm; 4.6 × 150 mm) The column was equilibrated with 12.5% methanol in 3 mM $CuSO_4$. The run time was 30 min with 0.8 mL/min flow rate. The detector was set at 240 nm. Retention times were: D-phenylalanine, 8.25 min; L-phenylalanine, 9.63 min; D-tyrosine, 5.54 min; L-tyrosine, 4.89 min; D-proline, 2.32 min; L-proline, 2.54 min.

Purification of Cbz-Deprotecting Enzyme

All the purification steps were carried out at room temperature using Cbz-L-phenylalanine as a substrate. Typical assay mixture contained 0.4 mL enzyme (cell extracts or fractions) and 0.5 mg of Cbz-L-phenylalanine. The reaction was carried out at 45 °C for 4 h. The reaction mixture was quenched with 0.4 mL of 50% acetonitrile containing 0.4% trifluoroacetic acid. The samples were filtered and analyzed by HPLC. Preparation of cell extract from 100 g of washed cells (*S. paucimobilis* SC 16113) was carried out as described above. The cell extract was loaded onto a Whatman DE-52 column (200 mL packed bed) equilibrated with buffer A. The column was washed with 200 mL of buffer A and eluted with a 1-L gradient of buffer A containing NaCl from 0.2 – 0.6 M. Fractions of 20 mL were collected. Enzyme does not bind to DE-52 column and activity was eluted in the wash buffer. The pooled wash from the DE-52 column containing the enzyme activity was precipitated with ammonium sulfate (516 g/L ammonium sulfate added). The resulting precipitated protein was collected by centrifugation at 15,000 rpm for 20 min at 4 °C, dissolved in buffer A containing 1 M ammonium sulfate and loaded on to a Pharmacia fast flow Phenyl Sepharose® column (50 mL bed volume) equilibrated with buffer A containing 132 g/L ammonium sulfate (1 M ammonium sulfate). The column was washed with 75 mL of buffer A containing 1 M ammonium sulfate and then with buffer A containing 0.5 M and 0.25 M ammonium sulfate and finally with buffer A without any ammonium sulfate. Fractions of 5 mL were collected. The most active fractions (fractions eluted from buffer A without ammonium sulfate) were pooled and concentrated by ultrafiltration to 8 mL using an Amicon YM -10 membrane. The concentrated material was loaded on to a Sephacryl S-200® column (400 mL) equilibrated with buffer A. The protein was eluted with buffer A at a flow rate of 0.8 mL/min. Fractions of 10 mL were collected. Active fractions containing enzyme activity were pooled. A unit of activity was defined as μmol of product formed per min per mg protein.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS/PAGE)

The active fractions from the Sephacryl S-200® column were evaluated by SDS-PAGE as described in the PhastSystem® procedure by Pharmacia[21] using the homogeneous 12.5% Phastgel. The enzyme samples (10 mL containing 10 μg protein) were added to a buffer containing 10 mM Tris-HCl, 1 mM EDTA, pH 8, 2.5% SDS and 5% β-mercaptoethanol. The mixture was heated at 100 °C for 5 minutes, and bromophenol blue was added to 0.01%. Gels were stained with silver stain and destained in 10% acetic acid solution. Markers with standard molecular weights were phosphorylase β (94,000), bovine serum albumin (67,000), ovalbumin

(43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

Determination of Molecular Weight

The molecular weight of the Cbz-deprotecting enzyme was determined by size exclusion chromatography using a Pharmacia Superose column® (15 cm × 1 cm). The column was equilibrated with buffer A. The enzyme (Sephacryl S-200 fraction) was applied to the column and eluted with the buffer A at a flow rate of 0.4 mL/min. Fractions of 1 mL were collected. A standard protein mixture containing thryglobulin (669,000 MW), ferritin (440,000 MW), human IgG (150,000 MW), human transferrin (81,000 MW), ovalbumin (43,000 MW), and myoglobin (17,600 MW) was also applied to the column and eluted with buffer A.

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