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Enzymatic synthesis of cyclic amino acids by *N*-methyl-L-amino acid dehydrogenase from *Pseudomonas putida*

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Abstract—A new enzymatic system for the synthesis of enantiomerically pure cyclic amino acids (CAA) from the corresponding diamino acids or racemic CAA is described. α,ω-Diamino acids were oxidized to α-keto acids with amino acid oxidases (AAO). The α-keto acids were spontaneously transformed into cyclic imino acids in the reaction medium. The resulting imines were reduced to the L-form CAA with *N*-methyl-L-amino acid dehydrogenase (NMAADH) from *Pseudomonas putida* ATCC12633 using NADPH as a cofactor. L-Form CAA were also obtained from racemic CAA using D-amino-acid oxidase and NMAADH. Using this method, a new compound [1,4]-thiazepane-3-carboxylic acid (Fig. 1) was synthesized from aminopropylcystein.

1. Introduction

Cyclic amino acids (CAA) are the components of many drugs. Thioproline is the starting material for the synthesis of the antagonists of α4β1 integrin.[1] L-Pipecolic acid is a component of several immunosuppressants, such as Tacrolimus.[2]

Several methods for the chemical preparation of optically active CAA have been reported. These include preparations of L-pipecolic acid from ethyl 6-bromohexanoate,[3] 1,4-thiazane-3-carboxylic acid from cysteine,[4,5] 3-morpholinecarboxylic acid from aziridine derivatives,[6] and azepane-2-carboxylic acid from *tert*-butyl 2-(*tert*-butyl)-3-methyl-4-oxo-1-imidazolinecarboxylate.[7] Enzymatic preparations of CAA have also previously been reported. The conversion of ornithine into L-proline by ornithine cyclase in *Clostridium* PA 3679;[8] the preparation of L-pipecolic acid from L-lysine by recombinant *Escherichia coli*;[9] and the production of 1,4-thiazane-3-carboxylic acid, piperazine-2-carboxylic acid, and 5-hydroxypiperidine-2-carboxylic acid by recombinant *E. coli*-expressed ornithine cyclase of the *Streptomyces* species[10] have been described. However, these chemical and enzymatic preparations have the disadvantage of requiring L-form amino acids as reaction substrates. This is because the enantiomeric purity of the product depends on the purity of the substrate; or in other words, these enzymes react only with L-form amino acids.

Recently, we identified a gene encoding a novel *N*-methyl-L-amino acid dehydrogenase (NMAADH) in *Pseudomonas putida* ATCC12633, and we succeeded in the functional expression of this enzyme in host *E. coli* BL21(DE3) cells.[11] This enzyme converts an α-keto acid and methylamine into their corresponding *N*-methyl-L-amino acid in an NADPH-dependent manner (Scheme 1). We have reported the production of *N*-methyl-L-phenylalanine from phenylpyruvic acid using this enzyme.[12] On further studying, we found that more suitable substrates for the reductive reaction of NMAADH were cyclic imino acids, such as Δ^1-piperidine-2-carboxylate **3a** and Δ^1-pyrrolidene-2-carboxylate **3b**. NMAADH was originally involved in the catabolism of D-lysine.[13] Cyclic imino acids are spontaneously produced in an aqueous medium from α-keto-ω-amino acids that can be synthesized irreversibly from their corresponding α,ω-diamino acids by using amino acid oxidases (AAO). For example, L-lysine was oxidized with

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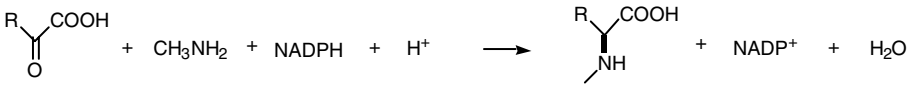
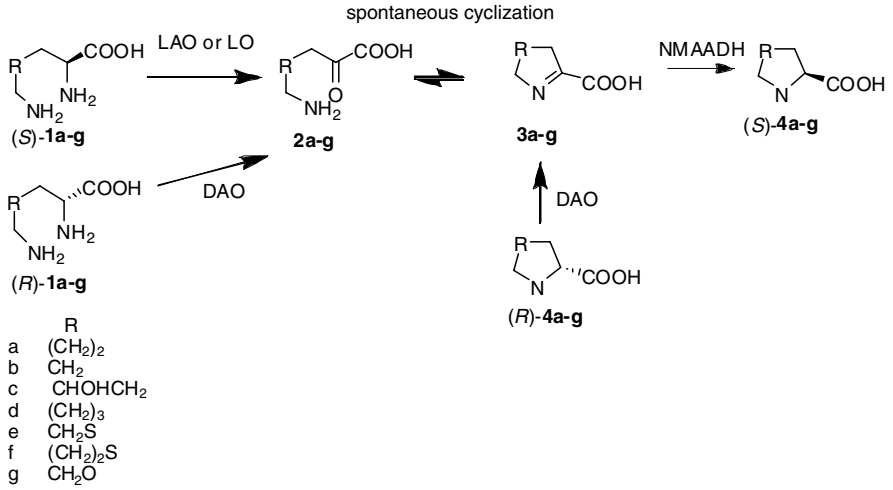


Scheme 1. The NMAADH-catalyzed production of *N*-methyl-L-amino acid with NADPH as cofactor.



Scheme 2. The production of CAA by the combination of the enzymes AAO and NMAADH.

L-amino-acid oxidase (LAO) or L-lysine oxidase (LO) into 2-keto-lysine (2-keto-6-amino-hexanoic acid). Subsequently, the ε-amino residue obtained reacted with its α-carbonyl residue, resulting in Δ^1-piperideine-2-carboxylate. In the case of D-amino acids, D-amino-acid oxidase (DAO) can be used for the preparation of cyclic imino acids. DAO also oxidizes D-form cyclic amino acids into cyclic imino acids. Therefore, cyclic imino acids can be obtained enzymatically from both enantiomers by using the appropriate AAO. It was suggested that a new production method for CAA could be achieved by combining two enzymatic reactions, namely, the oxidation of amino acids and the reduction of the resulting cyclic imino acids (Scheme 2).

Herein, we report a new method for the synthesis of enantiomerically pure CAA by using AAO and NMAADH as well as the synthesis of a new compound [1,4]-thiazepane-3-carboxylic acid (Fig. 1).

2. Results and discussion

2.1. Substrate specificity of AAO

For the production of various cyclic imino acids **3a**–**g**, the oxidative activities of LAO, LO, and DAO toward diamino acids **1a**–**g** and CAA **4a** and **4b** were measured. The results obtained are listed in Table 1. The substrate specificities of these enzymes were quite different, and LAO was found to have the widest specificity toward the diamino acids. In the case of DAO, D-form diamino acids were poor substrates but D-form CAA were good.

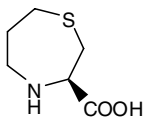


Figure 1. [1,4]-Thiazepane-3-carboxylic acid.

2.2. L-Pipecolic acid (*S*)-4a production from DL-pipecolic acid 4a or D-lysine (*R*)-1a or L-lysine (*S*)-1a

In our first study, we investigated the production of (*S*)-**4a** from Δ^1-piperidine-2-carboxylate **3a** because the latter was found to be the most suitable substrate for NMAADH. Using DAO, (*S*)-**4a** could be obtained with a 100% and 59% yield from DL-pipecolic acid **4a** and D-lysine (*R*)-**1a**, respectively. The enantiomeric purity of (*S*)-**4a** was 100% enantiomeric excess (ee) in each case. The low yield from (*R*)-**1a** was because of substrate specificity of DAO. Using LO, (*S*)-**4a** was obtained from (*S*)-**1a** with a 98% yield. The final concentration of (*S*)-**4a** in the reaction medium was 14 g/L. The enantiomeric purity of (*S*)-**4a** was 100% ee.

2.3. Combination of NMAADH and AAO

Table 2 shows the substrate specificity results when both NMAADH and AAO were used to synthesize CAA **4a**–**f** from diamino acids **1a**–**f**. The relative activities are shown compared with those of LO and NMAADH for (*S*)-**4a** production from (*S*)-**1a**. The relative activities of the combination of LAO and NMAADH were lower than expected. This indicated the inactivation or inhibition of NMAADH by LAO. We studied the effects of LAO and LO toward NMAADH. NMAADH was incubated with 1 units/mL LAO or 20 units/mL LO for 2 h. The resulting activity was 20% and 89%, respectively.

On comparing the results of Tables 1 and 2, the substrate specificity of the reductive activity of NMAADH on cyclic

Table 1. Substrate specificity of AAO

Substrate	Relative activity[a] (%)		Relative activity[b] (%)
	LAO	LO	DAO
(*S*)-**1a** or (*R*)-**1a**	379	100	3
(*S*)-**1b** or (*R*)-**1b**	335	35	20
(*S*)-**1c**	54	71	N.D.
(*S*)-**1d**	23	48	N.D.
(*S*)-**1e**	780	17	N.D.
(*S*)-**1f**	1097	15	N.D.
(*S*)-**1g**	14	8	N.D.
L-Phenylalanine	439	15	N.T.
D-Alanine	N.T.	N.T.	100
(*R*,*S*)-**4a**	N.D.	N.D.	50
(*R*)-**4b**	N.T.	N.T.	130
(*S*)-**4b**	N.D.	N.D.	N.D.

N.D.: not detected, N.T.: not tested.

[a] The number showed relative activities compared with the activity of LO toward L-lysine.

[b] The number showed relative activities compared with the activity of DAO toward D-alanine.

Table 2. Substrate specificity of the combination of the two enzymes

Substrate	Relative activity (%)	
	LAO and NMAADH	LO and NMAADH
(*S*)-**1a**	17	100
(*S*)-**1b**	7	22
(*S*)-**1c**	N.T.	36
(*S*)-**1d**	N.T.	40
(*S*)-**1e**	9	17
(*S*)-**1f**	24	18

The concentration of NMAADH, LAO, and LO were 0.9, 0.04, and 0.6 units/mL, respectively.

imines was estimated. The activity of NMAADH toward **3b** was 63% of its activity toward **3a**. This corresponds to the data obtained in our previous paper.[12]

To confirm the production of CAA by the combination of NMAADH and AAO, the reaction medium was incubated for more than 2 h after the addition of glucose for recycling the NADPH that was produced in the reaction. The reaction samples were analyzed by thin-layer chromatography (TLC) and compared with standard samples. In TLC, the diamino acids **1a**–**f** showed no mobility. Corresponding CAA were detected in each sample only in the case of the combination of NMAADH and LO. The colors of **4a**, **4b**, **4c**, **4d**, **4e**, and **4f** spots were violet, yellow, purple, brown, grayish blue, and magenta, respectively. The R_f (mobility relative to the solvent front) of the above amino acids was 0.22, 0.20, 0.18, 0.28, 0.32, and 0.30, respectively.

2.4. The isolation and detection of CAA

The prepared CAA were purified as described in the Material and methods section of this paper and were analyzed by liquid chromatography/mass spectrometry (LC–Mass) and ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR). The results of the LC–Mass analysis of (*S*)-**4a**–**f** were consistent with the *m*/*z* value of the quasimolecular ion $(M+H)^+$ peak of each compound. The ^{1}H NMR data for (*S*)-**4a**, **4b**, **4c**, **4d**, and **4e** obtained in this study corresponded to previously reported data. The amount of purified **4f** was not adequate for the ^{1}H NMR analysis, therefore, we conducted a further preparation of **4f**. The chiral analysis showed that all CAA were enantiomerically pure (ee >99%), with the exception of hydroxypipecolic acid **4c**. This was because the hydroxy group of **1c** was racemic, and both LO and NMAADH did not selectively recognize the hydroxy group.

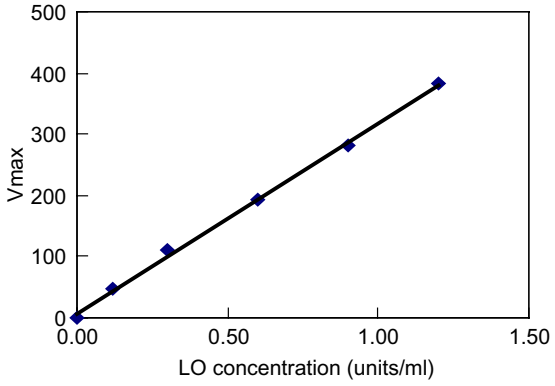


Figure 2. The effect of L-lysine oxidase (LO) concentration. The assay solution comprised of 100 mM Tris buffer pH 8.0 with 10 mM L-lysine, 440 units/mL catalase, 1.24 units/mL NMAADH, 0.01 mM FAD, and 0.32 mM NADPH.

2.5. Optimization of reactant concentrations

To improve the conversion, the reaction conditions were studied. The most suitable pH was found to be 8.0 (data not shown). The optimal substrate concentration was 30 mM in the case of (*S*)-**1a**. A high substrate concentration was found to inhibit the reaction. The effect of the concentration of LO was also studied (Fig. 2). The reaction rate increased with LO concentration.

2.6. The preparative scale reaction of (*S*)-4f

For further structural analysis, (*S*)-**4f** was synthesized for a preparative scale and analyzed. The structure was confirmed with high-resolution mass spectrometry (HRMS) and ^{1}H and ^{13}C NMR spectra (Fig. 1).

2.7. The preparative scale reaction of (*S*)-4g

The substrate specificity study of LO revealed that **1g** was a poor substrate. In order to synthesize (*S*)-**4g**, the amount of LO had to be increased; however, less than 50% of **1g** was converted. In order to prepare (*S*)-**4g** on a large scale, more suitable AAO should be screened and developed in the future.

3. Conclusion

In conclusion, we have demonstrated a new enzymatic synthesis of enantiomerically pure CAA from the corre-

sponding diamino acids **1** or racemic CAA by using a combination of AAO and NMAADH from *P. putida*. Using the appropriate AAO, both the enantiomers of **1** and the racemic **4** can be used as starting materials for this synthesis. LAO from snake venom was not suitable for this synthesis due to its strong inactivation of NMAADH. New compound (*S*)-**4f** was synthesized from (*S*)-*S*-aminopropylcystein (*S*)-**1f** using LO. A high concentration of LO was found to be effective in improving the reaction rate. LO is generally considered to be an expensive reagent; however, this enzyme can be easily prepared from *Trichoderma viride* according to the reported method.[14] Although many enzymes and cofactors were used for this reaction, the reaction itself was uncomplicated. However, the space–time yield of the system has to be improved before implementation. We have demonstrated that NMAADH is a promising catalyst for the development of biocatalytic routes for the production of chiral amino acids for use as pharmaceutical intermediates, for the production of *N*-methylamino acid, and for CAA preparation.

4. Experimental

4.1. Materials and methods

NMAADH from *P. putida* ATCC12633 and glucose dehydrogenase from *Bacillus subtilis* were over expressed in *E. coli* as described previously.[11,12] The crude enzyme was obtained by the disruption of these cells by sonication. LAO from snake venom typeVI (Sigma A-5147) was used. DAO from porcine kidney was purchased from Wako Chemicals, Japan. LO from *T. viride* was obtained from Seikagaku Kogyo, Japan. (*S*)-**1f** was synthesized from L-cysteine and bromopropylamine using the methods described in a previous paper.[15] Homolysine **1d** was synthesized according to methods previously described.[16] FAD was obtained from Nakalai Tesque, Japan. Catalase, horseradish peroxidase, NADPH, $NADP^+$, (*S*)-*S*-aminoethylcysteine (*S*)-**1e**, (*S*)-(+)-2-amino-3-(2-amino ethoxy)-propanoic acid monohydrochloride (*S*)-**1g**, and other chemicals were obtained from Sigma–Aldrich.

4.2. Analytical methods

The reaction medium was analyzed by TLC on silica gel (Merck, #1.05715 60F-254 0.25-mm silica gel plates) that was further developed with a mixture of acetonitrile/methanol/water (4:1:1). The standard samples of **4a** and **4b** were also analyzed simultaneously. The TLC plates were sprayed with ninhydrin and then heated. The enantiomeric purity and production yield were analyzed by high-performance liquid chromatography (HPLC) with a CHIRALPAK WH column (4.6 × 250 mm) (Daicel, Japan) and 2 mM $CuSO_4$ as an eluent (mobile phase) at a flow rate of 0.75 mL/min at 50 °C, or MCI GEL CRS10W column (4.6 × 50 mm) (Mitsubishi Chemical Corp., Japan) and 0.4 mM $CuSO_4$ as the eluent (mobile phase) at a flow rate of 0.5 mL/min at 40 °C. Both cases were monitored at 254 nm. The products were also analyzed with the LC–Mass system (Hewlett packard 1100MSD) with a UK-C18 column (250 × 4.6 mm: Imtakt Corp., Kyoto, Japan) and 10% acetonitrile as an eluent at a flow rate of 0.5 mL/min at 40 °C; they were monitored at 210 nm. The conditions of ionization were 20V API-ES and positive mode. The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL Lambda 400 spectrometer operating in D_2O. High-resolution mass spectra (HRMS) were taken on a JEOL JMS-700 mass spectrometer.

4.3. AAO activity assay

AAO activity was measured by quantifying the formation of quinoneimine dye in a coupled reaction with horseradish peroxidase. The assay solution comprised of 100 mM Tris buffer (pH 8.0), 0.74 mM 4-aminoantipyrine, 10 units/mL horseradish peroxidase, 0.01 mM FAD, 0.3 g/L *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine, and 10 mM amino acids. The reaction was started by the addition of an appropriate amount of the enzyme solution after pre-incubation of the mixture at 30 °C for 3 min. The increase in the absorbance at 550 nm was monitored by a UV–vis spectrophotometer with the temperature controlled at 30 °C.

4.3.1. Enzymatic synthesis of (*S*)-4a using DAO from (*R*)-1a or racemic 4a. Twenty microliters of the crude enzyme of NMAADH was added to a solution containing 10 mM (*R*)-**1a** or racemic **4a**, 100 mM glucose, 0.1 mM FAD, 1 mM $NADP^+$, 0.5 units/mL DAO, 50 units/mL catalase, and 100 mM Tris–HCl buffer (pH 8.0) in a 0.2 mL reaction volume. The reaction was conducted at 30 °C for 24 h. As a control, the reaction for the solution without NMAADH was carried out simultaneously. The medium was analyzed with TLC and HPLC.

4.3.2. Enzymatic synthesis of (*S*)-4a using LO from (*S*)-1a. One milliliter of the crude enzyme of NMAADH was added to a solution containing 1% (*S*)-**1a**, 100 mM glucose, 1 mM FAD, 0.2 mM $NADP^+$, 1.5 units/mL of LO, 14 units/mL catalase, and 100 mM Tris–HCl buffer (pH 7.5) in a 10 mL reaction volume. The reaction was carried out at 30 °C. The pH was occasionally adjusted from 5.1 to 7.6 using 10 M NaOH. After 5 h, 0.5% (*S*)-**1a**, 7 units/mL catalase, and 100 mM glucose were added to the reaction medium and the reaction was continued for 15 h. The medium was analyzed with TLC and HPLC.

4.4. Activity assay of the combination of AAO and NMAADH

The activity of the combination of the two enzymes was measured by monitoring the consumption of NADPH by a UV–vis spectrophotometer at 340 nm with the temperature controlled at 30 °C. The assay solution comprised of 100 mM Tris buffer (pH 8.0) with 10 mM substrate, 4% (v/v) NMAADH crude enzyme, 200 units/mL catalase, and 0.32 mM NADPH. The reaction was started by the addition of AAO. After monitoring the initial rate of consumption of NADPH, a 2% glucose solution was added for cofactor regeneration and incubated at 30 °C for 2 h. The formation of CAA was analyzed by TLC.

4.5. The isolation and purification of cyclic amino acids

For the isolation of CAA, two enzyme reactions (NMAADH and LO) were combined and carried out in 5 mL volume with a 2% glucose solution and incubated at 30 °C for 20 h. The products formed were purified with preparative thin-layer chromatography (PLC) on silica gel (Merck ART13793), which was then developed with a mixture of acetonitrile/methanol/water (4:1:1). The CAA were recovered with methanol, after which they were purified by ion-exchange column chromatography (SK1B: Mitsubishi Chemical Corp.) and then crystallized. The purified compounds were analyzed with LC–Mass and ^{1}H NMR.

4.5.1. Preparation of [1,4]-thiazepane-2-carboxylic acid (*S*)-4f. The reaction mixture containing 174.7 mg (*S*)-**1f**, 1 μmol FAD, 6.5 units LO, and 1230 units catalase in 8 mL of 100 mM Tris–HCl buffer (pH 8.2) was incubated for 5 h at room temperature, following which 2.33 mol glucose, 10 μmol $NADP^+$, and crude NMAADH (81 units of NMAADH) were added and incubated at 28 °C. The pH decreased during the reaction; therefore, it was adjusted occasionally from 7 to 8. The conversion was monitored by TLC analysis. After 3 days, the (*S*)-**1f** spot could not be detected. The reaction was stopped by adding 10 mL of methanol and 40 mL of acetonitrile. The medium was centrifuged to remove any precipitates and then filtered. The product was then purified with PLC and ion-exchange column chromatography. (*S*)-**4f** was crystallized from a methanol/acetone mixture.

4.5.2. [1,4]-Thiazepane-3-carboxylic acid (*S*)-4f. Yield: 70%; HRMS M^+ found (M^+ calculated for $C_6H_{11}O_2N_1S_1$): 161.0509 (161.05105); MS: *m*/*z* (relative intensity) = 161 (24) [M^+], 116 (100), 87 (24), 70 (19), 69 (17), 41 (21); $\delta = 4.01$ (1H, t, $J = 6.3$ Hz), 3.19–3.45 (3H, m), 3.10 (1H, dd, $J = 15.9$, 6.8 Hz), 2.63–2.82 (2H, m), 2.14 (2H, dt, $J = 10.9$, 5.1 Hz); ^{13}C NMR (100 MHz, D_2O): $\delta = 31.9$, 34.2, 34.5, 46.3, 64.8, 175.0.

4.5.3. Preparation of 3-morpholinecarboxylic acid 4g. The reaction mixture containing 159 mg (*S*)-**1g**, 1 μmol FAD, 6.5 units LO, and 1230 units catalase in 8 mL of 100 mM Tris–HCL buffer (pH 8.2), was incubated for 1 day at room temperature, after which 2.33 mol glucose, 10 μmol $NADP^+$, 5 units LO, and the crude enzyme of NMAADH (81 units of NMAADH) were added and incubated at 28 °C. The pH was occasionally adjusted from 7 to 8. The conversion was monitored with TLC analysis. After 3 days, approximately 50% of (*S*)-**1g** had reacted. The reaction was stopped by adding 20 mL of methanol and 20 mL of acetonitrile. The medium was centrifuged to remove any precipitates and filtered. The product was twice purified with PLC and with ion-exchange column chromatography. (*S*)-**4g** was crystallized from a methanol/acetone mixture.

4.5.4. 3-Morpholinecarboxylic acid (*S*)-4g. Yield: 35% LC–Mass; MS: $m/z = 132.15$; ^{1}H NMR (400 MHz, D_2O): $\delta = 4.04$ (1H, dd, $J = 11.6$, 3.0 Hz), 3.82 (1H, dt, $J = 12.4$, 3.6 Hz), 3.55–3.67 (3H, m), 3.13 (1H, dt, $J = 13.1$, 3.0 Hz), 2.98 (1H, ddd, $J = 13.4$, 10.1, 0.9 Hz).

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