Supplementary data

New enzymatic methods for the synthesis of primary α-aminonitriles and unnatural α-amino acids by oxidative cyanation of primary amine with D-amino acid oxidase from porcine kidney

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Supplementary methods

Chemical synthesis of 2MePGN, 2MePG, and F2MePG.

2MePGN

2MePGN was synthesized according to the method of R. G. Murray et al. Potassium cyanide (8.15 g, 125 mmol) and ammonium chloride (6.7g, 125 mmol) was suspended in dimethylsulfoxide/water (6 : 1, 100 mL) and acetophenone (7.5 g 62.5 mmol) was added to the solution. The reaction mixture was stirred at room temperature for 20 h. The products were extracted with ethylacetate and crystallization with 2N HCl in diethylethel. The purity of white crystals was checked by NMR. (49.5% yield including 4.4% acetophenone and δ: 1.65 (s, 3H,), 7.32–7.62 (m, 5H))

2MePG

2MePG was synthesized using the method of R. C. Atkinson et al. 5-Methyl-5-phenylhydantoin from Sigma-Aldrich (St. Louis, Missouri, USA) (5 g, 26.25 mmol) was suspended in H_2O (50 ml) and 8 equivalents of NaOH were added into the reaction. The reaction mixture was heated at 70°C for 48 h. After cooling on ice, the pH of the solution was adjusted to 2.0 with concentrated HCl and allowed to stand overnight. The

insoluble materials were removed by filtration and the desired product remained as an aqueous solution. The acidified solution was applied to a Dowex column and eluted by 10% ammonium solution. After the eluate was evaporated, the purity of 2MePG was checked by NMR. (34.7% yield and ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.48 – 7.33 (m, 5H), 1.81 (s, 3H).).

F2-MePG

4-Fluoro-5-methyl-5-phenylhydantoine was synthesized from 4-fluoro-acetophenone by the Bucherer-Bergs reaction. Ammonium carbonate (24.2 g, 252 mmol) and potassium cyanide (7 g, 108 mmol) were suspended in 120 ml 20% ethanol solvent. 4-Fluoro-acetophenone (5g, 36 mmol) was added to the reaction and heated at 50°C for 24 h. After cooling on ice, the pH of the solution was adjusted to 7.0 with concentrated HCl. The product was extracted by ethylacetate and evaporated to obtain the yellow oil after evaporation (2.2 g, 29.4% yield). The oily product was used for the next reaction to synthesize F2MePG. Two grams of hydantoine was suspended in H_2O (50 ml) and 8 equivalents of NaOH were added to the reaction. The solution was incubated at 70°C for 48 h. After cooling on ice, the pH of the solution was adjusted to 2.0 with concentrated HCl and allowed to stand overnight. The insoluble materials were removed

by filtration and the desired product remained as an aqueous solution. The acidified solution was applied to a Dowex column and eluted by 10% ammonium solution. After the eluate was evaporated, purity of F2MePG was checked by NMR. (8.6% yield and ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.35 – 7.27 (m, 2H), 7.03 – 6.93 (m, 2H), 1.48 (s, 3H).)

Assay of amine oxidase and nitrilase activity

The oxidase activity was assayed by measuring the rate of hydrogen peroxide formation at 30°C. The standard reaction mixture was composed of 20 mM (*R*)-MBA, 1.5 mM 4-aminoantipyrine, 2 mM phenol, 2 units of horseradish peroxidase, 100 mM KPB, pH 8.0, and an appropriate amount of enzyme, in a final volume of 1.0 ml. The assay of enzyme activity was initiated by addition of enzyme solution, and formation of hydrogen peroxide was monitored spectrophotometrically at 30°C for 5 min by measuring the absorbance at 505 nm. One unit of oxidase activity was defined as the amount of enzyme that catalyzes the production of one μmol hydrogen peroxide per min. The molar absorptivity value of 13.6 x 10^{3} M^{-1} cm^{-1} was used to calculate the enzyme activity.

Nitrilase activity was assayed by measuring the amount of 2PG produced from 2PGN. The substrate solution (0.5 ml) containing 20 mM 2PGN in 100 mM KPB buffer, pH

8.0 was preincubated at 30°C for 3 min, and the reaction was initiated by adding the enzyme solution. The reaction mixture incubated at 30°C for 5 min, then the reaction was stopped by adding 0.1 ml 2M $HClO_4$. The resulting precipitates were removed by centrifugation. The amounts of 2PG in the supernatant were analyzed by high-performance liquid chromatography (HPLC). One unit of nitrilase activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of 2PG from 2PGN per min.

Preparation of reaction products from Phe and (*R*)-MBA to identify the α-aminonitriles

D- or L-Phe (each 5 mM) was incubated at 20°C for 1 h with 2 U of pkDAO or CaLAO and 150 mM KCN solution adjusted to pH 9.0 with HCl in 1 ml reaction mixture. The reaction was stopped by 0.2 ml 2M $HClO_4$, and the precipitates were removed by centrifugation. The supernatant was used for product analysis by MS.

The (*R*)-MBA (5mM) was incubated with 2U mutant pkDAO in 150 mM KCN solution, adjusted to pH 9.0 with HCl at 30°C for 1h in a final volume of 1.0 ml. The reaction products were extracted with 500 μL hexane and identified by Gas chromatography–mass spectrometry (GC–MS) analysis.

Analysis of reaction products by HPLC

The α-aminonitrile reaction products from L-Phe, D-Phe and (*R*)-MBA were analyzed by HPLC (Waters, Tokyo, Japan) with a Crownpak CR-I (+) column (Daicel Co., Tokyo, Japan) at 25°C using a solution containing 60 mM $HClO_4$ and 20% acetonitrile (80:20) at a flow rate of 0.4 ml/min. The product amounts were measured at 200 nm using a UV detector.

The reaction products 2MePG and 2MePGN from (*R*)-MBA were also analyzed by a Crownpak CR(+) column (Daicel Co., Tokyo, Japan) at 30°C using a solution containing 60 mM $HClO_4$ and MeOH (0–10%) at a flow rate of 0.8 ml/min.

Identification of 2-amino-2-cyano-3-phenylpropanoic acid and unnatural α-amino acids from Phe and (*R*)-MBA, respectively

2-Amino-2-cyano-3-phenylpropanoic acid and unnatural α-amino acids (2MePG, F2MePG, 2EtPG) were identified using a Bruker-Daltonics instrument with an ESI source in the positive mode (Bruker-Daltonics, Bremen, Germany). Data evaluation was performed using the Generate Molecular Formula software suite of Bruker-Daltonics microTOF Data Analysis version 3.4. The samples were diluted with 0.1% formic acid for analysis in the positive mode by MS.

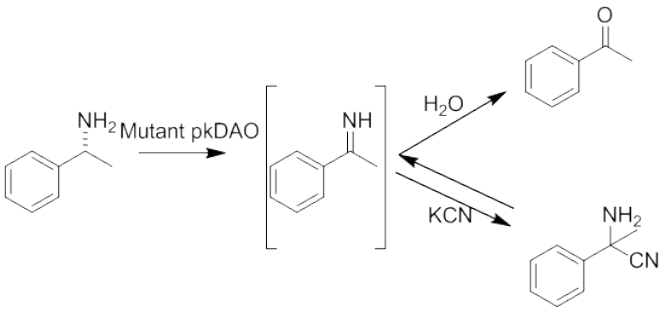
Identification of 2MePGN by GC–MS analysis

2MePGN from (*R*)-MBA were extracted from 1 ml reaction mixture with 0.5 mL hexane and separated by GC using a HP-5ms column (30 m x 0.25 mm; 0.25 μm, Agilent J & W) operated in the split-less mode at 60°C for 2 min. In this analysis, helium was used as the carrier gas at a flow rate of 1.00 ml/min. The MS of each compounds were analyzed to identify the peaks with reference to an MS database (Wiley 9th/NIST 2011 MS Library; Hewlett Packard Co).

Production of unnatural α-amino acids from primary amines

The unnatural α-amino acids (2MePG, F2MePG and 2EtPG) were synthesized under optimal condition containing 120–240 U of pkDAO, 600–1200U (with 2-PGN as a substrate) nitrilase AY487533, 5 mM each primary amine and 150 mM KCN, adjusted to pH 9.0 by HCl at 30°C for 4 h in 30 ml. The reaction was stopped by 6 ml 2 M HCl and centrifuged to remove insoluble materials. The supernatant was absorbed to a Dowex^{Tm} (50Wx8 50-100 Mesh (H) Cation Exchange Resin) column from Wako (Japan) and eluted with 10% ammonium solution. The eluate was evaporated and analyzed with nuclear magnetic resonance (NMR) and MS. ^{1}H NMR and ^{13}C NMR spectra were recorded using the Bruker Biospin AVANCE II 400 system (Bruker

Biospin, Rheinstetten, Germany).



Scheme S1. Putative reaction of 2MePGN with KCN by mutant pkDAO.

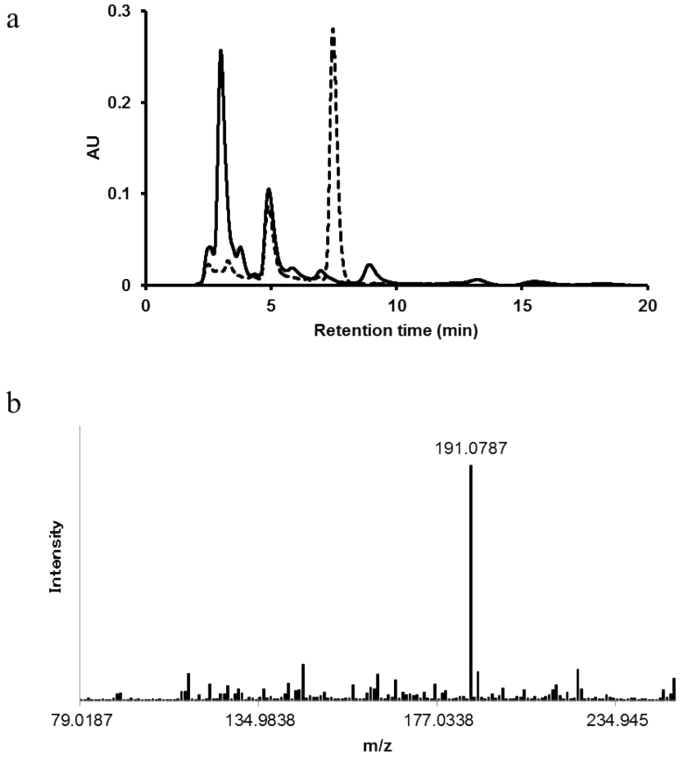
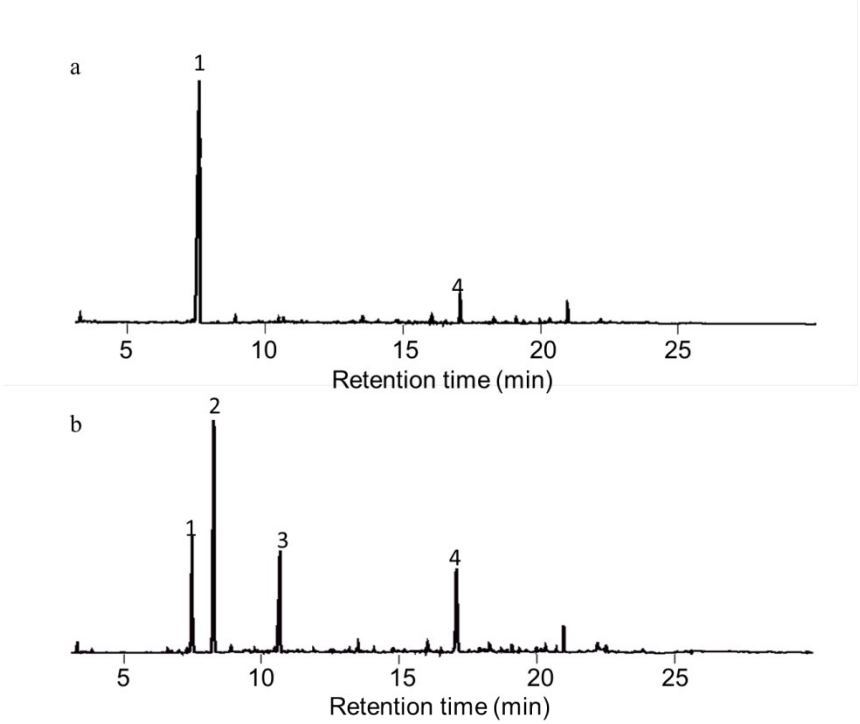


Figure S1. Enzymatic synthesis of 2-amino-2-cyano-3-phenylpropanoic acid from D- or L-phenylalanine (Phe) with amino acid oxidase.

(a) HPLC analysis of the oxidation products from D- or L-phenylalanine by incubation with L- or D-amino acid oxidase in 100 mM KPB, pH 8.0 (dotted line) or 100 mM KPB,

pH 8.0, containing 100 mM KCN (solid line). (b) Identification of 2-amino-2-cyano-3-phenylpropanoic acid synthesized from D- or L-Phe by cyanide addition. The enzyme reaction was stopped with $HClO_4$ and the supernatant was diluted with 0.1% formic acid then analyzed by a Bruker-Daltonics microTOF instrument.



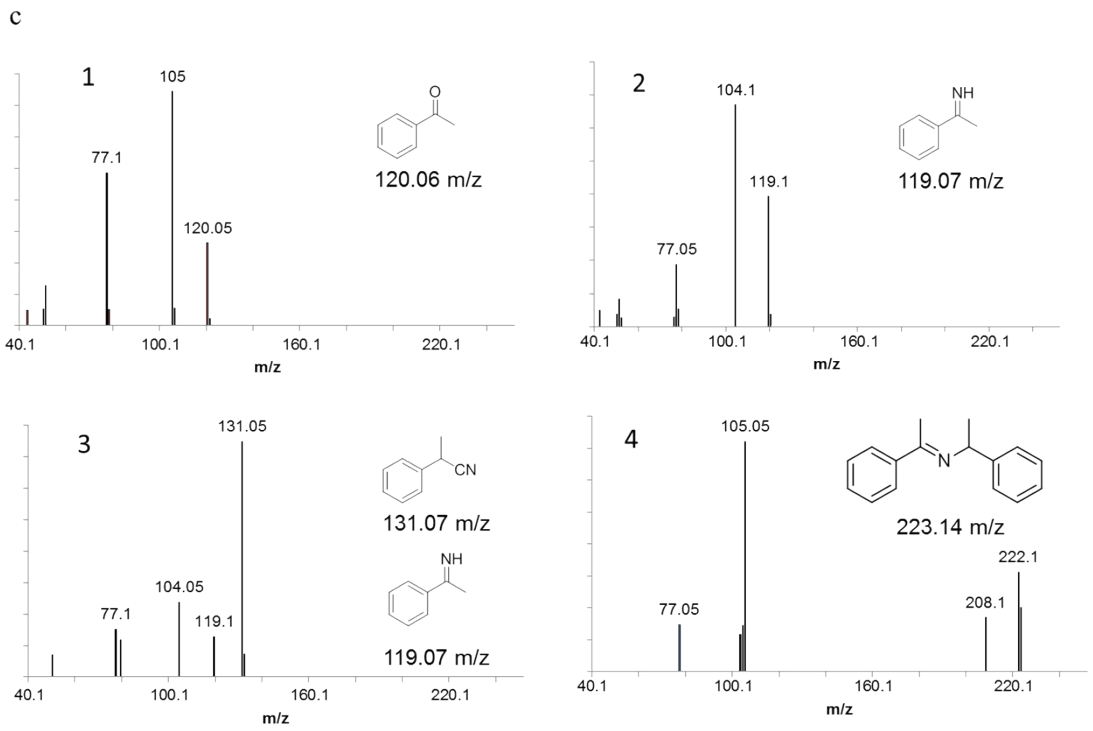


Figure S2. (a) GC spectrum of the reaction products from (*R*)-MBA by incubation with mutant pkDAO in KPB, pH 8.0. (b) GC spectrum of the reaction products from (*R*)-MBA by incubation with mutant pkDAO and 150 mM KCN at pH 9.0. (c) MS analysis of peaks 1–4 in figure 2b to identify the products from (*R*)-MBA.

Figure S3. Stability of 2MePGN and optimization of temperature and pH for cyanide addition with mutant pkDAO for production of 2MePGN.

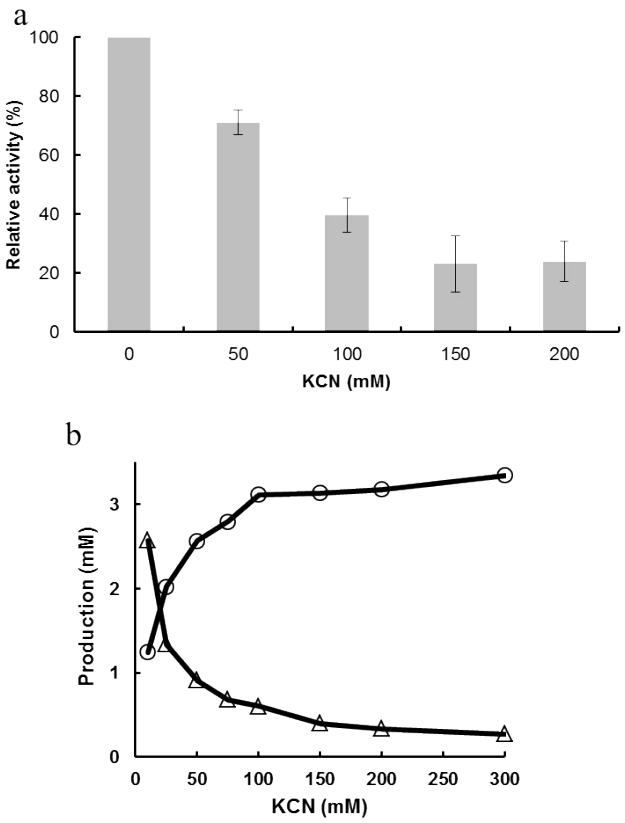


Figure S3. (a) Effect of KCN concentrations on pkDAO activity. pkDAO was incubated at 20°C for 1 h with 50–200 mM KCN at pH 9.0 adjusted by HCl and 100 mM Tris-HCl pH 9.0 was used for the control condition. The residual activity was measured using standard assay conditions (n=3). (b) Effect of cyanide concentration on production of 2MePGN. The reaction was performed at 20°C for 1 h with 10–300 mM KCN, pH 9.0 adjusted by HCl, 5 mM (*R*)-MBA and 2.6 U of pkDAO. The amount of 2MePGN and acetophenone were determined by HPLC (n=3). ○: 2MePGN, □: Acetophenone.

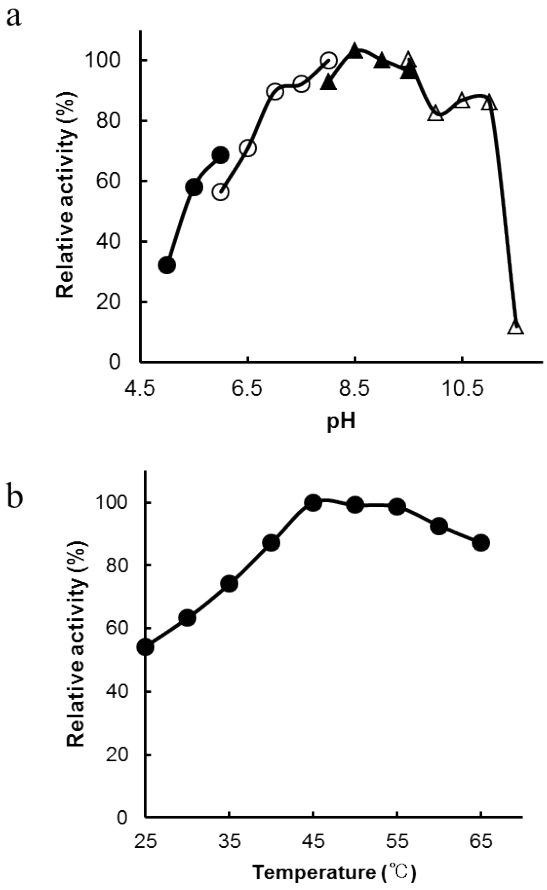


Figure S4. Effects of pH and temperature on nitrilase AY487533. (n=3) (a) Effect of pH on enzyme activity of nitrilase AY487533 for enzymatic production. The nitrilase activity of 2PGN was assayed under standard assay conditions, except the reaction pH values between pH 5.0 and 11.5. Citrate buffer pH 5.0–6.0, KPB buffer, pH 6.0–8.0, Tris-HCl buffer, pH 8.0–9.5. and glycine-KOH buffer pH 9.5–11.5 were used.

(b) Effect of temperature on enzyme activity of nitrilase AY487533. The nitrilase activity of 2PGN was assayed at the indicated temperature at pH 8.0.

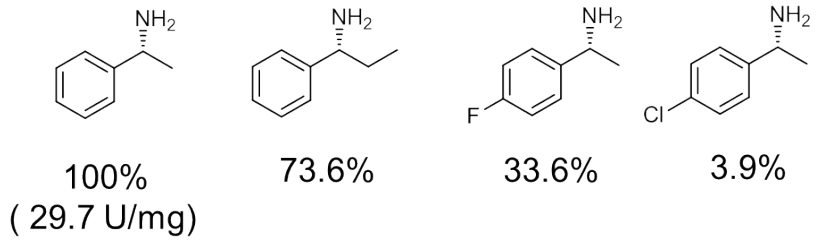


Figure S5. Substrate specificity of mutant pkDAO for primary amines (n=3).

The relative activity was measured by color development methods of oxidase activity measurement using purified mutant pkDAO and 20 mM of each primary amine at pH 8.0.

NMR spectra and MS spectra of enzymatically synthesized unnatural α-amino acids

The unnatural α-amino acids were synthesized by the new enzymatic reaction with mutant pkDAO and nitrilase AY487533. The products were purified by Dowex column chromatography, and identified by ^{1}H-NMR, ^{13}C-NMR (δ 42.62: Dimethylsulfone for internal standard) and a Bruker-Daltonics microTOF instrument.

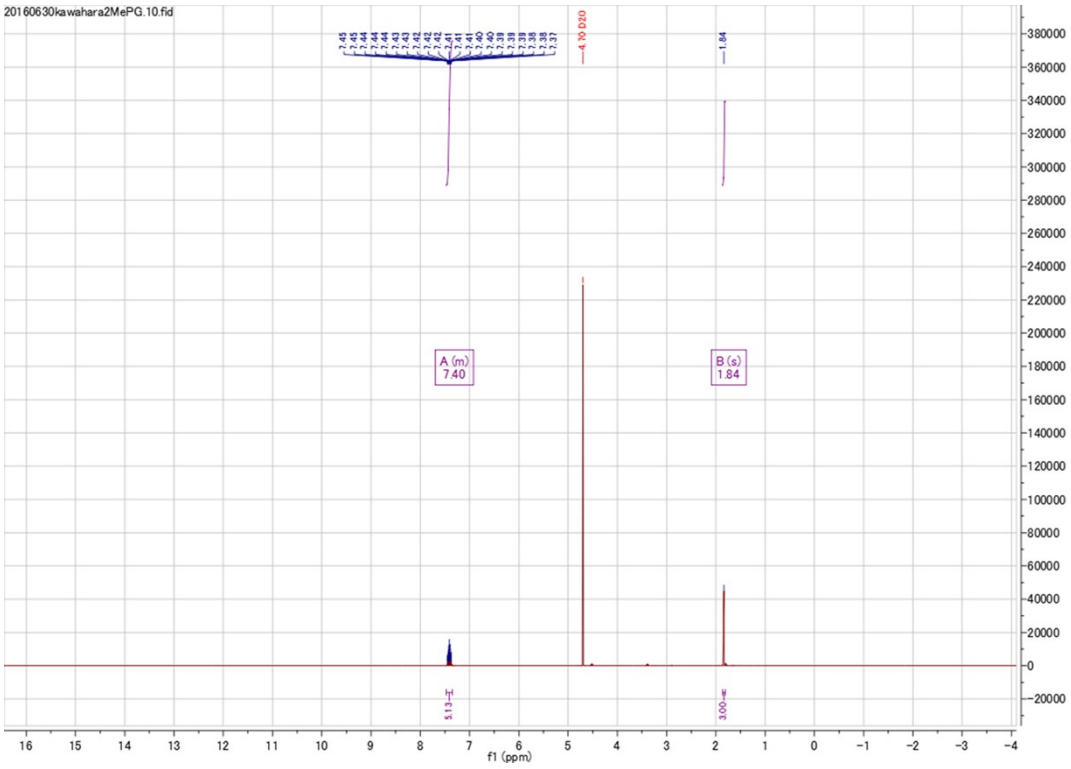
(a) Product from (*R*)-MBA, 2-MePG : ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.47 – 7.35 (m, 5H), 1.84 (s, 3H). ^{13}C NMR (100 MHz, Deuterium Oxide) δ 177.15, 138.65,

130.17, 126.88, 64.05, 22.28. MS (microTOF): m/z calcd for $C_9H_{11}NO_2$ $[M+H]^+$: 168.0855; found: 166.0863.

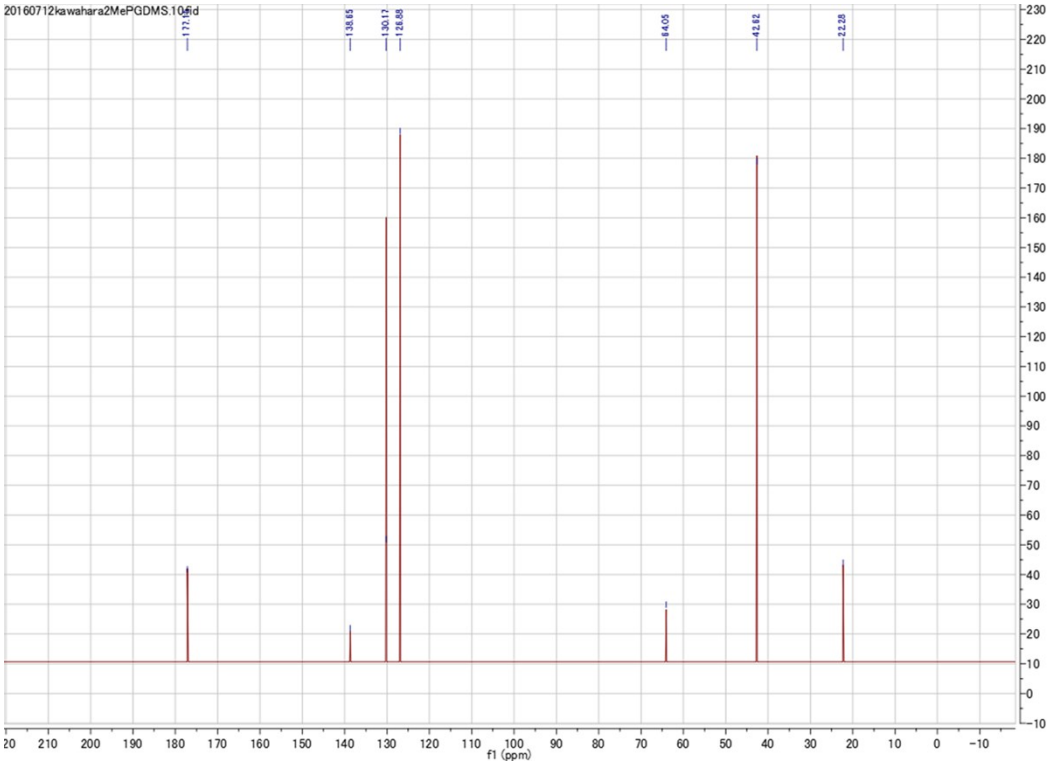
(b) Product from (*R*)-FMBA, F2-MePG : ^{1}H NMR (400 MHz, Deuterium Oxide) δ 7.48 – 7.38 (m, 2H), 7.18 – 7.07 (m, 2H), 1.82 (s, 3H). ^{13}C NMR (100 MHz, Deuterium Oxide) δ 177.15, 138.65, 130.17, 126.88, 64.05, 22.28. MS (microTOF): m/z calcd for $C_9H_{10}FNO_2$ $[M+H]^+$: 184.0768; found: 184.0767.

(c) Product from (*R*)-EBA, 2-EtPG : 1H NMR (400 MHz, Deuterium Oxide) δ 7.46 – 7.33 (m, 5H), 2.38 – 2.18 (m, 2H), 0.96 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, Deuterium Oxide) δ 177.15, 138.65, 130.19, 130.17, 126.88, 64.05, 22.28. MS (microTOF): m/z calcd for $C_{10}H_{13}NO_2$ $[M+H]^+$: 180.1019; found: 180.1014.

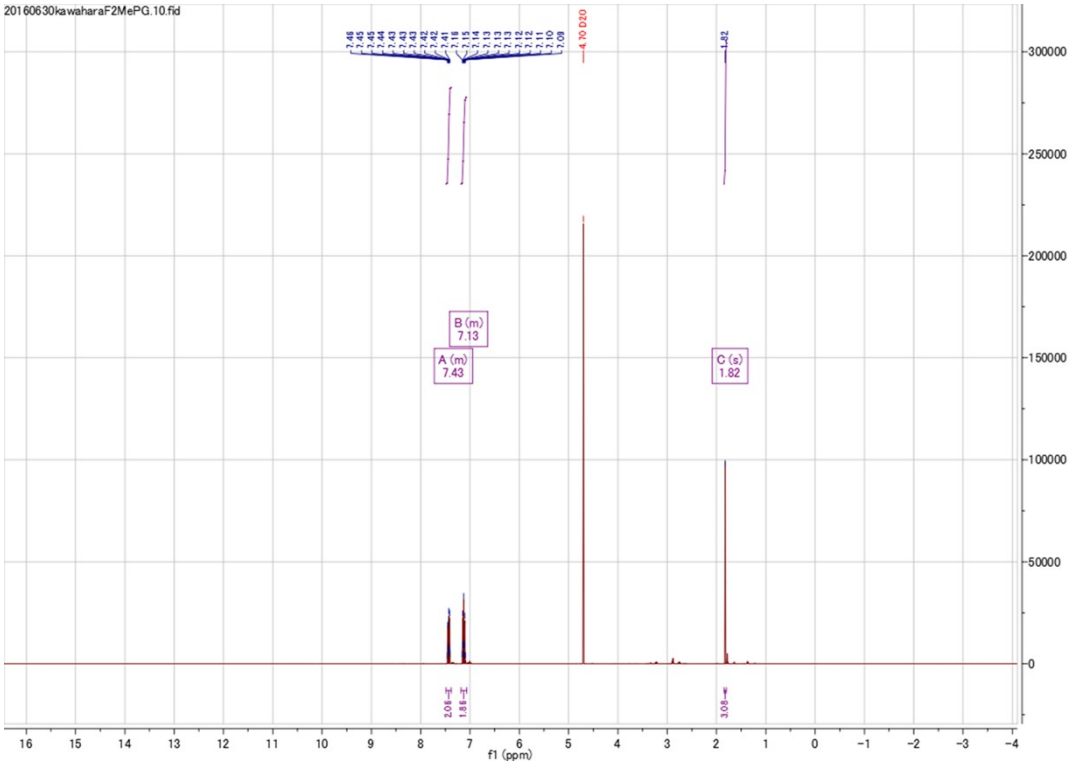
a ^{1}H-NMR (400 MHz, D_2O)



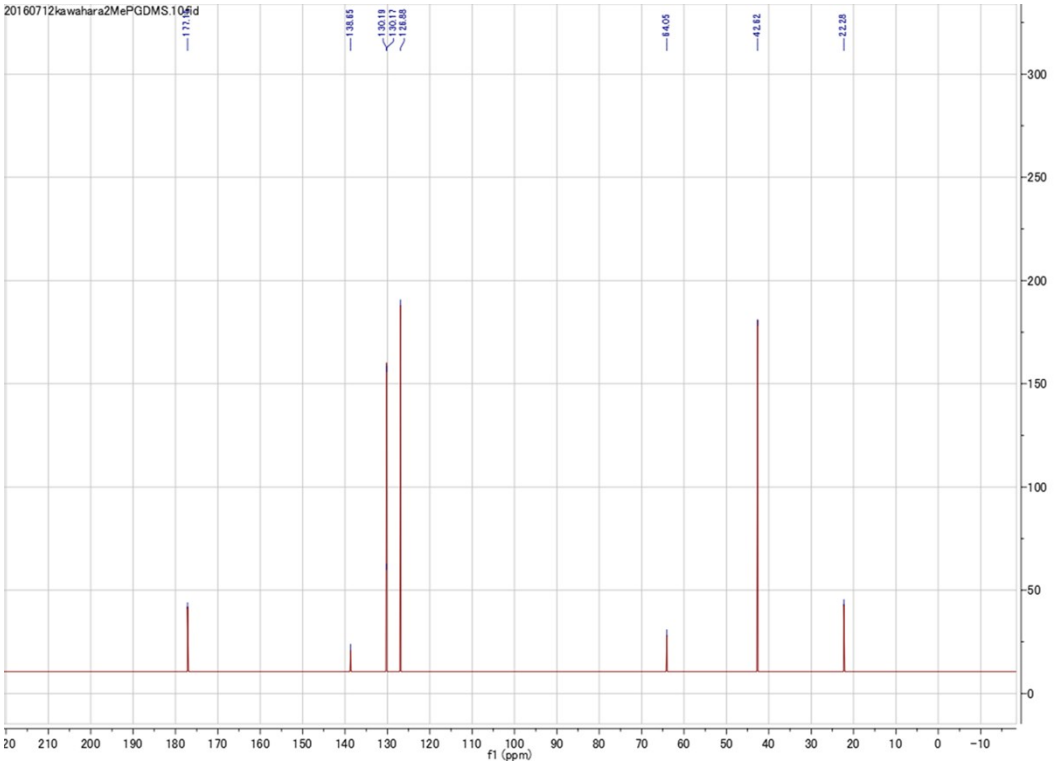
^{13}C-NMR (100 MHz, D_2O)



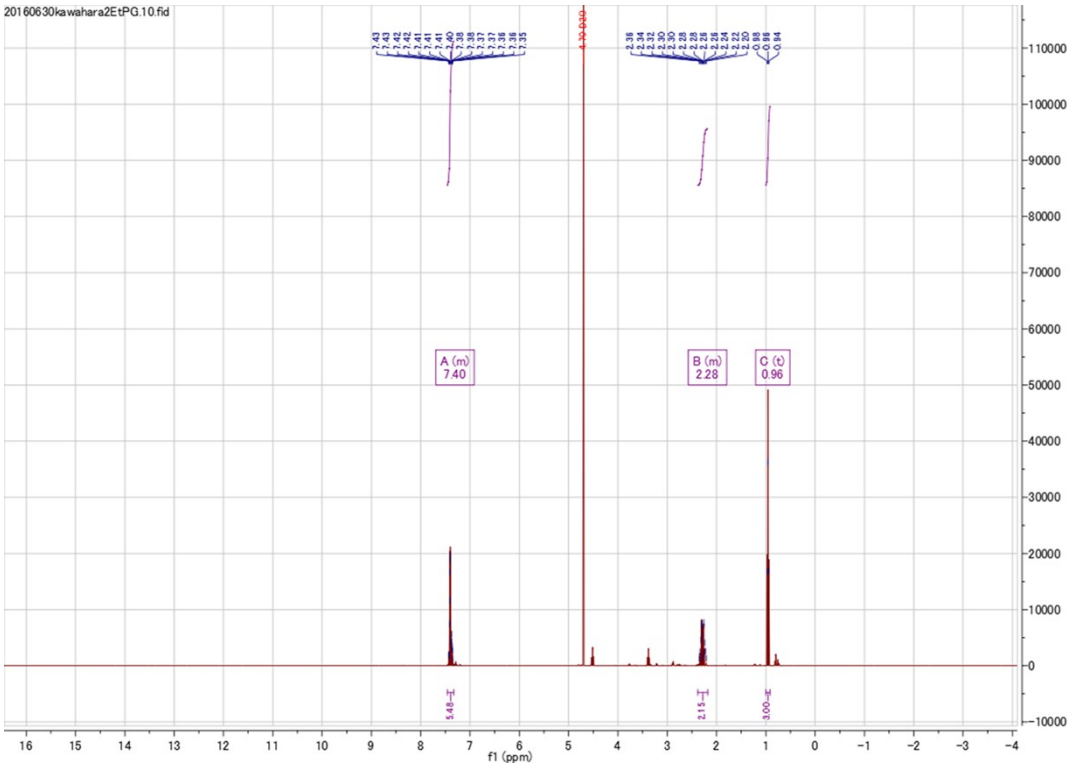
b ^{1}H-NMR (400 MHz, D_2O)



^{13}C-NMR (100 MHz, D_2O)



c ^{1}H-NMR (400 MHz, D_2O)



^{13}C-NMR (100 MHz, D_2O)

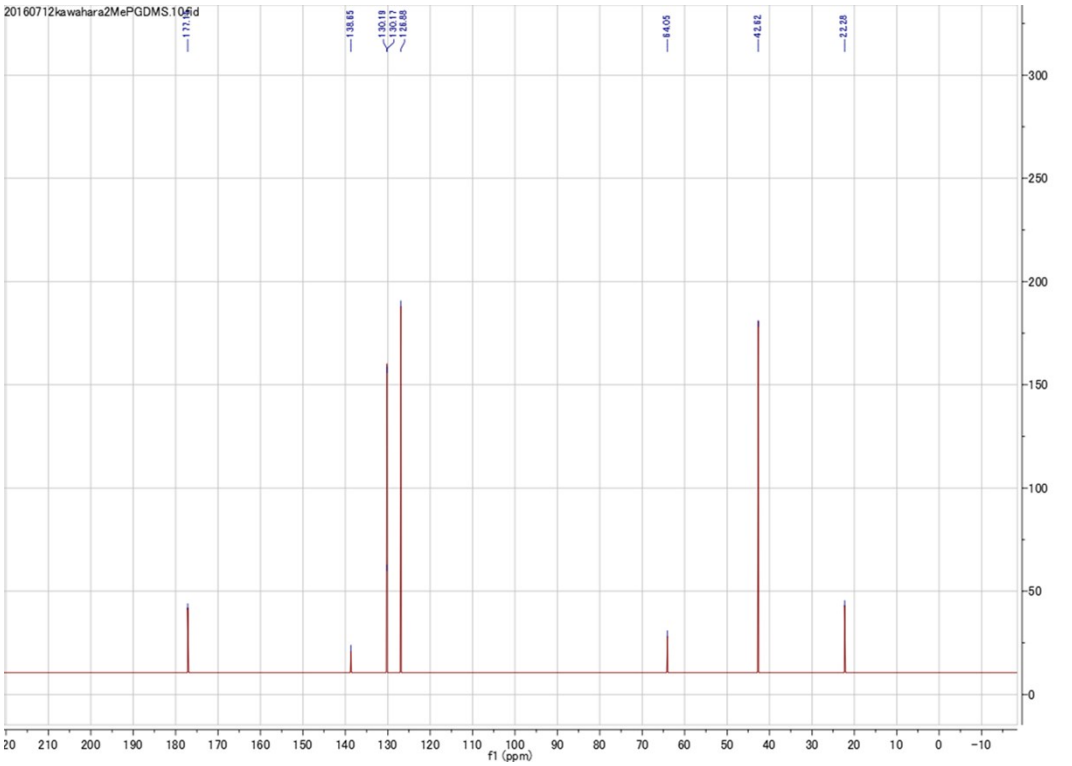
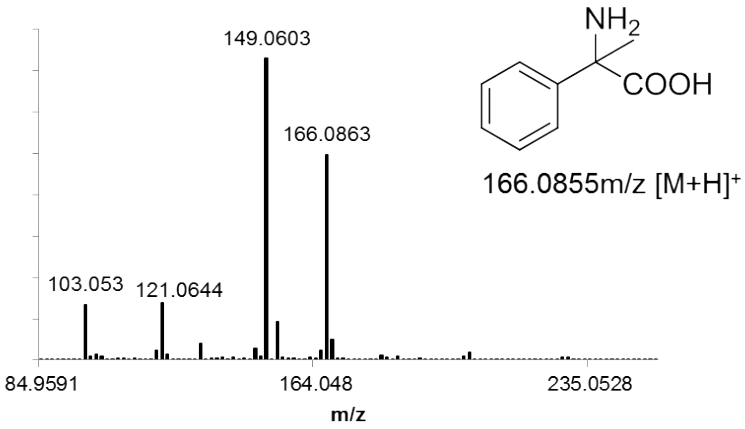


Figure S6. NMR data for identification of enzymatically synthesized unnatural α-amino acids. (a) 2MePG, (b) F-2MePG, (c) 2EtPG

a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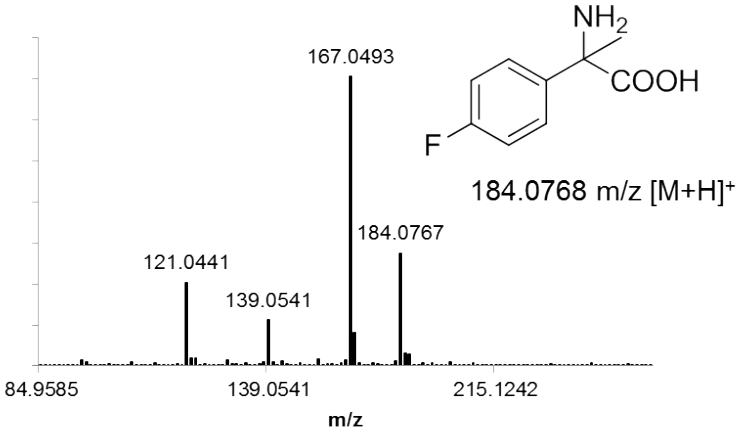
NH2
COOH
166.0855m/z [M+H]+

b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F
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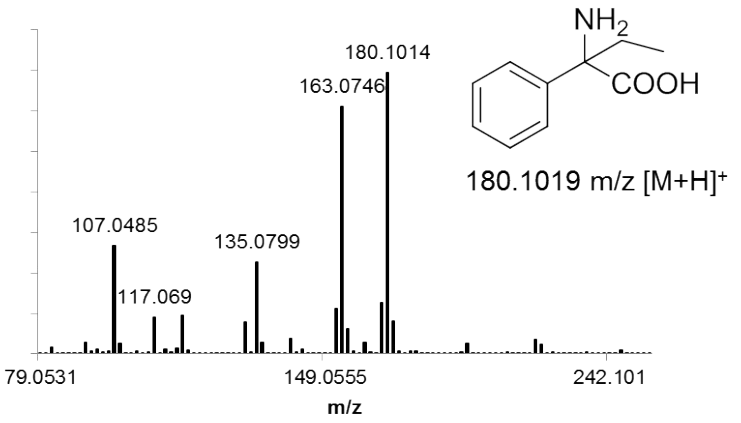
c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m/z
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Figure S7. MS analysis for identification of enzymatically synthesized unnatural α-amino acids.