Isolation and Structure of Pulcherrimine, a Novel Bitter-Tasting Amino Acid, from the Sea Urchin (*Hemicentrotus pulcherrimus*) Ovaries

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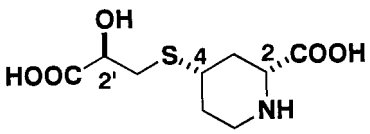
A novel sulfur-containing amino acid, pulcherrimine, has been isolated as a bitter principle from ovaries of the sea urchin *Hemicentrotus pulcherrimus*. The structure was elucidated as 4-(2′-carboxy-2′-hydroxy-ethylthio)-2-piperidinecarboxylic acid by spectroscopic and chemical methods. Absolute stereochemistry was determined by NOE experiments and chiral HPLC analysis. Pulcherrimine exhibited bitterness with a threshold value of 0.306 mM.

Keywords: *Sea urchin ovaries; bitter-tasting; amino acid*

INTRODUCTION

Sea urchin gonads are a prized food in Japan, especially consumed as sushi. Approximately 13 000 tons of sea urchin gonads are consumed annually in Japan (FAO, 1996). Five species of sea urchins are popular: *Strongylocentrotus intermedius*, *Strongylocentrotus nudus*, *Anthocidaris crassispina*, *Hemicentrotus pulcherrimus*, and *Pseudocentrotus depressus*. Accordingly, taste-active components of sea urchins have been well investigated since early times; glycine and alanine are known to be associated with umami (Hashimoto, 1965; Nishimura and Kato, 1988; Fuke and Konosu, 1991). However, some sea urchins become sporadically bitter, which poses a serious problem for the fishery industry. The bitter taste of sea urchin ovaries has been thought to be due to the presence of free amino acids such as valine, leucine, and isoleucine (Hashimoto, 1965; Fuke and Konosu, 1991). *H. pulcherrimus* from the coast of Onahama, Fukushima Prefecture, located 250 km north of Tokyo, are especially bitter and unmarketable. Only the ovaries are bitter; the testes are eatable (Murata et al., 1998). Free amino acid contents are not significantly different between ovaries and testes. Thus, the bitter principle was predicted to be different from the bitter amino acids mentioned above. Bioassay (sensory test)-guided fractionation yielded an unusual amino acid, named as pulcherrimine (**1**), as the cause of the bitterness. We describe the isolation and structure elucidation of this bitter principle.



Pulcherrimine (1)

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MATERIALS AND METHODS

Materials. Samples of the sea urchin *Hemicentrotus pulcherrimus* were collected from Onahama, Fukushima Prefecture, during the period from March to June, in 1997 and 1998, when most of the gonads were mature. After dissection, the ovaries were stored at −84 °C until used.

Apparatus. HPLC separations were performed with a Shimadzu LC-10ADvp liquid chromatograph equipped with a Shimadzu RID-6A refractive index detector, a YMC pack R&D ODS column, and a Reodyne injector.

ESI−MS and FABMS were recorded with a Micromass QUATTRO II and a JEOL JMX-SX102 mass spectrometer using thioglycerol as matrix, respectively. 1D and 2D NMR spectra were recorded on either a JEOL JNM-α600 (600 MHz for ^{1}H, 125 MHz for ^{13}C) or a JEOL GX-270 (270 MHz for ^{1}H, 67.5 MHz for ^{13}C) NMR spectrometer at 300K. ^{1}H chemical shifts were referenced to residual solvent peaks: TMS (δ_H 0.00) in $CDCl_3$ (internal standard), CD_2HOD (δ_H 3.30) in CD_3OD and D_2O (internal standard). ^{13}C chemical shifts were referenced to solvent peaks: δ_C 77.0 in $CDCl_3$, δ_C 49.0 in CD_3OD and D_2O (internal standard). The distortionless enhancement by polarization transfer (DEPT) experiments were performed using a transfer pulse of 135° to obtain positive signals for CH and CH_3 and negative signals for CH_2 groups. ^{1}H−^{1}H COSY, HMBC, and HMQC experiments were performed using conventional pulse sequences (Bax et al., 1986; Summers et al., 1986).

Optical rotation was measured on a JASCO DIP-371 digital polarimeter.

Analytical TLC was carried out on MERCK Kieselgel plates $60F_{254}$, 0.25 mm thick. Chromatograms were visualized by either ninhydrin or sulfuric acid.

Extraction and Isolation. Frozen ovaries (628 g) were homogenized and extracted with 80% aq EtOH (1.8 L × 3). The homogenate was centrifuged at 7500*g* for 15 min. The pellet was further extracted with 20% aq MeOH (0.9 L × 3). The 80% aq EtOH extracts were evaporated and partitioned between water and diethyl ether. The aqueous phase and 20% aq MeOH extracts were combined and concentrated under reduced pressure. Isolation of **1** was carried out by monitoring the bitterness using a brief sensory test. The taste of a small quantity of the solutions was determined by the authors. When fractions were eluted with 1% AcOH solutions, each solution was evaporated to eliminate AcOH and dissolved in an equal volume of distilled water for the sensory tests. The residue (34.7 g) was chromatographed on an ODS column (Cosmosil

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140C_{18}-prep, 50 × 500 mm) with distilled water. The active fractions were separated by gel filtration on Sephadex G-10 (Amersham Pharmacia Biotech Co. Ltd., 26.4 × 1000 mm) with distilled water. The bitter fraction (7.7 g) was fractionated by ODS column chromatography (Cosmosil 140C_{18}-prep, 30 × 800 mm) with 1% aq AcOH. The active fractions were combined (4.8 g) and purified by preparative HPLC on a YMC-Pack R&D ODS column (20 × 250 mm) with 1% AcOH (flow rate, 5 mL/min) monitoring with a refractive index detector to yield a bitter principle (**1**, 30.0 mg, 4.8×10^{-3} % based on wet weight) as amorphous white powder.

Pulcherrimine, 1. $[\alpha]\ ^{24}_{D}$ −16.5° (*c* 0.20, H_2O); TLC on silica gel, R_f 0.12 (*n*-BuOH/AcOH/H_2O, 4:1:2); ^{1}H NMR in D_2O/CD_3OD (40:1) at 600 MHz δ 4.19 (1H, dd, J = 6.7, 3.8 Hz, H2′), 3.64 (1H, dd, J = 12.7, 3.1, H2), 3.49 (1H, ddd, J = 13.1, 4.2, 2.3, H6eq), 3.08 (1H, m, H4), 3.06 (1H, dd, 13.8, 3.8, H1′b), 3.03 (1H, m, H6ax), 2.90 (1H, dd, J = 13.8, 6.7, H1′a), 2.58 (1H, ddd, J = 14.2, 6.2, 3.1, H3eq), 2.25 (1H, m, H5eq), 1.63 (1H, m, H5ax), 1.61 (1H, dd, J = 14.2, 12.7, H3ax). ^{13}C NMR in D_2O/CD_3OD (40:1) at 67.5 MHz δ 180.0s (COOH-2′), 174.2s (COOH-2), 72.3d (C2′), 60.0d (C2), 44.1t (C6), 39.6 (C4), 35.2t (C1′), 34.3t (C3), 29.8t (C5); ESIMS (rel ext): 248 [24, $(M-H)^-$], 195 (6), 159 (16), 145 (100), 133 (8), 131 (19), 89 (31), 87 (8), 59 (9); FABMS (rel ext): *m*/*z* 250 [25, $(M+H)^+$], 204 (15), 162 (22), 128 (48), 82 (98); HRFABMS (matrix, thioglycerol): obsd. $(M+H)^+$ *m*/*z* 250.0749 ($C_9H_{16}NO_5S$ Δ +0.5 mmu).

Boc Derivative, 2. Pulcherrimine (**1**, 1.8 mg, 0.007 mmol) was converted to triethylamine (TEA) salt by treating with 0.5 mL of TEA and 0.05 mL of H_2O twice. The salt in 0.5 mL of TEA/MeOH (1:1) was treated with $(Boc)_2O$ (5.0 mg, 0.030 mmol). The mixture was warmed to 50 °C and stirred for 24 h, and the reaction mixture was evaporated to afford a Boc derivative **2** (2.5 mg) as a yellow oil. ^{1}H NMR in CD_3OD at 270 MHz δ 4.50 (1H, brs, H2), 3.76 (1H, m, H6α), 3.35 (1H, m, H6*β*), 1.40 [9H, s, $-C(CH_3)_3$].

Dimethyl Ester Derivative, 3. A mixture of the Boc derivative **2** (1.25 mg, 0.0036 mmol), DMF (0.5 mL), MeI (1*μ*L, 0.016 mmol), and $NaHCO_3$ (5 mg, 0.06 mmol) was stirred overnight at room temperature. The reaction mixture was neutralized with 0.05 N HCl and extracted with diethyl ether. The ether layer was purified by column chromatography on silica gel (Wakogel C-300) with hexane/EtOAc (1:1) to afford a dimethyl ester **3** (0.2 mg). ^{1}H NMR in CD_3OD at 270 MHz δ 4.65 (1H, brs, H2), 4.40 (1H, dd, J = 6.2, 4.2 Hz, H2′), 3.82 (3H, s, $-OCH_3$), 3.75 (3H, s, $-OCH_3$).

Monoacetate, 4. The dimethyl ester **3** (0.2 mg) was acetylated with pyridine (100 *μ*L) and acetic anhydride (100 *μ*L) at room temperature for 7 h. The reaction mixture was evaporated to afford the monoacetate **4** quantitatively. ^{1}H NMR in CD_3OD at 270 MHz δ 5.21(1H, dd, J = 7.1, 4.9 Hz, H2′), 2.16 (3H, s, $-OCOCH_3$).

Chiral HPLC Analysis of Pipecolinic and Lactic acids. Pulcherrimine (**1**, 2.0 mg) was hydrogenolyzed at 60 °C in the presence of Raney Ni in H_2O for 2 days. The reaction mixture was then evaporated to dryness and dissolved in 5% aq MeCN containing 2mM $CuSO_4$. HPLC analysis was carried out with a Shimadzu SPD-10Avp equipped with UV−VIS detector on SUMICHIRAL OA-5000 (4.6 × 150 mm) with 5% aq MeCN containing 2mM $CuSO_4$ (detection, UV 254 nm; flow rate, 1.0 mL/min). Retention times: standard L-pipecolinic acid (4.667 min), D-pipecolinic acid (6.650 min), L-lactic acid (10.570 min), D-lactic acid (13.248 min); product from **1** (7.300 and 10.575 min). Absolute stereochemistry of pipecolinic and lactic acids obtained from **1** was assigned as D and L, respectively.

Taste Evaluation. The detection and recognition thresholds of the bitter principle were estimated according to the methods of Patton and Josephson (1957) and Bartoshuk (1978). The detection threshold is given as the minimum concentration at which difference from water can be detected. The recognition threshold is given as the minimum concentration at which the character can be recognized. A series of diluted solutions of **1** ranging from 0.032 to 4 mM was prepared using distilled water and evaluated by a panel of 7 persons. All data which were judged by 7 panelists were plotted, with the 50% level of positive responses being selected as the detection and recognition thresholds for **1**. Quinine sulfate was selected as a standard bitter substance for comparing the thresholds. Quinine sulfate solutions ranging from 0.0031 mM to 0.1 mM were used. The detection and recognition thresholds for quinine sulfate were estimated in the same manner as above.

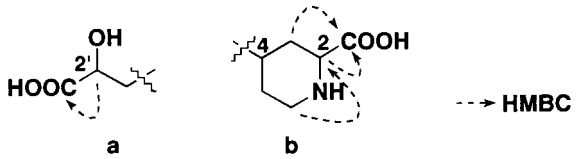


Figure 1. Partial structures and HMBC correlations.

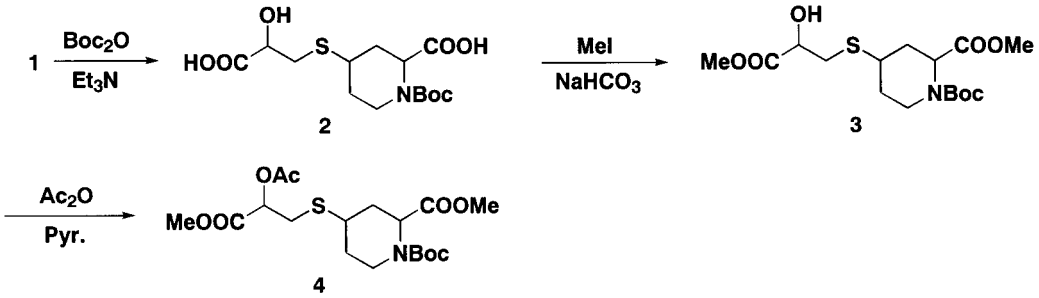
RESULTS AND DISCUSSION

Isolation of Pulcherrimine. The sea urchin ovaries (628 g wet weight) were extracted with 80% aq EtOH and then with 20% aq MeOH. The 80% aq EtOH was partitioned between water and diethyl ether. The aqueous layer and 20% aq MeOH extract were combined and separated by ODS flash chromatography, followed by gel-filtration on Sephadex G-10 with H_2O. Bitter fractions were further fractionated by MPLC and purified by HPLC on ODS with 1% aq AcOH to yield pulcherrimine (**1**, 30.0 mg) as amorphous white powder.

Pulcherrimine (**1**) had a molecular formula of $C_9H_{15}NO_5S$ as determined by HRFABMS [*m*/*z* 250.0749 $(M+H)^+$, Δ +0.5 mmu] and ^{13}C NMR data. The ^{1}H NMR spectrum displayed three methines [δ_H 4.19 (1H, dd, J = 6.7, 3.8 Hz), 3.08 (1H, m), and 3.64 (1H, dd, J = 12.7, 3.1)] and eight nonequivalent methylene signals [δ_H 3.49 (1H, ddd, J = 13.1, 4.2, 2.3), 3.06 (1H, dd, 13.8, 3.8), 3.03 (1H, m), 2.90 (1H, dd, J = 13.8, 6.7), 2.58 (1H, ddd, J = 14.2, 6.2, 3.1), 2.25 (1H, m), 1.63 (1H, m), and 1.61 (1H, dd, J = 14.2, 12.7)]. The ^{13}C NMR spectrum together with a DEPT experiment revealed that pulcherrimine (**1**) contained 3 × CH (δ_C 72.3, 60.0, and 39.6) and 4 × CH_2 (δ_C 44.1, 35.2, 34.3, and 29.8), in addition to two carbonyl carbons (δ_C 180.0 and 174.2). Pulcherrimine (**1**) was stable in the 6N HCl solution at 110 °C, suggesting that **1** had the absence of peptidic and esteric bonds. Therefore, two carbonyl carbons were both carboxyl groups. Interpretation of the COSY, HMQC, and HMBC spectra led to partial structures **a** and **b** (Figure 1). Chemical shift for C2′ (δ_H/δ_C 4.20/72.3) and an HMBC cross-peak between H2′ and a carboxyl carbon at δ 180.0 led to unit **a**. The chemical shifts of C2 and C6 were typical for nitrogen-substituted methine and methylene carbons, respectively. Interpretation of the COSY spectrum starting from a nitrogen-bearing methine at δ 3.64 (H2) led to connectivities from H2 to H6. HMBC cross-peaks between H6eq/C2, and H2 and H3ax/a carboxyl carbon at δ 174.2 resulted in unit **b**. The remaining 32 mass unit in the FAB mass spectrum corresponded to one sulfur atom, whose presence was evident from HRFABMS of pseudomolecular ion at *m*/*z* 250. An HMBC cross-peak between H1′ and C4, as well as chemical shifts for C1′ (δ_H/δ_C 2.90 and 3.06/35.2) and C4 (3.08/39.6), revealed connectivity between units **a** and **b** through a sulfide bond to establish the gross structure of **1**, 4-(2′-carboxy-2′-hydroxy-ethylthio)-2-piperidinecarboxylic acid.

Chemical transformation was carried out to confirm the proposed structure by the procedure shown in Scheme 1. The presence of two carboxyl groups was confirmed by production of the dimethyl ester upon treatment with MeI under a basic condition (Bocchi et al., 1979a and b) after protection with Boc_2O. Two

Scheme 1



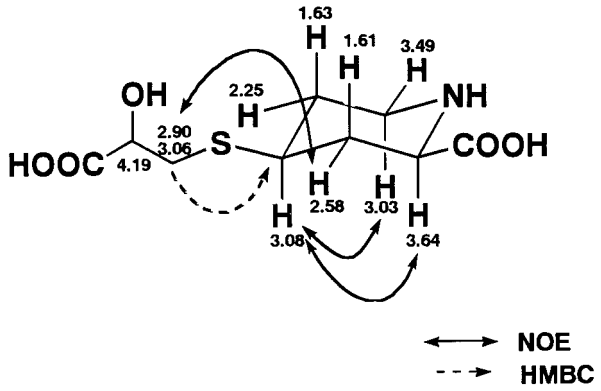


Figure 2. HMBC and NOE correlations for **1**.

singlet methyl signals were observed at δ 3.82 and 3.75 in the ^{1}H NMR spectrum. A hydroxyl group on C2′ was acetylated with Ac_2O/pyridine, which was evident from deshielded proton signals (δ 5.21).

The stereochemistry of pulcherrimine (**1**) was determined by the NOESY spectrum and chemical degradation. NOESY cross-peaks observed between H4/H2 and H4/H6β and coupling constants indicated their axial orientation, whereas a W-coupling between H3β and H5β suggested their equatorial relationships. Therefore, the piperidine ring was in a chair conformation having an ethylthio group on C4 and a carboxyl group on C2 as shown in Figure 2. To determine the absolute configuration, **1** was hydrogenolyzed on Raney Ni in H_2O at 60 °C to afford pipecolinic and lactic acids. Chiral HPLC analysis disclosed that the stereochemistry of pipecolinic and lactic acids was D and L, respectively. Accordingly, the absolute stereochemistry of **1** was 2′*R*, 2*R*, and 4*S*.

Taste Evaluation of Pulcherrimine (1). The detection and recognition threshold levels of pulcherrimine (**1**) were determined by tasting five solutions in which **1** was dissolved in distilled water. All panelists gave the same evaluation for the same taste character as bitterness, while there was some personal difference in the threshold concentration evaluation. All data generated by each panelist were plotted as shown in Figure 3 (Patton et al., 1957; Bartoshuk, 1978), which led to the detection and recognition thresholds of 0.192 mM and 0.306 mM, respectively. Similarly the detection and recognition thresholds of quinine sulfate were estimated to be 0.005 mM and 0.019 mM, respectively.

Thus, the pulcherrimine had a forty times higher detection threshold than that of quinine sulfate, a commonly available extremely bitter substance (Pfaffmann et al., 1971). The detection threshold of pulcherrimine was also compared with that of D- and L-amino acids reported previously by Shiffmann et al. (1981). Pulcherrimine had almost the same detection threshold

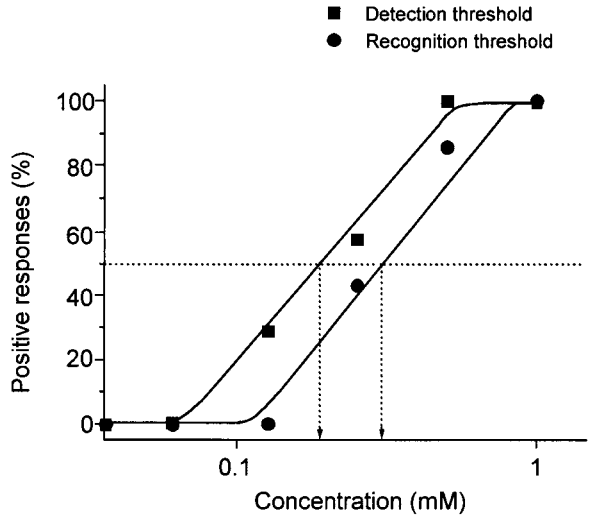


Figure 3. Taste threshold data for pulcherrimine using seven taste panelists.

as those of aspartic acid (D, 0.741; L, 0.182 mM) which exhibited slightly bitter taste. D- and L-Methionine, which had 10 times higher thresholds than that of **1**, were both reported to be bitter. D- and L-Threonine were tasteless and possibly bitter, respectively, and L-threonine had a hundred times higher detection threshold than that of pulcherrimine.

Pulcherrimine (**1**), 4*S*-(2′-carboxy-2′*R*-hydroxy-ethylthio)-2*R*-piperidinecarboxylic acid, was isolated for the first time as a bitter-tasting substance, although several bioactive compounds have been known from sea urchins: e.g., sulfonoglycolipid (Kitagawa et al., 1979), 1-methyl-5-thiol-L-histidine (Palumbo et al., 1982), ovothiol (Turner et al., 1986), temnosides A and B (Babu et al., 1997), and difucosylated neutral glycosphingolipid (Kubo et al., 1992). Pulcherrimine is a new type of sulfur-containing amino acid and tastes bitter like quinine, with a threshold of 0.306 mM. Further research into the mechanism of accumulation and the biosynthesis of **1** in ovaries is necessary.

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