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ISOLATIONS AND STRUCTURES OF NEW UREIDO AMINO ACIDS, LIVIDINE AND GRATELOUPINE, FROM RED ALGAE *GRATELOUPIA* C. AGARDH GENUS[1)]

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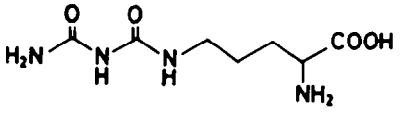
Abstract - Two new ureido amino acids, lividine (**1**) and grateloupine (**2**), were isolated from *Grateloupia livida* and *Grateloupia filicina* respectively. The structures of both amino acids were assumed mainly by NMR spectra and determined as N^{ω}-carbamoyl-L-citrulline for lividine and as *N*-carbamoyl-γ-aminobutyric acid for grateloupine by comparison with the synthetic compounds respectively.

A new amino acid, lividine (**1**), was obtained from aqueous extract of *Grateloupia livida* collected at Echizen seacoast, Fukui prefecture. The isolation of **1** was carried out through the ion-exchange column chromatography as shown in Fig. 1. Lividine gave a



1 lividine

G. livida (dried, 260 g)
| ground
| boiled in water for 30 min
| filtered

filtrate
| applied to Amberlite IRCG-120 column (NH_4^+ form, 6 x 42 cm)
| eluted with H_2O (2 l), 1M NH_4OH (2 l), and 2M NH_4OH (2 l) successively
| collected in each 200 ml fraction

fractions 14-18
| applied to Amberlite IRCG-50 column (NH_4^+ form, 3 x 34 cm)
| washed with H_2O (0.6 l) and then eluted gradiently with H_2O (1 l) to 1M NH_4OH (1 l)
| collected in each 20 ml fraction

fractions 21-62
| rechromatographed on Amberlite IRCG-50 column (NH_4^+ form, 6 x 76 cm)
| eluted with H_2O (1 l) and then 1M NH_4OH (1.5 l)
| collected in each 100 ml fraction

fractions 11-14
| concentrated *in vacuo*
| applied to preparative TLC (Merck silica gel, 0.5 mm thickness, developed with 1-BuOH:AcOH:H_2O = 4:1:2)

crude lividine (30 mg)

Fig. 1. Isolation of lividine.

positive Ehrlich reaction characteristic to mono-substituted ureido derivative as well as a positive ninhydrin reaction. Although both ^{1}H-NMR and ^{13}C-NMR spectra of **1** were quite similar to those of citrulline (**3**)[2], two carbon signals corresponding to the ureido carbonyl were recognized at 156.6 ppm and 158.1 ppm in the ^{13}C-NMR spectrum of lividine. (Table 1)

Acid hydrolysis of **1** gave citrulline, ornithine, and ammonia. A time-course of the hydrolysis was shown in Fig. 2. An increase in total amounts of citrulline and ornithine corresponded to a decrease in the amount of lividine. Moreover, the amount of ammonia formed during the hydrolysis was always equivalent to the sum of equimolar amount of citrulline and double molar amount of ornithine. All these results indicated that the structure of lividine should be assumed as N^{ω}-carbamoylcitrulline. This structure was soon confirmed clearly by the synthesis, a route of which is demonstrated in Fig. 3. The synthetic L-compound showed a good agreement with the natural product in all respects. (Table 2)

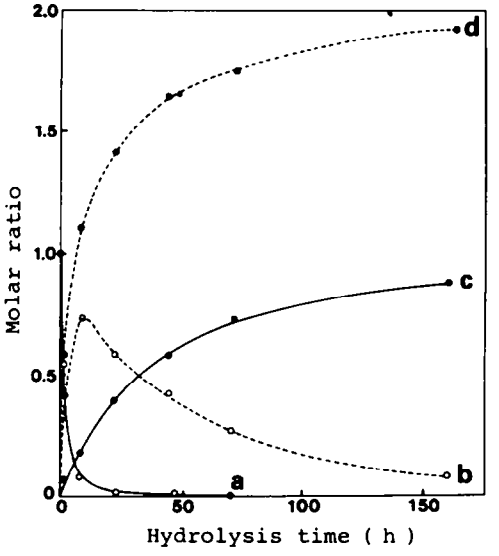
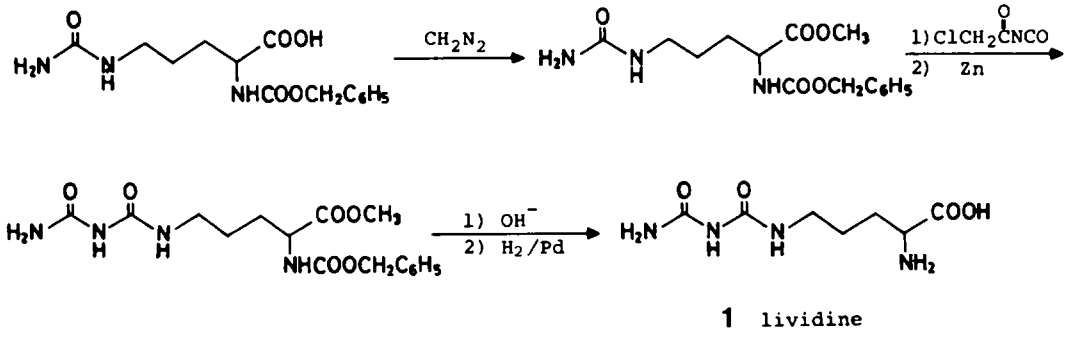


Fig. 2. Time-course of the hydrolysis of lividine.
a) lividine ; b) citrulline; c) ornithine; d) ammonia

Table 1. Comparison of the chemical shifts of lividine and citrulline in ^{13}C-NMR (22.5 MHz).

	δ (ppm from DSS)*	
	lividine[a]	citrulline[b]
-COOH	172.3	175.3
-NH-CO-NH-	158.1, 156.6	162.3
α-CH	53.3	55.4
δ-CH_2	39.4	40.1
β-CH_2	27.8	28.6
γ-CH_2	25.2	25.9

* The values of the chemical shifts are in DCl/D_2O for a) and in D_2O for b).



1 lividine

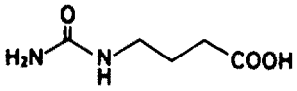
Fig. 3. Synthetic scheme of lividine.

Table 2. Comparison of natural and synthetic lividine.

		natural	synthetic
mp (°C, decomp)		225-228	230-231
Retention time in A.A.A.[a] (min)		133	133
Rf values on TLC[b]	A[c]	0.38	0.38
	B[d]	0.57	0.57
	C[e]	0.85	0.85
$[\alpha]_D^{14}$ in 2N HCl		+20°(*c* 0.66)	+19°(*c* 0.39)

a) amino acid analysis: analyzed on 55 cm column at 55°C with citrate buffer (pH 3.25-4.25).
b) Woelm silica gel F_{254} on glass plate (0.20 mm)
c) 1-butanol : acetic acid : water = 4 : 2 : 1
d) 1-propanol : 28% NH_4OH = 7 : 3
e) 95% ethanol : water = 7 : 3

Grateloupine (**2**), a second new amino acid from *Grateloupia* C. Ag. genus, was found in methanol extract of *G. filicina* collected at the same place where *G. livida* was grown. (Fig. 4) Grateloupine



2 grateloupine

was colored in yellow with Ehrlich reagent similarly to citrulline or lividine. Although this compound itself showed a negative ninhydrin reaction, γ-aminobutyric acid was produced in the acid hydrolysis. ^{1}H-NMR spectrum of **2** in DMSO-d_6 suggested the presence of a ureido group, namely $N\underline{H}_2CONH\underline{C}H_2$- protons at 5.38 ppm (2H, s) and 5.91 ppm (1H, t), respectively. A carbon signal corresponding to the ureido carbonyl, $NH_2\underline{C}O$-NH-, was observed at 162.3 ppm in the ^{13}C-NMR spectrum measured in 1N NaOD. Based on these facts, a structure of grateloupine was assumed as *N*-carbamoyl-γ-aminobutyric acid. This compound was easily prepared from γ-aminobutyric acid by carbamoylation with KCNO and confirmed to be identical with natural grateloupine not only in NMR spectrum

G. filicina (dried, 1.36 kg)
- ground
- kept for 1 month in 50% MeOH
- filtered

filtrate
- 1/3 of the filtrate was applied to Amberlite IRCG-120 column (NH_4^+ form, 6 x 40 cm)
- eluted with H_2O (2 l) and then 1M NH_4OH (2 l)

fractions 2-10 (each 200 ml)
- applied to Amberlite IRCG-4B column (CH_3COO^- form, 6 x 42 cm)
- washed with H_2O (1.2 l)
- eluted with 0.5M AcOH (1.2 l), 1M AcOH (1.2 l), and 2M AcOH (1.2 l) successively

fractions 18-21 (each 200 ml)
- applied to Amberlite IRCG-120 column (pyridine form, 3 x 37 cm)
- eluted with 0.1M pyridine formate (2 l), 0.2M pyridine formate (0.6 l), and 0.2M pyridine acetate (0.8 l) successively

fractions 21-50 (each 20 ml)
- kept to complete a precipitation
- precipitate was filtered

crude grateloupine (213 mg)

Fig. 4. Isolation of grateloupine.

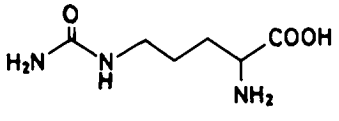
Table 3. Comparison of natural and synthetic grateloupine on TLC.

Developing solvent	Rf value [a]	
	natural	synthetic
1-butanol:acetic acid:water = 4:1:2	0.41	0.41
1-propanol:28% NH_4OH = 7:3	0.61	0.61
95% ethanol:water = 7:3	0.52	0.52

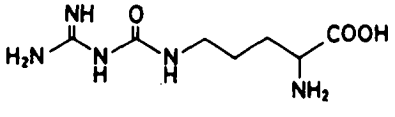
a) Merck CD-Alufolien silica gel 60 F_{254} plate.

but also chromatographically as shown in Table 3.

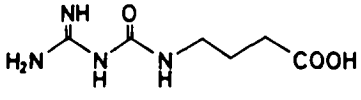
In addition to the above two new amino acids, we also isolated three known ureido amino acids, *i.e.*, citrulline, gigartinine (**4**),[3] and gongrine (**5**),[3b,4] from several species of *Grateloupia* C. Ag. such as *G. livida*, *G. filicina*, *G. turuturu*, and *G. okamurai*. Of these amino acids, lividine, citrulline, and gigartinine may be derived from ornithine biosynthetically. On the other hand, grateloupine and gongrine are derivatives of γ-aminobutyric acid. Although the fact that such ureido amino acids occurs particularly in *Grateloupia* C. Ag. genus seems to be very interesting in view of chemotaxonomy, their physiological roles or significances in algae body are not yet well known.



3 citrulline



4 gigartinine



5 gongrine

Experimental

All melting points are uncorrected. NMR spectra were obtained with a Varian XL-100-15 and JEOL FX-90Q spectrometers using TMS as an internal standard in DMSO-d_6 and DSS as an external standard in D_2O. The chemical shifts were given in δ value (ppm) from the standard. The specific rotations were measured with a Perkin-Elmer 141 polarimeter. Amino acid analyses were carried out with a Hitachi KLA-5 analyzer. Samples were hydrolyzed with constant boiling 6N HCl in a sealed tube at 110°C and run on 0.9 x 55 cm column at 55°C with citrate buffer of pH 3.25 to 4.25.

Purification of natural lividine.

Crude lividine (14 mg) obtained according to the procedure described in Fig. 1 was purified with paper electrophoresis (pH 3.5, 15 V/cm, 90 min). The part corresponding to a band of lividine was cut off and the amino acid was extracted from the paper with water. The extract was lyophilized to obtain lividine as powder (11 mg) which was reprecipitated from water to give 7.0 mg of pure lividine: mp 225-228°C (decomp); $[\alpha]_D^{14}$ +20° (*c* 0.66, 2N HCl). ^1H-NMR (D_2O, 100 MHz) δ=1.67 ($-CH_2-\underline{CH_2}-CH_2-$, m), 1.86 ($-\dot{C}H-\underline{CH_2}-CH_2-$, m), 3.22 ($-\underline{CH_2}-CH_2-$, t), 3.82 ($-\underline{CH}-\dot{C}H_2-$, t).

Found: C, 38.30; H, 6.46; N, 25.33%. Calcd for $C_7H_{14}N_4O_4$: C, 38.35; H, 6.44; N, 25.56%.

Methyl N^{α}-benzyloxycarbonyl-L-citrullinate.

To a solution of N^{α}-benzyloxycarbonyl-L-citrulline[5] (3.00g, 9.70

mmol) in 50 ml of methanol was added diazomethane in ether until a yellow color remained. After stirring for 1 h at room temperature, an excess of diazomethane was decomposed with acetic acid. The reaction mixture was then concentrated *in vacuo* and the residue was recrystallized from methanol and ether to give fine needles: yield 2.58g (82.2%), mp 146-147°C, $[\alpha]_D^{14}$ -15.2° (*c* 1.83, CH_3OH).

Found: C, 55.80; H, 6.55; N, 12.87%. Calcd for $C_{15}H_{21}N_3O_5$: C, 55.72; H, 6.55; N, 13.00%.

Methyl N^{α}-benzyloxycarbonyl-N^{ω}-carbamoyl-L-citrullinate.

Methyl N^{α}-benzyloxycarbonyl-L-citrullinate (1.60 g, 5.00 mmol) was suspended in 50 ml of dioxane under cooling in an ice bath. To the suspension was added monochloroacetyl isocyanate[6] (0.90 ml, 9.00 mmol). The reaction mixture was stirred for 10 min at 0°C and then for 1 h at room temperature. After additions of 50 ml of methanol and Zn dust (3 g), the suspension was stirred for 3 h. Zn dust was then removed off and the filtrate was concentrated *in vacuo*. The residue was dissolved in ethyl acetate, which was washed with saturated NaCl solution three times. The organic layer dried over $MgSO_4$ was concentrated *in vacuo*. To the residue was added ether to obtain crystalline product which was recrystallized from methanol and ether : Yield 1.83 g (83.6%), mp 129-130°C, $[\alpha]_D^{14}$ -12.4° (*c* 2.24, CH_3OH).

Found: C, 52.31; H, 6.01; N, 15.13%. Calcd for $C_{16}H_{22}N_4O_6$: C, 52.45; H, 6.05; N, 15.29%.

N^{ω}-Carbamoyl-L-citrulline (lividine).

Methyl N^{α}-benzyloxycarbonyl-N^{ω}-carbamoyl-L-citrullinate (0.23 g, 0.61 mmol) was dissolved in 0.80 ml of methanol. To the solution was added 2N NaOH (0.34 ml, 0.68 mmol) under ice cooling. The reaction mixture was stirred for 2 h at 0°C, neutralized with 2N HCl and then concentrated *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed with saturated NaCl solution. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. An oily residue (210 mg) thus obtained was dissolved in a mixture of 10 ml of methanol and 10 ml of 5% acetic acid. Hydrogen was bubbled into the solution in the presence of Pd black catalyst for 3 h. Catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in water and then lyophilized to obtain powdery product which was recrystallized from water: yield 77 mg (58%), mp 230-231°C (decomp), $[\alpha]_D^{14}$ +19° (*c* 0.39, 2N HCl).

Found: C, 38.52; H, 6.44; N, 25.37%. Calcd for $C_7H_{14}N_4O_4$: C, 38.35; H, 6.44; N, 25.56%.

Characterization of natural grateloupine.

Crude natural grateloupine was obtained according to the procedure as shown in Fig. 4. It was recrystallized from water: mp 178-179°C (decomp). ^{1}H-NMR (DMSO-d_6, 90 MHz) δ=1.56 ($-CH_2-\underline{CH}_2-CH_2-$, quintet), 2.19 ($-CH_2-\underline{CH}_2-COOH$, t), 2.95 ($-NH-\underline{CH}_2-CH_2-$, q), 5.38 ($\underline{NH}_2-CO-$, s), 5.91 ($-CO-\underline{NH}-CH_2-$, t). ^{13}C-NMR (NaOD, 22.5 MHz) δ=26.9 ($-CH_2-\underline{CH}_2-CH_2$), 35.6 ($-\underline{CH}_2-COOH$), 40.6 ($-NH-\underline{CH}_2-$), 162.3 ($NH_2-\underline{CO}-NH-$), 183.6 ($-\underline{COOH}$).

Found: C, 41.23; H, 6.91; N, 19.15%. Calcd for $C_5H_{10}N_2O_3$: C, 41.09; H, 6.90; N, 19.17%.

N-Carbamoyl-γ-aminobutyric acid (grateloupine).

To a solution of γ-aminobutyric acid (2.1 g, 20 mmol) in 60 ml of 6M acetic acid was added KCNO (16.2 g, 200 mmol) in 60 ml of water dropwise. The reaction mixture was stirred for 22 h at room temperature and then concentrated *in vacuo*. The residue was dissolved in water and applied to a column of Amberlite IRCG-120 (H^+ form, 3.5 x 33 cm). Grateloupine was eluted from the column with 1M AcOH. The eluate was concentrated *in vacuo* and the residue was recrystallized from water: yield

0.51g (17%), mp 178-179°C (decomp).

Found: C, 41.32; H, 6.72; N, 18.91%. Calcd for $C_5H_{10}N_2O_3$: C, 41.09; H, 6.90; N, 19.17%.

^1H- and ^{13}C-NMR spectra of the synthetic material were completely identical with those of natural compound. The identity of both compounds in TLC is shown in Table 3.

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