CHEMICAL STUDIES ON MARINE ALGAE
XIV. ON A NEW AMINO ACID, "CHONDRINE", ISOLATED FROM THE RED ALGA *Chondria crassicaulis*

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INTRODUCTION

Few studies have been made about the nitrogenous compounds in marine algae. Recently, however, B. Lindberg detected taurine and N-methyltaurines[1] in several red algae. S. Makisumi isolated arginylglutamine[2] from green alga, *Cladophora* species. Pharmacologically active substances, kainic acid[3] and domoic acid,[4] were isolated from *Digenea simplex* and *Chondria armata* by T. Takemoto and K. Daigo, respectively. The author detected citrulline in the red alga, *Porphyra tenera*[5] and it was isolated from *Rhodogrossum purchrum* and *Chondrus ocellatus*.[6] *l*-Citrulline and *d*-aspartic acid were also isolated from *Chondria armata*.[7]

The present writers' succeeding investigations on the free amino acids in red algae revealed the existence of an unknown amino acid in *Chondria crassicaulis*, which gave an intense blue colour with ninhydrin after development on paper chromatograms. This substance was isolated from ethanol extract by a technique of displacement chromatography using cationic resin Dowex 50-H,$^+$ and now it has been confirmed to be *l*-1-sulfoxythiazine-3-carboxylic acid. This substance was given the name "chondrine" from the name of this alga, *Chondria crassicaulis*.

EXPERIMENTAL

MATERIAL AND METHOD

The material (*Chondria crassicaulis* Harvey) was collected at Nanaehama, Hakodate on the 10th of Nov. 1958.

The method used for the isolation of this substance was practically the same as for *l*-citrulline from red algae already discribed.[6] All paper chromatograms were run on Tōyō No. 50 filter paper; the solvent used in this experiment were phenol saturated with water, n-butanol acetic acid water (4:2:1 & 4:1:5), collidine lutidine (1:3) saturated with water. As a spraying agent, 0.2 per cent ninhydrin solution in butanol was used, besides, the following specific tests were employed. A) Isatine solution for proline, B) Ehlrich's reagent[8] for citrulline, C) Plattner's reagent[9] for glycine and 1-sulfoxythiazine-3-carboxylic acid (chondrine).

ISOLATION

To fresh alga (15 kg.), 10 liters of hot water were added and the temperature maintained half an hour at 70° C until solution became muddy. After it became cool, 40 liters of ethanol were slowly added with stirring. Insoluble materials having been filtered off, the extract was concentrated *in vacuo*, decolourized with 100 g of acetic acid treated charcoal.[10] The colourless extract was passed through a large column of Dowex 50-H^+ (2500 ml, 20-50 mesh) to remove inorganic salts. Amino acids fraction was eluted with 0.2 N-NH_4OH. This fraction was applied to the two column system of Dowex 50-H^+ (200 and 60 ml, 100 mesh), washed with 3 liters of water and fractionated with 0.2 N-NH_4OH into 87 fractions 24 ml each. (Fig. 1). The unknown substance eluted in pure state at the top of common amino acids; only a small portion was eluted with aspartic acid. The fraction contaminated with aspartic acid (Fig. 1, Band II) was gathered, adjusted to pH 1.5 by addition of N-HCl, and was applied to a column of Amberlite IR-4B-OH^- (100 ml, 50-100 mesh). The column was washed with 500 ml. of water. The unknown substance was obtained in pure state in a nonabsorbable fraction, whereas aspartic acid remained in the column. Unknown substance fractions so obtained were concentrated *in vacuo*, and crystallized from ethanol. The unknown substance was crystallized 3 times from aqueous ethanol, and dried in a vacuum desiccator over H_2SO_4. Five g of white crystalline material were obtained (Fig. 2A).

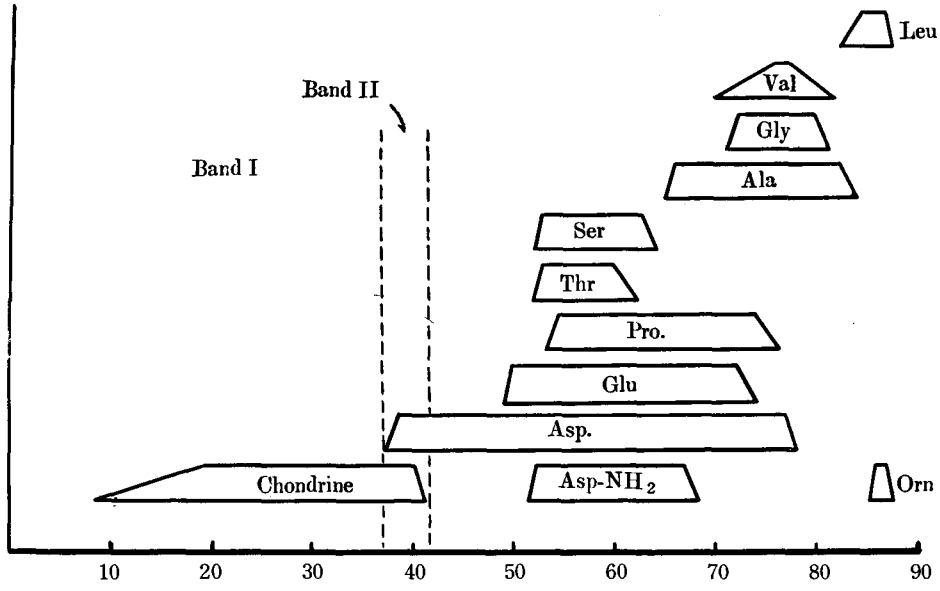


Fig. 1. Primary fractionation of the free amino acids in *Chondria crassicaulis* by a two column system of Dowex 50 X-4 (200 and 60 ml., 100-200 mesh); 0.2 N-NH_4OH was used as a displacement agent

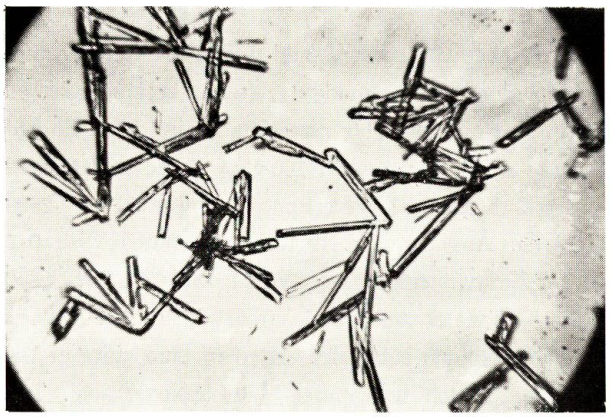


Fig. 2A. Unknown acid from *Chondria crassicaulis*

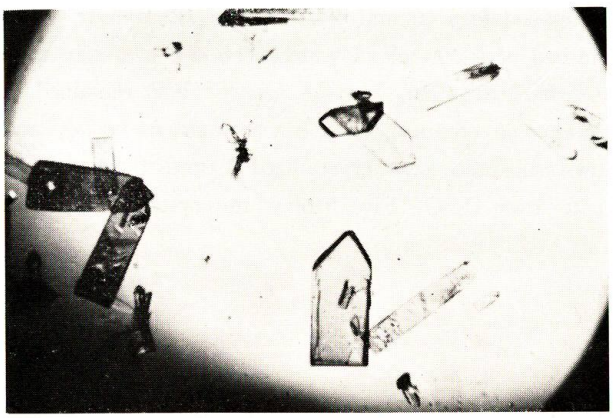


Fig. 2B. Chondrine Cu salt

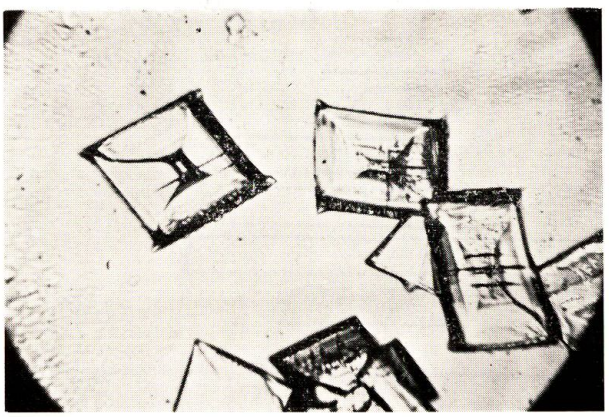


Fig. 2C. DL-N-Ethylalanine (Synthesis)

PROPERTIES OF THIS SUBSTANCE

The most striking property of this substance was its characteristic colour with ninhydrin on paper chromatograms. After development on paper chromatograms, it gave at first cobalt colour with ninhydrin (0.2 per cent in butanol), then deepened to intense blue and at last was changed into brown colour by prolonged heating. Rf values on various solvent systems are shown in Table 1. This substance was unstable for mineral acids and resultant from treatment with 6N-HCl at 100° C for 24 hrs in a sealed tube, the solution gave an intense red colour; paper chromatographically, besides the original substance, a new spot was obtained which no more gave a characteristic colour but a violet colour with ninhydrin (Table 1).

The unknown substance was very soluble in water and virtually insoluble in organic solvents such as ethanol, methanol or aceton. It did not show a definite melting point and slowly degraded above 210° C but it melted with decomposition at 255-257°C in a sealed tube. Molecular weight estimated by cryoscopic method gave 160. Elementary analysis agreed with the formula $C_5H_9O_3N$ S.

	C	H	O	N	S
Estimated	36.73%	5.56%	29.44%	8.63%	19.64%
Calculated	36.81	5.52	29.45	8.59	19.63

as $C_5H_9O_3NS$ O; by difference

Optical rotation gave values $(\alpha)_D^{16}=+20.91$ (C=2) in water and $(\alpha)_D^{12}=+30.15$ (C=2) in 6N-HCl.

This substance was led to a copper salt. Five hundred mg were dissolved in 10 ml of water and kept for 30 minutes on a boiling water bath with one g of copper carbonate. After an excess of copper carbonate, had been filtered off, copper salt was crystallized from ethanol (Fig. 2B).

	N	Cu
Estimated	6.26%	14.34%
Calculated	6.34	14.38

as $C_{10}H_{22}O_6N_2S_2Cu$

For the estimation of crystalline water, 200 mg of copper salt was dried *in vacuo* over P_2O_5 at 110°C. Estimated water correspond to 12.20 per cent of the salt so that this copper salt gave a formula $(C_5H_8O_3NS)_2Cu3H_2O$. It decomposed at 171°C in a sealed tube.

Thus this substance gave a copper salt, but gave no nitrogen when treated by Van Slyke method, so that a secondary amine group was assumed. But the reaction with sodium nitroprusside[11] for the secondary amine group was negative. On the other hand, it gave a strong red colour with quinhydron[12] characteristic for the secondary amine group. This substance (about 3 mg) was dissolved in 2 ml of 50 per cent ethanol. To the solution, 1 ml of 2.5 per cent quinhydron solution in methanol was added with stirring. Immediately, strong red colour was developed. When proline and hydroxyproline were tested for under the same condition, reactions seemed slightly insenstive because colour production occurred one or two minutes after the test solution had been added. This substance also showed positive with Plattner's reagent. After development on paper chromatograms, it was sprayed with 2 per cent p-nitrobenzoylchloride in benzene, next with pyridine; a bright brown spot immediately developed but faded in a little while.

For the estimation of carboxylic acid, phenolphthalein being used as an indicator, this substance was titrated with sodium hydroxide solution.

Unknown subst	N/50-NaOH titrated (f=0,998)	-COOH Estimated	Calcd. as $C_5H_9O_3NS$
19.0 mg	5.85 ml	27.66 %	27.61 %
16.6 mg	5,10 ml	27.60 %	

Negative result being obtained with diazo or ferric chloride reaction for the hydroxyl group, oxygen, other than carboxylic acid, might exist as a sulfoxide. But all attempts to obtain the reduction product with bisulfites were unsuccessful. This strong binding of oxygen, with the addition of a rather large specific optical rotation of this substance,

led to the conclusion that this substance might exist with a sulfur linkage. A reduced product was obtained by treatment with hydriodic acid, which could be converted to the original substance with hydrogen peroxide solution.

REDUCTION WITH HYDRIODIC ACID

Two g of this substance were dissolved in 5 ml of water and 15 g of hydriodic acid was added. The solution was boiled for 10 hours in an oil bath under a reflux. After removal of excess of hydriodic acid under a reduced pressure, 20 ml of N-NH_4OH was added. Excess of ammonia was again removed under a reduced pressure. The residue, dissolved in water, was applied to a column of Dowex 50-H^+ (40 ml), and washed with 500 ml of water. Amino acid was displaced with 0.2 N-NH_4OH and was crystallized from ethanol (yeild, 1.1 g).

This reduction product no longer gave a characteristic colour as an original substance, but gave a violet colour with ninhydrin (Table 1). Also it gave a bright brown colour with Plattler's reagent. This reduction product was practically the same as that obtained by treatment with 6N-HCl (Table 1).

The reduction product sublimated slowly above 190°C but in a sealed tube it was melted with decomposition at 262-263°C. Elementary analysis gave the formula $C_5H_9O_2NS$

	C	H	O	N	S
Estimated	40.79%	6.18%	21.73%	9.54%	21.76%
Calculated	40.82	6.12	21.77	9.52	21.77

as $C_5H_9O_2NS$ O ; by difference

Optical rotation gave a value $(\alpha)_D^{13} = -52.94$ (C=2) in water and $(\alpha)_D^{13} = -26.38$ (C=2) in 6N-HCl.

CONVERTION TO THE ORIGINAL SUBSTANCE

One g of this reduction product was dissolved in 6 ml of N-HCl. To the solution, 0.75 g of 30 per cent hydrogen peroxide solution was added. It was kept in a boiling water bath for an hour under a reflux and then overnight at room temperature. The solution was neutralized by addition of equimolar sodium hydroxide solution, desalted by being passed through a column of Dowex 50-H^+. Paper chromatographically it completely agreed with the original substance not only as to its Rf values but also as to it characteristic colour. Small unoxidized product being still encountered, the solution was refractionated on a column of Dowex 50-H^+, 0.2N-NH_4OH being used as a displacement agent. Eight hundred mg. of crystalline were obtained. Melting point gave 255-256°C and its copper salt melted at 171°C (sealed tube). Estimated N gave 8.60% (Calculated N, 8.59%). Optical rotation gave a value $(\alpha)_D^{11} = +19.97$ (C=1) in water.

REDUCTION WITH RANEY NICKEL

Two g of crystalline were dissolved in 40 ml of 50 per cent ethanol. The solution was boiled for 8 hours under a reflux with about 5 g of raney Ni prepared by the method of R. Mozingo.[13] Raney Ni was filtered off and it was washed with about 100 ml of dilute

sodium hydroxide solution. The filtrate was neutralized with hydrochloric acid, desalted by passage through a column of Dowex 50-H^+. The main reduction product, which gave a positive result with Plattner's reagent (Table 1, A) and small spot (Table 1, B), were observed on paper chromatograms. The latter, a negative result being obtained with Plattner's reagent, seemed to be an α-amino acid. The solution was co-chromatographed with known amino acids and it was found that alanine agreed well with it on various paper chromatograms.

To obtain the main reduction product with raney Ni, the solution was applied to a two column system of Dowex 50-H^+ (10 and 3 ml). Amino acids were displaced with 0.1 N-NH_4OH into 17 fractions, 5 ml each (Fig. 3). Fractions eluted with alanine were again fractionated with a small column of Dowex 50 and from the pure solution so obtained, 800 mg of the main reduction product was crystallized from ethanol. Elementary analysis gave the formula $C_5H_{11}O_2N$.

	C	H	O	N
Estimated	51.33%	9.35%	27.44%	11.88%
Calculated	51.26	9.46	27.32	11.95
as $C_5H_{11}O_2N$		O; by difference		

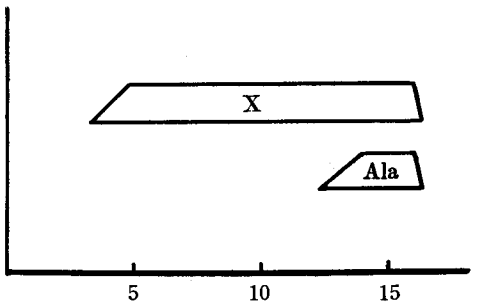


Fig. 3. Fractionation of reduced products with catalyst raney Ni. Two column system of Dowex 50 X-4, (10 and 3 ml, 100-200 mesh) was used and reduction products were fractionated into 17 fractions 5 ml each.

Specific optical rotation gave a value $(\alpha)_D^{15} = +10.41$ (C=1) in water. The reduction product melted with decomposition at 282-285°C in a sealed tube.

The main reduction product gave a copper salt and also gave a positive result with Plattner's reagent so that it seemed to be a N-alkyl-amino acid. On the other hand, by a reduction with raney Ni, a small amount of alanine was detected, so that the structure of N-ethylalanine was assumed. These considerations were confirmed by a synthesis of N-ethylalanine. An attempt to obtain an optically active form of this amino acid from *l*-alanine by a modified method of *d*-methyl-alanine described by E. Fischer[14] did not give a good result. But the use of Strecker's method[15] was successful.

SYNTHESIS OF DL-N-ETHYLALANINE

Cold ethylamine hydrochloride solution (42 g in 80 ml of water) was combined with potassium cyanate solution (30 g in 60 ml of water). To the solution, freshly prepared acetaldehyde (20 g) was dropwise added with stirring in an ice bath keeping the reaction mixture below 5° C. When acetaldehyde had been added, the reaction mixture was kept 3

days after transference into a brown bottle tightly closed. The reaction mixture was then transferred to a beaker, and was boiled with 50 ml of concentrated hydrochloric acid until concentration became difficult because of a precipitation of potassium chloride. After the solution became cool, insoluble salt was filtered off. The insoluble salt was washed with 100 ml of cold concentrated hydrochloric acid. After concentration of the solution *in vacuo* to dryness, the residue was extracted with 100 ml of methanol on a hot water bath under a reflux. Insoluble ammonium chloride was fitered off, and it was washed with methanol. The extract was concentrated *in vacuo* to a dryness, the residue was dissolved in 100 ml of water. To the solution excess of zinc carbonate was added to remove the chloride ion in the solution. The precipitate having been filtered off, it was washed with ice cold water. Filtrate was boiled for 30 minutes to remove ammonia and then saturated with hydrogen sulfide. Precipitate was filtered off. The solution was clarified with charcoal, concentrated *in vacuo*, crystallized from ethanol. Twelve g of white crystalline was obtained. (42 % theoretical, Fig. 2 C)

	C	H	O	N
Estimated	51.27%	9.37%	27.40%	11.96%
Calculated	51.26	9.46	27.32	11.95

as $C_5H_{11}O_2N$ O; by difference

Synthetically obtained N-ethylalanine melted with decomposition at 282-284°C in a sealed tube. As shown in Table 1, this substance was found to quite agree with the main reduction product with raney Ni on the various paper chromatograms.

Table 1. Rf values of chondrine and its degraded products in various solvents.

Substance	Solvent I	II	III	IV
Original	0.69	0.27	0.17	0.61
Reduction with HI	0.80	0.53	0.37	0.68
Treatment with 6N-HCl	0.69 0.80	0.27 0.53	0.17 0.37	0.61 0.68
Reduction with raney Ni (A) (B)	0.88 0.58	0.62 0.44	0.42 0.26	0.44 0.52
Oxidation with H_2O_2 (C) (D) (E) (F)	0.39 0.58 0.63	0.17 0.29 0.44 0.63	0.15 0.19 0.26 0.41	0.59 0.46 0.52
Alanine	0.58	0.44	0.26	0.52
DL-N-ethylalanine (Synthesis)	0.88	0.62	0.42	0.44
Taurine	0.39	0.17	0.15	0.59

Solvent I, Phenol saturated with water. Solvent II, n-Butanol acetic acid water (4:2:1). Solvent III, n-Butanol acetic acid water (4:1:5) upper phase. Solvent IV, Colidine lutidine (1:3) saturated with water.

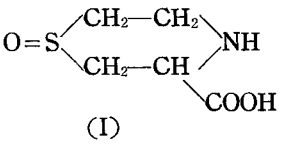
OXIDATION WITH HYDROGEN PEROXIDE SOLUTION

The unknown substance (160 mg) was dissolved in 1 ml of N-HCl and 1 g of 30 per cent hydrogen peroxide solution was added. The solution was boiled in an oil bath for 3 hours under a reflux. It was then passed through a small column of Dowex 2-OH^-, and developed on paper chromatograms. Three spots were detected in the solvent system of phenol water and colidine lutidine, whereas four spots were detected in the solvent system of n-butanol (Table 1, C-F). Spots C and E quite agreed with taurine and alanine respectively when the material was co-chromatographed with them. Furthermore, spot C gave a positive result with zenphel reaction,[16] so that spot C was confirmed to be taurine. On the other spots (D or F) no further investigation was performed.

CONCLUSION

For the isolation of this unknown substance, it was very efficient to use an ion exchange resin Dowex 50-H^+ and in one fractionation, an almost pure sample of the substance was obtained. Impurity could be removed by a refractionation on a small column of Dowex 50-H^+, but the use of Amberlite IR-4B was more efficient. Its characteristic colour with ninhydrin and a specific test with Plattner's reagent on paper chromatograms made it easy to identify this amino acid.

The unknown substance gave an empirical formula $C_5H_9O_3NS$. Negative results were obtained for the hydroxyl group, so that oxygen other than carboxylic acid was considered to exist as a sulfoxide. But the substance was stable against reduction with bisulfites and the reduction product was obtained by a treatment with hydriodic acid. From this strong binding of oxygen, a sulfur linkage amino acid was assumed. Rather large specific optical rotations of this substance and the reduction product also confirmed this assumption. Because this reduction product could be recovered to the original substance when the reduction product was oxidized with hydrogen peroxide solution, the existance of sulfoxide group was evident. On the other hand by a reduction with raney Ni, *l*-N-ethylalanine as the main reduction product was obtained, which was synthetically confirmed.



(I)

Furthermore, by oxidation with hydrogen peroxide solution the material gave, paper chromatographically, taurine, and other unknown spots. From these results, the substance isolated was thus confirmed to be *l*-1-sulfoxythiazine-3-carboxylic acid (structure I). This substance was given the name "chondrine" from the name of the alga, ***Chondria crassicaulis*** from which it was derived.

Though thiazine-3,5-carboxylic acid was synthetically obtained by R. D. Coghill,[17]

an amino acid which included the thiazine ring is thought not to have been detected in nature before.

T. Takemoto[3] isolated kainic acid from *Digenea simplex* and K. Daigo[4,7] isolated domoic acid, *l*-citrulline and *d*-aspartic acid from *Chondria armata*. It is very interesting that these algae belong to the same family, Rhodomelacease, especially, interesting that *Chondria armata* and *Chondria crassicaulis* belong to the same species. Though there is such a close relation between the two, not a trace of domoic acid was detected in the present experiment.

This new amino acid has not been detected in other algae so for examined.

SUMMARY

A new amino acid "chondrine" was isolated from the red alga *Chondria crassicaulis* by means of ion exchange resin Dowex 50-H^+. It was identified as *l*-1-sulfoxythiazine-3-carboxylic acid.

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ACKNOWLEGEMENT

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