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CLITHIONEINE, AN AMINO ACID BETAINE FROM *CLITOCYBE ACROMELALGA*

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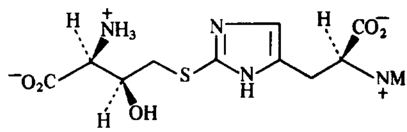
Key Word Index—*Clitocybe acromelalga*; Tricholomataceae; Basidiomycetes; new amino acid betaine; clithioneine.

Abstract—A new amino acid betaine, clithioneine, was isolated from a poisonous mushroom *Clitocybe acromelalga*, and its structure was established on the basis of spectral analyses and chemical degradation studies.

INTRODUCTION

Ingestion of the fungus *Clitocybe acromelalga* Ichimura (Japanese name; Dokusasako), found only in Japan, causes, after several days, a violent pain and red coloration in the fingers and toes, similar to acromelalgia or erythromelalgia, and the pain continues over 2–4 weeks. These characteristic properties prompted us to study the chemical constituents of the fungus, although it was difficult to reproduce the symptoms in experimental animals. Fractionation, carried out on the basis of the lethal effect in mice [1–3], enabled us to isolate a new betaine. In this paper, we describe the isolation and characterization of this compound, designated clithioneine (**1**) [4].



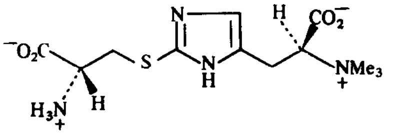
clithioneine **1**

RESULTS AND DISCUSSION

Clithioneine was obtained in a yield of 0.00023 % from fresh fruiting bodies by a combination of various chromatographic techniques as described in Experimental. It was a weakly basic substance judging from ion-exchange chromatographic behavior and PEP. It showed a dark yellow coloration with ninhydrin and a yellow coloration in the Pauly test. A molecular formula $C_{13}H_{22}N_4O_5S$ was obtained by the FD mass spectrum. In the IR spectrum the absorption bands due to hydroxyl, ammonium (3300–2200 cm^{-1}) and carboxylate groups (1620 cm^{-1}) were observed.

The presence and structure of an aromatic portion was deduced by the UV and NMR spectra. In the aromatic region of the ^{13}C NMR spectrum a doublet (δ121.9) and two singlet peaks were observed (δ136.6 and 141.7). Comparison of the δ values with those of ergothioneine disulfide suggested the presence of an imidazole nucleus, in particular, 2-thio-5-substituted imidazole [5, 6]. The partial structure was supported by the UV spectrum, that is, the characteristic bathochromic shift of the absorption maximum of **1** in acidic media (246 nm at pH 7 to 254 nm at pH 2) closely resembled that of S-(β-amino-β-carboxyethyl)-ergothioneine (**2**) [7]. Moreover, in the ^{1}H NMR spectrum, the chemical shift of the aromatic proton of **1** and **2** was the same (δ7.0), and the deduced structure well interpreted the weakly basic and Pauly-positive nature of clithioneine.

The formulae of the two substituents were clarified by NMR analysis. The ^{1}H NMR at 400 MHz of **1** (Table 1) clearly showed the proton sequences of (A) $■\text{-}CH_2\overset{■}{C}H\text{-}■$ and (B) $■\text{-}CH_2\overset{■}{C}H\overset{■}{C}H\text{-}■$, in which both terminal methines thought to be α-proton of either amino acid or betaine, judging from the δ values of the methines [8] and betaine moiety (δ3.26, 9H, *s*). Further evidence for these partial structures was given by the ^{13}C NMR; peaks due to two methylenes were observed at δ30.0 and 40.6, those for three methines at δ60.2, 71.6 and 80.6 and signals characteristic of methyls of betaine at δ54.8 [9]. Of these three methine signals, the one at 60.2 ppm was assignable to the α-carbon [10], and the next one at δ71.6 to a carbon bearing a secondary hydroxyl group and the remaining one to the α-carbon of betaine [6]. The question which of the portions [(A) or (B)] is connected to the betaine moiety was not settled, however. In order to solve this problem, ^{1}H NMR spectra were taken in acidic and basic solution. The shift values in those solutions of H_e were compatible with those of protein-amino acids [8], but the



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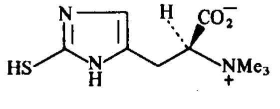

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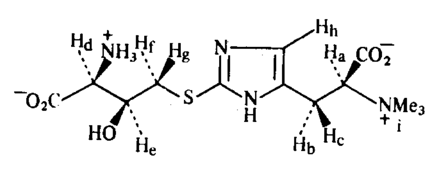

Table 1. 400 MHz ^1H NMR spectrum of clithioneine (DSS in D_2O)

	pH 2	pH 7	pH 12
H_a	4.21 (*dd*, 4.2, 12.3)	3.86 (*dd*, 3.9, 11.2)	3.87 (*dd*, 5.4, 9.3)
H_b	3.56 (*dd*, 4.2, 14.0)	3.24 (*dd*, 3.9, 13.2)	3.08 (*dd*, 5.4, 13.7)
H_c	3.47 (*dd*, 12.3, 14.0)	3.17 (*dd*, 11.2, 13.2)	2.97 (*dd*, 9.3, 13.7)
H_d	4.25 (*d*, 5.4)	3.84 (*d*, 4.3)	3.34 (*d*, 3.4)
H_e	4.39 (*ddd*, 3.9, 5.4, 9.3)	4.18 (*ddd*, 4.3, 4.8, 7.8)	3.96 (*ddd*, 3.4, 4.9, 8.3)
H_f	3.50 (*dd*, 3.9, 14.0)	3.23 (*dd*, 4.8, 14.2)	3.1–3.2 (*m*)
H_g	3.42 (*dd*, 9.3, 14.0)	3.11 (*dd*, 7.8, 14.2)	
H_h	7.42 (*s*)	7.02 (*s*)	6.85 (*s*)
H_i	3.34 (*s*)	3.26 (*s*)	3.20 (*s*)

Chemical shifts in δ-values (ppm); coupling constants in Hz are given in parentheses.

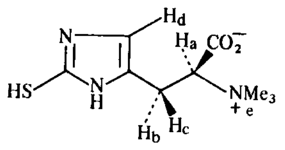

Table 2. 400 MHz ^1H NMR spectrum of ergothioneine (DSS in D_2O)

	pH 2	pH 7	pH 12
H_a	4.11 (*dd*, 3.9, 12.0)	3.88 (*dd*, 3.9, 11.7)	3.86 (*dd*, 4.9, 9.8)
H_b	3.36 (*dd*, 3.9, 14.2)	3.24 (*dd*, 3.9, 13.7)	3.08 (*dd*, 4.9, 13.7)
H_c	3.26 (*dd*, 12.0, 14.2)	3.15 (*dd*, 11.7, 13.7)	3.05 (*dd*, 9.8, 13.7)
H_d	6.86 (*s*)	6.77 (*s*)	6.66 (*s*)
H_e	3.30 (*s*)	3.26 (*s*)	3.22 (*s*)

Chemical shifts in δ values (ppm); coupling constants in Hz are given in parentheses.

feature of H_a was similar to that of the α-proton of ergothioneine (**3**) (Table 2). As a result, the portion (A) and (B) must be formulated as ■–$CH_2CH(\overset{+}{N}Me_3)CO_2^-$ and ■–$CH_2CH(OH)CH(\overset{+}{N}H_3)CO_2^-$, respectively.

The substituted positions of the imidazole ring were indicated by EIMS. The EIMS of **1** did not show a $[M]^+$ ion peak. This may be attributed to a smooth double McLafferty rearrangement [11] as shown in Fig. 1, and the fragmentation pattern was completely identical with that of **3**. Therefore, it was suggested that the portion (A) was attached to C-5 and (B) to C-2 (through the sulfur atom) of the imidazole ring.

Corroboration of the thus deduced structure was given by chemical degradation studies. Treatment of **1** with Raney-Ni afforded two ninhydrin-positive and two Pauly-positive products. By the spectroscopic and chromatographic comparison with authentic samples, it was shown that the former were threonine and α-amino-*n*-butyric acid, and the latter were hercynine [12] and recovered **1**. In addition, all the degradation products were identified as L-isomers by the CD spectral comparisons with authentic samples [13, 14].

These results clearly show the structure of clithioneine to be expressed by **1** including absolute configuration.

EXPERIMENTAL

The ^1H NMR (60 MHz and 400 MHz) and ^{13}C NMR (25 MHz) were recorded in D_2O using DSS as internal standard. The EIMS was recorded at 70 eV.

Cellulose TLC was carried out using the following solvents; (A) MeOH–pyridine–H_2O (15:1:5), (B) *n*-BuOH–HCO_2H–H_2O (6:1:5), and visualized by a UV lamp, ninhydrin and Pauly test [12]. Paper electrophoresis (PEP) was performed at pH 4.6 (pyridine–HOAc–H_2O, 3:3:994), 600 V, for 2 hr. Reversed-phase HPLC was performed using an ODS column.

Fruiting bodies of the mushroom were collected at Nagaoka city, Niigataken, Japan, frozen upon collection and stored at −20°.

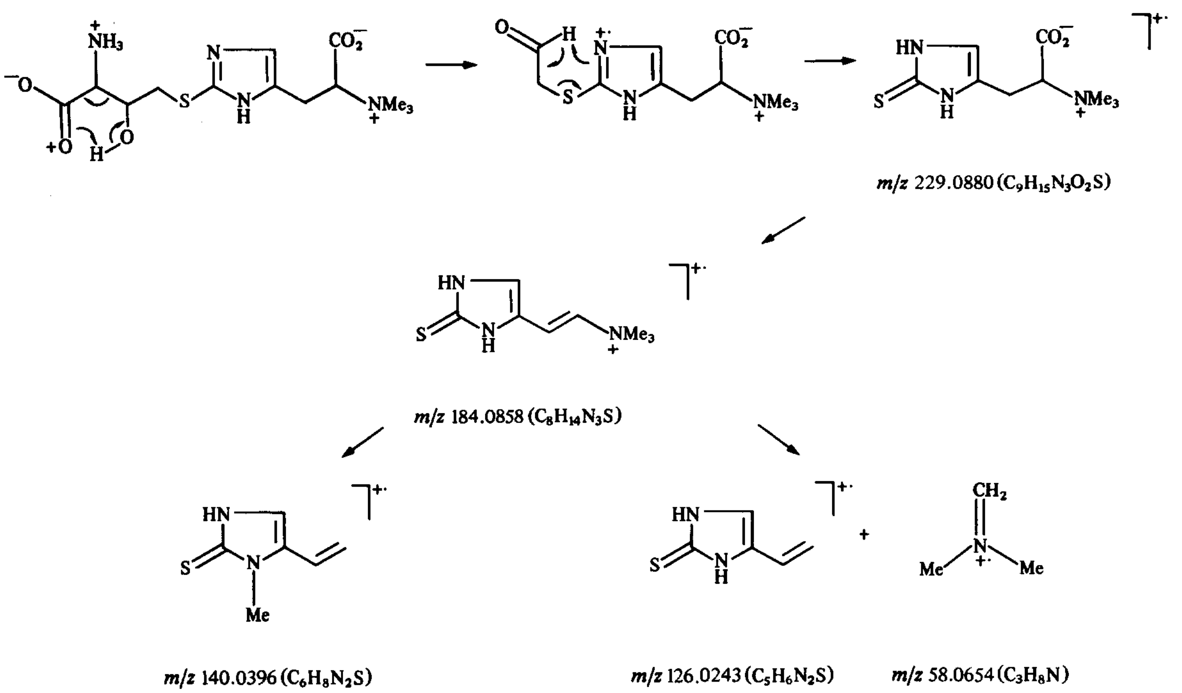

Fig. 1. EIMS of clithioneine.

Isolation of clithioneine. Frozen fruiting bodies (5.4 kg) were extracted with H_2O (3 x 10 l.) at 4° overnight. The combined extracts were evaporated *in vacuo* to about 1.8 l. To this turbid soln Me_2CO (7.2 l.) was added and after the mixture was left at 4° overnight, the supernatant was decanted and the ppt was dissolved in H_2O (750 ml), and dialysed against H_2O (3 x 6 l.) at 4° overnight. The combined dialysate was evaporated and the residue (82 g) was applied to a column of charcoal (82 g, H_2O, 2.5, 5, 10, 30% aq. EtOH, each 4 l.). PPC and PEP analysis of each fraction revealed the presence of clithioneine in 10% aq. EtOH eluate. The entire process was repeated twice more to yield 4 g of crude clithioneine fraction.

The crude clithioneine fraction was divided into two batches and each was subjected to chromatography on a weakly acidic ion-exchange resin (240 g, H^+ form). The eluate with 2.5% NH_4OH was evaporated and the residue (0.9 g) was subjected to electrophoresis on paper (46 x 20 cm, 40 sheets). A UV light-absorbing and ninhydrin-positive band at − 5 cm was cut off, and the strip was extracted with H_2O, the extract was evaporated and the residue (155 mg) was then placed on TLC plates of cellulose (20 x 20 cm, 15 plates) and developed by solvent A. Clithioneine was obtained by H_2O extraction of the band at R_f 0.38 (65 mg), and purified by reversed-phase HPLC (1.3 x 30 cm, 15 runs, 80% aq. EtOH) to yield 38 mg of pure sample as an amorphous powder, $[\alpha]_D^{24}$ $+44.2°$ (c 0.5, H_2O); m/z 347.1334 $[M + H]^+$ calc. for $C_{13}H_{23}N_4O_5S$ 347.1387; EIMS: Fig. 1; UV: λ_{max}^{pH7} 246 nm (ε16 000), λ_{max}^{pH2} 254 nm (ε17 300); λ_{max}^{pH12} 248 nm (ε15 000); ^1H NMR: Table 1; ^{13}C NMR: δ 30.0 (t, $-CH_2-$), 40.6 (t, $-CH_2S-$), 54.8 (q, $-\overset{+}{N}Me_3$), 60.2 (d, $-\underline{C}H{<}^{\overset{+}{N}H_3}_{CO_2^-}$), 71.6 ($d$, $-CH-OH$), 80.6 (d, $-\underline{C}H{<}^{CO_2^-}_{\underset{+}{N}Me_3}$), 121.9 ($d$, imidazole C-4), 136.6 (s, imidazole C-5), 141.7 (s, imidazole C-2), 173.4 (s, $-CO_2^-$), 174.7 (s, $-CO_2^-$).

Raney-Ni *treatment of clithioneine.* To a soln of clithioneine (11 mg) in 50% aq. EtOH ((0.6 ml) was added excess Raney-Ni and the mixture was refluxed for 2 hr. The reaction mixture was filtered through celite, and the filtrate was evaporated. The residue was applied to a column of charcoal (0.5 g) to separate the absorbed (10% aq. EtOH, 30 ml) and non-absorbed (H_2O eluate, 30 ml) fraction. The non-absorbed fraction was applied to a TLC plate of cellulose and developed by solvent B to afford two ninhydrin-positive fractions (R_f 0.53 and 0.20). The fraction of R_f 0.53 was purified by reversed-phase HPLC (1.3 x 30 cm, 80% aq. EtOH) to yield pure crystals of L-threonine (2.5 mg, 66%), which was identical with an authentic sample (NMR, CIMS, CD, TLC, PEP mobilities). L-α-Amino-*n*-butyric acid was isolated (0.5 mg, 15%) from the fraction of R_f 0.20 by similar purification process, and again it was identical with an authentic specimen. The absorbed fraction was purified in a similar way (cellulose TLC by solvent A, collection of bands R_f 0.63, 0.38 followed by reversed-phase HPLC by 80% aq. EtOH, and collection of Pauly-test positive fractions) to give L-hercynine (1.2 mg, 19%) and clithioneine (1 mg, 9%), which were identical with authentic samples (NMR, CIMS, CD, TLC, PEP mobilities).

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