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A CHLORO AMINO ACID FROM *AMANITA SOLITARIA*

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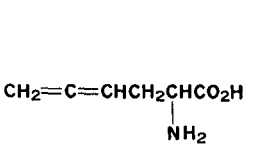
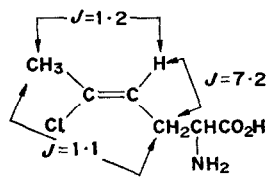
Key Word Index—*Amanita solitaria*; Basidiomycetes; *trans*-2-amino-5-chloro-4-hexenoic acid.

Abstract—The mushroom *Amanita solitaria* contains in excess of 1000 ppm 2(*S*)-amino-4,5-hexadienoic acid (I), 300 ppm *trans*-2-amino-5-chloro-4-hexenoic acid (II), and a chloride ion concentration (2000 ppm) significantly greater than that found in other basidiomycetes. I can be converted into II in hydrochloric acid, but II is not an artifact of isolation.

THE GENUS *Amanita* contains some of the most toxic mushrooms and has consequently been the subject of many chemical investigations.[1] In the course of a survey of the physiologically active compounds of *Amanita*, Benedict *et al.*[2] pointed out the presence of several unidentified amino acids in *A. solitaria* (Fr.) Secr. sensu D. E. Stuntz, a large mushroom belonging to a group (*A. solitaria*, *A. radicata*, *A. chlorinosma*) frequently described as having a pungent, unmistakable odor like chloride of lime or chlorine.[3] The major (*ca.* 0·1 %) new amino acid was identified as the allene 2(S)-amino-4,5-hexadienoic acid I.[4]



(I)



(II)

A second, minor constituent has now been isolated and identified as *trans*-2-amino-5-chloro-4-hexenoic acid (II). Identification of the chloro amino acid in a mixture containing leucine and isoleucine follows from the distinctive reaction of the amino acid with ninhydrin, from the NMR and MS of its mixture, and from its transformation chemistry. The low concentration of the chloro amino acid (300 ppm in fresh mushrooms) and the difficulty of separating it from the leucines required the initial identification in a mixture. Its identity is assured by the large amount of overlapping structural information, by its synthesis and by the identity of transformation products derived from the synthetic and natural amino acid. The brown ninhydrin reaction is characteristic of $\beta\gamma$- and $\gamma\delta$-unsaturated amino acids. Its chromatographic behavior in the presence of Cu^{2+}, and its mass spectral fragmentation pattern show that it is an α-amino acid. Absence of any amino acids other than leucine, isoleucine and norleucine after reduction indicates a six carbon skeleton. The MS of the amino acid mixture, its *N*-acetylation product, and its *N*-benzoylation product show the

[1] C. H. EUGSTER, *Progr. Chem. Nat. Prod.* **27**, 261 (1969); T. WIELAND, *Science*, **159**, 946 (1968).
[2] R. G. BENEDICT, V. E. TYLER and L. R. BRADY, *Lloydia* **29**, 333 (1966).
[3] C. F. AUSTIN, *Bull. Torrey Botan. Club*, **6**, 278 (1878); C. H. PECK, *ibid.* **27**, 609 (1900); W. A. MURRILL, *Mycologia* **5**, 78 (1913); W. C. COKER, *J. Elisha Mitchell Sci. Soc.* **33**, 1 (1917); A. H. SMITH, *Mushrooms in Their Natural Habitat*, p. 401, Sawyer's, Portland, Oregon (1949).
[4] W. S. CHILTON, G. TSOU, L. KIRK and R. G. BENEDICT, *Tetrahedron Letters* 6283 (1968).

characteristic $^{35}Cl/^{37}Cl$ doublets (Table 1). While covalent chlorine might have been introduced in benzoylation with benzoyl chloride, no chlorine could have been introduced in isolation of the free amino acid nor in *N*-acetylation with acetic anhydride. Consideration of chlorine-containing and chlorine-free mass spectral fragments precludes an α-chloro structure. The NMR spectrum of the chloro amino acid contains, in addition to signals ascribable to leucine and isoleucine, a *C*-methyl deshielded by a double bond and an electronegative group. The methyl signal is not split by vicinal protons but shows allylic coupling ($^4J = 1{\cdot}2$ Hz) with a vinyl hydrogen and homoallylic coupling ($^5J = 1{\cdot}1$ Hz) with methylene hydrogens. These relationships are confirmed by spin decoupling experiments. Assignment of α-methine and β-methylene signals follows from spin decoupling experiments and relative deshielding on shifting the pH of aqueous solutions from 11 to 1. Although the β-hydrogens are diastereotopic, their coupling constants with other hydrogens are indistinguishable. Accidental similarity of other coupling constants leads to an overly simplified spectrum at 100 mHz and pH 2: three ϵ-hydrogen quartet, 2·50 ppm; two β-hydrogen triplet of quartets, 3·17 ppm; α-hydrogen triplet, 4·48 ppm; γ-hydrogen triplet of quartets, 5·92 ppm.

TABLE 1. MS OF CHLORO AMINO ACID AND DERIVATIVES

Ion*	Compound II		*N*-Acetyl-II		*N*-Benzoyl-II	
	m/e	%	*m/e*	%	*m/e*	%
M	165	2	207	0·2	269	10
	163	6	205	1·0	267	30
M–OH	148	1				
	146	4				
M–Cl	128	14	170	6	232	78
$M-CO_2$					225	11
					223	34
$M-CO_2H$	120	6	162†	2	224†	8
	118	19	160†	3	222†	15
					179	41
$M-CH_2CH{=}CClCH_3$					178	41
M–acylamide			148	2	148	9
			146	7	146	24
$M-CO_2H$, Cl			125	8		
			124	10	134	29
$M-CO_2H$, ketene			120	4		
			118	16		
M–acylamide, Cl			111	50		
			99	29		
			88	29		
	82	17	82	43		
$(C_6H_5COHNH_2)^+$					122	100
$(H_2N{=}CHCO_2H)^+$	74	100				

* Ions identified by exact MS determination.
† Not analyzed at high resolution.

The chloro amino acid was synthesized by the acetamidomalonate alkylation route using commercial 1,3-dichloro-2-butene. The most convenient route to dichlorobutene leads to an equilibrium mixture in which the *trans* carbon skeleton predominates.[5] The

[5] L. F. HATCH and R. H. PERRY, *J. Am. Chem. Soc.* **77**, 1136 (1955).

assignment of *trans* isomer based on chemical evidence is confirmed by comparison of the NMR spectra of the major (90%) and minor isomer after separation by GLC. The major isomer has the larger homoallylic coupling constant expected for a *trans* relationship of homoallylic hydrogens.[6] The distinctive NMR spectrum of the β, γ and ϵ hydrogens of the natural chloro amino acid was indistinguishable from that of synthetic *trans*-2-amino-5-chloro-4-hexenoic acid.

The natural allenic amino acid can be converted into the chloro amino acid in hot 1 N HCl. This product was analyzed on an ion exchange analyzer adjusted to maximize the resolution of chloro amino acid and added norleucine. The synthetic chloro amino acid peak is barley resolved into two components (ratio 1:9), presumably *cis* and *trans* isomers. The minor isomer is not detectable in the NMR spectrum. The facility of addition of HCl to the allenic amino acid suggests that the chloro amino acid could be an artifact. However, the chloro compound is probably a true metabolite since the natural amino acid contains no *cis* isomer and since no addition to the allenic amino acid was observed in 0·1 N HCl plus 0·1 N NaCl over a period of 2 weeks at room temp. nor on heating I at reflux for 30 min in 1% HCl. Further, chromatograms prepared minutes after extraction of fresh mushrooms in the absence of added acid or chloride show the presence of the chloro amino acid.

TABLE 2. CHLORIDE CONTENT OF MUSHROOMS

Mushroom	% Cl^- range (dry wt)	ppm Cl^- (fr. wt)
Helvella lacunosa	0·03–0·06	
**Usnea* sp.	0·04–0·07	
Sparassis sp.	0·06–0·07	
**Cladonia* sp.	0·05	
Cantharellus cibarius	0·07–0·09	80
Armillaria mellea	0·08–0·12	
Lepista nuda	0·09	100
Lactarius spp.	0·09–0·14	100
Russula cerolens	0·10	
Nematoloma fasciculare	0·11–0·15	
Clitocybe spp.	0·17–0·24	
Pleurotus ostreatus	0·22–0·24	
Coprinus comatus	—	150
Amanita muscaria	0·34–0·60	
Entoloma sericeum	0·50–0·60	350
Boletus chrysenteron	0·56	940
Amanita pantherina	0·68–0·88	
A. solitaria (1966)	2·18	
A. solitaria (1971)	2·10–3·1	2000

* Lichens.

The occurrence of covalent chlorine in metabolites is infrequent. About one hundred examples are known.[7,8] The structures of the overwhelming majority suggest the intermediacy of a positive chlorine substitution route (SE_2). An electrophilic chlorination

[6] C. M. CIMARUSTI and J. WOLINSKI, *J. Org. Chem.* **31**, 4118 (1966).
[7] L. FOWDEN, *Proc. Roy. Soc. Lond.* **171B**, 5 (1968).
[8] W. B. TURNER, *Fungal Metabolites*, Academic Press, New York (1971).

mechanism has been demonstrated for the chloroperoxidase obtained from *Caldariomyces fumago*.[9] A route to II consistent with positive chlorination in *Amanita solitaria* would be electrophilic substitution of 4,5-dehydronorleucine (or, for example, its 2-keto equivalent) to give II followed by dehydrochlorination to give I. Thus in the positive chlorine route the sequence of biosynthesis would be II → I while, if the negative chlorine route (HCl addition) were followed, the sequence of biosynthesis would be I → II. A search for norleucine and dehydronorleucine by chromatography, amino acid analyzer and GLC failed to detect these compounds at the 100 ppm level in *A. solitaria*.

No direct evidence exists for the presence of free chlorine or hypochlorite in *A. solitaria*. However the fresh mushroom contains *ca.* 2000 ppm chloride, an abnormally high concentration (Table 2). Four per cent of the assayed chloride in *A. solitaria* is covalent chlorine of the amino acid. The concentration of chloride in terrestrial plants is generally in the range 50–500 ppm based on fr. wt.[10] A few plants have been reported to concentrate chloride in the 1000 ppm region. These cases involve irrigated crops in arid regions, plants adapted to haline soil, or other special situations. For example, tamarisk, which has salt extruding glands,[11] contains 3700 ppm chloride,[10] and the acidic digestive traps of the insectivorous plant *Nepenthe* contain about 1000 ppm HCl.[12] A survey of sixteen mushrooms and two lichens shows levels of chloride comparable to levels in higher plants except for *A. solitaria* and *Boletus chrysenteron*. The latter mushroom is reported to produce a chlorinated phenolic tetronic acid.[13] Biological formation of the carbon–chlorine bond is almost entirely confined to lichens, fungi and molds. Insufficient data exist to attempt to correlate presence of chloro metabolites with chloride concentration in these organisms. The *Amanita* species examined appear to concentrate chloride more than most mushrooms, but there is no indication that *A. pantherina* or *A. muscaria* contain chloro metabolites.

TABLE 3. CHROMATOGRAPHIC DATA OF NEW AMINO ACID

Amino acid	R_{leu} in BMAW	APW	ABW	AW
2-Amino-5-chloro-4-hexenoic acid	1·14	1·07	1·20	1·26
Norleucine	1·10	1·03	1·15	1·20
Leucine	1·00	1·00	1·00	1·00
Isoleucine	0·91	0·96	0·95	0·89

Key. BMAW, butanol–2-butanone–ammonia–water, 5:3:1:1; APW, isoamyl alcohol–pyridine–water, 7:7:6; ABW, *t*-amylalcohol–benzyl alcohol–1:1 saturated with water; AW, *t*-amyl alcohol saturated with water.

EXPERIMENTAL

Enrichment of chloro amino acid II. The amino acid was enriched by extraction of *A. solitaria* with 70% EtOH followed by elution of the amino acid complex from cellulose with *n*-BuOH–HOAc–H_2O (4:1:5). Fractions containing the amino acid and leucines were rechromatographed preparatively on paper (Whatman 3MM) eluted with the leucine-resolving system *t*-amyl alcohol saturated with H_2O (AW). The band containing the chloro amino acid was eluted and the recovered mixture was resubjected to preparative PC and column chromatography with AW eluent. Five cycles of rechromatography failed to remove all of the leucines. The best preparation contained about 60% chloro amino acid as estimated by NMR integration

[9] F. S. BROWN and L. P. HAGER, *J. Am. Chem. Soc.* **89,** 720 (1967).
[10] M. R. BLOCH, D. KAPLAN and J. SCHNERB, *Israel Res. Council Bull.* **8A,** 155 (1959).
[11] W. L. BERRY, *Am. J. Bot.* **57,** 1226 (1970).
[12] S. MORRISSEY, *Nature, Lond.* **176,** 1220 (1955).
[13] W. STEGLICH, W. FURTNER and A. PROX, *Z. Naturforsch.* **23B,** 1044 (1968).

of methyl groups, quantitative microhydrogenation, and Beckman Spinco Model 120 amino acid analyzer (pH 4·4, citrate buffer, Spinco sulfonated polystyrene resin).[14]

Characterization of II. Chloro amino acid II NMR (D_2O, *p*D 2): τ 4·08 (*t* of *q*, 1, J_{34} = 7·4 Hz, J_{46} = 1·2, –CCl=CH–, 5·52 (*t*, 1, J_{23} = 6·0, $-CHCO_2H$), 6·83 (*t* of *q*, 2, J_{36} = 1·1, $-CCH_2-$), 7·50 (~*q*, 3, $CH_3C=C-$). IR in KBr has bands of ammonium carboxylate zwitterion (3300, 2400, 2100 and 1600 cm^{-1}). MS Table 1. GLC retention times of trimethylsilyl derivatives: isoleucine and II 8·5 min, leucine 10 min, norleucine 11 min (20% SE-52 on acid-washed Chromosorb W at 70°). Elution order from amino acid analyzer: methionine, isoleucine, leucine, norleucine, *cis*-II, *trans*-II. R_{leu} in four solvents are given in Table 3.

2-Acetamido-5-chloro-4-hexenoic acid. Naturally-occurring II was acetylated with Ac_2O. HOAc was removed by repeated evaporation from H_2O. The residue was dissolved in acetone and chromatographed on preparative plates of silica gel in EtOAc–HOAc (9:1). The acetylated olefinic amino acid was located with $KMnO_4$ spray and eluted from the TLC plate. MS: Calcd. for $C_8H_{12}NO_3{}^{35}Cl$, 205·050; Found: 205·050. For the remainder of MS see Table 1.

Hydrogenation of enriched chloro amino acid. A solution of 12·6 mg enriched chloro amino acid in 3 ml aq. HOAc (50%, v/v) and 4 mg PtO_2 was hydrogenated at atmospheric pressure. H uptake of 2·28 ml corresponds to 55% chloro amino acid (2 mol H_2/mol). The reduced mixture contains norleucine (R_{leu} 1·22, AW), leucine and isoleucine (R_{leu} 0·91, AW). The presence of norleucine as the major amino acid after reduction is confirmed by automated amino acid analysis.

Separation of cis- *and* trans-1,3-*dichloro-2-butene.* Commercial dichlorobutene (Aldrich Chemical Company) was resolved by GLC at 70° on 60–80 mesh Chromosorb W coated with di-*n*-decylphthalate. *Trans* isomer retention time 10 min, *cis* 13 min. NMR *trans* isomer: 4·32 (*q* of *t*, 1, J_{12} = 7·6 Hz, J_{24} = 1·3, C=CH–), 5·86 (*q* of *d*, 2, J_{14} = 0·9, $-CH_2Cl$), 7·88 (*t* of d, 3, CH_3). *Cis* isomer: 4·11 (*q* of *t*, 1, J_{12} = 8·3, J_{24} = 1·2, C=CH–), 5·92 (*d* of *d*, 2, J_{14} = 0·4, $-CH_2Cl$), 7·99 (broad, 3, CH_3).

Synthetic trans-2-*amino-5-chloro-4-hexenoic acid.* Synthetic II was prepared by alkylation of ethyl acetamidomalonate according to Gershon and Scala[15] to give *trans*-2-acetamido-5-chloro-4-hexenoic acid, m.p. 117–118°. MS molecular ion: 205·0504. Calculated for $C_8H_{12}NO_3Cl$: 206·0506. The *N*-acetyl amino acid was deacetylated by heating at reflux in H_2O rather than in acid or base, to reduce complicating side reactions. The resultant synthetic *trans*-2-amino-chloro-4-hexenoic acid had R_{leu} 1·23 (AW, brown ninhydrin reaction) MS Table 1. *Anal.* Calcd. for $C_6H_{10}NO_2Cl$: C, 44·05; H, 6·16; N, 8·56. Found: C, 43·91; H, 6·11; N, 8·49.

Chloride assay. Mushroom samples having a dry wt. of 2–8 g were digested in 20 ml conc. HNO_3 and 10–20 ml 0·1 N $AgNO_3$ for 3 hr at 100° by which time the mushroom was completely disintegrated. A second portion of 20 ml conc HNO_3 was added and heating was continued for 3 more hr. The AgCl and 2–3% unoxidized lipids were removed. Excess silver ion was titrated with 0·1 N NH_4SCN to the clear point. Assay of controls gave: ionic chloride 100% recovery, *p*-nitrobenzylchloride 90%, 2-amino-5-chloro-4-hexenoic acid 88%, *o*-chlorobenzoic acid 0%, chloranil 0%. Assays on fresh samples were obtained by weighing and then drying with gentle heat before digesting. Sample sizes were selected to give the equivalent of 2–8 g dry wt. This procedure was necessary to minimize dilution of the oxidant and to reduce frothing.

Assay of covalent chlorine in A. solitaria *amino acid fraction.* The amino acids and ionic chlorine from 400 g of fresh *A. solitaria* were fractionated by absorption on a sulfonated polystyrene resin, H^+ form. New resin had been freshly regenerated with NaOH followed by H_2SO_4 avoiding any source of chloride ion. The column was washed with 5 column vol. of distilled water. The water eluate was made slightly basic with Na_2CO_3 and evaporated to dryness. The residue (ninhydrin negative) was used to assay ionic chloride. The column was then eluted with 1 N aq. NH_3. Five column vol. of ammoniacal eluate were reduced to about 3 g semi-crystalline amino acid mixture. Further elution of the column with aqueous ammonia did not elute any more material. An aliquot of the amino acid mixture was dissolved in H_2O and tested for ionic chloride with HNO_3 and $AgNO_3$. A clear solution with no AgCl precipitate was obtained. A $\frac{1}{8}$ aliquot subjected to the HNO_3-$AgNO_3$ oxidative procedure gave an appreciable AgCl precipitate and an assay for 3·5 mg chloride after oxidation, corresponding to 70 mg/kg covalent chlorine or 320 ppm chloro amino acid in the original sample. In a control experiment 56·2 mg synthetic II (=12 mg Cl) dissolved in 10 ml 0·1 N NaCl (=36 mg Cl) was subjected to the same separation and assay. Found: 11 mg covalent Cl.

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[14] Performed by Mr. R. GRANBERG, AAA Laboratory, Seattle.

[15] H. GERSHON and A. SCALA, *J. Org. Chem.* **26**, 2347 (1961).