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A TOXIC AMINO ACID, 2(*S*)3(*R*)-2-AMINO-3-HYDROXYPENT-4-YNOIC ACID FROM THE FUNGUS *SCLEROTIUM ROLFSII*

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Abstract—An amino acid, lethal to New Hampshire chickens (LD_{50}, 150 mg/kg) was isolated from dried sclerotia of the fungus *Sclerotium rolfsii* (Sacc.). Purification of the rather unstable compound was effected on a cation exchange column by means of displacement chromatography and the amino acid was crystallised from 80% methanol. A structure was assigned to the compound on the basis of available chemical and physical data, namely 2(*S*),3(*R*)-2-amino-3-hydroxypent-4-ynoic acid. Confirmation of this structure was gained by direct and indirect synthetic procedures.

INTRODUCTION

Sclerotium rolfsii is parasitic on a wide variety of plants [1–4], in warm climatic regions throughout the world [5]. Several aspects, closely related to its parasitic behaviour, have already been studied [6–8]. In spite of active interest in this fungus, only two reports have mentioned its toxic properties [9, 10]. Cultures of *S. rolfsii*, grown on sterilised yellow maize, were toxic to poultry and domestic mammals. Sub-lethal doses, ingested over a period of one week caused severe depression in growth rate and development of day-old New Hampshire chickens and Pekin ducklings. Acute symptoms were anorexia, muscular weakness, incoordination of movements, 'curl-toe paralysis', total paralysis of the legs followed by a coma and eventual death. No drastic localised histopathological lesions were observed [9]. Growth depression seems to be a typical syndrome caused by several vitamin antagonists and toxic amino acid analogues [11–14].

Recently Niimura *et al.* (1974) isolated two diastereomers, L-*threo* and L-*erythro*-2-amino-3-hydroxyhex-4-ynoic acid from the fruitbodies of the fungus *Tricholomopsis rutilans* (Fr.) Sing [15]. They did not comment on any possible physiological activity of these compounds. Acetylenic amino acids have also been isolated from seeds of the plant *Euphoria longan* and the authors reported a slight hypoglycaemia effect, caused by 2-amino-4-methylhex-5-ynoic acid in mice [16]. Another acetylenic amino acid, exhibiting antibiotic activity and closely related to the compounds isolated by Niimura *et al.* [15], was purified from culture filtrates of an unidentified *Streptomyces* species [17].

This report concerns the isolation and identification of a physiologically active acetylenic amino acid, 2(*S*) 3(*R*)-2-amino-3-hydroxypent-4-ynoic acid from the dried sclerotia of the plant parasite *S. rolfsii*.

RESULTS AND DISCUSSION

Experimental evidence indicated that the sclerotia of *S. rolfsii* contained the highest concentration of the physiologically active substance or substances. A typical isolation procedure is summarised in Table 1.

Table 1. Isolation of a toxic amino acid from *S. rolfsii*

Step No	Material	Procedure	Product	LD (mg/kg)	Enrichment factor
1	Fungus cultures	Dry in air on mosquito gauze	Sclerotia	36×10^3	—
2	Intact sclerotia	Water extraction (10°); filter and freeze-dry.	Residue 1	6.5×10^3	5.5
3	Residue 1	Decolourise with active carbon; filter and freeze-dry	Residue 2	3.6×10^3	1.8
4	Residue 2	Acidify aq. soln; adsorb on strong cation column; elute with with 0.15 M ammonia	Crystalline toxin	150	24.0
				Total enrichment	×238

Maximum production of sclerotia was achieved after a cultivation period of 20–30 days at 25° and coincided with maximum toxin production. Intact sclerotia were used, since it had been established that loss of toxicity occurred because of modification or breakdown by heat labile protein factors, liberated during water extraction of finely powdered, dried sclerotia.

The toxin was eluted from a strong cation exchange column just in front of L-aspartic acid. It gave a bright yellow to brown colour with ninhydrin on TLC (R_f 0.54 in *n*-BuOH–Py–H_2O (6:4:3) and 0.39 in *n*-BuOH–HIAc–H_2O (5:1:4)). The colour formation was completely inhibited by masking with Cu^{2+} ions and the compound was considered to be an α-amino acid [18]. The toxic compound proved to be quite unstable in neutral and alkaline solutions but was more stable in an acidic environment. Aqueous solutions of the toxin turned yellow to brown within minutes and diluted pyridine and ammonia enchanced this process. Decomposition was most likely caused by the combined action of light, O_2 and the catalytic action of bases in polar solvents (H_2O, DMF, DMSO).

Ammoniacal cuprous chloride precipitated the toxin as a cuprous acetylide derivative from aqueous solutions, strongly supporting the suggestion that a highly reactive acetylenic proton was present [19].

The crystalline toxin charred and decomposed above 209° and micro analysis suggested an empirical formula of $C_5H_7NO_3$. MS (electron impact and chemical ionisation) confirmed this composition and respectively gave a very low abundance M^+ of *m/e* 129 and a stable $M + 1$ peak of *m/e* 130.

A gradual shift in optical rotation from $-45.6° \pm 3°$ (*c*, 2.3 in H_2O) to an equilibrium value of $-76.9° \pm 3°$ took place within 24 hr. Oxidation in the aqueous environment might be responsible for this effect.

The UV spectrum for the pure, crystalline compound did not indicate any conjugated unsaturation. The IR spectrum was typical of α-amino acids and strong, sharp absorption bands at 3264 and 2134 cm^{-1}, respectively, suggested the unsymmetrical —C≡CH and symmetrical —C≡C— stretch of a terminal acetylenic group. The latter fact was finally confirmed by PMR in D_2O. The spectrum showed 3 types of protons: τ6.89 (*d*, J = 2.2 Hz, H—C≡C—), 6.08 (*d*, J = 3.5 Hz, —CH(ND_3^+) COO^-) and 4.98 (*dd*, J = 3.5 and 2.2 Hz, —CH(OD)—). The doublet due to the acetylenic proton and doublet of doublets assigned to the proton neighbouring the acetylenic group could be observed only immediately after the compound had been dissolved in D_2O. A slow, spontaneous, time dependent exchange of the acidic, acetylenic proton caused the doublet at 6.98τ to disappear completely while the doublet of doublets collapsed to a doublet (τ4.98, J = 3.5 Hz). PMR indicated that only the *threo*-form(s), namely the 2(*S*) 3(*R*)- and/or 2(*R*) 3(*S*)-diastereomer(s) of the toxic amino acid were present [15].

Catalytic reduction of the toxin afforded a saturated compound which proved to be identical to D,L-*threo*-β-hydroxynorvaline: a mixture of the 2(*R*)3(*S*)- and 2(*S*)3(*R*)-diastereomers of β-hydroxynorvaline. A synthetic preparation of β-hydroxynorvaline (a mixture of the 4 possible diasteriomers) confirmed the structure of the reduced toxin, when compared by means of IR and PMR spectra [20–23]. MS supported the chemical composition of $C_5H_{11}NO_3$ by giving a low abundance M^+ ion at *m/e* 133 electron impact and a $M + 1$ peak at *m/e* 134 (chemical ionisation). This data indirectly suggested the proposed structure of the toxic amino acid, namely 2(*S*)3(*R*)- or 2(*R*)3(*S*)-2-amino-3-hydroxypent-4-ynoic acid. Final confirmation of the structure was gained by synthesis, using a slightly modified version of the synthetic procedures used by Mix [23] and Niimura [15].

The absolute configuration of groups around the two asymmetric carbon atoms was determined, using D- and L-amino acid oxidases (D-AOD and L-AOD respectively) [23–26]. Oxidation of the toxic amino acid was monitored manometrically in a differential respirometer at 38°. O_2 uptake during oxidative deamination of *threo*-2-amino-3-hydroxipent-4-ynoic acid by L-AOD (9 μmol O_2/39 μmol amino acid/10 hr) was much lower, compared to the oxidation of either L- or D-isoleucine by L-AOD or D-AOD respectively (35.3 or 31.2 μmol O_2/39 μmol amino acid/10 hr), but still much higher than when the toxic amino acid was oxidised by D-AOD (1.8 μmol O_2/39 μmol amino acid/10 hr). Subsequent Moore–Stein analyses of reaction solutions after the 10 hr period of oxidation confirmed that *threo*-2-amino-3-hydroxypent-4-ynoic acid acted as a substrate for L-AOD and should therefore belong to the L-series.

After careful consideration of all the available information, it was finally concluded that the toxic metabolite isolated from the sclerotia of *S. rolfsii* was 2(*S*)3(*R*)-2-amino-3-hydroxypent-4-ynoic acid. X-ray crystallography, very recently completed by another group, strongly supported this conclusion [27].

EXPERIMENTAL

General. Mp's are uncorr. IR spectroscopy is in nujol, and PMR were run in D_2O using 2,2,3,3-tetradeutero-3-(TMSi)-propionate (Na salt) as internal standard. Evaporation of solvents was carried out *in vacuo* at 40°.

Fungus cultures were prepared in 250 ml conical flasks on yellow maize kernels (50 g kernels/50 ml H_2O) at 25–27° under artificial light [28] for 21–30 days. Afterwards the cultures were air dried (25–27°) on mosquito gauze and the dried sclerotia collected (yield, *ca* 5 g per flask). Toxicity was monitored by *ad libitum* administration of a 10% (w/w) infected, balanced ration (finely powdered sclerotia) to 3-day-old New Hampshire chickens (LD_{50}, 36 g/kg).

Isolation of toxic metabolite. Dried sclerotia (500 g) were soaked in H_2O (3 l). for 24 hr at 10° with occasional agitation. The suspension was filtered and washed (3 × 200 ml H_2O). The dark brown, clear filtrate was freeze-dried to yield a brown, hygroscopic residue (Residue 1, 85 g). Toxicity yield was determined as follows: an aq. soln of the toxic residue was administered *per os* into the crop of 3-day-old New Hampshire chickens by means of a feeding tube (LD_{50}, 6.5 g/kg). Residue 1 (85 g) was decolourised with activated charcoal (100 g suspended in H_2O) for 1 hr at 10°. The charcoal was removed by suction and the clear filtrate lyophilised to yield 46 g of a pure white residue (Residue 2; LD_{50}, 3.6 g/kg). This material tended to turn yellow, even when stored at low temp. in an evacuated desiccator, with a resultant loss in toxicity. Residue 2 (46 g) was dissolved in H_2O (500 ml), the pH of the soln adjusted to 2 with 0.1 M HCl and the acidic soln passed through a Dowex 50 X-8 (H^+) column at a flowrate of 30 ml/cm^2/hr. The column was eluted with 0.1 M HOAc (2 l.) to remove all non-cationic contaminants. Displacement chromatography was initiated by elution with 0.15 M ammonia, collecting fractions of 25 ml. The toxic amino acid was the very first ninhydrin positive fraction (yellow spot) to be eluted. Some overlapping with L-aspartic acid occured. Pure fractions were combined and concd *in vacuo*. Crystallisation was difficult, because of the continuous browning of toxin solns, in spite of repeated decolourisations with activated charcoal.

Recrystallisation from 80% MeOH eventually yielded 250 mg colourless crystals, barely 20% of what was expected from toxicity yield calculations (LD_{50}, 150 mg/kg). (Found: C, 46.49; H, 5.48; N, 10.82. $C_5H_7NO_3$ requires: C, 46.51; H, 5.46; N, 10.85). For PMR and MS see Results and Discussion.

Hydrogenation of the acetylenic amino acid. Pure, crystalline toxin (50 mg) was dissolved in 80% MeOH (100 ml) and shaken in the presence of 10% Pd/charcoal in a H_2 atm. (1.6 kg/cm^2) for 4 hr (Parr catalytic hydrogenator). The reaction mixture was filtered, the MeOH removed *in vacuo* and the aq. soln concd until crystallisation began. The soln was left in the cold until crystallisation was completed and the fine, white needles were filtered and dried. Recrystallisation from 80% MeOH yielded 40 mg (78%). Mp 218–219°. PMR τ8.96 (*t*, Me), 8.33 (*m*, —CH_2—), 6.29 (*d*, J = 4.2 Hz, —CH(OD)—), 5.97 (*m*, —CH(ND_3^+)COO^-). (Found: C, 45.18; H, 8.39; N, 10.55. $C_5H_{11}NO_3$ requires C, 45.10; H, 8.33; N, 10.52). For MS, see Results and Discussion.

Synthesis of 2-amino-3-hydroxypent-4-ynoic acid as diastereomeric mixture of racemates. Cu glycinate [29] was partly dissolved, partly suspended in M KOH (50 ml). Propiolaldehyde [30] (2 ml) was slowly added and the recation mixture was agitated at 0° under N_2 for 8 hr. HOAc (17 M, 10 ml) was added until the reaction mixture was slightly acidic to litmus paper. One vol. of H_2O was added, followed by finely powdered thioacetamide (0.6 g). The reaction mixture was stirred at room temp. for 1 hr. filtered through Whatman No 3 and the filtrate acidified to pH 2 with 0.1 M HCl. The acidic filtrate was passed through a Dowex 50X-8(H^+) column (15 cm × 1.5 cm), the column washed with 0.1 M HOAc (200 ml) and then eluted with 0.15 M ammonia; fractions of 5 ml were collected. Fractions containing the pure, glycine free, diastereomeric mixture were combined and concd *in vacuo*. The yellow to brown coloured concentrate was decolourised with charcoal and then lyophilised. The slightly yellow coloured, hygroscopic residue was dissolved in 0.4 ml H_2O, 1.6 ml MeOH added and the soln left in the cold to crystallise. A very low yield was obtained (110 mg, 11.9%). Mp >193° (decomp.) (Found: C, 46.42; H, 5.5; N, 10.89. $C_5H_7NO_3$ requires: C, 46.51; H, 5.46; N, 10.85) PMR: τ6.89 (*d*, J = 2.2 Hz, H—C≡C—, slow exchange with deuterium) and 6.94 (*d*, J = 2.2 Hz, H—C≡C—, slow exchange with deuterium), 6.08 (*d*, J = 3.5 Hz, —CH(ND_3^+)COO^-) and 6 (*d*, J = 4 Hz, —CH(ND_3^+)COO^-), 4.98 (*dd*, J = 3.5 and 2.2 Hz, —CH(OD)—) and 4.95 (*dd*, J = 4 and 2.2 Hz, —CH(OD)—). MS: M^+, 129 (EI); $M^+ + 1$, 130 (CI).

β-Hydroxynorvaline was synthesised by the same method as 2-amino-3-hydroxypent-4-ynoic acid using Cu glycinate (5 g) and propionaldehyde (15 ml). Column fractions containing the glycine free, diastereomeric mixture, D,L-*erythro*- and D,L-*threo*-β-hydroxynorvaline were combined and concd *in vacuo*. The mixture crystallised directly from the conc soln, was filtered, dried and recrystallised from 70% EtOH. (Yield 2.2 g; 70%). No attempt was made to separate the diastereomers, but they were identified by Moore–Stein analysis and TLC [21–23] Mp 217–219° PMR τ8.96 (*t*, Me), 8.33 (*m*, —CH_2—). 6.29 (*d*, J = 4.2 Hz, *threo*-CH(OD)—), 6.12 (*d*, J = 3.8 Hz, *erythro*-CH(OD)—), 5.97 (*m*, —CH(ND_3^+)COO^-). (Found: C, 45.16; H, 8.42; N, 10.58. $C_5H_{11}NO_3$ requires C, 45.10; H, 8.33; N, 10.52). MS: M^+, 133 (EI); $M^+ + 1$, 134 (CI).

Absolute configuration of 2-amino-3-hydroxypent-4-ynoic acid. Enzymic oxidations were carried out in a Gilson differential respirometer at 38°, using air as the gas phase [23–26]. (i) D-*amino acid oxidase* (D-*AOD*) *reaction.* The main compartment of the Gilson flasks contained: 39 μmol of the amino acid, Tris buffer (2 ml, 0.1 M at pH 9.2), catalase 32.5 U, 4×10^{-3} μmol FAD and H_2O. D-AOD (30 U/300 μl 2M $(NH_4)_2SO_4$) was placed in the side arm. The final vol. in each flask was 2.5 ml. The center well contained 200 μl 20% KOH. (ii) L-*Amino acid oxidase* (L-*AOD*) *reaction.* The main compartment of the flasks contained: 39 μmol of the amino acid, 2 ml Tris buffer (0.1 M, pH 7.7), 10^{-4} mol KCl, 4×10^{-3} μmol FAD, catalase 32.5 U, and H_2O. The side arm contained 3 U L-AOD in 600 μl 2 M $(NH_4)_2SO_4$. The total vol. per flask was 2.5 ml. Control expts were also run, leaving out the amino acid substrates while another set of controls checked the activity of the L-AOD and D-AOD with L- and D-isoleucine as standard substrates. The flasks were tipped after a 10 min equilibration period and readings were taken every 10 min for 10 hr or until O_2 uptake ceased.

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