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SHORT COMMUNICATION

α-AMINO-β-METHYLAMINOPROPIONIC ACID, A NEW AMINO ACID FROM SEEDS OF *CYCAS CIRCINALIS*

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Abstract—A new basic amino acid α-amino-β-methylaminopropionic acid was isolated from seeds of *Cycas circinalis* and its structure confirmed by synthesis. Preliminary experiments suggest that this compound is neurotoxic to higher animals.

INTRODUCTION

THOSE species of Cycadaceae which exist at the present time are "the surviving remnants of an ancient line of plants whose relatives were common in the early part of the Mesozoic Era and may have been ancestral to the flowering plants."[1] These palm-like plants are found in the tropics and sub-tropics. After suitable preparation, the roots, stems and seeds are used as human food, while the feathery leaves are eaten by domestic animals. Ingestion of the seeds or leaves of species from six of the nine genera of this family have been implicated in the development of toxic symptoms either in man or in higher animals.

From seeds and roots of several species have been isolated glycosides of methylazoxymethanol[2–3] which show hepatotoxic and carcinogenic properties when fed orally to experimental animals. The toxic agent apparently being the aglycone which is liberated from the parent glycoside by the action of enzymes synthesized by bacteria of the animal gut.

In addition to the carcinogenic and hepatotoxic effects, however, the ingestion of leaves of species of four of the genera have been reported to produce a neurological disorder in cattle involving the irreversible paralysis of the hindquarters[4].

The descriptions of this paralysis are remarkably similar to those of "classical lathyrism" in man and higher animals, a disease produced by eating seeds of *Lathyrus sativus*, *L. cicera* or *L. clymenum*[5] which contain the neurotoxin α-amino-β-oxalylaminopropionic acid.[6–8] The fact that carcinogenic and neurological effects do not necessarily occur simultaneously after the ingestion of Cycad material suggested that a toxic compound other than a methylazoxymethanol derivative might be responsible for the neurological symptoms. It therefore seemed worth while to look for α-amino-β-oxalylaminopropionic acid, αγ-diaminobutyric (another known plant neurotoxin[9]) or some similar compound in the neurotoxic species of this family.

[1] F. R. FOSBERG, *Proc. Third Conf. Toxicity Cycads, Fedn. Proc.* **23**, 1340 (1964).
[2] K. NISHIDA, A. KOBAYASHI and T. NAGAHAMA, *Bull. Agr. Chem. Soc. Japan* **19**, 77 (1965).
[3] N. V. RIGGS, *Chem. Ind.* 926 (1956).
[4] M. M. MASON, and M. G. WHITING, *Fedn. Proc.* **25**, 533 (1966).
[5] H. SELYE, *Rev. Can. Biol.* **16**, 3 (1957).
[6] P. R. ADIGA, S. L. N. RAO and P. S. SARMA, *Current Sci. (India)* **32**, 153 (1963).
[7] V. V. S. MURTI, T. R. SESHADRI and T. A. VENKITASUBRAMANIAN, *Phytochem.* **3**, 432 (1964).
[8] E. A. BELL, *Fed. Europ. Biochem. Soc. Abstr.* **1**, 53 (1964).
[9] C. RESSLER, P. A. REDSTONE and R. H. ERENBERG, *Science* **134**, 188 (1961).

When extracts of the kernels of *C. circinalis* were analysed by high voltage ionophoresis they were found to contain a basic compound whose ionic mobility at pH 3·6 resembled that of αγ-diaminobutyric acid. This compound also gave, like αγ-diaminobutyric acid, a brown–purple with ninhydrin. At pH 6·5 however the two compounds could be separated readily by ionophoresis and R_f values confirmed that the compound in the Cycad extract was not a known amino acid or peptide.

The present paper describes the isolation of this compound, its characterization as α-amino-β-methylaminopropionic acid, its synthesis, and evidence which suggests that it may be neurotoxic to higher animals.

EXPERIMENTAL AND RESULTS

Preliminary investigations showed that the unidentified basic compound in the seeds of *C. circinalis* was contained in the fresh white kernels (female gametophyte). The seed cases were therefore removed before making extracts.

Preparation of Extracts for Ionophoresis and Chromatography

Fresh kernels (100 mg) were ground with 50% ethanol (1 ml) and shaken for 2 hr at room temperature. After standing at the same temperature for a further 17 hr the suspension was centrifuged and the supernatant liquid used for analysis.

Ionophoresis

Ionophoresis was conducted on Whatman 3MM paper in buffer solutions of pH 1·9, pH 3·6 and pH 6·5 prepared as previously described,[10] and in boric acid–sodium borate buffer solutions adjusted to pH 9·0 and pH 10·0 by the addition of sodium hydroxide.[11] With the three buffers of lower pH a horizontal-ionophoresis method essentially that of Gross[12] was used, a potential difference of approx. 60 V/cm being applied for 30 min. At pH 9·0 and pH 10·0 a hanging-strip method was used, 5 V/cm being applied for 17 hr.

The mobility of the "unknown" at each value of pH was as follows. At pH 1·9 and at pH 6·5 the compound was positively charged and moved between arginine and lysine. At pH 3·6 it was positively charged and moved faster than lysine. At pH 9·0 it was uncharged (both arginine and lysine were positively charged). At pH 10·0 it was negatively charged and moved in the opposite direction to arginine and lysine which were still positively charged at this pH.

Paper Chromatography

One-dimensional chromatograms were prepared by the descending technique on Whatman No. 1 paper using 0·1 ml of extract. Solvents used were (1) *n*-butanol:acetic acid:water (12:3:5 by vol.); (2) phenol:water (4:1, w/v) in the presence of the vapour of aqueous NH_3 (sp. gr. 0·88); (3) *n*-butanol:pyridine:water (1:1:1 by vol.). The R_f values of the unknown in solvent (1) was 0·13, in solvent (2) 0·82 and in solvent (3) 0·42.

Colour Reactions

With ninhydrin the unidentified basic compound gave a brown–purple similar to the colour given by αγ-diaminobutyric acid. With Ehrlich's reagent it gave a pale yellow colour.

[10] E. A. BELL and A. S. L. TIRIMANNA, *Biochem. J.* **97**, 104 (1965).
[11] D. WALDRON-EDWARD, *J. Chromatog.* **20**, 556 (1965).
[12] D. GROSS, *J. Chromatog.* **5**, 194 (1961).

When treated successively with ninhydrin and Ehrlich's reagent on paper the compound gave a yellow which turned brown on prolonged standing. With *p*-nitrobenzoyl chloride[13] the compound gave a pink colour which faded after one or two minutes suggesting that it was an α-*N*-methylamino acid or a cyclic compound related to pipecolic acid. It was found however that α,β-diaminopropionic acid also gave a colour with this reagent indicating that it is not as specific as previously supposed.

Isolation of Amino Acid

The cases of nine seeds were removed and the white kernels (220 g) were homogenized with 50% ethanol (350 ml) and the suspension allowed to stand for 17 hr at room temperature before filtering. The extraction was repeated a second time and the combined filtrates after dilution with 5 vol. water were passed through a column (100×3 cm) of a strongly acidic cation exchange resin (Zeo-Karb 225) in the hydrogen form. After washing the column with water, the amino acids were eluted with 1·25 N HCl and the eluate collected in 50-ml fractions. Aspartic acid, glutamic acid, serine, alanine and valine were detected in successive fractions from 7 to 28. Fractions 29–37 contained little ninhydrin-reacting material, fractions 43–49 contained leucine, the "unknown" and traces of other ninhydrin-reacting compounds. When all the leucine had been removed (by fraction 49) the eluent was changed to 1·5 N HCl. Fractions 50–55 contained the "unknown" together with traces of other ninhydrin-reacting compounds, fractions 56–72 contained pure "unknown", and fractions 73–87 contained the "unknown" together with increasing concentrations of more basic amino acids.

Fractions 56–73 were combined and freeze-dried. The residue was recrystallized three times from aqueous ethanol to give colourless crystals (31 mg), m.p. 168° dec. (Found: C, 29·56; H, 6·58; N, 16·48; Cl, 22·85. $C_4H_{11}O_2N_2HCl$ required: C, 31·06; H, 7·11; N, 18·12; Cl, 22·97%.) No change was detectable in the compound after heating at 100° for 64 hr either in 6 N HCl or in 10% NaOH, neither did an aqueous solution of the isolated hydrochloride undergo change when shaken in an atmosphere of hydrogen in the presence of platinum oxide for a period of 17 hr. On melting, the compound underwent partial decomposition and three ninhydrin-reacting compounds were detected in the resulting mixture. These were identified chromatographically and by ionophoresis as the original "unknown", alanine and methylamine. The "unknown" gave a weak reaction with ninhydrin after pretreatment with cupric carbonate suggesting that it contained a free α-amino group.[14]

Absorption Spectrum

The "unknown" gave no absorption in the u.v. region of the spectrum. The i.r. absorption spectrum of the "unknown" (determined using the KBr-disk method) showed bands at:

3000 cm^{-1} (3·3 μ) [—OH stretching frequency of carboxyl group]; 1610 cm^{-1} (6·2 μ) and 1410 cm^{-1} (7·1 μ) [antisymmetrical and symmetrical stretching vibrations of the ionized carboxyl group of the dipolar ions]; 2090 cm^{-1} (4·8 μ) [N—H stretching frequency of $—NH_3^+$ ion]; 3420 cm^{-1} (2·9 μ) [N—H stretching frequency of —NH— group]; 1375 cm^{-1} (7·3 μ) [symmetrical CH_3 stretching frequency]. The assignments are made from the data of Greenstein and Winitz.[15]

[13] I. Smith, *Chromatographic and Electrophoretic Techniques*, Vol. I (2nd ed.), p. 99. Heinemann, London (1960).

[14] P. O. Larsen and A. Kjaer, *Biochem. Biophys. Acta* **38**, 148 (1960).

[15] J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 2, p. 1688. John Wiley, New York (1961).

Nuclear Magnetic Resonance Spectrum

The NMR spectrum (recorded in D_2O at 60 Mc/sec) showed three bands; a three-proton singlet (—NMe, at δ3·84 from trimethylsilylpropanesulphonic acid sodium salt); a two-proton doublet (C—CH_2—N, at δ3·47) and a one-proton triplet (—$\underset{|}{CH}$—N at δ4·10). The coupling J —CH—CH_2 was 7·5 c/s.

Synthesis of α-Amino-β-Methylaminopropionic Acid

From the chemical and physical evidence already presented the structure α-amino-β-methylaminopropionic acid was tentatively ascribed to the isolated compound. Confirmation was obtained by synthesis. α-Acetamidoacrylic acid (5 g) was dissolved in 50 ml of a solution of approximately 30% aqueous methylamine and allowed to stand at 40° for 72 hr.[16] Excess amine was removed by taking the reaction mixture to dryness under reduced pressure at room temperature. The residue was then boiled for 2 hr with 2 N HCl (≃ 25 ml), the solution again taken to dryness at room temperature and the residue recrystallized twice from aqueous alcohol (1·0 g), m.p. 165–167°. (Found: C, 32·16; H, 6·97; N, 18·10; Cl, 22·42. $C_4H_{11}O_2N_2$.HCl required: C, 31·06; H, 7·11; N, 18·12; Cl, 22·97%.) The synthetic compound moved with the isolated material in all chromatographic solvents and on ionophoresis at all values of pH. Like the isolated compound it decomposed on melting to give alanine and methylamine, its i.r. spectrum showed the same characteristic absorption maxima described for the isolated compound and its NMR spectrum was identical in all respects with that of the natural material.

Toxicity of α-Amino-β-Methylaminopropionic Acid in Chicks

Preliminary experiments show that the preparation of synthetic α-amino-β-methylaminopropionic acid described is markedly neurotoxic to chicks. Larger quantities of the natural compound are at present being isolated, so that fuller physiological investigations can be made.

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[16] I. F. EIGER and J. P. GREENSTEIN, *Arch. Biochem.* **19**, 467 (1948).