β -Pyrazol-1-ylalanine, an Amino Acid from Water-Melon Seeds (*Citrullus vulgaris*)

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Paper chromatography has been used extensively to study the free amino acids present in a wide variety of plant species. Comprehensive reviews describe the chemical and biological properties of many of these acids (Virtanen, 1957; Fowden, 1958, 1960; Hunt, 1959). This paper describes the isolation and identification of another acid, β -pyrazol-1-ylalanine (I), which forms a major component of the soluble nitrogen fraction of water-melon seeds. A preliminary account has been given by Noe & Fowden (1959). The possibility that pyrazole is used directly in the synthesis of the amino acid has been investigated by supplying young melon seedlings with pyrazole alone and in association with various amino acids.

ISOLATION AND IDENTIFICATION

The substance was encountered first as an unidentified ninhydrin-reacting spot on paper chromatograms prepared from 75% (v/v) ethanol extracts of ground water-melon seeds (*Citrullus vulgaris* var. Tom Watson). About 3 g. of the substance was isolated from an extract of 4.5 kg. of seeds. The empirical formula, $\text{C}_8\text{H}_{10}\text{O}_2\text{N}_3$, suggested that the substance was an isomer of histidine. The presence of an α -amino group was indicated by the compound's ability to chelate copper. On paper chromatograms it gave a yellow colour with Ehrlich reagent, and reacted slowly to give a blue colour after treatment with isatin. The Pauly reaction was negative. Degradation with hydriodic acid yielded alanine and what was probably an iodo derivative of a heterocyclic base which was not imidazole. The isolated material had a pK_A value of 2.2. This favoured the idea of a pyrazole nucleus since the pK_A values associated with the protonation of the tertiary nitrogen atom of imidazole and pyrazole are 7.03 and 2.53 respectively (Jacobs, 1957).

Nuclear magnetic resonance spectroscopy (see Appendix) established that a pyrazole ring formed

the heterocyclic component of the amino acid and that the alanine group was attached to the ring at a nitrogen and not at a carbon atom. The production of alanine during treatment with hydriodic acid provided other evidence in favour of the nitrogen-carbon linkage; this type of bond would be expected to undergo reductive fission far more readily than a carbon-carbon bond. Confirmation of the structure was obtained by synthesis of β -pyrazol-1-ylalanine, which was identical with the racemized natural product. The compound was readily separated from synthetic β -imidazol-1-ylalanine on paper chromatograms developed with butanol-acetic acid-water.

Shinano & Kaya (1957) isolated from press-juice of water melon a small quantity of an amino acid having the same empirical formula and m.p. as β -pyrazol-1-ylalanine. Noting that their compound was isomeric with histidine, they suggested that the amino acid was β -imidazol-1-ylalanine. Since water melon was the plant tissue used by Shinano & Kaya and in the present investigation, the two substances isolated were almost certainly identical, and, in view of the more critical evidence now presented, the assignment of the imidazolyl-alanine structure must be regarded as incorrect.

β -Pyrazol-1-ylalanine forms one of a group of aromatic or heterocyclic amino acids derived from alanine that have been characterized recently as higher-plant components. The group includes 2:4-dihydroxy-6-methylphenylalanine, isolated from seeds of *Agrostemma githago* (Schneider, 1958), β -(3-carboxy- γ -pyron-3-yl)alanine (stizolobic acid) isolated from *Stizolobium hassjoo* seedlings (Hattori & Komamine, 1959) and β -(uracil-3)-alanine (willardiine), isolated from seed of *Albizzia willardii* (Gmelin, 1959). In the last-named substance, the alanine is attached to a nitrogen atom of the uracil ring.

DISTRIBUTION

β -Pyrazol-1-ylalanine was the major free amino acid (approx. 1 g./kg.) in dry water-melon seeds. Citrulline (approx. 0.15 g./kg.) and glutamic acid (approx. 0.1 g./kg.) were also readily detected by chromatographic methods. During the early phase of seedling growth in the dark, many other free amino acids appeared as products of protein degra-

dation. Although some increase in the total amount of the pyrazolylalanine present in a seedling occurred during growth, concentrations of several other amino acids soon exceeded that of the pyrazolylalanine. β -Pyrazol-1-ylalanine is particularly characteristic of the dormant seed, and forms only a trace component of the vegetative tissues.

β -Pyrazol-1-ylalanine was detected as a minor component of extracts prepared from several other seeds of the family Cucurbitaceae, e.g. *Cucurbita pepo* var., *Blighia sapida* Kon. (vegetable marrow), *Cucurbita ficifolia*, *Cucumis melo* (melon), *Cucumis sativa* (cucumber), *Ecballium elaterium* (squirting cucumber), *Bryonia dioica* (white bryony) and *Echinocystis lobata*. The acid was present in these species in concentrations considerably less than that found in water-melon seed.

Data on the free amino acid fraction of numerous plant species indicate that β -pyrazol-1-ylalanine is not generally distributed in plants. Like many other recently identified non-protein amino acids, it may be confined to a relatively few plant species, of which the only examples so far known belong to the family Cucurbitaceae. However, the study of the free amino acid composition of plants has been directed largely to vegetative tissues and since the pyrazolylalanine has been found predominantly in seeds, studies on a wider range of seeds may reveal that this amino acid occurs in other families of plants.

BIOSYNTHESIS

The identification of β -pyrazol-1-ylalanine as a component of certain plants has added to the main types of naturally occurring heterocyclic compounds. There appears to be no previous report of the pyrazole-ring system occurring in natural products. Although the biosynthetic pathway leading to the formation of the pyrazole ring is unknown, biosynthesis from compounds such as hyponitrite or hydrazine, which contain linked nitrogen atoms, seems possible since several chemical syntheses of pyrazole utilize hydrazine as a starting material (Jacobs, 1957).

To test the possibility that pyrazole may be used directly for synthesis of β -pyrazol-1-ylalanine, seeds of *Cucumis melo* were used because they germinated more rapidly and consistently than the water-melon seed available. The seeds, after surface sterilization with weak sodium hypochlorite solution, were allowed to germinate in Petri dishes at 30° in the presence of the sterile solutions listed in Table 1. After 2 days, when the radicle had grown to about 1 cm. length, the seeds were planted in moistened vermiculite and grown for a further 5 days in the dark at 30°. The seeds were then killed and extracted with 75% (v/v) ethanol and the amounts of various amino acids present in each extract were determined by the paper-chromatographic technique of Fowden & Webb (1958) and Bryant & Fowden (1959). The results in Table 1 show that pyrazole may be incorporated, presumably as the intact ring system, into β -pyrazol-1-ylalanine. The quantity of the amino acid in seedlings supplied with pyrazole was approximately double that of similar plants not receiving pyrazole (Table 1). There was no further increase when alanine or serine was supplied in association with pyrazole. The results therefore do not support the idea that either alanine or serine is incorporated directly into the side chain of β -pyrazol-1-ylalanine in a manner similar to that by which tryptophan is known to be synthesized from indole and serine in higher plants (Polyanovskii & Kretovich, 1957; Mudd & Zalik, 1958; Greenberg & Galston, 1959). However, a more crucial test of this hypothesis is required.

EXPERIMENTAL

Chromatographic techniques. Paper chromatograms were run on Whatman no. 3 MM chromatographic-grade filter paper. The solvents used included: A, 75% (w/v) phenol solution in the presence of NH_3 solution; B, a one-phase mixture of butan-1-ol-acetic acid-water (90:10:29, by vol.); C, 70% (v/v) propan-1-ol; D, the upper phase of ethyl acetate-pyridine-water (2:1:2, by vol.); E, butan-1-ol saturated with 2N- NH_3 soln. and F, propan-1-ol-0.2N- NH_3 solution (3:1, v/v). A 0.1% (w/v) solution of ninhydrin in 95% (v/v) ethanol was normally used as a spraying agent.

Table 1. Concentrations of β -pyrazol-1-ylalanine in *Cucumis* seedlings supplied with various nitrogenous compounds during the first 2 days' growth

Results are expressed as $\mu\text{g.}$ of α -amino N present in pyrazolylalanine/g. fresh wt. of tissue.

Treatment	Concn. of pyrazolylalanine	Increase in concn. above that of appropriate control
Distilled water	8.1	—
0.125% Pyrazole	16.9	8.8
0.125% Pyrazole + 0.25% DL-alanine	15.2	8.8
0.125% Pyrazole + 0.25% DL-serine	15.4	9.1
0.25% DL-Alanine	6.4	—
0.25% DL-Serine	6.3	—

Occasionally the isatin, Sakaguchi, Ehrlich and persulphate dipping reagents of Jepson & Smith (1953*a, b*), were used.

Detection of β -pyrazol-1-ylalanine in water-melon seed. Paper chromatograms of aq. 75% (v/v) ethanol extracts of ground-seed material showed the presence of the pyrazolylalanine, giving a normal bluish purple colour with ninhydrin, that moved to a position nearly coincident with that of proline when solvent A followed by solvent B were used for development. The pyrazolylalanine was readily separated from the other amino acids of the seed extract on one-dimensional chromatograms in solvent B, and so its reactivity with reagents other than ninhydrin was easily checked. The pyrazolylalanine gave a strong yellow spot with Ehrlich reagent and reacted insensitively with isatin to produce a blue spot. No colour was produced on treatment with the Sakaguchi, persulphate or Pauly reagents. The amino acid chelated copper from a solution of copper acetylacetonate in chloroform.

Isolation of β -pyrazol-1-ylalanine. Ground water-melon seed (4.5 kg.) was stirred with 70 l. of aq. 75% (v/v) ethanol at 40–60° for 6 hr. and for a further 60 hr. at laboratory temperature. After filtration through Celite, the extract was evaporated *in vacuo* at about 35° to 4.5 l. Lipid material was extracted by shaking with 2 l. of ether, and the aqueous phase was adjusted to about pH 4.0 and diluted to 10 ml. The diluted extract was applied at a rate of 500 ml./hr. to a Zeo-Karb 215 (resin mesh 80–100) column, 80 cm. long, 6 cm. diam.

All the amino acids were absorbed. The column was washed with 20 l. of distilled water and eluted with 0.25-N-NH₃ solution (12 l.). When amino acids appeared in the eluate, 20 ml. fractions were collected. Resolution was not good, the pyrazolylalanine being present in a large proportion of the fractions, together with smaller amounts of other amino acids. These fractions were pooled and evaporated *in vacuo* to about 500 ml. The concentrate was treated with activated charcoal (Partridge, 1949) and then evaporated to about 100 ml. On cooling, the crude amino acid separated out as a white solid. After recrystallization from water, about 2 g. of pure β -pyrazol-1-ylalanine was obtained. A further quantity was obtained by treating the combined mother liquors on a column of Dowex-50 $\times$ 4 (mesh 100–200), 40 cm., diam. 2 cm. The amino acid was in the earliest ninhydrin-positive fractions eluted from this column. These fractions, which contained only traces of other amino acids, were combined and evaporated *in vacuo* to 30 ml. The crude material which separated on cooling was recrystallized from aq. 50% (v/v) ethanol to yield the pure amino acid (about 1 g.).

Properties. β -Pyrazol-1-ylalanine had m.p. 236–238° and $[\alpha]_D^{20} - 73^\circ$ in water (c. 3.4) (Found: C, 46.7; H, 5.8; N, 27.1. C₆H₈O₂N₂ requires C, 46.4; H, 5.8; N, 27.1%). When an 8 mm solution was titrated against 0.1-N-HCl, a pK_A of 2.2 was obtained, corresponding to the protonation of the tertiary nitrogen atom of the pyrazole ring. Histidine, titrated under comparable conditions, had an equivalent pK_A of 6.1. On paper electrophoresis the pyrazolylalanine at pH 7.0 moved towards the anode, whereas histidine remained at the origin.

Degradation. β -Pyrazol-1-ylalanine was completely destroyed when a 1% (w/v) solution of the amino acid in 55% (w/w) HI was heated at 120–130° for 18 hr. After removal of HI by repeated evaporation to dryness *in vacuo*, portions of the reaction mixture were applied to paper

chromatograms developed in all six solvents. Two spots were revealed on all chromatograms after treatment with ninhydrin. The slower-moving, bluish purple spot was inseparable from added alanine in all six solvents. The second spot, which was yellow-brown in colour, travelled at similar rates to the product obtained when pyrazole was treated with 55% (w/w) HI under comparable conditions. The second spot was probably given by an iodopyrazole (many reagents readily bring about substitution on the C-4 atom of the pyrazole ring).

Synthesis of β -pyrazol-1-ylalanine. The methyl ester of β -chloroalanine hydrochloride (25 g.), prepared by the method of Fischer & Raske (1907), was refluxed with 62 g. of silver pyrazole in 400 ml. of methanol for 48 hr. After removal of methanol, 500 ml. of 6-N-HCl was added and the mixture was refluxed for 2 hr. Precipitated AgCl was removed by filtration and thoroughly washed with hot water. The filtrate and washings were combined and evaporated to a sticky residue containing the pyrazolylalanine, serine and a little alanine. The pyrazolylalanine was separated from the other two amino acids on a Dowex-50 column; yield was about 0.2 g. (approx. 1% of theoretical), m.p. 236–238° (decomp.) (Found: C, 46.5; H, 5.9; N, 27.8. C₆H₈O₂N₂ requires C, 46.4; H, 5.8; N, 27.1%). For a better synthetic method see Finar & Utting (1960).

Identity of the natural and synthetic amino acids. The two substances showed identical chromatographic behaviours in all six solvents listed above. The nuclear magnetic resonance spectra of the two substances dissolved in strong sodium deuteroxide solution showed the same absorption maxima. The natural acid (500 mg.) was racemized by refluxing with 5 ml. of 5-N-Ba(OH)₂ solution for 36 hr. After removal of barium as the sulphate and carbonate, 150 mg. of DL-acid (a 2% solution in water showed no optical rotation) was obtained after crystallization. This racemized natural acid had an infrared spectrum identical with that of the synthetic acid (Fig. 1). The differences between the infrared spectra of the natural (–)-acid and those of either the racemized or synthetic preparations were quite small, and consisted mainly of small shifts in the frequencies of the major absorption peaks.

Fig. 1. Infrared spectra of β -pyrazol-1-ylalanine. (A) Natural (–) form; (B) racemized natural; (C) synthetic (racemic). All spectra were determined with liquid-paraffin mulls of the amino acid.

SUMMARY

1. A heterocyclic amino acid, (-)- β -pyrazol-1-ylalanine, was isolated from the seed of water melon (*Citrullus vulgaris*) and its structure confirmed by comparison with synthetic material.

2. DL- β -Pyrazol-1-ylalanine was synthesized in poor yield by condensation of silver pyrazole with the methyl ester of β -chloroalanine hydrochloride.

3. The amino acid was decomposed by treatment with 55% (w/w) hydriodic acid to give alanine and a substance tentatively identified as an iodo-pyrazole.

4. The distribution and possible method of biogenesis of the amino acid were examined briefly.

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APPENDIX

Nuclear Magnetic Resonance Spectrum of β -Pyrazol-1-ylalanine

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The results described above indicated that β -pyrazol-1-ylalanine probably contained an alanine side chain attached to a pyrazole or imidazole ring. The nuclear magnetic resonance spectrum of the amino acid was therefore compared with spectra of pyrazole, imidazole and some of their derivatives in order to identify the ring and to determine the position of attachment of the side chain. Hydrogen nuclear magnetic resonance spectra were obtained with a Varian V 4300B high-resolution spectrometer operating at 40 Mcyc. Compounds were examined as solutions in deuterium oxide or sodium deuteroxide in deuterium oxide contained in sample tubes of 5 mm. diam. Spectra were calibrated by the side-band technique, with a capillary tube containing water as an external reference.

The characteristics of the spectra due to the hydrogen nuclei in imidazole and pyrazole ring systems are summarized in Table 1. The spectrum

of imidazole consists of two peaks which show no fine structure. The separation between the peaks is 25 cyc./sec., the line of greater area being closer to the reference line. Substitution at the nitrogen atom does not produce any major change in the part of the spectrum due to the hydrogen atoms attached to the heterocyclic ring. In the spectrum of pyrazole, the two peaks both show fine structure, the smaller peak being a triplet and the larger a doublet. The separation between the two peaks is 52 cyc./sec., the peak of smaller area being closer to the reference line. Again, substitution at the nitrogen atom does not produce any marked change in the spectrum of the ring hydrogen atoms. As only small quantities of β -pyrazol-1-ylalanine were available, it was not possible to achieve the highest resolution in the spectrum, and the fine structure was not fully resolved. The spectrum of the heterocyclic hydrogen atoms of the new amino acid is summarized in Table 1.