α -(Methylenecyclopropyl)glycine from *Litchi* Seeds

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Two cyclopropyl derivatives are among a large group of newly characterized amino acids isolated from higher plants (reviews: Hunt, 1959; Fowden, 1958, 1960). These two compounds were identified as 1-aminocyclopropane-1-carboxylic acid, isolated from perry pears by Burroughs (1957) and from cowberries (*Vaccinium vitis-idaea*) by Vähätalo & Virtanen (1957), and β -(methylenecyclopropyl)-alanine (I, hypoglycin A), isolated from the fruits of the tropical plant *Blighia sapida* (Hassall, Reyle & Feng, 1954; Hassall & Reyle, 1955; Anderson *et al.* 1958; Holt & Leppla, 1958; Renner, Jöhl & Stoll, 1958; de Ropp *et al.* 1958; Wilkinson, 1958; Ellington, Hassall & Plimmer, 1958). This paper describes the isolation and identification of another related compound, α -(methylenecyclopropyl)glycine (II), from litchi (lychee) seeds, *Litchi chinensis*. The new compound is the lower homologue of hypoglycin A.

Like hypoglycin A, α -(methylenecyclopropyl)-glycine appears to exhibit hypoglycaemic activity when injected into animals.

ISOLATION AND IDENTIFICATION

α -(Methylenecyclopropyl)glycine was encountered first as an unidentified ninhydrin-reacting spot on paper chromatograms prepared from aqueous ethanol extracts of fresh litchi seeds. When ninhydrin-treated chromatograms were heated at about 70°, the initial colour of the spot was brown, but this changed to a normal purple colour in about 2 hr. if the chromatogram was kept at laboratory temperature. The new compound had R_f 0.74 in phenol-ammonia (solvent A below) and moved slightly faster than γ -aminobutyric acid in butan-1-ol-acetic acid-water (solvent B).

About 1.5 g. of the crude substance was isolated

from 24 kg. of fresh seeds. After recrystallization from 70% (v/v) ethanol, it had the empirical formula $\text{C}_6\text{H}_9\text{NO}_2$; this contained four hydrogen atoms fewer than a saturated, open-chain C_6 amino acid like leucine. Isatin and nitroprusside-acetaldehyde (Feigl, 1954) colour tests for imino nitrogen were negative. The compound was decomposed completely in 26 hr. by 6N-hydrochloric acid at 100°; three main ninhydrin-reacting products were formed but these were not identical with any commonly encountered amino acids. A negative result was obtained when the biuret peptide test was applied to a solution of the compound.

Structure (II) was indicated by an analysis of the products of hydrogenation of the new compound in the presence of Adams platinum oxide catalyst; the strained cyclopropane ring can be broken by hydrogenation (see general account, Rodd, 1953). The products formed after different reduction periods were separated on paper chromatograms developed in *tert.*-amyl alcohol (tech.)-acetic acid-water (solvent C). The earliest samples contained two ninhydrin-reacting products identified tentatively as *cis* and *trans* isomers of α -(methylenecyclopropyl)-glycine. The isomer produced in larger amount (III) ran slightly more slowly than the minor isomer (IV) on the chromatograms (see Fig. 1). Later samples contained three additional amino acids identified as leucine, isoleucine and norleucine. The progress of the hydrogenation is represented in Scheme 1. Addition of hydrogen (Scheme 1) occurred first at the ethylenic linkage and was followed by a reductive fission of the cyclopropane ring at position 1 to give norleucine, at position 2 to give isoleucine or at position 3 to give leucine. Only a compound possessing the carbon skeleton of structure (II) could yield this mixture of products.

The infrared spectra of the new compound and of the first reduction product [major isomer of α -(methylenecyclopropyl)glycine] support the presence of a terminal, exocyclic methylene group. Ettlinger & Kennedy (1956) reported that the terminal methylene group in Feist's acid (1-methylenecyclopropane-*trans*-2,3-dicarboxylic acid) was characterized by absorption peaks at 5.9, 7.1 and 11.0 μ . The spectrum of the new amino acid showed strong peaks at 5.9 and 11.0 μ ; absorption at 7.1 μ

Scheme 1. *cis*- and *trans*- α -(Methylecyclopropyl)glycine.

cannot be used as a diagnostic feature for the methylene group present in amino acids because their spectra normally possess several peaks in this region attributed to amino- and carboxyl-group absorptions. Conversion of the compound into α -(methylecyclopropyl)glycine led to the complete disappearance of the 5.9 and 11.0 μ absorption bands.

The nuclear magnetic resonance spectrum of the new compound determined with solutions in deuteriotrifluoroacetic acid showed that it did not contain a cyclopropene ring (this possibility was not eliminated by the hydrogenation experiments), and, when considered in association with the other evidence, it established (II) as the only tenable structure for the amino acid (see also Appendix).

Stereochemistry. The α and β carbon atoms of α -(methylenecyclopropyl)glycine are asymmetric and so four stereoisomers are possible. The configurations of these two carbon atoms in the natural isomer will be identical with those of the isoleucine (or alloisoleucine) molecule formed during hydrogenation. The isomer isolated after hydrogenation of α -(methylenecyclopropyl)glycine was shown to be isoleucine by paper chromatography. It had $[\alpha]_D^{25} + 43 \pm 6^\circ$ in 5*N*-hydrochloric acid (*c* 0.5); L-isoleucine has $[\alpha]_D + 39.5$ in 5*N*-hydrochloric acid (*c* 1.0). By the use of the Fischer convention the natural isomer then may be represented as L- α -(methylenecyclopropyl)glycine:

As yet it has not been possible to assign correct *cis* and *trans* configurations to the two isomeric α -(methylecyclopropyl)glycines.

DISTRIBUTION

Taxonomic significance may be attached to the fact that α -(methylenecyclopropyl)glycine and the structurally related hypoglycin A both occur in fruits of the family Sapindaceae. Hypoglycin A occurs both in the seed and in the edible portion of the *Blighia* fruits, particularly when they are unripe, but α -(methylenecyclopropyl)glycine could not be detected in the edible portion (the fleshy arils) of ripe *Litchi* fruits. Unripe fruits were not available for analysis. Neither α -(methylenecyclopropyl)glycine nor hypoglycin A could be detected in the seeds of four other species of Sapindaceae examined [*Dodonaea attenuata*, *D. physocarpa*, *Jagera pseudorhus* (whole fruits examined) and *Heterodendron diversifolium*, supplied by Dr H. G. McKee of Canberra]. The amino acids were not detected in leaf extracts prepared from 19 sapindaceous plants including *Litchi chinensis* (all the leaf samples were supplied by the Royal Botanic Gardens, Kew).

PHYSIOLOGICAL ACTIVITY

Hypoglycin A caused striking reductions in blood-sugar concentration when injected into rats, guinea pigs and kittens (Hassall *et al.* 1954; Hassall & Reyle, 1955). Rats died when their blood sugar fell to about 20 mg./100 ml., and, when observations were continued for 72 hr. after the injections, the LD₅₀ was about 90 mg./kg. body weight for fed animals. Patrick (1954) showed that the fall of blood sugar in rats was preceded by the almost complete disappearance of glycogen from the liver. Doses of 250 mg./kg. produced this total loss of glycogen within 6 hr. and the blood-sugar con-

centration had fallen to 46 mg./100 ml. (normal concentration about 100 mg./100 ml.).

The limited amounts of α -(methylenecyclopropyl)glycine available required the use of smaller animals, namely mice, in our experiments. The mice (weight about 30 g.) were starved for 22 hr. and then solutions of α -(methylenecyclopropyl)glycine were injected subcutaneously. The animals were killed 6.5 hr. after receiving the injections. Animals receiving 60, 130, 230 and 400 mg. of α -(methylenecyclopropyl)glycine/kg. body weight had blood-sugar concentrations of 83, 62, 57 and 35 mg./100 ml. (means of two determinations). Control animals into which an equivalent volume of water was injected had blood-sugar concentrations in the range 71–103 mg./100 ml. Determinations on other untreated mice after starvation for 23 hr. gave values between 55 and 112 mg./100 ml.

The general activity of the mice was related to the dose of α -(methylenecyclopropyl)glycine and animals receiving 230 or 400 mg./kg. were moribund at the end of the 6.5 hr. period. The liver-glycogen concentrations of all experimental animals were very low (about 0.1% of the fresh weight), and almost equal; values determined for control mice were higher but variable (0.25–0.9%). The livers of mice receiving the higher doses had an abnormal white appearance and were considerably enlarged when compared with those of control animals. However, liver sections of the experimental mice (examined by Professor Sir R. Cameron) showed no striking histological abnormalities.

These preliminary experiments suggest that α -(methylenecyclopropyl)glycine possesses hypoglycaemic activity but the use of a different animal species does not allow exact comparison with recorded hypoglycin A activities.

EXPERIMENTAL

Chromatographic techniques. Paper chromatograms were run on Whatman no. 3 MM chromatographic-grade paper. The solvents used were: A, 75% (w/v) phenol soln. in the presence of NH_3 ; B, a one-phase mixture of butan-1-ol-acetic acid-water (90:10:29, by vol.); C, upper phase of *tert.*-amyl alcohol-acetic acid-water (10:1:10, by vol.); D, upper phase of *tert.*-amyl alcohol-acetic acid-water (20:1:20, by vol.); E, water-saturated *tert.*-amyl alcohol; F, water-saturated butan-1-ol in the presence of 3% (w/v) NH_3 ; G, upper phase of ethyl acetate-pyridine-water (2:1:2, by vol.); H, a one-phase mixture of ethyl methyl ketone-butan-1-ol-8.5 N- NH_3 (3:5:2, by vol.); J, water-saturated benzyl alcohol. A 0.1% (w/v) solution of ninhydrin in 95% (v/v) ethanol was used normally for colour development. Occasionally the isatin dipping reagent of Jepson & Smith (1953) was used.

Detection of α -(methylenecyclopropyl)glycine. The compound could be detected readily on paper chromatograms

at all stages during the isolation procedure because it gave a characteristic colour reaction with ninhydrin (initially brown but rapidly becoming purple). The compound did not give a coloured spot when isatin-treated chromatograms were heated at 120° for 15 min. However, if these chromatograms were kept at room temperature for 24 hr., α -(methylenecyclopropyl)glycine gave an intense blue spot.

α -(Methylenecyclopropyl)glycine had the following R_{Lau} values: solvent A, 0.87; B, 0.68; C, 0.60; E, 0.56; G, 0.87; H, 0.69.

Isolation of α -(methylenecyclopropyl)glycine. Seeds (24 kg.) were removed from fully ripe *Litchi* fruits (225 kg.). The fresh seeds (water content about 50%) were crushed in a large mechanical pestle and mortar and then macerated with 95% (v/v) ethanol (36 l.) to give a final ethanol concentration of 70% (v/v). After standing at laboratory temperature for 3–5 days, the slurry was pressed at 300 lb./in.². The extract was filtered and evaporated *in vacuo* at 50° to remove most of the ethanol; the concentrate (4 l.) was diluted to 16 l. with water. A bulky precipitate, giving a strong carbohydrate reaction, was formed immediately and precipitation was completed by storing the diluted extract overnight at 4°; this was removed with a continuous rotary centrifuge and discarded. A rough estimate showed that the residual extract contained about 25 g. of mixed amino acids.

Preliminary fractionations of this extract on large columns of Zeo-Karb 215 and Dowex 50 (X4) removed non-cationic substances and effected a partial separation of amino acids. All fractions containing α -(methylenecyclopropyl)glycine were combined and these contained about 17 g. of mixed amino acids.

The combined extract was adjusted to pH 4.8 and diluted to 5 l. The amino acids were fractionated carefully on Dowex 50 (X4; 100–200 mesh) columns (upper, 44 cm. long, 3.2 cm. diam.; lower, 30 cm. long, 1.4 cm. diam.). The extract was applied to the columns at the rate of 200 ml./hr., when all amino acids were absorbed. The columns were washed with water (3.5 l.) and then the amino acids were eluted with 0.15 N- NH_3 soln. (flow rate about 150 ml./hr.). One-hundred-and-five fractions (17.5 ml.) were collected after the break-through of aspartic acid. α -(Methylenecyclopropyl)glycine was present in fraction nos. 14–45. Alanine was the only other major component of fraction nos. 14–29, and considerable amounts of valine were contained in fraction nos. 30–45. α -(Methylenecyclopropyl)glycine was divided about equally between these two groups of fractions, but only fraction nos. 14–29 were combined for the final isolation of α -(methylenecyclopropyl)glycine because valine cannot be separated readily from the new amino acid on paper chromatograms in solvent B. When the combined fractions were evaporated to dryness *in vacuo* at 50°, the yield of mixed amino acids was 4.6 g. This residue was recrystallized once from water and twice from 70% (v/v) ethanol to give a small sample (120 mg.) of pure α -(methylenecyclopropyl)glycine.

The residual amino acids present in the mother liquors were dissolved in a minimum volume of hot water and the solution was streaked across sheets of chromatographic-grade paper (24 in. wide); previously the sheets had been washed thoroughly with 5% (v/v) acetic acid soln. Each sheet received 0.2 g. of mixed amino acids, which were

separated by developing the chromatograms in solvent B for 40 hr. The bands of paper containing α -(methylenecyclopropyl)glycine were cut out and eluted with water. After evaporation the crude amino acid (1.35 g.) was obtained. Recrystallization from 70% (v/v) ethanol gave 570 mg. of pure acid.

Physical properties of α -(methylenecyclopropyl)glycine. The amino acid crystallized as lustrous plates not easily wetted by water. Its solubility in water at 25° was 38 g./l. The compound did not melt but decomposition began at about 202°. It had $[\alpha]_D^{21.5} + 83.4 \pm 1.5^\circ$ in water (c 3.0) and $[\alpha]_D^{25} + 110 \pm 2.5^\circ$ in 5N-HCl (c 2.5) (Found: C, 56.9; H, 7.2; N, 10.9. $C_6H_9NO_2$ requires C, 56.7; H, 7.1; N, 11.0%).

Stability of α -(methylenecyclopropyl)glycine to acid and alkali. The compound was stable to 3N-NaOH and to conc. HCl at room temperature for 4 and 45 hr. respectively. It was decomposed completely by treatment with 6N-HCl at 100° for 26 hr. Three ninhydrin-reacting substances were produced; these had R_{Leu} values of 0.43, 0.86 and 0.94 in solvent B. The slowest-moving substance reacted with ninhydrin to give a yellow spot initially, which changed gradually to violet, suggesting that it might be a chloro-amino acid. The other two products gave violet spots with ninhydrin. These three products were not studied further.

Small-scale hydrogenation of α -(methylenecyclopropyl)glycine. Hydrogen was bubbled through a 0.25% α -(methylenecyclopropyl)glycine soln. (0.3 ml.) containing Adams PtO_2 catalyst (20 mg.) at room temperature. Small portions were removed at intervals and spotted

directly on a chromatogram, which was developed in solvent C. Fig. 1 is a diagrammatic representation of the ninhydrin-reacting spots on the final chromatogram. Spots III and IV, which were probably geometrical isomers of α -(methylenecyclopropyl)glycine (see below), were formed in the first minute. Compound III was produced in larger amounts than compound IV. Further reduction gave three other compounds, V, VI and VII, which were inseparable from isoleucine, leucine and norleucine respectively. The identities of the leucine isomers were confirmed by additional chromatographic comparisons in solvents E, F, H and J, which were all capable of resolving the three isomers.

α -(Methylenecyclopropyl)glycine had disappeared from the mixture at 3.5 min. but the α -(methylenecyclopropyl)glycines were not reduced completely when hydrogenation was stopped after 45 min. Hydrogenation was less complete if the PtO_2 was reduced before addition of α -(methylenecyclopropyl)glycine.

Large-scale reduction of α -(methylenecyclopropyl)glycine. Hydrogen was bubbled for 3 hr. through a 2.6% (w/v) α -(methylenecyclopropyl)glycine soln. (2.25 ml.) containing PtO_2 (120 mg.) at 50°. The residual solution was streaked across sheets of filter paper (0.5 mg. of amino acids/cm.) previously washed successively with 1% NaOH, water, 5% (v/v) acetic acid and solvent B. These chromatograms were developed for 140 hr. at 15° in solvent D, which effected a good separation of all the reduction products. Bands of paper containing isoleucine and the major and minor isomers of α -(methylenecyclopropyl)glycine were cut from the chromatograms and the amino acids were eluted with water.

α -(Methylenecyclopropyl)glycine: major isomer (III). Since the eluate was liable to contain small amounts of impurities arising from the filter paper, the amino acid was purified by adsorption on and elution from a small Dowex 50 (X4) column (5.5 cm. long, 0.8 cm. diam.). After evaporation *in vacuo* at laboratory temperature, 18.5 mg. of this α -(methylenecyclopropyl)glycine isomer was obtained in the form of a monohydrate.

The isomer had the following R_{Leu} values: solvent B, 0.81; C, 0.68; D, 0.55; E, 0.56; F, 0.60; G, 0.87; H, 0.69; J, 0.64. It had $[\alpha]_D^{22} + 72.5 \pm 2.8^\circ$ in water (c 1.5) (Found: C, 49.2; H, 8.7; N, 9.0. $C_6H_{11}NO_2 \cdot H_2O$ requires C, 49.0; H, 8.9; N, 9.5%). The infrared spectrum of the compound was consistent with the presence of water of crystallization.

Isoleucine, leucine and norleucine were formed in about equal proportions when this isomer was hydrogenated in the presence of PtO_2 .

α -(Methylenecyclopropyl)glycine: minor isomer (IV). The eluate containing this isomer was purified with a similar Dowex 50 (X4) column (4.5 cm. long, 0.8 cm. diam.) and yielded 1.1 mg. of substance. The isomer gave a transient brown colour before turning violet on chromatograms treated with ninhydrin and it had these R_{Leu} values: solvent C, 0.76; D, 0.66; E, 0.67. It had $[\alpha]_D^{22.5} + 66 \pm 1.5^\circ$ in water (c 0.16).

Hydrogenation of the isomer gave leucine $\geq$ isoleucine $>$ norleucine as products.

Stereochemistry of α -(methylenecyclopropyl)glycine; isolation of L-isoleucine. The eluate containing the isoleucine isomer was purified on a Dowex 50 (X4) column (5.5 cm. long, 0.8 cm. diam.) and gave 2.64 mg. of purified material having $[\alpha]_D^{22.5} + 43 \pm 6^\circ$ in 5N-HCl (c 0.5). The isomer thus was either L-isoleucine $[[\alpha]_D + 39.5$ in 5N-HCl (c 1.0)] or

Fig. 1. The progressive reduction of α -(methylenecyclopropyl)glycine. Reduction times, given at the origin of the chromatogram, are in minutes. The chromatogram was developed in solvent C. Spots are: II, α -(methylenecyclopropyl)glycine; III, α -(methylenecyclopropyl)glycine, major isomer; IV, α -(methylenecyclopropyl)glycine, minor isomer; V, isoleucine; VI, leucine; VII, norleucine. The spot applied at S contained isoleucine, leucine and norleucine markers.

The spectrum was fully consistent with the proposed structure. The assignment of the peaks was as follows. The $\overset{+}{\text{NH}_3}$ protons 2.61 (on the τ scale); the olefinic hydrogens 4.30; the α -CH 6.13; the three cyclopropane protons gave a complex pattern centred at 8.41. This assignment was confirmed by the spectrum in deuteriotrifluoroacetic acid ($\text{CF}_3\cdot\text{CO}_2\text{D}$) in which the $\overset{+}{\text{NH}_3}$ peak disappeared and the multiplet structure of the α -proton peak collapsed to a broad doublet, giving $J \sim 7$ cyc./sec. The τ values given above agree entirely with the values found for these types of proton, e.g. the $\overset{+}{\text{NH}_3}$ protons of amino acids dissolved in trifluoroacetic