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NEWLY CHARACTERIZED AMINO ACIDS FROM *BLIGHIA UNIJUGATA*

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Abstract—Two amino acids have been isolated from seed of *Blighia unijugata*. The compounds were characterized as *trans*-α-(2-carboxymethylcyclopropyl)glycine and 2-amino-5-methyl-6-hydroxyhex-4-enoic acid following analysis of their hydrogenation products and NMR and mass spectra. The structures of the two compounds are discussed in relation to other C_7 amino acids occurring in members of the Sapindaceae and Hippocastanaceae.

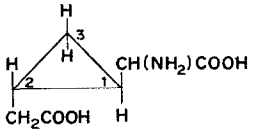
INTRODUCTION

IN A SERIES of papers published during the last 10 years,[1-7] we have described the isolation and characterization of a group of unusual amino acids from members of the Sapindaceae and Hippocastanaceae. The majority of these compounds contain six or seven carbon atoms, arranged as a branched-chain skeleton. This branching takes the form of a cyclopropane ring in several of the compounds. β-(Methylenecyclopropyl)alanine (hypoglycin A) from *Blighia sapida*[8] and α-(methylenecyclopropyl)glycine from *Litchi chinensis*[1] were among the earlier amino-acid isolates from species within the Sapindaceae; later isolations[2,3] included four more cyclopropane amino acids and also the γ-glutamyl peptides of hypoglycin A[8] and of *trans*-α-(carboxycyclopropyl)glycine.[4] Some of the compounds show ethylenic- or acetylenic-type unsaturation; 2-amino-4-methylhex-4-enoic acid forms the principal component of the soluble-nitrogen fraction of seed of *Aesculus californica*,[2] whilst three C_7 acetylenic amino acids occur in *Euphoria longan* seed.[5] Many of these compounds occur in the seed of various *Aesculus* and *Billia* species, and a summary of their structures and distribution in relation to the accepted taxonomy[9,10] of the bigeneric family, Hippocastanaceae, is available.[6]

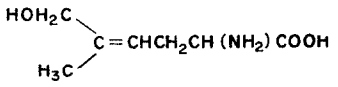
Recently, we have studied the amino acid complex present in the seed of another *Blighia* species, i.e. *B. unijugata*, collected in Ghana. Two amino acids, not previously described as natural products, were isolated and characterized as *trans*-α-(2-carboxymethylcyclo-

[1] D. O. GRAY and L. FOWDEN, *Biochem. J.* **82**, 385 (1962).
[2] L. FOWDEN and A. SMITH, *Phytochem.* **7**, 809 (1968).
[3] L. FOWDEN, A. SMITH, D. S. MILLINGTON and R. C. SHEPPARD, *Phytochem.* **8**, 437 (1969).
[4] L. FOWDEN and A. SMITH, *Phytochem.* **8**, 1043 (1969).
[5] M-L. SUNG, L. FOWDEN, D. S. MILLINGTON and R. C. SHEPPARD, *Phytochem.* **8**, 1227 (1969).
[6] L. FOWDEN, J. W. ANDERSON and A. SMITH, *Phytochem.* **9**, 2349 (1970).
[7] J. N. ELOFF and L. FOWDEN, *Phytochem.* **9**, 2423 (1970).
[8] E. V. ELLINGTON, C. H. HASSALL, J. R. PLIMMER and C. E. SEAFORTH, *J. Chem. Soc.* 80 (1959).
[9] J. W. HARDIN, *Brittonia* **9**, 173 (1957).
[10] J. W. HARDIN, *Brittonia* **12**, 26 (1960).

propyl)glycine (I) and 2-amino-5-methyl-6-hydroxyhex-4-enoic acid (II). The latter compound is a hydroxylated form of 2-amino-5-methylhex-4-enoic acid, earlier identified as a constituent of the fungus, *Leucocortinarius bulbiger*;[11] it is an isomer of 2-amino-4-methyl-6-hydroxyhex-4-enoic acid, which was characterized as one of a group of unusual amino acids present in seed of *A. californica*.[2]



(I)



(II)

RESULTS

Seed of *B. unijugata*, like that of *B. sapida*, contains hypoglycin A and hypoglycin B as major components of the soluble nitrogen fraction. *Trans*-α-(carboxycyclopropyl)glycine was not detected in the anionic fraction separated from the extract of *B. unijugata*, although this dicarboxylic amino acid forms a principal constituent of akee (*B. sapida*) seed. The two newly-identified amino acids were observed initially as unusual spots on a ninhydrin-treated two-dimensional chromatogram of a seed extract. Compound I was seen as a faint blue-purple spot present at a position associated with acidic amino acids, whilst compound II was

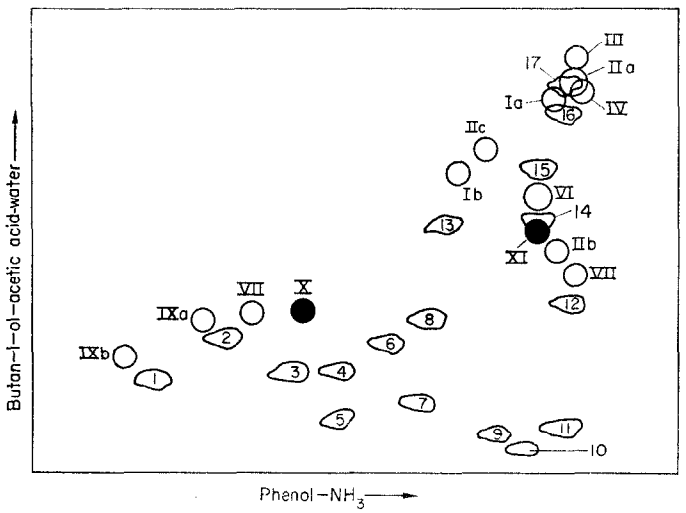


FIG. 1. DIAGRAMMATIC REPRESENTATION OF POSITIONS OF AMINO ACIDS (●) ISOLATED FROM *B. unijugata* IN RELATION TO CERTAIN STRUCTURALLY-SIMILAR COMPOUNDS FROM RELATED PLANT SPECIES.
Key to chromatographic spots: 1—aspartic acid; 2—glutamic acid; 3—serine; 4—glycine; 5—asparagine; 6—threonine; 7—glutamine; 8—alanine; 9—histidine; 10—lysine; 11—arginine; 12—proline; 13—tyrosine; 14—γ-aminobutyric acid; 15—valine; 16—phenylalanine; 17—leucine. (Ia) hypoglycin A; (Ib) hypoglycin B; (IIa) 2-amino-4-methylhex-4-enoic acid; (IIb) 2-amino-4-methyl-6-hydroxyhex-4-enoic acid; (IIc) γ-glutamyl peptide of IIa; (III) homoisoleucine; (IV) β-methyl-β-(methylenecyclopropyl)alanine; (VI) α-(methylenecyclopropyl)glycine; (VII) *cis*-α-(carboxycyclopropyl)-glycine; (VIII) *cis*-3,4-methanoproline; (IXa) *trans*-α-(carboxycyclopropyl)glycine; (IXb) γ-glutamyl peptide of IXa; (X) *trans*-α-(carboxymethylcyclopropyl)glycine; (XI) 2-amino-5-methyl-6-hydroxyhex-4-enoic acid.

recognized initially as a yellow spot, whose colour changed gradually to blue–purple, in a position partially overlapping that of γ-aminobutyric acid. Figure 1 illustrates these positions relative to those of some other unusual amino acids from *Blighia*, *Billia* and *Aesculus* species.

The two new compounds were separated from an aqueous-ethanolic extract of *Blighia unijugata* seed (4·5 kg) by coupling cation- and anion-exchange resin column techniques with preparative paper chromatographic methods.

Trans-α-(*Carboxymethylcyclopropyl*)*glycine* (I)

The isolation procedure leading to the separation of this compound involved an absorption step on to an anion-exchange resin, and so indicated the acidic nature of I. Elementary analysis suggested the empirical formula $C_7H_{11}NO_4$. The mass spectrum did not show a peak corresponding to the parent ion (i.e. at *m/e* 173), but a base peak at *m/e* 128 (i.e. M–COOH).

Hydrogenation of the compound, under weakly acidic conditions, in the presence of Adam's platinum catalyst resulted in a slow, and incomplete, conversion into two products. This behaviour was reminiscent of that shown by *cis*- and *trans*-isomers of α-(carboxycyclopropyl)glycine,[3] and unlike that of simple ethylenic or acetylenic amino acids. The reduction products were identified using chromatographic and electrophoretic methods, as *erythro*-3-methyl-2-aminoadipic acid and *threo*-4-methyl-2-aminoadipic acid, i.e. they arose by reductive 2,3- and 1,3-fissions within structure I. These structural assignments suggest a *trans* configuration of substituent groups about the cyclopropane ring. A *trans* configuration, as opposed to *cis*, is in agreement with the failure of I to give a lactam when autoclaved at pH 3 at 120° [under similar conditions, α-aminoadipic acid[12] and *cis*-α-(carboxycyclopropyl)glycine[3] are almost completely transformed into their lactams]. Oxidation of I by treatment with *N*-bromosuccinimide and silver oxide[13] gave an organic acid presumed to be *trans*-2-carboxymethylcyclopropane-1-carboxylic acid. An L-configuration at the α-carbon atom was indicated by specific optical rotation values.

These structural predictions were finally confirmed by a study of the NMR spectrum of the sodium salt of I. The spectrum showed the presence of four protons attached to a cyclopropane ring (complex multiplet at high field), a methylene group in a fragment $—CH_2—CH=$, and a single proton adjacent to one other, as in $=CH—CH=$. No olefinic protons were present. Spin decoupling experiments show that as expected from the multiplicities of the methylene and single proton signals, these protons were not adjacent to each other but were coupled to single protons on the cyclopropane ring. These results excluded all possible structural formulations other than that shown in I.

2-*Amino*-5-*methyl*-6-*hydroxyhex*-4-*enoic Acid* (II)

This compound eluted from a cation-exchange resin column (Dowex 50W) together with other neutral amino acids such as glycine, alanine, leucine, isoleucine and hypoglycin A. It was finally separated from other substances on preparative paper chromatograms developed in butan-1-ol-acetic acid–water. Elementary analysis indicated the formula $C_7H_{13}NO_3$.

Hydrogenation (Pt/H_2) of II at laboratory pressure and temperature produced a mixture

[11] G. DARDENNE, J. CASIMIR and J. JADOT, *Phytochem.* 7, 1401 (1968).
[12] J. P. GREENSTEIN and M. WINITZ, *Chemistry of the Amino Acids*, Vol. 3, p. 2457, Wiley, New York (1961).
[13] D. K. BLACK and S. R. LANDOR, *J. Chem. Soc.* C, 288 (1968).

of amino acids. The principal product was homoleucine (identified by paper chromatography and amino acid autoanalyser). A smaller amount of a second product, reacting blue–purple with ninhydrin, was present in the final reduction mixture: this substance moved only slightly faster than II on paper chromatograms, suggesting it resulted following saturation of an ethylenic linkage (i.e. it was presumed to be 2-amino-5-methylhex-4-enoic acid). At intermediate times, 2-amino-5-methylhex-4-enoic acid was recognized as a hydrogenation product, but this was rapidly further reduced to homoleucine. When 10% palladium on carbon was used as the catalyst during hydrogenation, the presumed 2-amino-5-methyl-6-hydroxyhexanoic acid represented the major reduction product. This behaviour during hydrogenation is an almost exact parallel of that recorded for the isomeric 2-amino-4-methyl-6-hydroxyhex-4-enoic acid.[2] It established the basic carbon skeleton of II, the positions of the doubly-bonded carbon atoms, and strongly suggested the presence of an allylic hydroxyl group on either C-6 or C-3. Oxidation of II with acidic permanganate produced aspartic acid in low yield, thereby eliminating the possibility that the hydroxyl group was on the C-3 atom.

The NMR spectrum of II was in accord with these structural features, and indicated that the hydroxyl was attached to a terminal carbon atom. The chemical shifts and multiplicities of the resonances assigned to the various protons are given in the Experimental. These compare very favourable with those measured previously for similar protons in 2-amino-4-methylhex-4-enoic acid and 2-amino-4-methyl-6-hydroxyhex-4-enoic acid,[14] and support in detail all features of structure II as written above. The nature of the *cis-trans* stereochemistry about the double bond has not been established for II.

DISCUSSION

The identification of compound I as another example of a cyclopropane amino acid was not surprising since the genus *Blighia* was already known to produce β-(methylenecyclopropyl)glycine (hypoglycin A) and *trans*-α-(carboxycyclopropyl)glycine. However, the structure finally assigned to I, on the basis of incontrovertible NMR evidence, was not the only one considered on biogenetic grounds. Previous work with members of the Sapindaceae and with *Billia* had resulted in the characterization of α-(methylenecyclopropyl)glycine and *trans*-α-(carboxycyclopropyl)glycine, i.e. compounds having the same basic carbon skeleton and differing only in the oxidation state of the single carbon substituent (methylene or carboxy) exocyclic to the cyclopropane ring. By analogy, compound I might have been *trans*-β-(carboxycyclopropyl)alanine showing a similar relationship to hypoglycin A. Although this was not the case, the possibility that *trans*-β-(carboxycyclopropyl)alanine is produced by this group of plants must still be regarded as being very real. Metabolically, *trans*-α-(carboxymethylcyclopropyl)glycine may be more closely related to *trans*-α-(carboxycyclopropyl)glycine than to hypoglycin A, but all three might be derived ultimately from a common precursor such as isoleucine (see earlier papers for a discussion of possible biogenetic relationships[2,15,16]).

The characterization of the second isolate from *B. unijugata* as 2-amino-5-methyl-6-hydroxyhex-4-enoic acid necessitates an extension of the biogenetic hypothesis recently advanced[16] to account for the synthesis of C_7 branched-chain amino acids in *Aesculus* and

[14] D. S. MILLINGTON and R. C. SHEPPARD, *Phytochem.* **7**, 1027 (1968).
[15] L. FOWDEN, in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 2, pp. 203–266, Wiley, London (1970).
[16] L. FOWDEN and M. MAZELIS, *Phytochem.* **10**, 359 (1971).

some sapindaceous species. In this instance, it is clearly unlikely that isoleucine serves as the initial biogenetic precursor; leucine might more logically undergo chain extension at the carboxyl end and so lead to the required type of branched C_7 skeleton. It is interesting to recall that a fungus was the source of the most closely related compound (2-amino-5-methylhex-4-enoic acid) so far described. Another fungus, *Amanita solitaria*, produces 2-aminohex-4,5-dienoic acid, an allenic amino acid suggested as a possible precursor of hypoglycin A;[17] however, when this allene was supplied, labelled with ^{14}C, to developing seeds of *A. californica*, it was not utilized as a precursor of 2-amino-4-methylhex-4-enoic acid.[16] Clearly, these unusual, structurally-similar compounds, can be elaborated by quite unrelated groups of plants that must nevertheless possess certain biogenetic systems in common.

EXPERIMENTAL

Chromatographic and electrophoretic methods. Descending paper chromatograms were run on Whatman No. 3MM or 4 paper using: (1) 75% (w/w) PhOH in the presence of NH_3 vapour; (2) *n*-BuOH–HOAc–H_2O (90:10:29, by vol.); and (3) *tert*-AmOH–HOAc–H_2O (20:1:20, by vol., upper phase). High voltage electrophoresis was performed on Whatman 3MM paper using a Locarte Co. (London) apparatus having 1 m plates. Separations were made at either pH 3·4 or 5·3 using appropriate pyridine–HOAc–H_2O buffers.[18]

Methyl substituted 2-aminoadipic acids. Synthetic samples[19] of 3- and 4-methyl-2-aminoadipic acids were kindly supplied by Dr. M. Takehara. Although racemic at the C-2 atom, electrophoresis indicated that the samples consisted of only one type (*threo*-) of diastereoisomer. Epimerization was effected by heating with 5 N $Ba(OH)_2$ for 48 hr, when each sample could be resolved into *threo*- and *erythro*-isomers.

A small sample of 5-methyl-2-aminoadipic acid was synthesized by a procedure based on that previously used to obtain γ-ethylglutamic acid.[20] 1,2-Dibromoethane was condensed successively with equimolar amounts of the sodium derivatives of dimethyl methylmalonate and ethyl acetamidocyanoacetate: after hydrolysis of the final ester with 6 N HCl, 5-methyl-2-aminoadipic acid was separated from the hydrolysate by absorption on to a Dowex-1 resin column.

Isolation of new compounds. B. unijugata seed (4·5 kg) was kindly provided by the University of Ghana Botanic Garden at Legon. After maceration, the seed material was first extracted with 75% (v/v) EtOH (25 l.), and then twice with $CHCl_3$–satd. H_2O (20 l.). The combined extracts were concentrated *in vacuo* at 40° to remove ethanol, adjusted to pH 4·5 and warmed briefly at 60° to precipitate protein and decolorize the solution. The clarified extract (pH 3) was applied to a Zeokarb 225 (×8) resin column (H^+ form, mesh 52–100, 110 × 10 cm) to absorb amino acids: after thorough washing to remove non-cationic materials, the amino acids were displaced with 0·5 N NH_3 and fractions (50 ml) were collected. The early ninhydrin-positive fractions, containing compound I together with the acidic amino acids, were pooled as were a middle group of fractions containing compound II and several other neutral amino acids. These two groups of combined fractions were treated separately as below.

The acidic amino acid fraction was concentrated and adjusted to pH 7 with NH_3. The solution was applied to a Dowex-1 (×10) resin column (acetate form, mesh 100–200, 120 × 5 cm). After washing, the column was eluted using a 0·5–1·5 N HOAc gradient, 50 ml fractions being collected. Compound I was eluted before any other amino acids, being contained in two fractions immediately prior to glutamic acid. These fractions were concentrated to small volume when I crystallized (140 mg). The compound was recrystallized from 50% (v/v) EtOH.

The neutral amino acid fraction was resolved further on a Dowex-50 (×8) column (H^+ form, 100–200 mesh, 100 × 2·5 cm); this procedure separated considerable quantities of alanine, valine and hypoglycin A from fractions containing compound II. These latter fractions were pooled to yield 2·5 g solids, of which II was still a relatively minor component. II was finally separated from other amino acids by preparative paper chromatography (Whatman No. 3MM paper and solvent 2), yield 96 mg. Recrystallization from 80% (v/v) EtOH gave 46 mg pure II.

Properties of the new amino acids. Trans-α-(carboxymethylcyclopropyl)glycine (I) had the following analysis: C, 48·8; H, 6·5; N, 8·0. $C_7H_{11}NO_4$ requires: C, 48·6; H, 6·4; N, 8·1%. The $[\alpha]_D^{20}$ values were +12° (c, 1 in water) and +45° (c, 0·5 in 5 N HCl). The NMR spectrum of the disodium salt measured at 100 MHz

[17] W. S. Chilton, G. Tsou, L. Kirk and R. G. Benedict, *Tetrahedron Letters* 6283 (1968).
[18] P. J. Peterson and L. Fowden, *J. Chromatog.* **48**, 575 (1970).
[19] M. Takehara and R. Yoshida, *Japan Chem. J.* **90**, 101 (1969).
[20] L. Fowden, *Biochem. J.* **98**, 57 (1966).

in D_2O/NaOD solution showed a four proton multiplet containing at least 22 lines centred at *ca.* 9·35 τ (cyclopropyl protons), a two proton octet (AB of ABX, J_{AB} = 15 Hz) centred at 7·85 τ (—C$\underline{H}_2$—CH=), and a one proton doublet (J = 8·5 Hz) at 7·40 τ (=C$\underline{H}$—CH=). Irradiation of the signal at 9·25 τ caused the signal at 7·40 τ to collapse to a singlet. The mass spectrum of I obtained at 200° showed no parent ion but a base peak at *m/e* 128 (M–COOH).

Two products were obtained after hydrogenation of I in dilute HOAc using Adam's Pt catalyst. These products were inseparable from 3- and 4-methyl-2-aminoadipic acids on paper chromatograms run in solvents 2 and 3: neither of the solvents effectively separated *threo-* and *erythro*-isomers of these two amino acids. 5-Methyl-2-aminoadipic acid moved faster than either hydrogenation product on chromatograms developed in solvent 3. When samples of the hydrogenation mixture and of the epimerized 3- and 4-methyl-2-aminoadipic acids were run on electrophoretograms at either pH 3·4 (100 v. cm^{-1}, 3 hr) or 5·3 (100 v. cm^{-1}, 1·5 hr), the two amino acids resulting after hydrogenation behaved identically with *erythro*-3- and *threo*-4-methyl-2-aminoadipic acids (moving −0·8 and 4·3 cm, respectively, towards the anode at pH 3·4, and 28·9 and 30·6 cm at pH 5·3). *Erythro-* and *threo*-configurations were assigned to the diastereoisomers of 3- and 4-methyl substituted 2-aminoadipic acid on the basis of their electrophoretic behaviour in comparison with that of the isomers of 3- and 4-methylglutamic acids, respectively.[3,21]

I was oxidized with *N*-bromosuccinimide and Ag_2O by a procedure described previously.[3] The organic acid produced moved slightly faster (R_f 0·88) than *trans*-cyclopropane-1,2-dicarboxylic acid (R_f 0·85) on paper chromatograms developed in solvent 3: *cis*-cyclopropane-1,2-dicarboxylic acid moved at R_f 0·65 under the same conditions. Authentic samples of *cis-* and *trans*-2-carboxymethylcyclopropane-1-carboxylic acid were not available, but the chromatographic behaviour was consistent with that expected of the *trans* isomer, by analogy with the behaviour of the *cis* and *trans* forms of the lower homologue.

2-*Amino*-5-*methyl*-6-*hydroxyhex*-4-*enoic acid* (II). The recrystallized material had the following analysis: C, 52·6; H, 8·3; N, 8·7. $C_7H_{13}NO_3$ requires: C, 52·8; H, 8·2; N, 8·8%. The 100 MHz NMR spectrum of the disodium salt showed a three proton singlet at 8·31 τ (Me. C≡), a two proton triplet at 7·36 τ (=CH—C$\underline{H}_2$—CH=), a one proton triplet at 6·22 τ (α-proton, =C$\underline{H}$—CH_2—), a two proton singlet at 6·02 τ (—OC$\underline{H}_2$—C≡), and a one proton triplet at 4·64 τ (=C$\underline{H}$—CH_2—).

Reduction of II (H_2/Pt) gave principally homoleucine as the final product: the identity was checked by comparison with authentic material using paper chromatography in solvent 3, which resolves homoleucine and homoisoleucine, and by Technicon amino acid autoanalyser. The identity of 2-amino-5-methylhex-4-enoic acid, formed as a transient intermediate during hydrogenation, was confirmed by the same procedures by reference to authentic material provided by Professor J. Casimir (Gembloux, Belgium).

Oxidation of II by 0·1% $KMnO_4$ in 1% (w/v) H_2SO_4 at 50° produced aspartic acid (isolated by absorption onto Zeokarb 225 and identified by paper chromatography using solvents 1–3.)

Acknowledgements—We wish to thank Mr. A. Smith for performing the Technicon amino acid assays, and the Agricultural Research Council for a grant to purchase the Autoanalyser.

[21] H. M. KAGAN and A. MEISTER, *Biochem.* **5**, 725 (1966).

Key Word Index—Blighia unijugata; Sapindaceae; cyclopropyl amino acids; hypoglycin A; *trans*-α-(2-carboxymethylcyclopropyl)glycine; 2-amino-6-hydroxy-5-methylhex-4-enoic acid.