Phytochemistry, 1969, Vol. 8, pp. 1227 to 1233. Pergamon Press. Printed in England.

ACETYLENIC AMINO ACIDS FROM *EUPHORIA LONGAN*

MAY-LIN SUNG and L. FOWDEN

Department of Botany, University College, London, W.C.1

and

D. S. MILLINGTON and R. C. SHEPPARD

The Robert Robinson Laboratories, Liverpool University, Oxford Street, Liverpool 7

(*Received* 20 *February* 1969)

Abstract—Three amino acids containing an acetylenic bond have been newly characterized from seed of *Euphoria longan*. Structures were assigned, largely on the basis of hydrogenation, NMR and mass spectrometric studies, as follows: 2-amino-4-methylhex-5-ynoic acid, 2-amino-4-hydroxymethylhex-5-ynoic acid, and 2-amino-4-hydroxyhept-6-ynoic acid. The possible biogenetic relationships existing between the two branched-chain C_7 compounds and other olefinic and cyclopropyl amino acids isolated earlier from species of the allied *Aesculus* and *Blighia* genera are discussed.

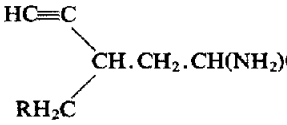
INTRODUCTION

PREVIOUS work relating to the amino acids of members of the family Sapindaceae is confined to the species *Blighia sapida* (akee), *Litchi chinensis* (litchi, lychee), *Aesculus californica* (California buckeye) and *A. parviflora*. There is no general agreement on the family affiliation of the genus *Aesculus* and some authorities prefer to establish it as the monogeneric family Hippocastanaceae but, for reasons of chemistry[1–3] and convenience, it seems rational to link it to the Sapindaceae, especially in regard to biogenetic considerations.

These three genera form the main sources of the group of cyclopropyl amino acids known as plant products. Two series of compounds exist, one group possessing a C_6 and the other a C_7 or C_8 carbon skeleton. Among the C_6 compounds are α-(methylenecyclopropyl)glycine from *L. chinensis*,[4] *cis*- and *trans*-isomers of α-(carboxycyclopropyl)glycine from *A. parviflora* and *B. sapida* respectively,[3] and *cis*-3,4-methanoproline from *A. parviflora*.[3] The second group is represented by β-(methylenecyclopropyl)alanine (hypoglycin A), the well-known hypoglycaemic factor present in the unripe edible fleshy aril and seed of *B. sapida* fruits,[5] and β-(methylenecyclopropyl)-β-methylalanine of *A. californica* seed,[1,2] a substance that may arise as a product of methyl group transfer to hypoglycin A. In addition to these cyclopropyl amino acids, a related group of branched-chain C_7 amino acids have been isolated from seed of *A. californica*;[1,2] 2-amino-4-methylhex-4-enoic acid is the main component of the soluble-nitrogen pool of these seeds, while its γ-glutamyl peptide, homoisoleucine and 2-amino-6-hydroxy-4-methylhex-4-enoic acid are present in much smaller amounts.

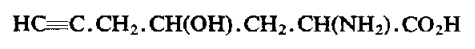
[1] L. FOWDEN and A. SMITH, *Phytochem.* **7**, 809 (1968).
[2] D. S. MILLINGTON and R. C. SHEPPARD, *Phytochem.* **7**, 1027 (1968).
[3] L. FOWDEN, A. SMITH, D. S. MILLINGTON and R. C. SHEPPARD, *Phytochem.* **8**, 437 (1969).
[4] D. O. GRAY and L. FOWDEN, *Biochem. J.* **82**, 385 (1962).
[5] E. V. ELLINGTON, C. H. HASSALL, J. R. PLIMMER and C. E. SEAFORTH, *J. Chem. Soc.* 89 (1959).

The present paper describes work on the amino acid complement of seed of *Euphoria longan*. *Euphoria* is generally considered as the genus most closely related to *Litchi*; with *Nephelium lappaceum* (rambutan), these species form a triumvirate of tropical fruits which were grouped together as the genus *Nephelium* in many old floras. However, the identification of three acetylenic amino acids in longan seeds indicates a clear phytochemical distinction from the other sapindaceous species, although the similar branched-chain C_7 skeleton of two of the isolates suggests biogenetic relationships which will be elaborated later. The structures assigned to the new compounds are 2-*amino*-4-*methylhex*-5-*ynoic acid* (Ia), 2-*amino*-4-*hydroxymethylhex*-5-*ynoic acid* (Ib) and 2-*amino*-4-*hydroxyhept*-6-*ynoic acid* (II).



(I)

a, R = H; b, R = OH



(II)

RESULTS

The three acetylenic amino acids were isolated from air-dried seed of *Euphoria longan* (5 kg) by ion-exchange chromatographic procedures. Details of their behaviour on paper chromatograms developed in three solvent systems are given in Table 1, together with similar data for related amino acids obtained as reduction products of Ia, Ib and II.

2-*Amino*-4-*Methylhex*-5-*Ynoic Acid* (*Ia*)

This compound runs coincidently with phenylalanine on two-dimensional chromatograms developed in phenol–NH_3 followed by butanol–acetic acid–water, and so it was not possible to assess its relative concentration in the initial seed extract. On the basis of the amount isolated, it represented the major acetylenic amino acid of *Euphoria* seed. After several recrystallizations, the best samples still contained some contaminating leucine.

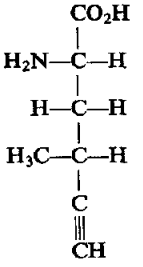
Assignment of structure was based on the following considerations. Elemental analysis indicated an empirical formula $C_7H_{11}NO_2$, and this was shown to be the molecular formula by mass spectrometry [molecular ion *m/e* 141, base peak *m/e* 96 (M-CO_2H)]. Hydrogenation using Adam's platinum catalyst was facile and gave homoisoleucine as the sole reduction product, so indicating the type of branching of the carbon skeleton. The NMR spectrum of Ia showed the presence of $\overset{+}{N}H_3.\underset{|}{C}H.CO_2^-$ and $CH_3.CH\langle$ structural units (confirmed by the mass spectrum), the complete absence of olefinic protons but the presence of resonances centred at 7·4 τ suggestive of a terminal acetylenic grouping $HC{\equiv}C{-}CH\langle$. This was confirmed by partial hydrogenation of Ia over a Lindlar catalyst (hydrogen uptake 0·88 mole) to the dihydro derivative (molecular ion *m/e* 143, base peak *m/e* 98), which now showed *three* olefinic protons in the NMR spectrum. These results can be accommodated only in structure Ia for the parent amino acid.

Optical rotation measurements on Ia and on the homoisoleucine obtained after reduction, made in water and 5 N-HCl solution, showed the shift to more positive rotations in acid associated with an L-configuration in amino acids. In an attempt to define the configuration

TABLE 1. R_f VALUES OF NEWLY CHARACTERIZED ACETYLENIC AMINO ACIDS OF *Euphoria* AND OF SOME DERIVED COMPOUNDS

	R_f in phenol–ammonia	R_{Leu} in butanol–acetic–water	R_{Leu} in *tert*-amyl alcohol–acetic–water	Ninhydrin colour
2-Amino-4-methylhex-5-ynoic acid (Ia)	0·84	0·81	0·75	Purple
2-Amino-4-hydroxymethylhex-5-ynoic acid (Ib)	0·70	0·35	0·26	Purple
2-Amino-4-hydroxyhept-6-ynoic acid (II)	0·75	0·47	0·45	Reddish brown → brownish purple v. quickly → purple
Homoisoleucine	—	1·13	1·33	Purple
2-Amino-4-hydroxymethyl-hexanoic acid	—	0·72	0·76	Purple
2-Amino-4-hydroxyheptanoic acid	—	0·85	1·05	Reddish brown → purple quickly
2-Amino-4-methylhex-5-enoic acid	—	1·08	1·20	Purple
2-Amino-4-hydroxymethylhex-5-enoic acid	—	0·53	0·46	Purple
2-Amino-4-hydroxyhept-6-enoic acid	—	0·71	0·75	Reddish brown → purple slowly
2-Aminoheptanoic acid	—	1·20	1·51	Purple
2-Amino-4-hydroxyhept-6-ynoic acid lactone	—	0·75	—	Yellowish brown → purple quickly

at the C-4 atom, the olefin derived from Ia, and its *N*-acetyl derivative, were separately treated with ozone followed by hydrogen peroxide: chromatography indicated several ninhydrin-positive products, one of which behaved like *erythro*-γ-methylglutamic acid (there was no evidence for the formation of *threo*-γ-methylglutamic acid). The tentative Fischer projection of the natural isomer of Ia then would be as III.



(III)

Ia was tested for hypoglycaemic activity against mice, but the results were indefinite. When the animals had been starved for 20 hr before experiment, the higher dose rates of Ia

(400 mg/kg body wt.), injected subcutaneously, caused a marked depression of blood sugar levels and general moribundness, but no significant effects were measured when the experiment was repeated on mice-pre-starved for only 5 hr.

2-Amino-4-Hydroxymethylhex-5-Ynoic Acid (Ib)

This compound formed the minor acetylenic amino acid in the seed.

The assignment of structure followed mainly from consideration of the NMR and mass spectra, as in the case of Ia. The mass spectrum of Ib did not show a molecular ion under normal operating conditions, but at higher sample pressures an M+1 ion at *m/e* 158 was observed. This behaviour is commonly seen in the mass spectra of alcohols.[6] In conjunction with the base peak at *m/e* 112 (M-CO_2H, cf. the analogous base peak at *m/e* 96 in the spectrum of Ia), this established the molecular weight as 157 ($C_7H_{11}NO_3$). The NMR spectrum was again suggestive of a terminal acetylene, and this was confirmed by partial hydrogenation with the appearance of vinyl protons in the spectrum. Other features in the spectra were in agreement with the replacement of the methyl group in Ia by hydroxymethyl in Ib.

Hydrogenation in the presence of Adam's platinum catalyst gave a main product, presumed to be 2-amino-4-hydroxymethylhexanoic acid, and two minor products. One of these minor products showed identical chromatographic behaviour with homoisoleucine. After the mixture of reduction products, obtained using platinum catalyst, had been treated further with red P/HI, homoisoleucine formed the major ninhydrin-positive product.

2-Amino-4-Hydroxyhept-6-Ynoic Acid (II)

This compound was easily recognized on chromatograms of the initial seed extract because the colour of its ninhydrin chromophore had a brownish tinge. During elution from cation-exchange resin columns, II was present in two distinct groups of fractions: a part was eluted in the early fractions along with glutamic acid and alanine, while the remainder was present in later fractions eluting between γ-aminobutyric acid and the basic amino acids. Such elution behaviour is reminiscent of that shown by other hydroxyamino acids capable of forming γ-lactones. When II was treated with 2 N-HCl at 100° for 2 hr, considerable lactone formation occurred (with ninhydrin the lactone initially gave a yellow colour, which changed to brownish-purple during 24 hr); subsequent treatment with 10 N-NH_3 completely reversed this change.

The unbranched nature of the carbon skeleton of II was indicated by its conversion into 2-aminoheptanoic acid. II was first hydrogenated to yield the saturated 2-amino-4-hydroxyheptanoic acid and then red P/HI was employed to replace the hydroxyl group by a proton.

The NMR spectrum of II was deceptively simple due to the superposition of resonances from different protons, and the main spectroscopic evidence in favour of the proposed structure came initially from a detailed study of the mass spectrum. Again no molecular ion was observed under normal operating conditions, but the appearance of an ion at *m/e* 158 at higher sample pressures and the intense ion at *m/e* 112 ($C_6H_{10}NO$) (formulae in parentheses were established by high resolution measurements) suggested that the compound was an alcohol of molecular weight 157. This was confirmed by the recognition[7] of metastable ions linking the (unobserved) molecular ion with fragments at *m/e* 139 ($C_7H_9NO_2$) (the highest observed ion) and *m/e* 112. The prominent ion at *m/e* 118 ($C_4H_8NO_3$) is reasonably accounted

[6] T. H. BEYNON, R. A. SAUNDERS and A. E. WILLIAMS, *The Mass Spectra of Organic Molecules*, p. 142, Elsevier, Amsterdam (1968).

[7] K. R. JENNINGS, *J. Chem. Phys.* **43**, 4176 (1965).

for by the loss of the propargyl group, $HC{\equiv}C{-}CH_2{-}$, and that at *m/e* 74 ($C_2H_4NO_2$) is characteristic of the $NH_2{-}\underset{|}{C}H{-}CO_2H$ moiety. On raising the source temperature to 230°, new ions appeared in the spectrum at *m/e* 239 ($C_{11}H_{15}N_2O_4$), 221 ($C_{11}H_{13}N_2O_3$), 209 ($C_{10}H_{13}N_2O_3$), and 196 ($C_9H_{12}N_2O_3$) which may be readily accounted for in terms of fragmentation of the diketopiperazine derived from II. These results led to the adoption of structure II for the amino acid, and the NMR spectrum (see Experimental section) and that of the dihydro derivative are readily interpreted on this basis.

DISCUSSION

The new compounds isolated from *Euphoria* seed extend the range of acetylenic compounds recognized as plant constituents beyond the present large group of hydrophobic polyacetylenes. Certain polyacetylenes are thought to be formed by desaturation and decarboxylation of related long-chain saturated fatty acids and a similar desaturation mechanism would seem likely for the acetylenic amino acids.[8] Homoisoleucine then would form the logical precursor of compound Ia, but no comparable saturated precursor for II is known as a plant constituent; the nearest related substance is a dicarboxy derivative, α-aminopimelic acid.

The slight differences seen within the structures of the branched-chain C_7 amino acids produced by *Blighia*, *Euphoria* and *Aesculus* species may be rationalized with their involvement either as sequential intermediates in a biosynthetic pathway or as products of alternative pathways commencing from a common precursor. The C_7 compounds of *Aesculus* include homoisoleucine and this amino acid would seem to form the most likely parent compound in these biosyntheses: previously we have postulated[1] that homoisoleucine may be formed by a chain-elongating mechanism, commencing from isoleucine, and involving steps akin to those yielding leucine from valine, or homomethionine from methionine as a pre-requisite for sinigrin production in *Brassica nigra*.[9] Desaturation and hydroxylation, catalysed by specific enzymes, would lead to Ia and Ib, and to 2-amino-4-methylhex-4-enoic acid and 2-amino-6-hydroxy-4-methylhex-4-enoic acid in *E. longan* and *A. californica*, respectively. Hydroxylation of the C_7 skeleton may favour cyclization to give the cyclopropyl ring of hypoglycin A, but some form of additional activation reaction might be necessary, e.g. the formation of a phosphate ester in an ATP-dependent reaction. If these biogenetic ideas are valid, then a new and rigorous examination of the trace amino acid constituents of *Blighia* might reveal the chemical nature of the open-chain C_7 precursors of hypoglycin A. An alternative biosynthetic route leading to hypoglycin A can be formulated starting from 2-aminohex-4,5-dienoic acid, an allenic compound recently isolated from *Amanita solitaria*:[10] cyclopropane ring formation might occur by a C_1-transfer from *S*-adenosylmethionine across the penultimate double bond, so giving hypoglycin A directly. Support for this idea would require the identification of the allenic amino acid as a component of sapindaceous plants.

EXPERIMENTAL

Chromatography

Descending paper chromatograms were prepared on Whatman No. 3MM paper using the following solvents: 75% (w/w) phenol in the presence of NH_3 vapour, butan-1-ol–acetic acid–water (90:10:29, v/v), or *tert*-amyl alcohol–acetic acid–water (10:1:10, v/v, upper phase).

[8] J. D. Bu'Lock, *Prog. Org. Chem.* **6**, 86 (1964).
[9] M. D. Chisholm and L. R. Wetter, *Can. J. Biochem.* **42**, 1033 (1964).
[10] W. S. Chilton, G. Tsou, L. Kirk and R. G. Benedict, *Tetrahedron Letters* 6283 (1968).

Isolation of Amino Acids

Euphoria seed (5 kg) was extracted with 75% (v/v) ethanol. After concentration and decolorization, the separation of the soluble amino acid fraction was effected by standard ion-exchange preparative procedures employing a series of Zeokarb 225 (×8), Dowex 50 (×8) and Dowex 1 (×10) columns. During elution from a Dowex 50 column, compound Ib was present in early fractions together with the acidic amino acids and some serine, threonine and proline. Compound II was eluted next, although this γ-hydroxyamino acid was not displaced as a sharp peak presumably because some lactone formation occurred under the acidic conditions prevailing during absorption onto a cation-exchange resin. Finally, Ia was eluted together with the less hydrophilic neutral amino acids such as leucine, valine and phenylalanine, and smaller amounts of II. Compound Ia (520 mg) and II (170 mg) were recovered from appropriate groups of fractions by evaporation and recrystallizations from aqueous ethanol. Before Ib could be crystallized (150 mg), the acidic amino acids present in pooled fractions containing Ib were removed by passing through a Dowex 1 column, and contaminating serine, threonine and proline were separated by preparative paper chromatography using butanol–acetic acid–water as solvent.

Hydrogenations

Hydrogenations were performed at laboratory temperature and pressure. When Adam's PtO_2 catalyst was used, a few mg of the unsaturated amino acid, together with an approximately equal amount of catalyst, were added to 5% acetic acid solution (2–4 ml), and then H_2 was bubbled continuously through the mixtures. Samples were withdrawn after various time intervals, and the reduction products separated on chromatograms developed with *tert*-amyl alcohol–acetic acid–water: this solvent was particularly effective in separating the various saturated C_7 amino acids from the corresponding olefinic and acetylenic derivatives.

The Lindlar catalyst was prepared by treating 5% palladium on $CaCO_3$ with lead acetate.[11] In order to effect the quantitative reduction of acetylenic amino acids to their olefinic derivatives, the catalyst (110–130 μg/μmole of amino acid) in 40% (v/v) ethanol (5 ml) containing one drop of freshly-distilled quinoline was shaken in a micro-hydrogenator for 1–1½ hr to saturate with H_2. Then the acetylenic amino acid was added and shaking was continued until further H_2 uptake, which was initially rapid, had become very slow.

Properties of the Acetylenic Amino Acids

The presumed products obtained after hydrogenation of each acetylenic amino acid are given above, and their chromatographic behaviour is recorded in Table 1.

2-*Amino*-4-*methylhex*-5-*ynoic acid* (*Ia*). The recrystallized material (containing a small amount of contaminating leucine) had the following analysis: C, 60·1; H, 8·2; N, 9·9. $C_7H_{11}NO_2$ required: C, 59·6; H, 7·8; N, 9·9% $[\alpha]_D^{20}$ values were −33° (c, 2 in water) and −27° (c, 1 in 5 N-HCl). The homoisoleucine produced after hydrogenation had $[\alpha]_D^{20}$ −1° (c, 1 in water) and +22° (c, 0·5 in 5 N-HCl): these values compare with literature determinations of $[\alpha]_D^{20}$ −13° (water) and +21° (5 N-HCl) for the natural homoisoleucine of *Aesculus californica* and −2° (water) and +24° (5 N-HCl) for homoisoleucine produced by catalytic hydrogenation of 2-amino-4-methylhex-4-enoic acid of *Aesculus californica*.[1]

The NMR spectrum of Ia (measured at 60 MHz in deuterium oxide solution) had resonances centred at 8·81 (doublet, CH_3—CH<, 8·07 (multiplet, >CH—CH_2—CH<, 7·43 (broad singlet with underlying broad multiplet, HC≡C—CH<, and 6·12 ppm (poorly resolved triplet, $\overset{+}{N}D_3$—$\underset{|}{C}H$—CO_2^-) (3-trimethylsilylpropan-1-sulphonic acid sodium salt = 10 ppm). The mass spectrum obtained at 200° (source temp.) and 70 eV contained the following ions whose intensities are given in parentheses relative to the base peak (= 100). Ions below *m/e* 41 and of intensity less than 5 per cent of that of the base peak (except for the molecular ion and that at *m/e* 126) are excluded. 41 (14), 42 (12), 43 (9), 44 (19), 46 (5), 51 (5), 53 (22), 54 (5), 55 (5), 67 (6), 68 (5), 69 (6), 74 (35), 75 (5), 80 (8), 81 (10), 86 (9), 94 (5), 96 (100), 97 (10), 126 (1), 141 (0·2).

2-*Amino*-4-*hydroxymethylhex*-5-*ynoic acid* (*Ib*). Found: C, 52·7; H, 7·2; N, 10·3. $C_7H_{11}NO_3$ required: C, 53·5; H, 7·0; N, 8·9 %. $[\alpha]_D^{20}$ values measured were −27° (c, 2 in water) and −13° (c, 1 in 5 N-HCl).

The NMR spectrum of Ib had resonances centred at 7·97 (quartet, >CH—CH_2—CH<, 7·35 (doublet, J = 2·5 Hz, HC≡C—CH<, 7·26 (broad multiplet, HC≡C—CH<, 6·31 (doublet, J = 6 Hz, DOCH_2—CH<, and 6·02 ppm (quartet, $\overset{+}{N}D_3$—$\underset{|}{C}H$—CO_2^-). The mass spectrum showed the following ions (those of intensity less than 10 per cent of the base peak are generally omitted): 41 (33), 42 (33), 43 (36), 44 (25), 46 (13), 51 (13), 53 (25), 54 (12), 57 (13), 65 (17), 67 (39), 68 (12), 74 (54), 75 (30), 77 (14), 80 (34), 81 (18), 82 (34), 87 (13), 88 (17), 93 (22), 94 (39), 95 (54), 112 (100), 127 (8), 139 (1·2).

[11] H. LINDLAR, *Helv. Chim. Acta* **35**, 446 (1952).

2-Amino-4-hydroxyhept-6-ynoic acid (II). Found: C, 52·8; H, 6·9; N, 10·2. $C_7H_{11}NO_3$ required: C, 53·5; H, 7·0; N, 8·9%. $[\alpha]_D^{20}$ −27° (c, 2 in water). Optical rotation measurements in 5 N-HCl suggested progressive lactone formation: initially $[\alpha]_D^{20}$ was −8° (c, 1 in 5 N-HCl) but after 3 hr the value was +3°. After hydrogenation with Pt catalyst, the derived saturated amino acid was heated with red P and 55% (w/v) HI at 130° for 5 hr. 2-Aminoheptanoic acid was the main product as indicated by chromatography against authentic material synthesized by the method of Albertson.[12]

The NMR spectrum of II had resonances at 7·90 (triplet, $\rangle CH—CH_2—CH\langle$), 7·57 (broad doublet, $HC{\equiv}C—CH_2—$), and 6·09 ppm (broad triplet, $\overset{+}{N}D_3—\underset{|}{C}H—CO_2^-$ and $—CH_2—CH(OD)—CH_2—$). The mass spectrum (at 200° source temp.) contained the following ions (those of intensity less than 5 per cent of that of the base peak are generally omitted): 41 (24), 42 (16), 43 (13), 44 (100), 45 (7), 46 (11), 55 (6), 56 (17), 67 (6), 68 (23), 69 (7), 72·04514 (17), 74·02420 (83), 75 (6), 94·06651 (7), 95·0495 and 95·0727 (6), 112·0768 (54), 113 (6), 118·0515 (13), 139·0621 (0·3). At 230°, the compound gave a mass spectrum containing all the above ions but with the following in addition: 196·0833, 209·0928, 221·0934, 239·1038.

Acknowledgements—We wish to thank Professor L. B. Thrower (Hong Kong) for arranging to supply *Euphoria* seed, and the California Foundation for Biochemical Research for a grant to support Miss Sung.

[12] N. F. ALBERTSON, *J. Am. Chem. Soc.* **68**, 450 (1946).