Phytochemistry, 1968, Vol. 7, pp. 809 to 819. Pergamon Press. Printed in England

NEWLY CHARACTERIZED AMINO ACIDS FROM *AESCULUS CALIFORNICA*

L. FOWDEN and A. SMITH

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

(*Received* 15 *November* 1967)

Abstract—Five amino acids have been isolated and newly characterized from seed of *Aesculus californica*. The major component of the free amino acid pool of the seed is 2-amino-4-methylhex-4-enoic acid. It is accompanied by smaller quantities of its γ-glutamyl peptide, the corresponding saturated amino acid (homoisoleucine), and its 6-hydroxy derivative. The fifth new compound has been assigned the structure β-(methylenecyclopropyl)-β-methylalanine. The properties of each compound are described.

The role of 2-amino-4-methylhex-4-enoic acid as an analogue of phenylalanine has been considered in relation to the activity of the phenylalanyl-sRNA synthetase enzymes of *Aesculus* and other plant species.

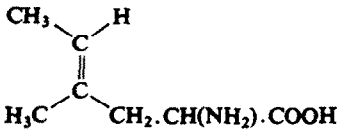
INTRODUCTION

EACH year several amino acids are isolated from higher plants and chemically characterized for the first time. Among some 150 non-protein amino acids so far recognized (see Fowden[1]) there are a number of examples of unsaturated compounds containing ethylenic linkages. These include γ-methyleneglutamic acid and γ-methyleneglutamine from peanuts[2] and tulips,[3] and 4-methyleneproline from seeds of loquat, *Eriobotrya japonica*.[4] The related saturated *erythro*-γ-methylglutamic acid and *trans*-4-methylproline either coexist with the unsaturates or are present in closely allied species (see review[1]). In other instances, e.g. γ-ethylideneglutamic acid from tulips[5] and the legume *Tetrapleura tetraptera*,[6] β-(methylenecyclopropyl)alanine (hypoglycin A) from *Blighia sapida*,[7] and α-(methylenecyclopropyl)-glycine from *Litchi chinensis*,[8] the corresponding methyl compounds are not yet known as natural products. Pipecolic acid and baikiain provide another example of related saturated and unsaturated compounds occurring in a single plant species, *Baikiaea plurijuga*.[9] The co-existence of structurally similar pairs of amino acids suggests that they may be interconverted in plants but strict experimental confirmation of this idea is still awaited.

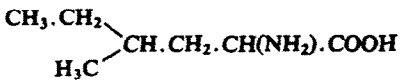
With the one exception of hypoglycin A, all the unsaturated amino acids mentioned are revealed on ninhydrin-treated chromatograms as spots coloured various shades of brown. The major component of the free amino acid fraction of seed of *Aesculus californica* gave a similar ninhydrin chromophore and this behaviour provided the first indication that it too was

[1] L. FOWDEN, *Ann. Rev. Biochem.* **33**, 173 (1964).
[2] J. DONE and L. FOWDEN, *Biochem. J.* **51**, 451 (1952).
[3] R. M. ZACHARIUS, J. K. POLLARD and F. C. STEWARD, *J. Am. Chem. Soc.* **76**, 1961 (1954).
[4] D. O. GRAY and L. FOWDEN, *Nature* **193**, 1285 (1962).
[5] L. FOWDEN, *Biochem. J.* **98**, 57 (1966).
[6] R. GMELIN and P. O. LARSEN, *Biochim. Biophys. Acta* **136**, 572 (1967).
[7] E. V. ELLINGTON, C. H. HASSALL, J. R. PLIMMER and C. E. SEAFORTH, *J. Chem. Soc.* 80 (1959).
[8] D. O. GRAY and L. FOWDEN, *Biochem. J.* **82**, 385 (1962).
[9] F. E. KING, T. J. KING and A. J. WARWICK, *J. Chem. Soc.* 3590 (1950).

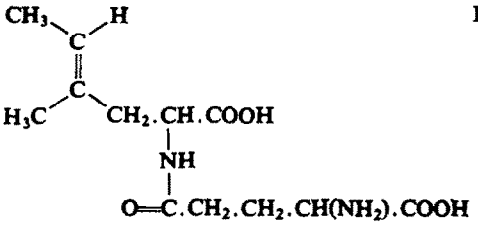
an unsaturated amino acid. After isolation, it has been characterized as 2-amino-4-methylhex-4-enoic acid (I), an unsaturated homoisoleucine isomer in which the two methyl groups bear a *cis* configuration about the double bond. In addition, γ-glutamyl-2-amino-4-methylhex-4-enoic acid (II) and 2-amino-4-methylhexanoic acid (III) have been identified as seed constituents, although the epimeric nature of III awaits elucidation. Lastly, small amounts of 2-amino-4-methyl-6-hydroxyhex-4-enoic acid (IV) and β-(methylenecyclopropyl)-β-methylalanine (V) have been isolated and structurally characterized using physical methods (see Addendum to this paper).



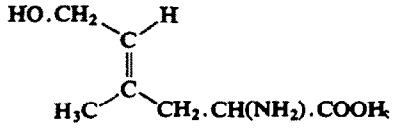
(I)



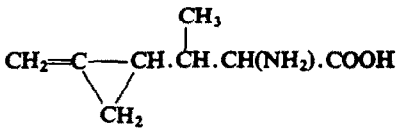
(III)



(II)



(IV)



(V)

RESULTS

The positions occupied by compounds I–V on a two-dimensional paper chromatogram (solvents phenol–ammonia followed by butan-1-ol–acetic acid–water) are shown diagrammatically in Fig. 1. When a chromatogram is prepared from an extract equivalent to 0·25 g fresh *Aesculus* seed, the spot attributable to I is predominant. It overlays the leucine area and is of such size and intensity that it not only masks leucine and isoleucine but also the much smaller amount of compound V present in the extract. Table 1 indicates the positions determined for the compounds in three solvent systems and also their normal ninhydrin colours.

The new compounds were isolated from a 75 per cent (v/v) ethanol extract of 8 kg fresh *Aesculus* seed from which the pericarp had been removed. The free amino acid fraction (85 g) was first separated from other components of the seed extract by absorption upon and elution from a large Zeokarb 225 cation-exchange resin column. Individual amino acids were separated subsequently using further ion-exchange resin column and paper chromatographic techniques (see Fig. 2).

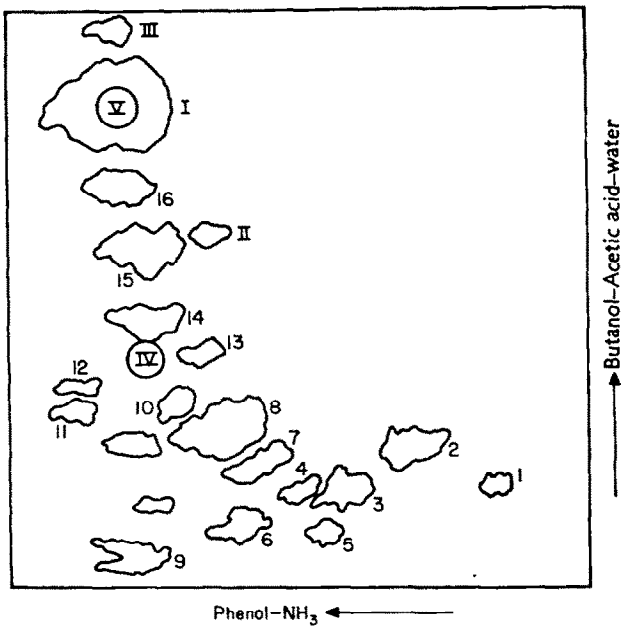


FIG. 1. TWO-DIMENSIONAL CHROMATOGRAM OF THE AMINO ACIDS PRESENT IN AN EXTRACT OF SEED OF *Aesculus californica*.

Key to chromatographic spots: 1, aspartic acid; 2, glutamic acid; 3, serine; 4, glycine; 5, asparagine; 6, glutamine; 7, threonine; 8, alanine; 9, basic amino acids; 10, β-alanine; 11, proline; 12, ethanolamine; 13, tyrosine; 14, γ-aminobutyric acid; 15, valine; 16, phenylalanine. I, 2-amino-4-methylhex-4-enoic acid; II, γ-glutamyl-2-amino-4-methylhex-4-enoic acid; III, 2-amino-4-methylhexanoic acid; IV, position of 2-amino-4-methyl-6-hydroxyhex-4-enoic acid; V, position of β-(methylenecyclopropyl)-β-methylalanine.

TABLE 1. R_f VALUES OF THE NEWLY CHARACTERIZED AMINO ACIDS OF *Aesculus*

Amino acid	R_f in phenol–NH_3	R_{Leu} in butanol–acetic–water	R_{Leu} in *tert*-amyl alcohol–acetic–water	Ninhydrin colour
2-Amino-4-methylhex-4-enoic acid (I)	0·85	0·99	1·00	Yellow–brown
γ-Glutamyl peptide of I (II)	0·68	0·75	0·69	Blue
2-Amino-4-methylhexanoic acid (III)	0·87	1·14	1·29	Blue–purple
2-Amino-4-methyl-6-hydroxy-hex-4-enoic acid (IV)	0·80	0·48	0·49	Yellow–brown
β-(Methylenecyclopropyl)-β-methylalanine (V)	0·85	1·00	1·06	Blue–purple
Lactone of I	0·86	0·96	—	Red–brown → purple (rapid)
γ-Hydroxy acid of III	0·83	0·79	—	Red–brown → purple (slow)

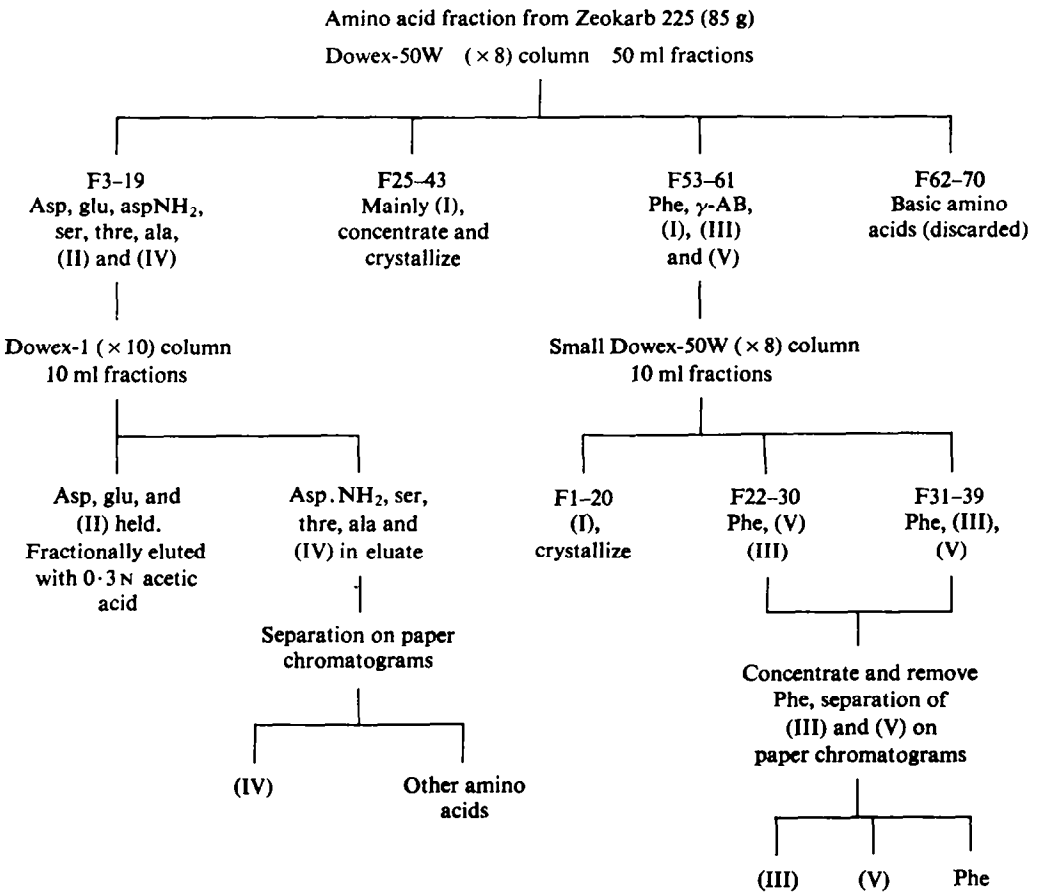


FIG. 2. FRACTIONATION STEPS USED TO ISOLATE COMPOUNDS I–V FROM AN EXTRACT OF *Aesculus* SEED.

2-*Amino*-4-*Methylhex*-4-*Enoic Acid* (*I*)

This substance accounted for about half of the total free α-amino-N of the seed and 26 g were eventually isolated.

Assignment of structure was based on the following evidence. Elementary analysis confirmed the empirical formula $C_7H_{13}NO_2$. Hydrogenation using Adam's platinum catalyst converted I into a compound chromatographically inseparable from compound III, analysing as $C_7H_{15}NO_2$. The reduction product was also inseparable from homoisoleucine (the "slowest" of the products obtained by hydrogenation of hypoglycin A after their separation on a chromatogram developed in *tert*-amyl alcohol–acetic acid–water solvent[10]), and so the C_7 skeleton of I is probably branched at the γ-carbon atom. The presence of a single C=C bond, indicated by hydrogenation and analysis, was further confirmed by the instability of I to treatment with acidic permanganate. Chromatographic methods indicated that several ninhydrin-reactive oxidation products were formed in small yield, but these were not identified. A γ,δ-unsaturation was indicated by the observed conversion of I into a γ-lactone on treatment with 6 *N*-hydrochloric acid, and by the subsequent conversion of the

[10] D. O. GRAY, J. BLAKE, D. H. BROWN and L. FOWDEN, *J. Chromatogr.* **13**, 276 (1964).

lactone into the corresponding hydroxyamino acid (2-amino-4-hydroxy-4-methylhexanoic acid) when treated with strong ammonia solution.

N.M.R. spectroscopy allowed a decision to be made between the two possible isomeric structures satisfying these findings, i.e. between 2-amino-4-methylhex-4-enoic acid and 2-amino-4-methylenehexanoic acid, because the spectrum obtained from I clearly indicated the presence of two methyl groups. Therefore DL-2-amino-4-methylhex-4-enoic acid was synthesized following the method of Edelson *et al.*,[11] and shown to behave identically with I when compared by chromatographic, and i.r. and NMR spectroscopic methods. The behaviour of the natural and synthetic compounds during hydrogenation and lactonization was also identical. Specific optical rotation values measured for I in water and in 6 N-hydrochloric acid showed the shift to more positive rotations in acid generally associated with an L-configuration in amino acids.

γ-Glutamyl-2-Amino-4-Methylhex-4-Enoic Acid (II)

This peptide was displaced by 0·3 *N*-acetic acid from a Dowex-1 column after glutamic and aspartic acids. The fractions containing II, uncontaminated by other ninhydrin-positive compounds, were evaporated to yield a glassy residue. This was redissolved in a small volume of water and lyophilized to give a fluffy, slightly hygroscopic, crystalline residue (1·6 g). Elementary analysis agreed with the formula $C_{12}H_{20}N_2O_5 . H_2O$, i.e. that of a monohydrate of γ-glutamyl-2-amino-4-methylhex-4-enoic acid. Although other γ-glutamyl peptides exist as monohydrates, e.g. γ-glutamyl-β-pyrazol-1-ylalanine,[12] the hygroscopic character of II leaves some doubt whether the 7·0 per cent loss of weight on heating at 105°, equivalent to one molecule of water of crystallization, is not simply fortuitous.

The peptide was completely hydrolysed by heating with *N*-hydrochloric acid for 2 hr at 100°; this lability to dilute mineral acid is typical of a γ-glutamyl peptide. When II was hydrolysed with 6 *N*-hydrochloric acid, the lactone of I was produced together with glutamic acid. The lactone was further characterized by conversion to the corresponding hydroxyamino acid during treatment with strong ammonia solution. Alkaline hydrolysis using *N*-barium hydroxide at 100° for 4 hr produced glutamic acid and I in equimolar amounts and, after separation on an anion-exchange resin column, the two products were confirmed by i.r. spectroscopy and established as L-isomers by polarimetry.

Dinitrophenylation of the peptide followed by acid hydrolysis gave dinitrophenylglutamic acid and the lactone of I (identified chromatographically), indicating that the peptide-N atom was derived from the α-amino group of I, i.e. a glutamyl link was confirmed.

2-*Amino-4-Methylhexanoic Acid (III)*

This compound (140 mg) was finally separated from V by preparative paper chromatography using *tert*-amyl alcohol–acetic acid–water as the solvent system. It was readily recrystallized from aqueous ethanol and analysed as $C_7H_{15}NO_2$. III was inseparable from the homoisoleucine, obtained by hydrogenating I, in all four solvents listed in the experimental section; in *tert*-amyl alcohol–acetic acid–water it had a slightly lower R_f than the isomeric homoleucine and homonorleucine (obtained together with homoisoleucine as products of hydrogenation of hypoglycin A).

[11] J. EDELSON, C. G. SKINNER, J. M. RAVEL and W. SHIVE, *J. Am. Chem. Soc.* **81**, 5150 (1959).
[12] P. M. DUNNILL and L. FOWDEN, *Biochem. J.* **86**, 388 (1963).

III gave an i.r. spectrum almost identical with that obtained from the reduction product of I, and the NMR spectra of these two preparations, although not identical, contained many common features indicating their close similarity. It is suggested that III and the reduction product of I are both homoisoleucines; III is likely to be a single isomer (i.e. either the normal L or the *allo*-L form, the L-configuration being indicated by optical rotation measurements in water and mineral acid), whereas reduced I may consist of a mixture of the two L-diastereoisomers. The solubility of III in water was less than that of reduced I; since isoleucine has a lower solubility than *allo*isoleucine, III may possess the same relative configurations about its two asymmetric carbon atoms as exists in isoleucine.

2-Amino-4-Methyl-6-Hydroxyhex-4-Enoic Acid (IV)

This substance reacts with ninhydrin to give a yellow–brown colour similar to that produced from I. IV was separated finally on paper chromatograms and, after elution, the solution of IV was decolorized and lyophilized to yield 210 mg of a hygroscopic solid.

Hydrogenation of IV gave a mixture of products. When Adam's platinum catalyst was employed during hydrogenation at laboratory temperature and pressure, the principal product was homoisoleucine (reduced I): a small quantity of I appeared as a transient reduction product. A smaller amount of a second ninhydrin positive compound (presumably 2-amino-4-methyl-6-hydroxyhexanoic acid) was present at the end of the hydrogenation procedure. This substance gave the more normal blue–purple colour with ninhydrin and moved only slightly faster than IV (R_{Leu} 0·56 compared with 0·49) on chromatograms developed with *tert*-amyl alcohol–acetic acid–water solvent. When 10 per cent palladium on carbon was used as a catalyst, the saturated hydroxyamino acid formed the major product of hydrogenation. It was more difficult to remove the hydroxyl group by hydrogenation once the double bond had been saturated, i.e. once formed, 2-amino-4-methyl-6-hydroxyhexanoate was converted only very slowly to homoisoleucine.

NMR and mass spectrometric methods were used to elucidate the structure of IV (see Addendum to this paper).

β-(Methylenecyclopropyl)-β-Methylalanine (V)

This substance was obtained only in small yield (65 mg) and the assignment of structure V was based largely upon the results of NMR and mass spectroscopic studies (see Addendum).

V moves slightly ahead of leucine on chromatograms developed in *tert*-amyl alcohol–acetic acid–water (R_{Leu} 1·06). Hydrogenation of V in the presence of platinum catalyst yielded two major amino acid products having R_{Leu} values of 1·20 and 1·41 and a minor one at R_{Leu} 1·53. This pattern of hydrogenation products resembled that obtained after hydrogenation of hypoglycin A and suggested that V also might contain a cyclopropyl ring. The R_{Leu} values of the products from V are slightly higher than those arising from hypoglycin A, a fact agreeing with the idea that they are C_8 amino acids. Probably the substance having R_{Leu} 1·20 is β-(methylcyclopropyl)-β-methylalanine, while the two faster-moving amino acids result by reductive splitting of the cyclopropyl ring at different points.

DISCUSSION

The occurrence of 2-amino-4-methylhex-4-enoic acid as the predominant compound of the free amino acid pool of *Aesculus* seed raises the intriguing question of the role, if any, that this (and the structurally related) amino acids play in the metabolism of the plant. In

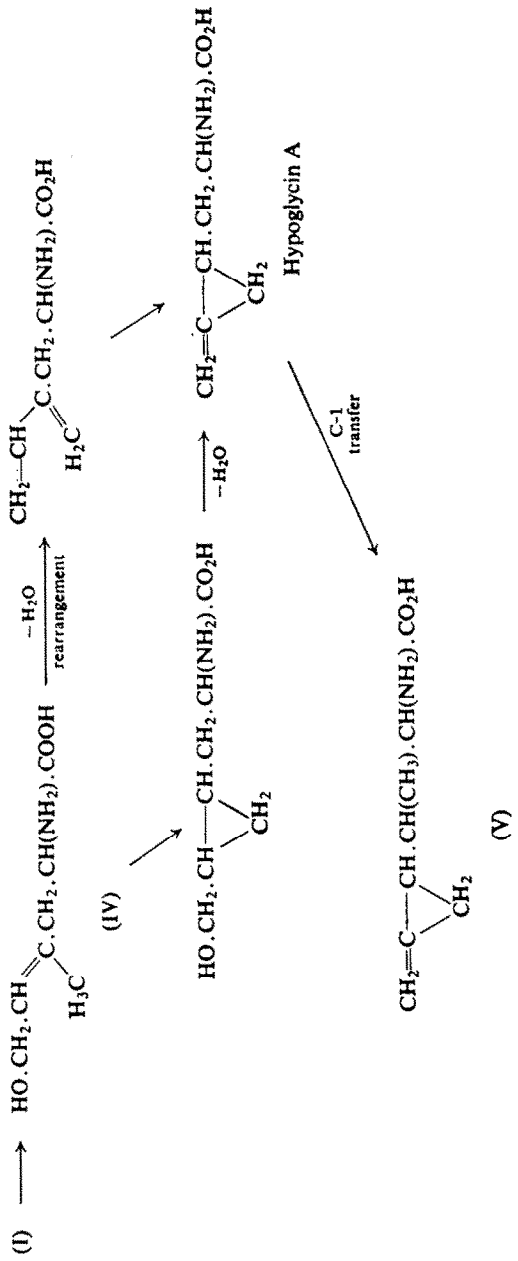
instances where other non-protein amino acids are encountered in particularly high concentrations in seeds, e.g. canavanine in many legumes,[13] the suggestion is made that the compounds may act as nitrogen storage materials which are degraded during the subsequent germination of the seeds making a pool of nitrogen readily available for the onset of biosynthetic processes. No study of the behaviour of 2-amino-4-methylhex-4-enoic acid during germination of *Aesculus californica* has yet been made, but it is noteworthy that seed of *A. hippocastanum* (horse chestnut) completely lacks this amino acid. Whatever the role of I may be, a significant proportion of the total nitrogen turnover occurring during seed formation and germination must be organized very differently in these two *Aesculus* species.

The fact that I is accompanied in the *Aesculus* seeds by smaller amounts of its γ-glutamyl peptides has a number of precedents among non-protein amino acids; for example hypoglycin A coexists with hypoglycin B [γ-glutamyl-β-(methylenecyclopropyl)alanine] in seeds of *Blighia sapida*,[7] while smaller quantities of γ-glutamyl-β-pyrazol-1-ylalanine are found together with β-pyrazol-1-ylalanine in many cucurbit seeds.[14] Indeed, γ-glutamyl peptides are prone to accumulate in seeds and other storage organs of plants, and are frequently hydrolysed relatively rapidly during the onset of new growth. Their biosynthesis from the constituent non-protein amino acid seems to occur by γ-glutamyl transfer from a suitable donor (e.g. glutathione), a process catalysed by a fairly unspecific transferase enzyme.[15]

Compounds III, IV and V probably also are biogenetically related to 2-amino-4-methylhex-4-enoic acid. Attention has been drawn previously to the frequent co-occurrence of structurally related saturated and unsaturated amino acids, but in no one instance does it seem certain which type of compound represents the primary product of biosynthesis. This is certainly the present situation in *Aesculus* when compounds I and III are considered. By analogy with other biosynthetic reactions, the formation of the 6-hydroxyamino acid (IV) probably results by hydroxylation of I. However, the most intriguing biogenetic possibility is that raised by the co-existence of V with compounds I and IV, because the unsaturated branched chain C_7 amino acids may be the precursors of amino acids containing the cyclopropyl ring system. The following reactions can be postulated as enzyme-catalysed steps in the conversion of I, via IV, into compound V. Cyclopropyl ring formation then would involve the formation of a new bond between C-5 and the 4-methyl C atom of IV. Alternative pathways may be envisaged depending upon whether cyclopropane ring formation precedes or follows dehydration. Unfortunately, there seem to be no strictly analogous chemical conversions. Cyclopropane ring formation has been observed to occur during reduction of allylic alcohols (cinnamyl derivatives) with lithium aluminium hydride,[16] but in this case the new C—C link involves the equivalent of C-4 and C-6 in IV. On the other hand, dehydration of IV under electron impact, probably with re-arrangement to a butadiene-type structure, was indicated by the mass spectrometric studies. To be speculative, the prior formation of the cyclopropane ring after activation of the allylic methyl group of IV might be favoured; dehydration of the intermediary hydroxymethyl compound would then yield the exocyclic methylene group of hypoglycin A. Finally, the β-methyl group of compound V presumably is inserted by a C_1 transfer from some suitable donor such as methionine.

If cyclopropyl ring formation does not occur from IV, then hypoglycin A and V on the

[13] B. L. Turner and J. B. Harborne, *Phytochem.* **6**, 863 (1967).
[14] P. M. Dunnill and L. Fowden, *Phytochem.* **4**, 933 (1965).
[15] J. F. Thompson, D. H. Turner and R. K. Gering, *Phytochem.* **3**, 33 (1964).
[16] M. J. Jorgenson and A. W. Friend, *J. Am. Chem. Soc.* **87**, 1815 (1965).


(I)
HO.CH2.CH
C.CH2.CH(NH2).COOH
H3C
(IV)
HO.CH2.CH
CH.CH2.CH(NH2).CO2H
CH2
−H2O
rearrangement
CH2—CH
C.CH2.CH(NH2).CO2H
H2C
−H2O
CH2=C
CH.CH2.CH(NH2).CO2H
CH2
Hypoglycin A
C-1
transfer
CH2=C
CH.CH(CH3).CH(NH2).CO2H
CH2
(V)

one hand, and I and IV, might share a common precursor such as tiglyl-CoA. If this were so, ring closure might occur at the C_5 level and subsequent addition of an acetyl-CoA residue then could give the C_7 skeleton of hypoglycin A and V: alternatively, tiglyl-CoA might condense directly with acetyl-CoA to give the open-chain structure of I and IV.

If the above scheme is essentially correct, then compounds I and IV become intermediates in the biosynthesis of hypoglycin A, which in turn is converted into compound V. It is possible that hypoglycin A exists in *Aesculus* seed for its chromatographic properties are such that it would fall in the same area as I and V on chromatograms, and so the presence of a minor amount could well have been overlooked during the fractionation procedures. From the standpoint of comparative biochemistry, it is noteworthy that the only known source of hypoglycin A is the species *B. sapida*, which is a member of the family (Sapindaceae) most closely allied to the Hippocastanaceae, in which the genus *Aesculus* is placed. Possibly similar biosynthetic pathways operate within the members of these two families, and the accumulation of different major products in related species may reflect only differing patterns in the activity of enzymes catalysing individual steps. Certainly, it will be interesting to learn whether 2-amino-4-methylhex-4-enoic acid, or its 6-hydroxy derivative, can act as a precursor of hypoglycin A in *B. sapida*.

2-Amino-4-methylhex-4-enoic acid has been synthesized previously by two distinct routes.[11,17] Shive and co-workers[11] also drew attention to the growth-inhibitory properties of this unsaturated amino acid upon cultures of *Leuconostoc dextranicum*. The inhibition could be reversed by incorporation of phenylalanine in the growth media. 2-Amino-4-methylhexanoic acid (homoisoleucine, III) also produced inhibition of *Escherichia coli* growth; in this case the compound was a leucine antagonist and normal growth could be restored by adding leucine to the culture media. Preliminary experiments in our laboratory indicate that I exhibits a weak growth-inhibitory action upon mung bean seedlings.

The *cis* configuration of the two methyl groups about the double bond confers on I a partial phenylalanine structure. The β-carbon atom and the group of four attached carbon atoms beyond are planar: they then appear like an incomplete phenyl ring attached to the β-carbon of the alanyl side-chain of the phenylalanine molecule. In a separate investigation,[18] it has been observed that a partially purified preparation of the phenylalanyl-sRNA synthetase from mung bean seeds can utilize I as a substrate: indeed, under the standard assay conditions used, the rate of ATP–$^{32}PP_i$ exchange measured in the presence of I can exceed that determined with the normal substrate phenylalanine, although a comparison of the K_m values calculated for the two amino acids reveals that the enzyme has a higher affinity for phenylalanine. These observations raise an intriguing question in relation to amino-acid activation in *Aesculus* seeds where the relative concentrations of I and phenylalanine (at least 20:1) would ensure that some 2-amino-4-methylhex-4-enoate-adenylate derivative was formed if the phenylalanine-activating enzyme of *Aesculus* exhibited similar substrate specificity to that of mung bean. Is I excluded from *Aesculus* seed protein by the existence in this species of a phenylalanyl-sRNA synthetase that discriminates against the amino acid by failing to activate it? This situation then would be comparable to that encountered previously with azetidine-2-carboxylic acid and the prolyl-sRNA synthetase of liliaceous species.[19] Alternatively, the phenylalanine-activating enzyme of *Aesculus* may effect the formation of an adenylate complex of I, but fail to catalyse the further transfer of I to t-RNA^{phe}.

[17] K. HEYNS, K. MOLGE and W. WALTER, *Chem. Ber.* **94**, 1015 (1961).
[18] I. K. SMITH and L. FOWDEN, *Phytochem.*, in press.
[19] P. J. PETERSON and L. FOWDEN, *Biochem. J.* **97**, 112 (1965).

EXPERIMENTAL

Paper Chromatography

Two-dimensional chromatograms were developed with 75% (w/w) phenol in the presence of NH_3 vapour, followed by a one-phase butan-1-ol–acetic acid–water mixture (90:10:29, v/v). Spots were revealed using 0·1% ninhydrin in ethanol as the chromogenic reagent. The upper phase of a *tert*-amyl alcohol–acetic acid–water mixture (10:1:10, v/v) was used for some one-dimensional chromatograms.

Extraction and Isolation of New Compounds

Fresh *Aesculus* seed (8 kg), lacking the pericarp, was macerated in 75% (v/v) ethanol (20 l.) and stored with intermittent stirring for 1 week. The slurry was pressed (6000 lb/in²) through a thick cloth and the expressed extract was filtered and concentrated in a vortex evaporator to 4 l. The concentrate was decolorized by treating with charcoal and the pH was adjusted to 4·4 to precipitate residual protein. After filtering, water was added to 10 l. and the whole was applied to a Zeokarb 225 (× 8) column (mesh 52–100, 100 cm × 10 cm). The column was thoroughly washed with water (60 l.) to remove non-cationic material and then the amino acids were eluted with N-NH_3, 50 ml fractions being collected. Fraction No. 1–17, containing only aspartic and glutamic acids and some asparagine, and No. 60–83, containing basic amino acids, were discarded. The remaining intermediate fractions were pooled and evaporated *in vacuo* to yield 85 g of mixed amino acids. The further fractionation steps have been outlined in Fig. 2.

Dowex-50 W (× 8) separation. The solution (1·5 l., pH 4·5) of the mixed amino acids was fractionated next on a Dowex 50 W (× 8) column (mesh 100–200, 100 cm × 5 cm). Elution was with 0·2 N-NH_3 and 70 ninhydrin-positive fractions (50 ml) were collected. No. 25–43 were pooled, evaporated and decolorized; after further concentration to 100 ml, compound I crystallized out and additional amounts were recovered from the mother liquors.

Dowex-1 (× 10) separation. Fraction No. 3–19 from the previous column, adjusted to pH 7, were applied to this type of column (mesh 100–200, 70 cm × 1·5 cm). IV, together with some asparagine, serine, threonine and alanine, was not held by the resin and was collected in the eluate at this stage. After thoroughly washing the column, 0·3 N-acetic acid was used to displace the acidic amino acids. Fractions (10 ml) were collected and glutamic acid, aspartic acid and II were clearly separated, being eluted in that order from the column. The fractions containing II were initially evaporated to dryness at low temperature, redissolved in a little water, decolorized and lyophilized to give a light, crystalline solid.

The solution containing IV and other amino acids was evaporated to give a solid residue (1·9 g). A concentrated solution of the amino acids was streaked across ten sheets of Whatman 3MM filter paper (width 23 in.) which were developed in butanol–acetic acid–water for 3 days. Bands of IV were well resolved from other amino acids and IV was eluted with hot water. The eluate was concentrated to about 50 ml, decolorized and lyophilized to give IV as a semi-crystalline solid.

Second Dowex-50 W (× 8) separation. Fraction No. 53–61 from the previous Dowex-50 column were pooled, adjusted to pH 4·5, and applied to this smaller column (mesh 100–200, 60 cm × 1·5 cm). 0·2 N-NH_3 was used to elute the amino acids, 50 fractions (10 ml) being collected. Further amounts of I were obtained from fractions 1–20. No. 22–30 contained phenylalanine, V and a little III; No. 31–39 contained phenylalanine, III and a small amount of V. These two groups of fractions were combined and concentrated when phenylalanine (1·5 g) crystallized and was removed. The remaining solution containing 1·75 g of mixed amino acids was streaked across eight sheets of Whatman 3MM filter paper and the chromatograms were developed in *tert*-amyl alcohol–acetic acid–water for 80 hr. The bands of III and V were cut from each chromatogram and the amino acids eluted with hot water. After concentrating, each solution was decolorized and evaporated to dryness *in vacuo* to yield the amino acids. III was recrystallized from aqueous ethanol but compound V was used directly in the further investigations.

Properties of the New Amino Acids

2-Amino-4-methylhex-4-enoic acid (I). The recrystallized material had the following analysis: C, 58·2; H, 8·9; N, 9·7. $C_7H_{13}O_2N$ required: C, 58·7; H, 9·1; N, 9·8%. The $[\alpha]_D^{20}$ values measured were $-61°$ (*c*, 2·4 in water) and $-36°$ (*c*, 1·2 in 6 N-HCl).

A solution of I in dil. acetic acid was hydrogenated at laboratory temperature and pressure using Adam's platinum catalyst to give homoisoleucine. (Found: C, 57·7; H, 10·3; N, 9·5. Calc. for $C_7H_{15}O_2N$: C, 57·9; H, 10·3; N, 9·7%.)

2-Amino-4-methylhex-4-enoic acid was synthesized by the method of Shive and co-workers.[11] Tiglyl aldehyde (100 g) was reduced to the corresponding alcohol (69 g) using $NaBH_4$. The tiglyl alcohol was converted into the related bromide (79 g) by treating with PBr_3. The bromide was condensed with the sodium derivative of ethyl acetamidocyanoacetate in absolute ethanol and then the condensation product (ethyl 2-acetamido-2-cyano-4-methylhex-4-enoate) was hydrolysed by refluxing with 5 N-barium hydroxide for 18 hr. Barium was precipitated as sulphate and the 2-amino-4-methylhex-4-enoic acid (22 g) was separated

from small amounts of glycine (arising by hydrolysis of residual acetamidocyanoacetate) on a Dowex-50 W column. The use of $Ba(OH)_2$ for hydrolysis, instead of conc. NaOH as used previously,[11] allowed the product to be recovered more easily and efficiently.

Natural and synthetic materials were shown to be identical by paper chromatographic, i.r. and NMR spectroscopic methods. The i.r. spectra prepared from nujol mulls of the two compounds were identical although a natural L-isomer was being compared with a synthetic DL-racemate. Both compounds were lactonized on treatment with 6 N-HCl, and the lactones were converted into the corresponding γ-hydroxy-amino acids when heated in a sealed ampoule with 0·880 NH_3.

The isomeric 2-amino-4-methylenehexanoic acid (kindly donated by Dr. W. Shive, Austin, Texas) was not separated from I with the chromatographic solvents used, but it gave a ninhydrin colour that was distinctly more yellow.

γ-Glutamyl-2-amino-4-methylhex-4-enoic acid (II). The lyophilized material analysed as: C, 48·8; H, 7·9; N, 9·7; loss at 105°, 7·0%. $C_{12}H_{20}O_5N_2.1H_2O$ required: C, 49·6; H, 7·6; N, 9·6; H_2O, 6·2%. The $[\alpha]_D^{20}$ value was +17° (*c*, 3 in H_2O).

II was hydrolysed with N-$Ba(OH)_2$ at 100° for 4 hr. After removal of barium as sulphate, aliquots of glutamic acid and I in the hydrolysate were separated on chromatograms developed in butan-1-ol–acetic acid–water and the amounts of each determined quantitatively by the method of Atfield and Morris.[20] The peptide (300 mg, 1·03 mmoles calculated as the monohydrate) gave glutamic acid (146 mg, 0·99 mmole) and I (154 mg, 1·08 mmoles). The remainder of the hydrolysate was applied to a Dowex-1 column: I passed through and was recovered from the initial eluate, while glutamic acid was displaced by N-acetic acid. Optical rotation measurements indicated that each product was the L-isomer, i.e. for I, $[\alpha]_D^{20}$ −58° (*c*, 2 in H_2O); for glutamic acid $[\alpha]_D^{20}$ +30° (*c*, 2 in 5 N-HCl).

Dinitrophenylation of II (5 mg) in 1% $NaHCO_3$ (3 ml) was performed in the dark with 1-fluoro-2,4-dinitrobenzene (10 μl) for 1 hr at 40°. Unchanged fluorodinitrobenzene was extracted with ether and, after acidification of the aqueous phase, the dinitrophenylated peptide was also transferred to ether. After evaporation, the residue was hydrolysed with 6 N-HCl at 100° for 3 hr. Chromatographic examination of the hydrolysis products revealed the lactone of compound I and dinitrophenylglutamic acid. No free glutamic acid was detected.

Homoisoleucine (III). Elementary analysis indicated: C, 57·4; H, 10·2; N, 9·4. $C_7H_{15}NO_2$ required: C, 57·9; H, 10·3; N, 9·7%. Optical rotations measured for III gave $[\alpha]_D^{20}$ −13° (*c*, 1 in H_2O) and +20·5° (*c*, 0·87 in 5 N-HCl), suggesting an L-isomer.

The product obtained after hydrogenating I in the presence of a platinum catalyst was inseparable from III when the two substances were co-chromatographed in each of the solvent systems listed above. The i.r. spectra of the two compounds differed very slightly (the magnitude of the differences was similar to that observed between L-isoleucine and *allo*-L-isoleucine[21]). The specific rotations measured for the reduction product of I were $[\alpha]_D^{20}$ −2° (*c*, 1 in H_2O) and +24° (*c*, 0·87 in 5 N-HCl).

2-Amino-4-methyl-6-hydroxyhex-4-enoic acid (IV). This substance had $[\alpha]_D^{20}$ −31° (*c*, 2·2 in H_2O) and +2° (*c*, 1·1 in 5 N-HCl) indicating an L-configuration. It was unstable when treated with 6 N-HCl at 100°. Probably lactonization involving loss of the γ,δ-double bond occurs, as with I, but the nature of the decomposition products have not been studied.

β-(Methylenecyclopropyl)-β-methylalanine (V). An L-configuration was suggested by $[\alpha]_D^{20}$ values of +1·5° (*c*, 2 in H_2O) and +45° (*c*, 1 in 5 N-HCl).

Acknowledgements—We should like to thank Professor G. W. Kenner (Liverpool) for his advice on structural problems concerning IV and V, Professor E. E. Conn (Davis, California) for supplying seed of *Aesculus californica*, and the Californian Foundation for Biochemical Research for a grant to initiate this work.

[20] G. N. Atfield and C. J. O. R. Morris, *Biochem. J.* **81**, 606 (1961).
[21] J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 3, p. 2072. Wiley, New York (1961).