Phytochemistry, 1972, Vol. 11, pp. 3521 to 3523. Pergamon Press. Printed in England.

NITROGENOUS CONSTITUENTS OF *BILLIA HIPPOCASTANUM* AND *ACER PSEUDOPLATANUS*

L. FOWDEN, HELEN M. PRATT and A. SMITH

Department of Botany and Microbiology, University College, London WC1

(*Received* 20 *July* 1972. *Accepted* 1 *August* 1972)

Key Word Index—*Billia hippocastanum*; *Acer pseudoplatanus*; hypoglycin A and B; α-(methylenecyclopropyl)glycine; γ-L-glutamyl-L-α-(methylenecyclopropyl)glycine; γ-glutamyl peptides.

Abstract—γ-L-Glutamyl-L-α-(methylenecyclopropyl)glycine has been characterized as a new plant constituent from seeds of *Billia hippocastanum* and fruits of *Acer pseudoplatanus*. The sycamore fruits also contained hypoglycins A and B, and α-(methylenecyclopropyl)glycine. The γ-glutamyl peptides of aspartic acid, asparagine, glutamic acid, valine, alanine and threonine were also isolated from *Billia* seed.

INTRODUCTION

PREVIOUS studies have revealed that a group of C_6 and C_7 branched-chain amino acids, some of which contain cyclopropyl residues, form characteristic constituents of seeds of many members of the families Sapindaceae and Hippocastanaceae (see summary in Fowden *et al.*[1]). Seeds of species which accumulate particular amino acids in high concentration also frequently contain the corresponding γ-glutamyl peptides: thus hypoglycin A and its γ-glutamyl peptide (hypoglycin B) occur together in seeds of *Blighia sapida* (Sapindaceae)[2] and *Billia hippocastanum* (Hippocastanaceae),[3] while other seeds contain associations of 2-amino-4-methylhex-4-enoic acid and its γ-glutamyl derivative (*Aesculus californica*, Hippocastanaceae),[4] or *trans*-α-(carboxycyclopropyl)glycine and the related peptide (*Blighia sapida*).[5]

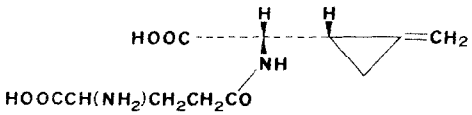
This paper reports new isolations and characterizations of some of the above amino acids and peptides, or of other similar compounds, from seed of *B. hippocastanum* and from mature fruits of *Acer pseudoplatanus* (sycamore, Aceraceae).

RESULTS AND DISCUSSION

In an earlier investigation,[3] the seed of *B. hippocastanum* was shown to be a rich source of hypoglycins A and B and to contain also α-(methylenecyclopropyl)glycine. A more detailed study showed the presence of L-γ-glutamyl-L-α-(methylenecyclopropyl)glycine (I) which represents a new constituent of plants. This peptide also has been isolated from the fruits of sycamore, where it occurs as a significant constituent in association with hypoglycins A and B and α-(methylenecyclopropyl)glycine. Several other γ-glutamyl derivatives were

[1] L. FOWDEN, J. W. ANDERSON and A. SMITH, *Phytochem.* **9**, 2349 (1970).
[2] E. V. ELLINGTON, C. H. HASSALL, J. R. PLIMMER and C. E. SEAFORTH, *J. Chem. Soc.* 80 (1959).
[3] J. N. ELOFF and L. FOWDEN, *Phytochem.* **9**, 2423 (1970).
[4] L. FOWDEN and A. SMITH, *Phytochem.* **7**, 809 (1968).
[5] L. FOWDEN and A. SMITH, *Phytochem.* **8**, 1043 (1969).

isolated from *Billia* seed: these included γ-glutamylglutamic acid, γ-glutamylaspartic acid and γ-glutamylasparagine (all recently characterized as components of *Acacia georginae* seed[6]), γ-glutamylvaline and γ-glutamylalanine (isolated earlier by Virtanen *et al.* from onion bulbs[7] and pea seedlings,[8] respectively), and γ-glutamylthreonine (tentative identification).



(I)

In this investigation of *B. hippocastanum* seed, and in a previous one utilizing *A. georginae* seed,[6] particular emphasis was placed upon ninhydrin-reactive components present in anionic fractions retained on Dowex 1 resin columns. It seems clear from the results that if a sufficiently large-scale and careful fractionation is followed, many other seed types may be expected to yield a considerable number of γ-glutamyl peptides. Presumably, γ-glutamyl transpeptidases of the type described by Thompson *et al.*[9] are present in most maturing seeds and catalyse the transfer of a glutamyl residue from a donor, such as glutathione, to a range of amino acids.

Another interesting feature of the results is that the potential to elaborate hypoglycin A and α-(methylenecyclopropyl)glycine extends to the Aceraceae. At one time, species now assigned to the Aceraceae, like those forming the Hippocastanaceae, were all grouped within an extended Sapindaceae. The present study provides chemical evidence, albeit limited in extent, that would support the suggestion of some taxonomists that the three families logically might be recombined.

Hypoglycins A and B and γ-glutamyl-α-(methylenecyclopropyl)glycine were present at lower concentrations in young leaves taken from 1- and 2-year-old sycamore plants, but were barely detectable in similar leaves from mature trees. The compounds could not be detected by chromatographic methods in free cell cultures of sycamore stem callus.

Fruits of sycamore contained the following minimum levels (g/kg) of cyclopropyl derivatives: hypoglycin A, 2; hypoglycin B, 5; γ-glutamyl-α-(methylenecyclopropyl)glycine, 1·5; and α-(methylenecyclopropyl)glycine, 0·5. Hypoglycins A and B, when given either orally or by intraperitoneal injection, produce hypoglycaemia and ultimately death in laboratory animals.[10] α-(Methylenecyclopropyl)glycine exhibits a toxicity comparable to that of hypoglycin A when injected into mice,[11] and presumably its γ-glutamyl peptide is also a hypoglycaemic agent. If 100 mg/kg hypoglycin A is accepted as an average LD_{50} value for normal rats, then the combined level of the four substances present in sycamore fruits is such that about 5 g of plant material would represent a lethal dose if ingested by a rat.

EXPERIMENTAL

Chromatographic and electrophoretic techniques used in the isolation and study of the amino acids and γ-glutamyl peptides were based on those used in an earlier investigation employing seed of *A. georginae*.[6] Seed (dry wt 1·92 kg) of *Billia hippocastanum* was collected in Costa Rica and kindly supplied by Dr. C. C.

[6] K. ITO and L. FOWDEN, *Phytochem.* **11,** 2541 (1972).
[7] A. I. VIRTANEN and E. J. MATIKKALA, *Hoppe-Seyler's Z. Physiol. Chem.* **322,** 8 (1960).
[8] A. I. VIRTANEN and A. M. BERG, *Acta Chem. Scand.* **8,** 1089 (1954).
[9] J. F. THOMPSON, D. H. TURNER and R. K. GERING, *Phytochem.* **3,** 33 (1964).
[10] R. BRESSLER, C. CORREDOR and K. BRENDEL, *Pharmac. Rev.* **21,** 105 (1969).
[11] D. O. GRAY and L. FOWDEN, *Biochem. J.* **82,** 385 (1962).

Moh (Centro de Ensenanza e Investigacion, Turrialba), whilst sycamore fruits (2·2 kg) were collected from local trees. Plant materials were macerated and extracted with 75% EtOH (6 l/kg), and then 3 × with $CHCl_3$–saturated H_2O (total vol. 30 l.). The amino acid and peptide fraction was obtained by absorption upon a cationic (Zeocarb-225) exchange resin column. Separation of the individual components was achieved using a series of Dowex 50 (cationic) or Dowex 1 (anionic) resin columns, followed if necessary by preparative paper chromatography.[6]

The following compounds were obtained from *B. hippocastanum* after crystallization from eluate fractions from a Dowex-1 column: hypoglycin B (1·34 g), γ-glutamylaspartic acid (96 mg), γ-glutamylglutamic acid (105 mg), γ-glutamylasparagine (85 mg) and γ-glutamyl-α-(methylenecyclopropyl)glycine (132 mg). Other fractions contained a mixture of γ-glutamyl derivatives, which were separated by preparative PC to yield crude preparations of: γ-glutamylalanine (170 mg), γ-glutamylvaline (80 mg) and γ-glutamylthreonine (70 mg). When fractionated by a similar procedure, the extract of sycamore yielded the following compounds as crystalline solids: hypoglycin B (8·1 g), γ-glutamyl-α-(methylenecyclopropyl)glycine (1·4 g), hypoglycin A (0·72 g), and α-(methylenecyclopropyl)glycine (0·31 g). The identities of compounds, other than γ-glutamyl-α-(methylenecyclopropyl)glycine and γ-glutamylthreonine (two new peptides), were confirmed by comparison with authentic samples[2,3,6,11] using IR, PC and electrophoretic techniques, and by amino acid autoanalyser procedures.

γ-Glutamyl-α-(methylenecyclopropyl)glycine. Crystalline material from sycamore (Found: C, 51·9; H, 6·3; N, 10·7. $C_{11}H_{16}N_2O_5$ requires: C, 51·6; H, 6·2; N, 10·9%) had $[\alpha]_D^{20}$ +61° (*c* 2, H_2O), and +16° (*c* 1, 5 N HCl). Treatment with 2 N HCl (100°, 3 hr) yielded glutamic acid and α-(methylenecyclopropyl)glycine in the molar ratio 1·0:1·05 (quantitative determination by the Cd–ninhydrin procedure[12]). Each product was crystallized and their identities confirmed by comparison (IR spectroscopy) with authentic samples. L-Configurations were established by polarimetry: for isolated glutamic acid, $[\alpha]_D^{20}$ +27° (*c* 1, 5 N HCl), compare lit.[13] of +31° for L-isomer; for isolated α-(methylenecyclopropyl)glycine, $[\alpha]_D^{20}$ +76° (*c* 1, H_2O), lit.[11] +83° for L-form.

Dinitrophenylation[14] of the peptide, followed by hydrolysis with 2 N HCl (100°, 4 hr), gave the dinitrophenyl derivative of glutamic acid and free α-(methylenecyclopropyl)glycine so the former acid was combined through a COOH, and the instability of the peptide to relatively mild HCl treatment indicated linkage through its γ-carboxyl group.

The peptide had an R_f 0·56 in 75% (w/w) phenol (in the presence of NH_3 vapour) and R_{Leu} 0·48 in BuOH–HOAc–H_2O (90:10:29).

γ-Glutamylthreonine was characterized tentatively, (i) by mild hydrolysis and identification of glutamic acid and threonine in equimolar amounts, and (ii) by dinitrophenylation and subsequent hydrolysis to establish the amino and carboxyl groups combined in the peptide link.

Electrophoretic mobilities (cm towards anode) measured during 90 min on Whatman No. 3 mm paper at pH 3·4 (see Ref. [6]) using 100 V/cm were as follows: glutamic acid 3·5, aspartic acid 14·6, γ-glutamyl-α-(methylenecyclopropyl)glycine 16·1, hypoglycin B 12·5, γ-glutamylvaline 11·0, γ-glutamylalanine 12·2, γ-glutamylthreonine 14·5, γ-glutamylasparagine 17·9, γ-glutamylglutamic acid 18·0, γ-glutamylaspartic acid 24·0.

Acknowledgement—The amino acid autoanalyzer used in this investigation was purchased using a grant from the Agricultural Research Council.

[12] G. N. Atfield and C. J. O. R. Morris, *Biochem. J.* **81,** 606 (1961).
[13] J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 3, p. 1929, Wiley, New York (1961).
[14] F. Sanger and E. O. P. Thompson, *Biochem. J.* **53,** 353 (1953).