Phytochemistry, 1972, Vol. 11, pp. 2541 to 2545. Pergamon Press. Printed in England.

NEW CHARACTERIZATIONS OF AMINO ACIDS AND γ-GLUTAMYL PEPTIDES FROM *ACACIA GEORGINAE* SEED

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(*Received* 13 *March* 1972)

Key Word Index—*Acacia georginae*; Leguminosae; non-protein amino acids; *S*-(2-hydroxy-2-carboxyethanethiomethyl)-L-cysteine; γ-glutamyl peptides.

Abstract—An extract of *Acacia georginae* seed has yielded several amino compounds not previously recorded as plant constituents. These include an acidic sulphur-containing amino acid, *S*-(2-hydroxy-2-carboxyethanethiomethyl)-L-cysteine, and the following γ-glutamyl peptides: γ-glutamylalbizziine, γ-glutamylasparagine, γ-glutamylglutamic acid and probably γ-glutamyldjenkolic acid sulphoxide. γ-Glutamylaspartic acid also was identified as a constituent of the seeds. Several other compounds, isolated in mg amounts, still await full characterization.

INTRODUCTION

SEEDS of many members of the Mimosaceae are particularly rich sources of amino acids whose distribution elsewhere within the plant kingdom is quite restricted. Several years ago, we made a comparative study of the free amino acids present in seeds of about forty species of the genus *Acacia*.[1] This survey indicated the co-existence of pipecolic acid and 4- and 5-hydroxypipecolic acids (recorded earlier for individual *Acacia* species[2–4]) as a common feature of the amino acid pattern of *Acacia* seeds. Albizziine and β-acetyl-α,β-diaminopropionic acid and various sulphur-containing amino acids also accumulated in many seeds. Among the sulphur compounds, djenkolic acid and its sulphoxide forms were encountered almost invariably, whilst *S*-carboxyethyl- and *S*-carboxyisopropyl-cysteines were found only slightly less frequently. A small number of species, all assigned to the subgeneric group Gummiferae by Bentham,[5] differed strikingly from all others in the nature of their main amino acid constituents; albizziine, acetyldiaminopropionic acid, *S*-carboxyethyl- and *S*-carboxyisopropyl-cysteines were absent from their seeds, but another sulphur compound (*N*-acetyldjenkolic acid) was uniquely stored in massive amounts. During the earlier survey, several other ninhydrin-positive compounds were noticed, but their identity was not established. Now we report the results of a more detailed study of the soluble nitrogenous constituents present in seed of one species, *A. georginae*. This species accumulates albizziine, the djenkolic acid complex (except *N*-acetyldjenkolic acid), and pipecolic acid and its hydroxylated forms,[1] but also contains a number of unidentified compounds.

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[1] A. S. SENEVIRATNE and L. FOWDEN, *Phytochem.* **7**, 1039 (1968).
[2] A. I. VIRTANEN and S. KARI, *Acta Chem. Scand.* **8**, 1290 (1954).
[3] A. I. VIRTANEN and S. KARI, *Acta Chem. Scand.* **9**, 170 (1955).
[4] J. W. CLARK-LEWIS and P. I. MORTIMER, *J. Chem. Soc.* 189 (1961).
[5] G. BENTHAM, in *Flora Australiensis*, Vol. 2, pp. 301–421, Lovell Reeve, London (1864).

A. georginae is also one of the few plant species known to synthesize fluoroacetate,[6] so it was conceivable that one or more of the unidentified compounds might be fluoroamino acids.

RESULTS AND DISCUSSION

When an extract of *A. georginae* seeds was examined by 2-dimensional paper chromatography, almost all the unidentified ninhydrin-reactive components were present in the general area associated with acidic amino acids. Subsequently, an extract was applied to an anion-exchange resin column to achieve a separation of acidic amino acids from the neutral and basic acid fraction. This confirmed that the unidentified materials all behaved as acidic compounds, and suggested that Dowex-1 resin would be effective in fractionation.

The amino acid complex present in an extract of 1·84 kg seed was separated from other soluble constituents by retention on a large cation-exchange resin column. The combined amino acid fraction was applied next to a Dowex-1 column, and the absorbed acidic amino acids were eluted by acetic acid (0·5–2 N). After combining appropriate fractions, several compounds were obtained in a pure state after recrystallization. In other cases, mixtures of compounds present in combined fractions were resolved by preparative paper chromatography. Finally, a sulphur-containing amino acid, *S*-(2-hydroxy-2-carboxyethanethiomethyl)-L-cysteine (a compound not previously recognized as a plant constituent), and the γ-glutamyl peptides of djenkolic acid sulphoxide, albizziine, asparagine, glutamic acid and aspartic acid were separated and characterized. Several other compounds designated as B_3, B_4, C_1 and C_2 were isolated by these techniques, but their identities remain uncertain. In addition, almost 100 g of djenkolic acid and its sulphoxide separated out from the mixture of amino acids not retained on the Dowex-1 column: these two compounds are very sparingly soluble in cold water.

The structure (I) assigned to the sulphur-containing amino acid was reached on the basis of elementary analysis, NMR spectroscopy, and synthesis from djenkolic acid. I then shows an analogy to djenkolic acid (II) and to dichrostachinic acid (III). The latter amino acid was isolated first from *Dichrostachys glomerata* by Gmelin,[7] and later[8] shown to be a constituent of certain species assigned to the related genera *Neptunia*, *Prosopis*, *Desmanthus* and *Leucaena* (from the tribes Adenanthereae and Mimoseae of the Mimosaceae[9]). I could be envisaged as an intermediate in a metabolic pathway leading from the more commonly occurring djenkolic acid to the less frequently encountered dichrostachinic acid.

$$HOOCCH(OH)CH_2SCH_2SCH_2CH(NH_2)COOH$$
(I)

$$HOOCCH(NH_2)CH_2SCH_2SCH_2CH(NH)_2COOH$$
(II)

$$HOOCCH(OH)CH_2SO_2CH_2SCH_2CH(NH_2)COOH$$
(III)

The NMR spectrum of I was fairly simple and was in full agreement with the proposed structure. It contained two *ABX* patterns in which the *AB* parts overlapped. This observation is consistent with two $>CH–CH_2–$ fragments arranged in the molecule such that the

[6] P. B. OELRICHS and T. MCEWAN, *Nature, Lond.* **190,** 808 (1961).
[7] R. GMELIN, *Hoppe-Seyler's Z. Physiol. Chem.* **327,** 186 (1962).
[8] I. K. SMITH, Ph.D. Thesis, University of London (1967).
[9] J. HUTCHINSON, *The Genera of Flowering Plants*, Vol. 1, Clarendon Press, Oxford (1964).

methylene groups are in similar environments. A two proton singlet supports the presence of an isolated $-CH_2-$ group.

I was synthesized by treating L,L-djenkolic acid with a limited quantity of sodium nitrite in acetic acid. The replacement of one of the $-NH_2$ groups of djenkolic acid by -OH was accompanied by marked racemization and almost equal amounts of the L,L- and D,L-forms of *S*-(2-hydroxy-2-carboxyethanethiomethyl)cysteine were obtained. The two isomeric forms were separated on paper chromatograms developed in *tert*-amyl alcohol–acetic acid–water, when the form presumed to have an L,L-configuration (on the basis of optical rotary dispersion studies) behaved similarly to the natural isolate. This isomer had an $[\alpha]_D^{20}$ similar to that recorded for the natural I: the circular dichroism curves measured for this isomer and the isolated material were also very similar, although curves for the two synthetic isomers were quite different. Furthermore, almost identical NMR spectra were obtained from the natural compound and the synthetic L,L (presumed)-isomer: very minor differences between the two spectra could be explained by a slight contamination of the synthetic L,L-form by remaining D,L-isomer.

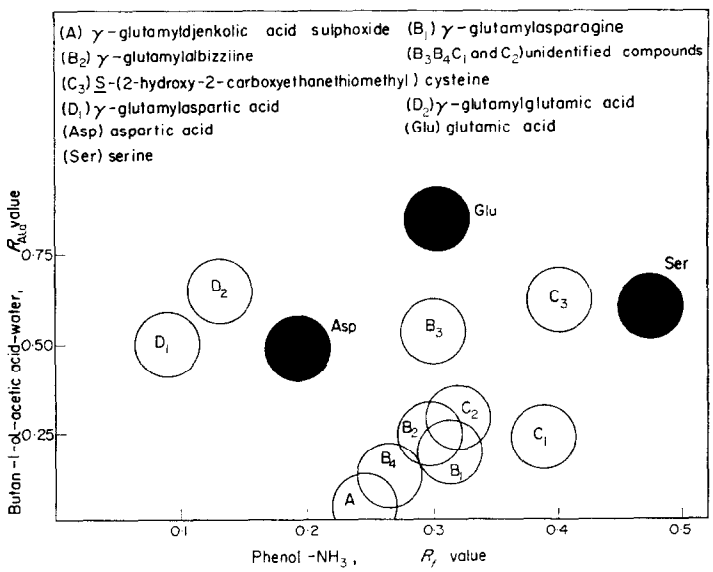

FIG. 1. ILLUSTRATES THE CHROMATOGRAPHIC BEHAVIOUR OF THE COMPOUNDS ISOLATED FROM *A. georginae*.

The glutamyl peptides were characterized by hydrolysis—all peptides were completely hydrolyzed by 2 N-HCl (100° for 2 hr), suggesting the presence of the labile γ-linkage rather than the more stable bond encountered in α-peptides. Dinitrophenylation[10] indicated that the α-amino group of glutamic acid was free in all compounds. The molar ratios of the hydrolysis products, separated by paper chromatography, were determined using a Cd–ninhydrin reagent and shown to be in a 1:1 ratio. Under these conditions, γ-glutamyl-asparagine gave aspartic acid after hydrolysis. The presence of an asparagine moiety was indicated by the formation of slightly less than 1 mol ratio of NH_3 during hydrolysis, by

[10] F. SANGER and E. O. P. THOMPSON, *Biochem. J.* **53**, 353 (1953).

subjecting the peptide to less rigorous hydrolytic conditions when asparagine appeared as a minor product, and by applying the Hofmann amide degradative procedure[11] to the peptide when α,β-diaminopropionic acid was identified as a product of acid hydrolysis. Albizziine obtained after hydrolysis of γ-glutamylalbizziine decomposed partially to yield several unidentified ninhydrin-positive compounds; when treated similarly with 2 N-HCl, free albizziine gave the same series of products, which then served to confirm the presence of an albizziine moiety in the original peptide.

γ-Glutamylaspartic acid has been described previously as a component of soybean seedling extracts, and γ-glutamylglutamic acid occurs in animal brain, but the three other γ-glutamyl derivatives do not seem to have been isolated previously. Their isolation from *Acacia* extends the known range of γ-glutamyl derivatives typically found in seeds, which often contain γ-glutamyl peptides of those amino acids present in high concentration within their tissues.

The compounds isolated, but still unidentified, were obtained in only small yield: B_3 (6 mg), B_4 (21 mg), C_1 (55 mg) and C_2 (22 mg). B_3 and C_1 still contained ninhydrin-positive contaminants. B_3 was stable when treated with 6 N HCl (100°, 15 hr), but the other compounds decomposed under these conditions, and C_1 and C_2 both appeared to yield B_3 as one of their hydrolysis products. B_4 and C_2 contained sulphur: analysis for sulphur was not performed on B_3 and C_1 because they were impure. Figure 1 illustrates the chromatographic behaviour of the compounds described; the positions of aspartic acid, glutamic acid and serine are shown for reference purposes.

EXPERIMENTAL

Chromatographic and electrophoretic methods. Descending paper chromatography was performed on Whatman Nos. 3MM or 4 filter paper using the following solvents: (1) 75% (w/w) phenol in the presence of NH_3 vapour; (2) *n*-BuOH–HOAc–H_2O (90:10:29, by vol.); and (3) *t*-AmOH–HOAc–H_2O (15:1:15, by vol., upper phase). High voltage (10 kV) paper electrophoresis was performed on Whatman 3MM paper using a Locarte Co. (London) apparatus having 1 m plates. Separations were obtained either at pH 3·4 (HOAc–pyridine–H_2O, 50:3:4947, by vol.) or at pH 5·3 (HOAc–pyridine–H_2O, 20:50:4930, by vol.). A 0·1% solution of ninhydrin in EtOH was used as the chromogenic reagent.

Isolation of compounds. Dry seed (1·84 kg) of *A. georginae* was ground to a fine meal and extracted initially with 75% (v/v) EtOH (18 l.): extraction was repeated with three 10-l. portions of $CHCl_3$-saturated water. The combined extracts were reduced *in vacuo* to 5 l. and decolorized with charcoal. The clarified extract (pH 3·5) was applied to a Zeokarb 225 (×8) resin column (85 × 8 cm, H^+ form) to absorb amino acids: after thorough washing, the amino acids were displaced by 0·5 N NH_3. The eluate containing amino acids was concentrated to about 1 l., adjusted to pH 7·0, and applied to a Dowex-1 (×10) resin column (115 × 5 cm, acetate form) to fractionate the acidic amino acid complex. During these steps, precipitates appeared in certain fractions obtained from the Zeokarb column, in the combined, concentrated extract prior to applying to the Dowex-1 column, and in the neutral amino acid fraction not retained by the Dowex-1 column. Five such precipitates were recovered as follows: (1) 7·2 g, almost pure djenkolic acid; (2) 28·9 g, mixture of djenkolic acid and its sulphoxide; (3) 21·8 g, almost pure djenkolic acid sulphoxide; and (4 and 5) 12·8 g and 28·5 g respectively, djenkolic acid sulphoxide with small contamination of other neutral amino acids. Acidic compounds were displaced from the Dowex-1 column with HOAc, 50 ml fractions being collected: 0·5 N HOAc was used to elute fraction Nos. 1–160, 1·0 N HOAc for Nos. 161–246, and 2·0 N HOAc for the remainder. The elution pattern of the different compounds was as follows: fraction Nos. 44–63, glutamic acid; 63–78, mainly γ-glutamyldjenkolic acid sulphoxide; 79–114, aspartic acid predominant; 115–167, γ-glutamylalbizziine, γ-glutamylasparagine, B_3 and B_4; 168–198, compounds C_1 and C_2; 199–246; mainly *S*-(2-hydroxy-2-carboxyethanethiomethyl)cysteine; and 247–341, γ-glutamylaspartic acid and γ-glutamylglutamic acid. Fractions within each group were combined for the crystallization of individual components, or for the separation of mixtures by employing preparative paper chromatographic techniques using appropriate solvents.

Characterization and properties of the isolated compounds. S-(*2-Hydroxy-2-carboxyethanethiomethyl*)-*cysteine* (8·2 g) was crystallized from aqueous solution after acetic acid had been removed from combined

[11] R. L. M. SYNGE, *Biochem. J.* **33**, 671 (1939).

fractions by repeated evaporation *in vacuo*. Found: C, 33·0; H, 5·1; N, 5·7; S, 25·2. $C_7H_{13}O_5NS_2$ requires: C, 32·9; H, 5·1; N, 5·5; S, 25·1%. $[\alpha]_D^{20}$ values were +39·3° (*c*, 1·2 in H_2O) and +21° (*c*, 1·3 in 5 N HCl). The c.d. curves of natural material, measured in water and in N HCl, closely resembled those determined for synthetic material (isomer *A*, see below). The NMR spectrum (in NaOD–D_2O solution) at 60 Mc/s showed resonances at 5·88 τ (quartet, one proton, $-C\underline{H}<^{OH}_{CO_2^-}$), 6·29 τ (singlet, two protons, $-S-C\underline{H}_2-S-$), 6·64 τ (quartet, one proton, $-C\underline{H}<^{NH_2}_{CO_2^-}$) and 7·12 τ [multiplet, four protons, $(>CH-C\underline{H}_2-)_2$]. The compound decomposed when heated with 6 N HCl at 100° for 15 hr. When a solution in 5% HOAc was hydrogenated (Pt/H_2), alanine and *S*-methylcysteine were identified as products. Synthetic material was obtained by treating djenkolic acid (isolated material) with nitrous acid. L,L-Djenkolic acid (2 g) was dissolved in 3 N HOAc (400 ml) and $NaNO_2$ (1·1 g in 10 ml H_2O) was added with cooling. The mixture was kept for 48 hr at room temp., and then HOAc was removed by vacuum evaporation. The residue was redissolved in water, adjusted to pH 7, and applied to a Dowex-1 column. Although separation of the synthetic isomers was not achieved during elution with HOAc, this step removed NaOAc. The mixed isomers (*A* and *B*) were resolved by streaking across paper chromatograms later developed in solvent 3. Synthetic isomer A behaved identically with the natural compound during chromatography and high voltage electrophoresis, and yielded an almost identical NMR spectrum. The $[\alpha]_D^{20}$ value for isomer A was +33·4° (*c*, 1·0 in H_2O), and for isomer *B*, −20·1° (*c*, 1·3 in H_2O). The c.d. curves of isomer A showed maxima at λ = 198 nm ($\Delta\epsilon$ value +2·74) in H_2O, and at λ = 200 nm ($\Delta\epsilon$, +1·09) in approx. N HCl (in acid, a shoulder was seen at λ = 223 nm, $\Delta\epsilon$ value +0·46). The curve determined for the natural isolate showed maxima and a shoulder at similar wavelengths. The c.d. curve for synthetic isomer B was quite different: in H_2O, maxima were seen at λ values of 199 and 218 nm ($\Delta\epsilon$ values of +2·71 and −0·60, respectively), whilst in acid solution maxima were now present at λ = 214 and 249 nm ($\Delta\epsilon$, −0·40 and +0·14, respectively). The maximum seen in all curves at about λ = 199 nm probably can be attributed to the common sulphur chromophore. The small $\Delta\epsilon$ values determined for isomer *B* in acid solution suggest that this may be the 'mesoid' type (D,L). The two asymmetric centres of the compound are not completely identical, so some dichroism may still be expected, although it would probably be small in comparison to that observed for the L,L isomer. IR spectra of the natural isolate and isomer *A* were not identical, presumably due to differences of crystalline form.

The γ-glutamyl derivatives. (i) *γ-Glutamyldjenkolic acid sulphoxide* (0·34 g) was slightly contaminated by an unidentified ninhydrin-positive compound (demonstrated by electrophoresis at pH 3·4). Found: C, 34·9; H, 6·0; N, 9·8; S, 15·0; loss at 100°, 4·8. $C_{12}H_{21}N_3O_8S_2.H_2O$ requires: C, 34·5; H, 5·5; N, 10·1; S, 15·3; H_2O, 4·3% $[\alpha]_D^{20}$ −36·8° (*c*, 1·2 in H_2O). Glutamic acid and djenkolic acid sulphoxide were produced by hydrolysis with 2 N HCl (100°, 2 hr) in a 1:0·96 molar ratio (determined after chromatographic separation by the method of Atfield and Morris[12]). (ii) *γ-Glutamylasparagine* (22 mg). Found: C, 38·2: H, 5·9; N, 14·8; loss at 100°, 6·0. $C_9H_{15}N_3O_6.H_2O$ requires: C, 38·6; H, 6·1; N, 15·1; H_2O, 6·5%. Hydrolysis with 2 N HCl (100°, 2 hr) gave glutamic acid, aspartic acid and NH_3 in molar ratios of 1:0·89:0·75, respectively. The presence of an asparagine moiety in the peptide was further supported by the formation of small amounts of asparagine after hydrolysis with N HCl for 20 min, and by the production of α,β-diaminopropionic acid after Hofmann amide degradation followed by acid hydrolysis. α,β-Diaminopropionic acid was confirmed by comparison with authentic material on electrophoretograms run at pH 3·4; using 100 V cm^{-1}, diaminopropionate travelled 57 cm towards the cathode in 70 min, moving faster than any other straight-chain α,ω-diamino acid. (iii) *γ-Glutamylalbizziine* (119 mg). Found: C, 38·1; H, 6·2; N, 20·1. $C_9H_{16}N_4O_6$ requires: C, 39·1; H, 5·8; N, 20·3%. $[\alpha]_D^{20}$ + 0·8° (*c*, 1·12 in H_2O). The peptide yielded glutamic acid (1:1·07 molar ratio) and some albizziine after hydrolysis with 2 N HCl (100°, 2 hr). The hydrolysate also contained other ninhydrin-positive compounds identical in nature to products derived from free albizziine treated in the same way. (iv) *γ-Glutamylglutamic acid* (10 mg). Hydrolysis (2 N HCl, 100°, 2 hr) gave glutamic acid as a sole product (molar ratio, 1:1·80). The isolated material behaved identically with authentic γ-glutamylglutamic acid on paper chromatograms (3 solvents) and during electrophoresis at pH 3·4 and 5·3. (v) *γ-Glutamylaspartic acid* (5·4 mg). $[\alpha]_D^{20}$ + 6·5° (*c*, 1·04 in H_2O). Hydrolysis (2 N HCl, 100°, 2 hr) gave glutamic acid and aspartic acid (1:0·99 molar ratio). Identity with authentic material was established by chromatography and electrophoresis as for (iv) above. Each of these peptides was subjected to dinitrophenylation following the procedure of Sanger and Thompson.[10] Subsequent hydrolysis and chromatographic examination showed that in all cases the amino group of glutamic acid carried the dinitrophenyl substituent, whilst the other component of the peptide was released unmodified.

Acknowledgements—Seed of *A. georginae* was supplied by the Plant Introduction Section of C.S.I.R.O., Canberra, Australia. Authentic samples of peptides were provided by Dr. T. Kasai (Sapporo, Japan) and Dr. A. Meister (New York). Dr. R. C. Sheppard (Cambridge) performed the NMR spectroscopy, and Mr. A. Smith gave technical assistance.

[12] G. N. Atfield and C. J. O. R. Morris, *Biochem. J.* **81**, 606 (1961).