ACTA CHEMICA SCANDINAVICA 16 (1962) 1511–1518

Amino Acids and γ-Glutamyl Derivatives in Seeds of *Lunaria annua* L.*

P. OLESEN LARSEN

Department of Organic Chemistry, The Royal Veterinary and Agricultural College, Copenhagen, Denmark

Unhydrolyzed seed extracts of *Lunaria annua* L. (family *Cruciferae*) have been investigated for ninhydrin-reacting constituents. γ-L-*Glutamyl-β-alanine*, not previously found in nature, has been isolated and identified on comparison with a synthetic sample. In addition, γ-L-glutamyl-β-aminoisobutyric acid, *m*-carboxy-L-phenylalanine and 3-(3-carboxy-4-hydroxyphenyl)-L-alanine (*m*-carboxy-L-tyrosine), all previously known from higher plants, have been isolated in crystalline form. The presence of still other amino acids, such as several of the ordinary protein constituents, as well as β-alanine, β-aminoisobutyric acid and *m*-carboxyphenylglycine, has been demonstrated by paper chromatography. A minor constituent is tentatively regarded as α- or γ-glutamyl-γ-aminobutyric acid, whereas two additional glutamic acid derivatives are still under consideration.

The *Lunaria* seed extract is the first reported source of the simultaneous occurrence of the three *m*-carboxy-substituted aromatic amino acids encountered so far in higher plants.

The occurrence of the above-mentioned and related compounds in the plant kingdom is briefly discussed.

In the course of investigations on the contents of free amino acids in certain higher plants, containing isothiocyanate-producing glucosides [1], seed extracts of the ornamental crucifer *Lunaria annua* L. (honesty) were examined. Paperchromatographic amino acid analysis revealed a complex picture (Fig. 1), in which, besides common amino acids, a number of unusual spots suggested the presence of other amino acids and peptides. Some of the spots could tentatively be assigned to known compounds, whereas others apparently represented new constituents.

In order to obtain detailed information on the composition of the free amino acids in *Lunaria* seed, investigations were initiated on a larger scale. The total amino acid fraction from 1 kg of seed flour was isolated by means of a strongly acid ion exchange resin and subdivided on other resins as outlined

* Presented in abstract before the *International Symposium on Organic Chemistry of Natural Products*, June 1962, Brussels.

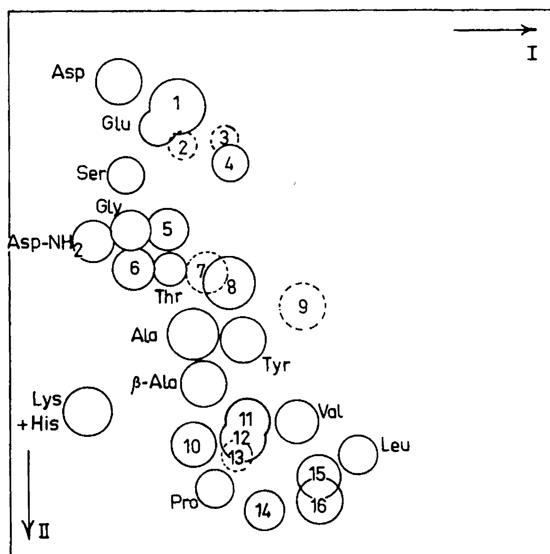

Fig. 1. Two-dimensional paper chromatogram of the amino acid fraction of *Lunaria annua* L. 1. Solvent: butanol:acetic acid: water (12:3:5), 2. Solvent: phenol:water:conc. ammonia (120:30:1). Spots marked by a dotted line are only detectable in highly concentrated fractions. 1: 3-(3-Carboxy-4-hydroxy-phenyl)-L-alanine (*m*-carboxy-L-tyrosine). 2: Unknown acid amino acid. 3: *m*-Carboxyphenylglycine. 4: *m*-Carboxy-L-phenylalanine. 5: γ-L-Glutamyl-β-alanine. 6: Unknown neutral amino acid. 7: Glutamyl-γ-aminobutyric acid. 8: γ-L-Glutamyl-β-aminoisobutyric acid. 9: Unknown acid glutamyl derivative. 10: Unknown neutral glutamic acid derivative. 11: β-Aminoisobutyric acid. 12: γ-Aminobutyric acid. 13: Ethanolamine. 14: Pipecolic acid. 15: γ-Guanidobutyric acid. 16: Unknown neutral glutamic acid derivative.

in Fig. 2. First, a weakly basic ion exchange resin in the acetate form was utilized to separate the amino acids into four fractions. The first of these contained neutral and basic amino acids and derivatives which showed no affinity to the resin. The second and third fraction included acidic compounds displaceable by acetic acid, whereas the fourth fraction contained amino acids sufficiently acid to be displaced only by hydrochloric acid.

In *the first fraction* the neutral and basic compounds were separated by means of a strongly acid resin in the ammonia form. Neutral compounds passed through the resin whereas basic constituents were bound and subsequently eluted with ammonia. The composition of these two fractions is indicated in Fig. 2. When not otherwise indicated the compounds were identified by R_F-values and co-chromatography with authentic samples. In addition, β-aminoisobutyric acid, β-alanine, γ-aminobutyric acid and lysine were controlled by a specific test for amino group position developed in this laboratory [2]. β-Aminoisobutyric acid and γ-aminobutyric acid were distinguished by paper chromatography in 2,4-lutidine:water, a solvent particularly suited for separation of the different aminobutyric and aminoisobutyric acids [3]. Asparagine,

Amino acid fraction

Weakly basic ion exchange resin on the acetate form

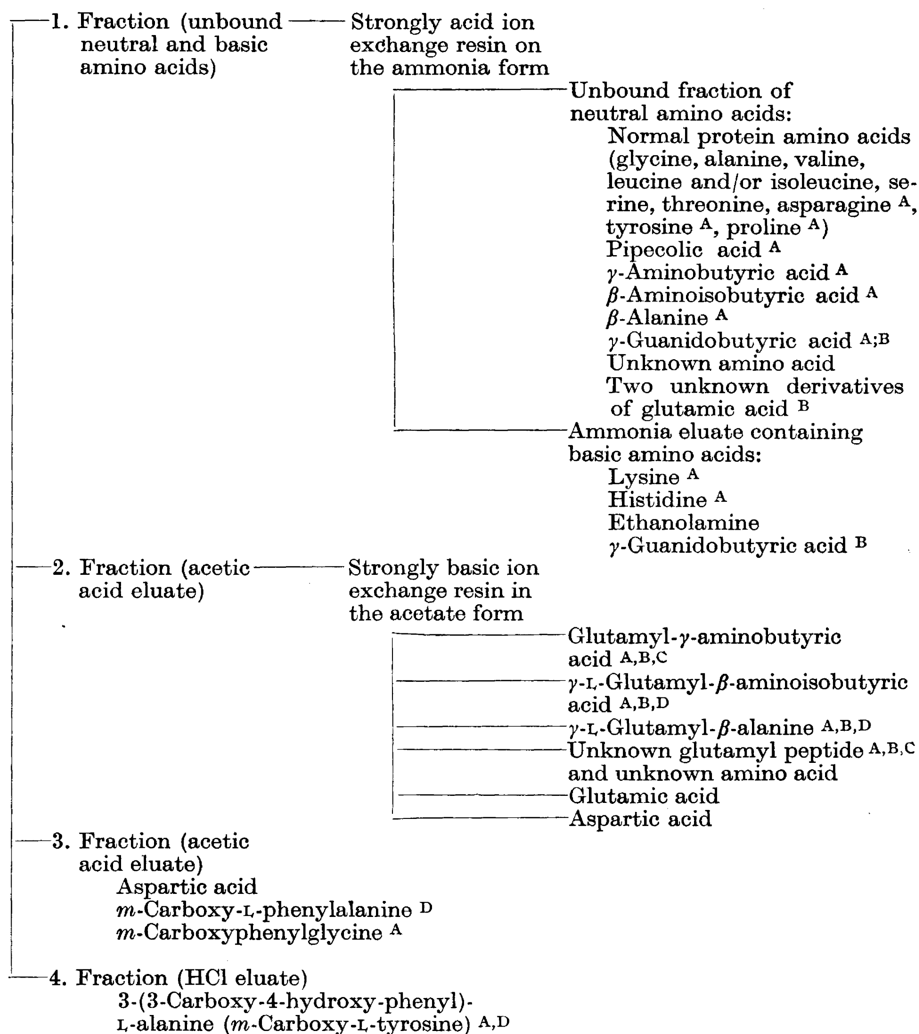

Fig. 2. Fractionation scheme for amino acids in seed extracts of *Lunaria annua* L. A) Identified by specific colour reaction. B) Identified by hydrolysis products. C) Identified by end group analysis. D) Identified by isolation and comparison of infra-red spectra and rotation values with those of authentic samples.

proline, pipecolic acid and β-alanine were recognized by their characteristic colours with ninhydrin. Tyrosine and histidine were identified by reaction with diazotized sulphanilic acid, and γ-guanidobutyric acid by a positive

Sakaguchi reaction coupled with the formation of γ-aminobutyric acid on hydrolysis with barium hydroxide.

The second fraction was shown by paper chromatography to contain aspartic and glutamic acid besides two unknown compounds. Further fractionation was accomplished by chromatography on a strongly basic ion exchange resin in the acetate form, resulting in only partial separation of the two unknown compounds but complete removal of aspartic and glutamic acid. The major component (*ca.* 300 mg) was purified by crystallization and identified as (—)-γ-L-glutamyl-β-aminoisobutyric acid on comparison with an authentic sample [4]. Moreover, the compound on hydrolysis afforded glutamic acid and β-amino-isobutyric acid, both identified by paper chromatography. The minor component, present in a total amount of about 100 mg, was purified by counter current distribution followed by preparative paper chromatography. A crystalline sample, slightly contaminated with γ-glutamyl-β-aminoisobutyric acid, was obtained, which on hydrolysis yielded glutamic acid and β-alanine, both identified by paper chromatography. The identity of the new compound as γ-L-glutamyl-β-alanine, not previously found in nature *, appeared from a critical comparison with a synthetic sample[5]. The infra-red spectra were identical, whereas a slight difference in rotation values could be attributed to a slight contamination with γ-glutamyl-β-aminoisobutyric acid.

β-Alanine and β-aminoisobutyric acid, found in the neutral fraction, could conceivably be artifacts produced by hydrolytic fission of the labile amide bonds in the γ-glutamyl derivatives during isolation of the amino acid fraction. That this was not the case, however, appeared from the fact that the two γ-glutamyl amino acids proved to be stable when subjected to the conditions employed during the preparation of the amino acid fraction.

The third fraction contained aspartic acid in addition to *m*-carboxy-phenylglycine and *m*-carboxy-L-phenylalanine. The latter (18 mg) was isolated in crystalline form and identified on comparison with an authentic sample [6]. The concentrated mother liquor contained *m*-carboxyphenylglycine as apparent from co-chromatography with a synthetic sample and by its specific colour reaction with ninhydrin.

The fourth fraction solely contained 3-(3-carboxy-4-hydroxyphenyl)-L-alanine (*m*-carboxy-L-tyrosine), isolated in substantial amounts (3.7 g) and again identified upon comparison with an authentic sample [1].

γ-L-Glutamyl-β-aminoisobutyric acid [4], as well as (—)-β-aminoisobutyric acid [7], have previously been isolated from bulbs of *Iris tingitana* var. Wedgewood **. (—)-β-Aminoisobutyric acid, of recently established absolute configuration [8], has formerly been observed in human urine [9,10] and in flatworms [11], whereas β-alanine is widely distributed in the plant kingdom and has been isolated from bulbs of *Iris tingitana* var. Wedgewood [12]. It may be significant that the two β-amino acids, occurring together in the same plant in the free state or as γ-glutamyl-derivatives, are regarded as products on the catabolic pathway of pyrimidines [13].

* See note added in proof.
** Although no precise author name is quoted, the employed plant material presumably represents a garden variety of *Iris tingitana* Boiss. *et* Reut.

Two other glutamine derivatives may be biogenetically related to γ-glutamyl-β-alanine, *viz.* N^5-ethyl-L-glutamine (theanine), isolated from tea leaves [14] and the fungus *Xenocomus badius* [15], and N^5-(2-cyanoethyl)-L-glutamine, occurring in various *Lathyrus*-species [16] and considered responsible for some of the toxic effects (lathyrism) observed in higher animals after ingestion of these plants.

m-Carboxy-L-phenylalanine and *m*-carboxyphenylglycine have formerly been isolated from *Iris tingitana* var. Wedgewood [6,17], whereas 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine is known as a constituent of seeds of *Reseda odorata* L.[1] For the first time, these three *m*-carboxy-substituted aromatic amino acids have now been identified in the same plant, possibly suggesting a biogenetic relationship between them. Whereas a search for 3-(3-carboxy-4-hydroxyphenyl)-L-alanine in *Iris tingitana* has been without avail [18], *m*-carboxyphenylalanine has been identified in this laboratory by paper chromatography, together with 3-(3-carboxy-4-hydroxy-phenyl)-alanine, in a number of species of *Resedaceae* including *Reseda odorata* L. *m*-Carboxyphenylglycine has not yet been observed in the same species, but may be present in very low concentrations, which, as in the case of *Lunaria annua*, would require highly concentrated fractions to permit detection. The strikingly similar pattern of amino acids and γ-glutamyl-derivatives in *Lunaria annua* L. and *Iris tingitana* var. Wedgewood is surprising in view of the taxonomic remoteness of *Cruciferae* and the monocotyledonous family *Iridaceae*.

In its amino acid picture seed material of *Lunaria annua* L. does not deviate from that of *L. rediviva* L. but is conspicuously different from the patterns observed on seed material of a number of other crucifers studied. Chemically, *Lunaria* species present other unusual features such as the contents of the alkaloid lunarine with as yet unknown structure, producing spermidine on hydrolysis [19]. The occurrence of γ-guanidobutyric acid and pipecolic acid in seeds of *Lunaria* is not too remarkable in view of the wide distribution of these compounds in higher plants [20,21].

Besides the common amino acids and those discussed above, other ninhydrin-reacting compounds were noticed in the amino acid extract of *Lunaria* seeds.

In the neutral fraction three unknown compounds, all with a normal ninhydrin reaction, were present in concentrations high enough to be readily observable on paper chromatograms of the original seed extract (Fig. 1). One of these appears to be an amino acid whereas the two other components may be derivatives of glutamic acid. Work is in progress to establish the identity of these three constituents.

When the second fraction from the weakly basic ion exchange resin was chromatographed on the strongly basic resin, three compounds present in very small amounts were observed. The first of these seems to be an amino acid, the second a glutamyl derivative of an unknown amino acid, whereas the third, after paperchromatographic purification, was hydrolyzed to give glutamic acid and γ-aminobutyric acid, both identified by paper chromatography. Dinitrophenylation of the unhydrolyzed material according to Sanger [22], followed by hydrolysis and paper chromatography of the amino acid fraction, resulted in the appearance of only γ-aminobutyric acid. Hence, it

seems safe to conclude that the compound is either γ-glutamyl-γ-aminobutyric acid or α-glutamyl-γ-aminobutyric acid, none of which have previously been found in nature.

EXPERIMENTAL

Microanalyses were performed by Mr. G. Cornali. Rotations were measured in a 1 dm tube. Infra-red absorption spectra were determined in potassium bromide pellets on a *Perkin-Elmer* "Infracord"-instrument.

The employed material of *Lunaria annua* L. has been stored for several years as seed flour. Comparison with a small specimen of fresh seed material (purchased from I. E. Ohlsen's Enke, Copenhagen), however, revealed no differences in the contents of ninhydrin-postive constituents.

The seed flour (1 kg) was refluxed with carbon tetrachloride (3 l) for 1 h and filtered off after cooling. This procedure was repeated, and the air-dried powder (700 g) was refluxed for 4 h with methanol:water (4 l, 7:3, v/v), cooled and filtered again. The extraction was repeated twice with fresh portions of solvent. The combined filtrates were evaporated *in vacuo* to a dark-brown syrup (220 g), which was partly dissolved in water (750 ml). The filtrate was divided into three equal portions, and the total amino acid fraction in each portion was bound on a strongly acid ion exchange resin (Zeokarb 225, 4 × 50 cm) in the acid form. After washing with water (1 l), the amino acids were eluted with ammonia (2 l, 1 N). The combined eluates from the three portions were evaporated to a dark-brown syrup (30 g). A substantial amount of ammonium acetate of unknown origin condensed in the receiver during the evaporation. The syrup was redissolved in water (450 ml) and again bound to the strongly acid resin and eluted with ammonia. This was done in order to remove some tarry material from the syrup. The final ammonia eluate was again concentrated to a brown syrup (19 g), dissolved in water (250 ml) and applied to a weakly basic resin (Dowex 3, 20—50 mesh, 3.2 × 40 cm) in the acetate form. The effluent was collected in 20-ml fractions. After washing with water (1 l), the column was eluted with acetic acid (2.6 l, 1 N) and subsequently with hydrochloric acid (3 l, 1 N). Fractions 4—30 contained the basic and neutral compounds (first fraction). Fractions 81—109 contained aspartic acid, glutamic acid, and the glutamyl derivatives (second fraction), whereas fractions 110—149 contained aspartic acid and *m*-carboxy-L-phenylalanine (third fraction). Finally, fractions 216—347 contained 3-(3-carboxy-4-hydroxyphenyl)-L-alanine (fourth fraction).

The first fraction was evaporated *in vacuo* to a brown syrup (8.2 g) which was dissolved in water and purified by passing through a column of alumina (Woelm, "annähernd neutral", Akt.-Stufe 1). Most of the coloured material was absorbed on the alumina, whereas the amino acids could be eluted with water, and the solution was concentrated to dryness (4.2 g). The amino acids were applied in aqueous solution (25 ml) to a strongly acid resin (Amberlite IR 120, 2.3 × 18 cm) in the ammonia form. Neutral amino acids were washed through with water (150 ml) and the basic compounds were eluted with ammonia (120 ml, 1 N). Both fractions were evaporated to dryness and the amino acid composition determined by paper chromatography (Fig. 2) (the neutral fraction weighed 3.4 g, the basic fraction 0.3 g).

The second fraction was evaporated *in vacuo* to a brown crystalline residue (1.2 g), which was dissolved in water (50 ml) and applied to a strongly basic ion exchange resin (Dowex 1-X8, 200—400 mesh, 3 × 76 cm) in the acetate form. The column was eluted with acetic acid of a concentration progressively increasing from zero to 0.6 N over a volume of 4 l. The effluate was collected in fractions of 250 drops (*ca.* 20 ml). Fractions 73—79 contained glutamyl-γ-aminobutyric acid, fractions 86—98 γ-L-glutamyl-β-aminoisobutyric acid, fractions 90—99 γ-L-glutamyl-L-alanine, and fractions 98—99 small amounts of two unknown compounds. Glutamic acid and aspartic acid appeared in later fractions.

The fractions 86—94 were evaporated *in vacuo* to a colourless crystalline solid (200 mg). After four crystallizations from water, pure γ-L-glutamyl-β-aminoisobutyric acid resulted (Found: C 46.50; H 7.04; N 12.07. Calc. for $C_9H_{16}N_2O_5$: C 46.54; H 6.95; N 12.06), $[\alpha]_D^{23}$ −1.8° (*c* 1.0, 1 N HCl) (determined 5 min. after preparation of the solution.

No change in rotation was observed during the first 15 min. For rotation of L-glutamine under the same conditions, *cf.* Ref.[23]), $[\alpha]_D^{27}$ $-20.0°$ (*c* 1.0, water (supersaturated)). Lit. value: $[\alpha]_D^{27}$ $-33°$ (*c* 1.0) [4], solvent: water [18]. For a sample of γ-L-glutamyl-β-aminoisobutyric acid from *Iris tingitana* var. Wedgewood [4], the rotation value $[\alpha]_D^{24}$ $-18.5°$ (*c* 1.0, water) was determined in this laboratory. The infra-red absorption spectrum was indistinguish-able from that of the authentic sample [4].

Fractions 95—98 were evaporated *in vacuo* to a colourless crystalline residue (190 mg) and combined with the first two mother liquors from the recrystallization of γ-glutamyl-β-aminoisobutyric acid (content 90 mg). The solid was dissolved in a mixture of water (15 ml), butanol (4 ml) and acetic acid (1 ml) and introduced into a Craig counter-current apparatus with 60 plates (lower and upper phase 10 ml each). The distribution was performed between the two phases of the system butanol:acetic acid:water (4:1:5). After 240 transfers, the tubes 10—34 contained γ-L-glutamyl-β-alanine, contaminated with γ-glutamyl-β-aminoisobutyric acid. The contents of these tubes were evaporated to dryness (70 mg), dissolved in water and chromatographed on Whatman paper No. 3 MM in butanol:acetic acid:water (12:3:5). γ-L-Glutamyl-β-alanine was eluted from the paper and evaporated to dryness (55 mg). Further purification was achieved by application to a strongly basic resin (Dowex 1-X 8, 200—400 mesh, 1 × 6 cm) on the acetate form followed by elution with acetic acid (1 N). The eluate was evaporated to dryness (38 mg) and three recrystallizations from aqueous ethanol afforded a reasonably pure specimen (Found: C 43.35; H 6.59; N 12.56. Calc. for $C_8H_{14}N_2O_5$: C 44.03; H 6.47; N 12.84), $[\alpha]_D^{23}$ $+ 5.5°$ (*c* 1.0, water), $[\alpha]_D^{24}$ $+ 23.0°$ (*c* 0.366, 0.1 N HCl) (determined 5 min. after preparation of the solution. No change in rotation was observed during the first 15 min.). No value for the rotation is recorded in the literature. A synthetic sample of γ-L-glutamyl-β-alanine [5] gave the value $[\alpha]_D^{25}$ $+ 25.8°$ (*c* 0.364, 0.1 N HCl). The infra-red absorption spectrum coincided with that of the synthetic product. Paper chromatography, however, revealed contamination with a small amount of γ-glutamyl-β-aminoisobutyric acid.

The third fraction was evaporated *in vacuo* to a brown oil (300 mg). After solution in water and treatment with charcoal a black resinous material (50 mg) was separated on addition of ethanol. Concentration of the mother liquor yielded *m*-carboxy-L-phenylalanine (18 mg) purified by recrystallization from water, $[\alpha]_D^{24}$ $-15.8°$ (*c* 0.95, water (supersat.)). Lit.value: $[\alpha]_D^{25}$ $-17.0°$ (*c* 1) [6], solvent: water [18] (Ref.[6] erroneously reports the opposite sign of rotation [18]). The infra-red absorption spectrum was identical with that of an authentic sample [6].

The fourth fraction was evaporated *in vacuo* to a brown crystalline residue (4.8 g) Solution in water (40 ml), treatment with charcoal and neutralisation with ammonia to pH 2.5 resulted in the precipitation of 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine (3.2 g). A second crop (460 mg) was obtained from the mother liquor. Recrystallization from water afforded a pure sample, $[\alpha]_D^{26}$ $-7.8°$ (*c* 0.9, 1 N NaOH). Lit.value $[\alpha]_D^{26}$ $-7.5°$ (*c* 0.9, 1 N NaOH) [1]. The infra-red absorption spectrum was indistinguishable from that of an authentic sample [1].

Paper chromatography. The following R_F-values were determined for the new compounds by descending chromatography on Whatman paper No. 1 at 22° in (1) butanol: acetic acid:water (12:3:5), and (2) phenol:water:conc.ammonia (120:30:1) (w/v/v)):

γ-L-Glutamyl-β-alanine:	(1) 0.28,	(2) 0.40.
Glutamyl-γ-aminobutyric acid:	(1) 0.36,	(2) 0.48.

The author wishes to thank professor A. Kjær for encouragement and help during the present work, and expresses his gratitude to Dr. O. Rosenlund Hansen for the employed *Lunaria* seed material, to Dr. J. F. Thompson for samples of *m*-carboxy-L-phenylalanine and γ-L-glutamyl-β-aminoisobutyric acid, to Dr. A. Meister for a specimen of γ-L-glutamyl-β-alanine and to Dr. Y. Robin for a specimen of γ-guanidobutyric acid. Support from *The Carlsberg Memorial Foundation for Brewer I.C. Jacobsen* is gratefully acknowledged.

Added in proof. After the conclusion of the present work, we were privately informed by Dr. J. F. Thompson that γ-L-glutamyl-β-alanine has been isolated from *Iris tingitana* [24]. A sample of the compound from this plant was identical with our product with regard to infra-red spectrum and rotation value.

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Received February 14, 1962.