

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

P. OLESEN LARSEN

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

In continuation of studies on the contents of ninhydrin-reacting constituents in unhydrolyzed seed extracts of *Lunaria annua* L., N⁵-(2-hydroxyethyl)-L-glutamine and 2-amino-4,5-dihydroxypentanoic acid, not previously found in nature, have been isolated and identified by comparison with synthetic samples. The synthetic sample of 2-amino-4,5-dihydroxypentanoic acid was a mixture of two diastereoisomeric compounds. The configuration at C₂ in the natural compound has been established as L (or S) by transformation to L-aspartic acid, whereas the configuration at C₄ is unknown.

In the isolation of the two compounds resort has been taken to untraditional principles for preparative ion-exchange chromatography using elution with volatile bases corresponding to acids with the same p*K*-values as those of the amino acids determining the elution order. In this way the capacity of the separation has been increased.

The chemical syntheses of N⁵-(2-hydroxyethyl)-L-glutamine, N⁵-(2-methylthioethyl)-L-glutamine, N⁶-(2-methylsulphinyloethyl)-L-glutamine, N⁵-(3-methylthiopropyl)-L-glutamine, N⁵-(3-methylsulphinylpropyl)-L-glutamine and N,N'-bis-(γ -L-glutamyl)cystamine and of N-methylpyrazole by conventional methods are described and R_F-values in paper chromatography for the compounds are recorded.

Paper chromatographic determination of the contents of free amino acids in other parts of *Lunaria annua* L. has demonstrated that only very few of the many non-protein amino acids present in seeds also occur in other parts of the plant.

Two-dimensional paper chromatography of an extract of seeds of *Lunaria annua* L. reveals a large number of ninhydrin reacting constituents a part of which only can be identified as amino acids occurring in proteins. Most of the constituents have been identified and isolated as reported in two previous communications,^{1,2} but two compounds, present in sufficiently large quantities to be easily recognizable on the chromatograms of the total amino acid frac-

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tion, remained unidentified.² The present paper reports the isolation and identification of these two components. This proved possible only by use of untraditional methods for preparative ion-exchange chromatography. Furthermore, a number of γ -glutamyl derivatives have been synthesized for the purpose of comparison. Finally, the contents of free amino acids in other parts of *Lunaria annua* L. have been determined by paper chromatography.

METHODS FOR PREPARATIVE ION-EXCHANGE CHROMATOGRAPHY OF AMINO ACIDS

Preparative ion-exchange chromatography on a strongly basic ion-exchange resin in the acetate form with acetic acid as an eluent is a satisfactory method for isolation of acidic amino acids.¹ Whereas the described methods for the analytical separation of neutral amino acids by ion-exchange chromatography as generally performed are very efficient, the preparative ion-exchange chromatography of these substances is considerably less satisfactory. Although the problem of removing the buffers used in the separation has been solved by the introduction of pyridine-acetic acid and pyridine-formic acid buffers, the capacity is still small. As demonstrated in the previous communication,² severe overlapping of the eluted amino acids easily occurs as a result of overloading. This problem may be solved, as previously proposed,³ by performing the preparative ion-exchange chromatography of neutral amino acids on strongly acidic ion-exchange resins with the functional groups occupied by and eluted with a monovalent base specifically selected for the purpose. The base should preferably satisfy the following four requirements:

1. The pK -value of the corresponding acid must be of the same magnitude as that characterizing the dissociation of the protonated amino acids, for normal α -amino acids about 2–2.5.
2. The base must be soluble in water.
3. The base must be unreactive towards amino acids, the ion-exchange resin, and water.
4. The base should preferably be volatile to facilitate its removal after the separation. This is not indispensable, however, since the base may also be removed by extraction with an organic solvent.³

By this means a greatly increased capacity is obtained. When the amino acid is adsorbed to the resin as a positive ion, the ion liberated is the acid corresponding to the base used. This acid will take the place of the amino acid ion in the acid-base equilibrium and therefore no alteration of the pH-value occurs. Consequently, the distribution coefficient is independent of concentration. In normal ion-exchange chromatography of amino acids, a sodium or pyridinium ion is liberated when the positively charged amino acid ion is adsorbed. None of the liberated ions are protolytically active in the pH-region (fixed by a buffer) of the system. Consequently, an increase in pH occurs when the amino acid is adsorbed to the resin, and a comparable decrease when the amino acid is liberated by displacement with a sodium or pyridinium ion, resulting in non-linear equilibrium isotherms and non-changing distribution coefficients. The main reason for the small capacity of the usual methods for ion-exchange chromatography of neutral amino

acids appears to be concentration-dependent distribution coefficients. Hence, only by using small amounts of amino acids and buffer systems with large capacity can good separations be obtained.

The method proposed here is based upon the same principle as the preparative ion-exchange chromatography of acidic amino acids on strongly basic ion-exchange resins in the acetate form with acetic acid as an eluent. Here, the amino acids are bound to the resin as negative ions and acetate ions are liberated. The pK -value of acetic acid is similar to the pertinent pK -values of the amino acids, that is those referring to the dissociation step which transforms the amino acids from compounds with total load zero into negative ions.

The method has obvious restrictions. If the amino acids possess widely differing pK -values it is difficult to achieve complete separation by use of only one base. In order to avoid a too rapid elution of the amino acids with lowest pK -values a very weak base is required with the result that the amino acids possessing the highest pK -values will appear very late, even when the concentration of the base is increased during the chromatography. It is both time- and base-consuming to change from one base to another during the chromatography, because all places on the resin are occupied with the first base. These problems may partly be overcome, however, by performing the separation step-wise, first attempting to separate the neutral amino acids into classes according to their pK -values.

In the present isolation two bases, 3-chloropyridine (b.p. 148° (Ref. 4); pK for the corresponding acid 2.84 (Ref. 5)) and N-methylpyrazole (b.p. 127°; pK for the corresponding acid 2.04 (Ref. 6)) were used. N-Methylpyrazole was synthesized by a modification of one of the methods described in the literature.³

ISOLATION AND IDENTIFICATION OF 2-AMINO-4,5-DI-HYDROXPENTANOIC ACID AND N⁵-(2-HYDROXYETHYL)-L-GLUTAMINE

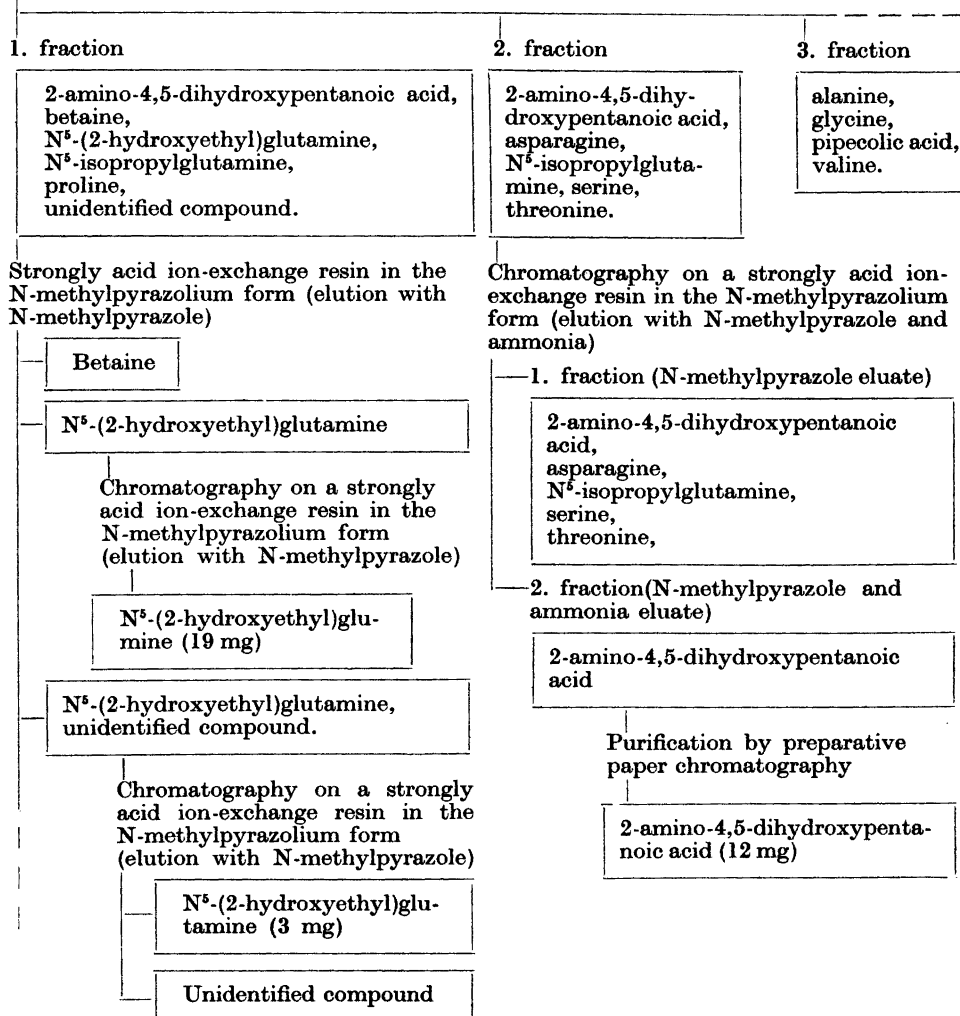
The separation procedure is outlined in Fig. 1.

The unknown compound previously referred to as No. 6^{1,2} (see Fig. 1 of the first communication in this series¹) formed a lactone on the strongly acid ion-exchange resins as expected from previous observations on its chemical properties.² Apparently, 3-chloropyridine was a base strong enough to open the lactone ring and elute the amino acid. With N-methylpyrazole as the base, however, it was difficult to elute the amino acid, presumably because this base is too weak to open the lactone ring. Ammonia was therefore employed as an eluent. Great losses occurred, and hence the amount of amino acid isolated corresponded only to a small fraction of the total amount present in the starting material.

The purified sample possessed the elemental composition C₅H₁₁NO₄. Its NMR-spectrum (in D₂O) indicated that the compound was a 2-amino-4,5-dihydroxypentanoic acid. A distorted triplet at 2.0 ppm (DOH as internal standard) was attributed to the methylene protons at C₃, split by the protons on C₂ and C₄. The C₂-proton appeared as a triplet at 3.9 ppm, split by two protons at C₃. This signal was overlapped by the signal for the proton on C₄,

Fig. 1. Fractionation scheme for amino acids in seed extracts of *Lunaria annua* L.

Disintegrated and defatted seeds
 Extraction with 70 % methanol
 Isolation of total amino acid fraction on a strongly acid ion-exchange resin in the acid form and elution with ammonia
 Removal of acid amino acids on a strongly basic ion-exchange resin in the acetate form
 Removal of basic amino acids on a strongly acid ion-exchange resin in the ammonia form
 Fraction of neutral amino acids (4 g)
 Strongly acid ion-exchange resin in the 3-chloropyridinium form (elution with 3-chloropyridine)



a complex pattern from 3.6 to 4 ppm. The two protons on C_5 also gave a complex pattern around 3.5 ppm with one intense peak (compare the NMR-spectra of propylene glycol and glycerol⁷). The integration of the spectrum was in accordance with this interpretation.

Again, the mass spectrum of the amino acid was in accordance with the proposed structure. Peaks with mass numbers higher than that of the molecular ion at 149 were observed, probably caused by bimolecular cyclodehydration of the molecule in the heated inlet in analogy with observations in mass spectra of other amino acids. Thus $2M-36$ was one of the dominating peaks. Other prominent peaks were: $M-18$ (loss of H_2O), $M-13$ (loss of CH_2OH), and $M-45$ (loss of $COOH$) (cf. Ref. 8).

By heating the amino acid with strong acid a lactone was produced as previously observed,² along with traces of aspartic acid. Treatment of the amino acid with permanganate resulted in the production of aspartic acid in nearly quantitative yield whereas exposure to hydroiodic acid and red phosphorus yielded only minute amounts of the expected product, norvaline. Aspartic acid, proline, and unidentified compounds were also observed in the reaction mixture, but most of the starting material was recovered unchanged (after opening of the formed lactone). The previously observed² production of formaldehyde by treatment with periodic acid⁹ was confirmed. All these observations are in accordance with the proposed structure.

No explanation is available, however, for the previously observed² production of acetaldehyde and ammonia upon treatment with periodic acid.^{9,10} Authentic material (*vide infra*) displayed the same behaviour. The identification of acetaldehyde and ammonia are based on colour reactions on paper chromatograms.^{9,10} Slightly different colours were obtained when threonine was subjected to the same treatments.

Syntheses of a mixture of the four stereoisomers of 2-amino-4,5-dihydroxypentanoic acid¹¹ as well as of a mixture of the two diastereoisomers with *L*-configuration at C_2 (Ref. 12) have been previously reported, the latter proceeding by reduction of the naturally occurring *L*-2-amino-5-hydroxy-4-oxopentanoic acid.¹² A sample of the latter mixture exhibited the same NMR-spectrum, mass spectrum, and paper chromatographic behaviour as our natural product, and the IR-spectra exhibited only minor differences. The diastereoisomeric mixture contained the two isomers in the molar ratio 10:7 as shown by quantitative ion-exchange chromatography. Paper electrophoresis in 10 % acetic acid also resolved the mixture into its two components. The isomers present in largest amounts was first eluted from the ion-exchange resin and was slowest moving in paper electrophoresis. Our natural product was a mixture of the same two diastereoisomers as revealed by ion-exchange chromatography and paper electrophoresis. The molar ratio was here 10:1.3, again with preponderance of the isomer with the lowest mobility in paper electrophoresis. It is reasonable to assume that the original natural product consists of this isomer and that a small amount of epimerization has taken place during the isolation. A similar isomerization has been observed during the isolation of the structurally related 2-amino-4-hydroxypentanoic acid.¹³ The configuration at C_2 of the natural compound has been determined as *L*-(or *S*) by rotatory dispersion curve measurement of the aspartic acid pro-

duced by oxidation of the compound with permanganate. The configuration at C_4 , however, remains undetermined. The small amounts available of the natural compound did not permit rotation determinations. Nevertheless, the established configuration at C_2 , combined with the known electrophoretic mobility of the natural compound, will permit determination of the configuration at C_4 of the natural compound provided this can be established on a synthetic preparation.

In attempts to isolate the unknown compound previously referred to as No. 10^{1,2} it was observed that more than one ninhydrin-reacting compound was present in the same area of the two-dimensional paper chromatograms. Some of the compounds were present only in minute amounts and only two in quantities sufficiently large for isolation. Whereas one of these proved identical with or closely related to a previously described constituent² (*vide infra*), the other, $C_7H_{14}N_2O_4$, appeared to be new to nature. Treatment with acid resulted in the production of two ninhydrin-reacting compounds, identified on basis of co-chromatography as glutamic acid and ethanolamine. The NMR-spectrum, recorded in D_2O , indicated that the compound was N^5 -(2-hydroxyethyl)glutamine. The four methylene protons in the glutamic acid moiety appeared as complex pattern of signals in the region around 2.3 ppm, identical with that observed in this region in the NMR-spectrum of glutamine. The four methylene protons in the ethanolamine moiety gave a complex pattern, partly overlapping the signal arising from the proton at C_2 in the glutamine moiety and located between 3 and 4 ppm. The integration of the spectrum was in accordance with this interpretation. The identity was further established by synthesis and comparison of infrared absorption spectra, NMR-spectra, and by co-chromatography. Rotatory dispersion curves established the configuration at C_2 of the natural compound as L.

A small amount of the previously described compound² was isolated, but no conclusive evidence for its structure has yet been obtained.

FREE AMINO ACIDS IN OTHER PARTS THAN SEEDS OF *Lunaria annua* L.

Investigations on the content of free amino acids, and other ninhydrin-reacting substances, in other parts of *Lunaria annua* L. than seeds gave an unexpected result. The investigations were performed on plants in the vegetative phase, divided into leaves and bulbs, and on flowering plants, divided into roots with bulbs, stems, leaves, and flowers with unripe seeds. The amino acid fractions were isolated by use of an ion-exchange resin, and two-dimensional paper chromatography was performed. Of all the compounds observed in the seeds only 2-amino-4,5-dihydroxypentanoic acid, γ -aminobutyric acid, pipercolic acid and protein amino acids occurred in significant quantities in the samples. In fact, 2-amino-4,5-dihydroxypentanoic acid was one of the dominating constituents in all the samples. Traces of 3-(3-carboxy-4-hydroxyphenyl)alanine occurred, whereas none of the other seed constituents could be detected. In the ripe seeds, harvested from plants grown for this experiment, all the compounds were refound.

SYNTHESES OF γ -GLUTAMYL DERIVATIVES OF AMINES

During the structural studies, a number of γ -glutamyl derivatives of amines were prepared. Two methods were applied: (1) the reaction between *p*-toluenesulphonyl-L-pyroglutamic acid,¹⁴ or its monohydrate,¹⁵ and the amine, with subsequent detosylation by sodium in liquid ammonia,¹⁶ a method known to give γ -glutamyl derivatives,¹⁷ (2) the reaction between carbobenzoxy-L-glutamic acid α -benzylester, ethyl chloroformate, followed by the amine,¹⁸ also used in the previously reported syntheses of γ -glutamyl derivatives.² Whereas the latter method was found suitable for the synthesis of N⁵-(2-methylthioethyl)-L-glutamine, the analogous N⁵-(3-methylthiopropyl)-L-glutamine could not be prepared in this way, because it was found impossible to remove the protecting groups by catalytic hydrogenation. However, both compounds were obtained by the first method, utilized also for the synthesis of N⁵-(2-hydroxyethyl)-L-glutamine. A synthesis of this compound, in low yield from L-pyroglutamic acid and ethanolamine has previously been reported.¹⁹ *p*-Toluenesulphonyl-L-pyroglutamic acid was also used for the synthesis of N,N'-bis-(γ -L-glutamyl)cystamine from cysteamine. Air oxidation caused disulfide formation in the first step. The removal of the tosyl group with sodium in ammonia caused reduction of the disulfide bond, reestablished, however, by air oxidation so that only the disulfide compound was isolated. A synthesis of N-(γ -L-glutamyl)cysteamine has been reported previously, yet without experimental details.²⁰ Co-chromatography established the identity between this preparation and our compound with free SH-group, and between the two disulfides obtained by air oxidation. The infrared absorption spectra of the two disulfides were identical.

Manometric Van Slyke determination of α -amino-N (Ref. 21) provided independent proof for the γ -glutamyl substitution of all compounds synthesized.¹⁸

Oxidation of the two thioethers was performed with H₂O₂ yielding the corresponding sulfoxides, N⁵-(2-methylsulphinyylethyl)-glutamine and N⁵-(3-methylsulphinylpropyl)glutamine. Since this step introduces a new chiral center, the oxidized product presumably represents a diastereoisomeric mixture. No attempts were made, however, to purify the compounds which were only characterized by their *R_F*-values.

DISCUSSION

As described in the present and two preceding communications,^{1,2} seeds of *Lunaria annua* L. contain several non-protein amino acids and γ -glutamyl derivatives. Among the numerous crucifer seeds investigated in this laboratory only those of *L. rediviva* L. showed a comparable complexity. 2-Amino-4,5-dihydroxypentanoic acid has, on the basis of paper chromatography, been identified also in seeds of the crucifers *Aubrietia erubescens* Griseb. and *Lepidium campestre* R.Br.

Formally, 2-amino-4,5-dihydroxypentanoic acid belongs to the series of natural amino acids with a straight five-carbon chain. Several of these contain a hydroxy group at C₄, such as 2-amino-4-hydroxypentanoic acid, recently

isolated from *Lathyrus odoratus*,¹³ γ -hydroxyarginine and γ -hydroxyornithine,²² γ -hydroxyglutamic acid, 4-hydroxyproline, and 4-*allo*-hydroxyproline.²³ Other amino acids with closely related structures are L-2-amino-5-hydroxypentanoic acid, isolated from *Canavalia ensiformis*,²⁴ and 2-amino-5-hydroxy-4-oxopentanoic acid, from *Streptomyces akiyoshiensis novo sp.*¹² The addition of N⁵-(2-hydroxyethyl)-L-glutamine to the rapidly growing list of naturally occurring γ -glutamyl derivatives^{23,25} can hardly be surprising in view of the widespread occurrence of ethanolamine in nature²⁶ and the previous identification of this aminoalcohol in *Lunaria annua* L.¹ The new compound is structurally related to N⁵-ethyl-L-glutamine (theanine), N⁵-ethyl-L-asparagine and N⁵-(2-hydroxyethyl)-L-asparagine, all occurring in higher plants.²³

EXPERIMENTAL

Isolations

The fraction of neutral amino acids (4 g) was obtained from 1 kg of seeds of *Lunaria annua* L. (purchased from I. E. Ohlsen's Enke, Copenhagen) by traditional methods including defatting with carbon tetrachloride, extraction with methanol:water (7:3), isolation of the total amino acid fraction on a strongly acid ion-exchange resin in the acid form with subsequent elution of the amino acids with ammonia, removal of acidic amino acids on a strongly basic ion-exchange resin in the acetate form, and removal of basic amino acids on a strongly acidic ion-exchange resin in the ammonium form.

The amino acids were dissolved in water (10 ml) and applied to a strongly acidic ion-exchange resin in the 3-chloropyridinium form (Dowex 50 W X 8, 200–400 mesh, 2.5 × 45 cm) (3-chloropyridine was obtained from Cilag-Chemie AG, Schaffhausen, Switzerland). The effluent was collected in fractions of each 200 drops, *ca.* 12 ml. The column was washed with water and from fraction 30 eluted with a saturated aqueous solution of 3-chloropyridine (*ca.* 0.25 M). Fractions 1–30 (1.3 g) contained amino acids with low pK₁-values, proline, N⁵-isopropylglutamine, 2-amino-4,5-dihydroxypentanoic acid, N⁵-(2-hydroxyethyl)glutamine, and the unidentified compound (*first fraction*). Fractions 31–60 (0.7 g) contained asparagine, serine, N⁵-isopropylglutamine, threonine, and 2-amino-4,5-dihydroxypentanoic acid (*second fraction*). In later fractions occurred alanine, glycine, pipercolic acid, and valine, whereas some of the amino acids present could not be eluted from the column with reasonable amounts of 3-chloropyridine.

The *first fraction* was used for purification of N⁵-(2-hydroxyethyl)glutamine. The amino acids were dissolved in water (10 ml) and applied to a strongly acidic ion-exchange resin in the N-methylpyrazolium form (Dowex 50 W X 8, 200–400 mesh, 2.5 × 45 cm) (N-methylpyrazole was prepared as described under Syntheses). The effluent was collected in fractions of each 200 drops, *ca.* 12 ml. The column was washed with water (20 ml) and then eluted with an aqueous solution of N-methylpyrazole (0.25 M). No compounds were eluted in fractions preceding fraction 76. Fractions 76–84 contained betaine, fractions 85–100 contained N⁵-(2-hydroxyethyl)glutamine, fractions 101–110 contained N⁵-(2-hydroxyethyl)glutamine and the unidentified compound. Proline, N⁵-isopropylglutamine, and 2-amino-4,5-dihydroxypentanoic acid occurred in later fractions.

Isolation of betaine from fractions 76–84. Purification was accomplished with a carbon column and a strongly acidic ion-exchange resin (elution with pyridine). The crystalline residue from the last column was identified as betaine by comparison of infrared absorption spectra and NMR-spectra in D₂O with those of an authentic sample.

Isolation of N⁵-(2-hydroxyethyl)-L-glutamine from fractions 85–100. Purification was accomplished by chromatography on a strongly acidic ion-exchange resin in the N-methylpyrazolium form (Dowex 50 X 8, 1.1 × 80 cm; the resin had been fractionated by sedimentation²⁷ and the particle size was 45–70 μ). The compounds were dissolved in water (2 ml) and the solution was applied to the column. Fractions of each 200 drops, *ca.* 12 ml, were collected. The column was washed with water (10 ml) and eluted with 0.25 M N-methylpyrazole. N⁵-(2-Hydroxyethyl)-L-glutamine occurred in fractions 35–

38. After determination of the NMR-spectrum, further purification was accomplished by use of a small carbon column and a strongly acidic ion-exchange resin (elution with pyridine). The crystalline residue from the last column (19 mg) was used for determination of the infrared absorption spectrum. A pure sample was obtained by recrystallization from ethanol:water. (Found (ultramicroanalysis): C 43.4; H 7.5; N 14.7. Calc. for $C_7H_{14}N_2O_4$: C 44.2; H 7.4; N 14.7. The substance was dried to constant weight at 50° and 0.04 mm Hg before analysis and no water of crystallization was noticed (compare the synthetic sample)). The natural product was identical with the synthetic sample (*vide infra*) with regard to infrared absorption spectra, NMR-spectra, and paper chromatographic behaviour. The rotatory dispersion curves measured in 95 % ethanol (c 0.10) were also identical, indicating that the natural product has the L-configuration. Acid hydrolysis produced glutamic acid and ethanolamine, identified by co-chromatography with authentic samples on paper.

Separation of N⁵-(2-hydroxyethyl)glutamine and the unidentified compound from fractions 101–110. The separation was performed on the column that was used for the purification of N⁵-(2-hydroxyethyl)glutamine from fractions 85–100. An additional sample of this compound (3 mg) was obtained from fractions 36–38 whereas the unidentified compound occurred in fractions 40–48.

The second fraction was used for purification of 2-amino-4,5-dihydroxypentanoic acid. The amino acids were dissolved in water (2 ml) and applied to the column that was used for purification of N⁵-(2-hydroxyethyl)glutamine. Asparagine, serine, N⁶-isopropylglutamine, and threonine occurred in fractions 1–78. 2-Amino-4,5-dihydroxypentanoic acid occurred in some of these fractions as well as in fractions 79–163. The column was then eluted with ammonia (1 M) to remove all residual 2-amino-4,5-dihydroxypentanoic acid. The amino acid was collected from the fractions not containing other amino acids and was purified by use of a strongly acidic ion-exchange resin (elution with ammonia), preparative paper chromatography in butanol:acetic acid:water, a small carbon column, and a strongly acid ion-exchange resin (elution with ammonia). The crystalline residue from the last column (12 mg) was used for determination of the NMR-spectrum and infrared absorption spectrum. A pure sample was obtained by recrystallization from ethanol:water. (Found (ultramicroanalysis): C 39.8; H 7.4; N 10.1. Calc. for $C_5H_{11}NO_4$: C 40.3; H 7.4; N 9.4).

Degradation studies of the natural 2-amino-4,5-dihydroxypentanoic acid and comparison with a synthetic sample. Attempts to reduce the natural compound with hydroiodic acid and red phosphorus were performed as described in the literature.²⁸ The amino acids were isolated from the reaction mixture by use of a strongly acidic ion-exchange resin (elution with pyridine). By use of two-dimensional paper chromatography and co-chromatography with authentic samples small amounts of aspartic acid, proline, α -aminobutyric acid, and norvaline were identified, but the dominating compound was unchanged 2-amino-4,5-dihydroxypentanoic acid.

The infrared absorption spectra of the natural product and the synthetic sample (obtained by catalytic reduction of the naturally occurring L-2-amino-5-hydroxy-4-oxopentanoic acid¹²) were nearly coinciding. The NMR-spectra and mass spectra and the paper chromatographic behaviour were identical.

Quantitative ion-exchange chromatography was performed on a Beckman Model 120 B Amino Acid Autoanalyzer with a 60 cm column, buffer pH 3.28, and a flow rate of 40 ml/h. The synthetic sample showed two peaks appearing after 83 and 95 min in the relative amounts 10:7. The natural compound showed the same two peaks but in the proportion 10:1.3.

Paper electrophoresis in 10 % acetic acid at 82 V/h resolved the synthetic mixture into two components in the course of 2 h. The natural compound showed the same two components with the slowest moving in largest amounts.

Production of L-aspartic acid by permanganate oxidation of 2-amino-4,5-dihydroxypentanoic acid. 4 mg dissolved in water (1 ml) was oxidized by addition of 100 μ l portions of a solution of potassium permanganate (1 %) in sulfuric acid (10 % (v/v)) at 60° until the violet colour persisted (2.1 ml permanganate solution in all). Excess permanganate was removed with ethylene glycol, sulfuric acid was removed by addition of excess of barium hydroxide (saturated solution), and barium ions were removed by addition of ammonium carbonate. After filtration, the solution was evaporated to dryness. The aspartic acid was purified by use of a strongly acid ion-exchange resin (elution with pyri-

dine) and a strongly basic ion-exchange resin (elution with acetic acid). The crystalline residue from the last column was shown by paper chromatography and co-chromatography to be pure aspartic acid. The rotatory dispersion curve was measured in 95 % ethanol (0.1 N with regard to HCl) at c 0.15 and had a positive Cotton effect identical with that of authentic L-aspartic acid, $[\alpha]_{223}^{25}$ 1133° (peak); $[\alpha]_{213}^{25}$ 0°.

Syntheses

*N*²-Carbobenzoxy-*N*⁵-(2-methylthioethyl)-L-glutamine benzyl ester (I). This compound was obtained from carbobenzoxy-L-glutamic acid α -benzylester (250 mg, Cyclo Chemical Corporation) and 2-methylthioethylamine (100 μ l, prepared as reported in the literature²⁹) by the same procedure as that previously described.³ The crude reaction product was recrystallized first from ethyl acetate-pentane and subsequently from methanol-water to give a pure sample (172 mg, 57 %), m.p. 119–120°, $[\alpha]_{\text{D}}^{24}$ –15.9° (c 1.5, CH₃OH). (Found: C 61.97; H 6.33; N 6.42; Calc. for C₂₃H₂₈N₂O₅S: C 62.14; H 6.35; N 6.31).

*N*⁵-(2-Methylthioethyl)-L-glutamine (II) from I. The synthesis was performed by catalytic hydrogenation of I (150 mg) by the same procedure as that previously described.³ The crude reaction product was recrystallized twice from ethanol-water to give a pure sample (20 mg, 27 %), m.p. 200.5–201.5°, $[\alpha]_{\text{D}}^{26}$ + 7.6° (c 1.4, H₂O). (Found: C 43.60; H 7.45; N 12.54. Calc. for C₈H₁₆N₂O₃S: C 43.62; H 7.32; N 12.72).

*N*³-Carbobenzoxy-*N*⁵-(3-methylthiopropyl)-L-glutamine benzyl ester (III). This compound was obtained from carbobenzoxy-L-glutamic acid α -benzylester (2.5 g) and 3-methylthiopropylamine (1 ml, prepared as reported in the literature³⁰) by the same procedure as that previously described.³ The crude reaction product was recrystallized first from ethyl acetate-pentane and subsequently from methanol-water to give a pure sample (1.76 g, 59 %), m.p. 106–107°, $[\alpha]_{\text{D}}^{25}$ –13.1° (c 1.3, CH₃OH). (Found: C 63.09; H 6.59; N 6.14; S 7.01. Calc. for C₂₄H₃₀N₂O₅S: C 62.86; H 6.59; N 6.11; S 6.99).

Attempts to hydrogenate III to *N*⁵-(3-methylthiopropyl)-L-glutamine were unsuccessful and only minute amounts of the desired reaction product could be identified by paper chromatography.

*N*²-(*p*-Toluenesulphonyl)-*N*⁵-(2-methylthioethyl)-L-glutamine (IV). A solution of 2-methylthioethylamine²⁹ (3.28 g) and *p*-toluenesulphonyl-L-pyroglutamic acid¹⁴ (5.10 g) in acetonitrile (60 ml) was refluxed for 2 h and evaporated to dryness. The residue was dissolved in water (75 ml) and hydrochloric acid (6 ml, 4 N) was added. The precipitate was isolated by filtration, dissolved in sodium hydrogen carbonate solution (60 ml, 5 %) and hydrochloric acid was added in excess. After cooling, the reaction product was isolated by filtration (4.43 g, 66 %). A pure sample was obtained after two recrystallizations from water, m.p. 181–181.5°, $[\alpha]_{\text{D}}^{23}$ + 30.6° (c 2.0, CH₃OH). (Found: C 48.00; H 6.08; N 7.34; S 17.32. Calc. for C₁₅H₂₂O₅S₂: C 48.10; H 5.92; N 7.48; S 17.12).

II from IV. To a solution of IV (3.6 g) liquid ammonia (200 ml) small pieces of sodium were added until a blue colour persisted in the solution (840 mg). Excess sodium was removed by addition of solid ammonium chloride and the solution was evaporated to dryness. The last traces of ammonia were removed by heating to 40° under reduced pressure. The residue was dissolved in water and strongly acidic ion-exchange resin (Amberlite IR 120) in the acid form was added to produce a pH-value of 3 in the solution. The solution with resin was transferred to a column of the same resin (1.5 × 40 cm in all). After washing with water, the reaction product was eluted with ammonia (1 N) and the eluate was evaporated to dryness (1.93 g, 96 %). A pure sample was obtained by two recrystallizations from ethanol-water, m.p. 199.2–200.6°, $[\alpha]_{\text{D}}^{22}$ + 7.5° (c 0.9, H₂O), $[\alpha]_{\text{D}}^{22}$ + 29.6° (c 0.9, 1 N HCl). (Found: C 42.87; H 7.32; N 12.68; S 14.76; α -amino-N 6.28. Calc. for C₈H₁₆N₂O₃S: C 43.62; H 7.32; N 12.72; S 14.56; α -amino-N 6.36). The two synthetic samples of II were identical with regard to infrared absorption spectra and paper chromatographic behaviour.

*N*²-(*p*-Toluenesulphonyl)-*N*⁵-(3-methylthiopropyl)-L-glutamine (V). This compound was obtained from *p*-toluenesulphonyl-L-pyroglutamic acid¹⁴ (5.66 g) and 3-methylthiopropylamine³⁰ (4.2 g) by the procedure described for the production of IV. The crude reaction product (5.50 g, 71 %) was recrystallized twice from water to give a pure sample, m.p. 156–156.5°, $[\alpha]_{\text{D}}^{22}$ + 31.4° (c 2.1, CH₃OH). (Found: C 49.47; H 6.33; N 7.32; S 16.47. Calc. for C₁₆H₂₄N₂O₅S₂: C 49.47; H 6.23; N 7.21; S 16.51).

*N*⁵-(3-Methylthiopropyl)-L-glutamine (VI). This compound was obtained from V (3.88 g) by the procedure described for the production of II from IV. The crude reaction product (2.16 g, 92 %) was recrystallized twice from ethanol-water to give a pure sample, m.p. 197.6–199.9°, $[\alpha]_D^{21} + 5.3^\circ$ (c 1.0, H₂O), $[\alpha]_D^{20} + 21^\circ$ (c 1.2, 1 N HCl). (Found: C 45.69; H 7.65; N 11.78; S 13.71; α -amino-N 5.97. Calc. for C₉H₁₈N₂O₃S: C 46.14; H 7.74; N 11.96; S 13.68; α -amino-N 5.98).

*N*²-(*p*-Toluenesulphonyl)-*N*⁵-(2-hydroxyethyl)-L-glutamine (VII). This compound was obtained from *p*-toluenesulphonyl-L-pyroglutamic acid, H₂O¹⁵ (6.04 g) and ethanolamine (3 g) by reflux in water (50 ml). For the isolation of the reaction product the same method was used as that described for the production of IV. The crude product (1.6 g, 23 %) was recrystallized twice from water to give a pure sample, m.p. 157.8–159.9°, $[\alpha]_D^{21} + 27.9^\circ$ (c 2.0, CH₃OH). (Found: C 48.69; H 5.95; N 8.17; S 9.29. Calc. for C₁₄H₂₀N₂O₆S: C 48.83; H 5.85; N 8.13; S 9.31).

*N*⁵-(2-Hydroxyethyl)-L-glutamine, H₂O (VIII). This compound was obtained from VII (1.3 g) by the procedure described for the production of II from IV. A pure sample (488 mg, 58 %) was obtained after two recrystallizations from ethanol-water, m.p. 188.5–191.0° (Lit. value 176° (Ref. 19), $[\alpha]_D^{19} + 5.8^\circ$ (c 1.8, H₂O) (Lit. value $[\alpha]_D^{15} + 5.4^\circ$ (c 0.5, H₂O)¹⁹). (Found: C 40.19; H 7.83; N 13.40; α -amino-N 6.68; H₂O (determined as weight loss by drying to constant weight at 50° and 0.1 mm Hg; the water of crystallization was regained when the anhydrous sample was exposed to the atmosphere) 8.59. Calc. for C₇H₁₄N₂O₄, H₂O: C 40.38; H 7.75; N 13.46; α -amino-N 6.73; H₂O 8.65).

N,N'-bis-(γ -*p*-Toluenesulphonyl)-L-glutamylcystamine (IX). This compound was obtained from *p*-toluenesulphonyl-L-pyroglutamic acid, H₂O¹⁵ (6.02 g) and cysteamine (3.1 g) by the procedure described for the production of VII. The crude reaction product (6.06 g, 84 %) was recrystallized three times from water to give a pure sample, m.p. 159–168°, $[\alpha]_D^{20} + 32.0^\circ$ (c 2.1, CH₃OH). (Found: C 46.60; H 5.59; N 7.64; S 17.72. Calc. for C₂₈H₃₈N₄O₁₀S₄: C 46.78; H 5.33; N 7.79; S 17.84).

That the product was a disulfide was inferred from the negative reaction for mercapto groups with sodium nitroprusside and the positive reaction after treatment with cyanide ions.³¹

N,N'-bis-(γ -L-Glutamyl)cystamine (X). This compound was prepared from IX (7.2 g) by the procedure described for the production of II from IV. The crude reaction product (3.49 g, 85 %) contained both the mercapto product and the disulfide as inferred from paper chromatography and reaction with sodium nitroprusside.³¹ Three recrystallizations from ethanol-water afforded a pure sample of the disulfide, m.p. 216.5–218°, $[\alpha]_D^{20} + 30.0^\circ$ (c 0.9, 1 N HCl). (Found: C 40.50; H 6.44; N 13.40; S 15.24; α -amino-N 6.83. Calc. for C₁₄H₂₂N₄O₆S₂: C 40.96; H 6.38; N 13.65; S 15.62; α -amino-N 6.82).

*N*⁵-(2-Methylsulphinylethyl)-L-glutamine (XI). This compound was prepared from II (5 mg), dissolved in water (500 μ l), by oxidation with hydrogen peroxide (3 μ l 30 % aqueous solution). Paper chromatographic analysis of the reaction mixture demonstrated the quantitative transformation of II to XI overnight at room temperature. XI was easy to recognize by its smaller *R_F*-value and its positive reaction for sulphoxide groups.³²

*N*⁵-(3-Methylsulphinylpropyl)-L-glutamine (XII). This compound was obtained from VI by the procedure described for the production of XI from II. Again the reaction product was easily recognizable by its smaller *R_F*-value and its positive reaction for sulphoxide groups.³²

N-Methylpyrazole. To a refluxing mixture of water (300 ml) and ethanol (200 ml) were simultaneous added, in the course of 2 h, 1,1,3,3-tetramethoxypropane (328 g, Aldrich Chemical Co.), and a solution of methylhydrazine (92 g, Aldrich Chemical Co.), in a mixture of concentrated hydrochloric acid (400 ml) and ethanol (300 ml). The reaction mixture was boiled under reflux for one additional hour, and the alcohols were removed by concentration under reduced pressure. Water (400 ml) and anhydrous sodium carbonate (400 g) was added and the two phases were separated after cooling. The lower phase was extracted three times with ether. The upper phase and the ether extracts were pooled, dried with anhydrous sodium sulphate, and, after removal of the ether, the product was distilled to give colourless material (103 g, 63 %), b.p. 123–126° (Lit. value 127° (Ref. 6)), n_D^{20} 1.4758 (Lit. value $n_{587.56}^{13.7}$ 1.487 (Ref. 33)), picrate m.p. 147–150° (Lit. value 148° (Ref. 33)).

Precautions should be taken in the mixing of the reactants in this synthesis to avoid a sudden and very vigorous reaction.

Paper chromatography

The following R_F -values were determined for the new compounds and for the sulphur-containing amines used in the syntheses by descending chromatography on Whatman paper No. 1 at 25° in (1) butanol: acetic acid: water (12:3:5 (v/v/v)) and (2) phenol: water: conc. ammonia (120:30:1 (w/v/v)). 2-Methylsulphinylethylamine and 3-methylsulphinylpropylamine were prepared from the corresponding thioethers by oxidation with hydrogen peroxide. In solvent 2, the amines had R_F -values close to 1.

	(1)	(2)	Colour developed with ninhydrin on the chromatograms
2-Methylthioethylamine	0.58		Greyish-blue
2-Methylsulphinylethylamine	0.32		Rose
3-Methylthiopropylamine	0.71		Brown
3-Methylsulphinylpropylamine	0.38		Yellow
Cysteamine	0.51		Brown
Cystamine	0.22		Rose
N ⁵ -(2-Methylthioethyl)-L-glutamine	0.50	0.91	Purple
N ⁵ -(2-Methylsulphinylethyl)-L-glutamine	0.26	0.92	Purple
N ⁵ -(3-Methylthiopropyl)-L-glutamine	0.57	0.95	Purple
N ⁵ -(3-Methylsulphinylpropyl)-L-glutamine	0.29	0.94	Purple
N ⁵ -(2-hydroxyethyl)-L-glutamine	0.28	0.78	Purple
N-(γ -L-glutamyl)cysteamine	0.41	0.81	Purple
N,N'-bis(γ -L-glutamyl)cystamine	0.18	0.80	Purple
2-Amino-4,5-dihydroxypentanoic acid	0.23	0.47	Purple

General methods, instrumentation, acknowledgements

Where not otherwise stated microanalyses were performed by Mr. G. Cornali, Copenhagen. Microanalyses designated 'Ultramicroanalysis' were performed at the Micro-analytical Laboratory, Institute for Medical Chemistry, University of Uppsala, Sweden.

Rotations were measured in a 1 dm tube. Determinations of rotations of γ -glutamyl derivatives in hydrochloric acid were made 5 min after preparation of the solutions. No change in rotation was observed during the first 15 min (*cf.* Ref. 1).

Mass spectroscopy was performed on an LKB 9000 instrument with direct inlet system at the Laboratory for Mass Spectroscopy, Wallenberg Laboratory, Karolinska Institutet, Stockholm, Sweden. Infrared absorption spectra were determined in potassium bromide pellets on a Perkin-Elmer Infracord instrument.

Melting points were determined in capillary tubes in an Anschutz-Hersberg apparatus equipped with fully immersed thermometers. The standard rate of heating was 2° per min.

Purification of the various compounds isolated was accomplished by use of preparative paper chromatography, ion-exchange resins, and small columns of deactivated carbon as previously described.²

All solvents from the various eluates were removed by evaporation under reduced pressure. The last traces of ammonia, pyridine, 3-chloropyridine, and N-methylpyrazole were removed by repeated evaporations with water.

Manometric Van Slyke determinations of α -amino-N were carried out at the Agricultural Research Department, Atomic Energy Commission Research Establishment, Risø, Roskilde, Denmark. The author wishes to express his gratitude to Miss I. Nørsgaard and Dr. H. Sørensen for these determinations and to the Department for its hospitality. The plants used for studies of the content of free amino acids in parts other than seeds of *Lunaria annua* L. were grown in greenhouses in the same Department.

Determination of rotatory dispersion curves were performed by the very kind service of Dr. J. Cymerman Craig.

NMR-Spectra were measured in D₂O on a Varian A-60 instrument at the Department of Organic Chemistry, The Technical University, Copenhagen. The assistance of Dr. C. Pedersen in recording the spectra is gratefully acknowledged.

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