Phytochemistry, 1974 Vol 13 pp 2803 to 2811 Pergamon Press Printed in England

FREE AMINO ACIDS AND γ-GLUTAMYL PEPTIDES IN SEEDS OF *FAGUS SILVATICA**

IB KRISTENSEN, PEDER OLESEN LARSEN and HILMER SØRENSEN

Department of Organic Chemistry, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Copenhagen, Denmark

(*Received* 21 *April* 1974)

Key Word Index—*Fagus silvatica*, Fagaceae, beechnuts, non-protein amino acids, γ-glutamyl peptides, N^5-acetylornithine, 3-(2-furoyl)alanine; 4-hydroxyproline, pipecolic acid, willardine, preparative ion exchange chromatography

Abstract—The contents of amino acids and peptides have been investigated in seeds of *Fagus silvatica* L (beechnuts) In addition to the common amino acids, the following compounds have been isolated and identified 4-hydroxyproline (probably the *cis*-L-isomer), N^5-acetylornithine, 3-(2-furoyl)-L-alanine, methionine sulfoxide (probably an artefact), pipecolic acid (probably partially racemized D-isomer), L-willardine (with a small amount of the D-isomer), *N*-(3-amino-3-carboxypropyl)azetidine-2-carboxylic acid, *N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]azetidine-2-carboxylic acid, 2(*S*),5(*S*),6(*S*)-5-hydroxy-6-methylpipecolic acid, 2(*S*),5(R),6(S)-5-hydroxy-6-methylpipecolic acid, γ-glutamylalanine, γ-glutamylglutamic acid, γ-glutamylisoleucine, γ-glutamylleucine, γ-glutamylmethionine sulfoxide (probably an artefact), γ-glutamylphenylalanine, γ-glutamyltyrosine, γ-glutamylvaline, glutathione, γ-glutamylwillardine, and γ-glutamylphenylalanylwillardine γ-Glutamylphenylalanine and willardine are the dominating components of the amino acid fraction

The isolations were performed by use of ion exchange chromatography, taking advantage of the different pK-values of the amino acids, mainly on acid resins in the 3-chloropyridinium form with aq 3-chloropyridine as eluant and on basic resins in the acetate form with aqueous acetic acid as eluant These methods in combination with preparative paper chromatography have permitted the isolation and identification of compounds present in amounts as low as 1/6000 of the dominant ninhydrin-reactive component The implications of the occurrence of this large variety of compounds in the Fagaceae are briefly discussed

INTRODUCTION

SEEDS of *Fagus silvatica* L. (beechnuts) have been used as food for man and animals since ancient times. They contain however toxic principles as reported in the literature [2 3] The nature of the toxic constituents has not been established, but it seems likely that water soluble low molecular weight compounds are involved.[2–5] It has been shown in recent years that several non-protein amino acids in seeds of leguminous plants including trees are toxic to insects and presumably protect the seeds against insect attacks.[6] Similar mechanisms may of course operate in other plant families. Finally, it has been reported on paper-chromatographic evidence that phenylglycine is present in the bleeding sap of

* Taken in part from the thesis of I Kristensen, Copenhagen (1973) [1]

[1] KRISTENSEN, I (1973) Free amino acids and γ-glutamyl peptides in *Fagus silvatica* L, Thesis, The Royal Veterinary and Agricultural University, Copenhagen, Denmark

[2] VAN EEKELEN, M and VAN DER LAAN, P J (1945) *Voeding* **1945**, 83

[3] HOTOVY, R (1947) *Klin Wochschr* **24–25**, 635

[4] BECKMAN, S and MANZ, A (1959) *Landwirtschaft Forsch* **12**, 165

[5] KRAUZE, S and DZIEDZIANOWICA, W (1959) *Nahrung* **3**, 213

[6] REHR, S S, BELL, E A, JANZEN, D H and FEENY, P P (1973) *Biochemical Systematics* **1**, 63

F silvatica L [7,8] Whereas phenylglycine never has been established as a constituent in higher plants, other phenylglycine derivatives from plants have been studied in this laboratory [9] For all these reasons it was decided to study the contents of free amino acids in beechnuts. No reports on this subject are available in the literature.

The present paper gives a general account of the result of these studies In addition separation principles are demonstrated which have permitted the isolation of nearly all free amino acids and oligopeptides with only small losses In this way compounds present in amounts down to about 1/6000 of the dominant ninhydrin-reactive component have been isolated and identified.

An account of part of this work has been given previously [1] During the studies a number of previously unknown constituents have been obtained Separate publications will describe the structure determination and properties of these compounds, i e *N*-(3-amino-3-carboxypropyl)azetidine-2-carboxylic acid and *N*-(*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl)azetidine-2-carboxylic acid,[10] γ-glutamylwillardine and γ-glutamylphenylalanylwillardine,[11] and 2(*S*),5(*S*),6(*S*)-5-hydroxy-6-methylpipecolic acid and 2(*S*),5(*R*),6(*S*)-5-hydroxy-6-methylpipecolic acid [12]

METHODS AND RESULTS

Two isolations, each from 2 3 kg of beechnuts, have been performed The second isolation was performed without the use of ammonia in the first ion exchange step in order to prevent decomposition of labile compounds, including furoylalanine (see below). In addition a small scale isolation was performed under very mild conditions in order to exclude the formation of artefacts and to prove that all the amino acids obtained in the first two isolations were true plant constituents.

The isolation procedure used in the second isolation is described in the Experimental The separation of the acid components depended to a large degree on ion exchange chromatography on a basic resin in the acetate form and with acetic acid as eluant This has been a standard procedure for the last 20 years. The separation of the neutral components was based on ion exchange chromatography on an acid resin in the 3-chloropyridinium form and with aq 3-chloropyridine as eluant, a principle used previously only in a few instances [13,14]

The isolation revealed a large number of compounds of which only those present in high concentrations could be identified on chromatograms of the crude seed extract (Table 1) The dominant components are γ-glutamylphenylalanine and willardine [3-(1-uracil)-alanine]. Of the protein amino acids, aspartic acid, glutamic acid, alanine, valine, and leucine + isoleucine were present in highest concentrations. Rather large amounts were also present of the 5-hydroxy-6-methylpipecolic acids but because of the weak ninhydrin reaction of these compounds[12] they did not appear on the first chromatograms Some of the other γ-glutamyl peptides were also present in rather high concentrations The fractions of

[7] DIETRICHS, H H and FUNKE H (1967) *Holzforschung* **21**, 102
[8] DIETRICHS, H H (1969) *Holzforschung* **23**, 177
[9] KJÆR, A and LARSEN, P O (1963) *Acta Chem Scand* **17**, 2397
[10] KRISTENSEN I and LARSEN, P O (1974) *Phytochemistry* **13**, 2791
[11] KRISTENSEN I and LARSEN, P O (1974) *Phytochemistry* **13**, 2799
[12] ETTLINGER M G, KRISTENSEN, I, LARSEN, P O and OLSEN C E In preparation
[13] LARSEN, P O (1967) *Acta Chem Scand* **21**, 1592
[14] FRIIS, P HEILBOE, P and LARSEN, P O (1974) *Acta Chem Scand* Ser B **28**, 317

TABLE 1 NON-PROTEIN AMINO ACIDS IDENTIFIED IN BEECHNUTS*

Basic
Ethanolamine, ornithine

Acidic
γ-Glutamylalanine, γ-glutamylglutamic acid, γ-glutamylisoleucine, γ-glutamylleucine, γ-L-glutamyl-L-phenylalanine, γ-glutamylphenylalanylwillardine, γ-glutamyltyrosine, γ-glutamylvaline, γ-L-glutamyl-L-willardiine, glutathione (probably the disulfide)

Neutral
N^5-Acetylornithine, β-alanine, γ-aminobutyric acid, *N*-(3-amino-3-carboxypropyl)azetidine-2-carboxylic acid, *N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]azetidine-2-carboxylic acid, 3-(2-furoyl)-L-alanine, 2(S),5(S),6(S)-5-hydroxy-6-methylpipecolic acid 2(S),5(R),6(S)-5-hydroxy-6-methylpipecolic acid, 4-hydroxyproline, pipecolic acid, L- and DL-willardiine (3-(1-uracil)alanine)

* Methionine sulfoxide and its γ-glutamyl derivative were also identified but are probably artefacts.

basic amino acids were rather small. However, some losses of arginine may have occurred. The weights indicated for the different fractions and for the isolated compounds in the Experimental gives a fairly reliable estimation of the quantitative composition of the free amino acid pool in beechnuts. However especially in fractions 1.1.8 and 1.5 large amounts of dark-coloured non-ninhydrin reactive material were present.

The methods used for the identification and determination of the various compounds are described in Experimental. Compounds known to be widely distributed in plants were only identified by paper chromatography, including cochromatography.

Methionine sulfoxide probably is an artefact, produced from methionine during the isolation, since the CD-curve indicates that the isolated material is a mixture of the two diastereoisomeric sulfoxides. It is therefore likely, that the γ-glutamylmethionine sulfoxide is also an artefact. Measurement of the CD-curve for the 4-hydroxyproline indicated a *cis*-L-configuration. Measurement of the CD-curve for the pipecolic acid indicated that the sample was partly racemized D-material.

The specific rotation found for willardiine ($[\alpha]_D^{26}$ −20·5° in HCl) was higher than that reported for material from *Acacia willardiana* ($[\alpha]_D^{22}$ −12·1° in HCl)[15] but in good agreement with that reported for synthetic resolved material of unknown optical purity ($[\alpha]_D^{20}$ −20° in HCl).[16] The low value for the material from *A. willardiana* may be due to partial racemization From the mother liquor of the isolate from *F. silvatica* a sample with $[\alpha]_D^{26}$ −1·4° and with an IR-spectrum identical with that of racemic willardiine was found. The spectra for optically active and racemic willardiine are distinctly different [16,17] There is no reason to believe that willardiine could have been partly racemized during the isolation. It is therefore likely that willardiine occurs as a mixture of the L-form with about 5% of the D-form in *F. silvatica* L. PMR-data for willardiine are reported in Experimental.

PMR-, ^{13}C-NMR-, UV- and rotation data for the isolated 3-(2-furoyl)alanine are reported in Experimental The PMR- and UV-data are in agreement with the literature.[18,19] The rotation values, not previously reported, establish the L-configuration

[15] GMELIN, R (1959) *Z Physiol Chem* **316,** 164
[16] DEWAR, J H and SHAW, G (1962) *J Chem Soc* 583
[17] KJAER, A, KNUDSEN, A and LARSEN, P O (1961) *Acta Chem Scand* **15,** 1193
[18] ICHIHARA, A., HASEGAWA, H, SATO, H, KOYAMA, M and SAKAMURA, S (1973) *Tetrahedron Letters* 37
[19] COUCHMAN, R, EAGLES, J, HEGARTY, M P, LAIRD, W M, SELE, R and SYNGE, R L M (1973) *Phytochemistry* **12,** 707

according to the Clough–Lutz–Jirgensons rule.[20] The compound was stable when boiled in 6 N HCl, producing only traces of aspartic acid In contrast it was unstable in boiling ammonia and therefore was partly decomposed in the first isolation, which used ammonia as eluant from the first ion exchange resin 3-(2-Furoyl)alanine has been isolated as a decomposition product from ascorbalamic acid (3-hydroxy-2-oxo-[3-(2,3,4-trihydroxytetrahydrofuran)]pyrrolidine-5-carboxylic acid) by treatment with hydrochloric acid [19] The conditions for the transformation of ascorbalamic acid are however rather drastic compared with our isolation procedure. Furthermore the conditions for isolation of ascorbalamic acid are rather similar to those used here for the isolation of the amino acid. It therefore seems safe to conclude that 3-(2-furoyl)-L-alanine is a true constituent of *F silvatica* L

The constituent amino acids in the γ-glutamyl peptides isolated were in all cases identified by paper chromatography after acid hydrolysis γ-Glutamyl peptides are more labile to acid than normal peptides Hydrolysis was routinely performed by treatment with 6 N HCl for 4 hr at 100° in a sealed ampoule This treatment resulted in complete hydrolysis γ-Glutamylphenylalanine was shown to be completely stable for 7 days at room temp. in 2 N HCl At 50° in 6 N HCl a half life of between 12 and 24 hr was found for this peptide

Various methods are available for determination of the nature of the linkage in peptides containing glutamic acid Standard methods can be used to determine the sequence of the peptides, but the differentiation between α- and γ-glutamyl derivatives poses a specific problem. The only three reliable chemical methods are synthesis, quantitative decarboxylation with ninhydrin,[21] and deamination with nitrous acid[22] However the last two methods are destructive and require considerable amounts of material For acid γ-glutamyl-α-amino acids the elution behaviour on strongly basic ion exchange resins in the acetate form can be used for differentiation since the pK_2-values determine the sequence of elution The γ-glutamyl-α-amino acids have lower pK_2-values than glutamic acid and are therefore eluted later from such resins than glutamic acid α-Glutamyl-α-amino acids are expected to have the same pK_2-values as glutamic acid and should therefore not be eluted later than glutamic acid It has however recently been demonstrated that PMR-spectroscopy at different pH-values corresponding to different ionization states of di- and tripeptides containing glutamic acid permits unequivocal differentiation between γ-glutamyl peptides on the one hand and α-glutamylpeptides and aminoacylglutamic acids on the other.[23] This method has been used to establish the γ-glutamyl linkage in most of the γ-glutamyl peptides isolated (see Experimental)

The identity of the γ-L-glutamyl-L-phenylalanine was established by comparison with authentic material.[24] The configuration of γ-glutamylwillardiine was established by isolation of hydrolysis products.[11] For the other peptides no determination of configuration was made, but L-configuration for both amino acids are likely in analogy with the configurations reported for most of the γ-glutamyl peptides found in plants. The recent identification of γ-L-glutamyl-D-alanine in pea seedlings[25] indicates however that the L-configuration of both amino acids cannot be taken for granted

[20] LUTZ, O and JIRGENSONS, B (1930) *Ber* **63**, 448
[21] SACHS, H and BRAND, E (1953) *J Am Chem Soc* **75**, 4608
[22] SACHS H and BRAND, E (1954) *J Am Chem Soc* **76**, 3601
[23] KRISTENSEN, I and LARSEN P O (1973) *Acta Chem Scand* **27**, 3123
[24] LARSEN P O and SØRENSEN, H (1968) *Acta Chem Scand* **21**, 2908
[25] FUKUDA, M, OGAWA, T and SASAOKA, K (1973) *Biochim Biophys Acta* **304**, 363

DISCUSSION

The isolation of more than 20 non-protein amino acids and peptides from beechnuts is perhaps not surprising considering the ever-increasing number of amino acids found in higher plants. However, most of these compounds have been found in only a relatively few plant families (e.g. the Leguminosae) and many plants contain only low concentrations of protein amino acids and a few ubiquitous compounds, like for example γ-aminobutyric acid, β-alanine, and ethanolamine. The rich variety of compounds found in beechnuts therefore calls for investigations in other members of the Fagaceae.

The question of toxicity of the beechnuts has not been answered by the present study. If however some of the compounds isolated are partly or wholly responsible for the toxicity the most likely candidates are of course those present in the highest concentrations, i.e. γ-glutamylphenylalanine and willardiine. No toxicity studies for these compounds are available. Since from an ecological point of view toxicity to insects is most likely it should be noted that γ-glutamylphenylalanine is a common constituent in insects [26]

Willardiine has not previously been found outside the family Leguminosae. The biosynthesis of willardiine has never been established, but it occurs in the leguminous plants together with a number of other alanine derivatives. Recent studies indicate a common biosynthetic origin of all of these compounds from *O*-acetylserine via reaction of enzymatically bound dehydroalanine with a nucleophile.[27] The occurrence of willardiine outside Leguminosae may therefore indicate a wider distribution of this biosynthetic pathway.

3-(2-Furoyl)alanine has recently been isolated from buckwheat (*Fagopyrum esculentum* Moench).[18] It is furthermore produced by chemical degradation from ascorbalamic acid, a compound widely distributed in higher plants [19] Ascorbalamic acid is assumed to be biosynthetically derived from ascorbic acid and a suitable alanine derivative. *O*-Acetylserine is a likely candidate for this alanine derivative, and this fits nicely with the co-occurrence of willardiine and furoylalanine provided that the latter is derived from ascorbalamic acid in the beechnuts.

Previously phenylglycine has been reported on paper chromatographic evidence to be present in the bleeding sap of *F. silvatica* L.[7,8] Furoylalanine and phenylglycine both produce a yellow colour with ninhydrin, and they occupy nearly identical positions on paper chromatograms in standard solvents. It therefore seems likely that the compound previously observed in the bleeding sap is furoylalanine.

Free *cis*-4-hydroxy-L-proline has been found in *Santalum* species[28] whereas *trans*-4-hydroxy-L-proline is a protein constituent in animals and has been identified as a cell-wall constituent in plants [29,30] The *cis*-configuration tentatively assigned to the hydroxyproline from beechnuts is in agreement with this pattern of distribution.

N^5-acetylornithine has been identified in numerous plants, most recently in minute amounts in sugar beets,[31] and its occurrence in a new species is therefore not surprising. The presence of ornithine in plants has not been reported very often, but ornithine is assumed to be universally distributed because of its role as an intermediate in the biosynthesis of arginine.

[26] BODNARYK, R P (1972) *Comp Biochem Physiol* **B43,** 587
[27] KJAER, A and LARSEN, P O (1973) *Biosynthesis* **2** (Specialist Reports, The Chemical Society), 71
[28] KUTTAN, R, PATTABHIRAMAN, K S V and RADHAKRISHNAN, A N (1974) *Phytochemistry* **13,** 453
[29] LAMPORT, D T A (1965) *Adv Bot Res* **2,** 151
[30] KUTTAN, R and RADHAKRISHNAN, A N (1970) *Biochem J* **119,** 651
[31] FOWDEN, L (1972) *Phytochemistry* **11,** 2271

Pipecolic acid has been identified in numerous higher plants. In those cases where the configuration has been determined it has invariably been the L-form.[32] The tentative assignment of predominant D-configuration to the isolate from beechnuts is therefore at first sight rather surprising. However, pipecolic acid invariably is formed from D-lysine and not from L-lysine in plants.[32]

γ-Glutamyl peptides and other γ-glutamyl derivatives occur widely in higher plants.[33,34] Most of the protein amino acids, numerous non-protein amino acids and a number of amines have been identified as the second constituent. No specific role has been assigned to the γ-glutamyl peptides, and their biosynthesis has not been clearly elucidated. The γ-glutamyl derivatives occur in different species in highly varying amounts but in reproducible patterns. It is therefore not likely that their occurrence merely reflects an ability to make the γ-glutamyl derivatives of amino acids that occur free.

γ-Glutamylamino acids have been proposed as intermediates in the transport of amino acids through intercellular barriers in animals.[35,36] It may hence be significant that in many cases those γ-glutamyl dipeptides occurring in highest concentrations in plants are those with a hydrophobic side chain in the second amino acid, i.e. γ-glutamylmethionine, γ-glutamylisoleucine, γ-glutamylleucine, γ-glutamylphenylalanine and γ-glutamylvaline.

Except γ-glutamylwillardiine and γ-glutamylphenylalanylwillardiine[11] all the γ-glutamyl peptides found in beechnuts have previously been identified in other plant species. γ-Glutamylmethionine sulfoxide has been isolated from *Phaseolus radiatus*, where it occurs together with γ-glutamylmethionine.[37] γ-Glutamylvaline has been identified in *Allium cepa*[38] and in *Iris* leaves.[39] γ-L-Glutamyl-L-alanine has been identified in *Iris* leaves.[39] As mentioned above γ-L-glutamyl-D-alanine has been isolated from pea seedlings.[25] γ-Glutamylisoleucine and γ-glutamylleucine have been isolated from *Allium cepa*.[38] The leucine derivative has also been found in *Phaseolus vulgaris*.[40] γ-Glutamylglutamic acid has been isolated from *Acacia georginae*.[41] γ-Glutamylphenylalanine and γ-glutamyltyrosine have been found in numerous plants, including *Aubrietia deltoidea*[24] and *Glycine max*.[42] Glutathione is generally assumed to be present in all living cells, but it has seldom been isolated from plant material.[33]

EXPERIMENTAL

General methods and instrumentation. IR spectra were determined in KBr pellets. Optical rotations were determined on a Perkin-Elmer Model 141 photoelectric polarimeter in 1-dm tubes. CD-curves were recorded on a Roussel-Jouan CD 185 Dichrograph using 1- or 2-mm cells and conc of 0.5 to 2 mg/ml. PMR-spectra were determined on a JEOL C-60 HL instrument in D_2O soln. Chemical shifts are given in ppm downfield from sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate. ^{13}C-NMR-spectra were recorded in D_2O on a Bruker WH 90 instrument using the pulse technique with Fourier transformation. Dioxane was used as internal standard and chemical shifts are given in ppm downfield from (TMS) = $\delta_{dioxane}$ + 67.4 ppm. PC was performed in a

[32] LEISTNER, E., GUPTA, R. N. and SPENSER, I. D. (1973) *J. Am. Chem. Soc.* **95**, 4040.
[33] WALEY, S. G. (1966) *Adv. Protein Chem.* **21**, 1.
[34] FOWDEN, L. (1970) in *Progress in Phytochemistry* (L. REINHOLD and Y. LIWSCHITZ, Eds.) Vol. 2, p. 203. Wiley, New York.
[35] ORLOWSKI, M. and MEISTER, A. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **67**, 1248.
[36] BODNARYK, R. P., BRUNET, P. C. J. and KOEPPE, J. K. (1974) *J. Insect Physiol.* **20**, 96[illegible]
[37] KASAI, T., SAKAMURA, S. and SAKAMOTO, R. (1972) *Agr. Biol. Chem.* **36**, 967.
[38] VIRTANEN, A. I. and MATIKKALA, E. J. (1961) *Suomen Kemistilehti* **B34**, 53.
[39] MORRIS, C. J., THOMPSON, J. F. and ASEN, S. (1964) *J. Biol. Chem.* **239**, 1833.
[40] MORRIS, C. J., THOMPSON, J. F. and ZACHARIUS, R. M. (1963) *J. Biol. Chem.* **238**, 650.
[41] ITO, K. and FOWDEN, L. (1972) *Phytochemistry* **11**, 2541.
[42] MORRIS, C. J. and THOMPSON, J. F. (1962) *Biochemistry* **1**, 706.

BuOH–HOAc–H_2O (12 3 5) (solvent 1), PhOH–H_2O–conc NH_3 (120 30 1) (w/v/v) (solvent 2), and PhOH–EtOH–H_2O–conc NH_3 (120 40 40 1) (w/v/v/v) (solvent 3) by the descending technique on Whatman No 1 paper Preparative PC was performed with the same solvents on Whatman No 3 MM paper After elution from preparative paper chromatograms the amino acids were routinely purified by passage through small ion exchange columns (*ca* 0 4 × 3 cm) Neutral amino acids were bound to Dowex 50W × 8, 200–400 mesh resin in the H^+-form and eluted with 0 2 N 3-chloropyridine or 1 N pyridine Acidic amino acids were first purified on this resin and afterwards bound to Dowex 1 × 8, 200–400 mesh resin in the AcO^--form and eluted with 1 N AcOH

Isolations All fractions were evaporated to dryness under reduced pressure, and the weights of the residues are reported according to the fraction numbers The residues were dissolved in water before application to ion exchange resins

Beechnuts (2 3 kg), harvested in Marselisborg Forest, Denmark, in 1969, were disintegrated in CCl_4 (6 l) and extracted three times by refluxing in CCl_4, cooling and filtering The air-dried residue (1 47 kg) was extracted 3× with 70% MeOH (4 l each time) by refluxing for 4 hr, cooling and filtering The combined MeOH–H_2O extracts were concentrated to leave a dark syrup (132 g) The residue was dissolved in H_2O (1 8 l) and centrifuged The supernatant was applied to Amberlite IR 120 resin (20–50 mesh, 5 × 80 cm, H^+) After washing with H_2O elution with 1 N pyridine (6 l) produced the fraction of acid and neutral amino acids (1) (14 3 g) The column was subsequently eluted with 1 N NH_3 (160 ml/hr, 20-ml fractions) to give two fractions of basic amino acids. (2) (160 mg) (fractions 238–247), (ornithine, histidine, lysine), and (3) (300 mg) (remaining ninhydrin-reactive fractions eluted with ammonia) (arginine, ethanolamine) (**1**) was applied to Dowex 1 × 4 resin (20–50 mesh, 2 5 × 80 cm, AcO^-, 160 ml/hr, 20-ml fractions) After washing with water (2 4 l) to give the fraction of neutral amino acids (1 1) (6 g), the column was eluted with 1 N AcOH, producing four fractions of acid amino acids, (1 2) (910 mg) (fractions 146–170) (glutamic acid, aspartic acid) (1 3) (800 mg) (fractions 171–220), (1 4) (4700 mg) (fractions 221–360), and (1 5) (1170 mg) (fractions 301–430) (glutathione, γ-glutamylglutamic acid, γ-glutamyl-tyrosine, γ-glutamylphenylalanylwillardiine) (**1.1**) was applied to Dowex 50W × 8 resin (200–400 mesh, 2 5 × 90 cm, 3-chloropyridinium form, 80 ml/hr, 15-ml fractions, elution with water (1 5 l), 0 1 N 3-chloropyridine (1 5 l), 0 2 N 3-chloropyridine (1 8 l), and 1 N pyridine) to give the following fractions (1 1 1) (800 mg) (fractions 57–100) (4-hydroxyproline, methionine sulfoxide, willardiine) (1 1 2) (1170 mg) (fractions 101–217) (asparagine, proline, serine, threonine, 5-hydroxy-6-methylpipecolic acids) (1 1 3) (760 mg) (fractions 218–230) (N^5-acetylornithine asparagine pipecolic acid, serine, 5-hydroxy-6-pepecolic acids) (1 1 4) (310 mg) (fractions 231–249) (alanine, valine N-(3-amino-3-carboxypropyl)azetidine-2-carboxylic acid) (1 1 5) (360 mg) (fractions 250–270) (alanine, glycine, valine) (1 1 6) (110 mg) (fractions 271–295) (isoleucine, leucine, N-[N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]azetidine-2-carboxylic acid) (1 1 7) (390 mg) (fractions 296–320) (3-(2-furoyl)alanine, isoleucine leucine), and (1 1 8) (700 mg) (β-alanine, γ-aminobutyric acid, phenylalanine tyrosine) Recrystallization from water of (1 1 1) yielded crystalline L-*willardiine* (500 mg), m p decomp from 202° [Lit[15] 204–6 (decomp)], $[\alpha]_D^{26}$ −20 5° (*c* 1 2, 1 N HCl), $\lambda_{max}^{0.2\,N\,HCl}$ 262 nm, ε 9300 (Lit[15] 262 nm, ε 8800) $\lambda_{max}^{0.2\,N\,NaOH}$ 266 nm, ε 7200 (Lit[15] 266 nm, ε 6600), $\lambda_{max}^{H_2O}$ 263 nm, ε 9400, PMR-spectrum in D_2O δ 7 62 (*d*, 1 H), 5 84 (*d*, 1 H, *J* 7 5 Hz), 3 95–4 40 (*m*, 3 H) The δ-values are in good agreement with those found for 1,3-dimethyl-uracil (7 61 and 5 84)[43] and the coupling constant for the vinylic protons is in agreement with the *cis*-arrangement IR identical with that of authentic L-willardiine[15] From the mother liquor a sample of DL-willardiine (20 mg), $[\alpha]_D^{26}$ −1 4° (*c* 0 9, 1 N HCl), was obtained IR identical with that of authentic DL-willardiine[16,17] After isolation of this sample, preparative PC in solvent 3 followed by ion exchange purification gave crystalline evaporation residues of 4-*hydroxyproline* (8 mg) and *methionine sulfoxide* (10 mg) 4-Hydroxyproline, which gave a yellow colour with ninhydrin, was chromatographically nearly homogeneous and identified by cochromatography with a sample of *trans*-4-hydroxy-L-proline The CD-curve exhibited by the sample showed a single broad positive maximum in water at 210 nm (Δε +0 41) The CD-curve was positive until 195 nm For *cis*-4-hydroxy-L-proline a single max at 205 nm (Δε +0 50) is reported, whereas two maxima are reported for *trans*-4-hydroxy-L-proline at 212 nm (Δε +0 28) and 190 nm (Δε −0 74)[44] Methionine sulfoxide was chromatographically nearly homogeneous and identified by cochromatography with a mixture of the two diastereoisomers (which are not separated in the solvents used) The CD-curve exhibited by the sample in water showed a positive maximum at 200 nm (Δε +0 43) After comparison with the CD-curves for the two diastereoisomeric L-methionine sulfoxides[45] it was concluded that the sample was a mixture of both (1 1 3) Was subjected to preparative PC in solvent 1 followed by solvent 3 and ion exchange purification to give N^5-*acetylornithine* (8 mg) Final purification was accomplished by crystallization from ethanol–water to give 2 5 mg of a homogeneous material PMR-spectrum in D_2O δ 2 (s, N-acetyl), 3 75 (*t*, α-proton), 3 20 (*t*, δ-protons), 1 6–2 (*m*, 4 remaining protons) The material was identified by cochromatography with authentic material, hydrolysis producing ornithine, and negative ninhydrin reaction after masking with cupric ions, indicating a free α-amino group and no free δ-amino group[46] *Pipecolic acid* was obtained by the same preparative PC

[43] BHACCA, N S, HOLLIS D P, JOHNSON, L F, PIER, E A and SHOOLERY, J N (1963) *NMR Spectra Catalog*, Varian

[44] FOWDEN, L, SCOPES, P M and THOMAS, R N (1971) *J Chem Soc C* 833

[45] LAUR, P Personal communication

[46] LARSEN P O and KJAER, A (1960) *Biochim Biophys Acta* **38,** 148

as an evaporation residue (3 mg). The chromatographically homogeneous material was identified by its blue ninhydrin colour and by cochromatography with authentic material. The CD-curve in 0·1 N HCl showed a negative max at 208 nm ($\Delta\epsilon$ −0·16) whereas a positive max at 208 nm ($\Delta\epsilon$ +0·76) is reported for L-pipecolic acid in this solvent[44] (1·1·7). Was subjected to preparative PC in solvent 1. Purification by ion exchange gave *3-(2-furoyl)-L-alanine* (40 mg). Final purification was accomplished by crystallization from water (yield 11 mg). M.p. decomp. above 150° (Lit.[18] 148–9°). $\lambda_{max}^{H_2O}$ 227 nm, ϵ 2640, and 278 nm, ϵ 15 400 (Lit.[19] 227 nm, ϵ 3040 and 278 nm, ϵ 15,300); $[\alpha]_D^{23}$ +14·5° (*c* 1·3, H_2O (supersaturated)); $[\alpha]_D^{23}$ +46·5° (*c* 1·1, 2 N HCl). The PMR-spectrum in D_2O showed the aromatic protons in an AMX-system (δ_A 7·88 (the proton at C_5), δ_M 7·53 (the proton at C_4) and δ_X 6·72 (the proton at C_3), J_{AM} 0·5 Hz, J_{AX} 1·8 Hz, J_{MX} 3·8 Hz). The α-proton occurred at δ 4·18 and the β-protons at δ 3·58 (*J* 6 Hz). These values are in good agreement with those reported in the literature.[45,46] The ^{13}C-NMR-spectrum in D_2O showed the presence of 8 carbon atoms with the following chemical shifts: 173·7 (COO⁻), 51·3 (α-C), 38·8 (β-C), 188·0 (C=O), 151·3 (ring C_2), 121·9 (ring C_3), 113·6 (ring C_4), 149·7 (ring C_5). The assignments are made on basis of comparison with literature values for aspartic acid[47] and furfural.[48] The IR-spectrum was identical with that obtained for an authentic sample of 3-(2-furoyl)-D,L-alanine.[20] (1·3) Was applied to a Dowex 1 × 8 resin (200–400 mesh, 2·5 × 90 cm, AcO⁻, 80 ml/hr, 20-ml fractions, elution with 1 N AcOH) to give the following fractions: (1·3·1) (150 mg) (fractions 36–40) (glutamic acid, γ-glutamylalanine, γ-glutamylmethionine sulfoxide, γ-glutamylvaline); (1·3·2) (140 mg) (fractions 41–55) (γ-glutamylisoleucine + γ-glutamylleucine); and (1·3·3) (380 mg) (fractions 56–70) (aspartic acid). (1·3·1) Was subjected to preparative PC in solvent 1. Ion exchange purification gave *γ-glutamylmethionine sulfoxide* as a crystalline evaporation residue. PMR-spectrum in D_2O: δ 2·75 (*s*, MeSO), 1·9–3·2 (*m*, 4 CH_2-groups), 3·89 (*t*, α-CH in glutamic acid), and 4·54 (α-CH in methionine sulfoxide). The δ-values for the α-protons in various ionization states have been published.[23] The same preparative PC followed by ion exchange purification gave *γ-glutamylvaline* as a crystalline evaporation residue. PMR in D_2O: δ 0·97 (*d*, 2 Me groups), 1·9–2·7 (*m*, 2 CH_2-groups in glutamic acid), and 4·24 (*d*, α-CH in valine). PMR in 6% trifluoroacetic acid in D_2O: δ 4·16 (*t*, α-CH in glutamic acid) and 4·28 (*d*, α-CH in valine). The preparative PC finally yielded a fraction containing glutamic acid and *γ-glutamylalanine*. Preparative PC of this fraction in solvent 2 followed by ion exchange purification yielded *γ-glutamylalanine* (0·5 mg) identified by hydrolysis. (1·3·2) Was subjected to preparative PC in solvent 1 followed by ion exchange purification to give an evaporation residue of *γ-glutamylisoleucine + γ-glutamylleucine* (46 mg). PMR in D_2O: δ 3·82 (*t*, α-CH in glutamic acid), 4·27 (*m*, α-CH in isoleucine + leucine). PMR in 6% trifluoroacetic acid in D_2O: δ 4·14 (*t*, α-CH in glutamic acid), 4·34 (*m*, α-CH in isoleucine + leucine). Hydrolysis followed by quantitative amino acid analysis on a Beckman Spinco Model 120 Analyser revealed the presence of glutamic acid, isoleucine, and leucine in the ratio of 3·2·1. (1·4) Was applied to a Dowex 1 × 8 resin (200–400 mesh, 2·5 × 90 cm, AcO⁻, 80 ml/hr, fractions of 20 ml, elution with 1 N AcOH) to give the following fractions: (1·4·1) (130 mg) (fractions 90–105) (γ-glutamylwillardiine); (1·4·2) (3000 mg) (fractions 140–200) (γ-glutamylphenylalanine); and (1·4·3) (70 mg) (fractions 280–320) (γ-glutamylglutamic acid). (1·4·2) By recrystallization from water gave *γ-L-glutamyl-L-phenylalanine* (2 g), $[\alpha]_D^{31}$ +17·7° (*c* 4, H_2O) (Lit.[42] $[\alpha]_D^{31}$ +16·9 (*c* 4 H_2O)). PMR in D_2O: δ 1·8–2·6 (*m*, CH_2-groups in glutamic acid), 2·7–3·5 (*m*, benzylic protons in phenylalanine), 3·70 (*t*, α-CH in glutamic acid), 4·65 (*q*, α-CH in phenylalanine), and 7·40 (broad singlet, aromatic protons). The δ-values for the α-protons in various ionization states have been published.[23] (1·4·3) By ion exchange purification gave *γ-glutamylglutamic acid* as a crystalline evaporation residue (70 mg). PMR in D_2O: δ 1·8–2·8 (*m*, 4 CH_2-groups), 3·92 (*t*, α-CH in γ-glutamyl part), and 4·42 (*t*, α-CH in acylglutamic acid). The δ-values for the α-protons in various ionization states have been published.[23] (1·5) Was subjected to preparative PC in solvent 1 to give *glutathione* (probably as disulfide) as an evaporation residue (5 mg). PMR in D_2O: δ 1·9–2·8 (*m*, two CH_2-groups in glutamic acid), 3·2 (*t*, CH_2 in cysteine), 4·0 (CH_2 in glycine), 3·85 (*t*, α-CH in glutamic acid). The α-proton in the cysteine residue was covered by the DOH-band. PMR in 6% trifluoroacetic acid in D_2O: δ 4·18 (*t*, α-CH in glutamic acid). PMR in 0·4 N NaOD in D_2O: δ 3·28 (*t*, α-CH in glutamic acid). Hydrolysis resulted in the production of glutamic acid, glycine, and a number of unidentified decomposition products of cystine. Hydrolysis after oxidation with performic acid resulted in complete conversion into cysteic acid, glutamic acid, and glycine. The same preparative PC followed by preparative PC with *isopropanol–H_2O–conc. NH_3* (8·1·1) as solvent and ion exchange purification gave *γ-glutamylcitrosine* as an evaporation residue (4 mg), identified by hydrolysis.

Partial isolation of amino acid fractions avoiding the use of heat. Beechnuts (100 g) were disintegrated in a mill and stirred with 70% MeOH at 5° for 48 hr. After filtration a second extraction was performed under the same conditions. The combined MeOH–H_2O extracts were conc. (7 g), dissolved in H_2O, and centrifuged. The supernatant was applied to Amberlite IR 120 resin (1·5 × 35 cm, H⁺). Elution was performed with 1 N pyridine to give a fraction of acid and neutral amino acids (1 g). Paper chromatographic examination of this fraction established the presence of willardiine, the 5-hydroxy-6-methylpipecolic acids, *N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]azetidine-2-carboxylic acid, 3-(2-furoyl)alanine, γ-glutamylglutamic acid, γ-glutamylphenylalanine, and several of the protein amino acids. This fraction was applied to Dowex 1 × 8 (20–50 mesh, 1·5 ×

[47] STOTHERS, J. B. (1972) *Carbon-13 NMR Spectroscopy*. Academic Press, New York.
[48] JOHNSON, L. F. and JANKOWSKI, W. C. (1972) *Carbon-13 NMR Spectra*. Varian.

35 cm, AcO^-) A fraction of neutral amino acids was obtained by washing with H_2O (300 mg) This fraction was applied to Amberlite IR 120 resin (0 8 × 17 cm, 3-chloropyridinium form) Three fractions were obtained by elution with water (200 mg), 0 2 N 3-chloropyridine (110 mg), and 1 N pyridine (30 mg) The different fractions were inspected by two-dimensional PC The presence of *N*-(3-amino-3-carboxypropyl)azetidine-2-carboxylic acid in the 3-chloropyridine eluate was established in this way

Acknowledgements—The authors are indebted to "Hedeselskabets Skovfrøcentral" for the beechnuts, to Professor L Fowden for a sample of N^5-acetylornithine, and to Dr R L M Synge for a sample of 3-(2-furoyl)-DL-alanine The assistance of Dr K Bock in recording the ^{13}C-NMR-spectra and of Dr P Laur in recording the CD-curves is gratefully acknowledged The ^{13}C-NMR-measurements were made at the Institute of Organic Chemistry and the CD-measurements at Chemical Laboratory A, both at the Technical University of Denmark, on instruments made available through grants from the Danish Natural Science Research Council The quantitative amino acid analysis was kindly performed by Mr E Lund, Institute for Protein Chemistry, Copenhagen We are finally indebted to Professor M G Ettlinger for a critical revision of the manuscript