**δ -Hydroxylysine, Canavanine and
Three α -Amino Acid Amides
from Ladino Clover Seed
(*Trifolium repens* L.
var. *giganteum*)[†]**

Takanori KASAI, Katsuhisa FURUKAWA
and Sadao SAKAMURA

Department of Agricultural Chemistry,
Faculty of Agriculture, Hokkaido
University, Sapporo

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The seeds of field crops belonging to *Leguminosae* contain a many kind of nitrogen-containing compounds and these are interesting components in view of food chemistry and chemotaxonomy. We have reported unusual amino acids and small peptides in seeds of field crops belonging to the *Lotoideae*, a subfamily of the *Leguminosae*.^{1,3)} This paper deals with the isolation and identification of five compounds cited in the title from the basic amino acids fraction of the ladino clover seed.

L-Leucine amide·HCl, L-phenylalanine amide·HCl, L-tyrosine amide·HCl, L-leucine-N-carboxyanhydride and L-phenylalanine-N-carboxyanhydride were prepared by the conventional method.^{4,5)} δ -Hydroxylysine (mixture of diastereomers), canavanine and L-tyrosine decarboxylase were purchased from Nakarai Chemical Ltd., Wako Pure Chemical Ltd. and Sigma Chemical Company, respectively. Developing solvent systems. Solvent I; *n*-butanol, acetic acid, water (4:1:2, v/v). Solvent II; isopropanol, acetic acid, water (7:2:1, v/v). Migration distance of each compound in paper chromatography was expressed as ratio to that of leucine (R_{10u}). Hitachi R-22 (90 MHz) for NMR spectra, Hitachi Model 285 for IR spectra, Hitachi RMS-4 for mass spectra and Hitachi KLA-2 for amino acid analysis were used.

Isolation of basic components. Basic amino acids fraction of ladino clover seeds (20 kg) obtained by a routine method using the cation exchange resin^{1,2)} was loaded on an Amberlite IR-120 (NH_4^+) (1 liter) column and eluted with 0.2 N ammonia water, eluates being collected in 15 g fractions. Several ninhydrin positive spots besides usual basic amino acids were detected on the paper chromatogram with solvent I and named as follows; CBU-1 (fractions 44~46, R_{10u} . 1.10), CBU-2

(frs. 47~50, R_{10u} . 0.72), CBU-4 (frs. 91~140, R_{10u} . 0.16) and CBU-5 (frs. 91~140, R_{10u} . 0.30). Each chromatographically pure component was obtained by preparative paper chromatography.

Identification of L-leucine amide (CBU-1a) and L-phenylalanine amide (CBU-1b). It was proved from some experiments such as hydrolysis, reaction with 2,4-dinitrofluorobenzene and measurements of IR and NMR spectra that CBU-1 was still mixture. CBU-1 was further separated into two components with the use of an active carbon column. Non-absorbed fraction and eluate from the column with solvent II were pure in each case and named CBU-1a and CBU-1b, respectively. MS of CBU-1a *m/e*: 131 ($M^+ + 1$, 0.8%), 86 (100%), 74 (3%), 73 (9%), 44 (71%). CBU-1a was easily hydrolyzed by a rather mild conditions (1 N HCl, 100°C, 3 hr) and gave one spot of leucine on a paper chromatogram. Configuration of leucine obtained after hydrolysis was determined to be L-form by the method of Manning and Moore.⁶⁾ CBU-1a had the same *Rf* value with authentic L-leucine amide in all solvent systems used so far. From these data, CBU-1a was identified as L-leucine amide. MS of CBU-1b *m/e*: 165 ($M^+ + 1$, 0.7%), 120 (100%), 103 (33%), 91 (42%), 77 (26%), 74 (5%), 73 (39%). CBU-1b gave one spot of phenylalanine on a paper chromatogram after hydrolysis (1 N HCl, 100°C, 3 hr) and the configuration of phenylalanine thus obtained was determined to be L-form by the method of Manning and Moore.⁶⁾ *Rf* value of CBU-1b was the same as that of authentic phenylalanine amide in all solvent systems experimented so far. CBU-1b was identified as L-phenylalanine amide from these data.

Identification of L-tyrosine amide (CBU-2). CBU-2 was crystallized as hydrochloride from water (83 mg). *Anal.* Found for CBU-2·HCl: C, 49.94; H, 6.12; N, 12.86. Calcd. for $\text{C}_9\text{H}_{12}\text{N}_2\text{O}_2\cdot\text{HCl}$: C, 49.88; H, 6.00; N, 12.93. MS *m/e*: 181 ($M^+ + 1$, 3%), 136 (70%), 107 (100%), 91 (83%), 77 (45%), 74 (48%), 73 (45%). NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 3.16 (2H, d, $J=7$ Hz), 4.24 (1H, t, $J=7$ Hz), 6.91 and 7.22 (both 2H, d, $J=8$ Hz). Hydrolyzate of CBU-2 (1 N HCl, 100°C, 3 hr) gave one spot of tyrosine on paper chromatogram. Tyrosine thus obtained yielded tyramine after incubation with L-tyrosine decarboxylase. CBU-2·HCl had the same IR spectrum and chromatographic behavior as those of authentic L-tyrosine amide hydrochloride. From these data, CBU-2 was identified as L-tyrosine amide.

Identification of L- δ -hydroxylysine (CBU-4). CBU-4 was crystallized as hydrochloride from water/ethanol (18.8 mg). *Anal.* Found for CBU-4·HCl: C, 36.47; H, 7.64; N, 14.35. Calcd. for $\text{C}_6\text{H}_{14}\text{N}_2\text{O}_8\cdot\text{HCl}$: C, 36.27; H, 7.56; N, 14.11. MS *m/e*: 145 ($M^+ - 17$, 3%), 126 (17%), 114 (7%), 109 (33%), 88 (16%),

[†] Amino Acid Composition of Ladino Clover. Part II. See reference 2) for Part I.

69 (28%), 56 (79%), 43 (100%). NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$ 1.67 (2H, m), 1.97 (2H, m), 2.92 (1H, dd, $J=9$ and 13 Hz), 3.13 (1H, dd, $J=3.5$ and 13 Hz), 3.78 (1H, t, $J=6$ Hz), 3.94 (1H, m). NMR spectrum and paper chromatographic behavior of CBU-4 were indistinguishable from those of authentic δ -hydroxylysine. But CBU-4 appeared as two peaks on an amino acid analyzer (conditions; Amberlite CG-120, 400 mesh, 0.9×50 cm. 0.38 N Na-citrate buffer, pH 4.26, 30 ml/hr 50°C). The authentic δ -hydroxylysine which contained about equal amount of normal and allo form gave also two peaks having the same elution times as those of two peaks of CBU-4. From these data, CBU-4 was identified as δ -hydroxylysine which contained both of normal and allo form. The ratio of normal to allo form was about one third on the basis of the peak areas on an amino acid analyzer chart. It was determined by measurement of optical rotation that both of normal and allo δ -hydroxylysine constituting CBU-4 with about one to three ratio were L-form. $[\text{M}]_{\text{D}}^{18} + 42.1$ ($c=0.5$ in 5 N HCl). (Lit.⁶⁾ $[\text{M}]_{\text{D}} + 50.9$, $c=2$ in 5 N HCl for allo form. $[\text{M}]_{\text{D}} + 28.9$, $c=2$ in 5 N HCl for normal form) Calcd. for the mixture of L-normal and L-allo form with one to three ratio; $(50.9 \times 3 + 28.9)/4 = 45.4$.

Identification of canavanine (CBU-5). CBU-5 gave positive reaction for pentacyanoammonioferrate reagent.⁷⁾ NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$ (pD<1): 2.37 (2H, q, $J=6$ Hz), 4.17 (2H, t, $J=6$ Hz), 4.26 (1H, t, $J=6$ Hz). Chromatographic behavior of CBU-5 was consistent with that of canavanine. The amount of isolated canavanine was about 16 mg on the basis of peak area of its purified fraction on an amino acid analyzer chart.

Although the amount of δ -hydroxylysine and canavanine actually isolated from the ladino clover seed is a rather small quantity, when based on their peak areas in the crude extract on an amino acid analyzer chart, the content of these two amino acids in the seeds seems to be much more. δ -Hydroxylysine, which was first found in gelatine hydrolyzate,⁶⁾ has been isolated in free state only from pods of unripe *Vicia faba*.¹⁰⁾ This is the second report on isolation of the compound from the plant material. Canavanine distribution had been restricted to the members of *Lotoideae*, a subfamily of *Leguminosae*,¹¹⁾ but it was identified recently in *Allium cepa*.¹²⁾ The argument that canavanine is a natural constituent of the edible fruiting body of mushroom,¹³⁾ however, has been denied.¹³⁾ As for three α -amino acid amides described in this paper, the possibility that these amides are artifacts produced during isolation procedures can not be excluded, it has been reported that lysine amide and ornithine amide are formed slowly when lysine and ornithine are allowed to stand in ammoniacal solution.¹⁴⁾ But the preparation procedure of the amino acids fraction employed at this work is

one of the conventional methods, and α -amino acid amides have never been encountered in the basic amino acids fractions of several plant materials examined by this method in our laboratory. Therefore, it is still obscure at this stage whether those α -amino acid amides are constituents of the seeds or artifacts produced during isolation procedures.

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REFERENCES

- 1) T. Kasai, S. Sakamura and R. Sakamoto, *Agr. Biol. Chem.*, **35**, 1603, (1971); *idem, ibid.*, **35**, 1607 (1971); *idem, ibid.*, **36**, 967 (1972); T. Kasai, S. Sakamura, S. Inagaki and R. Sakamoto, *ibid.*, **36**, 2621 (1972); T. Kasai, Y. Kishi, M. Sano and S. Sakamura, *ibid.*, **37**, 2923 (1973); T. Kasai and S. Sakamura, *J. Fac. Agr. Hokkaido Univ.*, **57**, 153 (1973).
- 2) T. Kasai, M. Ueda, S. Sakamura and K. Sakata, *Nippon Nôgeikagaku Kaishi*, **47**, 583 (1973).
- 3) T. Kasai, M. Sano and S. Sakamura, *ibid.*, **49**, 313 (1975).
- 4) "Chemistry of the Amino Acids," J. P. Greenstein and M. Winitz, John Wiley and Sons, Inc., 1961, p. 1187.
- 5) "Chemistry of the Amino Acids," J. P. Greenstein and M. Winitz, John Wiley and Sons, Inc., 1961, p. 860.
- 6) "Chemistry of the Amino Acids," J. P. Greenstein and M. Winitz, John Wiley and Sons, Inc., 1961, p. 1996.
- 7) W. R. Fearon and E. A. Bell, *Biochem. J.*, **59**, 221 (1955).
- 8) J. M. Manning and S. Moore, *J. Biol. Chem.*, **243**, 5591 (1968).
- 9) D. D. VanSlyke, A. Hillon, R. T. Dillon and D. A. Macfadyen, *Pecc. Soc. Exp. Biol. and Med.*, **38**, 548 (1938).
- 10) H. Inatomi, Y. Morita, M. Ozawa, Y. Suyama and F. Inukai, *Meiji Daigaku Nogakubu Kenkyu Hokoku*, No. **22**, 1 (1967).
- 11) B. L. Turner and J. B. Harborne, *Phytochem.*, **6**, 863 (1967).
- 12) M. R. Altamura, F. M. Robbins, R. E. Andreotti, L. Long and T. Hasselstron, *J. Agr. Food Chem.*, **15**, 1040 (1967).
- 13) G. A. Rosenthal and D. L. Davis, *Phytochem.*, **14**, 1117 (1975).
- 14) Y. Kakimoto and S. Akazawa, *J. Biol. Chem.*, **245**, 5751 (1970).
- 15) J. Mannhalter and H. Michl, *Monatsh.*, **105**, 889 (1974).