4-Hydroxypipecolic Acid and Pipecolic Acid in *Acacia* Species: Their Determination by High-Performance Liquid Chromatography, Its Application to Leguminous Plants, and Configuration of 4-Hydroxypipecolic Acid

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A new method for the determination of naturally occurring imino acids in plants was devised. This method comprises the formation of dinitrophenyl derivatives and their measurement by HPLC. The determination limits were 100 pmol for 4-hydroxypipecolic acid (HPA) and proline (Pro) and 10 pmol for pipecolic acid (Pip). The recoveries of HPA, Pro, and Pip from leaf homogenate of an *Acacia* species were 95.9 ± 2.8, 100.5 ± 1.7, and 101.1 ± 2.3%, respectively. HPA was found in leaves, seeds, and roots of five kinds of *Acacia* but not in leaves of other members of the legume family. The HPA content of *Acacia dealbata link* leaves was much higher in winter than in summer. From X-ray analysis, HPA was found to be hydrated by two water molecules that are situated in a vacant channel formed by the molecular arrangement of HPA. HPA had no remarkable physiological or pharmaceutical activities.

Keywords:　*4-Hydroxypipecolic acid; pipecolic acid; Acacia species; leguminous plants; determination*

INTRODUCTION

Proline, 3-hydroxyproline, 4-hydroxyproline, pipecolic acid (Pip), 4-hydroxypipecolic acid (HPA), 5-hydroxypipecolic acid, 4-aminopipecolic acid, and 4,5-dihydroxypipecolic acid are naturally occurring cyclic imino acids.

Collagen contains 4-hydroxyproline, and some types of collagen contain small amounts of the 3-OH form.

Pip occurs in legumes (Grobbelaar and Steward, 1953; Fowden, 1960; Evans *et al.*, 1977), rat urine (Rothstein and Miller, 1954), rat brain (Okano *et al.*, 1981; Nishio and Segawa, 1983), human blood (Gatfield *et al.*, 1968; Burton *et al.*, 1981; Hutzler and Dancis, 1983; Nakajima *et al.* 1987), human urine (Danks *et al.*, 1975; Lam *et al.*, 1984), cerebrospinal fluid (Zee *et al.*, 1992), and *Neurospora* species (Schweet *et al.*, 1954).

HPA has been found in *Acacia* species (Virtanen and Kari, 1955; Fowden, 1960; Evans *et al.*, 1977), *Calliandra calliandra* (Romeo *et al.*, 1983), and *Apocynaceae strophanthus scandens* (Schenk and Schütte, 1963).

5-Hydroxypipecolic acid occurs in legumes (Virtanen and Kari, 1954; Evans *et al.*, 1977; Despontin *et al.*, 1977; Hatanaka and Koneko, 1977; Bleecker and Romeo, 1982) and the flowers of *Trachycarpus forturei* (Murakoshi *et al.*, 1984).

4,5-Dihydroxypipecolic acid has been isolated from *Calliandra* species (Marlier *et al.*, 1972; Bleecker and Romeo, 1981, 1983; Romeo *et al.*, 1983) and other legumes (Shewry and Fowden, 1976; Marlier *et al.*, 1976; Evans *et al.*, 1977).

4-Aminopipecolic acid has been isolated from leaves of an oleander (Schenk and Schütte, 1963).

As can be seen from above, Pip and its analogues are widely present in leguminous plants. In this paper we describe a simple method for identifying and quantifying imino acids in those plants. We found that HPA occurred in relatively high concentrations in *Acacia* species. Six *Acacia* species are grown on an Okayama University farm. They are trees and grow quickly even if the soil is not fertile. There has been some interest in utilizing products from this plant as insecticides and fungicides. Because of this interest and because a sizeable amount of HPA could be easily isolated from *Acacia* species, we tested HPA for biological and pharmaceutical activities. Since the absolute configuration of HPA has not been confirmed, we have done so. Our results are reported here.

MATERIALS AND METHODS

Reagents. 2,4-Dinitrofluorobenzene (DNFB) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Pro was obtained from Wako Pure Chemical Industries (Osaka, Japan). Pip was from Nakarai Tesque Inc. (Kyoto, Japan). The strongly acidic cation exchanger Diaion SK-1B was purchased from Mitsubishi Chemical Industry Ltd. (Tokyo, Japan). Amberlite IRC-50 and IRA 93 were procured from Organo Japan Co. (Tokyo, Japan). Hyflo super-cel from Katayama Chemical Industry was used.

Isolation of HPA. HPA was prepared from leaves of *Acacia dealbata link*. Fresh leaves (150 g) were homogenized in 300 mL of H_2O with a blender. The homogenate was heated at 100 °C for 15 min. After cooling, the heated homogenate was filtered through Hyflo super-cel. The filtrate was applied onto a Diaion SK column (4.5 × 17 cm, H^+-type), which was then washed with 1 L of H_2O and eluted with 1 L of 2 N ammonia.

The eluant was concentrated by evaporation at 40 °C to a small volume and applied onto an Amberlite IRA 93 (weakly basic, free type) column (3.3 × 19 cm). The nonabsorbed fraction and the eluant (after the column was washed with 300 mL of H_2O) were combined and evaporated to dryness. The white crystals present in the residue were dissolved in

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minimum amounts of hot water, and alcohol was added to the solution until crystals began to form. The recrystallization from water and alcohol was repeated 5 times. The crystals were colorless, clear, and rod-shaped. If allowed to stand at room temperature, the crystals slowly turned to white powder. Samples dried over P_2O_5 and NaOH were used for elemental analysis and mass spectrometry. The calculated values for a substance having the chemical formula $C_6H_{11}NO_3$ were C, 49.64; H, 7.64; N, 9.65. The measured values were C, 49.51; H, 7.62; N, 9.63. $[\alpha]^{25}_D = -13.0$ ($c = 1.66\%$ in H_2O); MS m/z 146 (M$^+$ + 1), 128 (M$^+$ − OH), 100 (M$^+$ − COOH).

X-ray Structure Determination of HPA Dihydrate. The crystal used had dimensions of $0.25 \times 0.15 \times 0.50$ mm and was sealed in a capillary. The reflection data were measured by using Mo Kα radiation ($\lambda = 0.7107$ Å) at 50 kV and 200 mA (rotating anode). Lattice parameters were determined with 22 reflections in the range $18 < 2\theta < 20°$. Crystal data: $C_6H_{11}NO_3 \cdot 2H_2O$, $M_r = 181.19$; orthorhombic, space group $P2_12_12_1$; $a = 9.045(9)$, $b = 11.484(4)$, $c = 8.276(3)$ Å, $V = 860(1)$ Å^3; $Z = 4$, $D_x = 1.400$ g cm^{-3}; μ(Mo Kα) = 0.11 mm^{-1}. Intensities were measured up to sin θ/λ 0.65 Å^{-1} by using $\omega - 2\theta$ scan technique; the scan speed was 6° min^{-1} in ω and the scan range $1.10° \pm 0.30°$ tan θ in ω. Background was measured for 4 s on either side of the peak. Three standard reflections were monitored during the data collection for every 97 reflections with a fluctuation within 1.2% in F_o. To reduce the data, Lorentz and polarization corrections were applied, but no absorption correction was used. In total, 1319 reflections were measured over the ranges $h = 0–11$, $k = 0–14$, and $l = −1$ to 10, and 1171 were unique ($R_{int} = 0.015$). For refinement 886 reflections with I_o larger than 3σ (I_o) were used.

The structure was solved by the direct method MITHRIL (Gilmore, 1984) and refined (anisotropically for non-H atoms) by full-matrix least-squares: the quantity minimized was $\sum w(|F_o| - |F_c|)^2$, where w refers to weights, $\sigma^{-2}(F_o)$. H-atom positions were determined from difference Fourier mapping and refined isotropically by the least-squares method. Extinction correction was performed according to $I_{corr} = I_0[1 + (5.24 \times 10^{-6})I_c]$. The final values of R and R_w were 0.035 and 0.026, respectively ($S = 1.42$). In the last cycle of least-squares refinement $(\Delta/\sigma)_{max}$ was 0.04. In a final difference Fourier map the maximum and minimum $\Delta\rho$ values were 0.16 and −0.17 e Å^{-3}, respectively. The atomic scattering factors were taken from *The International Tables for X-Ray Crystallography* (Cromer and Weber, 1974). Computations were carried out at the X-Ray Laboratory of Okayama University by using the TEXRAY Structure Analysis Package from the Molecular Structure Corp. (The Woodlands, TX).

Leguminous Plants. The leaves of *Acacia cuitriformis A. Cunn., Acacia neriifolia A. Cunn., A. floribunda Willd, Acacia baileyana F. Muell.,* and *Acacia longifolia Willd*, which were planted on the Honjima farm of Okayama University, were kindly given to us. Leaves and other parts of *A. dealbata link* were also collected from trees growing naturally in Sanyo-Cho, Okayama. Other legume plants listed in Table 4 were collected in the medical herb garden of Okayama University.

HPLC. A Shimadzu liquid chromatograph, Model 3A (Kyoto, Japan), equipped with a Shimadzu variable-wavelength detector and a Shimadzu SGR-1A step gradient apparatus, was used for the assay of DNP cyclic imino acids. The detector was set at 380 nm.

A 150 × 4.6 mm (i.d.) Cosmosil 5C18-AR column was used with stepwise elution using a mixture of 10 mM potassium phosphate (pH 4.0) and acetonitrile (6% for 30 min, 9% for 15 min, and 40% for 15 min). The flow rate was 1.0 mL/min.

Determination of Pro, Pip, and HPA in Plants. The minced tissue of leaves, seeds, petioles, or roots was boiled for 5 min with 10 volumes of water based upon the tissue weight, followed by centrifugation at 1700*g* for 15 min. The extract (25 μL) was mixed with 10 μL of 10% Na_2CO_3, 10 μL of DNFB (liquid), and water to yield a final volume of 1 mL. The mixture was shaken at room temperature for 1 h. The mixture was then extracted twice with 1 mL of *n*-hexane. After 40 μL of 6 N HCl was added to the aqueous phase, the aqueous phase was extracted with 2 mL of ethyl acetate. The ethyl acetate
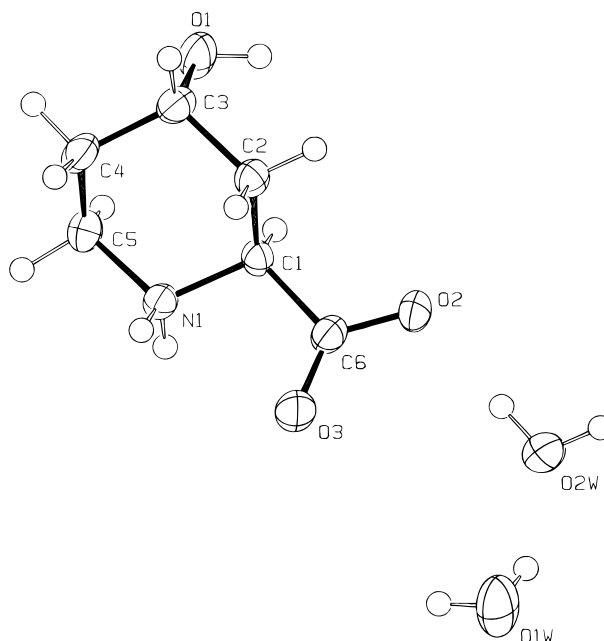

Figure 1. Configuration of 4-hydroxypipecolic acid dihydrate determined by X-ray analysis. The thermal ellipsoids are drawn at 50% probability for the non-H atoms; the H atoms are represented as spheres equivalent to $B = 1.0$ Å^2.

layer (1 mL) was evaporated using a Savant Speedvac concentrator (Model SUC-100H, New York, NY) at room temperature. The residue was dissolved in 1 mL of the mobile phase, of which 10 μL was injected into the chromatograph.

Preparation of DNP Derivatives of Pip and HPA. HPA (30 mg) was dissolved in 4 mL of 30% methanol (methanol: H_2O = 3:7 v/v) in a 5 mL vial. Na_2CO_3 (50 mg) and DNFB (60 μL) were added to the vial, which was mechanically shaken at room temperature for 1 h. After the reaction, the mixture was extracted with 5 mL of *n*-hexane and, subsequently, with 5 mL of benzene to remove DNFB. The water layer was acidified with 6 N HCl and extracted with 7 mL of ethyl acetate three times. The ethyl acetate layer was dried with Na_2SO_4, filtered, and evaporated to dryness. The residue was recrystallized from a mixture of methanol and water. The needle-shaped crystals had a mp between 185 and 189 °C. The R_f value was 0.22 on silica gel in 1:9 (v/v) acetic acid/benzene. Pip was derivatized in a manner similar to that above. However, dinitrophenyl (DNP)-Pip was not crystallized. The R_f value was 0.57 on silica gel TLC [1:9 (v/v) acetic acid/benzene]. The dried DNP-Pip was used as an analytical standard.

RESULTS

Configuration of HPA. The configuration of HPA as determined by X-ray analysis is depicted in Figure 1. It shows a zwitterion of *trans*-4-hydroxypipecolic acid with two water molecules.

The six-membered ring of the HPA ion has a chair conformation. The torsion angles characterizing the molecular configuration are as follows: C(1)−C(2)−C(3)−O(1), −65.1(3)°; C(1)−C(2)−C(3)−C(4), 54.5(4)°; N(1)−C(1)−C(2)−C(3), −55.1(3)°; and C(6)−C(1)−C(2)−C(3), −176.0(2)°.

As seen in Figure 1, the O(2) accepts a hydrogen bond from O(2W) [O···O 2.824(3) Å], and O(2W) accepts a hydrogen bond from O(1W) [O···O 2.938(4) Å]. In addition, O(2W) donates a hydrogen bond to O(1W) related by a 2_1 along the a axis [O···O 2.773(4) Å]. Thus, a hydrogen-bonded chain of the water molecules is formed to fill a vacant channel around this 2_1 axis. The N(1) donates a hydrogen bond to the carboxylate O(2)

related by a 2_1 along the *a* axis [N⋯O 2.808(4) Å] and the other hydrogen bond to O(2W) related by a 2_1 along the *b* axis [N⋯O 2.826(3) Å]. The hydroxyl O(1) donates a hydrogen bond to O(3) related by a 2_1 along the *a* axis [O⋯O 2.630(3) Å] and accepts a hydrogen bond from O(1W) related by the 2_1 [O⋯O 2.706(3) Å]. All functional groups capable of forming hydrogen bonds do participate in hydrogen bonds, and the O(2) and O(2W) accept two hydrogen bonds.

Reaction Conditions of Cyclic Imino Acids with DNFB. Two hundred nanomoles of Pro, Pip, and HPA was reacted with 20 μL of DNFB in a total volume of 1 mL for 1 h at room temperature at pH 8, 9, 10, or 11. The yields increased with pH and reached 101, 99.4, and 100% at pH 11, respectively. The extraction yields of DNP-Pro, DNP-Pip, and DNP-HPA with 2 mL of ethyl acetate from 1 mL of acidic aqueous solution were 102.3, 103.6, and 93.9%, respectively.

Calibration Curve. Various amounts of Pro, Pip, and HPA were derivatized with DNFB. The DNP-imino acids were washed with *n*-hexane, extracted with ethyl acetate, evaporated to dryness, and analyzed by HPLC (Figure 2). The calibration curves were linear from 100 pmol up to at least 25 nmol for Pro and HPA and from 10 pmol to 10 nmol for Pip. The relationships between the peak area (y, cm^2) and the amount of Pro or HPA are as follows: $y = 0.434x - 0.68$ ($r = 0.999$) for Pro and $y = 0.323x - 0.662$ ($r = 0.993$) for HPA. The peak height for Pip, y(cm) $= 0.925x + 0.06$ ($r = 0.999$) is expressed in nanomoles.

Recovery Test. Various amounts (0, 0.5, 1.0, 1.5, and 2.0 nmol) of Pro, Pip, and HPA were added to 25 μL leaf extract from *A. dealbata link*. The final volume was made up to 1 mL by adding water. The mixture was derivatized and extracted as usual. The results of recovery were 100.5 ± 1.7, 101.1 ± 2.3, and 95.9 ± 2.8% for Pro, Pip, and HPA, respectively ($n = 3$).

Distribution of Cyclic Imino Acids in Acacia and Leguminous Plants. As an example, a chromatogram of cyclic imino acids from a leaf extract of *A. dealbata link* is shown in Figure 2b. The imino acid contents in some tissues of *A. dealbata link* are summarized in Table 1. The HPA content of each tissue analyzed was higher than those of Pro and Pip and was especially higher in seed, leaf, and root. As shown in Table 2, HPA, Pro, and Pip contents in leaves of *A. dealbata link* were much higher in winter than in summer. The contents of cyclic imino acids in leaves of six *Acacia* species are shown in Table 3. HPA was present at about 1 order of magnitude higher concentration than Pro and Pip in leaves of *Acacia* species except *A. longifolia Willd*. We analyzed cyclic imino acids in leaves of some species of leguminous plants (Table 4). Pip was found in all leguminous plants tested, while HPA appears to be only present in *Acacia* and *Calliandra* species (Romeo *et al.*, 1984).

Pharmaceutical Potential of HPA. HPA was tested for antibacterial activity against seven strains of Gram-negative and six strains of Gram-positive bacteria, for antifungal activity against six strains of fungi, for antiviral activity against HSV-1, and for inhibition of IL-2 and HIV protease. The results of these tests were all negative. Electrophysiological activities were also tested in the presence of HPA. It has no effect on the electrical activity (Ca^{2+} current) of a spontaneously firing giant neuron and the periodically oscillating neuron identified in the subesophageal glia
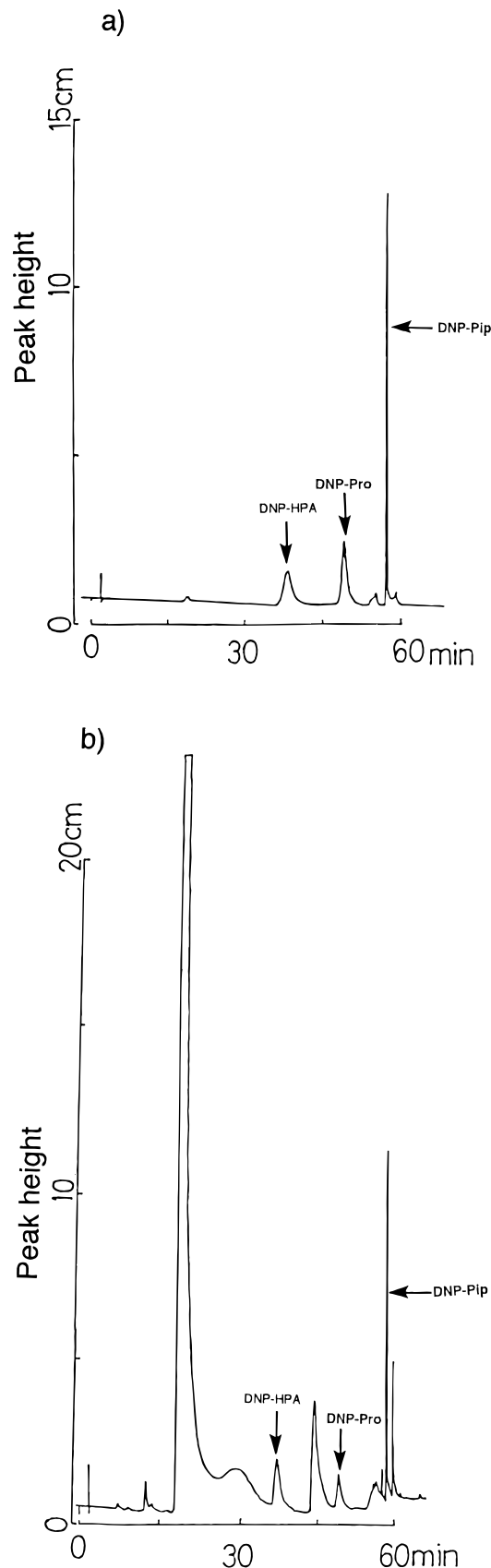

Figure 2. High-performance liquid chromatograms of DNP derivatives of HPA, Pro, and Pip. Imino acids were dissolved in 30% methanol (methanol:H$_2$O = 3:7 v/v) and derivatized with DNFB in alkaline conditions. The derivatives were extracted with ethyl acetate and then concentrated by evaporation. The residue was analyzed by HPLC (a). Fresh leaves of *A. dealbata link* were extracted in boiling water. The extract was reacted with DNFB, followed by evaporation and analysis (b).

Table 1. Distribution[a] of Cyclic Imino Acids in Different Tissues of *A. dealbata link*

	HPA	Pro	Pip
seed (*n* = 3)	8.95 ± 0.13	2.90 ± 0.49	0.40 ± 0.05
root (*n* = 3)	6.50 ± 0.30	0.99 ± 0.02	1.80 ± 0.10
stalk (*n* = 2)	1.20	0.14	0.40
leaf (*n* = 3)	6.86 ± 0.07	ND[b]	1.15 ± 0.01

[a] Micromoles per gram of wet weight. [b] Not detectable.

Table 2. Contents[a] of Cyclic Imino Acids in Leaves of *A. dealbata link* in Summer and Winter

	July	January
HPA	6.86 ± 0.07	11.60 ± 0.60
Pro	ND[b]	5.14 ± 0.67
Pip	1.15 ± 0.01	9.01 ± 0.40

[a] Micromoles per gram of wet weight. Means ± SD (*n* = 3). [b] Not detectable.

Table 3. Contents[a] of Cyclic Imino Acids in the Leaves of Various *Acacia* Species

	HPA	Pro	Pip
A. cuitriformis A. Cunn.	2.19 ± 0.25	0.26 ± 0.10	0.23 ± 0.01
A. nerifolia A. Cunn.	4.82 ± 0.55	0.55 ± 0.12	0.49 ± 0.03
A. floribunda Willd	3.23 ± 0.16	0.57 ± 0.03	0.53 ± 0.01
A. baileyana F. Muell.	6.26 ± 0.40	0.33 ± 0.02	0.39 ± 0.04
A. longifolia Willd	5.23 ± 0.34	2.17 ± 0.29	5.23 ± 0.99

[a] Micromoles per gram of wet weight. Means ± SD (*n* = 3).

Table 4. Contents[a] of Cyclic Imino Acids in the Leaves of Some Species of Leguminous Plants

	HPA	Pro	Pip
Canavalia gladiata (Jacga) DC.	ND[b]	0.35 ± 0.07	0.15 ± 0.01
Sophora flavescens Ait.	ND	0.83 ± 0.21	1.50 ± 0.01
Albizza julibrissin Durazz.	ND	0.96 ± 0.14	0.32 ± 0.02
Sophora japonica L.	ND	ND	0.11 ± 0.03
Cassia obtusifolia L.	0.09 ± 0.03	0.25 ± 0.01	2.51 ± 0.66
Cassia minosoides L. subsp. *nomame* (sieb) Ohashi	ND	2.22 ± 0.62	0.39 ± 0.05

[a] Micromoles per gram of wet weight. Means ± SD (*n* = 3). [b] Not detectable.

of an African giant snail. HPA also showed no effect on the cell membrane potential of other neurons of the snail.

DISCUSSION

Cyclic imino acids had been analyzed by paper chromatography until the end of the 1970's (Grobbelaar and Steward, 1953; Virtanen and Kari, 1955; Thompson and Morris, 1959; Schenk and Schütte, 1963; Shewry and Fowden, 1976). From the beginning of the 1960's, automatic amino acid analyzers have been commercially available. Imino acids were not detected at 570 nm, but rather at 440 nm, after ninhydrin reaction. More recently, to increase the detection sensitivity, *o*-phthalaldehyde or fluorescamine have been used as the labeling reagent in automatic amino acid analyzers. However, imino acids could not be determined by such labeling methods. If imino acids are oxidized with chloramine-T prior to the introduction of *o*-phthalaldehyde in the analyzer, they can be analyzed at the nanomole level (Bleecker and Romeo, 1982). When Pip is reacted with an acidic ninhydrin reagent and the developed color is detected at 570 nm using HPLC, it can be determined at least at the nanomole level. This method was applied to plasma in patients with liver disease (Hutzler and Dancis, 1983). Urine samples of patients with hyperpipecolatenemia were analyzed at 234 nm on a reversed-

phase HPLC column with a mobile phase containing L-aspartame and copper sulfate, resulting in the successful analysis of both D and L isomers (Lam *et al.*, 1984). This method appears to be more qualitative than quantitative. Even if Pip could be identified, the determination limit seems to be at the micromole level from estimations based upon published data.

Cyclic imino acids can be also determined by gas chromatography. Okano *et al.* used a gas chromatography−mass spectrometry method by which 20 pmol of Pip and Pro could be identified (Okano *et al.*, 1981). Pip in biological fluids was derivatized with methyl chloroformate and pentafluorobenzyl bromide, and the Pip derivative was determined by capillary gas chromatography with electron capture detection (Kok *et al.*, 1987; Zee *et al.*, 1992). The calibration curve was linear between 0.05 and 0.5 nmol of Pip. Urinary Pro and hydroxyproline were determined by a gas chromatograph equipped with a flame photometric detection system. The determination limits were 0.1 and 0.2 pmol for Pro and hydroxyproline, respectively (Kataoka *et al.*, 1993).

The currently available methods for assaying cyclic imino acids, which were summarized above, are insensitive, time-consuming, or expensive. These methods could not be used routinely and widely for each imino acid. We tried to establish a sensitive and simple determination method for cyclic imino acids in plant samples by taking advantage of their chemical properties. Cyclic imino acids react quantitatively with DNFB. DNP-imino acids are more extractable by organic solvent than are commonly occurring amino acids and have a relatively high molar absorbance. By the method presented here, 100 pmol of Pro and HPA and 10 pmol of Pip in legumes could be simply determined in the same HPLC run. We think that this method should be applicable to plasma or urine sample of patients with hyperpipecolatememia.

Since other imino acids such as 5-hydroxypipecolic acid and 4,5-dihydroxypipecolic acid were not available, we could not test them with our procedure.

As can be seen in Table 2, 1 g of fresh leaves of *A. dealbata link* collected in the winter contained 1.7 mg of HPA. The concentration is so high that a large quantity of the optically pure imino acid can be easily obtained from *Acacia* leaves. It was thought that these plants produce and accumulate HPA or Pip for some purpose, for example, in self-defense against fungi, bacteria, and virus.

Evans and Bell (1979) investigated the effect of the non-protein amino acids in the leaves of *Acacia* species on the feeding of tree locust nymphs. Romeo (1984) tested imino acids in leaves of *Calliandra* for insecticidal activity against a polyphagous. Brenner and Romeo (1986) reported that *Aspergillus* sp. was inhibited by Pip and 5-hydroxypipecolic acid and *Curvularia* sp. growth was stimulated by Pip. We also tested HPA for its pharmaceutical potential; however, as described under Results, HPA had no remarkable physiological or pharmaceutical activities.

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