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ACIDIC N^{α}-ACYLARGININE DERIVATIVES IN ARGININE-ACCUMULATING PLANT TISSUES

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Key Word Index—*Lilium maximowiczii*, *Smilax china*, *Vicia faba*, *Rumex obtusifolius*, Liliaceae, Leguminosae, Polygonaceae, acylarginine derivatives, N^{α}-(2-hydroxy-2-carboxymethylsuccinyl)-L-arginine, N^{α}-(2-hydroxysuccinyl)arginine

Abstract—Two new acidic N^{α}-acylarginine derivatives were isolated from arginine-accumulating plant tissues The first, N^{α}-(2-hydroxy-2-carboxymethylsuccinyl)-L-arginine, was isolated from bulbs of *Lilium maximowiczii*, whilst the second, N^{α}-(2-hydroxysuccinyl)arginine, was obtained from tubers of *Smilax china* and seeds of *Vicia faba* No acidic N^{α}-acylarginine derivative was detected, however, in roots of *Rumex obtusifolius* which contained fairly large amounts of arginine and malonic acid

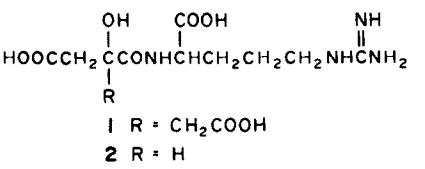
INTRODUCTION

Many plants utilize arginine as a main storage form of nitrogen [1] It is considered that plant tissues accumulating a large amount of arginine overcome the high p*I* value of this amino acid and maintain the pH of the physiological fluid around neutral by forming an ionic and/or organic bond between the amino acid and acidic compounds So far, however, only a few neutral or acidic derivatives of arginine such as γ-glutamylarginine [2] and arginosuccinic acid have been found in plant materials This paper reports the isolation of two new acidic derivatives from arginine-accumulating plant tissues

RESULTS AND DISCUSSION

Arginine was the most abundant amino acid in all four plant tissues studied (Table 1) The acidic amino acid fraction of three of them, bulbs of *L maximowiczii*, tubers of *S china* and seeds of *V faba*, contained a compound which gave a positive reaction with Sakaguchi reagent, but did not react with ninhydrin Each component was isolated using ion exchange chromatography and prep PC

The compound (**1**) from bulbs of *L maximowiczii* was hydrolysed completely to L-arginine and citric acid (6 M HCl, 120°, 3 hr) The L-arginine and citric acid were separated from each other by treatment with a Dowex 50(H^+) column, and the L-arginine identified by PC, HVE, FDMS, amino acid analysis and optical rotation FDMS(*m/z*) 175$[M+1]^+$ (100%), $[\alpha]_D^{20}$ + 12 1° (H_2O, *c* 0 7) Lit + 12 5° (H_2O, *c* 3 5) [3] Citric acid was identified by PC, HVE and FDMS FDMS (*m/z*) 193 $[M+1]^+$ (100%), 147 $[M-COOH]^+$ (22) The ^{1}H NMR spectrum of **1** contained signals corresponding to arginyl and citryl residues ^{1}H NMR (90 MHz in D_2O) δ1 38–2 07 (4H, *m*, arginyl β- and γ-CH_2), 2 69 and 2 89 (1H each, both *d*, *J* = 15 Hz, citryl one CH_2), 2 75 and 2 93 (1H each, both *d*, *J* = 15 Hz, citryl another CH_2), 3 17 (2H, *t*, *J* = 7 Hz, arginyl δ-CH_2) and 4 34 (partially overlap with DOH, arginyl α-CH) FDMS of **1** gave a parent ion corresponding to arginine conjugated with citric acid, i e *m/z* 349 $[M+1]^+$ (100%) Since **1** gave a positive reaction with Sakaguchi reagent and did not react with



1 R = CH_2COOH
2 R = H

Table 1 The arginine content and the most abundant organic acids in some arginine-accumulating plant tissues

	Bulbs of *L maximowiczii* (mg/100 g fr wt)	Tubers of *S china* (mg/100 g fr wt)	Seeds of *V faba* (mg/100 g dry wt)	Roots of *R obtusifolius* (mg/100 g fr wt)
Arginine	333	63	192	46
Citric acid	645	n d*	19	+
Malic acid	n d*	258	41	+
Malonic acid	n a†	n a†	n a†	100

*Not detected
†Not analysed

Table 2 Titration shift of the proton signals of the two methylene groups of the citryl residue in N^{α}-(2-hydroxy-2-carboxymethylsuccinyl)arginine (1) and of the methylene and methyne groups of the malyl residue in N^{α}-(2-hydroxysuccinyl)arginine (2)

	δ_{DSS}		
	pD < 1*	pD > 13*	Titration shift
Centre of one $C\underline{H}_2$ of the citryl residue in 1	2 95	2 63	0 32
Centre of the second $C\underline{H}_2$ of the citryl residue in 1	2 90	2 59	0 31
Centre of the $C\underline{H}_2$ of the malyl residue in 2	2 86	2 54	0 32
$C\underline{H}$ of the malyl residue in 2	4 56	4 48	0 08

*pD was adjusted by adding *ca* 37% DCl soln in D_2O or *ca* 40% NaOD soln in D_2O

ninhydrin, the α-amino group of arginine was acylated Measurement of the ^1H NMR spectra of 1 in acidic and basic medium showed that the β-carboxyl group of citric acid formed an amide bond with the α-amino group of arginine Both of the CH_2 groups of the citryl residue revealed a big titration shift when the acidic medium was rendered basic by addition of 40% NaOD in D_2O (Table 2) The structure of 1 was thus N^{α}-(2-hydroxy-2-carboxymethylsuccinyl)-L-arginine

The compound (2) from tubers of *S china* was hydrolysed to arginine and malic acid (3 M HCl, 110°, 3 hr) The arginine and malic acid were separated from each other by treatment with a Dowex 50 (H^+) column, and the arginine identified by PC, HVE and amino acid analysis Malic acid was identified by HVE and GC/MS after esterification with diethyl ether–diazomethane MS (*m/z*) 163 $[M+1]^+$ (1%), 103 $[M-COOMe]^+$ (100) The ^1H NMR spectrum of 2 contained signals corresponding to arginyl and malyl residues ^1H NMR (100 MHz in D_2O) δ1 40–2 00 (4H, *m*, arginyl β- and γ-CH_2), 2 63 and 2 77 (1H, *dd*, *J* = 7, 16 Hz and 1H, *dd*, *J* = 4, 16 Hz, respectively, malyl CH_2), 3 19 (2H, *t*, *J* = 6 Hz, arginyl δ-CH_2), 4 25 (1H, *dd*, *J* = 5, 7 Hz, arginyl α-CH) and 4 46 (1H, *dd*, *J* = 4, 7 Hz, malyl CH) FDMS of 2 gave a parent ion corresponding to arginine conjugated with malic acid i e *m/z* 581 $[2M+1]^+$ (3%), 291 $[M+1]^+$ (100), 273 $[291-H_2O]^+$ (72) Since 2 showed a positive reaction with Sakaguchi reagent and did not react with ninhydrin, the α-amino group of arginine was acylated as in compound 1 It was shown by measuring the titration shift in the ^1H NMR spectrum that the α-carboxyl group of malic acid formed an amide bond with the α-amino group of arginine Malyl CH_2 protons showed a big upfield shift when an acidic medium was turned basic by addition of 40% NaOD in D_2O, while the upfield shift of a malyl CH proton was slight (Table 2) The structure of 2 was established as N^{α}-(2-hydroxysuccinyl)arginine The configurations of the two chiral centres of 2 were not determined

A compound isolated from the acidic amino acid fraction of mature seeds of *V faba* which gave a positive reaction with Sakaguchi reagent and did not react with ninhydrin showed the same behaviour on PC and HVE as 2 ^1H NMR and FDMS spectra of the substance isolated from *V faba* seeds were also consistent with those of 2 FDMS (*m/z*) 291 $[M+1]^+$ (100%) The compound gave arginine and malic acid by hydrolysis (3 M HCl, 130°, 3 hr) which were identified by amino acid analysis and GC after diethyl ether–diazomethane treatment, respectively The compound isolated from *V faba* seeds was thus identified as 2

In bulbs of *L maximowiczii* and tubers of *S china*, the content of organic acid is fairly high and the most abundant organic acids are citric acid and malic acid, respectively (Table 1) Therefore, 1 and 2 are the amides which are formed between the most abundant amino acid and organic acid in bulbs of *L maximowiczii* and tubers of *S china*, respectively The content of organic acids is relatively low in seeds of *V faba* The difference in amount of malic acid and citric acid is not big (Table 2), but 1 could not be detected in seeds of *V faba* There was no spot which gave a positive reaction with Sakaguchi reagent and did not react with ninhydrin on PC and HVE of the acidic amino acid fraction from roots of *R obtusifolius*, although the tissue contained plenty of arginine and organic acids, especially malonic acid (Table 1) Amino acids and peptides in seeds of *V faba* [4, 5] and organic acids and peptides in *R obtusifolius* have been reported [6, 7] A survey of ninhydrin positive substances in the acidic amino acid fraction of *L maximowiczii* and *S china* is now in progress

EXPERIMENTAL

General methods PC *n*-BuOH–HOAc–H_2O (4 1 2) (solvent 1), PhOH–H_2O–conc NH_4OH (120 30 1, w/v/v) (solvent 2), HVE pH 6 5 (pyridine–HOAc–H_2O, 25 1 500, 100 V/cm)

Plant materials Bulbs of *L maximowiczii* and mature seeds of *V faba* were purchased at a local market Tubers of *S china* were collected in natural habitats on Shikoku Island, Japan, by Dr Tahara, Hokkaido University Roots of *R obtusifolius* were from the experimental farm of Hokkaido University

Quantitative analysis of organic acids and amino acids An aliquot of the effluent from a column of Amberlite IR-120 (H^+) loaded with the plant extract was concd, dissolved in MeOH, esterified with excess Et_2O–CH_2N_2 and analysed by GC using tridecane as int reference according to the method of ref [8] with slight modification GC conditions FID, 5% Reoplex 400 (2 m × 3 mm, glass column), N_2 60 ml/min, temp programme 80–210° at 5°/min, injector temp 240°, detector temp 255° Amino acids were analysed with an amino acid analyser

Isolation of 1 *from bulbs of* L maximowiczii *and hydrolysis* Bulbs of *L maximowiczii* (7 0 kg) were homogenized with 80% MeOH (20 l) The residue was placed in a column and extracted continuously with 70% MeOH (32 l) The extracts were combined, concd and dissolved in H_2O After centrifugation, the supernatant was applied to a column of Amberlite IR-120

(H^+, 2 l), which was thoroughly washed with H_2O The amino acid fraction was eluted with 2 M NH_4OH Fractions of 1 l each were collected Fractions 3–5 were concd and applied to a column of Dowex 1 × 4 (AcO^-, 500 ml) After the neutral and basic fraction was washed out with H_2O, acidic amino acids were eluted with 2 M HOAc Fractions of 500 ml each were collected Fractions 4 and 5 were concd, applied to a column of Sephadex G-10 (450 ml) and developed with H_2O Fractions of 15 ml each were collected Compound **1** (85 mg) was isolated as a colourless evaporation residue from fractions 11–20 by prep PC (solvent 1) followed by Dowex 50 (H^+) treatment Nearly the same amount of **1** was also contained in fraction 3 from Dowex 1 × 4 (AcO^-) as in fractions 4 and 5 judging from the intensity of the spot of **1** on PC, but fraction 3 was lost by accident The amount of **1**, therefore, should be *ca* 150 mg/7 kg fr wt See Results and Discussion for ^1H NMR and FDMS spectra of **1** Hydrolysate of **1** (6 M HCl, 120°, 3 hr) was concd and treated with a column of Dowex 50 (H^+) Citric acid was identified in the effluent and H_2O wash from the column by PC, HVE and FDMS See Results and Discussion for FDMS spectrum L-Arginine was identified in the 2 M NH_4OH eluate from the column by PC, HVE and amino acid analysis, FDMS and optical rotation See Results and Discussion for FDMS spectrum and $[\alpha]_D$ value

Isolation of **2** *from tubers of* S china *and hydrolysis* Tubers of *S china* (570 g) were sliced and extracted with 70% EtOH (9 l) The residue was extracted (twice) with 70% EtOH (5 l each) The extracts were combined, concd and dissolved in H_2O After filtration, the filtrate was applied to a column of Amberlite IR-120 (H^+, 250 ml), which was thoroughly washed with H_2O The amino acid fraction was eluted with 2 M NH_4OH and fractions of 500 ml each were collected, except for fraction 1 which was 200 ml Fractions 2–6 were concd and applied to a column of Dowex 1 × 4 (AcO^-, 100 ml) After the neutral and basic fractions had been washed out with H_2O, acidic amino acids were eluted with 2 M HOAc Fractions of 200 ml each were collected Fraction 1 was concd and applied to a column of Dowex 1 × 8 (AcO^-, 5 ml) and eluted with 0 2 M HOAc Fractions of 5 ml each were collected Compound **2** (4 9 mg) was obtained as a colourless evaporation residue from fractions 3–6 by prep PC (solvent 2) followed with Dowex 50 (H^+) treatment See Results and Discussion for ^1H NMR and FDMS spectra of **2** The hydrolysate of **2** (3 M HCl, 110°, 3 hr) was concd and treated with a Dowex 50 (H^+) column Effluent and H_2O wash from the column was concd, esterified with excess Et_2O–CH_2N_2 and analysed for organic acid by GC/MS, 5% Peg-20 M (1 m × 2 mm) He 30 ml/min, temp programme 110–150° at 8°/min, injector and jet separator at 230° and 260°, respectively, MS 24 eV Dimethyl malate was eluted at 2 4 min See Results and Discussion for MS Arginine was identified in 2 M NH_4OH eluate from the column of Dowex 50 (H^+) loaded with hydrolysate of **2** by PC, HVE and amino acid analysis

Isolation of **2** *from seeds of* V faba *and hydrolysis* Meal of *V faba* seeds (1 3 kg) was extracted with 70% EtOH (10 l) The residue was extracted (twice) with 70% EtOH (9 and 5 l) The extracts were combined, concd and dissolved in H_2O After centrifugation, the supernatant was applied to a column of Amberlite IR-120 (H^+, 1 l), which was thoroughly washed with H_2O The amino acid fraction was eluted with 2 M NH_4OH Fractions of 1 l each were collected Fractions 2–4 were concd and applied to a column of Dowex 1 × 4 (AcO^-, 50 ml) After neutral and basic fractions were washed out with H_2O, acidic amino acids were eluted with 2 M HOAc Fractions of 50 ml each were collected Fractions 2–7 were combined, put on a column of Sephadex G-10 (450 ml) and developed with H_2O Fractions of 10 ml each were collected Fractions 18–23 were concd, applied again to a column of Dowex 1 × 4 (AcO^-, 10 ml) and eluted with 0 2 M HOAc Fractions of 5 ml each were collected Fractions 8–13 were concd, put on a column of cellulose powder (600 ml) and developed with solvent 1 Fractions of 15 ml each were collected Compound **2** (1 9 mg) isolated as colourless evaporation residue from fractions 36–41 by prep PC (solvent 1) followed by Dowex 50 (H^+) treatment had the same behavior on PC and HVE as those of **2** Its ^1H NMR and FDMS spectra were also consistent with those of **2** See Results and Discussion for FDMS spectrum The hydrolysate of the isolated material (3 M HCl, 130°, 3 hr) was concd and treated with a Dowex 50 (H^+) column Effluent from the column was concd, esterified with excess Et_2O–CH_2N_2 and analysed for organic acid by GC (by the method described above) A peak corresponding to dimethyl malate was detected (16 min) The presence of malic acid in the effluent from the Dowex 50 (H^+) column was confirmed also by PC and HVE Arginine was identified in the 2 M NH_4OH eluate from the Dowex 50 (H^+) column by an amino acid analysis

Survey of acidic arginine derivative in roots of R obtusifolius Roots (1 9 kg) of *R obtusifolius* were sliced and extracted with 70% EtOH (19 l) The residue was homogenized with 70% EtOH (19 l) The combined extracts were concd and centrifuged The supernatant was applied to a column of Amberlite IR-120 (H^+, 500 ml), which was thoroughly washed with H_2O The amino acid fraction was eluted with 2 M NH_4OH and fractions of 1 l were collected Fractions 1–7 were concd and applied to a column of Amberlite IR-120 (NH_4^+, 500 ml), which was washed with H_2O until the wash gave a negative ninhydrin reaction The effluent and H_2O wash was concd and applied to a column of Dowex 1 × 8 (AcO^-, 50 ml) After neutral amino acids were washed out with H_2O, the acidic amino acid fraction was eluted with 2 M HOAc (1 l) and 8 M HOAc (200 ml) No spot giving a positive reaction to Sakaguchi reagent and a negative reaction to ninhydrin was detected on PC and HVE of the acidic amino acid fraction

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