Phytochemistry, Vol 23, No 1, pp 19–22, 1984
Printed in Great Britain

0031–9422/84 $3 00+0 00

ACIDIC N^{α}-ACYLARGININE DERIVATIVES IN APPLE AND PEAR TREES

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(*Revised received* 22 *April* 1983)

Key Word Index—*Malus pumila*, *Pyrus communis*, Rosaceae, acylarginine derivatives, N^{α}-(2-hydroxysuccinyl)arginine, N^{α}-(3-hydroxysuccinyl)arginine, N^{α}-oxalylarginine, N^{α}-succinylarginine, N^{α}-(2-carboxymethyl-2-hydroxysuccinyl)arginine

Abstract—The annual shoots of apple and pear trees which accumulated a high concentration of arginine during the dormant stage also contained N^{α}-acylarginine derivatives N^{α}-(2-Hydroxysuccinyl)arginine, N^{α}-(3-hydroxysuccinyl)arginine and N^{α}-oxalylarginine were found in apple trees, and N^{α}-succinylarginine and N^{α}-(2-carboxymethyl-2-hydroxysuccinyl)arginine, besides the former three, were found in pear trees N^{α}-(3-Hydroxysuccinyl)arginine, N^{α}-oxalylarginine and N^{α}-succinylarginine are new arginine derivatives

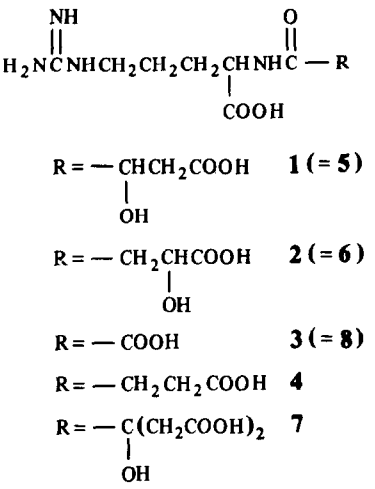
INTRODUCTION

Many plants utilize arginine as a storage form of nitrogen [1] It is considered that plant tissues accumulating a large amount of arginine overcome the high pI value of this amino acid to maintain the pH of the physiological fluid around neutral by forming an ionic and/or covalent bond between this amino acid and the acidic compound During a survey of the acidic arginine derivatives in the arginine-accumulating plant tissues, two new acidic arginine derivatives were isolated N^{α}-(2-hydroxysuccinyl)arginine from tubers of *Smilax china* and seeds of *Vicia faba* and N^{α}-(2-carboxymethyl-2-hydroxysuccinyl)arginine from bulbs of *Lilium maximowiczii* [2]

Perennial plants are classified into three groups according to the free amino acid pattern in twigs at the stage of wintering, group 1 accumulating arginine at the stage of wintering (namely, by cold stress), group 2 accumulating proline, and group 3 accumulating arginine and proline Apple (*Malus pumila*) and pear (*Pyrus communis*) trees belong to group 1 and accumulate a large amount of arginine in twigs every winter, namely by cold stress [3] The annual shoots (tips of branches grown for 1 year) of apple and pear trees during the stage of over-wintering were surveyed for acidic arginine derivatives in this study This paper reports the isolation of several acidic arginine derivatives, including three new compounds from the annual shoots of apple and pear trees

RESULTS AND DISCUSSION

The acidic amino acid fraction of the annual shoots of apple and pear trees at the stage of over-wintering contained three and five compounds, respectively, which gave a positive reaction with the Sakaguchi reagent, but which did not react with ninhydrin Those in apple branches were designated compounds **1**, **2** and **3** in order of elution from the Dowex 1 × 4 (AcO^-) column (**1** being the weakest acid), and those in pear branches were designated compounds **4**, **5**, **6**, **7** and **8** Each component was isolated, using ion exchange chromatography (IEC) and preparative PC, as a syrupy evaporation residue



Compound **1** showed the same behaviour on IEC, PC and HVE as that of N^{α}-(2-hydroxysuccinyl)arginine isolated from tubers of *S china* [2], and was hydrolysed to arginine and malic acid (3 M HCl, 110–120°, 3 hr) The ^{1}H NMR and FDMS spectra of **1** were also consistent with those of N^{α}-(2-hydroxysuccinyl)arginine Compound **1** was thus identified as N^{α}-(2-hydroxysuccinyl)arginine (see Experimental for ^{1}H NMR and FDMS spectra of **1**) Compound **2** had the same MW as **1** and also gave arginine and malic acid after hydrolysis (3 M HCl, 110–120°, 3 hr) FDMS of **2** *m/z* (rel int) 291 $[M+1]^+$ (100) The ^{1}H NMR spectrum of **2** was very similar to that of **1** ^{1}H NMR of **2** (100 MHz in D_2O) δ 1 40–2 00 (4H, *m*, arginyl β- and γ-CH_2), 2 56 and 2 74 (1H, *dd*, J = 9, 15 Hz and 1H, *dd*, J = 4, 15 Hz, respectively, malyl CH_2), 3 20 (2H, *t*, J = 7 Hz, arginyl δ-CH_2), 4 23 (1H, *dd*, J = 4, 7 Hz, arginyl α-CH) and 4 34 (1H, *dd*, J = 4, 9 Hz, malyl CH) Measurement of the ^{1}H NMR spectra (100 MHz) of **2** in acidic and basic media showed that the β-carboxyl group of malic acid formed an amide bond with the α-amino group of arginine The methine

protons of the malyl residue revealed a large titration shift of 0 30 ppm (from δ 4 62 to 4 32) when the acidic medium (pD < 1) was rendered basic (pD > 13) by the addition of 40% NaOD in D_2O, while the titration shift of the centre of the malyl CH_2 protons was 0 17 ppm (from δ 2 82 to 2 65) The structure of **2** was thus N^{α}-(3-hydroxysuccinyl)arginine Compound **3**, which was the most acidic arginine derivative in apple branches, showed no other signals than those of the arginyl residue in its ^{1}H NMR spectrum ^{1}H NMR of **3** (100 MHz in D_2O) δ 1 44–2 04 (4H, *m*, arginyl β- and γ-CH_2), 3 21 (2H, *t*, J = 6 Hz, arginyl δ-CH_2) and 4 26 (1H, *dd*, J = 5, 7 Hz, arginyl α-CH) Compound **3** was hydrolysed to arginine and oxalic acid (3 M HCl, 110–120°, 3 hr) Oxalic acid was separated from arginine by Dowex 50 (H^+) treatment and identified by its FDMS spectrum FDMS of acid component of **3** *m/z* (rel int) 91 $[M+1]^+$ (100) Compound **3** was thus N^{α}-oxalylarginine The FDMS spectrum of **3** supported this structure FDMS of **3** *m/z* (rel int) 247 $[M+1]^+$ (100%)

The acidity of **4** was weaker than that of **1** and eluted before glutamic acid from the Dowex 1 × 4 (AcO^-) column by 0 2 M HOAc (Table 1) The ^{1}H NMR spectrum of **4** revealed a broad singlet of succinyl CH_2 at δ 2 51 besides the signals of the arginyl residue ^{1}H NMR of **4** (100 MHz in D_2O) δ 1 44–1 96 (4H, *m*, arginyl β- and γ-CH_2), 2 51 (4H, *s*, succinyl 2 × CH_2), 3 19 (2H, *t*, J = 7 Hz, arginyl δ-CH_2) and 4 20 (1H, *dd*, J = 5, 7 Hz, arginyl α-CH) The hydrolysate of **4** (3 M HCl, 110–120°, 3 hr) was treated with a column of Dowex 1 × 4 (AcO^-) The eluate from the column with 2 M HOAc was applied onto a column of Dowex 50 (H^+) and washed with water Succinic acid was identified in the effluent from the column by PC and the FDMS spectrum FDMS of acid component of **4** *m/z* (rel int) 119 $[M+1]^+$ (81), 74 [119 $-COOH]^+$ (100) Compound **4** was thus N^{α}-succinylarginine The FDMS spectrum of **4** supported this structure FDMS of **4** *m/z* (rel int) 275 $[M+1]^+$ (100)

Compounds **5** and **6** showed the same behaviour on IEC, PC and HVE as that of **1** and **2**, respectively It was also proved by ^{1}H NMR and FDMS that **5** and **6** were the same compounds as **1** and **2**, respectively Compound **5** was thus identified as N^{α}-(2-hydroxysuccinyl)arginine and **6** as N^{α}-(3-hydroxysuccinyl) arginine (see Experimental for ^{1}H NMR and FDMS spectra of **5** and **6**) Compound **7** had the same behaviour on IEC, PC and HVE as that of N^{α}-(2-carboxymethyl-2-hydroxysuccinyl)arginine isolated from bulbs of *L maximowiczii* [2] The ^{1}H NMR and FDMS spectra of **7** also agreed with those of the citrylarginine It was confirmed by the measurement of titration shift of the ^{1}H NMR spectrum (90 MHz) that the β-carboxyl group of citric acid formed an amide bond with the α-amino group of arginine Both of the methylene protons of the citryl residue revealed a large titration shift of 0 34 ppm when the acidic medium (pD < 1) was rendered basic (pD > 13) by the addition of 40% NaOD in D_2O (from δ 2 90 to 2 56 for the centre of one CH_2 and from δ 2 95 to 2 61 for the centre of the other CH_2) Compound **7** was thus identified as N^{α}-(2-carboxymethyl-2-hydroxysuccinyl)arginine (see Experimental for ^{1}H NMR and FDMS spectra of **7**) Compound **8** was identified as N^{α}-oxalylarginine, because the chromatographic behaviour and spectral data of **8** were consistent with those of **3** (see Experimental for ^{1}H NMR and FDMS spectra of **8**) Pear trees contained one more acidic component which gave a positive reaction with the Sakaguchi reagent, but which did not react with ninhydrin Isolation and identification of this compound was, however, unsuccessful, mainly because of its very small concentration (see Experimental for further information on this compound)

The concentration of organic acids in the annual shoots of both apple and pear trees at the stage of wintering is shown in Table 2 Although citric acid was the second major organic acid in the annual shoots of apple trees, N^{α}-citrylarginine was not detected in that tissue The most abundant organic acid in the annual shoots of pear trees was citric acid, but the concentration of N^{α}-citrylarginine was much lower than that of N^{α}-malylarginines (sum of 2-hydroxysuccinyl- and 3-hydroxysuccinylarginines) Seeds of *V faba* contained both malic and citric acids, but no other acidic N^{α}-acylarginine derivatives other than N^{α}-malylarginine were detected in the seeds [2] It seems to be a general tendency that arginine is acylated more easily with malic acid than with other organic acids Although the content of succinic acid in the annual shoots of apple trees was slightly greater than in those of pear trees, N^{α}-succinylarginine was not detected in the acidic amino acid fraction of apple trees Compounds **2**(= **6**), **3**(= **8**) and **4** are new arginine derivatives The behaviour of these N^{α}-argininine derivatives on IEC, PC and HVE is sum-

Table 1 Behaviour of acidic N^{α}-acylarginine derivatives isolated from annual shoots of pear trees at the stage of wintering on ion exchange chromatography, PC and HVE

	Fractions eluted from Dowex 1 × 4 (AcO^-)*	PC	HVE
		R_{Glu} in solvent 1†	Relative mobility to Glu at pH 6 5†
N^{α}-Succinylarginine (**4**)	3–4	1 88	0 63
N^{α}-(2-Hydroxysuccinyl)arginine (**5** = **1**)	6–9	1 48	0 67
N^{α}-(3-Hydroxysuccinyl)arginine (**6** = **2**)	14–18	1 12	0 63
N^{α}-(2-Carboxymethyl-2-hydroxysuccinyl)arginine (**7**)	22	1 40	1 06
N^{α}-Oxalylarginine (**8** = **3**)	23–24	0 66	0 65

*Dowex 1 × 4 (AcO^-, 100 ml) Eluant 0 2 M HOAc (fractions 1–21, 50 ml each) followed by 2 M HOAc (fractions 22–24, 300 ml each) Glutamic acid and aspartic acid were eluted in fractions 8–12 and 18–22, respectively

†See Experimental for composition of solvent 1 and pH 6 5 buffer

Table 2 The content of amino acids and organic acids in the annual shoots of apple and pear trees at the stage of wintering

	Annual shoots of	
	Apple trees	Pear trees
	(mg/100 g fr wt)	
Aspartic acid	1 0	2 7
Threonine	1 6	1 6
Serine	1 7	2 2
Asparagine	12 7	21 5
Glutamic acid	4 8	6 0
Glutamine	0 8	1 2
Proline	2 6	3 1
Glycine	0 3	0 4
Alanine	5 5	7 3
Valine	0 8	2 0
Isoleucine	0 7	1 9
Leucine	0 7	1 9
Tyrosine	0 8	0 6
Phenylalanine	1 3	1 8
β-Alanine	0 3	1 1
γ-Aminobutyric acid	5 9	10 4
Ethanolamine	1 2	2 0
Tryptophan	0 4	2 3
Lysine	0 3	0 6
Histidine	0 1	1 3
Arginine	23 9	60 8
Lactic acid	3 4	3 2
Oxalic acid	3 7	5 1
Succinic acid	3 6	2 8
Malic acid	8 2	11 0
Citric acid	6 9	14 1

marized in Table 1 Arginine was the most abundant free amino acid in the annual shoots of both apple and pear trees, in accord with the results obtained by Sagisaka and Araki [3] (Table 2) Saccharopine was isolated from the acidic amino acid fraction of apple trees A spot corresponding to saccharopine found on PC on the acidic amino acid fraction of pear tree shoots was far weaker than that found in apple tree shoots (see Experimental for spectral data of saccharopine)

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), N^{α}-Acylarginine derivatives were visualized with Sakaguchi reagent (Soln a 0 1% 8-hydroxyquinoline in Me_2CO Soln b 0 3 ml liquid Br_2 in 100 ml 0 5 M NaOH Chromatogram was dipped through soln a and Me_2CO was allowed to evaporate, then it was sprayed with soln b) Amino acids were detected with 0 2% ninhydrin in Me_2CO and organic acids with the aniline–xylose reagent (1 g xylose and 1 ml aniline in 3 ml H_2O, diluted to 100 ml with MeOH)

Plant materials Annual shoots (grown during the preceding year, 1980) of healthy apple and pear trees were collected from 30 March to 3 April 1981, *ca* 40 days before budding at the experimental farm of Hokkaido University The length of the annual shoots of both apple and pear trees was *ca* 1 m

Isolation and identification of **1**, **2**, **3** *and saccharopine from annual shoots of apple trees* Annual shoots of apple trees at the stage of wintering (4 kg) were cut into small pieces and extracted with 70% EtOH (8 l) The extract was concd and dissolved in H_2O After filtration, the filtrate was applied to a column of Amberlite IR-120(H^+, 1 l) which was thoroughly washed with H_2O (10 l) 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^-, 100 ml) After the basic and neutral fractions were washed out with H_2O (1 l), acidic amino acids were eluted with 2 M HOAc Fractions of 100 ml each were collected Compounds **1** and **2** and saccharopine were present in fraction 2 Fractions 7 and 8 contained **3** Fraction 2 was concd and applied again to a column of Dowex 1 × 4 (AcO^-, 100 ml) The column was eluted with 0 2 M HOAc and fractions of 50 ml each were collected Fractions 6–8 contained α-aminoadipic acid and saccharopine Fractions 7 and 8 contained **1** besides these two non-protein amino acids Compound **1** (6 mg) was isolated by prep PC (solvent 1) followed by Dowex 50 (H^+) treatment Compound **1** (*ca* 1 mg) was hydrolysed (0 1 ml 3 M HCl, 110–120°, 3 hr) Arginine and malic acid were identified in the hydrolysate by PC and HVE ^1H NMR of **1** (100 MHz in D_2O) δ 1 43–2 15 (4H, *m*, arginyl β- and γ-CH_2), 2 62 and 2 76 (1H, *dd*, J = 7, 17 Hz and 1H, *dd*, J = 5, 17 Hz, respectively, malyl CH_2), 3 20 (2H, *t*, J = 6 Hz, arginyl δ-CH_2), 4 26 (1H, *dd*, J = 5, 7 Hz, arginyl α-CH) and 4 47 (1H, *dd*, J = 5, 7 Hz, malyl CH) FDMS of **1** *m/z* (rel int) 291 $[M+1]^+$ (100) Saccharopine was isolated as a crystalline evapn residue (*ca* 0 3 mg) by prep PC (solvent 1) followed by Dowex 50(H^+) treatment ^1H NMR of saccharopine (100 MHz in D_2O) δ 1 20–2 28 (8H, *m*), 2 44 (2H, *t*, J = 7 Hz), 3 07 (2H, *t*, J = 7 Hz), 3 61 (1H, *t*, J = 6 Hz) and 3 75 (1H, *t*, J = 6 Hz) FDMS of saccharopine *m/z* (rel int) 259 $[M+1-H_2O]^+$ (100), 214 $[259-COOH]^+$ (30) Conversion of saccharopine to pyrosaccharopine by heating at 110–120° for 20 hr in a sealed tube was confirmed by PC and amino acid analyser [4] Compound **2** (5 mg) eluted in fractions 16–18 was isolated by prep PC (solvent 1) followed by Dowex 50 (H^+) treatment *Ca* 1 mg **2** was hydrolysed (0 1 ml 3 M HCl, 110–120°, 3 hr) Arginine and malic acid were identified in the hydrolysate by PC and HVE (see Results and Discussion for ^1H NMR and FDMS spectra of **2**) Compound **3** (3 mg) was isolated from the fractions containing **3** by prep PC (solvent 1, then 2) followed by Dowex 50(H^+) treatment *Ca* 1 mg **3** was hydrolysed (0 1 ml 3 M HCl, 110–120°, 3 hr) The hydrolysate was treated with a column of Dowex 50(H^+) Oxalic acid was identified in the effluent from the column by the FDMS spectrum Arginine was identified in the 2 M NH_4OH eluate from the column by PC (see Results and Discussion for ^1H NMR and FDMS spectra of **3** and FDMS spectrum of acid component of **3**)

Isolation and identification of **4**, **5**, **6**, **7** *and* **8** *from annual shoots of pear trees* Annual shoots of pear trees at the stage of wintering (7 6 kg) were cut into small pieces and extracted with 70% EtOH (15 2 l) The extract was concd and dissolved in H_2O After filtration, the filtrate was applied to a column of Amberlite IR-120 (H^+, 1 l) which was thoroughly washed with H_2O (10 l) The amino acid fraction was eluted with 2 M NH_4OH and fractions of 1 l each were collected Fractions 1–7 were concd and applied to a column of Dowex 1 × 4 (AcO^-, 100 ml) After the basic and neutral fractions were washed out with H_2O (1 l), acidic amino acids were eluted with 2 M HOAc and fractions of 100 ml each were collected Fractions 1–8 were concd and applied again to a column of Dowex 1 × 4 (AcO^-, 100 ml) The column was eluted with 0 2 M HOAc (fractions 1–21, 50 ml each) followed by 2 M HOAc (fractions 22–24, 300 ml each) Fractions 3–4, 6–9, 14–18, 22 and 23–24 contained compounds **4**, **5**, **6**, **7** and

8, respectively Compound **4** (7 mg) was isolated from fractions 3 and 4 by prep PC (solvent 1) followed by Dowex 50(H^+) treatment *Ca* 2 5 mg **4** was hydrolysed (0 4 ml 3 M HCl, 110–120°, 3 hr) Arginine was identified in the hydrolysate by PC and HVE The hydrolysate was treated with Dowex 1 × 4(AcO^-) The resin was washed with H_2O and eluted with 2 M HOAc The eluate was concd and applied to Dowex 50(H^+), which was washed with H_2O Succinic acid was identified in the effluent from the column by PC and FDMS spectrum (see Results and Discussion for ^1H NMR and FDMS spectra of **4** and FDMS spectrum of acid component of **4**) Compound **5**(= **1**) (28 mg) was isolated from fractions 6–9 by prep PC (solvent 2) followed by Dowex 50(H^+) treatment ^1H NMR of **5** (90 MHz in D_2O) δ 1 37–2 13 (4H, *m*, arginyl β- and γ-CH_2), 2 54 and 2 70 (1H, *dd*, J = 7, 16 Hz and 1H, *dd*, J = 4, 16 Hz, respectively, malyl CH_2), 3 19 (2H, *t*, J = 7 Hz, arginyl δ-CH_2), 4 26 (1H, *dd*, J = 5, 7 Hz, arginyl α-CH) and 4 44 (1H, *dd*, J = 4, 7 Hz, malyl CH) FDMS of **5** *m/z* (rel int) 291 $[M+1]^+$ (56), 273 $[291-H_2O]^+$ (100) Slight shift to a higher magnetic field of malyl CH_2 protons of compound **5** compared to those of **1** was due to the difference of the PD values of their solns for ^1H NMR measurement The pD value of **5** in soln was slightly higher than that of **1** judging by pH test paper, probably because NH_3 used to elute **5** from Dowex 50(H^+) after prep PC was not completely removed Compound **6**(= **2**) (17 mg) was isolated from fractions 14–18 by prep PC (solvent 2) followed by Dowex 50(H^+) treatment ^1H NMR of **6** (90 MHz in D_2O) δ 1 37–1 99 (4H, *m*, arginyl β- and γ-CH_2), 2 54 and 2 74 (1H, *dd*, J = 8, 15 Hz and 1H, *dd*, J = 5, 15 Hz, respectively, malyl CH_2), 3 19 (2H, *t*, J = 6 Hz, arginyl δ-CH_2) and 4 28 (2H, *m*, arginyl α- and malyl CH) The signals of the arginyl α- and malyl CH protons could not be separated because the resolution of the ^1H NMR spectrum of **6** was poorer than that of **2** FDMS of **6** *m/z* (rel int) 291 $[M+1]^+$ (100) Compound **7** (11 mg) was isolated from fraction 22 by prep PC (solvent 1) followed by Dowex 50(H^+) treatment ^1H NMR of **7** (90 MHz in D_2O) δ 1 42–2 00 (4H, *m*, arginyl β- and γ-CH_2), 2 52 and 2 72 (1H each, both *d*, J = 15 Hz, citryl one CH_2), 2 60 and 2 76 (1H each, both *d*, J = 16 Hz, citryl other CH_2), 3 20 (2H, *t*, J = 6 Hz, arginyl δ-CH_2) and 4 31 (arginyl α-CH, partially overlapped with side band of DOH signal) FDMS of **7** *m/z* (rel int) 349 $[M+1]^+$ (100) See Results and Discussion for the measurement of titration shift with ^1H NMR spectra Fraction 22 contained another component besides **7** which gave a positive reaction with the Sakaguchi reagent, but did not react with ninhydrin The R_f value of this substance was very near to that of aspartic acid on PC (solvent 1) The component could not be isolated, however, because of its very low concn A crude preparation of this compound had large complicated multiplets between δ 1 and 3 and a small quartet at δ 3 31 (J = 7 Hz) in the ^1H NMR spectrum (100 MHz in D_2O) and a base peak at *m/z* 186 in the FDMS spectrum Compound **8**(= **3**) (7 mg) was isolated from fractions 23 and 24 by prep PC (solvent 2) followed by Dowex 50(H^+) treatment ^1H NMR of **8** (90 MHz in D_2O) δ 1 43–2 07 (*m*, arginyl β- and γ-CH_2), 3 21 (*t*, J = 7 Hz, arginyl δ-CH_2) and 4 31 (arginyl α-CH) The integral value of each proton and coupling pattern of arginyl α-CH could not be measured because the spectrum was very noisy FDMS of **8** *m/z* (rel int) 247 $[M+1]^+$ (100)

Quantitative analysis of organic acids and amino acids An aliquot of the extract was concd and dissolved in H_2O After filtration, the filtrate was applied to a column of Amberlite CG-120 (H^+) which was washed thoroughly with H_2O The effluent and H_2O-wash were concd and absorbed on a column of Amberlite CG-4B(OH^-) The eluate from the column with 2 M NH_4OH was treated with Amberlite CG-120 (H^+) again to remove NH_3 The effluent and H_2O-wash from the column were concd, esterified with excess Et_2O–CH_2N_2 and analysed by GC under the same conditions described previously [2] An aliquot of the extract was concd and applied to an amino acid analyser to determine the free amino acids without any other purification procedure

Acknowledgements—We express our gratitude to Dr T Yoshihara, Department of Agricultural Chemistry, Hokkaido University, for help in the extraction procedure of samples and analyses of organic acids, and to Mr K Watanabe of the same department for measurements of the FDMS spectra

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