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Journal of Chromatography A, 787 (1997) 288–294

JOURNAL OF CHROMATOGRAPHY A

Short communication

Identification of nonprotein amino acids from cycad seeds as N-ethoxycarbonyl ethyl ester derivatives by positive chemical-ionization gas chromatography–mass spectrometry

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Received 14 May 1997; accepted 11 July 1997

Abstract

Nonprotein amino acids from nine species of cycad seeds were analyzed as N-ethoxycarbonyl ethyl ester (ECEE) derivatives by positive chemical-ionization gas chromatography–mass spectrometry. Based on the retention times and mass spectrometry analyses, 12 nonprotein amino acids were identified in these seeds. In addition to the excitatory and putative neurotoxin β-N-methylamino-L-alanine (BMAA), the known neurotoxin β-N-oxalylamino-L-alanine (BOAA) was detected from the seeds of *Macrozamia moorei* and *M. communis*, and δ-N-oxalyl-ornithine was obtained from the *Cycas revoluta* seeds. A novel nonprotein amino acid named cycasindene, previously reported from *C. revoluta*, was also found in the seeds of members of the *C. angulata* and *C. rumphii* complex. Eight additional known nonprotein amino acids were also identified. This is the first report of the neurotoxin BOAA from cycad seeds. © 1997 Elsevier Science B.V.

Keywords: Cycad seeds; Amino acids; ECEE derivatives; Nonprotein amino acids

1. Introduction

Cycad seeds have been used as food and medicine by several native population in various tropical and subtropical areas in the world. Epidemiological studies on Guam and in the western Pacific have indicated that seeds of the Guam cycad *Cycas micronesica* K.D. Hill [1] might be a factor in the etiology of a neurodegenerative disorder known as amyotrophic lateral sclerosis-Parkinsonism dementia complex (ALS-PDC) [2]. Studies with primates and other animals have implicated β-N-methylamino-L-alanine (BMAA), a nonprotein amino acid from the seeds of the Guam cycad, in ALS-PDC [3]. Further studies on L-BMAA suggests that it can activate excitatory amino acid receptors producing toxic levels of intracellular calcium in newborn rat brain tissue [4]. Also, it is well known that livestock feeding on leaves and seeds of other cycad species developed hindlimb paralysis in Australia, Mexico, and elsewhere [2]. These previous findings led to our investigation of the nonprotein amino acids in several cycad species, especially those that have been used for food and medicine.

As part of this investigation, seed extracts of nine cycad species were profiled for protein and nonprotein amino acids analysis by GC–MS as their N-ethoxycarbonyl ethyl ester (ECEE) derivatives. The

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0021-9673/97/$17.00
PII S0021-9673(97)00789-9

ethylchloroformate-induced derivatization procedure involving simultaneous N(O,S)-derivatization with ethylchloroformate in water–ethanol–pyridine was first introduced by Hušek [5]. This method has been widely used for analysis of protein amino acids by gas chromatography [5,6]. In a comprehensive study, the fragmentation mechanisms and relative peak intensities of ECEE derivatives of protein amino acids has been reported by Huang et al. using electron-impact (EI) mass spectrometry [7]. To simplify the fragment identification and enhance sensitivity of detection, Vatankhah and Moini analyzed ECEE derivatives of amino acids by chemical-ionization mass spectrometry [8].

This paper describes the identification of protein and nonprotein amino acids from the seeds of nine species of cycads as N-ethoxycarbonyl ethyl ester derivatives by positive chemical-ionization gas chromatography–mass spectrometry.

2. Experimental

2.1. Materials

The sources of the cycad seeds are listed in Table 1. Seed vouchers are deposited in the Department of Botany, The University of Texas at Austin (Voucher Nos. Mabry-Pan 211–219). The 52 standard protein and nonprotein amino acids were obtained from Sigma (St. Louis, USA), RBI (Natick, MA, USA), and Tocris Cookson (Bristol, UK). A solution containing 2–3 μmol/ml of each of the amino acids in 25 m*M* HCl was employed. Ethylchloroformate was supplied by Sigma. Analytical cation-exchange resin AG 50W-X4 was purchased from Bio-Rad (Richmond, CA, USA). All other chemicals were of analytical reagent grade. The nonprotein amino acid cycasindene was recently isolated for the first time from the seeds of *Cycas revoluta* [9].

2.2. Sample preparation

Ground fresh seed endosperm (100 g) was extracted with 70% ethanol (100 ml ×3) at room temperature. The concentrated extract was applied to an AG 50W-X4 cation-exchange resin column (1.2× 20 cm column with 8 ml of exchange resin). The column was first eluted with Millipore water (100 ml) to remove organic acids and sugars. Then, the total amino acids were eluted with 0.5 *M* ammonium hydroxide (50 ml), and the eluate was concentrated to 2 ml with a vacuum rotatory evaporator below 40°C. Methanol (8 ml) was added to the amino acid concentrate to precipitate much of the major protein amino acids, such as glutamic acid, aspartic acid, alanine, and glycine. After removing the precipitate by centrifugation, the supernatant was dried under vacuum, then redissolved in 1 ml of millipore water. An aliquot of 20 μl of the solution was used for derivatization.

The N-ethoxycarbonyl ethyl ester derivatives of standard protein and nonprotein amino acids as well as plant samples were prepared according to the procedure reported previously [5], except the chloroform layer of ECEE derivatives was dried with a

Table 1
The sources of cycad seeds for the nonprotein amino acid GC–MS analysis

Cycad seeds	Collected or purchased
Cycas angulata	around the Robinson River, Northern Australia, Dec. 1994[a]
C. revoluta	Rockport, Texas, USA, Dec. 1995[b]
C. rumphii	complex Montgomery Foundation, Miami, FL, USA, Mar. 1996[c]
Dioon edule	'Select Seeds' Alamo, Texas, USA, 1995
Macrozamia communis	south of Sydney, New South Wales, Australia, July, 1994[a]
M. moorei	Carnarvon Ranges, Queensland, Australia, May, 1994[a]
Zamia fischeri	'Select Seeds' Alamo, Texas, USA, 1995
Zamia furfuraceae	'Select Seeds' Alamo, Texas, USA, 1995
Zamia integrifolia	'Select Seeds' Alamo, Texas, USA, 1995

[a]From Patricia B. Orriell, 'D. Orriell-Seed Exporters', 45 Frape Avenue, Mt Yokine, Perth, Western Australia 6060.
[b]Collected by Dr. Tom J. Mabry.
[c]From Dr. Terrence Walters, The Montgomery Foundation, 11901 Old Cutler Road, Miami, FL 33156, USA.

Table 2
Charateristic ion peaks in positive CI mass spectra of 52 ECEE derivatives of protein and nonprotein amino acids (order reflects relative retention on BPX5 column)

Amino acids	M_r	Derivatives $[M+1]^+$ (m/z)	Base peak (m/z)	Other important ions
γ-Aminobutyric acid (Gaba)	103	204	204	158, 232, 240
Alanine (Ala)	89	190	190	158, 218, 230
Sarcosine (Sar)	89	190	190	218, 230
Glycine (Gly)	75	176	175	204, 216
α-Aminobutyric acid (αAba)	103	204	204	158, 232, 244
β-Alanine (βAla)	89	190	190	158, 218, 230
β-Aminobutyric acid (βAba)	103	204	204	158, 232, 244
β-Aminoisobutyric acid (βAiba)	103	204	204	158, 232, 244
Valine (Val)	117	218	218	172, 246, 258
Norvaline (Nov)	117	218	218	172, 246, 258
O-Methyl-serine (OMSer)	119	220	220	174, 248, 260
Glutamic acid (Glu)	147	158[a]	158[a]	186, 198
Homoserine (HSer)	119	174[b]	174[b]	202, 214
Leucine(Leu)	131	232	232	186, 260, 272
Isoleucine (Ile)	131	232	232	186, 260, 272
Serine (Ser)	105	158[b]	158[b]	131, 186,
Threonine (Thr)	119	220	220	174, 248, 260
Proline (Pro)	115	216	216	169, 243, 255
Norleucine (Nol)	131	232	232	186, 260, 272
Pipecolic acid (Pip)	129	230	230	184, 258, 270
α-Aminoadipic acid (αAaa)	161	290	290	244, 318, 330
Aspartic acid (Asp)	133	262	262	216, 290, 302
N-Methylaspartic acid (NMA)	147	276	276	230, 304, 316
ϵ-Acetamidocaproic acid (ϵAaca)	173	202	202	156, 230, 242
α-Aminocaprylic acid (αAca)	159	260	260	214, 288, 300
Hydroxyproline (HPro)	131	232	232	186, 260, 272
Methionine (Met)	149	250	250	204, 278, 290
Ethionine (Eth)	163	264	264	218, 292, 304
β-N-Methylamino-L-alanine (BMAA)	118	291	291	245, 319, 331
Phenylalanine (Phe)	165	266	266	220, 294, 306
Diaminopropanoic acid (Dap)	104	277	277	231, 305, 317
Cysteine (Cys)	121	294	294	248, 322, 334
ω-Aminocaprylic acid (ω-Aca)	159	260	260	214, 288, 300
3-Aminobenzoic acid (3Abza)	137	238	238	192, 266, 289
Glutamine (Gln)	146	246	247	201, 275, 287
2,4-Diaminobutyric acid (DAba)	118	291	291	245, 332
Homophenylalanine (HPhe)	179	280	280	234, 308, 320
4-Aminobenzoic acid (4Abza)	137	238	238	192, 266, 289
β-N-Oxalylamino-L-alanine (BOAA)	176	305	305	259, 333, 345
Homocysteine (HCys)	135	308	308	262, 336, 348
Kainic acid (KA)	213	342	342	296, 370, 382
Lysine (Lys)	146	319	319	273, 347, 359
γ-N-Oxalyl-diaminobutyric acid (ODAB)	190	319	319	273, 347, 359
Histidine (His)	155	328	328	282, 356, 368
Hydroxy-lysine (HLys)	162	289[b]	289[b]	243, 317, 329
δ-N-Oxalyl-ornithine (OOrn)	204	333	333	287, 361, 373
Tyrosine (Tyr)	181	354	354	308, 382, 394
ω-N-Oxalyl-lysine (OLys)	218	347	347	301, 375, 387
Tryptophan (Trp)	204	304[c]	304[c]	259, 333, 345
Cycasindene (Cyc)	218	317[d]	317[d]	271, 345, 357
Domoic acid (DA)	311	468	468	422, 496
Dihydroxyphenylalanine (DOPA)	197	442	442	396, 470, 482

[a]A product of an internal cyclization resulting in formation of pyroglutamic acid. [b]Due to Mclafferty rearrangment forming a five- or six-membered ring. [c]$[M]^+$. [d]$[M-1]^+$.

stream of nitrogen gas at room temperature and redissolved in 20 μl of chloroform prior to GC–MS analysis.

2.3. GC–MS

A Varian 3400 gas chromatograph (Palo Alto, CA, USA) was interfaced to a Finnigan MAT TSQ-70 (San Jose, CA, USA) mass spectrometer. For GC separation, a BPX5 capillary column, 25 m long with 0.22 mm I.D. and 0.25-μm film coating (SGE, Austin, TX, USA) was employed. The mass spectrometer was run in a positive ionization mode under the following operating conditions: scan range of 150–550 u, scan rate of 1 scan/s, and an interface temperature of 270°C. Methane gas was used as CI reagent gas at a pressure of ca. 2 Torr. A 1-μl aliquot of each standard and sample solution was injected in a split mode (60:1) at an injector temperature of 270°C. The GC oven temperature was initially held at 130°C for 2 min and then programmed to 270°C at a rate of 5°C/min. Helium was used as the carrier gas with a head pressure of 10 p.s.i.

3. Results and discussion

The characteristic ion peaks of the N-ethoxycarbonyl ethyl ester derivatives of 52 standard protein and nonprotein amino acids are listed in Table 2. For most of the ECEE derivatives, a base peak of $[M+1]^+$ was observed, except $[M]^+$ for tryptophan and $[M-1]^+$ for cycasindene. The ion peaks $[M+29]^+$ and $[M+41]^+$ represent adducts with $C_2H_5^+$ and $C_3H_5^+$, respectively, as is typical when methane is used as the chemical ionization reagent gas. The fragment $[M-45]^+$ was assigned for the loss of an OEt group from the ECEE derivatives. These characteristic ion peaks were useful in distinguishing amino acid ECEE derivatives from impurities.

The base peak of ECEE derivatives of glutamic acid was m/z 158 instead of m/z 276, as a result of an internal cyclization in the formation of pyroglutamic acid [6]. Those amino acids with hydroxyl groups, such as serine, homoserine, and hydroxylysine showed base peaks of m/z 158, 174, and 289, respectively, due to a McLafferty rearrangement forming a five- or six-membered ring [7]. In addition to amino and carboxyl groups, both sulfhydryl and phenolic groups of amino acids also reacted with ethylchloroformate producing stable derivatives for detection. Therefore, the base peaks of ECEE derivatives of cysteine, homocysteine, tyrosine, and dihydroxyphenylalanine were obtained as m/z 294, 308, 354, and 442, respectively.

All 52 ECEE derivatives of amino acid standards showed excellent resolution on GC–MS. Because of the mild reaction conditions, oxalyl-nonprotein amino acids such as γ-N-oxalyl-diaminobutyric acid, ω-N-oxalyl-lysine, δ-N-oxalyl-ornithine, and the neurotoxin BOAA could be easily derivatized and analyzed by this method. Furthermore, other potential neurotoxins such as domoic acid and kainic acid were readily derivatized and separated on GC, and exhibited distinctive ECEE derivative MS fragmentation patterns. Therefore, this method could be

Table 3
Nonprotein amino acids in seeds of nine species of cycads

	C. angulata	*C. revoluta*	*C. rumphii*	*D. edule*	*M. Communis*	*M. moorei*	*Z. fischeri*	*Z. furfuracea*	*Z. integrifolia*
γ-Aminobutyric acid	+	+	+	+	+	+	+	+	+
β-Alanine	+	+	+	+				+	
β-Aminobutyric acid		+		+	+	+		+	+
Pipecolic acid	+	+	+	+	+	+	+	+	+
α-Aminoadipic acid	+	+		+	+	+	+		+
N-Methylaspartic acid	+	+			+	+			
ε-Acetamidocaproic acid				+					
β-*N*-Methylamino-L-alanine	+	+	+	+					
2,4-Diaminobutyric acid	+		+						
β-*N*-Oxalylamino-L-alanine					+	+			
δ-*N*-Oxalylamino-L-alanine		+							
Cycasindence	+	+	+						

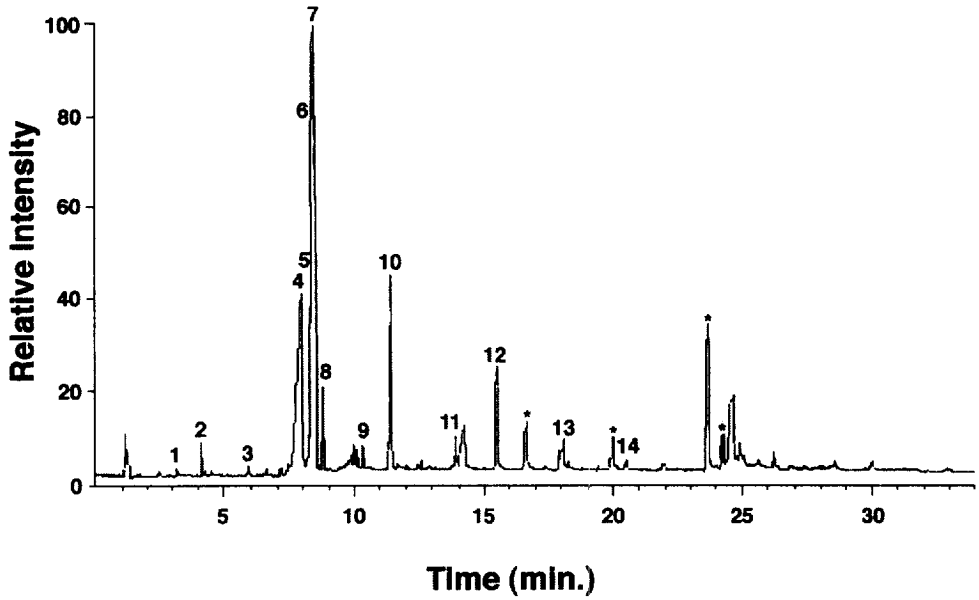

Fig. 1. Reconstructed ion chromatogram of ECEE derivatives of protein and nonprotein amino from the seeds of *M. moorei* separated on BPX5 (25 m×0.22 mm I.D.) capillary column. GC conditions are described in text. Peaks: (1) Gaba, (2) Ala, (3) βAba, (4) Glu, (5) Ser, (6) Thr, (7) Pro, (8) Pip, (9) αAaa, (10) Asp, (11) NMA, (12) Phe, (13) Gln, (14) BOAA. *Unidentified peaks.

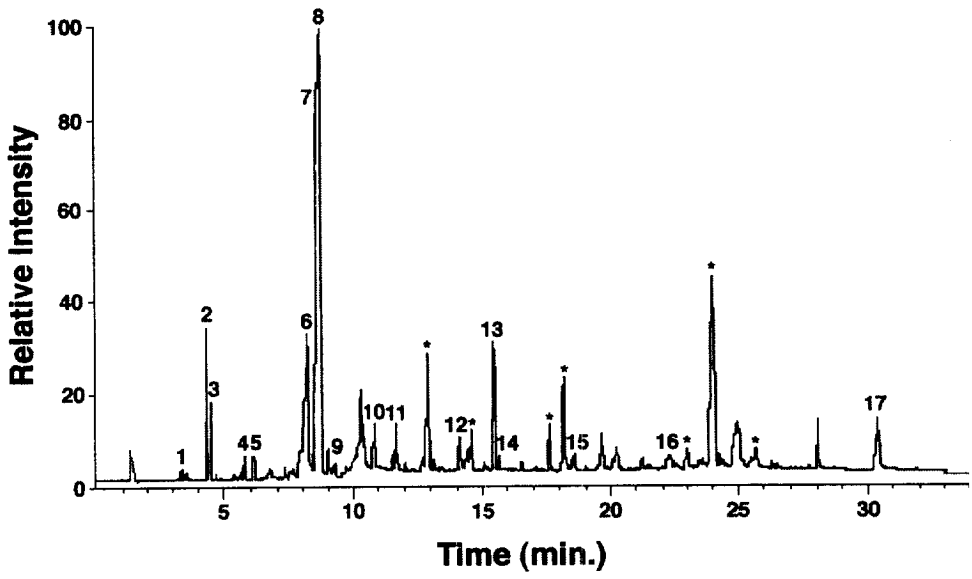

Fig. 2. Reconstructed ion chromatogram of ECEE derivatives of protein and nonprotein amino acids from the seeds of *C. revoluta* separated on BPX5 (25 m×0.22 mm I.D.) capillary column. GC conditions are described in text. Peaks: (1) Gaba, (2) Ala, (3) Gly, (4) βAba, (6) Glu, (7) Ser, (8) Pro, (9) Pip, (10) αAaa, (11) Asp, (12) NMA, (13) BMAA, (14) Phe, (15) Lys, (16) OOrn, (17) cycasindene. *Unidentified peaks.

useful for screening US food and medicinal plants for nonprotein amino acids that might be neurotoxic.

The GC–MS results of the ECEE derivatives of nonprotein amino acids in nine species of cycad seeds are given in Table 3. The reconstructed ion chromatograms of the ECEE derivatives of protein and nonprotein amino acids from *M. moorei* and *C. revoluta* are presented in Figs. 1 and 2. Based on the retention times and mass spectra, the ECEE derivatives of amino acids were identified by comparison with standards. The neurotoxin BOAA as well as five known nonprotein amino acids including β- and γ-aminobutyric acid, pipecolic acid, α-aminoadipic acid, and N-methylaspartic acid were detected in the seeds of *M. moorei*. From *C. revoluta* seeds, nine nonprotein amino acids including BMAA, δ-N-oxalyl-ornithine, cycasindene, β- and γ-aminobutyric acid, β-alanine, pipecolic acid, α-aminoadipic acid, and N-methylaspartic acid were identified. In addition, several nonprotein amino acids (marked with an asterisk) exhibited typical ECEE derivatives fragmentation patterns, but remain unidentified in extracts of seeds of both *M. moorei* and *C. revoluta*.

As shown in Table 3, BOAA was detected in the seeds of both *M. moorei* and *M. communis*, whereas δ-N-oxalyl-ornithine was only found in the seed of *C. revoluta*. BOAA is a potent neuroexcitatory amino acid that has been identified as the causative agent for human neurolathyrism [10]. BOAA was first isolated from the seeds of *Lathyrus sativus* [11] and was previously reported only in the seeds of legume species of *Lathyrus* and *Acacia* [12,13]. This is the first report of BOAA and δ-N-oxalyl-ornithine in cycad seeds.

BMAA was identified in the seeds of *C. revoluta*, *C. angulata*, the *C. rumphii* complex as well as the seeds of *Dioon edule*. The recently discovered nonprotein amino acid cycasindene was detected in the seeds of three *Cycas* species. Eight known nonprotein amino acids were also identified from the nine species of cycad seeds. The mass spectra of the ECEE derivatives of BOAA, BMAA, δ-N-oxalyl-ornithine, and cycasindene, compounds from the seeds of *M. Moorei* and *C. revoluta*, are shown in Figs. 3–6, respectively. The MS fragmentation patterns and relative peak intensities of the nonprotein amino acids obtained here were in agreement with data for standards.

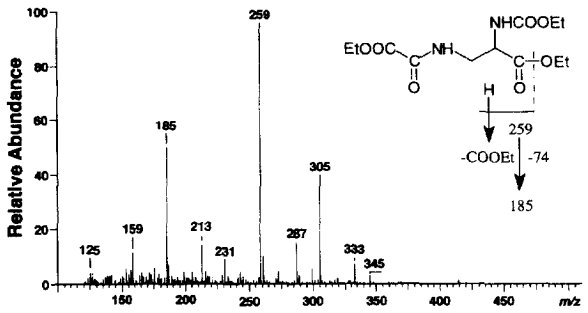

Fig. 3. Mass spectrum of ECEE derivatives of β-*N*-oxalylamino-L-alanine (BOAA, peak 14, M_r 304) from the seeds of *M. moorei*.

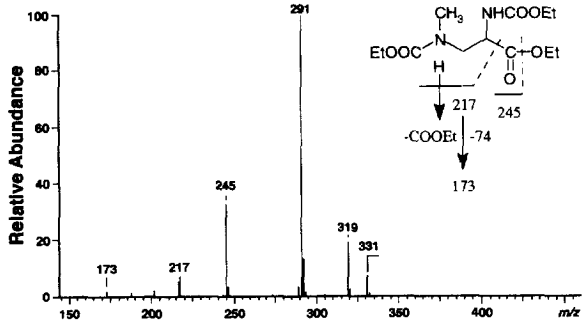

Fig. 4. Mass spectrum of ECEE derivatives of β-*N*-methylamino-L-alanine (BMAA, peak 13, M_r 290) from the seeds of *C. revoluta*.

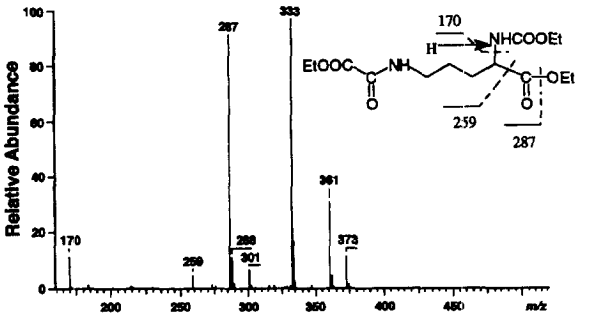

Fig. 5. Mass spectrum of ECEE derivatives of δ-*N*-oxalyl-ornithine (peak 16, M_r 332) from the seeds of *C. revoluta*.

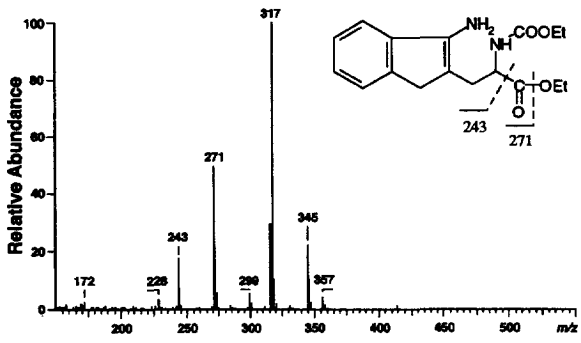

Fig. 6. Mass spectrum of ECEE derivatives of cycasindene (peak 17, M_r 318) from the seeds of *C. revoluta*.

Acknowledgments

The authors thank Dr. E. Arthur Bell and Dr. Chris Perera for providing standard nonprotein amino acids, Dr. Terrence Walters for providing seeds of members of *C. rumphii* complex, Dr. Ted Delevoryas for assistance with plant identification, and Dr. Delia Brownson for helpful suggestions. This work was supported by the National Institute on Aging (Grant AG-10637) and the Robert A. Welch Foundation (Grant F-130).

References

[1] K.D. Hill, Proc. 3rd International Conference on Cycad Biology, 1993, p. 139.
[2] M.G. Whiting, Economic Botany 17 (1963) 271.
[3] P.S. Spencer, P.B. Nunn, J. Hugon, A.C. Ludolph, S.M. Ross, D.N. Roy, R.C. Robertson, Science 237 (1987) 517.
[4] D.M. Brownson, Ph.D. Dissertation, The University of Texas at Austin, 1996, p. 108.
[5] P. Hušek, FEBS Lett. 280 (1991) 354.
[6] P. Hušek, J. Chromatogr. 552 (1991) 289.
[7] Z.H. Huang, J. Wang, D.A. Gage, J.T. Watson, C.C. Sweeley, J. Chromatogr. 635 (1993) 271.
[8] M. Vatankhah, M. Moini, Biol. Mass Spectrom. 23 (1994) 277.
[9] M. Pan, T.J. Mabry, J.M. Beale, B.M. Mamiya, Phytochemistry 45 (1997) 517–519.
[10] R.A. Chase, S. Pearson, P.B. Nunn, P.L. Lantos, Neurosci. Lett. 55 (1985) 89.
[11] P.R. Adiga, S.L.N. Rao, P.S. Sarma, Curr. Sci. 32 (1963) 153.
[12] J. Przybylska, T. Rymowicz, Genet. Pol. 6 (1965) 91.
[13] C.S. Evans, M.Y. Qureshi, E.A. Bell, Phytochemistry 16 (1977) 565.