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NONPROTEIN AMINO ACIDS FROM *CYCAS REVOLUTA*

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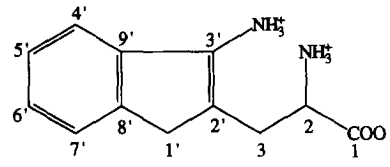
Key Word Index—*Cycas revoluta*; Cycadaceae; cycads; nonprotein amino acids; cycasindene; 3-[3′amino-indenyl-2′]-alanine; cycasthioamide; *N*-[glycinyl-alaninyl-11-thio]-5-one-pipecolic acid.

Abstract—Two nonprotein amino acids, cycasindene and cycasthioamide, along with eight known nonprotein amino acids, were isolated from the seeds of *Cycas revoluta* Thunb. The structures of cycasindene and cycasthioamide were elucidated as 3-[3′-amino-indenyl-2′]-alanine (**1**) and *N*-[glycinyl-alaninyl-11-thio]-5-one-pipecolic acid (**2**) by chemical and spectral methods.

INTRODUCTION

Studies with primates and other animals implicated the nonprotein amino acid L-BMAA (*β*-*N*-methylamino-L-alanine) in the neurologic disorder, prevalent among the Chamorro population of Guam, known as amyotrophic lateral sclerosis–parkinsonism dementia complex (ALS-PDC) [1]. L-BMAA was first isolated from the seeds of the Guam cycad, *C. circinalis* L. [2] (which is now recognized as *C. micronesica* K. D. Hill [3]). Further studies on the Guam cycad toxin suggested that the L-BMAA-carbamate, a product of L-BMAA and CO_2 at physiological pH, activated glutamate receptors producing toxic levels of intracellular calcium in neuronal cells [4]. These findings led to an investigation of the nonprotein amino acids in seeds from other *Cycas* species used as food or medicine. The present paper describes the isolation and structural elucidation of two new nonprotein amino acids, cycasindene (**1**) and cycasthioamide (**2**), from the seeds of *C. revoluta* Thunb.



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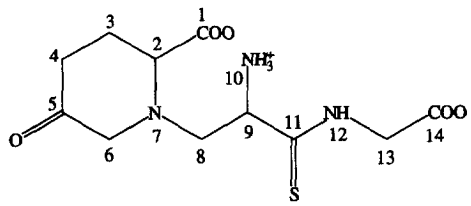

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RESULTS AND DISCUSSION

Compound **1** exhibited a yellow colour with ninhydrin reagent. The molecular formula of **1** was determined as $C_{12}H_{14}N_2O_2$ by high resolution FAB mass spectroscopy. 1D and 2D ^1H NMR experiments indicated nine protons in three separate spin systems: CH_2 CH_2CHN and an *ortho*-substituted benzene ring. The proton splitting pattern of **1** was similar to indene, but not indole in comparison with reference NMR data [5]. The ^{13}C NMR spectrum of **1** suggested the presence of carbons belonging to a carboxyl group (δ 170.57), an α-amino acid group (δ 54.33), a benzene ring and a quaternary double bond (δ 104.32 and 124.73, respectively). The correlation between H-1′ and C-2′, -3′, -8′ in the HMBC spectrum confirmed that **1** contained an indene ring. The very weak coupling between one of the protons at C-3 and C-1′ in the COSY spectrum indicated that the alanine side chain was attached to the 2′-position. This observation was in agreement with the HMBC experiment which showed ^{13}C-^1H correlations between C-1′ and H-2, as well as C-2 and H-1′. Based on the HMQC, HMBC

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Table 1. ^{1}H and ^{13}C NMR spectral data for compound **1** (D_2O–DCl, 500 MHz)*

C/H	^{1}H (*J* in Hz)	^{13}C	^{1}H–^{13}C HMBC correlation
1		170.57	
2	3.78 *dd* (5.0, 10.5)	54.33	C-1, C-3, C-1′
3	3.03 *dd* (5.0, 16.4)	21.13	C-2, C-2′, C-3′
	2.84 *dd* (10.5, 16.4)		C-2, C-2′, C-3′
1′	4.29 *d* (15.6)	40.05	C-2, C-2′, C-3′, C-8′
	4.04 *d* (15.6)		C-2′, C-3′, C-8′
2′		104.32	
3′		124.73†	
4′	7.27 *d* (8.0)	117.72	C-2′, C-5′, C-9′
5′	7.03 *t* (8.0)	122.31	C-4′, C-9′
6′	6.93 *t* (8.0)	119.46	C-7′, C-8′
7′	7.23 *d* (8.0)	113.34	C-6′, C-8′
8′		125.02†	
9′		136.11	

*Assignments confirmed by 2D experiments (COSY and HMQC).
†Interchangeable values.

Table 2. ^{1}H and ^{13}C NMR spectral data for compound **2** (D_2O, 500 MHz)*

C/H	^{1}H (*J* in Hz)	^{13}C	^{1}H–^{13}C HMBC correlation
1		178.74	
2	4.33 *m*	61.25	C-1, C-3
3	2.00 *m*	22.69	C-1, C-2, C-4
	2.43 *m*		C-1, C-2, C-4
4	2.54 *m*	29.79	C-2, C-3, C-5
5		179.91	
6	3.73 *d* (14.4)	42.54	C-2, C-5, C-8
	3.88 *d* (14.4)		C-2, C-5, C-8, C-9
7			
8	2.88 *dd* (15.0, 4.3)	30.94	C-6, C-9, C-11
	3.13 *dd* (15.0, 9.4)		C-6, C-11
9	4.33 *m*	51.89	C-8, C-11
10	6.98 *t*†		
11		168.01	
12	8.17 *s*†		
13	3.73 *dd* (17.2)	43.24	C-12, C-14
	3.88 *dd* (17.2)		C-12, C-14
14		175.81	

*Assignments confirmed by 2D experiments (COSY and HMQC).
†10% D_2O–90% H_2O.

and COSY spectra, **1** was identified as 3-[3′-amino-indenyl-2′]-alanine, named here as cycasindene.

Compound **2** also developed a yellow colour with ninhydrin reagent, but the yellow colour turned to red in a few hours. High voltage electrophoresis results demonstrated that **2** was an acidic amino acid. Compound **2** decomposed during acid hydrolysis, suggesting that it was not a peptide. The elemental composition of **2** was deduced as $C_{11}H_{17}N_3O_5S$ from a high resolution CI mass spectrum. 1D and 2D ^{1}H NMR spectra in D_2O indicated that **2** had 12 protons belonging to four isolated coupling systems: CH_2, CH_2, CH_2CHN, CH_2CH_2CHN. The ^{13}C NMR spectrum showed that **2** had four quaternary carbons: two carboxyl, one carbonyl, and one amide-type. The HMBC experiment established that two protons with the same chemical shift at δ 4.33 were correlated to two different carbons both of which had amino groups attached. The connections between carbons and protons were established from COSY, HMQC and HMBC spectra. Two moieties in **2**, the oxidized pipecolic acid ring and the side chain, were in agreement with MS fragments of *m/z* 142 and 161. A broad singlet for one proton at δ 8.17 was shown to be coupled to both protons at C-13 on the basis of PRESAT (1D Water Suppression) and COSY experiments in D_2O–H_2O (1:9). The latter spectrum verified that the proton at δ 8.17 belonged to an amide-type group and that the imino group (N-12) was connected to C-11 and C-13. The JUMPRET (Jump-and-Return Water Suppression, D_2O–H_2O) exhibited a triplet of three protons at δ 6.98 and a broad singlet for the proton at δ 8.17. The ratio of the

triplet was 1:1:1, indicative of quadrapole coupling with ^{14}N, thus implicating an ammonium ion (N-10) on the side chain. On the ^{15}N–^{1}H HMQC spectrum, the triplet was correlated to the ammonium ion N at δ 25, and the singlet at δ 8.17 was correlated to what must be a thioamide N at δ 117 [6]. From the MS and NMR data, **2** was concluded to be *N*-[glycinyl-alaninyl-11-thio]-5-one-pipecolic acid, named here as cycasthioamide. This is the first report of a thioamide nonprotein amino acid from plants.

EXPERIMENTAL

Plant material. Seeds of *C. revoluta* Thunb. were collected from Rockport, Texas, U.S.A., in December 1995. Seed and leaf vouchers are deposited in the Plant Resources Center, University of Texas at Austin, Voucher no. Mabry-Pan 210.

Extraction and isolation. Ground fresh endosperm (5.4 kg) was extracted with 80% EtOH at room temp and the extract was defatted with $CHCl_3$. The concd extract was applied to an AG 50W-X4 cation exchange resin column. The column was eluted with an ammonium hydroxide gradient (0.2–1 M). Twenty frs were collected. Each fr. was further chromatographed on a cellulose column, eluted with *iso*-PrOH–H_2O gradient (4:1–1:1). **1** (13 mg) was obtained from frs 8 and 9 of the ion exchange CC, and final purification by cellulose CC with *iso*-PrOH–H_2O (4:1). **2** (12 mg) was isolated from fr. 5 and final purification by cellulose CC with *iso*-PrOH–H_2O (2:1).

Eight known nonprotein amino acids were isolated and identified as α-aminoadipic acid, β-alanine, α-, β- and γ-aminobutyric acid, citrulline, BMAA and hydroxyarginine by comparison with authentic nonprotein amino acids using TLC, HVE (high voltage electrophoresis) and GC.

Cycasindene (**1**). Microcrystals. HRFABMS: *m/z* 217.0958 $[M-H]^+$, (calcd 217.0977) for $C_{12}H_{13}N_2O_2$. ^{1}H and ^{13}C NMR: Table 1.

Cycasthioamide (**2**). Amorphous powder. CIMS: *m/z* 302 $[M-H]^+$, 142, 161. HRCIMS: *m/z* 302.0805 $[M-H]^+$, (calcd 302.0810) for $C_{11}H_{16}N_3O_5S$. ^{1}H and ^{13}C NMR: Table 2.

Acid hydrolysis of **2**. Solns of **2**, each containing 0.5 mg **2**, in 0.1 ml each of 1 M, 2 M and 6 M HCl were heated at 100° for 2 hr. After cooling, each soln was concd to dryness. Each sample was analysed for amino acids by TLC and HVE. **2** was decomposed during acid hydolysis. Neither **2** nor any identifiable protein or nonprotein amino acids were detected in the hydrolysates.

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