Rhopeptin A: First Cyclopeptide Isolated from *Rhodobryum giganteum*

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Rhopeptin A was isolated as the first cyclopentapeptide from the moss *Rhodobyum giganteum* [SCHWAEGR.] PAR. This novel compound consists of proline, phenylalanine, and 3-hydroxyproline ring-bonded amino acid residues connected to a tyrosine fragment *via* an ether bridge. Attached to a 3-hydroxyproline unit is a side chain of pyroglutamic acid residue. The structure of the peptide was deduced from the 1D- and 2D-NMR and MS data.

Introduction. – Mosses represent a transition stage of evolution, as they combine the characteristics of algae and higher plants [1]. A plethora of structurally novel bioactive cyclopeptides have been reported from the marine cyanobacteria in the past few decades [2]. There are also a number of cyclopeptides and cyclopeptide alkaloids reported from higher seed plants [3]. However, no cyclopeptide was found in mosses so far. Through our study, a novel unprecedented cyclization mode of a peptide was discovered. *Rhodobryum giganteum* [SCHWAEGR.] PAR. (Bryaceae), a kind of mosses, has conventionally been used to treat CHD (cardiac heart disease) in China [4]. Pharmacological experiments revealed that *R. giganteum* enhances blood flow volume of coronary artery, improving atherosclerosis, high blood viscosity, and hypoxia, decreasing heart rate and blood pressure [5]. Within this current project, rhopeptin A, the first cyclopeptide was isolated from the BuOH extract of *R. giganteum*. Its structure was elucidated by means of 1D- and 2D-NMR, and MS data. Several cyclopeptide alkaloids with ring-bonded ether bridges were isolated by phytochemists, with styrylamine as ring-bonded fragment being their common structural feature [3][6]. Rhopeptin A is partially similar to this type of cyclopeptide alkaloids, but it has no N-terminus and owns an exocyclic COOH group [7].

Results and Discussion. – Rhopeptin A (*Fig. 1*) was obtained as a white amorphous powder that gave an $[M+Na]^+$ peak in the HR-ESI-MS at *m/z* 654.2567 (calc. 654.2534), consistent with the molecular formula $C_{33}H_{37}N_5O_8$, indicating 18 degrees of unsaturation. The IR spectrum of rhopeptin A exhibited bands characteristic of amino (3396 cm^{-1}), CO, and amide CO (1747, 1694, 1676, 1650, and 1635 cm^{-1}) groups. It gave a negative reaction with ninhydrin reagent but a positive one when hydrolyzed with 6N HCl, indicating a blocked N-terminus or a cyclopeptide. Amino acid analysis of the hydrolysate prepared from rhopeptin A with 6N HCl revealed the presence of phenylalanine (Phe), proline (Pro), tyrosine (Tyr), and glutamic acid (Glu) fragments.

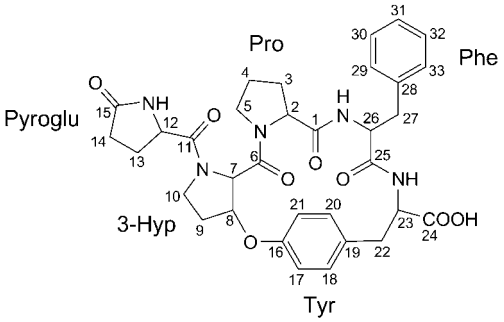

Fig. 1. *Cyclopeptide* (Rhopeptin A) *isolated from* Rhodobryum giganteum

Signals at δ(H) 8.81 (*s*), 8.22 (*d*, $J = 6.5$), and 8.14 (*d*, $J = 9.6$) disappeared in the ^{1}H-NMR spectrum (*Table*) after D_2O was added into C_5D_5N, indicating the presence of three exchangeable H-atoms. The ^{13}C-NMR spectrum (*Table*) displayed 33 C-atom signals, which were ascribed, by DEPT and HSQC techniques, to nine CH_2 and fifteen CH groups, and nine quaternary C-atoms. Six quaternary C-atoms (δ(C) 179.5, 175.7, 173.9, 172.4, 171.5, and 169.1) were assigned to CO groups. Careful analysis of ^{1}H-, ^{13}C-NMR, DEPT, HSQC, HMBC, ^{1}H,^{1}H-COSY, and NOSEY spectra, as well as comparison with those in literature, resulted in the complete assignments of ^{1}H- and ^{13}C-NMR data (*Table*).

The HMBC spectra showed rhopeptin A to have five partial structures comprising **a** (C(1) to C(5)), **b** (C(6) to C(10)), **c** (C(11) to C(15)), **d** (C(16) to C(24)), and **e** (C(25) to C(33)) (*Fig. 2*). From C(1) to C(5), signals indicated the Pro-residue features in ^{1}H- and ^{13}C-NMR with α position at tertiary C-atom C(2) and δ at C(5). The Pro residue of partial structure **a** was evidenced through HMBCs inside the pentacycle. A bridging O-atom at C(8) (δ(C) 81.2) in fragment **b** implied the 3-hydroxyproline (3-Hyp) structure. The HMBCs of H–C(7) with C(8), C(9), and C(10) established the 3-Hyp ring subunit, assigning α position to C(7) (δ(C) 64.6) and δ to C(10) (δ(C) 45.9). The low-field shift of δ(C) 179.5 suggested that C(15) was the lactam CO group, corresponding to the HMBCs of H–C(12) with C(13) to C(15), and HN–C(12) with C(12) to C(15). Therefore, the pyroglutamic acid (Pyroglu) residue of part **c** was established with the α tertiary C-atom C(12) (δ(C) 55.8). The aromatic C-atom signals at δ(C) 155.6, 135.0, 133.5, 130.6, 124.1, 120.5, and interior correlations indicated the presence of O-containing group on aromatic ring. The ^{1}H-NMR signals overlapping in C_5D_5N (δ(H) 7.28 – 7.33 and 7.37 – 7.40) were separated to four *doublet*s (δ(H) 7.55 (*d*, $J = 7.8$, 1 H), 7.46 (*d*, $J = 8.2$, 1 H), 7.40 (*d*, $J = 7.8$, 1 H), and 7.04 (*d*, $J = 8.2$, 1 H)) in a mixture C_5D_5N/D_2O. In the HMBC spectrum, signals of H_a–C(22) and H–C(23) correlated with that of C(24). These data suggested that the fragment **d** was a Tyr unit in α position at C(23) (δ(C) 53.8). The asymmetric signals of C(17), C(18), C(20), and C(21) in ^{1}H- and ^{13}C- spectra, and NOE cross-peaks H–C(21)/H–C(7), H–C(21)/H–C(8), and H–C(18)/H_a–C(22) pointed out that the Tyr residue belonged to the macrocycle. For part **e**, signals at δ(H) 7.30, 7.17, 7.08, and at δ(C) 139.9, 130.6, 129.4, 127.3 were attributed to Phe unit in α position at C(26), referring to the signals in aromatic field of ^{1}H- and ^{13}C-NMR, and the mutual correlations of Ph ring in HMBC spectra. The ^{1}H,^{1}H-COSY spectra revealed five partial structures indicated in bold bonds (*Fig. 2*).

Table. *^{1}H- and ^{13}C-NMR Data of Rhopeptin A* (recorded in C_5D_5N; δ in ppm, J in Hz)

Position	δ(H)	δ(C)
C(1)		172.4 (*s*)
H–C(2)	4.50 (*d*, $J = 7.7$)	62.0 (*d*)
H_a–C(3)	1.73 – 1.83 (*m*)	31.9 (*t*)
H_b–C(3)	2.63 – 2.68 (*m*)	
H_a–C(4)	0.72 – 0.83 (*m*)	22.6 (*t*)
H_b–C(4)	1.33 – 1.41 (*m*)	
H_a–C(5)	2.91 – 2.96 (*m*)	48.2 (*t*)
H_b–C(5)	3.38 – 3.46 (*m*)	
C(6)		169.1 (*s*)
H–C(7)	4.38 (*s*)	64.6 (*d*)
H–C(8)	5.12 (*d*, $J = 2.9$)	81.2 (*d*)
H_a–C(9)	2.36 – 2.40 (*m*)	32.7 (*t*)
H_b–C(9)	2.47 – 2.50 (*m*)	
H_a–C(10)	3.92 – 3.97 (*m*)	45.9 (*t*)
H_b–C(10)	3.99 – 4.05 (*m*)	
C(11)		173.9 (*s*)
H–C(12)	4.67 (*dd*, $J = 8.5, 2.8$)	55.8 (*d*)
HN–C(12)	8.81 (*s*)	
H_a–C(13)	2.42 – 2.46 (*m*)	25.6 (*t*)
H_b–C(13)	2.51 – 2.54 (*m*)	
H_a–C(14)	2.31 – 2.35 (*m*)	30.4 (*t*)
H_b–C(14)	2.55 – 2.58 (*m*)	
C(15)		179.5 (*s*)
C(16)		155.6 (*s*)
H–C(17)	6.71 (*d*, $J = 7.0$)	124.1 (*d*)
H–C(18)	7.28 – 7.33 (*m*)[a])	133.5 (*d*)
C(19)		135.0 (*s*)
H–C(20)	7.37 – 7.40 (*m*)[b])	130.6 (*d*)
H–C(21)	7.37 – 7.40 (*m*)[b])	120.5 (*d*)
H_a–C(22)	2.99 (*t*, $J = 13.6$)	38.7 (*t*)
H_b–C(22)	3.76 (*dd*, $J = 13.6, 4.8$)	
H–C(23)	5.61 (*ddd*, $J = 13.6, 9.6, 4.8$)	53.8 (*d*)
C(24)		175.7 (*s*)
HN–C(23)	8.14 (*d*, 9.6)	
C(25)		171.5 (*s*)
H–C(26)	4.75 (*ddd*, $J = 12.0, 6.5, 4.0$)	56.5 (*d*)
HN–C(26)	8.22 (*d*, $J = 6.5$)	
H_a–C(27)	3.19 (*dd*, $J = 14.6, 4.0$)	35.3 (*t*)
H_b–C(27)	3.59 – 3.66 (*m*)	
C(28)		139.9 (*s*)
H–C(29, 33)	7.28 – 7.33 (*m*)[a])	130.6 (*d*)
H–C(30, 32)	7.17 (*t*, $J = 7.5$)	129.4 (*d*)
H–C(31)	7.08 (*t*, $J = 7.5$)	127.3 (*d*)

[a]) [b]) Signals overlapped.

The HMBCs of C(1) with H–C(2), CH_2(3), and HN–C(26) revealed the connectivity of two partial structures **a** and **e**. The linkage of fragments **a** and **b** with

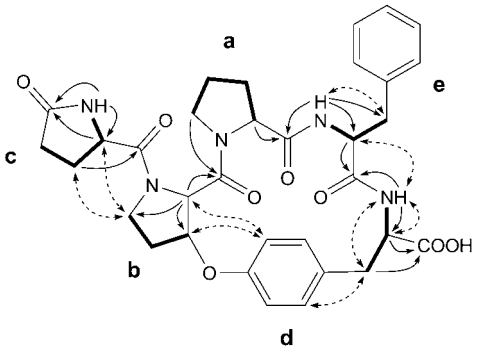

Fig. 2. *$^1H,^1H$-COSY* (bold), *key HMBC* (H → C), *and selected NOESY* (H ← -- → H) *correlations of rhopeptin A*

the quaternary C(6)-atom could be determined by the HMBCs of C(6) with H_b–C(5), H–C(7), and H–C(8). The connectivity of parts **b** and **c** through C(11) was evidenced by the critical HMBCs of H_a–C(13) with C(11). The sequence was also supported by the NOE cross-peaks of H_a–C(10) with H–C(12) and H_a–C(13). The HMBCs of HN–C(23) with C(25) and C(23), and the correlation between H–C(26) and C(25) confirmed the linkage of the fragments **d** and **e**, further evidenced by the NOE signals of HN–C(23) with H–C(23), H_a–C(22), and H–C(26). According to the degree of unsaturation, the ring-bonded ether bridge structure of rhopeptin A was determined. The residue sequence of rhopeptin A was supported by HR-ESI-MS and tandem ESI-MS. In HR-ESI-MS, accurate masses were determined for product ions by collisionally activated dissociation (CAD) of the protonated molecular ions. Expected fragment ions and sequence of part **a** – **e** were in accordance with experimentally determined ion peaks of *m/z* 632.2715 ($[M+H]^+$, $C_{33}H_{38}N_5O_8^+$; calc. 632.2715), 535.2185 ($[M+H-Pro]^+$, $C_{28}H_{31}N_4O_7^+$; calc. 535.2187), and 388.1497 ($[M+H-Pro\text{-}Phe]^+$, $C_{19}H_{22}N_3O_6^+$; calc. 388.1503). The exocyclic COOH group at C(24) was evidenced by the ion peak at 342.1445 ($[M+H-Pro\text{-}Phe-HCOOH]^+$, $C_{18}H_{20}N_3O_4^+$, calc. 342.1448). The segment **a** – **b** – **c** was difficult to dissociate, but confirmed by tandem ESI-MS of ions with the peaks at *m/z* 519 ($[M-H-Pyroglu]^-$), 424 ($[M-H-Pyroglu\text{-}(3\text{-}Hyp)]^-$), and 353 ($[M-H-Pyroglu\text{-}(3\text{-}Hyp)\text{-}Pro]^-$) in negative-ion mode.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200 – 300 and 400 – 600 mesh; *Qingdao Haiyang Chemical Group Co.*), *Sephadex LH-20* (*Amersham Biosciences*), *MCI* gel *CHP-20P* (*Mitsubishi Chemical Corporation*), and *ODS-A* (50 μm, *YMC*). TLC: *$HSGF_{254}$* (10 – 40 mm; Q*ingdao Haiyang Chemical Group Co.*). IR Spectra: *Perkin-Elmer* FT-IR spectrometer; in cm^{-1}. NMR Spectra: *Bruker-Advance-600* spectrometer; δ in ppm, with residual C_5H_5N (δ(H) 8.73, 7.58, and 7.21; δ(C) 149.9, 135.5, and 123.5) as internal standard, *J* in Hz; ^{1}H- and ^{13}C-NMR assignments were supported by ^{1}H,^{1}H-COSY, HMQC, HMBC, and NOESY experiments. HR-ESI-MS: *Bruker BioTOF-Q* time-of-flight mass

spectrometer equipped with an ESI source in positive-ion mode. Tandem ESI-MS: *Finnigan LCQ^{DECA}* ion-trap mass spectrometer equipped with an ESI source in negative-ion mode.

Plant Material. The whole plants of *R. giganteum* [SCHWAEGR.] PAR. were collected in Sichuan Province of P. R. China in September 2006 and identified by Assoc. Prof. *Y. Jia* (Institute of Botany, the Chinese Academy of Sciences, P. R. China). A voucher specimen (No. 060905-004) was deposited with the Herbarium of the Chengdu Institute of Biology, Chinese Academy of Sciences (P. R. China).

Extraction and Isolation of Cyclopeptide. The powdered and air-dried whole plants of *R. giganteum* (7.0 kg) were extracted with 95% EtOH at r.t. The syrup was suspended in H_2O, and extracted successively with petroleum ether (PE), AcOEt, and BuOH. The BuOH-soluble fraction was subjected to CC (*MCI* gel; 90% MeOH/H_2O), resulting in six fractions, *Frs. 1–6*. *Fr. 5* was submitted to CC (SiO_2; $CHCl_3$/MeOH with increasing polarity) to afford four fractions, *Frs. A–D*. *Fr. C* was futher seperated by CC (*ODS-A*; 70% MeOH/H_2O), followed by repeated CC (*Sephadex LH-20*; MeOH) to afford *rhopeptin A* (21 mg).

Data of Rhopeptin A. Amorphous powder. Ninhydrin reaction { – }. IR (KBr) 3396, 1747, 1694, 1676, 1650, 1635, 1523, 1506, 1441, 1327, 1226, 1183. ^{1}H- and ^{13}C-NMR: see the *Table*.

Amino Acid Analysis of Rhopeptin A. Hydrolysis of rhopeptin A (5 mg) was performed in a sealed tube at 110° with 6N HCl for 20 h, and amino analysis of its hydrolysates indicated the presence of Pro, Phe, and Glu residues (*Hitachi 835–50* Amino Acid Analyzer, Japan).

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