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단 신

Scolopendra Subspinipes Multilans L. Koch 로부터 정제된 신규 항균물질의 구조 결정

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Structural Characterization of a New Antibiotic Substance Purified from *Scolopendra Subspinipes Multilans* L. Koch

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INTRODUCTION

Scolopendra subspinipes multilans L. Koch has been used as a traditional medicine for many clinical purposes in Korea. Dried centipedes in combination with various herbal materials are usually used for sudden heart attack, toxicide, anticancer drug, and convolusions in the ancient oriental medicine[1-5]. As a folk clinic, the petroleum or oil extract of centipedes has been widely used for the infected or wounded part of body[1]. In spite of clinical usages of centipede and great attention in folk medicine, the systematic chemical investigation of its clinical effects is still lacking, and this has led us to establish the methods of extraction and purification, and to identify chemical structure of bioactive substance from centipedes.

EXPERIMENTAL

Purification of antibiotic substance. Centipedes were purchased from the farmers market at Kumsan, Chungchungbuk-Do, and Moran market at Sungnam City. The fresh and dried centipedes (112 mm × 0.7 g) were used after cutting its head and legs, and 200 g of dried centipedes were soaked in diethyl ether and stirred for 1 day at room temperature. Crude extract was filtered and concentrated up to 10 mL of final aliquot under N_2 gaseous condition, and subsequently subjected to column chromatography as follows.

First, silicic acid (Sigma, SIL-A-200) equilibrated with petroleum ether was packed in the column (2 × 9 cm), and 1 mL of diethyl ether extract was loaded into the column. The column was washed with 30 mL of petroleum ether and 20 mL of 25% diethyl ether mixed with petroleum ether, and the flow rate of 1.0 mL/min was maintained by vaccum regulator(Bio-rad, CA94547)[6]. Subsequent elution with pure diethyl ether gave rise to the fraction exhibiting antibiotic activity against *Klebsiella pneumoniae* ATCC 8308. The active antibiotic fraction examined by disk test was evaporated with N_2 gas and dissolved in 1 mL of Tris-HCl buffer, pH 7.4.

Second, cation exchange chromatography was carried out with Bio-Rad Econo system and high S cationic catridge (Bio-Rad, 732-0068) equilibrated with Tris-HCl buffer, pH 7.4[7-8]. One mL of active fraction from silicic acid column was loaded into the column, and antibiotic substance was eluted with Tris-HCl buffer, pH 7.4 at the flow rate of 1.0 mL/min. Antibiotic fractions were collected at 254

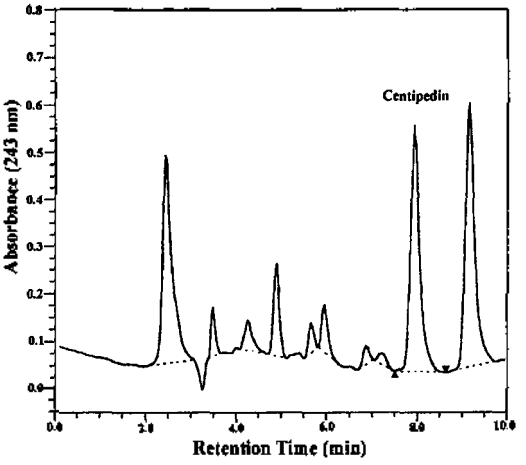

Fig. 1. Reverse-phase HPLC Chromatography. The fraction exhibiting antibiotic activity against *Klebsiella pneumoniae* ATCC 8308 was obtained at retention time 8 minutes.

nm, and active substance was extracted with pure diethyl ether. After evaporation under N_2 gas, antibiotic substance was dissolved in 30% of CH_3CN/H_2O and injected into the C-18 Capcell Pak HPLC column (4.6×250 mm, Shiseido) by using Waters 626LC pump and 486 TAD detector. The flow rate of the HPLC column was 1.0 mL/min, and absorbance was detected at 430 nm as shown in *Fig.* 1.

Characteristics of antibiotic substance. A new substance, Centipedin, exhibits the most significant antibiotic activity against gram-negative *Klebsiella pneumoniae* ATCC 8308 responsible for causing the lung and intestine infection. Two μg/mL of the minimal inhibitory concentration (MIC) value against *Klebsiella pneumoniae* ATCC 8308 was obtained. Structural characterizations were made by qualitative chemical indentification and other spectroscopic methods including UV, IR, Mass, and NMR. Highly purified Centipedin was also identified by thin layer chromatography plate prepared with silica gel 60 F_{254} (Merck). In this experiment, the first developing solvent was the mixture of chloroform : methanol : water (65:25:4, v/v) followed by petroleum ether : diethyl ether : 1-propanol (1:9:1, v/v) as the second developing solvent. Ninhydrin spray reagents prepared with 95 mL of 0.2% ninhydrin/n-butanol solution and 5 mL of 5% acetic acid/H_2O solution was applied to this TLC plate for the identification of amine group. In addition, Dragendorff test for this antibiotic and standard compounds including 8-hydroxyquinoline, picoline, pyridine was carried out to confirm if the substance has aromatic nitrogen. There was no color appeared on this Centipedin whereas other standard compounds exhibit redish color. Ninhydrin and Dragendorff test showed that there is no primary amine and aromatic nitrogen in the Centipedin. The pale yellowish solid powder, Centipedin has melting point of 122 °C and molecular weight (M^+, m/z=162.1) identified by mass spectrometric analysis.

Spectroscopic characterization. IR and NMR experiments were carried out for structural elucidation of Centipedin. A KBr pellet was made with 10 μg of highly purified Centipedin and 20 mg of KBr. IR spectrum recorded with Bio-Rad FTS 6000 has absorptions at 3471.2, 1705.3, 1630.2, 1364.4, 1260.1, 1092.1, and 737 cm^{-1}. Strong absorptions at 3471.2, and 1705.3 cm^{-1} exhibit that molecule has hydroxyl group and carbonyl group, respectively. NMR experiments were carried out by using Varian Unity 500 MHz and Mercury 300 MHz spectrometers under D_2O solvent at 22 °C. Ethanol was used as an external reference for the ^{13}C-NMR signal assignments. Five aromatic ^{1}H-NMR peaks were appeared at 8.49(H3, doublet), 7.62(H4, doublet), 7.43(H6, triplet), 7.33(H5, doublet), 7.06(H7, doublet) ppm with same integration value. A scalar coupling between 8.48 ppm and 7.61 ppm, and two scalar couplings from 7.43 ppm to 7.33 ppm and 7.06 ppm were observed in COSY experiment (*Fig.* 2). There were 9 signals observed to be 157.46(C1), 151.1(C8), 141.4(C3), 132.5(C9), 130.6(C10), 128.2(C6), 118.4(C4), 118.0 (C5), 109.4(C7) ppm in ^{13}C-NMR spectrum. Correlations between methine proton and carbon were identified with HMQC and DEPT spectrum. HMQC spectrum[9] shown in *Fig.* 3 gave proton-carbon one bond direct coupling with ^{1}H- and ^{13}C-NMR chemical shifts. A cross peak exhibits that a proton appeared at 7.06 ppm is attached to a carbon appeared at 109.4 ppm, and the rest of ^{1}H-

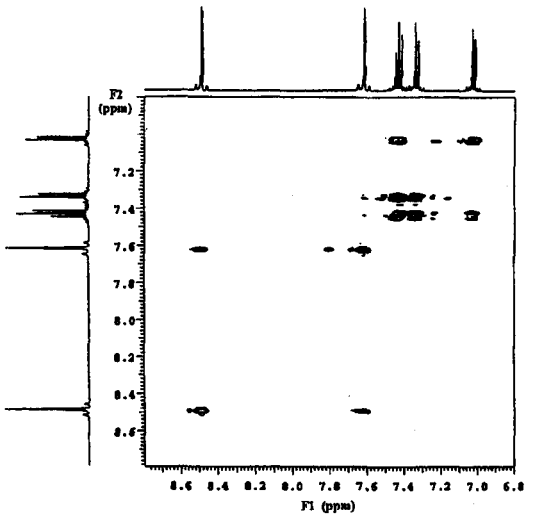

Fig. 2. A portion of COSY spectrum showing scalar connectivity. The direct coupling from two doublets (8.48 ppm, 7.61 ppm) and a direct coupling from triplet (7.43 ppm) to two doublets (7.33 ppm, 7.06 ppm) are shown.

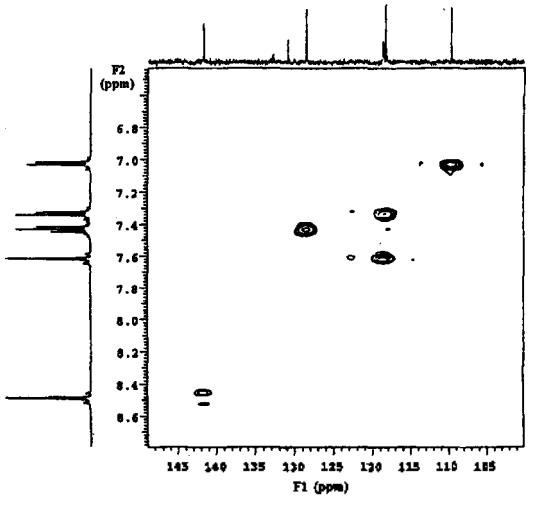

Fig. 3. A portion of HMQC spectrum showing proton-carbon one bond direct coupling. A cross peak exhibits that proton (^{1}H-NMR, 7.06 ppm) is attached to a carbon (^{13}C-NMR, 109.4 ppm).

and ^{13}C-NMR signal assignments were made for the directly coupled CH systems. A DEPT spectrum[10] giving carbon signals at 141.4, 128.2, 118.4, 118.0, and 109.4 ppm confirmed that all of these carbons couple with methine proton. Two bond (^{2}J) and three bond (^{3}J) away proton-carbon correlations were obtained in the HMBC spectrum. For example, correlations from H4 to three carbon atoms (C3, C9, C5), from H6 to two carbon atoms (C9, C10), from H5 to three carbon atoms (C9, C4, C7), and from H7 to two carbon atoms (C10, C4) could be assigned. In this manner, NMR signal assignments for four quarternary carbons and correlations with methine protons could be obtained, and subsequently these covalent linkages from HMBC spectrum enabled to confirm the ring system. By summarizing the physico-chemical measurements in combination with other various spectroscopic results of Mass, IR, and NMR data, the chemical structure could be accomplished to be 8-hydroxy-1H-2-benzopyran-1-one (*Fig.* 4).

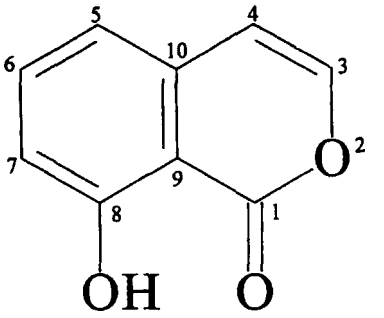

Fig. 4. A proposed chemical structure of a new antibiotic substance, Centipedin, was isolated from *Scolopendra subspinipes multilans* L. Koch.

RESULTS AND DISCUSSION

A new antibiotic substance was purified from *Scolopendra subspinipes multilans* L. Koch by using column chromatography and reverse-phase HPLC, and structural characterizations were made by various spectroscopic methods. The minimal inhibitory concentrations against broad microorganisms were measured in terms of the activity to inhibit microbial growth in liquid medium (*Table* 1). The most significant antibiotic activity was obtained against gram-negative *Klebsiella pneumoniae* ATCC 8308 responsible for causing the lung and intestine infection. Two μg/mL of the minimal inhibitory concentration value against *Klebsiella pne-*

Table 1. Minimal inhibitory concentrations (μg/mL) of Centipedin against representative laboratory strains

	Microorganism	MIC (μg/mL)
Gram⁻ Bacteria	*Klebsiella pneumoniae* ATCC 8308	2
	Vibrio cholerae (Ogawa strain)	64
	Proteus vulgaris NRRL B-123	16
	Pseudomonas aeroginosa ATCC 27853	128
	Escherichia coli ATCC 25922	256
Gram⁺ Bacteria	*Bacillus subtilis* ATCC 6633	32
	Micrococcus leuteus ATCC 9341	256
	Staphylococcus aureus ATCC 6538P	32
	Staphylococcus epidermidis ATCC 12228	128
Fungi	*Candida albicans* ATCC 10231	128
	Candida utilis ATCC 9255	-[a]
	Aspergillus niger ATCC 9642	-[a]

[a]-; Inhibition was not found up to 256 μg/mL of Centipedin.

umoniae ATCC 8308 was obtained. This antibiotic substance, 8-hydroxy-1H-2-benzopyran-1-one, showed a maximum UV absorption spectra at 243 nm and melting point of 122 °C. The compound exhibiting pale yellowish color at solid state was determined to have a molecular weight 162.1 by mass spectrometric analysis. In addition, ^{1}H- and ^{13}C-NMR, DEPT, 2D-NMR techniques including COSY, HMQC, HMBC enabled to establish the covalent linkage of this compound. Detailed methods of isolation, purification and results on antibiotic spectrum will be published elsewhere.

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REFERENCES

1. MBC, *The Encyclopedia of Korean Folk Medicine*; Kumpark Publishing Co: Seoul, Korea, 1987; p 240.
2. Mohamed, A. H.; Zaid, E.; El-Beih N. M.; Abd El-Aal, A. *Toxicol.* **1980**, *18*, 581.
3. Gomes, A.; Datta, A.; Sarange, B.; Kar, P. K.; Lahiri, S. C. *Indian J. Exp. Biol.* **1983**, *21*, 203.
4. Kosuge, T.; Yokota, M.; Sugiyama, K.; Yamamoto, T. *J. Pharm. Soc. Jpn.* **1985**, *105*, 791.
5. Bensky, D.; Gamble, A. *Chinese Herbal Medicine*; Eastland Press: Seattle, U.S.A., 1986; p 612.
6. Sweeley, C. C. *Methods in Enzymology*; Spinger-Verlag: New York, U.S.A., 1969; Vol. 14, p 255.
7. Scopes, R. K. *Protein purification, principles and practice*, 2nd Ed.; Spinger-Verlag: New York., 1987; p 30.
8. Harris, E. L. V.; Angal, S. *Protein Purification Methods, A Practical Approach*; IRL Press: Oxford, 1989; p 25.
9. Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093.
10. Bendall, M. R.; Doddrell, D. M.; Pegg, D. T. *J. Am. Chem. Soc.* **1981**, *103*, 4603.