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Mechanism of Antibiotic Action and Biosynthesis of Centipedin Purified from *Scolopendra subspinipes multilans* L. Koch (Centipede)

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The 8-hydroxyisocoumarin, named Centipedin, which has a significant antibiotic activity, was separated and solubilized with organic solvents, such as diethyl ether from centipede *Scolopendra subspinipes multilans* L. Koch. The Centipedin was purified by silicic acid column and high S cation exchange chromatography followed by reverse-phase HPLC. It was confirmed that Centipedin has a potent antibiotic effectiveness against Gram-negative *Klebsiella pneumoniae* ATCC 8308. The results showed that Centipedin blocks both DNA replication and RNA transcription during the growth of this pathogen *in vivo*. The biosynthesis of antibiotic 8-hydroxyisocoumarin was studied *in vivo* by feeding [^{14}C]-labelled compound as a precursor to live centipede, in which [^{14}C]acetate was the most efficiently incorporated into the Centipedin within 30 h after injection. Also, *in vitro* study on the biosynthesis of Centipedin showed that efficient incorporation of [^{14}C]acetate occurred at pH range 5.0–7.0 for 10 h incubation and decreased significantly after then. It is suggested that 8-hydroxyisocoumarin is one of the defense compounds acting on bacterial infection in *Scolopendra subspinipes*.

Keywords: Antibiotic mechanism, Biosynthesis of Centipedin, 8-Hydroxyisocoumarin.

Introduction

It has been well known that a centipede bite is toxic and causes intensive pain, oedema, necrosis, and sometimes even death (Venzmer, 1969; Faust, 1978). Various enzymes and histamine have been reported to be present in the venoms of centipede *Scolopendras* (Zaid, 1958; Freyvogel, 1972). Welsh and Batty (1963) found that venom of the poisonous Brazilian centipede *Scolopendra viridicornis* contained serotonin, 5-hydroxytryptamine, which was known to induce the release of epinephrine from the adrenal medulla (Reid, 1952; Hagen, 1959). The hyperglycaemia, simultaneously with liver and muscle glycogenolysis, was known to be mediated through 5-hydroxytryptamine from *Scolopendra* extract (Leonard and Day, 1960; Levine *et al.*, 1964; Mohamed *et al.*, 1980). The lethal protein toxin from *Scolopendra subspinipes* was characterized as a 60 kDa protein that produces a cardiac arrest and raises blood pressure by a vasoconstriction effect (Gomes *et al.*, 1983). The hemolymph of *Scolopendra cingulata* was tested for the existance of antibacterial substances using the Petri dish test, and its inhibition of growth of some Gram-negative and Gram-positive bacteria was found (Xylander and Nevermann, 1990). The defensive secretions from insects have yielded a diversity of small organic compounds. The defensive secretions of tenebrionid beetles consist most commonly of 1,4-benzoquinones with or without the admixture of 1-alkenes (Tschinkel, 1975).

The present investigation is the first case demonstrating an antibacterial substance, 8-hydroxyisocoumarin (Centipedin), from centipede *Scolopendra subspinipes*. Some of the physicochemical and pharmacological properties of Centipedin was demonstrated in order to understand the applicability to use as antibiotics in vertebrates.

Materials and Methods

The purification of Centipedin and identification of its molecular structure Crude Centipedin extract was obtained with diethyl ether after cutting pieces of centipede *Scolopendra*

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subspinipes. Further purification was performed through silicic acid column, high S cation exchange chrmotography and finally with reverse-phase HPLC (Fig. 1). The structure of antibiotic Centipedin was confirmed as 8-hydroxyisocoumarin (called Centipedin) determined by IR, mass spectroscopy, one-dimensional [^{1}H/^{13}C]-NMR and two-dimensional NMR techniques (Kim *et al.*, 1998b) (Fig. 2).

The effect of Centipedin on RNA synthesis in intact bacteria To study the effect of antibiotic Centipedin purified from centipede *Scolopendra subspinipes multilans* L. Koch on RNA synthesis in Gram-negative *Klebsiella pneumoniae* ATCC 8380 strain, radioactively labelled [α-^{32}P]UTP was added to the growing culture followed by addition of highly purified antibiotic Centipedin (Irschik *et al.*, 1995; Stella *et al.*, 1995). *Klebsiella pneumoniae* was grown at 37°C in nutrient broth (Difco, Detroit, USA) containing 0.3% of beef extract and 0.5% of peptone adjusted to pH 6.8. [α-^{32}P]UTP (5 mCi/l) was added to the 3 ml of culture broth at 0.26 OD_{600} and incorporated into *Klebsiella pneumoniae* for 20 min. Then, 60 μg of rifamycin as a positive control and 60 μg of Centipedin was added in 1 ml of the above culture broth, respectively. One ml of the other culture broth was used as a negative control under the same condition. At various times, up to 60 min, 0.1 ml of each sample was collected and added to 2 ml of ice-cold 5% TCA and filtered on glass fiber (Whatman GF/B, Kent, UK). Each glass fiber was placed in 10 ml of cocktail (Packard, Meriden, USA) and [^{32}P]UMP incorporation counted in a liquid scintillation counter (Packard, Tri-Carb 4000) (Park *et al.*, 1981).

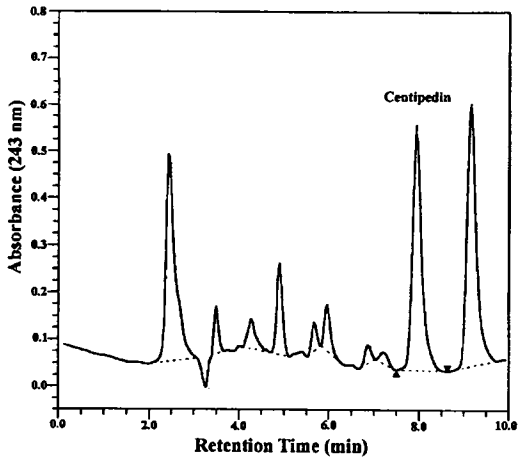

Fig. 1. Reverse-phase HPLC chromatography. The active fraction from high S cation exchange chromatography was applied to a Capcell Pak C18 HPLC column. Fractions were collected at a flow rate of 1.0 ml/min with 30% CH_3CN/H_2O. Retention time of Centipedin which has antibiotic activity against Gram-negative and Gram-positive bacteria, was about 8 min.

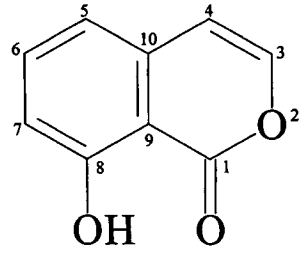

Fig. 2. Molecular structure of antibiotic Centipedin identified as 8-hydroxyisocoumarin.

The effect of Centipedin on DNA synthesis in intact bacteria *Klebsiella pneumoniae* ATCC 8308 was grown at 37°C in nutrient broth (Difco) containing 0.3% beef extract and 0.5% peptone adjusted to pH 6.8. [α-^{32}P]TTP (5 mCi/l) was added to the 3 ml of culture broth at 0.26 OD_{600} and incorporated into *Klebsiella pneumoniae* for 20 min. After 20 min of incorporation, culture broth was further divided into three parts: 1 ml of untreated culture broth as a negative control, 1 ml of culture broth treated with 80 μg of nalidixic acid as a positive control, and 1 ml of culture broth treated with 60 μg of Centipedin. At various times, up to 60 min, 0.1 ml of each sample was collected and added to 2 ml of ice-cold 5% TCA and filtered on glass fiber (Whatman, GF/B). Each glass fiber was placed in 10 ml of cocktail (Packard) and [^{32}P]TMP incorporation counted in a liquid scintillation counter (Packard, Tri-Carb 4000) (Park *et al.*, 1981).

Biosynthesis of Centipedin *in vivo* The biosynthesis of Centipedin, 8-hydroxyisocoumarin, was studied *in vivo* by feeding [^{14}C]-labelled compounds as a predictable precursor to live centipede *Scolopendra subspinipes multilans* L. Koch (Kumagai, 1994; Furiutami *et al.*, 1977). The live centipede was grown on soil that was put in a jar and covered with gauze in a dark place and fed boiled chicken. 5×10^5 cpm of [^{14}C]acetate, [^{14}C]alanine, and [^{14}C]tyrosine dissolved in sterile saline was injected into the body of centipedes, respectively. Each group was composed of 10 centipedes. At various times, up to 40 h, 2 centipedes of each bioreaction were collected and sliced to purify [^{14}C]Centipedin. [^{14}C]Centipedin was extracted with 10 ml of diethyl ether in a shaker for 24 h and then filtered and evaporated to 1 ml with N_2 gas at 37°C. [^{14}C]Centipedin was isolated by two-dimensional thin layer chromatography: one-dimensional developing solvent of petroleum ether:diethyl ether:1-propanol(1:9:1, v/v/v) and two-dimensional developing solvent of chloroform:methanol:water (65:25:4, v/v/v). [^{14}C]Centipedin, which has a R_f value of 0.66 on the TLC plate, was raked out and placed in 10 ml of cocktail and the radioactivity counted in a liquid scintillation counter (Park *et al.*, 1981).

Biosynthesis of Centipedin *in vitro* The biosynthesis of Centipedin was studied *in vitro* by adding [^{14}C]-labelled compound as a predictable precursor to the homogenized centipede *Scolopendra subspinipes multilans* L. Koch at different pH levels. Live centipede was sliced and homogenized in citrate buffer (pH 5.0), HEPES buffer (pH 7.0), or glycine buffer (pH 9.0). Each group was composed of 10 centipedes in 100 ml of each buffer and divided into five parts to detect [^{14}C]Centipedin at various times. [^{14}C]acetate, [^{14}C]alanine and [^{14}C]tyrosine with 1×10^6 cpm as predictable precursors dissolved in sterile saline, was added to each 20 ml of reaction mixture and incubated at 37°C. At various times, up to 30 h, each sample was mixed with 20 ml of diethyl ether, shaked vigorously and centrifuged at 3000 $\times$ *g* for 30 min. Diethyl ether extract was evaporated, and [^{14}C]Centipedin was isolated by two-dimensional TLC as described above.

Results and Discussion

The identification of the molecular structure of Centipedin as 8-hydroxyisocoumarin Centipedin is the first antibiotic identified in the centipede *Scolopendra subspinipes* and its structure is 8-hydroxyisocoumarin (Kim *et al.*, 1998b). Many efforts to identify the amino group, visinal hydroxyl group, phosphorus, and halogen had failed and these results indicate that no such groups are present as the molecular components. 8-hydroxyisocoumarin was confirmed as the structure of Centipedin by the following; identification of the hydroxyl group, carbonyl group, and aromatic ring was from IR spectroscopy, measurement of the molecular weight of 162.1 Da was from mass spectroscopy, and elucidation of the backbone structure was from one-dimensional [$^{1}H/^{13}C$]-NMR and two-dimensional NMR techniques, as shown in Fig. 2.

It has been known that venoms of centipede *Scolopendra* consist of many biologically active enzymes, histamine and serotonin were toxic in various biological activities (Reid, 1952; Zaid, 1958; Hagen, 1959; Welch and Batty, 1963; Freyvogel, 1972). Some protein toxin was also known to severely affect vasoconstriction (Gomes *et al.*, 1983) and another report using a Petri dish test showed that a hemolymph of *Scolopendra* contained antibacterial substances (Xylander and Nevermann, 1990). However, this is the first report in which the antibacterial substance, Centipedin (8-hydroxyisocoumarin), was identified and its molecular structure in centipede *Scolopendra subspinipes* determined.

The effect of Centipedin on RNA and DNA synthesis in intact bacteria A broad spectrum of antibiotic action was already studied with antibiotic Centipedin, 8-hydroxyisocoumarin, purified from centipede *Scolopendra subspinipes multilans* L. Koch against Gram-positive and Gram-negative bacteria and fungi (Kim *et al.*, 1998a). A significant antibiotic activity of Centipedin was obtained against Gram-negative *Klebsiella pneumoniae*. In addition, it had been shown that Centipedin blocked prokaryotic RNA transcription and a little of DNA replication *in vitro*.

To uncover the antibiotic's mechanism of action, a series of experiments can be carried out whose results will give information at three different levels (Gale *et al.*, 1981; Kerridge, 1986): in the intact cell, in a partially purified cell-free system, and in one or more purified enzyme systems. At the molecular level, antibiotics can inhibit cell growth by interfering with essential metabolic processes. To study the activity of antibiotic Centipedin in intact bacteria, a very useful procedure was used by adding radiolabelled RNA and DNA precursor followed by Centipedin to growing culture broth of *Klebsiella pneumonia* ATCC 8308 and observing RNA and DNA synthesis, respectively.

The effect of Centipedin on RNA synthesis in intact *Klebsiella pneumoniae* ATCC 8308 has been shown in Fig. 3. A known antibiotic, rifamycin (Sensi and Lancini, 1990), as a positive control, which inhibits specifically RNA polymerase, was used to compare with Centipedin at the same condition. In a negative control without Centipedin, the radioactivity of [α-^{32}P]UTP incorporated into the corresponding RNA molecule by *Klebsiella pneumoniae* ATCC 8308 could be an index of the rate of RNA synthesis. After 20 min of inhibition reaction by the antibiotic Centipedin, the activity of RNA synthesis was decreased for 40 min, almost the same as that of rifamycin. Stopping the synthesis of the macromolecule essential for cell growth affects all other cellular functions. Therefore, we observed the time course of the inhibition reaction to establish the primary effect which must be seen earlier (Franklin and Snow, 1989). These results indicate that the metabolic pathway of RNA synthesis is affected by the antibiotic Centipedin purified from centipede *Scolopendra subspinipes multilans* L. Koch.

The effect of Centipedin on DNA synthesis in intact *Klebsiella pneumoniae* ATCC 8308 has been shown in Fig. 4. [α-^{32}P]TTP was used to follow the synthesis of DNA in the intact cell. A known antibiotic, nalidixic acid, which specifically inhibits DNA synthesis, was used as a positive control (Hertzberg, 1990). After 20 min of the inhibition reaction by Centipedin, the activity of DNA synthesis was decreased compared with that of nalidixic acid. As in the results obtained from *in vitro* study using prokaryotic RNA and DNA polymerases, the *in vivo* study showed that the antibiotic action with Centipedin purified

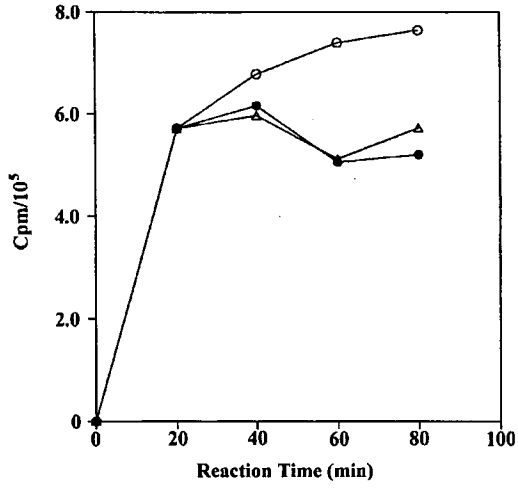

Fig. 3. The effect of Centipedin on RNA synthesis in *Klebsiella pneumoniae*. *Klebsiella pneumoniae* ATCC 8308 was grown at 37°C in nutrient broth. After 20 min of reaction treated with [α-^{32}P]UTP (5 mCi/l), 60 μg of antibiotic Centipedin (△—△) and 60 μg of rifamycin as a positive control (●—●) were added to 1 ml of each culture broth. The cells were collected at various times with control (○—○) and filtered on the glass fiber, Whatman GF/B. Radioactivity was measured in a liquid scintillation counter (Packard, Tri-Carb 4,000).

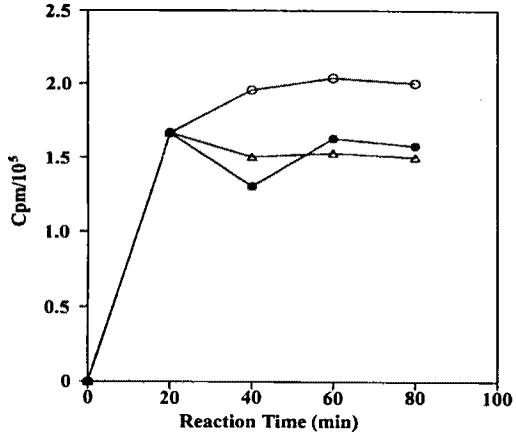

Fig. 4. The effect of Centipedin on DNA synthesis in *Klebsiella pneumoniae*. *Klebsiella pneumoniae* ATCC 8308 was grown at 37°C in nutrient broth. After 20 min of reaction treated with [α-^{32}P]TTP (5 mCi/l), 60 μg of antibiotic Centipedin (△—△) and 80 μg of nalidixic acid as a positive control (●—●) were added to 1 ml of each culture broth. The cells were collected at various times with control (○—○) and filtered on the glass fiber, Whatman GF/B. Radioactivity was measured in a liquid scintillation counter (Packard, Tri-Carb 4,000).

from centipede *Scolopendra subspinipes multilans* L. Koch accompanied blocking of RNA and DNA synthesis in intact bacteria.

Biosynthesis of Centipedin *in vivo* and *in vitro* To examine the precursor of Centipedin, 8-hydroxyisocoumarin, radiolabelled compounds such as [^{14}C]acetate, [^{14}C]alanine and [^{14}C]tyrosine were injected into the body of live centipede *Scolopendra subspinipes multilans* L. Koch, respectively. As shown in Fig. 5, by feeding [^{14}C]acetate *in vivo*, the maximum incorporation ratio of 0.27% into 8-hydroxyisocoumarin was obtained after 30 h. Radiolabelled amino acids such as tyrosine and alanine were not incorporated into 8-hydroxyisocoumarin. From the decreasing incorporation ratio activity of [^{14}C]acetate from 30 h of bioreaction, it can be assumed that biosynthesized 8-hydroxyisocoumarin is further metabolized by the catabolic pathway. *In vitro* study on the incorporation of [^{14}C]acetate into 8-hydroxyisocoumarin at different pH levels showed 0.16% of maximum incorporation ratio activity at pH 7.0 after 10 h of reaction (Fig. 6). The reaction *in vitro* at pH 9.0 rather than at pH 5.0 was not a good condition for biosynthesis of 8-hydroxyisocoumarin. From these results, we report that acetate is the precursor of the synthesis of Centipedin, 8-hydroxyisocoumarin in the body of centipede *Scolopendra subspinipes multilans* L. Koch.

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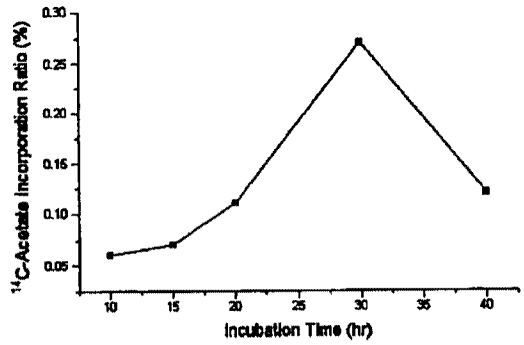

Fig. 5. Time course of [^{14}C]acetate incorporation into Centipedin *in vivo*. The biosynthesis of antibiotic Centipedin, 8-hydroxyisocoumarin, was studied by feeding [^{14}C]acetate, [^{14}C]alanine, and [^{14}C]tyrosine as predictable precursors to live *Scolopendra subspinipes multilans* L. Koch. [^{14}C]Centipedin was separated with diethyl ether followed by thin layer chromatography. Radiolabelled [^{14}C]Centipedin was counted by a liquid scintillation counter.

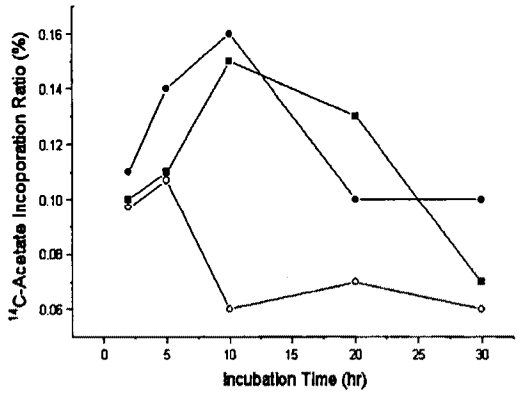

Fig. 6. Time course of [^{14}C]acetate incorporation into Centipedin *in vitro*. The biosynthesis of antibiotic Centipedin was studied with [^{14}C]acetate as a precursor in the homogenates of *Scolopendra subspinipes multilans* L. Koch. At pH 5.0 (■—■), pH 7.0 (●—●), and pH 9.0 (○—○), each reaction mixture was incubated at 37°C, and [^{14}C]Centipedin was separated with diethyl ether followed by thin layer chromatography. Radiolabelled [^{14}C]Centipedin was counted by a liquid scintillation counter.

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