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Mosquitocidal activity of anthraquinones isolated from symbiotic bacteria *Photorhabdus* of entomopathogenic nematode

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ABSTRACT

Two anthraquinones were isolated from the symbiotic bacteria *Photorhabdus temperata* of entomopathogenic nematodes *Heterorhabditis* spp. by repeated column chromatography. They were abundantly present in the culture medium and identified as 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone and 3-methoxychrysazine by spectral analysis. The isolated anthraquinones were highly lethal to larvae of *Culex pipiens pallens*. Our results suggest that anthraquinones might be useful as biopesticides for the biological control of mosquitoes.

Introduction

Mosquitoes are a serious pest and act as vectors for the transmission of various pathogens to livestock as well as humans. Despite the use of various pesticides and techniques to control mosquitoes, the spread of parasitic diseases to humans and animals is still a major health problem worldwide. Concerns over resistance development and environmental contamination due to the overuse of synthetic pesticides have prompted interest into alternative pest management strategies. As a result, researches have recently intensified the development of biological control techniques against mosquitoes using insect pathogens and natural enemies (Priest, 1992; Porter, 1996; Kumar et al., 2012).

The entomopathogenic nematodes *Heterorhabditis* and *Steinernema* are able to kill insects due to insecticidal compounds produced by their symbiotic bacteria, *Photorhabdus* and *Xenorhabdus*, respectively. After penetrating the bodies of insects through openings such as the mouth, anus, and spiracles, nematodes regurgitate symbiotic bacteria, which are housed in their intestine, directly into the hemocoel (Ciche and Ensign, 2003). These bacteria rapidly multiply and produce a range of proteins and metabolites that can kill the host insect within 24–48 h. Following this, nematodes multiply by ingesting the insect cadaver (Bowen et al., 1998; ffrench-Constant et al., 2003).

Numerous toxic factors have been identified from the symbionts of entomopathogenic nematodes. Many of these factors are pathogenic upon either injection into the hemolymph or oral ingestion (Bowen and Ensign, 1998; ffrench-Constant et al., 2007). Among them, the most well-known toxin is 'toxin complex' (Tc) from *P. luminescens*, which consists of at least 10 polypeptides grouped into three functional classes (Bowen et al., 1998). '*Photorhabdus* insect-related' (Pir) toxins consist of the binary toxins PirAB (Blackburn et al., 1998). Both Tc and Pir toxins are pathogenic upon both oral administration and hemolymph injection into insects. Other toxins include 'make caterpillars floppy' (Mcf) as well as proteins encoded by '*Photorhabdus* virulence cassettes' (PVCs), which only show injectable activity into the hemolymph (Daborn et al., 2001). In addition, symbiotic bacteria produce various secondary metabolites, such as antibiotics and pigment molecules, which are either insecticidal or protective against competing microorganisms (ffrench-Constant et al., 2007; Piel, 2009). For example, *X. szentirmaii* produces novel xenofuranones A and B (Brachmann et al., 2006), *X. nematophila* synthesizes benzylideneacetone (Ji et al., 2004), and *P. luminescens* produces the antibiotic 3,5-dihydroxy-4-isopropylstilbene, which has strong antifungal activity and inhibits the phenol oxidase of *Manduca sexta* (Richardson et al., 1988; Eleftherianos et al., 2007). Further, several anthraquinone pigments have been identified from symbiotic bacteria. Seven anthraquinone derivatives are described from various strains of *P. luminescens* and are specifically biosynthesized by a type II polyketide synthase (Brachmann et al., 2007).

Here, we isolated two anthraquinone derivatives from *P. temperata* and detected mosquitocidal activities. Our results suggest that

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anthraquinones might be useful as biopesticides for the biological control of mosquitoes.

Materials and methods

Mass culture of symbiotic bacteria

Entomopathogenic nematodes *Heterorhabditis* spp. were collected from soil in Kandong, Korea. Symbiotic bacteria were isolated from the hemolymph of *Galleria mellonella* larvae infected by nematodes. Isolated bacteria were streaked onto NBTA medium agar supplemented with 25 mg of bromothymol blue and 40 mg of triphenyl-2,3,5-tetrazolium chloride per liter. The resulting single-colony *P. temperata* strain was designated as Pt-Kandong. A base population of the strain (1×10^7 cells/mL) was incubated in 250 mL shaker flasks containing 50 mL of TSY broth (40 g of tryptic soy broth and 5 g of yeast extract per liter) under conditions of 28 °C, 200 rpm, and darkness for 72 h. Mass production of *P. temperata* was conducted in a 7 L jar fermenter under conditions of 28 °C, 100 rpm, and 0.5 VVM.

Insects

The colony of *Culex pipiens pallens* was obtained from the Korea National Institute of Health and maintained under conditions of 25 ± 2 °C, 70 ± 5% relative humidity, and a 16 h light/8 h dark (16L:8D) photoperiodic cycle. Larvae were reared in a plastic box ($15 \times 15 \times 10$ cm^3) with masticated tropical fish flakes (Prodac, Cittadella, Italy) as an artificial diet.

Isolation and identification of active compounds

Culture broth (9 L) was successively partitioned with ethyl acetate (EtOAc) and concentrated in a rotary evaporator (EYELA, Tokyo, Japan). The EtOAc-soluble fraction (114.1 g) was chromatographed on a silica gel column (10 × 110 cm, $CHCl_3$:MeOH = 100:1 → 20:1), yielding five fractions (Fr. 1–Fr. 5). Fraction 2 (13.8 g) was rechromatographed through a silica gel column (8 × 100 cm, Hexane:EtOAc = 100:1 → 20:1), yielding 15 subfractions (Fr. 2-1 to Fr. 2-15). Compound 1 (140.0 mg) was obtained from Fr. 2-2, whereas compound 2 (80 mg) was isolated from Fr. 2-9-3 following the silica gel column chromatography (3.5 × 50 cm, $CHCl_3$:MeOH = 80:1 → 30:1) of Fr. 2-9. The chemical structures of compounds 1 and 2 were determined by nuclear magnetic resonance (NMR) analysis. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker Avance Digital 400 spectrometer (Karlsruhe, Germany) at 400 and 100 MHz, respectively. Chemical shifts were given in δ (ppm) from internal control tetramethylsilane (TMS). Compounds 1 and 2 were in good accordance with the NMR data from the references, and they were identified as 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone and 3-methoxychrysazine, respectively (Fournier et al., 1975; Zembower et al., 1992). The structures of the isolated compounds are shown in Fig. 2.

Compound 1 (1,3-dimethoxy-8-hydroxy-9,10-anthraquinone): ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.99 (3H, s), 4.03 (3H, s), 6.78 (1H, d, J = 2.5 Hz), 7.28 (1H, dd, J = 8 and 1.3 Hz), 7.45 (1H, d, J = 2.5 Hz), 7.59 (1H, t, J = 8 Hz), 7.74 (1H, dd, J = 8 and 1.3 Hz), 13.14 (1H, s). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 187.73, 182.66, 165.40, 162.42, 137.58, 135.34, 132.55, 124.93, 118.83, 116.62, 115.08, 104.66, 103.95, 56.62, 56.07.

Compound 2 (3-methoxychrysazine): ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.92 (3H, s), 6.86 (1H, d, J = 4 Hz), 7.23 (1H, d, J = 4 Hz), 7.32 (1H, dd, J = 8 Hz), 7.63 (1H, dd, J = 8 and 4 Hz), 7.69 (1H, t), 11.29 (1H, s), 13.29 (1H, s). ^{13}C NMR (400 MHz, DMSO-d_6) δ: 187.00, 182.57, 164.96, 163.91, 161.88, 137.17, 135.92, 132.68, 124.80, 118.49, 116.87, 113.06, 107.32, 105.34, 56.72.

Bioassays

The toxicity of *Photorhabdus* was tested by allowing ingestion of the bacterial culture broth and extracts after fractionation. *C. pipiens pallens* third instar larvae (n = 20) were reared in transparent plastic tubes and treated with either a bacterial culture medium or purified extracts in water. Water was used as a control. The mortality of the larvae was observed over 7 days under room temperature conditions. Each set of experiments was carried out three times on different dates under similar environmental conditions.

Statistical analysis

To identify the significant effects of the bacterial culture medium on the mortality of larvae, analysis of variance (ANOVA) and multiple mean comparisons were performed using the general linear model (GLM) of the Statistical Analysis System program (SAS, 2003) version 9.1. Differences among the mean values were determined using DMRT at $p \leq 0.05$. Data were analyzed by a completely randomized design with two and five replications.

Results and discussion

Mosquitocidal compounds from the culture broth of *P. temperata* were identified. Specifically, we identified two anthraquinone compounds that were both highly toxic to mosquito larvae. To measure the toxicities of purified extracts of *P. temperata* culture medium, the culture broth of *P. temperata* was separated into EtOAc and water fractions, as shown in Fig. 1. Each fraction was tested against third instar larvae of *C. pipiens pallens*. The original culture medium of *P. temperata* was slightly lethal to mosquitoes within 24 h (Table 1). However, when fractions were treated to mosquito larvae at concentrations from 0.1 to 10%, significant mortalities were induced by both the EtOAc and water fractions (Table 1). Mortality induced by the 0.1% EtOAc fraction was 85.3% after 24 h of treatment, whereas 1% and 10% EtOAc fractions were completely lethal within 18 h and 12 h, respectively. Thus, the EtOAc fraction may contain various compounds toxic to mosquitoes. Of note, moderate mortality (46.7%) was induced by the 10% water fraction, suggesting that *P. temperata* produces various toxic compounds that are soluble in either water or solvent.

The EtOAc layer was then further separated into five fractions (Fr. 1–5). As Fr. 1 was not soluble in 1% ethanol, we determined the mosquitocidal activities of the four other fractions (Fr. 2–5). When each fraction was treated to mosquito larvae, complete mortality was induced by all fractions within 18 h, with Fr. 2 showing the most rapid effect (Table 1). All mosquitoes were dead within 6 h of treatment with the 1% Fr. 2 solution. We further purified Fr. 2 by silica gel column chromatography in order to determine active substances. The resultant compounds 1 and 2 were identified as 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone and 3-methoxychrysazine, respectively, by ^{1}H and ^{13}C NMR. Each compound induced high mortality to mosquito larvae, and compound 2 was more toxic to mosquitoes than compound 1 (Table 2). However, our results showed that treatments at concentrations of 0.1% for these two pure compounds were not rapidly lethal to mosquito larvae. Compound 2 was completely lethal after 4 days of treatment, whereas the mortality of compound 1 was 86.7% after 5 days of treatment (Table 2). This suggests the presence of other toxic compounds in the culture medium of *P. temperata*.

Among the two identified anthraquinones from *P. temperata* in this study, 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone has been previously identified in *P. luminescens* (Richardson et al., 1988; Li et al., 1995), whereas 3-methoxychrysazine has previously been purified from the plant *Xyris semifuscata* (Fournier et al., 1975). In addition, other anthraquinone derivatives have been identified from the symbiotic bacteria of entomopathogenic nematodes. Specifically,