Unusual Cytotoxic Phenethylamides from *Xenorhabdus nematophilus*

Seunguk Paik,* Young Hwan Park, Seong Il Suh,† Hyun Su Kim,‡ In Sun Lee,‡ Myung Kwang Park, Chun Soo Lee, and Sun Ho Park

Faculty of Chemical & Materials Engineering, Keimyung University, Taegu 704-701, Korea
†Department of Microbiology, School of Medicine, Keimyung University, Taegu 704-701, Korea
‡Faculty of Applied Sciences, Keimyung University, Taegu 704-701, Korea
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Three simple carboxamides incorporating the phenethylamine moiety have been isolated from strain XR-NC of a symbiotic bacterium *Xenorhabdus nematophilus*. Their structures were identified by spectroscopic data and synthesis. The compounds exhibited significant cytotoxicities against human cancer-cell line, *viz*. the gastric adenocarcinoma, colon adenocarcinoma and lung adenocarcinoma.

Keywords : *Xenorhabdus nematophilus*, Phenethylamides, Xenorhabdins.

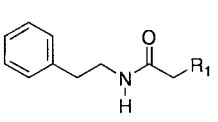
Introduction

Bacteria of the genus *Xenorhabdus* are known to be symbiotically associated with soil-dwelling, entomopathogenic nematodes of the genus *Steinernema*[1] and to produce several types of secondary metabolites exhibiting antibacterial and/or antifungal activities.[2] There have been previous reports of the isolation and characterization of antimicrobial metabolites including nematophins (indol derivatives[3]), xenorhabdins[4] (dithiolopyrrlones), hydroxystilbenes,[5] water-soluble xenocoumacins[6] (benzopyran-1-one derivatives) and anthraquinones[5] from cultures of the bacterial genus *Xenorhabdus*.

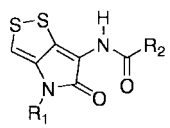
As part of our research on the development of bioinsecticides by using insect pathogenic nematodes, we also have investigated the secondary metabolites of five strains of *Xenorhabdus nematophilus* symbiotic to the nematodes of the genus *Steinernema*. Bioassay-guided isolation of antitumor agents among microbial metabolites from the XR-NC strain of *X. nematophilus* revealed unusual antitumor activities of carboxamides bearing phenethylamine moiety against human cancer cell lines. We report here the structure elucidation and antitumor activities of phenethylamide derivatives, which have been first isolated from natural sources[11] or *Xenorhabdus* species.

Results and Discussion

A symbiotic bacterium was isolated from entomopathogenic nematode *Steinernema glaseri* obtained from a soil sample collected at several places of the south Korea, and identified as *Xenorhabdus nematophilus* strain XR-NC.[7] The cell-free broth of the mass cultured *X. nematophilus* was extracted with ethyl acetate. The organic extract was subjected to flash chromatograph on C-18, and the fractions eluted with 50% and 75% MeOH were subjected to reverse-phase HPLC to afford three cytotoxic phenethylamides (**1-3**) with several xenorhabdin derivatives[4] (**4-6**) which were already reported in the literature.



1 $R_1 = C_6H_5$
2 $R_1 = CH_2CH_3$
3 $R_1 = CH(CH_3)_2$



4 $R_1 = CH_3$, $R_2 = CH_2CH_2CH_2CH_2CH_3$
5 $R_1 = CH_3$, $R_2 = CH_2CH_2CH_2CH(CH)_3$
6 $R_1 = H$, $R_2 = CH_2CH_2CH_2CH(CH)_3$

Figure 1

Compound **1** which was interestingly the major component of the organic extract was isolated as a white solid. The molecular formular ($C_{16}H_{17}NO$) of this compound was deduced from EIMS and NMR spectral data. The IR spectrum of **1** showed a strong absorption band at 1635 cm^{-1} which was characteristic of a carbonyl group of an amide. The ^1H NMR spectrum indicated the presence of phenyl rings (δ 7.31-7.18), two adjacent methylene signals (δ 3.45 and 2.72, J = 6.9 Hz), and a broad signal (δ 5.34, 1H) which could be assigned as an amide NH. Inspection of the ^{13}C Broad band and DEPT NMR spectra revealed the presence of a carbonyl carbon (δ 170.8), three methylenes (δ 35.7, 40.9, and 44.1), and 6 aromatic carbons which were shown to be two separate phenyl rings by COSY and HMBC spectra. The COSY, HMQC and HMBC spectra indicated 2-phenylethyl and phenylacetyl moieties, which were finally connected by an amide linkage by the correlation of the carbonyl carbon of phenylacetyl moiety with the terminal methylene protons of the 2-phenylethyl group. Therefore, the structure of **1** was established as *N*-phenethyl-2-phenylacetamide,[9] which was further confirmed by the synthesis of **1**.

Compound **2**, a minor component of the organic extracts, was isolated as a white solid. The molecular formular ($C_{12}H_{17}NO$) of this compound was deduced from EIMS and NMR spectral data. Spectral data of **2** were very similar to those of **1** in the region of the *N*-phenethylamide functionality; the ^1H NMR spectrum indicated the presence of a phenyl ring (δ 7.35-7.15), two adjacent methylene signals (δ 3.50 and 2.81, J = 7.2 Hz), and a broad signal of the NH at δ 5.66. The ^{13}C Broad band and DEPT NMR spectra revealed the presence of a carbonyl carbon (δ 173.0), four methylene car-

bons (δ 19.1, 35.6, 38.6, and 40.4), one methyl carbon (δ 13.7), and 6 aromatic carbons of a phenyl ring. The COSY, HMQC and HMBC spectra indicated a 2-phenylethyl moiety and a butyryl functionality which were finally connected by amide linkage by the correlation of the carbonyl carbon of the butyryl moiety with the terminal methylene protons of the 2-phenylethyl group. Therefore, the structure of **2** was established as *N*-phenethylbutyramide,[10] which was further confirmed by the synthesis of **2**.

Compound **3**, a minor component of organic extracts, was obtained as a white solid. The molecular formular ($C_{13}H_{19}NO$) of this compound was deduced from EIMS and NMR spectral data. Spectral data of **3** were also very similar to those of **2** in the region of the *N*-phenethylamide functionality; the ^{1}H NMR and COSY spectra suggested the presence of an ethylene unit (NH-CH_2-CH_2), an aliphatic isobutyl group and a broad NH signal of an amide at δ 5.53. The ^{13}C Broad band and DEPT NMR spectra revealed the presence of one carbonyl carbon (δ 172.4), one methine (δ 26.0), three methylenes (δ 35.7, 40.4 and 46.1), two methyl carbons (δ 22.4), and 6 aromatic carbons of a phenyl ring. Inspection of the COSY, HMQC and HMBC spectra indicated that a 2-phenylethyl moiety and an isovaleryl functionality were finally connected by an amide linkage by a correlation between a carbonyl carbon of the isovaleryl moiety with the terminal methylene protons of the 2-phenylethyl group. Hence, the structure of **3** was established as *N*-phenethyl isovaleramide,[11] which was further confirmed by the synthesis of **3**.

Bioassays of penethylamides **1**-**3** showed unusual cytotoxicities despite their simple structures against human cancer cell lines of the gastric adenocarcinoma (SNU668), cervical adenocarcinoma (HeLa), hepatoblastoma (HepG2), colon adenocarcinoma (HT-29) and lung adenocarcinoma (NCIH1703) (Table 1). Compound **1** exhibited much better activity than compounds **2** and **3**, and especially showed significant antitumor activity against the gastric adenocarcinoma cell line (SNU668) compared to etoposide as a reference.

Experimental Section

General Experimental Procedures. MS spectra (70 eV) were obtained with a Jeol JMS-700 instrument. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 and a Varian Unity plus-500 spectrometer using TMS as an internal reference. HPLC was conducted with a Rainin Dynamax SD-200 instrument equipped with a Rainin Dynamax UV-C detector. Analytical TLC was performed using Merck silica gel 60 PF_{254}.

Extraction and Isolation. The cell-free broth (3 L, pH = 8.2) of the cultured XR-NC strain of *X. nematophilus* was neutralized with conc. HCl and extracted with ethyl acetate (1 L × 3). After evaporation of the solvent, the crude extract (1.5 g) was flash chromatographed on a C_{18} column with 3 : 1, 1 : 1, 1 : 3 H_2O/MeOH mixtures, MeOH, and finally ethyl acetate. The 1 : 1 H_2O/MeOH fraction showed the strongest cytotoxicity against a panel of human tumor cell lines. The second and the third fractions were concentrated separately, and then subjected to HPLC (Dynamax C_{18}, 5μ, 21 × 250 mm, 8 mL/min; UV detection at 254 nm) using 65% MeOH as the eluent. The fractions containing compounds 1-3 were further purified by semipreparative reversed-phase HPLC (Dynamax C_{18}, 2μ, 10 × 250 mm, 5 mL/min; UV detection at 254 nm) using an isocratic system of 75% MeOH to afford phenylacetamide **1** (25 mg, t_R 5.53 min), butyramide **2** (10 mg, t_R 4.65 min), and isovaleramide **3** (*ca*. 3mg).

Bioassays. The MTT (3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide) assay was used with all the extracts, fractions, isolated pure and synthetic compounds as described in the literature.[8] Cytotoxicities to human cancer cell lines were evaluated by the independent measurement of IC_{50} values three times for gastric adenocarcinoma (SNU668), cervical adenocarcinoma (HeLa), hepatoblastoma (HepG2), colon adenocarcinoma (HT-29) and lung adenocarcinoma (NCIH1703) using etoposide as a reference.

***N*-Phenethyl-2-phenylacetamide (1)**: amorphous white solid; mp 93-94 °C {lit.[9] mp 92-3 °C}; IR (KBr) λ_{max} 3290, 1638, 1535 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.31-7.15 (8H, m), 7.03 (2H, d, *J* = 7 Hz), 5.34 (1H, brs, NH), 3.53 (2H, s, CH_2CO), 3.45 (2H, q, *J* = 6.9 Hz, $NHCH_2$), 2.72 (2H, t, *J* = 6.9 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.1, 138.9 (Ph C1'), 134.9 (Ph 1), 129.7 (Ph C2), 129.1 (Ph C3), 128.9 (Ph C2'), 128.7 (Ph C3') 127.5 (Ph C4), 126.6 (Ph C4'), 44.2 ($COCH_2$), 40.9 ($NHCH_2$), 35.7 (CH_2); EIMS (m/z, %) 240 (M^+, 19), 239 (64), 148 (14), 105 (62), 104 (59), 92 (100), 91 (83), 78 (8.4), 77 (8.4).

***N*-Phenethylbutyramide (2)**: amorphous white solid; mp 42-45 °C[10]; IR (KBr) λ_{max} 3295, 1636, 1540 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35-7.15 (5H, m), 5.64 (1H, brs, NH), 3.51 (2H, q, *J* = 7.2 Hz) 2.81 (2H, t, *J* = 6.8 Hz), 2.10 (2H, t, *J* = 7.2 Hz), 1.62 (2H, m), 0.91 (3H, t, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.0, 138.9, 128.7, 128.5, 126.4, 40.4 (CH_2), 38.6 (CH_2), 35.6 (CH_2), 19.1 (CH_2), 13.7 (CH_3); EIMS

Table 1. Cytotoxicities of compounds 1-3 and Etoposide (reference)

Compounds	IC_{50} (μg/mL)				
	HeLa[a]	SNU668[b]	HT-29[c]	NCIH1703[d]	HepG2[e]
1	125-250	31.3-62.5	62.5-125	62.5-125	125-250
2	250-500	62.5-125	250-500	250-500	250-500
3	125-250	125-250	125-250	125-250	125-250
Etoposide	125-250	62.5-125	125-250	31.3-62.5	125-250

[a]Cervical adenocarcinoma. [b]Gastric adenocarcinoma. [c]Colon adenocarcinoma. [d]Lung adenocarcinoma. [e]Hepatoblastoma.

(m/z, %) 191 (M^+, 71), 120 (8), 104 (100), 91 (37), 77 (20), 71 (55).

***N*-Phenethylisovaleramide (3)**: white powder; mp 67-70 °C; IR (KBr) λ_{max} 3250, 1638, 1540 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.32-7.18 (5H, m), 5.53 (1H, brs, NH), 3.52 (2H, q, J = 6.8 Hz) 2.81 (2H, t, J = 6.8 Hz), 2.07 (1H, m), 1.98 (2H, d, J = 7.2 Hz), 0.92 (6H, d, J = 6.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.4, 138.9, 128.7, 128.6, 126.4, 46.1 ($COCH_2$), 40.4 (CH_2NH), 35.7 ($\underline{CH_2}CH_2NH$), 26.0 (CH), 22.4 (CH_3); EIMS (m/z, %) 205 (M^+, 52), 104 (100), 91 (43), 85 (68), 77 (23), 57 (63).

Synthesis of 1. To a cooled (0 °C) solution of phenethylamine (6.3 mL, 50 mmol) and triethylamine (6.9 mL) in CH_2Cl_2 (30 mL) was added 6.6 mL of phenylacetyl chloride (50 mmol). The mixture was allowed to warm to room temperature and stirred for 4 h. The reaction mixture was poured into water (30 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were dried over Na_2SO_4, filtered, and concentrated *in vacuo* to afford 10.7 g (90%) of a yellowish solid, which was recrystallized from hexane and methylene chloride to yield **1** (9.5 g, 80%) as a white solid; ^1H and ^{13}C NMR data were identical to those of the natural product.

Synthesis of 2. To a cooled (0 °C) solution of phenethylamine (6.3 mL, 50 mmol) and triethylamine (6.9 mL) in CH_2Cl_2 (30 mL) was added 5.1 mL of butyryl chloride (50 mmol). The mixture was allowed to warm to room temperature and stirred for 4 h. The reaction mixture was poured into water (30 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting residue was chromatographed (30% EtOAc/hexane) to afford 7.6 g (80%) of **2** as a solid; ^1H and ^{13}C NMR data were identical to those of the natural product.

Synthesis of 3. To a cooled (0 °C) solution of phenethylamine (6.3 mL, 50 mmol) and triethylamine (6.9 mL) in CH_2Cl_2 (30 mL) was added 5.1 mL of isovaleryl chloride (50 mmol). The mixture was allowed to warm to room temperature and stirred for 4 h. The reaction mixture was poured into water (30 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting residue was chromatographed (30% EtOAc/hexane) to afford 7.5 g (80%) of **3** as a solid; ^1H and ^{13}C NMR data were identical to those of the natural product.

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