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Article

Cytotoxic Effects of a Diaza-Bicyclononandione Derivative Isolated from Broth Fermented by *Burkholderia anthina* on Cervical Cancer

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Abstract: An apoptosis inducing agent based on the activity-guided fractionation of the fermented broth of *Burkholderia anthina* KACC91391P, which was isolated from agricultural soils attacked by Phytophthora blight, was separated. The analysis in its structural elucidation based on the nuclear magnetic resonance and mass spectrometric data predicted two structures available. One of which was synthesized and its structural data compared with that of the isolated dipeptide. The biologically active compound was confirmed to a dipeptide, 2-(4-hydroxybenzyl)-1,4-diaza-bicyclo[3.3.1]nonan-3,9-dione. In addition, the cell cycle progression of human cervix carcinoma cells in the presence of the isolated dipeptide was measured as a means of determining its anticancer activity. The presence of the dipeptide altered the expression levels of cell cycle regulatory proteins, increased gene p21Waf1/Cip1 expression through an increase in p53, and decreased cyclin D1 expression..

Keywords: dipeptide; G2/M arrest; cyclin-dependent kinase inhibitor; apoptosis

1. Introduction

Since their first description in 1992 by Yabuuche et al., around 40 Burkholderia species have been discovered in a wide range of ecosystems, including soil, freshwater, seawater, and the respiratory tracks of animals [1,2]. Some of these species are valuable as biological control agents against plant diseases, while others can cause diseases of the human respiratory system [3,4]. The organisms themselves and their secondary metabolites are a rich source of biologically active compounds. A diverse range of secondary metabolites originating from microbial fermentation and microbial waste products have been applied over a wide range of research topics and particularly in the search for anticancer therapeutics [5,6]. Secondary metabolites from microorganisms are well-known sources for the discovery of new compounds [7]. Historically, soil has been a good repository from which to isolate microorganisms. For example, the antifungal antibiotic cepacidine A was isolated from *Pseudomonas cepacia* AF 2001 that had been isolated from the soil [8]. The anticancer agents benzyldihydroxyoctenone and phenylpyridineylbutenol were isolated from Streptomyces sp. KACC91015 [9,10]. Several anticancer agents such as doxorubicin, dactinomicina, mitomycin, and bleomycin originate from microorganisms and have been implemented in potential cancer treatments [11].

Peppers are used extensively in the preparation of kimchi, a traditional Korean food [12]. This strategy also provides an opportunity to identify microorganisms that inhibit Phytophthora blight caused by several pathogens including *Phytophthora capsici* [13]. Soil samples were screened for inhibitory effects on *P. capsici*, and the responsible microorganism thought to produce an antibiotic was isolated. This study sought to isolate and identify compounds with a biological activity against cervical cancer, the development of which is related to viral infection [14,15]. Cervical cancer is a malignant tumor that originates in the cervix uteri [16]. Worldwide, cervical cancer is the second most common and the fifth deadliest cancer in women [17]. If discovered early the proper treatment is surgery. However, chemotherapy is usually implemented because characteristic symptoms are not typically detected until the cancer reaches the more advanced stages. Cisplatin-based chemotherapies are widely used but limited by side effects that may include kidney or nerve damage,

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ototoxicity, and the disturbance of electrolyte levels [18]. Therefore, novel chemical agents that act on cervical cancer are sorely needed.

2. Materials and Methods

2.1. reagents

Reasoner's 2A (R2A) agar, cornmeal agar, and deuterated solvents were purchased from Sigma-Aldrich (MO, USA). Extraction solvents such as chloroform, ethyl acetate (EA), n-hexane, and n-butanol were acquired along with other chemicals from a local company in the Republic of Korea. Acetonitrile and methanol for high-performance liquid chromatography (HPLC) analyses and separations were obtained from Honeywell Burdick and Jackson (SK Chemicals, Korea).

2.2. cell culture

The human cervical carcinoma cell line (HeLa, CCL-2) was purchased from the American Type Culture Collection (ATCC, MD, USA). For cell cycle arrest, apoptosis analysis, and the analysis of Western blots, HeLa cells were cultivated at 37°C, 5% CO2 in accordance with the ATCC in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Hyclone, UT, USA).

2.3. screening and identification of bacterial strains KACC91391P and KACC40157

Soil was collected from a pepper field affected with Phytophthora blight in Jecheon, Korea. One gram of soil was suspended in 10 mL of sterilized water and diluted by 10^7. The diluted solution was transferred to an R2A medium plate and incubated for 8 days at 26°C. Among the resulting whole colonies, eight were isolated and purified. *P. capsici* (Korean Agricultural Culture Collection, KACC40157) was cultured in a liquid medium (17 g cornmeal agar, 1 L distilled water) for 3 days at 25°C and was spread onto a cornmeal agar plate and incubated for 3 days at 25°C. The eight colonies that had been isolated from the soil sample were inoculated onto the agar plate with *P. capsici*. As shown in Fig. 1A, the strain named 10H showed the best inhibitory effect against *P. capsici*. The bacterium was identified as *B. anthina* based on a 16S rRNA analysis. The strain was deposited in the Korean Agricultural Culture Collection (Suwon, Korea) as KACC91391P.

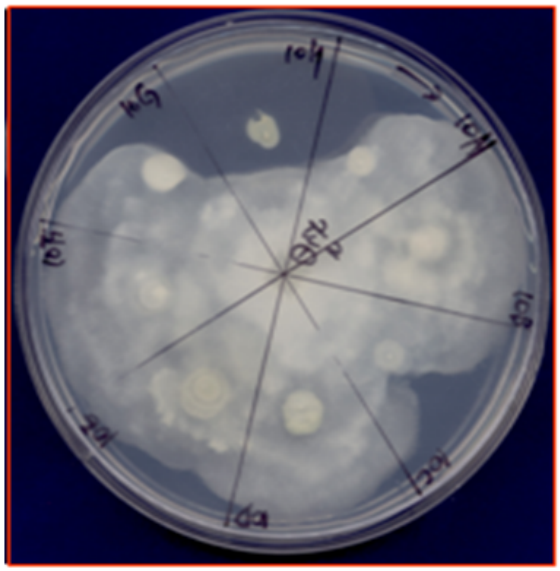

Fig. 1A. Plate showing inhibitory effects against *Phytohphthora capsica*.

2.3. production of the dipeptide

B. anthina strain 10H (KACC91391P) was inoculated into a seed R2A medium (10 mL cornmeal agar), and the culture was incubated on a shaking incubator at 150 rpm for 8 days at 26°C. The seed medium was inoculated into 10 L of R2A medium that was then transferred into 40 250-mL Erlenmeyer flasks and fermented under the conditions described above. The fermented broth was collected from the 40 250-mL Erlenmeyer

flasks and filtered through filter paper (Whatman International Inc., UK) to remove bacteria, cellular debris, and insoluble materials. The filtrate was sequentially liquid–liquid extracted with n-hexane, chloroform, EA, and n-butanol using in a shaker (275 rpm, EYELA, Tokyo Rikakikai Co., Ltd, Japan) for 24 h. The extracts of n-hexane, chloroform, EA, and n-butanol were collected as 130 mg, 92.4 mg, 104.4 mg, and 2690 mg, respectively [Fig. 1B]. The EA fraction showed the greatest inhibitory effect against HeLa cells, so it was separated further using preparative HPLC (Agilent Technologies, CA, USA), which was carried out on an HPLC system (1260 Infinity, Agilent Technologies) fitted with an RP-C18 column [Luna C-18 (II), 5 µm, 10.0 × 250 mm; Phenomenex, Torrance, CA]. Analytical HPLC was carried out at room temperature on an Agilent 1100 series fitted with an RP-C18 column (Gemini, 5 µm, 10.0 × 250 mm; Phenomenex) and a UV/VIS detector (Agilent Technologies, 260 nm). The eluent contained 10% acetonitrile and 0.1% trifluoroacetic acid and was run at a flow rate of 3.0 mL/min. Its peak was observed at 21.6 min in the chromatogram [Fig. 1C]. Fraction 3 obtained from the preparative HPLC run at a retention time of 21.5 min showed the greatest anticancer effect on HeLa cells. Based on NMR experiments, this fraction was consisted of a single compound named 10H-EA-F3. The fraction was dried in a freeze-drier for use in further experiments.

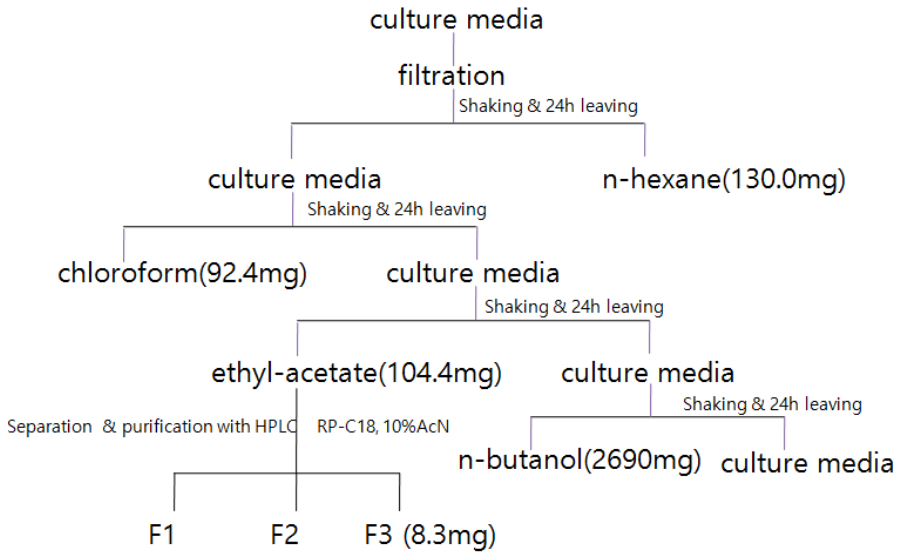

Fig. 1B. The schematic representation of the extraction and separation of a bioactive compound from culture media of *Phytohphthora capsica*.

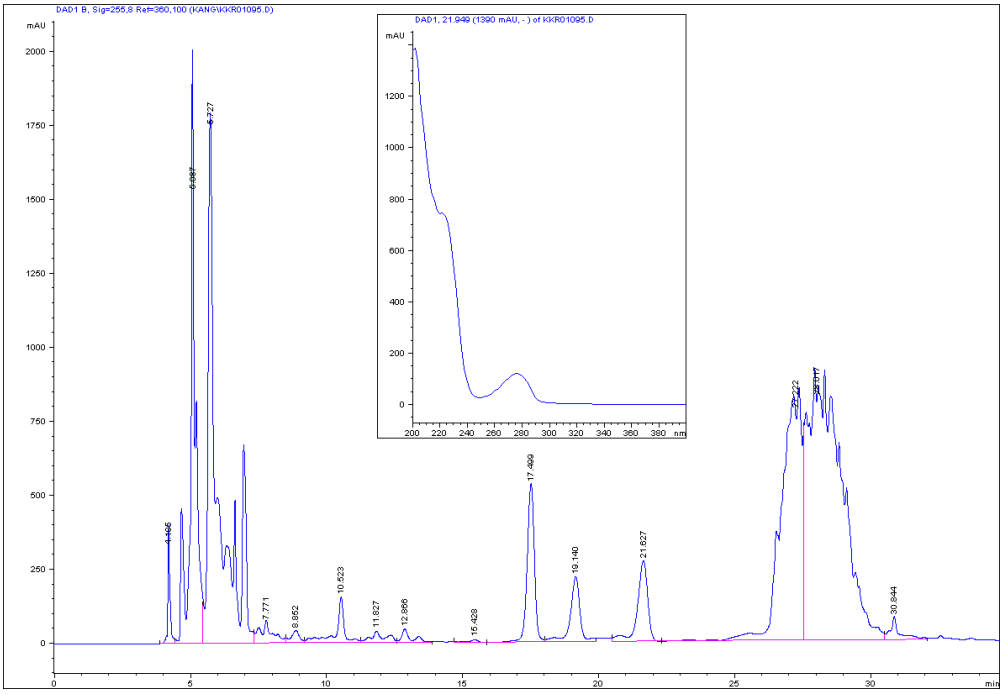

Fig. 1C. The HPLC chromatogram of bioactive compounds.

2.3. Western blot analysis

For Western blot analyses, HeLa cells (CCL-2, cervical cancer cell) were cultivated at 37°C in 5% CO2 as suggested by the ATCC. Cells (5 × 106) were seeded in DMEM (Gibco, CA, USA) with FBS (Gibco) and incubated for 24 h for them to adhere to the plate. The cells were treated with 10H-EA-F3 (20 μM) dissolved in 100% DMSO (Sigma-Aldrich) for 0, 3, 6, 12, or 24 h. Antibodies of cyclin D1 and GAPDH antibodies were purchased from Santa Cruz Biotechnology (CA, USA) and p21Waf1/Cip1 and p53 were obtained from Cell Signaling Technology (MA, USA). Cells were lysed in a buffer comprising 20 mM HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein extracts (20 μg each) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The blots were incubated with the corresponding primary antibodies and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Inc., NJ, USA) [19].

3. Results

3.1. isolation, separation and structural elucidation of fermented broth

A single compound (10H-EA-F3) based on cell-guided assay was obtained from a broth that had been fermented by bacterial strain KACC91391P, the microorganism isolated from Phytophthora-stricken soil, and identified using one- and two-dimensional nuclear magnetic resonance (NMR) techniques and mass spectrometry (MS) studies. However, two structural isomers were consistent with both the NMR and MS data. To determine the exact molecular conformation, the plausible structure was synthesized and its structural data compared with those of the isolated 10H-EA-F3. Based on the ^{1}H and ^{13}C NMR data, the 10H-EA-F3 that had been isolated from fermented broth was a single compound [Figs. 2 and 3]. The multiplicities of twelve ^{13}C peaks including four singlets, four doublets, and four triplets were determined by a comparison of the ^{13}C NMR spectrum with the distortionless enhancement by polarization transfer (DEPT) spectrum [Fig. 4]. Two peaks at 116.3 (C-3'/C-5') and 132.3 ppm (C-2'/C-6') were twice the intensity of their neighbors, and two proton peaks at 6.72 (H-3'/H-5') and 7.05 ppm (H-2'/H-6') corresponded to protons attached directly to carbon atoms in the heteronuclear multiple quantum coherence (HMQC) spectrum [Fig. 5]. Thus, 10H-EA-F3 contained 14 carbon atoms. The two aforementioned proton peaks were long-range-coupled to two carbon atoms with peaks at 127.8 (C-1') and 157.8 ppm (C-4') in the heteronuclear molecular bond correlation (HMBC) spectrum [Fig. 6], indicating the presence of a phenyl group. The ^{1}H peak at 7.05 ppm (H-2'/H-6') showed a cross peak correlation with the ^{1}H peak at 3.07 ppm (H-7') in the correlation spectroscopy (COSY) spectrum [Fig. 7], indicating that a methylene group was attached to the phenyl group to form a methylphenol group. A proton–proton cross-peak correlation between 3.07 (H-7') and 4.07 ppm (H-2) was observed in the COSY spectrum. HMQC data showed that a proton at 4.07 ppm was attached to the methine moiety at 60.2 ppm (C-2). HMBC data indicated that the ^{1}H peak at 3.07 ppm (H-7') was long-range-coupled to the ^{13}C peak at 167.1 ppm (C-3). COSY data specified that five proton peaks at 1.23 (H-6b), 1.80 (H-7), 2.11 (H-6a), 3.56 (H-8), and 4.37 ppm (H-5) were correlated with each other. Two of these protons, corresponding to peaks at 1.23 and 2.11 ppm, were attached to the carbon atom corresponding to the peak at 29.5 ppm (C-6). The remaining protons were attached to three carbon atoms at 22.9 (C-7), 46.1 (C-8), and 58.1 ppm (C-5). Thus, four carbon atoms were assigned to an n-butyl chain. COSY data showed that the ^{1}H peak at 4.37 ppm was correlated with the ^{1}H peak at 3.07 ppm (H-7'), indicating that the n-butyl group was attached to the methylphenol group. The remaining carbon atom at 170.9 ppm (C-9) was long-range-coupled to the proton at 1.23 ppm. The two carbon peaks at 167.1 and 170.9 ppm were upfield-shifted to a greater degree than carbonyl carbons and therefore assigned to peptide carbon atoms. In addition, the chemical shifts of two methine carbon atoms at 58.1 and 60.2 ppm, and another at 46.1 ppm, indicated the presence of nitrogen neighbors. The connectivities assigned above were confirmed by total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) spectra [Figs. 8 and 9]. A proposed structure of 10H-EA-F3 is shown in Fig. 10 in accordance with these data and named 2-(4-hydroxybenzyl)-1,4-diaza-bicyclo[3.3.1]nonan-3,9-dione. Table 1 shows all of the peak assignments of the ^{1}H and ^{13}C NMR data. Mass spectrometry (MS) was used to confirm these assignments. A molecular ion $(M+H)^{+}$

of 10H-EA-F3 was evident at m/z 261.12 [Fig. 11], indicating a calculated mass of m/z 260.1 and a molecular formula of $C_{14}H_{16}N_2O_3$.

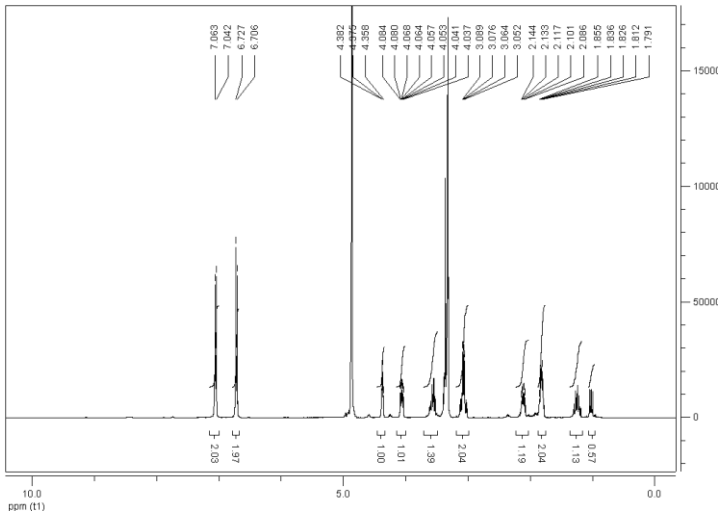

Fig. 2. The ^{1}H NMR spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

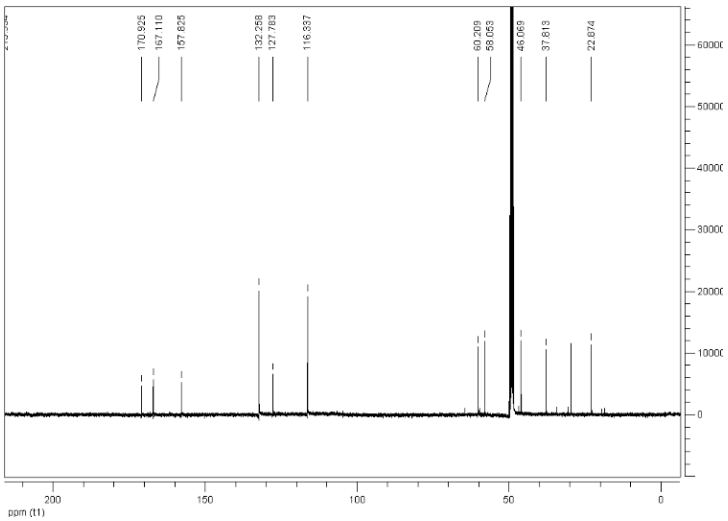

Fig. 3. The ^{13}C NMR spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

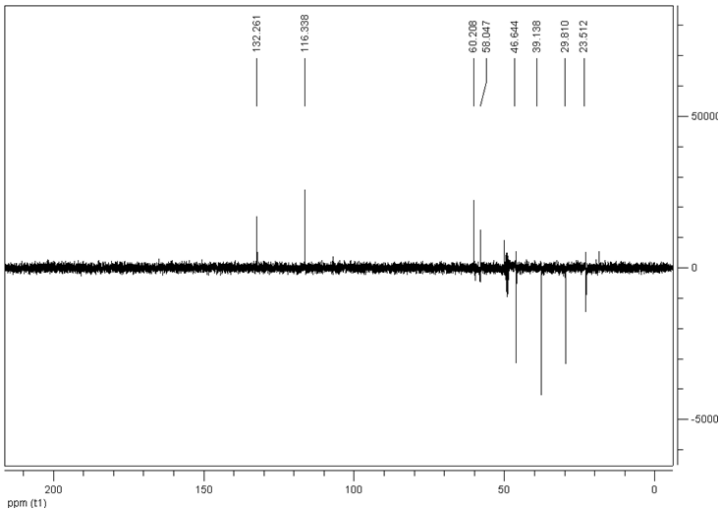

Fig. 4. The DEPT NMR spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

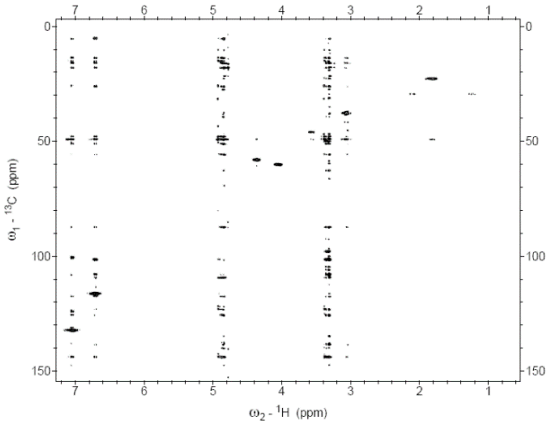

Fig. 5. The HMQC spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

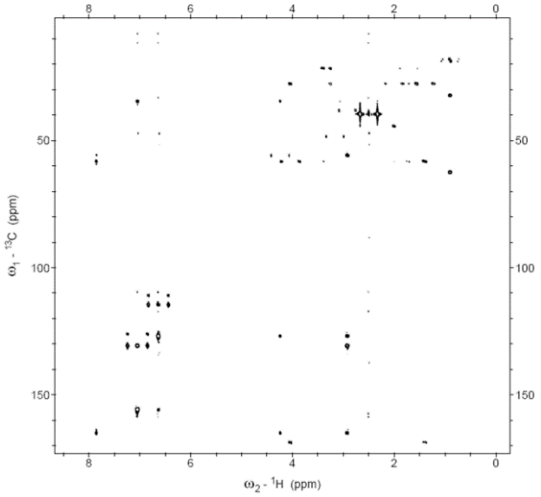

Fig. 6. The HMBC spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

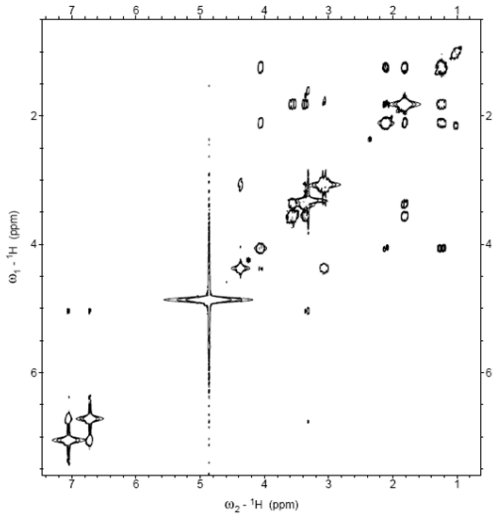

Fig. 7. The COSY spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

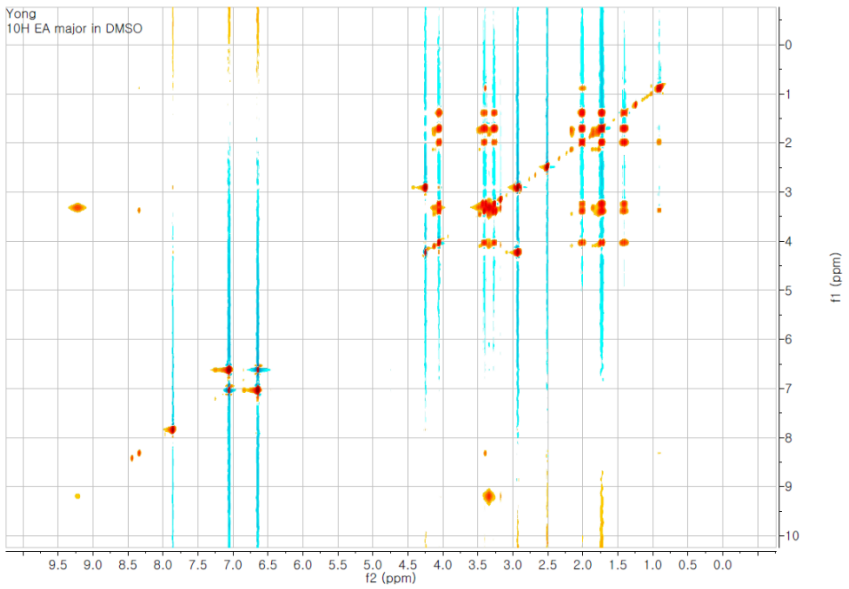

Fig. 8. The TOCSY spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

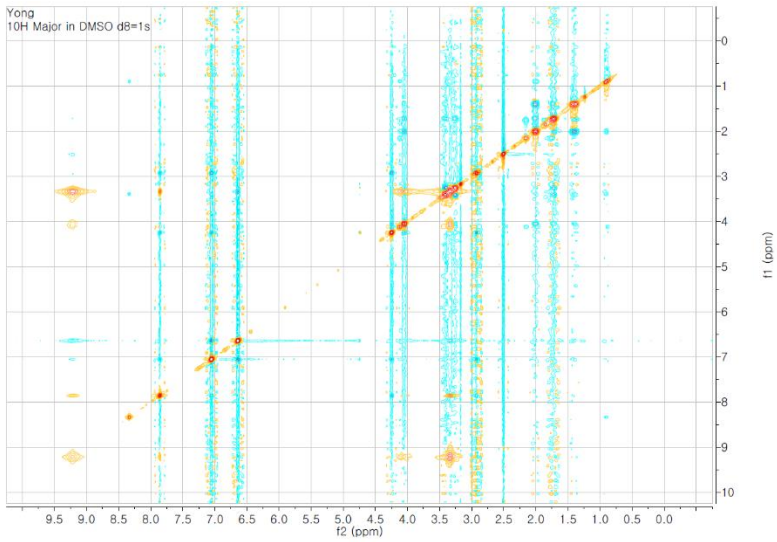

Fig. 9. The NOESY spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

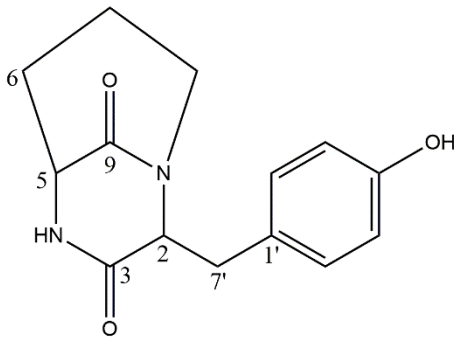

Fig. 10. The predictable structure of 2-(4-hydroxybenzyl)-1,4-diaza-bicyclo[3.3.1]nonan-3,9-dione determined by NMR spectrometry and mass spectrometry.

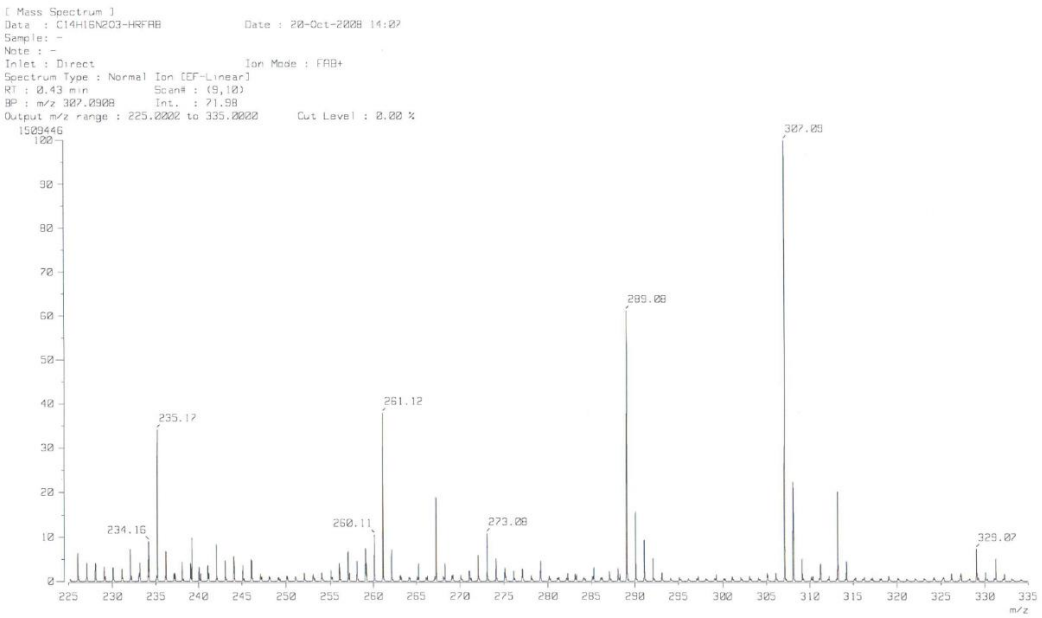

Fig. 11. The mass spectrum of the compound (10H-EA-F3) isolated from the broth fermented by bacterial strain KACC91391P.

Table 1. The assignments of the NMR data of 10H-EA-F3. (s, singlet; d, doublet; t, triplet; m, multiplet; δ, chemical shift; J, coupling constant)

δ of ^{13}C (ppm)	multiplicity	δ of ^{1}H (ppm, J Hz)	assignment
22.9	t	1.8(m)	6
29.5	t	2.11(m) 1.23(m)	7'
37.8	t	3.07(dd, J=4.62, 4.98)	7
46.1	t	3.56(m)	8
58.1	d	4.37(dd, J=4.62, 4.98)	5
60.2	d	4.07(dd, J=6.31, 10.85)	2
116.3	d(x2)	6.72(d, J= 8.55)	2'
127.8	s	-	1'
132.3	d(x2)	7.05(d, J= 8.55)	3'
157.8	s	-	4'
167.1	s	-	3
170.9	s	-	9

3.2. structural confirmation of 10H-EA-F3 via comparison with a synthetic version

During structural analyses of 10H-EA-F3, a second structure, 3-(4-hydroxybenzyl)-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione, shown in Fig. 12, was deemed plausible. The calculated mass of both structures is identical. The best means of determining the structure of 10H-EA-F3 would be to synthesize both compounds and compare their properties with those of the isolated compound. We could not synthesize 2-(4-hydroxybenzyl)-1,4-diaza-bicyclo[3.3.1]nonan-3,9-dione. Instead, its plausible structure, 3-(4-hydroxybenzyl)-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione could be synthesized as follows [Fig. 13A]. N-Boc-tyrosine and L-proline were dissolved in N,N-dimethylformamide (7 mL) and stirred for 24 h before adding 1-hydroxybenzotriazole (HOBT, 0.875 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI, 1.05 mmol) at room temperature. This produced methyl ester conjugates of N-Boc-L-tyrosine and L-proline (Fluka, MO, USA). Heating the conjugate to reflux with formic acid eliminated the tert-butoxycarbonyl protecting group (Boc) to form a cyclical compound. Reflux was continued with the addition of triethanolamine to yield a

complete cyclized compound that served as a reactant in subsequent reactions. The methyl ester conjugate consisted of N-Boc-tyrosine and L-proline and was obtained as a clear oil (57% yield) after purification through a silica column (eluent: 3:2, n-hexane:ethyl-acetate) and concentration under reduced pressure. 1-Butanol (1.5 mL) and toluene (5.5 mL) were added into the mixture of N-Boc-L-tyrosine and L-proline methyl ester conjugates, and the solution was refluxed with formic acid (1 mmol) under reduced pressure at 100°C for 12 h. The reactant was condensed under reduced pressure, methanol (5 mL) and triethylamine (TEA, 2 mmol) were added, and the mixture was refluxed at 80°C for 12 h. After concentrating the reaction mixture under reduced pressure, it was applied to a silica column for purification and separation. The eluent consisted of 20:1 dichloromethane:methanol. The final product (65% yield) was obtained as a white powder. NMR was employed for structural elucidation:

^{1}H NMR (400 MHz, CDCl3) δ(ppm) 7.11 (d, J = 8.36 Hz, 2H), 6.72 (d, J = 8.36 Hz, 2H), 5.27 (d, J = 8.89 Hz, 1H), 4.62 (m, 1H), 4.51 (dd, J = 3.87, 8.17 Hz, 1H), 3.73 (s, 3H), 3.64 (m, 1H), 3.28 (m, 1H) 3.02 (dd, J = 6.72, 13.8 Hz, 1H), 2.85 (dd, J = 5.99, 14.1 Hz, 1H), 2.18 (m, 1H), 1.95 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (100 MHz, CDCl3) δ(ppm) 17.9 (t), 26.7 (t), 35.1 (t), 40.0 (t), 56.9 (d), 62.7 (d), 115.8 (d, x2), 129.2 (s), 132.1 (d, x2), 155.7 (s), 164.8 (s), 169.1 (s).

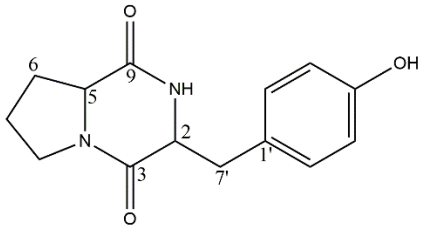

Fig. 12. The The predictable structure of 3-(4-hydroxybenzyl)-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione determined by NMR spectrometry and mass spectrometry.

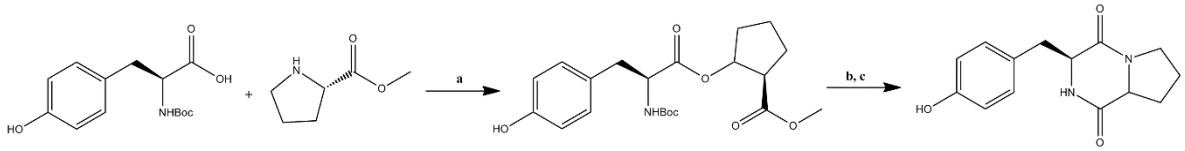

Fig. 13A. In order to clarify the predictable compounds, the synthetic scheme for 3-(4-hydroxybenzyl)-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione.

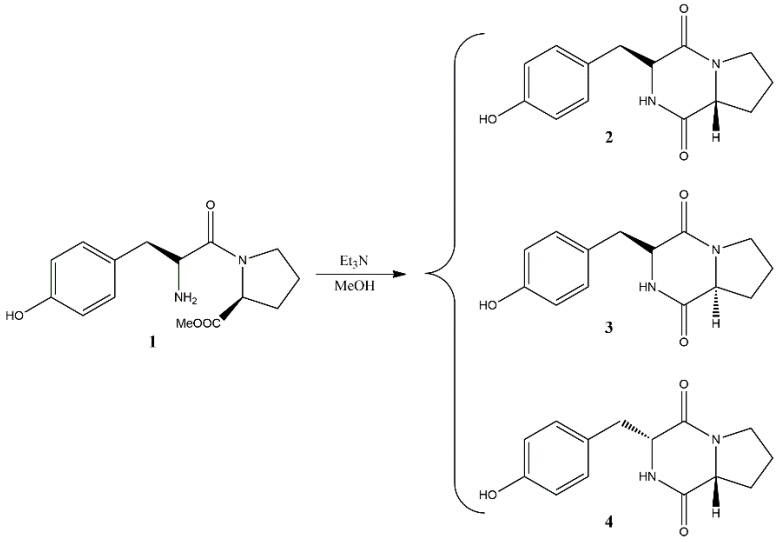

Fig. 13B. The synthetic scheme for epimers of 3-(4-hydroxybenzyl)-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione available.

A comparison of the ^{1}H NMR spectrum of the synthesized compound with that of the compound isolated from the fermented broth revealed they are not the identical compounds [Fig. 14A]. In addition, a comparison of their 13C NMR spectra showed the same results too [Fig. 14B]. As shown in the synthetic scheme [Fig. 13B], epimers can be produced, but their distinction is not necessary to identify 10H-EA-F3 because the NMR data

of 10H-EA-F3 and the synthesized compound are not identical. The structure of our synthesized compound differed from that of 10H-EA-F3. Thus, even two possible structures could be drawn based on the NMR and MS data, the compound isolated from the fermented broth should be 2-(4-hydroxybenzyl)-1,4-diaza-bicyclo[3.3.1]nonan-3,9-dione.

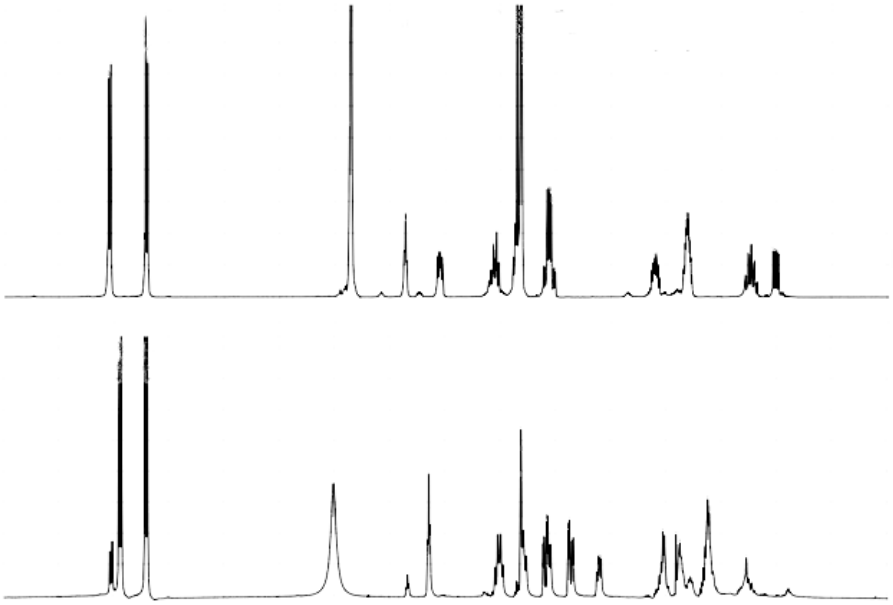

Fig. 14A. The Comparing NMR data between predictable structures, ^{1}H NMR of natural product isolated from KACC91391 (above), and synthetic compound 2 (below).

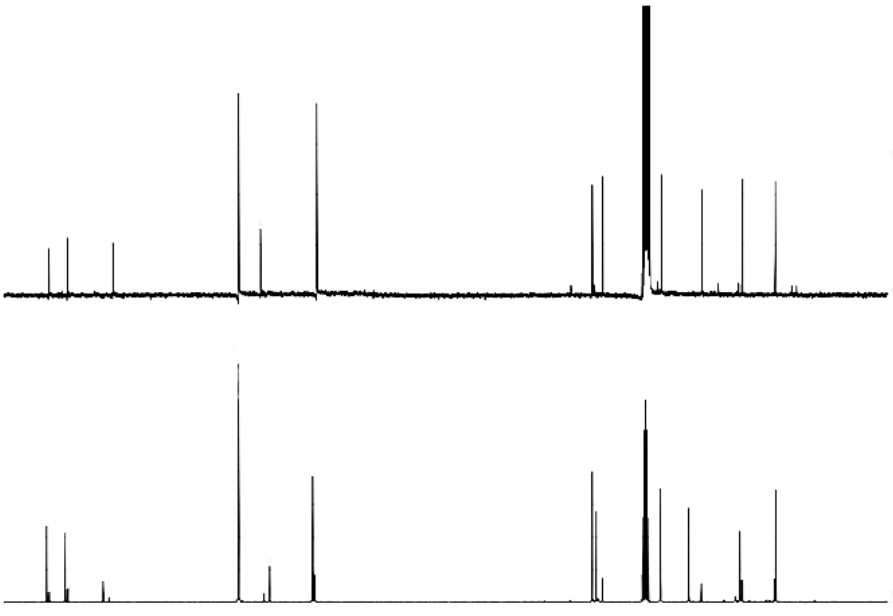

Fig. 14B. The Comparing NMR data between predictable structures, ^{13}C NMR data of natural product isolated from KACC91391 (above), and synthetic compound 2 (below).

3.3. effect of 2-(4-hydroxybenzyl)-1,4-diaza-bicyclo[3.3.1]nonan-3,9-dione on cell cycle progression

As mentioned before, to investigate the anticancer activities of the compound isolated from the fermented broth, 10H-EA-F3, first, the impact of early growth response-1 (Egr-1) on cell cycle progression was used to

assess its biological activity against HeLa human cervix carcinoma cells. Egr-1 is a transcriptional factor that is strongly correlated with cell growth, differentiation, and survival. Several observations have suggested that Egr-1 promotes growth and that blocking its activity may impede the progression of cancer [19>>20]. Fig. 15A shows that treatments with 10H-EA-F3 induced the expression of Egr-1 after 60 min, demonstrating that 10H-EA-F3 suppresses cervical cancer proliferation.

After treatments with 10H-EA-F3 for 0, 3, 6, 12, or 24 h, whole HeLa cells were harvested to assess the expression levels of proteins for cell proliferation. Fig. 15B shows that the p21Waf1/Cip1 expression level increased gradually after 12 h of treatment. Determining the mechanism of p21 Waf1/Cip1 expression requires a closer examination of p53 expression since the levels of these two proteins are highly correlated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the control protein in Western blot analyses used to quantify protein loading. Analyses of p53 expression followed the same method as that used for p21Waf1/Cip1 analyses. p53 expression increased in concert with p21Waf1/Cip1 levels in a time-dependent manner. Based on these results, p21 expression was induced by 10H-EA-F3 through its effect on p53 expression. Cell proliferation was blocked in the G1 phase by an increase in p21Waf1/Cip1 and p53 expression. The expression of cyclin D1 is also associated with the expression of p21Waf1/Cip1 and p53, and was assessed using the same method. The expression level of cyclin D1 changed after 3 h of 10H-EA-F3 treatment, indicating that 10H-EA-F3 induced p53 expression, which in turn promoted p21Waf1/Cip1. The increased p21Waf1/Cip1 expression then inhibited the proliferation process between phase G1 and phase S. This inhibitory effect decreased the expression levels of cyclin D1. The expression levels of p21Waf1/Cip1, p53, and cyclin D1 are shown as a function of time in Fig. 15B.

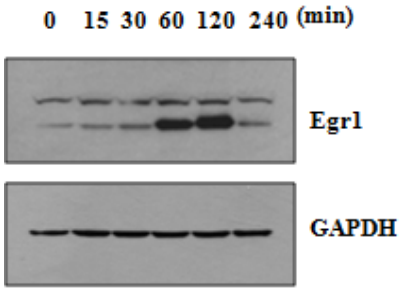

Fig. 15A. The treatment with 10H-EA-F3 induced the expression of Egr-1 after 60 min, demonstrating that 10H-EA-F3 suppresses cervical cancer proliferation.

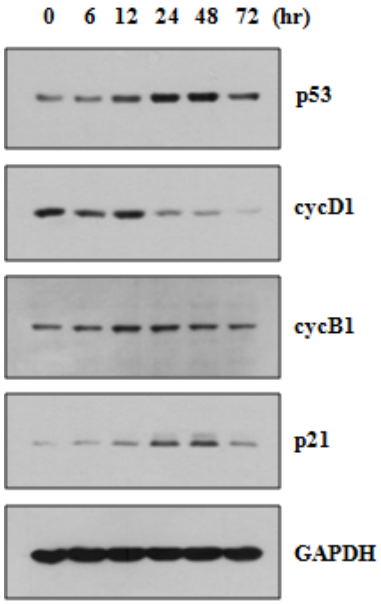

Fig. 15B. After treatments with 10H-EA-F3, Western blot analyses of p21, p53, cyclinD1, and cyclinB1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the control protein used to quantify protein loading.

4. Discussion

After In this study, a potentially useful microorganism was isolated from the soils of a pepper field infected with Phytophthora blight, and its secondary metabolites were screened for biological activity. However, the goal of this study was not to discover an antibiotic agent to be used against Phytophthora blight. This study focused on isolation and identification of biologically active compounds against differentiation of cervical cancer correlated with viral infection. Therefore, a broth fermented by the same microorganism that strongly inhibits *P. capsici* was tested for inhibitory activity on HeLa human cervical cancer cells. As a result, 10H-EA-F3, a novel compound isolated from a culture broth of *Burkholderia anthina*, exhibited an antiproliferative effect on cancer cells. The results of this study demonstrate that peptidomimetic compounds like 10H-EA-F3 can act as anticancer or antitumor agents. To the best of our knowledge, this is the first report of such findings. Rational derivative designs based on the skeleton of 2-(4-hydroxybenzyl)-1,4-diaza-bicyclo[3.3.1]nonan-3,9-dione will provide new approaches for obtaining novel anticancer agents. However, further study is needed to improve the selectivity of these compounds against cancer cells.

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