Emestrin B: Epipolythiodioxypiperazine from Marine Derived Fungus Emericella Nidulans

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***Abstract*—Marine fungi have become an important research subject of natural products with significant value due to its diversity in chemical structures and biological activities. Epipolythiodioxopiperazines (ETPs) which are characterized by the disulfide bridge or polysulfide dioxopiperazine six membered ring have been reported to have wide ranges of bioactivities. This research was aimed to isolate, identify and investigate anticancer properties of emestrin B produced by *Emericella nidulans* marine fungus. Emestrin B was isolated from mycelium of the *E.nidulans* fungus that cultivated on malt extract broth medium for 4 weeks by using repeated column chromatography. Elucidation of molecular structure using spectra data analysis of UPLC-ESI-ToF-MS, ^{1}H-NMR, and ^{13}C-NMR techniques concluded that the compound was emestrin B. The molecular formula of emestrin B was established as $C_{27}H_{22}N_2O_{10}S_3$ (m/z) 631.0525 $[M+H]^+$. Emestrin B was cytotoxic against T47D, HeLa, and WiDr cells with IC_{50} values of 0.16; 1.56; and 1.02 µg/ml, respectively. Based on flowcytometric analysis, emestrin B could induce apoptosis in T47D cells.**

***Index Terms*—Emestrin B, *Emericella nidulans*, Epipolythiodioxypiperazines, And Marine Fungus**

I. Introduction

Marine fungi have much attention as an important source of biologically active secondary metabolites [1] due to its diversity in chemical structures and biological activities [2]. Marine-derived fungi have different characteristic from terrestrial fungi, such as salt tolerance, and yield many unique secondary metabolites [3].

Majority of compounds that were isolated from marine-derived fungi strains are polyketide derived, alkaloids, terpenes, peptides and compounds of mixed biosynthesis. These compounds are representative groups of secondary metabolites produced by these fungi. The chemical diversity of marine-derived fungi secondary metabolites, along with the strains novelty, points this group of microorganisms as much interest for isolation of unusual bioactive natural products [4]. The genus of *Aspergillus* and *Penicillium* were major contributor to active compounds of marine fungi origin [5].

In our previous study, we found that crude extract of marine fungus strain MFW-39-08 mycelium, inhibited the growth of T47D (breast cancer) cell line. Based on morphological properties and molecular taxonomy, this fungus was identified as *Emericella nidulans*. An epipolythiodioxypiperazine, emestrin A, was isolated from mycelium of this fungus. Emestrin A exhibited anticancer properties against several cancer cell lines [6]. Continuing our previously study, the objective of this study was to isolate, identify and investigate another anticancer compound from mycelium of *Emericella nidulans* marine fungus.

II. Material and Methods

A. Fungus Material and Culture

Culture of *E.nidulans*, a fungus derived from the marine ascidia *Aplidium longithorax*, was maintained on malt extract agar. The fungus was cultured in static liquid culture of malt extract broth medium containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and seawater (salinity of 30 ppt). The fungus was cultured (1 L x 20 flasks) for 5 weeks at 27-29^{o}C.

B. Isolation of Emestrin B

The mycelium extract was fractionated by vacuum column on silica gel using n-hexane – EtOAc (8:1), n-hexane – EtOAc (1:1), EtOAc 100%, and MeOH 100%. Fraction 3 was then separated by silica gel vacuum column using *n*-hexane – EtOAc (8:1), (5:1), (1:1), EtOAc 100% and EtOAc – MeOH (5:1). Fraction 3.7 was then purified using silica gel preparative TLC to get emestrin B.

C. Compound Identification

Identification of bioactive compound was determined using UPLC-ESI-ToF-MS (Waters), ^{1}H-NMR, and ^{13}C-NMR (JEOL 500 Mhz).

D. Cytotoxic Bioassay

Three human cancer cell lines (T47D, HeLa and WiDr) were routinely grown and maintained in RPMI medium with 10% FBS and 1% penicillin/streptomycin. All cell

Manuscript received October 22, 2014; revised December 10, 2014.

doi: 10.12720/jomb.4.6.441-445

lines were incubated in a moisture-saturated atmosphere containing 5% CO_2. To each well of the 96-wells microplate containing 100 µL of cell suspension (1.5 $\times 10^4$ cells) was added 100 µL of active compound (dissolved in RPMI-1640 medium) and the plate was then incubated in a CO2 incubator at 37 ℃ for 24 h. After the addition of 100 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide saline solution (0.5 mg/mL) to each well of microplate, the plate was then incubated for 4 h under the same conditions in the CO_2 incubator. After incubation, the optical density was measured at 570 nm in a microplate reader (Thermo Scientific). The inhibition concentration 50 (IC_{50}) values defined as the concentrations of compound which inhibited 50% of the cell growth. IC_{50} value was determined by using Minitab probit analysis version 14.0.

E. DNA Fragmentation

The T47D cell was seeded at a final concentration of 10^6 cells/well in 6-wells microplate and incubated for 12 h in CO_2 incubator (37^{o}C, 5% of CO_2 flow). The active compound was added to the cells at final concentration of 1.0 µg/ml and then incubated for 6, 12 and 24 h. At the end of the incubation period, medium was collected from each of the treatment wells and attached cells were tripsynized (0.025% trypsin EDTA) from the microplate, followed by centrifugation at 10.000 rpm for 5 minutes to collectt the cells. Isolation of genomic DNA and DNA electrophoresis conducted according to reagent manufacturer (Apoptotic DNA Ladder Kit, Roche).

F. Flowcytometry Analysis

The analysis and discrimination between apoptosis and necrosis cancer cells was conducted using Annexin-V-FLUOS staining kit (Roche). After T47D cells treated with 1.0 µg/ml of emestrin B for 24 h, the cells were trypsinized, washed with PBS, and the cell pellet was resuspended in 100 µl of Annexin-V-FLUOS reagent. The cells were then incubated in dark room for 10 minutes at 20 – 25 ^{o}C. Apoptotic and necrotic cells were measured by FACS Calybur (Becton-Dickinson) flow cytometer.

III. Result

Cultivation of *Emericella nidulans* in 1 L x 20 flasks yielded 498.4 gram of mycelium. The mycelial extract of the fungus was separated by SiO_2 column chromatography and TLC preparative chromatography which yielded 20.0 mg emestrin B. Analysis of UPLC-ESI-qTOF-MS data determined the molecular formula of emestrin B as $C_{27}H_{21}N_2O_{10}S_3$.

The ^{1}H-NMR spectrum of emestrin B (Fig. 1) in $CDCl_3$ showed methoxy group (3 – 4 ppm) and olefinic proton/sp^2 (4 – 6 ppm) and aromatic proton (6 – 8 ppm). Two proton signals singlet derived methoxy proton (2 – NCH_3 dan 2”-OCH_3) detected at 3 – 4 ppm. The chemical shift of 2”-OCH_3 more downfield than 2 – NCH_3 caused 2”-OCH_3 containing oxygen atom that more electronegative than nitrogen atom.

The ^{13}C-NMR spectrum of emestrin B displayed 27 carbon signals. Chemical shift of 0 - 60 ppm indicated sp^3 carbon and chemical shift of 70 – 85 ppm showed atomic carbon bonded electronegative atom. Alkene and benzoic aromatic detected at 100 – 160 ppm (sp^2) whereas area of 160 – 180 ppm showed chemical shift of lactam carbonil and esther indicating structure of emestrin B. The summary of ^{1}H-NMR and ^{13}C-NMR of emestrin B was summarized in Table I.

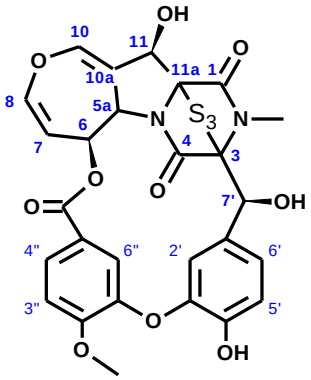

Figure 1. Molecular structure of emestrin B

TABLE I. ^{1}H AND ^{13}C NMR SPECTROSCOPIC DATA FOR EMESTRIN IN $CDCL_3$

Proton	**δ (ppm) ^{1}H-NMR**	**Carbon**	**δ (ppm) ^{13}C-NMR**
2-NMe	3.5395	1	165.69
5a-H	5.4034	2-NMe	29.04
6-H	5.3359	3	83.34
7-H	5.0143	4	165.40
8-H	6.3555	5a	59.17
10-H	6.8159	6	74.87
11-H	5.2387	7	110.01
2’-H	8.7162	8	139.16
5’-H	6.8354	10	143.18
6’-H	6.9288	10a	108.51
7’-H	4.8132	11	76.82
2”-OMe	4.0674	11a	78.14
3”-H	7.0287	1’	127.19
4”-H	7.8575	2’	123.39
6”-H	8.3517	3’	147.42
		4’	154.69
		5’	114.62
		6’	126.99
		7’	79.98
		1”	146.62
		2”	149.86
		2”-OMe	56.65
		3”	112.14
		4”	127.78
		5”	122.77
		6”	130.49
		7”	168.34

The MTT test was used for evaluating cytotoxic properties of emestrin B. The growth-inhibitory effects of emestrin B was tested in three cell lines, including T47D (breast cancer), HeLa (cervix cancer) and WiDr (colon cancer). Morphological changes in the cells caused by emestrin B were observed by microscopy as shown in Fig. 2. After 24 h treatment of 1.0 µg/ml emestrin B, the morphology change of T47D, HeLa and WiDr cells were observed. In contrast, there were no morphological

changes in untreated cells (control). Probit analysis showed that emestrin B had strong cytotoxicity activity to T47D, HeLa and WiDr cells with IC_{50} value of 0.16; 1.56; and 1.02 µg/ml, respectively. As emestrin B showed the best cytotoxic activity against T47D cells, we used T47D cells for further study.

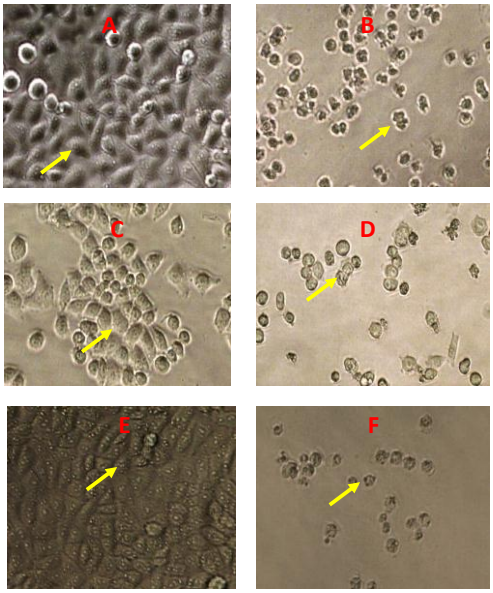

Figure 2. Effect of emestrin B (1 µg/ml) to HeLa (B), WiDr (D), and T47D (F) cells compare to untreated cells (control) of HeLa (A), WiDr (C) and T47D (E)

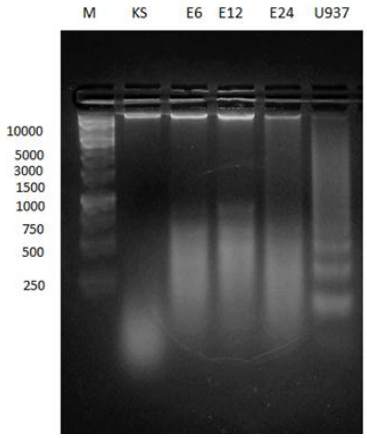

Figure 3. Analysis of DNA fragmentation in T47D cells treated with emestrin B. M (marker); KS (untreated cells); E6, E12, E24 (cells were treated for 6, 12 and 24 hours, respectively); U937 (apoptotic of U937 cells treated with camptothecin, positive control)

The T47D cells were exposed to emestrin B for 6, 12, and 24 h and then the DNA was extracted. Electrophoresis of DNA was performed and a typical DNA ladder pattern of apoptosis was observed. The DNA gel electrophoresis image showed that emestrin B was abble to cause DNA fragmentation, although it was not too clear, as early indication of apoptosis as seen in Fig. 3. In contrast, a clear DNA ladder was visible in the lyophilized apoptotic U937 cells as a positive control.

Since the induction of apoptosis in T47D cells by emestrin B cells was not clearly observed by analysis of DNA fragmentation, other analysis were used to determine whether the growth inhibition of T47D cells by emestrin B associated with the induction of apoptosis. In this research flow cytometry analysis was performed by using annexin V-propidium iodide staining. According to this method, T47D cells were treated by emestrin B for 24 hours. Doxorubicin was used to induce apoptosis in T47D cells as a positive control. Based on the flow cytometry analysis, emestrin B could induce apoptosis in T47D cells. Percentage of apoptotic cells treated with emestrin B was 74.1% whereas that treated with doxorubicin was 74.8% (Fig. 4).

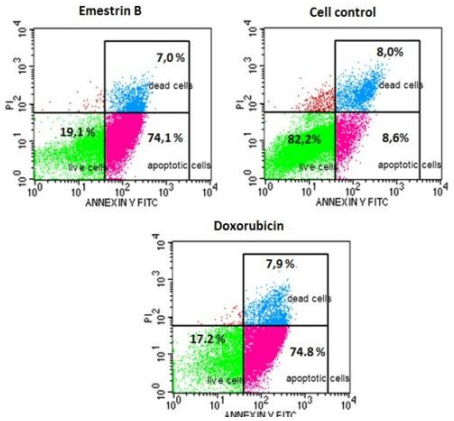

Figure 4. Apoptosis and necrosis were induced in T47D cells detected by annexin-PI staining. Viable cells : lower left quadrant, apoptotic cells : lower right quadrant and necrotic cells : upper right quadrant

IV. Discussion

Emestrin is a member of epipolythiodioxopipera-zines (ETPs) group which is a group of toxic secondary metabolites made only by fungi [7]. Emestrin A and B were originally isolated from mycelial acetone extract of *Emericella striata* [8], [9]. Emestrin A, the first reported example of 15-membered macrocyclic ETP with strong antifungal activity, was formally derived from two molecules of phenylalanine and one molecule of benzoic acid [10]. *Emericella nidulans*, isolated from the surface of marine ascidia *Aplidium longithorax*, also produced emestrin A which showed anticancer properties against several cell lines [6].

Recently, more than 14 different ETPs (excluding those with minor modifications) are known. The diversity of structures stems from the amino acids of the core ETP moiety, as well as the modifications of these amino acids. All natural ETP isolated to date contain at least one aromatic amino acid. A diverse range of filamentous ascomycetes produce ETPs. Five classes of ascomycetes (Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Saccharomycetes and Sordariomycetes) were known produce ETPs. At least two basidiomycetes, *Stereum hirsutum* and a *Hyalodendron* sp., produce ETPs epicorazine and hyalodendrin, respectively [7], [11]. Emestrin is action at the chemokine receptor has lead to its consideration as a possible treatment for autoimmune disorders including rheumatoid arthritis, atherosclerosis, multiple sclerosis, and infectious diseases [12], [13].

Some ETPs exhibit potent anti-tumor activity so which made them interesting to be explored. For example gliotoxin that showed not only immunosuppressive effect but also causes apoptotic and necrotic cell death in vitro. The toxicity of ETPs has made them attractive as potential therapeutic agents for diseases such as cancer [14]. An ETP, 11,11′-dideoxyverticillin, which is isolated from *Shiraia bambusicola*, exhibits potent cytotoxicity against a broad spectrum of cancer cell lines *in vitro* [15]. Furthemore, a novel 11′-deoxyverticillin A derivative (G226), exhibits potent cytotoxic activity against 9 breast cancer cell lines with a mean IC_{50} value of 48.5 nmol/L. The anticancer mechanism of G226 through triggering autophagy and caspase-dependent apoptosis [16].

In our previous study, we found that emestrin A inhibited the growth of T47D cells through the G phase of the cell cycle [6]. In this research, we assayed the ability of emestrin B to induce apoptosis in T47D cells through DNA fragmentation and flowcytometry analysis. The formation of distinct DNA fragments is a biochemical hall mark of apoptosis, with internucleosomal DNA cleavage activity as a major characteristic. In many systems, this DNA fragmentation was from activation of an end ogenous $Ca^{2+}$and Mg^{2+} dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments. These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit [17], [18].

Changes at the cell surface occur when a cell undergoes apoptosis. One of plasma membrane alteration is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cells. PS exposure therefore represent a useful assay for the apoptosis. PS present on the outer layer can be detected using Annexin V [19]. Annexin V is Ca^{2+}-dependent phospholipid binding protein with high affinity for PS. This protein can hence be used as a sensitive probe for PS exposure upon the outer layer of the cell membrane and is, therefore, suited to detect apoptosis cells. Necrotic cells also expose PS, and will therefore also bind Annexin V. To differentiate between apoptotic and necrotic cells, PI is often used in conjunction with Annexin V. PI will mark necrotic cells, but notapoptotic cells. In this assay, Annexin V binds the phospholipid PS, marking apoptotic and necrotic cells, while PI bind DNA, marking only necrotic cells [17].

V. Conclusion

Marine fungi *Emericella nidulans* produced emestrin B, a member of a epipolythiodioxopipera-
zines (ETPs) group that exhibited cytototoxic activity against T47D, HeLa and WiDr cells with IC_{50} value of 0.16; 1.56; and 1.02 μg/ml, respectively. Based on flowcytometry analysis, emestrin B could induced apoptosis in T47D cells.

Acknowledgment

This research was funded by Ministry of Marine and Fisheries Affairs, Republic of Indonesia. We thanks to Pathology Clinic Laboratory, Gadjah Mada University, Yogyakarta, Center for Assessments of Biotechnology-BPPT; Research Center for Chemistry-LIPI; and Ms. Asri Pratitis for the fungus isolation.

References

[1] J. W. Blunt, B. R. Copp, W. P. Hu, M. H. G. Munro, P. T. Northcote, and M. R. Prinsep, "Marine natural products," *Natural Product Reports*, vol. 24, pp. 31-86, 2007.
[2] M. P. L. Gresa, N. Cabedo, M. C. G. Mas, M. L. Ciavatta, C. Avila, J. Primo, and E. F. Terretonins, "Inhibitors of the mitochondrial respiratory chain from the marine-derived fungus *Aspergillus insuetus*," *J. Nat. Prod*., vol. 72, pp. 1348–1351, 2009.
[3] M. Ishino, K. Kinoshita, K. Takahashi, T. Sugita, M. Shiro, K. Hasegawa, and K. Koyama, "Phomactins K-M, three novel phomactin-type diterpenes from a marine-derived fungus," *Tetrahedron*, vol. 68, pp. 8572–8575, 2012.
[4] M. H. Kossuga, S. Romminger, C. Xavier, *et al*., "Evaluating methods for the isolation of marine-derived fungus strains and production of bioactive secondary metabolites," *Rev. Bras. Farmacogn. Braz. J. Pharmacogn*., vol. 22, no. 2, pp. 257-267, 2012.
[5] T. S. Bugni and C. M. Ireland, "Marine-derived fungi: A chemically and biologically diverse group of microorganisms," *Natural Product Reports*, vol. 21, pp. 143-163, 2004.
[6] M. Nursid, E. Chasanah, Murwantoko, and S. Wahyuono, "Isolation and identification of emestrin from emericella nidulans and investigation of its anticancer properties," *Microbiol. Indones*., vol. 5, no. 4, pp. 160-169, 2011.
[7] D. M. Gardiner, P. Waring, and B. J. Howlett, "The epipolythiodioxopiperazine (ETP) class of fungus toxins: distribution, mode of action, functions and biosynthesis," *Microbiology*, vol. 151, pp. 1021–1032, 2005.
[8] K. Nozawa, S. Udagawa, S. Nakajima, and K. Kawai, "Studies on fungus products: XIV, Emestrin B, a new ETP, from emericella striata," *Chem. Pharm. Bull*., vol. 35, no. 8, pp. 3460–3461, 1987.
[9] K. Seya, S. Nakajima, and K. Kawai, "Structure and absolute configuration of emestrin, a new macrocyclic epidithiodioxopiperazine from emericella striata," *J. Chem. Soc. Chem. Commun*., vol. 117, pp. 657–658, 1985.
[10] C. S. Jiang and Y. W. Guo, "Epipolythiodioxopiperazines from fungi: Chemistry and bioactivities," *Mini-Reviews in Medicinal Chemistry*, vol. 11, pp. 728-745, 2011.
[11] C. J. Guo, H. H. Yeh, Y. M. Chiang, J. F. Sanchez, S. L. Chang, K. S. Bruno, and C. C. C. Wang, "Biosynthetic pathway for the epipolythiodioxopiperazine acetylaranotin in aspergillus terreus revealed by genome-based deletion analysis," *J. Am. Chem. Soc.*, vol. 135, no. 19, pp. 7205–7213, 2013.
[12] K. B. Herath, H. Jayasuriya, J. G. Ondeyka, J. D. Polishook, G. F. Bills, *et al*., "Isolation and structures of novel fungus metabolites as chemokine receptor (CCR2) antagonists," *J. Antibiot*., vol. 58, no. 11, pp. 686-94, 2005.
[13] K. Terao, E. Ito, K. Kawai, K. Nozawa, and S. Udagawa, "Experimental acute poisoning in mice induced by emestrin, a new mycotoxin isolated from emericella species," *Mycopathologia*, vol. 112, no. 2, pp. 71-79, 1990.
[14] D. M. Vigushi, N. Mirsaidi, G. Brooke, C. Sun, P. Pace, *et al*., "Gliotoxin is a dual inhibitor of farnesyltransferase and geranylgeranyltransferase I with antitumor activity against breast cancer in vivo," *Med. Oncol.*, vol. 21, pp. 21–30, 2004.
[15] B. W. Son, P. R. Jensen, C. A. Kauffman, and W. Fenical, "New cytotoxic epidithiodioxopiperazines related to verticillin a from a marine isolate of the fungus penicillium," *Nat. Product Lett*., vol. 13, pp. 213–22, 1999.
[16] P. X. He, Y. S. Che, Q. J. He, Y. Chen, and J. Ding, "G226, a novel epipolythiodioxopiperazine derivative, induces autophagy and caspase-dependent apoptosis in human breast cancer cells in vitro," *Acta. Pharmacol. Sin.*, vol. 35, no. 8, pp. 1055–1064, 2014.

[17] M. Ranalli, A. Oberst, M. Corazzari, and V. D. Laurenzi, "Flow cytometric studies of cell death," in *Cell Proliferation and Apoptosis*, D. Hughes and H. Mehmet, Eds., Oxford: BIOS Scientific Publisher Limited, 2003.
[18] F. R. Yu, X. Z. Lian, H. Y. Guo, P. M. McGuire, R. D. Li, R. Wang, and F. H. Yu, "Isolation and characterization of methyl esters and derivatives from euphorbia kansui (Euphorbiaceae) and their inhibitory effects on the human SGC-7901 cells," *J. Pharmacol. Pharm. Sci*., vol. 8, no. 3, pp. 528-531, 2005.
[19] S. Elmore, "Apoptosis: A review of programmed cell death," *Toxicologic Pathology*, vol. 35, pp. 495–516, 2007.

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