

Sesquiterpene Aminoquinones, from a Marine Sponge, Induce Erythroid Differentiation in Human Chronic Myelogenous Leukemia, K562 Cells

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A new sesquiterpene aminoquinone, 5-*epi*-smenospongoringe, together with nine known sesquiterpene quinone/phenols, was isolated as differentiation-inducing substances to K562 cells into erythroblast from the marine sponge *Dactylosporgia elegans*. The structure–activity relationship study of these compounds clarified that the quinone skeleton is indispensable and the amino group plays an important role for their differentiation-inducing activity to K562 cells into erythroblast.

Key words 5-*epi*-smenospongoringe; erythroid differentiation; structure–activity relationship; K562 cells; marine sponge

Despite the various cancer therapeutic methods such as chemotherapy, which are being carried out clinically, cancer remains to be a serious and, in most cases, fatal disease. Chemotherapeutic drugs affect not only tumor cells but also proliferative normal cells such as bone marrow cells and result in serious side effects. To solve this problem, several other alternative therapeutic methods are being developed. The differentiation-induction therapy, in which tumor cells are differentiated to normal cells, seems to be one promising therapy. As a good example of differentiation inducer, ATRA (all-*trans*-retinoic acid) has shown superior therapeutic effect on acute promyelocytic leukemia (APL), which is unresponsive to conventional chemotherapeutic drugs.¹⁾ However, several kinds of leukemia such as human chronic myelogenous leukemia (CML) are unresponsive to ATRA. So, development of new differentiation inducers has been required.

As a part of our exploratory research of bioactive substances from marine organisms, we have been searching for new differentiation inducers to tumor cells and have found several inducers to ATRA-nonresponsive CML (K562 cells) or murine neuroblastoma (Neuro 2A cells) from marine sponges.^{2–4)} Recently, we have isolated from a marine sponge⁵⁾ a sesquiterpene aminoquinone, smenospongine (**4**), which induces differentiation of K562 cells into erythroblasts. Study on its action mechanism showed that smenospongine induced arrest of the cell cycle at the G1 phase and increased expression of p21 protein, a Cip1/Waf1 cdk (cyclin dependent kinase) inhibitor.⁵⁾ This interesting action of smenospongine led us to continue further study, and we isolated ten sesquiterpene quinones/phenol analogues from the same marine sponge, including a new compound named 5-*epi*-smenospongoringe (**1**). In this paper, the structure–activity relationship of these sesquiterpene quinones is discussed.

Results and Discussion

Chemical Structures of 5-*epi*-Smenospongoringe (1**) and the Analogues** The nine sesquiterpene quinone/phenols (**2**–**10**) together with a new compound named 5-*epi*-smenospongoringe (**1**) were isolated from the marine sponge *Dactylosporgia elegans*. (Fig. 1) Compounds **2**–**10** were identified as smenospongoringe,^{6,7)} 5-*epi*-smenospongoringe,^{6,8)} smenospongine,^{6,7,9)} 5-*epi*-smenospongidingine,⁶⁾ smenospongidingine,⁷⁾ 5-*epi*-ilimaquinone,⁶⁾ ilimaquinone,⁷⁾ dictyoceratin-

C,⁸⁾ and smenospondiol,^{6,7)} respectively, by comparison of the FAB-MS, ¹H- and ¹³C-NMR data with those of the authentic compounds.

The molecular formula of the new compound, 5-*epi*-smenospongoringe (**1**), was determined as C₂₅H₃₇NO₃ by HR-FAB-MS analysis. Compound **1** showed absorption bands ascribable to amino (3270 cm⁻¹) and carbonyl (1644 cm⁻¹) groups in the IR spectrum. The ¹H-NMR spectrum of **1** showed the signals assignable to two tertiary methyls (δ 1.06, 0.87, both s), three secondary methyls (δ 0.99, 6H d, $J=6.7$ Hz; δ 0.92, d, $J=6.1$ Hz), a benzylic methylene (δ 2.43 and 2.53, AB quartet, $J=13.8$ Hz), an exomethylene (δ 4.70, 4.67, both s), and an olefinic proton (δ 5.38, s). The ¹H- and ¹³C-NMR signals due to the decalin part and the quinone part in **1** were closely similar to those of 5-*epi*-smenospongidingine (**5**)⁶⁾ and smenospongoringe (**2**),⁷⁾ respectively. Then, it is presumed that compound **1** is a hybrid of 5-*epi*-smenospongidingine (**5**) and smenospongoringe (**2**).⁸⁾ This presumption that compound **1** is an epimer at the C-5 position of smenospongoringe (**2**) was further confirmed by nuclear overhauser effect spectroscopy (NOESY) analysis of both compounds **1** and **2**. Thus, the correlations between H-12 and H-10 for compound **1** and between H-12 and H-14 for compound **2** were found. Based on these evidences, compound **1** was determined as 5-*epi*-smenospongoringe as shown in Fig. 1.

Erythroid Differentiation of K562 Cells Induced by 5-

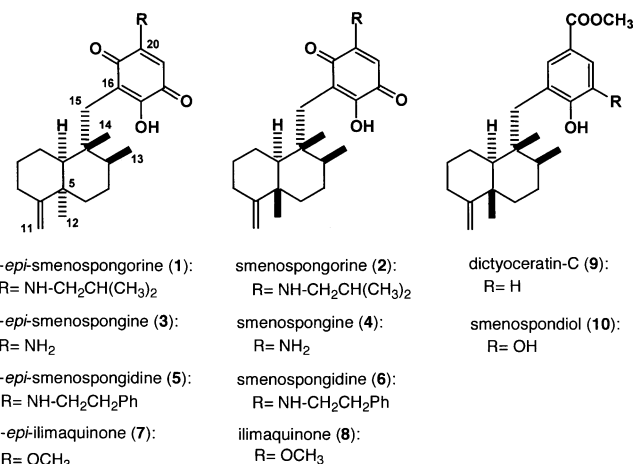


Fig. 1. Chemical Structures of 5-*epi*-Smenospongoringe and the Analogues

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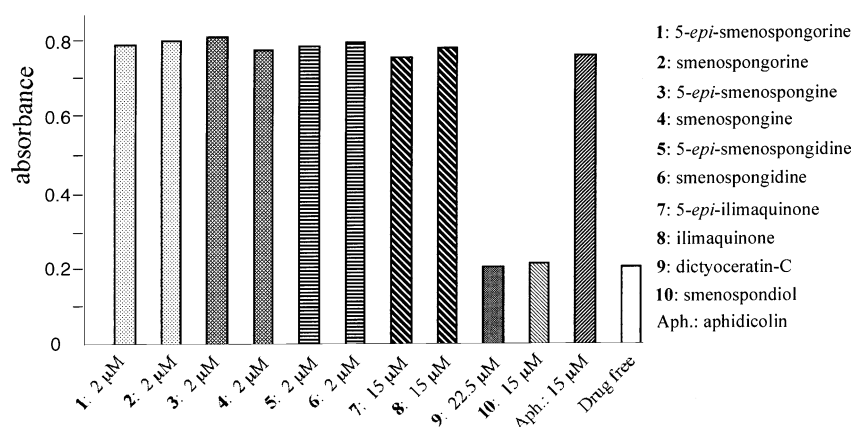


Fig. 2. The Induction of Hemoglobin Production by 5-*epi*-Smenospongorine and the Analogues

A suspension of 4×10^4 cells of K562 cells was treated with a testing sample and cultured for 4 d. The collected cells were treated with diaminofluorene solution and 3% hydrogen peroxide. The absorbance at 630 nm was measured by a microplate reader.

epi-Smenospongorine and the Related Compounds The differentiation-inducing activities of these compounds were evaluated by the induction of hemoglobin production in K562 cells. Thus, pseudo-peroxidase activity of hemoglobin was measured by the colorimetric assay using diaminofluorene. The lowest concentrations of each compound (1–8) indicating similar differentiation-inducing activity are shown in Fig. 2, respectively. And, the highest concentrations showing no cytotoxicity were chosen for the two inactive sesquiterpene phenols, 9 and 10. Firstly, each of six sesquiterpene aminoquinones (1–6) exhibited similar differentiation-inducing activity, which is stronger than that of aphidicolin, a well-known differentiation-inducing agent of K562 cells into erythroblasts.¹⁰ Secondly, compounds 9 and 10, which does not have quinone function, showed no activity. Thirdly, compounds 7 and 8 showed differentiation-inducing activities at the higher concentration than those of 1–6. On the basis of these structure–activity relationship studies, the following evidences were obtained. 1) The quinone structure is indispensable. 2) The amino group should play an important role. 3) The substituents at the amino group are not crucial. 4) The configuration at the C-5 in sesquiterpene part is not important.

So far, these sesquiterpene quinone/phenols have been reported to exhibit anti-microbial, anti-HIV, anti-malarial, and cytotoxic activities.^{9,11,12} In this study, we clarified that these sesquiterpene aminoquinones exhibit differentiation-inducing activity to K562 cells into erythroblasts. The clinical application of Glivec¹³ (a synthetic compound targeting Bcr-Abl (breakpoint cluster region-Abelson leukemia sequence),¹⁴ which has been recognized as the cause of CML) has provided light on a cure for this disease. However, the use of Glivec over extended periods caused drug-resistance in patients, and new types of differentiation inducers for CML have been required. These sesquiterpene aminoquinones having differentiation-inducing activities might be a promising agent for the treatment of CML.

Experimental

Materials Marine sponge *Dactylospongia elegans* was collected at West Flores, Indonesia in 2001. 2,7-Diaminofluorene and aphidicolin were purchased from Wako Pure Chemical Industries Co. Ltd.

Cell Lines Human chronic myelogenous leukemia cell line K562 was provided by RIKEN Cell Bank and routinely maintained in RPMI medium

Table 1. ¹H- and ¹³C-NMR Data for 5-*epi*-Smenospongorine (1)

No.	$\delta_c^a)$	δ (mult., J (Hz)) ^{b)}	No.	δ_c	δ (mult., J (Hz))
1	22.5	2.13 (m) 1.83 (m, overlapped)	11	105.7	4.70 (s) 4.67 (s)
2	25.0	1.77 (m) 1.68 (m)	12	33.1	1.06 (s)
3	32.9	2.43 (d, 13.8) 2.10 (m)	13	18.6	0.92 (d, 6.1)
4	153.6		14	18.3	0.87 (s)
5	39.5		15	32.0	2.53 (d, 13.8) 2.43 (d, 13.8)
6	37.9	1.99 (m) 1.13 (m)	16	113.7	
7	27.9 ^{c)}	1.84 (m, overlapped) 1.51 (m)	17	157.3	
8	39.3	1.22 (m)	18	178.4	
9	44.4		19	91.6	5.38 (s)
10	48.4	1.20 (m)	20	150.6	
			21	182.7	
			1'	50.4	2.99 (t, 6.4)
			2'	27.7 ^{c)}	1.96 (m)
			3', 4'	20.3	0.99 (d, 6.7)

a) 125 MHz in CDCl₃. b) 500 MHz in CDCl₃. c) Interchangeable.

supplemented with 10% fetal bovine serum, 100 μg/ml kanamycin, and 0.44 mg/ml glutamine at 37 °C in a humidified atmosphere of 5% CO₂.

Extraction and Isolation The frozen marine sponge (2.6 kg, wet weight) was cut into pieces and soaked in methanol overnight. The resulting methanol extract was subjected to solvent partition to give hexane, 90% methanol, *n*-BuOH, and H₂O phases. The 90% methanol phase was evaporated under reduced pressure to give 12 g of 90% MeOH extract. The 90% methanol extract (2 g), which exhibited differentiation-inducing activity, was separated by SiO₂ column (hexane–AcOEt–acetone–MeOH) to give three fractions [Fr. A (0.42 g), Fr. B (0.73 g), Fr. C (0.83 g)]. The active Fr. C was further separated by ODS column (Cosmosil ODS, MeOH–H₂O) and reversed-phase HPLC (CAPCELL PAK C18 UG120, 70% aq. MeOH–0.1% diethylamine) to obtain 1 (3 mg, 0.15% from the 90% MeOH extract), 2 (5 mg, 0.25%), 3 (19 mg, 0.95%), 4 (9 mg, 0.45%), 5 (3 mg, 0.15%), 6 (4 mg, 0.20%), 7 (5 mg, 0.25%), 8 (3 mg, 0.15%). The inactive Fr. A and Fr. B were also separated by ODS column (MeOH–H₂O) or HPLC (Cosmosil 5SL, hexane–AcOEt=7:1) to obtain 9 (20 mg, 1%) and 10 (17 mg, 0.85%).

5-*epi*-Smenospongorine (1): $[\alpha]_D^{23} + 23^\circ$ ($c=0.06$, CHCl₃). HR-FAB-MS m/z : Calcd for C₂₅H₃₈NO₃ ($[M+H]^+$): 400.2852; Found: 400.2842. UV λ_{max} (EtOH) nm (ϵ): 320 (9800), 205 (20500). IR (KBr): 3270, 1644 cm⁻¹. ¹H- and ¹³C-NMR: as shown in Table 1.

Induction of Hemoglobin Production in K562 Cells For the evaluation of hemoglobin production in K562 cells, pseudo-peroxidase activity of hemoglobin was measured by the colorimetric assay using diaminofluorene.² The suspension of K562 cells (4×10^4 cells/2 ml/well) was placed in a 24-well plate. After addition of 10 μl of the DMSO solution of the testing sample, the culture was incubated at 37 °C under 5% CO₂ atmosphere for 4 d. The culture medium was centrifuged at 3000 *g* for 3 min, and the resulting supernatant was removed. After addition of 50 μl of ultra-pure water, the

lysed cells were treated with 100 μ l of the diaminofluorene solution [5 mg of diaminofluorene was dissolved in 1 ml of the 90% acetic acid and Tris-HCl buffer (pH 7.5) (10:1) solution] and 10 μ l of 3% hydrogen peroxide and then kept for 30 min in the dark. The absorbance at 630 nm was measured by a microplate reader. The drug-untreated cells were used as the negative control.

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