Fluorescent image-based high-content screening of extracts of natural resources for cell cycle inhibitors and identification of a new sesquiterpene quinone from the sponge, *Dactylospongia metachromia*

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ABSTRACT

Natural products are important sources for drug development. Discovery of natural products that inhibit cell cycle progression significantly contributes to the progress of cancer biology and the development of new antitumor agents. In this study, cell cycle inhibitory activity was evaluated with our extract library of natural resources, including marine invertebrates, fungi, and bacteria, using HeLa/Fucci2 cells which allow classification of the cell cycle phases of living cells. Screening of the extract library revealed that the extract of the marine sponge *Dactylospongia metachromia* inhibited cell cycle progression at S/G2/M phases. Bioassay-guided fractionation afforded a new sesquiterpene quinone, neoisosmenospongine (**1**), and four known compounds, nakijiquinone I, N, and Q (**2**–**4**) and (–)-dictyoceratin-C (**5**). The chemical structure of **1** was elucidated by interpretating the NMR and mass spectroscopic data, and the absolute configuration was determined by comparison of the experimental and calculated ECD spectra. Fluorescent imaging of HeLa/Fucci2 cells revealed that **1**–**4** inhibited the cell cycle progression at S/G2/M phases. This study demonstrated that fluorescent image-based high-content screening using HeLa/Fucci2 cells is an effective approach for isolating cell cycle inhibitors from natural resources.

Keywords: Cell cycle, Fucci, Natural product, Marine sponge, Sesquiterpene quinone

1. Introduction

Cell proliferation is a fundamental biological process in eukaryotic cells. The cell cycle is divided into two stages, the interphase and the mitotic (M) phase, with the interphase consisting of G1, S, and G2 phases. During cell cycle progression, DNA in the chromosomes is replicated in the S phase and then divided into two genetically identical daughter cells in the M phase. The transition of cell cycle phases is regulated by the coordinated expression of various proteins including cyclin and cyclin-dependent kinases. Oncogenic stresses damage DNA, induce errors in DNA replication, and then elicit p53-dependent cell cycle arrest and apoptosis, whereas aberration of the normal signal transduction leads to uncontrolled and sustained cell proliferation.[1] Many anticancer drugs damage DNA and inhibit DNA replication in order to induce death of cancer cells.[2] Although the technique of combinatorial chemistry was developed in the 1990s and has been successfully used to optimize the structures of pharmaceutical agents, approximately 65% of anticancer drugs were developed from natural products[3] and discovery of cell cycle inhibitors from natural resources remains a promising approach for the discovery of new anticancer agents.[4]

In this study, we conducted a fluorescent image-based high-content screening to identify natural products that inhibit cell cycle progression in HeLa/Fucci2 cells. Conventional cell cycle analysis requires cell fixing and DNA staining with fluorescent dyes followed by the quantification of DNA content using a flow cytometer. Cell cycle analysis by flow cytometer is time-consuming and therefore unsuitable for high-

throughput screening. Fucci (Fluorescent Ubiquitination-based Cell Cycle Indicator) is a newly-developed fluorescent cell cycle probe, which allows visualization of the cell cycle progression of individual live cells.[5] Fucci2, the second generation of the Fucci probe, consists of dual-color fluorescent probes: a red fluorescent protein (mCherry) fused Cdt1 and a green fluorescent protein (mVenus) fused Geminin.[6] Cdt1, a DNA replication licensing factor, is expressed in G1 phase and degraded by the ubiquitin-proteasome system at G1/S phase transition, while Geminin is expressed in S/G2/M phases and degraded at the end of cytokinesis. HeLa/Fucci2 cells, stably expressing Fucci2 probe, accumulate the red fluorescent protein in their nuclei at G1 phase and the green fluorescent protein at S/G2/M phases.

We screened our in-house library composed of extracts of marine invertebrates, fungi, and bacteria for inhibitory activity toward cell cycles using HeLa/Fucci2 cells. Several extracts were found to inhibit cell cycle progression at S/G2/M or G1 phase, and among these the extract of the marine sponge *Dactylospongia metachromia*, collected in Indonesia, induced S/G2/M arrest. Herein, we report the fluorescent image-based high content screening of cell cycle inhibitors from natural resources, as well as the isolation, structure elucidation, and biological activities of a new sesquiterpene quinone, neoisosmenospongine (**1**), and four known compounds, nakijiquinones I, N, and Q (**2**–**4**)[7,8] and (–)-dictyoceratin-C (**5**).[9]

2. Results and discussion

2.1. Screening of cell cycle inhibitors from the extract library of natural resources

HeLa/Fucci2 cells in 96 well plates were incubated in the presence of crude extracts of natural resources for two days, and fluorescent microscopic images were taken (Fig. 1A). The number of cells expressing green or red fluorescence was automatically counted and the ratio of their numbers was calculated as a G/R value. The control cells showed a G/R value of 0.64. Samples with values larger than 1.20 were defined as inhibiting cell cycle progression at S/G2/M phases, and samples with values smaller than 0.40 were evaluated as inducing G1 arrest. Among the 5,687 extracts, 161 and 31 samples were found to induce S/G2/M and G1 arrest, respectively (Fig. 1B). Because cytotoxic samples do not show proper G/R values, 621 samples with cell viabilities less than 40% were excluded. In addition, 133 samples emitting autofluorescence were also excluded. This fluorescent image-based high-content screening using HeLa/Fucci2 cells successfully detected several extracts of natural resources that inhibited cell cycle progression.

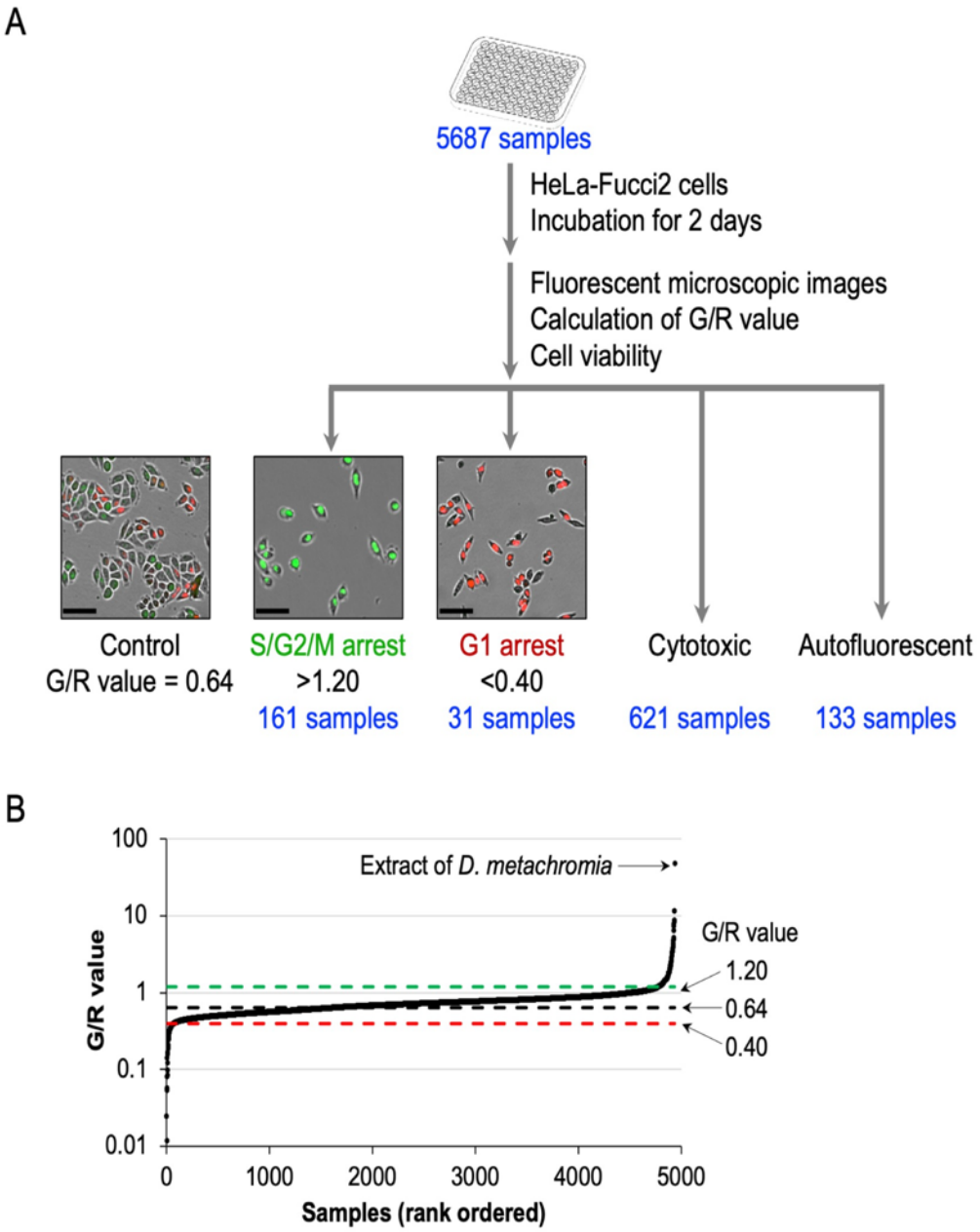

Fig. 1. Screening of cell cycle inhibitors from the extract library of natural resources using HeLa/Fucci2 cells. (A) Flow of the screening and fluorescent microscopic images of HeLa/Fucci2 cells treated with DMSO (control) and the extracts inducing S/G2/M and G1 arrest. Scale bar represents 100 μm. (B) Results of screening of cell cycle inhibitory activity with the extract library. G/R value is the ratio of the number of cells expressing green vs. red fluorescence. Black, green, and red dashed lines represent G/R values at 0.64, 1.20, and 0.40, respectively.

2.2. Isolation of cell cycle inhibitors from the marine sponge, D. metachromia

The extract of the marine sponge *D. metachromia* showed a G/R value of 48 (Fig. 1B), which indicated S/G2/M arrest. In order to identify the inhibitors, the extract was partitioned between water and EtOAc and the aqueous residue was successively extracted with *n*-BuOH. The EtOAc layer was concentrated and partitioned between *n*-hexane and 90% MeOH–H_2O. The *n*-BuOH and 90% MeOH–H_2O residues were subjected to column chromatography followed by HPLC to afford a new compound **1**, together with four known compounds, nakijiquinones I, N, and Q (**2**–**4**)[7,8] and (–)-dictyoceratin-C (**5**) (Fig. 2).[9]

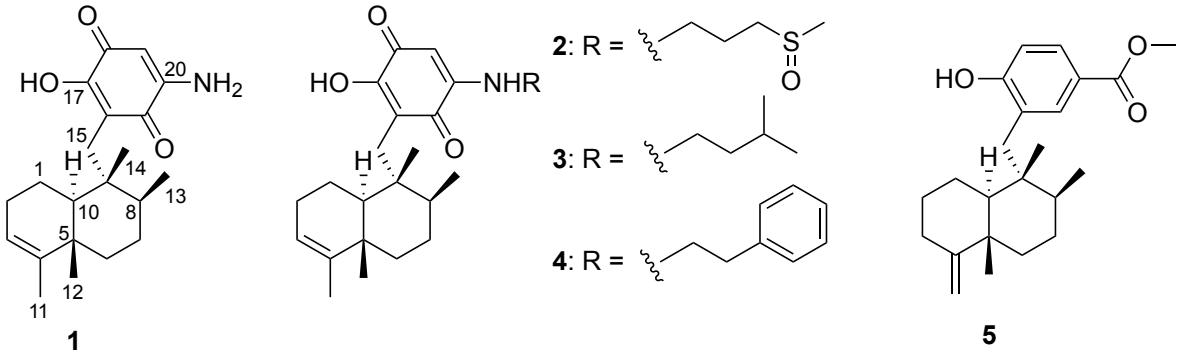

Fig. 2. Chemical structures of sesquiterpene quinone **1** and its derivatives (**2**–**5**) isolated from the sponge, *D. metachromia.*

2.3. Structure elucidation of cell cycle inhibitors

Compounds **2**–**5** were identified as nakijiquinones I, N, and Q[7,8] and (–)-dictyoceratin-C,[9] respectively, by comparing their spectroscopic data with the reported values. The molecular formula of **1** was established

as $C_{21}H_{29}NO_3$ by HRESIMS. ^1H and ^{13}C NMR spectra of **1** (Table 1) were similar to those of nakijiquinones **2**–**4**, indicating the presence of the sesquiterpene quinone framework in **1**. Interpretation of 1D and 2D NMR spectra of **1** confirmed a 4,9-friedodorimane rearranged sesquiterpene scaffold (Fig. 3A). A 2-amino-5-hydroxy benzoquinone moiety of **1** was assigned by HMBC correlations from H-19 (δ_H 5.60, s) to C-17 (δ_C 156.1) and C-21 (δ_C 183.1) and from H-15a (δ_H 2.55, d) and H-15b (δ_H 2.40, d) to C-16 (δ_C 35.9), C-17 (δ_C 156.1), and C-21 (δ_C 183.1) (Fig. 3A). The relative configuration of the sesquiterpene moiety was investigated on the basis of ^{13}C NMR chemical shifts and NOE correlations. Stothers' group originally showed that the configurations of the A/B ring junctions of steroids were differentiated by the ^{13}C chemical shift of the angular methyl carbon, C-19 (steroidal number);[10] the methyl signal of the *trans*-isomer is observed 11–12 ppm upfield from that of the *cis*-isomer. Subsequently, Minale's group showed that this difference is diagnostic of *cis*/*trans*-ring fusion in a series of derivatives of avarol, a rearranged sesquiterpenoid hydroquinone.[11] The ^{13}C chemical shift of C-12 (δ_C 20.1) of **1** matched that of isospongiaquinone (δ_C 20.2), which contains a *trans*-4,9-friedodorim-3-ene scaffold, but differed from that of 5-*epi*-isospongiaquinone (δ_C 32.2),[12] having a *cis* ring junction. The *trans* ring junction in **1** was supported by NOE correlations from H_3-12 to H-7β and H_3-14 and from H-10 to H-8, which revealed that Me-12 and Me-14 were β-oriented and H-10 and H-8 were α-orientated (Fig. 3B). Thus, the relative configuration of **1** was assigned to be 5*S**,8*S**,9*R**,10*S**.

Table 1

^{13}C and ^{1}H NMR data for **1** in $CDCl_3$.

no.	δ_C, type	δ_H (*J* in Hz)	HMBC
1α	19.9, CH_2	2.03, m	2, 3, 5, 10
1β		1.43, m	
2α	27.0, CH_2	1.84, m	3, 4
2β		1.96, m	
3	120.8, CH	5.10, s	1, 5, 11
4	144.1, C		
5	38.5, C		
6α	35.9, CH_2	1.02, m	8, 12
6β		1.59, m	8, 10
7α	27.9, CH_2	1.24, m	9
7β		1.32, m	5, 6, 8, 9, 13
8	37.7, CH	1.23, m	7
9	42.7, C		
10	47.6, CH	1.00, m	1, 2, 5, 9, 14
11	18.2, CH_3	1.50, s	3, 4, 5
12	20.1, CH_3	0.97, s	4, 5, 6, 10
13	17.7, CH_3	0.93, d (4.6)	7, 8, 9
14	17.3, CH_3	0.80, s	8, 9, 10, 15
15a	32.3, CH_2	2.55, d (14.2)	8, 9, 10, 14, 16, 17, 21
15b		2.40, d (14.2)	8, 9, 10, 14, 16, 17, 21
16	114.3, C		
17	156.1, C		
18	179.5, C		
19	95.5, CH	5.60, s	17, 21
20	150.9, C		
21	183.1, C		

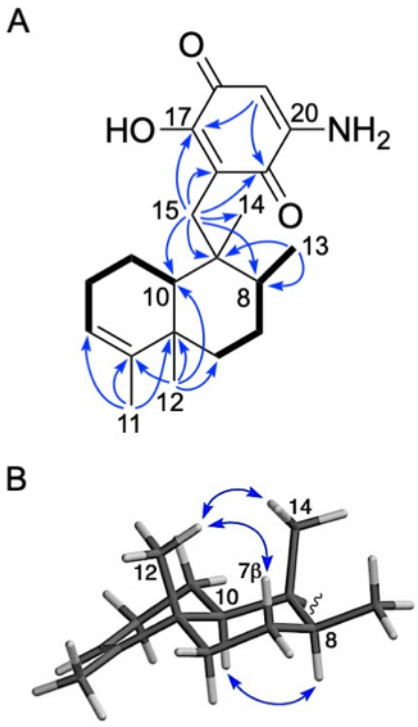

Fig. 3. (A) COSY (bold lines) and key HMBC (arrows) correlations of **1**. (B) Key NOE correlations and the relative configuration of the sesquiterpene moiety of **1**.

The absolute configuration of **1** was investigated using the experimental and calculated ECD spectra (Fig. 4). A negative Cotton effect around 200 nm for **1** matched with that of 5*S*,8*S*,9*R*,10*S*-**1**, and the absolute configuration of **1** was determined to be 5*S*,8*S*,9*R*,10*S*. We found that the chemical structure of **1** was identical to that of a previously reported compound (**13** in reference 13), which is a reaction product of the acid rearrangement of smenospongine. However, the ^{13}C chemical shifts of **13**[13] and **1** were not identical. The ^{1}H and ^{13}C NMR data of **13** were not assigned by 2D NMR, but the ^{13}C chemical shifts of four methyl

signals were observed at δ_C 17.8, 18.2, 20.6, and 32.8.[13] Compared to the reported ^{13}C chemical shifts of sesquiterpene quinones,[12,14] these methyl signals were indicated to be Me-14, Me-13, Me-11, and Me-12, respectively, of a *cis*-fused 4,9-friedodorim-3-ene derivative.[12,14] These data clearly showed that the structure of **13**[13] should be revised as a *cis* isomer. Unfortunately, the compound designated isosmenospongine[15] was reported as having the same structure as **13**.[13] Although no NMR data were reported for isosmenospongine, the authors referred to the publication of **13**[13] as a reference, and therefore the structure of isosmenospongine should be also revised as the *cis* isomer. Here, we report the correct structure of a new sesquiterpene quinone containing a 2-amino-5-hydroxy benzoquinone with a *trans*-4,9-friedodorim-3-ene skeleton elucidated by NMR spectroscopy and ECD calculation. Although **1** is the structural isomer of smenospongine,[16] the trivial name, isosmenospongine, was already used for the *cis* isomer, and we therefore named **1** neoisosmenospongine.

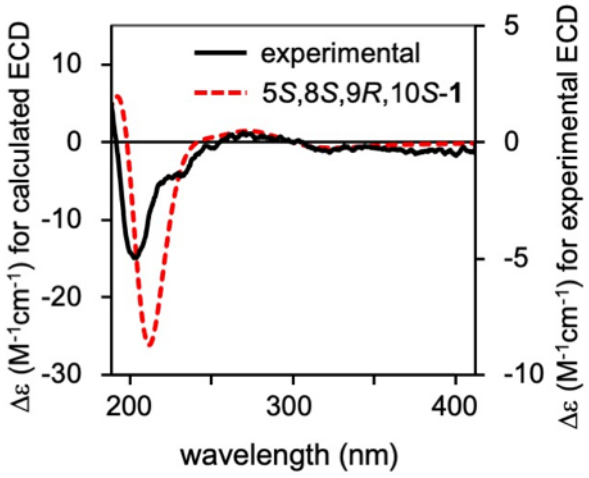

Fig. 4. Experimental ECD spectrum of **1** and calculated spectrum of 5*S*,8*S*,9*R*,10*S*-**1**.

*2.4. Cell cycle inhibitory activities of **1**–**5***

Compounds **1**–**5** moderately inhibited cell growth of HeLa/Fucci2 cells with IC_{50} values of 18, 16, 30, 36, and 13 μM, respectively. Their effects on cell cycle progression were observed under a fluorescent microscope. After treatment with **1**–**5** at concentrations of double their IC_{50} values for two days, **1**–**4** inhibited the cell cycle at S/G2/M phases, whereas **5** did not perturb cell cycle progression (Fig. 5A). Next, the cell cycle distribution in the presence of **1** was analyzed by flow cytometry using HeLa cells (Fig. 5B). After one-day incubation with **1** (15 μM), the percentage of cells at G1 phase (27.2%) was decreased compared to the control (59.7%), whereas those at S phase (46.5%) and G2/M phases (26.3%) were increased (26.1% and 14.2%, respectively). Thus, the fluorescent microscopic imaging of HeLa/Fuci2 cells

and the cell cycle analysis by flow cytometer confirmed that **1** inhibited cell cycle progression at S/G2/M phases.

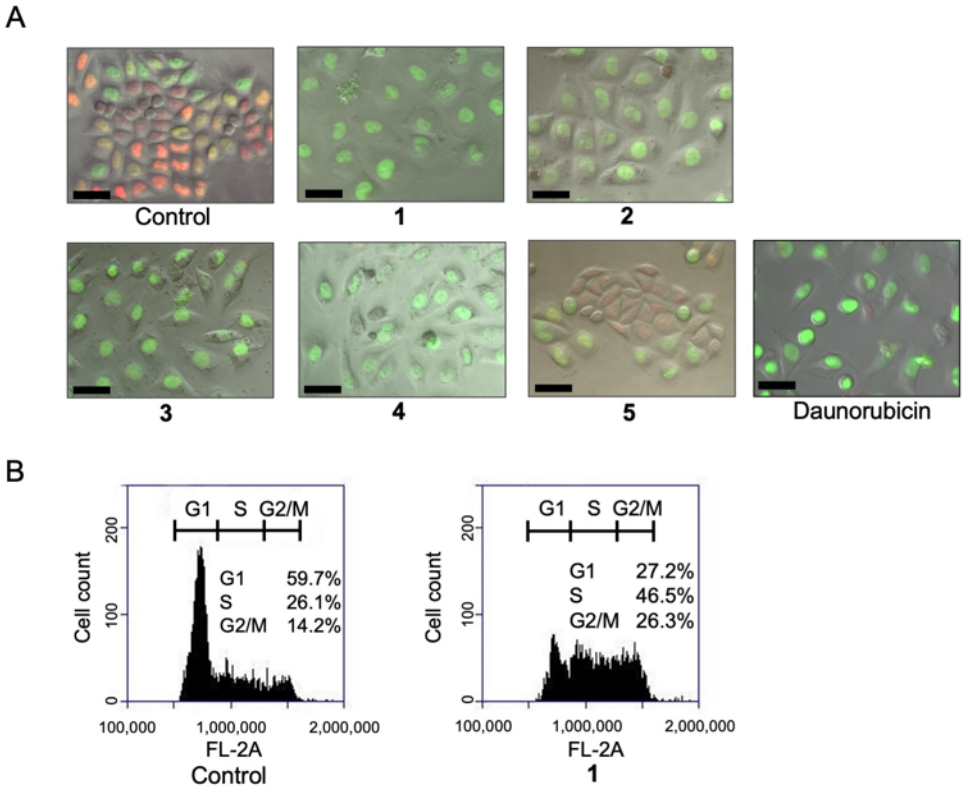

Fig. 5. Evaluation of cell cycle inhibitory effect of **1**–**5**. (A) Fluorescent microscopic images of HeLa/Fucci2 cells treated with DMSO (control), **1**–**5** at concentrations of double their IC_{50} values, or daunorubicin (0.2 μM) for two days. Scale bar represents 50 μm. (B) Flow cytometry analysis with HeLa cells treated with **1** (15 μM). After one-day incubation, cells were stained with propidium iodide and the DNA content and the distribution of the cell cycle phases were analyzed by flow cytometer.

3. Conclusion

In this study, we demonstrated fluorescent image-based high-content screening of an extract library of natural resources using HeLa/Fucci2 cells, and successfully identified several extracts that inhibited cell cycle progression. Using this method, we isolated the sesquiterpenoids inhibiting cell cycle progression at S/G2/M phases from the marine sponge, *D. metachromia*. A number of cognate sesquiterpene quinones/hydroquinones have been isolated from marine organisms,[17] and this class of compounds is known to exhibit broad biological activities. Some compounds were reported to inhibit cell cycle progression, but the mechanisms of their biological activities remained unclear.[18,19] 1,4-Benzoquinone and hydroquinone are known to generate reactive oxygen species to induce oxidative DNA damage.[20] A recent study suggested that sesquiterpene quinones damage DNA and thereby induce a DNA damage response.[21] The cell cycle inhibitory activities of **1**–**5** may be attributed to this oxidative DNA damage. Searching for cell cycle inhibitors from natural resources using our screening approach will lead to the discovery of new anticancer agents derived from natural products.

4. Experimental

4.1. General experimental procedures

Optical rotation was measured on a JASCO DIP-1000 polarimeter in $CHCl_3$. UV spectrum was measured on a JASCO V-550 spectrophotometer in MeCN. IR spectrum was recorded on a PerkinElmer

Frontier FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 600 NMR spectrometer in $CDCl_3$. Chemical shifts were referenced to the residual solvent peaks (δ_H 7.24 and δ_C 77.0 for $CDCl_3$). HRESIMS spectrum was measured on a Bruker Impact II mass spectrometer. Fluorescent microscopic images were obtained using a BioTek Cytation 1 cell imaging multi-mode reader and a Leica DMi8 microscope. Analysis of DNA content was conducted using a BD Accuri™ C6 flow cytometer. HeLa/Fucci2 cells were purchased from RIKEN BRC, Japan.

4.2. Animal material

The marine sponge was collected by scuba at a depth of 10 m at Bajotalawaan in North Sulawesi, Indonesia, in December 2006 and immediately soaked in EtOH. The sponge was identified as *Dactylospongia metachromia* by one of the authors (Y.I.). A voucher specimen (06M116) of the sponge has been deposited at the Department of Natural Medicines, Graduate School of Pharmaceutical Sciences, Kumamoto University.

4.3. Extraction and isolation

The marine sponge (wet weight 500 g) was extracted with EtOH and MeOH. The extract was concentrated and partitioned between EtOAc and H_2O, and the aqueous fraction was extracted with *n*-BuOH

(6.8 g). The EtOAc fraction (1.5 g) was partitioned between *n*-hexane and 90% MeOH–H_2O (0.9 g). The 90% MeOH–H_2O fraction was subjected to silica gel column chromatography with a stepwise gradient using CH_2Cl_2, CH_2Cl_2/MeOH (20:1, 10:1, and 5:1), MeOH, and CH_2Cl_2/MeOH/H_2O (6:4:1). The CH_2Cl_2 eluate (39 mg) was purified by silica gel HPLC (YMC-Pack R&D D-SIL-5 column, YMC Co., Ltd., 20 × 250 mm) with *n*-hexane/EtOAC (9:1) to yield **5** (8.3 mg). The CH_2Cl_2/MeOH (20:1) eluate (47 mg) was fractionated by ODS column chromatography with a stepwise gradient using MeOH/H_2O (1:1 and 4:1), MeOH, and CH_2Cl_2/MeOH/H_2O (6:4:1). The MeOH eluate (37 mg) was purified by silica gel HPLC (YMC-Pack R&D D-SIL-5 column, 20 × 250 mm) with *n*-hexane/EtOAc (4:1) to yield **1** (8.6 mg). The CH_2Cl_2/MeOH (10:1) eluate (211 mg) was subjected to ODS column chromatography with a stepwise gradient using MeOH/H_2O (3:2 and 4:1), MeOH, and CH_2Cl_2/MeOH/H_2O (6:4:1). The MeOH eluate (83 mg) was purified by ODS HPLC (COSMOSIL 5C18-AR-II column, Nacalai Tesque Inc., 20 × 250 mm) with MeOH/H_2O (17:3) to yield **1** (4.6 mg). The *n*-BuOH fraction was subjected to silica gel column chromatography with a stepwise gradient using CH_2Cl_2, CH_2Cl_2/MeOH (19:1, 9:1, and 4:1), MeOH, and CH_2Cl_2/MeOH/H_2O (6:4:1). The CH_2Cl_2 eluate (13.9 mg) was purified by silica gel HPLC (YMC-Pack R&D D-SIL-5 column, 20 × 250 mm) with *n*-hexane/EtOAC (9:1) to yield **2** (3.6 mg) and **3** (3.7 mg). The CH_2Cl_2/MeOH (19:1) eluate (77 mg) was fractionated by ODS column chromatography with a stepwise gradient using MeOH/H_2O (1:4, 2:3, 3:2, and 4:1), MeOH, and MeOH containing 0.02% CF_3COOH. The MeOH eluate (28 mg) was further

purified by ODS HPLC (COSMOSIL 5C18-MS-II column, 20 × 250 mm) with a linear gradient of 20–100% MeCN–H_2O containing 0.1% AcOH to yield **1** (8.5 mg) and **4** (2.6 mg).

*4.3.1. Neoisosmenospongine (**1**)*

Red-purple solid; $[\alpha]^{26}_D$ +70.0 (*c* 0.1, MeOH); UV (MeCN) λ_{max} (log ε) 314 (3.93) nm; IR (film) ν_{max} 3299, 2922, 2330, 2117, 1592, 1379, 1339, 1215 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 366.2039 $[M + Na]^+$ (calcd for $C_{21}H_{29}NNaO_3$, 366.2040).

*4.4. Conformational analysis and ECD calculation for **1***

Conformational analysis and ECD calculation were carried out as previously described.[22] ECD calculation was performed at the BHandHLYP/TZVP level. The calculated ECD spectrum was obtained after wavelength correction with the experimental UV absorption peak at 314 nm.

4.5. Cell culture

HeLa cells and HeLa/Fucci2 cells were cultured in Dulbecco's modified Eagle's medium (Fujifilm Wako, 041-29775) supplemented with 10% FBS (Capricorn Scientific, FBS-12B) and 1% penicillin-streptomycin (Fujifilm Wako, 168-23191) at 37 °C under an atmosphere of 5% CO_2.

4.6. MTT viability test

MTT assay was conducted as previously described[23] and absorbance at 570 nm was measured using a BioTek Cytation 1 cell imaging multi-mode reader.

4.7. Screening of natural product extracts with fluorescent imaging of HeLa/Fucci2 cells

HeLa/Fucci2 cells were suspended in FluoroBrite DMEM media (Thermo Fisher Scientific K.K., A1896701) supplemented with 10% FBS, 1% penicillin-streptomycin, and 4 mM L-glutamine. The cell suspension (0.2 mL, 25,000 cells/mL) was seeded into a 96-well plate in the presence of extracts of natural resources (1 μL) and incubated at 37 °C under an atmosphere of 5% CO_2 for two days. Fluorescent microscopic images were acquired using a BioTek Cytation 1 cell imaging multi-mode reader, and data were processed by Gen5 data analysis software (BioTek Instruments, Inc.).

4.8. Analysis of HeLa/Fucci2 cells by fluorescent imaging

The HeLa/Fucci2 cell suspension (0.1 mL, 25,000 cells/mL) in FluoroBrite DMEM media was seeded into a 96-well plate and incubated overnight. Sample solution (1 μL) in DMSO was added to each well and incubated for two days. Fluorescent microscopic images were obtained using a Leica DMi8 microscope.

4.9. Flow cytometry

Because HeLa/Fucci2 cells have fluorescent proteins, flow cytometry analysis was performed with HeLa cells. Cells were seeded into six-well plates at a concentration of 4×10^5 cells/well and incubated overnight at 37 °C under an atmosphere of 5% CO_2. Cells were then incubated for 48 h in the presence of **1** (15 μM). Cells were trypsinized and fixed with 70% ice-cold EtOH at 4 °C for 2 h. After washing with PBS, cells were treated with RNase (Sigma-Aldrich, R5000) solution (0.25 mg/mL in PBS) and incubated at 37 °C for 20 min. Propidium iodide solution (50 μg/mL in PBS) was added to the cell suspension and left at 4 °C for 30 min. After filtration with a 40 μm nylon mesh filter, the DNA content and cell cycle distribution were analyzed by a BD Accuri™ C6 flow cytometer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2020.xxxxx.

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