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Polyacetylenes from *Panax stipuleanatus* and their Cytotoxic Effects on Human Cancer Cells

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Panax stipuleanatus Tsai et Feng (Araliaceae) is an herb that grows in Southeast Yunnan, China, and North Vietnam. In China, it has traditionally been used as a tonic and in the treatment of bruises, bleeding, and muscular pain.[1] To date, oleanane-type triterpenoids have been reported to be major components of *P. stipuleanatus*,[1] and stipuleanosides R_1 and R_2 were isolated from a methanol extract of this plant.[2] In a previous investigation, we isolated eleven oleanane type triterpenoids from *P. stipuleanatus* and investigated their cytotoxicity on the HL-60 and HCT-116 cancer cell lines.

In the current study, two new polyacetylenes, stipudiol (**1**) and stipuol (**2**), together with a polyacetylene, (3*R*,9*R*,10*R*)-panaxytriol (**3**), a sesquiterpene, spathulenol (**4**), and a fatty acid, and 9-docosenoic acid methyl ester (**5**) were isolated from the rhizomes of this *P. stipuleanatus* (Fig. 1).

Stipudiol (**1**) was obtained as yellow oil. The ^{1}H-NMR spectrum contained the presence of proton signals at δ 5.27 (1H, d, J = 9.9 Hz), 5.49 (1H, d, J = 16.1 Hz), and 5.95 ppm (1H, ddd, J = 16.1, 9.9, 5.2 Hz), two oxygenated proton signals at δ 4.98 (1H, d, J = 5.2 Hz) and 4.20 ppm (1H, dt, J = 5.4, 6.2 Hz), and two olefinic proton signals at δ 5.78 (1H, d, J = 16.4 Hz) and 6.35 ppm (1H, dd, J = 6.2, 16.4 Hz). The ^{13}C-NMR contained 18 peaks, including four diyne carbon signals at δ 70.9, 73.6, 77.6, and 80.4 ppm, four olefinic carbon signals at δ 108.1, 117.3, 136.0, and 150.0 ppm, and two oxygenated carbon signals at δ 63.7 and 72.1. These signals were assigned to one methyl group, eight methylene groups, five methine carbon atoms, and four alkynyl carbon atoms, in accordance with correlations observed in HMQC spectra. These signals are similar to those of (3*R*,9*R*,10*R*)-panaxytriol (**3**), except for two olefinic signals at δ_C 108.1 and 150.0 ppm, which were assigned to C-8 and C-9 by analysis of HMBC spectral data (Fig. 2). Furthermore, long-range couplings were observed between one proton signal at δ 5.49 ppm (H-1) and two carbon signals at δ 136.0 (C-2) and 63.7 ppm (C-3), one proton signal at δ 4.98 ppm (H-3) and three carbon signals at δ 117.3 (C-1), 136.0 (C-2) and

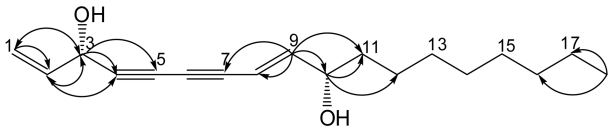

Figure 2. The selected HMBC correlations of compound **1**.

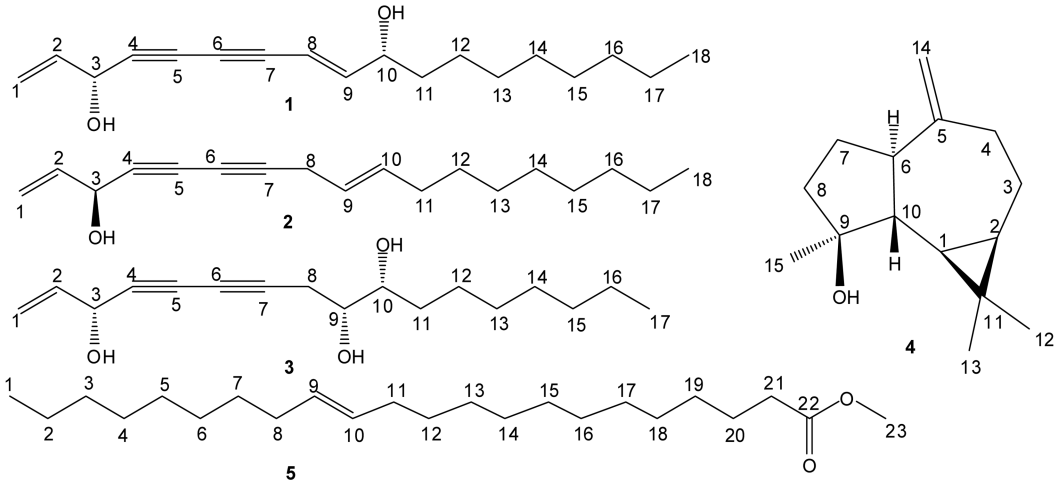

Figure 1. Structures of compounds **1-5** isolated from *P. stipuleanatus*.

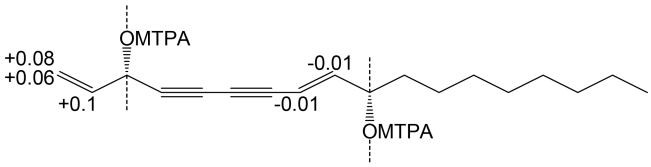

Figure 3. Results with the modified Mosher's method ($\Delta\delta_S$-δ_R) of compound **1**.

80.4 ppm (C-4), and one proton signal at δ 6.35 ppm (H-9) and four carbon signals at δ 77.6 (C-7), 108.1 (C-8), 72.1 (C-10), and 36.9 ppm (C-11). Negative ion mode APCI-MS revealed a peak at 275 $[M-H]^-$, and analysis of ^{13}C and DEPT-145 spectra data indicated a molecular formula of $C_{18}H_{26}O_2$. Compound **1** was subjected to the modified Mosher's method to yield (*S*)- and (*R*)-MTPA derivatives (**1a**, **1b**) for which the signals corresponding to H_2-1 and H-2 were observed at a lower field in **1a** than in **1b** (Fig. 3). In contrast, the signals for H-8 and H-9 were observed at a higher field in **1a** than in **1b**. Thus, the configurations at C-3 and C-10 in **1** were confirmed to be *R* and the structure was determined to be 1,8-octadecadiene-4,6-diyn-3*R*,10*R*-diol, named stipudiol. Previously, Kazuhiro Hirakura et al isolated and reported the 1,8-heptadecadiene-4,6-diyn-3,10-diol from roots of *P. ginseng*.[3] Compared with the compound from ginseng roots, compound **1** has one more methylene group. From all the above information, we concluded that compound **1** was isolated for the first time from nature.

Stipuol (**2**) was obtained as orange oil. The ^1H-NMR and ^{13}C-NMR spectral data were similar to those of (3*R*,9*R*,10*R*)-panaxytriol (**3**) with the exception of the hydroxyl group at the 9-position. The ^1H-NMR spectrum contained proton signals at δ 5.24 (1H, d, J = 10.3 Hz), 5.47 (1H, d, J = 17.1 Hz), and 5.96 ppm (1H, ddd, J = 17.1, 10.3, 5.5 Hz), oxygenated proton signals at δ 4.91 ppm (1H, d, J = 5.5 Hz) and two olefinic proton signals at δ 5.39 (1H, m) and 5.53 ppm (1H, m). The ^{13}C-NMR spectrum also contained 18 carbon signals including four olefinic carbon signals at δ 117.1, 122.0, 133.1, 136.2 ppm and one oxygenated carbon signal at δ 63.5 ppm. Negative ion mode ESI-MS revealed a peak at 257 $[M-H]^-$, and analysis of ^{13}C and DEPT-145 spectra data indicated a molecular formula of $C_{18}H_{26}O$. The absolute configuration of C-3 was determined as *S* according to the optical rotation value of compound **2** ($[\alpha]_D$ + 11.2), which is in good agreement with that of 3*S*-panaxydiol ($[\alpha]_D$ + 30.3) and 3*R*-panaxydiol ($[\alpha]_D$ – 57.5).[4] Based on these NMR spectra and compared with panaxynol,[5] compound **2** showed one more methylene group. Thus the compound **2** was identified as 1,9-octadecadie-ne-4,6-diyn-3*S*-ol, named stipuol.

The known compounds yielded experimental data in good agreement with previously reported NMR data and were identified as (3*R*,9*R*,10*R*)-panaxytriol (**3**),[6] spathulenol (**4**)[7] and 9-docosenoic acid methyl ester (**5**).[8]

Cytotoxic activity of compounds **3** has been reported by Yoshio Satoh.[9] The cytotoxic activities of the other compounds in this study were examined against human acute promyelocytic leukemia (HL-60) and human colon cancer (HCT-116) cells using a 3-(dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described by Mosmann.[9] Among them, compounds **1** and **2** showed significant cytotoxic activity, with IC_{50} values of 0.13 and 0.28 μM against HL-60 and 0.50 and 0.80 μM against HCT-116 cells, respectively (Table 1).

Table 1. Effects of isolated compounds on the growth of HL-60 and HCT-116 human cancer cells

Compound	IC_{50} (μM)[a]	
	HL-60 (leukemia)	HCT-116 (colon)
1	0.13 ± 0.15	0.50 ± 0.39
2	0.28 ± 0.08	0.80 ± 0.11
4	51.05 ± 0.60	>100
5	53.04 ± 0.63	69.66 ± 0.75
Mitoxantrone[b]	0.01 ± 0.01	2.68 ± 0.56

[a]Results are the means ± SD of three independent experiments in triplicate, and values < 100 μM are considered to be active. [b]Positive control.

To determine the cytotoxic mechanism, we investigated whether the inhibitory effects of compounds **1** and **2** on the proliferation of HL-60 and HCT-116 cells might arise from the induction of apoptosis. HL-60 and HCT-116 cells were treated with the IC_{50} concentration of compounds **1** and **2** for 24 h and the apoptotic characteristics were examined by Hoechst 33342 staining (Fig. 4). In all cases, critical markers of cell apoptosis, including nuclear morphological changes, chromatin condensation, membrane blebbing, and cell shrinkage, were observed. These results indicate that compounds **1** and **2** markedly inhibited the proliferation of HL-60 and HCT-116 cells by inducting apoptosis.

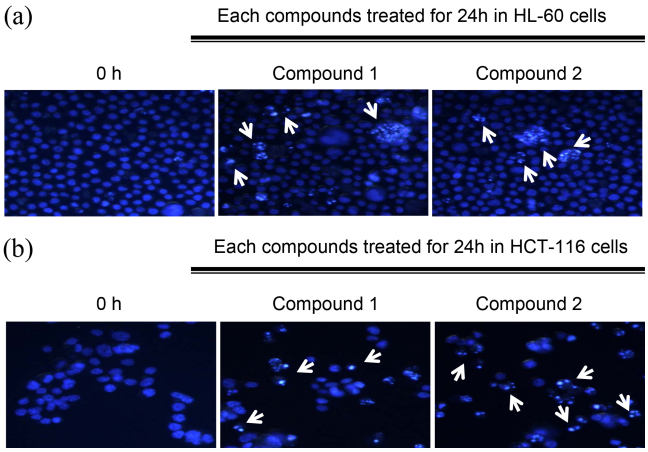

Figure 4. The degree of apoptosis represented as the fluorescent image of nuclei in HL-60 and HCT-116 cells by Hoechst 33342 staining. (a) The HCT-116 cells were treated with 0.50 μM of compound **1** and 0.80 μM of compound **2** for 24 hours. (b) The HL-60 cells were treated with 0.13 μM of compound **1** and 0.28 μM of compound **2** for 24 hours. DNA-specific fluorescent dye, Hoechst 33342 (culture medium at a final concentration of 10 μg/mL) was directly added to media and apoptotic bodies were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (magnification × 200).

Experimental

General Methods. Preparative HPLC was carried out using a Waters HPLC system (600 pump, 600 controller, and a 996 photodiode array detector). The ^{1}H-NMR (600 & 400 MHz) and ^{13}C-NMR (150 & 100 MHz) spectra were recorded on a Bruker AM 600 FT-NMR spectrometer and TMS was used as an internal standard. The electrospray ionization mass spectra were recorded on an Agilent 1200 LC-MSD Trap spectrometer. Column chromatography was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) and YMC RP-18 resins (30-50 μm, Fujisilisa Chemical Ltd.). Thin layer chromatography was performed on DC-Alufolien 60silica-gel F_{254} (Merck 1.05554.0001) or DC Platten RP_{18} F_{254s} (Merck 1.15685.0001) plates. Spots were visualized by spraying 10% H_2SO_4 aqueous and heating for 5 min.

Plant Material. The rhizomes of *P. stipuleanatus* were collected in Vietnam in 2007 and taxonomically identified by Dr. Tran Huy Thai, Institute of Ecology and Biological Resources, Vietnam. The reference specimen (VHKC-0044) has been deposited at the Institute of Natural Products Chemistry, VAST, Vietnam.

Extraction and Isolation. The rhizomes of *P. stipuleanatus* (2 kg) were extracted with MeOH at room temperature for 1 day (10 L × 3 times). The MeOH extract (200 g) was concentrated under vacuum to give a gummy residue, which was then suspended in H_2O (3 L). This solution was extracted with CH_2Cl_2 and EtOAc (3 L × 3 times) to give 11g, 4 g of a CH_2Cl_2-soluble fraction and EtOAc-soluble fraction, 146 g of an H_2O-soluble fraction. The CH_2Cl_2 soluble fraction was subjected to silica gel column chromatography, with elution with hexane-EtOAc (100:1-0:1) and 500 mL fractions being collected to give four fractions (1A-1D). Fraction 1A was subjected to silica gel column chromatography with a CH_2Cl_2-EtOAc (20:1) elution solvent to give seven fractions (2A-2G). Fraction 2C was subjected to silica gel column chromatography with a hexane-EtOAc (5:1) elution solvent to give four fractions (3A-3D). Compound **1** (7 mg) was obtained from fraction 3C by column chromatography (silica gel; CH_2Cl_2-EtOAc, 30:1). Fraction 1B was subjected to silica gel column chromatography with a CH_2Cl_2-EtOAc (10:1) elution solvent to give two fractions (4A and 4B). Compound **2** (171 mg) was isolated from fraction 4A by column chromatography (silica gel; hexane-EtOAc, 15:1). Compound **4** (15 mg) was isolated from fraction 4B by column chromatography (silica gel; Hexane-EtOAc, 20:1). Fraction 1C was subjected to silica gel column chromatography with a $CHCl_3$-EtOAc (10:1) elution solvent to give two fractions (5A and 5B). Compound **3** (53 mg) was obtained from fraction 5B by column chromatography (silica gel; hexane-EtOAc, 3:1). Fraction 1D was separated by LH-20 column chromatography eluted with a MeOH, to yield three sub-fractions (Fr. 6A-6C). Compound **5** (10 mg) was obtained from fraction 6B using a silica gel column chromatography (hexane-EtOAc, 20:1).

1,8-Octadecadiene-4,6-diyn-3*R*,10*R*-diol (1): Yellow oil; $[\alpha]_D^{20}$ – 38.5 (c 0.70, $CHCl_3$); ESI-APCI-MS *m/z* 275 $[M + H]^+$; ^{1}H-NMR ($CDCl_3$, 600 MHz): δ 5.49 (1H, d, *J* = 16.1 Hz, H-1a), 5.27 (1H, d, *J* = 9.9 Hz, H-1b), 5.95 (1H, ddd, *J* = 5.2, 9.9, 16.1 Hz, H-2), 4.98 (1H, d, *J* = 5.2 Hz, H-3), 5.78 (1H, d, *J* = 16.4 Hz, H-8), 6.35 (1H, dd, *J* = 6.2, 16.4 Hz, H-9), 4.20 (1H, dt, *J* = 5.4, 6.2 Hz, H-10), 1.54 (2H, m, H-11), 1.29 (12H, brs, H-12, 13, 14, 15, 16, 17), 0.89 (3H, t, *J* = 6.9 Hz, H-18); ^{13}C-NMR ($CDCl_3$, 150 MHz): δ 117.3 (C-1), 136.0 (C-2), 63.7 (C-3), 80.4 (C-4), 70.9 (C-5), 73.6 (C-6), 77.6 (C-7), 108.1 (C-8), 150.0 (C-9), 72.1 (C-10), 36.9 (C-11), 25.2 (C-12), 29.5 (C-13), 29.7 (C-14), 29.2 (C-15), 31.8 (C-16), 22.7 (C-17), 14.1 (C-18).

Preparation of (*S*)- and (*R*)-MTPA esters 1a and 1b from 1: A solution of **1** (1.0 mg) in dry pyridine (50 μL) was reacted with 5 μL (*S*)-(+)-MTPA chloride at room temperature for 30 min. The reaction mixture was dried by N_2 gas. The dried product was partitioned with CH_2Cl_2 and H_2O. The organic layer was dried (Na_2SO_4), filtered, and evaporated under reduced pressure. The residue was purified by preparative TLC silica gel (0.25 mm thickness) developed with CH_2Cl_2-MeOH (15:1), and the product was eluted with CH_2Cl_2-MeOH (5:1) to furnish the ester, **1a** (1.0 mg). Using a similar procedure, **1b** (1.0 mg) was prepared from **1** (1.0 mg) by having an esterification with (*R*)-(–)-MTPA chloride.

1,8-Octadecadiene-4,6-diyn-3*R*,10*R*-diol (*S*)-MTPA ester (1a): Yellow oil; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.64 (1H, d, *J* = 16.4 Hz, H-1a), 5.44 (1H, d, *J* = 10.2 Hz, H-1b), 5.98 (1H, ddd, *J* = 6.1, 10.2, 16.4 Hz, H-2), 5.80 (1H, d, *J* = 16.1 Hz, H-8), 6.28 (1H, dd, *J* = 7.0, 16.1 Hz, H-9), 1.57 (2H, m, H-11), 1.29 (12H, brs, H-12, 13, 14, 15, 16, 17), 0.89 (3H, t, *J* = 6.9 Hz, H-18), 7.27-7.36 (9H, m, aromatic protons), 7.39-7.47 (6H, m, aromatic protons).

1,8-Octadecadiene-4,6-diyn-3*R*,10*R*-diol (*R*)-MTPA ester (1b): Yellow oil; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.56 (1H, d, *J* = 16.5 Hz, H-1a), 5.38 (1H, d, *J* = 10.2 Hz, H-1b), 5.88 (1H, ddd, *J* = 5.8, 10.2, 16.5 Hz, H-2), 5.81 (1H, d, *J* = 15.6 Hz, H-8), 6.29 (1H, dd, *J* = 6.8, 15.6 Hz, H-9), 1.57 (2H, m, H-11), 1.29 (12H, brs, H-12, 13, 14, 15, 16, 17), 0.89 (3H, t, *J* = 6.9 Hz, H-18), 7.27-7.36 (9H, m, aromatic protons), 7.39-7.47 (6H, m, aromatic protons).

1,9-Octadecadiene-4,6-diyn-3*S*-ol (2): Orange oil; $[\alpha]_D^{20}$ + 11.2 (c 0.1, $CHCl_3$); ESI-MS *m/z* 257 $[M-H]^-$; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 5.47 (1H, d, *J* = 17.1 Hz, H-1a), 5.24 (1H, d, *J* = 10.2 Hz, H-1b), 5.96 (1H, ddd, *J* = 5.5, 10.2, 17.1 Hz, H-2), 4.91 (1H, d, 5.5, H-3), 3.03 (2H, d, *J* = 6.8 Hz, H-8), 5.39 (1H, m, H-9), 5.53 (1H, m, H-10), 2.02 (2H, q, *J* = 6.9, 7.5 Hz, H-11), 1.28 (10H, m, H-12, 13, 15, 16, 17), 1.35 (2H, m, H-14), 0.89 (3H, t, *J* = 6.8 Hz, H-18); ^{13}C-NMR ($CDCl_3$, 150 MHz): δ 117.1 (C-1), 136.2 (C-2), 63.5 (C-3), 74.3 (C-4), 71.3 (C-5), 64.1 (C-6), 80.3 (C-7), 17.7 (C-8), 122.0 (C-9), 133.1 (C-10), 27.2 (C-11), 29.3 (C-12), 29.2 (C-13), 29.2 (C-14), 29.2 (C-15), 31.9 (C-16), 22.7 (C-17), 14.2 (C-18).

(3*R*,9*R*,10*R*)-Panaxytriol (3)[5]: Colorless oil; $[\alpha]_D^{20}$ + 12.9 (c 0.095, $CHCl_3$); ESI-APCI-MS *m/z* 277 $[M-H]^-$; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 5.46 (1H, d, *J* = 15.8 Hz, H-1a), 5.24 (1H, d, *J* = 9.9 Hz, H-1b), 5.93 (1H, ddd, *J* = 4.8, 9.9,

15.8 Hz, H-2), 4.90 (1H, brs, H-3), 2.58 (2H, brs, H-8), 3.64 (1H, s, H-9), 3.58 (1H, s, H-10), 1.48 (4H, brs, H-11, 12), 1.28 (8H, brs, H-13, 14, 15, 16), 0.88 (3H, s, H-17).

Spathulenol (4)[6]: Yellow oil; $[\alpha]_D$ + 11.2 (c 1.05, $CHCl_3$); ESI-APCI-MS *m/z* 219 $[M-H]^-$; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.46 (1H, m, H-1), 0.64 (1H, M, H-2), 1.00 (1H, m, H-3a), 1.97 (1H, m, H-3b), 2.03 (1H, m, H-4a), 2.40 (1H, m, H-4b), 2.19 (1H, m, H-6), 1.64 (2H, m, H-7), 1.79 (2H, m, H-8), 1.30 (1H, m, H-10), 1.05 (3H, s, H-12), 1.03 (3H, s, H-13), 4.68 (2H, s, H-14), 1.27 (3H, s, H-15).

9-Docosenoic acid Methyl Ester (5)[7]: Colorless oil; $[\alpha]_D^{20}$ + 1.45 (c 1.05, $CHCl_3$); ESI-APCI-MS *m/z* 351 $[M-H]^-$; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.88 (3H, t, H-1), 2.00 (2H, m, H-8), 5.33 (1H, m, H-9), 1.62 (2H, brs, H-20), 2.30 (2H, t, H-21), 3.66 (3H, s, H-23).

Cell Cultures. HL-60 (human acute promyelocytic leukemia) and HCT-116 (human colorectal carcinoma) cell lines were obtained from KCLB (Korean Cell Line Bank) and used in cytotoxicity assay. The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS (v/v) and 2% penicillin-streptomycin, at 37 ºC in humid atmosphere containing 95% air and 5% CO_2. The exponentially growing cells were used throughout the experiments.

Cytotoxicity Tests. Human cancer cells (1.5-2.5 × 10^5 cells/mL) were treated for 3 d with 1, 10, 50, and 100 μM of the compounds. After incubation, 0.1 mg (50 μL of a 2 mg/mL solution) MTT (Sigma, Saint Louis, MO, U.S.A.) was added to each well and the cells were then incubated at 37 ºC for 4 h. The plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. Dimethylsulfoxide (150 μL) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech., U.S.A.). All the experiments were performed three times. The results were expressed as the percentage of inhibition that produced a reduction in the absorbance by the treatment of the compounds compared to the untreated controls. A dose-response curve was generated and the inhibitory concentration of 50% (IC_{50}) was determined for each compound as well as each cell line. Mitoxantrone (MX), an anticancer agent, was used as positive control.

Morphology Analysis. For the detection of apoptosis, the HCT-116 cells and the HL-60 cells were seeded at 1 × 10^5 cells/mL and 3 × 10^5 cells/mL on 24-well microplates. After 18 h of incubation to allow cell attachment, cells were treated with IC_{50} of compounds for 24 h. The cells were incubated in a Hoechst 33342 (10 μg/mL medium, final) staining solution at 37 ºC for 20 min. The stained cells were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (magnification×200).

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