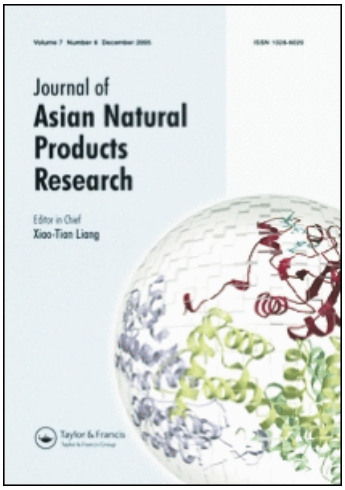

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Cheng-Bin Cui[ab]; Shao-Yu Yan[ac]; Bing Cai[a]; Xin-Sheng Yao[c]
[a] Beijing Institute of Biomedicine, Beijing, People's Republic of China [b] Marine Drug and Food Institute, Ocean University of Qingdao, Qingdao, People's Republic of China [c] Shenyang Pharmaceutical University, Shenyang, People's Republic of China

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CARBAZOLE ALKALOIDS AS NEW CELL CYCLE INHIBITOR AND APOPTOSIS INDUCERS FROM *CLAUSENA DUNNIANA* LEVL.

CHENG-BIN CUI[a,c,*], SHAO-YU YAN[a,b], BING CAI[a] and XIN-SHENG YAO[b]

[a]*Beijing Institute of Biomedicine, Beijing 100091, People's Republic of China;* [b]*Shenyang Pharmaceutical University, Shenyang 110016, People's Republic of China;* [c]*Marine Drug and Food Institute, Ocean University of Qingdao, Qingdao 266003, People's Republic of China*

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Carbazole alkaloids, 3-methylcarbazole (**1**), murrayafoline A (**2**), girinimbine (**3**), mahanimbine (**4**) and bicyclomahanimbine (**5**), were isolated for the first time from *Clausena dunniana* Levl. by bioassay-guided separation procedure and were identified by spectroscopic methods. Compounds **1**–**5** showed growth inhibitory activity (**1**, IC_{50} 25 μg/ml) on human fibrosarcoma HT-1080 cells and cell cycle M-phase inhibitory (**2**, MIC 0.78 μg/ml) and apoptosis inducing (**2**, MIC 1.56 μg/ml; **3**, MIC 25 μg/ml; **4**, MIC 20 μg/ml; **5**, MIC 30 μg/ml) activities on mouse tsFT210 cells, respectively, and **2**–**5** provided the first examples of carbazole alkaloids as new cell cycle inhibitors and apoptosis inducers.

Keywords: *Rutaceae*; *Clausena dunniana*; Carbazole alkaloid; 3-Methylcarbazole; Murrayafoline A; Girinimbine; Mahanimbine; Bicyclomahanimbine; Cell cycle inhibitor; Apoptosis inducer

INTRODUCTION

Cell cycle and apoptosis are tightly controlled bioprocesses and are well known as the sole pathway for cell proliferation and the best way to eliminate unnecessary cells in physiological conditions. Living cells in the human body are in a dynamic balance between their proliferation and death and the body regulates the balance by switching "on" or "off" the cell cycle and apoptosis according to the body's need to keep a homeostasis at the cell level. Under normal conditions, even some cancerous cells featured by DNA damage would be eliminated or repaired by the homeostatic system including the cell cycle and apoptosis processes. However, when the control process of cell cycle or apoptosis was deregulated, the undesired and unlimited proliferation of cancerous cells occurred, leading to an imbalance and subsequently developing to cancer. Chemical agents inhibiting cell cycle or inducing apoptosis might therefore be useful to regulate the balance and thus possess the potential to

*Corresponding author. Present address: Tianjin Institute for Biomedical Research (TIBiR), 3d Floor of D2-Building, Xinmao Technology Park, Huayuan Industrial District, Tianjin 300384, People's Republic of China. Tel.: +86-22-83712588. Fax: +86-22-83712688. E-mail: cuicb@sohu.com; cuichb@sina.com

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cure cancers. In fact, many anticancer drugs clinically used in cancer chemotherapy nowadays have been demonstrated to exert their anticancer effects by inhibiting cell cycle or inducing apoptosis even though the discovery and development of these drugs did not depend on their activities in apoptosis and cell cycle. From this viewpoint, we have undertaken the screening of new cell cycle inhibitors and apoptosis inducers from natural resources using mammalian tsFT210 cells [1–5].

In the course of the screening, we have examined more than a thousand Chinese medicinal herbs [5] and found that *Clausena dunniana* Levl. (family *Rutaceae*) possessed strong activity in inducing apoptosis and inhibiting the cell cycle at the G2/M phase on tsFT210 cells. *C. dunniana* Levl. is used as a folk medicine to cure certain cancers in China, and although a lot of research work had been done on the chemical constituents of several species of the genus *Clausena* [6,7], no research report had so far been seen on *C. dunniana* Levl. Thus, the chemical investigation was undertaken with particular attention focused on the active components of the title plant and we have now isolated five carbazole alkaloids **1–5** (Fig. 1) for the first time from *C. dunniana* Levl. as its active components by a bioassay-guided separation procedure. In this paper, the isolation, identification and biological activities of **1–5** were described.

RESULTS AND DISCUSSION

Isolation of Carbazole Alkaloids 1–5

Plant materials (5 kg) of *C. dunniana* Levl. were extracted with 70% aqueous alcohol to give an alcoholic extract (300 g). As the extract showed high activities in inducing apoptosis and inhibiting cell cycles at G2/M phase on tsFT210 cells, the following separation procedure was monitored by the same activities. The whole extract was partitioned between water and petroleum ether to obtain an active petroleum ether extract (25 g). This extract was then fractionated by silica gel vacuum liquid chromatography to afford four active fractions (**II–V**) eluted with petroleum ether–acetyl acetate (99:1–90:1), from which, compounds **1–5** were isolated, respectively through silica gel and ODS column chromatography or by preparative TLC separation.

Identification of 1–5

Compound **1**, colorless prisms, mp 178–179°C, gave a positive color reaction with Dragendorff reagent, indicating that **1** is an alkaloid. It gave *quasi*-molecular ion peak at *m*/*z* 182 $[M + H]^+$ in ESI–MS measurements, corresponding to the molecular formula, $C_{13}H_{11}N$. Its UV and IR absorptions (see "Experimental section") revealed that **1** is a carbazole alkaloid and the ^1H and ^{13}C NMR spectra of **1** in $CDCl_3$ (see "Experimental

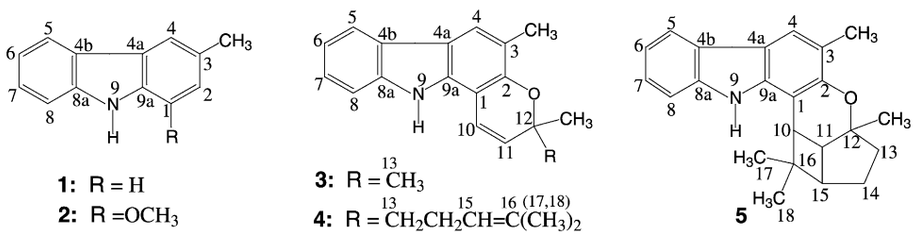

FIGURE 1 Structures of carbazole alkaloids **1–5**.

section") indicated that **1** is a methylcarbazole. The position of the methyl group at C-3 could be determined by detailed analysis of its ^{1}H and ^{13}C NMR spectra by means of 2D NMR techniques and thus **1** was identified as 3-methylcarbazole [8,9].

Compound **2**, yellowish oil (from *n*-hexane), gave the same color reaction and the UV and IR absorptions closely resembled those of **1**, indicating that **2** is also a carbazole alkaloid. In ESI–MS, **2** gave a *quasi*-molecular ion peak at *m/z* 212 $[M + H]^+$, corresponding to its molecular formula, $C_{14}H_{13}NO$, which contains one more CH_2O unit than **1**. The ^{1}H and ^{13}C NMR spectra of **2** in $CDCl_3$ resembled those of **1**, but they were characterized by the appearance of a new methoxy group (δ_H 4.07 s and δ_C 55.7) and two *meta*-coupled benzene proton signals in **2** instead of the signals due to 1,2,4-trisubstituted benzene protons in **1**. Thus **2** should be the methoxylated derivative of **1** and eventually **2** was identified as 1-methoxy-3-methylcarbazole (murrayafoline A) [10] by detailed analysis of its ^{1}H and ^{13}C NMR spectra.

Compound **3**, colorless plates, mp 176–177°C, and compound **4**, colorless needles, mp 95.5–96.5°C, both were considered to be carbazole alkaloids according to the same color reaction and their UV and IR absorptions resembled those of **1** and **2**. The ^{1}H NMR spectrum of **3** in $CDCl_3$ showed signals due to an isolated benzene proton and a 1,2-disubstituted benzene, *vicinal*-coupled olefin and three *tert*-methyl groups (see "Experimental section") and the ^{1}H NMR spectrum of **4** in $CDCl_3$ closely resembled that of **3** except for newly appeared signals due to a $CH_2CH_2CH{=}C(CH_3)_2$ group instead of a *tert*-methyl signal in **3**, suggesting that structures of **3** and **4** are different only in the $CH_2CH_2CH{=}C(CH_3)_2$ group. Correspondingly, **3** and **4** gave the *quasi*-molecular ion peaks at *m/z* 264 $[M + H]^+$ and at *m/z* 332 $[M + H]^+$, respectively in ESI–MS measurements, consistent with their molecular formula, $C_{18}H_{17}NO$ for **3** and $C_{23}H_{25}NO$ for **4**. Eventually, they were identified as girinimbine [11,12] (**3**) and mahanimbine [11,12] (**4**) on the basis of the ^{1}H and ^{13}C NMR spectral data analyzed by PFG ^{1}H–^{1}H COSY, PFG–HMQC and PFG–HMBC techniques.

Compound **5**, colorless prisms, mp 146.5–147.5°C, $C_{23}H_{25}NO$, gave the same color reaction and the UV and IR absorptions typical for carbazole alkaloids. In the ^{1}H and ^{13}C NMR spectra of **5** in CD_3OD, the signal patterns due to the carbazole skeleton closely resembled those of **3** and **4**, indicating the same substitution pattern on the carbazole skeleton, but the signals arising from alkyl moiety in **5** were quite different. The ^{1}H and ^{13}C NMR spectra were analyzed in detail with the aid of PFG ^{1}H–^{1}H COSY, PFG–HMQC and PFG–HMBC techniques, finally leading to the identification of compound **5** as bicyclomahanimbine [13].

Biological Activities of 1–5

The cell cycle inhibitory and apoptosis inducing activities were assayed for compounds **1–5** using tsFT210 cells by flow cytometry, accompanied with morphological observation of the cells and their nuclei under the light and fluorescent microscope, according to the previously reported method [2,5] with slight modification. Typical flow cytometric histograms for **1–5** are given in Fig. 2 and corresponding morphological observations respectively are given in Figs. 3 and 4 (each experiment was independently repeated more than 3 times to confirm the same result).

Compounds **2–5** significantly induced apoptosis of tsFT210 cells, as detected as sub-G_0/G_1 peaks in histograms (see Fig. 2), and the effects of **3** and **4** both at 50 μg/ml were especially dramatic as shown in Figs. 2–4, which is much stronger than the effect of cisplatin at 50 μg/ml used in the present study as a positive control on tsFT210 cells (data not given). Corresponding cell morphology showed typical characteristics of apoptosis, apoptotic cell bodies formed after treatment with **3** (50 μg/ml) and **5** (50 μg/ml) and typical cell shrinkage appeared after treatment with **4** (50 μg/ml) and **5** (50 μg/ml) were observed

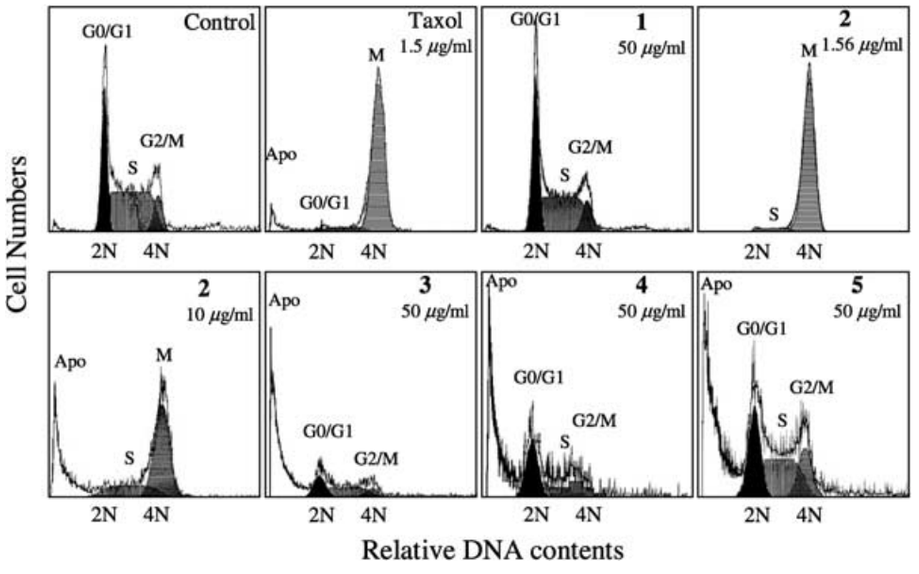

FIGURE 2 Flow cytometric histograms for compounds **1–5**. The tsFT210 cells in RPMI-1640 medium (20×10^5 cells/ml) were cultured at 32°C in a humidified atmosphere of 5% CO_2 and 95% air in the absence (control) or presence of taxol and **1–5** respectively at the concentrations indicated. The cell nuclei were stained with propidium iodide and then analyzed by flow cytometry. Open graphs indicate the raw data directly obtained from flow cytometry and filled figures indicate the data calculated by the computer software, WinCycle (Coulter).

clearly under the light microscope (see Fig. 3) and the chromatin condensation visualized as different density of fluorescence by staining of the cell nuclei with Hoechst 33258 reagent was also obvious under fluorescent microscope as shown in Fig. 4. Meanwhile, **2** inhibited also the cell cycle of tsFT210 cells at the M-phase at lower concentrations (lower than 20 μg/ml) and completely inhibited the cell cycle at the M-phase at the concentration of 1.56 μg/ml (see Figs. 2–4), while **3–5** showed only the apoptosis inducing activity even at lower

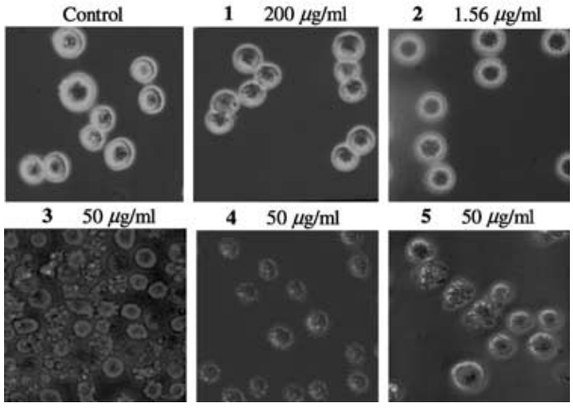

FIGURE 3 Photographs of tsFT210 cells treated with compounds **1–5**. Exponentially growing tsFT210 cells were cultured at 32°C for 17 hours in the absence (control) or presence of **1–5** respectively at the concentrations indicated and morphological characteristics of the cells were directly observed and photographed (× 200) under a light microscope.

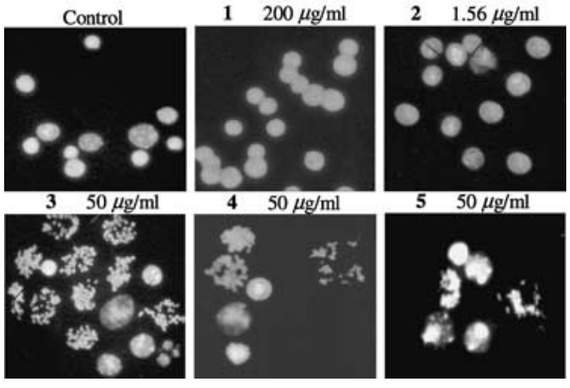

FIGURE 4 Fluorescent microscopic photographs of tsFT210 cells treated with **1–5**. Exponentially growing tsFT210 cells were cultured at 32°C for 17 hours in the absence (control) or presence of **1–5** respectively at the concentrations indicated. Then morphological characteristics of the cell nuclei were observed and photographed (×400) under a fluorescent microscope after staining of the cell nuclei with Hoechst 33258 reagent.

concentrations (data not given). In addition, at higher concentrations (100 μg/ml or more), **2–5** are a little cytotoxic and the higher concentration, the higher their cytotoxicity. Minimum inhibitory or inducing concentration (MIC) values are 0.78 μg/ml for **2** for inhibiting cell cycle at M-phase and 1.56 μg/ml for **2**, 25 μg/ml for **3**, 20 μg/ml for **4**, and 30 μg/ml for **5** for inducing apoptosis on tsFT210 cells.

On the other hand, compound **1** showed no cell cycle inhibitory and apoptosis inducing activities on tsFT210 cells (Figs. 2–4), however, it inhibited the growth of human fibrosarcoma HT-1080 cells with an IC_{50} value of 25 μg/ml which was determined by MTT method [14].

In present paper, five carbazole alkaloids **1–5** were isolated for the first time from *C. dunniana* Levl. as its bioactive components by bioassay-guided separation procedure. Compounds **1–5** showed growth inhibitory or cell cycle inhibitory and apoptosis inducing activities on cancer cells and the present result provided the first example of bioactive constituents of *C. dunniana* Levl. for its anticancer usage in Chinese people. On the other hand, although more than 80 carbazole alkaloids have been isolated from several species of the genus *Clausena* and widespread biological activities have been reported for those carbazole alkaloids [6,7], the apoptosis-inducing and cell-cycle-inhibitory activities have been hitherto reported for carbazole alkaloids. The apoptosis-inducing and cell-cycle-inhibitory activities were newly observed for carbazole alkaloids **2–5** in the present study and thus compounds **2–5** provide the first example of carbazole alkaloids as new cell cycle inhibitors and apoptosis inducers.

Compounds **1–5** are all 3-methylcarbazole derivatives and **1** is the simplest one in this class of carbazole derivatives. Structurally, **1** and **2** are different only in the substitution of 1-H in **1** by 1-OCH_3 in **2**, while compounds **3–5** are quite different, carrying a substituted hydropyran ring linked at the 1,2-positions of 3-methylcarbazole skeleton. Those structural differences in **1–5** are reflected well in their bioactivities, i.e. **1** showed no cell cycle-inhibitory and apoptosis-inducing activities on tsFT210 cells, while **2** showed both cell cycle-inhibitory and apoptosis-inducing activities and **3–5** showed only apoptosis-inducing activity on the same tsFT210 cells. It may appear, therefore, that the substitution at the 1- and/or 2-positions of 3-methylcarbazole is significant from bioactive consideration for 3-methylcarbazole derivatives for their cell cycle inhibitory and apoptosis inducing activities and the methoxy group at the 1-position in **2** is likely to be the most important structural factor for its activities on cell cycle and apoptosis.

EXPERIMENTAL SECTION

General Experimental Procedures

Thin-layer chromatography (TLC) was done on silica gel 60 F254 plates (0.25 mm thick, $20 \times 20\,cm^2$, Merck) or silica gel G plates (0.25 mm thick, $20 \times 20\,cm^2$, Qingdao Haiyang Chemical group Co., China) and the spots were detected under UV light (254 and 365 nm) or by the use of 10% aqueous sulfuric acid reagent and Dragendorff reagent. Vacuum liquid chromatography and column chromatography were carried out on a SYNTHWARE™ glass vacuum column ($5 \times 30\,cm^2$, Tianjin Synthware Glass Instruments Co., Tianjin, China) and a glass open column, respectively, and Sephadex LH-20 (Pharmacia) and SSC ODS–SS-1020T (Senshu Scientific Co., Ltd.) were used as adsorbents.

Melting points were measured using an XT-type micro melting point apparatus (Beijing Tech Instrument Co. Ltd., China) and are uncorrected. Optical rotations were determined on a JASCO P-1020 polarimeter in $CHCl_3$ solutions and UV spectra were recorded on a Shimadzu UV-2501PC UV–VIS recording spectrophotometer in MeOH solutions. IR spectra were taken on a Dynamin alignment FTS 175C Fourier transform infrared spectrophotometer in KBr discs. ESI–MS was measured on an Esquire LC mass spectrometer. ^1H and ^{13}C NMR spectra and 2D NMR spectra were taken on a JEOL Eclips-600 or Bruker AVANCE DRX-500 FT-NMR spectrometer using TMS as internal standard and chemical shifts are recorded in δ values.

Plant Material

The stems of *C. dunniana* Levl. were collected in the Mengla area in Yunnan province, China, in September 1998. The original plant was identified by Prof. Q.-S. Sun of Shenyang Pharmaceutical University, China, and a voucher specimen has been deposited at the Herbarium of the Shenyang Pharmaceutical University, China.

Extraction and Fractionation

Air-dried woods and barks (5 kg) from stems of *C. dunniana* Levl. were extracted with 70% ethanol by refluxing for 3 h (15 l × 3) to give a crude extract (300 g). The whole extract was partitioned between water (2 l) and petroleum ether (2 l) to obtain the active petroleum ether extract (25 g).

This extract (25 g) was subjected to vacuum liquid chromatography over silica gel H (300 g, bed $5 \times 30\,cm^2$) and eluted with petroleum ether (Pet. Ether)–ethyl acetate (EtOAc) in a stepwise manner to afford six fractions (**I**–**VI**), Fraction **I** (1.5 g) eluted with Pet. Ether, Fraction **II** (6.35 g) eluted by Pet. Ether–EtOAc (99:1–97:3), Fraction **III** (6.25 g) eluted by Pet. Ether–EtOAc (97:3–95:5), Fraction **IV** (1.25 g) eluted by Pet. Ether–EtOAc (95:5–93:7), Fraction **V** (0.35 g) eluted by Pet. Ether–EtOAc (93:7–90:10), and Fraction **VI** (9 g) eluted by Pet. Ether–EtOAc (90:10–50:50).

Isolation of Compounds 1–5

Fraction **II** (6.35 g) was subjected to silica gel column chromatography and eluted with *n*-hexane–acetone in a stepwise manner to obtain **3** (4.5 g) from a fraction eluted by *n*-hexane–acetone (95:5) as colorless plates from *n*-hexane solution.

Fraction **III** (6.35 g) was further separated by silica gel column chromatography eluting with *n*-hexane–acetone in a stepwise manner and from a fraction eluted by *n*-hexane–acetone (95:5), **4** (3.5 g) was obtained as colorless needles from *n*-hexane solution.

Fraction **IV** (1.25 g) was subjected to ODS column chromatography and eluted with CH_3CN-H_2O (7:3) to give **1** (15 mg) as colorless prisms from MeOH solution and **2** (500 mg) as yellowish oil from *n*-hexane solution, in turn, from early and later fractions, respectively.

Fraction **V** (0.35 g) was separated by preparative TLC (silica gel 60G plate) with *n*-hexane–MeOH (97:3) as eluting solvent to give **5** (25 mg) as colorless prisms from MeOH solution.

3-Methylcarbazole (1)

Colorless prisms from MeOH solution, $C_{13}H_{11}N$, mp 178–179°C. ESI–MS *m/z*: 182 $[M + H]^+$, 167 $[M + H - CH_3]^+$. UV λ_{max} nm (log ϵ) in MeOH: 342 (3.70), 328 (3.74), 296 (4.46), 259 (4.42), 246 (4.53), 235 (4.80), 230 (4.78). IR ν_{max} cm^{-1} (KBr): 3405 (NH), 3051 (aromatic protons), 2918, 2858 (CH_3), 1608, 1581, 1495, 1461 (aromatic rings), 1391, 1335, 1321, 1295, 1244, 930, 886, 807, 750, 728. ^{1}H NMR δ in $CDCl_3$: 8.05 (d, $J = 8.0$ Hz, 5-H), 7.91 (br s, NH), 7.88 (d, $J = 1.0$ Hz, 4-H), 7.40 (2H, AB type, 7-H and 8-H), 7.32 (d, $J = 8.0$ Hz, 1-H), 7.24 (dd, $J = 8.0$, 1.0 Hz, 2-H), 7.22 (m, 6-H), 2.54 (3H, s, 3-CH_3). ^{13}C NMR δ in $CDCl_3$: 139.89 (C-8a), 137.78 (C-9a), 128.78 (C-3), 127.19 (C-2), 125.67 (C-7), 123.61 (C-4a), 123.31 (C-4b), 120.26 (2 × C, C-4 and C-5), 119.26 (C-6), 110.56 (C-8), 110.25 (C-1), 21.42 (3-*C*H_3).

Murrayafoline A (2)

Yellowish oil from *n*-hexane solution, $C_{14}H_{13}NO$. ESI–MS *m/z*: 212 $[M + H]^+$, 197 $[M + H - CH_3]^+$, 181 $[M + H - OCH_3]^+$. UV λ_{max} nm (log ϵ) in MeOH: 341 (3.67), 328 (3.71), 291 (4.19), 251 (4.57), 241 (4.75), 222 (4.64). IR ν_{max} cm^{-1} (KBr): 3422 (NH), 3056 (aromatic protons), 2918, 2853 (CH_3), 1635, 1589, 1504, 1453 (aromatic rings), 1394, 1334, 1306, 1262, 1136, 1106, 828, 766, 748, 733. ^{1}H NMR δ in $CDCl_3$: 8.30 (br s, N*H*), 8.20 (br d, $J = 8.0$ Hz, 5-H), 7.66 (br s, 4-H), 7.54 (ddd, $J = 8.0$, 7.5, 1.0 Hz, 7-H), 7.49 (br d, $J = 8.0$ Hz, 8-H), 7.38 (ddd, $J = 8.0$, 7.5, 1.0 Hz, 6-H), 6.87 (br s, 1-H), 4.07 (3H, s, OCH_3), 2.70 (3H, s, CH_3). ^{13}C NMR δ in $CDCl_3$: 145.59 (C-1), 139.87 (C-8a), 129.76 (C-3), 128.40 (C-9a), 125.76 (C-7), 124.74 (C-4a), 123.90 (C-4b), 120.77 (C-5), 119.49 (C-6), 112.92 (C-4), 111.30 (C-8), 108.10 (C-2), 55.71 (O*C*H_3), 22.24 (*C*H_3).

Girinimbine (3)

Colorless plates (from *n*-hexane), $C_{18}H_{17}NO$, mp 176–177°C. ESI–MS *m/z*: 264 $[M + H]^+$, 286 $[M + Na]^+$, 304 $[M + H + K]^+$, UV λ_{max} nm (log ϵ) in MeOH: 357 (4.04), 342 (4.10), 327 (4.07), 287 (4.80), 236 (4.84). IR ν_{max} cm^{-1} (KBr): 3320 (NH), 2976, 2931 (CH_3), 1643, 1610, 1495, 1461 (aromatic ring), 1404, 1321, 1209, 1146, 1121, 1058, 882, 743. ^{1}H NMR δ in $CDCl_3$: 7.93 (dd, $J = 8.0$, 1.0 Hz, 5-H), 7.82 (br s, N*H*), 7.68 (s, 4-H), 7.37 (dd, $J = 8.0$, 1.0 Hz, 8-H), 7.32 (ddd, $J = 8.0$, 7.5, 1.0 Hz, 7-H), 7.19 (ddd, $J = 8.0$, 7.5, 1.0 Hz, 6-H), 6.60 (d, $J = 10$ Hz, 10-H), 5.69 (d, $J = 10$ Hz, 11-H), 2.36 (3H, s, 3-CH_3), 1.51 (6H, s, 13-CH_3 and 14-CH_3). ^{13}C NMR δ in $CDCl_3$: 149.88 (C-2), 139.56 (C-8a), 134.90 (C-9a), 129.42 (C-11), 124.30 (C-7), 124.01 (C-4b), 121.20 (C-4), 119.55 (C-6), 119.35 (C-5), 118.67 (C-3), 117.25 (C-10), 116.84 (C-4a), 110.43 (C-8), 104.48 (C-1), 75.89 (C-12), 27.66 (2 × C, C-13 and C-14), 16.05 (3-*C*H_3).

Mahanimbine (4)

Colorless needles (from *n*-hexane), $C_{23}H_{25}NO$, mp 95.5–96.5°C, $[\alpha]_D^{27}$ + 35.9 (*c* 1.0, $CHCl_3$). ESI–MS *m/z*: 332 $[M + H]^+$, 354 $[M + Na]^+$. UV λ_{max} nm (log ϵ) in MeOH: 357 (4.18), 342 (4.22), 329 (4.19), 287 (4.94), 238 (4.97). IR ν_{max} cm^{-1} (KBr): 3326 (NH), 2966, 2927, 2856 (CH_3 & CH_2), 1648 (C = C), 1612, 1496, 1460 (aromatic rings), 1445, 1324, 1157, 1058, 847, 747. ^{1}H NMR δ in $CDCl_3$: 7.93 (br d, J = 8.0 Hz, 5-H), 7.81 (br s, N*H*), 7.68 (s, 4-H), 7.37 (br d, J = 8.0 Hz, 8-H), 7.32 (td, J = 8.0, 1.0 Hz, 7-H), 7.19 (td, J = 8.0, 1.0 Hz, 6-H), 6.63 (d, J = 10 Hz, 10-H), 5.66 (d, J = 10 Hz, 11-H), 5.14 (tm J = 7.0 Hz, 15-H), 2.36 (3H, s, 3-CH_3), 2.20 (2H, m, 14-H_2), 1.79 (2H, t, J = 8.0 Hz, 13-H_2), 1.69 (3H, s, 17-H_3 in *cis*-relation with C-14), 1.61 (3H, s, 18-H_3 in *trans*-relation with C-14), 1.47 (3H, s, 12-CH_3). ^{13}C NMR δ in $CDCl_3$: 149.99 (C-2), 139.55 (C-8a), 134.94 (C-9a), 131.65 (C-16), 128.53 (C-11), 124.28 (C-15), 124.25 (C-7), 124.03 (C-4b), 121.24 (C-4), 119.54 (C-6), 119.32 (C-5), 118.47 (C-3), 117.53 (C-10), 116.71 (C-4a), 110.41 (C-8), 104.23 (C-1), 78.22 (C-12), 40.89 (C-13), 25.90 (12-*C*H_3), 25.66 (C-17 in *cis*-relation with C-14), 22.80 (C-14), 17.59 (C-18 in *trans*-relation with C-14), 16.05 (3-*C*H_3).

Bicyclomahanimbine (5)

Colorless prisms (from MeOH), $C_{23}H_{25}NO$, mp 146.5–147.5°C, $[\alpha]_D^{27}$ + 9.1 (*c* 1.0, $CHCl_3$). ESI–MS *m/z*: 332 $[M + H]^+$, 354 $[M + Na]^+$, 370 $[M + K]^+$. UV λ_{max} nm (log ϵ) in MeOH: 332 (3.85), 304 (4.44), 254 (4.66), 241 (4.85), 218 (4.76). IR ν_{max} cm^{-1} (KBr): 3478 (NH), 3062 (aromatic protons), 2953, 2933, 2860 (CH_3 & CH_2), 1613, 1580, 1492 (aromatic rings), 1459, 1384, 1341, 1306, 1216, 1145, 1113, 1066, 1029, 969, 923, 873, 770, 740. ^{1}H NMR δ in CD_3COD: 7.88 (br d, J = 8.0 Hz, 5-H), 7.65 (s, 4-H), 7.44 (br d, J = 8.0 Hz, 8-H), 7.23 (td, J = 8.0, 1.0, Hz, 7-H), 7.08 (td, J = 8.0, 0.5 Hz, 6-H), 3.46 (d, J = 9.0 Hz, 10-H), 2.71 (t, J = 9.0 Hz, 11-H), 2.54 (m, 15-H), 2.33 (3H, s, 3-*C*H_3), 2.09 (m, 13-H), 1.80 (m, 14-H), 1.74 (m, 14-H), 1.66 (m, 13-H), 1.59 (3H, s, 18-H_3), 1.38 (3H, s, 12-CH_3), 0.65 (3H, s, 17-H_3). ^{13}C NMR δ in CD_3COD: 151.67 (C-2), 141.66 (C-8a), 139.47 (C-9a), 125.14 (C-4b), 124.73 (C-7), 120.41 (C-3), 119.78 (C-4), 119.73 (C-5), 119.60 (C-6), 117.51 (C-4a), 111.78 (C-8), 108.74 (C-1), 85.01 (C-12), 48.12 (C-15), 40.93 (C-16), 40.78 (C-13), 40.49 (C-11), 38.13 (C-10), 34.89 (C-18), 26.70 (C-12), 26.26 (C-14), 19.11 (C-17), 16.81 (3-*C*H_3).

Cell Culture and Bioassay

Cell Line and Cell Culture

A mouse temperature-sensitive $p34^{cdc2}$ mutant cell line, tsFT210, was used for the bioassay. The tsFT210 cells were routinely maintained in RPMI-1640 medium supplemented with 10% FBS at 32°C under a humidified atmosphere of 5% CO_2 and 95% air.

Bioassay Procedure

Exponentially growing tsFT210 cells were harvested by centrifugation at 3000 rpm for 3 min at 4°C and suspended in fresh medium at a density of 2×10^5 cells/ml. 0.5 ml of the cell suspension was seeded into each well of the 24-well plate, 5 μl of sample solution was added into each well and the cells were cultured for 17 h at 32°C. Morphological characteristics of the cells treated with the samples were directly observed and photographed under an Olympus CK40 inverted system microscope with an Olympus PM-C35B camera system (Olympus, Japan). Then the cells were harvested by centrifugation at 3000 rpm for 3 min at 4°C, washed once with cold phosphate-buffered saline, and harvested again under the same

condition. The cells were stained with 150 μl of PI in water solution (PI 50 μg/ml, sodium citrate 0.1% and Nonidet P-40 0.2%) at 4°C for 30 min. The cells stained were diluted with the same volume of phosphate-buffered saline, analyzed by flow cytometer (EPICS® XL, Coulter Co., Hialeah, FL, USA), and the distribution within the cell cycle were calculated using the computing program, WinCycle (Coulter).

On the other hand, the cells treated with the samples were spread on glass slides and the cell nuclei were visualized by fluorescence staining with Hoechst 33258 reagent to examine their nuclear characteristics. An Olympus IX70 photographic system equipped with an Olympus IX70 inverted system microscope, an Olympus IX-FLA inverted reflected light fluorescence observation attachment, a PM-30 automatic photographic system, and a JVC TK-C1381 color video camera system was used for the observation of nuclear characteristics of the cells and to take photographs.

Assay for Growth Inhibitory Activity

Human fibrosarcoma HT-1080 cells were cultured at 37°C in DMEM medium supplemented with 10% FBS and the growth inhibitory activity was assayed for **1** by MTT method [14].

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