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CHEMICAL CONSTITUENTS FROM *Rauvolfia verticillata* AND BIOACTIVITIES RESEARCH

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*A new acridone alkaloid, named 9-hydroxynoracronycine (**1**), together with four known compounds, including coumarins, lignan, and indole alkaloid, was isolated and identified from the roots and rhizomes of* Rauvolfia verticillata. *The structure of the new compound was determined by spectroscopic means (UV, IR, MS, and NMR). The acridone alkaloid and coumarins were identified as new constituents of* Rauvolfia *genus. The cytotoxic activities of the new compound were tested against human breast cancer cell line MCF-7 and human promyelocytic leukemia HL-60. Results showed that compound **1** decreased MCF-7 cell proliferation in a statistically IC_{50} significant manner at 102.8 μmol/L. In addition, compound **1** exhibited moderate small intestine smooth muscle relaxation.*

Keywords: *Rauvolfia verticillata*, acridone alkaloid, indole alkaloid, cytotoxicity.

Rauvolfia is a genus of tropical trees belonging to the Apocynaceae family. It was transplanted in the south and southwest of China, especially in Guangxi and Yunnan Provinces. *Rauvolfia* is traditionally used as herbal remedies in China, India, Malaysia, and Indonesia [1]. Previous studies revealed that the roots and leaves of *Rauvolfia verticillata* (Lour.) Baill. were used as Chinese folk medicine to treat hypertension, inflammation, and fever [2, 3]. The extracts of the leaves and roots have been used as an antihypertensive preparation named verticilum. Previous investigations of this plant have shown that species of *Rauvolfia* are rich sources of bioactive indole alkaloids, such as reserpine [4–11]. Since our current interest includes all natural products, we investigated not only the indole alkaloids but also other types of chemical constituents with important biological activity in *Rauvolfia*.

In the present paper, the results of an investigation of the roots and rhizomes of *Rauvolfia* are reported, and the chemical characterization of a new acridone alkaloid, 9-hydroxynoracronycine (**1**), together with four additional known compounds, including two coumarins [12], seselin (**2**) and xanthyletin (**3**), a lignan [13], syringaresinol (**4**), and one indole alkaloid [4, 5], reserpine (**5**), from the fifth fraction of the chloroform extraction is described. Their structures are established by spectral data. The types of acridone alkaloids and coumarin compounds were isolated from *Rauvolfia* genus for the first time. According to the literature on acridone alkaloids [14–17], compound **1** has been evaluated for its biological activities, such as a cytotoxicity against MCF-7 and HL-60 cell lines, antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*, as well as their action on intestinal smooth muscle relaxation.

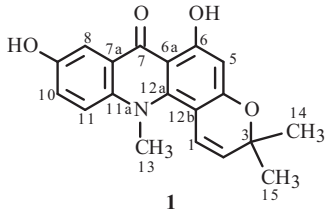

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TABLE 1. ^{1}H and ^{13}C NMR Data for Compound **1** (600 and 300 MHz, DMSO-d_6, δ, ppm, J/Hz)

C atom	δ_H	δ_C	HMBC (H→C)	C atom	δ_H	δ_C	HMBC (H→C)
1	6.69 (1H, d, J = 9.6)	120.6	12a, 4a, 3, 12b	9		148.7	
2	5.67 (1H, d, J = 9.6)	124.2	14, 15, 3, 12b	10	7.68 (1H, dd, J = 7.5, 1.8)	115.4	7, 8, 11a
3		76.8		11	7.22 (1H, d, J = 7.5)	123.7	7a, 9
4a		160.8		11a		136.7	
5	6.14 (1H, s)	97.1	4a, 6, 6a, 12b	12a		147.3	
6		163.8		12b		102.1	
6a		106.4		13	3.76 (3H, s)	48.6	11a, 12a
7		181.4		14	1.48 (3H, s)	26.8	2, 3, 15
7a		124.2		15	1.48 (3H, s)	26.8	2, 3, 14
8	7.27 (1H, d, J = 1.8)	120.2	10, 11a	6-OH	14.5 (1H, s)		5, 6, 6a

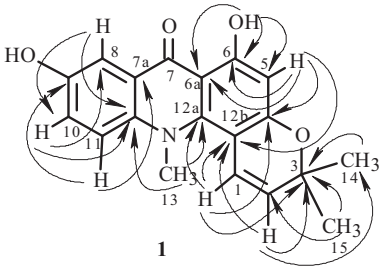

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Fig. 1. Selected key HMBC correlations for compound **1**.

Compound **1** was obtained as an orange amorphous crystal, mp 247–248°C. The compound exhibited a quasi-molecular ion at *m/z* (positive mode) 346.1066 $[M + Na]^+$ in its HR-TOF-MS spectrum, establishing its molecular formula as $C_{19}H_{17}NO_4$. This compound in MeOH displayed strong UV absorption at λ_{max} (log ε) 282 (1.22) and 237 (0.50) nm, consistent with the acridone molecule. The IR (KBr) spectrum showed an absorption band of the hydroxyl groups at 3282 cm^{-1}. An infrared peak at 1630 cm^{-1} and ^{13}C NMR resonance at δ_C 181.4 indicated the presence of a carbonyl group. The $FeCl_3$ test gave a deep green color, indicating the presence of the phenolic hydroxyl group. The ^{13}C NMR spectrum exhibited 19 carbon signals, and the ^{1}H NMR showed two singlet signals of three methyl groups at δ 3.76, 1.48, and 1.48, two hydroxyl protons at δ 14.47 and 10.47, and six aromatic protons at δ 7.68, 7.27, 7.22, 6.69, 6.14, and 5.67.

The ^{1}H NMR spectrum of **1** showed a pair of ortho aromatic protons at δ 6.69 (1H, d, J = 9.6 Hz) and 5.67 (1H, d, J = 9.6 Hz), which were assigned to H-1 and H-2. Three other aromatic protons, which resonated at 7.68 (dd, J = 7.5, 1.8 Hz), 7.27 (d, J = 1.8 Hz), and 7.22 (d, J = 7.5 Hz), indicated the presence of a 1,2,4-trisubstituted benzene ring, and they were assigned to H-10, H-8, and H-11, respectively. An aromatic proton singlet at 6.14 was unambiguously assigned to H-5. In high-field, three methyl protons, which resonated at 3.76 (3H, s) and 1.48 (6H, s), were assigned to H-13, H-14, and H-15, respectively.

The ^{13}C NMR spectrum showed 19 carbon signals, including a carbonyl group (δ 181.4), 14 aromatic carbons, three methyl group, and a quaternary carbon (76.8). These spectroscopic data together with HMBC correlation (Fig. 1) spectra supported the acridone basic structure of **1**. One methyl δ_H 3.76 (H-13) was attached to N-12 following analysis of HMBC correlation experiment, by ^{3}J correlations to C-11a and C-12a. The attachment of the other two methyls at C-3 was supported by the HMBC correlation observed between δ_H 1.48 (H-14, 15) and δ_C 76.8 (C-3) and 124.2 (C-2). On the basis of the foregoing, the structure of **1** was determined to be 9-hydroxynoracronycine, a new acridone alkaloid. Detailed assignments of ^{1}H, ^{13}C, and HMBC spectral data of **1** are shown in Table 1. The rest of the compounds were identified as seselin (**2**), xanthyletin (**3**), syringaresinol (**4**), and reserpine (**5**).

Biological Activities. Taking into account the unusual structural features of the acridone alkaloid (compound **1**) in *Rauvolfia* genus, we determined its possible functional role as a cancer preventive, antibacterial, and smooth muscle relaxant.

We initially test the effect of 46.4 μmol/L of compound **1** on growth of MCF-7 human breast cancer cells for 24 h with fresh compounds added. It significantly reduced cell number compared to the vehicle alone (0.1% DMSO-d_6).

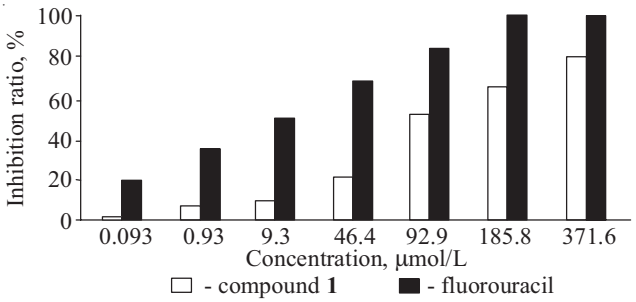

Fig. 2. The morphology of effect of compound **1** on breast cancer cell proliferation. MCF-7 cells were incubated in the medium alone for 24 h and then treated with vehicle (0.1% DMSO) or compound **1** at 0.093, 0.93, 9.3, 46.4, 92.9, 185.8, and 371.6 μmol/L for 48 h. The columns show the inhibition ratios at different concentrations of compound **1** and fluorouracil.

The effective concentration of compound **1** is shown in Fig. 2, where it is seen that compared to the vehicle control, there was a 51% reduction in cell number at 92.9 μmol/L. The observed change in the color of the cell from green to yellow or the red fluorescence appears to be due to increased membrane permeability and apoptosis. Compound **1** demonstrated cytotoxicity against MCF-7 human breast cancer cells with an IC_{50} value of 102.8 μmol/L. This result shows that *Rauvolfia* is a potential source of anti-breast cancer compounds. However, compound **1** was shown to be inactive against human promyelocytic leukemia HL-60, even when the dose was set at 309.6 μmol/L.

The antibacterial activity of disc-diffusion assay was used to determine the growth inhibition caused by the new compound against the following three representative bacterial strains: *Staphylococcus aureus* CMCC(B)26003 (Gram-positive), *Escherichia coli* CMCC(B)44102 (Gram-negative), and *Candida albicans* CMCC(F)98001 (fungus). Compound **1** exhibited no antibacterial activity against the three bacteria.

We investigated the relaxant effect of compound **1** on isolated small intestine smooth muscle of rabbits. Loperamide (1×10^{-4} mol/L) was used as a positive control. Due to the limited amount of compound **1**, we could not calculate its EC_{50} value, but compound **1** significantly attenuated the tension and amplitude of automatic contractions of isolated small intestine smooth muscles in a dose-dependent manner.

This study revealed the acridone alkaloid and coumarins in *Rauvolifia* genus, which suggests that *Rauvolifia* may have other pharmacological effects in addition its anti-hypertensive effect. Further studies of *Rauvolfia* genus are needed.

EXPERIMENTAL

General. IR spectrum was recorded on a Bruker IFS-55 IR spectrometer. UV spectrum was measured on a Shimadzu UV-2201 UV spectrometer in MeOH. ^1H and ^{13}C NMR experiments were recorded on a Bruker AV-300 instrument. HSQC and HMBC spectra were recorded on a Bruker AV-600 instrument using TMS as internal reference. High-resolution time-of-flight mass spectra (HR-TOF-MS) were measured on an Agilent 1200 LC/MSDTOF mass spectrometer, equipped with an ESI ion source, operating in positive-ion mode. Biological assays were measured on an IX71 inverted fluorescence microscope (Olympus Corporation, Japan) and an F039003 microplate reader (TECAN Corporation, Austria). The relaxant effect on isolated small intestine smooth muscle of rabbits was recorded on an RM6240B multi-channel physiological signal acquisition and processing system (Chengdu Instrument Production).

Column chromatography was carried out on silica gel (100–200 and 200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Sephadex LH-20 (Amersham Biosciences, Sweden), and LiChroprep RP-18 (40–60 μm, Merck). Fractions were monitored by TLC on silica gel plates (Merck precoated silica gel 60 F_{254}) and developed in the solvent systems chloroform–methanol and petroleum ether–acetone. Spots were visualized by heating after spraying with 10% H_2SO_4 and potassium heptaiodobismuthate.

Plant Material. The roots and rhizomes of *Rauvolfia verticillata* were collected in Yuanyang, Yunnan Province, People′s Republic of China, in October 2008, and identified by Prof. Jingming Jia. An authenticated voucher specimen (No. YN081004) has been deposited at the Herbarium of the College of Pharmacy, Shenyang Pharmaceutical University, Shenyang, People′s Republic of China.

Extraction and Isolation. Air-dried and finely powdered roots and rhizomes (12.1 kg) of *Rauvolfia verticillata* were sequentially extracted exhaustively with 70% ethanol four times at 70°C for 6 days, and the ethanol solutions were combined and concentrated in vacuo. The residue (2.1 kg) was suspended in H_2O and sequentially extracted four times each with petroleum ether, chloroform, EtOAc, and *n*-butanol to afford petroleum ether (90 g), chloroform (220 g), EtOAc (50 g), *n*-butanol (140 g), and H_2O-soluble (1.5 kg) fractions.

A portion of the chloroform-soluble extract was subjected to silica-gel CC (200–300 mesh, 1.5 kg) eluted with $CHCl_3$ (7.0 L), and then with a gradient of $CHCl_3$–MeOH by gradually increasing the polarity of the elution solvent system. The eluents were collected and monitored by TLC to give fractions A (6.8 g), B (17.9 g), C (35.2 g), D (38.7 g), E (35.3 g), F (23.5 g), G (21.5 g), and H (20.8 g).

Fraction E was subjected to silica-gel CC and eluted with a gradient of petroleum ether–acetone (98:2, 95:5, 93:7, 90:10, 80:20, 70:30, 60:40) to obtain compounds **2** (10.2 mg) and **3** (20.6 mg). Further subfractions rechromatographed on a silica gel column eluted with a gradient of chloroform–methanol and on an open ODS column and purified by Sephadex LH-20 CC afforded compounds **1** (5.5 mg), **4** (9.3 mg), and **5** (8 mg).

Seselin (2). ^{1}H NMR (300 MHz, $CDCl_3$, TMS, δ, ppm, J/Hz): 7.60 (1H, d, J = 9.5, H-4), 7.21 (1H, d, J = 8.4, H-5), 6.88 (1H, d, J = 10.1, H-9), 6.72 (1H, d, J = 8.4, H-6), 6.23 (1H, d, J = 9.5, H-3), 5.73 (1H, d, J = 10.1, H-10), 1.48 (6H, s, CH_3). ^{13}C NMR (300 MHz, $CDCl_3$, TMS, δ, ppm): 161.0 (C-2), 112.0 (C-3), 143.9 (C-4), 112.6 (C-4a), 130.7 (C-5), 113.5 (C-6), 156.3 (C-7), 109.3 (C-8), 150.1 (C-8a), 115.0 (C-9), 127.7 (C-10), 77.6 (C-11), 28.1 (CH_3).

Xanthyletin (3). ^{1}H NMR (300 MHz, $CDCl_3$, TMS, δ, ppm, J/Hz): 7.59 (1H, d, J = 9.5, H-4), 7.05 (1H, s, H-5), 6.72 (1H, s, H-8), 6.34 (1H, d, J = 9.9, H-10), 5.69 (1H, d, J = 9.9, H-9), 5.67 (1H, d, J = 9.5, H-3). ^{13}C NMR (300 MHz, $CDCl_3$, TMS, δ, ppm): 161.2 (C-2), 113.0 (C-3), 143.3 (C-4), 112.7 (C-4a), 118.5 (C-5), 120.8 (C-6), 155.4 (C-7), 104.4 (C-8), 156.8 (C-8a), 124.7 (C-9), 131.2 (C-10), 77.7 (C-11), 28.3 (CH_3).

Syringaresinol (4). ^{1}H NMR (300 MHz, $CDCl_3$, TMS, δ, ppm, J/Hz): 6.58 (4H, s, H-2, 2′, 6, 6′), 4.73 (2H, d, J = 4.2, H-7, 7′), 4.28, 3.90 (4H, m, H-9, 9′), 3.10 (2H, m, H-8, 8′). ^{13}C NMR (300 MHz, $CDCl_3$, TMS, δ, ppm): 132.1 (C-1, 1′), 102.7 (C-2, 2′), 147.1 (C-3, 3′), 134.3 (C-4, 4′), 147.1 (C-5, 5′), 102.7 (C-6, 6′), 86.1 (C-7, 7′), 54.3 (C-8, 8′), 71.8 (C-9, 9′), 56.4 (OCH_3).

Reserpine (5). ^{1}H NMR (300 MHz, $CDCl_3$, TMS, δ, ppm, J/Hz): 7.34 (3H, m, H-2′, 6′, 9), 6.85 (1H, d, J = 2.0, H-12), 6.78 (1H, dd, J = 8.5, 2.1, H-10), 5.06 (1H, m, H-3), 4.49 (1H, s, H-18), 3.93 (9H, s, C-3′, 4′, 5′-OCH_3), 3.83 (6H, s, 11-OCH_3, $COOCH_3$), 3.51 (3H, s, 17-OCH_3), 3.19 (2H, m, H-21), 3.19 (1H, m, H-5α), 3.05 (1H, m, H-5β). ^{13}C NMR (300 MHz, $CDCl_3$, TMS, δ, ppm): 172.8 (16-CO), 165.4 (1′-CO), 125.3 (C-1′), 130.2 (C-2), 106.8 (C-2′), 152.9 (C-3′), 53.7 (C-3), 142.3 (C-4′), 152.9 (C-5′), 51.7 (C-5), 106.8 (C-6′), 16.8 (C-6), 108.0 (C-7), 122.1 (C-8), 118.5 (C-9), 109.0 (C-10), 156.2 (C-11), 95.2 (C-12), 136.3 (C-13), 24.3 (C-14), 32.2 (C-15), 51.2 (C-16), 77.9 (C-17), 77.8 (C-18), 29.7 (C-19), 34.0 (C-20), 49.0 (C-21), 60.9 (4′-OCH_3), 60.7 (17-OCH_3), 56.2 (C-3′, 5′-OCH_3), 55.8 (11-OCH_3), 51.7 (CO-O$\underline{C}H_3$).

Cytotoxicity Assay. Cell culture: MCF-7 human breast cancer cells and HL-60 human promyelocytic leukemia were cultured in an RPMI 1640 nutritive medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. Fluorouracil was included as a reference substance.

Cell proliferation: tumor cells in the logarithmic growth phase were adjusted to 5×10^3 per well in a 96 well plate, and different concentrations of the tested samples were added after culturing for 24 h. Then 15 μL MTT (5 mg/mL) was added into the 96 well plate, and it was cultured in the CO_2 incubator for 48 h. Stock solutions were in 100% DMSO-d_6 and were diluted in the medium to a final concentration of 0.1% (v/v) DMSO-d_6 prior to treatment. The cells were fixed in DMSO-d_6 and stained with acridine orange (AO) and ethidium bromide (EB), and the cell morphology was observed using a fluorescence microscope. The absorbance (A) value was measured by the microplate reader at the wavelength of 492 nm. The cell inhibition rate was calculated according to the following formula: Inhibition ratio (%) = $[A_{492(control)} - A_{492(test)}]/[A_{492(control)} - A_{492(blank)}] \times 100$.

Antibacterial Assay. The antibacterial activity was assessed against three representative bacterial strains: *Staphylococcus aureus* CMCC(B)26003 (Gram-positive), *Escherichia coli* CMCC(B)44102 (Gram-negative), and *Candida albicans* CMCC(F)98001 (fungus) using the filter paper method.

Relaxant Effect on Intestine Smooth Muscles. Using routine experimental methods and isolated small intestine smooth muscle of rabbits, we investigated the effect of compound **1** on automatic contraction of small intestine smooth muscle. The contractile activity and signal changes in intestine smooth muscle were introduced into an RM6240 multi-channel physiological signal acquisition and processing system through a tension transducer.

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