Anti-HIV Lignans from *Schisandra micrantha*

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【**ABSTRACT**】 **AIM:** To find new natural compounds with anti-HIV activity from *Schisandra micrantha*. **METHORD:** The chemical constituents of *Schisandra micrantha* were isolated by various column chromatographic methods. Their structures were assigned by spectroscopic methods including 2D NMR experiments, and their anti-HIV-1 activities and cytotoxicities were tested by microtiter syncytium formation infectivity assay. **RESULT:** Four lignans, micrantherin A (**1**), gomisin K_3 (**2**), gomisin G (**3**), and vladinol F (**4**) were isolated from the leaves and stems of *Schisandra micrantha*. Vladinol F (**4**) was found to show potent anti-HIV-1 activity with IC_{50} value of 3.51 μg/mL and a selective index of 27.45. **CONCLUSION:** Micrantherin A (**1**) was a new lignan.

【**KEY WORDS**】 Schisandraceae, *Schisandra micrantha*; Lignan; Micrantherin A; Anti-HIV-1 activity

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1 Introduction

In the past two decades, a worldwide search has been made for new chemotherapeutic agents targeting the human immunodeficiency virus (HIV), the causative agent for acquired immune deficiency syndrome (AIDS). Twenty-five drugs, including nucleoside/nucleotide viral reverse transcriptase inhibitors (NRTIs)[1], non-nucleoside RT inhibitors (NNRTIs)[2], protease inhibitors (PIs)[2], and fusion (or entry) inhibitors (FIs)[3], are now approved for clinical use in the US. However, these drugs' efficiency is actually limited by the toxicity associated to such therapies and the appearance of HIV-1 resistance[4,5]. Therefore, current searches for new anti-HIV agents are focused on discovering compounds with novel structures and different mechanism of action. Many compounds with inhibitory HIV-1 activity have been screened out from natural products and provided a large reservoir for screening of anti-HIV-1 agents for their diversities[6].

The plants of the genus *Schisandra* are a rich source of lignans. Previous phytochemical studies on *Schisandra* species have resulted in the isolation of about 170 such compounds[7]. In our previous investigation, a cycloartane triterpenoid, nigranoic acid, was found to show remarkable inhibitory effect on several anti-HIV reverse transcriptase and polymerase assays[8]. In addition, schisantherin D demonstrated potent anti-HIV activity with EC_{50} value of 0.5 μg/mL, and a therapeutic index (TI) of 50.6, while the EC_{50} and TI values of interiotherin A were 3.1 μg/mL and 13.2, respectively[9]. Lancilactone C, isolated from *Kadsura*

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lancilimba, was found to shown anti-HIV activity with EC_{50} = 1.4 μg/mL and a TI of greater than 71.4[10]. A further examination of the 70% acetone extract of *S. micrantha* has led to the purification of a new lignan, micrantherin A (**1**), along with three known ones, gomisin K_3 (**2**)[11], gomisin G (**3**)[12], and vladinol F (**4**)[13]. Details of the isolation, structure determination of **1**, and inhibitory HIV-1 activity of compounds **1**～**4** are presented here.

2 Results & Discussion

Micrantherin A (**1**), was obtained as a yellow gum, and had the molecular formula $C_{28}H_{36}O_8$ as revealed by its HR-ESIMS ($[M + Na]^+$, *m/z* 523.2306, calcd for 523.2307). The UV spectrum, with maximum absorption at 241 (4.24) nm, and the IR spectrum, with bands at 3448 (OH), 1735 (ester), and 1647, 1598 cm^{-1} (aromatic), suggested that **1** was a dibenzocyclooctadiene lignan possessing a hydroxy group and an ester linkage[14]. The ^1H and ^{13}C NMR spectra revealed that **1** possessed five methoxyls and one phenolic hydroxyl group ($FeCl_3$ in EtOH, green) on the aromatic rings, an angeloyl group, and also a secondary methyl, a tertiary methyl and two methylenes on the cyclooctadiene ring[14]. The mass spectrum, with peaks at *m/z* 400 $[M-MeCH=C(Me)COOH]^+$ and 83 $[MeCH=C(Me)CO]^+$ supported the presence of an angeloyl group on **1**. The chemical shift of the angeloyl group (δ1.76, 3H, d, *J* = 6.5 Hz; 1.77, 3H, s; 5.97, 1H, m) in the ^1H NMR spectrum indicated that it was not shielded by the aromatic ring, suggesting it being attached to C-7 or C-8 of the cyclooctadiene ring[15,16]. In HMBC spectrum (Figure 1), H-6 (δ_H 2.73, d, *J* = 14.1 Hz; 2.48, dd, *J* = 7.8, 14.1 Hz) correlated to the secondary methyl (Me-18, δ_C 16.4), C-4 (δ_C 113.9) and C-16 (δ_C 124.5); H-9 (δ_H 2.38, 2.77, d, *J* = 13.7 Hz) correlated to the tertiary methyl (Me-17, δ_C 29.7), C-11 (δ_C 112.2) and C-15 (δ_C 123.3), H-7 (δ_H 1.86, m) correlated to C-5 (δ_C 136.0), which established the angeloyl group being located at C-8. In NOESY spectrum, the NOE correlation between H-3′ and H-7 further supported the above assignment (Fig 2).

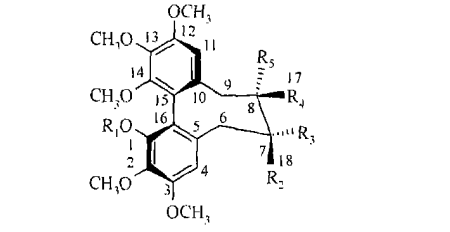
The phenolic hydroxyl group in **1** was located at C-1 position, which was supported by the presence of two upfield methoxyl signals at C-3 (δ 56.6) and C-12 (δ 56.4) in the ^{13}C NMR spectrum[17], and by comparison with that of isoschisandrin (**5**)[18,19]. In the ^{13}C NMR spectrum, the signals at the C-10～C-15 region in **1** appeared at the same chemical shifts as those of **5**, but the signal of C-1 (δ 151.7) in **5** was upfield shifted to δ 143.3 in **1**. The C-4, C-5 and C-16 signals were downfield shifted from δ 110.6, 133.7 and 122.9 in **5** to δ 113.9, 136.0 and 124.5 in **1**, respectively. These findings suggested that the 1-OCH_3 in **5** was replaced by 1-OH in **1**. This assignment was also supported by the HMBC correlations between H-4 (δ 6.88, s)/C-2 (δ 140.5), 2-OCH_3 (δ 3.84)/C-2 (δ 140.5), 3-OCH_3 (δ 3.91)/C-3 (δ 152.3), 12-OCH_3 (δ 3.87)/C-12 (δ 153.5), 13-OCH_3 (δ 3.84)/C-13 (δ 141.1), and 14-OCH_3 (δ 3.54)/C-14 (δ 152.9), respectively.

The stereochemical assignments in the cyclooctadiene ring were strengthened by the NOESY spectrum. The H-4 had NOE correlations with H-6β, 3-OCH_3, and 18-CH_3 [which was also correlated with H-6α]; the H-11 with H-9α and 12-OCH_3; the H-7β with Me-18 indicating a twist-boat-chair conformation (Fig 2)[19].

To determine the absolute configuration of the biphenyl group of **1**, the circular dichroism (CD) absorption values were examined. The CD spectrum showed positive Cotton effect around 225-255 nm, suggesting micrantherin A (**1**) possessed an R-biphenyl configuration as gomisin A, whose absolute structure has been established by X-ray analysis and shown to have R-configuration of biphenyl part[20,21].

The potencies of micrantherin A (**1**), gominsin K_3 (**2**), gominsin G (**3**), and vladinol F (**4**) in preventing the cytopathic effects of HIV-1 in C8166 cells, as well as compound-induced cytotoxicity in C8166 cells in parallel with the antiviral activity were evaluated. The results from the cell-based assays are summarized in Table2. Among these compounds, **4** possessed cytotoxicity with CC_{50} value of 96.36 μg/mL on tested human T

cell leukemia cell line C8166 at the assayed doses, and demonstrated potent anti-HIV-1 activity with EC_{50} value of 3.51 μg/mL and a selectivity index (SI) of 27.45. Micrantherin A (**1**) showed weak anti-HIV activity.



	R_1	R_2	R_3	R_4	R_5
1	H	H	CH_3	CH_3	OAng
5	CH_3	CH_3	OH	H	CH_3

Ang =

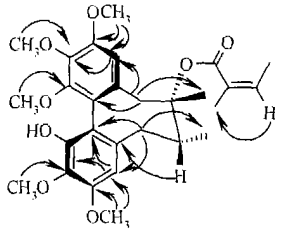


Fig **1** Selected HMBC correlations of **1**

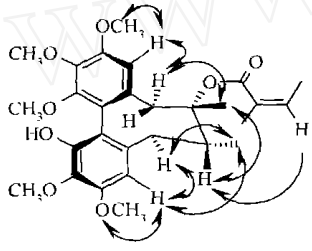


Fig **2** Key NOESY correlations assigned for **1**

3 Experimental

3.1 *General*

Optical rotations were measured in Horiba SEPA-300 High Sensitive Polarimeter. The CD spectra were recorded on a JASCO J-810 spectropolarimeter. IR spectra were obtained in KBr disc on a Bio-Rad Win infrared spectrophotometer. ESI-MS were measured on a VG Auto Spec-3000 MS spectrometer. ^1H, ^{13}C NMR and 2D NMR spectra were recorded on Bruker AM-400 and DRX-500 instruments with TMS as internal standard. Column chromatography was performed on silica gel (200-300 mesh), or on silica gel H (10～40 μm, Qingdao Marine Chemical Inc., China). Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10 % H_2SO_4 in EtOH.

3.2 *Plant material*

The leaves and stems of *S. micrantha* were collected in Dali, Yunnan, PR China, in November 2001. A voucher specimen (No. KIB 01-11-05) was deposited in the State Key Laboratory of Phytochemistry, Kunming Institute of Botany, and was identified by Prof. Wu Su-Gong.

3.3 *Extraction and Isolation*

The air-dried and powdered leaves and stems of *S. micrantha* (6.8 kg) were extracted with 70 % aqueous Me_2CO (20 L ×3, 24 h) at room temperature and the extract was partitioned successively with petroleum ether (3 L ×2) and EtOAc (5 L ×3), respectively. The petroleum ether extract (75 g) was separated into six fractions (Fr. Ⅰ～Ⅵ) through column chromatography (CC) over silica gel eluting with petroleum ether containing amounts of acetone (1∶0, 19∶1, 9∶1, 8∶2, 7∶3). Fr. Ⅲ (19 g, eluted with 10 % acetone) was chromatographed on silica gel using cyclohexane-isopropanol (100∶1) as elution to afford **1** (285 mg) and **2** (24 mg). The EtOAc extract (170 g) was subjected to CC over silica gel eluting with a $CHCl_3$-Me_2CO (1∶0-0∶1, 180 L) gradient system to give fractions 1-5. Fr. 2 ($CHCl_3$) was chromatographed on CC (silica gel, petrol-Me_2CO 19∶1) to give **3** (48 mg). Fr. 3 ($CHCl_3$-Me_2CO 10∶1, 11.5 g) was further purified by CC on silica gel eluted with petrol-EtOAc-Me_2CO (10∶1∶1) to yield **4** (175 mg).

3.3.1 *Micrantherin A* (**1**) Yellow gum. $[\alpha]_D^{17.9}$ + 16.2°(*c* 0.43, $CHCl_3$); IR (KBr) ν_{max} 3448 (br), 2935, 1735, 1647, 1598, 1578, 1494, 1456, 1401, 1321, 1225, 1127 cm^{-1}, UV (MeOH) λ_{max} (log ε) 241 (4.24) nm. CD (*c* = 0.015, $CHCl_3$) $\Delta OD_{256.4}$ + 80.049, $\Delta OD_{232.6}$ - 16.136, $\Delta OD_{227.8}$ + 30.257, ΔOD_{210} - 27.315 (mdeg). EI-MS *m/z* 500 (M^+, 100), 482 (8), 432 (6), 418 (42), 400 (8), 386 (15), 375 (8), 345 (15), 315 (40), 299 (15), 271 (5), 241 (4), 181 (1), 83 (42). HRESI-MS (positive ion mode) *m/z*: found 523.2306 $[M + Na]^+$

calc. 523.2307). ^{1}H (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) data see Table 1.

Table 1 ^{1}H and ^{13}C NMR Spectral Data for micrantherin A (1) in CD_3OD[a]

Position	δ_H(mult, J, Hz)	δ_C	position	δ_H(mult, J, Hz)	δ_C
1		143.3 s	14		152.9 s
2		140.5 s	15		123.3 s
3		152.3 s	16		124.5 s
4	6.88 (s)	113.9 d	17	1.25 (s)	29.7 q
5		136.0 s	18	0.89 (d, 7.1)	16.4 q
6α	2.48 (dd, 7.8, 14.1)	36.2 t	1′		166.9 s
6β	2.73 (d, 14.1)		2′		128.7 s
7	1.86 (m)	42.1 d	3′	5.97 (m)	138.6 d
8		73.5 s	2′-Me	1.77 (s)	20.6 q
9α	2.77 (d, 13.7)	42.6 t	3′-Me	1.76 (d, 6.5)	15.7 q
9β	2.38 (d, 13.7)		2-OCH_3	3.84 (s)	61.3 q
10		135.7 s	3-OCH_3	3.91 (s)	56.6 q
11	6.73 (s)	112.2 d	12-OCH_3	3.87 (s)	56.4 q
12		153.5 s	13-OCH_3	3.84 (s)	61.2 q
13		141.1 s	14-OCH_3	3.54 (s)	61.0 q

[a]Data were recorded on Bruker DRX-400 MHz spectrometer; assignments were confirmed by HMBC and ROESY.

Table 2 Anti-HIV-1 activity, cytotoxicity, selective index for 1～4[a].

Compound	Cytotoxity	Syncytium	
	CC_{50} (μg/mL)[b]	EC_{50} (μg/mL)[c]	SI[d]
1	99.02	24.54	4.04
2	30.76	3.83	8.03
3	56.01	5.74	9.57
4	96.36	3.51	27.45

[a]Data are expressed as means of three dependent measurements.

[b] Concentration required to reduce C8166 cells viability by 50 %.

[c]Concentration required to reduce HIV-1_{IIIB} induced syncytium formation by 50 % on C8166 cells.

[d]Selectivity index (SI): ratio CC_{50}/ EC_{50}.

3.3.2 *HIV-1 Inhibition assays*[22-24] Cytotoxicity assay. The cellular toxicity of compounds on C8166 cells was assessed by MTT colorimetric assay as described previously. Briefly, 100 μL of Vero cells was seeded onto a microtiter plate, 100 μL of various concentrations of compounds was added and incubated at 37 ℃ in a humidified atmosphere of 5 % CO_2 for 72 h. Discard 100 μL supernatant, MTT reagent was added and incubated for 4 h, 100 μL 50 % DMF-10 % SDS was added. After the formazum was dissolved completely, the plates were read on a Bio-Tek ELx 800 enzyme-linked immunosorbent assay (ELISA) reader at 595 nm/630 nm. The results were shown by absorbance values.

Inhibition assay for the cytopathic effects of HIV-1. Antimicrobial peptides serially diluted with RPMI-1640 medium were added to triplicate wells of a 96-well flatbottomed microtiter plate, then 4×10^4 C8166 cells and 200 $TCID_{50}$ (50 % tissue culture infectious dose) of HIV-1_{IIIB} stock solution were added immediately to each well. After incubation at 37 ℃ for 72 h without changing medium, syncytial cells from five different fields of each well were examined and counted under an inverted microscope (100 ×). The inhibition percentage of syncytial cell formation was calculated by percentage of syncytial cell number in the sample treated culture to that in infected control culture. The concentration of the antiviral sample reducing HIV-1 replication by 50 % (EC_{50}) was determined from the dose response curve. The selectivity index (SI) was calculated from the ratio of CC_{50}/ EC_{50}.

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小花五味子中抗 HIV 活性的木脂素类化合物

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【摘　要】 **目的**:从小花五味子中发现具有抗 HIV 活性的化学成分。**方法**:采用多种柱层析分离进行化合物的分离和纯化, 通过波谱分析鉴定化合物的结构, 化合物的抗 HIV-1 活性通过对 HIV-1 感染 C8166 细胞致细胞病变的抑制试验得到。**结果**:从小花五味子的茎藤部分分离得到了 4 个木脂素, 分别鉴定为 micrantherin A (**1**), gomisin K_3 (**2**), gomisin G (**3**) 和 vladinol F (**4**)。化合物 **4** 具有显著的抗 HIV-1 活性, IC_{50} = 3.51 μg/mL, 选择指数为 27.45。**结论**:化合物 **1** 为新的木脂素, 化合物 **4** 的抗 HIV-1 活性值得进一步研究。

【关键词】 五味子科; 小花五味子; 木脂素; micrantherin A; 抗 HIV 活性

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