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Anti-HIV-1 Activity of Lignans from the Fruits of *Schisandra rubriflora*

Wei-Lie Xiao[1], Rui-Rui Wang[2], Wei Zhao[1], Ren-Rong Tian[2], Shan-Zhai Shang[1], Liu-Meng Yang[2], Jian-Hong Yang[1], Jian-Xin Pu[1], Yong-Tang Zheng[2], and Han-Dong Sun[1]

[1]State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, Yunnan, China and [2]Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences and Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, Yunnan, China

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This study investigated the 70% aqueous acetone extract of the fruits of *Schisandra rubriflora* which led to the isolation of eight lignans, including a new isolate, rubrisandrin C (**1**), and seven known lignans (**2**-**8**) . The structure of **1** was established by extensive 1D and 2D NMR spectroscopy and its absolute stereochemistry was determined by CD spectrum. Compounds **1**-**5** and **7**-**8** were evaluated for their anti-HIV-1 activity that showed inhibitory activity on HIV-1_{IIIB} induced syncytium formation with EC_{50} values in the range of 2.26~20.4 μg/mL. Compounds **1** and **7** exerted their obvious protection of HIV-1_{IIIB} inducted MT-4 host cells lytic effects with a selectivity index of 15.4 and 24.6, respectively.

Key words: *Schisandra rubriflora,* Lignan, NMR, CD spectrum, Anti-HIV-1 activity

INTRODUCTION

Schisandra genus species is medicinally important and commonly used in Traditional Chinese Medicine for their diverse beneficial bioactivities. The fruits of *Schisandra* plants are commonly used in China as sedative and tonic agents. Previous studies have shown that lignans are the bioactive constituents of genus *Schisandra* species, which show various beneficial activities including antihepatitis, antitumor, and anti-HIV-1 activities (Sun et al., 1996; Kuo et al., 1997; Chen et al., 1998, 1999, 2001).

Schisandra rubriflora (Franch.) Rehd. et Wils, a climbing plant mainly distributed in southwest region of China, has been used as sedative and tonic agents in traditional Chinese medicine for a long time. Therefore, this species has attracted the interests of phytochemists and pharmacologists, which led to the discovery of a series of lignans (Chen et al., 2006; Li et al., 2004a, 2004b, 2005, 2008; Wang and Chen, 1985) and triterpenoids (Xiao et al., 2006, 2007a, 2007b), while some of them showed anti-HIV-1 activities (Chen et al., 2006; Xiao et al., 2006, 2007a). Since our previous phytochemical research led to the isolation of a series of highly oxygenated nortriterpenoids from *S. rubriflora* collected from Dali prefecture of Yunnan province in China (Xiao et al., 2006, 2007a, 2007b), we are interested in the investing the chemical constituents of its fruits. Our current chemical research led to the isolation of eight lignans from the 70% aqueous acetone extract of the fruits of this plant, including a new one, rubrisandrin C (**1**), and seven known ones, *i. e.* gomisin O (**2**) (Ikeya et al., 1991), angeloylgomisin P (**3**) (Ikeya et al., 1980), gomisin G (**4**) (Ikeya et al., 1979), Wulignan A_2 (**5**) (Liu et al., 1988), Epiwulignan A_1 (**6**) (Liu et al., 1988), 4,4'- Dihydroxy-3-methoxy-7,7'- epoxylignan (**7**) (Achenbach et al., 1987), and 3,3'-Dihydroxy-4,4',5,5'-tetramethoxylignan (**8**) (Ikeya et al., 1978). The structure of **1** was determined on the basis of extensive 1D and 2D NMR spectroscopy and its absolute stereochemistry was determined by CD spectrum. In this study, we report that the isolation and structural elucidation of new compound **1** and the anti-HIV-1 activity of all compounds **1**-**8**.

Correspondence to: Han-Dong Sun, State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, Yunnan, China
Tel: 86-871-5223251, Fax: 86-871-5216343
E-mail: hdsun@mail.kib.ac.cn

MATERIALS AND METHODS

General Experimental procedure

Optical rotations were measured with a Horiba SEPA-300 polarimeter. CD spectra were measured on JASCO J-810 spectropolarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D and 2D NMR spectra were recorded on Bruker AM-400 and DRX-500 spectrometers. Unless otherwise specified, chemical shifts (δ) were expressed in ppm with reference to the solvent signals. Mass spectra were performed on a VG Autospec-3000 spectrometer under 70 eV. Column chromatography was performed with silica gel (200-300 mesh, Qing-dao Marine Chemical, Inc.) and MCI gel CHP20P (75-150 μm, Mitsubishi Chemical Corporation). Semi-preparative HPLC was performed on a Hewlett Packard instrument (column: Zorbax SB-C18, 250 × 9.4 mm; DAD detector). Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

Plant material

The fruits of *S. rubriflora* were collected in August 2003 from Damang mountain, Dali Prefecture of Yunnan Province, China. The specimen was identified by Prof. Xi-Wen Li. A voucher specimen, No. KIB 2003-08-02, has been deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation

Air-dried and powdered fruits (4.2 kg) were extracted with 70% aqueous acetone (3 × 5 L) at room temperature and concentrated *in vacuo* to yield a crude extract (310 g), which was partitioned between H_2O and EtOAc. The EtOAc portion (180 g) was subjected to column chromatography over MCI gel eluting with 95% EtOH and concentrated *in vacuo*. The residue (126 g) was chromatographed on a silica gel column eluting with $CHCl_3$-CH_3OH (9:1, 8:2, 2:1, 1:1, and 0:1) to afford fractions I-VI. Fraction II (4.1 g) was further chromatographed on silica gel column to afford six sub-fractions II(a)-II(e). Sub-fraction II(b) was then purified by semi-preparative HPLC (CH_3OH:H_2O, 45:55) to yield compounds **2** (7 mg), **7** (19 mg), and **8** (10 mg). Fraction III (7.9 g) was further chromatographed on a silica gel column eluting with $CHCl_3$-Me_2CO (9:1, 8:2, 2:1, 1:1) to afford sub-fractions III(a)-III(g). Sub-fraction III(a) (2.0 g) was purified by crystallization and repeated chromatographed over silica gel, RP-18 and Sephadex LH-20 (CH_3OH), followed by semi-preparative (CH_3OH:H_2O, 35:65 and CH_3OH:CH_3CN:H_2O, 10:30:60) to yield compounds **1** (9 mg), **3** (11 mg), and **5** (10 mg). Sub-fraction III(c) (1.5 g) was chromatographed on Sephadex LH-20 (CH_3OH), and then followed by semi-preparative (CH_3OH:H_2O, 40:60) to yield compounds **4** (13 mg) and **6** (2 mg).

Rubrisandrin C (1)

Yellow powers; $[\alpha]_D^{25.3}$ + 8.26°(*c* 0.483, MeOH); UV (MeOH) λ_{max} (log ε) 240 (3.35), 326 (0.47), 346 (0.32) nm; CD (*c* 0.0512, MeOH) λ_{max} nm ($\Delta\varepsilon$) 250 (43.10), 224 (+18.46); IR (KBr) ν_{max} 3478, 3407, 2965, 1713, 1602, 1578, 1454, 1374, 1329, 1271, 1156, 1128, 1108, 1025, 997 cm^{-1}; positive FAB: 373 $[M+H]^+$; HRESIMS $[M+H]^+$ *m/z* 373.1659, calcd 373.1651; ^1H- and ^{13}C-NMR data see Table I.

Anti-HIV activity

The cytotoxic property and anti-HIV activities of **1-5** and **7-8** were tested using the method previously described, with AZT as a positive control (Wang et al., 2004a, 2004b).

For cytotoxicity assay, 4×10^4 cells were seeded per well on 96-well plate in the absence or presence of various gradient concentrations of compounds in triplicate. Then 96-well plates were incubated for 3 days in at 37°C, 5% CO_2 humidified incubator. The supernatants were discarded and MTT (5 mg/mL in PBS) was added to each well. After incubating for 4 h, 100 μL of 50% DMF-20% SDS was added. Following complete dissolution of formazan, the plates were read on a Bio-Tek Elx 800 ELISA reader at 595/630 nm. The 50% cytotoxic concentration (CC_{50}) was calculated.

For syncytia assay, 4×10^4 C8166, infected with virus HIV-1_{IIIB} at a multiplicity of infection (M.O.I.) of 0.15, were seeded on 96-well plate in the absence or presence of various gradient concentrations of compounds in triplicate. The final volume per well was 200 μL. Control assays were performed without the testing compounds in HIV-1_{IIIB} infected and uninfected cultures. After three days of culture, the cytopathic effect (CPE) was measured by counting the number of syncytia and the 50% effective concentration (EC_{50}) was calculated.

For protection assay of HIV-1_{IIIB} inducted MT-4 cells lytic effects, uninfected or HIV-infected (M.O.I. = 0.1) MT-4 cells were seeded in 96-well flat-bottomed microtiter culture plates with 100 μL of various gradient concentrations of compounds in triplicate. After 7 days of incubation at 37°C, 20 μL of MTT stock solu-

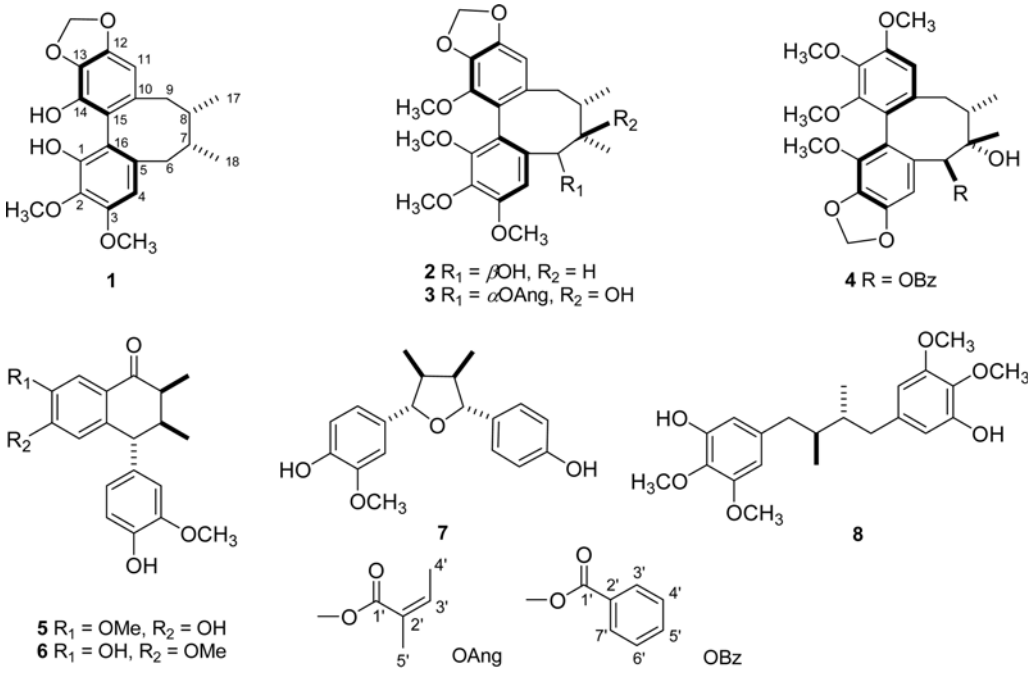

Fig. 1. Chemical structures of compounds **1**-**8**

Table I. ^{1}H and ^{13}C NMR assignments of **1**[a]

Position	δ_H (mult., J, Hz)	δ_C (mult.)	Position	δ_H (mult., J, Hz)	δ_C (mult.)
1	/	145.8 s	11	6.41 (s)	102.2 d
2	/	133.5 s	12	/	148.5 s
3	/	150.7 s	13	/	132.8 s
4	6.43 (s)	108.5 d	14	/	136.9 s
5	/	136.1 s	15	/	117.2 s
6α	2.47 (dd, 1.9, 13.6)	39.1 t	16	/	114.1 s
6b	2.55 (dd, 7.4, 13.6)		17	0.95 (d, 7.2)	21.4 q
7	1.89 (m)	33.5 d	18	0.72 (d, 7.2)	12.8 q
8	1.79 (m)	40.7 d	2-OCH_3	3.91 (s)	61.1 q
9α	2.15 (dd, 9.4, 13.2)	35.7 t	3-OCH_3	3.86 (s)	55.9 q
9b	2.03 (br d, 13.2)		OCH_2O	5.96 (d, 1.3)	101.3 t
10	/	138.2 s		5.95 (d, 1.3)	

[a]Spectra were recorded in C_5D_5N, chemical shifts (δ) are in ppm and J in Hz.

tion was added to each well. After 4 h of incubation at 37°C, 100 μL of the medium was carefully removed without disturbing the cells containing the formazan crystals. 100 μL 20% SDS-50% DMF was added. After the formazan dissolved completely, the plates were read on a Bio-Tek ELx 800 ELISA reader at 595 nm/630 nm. The 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}) was calculated.

RESULTS AND DISCUSSION

Rubrisandrin C (**1**), obtained as an amorphous powder, has the molecular formula $C_{21}H_{25}O_4$ as determined by HREIMS $[M+H]^+$ 373.1659 (calcd 373.1651). The ^{1}H- and ^{13}C-NMR spectra of **1** clearly indicated the presence of 12 aromatic carbons, two aromatic protons, one methylenedioxy group, and two methoxy groups, suggesting the presence of a biphenyl moiety (Ikeya et al., 1979). In addition, HMBC correlations of H-4 with C-5, C-6, and C-16 and of H-11 with C-9, C-10, and C-15, and ^{1}H-^{1}H COSY correlations of H-6/H-7/H-8/H-9 (Fig. 2), together with UV absorption bands at 208, 240, 326, and 346 nm, implied that **1** could be a dibenzocyclooctadiene lignan (Ikeya et al., 1979). HMBC correlations of the protons at δ_H 5.96 (OCH_2O) with C-12 and C-13 suggested that the methylenedioxy group was connected with C-12 and C-13, which was also supported by the obvious HMBC correlation observed from H-11 with C-12 and C-13. HMBC correlation of one methoxy group protons at δ_H 3.86 with C-3 and

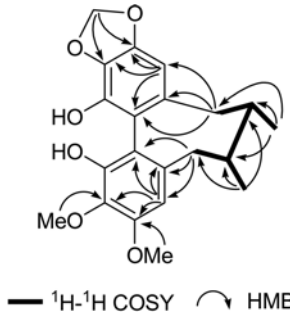

Fig. 2. Key HMBC and ^{1}H-^{1}H COSY correlations of **1**

the ROESY correlation of the protons of this methoxy group with H-4 determined this methoxy group was located at C-3 (Fig. 2). Another methoxy group was attached at C-2 was established by HMBC correlation of the protons of this methoxy group with C-2 and strong HMBC correlation of H-4 with C-2. In the cyclooctadiene ring, two secondary methyl groups (δ_H 0.95, d, J = 7.2 Hz; δ_H 0.72, d, J = 7.2 Hz) can be assigned to CH_3-17 and CH_3-18, respectively. The signals of two methylenes were assigned to C-6 and C-9 and two methines were assigned to be C-7 and C-8 by the analysis of HMBC and ^{1}H-^{1}H COSY correlations (Fig. 2). According to the molecular formula, each of aromatic carbons at C-1 and C-14 should be substituted by a hydroxy group, respectively.

Since the CD spectrum of dibenzocyclooctadiene lignan was dominated by the axial chirality of the biphenyl chromophore, the absolute configuration of biphenyl axis was determined by CD spectrum. The CD curve showed a negative Cotton effect around 250 nm and a positive one around 224 nm, suggesting that **1** possessed an *S*-biphenyl configuration (Ikeya et al., 1979). With the axial chirality defined, a ROESY experiment was used to establish the relative configuration of the remaining stereocenters (Fig. 3). The observed ROESY correlations of H-11 with H-8 and H-9, H-4 with H-6 and CH_3-17, H-6α with CH_3-17, and H-9 with CH_3-18 were consistent with a cyclooctadiene lignan with a twisted boat/chair conformation and the relative configurations of C-7 (*S*) and C-8 (*R*) (Fig. 3) (Choi et al., 2006). As a result, the structure of Rubrisandrin C (**1**) was determined as shown.

The cytotoxicities and anti-HIV activities of isolates **1-5**, **7-8** were tested by syncytium formation inhibitory assay, with AZT as a positive control to qualify the assay (CC_{50} > 1,000 μg/mL and EC_{50} 0.004 μg/mL). The assays included cytotoxicity in C8166 and MT-4 cells, inhibition of syncytium formation in HIV-1_{IIIB} infected C8166 cells, and effect in protecting HIV-1_{IIIB} infected MT-4 host cells from lytic effects *in vitro*. The tested compounds showed cytotoxicity with CC_{50} values in the range of 10.09~87.10 μg/mL on C8166 at the assayed doses. All tested compounds showed inhibitory activity on HIV-1_{IIIB} induced syncytium formation with EC_{50} values in the range of 2.26~20.4 μg/mL. The selectivity index of compounds **1-5** and **7-8** was 6.15, 5.75, 3.63, 3.58, 9.49, 2.83, and 4.46, respectively (Table II). As shown in Table III, compounds **1** and **7** exerted obvious protection of HIV-1_{IIIB} inducted MT-4 cells lytic effects with a selectivity index of 15.4 and 24.6, respectively. Compound **6** was not tested for its anti-HIV-1 activity due to the limited availability. AZT as a positive control with a selectivity index >277.8.

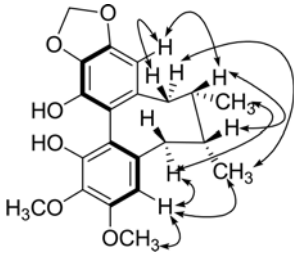

Fig. 3. Key ROESY correlations of **1**

Table II. Summary of cytotoxicity and anti-HIV-1 activity of **1-5**, **7-8**

Compound	Cytotoxicity CC_{50} (μg/mL)	Anti-HIV-1 activitiy EC_{50} (μg/mL)	Selectivity index CC_{50}/EC_{50}
1	34.28	5.57	6.15
2	31.62	5.50	5.75
3	47.97	16.6	3.63
4	72.44	20.4	3.58
5	87.10	9.18	9.49
7	26.98	9.55	2.83
8	10.09	2.26	4.46
AZT	>1000	0.004	>250000

Table III. Protective activities of tested compounds towards HIV-1_{IIIB} infected MT-4 cells

Compound	CC_{50} (μg/mL)	EC_{50} (μg/mL)	Selectivity index CC_{50}/EC_{50}
1	87.10	5.67	15.4
2	33.11	NP[a]	NP[a]
3	87.10	NP[a]	NP[a]
4	79.43	NP[a]	NP[a]
5	91.20	74.13	1.2
7	83.18	3.38	24.6
8	21.88	NP[a]	NP[a]
AZT	>1	0.0036	> 277.8

[a]NP means no protective activity.

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