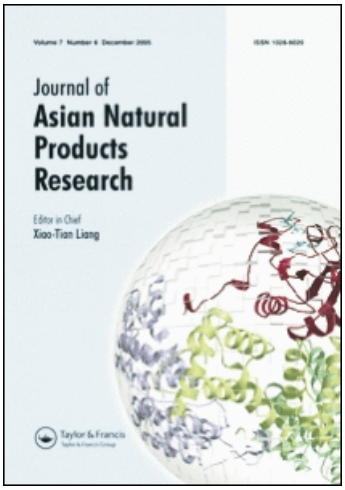

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A potent anti-HIV polyphenol from *Salvia yunnanensis*

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A potent anti-HIV polyphenol from *Salvia yunnanensis*

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A new polyphenol, designated as salvianolic acid N, was isolated from the aqueous extracts of the roots of *Salvia yunnanensis*. Its chemical structure was elucidated as 3-(3,4-dihydroxylphenyl)-2-{(E)-3-(1,8,9-trihydroxyl-dibenzo[b,-f]oxpin-3-yl)acryloxloxy}propanoic acid (1) on the basis of NMR and MS spectral analyses. The new polyphenol inhibited both HIV-1 IN *in vitro* and also reduced HIV-1 p24 antigen in MT-4 cell lines.

Keywords: *Salvia yunnanensis*; Labiatae; salvianolic acid N; anti-HIV activity

1. Introduction

Danshen is officially listed in the Chinese Pharmacopoeia. It is widely used in Traditional Chinese Medicine (TCM) for the treatment of cardiovascular diseases, hepatitis and hepatocirrhosis.[1] The polyphenols constitute the major part of the water-soluble components of Danshen and possess a variety of biological activities including antioxidant,[2] antitumour,[3] and antiviral.[4] Most of the polyphenols identified so far are in sequence designated as salvianolic acids A–M or yunnaneic acids A–H.[5,6] The total polyphenols purified from Danshen have been clinically used for the treatment of cardiovascular diseases in China.

Salvia yunnanensis is used as resources of Danshen in Yunnan province, China.[7] The water-soluble extracts of *S. yunnanensis* were found to have a potent effect against human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) in our laboratory. Subsequently, lithospermic acid and lithospermic acid B isolated from *S. yunnanensis* were found to be potent inhibitors of HIV-1 integrase.[4] During the later studies we found that the activity against HIV-1 is attributed principally to the presence of total polyphenols in *S. yunnanensis*. This prompted us to further study polyphenol chemical components in *S. yunnanensis* and resulted in the isolation of a new polyphenol, designated as salvianolic acid N (1) (Figure 1). Here we report the separation and structure elucidation of the new compound and its anti-HIV activity.

2. Results and discussion

Salvianolic acid N (**1**) was obtained as yellow, amorphous powder, and gave a dark green colour with $FeCl_3$ reagent. It was determined by high-resolution negative electrospray mass spectrometry (HRESI-MS) to have the molecular formula $C_{26}H_{20}O_{10}$, with a quasimolecular ion peak at *m/z* 491.0984 $[M - H]^-$. The IR spectrum exhibited absorption bands at 3345, 1664, 1657, 1607, and 1588 cm^{-1}, characteristic of the hydroxyl groups, the α,β-unsatured carbonyl and the aromatic rings. The ^1H NMR spectrum of **1** showed two doublets ($J = 16.0$ Hz) at δ 7.88 and 6.22 due to *trans*-olefinic protons conjugated with a carbonyl group and two doublets ($J = 8.5$ Hz) at δ 7.28 and 6.82 due to *cis*-olefinic protons. The appearance of three double doublets in the aliphatic region (δ 5.14, $J = 8.0$, 4.5 Hz; δ 3.05, $J = 15.0$, 4.5 Hz; δ 2.96, $J = 15.0$, 8.5 Hz) for a —CH(O)—CH_2— unit, along with ABX spin systems in the aromatic region suggested the presence of α,β-(3,4-dihydroxyphenyl) lactic acid moiety. In addition, a pair of singlets at δ 6.80 and 6.59 and two doublets ($J = 1.5$ Hz) at δ 6.78 and 6.77 revealed the presence of two *para* protons and two *meta* protons in the two separated benzol units. The ^{13}C NMR spectrum of **1** showed the presence of two carbonyl carbons, of which one was identical with carboxylic acid (δ 173.5) and another was a carboxyl ester (δ 168.1). In addition, three sets of 3,4-dihydroxyphenyl groups and a dibenzo[*b,f*]oxepin skeleton were deduced for **1** according to the presence of 18 aromatic carbons including seven tertiary carbons (δ 109.7–132.8) and 11 quaternary carbons, of which seven were phenoxyl carbons (δ 143.6–152.3). The compounds with dibenzo[*b,f*]oxepin skeleton were extremely rare in nature and some of them have been isolated from the aqueous extract of roots of *Salvia chinensis*, the roots of *Salvia mililtiorrhiza* and the leaves, stems, and pods of

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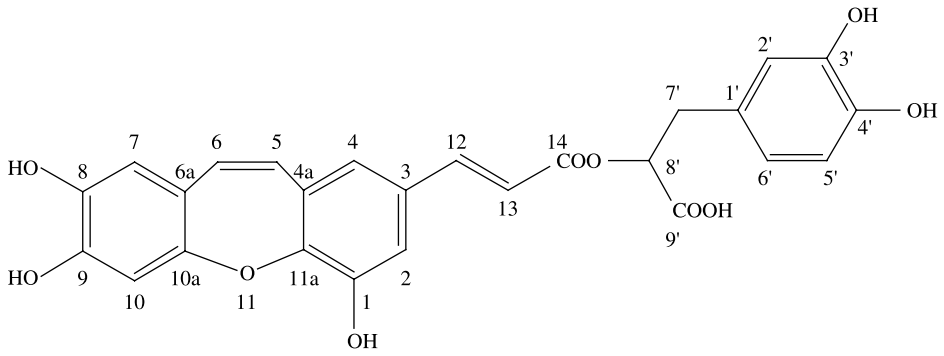

Figure 1. The structure of **1**.

Bauhinia pururea.[8–11] The dibenzo[*b,f*]oxepin skeleton for **1** was affirmed by HMBC correlation. Moreover, there were also two pairs of olefinic carbons (δ 143.7 and 117.4, δ 125.3 and 117.5), one oxygenated methine carbon (δ 74.7) and one methylene carbon (δ 37.9) present in the spectrum. The correlation peaks between protons and carbons appeared as illustrated in Figure 2. In the HMBC spectrum, the conjugated carboxyl carbon C-14 was correlated with the oxygen-bearing methine proton C-8′, indicating that the α,β-unsaturated carboxyl group was attached to the hydroxyl group of lactic acid moiety through ester linkage. On the basis of above evidence, the structure of **1** was elucidated as 3-(3,4-dihydroxylphenyl)-2-{(*E*)-3-(1,8,9-trihydroxyl-dibenzo[*b,f*]oxpin-3-yl)acryloxloxy}propanoic acid, named as salvianolic acid N. The full assignments of the ^{1}H NMR and ^{13}C NMR resonances of **1** (Table 1) were made using HMQC and HMBC experiments.

Salvianolic acid N (**1**) was evaluated for inhibitory activities against HIV-1 integrase (IN) and HIV-1 reverse transcriptase (RT) *in vitro* and anti-HIV-1 IIIB activity in MT-4 cell lines. As shown in Tables 2 and 3, the salvianolic acid N is inhibited on HIV-1 RT and IN, and the IC_{50} values were 67.10–193.39 μg/ml and 1.78–18.5 μg/ml, respectively. The non-toxic concentration of salvianolic acid N is also inhibitory on HIV-1 p24 antigen expression in MT-4 cell lines the TC_{50} value of cellular toxicity is 3.7–24.10 μg/ml, the IC_{50} of inhibition on P24 in MT-4 cell cultures were 0.649–4.28 μg/ml and the selective index (SI) were 5.63–5.70.

3. Experimental

3.1 General experimental procedures

UV spectra were obtained on a Shimadzu UV-260 spectrophotometer in MeOH. The optical values were determined on a Perkin–Elmer 341 polarimeter. IR spectra were recorded on a JASCO FT/IR-5300 spectrometer. NMR spectra were taken on a Varian Unity INOVA-500 spectrometer in MeOH-d_4 with tetramethylsilane (TMS) as an internal reference. ESI-MS, including HRESI-MS, was carried out on Waters Micromass ZQ 2000 mass spectrometer. HPLC was performed with a Shimadzu LC-10Avp instrument equipped with SPD-10Avp (UV–Vis) detector and YMC-Pack Pro C18 (5 μm, diameter 4.6 × 250 mm); solvent, MeOH/H_2O/CH_3COOH (25:75:0.005). Water and EtOH were used as eluent for Diaion HP20 CC, Sephadex LH-20 CC and ODS CC.

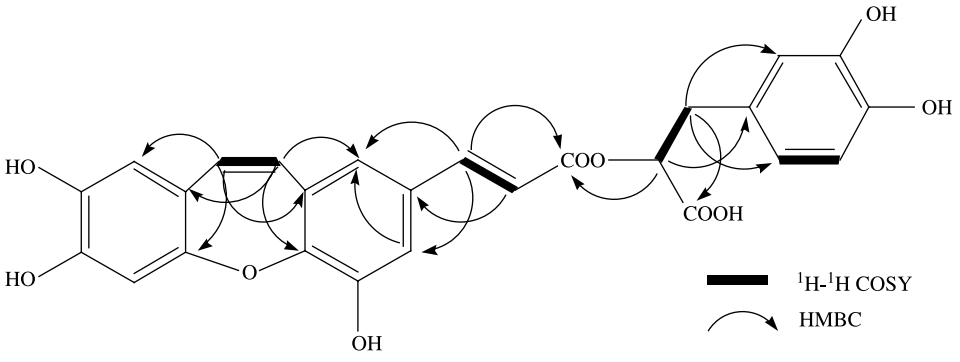

Figure 2. Key ^{1}H–^{1}H COSY and HMBC correlations of **1**.

Table 1. NMR spectral data of **1** in CD_3OD.

Position	δ_H	H → H	δ_C	HMBC (H → C)	
1				143.9	
2	6.77	d	1.5 (4)	124.8	C-1, C-3, C-12
3				132.5	
4	6.78	d	1.5 (2)	132.8	C-3, C-4a, C-5, C-12
4a				123.3	
5	7.28	d	8.5 (6)	125.3	C-4, C-4a, C-6, C-11a
6	6.82	d	8.5 (5)	117.5	C-5, C-6a, C-7, C-10a
6a				125.0	
7	6.80	s		109.7	
8				147.3	
9				148.3	
10	6.59	s		115.5	
10a				152.3	
11a				152.2	
12	7.88	d	16.0 (13)	143.7	C-3, C-13, C-14
13	6.22	d	16.0 (12)	117.4	C-3, C-12, C-14
14				168.1	
1′				129.3	
2′	6.70	d	1.5 (6′)	117.6	C-1′, C-7′
3′				146.2	
4′				145.3	
5′	6.66	d	8.0 (6′)	116.3	C-4′, C-6′
6′	6.57	dd	8.0,1.5 (5′, 2′)	121.8	C-1′, C-5′
7′	3.05	dd	4.5,15.0 (8′, $7'_\beta$)	37.9	C-1′, C-8′, C-9′
	2.96	dd	8.5,15.0 (8′, $7'_\beta$)		
8′	5.14	dd	4.5,8.0 ($7'_\beta$, $7'_\beta$)	74.7	C-1′,C-7′,C-9′, C-14
9′				173.5	

3.2 Plant material

The roots of *Salvia yunnanensis* were purchased from Yunnan Chinese Medicine Corporation, China, and identified by Chen Hubiao, a botany professor of Peking University Health Science Center. A voucher specimen (No. 200408 dali) has been deposited at the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, China. The roots of *Salvia yunnansis*, after washing with water and drying in the shade for several days, was comminuted.

3.3 Extraction and isolation

The dried roots of *S. yunnanensis* (4 kg) were extracted three times with 10 L of 80°C water for 30 min each time, and the conc. aq. extract was first partitioned into a water fraction (300 g) and a 50% ethanol fraction (160 g) by chromatography on a Diaion HP20 column. The 50% ethanol fraction (140 g) was further fractionated on a Sephadex LH20 column into three subfractions, I (0–30% ethanol) (90.8 g), II (30–50% ethanol) (37 g) and III (50–80% ethanol) (12 g). Subfraction III was then chromatographed on an ODS column using aqueous ethanol as eluent, and the eluates were collected in 20 ml volume and monitored by HPLC. The eluates containing the same pure compounds were combined, concentrated on a rotary evaporator, and freeze-dried. Purification of the residue by ODS column yielded salvianolic acid N (30 mg).

Table 2. The inhibition activity of **1** against HIV-1 RT and HIV-1 IN *in vitro*.

	IC_{50} (μg/ml)	
Compound	HIV-1 RT	HIV-1 IN
1	67.10–193.39	1.78–18.5

*3.3.1 Salvianolic acid N (**1**)*

Yellow amorphous powder, HPLC Rt 6.8 min, solvent, $MeOH/H_2O/CH_3COOH$ (25:75:0.005), $[\alpha]_D^{20}$ +41 (MeOH; *c* 0.08). UV λ_{max}^{MeOH} nm (log ε): 290 (4.32), 328 (4.34). ν_{max}^{KBr} cm^{-1}: 3402; 1740; 1612; 1585. ^1H NMR and ^{13}C NMR (MeOH-d_4) spectral data, see Table 1. HRESI-MS *m/z*: 491.0984 $[M - H]^-$ (calcd for $C_{26}H_{19}O_{10}$, 491.0978).

Table 3. The inhibition activity of **1** against p24 expression of HIV-1 IIIB in MT-4 cell lines.

Compound	TC_{50} (μg/ml)	IC_{50} (μg/ml)	SI
1	3.7–24.10	0.649–4.28	5.63–5.70

3.4 Anti-HIV activity

The anti-HIV screening procedures of **1** have been performed according to references.[12,13]

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