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Anti-AIDS Agents—XIX.[†] Neotripterifordin, a Novel Anti-HIV Principle from *Tripterygium wilfordii*: Isolation and Structural Elucidation

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Abstract—A new kaurane type diterpene lactone, neotripterifordin (**1**), has been isolated from the roots of *Tripterygium wilfordii*. The structure of **1** was elucidated by spectroscopic methods, which included the concerted application of a number of 2-D NMR techniques including ^1H–^1H COSY, phase-sensitive NOESY, HETCOR, and long-range HETCOR. Compound **1** showed potent anti-HIV replication activity in H9 lymphocyte cells with an EC_{50} of 25 nM and TI of 125.

Introduction

Tripterygium wilfordii Hook (Celastraceae) is a poison liana that is distributed in southern China. The ground parts and the root bark of this plant are used as an insecticide in Chinese folklore because of their high toxicity. After removal from the bark, the roots have been found to possess anti-tumor, anti-inflammatory, and immunosuppressive activities. A preparation of the roots, which is prepared by purification of the root extract and is commercially available as "Leigongteng Duodai", has been used for the treatment of various diseases such as dermatitis, rheumatoid arthritis, systemic acne rosacea, and nephritis with good results and without hormonal side effects.[2]

Many tricyclic diterpenes have been previously isolated from this plant. These include triptolide and tripdiolide as the major anti-tumor principles.[3] As a result of our continuing search for novel bioactive natural products as potent anti-AIDS agents, the EtOH extract of the roots of this plant was found to show significant anti-HIV activity. Bioactivity-guided fractionation of the active extract has now led to the isolation and characterization of a new potent anti-HIV principle, neotripterifordin (**1**). Two additional anti-HIV principles, tripterifordin (**2**)[4] and salaspermic acid,[5] were isolated and have been reported recently. We report herein on the structural elucidation and anti-HIV activity of **1**.

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Results and Discussion

The ethanolic extract of the roots of *T. wilfordii* was chromatographed on silica-gel. The active fractions were further chromatographed and afforded neotripterifordin (**1**) in 1.6×10^{-5}% yield.

Compound **1**, isolated as needles (mp 220–223 °C), had the molecular formula $C_{20}H_{30}O_3$ as found from its HRMS (m/z 318.2186). The IR spectrum showed the presence of hydroxyl (3430 cm^{-1}) and lactone (1725 cm^{-1}) groups. The ^1H NMR spectrum (Table 1) showed signals for two tertiary methyls at δ 0.91 and δ 1.37 and one oxymethylene at δ 4.04 and δ 4.25. The ^{13}C NMR spectrum (Table 1), DEPT, and HETCOR spectra indicated that **1** contains 20 carbons and 29 carbon-bound hydrogen atoms. The multiplicity of the carbon atoms was determined by a DEPT experiment. Three low-field signals correspond to one carbonyl (δ 174.92), one oxygenated methine (δ 79.63), and one oxymethylene (δ 76.71); the high-field region showed two methyls, nine methylenes, three methines, and three quaternary carbons. These data were consistent with the HRMS empirical formula and suggested that **1** was a tetracyclic diterpene bearing one lactone ring.

Compound **1** and tripterifordin (**2**) had the same molecular formula, and their mass spectra were almost identical. The IR spectrum of **1** closely resembled that of **2** (hydroxy 3430 cm^{-1} and lactone 1725 cm^{-1} groups). The ^1H and ^{13}C NMR spectra (Table 1) were also similar to those of **2**. This correspondence suggested that **1** possesses the same kaurane type skeleton found in **2**, and that compounds **1** and **2** are isomers.

Table 1. Proton and carbon resonance shift assignments for neotripterifordin (1) and tripterifordin (2)

Position	Neotripterifordin [1]		Tripterifordin [2][4]	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1	α 2.32, ddd, *J* = 13.0, 5.0, 2.5 β 1.12, ddd, *J* = 13.0, 12.0, 5.5	39.35, t	α 2.17, br. d, *J* = 12.5 β 1.05, m	40.6, t
2	1.68, m	20.75, t	1.71, m	20.9, t
3	α 1.65, m β 1.43, m	40.83, t	α 1.87, br. ddd, *J* = 1,2,12.5 β 1.49, ddd, *J* = 3,12.5,12.5	40.6, t
4	——	33.14, s	——	43.0, s
5	1.15, dd, *J* = 5.0,12.0	49.96, d	1.29, m	51.2, d
6	α 1.30, m β 1.79,dddd, *J* = 3, 5,12.5, 12.5	22.69, t	α 1.21, m β 1.81, br. ddd, *J* = 3,3,12.5	22.4, t
7	α 1.66, m β 1.46, ddd, *J* = 3,12.5,12.5	39.93, t	α 1.67, m β 1.47, ddd, *J* = 3,12.5,12.5	39.7, t
8	——	44.94, s	——	44.8, s
9	1.23, d, *J* = 9	53.40, d	1.20, m	50.3, d
10	——	47.92, s	——	38.7, s
11	α 2.17, dd, *J* = 7.0, 15.0 β 1.60, m	18.96, t	1.65, m	17.5, t
12	α 2.30, dddd, *J* = 3.5, 7, 12, 12 β 1.60, m	24.10, t	α 1.32, m β 1.64, m	26.0, t
13	1.86, br. dd, *J* = 3.5, 5	49.58, d	1.89, br. s	48.2, d
14	a 1.55, m b 1.65, m	35.49, t	a 1.73, m b 1.92, br. d, *J* = 12.5	37.9, t
15	α 1.63, d, *J* = 10.0 β 1.55, d, *J* = 10.0	56.78, t	α 1.64, d, *J* = 10.5 β 1.57, d, *J* = 10.5	57.8, t
16	——	79.63, s	——	79.0, s
17	1.37, s	24.48, q	1.40, s	24.5, q
18	0.91, s	24.10, q	1.20, s	23.1, q
19	a 4.04, d,*J* = 11.5 b 4.25,dd,*J* = 2.5,11.5	76.71, t	——	176.5, s
20	——	174.92, s	a 4.15, d,*J* = 12.0 b 5.21,dd,*J* = 2,12	73.9, t

Measured in $CDCl_3$. Multiplicity of signals: *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet; *br*, broad signal. δ in ppm, *J* in Hz.

By biogenetic consideration, the lactone ring in **1** could be located between C-19 and C-20, but with the carbonyl and the oxymethylene groups inverted from those of **2**. A comparison of the proton resonances of **1** with those of **2** revealed that some signals, especially those in rings A and C, were different due to this inversion. Thus, the signal of Me-18 was shifted upfield from δ 1.20 in **2** to δ 0.91 in **1**; the signals of the oxymethylene, H-19a and H-19b, were also shifted upfield Δ 0.11 ppm and Δ 0.96 ppm, respectively, in **1**

due to the inversion of the carbonyl and oxymethylene. Furthermore, upfield shifts of H-3α (Δ 0.22 ppm) and H-5 (Δ 0.14 ppm) and a downfield shift of H-1α (Δ 0.15 ppm) were observed. In addition, H-11α and H-12α in **1** are shifted downfield Δ 0.52 ppm and Δ 0.98 ppm respectively, due to the deshielding effect of the carbonyl at C-20. In the ^{13}C NMR spectrum, the carbon resonances of C-4 and C-10 in the two lactones were obviously different. The signal of the carbon adjacent to the carbonyl was shifted downfield about 9.5 ppm compared with that of the carbon adjacent to the oxymethylene carbon. [Compare C-4 (δ 33.14) and C-10 (δ 47.92) in **1** to C-4 (δ 43.0) and C-10 (δ 38.7) in **2**, respectively.] These spectral differences effectively supported the assignment of the carbonyl at C-20 and oxymethylene at C-19 in **1**. This assignment is further confirmed by long-range HETCOR (LR HETCOR) and phase-sensitive NOESY (PHNOESY) experiments. In the LR HETCOR experiment, the Me-18 protons (δ 0.91) showed two-bond correlation to the δ 33.14 (C-4) signal and three-bond coupling to δ 40.83 (C-3), δ 49.96 (C-5), and to δ 76.71 (C-19), which is the oxymethylene, but not to δ 174.92, which is the carbonyl. In turn, the oxymethylene protons were long-range coupled to C-3 (3-bond). In the PHNOESY experiment, a strong nOe response was observed between the three protons at C-18 (δ 0.91) and the two oxymethylene protons (δ 4.04 and δ 4.25). Of the two oxymethylene protons, one (H-19a, δ 4.04) showed a strong nOe response to H-3α (δ 1.65), and the other (H-19b, δ 4.25) to H-6α (δ 1.30). These correlations completely confirmed the assignment of the oxymethylene at C-19 and the carbonyl at C-20.

A hydroxyl group was assigned to C-16. This assignment is based on the LR HETCOR experiment. Thus, the protons of Me-17 were long-range coupled to C-16 (δ 79.63, 2-bond), C-13 (δ 49.58, 3-bond), and C-15 (δ 56.78, 3-bond).

The stereochemistry of **1** was ascertained by PHNOESY spectral evidence. The stereochemistry of Me-17 in **1** was assigned as the α configuration rather than β by careful examination of its PHNOESY spectrum and comparison with that of **2**. In the PHNOESY spectrum of **1**, the three protons of Me-17 only showed a strong nOe response to H-13; no nOe response was observed between Me-17 and H-11 or H-12, thereby establishing that the Me-17 and H-13 are in the same orientation and Me-17 is far away from H-11 or H-12. In addition, a strong nOe response was observed between H-9 (δ 1.23) and H-15β (δ 1.55) together with H-1β (δ 1.12) and H-7β (δ 1.46). Other nOe responses were observed between the protons that are similarly disposed and in close proximity as expected.

Similarly to compound **2**, the long-range *W*-coupling in **1** between H-19b and H-3β, H-1α and H-3α, and H-19a and H-5 were observed, indicating that they are also in the same plane and that ring A and the lactone are also in chair conformations.

The ^1H–^1H COSY, PHNOESY, HETCOR, and LR HETCOR spectra were used for the assignment of all proton and carbon resonances as shown in Table 1.

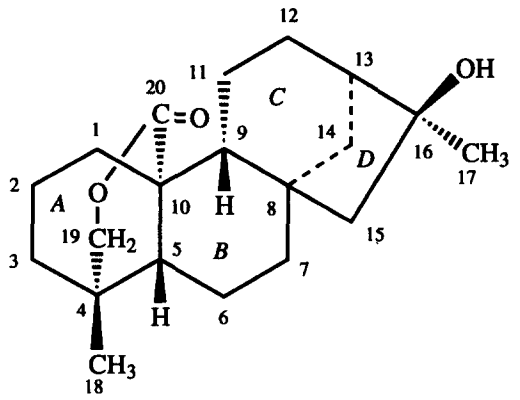
The evidence described above led to the elucidation of neotripterifordin (**1**) as shown in Figure 1. Because the amount of sample obtained was very small, the absolute stereochemistry of **1** remains to be determined.

Neotripterifordin (**1**) inhibits HIV replication in H9 lymphocyte cells with an EC_{50} of 25 nM. Neotripterifordin did not inhibit uninfected H9 cell growth at 3100 nM. The therapeutic index (TI) is 125. Neotripterifordin was much more active than tripterifordin (EC_{50} = 3100 nM). Therefore, the disposition of the lactone and the OH groups could play a crucial role with regard to the enhanced activity.

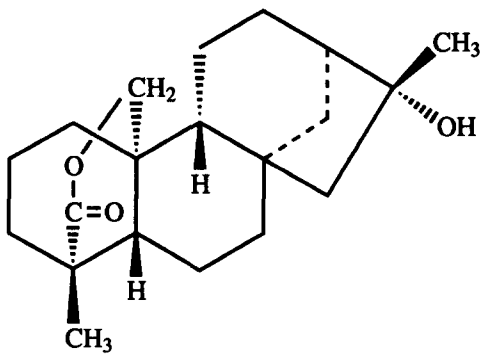
Experimental

General experimental procedure

The melting point was taken on a Fischer–Johns melting point apparatus and is uncorrected. The IR



1



2

Figure 1. Structures of neotripterifordin (**1**) and tripterifordin (**2**).

spectrum was recorded on a Perkin–Elmer 1320 spectrophotometer. The mass spectrum was determined on a VG 70-250 SEQ mass spectrometer. ^1H and ^{13}C NMR spectra were measured on Varian XL-400 and Bruker AC 300 spectrometers with TMS as an internal standard. Aldrich silica-gel 60 (5–25 μ) was used for CC and pre-coated silica-gel plates (Kieselgel 60 F_{254}, 0.25 mm thickness, Merck) were used for analytical TLC. Neotripterifordin was detected by spraying with 50% H_2SO_4 solution containing 1% anisaldehyde in 95% EtOH, followed by heating.

Plant material

The roots of *T. wilfordii* were collected in Fujian Province, China. The plant material was identified by Pharmacognosy Associate Professor Guan-Yun Gu, Vice-Chairman of Scientific and Technical Archives of Shanghai Medical University, Shanghai, China. A voucher specimen is available for inspection at the Department of Chemistry of Natural Drugs, School of Pharmacy, Shanghai Medical University, Shanghai, China.

Extraction and isolation of tripterifordin

The air dried roots (25 kg) of *T. wilfordii* were powdered and extracted with 95% EtOH. The EtOH extract was extracted with $CHCl_3$. The $CHCl_3$-soluble fraction was chromatographed in hexane over silica-gel with increasing polarity of $CHCl_3$. The active fraction (hexane:$CHCl_3$ 1:4) was further purified by flash chromatography employing hexane:toluene (1 → 0 and then 0 → 1) as eluant. Further repeated flash chromatography after elution with hexane:Et_2O (1 → 0 and then 0 → 1) afforded 4 mg (1.6×10^{-5}% yield) of neotripterifordin (**1**), which was purified by recrystallization.

Neotripterifordin (1)

White needles (acetone), mp 220–223 °C, R_f 0.50 (Et_2O). IR (KBr) 3430, 2975, 2930, 2870, 1725, 1450, 1410, 1380, 1350, 1135, 1095, 1050, 1035, 945, 870, 810 cm^{-1}; δ_H and δ_C (see Table 1); EIMS m/z 318.2186 (M^+ $C_{20}H_{30}O_3$, calcd 318.2195), 300 (67%), 285 (11), 275 (27), 260 (100%), 257 (24), 241 (8), 229 (15), 215 (21), 201 (14), 187 (27), 173 (13), 161 (11), 159 (17), 147 (19), 145 (30), 133 (24), 119 (25), 105 (28), 93 (28), 91 (37).

HIV inhibition assay

HIV inhibition was measured as described previously.[6,7] Briefly, H9 lymphocytes (3.5×10^6 cells mL^{-1}) were incubated in the presence or absence of HIV-1 (IIIB strain, 0.01–0.1 $TCID_{50}$ $cell^{-1}$) for 1 h at 37 °C. Cells were then washed thoroughly and resuspended at a final concentration of 2×10^5 cells mL^{-1} in the presence or absence of compound. After incubation for 3 days at 37 °C, the cell density of uninfected cultures was determined by cell count to assess toxicity of the drug. A p24 antigen capture assay was used to determine the level of virus released onto the medium of HIV infected cultures.

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