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THREE NEW ANTI-ROTAVIRUS CHROMENO[3,2-*c*]PYRIDINES FROM THE WHOLE PLANT OF *THALICTRUM SCABRIFOLIUM*

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Abstract – Three new chromeno[3,2-*c*]pyridines (**1-3**) were isolated from the whole plants of *Thalictrum scabrifolium*. Their structures were elucidated by spectroscopic methods, including extensive ^{1}H, ^{13}C, and 2D-NMR techniques. Compounds **1-3** were also tested for their anti-rotavirus activity, and they exhibited potent anti-rotavirus activity with therapeutic index (TI) valves of 23.7, 18.3, and 19.2 respectively.

The genus *Thalictrum*, a member of the Ranunculaceae family, comprises about 200 species widespread in temperate regions,[1,2] and around 67 species are recorded in the flora of China,[2] of which about 43 species have been used as traditional folk medicines to treat influenza, esoenteritis, cancer, dysentery, measles, and conjunctivitis.[3,4] Recent phytochemical and pharmacological investigations of *Thalictrum* plants uncovered that alkaloids are the main active components,[4-12] and many alkaloids with new efficacies have been isolated from the plants of this genus.

Thalictrum scabrifolium var. leve is a perennial herb with stem height of 25 - 40 cm. It is mainly distributed in Yulong, Jianchuan, and Heqing County, Yunnan Province, and grows on the hillside grassland at an altitude of 1900 - 2500 meters.[2] The whole plants of *T. scabrifolium* are used as substitutes for Rhizoma coptidis to treat enteritis, dysentery, nephritis, and the inflamme of the mouth and throat.[13] Since the use of traditional medicinal plants has been practiced for centuries and tested by a huge number of patients, the ethnopharmacologic information of *Thalictrum* plants can contribute greatly to the development of new drugs.

The chromenopyridine scaffold was regarded as the fusion of two main fragments (a chromene and a pyridine moiety).[14] Because both of the fragments display a wide range of biological activities, they

showed diverse biological activities, such as antitumor, anti-microbial, anti-fibrotic, topoisomerase inhibitory and anti-inflammatory activities, et al.[14-16] In order to extend the diversity of the library of alkaloids and discover more bioactive remarkable components from *Thalictrum* species, in the course of identifying bioactive compounds from local plants, we now investigated the chemical constituents of the whole plant of *T. scabrifolium* collected in Yulong in County, Lijiang Prefecture, Yunnan Province. As a results, three new chromeno[3,2-*c*]pyridine alkaloids (**1-3**) were isolated in this work. This paper describes the elucidation of the structures of these three compounds, and a preliminary evaluation of their anti-rotavirus activity.

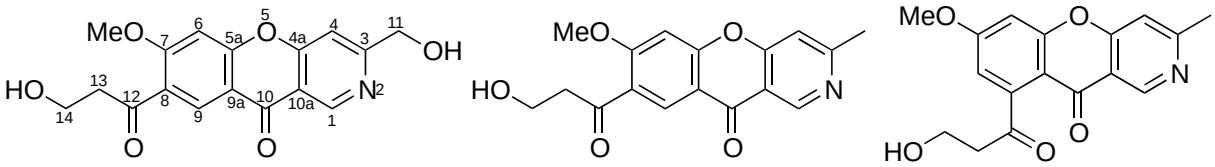

Figure 1. The new chromeno[3,2-*c*]pyridins from *T. scabrifolium*

A 95% aq. ethanol extract prepared from whole plants of *T. scabrifolium* was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9.0 with saturated Na_2CO_3 aq. and extracted with EtOAc again. The EtOAc-soluble alkaloidal materials were subjected repeatedly to column chromatography on silica gel and preparative HPLC to afford three new chromeno[3,2-*c*]pyridines, 3-hydroxymethyl-8-(3-hydroxypropanoyl)-7-methoxy-10*H*-chromeno[3,2-*c*]pyridin-10-one (**1**), 8-(3-hydroxypropanoyl)-7-methoxy-3-methyl-10*H*-chromeno[3,2-*c*]pyridin-10-one (**2**), and 9-(3-hydroxy-propanoyl)-7-methoxy-3-methy-10*H*-chromeno[3,2-*c*]pyridin-10-one (**3**). The structures of compounds **1-3** were shown in Figure 1, and the ^{1}H and ^{13}C NMR data of **1-3** were listed in Table 1.

Compound **1** was obtained as a pale-brown gum. The molecular formula $C_{17}H_{15}NO_6$ of **1** was assigned from its HRESIMS at *m/z* 352.0790 $[M+Na]^+$ (calcd 352.0797 for $C_{17}H_{15}NNaO_6$), with eleven degree of unsaturations. The IR absorption bands indicated the presence of hydroxyl (3422 cm^{-1}), carbonyl (1688, and 1666 cm^{-1}), and aromatic ring (1612, 1551, and 1482 cm^{-1}) groups. UV absorptions at 218, 246, 302, and 338 nm suggested a conjugated aromatic ring system. Its ^{1}H, ^{13}C, and DEPT NMR data displayed resonances for 17 carbons and 15 hydrogen atoms, which were ascribed to four *sp*2 aromatic methines [C-1, C-4, C-6, and C-9, including one nitrogen-bearing (C-1)], seven *sp*2 quaternary carbons (C-3, C-7, C-8, C-4a, C-5a, C-9a, and C-10a), one conjugated carbonyl (C-10), one methoxy group (δ_C 56.2, δ_H 3.79), one hydroxymethyl group (C-11, H_2-11), and one 3-hydroxypropanoyl

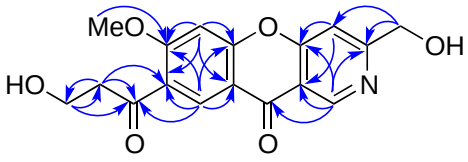

Figure 2. Key HMBC (↷) correlations of **1**

group (OH-CH_2-CH_2-CO-, C-12~C-14, H_2-12, and H_2-13).[17] On the basis of the carbon chemical shifts of these resonances, two ketone, five C=C groups, and a C=N bond in compound **1** was accounted for 8 of the 11 degrees of unsaturation. The still three rings should be needed to meet 11 degrees of unsaturation, and four *sp*2 aromatic methines, seven *sp*2 quaternary carbons, nitrogen atom, and conjugated carbonyl should be a fused to a chromeno-pyridine tricyclic ring structure.[16] In addition, the chromone moiety fused with a pyridine ring via C-4a and C-10a to form a chromeno[3,2-*c*]pyridine ring were also supported by the HMBC correlations (Figure 2) from H-1 to C-3, C-10, C-4a, C-10a, from H-4 to C-4a, C-10a, from H-6 to C-5a, C-9a, and from H-9 to C-10, C-5a, C-9a.

Since the chromeno[3,2-*c*]pyridine skeleton was determined, the positions of substituents (a hydroxymethyl, a 3-hydroxypropanoyl, and a methoxy group) can also be determined by further analysis of its HMBC data. The 3-hydroxypropanoyl group located at C-8 was supported by the HMBC correlation from H_2-13 to C-8, from H-9 to C-12. The HMBC correlations from the hydroxymethyl protons (H_2-11) to C-3, C-4, from H-4 to C-11 indicated that the hydroxymethyl group was located at C-3. Finally, the HMBC correlation from the methoxy proton (δ_H 3.77) to C-7 confirmed that the methoxy group was located at C-7. Thus, the structure of **1** was established, and gave the systematic name of 8-(3-hydroxypropanoyl)-7-methoxy-3-methyl-10*H*-chromeno[3,2-*c*]pyridin-10-one.

Table 1. ^{1}H NMR and ^{13}C NMR data of compounds **1-3** ($CDCl_3$, δ, ppm, *J*/Hz)

No.	Compound 1		Compound 2		Compound 3	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	151.4 d	9.15 s	149.8	9.13 s	151.2 d	9.10 s
3	160.4 s		162.8 s		160.8 s	
4	112.2 d	7.83 s	111.7	7.67 s	112.5 d	7.63 s
6	108.6 d	6.76 s	108.5	6.75 s	111.4 d	6.80 (d) 1.8
7	164.8 s		165.0 s		165.2 s	
8	120.2 s		120.4 s		107.9 d	7.10 (d) 1.8
9	128.1 d	8.17 s	128.3	8.14 s	139.8 s	
10	176.7 s		176.5 s		176.8 s	
4a	159.0 s		159.3 s		159.5 s	
5a	156.8 s		156.5 s		157.7 s	
9a	112.8 s		112.5 s		113.7 s	
10a	116.9 s		116.6 s		116.5 s	
11	66.5 t	5.15 s	25.8 q	2.44 s	25.6 q	2.48 s
12	201.3 s		201.1 s		201.3 s	
13	42.5 t	3.00 (t) 6.4	42.3 t	3.02 (t) 6.4	41.5 t	3.05 (t) 6.4
14	58.8 t	4.04 (t) 6.4	58.6 t	4.07 (t) 6.4	58.9 t	4.16 (t) 6.4
-OMe	56.2 q	3.77 s	56.4 q	3.78 s	56.3 q	3.75 s

8-(3-Hydroxypropanoyl)-7-methoxy-3-methyl-10*H*-chromeno[3,2-*c*]pyridin-10-one (**2**) was also obtained as brown gum with a molecular formula as $C_{17}H_{15}NO_5$, according to the ion peak of *m/z* 336.0845 ($[M+Na]^+$) in the HRESIMS. The UV and IR spectra of **2** were highly similar to those of **1**. The chemical shift differences resulted from the disappearance of a hydroxymethyl group and appearance of a methyl group (C-11 and H_3-11) in **2**. These changes indicated that the hydroxymethyl group at C-3 in **1** was converted into a methyl group in **2**. The HMBC correlation from to H_3-11 to C-3, C-4, from to H-4 to C-11 also supported the methyl group located at C-3. In addition, the positions of the 3-hydroxypropanoyl and methoxy group can also be determination by further analysis of its HMBC correlations. The structure of **2** was therefore defined.

9-(3-Hydroxypropanoyl)-7-methoxy-3-methy-10*H*-chromeno[3,2-*c*]pyridin-10-one (**3**) was obtained as a brown gum and showed a quasi-molecular ion at *m/z* 336.0842 $[M+Na]^+$ in the HRESIMS (calcd *m/z* 336.0848), corresponding to the molecular formula $C_{17}H_{15}NO_5$. The ^1H and ^{13}C NMR spectra of **3** were highly similar to those of **2**. These indicated that compounds **2** and **3** have very similar structures. The obvious chemical shift differences resulted from the proton signals on benzene ring. A pair of singlets at (δ_H 6.75 s and 8.14 s) in **1** were replaced by two doublets at δ_H 6.80 [(d) 1.8] and 7.10 [(d) 1.8] in **2**. These changes revealed that **3** should be a 3,7,9-trisubstituted chromeno[3,2-*c*]pyridine. In addition, the 3-hydroxypropanoyl group located at C-9, the methyl group located at C-3, and the methoxy group located at C-7, which were also be supported by the HMBC correlations from H_2-13 to C-9, from H_3-11 to C-3, C-4, and from the methoxy proton (δ_H 3.75) to C-7. Therefore, the structure of **3** was established as shown.

Table 2. Anti-rotavirus activity of compounds **1-3**

No.	CC_{50} (μg/mL)	EC_{50} (μg/mL)	TI (CC_{50}/EC_{50})
1	248.6	10.5	23.7
2	252.7	13.8	18.3
3	234.2	12.2	19.2
Ribavirin	275.6	11.8	23.4

CC_{50}: mean (50%) value of cytotoxic concentration; EC_{50}: mean (50%) value of effective concentration; TI: therapeutic index, CC_{50}/EC_{50}.

Since certain of the alkaloids from *Thalictrum* genus exhibit potential anti-viral activity,[5,6,18] compounds **1-3** were tested for their anti-rotavirus activity. Their ability to prevent the cytopathic effects of rotavirus in MA104 cells was tested according to our previous literatures,[19,20] and their effects were measured in parallel with the determination of antiviral activity using ribavirin as positive control. The results (Table 2) revealed that compounds **1-3** exhibited potent anti-rotavirus activity with therapeutic index (TI) valves of 23.7, 18.3, and 19.2 respectively.

EXPERIMENTAL

General Experimental Procedures. UV spectra were obtained using a Shimadzu UV-1900 spectrophotometer. A Bio-Rad FTS185 spectrophotometer was used for scanning IR spectra. ^1H, ^{13}C, and

2D-NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as internal standard. ESIMS and HRESIMS analyses were measured on Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. Chemical shifts (δ) are expressed in ppm with reference to the TMS signal. Semi-preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF C_{18} (2.12 mm × 25 cm) or Venusil MP C_{18} (2.0 mm × 25 cm) columns. Column chromatography was performed using silica gel (200 - 300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China), Sephadex LH-20 (Sigma-Aldrich, Inc, USA), or MCI gel (75 - 150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC visualized by spraying with 5% H_2SO_4 in ethanol and heating.

Plant Material. The whole plants of *Thalictrum scabrifolium* var. leve were collected in Yulong in County, Lijiang Prefecture, Yunnan Province, People's Republic of China, in September 2019. The identification of plant material was verified by Prof. Ning Yuan. A voucher specimen (Ynni-19-09-186) has been deposited in Key Laboratory of Chemistry in Ethnic Medicinal Resources, Yunnan Minzu University, P. R. China.

Extraction and Isolation. The air-dried and powdered whole plants of *T. scabrifolium* (2.8 kg) were extracted with 95% aq. EtOH, and the extract was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9 with saturated Na_2CO_3 aq., extracted with EtOAc, and removed the pigments with MCI gel. The purified EtOAc-soluble alkaloidal materials (41.5 g) were applied to silica gel column chromatography (150 - 200 mesh, 8 × 50 cm), eluting with $CHCl_3$/MeOH gradient system (10:0, 9:1, 8:2, 7:3, 6:4, 5:5) to give six fractions A-F. Further separation of fraction B (9:1, 6.22 g) by silica gel column chromatography (200 - 300 mesh, 5 × 50 cm), eluted with $CHCl_3$/Me_2CO (9:1-2:1), yielded mixtures B1–B7. Sub-fraction B1 (9:1, 1.42 g) was subjected to silica gel column chromatography (200 - 300 mesh, 2 × 50 cm) using petroleum ether/acetone, and then semi-preparative HPLC (68% MeOH/H_2O, flow rate 20 mL/min) to give the crude compounds. The crude compounds was applied to Sephadex LH-20 column (1.5 × 120 cm) eluting with MeOH to give **1** (14.2 mg), **2** (12.2 mg), and **3** (13.6 mg).

Anti-rotavirus Assay. The human rotavirus Wa group was used to infect the cell culture MA104 *in vitro*, the 50% cytotoxicity concentration (CC_{50}) and half maximal effective concentration (EC_{50}) were evaluated, and the ribavirin was used as positive control.[19,20] MA-104 cells (1×10^5 cells *per* well) were grown in 96-well plates for 48 h. The media were removed and replaced by new media containing serial dilutions of compounds under test. After incubation for 72 h, the media were discarded, and 5 μL of MTT solution was added to each well. Plates were then incubated at 37 ℃ for 4 h. The solution was removed, and 100 μL of 0.04 mol/L HCl-isopropanol were added to each well to dissolve formazan crystals. Using a microplate reader, the absorbance of each well was measured at 540 nm. After subtracting the

background absorbance at 655 nm, the 50% CC_{50} of each compound was estimated by regression analysis.

In the mixed treatment assay, each compound was mixed with a 0.01 multiplicity of infection (MOI) of the rotaviruses at various concentrations (1 - 160 μg/mL) and incubated at 4 ℃ for 1 h. The mixtures were inoculated in triplicates onto near confluent MA-104 cell monolayers (1×10^5 cells *per* well) for 1 h with occasional rocking. The solution was removed and the cells replaced with eagles minimum essential medium (EMEM) containing 1.0 μg/mL trypsin. The cells were incubated for 72 h at 37 ℃ under 5% CO_2 atmosphere until the cells in the control showed complete viral cytopathic effect (CPE) by light microscopy. EC_{50} was estimated by regression analysis.

3-Hydroxymethyl-8-(3-hydroxypropanoyl)-7-methoxy-10*H*-chromeno[3,2-*c*]pyridin-10-one (**1**): obtained as pale brown gum; UV (MeOH) λ_{max} (log ε) 218 (4.15), 246 (3.87), 302 (3.75), 338 nm (3.62); IR ν_{max} 3422, 3065, 2952, 1688, 1666, 1642, 1612, 1551, 1482, 1405, 1234, 1176, 1048, 859 cm^{-1}; positive ESIMS *m/z* 352 $[M+Na]^+$, positive HRESIMS *m/z* 352.0790 (calcd for $C_{17}H_{15}NNaO_6$, 352.0797).

8-(3-Hydroxypropanoyl)-7-methoxy-3-methyl-10*H*-chromeno[3,2-*c*]pyridin-10-one (**2**): obtained as pale brown gum; UV (MeOH) λ_{max} (log ε) 218 (4.20), 245 (3.82), 298 (3.79), 335 nm (3.68); IR ν_{max} 3405, 3070, 2958, 1682, 1664, 1638, 1615, 1563, 1472, 1412, 1239, 1170, 1054, 824 cm^{-1}; positive ESIMS *m/z* 336 $[M+Na]^+$, positive HRESIMS *m/z* 336.0845 (calcd for $C_{17}H_{15}NNaO_5$, 336.0848).

9-(3-Hydroxypropanoyl)-7-methoxy-3-methy-10*H*-chromeno[3,2-*c*]pyridin-10-one (**3**): obtained as pale brown gum; UV (MeOH) λ_{max} (log ε) 218 (4.25), 248 (3.89), 296 (3.72), 340 nm (3.75); IR ν_{max} 3408, 3062, 2960, 1680, 1668, 1644, 1610, 1561, 1467, 1408, 1235, 1164, 1059, 840 cm^{-1}; positive ESIMS *m/z* 336 $[M+Na]^+$, positive HRESIMS *m/z* 336.0842 (calcd for $C_{17}H_{15}NNaO_5$, 336.0848).

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