Supporting Information

Steroidal alkaloid with unprecedented triheterocyclic architecture

Zhong-Shun Zhou,[a, †] Yun-Li Zhao,[a, †] Bin-Yuan Hu,[a] Bei Wang,[c] Ya-Ping Liu,[b] Yan-Yan Zhu,[a] Ying-Jie He,[a] Zhao-Jie Wang,[a] Zhi Dai,[a] Li-Xing Zhao,[a] and Xiao-Dong Luo[a,b,*]

[a] *Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education and Yunnan Province, Yunnan Characteristic Plant Extraction Laboratory, School of Chemical Science and Technology, Yunnan University, Kunming, 650500, PR. China*

[b] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences Kunming, 650201, PR. China*

[c] *Key Laboratory of Herbal-Tebitan Drug Screening and Deep Processing of Gansu Province, School of Life Science and Engineering, Lanzhou University of Technology, Lanzhou 730050, PR.China*

[†] These authors contributed equally to this work.

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1. Experimental Section

1.1 general experimental procedure

Optical rotations were recorded on Autopol VI, Serial #91058. UV spectra were obtained on a Shimadzu serial UV-2700 spectrometer. IR spectra were obtained on a NICOLET iS10 infrared spectrophotometer using KBr pellets. NMR spectra were measured on a Bruker AVANCE NEO 400MHz spectrometer, with TMS as an internal standard. HRESIMS analyses were measured on Agilent 1290 UPLC/6545 Q-TOF mass spectrometer. Silica gel (200−300 mesh; Qingdao Marine Chemical Inc., PR China), C-18 silica gel (40−60 μm; Daiso Co., Japan), and Sephadex LH-20 (Amersham Pharmacia, Sweden) were used for column chromatography. Fractions were monitored by TLC on silica gel plates (GF254, Qingdao Haiyang Chemical Co., Ltd.). Semi-preparative HPLC was carried out using an Agilent 1260 liquid chromatograph equipped with an Agilent Zorbax SB-C18 column (250 mm × 9.4 mm, i.d., 5 μm).

1.2 Plant materials

The roots of *Veratrum stenophyllum* were collected in November 2018 in Dali city (Yunnan Province, China) and identified by Dr. Yi-Fen Wang, Kunming Institute of Botany, Chinese Academy of Science (Kunming, China). A voucher specimen (Wang 20181120) was deposited at the Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education and Yunnan Province, School of Chemistry Science and Technology, Yunnan University, Kunming, P. R. China.

1.3 Extraction and isolation

The air-dried and pulverized roots of *V. stenophyllum* (22.0 kg) were extracted with 90% CH_3OH (50 L×3, 12 h each) under reflux conditions and the solvent was evaporated under reduced pressure at 55.0 ℃ to yield the crude extract. The combined CH_3OH extracts were acidified with 0.5% hydrochloric acid to pH 2.5−3.0. After filtration, acidic aqueous fraction and non-alkaloid part were obtained. The acidic aqueous fraction was subsequently adjusted to pH 9.0−10.0 with 0.5% aqueous ammonia and then extracted with EtOAc to give total alkaloids (850.0 g). The extract

was subjected to silica gel (200−300 mesh, 9.0 kg) column chromatography (CC) and then eluted with CHCl3-CH_3OH (50:1, 20:1, 10:1, 5:1, and 0:1) to afford five fractions (Fr.A to Fr.E). Fr.C (60.0 g) was subjected to silica gel CC eluting with Petroleum-Acetone (3:1, 1:1, v/v) to give four subfractions (Fr.C.1–Fr.C.4). Fr.C.3 (2.3 g) was submitted to a C-18 column eluted with aqueous CH_3OH (70%–100%) to yield five fractions (Fr.C.3.1–Fr.C.3.5). Fr.C.3.4 (805.0 mg) was subjected to Sephadex LH-20 (CH_3OH) to yield three fractions (Fr.C.3.4.1–Fr.C.3.4.3). Compound **1** (15.0 mg) was purified from Fr.C.3.4.2 (78.5 mg) by semi-preparative HPLC (70% aqueous acetonitrile, t_R = 10.8 min; flow rate 2.5 mL/min), and the colorless needles crystal was obtained in CH_3OH.

1.4 Physical and chemical data

*Veratrazine A (**1**)*: colorless needle crystal (chloroform: CH_3OH); HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{32}H_{41}NO_7$, 552.2956; Found 552.2937; $[\alpha]_D^{22}$ +24.9 (*c* 0.12, CH_3OH); UV (CH_3OH) λ_{max} (log ε): 198 (2.71), 263 (1.04); IR (KBr) V_{max} 3436, 2930, 2854, 1744, 1626, 1436, 1375, 1309, 1229, 1105, 1048, and 812 cm^{-1}; ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectral data, see Table 1.

2. Biological evaluation

2.1 **Animals**

ICR male mice weighing 22–24 g were purchased from Kunming Medical University (License SCXK, 2015–0002). All animals were kept at 20–25 °C and constant humidity of 40–70% under a 12 h light-dark cycle and with standard laboratory food and water *ad libitum*. They were acclimatized to the laboratory environment for three consecutive days before the experiment in a specific-pathogen-free (SPF)-grade laboratory. The animal study was carried out following the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care.

2.2 **Chemicals**

Carrageenan was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tumor-necrosis factor α (TNF-α), Prostaglandin E2 (PGE2), and cyclooxygenase 2 (COX-2) kits were purchased from Shanghai Yuanye Bio-Technology Co. Ltd. (China). All other reagents were of the highest commercial grade available and purchased from Shanghai Aladdin Biochemical Technology Co. Ltd (China).

2.3 **Measurement of pro-inflammatory cytokine *in vitro***

The anti-inflammatory in vitro was performed according to the previous method we described elsewhere (Zhao et al., 2019). Murine macrophage RAW 264.7 cells were plated in 96-well plates at a density of 2×10^4 cells/well and cultivated in Dulbecco's modified Eagle's medium (DMEM) in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h. Cells were pretreated with compound **1** at a concentration of 5 μg/mL for 2 h, and then induced with 1.00 μg/mL lipopolysaccharide (LPS) for 24 h. Cell-free supernatant was collected for the quantification of COX-2, PGE2, and TNF-α using enzyme-linked immunosorbent assay (ELISA) kits according to the protocols of the manufacturer. A tetrazolium bromide reduction (MTT) assay was performed to study the effect of alkaloids on RAW 264.7 cell growth at the same concentration.

2.4 **Western blot analysis**

Total protein was extracted from the Murine macrophage RAW 264.7 cells. Protein

isolation and western blotting were performed as described previously. The protein concentration of the supernatant was measured and quantified using the bicinchoninic acid assay kit. Equal amounts of proteins (40 μg) were resolved by 8-12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) for 1 h. The normal protein blots were blocked with Tris-buffered saline with 0.1% Tween-20 (TBST) + 5% milk for 1 h (Beyotime Institute of Biotechnology, Nanjing, China). The membranes were incubated overnight at 4 °C with primer antibodies. The blots were washed 3 times with TBST (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) and incubated with horseradish peroxidase conjugated-secondary antibody for 1 h. Blots were washed 3 times with TBST. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Tiangen Biotech, Beijing, China) on a FluorChem E System (Protein Simple, Santa Clara, CA, USA).

2.5 **Anti-inflammatory study—carrageenan-induced paw edema model in vivo.**

The *in vivo* anti-inflammatory activity was conducted based on a previously described process (Li et al., 2016). Male mice were randomly divided into five groups: control, DEX (dexamethasone, 8.0 mg/kg), and compound **1** groups (2.0, 4.0, 8.0 mg/kg), 10 animals in each group., and the dosage regimen was determined according to the preliminary toxicity experiment. All groups were administered intraperitoneally with a volume of 10 mL/kg, and the control group was given an equal volume of saline solution by intraperitoneal injection. After administration for 30 min, 50.0 μL of 1% (w/v) carrageenan suspension in 0.9% saline was injected subcutaneously into the left hind paw to cause swelling. The paw size was measured with volume difference by a digital vernier caliper before (time 0) and at 4 h after carrageenan injection. Percentage inhibitions were calculated using the following formula:

$$\text{Percent inhibition} = \frac{\text{Average volume difference (control)} - \text{Average volume difference (test)}}{\text{Average volume difference (control)}} \times 100\%$$

2.6 Statistical Analysis.

Results were expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined using a two-tailed Student's test, $p < 0.05$ accepted as the

significance value.

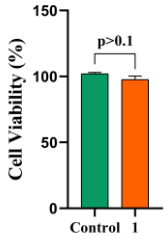


Figure S1. The cell viability of compound **1** in RAW 264.7 cell.

3. NMR, HRESIMS, IR, UV, and ORD of 1

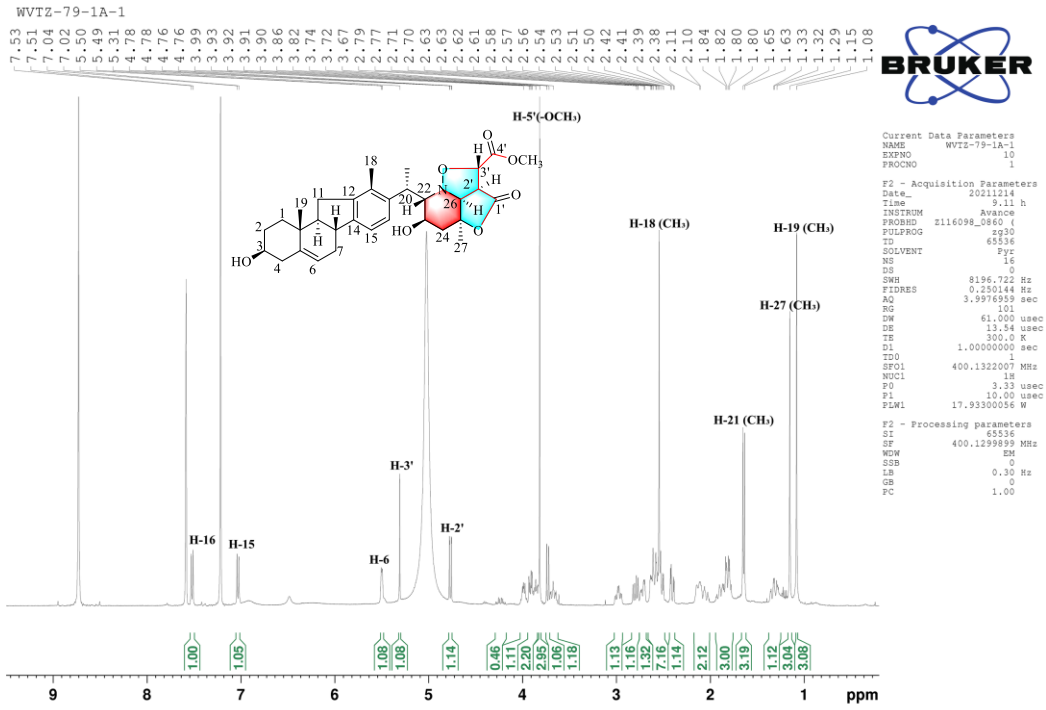


Figure S2. ^{1}H NMR (400 MHz) spectrum of compound **1** in $CDCl_3$.

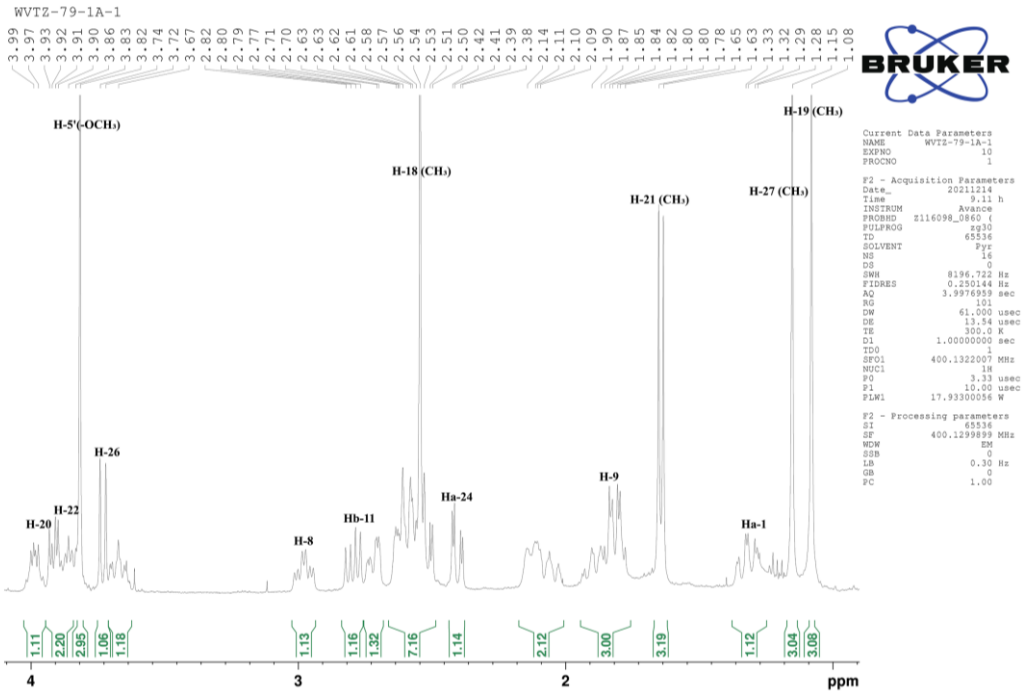


Figure S3. The magnification of ^{1}H NMR (400 MHz) spectrum of compound **1** in Pyridine-d_5.

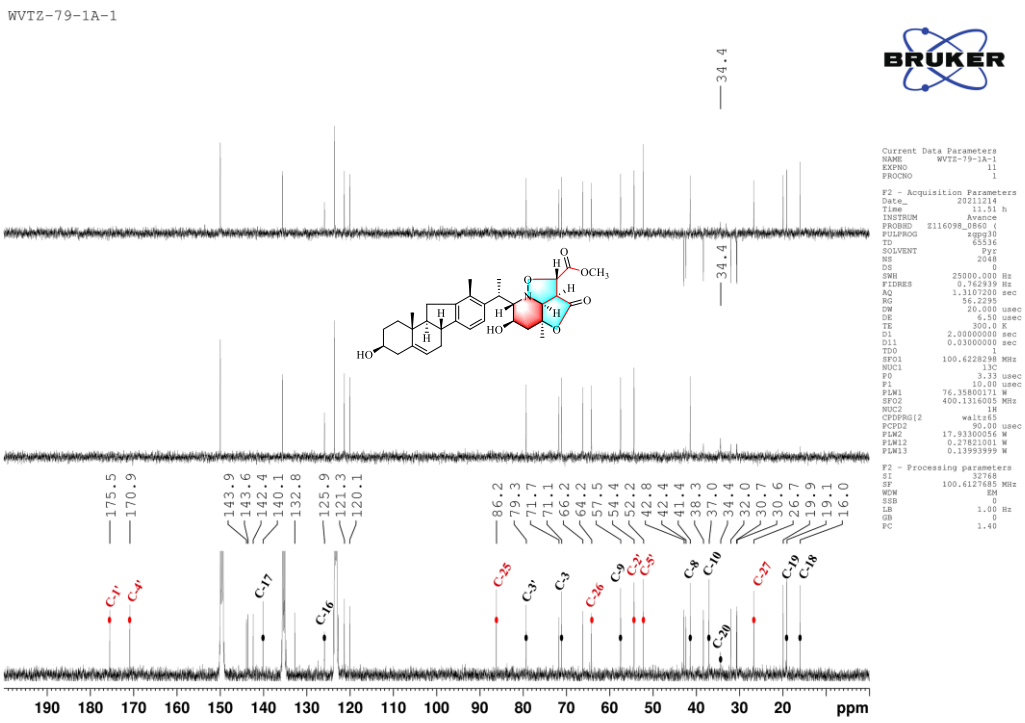


Figure S4. ^{13}C NMR (100 MHz) and DEPT spectra of compound **1** in Pyridine-d_5.

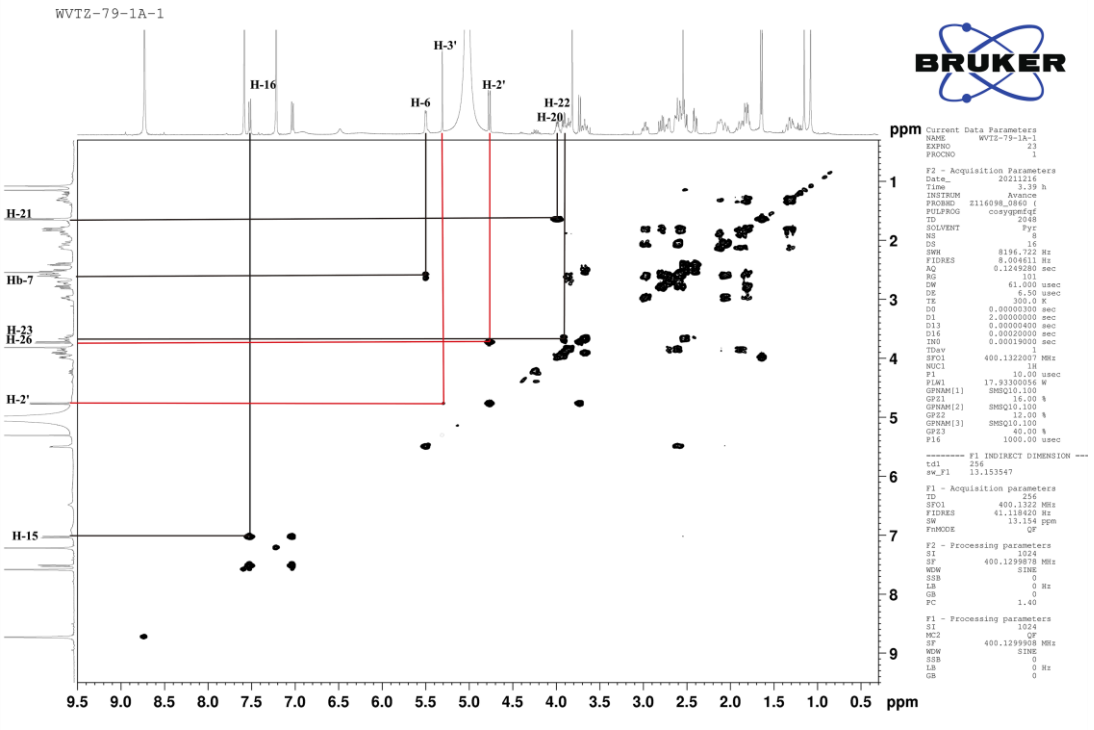


Figure S5. ^{1}H-^{1}H COSY spectrum of compound **1** in Pyridine-d_5.

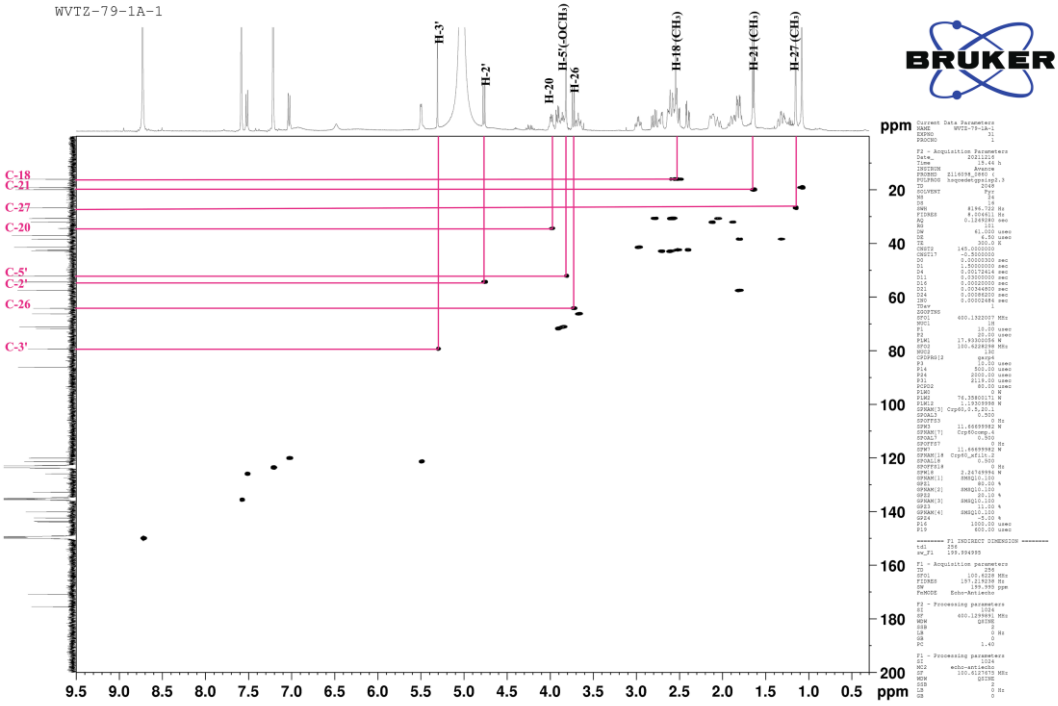


Figure S6. HSQC spectrum of compound **1** in Pyridine-d_5.

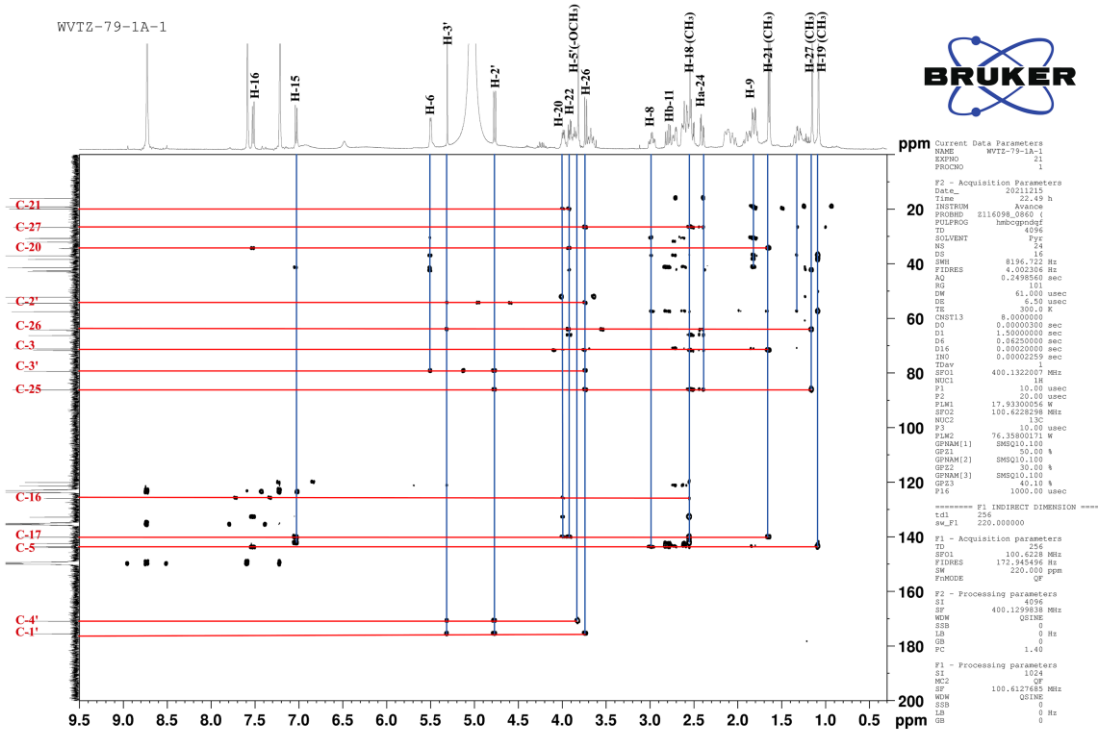


Figure S7. HMBC spectrum of compound **1** in Pyridine-d_5.

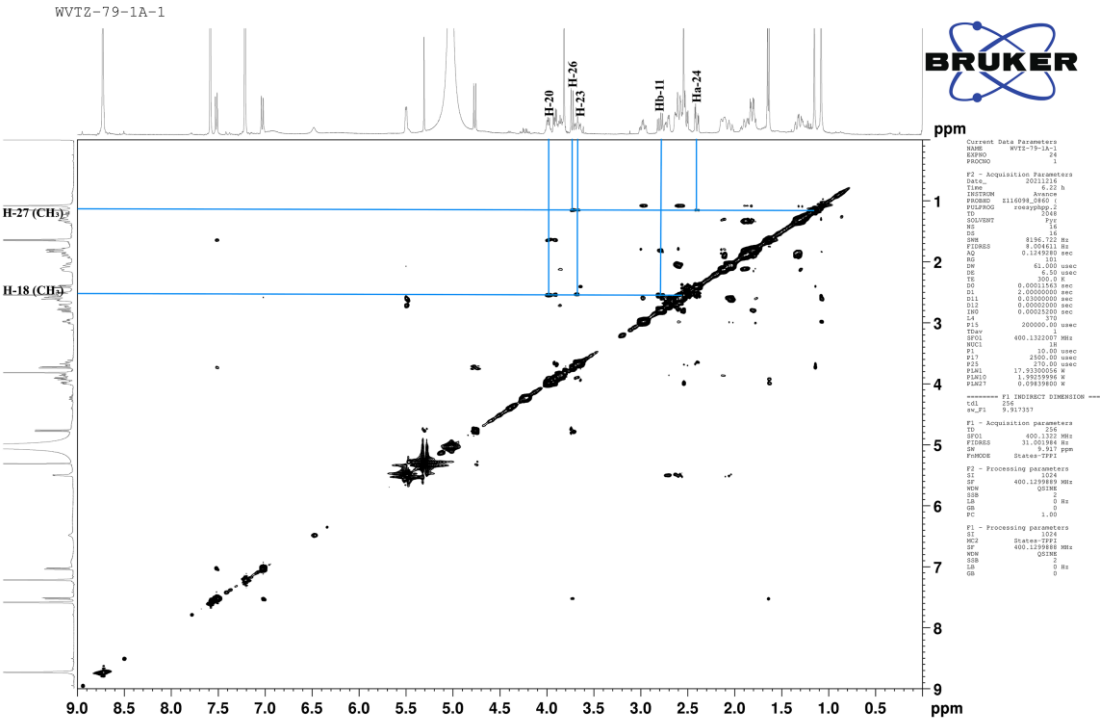


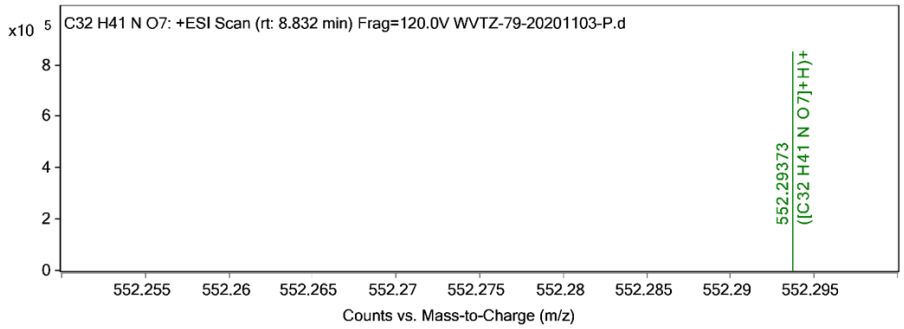
Figure 8. ROESY spectrum of compound **1** in Pyridine-d_5.

Qualitative Analysis Report

Data File	WVTZ-79-20201103-P.d	**Sample Name**	WVTZ-79
Sample Type	Sample	**Position**	P1-F8
Instrument Name	Instrument 1	**User Name**	
Acq Method	20201017-common test-P.m	**Acquired Time**	11/3/2020 3:30:19 PM (UTC+08:00)
IRM Calibration Status	Success	**DA Method**	20210819-QZH-P-YOUHUA1.m
Comment			
Sample Group		**Info.**	
Stream Name	LC 1	**Acquisition Time (Local)**	11/3/2020 3:30:19 PM (UTC+08:00)
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.09.00 (B9044.0)	**QTOF Driver Version**	8.00.00
QTOF Firmware Version	25.723	**Tune Mass Range Max.**	3200

Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
120	0	ESI



Spectrum Identification Results: + Scan (rt: 8.832 min) (WVTZ-79-20201103-P.d)

Best	ID Sourc	Nam	Formula	Specie	m/z	Scor	Diff (ppm	Score (MFG
☑	**MFG**		**C32 H41 N O**	**(M+H)+**	**552.2937**	**90.93**	**3.82**	**90.93**

Species	m/z	Score (iso. abund	Score (mass	Score (MS	Score (MFG)	Score (iso. spacing	Heigh	Ion Formul
(M+H)+	552.2937	97.25	84.25	90.93	90.93	96.69	850840.7	C32 H42 N O7

Height (Calc)	Height Sum% (Cal	Height % (Calc	m/z (Calc	Diff (mDa	Heigh	Height	Height Sum	m/z	Diff (ppm
834479.7	69.2	100	552.29558	1.8	850840.7	100	70.5	552.2937	3.34
298120.9	24.7	35.7	553.29892	2.6	282769	33.2	23.4	553.2963	4.73
63688.2	5.3	7.6	554.30179	3.2	62872.3	7.4	5.2	554.2985	5.86
10076.5	0.8	1.2	555.30456	2.7	9883.4	1.2	0.8	555.3018	4.91

--- End Of Report ---

Figure S9. HRESIMS of compound **1**.

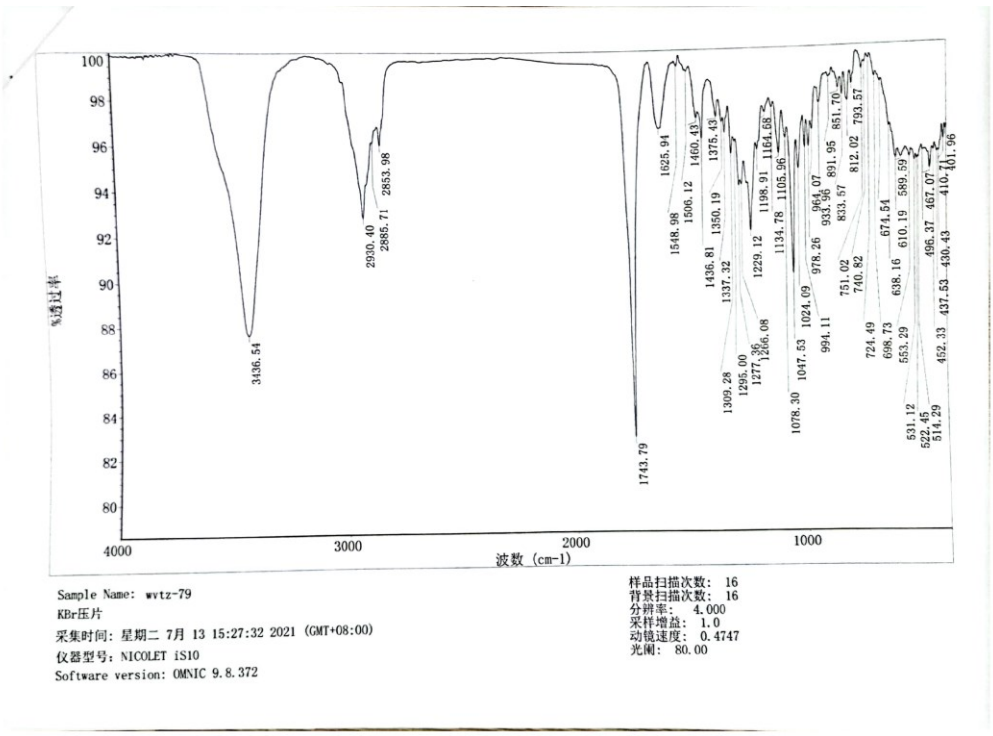
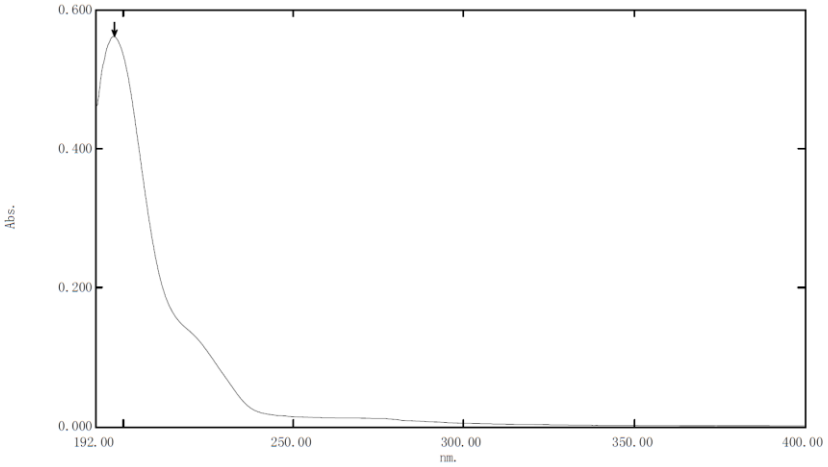


Figure S10. IR spectrum of compound **1**.

光谱选点检测报告

2021/03/02 20:45:55

数据集: WVTZ-79 - RawData



[测定属性]
波长范围(nm): 190.00 到 600.00
扫描速度: 中速
采样间隔: 0.5
自动采样间隔: 停用
扫描模式: 单个

No.	波长(nm)	吸收值	描述
1	197.50	0.562	
2	263.00	0.012	
3			

[仪器属性]
仪器类型: UV-2700 系列
测定方式: 吸收值
狭缝宽: 5.0 nm
积分时间: 0.1 秒
光源转换波长: 323.0 nm
检测器单元: 直接
S/R 转换: 标准
阶梯校正: OFF

[附件属性]
附件: 无

[数据处理参数]
阈值: 0.0010000
点: 4
内插: 停用
平均: 停用

[样品准备属性]
重量:
体积:
稀释:
光程长: 10mm
附加信息: 样品浓度：0.0060毫克/毫升
溶剂：甲醇

页 1 / 1

Figure S11. UV spectrum of compound **1**.

Rudolph Research Analytical

This sample was measured on an Autopol VI, Serial #91058
Manufactured by Rudolph Research Analytical, Hackettstown, NJ, USA.

Measurement Date : Monday, 01-MAR-2021

Set Temperature : OFF

Time Delay : Disabled

Delay between Measurement : Disabled

n	Average	Std.Dev.	% RSD	Maximum	Minimum
5	24.86	0.44	1.76	25.17	24.14

S.No	Sample ID	Time	Result	Scale	OR °Arc	WLG.nm	Lg.mm	Conc.g/100ml	Temp.
1	WVTZ-79	11:43:40 AM	24.74	SR	0.0287	589	100.00	0.116	21.7
2	WVTZ-79	11:43:49 AM	24.14	SR	0.0280	589	100.00	0.116	21.7
3	WVTZ-79	11:43:57 AM	25.17	SR	0.0292	589	100.00	0.116	21.7
4	WVTZ-79	11:44:05 AM	25.17	SR	0.0292	589	100.00	0.116	21.7
5	WVTZ-79	11:44:13 AM	25.09	SR	0.0291	589	100.00	0.116	21.7

Figure S12. Experimental ORD compound **1**.

4. Crystal data and structure refinement for compound 1

4.1. Crystal data

Crystal data for compound **1**: $C_{32}H_{41}NO_7$, M = 551.66, a = 11.4439(11) Å, b = 7.5246(7) Å, c = 17.3527(17) Å, α = 90°, β = 96.303(4)°, γ = 90°, V = 1485.2(2) Å^3, T = 298(2) K, space group $P2(1)$, Z = 2, μ(Cu Kα) = 0.701 mm^{-1}, 34656 reflections measured, 5292 independent reflections (R_{int} = 0.0612). The final R_1 values were 0.0340(I > 2σ(I)). The final $wR(F_2)$ values were 0.0948 (I > 2σ(I)). The final R1 values were 0.0495 (all data). The final $wR(F_2)$ values were 0.1045 (all data). The goodness of fit on F2 was 1.027. Absolute structure parameter = 0.05(6).

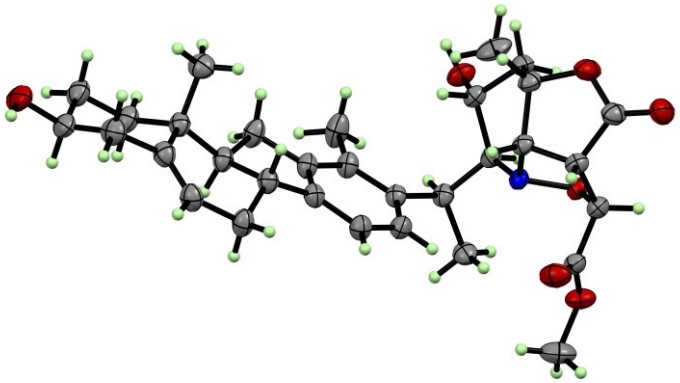


Figure S13 view of the molecules in a symmetric unit of compound **1**. Displacement ellipsoids are drawn at the 30% probability level.

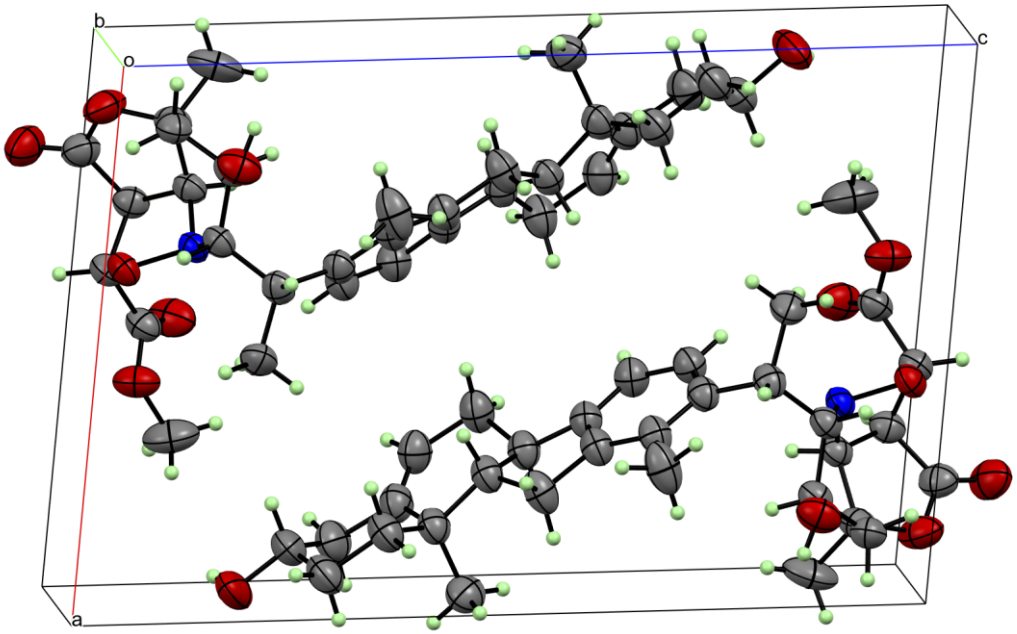


Figure S14 View of the pack drawing of compound **1**.

Table S1. Crystal data and structure refinement for **1**

Identification code	1	
Empirical formula	C32 H41 N O7	
Formula weight	551.66	
Temperature	298(2) K	
Wavelength	1.54178 Å	
Crystal system	Monoclinic	
Space group	$P2_1$	
Unit cell dimensions	a = 11.4439(11) Å	α= 90°.
	b = 7.5246(7) Å	β=96.303(4)°.
	c = 17.3527(17) Å	γ = 90°.
Volume	1485.2(2) $Å^3$	
Z	2	
Density (calculated)	1.234 Mg/m^3	
Absorption coefficient	0.701 mm^{-1}	
F(000)	592	
Crystal size	0.420 x 0.210 x 0.100 mm^3	
Theta range for data collection	3.886 to 68.497°.	
Index ranges	-13<=h<=13, -8<=k<=9, -20<=l<=20	
Reflections collected	34656	
Independent reflections	5292 [R(int) = 0.0612]	
Completeness to theta = 67.697°	99.6 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.7531 and 0.6217	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	5292 / 1 / 370	
Goodness-of-fit on F^2	1.027	
Final R indices [I>2sigma(I)]	R1 = 0.0340, wR2 = 0.0948	
R indices (all data)	R1 = 0.0495, wR2 = 0.1045	
Absolute structure parameter	0.05(6)	
Largest diff. peak and hole	0.174 and -0.160 $e.Å^{-3}$	

4.2. Accession Codes

CCDC 2152193 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge *via* www.ccdc.cam.ac.uk/data_request/cif, by emailing data_ request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.