HETEROCYCLES, Vol. 102, No. 3, 2021, pp. 506 - 515. © 2021 The Japan Institute of Heterocyclic Chemistry
Received, 9th December, 2020, Accepted, 12th January, 2021, Published online, 27th January, 2021
DOI: 10.3987/COM-20-14394

SYNTHESIS AND *IN VITRO* BIOLOGICAL EVALUATION OF CANANODINE

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Abstract – (±)-Cananodine was obtained by common reactions from (±)-rupestine G and was isolated and synthesized by our group. (+)-Cananodine and its enantiomer were obtained by chiral separation and their absolute configurations were determined by high-resolution mass spectrometry, nuclear magnetic resonance, and electronic circular dichroism spectroscopy. The optical rotation of both cananodine and its enantiomer were re-treated. Biological activity against Hep G2, Hela, and MDA-MB-231 were also investigated.

Guaipyridine sesquiterpene alkaloids are a collection of natural products with unusual structure consisting of a fused pyridine ring and seven-membered carbocycle.[1] Most of those alkaloids exhibit potent pharmacological activities and might serve as leads for further drug development.[2] Cananodine, a naturally occurring, representative guaipyridine sesquiterpene alkaloid, was isolated in small quantities (10 mg from 3.5 kg of fruit) from the perfumery tree *Cananga odorata* in 2001 by Hsieh *et al*.[3] The plant *Cananga odorata* was trivially known as the herb ylang-ylang, a Taiwanese folk medicine for treatment of tinea infections, malaria, and fever. The structure of cananodine was assigned by using

nuclear-magnetic-resonance (NMR) and mass-spectrometry (MS) techniques.[3] Per Hsieh's report, cananodine shows potent activity against human hepatocarcinoma cell lines (Hep G2 with IC_{50}=0.22 μg/mL and Hep G2.2.15 with IC_{50}=3.8 μg/mL) **(Figure 1)**.

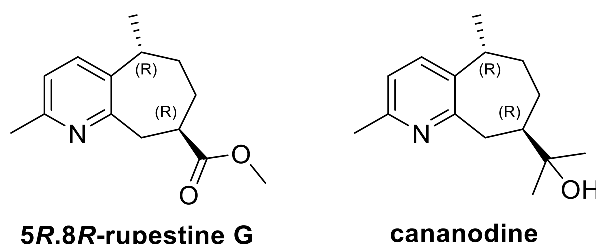


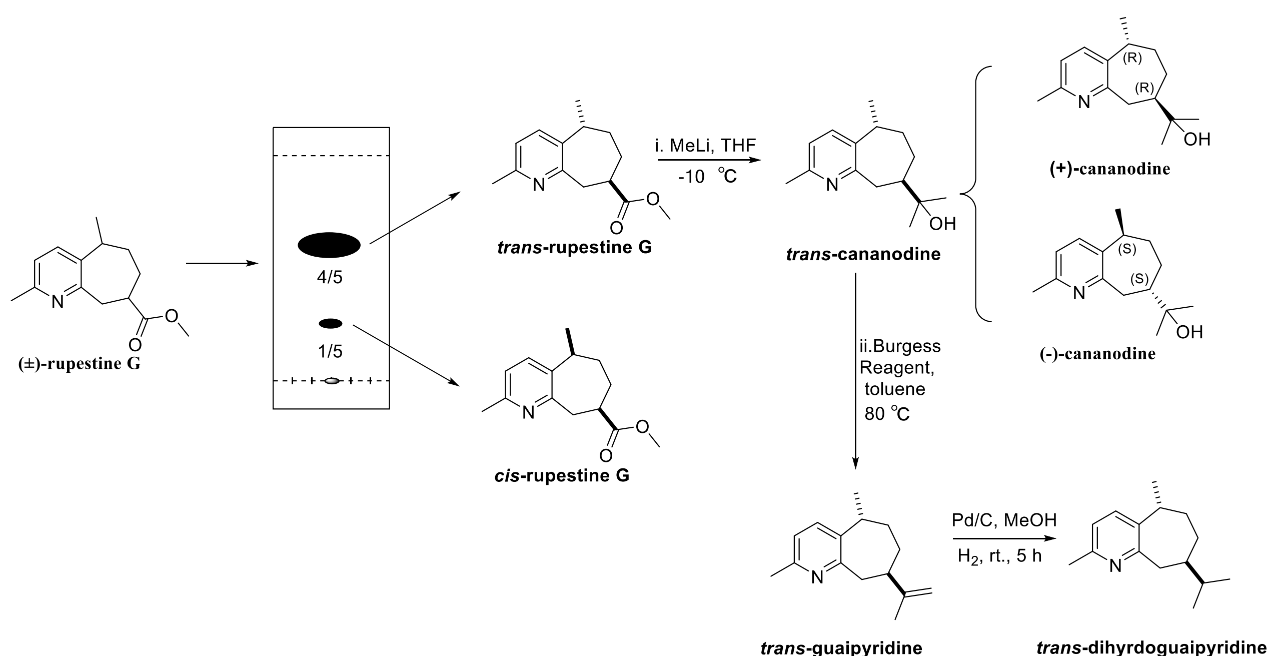
Figure 1. Structures of cananodine and 5*R*, 8*R*-rupestine G

Currently, despite the anticancer activity and unusual structure of cananodine, only two synthetic methods have been reported by Gavin and Vyvyan. The first total synthesis of (+)-cananodine was accomplished by Gavin in 2006.[4] This protocol used a chiral-pool-/chiral-auxiliary-based approach for the synthesis of a key oxazolidinone intermediate. Subsequent key steps involved diastereoselective oxazolidinone allylation, cycloheptenylmethanol formation by ring-closing olefin metathesis, and microwave-assisted decarboxylative Claisen rearrangement reaction. The optical rotation value obtained by Gavin is $[\alpha]_D^{21}$ +17.9 (*c* 1.34, $CHCl_3$), which differs significantly from that obtained by Wu's team, i.e., $[\alpha]_D^{25}$ –76.2 (*c* 0.06, $CHCl_3$).

The second method, developed by Vyvyan, synthesized cananodine using a remarkable intramolecular opening of a tri-substituted epoxide as the key step in construction of the seven-membered carbocycle of the target.[5] Despite the epoxide opening strategy giving all four stereoisomers of cananodine, unfortunately the yield is too low and the isomers were not separated to determine the difference in value of the above-mentioned optical rotation.

There are hundreds of natural products with seven-membered ring structures, like guaianes and several alkaloids, such as cananodine, guaipyridine, and rupestines.[6] Our group recently successfully carried out a nine-step total synthesis procedure of (±)-rupestine G with a yield of 18.9%.[7] The synthesis was started from commercially available compound 5-bromopicoline and employed a Suzuki reaction to build terminal diene moiety. The diene was further elaborated into the desired guaipyridine structure by an intramolecular ring closing metathesis (RCM) reaction. (±)-Rupestine G was obtained by several common

reactions after the RCM reaction. The structural similarity and pharmacological properties of cananodine and rupestine encourage establishment of the natural compound and its enantiomer to resolve the aforementioned optical rotation issue and lay a foundation for exploring the relevant biological activities.



Scheme 1. Synthesis of (±)-cananodine, *trans*-guaipyridine, and *trans*-dihydroguaipyridine

Scheme 1 would serve the purpose of synthesis of the racemic form of cananodine, *trans*-guaipyridine, and *trans*-dihydroguaipyridine. Rupestine G and its epimers were synthesized according to the procedure in the previously reported paper[7] as a mixture. The mixture was then isolated on a preparative TLC to give two pairs of diastereoisomers (approximately 4/5 *trans*-rupestine G and 1/5 *cis*-rupestine G). The methyllithium attacks the carbonyl group of *trans*-rupestine G,[8,9] and after hydrolysis it gives the target molecule *trans*-cananodine with total yield 12.6%. By dehydration with commercially available Burgess's reagent,[10,11] *trans*-guaipyridine (yield 9.4%) can be obtained. Catalytic hydrogenation was attempted to obtain *trans*-dihydroguaipyridine from guaipyridine and the disappearance of the double-bond peak was observed in the ^{1}H NMR spectra. However, the small amount of product prevents obtaining pure samples.

After obtaining *trans*-cananodine, it was further separated by chiral separation with a preparative high performance liquid chromatography (LC-20A, Shimadzu Corp., Japan) to give two optically pure isomers

(**Figure 2**). Absolute configurations of cananodine and its enantiomer were determined by comparison of experimental and calculated electronic circular dichroism (ECD) spectra. The optical rotation of cananodine and its enantiomer were tested for (+)-cananodine, with $[\alpha]_D^{25}$ +10.0 (*c* 0.06, $CHCl_3$), and for (−)-cananodine, with $[\alpha]_D^{25}$ −10.0 (*c* 0.06, $CHCl_3$).

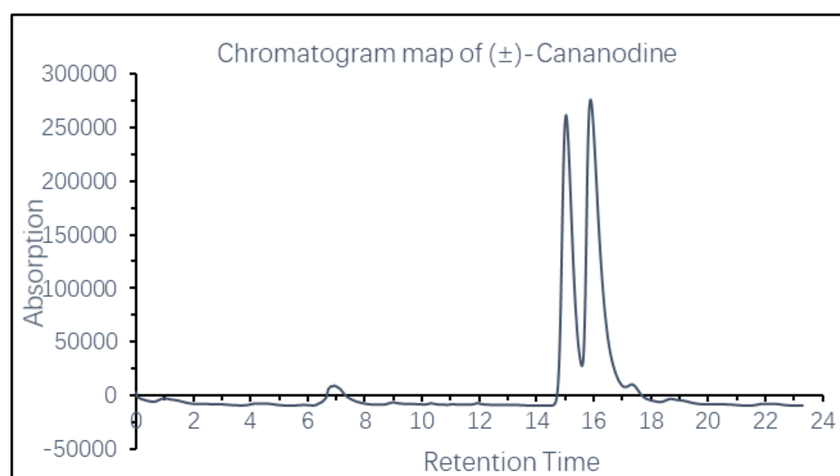


Figure 2. Chromatogram map of (±)-cananodine

The structures of these two isomers were intensively elucidated by extensive analysis of ^1H NMR, ^{13}C NMR, high-resolution–electrospray ionization–mass spectrometry (HR-ESI-MS), and ECD spectroscopy. The CD spectra of both compounds were measured and were the mirror image of each other, as shown in **Figure 3**, indicating their enantiomeric nature.

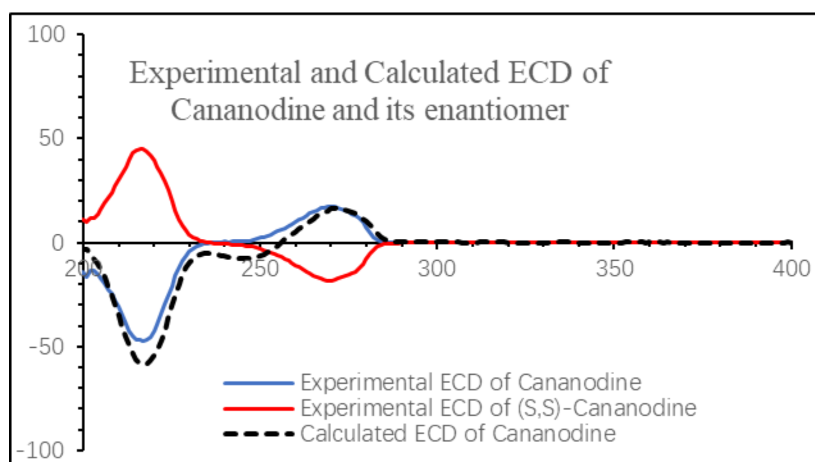


Figure 3. The experimental ECD and calculated ECD of cananodine and its enantiomer

The HR-ESI-MS spectra of the compound synthesized at *m/z* 234.1838 $[M+H]^+$ agrees with the reported natural product cananodine (*m/z* = 234.1775).[3] The ^{1}H and ^{13}C NMR data are identical to the previously published data.[5] The CD spectra of (+)-cananodine shows a CD pattern similar to the calculated data, i.e., a negative Cotton effect (CE) near 215 nm and a positive CE at 275 nm, which verifies that the absolute configuration of (+)-cananodine is 5*R*,8*R*. The ^{1}H and ^{13}C NMR data of (−)-cananodine is identical to that of the natural compound, but the CD spectra are opposite those of (+)-cananodine. Thus, the (−)-cananodine is confirmed to be 5*S*,8*S*-cananodine.

The success of synthesis and separation of (±)-cananodine impels continuous evaluation of the biological activity. (±)-Cananodine, (+)-cananodine, and the latter's enantiomer were tested for the cytotoxicity against Hela, MDA-MB-231, and HepG2 cell lines using the MTT colorimetric assay, with the well-known drug doxorubicin (DOX) as a positive control. In the present experiment, (+)-cananodine shows slightly weaker activity against human hepatocarcinoma cell lines than that in Hsieh's report. In addition to this, (+)-cananodine shows better activity against Hela and MDA-MB-231 than its enantiomer and the racemic one with IC_{50} values of 17.77 and 19.23 μg/mL (**Table 1**).

Table 1. Anticancer activity of cananodine and its isomer

Sample no.	Cell lines IC_{50}[a] (μM)		
	Hela[b]	MDA-MB-231[c]	Hep G2[d]
(±)-cananodine	113.98±6.06	130.35±4.15	385.58±4.59
(+)-cananodine	17.77±1.54	19.23±1.51	47.99±1.58
(−)-cananodine	73.20±4.59	170.85±4.29	313.98±4.36
DOX[e]	0.69±1.63	1.04±2.5	4.63±1.63

[a] IC_{50}: Concentration required to inhibit tumor cell proliferation by 50%.

[b] Hela: Cervical cancer.

[c] MDA-MB-231: Breast cancer.

[d] Hep G2: Human hepatocarcinoma cell.

[e] DOX: Doxorubicin used as positive control.

In summary, in this work the synthesis of *trans*-cananodine from commercially available 5-bromo-2-picoline has been achieved in total 12.6% yield using the method reported by our group. The separation of the enantiomer of *trans*-cananodine was successfully performed by preparative HPLC. The two optically pure isomers were fully characterized by ^{1}H, ^{13}C NMR, HRMS, and optical rotation value as well as experimental and ECD calculations. The optical rotation value of synthesized compound was $[\alpha]_D^{21}$ +10.0 (*c* 0.06, $CHCl_3$), and these data were consistent with those reported by Gavin. The biological activity was also measured. The synthetic approach demonstrated herein would be equally effective for the synthetic preparation of other guaipyridine sesquiterpene alkaloids, such as guaipyridine and dihydroguaipyridine.

EXPERIMENTAL

1. Material and methods

All reactions were performed in oven-dried flasks. Reagents and solvents were purchased from commercial vendors and used as received. Experiments were performed at ambient temperature. Reaction progress and purity of the compounds were monitored by TLC. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VNMRS 600 spectromerter and Varian 400-MR in $CDCl_3$ or DMSO-d_6 with TMS as an internal reference. Chemical shifts (δ) are reported in ppm relative to TMS (^{1}H NMR; δ= 0.00 ppm) and $CDCl_3$ (^{13}C NMR; δ=77.000 ppm) if not otherwise stated. Abbreviations s, d, t, m, def and br are used to designate singlet, doublet, triplet, multiplet, deformed and broad, respectively. The HR-ESI-MS data were collected with a QStar Elite mass spectrometer. Semi-preparative HPLC was conducted on a Shimadzu LC-20A instrument, with UV detection, using a CHIRALPAK ID (No. 84335, 10 mm id×250 mm, 5 µm silica gel, Daicel Chiral Technologies Co., LTD., China) column. As mobile phase (Flow rate, 5 mL/min), 98% *n*-hexane in EtOH was used (HPLC grade, Merck, Germany). The optical rotations were recorded on a Rudolph RS Autopol VI automatic polarimeter. ECD spectra were measured in EtOH on a JASCO J – 810 spectropolarimeter (Jasco, Tokyo, Japan). ECD calculations were performed by TmoleX 3.4 software (COSMOlogic GmbH & Co. KG, Germany).[12-15] Absolute configuration was assigned by using optical rotation spectra, circular dichroism spectroscopy and time dependent density functional theory calculations at BP/TZVPP level. The ground-state geometries were optimized with density functional theory calculations. All atoms were estimated with the basis set def-TZVP and the functional BP.

Electronic circular dichroism corresponding to the optimized structures were calculated using TDDFT method at BP/def-TZVP level. The results were subsequently optimized by Gaussian method.
Doxorubicin were purchased from BBI Inc. (Shanghai, China). The human cancer cell lines breast (T47D, MCF-7), cervical (Hela) were obtained from Chinese Type Culture Collection, CAS (Shanghai, China).
All compounds were dissolved in DMSO in a stock concentration of 10 mM. 200 mL of the cells Hep G2, MDA-MB-231 and cervical cancer cells Hela, were separately seeded in 96-well plates at the density of 3 $\times$ 10^3 cells/well. The cells grew for 24 h in anincubator (Binder, Germany) with 95% humidity and 5% CO_2. Thereafter, the cells were treated with 1, 10, 20, 30, and 50 mM of per compounds for 48 h. Twenty milliliters MTT (5 mg/mL) was then added to each cell and the plates were incubated at 37 °C. Absorbance was read at a wavelength of 570 nm using an enzyme-linked immu-nosorbent assay reader. The IC_{50} values were calculated for the inhibition rate. Inhibitionrate = (OD value of control group – OD value of experiment group)/(OD value of controlgroup – OD value of blank group).[16-18]

2. Synthesis of products

2.1 (±)-Cananodine

MeLi (3.1 M in diethoxymethane, 0.30 mL, 0.93 mmol) was added to a cold (-78 °C) solution of *trans*-rupestine G (50 mg, 0.22 mmol) in dry THF (5 mL). After 2 h, the reaction was quenched with sat. aq. soln. of NH_4Cl, and enough water was added to dissolve salts. The mixture was diluted with petroleum ether and the layers were separated. The ether layer was washed with brine and dried over $MgSO_4$. After evaporation, the residue was purified by preparative TLC (EtOAc/petroleum, 1/5) to yield the (±)-cananodine (46 mg, 0.20 mmol, 89%) as a colorless oil. The mixture (30 mg) was prepared 30 mg/mL solution in EtOH, and further separated by chiral separation with a Shimadzu LC-20A preparative-HPLC (CHIRALPAK ID-84335 used as chiral column, *n*-hexane/EtOH (98/2, *v*/*v*) used as mobile phase, flow rate was 5 mL/min) of 200 μL injection per time to give the two optically pure compound.

2.2 *trans*-Guaipyridine

A solution of *trans*-cananodine (10 mg, 0.05 mmol) in dry toluene (3 mL) was added under nitrogen to a suspension of Burgess' salt (15 mg, 0.06 mmol, 1.2 equiv.) in dry toluene (2 mL). The reaction mixture was stirred at 80 °C for overnight and concentrated *in vacuo*. The residue was taken up in water (10 mL)

and CH_2Cl_2 (25 mL), and the aqueous layer was separated and extracted with CH_2Cl_2 (3×25 mL). The combined organic layers were dried with $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc/petroleum, 1/5) to give *trans*-guaipyridine as a colourless oil (8 mg, 75%).

2.3 *trans*-Dihydroguaipyridine

To a solution of *trans*-guaipyridine (3 mg, 0.02 mmol) in MeOH (3 mL) was added Pd/C (5 mg, 0.5% Pd in C). The mixture was stirred for 5 h under H_2 in room temperature. After filtration of Pd/C, the reaction mixture was concentrated in vacuo to give *trans*-dihydroguaipyridine as a colourless oil. The ^{1}H NMR spectra were tested without purification of this compound.

3. Characterization data of products

3.1 Cananodine

Colourless oil; $[\alpha]_D^{25}$ +10.0 (*c* 0.06, $CHCl_3$); HRESI-MS, Calcd 233.1416, found $[M+H]^+$= 234.1838. IR (neat, film) ν_{max} 2986, 2883, 1734, 1608, 1458, 1188, 1158, 800 $cm^{-1.}$ ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (d, *J* = 7.7 Hz, 1H), 6.93 (d, *J* = 7.7 Hz, 1H), 3.64 (s, 3H), 3.36 (dd, *J* =14.6, 9.7 Hz, 1H), 3.31 (d, *J* = 14.6, 2.7 Hz, 1H), 3.04 – 2.95 (m, 1H), 2.49 (s, 3H), 2.70 – 2.61 (m, 1H), 2.17 – 2.07 (m, 1H), 2.01 – 1.93 (m, 1H), 1.86 – 1.74 (m, 2H), 1.32 (d, *J* = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.49, 157.19, 154.46, 137.68, 136.05, 121.16, 51.43, 41.80, 40.26, 37.41, 32.05, 28.96, 23.60, 18.66.

3.2 5*S*,8*S*-Cananodine

Colourless oil; $[\alpha]_D^{25}$ -10.0 (*c* 0.06, $CHCl_3$); HRESI-MS, Calcd 233.1416, found $[M+H]^+$= 234.1838. IR (neat, film) ν_{max} 2986, 2883, 1734, 1608, 1458, 1188, 1158, 800 $cm^{-1.}$ ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (d, *J* = 7.7 Hz, 1H), 6.93 (d, *J* = 7.7 Hz, 1H), 3.64 (s, 3H), 3.36 (dd, *J* =14.6, 9.7 Hz, 1H), 3.31 (d, *J* = 14.6, 2.7 Hz, 1H), 3.04 – 2.95 (m, 1H), 2.70 – 2.61 (m, 1H), 2.49 (s, 3H), 2.17 – 2.07 (m, 1H), 2.01 – 1.93 (m, 1H), 1.86 – 1.74 (m, 2H), 1.32 (d, *J* = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.49, 157.19, 154.46, 137.68, 136.05, 121.16, 51.43, 41.80, 40.26, 37.41, 32.05, 28.96, 23.60, 18.66.

3.3 Guaipyridine

Colourless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (d, *J* = 7.6 Hz, 1H), 6.89 (d, *J* = 7.6 Hz, 1H), 4.73 (s, 1H), 4.68 (s, 1H), 3.78 – 3.70 (m, 2H), 3.21 – 3.13 (m, 1H), 3.08-3.00 (m, 1H), 3.00 (s, 2H), 2.48 (s, 3H), 2.26 – 2.15 (m, 1H), 1.85 (d, *J* = 3.3 Hz, 1H), 1.78 (s,3H),1.31 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.66, 155.00, 151.29, 138.12, 137.09, 121.28, 109.28, 44.88, 38.61, 36.45, 33.55, 31.44, 24.37, 21.21, 18.88.

ACKNOWLEDGEMENT

This work was financially funded by Scientific Research Program of the Higher Education Institution of Xinjiang (XJEDU2019Y044) and West Light Foundation of the Chinese Academy of Sciences (No. 2016-QNXZ-B-6).

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