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Racemic immunosuppressive *seco*-aporphine derivatives from *Thalictrum wangii*

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ABSTRACT

Thallactones A (**1**) and B (**2**), enantiomeric aporphine alkaloids with rare cleaved rings A and B, as well as thaliglucine *N*-oxide (**3**) and their biosynthetically related precursor, northalphenine (**4**), were isolated from the whole plant of *Thalictrum wangii*. Their structures with absolute configurations were elucidated by spectral techniques and electronic circular dichroism (ECD). Moreover, compounds **1**, **3**, and northalphenine inhibited concanavalin A (Con A)-stimulated proliferation of mice splenocyte significantly in a dose-dependent manner.

1. Introduction

Immunosuppressive agents, as the mainstay of clinical approach therapies, have extensively increased the survival in organ transplantation [1], as well as decreased reaction in autoimmune disease [2] and tissue rejection [3]. The clinically available immunosuppressants, including azathioprine (AZA) [4], cyclosporin A (CSA) [5], tacrolimus (FK506) [6], methotrexate (MTX) [7] and rituximab [8], although effective, often present undesirable side effects including nephrotoxicity [9], hypertension [10], and gastrointestinal disturbances [11]. Therefore, searching for new immunosuppressant substances with high-efficiency and low-toxicity formulations remains tremendous challenging. Natural compounds, like tripterygium glycoside [12] (known as a herbal hormone) as a promising agent for the treatment of autoimmune myasthenia gravis [13], and berberine (BBR) [14], markedly increased the blood concentration of CsA in renal-transplant recipients in both clinical and pharmacokinetic studies [15].

The structures of aporphine alkaloids can be classified into two subtypes [16]: (1) Aporphines *sensu stricto* [17], basically, characterized as a heterocyclic aromatic skeleton system (rings A-D) with a nitrogen in the ring B [18], including simple aporphines [19], bis-aporphines [20], C-6/7 dehydroaporphines (Dehydroaporphines) [21] and oxoaporphines [22], of which completely dehydrogenated with a conjugated ketone group at C-7; (2) Hetero-ring B-*seco* aporphines derivative [16], phenanthrenes [23] and aristolactams [24], possessing a $CH_2CH_2NR_1R_2$ chain or losing the C-5, and generally containing a five-membered lactam ring, and about 10% aporphines belonging to this type were reported [17,18,25–28]. Recently, few derivatives featuring rare type backbone with significant biological activity were reported. For example, dactyllactone A [29] that we found last year and cited as "hot off press", was an aporphine alkaloid with a rearranged D ring and exhibited good anti-inflammatory property. Thus, searching for rare backbone aporphines as promising candidates seems great important.

Thalictrum wangii, a traditional herbal medicine, has been used to treat dysentery and enteritis [30]. Our previous study on the genus of *Thalictrum* plants showed that their main component, aporphine alkaloids, with bioactivities in antitumor and immunosuppressive activity [31,32]. As rare rings A and B cleaved aporphines, thallactones A (**1**) and B (**2**) with a reconstructed rings system, together with thaliglucine *N*-oxide (**3**) and their biosynthetic precursor northalphenine (**4**) [33] (Fig. 1) were obtained from the whole plants of title species. The isolation, structural elucidation, plausible biogenetic pathway, and bioactivity of these isolates are herein described.

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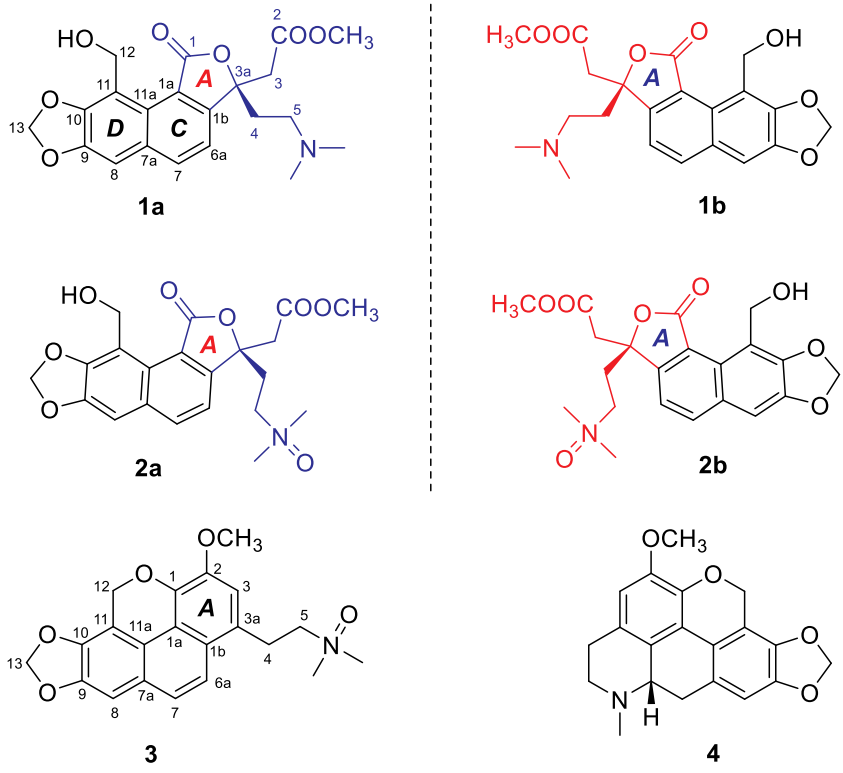


Fig. 1. Structures of alkaloids **1**–**4**.

2. Experimental

2.1. General information

Optical rotations, IR spectra, and UV spectra were measured on the P-1020, Bruker FT-IR Tensor 27 (KBr pellets), and Shimadzu UV-2401A spectrometers, respectively. NMR spectra were recorded on the Bruker 400 MHz and AV-600 MHz spectrometer with TMS as an internal standard. HREIMS analyses were carried out on Waters AutoSpec Premier P776 mass spectrometer. CD spectra were obtained on a JASCO 810 spectrometer. Column chromatography (CC) was performed using Silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, P. R. China). Sephadex LH-20 (GE Healthcare Bio-Sciences AB) and MCI-gel CHP 20P (75–100 μm) were purchased from Mitsubishi Chemical Co., Ltd. Fractions were monitored by TLC (GF_{254}, Qingdao Marine Chemical Co., Ltd., P. R. China) and spots were visualized by Dragendorff's reagent. Highly performance liquid chromatography (HPLC) was performed using the Waters 600 with semi-preparative (150 × 9.4 nm) and preparative C_{18} columns (250 × 21.2 mm).

2.2. Plant material

The whole plants of *T. wangii* were collected from Lijiang County (Yunnan Province, People's Republic of China), and identified by Mr. Jun Zhang (Yunnan University of Traditional Chinese Medicine). A voucher specimen (No. Luo_20170423) was deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Science.

2.3. Extraction and isolation

The air-dried and powdered plants of *T. wangii* (13.0 kg) were extracted with MeOH (90%) (25 L × 3) under reflux at 70 °C. After removal of the organic solvent under reduced pressure, the residue was dissolved in 0.3% (*v*/v) aqueous hydrochloric acid, the solution was subsequently basified with ammonia to pH 9–10, then extracted with EtOAc (5 L × 4) to give an alkaloidal extract (130.0 g). The extract was subjected to a silica gel column ($CHCl_3$/MeOH, 20:1 → 0:1) to afford four fractions (A → D). Fr. B (6.0 g) was subjected to column chromatography (CC) on silica gel ($CHCl_3$/MeOH, 9:1 → 7:3) to afford four sub-fractions (Fr. 1 → 4). Fr. 4 (2.5 g) was further purified on Sephadex LH-20 CC using MeOH isocratic elution to get a mixture (200.0 mg). The mixture was then separated on semi-preparative C_{18} HPLC column (MeOH/H_2O, 1:4 → 4:1 *v*/v, 3.0 mL/min) to produce thallactone A (**1**) (4.1 mg), thallactone B (**2**) (1.0 mg), and thaliglucine *N*-oxide (**3**) (1.5 mg). Compounds **1** and **2** were then successively separated by chiral semi-preparative HPLC with isopropanol-ethanol (7:3 v/v, 1.0 mL/min) to yield (+)-**1** (2.1 mg), (−)-**1** (1.4 mg), (+)-**2** (0.4 mg), and (−)-**2** (0.3 mg), respectively.

2.4. Spectroscopic data

2.4.1. Thallactone A (1)

White powder; $[\alpha]_D^{23}$ –9.4 (*c* 0.10, MeOH) for **1**, $[\alpha]_D^{21}$ +15.5 (*c* 0.10, MeOH) for (+)-**1**, $[\alpha]_D^{21}$ –13.7 (*c* 0.10, MeOH) for (−)-**1**; UV (MeOH) λ_{max} (log ε) 345 (3.14), 318 (3.07), 236 (3.80), and 225 (3.75) nm; IR (KBr) ν_{max} 3431, 2925, 1631, 1384, 1265, and 1077 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 402.1548 $[M + H]^+$ (calcd for $C_{21}H_{24}NO_7$, 402.1547).

2.4.2. Thallactone B (2)

White powder; $[\alpha]_D^{23}$ –5.0 (*c* 0.10, MeOH) for **2**, UV (MeOH) λ_{max} (log ε) 348 (3.86), 319 (3.79), 239 (4.51), 225 (4.45) and 195 (4.29) nm; IR (KBr) ν_{max} 3432, 2925, 1631, 1384, 1263, and 1048 cm^{-1}; ^1H

Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data of 1–3 (δ in ppm, J in Hz).

	1[b]		**2**[c]		**3**[b]	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		171.5		171.9		138.5
2		169.0		170.4		144.0
3	3.09 d (7.2) 3.09 d (7.2)	43.2	3.25 d (12.6) 3.25 d (12.6)	43.2	7.21 s	115.1
4	2.46 ddd (5.3, 10.3, 14.2) 2.18 ddd (4.5, 10.3, 14.2)	36.1	2.90[a]	32.9	3.70 m 3.70 m	27.0
5	2.27 m 1.96 m	53.7	3.30 m 2.68 m	66.1	3.55 m 3.55 m	71.5
7	7.97 d (8.2)	136.7	8.15 d (8.3)	138.3	7.51 d (9.2)	124.4
8	7.19 s	105.8	7.37 s	106.9	7.11 s	103.8
9		147.8		149.3		148.3
10		151.4		152.5		141.5
11		116.6		117.2		108.0
12	5.12 d (4.3) 5.12 d (4.3)	57.0	5.34 d (12.4) 5.23 d (12.4)	57.7	5.59 s 5.59 s	63.1
13	6.18 s 6.18 s	102.1	6.19 s 6.19 s	103.6	6.10 s 6.10 s	101.8
1a		120.6		122.2		119.1
1b		153.0		153.3		123.1
3a		84.2		84.3		125.7
6a	7.36 d (8.2)	116.7	7.57 d (8.3)	118.0	7.79 d (9.2)	120.5
7a		132.8		134.6		125.4
11a		127.0		127.6		119.5
6-*N*-Me$_1$	2.13 s	45.6	3.07 s	59.2	3.32 s	59.0
6-*N*-Me$_2$	2.13 s	45.6	3.12 s	58.7	3.23 s	59.0
C$_2$-OMe	3.54 s	52.1	3.47 s	52.3	4.01 s	56.9

[a] Overlapped.
[b] Recorded in CD_3Cl.
[c] Recorded in CD_3OD.

and ^{13}C NMR data, see Table 1; HRESIMS 440.1314 $[M + Na]^+$ (calcd for $C_{21}H_{23}NO_8Na^+$, 440.1316).

2.4.3. Thaliglucine N-oxide (3)

White powder; UV (MeOH) λ_{max} (log ε) 349 (4.17), 318 (4.54), 262 (5.13), 221 (4.97) and 200 (4.95) nm; IR (KBr) ν_{max} 3426, 2922, 1607, 1461, 1262, 1199, and 1039 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS m/z 368.1492 $[M + H]^+$ (calcd for $C_{21}H_{22}NO_5^+$, 368.1492).

2.5. Splenocyte proliferation assay

The *in vitro* immunosuppressive activities of **1**, **3**, and **4** were evaluated by Con A-stimulated splenocyte proliferation as described previously [32]. Briefly, the cells were seeded into 96-well flat-bottom microtiter plates (Nunc) at the density of 1×10^6 cell/mL, and exposed to the tested compounds at various concentrations in the presence of concanavalin A (Con A, 10.0 μg/mL), using the Con A-treated splenocytes as the experimental control, dexamethasone (DXM) as a positive control, and splenocytes without Con A treatment as the negative control. After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO_2, 10 μL of CCK-8 was added and incubated for another 4 h. The assays were conducted in three independent replicates, and the data were calculated as the mean of the three individual experiments. The viability of cells was evaluated using the CCK-8 assay by detecting absorbance at 450 nm on a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures/the absorbance value for non-stimulated cultures.

3. Results and discussion

Thallactone A **(1)**, a white powder, shared a molecular formula of $C_{21}H_{23}NO_7$ with 11 degrees of unsaturation determined by the HRESIMS ion at m/z 402.1548 $[M + H]^+$ (calcd for $C_{21}H_{24}NO_7$, 402.1547). The UV absorption maxima characteristic (318, 236, and 225 nm) reminiscent of aporphines possessing a ring B-*seco* system [34], and the IR spectrum showed the typical absorptions for the hydroxy group (3431 cm^{-1}) and conjugated ester (1630 cm^{-1}) functions.

The ^{1}H NMR data of **1** (Table 1) showed two *N*-methyls [δ_H 2.13 (s, *N*-(Me)$_2$)], a methoxy [δ_H 3.54 (s, -OMe)], three aromatic signals [δ_H 7.19 (s, H-8), 7.36 (d, J = 8.2 Hz, H-6a) and 7.97 (d, J = 8.2 Hz, H-7)], as well as the methylenedioxy protons [δ_H 6.18 (s, H$_2$–13)]. The ^{13}C NMR spectrum of **1** (Table 1) indicated a total of 21 carbon resonances, assignable to three methyls (δ_C 52.1, 45.6, and 45.6), five methylenes (δ_C 102.1, 57.0, 53.7, 43.2, and 36.1), three quaternary carbons (δ_C 171.5, 169.0, and 84.2), and remaining ten signals typically ascribed to a substituent naphthalene ring moiety. Compared with those of phenanthrene (ring B-*seco* aporphine) [34,35]. the above spectral evidences suggested that **1** might be a phenanthrene derivative.

In the HMBC spectrum of **1** (Fig. 2), the correlations of δ_H 5.12 (H$_2$−12) with δ_C 151.4 (C-10), 116.6 (C-11), and 127.0 (C-11a), of δ_H 6.18 (H$_2$−13) with δ_C 147.8 (C-9), 151.4 (C-10), as well as δ_H 7.19 (s, H-8) with δ_C 132.8 (C-7a), 127.0 (C-11a) suggested the presence of a penta-substituted aromatic ring D. In addition, the HMBC correlations of δ_H 7.36 (H-6a) with δ_C 120.6 (C-1a), 132.8 (C-7a), and δ_H 7.97 (H-7) with 153.0 (C-1b), 105.8 (C-8), and 127.0 (C-11a) supported the typical penta-substituted naphthalene system of rings C and D. Unlike other intact phenanthrenes [35], the correlation of δ_H 7.36 (s, H-6a) with δ_C 120.6 (C-1a), 153.0 (C-1b), and 84.2 (C-3a) in the HMBC spectrum suggested **1** to be an uncommon ring A reconstructed phenanthrene.

The remaining two degrees of unsaturation required by molecular formula together with a carbonyl group at δ_C 171.5 (C-1) and the high-field quaternary carbon at δ_C 84.2 (C-3a), which never appeared in other phenanthrenes, suggested the ring A as a reconstructed non-

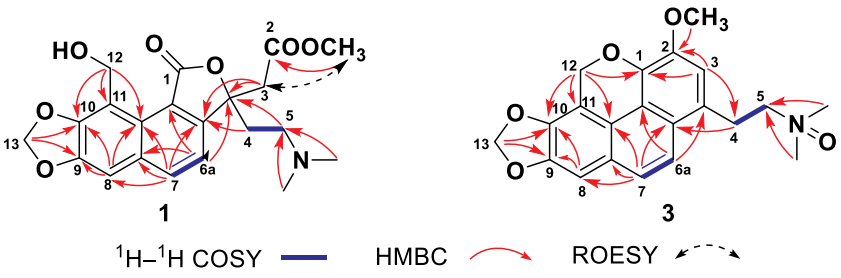


Fig. 2. Key 2D NMR correlations of **1** and **3**.

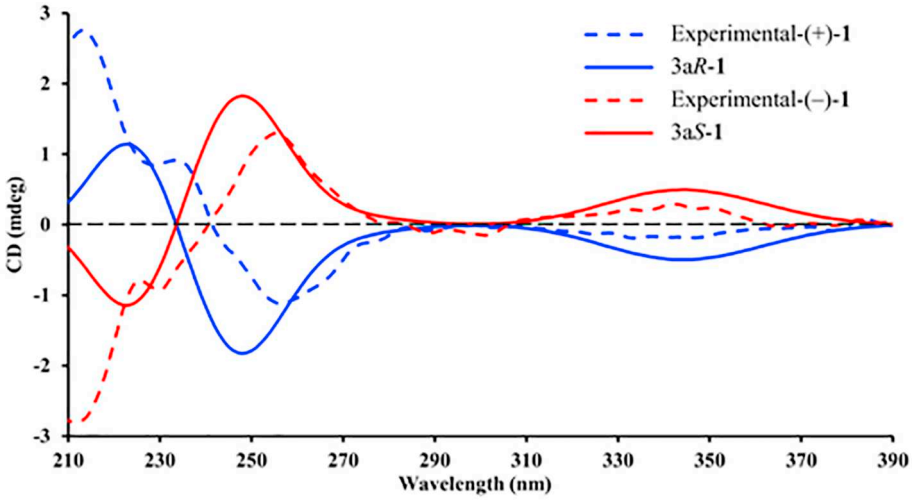


Fig. 3. Experimental and calculated ECD spectra of **1**.

aromatic system with five-membered lactone. Furthermore, HMBC correlation of δ_H 3.09 (H-3) with δ_C 153.0 (C-1b), 169.0 (C-2), and 84.2 (C-3a), along with NOE interrelation between δ_H 3.09 (H_2-3) and 3.54 (s, –$COOCH_3$) reaveled the existence of a C-3a methyl acetate unit. Besides, the key $^1H-^1H$ COSY spin system of δ_H 2.18, 2.46 (ddd, J = 5.3, 10.3, 14.2 Hz, H_2–4)/δ_H 1.96 and 2.27 (m, H_2–5), together with HMBC correlations of δ_H 2.13 (s, *N*-$(CH_3)_2$) with δ_C 53.7 (C-5), and of δ_H 1.96 (H-5) with δ_C 84.2 (C-3a), supported ring B-*seco* side chain [$CH_2CH_2N(CH_3)_2$] connected to C-3a. Compound **1** was then deduced to the rings A and B cleaved aporphine featuring a reconstructed rings system.

The optical rotation value of **1** $[\alpha]_D^{23}$ –9.4 (*c* 0.10, MeOH) suggested that compound **1** might be enantiomers. Subsequent separation using a Chiralpak IC column (Fig. S1) resolved this racemate into a pair of optically pure enantiomers **1a** and **1b** (Fig. 3), wherein the experimental circular dichroism (ECD) and optical rotation data of **1a** and **1b** clearly showed the enantiomeric relationship. Their absolute structures were determined by comparison of the experimental and calculated circular dichroism (CD) spectra using the time-dependent DFT. As depicted in Fig. 3, the calculated ECD spectrum of (3a*R*)-**1** matched the experimental plot of **1a**, which rationally indicated the absolute configuration of **1a** to be 3a*R*; while the theoretical CD curve of the 3a*S* enantiomer aligned with the experimental one of compound **1b**.

Thallactone B (**2**) displayed a molecular formula of $C_{21}H_{23}NO_8$ based on the HRESIMS peak at *m/z* 440.1314 $[M + Na]^+$, which was 16 Da more than that of **1**. The similar ^1H and ^{13}C NMR spectral data (Table 1) to those of **1** suggested **2** to be a thallactone A *N*-oxide. The assumption was indicated by a set of down-field chemical shifts of *N*-$(CH_3)_2$ (δ_H 3.12/3.07, δ_C 58.7/59.2) in **2**, and further supported by its 2D NMR spectra (Fig. S15–17). Likely, compound **2** appeared as enantiomers **2a** and **2b**. The absolute configurations of **2a** and **2b** (Fig. S30) were specifically clarified as 3a*R* and 3a*S* when compared with the calculated ECD curve of **1a** and **1b**.

Compound **3** was assigned a molecular formula of $C_{21}H_{21}NO_5$ from a prominent pseudo molecular ion peak at *m/z* 368.1492 $[M + H]^+$ in HRESIMS, indicating 11 degrees of unsaturation. The UV spectrum showed absorption maxima at 318, 262 and 221 nm, which characterized a heterocyclic aromatic basic skeleton, whereas the IR spectrum showed absorptions for hydroxy group (3426 cm^{-1}) and aromatic structure (1607, 1461, 1262, 1199, and 1039 cm^{-1}). Moreover, the ^1H and ^{13}C NMR spectral data of **3** (Table 1) were closely comparable to those of thaliglucine [36] suggesting a B-*seco* aporphine skeleton for **3**. The downfield shift signals of δ_H 3.70 (2H, H-4), 3.55 (2H, H-5) and 3.32, 3.23 (s, *N*-$(CH_3)_2$), as well as 16 Da more than that of thaliglucine, suggested **3** to be the thaliglucine *N*-oxide.

A plausible biogenetic pathway for **1**–**3** could be traced back to northalphenine (**4**), a major aporphine alkaloid isolated from the same plant (Scheme 1). Briefly, **4** went through a series of potential pre-functionalizations, including methylation and Hofmann elimination, to afford the critical thaliglucine. Further oxidation of thaliglucine may yield **3** (path 2) and **i** (path 1), respectively, and then **i** could be readily transformed to key intermediate **ii** by oxidation, featuring the cleavage of aromatic ring A. This might be followed by the nucleophilic addition of H_2O to the extra-ring double bonds to form **iii**. Then, compound **1** possessing a five-membered lactone ring A, might be derived from the key intermediate **iii** by esterification between C-1 and C-3a. Subsequently, **2** could be formed from **1** by *N*-oxidation.

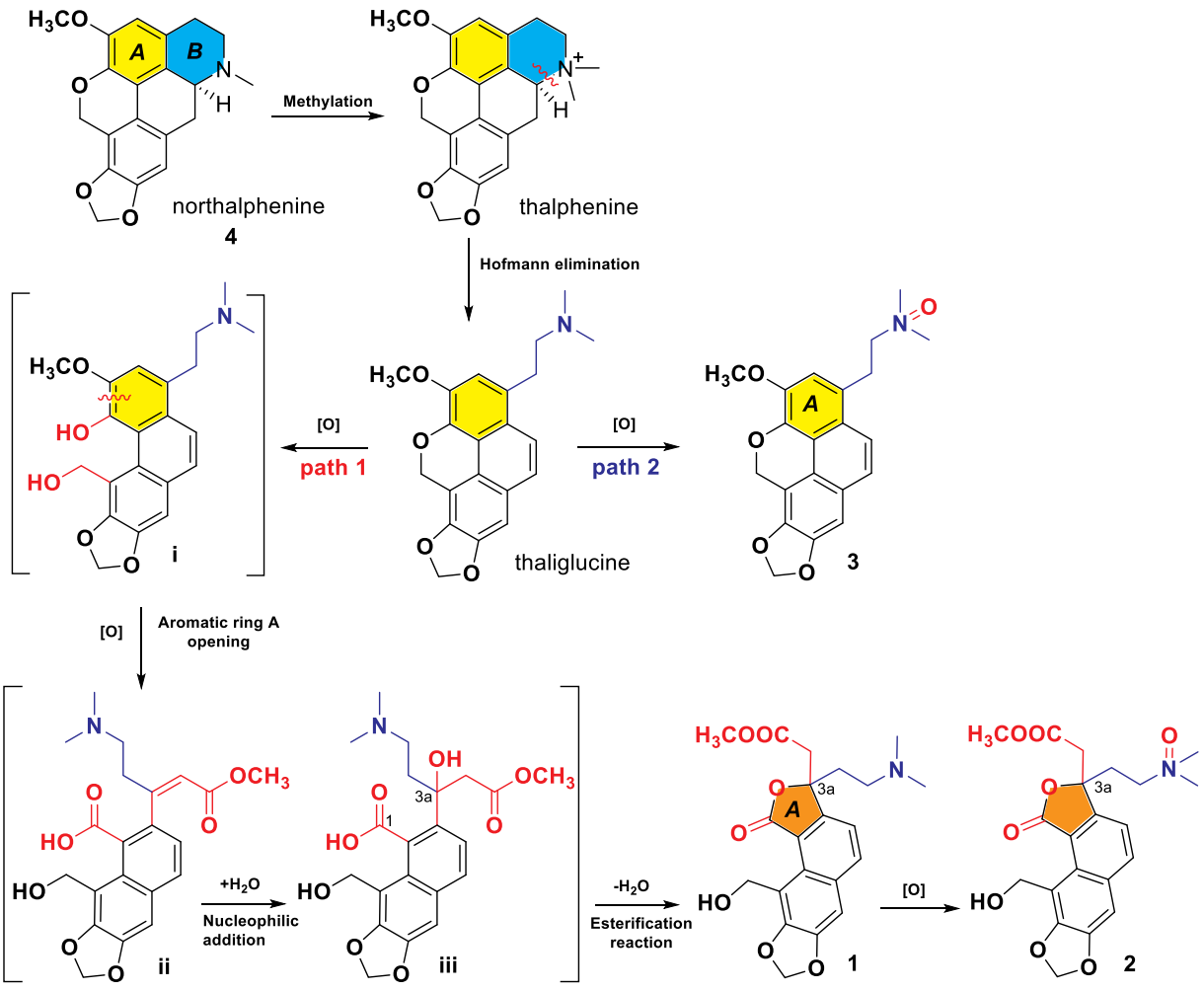
In the bioassay, the immunosuppressive activity of compounds **1**, **3**, and **4** was evaluated on mitogen-induced (Con A) splenocyte proliferation with dexamethasone (DXM) as a positive control [32,37] The results indicated that three different aporphines derivatives inhibited T lymphocyte significantly in a dose-dependent manner (Fig. 4). In detail, once aporphines with an *N*-oxide system, like compound **3**, exhibited significantly reduced the immunosuppressive effect. In contrast, without *N*-oxidation, aporphine appeared either with intact rings (**4**) or with ring cleaved and reconstructed (**1**), and there were no significant differences in their inhibitory effect on mitogen-stimulated splenocyte proliferation. It is worthy to note that their activities were even better than the positive control dexamethasone at a concentration between 25 and 50 μM.

4. Conclusions

Thallactones A (**1**) and B (**2**) enantiomeric aporphine derivatives, together with thaliglucine *N*-oxide (**3**) and northalphenine, were isolated from the whole plant of *T. wangii*. The novel structure of thallactone A (**1**) and its potent immunosuppressive bioactivity made it attractive to both chemists and biologists for total synthesis and further pharmacological evaluation.

Declaration of Competing Interest

The authors declare no conflicts of interest.



Scheme 1. Proposed biosynthetic pathway for **1–3**

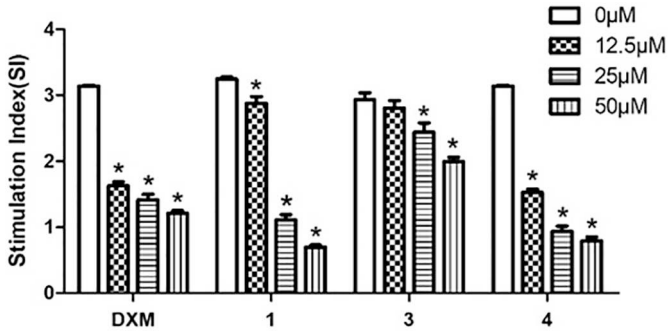


Fig. 4. Effect of compounds **1**, **3**, and **4** on Con A-stimulated splenocyte proliferation *in vitro*.
DXM was used as positive control. The values were presented as mean ± SD of triplicate. A statically significant difference was determined by ANOVA and Tukey test (*$p < .05$, compared with the control group).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2019.104445.

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