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Original Article

Two new terpenoids from Reduning Injection

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ABSTRACT

Objective: To study the antipyretic and anti-inflammatory constituents from the active fraction of Reduning (RDN) Injection.

Methods: In this study, the active fraction of RDN Injection was screened by the LPS-induced mouse endotoxin shock model. The chemical constituents were isolated by chromatography on HP-20 macroporous adsorptive resins, silica gel, ODS columns and reverse phase MPLC and HPLC repeatedly, and their structures were elucidated based on spectroscopic analysis (HR-ESI-MS, NMR, ECD) and chemical methods. Meanwhile, we evaluated the anti-inflammatory activities of the isolates by measuring their inhibitory effects on TNF-α production in LPS stimulated RAW 264.7 macrophages.

Results: The 95% ethanol eluate of RDN Injection by the macroporous adsorption resin column was proved to be the antipyretic and anti-inflammatory active fraction of this injection. A novel iridoid, named jasminoide A (**1**), and a new guaiane sesquiterpenoid, named (1*R*,7*R*,8*S*,10*R*)-7,8,11-trihydroxy-4-guaien-3-one (**2**), were isolated from Reduning injection, and compound **2** can inhibit TNF-α production with IC_{50} values of 72.24 μmol/L.

Conclusion: In this study, two new terpenoids were isolated from Reduning Injection, and compound **2** showed inhibitory activity against LPS-activated TNF-α production in RAW 264.7 cells *in vitro*.

1. Introduction

Traditional Chinese medicine prescription Reduning, which consists of three common used Chinese material medica, including *Lonicera japonica* Thunb (Jinyinhua), *Gardenia jasminoides* Ellis (Zhizi) and *Artemisia* annua L. (Qinghao), injection has the prominent effects of clearing heat, dispelling wind and detoxification. It has been proved to be widely used for the treatment of wind-heat cold, cough, upper respiratory tract infection (Zhao, Zhang, Wang & Zhu, 2010), and acute bronchitis (Wang, 2010) with good therapeutic effect in clinical practice. At present, a large number of chemical constituents with diverse structures, including iridoids (Ge et al., 2017; Li, 2013; Li et al., 2015a, Li et al., 2016), lignans (Li, 2013; Li et al., 2016), sesquiterpenoid (Li, 2013; Li et al., 2015a), flavanoids (Li, 2013, Li, Yu, Wang, Xiao, & Yao, 2014), coumarins (Li, 2013; Li, Yu, Wang, Xiao, & Yao, 2014) and organic acids (Ge et al., 2017; Li, 2013; Li, Yu, Wang, Xiao, & Yao, 2014, Li et al., 2015a) have been isolated from Reduning injection, and modern pharmacological studies showed that Reduning injection exhibited a broad range of biological activities, such as antipyretic (Chang et al., 2015a), anti-inflammatory activity (Chang et al., 2015b; Wang et al., 2015), antiviral (Cao et al., 2015; Tang et al., 2014; Wang et al., 2014; Zuo, Chen & Zhang, 2013), and so on.

Based on the previous studies, we determined that RDN-3 (95% ethanol elution fraction), which was isolated by HP-20 macroporous resin, was the antipyretic and anti-inflammatory active fraction of Reduning injection. As a continuing work for exploring anti-inflammatory agents, the 95% ethanol elution fraction of Reduning injection was separated and purified to obtain a novel iridoid, named jasminoide A (**1**), and a new guaiane sesquiter-

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penoid, named (1*R*,7*R*,8*S*,10*R*)−7,8,11-trihydroxy-4-guaien-3-one (**2**) (Fig. 1). Their structures were elucidated by the analyses of NMR, HR-ESI-MS, ECD and chemical methods. Anti-inflammatory (TNF-α) activity of two isolated compounds were evaluated *in vitro*.

2. Meterials and methods

2.1. General experimental procedures

1D and 2D NMR spectra were measured with a Bruker AV 400 and 500 spectrometers in CD_3OD solution (δ_H = 3.30 ppm, δ_C = 49.0 ppm) at room temperature. UPLC-ESI-Q/TOF-MS spectrum was acquired using an Agilent 6538 Q-TOF mass spectrometer. Analytical HPLC were performed on an Agilent 1100 series pump equipped with a DAD detector and a Gemini C18-MS-II column (5 μm; i.d. 250 × 4.6 mm, Phenomenex.). HPLC separations were performed using an Agilent 1260 series pump equipped with a MWD detector and a YMC C-18 (5 μm; YMC-Pack ODS-A, Japan) column (i.d. 20 × 250 mm). Semi preparative HPLC were performed using a Ulitmate 3000 series pump equipped with a YMC C-18 (5 μm; YMC-Pack ODS-A, Japan) column (i.d. 20 × 250 mm). Diaion HP-20 (Mitsubishi-Chemical, Japan), silica gel (100–200 mesh, 200–300 mesh; Qingdao Marine Chemical Ltd., China), octadecylsilanized (ODS) silica gel (50 μm; YMC Ltd., Japan) were used for column chromatography (CC). The rest of the reagents used for the analysis of pure (Nanjing Chemical reagent Limited by Share Ltd).

Reduning injection (lot number: 150,814) was produced by Jiangsu Kanion Pharmaceutical Co., Ltd., Lianyungang, China. LPS used for stimulation of RAW 246.7 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mouse TNF-α ELISA kit (Invitrogen).

2.2. Extraction and isolation

The concentrated Reduning injection (62.5 kg) was subjected to column chromatography (CC) over HP-20 macroporous adsorptive resins, eluted with water, 30% and 95% ethanol in successive to yield three fractions (A-C). Fraction C (500 g, 95% EtOH eluate) was separated over a silica gel column (SiO_2, 200–300 mesh, Φ 15 × 97 cm) eluted with a CH_2Cl_2−MeOH in gradient to yield 16 crude fractions (C1−C16). The subfraction C4 (4.9 g, CH_2Cl_2−MeOH, 98:2, eluant) was separated by CC over silica gel (200−300 mesh, Φ 5.4 × 47 cm) eluted with petroleum ether and ethyl acetate in gradient. The subfraction C4c1 (57.1 mg, petroleum ether and ethyl acetate, 9:1, eluant) was further purified by preparative HPLC (MeOH–H_2O, 3:2, eluant) to afford compound **1** (25.2 mg, t_R 22.0 min). Subfraction C7 (29.9 g, CH_2Cl_2−MeOH, 95:5, eluant) was separated by CC over ODS (50 μm, Φ 1.3 × 55 cm) eluted with MeOH–H_2O in gradient. Subfraction C7b1 (2.6 g, MeOH–H_2O, 2:3, eluant) was further purified by preparative HPLC (CH_3OH–H_2O, 1:1) to afford compound **2** (160.5 mg, t_R 28.5 min).

3. Results

Compound **1** was obtained as a white amorphous powder; [α]20 D −26.4 (*c* = 1.5, MeOH); UV (MeOH) λ_{max} nm (log ε): 235.2 (5.14); IR (KBr) ν_{max} cm^{-1}: 3253, 2932, 1704, 1602. Its molecular formula $C_{13}H_{18}O_5$ was determined by HR-ESI-MS (*m/z* 255.1234 $[M+H]^+$, calcd for 255.1227), with five degrees of unsaturation. The ^{13}C NMR (Table 1) and DEPT-135 spectra of **1** showed 13 carbon signals, including one carbonyl (δ_C 167.2), a double bond (δ_C 157.3 and 113.4), an oxygenated quaternary carbon (δ_C 107.6), three methines (δ_C 109.6, 60.3 and 53.0), four methylenes (δ_C 74.9, 64.0, 34.8 and 29.4), one methoxy carbon (δ_C 51.6), as well as a methyl group (δ_C 15.5). Thus, the three remaining degrees of unsaturation in **1** could be distributed to three rings. The structure of **1** was further demonstrated by analysis of 2D NMR spectra. In the HMBC spectrum, the correlations between H-3 and C-4/C-5/C-9/C-11, H-5/C-3, as well as 11–OCH_3/C-11 indicated the presence of a 2, 3-dihydrofuran unit (Part A in Fig. 1). A cyclopentane unit (Part B in Fig. 1) can be established by the HMBC correlations between C-9 and H-6/H-7/H-8/H-10, along with a proton spin system (H-5/H-6/H-7/H-8/H-10) in the ^1H–^1H COSY spectrum. The HMBC correlations between H-1 and C-8/C-9/C-10, between H-10 and C-1/C-8/C-9/C-12, along with the ^1H–^1H COSY correlations of H-12/H-13 showed the presence and the location of a tetrahydrofuran unit (Part C in Fig. 1). In the three structural fragments (Part A, B and C) that have been deduced, we can find that C-9 (δ_C 107.6) was shared carbon, as well as the HMBC correlations between H-1 and C-5, based on above data, compound **1** was thus identified as an unprecedented carbon skeleton for iridoids, which probably has arisen from rearrangements of the basic structure of iridoids. The cyclopentanopyran ring in iridoids was commonly H-5/H-9 β,β-*cis*-fused, while the allylic coupling of the H-3/H-5 resonances was 0.8 Hz (compared to 2.0–2.5 Hz for *trans*-fused iridoids) (Su and Jia, 2000). In the NOESY spectrum, H-8 (δ_H 2.57) exhibited a NOE interaction with H-5 (δ_H 3.33) and no correlation with H-10 (δ_H 4.84), suggesting a β-orientation of H-5 and H-8

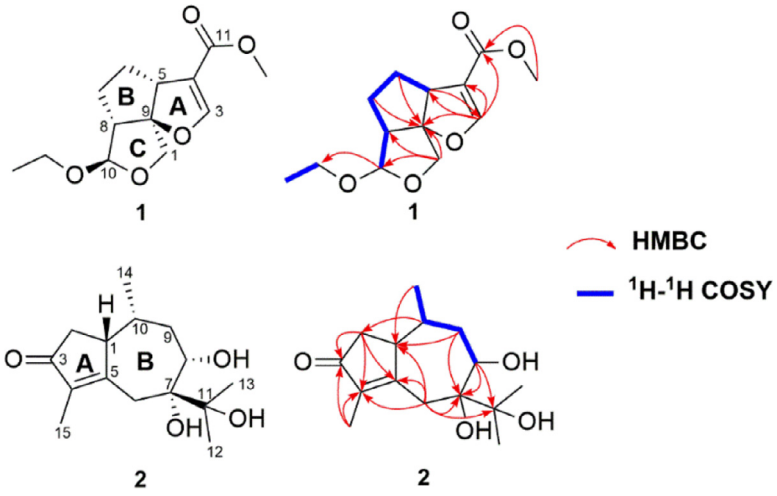


Fig. 1. Structures, key HMBC (→) and COSY (—) correlations of compounds **1** and **2**.

Table 1
NMR spectroscopic data (in CD_3OD) of **1** (^1H: 500 MHz; ^{13}C: 125 MHz) and **2** (^1H: 400 MHz; ^{13}C: 100 MHz).

Pos.	1			2		
	δ_C	δ_H (*J* in Hz)	HMBC	δ_C	δ_H (*J* in Hz)	HMBC
1	74.9	4.07, d (7.6)	C-5, 8, 9, 10	48.0	2.93, o	C-4, 5
		3.93, d (7.6)	C-5, 8, 9, 10			
2	/	/		38.3	2.33, dd (18.0, 6.0)	C-3, 4, 5
					2.06, dd (17.6, 4.0)	C-1, 3, 10
3	157.3	7.24, d (0.8)	C-4, 5, 9, 11	211.7		
4	113.4			141.7		
5	53.0	3.33, t (5.0)	C-1, 3, 4, 8	175.3		
		2.18, m	C-4, 5, 7, 8, 9		2.93, o	C- 1, 4, 5, 7, 8, 11
6	34.8			32.6		
		1.73, m	C-4, 8, 9		2.27, d (12.8)	C- 1, 4, 5, 7, 8, 11
7	29.4	1.93, m	C-5, 6, 8, 9, 10	79.0		
		1.53, m	C-5, 6, 8, 9, 10			
8	60.3	2.57, t (5.8)	C-6, 7, 9, 10	77.6	4.06, dd (10.8, 2.8)	C- 7, 9, 10, 11
9	107.6			36.4	1.57, m	C- 1, 8, 10
					1.10, dd (14.8, 2.8)	C- 1, 7, 10, 14
10	109.6	4.84, brs	C-1, 7, 8, 9, 12	30.8	2.19, m	C-1, 2, 5, 8, 9, 14
11	167.2			79.2		
12	64.0	3.73, m	C-10, 13	25.4	1.37, s	C- 7, 11, 13
		3.47, m	C-10, 13			
13	15.5	1.19, t (5.8)	C-12	26.7	1.37, s	C- 7, 11, 12
14				20.9	1.03, d (7.2)	C- 1, 9, 10
15				9.0	1.71, d (2.0)	C- 3, 4, 5
11–OCH_3	51.6	3.69, s	C-11			

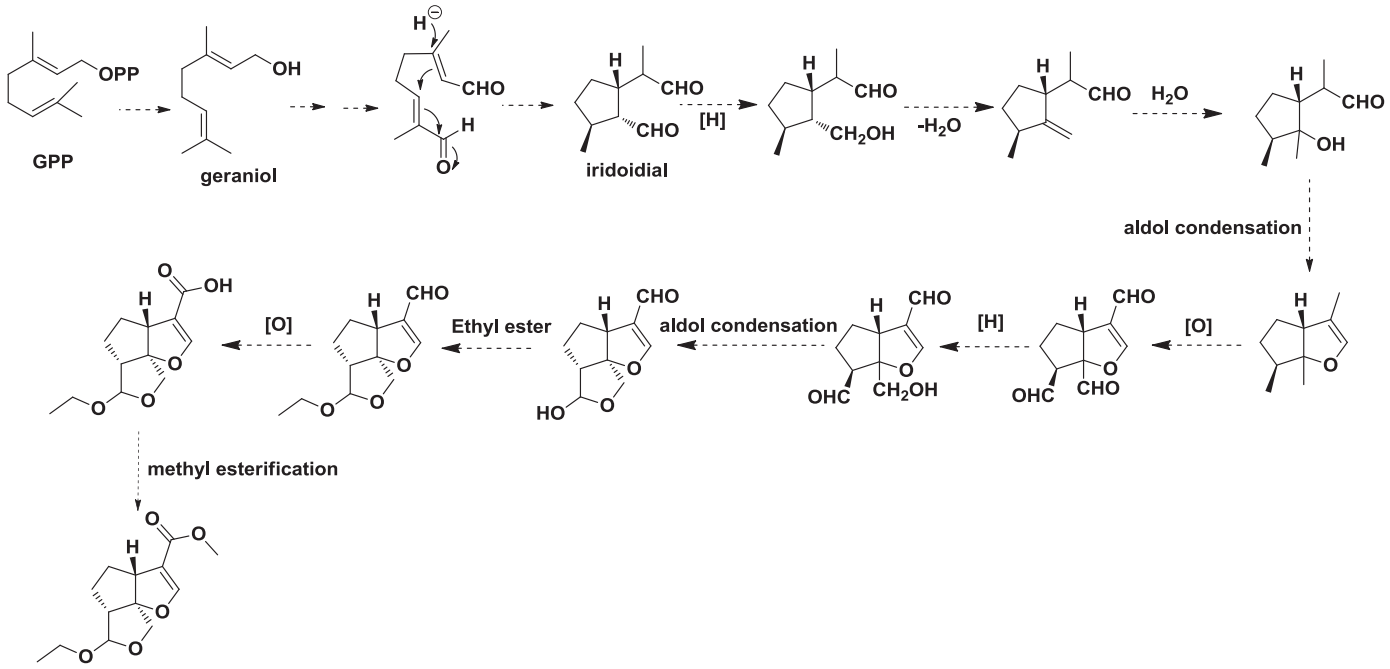


Fig. 2. Hypothetical biosynthetic pathway of compound **1.**

and α-orientation of H-10 (Ban et al., 2014). From the above data, the novel structure of **1** was elucidated and named as jasminoide A and a hypothetical biosynthetic pathway for **1** was also proposed (Fig. 2).

Compound **2** was obtained as a white amorphous powder; [α]20 D-28.2 (c = 1.5, MeOH); UV (MeOH) λ_{max} nm (log ε): 236.1 (4.66); IR(KBr) ν_{max} cm^{-1}: 3303, 2952, 1697, 1632, 1469, 1385, 1071; and the molecular formula of $C_{15}H_{24}O_4$ by HR-ESI-MS (*m/z* 269.1752 $[M+H]^+$, calcd for 269.1747, *m/z* 267.1605 $[M-H]^-$, calcd for 267.1602), with four degrees of unsaturation. The ^1H NMR spectrum of **2** displayed four methyl groups at δ_H 1.03 (3H, d, *J* = 7.2 Hz, H-14), 1.37 (6H, s, H-12, 13), 1.71 (3H, d, *J* = 2.0 Hz, H-15). The ^{13}C NMR (Table 1) and DEPT-135 spectra of **2** showed fifteen carbon signals, including one carbonyl (δ_C 211.7), a tetrasubstituted double bond (δ_C 141.7 and 175.3), two oxygenated quaternary carbons (δ_C 79.2, 79.0), three methines (δ_C 77.6, 48.0, 30.8), three methylenes (δ_C 38.3, 36.4, 32.6), as well as four methyl groups (δ_C 26.4, 25.4, 20.9, 9.0). The NMR data of **2** were similar to (1*S*, 7*R*, 8*R*, l0*S*)−7, 8, 11-trihydroxy-1-hydroperoxy-4-guaien-3-one (Li et al., 2015a) and (1*R*, 7*R*, 8*S*, l0*R*)−7, 8, 11-trihydroxy-guai-4-en-3-one 8- *O*-β-*D*-glucopyranoside (Machida et al., 2000). So, the gross structure of **2** was deduced as a guaiane sesquiterpenoid. The ^1H–^1H COSY spectrum of **2** indicated the presence of a proton spin systems (H-8/H-9/H-10/14-CH_3) as shown in Fig. S3. In the HMBC spectrum, the correlations of H-6 to C-1/C-5/C-7/C-8, H-9 to C-1/C-7, as well as 14-CH_3 to C-1/C-9 indicated the pres-

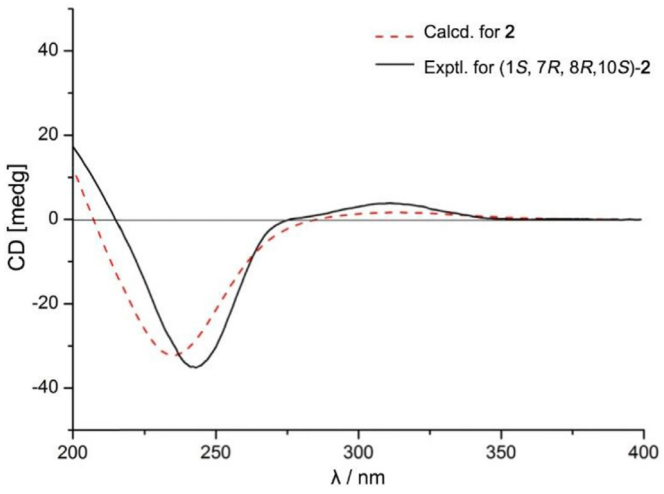


Fig. 3. Experimental and calculated ECD spectra of compound **2**.

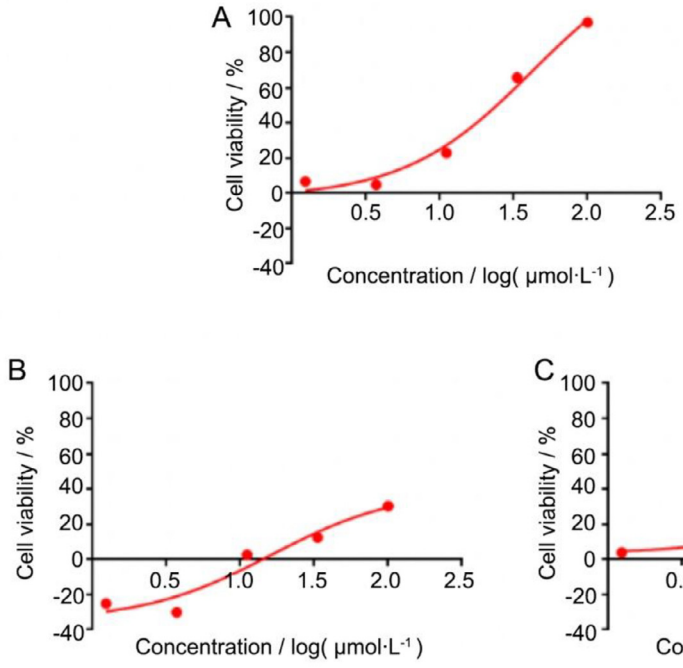
ence of a cycloheptane unit (Part B in Fig. 1). A substituted α, β-unsaturated cyclopentanone unit (Part A in Fig. 1) was established by the HMBC correlations between H-1/H-2 and C-3/C-4/C-5, between 15-CH_3 and C-2/C-4/C-5. In addition, The HMBC correlations of 12, 13-CH_3 to C-7/C-11 also showed the presence and the location of a hydroxyisopropyl. Based on the molecular formula evidence and low-field chemical shift [δ_C 79.0 (C-7), 77.6 (C-8), 79.2 (C-11)], three hydroxy groups were connected at C-7, C-8 and C-11, respectively. In summary, the gross structure of **2** was determined to be 7, 8, 11-trihydroxy-4-guaien-3-one.

The relative configuration of **2** was clarified by the NOESY experiment. The NOESY cross peaks were observed between H-10 /H-1 and H-8 suggesting that H-10, H-8 and H-1 were all on the same face (β) of the molecule. The hydroxyisopropyl was β-orientation, which was found in most sesquiterpenoids with a known stereochemistry (Li et al., 2015a). The absolute configuration at C-1 of **2** was determined by the CD spectrum. The CD spectrum of **2** (Fig. 3) showed a negative cotton effect at λ_{max} 249 nm and a positive cotton effect at λ_{max} 312 nm, which is opposite to (1*S*, 7*R*, 8*R*, l0*S*)−7, 8, 11-trihydroxy-1-hydroperoxy-4-guaien-3-one (Li et al., 2015b), suggesting a R configuration at C-1 for **2**. To confirm that, the electronic circular dichroism (ECD) computation was performed at the B3LYP/6–31G(d) level. As a result, the calculated ECD showed high agreement with the experimental spectrum as shown in Fig. 3. From the previous evidence, the absolute configuration of **2** was elucidated to be 1*R*, 7*R*, 8*S*, 10*R*.

The anti-inflammatory activities of **1**–**2** evaluated *in vitro* at doses of 100 μmol/L and celecoxib as a positive control (Fig. 4). IC_{50} value of anti-inflammatory activity in the presence or absence of test compounds were calculated by the expression of tumor Necrosis Factor α (TNF-α) on Raw 246.7 cells stimulated by LPS. Compound **2** could reduce TNF-α levels in LPS-activated RAW 264.7 macrophages with an IC_{50} of 72.24 μmol/L (celecoxib was used as a positive control, IC_{50} = 42.29 μmol/L).

4. Conclusions

In this study, a novel iridoid, named jasminoide A (**1**), and a new guaiane sesqniterpenoid, named (1*R*,7*R*,8*S*,10*R*)−7,8,11-trihydroxy-4-guaien-3-one (**2**), were isolated from 95% ethanol elution fraction of Reduning Injection. The structures of these compounds were elucidated based on spectroscopic analysis (HR-ESI-MS, NMR, ECD) and chemical methods. In order to confirm that the origin of compounds **1** – **2**, the crude methanol extracts of tree herbs (L. *japonica; G. jasminoides* and *A. annua*) in prescription Reduning were analyzed by UPLC-Q-TOF-MS/MS. The ion peak in *G. jasminoides* extract was consistent with **2** rather than **1**, which confirmed that possibly compound **1** is not naturally occurred. Anti-inflammatory (TNF-α) activity of the isolated compounds were evaluated *in vitro*, and compound **2** showed inhibitory activity against TNF-α production in LPS-induced RAW 264.7 mouse macrophage cell lines with IC_{50} value of 72.24 μmol/L.



Compounds	IC_{50} / (μmol·L^{-1})
1	> 100
2	72.24 ± 1.8
Celecoxib[a]	42.29 ± 2.6

[a] Positive control

Fig. 4. Inhibitory effect of celecoxib (A), compound **1** (B) and compound **2** (C) on TNF-α production induced by LPS in macrophages.

Declaration of Competing Interest

No potential conflict of interest was reported by the authors.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chmed.2019.11.006.

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