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Two novel nornemoralisin-type diterpenoids from *Aphanamixis polystachya* (Wall.) R. Parker

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ABSTRACT

Two novel heptanornemoralisin-type diterpenoids nornemoralisins A (**1**) and B (**2**), together with two known compounds nemoralisin (**3**) and nemoralisin A (**4**), were isolated from the stem bark and leaves of *Aphanamixis polystachya* (Wall.) R. Parker. Their structures were established through comprehensive analyses of NMR spectroscopic data and high resolution mass spectrometric (HR-ESI-MS) data. The absolute configurations of carbon stereocenters were elucidated by circular dichroism (CD) analyses. The four compounds were tested for their potential cytotoxic effects against ACHN, HeLa, SMMC-7721, and MCF-7 cell lines. Nornemoralisins A (**1**) and B (**2**) exhibited significant cytotoxicity on ACHN with an IC_{50} value of 13.9 ± 0.8 and 10.3 ± 0.4 μM, respectively, and other compounds failed to reveal obvious cytotoxicity on the tested cell lines, compared to positive control vinblastine (IC_{50}, 28.0 ± 0.9 μM).

1. Introduction

Aphanamixis polystachya (Wall.) R. Parker is a timber tree mainly growing in the low altitude tropical areas of Asia, such as China, India, Malaysia, and Indonesia [1]. This plant has been extensively investigated and has yielded a series of new limonoids from the fruits and seeds [1–4], a range of complex sesquiterpenes [5], diterpenoids [6–9], flavone glycoside [10], alkaloid [11], aphanamixin [12] and a keto fatty acid [13] from the stem bark and leaves. Some of its secondary metabolites displayed significant biological activities such as antitumor [3], antimicrobial [14], antifeedant [4,12], insecticidal [3], antimalarial and inhibit lipopolysaccharide-induced nitric oxide production activities [7].

Recently, our ongoing program towards the discovery of novel bioactive constituents from genus *Aphanamixis*, two novel diterpenoids nornemoralisins A (**1**) and B (**2**), together with two known homologues nemoralisin (**3**) and nemoralisin A (**4**), were isolated from the leaves and stem bark of *A. polystachya* collected from the Yunnan province of China. Herein, we report their isolation, structure elucidation, and *in vitro* cytotoxic evaluation against ACHN (human kidney cancer cells), HeLa (human cervical cancer cells), SMMC-7721 (human hepatoma cancer cells) and MCF-7 (human breast cancer cells).

2. Experimental

2.1. General experimental procedures

Optical rotations were obtained on an Autopol® IV polarimeter. CD spectra were recorded on an OLIS, Inc. DSM-1000 over a range of 190–1100 nm. IR spectra were measured on a Nicolet™ Avatar 360 FTIR spectrometer over a range of 400–4000 cm^{-1}. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were measured using a Bruker Corp. APEX II mass spectrometer. 1D- (^1H, ^{13}C, DEPT) and 2D- (^1H-^1H COSY, HSQC, HMBC) NMR experiments were performed on a Bruker Corp. AM-400 instrument operating at 400 MHz for ^1H and 100 MHz for ^{13}C, respectively. The chemical shifts (δ) are reported in ppm using tetramethylsilane (TMS) as an internal standard to referenced to solvent peaks at δ_H 7.26 and δ_C 77.16 ± 0.06 for $CDCl_3$ and coupling constants are in Hz.

Sephadex LH-20 (Amersham Pharmacia Biotech, Sweden), Reversed-phase (RP) C18 silica gel (100-200mesh, YMC, Japan), MCI gel (Mitsubishi Chemical Corporation, Japan), Macroporous resin HP-20 (Amersham Pharmacia Biotech, Sweden), and silica gel (200 − 300) mesh, Qingdao Marine Chemical Factory, China) were used for column chromatography (CC). Precoated silica gel plates (GF_{254} 10–40 μm, Qingdao Marine Chemical Factory, China) were used for TLC analyses and the compounds were visualized by spraying the TLC plates with 5%

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H_2SO_4 in EtOH (*v*/v) followed by heating.

2.2. Plant material

The stem bark and leaves of *A. polystachya* were collected in September 2011 from Mengla county, Yunnan province, China, and identified by Prof. Guo-Da Tao, Tropical Botanical Garden, Chinese Academy of Sciences. A voucher specimen (No. 201110AG) was deposited in the Institute of Organic Chemistry, Lanzhou University.

2.3. Extraction and isolation

The air-dried stem bark and leaves of *A. polystachya* (15.0 kg) were extracted with 95% EtOH (3 × 10.0 L) under 40 °C reflux for 3 × 2 h. The ethanol extract was filtered and concentrated under reduced pressure to yield a crude extract (1756 g), which was suspended in distilled H_2O (3 L) and then successively partitioned with petroleum ether (30–60 °C, 3 × 3 L), and ethyl acetate (3 × 3 L) to afford ethyl acetate -soluble fraction (903.5 g). The ethyl acetate -soluble fraction was subjected to Macroporous resin HP-20 eluting with a gradient of H_2O-MeOH (100:0, 7:3, 1:1, 1:4, 0:100, *v*/v) to give five fractions (Frs. A-E).

Fraction A (100.3 g) was fractionated by a silica gel column with petroleum ether/acetone (100:0, 50:1, 15:1, 5:1, 2:1, 1:1, 0:100); Seven fractions (Frs. A_1-A_7) were obtained on the basis of similar TLC profiles. Further chromatography of subfraction Fr. A_3 (1.0 g) on silica gel (200–300 mesh, 150.0 g) column eluting with petroleum ether/ethyl acetate (10:1–0:1) yielded three subfractions (Frs. $A_{3.1}$– $A_{3.3}$). Subfraction Fr. $A_{3.2}$ (0.4 g) was separated by Sephadex LH-20 (MeOH) column chromatography to yielded two subfractions (Frs. $A_{3.2.1}$–$A_{3.2.2}$), compounds **3** (68 mg) and **4** (96 mg) was obtained from Fr. $A_{3.2.1}$ (270 mg) by using silica gel (5 g) column chromatography eluting with petroleum ether/ethyl acetate (6:1–2:1).

Fraction B (200.3 g) was further separated by a silica gel column eluting with petroleum ether-acetone (50:1, 15:1, 5:1, 2:1, 1:1, 0:100, *v*/v). Five fractions (Frs. B_1-B_5) were obtained on the basis of similar TLC profiles. Subfraction FB_2 (20.3 g) was subjected to a Sephadex LH-20 column eluting with a mixture of CH_2Cl_2-MeOH (1:1, *v*/v), followed by reversed-phase C18 silica gel column eluting with H_2O-MeOH (2:1–1:3, v/v) to give three fractions (Frs. $B_{2.1}$, $B_{2.2}$, $B_{2.3}$). Fraction $B_{2.1}$ (2.2 g) was purified over a column chromatography of Sephadex LH-20 using MeOH as a mobile phase followed by silica gel chromatography eluting with petroleum ether- ethyl acetate (3:1, *v*/v) to afford compounds **1** (2 mg) and **2** (3.4 mg).

Compound **1**: pale yellow oil; $C_{13}H_{20}O_3$; [*α*]20 D = + 48.1 (*c* 1.0, in $CHCl_3$); IR (liquid film) ν_{max}: 3383.9, 2923.7, 2851.0, 2369.3, 1702.5, 1585.3, 1176.4 cm^{-1}; CD (*c* 1.0 × 10^{-3}, MeOH) λ_{max}nm (Δ*ε*): 225 (+0.53), 264 (−0.89); HR-ESI-MS *m/z* 225.1491 $[M + H]^+$ (calcd. for $C_{13}H_{20}O_3H$, 225.1485); ^1H (400 MHz, $CDCl_3$, *δ* ppm), ^{13}C (100 MHz, $CDCl_3$, *δ* ppm) NMR data, see Table 1.

Compound **2**: pale yellow oil, $C_{13}H_{18}O_3$; [*α*]20 D = + 62.6 (*c* 1.0, in $CHCl_3$); IR (liquid film) ν_{max}: 3391.3, 2924.8, 2852.3, 1701.3, 1588.9, 1175.5, 755.7 cm^{-1}; CD (*c* 1.0 × 10^{-3}, MeOH) λ_{max}nm (Δ*ε*): 226.5 (+1.51), 260 (−4.08), 294.5 (+1.47); HR-ESI-MS *m/z* 245.1151 $[M + Na]^+$ (calcd. for $C_{13}H_{18}O_3Na$, 245.1148); ^1H (400 MHz, $CDCl_3$, *δ* ppm), ^{13}C (100 MHz, $CDCl_3$, *δ* ppm) NMR data, see Table 1.

2.4. Cytotoxicity assay

Compounds **1–4** were evaluated for cytotoxicity against four tumor cell lines including ACHN (human kidney cancer cells), HeLa (human cervical cancer cells), SMMC-7721 (human hepatoma cell line) and MCF-7 (human breast cancer cells) with the MTT assay method [15–17]. The cells were incubated at Roswell Park Memorial Institute (RPMI) 1640 medium with 10% calf serum under the conditions of 37 °C and 5% CO_2. All data, shown in Table 2 were obtained from three independent experiments, were presented as mean ± standard deviation (SD). Vinblastine was used as a positive control.

Table 1
^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of compounds **1** and **2** ($CDCl_3$, *δ* ppm, *J*/Hz).

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
2	–	88.5	–	88.7
3	–	208.3	–	207.3
4	5.34 (s), 1H	100.0	5.36 (s), 1H	100.4
5	–	195.4	–	193.5
6	1.37 (s), 3H	23.0	1.36 (s), 3H	22.9
7	1.37 (s), 3H	23.0	1.36 (s), 3H	23.0
1′	1.23 (d, 6.89), 3H	17.7	1.28 (d, 6.9), 3H	17.5
2′	2.58–2.63 (m), 1H	35.7	2.83 (m, 6.9), 1H	34.9
3′	1.57–1.59 (m), 2H	21.2	2.45–2.58 (m, 1.4, 7.1), 2H	36.8
4′	1.57–1.59 (m), 2H	30.1	6.69 (dt, 15.8, 7.2,), 1H	143.5
5′	2.45 (t, 6.76), 2H	43.4	6.12 (dt, 15.8, 1.3), 1H	133.4
6′	–	207.6	–	198.0
7′	2.14 (s), 3H	33.6	2.23 (s), 3H	27.2

Table 2
Cytotoxicity of compounds **1–4** against four human cancer cell lines (IC_{50} *μ*M)[a].

Compounds	ACHN	HeLa	SMMC-7721	MCF-7
1	13.9 ± 0.8	19.3 ± 1.1	29.7 ± 0.8	31.4 ± 1.5
2	10.3 ± 0.4	1.6 ± 1.1	9.2 ± 0.7	11.3 ± 0.3
3	> 100	> 100	> 100	> 100
4	> 100	> 100	> 100	> 100
Vinblastine [b]	28.0 ± 0.9	0.01 ± 1.0	2.85 ± 1.3	7.5 ± 1.1

[a] The data represent the mean ± SD of three independent experiments.
[b] Positive control.

3. Results and discussion

3.1. Structure elucidation

Compound **1** was isolated as pale yellow oil with a molecular formula of $C_{13}H_{20}O_3$ deduced by HR-ESI-MS (found *m/z* 225.1491 $[M + H]^+$, calcd. *m/z* 225.1485 $[M + H]^+$) (Fig. S2), and it has 4 degrees of unsaturation. The IR spectrum (Fig. S1) showed absorptions for a carbonyl group (1702.5 cm^{-1}) and a double bond (1585.3 cm^{-1}).

In the ^1H NMR spectrum (Table 1), singlets of three tertiary methyl groups at δ_H 1.37, 1.37 and 2.14; one doublet of a secondary methyl group at δ_H 1.23; one triplet of a methylene group at δ_H 2.45; one multiplet of a methine group at δ_H 2.58–2.63; one singlet of an olefinic proton at δ_H 5.34 were clearly shown. Other signals occurred in a relatively high-field region (between δ_H 1.57 and 1.59) and mostly overlapped, resonating from either methine or methylene signals.

The ^{13}C NMR (Table 1) and DEPT-135 (Fig. S4) spectra exhibited 13 carbon resonances, which consisted of four methyl, three methylene, and two methine groups, including an olefinic methine group (δ_C 100.0). There were signals of four quaternary carbon atoms including two oxygenated ones (δ_C 88.5 and 195.4), and two keto carbonyl carbon atoms (δ_C 207.6 and 208.3).

By using a heteronuclear single-quantum correlation (HSQC) experiment (Fig. S6), all proton signals were accurately assigned to their respective carbon atoms.

In the ^1H-^1H COSY spectrum, correlations established the coupling relationships of H$_3$–1′/H-2′/ H$_2$–3′/ H$_2$–4′/ H$_2$–5′ (Fig. 2). In the heteronuclear multiple bond coherence (HMBC) spectrum, the ^1H-^{13}C correlations from H-7′ at *δ* 2.14 ppm to C-5′ (δ_C 43.4) and C-6′ (δ_C 207.6), the H-5′ at *δ* 2.45 showed correlations with C-6′ and C-7′ (δ_C 33.6), fragment A (Fig. 2) has been confirmed. The mutual ^1H-^{13}C correlations between H-6 and H-7 (δ_H 1.37) and their simultaneous

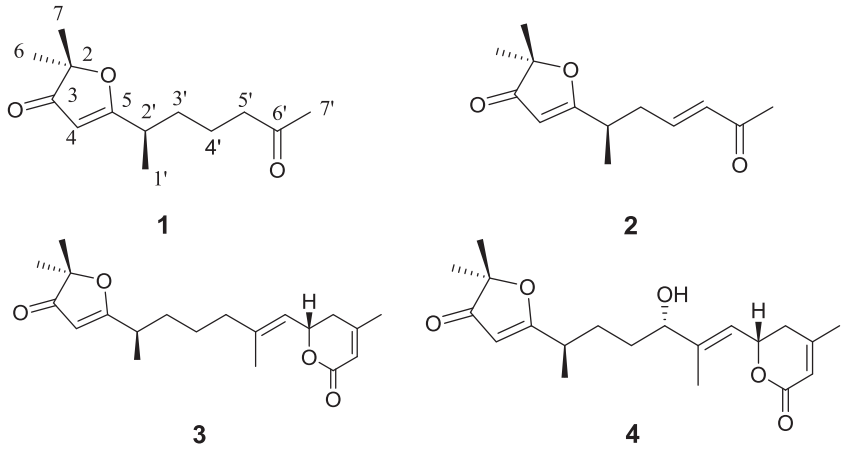
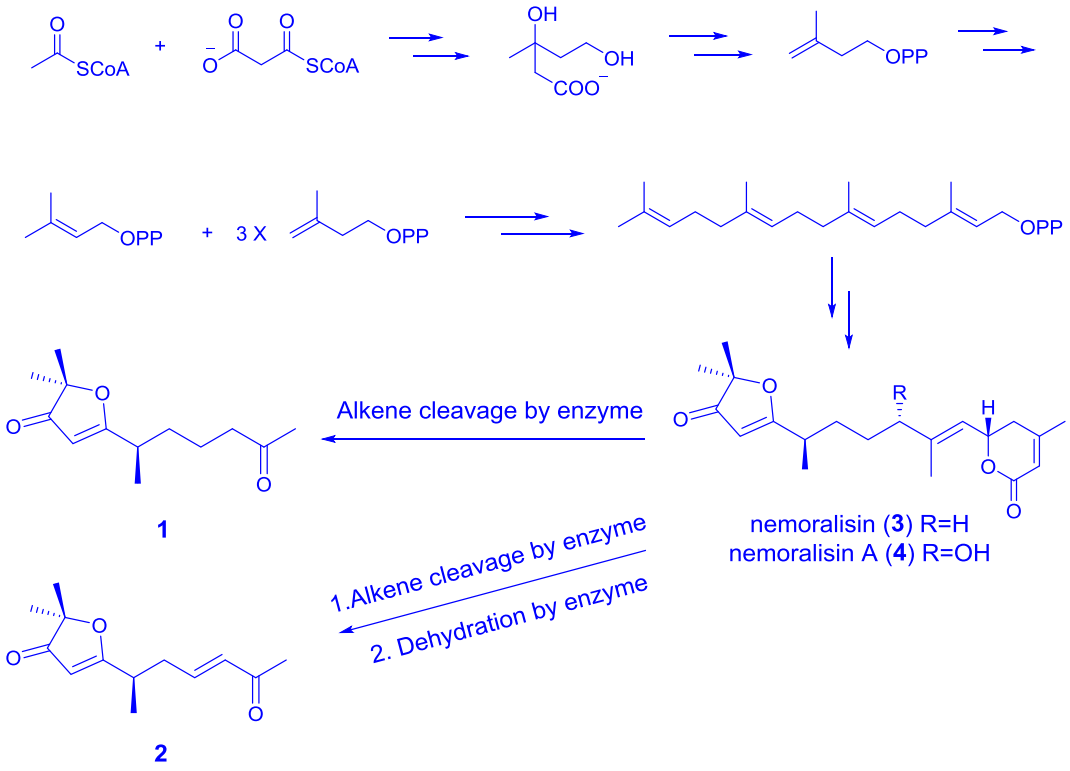


Fig. 1. Structures of compounds **1–4.**



Scheme 1. Possible biosynthetic pathways proposed for **1** and **2.**

correlations to C-2 (δ_C 88.5) and C-3 (δ_C 208.3), as well as the correlations from H-4 (δ_H 5.34) to C-2, C-3 and C-5 (δ_C 195.4), indicated the C-2 attached with C-4 (δ_C 100.0) through a keto carbon atom C-3 (δ_C 208.3). The molecular formula of **1**, which corresponded to four double-bond equivalents, implied the existence of an oxygen bridge in the compound. The chemical shifts of C-2 (δ_C 88.5), C-5 (δ_C 195.4), and the absence of 2-OH and 5-OH protons, indicated the oxygen bridge occurred between C-2 and C-5. Hence, fragment B (Fig. 2) was confirmed. Meanwhile, quaternary carbon (C-5) was attached to C-2′, which was supported by the HMBC correlations of H-4 (δ_H 5.34) with C-2′, H-2′ (δ_H 2.61) with C-5 (δ_C 195.4) and C-4 (δ_C 100.0), H-3′ (δ_H 1.58) with C-5 (δ_C 195.4) and H-1′ (δ_H 1.23) with C-5 (δ_C 195.4). Thus the planar structure of **1** was determined.

Compound **2** was obtained as pale yellow oil. Its molecular formula was determined as $C_{13}H_{18}O_3$ (five degrees of unsaturation) according to HR-ESI-MS. It exhibited a molecular ion peak at *m/z* 245.1151 $[M + Na]^+$ (calcd. for $C_{13}H_{18}O_3$, 245.1148) in HR-ESI-MS (Fig. S10). The molecular formula was only two hydrogen atoms less than that of **1**. The IR spectrum (Fig. S9) displayed characteristic carbonyl absorption (1701.3 cm^{-1}) and double bond absorption (1588.9 cm^{-1}).

The ^1H NMR spectrum (Table 1) shows three singlets for the methyl groups at δ_H 2.23 (3H, s, H-7′) and 1.36 (6H, s, H-6,7); one doublet for a methyl group at δ_H 1.28 (3H, d, J = 6.9 Hz, H-1′); one signal for an olefinic proton at δ_H 5.36 (1H, s, H-4), two signals for the trans double bond protons at δ_H 6.69 (1H, dt, J = 15.8, 7.2 Hz, H-4′) and 6.12 (1H, dt, J = 15.8, 1.3 Hz, H-5′); the ^{13}C NMR (Table1) and DEPT-135 spectra (Fig. S12) of compound **2** exhibited 13 carbon signals, including four methyl groups, one methylene unit, four methine moieties, and four quaternary carbon atoms, including one oxygenated quaternary carbon (δ_C 88.7, C-2) and two unsaturated ketone carbonyl groups (δ_C 198.0, C-6′; δ_C 207.3, C-3).

In comparison with **1**, the ^1H and ^{13}C NMR (Table 1) data of **2** revealed that both compounds **1** and **2** shared the same backbone except that the saturated bond in **1** between C-4′ and C-5′ was replaced by a

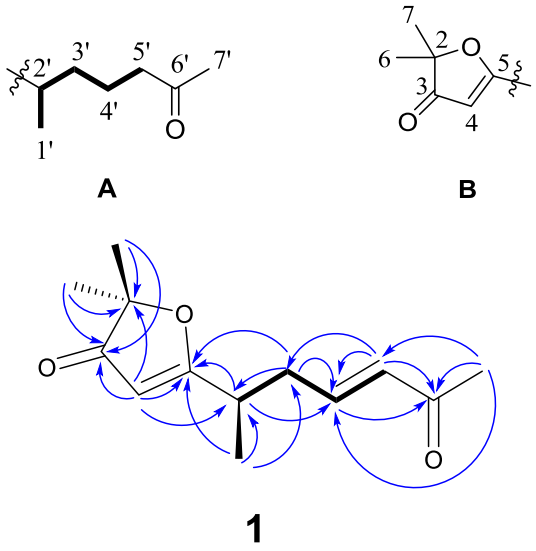


Fig. 2. ^{1}H-^{1}H COSY (thick black lines) and key HMBC (H → C) correlations of compound **1**.

double bond in **2**. Which was further supported by the ^{1}H-^{1}H COSY through correlations of H_3–1′/H-2′/H_2–3′/H-4′/H-5′ (Fig. S13) and HMBC correlations from H-7′ (δ_H 2.23) to C-5′ (δ_C 133.4) and C-6′ (δ_C 198.0), from H-5′ (δ_H 6.12) to C-6′ and C-7′ (δ_C 27.2) (Fig. S15). The larger coupling constant of 15.8 Hz between H-4′ and H-5′ revealed their *trans* relationship. The absolute configurations of compounds **1** and **2** determined by calculating electronic circular dichroism (ECD). Since the chiral position C-2′ of compounds **1** and **2** is on the chain, the two compounds are relatively flexible and have too many configurations. Since the ECD experiment spectrum of compounds **1** and **2** showed the same cotton effect at 225 nm, 260 nm and 295 nm, respectively (Fig. 3). Therefore, we selected compound **2,** which has fewer configurations than **1**, to calculate the ECD. The results (Fig. 3) showed that the calculated ECD curve for C-2′*R* matched well with the experimental spectrum. And finally, we determined the absolute configurations of C-2′ of both compounds to be *R*.

The structures of two known compounds were identified through comparison of their NMR data with literature reported data as nemoralisin (**3**) [18] and nemoralisin A (**4**) [19]. Compound **1** probably derived from oxidative cleavage of alkene from compound **3**, and compound **2** is formed by oxidative cleavage of the alkene and further dehydration from compound **4**. Alkene cleavage to give aldehydes or ketones is a very frequently used method in synthetic organic chemistry [20] and several enzymatic methods for alkene cleavage have been reported in the literature to establish safe, mild and selective oxidation methods [21]. There may be some biocatalytic alkene cleavage enzymes in the plant of *Aphanamixis polystachya*, and the alkene cleavage reactions could be performed under physiological reaction conditions-aqueous solution and the low temperature (Fig.1 and Scheme 1).

3.2. Cytotoxicity activity

Compounds **1**–**4** were evaluated for their *in vitro* cytotoxicity against ACHN, HeLa, SMMC-7721 and MCF-7 tumor cells using the MTT assay [15–17]. Nornemoralisin A (**1**) exhibited moderate inhibitory effect against all tested cell lines of with IC_{50} values of 13.9 ± 0.8, 19.3 ± 1.1, 29.7 ± 0.8 and 31.4 ± 1.5 μM, respectively, and nornemoralisin B (**2**) exhibited with IC_{50} values of 10.3 ± 0.4, 1.6 ± 1.1, 9.2 ± 0.7 and 11.3 ± 0.3 μM, respectively (Table 2). Compound **2** was more active than compound **1**, clearly indicating that the more conjugate system was important for the activity. Other compounds showed no activity at the concentrations 100 μM

4. Conclusions

To the best of our knowledge, nornemoralisins A (**1**) and B (**2**) are the first heptanornemoralisin-type diterpenoids among a number of nemoralisin-type diterpenes that have been reported today. The present results revealed that compounds **1** and **2** showed better inhibitory activities against ACHN cells than that of the positive control, and moderate cytotoxic activities against HeLa, SMMC-7721, and MCF-7 cell lines.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant

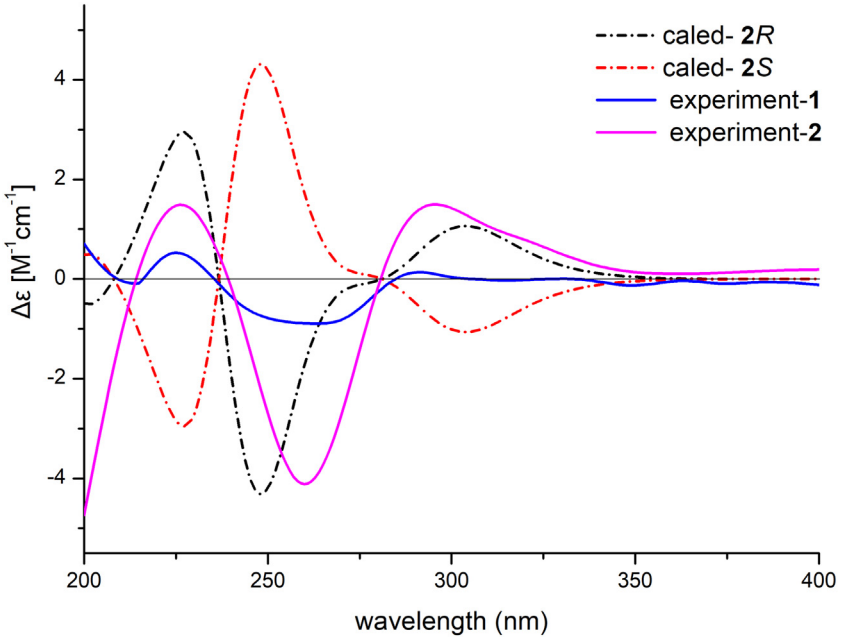


Fig. 3. Experimental curves of compounds **1**, **2**, and calculated ECD curves of compound **2**.

financial support for this work that could have influenced its outcome.

Acknowledgements

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Appendix A. Supplementary data

The original spectra of new compounds **1** and **2**, including ^{1}H, ^{13}C NMR, DEPT-135, 2D-NMR, HR-ESI-MS, IR and CD spectra were given as supporting information. Supplementary data to this article can be found online at [https://doi.org/10.1016/j.fitote.2019.104431].

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