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# Nicotabaflavonoidglycoside, the first example of cembranoid and flavonoid heterodimer from *Nicotiana tabacum*

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ABSTRACT

Nicotabaflavonoidglycoside (**1**), a novel cembrane-type diterpenoid and flavonoid heterodimer had been isolated from the leaves of *Nicotiana tabacum*. Its structure was elucidated as (1″*S*, 6″*S*) or (1″*R*, 6″*R*)-8-[6″-((−)-(1″*S*, 2″*E*, 4″*Z*, 7″*E*, 11″*E*)-cembra-2″, 4″, 7″, 11″-tetraenyl)]-rutin by comprehensive analyses of the NMR and HRESIMS spectra. Its absolute configurations of C-1″ and C-6″ were assigned as (1″*S*, 6″*S*) by its biogenesis and electronic circular dichroism (ECD). A possible biogenesis involving eliminate reaction of (1*S*, 2*E*, 4*S*, 6*R*, 7*E*, 11*E*)-2, 7, 11-cembratriene-4, 6-diol or its 4*R* isomer, as well as electrophilic substitution reaction of rutin was postulated.

## 1. Introduction

Cembranoids and flavonoids as important natural products with a wide range of biological activities have attracted attention of pharmacologists and synthetic chemists [1–4]. Natural cembranoids had shown diverse biological activities such as anti-tumor, anti-inflammatory, antibacterial, anti-metastatic, anti-pathogenic, neuroprotective, antifouling as well as anti-osteoporotic properties [1,5,6]; The first cembranoid, cembrene was isolated from the plants of *Pinus* and *Nicotiana*, and major cembranoids were obtained from the plants of *Nicotiana* genus, animals of gorgonians and alcyonaceans families such as the genera of *Sinularia*, *Euniceu*, *Sarcophyton* and so on [7–11]; A minority of cembranoids were isolated from *Mallotus hook-erianus*, *Anisomeles indica*, *Croton longissimus*, *Chandonanthus hirtellus*, *etc* [12–15]; In addition, some dimeric cembranoids were also achieved from soft corals of *Lobophytum pauciflorum*, *Sarcophyton glaucum*, *Sarcophyton trocheliophorum*. Flavonoids owing to huge diversity in the category and configuration, which had a wide varied activities such as anti-HSV (herpes simplex viruses), anti-oxidant, anti-inflammatory, anti-cancer, anti-cardiovascular diseases and so on, were widely distributed in almost all plants, especially in higher plants and fern [16–18]. Up to now, many heterodimers between monoterpene and flavonoid such as dicyclokuwanon EA, dicyclokuwanon EB, solophenols B and so on, together with few heterodimers between sesquiterpenoid and flavonoid such as solomonin, japonicasins A, japonicasins B as well as 6-farnesyl-3′, 4′, 5, 7-tetrahydroxyflavanone were isolated from natural resources, but the heterodimer between diterpenoid and flavonoid was not reported [19–22].

*Nicotiana tabacum* (Solanaceae) as an important economic crop because its leaves are used as raw material in tobacco production was proverbially cultivated in world. In Chinese folk medicines, people used its aerial plant as insecticide, sedative, diaphoretic, anesthetic and emetic agents [23]. Previous phytochemical investigations on *Nicotiana* plants showed that more than 2500 compounds had been identified in the plants of *N. tabacum* [1,10,24]. The main constituents of nonvolatile components in its leaves were diterpenoids, sesquiterpenoids, flavonoids and alkaloids. Cembrane-type diterpenoids, flavonoids as well as alkaloids were its representative constituents [25–27]. Up to now, more than seventy analogues of cembrane-type diterpenoids isolated from *N. tabacum* have been obtained, but the heterodimer of cembranoid and flavonoid has not been reported. In order to increase pharmacodynamic value of *N. tabacum*, further chemical and pharmacological investigation had been carried. As a result of (1″*S*, 6″*S*)-8-[6″-((−)-(1″*S*, 2″*E*, 4″*Z*, 7″*Z*, 11″*E*)-cembra-2″, 4″, 7″, 11″-tetraenyl)]-rutin, named nicotabaflavonoidglycoside (**1**) which is the first example cembranoid and flavonoid heterodimer was isolated. In this paper, isolation, structural elucidation, mass spectrometry (MS) fragmentation regularities and a possible biogenetic pathway of compound **1** were described.

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## 2. Experimental

### 2.1. General apparatus and chemicals

Optical rotations were obtained on a Jasco model 1020 polarimeter (Horiba, Tokyo, Japan). HRESIMS data were recorded on a LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan). UV spectra were conducted on a UV-2401A spectrophotometer (Shimadzu, Kyoto, Japan). Electronic circular dichroism (ECD) spectra were performed on an Applied Photophysics Chirascan instrument (Agilent, America). IR spectra were collected on a Bio-Rad FTS-135 spectrometer (Bio-Rad, Hercules, CA). 1D and 2D NMR spectra were acquired using Advance III-600 NMR spectrometers (Bruker, Bremerhaven, Germany) with TMS as internal standard. Semi-preparative HPLC was performed on a HPLC-AS20005 (Jiangshu Hanbang science and technology Ltd., Nanjing, China) using a reversed-phase (RP) $C_{18}$ column (10.0 × 250 mm, 5 μm, YMC). Silica gel (200–300 mesh, Qingdao Makall group Co., Ltd.; Qingdao, China), $C_{18}$ (Merck, Darmstadt, Germany) and Sephadex LH-20 (Amersham Bioscience, Sweden) were used for column chromatography.

### 2.2. Plant material

The leaves of *Nicotiana tabacum* Linn. were collected from Leye County of Guangxi Zhuang Autonomous Region of China, on June 15, 2016 and identified by lecturer Yuan-He Huang, Youjiang medical university for nationalities. A voucher specimen (No.20150615) was stored in the Laboratory of Cai-Yan Yang research group, the pharmaceutical school of Youjiang medical university for nationalities.

### 2.3. Extraction and isolation

The fresh leaves (26.5 kg) were extracted with 90% EtOH (100 l, 72 h) at room temperature 3 times and the combined solvent was evaporated *in vacuo*. The concentrate was partitioned between $H_2O$ and EtOAc. The residue (540 g) afforded by EtOAc layer was chromatographed on silica gel column (3.0 kg, 20.0 × 100 cm) using $CHCl_3$-MeOH-$H_2O$ (100:0:0–80:20:2, v/v/v) as eluent to afford Frs.1–8. Fr.7 (42.0 g) was fractionated by MCI CHP 20P gel column chromatography (CC) (310 g, 4.0 × 40 cm) using MeOH-$H_2O$ (from 20:80 to 100:0) as mobile phase to supply Frs.7.1–7.5. Fr.7.2 (14.0 g) was separated on a silica gel CC (300 g, 6.0 × 40 cm) eluted with EtOAc-MeOH-$H_2O$ (80:20:2), further was purified with Sephadex LH20 (50 g, 1.4 × 150 cm) and HPLC on reversed-phase $C_{18}$ column with MeOH (contain 20% THF)-$H_2O$ (80:20) as eluent to obtain compound **1** (3 mg).

Nicotabaflavonoidglycoside (**1**): yellow powder, $[\alpha]_D^{24}$: +24.5 (c 0.20, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 249 (4.40), 375 (3.90) nm; ECD ($CH_3OH$) $\Delta\varepsilon$ 196 + 11.14, $\Delta\varepsilon$ 208 − 5.00, $\Delta\varepsilon$ 214 − 2.84, $\Delta\varepsilon$ 226 − 9.28, $\Delta\varepsilon$ 250 + 7.97, $\Delta\varepsilon$ 257 + 7.18, $\Delta\varepsilon$ 264 + 8.41, $\Delta\varepsilon$ 266 + 8.47, $\Delta\varepsilon$ 306 − 1.24; IR (KBr) $\nu_{max}$ 3442, 1648, 1608, 1511, 1442, 1384, 1357, 1303, 1272, 1205, 1160, 1066 $cm^{-1}$; (−) HRESIMS *m/z* 879.3765 $[M-H]^-$ ($C_{47}H_{59}O_{16}$ calcd 879.3809), 903.3719 $[M+Na]^+$ ($C_{47}H_{60}O_{16}Na$ calcd 903.3774), 881.3896 $[M+H]^+$ ($C_{47}H_{61}O_{16}$ calcd 881.3954).

### 2.4. Acid hydrolysis and sugar identification

Compound **1** (2 mg) was hydrolyzed overnight by gently refluxing in 1.0 N HCl (MeOH-$H_2O$, 1:1, 5 ml). The reaction mixture was neutralized by $NaHCO_3$ and partitioned between $CHCl_3$ and $H_2O$. The $H_2O$ part was isolated on a silica gel CC (8 g, 1.0 × 40 cm) eluted with $CHCl_3$-MeOH-$H_2O$ (70:30:3) to produce glucose (0.1 mg) and rhamnose (0.1 mg). The relative configuration of glucose was determined to be D, and rhamnose to be L based on their $[\alpha]_D^{21}$ values [+50.0 (c 0.10, MeOH) of glucose and +4.3 (c 0.10, MeOH) of rhamnose] [28]. This was reinforced by PC experiment that their $R_f$ values were similar to those of authentic samples (BuOH-EtOAc-$H_2O$ 4:1:5, upper layer, $R_f$ values 0.43 of glucose and 0.65 of rhamnose; EtOAc-Py-$H_2O$ 10:4:0.5, $R_f$ values 0.52 of glucose and 0.64 of rhamnose).

## 3. Results and discussion

Nicotabaflavonoidglycoside, **1** had a molecular formula of $C_{47}H_{60}O_{16}$ with 18 degrees of unsaturation by its (−) HRESIMS which exhibited an $[M-H]^-$ at *m/z* 879.3765. Acid hydrolysis of **1** revealed that it contained one D-glucose and one L-rhamnose moieties. A characteristic flavonoid fragment was deduced from its UV (MeOH) spectrum [$\lambda_{max}$ (log $\varepsilon$) 249 (4.40), 375 (3.90) nm]. Its IR spectrum gave hydroxyl (3442 $cm^{-1}$), aromatic ring (1608 $cm^{-1}$), double bond (1648 $cm^{-1}$), ether bond (1272, 1205, 1160 and 1066 $cm^{-1}$) groups. Beside one glucosyl group and one rhamnosyl group, its $^1H$ NMR spectrum showed the presence of one pentasubstituted ($\delta_H$ 6.38, s) and trisubstituted [8.05, 1H, s; 7.06 and 7.78 (each 1H, d, $J$ = 8.2 Hz)] benzene rings, five olefinic protons (6.83, 1H, d, $J$ = 15.2 Hz; 6.08, 2H, d, $J$ = 10.2 Hz; 5.31, 1H, dd, $J$ = 15.2, 10.0 Hz and 5.06, 1H, d, $J$ = 10.0 Hz), five methyls [1.80, 1.66 and 1.62 (each 3H, s); 0.86 and 0.76 (each 3H, d, $J$ = 6.3 Hz)] while its $^{13}C$ NMR spectrum displayed 35 carbons including 5 methyls, 4 methenes, 12 methines and 14 quaternary carbons. Comparison of its NMR data (Table 1) with those of rutin indicated they possessed a similar skeleton except for an additional diterpene moiety (**DM**), H-8 which was $\delta_H$ 5.92 (s) in rutin but this disappeared in **1** along with C-8 which was changed from $\delta_C$ 94.9 (d) in rutin to 110.3 (s) in **1** [29]. Above analysis indicated compound **1** as a derivative of rutin with a **DM**, and **DM** was linked to C-8 of rutin. The NMR data due to the **DM** were similar to those of (−)-(1*R*, 2*E*, 4*Z*, 7*E*, 11*E*)-cembra-2, 4, 7, 11-tetrene (**CT**) except that H-6″ was turned from $\delta_H$ 3.04 (m) and 2.40 (m) in **CT** to 5.79 (t, $J$ = 10.2 Hz) in **DM** and C-6″ was transformed from $\delta_C$ 26.2 (t) in **CT** to 33.0 (d) in **DM** suggesting that **DM** was cembrene moiety and C-6″ of cembrene moiety was attached to C-8 of rutin [30,31]. Foregoing judgment was reinforced by HMBC cross peaks (Fig. 1) between H-6″ and C-7/C-8/C-9, H-6 and C-5/C-7/C-8/C-10 along with 5-OH ($\delta_H$ 12.44, s) and C-5/C-6/C-10.

Notably, the ROESY correlations (Fig. 1) of H-1″ (1.88)/H-3″ (6.83)/H-6″ (5.79) together with H-16″ (0.76)/H-2″ (5.31)/H-18″ (1.80)/H-5″ (6.08) were observed which hinted that H-1″, H-3″, H-6″ situated to the same face in the pseudoplanar of cembrene ring system, while H-16″, H-2″, H-18″ and H-5″ were directed to the opposite face (Fig. 2). This analysis indicated its stereochemistry of C-1″ and C-6″ both were *R* or *S* (Fig. 1). Then, its absolute configuration of 1″*S* and 6″*S* was speculated by its biogenesis (Scheme 1) that all cembranoids from *Nicotiana* genus except for cembra-3, 7, 11, 15-tetraene-6-ol had 1*S* configurations. Finally, to determine the absolute configuration of **1**, the comparison between the experimental and calculated ECD spectra of **1** was performed using the time-dependent density functional theory (TDDFT) method at the B3LYP/6–311 + + G (2d, p) level; Thus, the absolute configuration of 1″*S*, 6″*S* were assigned by that the calculated ECD spectrum for the 1″*S*, 6″*S* stereoisomer was consistent with the experimental ECD spectrum (Fig. 3) [32,33]. Thus the structure of nicotabaflavonoidglycoside was elucidated as (1″*S*, 6″*S*)-8-[6″-((−)-(1″*S*, 2″*E*, 4″*Z*, 7″*E*, 11″*E*)-cembra-2″, 4″, 7″, 11″-tetraenyl)]-rutin.

Studying the characterization of the fragmentation patterns from compound **1**, and comparing its fragmentation patterns with those of flavonoids will provide other valuable information for identification of compound **1**. The extensive MS fragmentation study on flavonoids and their derivatives had been conducted [34]. The tandem MS analyses of compound **1**, the negativeion mode of electrospray ionization (ESI) were selected because it easily provided extensive information. The deprotonated molecular ions [M-H] were readily observed for compound **1**. The accurate masses and corresponding assigned elemental compositions of the product ions were summarized (see Table S1 in

**Table 1**
$^{1}H$ (600 MHz) and $^{13}C$ (150 MHz) NMR Data of compound **1** in acetone-$d_6$ ($\delta$ in ppm, $J$ in Hz).

| No. | $\delta_H$ | $\delta_C$ | No. | $\delta_H$ | $\delta_C$ | No. | $\delta_H$ | $\delta_C$ |
|---|---|---|---|---|---|---|---|---|
| Rutin moiety | | | | | | | | |
| 2 | | 158.8, s | 1′ | | 123.0, s | 4‴ | 3.35, m | 74.0, d |
| 3 | | 135.8, s | 2′ | 8.05, s | 118.7, d | 5‴ | 3.37, m | 76.9, d |
| 4 | | 179.5, s | 3′ | | 145.7, s | 6‴ | 3.48, m<br>3.72, dd, 17.9, 6.8 | 67.8, t |
| 5 | | 160.8, s | 4′ | | 149.4, s | 1‴′ | 4.60, brs | 102.0, d |
| 6 | 6.38, s | 100.2, d | 5′ | 7.06, d, 8.2 | 116.2, d | 2‴′ | 3.66, d, 5.5 | 71.8, d |
| 7 | | 162.8, s | 6′ | 7.78, d, 8.2 | 122.5, d | 3‴′ | 3.46, m | 70.4, d |
| 8 | | 110.3, s | 1‴ | 5.16, d, 7.5 | 105.7, d | 4‴′ | 3.66, m | 72.1, d |
| 9 | | 154.5, s | 2‴ | 3.47, m | 75.5, d | 5‴′ | 3.47, m | 68.9, d |
| 10 | | 105.7, s | 3‴ | 3.47, m | 78.4, d | 6‴′ | 1.12, d, 5.5 | 18.1, q |
| 5-OH 12.44, s | | | | | | | | |
| Diterpene moiety (**DM**) | | | | | | | | |
| 1″ | 1.88, m | 49.6, d | 8″ | | 133.0, s | 15″ | 1.47, m | 33.4, d |
| 2″ | 5.31, dd, 15.2, 10.0 | 132.7, d | 9″ | a 2.94, t, 11.8<br>b 1.86, m | 31.4, t | 16″ | 0.76, d, 6.3 | 20.8, q |
| 3″ | 6.83, d, 15.2 | 131.0, d | 10″ | a 2.40, m<br>b 2.04, m | 24.1, t | 17″ | 0.86, d, 6.3 | 20.9, q |
| 4″ | | 133.0, s | 11″ | 5.06, d, 10.0 | 125.6, d | 18″ | 1.80, s | 21.2, q |
| 5″ | 6.08, d, 10.2 | 128.6, d | 12″ | | 131.7, s | 19″ | 1.62, s | 22.5, q |
| 6″ | 5.79, t, 10.2 | 33.0, d | 13″ | 2.02, m | 37.6, t | 20″ | 1.66, s | 15.0, q |
| 7″ | 6.08, d, 10.2 | 129.1, d | 14″ | a 1.86, m<br>b 1.19, m | 28.6, t | | | |

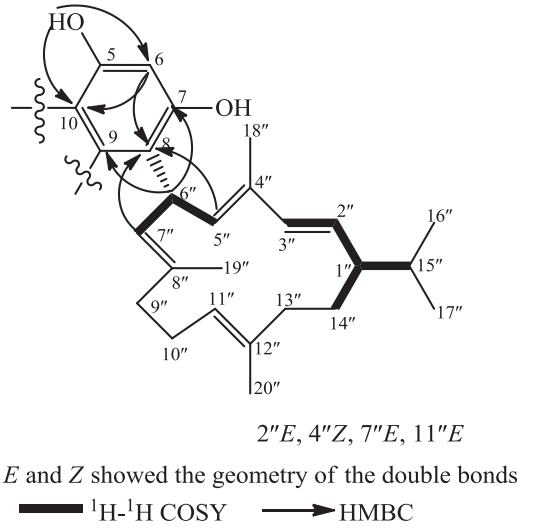



**Fig. 1.** The key COSY and HMBC correlations of **1**.

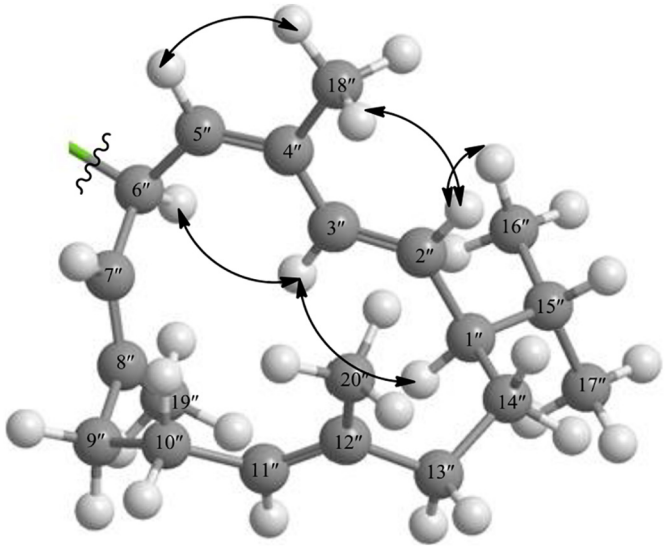


**Fig. 2.** The key ROESY correlations of **1**.

Supplementary data). The fragmentations in the subsequent multistage tandem $MS^n$ (see Table S1 in Supplementary data) and the $MS^n$ fragment pathways (see Scheme S1 in Supplementary data) analyses also indicated that compound **1** was a heterodimer of cembrene and rutin.

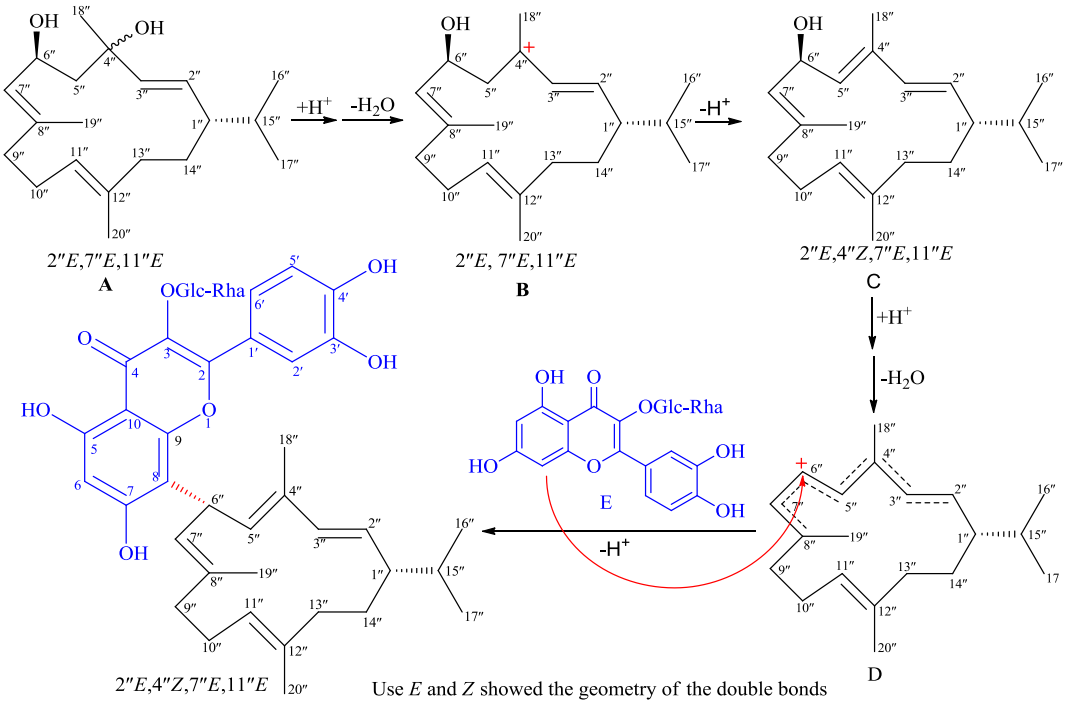
Nicotabaflavonoidglycoside, **1** was speculated a heterodimer of cembrene and rutin. The cembra-2,4,7,11-tetraen-6-ol, (1*S*, 2*E*, 4*S*, 6*R*, 7*E*, 11*E*)-2, 7, 11-cembratriene-4, 6-diol and its 4*R* isomer which were representative constituents of *N. tabacum*, were the intermediates and key metabolites in biosynthetic pathways of the most tobacco cembranoids [35,36]. Thus the plausible biogenesis of **1** from (1*S*, 2*E*, 4*S*, 6*R*, 7*E*, 11*E*)-2, 7, 11-cembratriene-4, 6-diol or its 4*R* isomer was postulated to explain its origin (Scheme 1). First, two reactions of elimination were involved in this pathway to provide cembra-2, 4, 7, 11-tetraene-6-ol or its 4*Z* isomer (C) and intermediates (B and D). Secondly, because the steric hindrance of cation of C-6″ was the minimum among cations of C-2″, C-4″, C-6″ and C-8″ as well as all directing effects of the aromatic electrophilic substitution reaction for A ring of rutin were consistent, the C-6″ of intermediate D attacked C-8 of rutin to convert compound **1**.

Compound **1** was tested for its anti-proliferative activities employing cervical cancer Hela and liver cancer HepG2 cell lines [37]. When its concentration was 20 μg/ml, the inhibition rate on Hela and HepG2 cell lines were 16.45% and 21.77%, respectively.

In summary, nicotabaflavonoidglycoside (**1**) is a structurally unique heterodimer of diterpenoid and flavonoid from *N. tabacum*. Notably, this dimeric feature of macrocyclic terpene and flavonoid is unprecedented among all heterodimers reported so far. The unique structure of this compound would make it an interesting target for realizing its partial synthesis or total synthesis. Moreover, more bioactivities of **1** possible possess such as neuroprotection, anti-cardiovasular diseases, anti-pathogen and so on was not been evaluated because its quantity was very small. Thus the partial synthesis or total synthesis will finally reveal the stereochemical puzzles encountered and bioactivities not been assayed in this report.

## Conflict of interest

The authors declare no conflicts of interest among all authors in this manuscript.




**Scheme 1.** Proposed biogenetic pathway of **1**.

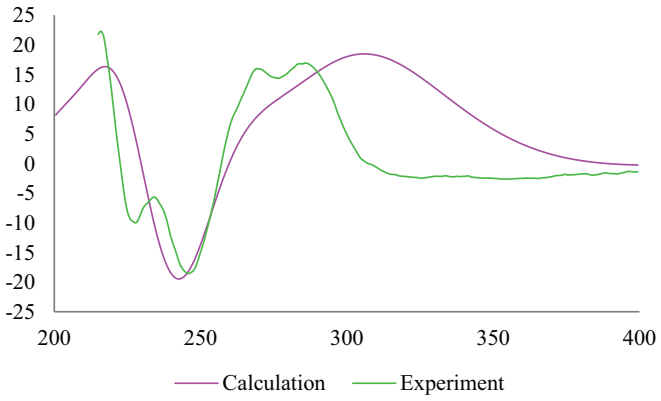



**Fig. 3.** Calculated and experimental ECD spectra of **1**.

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## Appendix A. Supplementary data

Supplementary data related to this article including the method of anti-proliferative activities assay, accurate masses and elemental compositions from negative ESI-IT-TOF $MS^n$ experiments, $MS_4$:879-571-421-335 and $MS^n$ fragment pathways, (+) HR-ESI-MS, (−) HR-ESI-MS, UV, IR, $^1H$ NMR, $^{13}C$ NMR, HSQC, $^1H$-$^1H$ COSY, HMBC, ROESY, ORD and ECD spectrum for compound **1** can be found online at https://doi.org/10.1016/j.fitote.2018.05.028.

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