Cembranoids From the Leaves of Nicotiana tabacum and Their Neuroprotective Activities

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Abstract
A new cembrane-type diterpenoid (1) and 9 known cembranoids (2-10) were isolated from the leaves of Nicotiana tabacum. The structure of the new metabolite was confirmed by extensive analyses of their spectroscopic data, including high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), 1D- and 2D nuclear magnetic resonance (NMR) spectroscopy, and infrared spectroscopy (IR). All of the isolated compounds were tested for their protective effects against oxygen-glucose deprivation (OGD)-induced neurotoxicity in SH-SY5Y cells. The new compound 1 exhibited a moderate protective effect against OGD-induced neurotoxicity at 10 μM.

Keywords
cembrane-type diterpenoid, Nicotiana tabacum, structural identification, oxygen-glucose deprivation, neuroprotective activities

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Introduction
Cembrane-type diterpenoids (cembranoid), comprising 4 isoprene units, are a kind of 14-member macrocyclic diterpene that have been isolated and identified mainly from marine organisms (eg soft corals and gorgonians) and terrestrial plants (eg tobacco, Pinus, myrrh, and frankincense).1,5 As reported previously, 105 cembranoids have been obtained from Nicotiana tabacum, which possess a wide range of biological functions,1 including antitumor, anti-inflammatory, antibacterial, anti-metastatic, anti-pathogenic, neuroprotective, anti-fouling, and anti-osteoporotic properties. However, studies of bioactivities of those metabolites to date have mainly focused on α-CBT-diol and β-CBT-diol.6 In our current study, we report the isolation, structure identification, and neuroprotective effects of a new cembranoid (1), along with 9 known ones (2-10), from the leaves of N. tabacum. The structure of compound 1 was evaluated as (13,25,3E,6R,7E,11E)-3,7,11-cembratriene-2,6-diol by spectroscopic methods (Figure 1), including high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), infrared spectroscopy (IR), 1D- and 2D nuclear magnetic resonance (NMR). All of the compounds were tested for their neuroprotective activities against oxygen-glucose deprivation (OGD)-induced cell injury in SH-SY5Y cells. Among these compounds, 1, 2, 5, and 7 at 10 μM attenuated SH-SY5Y cell damage induced by OGD. Compound 1 exhibited neuroprotective effects with a cell viability of 69.6% compared with that of the positive control L-3-n-butylphthalide (L-NBP), with a cell viability of 72.3%.

Results and Discussion
Compound 1, isolated as a colorless oil, has a molecular formula of C_{20}H_{34}O_{2}, which was determined by HR-ESI-MS (m/z 329.2458 [M + Na]⁺, calculated for C_{20}H_{34}O_{2}Na, 329.2457), corresponding to 4 indices of hydrogen deficiency. The IR spectrum (Supplemental Material) displayed characteristic absorptions of hydroxy groups (3314 cm⁻1). The 1H-NMR data (Table 1) of 1 showed 3 olefinic protons at δH 5.38 (1H, d, J = 8.4 Hz, H-3), 5.07 (1H, d, J = 9.0 Hz, H-7), and 4.95 (1H, m, and H-11) and 2 oxyxynem protons at δH 4.56 (2H, m, H-2, and H-6). In the upfield region, 2 doublet methyl protons at δH 0.94 (3H, d, J = 7.2 Hz, H-16) and 0.94 (3H, d, J = 7.2 Hz, H-10) and 3 singlet methyl protons at δH 1.62

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(3H, s, H-20), 1.61 (3H, s, H-19), and 1.60 (3H, s, H-18) were also observed. In the $^{13}$C-NMR spectrum, 20 carbon resonances were observed and classified by HSQC experiments as 5 methyl carbons [$\delta_{C} 21.9$ (C-16), 19.0 (C-17), 16.4 (C-18), 16.9 (C-19), 17.9 (C-20)], 5 methylene carbons [$\delta_{C} 48.9$ (C-5), 38.7 (C-9), 23.9 (C-10), 35.2 (C-13), 22.2 (C-14)], 2 methine carbons [$\delta_{C} 46.2$ (C-1), 29.5 (C-15)], and 2 oxygenated methine carbons [$\delta_{C} 68.4$ (C-2), 66.8 (C-6)]. The resonances at $\delta_{C} 131.3$ (C-3), 127.2 (C-7), and 123.4 (C-11) were attributed to 3 olefinic methine carbons, and 3 signals at $\delta_{C}$ 131.5 (C-4), 138.8 (C-8), and 134.0 (C-12) were assigned to 3 olefinic quaternary carbons. In combination with the aforementioned NMR data, 1 was determined as a cembranoid containing 3,7,11-triene-2,6-diol. The assignments for the cembrane-type diterpenoid skeleton were confirmed by heteronuclear multiple bond correlation (HMBC) and $^1$H−$^1$H correlation spectroscopy (COSY) experiments (Figure 2a). The $^1$H−$^1$H COSY correlations of H-3/H-2/H-1/H-14/H-13, H-1/H-15, and H-16/H-15/H-17 confirmed the presence of structural fragment A (C-3 − C-2 − C-1 − (C-15) − C-14 − C-13) and B (C-16 − C-15 − C-17). The $^1$H−$^1$H COSY cross-peaks of H-5/H-6/H-7 implied the fragment C (C-5 − C-6 − C-7), and the existence of fragment D (C-9 − C-10 − C-11) was confirmed by the spin-coupling sequence of H-9/H-10/H-11. Furthermore, in the HMBC spectrum, correlations from H-3 to C-5 and H-5 to C-4 demonstrated that fragments A and C were linked via C-4, while cross-peaks from H-7 to C-9, and H-10 to C-8 suggested the linkage of fragments C and D via C-8. Thus, the planar structure of compound 1 was established as 3,7,11-cembratriene-2,6-diol. The rotating frame overhauser effect spectroscopy (ROESY) correlations (Figure 2) from H-3 to H-5, H-7 to H-9, and H-11 to H-13, along with the chemical shift values at $\delta_{C}$ 16.4 (C-18), 16.9 (C-19), and 17.9 (C-20), determined the $E$ configuration for the 3 trisubstituted olefin. The ROESY cross-peak (Figure 2b) between H-2 and H-17 revealed that H-2 and the isopropyl group at C-1 were cofacial. As all cembranoids from the Nicotiana genus have a (1S,6R) configuration, the absolute configuration of compound 1 was tentatively determined as (1S,2S,3E,6R,7E,11E)-3,7,11-cembratriene-2,6-diol from its biogenesis.

![Figure 1. Cembranoids 1 to 10 isolated from the leaves of Nicotiana tabacum.](image)

![Table 1. $^1$H and $^{13}$C-NMR Spectroscopic Data of Compound 1 in CDCl$_3$-$d_2$ ($\delta$, ppm; $J$, Hz).](table)

| Position | $\delta_{H}$ ($J$, Hz) | $\delta_{C}$ | Position | $\delta_{H}$ ($J$, Hz) | $\delta_{C}$ |
|----------|------------------------|-------------|----------|------------------------|-------------|
| 1        | 1.25 (1H, m)           | 46.2        | 11       | 4.95 (1H, m Hz)        | 123.4       |
| 2        | 4.56 (1H, m)           | 68.4        | 12       | 1.92 (1H, m); 2.09 (1H, m) | 35.2       |
| 3        | 5.38 (1H, d, $J$ = 8.4 Hz) | 131.3   | 13       | 1.52 (1H, m); 1.67 (1H, m) | 22.2       |
| 4        | 2.49 (1H, dd, $J$ = 12.0, 3.0 Hz) | 2.14 (1H, m) | 14       | 1.78 (1H, m)        | 29.5       |
| 5        | 4.56 (1H, m)           | 66.8        | 15       | 0.94 (1H, d, $J$ = 7.2 Hz) | 21.9       |
| 6        | 5.07 (1H, d, $J$ = 9.0 Hz) | 127.2   | 16       | 0.97 (1H, d, $J$ = 7.2 Hz) | 19.0       |
| 7        | 138.8                  | 17          | 18       | 1.60 (3H, s)         | 16.4       |
| 9        | 2.11 (2H, m)           | 38.7        | 19       | 1.61 (3H, s)         | 16.9       |
| 10       | 2.27(1H, m); 2.11 (1H, m) | 23.9   | 20       | 1.62 (3H, s)         | 17.9       |

Abbreviation: NMR, nuclear magnetic resonance.
By comparing their observed and reported spectroscopic data, 9 known compounds were determined as
(1S,2E,4S,6R,7E,11E)-2,7,11-cembratriene-4,6-diol (2, α-CBT-diol), (1S,2E,4R, 6E,8S,11E)-2,6,11-cembratriene-4,8-diol (3), (1S,2E,4S,6E, 8S,11E)-2,6,11-cembratriene-4,8-diol (4), 4-O-methyl-(1S,2E,4R,6E,11E)-2,7,11-cembratriene-4,6-diol (5), (1S,2E,4S,6E,8R,11E)-2,6,11-cembratriene-4,8-diol (6), (1S,2E,4S,6E,8R,11E)-2,7,11-cembratriene-4,6-diol (7, β-CBT-diol), (1S,2E,4R,6E, 8R,11S,12E)-8,11-epoxy-2,6,12-cembratriene-4-ol (8), (1S,2E,4S, 6R,7E,10S, 11S)-10, 11-epoxy-2,7,12(20)-cembratriene-4,6-diol (9), and (1S,2E,4S,6R,7E,11R)-10,11-epoxy-2,7,12(20)-cembratriene-4,6-diol (10).

To the best of our knowledge, studies of the neuroprotective activities of cembranoids from tobacco to date have mainly focused on α-CBT-diol and β-CBT-diol. Thus, all of our isolates were evaluated for their neuroprotective effects against OGD-induced cell injury in SH-SY5Y cells. Compounds 1, 2, 5, and 7 at 10 μM attenuated SH-SY5Y cell damage (Figure 3). Among them, compound 1 exhibited neuroprotective effects with a cell viability of 69.6% compared with that of the positive control L- NBP (72.3%).

**Experimental**

**General Experimental Procedures**

IR spectra were measured on a Thermo Scientific Nicolet 5700 spectrometer, NMR spectra using a Bruker ascend 600 MHz spectrometer, and HR-ESI-MS on an AB SCIEX Triple TOF 6600 mass spectrometer. Preparative high-performance liquid chromatography (HPLC) was carried out on a Waters Prep 150 LC system using an YMC-Pack ODS column (250×10 mm, 5 μM). Column chromatography was performed on silica gel (200-300 mesh, Qingdao Marine Chemical Co., Ltd) and Sephadex LH-20 (Pharmacia Fine Chemicals) columns. Neuroprotective activity was performed in a super clean workbench (Suzhou Antai Airtech Co., Ltd). Cells were cultured in a Thermo Scientific Steri-Cycle CO₂ incubator. Cell counting was performed under an inverted microscope (Sunny Optical Technology Co., Ltd). The absorbance was detected with a microplate reader (Bio-Rad Laboratories, Inc) at 490 nm wavelength. All of the other chemicals and solvents were of analytical grade.

**Plant Material**

The leaves of *N tabacum* L. were collected from Sanmenxia city (Henan province, China) in August 2018 and authenticated by associate professor, Shen Huang, from Zhengzhou University of Light Industry. A voucher specimen (20180024) was stored in the laboratory of the Duobin Mao research group.

**Extraction and Isolation**

The fresh green leaves of *N tabacum* (960 kg) were rinsed 4 times with CH₂Cl₂. After the CH₂Cl₂ had been evaporated under reduced pressure, dark yellow residues (516 g) were obtained. The obtained extracts were subjected to chromatography on a silica gel column (2000×50 cm i.d.) and eluted successively with light petroleum/ethyl acetate (1000, 50:1, 30:1, 10:1, 5:1, 3:1, 1:1, and 0:1, v/v) as the mobile phase, and finally washed with MeOH to yield 9 fractions (fractions A-I). Fraction D (34 g) was separated on a Sephadex LH-20 column (120×10 cm i.d.) and eluted with a MeOH/CH₂Cl₂

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**Figure 2.** (a) ¹H−¹H COSY and key HMBC correlations of compound 1. (b) Selected ROESY correlations of compound 1.
Neuroprotective Activity Assays

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37 °C under a water-saturated atmosphere of 5% CO2 and 95% air.

Cells, in the log growth phase, were seeded in 96-well culture plates (1 × 10^5 cells/well in 100 μL) until 80% to 90% confluent for the next experiments. After pretreatment with compounds at 10^{-6} M for 2 h, SH-SY5Y cells were exposed to glucose-free DMEM in a hypoxia incubation chamber with 95% N2% and 5% CO2 for 1 h and then cultured for another 24 h under normal conditions. These cells were included in the OGD/R group compared with SH-SY5Y cells cultured with complete medium under normal conditions as the control group. Cell viability was evaluated by incubating with 1 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37 °C in an incubator. Media were then replaced with 100 μL DMSO and absorbance was read at 490 nm. Data were analyzed by one-way analysis of variance (ANOVA) and expressed as means ± SD with P<.05 regarded as significant.

Conclusion

A new cembranoid, (1S,2S,3E,6R,7E,11E)-3,7,11-cembra-triene-2,6-diol (1), was isolated from the leaves of N tabacum, along with 9 known ones (2-10). To the best of our knowledge, this is the second tobacco cembranoid that has been isolated with the E-configuration of a 3,4-double bond in its structure. Compound 1 exhibited neuroprotective effects against OGD-induced toxicity, with a cell viability of 69.6% compared with that of the positive control L-NBP (72.3%).

Declaration of Conflicting Interests

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Not applicable, because this article does not contain any studies with human or animal subjects.

Informed Consent
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Trial Registration
Not applicable, because this article does not contain any clinical trials.

Supplemental Material
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