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Isolation, synthesis, and neurite outgrowth-promoting activity of illicinin A from the flowers of *Illicium anisatum*

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ABSTRACT

Two new prenylated C_6–C_3 compounds, illicinin A (**1**) and (4*S*)-illicinone I (**2**), were isolated from the flowers of *Illicium anisatum*. The structures of the new compounds were elucidated by spectroscopic methods. The absolute structure of (4*S*)-illicinone I was determined by comparing its CD spectrum and specific rotation with those of (4*S*)-illicinone A (**4**). Illicinin A (**1**) and 4-allyl-2,6-dimethoxy-3-(3-methylbut-2-enyl)phenol (**3**) were found to exhibit neurite outgrowth-promoting activity at concentrations ranging from 0.1 to 10 μM in primary cultured rat cortical neurons. Illicinin A and its derivatives were synthesized for structure–activity relationship studies by employing sequential Stille reactions to introduce a prenyl and an allyl group to the benzene ring, thereby indicating that an allylphenyl moiety in the molecule of **1** is essential for its neurotrophic properties.

1. Introduction

In the course of our search for neurotrophic factor-like small molecules in natural products, honokiol (**5**), a biaryl compound with two allyl and two hydroxyl groups, has been shown to significantly enhance neurite outgrowth promotion and neuronal survival in primary cultured rat cortical neurons.[1] A further study on the relationship between neurite outgrowth-promoting activity and the structures of honokiol and its analogues revealed that the 4′-hydroxyl group and 5-allyl group in the structure of **5** are responsible for honokiol-mediated neurite outgrowth-promoting activity.[2] However, there remains the question of whether the biaryl group in **5** is necessary for its activity. As part of our chemical studies on the *Illicium* species,[3] we first investigated the chemical components of the flowers of *Illicium anisatum*, resulting in the isolation of two new prenylated C_6–C_3 compounds **1** and **2** named illicinin A and (4*S*)-illicinone I. The isolation of the allylphenol compound illicinin A (**1**) may help us to answer the above question.

In this paper, we deal with the isolation and structures of illicinin A (**1**) and (4*S*)-illicinone I (**2**) from the flowers of *I. anisatum* as well as the synthesis and neurite outgrowth-promoting activity of **1** and its analogues for structure–activity relationship studies (Fig. 1).

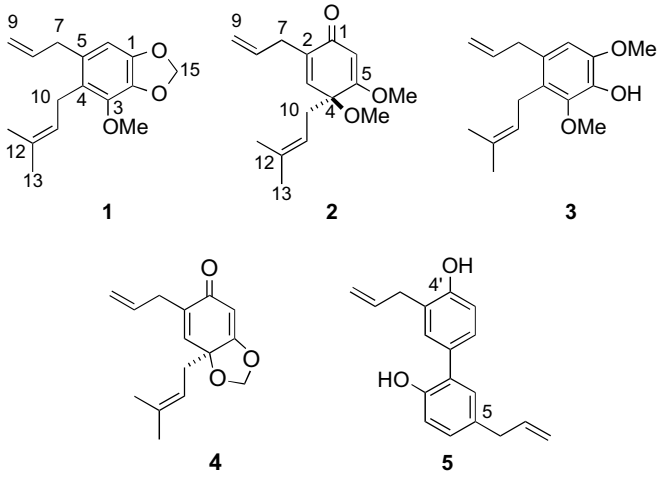

Figure 1. Prenylated C_6–C_3 compounds **1**–**4** isolated from the flowers of *I. anisatum* and honokiol (**5**).

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2. Results and discussion

2.1. Structure of new compounds 1 and 2

Compounds **1** and **2** were isolated from the methanol extract of the flowers of *I. anisatum* along with the previously known sesquiterpenes pseudoanisatin[4] and 6-deoxypseudoanisatin[5] and the prenylated C_6–C_3 compounds, (4*S*)-illicinone A (**4**),[6] (2*R*,4*S*)-illicinone B,[7] and 4-allyl-2,6-dimethoxy-3-(3-methyl-2-butenyl)phenol (**3**).[7]

Compound **1** has the molecular formula $C_{16}H_{20}O_3$ as established by high resolution (HR) EIMS (*m*/*z* 260.1446 [M^+]), and its IR spectrum revealed the presence of a benzene moiety at 1620 and 1478 cm^{-1}. The ^1H NMR spectrum of **1** contained signals due to an allyl group at δ_H 5.87 (1H, ddt, *J*=17.6, 12.8, 6.3 Hz), 4.98 (1H, dd, *J*=17.6, 1.9 Hz), 4.97 (1H, dd, *J*=12.8, 1.9 Hz), and 3.48 (2H, br d, *J*=6.3 Hz); a prenyl group at δ_H 5.29 (1H, t, *J*=6.6 Hz), 3.24 (2H, d, *J*=6.6 Hz), 1.71 (3H, s), and 1.65 (3H, s); a methylenedioxy group at δ_H 5.27 (2H, s); a methoxy group at δ_H 3.79 (3H, s); and an aromatic singlet signal at δ_H 6.49 (1H, s). These spectral data suggested that **1** consisted of a pentasubstituted benzene ring with allyl, prenyl, methylenedioxy, and methoxy groups. The disposition of the four substituents was elucidated by analysis of the NOESY and HMBC spectra (Fig. 2). The sole aromatic signal showed HMBC correlations with the oxygenated carbons (C-1 and C-2) resonating at δ_C 147.8 and 135.6. Both the C-1 and C-2 carbons had additional HMBC correlations with the methylenedioxy signal at δ_H 5.27, indicating that the methylenedioxy group was located at the C-1 and C-2 positions. The NOE cross-peak between the aromatic and the H-7 methylene signals provided compelling evidence for the allyl group being *ortho* to the sole aromatic proton. Sequential NOE correlations of H-7 to the H-10 methylene of the prenyl group and the H-10 to the methoxy signal indicated that the allyl, prenyl, and methoxy groups were placed in order. An additional HMBC, shown in Figure 2, confirmed the structure of **1** to be 1,2-methylenedioxy-3-methoxy-4-prenyl-5-allylbenzene.

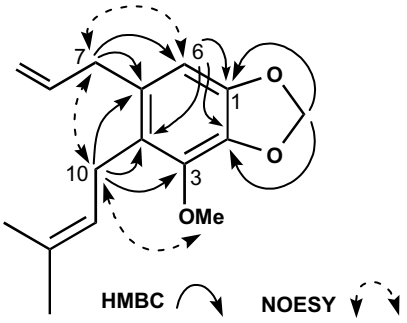


Figure 2. HMBC and NOESY correlations for **1**.

Compound **2** has the molecular formula, $C_{16}H_{22}O_3$, as established by HREIMS of its molecular ion peak at *m*/*z* 262. The IR absorption band at 1667 cm^{-1} revealed the presence of a conjugated carbonyl group. The ^1H NMR and ^{13}C NMR data of **2** were similar to those of (4*S*)-illicinone A (**4**).[5] However, the appearance of two singlet signals at δ_H 3.77 and 3.11 (3H for each) and the absence of the singlet signal at δ_H 5.87 (2H) present in **4** supposed that **2** was a structure with two methoxy groups at the C-4 and C-5 positions instead of the methylenedioxy group present in **4**. The respective HMBC correlation of the methoxy group at δ_H 3.11 and 3.77 with C-4 (δ_C 78.0) and C-5 (δ_C 172.8) supported the presumed structure for **2**. The CD spectrum of **2** showed the same negative cotton effect at 280 nm as that of **4**. The C-4 chiral center should have the same *S* configuration as **4**, and thus **2** was named (4*S*)-illicinone I.

2.2. Syntheses of illicinin A (1) and its analogues

According to our biological studies using primary cultured rat cortical neurons,[1] illicinin A (**1**) and compound **3** significantly promoted neurite outgrowth at concentrations ranging from 0.1 μM to 10 μM (Figs. 4 and 5). In order to further study their structure–activity relationship, illicinin A and its analogues (**1a**–**1d**) were synthesized (Fig. 3).

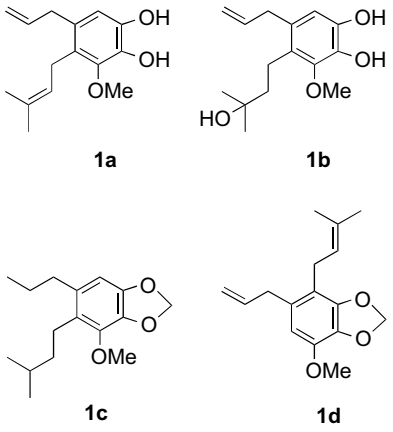
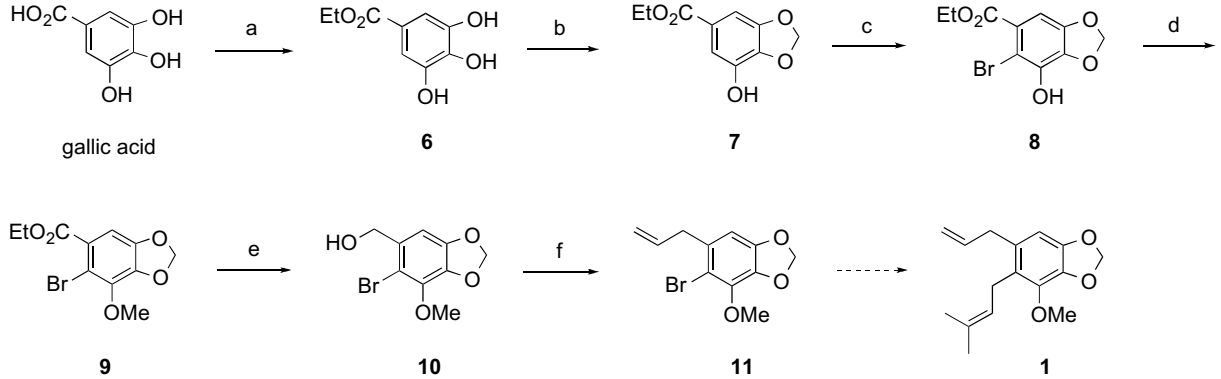


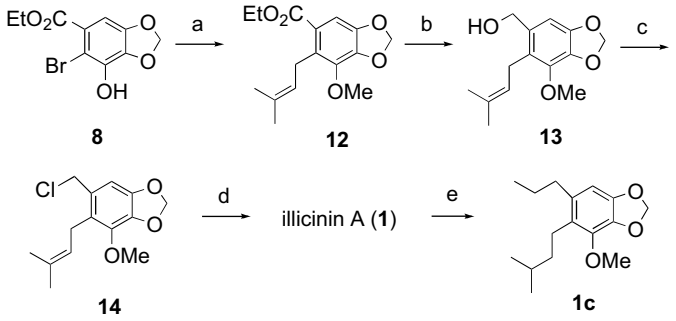
Figure 3. Synthesized derivatives **1a**–**1d**.

First, we explored the synthesis of illicinin A (**1**) based on a palladium-catalyzed Stille reaction that sequentially introduced an allyl and a prenyl group to the substituted benzene ring. The synthetic route is shown in Scheme 1.



Scheme 1. Reactions and conditions: (a) EtOH, H_2SO_4, reflux, 93%; (b) CH_2I_2, Cs_2CO_3, DMF, 120 °C, 91%; (c) Br_2, *t*-$BuNH_2$, tol, rt, 80%; (d) MeI, K_2CO_3, acetone, 100%; (e) DIBAL·THF, −78 °C, 100%; (f) $SOCl_2$, benzene, 70 °C, then CH_2=$CHSnBu_3$, 10 mol % $Pd(Ph_3P)_4$, DMF, 70 °C, 85%.

The synthesis commenced with the esterification of commercially available gallic acid into the ethyl ester **6**, the diol of which was protected as dioxymethane (**7**).[7] The *ortho*-position to the residual hydroxyl group was selectively brominated in the presence of *tert*-butylamine[8] to give the bromide **8** in 80% yield. After methylation of the hydroxyl group in **8**, the ester **9** was reduced with DIBAL to give the alcohol **10**, which was subjected to the first palladium-catalyzed Stille reaction.[9] Treatment of **10** with thionyl chloride provided an unstable chloromethyl group, which was not isolated and reacted with tributylvinyltin in the presence of 10 mol % $Pd(Ph_3P)_4$, giving rise to the desired ally compound **11** in 85% yield. However, no attempt to introduce the prenyl group using the Stille reaction produced illicinin A (**1**). The poor reactivity of **11** toward Pd(0) was presumably due to the presence of an electron-rich benzene. Hence, the order of the two kinds of Stille reactions was reversed; i.e., employing the first Stille reaction using tributylprenyltin to the ester group-bearing compound **8**. As expected, upon treatment of **8** with tributylprenyltin in the presence of $Pd(Ph_3P)_4$ the reaction proceeded smoothly to afford the prenyl-substituted derivative (Scheme 2). The ethyl ester group in **12** was reduced with $LiAlH_4$ to produce the alcohol intermediate **13** in 63% yield over two steps. Compound **13** was chlorinated with $SOCl_2$, and the resulting crude product **14** was reacted with tributylvinyltin in the presence of $Pd(Ph_3P)_4$ to give rise to illicinin A (**1**) in two steps and 78% overall yield. All spectroscopic data were identical with those of natural **1**, and thereby the structure of illicinin A was established.



Scheme 2. Reactions and conditions: (a) $Me_2C{=}CHCH_2SnBu_3$, 10 mol % $Pd(Ph_3P)_4$, DMF; (b) $LiAlH_4$, THF, rt, 63% (two steps); (c) $SOCl_2$, benzene, 70 °C; (d) $CH_2{=}CHSnBu_3$, 10 mol % $Pd(Ph_3P)_4$, DMF, 70 °C, 78% (two steps); (e) H_2, PtO_2, CH_2Cl_2, 50%.

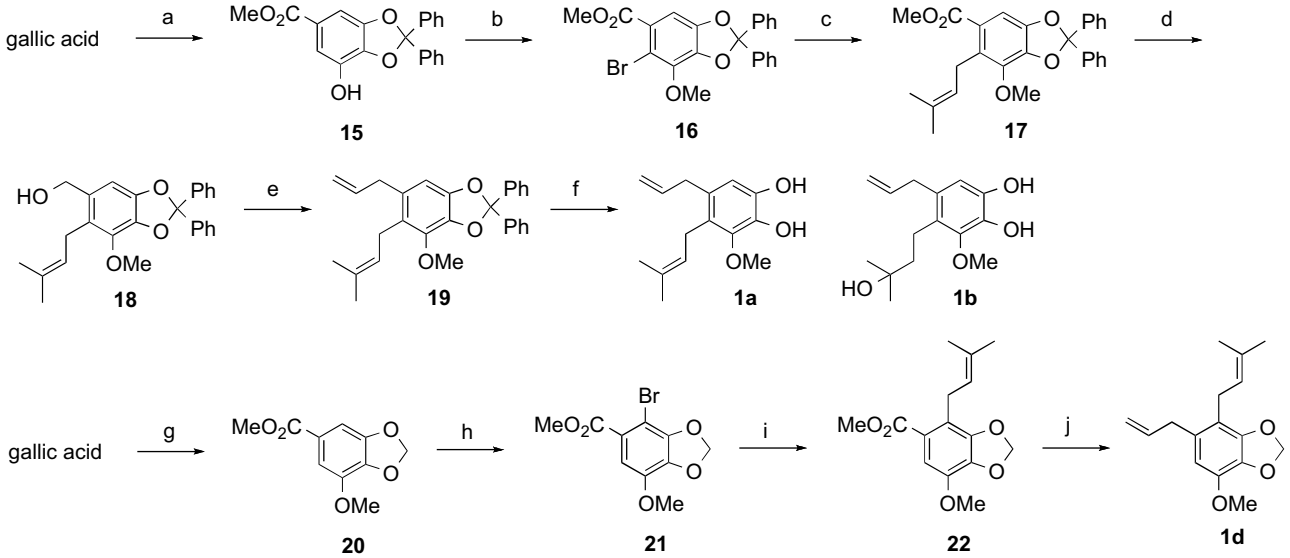
Next, our attention was directed toward preparation of derivatives **1a**–**1d** to define their structure–activity relationship. Attempts to derive **1a** from **1** have not been successful.[10] The synthesis of **1a** essentially followed the same route as that used for **1** starting from **15**[11] with a dioxydiphenylmethyl group as the protecting group of the vicinal diol (Scheme 3). Stepwise bromination and methylation of **15** gave rise to **16**, which was in turn reacted with tributylprenyltin in the presence of 10 mol % $PdCl_2$dppf in DMF at 70 °C to afford the prenylated compound **17** in 56% yield. The ester group of **17** was reduced to the alcohol **18**, and then it was converted to benzyl chloride, which was directly subjected to the second Stille reaction with tributylvinyltin in the presence of 10 mol % $Pd(Ph_3P)_4$, giving rise to **19** in a quantitative yield. Finally, treatment of **19** with AcOH/H_2O in the ratio 2:1 at 110 °C provided **1a** in 23% yield along with **1b** in 27% yield. When the ratio of AcOH/H_2O was changed to 5:1, the sole product **1a** was obtained in 57% yield.

Compound **1c** was easily derived from **1** by catalytic hydrogenation.

The regioisomer **1d**, which carries a prenyl substituent at the C-6 position of the gallic acid, was also synthesized from methylene-2,3-di-*O*-methyl-4-*O*-protected gallic acid **20** as follows. Upon treatment of compound **20** with bromine, bromination regioselectively took place to give rise to **21**. The methyl ester and bromide groups were converted to an allyl group and a prenyl group using successive Stille reactions with tributylvinyltin and allyltributyltin, respectively, to produce compound **1d**.

2.3. Neurite outgrowth-promoting activity of illicinin A and its analogues

We have continued to search for neurotrophic factor-like molecules from natural products, and some unique neurotrophic compounds such as merrilactone A[12] and jiadifenin[13] were found in *Illicium merrillianum* and *I. jiadifengpi*. Thus, the *Illicum* plants can be regarded as an important source of novel neurotrophic compounds. All compounds (**1**–**4**) isolated from the flowers of *I. anisatum* were evaluated for their neurite outgrowth-promoting activity in primary cultured rat cortical neuron cells. As a result,



Scheme 3. Reactions and conditions: (a) MeOH, H_2SO_4, reflux, and then Ph_2CCl_2, 170 °C, 97% (two steps); (b) Br_2, *t*-$BuNH_2$, tol, rt, and then MeI, K_2CO_3, acetone, reflux, 100%; (c) 10 mol % $PdCl_2$dppf, DMF, 70 °C, 56%; (d) $LiAlH_3$, THF, rt, 100%; (e) $SOCl_2$, benzene, 70 °C, and then $CH_2{=}CHSnBu_3$, 10 mol % $Pd(Ph_3P)_4$, DMF, 70 °C, 99% (two steps); (f) AcOH/H_2O (5:1), 110 °C; (g) Me_2SO_4, Na_2BO_4, EtOH, rt, 72%, then CH_2I_2, Cs_2CO_3, DMF, 120 °C, 91%; (h) Br_2, Fe, CH_2Cl_2, 0 °C, 73%; (i) $Me_2C{=}CHCH_2SnBu_3$, 10 mol % $Pd(Ph_3P)_4$, DMF, 125 °C, 54%; (j) 1. $LiAlH_4$, THF, rt, 2. $SOCl_2$, benzene, 70 °C, 3. $CH_2{=}CHSnBu_3$, 10 mol % $Pd(Ph_3P)_4$, DMF, 70 °C, 50% (three steps).

illicinin A (**1**) and 4-allyl-2,6-dimethoxy-3- (3-methyl-2-butenyl) phenol (**3**) showed neurite outgrowth-promoting activity at concentrations ranging from 0.1 μM to 10 μM as shown in Figures 4 and 5; whereas, **2** and **4** did not enhance neurite outgrowth at the same concentrations.

According to our previous studies on honokiol (**5**) and its analogues,[1,2,14] it is supposed that the biaryl moiety in **5** may contribute to its neurotrophic activity, because both *o*- and *p*-allyphenols, two structural units of **5**, are toxic against cultured neurons at 1 μM. Although the neurotrophic activity of **1** is less potent than that of honokiol, the present results suggested that an allylbenzene would be the basic structure behind the neurite outgrowth-promoting activity. In order to further understand the structure–activity relationship, the neurotrophic properties of the derivatives **1a**–**1d** were evaluated by measuring the longest neurites among rat cortical neurons cultured in the presence of each analogue. The neuronal cells were cultured in Neurobasal Medium (NBM) supplemented with B27[15] and the compounds (1 μM) for 6 d and were then visualized after being stained with anti-MAP 2 antibody. The longest neurite enhanced by each compound was calculated. The results are summarized in Figure 5. Among the screened compounds, **1c** decreased neurite outgrowth, and the others were comparable with illicinin A (**1**) (Fig. 5). These results indicate that an allyl function in **1** is essential for preserving neurotrophic activity. Among the active analogues, the extent of the activities of **1a**, **1b**, and **3**, which posses 1,2-free diol group slightly increased, implying that the presence of two vicinal oxy groups is important for neurotrophic activity, but their methylenedioxy forms were found to have nothing to do with the enhancement of its activity. In addition, the presence and the position of the prenyl group in **1** were found not to play an important role in neurotrophic activity.

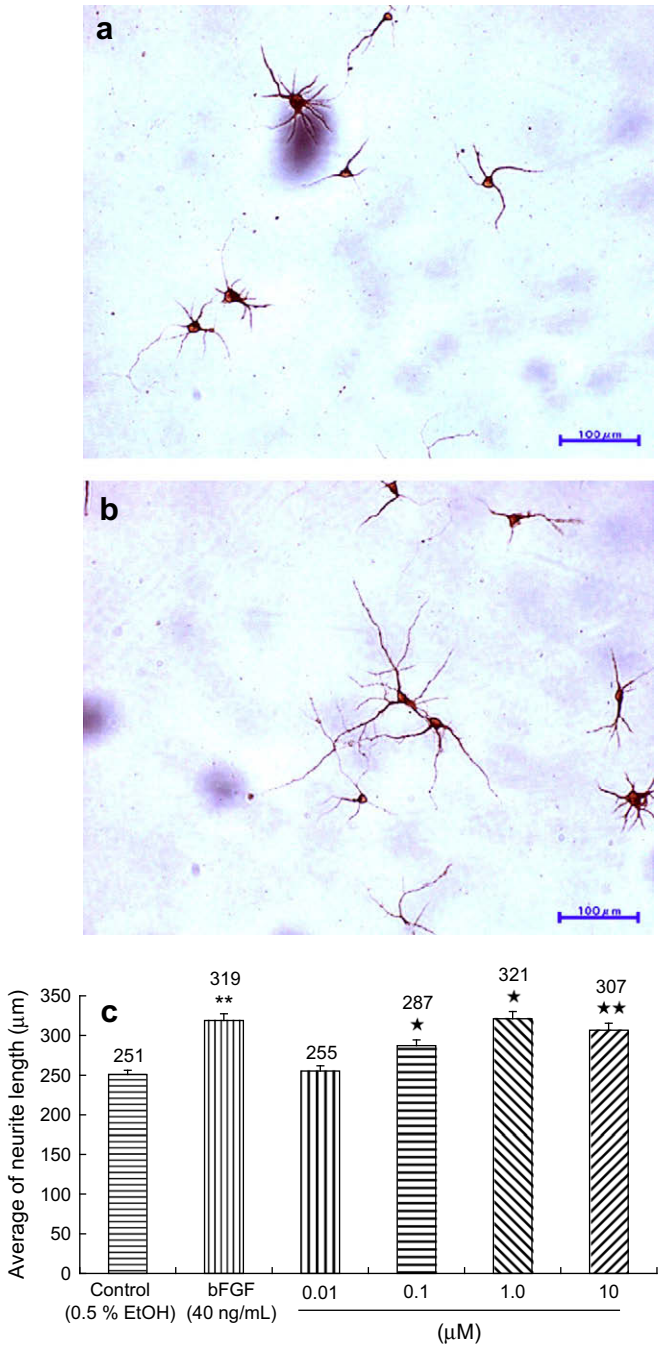


Figure 4. Neurotrophic effect of **1** in primary cultured cortical neurons. After the rat cortical neurons (9000 cells cm^{-2}) had been cultured for 6 d in the presence of 0.5% ethanol and illicinin A (**1**), the neurons were stained with anti-MAP 2 as described in the text; (a): morphology of the neurons in the presence of 0.5% ethanol as a vehicle control; (b): morphology of the neurons in the presence of **1** (1 μM); (c): quantitative analysis of neurite length (μm). bFGF (40 ng/mL) was used as a positive control. The data are expressed as means±SE (n=80); Student's t-test; *: $P<0.05$, **: $P<0.01$ versus control.

3. Summary

We have isolated two new prenylated C_6–C_3 compounds from the flowers of *I. anisatum*, and the aromatic compound illicinin A (**1**) has been found to promote neurite outgrowth in primary cultured rat cortical neurons. The structure of **1** has been confirmed by its total synthesis using two sequential Stille reactions to append allyl and prenyl groups onto its gallic acid ring. Illicinin A is the first example of a simple allylphenol derivative with neurotrophic properties and appears to be promising as a seed compound for the development of low-molecular neurotrophic factor-like compounds[16] via addition of a prenyl group to a variety of functional groups based on structure–activity studies. Additional structure–activity and mechanism studies of **1** are in progress and will be reported in due course.

4. Experimental

4.1. General information

Melting point was measured on a MPJ-2 instrument (uncorrected). Optical rotations were assessed on a Jasco DIP-1000 digital polarimeter. IR and UV spectra were measured on a Jasco FT-IR 5300 and a Shimadzu UV-300 spectrophotometer, respectively. CD spectra were measured on a Jasco J-725 instrument. NMR spectra were recorded on Varian Unity 200, Mercury 300, Unity 600, and JEOL JNM-ECP 400, JNM-GX 400 instruments. Chemical shifts are given as δ (ppm) with TMS as an internal standard. The MS spectra were recorded on a JEOL AX-500 instrument. Column chromatography was carried out on Merck silica gel 60 (70–230 mesh and 230–400 mesh), Wakogel C-300, or Sephadex LH-20. All organic layers were dried over anhydrous $MgSO_4$.

4.2. Extraction and isolation

The flowers of *I. anisatum* were collected in Tokushima, Japan and a voucher specimen (1742FR) is deposited in the Institute of Pharmacognosy (TBU). The dried material (1 kg) was crushed and extracted with MeOH at room temperature. The MeOH extract (137 g) mixed with Celite was subjected to silica gel column chromatography by elution with hexane, hexane/CH_2Cl_2 (1:1), CH_2Cl_2, CH_2Cl_2/EtOAc (1:1), EtOAc, and EtOAc/MeOH (7:3) to give frs. 1–6.

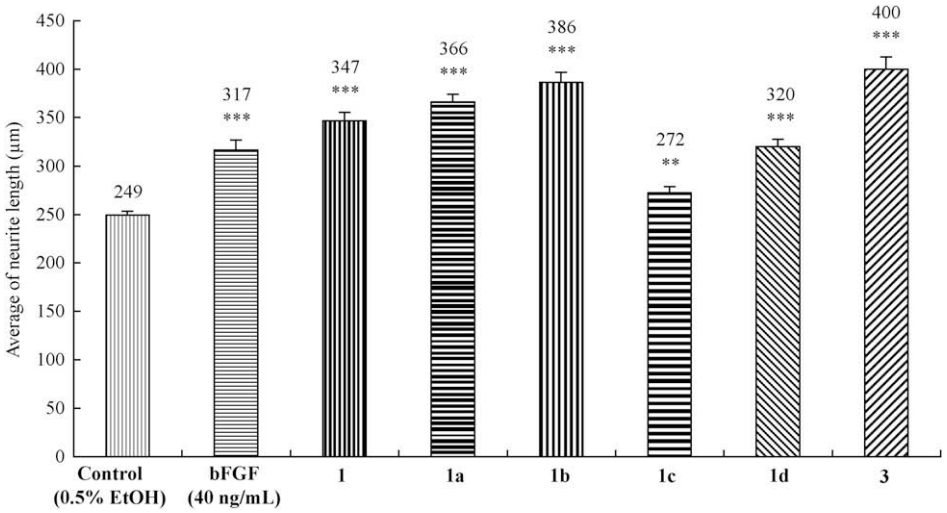


Figure 5. A comparison of the neurite length of rat cortical neurons induced by **1** and its analogues **1a**–**1d** at 1 μM. In each group, the mean length of the primary dendrite-like processes was determined in 80 neurons selected from random fields. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$ versus control. Data presented here were derived from one of two repeated experiments with similar results.

Fr. 1 (5.0 g) was further chromatographed on a silica gel eluted with $CHCl_3$, EtOAc, and MeOH gradiently to give frs. 7–19. Fr. 9 was separated by column chromatography on a Sephadex LH-20 column to yield frs. 20–22. Illicinin A (**1**, 2.1 mg) and **3** (10 mg) were obtained from fr. 21. Fr. 15 was separated by column chromatography on a Sephadex LH-20 eluted with MeOH to yield frs 23–32. (4*S*)-Illicinone I (**2**, 2.4 mg) and **4** (20 mg) were obtained from fr. 30 using a chromatographic method (silica gel, hexane/$CHCl_3$, 1:5).

*4.2.1. Illicinin **A** (**1**).* Colorless oil. EIMS *m*/*z* (rel int): 260 (66), 245 (17), 204 (100). HREIMS: M^+ calcd for $C_{16}H_{20}O_3$: 260.1413, found: 260.1446. UV (EtOH) λ_{max} nm (ε): 287 (2900), 225 (11,400). FTIR (cm^{-1}): 1620, 1478. ^1H NMR (400 MHz, C_6D_6) δ: 6.49 (1H, s, H-6), 5.87 (1H, ddt, *J*=17.6, 12.8, 6.3 Hz, H-8), 5.29 (1H, t, *J*=6.6 Hz, H-11), 5.27 (2H, s, H-15), 4.98 (1H, dd, *J*=17.6, 1.9 Hz, H-9), 4.97 (1H, dd, *J*=12.8, 1.9 Hz, H-9), 3.79 (3H, s, MeO), 3.48 (2H, d, *J*=6.3 Hz, H-7), 3.24 (2H, br d, *J*=6.6 Hz, H-10), 1.71 (3H, s, H-13), 1.65 (3H, s, H-14). ^{13}C NMR (100 MHz, C_6D_6) δ: 147.8 (C-1), 142.3 (C-3), 137.7 (C-8), 135.6 (C-2), 132.2 (C-5), 130.5 (C-12), 125.6 (C-4), 124.5 (C-11), 115.5 (C-9), 104.6 (C-6), 100.5 (C-15), 59.3 (MeO), 37.4 (C-7), 25.9 (C-13), 25.8 (C-10), 17.9 (C-14).

*4.2.2. (4S)-Illicinone I (**2**).* Colorless oil. $[\alpha]_D^{21.5}$ +9.1 (*c* 0.23, EtOH). CD (EtOH): $\Delta\varepsilon$ (287 nm) −2.3. EIMS *m*/*z* (rel int): 262 (M^+, 23), 194 (57), 67 (100). HREIMS: M^+ calcd for $C_{16}H_{22}O_3$: 262.1569, found: 262.1579. UV (EtOH) λ_{max} nm (ε): 236 (7100); IR (cm^{-1}): 1667, 1638, 1611. ^1H NMR (600 MHz, $CDCl_3$) δ: 6.22 (1H, t, *J*=1.4 Hz, H-3), 5.89 (1H, ddt, *J*=17.0, 10.4, 6.6 Hz, H-8), 5.68 (1H, s, H-6), 5.09 (1H, dd, *J*=10.4, 1.9 Hz, H-9), 5.08 (1H, dd, *J*=17.0, 1.9 Hz, H-9), 4.81 (1H, ddqq, *J*=8.2, 7.1, 1.1, 0.5 Hz, H-11), 3.77 (3H, s, MeO-5), 3.11 (3H, s, MeO-4), 3.09 (2H, m, H-7), 2.66 (1H, dd, *J*=13.7, 8.2 Hz, H-10), 2.45 (1H, dd, *J*=13.7, 7.1 Hz, H-10), 1.62 (3H, d, *J*=0.5 Hz, H-14), 1.55 (3H, d, *J*=1.1 Hz, H-13). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 186.9 (C-1), 172.8 (C-5), 141.8 (C-3), 140.0 (C-2), 136.0 (C-12), 135.1 (C-8), 116.8 (C-9), 116.3 (C-11), 104.9 (C-6), 78.0 (C-4), 55.9 (MeO-5), 52.6 (MeO-4), 37.2 (C-10), 32.8 (C-7), 25.9 (C-14), 17.9 (C-13).

4.3. Syntheses of illicinin A and its analogues

*4.3.1. 3,4,5-Trihydrobenzoic acid ethyl ester (**6**).* To a stirred solution of gallic acid (20 g) in EtOH (500 mL) was added 5 mL of condensed sulfuric acid. The mixture was stirred at 90 °C for 10 h and then cooled to room temperature. After the ethanol had evaporated, water was added. The aqueous solution was extracted with EtOAc. The combined organic layers were washed with H_2O and brine, dried, and concentrated in vacuo to give **6** (19.4 g) as a white solid (from hexane, mp 148–149 °C). ^1H NMR (200 MHz, CD_3OD) δ: 7.04 (2H, s), 4.86 (3H, br s), 4.26 (2H, q, *J*=7.1 Hz), 1.33 (3H, t, *J*=7.1 Hz). ^{13}C NMR (50 MHz, CD_3OD) δ: 168.6, 146.5, 139.7, 121.8, 110.0, 61.7, 14.6. IR (cm^{-1}): 3369, 1682. EIMS *m*/*z* (rel int.): 198 (54), 170 (19), 153 (100). HREIMS: M^+ calcd for $C_9H_{10}O_5$: 198.0528, found: 198.0524.

*4.3.2. 7-Hydroxy-benzo[1,3]dioxole-5-carboxylic acid ethyl ester (**7**).* To a solution of **6** (200 mg, 1.0 mmol) in dry DMF (10 mL) were added Cs_2CO_3 (362 mg, 1.01 mmol) and CH_2I_2 (0.9 mL, 1.0 mmol). The mixture was stirred at 120 °C for 1 h and then cooled to room temperature. The resulting mixture was poured into water. The aqueous layer was extracted with ether. The ether layer was sequentially washed with water, 20% $Cu(NO_3)_2$ solution, and brine then dried, filtered, and evaporated. The obtained residue was purified by column chromatography on silica gel eluted with hexane/EtOAc (4:1) to give **7** (191 mg, 91%) as a white powder (from hexane, mp 100–102 °C). ^1H NMR (200 MHz, $CDCl_3$) δ: 7.40 (1H, s), 7.14 (1H, s), 6.50 (1H, br s), 6.04 (2H, s), 4.34 (2H, q, *J*=7.0 Hz), 1.37 (3H, t, *J*=7.0 Hz). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 166.6, 148.9, 139.4, 138.6, 124.6, 114.2, 103.0, 102.3, 61.3, 14.2. IR (cm^{-1}): 3404, 1688. EIMS *m*/*z* (rel int.): 210 (67), 165 (100), 137 (17). HREIMS: M^+ calcd for $C_{10}H_{10}O_5$: 210.0528, found: 210.0519. Anal. Calcd for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80. Found: C, 57.31; H, 4.90.

*4.3.3. 6-Bromo-7-hydroxy-benzo[1,3]dioxole-5-carboxylic acid ethyl ester (**8**).* To a solution of *tert*-butylamine (0.5 mL, 4.8 mmol) in anhydrous toluene was added bromine (0.1 mL, 2.4 mmol) in 10 min at −78 °C under an argon atmosphere. After the mixture had been stirred for 1 h, a solution of **7** (500 mg, 2.4 mmol) in CH_2Cl_2 was added at −78 °C in 1 h. The reaction mixture was stirred at room temperature for 4 h and then concentrated in vacuo. The residue was purified by chromatography on silica gel ($CHCl_3$/EtOAc, 9:1) to give **8** (547.7 mg, 80%) as a colorless plate crystal (from hexane, mp 79–80 °C). ^1H NMR (300 MHz, $CDCl_3$) δ: 7.09 (1H, s), 6.09 (2H, s), 4.36 (2H, q, *J*=7.0 Hz), 1.39 (3H, t, *J*=7.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 165.1, 147.8, 137.5, 137.3, 124.5, 105.4, 104.6, 102.3, 61.6, 14.2. IR (KBr) (cm^{-1}): 3439, 1720. EIMS *m*/*z* (rel int.): 290 (88), 288 (88), 243 (100), 245 (97). HREIMS: M^+ calcd for

$C_{10}H_9O_5Br$: 287.9633, found: 287.9638. Anal. Calcd for $C_{10}H_9O_5Br$: C, 41.55; H, 3.14. Found: C, 41.34; H, 2.94.

*4.3.4. 6-Bromo-7-methoxybenzo[1,3]dioxole-5-carboxylic acid ethyl ester (**9**).* To a solution of **8** (733 mg, 2.5 mmol) in acetone were added K_2CO_3 (3.2 g, 22.8 mmol) and CH_3I (1.5 mL, 10.1 mmol). The reaction mixture was stirred for 2 h and then filtered and evaporated to give **9** (765 mg, 100%) as a yellow oil. ^1H NMR (200 MHz, $CDCl_3$) δ: 7.00 (1H, s), 6.04 (2H, s), 4.36 (2H, q, J=7.1 Hz), 4.02 (3H, s), 1.39 (3H, t, J=7.1 Hz). IR (cm^{-1}): 1726. EIMS m/z (rel int.): 304 (100), 302 (100), 276 (17), 274 (19), 259 (91), 257 (95). HREIMS: M^+ calcd for $C_{11}H_{11}O_5Br$: 301.9790, found: 301.9784.

*4.3.5. (6-Bromo-7-methoxybenzo[1,3]dioxole-5-yl)methanol (**10**).* To a solution of **9** (240 mg, 0.8 mmol) in THF (10 mL) was added *i*-Bu_2AlH (1.1 M solution in hexane, 1.6 mmol) at −78 °C, and the reaction mixture was stirred for 1 h. Water was carefully added, and the aqueous solution was extracted with EtOAc. The combined organic layers were washed with brine, dried, filtered, and evaporated to give the residue, which was purified by column chromatography on silica gel (hexane/EtOAc, 2:1) to give **10** (208 mg, 100%) as a white powder (from hexane, mp 61–63 °C). IR (cm^{-1}): 3370. ^1H NMR (300 MHz, $CDCl_3$) δ: 6.73 (1H, s), 5.96 (2H, s), 4.63 (2H, s), 4.02 (3H, s). ^{13}C NMR (75 Hz, $CDCl_3$) δ: 148.7, 140.3, 136.7, 134.1, 106.6, 103.3, 101.6, 65.2, 60.1. EIMS m/z (rel int.): 262 (M^+, 91), 260 (M^+, 100), 245 (21), 243 (21), 181 (52). HREIMS: M^+ calcd for $C_9H_9O_4Br$: 259.9684, found: 259.9672. Anal. Calcd for $C_9H_9O_4Br$: C, 41.41; H, 3.47. Found: C, 41.76; H, 3.70.

*4.3.6. 6-Allyl-5-bromo-4-methoxybenzo[1,3]dioxole (**11**).* To a solution of **10** (240 mg, 0.9 mmol) in benzene (10 mL) was added thionyl chloride (0.1 mL, 1.2 mmol), and the reaction mixture was stirred at 70 °C for 1.5 h. After being cooled down to room temperature, the solvent was removed under reduced pressure. To the residue, tributylvinyltin (0.3 mL, 0.9 mmol), dry DMF (20 mL), and $Pd(Ph_3P)_4$ (100 mg, 0.09 mmol) were added successfully, and the mixture was stirred at 70 °C for 1.5 h. The cooled reaction mixture was diluted with water and filtered. The resulting filtrate was extracted with ether. The organic layer was washed with 20% $Cu(NO_3)_2$ solution, water, and brine, before being filtered and dried. The organic layer was evaporated to give the residue, which was purified by column chromatography on silica gel (hexane/EtOAc, 7:1) to yield **11** (211 mg, 85%) as a yellow oil. ^1H NMR (300 MHz, $CDCl_3$) δ: 6.49 (1H, s), 5.94 (2H, s), 5.91 (1H, ddt, J=19.2, 12.9, 6.6 Hz), 5.10 (1H, dd, J=19.2, 1.6 Hz), 5.06 (1H, dd, J=12.9, 1.6 Hz), 4.20 (3H, s), 3.40 (2H, d, J=6.6 Hz). EIMS m/z (rel int.): 272 (100), 270 (100), 245 (40), 243 (40), 133 (75). HREIMS: M^+ calcd for $C_{11}H_{11}O_3Br$: 269.9891, found: 269.9884.

*4.3.7. 7-Methoxy-6-(3-methylbut-2-enyl)benzo[1,3]dioxole-5-carboxylic acid ethyl ester (**12**).* To a solution of **8** (1.5 g, 5.2 mmol) and tributyl(3-methylbut-2-enyl)tin (5.6 g, 20.8 mmol) in anhydrous DMF (30 mL) was added $Pd(Ph_3P)_4$ (644 mg, 0.5 mmol) under an argon atmosphere. The mixture was stirred at 125 °C for 12 h and then cooled to room temperature. The resulting mixture was poured into water and extracted with ether. The ether layer was sequentially washed with water, 20% $Cu(NO_3)_2$ solution, water, and brine and then dried, filtered, and evaporated. The residue was purified by column chromatography on silica gel eluted with hexane/EtOAc (4:1) to give **12** (890 mg) as an oil.

*4.3.8. [7-Methoxy-6-(3-methylbut-2-enyl)benzo[1,3]dioxol-5-yl]methanol (**13**).* To a solution of **12** (890 mg, 3 mmol) in THF (30 mL) was added $LiAlH_4$ (160 mg, 4.2 mmol). The reaction mixture was stirred at room temperature for 1 h. The excess $LiAlH_4$ was decomposed by adding a mixture of EtOAc and water. The organic layer was washed with brine, dried, filtered, and concentrated in vacuo. The resulting residue was purified by column chromatography on silica gel eluted with hexane/EtOAc (2:1) to give **13** (833 mg, 63% yield for two steps) as a white powder (from hexane, mp 61–63 °C). IR (cm^{-1}): 3370. ^1H NMR (300 MHz, $CDCl_3$) δ: 6.60 (1H, s), 5.87 (2H, s), 5.04 (1H, t, J=6.9 Hz), 4.52 (2H, s), 3.93 (3H, s), 3.32 (2H, d, J=6.9 Hz), 1.76 (3H, s), 1.67 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 147.2, 141.7, 136.6, 132.8, 131.6, 125.6, 123.7, 103.5, 100.9, 63.3, 59.8, 25.7, 24.7, 17.9. EIMS m/z (rel int.): 250 (100), 232 (41), 217 (90), 194 (90), 165 (21). HREIMS: calcd 250.1205 for $C_{14}H_{18}O_4$, found 250.1193. Anal. Calcd for $C_{14}H_{18}O_4$: C, 67.18; H, 7.25. Found: C, 66.11; H, 7.33.

*4.3.9. Synthesis of illicinin A (**1**).* Thionyl chloride (0.2 mL, 2.4 mmol) was added dropwise to a solution of **13** (200 mg, 0.8 mmol) in benzene. The mixture was stirred at 70 °C for 1.5 h. After being cooled down to room temperature, the resulting mixture was concentrated in vacuo to give a crude product, to which tributylvinyltin (0.2 mL, 2.4 mmol), dried DMF (20 mL), and $Pd(Ph_3P)_4$ (50 mg, 0.08 mmol) were added successively. The reaction mixture was stirred at 70 °C for 1.5 h and cooled to room temperature. The resulting mixture was poured into water, filtered, and extracted with ether. The organic layer was sequentially washed with water, 20% $Cu(NO_3)_2$ solution, water, and brine and then dried, filtered, and evaporated. The residue was purified by column chromatography on silica gel eluted with hexane/EtOAc (9:1) to give **1** (163 mg, 78%), the spectral data of which were identical to those of natural **1**.

*4.3.10. 7-Hydroxy-2,2-diphenylbenzo[1,3]dioxole-5-carboxylic acid ethyl ester (**15**).* Concd sulfuric acid (7.5 mL) was added to a solution of gallic acid (50 g) in MeOH (500 mL). The reaction mixture was stirred at 90 °C for 10 h and cooled to room temperature. The solvent was removed in vacuo, and water was added. After the aqueous solution had been extracted three times with EtOAc, the combined organic layers were washed with water and brine, dried, and concentrated in vacuo to give methyl ester (46 g, 93% yield). The methyl ester derivative (24.7 g, 134 mmol) was added to dichlorodiphenylmethane (25 mL, 134 mmol). The reaction mixture was stirred at 170 °C for 10 min. After being cooled to room temperature, the resulting mixture was added to benzene, and the organic layer was washed with water and brine, dried, and concentrated in vacuo to give **15** (45.3 g, 97% yield) as a solid. ^1H NMR (200 MHz, $CDCl_3$) δ: 7.47–7.54 (4H, m), 7.37–7.39 (6H, m), 7.30 (1H, d, J=1.4 Hz), 7.21 (1H, d, J=1.4 Hz), 5.17 (1H, br s), 3.85 (3H, s).

*4.3.11. 6-Bromo-7-methoxy-2,2-diphenylbenzo[1,3]dioxole-5-carboxylic acid ethyl ester (**16**).* Compound **16** (1.28 g, 100%) was synthesized from **15** (1 g, 2.9 mmol) according to the same procedure as described for the preparation of **8** from **7**. Colorless prism; mp 77–79 °C. IR (cm^{-1}): 1732. ^1H NMR (200 MHz, $CDCl_3$) δ: 7.52–7.56 (4H, m), 7.37–7.40 (6H, m), 7.08 (1H, s), 4.09 (3H, s), 3.87 (3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 166.3, 147.9, 140.8, 140.1, 139.4, 129.6, 128.5, 126.3, 119.1, 109.0, 105.6, 60.4, 52.4. EIMS m/z (rel int.): 442 (98), 440 (99), 411 (21), 409 (23), 365 (100), 363 (99), 165 (56). HREIMS: M^+ calcd for $C_{22}H_{17}O_5Br$: 440.0259, found: 440.0237. Anal. Calcd for $C_{22}H_{17}O_5Br$: C, 59.88; H, 3.88. Found: C, 59.90; H, 4.0.

*4.3.12. 7-Methoxy-6-(3-mehtylbut-2-enyl)-2,2-diphenylbenzo[1,3]-dioxole-5-carboxylic acid ethyl ester (**17**).* Compound **17** (59 mg, 56%) was synthesized from **16** (100 mg, 0.2 mmol) according to the same procedure as described for the preparation of **12**. Yellow oil. IR (cm^{-1}): 1723. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.52–7.59 (4H, m), 7.31–7.47 (6H, m), 7.14 (1H, s), 5.12 (1H, t, J=6.6 Hz), 4.01 (3H, s), 3.81 (3H, s), 3.63 (2H, d, J=6.6 Hz), 1.75 (3H, s), 1.66 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 167.6, 146.5, 141.6, 139.7, 131.4, 131.2, 130.0, 129.3, 128.3, 126.3, 123.5, 117.8, 105.1, 59.9, 51.9, 25.9, 25.8, 17.9. EIMS m/z (rel

int.): 430 (68), 398 (43), 387 (67), 375 (100), 359 (20), 321 (23). HREIMS: calcd for $C_{27}H_{26}O_5$: 430.1780, found: 430.1785.

*4.3.13. [7-Methoxy-6-(3-methylbut-2-enyl)-2,2-diphenylbenzo[1,3]-dioxole-5-yl]methanol (**18**).* Compound **18** (374 mg, 100%) was prepared from **17** (400 mg, 0.9 mmol) according to the method described for the preparation of **13**. Yellow oil. IR (cm^{-1}): 3428. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.54–7.59 (4H, m), 7.31–7.39 (6H, m), 6.67 (1H, s), 5.04 (1H, t, J=6.9 Hz), 4.53 (2H, d, J=5.8 Hz), 4.04 (3H, s), 3.33 (2H, d, J=6.9 Hz), 1.75 (3H, s), 1.66 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 167.6, 146.5, 141.6, 139.7, 131.4, 131.2, 130.0, 129.3, 128.3, 126.3, 123.5, 117.9, 105.1, 59.9, 51.9, 25.9, 17.9. EIMS m/z (rel int.): 402 (9), 384 (100), 369 (44), 307 (39), 182 (25). HREIMS: M^+ calcd for $C_{26}H_{26}O_4$: 402.1831, found: 402.1870.

*4.3.14. 6-Allyl-4-methoxy-5-(3-methylbut-2-enyl)-2,2-diphenylbenzo-[1,3]dioxole (**19**).* Compound **19** (129 mg, 99%) was prepared from **18** (130 mg, 0.32 mmol) according to the same procedure described for the preparation of **1** from **13**. Yellow oil. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.55–7.59 (4H, m), 7.32–7.36 (6H, m), 6.45 (1H, s), 5.88 (1H, ddt, J=16.8, 12.1, 6.3 Hz), 5.01 (1H, t, J=6.3 Hz), 5.01 (1H, dd, J=16.8, 1.4 Hz), 4.96 (1H, dd, J=12.1, 1.4 Hz), 4.02 (3H, s), 3.25 (4H, d, J=6.3 Hz), 1.73 (3H, s), 1.65 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 146.7, 141.4, 140.5, 137.3, 135.0, 131.7, 130.9, 129.0, 128.2, 126.4, 125.2, 123.5, 116.5, 115.5, 104.1, 59.8, 37.2, 25.7, 25.1, 17.9. EIMS m/z (rel int.): 412 (100), 398 (15), 370 (15), 357 (61), 335 (21). HREIMS: M^+ calcd for $C_{28}H_{28}O_3$: 412.2038, found: 412.2034.

*4.3.15. 5-Allyl-3-methoxy-4-(3-methylbut-2-enyl)benzene-1,2-diol (**1a**) and 5-allyl-4-(3-hydroxy-3-methylbutyl)benzene-1,2,3-triol (**1b**).* A mixture of **14** (100 mg) and 5 mL of acetic acid/H_2O (2:1) was stirred at 110 °C for 1 h. After being cooled to room temperature, the resulting mixture was concentrated in vacuo and purified by column chromatography on silica gel eluted with $CHCl_3$/EtOAc (4:1) to give **1a** (23% yield) and **1b** (27% yield). Compound **1a**: yellow oil. IR (cm^{-1}): 3416. ^1H NMR (300 MHz, CD_3OD) δ: 6.39 (1H, s), 5.89 (1H, ddt, J=16.5, 12.4, 6.3 Hz), 5.00 (1H, dd, J=16.5, 1.9 Hz), 4.98 (1H, m), 4.97 (1H, dd, J=12.4, 1.9 Hz), 3.72 (3H, s), 3.24 (4H, d, J=6.3 Hz), 1.74 (3H, s), 1.66 (3H, s). ^{13}C NMR (75 MHz, CD_3OD) δ: 147.9, 145.2, 139.2, 137.6, 131.3, 130.1, 125.7, 125.5, 115.2, 113.4, 61.0, 37.7, 25.9, 25.9, 18.0. EIMS m/z (rel int.): 248 (77), 233 (16), 205 (28), 192 (100), 159 (46). HREIMS: M^+ calcd for $C_{15}H_{20}O_3$: 248.1412, found: 248.1398.

Compound **1b**: yellow oil. IR (cm^{-1}): 3412. ^1H NMR (300 MHz, CD_3OD) δ: 6.38 (1H, s), 5.90 (1H, ddt, J=16.5, 12.6, 6.3 Hz), 4.99 (1H, dd, J=16.5, 1.9 Hz), 4.96 (1H, dd, J=12.6, 1.9 Hz), 3.78 (3H, s), 3.24 (2H, d, J=6.3 Hz), 2.58 (2H, m), 1.57 (2H, m), 1.25 (6H, s). EIMS m/z (rel int.): 266 (9), 248 (54), 233 (8), 192 (100), 177 (22), 161 (38). HREIMS: M^+ calcd for $C_{15}H_{22}O_4$: 266.1518, found: 266.1542.

*4.3.16. 4-Methoxy-5-(3-methylbutyl)-6-propylbenzo[1,3]dioxole (**1c**).* A solution of illicinin A (**1**) (13.6 mg, 0.05 mmol) in CH_2Cl_2 (5 mL) was stirred over PtO_2 (5 mg) under an atmosphere of hydrogen for 12 h. The resulting mixture was purified by column chromatography on silica gel (hexane/EtOAc, 9:1) to give **1c** (6.8 mg, 50%) as a yellow oil. ^1H NMR (200 MHz, $CDCl_3$) δ: 6.40 (1H, s), 5.86 (2H, s), 3.96 (3H, s), 2.43–2.56 (4H, m), 1.50–1.66 (1H, m), 1.26–1.50 (4H, m), 0.95 (6H, d, J=6.6 Hz), 0.87–1.00 (3H, m). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 146.7, 141.6, 134.7, 134.3, 126.4, 103.7, 100.5, 59.6, 40.2, 35.0, 29.7, 24.8, 24.1, 22.5, 14.2. EIMS m/z (rel int.): 264 (38), 208 (13), 207 (100), 179 (20). HREIMS: M^+ calcd for $C_{16}H_{24}O_3$: 264.1714, found: 264.1697.

*4.3.17. 7-Methoxybenzo[1,3]dioxole-5-carboxylic acid methyl ester (**20**).* An aqueous solution of $Na_2BO_4 \cdot 10H_2O$ (10.36 g, 27.17 mmol) was slowly added to a solution of gallic acid methyl ester (5 g, 27.2 mmol) in EtOH (12.5 mL) for 30 min. After being stirred for 30 min, K_2CO_3 (3.75 g, 27.17 mmol) and $(CH_3)_2SO_4$ (2.57 mL, 27.17 mmol) were added. The reaction mixture continued to be stirred at room temperature for 15 h, and was then acidified with 2 M HCl and extracted with EtOAc. The combined organic layers were washed with water and brine, dried, filtered, and concentrated. The residue was purified by column chromatography on silica gel (benzene/ethyl acetate/CH_3CO_2H, 8:2:0.5) to a methyl ester (3.87 g, 72%), which was produced as a solid; mp 112–113 °C (from hexane). IR (cm^{-1}): 3374, 1696. ^1H NMR (300 MHz, CD_3OD) δ: 7.16 (1H, d, J=1.9 Hz), 7.12 (1H, d, J=1.9 Hz), 4.88 (2H, s), 3.81 (3H, s), 3.79 (3H, s). EIMS m/z (rel int.): 198 (78), 183 (5), 167 (100), 153 (10). Anal. Calcd for $C_9H_{10}O_5$: C, 54.55; H, 5.09. Found: C, 54.26; H ,4.98. The monomethyl ester (1.5 g, 7.58 mmol) was treated in a manner similar to that described for the preparation of **4**. The resulting product was purified by column chromatography on silica gel eluted with benzene/EtOAc/AcOH (9:1:0.5) to afford **20** (1.45 g, 91%) as a white powder (from hexane, mp 70–71 °C). ^1H NMR (300 MHz, $CDCl_3$) δ: 7.33 (1H, d, J=1.1 Hz), 7.21 (1H, d, J=1.1 Hz), 6.06 (2H, s), 3.94 (3H, s), 3.89 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 166.4, 148.6, 143.3, 139.4, 124.4, 109.9, 103.9, 102.3, 56.6, 52.2. IR (KBr) (cm^{-1}): 1729. EIMS m/z (rel int.): 210 (82), 179 (100), 151 (18), 135 (3). HREIMS: M^+ calcd for $C_{10}H_{10}O_5$: 210.0529, found: 210.0518. Anal. Calcd for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80. Found: C, 49.73; H, 3.95.

*4.3.18. 4-Bromo-7-methoxybenzo[1,3]dioxole-5-carboxylic acid methyl ester (**21**).* A mixture of **20** (3.12 g, 14.86 mmol) and Fe (320 mg) was suspended in CH_2Cl_2 (45 mL). Upon cooling to −78 °C, bromine (0.73 mL, 14.8 mmol) was added. The reaction mixture was warmed to 0 °C and stirred for 4 h. The organic layer was washed with satd $Na_2S_2O_3$ solution, water, and brine, and then dried and filtered. The solvent was removed under a vacuum. The residue was purified by column chromatography on silica gel eluted with benzene to afford **21** (3.11 g, 73% yield) as a needle crystal (from hexane, mp 104–105 °C). IR (cm^{-1}): 1725. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.27 (1H, s), 6.15 (2H, s), 3.95 (3H, s), 3.93 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 165.4, 148.0, 142.2, 138.3, 124.2, 112.4, 102.4, 94.4, 56.7, 52.2. EIMS m/z (rel int.): 290 (98), 288 (99), 259 (99), 257 (100), 210 (25), 179 (27). HREIMS: M^+ calcd for $C_{10}H_9O_5Br$: 287.9634, found: 287.9620. Anal. Calcd for $C_{10}H_9O_5Br$: C, 41.55; H, 3.14. Found: C, 47.26; H, 3.95.

*4.3.19. 7-Methoxy-4-(3-methylbut-2-enyl)benzo[1,3]dioxole-5-carboxylic acid methyl ester (**22**).* Compound **22** was prepared through the same procedure as described for the preparation of **17**. Compound **21** (280 mg) gave **22** (146 mg, 54% yield) as a colorless oil. IR (cm^{-1}): 1729. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.22 (1H, s), 6.04 (2H, s), 5.20 (1H, t, J=6.9 Hz), 3.90 (3H, s), 3.86 (3H, s), 3.60 (2H, d, J=6.9 Hz), 1.68 (3H, s), 1.67 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 167.2, 147.7, 141.1, 138.1, 132.1, 123.0, 122.3, 111.3, 101.9, 56.6, 51.9, 25.7, 17.9, 17.5. EIMS m/z (rel int.): 278 (M^+,100), 246 (75), 235 (55), 231 (79), 223 (63), 207 (29). HREIMS: M^+ calcd for $C_{15}H_{18}O_5$: 278.1142, found: 278.1159.

*4.3.20. 5-Allyl-7-methoxy-4-(3-methylbut-2-enyl)benzo[1,3]dioxole (**1d**).* Compound **1d** (68 mg, 50% yield in three steps) was prepared from **22** (148 mg, 0.47 mmol) according to the same procedures used for the preparation of illicinin A (**1**) from **12**. Yellow oil. ^1H NMR (300 MHz, $CDCl_3$) δ: 6.39 (1H, s), 5.90 (1H, m), 5.36 (2H, m), 5.11 (1H, m), 5.06 (1H, m), 4.99 (1H, m), 3.51 (3H, s), 3.47 (2H, d, J=6.2 Hz), 3.39 (2H, d, J=7.0 Hz), 1.76 (3H, s), 1.67 (3H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 147.3, 141.7, 137.4, 132.4, 131.9, 122.4, 115.6, 115.4, 108.7, 101.2, 93.0, 56.6, 36.7, 25.6, 24.9, 17.8. EIMS m/z (rel int.): 260 (M^+, 94), 245 (21), 204 (100), 203 (53), 173 (30). HREIMS: M^+ calcd for $C_{16}H_{20}O_3$: 260.1412, found: 260.1388. Anal. Calcd for $C_{16}H_{20}O_3$: C, 67.18; H, 7.25. Found: C, 67.89; H, 7.65.

4.4. Cell culture procedure for neurite outgrowth-promoting activity screening[1,16]

All operations were carried out under sterile conditions. The neuronal cells were separated from the cerebral hemispheres of

fetal 18 days SD rats (Japan SLC, Inc.), suspended in 10% FBS/DMEM, and then seeded at 9000 cells cm^{-2} into poly-L-lysine-coated 24 well-culture plates. After 24 h, the medium was changed for serum-free Neurobasal Medium (NBM) supplemented with B27 in the presence or absence of the compounds at different concentrations. bFGF (basic Fibroblast Growth Factor, 40 ng mL^{-1}) was used as a positive control. After being incubated for 6 days, the cells were fixed in 4% paraformaldehyde/PBS for anti-MAP 2 immunohistochemical staining. The neurite outgrowth induced by the compounds was analyzed using mean neurite length under a microscope. Eighty neurons, which did not grow on or near glial cells, showed no network-formation with more than two cells, and were well stained with anti-MAP 2, were selected for the measurement of each sample. The length of the longest neurite of each neuron was measured and calculated by using Lumina Vision and MacSCOPE software. The data are expressed as means±SE (n=80); Student's t-test; $^{*}P<0.05$, $^{**}P<0.01$ versus control.

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