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Caffeoyl Triterpenoid Esters as Potential Anti-ischemic Stroke Agents from *Celastrus orbiculatus*

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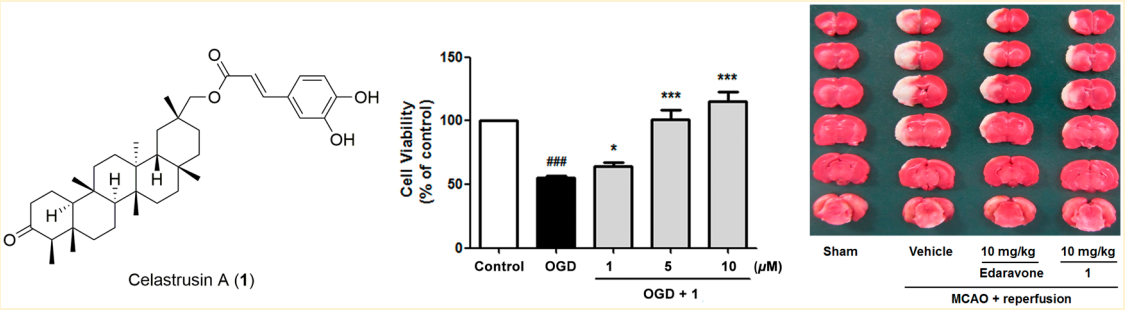
Supporting Information



ABSTRACT: Three new triterpenoids, celastrusins A–C (**1**–**3**), together with 3-*O*-caffeoyl-α-amyrin (**4**) were isolated from the root bark of *Celastrus orbiculatus*. Their structures were identified by spectroscopic analysis, X-ray crystallography using Cu Kα radiation, and the comparison of both observed and reported spectroscopic data. An in vitro bioassay revealed that the caffeoyl triterpenoid esters **1**, **3**, and **4** possess neuroprotective effects against oxygen-glucose deprivation (OGD) induced SH-SY5Y cell damage. Further animal studies indicated that compound **1** significantly reduced brain infarction after transient middle cerebral artery occlusion (MCAO) in rats using a 10 mg/kg (i.v.) dose.

Stroke ranks as the second highest leading killer in the world.[1] There are two broad categories of stroke: ischemic, due to lack of blood flow, and hemorrhagic, due to bleeding in the brain. According to a recent study, ischemic strokes account for approximately 87% of all strokes.[2] Effective therapeutic strategies remain a challenge, despite advances made in understanding the pathophysiology of cerebral ischemia. Most marketed drugs for stroke prevention act as antiplatelet agents (aspirin, among others), anticoagulants (warfarin), HMG-CoA reductase inhibitors (statins), ACE inhibitors (ramipril), or angiotensin II blockers (sartans). Currently, the only FDA-approved drug for the treatment of ischemic stroke, recombinant tissue-plasminogen activator (rt-PA), is still restricted by its short time window, narrow eligible range, and risk of hemorrhage.[3−5] Therefore, discovering new lead compounds that are both safe and effective for minimizing postischemic damage is a priority in the field of antistroke drug development.

Natural products have been shown to be reliable resources in drug discovery and development processes. In the area of stroke research, natural products have played a significant role in providing potentially valuable compounds for drug discovery, such as butylphthalide from *Apium graveolens*[6] and ginkgolide B from *Ginkgo biloba*,[7] among others. *Celastrus orbiculatus* Thunb. (Celastraceae) is widely distributed in China. The roots and fruits have been used to treat snakebite, arthritis, insomnia, neurasthenia, palpitation, and amnesia.[8] Previous investigations showed β-dihydroagarofuran-type sesquiterpenoids as the major secondary metabolites of the seeds and the presence of triterpenoids mainly in the roots. Bioassays of these terpenoids were mainly focused on their antitumor[9,10] and anti-inflammatory[11,12] aspects. Inspired by the traditional use of *C. orbiculatus* and *C. paniculatus* seeds in China and India for memory-enhancing effects, neuroprotective assays of components isolated from the genus *Celastrus* were undertaken. According to recent investigations, dimeric trinorditerpenoids from the root barks of *C. orbiculatus* were found to exhibit significant neuroprotective effect against H_2O_2-induced injury of PC12 cells.[13] Furthermore, β-dihydroagarofuran-type sesquiterpenoids from the seeds of *C. flagellaris* and *C. angulatus* were shown to rescue Aβ_{25-35}-induced SH-SY5Y cells from viability reduction, some of which were also observed to possess significant cognition-enhancing effects in rats with doses of 20 mg/kg (p.o.).[14] Herein the structure elucidation of caffeoyl triterpenoid esters isolated from *C. orbiculatus* and their pharmacological evaluation against postischemic brain damage are reported.

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RESULTS AND DISCUSSION

Celastrusin A (**1**) was obtained as a white, amorphous powder, and its molecular formula was determined as $C_{39}H_{56}O_5$ based on the HREIMS molecular ion at m/z 604.4131 and ^{13}C NMR data. The ^1H and ^{13}C NMR data of celastrusin A (**1**) showed characteristic signals for one caffeoyl group and one triterpenoid moiety [an aliphatic carbonyl (δ_C 214.3), six sp^3 quaternary carbons, four methines, 12 methylenes, including an oxymethylene at δ_C 75.4/δ_H 3.87 and 3.88, and seven methyl groups, including six tertiary methyls and one secondary methyl] (Table 1). In the HMBC spectrum of **1**, cross-peaks were observed between H_2-2 (δ_H 2.39, 2.30), H-4 (δ_H 2.25), H_3-23 (δ_H 0.88) and C-3 (δ_C 214.3); between H_3-23 (δ_H 0.88) and C-5 (δ_C 42.4); between H_3-24 (δ_H 0.73) and C-4 (δ_C 58.4), C-6 (δ_C 41.7); between H_3-25 (δ_H 0.86) and C-10 (δ_C 59.5), C-8 (δ_C 53.4), C-11 (δ_C 35.7); between H_3-26 (δ_H 1.03) and C-8 (δ_C 53.4), C-15 (δ_C 32.7); between H_3-27 (δ_H 1.04) and C-18 (δ_C 42.0), C-12 (δ_C 30.7); between H_3-28 (δ_H 1.22) and C-18 (δ_C 42.0), C-22 (δ_C 39.4), C-16 (δ_C 35.9); and between H_3-30 (δ_H 1.10) and C-29 (δ_C 75.4), C-19 (δ_C 30.2), C-21 (δ_C 28.3) (Figure 2), which enabled confirmation of the presence of 29-hydroxyfriedelin as its triterpenoid moiety.[15] NOE correlations between H_3-25 (δ_H 0.86) and H_3-24 (δ_H 0.73) and H_3-27 (δ_H 1.04); between H-18 (δ_H 1.65) and H_3-27 and H_3-28 (δ_H 1.22); and between H-8 (δ_H 1.39) and H_3-26 (δ_H 1.03) and H-10 (δ_H 1.53) indicated a *trans* A/B/C/D ring fusion and a *cis* D/E ring fusion. The NOESY cross-peaks of H_3-30/H_3-28 and H-4/H-10 revealed their axial orientations (Figure 2). Its absolute configuration was defined by X-ray diffraction analysis of its acetylated derivative **1a**, crystallized from acetonitrile (Figure 3) as (4*R*,5*S*,8*S*,9*S*,10*S*,13*S*,-14*R*,17*S*,18*R*,20*R*)-3-oxo-29-(6′,7′-di-*O*-acetyl)-caffeoyloxyfriedelin.

Compound **2** gave an $[M]^+$ peak at m/z 588.4176 in its HREIMS, 16 Da less than that of **1** and consistent with a molecular formula of $C_{39}H_{56}O_4$. Comparison of the ^1H and ^{13}C NMR data of **2** and **1** revealed the absence of the 6′-OH group on the aromatic nucleus in **2**, as evidenced by the appearance of an A_2B_2 spin system in **2** (δ_H 7.43 and 6.88, each 2H, d, J = 8.2 Hz) instead of the ABX spin system in **1** (Table 1). In addition, hydrolysis of **2** with 10% KOH yielded 29-hydroxyfriedelin and *p*-hydroxycinnamic acid, as identified by comparison with authentic samples. Thus, the structure of **2** was identified as 29-*O*-*p*-hydroxycinnamoylfriedelin as shown in Figure 1 and named celastrusin B.

The molecular formula of **3** was deduced as $C_{39}H_{58}O_5$ on the basis of HREIMS (m/z 606.4292) and ^{13}C NMR data. Compound **3** possessed a caffeoyl moiety according to the UV absorption bands (λ_{max} 330, 244, and 217 nm) and NMR data. The remaining ^{13}C NMR signals of **3** (five sp^3 quaternary carbons, six sp^3 tertiary carbons, 11 sp^3 secondary carbons, and eight methyl groups) suggested a pentacyclic triterpenoid skeleton different from those of both **1** and **2**. ^2J correlations between the eight methyl signals and their attached carbons, as well as ^3J couplings in the HMBC spectrum (Figure 4), led to the deduction of an α-amyrin-type triterpenoid moiety in the structure of **3**.[16,17] The downfield shift of H-3/C-3 (δ_H 4.45/δ_C 81.1) and the HMBC correlation between H-3 (δ_H 4.45) and C-1′ (δ_C 168.0) localized the caffeoyl group at C-3. The coupling constants of H-3 (δ_H 4.45, dd, J = 9.9, 6.0 Hz) suggested its α-orientation. In an NOE experiment (Figure S31, Supporting Information), selective irradiation of the signal at δ_H 0.94 (H_3-30) enhanced the signals at δ_H 1.57 (H_3-27) and δ_H 1.43 (H-19), revealing the β-orientation of the 20-OH function. Therefore, compound **3**, celastrusin C, was characterized as 3β-caffeoyloxy-20β-hydroxyursane (Figure 1).

3β-Caffeoyloxy-Δ^{12}-ursene (**4**) was also isolated and identified through comparing its spectroscopic data to those reported (Figure 1).[18]

Table 1. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds 1−3 ($CDCl_3$)

	1 and **2**		**3**	
position	δ_H, multiplicity (J in Hz)	δ_C	δ_H, multiplicity (J in Hz)	δ_C
1	1.96, m; 1.69, m	22.5	1.59, m; 1.05, m	38.2
2	2.39, m; 2.30, m	41.4	1.56, m	23.7
3		214.3	4.45, dd (9.9, 6.0)	81.1
4	2.25, m	58.4		37.9
5		42.4	0.70, m	55.2
6	1.74, m; 1.27, m	41.7	1.40, m; 1.27, m	18.2
7	1.49, m; 1.39, m	18.4	1.27, m	34.2
8	1.39, m	53.4		41.2
9		37.5	1.16, m	49.3
10	1.53, m	59.5		36.8
11	1.61, m; 1.37, m	35.7	1.19, m; 1.10, m	40.0
12	1.45, m; 1.23, m	30.7	1.34, m; 1.18, m	21.2
13		40.0	1.05, m	38.7
14		38.4		42.9
15	1.47, m; 1.22, m	32.7	1.61, m; 0.85, m	26.4
16	1.47, m; 1.26, m	35.9	1.88, m; 1.03, m	28.4
17		30.5		35.4
18	1.65, m	42.0	0.92, m	47.8
19	1.32, m; 1.24, m	30.2	1.43, m	41.4
20		32.0		75.1
21	1.46, m; 0.90, m	28.3	1.35, m; 1.05, m	37.2
22	1.44, m; 1.05, m	39.4	1.70, m; 0.90, m	38.2
23	0.88, d (6.6)	7.0	0.75, s	27.8
24	0.73, s	14.8	0.79, s	16.5
25	0.86, s	18.0	0.76, s	15.8
26	1.03, s	20.7	0.91, s	16.0
27	1.04, s	18.6	0.82, s	14.8
28	1.22, s	32.2	0.77, s	18.2
29	3.87, brs	75.4	0.92, d (6.6)	17.1
30	1.10, s	26.6	0.94, s	20.8
	Caffeoyl moiety of **1**		Caffeoyl moiety of **3**	
1′		168.2		168.0
2′	6.30, d (15.9)	115.9	6.09, d (15.9)	115.9
3′	7.59, d (15.9)	144.9	7.39, d (15.9)	145.0
4′		127.7		126.6
5′	7.13, d (1.8)	114.4	6.91, d (2.1)	113.9
6′		144.0		144.9
7′		146.5		147.4
8′	6.89, d (8.2)	115.6	6.68, d (8.2)	115.2
9′	7.02, dd (8.2, 1.8)	122.6	6.81, dd (8.2, 2.1)	121.8
	p-Hydroxycinnamoyl moiety of **2**			
1′		168.1		
2′	6.32, d (15.7)	115.2		
3′	7.64, d (15.7)	144.6		
4′		126.7		
5′/9′	7.43, d (8.3)	130.0		
6′/8′	6.88, d (8.3)	115.9		
7′		158.4		

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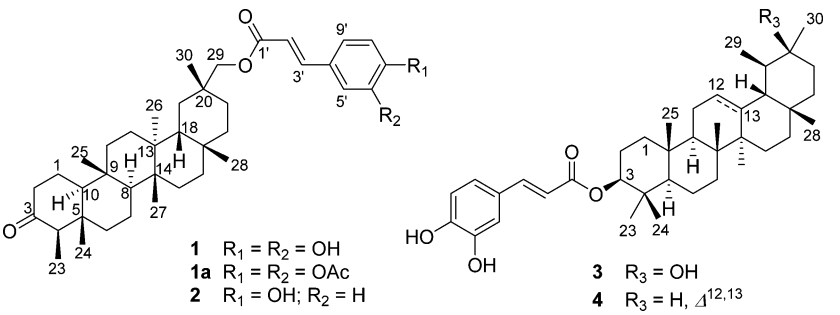

Figure 1. Structures of compounds **1**−**4**.

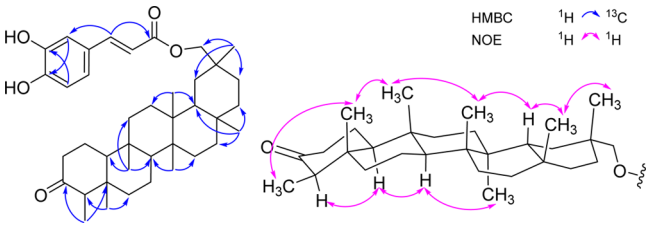

Figure 2. Key HMBC and NOESY correlations of **1**.

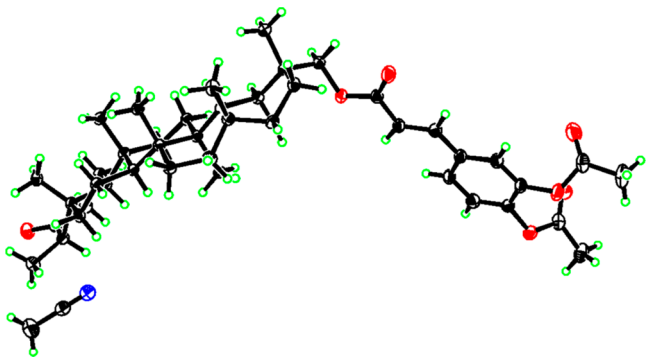

Figure 3. ORTEP drawing of **1a**.

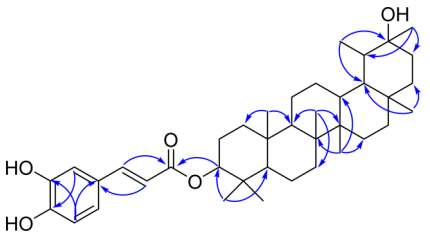

Figure 4. Key HMBC correlations of **3**.

Compounds **1**−**4** were evaluated for their neuroprotective effects against oxygen-glucose deprivation (OGD)-induced damage of SH-SY5Y cells. Exposing SH-SY5Y cells to OGD for 1 h and reoxygenating in normoxic and normoglycemic conditions for 24 h induced a significant decrease in cell viability (56.18 ± 3.34%, $p < 0.001$ vs control). Compounds **1**−**4** were added at the beginning of reoxygenation to obtain a final concentration of 10 μM for evaluating efficacy. Compared with the inactive compound **2**, compounds **1**, **3**, and **4**, which have a caffeoyl moiety in their structures, exhibited more potent activity (Figure 5). Among the three bioactive compounds, **1** most significantly protected SH-SY5Y cells from OGD damage. Therefore, compound **1** was selected for further investigations.

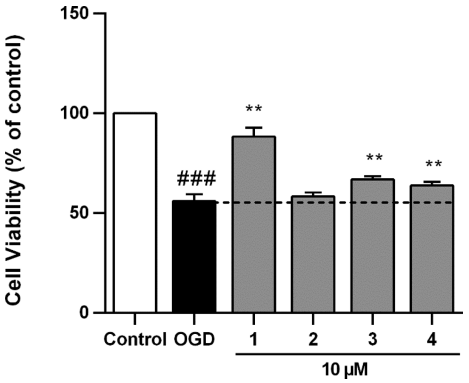

Figure 5. Effect of compounds **1**−**4** on SH-SY5Y cells subjected to OGD. The cell viability of the control was taken as 100%, and the average value of cell viability under OGD exposure was 56.18 ± 3.34%. The data are expressed as the mean ± SEM ($n = 3$), $^{###}p < 0.001$ vs control, $^{**}p < 0.01$ vs OGD group.

Dose-Dependent Effects of 1 in Protecting SH-SY5Y Cells from OGD Damage. SH-SY5Y cells were subjected to OGD for 1 h and reoxygenation for 24 h, which resulted in dendrite fragmentation and simultaneous decreases in cell number (Figure 6A) and cell viability (55.05 ± 2.03%, $p <$ 0.001 vs control) (Figure 6B). However, post-treatment with compound **1** effectively reduced the OGD-induced morphological damage in SH-SY5Y cells (Figure 6A). In accordance with the result from morphological observations, compound **1** treatment notably attenuated OGD-induced injury in a dose-dependent (1 to 10 μM) manner with maximal protection at 10 μM (115.41 ± 7.16%, $p < 0.001$ vs OGD group) (Figure 6B). Additionally, compound **1** showed no significant effects on normal SH-SY5Y cells at those experimental concentrations (Figure S1, Supporting Information).

Compound 1 Attenuated Brain Injury after Transient Middle Cerebral Artery Occlusion (MCAO) in Rats. With the best neuroprotective activity found in the bioassay, compound **1** was further evaluated for its in vivo neuroprotective activity using a classic MCAO model for ischemic stroke study. As shown in Figure 7, the MCAO insult produced a large infarct (29.45 ± 3.65%), as shown in the white region of rat brain sections. Compound **1** was administered intravenously immediately 2 h after the onset of ischemia. As a result, post-treatment of **1** at the dose of 10 mg/kg reduced infarct volume by 14.20 ± 4.54% ($p < 0.05$ vs MCAO group). Edaravone was used as a positive control. Consistent with its protective effect on infarct areas, **1** also produced significant attenuation in neurological scores ($p < 0.05$ vs MCAO group).

The above experiments suggest that further exploration of both the pharmacological mechanism of action of **1** and the

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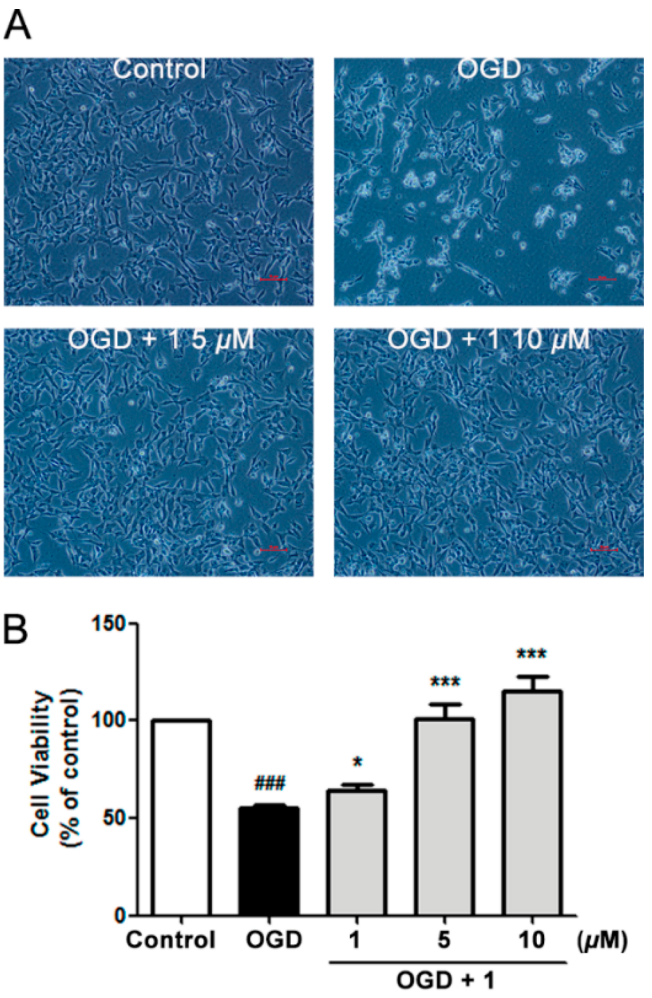

Figure 6. Compound **1** attenuated OGD-induced SH-SY5Y cell injury. (A) Morphology of SH-SY5Y cells after different treatments. (B) Cell viability was measured by MTT assay after treatment with different concentrations of **1** and OGD exposure. The data are presented as the percentage of surviving cells relative to control cells from three independent experiments and as the mean ± SEM. $^{###}p < 0.001$ vs control, $^{*}p < 0.05$, $^{***}p < 0.001$ vs OGD group.

structure−activity relationship of caffeoyl triterpenoid esters as potential lead compounds against ischemic stroke is warranted.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured on an SGW X-4B melting instrument and are uncorrected. Optical rotations were measured with a PerkinElmer 341 polarimeter in MeOH. MS data were measured with a Finnigan LCQ/DECA or Finnigan LTQ mass spectrometer. UV spectra were measured using a Varian Cary 50 UV/vis spectrophotometer. IR spectra were measured with a PerkinElmer 577 instrument. NMR spectra were recorded on a Bruker AVANCE III 500 instrument with tetramethylsilane as the reference. Semipreparative HPLC separations were conducted using a Unimicro 2010 instrument and YMC-Pack ODS-A column (250 × 20 mm, 5 μm; YMC Co., Ltd., Kyoto, Japan). Low-pressure column chromatography (CC) was conducted using silica gel (200−400 mesh; Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) and Sephadex LH-20 (GE Healthcare Biosciences AB, Uppsala, Sweden) as packing materials.

Plant Material. The root barks of *Celastrus orbiculatus* were collected in the suburb of Huaihua, Hunan Province. A voucher specimen (No. SIMM0906-2) has been identified by Prof. Jin-Gui Shen of SIMM, CAS.

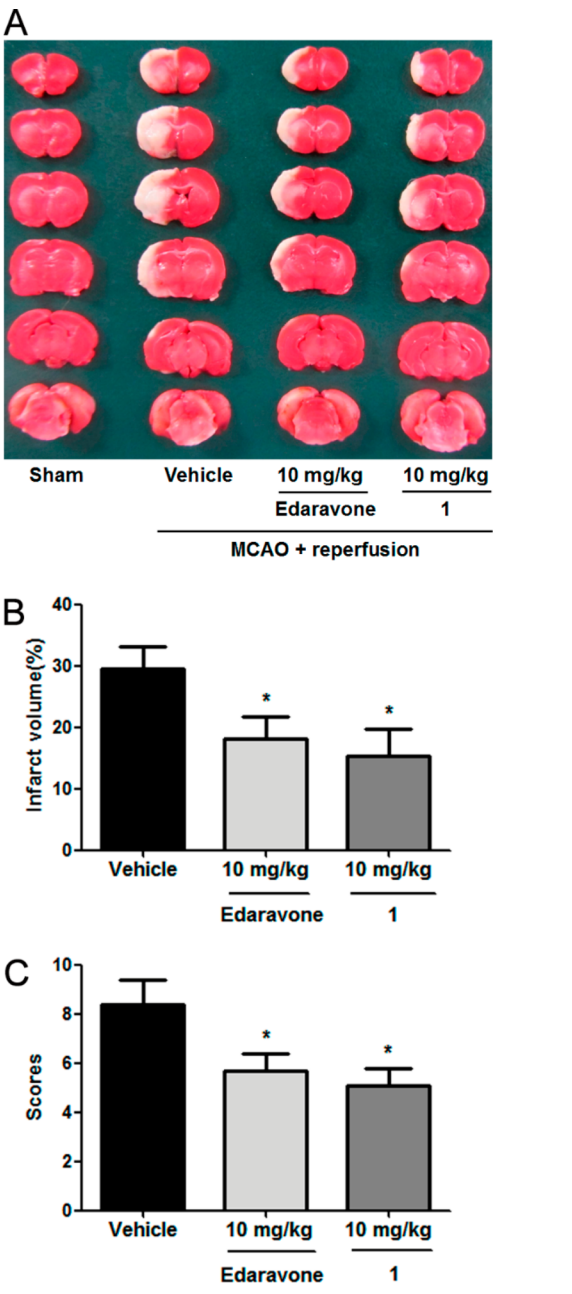

Figure 7. Effect of compound **1** on infarct area and neurological function after 2 h of middle cerebral artery occlusion (MCAO) and 24 h of reperfusion. (A) Triphenyltetrazolium chloride-stained coronal sections from representative rats given either vehicle or **1**. (B) Quantification of the infarct volume. (C) Effect of **1** on neurological score outcomes. $^{*}p < 0.05$ vs vehicle-treated MCAO group, $n = 11-14$.

Extraction and Isolation. The EtOAc fraction of the 95% EtOH extract of the root barks of *C. orbiculatus* (20 kg) was separated over a silica gel column eluted with a mixture of petroleum ether/EtOAc (10:1 to 1:1, v/v) to give Fr. 1−4.[13] Fr. 2 was further separated using RP-18 CC and eluted with MeOH/H_2O (3:2 to 1:0, v/v) to obtain four subfractions (Fr. 2a−2d). Fr. 2d was purified by semipreparative HPLC (MeCN/H_2O, 9:1) to give compounds **2** (0.028 g) and **4** (0.035 g). Fr. 3 was subjected to CC over MCI and eluted with a gradient of MeOH/H_2O (1:4 to 10:0, v/v) to yield fractions 3a−3c. Fr. 3c was separated by Sephadex LH-20 with 95% EtOH as eluent to yield **1** (4.0 g) and **3** (0.03 g).

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Celastrusin A (1): canary yellow, amorphous powder; $[\alpha]_D^{22}$ −23 (*c* 0.1, acetone); UV (MeOH) λ_{max} 329, 244, and 218 nm; IR (KBr) ν_{max} 3369, 2935, 2872, 1701, 1678, 1630, 1599, 1518, 1261, and 754 cm^{-1}; 1D NMR data, see Table 1; HREIMS *m*/*z* 604.4131 $[M]^+$ (calcd for $C_{39}H_{56}O_5$, 604.4128).

(4R,5S,8S,9S,10S,13S,14R,17S,18R,20R)-3-Oxo-29-(6′,7′-di-O-acetyl)caffeoyloxyfriedelin (1a): colorless block crystal; mp 218−219 °C; ^1H NMR (500 MHz, $CDCl_3$) δ_H 7.62 (d, *J* = 16.0 Hz, H-3′), 7.41 (dd, *J* = 8.4, 2.1 Hz, H-9′), 7.38 (d, *J* = 2.1 Hz, H-5′), 7.22 (d, *J* = 8.4 Hz, H-8′), 6.41 (d, *J* = 16.0 Hz, H-2′), 3.88 (brs, H-29), 2.31 and 2.30 (s, CH_3CO-), 1.22 (s, H-28), 1.10 (s, H-30), 1.04 (s, H-27), 1.03 (s, H-26), 0.88 (d, *J* = 6.6 Hz, H-23), 0.87 (s, H-25), 0.72 (s, H-24); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 213.3 (C-3), 168.2 (C-1′), 168.1 and 167.1 (each $CH_3\underline{C}O-$), 143.6 (C-7′), 142.6 (C-6′), 142.6 (C-3′), 133.5 (C-4′), 126.6 (C-8′), 124.1 (C-5′), 122.8 (C-9′), 119.7 (C-2′), 75.4 (C-29), 59.6 (C-10), 58.3 (C-4), 53.4 (C-8), 42.3 (C-5), 42.0 (C-18), 41.7 (C-6), 41.4 (C-2), 40.0 (C-13), 39.3 (C-22), 38.4 (C-14), 37.6 (C-9), 35.9 (C-16), 35.7 (C-11), 32.69 (C-15), 32.23 (C-28), 32.0 (C-20), 30.7 (C-12), 30.5 (C-17), 30.2 (C-19), 28.3 (C-21), 26.6 (C-30), 22.4 (C-1), 20.8 (2 × $\underline{C}H_3CO-$), 20.7 (C-26), 18.6 (C-27), 18.4 (C-7), 18.0 (C-25), 14.8 (C-24), 7.0 (C-23); HREIMS *m*/*z* 688.4336 $[M]^+$ (calcd for $C_{43}H_{60}O_7$, 688.4339). The crystallographic data of **1a** have been deposited in the Cambridge Crystallographic Data Center (CCDC) with the entry number 1454973.

Celastrusin B (2): canary yellow, amorphous powder; $[\alpha]_D^{22}$ −8 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} 313, 227 nm; IR (KBr) ν_{max} 3365, 2933, 2872, 1705, 1635, 1608, 1587, 1514, 1456, 1388, 1275, 1169, 982, and 829 cm^{-1}; 1D NMR data, see Table 1; HREIMS *m*/*z* 588.4176 $[M]^+$ (calcd for $C_{39}H_{56}O_4$, 588.4179).

Celastrusin C (3): canary yellow, amorphous powder; $[\alpha]_D^{22}$ +31 (*c* 0.1, $CHCl_3$/MeOH, 1:1); UV (MeOH) λ_{max} 330, 244, and 217 nm; IR (KBr) ν_{max} 3535, 3431, 2935, 1685, 1633, 1603, 1529, 1446, 1386, 1275, 1192, 978, and 758 cm^{-1}; 1D NMR data, see Table 1; HREIMS *m*/*z* 606.4292 $[M]^+$ (calcd for $C_{39}H_{58}O_5$, 606.4284).

Cell Culture. Human neuroblastoma SH-SY5Y cells were cultured in a 1:1 mixture of Eagle's minimum essential medium and F12 medium (Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere of 5% CO_2 at 37 °C. SH-SY5Y cells (2 × 10^4 cells/100 μL per well) were seeded into 96-well plates.

OGD Exposure. Neuronal ischemia/reperfusion was induced by OGD and reoxygenation.[19] The culture medium of the OGD group was replaced with glucose-free EBSS buffer (mmol/L: 116 NaCl, 5.4 KCl, 0.8 $MgSO_4{\cdot}7H_2O$, 1.0 $NaH_2PO_4{\cdot}2H_2O$, 26.2 $NaHCO_3$, 0.01 glycine, 8.0 $CaCl_2{\cdot}2H_2O$, pH 7.4) after washing twice and transferred to a hypoxia chamber (H35 Hypoxystation, Don Whitley Scientific) in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 at 37 °C. OGD was terminated after 1 h by returning to normal cultured conditions and reoxygenated for another 24 h. The control group was treated without OGD insult. Compounds were added at the beginning of reoxygenation.

Cell Viability Assay. Cell viability was measured by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) assay. After 24 h of reoxygenation, 10 μL of MTT (5 mg/mL) was added to each well and incubated at 37 °C in the dark for 3 h. All culture medium was replaced with 100 μL of DMSO to dissolve the resulting formazan. The absorbance of each well was assessed using a DTX 800 multimode detector (Beckman Coulter, Fullerton, CA, USA) at 490 nm.

MCAO Model in Rats. Focal cerebral ischemia was induced in male Sprague−Dawley rats (SD rats, Shanghai Laboratory Animal Center, Chinese Academy of Sciences, China) using the MCAO model described previously with minor modification.[20] Rats weighing 230−280 g were subjected to MCAO. In brief, rats were anesthetized with chloral hydrate (400 mg/kg, i.p.). MCAO was induced using a 4−0 monofilament nylon suture (Beijing Sunbio Biotech Co., China), inserted into the left internal carotid artery and positioned to occlude the MCA origin through the proximal external carotid artery. The suture was withdrawn to restore blood flow (reperfusion) after 2 h of MCAO. Saline, edaravone (10 mg/kg), and compound **1** (10 mg/kg) were administered intravenously immediately after reperfusion. The animal's body temperature was maintained at 37.0 ± 0.5 °C during the whole surgical procedure.

Neurological Deficit Evaluation. After 24 h of reperfusion, the neurological deficits were tested according to the modified Neurological Severity Score (mNSS) evaluations.[21] The mNSS was scored on an 18-point scale including motor, visual, and tactile responses.[22] A higher score represents worse neurological performance.

Brain Injury Measurement. After mNSS evaluation, rat brain coronal sections (2 mm thick) were prepared and stained with 1% triphenyltetrazolium chloride (Sinophar Chemical Reagent Co., Shanghai, China) for 15 min followed by fixation in paraformaldehyde solution (4%) for 24 h. The red staining represented normal and healthy brain tissue, and the white represented infarct tissue. After scanning with a digital camera, different brain areas were analyzed and calculated using an image analysis system (Image-ProPlus). The infarct volumes were calculated as follows: (area of right hemisphere − area of noninfarct left area)/right hemisphere × 100%.[23]

Statistical Analysis. All data were reported only if at least three independent experiments showed consistent results. The data were expressed as the mean ± SEM and analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons or by Student's *t*-test for single comparison. Statistical significance was established using a *p* value of <0.05.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00314.

Spectra of new compounds (PDF)

Crystallographic data file of diacetylated celastrusin A (**1a**) (CIF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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