Dimeric Diarylheptanoids with Neuroprotective Activities from Rhizomes of *Alpinia officinarum*

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**ABSTRACT:** Two novel dimeric diarylheptanoids, alpinidinoids A [(±)-1] and B (2), with two unusual coupling patterns, together with a naturally occurring diarylheptanoid dimer possessing a rare pyridine ring linkage (alpinidinoid C, 3), were isolated from the rhizomes of *Alpinia officinarum*. Their structures including absolute configurations were determined by extensive spectroscopic methods and theoretical calculations. All isolates were examined for their neuroprotective activities against oxygen-glucose deprivation and reoxygenation (OGD/R) damage in primary cortical neurons. Remarkably, the dextrorotatory enantiomer of alpinidinoid A [(+)-1] significantly ameliorated OGD/R-induced neuronal apoptosis, which was dependent on the activation of the AKT/mTOR signaling pathway.

**INTRODUCTION**

Brain ischemia is a very common cause of mortality and disability worldwide, which generally occurs due to the critical reduction of brain blood supply.1-2 The major manifestation of brain ischemia is ischemic stroke, ranking the second cause of death globally. It is estimated that 17 000 000 people suffer from a stroke each year and about 80% of these conditions are caused by cerebral ischemia.3,4 Reperfusion to ischemic brains is an approach to reverse brain damage. Nonetheless, reperfusion also leads to secondary tissue damage accompanied by death and dysfunction of brain cells, called ischemia/reperfusion (I/R) injury.5,6 Multiple studies indicate that apoptosis is a major form of cell death after cerebral I/R, and the prosurvival AKT/mTOR signaling pathway is inhibited during neuronal apoptosis.7-9 Thus, the activators of the AKT/mTOR pathway could be potential therapeutic agents for brain ischemia.

As reported in numerous reports, natural products derived from food exhibit significant effects on the treatment of cerebral ischemic injury, revealing a potent resource of neuroprotective agents.10-12 The plant *Alpinia officinarum* Hance, belonging to family Zingiberaceae, is an important edible medicinal plant widely cultivated in the tropical and subtropical areas of Asia.13 The rhizomes of *A. officinarum*, commonly known as lesser galangal, are not only widely utilized as an important food spice and flavoring agent but also used as a traditional Chinese medicine in China for the treatment of gastrointestinal diseases.14,15 Phytochemical investigations have revealed that diarylheptanoids are the characteristic constituents of the rhizomes of *A. officinarum*, which exhibit a variety of biological activities, such as antioxidant,16 antibacterial,17 anticancer,18 antiemetic,19 and anti-inflammatory activities.20 As a result of our continuing efforts to discover natural products with nervous system activities, our previous studies on the title plant had resulted in the discovery of a series of diarylheptanoids with promising neuroprotective effects, including promoting neuronal differentiation and neurite outgrowth, against Aβ-induced damage and against 1-methyl-4-phenylpyridinium (MPP+)-induced injury in neuronal cells or primary cortical neurons.21-24 Recently, we also found that 7-(4-hydroxy-3-methoxyphenyl)-1-phenyl-4E-hepten-3-one protects cortical neurons against oxygen-glucose deprivation and reoxygenation (OGD/R) injuries.25 In the current study, a pair of new dimeric diarylheptanoid enantiomers [(±)-alpinidinoid A, (±)-1], a new axially symmetrical diarylheptanoid dimer (alpinidinoid B, 2), and a new naturally occurring dimeric diarylheptanoid with a rare pyridine ring linkage (alpinidinoid C, 3) were isolated from the nonpolar fraction of *A. officinarum* (Figure 1).

Notably, the two monomeric diarylheptanoid motifs in 1 were connected through the C-4–C-3‴ and C-5–O–C-5‴ bonds to form a tetrahydropyran ring, which is unprecedented in dimeric diarylheptanoids. In 2, the two monomeric diary-
Alpinidinoid A (1) was isolated as a yellow oil. The molecular formula of 1 was determined as C_{38}H_{42}O_{3} by its HR-ESI-MS data (m/z 545.3055 [M + H]^+), calcld for C_{38}H_{42}O_{3}: 545.3056, indicating 18 degrees of unsaturation in its molecular. The UV spectrum revealed the absorption bands at λ_{max} 208 and 262 nm. The IR spectrum indicated the presence of hydroxyl (3448 cm⁻¹), carbonyl (1687 cm⁻¹), and aromatic ring (1601 and 1493 cm⁻¹) functional groups in 1. Analysis of ¹H and ¹³C NMR spectral data (Table 1) revealed the presence of a ketone carbonyl (δ_{C} 215.9), four monosubstituted benzene rings, and nine alkyl methylene groups. The HMB transfer correlations between H₂-1 and C-3′/C-2′ (C-6′), between H₂-2 and C-1′, between H-4 and C-2, between H₂-6 and C-1′, and between H₂-7 and C-2′ (C-6′) allowed the construction of a diarylheptanoid motif 1a. Meanwhile, the HMBC correlations between H₂-1′ and C-3″/C-2″ (C-6″), between H₂-2′ and C-1″/C-4″, between H₂-6′ and C-1″, and between H₂-7′ and C-2″ (C-6″) led to the establishment of another diarylheptanoid moiety 1b. Furthermore, the HMB cross-peaks between H₂-2″/H₂-4″ and C-4 and between H-5 and C-5″, together with the molecular formula information and the obvious down-field shifts of C-5 (δ_{C} 74.1) and C-5″ (δ_{C} 72.0), indicated that substructures 1a and 1b were connected via the C-4–C-3″ bond and the oxygen bridge between C-5 and C-5″ to form a tetrahydropyran ring (Figure 2).

The relative configuration of the partial structure of 1 could be deduced on the basis of the coupling constant of vicinal protons and the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum. The large coupling constants of J_{H4,H5} (9.8 Hz) and J_{H4',H5'} (11.2 Hz) indicated that H-4 and H-5 as well as H-4″β and H-5″ were trans-related, respectively. In the NOESY spectrum, the NOE correlation between H-4 and H-4″β was observed, suggesting the same orientation of H-4 and H-4″β (Figure 3). Based on the aforementioned spectroscopic evidence, the relative configurations of three chiral centers (C-4, C-5, and C-5″) in 1 were
chemical shifts of the two plausible relative configurations, (+)-1 and (-)-1 were determined by comparison of their experimental spectra and estimated ECD curves employing the time-dependent density functional theory (TDDFT) method. The calculated ECD curves of 4S, SS, 3”R, 5”R-1 and 4R, SR, 3”S, 5”S-1 revealed good accordance with the measured spectra of (+)-1 and (-)-1, respectively (Figure S3A). Therefore, the absolute configurations of (+)-1 and (-)-1 were defined, as shown in Figure 1.

Alpinidinoid B (2) was isolated as a yellow oil with an optical rotation value of [α]D 20 + 24 (c 0.15, MeOH). The molecular formula of 2 was identified as C38H42O3 on the basis of its HR-ESI-MS data with m/z 547.3212 [M + H]+ (calcd for C38H43O3: 547.3212). However, inconsistent with its molecular formula information, the 1H and 13C NMR spectra of 2 only showed the signals corresponding to 21 protons and 19 carbons, suggesting that 2 is a dimeric diarylheptanoid with a highly symmetrical structure (Table 2). Comprehensive analysis of the two-dimensional (2D) NMR data of 2 led to the establishment of a partial diarylheptanoid unit in 2 (Figure 2), which was close to a known monomeric diarylheptanoid (5S, 5-hydroxy-1,7-diphenyl-3-heptanone).17 Besides, the HMBC correlation between H-5 (H-5‴) and C-5‴ (C-5) was observed. Considering the obvious down-field shift of C-5 (C-5‴, δC 72.4), along with the molecular formula information, the gross structure of 2 could be determined to be a symmetrical diarylheptanoid dimer, in which the two monomeric units were linked through C-S−O–C-S‴ bonds.

Due to the obvious positive specific rotation and measurable ECD Cotton effects, we could exclude the possibility that compound 2 was a mesomer. Therefore, the above-mentioned evidence demonstrated the presence of a C2 symmetry axis in the molecule of 2, and the following chiral HPLC analysis indicated that 2 was obtained as an optically pure compound. To further clarify the absolute configuration of 2, quantum chemical calculation of ECD curves of two possible stereoisomers of 2, (SS, S‴S)-2 and (SR, S‴R)-2, were subsequently performed by utilizing the TDDFT method. Excellent agreement was found between the theoretical ECD curve for (SS, S‴S)-2 and the experimental one of 2 (Figure S5B). Thus, the absolute configuration of 2 was determined to be SS, S‴S (Figure 1).

The HR-ESI-MS data of 3 showed a protonated molecular ion peak at m/z 524.2936 [M + H]+ (calcd for C39H44NO2 S24.2953), which was consistent with the molecular formula of C39H44NO. The UV spectrum of 3 exhibited absorption maxima at 208 and 269 nm. The IR spectrum suggested the existence of the carbonyl group (1695 cm−1) and aromatic ring (1590 and 1449 cm−1) in 3. Similar to 1, the 1H and 13C NMR spectra of 3 revealed the presence of characteristic signals corresponding to two diarylheptanoid moieties, including a ketone carbonyl (δC 208.0), four monosubstituted benzene rings (δH 7.06–7.11 (4H, overlapped), 7.14–7.19 (4H, overlapped), and 7.20–7.30 (12H, overlapped)); δC 142.7, 141.9 × 3, 129.5 × 2, 129.34 × 6, 129.27 × 2, 129.2 × 2, 129.1 × 4, 126.9, 126.8, 126.69, and 126.68), four sp2 quaternary carbons (δC 161.4, 155.5, 147.2, and 138.8), one olefinic methine (δH 6.97 (1H, br s); δC 121.8), along with eight alkyl methylene groups.

The 1H–1H COSY spectrum of 3 revealed the existence of seven spin-coupling systems (Figure 2). To elucidate the planar structure of 3, the HMBC correlations were further interpreted. On the one hand, the HMBC cross-peaks between H2-1 and C-3/C-2’ (C-6’), between H2-2 and C-1’, between...
H₂-6 and C-4/C-1″, and between H₂-7 and C-5/C-2″ (C-6″) resulted in the formation of a diarylheptanoid moiety, as shown in Figure 2 (3a). On the other hand, the HMBC correlations between H₂-1‴ and C-3‴/C-2‴′ (C-6‴′), between H₂-2‴ and C-1‴′/C-4‴, between H₂-6‴ and C-4‴/C-1‴″, and between H₂-7‴ and C-5‴/C-2‴″ (C-6‴″) allowed the assignment of the second diarylheptanoid moiety 3b (Figure 2). Furthermore, the HMBC correlations between H-6‴ and C-4 and between H-4‴ and C-4, combined with the molecular formula information, indicated that the two diarylheptanoid motifs 3a and 3b were connected via C-4−C-5‴ and C-5−N–C-3‴ bonds to form an additional pyridine ring (Figure 2). Structurally, the two diarylheptanoid monomers of 3 were connected via a pyridine ring, which is very rare in naturally occurring dimeric diarylheptanoids. So far, only two analogues, officinaruminane A and officinone B, have been reported. When we searched the structure of 3 through SciFinder Scholar, we found that this compound has been registered as a synthetic chemical with a CAS registry number of 2181082-48-2. However, this is the first report that this compound was obtained from a natural source. Therefore, we gave a trivial name alpinidinoid C for this new natural product.

(+)1 Protects Cortical Neurons against OGD/R-Induced Damage. To test the nontoxic dosages of these compounds, cortical neurons were treated with compounds at different concentrations (1−10 μM) for 24 h, and cell viability was examined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We found that (+)1 and (−)1 were not toxic at concentrations up to 5 μM, whereas compounds 2 and 3 had toxicity at concentrations larger than 1 μM (Figure 6A). To examine neuroprotective activities of these compounds, cortical neurons were pretreated with compounds for 4 h before OGD/R and then cell viability was measured. Compared to the control group, OGD/R reduced cell survival by 30% (Figure 6B). However, the decreased cell viability was significantly reversed by treatment with (+)1 (Figure 6B). These results suggest that pretreatment with (+)1 protects cortical neurons from OGD/R-induced damage, but compounds (−)1, 2, and 3 do not have this effect.

(+)1 Reduces OGD/R-Induced Cell Apoptosis. To examine apoptosis after OGD/R and to determine whether (+)1 could inhibit apoptosis following OGD/R, we tested the apoptosis rate of cortical neurons with flow cytometry. The
viability was measured by the MTT assay.

According to the results in Figure 8A, B, OGD/R increased the expression of cleaved-caspase-3, an important apoptosis-related protein, is regulated by (+)-U0126. Cortical neurons pretreated with (+)-U0126 for 4 h were subjected to OGD/R, and cleaved-caspase-3 was detected in neuron homogenates by western blotting. According to the results in Figure 8A, B, OGD/R increased the level of cleaved-caspase-3, which was significantly down-regulated in the OGD/R group treated with (+)-1. The AKT/mTOR signaling pathway plays an essential role in inhibiting apoptosis. To explore the neuroprotective molecular mechanism of (+)-1, we examined the phosphorylation levels of AKT and mTOR, which reflect the active states of the two proteins. We found that OGD/R markedly inhibited the phosphorylation of AKT and mTOR, which could be reversed by (+)-1 pretreatment (Figure 8A–D). By contrast, (-)-1 had no effects on either cleaved-caspase-3 or AKT/mTOR phosphorylation. These results indicate that the AKT/mTOR signaling pathway could be involved in the (+)-1-mediated neuroprotective effect in OGD/R-exposed cortical neurons.

Neuroprotective Effect of (+)-1 is Mediated through the PI3K/AKT/mTOR Signaling Pathway. To verify whether the PI3K/AKT/mTOR pathway is critical in (+)-1-mediated neuronal protection, we examined whether the PI3K-specific inhibitor LY294002 (10 μM) and the mTOR inhibitor Torin1 (30 nM) affect the activity of (+)-1. The ERK inhibitor U0126 (10 μM) was also examined as a control. Indeed, western blotting results revealed that LY294002 and Torin1 canceled the effect of (+)-1 on cleaved-caspase-3 inhibition after OGD/R (Figure 9A, B). Consistently, the cell viability assay also confirmed that LY294002 and Torin1 abrogated the neuroprotection effect of (+)-1 (Figure 9C). By contrast, U0126 exerted no interfering actions on (+)-1, suggesting that

Table 2. 1H and 13C NMR Spectroscopic Data for 2 (in CDCl3, δ in ppm, J in Hz)ab

| no. | δH | δC |
|-----|-----|-----|
| 1/1‴ | 29.5 | 2.89 (t, 7.5) |
| 2/2‴ | 45.5 | 2.78 (dt, 17.5, 7.5) |
| 3/3‴ | 208.7 | b: 2.72 |
| 4/4‴ | 47.4 | a: 2.71 (dd, 16.4, 6.7) |
| 5/5‴ | 72.4 | b: 2.34 (dd, 16.4, 5.2) |
| 6/6‴ | 36.2 | 1.75 (m) |
| 7/7‴ | 31.5 | 2.61 (m) |
| 1/1‴ | 141.0 | |
| 2/2‴, 6/6‴ | 128.5b | 7.16–7.20 |
| 3/3‴, 5/5‴ | 128.4b | 7.26–7.29 |
| 4/4‴ | 126.1 | 7.16–7.20 |
| 1/1‴ | 141.8 | |
| 2/2‴, 6/6‴ | 128.4b | 7.14 |
| 3/3‴, 5/5‴ | 128.5b | 7.26–7.29 |
| 4/4‴ | 125.9 | 7.16–7.20 |

abOverlapped resonances are reported without designating multiplicity.

Image 60x249 to 300x488

Figure 6. (+)-1 protects cortical neurons against OGD/R-induced cell damage. (A) Cytotoxicity was measured by the MTT assay. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs control. (B) Cell viability was measured by the MTT assay. *p < 0.05 vs dimethyl sulfoxide (DMSO) group. The data represent the mean ± standard error of mean (SEM) of three independent experiments.

Figure 7. (+)-1 inhibits OGD/R-induced neuronal apoptosis. (A) Cortical neurons were pretreated with 5 μM (+)-1 for 4 h followed by 4 h OGD and 24 h reoxygenation. Apoptosis neurons were detected with Annexin V/PI staining by flow cytometry. (B) Apoptosis rate of cortical neurons measured by flow cytometry. **p < 0.01, vs Control. *p < 0.01, vs OGD/R group. The data represent the mean ± SEM of three independent experiments.

Figure 8. (+)-1 reverses OGD/R-induced upregulation of cleaved-caspase-3 and downregulation of the AKT/mTOR signaling pathway in cortical neurons. (A) Levels of phosphorylated p-AKT, p-mTOR, and cleaved-caspase-3 (C-Casp-3) in OGD/R-damaged neurons are reversed by (+)-1 pretreatment. (B–D) Relative band density of C-Casp-3 (B), p-AKT (C), and p-mTOR (D) were measured with ImageJ and normalized to that of GAPDH. **p < 0.01, ***p < 0.001, vs Control. *p < 0.05, **p < 0.01, ***p < 0.001, vs OGD/R group. Data are presented as mean ± SEM from at least three independent experiments.
ERK does not participate in the function of (+)-1. Therefore, (+)-1 has a protective effect in cortical neurons against OGD/R through the PI3K/AKT/mTOR signaling pathway.

**CONCLUSIONS**

In conclusion, the phytochemical investigation of the petroleum ether-soluble fraction of *A. officinarum* resulted in the isolation and identification of three dimeric diarylheptanoids, including a pair of new dimeric diarylheptanoid enantiomers [(±)-alpinidinoid A, (±)-1] and a new axysymmetrical diarylheptanoid dimer (alpinidinoid B, 2) possessing two unusual connecting manners, and a new naturally occurring dimeric diarylheptanoid with a rare pyridine ring linkage (alpinidinoid C, 3). Among them, (+)-1 exhibits protective effects against OGD/R-induced primary cortical neurons apoptosis. Moreover, (+)-1 significantly decreased the expression of cleaved-caspase-3. The protective effect of (+)-1 is achieved via activating the PI3K/AKT/mTOR signaling pathway. Therefore, this dimeric diarylheptanoid from the widely used *A. officinarum* holds beneficial potential to be used as a candidate agent for treating cerebral ischemic injury.

**MATERIALS AND METHODS**

**General Experimental Procedures.** Details on instruments for structural elucidation (UV, IR, optical rotations, ECD, NMR, HR−ESI−MS), along with the materials for isolation (silica gel, octadecyl silica, liquid chromatograph system, and solvents), have been described in our previous work.23

**Plant Material.** The rhizomes of *A. officinarum* Hance (Zingiberaceae) were cultivated and collected from Longtang Town, Xuwen County, Guangdong Province of People’s Republic of China, in December 2013. The plant material was identified by Prof. Guang-Xiong Zhou (Institute of Traditional Chinese Medicine & Natural Products, Jinan University), and a voucher specimen (No. 20131211) has been stored in the Institute of Traditional Chinese Medicine & Natural Products, College of Pharmacy, Jinan University, Guangzhou.

**Extraction and Isolation.** The general extraction and partition procedures of the rhizomes of *A. officinarum* were described in our previous report.23 In this work, the petroleum ether-soluble fraction (500 g) was subjected to a silica gel column using a gradient mixture of petroleum ether and EtOAc (1:0→2:1, v/v) as eluent to afford 12 major fractions (1−12). Fraction 3 (56 g) was separated on a silica gel column with a cyclohexane−EtOAc mixture (1:0→0:1, v/v) as eluent to obtain seven subfractions (3A−3G). Subfraction 3C (6.4 g) was subjected to an ODS column eluted with a gradient mixture of MeOH and H₂O (30:70→100:0, v/v) to afford five subfractions (3C-1−3C-5). Subfraction 3C-3 (1.5 g) was further separated by a Sephadex LH-20 column (CHCl₃−MeOH, 50:50, v/v) and subsequently purified by reversed-phase preparative HPLC using MeOH−H₂O (90:10, v/v, 6 mL/min) as the mobile phase to yield compound 1 (6.8 mg). Fraction 4 (61 g) was subjected to a silica gel column eluted with a gradient mixture of petroleum ether and EtOAc (100:5→1:1, v/v) to afford five subfractions (4A−4E). Subfraction 4B (7.6 g) was then separated on an ODS column with MeOH−H₂O (20:80→90:10, v/v) as eluent to yield five subfractions (4B-1−4B-5). Subfraction 4B-2 (1.1 g) was subsequently purified by reversed-phase preparative HPLC, using CH₃CN−H₂O (75:25, v/v, 6 mL/min) as the mobile phase to afford compound 2 (4.8 mg). Compound 3 (4.5 mg) was afforded from subfraction 4B-3 (0.75 g) by preparative HPLC using CH₃CN−H₂O (80:20, v/v, 6 mL/min) as the mobile phase.

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**Figure 9.** (+)-1 protects OGD/R-induced cortical neuronal injury through the PI3K/AKT/mTOR signaling pathway. (A) Treatment of LY294002 (LY) and Torin1 blocked the effect of (+)-1 to attenuate the expression levels of C-Casp-3 in OGD/R-damaged neurons. (B) Relative band density of C-Casp-3 was measured with ImageJ and normalized to that of GAPDH. $^{**}p < 0.01$, vs control. $^{***}p < 0.01$, vs OGD/R group. $^{###}p < 0.001$, vs OGD/R+ (+)-1 group. (C) Cell viability was measured by MTT. $^{##}p < 0.01$, vs control. **$p < 0.01$, vs OGD/R group. $^{##}p < 0.05$, $^{###}p < 0.01$, vs OGD/R+ (+)-1 group. Data are presented as mean ± SEM from at least three independent experiments.
Chiral Resolution of Enantiomers. Chiral resolution of (+)-1 was carried out on an Agilent 1260 liquid chromatography system equipped with a DAD detector and a Phenomenex Cellulose-4 column (4.6 mm × 250 mm, i.d. 5 μm, Phenomenex, CA). A pair of enantiomers (+)-1 (tR 11.9 min, 1.6 mg) and (−)-1 (tR 12.4 min, 1.5 mg) were obtained, respectively, by employing MeOH–H2O (80:20, v/v) as the mobile phase, with a flow rate of 1 mL/min.

(+)-Alpinidinoid A (1). Yellow oil; [α]D25 + 0 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 208 (3.43), 262 (1.89) nm; IR (KBr) νmax 2929, 2928, 1627, 1601, 1493, 1454, 1385, 1117, 1063, 746, 699 cm−1; 1H NMR (CDCl3, 500 MHz) and 13C NMR (CDCl3, 125 MHz) data, see Table 1; HR–ESI−MS m/z 545.3056 [M + H]− (calcd for C38H41O3, 524.2936)−.

(−)-Alpinidinoid A (1). Yellow oil; [α]D25 − 28 (c 0.16, MeOH); ECD (MeOH, Δε) λmax 229 (+14.83), 253 (−2.24), 305 (±2.96) nm.

Alpinidinoid B (2). Yellow oil; [α]D25 +24 (c 0.15, MeOH); UV (MeOH) λmax (log ε) 209 (3.66), 260 (2.09) nm; IR (KBr) νmax 2923, 2911, 1645, 1601, 1499, 1454, 1365, 1069, 746, 700, 481 cm−1; ECD (MeOH, Δε) λmax 259 (−19.05), 322 (±3.48) nm; 1H NMR (CDCl3, 600 Hz) and 13C NMR (CDCl3, 150 MHz) data, see Table 2; HR−ESI−MS m/z 547.3212 [M + H]+ (calcd for C39H43O3, 547.3212).

Alpinidinoid C (3). Yellow oil; UV (MeOH) λmax (log ε) 208 (3.59), 269 (2.58) nm; IR (KBr) νmax 3477, 2928, 1695, 1631, 1590, 1552, 1449, 1387, 1076, 1028, 746, 700 cm−1; 1H NMR (acetone-d6, 500 MHz) and 13C NMR (acetone-d6, 125 MHz) data, see Table 1; HR−ESI−MS m/z 542.2936 [M + H]+ (calcd for C38H40NO, 524.2953).

Computational Methods. The Gaussian 09 program package was employed to perform the calculation.26 The general computational methods for conformational analysis, energy optimization, and NMR and ECD simulation were similar to those in our previous report.27 Differently, the NMR data of all the selected conformers were calculated with the GIAO/mPW1PW91/6-311+G(d,p) methods in the gas phase. Also, the overall simulated CD curves were generated by the Boltzmann distribution of each conformer using SpecDis 1.71 software,26 and the theoretical NMR data were analyzed by using linear regression and DP4+ probability.28

Culture of Primary Cortical Neurons. Primary cortical neurons were prepared from E18 Sprague–Dawley rat embryos as previously described.29,30 Briefly, cortical neurons were dissociated for 15 min at 37 °C in 5 mL of calcium and magnesium-free Hank’s balanced salt solution (CMF-HBSS) (Life Technologies, CA) with 0.05% trypsin. Then, cells were centrifuged at 1300 rpm for 5 min, and the neurons were resuspended with Neurobasal medium (Life Technologies, CA) supplemented with 2% B27 (Life Technologies, CA). Cortical cells were seeded onto 96-well plates (1 × 104 per well) precoated with poly-l-lysine (Sigma-Aldrich, St. Louis, MO) for the MTT assay, and 35 mm dishes (8 × 103 per dish) for western blot analysis. The neurons were cultured for 7 days (7 days in vitro; DIV) for experiments.

Oxygen-Glucose Deprivation and Reoxygenation (OGD/R) Model and Compound Treatment. Neurons were randomly divided into control group, OGD/R group, and OGD/R++1 group. Cortical neurons were pretreated with 5 μM (+)-1 for 4 h in a normal medium. Then, the culture media were changed into glucose-free Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) and the cortical neurons were placed in a Modular Incubator Chamber (MIC-101) (Billups-rothenberg, Inc.) filled with a mixed gas of 95% N2 and 5% CO2 for 4 h. Afterwards, reoxygenation was simulated by replacing the glucose-free medium with a normal culture medium for 24 h. Control cells were not exposed to OGD/R. Cell survival was determined by the MTT assay.

Measurement of Cell Viability. Cell viability was performed by the MTT assay.21,23 Briefly, the culture medium was removed and MTT (5 mg/mL) was added to cells for 4 h, and then MTT was replaced with DMSO to dissolve formazan. The solution was centrifuged for 3 min at 3000 rpm. The absorbance values were detected at 595 nm in a microplate reader (Beckman coulter). Cell viability was presented as a percentage of the control group.

Detection of Apoptosis Using Flow Cytometry. Briefly, primary cortical neurons were collected by centrifugation, washed twice using cold phosphate buffered saline (PBS) buffer, and resuspended with 1× Annexin-binding buffer. Next, cells were incubated with Annexin V-FITC and PI for 15 min at room temperature in the dark. Finally, the antiapoptosis effect was measured by flow cytometry according to the manufacturer’s instructions (Abcam, U.K.).

Western Blot Analysis. At DIV 7, cells were pretreated with (+)-1 for 4 h and were insulted with OGD/R in glucose-free DMEM (Life Technologies). The cells were washed twice with cold PBS, collected in the radioimmunoprecipitation assay (RIPA) buffer accompanied with a protease inhibitor (Bimake), and lysed on ice for 30 min. Cell lysis was centrifuged at 4 °C, 12,000 rpm for 15 min. The protein concentration in the supernatant was determined with a BCA assay kit (Beyotime Biotechnology, China). The sodium dodecyl sulfate (SDS) sample buffer was added for denaturing the proteins, which were separated by 8–12% SDS–polyacrylamide gel electrophoresis (PAGE). The anti-rabbit primary antibodies used in Western Blot Analysis were p-AKT (1:1000, Cell Signaling Technology), AKT (1:1000, Cell Signaling Technology), p-mTOR (1:1000, Cell Signaling Technology), mTOR (1:1000, Cell Signaling Technology), cleaved-caspase-3 (1:1000, Cell Signaling Technology), or GAPDH (1:5000, Abbkine).

Statistical Analysis. Data processing and analysis were performed by using GraphPad Prism 5 software. All data were expressed as means ± standard error of mean (SEM) of at least three independent experiments. The difference between groups was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test.
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