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Note

An Efficient Regioselective Synthesis of 8-Formylhomoisoflavonoids with Neuroprotective Activity by Enhancing Autophagy

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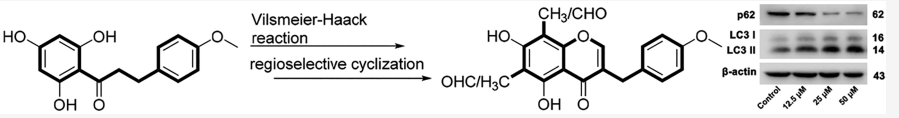

ABSTRACT: 6-Formylisoophiopogonone B (**7a**) and 8-formylophiopogonone B (**7b**), two natural products isolated from *Ophiopogon japonicus*, represent a subgroup of rare 6/8-formyl/methyl-homoisoflavonoid skeletons. Herein we report an efficient method for the synthesis of these formyl/methyl-homoisoflavonoids. The synthesized compounds were evaluated for their neuroprotective effects on the MPP^+-induced SH-SY5Y cell injury model and showed marked activity. Exploration of the neuroprotective mechanisms of compound **7b** led to an increased expression of autophagy marker LC3-II and down-regulation of autophagy substrate p62/SQSTM1. Molecular docking studies showed that **7b** may prevent the inhibition of the classic PI3K-AKT-mTOR signaling pathway by interfering with the human HSP90AA1.

Homoisoflavonoids are a special subclass of flavonoids that contain an additional carbon between the B and C rings of the isoflavonoid core (Figure 1).[1,2] To date, more than 240

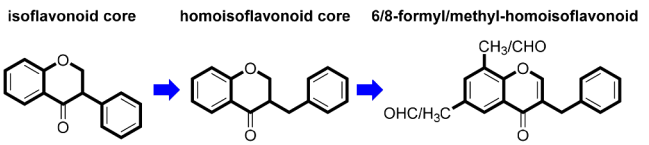

Figure 1. Isoflavonoid and homoisoflavonoid carbon skeletons.

naturally occurring homoisoflavonoids have been isolated from several plant genera, such as *Polygonatum*, *Ophiopogon*, *Muscari*, *Eucomis*, and *Caesalpinia*, in which many plant species have been applied in traditional herbal medicines.[3,4] From a structure diversity point of view, a subclass of rarely occurring C-6/C-8 methylated homoisoflavonoids were subsequently discovered, mostly from the genus *Ophiopogon*. These natural compounds have attracted growing attention due to their various biological activities, especially antitumor,[5] anti-inflammation,[6] antioxidation,[7,8] antipathogen,[9] and cardiovascular protection.[10] Accordingly, significant efforts have been devoted to the synthetic approach of these chromone derivatives.[11−16] Among the synthesized homoisoflavonoids, some were found to be potent and selective MAO-B or cholinesterase inhibitors,[17−19] indicating potential neuroprotective activity of these natural-product-like compounds. In our prior investigations, we have reported the isolation of a series of homoisoflavonoids from *O. japonicus*[20] and *P. cyrtonema*[21] with antioxidative, cytotoxic, and myocardial-protective activities. Subsequently, using a readily prepared chalcone as a key intermediate, 20 homoisoflavonoid derivatives with different substitutions on rings A and B were also synthesized through one-carbon extension annulation using $DMF/MeSO_2Cl$.[22] Structure−activity relationship studies showed that the 6/8-methyl or formyl groups are crucial for their bioactivities, especially in their myocardial- and neuroprotective effects. With our ongoing interest in constructing the homoisoflavonoid-related scaffold, a series of quinazolinone derivatives were synthesized by a four-step sequence with adoption of the scaffold hopping principle.[23] For the C-6/C-8 methyl- or formyl-homoisoflavonoids, a concise regioselective strategy was developed with a key step to introduce the 8-formyl group and to form ring C simultaneously. Herein, we report the regioselective total synthesis of formyl-containing homoisoflavonoids and derivatives in high yield. An in vitro assay and a mechanism of action study on the MPP^+-induced SH-SY5Y cell injury model indicated that some of them showed promising neuroprotective activity via enhancing autophagy.

As shown in Scheme 1, synthesis of 6-formylisoophiopogonone B (**7a**) and 8-formylophiopogonone B (**7b**) was first attempted starting from the commercially available 2′,4′,6′-trihydroxyacetophenone (**1**), which were protected using benzyl chloride in the presence of K_2CO_3 to afford diprotected acetophenone **2** in good yield. The remaining hydroxy group did not react due to the hydrogen bond with the adjacent

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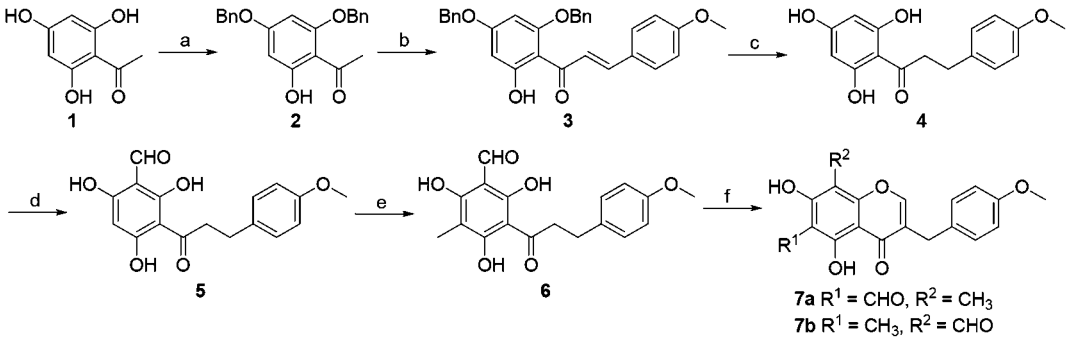
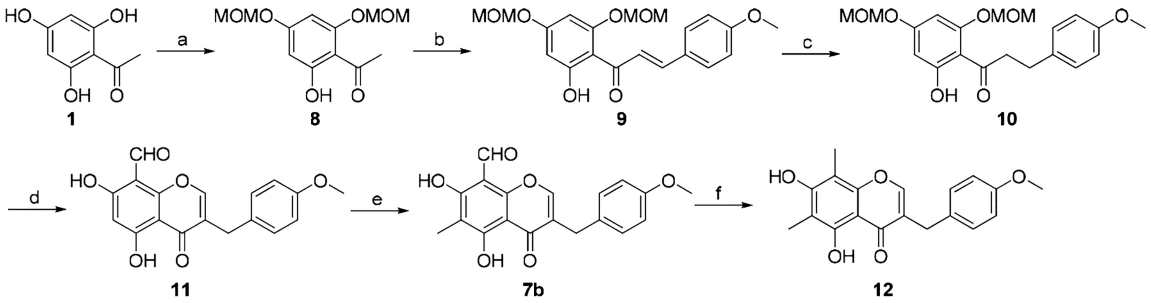
Scheme 1. Synthesis of 6-Formylisoophiopogonone B and 8-Formylophiopogonone B[a]



[a]Reagents and conditions: (a) BnCl, K_2CO_3, DMF, 70 °C, 16 h, 62%; (b) *p*-anisaldehyde, NaH, DMF, 0 °C, 30 min, 95%; (c) 10% Pd/C, H_2, rt, 12 h, 96%; (d) $POCl_3$, DMF, rt, 5 h, 80%; (e) MeI, MeOH, KOH, 70 °C, 2 h, 42%; (f) $MeSO_2Cl$, DMF, $BF_3{\cdot}OEt_2$, 80 °C, 2 h, 24% yield for **7a** and 41% yield for **7b**.

Scheme 2. Selective Synthesis of 8-Formylophiopogonone B[a]



[a]Reagents and conditions: (a) K_2CO_3, acetone, MOMCl, rt 3 h, 65%; (b) *p*-anisaldehyde, NaH, DMF, rt, 30 min, 90%; (c) 10% Pd/C, H_2, rt, 3 h, 98%; (d) $POCl_3$, DMF, rt, 5 h, 61%; (e) MeI, MeOH, KOH, 70 °C, 2 h, 42%; (f) Zn/Hg, HCl, CH_3OH, 62%.

carbonyl group. Next, chalcone **3** was assembled by an aldol condensation of di-*O*-benzylacetophenone (**2**) and 4-methoxybenzaldehyde. The resulting chalcone was reduced with 10% palladium/carbon, affording the corresponding deprotected dihydrochalcone **4** in 98% yield. Using $POCl_3$ and DMF, the formyl moiety was introduced at C-3 (A ring) of the dihydrochalcone **4** by a Vilsmeier−Haack reaction. Subsequently, methylation of product **5** with MeI and KOH afforded compound **6** in 50% yield. With the key intermediate **6** in hand, the final cyclization proceeded smoothly with $MeSO_2Cl$ in the presence of catalytic $BF_3{\cdot}OEt_2$, to furnish the natural products 6-formylisoophiopogonone B (**7a**) and 8-formylophiopogonone B (**7b**) in 24% and 41% yields, respectively.

Considering the promising neuroprotective activity of 8-formylophiopogonone B (**7b**), the regioselective synthesis of this compound was attempted. With the same starting material, the modified synthetic route began with a different phenolic protecting group by replacing the benzyl group with the methoxymethyl (MOM) group (Scheme 2, step a). The di-MOM-protected acetophenone **8** was subjected to the same aldol condensation/reduction sequence to afford the dihydrochalcone **10** in 90% yield, over two steps. Treatment of compound **10** with excess $POCl_3$ in the presence of DMF gave the homoisoflavonoid **11** in 65% yield.[24] This conversion is highly efficient because the annulation, deprotection of the MOM groups, and introduction of the formyl group occurred in one step. A plausible mechanism for regioselective formation of the C ring is proposed in the Supporting Information. Finally, the target compound 8-formylophiopogonone B (**7b**) was prepared by methylation with MeI and KOH. To facilitate the structure−activity relationship analysis, the natural product **7b** was reduced by a classical Clemmensen reduction to give the dimethyl derivative **12** in 85% yield.

To investigate the effects of different substituents on the activity, compounds **15a** and **15b** with C-6 and C-8 benzyl groups were prepared via similar procedures to those in Scheme 1. Under more vigorous reaction conditions, the benzylation product **13** was formed in 48% yield, in which an additional benzyl group was introduced on ring A compared to compound **2**. Having the benzylation product **13** in hand, we proceeded to the synthesis of dihydrochalcone **14** using the aforementioned condensation/reduction/formylation sequence. Not surprisingly, a mixture of homoisoflavonoids **15a** and **15b** was formed in the final cyclization step using $DMF/MeSO_2Cl$. These two products were separated by flash chromatography on silica gel.

Using the MPP^+-injured human neuroblastoma cell line SH-SY5Y as a model system, the synthesized homoisoflavonoid derivatives were screened for their neuroprotective capacity. As illustrated in Table 1, most of these compounds showed neuroprotective capacity at 5 μM, and the natural product 8-formylophiopogonone B (**7b**) showed the best protective activity. The A-ring formyl group is crucial for these structures to maintain their neuroprotective activity. Additionally, the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay on the SH-SY5Y cell line was conducted to test the potential cytotoxic effects of the active

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Table 1. Evaluation of Neuroprotective Efficacy and Cytotoxicity of the Synthesized Homoisoflavonoids

	neuroprotective efficacy: cell viability		cytotoxicity: inhibitory rate
compd	MPP^+	MPP^+ + comp @ 5 μM	comp @ 50 μM
7a	55.13	61.33 ± 1.08	11.25%
7b	53.24	62.08 ± 1.21	16.60%
11	55.86	60.45 ± 1.17	8.21%
13	52.72	51.68 ± 2.33	−1.97%
15a	49.57	52.32 ± 1.28	5.54%
15b	48.62	53.68 ± 1.49	10.40%

compounds. After incubation for 24 h, the compounds showed no significant effect on cell viability at the high-dose level (50 μM).

Based on our previous findings that the homoisoflavonoids may protect nerve cells by inducing autophagy,[22] the effect of 8-formylophiopogonone B (**7b**) on autophagy was further studied. As summarized in Figure 2A, monodansylcadaverine (MDC) was applied to compound **7b**-treated cells, which is a probe for autophagic vacuoles. According to the results of flow cytometry analysis, autophagy ratios were increased after SH-SY5Y cells were treated with compound **7b**. Quantitative analysis of autophagy was further pursued, and it was found that MDC positive ratios were notably increased after treatment with compound **7b** at high-dose level (50 μM). Furthermore, an enhanced expression of autophagy marker LC3-II and down-regulation of autophagy substrate p62/SQSTM1 were also observed after treatment with compound **7b** (Figure 2B).

The autophagy-inducing effects of the test compounds were further investigated by docking and reverse docking studies. As summarized in Table 2, a pool of potential targets was constructed with the help of the reverse docking procedure, Swiss Target Prediction. Human HSP90AA1, a classic autophagy biomarker, appeared frequently in the predicted targets. Subsequently, the binding conformations of the most active homoisoflavonoid, **7b**, to HSP90AA1 was predicted by molecular docking modeling utilizing the Libdock method. As shown in Figure 3, **7b** interacted with the active site of HSP90AA1 (PDB: 6N8X) through a hydrogen bond with LEU103 and THR184 amino acid residues. Overall, compound **7b** may rescue the inhibition of the classic PI3K-AKT-mTOR signaling pathway on autophagy by interfering with HSP90AA1.

In conclusion, an efficient total synthesis of the rare 6/8-formyl homoisoflavonoids, especially regioselective synthesis of 8-formylophiopogonone B (**7b**), was accomplished in good yield. The 8-formylhomoisoflavonoid **7b** showed promising neuroprotective activity by inducing autophagy on the MPP^+-induced SH-SY5Y cell injury model. Increased expression of autophagy marker LC3-II and down-regulation of autophagy substrate p62/SQSTM1were observed in the mechanism of action study. Further docking experiments revealed that these neuroprotective homoisoflavonoids may involve autophagy regulation via the PI3K-AKT-mTOR signaling pathway. Thus, compound **7b** represents a lead structure for developing new drug candidates against neurodegenerative disorders.

EXPERIMENTAL SECTION

General Experimental Procedures. All anhydrous solvents were purchased from Sigma-Aldrich (Shanghai, China) and directly used without further purification. Unless otherwise stated, reagents were commercially available and used as purchased without further purification. Chemicals were purchased from Sigma-Aldrich (Shanghai, China), TCI China (Shanghai, China), Acros (Beijing, China), Alfa Aesar (Shanghai, China), or J&K (Shanghai, China). ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker AVANCE III 500 MHz spectrometer (Bruker Co., Switzerland) with TMS as the internal standard. The chemical shifts are expressed in δ values (ppm), and the coupling constants (J) are reported in Hz. HRMS data were obtained on an Agilent Q-TOF 1290 LC/6224 MS system using ESI in positive or negative mode. The progress of reactions was monitored by TLC on silica gel and visualized by short-wave ultraviolet light. Flash chromatography was performed with Qingdao Haiyang flash silica gel (200−300 mesh).

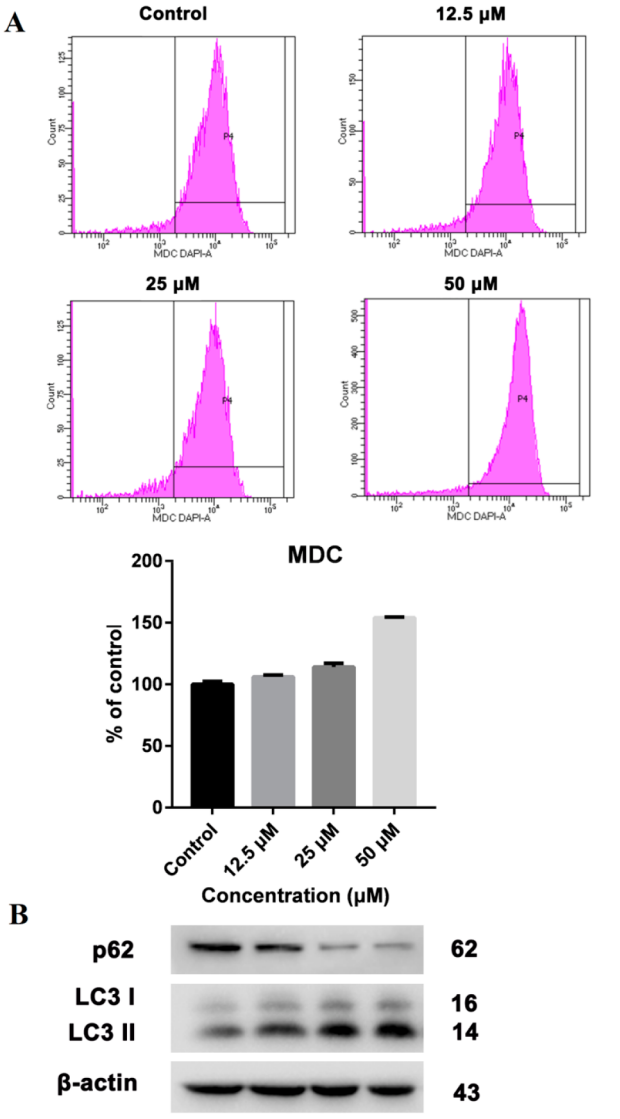

Figure 2. 8-Formylophiopogonone B (**7b**) induces autophagy in SH-SY5Y cells. (A) Flow cytometry analysis of MDC staining was used to determine autophagy ratios after **7b** treatment for concentrations of 0, 12.5, 25, and 50 μM. (B) Western blot analysis of the expression level of p62 and LC3 after treatment with **7b** for concentrations of 0, 12.5, 25, and 50 μM.

Synthesis of 7a, 7b, 11, 12, 15a, and 15b. The reaction conditions for the synthesis of the above-mentioned compounds are shown in Schemes 1−3. The intermediates were prepared as follows.

*1-[2,4-Bis(benzyloxy)-6-hydroxyphenyl]ethan-1-one (**2**).* To a solution of ketone **1** (3.36 g, 20 mmol, 1 equiv) in DMF (28 mL)

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Table 2. Selection of the Human Protein Targets Found by Swiss Target Prediction for Compounds 7a, 7b, 11, 15a, and 15b

compd	reverse docking procedure targets
7a	HSP90AB1, HSP90AA1, HSP90B1, GCGR, NQO1, CNR1, PDE10A, GPR55, ALPL, CYP11B1, CYP11B2, CFTR, NOS2, EGLN1, DNASE1L3, CNR2, PDK1, SIRT1, KIT, OPRD1, ABCB1, PCSK7, CYP2C9, SHBG
7b	HSP90AB1, HSP90AA1, HSP90B1, GCGR, NQO1, CNR1, PDE10A, GPR55, ALPL, CYP11B1, CYP11B2, CFTR, NOS2, EGLN1, DNASE1L3, CNR2, PDK1, SIRT1, KIT, OPRD1, CDK1, CDK4, IKBKB
11	NQO1, CNR1, CNR2, GPR55, CYP11B1, CYP11B2, MAOB
15a	HSP90AB1, HSP90AA1, HSP90B1, GCGR, NQO1, CNR1, CALCA, GPR55, MELK, CYP11B1, CYP11B2, CFTR, NOS2, ABCB1, CTNNB1, CNR2, PDK1, PRKCG, PRKCD, PRKCA, PRKCB, PRKCE, PRKCH, AGTR1, BCL2L1, PLAU, CCR5, MPI, TNF, MAPK3, CDK6, GSK3B, F10
15b	HSP90AB1, HSP90AA1, CXCR2, NQO1, CXCR1, CNR1, CNR2, KCNMA1, GPR55, CYP11B1, CYP11B2

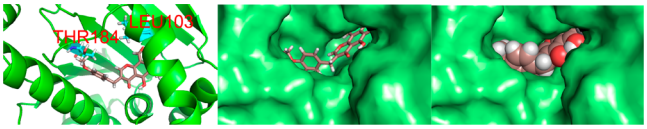


Figure 3. Compound **7b** docked in the active site of HSP90AA1.

were added K_2CO_3 (7 g, 50 mmol, 2.5 equiv) and benzyl chloride (4.5 mL, 40 mmol, 2 equiv) at 0 °C. After stirring at 70 °C for 12 h, the reaction mixture was diluted with water (30 mL) and the resulting mixture was extracted with EtOAc (3 × 15 mL). The organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 4:1) to give the product (4.31 g, 62% yield) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 14.02 (1H, s), 7.44−7.31 (10H, m), 6.17 (1H, d, J = 2.3 Hz), 6.10 (1H, d, J = 2.3 Hz), 5.06 (2H, s), 5.06 (2H, s), 2.56 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 203.2, 167.6, 165.1, 162.0, 135.9, 135.6, 128.8, 128.7, 128.5, 128.4, 128.0, 127.7, 106.4, 94.8, 92.4, 71.1, 70.3, 33.3; HRESIMS m/z $[M + H]^+$ 349.1438 (calcd for $C_{22}H_{21}O_4$, 349.1434).

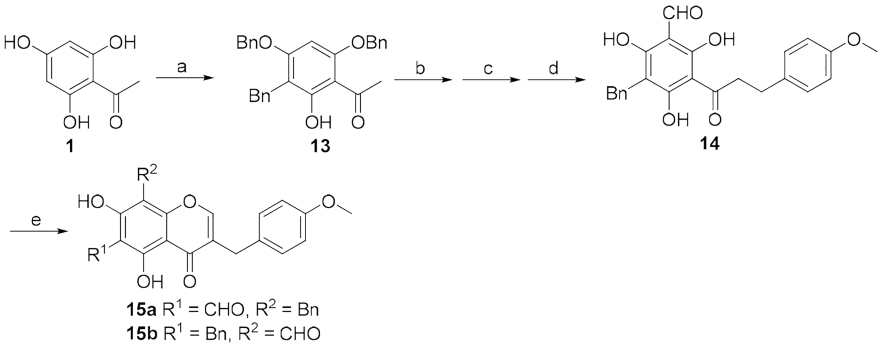
(E)-1-[2,4-Bis(benzyloxy)-6-hydroxyphenyl]-3-(4-methoxyphenyl)prop-2-en-1-one (**3**). To a stirred solution of **2** (3.48 g, 10 mmol, 1 equiv) in DMF (20 mL) were added NaH (0.36 g, 15 mmol, 1.5 equiv) and anisaldehyde (1.36 g, 10 mmol, 1 equiv) at 0 °C. After stirring for 30 min, the reaction mixture was quenched with water (20 mL), and the resulting mixture was extracted with EtOAc (10 mL × 3). The organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 5:1) to give chalcone **3** (4.43 g, 95% yield) as a yellow solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 14.72 (1H, d, J = 1.1 Hz), 7.80 (1H, d, J = 15.5 Hz), 7.71 (1H, d, J = 15.5 Hz), 7.54−7.49 (2H, m), 7.48−7.32 (8H, m), 7.02 (2H, d, J = 8.8 Hz), 6.71 (2H, d, J = 8.8 Hz), 6.22 (1H, d, J = 2.4 Hz), 6.17 (1H, d, J = 2.4 Hz), 5.10 (2H, s), 5.06 (2H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 192.6, 168.8, 165.1, 161.7, 161.2, 142.8, 135.9, 135.6, 130.2, 129.0, 128.8, 128.8, 128.6, 128.4, 128.2, 127.7, 125.2, 114.1, 106.4, 95.1, 92.5, 71.4, 70.3, 55.4; HRESIMS m/z $[M + H]^+$ 467.1836 (calcd for $C_{30}H_{27}O_5$, 467.1853).

3-(4-Methoxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one (**4**). Under a nitrogen atmosphere, to a stirred solution of chalcone **3** (2.33 g, 5 mmol, 1 equiv) in THF (20 mL) was added Pd/C (0.15 g, 6.4%) at room temperature. The reaction mixture was stirred at room temperature under 1 atm H_2 for 16 h. After filtering off the solids, the solvent was removed under reduced pressure. The crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 4:1) to give the product (1.38 g, 96% yield) as a white solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.23 (2H, s), 10.35 (1H, s), 7.15 (1H, d, J = 8.6 Hz), 6.84 (d, J = 8.6 Hz, 1H), 5.80 (1H, s), 3.71 (1H, s), 3.24 (2H, t, J = 7.7 Hz), 2.81 (2H, t, J = 7.7 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 204.1, 164.6, 164.2, 157.4, 133.5, 129.2, 113.7, 103.7, 94.6, 54.9, 45.3, 29.3; HRESIMS m/z $[M + H]^+$ 289.1065 (calcd for $C_{16}H_{17}O_5$, 289.1071).

2,4,6-Trihydroxy-3-[3-(4-methoxyphenyl)propanoyl]-benzaldehyde (**5**). Under a nitrogen atmosphere, to a stirred solution of dihydrochalcone **4** (1.44 g, 5 mmol, 1 equiv) in EtOAc (20 mL) were added DMF (0.52 mL, 5.5 mmol, 1.1 equiv) and $POCl_3$ (0.73 mL, 5.5 mmol, 1.1 equiv) at room temperature. After stirring for 3 h, the reaction mixture was quenched with water (30 mL) and the resulting mixture was extracted with EtOAc (3 × 15 mL). The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 7:1) to give the product (1.26 g, 80% yield) as a yellow solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 14.83 (1H, s), 13.65 (1H, s), 12.24 (1H, s), 9.99 (1H, s), 7.16 (2H, d, J = 8.6 Hz), 6.84 (2H, d, J = 8.6 Hz), 5.90 (1H, s), 3.71 (3H, s), 3.29 (2H, t, J = 7.6 Hz), 2.83 (2H, t, J = 7.6 Hz); ^{13}C NMR (125 MHz, DMSO-d_6) δ 204.8, 191.9, 170.6, 169.6, 167.4, 157.5, 133.0, 129.3, 113.8, 103.9, 103.3, 94.5, 55.0, 45.4, 28.8; HRESIMS m/z $[M + H]^+$ 317.1015 (calcd for $C_{17}H_{17}O_6$, 317.1020).

2,4,6-Trihydroxy-3-[3-(4-methoxyphenyl)propanoyl]-5-methylbenzaldehyde (**6**). Under a nitrogen atmosphere, to a stirred solution of compound **5** (2.1 g, 6.64 mmol, 1 equiv) in MeOH (34 mL) were added KOH (747 mg, 13.3 mmol, 2 equiv) and MeI (1.89 g, 13.3

Scheme 3. Synthesis of Homoisoflavonoids 15a and 15b[a]



[a]Reagents and conditions: (a) BnCl, K_2CO_3, DMF, 90 °C, 24 h, 48%; (b) *p*-anisaldehyde, NaH, DMF, 0 °C, 30 min, 92%; (c) 10% Pd/C, H_2, rt, 12 h, 95%; (d) $POCl_3$, DMF, rt, 5 h, 75%; (e) $MeSO_2Cl$, DMF, $BF_3{\cdot}Et_2O$, 80 °C, 2 h.

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mmol, 2 equiv). After stirring at 70 °C for 2 h, the reaction mixture was quenched with water (30 mL), and the resulting mixture was extracted with EtOAc (3 × 15 mL). The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 9:1) to give the product (921 mg, 42% yield) as a yellow solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.05 (1H, s), 7.17 (2H, d, J = 8.7 Hz), 6.84 (2H, d, J = 8.7 Hz), 3.71 (3H, s), 3.35 (2H, t, J = 7.6 Hz), 2.85 (2H, t, J = 7.6 Hz), 1.94 (3H, s); HRESIMS m/z $[M + H]^+$ 331.1159 (calcd for $C_{18}H_{19}O_6$, 331.1176).

*6-Formylisoophiopogonone B (**7a**).* Under a nitrogen atmosphere, to a stirred solution of **6** (1.44 g, 5 mmol, 1 equiv) in DMF (20 mL) were added $BF_3{\cdot}Et_2O$ (0.69 mL, 5.5 mmol, 1.1 equiv) and $MeSO_2Cl$ (0.46 mL, 6 mmol, 1.2 equiv) at 0 °C. After stirring at 80 °C for 2 h, the reaction was diluted with water (30 mL) and the resulting mixture was extracted with EtOAc (3 × 15 mL). The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude material was purified by flash chromatography on silica gel (eluted with CH_2Cl_2/MeOH = 9:1) to give the product (408 mg, 24% yield) as a yellow solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 14.04 (1H, s), 12.62 (1H, s), 10.34 (1H, s), 7.53 (1H, s), 7.19 (2H, d, J = 8.6 Hz), 6.87 (2H, d, J = 8.6 Hz), 3.80 (3H, s), 3.70 (2H, s), 2.10 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 192.8, 182.3, 165.9, 165.2, 159.8, 158.6, 153.8, 130.1, 129.4, 123.6, 114.3, 106.2, 104.1, 103.0, 55.4, 30.0, 6.5 (for detailed assignments, see ref 20); HRESIMS m/z $[M + H]^+$ 341.1013 (calcd for $C_{19}H_{17}O_6$, 341.1020). The 8-formylophiopogonone B (**7b**) isomer was simultaneously obtained in 42% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.78 (1H, s), 12.97 (1H, s), 10.21 (1H, s), 7.54 (1H, s), 7.19 (2H, d, J = 8.6 Hz), 6.88 (2H, d, J = 8.6 Hz), 3.80 (3H, s), 3.73 (2H, s), 2.09 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.8, 181.0, 167.5, 165.9, 158.7, 158.4, 152.7, 130.2, 129.3, 125.3, 114.4, 108.5, 104.5, 102.5, 55.4, 30.1, 6.5 (for detailed assignments, see ref 20); HRESIMS m/z $[M + H]^+$ 341.1013 (calcd for $C_{19}H_{17}O_6$, 341.1020).

*1-[2-Hydroxy-4,6-bis(methoxymethoxy)phenyl]ethan-1-one (**8**).* Under a nitrogen atmosphere, to a stirred solution of ketone **1** (1 g, 5.95 mmol, 1 equiv) in acetone (20 mL) were added K_2CO_3 (5 g, 36 mmol, 6 equiv) and MOMCl (1.08 mL, 14.28 mmol, 2.4 mmol) slowly. After stirring at room temperature for 3 h, the reaction mixture was diluted with water (30 mL), and the resulting mixture was extracted with EtOAc (3 × 15 mL). The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 10:1) to give the product (990 mg, 65% yield) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.70 (1H, s), 6.23−6.21 (2H, m), 5.23 (2H, s), 5.14 (2H, s), 3.50 (3H, s), 3.44 (3H, s), 2.63 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 203.3, 166.9, 163.5, 160.4, 106.9, 97.1, 94.5, 94.0, 56.8, 56.5, 33.0; HRESIMS m/z $[M + H]^+$ 257.1003 (calcd for $C_{12}H_{17}O_6$, 257.1020).

*(E)-1-[2-Hydroxy-4,6-bis(methoxymethoxy)phenyl]-3-(4-methoxyphenyl)prop-2-en-1-one (**9**).* Compound **9** was prepared according to the procedure described for **3** in 90% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.93 (1H, s), 7.81 (1H, d, J = 15.6 Hz), 7.80 (1H, d, J = 15.6 Hz), 7.56 (2H, d, J = 8.8 Hz), 6.93 (2H, d, J = 8.8 Hz), 6.32 (1H, d, J = 2.3 Hz), 6.24 (1H, d, J = 2.3 Hz), 5.29 (2H, s), 5.18 (2H, s), 3.85 (3H, s), 3.54 (3H, s), 3.48 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 193.0, 167.4, 163.4, 161.6, 160.0, 142.8, 130.2, 128.3, 125.1, 114.6, 107.7, 97.7, 95.3, 94.9, 94.2, 57.0, 56.6, 55.5; HRESIMS m/z $[M + H]^+$ 375.1421 (calcd for $C_{20}H_{23}O_7$, 375.1438).

*1-[2-Hydroxy-4,6-bis(methoxymethoxy)phenyl]-3-(4-methoxyphenyl)propan-1-one (**10**).* Compound **10** was prepared according to the procedure described for **4** in 98% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.71 (1H, s), 7.15 (2H, d, J = 8.6 Hz), 6.84 (2H, d, J = 8.6 Hz), 6.28 (1H, d, J = 2.3 Hz), 6.25 (1H, d, J = 2.3 Hz), 5.23 (2H, s), 5.17 (2H, s), 3.79 (3H, s), 3.47 (6H, d, J = 1.3 Hz), 3.35−3.32 (2H, m), 2.98−2.95 (2H, m); HRESIMS m/z $[M + H]^+$ 377.1599 (calcd for $C_{20}H_{25}O_7$, 377.1595).

*5,7-Dihydroxy-3-(4-methoxybenzyl)-4-oxo-4H-chromene-8-carbaldehyde (**11**).* Under a nitrogen atmosphere, to a stirred solution of **10** (3.76 g, 10 mmol, 1 equiv) in EtOAc (50 mL) were added DMF (2.08 mL, 22 mmol, 2.2 equiv) and $POCl_3$ (2.92 mL, 22 mmol, 2.2 equiv) slowly at room temperature. After stirring at room temperature for 5 h, the reaction mixture was quenched with water (50 mL), and the resulting mixture was extracted with EtOAc (3 × 25 mL). The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 9:1) to give the product (1.99 g, 61% yield) as a yellow solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.55 (1H, s), 12.69 (1H, s), 10.19 (1H, s), 7.56 (1H, t, J = 1.2 Hz), 7.19 (2H, d, J = 8.6 Hz), 6.88 (2H, d, J = 8.6 Hz), 6.26 (1H, s), 3.79 (3H, s), 3.72 (2H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.8, 180.9, 169.6, 168.6, 160.1, 158.7, 152.8, 130.1, 129.0, 125.5, 114.4, 105.0, 103.0, 99.9, 55.4, 30.0; HRESIMS m/z $[M + H]^+$ 327.0843 (calcd for $C_{18}H_{15}O_6$, 327.0863).

*5,7-Dihydroxy-3-(4-methoxybenzyl)-6,8-dimethyl-4H-chromen-4-one (**12**).* To a solution of **7b** (100 mg, 0.3 mmol, 1 equiv) in MeOH (5 mL) was added 3 N HCl (3 mL, 3 mmol, 10 equiv). After warming the mixture to 90 °C, amalgamated Zn was added slowly. After stirring vigorously under reflux for 12 h, the reaction mixture was filtered. The filtrate was concentrated under reduced pressure and the crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 7:1) to give the product (60 mg, 62% yield) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.94 (s, 1H), 7.52 (1H, s), 7.19 (2H, t, J = 1.2 Hz), 6.86 (2H, dd, J = 9.1, 2.5 Hz), 3.79 (3H, s), 3.72 (2H, s), 2.18 (3H, s), 2.16 (3H, s). The NMR spectral data match the previously published data.[23]

*1-[3-Benzyl-4,6-bis(benzyloxy)-2-hydroxyphenyl]ethanone (**13**).* To a solution of ketone **1** (1.18 g, 10 mmol, 1 equiv) in DMF (15 mL) were added K_2CO_3 (3.5 g, 25 mmol, 2.5 equiv) and benzyl chloride (2.3 mL, 20 mmol, 2 equiv) at 0 °C. After stirring at 90 °C for 24 h, the reaction mixture was diluted with water (30 mL) and the resulting mixture was extracted with EtOAc (3 × 15 mL). The organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude material was purified by flash chromatography on silica gel (eluted with hexanes/EtOAc = 5:1) to give the product (2.1 g, 48% yield) as a white solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.06 (1H, s), 7.23 (2H, t, J = 7.5 Hz), 7.15 (5H, dd, J = 15.7, 7.2 Hz), 6.83 (2H, d, J = 8.5 Hz), 3.89 (2H, s), 3.70 (3H, s), 3.34 (2H, t, J = 7.6 Hz), 2.84 (2H, t, J = 7.6 Hz); ^{13}C NMR (125 MHz, DMSO-d_6) δ 205.3, 192.9, 170.1, 167.8, 164.9, 157.5, 140.2, 133.0, 129.3, 128.2, 128.0, 125.7, 113.8, 106.6, 104.5, 103.5, 55.0, 45.7, 28.9, 26.8; HRESIMS m/z $[M + H]^+$ 439.1911 (calcd for $C_{29}H_{27}O_4$, 439.1904).

*8-Benzyl-5,7-dihydroxy-3-(4-methoxybenzyl)-4-oxo-4H-chromene-6-carbaldehyde (**15a**).* Compound **15a** was prepared according to the procedure described for **7a** in 31% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 14.18 (1H, s), 12.79 (1H, s), 10.39 (1H, s), 7.53 (1H, s), 7.30−7.26 (4H, m), 7.21 (2H, d, J = 8.6 Hz),− 7.20 (1H, m), 6.92 (2H, d, J = 8.6 Hz), 4.04 (2H, s), 3.84 (3H, s), 3.73 (2H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 192.8, 182.2, 166.5, 165.2, 159.8, 158.6, 153.8, 139.7, 130.2, 129.2, 128.5, 128.5, 126.3, 123.8, 114.3, 106.5, 106.3, 104.1, 55.4, 30.0, 27.3 (for detailed assignments, see the Supporting Information); HRESIMS m/z $[M + H]^+$ 417.1316 (calcd for $C_{25}H_{21}O_6$, 417.1333).

*6-Benzyl-5,7-dihydroxy-3-(4-methoxybenzyl)-4-oxo-4H-chromene-8-carbaldehyde (**15b**).* Compound **15b** was attained in the same steps as for the synthesis of **15a** in 43% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 14.02 (1H, s), 13.17 (1H, s), 10.30 (1H, s), 7.63 (1H, t, J = 1.1 Hz), 7.47 (2H, d, J = 7.2 Hz), 7. Twenty-seven (2H, m), 7.21 (2H, d, J = 8.6 Hz), 7.19 (1H, m), 6.97 (2H, d, J = 8.6 Hz), 4.08 (2H, s), 3.90 (3H, s), 3.82 (2H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.9, 181.0, 167.3, 165.9, 158.7, 158.7, 152.7, 140.1, 130.1, 129.2, 128.9, 128.4, 126.2, 125.3, 114.4, 112.1, 104.7, 102.6, 55.4, 30.1, 27.2 (for detailed assignments, see Supporting Information); HRESIMS m/z $[M + H]^+$ 417.1316 (calcd for $C_{25}H_{21}O_6$, 417.1333).

Experimental Procedure for Biological Screening. *Cell Culture.* The human neuroblastoma cell line (SH-SY5Y) was

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purchased from American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. MPP^+ was dissolved in 10 mM DMEM stock solution right before use and diluted to the appropriate concentration in treatment medium. SH-SY5Y cells were seeded at a density of 1.0×10^6 cells/mL and incubated with each compound for 16 h prior to adding MPP^+ to the medium. Cells were treated with MPP^+ at 50 μM and cultured for the last 8 h of each compound treatment.

In Vitro Cytotoxicity Assay. The cytotoxic effects of all compounds were determined using the standard MTT assay. SH-SY5Y cells were harvested from culture flasks, resuspended in cell culture medium, and plated at a density of 1.0×10^6 cells/mL onto a 96-well culture plate. Cells were incubated for 72 h (5% CO_2). Activation solution (20 μL) was added to the MTT reagent. The reaction solutions were added to each well. The plate was incubated for 2 h and shaken gently to evenly distribute the dye in the wells. Absorbance was measured at a wavelength of 490 nm.

Autophagy Assay. The SH-SY5Y cells were treated with or without 5 μM of each compound for 24 h and cultured with 0.05 mM MDC at 37 °C for 30 min. The fluorescence intensity of cells was analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were transfected with GFP-LC3 plasmid using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The fluorescence of GFP-LC3 was observed under a fluorescence microscope. Electron microscopy analysis was also performed to observe the autophagic vacuoles (Hitachi 7000, Japan).

Monodansylcadaverine Staining Assays. Treated cells were stained with MDC (0.05 mmol/L) for 15 min. The cells were collected, washed with PBS three times, and resuspended. MDC positive ratios were measured by a Beckman CytoFLEX FCM flow cytometer.

Western Blot. Proteins were extracted with lysis buffer solution. The lysates were centrifuged at 12000*g* for 10 min at 4 °C, the supernatants were transferred to a new tube, and the protein concentration was determined with a BCA kit (Beyotime, Jiangsu, China). The solubilized protein was run on 12% SDS-polyacrylamide gels and blotted electrophoretically onto a nitrocellulose membrane. The membrane was sequentially incubated with a 5% skimmed milk (TBST, TBS/0.1% Tween-20) solution at room temperature for 1 h and then with a specific primary antibody at 4 °C overnight. The membrane was washed three times with TBST for 10 min each time and subsequently incubated with secondary horseradish peroxidase-conjugated and goat anti-rabbit-mouse IgG (CST) at room temperature for 2 h. Antibody binding was detected using an Immobilon Western chemiluminescent HRP substrate reagent and visualized on a Biorad gel imaging system.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00830.

Additional information (PDF)

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Notes

The authors declare no competing financial interest.

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