3,4-seco-28-Nor-oleanane triterpenes from Camellia japonica protect from neurotoxicity in a rotenone model of Parkinson’s disease

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A natural material extract library (Korea Bioactive Natural Material Bank) was screened with respect to their protective effects on neuronal cells, and a 70% ethanol extract from the flowers of Camellia japonica turned out to be a potential hit. Bioassay-guided fractionation of this active extract led to the isolation of six new 3,4-seco-28-nor-oleanane triterpenoids (1–6). The molecular structures of these new triterpenoids were elucidated through extensive spectroscopic analyses, including high-resolution MS and 1D- and 2D-NMR data. In a rotenone model of Parkinson’s disease (PD), compounds 3–6 effectively protected against neurotoxicity in the human dopaminergic SH-SY5Y cell line. Among these 3,4-seco-28-nor-oleanane triterpenoids, 4,17β,29-trihydroxy-16-oxo-3,4-seco-28-norolean-12-en-3-oxo acid n-butyl ester (5) exerted the strongest neuroprotective effect by suppressing the expression of α-synuclein and the intracellular production of reactive oxygen species (ROS) induced by rotenone treatment. In addition, compound 5 induced autophagy-associated protein 1A/1B-light chain 3 (LC3), which is known as an autophagy biomarker. These results suggest a new class of chemical entities for developing bioactive compounds for PD therapy.

1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder of the central nervous system (CNS) worldwide.1–3 This incurable disease is physiologically caused by a deficit in brain dopamine and the diminution of dopaminergic neurons in substantia nigra,4,5 and PD patients present clinical symptoms such as rigidity, tremors, bradykinesia, and postural instability.6 Worldwide, approximately 1–2% of people aged 65 years or older, that is, approximate five million people, are affected by PD.7,8 In recent years, this tendency has become more serious due to the rapid aging of the global population.9 Diagnosing this disease has long been a challenge due to the complicated and progressive symptoms among different stages and the confusion with the symptoms of other CNS disorders.10 The drugs used clinically for treating PD mainly include dopamine agonists and monoamine oxidase inhibitors.11 However, these approaches only relieve the PD symptoms; they do not inhibit or reverse the neurodegeneration.12

To date, the overall underlying mechanism of PD has remained elusive. However, it has been reported that mitochondrial complex I plays a critical role in the pathogenesis of PD.13 The increase in intracellular ROS production by mitochondrial dysfunction and α-synuclein oligomerization are considered to be causes or characteristics of PD lesions.14 Rotenone, an inhibitor for mitochondrial complex I, is able to induce α-synuclein oligomerization and is able to produce intracellular ROS.15,16 Through this function, rotenone has been reported to be an effective reagent for mimicking the biochemical process of PD, and this model has been used to screen potential active compounds for PD treatment.17 Considering the interrelationship between natural products and PD therapy, our group attempted to screen the Korea Bioactive Natural Material Bank (KBNMB) using a rotenone-induced Parkinson’s disease model to search for new bioactive natural products with the potential for treating PD.
During this process, we found as a potential hit a 70% ethanol extract from the flowers of *Camellia japonica*. The plant *C. japonica* L., which belongs to the Theaceae family, is widely cultivated in Korea, China, and Japan.\(^{18,19}\) In addition to its use as an ornamental or garden tree, *C. japonica* has important uses in traditional medicine. The flower buds of this plant have been used for treating vomiting of blood and bleeding, and showed anti-inflammatory, tonic, and tonic activities.\(^{26}\) Previous phytochemical studies in our lab have found potential PTP1B (protein tyrosine phosphatase 1B) inhibitors\(^{22}\) and anti-PEDV (porcine epidemic diarrhea virus) active compounds\(^{21}\) from the fruit peels and flowers of this plant, respectively. Bioassay-guided fractionation using a rotenone-sensitive model resulted in the isolation of six new 3,4-seco-28-nor-oleanane triterpenoids (1–6) from the flowers of *C. japonica* (Fig. 1).

In this report, we will describe the isolation and structural elucidation of the purified compounds 1–6, as well as their protective effects on SH-SY5Y neuronal cells in a rotenone model of Parkinson’s disease.

2. Results and discussion

Using diverse chromatographic methods, including silica gel, RP-C<sub>18</sub>, Sephadex LH-20, and HPLC, six new 3,4-seco-28-nor-oleanane triterpenoids (1–6) were purified from a 70% ethanol extract from the flowers of *C. japonica* through bioassay-guided fractionation.

**Compound 1** was isolated as a white amorphous powder with [\(\alpha\)]<sub>D</sub>\(^{25}-20.6\) (c 0.1, MeOH). Its molecular formula was assigned as C<sub>29</sub>H<sub>46</sub>O<sub>7</sub>, as determined from the HRESIMS peak at m/z 529.3140 [M+Na]<sup>+</sup> (calc 529.3136), which suggested seven degrees of unsaturation. The IR absorptions indicated the existence of hydroxy (3390 cm<sup>-1</sup>), carbonyl (1710 cm<sup>-1</sup>), and olefin (1624 cm<sup>-1</sup>) functionalities. The <sup>1</sup>H and <sup>13</sup>C NMR data (Table 1) showed one oxygenated methene (\(\delta_H\) 3.60, 2H, s; \(\delta_C\) 74.1 (CH₂)), one oxymethine (\(\delta_H\) 4.19, 1H, dd, J = 11.0, 4.5 Hz; \(\delta_C\) 72.9 (CH)), two oxygenated quaternary carbons (\(\delta_C\) 77.1 (C) and 74.8 (C)), one trisubstituted double bond (\(\delta_H\) 5.69, 1H, t, J = 3.5 Hz; \(\delta_C\) 142.6 (C) and 125.4 (CH)), one ketone group (\(\delta_C\) 216.6 (C)), and one carboxylic carbon signal (\(\delta_C\) 177.5 (C)). Moreover, there were six carbon signals in the <sup>13</sup>C NMR spectrum assigned to methyl signals, which correspond to the NMR spectrum assigned to methyl signals, which correspond to the 1H NMR peaks at \(\delta_H\) 1.68, 1.55, 1.48, 1.34, and 1.25 (all s, each 3H). The above results indicated that compound 1 was a 3,4-seco-28-nor-oleanane triterpenoid.**\(^{23}\)** Compared with camelliaolean B,\(^{20}\) there were no methoxyl signals in the NMR spectra of compound 1 (Table 1), which indicated that a carbonyl group should be connected to C-2 in compound 1. This proposal was also confirmed by HMBC correlations (Fig. 2) from H-1 (\(\delta_H\) 3.05, 2.21) and H-2 (\(\delta_H\) 3.06, 2.66) to C-3 (\(\delta_C\) 177.5 (C)). In addition, the existence of one oxygenated methene group (\(\delta_H\) 3.60, 2H, s; \(\delta_C\) 74.1 (CH₂)) in compound 1, together with the molecular formula, indicated the existence of one additional hydroxy group, which was bonded to C-29 (\(\delta_C\) 74.1 (CH₂)) on the basis of HMBC correlations from H₂-29 (\(\delta_H\) 3.60) and H₃-30 (\(\delta_H\) 1.25) to C-19 (\(\delta_C\) 43.6), C-20 (\(\delta_C\) 37.1), and C-21 (\(\delta_C\) 32.7), as well as ROESY correlations from H-18 (\(\delta_H\) 3.30) to H₃-30 (\(\delta_H\) 1.25). The assignments of the other functionalities were determined based on HMBC and ROESY experiments (Fig. 2). The ROESY correlations from H-7 (\(\delta_H\) 4.19) to H-5 (\(\delta_H\) 1.94) and H-27 (\(\delta_H\) 1.55), together with the coupling patterns of H-5 (dd, J = 10.0, 3.1 Hz) and H-7 (dd, J = 11.0, 4.5 Hz), determined the a-orientation of H-5 and \(\beta\)-orientation of OH-7. The chemical shifts of C-16, C-17, C-18, and C-22 (\(\delta_C\) 161.3, 154.6, 125.4, and 122.6 (CH)), which were similar to those of camelliaolean B (\(\delta_C\) 161.5, 154.7, 125.4, and 122.2 (CH)), were used to determine the \(\beta\)-orientation of OH-17. Finally, compound 1 was elucidated as 4,7,17,19-tetrahydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oyl acid.

**Fig. 1.** Chemical structures of compounds 1–6 from *Camellia japonica*.
hydroxylation at Me-29 and the presence of an oxygenated n-butyl group in compound \( \beta \) (J=6.6 Hz), 1.59 (2H, m), 1.39 (2H, m), 0.95 (3H, t, J=7.2 Hz); \( \delta_C \) 65.4 (CH\( _2 \)), 31.8 (CH\( _2 \)), 20.2 (CH\( _2 \)), and 14.1 (CH\( _3 \)). An HMBC experiment revealed that this oxygenated n-butyl group was connected to C-3 based on the correlation of H\( _2 \)-2′ (\( \delta_H \) 4.04 (2H, t, J=6.6 Hz) to C-3 (\( \delta_C \) 176.8). Other functionalities were also determined as analyzed by HMBC correlations. Following the same procedure as for compounds 1 and 2, the orientation of both H-5 and H-7 was deduced from the coupling patterns of H-5 (dd, J=10.8, 3.2 Hz) and H-7 (dd, J=12.0, 4.8 Hz) and from ROESY correlations between H-7 (\( \delta_H \) 3.59) to H-5 (\( \delta_H \) 1.42) and H-27 (\( \delta_H \) 1.13). The similarity of the chemical shifts of C-16, C-17, C-18, and C-22 in compound 3 and camellialean B was used to determine the \( \beta \)-orientation of OH-17. Thus, compound 3 was identified as \( 4,7,17 \)-trihydroxy-16-oxo-3,4-seco-28-norolean-12-en-3-0ic acid n-butyl ester.

Compound 4, a white amorphous powder with \( J_\text{H} = 25 \)°C, 34.8 (c 0.1, MeOH), possessed a molecular formula of C\( _{40} \)H\( _{44} \)O\( _{6} \), as suggested by HRESIMS peaks at m/z 487.3423 [M+H\( ^+ \)] (calcd 487.3423) and 469.3317 [M−2H\( _2 \)O+H\( ^+ \)] (calcd 469.3318). The IR spectrum of compound 4 presented absorptions that are characteristic of hydroxyl (3442 cm\(^{-1}\)), carbonyl (1715 cm\(^{-1}\)), and olefin (1665 cm\(^{-1}\)) functionalities. Its \( ^{1} \)H and \( ^{1} \)C NMR data (Table 2) showed an oxymethene (\( \delta_H \) 3.16, br s; \( \delta_C \) 73.9 (CH\( _2 \))), two oxygenated quaternary carbons (\( \delta_C \) 77.2, 75.4), one trisubstituted double bond signal (\( \delta_H \) 5.43, t, J=3.5 Hz; \( \delta_C \) 142.6, 124.9), and two carboxyl carbons (\( \delta_C \) 213.9, 175.2). These observations indicated that compound 4 shared a similar structure with compound 2. The only difference was the disappearance of the oxymethine signals (\( \delta_H \) 3.74, 1H, dd, J=11.3, 4.1 Hz; \( \delta_C \) 73.4 (CH\( _2 \))) in compound 2, suggesting the absence of OH-7 in compound 4. Other functionalities were determined based on HSQC and HMBC experiments. The relative configuration of compound 4 was determined through a ROESY experiment and comparison of NMR data with analogs. The ROESY correlations from H-18 (\( \delta_H \) 2.82) to H-30 (\( \delta_H \) 0.96) indicated the attachment of one hydroxy group to C-29 (\( \delta_C \) 73.9 (CH\( _2 \))). The \( \beta \)-orientation of OH-17 was supported by the similarity of the chemical shifts of C-16, C-17, C-18, and C-22 (\( \delta_C \) 1625.0, \( \delta_C \) 176.1, \( \delta_C \) 52.3, and \( \delta_C \) 20.2 in CDCl\( _3 \)) of compound 4 and those of camellialean B (\( \delta_C \) 1621.5, \( \delta_C \) 176.3, \( \delta_C \) 52.5, and \( \delta_C \) 20.2 in CDCl\( _3 \)). The chemical shifts of C-5,9,10/6/10 in compound 4 (\( \delta_C \) 75.4,52.3/23.1/41.9 in acetone-\( \delta_d \)) and camellialean A (\( \delta_C \) 75.4/52.3/23.1/41.9 in acetone-\( \delta_d \))
52.4/23.2/41.8 in acetone-\textit{d}6\textsubscript{30} were used to determined H-5 as \textit{a}-oriented, which was also supported by the coupling pattern of H-5 (\textit{d}d, \textit{J}=11.0, 3.0 Hz) and the ROESY correlations from H-9 (\textit{d}0.1, 1.81) to H-5 (\textit{d}0.1, 1.41) and H-\textit{27} (\textit{d}0.1, 1.16). Thus, compound 4 was determined to be 4,17\textbeta,29-trihydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid methyl ester.

Compound 5 was isolated as a white amorphous powder with \textit{[\textalpha\textsubscript{D}]}\textsubscript{25}+27.2 (c 0.1, MeOH) and was assigned a molecular formula of \textit{C}_{23}H_{35}O_{5} based on a positive HRESIMS peak at \textit{m/z} 529.3880 [M\textsuperscript{+}\textsuperscript{25+}–H\textsuperscript{2}O–H\textsuperscript{+}]\textsuperscript{+} (calcd 529.3893). The IR peaks at 3394, 1730, and 1614 cm\textsuperscript{-1} suggested the presence of hydroxy, carbonyl, and olefin functionalities, respectively. Compound 5 shared a similar NMR pattern (Table 2) with compound 4. The characteristic difference is the replacement of the methoxyl group in 4 (\textit{d}8.37, s; \textit{\delta} 51.4 (CH\textsubscript{3})) by an oxygenated \textit{n}-butyl group in 5 (\textit{d}8.37, 4.04, 2H, t, J=6.6 Hz), 1.59 (2H, m), 1.39 (2H, m), 0.95 (3H, t, J=7.8 Hz); \textit{\delta} 65.4 (CH\textsubscript{2}), 31.8 (CH\textsubscript{2}), 20.2 (CH\textsubscript{2}), and 14.1 (CH\textsubscript{3})), indicating \textit{n}-butyl esterification of the carboxylic group at C-3. This proposal was further supported by the HMBC correlation from H\textsubscript{2}-1\textsuperscript{1} (\textit{d}8.37, 4.04) to C-3 (\textit{d}176.7). Following the same procedure as compound 4, other functionalities and the relative configuration of 5 were determined via HSQC and HMBC experiments. The ROESY correlations from H-9 (\textit{d}8.37, 1.78) to H-5 (\textit{d}8.37, 1.39) and H-\textit{27} (\textit{d}8.37, 2.82) to H-30 (\textit{d}8.37, 0.97) indicated the \textit{a}-orientation of H-5 and the hydroxylation at Me-29, respectively. The \textit{\beta}-orientation of OH-17 was supported by the similar chemical shifts of C-16/17/18/22 (\textit{\delta} \text{C} 16.215.2, \text{C} 17.76.3, \text{C} 18.52.3, and \text{C} 22 30.1 in CDCl\textsubscript{3}) of compound 5 and those of camelliaeol B (\textit{\delta} \text{C} 16 215.1, \text{C} 17 76.3, \text{C} 18 52.5, and \text{C} 22 30.1 in CDCl\textsubscript{3})\textsuperscript{20}. Finally, compound 5 was elucidated as 4,17\textbeta,29-trihydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid \textit{n}-butyl ester.

Compound 6 was isolated as a white amorphous powder with \textit{[\textalpha\textsubscript{D}]}\textsubscript{25}+32.1 (c 0.1, MeOH), and it had a molecular formula of \textit{C}_{29}H_{44}O_{5} based on a positive HRFABMS peak at \textit{m/z} 473.3283 [M+H\textsuperscript{+}]\textsuperscript{+} (calcd 473.3267). The IR absorptions indicated the presence of hydroxy (3393 cm\textsuperscript{-1}), carbonyl (1714 cm\textsuperscript{-1}), and olefin (1596 cm\textsuperscript{-1}) functionalities. The \textit{H} and \textit{13C} NMR spectra showed one oxymethene group (\textit{d}8.24, 1H, \textit{d}, \textit{J}=10.0 Hz), 3.14 (1H, \textit{d}, \textit{J}=10.0 Hz); \textit{\delta} 73.8 (CH\textsubscript{2})), one oxygenated quaternary carbon (\textit{\delta} 77.1), one trisubstituted olefin system (\textit{d}8.52, 1H, t, \textit{J}=3.5 Hz; \textit{\delta} \text{C} 143.0, 124.4), one terminal double bond (\textit{d}8.89 (br s), 4.74 (br s); \textit{\delta} 148.3 (C), 114.0 (CH\textsubscript{2})), and two carbonyl carbons (\textit{\delta} \text{C} 213.9, 174.9). Further analysis indicated that the NMR patterns of compounds 1 and 6 were similar. However, the CH-7 signals (\textit{d}8.19, 1H, dd, \textit{J}=11.0, 4.5 Hz; \textit{\delta} 72.9) and the C-4 signal at \textit{\delta} 74.8 in compound 1 disappeared, whereas one terminal double bond (\textit{d}8.89 (br s), 4.74 (br s); \textit{\delta} 148.3 (C), 114.0 (CH\textsubscript{2})) was observed in compound 6, which suggested the absence of OH-7 and the presence of a \textit{\Delta}4,\textit{\textbeta},29-dihydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid system in 6. This proposal was confirmed by HSQC and HMBC experiments, which also determined other functionalities in compound 6. The \textit{a}-orientation of H-5, the hydroxylation of Me-29, and the relative configuration at C-17 were determined similar to those of compounds 1, 2, 4, and 5, as analyzed by the ROESY correlation from H-9 (\textit{d}8.02, 1H, \textit{d}, \textit{J}=2.07) and H-\textit{27} (\textit{d}8.19), and from H-18 (\textit{d}8.02) to H-30 (\textit{d}8.06) and comparing the chemical shifts of C-16/17/18/22 with those of compounds 1, 2, 4, 5, and camelliaeol B. Therefore, compound 6 was determined to be 17\textbeta,29-dihydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid.

Recently, a rotenone model of Parkinson’s disease has been established to search for candidates to treat PD\textsuperscript{17,22} in which human dopaminergic SH-SY5Y cells are exposed to 2.5 \textmu M of rotenone, a mitochondrial complex I inhibitor. The protection against
neurotoxicity of compounds 1–6 was examined with this model. In the first step, the cytotoxicities of compounds 1–6 were determined to estimate the optimum concentrations that are not toxic to SH-SY5Y cells. Consequently, cytotoxicity was not observed when cells were treated with less than 20 μM of compounds 1, 2, and 4–6, except for compound 3, as shown in Fig. 3A. Compound 3 was used at low concentrations of less than 10 μM because of its cytotoxicity to evaluate protective action against rotenone-induced dopaminergic cell death in SH-SY5Y cells. Therefore, cytotoxicity was not observed when cells were treated with less than 20 μM of compounds 1, 2, 4–6, and 1–5 μM of compound 3 were used to evaluate their neuroprotective effects on a rotenone model. As shown in Fig. 3B, pretreatment of compounds 3–6 for 24 h increased cell viability against cell death resulting from treatment by 2.5 μM rotenone in a concentration-dependent manner. Furthermore, the neuroprotective properties of compounds 3 and 5 were also analyzed by Western blot analysis (Supplementary data Fig. S25). Considering the cytotoxicities of compounds 1–6 and their protection against neurotoxicity, compound 5 was selected for further evaluation on rotenone-induced neurotoxicity in SH-SY5Y cells.

An abnormal accumulation of α-synuclein in the brain is a major feature of Parkinson’s disease.15,16 Therefore, α-synuclein could be a promising target for treating PD.23–26 Because rotenone is able to affect α-synuclein pathology, we focused on the change of α-synuclein expression in the presence or absence of compound 5 in SH-SY5Y cells. As shown in Fig. 4A and B, treatment with rotenone elevated the mRNA and protein expression level of α-synuclein in the presence of compound 5, whereas pretreatment with compound 5 significantly attenuated the increase of α-synuclein induced by rotenone treatment in a concentration-dependent manner. Multiple studies have shown that over-expression of α-synuclein is potentially related with the increase of reactive oxygen species (ROS) by mitochondrial dysfunction, finally leading to neuronal cell death.25,27 To examine the inhibitory action of ROS by compound 5, intracellular ROS generation was determined using a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay in SH-SY5Y cells.

### Table 2

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a. Recorded in acetone-d₆, and at 500 MHz.
b. Recorded in methanol-d₄ and at 600 MHz.

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cells, as presented in Fig. 5A.28,29 Treatment with rotenone significantly induced the ROS production compared with the non-treatment group. However, pretreatment with compound 5 at various concentrations effectively decreased the ROS production induced by rotenone treatment in a concentration-dependent manner.

Furthermore, we examined the autophagy regulation by rotenone and compound 5. Several studies have recently reported that autophagy is responsible for the clearance of α-synuclein induced by rotenone treatment. Considering that the autophagosomes which possess the LC3 in the membrane of vacuoles colocalized with α-synuclein-overexpressed aggregation as well as autophagic vacuoles were increased in rotenone-induced PD models,30 autophagy induced by the treatment of compound 5 might contribute to the reduction of the α-synuclein expression. The cells used in this study were stably transfected with GFP-LC3 vector, a commonly used autophagy marker,31,32 and sequentially treated with compound 5 and rotenone. While rotenone treatment caused chromosome condensation, compound 5 recovered rotenone-induced chromosome condensation. Moreover, the formation of autophagosomes was observed upon treatment with compound 5, suggesting that autophagy is modulated by the treatment with compound 5 (Fig. 5B).

Because Parkinson’s disease shows accumulation of abnormal aggregated protein (α-synuclein) in neuronal cells, its clearance has been focused on as a treatment marker of this disease.33 The sesquiterpene lactone reynosin from Laurus nobilis34,35 and xanthones from the root bark of Cudrania (Maclura) tricuspidata2 showed neuroprotective effects in human neuroblastoma SH-SY5Y cells. In particular, reynosin protected against cell death from dopamine-induced toxicity in SH-SY5Y cells by down-regulating the over-expression of α-synuclein. Until present, there have been only two 3,4-seco-28-nor-oleanane triterpenoids reported from C. japonica without activity, and they were isolated from the fruit peel of C. japonica by our group in 2013.20 Herein, six new 3,4-seco-28-nor-oleanane triterpenoids (1–6) were isolated from the flowers of C. japonica, and compounds 3–6 exhibited potential protective effects on SH-SY5Y neuronal cells in a rotenone model of Parkinson’s disease through autophagy activation.

3. Experimental

3.1. General

Optical rotation values were obtained using a JASCO P-2000 polarimeter (JASCO International Co. Ltd., Tokyo, Japan). IR spectra
were acquired using a Nicolet 6700 FT-IR spectrometer (Thermo Electron Corp., Waltham, MA, USA). NMR spectra were recorded on a Bruker Advance 500 or 600 MHz spectrometer. HRESIMS and HRFABMS were obtained using an Agilent 6530 Q-TOF (Agilent Technologies, Inc., Santa Clara, CA, USA) and JEOL JMS 700 (JEOL, Ltd., Tokyo, Japan) spectrometer, respectively. For column chromatography (CC), silica gel (63–200 μm particle size) and RP-C18 (40–63 μm particle size) were purchased from Merck (Darmstadt, Germany), while Sephadex LH-20 was purchased from Sigma-Aldrich (St. Louis, MO, USA). RP-18 F254 and silica gel 60 F254 plates, from Merck (Darmstadt, Germany), were used for TLC analysis. A Gilson HPLC system, equipped with an Optima Pak C18 column (10×250 mm, 10 μm particle size; RS Tech, Seoul, Korea), was used for the purification of compounds, with a flow rate of 2 mL/min and UV detection at 205 and 254 nm. In all fractionation processes, analytical-grade solvents were used.

### 3.2. Plant material

The flowers of *C. japonica* were collected from Gwangju city in February, 2013, and were authenticated by Prof. Won Keun OH at Seoul National University. A voucher specimen (SNU2013-02) was deposited at the College of Pharmacy, Seoul National University, Seoul, Republic of Korea.

### 3.3. Extraction and isolation

*C. japonica* flowers (0.5 kg) were dried and extracted with 70% ethanol three times (each for 2 days) at room temperature. A concentrated crude residue (56 g) was first suspended in deionized water (3 L, 40 °C) and then successively partitioned against n-hexane, EtOAc, and n-BuOH. Screening in a rotenone model of Parkinson’s disease indicated that the n-BuOH fraction exhibited potential protective effects on SH-SY5Y neuronal cells, and this assay was used in the entire bioactivity-guided fractionation process. The n-BuOH fraction (11.2 g) was chromatographed over silica gel CC (5×40 cm) eluting with EtOAc:MeOH (from 20:1 to 1:1) to afford eight subfractions (CJF1–CJF8) according to the TLC profile. Fraction CJF3 (1.1 g) was further subjected to RP-C18 CC (1.0×20 cm) eluting with n-hexane:EtOAc (from 20:1 to 1:1) to provide six subfractions (CJF3.1–CJF3.6). Compounds 4 (6.3 mg; tR = 39.2 min) and 6 (13.2 mg; tR = 36.1 min) were purified from CJF4.3 [301 mg; HPLC condition: mobile phase: MeOH:H2O (68:32)] and CJF4.4...
Fraction CJF5 (2.0 g) was separated using a Sephadex LH-20 CC (2.0×60 cm) eluting with 90% MeOH in H2O to yield 10 subfractions (CJF5.1–CJF5.10). Fraction CJF5.6 (325 mg) was purified using HPLC [mobile phase: MeOH:H2O (58:42)] to afford compound 2 (4.2 mg; tR=40.6 min), and compound 1 (3.8 mg; tR=27.6 min) was obtained from CJF5.7 (169 mg) via HPLC [mobile phase: MeOH:H2O (55:45)].

3.3.1. 4,7β,17β,29-Tetrahydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid (1). White amorphous powder; [α]D25 –20.6 (c 0.1, MeOH); IR (KBr) νmax 3390, 2981, 1710, 1624, 1181, 910 cm−1; 1H and 13C NMR data, Table 1; HRESIMS m/z 529.3140 [M+Na]+ (calcd for C29H46O7Na, 529.3136).

3.3.2. 4,7β,17β,29-Tetrahydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid methyl ester (2). White amorphous powder; [α]D25 –24.1 (c 0.1, MeOH); IR (KBr) νmax 3394, 2920, 1715, 1631, 1032, 903 cm−1; 1H and 13C NMR data, Table 1; HRESIMS m/z 1063.6682 [2M+Na]+ (calcd for C60H96O14Na, 1063.6692).

3.3.3. 4,7β,17β-Trihydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid n-butyl ester (3). White amorphous powder; [α]D25 –26.4 (c 0.1, MeOH); IR (KBr) νmax 3420, 2958, 1714, 1642, 1008, 851 cm−1;
3.4. Cytotoxicity assay

SH-SY5Y cells (human dopaminergic neuroblastoma cell line) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) and Ham’s F12 Nutrient Mixture (F12) (1:1, v:v) supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin and incubated at 37 °C under 5% CO₂. The cytotoxicity assay was performed by staining with (3,4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to measure cell viability.16 Briefly, cells were seeded onto 48-well plates and grown to 70–80% confluence. Subsequently, the cells were pretreated with various concentrations of compounds 1–6 dissolved in low-serum medium supplemented with 5% FBS for 24 h, and then 2.5 μM rotenone was added to the cells for 12 h. Then, 40 μL of a 2 mg/mL MTT solution was added to each well and incubated for 4 h at 37 °C in the dark. After discarding the medium, 250 μL of DMSO was added to each well to dissolve the MTT formazan crystals. The absorbance was measured at 550 nm using an absorbance microplate reader (VersaMax™, Randor, PA, USA).

3.5. Quantitative real-time PCR

Cells were seeded onto a 6-well plate and incubated for 1–2 days. When the cell confluence reached approximately 70–80%, the growth medium was replaced with low-serum medium in the presence of various concentrations of compound 5. After 24 h, the cells were treated with 2.5 μM rotenone and incubated for 12 h. Total RNA was isolated from the cells using the TRIzol method. The total RNA was reverse transcribed using random primer (iNTRON Biotechnology, INC, Korea) according to the manufacturer’s instruction. Real-time PCR was performed using selective primers for α-synuclein (forward: 5′-AAC GAC CAG TTG GGC AAG AA-3′, reverse: 5′-CCA CAG GCA TAT CTT CCA GAT TCC-3′) and 18S ribosomal RNA (forward: 5′-GCT TAA TTT GAC TCA ACA CGG GA-3′, reverse: 5′-AGC TAT CAA TCT GTC AAT CTT GTC-3′) and conducted using 2 μL of cDNA and Maxima SYBR Green qPCR master mix 2X (Thermo sci., Rockford, IL, USA). The cycling conditions for real-time PCR were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time PCR was conducted using a StepOnePlus Real-Time PCR System (Applied Biosystems). The data were analyzed using StepOne software v2.3.

3.6. Western blot analysis

The cultures were prepared using methods similar to those for quantitative real-time PCR. After 12 h, the cells were washed with cold PBS and stored at −80 °C. For whole cell lysate, the cells were lysed on ice in 100 μL lysis buffer [50 mM Tris–HCl (pH 7.6), 120 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 50 mM NaF] and centrifuged at 14,000×g for 20 min. Supernatants were collected from the lysates, and protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Inc, CA, USA). Aliquots of lysates were boiled for 5 min and electrophoresed on 15% SDS-polyacrylamide gels. Protein in the gels were electronically transferred to nitrocellulose membranes (PVDF 0.45 μm, Immobilon-P, USA). The membranes were then incubated with primary antibodies α-synuclein (SC-12767, Santa Cruz Biotechnology, INC,) and mouse monoclonal actin antibody. The membranes were further incubated with secondary antibodies. Finally, they were detected using an enhanced chemiluminescence western blot detection kit (Thermo sci., Rockford, IL, USA) and quantified by LAS 4000 luminessent image analyzer (Fuji Film, Tokyo, Japan).

3.7. Measurement of intracellular ROS levels

The fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFDA), a well-known ROS indicator, was used to monitor intracellular ROS production in SH-SY5Y cells. Briefly, SH-SY5Y cells were seeded onto 96-well plates and grown to 70–80% confluence. Then, the growth medium was replaced with low-serum medium supplemented with 5% of FBS in the presence or absence of various concentrations of compound 5. After 24 h of incubation, the cells were treated with 2.5 μM rotenone and incubated for 12 h at 37 °C. The cells were then incubated with 20 μM of DCFH–DA (D6883, Sigma–Aldrich, St. Louis, MO, USA) for 30 min at 37 °C in PBS supplemented with 0.5% FBS and then washed twice with PBS. The fluorescence intensity of the cells were monitored using a fluorescence microplate reader (Spectra Max GEMINI XPS, Molecular Devices, Sunnyvale, CA, USA) with excitation at 488 nm and emission at 530 nm.

3.8. Confocal immunostaining

HEK293 cells were grown in DMEM (Welgene, Gyeongsangbuk-do, Korea) supplemented with 10% FBS (Gibco, Grand Island, NY, USA). A HEK293 stable cell line expressing GFP-LC3 was generated as described previously29 using a GFP-LC3 plasmid provided by T. Yoshimori.31 For immunostaining, cells were grown on sterilized glass coverslips. After drug treatment, the cells were fixed with 4% paraformaldehyde. The slides were stained with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) and mounted in mounting medium (Vector, Burlingame, CA, USA). Images were captured using a Carl Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochem, Germany).

3.9. Statistical analysis

Statistical calculations were examined by analysis of variance (ANOVA), followed by Tukey’s range test, conducting in SPSS Statistics 20 (SPSS, Inc., Chicago, IL, USA). The results are presented as the means±SD of two to three independent experiments (* p<0.05, ** p<0.01, and *** p<0.001 compared to the rotenone treatment.

Acknowledgements

This work was supported in part by grants from the Korea Bio-active Natural Material Bank (NRF-2012M3A9B8021570) and from the Procurement and Development of Foreign Biological Resources

1H and 13C NMR data, Table 1; HRESIMS m/z 569.3819 [M+Na]+ (calcd for C34H46O9Na, 569.3813).

3.4. 4,17β,29-Trihydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid methyl ester (4). White amorphous powder; 1H NMR data, Table 2; HRESIMS m/z 487.3423 [M–H2O+H]+ (calcd for C30H45O5, 487.3423) and 469.3317 [M–2H2O+H]+ (calcd for C30H43O5, 469.3318).

3.5. 4,17β,29-Trihydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid n-butyl ester (5). White amorphous powder; 1H NMR data, Table 2; HRESIMS m/z 529.3880 [M–H2O+H]+ (calcd for C31H47O5, 529.3893).

3.3. 17β,29-Dihydroxy-16-oxo-3,4-seco-28-nor-olean-12-en-3-oic acid methyl ester (3). White amorphous powder; max 3393, 2925, 1714, 1596, 1027, 951 cm⁻¹; 1H and 13C NMR data, Table 2; HRESIMS m/z 473.3283 [M+Na]+ (calcd for C29H45O5, 473.3267).

3.6. Steady-state fluorescence measurements of compounds 2, 3, 4, 5, and 6. These compounds were used to inhibit the formation of Aβ fibrils. The fluorescence intensity of the cells were monitored using a fluorescence microplate reader (Spectra Max GEMINI XPS, Molecular Devices, Sunnyvale, CA, USA) with excitation at 488 nm and emission at 530 nm.

3.7. Measurement of intracellular ROS levels

The fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFDA), a well-known ROS indicator, was used to monitor intracellular ROS production in SH-SY5Y cells. Briefly, SH-SY5Y cells were seeded onto 96-well plates and grown to 70–80% confluence. Then, the growth medium was replaced with low-serum medium supplemented with 5% of FBS in the presence or absence of various concentrations of compound 5. After 24 h of incubation, the cells were treated with 2.5 μM rotenone and incubated for 12 h at 37 °C. The cells were then incubated with 20 μM of DCFH–DA (D6883, Sigma–Aldrich, St. Louis, MO, USA) for 30 min at 37 °C in PBS supplemented with 0.5% FBS and then washed twice with PBS. The fluorescence intensity of the cells were monitored using a fluorescence microplate reader (Spectra Max GEMINI XPS, Molecular Devices, Sunnyvale, CA, USA) with excitation at 488 nm and emission at 530 nm.

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Supplementary data

Supplementary data (High-resolution mass data and NMR spectra (1H, 13C, and HMBC) of compounds 1–6, and the figure shows that compounds 3 and 5 decreased the expression of α-synuclein induced by rotenone treatment as analyzed by Western blot in SH-SY5Y cells. These materials are available free of charge via the Internet at http://www.elsevier.com.) associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2016.04.045.

References and notes