Neuritogenic Activities of 1-Alkyloxygenipins

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We designed 1-alkyloxygenipins with the aim of improving the stability of genipins based on the structural and electronic properties of genipins, and prepared 1-alkyloxygenipins and examined their neuritogenic activities in PC12h cells. All genipin-derivatives exhibited electronic properties similar to those of genipin and induced significant neurite outgrowth. These compounds will be classified as nitric oxide synthase (NOS) activators (neuritogenic active compounds) since their lowest unoccupied molecular orbital (LUMO)-energies are similar to that of tetrahydrobiopterin (H4B). (1*R*)-*iso*Propyloxygenipin showed activity comparable to that of genipin, and unlike the parent compound genipin, it was found to be physiologically stable in rat liver homogenate.

Key words genipin; 1-*iso*propyloxygenipin; neuritogenic activity; neuronal nitric oxide synthase; tetrahydrobiopterin

Iridoids have been reported to possess various biological activities, such as anti-microbial, anti-inflammatory, and antioxidant activities.[1)] Furthermore, we have previously reported that several iridoid compounds, and their hydrolysates, have neuritogenic activity in PC12h cells,[2—4)] a subclone of a rat pheochromocytoma cell.[5)] Genipin, the aglycon of geniposide (gardenia jasminoides), is a particularly potent inducer of neurite outgrowth. We have previously suggested that genipin directly binds to neuronal nitric oxide synthase (nNOS) and activates, and consequently induces neurotrophic factor-like activity, similar to that of nerve growth factor.[4,6)] Subsequently, we found that the structural and electronic properties of genipin were comparable to those of tetrahydrobiopterin (H4B), an essential cofactor in neuronal nitric oxide synthase, by molecular orbital calculations as well as by superimposing and docking studies using computer-based molecular modeling techniques, and that the interactive domain of genipin is the H4B binding domain in nNOS.[7)] In the meantime, since the C_1 atom forms a hemiacetal structure, it is known that the dehydropyran ring in genipin is easily broken and that the dialdehyde produced reacts easily with amino and/or SH residues in physiological conditions (Fig. 1).[8)]

The present investigation was undertaken to obtain new genipin-derivatives that are both stable and have neuritogenic activity. This paper deals with neuritogenic activity and molecular docking studies of 1-*iso*propyloxygenipin (1*R*)-**4**, designed using theoretical calculations.

Results and Discussion

Gibbs Free Energy and HOMO-Energy of 1-Alkyloxygenipins As the formation of dialdehyde from fission of the dehydropyran ring of genipin should be easily predictable, 1-alkyloxygenipins (**1**—**6**), which do not readily decompose, were selected as stable genipin derivatives (Fig. 1). The stability of **1**—**6** is supported by a calculation (Gibbs free energy) using a Gaussian03 program (Table 1). Further, we have previously reported that the neuritogenic activity of genipin is closely related to their lowest unoccupied molecular orbital (LUMO)-energy.[4)] A plot of the highest occupied molecular orbital (HOMO)-energy *versus* the LUMO-energy for **1**—**6** showed similar electronic features to that of genipin, and their LUMO-energies are as close as possible to that of H4B (Fig. 2). Therefore, the following experiments were performed using 1-alkyloxygenipins (**1**—**6**).

Synthesis of 1-Alkyloxygenipins 1-Alkyloxygenipins (**1**—**6**) were obtained by reaction of genipin with each alco-

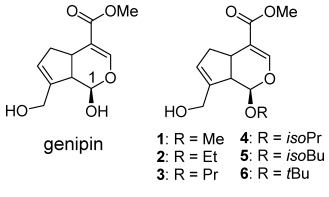

Fig. 1

Table 1. Program: Gaussian03 Method: DFT (B3LYP/6-31G*)

Compd.	Gibbs free energy (a.u.)
Genipin	−803.465
1	−842.747
2	−882.040
3	−921.291
4	−921.330
5	−960.579
6	−960.545

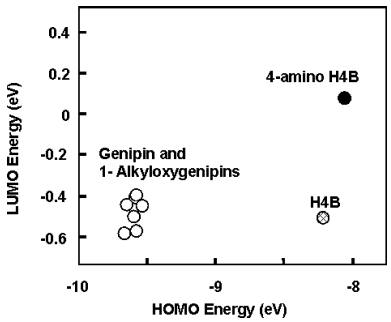

Fig. 2. Parameters Calculated by PM5 for Genipin and Their Derivatives, H4B and 4-Amino-H4B

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hol in the presence of acid (Chart 1). The structures of **1**—**6** were confirmed by NMR spectra to be mixtures of (*R*)- and (*S*)-isomers at 1-position.

Neuritogenic Activity for 1-Alkyloxygenipins Figure 3 presents the concentration-dependent neuritogenic activity for **1**—**6** in PC12h cells. The neuritogenic activity increased slightly in terms of the extension of the side chain of 1-alkyloxy substituents of **1**—**6**. The most effective among the 6 compounds was **4**, whose activity was similar to that of genipin. Thus, these results suggest that **4** was the most suitable compound for the induction of neurite outgrowth.

Stability of 1-*iso*Propyloxygenipin (4) **4** was chosen as a typical compound among 1-alkyloxygenipins for the measurement of stability. As shown in Fig. 6, under incubation in culture medium (Dulbecco's modified Eagle's medium (DMEM)) at 37 °C, the peak area of genipin had decreased to below half after 1 h of incubation, and disappeared completely after 6 h (data not shown). In contrast to this, a significant decrease in the peak area of **4** was not observed by incubation with rat liver homogenate. Moreover, after incubation of **4** with DMEM, a decrease in the peak area was not observed (data not shown) (Fig. 6C). These results indicated that **4** was suitable for studying the ADME system and the target molecule.

Isolation, Neuritogenic Activity and Docking Study of 1-*iso*Propyloxypgenipin (1*R*)-4 Since **4**, the most effective genipin-derivative, was a mixture of (*R*)- and (*S*)-isomers, the separation of **4** was carried out using HPLC (COSMOSIL 5C8-AR-300). Both the *R*-isomer (1*R*)-**4** and *S*-isomer (1*S*)-**4** separated were assigned by analysis of the coupling constants between C_1-H and C_{7a}-H using ^{1}H-NMR. Thus, the C_1-H of (1*R*)-**4** determined a doublet at 4.61 ppm of 8.7 Hz, and C_1-H of (1*S*)-**4** showed a doublet at 5.15 ppm of 3.2 Hz (Fig. 4). (1*R*)-**4** inhibited neuritogenic activity in Neuro2a cells more potently than (1*S*)-**4**. (1*R*)-**4** exhibited neuritogenic activity comparable to that of genipin (Fig. 5). We have previously suggested that the neuritogenic activity of genipin was induced by its direct-binding to and following activation of nNOS. We have proposed that this may be caused by the genipin-H4B binding domain interaction by docking analysis.[7] Thus, docking simulation of (1*R*)-**4** in nNOS-H4B binding domain (PDID: 1QW6) was carried out. The results of analysis in the H4B binding domain of (1*R*)-**4**, which exhibited the strongest activation, indicated the ester group at 4-position and the hydroxymethyl group at 7-position in (1*R*)-**4** acted with Trp678, Arg596, and Val677 (Fig. 7). A plot of the neuritogenic activity *versus* docking scores (free binding energy between **1**—**4** and H4B binding domain) for **1**—**4** is shown in Fig. 8. Binding affinities indicated a high correlation with neuritogenic activity, and neuritogenic activity was increased with an elongation of alkyl chain at 1-position. From the X-rays crystal structure of nNOS-H4B, a relatively planar molecular as H4B is probably desirable for interaction with nNOS-H4B binding domain because H4B is formed the π-stacking interaction with Trp678, and the space around the

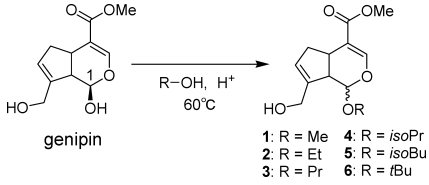

Chart 1

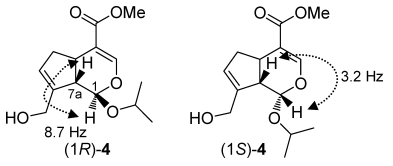

Fig. 4

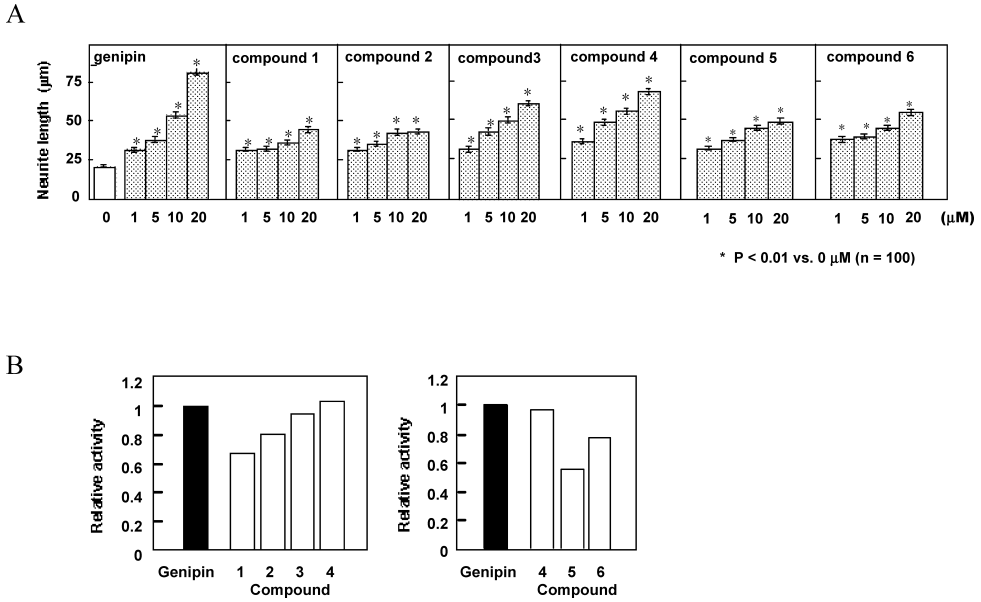

Fig. 3. Neuritogenic Activities of Genipin-Derivatives in PC12h Cells

Cells were cultured with a test compound (20 μM) or without (control) as indicated for 2 d. (A) Neuritogenic activity. Neurite outgrowth was measured in 100 cells in each group as described in Materials and Methods. The values shown are the mean±S.E.M. $*p<0.01$ *vs.* control. (B) Comparison of the relative activity in experiments carried out at the same time.

heme in nNOS is small. As all the carbon atoms at C3, C4 and C4-ester carbonyl carbon of genipin lie on the same plane, genipin may interact by the π-stacking interaction with nNOS-H4B binding domain. Further, 1-alkyloxy groups may be favorably located to the wide space in nNOS-H4B binding domain by both of an elongation of alkyl chain and a chain branching at 1-position. As above result, it probably considered that binding affinities of the ester group at 4-position (planar region) and the residues around the heme improved by the conversion from hemiacetal to acetal structure at 1-position.

Conclusion

The stability of genipin has been improved by converting the hydroxyl group at the 1-position to an alkyloxy group (conversion from hemiacetal to acetal structure). The present results suggest that 1-alkyloxygenipins retained approximately the same neuritogenic activity as genipin, and act on nNOS in a manner similar to that of genipin. Among them, (1*R*)-**4** demonstrated the most potent neuritogenic activity and the highest binding affinity. Therefore, we believe the search for and identification of stable genipin-derivatives will be useful for the development of neurotrophic compounds.

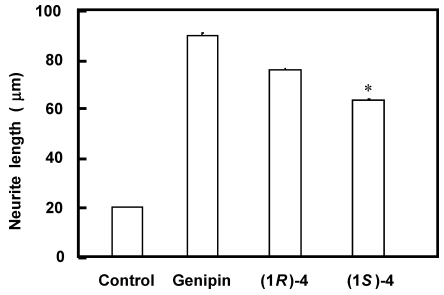

Fig. 5. Comparison of Neuritogenic Activity of (1*R*)-, (1*S*)-Derivatives of Compound **4** in PC12h Cells

Cells were cultured with a test compound (20 μM) or without (control) as indicated for 2 d. The neurite length was measured as described in Fig. 3. The values shown are the mean±S.E.M. $*p<0.01$ *vs.* treatment with (1*R*)-derivative.

Experimental

High resolution mass spectra (MS) were measured with a JEOL-DX300. Nuclear magnetic resonance spectra (^{1}H-NMR and ^{13}C-NMR) were recorded with a JNM-ECP400. Chemical shifts are quoted in parts per million (ppm) with tetramethyl silane as an internal standard. Coupling constants (*J*) are given in hertz (Hz).

Synthesis of 1-Alkyloxygenipins (1—6). General Procedure A mixture of genipin (10 g, 0.044 mol), alcohol (200 ml), and conc. HCl (1 drop) was stirred at 60 °C for 3 h. The reaction mixture was neutralized with 1 M

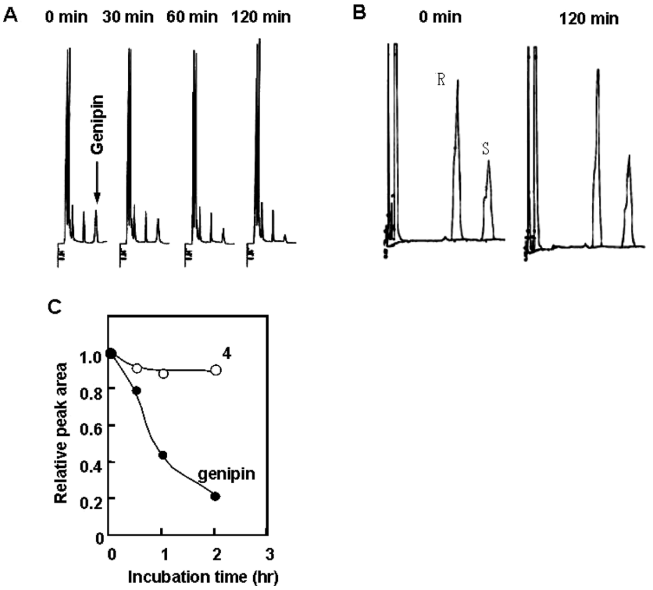

Fig. 6. Comparison of the Stability of Genipin and 1-*iso*Propyloxygenipin (**4**)

Genipin or **4** was incubated in culture medium or rat liver homogenate for 120 min at 37 °C, respectively. The medium incubated with genipin and the supernatants from the homogenates incubated with **4** were then applied to HPLC as described in Materials and Methods. (A) Elution profile of genipin after incubating in culture medium at 37 °C. (B) Elution profile of **4** after incubating in rat liver homogenate at 37 °C. (C) Comparison of the relative peak area for genipin and **4**. The values shown are relative to the value at time 0 h in (A) and (B), respectively.

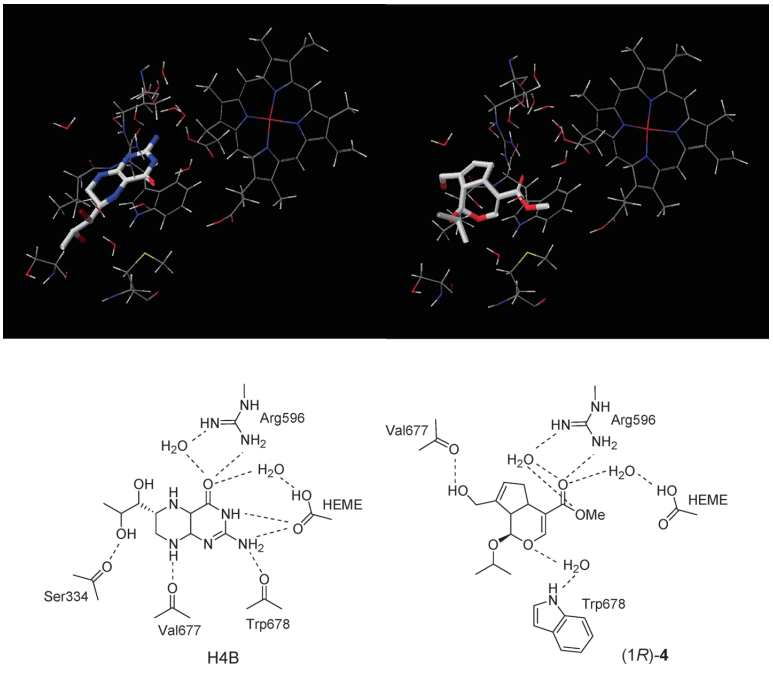

Fig. 7. The Preferred Pose Obtained for (1*R*)-**4** in nNOS-H4B Binding Domain

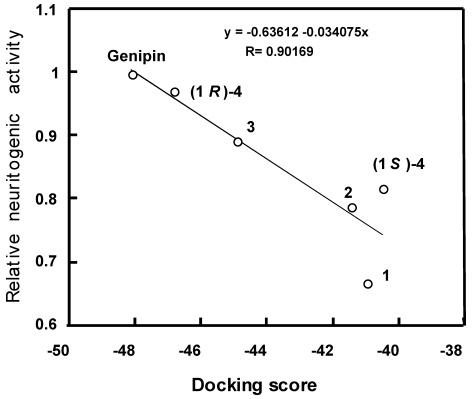

Fig. 8. Correlation between Docking Score and Neuritogenic Activity

The values of the plotted neuritogenic activities used a relative values in Figs. 3 and 5. The docking scores were calculated using a Scigress Explorer program (Fujitsu Co.).

aq. NaOH and then concentrated *in vacuo*. The residue was extracted with AcOEt. The extracts were washed with brine, dried, and evaporated *in vacuo*. The residue was purified by silica gel column chromatography using $CHCl_3$–AcOEt (3 : 1) as an eluent to give 1-alkyloxygenipin as a mixture of (*R*)- and (*S*)-isomers at the 1-position.

1-Methyloxygenipin (**1**): Yields 62%. ^{1}H-NMR ($CDCl_3$) δ: 2.05—2.26 (2H, m), 2.59—2.63 (2H, m), 2.74—2.92 (2H, m), 3.04—3.23 (2H, m), 3.43 (3H, s), 3.59 (3H, s), 3.73 (3H, s), 4.19 (1H, d, J=13.2 Hz), 4.25 (2H, s), 4.26 (1H, d, J=13.2 Hz), 4.49 (1H, d, J=8.1 Hz), 5.07 (1H, d, J=3.3 Hz), 5.80 (1H, s), 5.84 (1H, s), 7.46 (1H, s), 7.52 (1H, s). ^{13}C-NMR δ: 34.24 (d), 35.97 (d), 38.65 (t), 38.90 (t), 46.40 (d), 46.44 (d), 51.21 (q), 51.29 (q), 56.63 (q), 57.10 (q), 61.23 (t), 61.30 (t), 100.46 (d), 102.68 (d), 111.02 (s), 111.59 (s), 129.07 (d), 130.68 (d), 140.85 (s), 142.82 (s), 151.20 (d), 152.67 (d), 167.81 (s), 167.88 (s). HR-MS *m*/*z*: 240.0999 (Calcd for $C_{12}H_{16}O_5$: 240.0998).

1-Ethyloxygenipin (**2**): Yields 75%. ^{1}H-NMR ($CDCl_3$) δ: 1.21 (3H, t, J=7.1 Hz), 1.28 (3H, t, J=7.1 Hz), 2.06—2.10 (1H, m), 2.22—2.26 (1H, m), 2.57—2.91 (4H, m), 3.09—3.23 (2H, m), 3.65 (2H, t, J=7.3 Hz), 3.72 (3H, s), 3.73 (3H, s), 4.05 (2H, t, J=7.3 Hz), 4.15 (1H, d, J=13.2 Hz), 4.25 (1H, d, J=13.2 Hz), 4.26 (2H, s), 4.55 (1H, d, J=8.7 Hz), 5.11 (1H, d, J=3.2 Hz), 5.75 (1H, s), 5.84 (1H, s), 7.45 (1H, s), 7.52 (1H, s). ^{13}C-NMR δ: 14.95 (q), 14.96 (q), 34.75 (d), 36.28 (d), 38.48 (t), 38.93 (t), 46.52 (d), 46.99 (d), 51.18 (q), 51.28 (q), 61.16 (t), 61.35 (t), 65.02 (t), 65.73 (t), 99.43 (d), 101.61 (d), 111.88 (s), 111.46 (s), 129.04 (d), 130.43 (d), 140.77 (s), 142.98 (s), 151.93 (d), 152.25 (d), 167.82 (s), 167.86 (s). HR-MS *m*/*z*: 254.1155 (Calcd for $C_{13}H_{18}O_5$: 254.1154).

1-Propyloxygenipin (**3**): Yields 74%. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7.4 Hz), 0.96 (3H, t, J=7.4 Hz), 1.57 (2H, sext., J=7.4 Hz), 1.67 (2H, sext., J=7.4 Hz), 2.04—2.10 (1H, m), 2.22—2.28 (1H, m), 2.50—2.62 (1H, m), 2.72—2.78 (1H, m), 2.86—2.92 (1H, m), 3.08—3.10 (1H, m), 3.15—3.22 (1H, m), 3.51 (2H, t, J=7.4 Hz), 3.54 (2H, t, J=7.4 Hz), 3.72 (3H, s), 3.73 (3H, s), 4.18 (1H, d, J=13.1 Hz), 4.22 (1H, d, J=13.1 Hz), 4.23 (2H, s), 4.54 (1H, d, J=8.5 Hz), 5.10 (1H, d, J=3.0 Hz), 5.76 (1H, s), 5.83 (1H, s), 7.45 (1H, s), 7.52 (1H, s). ^{13}C-NMR δ: 10.49 (q), 10.61 (q), 22.77 (t), 22.79 (t), 34.72 (d), 36.28 (d), 38.55 (t), 38.96 (t), 46.48 (d), 46.87 (d), 51.16 (q), 51.25 (q), 61.24 (t), 61.40 (t), 71.19 (t), 71.79 (t), 99.69 (d), 101.75 (d), 110.89 (s), 111.44 (s), 128.82 (d), 130.45 (d), 140.89 (s), 143.13 (s), 151.82 (d), 152.23 (d), 167.82 (s), 167.87 (s). HR-MS *m*/*z*: 268.1310 (Calcd for $C_{14}H_{20}O_5$: 268.1311).

1-*iso*Propyloxygenipin (**4**): Yields 67%. ^{1}H-NMR ($CDCl_3$) δ: 1.17 (3H, d, J=6.0 Hz), 1.21 (3H, d, J=6.0 Hz), 1.22 (3H, d, J=6.0 Hz), 1.30 (3H, d, J=6.0 Hz), 2.04—2.11 (1H, m), 2.23—2.27 (1H, m), 2.57—2.61 (1H, m), 2.70—2.76 (1H, m), 2.86—2.93 (1H, m), 3.10—3.12 (1H, m), 3.16—3.23 (1H, m), 3.20—3.25 (1H, m), 3.72 (3H, s), 3.73 (3H, s), 4.02 (1H, q, J=6.0 Hz), 4.11 (1H, q, J=6.0 Hz), 4.18 (1H, d, J=13.0 Hz), 4.26 (1H, d, J=13.0 Hz), 4.27 (2H, s), 4.61 (1H, d, J=8.7 Hz), 5.15 (1H, d, J=3.2 Hz), 5.73 (1H, s), 5.82 (1H, s), 7.45 (1H, s), 7.51 (1H, s). ^{13}C-NMR δ: 21.41 (q), 21.44 (q), 23.28 (q), 23.45 (q), 35.13 (d), 36.45 (d), 38.40 (t), 38.98 (t), 46.57 (d), 47.46 (d), 51.14 (q), 51.25 (q), 61.13 (t), 61.34 (t), 71.37 (d), 72.15 (d), 97.97 (d), 99.95 (d), 110.81 (s), 111.39 (s), 128.55 (d), 130.20 (d), 140.85 (s), 143.32 (s), 152.35 (d), 152.39 (d), 167.88 (s), 167.90 (s). HR-MS *m*/*z*: 268.1311 (Calcd for $C_{14}H_{20}O_5$: 268.1311).

1-*iso*Butyloxygenipin (**5**): Yields 61%. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, d, J=6.6 Hz), 0.92 (3H, d, J=6.6 Hz), 0.94 (3H, d, J=6.6 Hz), 0.95 (3H, d, J=6.6 Hz), 1.83 (1H, sext., J=6.6 Hz), 1.94 (3H, t, J=6.6 Hz), 2.04—2.10 (1H, m), 2.23—2.28 (1H, m), 2.73—2.78 (1H, m), 2.86—2.91 (1H, m), 3.06—3.07 (1H, m), 3.14—3.23 (3H, m), 3.33 (1H, dd, J=6.6, 9.1 Hz), 3.59 (1H, dd, J=6.6, 9.1 Hz), 3.72 (3H, s), 3.73 (3H, s), 4.19 (1H, d, J=13.1 Hz), 4.27 (2H, s), 4.29 (1H, d, J=13.1 Hz), 4.53 (1H, d, J=8.5 Hz), 5.10 (1H, d, J=3.2 Hz), 5.77 (1H, s), 5.83 (1H, s), 7.45 (1H, s), 7.51 (1H, s). ^{13}C-NMR δ: 19.19 (q), 19.24 (q), 19.28 (q), 19.34 (q), 28.43 (d), 28.46 (d), 34.56 (d), 36.22 (d), 38.62 (t), 38.94 (t), 46.36 (d), 46.61 (d), 51.15 (q), 51.23 (q), 61.25 (t), 61.40 (t), 99.68 (d), 101.78 (d), 110.87 (s), 111.37 (s), 128.62 (d), 130.51 (d), 140.91 (s), 143.16 (s), 151.64 (d), 152.25 (d), 167.82 (s), 167.90 (s). HR-MS *m*/*z*: 282.1469 (Calcd for $C_{15}H_{22}O_5$: 282.1467).

1-*tert*Butyloxygenipin (**6**): Yields 61%. ^{1}H-NMR ($CDCl_3$) δ: 1.29 (9H, s), 1.31 (9H, s), 2.06—2.12 (1H, m), 2.25—2.29 (1H, m), 2.57—2.60 (1H, m), 2.68—2.72 (1H, m), 2.85—2.91 (1H, m), 3.07—3.09 (1H, m), 3.16—3.21 (1H, m), 3.25—3.27 (1H, m), 3.71 (3H, s), 3.74 (3H, s), 4.19 (1H, d, J=13.1 Hz), 4.26 (1H, d, J=13.1 Hz), 4.29 (2H, s), 4.75 (1H, d, J=8.5 Hz), 5.24 (1H, d, J=2.7 Hz), 5.76 (1H, s), 5.81 (1H, s), 7.45 (1H, s), 7.51 (1H, s). ^{13}C-NMR δ: 28.47 (q), 28.67 (q), 35.88 (d), 36.85 (d), 38.20 (t), 38.94 (t), 46.69 (d), 48.76 (d), 51.12 (q), 51.21 (q), 61.11 (t), 61.41 (t), 77.24 (s), 77.40 (s), 95.18 (d), 96.11 (d), 110.63 (s), 111.18 (s), 127.97 (d), 130.12 (d), 140.93 (s), 143.71 (s), 152.71 (d), 153.16 (d), 167.96 (s), 167.85 (s). HR-MS *m*/*z*: 282.1468 (Calcd for $C_{15}H_{22}O_5$: 282.1467).

The Gibbs free energy values of 1-alkyloxygenipins (**1**—**6**) were determined using B3LYP contained in a Gaussian03 software package together with the 6-31G* basis set function of the density functional theory.

Calculations of the HOMO-energy and LUMO-energy of the 1-alkyloxygenipins (**1**—**6**), genipin, H4B, and 4-amino-H4B (NOS inhibitor) were carried out using the PM5 method in the MOPAC program.

Docking simulations were performed using a Scigress Explorer ver.7.5 parameter set developed by Fujitsu Co. (Chiba, Japan).

For the evaluation of neurite outgrowth, PC12h cells were grown in DMEM supplemented with 5% (v/v) horse serum and 5% (v/v) precolostrum calf serum as previously reported.[1)] Briefly, PC12h cells were plated in 35 mm culture dishes coated with collagen at a density of 4×10^4 cells in 2 ml of medium per dish. After 24 h of culturing, the growth medium was replaced by DMEM/Ham's F12 (1 : 1) supplemented with sodium selenate, transferrin, insulin, and progesterone together with either vehicle (DMSO, unless otherwise noted) in the case of the control or one of the 1-alkyloxygenipins (**1**—**6**). Neurite outgrowth of the PC12h cells was evaluated after treatment for 24 h, unless otherwise specified, by measuring the longest neurite of individual cells as previously reported.[2)] The average neurite length of 100 cells in each treatment was calculated. Statistical analyses were performed using ANOVA and Student's *t*-test.

To evaluate the physiological stability of 1-isopropyloxygenipin (**4**), we observed the effect of incubation with rat liver homogenate prepared from Wister rats. Reverse-phase high performance liquid chromatography (HPLC) was performed on a Cosmosil column (5C8, 120 Å, 0.46×25 cm) using a Waters 610 Fluid Unit (Millipore Waters) with a 468 Tunable Absorbance Detector. Eluent was used with various % of acetonitrile containing 0.1% trifluoroacetic acid (TFA) (flow rate, 1 ml/min; detection, UV at 240 nm). Genipin or **4** was incubated with DMEM or the rat liver homogenate, respectively, before it was applied to HPLC.

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References

1) Dinda B., Debnath S., Harigawa Y., *Chem. Pharm. Bull.*, **55**, 689—728 (2007).
2) Yamazaki M., Chiba K., Mohri T., *Biol. Pharm. Bull.*, **19**, 791—795 (1996).
3) Chiba K., Yamazaki M., Kikuchi M., Machida K., Kikuchi M., *J. Health Sci.*, **52**, 743—747 (2006).
4) Yamazaki M., Chiba K., Mohri T., Hatanada H., *J. Neurochem.*, **79**, 45—54 (2001).
5) Hatanaka H., *Brain Res.*, **222**, 225—233 (1981).
6) Ohkubo T., Yamazaki M., Yoshida A., Chiba K., Mohri T., *J. Health Sci.*, **50**, 193—196 (2004).
7) Suzuki H., Yamazaki M., Chiba K., Sawanishi H., *J. Health Sci.*, **53**, 1—4 (2007).
8) Kawata Y., Hattori M., Akao T., Kobashi K., Namba T., *Planta Med.*, **57**, 536—542 (1991).