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Structure–Activity Relationships of Neuritogenic Gentiside Derivatives

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Neurotrophic factors are target-derived peptides that play an important role in the development and survival of responsive neuronal populations. Nerve growth factor (NGF), one of the most important neurotrophic factors, is essential for neuronal differentiation, growth, survival, function maintenance, and prevention of aging in the central and peripheral systems.[1,2] However, because of its large molecular size and hydrophilic properties, NGF cannot pass through the blood–brain barrier, limiting its use as a therapeutic agent. Therefore, synthetic low-molecular weight compounds that possess equivalent or better neuritogenic activity compared with NGF are promising agents for the treatment of neurodegenerative diseases, such as Alzheimer's disease.[3] The PC12 cell line, cloned from rat pheochromocytoma, is widely used as a model system to evaluate the biological activity of neuritogenic substances.[4–7]

Recently, screening for neuritogenic substances from traditional Chinese medicine resulted in the isolation of 11 novel alkyl benzoates (gentisides A–K) from *Gentiana rigescens* (Franch.).[8,9] These compounds are structurally different from one another through varying alkyl chain lengths and the presence or absence of an isobutyl or isopropyl group at the end of the alkyl chain. The structure–activity relationships within gentisides reveal that the alkyl chain length is important for activity, but structural diversity at the end of the alkyl chain is not. Gentisides D, E, and F have similar alkyl chain lengths. In spite of the different structures at the end of the alkyl chain, these compounds exhibit similar neuritogenic activities at the optimum concentration of 3 μM. Gentiside E, which has a straight alkyl chain of 18 carbon atoms, exhibits higher activity (74% at 3 μM) than gentiside K, which has 24 carbon atoms in the alkyl chain (48% at 30 μM).[9]

To study the structure–activity relationships and discover lead compounds for drug development, a series of gentiside derivatives were designed and synthesized. First, 2,3-dihydroxybenzoates **1a**–**j** (Table 1) with different alkyl chain lengths were synthesized to find the optimum length of the alkyl chain. Second, tetradecylbenzoates **1k**–**u** (Table 1) were prepared to study the importance of the number and position of the hydroxy groups on the benzene ring. Third, to find the best linkage group between the benzene ring and alkyl chain, tetradecyl-benzamides **1v**–**y**, phenylketones **2a**–**d**, 2,3-dihydroxyphenyl tetradecanoate (**3a**) and 3-(tetradecyloxy)benzene-1,2-diol (**3b**) were synthesized. Full experimental details can be found in the Supporting Information.

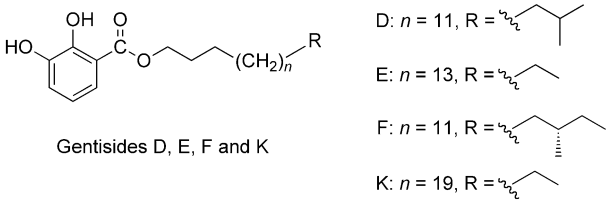
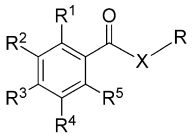
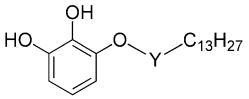


Table 1. Structures of gentiside derivatives **1a**–**y**, **2a**–**d** and **3a**–**b**.



1a–y, **2a–d**



3a Y = CO, **3b** Y = CH_2

Compd	R^1	R^2	R^3	R^4	R^5	X	R
1a	OH	OH	H	H	H	O	C_2H_5
1b	OH	OH	H	H	H	O	C_5H_{11}
1c	OH	OH	H	H	H	O	C_8H_{17}
1d	OH	OH	H	H	H	O	$C_{10}H_{21}$
1e	OH	OH	H	H	H	O	$C_{12}H_{25}$
1f	OH	OH	H	H	H	O	$C_{14}H_{29}$
1g	OH	OH	H	H	H	O	$C_{16}H_{33}$
1h	OH	OH	H	H	H	O	$C_{18}H_{37}$
1i	OH	OH	H	H	H	O	$C_{20}H_{41}$
1j	OH	OH	H	H	H	O	$C_{22}H_{45}$
1k	OH	H	H	H	H	O	$C_{14}H_{29}$
1l	OH	H	H	H	OH	O	$C_{14}H_{29}$
1m	OH	H	OH	H	H	O	$C_{14}H_{29}$
1n	OH	H	H	OH	H	O	$C_{14}H_{29}$
1o	H	OH	H	OH	H	O	$C_{14}H_{29}$
1p	H	OH	OH	H	H	O	$C_{14}H_{29}$
1q	OH	OH	OH	H	H	O	$C_{14}H_{29}$
1r	H	OH	OH	OH	H	O	$C_{14}H_{29}$
1s	OCH_3	OCH_3	H	H	H	O	$C_{14}H_{29}$
1t	OH	OCH_3	H	H	H	O	$C_{14}H_{29}$
1u	OCH_3	OH	H	H	H	O	$C_{14}H_{29}$
1v	OH	OH	H	H	H	NH	$C_{14}H_{29}$
1w	H	OH	OH	H	H	NH	$C_{14}H_{29}$
1x	H	OH	OH	OH	H	NH	$C_{14}H_{29}$
1y	OCH_3	OH	H	H	H	NH	$C_{14}H_{29}$
2a	H	OCH_3	OCH_3	H	H	CH_2	$C_{12}H_{25}$
2b	H	OH	OH	H	H	CH_2	$C_{12}H_{25}$
2c	OCH_3	OCH_3	H	H	H	CH_2	$C_{13}H_{25}$
2d	OH	OH	H	H	H	CH_2	$C_{13}H_{25}$

Ethyl-2,3-dihydroxybenzoate **1a** and pentyl-2,3-dihydroxybenzoate **1b** were prepared by reacting 2,3-dihydroxybenzoic

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acid with the corresponding alcohol in the presence of a catalytic amount of concentrated sulfuric acid. Compounds **1c–k**, **1m–n**, **1p** and **1r** were synthesized by esterification of substituted benzoic acid with the corresponding alcohol in dry tetrahydrofuran (THF) using *N*,*N*′-dicyclohexylcarbodiimide (DCC).[10] 2,3-Dihydroxyphenyl tetradecanoate (**3a**) was prepared from pyrogallic acid and tetradecanoic acid also using DCC as a coupling agent. Compounds **1l**, **1s**, and **1t** were obtained using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) as an activating reagent. Tetradecyl 3,5-dihydroxybenzoate (**1o**) and tetradecyl 2,3,4-trihydroxybenzoate (**1q**) were prepared by a Mitsunobu reaction using diethyl azodicarboxylate (DEAD) as the condenser.[11] Methylation of compound **1f** gave tetradecyl 3-hydroxy-2-methoxybenzoate (**1u**).[12]

Benzamides **1v**, **1w** and **1x** were synthesized by amidation of 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid or 3,4,5-trihydroxybenzoic acid with tetradecan-1-amine in the presence of DCC and 1-hydroxybenzotriazole hydrate (HOBt).[13] 3-Hydroxy-2-methoxy-*N*-tetradecylbenzamide (**1y**) was prepared via a methylation reaction according to the procedure reported for **1u**. The 3,4-dimethoxyphenyl ketone **2a** was prepared using veratrole through a Friedel–Crafts reaction. 2,3-Dimethoxyphenyl ketone **2c** was obtained by a Grignard reaction.[14] Deprotection of the methoxyphenyl ketones **2a** and **2c** with boron tribromide yielded hydroxyphenyl ketones **2b** and **2d**, respectively. 3-(Tetradecyloxy)-benzene-1,2-diol (**3b**) was prepared by a Mitsunobu reaction between pyrogallol and 1-tetradecanol.[15] All compounds were purified by silica gel open column chromatography.

The neuritogenic activities of the synthesized gentiside derivatives were evaluated in PC12 cells and compared with a negative control (0.5% DMSO) and a positive control (NGF at the optimum concentration of 40 ng mL^{-1}). The dose-dependent response of the NGF-mimicking activity of each compound was tested. The concentration that induced the highest percentage of neurite outgrowth (maximum activity) of PC12 cells is reported. Figure 1 shows the maximum neuritogenic activity of gentiside analogues **1a–y**, **2a–d** and **3a–b**.

Figure 1a shows the maximum neuritogenic activity of compounds **1a–j**. Compounds **1a** and **1b**, which have short alkyl chain lengths (C_2 and C_5), exhibited 38% (10 μM) and 37%

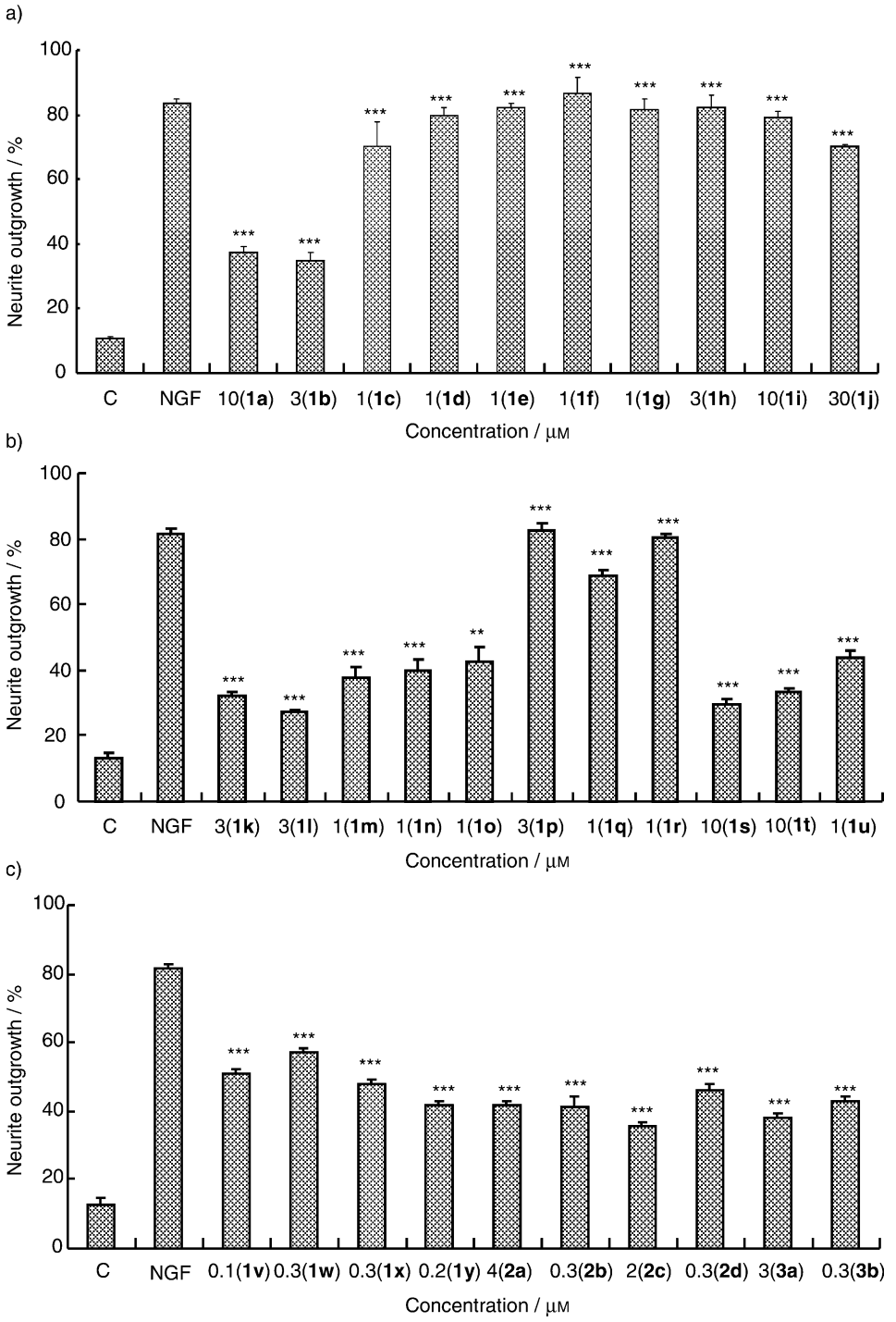

Figure 1. Maximum NGF-mimicking activity of gentiside derivatives **1a–y**, **2a–d**, and **3a–b** at the optimum concentration. Values represent the mean neurite outgrowth (%) ± SD ($n = 3$). C: control (0.5% DMSO); NGF: positive control (40 ng mL^{-1}). **1a–y**, **2a–d**, **3a–b**: *** $p < 0.001$.

(3 μM) neuritogenic activity, respectively. Compounds **1c**–**g**, with alkyl chain lengths of 8 to 16 carbon atoms, showed maximum activities of 71%, 80%, 82%, 87%, and 80%, respectively at 1 μM. Compounds **1h**–**j**, with alkyl chain lengths of 18 to 22 carbon atoms exhibited maximum activities of 81% (3 μM), 78% (10 μM), and 70% (30 μM), respectively. These results reveal that alkyl chain length had significant effects on neuritogenic activity. Tetradecyl benzoate (**1f**) exhibited the highest neuritogenic activity at 1 μM. Therefore, a straight chain of 14 carbon atoms was considered to be the optimum structure for biological activity.

After determination of the best alkyl chain length, the synthetic strategy was targeted to find the optimum number and position of hydroxy groups on the benzene ring. Tetradecyl monohydroxybenzoate (**1k**), tetradecyl 2,6-dihydroxybenzoate (**1l**), tetradecyl 2,4-dihydroxybenzoate (**1m**), tetradecyl 2,5-dihydroxybenzoate (**1n**), and tetradecyl 3,5-dihydroxybenzoate (**1o**) exhibited maximum activities of 32% (3 μM), 28% (3 μM), 38% (1 μM), 40% (1 μM), and 43% (1 μM), respectively (Figure 1b). Tetradecyl 3,4-dihydroxybenzoate (**1p**), tetradecyl 2,3,4-trihydroxybenzoate (**1q**) and tetradecyl 3,4,5-trihydroxybenzoate (**1r**) showed maximum activities of 83% (3 μM), 69% (1 μM) and 80% (1 μM), respectively (Figure 1b). These results reveal that at least two *ortho*-hydroxy groups on the benzene ring are necessary for significant neuritogenic activity. Furthermore, the maximum activity of 29% (10 μM) for **1s**, 33% (10 μM) for **1t**, and 44% (1 μM) for **1u** indicate that the presence of hydroxy groups is important for neuritogenic activity (Figure 1b).

Finally, suitable groups linking the benzene ring and the alkyl chain were investigated. Compounds **1v**–**y**, **2a**–**d** and **3a**–**b** induced maximum activities of 51% (0.1 μM), 57% (0.3 μM), 48% (0.3 μM), 42% (0.2 μM), 42% (4 μM), 41% (0.3 μM), 36% (2 μM), 46% (0.3 μM), 38% (3 μM), and 43% (0.3 μM), respectively (Figure 1c). These results indicate that an ester linkage, rather than a ketone, amido, ether, or modified ester linkage, is optimal for neuritogenic activity.

Taking the above results and structural features into consideration, tetradecyl 2,3-dihydroxybenzoate (**1f**), tetradecyl 3,4-dihydroxybenzoate (**1p**), and tetradecyl 3,4,5-trihydroxybenzoate (**1r**) were selected as lead compounds and renamed ABG-001, ABG-002, and ABG-003, respectively. ABG-001 (**1f**) exhibited the highest neuritogenic activity against PC12 cells of all synthesized compounds. Thus, this compound was further evaluated.

ABG-001 (**1f**) showed dose-dependent activity when evaluated in the concentration range of 0.03 to 1 μM (Figure 2). The maximum neuritogenic activity of ABG-001 (**1f**) at the best concentration of 1 μM was comparable to that seen for NGF at 40 ng mL^{-1}, the best concentration for this protein. Significant neuritogenic activity ($p < 0.01$) of ABG-001 (**1f**) was observed even at 0.03 μM (Figure 2). The solvent control also induced some neurite outgrowth. Figure 3 shows the morphological changes of PC12 cells after treatment with ABG-001 (**1f**) in comparison with the solvent control (0.5% DMSO), and the positive control (NGF). Control cells (without any test compound) showed very few short neurite outgrowth (Figure 3a). When treated with ABG-001 (**1f**) at 1 μM, the cells extended long multipolar neurite outgrowths 48 h after treatment (Figure 3b), which were similar to those produced following treatment with NGF at 40 ng mL^{-1} (Figure 3c). At a dose even as low as 0.03 μM, ABG-001 (**1f**) induced significant neurite outgrowth (Figure 3d). However, 2,3-dihydroxybenzoic acid only or tetradecan-1-ol did not give rise to obvious neurite outgrowth in PC12 cells (data not shown). These results reveal that both the hydrophobic alkyl chain and the 2,3-dihydroxybenzoic acid moiety are necessary for neuritogenic activity.

Subsequently, the signaling pathway of ABG-001 (**1f**)-induced neuritogenesis was investigated. The cellular mechanism of NGF-induced neuritogenesis has been well investigated.[16-18] NGF first binds to the transmembrane-specific receptor, tyrosine receptor kinase A (TrkA) to induce phosphorylation of the specific tyrosine residues located at the intracellular domain, leading to recruitment and activation of a number of kinases. Among them, the mitogen-activated protein (MAP)

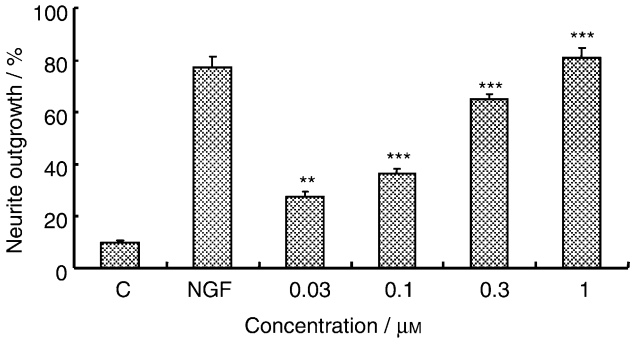

Figure 2. Dose-dependent response of the NGF-mimicking activity of ABG-001 (**1f**) 48 h after treatment. C: control (0.5% DMSO); NGF: positive control (40 ng mL^{-1}). **1f** at 0.03 μM, ** $p < 0.01$; 0.1, 0.3, 1 μM: *** $p < 0.001$.

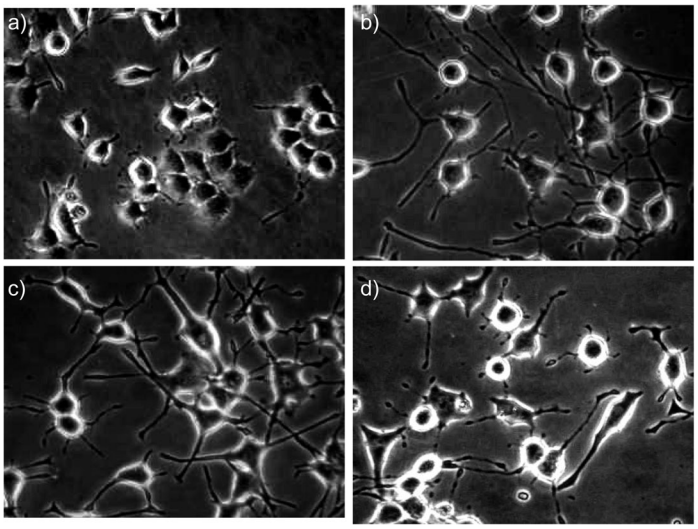

Figure 3. Photomicrographs of PC12 cells under a phase-contrast microscope 48 h after treatment: a) solvent control (0.5% DMSO); b) ABG-001 (**1f**) (1 μM); c) NGF (40 ng mL^{-1}); d) ABG-001 (**1f**) (0.03 μM).

kinase extracellular signal-regulated kinase (ERK) is a key enzyme during NGF-induced neurite outgrowth. To investigate the cellular events of significant neurite outgrowth induced by ABG-001 (**1 f**), specific inhibitor tests were performed to find the possible target kinases for ABG-001-induced neuritogenesis. Therefore, the TrkA-specific inhibitor K252a[19] and the ERK-specific inhibitor U0126[20] were applied to examine whether TrkA and ERK kinases are necessary for neuritogenesis induced by ABG-001 (**1 f**). The results show that the percentage of neurite outgrowth induced by ABG-001 (**1 f**) did not significantly decrease when treated with K252a at various concentrations (data not shown). However, U0126 treatment led to a reduction of neuritogenesis induced by ABG-001 (**1 f**) from 89% to 9%. To confirm these results, phosphorylation of TrkA and ERK in PC12 cells was examined using Western blot analysis after treatment with ABG-001 (**1 f**) at 1 μM. Sustained and strongly enhanced ERK phosphorylation was observed over 28 h when PC12 cells were treated with NGF at 40 ng mL^{-1} (Figure 4). However, ABG-001 (**1 f**) treatment resulted in sustained and significantly enhanced ERK phosphorylation 16 h after treatment (Figure 4). At 1 μM, ABG-001 (**1 f**) did not activate TrkA (data not shown). Phosphorylation levels induced by ABG-001 (**1 f**) were slightly lower and more delayed than those induced by NGF. These results reveal that significant neuritogenesis induced by ABG-001 (**1 f**) was dependent on the sustained activation of ERK in PC12 cells.

In conclusion, a series of gentiside derivatives were prepared through effective and convenient synthetic methods. Most of these derivatives are new compounds. Their structures were confirmed by NMR (^{13}C NMR for new compounds only), IR, and high-resolution ESI-TOF-MS (Supporting Information). The length of the alkyl chain, number and position of hydroxy groups on the benzene ring, and the type of linker between the benzene ring and alkyl chain had distinct effects on the neuritogenic activities of these derivatives. ABG-001 (**1 f**), ABG-002 (**1 p**), and ABG-003 (**1 r**), with 14 carbons, two or three close hydroxy groups on the benzene ring, and an ester linkage, exhibited significant activities at 1 or 3 μM. Furthermore, ABG-001 (**1 f**) induced significant neurite outgrowth via activation of the ERK signaling pathway. This compound was examined using specific-inhibitor experiments and Western blot analysis. Intensive studies on these neuritogenic compounds are currently underway in our lab.

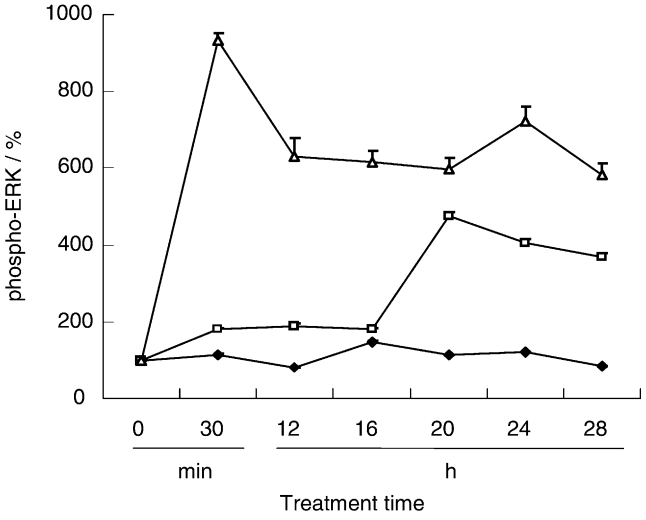

Figure 4. Phosphorylation of ERK in the presence of control, ABG-001 (1 μM) and NGF (40 ng mL^{-1}). The phosphorylation levels (%) were measured using a luminescent image analyzer after Western blotting and normalized to the control value at 0 min (100%) for phospho-ERK. ◆ = control; □ = **1 f** (1 μM); △ = NGF (40 ng mL^{-1}).

Experimental Section

The experimental details of compound syntheses, characterization data, biological evaluation, and mechanism studies are available in the Supporting Information.

Acknowledgements

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Keywords: ABG-001 · biological activity · gentiside derivatives · PC12 cells · structure–activity relationships

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