



Synthesis and biological evaluation of pyrethroid insecticide-derivatives as a chemical inducer for *Bdnf* mRNA expression in neurons

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) plays a fundamental role in neuronal synaptic plasticity. A decrease of plasticity in the brain may be related to the pathogenesis of neurodegenerative or psychiatric disorders. Pyrethroid insecticides, which affect sodium channels in neurons, are widely used to control insect pests in agriculture and in the home. We previously found that deltamethrin (DM), a type II pyrethroid, increased *Bdnf* mRNA expression in cultured rat cortical neurons. However, the cyano group at the α -position of type II pyrethroids is likely susceptible to hydrolytic degradation and, its degraded product, hydrogen cyanide, could generate a cellular toxicity in the human body. To determine if the cyano group is required for the *Bdnf* exon IV–IX (*Bdnf* *eIV*–IX) mRNA expression induced by type II pyrethroids, for this study we synthesized a series of derivatives, in which the cyano group at the α -position was replaced with an ethynyl group. Then we added various substituents at the terminal position of the ethynyl group, and biologically evaluated the effects of these derivatives on *Bdnf* *eIV*–IX mRNA expression. These ethynyl derivatives induced the *Bdnf* *eIV*–IX mRNA expression in a concentration-dependent manner, at varying levels but lower levels than that evoked by DM. The mechanisms for the *Bdnf* induction and the morphological changes of neurons were the same whether the cyano or ethynyl group was included in the compounds.

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1. Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that plays a fundamental role in a variety of neuronal functions, such as cell survival, differentiation and synaptic plasticity. Recently, BDNF has emerged as a key target in the treatment of several neuronal disorders. Indeed, a decrease in BDNF-mediated trophic support has been detected in patients with depression¹ and Alzheimer's disease.² Since BDNF has low permeability through the blood–brain barrier,³ low-weight chemical compounds that induce its expression in the brain are potentially useful in the treatment of neuronal disorders. The BDNF gene (*Bdnf*) consists of eight 5' exons

(*eI*–*VIII*) and a common 3' exon (*eIX*), encoding a preproBDNF protein. Alternative splicing produces several types of transcripts between each of the 5' exons and *eIX*.⁴ Transcripts containing *eI* or *eIV* (*eI*–IX and *eIV*–IX mRNA) are expressed in an activity-dependent manner.⁵ Specifically, *Bdnf* *eIV*–IX mRNA is the most abundantly and widely expressed subtype in the rat brain.⁶

Pyrethroids are highly active synthetic derivatives of natural pyrethrins which are toxins present in the flowers of *Chrysanthemum cinerariaefolium*. Pyrethroids are used to control insect pests particularly in the home. Pyrethroids are classified into two major groups on the basis of chemical structure as shown in Figure 1: type II pyrethroids have an *O*-acyl cyanohydrin substructure at the benzylic position (α -position) (i.e., cypermethrin and deltamethrin), whereas type I do not (i.e., permethrin).⁷ Both types of pyrethroids prolong the opening of the voltage-sensitive sodium channel (VSSC) and raise a prolonged sodium tail current in mammalian as well as invertebrate neurons.^{8,9}

Previously, we found that deltamethrin (DM), a type II pyrethroid, increased *Bdnf* *eIV*–IX mRNA expression not only in vitro but also in vivo.^{10,11} Though their effects varied, type II pyrethroids induced *Bdnf* *eIV*–IX mRNA expression, while type I pyrethroids did not. However, type II pyrethroids possessing the cyano group at the α -position are highly susceptible to hydrolytic degradation

Abbreviations: APV, D-(–)-2-amino-5-phosphonopentanoic acid; BDNF, brain-derived neurotrophic factor; DM, deltamethrin; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L-VDCC, L-type voltage-dependent calcium channel; MAPK, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate; RT-PCR, reverse transcription-polymerase chain reaction; TTX, tetrodotoxin; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; VSSC, voltage-sensitive sodium channel.

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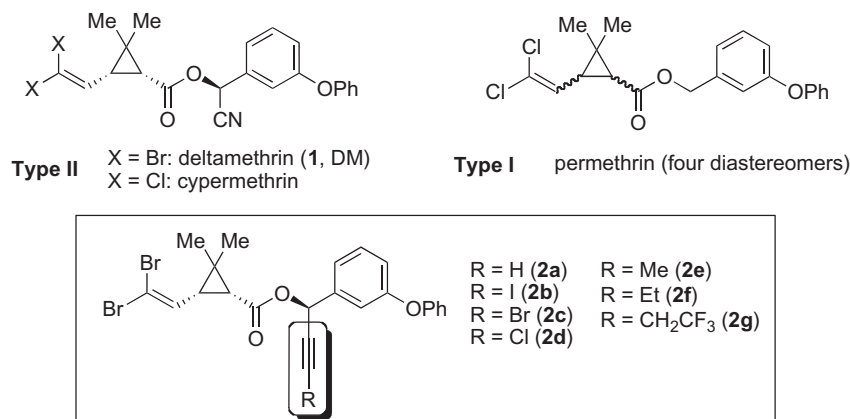


Figure 1. Pyrethroids including deltamethrin (DM) and its ethynyl analogues.

compared with the type I pyrethroids. This degradation yields an aldehyde and hydrogen cyanide, which brings about structural instability. Hydrogen cyanide generates a cellular toxicity in the living body because the cyanide ion halts cellular respiration by inhibiting the mitochondrial enzyme, cytochrome c oxidase. In this study, therefore, we aimed to determine if the cyano group at the α -position in type II pyrethroids is required for the induction of *Bdnf eIV-IX* mRNA expression and, if not, whether non-cyano derivatives of DM could induce the expression via the same mechanisms as DM. For this purpose, we synthesized several derivatives of DM, by replacing the cyano group with an ethynyl group possessing various substituents at its terminal to improve the chemical stability. These changes also avoid a production of a harmful hydrolysate. We then evaluated the effects of these derivatives on *Bdnf eIV-IX* mRNA expression and the morphological changes in neurons.

2. Results and discussion

2.1. Chemistry

Figure 1 shows the chemical structure of DM (**1**), a representative type II pyrethroid, which contains an *O*-acyl cyanohydrin substructure at the benzylic position (α -position). This functional group is likely to be susceptible to hydrolytic (or some other nucleophilic) degradation to yield an aldehyde and hydrogen cyanide as mentioned above. Here we envisaged that replacement of the cyano group (a carbon–nitrogen triple bond) with an ethynyl group (a carbon–carbon triple bond) would increase the stability of the compound, leading to more promising and safer candidates for an agent to treat neuronal disorders. We used the ethynyl derivative **2a** and its analogues, **2b–g**, possessing various substituents at the terminal of the ethynyl group, as targets in this study (Fig. 1).

3-Phenoxybenzaldehyde (**3**) was reacted with lithium trimethylsilylacetylide in THF to afford an adduct alcohol, which was then oxidized with PDC in the presence of molecular sieves in CH₂Cl₂ (with a 94% yield in 2 steps). The resulting ketone **4** was subjected to asymmetric reduction using (*S*)-2-methyl-CBS-oxazaborolidine as a catalyst to give an optically active alcohol (**5**) with an 85% yield. The TMS group in **5** was removed upon treatment with K₂CO₃ in methanol quantitatively, and the alcohol **6a** thus obtained was condensed with a known chiral cyclopropanecarboxylic acid (**7**) using carbonyldiimidazole and a catalytic amount of sodium hydride to furnish the target ethynyl derivative **2a** (Scheme 1).

Introduction of halogen or alkyl substituents into the terminal alkyne was achieved according to Scheme 2. Iodination and bromination were carried out through the reaction of **6a** with I₂/KOH (39%)¹² and the reaction of **5** with NBS/AgNO₃ (70%),¹³ respectively. On the other hand, chlorination involved a somewhat circuitous

route. That is, the hydroxyl group of **6a** was protected as a TBS ether (quant.) and the acetylide anion generated from **8** and *n*-BuLi was treated with NCS to form the chlorinated product **9d** (69%),¹⁴ which was then deprotected by exposure to TBAF (92%). In addition, methyl, ethyl, and trifluoroethyl substituents were introduced via the same pathway. Thus, the alcohols **6b–g** were synthesized successfully. The target compounds **2b–g** were obtained by esterification between the alcohols **6b–g** and carboxylic acid **7** under the same reaction conditions as for the synthesis of **2a**. In this way, the target ethynyl derivatives having various substituents were synthesized by efficient pathways.

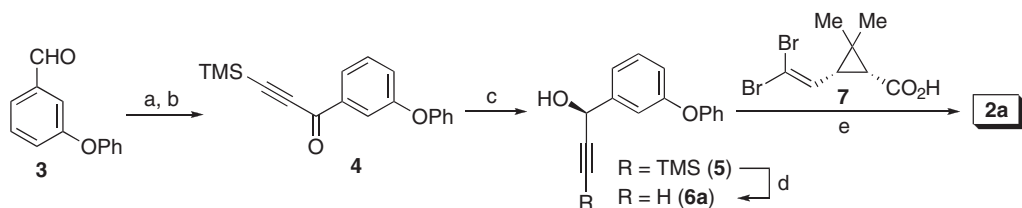
2.2. Biology

2.2.1. The *Bdnf eIV-IX* mRNA expression induced by ethynyl derivatives

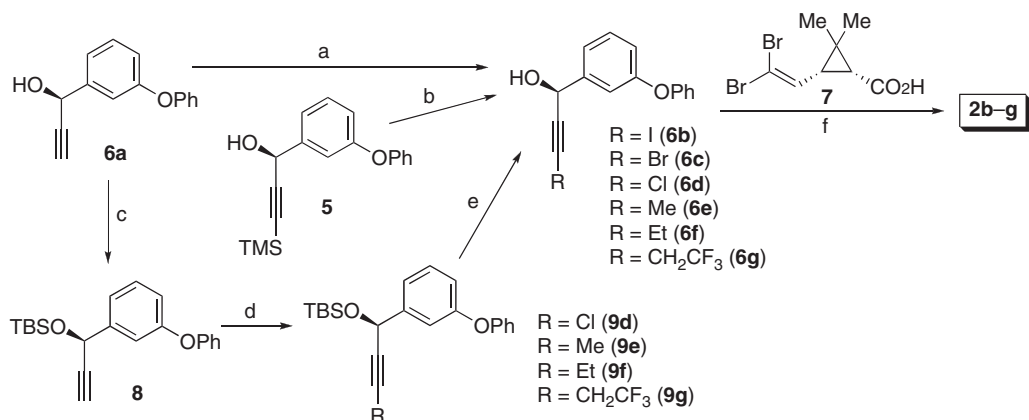
The inducibility of *Bdnf eIV-IX* mRNA expression in cultured rat cortical neurons was evaluated using real-time RT-PCR with all the derivatives synthesized here. Initially, we monitored the level of *Bdnf eIV-IX* mRNA expression induced by the parental ethynyl derivative, **2a**. As shown in Figure 2, the expression of *Bdnf eIV-IX* mRNA was increased by addition of **2a**. While the optimum concentration of DM was 1 μ M, the maximum induction by **2a** was obtained at 10 μ M (Fig. 2A), indicating that the inductive activity of **2a** is less than one tenth lower than that of DM. A time-dependent increase in *Bdnf eIV-IX* mRNA expression was observed until 3 h after starting the incubation but declined at 6 h (Fig. 2B(a)). However, the increased level of expression seen at 3 h returned at 12 h and was maintained until 24 h (Fig. 2B(b)).

To examine whether or not the increase in *Bdnf eIV-IX* mRNA expression induced by **2a** is regulated similarly as that induced by DM,^{10,11} we added several kinds of inhibitors to antagonize the effect of **2a**. The pretreatment of cortical cells with TTX, a potent antagonist of VSSC, completely decreased the expression obtained by **2a**. The pretreatment of cells with nifedipine, a blocker of L-type voltage-dependent calcium channels, but not with APV, an antagonist for the NMDA receptor, also inhibited the increase. The addition of U0126, a potent inhibitor of extracellular signal-regulated kinase 1/2 (ERK1/2), also markedly decreased the expression, indicating the involvement of the ERK/MAPK pathway in the increase in *Bdnf eIV-IX* mRNA expression. These responses induced by **2a** were consistent with those by DM,¹¹ though its inductive level was lower than DM's. These data are provided as Supplementary data.

To investigate the effect of hydrolysates on *Bdnf eIV-IX* mRNA expression, we measured the mRNA levels using cyclopropanecarboxylic acid **7** or an aldehyde moiety **3** produced from pyrethroids. These compounds did not increase the expression (data not



Scheme 1. Reagents and conditions: (a) TMS-acetylene, *n*-BuLi, THF; (b) PDC, MS4A, CH₂Cl₂; (c) (*S*)-2-methyl-CBS-oxazaborolidine, BH₃, THF; (d) K₂CO₃, MeOH; (e) (Im)₂C=O, cat. NaH, CH₂Cl₂.



Scheme 2. Reagents and conditions: (a) I₂, KOH, MeOH/H₂O (**6b**); (b) NBS, AgNO₃, acetone (**6c**); (c) TBSOTf, 2,6-lutidine, CH₂Cl₂; (d) (i) *n*-BuLi, THF (ii) NCS (**9d**), MeI (**9e**), EtI (**9f**), CF₃CH₂I (**9g**); (e) TBAF, THF (**6d–g**); (f) (Im)₂C=O, cat. NaH, CH₂Cl₂.

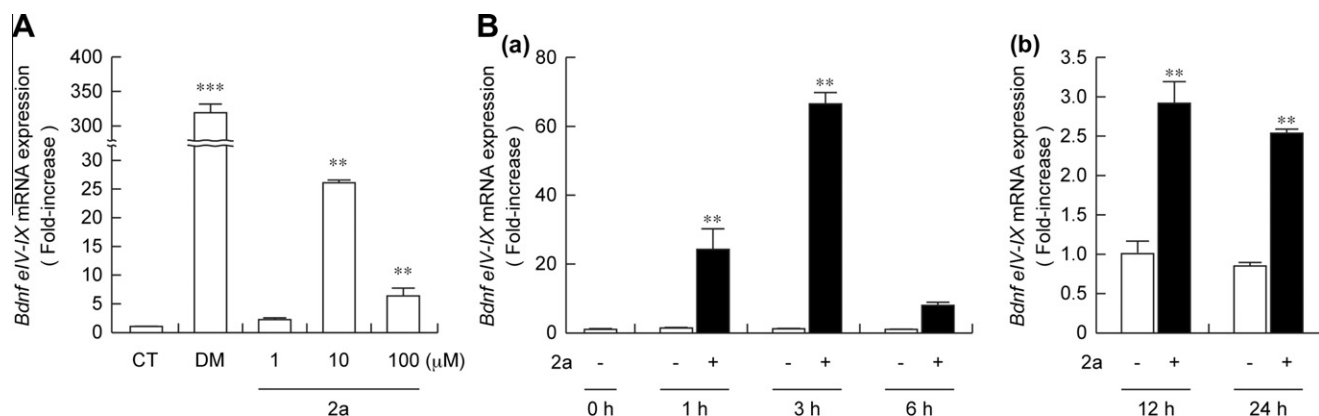


Figure 2. The effects of the test compound **2a** on *Bdnf eIV-IX* mRNA expression in cultured cortical neurons at 1–100 μM. (A) The cells were treated with DM or **2a** and then incubated for another 3 h before total RNA was extracted. One micromolar DM was used as a positive control. The ratio of mRNA expression relative to the control is shown. Data are presented as the mean ± S.E.M. (number of experiments *n* = 3–4). ***p* < 0.01 and ****p* < 0.001 compared with the control (CT). ****p* < 0.001 versus the control, using a Student's *t*-test with F test. ***p* < 0.01 compared with the control, using a one-way ANOVA with the Scheffe's F test. (B) Continuous induction of *Bdnf eIV-IX* mRNA expression by **2a** in rat cortical neurons. Ten micromolar **2a** was added and the cells were incubated for the periods indicated before total RNA was prepared. The ratio of mRNA expression relative to the control (**2a** (–)) at 0 h (a) or 12 h (b) is shown. The values represent the mean ± S.E.M. (number of experiments *n* = 3–4). ***p* < 0.01 compared with the control at the same time point, using a one-way ANOVA with the Scheffe's F test.

shown), indicating that pyrethroids are inactivated after their hydrolysis.

2.2.2. The effect of substituents at the terminus of the ethynyl group on *Bdnf eIV-IX* mRNA expression

Next, we modified the terminal hydrogen of the ethynyl group with several substituents. The halogenated compounds (**2b–d**) significantly increased *Bdnf eIV-IX* mRNA expression in a concentration-dependent manner (Fig. 3A). However, at 10 μM, the increase induced by the halogenated compounds was lower than that induced by **2a** (Fig. 3A). On the other hand, the ethynyl derivatives

possessing an alkyl group (**2e–g**) exhibited increases in *Bdnf eIV-IX* mRNA expression with a maximum at 10 μM (Fig. 3B). In particular, the methyl derivative **2e** exhibited greater potency at 10 μM than **2a**.

Based on these observations, a substituent containing a triple bond at the α-position effectively induces the expression of *Bdnf* mRNA, with the cyano group having the greatest effect. In addition, the size of the substituent at the terminal of the ethynyl group affects the action toward sodium channels, resulting in the difference in the levels of *Bdnf eIV-IX* mRNA expression among the derivatives.

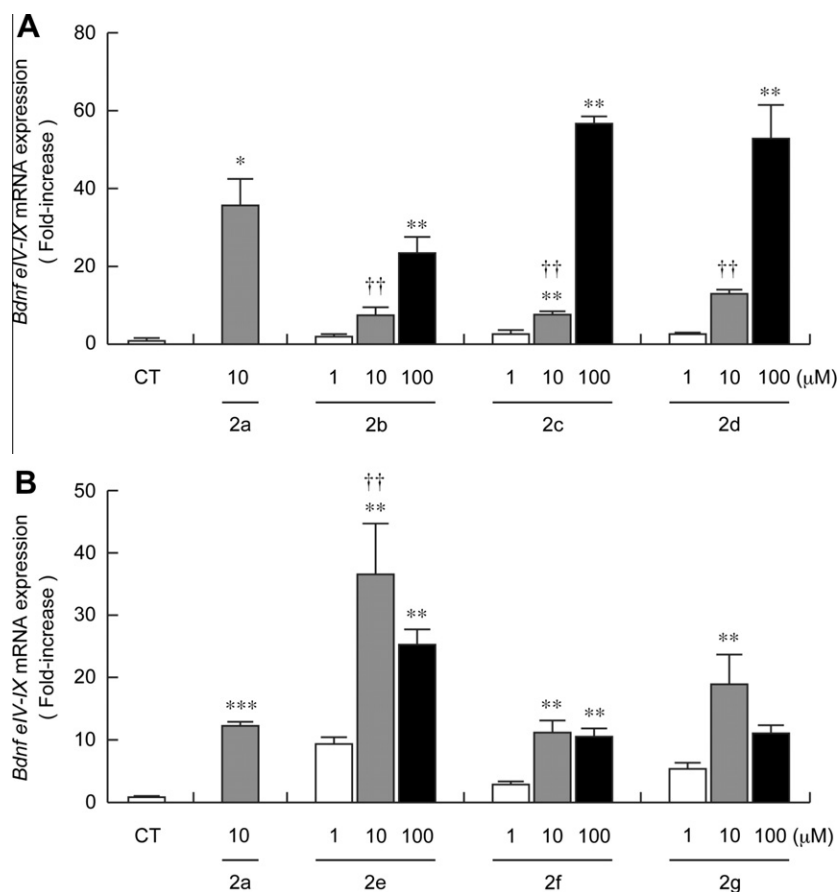


Figure 3. The *Bdnf eIV-IX* mRNA-inducing effects of **2a** and its derivatives **2b–g** in cultured cortical neurons at 1–100 μ M. The cells were treated with **2a**, **2b–d** (A), or **2e–g** (B) and incubated for another 3 h before total RNA was extracted. The ratio of mRNA expression relative to the control is shown. The data are presented as the mean \pm S.E.M. (number of experiments $n = 3–4$). * $p < 0.05$ and *** $p < 0.001$ comparing **2a** with the control using the Student's *t*-test with the F test. ** $p < 0.01$ compared the control with **2b**, **2c**, **2d**, **2e**, **2f** and **2g**, and †† $p < 0.01$ compared **2a** versus **2b**, **2c**, **2d** and **2e** at the same concentration (10 μ M), using one-way ANOVA with the Scheffe's F test.

2.2.3. Morphological changes of cortical neurons stimulated with the ethynyl derivative **2a**

Also, we examined the effect of **2a** on the neuronal morphology. First, we introduced the GFP expression vector into cultured cortical neurons. Then, we cultured the cells with 10 μ M **2a** for 6 h, and then, processed the culture for the fluorescence microscopic observation of GFP-positive cells (Fig. 4A) and immunostaining with anti-MAP2 antibody (data shown in Supplementary data). To examine the morphological changes in neurites, we performed Sholl analysis to measure the neurite complexity (Fig. 4B(a)), and also measured the length of neurites (Fig. 4B(b)).¹¹ As shown in Figure 4A, the addition of **2a** increased the number of neurite crossings and the length of neurites in cultures harvested 6 h after the incubation in a manner similar to DM (Fig. 4B). The effect of **2a** on the morphological changes of neurites was detected at almost the same level as that of DM, though their effects on the induction of *Bdnf eIV-IX* mRNA expression were different (Fig. 2). These data suggest that the level of BDNF expression induced by **2a** is sufficient to cause the morphological changes.

3. Conclusion

In this study, we synthesized a series of ethynyl analogues of DM, to confer less susceptibility to hydrolytic degradation than DM. We found that the presence of a ethynyl group at the α -position was sufficient to preserve the activity of type II pyrethroids to induce *Bdnf eIV-IX* mRNA expression and morphological changes in neurons. These changes indicate that not only the cyano but also

the ethynyl group can stimulate neurons, although the efficiencies on the inductive level were different. Furthermore, the same mechanisms for inducing *Bdnf eIV-IX* mRNA expression operate whether the cyano or ethynyl group is present. Changing the substituent at the terminus of the ethynyl group affected the extent of the expression, suggesting that it is possible to control the inductive level of *Bdnf eIV-IX* mRNA in neurons by changing substituents. Thus, the conversion from a cyano to an ethynyl group in the DM moiety would increase the safety of the compound in the living body, leading to more promising candidates for an agent to treat neuronal disorders.

4. Experimental section

4.1. Chemistry

All reagents were purchased from commercial sources and used as received. Anhydrous solvents were obtained from commercial sources or prepared by distillation over CaH_2 or P_2O_5 . ^1H and ^{13}C NMR spectra were obtained on a Varian Gemini 300 (300 MHz for ^1H and 75.46 MHz for ^{13}C), using chloroform as an internal reference. Mass spectra were measured on a JEOL D-200 or JEOL AX 505 mass spectrometer, and the ionization method was via electron impact (EI, 70 eV). IR spectra were recorded on a JASCO FT/IR-460Plus spectrometer. Column chromatography was carried out by employing Cica Silica Gel 60 N (spherical, neutral, 40–50 μ m). The carboxylic acid **7** was prepared by the hydrolysis of commercially available deltamethrin.

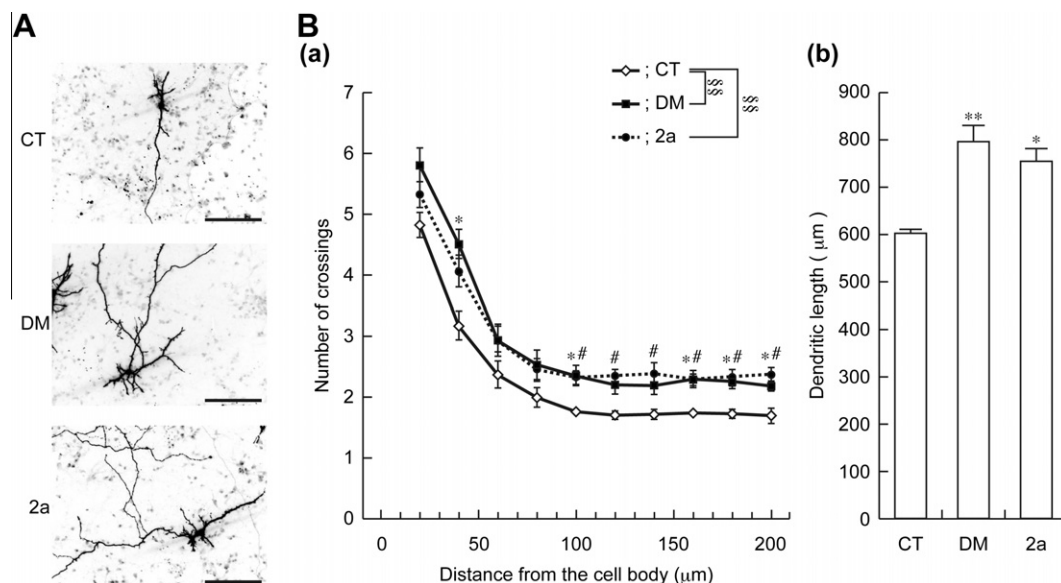


Figure 4. The effect of **2a** on neuronal morphology. At 4 days in culture, rat cortical neurons were transfected with a GFP plasmid for immunostaining. Two days after transfection, the neurons were treated with 1 μM DM or 10 μM **2a** and fixed after 6 h. Representative images of untreated or pretreated neurons are shown (A). A score for the number of neurites was obtained for each cell by placing a 20–200 μm-radius circle (20 μm interval) over the center of the cell and counting the number of times neurites emanating from the cell body intersected the circles (B(a)). The values represent the mean ± S.E.M ($n = 3$ experiments). * $p < 0.05$ control versus DM, # $p < 0.05$ control versus **2a**, using a one-way ANOVA with the Scheffe's F test. ** $p < 0.05$ compared with the control, using a repeated measure ANOVA with the Bonferroni/Dunn test. Scale bar, 100 μm. The length of neurites was also measured by tracing all of the neurites starting from the cell body and extending within a 400 μm diameter (B(b)). The values represent the mean ± S.E.M ($n = 3$). * $p < 0.05$ and ** $p < 0.01$ versus the control, using a one-way ANOVA with the Scheffe's F test.

4.1.1. 1-Phenoxy-3-(3-trimethylsilyl-2-propynyl)benzene (**4**)

To a solution of trimethylsilylacetylene (982 mg, 12 mmol) in anhydrous THF (25 mL) was added *n*-BuLi (1.6 M hexane solution, 6.25 mL, 10 mmol) at -78°C and the mixture was stirred at the same temperature for 15 min. A solution of 3-phenoxybenzaldehyde (2.023 g, 10 mmol) in anhydrous THF (25 mL) was added, and the reaction mixture was warmed to room temperature for 20 min. The reaction was quenched by the addition of a sat. NH_4Cl solution and after extraction with CH_2Cl_2 , the organic layer was dried over MgSO_4 . Evaporation of the solvent gave a crude adduct alcohol (3.096 g, quant.) in an almost pure form. The crude alcohol (100 mg, 0.337 mmol) was dissolved in anhydrous CH_2Cl_2 (2 mL), and MS4A (100 mg) and PDC (389 mg, 1.01 mmol) were added. After 2 h of stirring at room temperature, the precipitates were filtered off through a celite pad, and the filtrate was concentrated. The residue was subjected to column chromatography to afford the ketone **4** (93 mg, 94%) as a yellow oil. ^1H NMR (300 MHz, CDCl_3): δ 7.88–7.04 (9H, m), 0.27 (9H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 176.67, 157.78, 155.94, 137.90, 129.84, 129.81, 124.03, 123.92, 119.44, 118.42, 100.77, 100.49, 53.43, -0.69 ; IR (neat): 2152 ($\text{C}\equiv\text{C}$), 1649 cm^{-1} ($\text{C}=\text{O}$); MS (EI): m/z 294 (M^+); HRMS (EI) Calcd for $\text{C}_{18}\text{H}_{18}\text{O}_2\text{Si}$: 294.1094 (M^+), found: 294.1076.

4.1.2. (–)-1-Phenoxy-3-(3-trimethylsilyl-1-hydroxy-2-propynyl)benzene (**5**)

A solution of BH_3 -THF complex (1 M in THF, 5.1 mL, 5.1 mmol) was diluted with anhydrous THF (8 mL), and (S)-2-methyl-CBS-oxazaborolidine (1 M in THF, 0.34 mL, 0.34 mmol) was added at 0°C . After 15 min of stirring at 0°C , a solution of **4** (1 g, 3.4 mmol) in anhydrous THF (9 mL) was added dropwise, and the mixture was stirred at 0°C for 70 min. Methanol (10 mL) was added and the reaction mixture was diluted with Et_2O . The mixture was washed with 10% HCl and brine successively, and the organic layer was dried over MgSO_4 . Evaporation of the solvent gave a residue, which was chromatographed on silica gel to afford the alcohol **5** (859 mg, 85%) as a yellow oil. We consider that the optical purity of **5** would be practically high because condensation of **6a** (desilylation of **5**)

with optically pure **7** afforded ester **2a** in high yield (94%) without any other diastereomers, although the specific rotation value of **6a** was rather low compared with that reported, estimated as 65% ee (vide infra). ^1H NMR (300 MHz, CDCl_3): δ 7.61–6.90 (9H, m), 5.42 (1H, s), 2.13 (1H, br), 0.17 (9H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 157.36, 156.57, 142.07, 129.71, 129.63, 123.35, 121.20, 119.07, 118.32, 116.64, 91.58, 64.46, 53.43, -0.16 ; IR (neat): 3354 ($-\text{OH}$), 2173 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 296 (M^+); HRMS (EI) Calcd for $\text{C}_{18}\text{H}_{20}\text{O}_2\text{Si}$: 296.1238 (M^+), found: 296.1233; $[\alpha]_{\text{D}} -9.41$ ($c = 0.780$, CHCl_3).

4.1.3. (–)-1-(1-Hydroxy-2-propynyl)-3-phenoxybenzene (**6a**)

To a solution of the alcohol **5** (120 mg, 0.404 mmol) in methanol (6 mL) was added K_2CO_3 (111 mg, 0.808 mmol) and the reaction was continued at room temperature for 0.5 h. The mixture was diluted with water and extracted with CH_2Cl_2 , and the organic layer was dried over MgSO_4 . Evaporation of the solvent gave a terminal ethynyl product **6a** (93 mg, quant.) as an almost pure yellowish oil, which was identified by ^1H NMR spectroscopy. ^1H NMR (270 MHz, CDCl_3): δ 7.30–6.88 (9H, m), 5.36 (1H, s), 2.57 (1H, s), 2.07 (1H, s); MS (EI): m/z 224 (M^+); $[\alpha]_{\text{D}} -10.36$ ($c = 2.15$, benzene) (lit.¹⁵ $[\alpha]_{\text{D}} -16$ (1% in benzene)).

4.1.4. (–)-1-(3-Phenoxyphenyl)-2-propynyl 2-(2,2-dibromoethenyl)-3,3-dimethylcyclopropanecarboxylate (**2a**)

To a solution of the carboxylic acid **7** (57 mg, 0.19 mmol) in anhydrous CH_2Cl_2 (5 mL) was added a solution of 1,1-carbonyldiimidazole (45 mg, 0.29 mmol) in anhydrous CH_2Cl_2 (0.6 mL) at room temperature, and the mixture was stirred for 50 min. A solution of the alcohol **6a** (129 mg, 0.574 mmol) in anhydrous CH_2Cl_2 (2 mL), and then a small amount of NaH were added. After 30 min, the reaction mixture was diluted with CH_2Cl_2 and washed with 10% HCl, sat. NaHCO_3 , and brine successively. The organic layer was dried over MgSO_4 . Evaporation of the solvent gave a residue, which was chromatographed on silica gel to afford the ester **2a** (90 mg, 94%) as a pale yellow oil. ^1H NMR (300 MHz, CDCl_3): δ 7.38–6.98 (9H, m), 6.64 (1H, d, $J = 8.4$ Hz), 6.41, (1H, d, $J = 1.8$ Hz),

2.66 (1H, d, $J = 2.4$ Hz), 1.99 (1H, t, $J = 8.4$ Hz), 1.90 (1H, d, $J = 8.4$ Hz), 1.23 (3H, s), 1.20 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 168.92, 157.40, 156.52, 138.27, 132.98, 129.89, 129.70, 123.48, 122.13, 122.06, 118.97, 117.71, 89.77, 79.85, 75.67, 64.77, 35.95, 31.68, 28.32, 28.07, 15.11; IR (neat): 2358 ($\text{C}\equiv\text{C}$), 1732 cm^{-1} ($\text{C}=\text{O}$); MS (EI): m/z 502, 504, 506 (M^+); HRMS (EI) Calcd for $\text{C}_{23}\text{H}_{20}\text{Br}_2\text{O}_3$: 501.9781 (M^+), found: 501.9780, $[\alpha]_{\text{D}}^{25}$ 7.06 ($c = 1.15$, benzene).

4.1.5. (–)-1-(1-Hydroxy-3-iodo-2-propynyl)-3-phenoxybenzene (6b)

A mixture of the alkyne **6a** (100 mg, 0.446 mmol), KOH (1 M aqueous solution, 1.12 mL, 1.12 mmol), and I_2 (125 mg, 0.491 mmol) in methanol (2.2 mL) was stirred for 80 min at room temperature. Water was added, and the aqueous mixture was extracted with CH_2Cl_2 and dried over MgSO_4 . Evaporation of the solvent gave a residue, which was chromatographed on silica gel to afford the iodide **6b** (62 mg, 39%) as a pale yellow oil. ^1H NMR (300 MHz, CDCl_3): δ 7.39–6.94 (9H, m), 5.55 (1H, s), 2.29 (1H, br); ^{13}C NMR (75 MHz, CDCl_3): δ 157.45, 156.59, 141.85, 129.89, 129.70, 123.44, 121.11, 119.03, 118.53, 116.80, 93.73, 65.64, 4.78; IR (neat): 3375 ($-\text{OH}$), 2184 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 350 (M^+); HRMS (EI) Calcd for $\text{C}_{15}\text{H}_{11}\text{IO}_2$: 349.9804 (M^+), found: 349.9799; $[\alpha]_{\text{D}}^{25}$ –6.03 ($c = 1.41$, benzene).

4.1.6. (–)-1-(3-Bromo-1-hydroxy-2-propynyl)-3-phenoxybenzene (6c)

A mixture of the alkyne **5** (40.2 mg, 0.136 mmol), NBS (29 mg, 0.163 mmol), and AgNO_3 (1.6 mg, 0.009 mmol) in acetone (1.25 mL) was stirred for 70 min at room temperature in a light-omitted flask. Water was added, and the aqueous mixture was extracted with Et_2O and dried over MgSO_4 . Evaporation of the solvent gave a residue, which was chromatographed on silica gel to afford the bromide **6c** (28.6 mg, 70%) as a yellow oil. ^1H NMR (300 MHz, CDCl_3): δ 7.39–6.95 (9H, m), 5.44 (1H, s), 2.32 (1H, br); ^{13}C NMR (75 MHz, CDCl_3): δ 157.48, 156.62, 141.72, 129.91, 129.70, 123.44, 121.07, 119.02, 118.57, 116.79, 79.49, 65.09, 47.63; IR (neat): 3358 ($-\text{OH}$), 2213 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 302, 304 (M^+); HRMS (EI) Calcd for $\text{C}_{15}\text{H}_{11}^{79}\text{BrO}_2$: 301.9942 (M^+), found: 301.9925; $[\alpha]_{\text{D}}^{25}$ –6.91 ($c = 1.26$, benzene).

4.1.7. (–)-1-(1-tert-Butyldimethylsilyloxy-2-propynyl)-3-phenoxybenzene (8)

To a solution of the alcohol **6a** (263 mg, 1.17 mmol) in anhydrous CH_2Cl_2 (7 mL) were added 2,6-lutidine (252 mg, 2.34 mmol) and then TBSOTf (465 mg, 1.76 mmol), and the resulting mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with CH_2Cl_2 , and washed with 10% HCl, sat. NaHCO_3 , and brine successively, and the organic layer was dried over MgSO_4 . Evaporation of the solvent gave a residue, which was chromatographed on silica gel to afford the TBS ether **8** (397 mg, quant.) as a yellow oil. ^1H NMR (300 MHz, CDCl_3): δ 7.36–6.91 (9H, m), 5.45 (1H, s), 2.55 (1H, d, $J = 2.1$ Hz), 0.90 (9H, s), 0.16 (3H, s), 0.12 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 157.32, 156.78, 143.19, 129.62, 129.50, 123.27, 120.52, 119.07, 117.85, 116.14, 84.33, 73.75, 64.19, 25.82, 18.37, –4.43, –4.89; IR (neat): 3307 ($\text{C}\equiv\text{C}-\text{H}$), 2124 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 338 (M^+); HRMS (EI) Calcd for $\text{C}_{21}\text{H}_{26}\text{O}_2\text{Si}$: 338.1702 (M^+), found: 338.1704; $[\alpha]_{\text{D}}^{25}$ –4.72 ($c = 1.00$, benzene).

4.1.8. General procedure for the synthesis of 9d–g

A solution of the alkyne **8** (100 mg, 0.296 mmol) in anhydrous THF (2 mL) was cooled to -78°C , $n\text{-BuLi}$ (1.6 M hexane solution, 0.277 mL, 0.444 mmol) was added, and the mixture was stirred at the same temperature for 15 min. NCS (for **9d**, 2 equiv), MeI (for **9e**, excess amount), EtI (for **9f**, excess amount), or $\text{CF}_3\text{CH}_2\text{I}$

(for **9g**, excess amount) was added, and the mixture was warmed to room temperature. After 1.5 h of stirring, the reaction was quenched by addition of a sat. NH_4Cl solution, and the aqueous solution was extracted with CH_2Cl_2 and dried over MgSO_4 . Evaporation of the solvent gave a residue, which was chromatographed on silica gel to afford the products **9d–g** as pale yellow oils.

Compound 9d: Yield 69%; ^1H NMR (300 MHz, CDCl_3): δ 7.38–6.92 (9H, m), 5.46 (1H, s), 0.91 (9H, s), 0.16 (3H, s), 0.12 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 157.36, 156.72, 143.06, 129.65, 129.54, 123.34, 120.46, 119.10, 117.87, 116.04, 69.85, 64.64, 64.38, 25.82, 18.35, –4.43, –4.90; IR (neat): 2236 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 372, 374 (M^+); HRMS (EI) Calcd for $\text{C}_{21}\text{H}_{25}^{35}\text{ClO}_2\text{Si}$: 372.1312 (M^+), found: 372.1278; $[\alpha]_{\text{D}}^{25}$ –8.06 ($c = 0.555$, benzene).

Compound 9e: Yield 89%; ^1H NMR (300 MHz, CDCl_3): δ 7.37–6.90 (9H, m), 5.45 (1H, d, $J = 1.8$ Hz), 1.86 (3H, d, $J = 2.1$ Hz), 0.92 (9H, s), 0.16 (3H, s), 0.13 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 157.17, 156.88, 144.34, 129.58, 129.34, 123.18, 120.60, 119.03, 117.56, 116.22, 81.92, 79.91, 64.57, 25.91, 18.41, 3.80, –4.34, –4.84; IR (neat): 2228 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 352 (M^+); HRMS (EI) Calcd for $\text{C}_{22}\text{H}_{28}\text{O}_2\text{Si}$: 352.1859 (M^+), found: 352.1841; $[\alpha]_{\text{D}}^{25}$ –4.92 ($c = 0.62$, benzene).

Compound 9f: Yield 54%; ^1H NMR (300 MHz, CDCl_3): δ 7.36–6.89 (9H, m), 5.45 (1H, s), 2.22 (2H, qd, $J = 7.5$, 2.1 Hz), 1.13 (3H, t, $J = 7.4$ Hz), 0.90 (9H, s), 0.15 (3H, s), 0.13 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 157.17, 156.86, 144.28, 129.57, 129.31, 123.16, 120.62, 119.05, 117.53, 116.22, 87.74, 80.07, 64.54, 25.92, 18.40, 13.75, 12.59, –4.24, –4.79; IR (neat): 2230 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 366 (M^+); HRMS (EI) Calcd for $\text{C}_{23}\text{H}_{30}\text{O}_2\text{Si}$: 366.1966 (M^+), found: 366.2015; $[\alpha]_{\text{D}}^{25}$ –20.12 ($c = 0.52$, benzene).

Compound 9g: Yield 82%; ^1H NMR (300 MHz, CDCl_3): δ 7.37–6.91 (9H, m), 5.57 (1H, s), 0.90 (9H, s), 0.15 (3H, s), 0.12 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 157.33, 156.70, 143.08, 129.63, 129.50, 123.32, 120.52, 119.12, 119.07, 117.84, 116.07, 94.83, 65.79, 64.19, 25.84, 18.40, 3.14, –4.42, –4.87; IR (neat): 2182 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 420 (M^+); HRMS (EI) Calcd for $\text{C}_{23}\text{H}_{27}\text{F}_3\text{O}_2\text{Si}$: 420.1733 (M^+), found: 420.1602; $[\alpha]_{\text{D}}^{25}$ –22.24 ($c = 0.505$, benzene).

4.1.9. General procedure for the synthesis of 6d–g

To a solution of the TBS ether **9d–g** (0.14 mmol) in anhydrous THF (2 mL) was added TBAF (1 M THF solution, 0.28 mL, 0.28 mmol), and the mixture was stirred at 0°C for 0.5 h. The reaction mixture was diluted with CH_2Cl_2 and washed with brine, and the organic layer was dried over MgSO_4 . Evaporation of the solvent gave a residue, which was chromatographed on silica gel to afford the alcohols **6d–g** as yellow oils.

Compound 6d: Yield 92%; ^1H NMR (300 MHz, CDCl_3): δ 7.39–6.95 (9H, m), 5.43 (1H, s), 2.27 (1H, br); ^{13}C NMR (75 MHz, CDCl_3): δ 157.45, 156.60, 141.80, 129.91, 129.70, 123.44, 121.06, 118.99, 118.55, 116.77, 77.21, 68.75, 65.72, 64.49; IR (neat): 3335 ($-\text{OH}$), 2238 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 258, 260 (M^+); HRMS (EI) Calcd for $\text{C}_{15}\text{H}_{11}^{35}\text{ClO}_2$: 258.0448 (M^+), found: 258.0424; $[\alpha]_{\text{D}}^{25}$ –6.63 ($c = 0.975$, benzene).

Compound 6e: Yield quant.; ^1H NMR (300 MHz, CDCl_3): δ 7.38–6.92 (9H, m), 5.40 (1H, d, $J = 2.1$ Hz), 2.11 (1H, br), 1.90 (3H, d, $J = 2.1$ Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 157.30, 156.80, 143.08, 129.73, 129.65, 123.29, 121.19, 118.94, 118.27, 116.93, 83.28, 78.86, 64.46, 3.82; IR (neat): 3381 ($-\text{OH}$), 2228 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 238 (M^+); HRMS (EI) Calcd for $\text{C}_{16}\text{H}_{14}\text{O}_2$: 238.0994 (M^+), found: 238.0974; $[\alpha]_{\text{D}}^{25}$ –6.03 ($c = 1.36$, benzene).

Compound 6f: Yield 92%; ^1H NMR (300 MHz, CDCl_3): δ 7.37–6.93 (9H, m), 5.41 (1H, s), 2.23 (2H, qd, $J = 7.5$, 1.9 Hz), 1.15 (3H, dd, $J = 7.2$, 7.8 Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 157.30, 156.73, 143.06, 129.70, 129.63, 123.29, 121.20, 118.99, 118.24, 116.83, 83.04, 78.97, 64.41, 13.77, 12.57; IR (neat): 3366 ($-\text{OH}$), 2229 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 252 (M^+); HRMS (EI) Calcd for $\text{C}_{17}\text{H}_{16}\text{O}_2$: 252.1150 (M^+), found: 252.1128; $[\alpha]_{\text{D}}^{25}$ –8.47 ($c = 0.41$, benzene).

Compound 6g: Yield 78%; ^1H NMR (300 MHz, CDCl_3): δ 7.39–6.94 (9H, m), 5.55 (1H, s), 2.26 (1H, br); ^{13}C NMR (75 MHz, CDCl_3): δ 157.49, 156.62, 141.86, 129.91, 129.71, 123.45, 121.11, 119.05, 118.55, 116.80, 93.75, 65.69, 4.73; IR (neat): 3363 (–OH), 2184 cm^{-1} ($\text{C}\equiv\text{C}$); MS (EI): m/z 306 (M^+); HRMS (EI) Calcd for $\text{C}_{17}\text{H}_{13}\text{F}_3\text{O}_2$: 306.0868 (M^+), found: 306.0901; $[\alpha]_{\text{D}} -4.61$ ($c = 0.89$, benzene).

4.1.10. Synthesis of 2b–g

According to the procedure used for the synthesis of **2a**, the carboxylic acid **7** was reacted with the alcohols **6b–g** (3 equiv) to afford compounds **2b–g** as pale yellow oils.

Compound 2b: Yield 87%; ^1H NMR (300 MHz, CDCl_3): δ 7.39–6.96 (9H, m), 6.75 (1H, d, $J = 8.7$ Hz), 6.51 (1H, s), 1.99 (1H, t, $J = 8.4$ Hz), 1.89 (1H, d, $J = 8.7$ Hz), 1.22 (3H, s), 1.18 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 168.88, 157.46, 156.49, 138.45, 132.98, 129.92, 129.75, 123.57, 122.06, 119.07, 118.94, 117.66, 90.35, 89.86, 66.14, 36.02, 31.67, 28.33, 28.11, 15.13, 6.02; IR (neat): 3055 ($\text{C}=\text{C}-\text{H}$), 2191 ($\text{C}\equiv\text{C}$), 1731 cm^{-1} ($\text{C}=\text{O}$); MS (EI): m/z 628, 630, 632 (M^+); HRMS (EI) Calcd for $\text{C}_{23}\text{H}_{19}^{79}\text{Br}_2\text{O}_3$: 627.8746 (M^+), found: 627.8723; $[\alpha]_{\text{D}} -19.05$ ($c = 0.365$, benzene).

Compound 2c: Yield 61%; ^1H NMR (300 MHz, CDCl_3): δ 7.39–6.96 (9H, m), 6.75 (1H, d, $J = 8.1$ Hz), 6.41 (1H, s), 1.99 (1H, dd, $J = 8.7, 8.1$ Hz), 1.89 (1H, d, $J = 8.7$ Hz), 1.22 (3H, s), 1.19 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 168.90, 157.46, 156.51, 138.27, 132.95, 129.94, 129.73, 123.57, 122.01, 119.05, 118.95, 117.64, 89.88, 76.39, 65.63, 48.61, 36.02, 31.65, 28.33, 28.12, 15.13; IR (neat): 3055 ($\text{C}=\text{C}-\text{H}$), 2221 ($\text{C}\equiv\text{C}$), 1731 cm^{-1} ($\text{C}=\text{O}$); MS (EI): m/z 580, 582, 584, 586 (M^+); HRMS (EI) Calcd for $\text{C}_{23}\text{H}_{19}^{79}\text{Br}_3\text{O}_3$: 579.8884 (M^+), found: 579.8863; $[\alpha]_{\text{D}} -12.83$ ($c = 0.395$, benzene).

Compound 2d: Yield 65%; ^1H NMR (300 MHz, CDCl_3): δ 7.39–6.97 (9H, m), 6.76 (1H, d, $J = 8.1$ Hz), 6.40 (1H, s), 2.00 (1H, dd, $J = 8.1, 8.7$ Hz), 1.89 (1H, d, $J = 8.4$ Hz), 1.23 (3H, s), 1.19 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 168.92, 157.48, 156.51, 138.34, 132.95, 129.96, 129.75, 123.57, 122.00, 119.05, 118.95, 117.76, 117.63, 89.88, 65.77, 65.16, 36.02, 31.67, 28.35, 28.12, 15.13; IR (neat): 3056 ($\text{C}=\text{C}-\text{H}$), 2245 ($\text{C}\equiv\text{C}$), 1732 cm^{-1} ($\text{C}=\text{O}$); MS (EI): m/z 536, 538, 540 (M^+); HRMS (EI) Calcd for $\text{C}_{23}\text{H}_{19}^{79}\text{Br}_2^{35}\text{ClO}_3$: 535.9390 (M^+), found: 535.9378; $[\alpha]_{\text{D}} -5.55$ ($c = 1.305$, benzene).

Compound 2e: Yield 84%; ^1H NMR (300 MHz, CDCl_3): δ 7.38–6.95 (9H, m), 6.78 (1H, d, $J = 8.4$ Hz), 6.38 (1H, d, $J = 2.4$ Hz), 1.97 (1H, dd, $J = 8.7, 8.1$ Hz), 1.91–1.87 (4H, m), 1.22 (3H, s), 1.19 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 169.13, 157.30, 156.65, 139.47, 133.19, 129.75, 129.66, 123.40, 122.08, 118.97, 118.68, 117.74, 89.51, 84.22, 75.59, 65.55, 35.89, 31.83, 28.36, 27.93, 15.13, 3.95; IR (neat): 3055 ($\text{C}=\text{C}-\text{H}$), 2240 ($\text{C}\equiv\text{C}$), 1730 cm^{-1} ($\text{C}=\text{O}$); MS (EI): m/z 516, 518, 520 (M^+); HRMS (EI) Calcd for $\text{C}_{24}\text{H}_{22}^{79}\text{Br}_2\text{O}_3$: 515.9936 (M^+), found: 515.9955; $[\alpha]_{\text{D}} -9.38$ ($c = 0.67$, benzene).

Compound 2f: Yield 52%; ^1H NMR (300 MHz, CDCl_3): δ 7.37–6.95 (9H, m), 6.77 (1H, d, $J = 8.1$ Hz), 6.41 (1H, s), 2.27 (2H, qd, $J = 7.6, 2.4$ Hz), 1.97 (1H, t, $J = 8.4$ Hz), 1.89 (1H, d, $J = 8.4$ Hz), 1.21 (3H, s), 1.19 (3H, s), 1.14 (3H, dd, $J = 7.8, 7.2$ Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 169.13, 157.33, 156.64, 139.49, 133.24, 129.75, 129.68, 123.42, 122.13, 119.03, 118.71, 117.69, 89.92, 89.46, 75.71, 65.55, 35.89, 31.89, 28.38, 27.93, 15.13, 13.61, 12.65; IR (neat): 3055 ($\text{C}=\text{C}-\text{H}$), 2239 ($\text{C}\equiv\text{C}$), 1729 cm^{-1} ($\text{C}=\text{O}$); MS (EI): m/z 530, 532, 534 (M^+); HRMS (EI) Calcd for $\text{C}_{25}\text{H}_{24}^{79}\text{Br}_2\text{O}_3$: 530.0093 (M^+), found: 530.0059; $[\alpha]_{\text{D}} -11.22$ ($c = 0.45$, benzene).

Compound 2g: Yield 74%; ^1H NMR (300 MHz, CDCl_3): δ 7.39–6.96 (9H, m), 6.75 (1H, d, $J = 8.4$ Hz), 6.52 (1H, s), 1.99 (1H, t, $J = 8.4$ Hz), 1.89 (1H, d, $J = 8.4$ Hz), 1.22 (3H, s), 1.18 (3H, s); ^{13}C NMR (75 MHz, CDCl_3): δ 168.88, 157.46, 156.49, 138.45, 132.97, 129.92, 129.73, 123.55, 122.06, 119.07, 118.94, 117.77, 117.66, 90.35, 89.85, 66.14, 36.02, 31.67, 29.77, 28.33, 28.11, 15.13, 5.99; IR (neat): 3055 ($\text{C}=\text{C}-\text{H}$), 2191 ($\text{C}\equiv\text{C}$), 1731 cm^{-1} ($\text{C}=\text{O}$); MS

(EI): m/z 503 ($\text{M}^+ - \text{CH}_2\text{CF}_3$), 505, 507; HRMS (EI) Calcd for $\text{C}_{23}\text{H}_{21}^{79}\text{Br}_2\text{O}_3$: 502.9858 ($\text{M}^+ - \text{CH}_2\text{CF}_3$), found: 502.9860; $[\alpha]_{\text{D}} -19.92$ ($c = 0.42$, benzene).

4.2. Biological activity

4.2.1. The preparation of the test compound solutions

The test compounds were dissolved in dimethylsulfoxide (DMSO) and then diluted with suitable medium until the final concentration of DMSO was 0.1%.

4.2.2. Cells

A primary culture of rat cortical cells was prepared from Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan) at embryonic day 17 (E17), as described previously.^{10,11} In brief, small pieces of cerebral cortex were dissected by enzymatic treatment with trypsin and DNase I and suspended by pipetting, and the cells were seeded at 2.5×10^6 cells in a 35 mm-diameter poly-ethylenimine-coated dish (Iwaki, Tokyo, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin for 3 days, and then the medium was replaced with serum-free DMEM.

4.2.3. RNA isolation and reverse transcription

Total cellular RNA was extracted by the acid guanidine phenol-chloroform method. The isolation of RNA from cultured cells was described in detail previously.^{10,11} In brief, total cellular RNA was extracted using TRIsure (BIOLINE, London, UK) and treated with RNase-free DNase I (TaKaRa, Shiga, Japan) to prevent contamination by genomic DNA. After the removal of DNase I, total RNA was quantified with a Beckman spectrophotometer (Beckman Coulter, Fullerton, CA). One microgram of RNA was used for reverse transcription with SuperScript II (Invitrogen).

4.2.4. Quantitative PCR analysis

Quantitative PCR was performed using the Stratagene Mx3000p Real-Time PCR system (La Jolla, CA, USA) as described previously.^{10,11} In brief, PCR was performed in 20 μL of a reaction mixture containing $1 \times$ SYBR Green QPCR master mix (Stratagene), 2 μL cDNA solution, and 0.2 μM primer pairs. The thermal profile for real-time PCR was as follows: initial denaturation at 95 $^\circ\text{C}$ for 10 min, followed by 40 cycles of denaturation at 95 $^\circ\text{C}$ for 45 s, annealing at 57–59 $^\circ\text{C}$ (dependent on T_{m} $^\circ\text{C}$ of primers) for 45 s, and extension at 72 $^\circ\text{C}$ for 1 min. Standard curves were generated for each gene using a plasmid DNA dilution series containing the target sequences. The *Bdnf* mRNA levels were computed from the threshold cycle (C_{t}) value and normalized to the concentration of internal control *Gapdh* mRNA. The primer sequences for measuring each mRNA were rat *eIV*-containing *Bdnf* mRNA sense, 5'-TCGGCCA CCAAAGACTCG-3' and antisense, 5'-GCCCATTCACGCTCTCCA-3'; *Gapdh* mRNA sense, 5'-TCCATGACAACCTTTGGCATCGTGG-3' and antisense, 5'-GTTGCTGTTGAAGTCACAGGAGAC-3'.

4.2.5. Immunostaining and morphological analysis

The procedure was performed as described previously.¹¹ To monitor the effect of DM on the morphology of cortical neurons, a green fluorescent protein (GFP) expression vector (1 $\mu\text{g}/\text{well}$) was transfected at 4 days in culture using the calcium phosphate precipitation method. At 6 days in culture, after treatment with the drugs for 6 h, cells were fixed by placing them in PBS containing 4% formaldehyde and 4% sucrose for 15 min. Then the cells were permeabilized by placing them in PBS containing 0.3% Triton X-100, 3% bovine serum albumin, and 3% normal goat serum for 1 h. The cells were incubated with anti-GFP and microtubule-

associated protein 2 (MAP2) primary antibodies in the presence of blocking solution (PBS with 3% BSA and 3% normal goat serum) at 4 °C overnight. After being washed in PBS, the cells were incubated for 1 h at room temperature with Alexa488- and Alexa594-conjugated secondary antibodies against rabbit or mouse IgG diluted in blocking solution. After another wash, nuclei were counter-stained with 300 nM 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Finally, coverslips were mounted with Fluoromount (Diagnostic BioSystems, Pleasanton, CA). The rabbit polyclonal antibodies used were anti-GFP (1:500; Invitrogen) and Alexa 488-conjugated anti-rabbit IgG (1:1000, Invitrogen). The mouse monoclonal antibodies used were anti-MAP2 (1:1000, Sigma) and Alexa 594-conjugated anti-mouse IgG (1:1000, Invitrogen).

After immunostaining, the cells were processed for microscopy (BX50-34-FLA-1, Olympus, Tokyo, Japan), as described previously (Sholl analysis). A series of concentric circles at 20- μ m intervals were centered on the cell body and the number of intersections with GFP-positive processes was recorded as an index of neurite complexity¹¹; that is, the increase in the number of the intersections represents the increase in neurite complexity. The length of neurites was measured by tracing all of the neurites starting from the cell body and extending within a 400- μ m diameter using Image J software. More than 96 neurons were evaluated from three independent experiments ($n=3$). We confirmed that the GFP-positive cells used for the morphological analysis were MAP2-positive cells (Supplementary data). Notably, we measured all kinds of neurons without any selection of cells for these measurements.

4.2.6. Statistics

All data were expressed as the mean \pm S.E.M. for the separate experiments performed in duplicate, as indicated in the figure captions. Statistical analyses were performed using the Student's *t*-test followed by the *F*-test or a one-way analysis of variance (ANOVA)

with the Scheffe's *F* test, or repeated measure ANOVA with the Bonferroni/Dunn test (see figure captions).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bmc.2012.02.048](https://doi.org/10.1016/j.bmc.2012.02.048).

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