Synthesis and Biological Activities of Neoechinulin A Derivatives: New Aspects of Structure–Activity Relationships for Neoechinulin A

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We synthesized a series of neoechinulin A derivatives and examined the structure–activity relationships in terms of their anti-nitration and anti-oxidant activities as well as their cytoprotective activity against peroxynitrite from SIN-1 (3-(4-morpholinyl)sydnonimine hydrochloride) using PC12 cells. Our results showed that the C-8/C-9 double bond, which constitutes a conjugate system with indole and diketopiperazine moieties of neoechinulin A is essential for anti-nitration and anti-oxidant activities as well as protection against SIN-1 cytotoxicity. The presence of an intact diketopiperazine moiety is an additional requirement for anti-nitration activity but not for the cytoprotective action. Our results suggest that the antioxidant activity or electrophilic nature of the C-8 carbon, both of which are afforded by the C-8/C-9 double bond, may play a role in the cytoprotective properties of this alkaloid.

Key words neoechinulin A; structure–activity relationship; reactive oxygen species; reactive nitrogen species; antioxidant

Peroxynitrite ($ONOO^-$), generated *in vivo* from the reaction of nitric oxide (NO) with the superoxide anion ($O_2^{-\cdot}$), is a potent oxidant that can cause severe cell damage.[1] Specifically, peroxynitrite promotes the oxidation of biomolecules such as lipids, proteins and nucleic acids,[2—4] as well as the nitration of tyrosine residues in proteins.[5,6] Furthermore, it has been suggested that peroxynitrite formation plays a role in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.[7—9]

Neoechinulin A (**1**), an isoprenyl indole alkaloid, can protect neuronal PC12 cells from $ONOO^-$-induced death.[10—12] We have previously shown that the biological effects, rather than scavenging activity against $ONOO^-$, are likely to play a role in the cytoprotective action of neoechinulin A.[12] However, the precise molecular mechanism remains elusive. To investigate the potential mechanism of action, we have designed and prepared a series of neoechinulin A analogues (**2**—**6**). We then examined the structure–activity relationships of these analogues in terms of their anti-nitration and anti-oxidant activities as well as their cytoprotective activity against $ONOO^-$ derived from SIN-1 (3-(4-morpholinyl)sydnonimine hydrochloride) using PC12 cells (Fig. 1). The results showed that: 1) the presence of the C-8/C-9 double bond is indispensable for anti-nitration and anti-oxidant activities as well as cytoprotective activity of neoechinulin A against $ONOO^-$ toxicity; 2) in conjunction with the C-8/C-9 double bond, the presence of an intact diketopiperazine moiety is essential for the anti-nitration activity but not for anti-oxidant or cytoprotective activity.

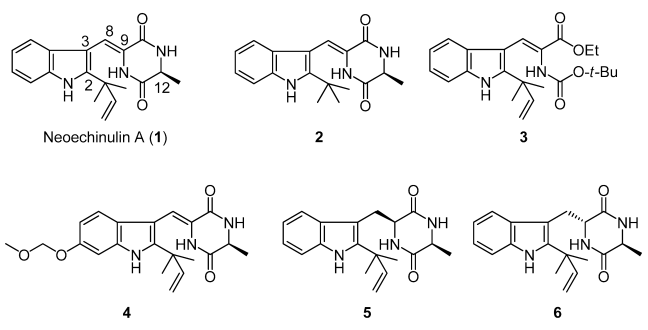

Fig. 1. Structure of Neoechinulin A (**1**) and Its Analogues (**2**—**6**)

Results and Discussion

Compound **2** was synthesized from 2-*tert*-butyl-1*H*-indole (**7**)[13] (Chart 1). Methoxy methyl (MOM) protection of **7**, followed by the Vilsmeier reaction, gave aldehyde **9**. A coupling reaction of the aldehyde **9** with diketopiperazine **10** using *t*-BuOK in DMF afforded **11**.[14] Subsequent deprotection of protective groups provided the desired product **2**.[15]

Compound **3** was prepared by coupling of aldehyde **12** with *N*-Boc-Gly-OEt, followed by treatment of the resulting

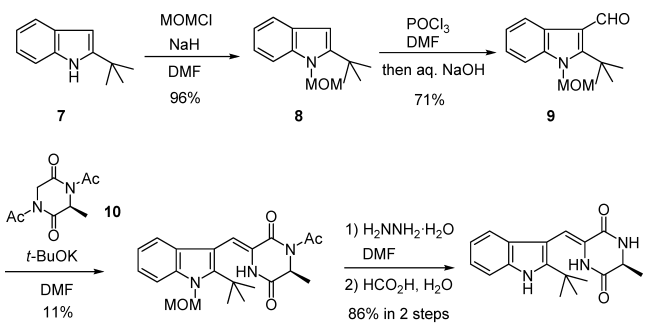

Chart 1. Synthesis of Compound **2**

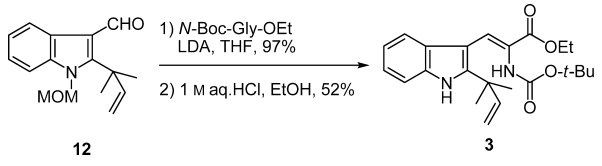

Chart 2. Synthesis of Compound **3**

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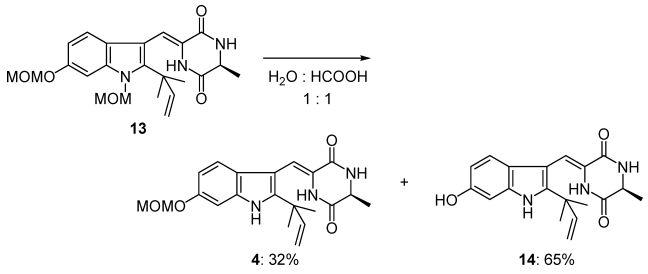

Chart 3. Synthesis of Compound **4**

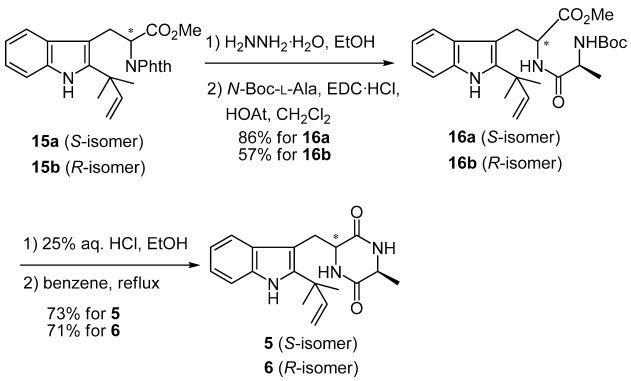

Chart 4. Synthesis of Compounds **5** and **6**

adduct with 1 M aqueous HCl solution (Chart 2).

Compound **4** was obtained as a byproduct by the deprotection of bis-MOM-groups in **13**[16] with HCO_2H (Chart 3).

Preechinulin (**5**)[17—21] was synthesized starting from isoprenyl L-tryptophan derivative **15a** (Chart 4). Deprotection of *N*-phthaloyl group in **15a**[22—24] with hydrazine and coupling of the resulting amine with *N*-Boc-L-Ala gave **16a**. Deprotection of *N*-Boc group in **16a** and thermal cyclization afforded preechinulin (**5**).[17—21] Our synthetic preechinulin had a specific rotation $[\alpha]_D^{23}$ +23.4 (c=0.09, AcOH) [lit. synthetic product: $[\alpha]_D^{20}$ +22.3 (AcOH),[17] natural one: $[\alpha]_D^{24}$ +46 (AcOH)[20]], although the ^{1}H-NMR data of our synthetic preechinulin are slightly different from those reported in literature.[17,20] A diastereomer (**6**) of preechinulin was prepared from isoprenyl D-tryptophan derivative **15b** according to the same procedure.[17,21]

The effects of neoechinulin A (**1**) and its analogues (**2**—**6**) on nitrotyrosine formation by $ONOO^-$ were examined using SIN-1 as a $ONOO^-$ generator and BSA as a target (Fig. 2). Consistent with our previous study,[12] an inhibitory effect could be observed with neoechinulin A at 20 μM, and almost complete inhibition could be attained at 100 mM. Compounds **2** and **4** also inhibited nitrotyrosine formation to a similar extent to that observed for neoechinulin A. In contrast, neither compounds **3**, **5**, nor **6** showed detectable inhibition even at a concentration of 100 mM under our assay conditions. The order of inhibitory potency was as follows: **1**=**2**=**4**>**3**=**5**=**6**.

We next measured the effects on lipid peroxidation using rat brain homogenates. Compounds **1**, **2** and **3** all inhibited the formation of malondialdehyde (MDA) in a similar concentration-dependent manner, each with an IC_{50} value of about 100 μM (Fig. 3). Compound **4**, which contains a methoxymethyloxy moiety on the indole ring, inhibited MDA formation by >80% even at 20 μM. In contrast, compounds **5** and **6**, which possess a single carbon bond between C8–C9, showed only a marginal inhibitory effect at the highest concentration of 200 μM. None of the test compounds used in this study showed an interference effect on the thiobarbituric acid reaction (data not shown), thus verifying the antioxidative effects of the compounds. The order of inhibitory potency was as follows: **4**>**1**=**2**=**3**>**5**=**6**.

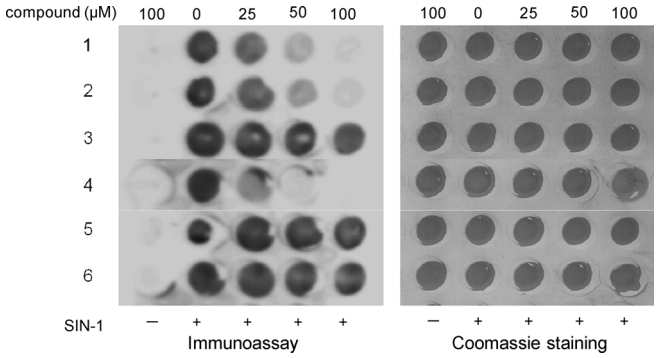

Fig. 2. Effects of Neoechinulin A and Its Analogues on Tyrosine Nitration in BSA Induced by $ONOO^-$

BSA was exposed to SIN-1 in the absence or presence of indicated compound for 2 h at 37 °C, and nitrotyrosine levels were then evaluated by dot blot immunoassay using an anti-nitrotyrosine antibody. One representative result is shown. Equal loading of BSA was confirmed by Coomassie staining of another membrane prepared in parallel. One representative result is shown.

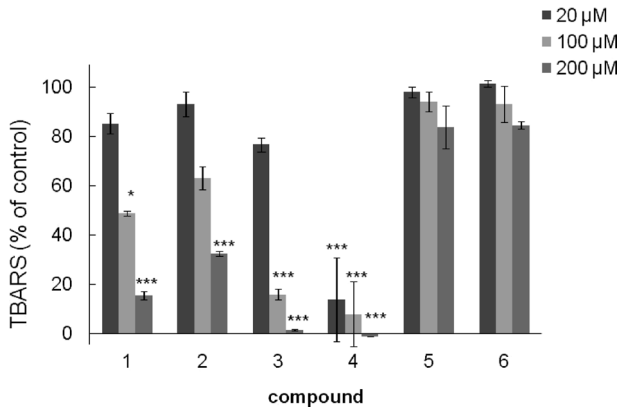

Fig. 3. Effects of Neoechinulin A and Its Analogues on Lipid Peroxidation in Rat Brain Homogenates

Rat brain homogenates were kept on ice or incubated at 37 °C for 90 min with or without 100 μM of each test compound. The degree of lipid peroxidation was evaluated by means of thiobarbituric acid-reactive substance (TBARS) formation. The final concentration of DMSO in the autoxidation reaction was 1%. Values are mean±S.D. from 3 replicates in a representative experiment. * p<0.05, ** p<0.01, *** p<0.001.

To evaluate the cytoprotective activity against $ONOO^-$, we first assessed inherent toxicity of the test compounds toward nerve growth factor (NGF)-differentiated PC12 cells (Fig. 4). Cells were incubated with each test compound for 24 h, and cell viability was then measured. All the compounds except **3** at 100 μM showed no or, if any, marginal (<20%) cytotoxicity. For 100 μM of **3**, cell viability was reduced to 10% that of the control. However, at concentrations of **3** below 50 μM, cell viability was less than 20% that of the control.

Finally the cytoprotective activity of the test compounds against $ONOO^-$ toxicity was measured in NGF-differentiated PC12 cells (Fig. 5). Treatment with 1 mM SIN-1 for 24 h decreased the viability of control cells to 10% of untreated cells. However, cell viability increased to 50% when cells were treated with neoechinulin A at 100 μM prior to exposure

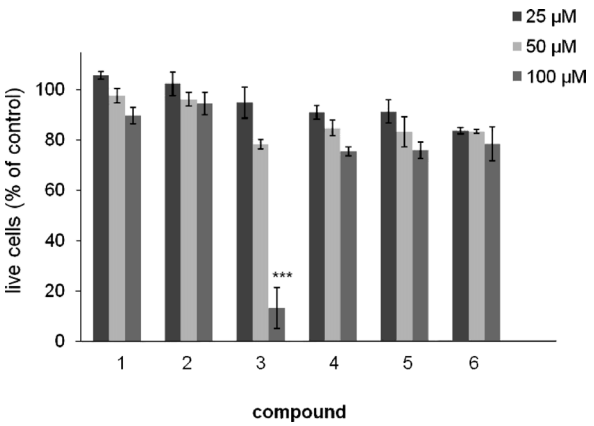

Fig. 4. Cytotoxicity of Neoechinulin A and Its Analogues in PC12 Cells

NGF-differentiated PC12 cells in 96 well plates were incubated without or with indicated compound for 24 h, and live cells in the plates were determined by LDH assay. Values are mean±S.D. from 5 wells in a representative experiment. *** $p<0.001$.

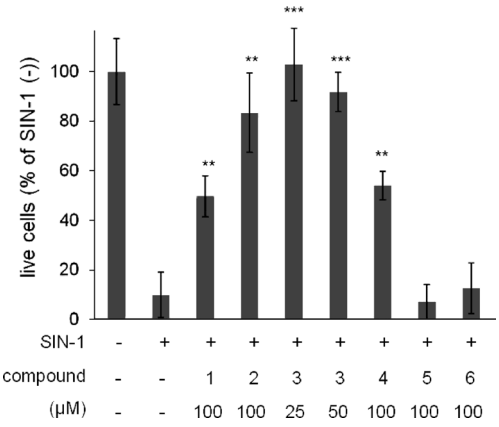

Fig. 5. Effects of Neoechinulin A and Its Analogues on PC12 Cell Death Induced by $ONOO^-$ NGF-Differentiated PC12 Cells in 96 Well Plates Were Pretreated with or without Indicated Compound for 24 h, and Then Exposed to SIN-1 for an Additional 24 h

The numbers of live cells on the plates were determined using the LDH assay. Values are mean±S.D. from 5 wells in a representative assay. ** $p<0.01$, *** $p<0.001$.

to $ONOO^-$ as reported previously.[11] Both **2** and **4** at 100 μM protected the cells by a similar extent to that observed for neoechinulin A. However, **3** at 100 μM apparently exacerbated the toxicity (data not shown), which possibly resulted from the intrinsic toxicity of the compound itself (see Fig. 3). Nevertheless, **3** at nontoxic concentrations of 50 and 25 μM showed a significant level of protection, which was more potent than that for 100 μM neoechinulin A. In contrast, neither **5** nor **6** could rescue cells from the oxidative insult. The order of inhibitory potency was as follows: **2**=**3**>**1**=**4**>**5**=**6**.

Our present results strongly indicate that the C-8/C-9 double bond of neoechinulin A is essential for the anti-nitration activity, anti-oxidant activity, and the cytoprotective activity against $ONOO^-$ from SIN-1 in PC12 cells (*i.e.*, compounds **5** and **6** displayed a complete loss of these activities). However, the presence of the C-8/C-9 double bond is not sufficient for anti-nitration activity as compound **3** failed to demonstrate the activity. In this regard, our previous study revealed that an acyclic analogue of neoechinulin A (alanyl dehydrotryptophan) still retained anti-nitration activity.[12] It is likely that an intact diketopiperazine ring (otherwise, intact alanyl dehydrotryptophan) is also an essential structural feature in addition to the C-8/C-9 double bond. Alternatively, the lack of anti-nitration activity with **3**, but not with the acyclic analogue, suggests that the redox potential or electron density of the dehydrotryptophan moiety might exert an influence on the anti-nitration activity.[25] Nonetheless, compound **3** possessed more potent cytoprotective activity than neoechinulin A (**1**), indicating that anti-nitration activity is not responsible for the cytoprotective activity of compounds in SIN-1 induced cell death. This also indicates that the presence of the diketopiperazine moiety is not required for the observed cytoprotective action.

In this study, all the compounds that had antioxidant activity in lipid peroxidation assay (compounds **1**, **2**, **3**, **4**) also showed cytoprotection against SIN-1, suggesting that the presence of the dehydrotryptophan moiety or the C-8/C-9 double bond is important for both anti-oxidation and cytoprotection activities. Because the C-8/C-9 double bond, together with the indole ring and diketopiperazine, constitute a conjugated system that extends the entire molecule, this structural feature is likely a requirement for the cytoprotection. This interpretation is also supported by our previous finding that acyclic analogue of neoechinulin A (aranyl dehydrotryptophan), which has the C-8/C-9 double bond but is devoid of a carbonyl group conjugated with the double bond, lost cytoprotective activity.[12] Taken together, the following mechanisms are predictable for those compounds: 1) they directly react with oxidative species such as alkyl radical ($R^\cdot$) and hydroperoxide (ROOH), 2) they react at the C-8 with biomolecules such as glutathione and proteins as a Michael acceptor. In support of the latter possibility, we found that treatment of PC12 cells with neoechinulin A for 3 h decreased cellular glutathione contents by 50% (data not shown). It is noteworthy that some antioxidant compounds and electrophilic compounds are an activator of the treascription factor Nrf-2, whose activation induces expression of a set of antioxidant and phase II detoxification enzyme genes through binding to the electrophile/antioxidant response element in their promoter regions (see reviews,[26,27]) thereby affording cytoprotection against subsequent oxidative insults.[28,29] Our previous studies demonstrated that preincubation of cells with neoechinulin A for at least 12 h was essential to confer cells cytoprotection against SIN-1, suggesting biological effect(s) was responsible for the cytoprotection. Whether the antioxidant activity against lipid peroxidation or electrophilic properties of the C-8 center (Michael acceptor), both of which are engendered by the C-8/C-9 double bond, is responsible for the "biological effect" is an important issue. Studies are underway to further clarify this point. In conclusion, the present study demonstrates that that C-8/C-9 double bond of neoechinulin A plays a crucial role in the cytoprotective action against $ONOO^-$ cytotoxicity.

Experimental

All non-aqueous reactions were carried out by using freshly distilled solvents under argon atmosphere. THF was distilled from sodium/benzophenone prior to use. *N*,*N*-Dimethylformamide was distilled from calcium hydride prior to use. All other reagents were commercially available and used without further purification. All reactions were monitored by TLC, which was carried out on Silica Gel 60 F_{254} plates (E. Merck). Flash chromatography separations were performed on PSQ 100B (Fuji Silysia Co., Ltd., Japan).

The NMR spectra (^1H and ^{13}C) were determined on a Bruker 600 MHz or

400 MHz spectrometer (Avance DRX-600, Avance DRX-400) or a JEOL 400 MHz spectrometer (JNM-LD400), using $CDCl_3$ (with TMS for ^{1}H-NMR and chloroform-*d* for ^{13}C NMR as the internal reference) solution, unless otherwise noted. Chemical shifts were expressed in parts per million (ppm) and coupling constants are in hertz. Melting points were determined with Yanaco MP-3S and were uncorrected. Optical rotations were recorded using a JASCO P-1030 digital polarimeter at room temperature, using sodium D line. Infrared spectra (IR) were recorded on a Jasco FT/IR-410 spectrometer using NaCl (neat) or KBr pellets (solid), and were reported on wavenumbers (cm^{-1}). Mass spectra (MS) were obtained on an Applied Biosystems mass spectrometer (APIQSTAR pulsar i) under high resolution conditions, using poly (ethylene glycol) as internal standard.

All animals were handled according to the guidelines for animal experimentation of the Tokyo University of Science.

Cells and Chemicals PC12 cells, a rat pheochromocytoma cell line, were routinely maintained in DMEM containing 5% horse serum, 10% bovine calf serum, and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C. DMEM medium, RPMI medium, NADH, sodium pyruvate, 2-thiobarbituric acid, buthylated hydroxytoluene, trichloroacetic acid were purchased from Wako Chemicals (Osaka, Japan). SIN-1 (3-(4-morpholinyl)sydnonimine hydrochloride) was from Dojindo (Kumamoto, Japan). Polyclonal anti-nitrotyrosine antibody, and horseradish peroxidase-coupled anti-rabbit IgG polyclonal antibody were from UPSTATE (Lake Placid, NY, U.S.A.) and GE Health Care (Little Chalfont, U.K.), respectively.

2-*tert*-Butyl-1-methoxymethyl-1*H*-indole (8) To a solution of **7**[13] (50 mg, 0.29 mmol) in DMF (3 ml) was adde NaH (60% dispersion in mineral oil, 14 mg, 0.35 mmol) at 0 °C, and the mixture was stirred for 20 min. MOMCl (26 μl, 0.35 mmol) was added to the mixture at 0 °C, and the mixture was stirred overnight at room temperature (rt). The reaction was quenched by the addition of saturated NH_4Cl solution. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was chromatographed on silica gel (hexanes : EtOAc = 9 : 1) to afford **9** (60 mg, 96%). ^{1}H-NMR ($CDCl_3$) δ: 1.47 (9H, s), 3.36 (3H, s), 5.59 (2H, s), 6.35 (1H, s), 7.09 (1H, dd, J=7.7, 7.3 Hz), 7.17 (1H, dd, J=8.2, 7.3 Hz), 7.40 (1H, d, J=8.2 Hz), 7.52 (1H, d, J=7.7 Hz).

2-*tert*-Butyl-1-methoxymethyl-1*H*-indole-3-carbaldehyde (9) Phosphorus trichloride (39 ml, 0.42 mmol) was added to DMF (1.5 ml) at 0 °C, and the mixture was stirred for 30 min. A solution of **8** (60 mg, 0.28 mmol) in DMF (1.5 ml) was added to the mixture at 0 °C, and the mixture was stirred for 1 h at rt. Then the mixture was cooled to 0 °C, 1.0 M aqueous NaOH (5.5 ml) was added to the mixture, and the whole mixture was stirred at rt for 1 h. The reaction was quenched by the addition of saturated NH_4Cl solution. The aqueous layer was extracted with CH_2Cl_2. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was chromatographed on silica gel (hexanes : EtOAc = 4 : 1) to afford **9** (49 mg, 71%) as a white solid. mp 54—56 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.70 (9H, s), 3.41 (3H, s), 5.66 (2H, s), 7.25—7.32 (2H, m), 7.36—7.40 (1H, m), 8.46—8.51 (1H, m), 10.73 (1H, s); ^{13}C-NMR ($CDCl_3$) δ: 33.0 (3C), 36.2, 56.0, 75.6, 109.6, 116.9, 122.6, 123.3, 123.8, 126.5, 137.9, 156.3, 188.5; IR (KBr) cm^{-1}: 2928, 1719, 1639, 1471, 1377. HR-ESI-MS *m/z*: 268.1303 (Calcd for $C_{15}H_{19}NO_2Na$: 268.1308).

(3*Z*)-1-Acetyl-3-[(2-*tert*-butyl-1-methoxymethyl-1*H*-indol-3-yl)methylene]-2,5-piperazinedione (11) To a solution of **9** (76 mg, 0.31 mmol) and **10**[14] (132 mg, 0.62 mmol) in DMF (3 ml) was added *t*-BuOK (70 mg, 0.62 mmol) at 0 °C, and the mixture was stirred at rt for 4 h. The reaction was quenched by the addition of saturated NH_4Cl solution. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was purified by a preparative TLC (hexanes : EtOAc = 1 : 1) to afford **11** (14 mg, 11%) as a yellow solid. ^{1}H-NMR ($CDCl_3$) δ: 1.55 (9H, s), 1.58 (3H, d, J=7.0 Hz), 2.65 (3H, s), 3.43 (3H, s), 5.11 (1H, q, J=7.0 Hz), 5.63 (2H, s), 7.13—7.18 (3H, m), 7.44 (1H, d, J=8.3 Hz), 7.47 (1H, s), 8.41 (1H, br s); ^{13}C-NMR ($CDCl_3$) δ: 20.0, 23.9, 27.1, 31.9 (3C), 52.9, 55.9, 75.5, 104.7, 110.3, 118.1, 118.6, 121.7, 123.2, 124.9, 125.4, 138.4, 147.2, 160.6, 166.3, 172.1; HR-ESI-MS *m/z*: 420.1874 (Calcd for $C_{22}H_{27}N_3O_4Na$: 420.1893).

(3*Z*)-3-[(2-*tert*-Butyl-1-methoxymethyl-1*H*-indol-3-yl)methylene]-2,5-piperazinedione (2) To a solution of **11** (14 mg, 36 μmol) in DMF (0.35 ml) was added hydrazine monohydrate (3.2 μl, 53 μmol) at 0 °C, and the mixture was stirred at rt for 4.5 h. The reaction was quenched by the addition of saturated NH_4Cl solution. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue.

To the solution of the residue in H_2O (0.6 ml) was added formic acid (0.6 ml) at 0 °C, and the mixture was stirred at rt for 4 h. The reaction was quenched by the addition of saturated $NaHCO_3$ solution. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was purified by a preparative TLC (hexanes : EtOAc = 1 : 3) to afford **2** (9.9 mg, 86%) as a white amorphous solid. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (9H, s), 1.61 (3H, d, J=7.0 Hz), 4.32 (1H, qd, J=7.0, 1.8 Hz), 6.05 (1H, s), 7.16 (1H, td, J=7.3, 1.1 Hz), 7.20 (1H, td, J=7.3, 1.1 Hz), 7.26 (1H, d, J=7.3 Hz), 7.38 (1H, d, J=7.3 Hz), 7.43 (1H, s), 8.33 (1H, s), 10.7 (1H, s); ^{13}C-NMR ($CDCl_3$) δ: 20.9, 30.0 (3C), 33.1, 51.7, 102.2, 111.2, 112.1, 118.7, 121.1, 122.2, 124.6, 126.1, 134.1, 146.6, 159.6, 165.5; IR (KBr) cm^{-1}: 3359, 2961, 2925, 2854, 1673, 1428; HR-ESI-MS *m/z*: 334.1544 (Calcd for $C_{18}H_{21}N_3O_2Na$: 334.1531).

Ethyl 2-(*tert*-Butyloxycarbonylamino)-3-[(*Z*)-2-(1,1-dimethyl-2-propenyl)-1*H*-indol-3-yl]acrylate (3) To a solution of diisopropylamine (0.22 ml, 1.6 mmol) in THF (1.6 ml) was added a 1.56 M *n*-BuLi solution in hexane (1.0 ml, 1.56 mmol) at −78 °C, and the mixture was stirred at −78 °C for 1 h. To the resultant solution of LDA was added a solution of *N*-Boc-Gly-OEt (159 mg, 0.78 mmol) in THF (0.8 ml), and the mixture was stirred at −78 °C for 30 min. A solution of **12** (84 mg, 0.33 mmol) in THF (0.8 ml) was added to the mixture, and the mixture was stirred at −78 °C for 15 min. The reaction was quenched by the addition of saturated NH_4Cl solution. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was chromatographed on silica gel (hexanes : EtOAc = 5 : 1) to afford the adduct (146 mg, 97%) as an inseparable diastereomeric mixture as a white amorphous solid.

To a solution of the adduct (103 mg, 0.22 mmol) in EtOH (20 ml) was added a 1.0 M aqueous solution of HCl (6 ml), and the mixture was stirred at rt for 7 h. The reaction was quenched by the addition of saturated $NaHCO_3$ solution. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was purified by a preparative TLC (hexanes : EtOAc = 2 : 1) to afford **3** (46 mg, 52%) as a yellow amorphous solid. ^{1}H-NMR ($CDCl_3$) δ: 1.34 (9H, s), 1.36 (3H, t, J=7.2 Hz), 1.52 (6H, s), 4.32 (2H, q, J=7.2 Hz), 5.18 (1H, d, J=17.5 Hz), 5.19 (1H, d, J=10.3 Hz), 5.93 (1H, s), 6.04 (1H, dd, J=17.5, 10.3 Hz), 7.08 (1H, t, J=7.4 Hz), 7.15 (1H, t, J=7.4 Hz), 7.32 (1H, d, J=7.4 Hz), 7.43 (1H, d, J=7.4 Hz), 7.47 (1H, s), 8.44 (1H, s); ^{13}C-NMR ($CDCl_3$) δ: 14.2, 27.5 (2C), 28.0 (3C), 39.1, 61.2, 80.3, 105.9, 110.8, 112.9, 120.5 (2C), 121.9, 123.2, 124.4, 126.6, 134.2, 144.2, 144.4, 153.1, 165.4; IR (KBr) cm^{-1}: 3357, 2978, 1703, 1628; HR-ESI-MS *m/z*: 421.2127 (Calcd for $C_{23}H_{30}N_2O_4Na$: 421.2097).

(3*Z*)-3-[[2-(1,1-Dimethyl-2-propenyl)-6-methoxymethyloxy-1*H*-indol-3-yl]methylene]-2,5-piperazinedione (4) To a suspension of **13**[16] (81 mg, 0.19 mmol) in H_2O (2 ml) was added formic acid (2 ml) at rt, and the mixture was stirred at rt for 4.5 h. The reaction was quenched by the addition of saturated NH_4Cl solution. The aqueous layer was extracted with THF–EtOAc solution. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was chromatographed on silica gel ($CHCl_3$: MeOH = 5 : 1) to afford **4** (25 mg, 34%) as a white solid and **14**[16] (42 mg, 65%) as a white amorphous solid. **4**: mp 204—206 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.50 (6H, s), 1.60 (3H, d, J=6.8 Hz), 3.50 (3H, s), 4.30 (1H, q, J=6.8 Hz), 5.15 (1H, d, J=17.2 Hz), 5.19 (1H, d, J=9.2 Hz), 5.19 (2H, s), 6.04 (1H, dd, J=17.2, 9.2 Hz), 6.90 (1H, dd, J=8.8, 2.0 Hz), 6.98 (1H, br s), 7.08 (1H, d, J=2.0 Hz), 7.16 (1H, d, J=8.8 Hz), 7.18 (1H, s), 7.51 (1H, s), 8.35 (1H, s); ^{13}C-NMR ($CDCl_3$) δ: 20.7, 27.3, 27.4, 39.2, 51.6, 55.8, 95.1, 98.4, 102.8, 111.9, 112.2, 113.1, 119.5, 121.2, 124.4, 134.9, 143.0, 144.4, 153.8, 160.0, 165.9; IR (KBr) cm^{-1}: 3344, 2966, 1684, 1635; HR-ESI-MS *m/z*: 406.1740 (Calcd for $C_{21}H_{25}N_3O_4Na$: 406.1737).

Methyl (2*S*)-2-[(2*S*)-2-*tert*-butyloxycarbonylaminopropylamino]-3-[2-(1,1-dimethyl-2-propenyl)-1*H*-indol-3yl]propionate 16a To a solution of **15a**[22,23] (350 mg, 0.84 mmol) in EtOH (8.5 ml) was added hydrazine monohydrate (0.14 ml, 2.94 mmol), and the mixture was stirred at rt for 3 d. The mixture was filtrated and washed with MeOH, and the resultant filtrate was evaporated to give a crude amine.

To a solution of the amine (277 mg), *N*-Boc-L-Ala-OH (318 mg, 1.68 mmol) and 1-hydroxy-7-azabenzotriazole (HOAt) (229 mg, 1.68 mmol) in CH_2Cl_2 (14 ml) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC · HCl) (322 mg, 1.68 mmol) was added at −15 °C, and the mixture was stirred for 1.5 h. The reaction was quenched by the ad-

dition of saturated NH_4Cl solution. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was chromatographed on silica gel (hexanes : EtOAc = 3 : 1) to afford **16a** (333 mg, 86%) as a white amorphous solid. ^{1}H-NMR ($CDCl_3$) δ: 1.44 (12H, m), 1.57 (3H, s), 1.58 (3H, s), 3.23 (1H, dd, J=14.2, 8.6 Hz), 3.34 (1H, dd, J=14.2, 7.1 Hz), 3.56 (3H, s), 4.07 (1H, br s), 4.75 (1H, br s), 4.83 (1H, br m), 5.20 (1H, d, J=10.5 Hz), 5.21 (1H, d, J=17.4 Hz), 6.16 (1H, dd, J=17.4, 10.5 Hz), 6.55 (1H, d, J=7.7 Hz), 7.11 (2H, m), 7.28 (1H, d, J=7.5 Hz), 7.50 (1H, d, J=8.1 Hz), 7.95 (1H, s); ^{13}C-NMR ($CDCl_3$) δ: 18.2, 22.6, 27.6 (3C), 28.3 (2C), 34.7, 52.3, 53.4 (2C), 65.8, 105.3, 110.6, 112.4, 118.0, 119.5, 121.6, 128.3, 129.8, 134.0, 140.6, 145.8, 172.0, 172.7; IR (KBr) cm^{-1}: 3354, 2978, 1703 1510, 1456; HR-ESI-MS m/z: 480.2468 (Calcd for $C_{25}H_{35}N_3O_5Na$: 480.2468).

Preechinulin ((3*S*,6*S*)-3-[2-(1,1-Dimethyl-2-propenyl)-1*H*-indol-3-yl]-methyl-2,5-piperazinedione) 5 To a solution of **16a** (27 mg, 0.060 mmol) in EtOH (4.2 ml) was added a 25% aqueous solution of HCl (2.1 ml), and the mixture was stirred at rt for 2.5 h. The mixture was evaporated to give a crude amine.

A solution of the crude amine in benzene (30 ml) was refluxed for 7 d. The mixture was evaporated to give a crude residue, which was purified by a preparative TLC (hexanes : toluene : isopropylamine = 5 : 3 : 2) to afford **5** (14 mg, 73%) as a white solid. $[\alpha]_D^{23}$ +23.4 (c=0.09, AcOH) [lit. $[\alpha]_D^{20}$ +22.3 (AcOH),[17] $[\alpha]_D^{24}$ +46 (AcOH)[20]]; mp >250°C; ^{1}H-NMR (DMSO-d_6) δ: 1.22 (3H, d, J=7.1 Hz), 1.47 (3H, s), 1.48 (3H, s), 3.06 (1H, dd, J=14.4, 9.0 Hz), 3.30—3.35 (1H, m), 3.75—3.80 (1H, m), 3.91—3.98 (1H, m), 5.03 (1H, d, J=10.5 Hz), 5.06 (1H, d, J=17.5 Hz), 6.17 (1H, dd, J=17.5, 10.5 Hz), 6.92 (1H, t, J=7.4 Hz), 7.01 (1H, t, J=7.4 Hz), 7.31 (1H, d, J=7.4 Hz), 7.41 (1H, d, J=7.4 Hz), 7.52 (1H, s), 8.17 (1H, s), 10.54 (1H, s); IR (KBr) cm^{-1}: 3374, 3209, 2966, 1677, 1458; HR-ESI-MS m/z: 348.1689 (Calcd for $C_{19}H_{23}N_3O_2Na$: 348.1682).

Methyl (2*R*)-2-[(2*S*)-2-*tert*-Butyloxycarbonylaminopropylamino]-3-[2-(1,1-dimethyl-2-propenyl)-1*H*-indol-3yl]propionate 16b To a solution of **15b**[22,23] (27 mg, 65 μmol) in EtOH (1.0 ml) was added hydrazine monohydrate (12 μl, 250 μmol), and the mixture was stirred at rt for 3 d. The mixture was filtrated and washed with MeOH, and the resultant filtrate was evaporated to give a crude amine.

To a solution of the amine (28 mg), *N*-Boc-L-Ala-OH (37 mg, 0.19 mmol) and 1-hydroxy-7-azabenzotriazole (HOAt) (26 mg, 0.19 mmol) in CH_2Cl_2 (14 ml) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·Cl) (37 mg, 0.19 mmol) was added at −15 °C, and the mixture was stirred for 1.5 h. The reaction was quenched by the addition of saturated NH_4Cl solution. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, and dried over Na_2SO_4. The organic layer was evaporated to give a crude residue, which was chromatographed on silica gel (hexanes : EtOAc = 4 : 1) to afford **16b** (17 mg, 57%) as a white amorphous solid. ^{1}H-NMR ($CDCl_3$) δ: 1.43 (9H, m), 1.47 (3H, d, J=5.6 Hz), 1.57 (3H, s), 1.58 (3H, s), 3.28 (1H, m), 3.31 (1H, m), 3.53 (3H, s), 4.09 (1H, br s), 4.83 (1H, m), 4.93 (1H, br s), 5.20 (1H, d, J=10.8 Hz), 5.20 (1H, d, J=17.4 Hz), 6.14 (1H, dd, J=17.4, 10.8 Hz), 6.65 (1H, br s), 7.10 (2H, m), 7.27 (1H, *d*, J=7.4 Hz), 7.51 (1H, d, J=7.7 Hz), 7.95 (1H, s).

((3*R*,6*S*)-3-[2-(1,1-Dimethyl-2-propenyl)-1*H*-indol-3-yl]methyl-2,5-piperazinedione 6 To a solution of **16b** (33 mg, 0.075 mmol) in EtOH (1.5 ml) was added a 25% aqueous solution of HCl (0.75 ml), and the mixture was stirred at rt for 2.5 h. The mixture was evaporated to give a crude amine.

A solution of the crude amine (26 mg) in benzene (20 ml) was refluxed for 7 d. The mixture was evaporated to give a crude residue, which was purified by a preparative TLC (hexanes : toluene : isopropylamine = 5 : 3 : 2) to afford **6** (17 mg, 71%) as a white solid. [a]$_D^{23}$ −46.1 (c=0.09, AcOH) [lit. $[\alpha]_D^{20}$ −68 (AcOH)[17]; mp 237—240 °C (decomp.)]; ^{1}H-NMR (DMSO-d_6) δ: 1.20 (3H, d, J=6.9 Hz), 1.46 (3H, s), 1.47 (3H, s), 3.11 (1H, dd, J=14.5, 8.9 Hz), 3.23 (1H, dd, J=14.5, 5.1 Hz), 3.80—3.85 (1H, m), 3.89 (1H, q, J=6.9 Hz), 5.03 (1H, d, J=10.6 Hz), 5.07 (1H, d, J=17.4 Hz), 6.15 (1H, dd, J=17.4, 10.6 Hz), 6.91 (1H, t, J=7.5 Hz), 7.00 (1H, t, J=7.5 Hz), 7.30 (1H, d, J=7.5 Hz), 7.40 (1H, d, J=7.5 Hz), 7.80 (1H, s), 8.14 (1H, s), 10.52 (1H, s); IR (KBr) cm^{-1}: 3345, 2969, 1675, 1458; HR-ESI-MS m/z: 348.1679 (Calcd for $C_{19}H_{23}N_3O_2Na$: 348.1682).

Nitrotyrosine Assay The effects of test compound on nitration of tyrosine residues in BSA were measured as described previously[12] with some modifications. Briefly, test compound in DMSO was mixed with 0.6 mg/ml BSA in phosphate buffered saline (PBS), and then mixed with an equal volume of 400 μM SIN-1 dissolved in PBS. After a 2 h incubation at 37 °C, the samples (1 μg BSA) were loaded onto a PVDF membrane, followed by treatment with 5% (v/v) 2-mercaptoethanol in PBS for 10 min. After washing with PBS, the membrane was blocked with 1% (w/v) skim milk in PBS. The levels of nitrotyrosine in BSA on the membrane were detected with an anti-nitrotyrosine antibody, followed by a horse-radish peroxidase-coupled secondary antibody, and chemimuminescence detection using an ECL kit (GE Healthcare). The signal was recorded using a CCD camera (Fuji Film, Japan).

Lipid Peroxidation Assay Rat brains were homogenized in 10-fold volume (w/v) of 0.1 M sodium phosphate buffer (pH 7.4) using a Teflon homogenizer, and centrifuged at 200×***g*** for 5 min to remove debris. To 100 μl of homogenates, 1 μl of test compounds in DMSO were added, and aerobically incubated at 37 °C for 90 min. Then the samples were mixed with 400 μl of thiobarbituric acid reaction solution (15% (w/v) trichloroacetic acid, 3.75 mg/ml thiobarbituric acid, 0.56 mg/ml buthylated hydroxytoluene, 0.25 M HCl), and heated at 90 °C for 20 min. Insoluble materials were precipitated by centrifugation, and the absorbance at 535 nm of the supernatants were measured in a 96-well plate on the Spectra Max microplate reader (Molecular Devices).

Cell Viability Assay The effects of test compounds on SIN-1-induced cytotoxicity were measured as described previously[12] with some modifications. NGF-differentiated PC-12 cells in 96-well culture plates were exposed to SIN-1 for 24 h, and live cells in the wells of the plates were lysed in 50 μl of lysis buffer (0.2% (v/v) Triton X-100, 1 mM diethylene pentaacetic acid in PBS). The aliquot of the lysate was mixed with NADH and pyruvate in PBS at final concentrations of 200 μM and 0.5 mM, respectively in a total volume of 200 μl in 96-well assay plates. NADH oxidation was monitored at 340 nm at 25 °C on the Spectra Max plate reader. The viability (%) was expressed with respect to the LDH activity of untreated cells.

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