

## **Parguerol and Isoparguerol Isolated from the Sea Hare, *Aplysia kurodai*, Induce Neurite Outgrowth in PC-12 Cells**

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Received: 21 August 2004 / Accepted: 10 October 2004 / Published: 25 November 2004

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**Abstract:** The extract of the sea hare, *Aplysia kurodai*, showed neurotrophic activity toward rat pheochromocytoma (PC-12) cells. Bioassay-guided purification afforded two active compounds, which were subsequently identified to be parguerol and isoparguerol by spectroscopic analysis. It was found that both parguerol and isoparguerol induced neurite outgrowth in PC-12 cells at concentrations of 25 and 50  $\mu$ M, respectively.

**Keywords:** neurotrophic, parguerol, isoparguerol, sea hare.

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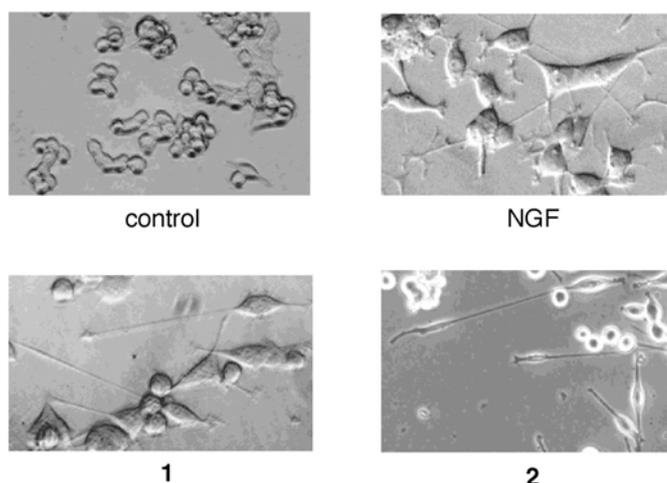
### **Introduction**

It is well known that neurons are terminally differentiated cells. Neurotrophic factors, such as nerve growth factor (NGF) [1], brain-derived neurotrophic factor (BDNF) [2], neurotrophin 3 (NT-3) [3], and glia-derived neurotrophic factor (GDNF) [4], are necessary for the functional maintenance and organization of neurons. Among various neurotrophic factors, NGF has been extensively investigated and found to show pleiotrophic effects, such as the induction of neuronal differentiation, neural cell survival, and prevention of apoptosis of neurons, in both central and peripheral nervous systems [1]. In rat pheochromocytoma (PC-12) cells, which have been used as an *in vitro* model system to study the mechanisms of neuronal differentiation, NGF induces their differentiation, leading to the extension of neurites and the development of the characteristics of sympathetic neurons [5]. In the course of our search for new neurotrophic substances from natural resources [6, 7], we succeeded in isolating two neurotrophic substances and identified them to be parguerol (**1**) [8] and isoparguerol (**2**) [8]. Here we report the isolation, identification, and neurotrophic activities of the two neurotrophic substances, **1** and **2**, together with inactive



(intensity, 1:1)  $[M + Na]^+$  in the FABMS supported identification of **3** as deoxyparguerol acetate. Although **3** was derived from deoxyparguerol (**4**) [8], this is the first isolation of **3** from natural resources.

Parguerol (**1**) and isoparguerol (**2**) showed significant neurotrophic effects on PC-12 cells (Fig. 1): More than 70% of the cells exhibited neurite outgrowth by treatments with **1** and **2** at concentrations of 25 and 50  $\mu\text{g/mL}$ , respectively, for 2 days. On the other hand, deoxyparguerol acetate (**3**) was unable to induce neurite extension even at a concentration of 100  $\mu\text{g/mL}$ , although it possesses the same skeleton as that of **1** and **2**. It should be noted that both **1** and **2** induce bipolar (two-neurite-bearing) morphology, while NGF (a positive control) induces multipolar (multiple-neurite-bearing) morphology. The relationship between the morphological change and the cellular event has been extensively studied and elucidated in the case of murine neuroblastoma Neuro 2A cells [9]. However, such relationship remains obscure in the case of PC-12 cells. Further pharmacological study is needed to understand the mechanism of **1**- and **2**-induced morphological changes in PC-12 cells.



**Fig. 1.** Effects of **1**, **2**, and NGF on neurite outgrowth in PC-12 cells. PC-12 cells were incubated for 2 days in the absence (control) or presence of NGF (positive control) at 50 ng/mL, **1** at 25  $\mu\text{g/mL}$ , or **2** at 50  $\mu\text{g/mL}$ .

It is well known that the content of sea hare digestive glands is dependent on diet that this animal ingests [10-13]. A large number of cognate metabolites have been isolated from both the red alga of the genus *Laurencia* and the sea hare that preys on the red alga. Metabolites isolated from sea hares exhibit various biological activities, for example, cytotoxic [14, 15], algicidal [15], antifungal [15, 16], and ichthyotoxic [16] activities: It can be inferred that sea hares contain such metabolites for the sake of their self-defense. This is the first report of sea hare metabolites that show neurotrophic activity.

## Conclusions

We have isolated parguerol (**1**), isoparguerol (**2**), and deoxyparguerol acetate (**3**) from the sea hare, *Aplysia kurdai*. In rat pheochromocytoma (PC-12) cells, **1** and **2** showed significant neurotrophic activities at concentrations of 25 and 50  $\mu\text{g/mL}$ , respectively, while **3** did not show such effect even at a concentration of 100  $\mu\text{g/mL}$ .

NGF activates neuronal function and prevents neuronal death in the brain. When peripherally administered, however, exogeneously administered NGF is thought to be unable to cross the blood-brain barrier. Therefore, low-molecular-weight NGF-like substances such as **1** or **2** are useful for treatment of various neuronal degenerative disorders.

## Experimental

### General

NMR spectra were recorded on a JEOL GSX500 in  $\text{CDCl}_3$ . All chemical shifts were reported with respect to  $\text{CDCl}_3$  ( $\delta$  7.26). Mass spectra were measured on a JEOL SX-102 mass spectrometer.

### Extraction and Isolation

The frozen material (1.7 kg, wet wt) was extracted with MeOH. The extract was concentrated under reduced pressure and extracted with EtOAc. The EtOAc layer (1.4 g) was subjected to silica gel chromatography with a stepwise gradient of hexane/EtOAc/MeOH. The most potent neurotrophic fraction (216.3 mg) eluted with EtOAc/MeOH (1:1) was purified by silica gel chromatography with  $\text{CHCl}_3/\text{MeOH}$  (9:1) followed by gel permeation on Sephadex LH-20 to afford parguerol (**1**, 1.11 mg,  $6.5 \times 10^{-5}\%$ ), isoparguerol (**2**, 0.69 mg,  $4.1 \times 10^{-5}\%$ ), and deoxyparguerol acetate (**3**, 0.60 mg,  $3.5 \times 10^{-5}\%$ ).

**Parguerol (1):**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.08 (1H, t,  $J = 5.4$  Hz, H-18endo), 0.88 (1H, dd,  $J = 10.2, 5.4$  Hz, H-18exo), 1.04 (1H, m, H-3), 1.07 (3H, s, H<sub>3</sub>-17), 1.14 (3H, s, H<sub>3</sub>-20), 1.14 (2H, m, H-1 and H-5), 1.49 (1H, m, H-14), 1.82 (1H, br d,  $J = 18.6$  Hz, H-12), 1.92 (1H, t,  $J = 15.6$  Hz, H-1), 1.92 (1H, m, H-6), 2.07 (3H, s,  $\text{CH}_3\text{COO}$ ), 2.27 (1H, m, H-8), 2.41 (1H, m, H-6), 2.42 (1H, m, H-12), 3.18 (1H, dt,  $J = 4.9, 11.7$  Hz, H-7), 3.39 (1H, d,  $J = 11.7$  Hz, H-19), 3.55 (1H, d,  $J = 11.7$  Hz, H-19), 3.84 (1H, dd,  $J = 12.7, 9.3$  Hz, H-16), 3.95 (1H, dd,  $J = 12.7, 2.9$  Hz, H-16), 4.28 (1H, dd,  $J = 9.3, 2.9$  Hz, H-15), and 5.36 (2H, d,  $J = 4.9$  Hz, H-2 and H-11). FABMS (positive, glycerol matrix)  $m/z$  479/481 (intensity, 1:1)  $[\text{M} + \text{Na}]^+$ .

**Isoparguerol (2):**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.09 (3H, s, H<sub>3</sub>-17), 1.22 (3H, s, H<sub>3</sub>-20), 1.44 (1H, m, H-14), 1.67 (1H, dd,  $J = 15.1, 4.9$  Hz, H-1), 1.76 (1H, t,  $J = 15.1$  Hz, H-14), 1.86 (1H, m, H-6), 1.86 (1H, m, H-12), 2.03 (3H, s,  $\text{CH}_3\text{COO}$ ), 2.10 (1H, br d,  $J = 15.1$  Hz, H-1), 2.28 (1H, m, H-8), 2.45 (1H, br

d,  $J = 18.6$  Hz, H-12), 2.53 (1H, t,  $J = 14.7$  Hz, H-6), 3.28 (1H, dt,  $J = 4.9, 11.7$  Hz, H-7), 3.85 (1H, dd,  $J = 12.7, 9.8$  Hz, H-16), 3.92 (1H, dd,  $J = 12.7, 2.9$  Hz, H-16), 4.29 (1H, dd,  $J = 9.8, 2.9$  Hz, H-15), 4.96 (1H, d,  $J = 4.0$  Hz, H-2), and 5.45 (1H, d,  $J = 6.3$  Hz, H-11). FABMS (positive, glycerol matrix)  $m/z$  479/481 (intensity, 1:1)  $[M + Na]^+$ .

**Deoxyparguerol acetate (3):**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.01 (1H, m, H-18endo), 0.68 (1H, m, H-18exo), 1.02 (3H, s, H<sub>3</sub>-19), 1.05 (3H, s, H<sub>3</sub>-17), 1.16 (3H, s, H<sub>3</sub>-20), 1.90 (1H, br d,  $J = 15.8$  Hz, H-1), 2.07 (3H, CH<sub>3</sub>COO), 2.09 (3H, CH<sub>3</sub>COO), 2.13 (3H, CH<sub>3</sub>COO), 2.42 (1H, br d,  $J = 18.6$  Hz, H-12), 4.28 (2H, d,  $J = 9.8$  Hz, H<sub>2</sub>-16), 4.40 (1H, dt,  $J = 4.9, 11.7$  Hz, H-7), 4.52 (1H, dd,  $J = 9.8, 2.9$  Hz, H-15), 5.31 (1H, br d,  $J = 5.0$  Hz, H-2), and 5.39 (1H, br d,  $J = 6.5$  Hz, H-11). FABMS (positive, glycerol matrix)  $m/z$  547/549 (intensity, 1:1)  $[M + Na]^+$ .

### Neurite Outgrowth Assay

Neurite outgrowth assay was carried out using rat pheochromocytoma (PC-12) cells.<sup>5</sup> PC-12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5% horse serum, penicillin (50 units/mL), and streptomycin (50  $\mu\text{g/mL}$ ) in an incubator under 5% CO<sub>2</sub> at 37°C. PC-12 cells were seeded onto 24-well plates ( $1 \times 10^5$  cells/mL) and cultivated for a day. The medium was replaced with that containing **1-3** or 5 ng/mL NGF (a positive control), and PC-12 cells were further cultivated for 2 days and observed under a phase-contrast microscope. The percentage of the cells with neurites was determined by counting 300 cells.

### Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture, and Technology of Japan.

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*Samples Availability:* Not available.