# *Brief Articles*

## Phenanthroindolizidine Alkaloids as Cytotoxic Substances in a Danaid Butterfly, *Ideopsis similis*, against Human Cancer Cells

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We previously reported the presence of cytotoxic substances in extracts of the Danaid butterfly, *Ideopsis similis*. In the present study, we isolated cytotoxic substances against a human gastric cancer cell line, TMK-1, in *I. similis* pupae, with an activity similar to that of the adult butterfly. The basic fraction, prepared from a methanol extract, accounted for 83% of the cytotoxic activity. Two major cytotoxic substances were purified by HPLC, and one was determined to be a new phenanthroindolizidine alkaloid, *trans*-(+)-3,14α-dihydroxy-6,7-dimethoxyphenanthroindolizidine (**1**), and the other a known compound, *trans*-(+)-3,14α-dihydroxy-4,6,7-trimethoxyphenanthroindolizidine (**2**). The $IC_{50}$ values for TMK-1 cells were 0.5 ng/mL and 0.7 ng/mL, respectively. These two compounds showed similar cytotoxic potential with four other cancer cell lines including cervical, lung, and colon carcinomas and leukemia. Quantitative analyses indicated the presence of each of the two phenanthroindolizidine alkaloids at levels of 11−74 μg in each larva, pupa, or adult of *I. similis*. However, **1** was not detected in the leaves of *Tylophora tanakae*, a host plant for larvae of *I. similis*, and the level of **2** (2 μg per gram of leaves) was far less than that in the larvae. Since the leaves of *T. tanakae* are known to contain various phenanthroindolizidines, compounds **1** and **2** are presumably metabolically converted from such alkaloids in larvae of *I. similis*.

### Introduction

Natural products are important sources of pharmaceuticals. Indeed, a number of the drugs have been established after screening of plants and fungi. Insects might also be valuable sources of active materials, since there are a tremendous number of species with physiological systems different from those of mammals. We previously discovered the presence of a protein, pierisin-1, which induces apoptotic cell death in human carcinoma cells, in the pupae of the cabbage butterfly, *Pieris rapae*.[1−4] The pupae of another cabbage butterfly, *Pieris brassicae*, also contained a similar cytotoxic substance, pierisin-2.[5,6] Subsequently, we reported that cytotoxic activity was found in a kind of Danaidae, *Ideopsis similis* (see Supporting Information), by screening chloroform−methanol extracts from 92 kinds of butterflies.[7] Specimens of the adult butterfly demonstrated an $IC_{50}$ at a dilution of $5 \times 1/10^6$ for human gastric carcinoma TMK-1 cells. Cytotoxicity was also observed in the larvae and pupae of *I. similis*, at almost the same potency as in the adult. The responsible substance (or substances) was extractable with various organic solvents and proved to be heat-stable, suggesting a nonproteinous nature.

In the present study, two major cytotoxic active substances were purified from pupae of *I. similis*, and their structures and cytotoxicities against human cancer cells were determined. In addition, these compounds were quantified in the larvae, pupae, and adults of *I. similis* and in leaves of *Tylophora tanakae*, which is the host plant for caterpillars of *I. similis*. On the basis of the data, the origin of the cytotoxic substances in *I. similis* is discussed.

### Results

*n*-Hexane, acid-neutral, basic, and water fractions were obtained from the methanol extract of five pupae of *I. similis*. The recoveries of cytotoxic activity against TMK-1 cells in each fraction were 9, 2, 83, and 1%, respectively. Since most of the responsible substance (or substances) was present in the basic fraction, the active principles in this fraction were separated by HPLC on an ODS column using a gradient solvent system. Figure 1 shows HPLC profiles for UV absorbance and cytotoxicity against TMK-1 cells. Significant cytotoxicity was seen in fractions with retention times of 30−32 min

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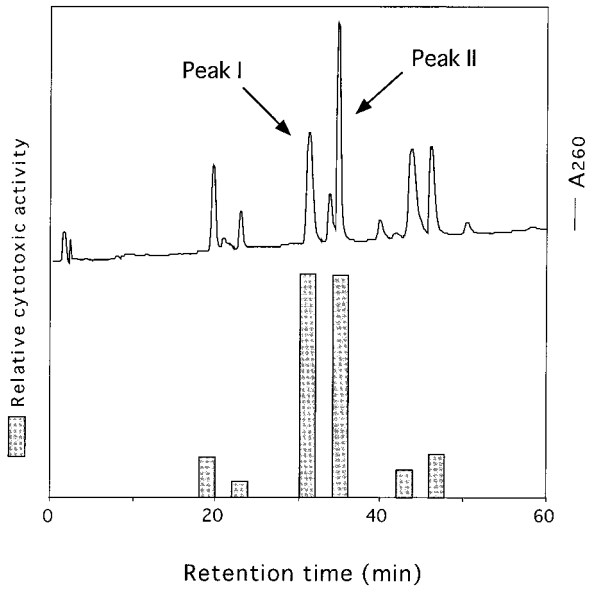
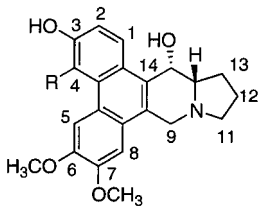



**Figure 1.** Purification of cytotoxic compounds by HPLC.

(peak I) and 34−36 min (peak II). Compound **1** in peak I and compound **2** in peak II accounted for 34% and 34% of the total cytotoxicity of the methanol extract, respectively. Under two different HPLC conditions, peaks I and II were again detected as single peaks. With compounds **1** and **2** from five pupae as markers, large quantities of compound **1** (2.2 mg) and compound **2** (3.7 mg) were isolated from 50 pupae of *I. similis*.




**1** R = H
**2** R = $OCH_3$

Compound **1** showed positive rotation. On the basis of TLC examination using ferrous chloride solution, it was deduced to possess phenolic hydroxy groups. High-resolution MS analysis of the molecular ion peak $M^+$ (*m*/*z* 365) revealed that the molecular formula of compound **1** was $C_{22}H_{23}NO_4$. In the $^{13}C$ NMR spectrum, 22 signals for carbon were observed, and 14 signals ($\delta_C$ 103.8−155.3) were assigned to be aromatic carbons (Table 1). Table 2 shows $^1H$ NMR spectral data for compound **1**. A hydroxymethine proton ($\delta_H$ 4.90) at the benzyl position, two methoxy groups ($\delta_H$ 3.91 and 3.99), and five aromatic protons ($\delta_H$ 7.09, 7.16, 7.91, 7.92, and 8.13) were observed. With the aid of homocorrelation spectroscopy ($^1H$-$^1H$ COSY), and of heteronuclear multiple quantum coherence (HMQC) between $^1H$-$^{13}C$ correlations, compound **1** was deduced to be a 3,6,7,14-tetraoxygen-substituted phenanthroindolizidine alkaloid. The results of the nuclear Overhauser effect spectroscopy (NOESY) spectrum suggested the presence of 3,-14-dihydroxy-6,7-dimethoxy substitution. From the above observations, the perfect chemical structure of compound **1** was concluded to be *trans*-(+)-3,14α-dihydroxy-6,7-dimethoxyphenanthroindolizidine (*trans*-(+)-9,11,-12,13,13a,14-hexahydro-6,7-dimethoxy-dibenzo[*f*,*h*]-pyrrolo[1,2-*b*]isoquinoline-3,14-diol) (**1**). This is a newly registered compound.

**Table 1.** $^{13}C$ NMR Spectral Data for Compound **1**[a,b]

| position | chemical shift ($\delta$ ppm) | position | chemical shift ($\delta$ ppm) |
|---|---|---|---|
| 1 | 126.4 | 3,6,7 and ring C | 123.3 |
| 2 | 116.2 | | 124.0 |
| 4 | 106.0 | | 124.2 |
| 5 | 103.9 | | 125.3 |
| 8 | 103.8 | | 129.7 |
| 9 | 53.6 | | 130.6 |
| 11 | 54.9 | | 148.5 |
| 12 | 21.6 | | 149.1 |
| 13 | 23.9 | | 155.3 |
| 13a | 64.9 | 6-$OCH_3$ | 55.5 |
| 14 | 63.6 | 7-$OCH_3$ | 55.5 |

[a] The spectrum was measured in DMSO-$d_6$. [b] Signal assignments were determined based on HMQC spectra.

**Table 2.** $^1H$ NMR Data for Compounds **1** and **2**[a,b]

| position | compound **1**[c] | compound **2**[d] |
|---|---|---|
| 1 | 8.13 (1H, d, $J$ = 9.0) | 8.15 (1H, d, $J$ = 9) |
| 2 | 7.09 (1H, dd, $J$ = 9.0, 1.8) | 7.34 (1H, d, $J$ = 9) |
| 4 | 7.92 (1H, d, $J$ = 1.8) | |
| 5 | 7.91 (1H, s) | 9.00 (1H, s) |
| 8 | 7.16 (1H, s) | 7.07 (1H, s) |
| 9 | 3.43, 4.50 (1H each, ABq, $J$ = 15.0) | 3.59, 4.46 (1H each, ABq, $J$ = 15) |
| 11 | 2.36, 3.30 (1H each, m) | 2.61, 3.41 (1H each, m) |
| 12 | 1.75−1.85 (2H, m) | 1.96 (2H, m) |
| 13 | 1.82, 2.17 (1H each, m) | 2.35, 2.50 (1H each, m) |
| 13a | 2.39 (1H, m) | 2.90 (1H, m) |
| 14 | 4.90 (1H, d, $J$ = 9.6) | 5.10 (1H, m) |
| 4-OCH3 | | 3.84 (3H, s) |
| 6-OCH3 | 3.99 (3H, s) | 4.08 (3H, s) |
| 7-OCH3 | 3.91 (3H, s) | 4.04 (3H, s) |
| 14-OH | 4.59 (1H, d, $J$ = 9.6) | |

[a] Chemical shifts are presented as ppm. [b] Signal patterns are indicated as follows: s, singlet; d, doublet; dd, double doublet; m, multiplet; ABq, AB quartet. [c] Spectra were measured in DMSO-$d_6$ at 600 MHz. [d] Spectra were measured in $CDCl_3$ at 270 MHz.

Compound **2** showed a positive rotation. Based on TLC examination, it was deduced to possess phenolic hydroxy groups. The UV spectrum showed similar absorption maxima to those of compound **1**, suggesting the presence of a phenanthroindolizidine skeleton. High-resolution MS analysis of the molecular ion peak $M^+$ (*m*/*z* 395) revealed that the molecular formula of compound **2** was $C_{23}H_{25}NO_5$. Table 2 shows the $^1H$ NMR spectral data for compound **2**. These spectral data were very similar to those for compound **1**, except that the signal corresponding to the H-4 proton disappeared, a signal corresponding to a methoxy group was detected, and a signal for the H-5 proton was shifted downfield. On the basis of these results, compound **2** was identified to be *trans*-(+)-3,14α-dihydroxy-4,6,7-trimethoxyphenanthroindolizidine (*trans*-(+)-9,11,12,13,13a,14-hexahydro-4,6,7-trimethoxy-dibenzo[*f*,*h*]pyrrolo[1,2-*b*]isoquinoline-3,14-diol) (**2**), which has been previously isolated from leaves of *T. tanakae*.[8]

When TMK-1 cells were treated with **1** or **2** at doses of 0.01−10 ng/mL for 96 h at 37 °C, both compounds showed cytotoxic activities in a dose-dependent manner. The $IC_{50}$ values were estimated to be 0.5 ng/mL for the former compound and 0.7 ng/mL for the latter. These two alkaloids showed similar cytotoxic activities against

four other cancer cell lines. When **1** was used as a test compound, $IC_{50}$ (ng/mL) values were 0.4 for A549 (lung cancer cell line), 0.5 for DLD-1 (colon cancer cell line), 0.8 for HeLa (cervical cancer cell line), and 0.4 for K562 (leukemia cell line) cancer cells. In the case of **2**, $IC_{50}$ (ng/mL) values were 0.5 for A549, 0.8 for DLD-1, 1.0 for HeLa, and 0.5 for K562 cancer cells.

Next, **1** and **2** were quantified in basic fractions from the larvae, pupae, and adults of *I. similis* and the leaves of *T. tanakae*. These levels were 47 and 46 $\mu$g per larva, 44 and 74 $\mu$g per pupa, and 11 and 26 $\mu$g per adult, respectively. On the other hand, **1** was not detected in the basic fraction from the leaves of *T. tanakae*: if present, its level must be less than 0.2 $\mu$g per gram of wet weight of leaves. Compound **2** was detected at a level of 2 $\mu$g per gram of wet weight of the leaves of *T. tanakae*, a level far less than in the larvae, pupae, and adults of *I. similis*.

## Discussion

In the present study, two cytotoxic substances **1** and **2** were isolated from the pupae of *I. similis*, and their in vitro cytotoxic potentials were shown to be comparable to that of Taxol, which is used clinically as a cancer chemotherapeutic agent.[9] These two phenanthroindolizidine alkaloids accounted for around 70% of the total cytotoxicity against TMK-1 cells in the pupae of *I. similis*.

It is known that *I. similis* feeds on leaves of *T. tanakae* during its larval stage. We previously reported that chloroform−methanol extracts of such leaves show cytotoxic activity with an $IC_{50}$ value at $1/10^5$ dilution.[7] Moreover, several phenanthroindolizidine alkaloids have been isolated from *T. tanakae*, and a mixture of (+)-isotylocrebrine and (−)-7-demethyltyrophorine has been shown to possess oviposition-stimulant activity in *I. similis*.[8,10,11] To study the origin of these cytotoxic substances in *I. similis*, **1** and **2** were quantified in the larvae, pupae, and adults of *I. similis* and also in the leaves of *T. tanakae*, all of which were collected in the same season and area of Ishigaki island in Okinawa prefecture. The absence or low levels of the alkaloids in the plant clearly contrasted with the observed high levels of 11−74 $\mu$g in each different stage of the butterfly. It has been estimated that a Danaid butterfly, *Danaus plexippus*, consumes about 5 g of milkweed during its larval stage.[12] It is possible to assume that this feeding amount is applicable in the case of the larvae of *I. similis*. Thus, it is most likely that **1** and **2** are metabolically converted from other plant-derived phenanthroindolizidine alkaloids in the larvae. It is also conceivable that some **2** is efficiently accumulated in the larvae.

The present study demonstrated that **1** and **2** exert potent cytotoxic activities against five human cancer cell lines. It is of interest to examine whether the cytotoxic activities of the two alkaloids might be a general phenomenon with effects on various kinds of malignant cells and normal cells. Moreover, it is most likely that the alkaloids might be effective as chemoprotective factors against natural enemies in *I. similis*. It is very important to elucidate the biological roles of **1** and **2** in *I. similis*. An understanding of their biological roles should be very helpful for developing these compounds as candidate anticancer drugs.

## Experimental Section

UV absorption spectra were measured with a Tosoh PD-8020 photodiode array detector and a Beckman DU 640 spectrophotometer. EI-MS and high-resolution EI-MS were performed with a JEOL JMS-SX 102A spectrophotometer. $^1$H NMR spectra were taken as solutions in $CDCl_3$ with TMS as an internal standard on a JEOL EX-270 (270 MHz) spectrophotometer and as solutions in DMSO-$d_6$ with DMSO ($\delta_H$ 2.5) in the solvent as an internal standard on a JEOL α-600 (600 MHz) Fourier transform spectrophotometer. Chemical shifts are shown in ppm. $^{13}$C NMR spectra were taken as solutions in DMSO-$d_6$ with an α-600 (125 MHz) Fourier transform spectrophotometer. Chemical shifts are shown in ppm using the solvent ($\delta_C$ 39.7) as an internal standard. To identify the two isolated alkaloids, TLC plates (silica gel 60 $F_{254}$, Merck, Germany) ($CHCl_3$:$CH_3OH$ = 5:1, v/v) and 2% ferrous chloride solution were used.

**Materials.** Larvae (fifth instar), pupae, and adults of *I. similis*, collected in the spring from the Ishigaki island of Okinawa prefecture, were purchased from Hiroshima-Mikado Co. Ltd. (Hiroshima, Japan). The specimens were stored at −80 °C in a freezer until use. Fresh leaves of *T. tanakae* were also collected in the same season and area of Ishigaki island. All chemicals used were analytical grade.

**Isolation of Cytotoxic Compounds in the Pupae of *I. similis*.** Five pupae (2.8 g) of *I. similis* were squeezed and extracted with methanol (40 mL) under ultrasonication for 20 min three times at room temperature. The solution was evaporated to dryness, and the residue was partitioned with *n*-hexane (10 mL) and water (10 mL). The aqueous layer was made acidic (pH 1) with hydrochloric acid and extracted three times with ethyl acetate (10 mL). The aqueous layer was then made alkaline (pH 9) with ammonia solution and extracted three times with ethyl acetate (10 mL). Thus, the *n*-hexane fraction (67 mg), acid-neutral fraction (13.7 mg), basic fraction (2.4 mg), and residual water fraction were obtained. These fractions were subjected to cytotoxicity testing with human gastric carcinoma TMK-1 cells.[13] An aliquot of a solution of the basic fraction was injected into an ODS column (J'sphere ODS-80H, 4 $\mu$m particle size, 4.6 mm × 150 mm; YMC Co. Ltd., Kyoto, Japan) with a linear gradient of 20−50% acetonitrile for 0−60 min in 20 mM monoethanolamine-acetic acid buffer (pH 9.6). The cytotoxicity of each 2 min fraction (2 mL) against TMK-1 cells was then tested. Significant cytotoxicity was observed in fractions with retention times of 30−32 min (peak I) and 34−36 min (peak II). Compound **1** in peak I and compound **2** in peak II were confirmed to be homogeneous under two other HPLC conditions. In one HPLC condition, an ODS column (Shiseido Capcell Pak C18, 5 $\mu$m particle size, 4.6 mm × 250 mm; Shiseido Co. Ltd., Tokyo, Japan) was used, and the applied materials were eluted with 15% acetonitrile−20 mM phosphate buffer (pH 5). In the other HPLC condition, the samples were analyzed with the same ODS column, eluted with 35% acetonitrile−20 mM monoethanolamine-acetic acid buffer (pH 9.6). Under the above HPLC conditions, the mobile phase was pumped in at a flow rate of 1 mL/min, and UV absorbance of the eluate at 260 nm was monitored at ambient temperature.

**Preparation of Large Quantities of Cytotoxic Compounds 1 and 2.** Large amounts of cytotoxic compounds **1** and **2**, for structural determination, were isolated from 50 pupae (32 g) of *I. similis*, using the original compounds obtained from the five pupae as standard markers, by the following procedures. The 50 pupae were squeezed and extracted with methanol (100 mL) under ultrasonication for 20 min three times at room temperature. The solution was evaporated to dryness, and the basic fraction was obtained using a method similar to that in the case of five pupae and purified by HPLC. An aliquot was injected into an ODS column (J'sphere ODS-80H, 4 $\mu$m particle size, 10 mm × 150 mm; YMC Co. Ltd.), and the materials were eluted with a linear

gradient of 20−50% acetonitrile for 0−60 min in 20 mM monoethanolamine-acetic acid buffer (pH 9.6) at a flow rate of 4 mL/min. The UV absorbance of the eluate at 260 nm was monitored at ambient temperature. The amounts of compounds **1** and **2** isolated from the 50 pupae were 2.2 and 3.7 mg, respectively.

**Quantification of Compounds 1 and 2 in Larvae, Pupae, and Adults of *I. similis* and Leaves of *T. tanakae*.** Basic fractions were prepared from the methanol extracts of 3 larvae (2.0 g), 5 pupae (2.8 g), and 19 adults (3.8 g) of *I. similis* and also from that of fresh leaves of *T. tanakae* (6.2 g). The levels of compounds **1** and **2** in these four basic fractions were analyzed by HPLC as follows. An aliquot of the methanol solution of basic fraction was injected into a Shiseido Capcell Pak C18 ODS column and then eluted with a linear gradient of 10−25% acetonitrile in 20 mM phosphate buffer (pH 5) for 0−80 min and 25% acetonitrile in 20 mM phosphate buffer (pH 5) for 80−100 min at a flow rate of 1 mL/min. The UV absorbance of the eluted materials at 254 nm was monitored at ambient temperature. In addition, compounds **1** and **2** were identified by their UV spectra using a Tosoh PD-8020 photodiode array detector.

**Cytotoxicity Assay.** Three human cancer cell lines, A549 (lung cancer cell line), DLD-1 (colon cancer cell line), and K562 (leukemia cell line) were obtained from the American Type Culture Collection (Rockville, MD). The cervical cancer cell line, HeLa, was from RIKEN Cell Bank (Tsukuba, Japan). In the case of K562 floating cells, a 100 $\mu$L aliquot of cell suspension in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD) containing $2.5 \times 10^3$ cells was added to each well of a 96-well plate, which was immediately treated with diluted test samples for 96 h at 37 °C in 5% $CO_2$ in air. When cells growing in monolayers including A549, DLD-1, HeLa, and TMK-1 were investigated, a 100 $\mu$L aliquot of cell suspension in the same medium containing $1 \times 10^3$ cells was added to each well of a 96-well plate, the medium was changed after 24 h, and the cells were then treated with diluted test samples for 96 h at 37 °C in 5% $CO_2$ in air. After these treatments, cytotoxic effects were measured with a WST-1 {2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, Dojindo Laboratories, Kumamoto, Japan} assay.

***trans*-(+)-3,14α-Dihydroxy-6,7-dimethoxyphenanthroindolizidine (1).** This compound, obtained as a white solid, showed a positive rotation {$[\alpha]_D^{27}$ +20.8° ($c$ = 0.1, $CHCl_3$−$CH_3OH$ (1:1))}. The data from the $^1$H NMR and $^{13}$C NMR spectra are shown in Tables 1 and 2. NOE correlations were observed between $\delta_H$ 8.13 and $\delta_H$ 4.90, $\delta_H$ 8.13 and $\delta_H$ 7.09, $\delta_H$ 7.92 and $\delta_H$ 7.91, $\delta_H$ 7.91 and $\delta_H$ 3.99, $\delta_H$ 7.16 and $\delta_H$ 3.91, $\delta_H$ 7.16 and $\delta_H$ 4.50, and $\delta_H$ 3.91 and $\delta_H$ 3.99. UV ($CH_3OH$−$CHCl_3$) nm $\lambda$max 259, 285, 314; EI-MS $m/z$ = 365 ($M^+$), 296 ($M^+ - 69$), EI-HRMS calcd for $C_{22}H_{23}NO_4$ 365.1627, found 365.1617.

***trans*-(+)-3,14α-Dihydroxy-4,6,7-trimethoxyphenanthroindolizidine (2)**. This compound, obtained as a white solid, showed a positive rotation {$[\alpha]_D^{27}$ +24.0° ($c$ = 0.1, $CHCl_3$−$CH_3OH$ (1:1))}. The data from the $^1$H NMR spectrum are shown in Table 1. UV ($CH_3OH$−$CHCl_3$) nm $\lambda$max 261, 319; EI-MS $m/z$ = 395 ($M^+$), 296 ($M^+ - 69$), EI-HRMS calcd for $C_{23}H_{25}NO_5$ 395.1733, found 395.1732. (Literature values are as follows:[10] $^1$H NMR ($CD_3OD$−$CDCl_3$ (400 MHz)) $\delta$ 1.92−2.02, 2.32 (m, 1H), 2.41 (q, $J$ = 9), 2.52 (m, 1H), 3.34 (td, $J$ = 9, 2), 3.41 (d, $J$ = 15), 3.89 (s), 3.97 (s, 3H), 4.08 (s), 4.17 (d, $J$ = 15), 5.05 (d, $J$ = 2), 6.87 (s), 7.29 (d, $J$ = 9), 8.09 (d, $J$ = 9), 9.19 (s, 1H); UV ($CHCl_3$) nm $\lambda$max 243, 262, 277, 285, 305, 317; FAB-MS $m/z$ 396.1813.)

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**Supporting Information Available:** Figure of pupae and a female adult of *I. similis*. This material is available free of charge via the Internet at http://pubs.acs.org.

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