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Cytotoxic Antifeedant from *Dionaea muscipula* Ellis: A Defensive Mechanism of Carnivorous Plants against Predators

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We revealed that *Dionaea muscipula* Ellis accumulates a great amount of cytotoxic naphthoquinone derivatives, of which the main component is plumbagin (**1**). It was also revealed that **1** showed strong antifeedant activity against *Spodoptera litura*. These results strongly suggest that **1** in *D. muscipula* operates as an antifeedant which prevents them from being consumed by predators. The accumulation of a great amount of **1** would be an important strategy of carnivorous plants for their survival. Also, we discuss the structure–activity relationship of the cytotoxicity of naphthoquinones using isolated naphthoquinones and their derivatives.

Dionaea muscipula Ellis, known as Venus's flytrap, is the most famous carnivorous plant (Fig. 1). Charles Darwin claimed that it is "the most wonderful plant in the world".[1] *D. muscipula* can survive under nutritionally poor soil conditions by capturing and digesting prey, such as insects for a nitrogen source by using their trap-like leaves called trap lobes. However, it is also believed that *D. muscipula* attracts some insects that are predators, such as herbivorous insects, worms, etc. This is a serious problem for their survival because if their leaves were wounded by such predators, their leaves would be damaged by their own strongly acidic digestive enzymes. Thus, we assumed that there would be some mechanism for protection from predation in *D. muscipula*. Some plants and animals are known to have strong antifeedant or toxic compounds for their protection from predation. The most famous example for the latter case is tetrodotoxin in puffer fish.[2] We searched for the toxic compounds in *D. muscipula* according to the cytotoxicity against the P388 cell line. As a result, we found that *D. muscipula* contains a great amount of plumbagin (**1**), which shows strong cytotoxicity (IC_{50}: 0.01 μg/mL for P388, 0.11 μg/mL for HCT116). **1** also showed strong antifeedant activity in the bioassay using *Spodoptera litura*. Moreover, **1** is widely present in other carnivorous plants.[3]

Fig. 1. *D. muscipula*, the most famous carnivorous plant in the world.

In this paper, we discuss the identification of **1** as an antifeedant of carnivorous plants that is important for the survival, isolation, and structure determination of some analogs of **1** from *D. muscipula*, and structure–activity relationship study of naphthoquinone compounds relating **1**.

Results and Discussion

Fresh leaves of *D. muscipula* were immersed in 10% MeOHaq. After filtration, the extract was concentrated in vacuo. After this evaporation, a yellow oil was collected from the round bottom flask of the rotary evaporator by steam distillation. The yellow oil gave orange crystals by extensive pumping. The concentrated aqueous layer was partitioned with hexane, ethyl acetate, and 1-butanol. Moderate cytotoxicity against cancer cells, P388 murine lymphocytic leukemia and HCT116 human colon carcinoma, was observed in the hexane layer (IC_{50}: 0.01 μg/mL for P388), EtOAc layer (IC_{50}: 6.3 μg/mL for P388), and 1-BuOH layer (IC_{50}: 15.8 μg/mL for P388). However, the orange crystal collected by steam distillation showed strong cytotoxicity against cancer cells (IC_{50}: 0.01 μg/mL for P388, 0.11 μg/mL for HCT116).

This orange crystal was found to be completely pure plumbagin (**1**) by ^{1}H and ^{13}C NMR experiments.[4]

On the other hand, the hexane layer which showed the strongest cytotoxicity on the bioassay using the P388 cell-line was expected to contain some bioactive substance of strong activity in addition to **1**. From HPLC analysis, the hexane layer was proven to contain **1**. Thus, the cytotoxicity of the toxic compounds would be masked by the strong cytotoxicity of **1**. Therefore, to detect other cytotoxic compounds, we removed **1** from the hexane layer. The hexane layer (1.6 g) was evaporated in vacuo to remove **1** by steam distillation. Thus, we obtained 1.4 g of **1** from 1.6 g of the hexane layer. The content of **1** in *D. muscipula* reached 0.5% weight of the fresh trap lobes. The residual hexane fraction was separated using silica-gel and reversed-phase column chromatography, followed by re-

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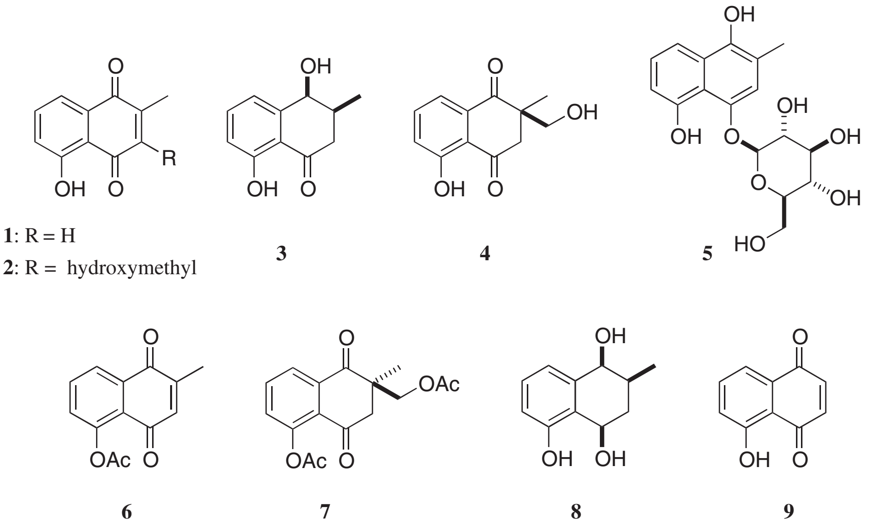

Fig. 2. Plumbagin and its analogs.

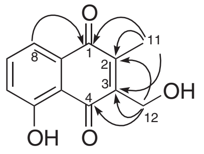

Fig. 3. Selected HMBC correlations of **2**.

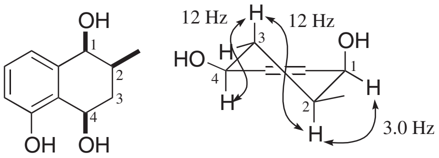

Fig. 4. Relative stereochemisty in **9**.

Table 1. Cytotoxicities of Plumbagin and Its Analogs[a)]

Compounds	$IC_{50}/\mu g\,mL^{-1}$	Compounds	$IC_{50}/\mu g\,mL^{-1}$
1	0.01	**6**	3.0
2	0.08	**7**	0.5
3	0.4	**8**	25
4	0.06	**9**	7.1
5	2.0		

a) Against P388 cells.

versed-phase HPLC. Fractionation was carried out based on the characteristic UV absorption (400 nm) of the naphthoquinone moiety. From this, we obtained a novel naphthoquinone compound, 3-hydroxymethylplumbagin (**2**), in addition to the known naphthoquinones, such as isoshinanolone (**3**)[5] and diomuscinone (**4**)[6] (Fig. 2). Also, the known naphthoquinone compound, hydroplumbagin β-D-glucopyranoside (**5**),[7] was obtained from the EtOAc fraction.

The structure of **2** was determined by using FAB MS, ^1H, and ^{13}C NMR experiments. The existence of the naphthoquinone skeleton in **2** was determined by the comparison of the chemical shifts in ^1H and ^{13}C NMR spectra with those of **1**. The NMR spectra also suggested the existence of a methyl and hydroxymethyl group. HMBC (Heteronuclear Multiple-Bond Correlation) correlations from H-11 to C-1, C-2, and C-3 and from H-8 to C-1 indicated that a methyl group was connected to the C-2 position (Fig. 3). Correlations from H-12 in the hydroxymethyl group to C-2, C-3, and C-4 revealed the connectivity of the hydroxymethyl group and C-3. The structure of **2** was determined as shown in Fig. 3.

At this point, we obtained some analogs of naphthoquinone compounds. Then, we discuss the structure–activity relationship on this type of cytotoxic compounds. We prepared some derivatives of naphthoquinones from isolated compounds. Two acetylated analogs, acetylplumbagin (**6**) and diacetyldiomuscinone (**7**), were prepared from **1** and **4**, respectively. Also, reduced analog **8**, from **3** that lost the carbonyl group, was prepared. Compound **8** was obtained as a single isomer, and the relative configuration was determined as shown in Fig. 4. In the ^1H NMR spectrum, the axial proton of H-3 was observed as a quartet signal with a large coupling constant (12 Hz). H-2 and H-4 were determined to have a *trans* diaxial relationship. This implies the methyl group at C-2 and the hydroxy group at C-4 should be in a *syn* relationship. Also, because of the small coupling constant (3.0 Hz) between H-1 and H-2, the relative configuration between them was determined to be *syn*.

The bioassay using the P388 cell-line using all of these naphthoquinone analogs was carried out. Cytotoxicities of these naphthoquinone compounds are summarized in Table 1. Compound **1** showed the strongest bioactivity (IC_{50}: 0.01 μg/mL). Compounds **2** and **4** also showed significant cytotoxicities (IC_{50}: 0.08 and 0.06 μg/mL, respectively), however, it was reported by Zhang et al. that jugrone (**9**) showed a weak cytotoxicity (7.1 μg/mL) against P388 cells.[8] These results indicate that the methyl group at the H-2 position is important, but the double bond is not necessary for strong cytotoxicity. On the other hand, compound **3**, with the reduced carbonyl group at the 1-position and a double bond, showed moderately strong cytotoxicity (IC_{50}: 0.4 μg/mL). Compound **8** also showed weak cytotoxicity. Thus, two carbonyl groups within the naphthoquinone structure are required for strong cytotoxicity. Also, a significant reduction of activity was induced by the acetylation of the phenolic hydroxy groups of **1** and **4**. Thus, the phenolic hydroxy group is also important for cytotoxicity. Hydroplumbagin β-D-glucopyranoside (**5**), which cannot isomerize to a naphthoquinone skeleton, also showed weak cytotoxicity (IC_{50}: 2.0 µg/mL). These results revealed that the resonance between the phenol moiety and the two carbonyl groups, and the existence of a methyl group at C-2, are indispensable for strong cytotoxicity. Interestingly, the naphthoquinone skeleton itself is not necessary for strong bioactivity.

From these results, it was revealed that *D. muscipula* contains some cytotoxic naphthoquinones in their trap lobes. In particular, the content of **1** reached 0.5% by weight of fresh trap lobes. The accumulation of a great amount of **1** in *D. muscipula* strongly suggests some important biological significance.

Next, we examined the antifeedant activity of cytotoxic naphthoquinone isolated from *D. muscipula* by using the larvae of *Spodoptera litura*, a moth most widely used for antifeedant tests. The bioassay was carried out as follows. A trap leaf of *D. muscipula* was extracted with hexane and then MeOH to remove **1** completely, as checked by HPLC. The HPLC analysis of the resulting extract showed that only **1** was detected in the hexane extract, and **1** was not detected in the MeOH extract. The resulting **1**-free trap lobes and intact trap lobes of *D. muscipula* were used for the bioassay. Additionally, a bioassay using intact lettuce leaves and lettuce leaves on which a coat of **1** (0.5% from the fresh weigh) was present were also carried out. We compared the area of leaves on which *S. litura* ate in these two cases.

The result of the antifeedant bioassay is shown in Table 2. The **1**-free leaves of *D. muscipula* were moderately eaten by the *S. litura* worm. On the other hand, intact leaves significantly inhibited the feeding of *S. litura* (Fig. 5). A similar result was also obtained in a bioassay using intact lettuce leaves and the lettuce leaves with **1** (Fig. 6). These results clearly showed that **1** has strong antifeedant activity against the worm of *S. litura*, and, thus, indicated that **1** operates as an antifeedant in the trap lobes of *D. muscipula*.

We revealed that *D. muscipula* accumulates a great amount of cytotoxic naphthoquinone derivatives, of which the main component is **1**. It was also revealed that **1** showed strong antifeedant activity against *S. litura*. These results strongly suggest that **1** in *D. muscipula* operates as an antifeedant which prevents them from being eaten by predators. Moreover, it is known that other carnivorous plants, such as *Drosera*, *Nepenthes*, and *Ardorovanda*, also contain **1**. Similar to the case of *D. muscipula*, it is believed that **1** would also operate as an antifeedant in these carnivorous plants. Because of the serious damage suffered from the feeding by many predators, the accumulation of a great amount of **1** would be an important strategy of carnivorous plants for their survival.

Table 2. Antifeedant Activity of **1** against *S. litura*

Entry	Leaves	Consumption area[a)] /%
1	intact trap leaves (containing **1**)	0.1
2	treated trap leaves (**1**-free)	90
3	intact lettuce leaves	100
4	lettuce leaves coated with **1** (0.5%)	0

a) Consumption area was calculated by using the Image J.

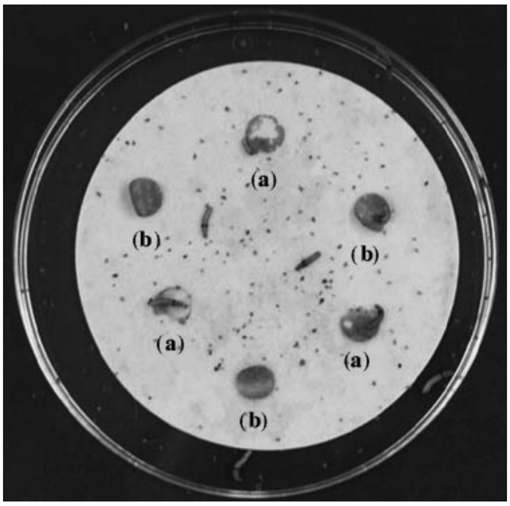

Fig. 5. The antifeedant bioassay: (a) **1**-free leaves of *D. muscipula*, (b) intact leaves.

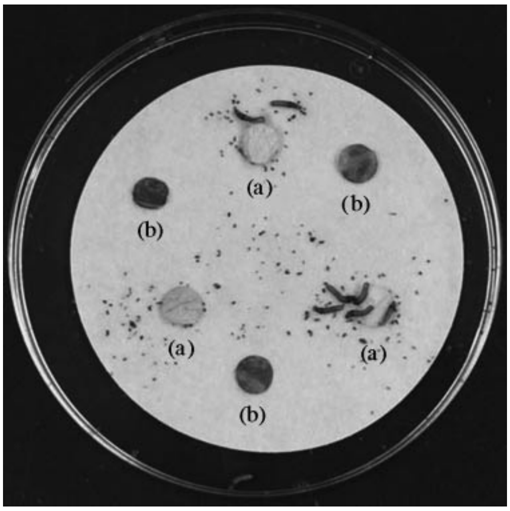

Fig. 6. The antifeedant bioassay: (a) intact lettuce leaves, (b) lettuce leaves with **1**.

Experimental

General. ^1H NMR (400 MHz and 270 MHz) spectra and ^{13}C NMR (100 MHz) spectra were recorded on Jeol A400 NMR and EX270 spectrometers, respectively, using TMS as an internal standard. The FAB-MS and HR–FABMS were measured on a Jeol JMS-700 spectrometer, using glycerol or *m*-nitrobenzyl alcohol as a matrix. The IR spectra were recorded by a Jasco FT/IR-410 spectrometer. The specific rotations were measured by a Jasco DIP-360 polarimeter. The HPLC purification was performed using a Develosil ODS-HG-5 (ϕ 20 × 25 mm, Nomura chemicals Co Ltd.) on a Jasco 970 system containing a 970 UV detector. The solvents used for HPLC were available from Kanto Chemical Co. and were filtered through a Toyo Roshi membrane filter (cellulose acetate of 0.5 µm pore size, 47 mm dia.). Silica-gel column chromatography was performed on Silica-gel 60N (Kanto Chemical Co. Ltd.). Reversed-phase column chromatography was performed on a Cosmosil 75C18-OPN (Nakalai Tesque Co. Ltd.). TLC was performed on Silica gel F_{254} (0.25 mm, MERCK).

Plant Materials. *D. muscipula* used for the extraction and the antifeedant bioassay were purchased from Yoneyama Plantation (Japan).

Extraction and Isolation. Fresh leaves of *D. muscipula* (1.01 kg) were successively extracted with 10% MeOHaq (6.3 L) at 4 °C for a week, hot water (2.5 L) for 10 min, and MeOH (5.2 L) at 4 °C for a week. The resulting 10% MeOHaq extract was evaporated in vacuo. While the extract was being concentrated, plumbagin (**1**, 321 mg) was obtained by steam distillation. The obtained aqueous solution was partitioned with hexane (300 mL × 3), ethyl acetate (300 mL × 3), and then 1-butanol (300 mL × 3). Water was added to the hexane extract (1.6 g) for steam distillation. After evaporation, 1.4 g of **1** was obtained as a distillate. The residue was chromatographed with a silica-gel column (ϕ 55 × 470 mm) using hexane–ethyl acetate (100:0, 80:20, 60:40, 50:50, 40:60, 20:80, 0:100) to give **1** (19.4 mg) and a cytotoxic fraction (24.3 mg). Further purification was carried out by HPLC using a Develosil ODS HG-5 column with 30% CH_3CNaq to give **2** (1.0 mg).

Quantitative Analysis of 1 in the Trap Lobe of *D. muscipula*. Fresh leaves of *D. muscipula* (10 g) were chopped and extracted with hexane (100 mL) at 4 °C for a week. After filtration, the extract was dried over absolute sodium sulfate and evaporated in vacuo to give **1** (45.7 mg, 0.5% from the fresh weigh of trap lobes).

Plumbagin (1). ^{1}H NMR (400 MHz, $CDCl_3$, rt) δ 11.98 (1H, s), 7.64 (1H, dd, $J = 1.8, 7.8$ Hz), 7.61 (1H, dd, $J = 7.6, 7.8$ Hz), 7.26 (1H, dd, $J = 1.8, 7.6$ Hz), 6.82 (1H, q, $J = 1.6$ Hz), 2.19 (3H, d, $J = 1.6$ Hz).

3-Hydroxymethyl Plumabagin (2). ^{1}H NMR (400 MHz, CD_3OD, rt) δ 7.66 (1H, dd, $J = 2.5, 7.4$ Hz), 7.60 (1H, t, $J = 7.4$ Hz), 7.26 (1H, dd, $J = 2.5, 7.4$ Hz), 4.64 (2H, s), 2.27 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$, rt) δ 191.0, 184.6, 161.4, 146.1, 142.7, 136.5, 132.0, 124.2, 119.4, 114.7, 57.4, 12.5; IR (KBr) 3447, 2921, 1732, 1630, 1614, 1455, 1352, 1265, 1005, 837, 766, 732 cm^{-1}; FAB MS m/z 219 $[M + H]^+$; HR FAB MS m/z 241.0502 $[M + Na]^+$ (calcd for $C_{12}H_{10}O_4Na$ m/z 241.0477).

Isoshinanolone (3). ^{1}H NMR (270 MHz, $CDCl_3$, rt) δ 12.4 (1H, s), 7.48 (1H, dd, $J = 7.4, 8.6$ Hz), 7.26 (1H, s), 6.94 (1H, dd, $J = 1.0, 8.6$ Hz), 6.92 (1H, br d, $J = 7.4$ Hz), 4.75 (1H, d, $J = 2.7$ Hz), 2.87 (1H, dd, $J = 10.8, 17.5$ Hz), 2.57 (1H, dd, $J = 4.1, 17.5$ Hz), 2.44 (1H, m), 1.18 (3H, d, $J = 6.6$ Hz).

Diomuscinone (4). ^{1}H NMR (270 MHz, $CDCl_3$, rt) $\delta = 12.0$ (1H, s), 7.65 (1H, dd, $J = 7.9, 8.2$ Hz), 7.56 (1H, dd, $J = 1.2, 7.9$ Hz), 7.27 (1H, dd, $J = 1.2, 8.2$ Hz), 4.01 (1H, dd, $J = 4.9, 11.1$ Hz), 3.50 (1H, dd, $J = 6.9, 11.1$ Hz), 3.40 (1H, d, $J = 17.0$ Hz), 2.75 (1H, d, $J = 17.0$ Hz), 2.30 (1H, br s), 1.25 (3H, s).

Hydroplumbagin β-D-Glucopyranoside (5). ^{1}H NMR (270 MHz, CD_3OD, rt) δ 7.63 (1H, dd, $J = 1.0, 8.5$ Hz), 7.25 (1H, dd, $J = 7.6, 8.5$ Hz), 7.20 (1H, s), 6.73 (1H, dd, $J = 1.0, 7.6$ Hz), 4.99 (1H, d, $J = 7.6$ Hz), 3.95 (1H, dd, $J = 2.0, 12.0$ Hz), 3.73 (1H, dd, $J = 5.8, 11.9$ Hz), 3.60–3.40 (4H, m), 2.34 (3H, s).

5-*O*-Acetylplumbagin (6). Plumbagin **1** (20 mg, 0.11 mmol) was dissolved in pyridine (1.0 mL), and acetic anhydride (0.79 mL) was added to this solution. The reaction mixture was stirred for 10 h at room temperature, and then evaportated in vacuo. The residue was purified by silica-gel column chromatography with hexane–ethyl acetate (100:0, 60:40, 50:50, 40:60, 0:100) to give **6** (21.7 mg, 93%). ^{1}H NMR (270 MHz, $CDCl_3$, rt) δ 8.03 (1H, dd, $J = 1.2, 8.0$ Hz), 7.70 (1H, t, $J = 8.0$ Hz), 7.33 (1H, dd, $J = 1.2, 8.0$ Hz), 6.68 (1H, q, $J = 1.3$ Hz), 2.42 (3H, s), 2.14 (3H, d, $J = 1.3$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$, rt) δ 184.8, 183.6, 169.4, 149.2, 146.9, 136.9, 134.4, 133.8, 129.4, 125.1, 123.4; IR (film) 1756, 1658, 1591, 1362, 1252 cm^{-1}; FAB MS m/z 253 $[M + Na]^+$; HR FAB MS m/z 253.0453 $[M + Na]^+$ (calcd for $C_{13}H_{10}O_4Na$ m/z 253.0477).

Diacetyldiomuscinone (7). Compound **4** (2.0 mg, 9.0 μmol) was dissolved in pyridine (1.0 mL), and acetic anhydride (0.5 mL) was added to this solution at room temperature. After stirring for 10 h, the reaction mixture was concentrated in vacuo. The residue was purified by silica-gel column chromatography with hexane–ethyl acetate (100:0, 60:40, 50:50, 40:60, 0:100) to give **7** (2.0 mg, 85%). ^{1}H NMR (270 MHz, $CDCl_3$, rt) δ 8.04 (1H, dd, $J = 1.3, 7.9$ Hz), 7.73 (1H, t, $J = 7.9$ Hz), 7.37 (1H, dd, $J = 1.3, 7.9$ Hz), 4.41 (1H, d, $J = 10.5$ Hz), 4.03 (1H, d, $J = 10.5$ Hz), 3.14 (1H, d, $J = 15.3$ Hz), 2.81 (1H, d, $J = 15.3$ Hz), 2.34 (3H, s), 1.93 (3H, s), 1.27 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$, rt) δ 197.5, 193.9, 170.4, 169.6, 148.6, 135.4, 134.8, 129.9, 129.7, 125.9, 68.7, 48.6, 29.7, 21.4, 21.0, 20.5; IR (film) 1747, 1697, 1594, 1373 cm^{-1}; $[\alpha]_D^{22}$ $-5°$ (c 0.10, $CHCl_3$); FAB MS m/z 305 $[M + Na]^+$; HR FAB MS m/z 327.0871 $[M + Na]^+$ (calcd for $C_{16}H_{16}O_6Na$ m/z 327.0845).

Reduction of 3. Compound **3** (2.0 mg, 0.010 mmol) was dissolved in EtOH (0.5 mL), and then sodium tetrahydroborate (1.5 mg, 0.040 mmol) was added at 0 °C. After stirring for 10 h at room temperature, the reaction was quenched by adding water (0.5 mL), and the reaction mixture was then concentrated in vacuo. The residue was purified by ODS column chromatography (Cosmosil $75C_{18}$-OPN, ϕ 5 × 20 mm, $MeOH/H_2O = 30:70$) and then HPLC (Develsil ODS-HG-5, 50% MeOHaq) to give **8** (0.5 mg, 23%). ^{1}H NMR (400 MHz, D_2O, rt) δ 7.31 (1H, t, $J = 8.0$ Hz), 6.97 (1H, d, $J = 8.0$ Hz), 6.80 (1H, d, $J = 8.0$ Hz), 4.87 (1H, dd, $J = 7.2, 12.0$ Hz), 4.54 (1H, d, $J = 2.3$ Hz), 1.90–2.20 (2H, m), 1.75 (1H, ddd, $J = 12.0, 12.0, 12.0$ Hz), 1.24 (3H, d, $J = 7.2$ Hz); ^{13}C NMR (100 MHz, D_2O, rt) δ 161.6, 136.1, 127.2, 124.5, 118.3, 114.5, 69.4, 65.5, 47.7, 30.9, 16.0; IR (film) 3208, 1595, 1475, 1275 cm^{-1}; $[\alpha]_D^{22}$ $+ 13°$ (c 0.03, MeOH); FAB MS m/z 217 $[M + Na]^+$; HR FAB MS m/z 217.0906 $[M + Na]^+$ (calcd for $C_{11}H_{14}O_3Na$ m/z 217.0841).

Cytotoxicity Test. Growing cells of P388 murine lymphocytic leukemia were suspended in RPMI-1640 medium containing 10% fetal bovine serum, 10 μM 2-hydroxyethyl disulfide, and kanamycin (100 g/mL) at 2×10^4 cells/mL, and samples dissolved in MeOH were added. The mixture was incubated at 37 °C for 4 days in a CO_2 incubator in a humidified atmosphere containing 5% CO_2. The cells were counted by the MTT method.[9] The IC_{50} value (concentration required for 50% inhibition of cell growth) was determined using the growth curve.

Antifeedant Bioassay. The antifeedant potential of leaves of *D. muscipula* was tested with the third instar larvae of *S. litura* by a leaf-disk-choice test. Untreated trap lobes were bored (ϕ 7 mm) and dried for 1 h. The treated disks (**1**-free disks) were prepared by boring and extracting with hexane for 51 h at room temperature to remove **1** completely. Then, the leaves were dried for 1 h to remove hexane. The resulting hexane layer was evaporated and analyzed by HPLC using Develosil ODS-HG-5 with 80% MeOHaq to identify the components in this layer. Three untreated leaf discs and three treated leaf discs were placed on moist filter paper in a ϕ 8.5 cm petri dish with 10 larvae per dish. Tests were performed under darkness at 27 °C for 24 h. Images of the leaf discs were produced using a Canon Canonscan D2400U. These images were analyzed using the Image J (available from the National Institute) to get the consumed area.

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