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Mechanism of antifeedant activity of plumbagin, a compound concerning the chemical defense in carnivorous plant

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Abstract—*Dionaea muscipula* Ellis accumulates a large amount of plumbagin (**1**), which operates as an antifeedant against predators. Content of **1** reached 0.5% weight of the fresh trap lobes. It was found that other carnivorous plants also accumulated similar naphthoquinone-type strong antifeedant. Thus, naphthoquinone analogs are widely used as an antifeedant among the carnivorous plants. By using several analogs of **1**, we clarified that both the high volatility and high redox potential of **1** are important for its strong antifeedant activity. It was known that plumbagin stimulates the mitochondrial electron transport system as a result of intercepting electrons. These results suggested that the *Droseraceae* family possesses a universal defensive mechanism against predators, that is, accumulation of volatile naphthoquinone with high redox potential as defensive substance. Thus, it is estimated that highly volatile naphthoquinone of moderately high redox potential would be used as an antifeedant of weak toxicity.

Dionaea muscipula Ellis, known as Venus' flytrap, is the most famous carnivorous plant (Fig. 1).[1] *D. muscipula* can survive under nutritionally poor soil conditions by capturing and digesting insects as nitrogen source using their trap lobes. *D. muscipula* can capture and digest even some predators, such as herbivorous insects, worms, etc. However, it is interesting that *D. muscipula* is never preyed on by predators. If their leaves were wounded by such predators, the leaves would be seriously damaged by their own digestive enzymes, which are secreted when they prey on insects.

Figure 1. *Dionaea muscipula* Ellis.

Recently, we revealed the defensive mechanism of *D. muscipula* against predation.[2] *D. muscipula* accumulates a great amount (0.5% weight of fresh trap lobes) of cytotoxic plumbagin (**1**) as a strong antifeedant. This result strongly suggested that **1** in *D. muscipula* operates as a defensive substance, which prevents them from being fed on by predators. Analogs of **1** with high antifeedant activity and low cytotoxicity would be useful as an antifeedant, which is used in agricultural application. For this purpose, the investigation on the mechanism of antifeedant activity of **1** is important. However, no research was carried out on the mode of action of strong antifeedant **1**. In this letter, we examined the mechanism of antifeedant activity of **1** using its analogs and found that the antifeedant activity of **1** is due to the high volatility and high redox potential.

To study the molecular mechanism for antifeedant activity of **1**, we first examined the structure–activity relationship using **1**, its analogs (**2**, **4**, and **14**) isolated from *D. muscipula*[2] structurally modified analogs (**3**, **5**, **10**) prepared from **1** and **4**, respectively,[2] commercially available 7-methyljuglone (**8**) and juglone (**11**). The antifeedant bioassay against *Spodoptera litura* was carried out using these compounds (Table 1).[2] Compounds **1**

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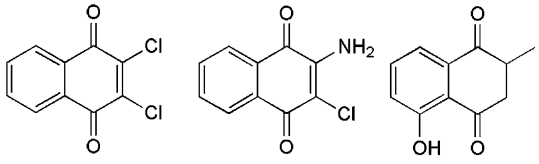
Table 1. Antifeedant activities of plumbagin and its analogs

Compound	Antifeedant activity(ppm)	Compound	Antifeedant activity (ppm)
1	5	**8**	10
2	50	**9**	>100
3	>100	**10**	>100
4	>100	**11**	5
5	>100	**12**	50
6	1	**13**	>100
7	50		

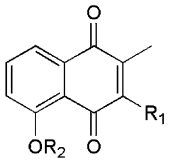
and **11** showed significant antifeedant activity at 5 ppm against *S. litura*. However, analogs with a reduced quinone moiety (**4**, **5**, **10**, **13**) did not show bioactivity at more than 100 ppm. This result suggested the importance of the quinone moiety for antifeedant activity. And acetylplumbagin (**3**), which has a masked phenolic hydroxyl group did not show bioactivity at more than 100 ppm. Thus, the importance of the phenolic hydroxyl group was also suggested. However, **2**, which has a hydroxymethyl group at the 3-position was effective at as weak as 50 ppm, although it has both a quinone skeleton and a free phenolic hydroxyl group. Further antifeedant bioassay using commercially available naphthoquinone menadione (**7**) without a phenolic hydroxyl group and naphthazarin (**12**) with an additional phenolic hydroxyl group was carried out to evaluate the importance of the phenolic hydroxyl group (Table 1). In the bioassay, both compounds were effective at as weak as 50 ppm. From these results, we assumed that the reactivity of naphthoquinone would be concerned with antifeedant activity.[3] We supposed that obstruction of feeding started from Michael addition or reduction of the quinone moiety, considering the reactivity of the naphthoquinone skeleton.

First, we examined the connectivity between antifeedant activity and Michael reactivity of plumbagin analogs. The phenolic hydroxyl group would activate α,β-unsaturated ketone by hydrogen bonding. It was already known that warburganal,[4] a strong antifeedant of α,β-unsaturated aldehyde isolated from the bark of *Warburgia* species, lost its antifeedant activity, when it was treated with L-cystein.[5] Thus, strong antifeedant activity of warburganal would be due to the Michael addition with protein in the worm body. As a model experiment, glutathione (**14**),[6] famous peptidyl Michael donor, was added to naphthoquinone compounds and then the production of the Michael adduct was examined (Table 2). Plumbagin (**1**), a strong antifeedant, gave a moderate amount of the Michael adduct[7] (29% yield). Compounds **3** and **7**, which were weak antifeedants, gave only a small amount of adducts (from **3**, 1.4% yield; from **7**, 2.9% yield). Also, weak antifeedant **12** gave no Michael adduct at all. However, strong antifeedant **11** gave only a small amount of Michael adduct (2.3% yield), and its yield was comparable to the case of **3** and **7**. As a result, we concluded that antifeedant activity of **1** cannot be explained by the Michael reactivity of naphthoquinones.

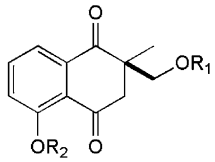


15 **16** **17**

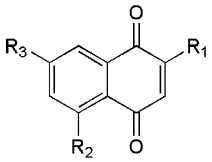
Next, we examined the relationship between antifeedant activity and the redox potential of naphthoquinones. Antifeedant activities and redox potentials of naphthoquinones, **1**, **3**, **6**, **9**, **11**–**12**, **15**, and **16** were measured. A well-defined two-electron redox wave was observed in the cyclic voltammogram of **1** [0.1 mM, 0.1 M phosphate buffer (pH 6.3)]. Similar two-electron redox waves were observed for other analogs. The standard redox potential, E_0, and the difference of cathodic peak potential and anodic peak potential, ΔE_p, are summarized in Table 3. Though the ΔE_p value of **7**, which is a weak



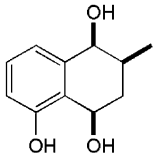
1: $R_1 = H, R_2 = H$
2: $R_1 = CH_2OH, R_2 = H$
3: $R_1 = H, R_2 = Ac$



4: $R_1 = H, R_2 = H$
5: $R_1 = Ac, R_2 = Ac$



6: $R_1 = H, R_2 = H, R_3 = H$
7: $R_1 = CH_3, R_2 = H, R_3 = H$
8: $R_1 = H, R_2 = OH, R_3 = CH_3$
9: $R_1 = OH, R_2 = H, R_3 = H$



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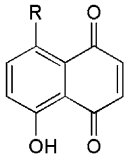

11: R = H
12: R = OH

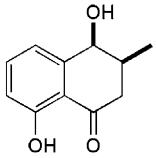

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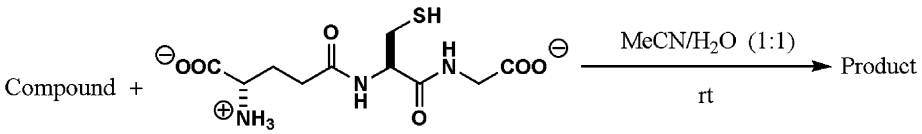
Table 2. Michael addition and antifeedant activities



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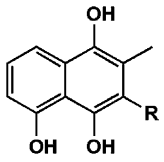
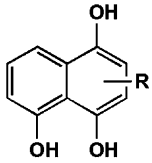
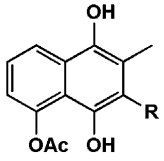
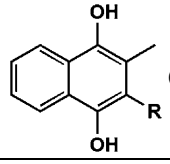
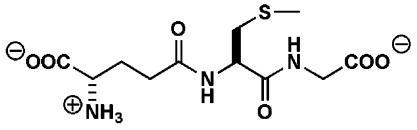
Compound	Product (yield)	Antifeedant activity (ppm)	Compound product (yield)	Antifeedant activity (ppm)
1	 (29%)	5	**11**	 (2.3%)	5
3	 (1.4%)	>100	**12**	ND	50
7	 (2.9%)	50	R =		

Table 3. Redox potential and antifeedant activities of plumbagin and its analogs

Compound	Redox potential (V)		E_0 (V)	ΔE_p (mV)	Antifeedant activity (ppm)
1	−0.238	−0.273	−0.26	35	5
3	−0.244	−0.273	−0.26	29	>100
6	−0.117	−0.166	−0.14	49	1
7	−0.160	−0.246	−0.20	86	50
8	−0.198	−0.233	−0.22	35	10
9	−0.275	−0.321	−0.30	46	>100
11	−0.174	−0.201	−0.19	27	5
12	−0.264	−0.291	−0.28	27	50
15	−0.134	−0.150	−0.14	16	20
16	−0.352	−0.384	−0.37	32	>100

antifeedant, was high (86 mV), possibly due to slow redox reaction, ΔE_p values of other naphthoquinones were close to the theoretical value (ca. 30 mV) for a two-electron reversible wave. Thus, positive correlation between redox potential and antifeedant activities of naphthoquinones was observed in some cases, such as **1**, **7**, **8**, **11**, and **15**. However, as seen in the cases of **3**, **6**, **9**, **12**, and **16** there were several exceptions. From these results, it was concluded that antifeedant activity of **1** cannot be fully explained only by the simple redox potentials of naphthoquinones.

In the course of handling some samples of naphthoquinone, we noted all antifeedant naphthoquinones were highly volatile. It was supposed that volatility of the antifeedant, not only redox potentials, was essential for antifeedant activity. When antifeedant activity is monitored in the bioassay using *S. litura*, there would be two steps in the development of antifeedant activity. The first one is an absorption of the vapor of **1** from the atmosphere, and the second one would be redox reaction of naphthoquinones in the worm body.

We made an estimation of the volatility of naphthoquinones (**1**, **3**, **6**–**9**, **11**–**12**, and **15**–**16**): the vials with sample (5.0 mg) with beforehand-weighed cap were made airtight and after 2 days the caps were weighed to calculate the increase due to the sublimed naphthoquinone. We used the increase in weight as the index of volatility (Table 4). Interestingly, strong antifeedants such as **1**, **8**, **11**, and **6** were highly volatile. As a result, it was found that the order of volatility was **1** > **11** ≃ **8** > **6**. Antifeedant activity of volatile and reductive naphthoquinone (**6**) was as strong as that of **1** and **8**. Nonvolatile and reductive 2,3-dichloronaphthoquinone (**15**) showed moderate antifeedant activity. Moreover, 2-amino-3-chloronaphthoquinones (**16**) and Lawson (**9**), which is used as a standard of high redox potential, have low volatility, and showed weak antifeedant activity. These results suggested that strong antifeedant activity could

Table 4. Volatility and antifeedant activities of plumbagin and its analogs

Compound	Volatility (μg)	Antifeedant activity (ppm)
1	31	5
3	<3	>100
6	17	1
7	<3	50
8	20	10
9	<3	>100
11	21	5
12	5	50
15	<3	20
16	<3	>100

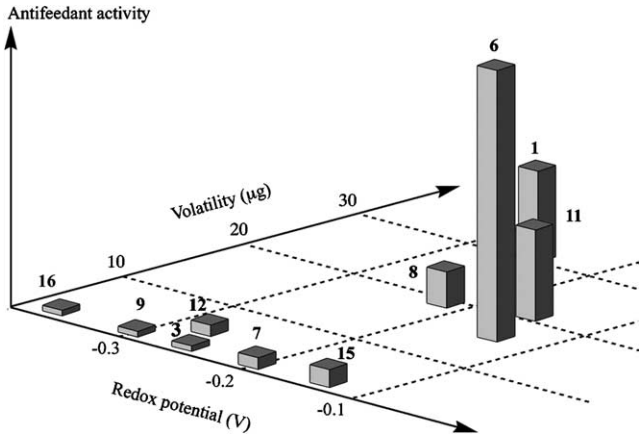


Figure 2. Relationship among redox potential, volatility and antifeedant activity in plumbagin and its analogs.

be explained by combining volatility and standard redox potentials (E_0) of naphthoquinones as seen in the cases of **1**, **6**, and **11** (Fig. 2). As seen in **1** in Figure 2, volatility would be a more important factor for the strong antifeedant activity than the redox potential. Antifeedant naphthoquinones absorbed by *S. litura* would be concerned with the redox reaction in the worm body. Then, we tried to detect the reduced **1** in the body of *S. litura*, which was bred in the atmosphere of **1** to confirm this hypothesis.

The third instar larvae of *S. litura* were bred in the atmosphere of **1**. Volatile **1** gradually vaporized and was adsorbed by the larvae through respiration. And then these larvae were extracted with MeOH, filtered (PTFE membrane filter), and concentrated. The obtained extract of **1**-treated larvae was analyzed by HPLC (Develosil ODS-HG-5, 60% MeOHaq containing 0.1% TFA). Similarly, an extract of **1**-free larvae was also prepared as a control and analyzed by HPLC. Compared with the result using **1**-free larvae, HPLC analysis of the extract of the **1**-treated larvae gave a peak corresponding to **1** and an additional new peak at rt 10 min (Fig. 3). This peak was identified to be 1,4-diketone **17** by the coinjection of an authentic sample, which was prepared by catalytic hydrogenation of **1** followed by Dess–Martin oxidation[8] (Scheme 1). ^{1}H NMR study showed that the structure of **17** was determined to be 1,4-diketone, not 1,4-hydroquinone.[9] We clarified that volatile **1** was reduced in the body of *S. litura* into **17**. It was also found that **17** was gradually oxidized by air to afford **1** again. This result strongly suggests that volatility and high standard redox potential of **1** are important for its antifeedant activity.

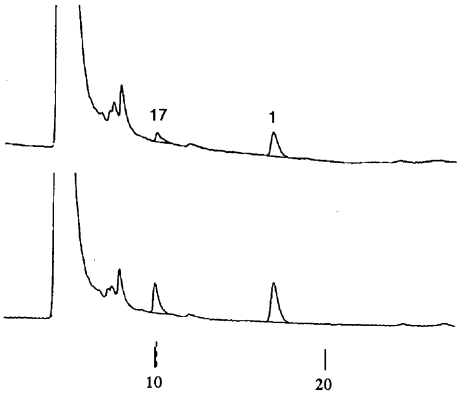
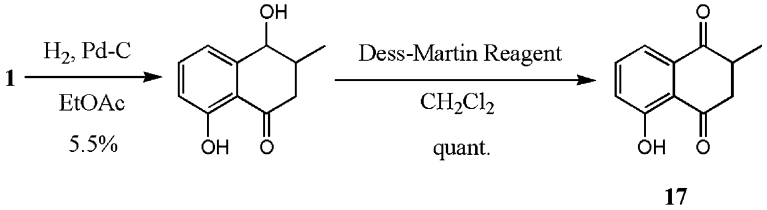


Figure 3. HPLC analysis of **17** and **1** in the extract of *S. litura* (upper: HPLC charomatogram of the extract of *S. litura* bred in the atmosphere of **1**; under: HPLC charomatogram of the extract of *S. litura* coinjected with authentic **17**) [conditions; column: Develosil ODS-HG-5 (ϕ4.6 × 250 mm), mobile phase: 60% MeOH aq containing 0.1% TFA. Flow: 0.8 mL/min, detection: 254 nm].

It was known that some naphthoquinones showed potent inhibition of respiratory electron transport. It was already known that naphthoquinones isolated from *Fusarium* sp. stimulated the bacterial respiratory chain and generated a superoxide anion.[10] As described above, it was strongly suggested that strong antifeedant activity of **1** would be due to the stimulation of the mitochondrial electron transport system of *S. litura* as a result of intercepting electrons from the respiratory chain.

Our results suggested that volatility would be a more important factor for the strong antifeedant activity than the redox potential (Fig. 2). Thus, it is estimated that highly volatile naphthoquinone of moderately high redox potential would be used as an antifeedant of weak



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Scheme 1. Preparation of **17** from **1**.

toxicity. Studies searching for strong antifeedants of weak toxicity are now in progress.

Acknowledgements

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7. Michael adduct from **1**: ^{1}H NMR (400 MHz, D_2O, rt): 7.61 (1H, t, J = 6.8 Hz), 7.47 (1H, d, J = 6.8 Hz), 7.25 (1H, d, J = 6.8 Hz), 4.57 (1H, dd, J = 4.1, 6.8 Hz), 3.69 (1H, t, J = 8.2 Hz), 3.57 (2H, d, J = 10.9 Hz), 3.54 (1H, dd, J = 8.2, 13.6 Hz), 3.39 (1H, dd, J = 8.2, 13.6 Hz), 2.44 (2H, ddd, J = 4.1, 6.8, 12.3 Hz), 2.27 (3H, s), 2.07 (2H, dd, J = 4.1, 6.8 Hz) ppm.
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9. **17**: ^{1}H NMR (400 MHz, $CDCl_3$, rt): 7.91 (1H, s), 7.68 (1H, t, J = 7.5 Hz), 7.49 (1H, d, J = 7.5 Hz), 7.25 (1H, d, J = 7.5 Hz), 3.23 (1H, m), 3.08 (1H, dd, J = 5.3, 11.3 Hz), 2.93 (1H, dd, J = 11.3, 16.5 Hz), 1.28 (3H, d, J = 6.3 Hz) ppm.
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