Naturally Occurring Antidotes against Benzimidazole Fungicides

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TLC bioautography using precoated glass thin-layer plates impregnated with benomyl or carbendazim (MBC), and *Cladosporium herbarum* as a test fungus was evaluated as a facil way to detect plant secondary metabolites antidoting against benzimidazole fungicides. In addition to emodin and α-tocopherol from *Polygonum sachalinense,* three phenolics, 3,5-dihydroxy-4-methylstilbene and 5-methoxy-6,7-methylenedioxyflavone from *P. lapathifolium,* and 2,6-dimethoxybenzoquinone from *P. thunbergii* were isolated and characterized as new benzimidazole antidotes. Emodin exhibited the antidoting activity not only against benomyl but also against carbendazim (MBC), thiabendazole (TBZ), thiophanate-methyl and nocodazole. Furthermore, emodin showed antidoting activity against MBC in the wild-type *Neurospora crassa* and against diethofencarb in the mutant of *N. crassa* resistant to benzimidazole fungicides but highly susceptible to diethofencarb.

Introduction

A benzimidazole fungicide, benomyl (**1**), one of the best known synthetic and systemic fungicides, is believed to suppress the development of numerous species of fungi except Porosporae and Annellosporae (Ascomycetes), Oömycetes and other Phycomycetes by inhibiting cell division when it binds to β-tubulin and thereby interfering with microtubular subunit assembly.

Interested in plant metabolites our preliminary experiments showed some interactions with xenobiotics. So we established a simple bioassay system to find agents in plant extracts antidoting against benomyl (**1**). By this assay system in combination with TLC plates developed with wild plant extracts, benomyl and the test fungus *Cladosporium herbarum,* compounds were detected exhibiting antidoting activity against benomyl (**1**) checking methanol extracts from 22 species of wild plants. One of those extracts from *Polygonum thunbergii* Sieb. *et* Zucc. (Polygonaceae) gave a spot on thin layer plates on which *C. herbarum* could grow even after the plates were treated with benomyl (**1**) in excess amounts of the minimum inhibitory dosage. Therefore, the further survey of benomyl antidotes was focused on Polygonaceae species.

As the first naturally occurring benomyl antidotes, α-tocopherol (**6**) and emodin (**7**) were isolated from *P. sachalinense* Fr. Schm. and chemically characterized [1]. The present study revealed the presence of three more antidoting compounds (**8–10**) in Polygonaceae plants.

To examine the character of Polygonaceae benomyl antidotes, their activity was tested against some benzimidazole fungicides (**2–5**) with *C. herbarum* and against diethofencarb with a benomyl-resistant mutant of *Neurospora crassa* [2, 3] which showed clearly negatively correlated cross-resistance to diethofencarb.

Results and Discussion

Preliminary screening test for benomyl antidotes

The following 22 plants (18 families) were collected, and their aerial parts were extracted to be applied to the benomyl antidote test as shown in the Experimental section. Out of 22 plants, a benomyl-antidotal substance was found only in *Polygonum thunbergii* (Polygonaceae). The following were assayed without exhibiting antidote activity: Borraginaceae, *Myosotis sylvatica;* Caryophyllaceae, *Stellaria media;* Chenopodiaceae, *Spinacia oleracea;* Compositae, *Crysanthemum leucanthemum* and

Abbreviations or systematic names of pesticides: benomyl, [1-(butylaminocarbonyl)-1 H-benzimidazol-2-yl]carbamic acid methyl ester; carbendazim (MBC), 1 H-benzimidazol-2-ylcarbamic acid methyl ester; diethofencarb, 3,4-diethoxyphenylcarbamic acid isopropyl ester; DMF, N,N-dimethylformamide; nocodazole, [5-(2-thienylcarbonyl)-1 H-benzimidazol-2-yl]carbamic acid methyl ester; thiabendazole (TBZ), 2-(4-thiazolyl)-1 H-benzimidazole; thiophanate-methyl; [1,2-phenylenebis(iminocarbonothioyl)]biscarbamic acid dimethyl ester.

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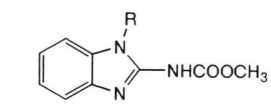
Erygeron annuus; Cruciferae, *Capsella bursa-pastoris;* Equisetaceae, *Equisetum arvense;* Geraniaceae, *Geranium* sp.; Hippocastanaceae, *Aesculus turbinata;* Labiatae, *Ajuga yezoensis* and *Lamium album;* Liliaceae, *Trillium kamtschaticum;* Magnoliaceae, *Liriodendron tulipifera;* Osmundaceae, *Osmundastrum cinnamomeum;* Papaveraceae, *Corydalis ambigua;* Polypodiaceae, *Onaclea sensibilis;* Scrophulariaceae, *Veronica aravensis* and *V. persica;* Violaceae, *Viola grypoceras;* and Umbelliferae, *Heracleum lanatum* subsp. *lanatum* and *Trilis japonica.* An aliquot of the *P. thunbergii* extract equivalent to 125 mg of fresh plant was enough to express the antidotal activity on thin-layer plates.

Benomyl antidotes in Polygonaceae plants

Since we could find a benomyl-antidoting substance in *P. thunbergii,* the further survey of the active principles was focused upon Polygonaceae plants. Polygonaceae (Polygonales) consists of about 30 genera with 750 species. Nine Polygonaceae plants tested in the present study are listed in Table I, which also show the presence of some benomyl antidotes more or less. Nothing was found in two cultivated species *Fagopyrum esculentum* and *F. tataricum.*



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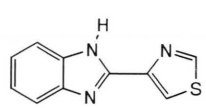

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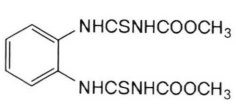

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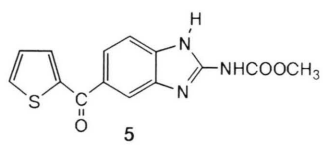

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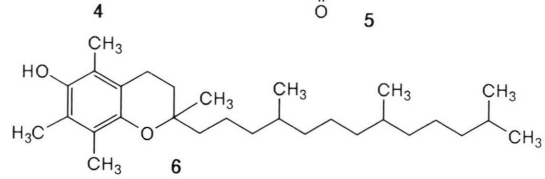

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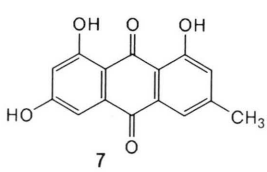

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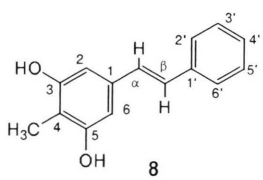

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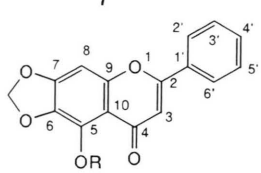
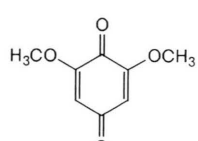


9: R=CH$_3$
11: R=H



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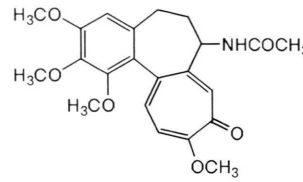
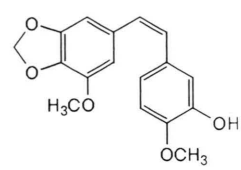


12: colchicine



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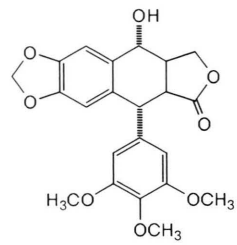
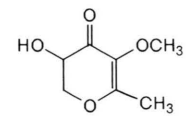
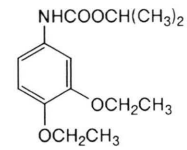


14: podophyllotoxin



15: streptopyrone



16: diethofencarb

1. Two antidotes from *P. sachalinense* Fr. Schm.

As reported in our earlier paper, a preliminary account for isolation and identification of α-tocopherol (**6**) and emodin (**7**) has been presented elsewhere [1].

2. Antidotes from *P. lapathifolium* L.

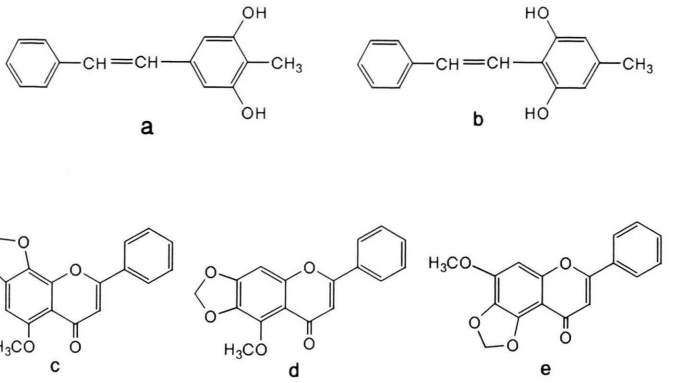
The lower running benomyl antidote PN-1 of *P. lapathifolium* L. subsp. *nodosum* (Table I) has a molecular formula of $C_{15}H_{14}O_2$ as found by high resolution mass spectroscopy, and those protons were attributed by ^{1}H NMR to a phenyl group (5 H), disubstituted olefin protons (2 H, J = 16.3 Hz, *trans*), 1,3,4,5-tetrasubstituted benzene (2 H), phenolic OH (2 H), and a methyl group (3 H) on the benzene ring. A symmetric structure of the tetrasubstituted benzene was evident from the ^{1}H and ^{13}C NMR data.

Possible structures for PN-1 was restricted to two **a** and **b.** The structure **a** was preferred to **b** for PN-1 on the basis of NOE experiments using the di-O-methyl derivative. As shown in Table II, irradiation at δ 6.87 (2 H, singlet, aromatic) attributed to A-ring enhanced both OMe and olefinic protons. The result clearly ruled out the structure **b** for

Table I. Polygonaceae benomyl antidotes: TLC detection using *Cladosporium herbarum.*

Plant	Antidoting activity	Antidoting substances on TLC	TLC solvent*
Fagopyrum esculentum Moench.	–		H–E = 1:1, C–M = 25:1
F. tataricum (L.) Gaerth.	–		H–E = 1:1, C–M = 25:1
Polygonum arenastrum Boreau	+	0.96	C–M = 70:1
P. lapathifolium L. subsp. *nodosum*	++	0.27, 0.32, 0.41	C–M = 70:1
P. longisetum De Bruyn	+	0.22	H–E–F = 600:600:1
P. sachalinense Fr. Schm.	++	0.82, 0.96	H–E–F = 400:800:1
P. thunbergii Sieb. *et* Zucc.	++	0.26	H–E = 1:1
Rumex acetocella L.	+	0.37	C–M = 70:1
R. obtusifolius L.	++	0.31, 0.74, 0.81, 0.90	H–E–F = 400:800:1

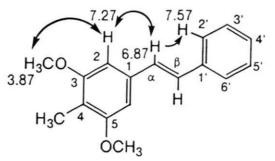
* C, chloroform; E, ethyl acetate; F, formic acid; H, hexane; M, methanol.



PN-1. The compound **a** was chemically synthesized according to Scheme 1 and confirmed to be identical with the isolated PN-1 in TLC and ^{1}H NMR spectroscopy. Thus PN-1, the third benomyl antidote was identified as a new naturally occurring stilbenoid, 3,5-dihydroxy-4-methylstilbene (**8**).

The molecular formula $C_{17}H_{12}O_5$ for PN-2 was deduced by HR-EI mass spectrometry (M^+ *m/z* 296.0685). UV λ_{max} in methanol at 272 and 308 nm, and ^{1}H NMR absorption at δ 6.67 attributable to flavone H-3 were indicative of flavone nature of PN-2. The presence of 2H resonated around δ 7.84–7.86 and 3H around δ *ca.* 7.5 were attributed to a phenyl group (B-ring) [4] and remaining protons were reasonably assigned to H-3 (δ 6.67), an A-ring proton (δ 6.74), a methoxy group (δ 4.13, 3H) and a methylenedioxy group (δ 6.07, 2H). Since flavone H-5 resonates around δ 7.9–8.2 [5] and almost all of flavone C-7 are oxygenated, the A-ring proton should be attributed to H-6 or H-8.

Table II. NOE experiment for the dimethyl ether of PN-1 from *Polygonum lapathifolium.*

Structure	Irradiated signal [δ ppm]	Enhanced signal [δ ppm]
	2.10 (OCH_3–C-4)	No
	3.87 (OCH_3-3 and 5)	6.87 (H-2 and 6)
	6.87 (H-2 and 6)	3.87 (OCH_3-3 and 5) 7.27 (H-α)
	7.27 (H-α)	6.87 (H-2 and 6) 7.57 (H-2′ and 6′)

Therefore, three structures **c–e** are probable for PN-2, and the most preferable one was **d** according to ^{1}H NMR solvent effects on the methoxy proton shift value. When a methoxy group substituted *ortho* to an unsubstituted carbon, the chemical shift value in $CDCl_3$ is lower than that in C_6D_6 by 0.5–0.8 ppm [OMe: δ ($CDCl_3$) – δ (C_6H_6) = Δ 0.5–0.8 ppm] [6]. In the case of PN-2, the methoxy group resonated at δ 4.13 in $CDCl_3$ shifted to δ 4.09 (Δ 0.04) by changing the solvent to C_6D_6, which means that both carbons *ortho* to the methoxylated one are substituted. The result was compatible only with the substitution pattern of **d.** The proposed compound was chemically synthesized and revealed to be identical to PN-2 in chromatographic and spectroscopic properties.

Thus the second antidote from *P. lapathifolium* was identified to be 5-methoxy-6,7-methylenedioxyflavone (**9**), which has been isolated from *Physalis minima* (Soranaceae) [7].

Interestingly, this compound is a 5-O-methyl ether of cochliophilin A (**11**) which has been isolated from the host plant spinach *(Spinacia oleracea)* as a potent attractant for zoospores of pathogenic *Aphanomyces cochlioides* [4].

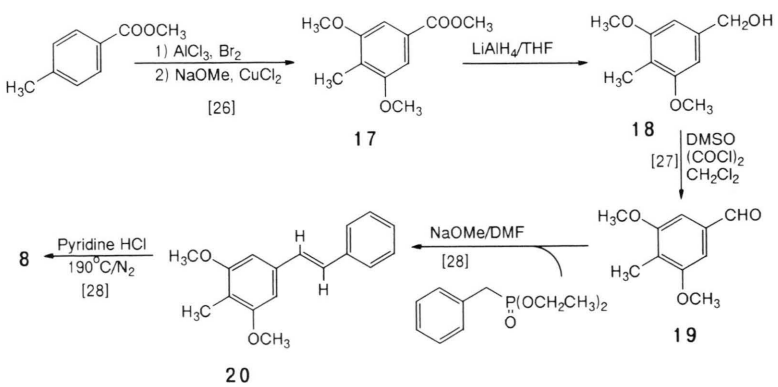
3. Antidote from *P. thunbergii*

The structure of PT-1, an benomyl antidote from *P. thunbergii* was estimated to be dimethoxy-substituted symmetric *p*-benzoquinone because of its molecular formula $C_8H_8O_4$ (HR-MS, M^+ *m/z* 168.0405), UV λ_{max} in methanol 277 nm and another weak band at *ca.* 380 nm, and ^{1}H NMR ($OCH_3 \times 2$ and $=CH- \times 2$, both singlets). The possibility of 2,5-dimethoxy-*p*-benzoquinone structure for PT-1 was discounted by the direct comparison with the isolate in mass and ^{1}H NMR spectroscopies. Thus PT-1 was deduced to be 2,6-dimethoxy-*p*-benzoquinone (**10**). This compound has been isolated from *Caesalpinia pulcherrima* [8], *Peddiea fischeri* [9] and *Phyllostachys heterocycla* var. *pubescens* [10] and known as an antimicrobial [10, 11].

Antidotal properties of emodin

Direct TLC bioautography using thin-layer plates impregnated with benomyl revealed the presence of some benomyl antidotes in Polygonaceae plants. The active principles identified are α-tocopherol (**6**), emodin (**7**), 3,5-dihydroxy-4-methylstilbene (**8**), 5-methoxy-6,7-methylenedioxyflavone (**9**) and 2,6-dimethoxy-*p*-benzoquinone (**10**), which are all belonging to phenolics. It is possible that the mode of action of benomyl antidotes is closely related to that of benomyl which is believed to demonstrate the antifungal activity through its interaction with β-tubulin. As a matter of fact, it is quite difficult to find any structural similarity in skeletons or functional groups among benomyl antidotes to those of benomyl (**1**) or carbendazim (= MBC, **2**), an active principle of benomyl [12]. However, Polygonaceae benomyl antidotes seem to possess some similarities in part or gross structure to those of colchicine (**12**) [13], combretastatin (**13**) [14, 15], podophyllotoxin (**14**) [16] and streptopyrone (**15**) [17] which interact with tubulin as with carbendazim (**2**) [18].

To know more about the benomyl antidotes, the antidoting activity of emodin (**7**) were examined against other benzimidazole fungicides than



Scheme 1. Synthetic route for 3,5-dihydroxy-4-methylstilbene.

benomyl. This test was carried out on agar plates containing fungal spores or mycelia and a fungicide in excess amounts of its minimum inhibitory concentration by using paper disks charged with an emodin solution. In this bioassay *Botrytis cinerea* and *Aspergillus nidulans* together with *C. herbarum* were used as test fungi mainly because of the difference in susceptibility to each fungicide. As shown in Table III, emodin exhibited antidoting activity against 5 benzimidazole fungicides, **1,** carbendazim (MBC, **2**), thiabendazole (TBZ, **3**), thiophanate-methyl (**4**) [18] and nocodazole (**5**) tested in the present experiment. Emodin (**7**) is thus evaluated as a benzimidazole antidote.

Recently, a carbamate fungicide diethofencarb (**16**) taking advantage of a negatively correlated cross-resistance phenomenon has been developed as a potent fungicide against benzimidazole-resistant fungi [2, 3]. Studies on the mode of action of diethofencarb (**16**) revealed that the β-tubulin in a benzimidazole-resistant strain of *Neurospora crassa* F 914 exhibited an affinity not to benzimidazoles but to diethofencarb (**16**), whilst the β-tubulin in a *N. crassa* wild-type strain possessed an inversed affinity [3, 20]. Therefore, an experiment using two combination systems, (1) benzimidazole-resistant strain – diethofencarb (**16**) – emodin (**7**), and (2) benzimidazole-susceptible strain – carbendazim (**2**) – emodin was conducted to obtain data whether emodin could exhibit the antidotal activity in both systems.

The results are summarized in Table IV indicating that emodin (**7**) can also act as an antidote against diethofencarb (**16**) in a benzimidazole-resistant strain. These results (Tables III and IV) are indicative of the versatility of emodin in the antidotal action of fungitoxins possessing an affinity to β-tubulin, and may suggest that the mode of antidotal action of emodin is closely related to the function of β-tubulin.

Anthraquinones including emodin (**7**) and flavones structurally closely related to 5-methoxy-6,7-methylenedioxyflavone (**8**) have been reported to be inhibitors of protein tyrosine kinase which regulate cell division and tumorigenesis [21, 22]. Further investigations concerning the analyses of structure and antidotal activity relationships and the direct interaction of benzimidazole antidotes with β-tubulin in both systems with and without a benzimidazole fungicide must be necessary to disclose the mode of action of benzimidazole antidotes [23].

Experimental

General

Analytical and preparative thin-layer separations were carried out on Merck pre-coated silica gel plates (F 254, layer thickness 0.25 or 0.50 mm). Wako-gel C-200 (silica gel) was used for column chromatography. ^1H and ^{13}C NMR spectra were determined in acetone-d_6 or $CDCl_3$ (int. standard, TMS) at 500 and 125 MHz, respectively on a Bruker AM 500. Mass and UV spectra were recorded on a JEOL DX 500 and a Hitachi U-3210, respectively.

Chemicals

Benomyl and diethofencarb were kindly supplied by Du Pont Japan Ltd. and Sumitomo Chemical Company Ltd., respectively. Carbendazim (MBC), thiabendazole (TBZ), thiophanate-methyl were commercially available. Nocodazole was prepared by the method of Raeymaekers *et al.* [24].

Table III. Antidoting activity of emodin (**7**) against benzimidazole fungicides.

Fungus tested	Fungicide [dose: ppm]	Antidoting activity of emodin* [dose: μg/disk]
Cladosporium herbarum	benomyl (0.2)	++ (5.4)
	carbendazim (0.2)	++ (5.4)
	thiabendazole (0.8)	++ (2.0)
Botrytis cinerea	thiophanate-methyl (2.0)	+ (5.4)
Aspergillus nidulans	nocodazole (0.5)	++ (5.4)

* Paper disk method; +, growth zone, 2–3 mm from the edge of the paper disks; ++, growth zone, more than 5 mm from the edge of paper disks.

Table IV. Antidoting activity of emodin (**7**) in *Neurospora crassa* against carbendazim (**2**) and diethofencarb (**16**).

Fungicide in agar plates [ppm]	Emodin [dose: μg/disk]	Growth of *Neurospora crassa** Wild type	Strain F 914
Carbendazim (0.2)	0	0	G
	5.4	9.7	G
Diethofencarb (0.1)	0	G	0
	5.4	G	10.0

* Paper disk method; G, natural growth; 0, completely inhibited, and figures mean the width of the growth zone from the edge of paper disks. Results of duplicated assays were averaged.

Fungi

Cladosporium herbarum AHU 9262 highly susceptible to benomyl and carbendazim (MBC) was used as a test fungus for detecting antidoting agents. *Neurospora crassa* wild-type susceptible to benzimidazole fungicides and *N. crassa* F 914 resistant to benzimidazoles but highly susceptible to diethofencarb were kindly supplied by Dr. M. Fujimura [2, 3]. *Aspergillus nidulans* and *Botrytis cinerea* were also used in the present study. Fungi were cultured in a PYG medium (3% glucose, 1% peptone and 0.1% yeast extract). The medium for TLC bioautography is found in ref. [25].

Plant material

The plants used in the present study were collected in Sapporo, Northern Japan, randomly in the summer season and for the screening test, 10 g (fresh weight) of the aerial parts were dipped in 150 ml of MeOH for 3–4 days. The extract was concentrated to dryness and the residue was partitioned between EtOAc and water. The EtOAc layer was concentrated to 2 ml.

To isolate the antidoting agents, *Polygonum lapathifolium* L. subsp. *nodosum* and *P. thunbergii* were collected in large amounts and extracted with MeOH.

Bioassay of benomyl (benzimidazole) antidotes

1. TLC bioautography

A little portion of the concentrated plant extract, 25 μl out of 2 ml of concentrated EtOAc extract from 10 g of a fresh plant for screening tests and 10 μl for monitoring antidotes, was subjected to silica gel 60 F254 thin-layer plates (20 × 20 cm, thickness 0.25 mm). Two plates each charged with the same plant extract were developed 15–16 cm in hexane–EtOAc = 3 : 2 and $CHCl_3$–MeOH = 25 : 1, respectively, and air-dried well to be free from the solvents. Then, the plates were passed through a 0.3 ppm benomyl solution in hexane over 3 sec, and the plates freed from hexane were subjected to TLC bioautography devised by Homans and Fuchs [25]. When the plant extract contained an antidotic substance, *C. herbarum* inoculated as a test fungus can grow around the spot corresponding to the active agent and afford a black growing zone of a size due to its activity, whilst a control lane (charged only EtOAc), the background and around inactive constituents remain unchanged.

2. Paper disk method on agar plates

To 10 ml of PYG agar medium (0.8% agar) at 50 °C were added 1 ml of *C. herbarum* spore suspension and 100 μl of a 20 ppm benomyl (or other benzimidazole fungicide) solution in acetone, and solidified in a 9 cm i.d. petri dish. A plant extract (25 μl) or a test solution (pure material in acetone) was charged on a paper disk (Toyo Paper Disk ⟨Thick⟩ 8 mm i.d.) and dried *in vacuo*. Those disks in association with control ones treated with solvent alone (acetone or EtOAc) were put onto the agar plates and conditioned for 1 h at room temperature before incubation at 25 °C for 24–48 h. When the paper disk contained an antidoting substance, *C. herbarum* or other test fungus could grow only around the relevant disk of a size due to the activity. The activity was quantified by measuring the distance from the edge of the paper disk to that of the growing zone.

Extraction and isolation of Polygonaceae benomyl antidotes

1. PN-1 (3,5-dihydroxy-4-methylstilbene, **8**) and PN-2 (5-methoxy-6,7-methylenedioxyflavone, **9**) from *P. lapathifolium*

Aerial parts of *P. lapathifolium* L. subsp. *nodosum* were extracted with MeOH (12.8 kg fresh material in 40 l of MeOH) twice and combined extracts were concentrated to 1000 ml. The concentrate was extracted with EtOAc (equal volume × 3) and the combined EtOAc extracts were washed with 5% $NaHCO_3$ aq. (½ volume × 3). The phenolic and neutral constituents (82 g) which contained benomyl antidoting agents were column chromatographed over 500 g of silica gel and eluates with $CHCl_3$–MeOH = 10:1 were collected from the 2nd to 6th fraction (1 l each). The concentrate from these fractions (42 g) was rechromatographed over silica gel (400 g) and eluted with $CHCl_3$–MeOH = 100:1 to give seven fractions (400 ml each). Three antidotes originally detected were all contained from the 2nd to 7th fractions (R_f: PN-1, 0.73; PN-2, 0.20; and PN-3, 0.08 in hexane–EtOAc = 1:1). The weak PN-3 was missed in the following fractionation. The PN-1 showed antifungal activity at inner zone of the TLC spot where the concentration is relatively high, and then, antidoting activity was observed as a doughnut zone.

The combined eluates were concentrated to give 8 g of gum, which was further fractionated by silica gel column chromatography in a mixture of hexane–EtOAc. PN-1 was eluted with hexane–EtOAc = 5:1 in the fractions from 5th to 10th (100 ml each) to give 710 mg of crude material, whilst PN-2 was eluted with EtOAc–hexane = 1:1. The PN-1 was eventually isolated by subsequent column chromatography over 10 g of silica gel in $CHCl_3$–MeOH = 100:1. Every 10 g of the eluate was collected, and PN-1 was detected in the column fractions from 12th to 15th which were concentrated to yield 32 mg of 3,5-dihydroxy-4-methylstilbene as a pale yellow powder.

The PN-2 containing fractions (2.5 g) were further chromatographed over 18 g of silica gel in $CHCl_3$–MeOH = 100:1 and except the first four fractions (50 ml each), 700 ml of the eluate was collected. The concentrated eluate (210 mg) was subjected to preparative TLC successively in hexane–EtOAc = 1:2 (R_f 0.34) and in $CHCl_3$–MeOH = 40:1 (R_f 0.42) to yield 3 mg of purified PN-2.

2. PT-1 (2,6-dimethoxy-*p*-benzoquinone, **10**) from *P. thunbergii*

The aerial part of *P. thunbergii* Sieb. et Zucc. (13 kg, fresh) were extracted with *ca.* 60 l of MeOH and the extracts were subjected to fractional extraction as usual to yield the fraction of neutral and phenolic constituents soluble in EtOAc. The concentrated fraction (39 g) was mounted on the top of silica gel column (300 g) and eluted with EtOAc to remove highly polar substances. The constituents in the EtOAc eluate (8.2 g) was further fractionated by silica gel (160 g) column chromatography. An active principle was eluted with hexane–EtOAc = 1:1–4 (four fractions, 600 ml each, solute 4 g in total). At this point, antidotal activity was detectable by using 3 µl of the eluate on TLC plates at R_f 0.2–0.3 in hexane–EtOAc = 1:1. The combined fractions were rechromatographed over silica gel in benzene–EtOAc (5:1), and the active principle was eventually purified by preparative TLC in $CHCl_3$–MeOH–HCOOH = 50:1:0.05 (R_f *ca.* 0.4) to yield pure PT-1 in poor yield (*ca.* 1 mg).

Physicochemical properties of Polygonaceae benomyl antidotes

1. The benomyl antidotes, α-tocopherol (**6**) and emodin (**7**) from *Polygonum sachalinense* Fr. Schm., isolation and their structure elucidation, have already been accounted in our previous paper [1].

2. Antidotes from *Polygonum lapathifolium* L. subsp. *nodosum*

3,5-Dihydroxy-4-methylstilbene (*PN-1,* **8**). Pale yellow powder, m.p. 290–294 °C. $UV_{365\,nm}$ fluorescence: white-blue. Gibbs test: (+), deep blue. HR-MS: M^+ *m/z* 226.0985 ($C_{15}H_{14}O_2$ requires 226.0984). EI-MS *m/z* (%): 228 ($[M+2]^+$, 21), 227 ($[M+1]^+$, 72), ($[M]^+$, 100), 225 (23), 211 (16), 210 (11), 179 (16), 165 (12), 128 (10), 89 (11), 77 (11). UV λ_{max} nm (rel. int.): MeOH 212 (53), 220 sh (46), 234 sh (47), 239 (50), 245 sh (43), 303 sh (95), 314 (100), 329 sh (70). ^1H NMR δ_{TMS} (acetone-d_6, 500 MHz, *J* = Hz): 8.17 (2 H, br. s, OH), 7.53 (2 H, br. d-like, *J* = *ca.* 7.7, H-2′ and 6′), 7.34 (2 H, br. t-like, *J* = *ca.* 7.7, H-3′ and 5′), 7.24 (1 H, br. t, *J* = *ca.* 7.7, H-4′),

7.04 and 6.99 (both 1H, two d, J = 16.3, H-α and H-β), 6.6 (2H, s, H-2 and 6), 2.10 (3H, s, CH_3-4). ^{13}C NMR δ_{TMS} (acetone-d_6, 125 MHz): 138.5 (C-1), 105.8 and 105.7 (C-2 and 6), 157.3 and 157.2 (C-3 and 5), 111.7 (C-4), 129.9 and 128.0 (C-α and β), 8.7 ($\underline{C}H_3$–C-4), 136.4 (C-1′), 127.2 (C-2′ and 6′), 129.5 (C-3′ and 5′), 128.2 (C-4′).

Methylation of PN-1 in dried acetone containing K_2CO_3 and excess amounts of dimethyl sulfate was effected by refluxing for 2 h. Working-up as usual yielded the corresponding di-O-methyl derivative, which run at R_f 0.99 on silica gel TLC in $CHCl_3$–MeOH = 50:1, whilst PN-1 run at R_f 0.32. FD-MS: *m/z* 254 (M^+, 100%). ^1H NMR δ_{TMS} (acetone-d_6, 500 MHz, J = Hz): 7.57 (2H, d, J = 7.5, H-2′ and 6′), 7.36 (2H, br. t, J = 7.5, H-3′ and 5′), 7.27–7.19 (3H, m, H-α, H-β and H-4′), 6.87 (2H, s, H-2 and 6), 3.87 (6H, s, CH_3O-3 and 5), 2.10 (3H, s, CH_3-4). NOE experiment was carried out under the same condition and the results are summarized in Table II.

5-Methoxy-6,7-methylenedioxyflavone (*PN-2,* **9**). Pale yellow powder, m.p. 280–282 °C (uncorr.). $UV_{365\,nm}$ fluorescence: white-yellow. Gibbs test: (–). FD-MS: *m/z* 297 ($[M+1]^+$, 32), 296 (M^+, 100). EI-MS *m/z* (%): 296 (M^+, 50), 295 (28), 268 (100), 267 (28), 250 (90), 166 (86), 164 (83), 136 (32), 120 (80), 105 (25), 102 (24), 77 (29), 69 (27), 53 (48). UV λ_{max} nm (rel. int.): MeOH 215 (100), 272 (69), 308 (56). ^1H NMR δ_{TMS} ($CDCl_3$, 500 MHz): 7.84–7.86 (2H, m, H-2′ and 6′), *ca.* 7.5 (3H, m, H-3′, 4′ and 5′), 6.74 (1H, s, H-8), 6.67 (1H, s, H-3), 6.07 (2H, s, $-OCH_2O-$), 4.13 (3H, s, OCH_3-5). ^{13}C NMR δ_{TMS} ($CDCl_3$, 125 MHz): 177.6 (C-4), 160.9 (C-2), 154.8 (C-9), 153.0 (C-5), 141.5 (C-7), 134.9 (C-6), 131.5 (C-1), 131.3 (C-4′), 129.0 (C-2′ and 6′), 125.9 (C-3′ and 5′), 113.0 (C-10), 108.4 (C-3), 102.1 ($-OCH_2O-$), 93.3 (C-8), 61.2 (OCH_3).

3. Antidote from *P. thunbergii*

2,6-Dimethoxy-p-benzoquinone (*PT-1,* **10**). Pale yellow powder. $UV_{365\,nm}$ fluorescence: pale purple. HR-MS: M^+ *m/z* 168.0405 ($C_8H_8O_4$ requires 168.0422). EI-MS *m/z* (%): 168 (M^+, 63), 149 (16), 140 (13), 138 (33), 125 (17), 112 (13), 97 (15), 85 (16), 83 (20), 81 (11), 80 (58), 73 (14), 71 (28), 70 (13), 69 (100), 60 (12), 59 (27), 57 (51). UV λ_{max}: MeOH 277 nm and weak broad absorption at *ca.* 380 nm. ^1H NMR δ_{TMS} (500 MHz, acetone-d_6): 5.89 (2H, s, H-3 and 5), 3.82 (6H, s, OCH_3-2 and 6).

Preparation of reference compounds

1. 3,5-Dihydroxy-4-methylstilbene (**8**)

Commercially available methyl 4-methylbenzoate was derivatized into methyl 3,5-dimethoxy-4-methylbenzoate (**17**) according to Manchand *et al.* [26]. **17:** MS *m/z* (%): 210 (M^+, 100), 179 (74), 151 (34), 91 (33). ^1H NMR δ_{TMS} (DMSO-d_6): 7.18 (2H, s, H-2 and 6), 3.85 (3H, s, $COOCH_3$), 3.83 (6H, s, OCH_3-3 and 5), 2.05 (3H, s, CH_3-4). The benzoate **17** was reduced to the corresponding benzyl alcohol with $LiAlH_4$/THF as usual.

3,5-Dimethoxy-4-methylbenzyl alcohol (**18**): MS *m/z* (%): 182 (M^+, 100), 167 (22), 153 (39), 151 (34), 139 (27), 91 (33), 77 (26). ^1H NMR: benzyl protons in DMSO at δ 4.46 (2H, d, J = 5.7 Hz).

Swern oxidation [27] of **18** (430 mg) afforded 3,5-dimethoxy-4-methylbenzaldehyde (**19**) in 97% yield. **19:** MS *m/z* (%): 180 (M^+, 100), 179 (33), 151 (23), 91 (30), 77 (24). ^1H NMR δ_{TMS} ($CDCl_3$): 9.90 (1H, s, –CHO), 7.05 (2H, s, H-2 and 6), 3.90 (6H, s, OCH_3-3 and 5), 2.15 (3H, s, CH_3-4). The aldehyde **19** was coupled with diethyl benzylphosphonate prepared from benzyl bromide and triethyl phosphite as follows [28].

To the mixture of NaOMe (120 mg) in 1.5 ml of dry DMF was added subsequently 579 mg of diethyl benzylphosphonate (**20**) and 200 mg of **19** dissolved in 2 ml of dry DMF with stirring at 0 °C under N_2 gas. After 2 h stirring at room temperature, to the reaction mixture was added 6 ml of 33% aq. MeOH to yield 240 mg of 3,5-dimethoxy-4-methylstilbene (**21**) as a colourless solid. **21:** MS *m/z* (%): 254 (M^+, 100), 179 (16), 178 (18). ^1H NMR δ_{TMS} (CD_3OD, J = Hz): 7.54 (2H, dd, J = 8.4 and 1.9, H-2′ and 6′), 7.33 (2H, br. t, J = *ca.* 8, H-3′ and 5′), 7.22 (1H, br. t, J = *ca.* 8, H-4′), 7.13 (2H, s, H-α and β), 6.78 (2H, s, H-2 and 6), 3.86 (6H, s, OCH_3-3 and 5), 2.03 (3H, s, CH_3-4).

Finally, **21** (200 mg) was mixed with 1.0 g of pyridine hydrochloride and heated at 190 °C for 90 min under N_2 gas [28]. The reaction mixture diluted with 10 ml of 2 N HCl was extracted with ether to give 180 mg of the crude material, which was purified by silica gel column chromatography eluting with $CHCl_3$ to afford 85 mg of pure 3,5-dihydroxy-4-methylstilbene (**8**).

2. 5-Methoxy-6,7-methylenedioxyflavone (**9**)

As shown in our previous paper [4], cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, **11**), a potent attractant for zoospores of *Aphanomyces cochlioides,* was synthesized according to the known methods [29, 30]. To the solution of **11** (43 mg) in 1 ml of 1,3-dimethyl-2-imidazolidinone was added 40 mg of NaH (60%), followed by 0.5 ml of CH_3I. The mixture was stirred for 24 h at room temperature, at which point methanol was added to decompose the excess NaH. The reaction mixture was acidified to yield 36 mg (80% yield) of the 5-O-methylation product (**9**).

3. 2,5-Dimethoxy-*p*-benzoquinone

2,5-Dimethoxy-*p*-benzoquinone, an isomer of **10,** was prepared from 2,5-dihydroxybenzoquinone as described to the methylation of **11** in a poor yield. MS *m/z* (%): 168 (M^+, 6), 153 (24), 139 (60), 138 (11), 125 (20), 111 (17), 95 (34), 69 (100). ^1H NMR δ_{TMS} (500 MHz, acetone-d_6): 5.94 (2 H, s, H-3 and 6), 3.85 (6 H, s, OCH_3-2 and 5).

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