Aspterric Acid and 6-Hydroxymellein, Inhibitors of Pollen Development in *Arabidopsis thaliana*, Produced by *Aspergillus terreus*

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Aspterric Acid, 6-Hydroxymellein, *Arabidopsis thaliana*, *Aspergillus terreus*

Aspterric acid (**1**) and 6-hydroxymellein (**2**), inhibitors of pollen development in *Arabidopsis thaliana*, have been isolated from the fungus *Aspergillus terreus*. **1** and **2** inhibited the pollen development at concentrations of 38 and 52 μM, respectively. The microscopic examination of pollen development suggested that the inhibition by the treatment with **1** caused at meiosis and the inhibition by the treatment with **2** caused at microspore stage. **1** and **2** could be useful agents for the molecular investigation of anther and pollen development in higher plants.

Arabidopsis thaliana is currently a model system for plant molecular biology of male sterility and pollen development in dicotyledonous plants (Vieira *et al*., 1990). This plant has been developed as a convenient screening plant to detect plant growth regulators that induce changes in the development of flowers, because the rapid growth, small size and uniformity of this plant enables any changes in the lifecycle from germination through flowering and senescence to be observed (Clark and Meyerowitz, 1994; Dawson *et al*., 1994; Dawson *et al*., 1993; Dennis and Surridge, 2000; Brown, 1972). In the course of our screening search for the regulators of plant reproductive development among fungal metabolites, using bioassay method with *A. thaliana*, suitable for use as tools to analyze plant reproductive functions, we found the presence of inhibitors of pollen development in cultures of the fungus *Aspergillus terreus*. Bioassay-guided fractionation led to isolation of aspterric acid (**1**) (Tsuda *et al*., 1978; Harayama *et al*., 1983; Harayama *et al*., 1987) and 6-hydroxymellein (**2**) (Ayer *et al*., 1987; Krohn *et al*., 1997) as active compounds. In this report, we describe the biological activities of **1** and **2** as inhibitors of pollen development in *Arabidopsis*.

Materials and Methods

Instruments

Melting points were determined using a Yanagimoto micromelting point apparatus. IR spectra were recorded on a JASCO FT IR-7000 spectrometer and UV spectra on a SHIMAZU UV-2200 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded with a JEOL JNM-ESP 500 NMR spectrometers at 500 and 125 MHz, respectively. Chemical shifts are expressed in δ values with solvents as internal standards. MS spectra were recorded with a JEOL JMS-SX 102 apparatus.

Isolation and purification of aspterric acid (**1**) *and 6-hydroxymellein* (**2**)

Aspergillus terreus was cultured stationarily in a malt extract medium at 24 °C for 28 days. The culture broth (40 l) was filtered, and the filtrate was adjusted to pH 2.0 with 2 N HCl, before being extracted twice with EtOAc. The combined solvents were concentrated *in vacuo*, and the resulting residue (28.8 g) was first fractionated by column chromatography on silica gel (hexane–acetone). Fraction 6 (172 mg), obtained by elution with 30%

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acetone, was further purified by preparative TLC ($CHCl_3$–MeOH, 98:2, v/v) and the solid was recrystallized from EtOAc to afford 17 mg of **2** as colorless plate. Fractions 7–10 (5808 mg), obtained by elution with 30% acetone, was chromatographed on a silica gel column ($CHCl_3$–MeOH). The active fraction eluted with 1% MeOH was further purified by preparative TLC ($CHCl_3$–MeOH–AcOH 95:5:2, v/v/v) and the solid was recrystallized from benzene to afford 10 mg of **1** as colorless powder.

Aspterric acid (**1**). M.p. 158–159 °C. – IR (KBr): ν = 3402 (OH), 2934 (C=C), 1722 (O–C=O), 1462, 1439, 1327, 1244 cm^{-1}. – ^1H NMR (500 MHz, $CDCl_3$): δ = 1.52 (m, 1H, 9-H), 1.61 (s, 3H, 15-H), 1.71 (s, 3H, 14-H), 1.72 (m, 1H, 9-H), 1.76 (m, 1H, 5-H), 2.04 (m, 1H, 4-H), 2.15 (m, 1H, 1-H), 2.18 (m, 1H, 5-H), 2.29 (m, 1H, 1-H), 2.3–2.4 (m, 3H, 4-H, 6-H, 8-H), 2.42 (m, 1H, 8-H), 3.50 (d, *J* = 8.0 Hz, 1H, 13-H), 3.94 (d, *J* = 8.0 Hz, 1H, 13-H), 4.30 (d, *J* = 8.8 Hz, 1H, 2-H). – $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$): δ = 20.8 (q, C-14), 23.3 (q, C-15), 23.6 (t, C-8), 32.1 (t, C-4), 33.7 (t, C-9), 36.2 (t, C-1), 37.4 (t, C-5), 53.0 (s, C-7), 55.4 (d, C-6), 74.9 (s, C-3), 76.3 (t, C-13), 82.5 (d, C-2), 125.3 (s, C-11), 134.3 (s, C-10), 177.9 (s, C-12). – MS (EI): *m/z* (%) = 266 (100) [M^+], 251 (35), 248 (10), 230 (7), 220 (16), 204 (48), 191 (60). – HRMS *m/z* (M^+): Calcd. for $C_{15}H_{22}O_4$: 266.1518, found: 266.1513.

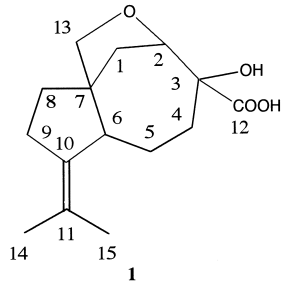
6-Hydroxymellein (**2**). M.p. 155–159 °C. – UV/vis (EtOH): λ_{max} (lg ε) = 216 (4.12), 269 (3.97), 304 nm (3.61). – IR (KBr): ν = 3225 (OH), 2974 (C=C), 1655 (O–C=O), 1585 (C=C), 1477, 1386, 1257, 1221 cm^{-1}. – ^1H NMR (500 MHz, acetone-d_6): δ = 1.51 (d, *J* = 6.2 Hz, 3H, 3-CH_3), 2.84 (br. s, 1H, 4-H), 2.85 (d, *J* = 5.5 Hz, 1H, 4-H), 4.66 (dq, *J* = 5.5, 6.2 Hz, 1H, 3-H), 6.20 (d, *J* = 2.3 Hz, 1H, 7-H), 6.31 (d, *J* = 2.3 Hz, 1H, 5-H), 11.22 (s, 1H, 8-OH). – $^{13}C\{^1H\}$ NMR (125 MHz, acetone-d_6): δ = 20.6 (q, *C*H_3-3), 34.9 (t, C-4), 76.2 (d, C-3), 101.5 (s, C-8a), 101.8 (d, C-7), 107.3 (d, C-5), 143.0 (s, C-4a), 165.0 (s, C-8), 165.1 (s, C-6), 170.5 (s, C-1). – MS (EI): *m/z* (%) = 194 (100) [M^+], 176 (20), 150 (81), 148 (8), 122 (7). – HRMS *m/z* (M^+): Calcd. for $C_{10}H_{10}O_4$: 194.0579, found: 194.0580.

Bioassay for the growth of Arabidopsis thaliana

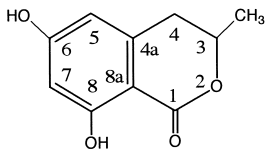
Bioassay with *Arabidopsis thaliana* was carried out according to the reported procedure of Brown (1972). Seeds of *A. thaliana* had been surface-sterilized with 70% ethanol for 30 sec and rinsed five time with sterile distilled water. A single seed placed in each test tube containing 10 ml of solid medium and a defined amount of the test compound. Triplicate experiments were conducted. The test tubes plugged with cotton wool were incubated at 25 °C under continuous light (8,000 lx). Date of bolting and first flowering, and the stem length treated with compound **1** and **2** were recorded and the mean values of those were compared with an untreated control. In addition, anthers from freshly opened flowers was stained with acetocarmine and observed under a light microscope.

Histological observation of anthers

Fixing and embedding in paraffin of anthers were carried out according to the reported procedure (Grima-Pettenati *et al.*, 1989; Regan and Moffatt, 1990). Young buds from the primary inflorescences of an untreated and a treated plant were collected. The buds were fixed in 70% ethanol–formaldehyde–acetic acid (90:5:5, v/v/v) then dehydrated. After dehydration, the buds were gradually infiltrated with paraffin and left at room temperature until paraffin solidified. Sections



1



2

Fig. 1. Structures of aspterric acid (**1**) and 6-hydroxymellein (**2**).

(10 μm) were made on a microtome and deparaffinized before staining. After deparaffinized, the sections were stained with toluidine blue and observed under a light microscope.

Results and Discussion

The EtOAc-soluble acidic fraction (28.8 g) was purified with a silica gel column chromatography and preparative TLC, and final purification by recrystallization afforded two active compounds **1**

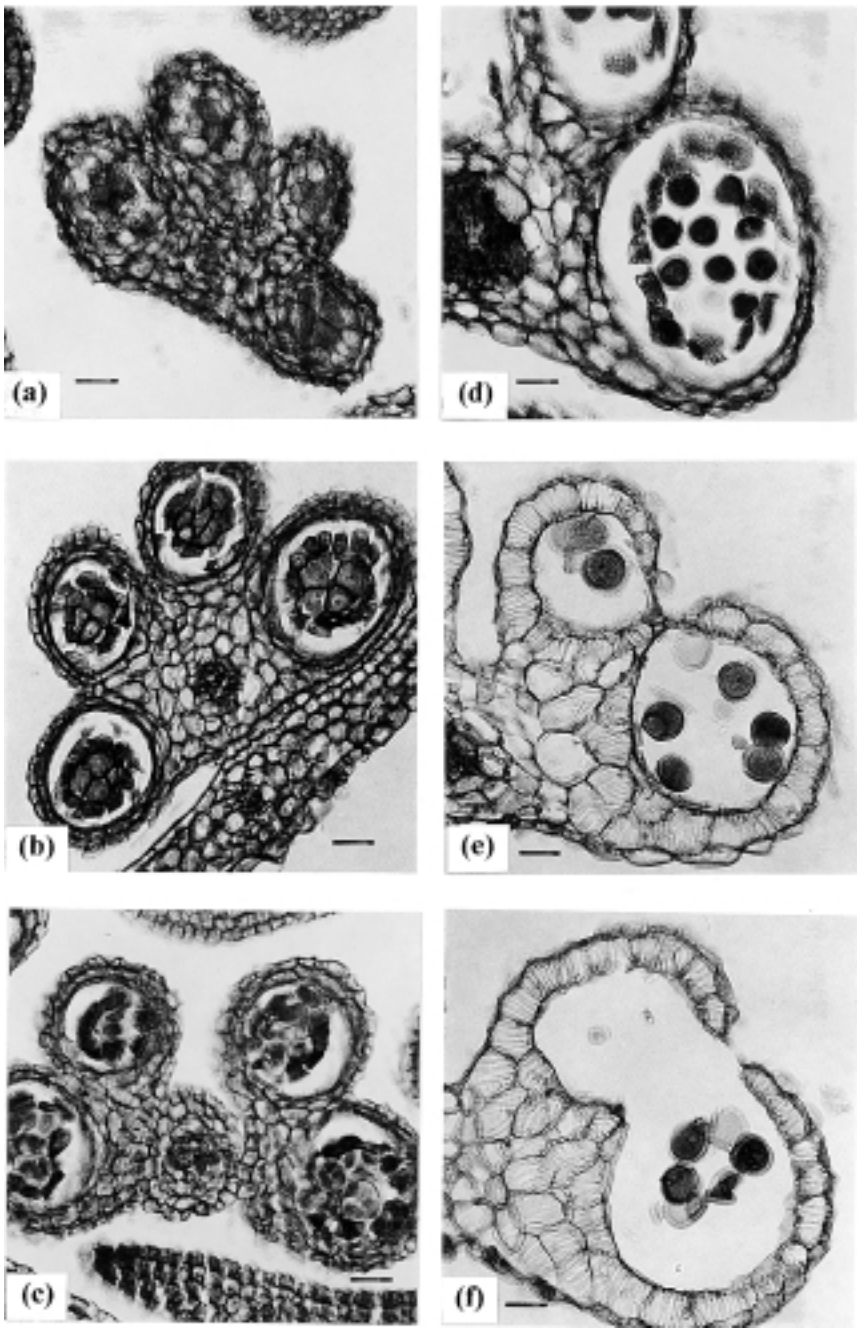


Fig. 2. Pollen development in the untreated flowers. (a) pollen mother cells (PMCs), (b) PMCs in meiosis, (c) tetrads, (d) microspores, (e) mature pollen, and (f) dehisced anther. Bars = 20 μm.

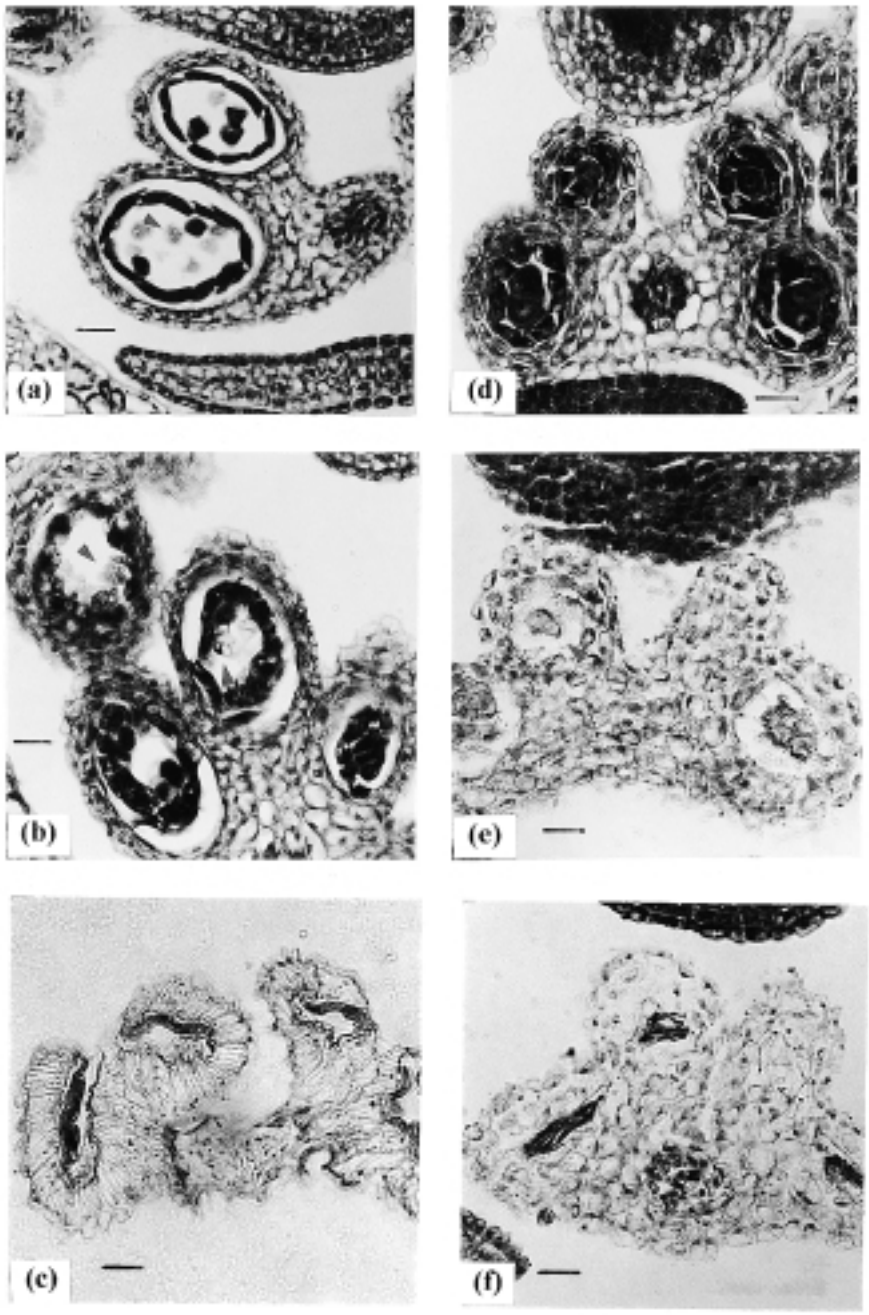


Fig. 3. Inhibitioin of pollen development by the treatment with aspterric acid (**1**) or 6-hydroxymellein (**2**). (a) to (c) sections of anthers treated with **1**, (d) to (f) sections of anthers treated with **2**; (a) aberrant PMCs in meiosis, (b) aberrant PMCs in meiosis, (c) degenerate anther locules, (d) normal PMCs in meiosis, (e) adhered and collapsed microspores, and (f) degenerate anther locules. Aberrations of PMCs and microspores are indicated by the arrowhead. Bars = 20 μm.

(10 mg) and **2** (17 mg). **1** and **2** were identified as aspterric acid and 6-hydroxymellein (Fig. 1) by comparing the physicochemical properties with those reported (Tsuda *et al.*, 1978; Krohn *et al.*, 1997).

The condition of pollen in anthers from mature flowers of *A. thaliana* treated with 38 μM of **1**, 52 μM of **2** and an untreated control was compared by a light microscope. While the pollen grains within the untreated anthers were densely stained, the anther treated with **1** as well as **2** contained collapsed structures and no pollen. In order to identify the stage during which pollen development became aberrant in the treated plants, individual buds of the primary inflorescence of the treated and the untreated plants were examined at each stage of development. A series of micrographs of buds at different stages of development were obtained from the untreated plants, the plants treated with **1** or **2** (Figs 2, 3). In the untreated anthers, pollen mother cells (PMCs), meiotic stages, tetrads, microspores, and mature pollen were observed in accordance with pollen development (Fig. 2). This pollen development in the untreated anthers appeared normal by comparing their micrographs with those reported (Dawson *et al.*, 1994; Dawson *et al.*, 1993). On the other hand, **1** induced aberrations in PMCs at meiosis (Fig. 3a, b). The PMCs were abnormally granular and became vacuolation at meiosis. Then, PMC-derived material degenerated leaving an empty anther before anthesis (Fig. 3c). By the treatment with **2**, PMCs appeared normal during meiosis (Fig. 3d). However, the microspores adhere, collapse, and the cytoplasm was absent (Fig. 3e). Before anthesis, the microspores completely degenerated in the anther locule (Fig. 3f). These results of microscopic examination suggested that inhibition of pollen development by the treatment with **1** caused at meiosis and the inhibition by the treatment with **2** caused at the microspore stage.

Table I shows the growth response of *A. thaliana* to **1** and **2**. **1** and **2** exhibited no effect on the growth at concentrations of 11 and 15 μM, respectively. **1** delayed the bolting and flowering times at concentrations of 11 and 38 μM. In particular, treatment with 38 μM of **1** delayed those for 12 days. Furthermore, **1** completely inhibited the growth of the seedlings at a concentration of 113 μM. On the other hand, the bolting and flowering times of plants treated with 15 or 52 μM of **2** were similar to those of untreated control. However, treatment with 155 μM of **2** delayed the bolting and flowering times for 10 days. **1** reduced stem length at flowering at a concentration of 38 μM. Similarly, **2** reduced that at concentrations of 52 and 155 μM.

Table I. Response of *Arabidopsis thaliana* to aspterric acid (**1**) and 6-hydroxymellein (**2**).

Treatment		Period of growth to bolting [days]	Period of growth to first flowering [days]	Stem length to first flowering [mm]
1	11 μM	20	22	26
	38	28	30	7
	113	–	–	–
2	15	16	18	28
	52	16	20	9
	155	26	28	9
Control		16	18	29

Aspterric acid (**1**) is a carotane-type sesquiterpene, but its biological activity has not been reported previously (Tsuda *et al.*). 6-Hydroxymellein (**2**), which is a dihydroisocoumarin compound such as mellein and kigelin (Krohn *et al.*, 1997; Govindachari *et al.*, 1971), shows weak inhibitory activity against fungi, but its plant growth activity has not been reported previously (Krohn *et al.*, 1997). This is first report about these two compounds as plant growth regulators to inhibit pollen development. Our findings suggest that aspterric acid and 6-hydroxymellein could be useful agents for the molecular investigation of anther and pollen development in higher plants.

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Ayer W. A., Attah-Poku S. K., Browne L. M. and Orszanska H. (1987), The Chemistry of the Blue Stain Fungi. Part 3. Some Metabolites of *Ceratocystis minor* (Hedgcock) Hunt. Can. J. Chem. **65**, 765–769.

Brown B. T (1972), A new screening procedure for detecting plant growth regulating compounds. Pestic. Sci. **3**, 161–168.

Clark S. E. and Meyerowitz E. M. (1994), In: *Arabidopsis* (Meyerowitz E. M. and Somerville C. R., ed.). Cold Spring Harbor Laboratory Press, New York.

Dawson J., Wilson Z. A., Aarts M. G. M., Braithwaite A. F., Briarty L. G. and Mulligan B. (1993), Microspore and pollen development in six male-sterile mutants of *Arabidopsis thaliana*. Can. J. Bot. **71**, 629–638.

Dawson J., Wilson Z. A., Briarty L. G. and Mulligan B. J. (1994), In: *Arabidopsis*. An Atlas of Morphology and Development (Bowman J., ed.). Springer-Publ. New York, Inc..

Dennis C. and Surridge C. (2000), *Arabidopsis thaliana* genome. Nature **408**, 791–826.

Govindachari T. R., Patankar S. J. and Viswanathan N. (1971), Isolation and structure of two new dihydroisocoumarins from *Kigelia pinnata*. Phytochemistry **10**, 1603–1606.

Grima-Pettenati J., Chriqui D., Sarni-Manchado P. and Prinsen E. (1989), Stimulation of lignification in neoformed calli induced by *Agrobacterium rhizogenes* on bean hypocotyls. Plant Science **61**, 179–188.

Harayama T., Sakurai K., Tanaka K., Hashimoto Y., Fukushi H. and Inubushi Y. (1987), Synthetic studies on *dl*-aspterric acid, a carotane-type sesquiterpene. II. Synthesis of *dl*-aspterric acid. Chem. Pharm. Bull. **35**, 1434–1442.

Harayama T., Shinkai Y., Hashimoto Y., Fukushi H. and Inubushi Y. (1983), Synthesis of *dl*-aspterric acid, a carotane type sesquiterpene. Tetrahedron Lett. **24**, 5241–5244.

Krohn K., Bahramsari R., Florke U., Ludewig K., Kliche-Spory C., Michel A., Aust H. Draeger S., Schulz B. and Antus S. (1997), Dihydroisocoumarins from fungi: isolation, structure elucidation, circular dichroism and biological activity. Phytochemistry **45**, 313–320.

Regan S. M. and Moffatt B. A. (1990), Cytochemical analysis of pollen development in wild-type *Arabidopsis* and a male-sterile mutant. Plant Cell **2**, 877–889.

Tsuda Y., Kaneda M., Tada A., Nitta K., Yamamoto Y. and Iitaka Y. (1978), Aspterric acid, a new sesquiterpenoid of the carotane group, a metabolite from *Aspergillus terreus* IFO-6123. *X*-ray crystal and molecular structure of its *p*-bromobenzoate. J. C. S. Chem. Commun. 160–161.

Vieira M. L. C., Briarty L. G. and Mulligan B. J. (1990), A method for analysis of meiosis in anthers of *Arabidopsis thaliana*. Ann. Bot. **66**, 717–719.