



PHYTOTOXICITY OF AUSTRALIFUNGIN, AAL-TOXINS AND FUMONISIN B₁ TO *LEMNA PAUSICOSTATA*

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Abstract—AAL-toxins and fumonisin B₁ are phytotoxic to susceptible plants by inhibiting the enzyme ceramide synthase. Australifungin, which is structurally unrelated to these toxins, inhibits the same enzyme in the sphingolipid biosynthesis pathway of animals. In duckweed (*Lemna pausicostata*) cultures, 5 μ M australifungin caused accumulation of the sphingolipid precursors, phytosphingosine and sphinganine, although less so than AAL-toxin T_A or FB₁ at 1 μ M. Phytosphingosine and sphinganine began to accumulate after 12 h, followed by increased electrolyte leakage at 24 h. Electrolyte leakage with 5 μ M australifungin was somewhat less than 1 μ M fumonisin B₁ or AAL-toxin T_A. Morphological effects were not identical; fumonisin B₁ and AAL-toxin T_A mainly caused bleaching, while australifungin caused clumping of duckweed fronds. Our study shows that australifungin inhibits sphingolipid synthesis in plants, but is about eight times less potent than AAL-toxin T_A or fumonisin B₁. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

AAL-toxins and fumonisins (Fig. 1), two series of fungal secondary metabolites which share structural analogy with sphingosine, have been identified as potent phytotoxins during the course of studies on the biological control of weeds [1–5]. AAL-toxins were first isolated from *Alternaria alternata* f. sp. *lycopersici* [6, 7] as host-specific toxins [8–10], causing stem-canker disease on susceptible tomato biotypes, with the genotype *asc/asc* [11]. They are produced in five forms, AAL-toxin T_A (1), T_B (2), T_C, T_D (3) and T_E (4), each with two isomers. The fumonisins, the most abundant of which is fumonisin B₁ (FB₁) (7), are produced by *Fusarium moniliforme* [12], a ubiquitous contaminant of stored corn. AAL-toxins and FB₁ act by inhibiting the enzyme, ceramide synthase, in the sphingolipid biosynthesis pathway [13–15], resulting in accumulation of free sphingoid bases, including phytosphingosine (sphingosine) and sphinganine, both in plants and animals [14–16]. Relatively few phytotoxic mechanisms have been exploited in the development of commercial herbicides [1]. The dis-

covery of new, very effective phytotoxic mechanisms provide the potential to develop new classes of herbicides. However, an essential requirement for a useful herbicide is low mammalian toxicity. This is a particular concern with the fumonisins, which have been shown to cause leukoencephalomalacia in horses [17], pulmonary edema in swine [18] and cancer in rats [19]. Successful use of ceramide synthase inhibition as the basis of a new class of herbicides will require the development of analogues with reduced mammalian toxicity. Initial studies on structure-activity relationships of AAL-toxins and fumonisins for plant and mammalian toxicity [20] indicate that this may be possible. Further understanding of the phytotoxic mechanism provided by ceramide synthase inhibition was sought to guide the effort to develop analogues with increased phytotoxicity and/or reduced mammalian toxicity. The discovery of australifungin (5) [13], an antifungal antibiotic produced by the fungus, *Sporormiella australis*, which represents a new structural class of ceramide synthase inhibitor with no structural homology to sphingosine, has made available a new tool for investigating ceramide synthase inhibition as a phytotoxic mechanism. In the present study, we have compared the toxicity to 5 with that of sphingosine-analogue mycotoxins in model systems for phytotoxicity and mammalian toxicity.

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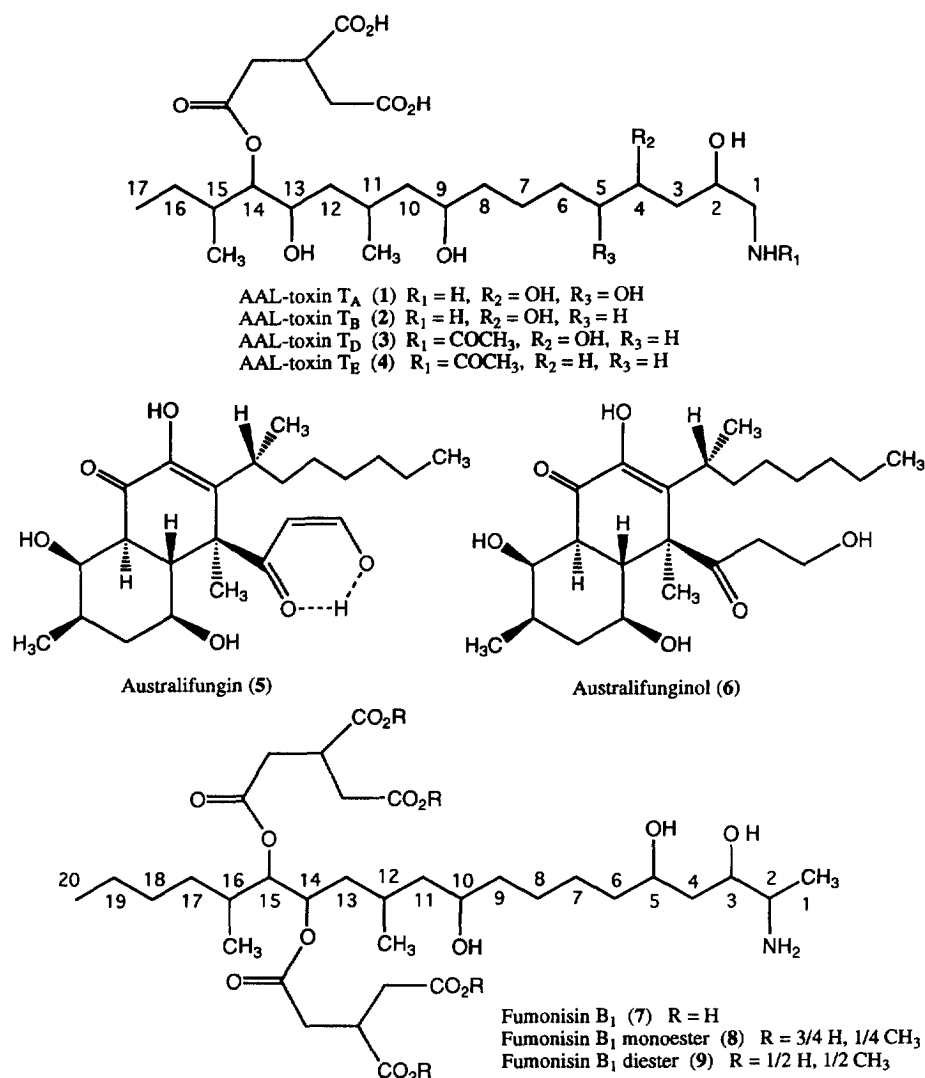


Fig. 1. Structures of ceramide synthase inhibitors used.

RESULTS AND DISCUSSION

In the duckweed bioassay compounds **1** and **7** were more active as phytotoxins than **5** (Table 1, Figs 2A–C). For example, **1** and **7** at $1 \mu M$ caused an increase in conductivity of 411 and $429 \mu S cm^{-1}$, respectively, chlorophyll reductions of 54% and 53%, respectively, and growth inhibitions of 54% and 60%, respectively, while the 5 and $10 \mu M$ solutions caused more severe symptoms. Compound **5** at 5 and $10 \mu M$ showed clear toxic effects on duckweed, causing an increase in conductivity (380 and $449 S cm^{-1}$), chlorophyll loss (24% and 50%) and growth inhibition (35% and 67%), respectively, although $1 \mu M$ produced no effect. Australifunginol (**6**), a structural analogue of **5**, was not toxic to duckweed at any concentration tested (Table 1). A control group of dilutions of the solvent (0.5% ethanol) also showed no toxic effects. In all cases, the first phytotoxic effect observed was cellular leakage, which started 24 h after initial treatment and increased

with time and concentrations (Fig. 2). Compounds **1** and **7** bleached duckweed fronds at all concentrations tested (1, 5, and $10 \mu M$), beginning within 48 h, and leading to separation of fronds and mortality. Compound **5** at $10 \mu M$ bleached duckweed fronds within 48 h and also caused clumping of fronds and mortality. At $5 \mu M$, **5** produced similar symptoms of phytotoxicity as the $10 \mu M$ concentration, but less severe. The middle of the three fronds of duckweed showed bleaching while the others remained green and fronds clumped in the center of the dish. Lower concentrations of **5** stimulated growth slightly after 14 days (Fig. 2C) ($P < 0.05$). These results showed that **5** is phytotoxic, but less active than **1** and **7**.

Ceramide synthase inhibitors were tested for reversibility on duckweed (Fig. 3). In preliminary experiments, **5** at $5 \mu M$ showed toxic effects after 72 h, but when the toxin-containing medium was removed and replaced with toxin-free medium, duckweed tissues began to recover within 7 days of initial

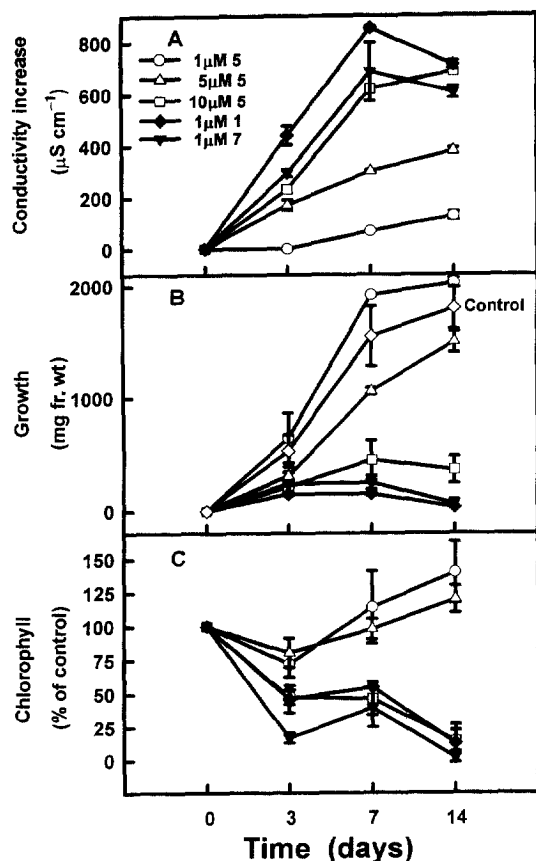


Fig. 2. Effects of ceramide synthase inhibitors (1, 7 and 5) on duckweed tissues measured as (A) cellular leakage determined by change in conductivity of bathing media relative to that of the control, (B) growth determined by the gain in fresh weight, and (C) extractable chlorophyll content measured spectrophotometrically. Effects of 1 and 7 at 5 μ M and 10 μ M were the same as at 1 μ M. Values given are means of three replicates $\pm$ standard deviation.

treatment. After 14 days, duckweed initially treated with 5 at 1 or 5 μ M contained significantly more chlorophyll than controls ($P < 0.05$). No recovery was noted in duckweed initially treated for 72 h with 10 μ M 5 or with 1 μ M or greater 1 or 7. In a second reversibility experiment, duckweed tissues pre-treated 24, 48 or 72 h with 5 μ M 5 initially exhibited toxic effects (photobleaching and increased conductivity in media), but began to recover within 7 days after removal from the toxin-containing medium (Fig. 3). Duckweed tissues were not affected by exposure to 1 or 7 at 1 μ M for 24 h but after the same treatment for 48 h, they showed limited recovery within 14 days after removal of the toxin-containing medium (Fig. 3). No recovery was seen in duckweed tissues treated with 1 or 7 at 1 or 5 μ M for 72 h (Fig. 3).

Compounds 1, 5 and 7 at 1, 5 and 1 μ M, respectively, caused increases in free phytosphingosine (Pso) and free sphinganine (Sa), while the controls and 6 at 50 μ M showed no change in free sphingoid base content. All three toxins caused greater increases in Pso than those of Sa. The amounts of both sphingoid

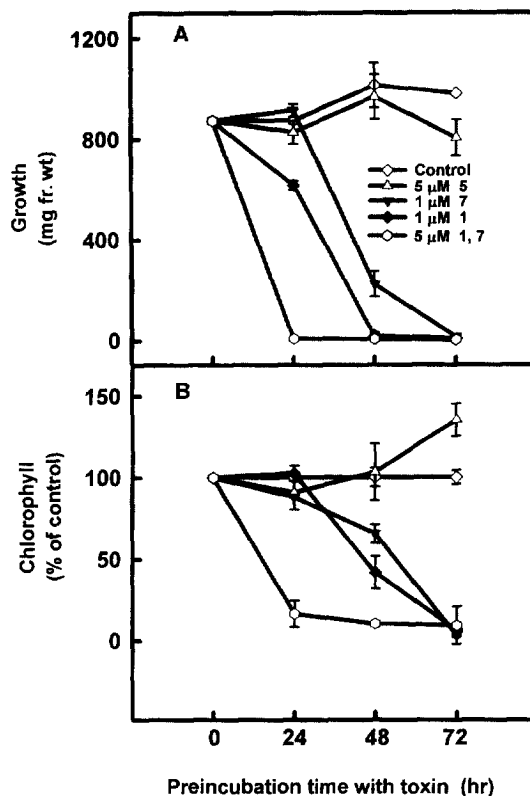


Fig. 3. Time-course of reversibility of toxic effects of ceramide synthase inhibitors in duckweed pretreated for 0 to 72 h with 5 (5 μ M), 7 (1 and 5 μ M) or 1 (1 and 5 μ M), followed by wash-out and culturing for 14 days in toxin-free medium. Recovery is shown as (A) growth determined by the gain in fresh weight and (B) extractable chlorophyll content measured spectrophotometrically. Values given are means of three replicates $\pm$ standard deviation.

bases rose within 12 h of initial treatment and increased with time, reaching their peak 24 h for Sa and 48 h for Pso, then dropping at 72 h for all toxins. The increases in Pso and Sa in duckweed treated with 1 or 7 at 1 μ M were higher than those treated with 5 at 1 μ M by five–eight fold. Compound 5 caused an increase in the amount of free sphingoid bases as early as 12 h and an increase in electrolyte leakage at 24 h, as in the case of 1 or 7 (Fig. 4).

Compounds 1 and 2 were more active against duckweed than 3 and 4, as measured by electrolyte leakage, chlorophyll reduction and growth inhibition at 1 μ M (Table 1). Compounds 1 and 2 were equally phytotoxic to duckweed (Table 1). Compounds 3 and 4 at 1 μ M showed no effect, but 3 and 4 at 100 μ M caused phytotoxicity to duckweed, 3 more so than 4 (Table 1). FB₁ monoester (8), the monomethyl ester of 7, exhibited phytotoxicity similar to 1 and 7, whereas FB₁ diester (9), the dimethyl ester of 7, was much less phytotoxic than 1 and 7 (Table 1).

With NIH3T3 mouse fibroblasts, 5 was more cytotoxic than 6 and both were more cytotoxic than 7. Compounds 5 and 7 were more cytotoxic than 6 against H4TG rat hepatoma and MDCK dog kidney

Table 1. Phytotoxicity of AAL-toxins, fumonisin B₁, australifungin and some derivatives and analogues against duckweed

Toxin	EC ₅₀ *		
	Conductivity ($\mu\text{S cm}^{-1}$)	Chlorophyll reduction	Growth inhibition
AAL-toxin T _A (1)	0.38	0.71	0.37
AAL-toxin T _B (2)	0.41	0.31	0.22
AAL-toxin T _D (3)	4.80	17.0	2.20
AAL-toxin T _E (4)	> 200	> 200	191
Australifungin (5)	4.10	9.80	6.70
Australifunginol (6)	Inactive	Inactive	Inactive
Fumonisin B ₁ (7)	0.81	0.98	0.83
FB ₁ monoester (8)	0.73	0.85	1.30
FB ₁ diester (9)	48.0	22.0	> 20

* Phytotoxic responses measured after 72 h.

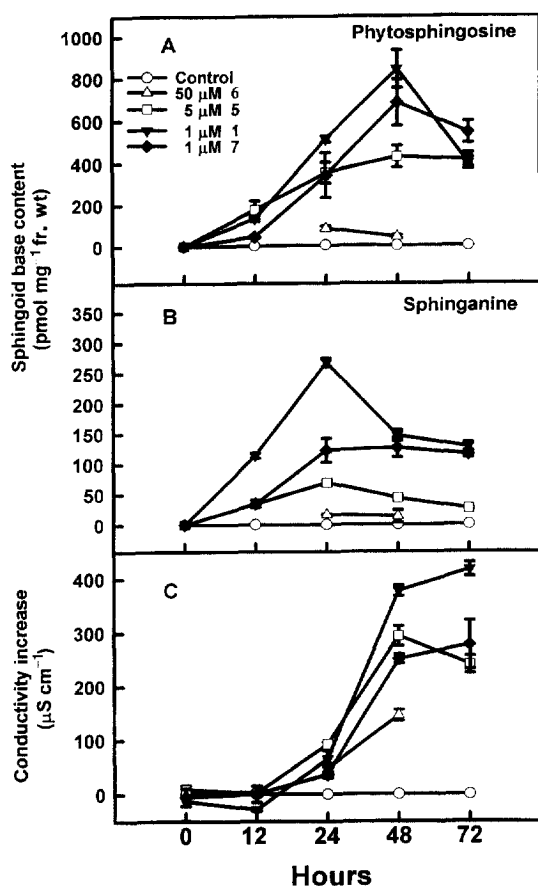


Fig. 4. Time-course of sphingoid free base contents for (A) phytosphingosine and (B) sphinganine, in duckweed cultured treated with 1 μM 1, 5 μM 5, 50 μM 6, 1 μM 7 and 0.5% ethanol as control. Cellular leakage (C) was determined in the same experiment by change in electrical conductivity of the bathing media relative to that of the control. Values given are means of three replicates $\pm$ standard deviation.

cell lines, but both 5 and 6 were cytotoxic, with IC₅₀ values ranging from 12.7 to 50.4 μM (Table 2).

The significance of the difference between the morphological effects of the toxins is unknown. Compounds 1 and 7 are substrate analogues, whereas 5

Table 2. Cytotoxicity of australifungin, australifunginol, fumonisin B₁ and AAL-toxin T_A against cultured mammalian cell lines

Sample	IC ₅₀ (μM)		
	NIH3T3*	H4TG†	MDCK‡
1	200.0	25.0	25.0
5	38.9	21.3	12.7
6	108.0	50.4	41.7
7	> 200	16.0	29.0

* Contact-inhibited NIH Swiss mouse embryo fibroblasts (NIH3T3).

† 6-Thioguanine-resistant rat hepatoma cells (H4TG).

‡ Madin-Darby canine kidney cells (MDCK).

bears no obvious structural resemblance to substrates of ceramide synthase. It is possible that 1 and 5 inhibit different isozymes or other forms of ceramide synthase. The plant also may metabolize 5 to non-toxic products differently from the sphingosine analogue toxins or the toxins may distribute differently in plant tissues.

Our studies establish that different chemical classes of ceramide synthase inhibitors are effective as phytotoxic agents and that the relative toxicity in plant and mammalian bioassay systems varies with structure. Additional studies on the relationship of structure to both plant and mammalian toxicity will be needed to establish the potential usefulness of ceramide synthase inhibitors as herbicides.

EXPERIMENTAL

Source of phytotoxins

Compounds 5 and 6, prepared from *S. australis* [13], were provided by S. M. Mandala. Compound 1 was prepared from *A. alternata* SWSL#1 [21]. Compound 7 was prepared from *F. moniliforme* JW#1 [22].

Compounds **8** and **9** prepared from *F. moniliforme* NRRL-A28160 (unpublished results) were provided by R. F. Vesonder. Compounds (**1–4**) prepared from *A. alternata* f. sp. *lycopersici* [2] were provided by D. G. Gilchrist. AAL-toxin T_c was not available.

Plant materials

Cultures of *Lemna paucicostata* Hegelm. 6746 were initiated with inoculation of 30 fronds (10 colonies) onto 500 ml of a mineral growth medium [23].

Duckweed bioassays

Assays were carried out as described in detail in ref. [24], using 10 colonies of 3 fronds each incubated at 25° under continuous light with toxin dilns prepared from stocks dissolved in EtOH or with solvent controls. Phytotoxicity was observed visually (bleaching and necrosis) and quantified by (i) cellular leakage monitored with a conductivity meter or (ii) bleaching measured by extracting total chlorophyll with DMSO [25] and spectrophotometry [26]. Growth inhibition was measured as the gain in fr. wt relative to controls. Tests were performed twice with three replicates for each expt. Reported results are EC₅₀ values estimated graphically.

Determination of free sphingoid bases (phyto-sphingosine and sphinganine)

Determining the accumulation of free sphingoid bases and correlating the time-course profile for free sphingoid base elevation with cellular leakage, were conducted as described for bioassays, except that 50 colonies of 3 fronds of duckweed were used in 6-cm diameter polystyrene Petri dishes with 5 ml of each toxin concn or vehicle control (0.5% EtOH). At appropriate times, duckweed fronds were removed, dried, frozen in liquid N₂ and stored at –80° until analyzed. Extraction, clean-up and determination of free sphingoid bases by HPLC was as described previously [15, 16].

Reversibility of toxin effects

Determined by initially treating cultures of 10 duckweeds, consisting of 3 fronds each, with a range of toxin concns in 3 ml medium in 3.6 cm Petri dishes for various time periods up to 72 h under normal culture conditions in continuous light. Cultures were washed, placed in toxin-free medium and cultured for an additional 14 days under normal culture conditions in continuous light. Conductivity, chlorophyll content and growth of duckweed cultures were measured, as described above, after 3, 7 and 14 days and compared with control groups. Experiments were repeated in triplicate.

Cytotoxicity bioassays

Three mammalian permanent cell lines were used to compare the cytotoxicity of **5–7**. Sources, growth media and cytotoxicity assay procedure were as previously described [20, 27].

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