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Phytotoxic Ophiobolins Produced By *Helminthosporium Gramineum Rabenh*, A Potential Bioherbicide For Control Of Barnyardgrass (*Echinochola Crus-Galli*)

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ABSTRACT

3-Anhydroophiobolin B, ophiobolin A, 3-anhydro-6-epiophiobolin A and 3-anhydro-6-epiophiobolin B were isolated from mycelia and culture filtrate of *helminthosporium gramineum rabenh*, a fungus phytopathogenic to barnyardgrass (*Echinochloa crus-galli*). Their structures were identified by detailed analysis of the NMR spectral data and by comparison with reported data. Root growth inhibition bioassays showed that all four ophiobolins inhibited root growth of barnyardgrass and rice at high concentration (500μg ml^{-1}) but only ophiobolin A inhibited rice root growth at low concentration. Root growth of barnyardgrass was much more susceptible to ophiobolin A than was rice. Ophiobolin A was phytotoxic to some but not all of the plant species tested by leaf bioassay in vivo. However, 3-anhydroophiobolin B, 3-anhydro-6-epiophiobolin A, and 3-anhydro-6-epiophiobolin B exhibited no phytotoxicity to any of the test species in vivo. Clarification of the structure–activity relationships among these ophiobolin analogs may provide useful chemical information.

KEYWORDS

Helminthosporium gramineum Rabenh;
Phytotoxin;
Ophiobolins;
SAR;
Bioherbicide.

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INTRODUCTION

Weeds pose a serious constraint to agricultural production. According to an estimate, in US alone, weeds cause a loss of around 12% costing to nearly US $ 33 billion and it is even more in developing countries[1]. Furthermore, the increasing use of herbicides has resulted in a dramatic increase in the herbicidal resistance among weeds. Over 311 weed resistant biotypes belonging to 183 species (110 dicots and 73 monocots) have been identified world over[2]. Concern is now being expressed throughout the world about the environmental impact, and effects of the widespread use of chemical herbicides. Kupatt et al.[3] pointed that in order to reduce heavy reliance on herbicides there is a need to move to low-input sustainable agriculture as a component of integrated weed management. In this regard, there has been a rising interest in the discovery of environmentally friendly bioherbicidal compounds and biocontrol agent for weed control in sustainable agriculture[4,5]. Since the commercially successful development of bialaphos, produced by (*Streptomyces hygroscopicus*), as a herbicide for barnyard millet[6], phytotoxins produced by biocontrol agents have been considered as potential herbicides that are selective, easily degradable and environmentally friendly[7-9].

Barnyardgrass (*Echinochloa crus-galli*) is one of the most severely yield-limiting weeds in rice cultivation systems[10]. In the course of our screening of bioherbicidal agent (BCA) for paddy weeds control, a fungus strain, (*Helminthosporium gramineum Rabenh*) which was isolated from the naturally infected barnyardgrass plant, has potential as BCA for control of barnyardgrass in rice[11]. On account of the possible use of phytotoxins as naturally occurring and safe herbicides, it is of interest to acertain the production of phytotoxic metabolite by active strain. The objectives of the present study were to isolate and identify the phytotoxic secondary metabolites produced by this fungus strain and to evaluate the potential of these compounds to be developed as a bioherbicide.

EXPERIMENTAL

Pathogenic fungus and culture conditions

Monoconidial isolates of the pathogenic fungus *H.gramineum Rabenh*. were maintained on potato dextrose agar (PDA) slants in test tubes at 4°C as stock cultures. Small pieces of mycelium from the stock culture were aseptically transferred to the centre of fresh PDA plates and incubated in the dark at 27±1°C for 7 days. Agar plugs with mycelium(7mm diameter) taken from the margin of these young colonies were placed in 250mL Erlenmeyer flasks containing 50ml of potato dextrose broth (PDB) liquid medium. The flasks were incubated in the dark at 25-30°C for 14 days.

Isolation and purification of phytotoxic compounds

The isolation of phytotoxic compounds was guided by root growth inhibition bioassay. PDB cultures were filtered through four layers of cheesecloth to separate the mycelia and the culture filtrate.

1. Compound (1)

The mycelia were extracted with EtOAc (3×2 l). The combined mycelial extracts were dried (Na_2SO_4) and evaporated under reduced pressure to yield an oily dark-green residue with high phytotoxic activity and was then stored at 4°C. Two days later, a crude solid containing compound **(1)** was found in the mycelial extracts. The crude **(1)** was separated from the mycelial extracts by filtering through analytical filter paper in a Büchner funnel under vacuum. Leaf bioassay showed that crude **(1)** was phytotoxic against barnyardgrass. The crude **(1)** was then dissolved in a minimal volume of $CHCl_3$ and subjected to silica gel (Merck, Kieselgel 60, 230-400mesh) column chromatography eluting with $CHCl_3$-MeOH (150:1, v/v). Fractions (10-ml) were collected and tubes 22-25 contained biologically active compound **(1)**. The active fractions were evaporated under reduced pressure at 40°C and re-dissolved in $CHCl_3$. Compound **(1)** was further purified by re-crystallization from $CHCl_3$ by adding a little EtOH into the $CHCl_3$ solution, whereby colorless crystals of **(1)** were obtained.

2. Compound (2)

The culture filtrate was concentrated to half its original volume by evaporation under reduced pressure at 40°C. The concentrated filtrate (8L) was ex-

tracted with EtOAc(3×3 l). The organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure to give a red-brown oil residue with high phytotoxicity. The crude residue was dissolved in a minimal amount of mixed solvent (n-Hexane: EtOAc, 1:1, v/v) for crystallization of compound **(2)**. The crude **(2)** was separated from the culture broth extract by filtering through analytical filter paper in a Buchner funnel under vacuum. Root growth inhibition bioassay showed that crude **(2)** was phytotoxic against barnyardgrass. Crude **(2)** was further purified by recrystallization from the mixed solvent, and colorless needle crystals of 2 were obtained.

3. Compounds (3) and (4)

The mycelia and culture filtrate extracts were combined after removal of compounds **(1)** and **(2)**, and were subjected to silica gel (Merck, Kieselgel 60, 230-400mesh) column chromatography, eluted with a gradient of n-Hexane-EtOAc (9:1→0:10, v/v) and MeOH. The fractions (50ml each) were pooled on the basis of their TLC profiles and evaporated under reduced pressure and then subjected to bioassay. The n-Hexane-EtOAc (6:4) fractions 28-37 and 38-50 exhibited activity. Each set was further purified by silica gel column chromatography. The first set (28-37) was subjected to silica gel (TLC silica gel H, Qingdao, China) column chromatography eluted with $CHCl_3$-MeOH (250:1, v/v). Fractions (10-ml) were collected and the bioactive compound **(3)** was found in tubes 10-12 as a reddish orange solid. The second set (38-50) was further purified in the same manner as the first set but eluted with $CHCl_3$-MeOH (150:1, v/v). The colorless amorphous solid bioactive compound **(4)** was found in tubes 15-17.

Structural elucidation

The structures of compounds were identified by spectroscopic methods and by comparison with reported data. NMR spectra, including COSY, HMQC, HMBC, DEPT and NOESY experiments, were recorded on an AVANCE 500 spectrometer in $CDCl_3$ at 500 MHz(^{1}H) or 125 MHz(^{13}C), using TMS as an internal standard. TOF-ESI-MS was performed on a Micromass LCT spectrometer.

Leaf bioassay

A simple leaf-puncture assay was used as a rapid guide in isolating the suspected phytotoxins. The most recently expanded leaf of barnyardgrass was detached from plants (2-4 weeks old). A 3-µl droplet of a 1% DMSO solution was placed on a leaf blade. The solution was placed over a needle puncture wound to enhance access to the leaf tissue. The leaves were placed on moist filter paper in a sealed petri dish at 27±1°C for 48 h in the dark. Each test (concentration) was repeated at least 3 times on individual leaves and the data (size of the lesion developing after 48h) was averaged. The control solution was 1% DMSO.

Root growth inhibition

Barnyardgrass seeds were collected from natural agricultural Echinochloa populations on the China National Rice Research Institute (CNRRI) farm 1 year before the experiment and stored at -20°C. The rice cultivars used were Xiushui 11, Zaodao 03-133 and Zajiaodao 187, representing the japonica, indica and hybrid rice types, respectively. Germinated seeds (barnyardgrass or rice) with primary roots 2 mm long were selected and placed in 7cm diameter petri dishes (10 germinated seeds per dish). Dishes contained 2ml of toxin solutions in 1% DMSO at concentrations of 0, 10, 50, 100, or 500mg ml^{-1}. The dishes were incubated in a chamber at 27±1°C for 2 days. Then the root length was measured. Percent root growth inhibition was obtained by comparing the root length of seedlings in the presence of toxins with that of controls. All bioassays were conducted twice with six replications in a completely randomized design. ANOVA with Duncan's multiple range-test was performed.

Host selectivity of compounds

Selectivity of compounds was evaluated *in vivo* in pots (15cm×20cm) against 9 crops and 10 weeds during post-emergence under greenhouse conditions (number of seedlings per pot shown in parenthesis): *Oryza sativa* (10) (japonica rice Xiushui 11, indica rice Zaodao 03-133, and hybrid rice Zajiaodao 187), *Zea mays* (10), *Triticum aestivum* (30), *Brassica campestris* (10), *Glycine max* (10), *Lycopersicum esculentum* (10), *Brassica chinensis* (15), *Digitaria sanguinalis* (30), *Echinochloa crus-galli* (30), *Leptochloa chinensis* (30), *Cyperus serotinus* (20), *Setaria viridis* (30), *Eleusine in-*

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dica (15), *Monochoria vaginalis* (15), *Alternanthera philoxeroides* (15), *Stellaria media* (10), and *Marsilea quadrifolia* (15). Compounds **(1)**, **(2)**, **(3)**, and **(4)** were dissolved at 500, 100, 50 and 10μg ml^{-1} in 1% DMSO containing 0.05% Tween-20 and then the solution was sprayed on plants (2-4 weeks old) in pots at 50 ml m^{-2} with a hand sprayer. DMSO (1%) solution containing 0.05% Tween-20 served as untreated control. Phytotoxicity was recorded 14 days after treatment.

RESULTS AND DISCUSSION

Isolation and identification of compounds

Compounds **(1)**, **(2)**, **(3)** and **(4)** were successively isolated from the culture filtrate (16 l) and mycelia (dry weight 169.8g after extraction with EtOAc) in yields of 55, 157, 248 and 52 mg, respectively.

Detailed analysis of the NMR spectral data and comparison with reported data[12-16] identified the structures (Figure 1) of compounds **(1)**, **(2)**, **(3)**, and **(4)** as 3-anhydroophiobolin B**(1)**, ophiobolin A**(2)**, 3-anhydro-6-epiophiobolin A**(3)**, and 3-anhydro-6-epiophiobolin B**(4)**, respectively. The complete assignments (TABLE 1) of proton and ^{13}C-NMR signals of compounds were confirmed by analyzing a combination of ^{1}H-^{1}H COSY, HMQC, HMBC, DEPT and NOESY data.

3-Anhydroophiobolin B **(1)** was previously prepared by Canonica and coworkers[13] by chemical conversion. This is the first time that **(1)** has been isolated from mycelia of *H.gramineum* and the relative stereochemistry of **(1)** has been confirmed.

The assignments of ophiobolin A **(2)** were almost identical to those previously reported by Li et al.[14] except for the reversing assignments for C-13 and C-16. The HMBC spectrum of **(2)** showed a long-range ^{1}H-^{13}C correlation between the C-23 methyl group and the protons at C-16 which allowed assignment of C-16. Also, there was a long-range ^{1}H-^{13}C correlation between the C-22 methyl group and the protons at C-13, which allowed assignment of C-13. The NOESY spectrum of **(2)** showed clear NOE correlation between H-12 and H-13; H-17 and H-16 which also confirmed the assignments of C-13 and C-16.

3-Anhydro-6-epiophiobolin B **(4)** was previously isolated from cultural filtrates of *Cochliobolus heterostrophus* by Shen et al.[15]. However, 3-anhydro-6-epiophiobolin B**(4)** changed into di-6-epi-3-anhydroophiobolin B and 6-epi-3-anhydro-$\Delta^{10(14)}$-ophiobolin B during HMBC spectrum acquisition in CH_2Cl_2 (Shen et al., 1999). This is the first time that the relative stereochemistry of **(4)** has been confirmed by detailed analysis of 2D NMR of **(4)**.

Phytotoxic activities of compounds

The effects of the four ophiobolins at concentrations of 500, 100, 50 and 10μg ml on root growth of barnyardgrass and rice are presented in TABLE 2. All four ophiobolins inhibited the growth of barnyardgrass at 500μg ml^{-1}. Ophiobolin A **(2)** was the most active compound and completely inhibited the growth of barnyardgrass at 50μg ml^{-1}, whereas the other three ophiobolins showed no or low inhibitory effects at 100μg ml^{-1}. Ophiobolin A **(2)** also significantly inhibited the root growth of the three selected rice cultivars. However, root growth of barnyardgrass was much more susceptible to ophiobolin A**(2)** than was rice. Compounds **(1)**, **(3)**,

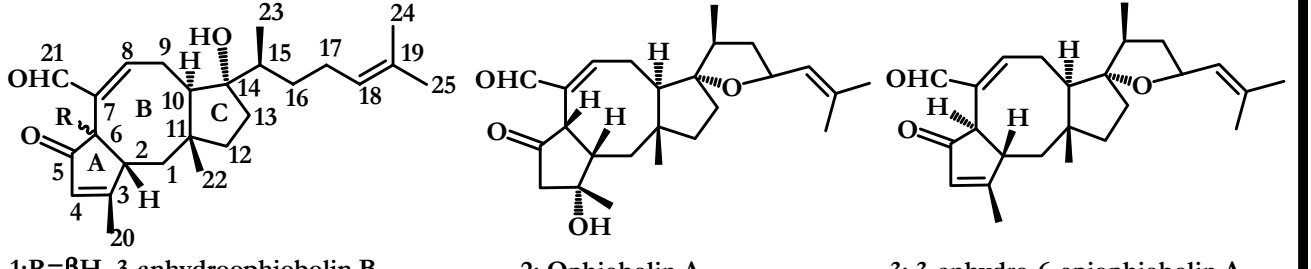

Figure 1: Structural formulae of compounds (1–4)

TABLE 1: NMR data for 3-anhydroophiobolin B (1), ophiobolin A (2), 3-anhydro-6-epiophiobolin A (3) and 3-anhydro-6-epiophiobolin B (4)

Carbon	(1)		(2)		(3)		(4)	
	δ_C[a]	δ_H[b]	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	36.2	A 1.91 (m) B 1.38(m)	36.0	~1.71 (m)	46.8	A 1.42 (m) B 1.98 (dd,13.8,9.6)	46.1	A 1.34 (m) B 2.10 (m)
2	48.8	3.19 (d,4.5)	51.0	2.36 (m)	49.2	2.65 (d,3.5)	48.8	2.68 (d,13)
3	176.7	-	77.0	-	177.2	-	177.2	-
4	131.2	6.11 (s)	55.6	A 2.81 (d,19.2) B 2.50 (d,19.2)	130.4	6.02 (t,1.4)	130.2	6.04 (s)
5	207.3	-	217.9	-	207.0	-	207.0	-
6	48.3	4.13 (d,7.1)	49.1	3.26 (d,10.6)	49.3	3.42 (d,4.0)	49.8	3.45 (d,4.2)
7	138.5	-	142.5	-	141.1	-	140.6	-
8	158.8	7.07	163.7	7.21 (t,8.6)	155.0	6.81 (dd,6.5,2.3)	155.3	6.78 (dd,6.3,2.4)
9	27.4	A 2.73 (m) B 2.45 (m)	24.3	A 2.42 B 2.25 (m)	29.8	A 2.83 (dt,19.9,2.8) B 2.35 (m)	31.5	α 2.80 B 2.10 (m)
10	52.8	2.27 (s)	61.3	1.76 (m)	53.8	2.67 (d,3.8)	54.5	2.56 (dd,4.3,2.4)
11	46.1	-	43.4	-	42.5	-	44.7	-
12	38.1	A 1.78 (m) B 1.25 (m)	41.6	A 1.73 (m) B 1.42	41.8	~1.42 (m)	41.4	A 2.00 (m) B 1.45 (m)
13	33.8	A 1.38 (m) B 1.64 (m)	30.9	A 1.63 (m) B 2.04 (m)	30.6	A 1.60 (m) B 1.68 (m)	35.2	A 1.45 (m) B 1.59 (m)
14	86.5	-	95.4	-	96.1	-	88.3	-
15	39.5	1.41 (m)	37.6	2.18 (m)	35.4	2.23 (m)	37.1	1.45 (m)
16	32.7	1.00 (m) 1.66 (m)	43.9	1.67 (m) 1.77 (m)	42.2	1.72 (m) 1.78 (m)	32.6	1.20 (m) 1.47 (m)
17	26.3	1.90 (m) 2.08 (m)	71.6	4.43 (m)	72.0	4.59 (dd,15.6,7.2)	26.2	1.98 (m) 2.12 (m)
18	124.3	5.09	126.1	5.15 (dt,8.6,1.3)	126.8	5.13	124.1	5.16 (d,7.3)
19	131.7	-	137.0	-	135.1	-	132.0	-
20	18.5	2.23 (s)	26.3	1.37 (s)	17.1	2.04 (s)	17.1	2.07 (s)
21	194.5	9.41 (s)	196.8	9.24 (s)	192.7	9.31 (s)	192.8	9.32 (s)
22	23.6	0.78 (s)	18.6	0.82 (s)	22.3	0.87 (s)	22.5	0.88 (s)
23	16.1	0.89 (d,6.7)	18.9	1.09 (d,7.2)	16.2	1.04 (d,6.9)	14.8	0.98 (d,6.6)
24	17.7	1.60 (s)	18.9	1.71 (d,1.1)	18.1	1.65 (d,1.0)	17.7	1.63 (s)
25	25.7	1.69 (s)	26.6	1.74 (d,1.1)	25.8	1.70 (d,0.9)	25.7	1.71 (s)

[a] In ppm downfield from TMS at 125 MHz in $CDCl_3$. The resonances designated by * in 2 reflect differences from previous assignments in reference 14; [b] In ppm downfield from TMS at 500 MHz in $CDCl_3$. Mult. and coupling constants are in parentheses.

and **(4)** inhibited root growth of rice only at high concentration(500μg ml^{-1}).

Data in a column followed by the same letter are not significantly different at the 0.05 levels by Duncan's multiple-range test.

Selectivity of compounds

Ophiobolin A**(2)** displayed some levels of selective phytotoxicity towards the crops and weeds tested *in vivo*(TABLE 3). However, 3-anhydroophiobolin B **(1)**, 3-anhydro-6-epiophiobolin A**(3)**, and 3-anhydro-6-epiophiobolin B**(4)** exhibited no phytotoxicity towards any of the test plants *in vivo* (data not shown). At 500μg ml^{-1}, ophiobolin A**(2)** produced red-brown lesions on leaves of seven plant species in vivo, namely one crop (Glycine max) and six weeds

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TABLE 2: Effects of ophiobolins on root growth of barnyardgrass and rice by petri dish bioassay

Test chemicals	Concentration (μg ml^{-1})	Inhibition (%)			
		Barnyardgrass	Xiushui11 (japonica rice)	Zaodao03-133 (indica rice)	Zajiaodao 187 (hybrid rice)
3-Anhydroophiobolin B **(1)**	500	51.5c	48.2d	24.1c	29.1d
	100	-3.7f	-19.9j	-17.8f	-12.8g
	50	-1.0ef	-1.5h	-1.5e	-3.3efg
	10	-0.5ef	-0.3h	1.1de	-0.5ef
Ophiobolin A **(2)**	500	100a	100.0a	100a	100a
	100	100a	100.0a	94.6a	96.1a
	50	100a	76.3b	61.3b	71.4b
	10	73.9b	15.4f	9.8d	5.3e
3-Anhydro-6-epiophiobolin A **(3)**	500	69.4b	67.7c	55.9b	40.9c
	100	29.4d	30.2e	21.7c	-8.1fg
	50	0.4ef	-7.1i	-8.0ef	-5.5efg
	10	-0.2ef	-0.9h	-1.3e	-0.7ef
3-Anhydro-6-epiophiobolin B **(4)**	500	49.3c	49.8d	31.5c	35.8cd
	100	6.7e	10.1g	0.1de	-14.8g
	50	3.0ef	-16.1j	-14.4f	-5.6efg
	10	0.9ef	0.3h	-1.0e	1.35ef

(*Echinochloa crus-galli, Cyperus serotinus, Eleusine indica, Monochoria vaginalis, Alternanthera philoxeroides, and Marsilea quadrifolia*). Among these seven susceptible plants, *Glycine max, Monochoria vaginalis*, and *Marsilea quadrifolia* were most sensitive to ophiobolin A**(2)**; at an application level of 10μg ml^{-1}, ophiobolin A caused red-brown lesions on leaves of all three, although it did not inhibit the growth of soybean. Although ophiobolin A**(2)** caused severe red-brown lesions on *Monochoria vaginalis* and *Marsilea quadrifolia* leaves, it did not kill weeds even at the high concentration. Our results indicate that ophiobolin A**(2)** may not be suitable for direct development into a herbicide.

The phytotoxic activity of ophiobolin A**(2)** on weeds and rice in both the root growth inhibition assay and the leaf assay *in vivo* was much higher than that of 3-anhydroophiobolin B**(1)**, 3-anhydro-6-epiophiobolin A**(3)** and 3-anhydro-6-epiophiobolin B**(4)**. The significant difference in bioactivity between ophiobolin A and its analogs is interesting from the point of view of a structure–activity relationship because their chemical structures are similar. Unlike ophiobolin A**(2)**, in 3-anhydroophiobolin B**(1)**, 3-anhydro-6-epiophiobolin A**(3)** and 3-anhydro-6-epiophiobolin B**(4)**, there are no -OH groups on carbon 3. Kim et al.[17] reported a much greater inhibition of photosynthesis by ophiobolin A and 6-epiophiobolin A than by their 3,4-dehydroated derivatives (3-anhydroophiobolin A and 3-anhydro-6-epiophiobolin A). Leung et al.[18] compared the potency of several ophiobolin analogs in inhibiting maize calmodulin and found that the order of potency was ophiobolin A>6-epiophiobolin A>3-anhydroophiobolin A. The results of our experiments combined with reported results indicated that the -OH group attached to C-3 of ophiobolins may be important in the phytotoxic activity of ophiobolin analogues. Still, these conjectures regarding the structure–activity relationships of ophiobolin analogs require further study.

There has been considerable research interest in phytotoxins produced by plant pathogens of crop plants[9,19]. Phytotoxins from weed pathogens have received less attention. However, phytotoxins produced by weed pathogens have the potential to be used directly on the target weed species or used as building blocks for novel herbicides[20]. In addition, rational modifications of the original structure could illuminate the relationships between molecular structure, biological activity, and host specificity[4]. Although ophiobolin A and its 3,4-dehydrated analogs

TABLE 3: Reaction of some crop and weed plants to ophiobolin A (2) *in vivo*

Plant	Ophiobolin A (μg ml^{-1})*				
	500	100	50	10	0
Oryza sativa (japonica rice Xiushui 11)	-	-	-	-	-
Oryza sativa (indica rice Zaodao 03-133)	-	-	-	-	-
Oryza sativa (hybrid rice Zajiaodao 187)	-	-	-	-	-
Zea mays (Corn)	-	-	-	-	-
Triticum aestivum (Wheat)	-	-	-	-	-
Brassica campestris (Oilseed Rape)	-	-	-	-	-
Glycine max (Soybean)	+	+	+	-	-
Lycopersicum esculentum (Tomato)	-	-	-	-	-
Brassica chinensis (Pai-tsai)	-	-	-	-	-
Digitaria sanguinalis (Common Crabgrass)	-	-	-	-	-
Echinochloa crus-galli (Barnyardgrass)	+	-	-	-	-
Leptochloa chinensis (Chinese Sprangletop)	-	-	-	-	-
Cyperus serotinus (Late Juncellus)	+	-	-	-	-
Setaria viridis (Green Foxtail)	-	-	-	-	-
Eleusine indica (Goosegrass)	+	+	-	-	-
Monochoria vaginalis (Sheathed Monochoria)	+	+	+	-	-
Alternanthera philoxeroides (Alligator Alternanthera)	+	-	-	-	-
Stellaria media (Common Chickweed)	-	-	-	-	-
Marsilea quadrifolia (Pepperwort)	+	+	+	+	-

may not be suitable for use directly as bioherbicides, the as yet unknown structure-activity relationships of these analogs may provide useful chemical information.

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