Germination and Growth Inhibition of Annual Ryegrass (*Lolium multiflorum* L.) and Alfalfa (*Medicago sativa* L.) by Loline Alkaloids and Synthetic *N*-Acylloline Derivatives

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Three naturally occurring loline derivatives (*N*-formylloline, *N*-acetylloline, and *N*-methylloline) and 15 new *N*-acylloline derivatives were synthesized from loline (hexahydro-*N*-methyl-2,4-methano-4*H*-furo[3,2-*b*]pyrrol-3-amine). These compounds were all tested for phytotoxic activity. Several concentrations of each were applied exogenously to alfalfa (*Medicago sativa* L.) and to annual ryegrass (*Lolium multiflorum* L.) seeds to determine dose–response relationships. Of the endogenous compounds only *N*-formylloline demonstrated significant phytotoxicity by inhibiting germination of annual ryegrass. Eight of the synthetic *N*-acyl derivatives of loline were phytotoxic and exhibited 50% reductions in germination and seedling length with doses of less than 1.5×10^{-7} mol/seed. *N*-Dodecanoyl- and *N*-(*p*-*n*-hexylbenzoyl)loline were the most phytotoxic derivatives tested and exhibited ID_{50} values of 5×10^{-8} mol/seed for the reduction of annual ryegrass seedling length. The *N*-dodecanoyl and *N*-tetradecanoyl derivatives were the most active; activity decreased as the chain length of the acyl groups became either longer or shorter. The loline derivatives were only slightly selective as exhibited by general 2-fold greater toxicity against annual ryegrass over alfalfa.

Numerous secondary plant constituents inhibit the growth and development of other organisms. Constitutive and induced compounds protect the donor plant from stress imposed by insects, fungi, herbivores, and competing plants (Bailey, 1982; Rice, 1984; Powell and Spencer, 1988). Saturated pyrrolizidine alkaloids of the loline type (Petroski et al., 1989; Yates et al., 1989) occur at relatively high concentrations in tall fescue (*Festuca arundinacea* Schreb.) that is infected with *Acremonium coenophialum* Morgan-Jones and Gams, but they have not been detected in tall fescue in the absence of this fungal endophyte. The lolines have not been considered contributors to the observed allelopathy of tall fescue (Peters, 1968; Peters and Mohammed Zam, 1981; Walters and Gilmore, 1976). The objectives of this study were to prepare and test a number of loline derivatives for phytotoxicity against, and selectivity between, annual ryegrass (*Lolium multiflorum* L.) and alfalfa (*Medicago sativa* L.) seed germination and seedling growth.

EXPERIMENTAL PROCEDURES

Materials. *N*-Formylloline, *N*-acetylloline, norloline, *N*-formylnorloline, and *N*-methylloline were prepared from pure loline as described previously (Petroski et al., 1989). Juglone and plumbagin were purchased from Sigma Chemical Co. Cinmethylin [*exo*-1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)-methoxy]-7-oxabicyclo[2.2.1]heptane] was a gift from E. I. du Pont de Nemours and Co., Wilmington, DE. Seed of alfalfa (*M. sativa* L. cv. Vernal) and annual ryegrass (*L. multiflorum* L.) were purchased from Kelly Seed Co., Peoria, IL. Seed of annual ryegrass was checked for the presence of endogenous loline alkaloids by capillary gas chromatography (Yates et al., 1989).

Preparation of N-Acyllolines. Each appropriate acid chloride (10 mmol) was added separately to a solution of loline (1.54 g, 10 mmol) in 10 mL of $CHCl_3$ and stirred at 25 °C for 48 h. The reaction mixture and a 20-mL aqueous wash of the reaction flask were then poured into a 100-mL beaker, and the pH of the aqueous phase was adjusted to 10 with NaOH. The $CHCl_3$ phase was collected, and the alkaline aqueous solution was extracted three more times with 20 mL of $CHCl_3$. The $CHCl_3$ extracts were combined and dried over anhydrous Na_2SO_4, and then $CHCl_3$ was removed by evaporation, in vacuo, to afford the desired product. The purity of each loline derivative (Table I) was determined by capillary GC using conditions described previously for the analysis of loline derivatives that occur naturally (Petroski et al., 1989). *N*-Benzoylloline was recrystallized from carbon tetrachloride–cyclohexane. *N*-Octanoylloline, *N*-decanoylloline, *N*-dodecanoylloline, *N*-tetradecanoylloline, *N*-hexadecanoylloline, and *N*-(*p*-*n*-hexylbenzoyl)loline were each chromatographed on silica gel (10 g) gravity columns using $CHCl_3$ to elute impurities, followed by $CHCl_3$–MeOH (80:20) to elute the purified product. The structure of each compound was confirmed by IR, NMR, and GC/MS. All loline derivatives were >99% single components as determined by capillary GC.

Test Compound Solutions. A 5 mM stock solution of loline and several loline derivatives were prepared by dissolving the appropriate weight in $CHCl_3$ and diluting with hexane to 1:99 (v/v) $CHCl_3$–hexane. A dilution series was made from the stock solution of each test compound with the same solvent composition to give a dosage series that ranged in concentration from 0.05 to 5 mM.

Seed Pretreatment. Alfalfa seeds were imbibed for 4 h on two layers of 9-cm-diameter Whatman No. 1 filter paper that was saturated with water. The preimbibition treatment was necessary to select seeds permeable to water, so that failure to germinate could more accurately be attributed to a test compound effect. Annual ryegrass seeds were not preimbibed because the seeds exhibited no hardseededness.

Bioassay. The bioassay used to determine phytotoxicity of each exogenously applied loline derivative was similar to that described previously (Dornbos and Spencer, 1990). Bactoagar (4.4 g; Difco Laboratory, Detroit, MI) was dissolved in water (400 mL) by autoclaving at 121 °C for 20 min and then diluted with an additional 400 mL of water. One milliliter of the resulting agar solution was pipetted into each 4-mL glass vial and allowed to harden. Test compounds, contained in 1 mL of sol-

vent and solvent controls, were carefully layered on top of the hardened agar, and then the solvent was allowed to passively evaporate in a ventilated hood.

Five alfalfa or annual ryegrass seeds were placed on the agar in each vial. Alfalfa, a dicot, and annual ryegrass, a monocot, were selected as test species to indicate any broad spectrum inhibition by the alkaloids tested. The vials were capped (small holes were punched in the vial caps) and placed in a growth chamber in darkness at a constant temperature of 30 °C. After 3 days, seeds with a radicle protruding through the seed coat were counted as germinated. The length of each seedling (root plus shoot) was measured to the nearest millimeter.

All experiments were conducted as completely randomized designs with four or six replications. Treatment effects were considered significant when $P \leq 0.05$ as determined by the General Linear Model (Standard Anova) procedure of the Statistical Analysis System (SAS Institute, Inc., Box 8000, Cary, NC 27511). A regression equation describing the linear reduction of germination percentage and seedling length by each compound was calculated if the germination percentage or seedling length was reduced significantly by increased doses. Doses that caused 50% inhibition (ID_{50}) in germination and in seedling growth were then calculated from each regression equation.

RESULTS AND DISCUSSION

Endogenous loline alkaloids were not detected in annual ryegrass seed used for the germination tests. Mean germination percentages and seedling lengths of untreated seeds were 93% and 2.9 cm for alfalfa and 86% and 2.7 cm for annual ryegrass, respectively. Inhibitory doses (ID_{50}) were calculated from statistically significant ($P \leq 0.05$) regression equations that described the dose response of each exogenously applied loline derivative (Table I).

Norloline and *N*-formylnorloline exhibited no phytotoxicity against either test species at doses up to 1×10^{-6} mol/seed (data not shown). *N*-Acetylloline and *N*-methylloline also exhibited no phytotoxicity at doses between 0.1×10^{-8} and 100×10^{-8} mol/seed, but *N*-formylloline inhibited germination of annual ryegrass; the ID_{50} value was 77×10^{-8} mol/seed (Table I).

Eight synthetic *N*-acyl derivatives of loline were significantly phytotoxic against seedling growth of alfalfa and annual ryegrass (Table I). In addition to several straight-chain derivatives, the cinnamoyl and *N*-(*p*-*n*-hexylbenzoyl) derivatives were also phytotoxic against both species. ID_{50} values for seedling length varied upward from 5×10^{-8} mol/seed, and seedling length was a more sensitive indicator of phytotoxicity than was germination percentage.

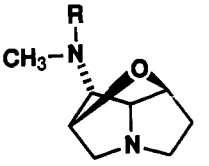
An interesting structure–activity relationship was evident when chain length of the acyl group for the loline derivatives and seedling length inhibition for these derivatives were compared (Figure 1). Maximum phytotoxicity was observed with loline derivatives having a straight *N*-acyl chain length between 9 and 14 carbons. The derivatives exhibited progressively less activity if the carbon chain length was longer or shorter.

A similar phenomenon was noted previously in the nornicotine series as nornicotine, *N'*-formylnornicotine, and *N'*-acetylnornicotine were all reported as noninhibitory to tobacco seed germination (Matsuzaki et al., 1988). Among those *N'*-acylnornicotines without hydroxy groups, *N'*-hexanoylnornicotine, *N'*-octanoylnornicotine, and *N'*-decanoylnornicotine had high inhibiting activity against tobacco seed germination.

With both loline and nornicotine, the parent compounds (N-unacylated derivatives) and derivatives having relatively short *N*-acyl chains demonstrated little or no phytotoxicity, while derivatives having *N*-acyl chain lengths of 6–10 carbons (nornicotines) or 9–14 carbons (lolines) demonstrated high levels of phytotoxicity. The intermediate chain length *N*-acyl moieties may contribute directly to phytotoxicity of these alkaloid derivatives or indirectly by facilitating their absorption (uptake) by seeds and seedlings. In our bioassay, loline derivatives were exogenously applied to the seeds and seedlings. An intermediate chain length *N*-acyl function may make the loline derivative sufficiently lipophilic to move across cell membranes into the developing seedlings.

Phytotoxicity of the loline derivatives was tested against a broad-leaved plant and against a grass to detect any general selectivity. To facilitate comparison of seeds from different plant species that vary in mass, dose was calculated in moles per gram of seed dry weight. The ID_{50}

Table I. Inhibition of Alfalfa and Annual Ryegrass Seed Germination and Seedling Growth by Loline and Selected Derivatives[a]



compd[b]	R	germination %, ID_{50}		seedling length,[c] ID_{50}	
		alfalfa	annual ryegrass	alfalfa	annual ryegrass
1	H	>	>	>	>
2	formyl	>	77	>	>
3	acetyl	>	>	>	>
4	propionyl	>	>	>	>
5	butyryl	>	90	>	54
6	*n*-hexanoyl	>	147	66	15
7	*n*-octanoyl	101	42	19	19
8	*n*-nonanyl	47	34	12	6
9	*n*-decanoyl	43	11	16	7
10	*n*-dodecanoyl	>	6	7	5
11	*n*-tetradecanoyl	51	47	8	6
12	*n*-hexadecanoyl	>	>	27	30
13	methyl	>	124	>	>
14	isobutyryl	>	144	>	>
15	cyclopropanecarboxyl	>	>	91	>
16	benzoyl	>	>	50	71
17	cinnamoyl	48	97	35	49
18	*p*-*n*-hexylbenzoyl	86	6	8	5
19	3-methyl-2-butenoyl	>	>	107	>

[a] Values are expressed as the dose ($\times 10^{-8}$ mol/seed) that gives 50% inhibition of seed germination or seedling length (ID_{50}). > indicates that a toxic dose may exist at doses greater than those used in our bioassay. As such higher doses, the compound would not be considered useful as a potential herbicide. [b] Compounds 4–12 and 14–19 are new compounds. The structure of each compound was confirmed by IR, NMR, and GC/MS. Supplemental spectral data and statistical data are available from the authors upon request. [c] The length of each seedling (root plus shoot) was measured to the nearest millimeter.

Table II. Alfalfa and Annual Ryegrass Seedling Growth Inhibition (ID_{50}) by *N*-Dodecanoylloline and Known Phytotoxins

compd	seedling growth[a] ID_{50}, $\times 10^{-8}$ mol/seed	
	alfalfa	annual ryegrass
N-dodecanoylloline[b]	7	5
juglone	4	6
plumbagin	4	6
cinmethylin		1[c]

[a] The length of each seedling (root plus shoot) was measured to the nearest millimeter. [b] Maximum activity was observed in loline derivatives having a straight *N*-acyl chain length between 6 and 14 carbons. A representative, *N*-dodecanoylloline, was chosen for this comparison. [c] Cinmethylin caused nearly a 100% reduction in seedling length at 1×10^{-8} mol/seed, the lowest dose tested.

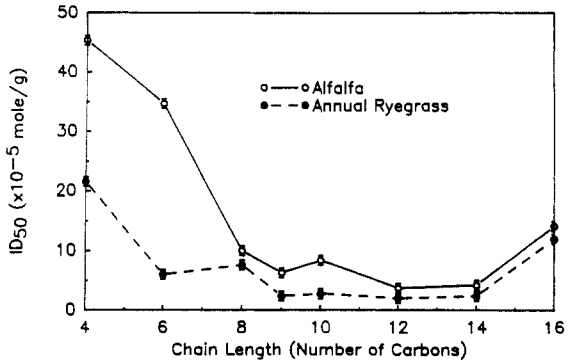


Figure 1. Loline derivatives: Structure–activity relationship between straight *N*-acyl chain length and seedling length inhibition. (—) $L.S.D._{0.05}$.

of (*p*-*n*-hexylbenzoyl)loline against germination of annual ryegrass was 2.4×10^{-5} mol/g, and that of alfalfa was 43.3×10^{-5} mol/g. This compound is nonselectively phytotoxic to alfalfa and annual ryegrass seedling length exhibiting ID_{50} values of 2.0×10^{-5} and 4.2×10^{-5} mol/g of seed, respectively. The seedling growth ID_{50} values for *N*-octanoylloline, *N*-nonanoylloline, *N*-decanoylloline, *N*-dodecanoylloline, *N*-tetradecanoylloline, and *N*-(*p*-hexylbenzoyl)loline were generally 2-fold lower for annual ryegrass than for alfalfa. *N*-(Cyclopropanecarboxoyl)-loline was slightly selective against alfalfa seedling length. Compounds that were phytotoxic, but nonselective, included *N*-(3-methyl-2-butenoyl)loline (seedling growth inhibition) and *N*-cinnamoylloline (both seed germination and seedling growth inhibition).

Phytotoxic activity against seedling length of a representative straight-chain *N*-acylloline derivative, *N*-dodecanoylloline, was compared with the activity of two known allelochemicals and a commercial grass herbicide (Table II). The phytotoxicity of *N*-dodecanoylloline to both alfalfa and annual ryegrass was comparable to that of juglone and plumbagin (Dornbos and Spencer, 1989). Cinmethylin demonstrated little activity against alfalfa in bioassay but, at 1×10^{-9} mol/seed, it was 100% effective against annual ryegrass seedlings. Cinmethylin was more toxic than any of the lolines tested.

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