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Allelochemicals and Activities in a Replanted Chinese Fir (*Cunninghamia lanceolata* (Lamb.) Hook) Tree Ecosystem

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Autotoxicity is a major reason for replant problems in managed tree ecosystems. Studies have related phenolics-based allelochemicals to autotoxicity. We selected a 20-year-old replanted Chinese fir [*Cunninghamia lancealata* (Lamb.) Hook] tree ecosystem to isolate, identify, determine the biological activity of, and quantify soil phytotoxins. Eight common phenolics (coumarin, vanillin, isovanillin, and *p*-hydroxybenzoic, vanillic, benzoic, cinnamic, and ferulic acids), friedelin, and a novel cyclic dipeptide (6-hydroxy-1,3-dimethyl-8-nonadecyl-[1,4]-diazocane-2,5-diketone) were obtained by using the bioassay-guided isolation technique from toxic soil of the replanted Chinese fir tree ecosystem. Chemical structures were determined by spectroscopic means, including 2D-NMR (COSY, HMQC, HMBC, and NOESY) experiments. High concentrations of soil phenolics and friedelin were observed in the natural evergreen broadleaf forest (CK) rather than in the Chinese fir tree ecosystem. The phenolics and friedelin were not phytotoxic to Chinese fir trees. However, the cyclic dipeptide inhibited Chinese fir growth at soil concentrations determined in the replanted Chinese fir tree ecosystem. There was a significantly higher soil concentration of cyclic dipeptide in the replanted Chinese fir tree ecosystem than in a fresh Chinese fir tree ecosystem. The results suggest that phenolics and friedelin are not key allelochemicals since they are weakly phytotoxic and are detected in low concentrations in the replanted Chinese fir tree ecosystem, while cyclic dipeptide is a highly active allelochemical with a phytotoxic effect that limits offspring growth in the replanted Chinese fir tree ecosystem. The discovery of cyclic dipeptide, as well as a further understanding of its potential action mechanism in the replanted Chinese fir tree ecosystem, may contribute to solving the replant problems in managed tree ecosystems.

KEYWORDS: Autotoxicity; Chinese fir; cyclic dipeptide; phenolic acid; replant problem; soil phytotoxin

INTRODUCTION

Autotoxicity is a type of intraspecific allelopathy that a plant exhibits to inhibit the growth of other plants of its own species. This process involves the release of allelochemicals or phytotoxins into the environment (*1*). Autotoxicity is a major reason why natural forests and managed tree ecosystems fail to regenerate, causing replant problems (*2*−*4*). To understand chemically mediated allelopathy and autotoxicity in managed tree ecosystems, identification of allelochemicals or phytotoxins and their potential action mechanisms in a few typical tree ecosystems with replant problems is required.

Chinese fir [*Cunninghamia lancealata* (Lamb.) Hook] trees are a native species that has been widely planted in mountainous tropical and subtropical areas in China for more than 1000 years (*5*). Today, Chinese fir is commercially planted in south China and has become an important economic commodity for industrial wood production. However, the establishment and productivity decline of replanted Chinese fir tree ecosystems has remained a significant problem in China (*6*). An increasing number of studies have shown that autotoxicity plays an important role in the replant problem from Chinese fir tree ecosystems (*7*−*12*). Extracts of soil collected in replanted Chinese fir tree ecosystems have been shown to significantly reduce the growth of Chinese fir seedlings, inhibit soil nonpathogenic fungi growth, and lower soil respiration and net soil nitrogen mineralization (*7, 8*). Several phenolic acids including *p*-hydroxybenzoic, gallic, coumaric, ferulic, vannilic, and protocatechuic acids were regarded as allelochemicals to be responsible for the auto-

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toxicity in replanted Chinese fir tree systems (*9*−*12*). However, these phenolic acids were extracted and identified from Chinese fir tissues rather than soil samples. In addition, the inhibitory experiments on the growth of Chinese fir seedlings were completed with authentic phenolic acids at arbitrary concentrations rather than phenolics identified from soil at actual quantities in replanted Chinese fir tree systems.

Phenolic acids are ubiquitous compounds in almost all higher plants and forest soil. Therefore, it is expected that Chinese fir tissues and soil contain these phenolic acids. Although phenolic acids were identified as model allelopathic agents in an attempt to explain allelopathy or autotoxicity in natural forests and managed tree ecosystems (*13*−*16*), such phenolics-based allelochemicals have been argued due to the effects of known phenolic acids with arbitrary concentrations and the neglect of other potent phytotoxins in certain natural forests and managed tree ecosystems (*17*). Therefore, a complete description of using a bioassay-guided isolation technique and subsequent structure determination of allelochemicals would be very important to build strong evidence for autotoxicity and allelopathy in natural forests and managed tree ecosystems. Accordingly, phenolic acids identified from Chinese fir tissue are unlikely to explain the autotoxicity of replanted Chinese fir tree systems, and replanted Chinese fir tree systems should contain other allelochemicals awaiting detection and identification. To address this lack of knowledge, the present study was to undertake the isolation, identification, determination of the biological activity, and quantification of soil phytotoxins in a 20-year-old replanted Chinese fir tree system, with an attempt to provide a convincing example of autotoxicity and complete the identification of a highly phytotoxic allelochemical in a managed tree ecosystem with a replant problem.

MATERIALS AND METHODS

Instruments. High-resolution mass spectrometry experiments were carried out with IonSpec Ultima FTMS and FABMS instruments with a VG-ZAB-HS. IR spectra were recorded on a Bruker FT-IR infrared spectrophotometer. Optical rotation was measured with a Perkin-Elmer model-241 MC polarimeter. The NMR spectra were measured with Bruker AM-300 NMR and Bruker 600 NMR spectrometers. All chemical shifts are reported as δ values relative to the peak for TMS.

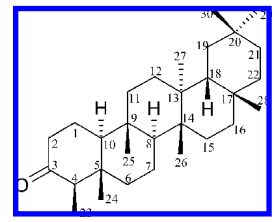
Soil Sampling Sites. Soil was collected from the Huitong Experimental Station of Forest Ecology, Chinese Academy of Science (Hunan Province, China; 26°40′−27°09′ N, 109°26′−110°08′ E). The experimental station is in a subtropical climate zone, with a mean annual temperature of 16.5 °C and a mean annual rainfall of 1250 mm. The evergreen broadleaf forest is the natural type of this area. The dominant tree species are *Castanopsis hystrix*, *Cyclobalanopsis glauca*, *Machilus pauhoi*, *Liquidambar formosana*, and *Juglans cathayensis* with shrub layer species of *Loropetalum chinensis*, *Eurya chinensis*, *Lindera glauca*, *Camellia oleosa*, *Camellia salicifolia*, *Rhus semialata*, and *Indocalamus tessellates*. The herbaceous species are rarely observed in this forest system.

Chinese fir tree ecosystems were first built after clear-cutting and slash-burn practices of the natural evergreen broadleaf trees in 1954. Replanting of Chinese fir trees took place in 1986. Fresh Chinese fir tree ecosystems were built after clear-cutting of the natural evergreen broadleaf forests in 1983. From 2005 to 2007, the soil was randomly sampled at 0−20 cm depth from the three tree ecosystems described above. There were no significant differences in pH, organic matter content, and nutrient status among soil samples collected from the three tree ecosystems (pH 4.16 ± 0.04, organic matter 41.10 ± 3.48 g kg^{-1}, total N 1.93 ± 0.21 g kg^{-1}, NH_4 N 8.33 ± 1.50 mg kg^{-1}, NO_3 N 3.26 ± 0.26 mg kg^{-1}, total P 0.16 ± 0.04 g kg^{-1}, available P 0.76 ± 0.09 mg kg^{-1}, total K 15.44 ± 2.27 g kg^{-1}, available K 46.60 ± 8.12 mg kg^{-1}). All soil samples were sieved (2 mm mesh) to remove plant residues and homogenized for a series of experiments as described below.

Soil Phytotoxicity. A randomized complete block design was used with 12 testing blocks, 1 control block in the experiment, and 6 replications per block. One-year-old Chinese fir seedlings were transplanted into 30 × 40 cm pots (one seedling per pot) with 15 kg of the soil collected from the three tree systems described above. All pots were placed in a greenhouse and watered by tap as needed. The pots were randomized once a month in the greenhouse. After one year, the height, basal diameter, and above-ground and underground biomasses of the Chinese fir seedlings in each pot were measured and recorded.

Isolation and Identification of Soil Allelochemicals. Soil allelochemicals in the replanted Chinese fir tree ecosystem were isolated with the bioassay-guided method (*18, 19*). A total of 1000 kg of soil was collected from the replanted Chinese fir tree system described above. The soil samples (20 kg × 50) were each extracted with 75% aqueous EtOH at room temperature, shook for 48 h, and filtered. The combined filtrates were concentrated in vacuo, and the concentrated extract was partitioned three times with EtOAc and then *n*-ButOH. Both EtOAc and *n*-ButOH phases were subsequently evaporated in vacuo, and their residues were subjected to silica gel CC. The EtOAc extract was eluted stepwise with a mixture of petroleum ether/EtOAc/MeOH (100:0:0, 98:2:0, 80:20:0, 50:50:0, and 0:50:50, v/v/v) to yield five fractions. Among them, three fractions (1, 2, 3) showed inhibitory activity using a lettuce (*Lactuca sativa* L.) bioassay. Fraction 1 eluted with petroleum ether/EtOAc/MeOH (98:2:0, v/v/v) was recrystallized with *n*-hexane/EtOAc to obtain compound **1**. Fraction 2 eluted with petroleum ether/EtOAc/MeOH (80:20:0, v/v/v) was further purified by silica gel CC with *n*-hexane/EtOAc (4:1, v/v) to give compound **2**. Fraction 3 eluted with petroleum ether/EtOAc/MeOH (0:50:50, v/v/v) was subjected to silica gel CC with EtOAc and yielded coumarin, vanillin, and isovanillin. The *n*-ButOH extract described above was eluted with EtOAc containing increasing amounts of MeOH to yield *p*-hydroxybenzoic, vanillic, benzoic, cinnamic, and ferulic acids.

*Data for Friedelin (***1***).* Colorless needle crystals (*n*-hexane/EtOAc). Mp: 263−264 °C. $[\alpha]_D^{20}$: −27.8° ($CHCl_3$, *c* 0.009). EI-MS ($C_{30}H_{50}O$): *m*/*z* 426 (M^+, 9.2), 411 (3.2), 341 (3.3), 302 (9.7), 273 (24.1), 232 (17.9), 218 (24.6), 205 (32.5), 163 (42.0). ^{1}H NMR (400 MHz, $CDCl_3$): 0.73 (3H, s, CH_3-24), 0.87 (3H, s, CH_3-25), 0.89 (3H, d, J = 6.9 Hz, CH_3-23), 0.95 (3H, s, CH_3-29), 1.00 (3H, s, CH_3-30), 1.01 (3H, s, CH_3-26), 1.05 (3H, s, CH_3-27), 1.18 (3H, s, CH_3-28), 1.96 (2H, m, H-1), 2.31 (2H, m, H-2), 2.25 (1H, d, J = 10.0 Hz, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): 22.3 (C-1), 41.5 (C-2), 213.2 (C-3), 58.2 (C-4), 42.1 (C-5), 41.3 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.5 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.7 (C-15), 36.0 (C-16), 30.0 (C-17), 42.8 (C-18), 35.3 (C-19), 28.2 (C-20), 32.4 (C-21), 39.2 (C-22), 6.8 (C-23), 14.7 (C-24), 17.9 (C-25), 20.2 (C-26), 18.6 (C-27), 32.1 (C-28), 31.8 (C-29), 35.0 (C-30).



*Data for 6-Hydroxy-1,3-dimethyl-8-nonadecyl-[1,4]-diazocane-2,5-diketone (***2***).* White amorphous solid. Mp: 136−138 °C. $[\alpha]_D^{20}$: −3.3° ($CHCl_2$, *c* 0.009). The molecular formula was determined to be $C_{27}H_{52}O_3N_2$ by HR-MS and accurate mass spectrometry (HR-ESI, *m*/*z* 453.1679 $[M + H]^+$). IR (ν_{max} (KBr), cm^{-1}): 3427 (OH, NH), 2918, 2955, 2853 (C−H), 1728 (C═O), 1641 (C═O). NMR data are listed in **Table 1**.

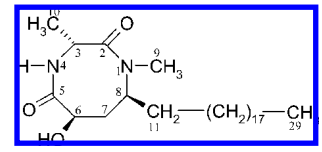


Table 1. NMR Data of Cyclic Dipeptide **2**[a]

position	δ_H	δ_C	key HMBC
1			
2		173.5	C-2/H-3, H-10, H-8
3	4.62 (1H, m)	47.8	
4	6.30 (1H, J = 7.2 Hz, NH)		
5		175.3	C-5/H-3, H-6
6	3.82 (1H, m), 3.08 (1H, d, J = 1.8 Hz, OH)	72.3	
7	1.73 (1H, m), 1.69 (1H, m)	34.0	
8	2.17 (1H, m)	51.8	
9	3.77 (3H, m)	52.3	
10	1.44 (3H, d, J = 7.2 Hz)	18.4	
11	1.39 (2H, q, J = 7.2 Hz)	31.9	
	1.27 (34H, br s, nCH_2)	29.7−29.3, 27.8, 26.1, 25.9, 22.6	
29	0.89 (t, J = 7.2 Hz,3H)	14.0	

[a] $CDC1_3$; ^{13}C NMR, 150 MHz; ^1H NMR, 600 MHz.

Quantification of Soil Allelochemicals. Each soil sample collected from the three tree ecosystems described above was divided into three groups, respectively. One group was for quantitative analysis of phenolic compounds. The other two groups were for quantitative analysis of nonphenolic compounds **1** and **2**.

For quantitative analysis of phenolic compounds, 20 g of soil was extracted with 50 mL of 1 N NaOH and shaken for 12 h at room temperature (*20*). The filtrate was adjusted to pH 2.5 using HCl followed by centrifugation at 1200*g* for 20 min. The phenolics were extracted from the acidified solution with EtOAc and redissolved in EtOH before being analyzed by HPLC (*21*). The quantitative analysis was carried out with an Agilent 1100 HPLC instrument equipped with a C_{18} reversed-phase column (Agilent ZORBAX SB-C_{18}, 4.6 × 150 mm, 5 μm). The optimum efficiencies of separation were obtained using linear gradients of a mobile phase of acetonitrile: 0.5% acetic acid starting from 10:90 and changing to 36:64 in 30 min. The hold time was 2 min. The flow rate was 1.0 mL min^{-1} at a 25 °C column temperature. The injection volume was 10 μL. Detection was performed by using a diode array detector at 274 nm. The phenolics in the soil samples were each quantified by interpolating the peak areas on the HPLC chromatograms to a standard curve constructed by the peak area of authentic phenolic compounds.

Quantitative analysis of **1** was conducted as described by Hanisch et al. (*22*) with some modifications. Briefly, 10 g of soil were ultrasonically extracted with MeOH/$CHCl_2$ (1:3, v/v; 150 mL), MeOH/$CHCl_2$ (1:9, v/v; 100 mL), and finally $CHCl_2$ (2 × 100 mL) until the extracts were colorless. The extracts were evaporated to dryness, and the residues were dissolved with 1 mL of $CHCl_3$. The samples were subjected to GC for quantitative analysis. GC analysis was performed on a Hewlett-Packard 5890 instrument with a split injector at 280 °C and a flame ionization detector (FID) at 300 °C. The injection volume was 1 μL, and the split ratio was 1:10. Nitrogen was employed as the carrier gas, and the flow rate was 1 mL min^{-1}. The HP-5 capillary column was 30 m × 0.32 mm with a 0.25 μm film of a cross-linked methyl (95%) phenyl (5%) polysiloxane stationary phase. The column temperature was programmed from 200 °C at 25 °C min^{-1} to 300 °C and maintained for 30 min. Compound **1** in the soil samples was quantified by interpolating the peak area on the GC chromatograms to a standard curve constructed by the peak area of pure **1**.

For quantitative analysis of **2**, 50 g of soil was extracted with MeOH/$CHCl_2$ (3:1, v/v; 3 × 100 mL). The extracts were evaporated, and the residues were dissolved with 1 mL of MeOH. Through a 0.25 μm nylon syringe, the filtrate was quantitatively analyzed with an Agilent 1100 HPLC instrument equipped with a C_{18} reversed-phase column (Agilent ZORBAX SB-C_{18}, 4.6 × 150 mm, 5 μm). The mobile phase was acetonitrile with 0.1% trifluoroacetic acid, eluted at a flow rate of 1 mL·min^{-1} and detected at 200 nm (diode array detector). The injection volume was 5 μL at a 60 °C column temperature. Compound **2** in the soil samples was quantified by interpolating the peak area on the HPLC chromatograms to a standard curve constructed by the peak area of pure **2** isolated from Chinese fir tree systems.

The average recoveries of the compounds described above added into the soil were 60.0% (**1**), 99.1% (**2**), 75.5% (coumarin), 68.5% (vanillin), 70.5% (isovanillin), 80.9% (*p*-hydroxybenzoic acid), 80.0% (vanillic acid), 90.8% (benzoic acid), 80.2% (cinnamic acid), and 65.4% (ferulic acid), which were used to correct their determined concentrations.

Bioassays. The effect of the identified soil allelochemicals described above on Chinese fir growth was determined by literature methods (*10, 11*) with modifications. Chinese fir seeds were sterilized using a 3% hydrogen peroxide solution for 30 min and then rinsed with distilled water. Sterile seeds were soaked in distilled water at 25 °C for 4 days. Twenty per-germinated seeds were uniformly placed in a 9 cm Petri dish with one layer of filter paper. The treated dishes were incubated with individual allelochemicals or a mixture of identified allelochemicals (3 mL) at soil concentrations determined in the replanted Chinese fir tree ecosystem (**Table 2**). In addition, **1** and **2** were applied at more concentrations ranging from 3 to 40 nmol·mL^{-1} for their dose−response curves and inhibition thresholds (*23*). The control dishes received 3 mL of distilled water only.

All dishes were placed in a controlled environmental chamber (3 m^3) with approximately 350 (μmol/m^2)/s light intensity at 25−28 °C and 70% relative humidity. The dishes were randomized once a day. After 15 days, the radicle length of the Chinese fir in the treated and control dishes was measured and the inhibition (%) at different concentrations tested was obtained from the comparison of the radicle lengths between the treated and control dishes. The same manipulation was conducted three times for each determination under identical conditions.

RESULTS AND DISCUSSION

Pot experiments showed that the soil of the planted Chinese fir trees significantly reduced the height, basal diameter, and biomass of Chinese fir seedlings when compared to the natural evergreen broadleaf forest soil. In particular, there was a higher autoinhibition in the soil of the replanted Chinese fir trees than in the fresh Chinese fir tree soil (**Table 3**). These results indicated that the soil of the replanted Chinese fir trees was toxic, limiting offspring, which agreed with previous studies that extracts of soil collected from replanted Chinese fir tree ecosystems significantly inhibited the growth of Chinese fir seedlings (*7, 9−11*). This implied that the soil of the replanted Chinese fir trees contained phytotoxins that inhibit the growth of Chinese fir seedlings.

To completely identify soil phytotoxins (allelochemicals), large quantities of toxic soil were collected from a 20-year-old replanted Chinese fir tree ecosystem. The advantage of using 1000 kg of soil is that the combined extracts could be enough for further isolation by using the bioassay-guided method (*18, 19*). Subsequently, eight phenolic compounds including coumarin, vanillin, isovanillin, *p*-hydroxybenzoic acid, vanillic acid, benzoic acid, cinnamic acid, and ferulic acid were easily obtained and identified. Besides these common phenolics, two other compounds, **1** and **2**, were isolated from replanted Chinese fir plantation soil. Their structures were identified by spectroscopic means described below.

Compound **1** was identical to friedelin by comparison of its IR and NMR data with the literature (*24*). Friedelin is widely distributed in many kinds of plant tissues (*25−27*). However, this is the first report of isolation and identification of friedelin from forest soil. HR-ESI-MS of **2** exhibited a pseudomolecular ion peak, $[M + H]^+$, at *m/z* 453.1679, suggesting a molecular formula of $C_{27}H_{52}O_3N_2$, and the unsaturated degree was 3. The IR spectra showed the presence of OH and NH group (3427

Table 2. Concentrations (nmol · g^{-1} of soil) of Identified Allelochemicals in the Three Tree Ecosystems Tested[a]

compound	evergreen broadleaf forest ecosystem	fresh Chinese fir tree ecosystem	replanted Chinese fir tree ecosystem
p-hydroxybenzoic acid	23.35 ± 5.65 a	50.56 ± 5.00 b	51.11 ± 12.72 b
vanillin	16.14 ± 0.42 ab	15.47 ± 1.55 a	19.47 ± 2.54 b
vanillic acid	28.66 ± 3.28 a	27.61 ± 3.25 a	24.13 ± 3.64 a
ferulic acid	12.95 ± 2.69 a	6.30 ± 1.39 b	4.72 ± 1.9 b
benzoic acid	43.82 ± 9.24 a	19.23 ± 2.97 b	17.04 ± 4.66 b
iso-vanillin	1.89 ± 0.11 a	2.37 ± 0.20 b	2.18 ± 0.24 ab
coumarin	2.76 ± 0.63 a	0.95 ± 0.40 b	0.56 ± 0.31 b
cinnamic acid	0.94 ± 0.08 a	0.72 ± 0.16 a	0.46 ± 0.12 b
total of eight phenolics	130.51 ± 20.03 a	123.22 ± 10.31 a	119.68 ± 20.25 a
friedelin (**1**)	43.69 ± 16.80 a	3.83 ± 0.32 b	3.14 ± 0.74 b
cyclic dipeptide **2**	ND	5.56 ± 0.38 ab	6.15 ± 0.75 b

[a] ND = not detected. Means ± SE (n = 12) in a row with different letters indicate significant differences at p = 0.05, ANOVA with Duncan's multiple-range test.

Table 3. Effects of Soil Collected from the Three Tree Ecosystems Tested on the Growth of Chinese Fir Seedlings[a]

			biomass (g of dry weight)	
tree ecosystem	seedling height (cm)	basal diameter (cm)	above ground	underground
evergreen broadleaf forest ecosystem	50.79 ± 8.78 a	1.13 ± 0.15 a	41.91 ± 11.42 a	22.19 ± 7.24 a
fresh Chinese fir tree ecosystem	39.58 ± 3.08 b	0.92 ± 0.09 b	22.35 ± 3.52 b	15.02 ± 1.14 b
replanted Chinese fir tree ecosystem	37.88 ± 6.64 b	0.81 ± 0.10 c	18.80 ± 6.17 c	12.56 ± 0.87 c

[a] Means ± SE (n = 12) in a column with different letters indicate significant differences at p = 0.05, ANOVA with Duncan's multiple-range test.

cm^{-1}, wide peak) as well as two carbonyl groups (1728 and 1641 cm^{-1}). In the DEPT experiment, the carbon resonances were assigned to 3 methyls, 19 methylenes, 3 methines, and 2 sp^2 carbonyl group carbons (δ_C 173.5 and 175.3). The results showed a cyclic system in **2**. The ^1H NMR spectrum indicated the peptide nature of **2** by showing signals for exchangeable protons at δ_H 6.30 (1H, J = 7.2 Hz, NH) and δ_H 3.08 (1H, d, J = 1.8 Hz, OH) and an *N*-Me singlet at δ 3.77 (3H, m). The carbonyl group carbon signals at δ_C 173.5 and 175.3 supported this information. Additionally, the signals at δ_H1.27 (br s, nCH_2) revealed the presence of a long chain. The COSY experiment clearly exhibited two spin systems. For the first system, the proton signal at δ_H 4.86 (1H, m, H-3) showed $^1H-^1H$ correlations to methyl resonances at δ_H 1.44 (3H, d) and 6.30 (1H, J = 7.2 Hz, NH). In the second spin system, an oxygen-bearing methine proton at δ_H 3.82 (1H, m) was correlated with a methylene with signals at δ_H 1.73 (1H, m) and 1.69 (1H, m) which showed correlation with δ_H 2.17 (1H, m) corresponding to a nitrogen-bearing carbon, δ_C 51.8. The structure of **2** was finally determined from the HMBC experiment, which allowed the partial structures to be linked together. The attachment of the methine C-3 to the carbonyl group C-2 was shown through ^2J correlations of H-3 and ^3J correlations of H-10 (δ_H 1.44, d, J = 7.2 Hz, 3H) to C-2 and δ_H 6.30 (1H, NH) to C-2. The oxygen-bearing methine (δ_C 72.3, δ_H 3.82) was attached to C-5 through ^2J correlation of H-6 (δ_H 3.82) to C-5. The remaining nitrogen-bearing machine was attached to C-2 and gave the final structure. All of the ^1H and ^{13}C NMR signals were unambiguously assigned on the basis of COSY and HMQC and HMBC experiments (**Table 1**).

The relative stereochemistry of **2** was determined by a NOESY experiment; the NOE correlations from H-6, δ_H 3.82, to H-8, δ_H 2.17, indicated that these protons were on the same face of the eight-membered ring and were assigned as the α-protons. On the other hand, H-10 showed no NOE responses with H-8 and H-6. Compound **2** was subsequently identified as a novel cyclic dipeptide, 6-hydroxy-1,3-dimethyl-8-nonadecyl-[1,4]-diazocane-2,5-diketone. Although many dipeptides have been isolated and identified from plant and microbial species (*28, 29*), to the best of our knowledge, this novel cyclic dipeptide has never been reported in the literature.

Quantitative analysis showed that soil samples collected from the three tree ecosystems contained eight phenolic compounds and friedelin (**1**) identified from the replanted Chinese fir tree ecosystem, but there were significant differences in their concentrations among the soil samples (**Table 2**). Unexpectedly, high concentrations of soil phenolics, except *p*-hydroxybenzoic acid, were observed in the natural evergreen broadleaf forest (CK) rather than in the Chinese fir tree ecosystems. Similar results were observed for the soil concentrations of friedelin. The soil concentration of friedelin in the natural evergreen broadleaf forest was more than 10-fold higher than those in the Chinese fir tree ecosystems. Soil of the planted Chinese fir trees contained cyclic dipeptide **2**. In particular, there was a significant higher soil concentration of cyclic dipeptide in the replanted Chinese fir tree ecosystem than in the fresh Chinese fir tree ecosystem. However, cyclic dipeptide was not detected in the natural evergreen broadleaf forest soil (**Table 2**).

Bioassays showed that individual phenolics and mixtures of the eight phenolics at their soil concentrations determined in the replanted Chinese fir tree ecosystem stimulated rather than inhibited the growth of Chinese fir. Similarly, friedelin and its mixture with the eight phenolics stimulated the growth of Chinese fir at the determined soil concentrations. However, cyclic dipeptide and its mixture with phenolics and/or friedelin could significantly inhibit the growth of Chinese fir at the determined soil concentrations (**Figure 1**). It is well-known that the effect of phenolic acids on plant growth is dose-dependent and their plant growth inhibition or stimulation depends on the concentration tested (*30*). The soil concentration of phenolics in the replanted Chinese fir tree ecosystem is not enough to start inhibition. So far, there has been no report on the effect of friedelin and cyclic dipeptide on plant growth. Dose−response curves showed that friedelin at low concentrations stimulated the growth of Chinese fir but growth inhibition was observed at the high concentrations tested. The inhibition threshold, the lowest concentration required to have an observable inhibition (*23*), was 8.6 nmol g^{-1}, while cyclic dipeptide always inhibited the growth of Chinese fir, even at low concentrations. Cyclic dipeptide had a much higher inhibitory activity and lower inhibition threshold (1.2 nmol g^{-1}) on Chinese fir than friedelin (**Figure 2**).

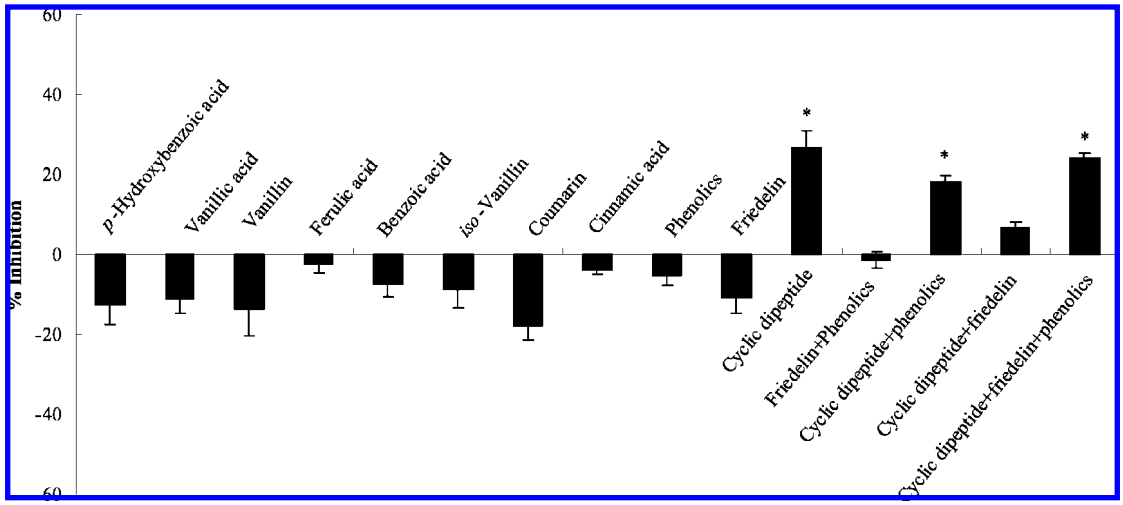


Figure 1. Activity of individual phenolics, friedelin, and cyclic dipeptide and their mixtures on Chinese fir growth. The concentrations tested are the same as the concentrations determined in the replanted Chinese fir tree ecosystem soil (**Table 2**). Means ± SE from independent experiments with three replicates for each determination are shown. The asterisk on the black bar indicates significant differences at $p = 0.05$, ANOVA with Duncan's multiple-range test.

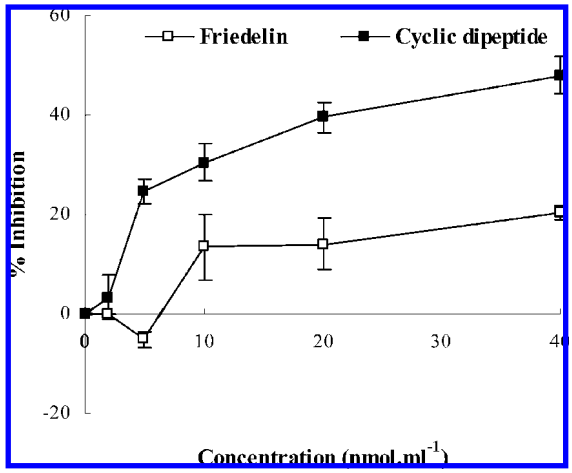


Figure 2. Dose−response curves of friedelin and cyclic dipeptide on Chinese fir growth. Means ± SE from independent experiments with three replicates for each determination are shown.

Previous studies have regarded that phenolic compounds are major allelochemicals causing allelopathy or autotoxicity in natural forests and managed ecosystems (*13*−*15*). Several phenolic acids were identified as potent allelochemicals from replanted Chinese fir tree ecosystems (*9*−*12*). Data generated in this study argued such common-phenolics-based allelochemicals are not responsible for autotixicity of the replanted Chinese fir tree ecosystem. Although these phenolics were detected in Chinese fir tree ecosystems, their soil concentrations are significantly lower than those of the natural evergreen broadleaf forest in which no autotoxicity occurred. Particularly, both individual phenolics and mixtures of these phenolics at their determined soil concentrations in the replanted Chinese fir tree ecosystem stimulated rather than inhibited Chinese fir trees. In addition, these phenolics are widely distributed in natural forests and managed ecosystems and are not specific to Chinese fir tree ecosystems. Accordingly, these phenolics may play some roles in the replanted Chinese fir tree ecosystem, but they are not key allelochemicals to explain the productivity decline of the replanted Chinese fir tree ecosystem. In fact, phenolic acids are utilized by soil microorganisms as a carbon resource (*31, 32*) and interfere with soil nitrogen availability in forest ecosystems (*15, 33*).

Friedelin has shown antimicrobial, anticandidal, anti-inflammatory, and anticancer activities (*25*−*27*). Friedelin showed weak inhibitory activity on Chinese fir at the high concentrations tested, but it never reached the phytotoxic level in the replanted Chinese fir tree ecosystem. On the contrary, the natural evergreen broadleaf forest soil contained friedelin in sufficient quantity. This implied that friedelin is not involved in direct inhibition of Chinese fir trees. However, potential actions and implications of friedelin in the natural evergreen broadleaf forest and Chinese fir tree ecosystems remain obscure.

Dipeptide and nonprotein amino acids isolated from plant and microbial species have been extensively investigated in relation to their herbicidal activities (*19, 34*−*36*). Hydantocidin derived from *Streptomyces hygroscopicus*, one cyclic dipeptide, completely killed or seriously damaged the troublesome perennials, such as purple nutsedge, field bindweed, horsenettle, and yellow nutsedge (*34*). A novel cyclic dipeptide, 6-hydroxy-1,3-dimethyl-8-nonadecyl-[1,4]-diazocane-2,5-diketone, isolated from toxic soil of the replanted Chinese fir tree ecosystem is significantly more phytotoxic than phenolics and friedelin (**Figure 1**). This cyclic dipeptide is a highly active allelochemical and reached the phytotoxic level, which provides clear evidence of autotoxicity in the replanted Chinese fir tree ecosystem. Productivity decline of the replanted Chinese fir tree ecosystem involves multiple interactions of allelochemicals, soil nutrient status, and nutrient cycling as well as microbial associations and other environmental factors. However, the discovery of the cyclic dipeptide, as well as a further understanding of its potential action mechanism in the replanted Chinese fir tree ecosystem, could lead to new insight into the regeneration failure and replant problem in natural and managed tree ecosystems.

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