Antinematodal Activities of Ingenane Diterpenes from *Euphorbia kansui* and their Derivatives against the Pine Wood Nematode

(*Bursaphelenchus xylophilus*)

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Under the bioassay-guided method, two diterpenes, 3-O-(2\(^\prime\),3\(^\prime\)-dimethylbutanoyl)-13-O-dodecanoyl ingenol (1) and 3-O-(2\(^\prime\),3\(^\prime\)-dimethylbutanoyl)-13-O-decanoyl ingenol (2) isolated from *Euphorbia kansui*, showed a pronounced antinematodal activity against the nematode *Bursaphelenchus xylophilus* at the same minimum effective dose (MED) of 5 \(\mu\)g per cotton ball and still displayed antinematodal activity at a dose of 2.5 \(\mu\)g per cotton ball. Compounds 3–6 were obtained, and the structure of the new compound 6 was elucidated based on 1D- and 2D-NMR analyses and physicochemical data. Preliminary structure-biological activity relationships of ingenane-type compounds were deduced.

**Key words:** *Euphorbia kansui*, Antinematodal, Ingenane Diterpenes, *Bursaphelenchus xylophilus*

**Introduction**

As a causal organism of pine wilt disease, *Bursaphelenchus xylophilus* Steiner & Bührer (Aphelenchoididae family) is widely distributed in Japan, in China, and to some extent in the United States, with the result that many pine species are seriously infested (Mamiya, 1983; Yang and Wang, 1989; Sutherland \textit{et al.}, 1991). Current control strategies rely mainly on the application of synthetic insecticides. The broad-spectrum toxicity of these compounds has encouraged people to search for naturally occurring compounds from higher plants which would be active against the pine wood nematode, *B. xylophilus*, and which might well be safer for humans and the environment than synthetic nematicides. Some active compounds inhibiting *B. xylophilus* have been obtained, such as polyacetylenes (Kawazu \textit{et al.}, 1980a), sesquiterpene α-humulene (Suga \textit{et al.}, 1993), alkaloids (Matsuda \textit{et al.}, 1991; Zhao, 1999), phenols (Alen \textit{et al.}, 2000a), and plant essential oils (Park \textit{et al.}, 2005).

In the course of the authors’ search for substances from Chinese plants which are biologically active against the nematode *B. xylophilus*, three antinematodal diterpenes from the roots of *Euphorbia kansui* (Shi \textit{et al.}, 2007), which is distributed throughout the north of China, have previously been isolated and identified. Its roots are used in traditional Chinese medicine as a remedy for edema, ascites (Pharmacopoeia Commission, 1995), and cancer (Xia and Li, 1999; Xu \textit{et al.}, 1997). Reported effects of the ingenane-type compounds isolated from the plant include stimulating the expression of the macrophage Fc receptor (Matsumoto \textit{et al.}, 1992), antileukemic activity, cytotoxicity, and antiviral activity (Wu \textit{et al.}, 1991; Zheng \textit{et al.}, 1998). They induce cell division arrest (Wang \textit{et al.}, 2002, 2003), inhibit the growth of cancer cells (Miyata \textit{et al.}, 2004), and suppress IgE-mediated mast cell activity (Nunomura \textit{et al.}, 2006). However, to the authors’ knowledge, no other studies have reported the use of these compounds for pest control apart from our own earlier antinematodal research (Shi \textit{et al.}, 2007). As part of ongoing efforts to identify antinematodal compounds from this species, the present paper reports the isolation and characterization of two other antinematodal compounds from *E. kansui* and the establishment of a preliminary structure-activity relationship.
Materials and Methods

General experimental procedures

$^1$H and $^{13}$C NMR spectra were recorded using a Varian Unity Inova AS 600 (CA, USA) instrument at 600 MHz and 150 MHz, respectively. The standard pulse sequences programmed for the instrument were used for 2D measurements [correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple quantum coherence (HMQC)]. FAB-mass spectra were performed on a Shimadzu (Tokyo, Japan) 9020-DF mass spectrometer with 3-NBA as a matrix, and ESI-mass spectra were recorded by a Perkin Elmer model API III (ON, Canada) instrument. Specific rotation data were collected using a Jasco (Tokyo, Japan) DIP-360 polarimeter, IR (KBr) spectra were determined using an Avatar 360 T2 (Thermo Nicolet, MA, United States), and UV spectra were determined using a Shimadzu (Tokyo, Japan) Multi-Spec-1500 spectrophotometer. High-performance liquid chromatography (HPLC) was performed using a Hitachi L-6250 Intelligent Pump (Tokyo, Japan) with an inertsil ODS-3 column (5 µm, 4.6 × 250 mm, GL Sciences, Tokyo, Japan) and an L-7420 UV-Vis detector. Column chromatography (CC) was performed on silica gel 60 F plates (Merck, 0.25 mm and 1.00 mm, respectively), and reverse-phase TLC was performed on silica gel 60 F$_{254}$ plates (Merck, 0.25 mm and 1.00 mm, respectively), and reverse-phase TLC was performed on RP-18 F$_{254s}$ (Merck, Darmstadt, Germany).

Plant material

The dried roots of Euphorbia kansui Liou were purchased from the Traditional Chinese Medicine Supermarket in Xi’an, Shaanxi Province, China, and a voucher specimen was deposited at the College of Life Science of Northwest University, Xi’an, China.

Antinematodal bioassay

The bioassay (Kawazu et al., 1980b; Alen et al., 2000b) was performed against the phytopathogenic nematode Bursaphelenchus xylophilus. Estimation of the antinematodal activity was carried out according to the method described by Alen et al. (2000b). The antinematodal effect (active or inactive) was determined by observing whether or not the mycelia of Botrytis cinerea were consumed by nematodes, by comparing with the corresponding equivalent solvent as a control. The minimum effective dose (MED) was defined as the lowest dose of the tested sample inhibiting the nematode completely from consuming the fungal mat.

Extraction and isolation

The dried roots (10 kg) of E. kansui were ground and macerated with 20 l EtOH for two weeks to obtain the residue (239.68 g), followed by separation with hexane and EtOAc. The three fractions obtained (hexane fraction, EtOAc fraction, and water fraction) were examined for their bioactivity, and the hexane fraction was found to have the greatest activity against the nematodes at a dose of 5 mg. The hexane fraction (113.82 g) was applied to a silica gel chromatography column eluted with a graded series of hexane and EtOAc, monitored by TLC with a sulfuric acid reagent, combined by virtue of the similar spots to yield six fractions. One of the active fractions showed the highest activity against the nematodes at a dose of 5 mg. This fraction (11.82 g) was subjected to a silica gel column eluted with hexane/EtOAc (60:40, v/v) and EtOAc to yield five fractions. The active fraction (4.087 g) was subjected to a silica gel column eluted with CHCl$_3$/MeOH (97:3, v/v) to yield four fractions. The active fraction obtained (1.83 g) was repeatedly separated by reverse-phase CC with a gradient of MeOH and H$_2$O, monitored by octadecylsilyl (ODS) TLC. The active fraction obtained (200 mg) was chromatographed by preparative TLC developed with CHCl$_3$/MeOH (96:4, v/v) to yield compound 1 (110 mg). The other active fraction (50 mg) was purified by preparative TLC developed with CHCl$_3$/MeOH (96:4, v/v) followed by reverse-phase TLC developed with MeOH/H$_2$O (85:15, v/v), yielding compound 2 (11 mg).

3-O-(2"",3""-Dimethylbutanoyl)-13-O-dodecanoyl-ingenol (1): Colourless resin; $[\alpha]_D^{23} = -26^\circ$ (c 0.2, CHCl$_3$). – UV (EtOH): $\lambda_{max}$ (log $\varepsilon$) = 230 (4.46), 269 nm (4.29). – ESIMS: $m/z$ = 645 [M+H]+, 511 [M + H – H$_2$O – C$_8$H$_7$COOH]$^+$. – IR (KBr): $\nu_{max}$ = 3420, 2930, 1725 cm$^{-1}$. – $^1$H NMR (CDCl$_3$): $\delta$ = 0.87 (3H, t, $J = 7.2$ Hz, H-12""), 0.91 (3H, d, $J = 6.6$ Hz, H-14""), 0.95 (3H, d, $J = 6.6$ Hz, 3""-CH$_3$), 0.96 (3H, d, $J = 7.0$ Hz, 18-H), 1.05 (3H, 269 nm (4.29). – ESIMS: $m/z$ = 645 [M+H]+, 511 [M + H – H$_2$O – C$_8$H$_7$COOH]$^+$. – IR (KBr): $\nu_{max}$ = 3420, 2930, 1725 cm$^{-1}$. – $^1$H NMR (CDCl$_3$): $\delta$ = 0.87 (3H, t, $J = 7.2$ Hz, H-12""), 0.91 (3H, d, $J = 6.6$ Hz, H-14""), 0.95 (3H, d, $J = 6.6$ Hz, 3""-CH$_3$), 0.96 (3H, d, $J = 7.0$ Hz, 18-H), 1.05 (3H,
Preparation of compounds 3–6

20-O-Acetyl-[3-O-(2′E,4′Z)-decadienoyl]-ingenol (15 mg) obtained according to the method previously described (Shi et al., 2007) was treated with 5% KOH/Methanol (2 ml) at room temperature for 2 h, monitored by TLC, and then neutralized with acetic acid. The product obtained was purified by preparative TLC (CHCl₃/Methanol 95:5, v/v) to yield compound 3 (6 mg).

Compound 3 (9 mg) was acetylated with Ac₂O/pyridine at 30 °C for 12 h, then purified by preparative TLC (CHCl₃/Methanol 97:3, v/v) to yield compound 4 (7 mg).

Compound 1 (30 mg) was hydrolyzed and acetylated to produce compound 5 (6 mg) and compound 6 (9 mg), respectively, using the same methods as described above.

Ingenol (3): Colourless oil; [α]D = −54.0° (c 0.2, CHCl₃). – UV (EtOH): λmax (log ε) = 203 (3.75), 248 nm (3.02). – ESIMS: m/z = 366 [M + NH₄]⁺, 348 [M + NH₄ + 18]⁺. – IR (KBr): νmax = 3378, 2932, 2874, 1717, 1649, 1460, 1437 cm⁻¹. – ¹H NMR (CDCl₃): δ = 0.70 (1H, ddd, J = 12.5, 8.5, 6.0 Hz, H-13), 0.94 (1H, dd, J = 12.5, 8.5 Hz, H-14), 0.97 (3H, d, J = 7.2 Hz, H-18), 1.06 (3H, s, H-16), 1.85 (3H, d, J = 1.2 Hz, H-19), 2.27 (2H, ddd, J = 15.6, 9.0, 3.0 Hz, H-12), 2.32 (1H, m, H-11), 3.81 (1H, s, H-5), 4.11 (1H, dd, J = 12.5, 4.2 Hz, H-8), 4.17 (2H, d, J = 12.6 Hz, H-20), 4.40 (1H, s, H-3), 5.94 (1H, d, J = 1.8 Hz, H-1), 6.05 (1H, d, J = 4.2 Hz, H-7). – ¹³C NMR (CDCl₃) δ = 15.4 (C-17), 15.5 (C-19), 17.3 (C-18), 22.9 (C-14), 23.1 (C-13), 24.0 (C-15), 28.5 (C-16), 30.8 (C-12), 39.8 (C-11), 44.0 (C-8), 67.2 (C-20), 72.4 (C-10), 75.3 (C-5), 80.5 (C-3), 84.3 (C-4), 127.4 (C-7), 130.0 (C-1), 138.8 (C-2), 140.3 (C-6), 207.8 (C-9).

3,5,20-O-Triacetylingenol (4): Colourless crystals; [α]D = −6.0° (c 0.2, CHCl₃). – UV (EtOH): λmax (log ε) = 204 nm (3.13). – ESIMS: m/z = 492 [M + NH₄]⁺. – IR (KBr): νmax = 3326, 2928, 1746, 1717, 1462, 1377, 1230 cm⁻¹. – ¹H NMR (CDCl₃): δ = 0.71 (1H, m, H-13), 0.96 (1H, m, H-14), 0.99 (3H, d, J = 7.0 Hz, H-18), 1.06 (3H, s, H-16), 1.08 (3H, s, H-17), 1.25 (1H, m, H-12b), 2.00 (3H, d, J = 3.0 Hz, H-19), 2.04 (3H, d, J = 3.0 Hz, 20-COMe), 2.13 (3H, t, J = 2.7 Hz, 3-COMe), 2.21 (3H, d, J = 2.8 Hz, 5-COMe), 2.29 (1H, m, H-12a), 2.51 (1H, m, H-11), 4.18 (1H, d, J = 12.6 Hz, H-20b), 4.24 (1H, dd, J = 11.6, 4.4 Hz, H-8), 4.59 (1H, d, J = 12.6 Hz, H-20a), 4.96 (1H, s, H-3), 5.38 (1H, s, H-5), 6.08 (1H, d, J = 1.8 Hz, H-1), 6.24 (1H, d, J = 4.4 Hz, H-7). – ¹³C NMR (CDCl₃): δ = 15.4 (C-19), 15.5 (C-17), 17.0 (C-18), 20.8 (5-COMe), 20.9 (20-COMe), 21.2 (3-COMe), 22.9 (C-14), 23.1 (C-13), 24.4 (C-15), 28.4 (C-16), 31.1 (C-12), 38.6 (C-11), 43.6 (C-8), 65.8 (C-20), 71.9 (C-10), 74.8 (C-5), 82.2 (C-3), 85.8 (C-4), 131.9 (C-7), 132.2 (C-1), 133.2 (C-2), 135.4 (C-6), 172.5 (3-CO), 170.7 (5-CO), 170.7 (20-CO), 205.4 (C-9).

13-O-Dodecanoylingenol (5): Colourless resin; [α]D = −54.0° (c 0.2, CHCl₃). – UV (MeOH): λmax (log ε) = 204 (3.94), 241 nm (3.49). – ESIMS: m/z = 564 [M + NH₄]⁺. – IR (KBr): νmax = 3384, 2928, 2858, 1742, 1460, 1379, 1118 cm⁻¹. – ¹H NMR (CDCl₃): δ = 0.87 (3H, t, J = 7.2 Hz, H-12′), 0.96 (3H, d, J = 7.2 Hz, H-18), 1.07 (3H, s, H-16), 1.22 (3H, s, H-17), 1.25 (1H, m, H-14), 1.25 (16H, superimposed, H-4′ to H-11′), 1.55 (2H, m, H-3′),
1.80 (3H, d, J = 1.2 Hz, H-19), 2.19a (1H, m, H-12b), 2.19b (2H, m, H-3'), 2.44 (1H, m, H-11), 2.72 (1H, dd, J = 16.8, 3.0 Hz, H-12a), 3.83 (1H, s, H-5), 4.05 (1H, dd, J = 11.4, 4.2 Hz, H-8), 4.11 (1H, d, J = 12.6 Hz, H-20b), 4.19 (1H, d, J = 12.6 Hz, H-20a), 4.41 (1H, s, H-3), 5.90 (1H, m, H-1), 6.03 (1H, d, J = 4.2 Hz, H-7) (overlapping signal). – 13C NMR: see Table I.

5,20-O-Diacetyl-3-O-(2"",3""-dimethylbutanoyl)-13-O-dodecanoylingenol (6): Colourless resin; [α]D2 2 – 20.0° (c 0.2, CHCl3). – UV (MeOH): λmax (log ε) = 203 nm (4.12). – ESIMS: m/z = 746 [M + NH4]⁺. – IR (KBr): νmax = 3456, 2974, 2942, 1725, 1657, 1586, 1377, 1313, 1234, 1036 cm⁻¹. – 1H NMR (CDCl3): δ = 0.87 (3H, t, J = 7.2 Hz, H-12'), 0.91 (3H, d, J = 7.2 Hz, 3""-CH₃), 1.00 (3H, d, J = 7.2 Hz, H-18), 1.06 (3H, s, H-16), 1.12 (3H, d, J = 7.2 Hz, 2""-CH₃), 1.18 (3H, s, H-17), 1.26a (1H, m, H-14), 1.26b (16H, superimposed, H-4' to H-11'), 1.55 (2H, m, H-3'), 1.75 (3H, d, J = 1.8 Hz, H-19), 1.87 (1H, m, H-3''), 2.00 (3H, s, 20-COMe), 2.20a (1H, m, H-12b), 2.20b (2H, m, H-2'), 2.23 (3H, s, 5-COMe), 2.25 (1H, m, H-2''), 2.60 (1H, m, H-11), 2.68 (1H, dd, J = 16.8, 3.3 Hz, H-12a), 4.19 (1H, d, J = 12.6 Hz, H-20b), 4.21 (1H, m, H-8), 4.59 (1H, d, J = 12.6 Hz, H-20a), 4.94 (1H, s, H-5), 5.42 (1H, s, H-3), 6.07 (1H, m, H-1), 6.21 (1H, d, J = 4.2 Hz, H-7) (overlapping signal). – 13C NMR: see Table I.

Results and Discussion

Using the bioassay-directed method, the active hexane fraction from an ethanol extract of the roots of E. kansui was subjected to silica gel CC and reverse-phase CC to yield compounds 1 and 2.

Compound 1 was obtained as a colourless resin. The protonated molecular peak at m/z 617 [M + H]⁺ in the FAB-mass spectrum, together with 1H and 13C NMR spectral data, suggested the molecular formula C₃₅H₅₆O₈. The 1H NMR and 1H-1H COSY spectral data for 2 were almost identical with those for 1, and they had the same Rf value (0.42) obtained from TLC (silica gel 60 F₂₅₄, CHCl₃/MeOH 1: 9, v/v). However, the Rf values of compound 2 (Rf: 0.22) and compound 1 (Rf: 0.11) appeared clearly different in ODS TLC (RP-18 F₂₅₄, H₂O/MeOH 1: 9, v/v). The DEPT and 13C NMR spectral data revealed that the structure of 2 was composed of eight methyl, ten methylene, nine methine, two ester carbonyl groups, a carbonyl (ketone) group and six quaternary carbon atoms, showing two methylene groups less than the structure of 1 (Table I). In addition, the ion at m/z 427 [M + H - H₂O - C₉H₁₈COOH]⁺ in combination with the NMR spectral data of 2 indicated that one of two acyl residues was a decanoyl group instead of a dodecanoyl group as in compound 1. On the basis of these spectral data, compound 2 was identified as 3-O-(2"",3""-dimethylbutanoyl)-13-O-dodecanoylingenol (Fig. 1). The structure of compound 2 has been reported from previous research (Ott and Hecker, 1981; Nunomura et al., 2006). As 1H NMR assignments for it are incomplete, and since 13C NMR, IR, and optical
rotation data are not available, they are fully described here.

Alkaline hydrolysis of 20-O-acetyl-[3-O-(2',3',
4'-Z)-decadienoyl]-ingenol with 5% KOH in
MeOH at room temperature yielded a colourless
oil which was identified as ingenol (3) by comparison
with literature data (Appendino et al., 1999).

Compound 3 was treated with acetic anhydride/
pyridine and yielded compound 4. The structure of
4 was characterized as 3,5,20-O-triacetylingenol
with the aid of HMBC and HMQC data and com-
parisons with data reported in the literature
(Zechmeister et al., 1970). These 13C NMR data are
reported here by the authors for the first time.

De-esterification of 1 by the above-mentioned
method gave rise to the tetrahydroxy derivative 5.
In comparison with the NMR data for 3-O-(2',3',
dimethylbutanoyl)-13-O-dodecanoyl ingenol (1),
H-3 in compound 5 resonated at δ 4.41 (s) with C-
3 (δ 80.3), exhibiting an upfield shift of 1.01 ppm
(2.2 ppm), indicating that the 2,3-dimethylbutano-
ester attached at C-3 underwent hydrolysis. On
the contrary, C-13 resonated at δ 68.8, shifting up-
field by only 0.2 ppm as compared to the NMR
data for 1, suggesting that the dodecanoate ester
could not undergo hydrolysis. According to NMR
and MS spectral data, the structure of 5 was as-
signed as 13-O-dodecanoyl ingenol with the aid of
other research results (Wu et al., 1991; Matsumoto
et al., 1992).

With acetic anhydride/pyridine, compound 1
yielded the diacetoxy derivative 6. In the HMBC
spectrum, the correlations between H-5 and C-10,
C-6 and 5-OCO, between H-20a and C-6, C-7 and
20-OCO, and between H-20b and C-6, C-7 and 20-
OCO demonstrated that two acetyl groups were
attached at C-5 and C-20. C-5 and C-20 resonated
at δ 74.8 and 65.7, shifting upfield by 1.9 ppm and
1.4 ppm, respectively, compared to the NMR
data for 1, suggesting that the dodecanoate ester
could not undergo hydrolysis. According to NMR
and MS spectral data, the structure of 5 was as-
signed as 13-O-dodecanoyl ingenol with the aid of
other research results (Wu et al., 1991; Matsumoto
et al., 1992).

The six compounds were analyzed for their anti-
nematodal activity against B. xylophilus. Com-
ounds 1 and 2 showed the same level of antine-
motodal activity with an MED of 5 μg per cotton
ball (Table II).

Table II. Antinematodal activities of compounds 1 and
2 against B. xylophilus.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose/cotton ball</th>
<th>Minimum effective dose (MED)/cotton ball</th>
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<tr>
<td></td>
<td>10 μg</td>
<td>5 μg</td>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
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</table>

Each compound tested was evaluated in triplicate.
In the current research, derivatives 3–6 showed weaker or no antinematodal activity against *B. xylophilus* (Table III). It has been observed that ingenol (3) and 3,5,20-O-triacetylingenol (4) displayed antinematodal activity starting at 100 μg/cotton ball.

Interestingly, compound 5 was found to exhibit antinematodal activity in a range of 2.5 to 200 μg/cotton ball, whereas compound 6 appeared to be inactive (Table III). The antinematodal bioassay results for compounds 1–6 and their structural characteristics, along with those for the three antinematodal compounds reported previously (Shi et al., 2007), have led to the establishment of a preliminary structure-activity relationship. It is hypothesized that the compounds have the identical basic ingenane-type skeleton, but the structural differences among the compounds occurring at C-3, C-5, and C-20 positions of the ring resulted in the antinematodal differences. If two hydroxy groups at C-3 and C-5 of the ring simultaneously remained hydroxy-free or were esterified, they exhibited weaker antinematodal activity or were inactive; if one hydroxy group at C-3 or C-5 of the ring remained free, the compound displayed a pronounced antinematodal activity (Tables II and III). In addition, whether or not the hydroxy group at C-20 was esterified appeared to have no effect on the antinematodal activity (Table II). Evidently, the presence of one free hydroxy group at C-3 or C-5 may play an important role in the antinematodal activity.

*E. kansui*, a plant with a history of medicinal use as an anticancer agent, has been received increasing attention because of its inhibitory activity with regard to cellular proliferation and enzyme activity (Wang et al., 2002; Miyata et al., 2006). From this plant, many diterpenes with different kinds of biological activity have been isolated. For example, Opferkuch and Hecker (1982) have reported that the ingenol-3 esters from *E. kansui* showed irritant and tumour-promoting activities, while the ingenol-20 esters were inactive. In the mean time, it has also been found that C-20 esterification makes the tumour-promoting activity disappear while keeping the antileukemic activity intact (Opferkuch and Hecker, 1982; Wu et al., 1991). The compounds, if modified, will have the active agent chosen for application to control the nematode *B. xylophilus*. This study also suggests that the extracts of the root of this plant may be used directly as an antinematodal agent in the field, providing safe and economical benefits. Furthermore, the preliminary structure-activity relationship study will supply data to perform further research to identify the antinematodal mechanism of the ingenane-type compounds and to synthesize naturally based ingenane-type antinematodal compounds. How derivatives with lengthened unsaturated and saturated side chains at the C-3 and C-5 positions affect the antinematodal activity will be studied by future work.

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oils against the pine wood nematode (Bursaphelenchus xylophilus).) Nematology 7, 767–774.


