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″,3″-dimethylbutanoyl)-13-*O*-dodecanoylingenol (**1**) and 3-*O*-(2″,3″-dimethylbutanoyl)-13-*O*-decanoylingenol (**2**) isolated from *Euphorbia kansui*, showed a pronounced antinematodal activity against the nematode *Bursaphelenchus xylophilus* at the same minimum effective dose (MED) of 5 μg per cotton ball and still displayed antinematodal activity at a dose of 2.5 μg 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 *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 *et al.*, 1980a), sesquiterpene α-humulene (Suga *et al.*, 1993), alkaloids (Matsuda *et al.*, 1991; Zhao, 1999), phenols (Alen *et al.*, 2000a), and plant essential oils (Park *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 *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 *et al.*, 1997). Reported effects of the ingenane-type compounds isolated from the plant include stimulating the expression of the macrophage Fc receptor (Matsumoto *et al.*, 1992), antileukemic activity, cytotoxicity, and antiviral activity (Wu *et al.*, 1991; Zheng *et al.*, 1998). They induce cell division arrest (Wang *et al.*, 2002, 2003), inhibit the growth of cancer cells (Miyata *et al.*, 2004), and suppress IgE-mediated mast cell activity (Nunomura *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 *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.

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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 (Merck, 0.040–0.063 mm) and reverse-phase silica gel (Millipore Preparative C18, 125 Å, 55–105 μm). Thin-layer chromatography (TLC) and preparative TLC were 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_2O, 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_2O (85 : 15, v/v), yielding compound **2** (11 mg).

*3-O-(2″,3″-Dimethylbutanoyl)-13-O-dodecanoyl-ingenol (**1**)*: Colourless resin; $[\alpha]_D^{22}$ −26° (*c* 0.2, $CHCl_3$). – UV (EtOH): λ_{max} (log ε) = 230 (4.46), 269 nm (4.29). – ESIMS: m/z = 645 $[M+H]^+$, 511 $[M + H - H_2O - C_5H_{11}COOH]^+$. – IR (KBr): ν_{max} = 3420, 2930, 1725 cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 0.87 (3H, t, J = 7.2 Hz, H-12′), 0.91 (3H, d, J = 6.6 Hz, H-4″), 0.95 (3H, d, J = 6.6 Hz, 3″-CH_3), 0.96 (3H, d, J = 7.0 Hz, 18-H), 1.05 (3H, s, H-16), 1.13 (3H, d, J = 6.6 Hz, 2″-CH_3), 1.18 (3H, s, H-17), 1.23 (1H, m, H-14), 1.25 (16H, superimposed, H-4′ to H-11′), 1.54 (2H, m, H-3′), 1.77 (3H, d, J = 1.8 Hz, H-19), 1.90 (1H, m, H-3″), 2.19^a (1H, m, H-12b), 2.19^a (2H, m, H-2′), 2.30 (1H, m, H-2″), 2.62 (1H, m, H-11), 2.71 (1H, dd, J = 16.8, 3.4 Hz, H-12a), 4.04 (1H, s, H-5), 4.09 (1H, dd, J = 12.5, 4.2 Hz, H-8), 4.14 (2H, s, H-20), 5.45 (1H, s, H-3), 6.00 (1H, d, J = 0.6 Hz, H-1), 6.01 (1H, d, J = 4.2 Hz, H-7) (^{a}overlapping signal). – ^{13}C NMR: see Table I.

*3-O-(2″,3″-Dimethylbutanoyl)-13-O-decanoylingenol (**2**)*: Colourless resin; $[\alpha]_D^{22}$ –27° (c 0.2, $CHCl_3$). – UV (EtOH): λ_{max} (log ε) = 208 nm (4.02). – FABMS: m/z = 617 $[M + H]^+$, 483 $[M + H - H_2O - C_5H_{11}COOH]^+$, 427 $[M + H - H_2O - C_9H_{19}COOH]^+$. – IR (KBr): ν_{max} = 3461, 2971, 2930, 2856, 1733, 1461, 1383, 1284 cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 0.87 (3H, t, J = 7.2 Hz, H-10′), 0.93 (3H, d, J = 6.6 Hz, H-4″), 0.96 (3H, d, J = 6.6 Hz, 3″-CH_3), 0.97 (3H, d, J = 7.2 Hz, H-18), 1.06 (3H, s, H-16), 1.15 (3H, d, J = 6.6 Hz, 2″-CH_3), 1.19 (3H, s, H-17), 1.24 (1H, m, H-14), 1.25 (12H, superimposed, H-4′ to H-9′), 1.55 (2H, m, H-3′), 1.78 (3H, d, J = 0.6 Hz, H-19), 1.92 (1H, m, H-3″), 2.21^a (1H, m, H-12b), 2.21^a (2H, m, H-2′), 2.32 (1H, m, H-2″), 2.61 (1H, m, H-11), 2.72 (1H, dd, J = 16.8, 3.6 Hz, H-12a), 4.05 (1H, s, H-5), 4.09 (1H, dd, J = 12.5, 4.2 Hz, H- 8), 4.14 (2H, s, H-20), 5.44 (1H, s, H-3), 6.01 (1H, d, J = 1.2 Hz, H-1), 6.03 (1H, d, J = 4.2 Hz, H-7) (^{a}overlapping signal). – ^{13}C NMR: see Table I.

*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/MeOH (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 ($CHCl_3$/MeOH 95:5, v/v) to yield compound **3** (6 mg).

Compound **3** (9 mg) was acetylated with Ac_2O/pyridine at 30 °C for 12 h, then purified by preparative TLC ($CHCl_3$/MeOH 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; $[\alpha]_D^{22}$ –34.0° (c 0.2, $CHCl_3$). – UV (EtOH): λ_{max} (log ε) = 203 (3.75), 248 nm (3.02). – ESIMS: m/z = 366 $[M + NH_4]^+$, 348 $[M + NH_4 - 18]^+$. – IR (KBr): ν_{max} = 3378, 2932, 2874, 1717, 1649, 1460, 1437 cm^{-1}. – ^{1}H NMR ($CDCl_3$): 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). – ^{13}C NMR ($CDCl_3$) 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; $[\alpha]_D^{22}$ –6.0° (c 0.2, $CHCl_3$). – UV (EtOH): λ_{max} (log ε) = 204 nm (3.13). – ESIMS: m/z = 492 $[M + NH_4]^+$. – IR (KBr): ν_{max} = 3326, 2928, 1746, 1717, 1462, 1377, 1230 cm^{-1}. – ^{1}H NMR ($CDCl_3$): 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). – ^{13}C NMR ($CDCl_3$): 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).

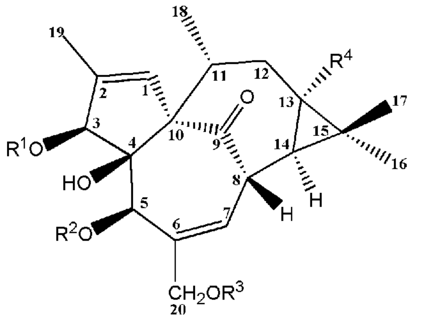
*13-O-Dodecanoylingenol (**5**)*: Colourless resin; $[\alpha]_D^{22}$ –54.0° (c 0.2, $CHCl_3$). – UV (MeOH): λ_{max} (log ε) = 204 (3.94), 241 nm (3.49). – ESIMS: m/z = 564 $[M + NH_4]^+$. – IR (KBr): ν_{max} = 3384, 2928, 2858, 1742, 1460, 1379, 1118 cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 0.87 (3H, t, 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^a (1H, m, H-14), 1.25^a (16H, superimposed, H-4′ to H-11′), 1.55 (2H, m, H-3′), 1.80 (3H, d, J = 1.2 Hz, H-19), 2.19^{a} (1H, m, H-12b), 2.19^{a} (2H, m, H-2′), 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) (^{a}overlapping signal). – ^{13}C NMR: see Table I.

5,20-O-Diacetyl-3-O-(2″,3″-dimethylbutanoyl)-13-O-dodecanoylingenol (***6***): Colourless resin; $[\alpha]_D^{22}$ −20.0° (c 0.2, $CHCl_3$). – UV (MeOH): λ_{max} (log ε) = 203 nm (4.12). – ESIMS: m/z = 746 $[M + NH_4]^+$. – IR (KBr): ν_{max} = 3456, 2974, 2942, 1725, 1657, 1605, 1586, 1377, 1313, 1234, 1036 cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 0.87 (3H, t, J = 7.2 Hz, H-12′), 0.91 (3H, d, J = 7.2 Hz, 3″-CH_3), 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″-CH_3), 1.18 (3H, s, H-17), 1.26^{a} (1H, m, H-14), 1.26^{a} (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), 2.20^{a} (1H, m, H-12b), 2.20^{a} (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) (^{a}overlapping signal). – ^{13}C 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 645 $[M + H]^+$ in the ESI mass spectrum, together with ^{1}H and ^{13}C NMR spectral data, suggested that compound **1** had the molecular formula $C_{38}H_{60}O_8$. The IR spectrum of the compound indicated the presence of a hydroxy group (3420 cm^{-1}) and a ketone (1725 cm^{-1}). The ^{13}C NMR spectrum and DEPT data revealed that the structure of **1** consisted of eight methyl, twelve methylene, nine methine, two ester carbonyl groups, a carbonyl (ketone) group and six quaternary carbon atoms. A review of the literature survey and a comparison of the spectral data of **1** with those for ingenane-type diterpenes revealed that compound **1** was 3-*O*-(2″,3″- dimethylbutanoyl)-13-*O*-dodecanoylingenol (Matsumoto *et al.*, 1992) (Fig. 1 and Table I).



1: $R^1 = COCH(CH_3)CH(CH_3)_2$, $R^2 = H$, $R^3 = H$, $R^4 = OCO(CH_2)_{10}CH_3$
2: $R^1 = COCH(CH_3)CH(CH_3)_2$, $R^2 = H$, $R^3 = H$, $R^4 = OCO(CH_2)_8CH_3$
3: $R^1 = H$, $R^2 = H$, $R^3 = H$, $R^4 = H$
4: $R^1 = COCH_3$, $R^2 = COCH_3$, $R^3 = COCH_3$, $R^4 = H$
5: $R^1 = H$, $R^2 = H$, $R^3 = H$, $R^4 = OCO(CH_2)_{10}CH_3$
6: $R^1 = COCH_3$, $R^2 = COCH_3$, $R^3 = COCH_3$, $R^4 = OCO(CH_2)_{10}CH_3$

Fig. 1. Chemical structure of compounds **1**–**6**.

Compound **2** was obtained as a colourless resin. The protonated molecular peak at m/z 617 $[M + H]^+$ in the FAB-mass spectrum, together with ^{1}H and ^{13}C NMR spectral data, suggested the molecular formula $C_{36}H_{56}O_8$. The ^{1}H NMR and ^{1}H-^{1}H COSY spectral data for **2** were almost identical with those for **1**, and they had the same R_f value (0.42) obtained from TLC (silica gel 60 F_{254}, $CHCl_3$/MeOH 96:4, v/v). However, the R_f values of compound **2** (R_f: 0.22) and compound **1** (R_f: 0.11) appeared clearly different in ODS TLC (RP-18 F_{254s}, H_2O/MeOH 1:9, v/v). The DEPT and ^{13}C 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 $[M + H - H_2O - C_9H_{19}COOH]^+$ 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*-decanoylingenol (Fig. 1). The structure of compound **2** has been reported from previous research (Ott and Hecker, 1981; Nunomura *et al.*, 2006). As ^{1}H NMR assignments for it are incomplete, and since ^{13}C NMR, IR, and optical

Table I. ^{13}C NMR data of compounds **1**, **2** and derivatives **5** and **6** [$CDCl_3$, TMS, δ (ppm)].

C	**1**	**2**	**5**	**6**
1	131.4	131.4	129.0	130.5
2	136.2	136.2	139.3	136.0
3	82.5	82.5	80.3	81.7
4	84.5	84.4	84.0	85.7
5	76.7	76.7	75.2	74.8
6	139.6	139.5	140.7	133.8
7	127.2	127.3	126.2	129.7
8	42.6	42.6	43.2	42.9
9	205.9	205.9	206.5	204.7
10	71.8	71.8	72.6	71.8
11	37.4	37.4	38.6	37.9
12	35.0	35.0	35.0	35.0
13	69.0	69.0	68.8	69.1
14	28.2	28.2	28.2	28.0
15	30.3	30.3	30.2	30.7
16	22.4	22.5	22.5	22.2
17	16.7	16.7	16.7	16.8
18	18.2	18.2	18.4	18.0
19	15.4	15.5	15.4	15.3
20	67.1	67.2	66.8	65.7
1′	174.1	174.1	174.1	174.1
2′	34.4	34.4	34.4	34.4
3′	24.8	24.8	24.8	24.8
4′	29.5 a	29.6 a	29.6 a	29.6 a
5′	29.5 a	29.4 a	29.6 a	29.6 a
6′	29.4 a	29.2 a	29.4 a	29.4 a
7′	29.3 a	29.2 a	29.3 a	29.3 a
8′	29.2 a	31.8	29.2 a	29.2 a
9′	29.2 a	22.6	29.2 a	29.2 a
10′	31.9	14.1	31.9 a	31.9 a
11′	22.6	22.7 a	22.7 a	
12′	14.1	14.1	14.1	
1″	177.6	177.6	178.0	
2″	46.5	46.4	46.7	
3″	31.0	31.0	30.9	
4″	20.7	20.7	20.8	
2″–CH3	14.0	14.0	14.2	
3″–CH3	19.1	19.1	19.2	
20-COMe				170.7
				20.9
5-COMe				170.8
				20.8

Signals with the letter a are uncertain about their precise position within the spectrum because the proton signals superimposed.

rotation data are not available, they are fully described here.

Alkaline hydrolysis of 20-*O*-acetyl-[3-*O*-(2′*E*, 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 comparisons with data reported in the literature (Zechmeister *et al.*, 1970). These ^{13}C 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*-dodecanoylingenol (**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-dimethylbutanoate ester attached at C-3 underwent hydrolysis. On the contrary, C-13 resonated at δ 68.8, shifting upfield 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 assigned as 13-*O*-dodecanoylingenol 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 compound **1** (Table I), further supporting the assertion that the acetylation of **1** occurred at C-5 and C-20. On the basis of ^{1}H and ^{13}C NMR, HMBC, HMQC, ^{1}H-^{1}H COSY and ESIMS spectral data, the structure of compound **6** was established as 5,20-*O*-diacetyl-3-*O*-(2″,3″-dimethylbutanoyl)-13-*O*-dodecanoylingenol, to the best of the authers' knowledge. This could be a novel compound because no report of it has been found in the literature.

The six compounds were analyzed for their antinematodal activity against *B. xylophilus*. Compounds **1** and **2** showed the same level of antinematodal activity with an MED of 5 μg per cotton ball (Table II).

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

Compound	Dose/cotton ball			Minimum effective dose (MED)/cotton ball
	10 μg	5 μg	2.5 μg	
1	+	+	±	5 μg
2	+	+	±	5 μg

Each compound tested was evaluated in triplicate.

Compound	Dose/cotton ball						
	200 μg	100 μg	40 μg	20 μg	10 μg	5 μg	2.5 μg
3	±	±	–	–	–	–	–
4	±	±	–	–	–	–	–
5	±	±	±	±	±	±	±
6	–	–	–	–	–	–	–

Table III. Antinematodal activities of derivatives **3**–**6** against *B. xylophilus.*

Each compound tested was evaluated in triplicate.

– indicates that the mycelia were completely consumed by nematodes.

± indicates that only part of the mycelia was consumed by nematodes.

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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