

Insecticidal Compounds from *Tripterygium wilfordii* Active against *Mythimna separata*

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Z. Naturforsch. **59c**, 421–426 (2004); received December 9, 2003/January 12, 2004

In the course of screening for novel naturally occurring insecticides from plants, the ethanol extract of the root bark of *Tripterygium wilfordii* Hook f. was found to show insecticidal activity against larvae of *Mythimna separata* Walker. Three active compounds were isolated by bioassay-guided fractionation of the extract and characterized as triptolide (**1**), triptonide (**2**) and euonine (**3**) by IR, ¹H and ¹³C NMR and mass spectral analysis. Compounds **1** and **2** showed strong contact activity against 3rd or 5th larvae of *M. separata* (LD₅₀ 1.6 µg/insect for **1**, 2.9 µg/insect for **2**, no contact activity for **3**; LD₅₀ is the lethal dose for 50% mortality). The antifeedant activity against the 3rd larvae of *M. separata* after a 24-h treatment was demonstrated; **1**, **2** and **3** gave EC₅₀ (effective concentration causing 50% antifeedance) values of 0.25, 0.35 and 0.02 mM, respectively. **1** and **2** were inferior to the positive control represented by toosendanin (12 α -acetoxyamoorastatin), **3** was superior to toosendanin. For the ingested toxicity against *M. separata*, **1** had the more potent activity with an KD₅₀ value of 13.5 µg/g (insect body weight) than toosendanin. This is the first report on insecticidal activity of these three compounds.

Key words: *Tripterygium wilfordii*, Insecticidal Compounds, *Mythimna separata*

Introduction

The Thunder God Vine, *Tripterygium wilfordii* Hook f. belongs to the Celastraceae family. Species of the Celastraceae have got a lot of attention due to the range of biological activities (Fujita *et al.*, 2000). *T. wilfordii* has been used as an anticancer agent and insecticide for hundreds of years in China (Li *et al.*, 1997). Recently, some important pharmacologic activities, including antifertility (Bai and Shi, 2002), antirheumatoid arthritis (Cibere *et al.*, 2003), immunosuppressive reaction (Fidler *et al.*, 2002), were observed for *T. wilfordii*. A number of diterpenoids have been isolated and reported from this plant (Fujita *et al.*, 2000; Li *et al.*, 1997; Xu *et al.*, 1997; Takaishi *et al.*, 1997; Guo *et al.*, 1999).

Early investigations on insecticidal activities of this plant demonstrated that *T. wilfordii* shows repellent and ingested toxicity to *Pieris rapae* L., *Palearcita vernata* (Peck), *Plutella maculipennis* (Curt.), *Rhaphidopalpa chinensis* Weise, and contact toxicity to *Oregma lanigera*, *Sylepta derogata* Fab., *Hellula undalis* Fab. The investigation of its

insecticidal chemical constituents can be traced back to the 1950s, a period in which four insecticidal sesquiterpene alkaloids had been reported from this plant (Beroza and Botteger, 1954). In this paper, bioassay-guided isolation, identification and insecticidal activities of three compounds from the root bark of *T. wilfordii* are described.

Results and Discussion

T. wilfordii was extracted with 95% ethanol under reflux for 12 h. After filtration, the solution was evaporated to obtain a brown residue that was subsequently re-dissolved in water yielding water-soluble and -insoluble parts. The isolation scheme for the biologically active constituents is shown in Fig. 1. The three insecticidal active constituents were identified as triptolide (**1**), triptonide (**2**), and euonine (**3**), respectively, by means of their IR, MS and NMR spectral data (Fig. 2).

The contact toxicities of the three constituents from *T. wilfordii* against larvae of *M. separata* were examined by topical application and are shown in Table I. Compounds **1** and **2** showed po-

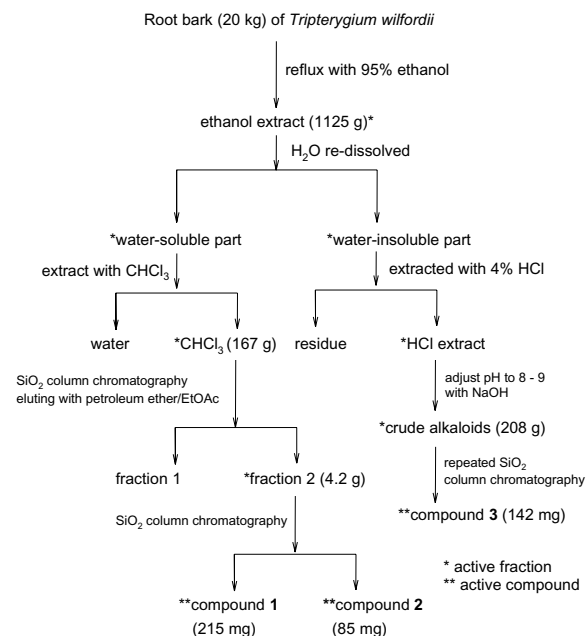


Fig. 1. Scheme for isolation of insecticidal compounds from *Tripterygium wilfordii*.

tent contact activity in a dose-dependent manner against 3rd or 5th larvae of *M. separata*; while compound **2** was less active than compound **1**, compound **3** showed no contact activity. Compounds **1** and **2** were superior to toosendanin in this assay (toosendanin showed almost no contact activity). Using regression and correlation analysis, the results indicate that the correlation was significant between concentration and effectiveness against 3rd and 5th larvae of *M. separata*, with coefficients of correlation (r) > 0.98 (48 h).

The antifeedant activity assay (Table II) showed that compounds **1**, **2** and **3** had relatively good

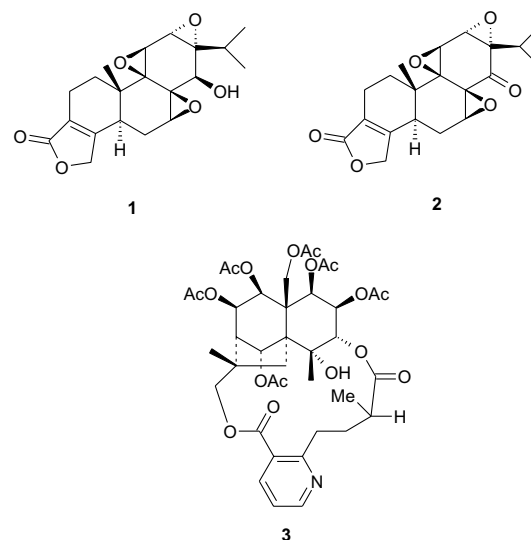


Fig. 2. The structures of insecticidal constituents triptolide (**1**), triptonide (**2**) and euonine (**3**) from *Tripterygium wilfordii*.

antifeedant activity against the larvae of *M. separata*, with compound **3** about 12.5 times as active as compound **1** and 17.5 times as compound **2**. After a 24-h treatment, the median antifeedant concentration (EC₅₀) of compounds **1**, **2** and **3** were 0.25, 0.35, 0.02 mM and 0.30, 0.50, 0.03 mM after a 48-h treatment, respectively. Compounds **1** and **2** were found inferior to toosendanin, compound **3** was superior to toosendanin.

The bioassay for ingested toxicity was generally performed by the sandwich method. The ingested toxicity assay (Table III) showed that compounds **1** and **3** had stronger ingested toxicity than com-

Table I. 48-h contact toxicity of compounds **1**, **2**, and **3** against the 3rd and 5th instar larvae of *M. separata*.

Compound	3 rd instar larvae of <i>M. separata</i>				5 th instar larvae of <i>M. separata</i>			
	LD ₅₀ [μ g/insect] ^a	95% FL ^b	Slope	Ratio ^c	LD ₅₀ [μ g/insect] ^a	95% FL ^b	Slope	Ratio ^c
1	1.6	1.4–2.1	1.6	0.6	2.2	1.8–2.5	1.2	0.5
2	2.9	2.3–3.2	1.1	1	4.5	3.6–4.8	0.9	1
3	no contact activity				no contact activity			
Toosendanin	no contact activity				no contact activity			

^a LD₅₀ value (death dose for 50% mortality) determined by log-probit analysis.

^b Fiducial limits.

^c LD₅₀ value of test compounds divided by LD₅₀ value of compound **2**.

Table II. Antifeedant activity of compounds **1**, **2**, and **3** against the 3rd instar larvae of *M. separata*.

Compound	Antifeedant activity at							
	EC ₅₀ [mM] ^a	24 h			48 h			
		95% FL ^b	Slope	Ratio ^c	EC ₅₀ [mM] ^a	95% FL ^b	Slope	Ratio ^c
1	0.25	0.22–0.27	1.4	5.0	0.30	0.26–0.32	1.4	4.3
2	0.35	0.32–0.37	2.1	7.0	0.50	0.40–0.51	1.2	7.1
3	0.02	0.01–0.03	1.2	0.4	0.03	0.02–0.04	1.5	0.4
Toosendanin	0.05	0.04–0.07	1.8	1	0.07	0.06–0.08	2.1	1

^a EC₅₀ value (50% effective concentration) determined by log-probit analysis.

^b Fiducial limits.

^c EC₅₀ value of test compounds divided by EC₅₀ value of toosendanin.

Table III. 24-h ingested toxicity of compounds **1**, **2** and **3** against the 5th instar larvae of *M. separata*.

Compound ^a	No. of insects in the intermediate zone ^b	Minimum dosage [$\mu\text{g/g}$]	Maximum dosage [$\mu\text{g/g}$]	Average dosage [$\mu\text{g/g}$]	KD ₅₀ [$\mu\text{g/g}$] ^c	Ratio ^d
1	Survival	16	5.9	22.6	10.9	13.5
	Narcosis	29	5.7	23.0	16.0	
2	Survival	19	57.3	128.6	108.8	134.5
	Narcosis	26	59.2	132.9	160.2	
3	Survival	35	17.8	44.4	28.5	29.0
	Narcosis	42	17.2	45.2	29.7	
Toosendanin	Survival	35	41.8	96.9	74.2	74.5
	Death	31	41.3	99.5	74.7	

^a Ingested toxicity of the compounds were tested by the sandwich method mentioned in the context using 200 larvae of *Mythimna separata*.

^b Intermediate zone: larvae after treated for 24 h.

^c KD₅₀ value (dose required to knock down 50% of the population) was calculated by the formula $(A + B)/2$, within which *A* represents the average dosage of the survived larvae in the intermediate zone and *B* represents the average dosage of narcotized larvae in the intermediate zone.

^d KD₅₀ value of test compounds divided by LD₅₀ value of toosendanin.

pound **2**, with compound **1** about twice as active as compound **3**. The median lethal dose (KD₅₀) of compounds **1**, **2** and **3** were 13.5, 134.5 and 29.0 $\mu\text{g/g}$ (insect body weight), respectively, compounds **1** and **3** were superior, compound **2** was inferior to toosendanin.

Botanical insecticides play an important role in controlling pests. In China, *Tripterygium wilfordii* Hook. is a widely used insecticidal plant, whose crude extract showed strong activities against many species of insects. Although five alkaloid compounds with insecticidal activities were reported from this plant, no further information about its insecticidal constituents may be available. From the results presented here, the activity against *Mythimna separata* shown by compounds **1**, **2** and **3** was comparable to the commercially available limonoid toosendanin.

Experimental

General

¹H and ¹³C NMR spectra were measured with a Bruker AM-400 spectrometer using tetramethylsilane as internal standard. Infrared spectra (IR) were recorded on a Nicolet AVATR 360 FT-IR spectrometer. EI-MS, FAB-MS and HR-MS spectra were obtained on a HP 5890, ZAB-HS and HP 5859 spectrometer, respectively.

Materials

The air-dried root barks of *Tripterygium wilfordii* Hook. were collected in Tanning County of Fujian Province, P. R. China, in November 2001. The plant was identified by Dr. X. L. He, and a voucher specimen was deposited at the Herbarium of Kunming Institute of Botany, The Chinese

Academy of Sciences. *Mythimna separata* Walker was reared with leaf of wheat or corn in glass containers (24 cm × 30 cm × 20 cm) at 25 ± 1 °C and 70–80% relative humidity (RH) under a 12/12 h light/dark cycle in the laboratory, the 3rd or 5th larvae were used for insecticidal activity tests. Toosendanin (> 95%) was used as positive control and purchased from Yi Bing Pesticides Factory of Sichuan Province (Yibing), P. R. China.

Isolation of active principle

Insecticidal compounds were isolated from the root bark of *T. wilfordii* by bioassay-guided fractionation as outlined in Fig. 1. Air-dried root bark (20 kg) of *T. wilfordii* was extracted with 95% ethanol (4 × 50 l) under reflux for 12 h. After filtration, the solution was evaporated to yield 1125 g of crude extract, which was subsequently re-dissolved with 5 l H₂O, yielding water-soluble and -insoluble parts.

The water solution was extracted with CHCl₃ (4 × 2 l) to give a bioactive brown residue (167 g) which was then subjected to silica gel chromatography, eluted with gradient petroleum ether/EtOAc (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, v/v). The fraction (4.2 g) eluted with petroleum ether/EtOAc (5:5, v/v) was further purified by repeated column chromatography (silica gel) eluting with CHCl₃ to afford the bioactive compounds **1** (215 mg) and **2** (85 mg).

The water-insoluble part was suspended with 50 g/l HCl (4 l) to get a brown solution after filtration, the pH was adjusted by dilute 10% NaOH to 8–9 to precipitate the total alkaloids. After filtration, washing with water for 6 times and drying, 208 g of crude total alkaloid extract was obtained a white powder. Subsequently, the crude alkaloids were subjected to silica gel column chromatography. The silica gel was first mixed with 1% hydrochloric acid and packed in column, and eluted with diethyl ether to get 3 g bioactive fraction. This fraction was further purified by silica gel chromatography (eluted with CHCl₃/acetone 9:1, v/v) to afford 142 mg bioactive compound **3**.

Bioassay for contact toxicity against larvae of Mythimna separata

The contact toxicities against larvae of *M. separata* of the three isolated compounds were determined by topical application with a microapplicator equipped with a 50 µl syringe (Miyazawa *et al.*,

1999). Immediately prior to treatment, insects were anaesthetized with carbon dioxide. Insects were treated with each of the compounds to be tested dissolved in acetone at concentrations of 1, 1.5, 2, 2.5, 3 g/l for **1**, 1, 2, 3, 4, 5 g/l for **2**, 2, 4, 6, 8, 10 g/l for **3** and toosendanin (positive control), respectively. Compound solutions (1 µl) were then delivered to the dorsal thorax of each insect using a Hamilton microapplicator. Each dose was replicated three times, with 20 insects per replicate in each Petri dish. In addition, control batches of insects were treated with acetone (control). After the treatment, insects were transferred into Petri dishes with fresh leaves. The Petri dishes were kept at 25–26 °C and relative humidity of 60–65%. Mortality was then examined after 48 h and the percentage kill was corrected for control mortality by Abbot's formula and the LD₅₀ values were calculated using log-probit analysis.

Bioassay for antifeedant activity against larvae of Mythimna separata

The antifeedant activity of the isolated compounds was tested by a conventional leaf disk method against third-instar larvae of *M. separata* (Abdelgaleil *et al.*, 1999). Five leaf discs (12 mm in diameter) of corn were cut and immersed in acetone solution of the compounds to be tested (five concentrations) for 2 s. After evaporation of the solvent, the five treated leaf discs as well as the other five control ones (immersed only in acetone) were placed together in one Petri dish (9 cm in diameter) close to the wall. Ten larvae were then placed in the center of the dish. Five different concentrations of each compound were assayed and three replicates of each concentration carried out. The percentage of antifeedance was then calculated by the results obtained after a 48-h treatment using the following equation: Antifeedance = $(C - T)/C \times 100$, where *C* is the weight of the leaf discs consumed in the control and *T* is the weight of the leaf discs consumed in the treatment. The ED₅₀ (effective doses causing 50% antifeedance) values were calculated by log-probit analysis.

Bioassay for ingested toxicity against larvae of Mythimna separata

The ingested toxicities of the isolated compounds against larvae of *M. separata* were determined by use of the sandwich method (Ellisor and Floyd, 1938). Leaf discs (10 mm in diameter) were

cut from corn leaves by means of a cork borer and kept in a moist chamber. Acetone solutions (2 μ l) of each compound to be tested at concentrations of 1 and 1.5 g/l were delivered to each leaf disc using a 50- μ l microapplicator. The side of disc treated with the test compounds was stuck with another uninoculated leaf disc together using starch paste in the form of a sandwich. Precisely weighed, 4 h pre-starved 5th instar larvae of *M. separata* were separately fed for 2 h with the treated leaf discs (one insect per Petri dish and totally 200 dishes were used), and the inoculated leaf discs were then replaced with fresh leaves to continuously rear the insects. 24 h after treatment the measurement of the leaf disc areas eaten by the insects, the corresponding amount of the compounds consumed by individual insects were subsequently converted to μ g per gram of insect body weight and the toxicity of the compounds were eventually evaluated by the median knock-down doses of the tested samples calculated with the method of Ellisor and Floyd (1938). All tested pests were arranged sequentially with the corresponding amount of the compounds consumed by individual insects and divided into narcosis zone (symptoms: the larvae were narcotized and could not move, the bodies were immobile and soft, the response disappeared completely), intermediate zone (partial narcosis and partial survival) and survival zone (all survive). The KD₅₀ (the dose required to knock down 50% of the population) values were calculated by the formula $(A + B)/2$, within which *A* represents the average dosage of the survived larvae in intermediate group and *B* represents the average dosage of narcotised larvae in intermediate zone.

Triptolide (1): C₂₀H₂₄O₆. – M.p. 226–227 °C (lit. 226–227 °C), $[\alpha]_D^{20} - 152^\circ$ (c, 0.3 in CH₂Cl₂). – IR: ν 3454 (OH), 1768 (lactone), 1679 cm⁻¹. – EIMS: $m/z = 360, 343, 313, 299, 271, 259, 241, 217$. – ¹H NMR (CDCl₃): $\delta = 0.8$ (3H, d, *J* = 7 Hz, 16-CH₃), 1.0 (3H, d, *J* = 7 Hz, 17-CH₃), 1.1 (3H, s, 20-CH₃), 3.4 (1H, *J* = 5 Hz, 7-H), 3.40 (1H, dd, *J* = 11, 1 Hz, 14-H), 3.52 (1H, dd, *J* = 3, 1 Hz, 12-H), 3.89 (1H, d, *J* = 3 Hz, 11-H), 4.70 (2H, m, 19-CH₂). – ¹³C NMR (DEPT): $\delta = 13.6$ (C-20, CH₃), 16.8 (C-16, CH₃), 17.0 (C-1, CH₂), 17.7 (C-17, CH₃), 23.4 (C-2, CH₂), 28.2 (C-15, CH), 29.8 (C-6, CH₂), 35.8 (C-10, C), 40.4 (C-5, CH), 54.5 (C-7, CH), 56.7 (C-11, CH), 60.0 (C-12, CH), 60.7 (C-13, C), 5.7 (C-8, C), 66.2 (C-9, C), 69.9 (C-19, CH₂), 73.4 (C-14, CH), 125.5 (C-3, C), 125.9 (C-4, C), 173.1 (C-18, C) (Kupchan *et al.*, 1972).

Triptonide (2): C₂₀H₂₂O₆. – M.p. 247–251 °C (lit. 252–253 °C). – $[\alpha]_D^{20} - 170^\circ$ (c, 0.1 in CH₂Cl₂). – IR: ν 1764 (lactone), 1680, 1723 cm⁻¹ (ketone). – FABMS (M+1): m/z 359. – ¹H NMR (CDCl₃): $\delta = 0.83$ (3H, *J* = 7 Hz, 16-CH₃), 0.96 (3H, d, *J* = 7 Hz, 17-CH₃), 1.10 (3H, s, 20-CH₃), 3.41 (1H, d, *J* = 5 Hz, 7-H), 3.81, 4.11 (d, *J* = 3 Hz, 11, 12-H), 4.69 (2H, m, 19-CH₂) (Kupchan *et al.*, 1972).

Euonine (3): C₃₈H₄₇NO₁₈, colorless prism crystals. – M.p. 148–153 °C; $[\alpha]_D^{20} - 2.5^\circ$ (c, 2 in CHCl₃). – IR: ν 3590, 3451 (OH), 3008 (aromatic = C-H), 1751 (ester), 1650, 1571 (piperidyl ring), 1438, 1263, 1044, 680 cm⁻¹. – HRMS: $m/z = 867.3016$. ¹H-NMR (CDCl₃): $\delta = 1.82, 1.98, 2.12, 2.28$ (12H, s, 4 × CH₃COO-), 2.16 (6H, s, 2 × CH₃COO-), 1.63 (3H, s, 14-CH₃), 1.55 (3H, s, 12-CH₃), 1.18 (3H, d, *J* = 6 Hz, 18-CH₃), 5.17 (1H, dd, *J* = 3.4 Hz, 2-H), 5.59 (1H, d, *J* = 4 Hz, 1-H), 4.92 (1H, d, *J* = 3.5 Hz, 3-H), 6.90 (1H, s, 5-H), 2.32 (1H, *J* = 4.03 Hz, 6-H), 5.54 (1H, dd, *J* = 6.4 Hz, 7-H), 5.34 (1H, d, *J* = 5.8 Hz, 8-H), 4.45, 5.23 (2H, ABq, *J* = 13.2 Hz, 11-CH₂), 3.76, 5.74 (2H, ABq, *J* = 12.1 Hz, 15-CH₂), 3.10 (2H, m, 16-CH₂), 2.35, 2.47 (2H, m, 17-CH₂), 5.36 (1H, m, 18-H), 7.41, 8.33, 8.72 (3H, q, 2', 3', 4'-H), 4.94 (1H, 4-OH, D₂O exchangeable). – ¹³C NMR (DEPT): $\delta = 73.5$ (C-1, CH), 69.3 (C-2, CH), 77.3 (C-3, CH), 69.8 (C-4, C), 73.7 (C-5, CH), 51.1 (C-6, CH), 69.0 (C-7, CH), 70.9 (C-8, C), 52.1 (C-9, C), 93.8 (C-10, C), 60.1 (C-11, CH₂), 22.7 (C-12, CH₃), 84.4 (C-13, C), 17.9 (C-14, CH₃), 70.3 (C-15, CH₂), 33.1 (C-16, CH₂), 33.2 (C-17, CH₂), 38.4 (C-18, CH), 21.5 (C-19, CH₃), 175.1 (C-20, C), 170.2 (C-21, C), 166.7 (C-22, C), 152.8 (C-2', CH), 121.2 (C-3', CH), 139.0 (C-4', CH), 124.5 (C-5', C), 164.8 (C-6', C), 1-Ac (20.5, CH₃, 169.3, C=O), 2-Ac (20.5, CH₃, 169.0, C=O), 5-Ac (21.0, CH₃, 168.7, C=O), 7-Ac (21.3, CH₃, 169.8, C=O) 8-Ac (20.5, CH₃, 170.1, C=O), 11-Ac (21.3, CH₃, 170.1, C=O) (Li and Strunz, 1990).

Acknowledgements

This work was supported by National Natural Science Foundation of China (30225048) and grant no. 003518 from the Ph. D. foundation of the Ministry of Education of China. We are grateful to Dr. J. W. Tan and Prof. S. Lin for useful discussion during the preparation of the manuscript.

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