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ORIGINAL ARTICLE

New nemadectin congener from *Streptomyces microflavus* neau3: fermentation, isolation, structure elucidation and biological activities

Wen-Sheng Xiang[1], Ji-Dong Wang[1,2], Ming Wang[1] and Xiang-Jing Wang[1]

Bioactivity-guided fractionation of *Streptomyces microflavus* neau3 fermentation broth was used to determine the chemical identity of bioactive constituents. The structure of the new compound 1 was determined on the basis of spectroscopic analysis, including 1D and 2D NMR, as well as HRESI-MS, ESI-MS, UV and IR, and compared with the data reported in the literature. The acaricidal and nematocidal activities of the isolated compound 1 and its previously reported analogs 2, 3, 4 and 5 were evaluated. The results demonstrate that compounds 1, 2, 3, 4 and 5, especially compound 1, have potential as antiparasitics with acaricidal and nematocidal activities. These lactones shed new insights into the structural diversity of nemadectin and milbemycin analog libraries that are potentially accessible by combinatorial biosynthesis.

INTRODUCTION

Microbial metabolites have attracted increasing attention as potential pesticides. They are expected to overcome the resistance and pollution that have accompanied the use of synthetic pesticides.[1,2] Several microbial metabolites, such as the avermectin and milbemycin families, have been proven to be potent preventatives against a variety of pests such as insects and parasites. Moreover, they are the biggest-selling and arguably most effective acaricides and anthelmintics currently available.[3–5] In addition, blasticidin, polyoxin, kasugamycin, validamycin and mildiomycin as fungicides, bialaphos as herbicide, and spinosad as insecticide have been widely used.[6] The excellent activities of these compounds suggest that other desirable pesticides will be discovered from microbial metabolites.[7–10]

We conducted experiments to screen for new metabolites as pesticides and antiparasitic veterinary drugs or as semi-synthetic intermediates. As a result, a new compound 1 (Figure 1) was isolated from the fermentation broth of *Streptomyces microflavus* neau3 strain, which was newly obtained from a soil sample. Compound 1 is reminiscent of nemadectin and seco-milbemycin compounds 2, 3, 4 and 5 (Figure 1) produced by *S. bingchenggensis*.[11,12] At the position of C-25, compound 1 has the same substituent as nemadectin, whereas it is different from compounds 2, 3, 4 and 5. Moreover, nemadectin is a 16-membered lactone, whereas compounds 1, 2, 3, 4 and 5 are six-membered lactones. This paper describes the fermentation, isolation, identification, and acaricidal and nematocidal activities of compound 1. In the course of this work, we also determined the acaricidal and

nematocidal activities of compounds 2, 3, 4 and 5 (Figure 1), which we previously reported.[11,12]

RESULTS AND DISCUSSION

Isolation and structural elucidation

Using bioactivity-guided separation of the components in the methanol extract of *S. microflavus* neau3 fermentation broth, compound 1 was afforded by twice silica gel column chromatography and semi-preparative HPLC. The two seco-milbemycins from the *S. bingchenggensis*, analogous of compound 1, were obtained using the same approach, demonstrating that the two new seco-milbemycins and corresponding milbemycin β_{14} were not converted to each other during purification by silica gel column and storage in MeOH extract at room temperature.[11]

Compound 1 was isolated as colorless oil. Its molecular formula was established as $C_{36}H_{50}O_7$ on the basis of HRESI-MS (m/z 617.3447 [M+Na]$^+$, calcd 617.3449 for $C_{36}H_{50}NaO_7$). The IR spectrum of compound 1 showed absorption bands assignable to the hydroxyl group (3447 cm^{-1}) and an ester carbonyl (1705 cm^{-1}). The ^{1}H NMR (400 MHz, CDCl$_3$) data (Table 1) of compound 1 showed one *trans* double bond at δ 5.90 (dd, $J=14.9, 7.0$ Hz) and δ 6.24 (dd, $J=14.9, 11.2$ Hz), one aromatic methyl at δ 2.24 (s), two vinylic methyls at δ 1.63 (br s) and 1.58 (d, $J=0.8$ Hz), and four aliphatic methyls at δ 0.79 (d, $J=6.9$ Hz), 0.93 (d, $J=6.6$ Hz), 0.98 (d, $J=6.6$ Hz) and 1.08 (d, $J=6.6$ Hz). Its ^{13}C NMR and DEPT data (Table 1) revealed an ester carbonyl at δ 165.6 (s), a ketal at δ 99.8 (s), four oxygenated methines,

[1]School of Life Science, Northeast Agricultural University, Harbin, PR China and [2]Zhejiang Hisun Pharmaceutical, Taizhou, PR China
Correspondence: Dr X-J Wang, School of Life Science, Northeast Agricultural University, No. 59 Mucai Street, Xiangfang District, Harbin 150030, PR China.
E-mail: wangxiangjing2008@yahoo.com.cn
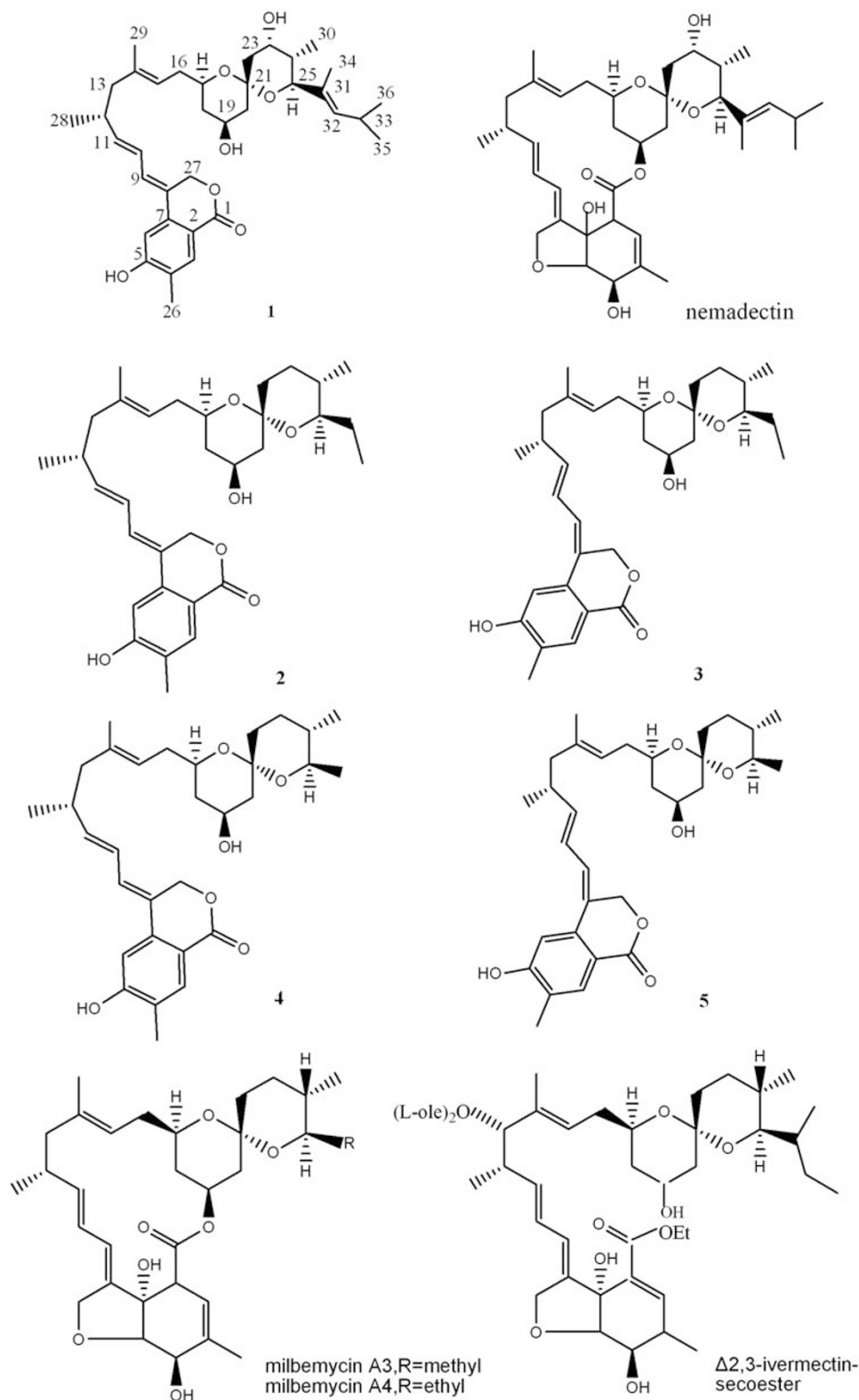
Figure 1 The structures of compounds 1, 2, 3, 4, 5, $\Delta^{2,3}$-ivermectin-secoester, nemadectin and milbemycin.

one oxygenated methylene, in addition to 14 sp^2 carbons, 5 aliphatic methylenes, 3 aliphatic methines and 7 methyls. Comparison of the ^{1}H and ^{13}C NMR data of compound 1 with those of seco-milbemycin A (compound 2, Figure 1)[11] suggested that compound 1 was structurally related to compound 2. The differences between compounds 1 and 2

could be explained by replacement of a methyl carbon and two methylene carbons in compound 2 by three methyl carbons, two methine carbons (one oxygenated) and two olefinic carbons in compound 1. The placement of the seven carbons in compound 1 was revealed by the ^{1}H–^{1}H COSY and HMBC correlations (Figure 2).

Table 1 ^{1}H and ^{13}C NMR data of compound 1 (coupling constants in parentheses)

Number	Proton	Carbon	Number	Proton	Carbon
1		165.6 s[a]	19	4.17 m	64.6 d
2		114.9 s	20	2.05 m	44.1 t
3	7.82 s	133.3 d		1.38 br t (11.8)	
4		126.1 s	21		99.8 s
5		160.3 s	22	1.98 m	41.0 t
6	6.97 s	107.5 d		1.73 m	
7		138.3 s	23	3.80 m	69.5 d
8		124.0 s	24	1.63 m	35.9 d
9	6.51 d (11.2)	127.7 d	25	3.69 (10.9)	76.7 d
10	6.24 dd (14.9, 11.2)	122.1 d	26	2.24 s	15.6 q
11	5.90 dd (14.9, 7.0)	146.3 d	27	5.08 br s	66.6 t
12	2.54 m	34.4 d	28	1.08 d (6.6)	19.7 q
13	2.17 m	47.8 t	29	1.63 br s	16.3 q
	1.98 m		30	0.79 d (6.9)	13.9 q
14		136.1 s	31		130.7 s
15	5.13 m	121.7 d	32	5.13 d (7.9)	137.4 d
16	2.28 m	34.1 t	33	2.54 m	26.8 d
17	3.71 m	69.1 d	34	1.58 d (0.8)	11.0 q
18	2.05 m	40.0 t	35	0.93 d (6.6)	22.8 q
	1.23 m		36	0.98 d (6.6)	22.8 q

[a]By DEPT sequence.

Figure 2 The key ^{1}H–^{1}H COSY, HMBC and NOESY correlations of compound 1.

In the HMBC spectrum of compound 1, the correlation from H-30 to C-23 indicated that the oxygenated methine was C-23. The correlations of H-35, H-36 and H-33 in the ^{1}H–^{1}H COSY spectrum and the HMBC correlations between H-35, H-36 and C-32, and between H-34, C-31 and C-32 connected the remaining six carbons as the 4-methyl-2-pentenyl moiety by analogy with the structure of nemadectin.[13,14] The correlation from H-34 to C-25 in the HMBC spectrum supported the linkage of the 4-methyl-2-pentenyl moiety with C-25 by C-31. Thus, the gross structure of compound 1 was established.

Table 2 Acaricidal and nematocidal activities of compounds 1, 2, 3, 4 and 5 compared with milbemycin A_3/A_4[a] and doramectin

	IC_{50} ($\mu g \, ml^{-1}$)[b]		
Compounds	Adult mite	Mite egg	Caenorhabditis elegans
1	24.9 ± 1.1	>100	15.4 ± 1.0
2	57.8 ± 1.4	>100	37.6 ± 1.6
3	63.2 ± 1.2	>100	45.3 ± 1.6
4	90.5 ± 1.7	>100	75.9 ± 1.2
5	98.5 ± 1.3	>100	81.4 ± 1.3
Milbemycin A_3/A_4	5.4 ± 1.7	50.1 ± 1.2	9.8 ± 1.2
Doramectin	7.3 ± 1.5	>100	2.7 ± 1.4

[a]Milbemycins A_3 and A_4 mixtures, 30:70 (in volume).
[b]Values are the means ± s.d.'s of three independent experiments.

The *E* configuration of the Δ^{10} olefin of compound 1 was assigned by the large coupling constant (14.8 Hz) between H-10 and H-11. The NOESY correlation signal between H-29 and H-16 revealed that the double bond of C-14 and C-15 was *E*. Especially, the evident NOESY signals between H-27 and H-10, and between H-6 and H-9 showed the presence of a *trans* (*E*) double bond between C-8 and C-9 (Figure 2).
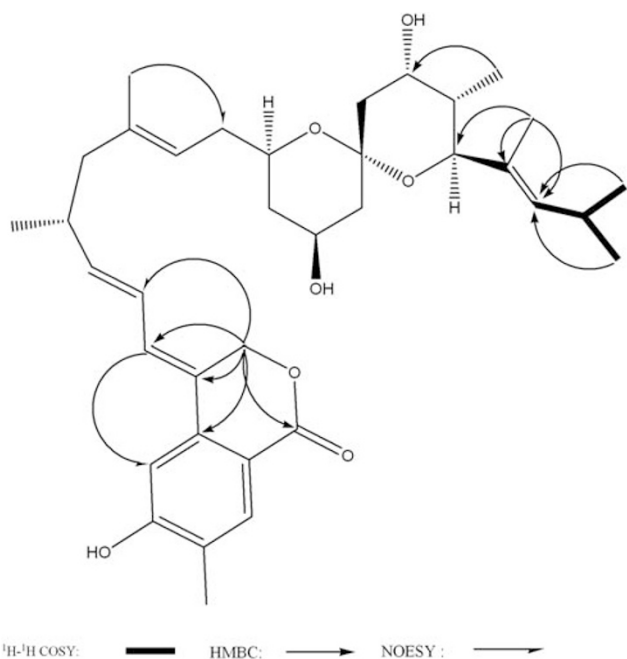
The configurations of the chiral centers and the 4-methyl-2-pentenyl moiety in compound 1 were assigned by analogy with nemadectin.[13] More research is needed in the future to obtain the absolute configuration of compound 1.

We have determined the 16S rDNA sequence of the strain (DQ 924968.1), which produces compound 1. This shared 99.51, 99.24, 99.10 and 99.37% identity with the 16S rDNA sequences of *S. microflavus* strains HBUM 174011 (FJ532427.1), HBUM 174884 (FJ481633.1), HBUM 174246 (FJ486435.1) and HBUM 174098 (FJ 486343.1), respectively, thereby characterizing strain neau3 as a *S. microflavus*. Compared with nemadectin producers *S. cyaneogriseus* ssp. *noncyanogenus*[13] and *S. thermoarchaensis*,[15] *S. microflavus* neau3 belongs to a distinct species. Although compound 1 is analogous to the seco-milbemycins (compounds 2, 3, 4 and 5, Figure 1)[11,12] produced by *S. bingchenggensis*, the 16S rDNA sequence of *S. microflavus* neau3 (DQ 924968.1) only shared 93.81% identity with that of *S. bingchenggensis* (DQ449953).

Acaricidal and nematocidal activities of the new compound 1 and compounds 2, 3, 4 and 5

Compound 1 from the *S. microflavus* neau3 and compounds 2, 3, 4 and 5 from *S. bingchenggensis* were tested for their acaricidal and nematocidal activities. The acaricidal and nematocidal capacities of these compounds were compared with the two known standard acaricides and nematocides, milbemycin A_3/A_4 and doramectin, in the same assay. As shown in Table 2, although compounds 1, 2, 3, 4 and 5 showed lower acaricidal potencies against adult mites and nematocidal activities against *Caenorhabditis elegans* than doramectin and milbemycin A_3/A_4, compound 1 possessed relatively higher acaricidal and nematocidal activities.

Although compound 1 is an analog of nemadectins and milbemycins, we were surprised at these findings. Thus, we determined the bioactivities of compounds 2, 3, 4 and 5, which we previously reported.[11,12] These seco-compounds showed unexpected potencies. It is also reported that $\Delta^{2,3}$-ivermectin-secoester (Figure 1) has a high leishmanicidal activity.[16]

The discovery of compounds 1, 2, 3, 4 and 5 provides new insights into the biosynthesis of nemadectin and milbemycin. Homologous thioesterases[17] from *S. microflavus* neau3 and *S. bingchenggensis* presumably are responsible for cyclizations resulting in both 16- and (or) 6-membered lactones. Therefore, this represents new opportunities for macrolide modifications and a new analog design to further expand the size and diversity of nemadectin and milbemycin analog libraries.

METHODS

General procedures and reagents

UV spectra were obtained on a Varian CARY 300 BIO spectrophotometer (Varian, Palo Alto, CA, USA). IR spectra were recorded on a Nicolet Magna FT-IR 750 spectrometer (Nicolet, Madison, WI, USA); ^{1}H and ^{13}C NMR spectra were measured with a Bruker DRX-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts are reported as p.p.m. (δ), using the residual CHCl$_3$ (δ_H 7.26; δ_C 77.0) as an internal standard, and coupling constant (J) in Hz. ^{1}H and ^{13}C NMR assignments were supported by ^{1}H–^{1}H COSY, HMQC and HMBC experiments. The ESI-MS and HRESI-MS spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer (Micro, Milford, MA, USA). Optical rotation was measured on a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Fremont, CA, USA). Column chromatography was carried out on silica gel (100–200 mesh, Qingdao Haiyang Chemical Group, Qingdao, Shandong, China). Semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250×9.4 mm i.d.; Agilent, Palo Alto, CA, USA) was further performed to obtain the pure compound. Spots were detected on TLC under UV or by heating after spraying with sulfuric acid–ethanol, 5:95 (v/v). All chemicals used in the study, such as methanol (MeOH), ethyl acetate (EtOAc), petroleum ether (60–90 ºC) and acetone, were analytical grade.

Microorganism

The producing organism, *S. microflavus* neau3, was isolated from a farmland in Laifeng, Hubei province, China, by using selective media humic acid–vitamin medium,[18] with 20 μg ml^{-1} nalidixic acid. *S. microflavus* neau3 has been deposited at the China General Microbiological Culture Collection Center (Accession No.: CGMCC 3316), Institute of Microbiology, Chinese Academy of Sciences. The 16S rDNA sequence (Accession No.: DQ 924968.1 in GenBank, National Center for Biotechnology Information) was determined.

Fermentation

The strain was maintained on the medium containing glucose (Beijing Ao Bo Xing, Beijing, China) 10 g, maltose (Beijing Ao Bo Xing) 3 g, yeast extract powder (Beijing Ao Bo Xing) 3 g, K$_2$HPO$_4$ 3H$_2$O 0.5 g, MgSO$_4$ 7H$_2$O 0.5 g, NaCl 0.5 g, KNO$_3$ 1 g and agar 20 g in 1.0 l tap water, pH 7.0. The seed medium consisted of glucose (Beijing Ao Bo Xing) 5 g, maltodextrin (Beijing Ao Bo Xing) 5 g, soluble amylum (Beijing Ao Bo Xing) 1 g, yeast autolysate (Beijing Ao Bo Xing) 5 g, beef extract (Beijing Ao Bo Xing) 1 g and casein peptone (Beijing Ao Bo Xing) 1 g in 1.0 l water and pH 7.0. All the media were sterilized at 121 °C for 20 min. Slant culture was incubated for 8–9 days at 28 °C.

A total of 10 ml of sterile water was added to the slant medium. The spores were scraped and transferred to the sterile tube of beading. The spore suspension was then filtered through six layers of sterile filter cheesecloth and adjusted to 10^7–10^8 c.f.u. ml^{-1}. In all, 2.0 ml of the spore suspension was inoculated into a 250-ml flask comprising 25 ml of seed medium and incubated at 28 °C for 46 h at 250 r.p.m. Then, 8.0 ml of the culture was transferred into a 1-l Erlenmeyer flask containing 100 ml of the producing medium consisting of glucose (Beijing Ao Bo Xing) 1%, lactose (Beijing Ao Bo Xing) 2.5%, cotton seed powder (Beijing Ao Bo Xing) 2.5% and CaCO$_3$ 0.03%, pH 7.0, before sterilization. Fermentation was carried out at 28 °C for 7–8 days on a rotary shaker at 250 r.p.m.

Isolation and purification of compound 1

A total of 30 l broth from 300 producing fermentations was filtered. The resulting cake was washed with water, and both filtrate and wash were discarded. The washed cake was extracted twice for about 24 h with MeOH (10 l). The MeOH extract was evaporated under reduced pressure to approximately 2 l at 45 °C. The resulting concentrate was extracted three times using an equal volume of EtOAc. The combined EtOAc phase was concentrated under reduced pressure to yield 25 g of oily substances. The residual oily substance was chromatographed on silica gel (90×5 cm i.d.; Qingdao Haiyang Chemical Group; 100–200 mesh) and eluted with a petroleum ether (60–90 °C)–acetone mixture (95:5, 75:25, 50:50, v/v; each 3 l) of increasing polarity to afford three fractions. Fraction III eluted with a mixture of petroleum ether–acetone (50:50, v/v) had the acaricidal activity against adult mites. This was further separated over silica gel column chromatography (70×2.5 cm i.d.) eluted with CHCl$_3$–MeOH (95:5, 90:10 and 85:15, v/v; each 2 l) to give three subfractions. Subfraction II was eluted with a mixture of CHCl$_3$–MeOH (90:10, v/v) having acaricidal activity, and it was separated by semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250×9.4 mm i.d.; 1.5 ml min^{-1}; 260 nm; Agilent) by eluting with CH$_3$OH–H$_2$O (85:15, v/v) to afford compound 1 (Figure 1; t_R 10.2 min, 30 mg).

Compound 1 (C$_{36}$H$_{50}$O$_7$)

Seco-nemadectin, colorless oil; $[\alpha]^{25}_D$ +45.1 (c. 0.10, EtOH); UV (EtOH) λ_{max} nm (log ε): 270 (4.16), 296 (4.03); IR (KBr), ν_{max} cm^{-1} 3447, 2961, 2927, 1705, 1613, 1457, 1383, 1282, 1162, 997; ^{1}H NMR (CDCl$_3$, 400 MHz) δ ; ^{13}C NMR (CDCl$_3$, 100 MHz) δ (see Table 1); ESI-MS m/z 593 [M-H]$^-$; HRESI-MS m/z 617.3447 [M+Na]$^+$, calcd for C$_{36}$H$_{50}$NaO$_7$ 617.3449.

Acaricidal activity against adult mites and bioactivity-guided fractionation

MeOH solutions containing 0.1% of the individual pure compounds were diluted 10-fold with water containing 0.01% of detergent to prepare 100 μg ml^{-1} solutions. Then appropriate further dilutions were prepared. Two-spotted spider mites, sensitive to organophosphorus insecticides, were inoculated on the primary leaves of cowpea plants. One day after inoculation, the leaves of the cowpea plants were soaked in the sample solutions for 1–2 s. Then the leaves were kept at 25 °C. After 3 days, the survival of the adult insects was determined with a binocular microscope and the mortality (%) was calculated. Two-spotted spider mites were also used for bioactivity-guided fractionation.

Acaricidal activity against mite eggs

Sample solutions containing 100, 50, 30 and 10 μg ml^{-1} of individual compounds were prepared. Female adult two-spotted spider mites were allowed to lay eggs on the primary leaves of cowpea plants. The adult mites were removed to obtain test leaves each bearing about 40 eggs. In a manner similar to the preceding example, the test leaves were soaked in the sample solutions for 1–2 s. After 10 days at 25 °C the number of unhatched eggs was counted, and the unhatched egg rates (%) were calculated.

Nematocidal activity

MeOH solutions containing 0.1% of individual compounds were diluted 10-fold with water to prepare solutions containing 100 μg ml^{-1}. Then appropriate amounts of the solutions were added to 1-l portions of an aqueous suspension containing living nematodes *C. elegans*. The mixtures were left at 25 °C for 15 h after shaking. The number of nematodes that were immobilized and the total number of nematodes tested were counted under a stereoscopic microscope. Immobilized rates (%) against the total number of tested nematodes were calculated.

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