15 p. 2 fig. electronically pre-edited, REVISED version **Dr. K.-Q. Zhang**

Setzer: Nur Änderungen sind explizit angegeben

C07042. Nematicidal Metabolites Produced by the Endophytic Fungus *Geotrichum* sp. AL4

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Note for the Authors

1. The ORD data of achiral **4** (!) were deleted in the *Exper. Part*
2. Please do not list NMR data according to atom numbering, when in the *Exper. Part*. This in only done in Tables. Otherwise, list according to chemical shift.

From the endophytic fungal strain *Geotrichum* sp. AL4, cultivated from the leaves of the neem tree (*Azadirachta indica*), four compounds, **1** – **4**, were isolated from the AcOEt extract, including two new, chlorinated, epimeric 1,3-oxazinane derivatives. All compounds were assessed for their nematicidal activities against the nematodes *Bursaphelenchus xylophilus* and *Panagrellus redivivus*, and three out of the four isolates showed noticeable bioactivities.

Introduction. – Endophytes are commonly found in almost all plants. These fungi are important sources of biologically active natural products. Many compounds of endophytic microbes have been used in medicine, agriculture, and industry [1 – 4]. Endophytes also play important roles in the ecology of plant communities [5]. Among them, endophytes in plants with medicinal and pesticidal properties have attracted significant attention in recent years.

Azadirachta indica A. JUSS. (Meliaceae), commonly called '*neem*', is one of the most widely used medicinal plants. It is widely distributed in Asia, Africa, and other tropical parts of the world. This tree exhibits diverse biological activities, and almost every part has been used for medicinal purposes [6 – 8]. Besides medicinal applications, there are also reports about the antiparasitic activity of *A. indica*, including nematicidal activity [9 – 11]. Whereas some investigations on endophytes from this tree have been performed [12] [13], there are only few reports on their metabolites.

Herein, we describe the isolation, structure elucidation, and nematicidal properties of the endophytic metabolites **1** – **4** obtained from the mycelial extract of the strain *Geotrichum* sp. AL4, which was cultivated from the leaves of *A. indica*.

Formeln v. S. 14

Results and Discussion. – 1. *Structure Elucidation*. Compound **1** was obtained as a colorless, amorphous solid. Its ^{13}C-NMR (DEPT) data (*Table 1*) showed only seven signals, including two Me, one CH_2, and three CH groups, as well as one quaternary C-

atom. In the ^{1}H-NMR spectrum, the Me signals appeared at δ(H) 2.13 (*s*) and 0.77 (*d*, *J* = 7.2 Hz). An HMBC experiment (*Table 1*) showed correlations between the Me H-atoms and the C-atoms[1]) at δ(C) 207.0 (C(2)), 53.0 (C(5')), and 42.5(C(4')), between δ(H) 5.07 (H–C(2')) and δ(C) 12.3 (4'-Me), 42.5 (C(4')), 53.0 (C(5')), and 66.1 (C(6')), between δ(H) 2.53 (H–C(4')) and δ(C) 12.3 (4'-Me), 53.0 (C(5')), 66.1 (C(6')), and 104.0 (C(2')), between δ(H) 3.60 (H–C(5')) and δ(H) 12.3 (4'-Me), 42.5 (C(4')), 66.1 (C(6')), and 207.0 (C(2)), and between δ(H) 4.18/3.94 (CH_2(6')) and δ(C) 42.5 (C(4')), 53.0 (C(5')), 104.0 (C(2')), and 207.0 (C(2)).

Table 1 v. S. 11

The ^{1}H,^{1}H-COSY spectrum of **1** showed that H–C(5') correlated with CH_2(6'), and H–C(4') correlated with H–C(5') and 4'-Me. The relative configuration of **1** was deduced from ROESY experiments, which indicated correlations between Me-4' and both H–C(2') and H–C(5'). From the above data, compound **1** was identified as 1-[(2*R**,4*S**,5*S**)-2-chloro-4-methyl-1,3-oxazinan-5-yl]ethanone – a new chlorinated oxazinane derivate.

Compound **2** was obtained as a colorless, amorphous solid. The ^{13}C-NMR (DEPT) data (*Table 1*) only showed seven carbon signals: two Me, one CH_2, and three CH groups, and one quaternary C-atom. The IR and NMR data of **2** were very similar to those of compound **1**. An HMBC experiment (*Table 1*) showed correlations between

[1]) Arbitrary atom numbering.

δ(H) 2.13 (H–C(1)) and δ(C) 208.3 (C(2)), 56.2 (C(5')), and 42.3 (C(4')), between δ(H) 5.26 (H–C(2')) and δ(C) 12.4 (4'-Me), 42.3 (C(4')), 56.2 (C(5')), and 68.3 (C(6')), between δ(H) 2.53 (H–C(4')) and δ(C) 12.4 (4'-Me), 56.2 (C(5')), 68.3 (C(6')), and 99.9 (C(2')), between δ(H) 3.07 (H–C(5')) and δ(C) 12.4 (4'-Me), 42.3 (C(4')), 68.3 (C(6')), and 208.3 (C(2)), and between δ(H) 4.17/3.82 (CH_2(6')) and δ(C) 42.3 (C(4')), 56.2 (C(5')), 99.9 (C(2')), and 208.3 (C(2)). After careful analysis of the ROESY spectrum, there were differences in the NOEs of **2** compared to **1**: Me-4' was correlated with H–C(2'), and H–C(4') was correlated with H–C(5'). So, compound **2** was determined to be an epimer of **1**, corresponding to 1-[(2*R**,4*S**,5*R**)-2-chloro-4-methyl-1,3-oxazinan-5-yl]ethanone.

The structures of the two known compounds were determined as [2,3-dihydro-2-(1-methylethenyl)-1-benzofuran-5-yl]methanol (**3**) [14] and 1-(2,4-dihydroxyphenyl)ethanone (**4**) [15] by spectroscopic comparison with literature data.

2. *Biological Activity*. Compounds **1** – **4** were assessed for their nematicidal activities. Compounds **1**, **2**, and **4** showed noticeable activities against the two tested nematodes (*Table 2*). In contrast, compound **3** did not show any nematicidal activity at a concentration of 100 µg/ml after 48 h of exposure. The two new compounds exhibited similar activities, both killing *ca*. 50% of *Panagrellus redivivus* and 60% of *Bursaphelenchus xylophilus* within 48 h at 100 µg/ml. Compound **4** is commonly isolated from plants. However, this is the first report of this compound from a microbial source showing nematicidal activity.

Table 2 v. S. 12

The positive control, avermectin (0.5 µg/ml), caused a high mortality (89.2%) of *B*. *xylophilus* after 48 h, but was inactive against *P*. *redivivus*. The observed activities of the isolates are certainly weak compared to that of commercial avermectin (in the case of *B*. *xylophilus*). However, their effect is quite astonishing when considering their very simple chemical structures, compound **4** even lacking a stereogenic center. A series of simple aromatic fungal compounds – including *para*-anisaldehyde, *para*-anisyl alcohol, 1-(4-methoxyphenyl)propane-1,2-diol, '2-hydroxy-(4'-methoxy)propiophenone' [16], '3-*para*-anisoloxypropionate' [17], flavipin [18], 3,5-dihydroxy-4-(3-methyl-but-2-enyl)benzene-1,2-dicarbaldehyde, and butyl 2,4-dihydroxy-6-methylbenzoate – were also found to exhibit weak-to-moderate activities against different nematodes [19].

The biological roles of most endophytic microbes and their plant hosts are not well-studied [20], but as one of the main biological resources, endophytic microbes have been used to search for new agricultural chemicals. 3-Hydroxypropanoic acid was obtained as a nematicidal compound from several endophytic fungi isolated from above-ground plant organs [21]. It has been reported that *A*. *indica* possesses nematicidal activity [9 – 11], but no investigations about this nematicidal activity from its endophyte have been performed so far. Most of the compounds from the plant are triterpenoids and, as main active constituents, tetranor-triterpenoids. The endophyte *Geotrichum* sp. AL4 from *A*. *indica* produces different types of compounds; and the

present work describes, for the first time, the isolation of a nematicidal endophyte with novel active components from the widely used neem tree.

This work was partially supported by the *Science and Technology Department of Yunnan Province* (No. 2005NG05 and 2005NG03), the *Natural Science Foundation of Yunnan Province* (No. 2004C0003Q), the *Key Applied Foundation Program of Yunnan Province* (No. 1999C0001Z), and the *Yunnan Provincial Education Department Program* (No. 5Z0226B).

Experimental Part

General. TLC: Precoated silica gel *G* plates (*Qingdao Marine Chemical Factory*, Qingdao, China). Column chromatography (CC): *Sephadex LH-20* (*Pharmacia*); silica gel *G* (200 – 300 mesh), or silica gel *H* (*Qingdao Marine Chemical Factory*). Optical rotation: *Jasco DIP*-370 polarimeter. IR Spectra: *Paragon 1000pc* spectrometer, as KBr pellets; in cm^{-1}. NMR Spectra: *Bruker DRX-500* spectrometer; δ in ppm rel. to Me_4Si, *J* in Hz. ESI-MS: *Finnigan LCQ-Advantage* mass spectrometer; in *m*/*z*.

Microbial Material. *Azadirachta indica* A. JUSS. was collected at Xishuangbanna, Yunnan Province, P. R. China. The leaves of the plant were washed with running tap water, sterilized successively with 75% EtOH (1 min) and 0.12% sodium hypochlorite (4 min), then rinsed in sterile H_2O (5×), and finally cut into small pieces, which were incubated at 26° on *YMG* medium (4 g yeast extract, 10 g malt

extract, 4 g glucose, and 15 g agar in H_2O (1 l)). The plant material was cultured until colony or mycelium appeared, surrounding the leaf segments. A specific strain (*Geotrichum* sp. AL4) was isolated from the sterilized leaves, and deposited at the Key Laboratory for Conservation and Utilization of Bio-Resources of Yunnan Province, P. R. China. The isolated strain was grown in shake culture (120 ml per 250 ml triangular flask) on *PDB* medium (200 g potato and 20 g dextrose in H_2O (1 l)). After fermentation for 10 d at 26° at 140 r.p.m., the cultures (10 l) were filtered to obtain the mycelia.

Extraction and Isolation. The mycelia were dried in an oven at 50°, and then extracted with AcOEt (3×). The extract was concentrated under reduced pressure, and the resulting residue was subjected to bioassay-guided chromatographic separation. The residue (2.42 g) was first subjected to CC (50 g *Sephadex LH-20*; acetone) to yield eight fractions (*Fr. A1 – A8*). *Fr. A3* (150 mg) was re-subjected to CC (10 g silica gel *G*; petroleum ether (PE)/acetone 9:1 → 5:1) to afford seven subfractions (Fr. A3.1 – A3.7). *Fr. A3.2* (15 mg) was purified further by CC (1.5 g silica gel *H*; $CHCl_3$) to afford **3** (8 mg). *Fr. A3.3* (20 mg) was also subjected to CC (1. 2 g silica gel *G*, PE/AcOEt 9:1; 2. 10 g *Sephadex LH-20*, acetone) to yield **4** (10 mg). *Fr. A3.4* (120 mg) was purified by repeated CC (1. 20 g *Sephadex LH-20*, acetone; 2. 10 g silica gel *G*, PE/AcOEt 7:1) to furnish **1** (30 mg) and **2** (20 mg).

*1-[(2*R**,4*S**,5*S**)-2-Chloro-4-methyl-1,3-oxazinan-5-yl]ethanone* (**1**). Colorless, amorphous solid. $[\alpha]_D^{25} = +25.00$ (c = 0.1, $CHCl_3$). IR (KBr): 3416, 2974, 1712, 1366.

^{1}H-, ^{13}C-, and 2D-NMR: see *Table 1*. EI-MS: 177 (M^+, $C_7H_{12}ClNO_2^+$).

*1-[(2*R**,4*S**,5*R**)-2-Chloro-4-methyl-1,3-oxazinan-5-yl]ethanone* (**2**). Colorless, amorphous solid. $[\alpha]_D^{25}$ = +18.00 (*c* = 0.09, $CHCl_3$). IR (KBr): 3412, 2972, 1715, 1378. ^{1}H-, ^{13}C-, and 2D-NMR: see *Table 1*. EI-MS: 177 (M^+, $C_7H_{12}ClNO_2^+$).

[2,3-Dihydro-2-(1-methylethenyl)-1-benzofuran-5-yl]methanol (**3**). Colorless powder. $[\alpha]_D^{25}$ = +9.50 (*c* = 0.2, $CHCl_3$). IR (KBr): 3408, 2986, 1624, 1500, 1448, 1246, 1012. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.21 (*s*, H–C(4)); 7.14 (*d*, *J* = 8.0, H–C(6)); 6.81 (*d*, *J* = 8.1, H–C(7)); 5.23 (*t*, *J* = 8.7, H–C(2)); 5.12 (*s*, H–C(3')); 3.39 (*dd*, *J* = 9.6, 15.6, H_α–C(3)); 3.09 (*dd*, *J* = 8.1, 15.6, H_β–C(3)); 4.95 (*s*, H–C(3')); 4.61 (*s*, CH_2(4')); 1.81 (*s*, Me). ^{13}C-NMR (125 MHz, $CDCl_3$): 160.6 (C(7a)); 144.9 (C(2')); 129.2 (C(5)); 128.4 (C(6)); 128.0 (C(3a)); 125.5 (C(4)); 112.9 (C(3')); 109.9 (C(7)); 86.9 (C(2)); 66.3 (C-(4')); 35.5 (C(3)); 18.1 (Me). ESI-MS: 191 ($[M + H]^+$).

2',4'-Dihydroxyacetophenone (*= 1-(2,4-Dihydroxyphenyl)ethanone*; **4**). Colorless powder. IR (KBr): 3304, 1640, 1610, 1522, 1448. ^{1}H-NMR (500 MHz, $CDCl_3$): 12.71 (*s*, OH); 7.64 (*d*, *J* = 8.6, H–C(6)); 6.54 (*s*, H–C(3)); 6.42 (*d*, *J* = 8.6, H–C(5)); 2.56 (*s*, Me). ^{13}C-NMR (125 MHz, $CDCl_3$): 203.4 (C=O); 165.5 (C(4)); 163.4 (C(2)); 133.6 (C(6)); 114.6 (C(1)); 108.4 (C(5)); 103.9 (C(3)); 26.6 (Me). ESI-MS: 151 ($[M - H]^-$).

Nematicidal-Activity Assay. The saprophytic nematode *Panagrellus redivivus* (LINN.) Goodey and the pine-wood nematode *Bursaphelenchus xylophilus* (STEINER et BUHRER) NICKLE were cultured according to reference [22]. Each compound was dissolved in acetone, and diluted to 100 and 50 μg/ml with sterile H_2O for the assay. As

a standard, avermectin (pos. control) and 5% acetone (neg. control) were used. The numbers of active and inactive nematodes were counted after 24 and 48 h, resp. The nematodes were considered to be dead when they showed no response to physical stimuli, and the toxicity was estimated based on the percentage of dead nematodes. Each treatment was replicated three times (n = 3), and the mean percentage mortality was calculated. The experiments were repeated twice.

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Received February 5, 2007

Table 1. *^{1}H-, ^{13}C-, and 2D-NMR Data of* **1** *and* **2**. At 500/125 MHz, resp., in $CDCl_3$; chemical shifts δ in ppm, coupling constants *J* in Hz. Arbitrary atom numbering (see chemical formulae).

Position	**1**			**2**		
	δ(C)	δ(H)	HMBC (H→C)	δ(C)	δ(H)	HMBC (H→C)
1	30.2 (*q*)	2.13 (*s*)	2, 4', 5'	30.2 (*q*)	2.13 (*s*)	2, 4', 5'
2	207.0 (*s*)	–	–	208.3 (*s*)	–	–
2'	104.0 (*d*)	5.07 (*s*)	4'-Me, 6', 4', 5'	99.9 (*d*)	5.26 (*s*)	6', 4', 5'
4'	42.5 (*d*)	2.47 – 2.53 (*m*)	4'-Me, 5', 6', 2'	42.3 (*d*)	2.46 – 2.53 (*m*)	4'-Me, 5', 6', 2'
5'	53.0 (*d*)	3.56 – 3.60 (*m*)	4'-Me, 4', 6', 2	56.2 (*d*)	3.02 – 3.07 (*m*)	4'-Me, 4', 6', 2
6'	66.1 (*t*)	4.18 (*t*, *J* = 7.2)	5', 6', 2', 2	68.3 (*t*)	4.17 (*t*, *J* = 7.2)	5', 6', 2', 2

		3.94 (*t*, *J* = 4', 5', 2', 2 7.3)			3.82 (*t*, *J* = 5', 6', 2', 2 7.2)
4'-Me	12.3 (*q*)	0.77 (*d*, *J* = 4', 5', 2' 7.2)	12.4 (*q*)		1.03 (*d*, *J* = 4', 5', 2', 2 7.3)

Table 2. *Nematicidal Activities of* **1**, **2**, *and* **4**. All averaged values (n = 3; ± S.D.) refer to % mortality at the time indicated. Compound **3** was inactive in this assay.

Compound	Concentration [µg/ml]	*Panagrellus redivivus*		*Bursaphelenchus xylophilus*	
		24 h	48 h	24 h	48 h
1	100	39.2 ± 1.8	53.3 ± 2.1	54.8 ± 2.2	62.3 ± 2.4
	50	27.4 ± 1.2	41.3 ± 1.7	25.6 ± 1.3	35.5 ± 1.6
2	100	36.7 ± 1.7	47.2 ± 2.0	52.3 ± 2.3	64.2 ± 2.7
	50	22.3 ± 1.0	36.8 ± 1.3	27.8 ± 1.2	39.6 ± 1.7
4	100	45.3 ± 1.8	55.8 ± 2.6	41.9 ± 1.6	54.8 ± 2.3
	50	26.7 ± 1.3	45.8 ± 1.6	27.6 ± 1.5	37.9 ± 1.7
Avermectin[a])	0.5	0	0	83.6 ± 3.4	89.2 ± 3.6
	0.25	0	0	54.2 ± 2.1	62.3 ± 2.6
Acetone[b])		0	0	0	0

[a]) Pos. control (towards *B. xylophilus*). [b]) 5% (*v*/*v*) Acetone in H_2O (neg. control).