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Nematicidal Resorcylides from the Aquatic Fungus *Caryospora callicarpa* YMF1.01026

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Abstract This study investigated metabolites with activities against plant parasite nematodes from the fresh-water fungus *Caryospora callicarpa* YMF1.01026. We obtained three novel tetradecalactone metabolites, caryospomycins **A**–**C**, with such activities. The chemical structures of these were determined through NMR spectroscopic analysis and were found to belong to the 14-membered macrolides with a fused 1,2-dimethoxy-4-hydroxybenzene ring, a rare structure among the resorcylides. In the *in vitro* tests, all three compounds exhibited moderate killing activity against the nematode *Bursaphelenchus xylophilus*. To our knowledge, this is the first report of secondary metabolites in the aquatic fungal genus *Caryospora*.

Keywords *Caryospora carllicarpa* · Freshwater fungi · Nematicidal · Resorcylide

Introduction

The annual global loss in agriculture due to damages by plant-parasitic nematodes has been estimated as US $100 billion worldwide (Sasser and Freckman 1987). To reduce economic loss, several procedures for nematode control have been developed that use biological control agents and organic amendments. However, none of the procedures is ideal, and

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there is an urgent need for alternative, environmentally friendly measures, including natural nematocidal compounds (Noling and Becker 1994). One way to search for such compounds is to screen naturally occurring fungi. Fungi are important sources of naturally occurring antibiotics and pesticides. In addition, fungal secondary metabolites often have low plant and human toxicity and can be easily biodegraded (Siddiqui and Mahmood 1996). Compounds with nematicidal activity include alkaloids, peptides, terpenes, and fatty acids (Anke and Sterner 1997; Dong et al. 2005a, b, 2006). Omphalotin A, a cyclic dodecapeptide isolated from *Omphalotus olearius* (Mayer et al. 1997; Sterner et al. 1997), under *in vitro* conditions, out-performs known nematicides such as ivermectin in both potency and selectivity (Mayer et al. 1999).

These findings prompted us to conduct an evaluation of other little characterized members of freshwater fungi. Specifically, we were interested in the extracts of strain YMF1.01026 that belongs to species *Caryospora callicarpa* (Cai et al. 2002). This strain has shown high nematicidal activities towards the pine wood nematode, *Bursaphelenchus xylophilus* (Dong et al. 2004). In addition, there is so far no record of any previous investigation of the chemistry of this genus. The genus belongs to subfamily Zopfiaceae of the Dothideomycetidae and includes 15 species (*Caryospora australiensis*, *C. callicarpa*, *Caryospora cariosa*, *Caryospora coffeae*, *Caryospora langloisii*, *Caryospora lichenopsis*, *Caryospora mangrovei*, *Caryospora masonii*, *Caryospora minima*, *Caryospora minor*, *Caryospora nuclearia*, *Caryospora olearum*, *Caryospora phyllostachydis*, *Caryospora putaminum*, and *Caryospora striata*). In ths study, we report the bioassay-guided fractionation of extracts of *C. carllicarpa* YMF1.01026 that were tested against pine wood nematode, *B. xylophilus*.

Methods and Materials

Culture and Fermentation of C. callicarpa YMF1.01026 The fungal strain *C. callicarpa* YMF1.01026 was initially isolated from a submerged woody substrate collected from a freshwater habitat in Yunnan Province, China. This strain is deposited in the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Yunnan Province, the People's Republic of China (culture collection number YMF1.01026). The strain was maintained on PDA medium (potato 200 g, sucrose 20 g, agar 18 g, and water 1,000 ml) and was grown on wheat at 26°C for a period of 30 d before being processed for extracts.

Extraction and Isolation of Compounds Mycelial cultures were lyophilized and extracted with CH_3OH. The CH_3OH solution, after concentrated through vacuuming, was sequentially extracted ×3, each with petroleum ether and EtOAc. The combined EtOAc solution, upon evaporation, yielded a deep-brown syrup (~3.2 g). The syrup had *in vitro* nematicidal activity against pine wood nematodes *B. xylophilus*. This syrup was loaded onto a silica gel column [200 g Silica gel G (200–300 mesh), 3.6 cm i.d.×150 cm] and eluted by using solvent mixtures containing petroleum ether (bp 60–90°C)-EtOAc with increasing polarities (95:5 to 10:90). The resulting fractions were monitored by TLC (Silica gel G, 0.25-mm film thickness; Qingdao Marine Chemical Ltd., Qingdao, China) and reduced to one active fraction, **FV** (430 mg). The **FV** fraction, obtained on elution with petroleum (bp 60–90°C)-EtOAc (30:37), was further separated on a Sephadex LH-20 gel column and eluted with CH_3COCH_3 to yield pure **1** (16 mg), **2** (11 mg), and **3** (8 mg), respectively (Fig. 1).

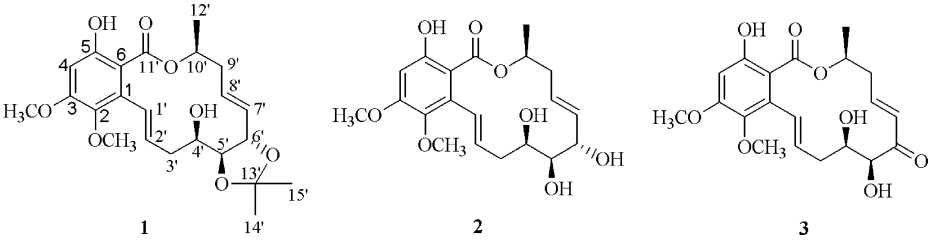

Fig. 1 Structures of caryospomycin **A**–**C** (**1**–**3**)

Identification of Compounds The structures of caryospomycins isolated from the cultures of *C. carllicarpa* YMF1.01026 were determined by spectroscopic analysis. Infrared (IR) spectra were obtained in KBr pellets with a Bio-Rad FTS-135 spectrophotometer (Bio-Rad, Richmond, CA, USA). UV spectra were taken on a Shimadzu double-beam 210A spectrophotometer (Shimadzu, Kyoto, Japan). Optical rotations were measured with a Horiba SEPA-300 polarimeter (Horiba, Tokyo, Japan). MS was performed on an Autospec-3000 spectrometer (VG, Manchester, England). Nuclear magnetic resonance (NMR) spectra were recorded on DRX-500 NMR (Bruker, Karlsruhe, Germany) spectrometers, with TMS as an internal standard and coupling constants were represented in Hertz.

Caryospomycin A (**1**): white amorphous powder (CH_3COCH_3); [α]+ 62.1° (CH_3OH; *c* 0.31); UV (CH_3OH) λ_{max} (log ε) 320.2 (4.01), 267.0 (4.22), 229.8 (4.70), 204.4 (4.71) nm; IR (film) ν_{max} 3,441, 2,959, 2,931, 2,875, 1,728, 1,648, 1,598, 1,472, 1,448, 1,381, 1,360, 1,316, 1,246, 1,224, 1,169, 1,126, 1,057, 1,041, 1,018, 969, 834, 743, 606; FABMS *m*/*z* (rel. int) 433$[M\text{-}H]^+$(3), 339 (40), 325 (100), 311 (60), 281 (42), 255 (5); EIMS *m*/*z* (rel. int) 434 $[M]^+$ (58), 416 $[M\text{-}18]^+$ (1), 372 (10), 296 (14), 267 (15), 249 (38), 223 (60), 221 (88), 219 (100), 205 (98), 195 (45), 193 (42), 167 (25), 109 (50), 95(58), 55(42); HRMS (ESI-TOF) *m*/*z*: 457.1844 $[M+Na]^+$ (calcd for $C_{23}H_{30}O_8Na$, 457.1838).

Caryospomycin B (**2**): white amorphous powder (CH_3COCH_3); [α]+ 30.0° (CH_3OH; *c* 0.76); UV (CH_3OH) λ_{max} (log ε) 319.0 (3.83), 267.2 (4.01), 225.4 (4.54) nm; IR (film) ν_{max} 3,424, 2,929, 2,856, 1,726, 1,648, 1,597, 1,474, 1,447, 1,383, 1,358, 1,308, 1,248, 1,226, 1,202, 1,169, 1,136, 1,063, 1,021, 965, 887, 610; FABMS *m*/*z* (rel. int) 393$[M\text{-}H]^+$(100); HRMS (ESI-TOF) *m*/*z*: 393.1554 $[M\text{-}H]^-$ (calcd for $C_{20}H_{25}O_8$, 393.1549).

Caryospomycin A (**3**): white amorphous powder (CH_3COCH_3); [α]+ 58.1° (CH_3OH; *c* 0.54); UV (CH_3OH) λ_{max} (log ε) 320.3 (3.97), 267.0 (4.07), 225.3 (4.60) nm; IR (film) ν_{max} 3,431, 2,939, 2,865, 1,728, 1,648, 1,598, 1,472, 1,448, 1,381, 1,360, 1,306, 1,245, 1,225, 1,200, 1,166, 1,059, 1,021, 969, 854, 609; FABMS *m*/*z* (rel. int) 391$[M\text{-}H]^+$(100); HRMS (ESI-TOF) *m*/*z*: 391.1403 $[M\text{-}H]^-$ (calcd for $C_{20}H_{23}O_8$, 391.1393).

Nematode Culture and Nematicidal Study Nematicidal activity was determined by using a microtiter plate assay as described previously (Dong et al. 2004). The test organism was the pine wood nematode *B. xylophilus*. This species has been maintained in our laboratory and used as a model target for *in vivo* nematocidal assays. For this assay, *B. xylophilus* was first grown on PDB agar media containing a strain of the fungus *Botrytis cinerea* in disposable Petri dishes prewetted with 2–4 ml of physiological saline. Cultures were then stored at room temperature and subcultured before assay. The assay was conducted in Corning polystyrene 96-well plates. Nematodes were added to 1 ml physiological saline in a scintillation vial and diluted until the nematode counts were 20–25 in a 48-μl aliquot. A solution (48 μl) containing nematodes was delivered to each of three wells for each

treatment. Two microliters of DMSO (5%) or DMSO (5%) plus the test compounds were added to each well. Plates were covered, parafilmed, and kept in a humid chamber. The numbers of live and dead nematodes were counted under a binocular microscope after various incubation times. Toxicity was inferred by using the mean percentage of dead organisms. Nematodes were considered dead if they gave no response to physical stimuli such as mechanical stirring or pricking with a pointed needle.

Statistical Analysis To evaluate nematicidal activity of compounds at different concentrations of 50, 80, 100, and 200 ppm, and different exposure times of 12, 24, and 36 hr, data were subjected to independent sample *F* test using ANALYZE (SPSS/version11.0 software, USA). Data on proof mortality were changed to $\sin^{1/2}$(M) before analysis.

To describe nematicidal effects of compound against *B. xylophilus*, LC_{50} was calculated according to probit analysis (Sporleder et al. 2005). Regression analyses were also conducted by SPSS for linear model. Data on proof mortality of nematodes were transformed into probit value, and concentrations (C) of compound were also changed to $\log_{10}$ (C) before analysis.

Results and Discussion

The molecular formula of caryospomycin A (**1**) was determined as $C_{23}H_{30}O_8$ by HRMS (ESI-TOF) and ^{13}C NMR data, indicating the presence of nine unsaturated bonds in the molecule. The IR (KBr) spectrum showed absorptions at 3,441, 1,728, 1,648, 1,598, 1,472, 1,448, 1,381, 1,360, 1,316, 1,246, 1,224, 1,169, 1,126, 1,057, 1,041, 1,018, 969, 834, 743, and 606 cm^{-1} that were attributable to hydroxyl, ester carbonyl, olefin, ether functions, and the aromatic ring. The UV spectrum maxima near 305, 265, and 220 were typical of the 4-methoxy resorcylic acid lactone macrolide chromophore present in the radicicol derivatives (Mirrington et al. 1964; Nair et al. 1981). The ^1H NMR spectrum (Table 1) showed signals attributable to the presence of one secondary methyl [δ_H 1.44 (3H, d, J=6.4 Hz)], two tertiary methyls [δ_H 1.34 (3H, s, acetonide), 1.28 (3H, s, acetonide)], and two oxymethyls [δ_H 3.88 (3H, s), and 3.56 (3H, s)]. An exchangeable 1H singlet at δ_H 11.1 was assigned to the chelated phenolic hydrogen (Nair et al. 1981; Isaka et al. 2002). Additional resonances were observed in the ^1H and ^{13}C NMR spectra for two *trans* 1,1-disubstituted double bonds [δ_H 6.75 (1H, d, J=15.9 Hz), 5.96 (1H, dt, J=15.6, 5.7, 3.5 Hz), 5.55 (1H, dd, J=8.6, 15.4 Hz), and 5.99 (1H, dt, J=15.4, 5.3, 3.5 Hz); δ_C 127.2 (d), 131.5 (d), 133.9 (d), and 129.4 (d)], and four oxymethine carbons [δ_H 3.86 (1H, dd, J=8.0, 2.0 Hz), 4.15 (1H, m), 4.58 (1H, t, J=8.3 Hz), 5.41 (1H, m); δ_C 69.2 (d), 72.0 (d), 76.1 (d), 82.3 (d)]. The ^{13}C NMR spectra also revealed resonances consistent with one lactone carbonyl [δ_C 171.7 (s)], one acetonic carbon [δ_C 108.7 (s)], and one penta-substituted benzene ring [δ_H 6.46 (1H, s); δ_C 100.2 (d), 105.1 (s), 133.6 (s), 141.4 (s), 159.5 (s), 160.8 (s)]. From this analysis, coupled with the degrees of unsaturations (9), it became clear that **1** was a resorcylic macrolide with two *trans*-disubstituted olefins.

The HMQC analysis revealed the assignment of each direct C–H bonding in **1** as summarized in Table 1. The ^1H–^1H correlations obtained from the ^1H–^1H COSY exhibited one big spin system, substructure **1a**, as depicted in Figs. 2 and 3. The HMBC correlations from O**H** at C-5 (δ_H 11.1, s) to C-6 (δ_C 105.1), C-5 (δ_C 160.8), C-4 (δ_C 100.2), from the aromatic proton H-4 (δ_H 6.46, s) to C-6 (δ_C 105.1), C-5 (δ_C 160.8), C-3 (δ_C 159.5), C-2 (δ_C 141.4), and C-11′ (δ_C 171.1), from one oxymethyl OC**H**$_3$-3 (δ_H 3.88, s) to C-4 (δ_C 100.2), C-3 (δ_C 159.5), and from the second oxymethyl OC**H**$_3$-2 (δ_H 3.56, s) to C-2

Table 1 ^{13}C and ^{1}H NMR data of compounds **1**–**3**[a] (Acetone-d_6)

Number	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	133.6 s		134.3 s		133.5 s	
2	141.4 s		141.0 s		141.1 s	
3	159.5 s		159.2 s		159.4 s	
4	100.2 d	6.46 (s)	100.1 d	6.45 (s)	100.4 d	6.48 (s)
5	160.8 s		160.0 s		160.3 s	
6	105.1 s		105.8 s		104.2 s	
1′	127.2 d	6.75 (d, 15.9)	126.0 d	6.67 (d, 16.0)	126.0 d	6.61 (d, 15.9)
2′	131.5 d	5.96 (dt, 15.6, 5.7, 3.5)	133.3 d	5.95 (dt, 15.6, 6.2, 3.0)	133.5 d	5.91 (dt, 15.6, 6.3, 3.0)
3′	38.0 t	2.71 (H_α, m)	37.9 t	2.41 (H_β, m), 2.56 (H_α, m)	39.0 t	2.36 (H_β, m), 2.91 (H_α, m)
4′	69.2 d	4.15 (m)	73.5 d	3.73 (m)	75.0 d	3.87 (m)
5′	82.3 d	3.86 (dd, 8.0, 2.0)	78.6 d	3.53 (dd, 8.2, 2.0)	79.8 d	4.52 (d, 3.5)
6′	76.1 d	4.58 (t, 8.3)	73.9 d	4.13 (t, 7.8)	198.0 s	
7′	133.9 d	5.55 (dd, 8.6, 15.4)	134.2 d	5.65 (dd, 7.6, 15.5)	132.3 d	6.43 (d, 15.4)
8′	129.4 d	5.99 (dt,15.4, 5.3, 3.5)	128.4 d	5.94 (dt, 14.2, 5.5, 3.9)	142.6 d	7.00 (dt, 15.5, 6.0, 3.5)
9′	37.8 t	2.50 (H_β, m), 2.56 (H_α, m)	37.4 t	2.54 (m)	38.2 t	2.80 (m)
10′	72.0 d	5.41 (m)	73.2 d	5.28 (m)	73.4 d	5.32 (m)
11′	171.7 s		171.9 s		171.8 s	
12′	19.3 q	1.44 (d, 6.4)	19.8 q	1.42 (d, 6.3)	19.8 q	1.48 (d, 6.2)
13′	108.7 s					
14′	27.1 q	1.34 (s)				
15′	27.4 q	1.28 (s)				
2-OCH3	60.1 q	3.56 (s)	60.1 q	3.54 (s)	60.1 q	3.57 (s)
3-OCH3	56.2 q	3.88 (s)	56.2 q	3.87 (s)	56.0 q	3.87 (s)
5-OH		11.1 (s)		11.2 (s)		11.3 (s)

[a] Assignments of the ^{13}C and ^{1}H signals were made on the basis of HMQC.

(δ_C 141.4) established one penta-substituted benzene ring, substructure **1b**, as depicted in Fig. 3. Furthermore, HMBC cross peaks of the olefinic proton at H-1′ (δ_H 6.75, d) with C-6 (δ_C 105.1), C-1 (δ_C 133.6), C-2 (δ_C 141.4), C-2′ (δ_C 131.5), and C-3′ (δ_C 38.0), the olefinic proton H-2′ (δ_H 5.96, dt) with C-3′ (δ_C 38.0), C-4′ (δ_C 69.2), C-1′ (δ_C 127.2), and C-1 (δ_C 133.6), the oxymethine proton H-10′ (δ5.41, m) with C-11′ (δ_C 171.7), C-12′ (δ_C 19.3), C-8′ (δ_C 129.4), and C-7′ (δ_C 133.9) established the reasonable connection patterns of C-1 with C-1′ and C-10′ with C-11′ through an oxygen atom, and permitted fragments **1a** and **1b** to be joined together. Finally, the HMBC correlations of two geminal methyl groups, C**H**$_3$-14′ and C**H**$_3$-15′ to C-13′ identified the occurrence of the acetonide moiety **1c**. Based on the ^{13}C NMR chemical shifts values (δ_C 27.1 and δ_C 27.4) of two geminal methyl

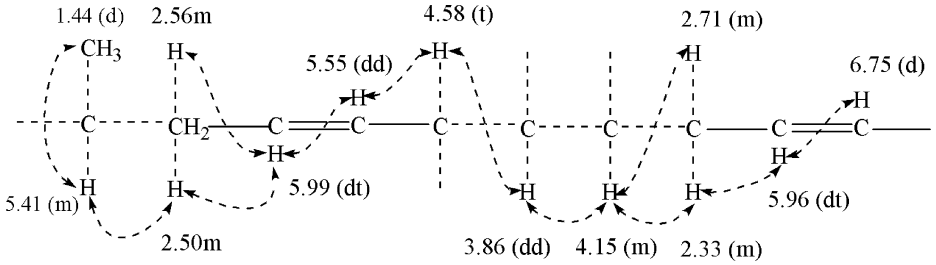

Fig. 2 Substructure **1a**, ^{1}H NMR data, and ^{1}H–^{1}H correlations (dashed arrows) of caryospomycin **A** (**1**)

groups, and the strong cross peak at H-5′ (δ_H 3.86, dd) with C**H**$_3$-15′ (δ_H 1.28, s) and the weak cross peak at H-6′ (δ_H 4.58, t) with C**H**$_3$-14′ (δ_H 1.34, s) in the NOESY spectrum, the acetonide carbon (C-13′, δ_C 108.7, s) was connected to C-5′ (δ_C 82.3) and C-6′ (δ_C 76.1) with *trans* stereochemistry (Adamczeski et al. 1988; Rychnovsky and Skalitzky 1990). Thus, the structure of caryospomycin **A** was established as **1**.

Both coupling constants between H-1′ (δ_H 6.75) and H-2′ (δ_H 5.96), H-7′ (δ_H 5.55) and H-8′ (δ_H 5.99) olefinic protons were measured as near 15 Hz, indicating the *E* geometry of the carbon–carbon double bonds in the diene at C-1′(2′) and C-7′(8′), because the *cis* coupling constant in comparable hypothemycin is 11 Hz (Nair and Carey 1980; Nair et al. 1981). The relative stereochemistries of C-10′ (δ_C 72.0), C-6′ (δ_C 76.1), C-5′ (δ_C 82.3), and C-4′ (δ_C 69.2) in **1** was deduced from the analysis of NOESY correlations with supporting information from vicinal coupling constants (Table 1). A NOESY experiment on **1** showed cross-peaks between H-1′ (δ_H 6.75)/$H_\beta-3'$(δ_H 2.33), $H_\beta-3'$(δ_H 2.33)/H-6′ (δ_H 4.58), H-6′ (δ_H 4.58)/C**H**$_3$-14′ (δ_H 1.34), H-4′(δ_H 4.12)/$H_\alpha-3'$(δ_H 2.33), H-4′(δ_H 4.12)/H-5′(δ_H 3.86), H-5′(δ_H 3.86)/C**H**$_3$-15′ (δ_H 1.28), H-7′ (δ_H 5.55)/$H_\beta-9'$(δ_H 2.50), H-7′ (δ_H 5.55)/$H_\alpha-9'$(δ_H 2.56), H-8′(δ_H 5.99)/$H_\alpha-9'$(δ_H 2.56), and $H_\alpha-9'$(δ_H 2.56)/H-10′(δ_H 5.41) (Fig. 4). This result demonstrated that $H_\alpha-3'$, H-4′, H-5′, $H_\alpha-9'$, C**H**$_3$-15′, and H-10′ were α-oriented, while $H_\beta-3'$, H-6′, C**H**$_3$-14′, $H_\beta-9'$, and C**H**$_3$-12′ possessed β-orientation. The 2.0 H-4′/H-5′ coupling constant and the almost 8 Hz H-5′/H-6′ coupling constant further supported the axial configurations for H-4′ and H-5′, as well as the equatorial configuration for H-6′. From the ^{1}H NMR, COSY, and NOESY spectra, a

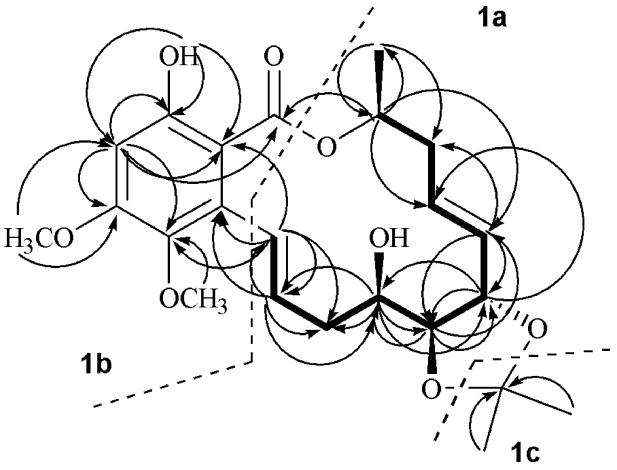

Fig. 3 Gross structure, ^{1}H–^{1}H correlations (bold lines), key HMBC correlations (plain arrows) of caryospomycin **A** (**1**)

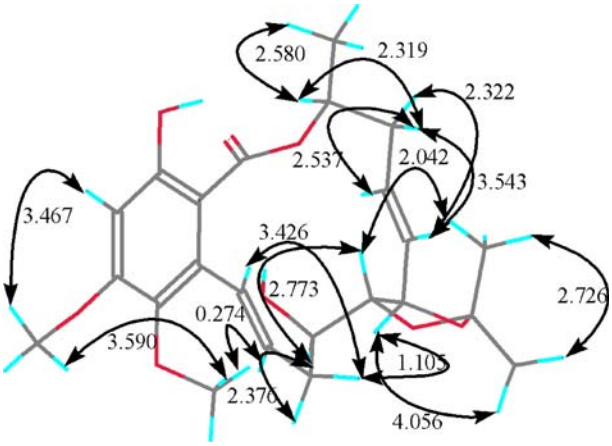

Fig. 4 Key NOESY correlations of **1** and corresponding interatomic distance (A), and some protons hidden for concision of the picture

computer-generated 3D structure of **1** was obtained by using the molecular modeling program, CS CHEM 3D V 4.0, with MM2 force-field calculations for energy minimization (Fig. 4). The calculated distances between $H-1'/H_\beta-3'$, $H_\beta-3'/H-6'$, $H-4'/H_\alpha-3'$, H-4′/H-5′, H-5′/C**H**$_3$-15′, $H-7'/H_\beta-9'$, $H-7'/H_\alpha-9'$, $H-8'/H_\alpha-9'$, and $H_\alpha-9'/H-10'$ were all less than 4.00 Å, except that the calculated distance between H-6′ and C**H**$_3$-14′ was slightly higher, at 4.056 Å. This high value is consistent with the observed weak cross peak at H-6′ with C**H**$_3$-14′ and the observed strong cross peaks from each of these proton pairs. Thus, the relative overall stereochemistry for compound **1** was deduced as 1′*E*, 4′*R**, 5′*S**, 6′*S**, 7′*E*, 10′*R**.

The molecular formula of caryospomycin B (**2**), $C_{20}H_{26}O_8$, was established from a high-resolution MS (ESI-TOF) measurement of the $[M–H]^-$ peak at *m/z* 393.1554, indicating the presence of eight unsaturated bonds in the molecule. The UV, IR, and NMR spectra were similar to those obtained for **1**, indicating that they were structurally related. The most striking differences in the NMR data between **1** and **2** compared were the disappearance of the acetonide moiety signals (δ_C 108.7, 27.1 q, and 27.4 q; δ_H 1.34 s and 1.28 s) in **2** but their presence in **1**. In the ^{13}C NMR data, signals of the C-5′ and C-6′ oxymethine carbons were noticeably shifted upfield from δ_C 82.3 and 76.1 in **1** to δ_C 78.6 and 73.9 in **2**, while the C-4′ oxymethine carbon signal was noticeably shifted downfield from δ_C 69.2 in **1** to δ_C 73.5 in **2** (Table 1). Corresponding differences were observed in the ^1H NMR data, in which signals of the oxymethine protons were shifted upfield from δ_H 4.15 (1H, m, H-4′), 3.86 (1H, dd, *J*= 8.0, 2.0 Hz, H-5′), and 4.58 (1H, t, *J*=8.3 Hz, H-6′) in **1** to δ_H 3.73 (1H, m, H-4′), 3.53 (1H, dd, *J*=8.2, 2.0 Hz, H-5′), and 4.13 (1H, t, *J*=7.8 Hz, H-6′) in **2**. These spectral changes were consistent with the absence of the acetonide moiety from C-13′ to C-15′ in **2**. Despite the spectral differences, however, the combined 2D NMR experiments showed that **2** had the same proton-proton and proton-carbon correlations as **1** throughout the entire molecule. Thus, the structure of **2** was determined to be a deacetonided derivative of **1**.

The molecular ion $[M]^+$ of caryospomycin C (**3**) is 2 mass units lower than compound **2**, and its molecular formula was determined as $C_{20}H_{24}O_8$ by HRMS (ESI-TOF) and NMR data. One noticeable difference in the ^{13}C NMR data between **2** and **3** was the addition of a ketone functionality at δ_C 198.0 in **3**, and the absence of an oxymethine carbon at this position, an observation suggesting that the oxymethine carbon of **2** was replaced by the ketone functionality in **3**. In addition, the ^{13}C chemical shifts of the C-7′/C-8′ double band and the C-4′, C-5′ oxymethines were also changed from δ_C 134.2 (CH, C-7′), 128.4 (CH,

C-8′), 73.5 (CH, C-4′), and 78.6 (CH, C-4′) in **2** to δ_C 132.3 (CH, C-7′), 142.6 (CH, C-8′), 75.0 (CH, C-4′), and 79.8 (CH, C-4′), respectively, in **3**. Corresponding differences were observed in the ^{1}H NMR data, in which signals of the olefinic and oxymethine protons were shifted downfield from δ_H 5.94 (dt, *J*=14.2, 5.5, 3.9 Hz, H-8′), 5.65 (dd, *J*=7.6, 15.5 Hz, H-7′), 3.53 (dd, *J*=8.2, 2.0 Hz, H-5′), 3.73 (m, H-4′) in **2** to 7.00 (dt, *J*=15.5, 6.0, 3.5 Hz, H-8′), 6.43 (d, *J*=15.4 Hz, H-7′), 4.52 (d, *J*=3.5 Hz, H-5′), 3.87 (m, H-4′) in **3**. Based on these spectral changes, the newly appearing ketone carbonyl was placed in between C-5′ and C-7′ neighboring the C-7′/C-8′ double band. This was confirmed by HMBC correlations between the protons at δ_H 6.43 (H-7′), 7.00 (H-8′), and 3.87 (H-4′), and the ketone carbonyl signal at δ_C 198.0. Thus, the structure of caryospomycin **C** was determined to be an analogue of **2** but with a different oxidation state at C-6′, as shown in Fig. 1.

Nematicidal Activity The nematicidal activities of caryospomycin **A**–**C** are shown in Table 2. Caryospomycin **A**–**C** displayed moderate nematicidal activities against *B. xylophilus*. Nematicidal effects varied with concentration and exposure time. Activity differed significantly between different exposure times of 12, 24, and 36 h at the same concentration (Table 3), and different concentration of 50, 80, 100, and 200 ppm at the same exposure time (Table 4). Probit value of proof mortality showed linear type of increase with increasing Log_{10} (concentrations). The LC_{50} values of compounds **1-3** against *B. xylophilus* were 103.1, 105.8, and 105.1 ppm, at 36 hr, respectively.

The study demonstrated the presence in *C. carllicarpa* YMF1.01026 of three new nematicidal metabolites, all of which belonged to the resorcylic 14-membered macrolide family. This family of compounds includes many well-characterized chemicals such as zearalenone isolated from *Gibberella zeae* and *Fusarium culmorum* (Stob et al. 1962; Richardson et al. 1985); radicicol (also named monorden) from *Chaetomium chiversii* (Turbyville et al. 2006), *Cylindrocarpon radicicola* (Evans and White 1966), *Monocillium nordinii* (Ayer et al. 1980), *Penicillium luteo-aurantium* (Nozawa and Nakafima 1979), *Neocosmospora tenuicristata* (Cutler et al. 1987), *Verticillim chlamydosporium* (Khambay et al. 2000), and *Humicola* sp. F02942 (Wicklow et al. 1998; Arai et al. 2003); monorcillins from *Monocillium nordinii* (Ayer and Pena-Rodriguez 1987; Ayer et al. 1980), *Diheterosporia chlamydosporia* (Espenshade and Calton 1979), *Humicola fuscoatra* NRRL22980 (Wicklow et al. 1998), *Humicola* sp. (Arai et al. 2003; Yamamoto

Table 2 Effect of caryospomycin **A**–**C** on the proof mortality of *B. xylophilus in vitro*

Compounds	Percent mortality (hr)	Concentrations (ppm)				LC_{50} (ppm)
		200	100	80	50	
1	12	14.5	7.9	6.8	4.4	1011.6
	24	57.1	40.3	29.7	20.1	164.7
	36	72.4	50.3	41.3	33.5	103.1
2	12	16.7	13.4	7.6	5.1	1255.6
	24	47.8	42.1	31.3	12.6	185.9
	36	67.5	54.7	50.1	23.7	105.8
3	12	18.8	16.1	8.3	4.4	620.3
	24	38.5	30.6	19.6	20.0	442.1
	36	64.2	57.8	50.7	25.5	105.1

Table 3 Influence of different exposure time (12, 24, and 36 hr) on the mortality of nematodes at each concentration of compounds **1**–**3**

Compounds	Concentrations (ppm)			
	200[a]	100	80	50
1	212.6*[b]	320.4*	166.797*	156.3*
2	602.4*	235.4*	273.4*	104.3*
3	489.5*	375.6*	468.1*	103.5*

*$P<0.001$

[a] Concentrations (mg l^{-1}) of the compound

[b] Values were *f* values from independent sample ANOVA

et al. 2003), and *Paraphaeosphaeria quadriseptata* (Wijeratne et al. 2004); pochonins from *Pochonia chlamidospora* (Hellwig et al. 2003); hypothemycin from *Hypomyces trichothecoides* (Nair and Carey 1980; Nair et al. 1981; Agatsuma et al. 1993); and aigialomycins from *Aigialus parvus* (Isaka et al. 2002). All of these compounds have been reported to have a wide range of biological activities such as antifungal, antitumor, antiprotozoan, antimalarial, antiviral, and antiparasitic functions. Among them, zearalenone and radicicol are probably the two most noteworthy. Specifically, zearalenone possesses anabolic, estrogenic, and antibacterial activities. Compounds exhibiting anabolic activities can be employed as cattle-growth stimulants (Lone 1997) as well as in treatments of menopausal and postmenopausal syndromes (Utian 1973). The compound radicicol exhibits various biological activities including antifungal, antibiotic, and antimalarial functions (with *in vivo* efficacy). In addition, radicicol inhibits various kinases and the Heat-shock protein Hsp 90 (Janin 2005). There have been over 200 publications and patents associated with this compound (Jayasuriya et al. 2005). Among its various activities, the inhibition against Hsp90 has attracted significant attention in recent years. Specifically, this compound is capable of suppressing the transformed phenotype caused by various oncogenes such as *src*, *ras*, and *raf*. These suppressive functions are linked to radicicol's high-affinity binding (20 nM) and inhibition of the Hsp90 molecular chaperone. This "antichaperone" activity might help reduce the level of oncogenic proteins, and could thus be of clinical interest. Consequently, the resorcylic 14-membered macrolides have been the subject of many preparative and biosynthetic studies (Nicolaou et al. 1998; Furstner et al. 2000; Burckhardt and Ley 2002; Yang and Danishefsky 2003; Moulin et al. 2005). Our finding that the widely distributed fresh water fungus *C. carllicarp* YMF1.01026 can

Table 4 Influence of different concentration (50, 80, 100, and 200 ppm) on the mortality of nematodes at each exposure time

Compounds	Exposure time (hr)		
	12[a]	24	36
1	19.1*[b]	78.3*	111.1*
2	33.6*	193.2*	166.0*
3	86.5*	72.8*	171.8*

*$P<0.001$

[a] Times of the exposure

[b] Values were *f* values from independent sample ANOVA

also produce resorcylic 14-membered macrolides may be of significant biomedical importance. Further studies are required to examine the resorcylide biosynthetic capacities of this fungus and to obtain sufficient quantities of caryospomycins **A**–**C** as well as their analogues to fully evaluate their biological activities.

The present study demonstrated that fungi inhabiting freshwater environments can produce nematicidal metabolites. The occurrence of nematicidal substance in freshwater fungi might be linked to their survival strategies. For example, nematicidal compounds could help fungi obtain nutrients (e.g., organic nitrogen compounds) from dead nematodes, similar to the proposed functions of these groups of compounds in some wood-inhabiting Basidiomycetes like *Pleurotus* species (Thorn and Barron 1984; Barron 1992). In freshwater ecosystems, submerged woody substrata constitute the main energy input source (Wong et al. 1998). Wood is, however, a substrate notably deficient in nitrogen, and thus the nitrogen utilized by freshwater fungi might need to come from other sources. Nematodes are cosmopolitan organisms and are adapted to live in both terrestrial and aquatic environments. They have been shown to be an integral part of various ecosystems, providing food and energy for small invertebrates or fungi (Dropkin 1980). With their high nitrogen contents, nematodes are considered to play an important role in providing nitrogen to other organisms in freshwater ecosystems. Several nematophagous fungi have previously shown to be associated with nematodes found in wood that was submerged in freshwater. These fungal species included *Dactylella ellipsospora* Grove (Hyde and Goh 1998) and *Dactylella aquatica* (Ingold) Ranzoni (Kane et al. 2002). Similarly, freshwater fungi producing nematicidal compounds might also obtain their nitrogenous nutrients from nematodes living in submerged wood. The ability for these fungi to produce nematicides that can kill nematodes and release nutrients could be highly advantageous for these fungi in their natural environments.

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