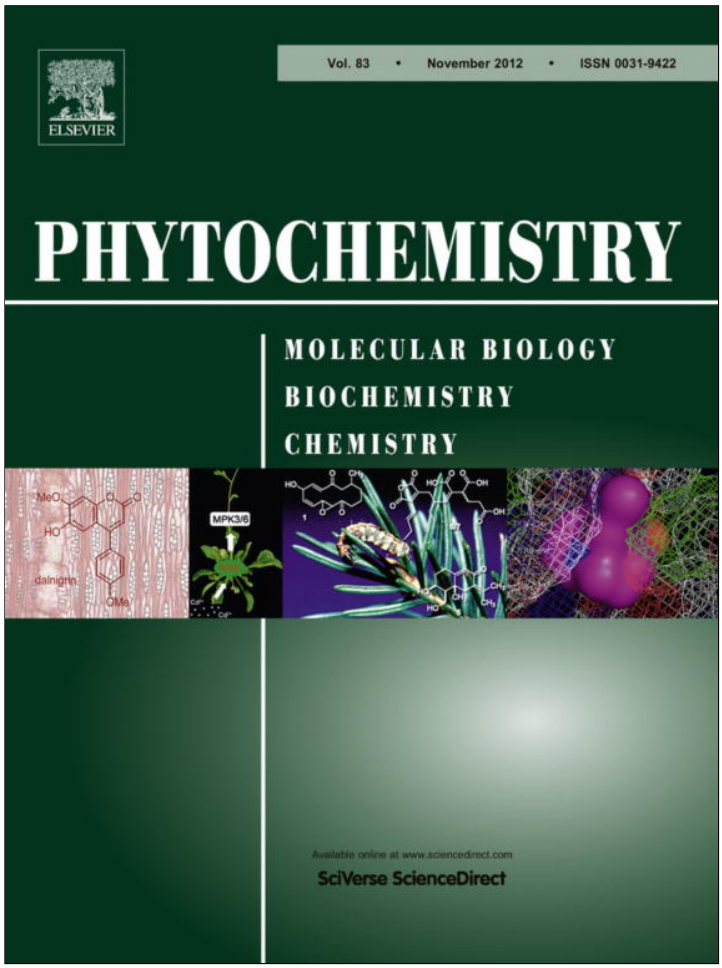

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Zoosporicidal metabolites from an endophytic fungus *Cryptosporiopsis* sp. of *Zanthoxylum leprieurii*

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ABSTRACT

Two polyketides, cryptosporiopsin A (**1**) and hydroxypropan-2′,3′-diol orsellinate (**3**), and a natural cyclic pentapeptide (**4**), together with two known compounds were isolated from the culture of *Cryptosporiopsis* sp., an endophytic fungus from leaves and branches of *Zanthoxylum leprieurii* (Rutaceae). The structures of these metabolites were elucidated on the basis of their spectroscopic and spectrometric data. Cryptosporiopsin A and the other metabolites exhibited motility inhibitory and lytic activities against zoospores of the grapevine downy mildew pathogen *Plasmopara viticola* at 10–25 μg/mL. In addition, the isolated compounds displayed potent inhibitory activity against mycelial growth of two other peronosporomycete phytopathogens, *Pythium ultimum*, *Aphanomyces cochlioides* and a basidiomycetous fungus *Rhizoctonia solani*. Weak cytotoxic activity on brine shrimp larvae was observed.

1. Introduction

The peronosporomycetes genera such as *Phytophthora*, *Plasmopara*, *Aphanomyces*, and *Pythium* are devastating pathogens implicated in plant, animal, and human diseases (Agrios, 1997). They infect their hosts through asexually produced biflagellate motile zoospores. These zoospores locate infection sites on their host guided by host cues and then rapidly change into round cystospores followed by germination to form hyphal germ tubes, which initiate infection (Islam and Tahara, 2001). The motility of zoospores is critical for successful disease cycles, and thus disruption of their motility eliminates infection by these pathogens (Judelson and Blanco, 2005; Islam et al., 2011). Among the peronosporomycetes, *Plasmopara viticola* is an obligate biotrophic parasite, which causes downy mildew in grapevine worldwide (Werner et al., 2002). This fungus-like microorganism spreads very rapidly through asexually produced airborne sporangia, which release biflagellate motile zoospores on the surface of wet grapevine leaves (Riemann et al., 2002). Due to the unique cell biology, zoospores of the fungus-like peronosporomycetes are insensitive to most of the fungicides. Therefore, novel strategies to manage these economically important phytopathogens are needed.

In the course of our search for new and environmentally benign secondary metabolites regulating the motility and viability of zoospores of *P. viticola*, we found that the extract from cultures of an endophytic fungus *Cryptosporiopsis* sp. isolated from the healthy leaves, branches and stems of *Zanthoxylum leprieurii* (Rutaceae) impaired the motility of zoospores of the grapevine downy mildew pathogen *P. viticola* at 100 μg/mL. The extract also displayed significant growth inhibitory activities against three plant pathogens, *Aphanomyces cochlioides*, *Pythium ultimum* and *Rhizoctonia solani*, and cytotoxic activity against brine shrimp larvae. This report describes the isolation and characterization of two new polyketides, cryptosporiopsin A (**1**) and hydroxypropan-2′,3′-diol orsellinate (**3**), two pentapeptides (**4** and **5**), and (−)-phyllostine (**6**) as the active components from the extract of the fungus *Cryptosporiopsis* sp. strain CAFT122-1, which was also isolated for the first time from the medicinal plant *Z. leprieurii*. Bioassay results of the metabolites against motility and viability of zoospores, mycelial growth of fungi and peronosporomycete phytopathogens, and cytotoxicity against brine shrimp larvae are also discussed.

2. Results and discussion

The endophytic fungus *Cryptosporiopsis* sp. was isolated from *Z. leprieurii* and grown on solid rice medium for four weeks. Exhaustive extraction and chromatographic separation of the

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extract yielded cryptosporiopsin A (**1**), hydroxypropan-2′,3′-diol orsellinate (**3**), and the pentapeptide **4**, along with two known fungal metabolites **5** and **6** (Fig. 1).

Cryptosporiopsin A (**1**) was obtained as colorless oil. The molecular formula $C_{19}H_{21}O_6Cl$ was assigned on the basis of (+)-HRESIMS and NMR spectra (Table 1). The NMR data of **1** were similar to those of resorcylic acid lactones, e.g. ponchonin D (**2**) (Hellwig et al., 2003). The ^{1}H NMR spectrum revealed an aromatic proton singlet at δ 6.63 (δ_C 99.2) of a pentasubstituted benzene derivative, of which two of the respective carbon signals (δ_C 154.9 and 155.6) were seen at low field, pointing to phenolic carbons as in **2**.

From the ^{1}H–^{1}H COSY and TOCSY spectra, two extended proton spin systems were identified, from H-1 to H-4, and from H-6 to H-9. They were connected via the carbonyl group C-5, as the HMBC correlations from H-3, H-4, H-6 and H-7 to the ketone carbonyl at δ_C 209.8 indicated. Two proton signals of a *trans* configured double bond in this fragment correlated with another carbonyl group at δ_C 193.6, forming an α,β-unsaturated ketone. This chain was connected with a benzoic acid moiety via an ester bond, as the shifts of C-2/H-2 and HMBC cross signal between H-2 and C-18 indicated. The methylene group C-11 was connected with the benzene unit, forming a resorcylic acid lactone (HMBC correlations see Fig. 2). The proton H-15 and the methylene group at H_2-11 correlated to the same carbon atoms C-13 (where the chlorine should be attached) and C-17 as in ponchonin D (**2**). In addition, correlations were observed from H_2-11 to C-10, C-14 and C-17. According to the methoxy signal at δ_H 3.71 and its correlation with the oxygenated carbon at δ_C 155.6, one of the phenolic oxygens at C-14 or C-16 must be methylated. The other one is forming a non-chelated OH group, as the sharp singlet of chelated *o*-hydroxy-resorcylic acid lactones (Hellwig et al., 2003) was missing, and as weak long-range correlations of the methoxy group with C-15 and C-17 appeared. As all sp^3 carbons were fully assigned, the chlorine atom must be placed at C-13 as in ponchonin D (**2**) and related resorcylic acid derivatives; this agrees very well with the chemical shifts (see Table 1). On this basis, the structure of cryptosporiopsin A was derived as **1** (Fig. 1). Besides the few lasiodiplodin derivatives, resorcylic acid monomethyl ethers with a non-chelated OH group as in **1** are very rare (Matsuura et al., 1998).

The molecular formula of hydroxypropan-2′,3′-diol orsellinate (**3**) was established as $C_{11}H_{14}O_6$ by (+)-HRESIMS and NMR data (Table 2). Its UV spectrum revealed strong absorption bands at 216 and 292 nm, which suggested the presence of a benzene ring. In the ^{1}H spectrum, resonances for one methyl, two coupled aromatic protons and a pattern of signals between δ_H 3.61 and 4.41 characteristic of a monosubstituted glycerol moiety were observed (Table 2). Analysis of ^{13}C and 2D NMR data revealed close similarities with 1-*O*-(2,4-dihydroxy-6-methylbenzoyl)-glycerol (Hui-Jiao et al., 2010). The only difference observed was the negative value of the specific optical rotation ($[\alpha]_D^{20}$ −17) of compound **3** and since it has only one stereogenic center (C-2′), the stereochemistry at C-2′ was established by comparing the obtained specific optical rotation with that of 1-*O*-(2,4-dihydroxy-6-methylbenzoyl)-glycerol ($[\alpha]_D^{25}$ +15). Further comparison with other compounds also featuring the monosubstituted glycerol moiety such as variecolorquinone A (Wang et al., 2007), peyssonenynes A (McPhail et al., 2004), (+)-variecolorquinones A (Du et al., 2008) and (2′*S*)-2′,3′-dihydroxypropyl 1,6,8-trihydroxy-3-methylanthraquinone-2-carboxylate (Segawa et al., 1999) have led to the tentative assignment

Table 1
NMR data of cryptosporiopsin A (**1**)[a] and ponchonin D (**2**)[a].

No	δ_C	**1** δ_H (mult. *J* in [Hz])	HMBC (H → C)	**2** δ_C
1	19.9	1.21 (3H, d, 6.0)	2, 3	19.0
2	70.9	4.97 (1H, m)	1, 3, 4, 18	71.5
3	28.1	1.57, 1.77 (2H, 2 m)	2, 4, 5	37.9
4	38.9	2.47, 2.50 (2H, 2 m)	2, 3, 5, 6	127.7
5	209.9	–	–	131.5
6	40.2	2.54, 2.56 (2H, 2 m)	8	30.6
7	28.7	2.42 (2H, m)	6, 8	30.6
8	146.8	6.91 (1H, dt, 15.8, 7.2)	6, 7, 9, 10	147.5
9	129.3	5.96 (1H, d, 15.8)	6, 8, 10, 11	128.7
10	193.6	–	–	195.2
11	41.9	H_a: 4.04 (1H, d, 17.3)	10, 12, 13, 17	44.4
		H_b: 3.88 (1H, d, 17.3)	10, 12, 13, 17	
12	132.0	–	–	133.6
13	116.2	–	–	114.4
14	154.9	–	–	156.7
15	99.2	6.63 (1H, s)	13, 14, 16, 17, 18	103.0
16	155.6	–	–	155.7
17	113.4	–	–	112.9
18	166.1	–	–	167.9
19	55.9	3.71 (3H, s)	16, (15, 17)	–

[a] ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 at 300 and 125 MHz, respectively; TMS was used as internal standard.

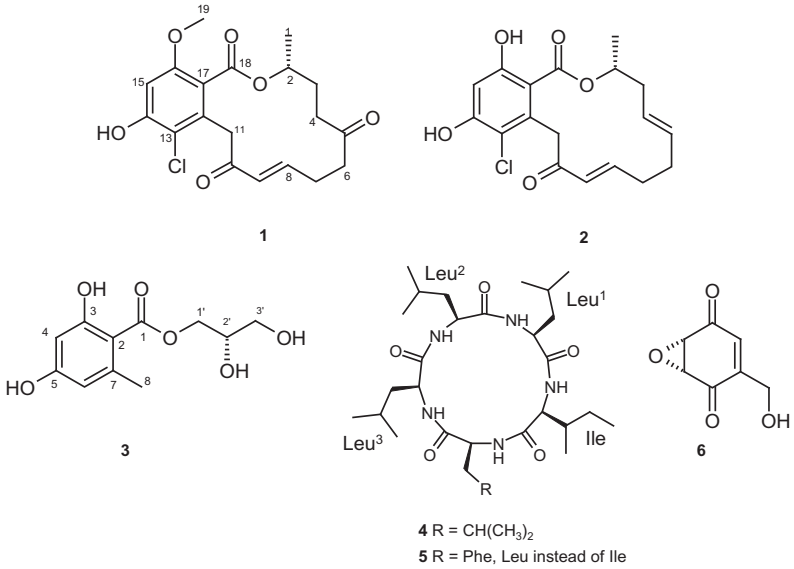

Fig. 1. Structures of compounds **1**–**6**.

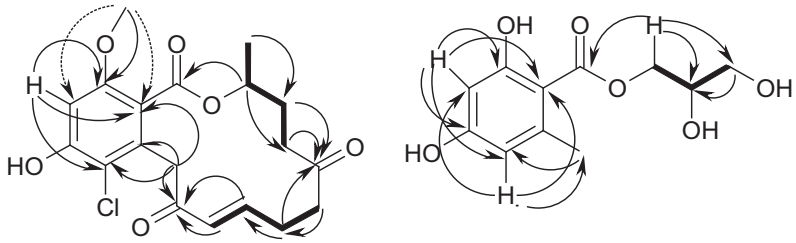

Fig. 2. Selected ^{1}H–^{1}H COSY (—) and HMBC (⌒) correlations of **1** and **3**.

Table 2
NMR data of hydroxypropan-2′,3′-diol orsellinate (**3**)[a].

No	δ_C	δ_H (mult., *J* in [Hz])	^{1}H–^{1}H COSY
1	172.7	–	–
2	105.6	–	–
3	166.0	–	–
4	101.7	6.14 (1H, d, 2.5)	H-6
5	164.0		–
6	112.6	6.20 (1H, d, 2.5)	H-4
7	144.6		–
8	24.6	2.50 (3H, s)	–
1′	67.0	H_a: 4.41 (1H, dd, 10.7, 6.1)	H-2′, H-3′
		H_b: 4.30 (1H, dd, 10.7,4.5)	H-2′, H-3′
2′	71.0	3.97 (1H, m)	H-1′, H-3′
3′	64.3	3.61 (2H, m)	H-2′, H-1′

[a] ^{1}H and ^{13}C NMR spectra were recorded in MeOH-d_4 at 300 and 125 MHz, respectively; TMS was used as internal standard.

of the absolute configuration of **3** at C-2′ as (*S*). Thus, the structure of **3** was elucidated as (2′*S*)-hydroxypropan-2′,3′-diol orsellinate (**3**), which is closely related to globosumones A-C isolated from the endophytic fungus *Chaetomium globosum* (Bashyal et al., 2005).

Compound **4** was isolated as white amorphous powder with the molecular formula $C_{30}H_{55}N_5O_5$, obtained by (+)-HRESIMS of the $[M + Na]^+$ *pseudo*molecular ion. It showed a positive reaction in the chlorine/tolidine test, characteristic for peptides. The ^{13}C and ^{1}H NMR data (Table 3) displayed the presence of each five amide carbonyls, α-methine carbons, amide protons (NH), and α-protons, respectively. Additional high-field signals due to methines, methylenes, and methyl protons were observed.

The amide proton (NH) at δ_H 8.37 correlated in the ^{1}H–^{1}H COSY spectrum with the α-proton at δ_H 3.32, which in turn showed cross peaks with a *sec*-butyl group, and thus an isoleucine (Ile) residue was assigned. Each of the other four amide protons showed correlations in the ^{1}H–^{1}H COSY spectrum in the same way, which in turn defined four leucine residues. According to the empirical formula, the peptide must be cyclic. This was further confirmed by HMBC key correlations mainly from the NH protons to the amide carbonyl of the neighboring amino acid, which established the sequence as *cyclo*-(Ile-Leu-Leu-Leu-Leu) (Table 3). The structure of **4** was further confirmed by ESI-MS/MS experiments using collision induced dissociation in a quadrupole ion trap (Fig. 3). A sequential cleavage of three Leu/Ile residues from the isolated $[M + H]^+$ ion at *m*/*z* 566 was clearly observed and verified by further studies by means of MS^3 and MS^4. All ion compositions were determined by high-resolution CID-MS/MS measurements of the $[M + Na]^+$ ions using Fourier-transform ion cyclotron resonance mass spectrometry (Table 4).

The configuration of the amino acid residues in **4** was established by acid hydrolysis, followed by derivatization with Marfey's reagent (FDAA) (Marfey, 1984) and subsequent HPLC analysis of the derivatives. Comparison with amino acid standards derivatized with L- and D-FDAA showed that isoleucine and the four leucine residues all had the L-configuration. Although compound **4** was previously reported as a synthetic intermediate for varcunamide (Sakurai and Okumura, 1979), no ^{13}C NMR data or complete sequencing data using MS/MS analysis were reported. Compound **4** is reported here for the first time as natural product.

A second cyclic pentapeptide was identified in a similar way as *cyclo*-(L-Phe-L-Leu-L-Leu-L-Leu-L-Leu) (**5**), previously isolated from cultures of the mangrove endophytic fungus No. 2524 (Li et al., 2004). It differs from **4** only by replacement of L-isoleucine by phenyl alanine in **5** (see Fig. 4 and Fig. S12–S14, Supplementary data). The benzoquinone epoxide, (−)-phyllostine (**6**), was identified on the basis of ^{1}H NMR, ^{13}C NMR, and MS data and confirmed by comparison with reported data (Yoshida et al., 1999; Sakamura et al., 1971).

The crude extract and the isolated compounds from *Cryptosporiopsis* sp. strain CAFT122-1 exhibited motility inhibitory activity followed by lysis of *P. viticola* zoospores in a dose- and time-dependent manner (Table 5). In sterilized water, zoospores of *P. viticola* swim normally for several hours in a helical fashion. However, zoospores exposed to compounds **1** or **3**–**6** swam very slowly or spun in tight circles or lysed depending on the dose of the test compounds. The minimum inhibitory concentration (MIC) for motility inhibition by the crude extract, cryptosporiopsin A (**1**) and the pentapeptide (**5**) was 10 μg/mL. At 25 μg/mL, cryptosporiopsin A (**1**), the pentapeptides **4** and **5**, and (−)-phyllostine (**6**) completely inhibited motility of zoospores within 30 min (Table 5). All the immobilized zoospores lysed within 60 min. However, hydroxypropan-2′,3′-diol orsellinate (**3**) displayed moderate motility inhibitory and lytic activities against the zoospores at 25 μg/mL. Among the tested compounds, cryptosporiopsin A (**1**) showed the highest motility inhibitory and lytic activities followed by pentapeptides **4** and **5**, (−)-phyllostine (**6**), and hydroxypropan-2′,3′-diol orsellinate (**3**) in order of decreasing activity (Table 5).

Motility of zoospores is critical for the completion of disease cycles in peronosporomycete phytopathogens (Latijnhouwers et al., 2004; Islam et al., 2011). It has recently been reported that motility of zoospores is likely to be maintained by protein kinase C, and PKC inhibitors significantly suppressed the downy mildew disease on grapevine leaves artificially inoculated with freshly harvested sporangia of *P. viticola* (Islam et al., 2011). Motility inhibitory and lytic activities of zoospores by secondary metabolites from non-host plants and environmental microorganisms have been previously reported (Begum et al., 2002; Islam et al., 2002, 2011; Islam and Tiedemann, 2011). This is, however, the first report on such activities of secondary metabolites from an endophytic fungus, *Cryptosporiopsis* sp. against *P. viticola*.

Cryptosporiopsin A (**1**) and compounds **3**–**6** were routinously also evaluated for cytotoxicity against brine shrimp larvae (*Artemia salina*) (Takahashi et al., 1989). All compounds showed weak toxicity at a concentration of 10 μg/mL, with mortality rates of 20.1%, 10.2%, 12.3%, 10% and 27.3%, respectively. The newly isolated metabolites **1**, **3** and **4** and the known compounds **5** and **6** exhibited significant inhibitory effects on three plant pathogenic fungi *P. ultimum*, *A. cochlioides*, and *R. solani* with a minimum inhibitory

Table 3
NMR data of cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu) (**4**)[a].

Unit		δ_C	δ_H (mult., *J* in [Hz])	Selected HMBC
Ile	1	171.1 (C_q)		
	2	63.0 (CH)	3.32 (1H, dd, 7.4, 11.0)	1, 3, 4
	3	33.0 (CH)	2.32 (1H, m)	1, 2, 4, 5
	4	25.1 (CH_2)	1.40 (1H, m)	3, 5
			1.55 (1H, m)	
	5	9.8 (CH_3)	0.79 (3H, t, 7.6)	4, 3
	6	15.4 (CH_3)	0.82 (3H, d, 6.5)	5, 4, 3
	NH		8.37 (1H, d, 7.4)	Leu^1-1; 2, 3
Leu^1	1	170.7 (C_q)		
	2	51.7 (CH)	4.33 (1H, dd, 7.4, 8.0)	1, 3, 4
	3	40.14 (CH_2)	1.51 (2H, m)	1, 2, 4
	4	24.6 (CH)	1.47 (1H, m)	2, 3, 5, 6
	5	22.1 (CH_3)*	0.83 (3H, d, 6.5)	3, 4, 6
	6	21.7 (CH_3)*	0.86 (3H, d, 6.5)	3, 4, 5
	NH		7.20 (1H, d, 8.3)	Leu^2-1; 2
Leu^2	1	171.2 (C_q)		
	2	52.0 (CH)	4.09 (1H, dd, 7.7, 7.9)	1, 3, 4
	3	40.09 (CH_2)	1.48 (1H, m)	1, 2, 4
			1.51 (1H, m)	
	4	24.2 (CH)	1.49 (1H, m)	2, 3, 5, 6
	5	23.0 (CH_3)*	0.79 (3H, d, 6.5)	3, 4, 6
	6	20.6 (CH_3)*	0.87 (3H, d, 6.5)	3, 4, 5
	NH		8.55 (1H, d, 8.0)	Leu^3-1; 2, 3
Leu^3	1	171.4 (C_q)		
	2	51.9 (CH)	4.18 (1H, dd, 7.4, 6.8)	1, 3, 4
	3	38.7 (CH_2)	1.52, 1.38 (2H, 2 m)	1, 2, 4
	4	24.5 (CH)	1.61 (1H, m)	3, 2, 5
	5	22.0 (CH_3)*	0.89 (3H, d, 6.5)	3, 4
	6	22.8 (CH_3)*	0.92 (3H, d, 6.5)	3, 4, 5
	NH		8.69 (1H, d, 6.5)	Leu^4-1; 2, 3
Leu^4	1	172.6 (C_q)		
	2	50.2 (CH)	4.45 (1H, dd, 7.5, 9.1)	1, 3, 4
	3	41.7 (CH_2)	1.38 (2H, m)	1, 2, 4
	4	24.3 (CH)	1.60 (1H, m)	2, 3, 5, 6
	5	22.4 (CH_3)*	0.80 (3H, d, 6.5)	3, 4, 6
	6	22.5 (CH_3)*	0.88 (3H, d, 6.5)	3, 4, 5
	NH		7.86 (1H, d, 9.2)	Ile-1,2; 1, 2

[a] ^1H and ^{13}C NMR spectra were recorded in DMSO-d_6 at 300 and 125 MHz, respectively; TMS was used as internal standard. *Assignment of methyl signals might be interchangeable.

concentration of 20–40 μg per paper disk by the agar diffusion method (Table 6).

3. Conclusion

Two new polyketides, cryptosporiopsin A (**1**) and hydroxypropan-2′,3′-diol orsellinate (**3**), two cyclic pentapeptides **4** and **5**, and (−)-phyllostine (**6**) have been isolated from the culture of an endophytic fungus *Cryptosporiopsis* sp. strain CAFT122-1 Cryptosporiopsin A (**1**) and compounds **3**–**6** exhibited motility inhibition followed by lysis of *P. viticola* zoospores. Compounds **1** and **3**–**6** also inhibited mycelial growth of two other peronosporomycete phytopathogens, *P. ultimum* and *A. cochlioides* and a basidiomycetous fungus *R. solani*. Weak cytotoxic activity against brine shrimp larvae was observed. Further studies on the mode of action of the motility inhibitory and zoosporicidal activities of these metabolites and their effects on other phytopathogenic peronosporomycetes are needed for considering their practical use as naturally occurring antiperonosporomycetal agents.

4. Experimental

4.1. General experimental procedures

The NMR spectra were measured on a Bruker AMX 300 (300.135 MHz), a Varian Unity 300 (300.145 MHz) and a Varian Inova 600 (599.740 MHz) spectrometer. Optical rotation was measured on a Perkin-Elmer polarimeter, model 241. UV/VIS spectra were recorded on a Perkin-Elmer Lambda 15 UV/VIS spectrometer. ESI-MS was recorded on a Finnigan LCQ ion trap instrument, and collision induced dissociation (CID) MS/MS was carried out with helium as collision gas. ESI-HRMS spectra were acquired on a Fourier-transform ion cyclotron resonance mass spectrometer APEX IV (Bruker Daltonik) and argon was used as collision gas for CID-MS/MS under high-resolution conditions. Samples were introduced via a syringe pump. IR spectra were recorded on a Perkin-Elmer (Model 1600) FTIR spectrometer. Flash chromatography was carried out on silica gel (230–400 mesh). R_f-values were measured on Polygram SIL G/UV254 (Macherey–Nagel & Co.). Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex; Amersham Biosciences, Ltd., purchased from Sigma–Aldrich Chemie, Steinheim, Germany).

4.2. Fungal material and identification

Healthy leaves, branches, and stems of *Zanthoxylum leprieurii* (Rutaceae) were collected at Mont Kala near Yaoundé, Cameroon. The plant samples were first washed with tap water to remove dust and debris, and then air-dried on sterile filter paper. The cleaned material was cut into small pieces using a blade. Sterile conditions were maintained for the isolation of endophytes and all the work was performed in a laminar flow hood to avoid con-

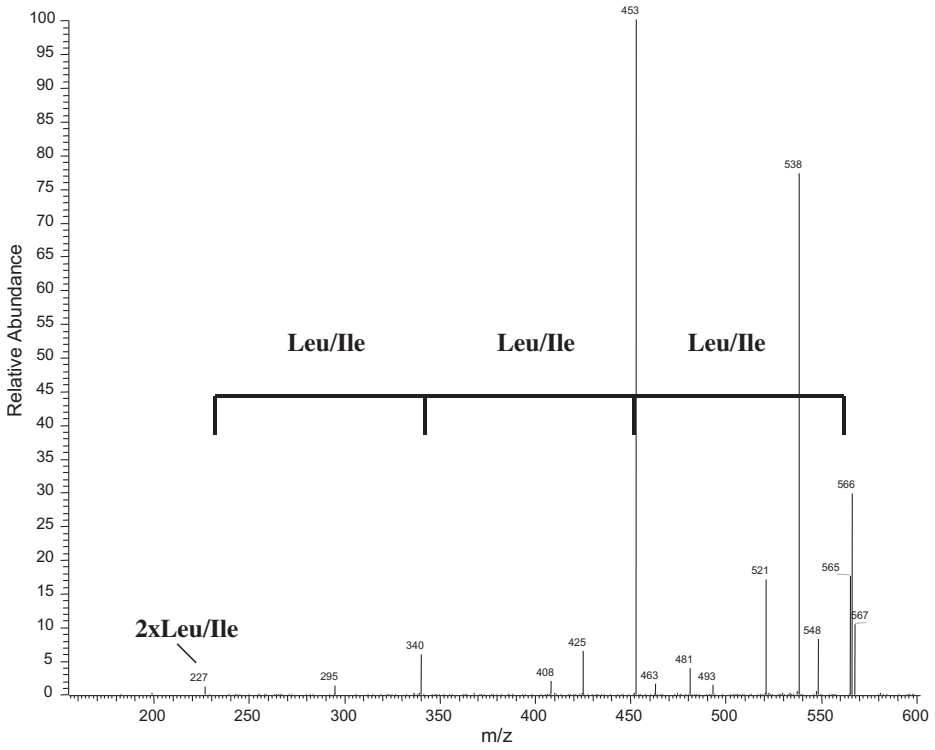

Fig. 3. CID-MS/MS spectrum of **4**, precursor ion $[M + H]^+$ at *m/z* 566.

Table 4
High-resolution CID-MS/MS fragmentation of cyclopeptides **4** and **5** (precursor ions $[M + Na]^+$).

Cyclopeptide **4**			Cyclopeptide **5**		
Precursor ion *m/z* 588.4 $[M + Na]^+$			Precursor ion *m/z* 622.4 $[M+Na]^+$		
m/z	Elemental composition	Fragmentation	*m/z*	Elemental composition	Fragmentation
560.4161	$C_{29}H_{55}N_5O_4Na^+$	- CO	594.4011	$C_{32}H_{53}N_5O_4Na^+$	- CO
447.3311	$C_{23}H_{44}N_4O_3Na^+$	- CO-Leu/Ile	481.3155	$C_{26}H_{42}N_4O_3Na^+$	- CO-Leu/Ile
362.2415	$C_{18}H_{33}N_3O_3Na^+$	- 2 × Leu/Ile	447.3321	$C_{23}H_{44}N_4O_3Na^+$	- CO-Phe
334.2468	$C_{17}H_{33}N_3O_2Na^+$	- CO-2 × Leu/Ile	368.2311	$C_{20}H_{31}N_3O_2Na^+$	- CO-2 × Leu/Ile
249.1574	$C_{12}H_{22}N_2O_2Na^+$	- 3 × Leu/Ile	334.2459	$C_{17}H_{33}N_3O_2Na^+$	- CO-Phe-Leu/Ile

tamination. Surface sterilization of the samples was achieved with 95% EtOH for 30 s, 10% sodium hypochlorite for 10 min, 70% EtOH for 2 min, and then dried aseptically. The inner tissues were placed on isolation media (water agar; WA) in Petri dishes supplemented with 100 mg/L chloramphenicol to suppress bacterial growth, and incubated at 25 °C until the outgrowth of endophytes was discerned. Individual fungal colonies were removed and transferred onto sterile potato dextrose agar (PDA: cooking water from 200 g potatoes/L, enriched with glucose 40 g/L, and agar-agar 20 g/L) and potato sucrose agar (PSA: potato cooking water (200 g potatoes/L), sucrose 40 g/L, agar-agar 20 g/L) or M_2 agar (malt extract 10 g/L, yeast extract 4 g/L, glucose 4 g/L, and agar-agar 15 g/L) and periodically checked for purity. Each isolate was kept in a slant agar tube for future investigations. The endophytic fungus isolate CAFT122-1 was identified by one of the authors (S.D.) on the basis of its 16S rRNA gene sequence and deposited in the microbial collection at the Institute of Organic and Biomolecular Chemistry, Georg-August University of Göttingen, Germany.

4.3. Fermentation and isolation of compounds

The fungus isolate CAFT122-1 was cultured on slants of potato dextrose agar (PDA) at 25 °C for 7 days and then agar plugs were used to inoculate 20 Erlenmeyer flasks, each containing a sterilized medium of 100 g of rice and 100 mL of water and incubated at room temperature under static conditions for 30 days. The culture was extracted three times with ethyl acetate (EtOAc) and the filtrate was concentrated to dryness *in vacuo* to afford a dark brown gum (10.7 g). A part of the extract (8.2 g) was separated into eight fractions (F_1–F_8) by column chromatography on silica gel, eluted with dichloromethane–methanol (CH_2Cl_2–MeOH) mixtures of increasing polarity. The third fraction eluted with CH_2Cl_2–MeOH (95:5) was chromatographed by silica gel column chromatography eluting with *n*-hexane–EtOAc with increasing polarity to afford cryptosporiopsin A (**1**) (10.5 mg), hydroxypropan-2′,3′-diol orsellinate (**3**) (2.7 mg), metabolite **6** (27.9 mg) as very unstable compound, and ergosterol. Fraction F_5 eluted with CH_2Cl_2–15% MeOH was applied to Sephadex LH-20 eluting with MeOH to afford compound **4** (17.2 mg) and compound **5** (40.9 mg).

4.4. Cryptosporiopsin A (*1*)

Colorless oil; $[\alpha]^{20}_D = +11$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 225 (3.24), 273 (3.11). IR (KBr) ν_{max}: 3435, 2932, 1714, 1598, 1557, 1253, 1025 cm^{-1}; ^1H NMR (DMSO-d_6), ^{13}C NMR (DMSO-d_6) and 2D NMR see Table 1 and Fig. S1–S4, Supplementary data; (+)-ESIMS *m/z* 403 ($[M + Na]^+$, 100); HRESIMS *m/z* 403.0919 $[M + Na]^+$ (calc. for $[C_{19}H_{21}O_6ClNa]^+$, 403.0919).

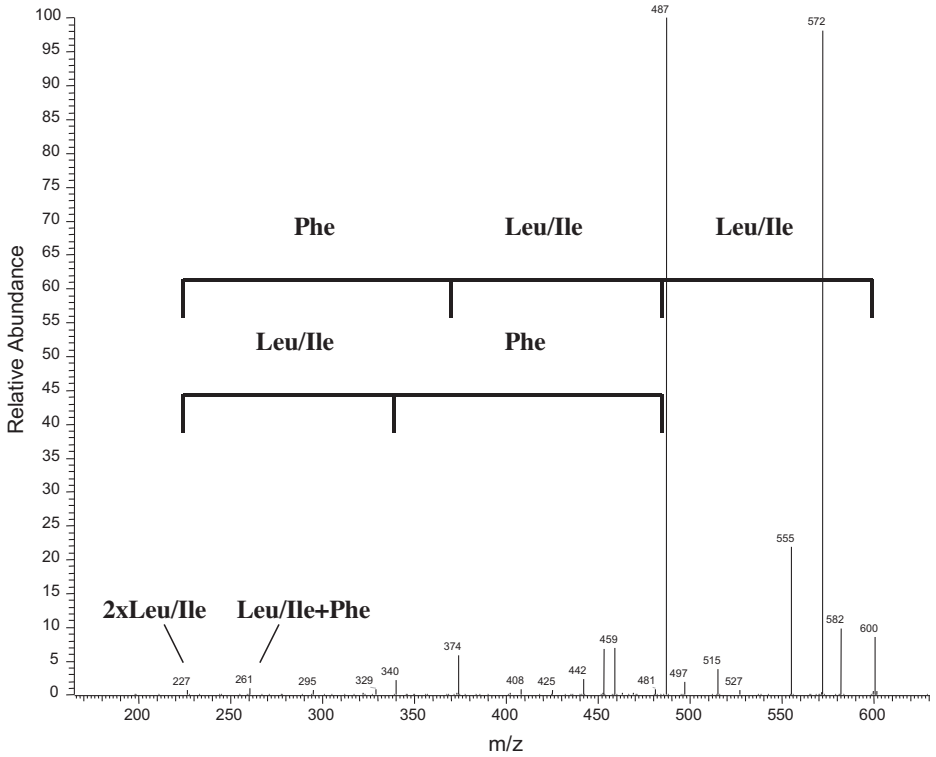

Fig. 4. CID-MS/MS spectrum of **5**, precursor ion $[M + H]^+$ at *m/z* 600.

Table 5
Motility inhibitory and zoosporicidal activities of compounds **1** and **3–6** against the downy mildew pathogen *P. viticola*.

	Dose (μg/mL)	Motility inhibitory and lytic activity (%)[a]							
		15 min		30 min		45 min		60 min	
		Halted	Lysis	Halted	Lysis	Halted	Lysis	Halted	Lysis
1	5.0[b]	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	10.0[c]	66 ± 5	0 ± 0	88 ± 7	32 ± 3	92 ± 4	73 ± 8	94 ± 5	84 ± 7
	25.0	79 ± 6	0 ± 0	92 ± 5	71 ± 7	99 ± 1	88 ± 6	100 ± 0	98 ± 2
	50.0	82 ± 7	0 ± 0	100 ± 0	100 ± 0	nt	nt	nt	nt
3	10.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	25.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	50.0	0 ± 0	0 ± 0	98 ± 2	88 ± 7	100 ± 0	100 ± 0	nt	nt
4	5.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	10.0	0 ± 0	0 ± 0	26 ± 8	0 ± 0	58 ± 5	53 ± 8	99 ± 1	93 ± 5
	25.0[d]	38 ± 6	0 ± 0	82 ± 5	0 ± 0	96 ± 5	78 ± 6	100 ± 0	100 ± 0
	50.0	80 ± 6	40 ± 7	99 ± 1	97 ± 3	100 ± 0	100 ± 0	nt	nt
5	5.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	10.0	60 ± 5	0 ± 0	88 ± 5	56 ± 9	100 ± 0	86 ± 5	100 ± 0	100 ± 0
	50.0	81 ± 7	0 ± 0	100 ± 0	100 ± 0	nt	nt	nt	nt
6	5.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	10.0	0 ± 0	0 ± 0	55 ± 6	29 ± 4	73 ± 7	51 ± 9	100 ± 0	100 ± 0
	25.0	41 ± 5	0 ± 0	77 ± 4	54 ± 8	92 ± 6	78 ± 6	100 ± 0	100 ± 0
	50.0	100 ± 0	0 ± 0	100 ± 0	88 ± 7	100 ± 0	100 ± 0	nt	nt
c.e	10.0	0 ± 0	0 ± 0	33 ± 8	0 ± 0	44 ± 6	0 ± 0	61 ± 6	0 ± 0
	25.0	72 ± 8	0 ± 0	86 ± 5	37 ± 6	93 ± 5	57 ± 4	96 ± 3	62 ± 6
	50.0	98 ± 2	28 ± 5	99 ± 1	62 ± 7	100 ± 0	80 ± 6	100 ± 0	89 ± 5

[a] Data presented here are average value of at least three replications in each dose of compound.
[b] Zoospores were moving very fast (stimulant activity).
[c] Aggregated and encysted.
[d] Zoospores moved to bottom of Petri dish and aggregated, c.e = crude extract.

4.5. Hydroxypropan-2′,3′-diol orsellinate (**3**)

Colorless oil; $[\alpha]^{20}_D = -17$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 216 (3.80), 292 (3.11); IR (KBr) ν_{max}: 3442, 2935, 1717, 1630, 1653, 1442, 1380, 1413, 1260, 1112 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2 and Fig. S5–S7, Supplementary data; (+)-ESIMS *m/z* 265 ($[M + Na]^+$); HRESIMS *m/z* 265.06830 $[M + Na]^+$ (calc. for $[C_{11}H_{14}O_6Na]^+$, 265.06826).

Table 6
Inhibitory concentrations of isolated compounds and crude extract against three plant pathogenic fungi, and mortality rates (%) for brine shrimp larvae (*A. salina*) at 10 μg/mL.

Compound	*A. cochlioides*	*P. ultimum*	*R. solani*	*A. salina* (%)
1*	20	40	50	20.1
3	20	40	>100	10.2
4	20	>100	>100	12.3
5	>100	>100	>100	10
6	50	40	>100	27.3
Crude extract	++	++	++	90

* Our cryptosporiopsin A (**1**) was contaminated with some β-hydroxypropionic acid or with a polymer thereof, a compound eluted from preparative TLC plates with solvents of higher polarity. However, as the biological activity of fractions containing cryptosporiopsin A (**1**) proportionally increased with the purity of this compound, we are convinced that the biological activity of cryptosporiopsin presented in this paper represents activity of **1** not of the impurity.

4.6. Cyclo-(L-Ile-L-Leu-L-Leu-L-Leu-L-Leu-L-Leu) (**4**)

White powder; $[\alpha]^{20}_{D} = -43$ (*c* 0.13, MeOH); IR (KBr) ν_{max}: 3412, 2966, 1717, 1700, 1653, 1568, 1458, 1414, 1247, 1031, 729 cm^{-1}; ^{1}H NMR (DMSO-d_6), ^{13}C NMR (DMSO-d_6) and 2D NMR see Table 3 and Fig. S8–S11, Supplementary data; (+)-ESIMS *m*/*z* (%): 588 ($[M + Na]^+$, 40), 1153 ($[2\ M + Na]^+$, 100); (+)-HRESIMS *m*/*z* 566.4274 $[M + H]^+$ (calc. for $[C_{30}H_{56}N_5O_5]^+$, 566.4276).

4.7. Biological activities

4.7.1. Antifungal bioassay

The pathogenic fungi *P. ultimum*, *A. cochlioides*, and *R. solani* were made available from our culture collection. The samples were dissolved in CH_2Cl_2/10% MeOH at a concentration of 1 mg/mL. Aliquots were soaked on paper disks (9 mm ∅, Schleicher & Schüll, Germany) and dried for 1 h at room temperature under sterile conditions. The paper disks were placed on PDA agar plates previously inoculated with the test fungi and then incubated for 48 h at 28 °C in the dark. Pure compounds were tested at 20–100 μg/paper disk. The diameters of inhibition zones were then measured.

4.7.2. Brine shrimp microwell cytotoxicity assay

The test was performed according to our previous descriptions (Sajid et al., 2009).

4.7.3. Motility inhibitory and zoosporicidal assay

A strain of *P. viticola* was obtained from Prof. Beate Berkelmann-Löhnertz, Geisenheim Research Center, Germany. This strain was originally isolated from infected leaves of grapevine (*Vitis vinifera*) cv. Riesling in 1996 and since then propagated on fresh leaves of grapevine cv. Mueller-Thurgau. Stock solutions of each isolated compound were prepared in small amounts of dimethyl sulfoxide. Then a series of concentrations of each compound was prepared in distilled water. The bioassays for motility of zoospores were carried out following protocols described earlier (Islam et al., 2002, 2011; Islam and Tiedemann, 2008). Briefly, 40 μL of sample solution in appropriate concentration was directly added to 360 μL of motile zoospore suspension (ca. 1×10^5/mL) of *P. viticola* taken in a cavity of a plant tissue culture multi-well plate to make a final volume of 400 μL and then quickly mixed with a glass rod. The final concentration of DMSO in the zoospore suspension never exceeded 1% (v/v), a condition that does not affect zoospore motility. A 1% aqueous DMSO solution was used as control. The motility inhibition and lysis of zoospores at varying doses of the test compounds were observed under a light microscope at 10× magnification. Quantification of time-course of motility inhibition and lysis of zoospores was carried out as described earlier (Islam et al., 2002, 2011). Each treatment was replicated three times. The mean values ± SE (standard error) of percentage motility inhibition and lysed zoospores in each treatment were calculated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.06.006.

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