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Bioactive metabolites from the endophytic fungus *Ampelomyces* sp. isolated from the medicinal plant *Urospermum picroides*

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Abstract

Extracts of cultures grown in liquid or on solid rice media of the fungal endophyte *Ampelomyces* sp. isolated from the medicinal plant *Urospermum picroides* exhibited considerable cytotoxic activity when tested *in vitro* against L5178Y cells. Chromatographic separation yielded 14 natural products that were unequivocally identified based on their ^{1}H and ^{13}C NMR as well as mass spectra and comparison with previously published data. Six compounds (**2**, **4**, **5**, **7**, **9** and **11**) were natural products. Both fungal extracts differed considerably in their secondary metabolites. The extract obtained from liquid cultures afforded a pyrone (**2**) and sulfated anthraquinones (**7** and **9**) along with the known compounds **1**, **3**, **6** and **8**. When grown on solid rice medium the fungus yielded three compounds **4**, **5** and **11** in addition to several known metabolites including **6**, **8**, **10**, **12**, **13** and **14**. Compounds **4**, **8** and **10** showed the strongest cytotoxic activity against L5178Y cells with EC_{50} values ranging from 0.2–7.3 μg/ml. Furthermore, **8** and **10** displayed antimicrobial activity against the Gram-positive pathogens, *Staphylococcus aureus*, *S. epidermidis* and *Enterococcus faecalis* at minimal inhibitory concentrations (MIC) of 12.5 μg/ml and 12.5–25 μg/ml, respectively. Interestingly, **6** and **8** were also identified as constituents of an extract derived from a healthy plant sample of the host plant *U. picroides* thereby indicating that the production of bioactive natural products by the endophyte proceeds also under *in situ* conditions within the host plant.

Keywords: *Ampelomyces*; Endophytic fungi; Anthraquinones; Pyrones; Chromones; Cytotoxic activity; Antibacterial activity

1. Introduction

Fungal endophytes are microorganisms that colonize living, internal tissues of plants without causing any immediate, overt negative effects (Bacon and White, 2000). They have proven to be promising sources of new and biologically active natural products which are of interest for specific medicinal or agrochemical applications (Strobel, 2002).

In this study we investigated the fungal endophyte *Ampelomyces* sp. isolated from flowers of *Urospermum picroides* growing in the wild in Egypt. *U. picroides* is a typical constituent of the traditional Mediterranean diet and its extract shows anti-inflammatory activities (Ebel, 2006; Strzelecka et al., 2005).

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Historically, pycnidial fungi belonging to the genus *Ampelomyces* were among the first mycoparasites to be studied in detail and were also the first fungi used as biocontrol agents of plant parasitic fungi (Yarwood, 1932; Sundheim and Krekling, 1982). Biocontrol agents that can supplement conventional chemical fungicides are a valuable contribution to disease management, especially for a disease like powdery mildew (Shishkoff and McGrath, 2002). The interactions between host plants, powdery mildew fungi and *Ampelomyces* mycoparasites are one of the clearest cases of tritrophic relationships in nature (Kiss et al., 2004). While it seems likely that fungal metabolites are involved in many reported interspecies interactions, *Ampelomyces* mycoparasites attracted our attention because they have rarely been studied chemically. However, the biofungicide AQ10, a pelleted formulation of conidia of *Ampelomyces quisqualis*, is intended for use as part of an integrated management program for powdery mildew (Shishkoff and McGrath, 2002). Recently, 3-chloro-2,5-dihydroxybenzyl alcohol, a potent antifouling and antimicrobial compound, was isolated from a marine-derived *Ampelomyces* sp. using a bioassay-guided isolation and purification procedure. The compound effectively inhibited larval settlement of the tubeworm *Hydroides elegans* and of cyprids of the barnacle *Balanus amphitrite*. In addition, the growth of 13 out of 15 marine bacterial species was strongly inhibited by this compound when tested in disc diffusion bioassay (Kwong et al., 2006). These results as well as our findings reported in the present investigation indicate that fungi of the genus *Ampelomyces* are interesting sources for new, bioactive metabolites that warrant further studies.

2. Results and discussion

Extracts of *Ampelomyces* sp. grown in liquid culture afforded a new pyrone, ampelopyrone (**2**), two new sulfated derivatives (**7** and **9**) of macrosporin (**6**) and 3-*O*-methylalaternin (**8**) together with the known compounds methyltriacetic lactone (**1**), citreoisocoumarin (**3**), macrosporin (**6**) and 3-*O*-methylalaternin (**8**). In contrast those from the same fungus grown on solid rice medium gave two new isocoumarins, desmethyldiaportinol (**4**) and desmethyldichlorodiaportin (**5**), and a new hexahydroanthronol, ampelanol (**11**), as well as the known compounds **6**, **8**, altersolanol A (**10**), alterporriols D and E (**12** and **13**) and altersolanol J (**14**).

Compound **2** was isolated as a viscous yellow oil whose HRESI-MS exhibited a strong peak at *m*/*z* 227.0910 $[M+H]^+$ indicating a molecular formula of $C_{11}H_{14}O_5$. Its UV spectrum showed a close similarity to that of methyltriacetic lactone (**1**). The ^1H and ^{13}C NMR spectra (Table 2) indicated the presence of three methyl groups, belonging to a methyl group located at the α-position of the carbonyl group of the conjugated lactone at δ_H 1.71 and δ_C 8.4 (3-CH_3), an acetoxy methyl group at δ_H 1.94 and δ_C 20.8 (H_3/C-11), and a secondary methyl group at δ_H 1.19 (*d*, $J = 6.3$ Hz) and δ_C 19.4 (H_3/C-9). The last was part of an esterified 2-hydroxypropyl group as evident from the COSY spectrum, which in turn was attached at C-6 of the cyclic lactone system, as shown from the HMBC correlation of H_2-7 to C-5 (Table 2). As in methyltriacetic lactone, a vinyl proton singlet at δ_H 5.97 assigned to H-5 and the corresponding vinyl carbon at δ_C 101.2 were observed. The ^{13}C NMR spectrum also showed a quaternary carbon at δ 96.7 corresponding to C-3, and three oxygenated quaternary carbons at δ 165.5, 164.7 and 158.5, corresponding to C-4, C-2 and C-6, respectively. The α-pyrone core structure found in **2** was confirmed by the HMBC correlations of 3-CH_3 to C-2, C-3 and C-4 and of H-5 to C-3 (Table 2), as well as by comparison of the NMR data with those obtained for methyltriacetic lactone (Fehr et al., 1999). Thus, **2** was identified as a new natural product for which we suggest the name ampelopyrone.

Compound **7** was isolated in the form of yellow crystals. The HRESI-TOF MS exhibited a prominent pseudomolecular ion peak at *m*/*z* 408.9970 $[M-H+2Na]^+$ indicating a molecular formula of $C_{16}H_{12}O_8S$ (for the corresponding free acid). Comparison of the ^1H, ^{13}C NMR and HMBC data (Table 4) with those measured for macrosporin (**6**) showed good accordance except for the downfield shifts observed for H-8, C-6, and C-8 as well as the upfield shift of C-7, indicating the presence of a sulfate substitution at C-7 (Ragan, 1978). This was further corroborated by a prominent fragment at *m*/*z* 283 formed through loss of 80 mass units in the mass spectrum of **7** upon negative ionization, and the hypsochromic shift in the UV spectrum of **7** compared to that of **6** (Plasencia and Mirocha, 1991). Compound **7** was thus identified as the new natural product macrosporin-7-*O*-sulfate.

Compound **9** was obtained as orange crystals. The HRESI-TOF MS exhibited a prominent pseudomolecular peak at *m*/*z* 424.9910 $[M-H+2Na]^+$ consistent with the molecular formula $C_{16}H_{12}O_9S$ (for the corresponding free acid). The NMR spectra (Table 4) were reminiscent of those obtained for 3-*O*-methylalaternin (**8**), but exhibiting similar chemical shift differences as discussed above for macrosporin (**6**) and its 7-*O*-sulfate (**7**). Accordingly, in the ESI mass spectrum the characteristic loss of 80 mass units due to a sulfate group was observed, while the UV spectrum of **9** showed a hypsochromic shift compared to that of **8**. Compound **9** was thus identified as the new natural product 3-*O*-methylalaternin-7-*O*-sulfate.

The known compounds (**1**, **3**, **6** and **8**) isolated from the extract of the liquid culture of *Ampelomyces* sp. were identified by their UV, ^1H, ^{13}C NMR, and mass spectral data and by comparison with published data (Fehr et al., 1999; Akira et al., 1998; Suemitsu et al., 1984; Lee et al., 1998).

The EtOAc extract of rice cultures of *Ampelomyces* sp. yielded 11 compounds (**4**–**14**). Compounds **10** and **12**–**14**

Table 1
Cytotoxicity and antimicrobial activities of *Ampelomyces* extracts and isolated compounds

Samples tested	L5178Y growth in% (Conc. 10 μg/ml)	EC_{50} (μg/ml)	MIC (μg/ml)				
			S. epi[a]	*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*
Ampelomyces liquid *n*-BuOH	98.3		n.d.	n.d.	n.d.	n.d.	n.d.
Ampelomyces liquid EtOAc	108.2		n.d.	n.d.	n.d.	n.d.	n.d.
Ampelomyces liquid MeOH	65.9		n.d.	n.d.	n.d.	n.d.	n.d.
Ampelomyces rice EtOAc	0.3		n.d.	n.d.	n.d.	n.d.	n.d.
1	100.5		>50.0	>50.0	>50.0	>50.0	>50.0
2	102.6		>50.0	>50.0	>50.0	>50.0	>50.0
3	99.5		>50.0	>50.0	>50.0	>50.0	>50.0
4	−0.4	7.30	n.d.	n.d.	n.d.	n.d.	n.d.
5	41.4		n.d.	n.d.	n.d.	n.d.	n.d.
6	54.5		>50.0	>50.0	>50.0	>50.0	>50.0
7	74.8		>50.0	>50.0	>50.0	>50.0	>50.0
8	4.9	1.25	12.5	12.5	12.5	>50.0	>50.0
9	93.6		>50.0	>50.0	>50.0	>50.0	>50.0
10	0.0	0.21	12.5	25.0	12.5	>50.0	>50.0
11	69.1		>50.0	n.d.	n.d.	n.d.	n.d.
12	64.8		>50.0	>50.0	>50.0	>50.0	>50.0
13	92.1		>50.0	>50.0	>50.0	>50.0	>50.0
14	35.7		>50.0	>50.0	>50.0	>50.0	>50.0
Gentamycin (control)	n.d.		n.d.	n.d.	25.0	1.6	1.6
Tetracycline (control)	n.d.		0.4	n.d.	n.d.	n.d.	n.d.

n.d. – not determined.
[a] *S. epidermidis*.

Table 2
^1H, ^{13}C NMR, COSY and HMBC data of compound **2** at 500 (^1H) and 125 (^{13}C) MHz

Nr.	2			
	δ_H (DMSO-d_6)	COSY	HMBC	δ_C (DMSO-d_6)
2				164.7[a]
3				96.7
4				165.5[a]
5	5.97, *s*	7	3,6,7	101.2
6				158.5
7	2.65, *m*	5,8	5,6,8	38.5
8	5.02, *m*	7,9		67.6
9	1.19, *d* (6.3)	8	7,8	19.4
10				169.6
11	1.94, *s*		10	20.8
3-CH_3	1.71, *s*		2,3,4	8.4
4-OH	4.02, *br s*			

[a] May be interchanged.

were identified by their UV, ^1H, ^{13}C NMR, $[\alpha]_D$ values and mass spectral data and comparison with published data as altersolanol A (**10**) (Yagi et al., 1993; Okamura et al., 1993, 1996), alterporriol D (**12**) (Lazarovits et al., 1988; Suemitsu et al., 1989), alterporriol E (**13**) (Lazarovits et al., 1988; Suemitsu et al., 1989) and altersolanol J (**14**) (Höller et al., 2002).

Compound **4** was isolated as a viscous yellow oil and showed UV absorbances characteristic of an isocoumarin derivative (Larsen and Breinholt, 1999). The HRESI-MS exhibited a prominent peak at *m/z* 275.0540 $[M+Na]^+$ indicating the molecular formula $C_{12}H_{12}O_6$. The ^1H NMR spectrum (Table 3) displayed characteristic signals attributable to protons H-4, H-5 and H-7, appearing at δ 6.43 (*s*), 6.42 (*d*, $J = 2.2$ Hz) and 6.37 (*d*, $J = 2.2$ Hz), respectively, in a 3,6,8-trisubstituted isocoumarin ring system. The downfield singlet signal (δ 11.14) indicated the presence of a strongly hydrogen-bonded phenolic proton at C-8. ^1H NMR, COSY and NOE spectra (Table 3) confirmed the substitution pattern and demonstrated the presence of a CH_2CHCH_2 fragment consisting of two methylene protons detected at δ 2.76 (*dd*, $J = 14.5$, 3.7 Hz, H-9 A) and δ 2.52 (*dd*, $J = 14.5$, 8.8 Hz, H-9B), an oxymethine proton at δ 4.02, and a oxymethylene group at δ 3.55 (*d*, $J = 5.3$ Hz). The attachment of the side chain at C-3 was confirmed by the NOE correlation of H-4 to H_2-9 (Table 3). Comparison of UV, ^1H, ^{13}C NMR and mass spectral data with literature data indicated the similarity of **4** to the known compound diaportinol, which possesses a methoxyl group at C-6 (Larsen and Breinholt, 1999). The absolute stereochemistry of **4** was assumed to be identical to diaportinol and dichlorodiaportin based on the $[\alpha]_D$ values (Larsen and Breinholt, 1999). Thus **4** was identified as a new natural product and was given the name desmethyldiaportinol.

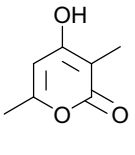
Compound **5** was isolated as a viscous yellow oil and, like **4**, showed UV absorbances characteristic of an isocoumarin derivative. The HRESI-MS exhibited a prominent peak at *m/z* 326.9800 $[M+Na]^+$ indicating the molecular formula $C_{12}H_{10}O_5Cl_2$. Pseudomolecular ion peaks at the nominal masses *m/z* 327.0, 329.0 and 331.0, together with corresponding signals in the negative ESI-MS at *m/z* 303.5, 305.4 and 307.4 $[M-H]^-$, displayed the distinctive isotope pattern caused by two chlorine atoms in the molecule. The ^1H NMR spectrum (Table 3) showed characteristic signals assigned to protons

Table 3
^{1}H NMR, COSY and NOE data of compounds **4** and **5** at 500 MHz

Nr.	**4**				**5**			
	δ_H (MeOD)	δ_H (acetone-d_6)	COSY	NOE	δ_H (MeOD)	δ_H (acetone-d_6)	COSY	NOE
4	6.38, *s*	6.43, *s*	9A	5,9A,9B	6.41, *s*	6.50, *s*	9A	5,9B,9A
5	6.29, *s*	6.42, *d* (2.2)	7	4	6.30, *s*	6.42, *d* (2.0)	7	4
7	6.29, *s*	6.37, *d* (2.2)	5		6.30, *s*	6.38, *d* (2.0)	5	
9A	2.74, *dd* (14.6,4.1)	2.76, *dd* (14.5,3.7)	4,9B,10		2.96, *dd* (14.8,3.1)	2.98, *dd* (14.5,3.4)	4,9B,10	
9B	2.52, *dd* (14.6,8.8)	2.52, *dd* (14.5,8.8)	9A,10		2.72, *dd* (14.8,9.4)	2.76, *dd* (14.5,9.2)	9A,10	
10	3.99, *m*	4.02, *m*	9A,9B,11		4.29, *ddd* (9.4,3.4,3.1)	4.39, *ddd* (9.2,3.4,3.1)	9A,9B,11	
11	3.54, *d* (5.3)	3.55, *d* (5.3)	10		6.02, *d* (3.4)	6.20, *d* (3.1)	10	10
8-OH		11.14, *brs*						

H-4, H-5 and H-7 observed at δ 6.50 (*s*), 6.42 (*d*, $J = 2.0$ Hz) and 6.38 (*d*, $J = 2.0$ Hz), respectively, indicating a 3,6,8-trisubstituted isocoumarin ring system, similar to desmethyldiaportinol (**4**). ^{1}H NMR, COSY and NOE spectra (Table 3) confirmed the substitution pattern and demonstrated the presence of an analogous $CH_2CH(OH)CH$ substructure as described above for **4**, with two methylene protons detected at δ 2.98 (*dd*, $J = 14.5$, 3.4 Hz, H-9A) and 2.76 (*dd*, $J = 14.5$, 9.2 Hz, H-9B), an oxymethine proton at δ 4.39, and a methine group at δ 6.20 (*d*, $J = 3.4$ Hz). Thus, the ^{1}H NMR data of **5** were very similar to those of desmethyldiaportinol (**4**), except for the marked downfield shift of H-11 (δ 6.20 compared to 3.55) which indicated dichlorosubstitution at this position. This was also in agreement with the fragmentation pattern observed in the negative ESI mass spectrum which showed the subsequent loss of both chlorine atoms (see Section 3). The attachment of the side chain at C-3 was confirmed by the NOE correlation of H-4 to H_2-9 (Table 3). The absolute stereochemistry of **5** was assumed to be identical to dichlorodiaportin based on the $[\alpha]_D$ value (Larsen and Breinholt, 1999). Thus, **5** was identified as the 10-deoxy-10,10-dichloro congener of **4** and represents a new natural product for which we propose the name desmethyldichlorodiaportin.

Compound **11** was isolated in the form of white crystals. The HRESI-MS exhibited a prominent peak at *m/z* 341.1230 $[M+H]^+$, four mass units greater than altersolanol A (**10**), indicating a molecular formula $C_{16}H_{20}O_8$. The ^{1}H NMR spectrum (Table 5) contained five exchangeable alcoholic hydroxyl groups, two doublets at δ 5.49 and 5.06, a broad singlet at δ 4.41 and two singlets at δ 4.46 and 4.21, assigned to 9-OH, 1-OH, 3-OH, 4-OH, and 2-OH, respectively. In addition, a singlet of a chelated phenol appeared at δ 12.57 which was likewise exchangeable and was attributed to 5-OH. A singlet corresponding to an aliphatic methyl group was detected at δ 1.20 (2-CH_3), while four oxymethine protons resonated at δ 4.67 (H-9), 3.82 (H-4), 3.76 (H-1) and 3.36 (H-3). Similar to compounds **6**–**10**, the *meta*-coupled protons H-8 and H-6 appeared at δ 6.72 and 6.35, respectively, while the aromatic methoxyl group was detected at δ 3.83. In the COSY spectrum, the low field aryl proton H-8 exhibited a long range correlation to a *peri*-proton (H-9), which in turn coupled to both the hydroxyl signal at δ 5.49 (9-OH) and the ring junction proton at δ 2.30 (H-9a). These results indicated that the quinone carbonyl at C-9 in **10** had been reduced to a hydroxyl group, while the double bond between C-9a and C-4a, present in **10,** had also been reduced, which is in accordance with the observed increase of the molecular weight of compound **11** and the absence of colour for this compound. The complete aliphatic spin system comprising H-9, H-9a, H-1, H-4a, H-4 and H-3, together with the corresponding hydroxyl functions, was clearly discernible in the COSY spectrum of **11** (Table 5). Furthermore, in the HMBC spectrum (Table 5) the correlations attributed to the two protons at the ring junction, *i.e.* H-9a (to C-4a and C-9) and H-4a (to C-4, C-9, C-9a, and C-10), as well as the correlation of H-8 to C-9 fully supported the assignment of the planar structure as depicted. The relative stereochemistry of **11** was deduced from the coupling constants observed in the ^{1}H NMR spectrum as well as from the correlations detected in the ROESY spectrum (Table 5). The large values of J_{3-4} (9.4 Hz), J_{4-4a} (9.4 Hz), J_{4a-9a} (13.2 Hz) and J_{9-9a} (10.5 Hz) could only be explained by a series of mutual diaxial relationships and thus proved that all of these hydrogens were axially positioned, while the corresponding 2.2 Hz coupling between H-9a and H-1 indicated an equatorial position for the latter. Correlations of H-9 to 1-OH and H-4a, 2-CH_3 to both 1-OH and H-3, as well as H-4a to H-3 and 4-OH in the ROESY spectrum, indicated their position on the β-face of the molecule. On the other hand, correlations of H-4 to 2-OH and 3-OH, and of 9-OH to both H-1 and H-9a, as well as of H-9a to 2-OH indicated their α-orientation (Table 5). These data indicated a chair conformation for cyclohexane ring in **11** with the relative stereochemistry as depicted. Thus, **11** was identified as a new natural product for which we propose the name ampelanol. The structure was further confirmed by comparing NMR data of **11** to those reported for altersolanol A (**10**) (Yagi et al., 1993; Okamura et al., 1993, 1996), and tetrahydroaltersolanol B (Stoessl and Stothers, 1983), in which the 1- and 4-OH groups are absent.



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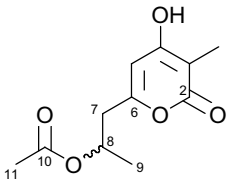

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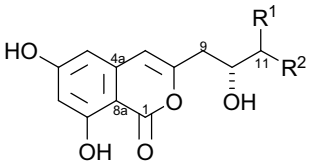
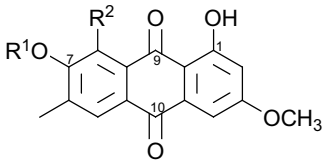
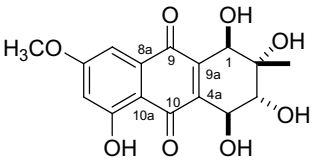


3 (R^1= $COCH_3$, R^2 = H)
4 (R^1= OH, R^2 = H)
5 (R^1= R^2 = Cl)



6 (R^1= R^2 = H)
7 (R^1= $SO_3^-Na^+$, R^2 = H)
8 (R^1= H, R^2 = OH)
9 (R^1= $SO_3^-Na^+$, R^2 = OH)



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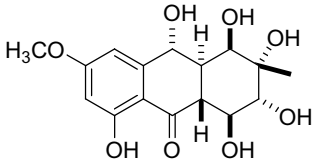
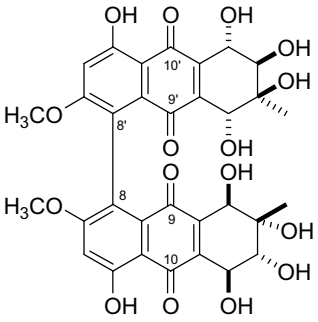
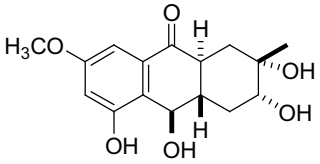


11



12, **13**:atropisomers



14

The differences in the chemical composition also resulted in a clear difference in the cytotoxic activity toward L5178Y mouse lymphoma cells of the two EtOAc extracts, with the one obtained from solid rice cultures being far more active than the one from liquid cultures (Table 1). The corresponding results obtained for the pure natural products **1**–**14** showed that the tetrahydroanthraquinone, altersolanol A (**10**) was the most active compound. The anthronol derivatives, ampelanol (**11**) and altersolanol J (**14**) showed only moderate to weak activities, suggesting that the *para*-quinone moiety is of great importance for the cytotoxic activity. Furthermore, the monomer **10** was far more active than its dimers **12** and **13**. 3-*O*-Methylalaternin (**8**) was the second most active derivative, while macrosporin (**6**) only showed moderate activity, indicating that the 1,8-dihydroxy substitution in the case of the anthraquinones greatly increased cytotoxic activity. Sulfate substitution at 7-OH found in **9** resulted in a reduced activity compared to **8** indicating a possible contribution of this hydroxyl group to the cytotoxic activity of these compounds or a reduced uptake of the more polar derivatives by the lymphoma cells. For the isocoumarins tested only **4** exhibited moderate activity against lymphoma cells, whereas the pyrones were inactive.

The increasing use of antibiotics for antibacterial therapy has initiated a rapid development of antibiotic resistance in microorganisms. Of particular concern are human infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant *Enterococcus* (Menichetti, 2005). Thus, the search for new antimicrobial agents is of utmost importance. The antimicrobial potential of the isolated compounds was investigated using the minimal inhibitory concentration (MIC) assay. The following multiresistant indicator strains were tested: *Staphylococcus epidermidis*, *S. aureus*, *Enterococcus faecalis* (Gram-positive) and *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative). Two substances, 3-*O*-methylalaternin (**8**) and altersolanol A (**10**) exhibited antimicrobial activity against Gram-positive pathogens (Table 1). 3-*O*-Methylalaternin showed activity with a MIC of 12.5 μg/ml against *S. epidermidis*, *S. aureus*, *E. faecalis*. Altersolanol A (**10**) featured a MIC value of 12.5 μg/ml against *S. epidermidis* and *E. faecalis*, and 25 μg/ml against*S. aureus*. Altersolanol A (**10**) was reported in the literature to inhibit the growth of Gram-positive bacteria and *P. aeruginosa* IFO 3080 when tested using the broth dilution method (Yagi et al., 1993). It was found that the compound acts as an electron acceptor

in the bacterial membrane and thus inhibits bacterial growth (Haraguchi et al., 1992). Therefore, the antibacterial activity of **10** is probably not due to its cytotoxic activity.

The crude MeOH extract of the host plant *U. picroides* was fractionated over Diaion HP20 and the fractions obtained were analyzed by LC/MS, for the presence of the identified fungal metabolites. Co-elution studies with the corresponding pure metabolites and the respective plant fractions were carried out to increase the specificity of the method and spectra were evaluated for matching of retention times, the presence of the molecular ions of the target compounds, as well as patterns of MS and MS/MS spectra of the pure substances and the substances detected in the host plant fractions. Macrosporin (**6**) and 3-*O*-methylalaternin (**8**) could be unequivocally detected in fraction 5 (obtained with 100% acetone) (Fig. 1). Interestingly, these substances were produced both in liquid cultures of *Ampelomyces* sp. and in cultures grown on solid rice medium, while any of the compounds obtained from only one of the two cultures were not detected in the host plant fractions. These results suggest the possible production of such metabolites by the endophytic fungus under its normal physiological conditions of growth within the tissues of the healthy plants, implying their possible contribution to the mutualistic interaction between the endophyte and its host plant. It is worth mentioning that, apart from a few studies which reported more or less circumstantial isolations of typical fungal metabolites from plant sources, the presence of secondary metabolites of endophytic fungi in the same host plants from which the respective fungi had originally been isolated has rarely been documented. Strictly speaking, the general hypothesis that the presence of endophytes enhances host plant fitness and competitiveness in stressful environments by producing functional metabolites had so far only been proven for grass-endophyte associations. Our study, however, showed that this could also be true for other plant-endophyte associations, supported by the unequivocal detection of fungal metabolites in the host plant. It may also be hypothesized that "typical" fungal metabolites reported previously from other plants may likewise originate from endophytic fungi colonizing these plants. For example, bipendensin was originally isolated in very small amounts from wood samples of the African tree *Afzelia bipendensis*, but four years later was obtained from an unidentified *Coniothyium* fungus (Connolly, 1991; Krohn et al., 1994). Our current findings therefore add an important contribution to questions related to the ecological function of secondary metabolites produced by endophytic fungi, which could lead to a better understanding of this interesting group of organisms as well as help in the specific search for new bioactive substances with pharmaceutical potential.

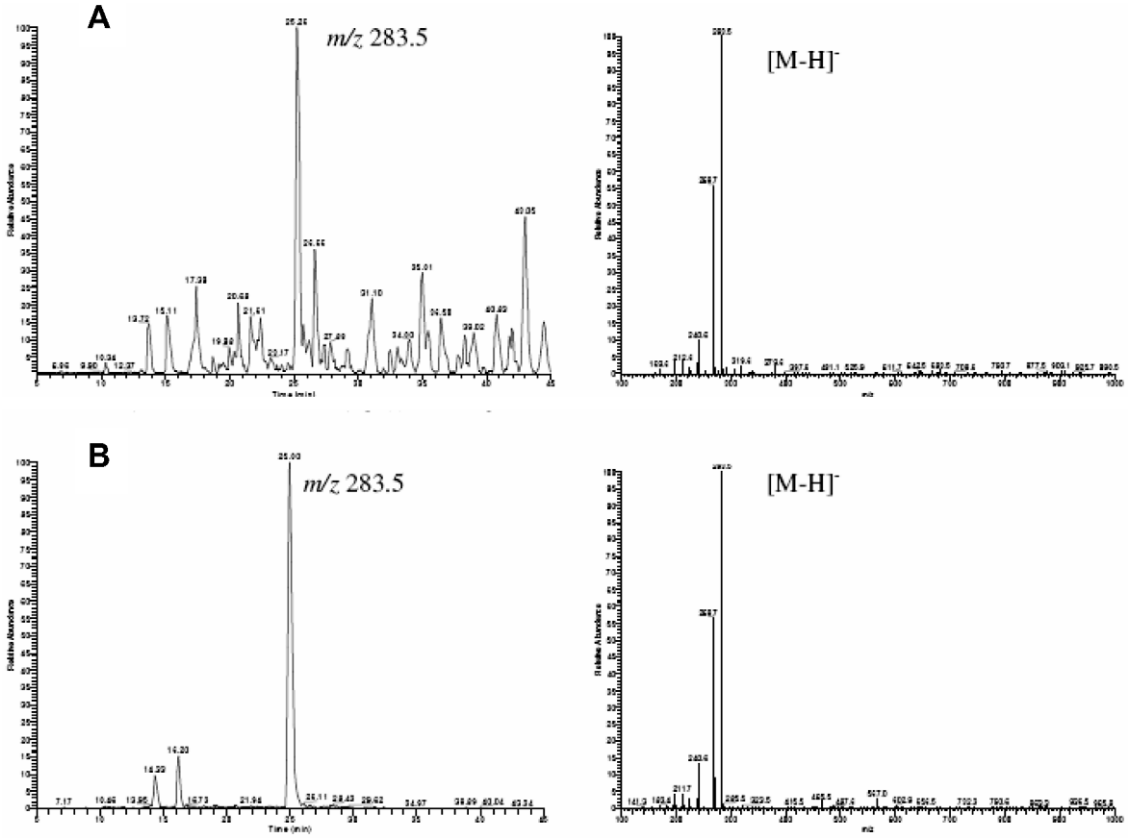


Fig. 1. Detection of macrosporin (**6**) by LC/MS (**A**, in *Urospermum picroides*, fraction 5; **B**, reference). Mass chromatograms at *m/z* 283 are shown, corresponding to the pseudomolecular ion $[M-H]^-$ of **6** (left), together with the corresponding mass spectrum taken at the apex of the respective main peaks (right).

3. Experimental

3.1. General remarks

^{1}H and ^{13}C NMR spectra were recorded on Bruker ARX 500 or AVANCE DMX 600 NMR spectrometers. ESI-MS was conducted on a Finnigan LCQ-Deca mass spectrometer and HRESI-MS spectra were obtained on a Micromass Qtof 2 mass spectrometer. Solvents were distilled before use, and spectral grade solvents were used for spectroscopic measurements. HPLC analysis was performed using a HPLC (Dionex P580) system coupled to a photodiode array detector (UVD340S). Routine detection was at 235, 254, 280 and 340 nm. The separation column (125 × 4 mm, L × ID) was prefilled with Eurospher-10 C18 (Knauer, Germany) using a linear gradient of MeOH and 0.02% H_3PO_4 in H_2O and a flow rate of 1 ml/min. UV data (λ_{max}) for individual compounds were extracted from the online UV spectra provided by the instrument software. TLC plates with silica gel F_{254} (Merck, Darmstadt, Germany) were used for monitoring of fractions using *n*-hexane:EtOAc (95:5 and 90:10), CH_2Cl_2:MeOH (95:5, 90:10, 85:15 and 80:20) as well as CH_2Cl_2:MeOH:EtOAc (90:10:5 and 80:20:10). Detection was at 254 and 366 nm or by spraying the plates with anisaldehyde reagent. Optical rotations were determined on a Perkin–Elmer-241 MC polarimeter.

3.2. Fungal material

The fungus *Ampelomyces* sp. was isolated from fresh healthy flowers of *Urospermum picroides* (L.) F.W. Schmidt growing in the wild. The plant was collected in April 2004 near Alexandria, Egypt. A voucher specimen has been deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University. Voucher specimens were identified by Prof. Dr. Amin El-Sayed Ali, Department of Crops, Faculty of Agriculture, Alexandria University. Following surface sterilization with 70% EtOH for 1 min the flowers were rinsed in sterile water. To distinguish remaining epiphytic fungi from endophytic fungi, an imprint of the flower surface on biomalt agar was made. Small tissue samples from inside the flowers were aseptically cut and pressed onto agar plates containing an antibiotic to suppress bacterial growth (composition of isolation medium: 15 g/l malt extract, 15 g/l agar and 0.2 g/l chloramphenicol in distilled water, pH 7.4–7.8). After incubation at room temperature the fungal strain under investigation was found to grow exclusively out of the plant tissue, but not on the agar plates taken from the imprint of the flower surface. From the growing cultures pure strains of *Ampelomyces* sp. were isolated by repeated re-inoculation on malt agar plates.

3.3. Identification of fungal cultures

Fungal cultures were identified according to a molecular biological protocol by DNA amplification and sequencing of the ITS region as described previously (Wang et al., 2006). The sequence data has been submitted to GenBank, accession number EU143251. The fungal strain was identified as *Ampelomyces* sp., however, due to the lack of similar sequences in the GenBank, identification of the strain to the species level was not possible.

3.4. Cultivation

Mass growth of the fungus for the isolation and identification of new metabolites was carried out in Erlenmeyer flasks (1 l each). The fungus was grown in liquid Wickerham medium (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, distilled water added up to 1000 ml, pH adjusted to 7.2–7.4) or on rice solid medium (to 100 g commercially available rice was added 100 ml of distilled water and kept overnight prior to autoclaving) at room temperature under static conditions for 21 or 30 days, respectively.

3.5. Extraction and isolation

For the liquid culture, fungal mycelia were separated from culture media and soaked in MeOH overnight. Cells were destroyed using Ultra Turrax® for 10 min, followed by filtration and exhaustive extraction. The MeOH extract (0.8 g) was subjected to solvent–solvent partitioning using *n*-hexane, CH_2Cl_2 and EtOAc. CC of the EtOAc fraction over Sephadex LH-20 (MeOH) gave **6** and **8**.

The culture media were successively extracted with EtOAc and *n*-BuOH. The EtOAc portion (0.5 g) was separated by VLC on silica gel 60 using stepwise elution with mixtures of *n*-hexane, EtOAc, and MeOH, respectively.

The fraction obtained at 25% *n*-hexane in EtOAc was chromatographed over Sephadex LH-20 (50% MeOH:CH_2Cl_2) to give further amounts of **6** and **8**. The fraction obtained at 25% MeOH in EtOAc was chromatographed over Sephadex LH-20 (MeOH) and further separated by preparative HPLC (Varian, Prep-Star 218) on a Microsorb 60-8 C18 column (250 × 21.4 mm, L × ID, Varian Dynamax) using the following gradient (MeOH, H_2O, flow rate 20 ml/min): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100% MeOH, to yield **1** and **2**. For **3**, further purification was performed using semi-preparative HPLC (Merck Hitachi L-7100) on a Eurosphere 100-10 C18 column (300 × 8 mm, L × ID, Knauer, Germany), using the following gradient (MeOH, H_2O, flow rate 5 ml/min): 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100% MeOH. The eluted peaks were detected by the online UV detector.

The solid state rice culture was extracted with EtOAc. The crude extract was dried and partitioned between *n*-hexane and 90% MeOH. The 90% MeOH soluble material (1.4 g) was fractionated by VLC on silica gel 60 using stepwise elution with mixtures of *n*-hexane, EtOAc, CH_2Cl_2, and MeOH, respectively.

The fractions obtained at 75% and 25% *n*-hexane in EtOAc yielded further amounts of **6** and **8**, respectively. The fractions eluted at 20% (fraction 1) and 60% (fraction 2) MeOH in CH_2Cl_2 were chromatographed over Sephadex LH-20 (MeOH) and then further separated by preparative and semi-preparative HPLC (see above), resulting in the isolation of **4**, **5** and **10** from fraction 1, and **7**, **9**, **11** and **14** from fraction 2.

The fraction eluted at 20% CH_2Cl_2 in MeOH was separated by VLC on silica gel 60 (CH_2Cl_2:MeOH gradient elution), followed by preparative HPLC (see above) to yield further amounts of **11** as well as **12** and **13**.

Overall yields of compounds were: **1**, 4.2 mg, **2**, 2.1 mg, **3**, 0.5 mg, **4**, 1.5 mg, **5**, 1.0 mg, **6**, 21.6 mg, **7**, 7.1 mg, **8**, 3.5 mg, **9**, 2.5 mg, **10**, 15.7 mg, **11**, 18.6 mg, **12**, 10.5 mg, **13**, 12.3 mg, **14**, 1.8 mg.

3.5.1. Ampelopyrone (*2*)

Viscous yellow oil; $[\alpha]_D^{20}$ +67 (*c* 0.2, MeOH); UV λ_{max}^{MeOH} 203.2, 288.4 nm; ^1H and ^{13}C NMR, see Table 2; ESI-MS pos *m/z* (rel. int.): 227.0 $[M+H]^+$ (25); ESI-MS neg *m/z* (rel. int.): 225.2 $[M-H]^-$ (100); HR-ESIMS pos $[M+H]^+$ (found 227.0910; calculated for $C_{11}H_{15}O_5^+$, 227.0914).

3.5.2. Desmethyldiaportinol (*4*)

Viscous yellowish oil; $[\alpha]_D^{20}$ +51 (*c* 0.5, MeOH); UV λ_{max}^{MeOH} 244.0, 277.3, 326.1 nm; ^1H and ^{13}C NMR, see Table 3; ESI-MS pos *m/z* (rel. int.): 527.1 $[2M+Na]^+$ (100), 275.3 $[M+Na]^+$ (90); ESI-MS neg *m/z* (rel. int.): 251.3 $[M-H]^-$ (100); HR-ESIMS pos $[M+Na]^+$ (found 275.0540; calculated for $C_{12}H_{12}NaO_6^+$, 275.0526).

3.5.3. Desmethyldichlorodiaportin (*5*)

Viscous yellowish oil; $[\alpha]_D^{20}$ +19 (*c* 0.3, MeOH); UV λ_{max}^{MeOH} 244.2, 277.5, 326.9 nm; ^1H and ^{13}C NMR, see Table 3; HRESI-MS pos *m/z* (rel. int.): 327.0 $[M+H]^+$ (100), 329.0 (62), 331.0 (18); ESI-MS neg *m/z* (rel. int.): 303.5 $[M-H]^-$ (100), 305.4 (66), 307.4 (11), 267.6 $[(M-H) - HCl]^-$ (18), 269.6 (6), 231.6 $[(M-H) - 2\ HCl]^-$ (28), 191.6 $[(M-H) - C_2H_2Cl_2O]^-$ (26); HR-ESIMS pos $[M+Na]^+$ (found 326.9800; calculated for $C_{12}H_{10}Cl_2NaO_5^+$, 326.9797).

3.5.4. Macrosporin-7-O-sulfate (*7*)

Yellow crystals; UV λ_{max}^{MeOH} 203.1, 267.3, 278.3, 420.0 nm; ^1H and ^{13}C NMR, see Table 4; ESI-MS pos *m/z* (rel. int.): 365.0 $[M+H]^+$ (100), 285.3 $[(M+H)–SO_3]^+$ (30); ESI-MS neg *m/z* (rel. int.): 363.2 $[M-H]^-$ (30), 283.4 $[(M-H)–SO_3]^-$ (100); HR-ESIMS pos $[M-H+2Na]^+$ (found 408.9970; calculated for $C_{16}H_{11}Na_2O_8S^+$, 408.9965).

3.5.5. 3-O-Methylalaternin-7-O-sulfate (*9*)

Orange crystals; UV λ_{max}^{MeOH} 226.3, 273.1, 438.4 nm; ^1H NMR, see Table 4; ^{13}C NMR spectral data (125 MHz, MeOD, all derived from HMBC spectrum): δ 122.5 (C-5), 143.5 (C-6), 146.0 (C-7), 167.0 (C-3), remaining signals not detected due to low signal to noise ratio; ESI-MS pos *m/z* (rel. int.): 381.0 $[M+H]^+$ (100), 301.2 $[(M+H)–SO_3]^+$ (40); ESI-MS neg *m/z* (rel. int.): 379.2 $[M-H]^-$ (50), 299.5 $[(M-H)–SO_3]^-$ (100); HR-ESIMS pos $[M-H+2Na]^+$ (found 424.9910; calculated for $C_{16}H_{11}Na_2O_9S^+$, 424.9919).

3.5.6. Ampelanol (*11*)

White crystals; $[\alpha]_D^{20}$ −64 (*c* 0.5, MeOH); UV λ_{max}^{MeOH} 218.1, 231.6, 283.4, 318.0 nm; ^1H and ^{13}C NMR, see Table 5; ESI-MS pos *m/z* (rel. int.): 341.1 $[M+H]^+$ (100); ESI-MS neg *m/z* (rel. int.): 339.3 $[M-H]^-$ (100); HR-ESIMS pos $[M+H]^+$ (found 341.1230; calculated for $C_{16}H_{21}O_8^+$, 341.1231).

Table 4
^1H, ^{13}C NMR and HMBC data of compounds **6**, **7** and **9** at 500 (^1H) and 125 (^{13}C) MHz

Nr.	**6**			**7**			**9**
	δ_H (DMF-d_7)	HMBC	δ_C (DMF-d_7)	δ_H (MeOD)	HMBC	δ_C (MeOD)	δ_H (MeOD)
1			165.8			166.0	
2	6.80, *d* (2.5)	1,3,4,9a	106.0	6.74, *d* (2.5)	1,3,4,9a	107.0	6.77, *d* (2.5)
3			167.0			167.0	
4	7.19, *d* (2.5)	2,9a,10	107.8	7.27, *d* (2.5)	2,3,9a,10	108.0	7.31, *d* (2.5)
4a			136.0				
5	7.95, *s*	CH_3,7,8a,9[a],10	130.8	8.06, *s*	CH_3,7,8a,9[a],10	131.0	7.65, *s*
6			133.0			138.0	
7			162.5			156.0	
8	7.67, *s*	6,7,8a,9,10[a],10a	111.8	8.36, *s*	6,7,8a,9,10[a],10a	119.0	
8a			134.1			134.0	
9			187.6			187.0	
9a			111.1			111.0	
10			181.2			182.0	
10a			126.0			130.0	
CH_3	2.34, *s*	5,6,7,8[a]	16.4	2.46, *s*	5,6,7,8[a]	17.0	2.52, *s*
OCH_3	4.00, *s*	3	56.7	3.92, *s*	3	56.0	3.94, *s*

[a] Weak.

Table 5
^{1}H, ^{13}C NMR, COSY, ROESY and HMBC data of compound **11** at 500 (^{1}H) and 125 (^{13}C) MHz

Nr.	**11**				
	δ_H (DMSO-d_6)	COSY	ROESY	HMBC	δ_C (DMSO-d_6)
1	3.76, *br s*	9a,1OH	CH_3,9,9a,9OH		71.2
1-OH	5.06, *d* (4.7)	1	CH_3,9		
2					72.9
2-OH	4.21, *s*	CH_3	CH_3,4,9a	1	
3	3.36, *d* (9.4)	4,3OH	CH_3,4a	4	74.0
3-OH	4.41, *br s*	3	4		
4	3.82, *m*	3,4a,4OH	2OH,3OH	3,10	70.7
4-OH	4.46, *s*	4	4a	3	
4a	2.63, *dd* (13.2, 9.4)	4	3,9,4OH	4,9,9a,10	48.1
5					164.2
5-OH	12.57, *s*		6	5,6,10a	
6	6.35, *d* (1.8)	8	OCH_3,5OH	5,7,8,10a	99.0
7					166.0
8	6.72, *d* (1.8)	6,9	OCH_3,9,9OH	6,7,9,10a	104.6
8a					152.0
9	4.67, *dd* (10.5, 6.2)	8,9a,9OH	1,4a,8,1OH		66.4
9-OH	5.49, *d* (6.2)	9	1,8,9a		
9a	2.30, *ddd* (13.2, 10.5, 2.2)	1,4a,9	1,2OH,9OH	4a,9	44.5
10					206.1
10a					109.1
CH_3	1.20, *s*	2OH	1,3,1OH,2OH	1,2,3	23.8
OCH_3	3.83, *s*		6,8	7	55.6

3.6. Determination of minimal inhibitory concentration

Assays were carried out as described previously (Teeyapant et al., 1993). The minimal inhibitory concentration (MIC) of a substance was defined as the lowest concentration where bacterial growth was inhibited (Andrews, 2001).

3.7. Cell proliferation assay

Cytotoxicity was tested against L5178Y mouse lymphoma cells using the microculture tetrazolium (MTT) assay (Carmichael et al., 1987) as described earlier (Ashour et al., 2006). All experiments were carried out in triplicate and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments.

3.8. Extraction and fractionation of plant material

The plant sample (*U. picroides* stems, leaves and flowers) was frozen at −80 °C followed by freeze drying. The freeze dried sample was ground and extracted with 90% MeOH overnight with shaking and the resulting extract was dried. The dried residue was subjected to partitioning between *n*-hexane and 90% MeOH. The 90% MeOH soluble fraction was fractionated over Diaion HP-20 using H_2O:MeOH and MeOH:acetone gradient elution.

3.9. LC/MS analysis

Fractions of *U. picroides* were analyzed by HPLC and LC/MS. The resulting chromatograms were searched for the specific most intense pseudomolecular ions (“extracted ion chromatograms”) of the respective compounds isolated from *Ampelomyces* sp. cultures. Co-elution studies with the corresponding pure metabolites and the respective plant fractions were carried out and retention times, extracted ion chromatograms of the target compounds, MS as well as MS/MS spectra were compared. Specifically, the following pseudomolecular ions, each corresponding to the respective $[M-H]^-$, were used for successful detection of secondary metabolites in the host plant extract: macrosporin (**6**), R_t 25.0 min, *m/z* 283; 3-*O*-methylalaternin (**8**), R_t 23.1 min, *m/z* 299.

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