Protein kinase inhibitors and other cytotoxic metabolites from the fungal endophyte *Stemphylium botryosum* isolated from *Chenopodium album*

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The endophytic fungus *Stemphylium botryosum* was isolated from leaves of the medicinal plant *Chenopodium album* collected in Egypt. Extracts of the fungus grown on rice exhibited considerable cytotoxicity when tested *in vitro* against L5178Y mouse lymphoma cells. Upon chemical investigation they afforded the macrocyclic lactones curvularin (1) and dehydrocurvularin (2), as well as altersolanol A (3), tetrahydroaltersolanol B (4), stemphyperylenol (5) and macrosporin (6). The structures of all isolated compounds were determined by 1D and 2D NMR spectroscopy and mass spectrometry as well as by comparison with published data. Compounds 1-3 exhibited considerable cytotoxicity against L5178Y cells with EC_{50} values of 16, 1.4 and 0.6 μM, respectively, whereas the remaining compounds showed only modest activity. All compounds were further tested for protein kinase inhibitory activity in an assay involving 24 different kinases. Compound 3 was the most potent inhibitor displaying EC_{50} values ranging between 1.9 and 29.4 μM toward individual kinases, followed by 6 (EC_{50} = 2.3 - 27.1 μM). Compounds 4 and 5 showed moderate activity, while 1 and 2 were inactive.

Key words – cytotoxic activity – endophytic fungi – natural products – protein kinases

Introduction

Endophytic fungi are a polyphyletic group of highly diverse, primarily ascomycetous fungi that occur within healthy plant tissues without causing any immediate overt effects (Bacon & White 2000, Hyde & Soytong 2008). Each plant, of the nearly 300,000 species of higher plants existing on earth, was found to contain a diversity of endophytes. Their relationships with host plants range from symbiotic to slightly pathogenic (Strobel & Daisy 2003). Thus, they reside inside their host plant assuming a quiescent (latent) state either for the whole lifetime of the infected plant

tissue or for an extended period of time until the association is disturbed by either a weakening of plant defence or an increase in fungal virulence (Schulz et al. 2002, Sieber 2007, Rodriguez & Redman 2008). Endophytic fungi are, furthermore, believed to contribute to host plant adaptation to biotic and abiotic stress factors (Redman et al. 2002, Arnold et al. 2003, Waller et al. 2005, Zhang et al. 2006, Akello et al. 2007, Bae et al. 2009), which in many cases has been correlated with fungal natural products (Saikkonen et al. 1998, Tan & Zou 2001, Strobel et al. 2004, Zhang et al. 2006).

In this study, we have investigated the endophytic fungal strain *Stemphylium botryosum* (Pleosporaceae) which was isolated from healthy leaves of the traditional medicinal plant *Chenopodium album* (Amaranthaceae), a plant growing wild in Egypt. *C. album* is used for its anthelmintic properties and the seed oil is believed to be effective against many forms of intestinal parasites. The plant was also used in the past as an oral contraceptive (Laszlo & Henshaw 1954). In the Indian Himalayan Region it is used for treating liver diseases (Samant & Pant 2006).

In 1833, the genus *Stemphylium* was established by Wallroth, with *S. botryosum* as the type species (Wallroth 1833). Both saprotrophic and pathogenic forms of *Stemphylium* occur in a wide range of plants and many species are economically important pathogens of agricultural crops. They are known as the causal agents of foliar diseases such as the leaf spot of lettuce, onion, garlic, tomato and potato, which are diseases of economic importance in many countries (Camara et al. 2002). Furthermore, *Stemphylium* species are known sources of bioactive compounds including the cytotoxic and protein kinase inhibiting alterporriols G and H (Debbab et al. 2009), the antibacterial perylenequinones, stemphyltoxins I-IV (Arnone et al. 1986), as well as the phytotoxic chromone glucoside, stemphylin (Barash et al. 1975).

Methods

General Experimental Procedures

Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. ^{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. 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 CH_2Cl_2:MeOH:EtOAc (90:10:5 and 80:20:10) solvent systems. Detection was at 254 and 366 nm or by spraying the plates with anisaldehyde reagent.

Fungal material

Stemphylium botryosum was isolated from fresh healthy leaves of *Chenopodium album* L. 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. Following surface sterilization with 70% EtOH for 1 min the leaf samples were rinsed in sterile water. To distinguish the remaining epiphytic from endophytic fungi, an imprint of the leaf surface on biomalt agar was performed. Small tissue samples from inside the leaves were cut aseptically 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, adjusted with 10% NaOH or 36.5% HCl). After incubation at room temperature, the fungal strain under investigation was found to grow exclusively out of the leaf tissue, but not on the agar plates taken from the imprint of the leaf surface. From the growing cultures pure strains of *S. botryosum* were isolated by repeated reinoculation on malt agar plates.

Identification of fungal cultures

The fungus (strain no. I7L2) was identified using a molecular biological protocol by DNA amplification and sequencing of the ITS region as described previously (Wang et al. 2006). A voucher strain is kept at the authors' laboratory (P.P.).

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 on rice solid medium (to 100 g commercially available rice was added 100 mL of distilled water and kept overnight prior to autoclaving, 6 flasks) at room temperature under static conditions for 30 days.

Extraction and isolation

Cultures were extracted with EtOAc, and the concentrated residue (1.2 g) partitioned between n-hexane and 90% MeOH. The 90% MeOH-soluble material (0.7 g) was then fractionated by vacuum-liquid chromatography (VLC) on silica gel 60 using CH_2Cl_2/MeOH gradient elution. Further purification was achieved by preparative HPLC (Varian, PrepStar 218) on a Microsorb 60-8 C18 column (250 × 21.4 mm, L × ID) using MeOH/H_2O gradient as the mobile phase. Yields of compounds were as follows: **1** 1.9 mg, **2** 15.5 mg, **3** 3.3 mg, **4** 5.6 mg, **5** 3.9 mg, and **6** 3.7 mg.

Curvularin (1) yellow powder; $[\alpha]_D^{20}$ - 28.5º (c 0.4, EtOH); UV λ_{max} (PDA) 202.5, 222.0, 271.9 and 298.9 nm; ^{1}H NMR (MeOD, 500 MHz) δ 6.24 (1H, d, J = 2.2 Hz, H-6), 6.21 (1H, d, J = 2.2 Hz, H-4), 4.92 (1H, m, H-15), 3.85 (1H, d, J = 15.7 Hz, H-2A), 3.61 (1H, d, J = 15.7 Hz, H-2B), 3.20 (1H, ddd, J = 15.2, 8.8, 2.8 Hz, H-10A), 2.73 (1H, ddd, J = 15.2, 9.7, 2.8 Hz, H-10B), 1.74 (1H, m, H-11A), 1.59 (1H, m, H-14A), 1.57 (1H, m, H-11B), 1.47 (1H, m, H-13A), 1.43 (1H, m, H-14B), 1.39 (1H, m, H-12A), 1.30 (1H, m, H-13B), 1.26 (1H, m, H-12B), 1.12 (3H, d, J = 6.3 Hz, CH$_3$-15); ^{13}C NMR (Derived from HMBC spectrum.) (MeOD, 125 MHz) δ 173.0 (C-1), 161.5 (C-5), 137.5 (C-3), 121.0 (C-8), 112.6 (C-4), 102.9 (C-6), 73.9 (C-15), 40.6 (C-2), 33.0 (C-14), 20.5 (CH$_3$-15); ESIMS positive m/z 293 [M + H]$^+$ (100), 607 [2M + Na]$^+$ (90), negative m/z 291 [M - H]$^-$ (100).

Dehydrocurvularin (2) brown powder; $[\alpha]_D^{20}$ - 79.8º (c 3.0, EtOH); UV λ_{max} (PDA) 204.1, 228.4 and 287.6 nm; ^{1}H NMR (MeOD, 500 MHz) δ 6.57 (1H, ddd, J = 15.4, 8.2, 5.6 Hz, H-11), 6.49 (1H, d, J = 15.4 Hz, H-10), 6.28 (1H, d, J = 2.0 Hz, H-4), 6.24 (1H, d, J = 2.0 Hz, H-6), 4.79 (1H, m, H-15), 3.72 (1H, d, J = 16.7 Hz, H-2A), 3.44 (1H, d, J = 16.7 Hz, H-2B), 2.38 (1H, m, H-12A), 2.29 (1H, m, H-12B), 1.96 (1H, m, H-13A), 1.85 (1H, m, H-14A), 1.58 (1H, m, H-14B), 1.56 (1H, m, H-13B), 1.18 (3H, d, J = 6.6 Hz, CH$_3$-15); ^{13}C NMR (MeOD, 125 MHz) δ 200 (C-9), 173.0 (C-1), 162.2 (C-7), 162.0 (C-5), 154.3 (C-11), 137.0 (C-3), 133.3 (C-10), 117.8 (C-8), 112.2 (C-4), 102.8 (C-6), 74.2 (C-15), 42.4 (C-2), 35.1 (C-14), 34.2 (C-12), 25.4 (C-13), 20.3 (CH$_3$-15); ESIMS positive m/z 291 [M + H]$^+$ (20), negative m/z 289 [M - H]$^-$ (100).

Altersolanol A (3) orange yellow crystals; $[\alpha]_D^{20}$ - 149 (c 1.2, EtOH); UV λ_{max} (PDA) 220.5, 269.5 and 434.5 nm; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 12.11 (1H, brs, OH-5), 7.00 (1H, brs, H-8), 6.81 (1H, brs, H-6), 5.67 (1H, d, J = 5.9 Hz, OH-1), 5.03 (1H, d, J = 5.3 Hz, OH-4), 4.88 (1H, d, J = 6.3 Hz, OH-3), 4.47 (1H, m, H-4), 4.46 (1H, s, OH-2), 4.31 (1H, d, J = 3.7 Hz, H-1), 3.89 (3H, s, OCH$_3$-7), 3.63 (1H, dd, J = 6.3, 5.6 Hz, H-3), 1.23 (3H, s, CH$_3$-2); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 188.5 (C-10), 183.7 (C-9), 165.4 (C-7), 163.2 (C-5), 144.5 (C-4a), 142.1 (C-9a), 133.3 (C-8a), 109.5 (C-10a), 106.6 (C-8), 105.9 (C-6), 73.8 (C-3), 72.9 (C-2), 68.5 (C-1, C-4), 56.2 (OCH$_3$-7), 22.3 (CH$_3$-2); ESIMS positive m/z 337 [M + H]$^+$ (100), 690 [2M + NH$_4$]$^+$ (50), negative m/z 335 [M - H]$^-$ (100).

Tetrahydroaltersolanol B (4) yellowish white powder; $[\alpha]_D^{20}$ - 27.0º (c 0.18, MeOH); UV λ_{max} (PDA) 216.1, 231.0, 281.7 and 320.0 nm; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 12.89 (1H, s, OH-5), 6.68 (1H, dd, J = 2.3, 1.2 Hz, H-8), 6.34 (1H, d, J = 2.3 Hz, H-6), 5.61 (1H, brs, OH-9), 4.43 (1H, brs, OH-3), 4.26 (1H, brd, J = 10.8 Hz, H-9), 3.80 (3H, s, OCH$_3$-7), 3.79 (1H, brs, OH-2), 3.28 (1H, dd, J = 12.2, 4.2 Hz, H-3), 2.44 (1H, m, H-4a), 2.15 (1H, dd, J = 13.2, 3.4 Hz, H-1A), 2.11 (1H, ddd, J = 12.2, 4.2, 3.7 Hz, H-4A), 1.94 (1H, dddd, J = 12.1, 11.6, 10.8, 3.4 Hz, H-9a), 1.45 (1H, q, J = 12.2

Hz, H-4B), 1.19 (1H, dd, J = 13.2, 12.1 Hz, H-1B), 1.15 (3H, s, CH$_3$-2); ESIMS positive m/z 309 [M + H]$^+$ (100), 639 [2M + Na]$^+$ (30), negative m/z 307 [M - H]$^-$ (100).

Stemphyperylenol (5) reddish brown powder; $[\alpha]_D^{20}$ + 411° (c 0.2, MeOH); UV λ_{max} (PDA) 217.4, 261.3 and 342.0 nm; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 12.02 (2H, s, OH-4/10), 8.01 (2H, d, J = 8.8 Hz, H-6/12), 6.85 (2H, d, J = 8.8 Hz, H-5/11), 5.75 (2H, brs, OH-1/7) 4.59 (2H, m, H-1/7), 3.71 (2H, d, J = 8.8 Hz, H-6b/12b), 3.14 (2H, dd, J = 15.4, 11.9 Hz, H-2A/8A), 2.91 (2H, dd, J = 15.4, 4.4 Hz, H-2B/8B); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 203 (C-3/9), 159.1 (C-4/10), 143.0 (C-3b/9b), 134.6 (C-6/12), 129.9 (C-6a/12a), 114.8 (C-3a/9a), 114.4 (C-5/11), 66.5 (C-1/7), 46.8 (C-2/8), 44.6 (C-6b/12b); ESIMS negative m/z 351 [M - H]$^-$ (30), 334 [M – H$_2$O]$^-$ (100).

Macrosporin (6) yellow crystals; UV λ_{max} (PDA) 225.4, 284.4 and 380.2 nm; ^{1}H NMR (DMF-d_7, 500 MHz) δ 7.95 (1H, s, H-5), 7.67 (1H, s, H-8), 7.19 (1H, d, J = 2.5 Hz, H-4), 6.80 (1H, d, J = 2.5 Hz, H-2), 4.00 (3H, s, OCH$_3$-3), 2.34 (3H, s, CH$_3$-6); ^{13}C NMR (DMF-d_7, 125 MHz) δ 187.6 (C-9), 181.2 (C-10), 167.0 (C-3), 165.8 (C-1), 162.5 (C-7), 136.0 (C-4a), 134.1 (C-8a), 133.0 (C-6), 130.8 (C-5), 126.0 (C-10a), 111.8 (C-8), 111.1 (C-9a), 107.8 (C-4), 106.0 (C-2), 56.7 (OCH$_3$-3), 16.4 (CH$_3$-6); ESIMS positive m/z 285 [M + H]$^+$ (100), negative m/z 283 [M - H]$^-$ (100).

Cell Proliferation Assay

Cytotoxicity was tested against L5178Y mouse lymphoma cells using a microculture tetrazolium (MTT) assay and compared to that of untreated controls as described previously (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. The depsipeptide kahalalide F isolated from *Elysia grandifolia* (Ashour et al. 2006) was used as positive control.

Biochemical protein kinase activity assay

All biochemical protein kinase activity assays were performed in 96-well FlashPlates from Perkin-Elmer/NEN (Boston, MA) in a 50 μL reaction volume. The reaction cocktail contained 20 μL of assay buffer, 5 μL of ATP solution (in H$_2$O), 5 μL of test compound (in 10% DMSO), 10 μL of substrate, and 10 μL of purified recombinant protein kinase. The assay for all enzymes contained 60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl$_2$, 3 mM MnCl$_2$, 3 μM Na-orthovanadate, 1.2 mM DTT, 50 μg/mL PEG20000, and 1 μM [γ-33P]-ATP (ca. 5 × 105 cpm per well). The following substrates were used: GSK3(14-27), AKT1; tetra (LRRWSLG), Aurora A, B; MEK1 KM, B-Raf; histone H1, CDK2/CycA; Rb-CTF, CDK4/CycD1; Poly(Glu,Tyr)4:1, EGF-R, EPHB4, ERBB2, FAK, IGF1-R, SRC, VEGF-R2, VEGF-R3, Tie2; Poly(Ala,Glu,Lys,Tyr), FLT3, INS-R, Met, PDGF-Rb; casein, PLK1, CK2a1. Autophosphorylation was measured for ARK5, COT, and SAK.

Results and discussion

From extracts of *Stemphylium botryosum* grown on solid rice medium the macrocyclic lactones curvularin (**1**) and dehydrocurvularin (**2**), as well as altersolanol A (**3**), tetrahydro-altersolanol B (**4**), stemphyperylenol (**5**) and macrosporin (**6**) were obtained by column chromatography including preparative HPLC.

ESI-MS indicated a molecular weight of 292 and 290 g/mol for compounds **1** and **2**, respectively, with a difference of 2 mass units. The ^{1}H NMR spectra of both compounds showed a pair of *meta*-coupled aromatic protons as well as a diastereotopic methylene function. In addition, the spectrum of **1** contained an extended aliphatic spin system which could only be completely assembled with help of the COSY spectrum due to a significant degree of overlap, whereas that of **2** included an ABX$_2$ system, with two olefinic protons forming the AB part (δ_H 6.49 and 6.57), and a coupling constant of 15.4 Hz consistent with a *E*-configuration of the double bond. These olefinic protons were found to be part of an extended spin system, as in the case of **1**, consisting of an aliphatic methyl group adjacent to an oxygenated methine group, which was further connected to a chain of methylene groups. The spectrum of **1** showed five methylene groups, four of which appear as a complex set of multiplets resonating between 1.26 and 1.74 ppm, while the downfield chemical shift of the last one (δ_H 3.20 and 2.73) indicated its position adjacent to a carbonyl

function. In contrast, the spectrum of **2** indicated two methylene groups showing close similarity to the respective signals observed for **1**, as well as a downfield methylene group adjacent to the double bond. In the HMBC spectra of **1** and **2**, CH_2-2 displayed correlations to C-3, C-4, C-8, and the ester carbonyl at δ_C 173.0 (C-1), the oxygen atom of which had to be connected to H-15. Overall, the substructures established so far suggested that **1** was identical to the known curvularin, a macrocyclic lactone rather widespread in fungi, while **2** was identified as its known 10-dehydro congener, dehydrocurvularin. This assumption was confirmed by comparison of the spectral properties of **1** with data reported in the literature (Munro et al. 1967, Lai et al. 1989, Ghisalberti et al. 1993). Based on the almost identical values obtained for the [α]$_D$ it was possible to deduce the absolute configuration at C-15 to be *S* in both compounds (Munro et al. 1967, Ghisalberti et al. 1993). In spite of its occurrence within several fungal genera, this is the first report for the isolation of curvularin from the genus *Stemphylium*. Dehydrocurvularin, however, was previously reported from *Stemphylium radicinum* (Grove 1971). Both compounds have been obtained from *Curvularia* sp. (Birch et al. 1959, Munro et al. 1967), *Alternaria* sp. (Robeson & Strobel 1981, 1985, Arai et al. 1989) and *Penicillium* sp. (Lai et al. 1989).

ESI-MS of compounds **3** and **4** indicated a molecular weight of 336 and 308 g/mol, respectively. The UV spectrum of **3**, revealing absorbances at λ_{max} (MeOH) 220.5, 269.5 and 434.5 nm, suggested a quinone as the basic structure, while inspection of the NMR data indicated a molecular formula of $C_{16}H_{16}O_8$. The ^{1}H NMR spectrum displayed exchangable signals corresponding to four hydroxyl groups, three of which appearing as doublets, one as a singlet, in addition to a broad singlet indicating a chelated phenolic group. Furthermore, a singlet was detected at δ_H 1.23 corresponding to an aliphatic methyl group, together with three carbinolic protons (H-1, H-3 and H-4). The pair of *meta*-coupled aromatic protons were comparable to the respective signals of metabolites **1** and **2**, whereas additionally the signal of a methoxy group linked to an aromatic ring was observed. The nature of the

aliphatic ring system was evident from the COSY spectrum establishing the planar structure of **3** as identical to altersolanol A (Stoessl 1969). Moreover, correlations of 2-CH$_3$ with H-3, 1-OH, and 4-OH in the ROESY spectrum indicated that the relative stereochemistry also corresponded to that of altersolanol A, which was also in agreement with the observed coupling constants. This assignment was further corroborated by comparison of UV, NMR and mass spectral data, as well as [α]$_D$ value to published data for altersolanol A (Yagi et al. 1993, Okamura et al. 1993, 1996). Altersolanol A was previously isolated from several *Alternaria* species (Stoessl et al. 1983, Lazarovits et al. 1988, Yagi et al. 1993, Okamura et al. 1993, 1996). In contrast, the ^{1}H NMR spectrum of **4** showed only three exchangeable alcoholic hydroxyl groups as well as two carbinolic protons assigned to H-9 and H-3. Furthermore, a singlet corresponding to an aliphatic methyl group (δ_H 1.15), a chelated phenolic OH group, two *meta*-coupled aromatic protons (H-8 and H-6), and a methoxy group linked to an aromatic ring represented features already familiar from the spectra of **3**. Importantly, H-8 exhibited a second splitting of 1.2 Hz, indicating a long range coupling with a *peri*-proton (H-9), which was confirmed by the corresponding correlation observed in the COSY spectrum. H-9 coupled to the proton situated at the junction of the *trans*-decaline-like ring system (H-9a), which in turn coupled to a diastereotopic methylene group (CH$_2$-1) as well as to the other ring junction proton (H-4a). Accordingly, the quinone carbonyl corresponding to that at C-9 in **3** had been reduced to a hydroxyl group at the respective position in **4**, while the double bond between C-9a and C-4a in **3** was likewise reduced in **4**, as indicated by the loss of color for this compound compared to **3**. Further protons of the aliphatic spin system (CH$_2$-4 and H-3) were clearly discernible in the COSY spectrum. The relative stereochemistry of **4** (with the exception of C-2) could be resolved by analysis of the coupling constants of signals in the high field region. The large values of J_{4a-9a} (11.6 Hz) and J_{9a-9} (10.8 Hz) indicated their respective diaxial relationship. The value for J_{3-4ax} (12.2 Hz) furthermore indicated an axial position for H-3.

In addition, spectroscopical properties of **4** proved virtually identical to those published for tetrahydroaltersolanol B (Stoessl & Stothers 1983), thus confirming its identity. Tetrahydro-altersolanol B had previously been reported from culture filtrates of *Alternaria solani* (Stoessl & Stothers 1983).

UV absorbances recorded for compound **6** suggested an anthraquinone as the basic structure (λ_{max} (MeOH) 225.4, 284.4 and 380.2 nm). The ESI-MS indicated a molecular weight of 284 g/mol, while examination of the NMR spectra suggested the molecular formula $C_{16}H_{12}O_5$. In the ^{13}C NMR spectrum reso-nances at δ_C 187.6 and 181.2 indicated a quinone. The NMR spectra of **6** revealed the same aromatic signals observed for compounds **3** and **4**, in addition to an oxygenated aromatic carbon, as well as two aromatic proton singlets which were assigned as H-5 and H-8 and an aromatic methyl group, thereby assembling the other aromatic ring in the structure. The compound was thus identified as the known macrosporin, which was corroborated by interpretation of HMBC spectra and compari-son of the spectral data of **6** with those published for macrosporin (Suemitsu et al. 1984, 1989). Macrosporin was previously reported from several *Alternaria* species (Stoessl et al. 1983, Lazarovits et al. 1988, Suemitsu et al. 1989) as well as from *Phomopsis juniperovora* (Wheeler & Wheeler 1975), *Dactylaria lutea* (Becker et al. 1978), *Dichotomophthora lutea* (Hosoe et al. 1990) and *Pleospora* sp. (Ge et al. 2005).

ESI-MS of compound **5** indicated a molecular weight of 352 g/mol. The NMR spectra containing signals due to only eight protons and ten carbon atoms, indicated that the molecule was a symmetrical C_{10} dimer. The ^1H resonances were assigned to an AX spin system of *ortho*-coupled aromatic protons (H-6/H-12 and H-5/H-11), a chelated phenolic hydroxyl group (4-OH/10-OH), and a CH$_2$-CHOH-CH fragment. The respective substruc-tures were also assembled on the basis of the COSY spectrum. The ^{13}C resonances were attributed to a tetra-substituted aromatic ring and a carbonyl carbon atom, the remaining signals were assigned to one methylene and two methane sp^3 carbons, one of which was oxygen-bearing. From the HMBC spectrum, it was possible to place the chelated phenolic hydroxyl group at C-4, since this carbon showed HMBC correlations to H-5, H-6 and 4-OH. Consequently, the carbonyl group was located at C-3. Furthermore, CH_2-2/8 dis-played correlations with C-3/9, thereby establishing the C2-C3/C8-C9 bonds. The correlations of H-6b/12b to C-6a/12a and of H-6/12 to C-6b/12b indicated the connection of the two halves of the molecule and gave the planar structure of **5**. This was further confirmed by the long-range coupling between the aromatic (H-6/12) and benzylic (H-6b/12b) protons observed in the COSY spectrum. The relative stereochemistry was derived from detailed examination of the coupling constants. The large values of $J_{1/7\text{-2ax/8ax}}$ (11.9 Hz) and $J_{1/7\text{-12b/6b}}$ (8.8 Hz) indicated the diaxial (= *trans*) position of H-1/7 with regard to both H-12b/6b and H-2ax/8ax, suggesting that the cyclo-hexanone rings preferentially adopted a half-chair conformation with the hydroxyl groups being in an equatorial position and H-6b/H-12b in an axial position. The obtained data were in agreement with the spectral data published for stemphyperylenol (Arnone et al. 1986), con-firming that **5** and the latter were identical. Stemphyperylenol had previously been des-cribed from *Alternaria cassiae* (Hradil et al. 1989) and *Stemphylium botryosum* var. Lactu-cum (Arnone et al. 1986).

Table 1. Cytotoxicity assay for the isolated compounds.

Compound	L5178Y growth in % (at 10 μg/mL)	EC$_{50}$ (μM)
1	6.6	16.0
2	-3.8	1.4
3	0.0	0.6
4	38.9	
5	31.6	
6	54.5	

The isolated compounds were subjected to bioassays aimed at determining their cytotoxicity toward L5178Y mouse lymphoma cells and their protein kinase inhibitory profiles in an assay involving 24 different kinases (Tables 1 and 2). Altersolanol A (**3**) exhibited significant growth inhibition of L5178Y cells with an EC$_{50}$ value of 0.6 μM, followed by

dehydrocurvularin (**2**) and curvularin (**1**) showing EC_{50} values of 1.4 and 16.0 μM, respectively. In contrast, compounds **4 - 6** showed only moderate activity in the assay (Table 1). Interestingly, testing altersolanol A (**3**) in the biochemical protein kinase activity assay paralleled the pronounced activity of the compound as found in the MTT assay (Table 2). Among the 24 different enzymes tested, Aurora-B and CDK4/CycD1 were most susceptible to **3** (EC_{50} values 2.2 and 1.9 μM). In contrast, different activity patterns were observed for the remaining compounds. Macrosporin (**6**) was only moderately active in

the MTT assay, but was a strong kinase inhibitor similar to **3**. EC_{50} values of **6** against Aurora-B and FLT3 kinases amounted to 2.2 and 2.9 μM (Table 2). Compounds **4** and **5** showed only weak activity against individual kinases, whereas curvularin (**1**) and dehydro-curvularin (**2**) were inactive towards all tested protein kinases in spite of their pronounced activity in the MTT assay. Interestingly, curvularin (**1**) was reported to show remarkable cytotoxic activity towards sea urchin embryo-genesis, blocking cell division at concen-trations of 8.5 μM by specifically disordering microtubule centers and inducing barrel-

Table 2. EC_{50} values of compounds **3** and **6** against selected protein kinases[a].

Compound	AKT1	Aurora-A	Aurora-B	B-RAF-VE	CDK4/CycD1	COT	EGF-R	ERBB2	FAK	IGF1-R	SRC	VEGF-R2	VEGF-R3	FLT3	INS-R	MET	PLK1	SAK	TIE2
3	19.9	3.8	2.2	n.a.	1.9	n.a.	17.8	n.a.	4.4	10.4	7.1	21.7	29.4	14.2	n.a.	n.a.	14.2	7.4	26.4
6	n.a.	6.6	2.2	22.5	n.a.	27.1	9.5	25.7	n.a.	n.a.	n.a.	8.4	n.a.	2.9	12.3	15.1	n.a.	15.1	6.6

[a] EC_{50} values are listed in μM. n.a.: not active, i.e., $EC_{50} > 10$ μg/mL.

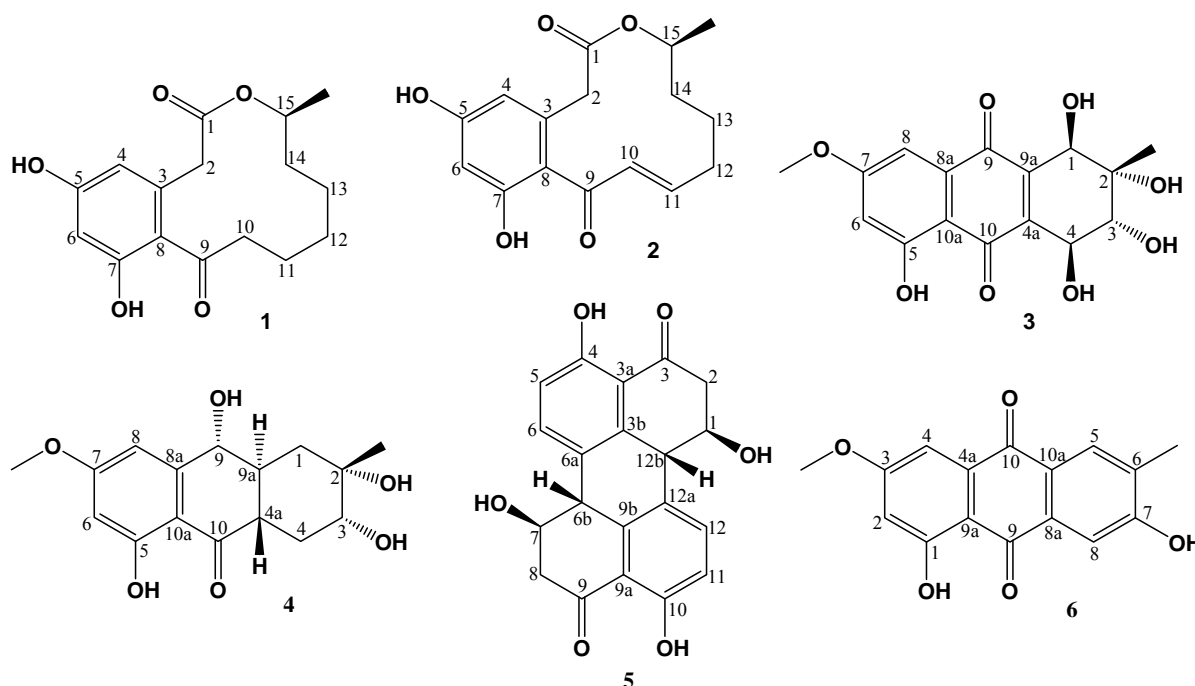**Fig. 1** – Chemical structures of compounds **1 – 6**.

shaped spindles (Kobayashi et al. 1988). Furthermore, curvularin macrolides were recently found to be inhibitors of the heat shock protein 90 (HSP 90), an ATP-dependent chaperone which is integrally involved in cell signaling, proliferation, and survival, thus representing a promising target for the treatment of cancer (Agatsuma et al. 2004, Janin 2005). Accordingly, the results obtained when testing **1** and **2** for cytotoxic activity toward L5178Y mouse lymphoma cell line and their lack of activity in the protein kinase inhibition assay are in accordance with literature data and strongly point to a mechanism of cytotoxic activity not involving interactions with protein kinases.

Acknowledgements

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