ORIGINAL ARTICLE

Cladosporinone, a new viriditoxin derivative from the hypersaline lake derived fungus *Cladosporium cladosporioides*

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A new cytotoxic viriditoxin derivative, cladosporinone (1), along with the known viriditoxin (2) and two viriditoxin derivatives (3 and 4) were obtained from the fungus *Cladosporium cladosporioides* isolated from the sediment of a hypersaline lake in Egypt. The structure of the new compound (1) was determined by 1D and 2D NMR measurements as well as by high-resolution ESIMS and electronic circular dichroism spectroscopy. All isolated compounds were studied for their cytotoxicity against the murine lymphoma cell line L5187Y and for their antibiotic activity against several pathogenic bacteria. Viriditoxin (2) was the most active compound in both bioassays. Compound 1 also exhibited strong cytotoxicity against the murine lymphoma cell line L5187Y with an IC_{50} value of $0.88 \mu\text{M}$, whereas its antibiotic activity was weak.

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INTRODUCTION

Since the discovery of penicillin by Sir Alexander Fleming in 1928, several fungal-derived natural products have been discovered and developed as new drugs, such as the antibacterial agent cephalosporin C from *Cephalosporium acremonium*,^{1–3} the immunosuppressive drug cyclosporine from *Tolypocladium inflatum*^{4,5} or the cholesterol-lowering agent lovastatin from *Aspergillus terreus*.⁶ However, the frequent re-isolation of known metabolites from fungi has turned the interest of natural product chemists to hitherto less investigated ecological niches, such as arctic glaciers, deep-sea hydrothermal vents or hypersaline lakes.^{7,8} Fungi that live at elevated temperatures, at an acidic or alkaline pH, high pressure, high-salt concentration and/or low-nutrient concentration are called extremophiles, and have developed unique metabolic mechanisms to produce bioactive secondary metabolites as a response to environmental stress.^{9–11} Thus, extremophilic fungi are attracting considerable attention in recent years as new promising sources for biologically active compounds with potential pharmaceutical applications.^{12–13}

During our ongoing search for new bioactive compounds from fungi,^{14–17} the extract of the fungus *Cladosporium cladosporioides*, isolated from the sediment of the hypersaline lake El Hamra located in Wadi el Natrun (Egypt), caused complete growth inhibition of the murine lymphoma cell line L5187Y when assayed at a dose of $10 \mu\text{g ml}^{-1}$. Bioassay-guided fractionation of the extract afforded a

new viriditoxin derivative (1), together with viriditoxin (2) and two previously reported viriditoxin congeners (3 and 4).^{18,19} Viriditoxin (2) was first isolated from *Aspergillus viridinutans* as a mycotoxin, whereas it was later also obtained from *Aspergillus brevipes*, *Aspergillus fumigatus*, *Paecilomyces variotii* and *Spicaria divaricata*.^{18–20} Details on the isolation and structure elucidation of 1, its biological activity and the plausible biogenesis of the isolated compounds are reported herein.

RESULTS AND DISCUSSION

The ethyl acetate (EtOAc) extract of the sediment-derived fungus *C. cladosporioides* following cultivation on solid rice medium was partitioned between *n*-hexane and 90% aqueous methyl alcohol (MeOH). The resulting MeOH phase was fractionated by vacuum liquid chromatography on silica gel, followed by size exclusion chromatography over Sephadex LH-20 and separation on diol-functionalized silica. Final purification was achieved by semipreparative reversed-phase HPLC to yield a new viriditoxin derivative (1), along with three known compounds including viriditoxin (2) and its derivatives (3) and (4) (Figure 1).

Compound 1 was isolated as a red, amorphous powder. Its molecular formula was established as $\text{C}_{33}\text{H}_{30}\text{O}_{14}$ based on the prominent ion peak observed at $m/z = 651.1706$ $[\text{M}+\text{H}]^+$ in the high-resolution ESIMS spectrum. The ^1H and ^{13}C NMR data of 1

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Figure 1 Structures of cladosporinone (1), viriditoxin (2) and viriditoxin derivatives (3 and 4).

Table 1 NMR spectroscopic data of **1** [DMSO-*d*₆] (¹H NMR in 600 MHz, ¹³C NMR in 150 MHz, δ in p.p.m.)

1							
Position	δ_H	δ_C	HMBC		δ_H	δ_C	HMBC
1		169.90		9'-OH	12.81 s		C-8', 9', 9a'
3	4.94 m	75.7		3'	3.84 m	65.6	
4	2.98 dd (16.4, 2.9) 2.89 dd (16.4, 11.8)	31.8	C-10a, 5, 11, 3	4'	2.36 dd (13.5, 5.3) 2.11 dd (13.5, 8.1)	37.1	C-3', 10a', 4a', 5', 11'
4a		133.9		4a'		136.4	
5	6.41 s	112.8	C-4, 10a, 9a, 5a, 6	5'	6.58 s	138.7	C-4', 9a', 6', 10a', 4a'
5a		138.6		5a'		134.9	
6		106.6 ^a		6'		120.3	
7		160.1		7'		166.9	
8	6.84 s	97.8	C-9, 6, 9a	8'	6.70 s	100.3	C-9a', 6', 9'
9		159.1 ^a		9'		168.0	
9a		107.6		9a'		109.2	
10		162.6		10'		179.9	
10a		99.1		10a'		180.8	
11	2.81 t (6.1)	38.7	C-4, 3, 12	11'	2.32 dd (15.2, 5.3) 2.13 dd (15.2, 8.6)	41.1	C-4', 12', 3'
12		169.87		12'		171.5	
12-OCH ₃	3.63 s	51.7	C-12	12'-OCH ₃	3.49 s	51.1	C-12'
7-OCH ₃	3.77 s	56.1	C-7,	7'-OCH ₃	3.72 s	56.8	C-7'

^aAssignments may be interchanged.

(Table 1) were similar to those of the co-isolated viriditoxin (2). Accordingly, the ¹H NMR spectrum of **1** revealed signals corresponding to four methylene groups (H-4, δ_H 2.98 and 2.89; H-11, δ_H 2.81; H-4', δ_H 2.36 and 2.11 and H-11', δ_H 2.32 and 2.13), four methoxy groups (12-OCH₃, δ_H 3.63; 7-OCH₃, δ_H 3.77; 12'-OCH₃, δ_H 3.49 and 7'-OCH₃, δ_H 3.72), two oxymethine protons (H-3, δ_H 4.94 and H-3', δ_H 3.84) and four singlets in the aromatic region (H-8, δ_H 6.84; H-8', δ_H 6.70, H-5', δ_H 6.58 and H-5, δ_H 6.41) (Table 1). In addition, one exchangeable proton detected at δ_H 12.81 was attributable to a chelated hydroxy group. The ¹³C and DEPT spectra revealed the presence of 19 *sp*² quaternary carbons, including five carbonyl groups (δ_C 169.90, C-1; δ_C 169.87, C-12; δ_C 179.9, C-10'; δ_C 180.8, C-10a' and δ_C 171.5, C-12').

Further inspection of the 2D NMR (COSY, HSQC and HMBC) data allowed us to establish the planar structure of **1**. The HMBC correlations from H-8 to C-9, C-9a and C-6, from H-5 to C-4, C-9a, C-10a, C-5a and C-6, and from H₂-4 to C-10a, C-5, C-11 and C-3 indicated the presence of a 3,4-dihydro- α -naphthopyrone structure. Further correlations from 7-OCH₃ to C-7, from 12-OCH₃ to C-12 and from H₂-11 to C-4, C-3 and C-12 fully supported the semi-viriditoxin moiety in the structure of **1** (Figure 2). Analysis of the 1D and 2D NMR data revealed that **1** also contained a 1,2-naphthoquinone moiety as confirmed by the HMBC correlations from H-8' to C-6', C-9' and C-9a' and from H-5' to C-9a', C-6', C-10a', C-4' and C-4a'. In addition, a 3'-OH-methyl butyrate side chain was located at C-4a' of the 1,2-benzoquinone ring, as supported by the COSY

Figure 2 Selected HMBC and COSY correlations of **1**.

Figure 3 ECD spectrum of **1** in acetonitrile. $\Delta\epsilon$ values were multiplied by 5 above 314 nm for better visualization.

correlations from H-3' to H₂-4' and H₂-11', and by the HMBC correlations from H-4' to C-3', C-4a', C-10a', C-5' and C-11', from H-11' to C-3', C-4' and C-12', and from 12'-OCH₃ to C-12' (Figure 2). In a similar manner to viriditoxin, the HMBC correlations from 7'-OCH₃ to C-7' and from the chelated proton 9'-OH to C-8', C-9' and C-9a', indicated the presence of 7'-methoxy and 9'-hydroxy substituents in the aromatic ring, leaving only C-6' for the connection of the two monomers through a 6',6'-biaryl linkage. Thus, the planar structure of **1** was elucidated as shown in Figure 2.

In an attempt to determine the absolute configuration of C-3', the modified Mosher's method was applied, however, this reaction failed to give the corresponding Mosher's ester, probably, due to the chelated hydroxy group. The axial chirality of **1** was determined by comparing its electronic circular dichroism (ECD) spectrum (Figure 3) with that of the related viriditoxin (**2**), which had a negative exciton couplet (275 nm -194.7 , 255 nm $+174.0$) in chloroform.¹⁸ The negative couplet of viriditoxin derives from coupling of the two ¹B_b transitions of the interacting 6,6'-linked naphthopyrone chromophores having a counter-clockwise twist with a dihedral angle of about 90°. Although one of the naphthalene ring is replaced by a 1,2-naphthoquinone residue in **1**, the alignment of the interacting electric transition moments are not expected to alter significantly, and thus similarly to viriditoxin, the negative exciton couplet of **1** derives from (aR) axial chirality of the biaryl system. As the ECD spectrum is governed predominantly by the axial chirality element, the central chirality of the fused α -pyrone ring of **1** could not be determined from ECD. However, this configuration was determined earlier for semi-viriditoxin, the naphthopyrone monomer of viriditoxin, as (S)²¹ and it was also confirmed by stereoselective synthesis.²² Thus, **1** was identified as a new natural heterodimer for which the name cladosporinone is proposed.

The known compounds viriditoxin (**2**) and its analogs (**3** and **4**) were identified on the basis of NMR, mass spectrometry and optical rotation data analysis, as well as by comparison with the literature.^{18,19} The axial chirality of **3** and **4** had been deduced earlier as (aR) by comparison of their ECD curves with those of **2**.^{18–20} Several attempts were undertaken to influence the pattern of metabolites produced by *C. cladosporioides* either by changing the medium (OSMAC approach²³) or by co-culturing the fungus in the presence of several bacteria, such as *Bacillus subtilis* 168 trpC2, *Bacillus cereus* T, *Streptomyces lividans* TK24 and *Streptomyces coelicolor* A2(3). Mixed fermentation of fungi and bacteria has repeatedly been shown to activate silent fungal biogenetic gene clusters, thus causing the accumulation of compounds not detected in axenic fungal cultures.^{24,25} However, none of these attempts caused a significant change in the metabolite pattern on *C. cladosporioides* as shown by HPLC analysis of the respective extracts and by comparison to extracts of the fungus grown only on rice.

A plausible biogenetic pathway of compounds **1–4** includes a head-to-tail combination of one acetyl-CoA (starter unit) and seven malonyl-CoA (extender units) moieties, a process that is catalyzed by multidomain enzymes called polyketide synthases. Then, the octaketide chain would undergo regiospecific aromatization, reduction or cyclization reactions to form precursor B. Dehydration and lactonization of the latter (B) and subsequent methoxylation would afford the monomeric precursors C and D, respectively. Oxidative coupling of the different monomers at C-6/C-6' would result in the formation of **2–4**, as shown in Figure 4. An alternative biosynthetic route would include oxidative decarboxylation of precursor B to form the 1,2-naphthoquinone monomer, followed by oxidative coupling with monomer D to afford the new metabolite **1** (Figure 4).²⁶

Compounds **1–4** were assayed for their cytotoxicity toward L5178Y (murine lymphoma) cells. Viriditoxin (**2**) exhibited the most potent cytotoxicity against the tested cell line, with an IC₅₀ of 0.1 μ M, followed by compound **3** with an IC₅₀ of 0.25 μ M and the new compound **1** with an IC₅₀ of 0.88 μ M (Table 2). Compound **4** was inactive in this bioassay. The loss of one or both methylated carboxyl groups at C-12 and C-12' as in **3** and **4** weakens the cytotoxicity of the respective derivatives when compared to **2**. Hydrolysis of one of the two lactone rings followed by oxidative decarboxylation as in **1** likewise attenuates the cytotoxicity as evident when compared to **2**. In addition, compounds **1–4** were evaluated for their antimicrobial activities against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25213, and *Pseudomonas aeruginosa* ATCC 27853 (Table 2). Interestingly, all compounds showed selective activity against *S. aureus* ATCC 29213, with viriditoxin (**2**) being by far the most active compound exhibiting a MIC of 0.015 μ g ml⁻¹ (0.023 μ M), thus confirming the results of earlier studies.^{27–29}

EXPERIMENTAL PROCEDURE

General experimental procedures

Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter (Perkin-Elmer, Waltham, MA, USA). ¹H, ¹³C, and 2D NMR spectra were recorded at 25 °C in DMSO-*d*₆ on Bruker AVANCE DMX 600 NMR spectrometers (Bruker BioSpin GmbH, Rheinstetten, Germany). Chemical shifts were referenced to the solvent residual peaks, $\delta_H = 2.50$ p.p.m. for ¹H and $\delta_C = 39.51$ p.p.m. for ¹³C. Mass spectra (ESI) were recorded with a Finnigan LCQ Deca mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany), and HRMS (ESI) spectra were obtained with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer (Thermo Fisher Scientific GmbH). Solvents were distilled before use and spectral grade solvents were

Figure 4 Proposed biosynthetic pathway of 1–4.**Table 2** Bioactivities of tested compounds 1–4

		Compound			
Bioactivity		1	2	3	4
Cytotoxicity	L5178Y, IC ₅₀ in μM	0.88	0.1	0.25	Inactive
Antimicrobial activity, MIC ($\mu\text{g ml}^{-1}$)	<i>Staphylococcus aureus</i>	64	0.015	2	16
	ATCC 29213				
	<i>Escherichia coli</i> ATCC 25213	>64	>64	>64	>64
	<i>Pseudomonas aeruginosa</i> ATCC 27853	>64	>64	>64	>64

used for spectroscopic measurements. HPLC analysis was performed with a Dionex UltiMate3400 SD (Dionex Softron GmbH, Munich, Germany) with a LPG-3400 s.d. Pump (Dionex Softron GmbH) coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280 and 340 nm. The separation column (125 $\times$ 4 mm) was prefilled with Eurosphere-10 C18 (Knauer, Germany), and the following gradient was used (MeOH, 0.02% HCOOH in H₂O): 0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH). Semipreparative HPLC was performed using a Merck Hitachi HPLC System (Merck KGaA, Darmstadt, Germany; UV detector L-7400; Pump L-7100; Eurosphere-100 C18, 300 $\times$ 8 mm, Knauer, Germany). Column chromatography included LH-20 Sephadex (0.25–0.1 mm mesh size, GE Healthcare Europe GmbH, Freiburg, Germany), Merck MN Silica gel 60 M

(0.04–0.063 mm, Merck KGaA) and Diol-functionalized silica gel spherical (40–75 μm , GE Healthcare Europe GmbH). TLC plates with silica gel F254 (Merck KGaA) were used to monitor fractions; detection was under UV at 254 and 366 nm or by spraying the plates with anisaldehyde reagent followed by heating.

Fungal material

The fungus *C. cladosporioides* was isolated from the sediment of the hypersaline lake El Hamra located in Wadi el Natrun of Egypt, which was collected in November 2012. The isolation was performed as described before.³⁰

Identification of fungal cultures

The fungus was identified as *C. cladosporioides* according to a molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (ITS) region as described previously.³¹ The sequence data was submitted to the GenBank, with accession number KT899331. The fungal strain is kept in one of the author's laboratory (PP).

Fermentation

The fungus was cultivated on solid rice medium in six Erlenmeyer flasks. Solid rice medium was prepared by adding demineralized water (110 ml) to rice (100 g) in an Erlenmeyer flask, followed by autoclaving (121 $^{\circ}\text{C}$, 20 min). The fungus, which nearly covered the whole surface of a petri dish, was inoculated onto this sterile rice medium under the clean bench and was allowed to grow (20 $^{\circ}\text{C}$) for 35 days. Attempts to optimize the fermentation conditions were performed by adding 3.5% sea salt to the rice medium or by adjusting the pH

of the medium to 9 or 11 by adding sodium hydroxide. In addition, co-cultivation experiments of *C. cladosporioides* with *B. subtilis* 168 trpC2, *B. cereus* T, *S. lividans* TK24 and *S. coelicolor* A2(3) were performed on rice medium as described before.^{24,25}

Extraction and isolation

Fungal cultures were extracted with EtOAc. The crude extract was evaporated to dryness, and then subjected to liquid–liquid partitioning between *n*-hexane and 90% aqueous MeOH. The 90% MeOH fraction amounted to 4.9 g, and was further separated by vacuum liquid chromatography (4.5 × 30 cm) on silica gel, using solvents in a gradient of increasing polarity—*n*-hexane–ethyl acetate–dichloromethane–methanol—to obtain a total of 11 fractions. The fractions eluted with *n*-hexane–ethyl acetate (30:70–100% EtOAc, 126 mg) and DCM–MeOH (90:10, 153 mg) were then subjected to column chromatography (3.5 × 60 cm) using Sephadex LH-20 as stationary phase and DCM–MeOH (50:50v/v) as mobile phase to remove pigments. The bioactive fraction (87 mg) was further subjected to column chromatography (2.5 × 50 cm) over diol-functionalized silica using DCM–MeOH (99:1v/v) as eluting solvent. Fractions were combined according to TLC monitoring (53 mg) and purified by reversed-phase HPLC (silica-based, semipreparative column), with gradient elution MeOH–H₂O from 50:50 to 60:40 in 30 min, to yield the four compounds **1** (5.3 mg), **2** (21 mg), **3** (4.4 mg) and **4** (3.9 mg).

Cladosporinone (**1**): red, amorphous powder; $[\alpha]_D^{25} = -154^\circ$ (*c* 0.35, MeOH); UV (MeCN) $\lambda_{\max}$ log ϵ : 440sh (2.8), 383 (3.1), 334sh (2.9), 264 (3.9), 222sh (3.7). ECD (MeCN, λ (nm) ($\Delta\epsilon$), $c = 2.56 \times 10^{-4}$ M): 471 (−1.8), 386 (1.5), 359 (−0.4), 333 (3.8), 289sh (−9.1), 268 (−91.6), 246 (64.3), 200 (−6.2); ¹H (600 MHz) and ¹³C (150 MHz) NMR, see Table 1; ESI-MS *m/z* 651.0 [M+H]⁺, 649.4 [M−H][−]; HRESIMS *m/z* 651.1706 [M+H]⁺ (calcd for C₃₃H₃₁O₁₄, 651.1708).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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