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The isolation, structure determination and cytotoxicity of the new fungal metabolite, trichodermamide C

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Abstract—Chemical investigations of a culture broth from the endophytic fungus *Eupenicillium* sp. afforded the new modified dipeptide trichodermamide C **1**. The structure of **1** was established following the analysis of NMR, UV, IR, MS and X-ray diffraction data. Trichodermamide C was shown by high content screening to display cytotoxicity towards the human colorectal carcinoma HCT116 and human lung carcinoma A549 with IC_{50} values of 0.68 and 4.28 μg/ml, respectively.

Microfungi have been the sources of numerous new and bioactive secondary metabolites over the decades. Although much scientific research is currently focused on marine-derived microfungi,[1] terrestrial fungi still continue to provide natural products chemists with new and biologically interesting compounds. Recent examples include chloriolide A, a 12-membered macrolide from *Chloridium virescens* var. *chlamydosporum*,[2] the anticancer berkelic acid from *Penicillium* sp.,[3] and the insecticidal paraherquamides H and I from *Penicillium cluniae*.[4] As part of our continuing research on the bioactive chemistry of endophytic fungi from Australian plants we examined the outer bark of the rainforest tree *Glochidion ferdinandi* (family Euphorbiaceae) for its fungal content.[5–8] These investigations afforded several microfungal strains, one of which was identified as *Eupenicillium* sp.[9] We recently reported the isolation of two new polyketides from this particular strain,[9] however during these studies we also purified another secondary metabolite that we had difficulty assigning a chemical structure. X-ray quality crystals of this unknown metabolite have recently been obtained and this has enabled a complete stereostructure to be assigned. Herein we describe the isolation and structure elucidation of a new modified dipeptide, which we have named trichodermamide C **1**. The high content screening results obtained by dosing two cancer cell lines (HCT116 and A549) with compound **1** are also reported.

The fungus *Eupenicillium* sp.[9] was grown in shaken malt extract broth and the culture was extracted with EtOAc. This crude extract was initially fractionated by C18 flash chromatography using H_2O and increasing amounts of MeOH. An early eluting fraction was further purified by phenyl HPLC (MeOH/H_2O) to yield trichodermamide C **1** (2.8 mg).

Trichodermamide C **1** was assigned the molecular formula $C_{21}H_{22}N_2O_9$ on the basis of HRESIMS[10] and NMR data (Table 1). Analysis of the IR spectrum for trichodermamide C suggested the presence of hydroxyl group(s) (3600–3200 cm^{-1}), a lactone (1717 cm^{-1}) and an amide (1654 cm^{-1}) moiety. The ^1H NMR spectrum of **1** contained three exchangeable singlets [δ 5.15 (1H), 5.12 (1H), 5.00 (1H)], two mutually-coupled aromatic doublets [δ 7.40 (d, J = 9.0 Hz, 1H) and 7.16 (d, J = 9.0 Hz, 1H)], one low field aromatic singlet [δ 8.02 (1H)], two mutually-coupled olefinic protons [δ 5.41 (d, J = 10.2 Hz, 1H) and 5.35 (d, J = 10.2 Hz, 1H)], three aliphatic methine signals [δ 4.12 (1H), 3.75 (1H), 3.68 (1H)], one methylene moiety [δ 2.38 (1H) and 2.01 (1H)], two methoxyls [δ 3.91 (3H), 3.83 (3H)] and a *N*-methyl singlet [δ 3.21 (3H)]. The ^{13}C NMR spectrum displayed 21 signals of which 13 resonated between 109 and 166 ppm. HMBC data (Table 1) readily allowed the construction of a coumarin system substituted with methoxyl groups at C-7′ and C-8′ and an *N*-methyl amide group attached to C-2′. The remaining protons

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Table 1. NMR data for trichodermamide C **1**[a]

Position	δ_C	δ_H (mult., J in Hz)	COSY (H No.)	HMBC (C No.)	ROESY (H No.)
1	165.4				
2	151.8				
3a	24.3	2.01 (brd, 19.2)	3b	2, 5	3b, 6, 7, 8
3b		2.38 (br s)	3a	1, 4	3a, 5
4	67.2				
4-OH		5.00 (br s)			5, 9
5	73.4	4.12 (br s)	5-OH, 6, 7, 8	4, 6, 7	3b, 4-OH, 5-OH, 6, 7, 9
5-OH		5.15 (br s)	5	4, 5, 6	5
6	129.9	5.41 (d, 10.2)	5, 7, 8	4, 7, 8	3a, 5, 8
7	127.8	5.35 (br d, 10.2)	5, 6		3a, 5, 8, 9
8	66.6	3.68 (br s)	5, 6, 8-OH, 9		3a, 6, 7, 8-OH
8-OH		5.12 (br s)	8		8, 9
9	82.8	3.75 (d, 7.2)	8	3, 5, 8	4-OH, 5, 6, 7, 8-OH
1′	158.5				
2′	127.8				
3′	137.0	8.02 (s)		1′. 9′, 8′, 2′, 5′, 4′, 12′	5′, 12′
4′	113.5				
5′	123.5	7.40 (d, 9.0)	6′	7′, 9′, 3′, 8′, 4′, 6′	3′, 6′
6′	109.9	7.16 (d, 9.0)	5′, 10′	7′, 9′, 8′, 5′, 4′	5′, 10′
7′	155.0				
8′	135.2				
9′	145.9				
10′	56.4	3.91 (s)	6′	7′, 6′	6′
11′	60.8	3.83 (s)		8′	
12′	36.3	3.21 (s)		1, 2′	3′

[a] Spectra were recorded in DMSO-d_6 at 30 °C.

in **1** were assigned to a tetra-oxygenated cyclohexene system following gCOSY, HMBC and ^{1}H NMR data analysis. With the knowledge that all the atoms of **1** were now accounted for except for one N, and that 2° of unsaturation were still required, a 1,2-oxazine system was established. A weak $^3J_{CH}$ correlation from H-3b (δ 2.38) to the amide carbonyl C-1 (165.4 ppm) established the link between the coumarin and oxazine portions of trichodermamide C. Furthermore, ROESY data analysis allowed the relative configuration about the cyclohexene system of **1** to be assigned. Several natural products related to **1** have been reported in the literature. Examples include trichodermamides A **2** and B **3** from the marine-derived fungus *Trichoderma virens*,[11] and aspergillazine A **4**, from the terrestrial fungal strain *Aspergillus unilateralis*[12] (Fig. 1). While compounds **2**–**4** all share a dimethoxylated coumarin system and a secondary amide functionality, only trichodermamides A **2** and B **3** contain the rare cyclic *O*-alkyl-oxime functionality that is also present in **1**. The structure of trichodermamide A **2** had been determined by X-ray diffraction analysis and the amide stereochemistry assigned as *trans*.[11] The NMR data for the oxazine–cyclohexene portion of trichodermamide C showed only minor differences with trichodermamide A. At this stage it appeared that we had isolated the *N*-methyl analogue of trichodermamide A, however the stereochemistry of the tertiary amide in **1** remained unassigned. Crystallization studies on **1** were undertaken and crystals suitable for X-ray diffraction analysis were eventually obtained from a MeOH/H_2O mix.[13] The ORTEP diagram for trichodermamide C is shown in Figure 2.[14] The X-ray structure confirmed the oxazine–cyclohexene and coumarin components of **1** and established the *N*-methyl amide as having a *cis* configuration in the crystalline state, with the C10–N11–C11–O11 torsion angle measured as −3.7(11)°. The oxazine–cyclohexene and coumarin groups are further linked by strong O–H···O hydrogen bonding between the hydroxyl group (O18), the solvated water molecule (O44) and the methyl ether oxygen (O18′).

The absolute stereochemistry of trichodermamide A was determined to be 4*S*, 5*R*, 8*R* and 9*S* using the modified

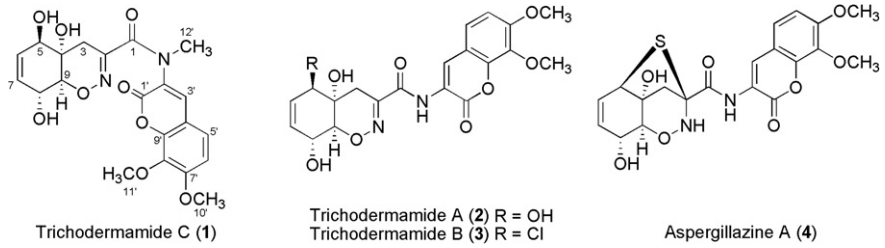

Figure 1. Chemical structures of trichodermamides A–C and aspergillazine A.

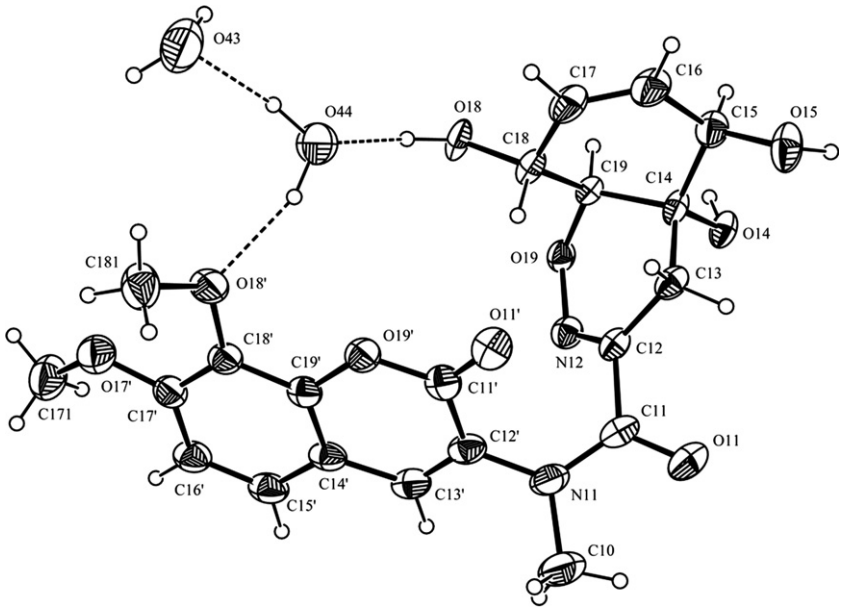

Figure 2. ORTEP diagram of trichodermamide C **1** dihydrate.

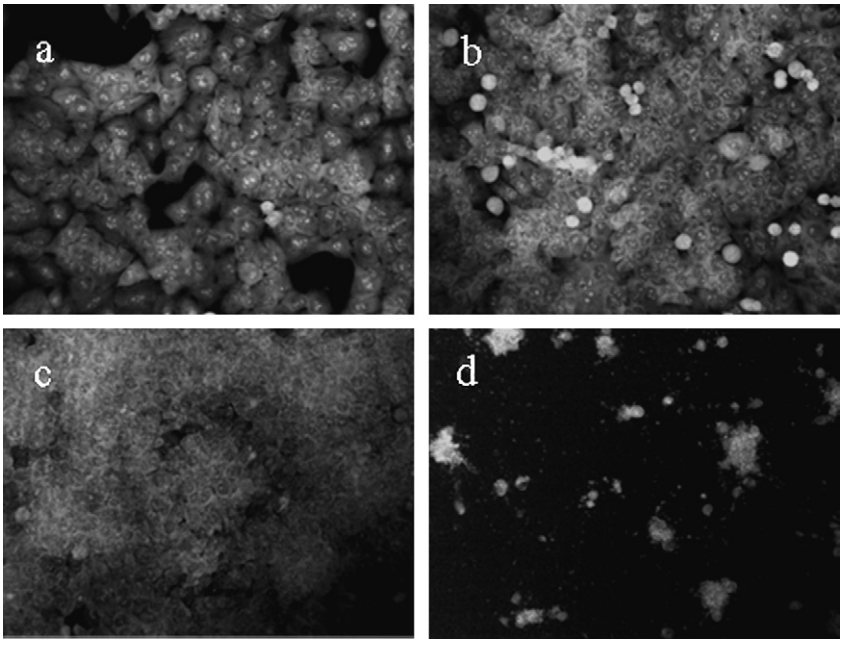

Figure 3. Representative images of A549 (a and b) and HCT116 (c and d) cells captured with the Evotec Opera using a ×20 objective. Images a and c show untreated cells whilst b and d show cells treated with 26.78 μg/ml trichodermamide C at 24 h. All the cells were stained with YO-PRO-1, a marker for late-stage apoptosis.

Mosher's method.[11,15] Based on biosynthetic grounds we propose that trichodermamide C has the same absolute stereochemistry as trichodermamide A.

In the original paper for trichodermamides A and B both compounds were tested for cytotoxicity against the human colorectal carcinoma HCT116. Interestingly, only the chlorinated metabolite, trichodermamide B, displayed significant activity with an IC_{50} of 0.32 μg/ml.[11]

Due to our interest in the discovery of new natural products exhibiting anticancer activity, and the structural similarity between trichodermamides B and C, we decided to test the new natural product **1** against the colorectal carcinoma cell line HCT116 using a high content imaging system [Opera (PerkinElmer)]. The Opera was utilised due to its ability to simultaneously capture and quantify confocal images to measure a vast array of cellular changes. In this assay, three fluorescent markers were employed to investigate changes to nuclear

morphology, and membrane permeability, which all undergo significant changes during cell death. Nuclear morphology was visualized using the DNA/RNA binding dyes DRAQ5, YO-PRO-1 and propidium iodide. DRAQ5 is a cell permeant dye and therefore stained all cells, however YO-PRO-1 and propidium iodide are membrane impermeant. During the late stages of apoptosis, or programmed cell death, the permeability of the cell membrane increases allowing nuclear staining with YO-PRO-1. Propidium iodide however, remains cell impermeant until the cell has died.[16] In these high content screening assays trichodermamide C was shown to display cytotoxicity towards the HCT116 cells with an IC_{50} value of 0.68 μg/ml at 24 h. Due to its morphology, the HCT116 cells showed very low contrast between nuclear and cytoplasmic staining, which makes the HCT116 cell line difficult to analyze accurately in high content imaging assays. For this reason cytotoxicity assays were also carried out in the A549 lung carcinoma cell line for comparison. Trichodermamide C was found to be less active in this cell line, although staining by both YO-PRO-1 and propidium iodide was observed after treatment for 24 h, and an IC_{50} value of 4.28 μg/ml was determined for **1**. Interestingly, whilst the IC_{50} values obtained indicate that trichodermamide C has only a moderate cytotoxic effect, the captured images illustrate a marked difference in cytotoxicity between the two cell lines (Fig. 3). In the A549 cells trichodermamide C showed increased YO-PRO-1 nuclear staining at the highest dose at 24 h (Fig. 3b). In the HCT116 cells, however, trichodermamide C was far more cytotoxic, with almost all the cells dead after 24 h (Fig. 3d). This was confirmed by propidium iodide staining, also at 24 h. This may indicate some selectivity or a different mechanism of action between the two cancer cell lines tested. However, further work would be required to determine whether this was due to activation of apoptotic pathways in the HCT116 and A549 cell line or caused by non specific cell death.

In conclusion, this letter reports the isolation, structure elucidation, and cytotoxicity of the new modified dipeptide trichodermamide C **1**. Compound **1** represents only the fourth natural product to be reported that contains a 1,2-oxazine system.[17]

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Supplementary data

Supplemental material available: ^{1}H and ^{13}C NMR spectra, (+)-LRESIMS data, general experimental details, collection and identification of fungus, fermentation, extraction and isolation, crystallographic data, biological assays and high-content screening images. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.090.

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10. Stable clear needles (6:4 MeOH:H_2O); mp 170–172 °C; $[\alpha]_D^{23}$ + 56° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.99), 240 sh (3.75), 329 nm (3.87); IR ν_{max} (film) 3600–3200, 1717, 1654, 1608, 1507, 1464, 1431, 1396, 1369, 1322, 1288, 1106, 1083, 777 cm^{-1}; ^{1}H and ^{13}C NMR data see Table 1; (+)-LRESIMS *m/z* (rel. int.): 262 $[M-C_8H_{10}NO_4]^+$ (25), 447 $[M+H]^+$ (50), 469 $[M+Na]^+$ (100); (+)-HRESIMS *m/z* 469.119572 ($C_{21}H_{22}N_2O_9Na$ $[M+Na]^+$ requires 469.121751).
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