

## ORIGINAL ARTICLE

# Cytotoxic epipolythiodioxopiperazine alkaloids from filamentous fungi of the Bionectriaceae

Mario Figueroa<sup>1</sup>, Tyler N Graf<sup>1</sup>, Sloan Ayers<sup>1</sup>, Audrey F Adcock<sup>2</sup>, David J Kroll<sup>2</sup>, Jilai Yang<sup>3</sup>, Steven M Swanson<sup>3</sup>, Ulyana Munoz-Acuna<sup>4,5</sup>, Esperanza J Carcache de Blanco<sup>4,5</sup>, Rajesh Agrawal<sup>6</sup>, Mansukh C Wani<sup>7</sup>, Blaise A Darveaux<sup>8</sup>, Cedric J Pearce<sup>8</sup> and Nicholas H Oberlies<sup>1</sup>

Bioactivity-directed fractionation of the organic extracts of two filamentous fungi of the Bionectriaceae, strains MSX 64546 and MSX 59553 from the Mycosynthetix library, led to the isolation of a new dimeric epipolythiodioxopiperazine alkaloid, verticillin H (1), along with six related analogs, Sch 52900 (2), verticillin A (3), gliocladicillin C (4), Sch 52901 (5), 11'-deoxyverticillin A (6) and gliocladicillin A (7). The structures of compounds 1–7 were determined by extensive NMR and HRMS analyses, as well as by comparisons to the literature. All compounds (1–7) were evaluated for cytotoxicity against a panel of human cancer cell lines, displaying IC<sub>50</sub> values ranging from 1.2 μM to 10 nM. Compounds 1–5 were examined for activity in the NF-κB assay, where compounds 2 and 3 revealed activity in the sub-micromolar range. Additionally, compounds 1, 3 and 4 were tested for EGFR inhibition using an enzymatic assay, while compound 3 was examined against an overexpressing EGFR<sup>+</sup>ve cancer cell line.

*The Journal of Antibiotics* (2012) 65, 559–564; doi:10.1038/ja.2012.69; published online 12 September 2012

**Keywords:** cytotoxicity; epipolythiodioxopiperazine alkaloids; EGFR; fungus; NF-κB

## INTRODUCTION

Dimeric epipolythiodioxopiperazine (ETP) alkaloids are bioactive secondary metabolites reported to have potent cytotoxic<sup>1–10</sup> and antibacterial activities,<sup>11–16</sup> along with antiparasitic,<sup>17</sup> nematicidal,<sup>18</sup> antiviral<sup>19</sup> and immunosuppressive properties.<sup>20</sup> This class of compounds has been isolated largely from several terrestrial and marine fungi, including species of the *Leptosphaeria*, *Chaetomium*, *Tilachlidium*, *Verticillium*, *Gliocladium* and *Penicillium* genera.<sup>21</sup> The densely functionalized and stereochemically complex core of these alkaloids, coupled with their potent biological activities, presents an attractive target for drug leads. Although the ETP alkaloids have been known for over four decades, with chaetococin<sup>22</sup> and verticillin A<sup>16,23,24</sup> first described in 1970, only 23 other natural analogs have been described since then. The first total synthesis (11,11'-dideoxyverticillin A),<sup>25</sup> which stymied chemists for nearly 40 years, was hailed in *Science*.<sup>26</sup> Ongoing studies by this same group<sup>27</sup> resulted in a synthetic strategy to generate the di-, tri- and tetrasulfides, (+)-chaetocin A, (+)-chaetocin C and (+)-12,12'-dideoxytetracin A, respectively, with complete stereochemical control in thiolation and precision in the degree of sulfidation. The mechanism of action for the potent cytotoxicity of the ETPs has not been fully elucidated.

However, several different activities have been described, including: generation of reactive oxygen species through oxidation of the polysulfide bridge,<sup>2</sup> intracellular induction of the *c-fos* proto-oncogene,<sup>28</sup> inhibition of the epidermal growth factor receptor (EGFR) tyrosine kinase activity<sup>3</sup> and more recently, apoptosis activation by BNIP3 (19 kDa interacting protein-3) upregulation and/or TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) sensitizer.<sup>29</sup> Interest in these compounds remains high, because of both their potent biological activities and interesting chemistry.

As part of ongoing studies to discover new anticancer drug leads from filamentous fungi,<sup>30,31</sup> the organic-soluble extracts of solid substrate fermentation of fungi of the Bionectriaceae, cultures MSX 64546 and MSX 59553 from the Mycosynthetix library, exhibited cytotoxic activity against MCF-7 (breast), H460 (large cell lung) and SF-268 (astrocytoma) human cell lines that was indicative of IC<sub>50</sub> values <2 μg ml<sup>-1</sup> for the crude extracts. Preliminary LC-HRMS dereplication procedures suggested the presence of the known ETP, verticillin A (3), as well as characteristic fragmentation patterns for related and possibly new ETPs, both of which likely contributed to the cytotoxicity of the extracts. Therefore, bioactivity-directed

<sup>1</sup>Department of Chemistry and Biochemistry, University of North Carolina at Greensboro, Greensboro, NC, USA; <sup>2</sup>Department of Pharmaceutical Sciences, BRITE, North Carolina Central University, Durham, NC, USA; <sup>3</sup>Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, IL, USA; <sup>4</sup>Division of Pharmacy Practice and Administration, College of Pharmacy, The Ohio State University, Columbus, OH, USA; <sup>5</sup>Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH, USA; <sup>6</sup>Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Denver, Aurora, CO, USA; <sup>7</sup>Natural Products Laboratory, Research Triangle Institute, Research Triangle Park, NC, USA and <sup>8</sup>Mycosynthetix Inc., Hillsborough, NC, USA

Correspondence: Dr NH Oberlies, Department of Chemistry and Biochemistry, University of North Carolina at Greensboro, 435 Sullivan Sciences Building, Greensboro, NC 27402, USA.

E-mail: Nicholas\_Oberlies@uncg.edu

Received 24 April 2012; revised 6 July 2012; accepted 12 July 2012; published online 12 September 2012

fractionations were initiated using H460 cancer cells to monitor the purifications, and this led to the isolation of a new ETP, verticillin H (1), along with six known verticillin-type compounds, Sch 52900 (2), verticillin A (3), gliocladicillin C (4), Sch 52901 (5), 11'-deoxyverticillin A (6) and gliocladicillin A (7). All pure isolates were assessed for cytotoxicity against additional cancer cell lines. Compounds 1–5 were examined in an NF- $\kappa$ B inhibition assay, while compounds 1, 3 and 4 were tested *in vitro* for EGFR inhibition using an enzymatic assay, and 3 was examined against an overexpressing EGFR<sup>+</sup> cancer cell line.

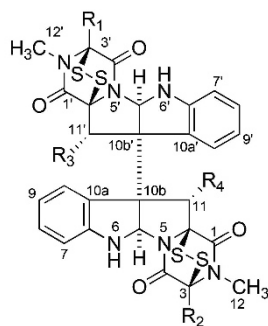
## RESULTS AND DISCUSSION

### Identification of ETPs

Bioactivity-directed purification of the organic extracts of fungal strains MSX 64546 and MSX 59553 led to the isolation of seven cytotoxic ETPs (1–7; see Figure 1), including the new compound, verticillin H (1). An LC-HRMS dereplication procedure (Figure 2), which utilized molecular formula, exact mass and UV maxima (204, 240 and 301 nm) as search criteria in the Dictionary of Natural Products and SciFinder databases, coupled with NMR analysis of pure isolates (Supplementary Table S1), confirmed that compounds 2–7 belong to the ETP class of fungal natural products. The data for the known compounds matched the literature for Sch 52900 (2),<sup>28</sup> verticillin A (3),<sup>23</sup> gliocladicillin C (4),<sup>32</sup> Sch 52901 (5),<sup>28</sup> 11'-deoxyverticillin A (6),<sup>9</sup> and gliocladicillin A (7);<sup>1</sup> Supplementary Table S1 compiles the <sup>1</sup>H and <sup>13</sup>C data for these compounds in CDCl<sub>3</sub>.

### Structure elucidation of verticillin H (1)

The HRMS data of compound 1 yielded a molecular formula C<sub>32</sub>H<sub>32</sub>N<sub>6</sub>O<sub>6</sub>S<sub>4</sub>. The IR data suggested the presence of carbonyl (1673 cm<sup>-1</sup>), hydroxy (3402 cm<sup>-1</sup>) and aromatic (1685 and 1429 cm<sup>-1</sup>) moieties. The UV spectrum of 1 exhibited absorption maxima at 204, 240 and 301 nm, as observed with similar epipolythiodioxopiperazine analogs.<sup>9,15,23,24</sup> Although the <sup>13</sup>C NMR spectrum showed resonances for 16 carbons (Table 1), inspection of the HRMS data, which suggested 32 carbons, revealed a symmetrical dimeric structure. Detailed analysis of HMBC and HSQC data for compound 1 (Table 1) showed similarities to verticillin A (3);



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
Verticillin H (1)	CH <sub>2</sub> CH <sub>3</sub>	CH <sub>2</sub> CH <sub>3</sub>	OH	OH
Sch 52900 (2)	CH(OH)CH <sub>3</sub>	CH <sub>3</sub>	OH	OH
Verticillin A (3)	CH <sub>3</sub>	CH <sub>3</sub>	OH	OH
Gliocladicillin C (4)	CH <sub>2</sub> CH <sub>3</sub>	CH(OH)CH <sub>3</sub>	OH	OH
Sch 52901 (5)	CH <sub>2</sub> CH <sub>3</sub>	CH <sub>3</sub>	OH	OH
11'-Deoxyverticillin A (6)	CH <sub>3</sub>	CH <sub>3</sub>	H	OH
Gliocladicillin A (7)	CH <sub>3</sub>	CH(OH)CH <sub>3</sub>	H	H

**Figure 1** Structure of compounds (1–7) isolated from fungi MSX 64546 and MSX 59553.

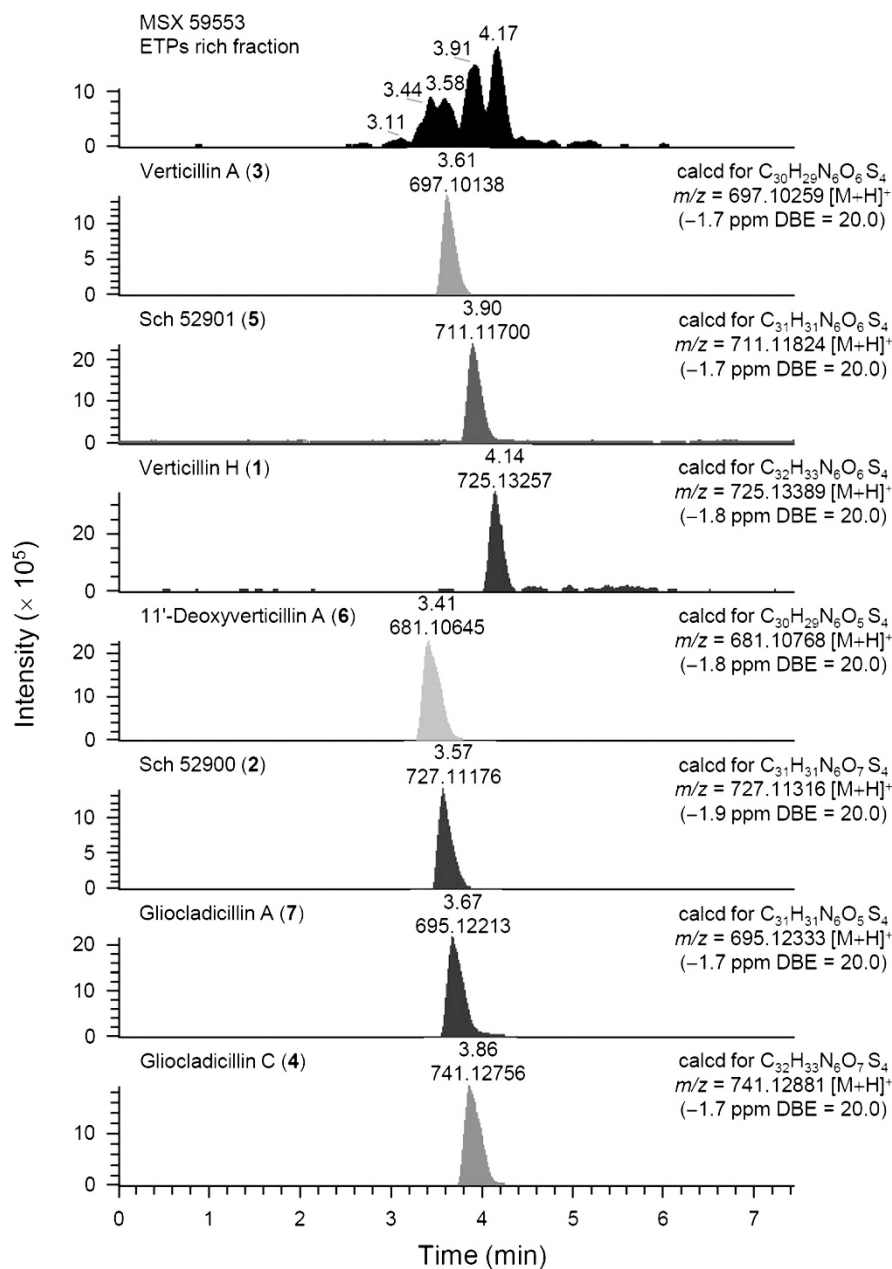
however, the molecular weight of 1 was 28 greater than that of 3. This suggested the presence of an additional methylene unit in each half of the molecule, which was evidenced by the resonances at  $\delta_{\text{H}}$  2.4, 2.1 (for H<sub>2</sub>-13/H<sub>2</sub>-13') that were coupled to a methyl doublet of doublets at  $\delta_{\text{H}}$  1.2 (H<sub>3</sub>-14/H<sub>3</sub>-14'). In addition, HMBC correlations of the methyl proton signals H<sub>3</sub>-14/H<sub>3</sub>-14' with the quaternary carbon C-3/C-3' ( $\delta_{\text{C}}$  77.0), the secondary carbon C-13/C-13' ( $\delta_{\text{C}}$  24.6) and the amide carbonyl C-4/C-4' ( $\delta_{\text{C}}$  161.5), established the connection of the ethyl side chain to position C-3 and C-3' in each monomeric unit. In turn, these two units were connected via the C-10b to C-10b' bond by virtue of HMBC correlations of the methine proton H-5a/H-5a' ( $\delta_{\text{H}}$  5.7) with the quaternary carbons C-6a/C-6a' ( $\delta_{\text{C}}$  148.9), C-10b/C-10b' ( $\delta_{\text{C}}$  65.8) and C-11a/C-11a' ( $\delta_{\text{C}}$  77.5), respectively; such a connection was consistent with other ETP alkaloids in the literature.<sup>9,11,15,24</sup> All other NMR data and HMBC correlations for 1 were consistent with the structural assignment (Figure 1), and compound 1 was ascribed the trivial name verticillin H. The absolute configuration of 1 was determined by comparison of the optical rotation values and CD spectra with those reported for several verticillin analogs.<sup>9,11,15</sup>

### Biological activity

**Inhibition of cancer cell proliferation *in vitro*.** All isolates (1–7) were evaluated for cytotoxic activity against HT-29, H460, SF-268, MCF-7 and MDA-MB-435 cell lines (see Table 2). Previously, Erkel *et al.*<sup>20</sup> reported the cytotoxicity of 2 against HL60 (leukemia) cells; Katagiri *et al.*<sup>23</sup> described the effect of 3 against the Ehrlich ascites carcinoma in mice and HeLa cells; and Che *et al.*<sup>32</sup> showed that 4 exhibited high cytotoxic activity on A549 (adenocarcinoma) and HepG2 (hepatocellular carcinoma) human cell lines. However, this is the first report of the simultaneous evaluation of 1–7 against all the cancer cell lines listed above. In general, all of the compounds displayed potent cytotoxicity, approaching the activity of the positive controls. However, as suggested by Gardiner *et al.*,<sup>21</sup> side chain modifications did not change the cytotoxicity of these compounds drastically.

**Transcription factor NF- $\kappa$ B assay.** Phal *et al.*<sup>33</sup> reported the inhibition of NF- $\kappa$ B in cells by glyotoxin, the first monomeric ETP characterized from *Gliocladium fimbriatum*.<sup>21</sup> The inhibitory effect of this compound was attributed to a possible interaction within an essential thiol residue of the cysteine rich extracellular domains of the protein.<sup>21,33</sup> As NF- $\kappa$ B factor is an integral part of the inflammatory immune response and controls expression of some cytokines, its inhibition may account for the cytotoxic and immunosuppressive properties of dimeric ETPs. Compounds 1–5 were examined for NF- $\kappa$ B inhibitory activity (Table 2). Compounds 2 and 3 inhibited the specific binding ability of activated p65 subunits of NF- $\kappa$ B in the nucleus of HeLa cells with IC<sub>50</sub> values of 0.5 and 0.1  $\mu$ M, respectively, being approximately a half-order of magnitude less potent than the positive control (rocgalamide). These results were in agreement with the recently reported effect of verticillin A (3) as a sensitizer of human colon carcinoma cells to TRAIL-induced apoptosis.<sup>29</sup>

**EGFR activity.** 11,11'-Dideoxyverticillin, a structurally-related ETP isolated from the traditional Chinese medicinal herb *Shiraiia bambusicola*,<sup>3</sup> showed tyrosine kinase inhibitory activity in a cell-free ELISA assay against the EGFR, VEGFR-1/Flt-1 (the vascular endothelial growth factor receptor-1/fms-like tyrosine kinase-1) and HER2/ErbB-2 (the human EGFR-2), with activity in the nM range for the former two and  $\mu$ M range for the latter. To the best of our knowledge, those



**Figure 2** LC-UV-HRMS dereplication for compounds 1–7 and the epipolythiodioxopiperazines (ETPs) rich fraction from fungus MSX 59553. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

studies represent the first report of enzymatic inhibition of dimeric ETPs. Based on those results, compounds 1, 3 and 4 were tested for EGFR inhibition using a luminescence kinase assay, but none of these substances were found to be active ( $IC_{50}$  values  $>10 \mu M$ ; positive control lapatinib,  $IC_{50}$  13 nM; Figure 3). To further assess whether these compounds exert their cytotoxic activity in an EGFR-dependent manner by targeting EGFR expression/activity in a cellular system, compound 3 was tested for cytotoxicity against EGFR<sup>+</sup> and EGFR<sup>-</sup> (EGFR<sup>+</sup> and HuTu-80, respectively) cell lines. Figure 4 (top panel) shows western immunoblot data that indicates no EGFR protein versus very high EGFR protein levels in HuTu-80 and EGFR<sup>+</sup> cells, respectively. However, such a large difference in EGFR levels among the two cell lines did not result in any biological differences in the activity of compound 3 in these two cell lines (Figure 4; lower panels).

Together, these observations suggest that the agent has strong cytotoxic activity independent of cellular EGFR levels. We have not identified 11,11'-dideoxyverticillin in the cultures we have studied to date. Hence, we have not been able to determine if the literature data are reproducible.

## CONCLUSIONS

Using bioactivity-directed fractionation and a LC-UV-HRMS dereplication protocol, one new compound, along with six known ETPs were isolated from the organic extracts of two fungi of the Bionectriaceae (strains MSX 64546 and MSX 59553). The isolated ETPs were potently cytotoxic against a panel of human cell lines in culture. Compounds 1–5 were tested for NF- $\kappa$ B inhibitory activity, with compounds 2 and 3 having  $IC_{50}$  values in the sub-micromolar

**Table 1** NMR data for compound **1** (in CDCl<sub>3</sub>, 500 (<sup>1</sup>H) and 125 (<sup>13</sup>C) MHz, chemical shifts in  $\delta$ , coupling constants in Hz)

Position	$\delta_C$	$\delta_H$ , Mult. (J in Hz)	HMBC (H→C)
1, 1'	167.3	—	—
3, 3'	77.0	—	—
4, 4'	161.5	—	—
5a, 5a'	82.1	5.7, s	6a, 10a, 10b, 11a
6a, 6a'	148.9	—	—
7, 7'	110.9	6.7, d (8.0)	8, 9, 10a
8, 8'	130.1	7.1, ddd (8.0, 7.6, 1.1)	6a, 10
9, 9'	120.5	6.8, ddd (8.0, 7.6, 1.1)	7, 8, 10a
10, 10'	128.3	7.8, d (7.6)	6a, 10a
10a, 10a'	129.5	—	—
10b, 10b'	65.8	—	—
11, 11'	83.3	5.2, s	5a, 10b
11a, 11a'	77.5	—	—
12, 12'	28.1	3.0, s	1, 3
13, 13'	24.6	a 2.4, b 2.1, dd (14.5, 7.3)	3, 4, 14
14, 14'	9.9	1.2, d (7.3)	3, 13
OH-11, OH-11'		5.1, s	—
NH-6, NH-6'		5.6, s	5a

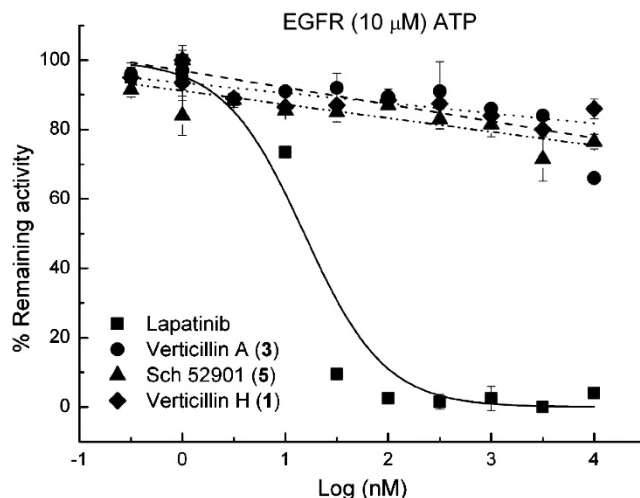
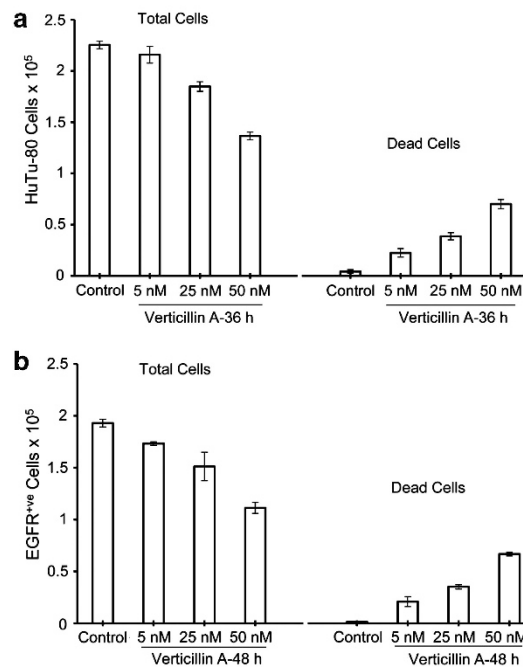
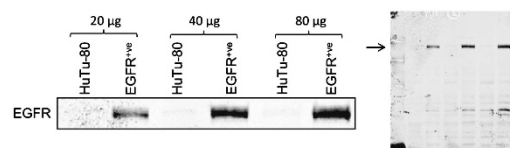
Abbreviations: Mult., multiplicity.

**Table 2** Cytotoxicity against a panel of human tumor cell lines and NF- $\kappa$ B inhibitory activity of compounds isolated from MSX 64546 and MSX 59553

Compound	Cytotoxic activity					NF- $\kappa$ B inhibitory activity IC <sub>50</sub> values (in $\mu$ M)
	IC <sub>50</sub> values (in $\mu$ M) <sup>a</sup>					
	HT- 29	SF- H460	MCF- 268	MDA- 7	MDA- MB-435	
Verticillin H ( <b>1</b> )	0.04	0.30	0.33	0.49	0.10	> 10
Sch 52900 ( <b>2</b> )	0.01	0.29	0.37	0.58	0.48	0.5
Verticillin A ( <b>3</b> )	0.02	0.20	0.25	0.37	0.07	0.1
Gliocladicillin C ( <b>4</b> )	0.03	0.52	0.38	0.61	0.08	> 10
Sch 52901 ( <b>5</b> )	0.19	1.20	0.75	1.11	0.03	> 10
11'-Deoxyverticillin ( <b>6</b> )	NT	0.01	0.04	0.03	NT	NT
Gliocladicillin A ( <b>7</b> )	NT	0.03	0.09	0.09	NT	NT
Camptothecin <sup>b</sup>	NT	0.01	0.01	0.13	NT	NT
Silvestrol <sup>b</sup>	0.01	NT	NT	NT	0.01	NT
Rocaglamide <sup>b</sup>	NT	NT	NT	NT	NT	0.075

Abbreviations: NF- $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NT, not tested.<sup>a</sup>IC<sub>50</sub> values were determined as the concentration required to reduce cellular staining with sulforhodamine B by 50% relative to untreated controls following 72 h of continuous exposure.<sup>b</sup>Positive controls.

range. Based on literature precedence, some of the isolates were examined for activity vs EGFR; however, no activity was observed when tested against both enzyme-based and cell-based assays. Regardless, the potent cytotoxicity of these compounds, which was on the same order of magnitude as the positive control, camptothecin, coupled with the promising NF- $\kappa$ B activity, warrants further exploration of the ETPs.

**Figure 3** Inhibitory effects of compounds **1**, **3** and **5** and the positive control, lapatinib, on epidermal growth factor receptor (EGFR) activity.**Figure 4** Epidermal growth factor receptor (EGFR) level of expression on HuTu-80 and EGFR<sup>+</sup> cell lines and cytotoxicity after treatment with compound **3** (HuTu-80 (a) and EGFR<sup>+</sup> (b)).

## METHODS

### General

Optical rotations, UV spectra, IR spectra and CD spectra were obtained on a Rudolph Research Autopol III polarimeter (Rudolph Research, Hackettstown, NJ, USA), a Varian Cary 100 Bio UV-vis spectrophotometer (Varian Inc., Walnut Creek, CA, USA), a Perkin-Elmer Spectrum One with Universal ATR

attachment (Perkin-Elmer, Waltham, MA, USA), and an Olis DSM 17 CD spectrophotometer (Olis, Bogard, GA, USA), respectively. NMR experiments were conducted in CDCl<sub>3</sub> using a JEOL ECA-500 (operating at 500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C; JEOL Ltd., Tokyo, Japan). HRESIMS data were measured using an electrospray ionization (ESI) source coupled to a Q-TOF Premier mass spectrometer (Waters Corp., Milford, MA, USA) or a LTQ Orbitrap XL system (Thermo Fisher Scientific, San Jose, CA, USA) in both positive and negative ionization modes and by either direct injection or via a liquid chromatographic/autosampler system that consisted of a Acquity UPLC system (Waters Corp.). Flash chromatography was conducted using a Combi-Flash Rf system using a RediSep Rf Si-gel Gold column (both from Teledyne-Isco, Lincoln, NE, USA). HPLC was carried out on Varian Prostar HPLC systems equipped with Prostar 210 pumps and a Prostar 335 photodiode array detector, with data collected and analyzed using Galaxie Chromatography Workstation software (version 1.9.3.2, Varian Inc.). For preparative HPLC, a Synergi Max-RP (4 μm; 250 × 21 mm; Phenomenex, Torrance, CA, USA) or a YMC ODS-A (5 μm, 250 × 20 mm; Waters Corp.) column was used. For semipreparative HPLC, a YMC ODS-A (5 μm, 250 × 10 mm; Waters Corp.) column was used. A Gemini-NX (5 μm; 250 × 4.6 mm; Phenomenex) column was used for analytical HPLC. For UPLC analysis, a BEH C18 (1.7 μm; 50 × 2.1 mm; Waters Corp.) column was used.

### LC-UV-HRMS dereplication procedure

**UPLC-HRESIMS conditions.** Sample concentration, 1.0 mg ml<sup>-1</sup> MeOH-Dioxane (1 : 1) solution; mobile phase (UPLC Acquity system): gradient elution of CH<sub>3</sub>CN–0.1% aqueous formic acid (0–1.0 min, 20 : 80; 1.0–5.0 min, from 20: 80–100% CH<sub>3</sub>CN and hold for 2 min); UV detection wavelengths, 210 and 254 nm; flow rate, 0.3 ml min<sup>-1</sup>; injection volume, 3 μl. ESI conditions (Thermo LTQ Orbitrap XL system): capillary temperature, 275 °C; sheath gas, 15; auxiliary gas, 5; sweep gas, 2; source voltage, 4.50 kV; capillary voltage, 46 V; tube lens, 115 V. Mass range was set to optimally pass ions from *m/z* 100–2000. Data were acquired in centroid mode during the LC run.

**Data analysis.** Using a combination search of proposed molecular formulas, exact mass and UV maxima corresponding to the major peaks in the Dictionary of Natural Products (Chapman and Hall, London, UK) and SciFinder databases (Chemical Abstracts Service, Columbus, OH, USA), the samples with unknown molecular formulas were designated for further purification.

### Producing organisms and fermentations

Mycosynthetic fungal strains 64546 and 59553 were isolated by Dr Barry Katz in May of 1992 and January of 1992 from leaf litter and leaves, respectively. DNA analyses were performed by MIDI Labs (Newark, DE, USA), and the D2 variable region of the large subunit rRNA was sequenced and compared with their database, which suggested that these fungi belong to the Bionectriaceae family (order Hypocreales); the sequences were deposited in Genbank (accession numbers JQ749727 and JQ749725 for MSX 64546 and MSX 59553, respectively). The cultures were stored on malt extract slants and transferred periodically. Fresh cultures were grown on similar slants, and a piece of each culture was transferred individually to a medium containing 2% soy peptone, 2% dextrose and 1% yeast extract (YESD media). Following incubation (7 days) at 22 °C with agitation, the cultures were used to inoculate 50 ml of rice media, prepared using rice to which was added a vitamin solution and twice the volume of rice with H<sub>2</sub>O, in 250 ml Erlenmeyer flasks. These were incubated at 22 °C until the cultures showed good growth (~14 days) to generate the screener cultures. The scale-up cultures were treated in a similar manner but grown in 2.8-L Fernbach flasks (Corning, Inc., Corning, NY, USA) containing 150 g rice and 300 ml H<sub>2</sub>O. These were inoculated using the seed cultures grown in the YESD media and were incubated at 22 °C for 14 days.

### Extraction and isolation

To the small-scale cultures on rice were added 60 ml of 1 : 1 MeOH–CHCl<sub>3</sub>, and the mixtures were shaken for 16 h on a reciprocating shaker. The solutions were filtered, and equal volumes of H<sub>2</sub>O and CHCl<sub>3</sub> were added to the filtrate to bring the total volume to 250 ml. The solutions were stirred vigorously for

1 h, partitioned in a separatory funnel, and the bottom, organic layers were concentrated by rotary evaporation to dryness. Then, each extract was defatted by stirring vigorously for 1 h in a mixture of 50 ml MeOH, 50 ml CH<sub>3</sub>CN and 100 ml hexane and then partitioned in a separatory funnel. The bottom layers were collected and evaporated to dryness. The scale-up solid fermentations on rice were extracted using the same procedures, except that the extraction volumes were adjusted as follow: 500 ml of 1 : 1 MeOH–CHCl<sub>3</sub> mixture, equal volumes of H<sub>2</sub>O and CHCl<sub>3</sub> to a total volume of 2 l, and a mixture of 150 ml MeOH, 150 ml CH<sub>3</sub>CN and 200 ml hexane for the defatting process. Each defatted large-scale extract (602 mg and 3.2 g for MSX 64546 and MSX 59553, respectively) was adsorbed onto a minimal amount of Celite 545 (Acros Organics, Geel, Belgium) and dried with mixing via a mortar and pestle. The materials were fractionated separately at 40 ml min<sup>-1</sup> on a RediSep Rf Gold Si-gel column (40 g), first with 100% hexanes for 0.7 column volumes (CV) followed by a gradient of 100% hexanes to 100% CHCl<sub>3</sub> over 8.9 CV. The elution continued with 100% CHCl<sub>3</sub> for 7.4 CV, then with a gradient of MeOH in CHCl<sub>3</sub> (0–2% over 9.7 CV, then 2–5% over 5.2 CV, then 5–10% over 5.2 CV, then 10–20% over 3.7 CV, then 20–100% over 2.2 CV. MeOH (100%) was finally held for a further 6.7 CV). Fractions were collected every 25 ml and pooled according to UV and ELSD profiles. From MSX 64546, compounds 1–5 were present in fractions 19–29, which were combined and evaporated (229.8 mg; IC<sub>50</sub> values <2.0 μg ml<sup>-1</sup> against H460 cells). This pooled fraction was then subjected to preparative HPLC (Synergi Max-RP column, 20–100% CH<sub>3</sub>CN in 0.1% aqueous formic acid over 30 min; fractions collected every 0.5 min). Further purification of the subsequent fractions by semipreparative HPLC (various ratios of CH<sub>3</sub>CN in 0.1% aqueous formic acid) afforded compounds 1 (1.6 mg), 2 (0.8 mg), 3 (2.3 mg), 4 (2.5 mg) and 5 (4.9 mg). From MSX 59553, compounds 1, 3 and 5 were present in fractions 34–56, while compounds 2, 4, 6 and 7 were present in fractions 57–61. These two sets of fractions were combined and evaporated individually (108.5 and 132.7 mg, respectively; IC<sub>50</sub> values <2.0 μg ml<sup>-1</sup> against H460 cells). The combined pool of the former (fractions 34–56) was then subjected to preparative HPLC (YMC ODS-A, 20–100% CH<sub>3</sub>CN in 0.1% aqueous formic acid over 30 min; fractions collected every 0.5 min). Further purification of the subsequent fractions by semipreparative HPLC (various ratios of CH<sub>3</sub>CN in 0.1% aqueous formic acid) afforded compounds 1 (3.0 mg), 3 (2.1 mg) and 5 (5.5 mg). The combined pool of the latter (fractions 57–71) was subjected to preparative HPLC using the same conditions and column. Further purification of the subsequent fractions by semipreparative HPLC (various ratios of CH<sub>3</sub>CN in 0.1% aqueous formic acid) afforded compounds 2 (8.5 mg), 4 (15.5 mg), 6 (6.0 mg) and 7 (11.8 mg).

### Verticillin H (1)

Compound 1 was isolated as a white amorphous solid (4.6 mg); [ $\alpha$ ]<sub>D</sub><sup>25</sup> +440 (c 0.1 CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 204 (4.79), 240 (2.84) and 301 (3.79) nm; IR (diamond)  $\nu_{\max}$  3522, 2939, 1685, 1429 and 1376 cm<sup>-1</sup>; CD (MeOH)  $\lambda_{\max}$  ( $\Delta\epsilon$ ) 235 (+62.1), 270 (–5.9), 304 (+8.6), and 373 (–1.2) nm; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz), see Table 1 and Supplementary Information (Supplementary Figures S1–S6); HRESIMS (positive mode) *m/z* 725.1326 [M + H]<sup>+</sup> (calculated for C<sub>32</sub>H<sub>33</sub>N<sub>6</sub>O<sub>6</sub>S<sub>4</sub>, 725.1339), (negative mode) *m/z* 723.1189 [M–H]<sup>–</sup> (calculated for C<sub>32</sub>H<sub>31</sub>N<sub>6</sub>O<sub>6</sub>S<sub>4</sub>, 723.1193).

### Cytotoxicity assay

The cytotoxicity measurements against the MCF-7<sup>34</sup> human breast carcinoma (Barbara A Karmanos Cancer Center, Detroit, MI, USA), NCI-H460<sup>35</sup> human large cell lung carcinoma (HTB-177, American Type Culture Collection (ATCC), Manassas, VA, USA), SF-268<sup>36</sup> human astrocytoma (NCI Developmental Therapeutics Program), HT-29<sup>37</sup> human colorectal adenocarcinoma (HTB-38, ATCC) and the MDA-MB-435<sup>38</sup> human melanoma (HTB-129, ATCC) cell lines were performed as described in detail previously.<sup>39</sup>

### NF- $\kappa$ B p65 assay

An ELISA based NF- $\kappa$ B inhibitory assay was performed as described previously.<sup>39</sup> Rocaglamide (Enzo Life Sciences, Inc., Farmingdale, NY, USA) was used as a positive control (IC<sub>50</sub> value of 0.075 μM).

**EGFR enzymatic assay**

Measurement of EGFR tyrosine kinase inhibitor activity for compounds 1–7 was performed by BPS Biosciences (San Diego, CA, USA). Detailed experimental descriptions are provided in the Supporting Information as well as via protocols available at <http://www.bpsbioscience.com>.

**EGFR<sup>+</sup> cell assay**

Human EGFR positive (EGFR<sup>+</sup>) and EGFR negative (HuTu-80) cells were cultured under standard conditions, and total cell lysates were prepared from sub-confluent cultures and analyzed by standard immunoblotting for total EGFR levels as described earlier.<sup>40</sup> In other studies, both cell lines were treated with indicated concentrations of the agent and after desired time, both total and dead cell numbers were determined using a hemocytometer, using methods described previously.<sup>40</sup>

**ACKNOWLEDGEMENTS**

This research was supported by grants P01 CA125066 and R01 CA102514 (to RA) from the National Cancer Institute/National Institutes of Health, Bethesda, MD, USA. Mycology technical support was provided by Maurica Lawrence. The high resolution mass spectrometry data were acquired at the David H Murdock Research Institute, Kannapolis, NC, USA and the Triad Mass Spectrometry Laboratory at the University of North Carolina at Greensboro. EGFR tyrosine kinase assays were performed by BPS Biosciences (San Diego, CA, USA). We thank Mallikarjuna Gu in the Agarwal laboratory for his technical assistance in conducting the EGFR<sup>+</sup> cell assays.

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Supplementary information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)