**ABSTRACT:** The cyclic tetrapeptide 1-alaninechlamydocin was purified from a Great Lakes-derived fungal isolate identified as *Tolypocladium* sp. Although the planar structure was previously described, a detailed analysis of its spectroscopic data and biological activity are reported here for the first time. Its absolute configuration was determined using a combination of spectroscopic (1H−1H ROESY, ECD, and X-ray diffraction) and chemical (Marfey’s analysis) methods. 1-Alaninechlamydocin showed potent antiproliferative/cytotoxic activities in a human pancreatic cancer cell line (MIA PaCa-2) at low-nanomolar concentrations (GI₅₀ 5.3 nM, TGI 8.8 nM, LC₅₀ 22 nM). Further analysis revealed that 1-alaninechlamydocin induced G2/M cell cycle arrest and apoptosis. Similar to other cyclic epoxytetrapeptides, the inhibitory effects of 1-alaninechlamydocin are proposed to be produced primarily via inhibition of histone deacetylase (HDAC) activity.

Histone deacetylases (HDACs) are important regulators of gene expression and have been implicated as key participants in a variety of diseases. HDAC inhibitors are used and/or being tested for the treatment of cancer, asthma and chronic respiratory conditions, Alzheimer’s disease, schizophrenia, Niemann-Pick type C disease, and others. To date, three HDAC inhibitors, vorinostat (SAHA), resminostat (4SC-201), and romidepsin (FK228), have been approved by the FDA for the treatment of cancer with other HDAC inhibitors currently under clinical assessment. Many of the compounds in clinical development, as well as those being used as HDAC-targeting molecular tools, are derived from natural sources including microorganisms.

Naturally occurring HDAC inhibitors can be classified into four major structural groups based on their putative pharmacophores: hydroxamic acids (e.g., trichostatins), thiols/protected thiols (e.g., FR901375, FK228, spiruchostatins A and B, and largazole), cyclic tetrapeptides (e.g., apicidin, FR235222, azumamides A–E, chlamydocin, microsporins A and B, and trapoxins), and compounds with mixed functionalization (e.g., depudacin and psammaplin A). Most of these naturally occurring HDAC inhibitors are proposed to directly chelate the active site Zn²⁺ ions of the enzymes with the exception of the epoxides, which are reported to form covalent bonds with the HDACs.

Our research group is focused on investigating the chemical diversity of fungi to generate new and therapeutically useful bioactive compounds. In our investigation of fungal natural products that are active against human pancreatic carcinoma cell lines, a potent HDAC inhibitor, 1-alaninechlamydocin (1), was obtained from a Great Lakes-derived fungal isolate identified as *Tolypocladium* sp. Structurally, 1-alaninechlamydocin (1) belongs to the cyclic epoxytetrapeptide family of HDAC inhibitors that include the trapoxins, HC toxin, Cyl-1 and Cyl-2, and
Although the planar structure of compound 1 was reported by Kim et al. in 1992, details of its absolute configuration and assessment of its biological activities had not been described. In this paper, we provide a report of the isolation, 1H and 13C NMR assignments, absolute configuration, and in vitro activities (HDAC inhibition, antiproliferation/cytotoxicity, cell cycle arrest, and apoptosis induction) of compound 1.

Compound 1 was isolated as an opaque white, optically active \([\alpha]_D^{24} = -80 (c 0.1, 
MeOH)\) crystalline solid. The molecular formula was determined to be C27H36N4O6 based on the HRESIMS data (m/z 513.2710, [M + H]+). A search of fungal-derived natural products with this molecular formula in the Dictionary of Natural Products led to the identification of a known cyclic tetrapeptide, 1-alaninechlamydocin (1); however, no 1H or 13C NMR data had been reported for the compound. Therefore, we proceeded to independently verify the planar structure, as well as determine the absolute configuration of 1, by means of spectroscopic analysis. In CDCl3, the 1H and 13C NMR spectra (Table 1) of 1 were composed of two sets of resonances (1D 1H COSY, HSQC, and HMBC) spectra confirmed both sets of resonances represented the same planar structure as two major configurational stereoisomers (Figure 1). The Phe–Pro amide bond bore a trans configuration in steroisomer A, which converted to a cis configuration in steroisomer B as determined by the 1H–1H ROESY correlation data (Figure 1).

Table 1. 1H and 13C NMR Data for 1 in CDCl3 (400 and 100 MHz, \(\delta \) ppm)

| no. | \(\delta_C\) | \(\delta_H (J \text{ in Hz})\) | \(\delta_C\) | \(\delta_H (J \text{ in Hz})\) |
|-----|-------------|-------------------------------|-------------|-------------------------------|
| 1   | 171.8       | 59.6 (4.98, br s)             | 136.7       | 174.9                         |
| 2   | 58.0        | 4.66, dd (7.6, 1.6)           | 59.6        | 4.98, br s                    |
| 3   | 24.9        | 2.28, m                       | 33.0        | 2.40, m                       |
|     | 1.68, m     |                               | 2.20, m     |                               |
| 4   | 24.9        | 2.13, m                       | 20.8        | 1.85, m                       |
|     | 1.75, m     |                               | 1.58, m     |                               |
| 5   | 47.0        | 3.81, m                       | 48.5        | 3.40, m                       |
|     | 3.12, m     |                               | 3.07, m     |                               |
| 6   | 173.0       | 53.6 (4.71, br s)             | 58.6        | 3.30, m                       |
|     |             | 2.95, dd (5.7, 13.4)          | 37.6        | 3.00, m                       |
| 7   | 136.8       |                               | 136.7       |                               |
| 8   | 129.1       | 7.20, m                       | 129.1       | 7.20, m                       |
| 9   | 128.7       | 7.26, m                       | 128.4       | 7.26, m                       |
| 10  | 126.9       | 7.15, m                       | 126.9       | 7.15, m                       |
| 11  | 128.7       | 7.26, m                       | 128.4       | 7.26, m                       |
| 12  | 129.1       | 7.20, m                       | 129.1       | 7.20, m                       |
| 13  |             | 7.33, d (10.2)                |             |                               |
| 14  | 174.9       |                               | 174.9       |                               |
| 15  | 56.4        | 3.74, m                       | 52.2        | 4.41, br s                    |
| 16  | 15.8        | 1.70, d (7.6)                 | 16.8        | 1.10, d (7.6)                 |
| 17  | 6.75, d (6.2)|                               |             |                               |
| 18  | 174.9       |                               |             |                               |
| 19  | 54.4        | 4.21, ddd (7.6, 7.6, 10.2)    | 59.5        | 3.85, m                       |
| 20  | 29.0        | 1.77, m                       | 29.6        | 1.78, m                       |
|     |             | 1.60, m                       |             |                               |
| 21  | 28.7        | 1.30, m                       | 28.6        | 1.33, m                       |
|     |             | 1.28, m                       |             | 1.28, m                       |
| 22  | 25.4        | 1.30, m                       | 26.1        | 1.41, m                       |
|     |             | 1.30, m                       |             | 1.30, m                       |
| 23  | 22.8        | 1.54, m                       | 22.7        | 1.54, m                       |
| 24  | 36.4        | 2.30, m                       | 36.4        | 2.30, m                       |
|     |             | 2.40, m                       |             | 2.40, m                       |
| 25  | 207.6       |                               | 207.6       |                               |
| 26  | 53.5        | 3.40, dd (2.5, 4.6)           | 53.5        | 3.40, dd (2.5, 4.6)           |
| 27  | 46.2        | 2.83, dd (2.5, 5.8)           | 46.2        | 2.83, dd (2.5, 5.8)           |
| 28  | 29.6        | 4.64, dd (4.6, 5.8)           | 29.6        | 4.64, dd (4.6, 5.8)           |
| 29  | 7.11, d (10.2)|                               |             |                               |

**Note:** Not detected. **Overlapped.**

configurations (\(\Delta_H^< \text{trans} < \Delta_H^> \text{cis}\)) based on a statistical analysis of 13C chemical shifts of over 1000 protein-embedded proline residues. The cis–trans isomerization of the Phe–Pro peptide bond has been previously reported in chlamydocin, the aminoisobutyric acid (Aib) analogue of 1.\(^{1,22}\)

In order to determine the absolute configuration of 1, the compound was subjected to acidic hydrolysis followed by Marley’s analysis (Figure S1, S1). Accordingly, the absolute configurations of C-2, C-7, and C-17 were assigned as R, S, and S, respectively. Furthermore, the ECD spectrum of 1 exhibited a negative Cotton effect at 290 nm (Figure S2, S1), which indicated an S configuration for the C-28 epoxy.\(^{23}\) In addition, a single crystal was obtained from a concentrated MeOH solution of 1 that was suitable for X-ray diffraction analysis. The X-ray diffraction data confirmed the proposed structural assignments of 1, as well as provided evidence for the absolute configuration of C-21 as S.
The antiproliferative/cytotoxic activities of 1 were evaluated in two human pancreatic carcinoma cell lines, MIA PaCa-2 and Panc-1, as well as an immortalized human pancreatic ductal cell line, hTERT-HPNE. Compound 1 showed potent antiproliferative and cytotoxic activities against MIA PaCa-2 cells, with GI<sub>50</sub> and LC<sub>50</sub> values of 5.3 and 22 nM, respectively (Figure 2A and B). The cyclic-epoxytetrapeptide HDAC inhibitor trapoxin A showed comparable potency to 1, while another two HDAC inhibitors, SAHA and apicidin, were significantly less active than 1 in the same cell line (Figure 2A). Compound 1 also inhibited the proliferation of Panc-1 and hTERT-HPNE cells at low-nanomolar concentrations, but did not induce cytotoxicity in either cell line at concentrations up to 10 μM (Figure 2B). The positive control SAHA was much less active than 1, but it too showed a similar pattern of differential antiproliferative and cytotoxic activity in the same three cell lines (Figure 2B). Flow cytometry experiments revealed that when MIA PaCa-2 cells were exposed to 20 nM 1 for 20 h, the population of cells in the G1 phase decreased by >50%, which was accompanied by a doubling of the percentage of cells in G2/M phase relative to G1 phase. This further supported the hypothesis that 1 exerts its observed biological effects through inhibition of HDAC function.

Prior concern had been raised regarding the serum stability of 1, we evaluated the compound’s in vitro activity following incubation in serum for an extended period of time. No loss of potency against MIA PaCa-2 cells was observed for 1 (25 nM) when it was incubated in human serum for up to 30 min (data not shown). Additional in vitro and in vivo studies will be needed to confirm whether 1 is subject to epoxide degradation similar to that reported for chlamydacin.

In summary, the cyclic epoxytetrapeptide 1-alaninechlamydacin (1) was purified from a Great Lakes-derived Tolypocladium isolate. The metabolite bears an epoxynketone moiety similar to other HDAC inhibitors including the trapoxins and chlamydacin. Compound 1 displayed a pattern of antiproliferative/cytotoxic activities that were similar to but much more potent than the HDAC inhibitor SAHA. It induced cell cycle (G2/M phase) arrest and apoptosis consistent with other HDAC inhibitors. The in vitro antiproliferative potency of 1 is equivalent to its potency as an HDAC inhibitor in cellular lysates, indicating that HDAC inhibition is the major mode of action responsible for the in vitro biological effects attributed to this compound.
#### EXPERIMENTAL SECTION

**General Experimental Procedures.** Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter. NMR data were obtained on a Varian VNMR spectrometer (400 MHz for $^1$H, 100 MHz for $^{13}$C) with a broadband probe at 25 ± 0.5 °C. Electrospray-ionization mass spectrometry data were collected on an Agilent 6538 high-mass-resolution QTOF mass spectrometer. The ECD spectrum was obtained with a model 202-01 AVIV circular dichroism spectrometer. All solvents were of ACS grade or better.

**Strain Information.** The *Tolypocladium* sp. isolate was obtained from a sandy sediment collected at a depth of approximately 350 feet in Lake Superior offshore from Hancock, Michigan, USA. The isolate was identified as a *Tolypocladium* sp. based on sequence data generated for the ribosomal internal transcribed spacer region and the 5.8S rRNA genes (ITS1-5.8S-ITS2)28 (GenBank accession KJ571609).

**Isolation and Purification of Compound 1.** Spores and mycelia were inoculated into 50 Erlenmeyer flasks (1 L) containing 200 mL of mashed potatoes dextrose broth (10 g/L Great Value mashed potatoes, a brand of instant mashed potatoes distributed by Great Value). The flasks were shaken at 135 rpm for 9 days at room temperature on an Innova 5000 shaker. The culture was extracted three times with equal volumes of ethyl acetate, and the organic solvent was evaporated in vacuo to generate the crude extract (4.5 g). The extract was separated into six fractions (1–6) by HP20SS column chromatography (eluted with a gradient of MeOH–H$_2$O). The eluent for fraction 3 (eluted with 70% MeOH in H$_2$O) was evaporated in vacuo, yielding compound 1 (56 mg, 1.24% yield).

**X-ray Crystal Structure Analysis of 1.** A colorless block-shaped crystal of dimensions 0.520 × 0.400 × 0.160 mm was selected for structural analysis. Intensity data for this compound were collected using a diffractometer with a Bruker APEX CCD area detector and graphite-monochromated Mo K radiation ($\lambda = 0.71073$ Å). The sample was cooled to 100(2) K. Cell parameters were determined using a diode array detector and the crude extract (4.5 g). The extracted material was dissolved in 20 mL of DMSO stock in 18 L of serum) for 5, 10, 15, and 30 min. After incubation was complete, 1 mL aliquots of the resulting stock solutions were aspirated and transferred to separate wells of a new 96-well plate seeded with 5000 MIA PaCa-2 cells per well. The cells were grown for 48 h before viability was determined by MTT assay.

**LC-MS Analysis of Marfey’s reaction products of 1, ECD spectrum of 1, flow cytometry analysis of 1 in MIA PaCa-2 cells, and NMR spectra of 1 and 2.** LC-MS data for this compound were collected using a Finnigan ISQ mass spectrometer (San Antonio Area Foundation Grant (A.L.R.). The authors are grateful for the help provided by J. Shanle for coordinating the
collection of sediment samples with the captain (J. Ylitalo) and crew of the Sue Plus Two.

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