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Note

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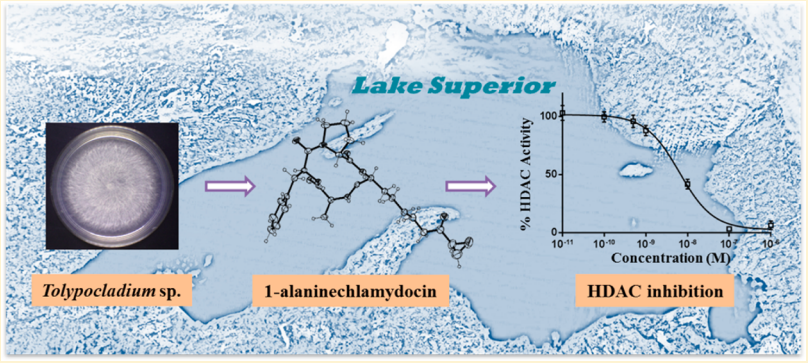
A Potent HDAC Inhibitor, 1-Alaninechlamydocin, from a *Tolypocladium* sp. Induces G2/M Cell Cycle Arrest and Apoptosis in MIA PaCa-2 Cells

Lin Du,[†,‡,⊥] April L. Risinger,[§,▽,⊥] Jarrod B. King,[†,‡] Douglas R. Powell,[†] and Robert H. Cichewicz*[,†,‡]

[†]Department of Chemistry and Biochemistry, Stephenson Life Sciences Research Center, [‡]Natural Products Discovery Group, and Institute for Natural Products Applications and Research Technologies, University of Oklahoma, Norman, Oklahoma 73019-5251, United States

[§]Department of Pharmacology and [▽]Cancer Therapy & Research Center, University of Texas Health Science Center, San Antonio, Texas 78229, United States

Supporting Information



ABSTRACT: The cyclic tetrapeptide 1-alaninechlamydocin was purified from a Great Lakes-derived fungal isolate identified as a *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 (^{1}H−^{1}H 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 (GI_{50} 5.3 nM, TGI 8.8 nM, LC_{50} 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.[1] HDAC inhibitors are used and/or being tested for the treatment of cancer,[2] asthma and chronic respiratory conditions,[3] Alzheimer's disease,[4] schizophrenia,[5] stroke,[6] spinal muscular atrophy,[7] Niemann-Pick type C disease,[8] and others.[1] 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.[9] 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., depudecin and psammaplin A).[10] Most of these naturally occurring HDAC inhibitors are proposed to directly chelate the active site Zn^{2+} ions of the enzymes with the exception of the epoxides, which are reported to form covalent bonds with the HDACs.[10]

Our research group is focused on investigating the chemical diversity of fungi to generate new and therapeutically useful bioactive compounds.[11−13] 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 a *Tolypocladium* sp. Structurally, 1-alaninechlamydocin (**1**) belongs to the cyclic epoxytetrapeptide family of HDAC inhibitors that include the trapoxins,[14,15] HC toxin,[16] Cyl-1 and Cyl-2,[17] and

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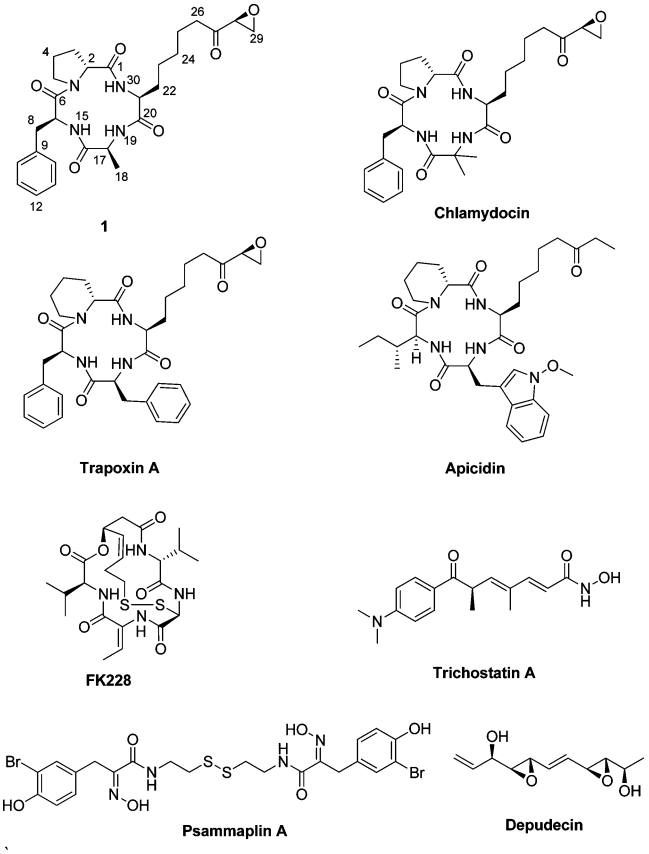
WF-3161.[18] Although the planar structure of compound **1** was reported by Kim et al. in 1992,[19] details of its absolute configuration and assessment of its biological activities had not been described. In this paper, we provide a report of the isolation, ^{1}H and ^{13}C NMR assignments, absolute 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 {$[\alpha]^{24}_{D}$ −80 (*c* 0.1, MeOH)} crystalline solid. The molecular formula was determined to be $C_{27}H_{36}N_4O_6$ 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 ^{1}H or ^{13}C 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 $CDCl_3$, the ^{1}H and ^{13}C NMR spectra (Table 1) of **1** were composed of two sets of similar resonances in a ∼1:1 ratio. An investigation of the 1D (^{1}H and ^{13}C) and 2D NMR (^{1}H−^{1}H 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 ^{1}H−^{1}H ROESY correlation data (Figure 1). As a result of the isomerization of the Phe−Pro amide bond, the ^{13}C NMR resonances of C-3 and C-4 shifted substantially ($\delta_{C\text{-}3}$ 24.9 ppm and $\delta_{C\text{-}4}$ 24.9 ppm for isomer A; $\delta_{C\text{-}3}$ 33.0 ppm and $\delta_{C\text{-}4}$ 20.8 ppm for isomer B). Similar chemical shift differentials ($\delta^{13}C^{\beta} - \delta^{13}C^{\gamma}$) have been used as indicators of Xaa−Pro peptide bond configurations ($\Delta_{\beta\gamma}$ *trans* < $\Delta_{\beta\gamma}$ *cis*) based on a statistical analysis of ^{13}C chemical shifts of over 1000 protein-embedded proline residues.[20] The *cis*−*trans* isomerization of the Phe−Pro peptide bond has been previously reported in chlamydocin, the aminoisobutyric acid (Aib) analogue of **1**.[21,22]

Table 1. ^{1}H and ^{13}C NMR Data for 1 in $CDCl_3$ (400 and 100 MHz, δ ppm)

	isomer A		isomer B	
no.	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	171.8		–[a]	
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		
6	173.0		–[a]	
7	53.6	5.07, ddd (10.2, 10.2, 5.7)	58.6	4.71, br s
8	36.0	3.22, dd (10.2, 13.4)	37.6	3.30, m
		2.95, dd (5.7, 13.4)		3.07, m
9	136.8		136.7	
10	129.1	7.20, m[b]	129.1	7.20, m[b]
11	128.7	7.26, m[b]	128.4	7.20, m[b]
12	126.9	7.15, m[b]	126.9	7.15, m[b]
13	128.7	7.26, m[b]	128.4	7.20, m[b]
14	129.1	7.20, m[b]	129.1	7.20, m[b]
15		7.33, d (10.2)		–[a]
16	174.9		174.9	
17	56.4	3.74, m	52.2	4.41, br s
18	15.8	1.70, d (7.6)	16.8	1.10, d (7.6)
19		6.75, d (6.2)		–[a]
20	174.9		–[a]	
21	54.4	4.21, ddd (7.6, 7.6, 10.2)	59.5	3.85, m
22	29.0	1.77, m	29.6	1.78, m
		1.60, m		
23	28.7	1.30, m	28.6	1.33, m
				1.28, m
24	25.4	1.30, m	26.1	1.41, m
				1.30, m
25	22.8	1.54, m	22.7	1.54, m
26	36.4	2.30, m	36.4	2.30, m
		2.40, m		2.40, m
27	207.6		207.6	
28	53.5	3.40, dd (2.5, 4.6)	53.5	3.40, dd (2.5, 4.6)
29	46.2	2.83, dd (2.5, 5.8)	46.2	2.83, dd (2.5, 5.8)
		2.96, dd (4.6, 5.8)		2.96, dd (4.6, 5.8)
30		7.11, d (10.2)		–[a]

[a]Not detected. [b]Overlapped.

In order to determine the absolute configuration of **1**, the compound was subjected to acidic hydrolysis followed by Marfey's analysis (Figure S1, SI). 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, SI), 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*.

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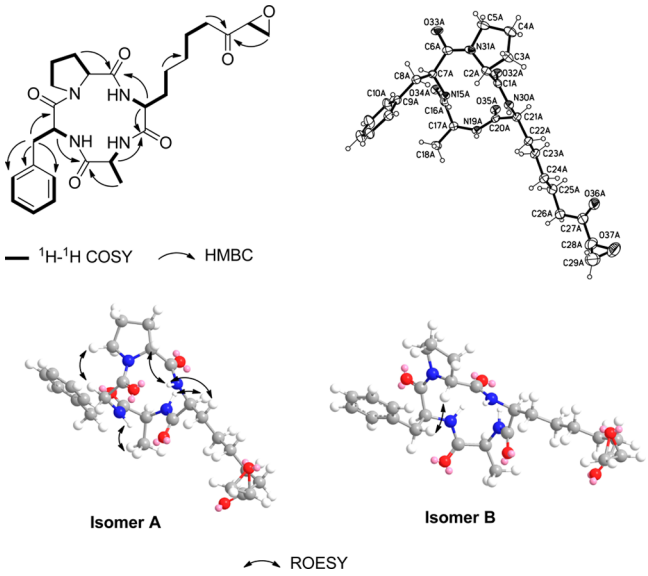


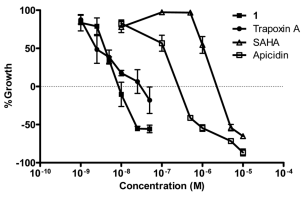
Figure 1. Selected 2D NMR (^{1}H−^{1}H COSY, HMBC, and ^{1}H−^{1}H ROESY) correlations of **1** and ORTEP structure generated from the X-ray diffraction data for a single crystal of **1**.

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_{50} and LC_{50} 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 the vehicle-treated cells (Figures 2C and S3, SI). At this same concentration, **1** also induced significant cleavage of poly ADP-ribose polymerase (PARP) (Figure 2D), which is an indicator of early stage apoptosis.[24] The G2/M-phase-arrest effects and apoptosis-induction activity of **1** is consistent with its antiproliferative/cytotoxic effects (Figure 2A and B).

Due to the structural similarity of **1** to the known histone deacetylase inhibitor chlamydocin,[25] we hypothesized that it would have a similar mechanism of action. Inhibition of HDAC−peptide−substrate deacetylation in the presence of HeLa cell lysate was investigated. Compound **1** exhibited potent inhibition of HDAC activity with an IC_{50} value of 6.4 nM (Figure 2E). 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 a structural analogue of **1**, chlamydocin.[26] *In vivo* and supporting *in vitro* pharmacokinetics experiments of chlamydocin suggested a short half-life ($t_{1/2}$) of 8.7 min in freshly prepared rat serum and 0.68−3.6 min in whole blood. To investigate the

(A)



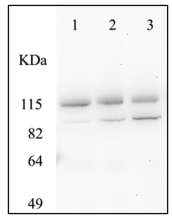
(B)

Compound	MIA PaCa-2			Panc-1			hTERT-HPNE		
	GI_{50} (nM)	TGI (nM)	LC_{50} (nM)	GI_{50} (nM)	TGI (nM)	LC_{50} (nM)	GI_{50} (nM)	TGI (nM)	LC_{50} (nM)
1	5.3	8.8	22	14	50	>10^4	2.0	660	>10^4
SAHA	790	2350	4850	678	3260	>10^4	521	>10^4	>10^4

(C)

	% G1	% G2/M	% S	Cv
0 nM	50.2	19.2	30.6	4.8
8 nM	46.0	19.4	34.6	5.2
20 nM	19.6	45.4	35.0	5.5

(D)



(E)

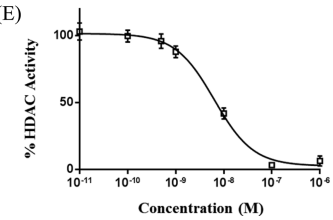


Figure 2. Antiproliferative and cytotoxic activities of **1** and related mechanism studies. (A) Concentration-dependent-response curves of **1** and other HDAC inhibitors in MIA PaCa-2 cells using the MTT assay. The dashed line at 0% growth indicates the cell density at the time of drug addition, with 100% growth representing vehicle-treated controls and negative growth values indicative of cytotoxicity. Data are expressed as the means of triplicate wells with standard derivation. (B) GI_{50}, TGI, and LC_{50} values of **1** and SAHA in MIA PaCa-2, Panc-1, and hTERT-HPNE cells. (C) Cell cycle distribution of MIA PaCa-2 cells 20 h after the addition of **1**. Cv, coefficient of variation. (D) SDS-PAGE of full-length PARP (upper band) and cleaved PARP (lower band) from MIA PaCa-2 cells after treatment (20 h) with vehicle (lane 1), 8 nM **1** (lane 2), and 20 nM **1** (lane 3). The approximate molecular weights of proteins are indicated by the scale on the left side of the gel. (E) Concentration-dependent inhibition of histone deacetylase (HDAC) activity by **1**. Data are expressed as the mean response for each concentration tested in triplicate (±standard error).

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 chlamydocin.

In summary, the cyclic epoxytetrapeptide 1-alaninechlamydocin (**1**) was purified from a Great Lakes-derived *Tolypocladium* isolate. The metabolite bears an epoxyketone moiety similar to other HDAC inhibitors including the trapoxins[14] and chlamydocin.[15] 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.[27] 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.

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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 broad band 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 aproximately 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 Wal-Mart Stores, Inc., 5 g/L D-glucose, and 2 g/L $NaNO_3$). 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_2O). The eluent for fraction 3 (eluted with 70% MeOH in H_2O) 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 (λ = 0.71073 Å). The sample was cooled to 100(2) K. Cell parameters were determined from a nonlinear least-squares fit of 9894 peaks in the range 2.27° < θ < 27.98°. A total of 128 755 data were measured in the range 1.459° < θ < 28.381° using ϕ and ω oscillation frames. The data were corrected for absorption by the empirical method (SADABS, Bruker AXS Inc.), giving minimum and maximum transmission factors of 0.955 and 0.986. The data were merged to form a set of 22 017 independent data with R(int) = 0.0521 and a coverage of 99.8%. The X-ray crystallographic data for **1** have been deposited with the Cambridge Crystallographic Data Center under accession number CCDC 999342. These data can be accessed free of charge at http://www.ccdc.cam.ac.uk/.

Marfey's Analysis of FDAA Derivatives of Compound 1. Compound **1** (0.3 mg) was suspended in 6 M HCl (500 μL) and heated overnight at 110 °C. The hydrolysate was evaporated under N_2 and treated with 1 M $NaHCO_3$ (20 μL) and 1% FDAA−acetone (100 μL) at 45 °C for 1 h. The reactants were neutralized with 1 M HCl (20 μL) and diluted with MeCN (500 μL) prior to LC-MS analysis. FDAA derivatives of amino acid standards were prepared in a similar manner. Aqueous solutions of amino acid standards (50 mM, 50 μL) were reacted, neutralized, and diluted following the same procedure used for compound **1**. The derivatized products of compound **1** and the amino acid standards were analyzed by LC-MS (solvent A: H_2O with 0.1% formic acid; solvent B: CH_3CN, 0−15 min, 10−100% B).

Antiproliferation/Cytotoxicity Assay. Concentration-dependent inhibition studies of two pancreatic cancer cell lines, MIA PaCa-2 and Panc-1, and the immortalized human pancreatic ductal cell line, hTERT-HPNE, were assessed using the MTT assay.[29] MIA PaCa-2, Panc-1, and hTERT-HPNE cell lines were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI media supplemented with 5% FetalClone III (Hyclone) and penicillin/streptomycin (50 U/mL, 50 μg/mL). The hTERT-HPNE cell line was also supplied with 10 ng/mL of recombinant human EGF (Life Technologies). Cell lines were used within 3 months of resuscitation from liquid nitrogen. For all tests, the final concentration of DMSO did not exceed 1% volume. Assays were performed by exposing cells to compounds for 48 h prior to analysis. Cell densities were measured colorimetrically with a microplate reader (Infinite M200, Tecan Group Ltd.) and compared to data from vehicle-treated wells, which were normalized to 100% growth. Cytotoxicity was determined by comparisons of the final cell densities to the cell densities at the time of drug addition (0% growth). The concentrations of **1** that caused 50% growth inhibition (GI_{50}), total inhibition of cell growth (TGI), and 50% cytotoxicity (LC_{50}) were calculated by nonlinear regression analysis using GraphPad Prism software.

Flow Cytometry. The cell cycle profiles of MIA PaCa-2 cells after treatment with **1** at 8 or 20 nM for 20 h were compared to vehicle-treated cells after propidium iodide (PI) staining.[30] Flow cytometry was performed on cells stained with PI and analyzed on a BD Biosciences LSR II flow cytometer. Cell cycle distributions were calculated using ModFit software (Verity).

PARP Cleavage. Total cell lysates were generated from MIA PaCa-2 cells treated with **1** at 8 or 20 nM for 20 h. Lysates were run on SDS-PAGE and immunoblotted with a PARP antibody that recognizes both full-length and cleaved forms of the PARP protein (Cell Signaling #9542). PARP was detected using an HRP-secondary antibody (GE Healthcare) and analyzed on a Geliance imager (PerkinElmer).

HDAC Assay. Histone deacetylase activity was measured using a fluorometric histone deacetylase kit (CS1010, Sigma-Aldrich). Stock solutions of **1** were made in assay buffer at 20-fold of the final concentration and added to 100 μL of assay buffer with 100 μM HDAC substrate and 15 μL of HeLa cell lysate. After 30 min incubation at 30 °C, the developer solution was added, which allowed for the release of the fluorescent reporter from the deacetylated substrate. Fluorescence was detected using a Gemini fluorescence plate reader (Molecular Devices) with an excitation of 355 nm and emission of 460 nm. Measurements were taken from triplicate wells and normalized to the fluorescence of the deacetylated substrate in the absence of **1**. Nonlinear regression analysis of the data was performed using GraphPad Prism software to calculate the IC_{50} value.

Serum Stability Assay. Stock solutions of 1-alaninechlamydocin (**1**) were prepared by incubating the compound at room temperature with human serum (type AB, Atlanta Biologicals #S40190) in a 1:10 ratio (2 μL of DMSO stock in 18 μL of serum) for 5, 10, 15, and 30 min. After incubation was complete, 1 μL aliquots of the resulting stock solutions were aspirated and transferred to separate wells of a new sterile 96-well plate seeded with 5000 MIA PaCa-2 cells per well. The final concentration of **1** in wells of the test plate was 25 nM. The cells were grown for 48 h before viability was determined by MTT assay.

ASSOCIATED CONTENT

Supporting Information

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** (^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HSQC, HMBC, and ^{1}H−^{1}H ROESY) are available. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: rhcichewicz@ou.edu.

Author Contributions

⊥L. Du and A. L. Risinger contributed equally.

Notes

The authors declare no competing financial interest.

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