JOURNAL OF NATURAL PRODUCTS

Article

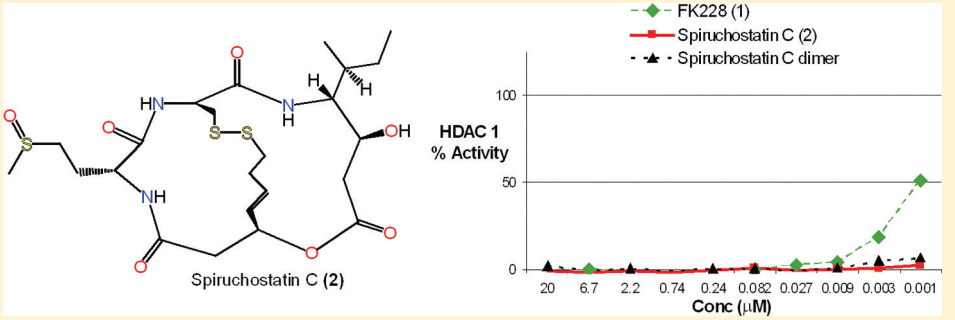
pubs.acs.org/jnp

Histone Deacetylase Inhibitors from *Burkholderia thailandensis*

Paul Klausmeyer,* Suzanne M. Shipley, Karina M. Zuck, and Thomas G. McCloud

[†]Natural Products Support Group, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland 21702, United States

Supporting Information



ABSTRACT: Bioactivity-guided fractionation of an extract of *Burkholderia thailandensis* led to the isolation and identification of a new cytotoxic depsipeptide and its dimer. Both compounds potently inhibited the function of histone deacetylases 1 and 4. The monomer, spiruchostatin C (**2**), was tested side by side with the clinical depsipeptide FK228 (**1**, Istodax, romidepsin) in a murine hollow fiber assay consisting of 12 implanted tumor cell lines. Spiruchostatin C (**2**) showed good activity toward LOX IMVI melanoma cells and NCI-H522 non small cell lung cancer cells. Overall, however, FK228 (**1**) showed a superior in vivo antitumor profile in comparison to the new compound.

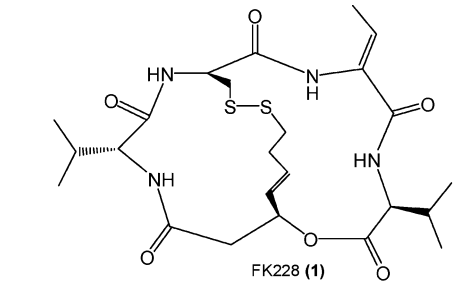
Histone deacetylases (HDACs) are enzymes involved in the regulation of eukaryotic and prokaryotic RNA synthesis through the removal of acetyl groups from ε-*N*-acetyl lysine on a histone, thereby restricting DNA transcription. As of 2008, four classes of HDACs consisting of 18 isoforms have been identified,[1] and attempts are being made to characterize the role of individual isoforms. Natural product inhibitors of histone deacetylases (HDACi) are now finding applications as anticancer agents,[2−4] with the recent FDA approval of the class I HDAC inhibitor FK228 (**1**; Istodax, Celgene Corp.) for the treatment of human cutaneous T-cell lymphoma. Continuing research in this area is aimed at finding compounds that specifically inhibit individual HDAC isoforms that have not previously been targeted and that may be used to treat other types of cancer or as experimental tools in assigning functions to individual HDAC isoforms.[3] In a recent publication,[5] the biological activity and structure elucidation of the bicyclic depsipeptides thailandepsins A and B from the gram-negative bacteria *Burkholderia thailandensis* were described. In this work, solvent extraction of the total fermentation broth of *B. thailandensis* followed by activity-guided fractionation of the organic solvent soluble material has led to the isolation of a new antineoplastic compound having potent HDAC inhibitory activity, spiruchostatin C (**2**).

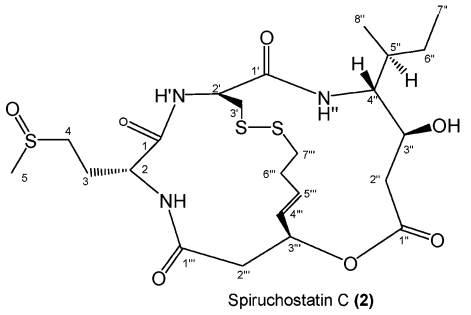
RESULTS AND DISCUSSION

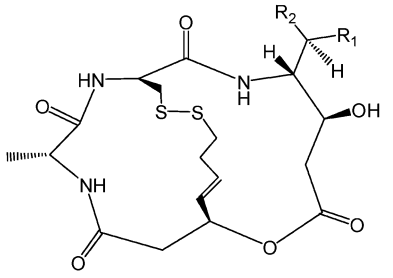
The ethyl acetate (EtOAc) extract of *B. thailandensis* was subjected to semipreparative scale HPLC dereplication, a technique developed in our laboratory for identifying bioactive compounds in molecular-targeted screens.[6,7] A 2.5% amount of each of the 84 × 8 mL fractions was submitted for testing at a single dose in the NCI 60 primary anticancer screen.[8,9] Cytotoxic activity was found in multiple zones of the chromatogram (see the Supporting Information), including two well-resolved components tentatively identified by UV and MS data as the known compounds PC-3[10] and YM-30059.[11] An early-eluting fraction also had cytotoxic activity, which upon LC/MS analysis was shown to consist of a major component having MW 563. Careful analysis of the isotopic pattern of the MS spectrum suggested that the structure contained three sulfur atoms. High-resolution mass spectrometry of **2** led to the molecular formula $C_{23}H_{37}N_3O_3S_3$. The UV absorbance spectrum of **2** displayed a weakly discernible maximum at 270 nm, suggesting the absence of a conjugated π-electron system. To obtain sufficient material for structure elucidation and for biological screening, a 20 L culture of *B. thailandensis* was undertaken, which yielded 12.4 mg of **2**, a 0.12% yield from the EtOAc extract (10.29 g) or 0.62 mg/L from the bacterial broth.

The ^{1}H NMR spectrum of **2** revealed a mixture of two compounds, occurring in a 55:45 ratio, that were inseparable on C_{18}, silica, cyano, phenyl, and chiral HPLC columns. Raising the temperature of the NMR cryoprobe did not cause the two sets of peaks to merge, and so the data were interpreted and assigned for one component of the mixture. The ^{1}H NMR spectrum of **2** showed three doublets for exchangeable protons (δ_H 8.88, 7.53, and 6.92 ppm, J = 2.0, 7.0, and 8.5 Hz,

Received: June 21, 2011
Published: October 3, 2011







respectively) (Table 1). However, four ester/amide carbonyls were deduced by analysis of the HMBC (δ_C 172.1, 171.7, 170.9, 169.3 ppm). From these data, a sulfur-containing peptide containing three amino acid residues and an ester-linked fragment were considered the motif upon which the active structure was based.

The partial amino acid composition of **2** was deciphered by acid hydrolysis followed by HILIC HPLC of the hydrolysate. In comparison to the retention times of 21 of the most commonly found L amino acids, methionine was indicated, yet the NMR spectrum lacked the characteristic singlet at δ_H ~2.1 of the methionine methyl group. Rather, an HSQC correlation between δ_C 38.4 and δ_H 2.70 (3H, s) suggested a methyl arising from a vicinal sulfoxide group. Under anoxic conditions, acid hydrolysis of peptides that contain methionine sulfoxide results in the release of methionine.[12] The presence of methionine sulfoxide in **2** was later confirmed by HILIC chromatography of a standard and its configuration assigned as D by FDAA derivatization followed by chromatographic retention time matching of the FDAA-derivatized D- and L-methionine standards. Sulfoxide functionalities have stable chiral centers that form enantiomeric pairs, which may explain the complexity of the NMR spectra of **2**.

Since no other amino acids were readily identified in the hydrolysis mixture by HPLC analysis, and due to the complexity of the NMR data, other techniques were applied in the elucidation of **2**. The possibility that **2** possessed structural homology with FK228[13] (**1**) suggested that the unassigned sulfur atoms might originate from cysteine that had condensed to form a disulfide bridge. Addition of Ellman's reagent to **2** did not produce a color change, indicating an absence of sulfhydryl groups. However, **2** tested positive for sulfhydryl when first treated with zinc catalyst, followed by addition of Ellman's reagent. Cysteine is not detected by acid hydrolysis/FDAA derivatization unless the compound is first treated with performic acid to break the −S−S− linkage, resulting in the disulfhydryl form of the molecule. Acid hydrolysis then liberates cysteine as cysteic acid, which is then derivatized with the advanced Marfey's reagent FDVA prior to HPLC determination.[14] Using this indirect method, D-cysteine was detected in the hydrolysate. The sulfur-bound β-methylene carbon of cysteine (δ_C 39.6, δ_H 3.10, 3.28) was detected by a COSY correlation to H-2′, supporting the findings of the advanced Marfey's analysis.

Substructures of **2** that were not decipherable by Marfey's analysis were elucidated through the 1-D and 2-D NMR data.

Table 1. NMR Spectroscopic Data ($CDCl_3$ + MeOH-d_4 (9:1)) for Spiruchostatin C (2)

	position	δ_C,[a] type	δ_H (J in Hz)	HMBC[b]
Met-sul[c]	1	170.9, C=O		2, 3, NH, NH′
	2	55.8, CH	4.15, m	4
	3	24.5, CH_2	2.33, m	2, 4
			2.51, m	
	4	50.4, CH_2	2.87, m	2, 3, 5
			3.14, m	
	5	38.4, CH_3	2.70, s	4
	NH		8.88, br d	
Cys	1′	169.3, C=O		2′, 3′, 4″, NH″
	2′	55.5, CH	4.84, m	3″
	3′	39.6, CH_2	3.10, m	
			3.28, m	
	NH′		6.92, d (8.5)	
AHHP[d]	1″	171.7, C=O		2″, 3″
	2″	40.0, CH_2	2.62, m	3″, 4″
			2.70, m	
	3″	67.9, CH	4.63, dt (9.4, 3.7)	2″, 4″
	4″	61.6, CH	3.00, ddd (9.4, 7.3, 5.5)	3″, 5″, 6″, 8″
	5″	36.4, CH	2.09, m	3″, 4″, 6″, 7″
	6″	27.3, CH_2	1.24, m	4″, 5″, 7″, 8″
			1.56, m	
	7″	11.6, CH_3	0.92, t (7.5)	5″, 6″, 8″
	8″	15.4, CH_3	0.93, d (7.0)	4″, 5″, 6″
	NH″		7.53, d (7.0)	
	OH″			
acyl	1‴	172.1, C=O		2, 2‴, NH
	2‴	40.6, CH_2	2.67, m	4‴
			3.21, dd (12.5, 7.0)	
	3‴	71.2, CH	5.52, br s	2‴, 4‴
	4‴	130.5, CH	5.89, d (15.0)	2‴
	5‴	132.1, CH	6.10, m	2‴
	6‴	32.0, CH_2	2.44, m	4‴
			2.65, m	
	7‴	41.8, CH_2	2.71, m	
			3.39, m	

[a] δ_C determined by 2D experiments. [b] HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon. [c] Methionine sulfoxide. [d] AHHP = 4-amino-3-hydroxy-5-methylheptanoic acid.

dx.doi.org/10.1021/np200532d | J. Nat. Prod. 2011, 74, 2039–2044

The TOCSY spectrum showed two additional spin systems, one system including two sets of methylene protons (δ_H 1.24/1.56 and δ_H 2.62/2.70), a methine bound to a heteroatom (δ_H 4.63), a methine proton (δ_H 3.00), two methyl groups (δ_H 0.92 and 0.93), and one of the amide protons (δ_H 7.53). COSY correlations revealed the sequence of this group of aliphatic hydrogens, and HMBC correlations from H-2″ and H-3″ to the C-1″ carbonyl established the structure of the fragment as 4-amino-3-hydroxy-5-methylheptanoic acid (AHHP). The second spin system consisted of three sets of methylene protons (δ_H 2.44/2.65, δ_H 2.67/3.21, and δ_H 2.71/3.39), a methine bound to a heteroatom (δ_H 5.52), and a disubstituted alkene (δ_H 5.89, 6.10). COSY correlations allowed for the assignment of the C-2‴ through C-6‴ fragment, while the C-7‴ sulfur-bound methylene group was oriented on the basis of TOCSY correlations, also to C-2‴ through C-6‴. An HMBC correlation linked the H-2‴ methylene protons to the C-1‴ carbonyl, while the C-3‴ HSQC correlation to δ_H 5.52 suggested linkage to an oxygen or nitrogen heteroatom. Molecular weight considerations and the absence of a COSY signal between an amide proton and δ_H 5.52 confirmed the presence of the ester. The data indicated the structure of this acyl fragment as 3-hydroxy-7-thio-hept-4,5(*E*)-enoic acid (HTH).

The linkage of the four subunits of **2** was determined by analysis of HMBC (J_{C-H} = 8 Hz) data (Figure 1). Each of the three amide protons had HMBC correlations to the carbonyl groups of adjacent fragments, thereby establishing the linkage of methionine sulfoxide to both cysteine and the HTH fragment. Unfortunately, HMBC correlations were not seen between the C-3′ group of the cysteinyl across the disulfide bridge to the C-7‴ group of the HTH fragment or from the C-3‴ methylene protons of the HTH fragment to the C-1″ carbonyl of the AHHP subunit. However, the proton and carbon chemical shifts for the 3′-, 3‴-, and 7‴-positions are in agreement with those published for both natural[15] and synthetic[16] spiruchostatins A and B. FK228 (**1**) isolated from *Chromobacterium violaceum,* spiruchostatins isolated from a *Pseudomonas* sp., and this new compound are all bicyclic depsipeptides, with the name spiruchostatin C (**2**) chosen for the new compound because of its close structural similarity to spiruchostatin A.

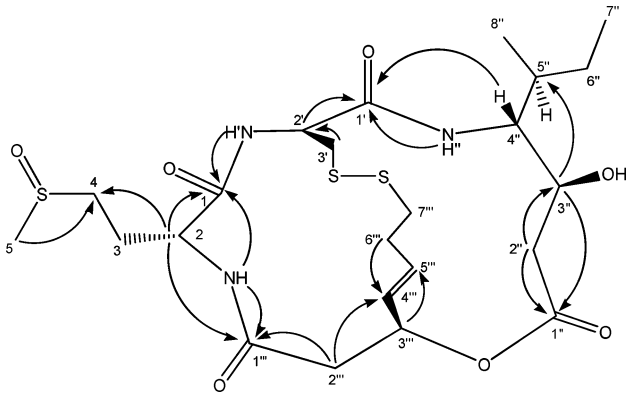


Figure 1. Key HMBC correlations for **2** optimized for 8 Hz.

In the isolation of **2**, a minor peak was noted arising from a compound having a monoisotopic mass of 1126 amu, or twice the molecular weight of **2**. This compound was isolated and subjected to acid hydrolysis followed by amino acid analysis. The ESI-MS chromatogram of the hydrolysate of the MW 1126 substance was essentially identical (Supporting Information) with that of the hydrolysate of **2**, which suggested the formation of the larger species from two subunits of **2**. Several experiments were tried to determine if the dimer had resulted from complexation with a metal ion. The LC/MS spectrum for this compound showed no other signals beyond the commonly occurring H^+, NH_4^+, Na^+, and K^+ molecular ion adducts. Treatment of the substance with 3.4 mM EDTA failed to transform the sample, suggesting that a metal ion is not a component of the dimer. Interestingly, the circular dichroism spectra of **2** and the dimer are significantly different (Supporting Information), and the compounds might therefore be expected to possess different biological activity profiles, which proved not to be the case in the HDACi screen. It is likely that both **2** and its dimer undergo reductive transformation to the disulfhydryl monomer when inside the cell, similar to the behavior reported for FK228 prodrug.[17]

An advantage of the NCI 60 human tumor cell line screen[8,9] lies in its ability to identify selective toxicity toward different tumor cell types. Both **1** and **2** exert differential cytotoxicity both within and among cell panels. In the testing of **2**, 44 of 57 (Supporting Information) dose response curves level off at the

Table 2. Comparative Analysis of the NCI 60 Data for Compounds 1 and 2

		TGI (μM)			LC_{50} (μM)		
cell panel	cell line	FK228 (**1**)	Spir C (**2**)	TGI ratio[a]	FK228 (**1**)	Spir C (**2**)	LC_{50} ratio[b]
renal	UO-31	1.55	0.32	4.9			
renal	ACHN	0.73	0.18	4.0	5.37		
breast	MDA-MB-468	0.090	0.023	3.9	4.14		
renal	RXF 393	0.062	0.016	3.8	0.30	0.17	1.8
colon	COLO 205	0.031	0.008	3.8	0.092	0.015	6.0
melanoma	UACC-257	0.095	0.033	2.9	5.46		
CNS	U251	0.031	0.012	2.7	0.13	0.037	3.6
breast	MDA-MB-231/ATCC	0.35	0.15	2.4	4.08		
renal	A498	0.028	0.012	2.4	0.20	0.055	3.6
CNS	SF-295	0.25	0.11	2.3	10.2		
non small cell lung	NCI-H322M	0.23	0.10	2.3	9.02		
renal	CAKI-1	1.89	0.88	2.1	20.9		
renal	SN12C	0.34	0.16	2.1	1.50		

[a]TGI ratio = TGI(**1**)/TGI(**2**). [b]LC_{50} ratio = LC_{50}(**1**)/LC_{50}(**2**).

dx.doi.org/10.1021/np200532d | *J. Nat. Prod.* 2011, 74, 2039–2044

higher concentrations before the LC_{50} value is reached. The means of the total growth inhibition (TGI) and LC_{50} values across all 60 cell lines were 0.33 and 3.5 μM for **1** and 0.83 and 7.7 μM for **2**, indicating greater overall cytotoxic activity of **1**. However, when the TGI values for each cell line are examined individually, **2** shows at least 2-fold greater relative potency over **1** toward 13 cell lines (Table 2). The lower LC_{50} values toward COLO 205 (0.015 μM), U251 (0.037 μM), and A498 (0.055 μM) indicate these cell lines are highly susceptible to **2** and should be targeted for further development of the compound.

Structural homology among FK228 (**1**) and the *B. thailandensis* isolates prompted further biological testing in an HDAC inhibitory screen. Against class I HDACs, both compound **2** and its dimer showed greater inhibition toward both HDAC1 and HDAC8, in comparison to **1** (Figure 2). When tested against the class II type HDAC4, **2** and its dimer exhibited greater inhibition than **1**. Downregulation of HDAC4 expression in HCT116 cells has been linked to reduced cell growth and increased apoptosis in vitro and to a reduction in growth of xenograft tissue.[18] Both **1** and **2** showed equal activity toward HCT116 cells in the NCI 60; therefore, a direct correlation between the HDACi results and the NCI 60 screening results cannot be made from the existing data. Overall, the data indicate lesser selectivity of **2** toward class I and II HDACs yet greater potency of **2** over that of the FDA-approved compound **1**.

Spiruchostatin C was assayed side by side with FK228 in an in vivo murine hollow fiber assay. Neither compound produced *T*/*C* values of less than 50% when tumors were implanted subcutaneously (Supporting Information), yet when implantation was intraperitoneal, FK228 showed superior antitumor activity overall, with lower *T*/*C* values in 23 of the 24 cell lines that were tested. No further in vivo assays were conducted on **2**, as the results suggested that the new compound would likely provide no therapeutic advantage over the currently used clinical compound.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was determined with a Perkin-Elmer 241 polarimeter. UV spectra were acquired on a Waters 2996 photodiodearray detector. CD spectra were acquired with a Jasco CD-1595 circular dichroism chiral detector. NMR spectra were recorded with a Varian 500 MHz INOVA spectrometer in $CDCl_3/CD_3OD$ with TMS as internal standard. High-resolution mass spectra were obtained with a Waters LCT Premier TOF mass spectrometer. HPLC-MS equipment consisted of a Waters 600 pump, a Waters ZQ electrospray mass spectrometer, a Waters 2996 photodiodearray spectrometer, and a Sedex 75 evaporative laser light scattering detector. C_{18} HPLC was used to fractionate crude organic extracts in the initial dereplication experiment. Scale-up purification was accomplished on Sephadex LH-20 (Pharmacia) and Microsorb C_{18} bonded-phase HPLC columns (Varian). Analytical HPLC was performed using an Xterra C_{18} HPLC column (Waters).

Organism. *Burkholderia thailandensis*, strain E264, was purchased from the American Type Culture Collection (ATCC, No. 700388) and maintained on nutrient agar at room temperature.

Fermentation. A seed culture medium consisting of glucose (10 g/L) and meat extract (20 g/L) was sterilized in 500 mL Erlenmeyer flasks at 120 °C for 15 min. Nutrient agar plugs were inoculated into each flask. The flasks were incubated for 24 h at 26.5 °C on a rotary shaker (200 rpm). The resultant seed culture was used to inoculate the production medium consisting of glucose (10 g/L), meat extract (BD Difco, #212610; 15 g/L), monopotassium phosphate (11 g/L), disodium phosphate (7.2 g/L), ammonium sulfate (1 g/L), magnesium sulfate (0.06 g/L), and DL-phenylalanine (0.5 g/L) in 1 L Erlenmeyer flasks sterilized at 120 °C for 15 min. Fermentation was carried out for 6 days at 26.5 °C, with an initial pH of 6.0. Upon harvest, the pH had increased to 7.0, and the broth appeared brown with moderate foam.

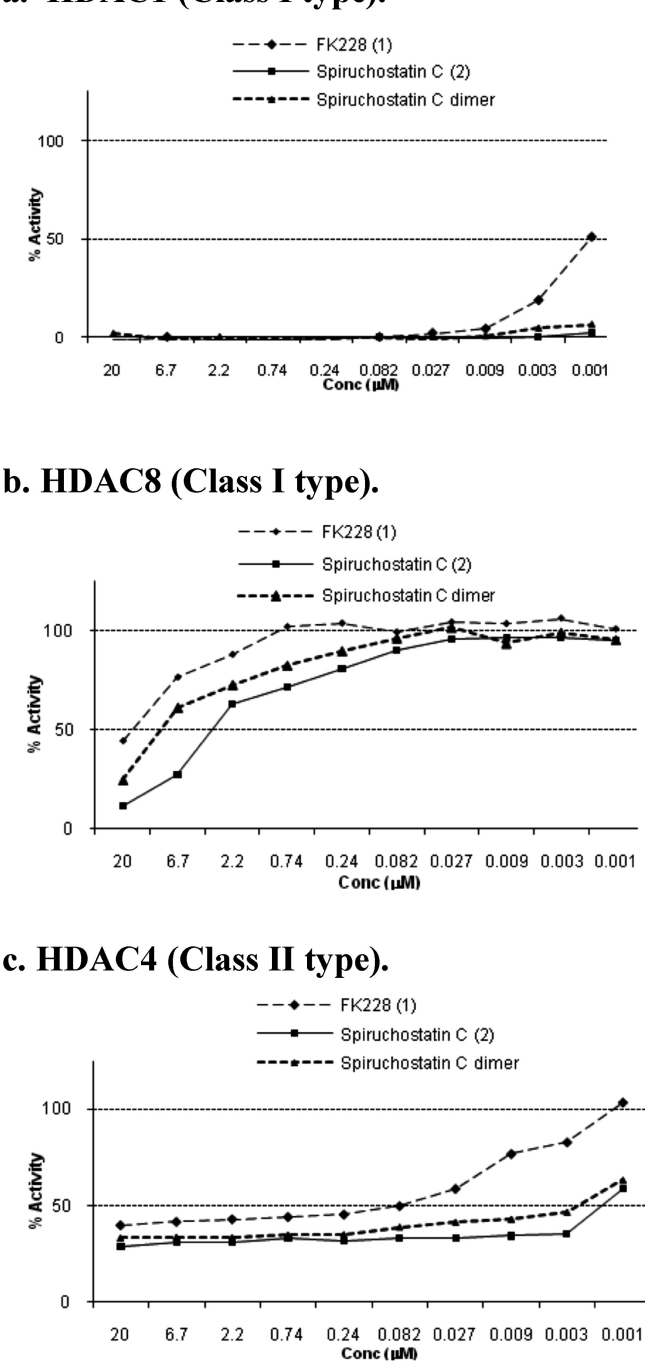


Figure 2. HDAC inhibition assay results.

Extraction. The total fermentation broth (20 L) was homogenized with an Omni Macro Homogenizer and then partitioned against an equal volume of EtOAc. Centrifugation at 5000 rpm facilitated the separation of the aqueous and organic layers. The EtOAc was removed by rotary evaporation and dried, resulting in 0.91 g of crude extract.

HPLC Dereplication. The EtOAc extract was subjected to dereplication analysis by the preparative C_{18} LC/MS method described in previous publications,[6,7] with 84 HPLC fractions tested in the NCI 60. Multiple zones of toxicity were identified within the chromatographic space.

dx.doi.org/10.1021/np200532d | *J. Nat. Prod.* 2011, 74, 2039–2044

Isolation of the Bioactive Compounds. The EtOAc extract was partitioned between 1 L hexane and 1 L MeOH−H_2O (9:1). After removal of the hexane-soluble material (89 mg), H_2O was added to the methanolic layer, bringing the amount of H_2O to 66%. This solution was then partitioned against methyl *tert*-butyl ether (MeOtBE), yielding 300 mg of MeOH (aq) solubles and 452 mg of MeOtBE solubles. The methanolic-soluble material was loaded onto a 100 cm × 2.5 cm bed of Sephadex LH-20 and eluted over 13 h with 1.5 mL/min MeOH. The fraction enriched in **2** was then purified on a C_8 semipreparative HPLC column eluted with a MeCN−H_2O gradient: 0−5 min (3:7), 45−60 min (1:1), flow rate 10 mL/min.

Sulfhydryl Detection with Ellman's Reagent. A 4 mg/mL stock solution of Ellman's reagent (Pierce) was made in a reaction buffer containing 0.1 M Na_3PO_4 (aq) pH 8 and 1 mM EDTA. A positive control solution containing 0.1 mM DL-cysteine turned bright yellow upon addition of Ellman's stock solution. Compound **2** was assayed both prior to and after reduction with zinc dust.[19] To reduce the disulfide bridge, 10 μg of **2** was dissolved in 0.2 mL of MeCN−H_2O (1:1) containing 1% trifluoroacetic acid. To this solution was added 60 mg of zinc dust and the mixture incubated at room temperature for 10 min, after which the supernatant liquid was added to Ellman's reagent.

Oxidation, Hydrolysis, and Amino Acid Analysis. Oxidation of samples was required prior to acid hydrolysis in order to cleave the disulfide bridge of **2**. A 0.2 mg portion of each sample was treated with a formic acid−H_2O_2 mixture. A 0.1 mg aliquot of each oxidized compound was transferred in MeOH to a hydrolysis tube, with removal of the solvent under a stream of nitrogen gas. Each sample was dissolved in 0.5 mL of 6 N HCl (aq), degassed, and sealed under vacuum. The samples were then incubated at 110 °C in an oil bath for 18 h, after which time solvent was blown off under a stream of nitrogen gas. The samples were redissolved in MeOH, transferred from the hydrolysis tube to a tared 8 mL vial, and dried again under nitrogen to aid in the evaporation of residual HCl. Each hydrolysate was dissolved in MeOH @ 0.5 mg/mL and analyzed by HILIC HPLC with a MeCN−H_2O−0.1% GAA mobile phase gradient: 0−5 min (4:1), 30 min (1:1), 0.7 mL/min. Identification of amino acids in the hydrolysates was determined by comparison of retention time and mass spectra to those of a standard amino acid mixture (Sigma #9416).

Determination of Configuration by Marfey's Analysis. The hydrolysate of **2** and the amino acid standards L-Met, D/L-Met, L-Met sulfoxide, D/L-Met sulfoxide, L-Cys, D/L-Cys, L-cysteic acid, and D/L-cysteic acid were dissolved in MeOH−H_2O (4:1, @ 1 mg/mL) and transferred (25 μL) into separate HPLC autosampler vials. All traces of solvent were removed under vacuum. To each sample was added 15 μL of 6% TEA and 7.5 of μL FDAA (Marfey's reagent, Pierce). The samples were heated to 40 °C for 1 h and then diluted 10-fold with MeCN−H_2O (1:1) prior to C_{18} HPLC analysis with CH_3CN/5% aqueous HOAc (1:9 to 1:1). During acid hydrolysis cysteine is converted to cysteic acid, whose FDAA derivative is poorly retained during C_{18} analysis. When FDVA (advanced Marfey's reagent, Pierce) was used to derivatize the hydrolysis mixture and amino acid standards, retention and identification became possible. Experimental conditions and solvents were identical with those used in the FDAA procedure.

*Spiruchostatin C (NSC 754830) (**2**):* white powder; UV (MeOH) end absorbance; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 203 (−86), 211 (−73), 241 (+11) nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 564.1875 $[M + H]^+$ (calcd for $C_{23}H_{38}N_3O_7S_3$ 564.1872).

NCI 60 Human Tumor Cell Line Screen. Details of the NCI 60 have previously been described.[8,9]

HDAC Inhibition Screen. Cell-free human histone deacetylase inhibition assays were performed by Reaction Biology, Inc. http://www.reactionbiology.com/. Human HDAC1 (GenBank Accession No. NM_004964), Human HDAC4 (GenBank Accession No. NM_006037), and Human HDAC8 (GenBank Accession No. NM018486) were employed in these assays. Fluorogenic peptides derived from p53 residues 379−382 were used as substrates for assaying HDAC inhibition. Specifically, RHKKAc was used for assaying HDAC1 and 8 inhibition and RHKAcKAc for HDAC4. Compounds **1** (American Custom Chemicals Corp.) and **2** were tested at 10 doses in duplicate with 3-fold serial dilution starting at 20 μM. The assay was conducted in two steps. First, the fluorogenic substrate with acetylated lysine side chain was incubated with compound **1** or **2** to produce deacetylated substrate. The reaction mixture was then digested by a proprietary developing mixture to produce the fluorescent signal measured at 360 nm excitation and 460 nm emission wavelengths, which is proportional to the amount of deacetylated substrate.

Murine Hollow Fiber Assays. The in vivo cultivation of tumor cells in hollow fibers has previously been described.[20,21] In brief, a standard panel of 12 human tumor cell lines is encapsulated within polyvinylidene fluoride hollow fibers having a molecular weight exclusion of 500 000 Da. A total of 3 different tumor lines are prepared for each experiment so that each mouse receives 3 intraperitoneal implants (1 of each tumor line) and 3 subcutaneous implants (1 of each tumor line). On the day of implantation, samples of each tumor cell line preparation are quantitated for viable cell number by a stable end point MTT assay so that the time zero cell mass is known. Drugs were administered IP at both 2.4 mg/kg/dose and at 1.6 mg/kg/dose on days 1−4. These doses were selected on the basis of previous studies with FK228, which exhibited antitumor activity at these doses. The fibers were retrieved and viable cell mass determined on day 5. *T*/*C* values were calculated against control animals that received injections of vehicle only.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures and a table giving a fractionation tree diagram, 1-D and 2-D NMR spectra of **2**, and in vitro and in vivo test results for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: (301) 228-4780. Fax: (301) 228-4361. E-mail: klausmeyerp@mail.nih.gov.

■ ACKNOWLEDGMENTS

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government. This research was supported (in part) by the Developmental Therapeutics Program in the Division of Cancer Treatment and Diagnosis of the National Cancer Institute. We gratefully acknowledge Q. Van for NMR experiments, the NCI 60 team for in vitro biological assay results, M. Hollingshead, M. Ahalt-Gottholm, and V. Brinsfield for the murine hollow fiber assay results, and J. Britt for robotics and computer support.

■ REFERENCES

(1) Bieliauskas, A. V.; Pflum, M. K. H. *Chem. Soc. Rev.* **2008**, *37*, 1402−1413.
(2) Yoshida, M.; Furumai, R.; Nshiyama, M.; Komatsu, Y.; Nishino, N.; Horinouchi, S. *Cancer Chemother. Pharmacol.* **2001**, *48*, S20−S26.
(3) Minucci, S.; Pelicci, P. G. *Nat. Rev. Cancer* **2006**, *6*, 38−51.
(4) VanderMolen, K. M.; McCulloch, W.; Pearce, C. J.; Oberlies, N. H. *J. Antibiot.* **2011**, *64*, 525−531.
(5) Wang, C.; Henkes, L. M.; Doughty, L. B.; He, M.; Wang, D.; Meyer-Almes, F.-J.; Cheng, Y.-Q. *J. Nat. Prod.* **2011**, DOI:10.1021/np200324x.

(6) Klausmeyer, P.; McCloud, T. G.; Scudiero, D. A.; Melillo, G.; Cardellina, J. H. II; Shoemaker, R. H. *Planta Med.* **2007**, *73*, 49−52.
(7) Klausmeyer, P.; McCloud, T. G.; Uranchimeg, B.; Melillo, G.; Scudiero, D. A.; Cardellina, J. H. II; Shoemaker, R. H. *Planta Med.* **2008**, *74*, 258−263.
(8) Scudiero, D. A.; Shoemaker, R. H.; Paul, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827−4833.
(9) Shoemaker, R. H. *Nat. Rev. Cancer* **2006**, *6*, 813−823.
(10) Moon, S.-S.; Kang, P. M.; Park, K. S.; Kim, C. H. *Phytochemistry* **1996**, *42*, 365−368.
(11) Kamigiri, K.; Tokunaga, T.; Shibazaki, M.; Setiawan, B.; Rantiatmodjo, R. M.; Morioka, M.; Suzuki, K. *J. Antibiot.* **1996**, *49*, 823−825.
(12) Takahashi, K. *J. Biochem.* **1973**, *74*, 1083−1089.
(13) Shigematsu, N.; Ueda, H.; Takase, S.; Tanaka, H. *J. Antibiot.* **1994**, *47*, 311−314.
(14) Szabó, S.; Szókán, G.; Khlafulla, A. M.; Almás, M.; Kiss, C.; Rill, A.; Schön, I. *J. Pept. Sci.* **2001**, *7*, 316−322.
(15) Masuokaa, Y.; Nagaia, A.; Shin-yaa, K.; Furihatab, K.; Nagaic, K.; Suzukic, K.; Hayakawaa, Y.; Seto, H. *Tetrahedron. Lett.* **2001**, *42*, 41−44.
(16) Narita, K.; Kikuchi, T.; Watanabe, K.; Takizawa, T.; Oguchi, T.; Kudo, K.; Matsuhara, K.; Abe, H.; Yamori, T.; Yoshida, M.; Katoh, T. *Chem. Eur. J.* **2009**, *15*, 11174−11186.
(17) Furumai, R.; Matsuyama, A.; Kobashi, N; Lee, K.-H.; Nishiyama, M.; Nakajima, H.; Tanaka, A.; Komatsu, Y.; Nishino, N.; Yoshida, M.; Horinouchi, S. *Cancer Res.* **2002**, *62*, 4916−4921.
(18) Wilson, A. J.; Byun, D. -S.; Nasser, S.; Murray, L. B.; Ayyanar, K.; Arango, D.; Figueroa, M.; Melnick, A.; Kao, G. D.; Augenlicht, L. H.; Mariadason, J. M. *MBoC* **2008**, *19*, 4062−4075.
(19) Erlandsson, M.; Hallbrink, M. *Int. J. Pept. Res. Ther.* **2005**, *11*, 261−265.
(20) Hollingshead, M. G.; Alley, M. C.; Camalier, R. F.; Abbott, B. J.; Mayo, J. G.; Malspeis, L.; Grever, M. R. *Life Sci.* **1995**, *57*, 131−141.
(21) http://dtp.nci.nih.gov/branches/btb/hfa.html.