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Aniline-Tetramic Acids from the Deep-Sea-Derived Fungus *Cladosporium sphaerospermum* L3P3 Cultured with the HDAC Inhibitor SAHA

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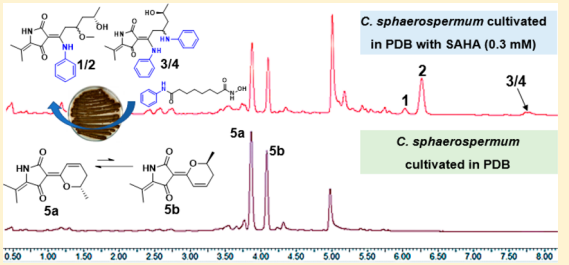
Supporting Information

ABSTRACT: Four new tetramic acids, cladosins H−K (**1**−**4**), and a related known compound, cladodionen (**5**), were isolated from the culture of the Mariana Trench (depth 6562 m) sediment-derived fungus *Cladosporium sphaerospermum* L3P3 treated with the histone deacetylase inhibitor SAHA (suberanilohydroxamic acid). Interestingly, compounds **1**−**5** existed as equilibrium *E*/*Z* mixtures and **1**−**4** were the first cases of tetramic acids containing aniline moieties. Their structures including absolute configurations were elucidated through a combination of NMR, MS, and Mosher's method, together with the consideration of biogenetic origins. Incubation experiments of exogenous aniline and *N*-phenyloctanamide revealed that the aniline moiety in cladosins H−K (**1**−**4**) is probably derived from the degradation of SAHA, indicating that the well-known histone deacetylase inhibitor SAHA could be metabolized by L3P3 and provide aniline as a precursor for biotransformation of chemically reactive polyketides. The cytotoxicity of **1**−**5** was evaluated against the PC-3, MGC-803, SH-SY5Y, HCT-116, K562, and HL-60 cell lines, and compound **2** showed promising cytotoxicity against the HL-60 cell line with an IC_{50} value of 2.8 μM.



Genomic analyses of fungi have revealed that many secondary metabolite biosynthetic gene clusters are transcriptionally suppressed under standard laboratory cultivation conditions.[1] The activation of these gene clusters to generate new structures for drug leads has attracted extensive attention.[2] Several strategies have been developed to better harness the biosynthetic repertoire of fungi, including epigenetic modifications.[3] One of the most popular epigenetic modifications is to inhibit histone deacetylase (HDAC) by adding small molecular inhibitors, such as suberanilohydroxamic acid (SAHA, commercial name vorinostat).[4] However, in addition to the expected activations of gene clusters encoding secondary metabolites,[5] HDAC inhibitors may also be directly modified by the target microorganisms to new chemical entities via biotransformation.[6] Interestingly, the degraded/metabolized products of exogenous HDAC inhibitors can then further contribute to the molecular output of novel host systems.

During our efforts to tap the potential of marine-derived fungi for producing new bioactive molecules, a SAHA-based epigenetic modification strategy was applied for screening fungal strains.[7] Among them, the fungus *Cladosporium sphaerospermum* L3P3, which was isolated from the sediment collected from the Mariana Trench (depth 6562 m), was selected for its special metabolic profile enhanced by culturing with the SAHA modifier. Chemical investigation of fermentation extracts led to the discovery of four new aniline-tetramic acid adducts, cladosins H−K (**1**−**4**), and a related known compound, cladodionen (**5**). The aniline moiety in compounds **1**−**4** was inferred to be derived from biotransformation of SAHA by strain L3P3. Interestingly, all of the compounds **1**−**5** existed as two tautomeric forms differing in the configurations of the enamine, which was similar to those of cladosins A (**6**)−D (Figure S1, Supporting Information), a group of tautomeric structures from our previous study.[8] Herein, we report the details of the isolation, structure elucidation, and biological activities of these new compounds.

RESULTS AND DISCUSSION

The fungus *C. sphaerospermum* L3P3 was cultured in potato-based marine medium with 300 μM SAHA at 28 °C and agitated at 180 rpm for 6 days (40 L). The EtOAc extract (5.0 g) of the fermentation broth was separated by MPLC and semipreparative HPLC, which led to the isolation of

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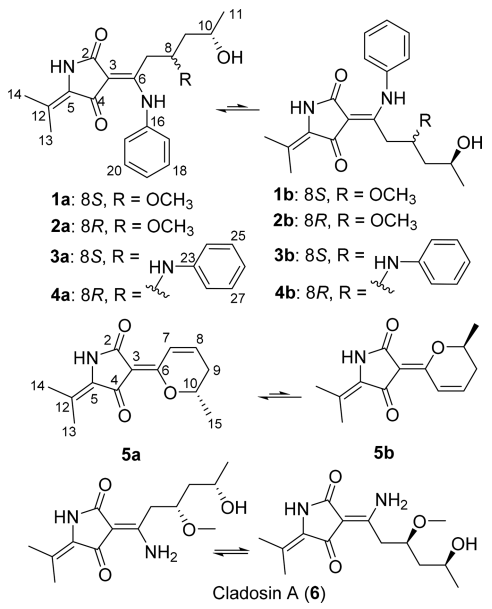

compounds **1** (4.0 mg), **2** (9.0 mg), **3** (5.0 mg), and **4** (5.0 mg), as well as **5** (50.0 mg).

Both cladosins H (**1**) and I (**2**) were obtained as an inseparable mixture of isomers, respectively, with the ratio 5:3 (**1a**:**1b** and **2a**:**2b**) deduced from the ^{1}H spectra, similar to those of cladosins A−D.[8] Compounds **1** and **2** have the same molecular formula of $C_{20}H_{26}N_2O_4$ according to the protonated molecule HRESIMS peaks at *m*/*z* 359.1967 and 359.1966, indicating nine degrees of unsaturation. The 1D NMR data (Table 1) of **1** and **2** were highly similar. Each of them suggested the presence of four methyls with one methoxy group, two methylenes, five sp^2 methines, two sp^3 methines, and seven nonprotonated carbons including an amide carbonyl and an α/β-unsaturated ketone carbon. Detailed analysis of the NMR data of **1** and **2** revealed **1** and **2** shared the same planar structure, which contains one extra phenyl group compared with the known compound cladosin A (**6**).[8] Key COSY correlations from H-17 through H-21 together with HMBC correlations from H-15 (NH) to C-3, C-7, C-17, and C-21 further proved the presence of a phenyl group and attached it to the 15-N atom (Figure 1). Similar to cladosin A,[8] the

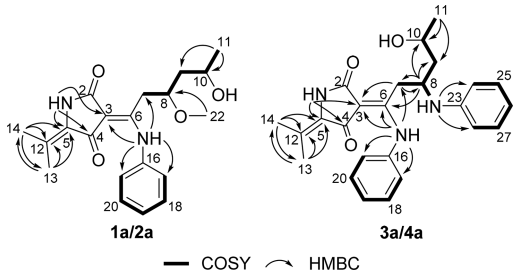

Figure 1. Selected 2D NMR correlations of **1a**−**4a**.

configurations of the $\Delta^{3(6)}$ in **1a**/**2a** (major) and **1b**/**2b** (minor) were assigned as exoform A ($\Delta^{3(6)}$: *E*) and exoform B ($\Delta^{3(6)}$: *Z*) based on the NMR evidence, in which the hydrogen-bonded carbonyl downfield shifted by 3−4 ppm, when compared to that of the corresponding free carbonyl.[9] The relative configurations of the 1,3-diol moiety of cladosins were indicated by the characteristic hydrogen signals of methylene protons (CH_2 between the 1,3-diol), which were overlapped in *anti*-1,3-diol while appeared as two sets of multiplets in the *syn*-1,3-diol system.[8,10,11] In compounds **1** and **2**, the difference between them was revealed by the minor

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data for 1 and 2 in DMSO-d_6

	1a		1b		2a		2b	
no.	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
NH-1		9.29, s		9.66, s		9.36, s		9.66, s
2	167.7, C		171.5, C		168.0, C		171.5, C	
3	99.2, C		97.6, C		98.9, C		97.5, C	
4	186.6, C		182.9, C		186.5, C		183.1, C	
5	119.8, C		120.0, C		120.3, C		120.3, C	
6	167.7, C		167.5, C		168.2, C		168.0, C	
7	32.0, CH_2	3.11−3.20, m	32.5, CH_2	3.11−3.20, m	32.1, CH_2	3.05−3.09, m	32.5, CH_2	3.06−3.10, m)
		3.26−3.30, m		3.26−3.30, m		3.35, dd (5.4, 12.8)		3.29, dd (5.4, 12.8
8	77.3, CH	3.52−3.61, m	77.1, CH	3.52−3.61, m	78.0, CH	3.46−3.49, m	77.6, CH	3.46−3.49, m
9	44.4. CH_2	1.20−1.36, m	44.5, CH_2	1.20−1.36, m	43.9, CH_2	1.25−1.30, m	43.9, CH_2	1.25−1.30, m
						1.54−1.58, m		1.54−1.58, m
10	62.8, CH	3.52−3.61, m	62.8, CH	3.52−3.61, m	63.5, CH	3.64−3.67, m	63.5, CH	3.64−3.67, m
11	24.7, CH_3	0.94−0.97, m	24.8, CH_3	0.94−0.97, m	23.5, CH_3	0.93−0.95, m	23.6, CH_3	0.93−0.95, m
12	129.8, C		130.5, C		129.7, C		130.5, C	
13	21.4, CH_3	1.80, s	21.4, CH_3	1.78, s	21.4, CH_3	1.80, s	21.4, CH_3	1.78, s
14	18.5, CH_3	2.19, s	18.4, CH_3	2.17, s	18.6, CH_3	2.19, s	18.5, CH_3	2.16, s
NH-15		12.84, s		12.17, s		12.89, s		12.22, s
16	136.9, C		136.9, C		136.8, C		136.8, C	
17	126.6, CH	7.34−7.42, m	126.5, CH	7.34−7.42, m	126.4, CH	7.40−7.48, m	126.3, CH	7.40−7.48, m
18	129.8, CH	7.45−7.49, m	129.8, CH	7.45−7.49, m	129.8, CH	7.40−7.48, m	129.8, CH	7.40−7.48, m
19	127.9, CH	7.34−7.42, m	127.8, CH	7.34−7.42, m	127.9, CH	7.34−7.37, m	127.8, CH	7.34−7.37, m
20	129.8, CH	7.45−7.49, m	129.8, CH	7.45−7.49, m	129.8, CH	7.40−7.48, m	129.8, CH	7.40−7.48, m
21	126.6, CH	7.34−7.42, m	126.5, CH	7.34−7.42, m	126.4, CH	7.40−7.48, m	126.3, CH	7.40−7.48, m
22	57.1, CH_3	3.00, s	57.3, CH_3	3.08, s	56.8, CH_3	3.00, s	56.8, CH_3	3.02, s

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discrepancies in the ^{1}H NMR data (Table 1), where the chemical shifts for the H_2-9 protons were overlapped at 1.20−1.36 ppm in **1**, while in **2** they appeared as two sets of multiplets at 1.25−1.30 and 1.54−1.58 ppm, which indicated an *anti*-1,3-diol unit in **1** and a *syn*-1,3-diol system in **2**.[8,10,11] Thus, the relative configurations of compounds **1** and **2** were determined as 8*S**, 10*S** and 8*R**, 10*S**, respectively. The absolute configurations of C-10 were both determined as 10*S* by interpretation of the ^{1}H NMR chemical shift differences ($\Delta\delta^{R-S}$) between their MPA esters (Figure 2). Accordingly, the absolute configurations of **1** and **2** were determined as 8*S*, 10*S* and 8*R*, 10*S*, respectively.

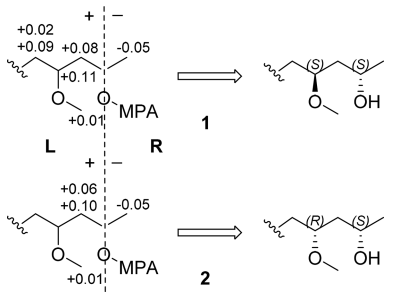

Figure 2. $\Delta\delta^{R-S}$ values for the MPA ester derivatives of **1** and **2**.

Cladosins J (**3**) and K (**4**) were both obtained as inseparable mixtures, respectively, with the ratio of 5:3. They showed the same molecular formula of $C_{25}H_{29}N_3O_3$ based on the protonated molecule HRESIMS peaks at *m*/*z* 420.2273 and 420.2275. The highly similar 1D NMR data (Table 2) of **3** and **4** indicated the same planar structure. Comparison of the ^{1}H and ^{13}C NMR spectra of compounds **1**−**4** revealed that the 8-OCH_3 in **1** and **2** was replaced by an aniline moiety in compounds **3** and **4**, which was further supported by the COSY correlations of H-7/H-8/H-9, H-8/H-22 (NH), and from H-24 to H-28, together with the HMBC correlations from H-22 (NH) to C-24 and C-28 (Figure 1). The configurations of the $\Delta^{3(6)}$ in **3a**/**4a** (major) and **3b**/**4b** (minor) were also assigned as the exoform A ($\Delta^{3(6)}$: *E*) and exoform B ($\Delta^{3(6)}$: *Z*), respectively, according to the chemical shifts of C-2 and C-4.[9] The relative configurations of compounds **3** and **4** were suggested as 8*S**, 10*S** and 8*R**, 10*S**, based on the NMR signals of the H_2-9 protons (**3**: δ_H overlapped at 1.39−1.51 ppm; **4**: two sets of multiplets at 1.38−1.41 and 1.63−1.68 ppm, respectively).[8,10,11] Considering that they share the same biogenetic origin as **1** and **2**, the absolute configurations of compounds **3** and **4** were suggested to be 8*S*, 10*S* and 8*R*, 10*S*, respectively.

The rare aniline-containing structures of **1**−**4** prompted our interest in investigating their routes of formation. As we compared the extracts from culture media with and without SAHA, two additional peaks (**5a** and **5b**) with similar UV

Table 2. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data for 3 and 4 in DMSO-d_6

	3a		3b		4a		4b	
no.	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
NH-1		9.37, s		9.64, s		9.43, s		9.66, s
2	168.2, C		171.5, C		168.2, C		171.5, C	
3	99.9, C		98.3, C		99.8, C		98.4, C	
4	186.5, C		183.4, C		186.4, C		183.5, C	
5	119.8, C		118.9, C		120.1, C		118.9, C	
6	168.3, C		168.1, C		168.3, C		168.2, C	
7	32.0, CH_2	3.06−3.13, m	32.0, CH_2	3.06−3.13, m	32.4, CH_2	3.04−3.12, m	32.4, CH_2	3.04−3.12, m
		3.41−3.47, m		3.41−3.47, m		3.44−3.52, m		3.44−3.52, m
8	48.5, CH	3.58−3.90, m	48.6, CH	3.58−3.90, m	49.0, CH	3.45, m	48.8, CH	3.45, m
9	43.3, CH_2	1.39−1.51, m	43.0, CH_2	1.39−1.51, m	43.3, CH_2	1.38−1.41, m	43.0, CH_2	1.38−1.41, m
						1.63−1.68, m		1.63−1.68, m
10	62.9, CH	3.55−3.78, m	62.9, CH	3.55−3.78, m	63.9, CH	3.60, m	63.9, CH	3.60, m
11	25.0, CH_3	0.94−0.96, m	25.0, CH_3	0.94−0.96, m	23.4, CH_3	0.86−0.88, m	23.3, CH_3	0.86−0.88, m
12	130.1, C		129.7, C		129.6, C		130.5, C	
13	21.4, CH_3	1.81, s	21.4, CH_3	1.79, s	21.4, CH_3	1.80, s	21.4, CH_3	1.80, s
14	18.6, CH_3	2.19, s	18.4, CH_3	2.20, s	18.6, CH_3	2.19, s	18.5, CH_3	2.20, s
NH-15		12.74, s		12.04, s		12.78, s		12.08, s
16	136.8, C		136.8, C		136.8, C		136.8, C	
17	127.4, CH	7.37−7.41, m	127.4, CH	7.37−7.41, m	127.1, CH	7.37−7.49, m	127.1, CH	7.37−7.49, m
18	130.1, CH	7.50−7.54, m	130.1, CH	7.50−7.54, m	130.2, CH	7.52−7.54, m	130.2, CH	7.52−7.54, m
19	128.3, CH	7.50−7.54, m	128.2, CH	7.50−7.54, m	128.3, CH	7.46−7.49, m	128.2, CH	7.46−7.49, m
20	130.1, CH	7.50−7.54, m	130.1, CH	7.50−7.54, m	130.2, CH	7.52−7.54, m	130.2, CH	7.52−7.54, m
21	127.4, CH	7.37−7.41, m	127.4, CH	7.37−7.41, m	127.1, CH	7.37−7.49, m	127.1, CH	7.37−7.49, m
NH-22		5.35, d (8.0)		5.29, d (8.0)		5.42, d (8.0)		5.38, d (8.0)
23	148.2, C		148.2, C		147.9, C		147.9, C	
24	112.3, CH	5.98, d (7.9)	112.3, CH	5.98, d (7.9)	112.3, CH	6.04, d (7.9)	112.3, CH	6.00, d (7.9)
25	129.1, CH	6.82−7.00, m	129.1, CH	6.82−7.00, m	129.2, CH	6.84−6.88, m	129.2, CH	6.84−6.88, m
26	116.0, CH	6.37−6.46, m	115.6, CH	6.37−6.46, m	115.9, CH	6.40−6.43, m	114.3, CH	6.40−6.43, m
27	129.1, CH	6.82−7.00, m	129.1, CH	6.82−7.00, m	129.2, CH	6.84−6.88, m	129.2, CH	6.84−6.88, m
28	112.3, CH	6.04, d (7.9)	112.3, CH	6.00, d (7.9)	112.3, CH	6.04, d (7.9)	112.3, CH	6.00, d (7.9)

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absorption to those of **1**−**4** were observed (Figure 3), suggesting compound **5** could be a biogenetically related

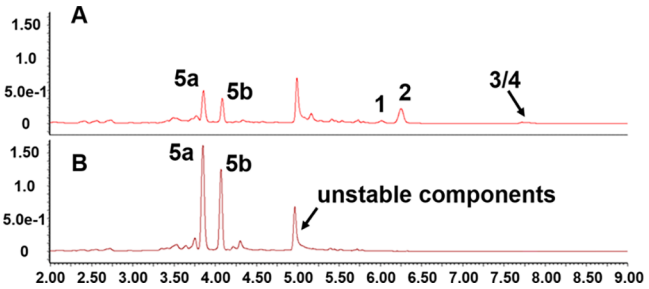

Figure 3. UPLC profile of extracts detected by UV absorption at 280 nm from *Cladosporium sphaerospermum* cultivated in PDB with SAHA (A; 300 μM) and without (B) SAHA.

derivative. The NMR data and the specific rotation of compound **5** were identical to those for the recently reported cladodionen.[12]

Further comparison of the structures between compounds **1**−**4** and **5** revealed their core skeletons were all assembled from a C8 polyketide unit and a valine residue,[8] while the major difference among them was the number and position of anilines attached to the core structures. Interestingly, in compound **5**, instead of aniline added to the C8 chain, a methyl-substituted pyran ring was formed via an intramolecular reaction. On the basis of those findings, we proposed compounds **1**−**4** and **5** were derived from the same biogenetic process, where SAHA contributed as a source of aniline, which was captured and incorporated into synthesizing compounds **1**−**4**.

To better understand the SAHA impact upon formation of the aniline-containing structures, *N*-phenyloctanamide (*N*-POA), a SAHA analogue, was chemically synthesized and added into the culture of *C. sphaerospermum*, and compounds **1**−**4** were observed by UPLC-MS (Figure 4, Figure S42-1, Supporting Information). The result indicated that the roles of SAHA and *N*-phenyloctanamide were probably to provide free aniline precursor from molecular degradation, which was

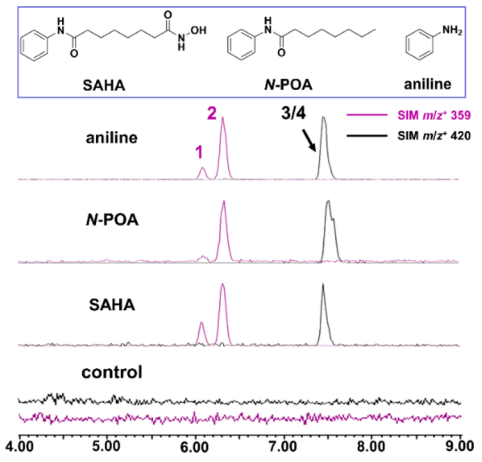

Figure 4. UPLC-MS analysis (protonated molecule peak at m/z 359 of **1**/**2** and m/z 420 of **3**/**4**) of extracts of *C. sphaerospermum* cultivated in PDB medium with *N*-POA (300 μM), with SAHA (300 μM), with aniline (300 μM), and without SAHA/aniline/*N*-phenyloctanamide (*N*-POA) (control).

required by the production of new compounds **1**−**4**. This assumption was further validated by incubation experiments with free aniline added to the culture of *C. sphaerospermum*, during which compounds **1**−**4** were also observed by UPLC-MS (Figure 4). Considering that aromatic amines can act as nucleophiles,[13] we speculated that the aniline-containing tetramic acid adducts formed from a nonenzymatic process. For this, SAHA, *N*-POA, and aniline were added to the medium containing inactivated enzymes from *C. sphaerospermum*. The results showed that compounds **3** and **4** together with a proposed intermediate **A** (Figure 6) were tentatively

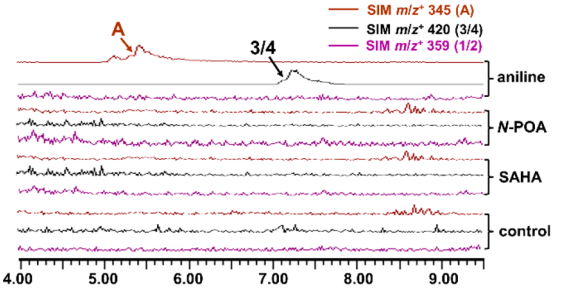

Figure 5. UPLC-MS analysis (28 °C for 24 h detected by UPLC-MS (protonated molecule peak at m/z 359 of **1**/**2**, m/z 345 of intermediate **A**, m/z 420 of **3**/**4**)) of fifth day fermentation broth extracts from *C. sphaerospermum* (enzym-deactivated by filtration and heat treatment) incubated with aniline (300 μM), with *N*-phenyloctanamide (300 μM), with SAHA (300 μM), and without SAHA/aniline/*N*-phenyloctanamide (*N*-POA) (control).

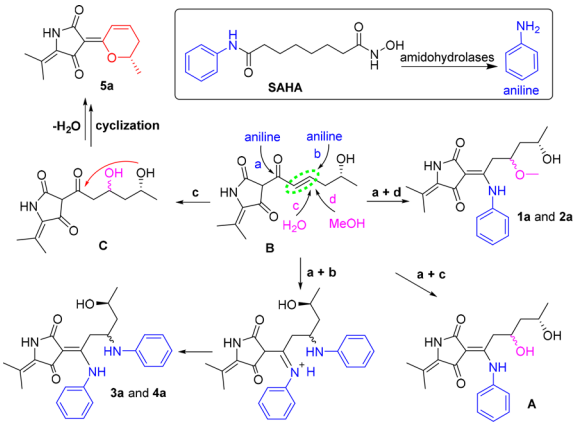

Figure 6. Plausible formation process of compounds **1**−**5**.

detected by UPLC-MS in aniline-added medium (Figure 5, Figure S42-2, Supporting Information), indicating aniline modifications took place via nonenzymatic events. In comparison, the same medium with inactivated enzymes enriched with SAHA or *N*-POA failed to provide any aniline-containing adducts, which indicated the conversion process of SAHA and SAHA analogues is likely an enzymatic process. Furthermore, when SAHA was incubated in the cell-free fungal broth obtained by filter sterilization, neither aniline nor compounds **1**−**4** could be detected, which indicated that the hydrolysis of SAHA was carried out inside the fungal cells (Figure S42-3, Supporting Information). This result was consistent with a previous study that proposed that an enzyme (amidohydrolases)-catalyzed hydrolysis should take place on SAHA analogues to release free aniline.[6] Based on the above experiments, a plausible biotransformation process of **1**−**5** was

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proposed. Compounds **1**−**5** were proposed to be generated from the key intermediate **B**, and the different epimers at C-8 were proposed to be generated by the nonstereoselective Michael-type addition of different nucleophiles (aniline and methanol) under weak acidic conditions (Figure 6).

The cytotoxicities (Table 3) of **1**−**5** were evaluated against the PC-3, MGC-803, SH-SY5Y, and HCT-116 cell lines using the sulforhodamine B (SRB) method,[14] and the K562 and HL-60 cell lines were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[15] Compounds **2**−**4** showed cytotoxicity at various levels against the K562 and HL-60 cell lines, while compound **1** was inactive (IC_{50} > 10 μM). Compound **1**, with the 8*S* configuration, showed less activity than compound **2**, with the 8*R* configuration, indicating the absolute configuration of C-8 was important for cytotoxicity. Meanwhile, by comparing activities of compounds **2**−**4** to the noncytotoxic analogues cladosins A−D,[8] we also discovered the aniline moiety was essential for enhancing cytotoxicity.

Table 3. Cytotoxicities of Compounds 1−5 against Six Cancer Cell Lines (IC_{50} μM)

compound	K562	HL-60	HCT-116	PC-3	SH-SY5Y	MGC-803
1	>30	>30	>30	>30	>30	>30
2	4.1	2.8	11	13	12	19
3	6.8	7.8	>30	>30	>30	>30
4	5.9	7.5	14	18	>30	>30
5	4.5	6.6	12	11	15	22
ADM[a]	0.3	0.2	0.2	1.0	0.1	0.2

[a]ADM = doxorubicin (positive control).

Cladosins represent a rare subclass of naturally occurring tetramic acid congeners and are only found in fungi.[8,11,12] Given the chemically active intermediates (such as intermediate **B**) probably appeared in the biosynthetic process (Figure 6), this group of compounds may be promising intermediates to generate diversified compounds by nonenzymatic conversions. In this paper, we successfully obtained four aniline-cladosin adducts named cladosins H−K (**1**−**4**) from the deep-sea-derived fungus *C. sphaerospermum* cultured with the HDAC inhibitor SAHA. On the basis of further incubation experiments with *N*-phenyloctanamide (SAHA analogue) and aniline, we inferred the formation of those adducts involved both enzymatic degradation of SAHA and nonenzymatic steps. Overlapping in time frame with the work presented here, Adpressa and co-workers also reported a similar phenomenon observed with the endophytic ascomycete *Chalara* sp. 6661, in which aniline derived from SAHA was embedded into a xanthone containing a reactive aldehyde intermediate.[6] The biotransformation of SAHA by different fungal genera (at least *Chalara* and *Cladosporium*) suggests maybe a common catabolism/degradation reaction takes place in response to these foreign aniline-containing chemical intruders for self-defense or biosynthetic purposes.[6,16] Compared with the noncytotoxic cladosin counterparts, most of the aniline-containing compounds showed promising cytotoxicity, which suggests conversions of intermediates in fungi could be an economic and efficient method for generating pharmaceutically meaningful structures.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Waters 2487, while the electronic circular dichroism (ECD) spectrum was measured on a JASCO J-815 spectropolarimeter. ^{1}H NMR, ^{13}C NMR, DEPT, and 2D NMR spectra were recorded on an Agilent 500 MHz DD2 spectrometer. HRESIMS and ESIMS data were obtained using a Thermo Scientific LTQ Orbitrap XL mass spectrometer. MPLC was performed using a C_{18} column (Agela Technologies, YMC-Pack ODS-A, 3 × 40 cm, 5 μm, 20 mL/min). Preparative HPLC collection used a C_{18} column (Waters, YMC-Pack ODS-A, 10 × 250 mm, 5 μm, 3 mL/min). LC-MS was perfomed using an Acquity UPLC H-Class coupled to a SQ Detector 2 mass spectrometer (Waters) using a BEH C_{18} column (1.7 μm, 2.1 × 50 mm).

Fungal Material. The fungal strain was isolated from a marine sediment sample collected from the Mariana Trench (depth 6562 m, 10°57.722′ N/142°19.617′ E) and identified as *Cladosporium sphaerospermum* based on sequencing of the ITS region (GenBank no. MF418012) with 100% similarity to *C. sphaerospermum*. The strain was deposited at the Key Laboratory of Marine Drugs, the Ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao, People's Republic of China.

Fermentation and Extraction. Erlenmeyer flasks (500 mL) each containing 150 mL of fermentation media were directly inoculated with spores. The media contained potato (200 g) and glucose (20 g) dissolved in 1 L of naturally collected seawater (Huiquan Bay, Yellow Sea) in the presence of 0.3 mM SAHA (as indicated). The 270 flasks were cultured at 28 °C on a rotary platform shaker at 180 rpm for 6 days. The whole fermentation broth (40 L) was filtered through cheese cloth to separate the supernatant from the mycelia. The supernatant was extracted with EtOAc (3 × 30 L) and evaporated under reduced pressure to give a gum (5.0 g).

Purification. The extract was separated by MPLC (MeOH−H_2O, 40% to 100%, 35 min) to give nine fractions (fraction 1 to fraction 9). Fraction 7 was separated by semipreparative HPLC eluting with MeOH−H_2O (65:35) to obtain compound **5** (50.0 mg, t_R = 8.5 min), compound **1** (4.0 mg, t_R = 20.5 min), and compound **2** (9.0 mg, t_R = 22.3 min). Fraction 8 was separated by semipreparative HPLC eluted with MeOH−H_2O (70:30) to obtain compound **3** (5.0 mg, t_R = 17.0 min) and compound **4** (5.0 mg, t_R = 20.0 min).

*Cladosin H (**1**):* pale yellow oil, $[\alpha]^{25}_D$ +18.4 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 249 (1.96) 285 (2.35), 322 (2.01) nm; ECD (1 mM MeOH) λ_{max} ($\Delta\varepsilon$) 225 (+1.72), 275 (−1.05), 325 (−0.84) nm; IR (KBr) ν_{max} 3200, 2927, 1670, 1579, 1384, 1264 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HRESIMS *m*/*z* 359.1967 $[M + H]^+$ (calcd for $C_{20}H_{27}N_2O_4$, 359.1965).

*Cladosin I (**2**):* pale yellow oil, $[\alpha]^{25}_D$ +30.5 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 249 (1.96) 285 (2.35), 322 (2.01) nm; ECD (1 mM MeOH) λ_{max} ($\Delta\varepsilon$) 225 (−1.67), 275 (+1.02), 325 (+0.78) nm; IR (KBr) ν_{max} 3200, 2928, 1669, 1578, 1383, 1265 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HRESIMS *m*/*z* 359.1966 $[M + H]^+$ (calcd for $C_{20}H_{27}N_2O_4$, 359.1965).

*Cladosin J (**3**):* pale yellow oil, $[\alpha]^{25}_D$ +16.5 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 221 (3.47), 286 (2.01), 325 (1.78) nm; IR (KBr) ν_{max} 3199, 2928, 1668, 1578, 1383, 1264 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2; HRESIMS *m*/*z* 420.2275 $[M + H]^+$ (calcd for $C_{25}H_{30}N_3O_3$, 420.2282).

*Cladosin K (**4**):* pale yellow oil, $[\alpha]^{25}_D$ +18.4 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 221 (3.47), 286 (2.01), 325 (1.78) nm; IR (KBr) ν_{max} 3442, 2926, 1669, 1578, 1384, 1262 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 2; HRESIMS *m*/*z* 420.2273 $[M + H]^+$ (calcd for $C_{25}H_{30}N_3O_3$, 420.2282).

*Cladodionen (**5**):* pale yellow oil, $[\alpha]^{25}_D$ −16.0 (*c* 0.15, MeOH) ($[\alpha]^{25}_D$ −10.8 (*c* 0.3, MeOH), reported data[12]); UV (MeOH) λ_{max} (log ε) 248 (1.65), 281 (2.01), 324 (3.21) nm; ECD (1 mM MeOH) λ_{max} ($\Delta\varepsilon$) 255 (−3.23), 280 (+2.37), 322 (−6.99) nm; HRESIMS *m*/*z* 234.1125 $[M + H]^+$ (calcd for $C_{13}H_{16}NO_3$, 234.1125).

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Preparation of MPA Esters of 1 and 2. The sample of cladosins H and I (**1** and **2**) (0.5 mg each) was treated with (*R*)- or (*S*)-MPA (0.4 mg) with dicyclohexylcarbodiimide (DCC) (0.5 mg) and dimethylaminopyridine (DMAP) (0.3 mg) in dry CH_2Cl_2 (0.5 mL). The reaction was stirred at room temperature (rt) under a nitrogen atmosphere for 2 h. The organic phase was evaporated to dryness and separated by semipreparative HPLC with MeCN−H_2O (45:55) to obtain the (*R*)-MPA ester and (*S*)-MPA ester (**1** and **2**), respectively.

Cladosin H (1) (R)-MPA ester: ^{1}H NMR (DMSO-d_6) exoform A (exoform B) δ 12.82 (12.16), 9.40 (9.73), 7.36−7.49, 7.29−7.33, 4.84−4.89, 4.73, 3.37−3.42, 3.28 (3.26), 3.09−3.20, 2.82 (2.85), 2.20 (2.19), 1.81 (1.80), 1.55−1.65, 1.38−1.45, 0.96 (0.99); HRESIMS *m/z* 507.2478 $[M + H]^+$ (calcd for $C_{29}H_{35}N_2O_6$, 507.2490).

Cladosin H (1) (S)-MPA ester: ^{1}H NMR (DMSO-d_6) exoform A (exoform B) 12.78 (12.11), 9.40 (9.73), 7.42−7.46, 7.28−7.37, 7.21−7.24, 4.77 (4.79), 4.72−4.74, 3.27, 3.12−3.16, 2.98−3.06, 2.92−2.95, 2.54 (2.55), 2.21 (2.24), 1.83 (1.82), 1.56−1.65, 1.31−1.39, 1.11 (1.13); HRESIMS *m/z* 507.2490 $[M + H]^+$ (calcd for $C_{29}H_{35}N_2O_6$, 507.2490).

Cladosin I (2) (R)-MPA ester: ^{1}H NMR (DMSO-d_6) exoform A (exoform B) 12.80 (12.16), 9.33 (9.69), 7.31−7.46, 4.81−4.85, 4.77 (4.78), 3.28−3.40, 3.26 (3.24), 2.94 (2.99), 2.19 (2.15), 1.80 (1.78), 1.62−1.68, 1.48−1.55, 0.92 (0.94); HRESIMS *m/z* 507.2491 $[M + H]^+$ (calcd for $C_{29}H_{35}N_2O_6$, 507.2490).

Cladosin I (2) (S)-MPA ester: ^{1}H NMR (DMSO-d_6) exoform A (exoform B) 12.77 (12.11), 9.34 (9.68), 7.41−7.45, 7.31−7.36, 7.28 (7.27), 4.80−4.88, 4.77, 3.25, 2.94−3.07, 2.85 (2.89), 2.19 (2.16), 1.80 (1.78), 1.53−1.58, 1.42−1.49, 1.07 (1.09); HRESIMS *m/z* 507.2497 $[M + H]^+$ (calcd for $C_{29}H_{35}N_2O_6$, 507.2490).

Preparation of *N*-Phenyloctanamide. Aniline (50.0 mg) was dissolved in CH_2Cl_2 (3.0 mL) containing DMAP (13.0 mg), followed by adding Et_3N (111 μL). Octanoyl chloride (100 μL) was then added, and after stirring 2 h at rt, the product was filtered through a small plug of silica eluting with CH_2Cl_2 (15 mL) prior to solvent evaporation in vacuo. The product was purified by RP-HPLC eluting with MeOH−H_2O (75:25) to obtain *N*-phenyloctanamide.

N-Phenyloctanamide: white powder; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.81 (s, 1H), 7.56 (d, J = 7.8 Hz, 2H), 7.25 (t, J = 7.9 Hz, 2H), 6.99 (t, J = 7.4 Hz, 1H), 2.27 (t, J = 7.5 Hz, 2H), 1.56 (dd, J = 13.4, 6.5 Hz, 2H), 1.31−1.19 (m, 8H), 0.84 (t, J = 6.9 Hz, 3H); ESIMS *m/z* 220.55 $[M + H]^+$.

SAHA and Analogues Incubation Studies. Incubation studies with SAHA, *N*-phenyloctanamide, and aniline (each at 300 μM) were carried out in 500 mL flasks containing 150 mL of the potato dextrose broth (PDB) medium (a separate flask containing PDB medium without any additive served as a control). Cultures were each inoculated with spores of *C. sphaerospermum*. After 6 days, cultures were clarified via filter paper to separate the supernatant from mycelia. The supernatant was extracted with EtOAc and evaporated under reduced pressure to give an extract. Dried extracts were resuspended in 2 mL of MeOH and analyzed by UPLC-MS.

Enzyme Inactivation Experiment. The fungus *C. sphaerospermum* was cultivated in an Erlenmeyer flask (500 mL) containing 150 mL of fermentation media for 5 days. The supernatant of the fermentation broth was clarified by a 3 kDa filter, collected in a separate Erlenmeyer flask (100 mL), and then heated to 110 °C in an oil bath for 5 min to denature and/or remove enzymes.[17] The collected supernatant without enzymes was divided into three portions (each contain 20 mL), with SAHA (300 μM) added to one portion, aniline (300 μM) added to another portion, and nothing added to the third portion (control), respectively. After stirring at 28 °C for 24 h, the supernatant was extracted with EtOAc and evaporated under reduced pressure to give an extract. Dried extracts were dissolved in 1 mL of MeOH and analyzed by UPLC-MS.

Bioactivity Evaluation. Cytotoxic activities of **1**−**5** were evaluated against PC-3, MGC-803, SH-SY5Y, and HCT-116 cell lines by the SRB method;[14] the K562 and HL-60 cell lines were analyzed by the MTT method.[15]

In the MTT assay, moderate cell suspensions were mixed with 0.8% Trypan blue solution by equal volume. Cell viability was detected by counting the living cells on the cell counting plate. Then, cell suspensions, 90 μL, at a density of 8000 cells per well were plated in 96-well microtiter plates and exposed to 10 μL of test solution in each well for 72 h. Then, 100 μL of each of the three linked dissolved solutions was added to each well, which were incubated for 10 h. Absorbance was then determined on a Spectra Max Plus plate reader at 570 nm.

In the SRB assay, the cells were washed with 1 mL of phosphate-buffered saline twice. Then 1 mL of pancreatin was added to 2 mL of medium to digest parietal cells for 2 min, preparing the single-cell suspensions. Cell viability was detected by the Trypan blue cell counting assay. Then, 6000 cells per well, 90 μL, were seeded in 96-well microtiter plates and exposed to 10 μL of test solution in each well for 72 h. The cells were fixed with 12% trichloroacetic acid, and the cell layer was strained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm. Doxorubicin hydrochloride was used as the positive control.[18]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00289.

Structures of cladosins A−K and cladodionen, MS, IR, and 1D and 2D NMR spectra for compounds **1**−**4**, and UPLC-MS analyses (PDF)

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Notes

The authors declare no competing financial interest.

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