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Structures of Nahuoic Acids B–E Produced in Culture by a *Streptomyces* sp. Isolated from a Marine Sediment and Evidence for the Inhibition of the Histone Methyl Transferase SETD8 in Human Cancer Cells by Nahuoic Acid A

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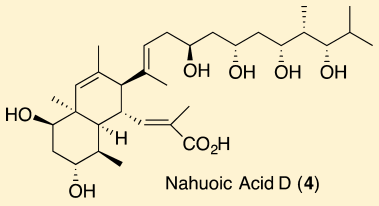
Supporting Information

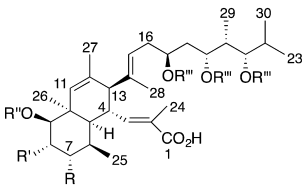
ABSTRACT: Nahuoic acids A–E (**1**–**5**) have been isolated from laboratory cultures of a *Streptomyces* sp. obtained from a tropical marine sediment. The structures of the new polyketides **2**–**5** were elucidated by analysis of spectroscopic data of the natural products and the chemical derivatives **6** and **7**. Nahuoic acids **1**–**5** are in vitro inhibitors of the histone methyltransferase SETD8, and nahuoic acid A (**1**) and its pentaacetate derivative **8** inhibit the proliferation of several cancer cells lines in vitro with modest potency. At the IC_{50} for cancer cell proliferation, nahuoic acid A (**1**) showed selective inhibition of SETD8 in U2OS osteosarcoma cells that reflect its selectivity against a panel of pure histone methyl transferases. A cell cycle analysis revealed that the cellular toxicity of nahuoic acid A (**1**) is likely linked to its ability to inhibit SETD8 activity.



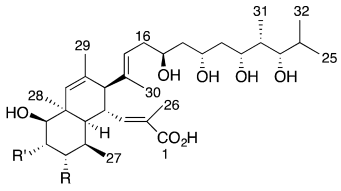
Nahuoic Acid D (**4**)

INTRODUCTION

Histone methyl transferases catalyze the transfer of one to three methyl groups from the cofactor *S*-adenosylmethionine to the side-chain amino groups of lysine residues in the histone proteins that make up the core of nucleosomes, the basic building blocks of chromatin.[1] These methylation events play a key role in the epigenetic regulation of gene expression. SETD8 is a lysine methyltransferase that monomethylates the side-chain amino group of lysine 20 of histone H4 (H4K20),[2,3] and it also monomethylates lysine 248 of proliferating cell nuclear antigen (PCNA)[4] and lysine 382 of p53/TP53.[5] SETD8 is overexpressed in various cancers, and it has been suggested that aberrant over-methylation by SETD8 might be involved in carcinogenesis.[6,7] Recently, we reported the isolation of the polyketide nahuoic acid A (**1**) from cultures of a *Streptomyces* sp. obtained from a marine sediment collected in Papua New Guinea.[8] Nahuoic acid A (**1**) was found to be a selective and SAM competitive inhibitor of SETD8. Further investigations of the nahuoic acid A producing *Streptomyces* sp. cultures have resulted in the isolation of the new analogues nahuoic acids B (**2**), C (**3**), D (**4**), and E (**5**). Herein, we describe the structure elucidation of nahuoic acids B–E (**2**–**5**), their ability to inhibit SETD8 in vitro, evidence showing the ability of nahuoic acid A (**1**) and its pentaacetate **8** to inhibit the proliferation of human cancer cell lines in vitro with modest potency, and the selective inhibition of SETD8 by nahuoic acid A (**1**) in human U2OS osteosarcoma cancer cells.



1 R=OH, R'=H, R''=H, R'''=H
2 R=H, R'=OH, R''=H, R'''=H
3 R=OH, R'=OH, R''=H, R'''=H
6 R=H, R'=OAc, R''=Ac, R'''=Ac



4 R=OH, R'=H
5 R=H, R'=OH

RESULTS AND DISCUSSION

Large-scale cultures of the nahuoic acid A (**1**) producing *Streptomyces* sp. were grown as lawns on solid agar containing marine medium as previously described.[8] EtOAc extracts of the combined cells and solid agar media were fractionated using sequential application of Sephadex LH20 chromatography, step-gradient Si Gel flash chromatography, and reversed-phase HPLC to give nahuoic acids A (**1**), B (**2**), C (**3**), D (**4**), and E (**5**).

The first sample of nahuoic acid B (**2**) obtained from the reversed-phase HPLC fractionation step was collected as a single

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Table 1. ^{1}H NMR Data for Nahuoic Acids A (1), B (2), and C (3) Recorded in DMSO-d_6 and Nahuoic Acid B Pentaacetate (6) Recorded in C_6D_6 at 600 MHz

	δ, multiplicity (J Hz)			
position	nahuoic acid A (**1**)	nahuoic acid B (**2**)	nahuoic acid C (**3**)	nahuoic acid B pentaacetate (**6**)
1				
2				
3	6.41, d (10.2)	6.37, bd (10.9)	6.41, bd (10.3)	6.70, d (10.9)
4	3.54[a]	3.40[a]	3.40[a]	3.21, ddd (10.9, 10.9, 10.9)
5	1.52, dd (11.6, 2.9)	1.34, dd (10.9, 2.0)	1.47[a]	1.36[a]
6	1.92[a]	2.41 m	2.34 m	2.34 m
7_{ax}	3.51, td (11.0, 3.7)	1.16, bd (13.4) 1.61[a]	3.52, dd (11.7, 2.1)	1.64[c]
7_{eq}				1.53[a]
				1.64[a]
8_{ax}	1.62[a]	3.78, m	3.74, bs	5.18[a]
8_{eq}	1.87, bd (13.6)			
9	3.47, m	3.21, bs	3.40, bs	5.04, bs
10				
11	5.15, bs	5.14, bs	5.13, bs	5.17[a]
12				
13	2.23, bd (8.9)	2.22, bd (9.1)	2.22, bd (9.4)	2.25[a]
14				
15	4.97, m	4.98, m	4.98, m	5.21, bt (7.3)
16a	1.91[a]	1.91, m	1.91, m	2.25[a]
16b	2.03, m	2.04, m	2.03, m	2.41, m
17	3.57, m	3.58, m	3.57, m	5.25, m
18a	1.17, bt (10.6)	1.18[a]	1.17[a]	1.78[a]
18b	1.42[a]	1.43[a]	1.42[a]	2.12, m
19	3.77, dm (9.8)	3.77[a]	3.77, dm (9.5)	5.29, m
20	1.39[a]	1.39, m	1.39[a]	2.21, m
21	3.12, dd (7.2, 4.0)	3.12, dd (7.0, 4.0)	3.12, dd (7.1, 4.0)	5.11, bt (5.0)
22	1.63[a]	1.63, m	1.63, m	2.08, m
23	0.76, d (6.5)	0.77, d (6.7)	0.76, d (6.6)	0.96, d (7.0)
24	1.58, s	1.57, bs	1.58, bs	1.93, s
25	0.85, d (7.2)	0.80, d (7.3)	0.85, d (7.4)	0.95, d (7.4)
26	1.01, s	1.14, s	1.14, s	1.16, s
27	1.46, bs	1.46, bs	1.45, bs	1.49, bs
28	1.40, bs	1.41, bs	1.39, bs	1.36, bs
29	0.76, d (6.7)	0.76, d (6.8)	0.76, d (6.7)	1.04, d (6.9)
30	0.83, d (6.7)	0.83, d (6.6)	0.83, d (6.8)	0.88, d (6.6)
1-COO*H*	11.81, br	11.81, br	NO	NO
7-O*H*	4.28−4.35 br		NO	
8-O*R*		NO	NO	1.60, s
9-O*R*	4.28−4.35 br	4.30 bs	NO	1.64, bs
17-O*R*	4.28−4.35 br	NO	NO	1.85, s
19-O*R*	4.28−4.35 br	NO	NO	1.85, s
21-O*R*	4.28−4.35 br	NO	NO	1.77, s

[a]Multiplicity not determined due to overlapping signals; chemical shifts determined from 2D data. NO: not observed.

sharp peak but was found by NMR analysis to contain a coeluting chemically unrelated metabolite that has not been identified. A positive-ion HRESIMS of this impure sample gave a strong $[M + Na]^+$ ion at m/z 545.3455 that was appropriate for a molecular formula of $C_{30}H_{50}O_7$, the same as nahuoic acid A (**1**). The impure sample of B (**2**) was reacted with acetic anhydride in pyridine, and the reaction mixture was purified by reversed-phase HPLC to give the pentaacetate **6** that gave an $[M - H]^-$ ion in the (−)-HRESIMS at m/z 731.4016 appropriate for a molecular formula of $C_{40}H_{60}O_{12}$. Conversion of nahuoic acid B (**2**) to the corresponding pentaacetate **6** would account for the addition of 10 carbon, 10 hydrogen, and 5 oxygen atoms compared with the molecular formula $C_{30}H_{50}O_7$ determined by HRMS for the impure sample. Five new methyl singlets at δ 1.60, 1.64, 1.77, 1.85, and 1.85, in addition to the observed downfield shifts of five carbinol methine resonances at δ 5.04, 5.11, 5.18, 5.25, and 5.29 in the ^{1}H NMR spectrum of **6**, confirmed the formation of a pentaacetate (Table 1).

Detailed analysis of the 1D and 2D NMR data obtained for **6** (Figure 1 and Tables 1 and 2; Supporting Information) supported the pentaacetylation and showed that **1** and **2** were identical in all respects except for the positions of hydroxylation at C-7 and C-8 in the decalin ring system. A correlation in the HMBC spectrum of **6** between the resonance assigned to H-8 at δ 5.18 and an acetate carbonyl at δ 168.6 established that in **6** an acetate, and by analogy a hydroxy in **2**, was located at C-8 in **2** instead of C-7 as in **1**. A series of tROESY correlations and the scalar coupling data obtained for **6** established that the C-5 to

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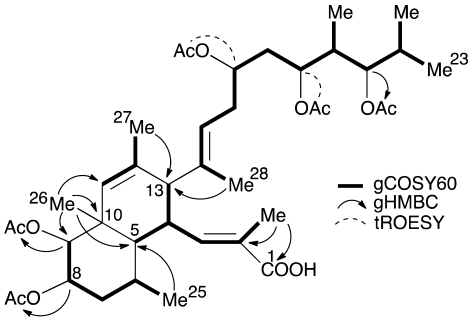

Figure 1. Selected gCOSY60, gHMBC, and tROESY correlations observed for pentaacetate **6**.

C-10 cyclohexane ring was in a chair conformation and that the relative configurations about the decalin ring system at the common stereogenic centers C-4, C-5, C-6, C-9, C-10, and C-13 were the same as those in nahuoic acid A (**1**).[8] tROESY correlations between Me-26 (δ 1.16) and the C-8 acetate methyl (δ 1.60), and between H-8 (δ 5.18) and each of H-7_{ax} (δ 1.64), H-7_{eq} (δ 1.53) and H-9 (δ 5.04) established that the C-8 acetate was axial. The relative configurations at the side-chain carbons C-17, C-19, C-20, and C-21 were assumed to be the same as the corresponding centers in nahuoic acid A (**1**), leading to the assignment of the absolute configuration of **6** as 4*R*,5*S*,6*S*,8*S*,9*S*,10*R*,13*R*,17*S*,19*R*,20*S*,21*S*. A pure sample of nahuoic acid B (**2**) was obtained from a subsequent *Streptomyces* sp. culture, and analysis of the 1D and 2D NMR data (Tables 1 and 2; Supporting Information) obtained for this sample was in full agreement with the structure assigned to **2** using the NMR data of the pentaacetate **6**.

Nahuoic acid C (**3**) was obtained as an optically active oil that gave a $[M + Na]^+$ ion in the HRESIMS at *m*/*z* 561.3398 appropriate for a molecular formula of $C_{30}H_{50}O_8$ that differs from the formulas of **1** and **2** by the addition of an oxygen but still requires the six sites of unsaturation present in nahuoic acids A (**1**) and B (**2**). The NMR spectra obtained for **3** (Tables 1 and 2) were very similar to the spectra of **1** and **2** except that there was an additional carbinol methine and the corresponding disappearance of an aliphatic methylene observed in the NMR spectra of **3** compared with the NMR spectra of **1** and **2**. An additional secondary alcohol at one of the three methylenes in the structures **1** or **2** would account for the observed differences in their NMR spectra. Correlations observed in the gradCOSY60 spectrum of **3** between a resonance at δ 3.74 and the resonances assigned to H-7 and H-9 at δ 3.52 (dd, J = 11.7, 2.1 Hz) and δ 3.40 (bs), respectively, established that the sixth hydroxyl substituent in **3** was at C-8 ($^{13}C/^{1}H$: δ 74.7/3.74). A series of tROESY correlations and the J coupling data obtained for **3** established that the cyclohexane ring encompassing C-5 to C-10 was in a chair conformation with H-7 axial and the relative configurations about the decalin ring system at the common stereogenic centers C-4, C-5, C-6, C-7, C-9, C-10 and C-13 were the same in nahuoic acid C (**3**) as in nahuoic acid A (**1**).[8] A small scalar coupling of 2.1 Hz between H-7 (δ 3.52) and H-8 (δ 3.74), and tROESY correlations observed between H-8 and both the axially orientated H-7 and the equatorial H-9 (δ 3.40), established that H-8 was also equatorial with the hydroxyl at C-8 in an axial orientation. The configurations at the side chain carbons C-17, C-19, C-20, and C-21 were assumed to be the same as those seen in nahuoic acid A (**1**), leading to assignment of the absolute configuration of **3** as 4*R*,5*S*,6*S*,7*S*,8*S*,9*S*,10*R*,13*R*,17*S*,19*R*,20*S*,21*S*.

Nahuoic acid D (**4**) was obtained as an optically active oil that gave a $[M + Na]^+$ ion in the HRESIMS at *m*/*z* 589.3712 appropriate for a molecular formula of $C_{32}H_{54}O_8$ that differs from the formula of **1** by the addition of C_2H_4O but still requires six sites of unsaturation. The ^{1}H and ^{13}C NMR spectra (Table 3; Supporting Information) obtained for **4** revealed the presence of an additional alkyl methylene ($^{1}H/^{13}C$: δ 1.28/44.0) and an additional secondary alcohol ($^{1}H/^{13}C$: δ 3.75/65.9), which accounted for the difference in the masses of **1** and **4**. Other than these two differences, the NMR spectra obtained for **4** were remarkably similar to the spectra obtained for **1** (Tables 1−3). Detailed analysis of the 2D NMR data obtained for **4** established

Table 2. ^{13}C NMR Data for Nahuoic Acids A (1), B (2), and C (3) Recorded in DMSO-d_6 and Nahuoic acid B Pentaacetate (6) Recorded in C_6D_6 at 150 MHz

	δ					δ			
position	1	2	3	6	position	1	2	3	6
1	169.2	169.2	169.2	169.3	19	70.0	70.0	70.0	72.4
2	124.3	124.5	124.4	125.1	20	39.7	39.7	39.7	38.2
3	149.1	149.0	149.0	149.7	21	78.6	78.6	78.6	78.7
4	36.0	35.4	36.2	36.3	22	30.3	30.3	30.3	30.4
5	50.0	48.8	50.6	49.1	23	19.5	19.5	19.5	19.5
6	38.3	24.6	30.6	25.9	24	12.9	13.0	13.0	13.4
7	66.2	32.1	68.4	30.2	25	18.1	22.0	17.9	21.9
8	40.2	71.0	74.7	71.5	26	27.6	27.6	27.7	27.6
9	72.7	73.6	75.7	73.4	27	21.6	21.7	21.7	21.6
10	41.1	40.7	40.5	40.8	28	12.3	12.2	12.1	12.9
11	132.5	133.6	133.1	131.6	29	7.1	7.2	7.2	11.8
12	132.7	131.6	131.6	133.4	30	18.4	18.5	18.5	17.3
13	56.8	56.2	56.5	57.3	8-OR				168.6, 20.7
14	135.1	134.9	134.9	137.1	9-OR				168.4, 21.0
15	125.3	125.3	125.4	123.7	17-OR				169.9[a], 20.9[b]
16	36.7	37.0	37.0	33.0	19-OR				170.5[a], 20.8[b]
17	67.0	67.1	67.1	69.2	21-OR				171.2, 20.5
18	41.7	41.7	41.7	33.8					

[a,b]Assignments within a column maybe interchanged.

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Table 3. NMR Data for Nahuoic Acids D (4) and E (5) Recorded in DMSO-d_6 at 600 MHz

	nahuoic acid D (**4**)		nahuoic acid E (**5**)	
position	δ_C	δ_H, multiplicity (J Hz)	δ_C	δ_H, multiplicity (J Hz)
1	169.2		169.2	
2	124.3		124.5	
3	149.1	6.41, d (11.1)	149.1	6.37 bd (11.2)
4	36.1	3.54[a]	35.5	3.40[a]
5	50.1	1.52, dd (11.5, 2.6)	48.9	1.34, bd (12.0)
6	38.3	1.91[a]	24.6	2.41, m
7_{ax}	66.2	3.51, td (11.0, 4.1)	32.1	1.61[a]
7_{eq}				1.16, bd (13.7)
8_{ax}	39.9	1.62[a]	71.0	4.64, bs
8_{eq}		1.87, bd (9.9)		
9	72.7	3.47, bs	73.7	3.21, bs
10	41.1		40.7	
11	132.4	5.15, bs	133.6	5.14, bs
12	132.4		131.6	
13	56.8	2.22, bd (9.0)	56.2	2.23, bd (9.5)
14	135.0		135.1	
15	125.2	4.98, m	125.2	4.98, m
16a	36.8	1.92[a]	36.9	1.92 m
16b		2.02, m		2.03, m
17	66.8	3.61, m	66.9	3.61, m
18	44.0	1.28, m	44.0	1.29, td (8.7, 2.1)
		1.28, m		1.29, td (8.7, 2.1)
19	65.9	3.75, m	66.0	3.76[a]
20a	42.3	1.42, bt (6.4)	42.4	1.42, bt (6.6)
20b		1.42, bt (6.4)		1.42 bt (6.6)
21	72.0	3.72, m	72.0	3.72, m
22	38.6	1.49[a]	38.7	1.49, m
23	78.1	3.14, dd (7.1, 4.1)	78.2	3.14, m
24	30.3	1.64, m	30.4	1.64, m
25	19.5	0.77, d (6.9)	19.6	0.78, d (6.8)
26	12.9	1.58, s	13.0	1.58, bs
27	18.1	0.85, d (5.7)	22.0	0.80, d (7.3)
28	27.6	1.01, s	27.6	1.14, s
29	21.6	1.46, bs	21.7	1.46, bs
30	12.2	1.39, bs	12.2	1.40, bs
31	7.1	0.77, d (6.9)	7.1	0.78, d (6.8)
32	18.3	0.84, d (6.3)	18.4	0.84, d (6.5)
1-COO*H*		NO		NO
7-O*H*		4.28−4.39		
8-O*H*				4.64 bs
9-O*H*		4.28−4.39		4.29 bs
17-O*H*		4.28−4.39		4.29 bs
19-O*H*		4.28−4.39		4.39 bs
21-O*H*		4.28−4.39		4.51 bs
23-O*H*		4.28−4.39		4.32bs

[a]Multiplicity not determined due to overlapping signals; chemical shifts determined from 2D data. NO: not observed.

that the structural differences in **1** and **4** were in the acyclic side chain attached at C-13 of the decalin ring system (Figure 2). In the gCOSY60 NMR spectrum the methylene multiplet at δ 1.28 (H-18) correlated to both the methine resonance of the additional secondary alcohol at δ 3.75 (H-19) and to the resonance at δ 3.61 assigned to the carbinol methine of C-17. H-19 in turn showed vicinal coupling to the methylene resonance at δ 1.42 that was assigned to H_2-20 (Table 3). The ^{1}H and ^{13}C NMR chemical shifts and the ^{1}H scalar coupling patterns assigned to the fragment from H_2-20 to Me-25, including the resonances for Me-31 and Me-32, in **4** were very similar to those observed for the corresponding fragment extending from H_2-18 to Me-23 including Me-29 and Me-30 in **1** (Tables 1−3), indicating that these fragments were closely related in **1** and **4**. The observed scalar coupling constants and tROESY data demonstrated that the relative configurations about the decalin ring system and the configurations of the $\Delta^{2,3}$ and $\Delta^{14,15}$ olefins in **4** were the same as in **1**.[8]

Nahuoic acid D (**4**) was treated with 2,2-dimethoxypropane and PPTS, and the crude reaction mixture was treated with *p*-bromophenacyl bromide and Et_3N in DMF as shown in Scheme 1 to give ester **7** as a major product in an attempt to make a crystalline derivative for X-ray diffraction analysis. Unfortunately, ester **7** failed to crystallize. The Me-42, Me-43, Me-45,

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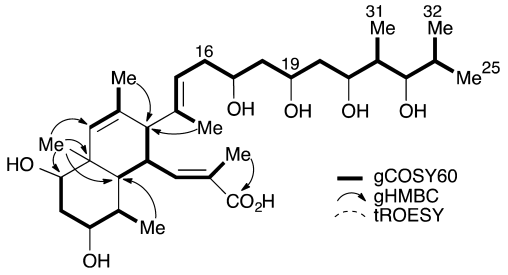


Figure 2. Selected gCOSY60 and gHMBC correlations observed for nahuoic acid D (**4**).

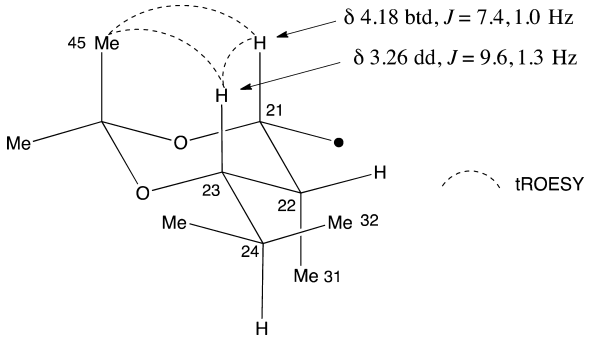


Figure 3. Selected scalar coupling and tROESY correlations in the C-25 to C-21 fragment of the bisacetonide ester **7**.

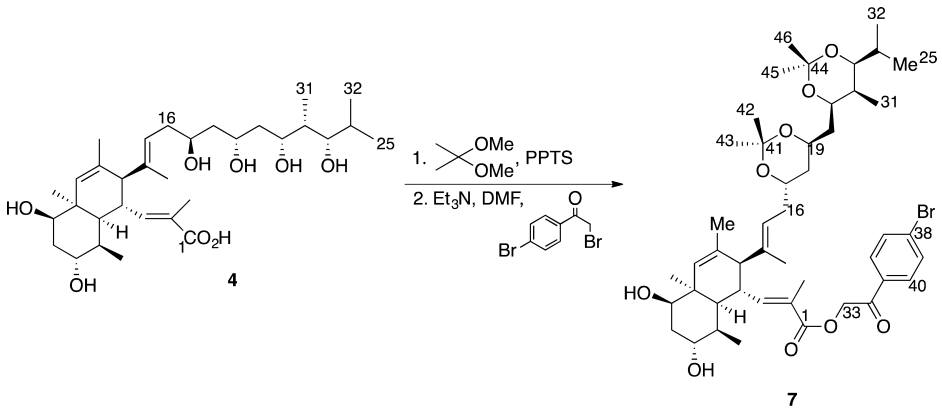
and Me-46 methyl carbons of the C-17/C-19 and the C-21/C-23 acetonides in **7** were assigned the ^{13}C NMR chemical shifts δ 25.2, 25.1, 19.8, and 30.5 (C_6D_6) (Supporting Information), respectively, which according to Rychnovsky's rules demonstrated that the C-17 and C-19 alcohols in **4** were *anti* while those at C-21 and C-23 were *syn*.[9] tROESY data obtained for ester **7** confirmed that the C-21/C-23 dioxane ring was in a chair conformation as predicted from the ^{13}C NMR data according to Rychnovsky's rules (Figure 3).[9] The scalar coupling observed in the H-23 resonance (dd, J = 9.6, 1.3 Hz) of **7** was identical to the scalar coupling in the corresponding H-21 resonance (dd, J = 9.6, 1.7 Hz) of the C-19/C-21 acetonide of nahuoic acid A (**1**),[8] which has an axial methyl at C-20 and the 19*R*,20*S*,21*S* configuration. Me-31 has a carbon chemical shift of δ 5.4 in **7**, confirming that it is axial. Therefore, the relative configurations at C-21, C-22, and C-23 in **7** were assigned as 21*R**,22*S**,23*S** (Figure 3).

Comparison of the ^1H and ^{13}C NMR chemical shifts for the C-13−C-17 fragments of nahuoic acids A (**1**) and D (**4**) showed that they were virtually identical, indicating that the configurations at C-13 and C-17 were 13*R*,17*S* in both **1**[8] and **4** (Tables 1−3 and Figure 4). As outlined above, Rychnovsky's rules showed that the C-17 and C-19 hydoxy substituents in **4** were *anti*, leading to the assignment of the C-19 configuration as *R*. The Rychnovsky acetonide analysis of ester **7** described above also established the relative configurations at C-21 to C-23 as 21*R**,22**S*,23*S** in **4**, which is identical to their relative configurations in **1**.[8] Comparison of the ^1H and ^{13}C NMR chemical shifts for the C-17 to C-23 fragment in nahuoic acid A (**1**) with the chemical shifts for the corresponding C-19−C-25 fragment in **4** (Figure 4) shows that they are significantly different at C-21 (**4**)/C-19 (**1**) and C-22 (**4**)/C-20 (**1**) consistent with opposite relative configurations between C-17 and C-19 in **1** (*anti*) and C-19 and C-21 in **4** (*syn*). Nahuoic acids A (**1**) and D (**4**) are both polyketides whose putative biogenesis starts with an isobutyrate unit followed by condensation of a propionate residue to generate identical terminal side-chain fragments in **1** (C-19 to C-23 and C-29/C-30) and in **4** (C-21 to C-25 and C-31/C-32). The pathways diverge at this point, with one extra acetate unit providing the source of the C-19 and C-20 carbons in **4**. From there, the remainder of the pathways to **1** and **4** leading to the linear side chain C-14 to C-18 fragments (including C-28 in **1** and C-30 in **4**), the decalin ring systems, and the C-4 side chain substituents are identical. The above biogenetic arguments require that the side chain stereogenic centers at C-21, C-22, and C-23 in **4** have the same relative configurations compared with the decalin fragment as was previously determined for the C-19, C-20, and C-21 side-chain stereogenic centers and the decalin fragment in nahuoic acid A (**1**) using Mosher ester analysis at C-7 and C-17 in the C-19/C-21 monoacetonide derivative of **1**.[8] This led to the complete absolute configuration assignment of 4*R*,5*S*,6*R*,7*R*,9*R*,10*R*,13*R*,17*S*,19*R*,21*R*,22*S*,23*S* for nahuoic acid D (**4**) (Figure 4).

Nahuoic acid E (**5**) was obtained as an optically active oil that gave a $[M + Na]^+$ ion in the HRESIMS at m/z 589.3710 appropriate for a molecular formula of $C_{32}H_{54}O_8$, the same as nahuoic acid D (**4**). The NMR spectra obtained for **5** were remarkably similar to the spectra obtained for **4**, except that in **5** there was now hydroxylation at C-8 rather than C-7. In all other respects, **5** was the same as **4** with the C-8 hydroxy axial as in **2** (Table 3; Supporting Information) and the absolute configuration 4*R*,5*S*,6*S*,8*S*,9*S*,10*R*,13*R*,17*R*,19*S*,21*R*,22*S*,23*S*.

Table 4 shows the IC_{50} values for in vitro inhibition of the histone methyltransferase SETD8 by nahuoic acids A−E (**1**−**5**).

Scheme 1. Derivatization of Nahuoic Acid D (4)



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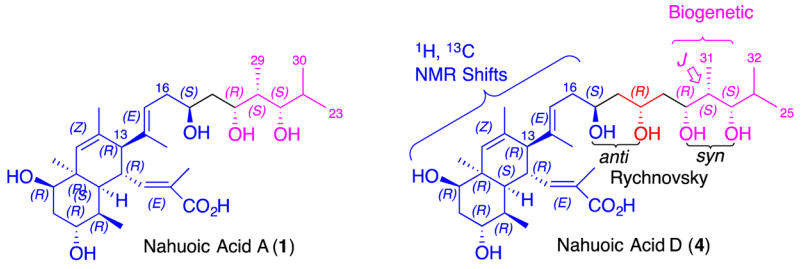

C#	13	14	15	16	17	18	19	20	21	22	23	24	25	30	31	32
4	56.8	135.0	125.2	36.8	66.8	44.0	65.9	42.3	72.0	38.6	78.1	30.3	19.5	12.2	7.1	18.3
C#	13	14	15	16	17	18	19	-	-	20	21	22	23	28	29	30
1	56.8	135.1	125.3	36.7	67.0	41.7	70.0	-	-	39.7	78.6	30.3	19.5	12.3	7.1	18.4

Figure 4. Assignment of the absolute configuration of nahuoic acid D (**4**) using (i) a comparison of ^{1}H, ^{13}C NMR chemical shifts and NOE data between nahuoic acids A (**1**) and D (**4**) (blue fragment), (ii) Rychnovsky's acetonide analysis, and (iii) biogenetic arguments (magenta fragment).

Table 4. In Vitro Inhibition of SETD8 by Nahuoic Acids A–E (1–5)

compd	IC_{50} (μM)	hill slope
nahuoic acid A (**1**)	8	1.4
nahuoic Acid B (**2**)	27	1.7
nahuoic Acid C (**3**)	41	1.5
nahuoic Acid D (**4**)	76	1.7
nahuoic Acid E (**5**)	13	1.7

There is roughly 1 order of magnitude difference in IC_{50} values between the most active inhibitor nahuoic acid A (**1**) and the weakest inhibitor nahuoic acid D (**4**).

Given the ability of nahuoic aid A (**1**) to inhibit SETD8 in vitro, we sought to determine whether this natural product has cytotoxic activity in human cancer cells. To address this question, growth inhibition assays were performed by exposing osteosarcoma U2OS cells to increasing concentrations of nahuoic acid A (**1**), as loss of SEDT8 activity in U2OS cells is known to trigger cell proliferation defects.[10,11] The effects of the prodrug versions nahuoic acid A pentacetate (**8**) and methylnahuoic A pentaacetate (**9**), prepared in an attempt to improve cell permeability of the natural product, were also evaluated. As shown in Figure 5 (upper panel), exposure to the nahuoic acid A (**1**) or the nahuoic acid A pentaacetate (**8**) results in a significant decrease in U2OS cell proliferation with an apparent IC_{50} value of 65 ± 2 μM for nahuoic acid A (**1**) and 39 ± 4 μM for nahuoic acid A pentaacetate (**8**). In contrast, treatment with the methylnahuoic acid A pentaacetate (**9**) has a weak effect on cell proliferation, indicating that this prodrug version was largely inactive. Interestingly, the cytotoxic effect of the nahuoic acid A compounds was not specific to U2OS cells as it was also observed in SUM159 (IC_{50} = 45 μM) and MDA-MB-436 (IC_{50} = 85 μM) breast cancer cell lines for the nahuoic acid A pentaacetate (**8**) (Figure 5, lower panel).

We next determined whether the cytotoxicity of nahuoic acid A (**1**) is related to its ability to inhibit SETD8 activity. To this end, we measured the levels of histone H4-K20 methylation by immunoblotting with specific antibodies. SETD8 is the unique enzyme responsible for the monomethylation of histone H4-K20 (H4K20me1), which exists on its own in active chromatin and also serves as a substrate for the lysine methyltransferases SUV4-20H to induce H4-K20 trimethylation (H4K20me3) in heterochromatin regions.[12] Depletion of SETD8 upon

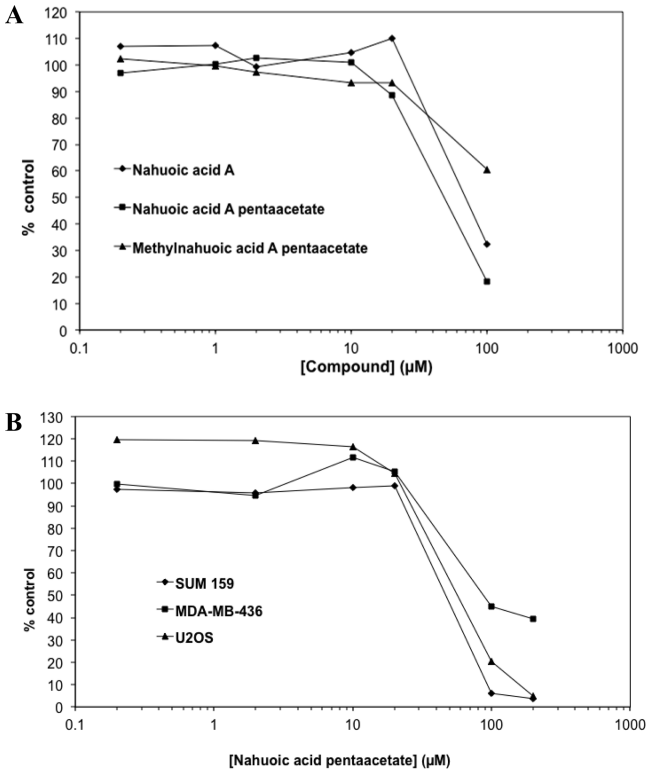

Figure 5. (A) Representative cytotoxicity curves in U2OS osteosarcoma cell line upon exposure to increasing concentrations of nahuoic acid A (◆) and its derivatives nahuoic acid A pentaacetate (■) and methylnahuoic acid A pentaacetate (▲). (B) Cytotoxicity curves in U2OS osteosarcoma cell line (▲), SUM159 (◆), and MDA-MB-436 (■) breast cancer cell lines upon exposure to increasing concentrations of nahuoic acid A pentaacetate. Cytotoxicity was evaluated using the sulforhodamine B technique.

siRNA treatment leads to a decrease in both H4K20me1 and H4K20me3 levels.[10,11] Similarly, treatment of U2OS cells with 65 μM of nahuoic acid A (**1**) led to a significant decrease in the levels of both H4K20me1 and H4K20me3 within 48 h, whereas no significant change was observed for other methylated histone marks such as H3K4me3 and H3K27me3 at this time (Figure 6). These results indicate that this natural product can penetrate into human cancer cells and can induce a partial but specific inhibition of SETD8 activity.

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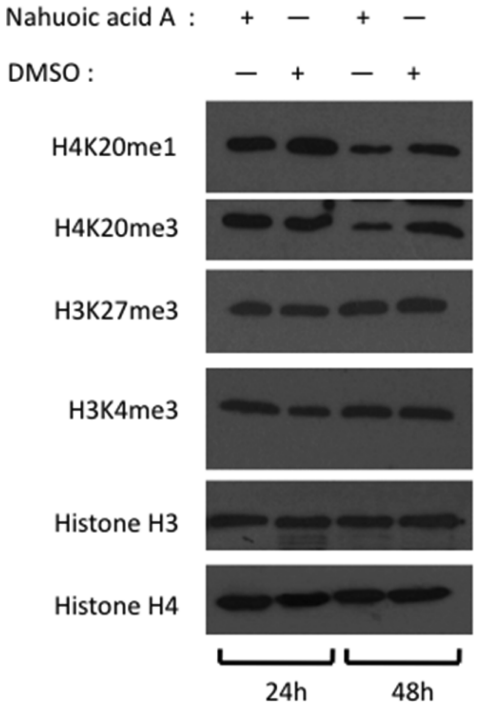

Figure 6. Immunoblot analysis of the levels of histones H3 and H4 and the levels of H4K20me1, H4k20me3, H3K27me3, and H3K4me3 chromatin marks, 24 and 48 h after exposure to 65 μM (IC_{50}) of nahuoic acid A or DMSO.

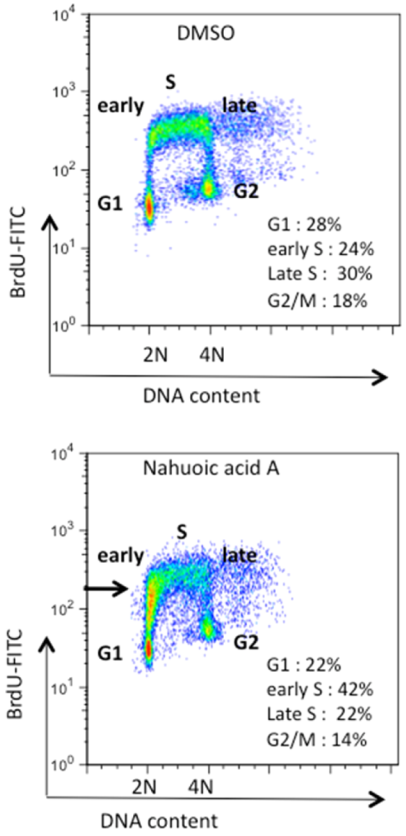

Figure 7. Cell-cycle profile of U2OS cells with DNA content (x axis) and BrdU incorporation levels (y axis) revealed by BrdU-FITC antibody, 3 days after treatment with DMSO or nahuoic acid A (65 μM). G1 cells appeared as cells with 2N DNA content and negative for BrdU, while G2/M cells appeared as cells with 4N DNA content and negative BrdU. Replicating (S phase) cells appear as BrdU positive cells in early S (2N DNA content) and late S (4N DNA content) stages. The black arrow points to the accumulation of U2OS cells at the S phase entry upon nahuoic acid A treatment. The percentage of cells in different cell-cycle phases is indicated for each panel.

To determine whether the SETD8 inhibition induced by the nahuoic acid A (**1**) is sufficient to perturb the cellular functions of this enzyme and perhaps explain the cellular toxicity of this product, the cell-cycle profiles of U2OS cells were analyzed for BrdU incorporation and DNA content by flow cytometry 3 days after DMSO or nahuoic acid A (**1**) treatment. Previous studies have shown that a decrease in levels of H4K20me upon RNAi-mediated SETD8 depletion is characterized by defects in DNA replication (S phase) initiation and progression.[10,11] Consistent with these previous results and compared to control DMSO-treated cells, we observed that U2OS treated with nahuoic acid A (**1**) displayed an abnormal accumulation at the entry of S phase, which is observed by the increase in percentage of BrdU-positive cells with a 2N DNA content (Figure 7). This S-phase phenotype is characteristic of defects in DNA replication process observed in SETD8 depleted U2OS cells,[10,11] thereby suggesting that the cellular toxicity of nahuoic acid A is likely linked to its ability to inhibit SETD8 activity.

CONCLUSIONS

Nahuoic acids B–E (**2**–**5**) represent new members of the nahuoic acid family of polyketides. They differ from nahuoic acid A (**1**) in the hydroxylation patterns on the decalin ring system and in the length and functionality of the C-13 side chain. Nahuoic acid A (**1**) has previously been shown to be a selective SAM competitive inhibitor of the histone methyl transferase SETD8, and the data presented above show that nahuoic acids B–E (**2**–**5**) also inhibit SETD8 with an order of magnitude range of potencies. Given the emerging role of SETD8 in cancer biology, the further characterization of the potential of nahuoic acid A (**1**) and its derivatives to act as a cell biology tools and therapeutic leads becomes of great interest. As part of our continuing exploration of the biological activity of nahuoic acid A (**1**), it has been shown that **1** and its pentaacetate analogue **8** inhibit proliferation of several cancer cell lines in vitro with modest potencies. Furthermore, at the IC_{50} for inhibition of cancer cell proliferation, nahuoic acid A (**1**) showed selective inhibition of SETD8 in U2OS osteosarcoma cells that reflected its selectivity against a panel of pure histone methyl transferases. A cell-cycle analysis revealed that the cellular toxicity of nahuoic acid A (**1**) is likely linked to its ability to inhibit SETD8 activity. There are no other known SAM competitive selective SETD8 inhibitors.[13−16] Therefore, despite their relative lack of potency, nahuoic acid A (**1**) and the new analogues B–E (**2**–**5**) represent interesting lead compounds for the development of more potent SETD8 inhibitory cell biology tools and experimental therapeutic agents.

EXPERIMENTAL SECTION

General Experimental Methods. The ^{1}H and ^{13}C NMR spectra were recorded on a 600 MHz spectrometer with a 5 mm cryoprobe. ^{1}H chemical shifts are referenced to the residual C_6D_6, DMSO-d_6, or C_5D_5N (δ 7.15, 2.49, and 7.22 ppm, respectively), and ^{13}C chemical shifts are referenced to the C_6D_6, DMSO-d_6, or C_5D_5N solvent peak (δ 128.0, 39.5, and 123.87 ppm, respectively). Low- and high-resolution MS were recorded using ESI ionization and a TOF mass analyzer. Merck type 5554 silica gel plates and Whatman MKC18F plates were used for

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analytical thin-layer chromatography. All solvents used for HPLC were HPLC grade.

Extraction of *Streptomyces* sp. and Isolation of Nahuoic Acids A−E (1−5). As described previously,[8] the strain RJA2928 was cultured on 60 pans of solid agar, equivalent to 24 L volume of the marine medium 1 (10.0 g of soluble starch, 4.0 g of yeast extract, 2.0 g of peptone, 0.001g of $FeSO_4.7H_2O$, 0.001 g of KBr, 18.0 g of agar, 1 L pf seawater) at rt for 14 days. The mature cultures were sliced into small squares containing the streptomyces biomass and the media and extracted twice with EtOAc. The combined EtOAc extracts were concentrated in vacuo and partitioned between H_2O (900 mL) and EtOAc (3 × 300 mL). The EtOAc-soluble material was chromatographed on Sephadex LH20 with 4:1 methanol/CH_2Cl_2 as eluent. An early eluting fraction, responsible for the MRSA inhibitory activity, was found to contain a mixture of elaiophylin and nigericin acetyl methyl ester and related analogues. A later eluting fraction was subjected to Si gel flash chromatography (step gradient: 19:1 hexanes/EtOAC to EtOAc to 1:9 MeOH/EtOAc and to MeOH, 10 g Sep pak). The 1:9 MeOH/EtOAc and MeOH fractions contained a mixture of padanamides (actinoramide A)[17] and nahuoic acids and were combined. Pure samples of nahuoic acids A (**1**) (11.5 mg) and D (**4**) (5.0 mg) and impure samples of B (**2**), C (**3**), and E (**5**) were obtained from this fraction via C_8 reversed-phase HPLC using a Phenomenex, 5 μm, Luna 25 × 1.0 cm column, with 7:3 (0.05% TFA/H_2O)/MeCN as eluent. The HPLC fractions containing nahuoic acids B (**2**) and E (**5**) were further purified on C_{18} reversed-phase HPLC using an InertSustain, 5 μm, 25 × 0.46 cm column, with 3:1 (0.05% TFA/H_2O)/MeCN as eluent, and the fraction containing C (**3**) was purified using the same column with 77:33 (0.05% TFA/H_2O)/MeCN as eluent. This resulted in clean samples of nahuoic acids B (**2**) (1.2 mg), C (**3**) (2.1 mg), and E (**5**) (0.5 mg).

*Nahuoic Acid B (**2**).* Isolated as a pale yellow oil: $[\alpha]^{25}_D$ likely positive but unable to confidently assign a value at the concentration used (*c* 0.8, MeOH); UV [3:1 (0.05%TFA/H_2O)/MeCN] λ_{max} 227 nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; positive-ion HRESIMS $[M + Na]^+$ *m*/*z* 545.3455 (calcd for $C_{30}H_{50}O_7Na$, 545.3454).

*Nahuoic Acid C (**3**).* Isolated as a pale yellow oil: $[\alpha]^{25}_D$ −6.6 (*c* 1.4, MeOH); UV [77:33 (0.05%TFA/H_2O)/MeCN] λ_{max} 227 nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; positive-ion HRESIMS $[M + Na]^+$ *m*/*z* 561.3398 (calcd for $C_{30}H_{50}O_8Na$, 561.3403).

*Nahuoic Acid D (**4**).* Isolated as a pale yellow oil: $[\alpha]^{25}_D$ −5.9 (*c* 1.0, MeOH); UV [7:3 (0.05%TFA/H_2O)/MeCN] λ_{max} 227 nm; ^{1}H and ^{13}C NMR, see Table 3; positive-ion HRESIMS $[M + Na]^+$ *m*/*z* 589.3712 (calcd for $C_{32}H_{54}O_8Na$, 589.3716).

*Nahuoic Acid E (**5**).* Isolated as a pale yellow oil: $[\alpha]^{25}_D$ +53.5 (*c* 0.3, MeOH); UV [3:1 (0.05%TFA/H_2O)/MeCN] λ_{max} 227 nm; ^{1}H and ^{13}C NMR, see Table 3; positive-ion HRESIMS $[M + Na]^+$ *m*/*z* 589.3710 (calcd for $C_{32}H_{54}O_8Na$, 589.3716).

Preparation of the Pentaacetate of Nahuoic Acid B (6). On our first attempt to isolate the nahuoic acids, nahuoic acid B (**2**) (<0.8 mg, <1.5 μmol) was collected as a single sharp peak; however, in our hands, the sample was contaminated with coeluting material. In order to aid the purification, nahuoic acid B (**2**) was treated with acetic anhydride in pyridine (1:1, 1 mL) along with a catalytic amount of DMAP and stirred at rt for 17 h. After drying, the resulting gum was purified via C_{18} reversed-phase HPLC using a InertSustain, 5 μm, 25 × 0.46 cm column, with 3:2 MeCN/(0.05% TFA/H_2O) as eluent to yield 0.1 mg of the pentaacetate **6**.

*Nahuoic Acid B Pentaacetate (**6**).* Isolated as a clear glass: $[\alpha]^{25}_D$ +166.7° (*c* 0.07, MeOH); UV (3:2 MeCN/(0.05%TFA/H_2O)) λ_{max} 227 nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; negative ion HRESIMS $[M - H]^-$ *m*/*z* 731.4016 (calcd for $C_{40}H_{59}O_{12}$, 731.4007).

Preparation of Ester Derivative 7. Nahuoic acid D (**4**) (0.2 mg, 0.4 μmole) along with a catalytic amount of pyridinium *p*-toluenesulfonate (~0.1 mg) was dissolved in a 0.8 mL solution of 1:1 dimethoxypropane/CH_2Cl_2 and stirred at 37−40 °C for 17 h. The reaction was quenched with saturated aqueous $NaHCO_3$, and the resulting solution extracted with EtOAc (3 × 0.5 mL). The combined EtOAc extracts were evaporated to dryness and without purification the resulting residue was stirred with *p*-bromophenacyl bromide (1.0 mg) dissolved in 7:3 DMF/Et_3N (1.0 mL) at 45−50 °C for 17 h. The reaction was quenched with saturated aqueous $NaHCO_3$ and the resulting solution extracted with EtOAc (3 × 0.75 mL). The EtOAc soluble material was fractionated by Si gel flash chromatography (step gradient: 19:1 hexanes/EtOAC to EtOAc to 1:9 MeOH/EtOAc, 2 g of Sep pak). The 1:1 hexanes/EtOAc fraction contained derivative **7**. A pure sample of **7** (<0.1 mg) was obtained from this fraction via C_{18} reversed-phase HPLC using a CSC-Inertsil 150A/ODS 2.5 μm 25 × 0.94 cm column with 85:15 MeCN/H_2O as eluent.

*Ester Derivative **7**.* Isolated as a clear glass; UV (7:3 (MeCN/H_2O) λ_{max} 257 nm; ^{1}H NMR (C_6D_6) δ 7.26 (d *J* = 8.4 Hz) (H-36/40), 7.09 (d *J* = 8.4 Hz) (H-37−39), 6.89 (bd *J* = 10.1 Hz) (H-3), 5.33 (bt *J* = 6.8 Hz) (H-15), 5.04 (d *J* = 16.4 Hz) (H-33b), 4.89 (bs) (H-11), 4.89 (d *J* = 16.4 Hz) (H-33a), 4.18 (btd *J* = 7.4, 1.0 Hz) (H-21), 4.14 (dddd *J* = 6.2, 6.2, 6.2, 6.2 Hz) (H-19), 3.95 (dddd *J* = 7.4, 7.4, 7.4, 7.4 Hz) (H-17), 3.88 (td *J* = 11.3, 4.2 Hz) (H-7), 3.59 (ddd *J* = 10.1,10.1,10.1 Hz) (H-4), 3.37 (bs) (H-9), 3.26 (dd *J* = 9.6, 1.3 Hz (H-23), 2.44 (m) (H-16b), 2.29 (8_{eq}), 2.27 (H-16a), 2.26 (H-13), 2.12 (dt, *J* = 13.6, 7.4 Hz) (20b), 2.02 (bs) (H-26), 2.01 (H-6), 1.75 (H-24), 1.74 (H-18b), 1.67 (H-18a), 1.66 (H-20a), 1.65 (8_{ax}), 1.50 (bs) (H-29), 1.50 (s) (H-46), 1.49 (H-5), 1.48 (s) (H-43), 1.47 (H-22), 1.46 (bs) (H-30), 1.43 (s) (H-42), 1.34 (s) (H-45), 1.22 (d *J* = 7.3 Hz) (H-27), 1.05 (d *J* = 6.8 Hz) (H-25), 0.95 (d *J* = 6.8 Hz) (H-31), 0.87 (s) (H-28), 0.68 (d *J* = 6.8 Hz) (H-32) ppm; ^{1}H NMR (C_5D_5N) δ 7.98 (d *J* = 8.4 Hz) (H-36/40), 7.67 (d *J* = 8.4 Hz) (H-37−39), 7.16 (bd *J* = 10.5 Hz) (H-3), 5.79 (d *J* = 16.4 Hz) (H-33b), 5.74 (d *J* = 16.4 Hz) (H-33a), 5.44 (H-11), 5.42 (H-15), 4.46 (td *J* = 10.3, 4.0 Hz) (H-7), 4.28 (bt *J* = 6.4 Hz) (H-21), 4.22 (H-19), 4.21 (H-4), 4.04 (dddd *J* = 6.5, 6.5, 6.5, 6.5 Hz) (H-17), 4.00 (bs) (H-9), 3.42 (bd *J* = 10.0 Hz (H-23), 2.74 (bd *J* = 13.5 Hz) (8_{eq}), 2.56 (bd *J* = 8.1 Hz) (H-13), 2.52 (H-6), 2.51 (H-16b), 2.38 (m) (H-16a), 2.30 (ddd *J* = 13.5, 10.3, 3.8 Hz) (8_{ax}), 2.14 (ddd, *J* = 13.3, 6.4, 6.4 Hz) (20b), 2.02 (bs) (H-26), 1.80−1.88 (H-18a/18b), 1.77 (bs) (H-30), 1.75 (H-24), 1.75 (H-20a), 1.72 (bs) (H-29), 1.60 (H-22), 1.90 (bdd *J* = 11.3, 3.5 Hz) (H-5), 1.60 (d *J* = 7.1 Hz) (H-27), 1.51 (bs) (H-43), 1.51 (bs) (H-46), 1.47 (bs) (H-42), 1.47 (s) (H-45), 1.25 (s) (H-28), 1.04 (d *J* = 6.4 Hz) (H-25), 0.96 (d *J* = 6.8 Hz) (H-31), 0.77 (d *J* = 6.7 Hz) (H-32) ppm; a complete ^{13}C NMR spectrum is not available due to limited material; partial ^{13}C NMR (data obtained form HSQC and HMBC data, C_6D_6) δ 150.6 (C-3), 132.0 (C-37/39), 130.9 (C-11), 129.4 (C-36/40), 126.3 (C-15), 79.6 (C-23), 74.2 (C-9), 69.9 (C-21), 68.1 (C-7), 66.9 (C-17), 66.1 (C-33), 63.5 (C-19), 56.6 (C-13), 49.9 (C-5), 39.3 (C-20), 38.9 (C-6), 38.4 (C-8), 38.4 (C-18), 36.9 (C-4), 35.2 (C-16), 32.6 (C-22), 30.5 (C-46), 29.6 (C-24), 27.2 (C-28), 25.2 (C-42), 25.1 (C-43), 21.9 (C-29), 20.0 (C-25), 19.8 (C-45), 18.2 (C-27), 17.5 (C-32), 13.7 (C-26), 12.6 (C-30), 5.1 (C-31), ppm; ^{13}C NMR (C_5D_5N) δ 152.3 (C-3), 133.5 (C-11), 133.0 (C-37/39), 130.4 (C-36/40), 125.7 (C-15), 79.8 (C-23), 74.6 (C-9), 70.3 (C-21), 67.9 (C-7), 67.4 (C-17), 67.2 (C-33), 64.1 (C-19), 57.7 (C-13), 51.2 (C-5), 41.2 (C-8), 40.2 (C-6), 39.6 (C-20), 38.7 (C-18), 37.6 (C-4), 35.6 (C-16), 32.8 (C-22), 30.7 (C-46), 29.8 (C-24), 28.2 (C-28), 25.5 (C-42), 25.4 (C-43), 22.5 (C-29), 21.2 (C-25), 21.1 (C-45), 19.3 (C-27), 17.6 (C-32), 13.9 (C-26), 13.1 (C-30), 5.4 (C-31) ppm; positive-ion HRESIMS $[M + Na]^+$ *m*/*z* 865.3852 (calcd for $C_{46}H_{67}O_9BrNa$, 865.3866).

Preparation of the Pentaacetate of Nahuoic Acid A (8). Nahuoic acid A (**1**) (1.0 mg, 1.9 μmol) was treated with acetic anhydride in pyridine (1:1, 1 mL) along with a catalytic amount of DMAP and stirred at rt for 5 days. After drying, the resulting gum was purified via C_{18} reversed-phase using a InertSustain, 5 μm, 25 × 0.46 cm column, with 3:2 MeCN/(0.05% TFA/H_2O) as eluent to yield 0.5 mg of the pentaacetate **8**.

*Nahuoic Acid A Pentaacetate (**8**).* Isolated as a clear glass; UV (3:2 MeCN/(0.05%TFA/H_2O)) λ_{max} 227 nm; ^{1}H NMR spectrum see the Supporting Information; a ^{13}C NMR spectrum is not available due to limited material; positive-ion HRESIMS $[M + Na]^+$ *m*/*z* 755.3999 (calcd for $C_{40}H_{60}O_{12}Na$, 755.3982).

Preparation of the Methyl Ester of the Pentaacetate of Nahuoic Acid A (9). Nahuoic acid A (**1**) (0.6 mg, 1.2 μmole) was treated with acetic anhydride in pyridine (1:1, 1 mL) along with a catalytic amount of DMAP and stirred at rt for 16 h. After drying

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and without further purification, the resulting gum was treated with diazomethane that was generated in situ by the addition of 0.5 mL of 2.0 M trimethylsilyldiazomethane in hexanes to 0.5 mL of anhydrous MeOH in 0.6 mL of C_6H_6. The reaction mixture was allowed to stir for 16 h at rt. After evaporation of the reagents, the sample was purified via C_{18} reversed-phase HPLC using a InertSustain, 5 μm, 25 × 0.46 cm column, with 7:3 MeCN/(0.05% TFA/H_2O) as eluent to yield 0.5 mg of the methyl ester of the pentaacetate of nahuoic acid A (**9**).

*Methyl Ester of the Pentaacetate of Nahuoic Acid A (**9**).* Isolated as a clear glass: UV (7:3 MeCN/(0.05%TFA/H_2O)) λ_{max} 226 nm; ^{1}H NMR, see the Supporting Information; ^{13}C NMR, not available due to limited material; positive-ion HRESIMS $[M + Na]^+$ m/z 769.4131 (calcd for $C_{41}H_{62}O_{12}Na$, 769.4139).

IC_{50}'s for in Vitro SETD8 Inhibition. See reference 8, Supporting Information.

Cell Culture. U2OS and MDA-MB-436 Cells were grown in DMEM (Dubelcco's Modified Eagle's Medium) with 10% fetal bovine serum supplemented with penicillin and streptomycin. SUM159 cells were grown in Ham's F-12 with 5% FBS and supplemented with glutamine, insulin (10 μg/mL), hydrocortisone (1 μg/mL), and penicillin and streptomycin. For the treatment with nahuoic acid A (**1**) and its derivatives, these products were resuspended in DMSO at 0.5 mg/mL and directly diluted into culture medium at the appropriate concentration.

Immunoblot Analysis. After treatment with nahuoic acid A (**1**) or DMSO, cells were harvested and washed with phosphate-buffered saline (PBS) before lysis in Laemmli SDS-PAGE buffer and protein quantification by Bradford assay. Equal amounts of protein were then resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the following antibodies: anti-histone H4, H4K20me1, and anti-K20me3 (1:1000; cell signaling), anti-histone H3 (1:5000, Diagenode), anti-H3K4me3 and anti-H3K27me3 (1:1000, cell signaling).

Flow Cytometry. Cells were incubated with 50 μM BrdU for 1 h, fixed with a 70% ethanol solution, and then permeabilized with 0.2% Triton X-100 for 10 min. Cells were then treated with 0.2 N HCl before staining with mouse antibody to BrdU (1:30 diluted in PBS with 0.2% Tween20, 1% bovine serum albumin) for 1 h at room temperature, followed by 2 h incubation with an FITC-conjugated antibody (BD; 1:300). DNA was then counterstained by overnight incubation with 7-aminoactinomycin D (7AAD, 1:50) in the presence of RNase (50 μg/mL). Cell cycle profiles were acquired with a flow cytometer and analyzed with the FlowJo software.

Cytotoxicity Assay. Cytotoxicity studies were performed using the sulforhodamine B assay based on the percentage of cell growth compared to a control after exposure to increasing drug concentrations. The cytotoxicity of each drug was evaluated by the IC_{50} value. On day 1, 2000 U2OS or SUM159 cells and 4000 MDA-MB-436 cells were seeded onto 96-well plates in a volume of 150 μL medium per well. On day 2, they were exposed to increasing (0.5−100 μM) concentrations of nahuoic acid A (**1**) and its derivatives for 24 h. After drug exposure, the medium in control and treated wells was removed, and cells were washed with PBS and grown in fresh medium for three doubling times. Cells were then fixed overnight with ice-cold 12.5% trichloroacetic acid, washed with tap water, and air-dried. Fixed cells were dyed with 0.4% sulforhodamine B in 1% acetic acid solution, washed with 1% acetic acid, and air-dried. Sulforhodamine B was then extracted with 10 mM Tris buffer (pH 10.5), and 540 nm optical density was quantitated using a microplate reader.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b02569.

1D and 2D NMR spectra for **2**−**9** (PDF)

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Notes

The authors declare no competing financial interest.

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