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Three new cyclostellettamines, which inhibit histone deacetylase, from a marine sponge of the genus *Xestospongia* ☆

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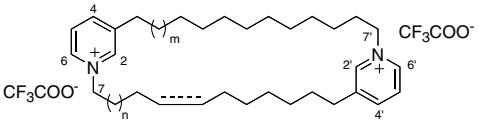
Abstract—Three new cyclostellettamines, cyclostellettamine G (**1**), dehydrocyclostellettamines D (**2**), and E (**3**), were isolated together with the known cyclostellettamine A (**4**) from a marine sponge of the genus *Xestospongia* as histone deacetylase inhibitors. Their structures were determined by spectral and chemical methods. They inhibit histone deacetylase derived from K562 human leukemia cells with IC_{50} values ranging from 17 to 80 μM.

Histone deacetylase enzymes (HDACs), which catalyze deacetylation of the acetylated lysine residues of histones are an emerging therapeutic target for the treatment of cancer.[1–4] It has been shown that HDAC inhibitors affect differentiation, growth arrest, and apoptosis in transformed cells. In fact, such inhibitors as SAHA (hydroxamate), MS-275 (benzamide), and FK228 (cyclic peptide) are currently under clinical trials.[2,3] In our program to discover novel HDAC inhibitors from Japanese marine invertebrates, we found the aqueous extract of a marine sponge of the genus *Xestospongia* collected off Shishijima Island, Southern Japan, inhibited HDAC. Bioassay-directed fractionation of the extract gave three new congeners of cyclostellettamines, cyclic 3-alkyl pyridinium dimers; we first isolated cyclostellettamines as muscarinic acetylcholine receptor antagonists from a marine sponge *Haliclona* sp.[5]

The EtOH extract of the frozen sponge[6] (500 g, wet wt) was partitioned between Et_2O and H_2O. The aqueous layer was extracted with *n*-BuOH, and the *n*-BuOH layer was further partitioned between CH_2Cl_2 and 60% MeOH. The latter layer was subsequently fractionated by ODS (aqueous MeOH), TSK G3000S (aqueous MeCN–5% AcOH), and Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) chromatographies to give a fraction enriched with cyclostellettamines (1.47 g). A portion of the mixture was further purified by HPLC on phenylethyl-SiO_2 (MeCN–H_2O, 52:48, containing 0.1% TFA) and finally by HPLC on C_{30}-SiO_2 (aqueous *n*-PrOH containing 0.1% TFA) to yield cyclostellettamine G (**1**: 0.5 mg; 3.1×10^{-3}%),[7] dehydrocyclostellettamine D (**2**: 1.5 mg; 9.4×10^{-3}%),[8] and dehydrocyclostellettamine E (**3**: 1.3 mg; 8.2×10^{-3}%),[9] together with the known cyclostellettamine A (**4**: 2.8 mg; 1.8×10^{-4}%) each as the TFA salt.



1: m=1, n=1
2: m=1, n=4, Δ^{13}
3: m=2, n=4, Δ^{13}
4: m=1, n=2

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The molecular formula of **1** was established as $(C_{33}H_{54}N_2)^{2+}\cdot(CFCOO^-)_2$ on the basis of FABMS data with ion peaks at m/z 477 $[M-H]^+$ and m/z 591 $[M-H+CF_3CO_2H]^+$, arising from the cleavage of a C–N bond.[5] The ^1H NMR spectrum of **1** was almost identical to that of **4**.[5] The UV absorption at 268 nm (ε 4700) was reminiscent of pyridine rings, which was corroborated by four aromatic ^1H NMR signals [δ 8.88 (2H, br s, H-2/2′), 8.45 (2H, d, $J = 8.1$ Hz, H-4/4′), 8.02 (2H, dd, $J = 8.1$, 6.2 Hz, H-5/5′), 8.81 (2H, d, $J = 6.2$ Hz, H-6/6′)].[5] Analysis of 2D NMR spectra led to a pair of 1,3-disubstituted pyridinium units, which were substituted by methylene chains. The lengths of the methylene chains were determined on the basis of the FAB-MS/MS data, which gave two intense peaks at m/z 232 and 246. Because these daughter ions were attributable to a pair of monomeric products generated via Hoffmann-type elimination[10] from their parent ion, **1** was suggested to have C_{11} and C_{12} chains.

The molecular formulas of **2** and **3** were determined to be $C_{36}H_{58}N_2$ and $C_{37}H_{60}N_2$, respectively, indicating that they both have one more unsaturation than **1**. The ^1H NMR spectra of **2** and **3** were almost identical and consisted of signals for 1,3-disubstituted pyridine, methylene chains, two degenerated olefinic protons [**2**: δ 5.31 (2H, m, H-13/14); **3**: 5.33 (2H, m, H-13/14)], and a pair of allylic methylene protons [**2**: δ 1.98 (4H, m, H-12/15); **3**: 2.00 (4H, m, H-12/15)]. Therefore, both **2** and **3** had one double bond in the alkyl chains. The *Z*-geometry of the double bond in **2** and **3** was deduced from the ^{13}C chemical shift values of the allylic methylene [**2**: δ 28.1; **3**: 28.0].[11]

Two prominent daughter ions at m/z 246 and 272 in the tandem FAB mass spectrum of **2** demonstrated the presence of a C_{12}-saturated chain and a C_{14}-chain with one double bond. The location of the double bond, which could not be assigned by NMR data due to severely overlapped methylene signals, was determined by an analysis of the FABMS data after oxidation of the double bond. Compound **2** was treated with a mixture of OsO_4 and $NaIO_4$ and the reaction mixture was subjected to FABMS analysis. The mass spectrum gave an intense ion at m/z 549, in accordance with the introduction of two oxygen atoms.[12] In sharp contrast to the FABMS of **2**, which was virtually devoid of fragment ions, two sequential fragment ions were observed at m/z 358 and 437. Being an even number the ion at m/z 358 should contain one nitrogen atom, while the ion at m/z 437 contain two nitrogen atoms, which was consistent with the Δ^{12} double bond (Fig. 1). The lengths of the alkyl chains and the location of the double bond in **3** were similarly determined.[13]

New cyclostellettamines and cyclostellettamine A inhibited histone deacetylase from K562 human leukemia cells with IC_{50} values of 17–80 μM (Table 1).[14] The

Table 1. HDAC inhibitory activity and cytotoxicity against P388, HeLa, and 3Y1 cells of **1**–**4** (IC_{50}, μM)

	1	2	3	4
HDAC	80	17	30	61
P388	2.7	1.3	1.3	2.1
HeLa	2.8	0.60	1.8	1.8
3Y1	11	4.3	3.2	7.2

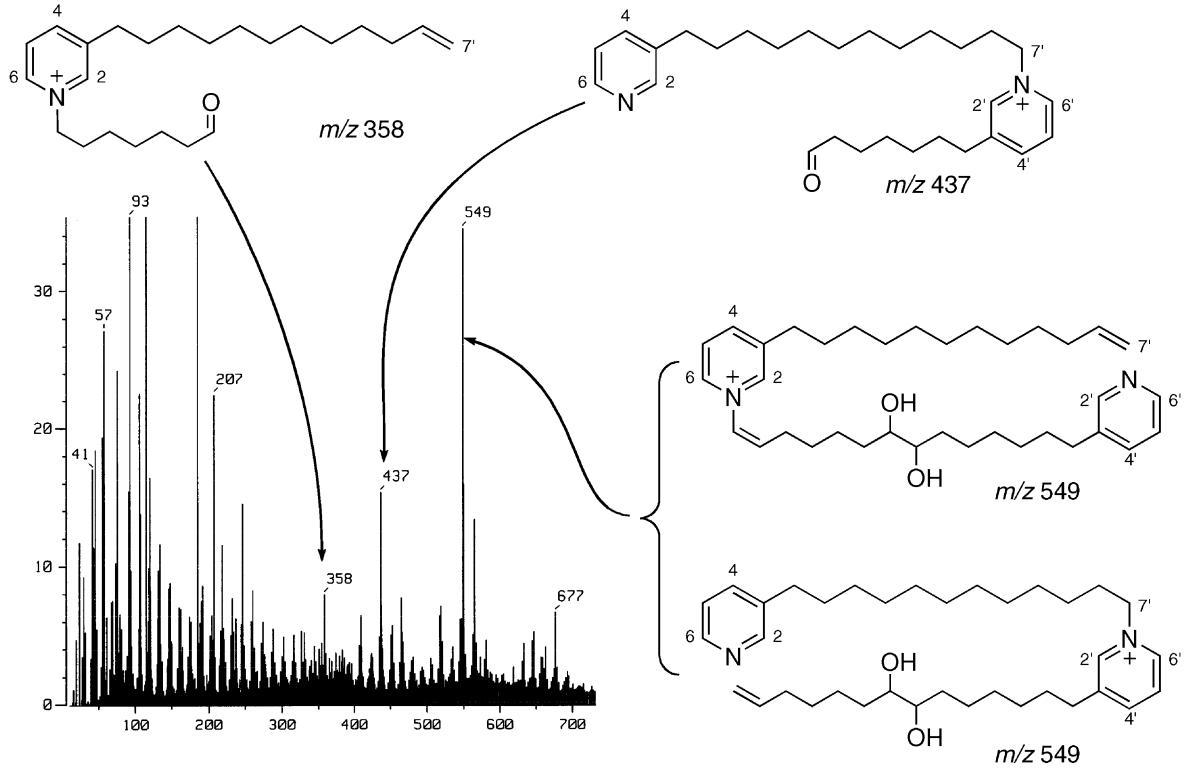

Figure 1. The FABMS spectrum for the oxidation products of **2**.

cyclostellettamines showed moderate cytotoxicity against HeLa human cervix carcinoma, P388 mouse leukemia, and 3Y1 rat fibroblastic cells (Table 1). Although 1,3-alkyl pyridinium salts (1,3-APS) are reported to exhibit a wide range of bioactivities, such as cytotoxic,[17] toxic,[17] hemolytic,[17,18] ichthyotoxic,[17] antimicrobial,[17,19] antifeedant,[20] neurotoxic,[18] and antifouling,[19] this is the first report for their HDAC inhibitory activity.[21]

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6. The sponge was collected at a depth 15 m off Shishijima Island in The Amakusa Islands, Southern Japan (N 32°17′, E 130°12′), during the cruise on R/V Toyoshiomaru of Hiroshima University in July, 1999. The massive lobate beige sponge was identified as *Xestospongia* sp., which is probably close to *X. exigua*, *X. subtriangularis*, and *X. proxima*. A voucher specimen (ZMA POR 16161) was deposited at the Zoological Museum of the University of Amsterdam.
7. Compound **1**: colorless solid; HRFABMS (pos, glycerol–thioglycerol–HCl=100:100:0.1) m/z 477.4195 $[M-H]^+$ ($\Delta-1.4$ mmu, calcd for $C_{33}H_{53}N_2$); UV (MeOH) λ_{max} 203 (ε 13000), 216 (sh) (6400), 268 (4700), and 274 (sh) (4000) nm; ^{13}C NMR (CD_3OD, 125 MHz, 300 K) δ 145.3 (C-2/2′), 145.4 (C-3/3′), 147.1 (C-4/4′), 129.3 (C-5/5′), 143.7 (C-6/6′), 62.9 (C-7/7′), 32.1 (C-8/8′), 26.7 (C-9/9′), 30.1–30.5 (C-10–14/10′–15′), 29.8 (C-15/16′), 31.2 (C-16/17′), 33.3 (C-17/18′); ^{1}H NMR (CD_3OD, 600 MHz, 300 K) δ 8.88 (2H, br s, H-2/2′), 8.45 (2H, d, $J=8.1$ Hz, H-4/4′), 8.02 (2H, dd, $J=6.2$, 8.1 Hz, H-5/5′), 8.81 (2H, d, $J=6.2$ Hz, H-6/6′), 4.61 (4H, t, $J=6.7$ Hz, H-7/7′), 1.99 (4H, m, H-8/8′), 1.31 (H-9/9′), 1.22–1.31 (H-10–14/10′–15′), 1.35 (H-15/16′), 1.73 (4H, quint, $J=7.2$ Hz, H-16/17′), 2.89 (4H, dt, $J=1.9$, 7.3 Hz, H-17/18′).
8. Compound **2**: colorless solid; HRFABMS (pos, glycerol–thioglycerol–HCl = 100:100:0.1) m/z 517.4503 $[M-H]^+$ ($\Delta-1.9$ mmu, calcd for $C_{36}H_{57}N_2$); UV (MeOH) λ_{max} 203 (ε 18,000), 215 (sh) (9000), 269 (7700), and 274 (sh) (6500) nm; ^{13}C NMR (CD_3OD, 125 MHz, 300 K) δ 145.2 (C-2/2′), 145.7 (C-3/3′), 146.9 (C-4/4′), 129.0 (C-5/5′), 143.4 (C-6/6′), 62.8 (C-7/7′), 32.2 (C-8/8′), 26.8 (C-9/9′), 30.0–30.5 (C-10/17/10′–15′), 30.3 (C-11/16), 28.1 (C-12/15), 130.7 (C-13/14), 29.3 (C-18/16′), 31.3 (C-19/17′), 33.3 (C-20/18′); ^{1}H NMR (CD_3OD, 600 MHz, 300 K) δ 8.89 (2H, br d, $J=1.5$ Hz, H-2/2′), 8.45 (2H, dd, $J=7.7$, 1.4 Hz, H-4/4′), 8.01 (2H, dd, $J=7.9$, 6.1 Hz, H-5/5′), 8.81 (2H, ddd, $J=6.1$, 1.5, 1.2 Hz, H-6/6′), 4.61 (4H, br t, $J=7.0$ Hz, H-7/7′), 2.01 (4H, m, H-8/8′), 1.28 (H-9/9′), 1.26–1.34 (H-10/17/10′–15′), 1.33 (H-11/16), 1.98 (4H, m, H-12/15), 5.31 (2H, m, H-13/14), 1.32 (H-18/16′), 1.73 (4H, m, H-19/17′), 2.89 (4H, br t, $J=7.3$ Hz, H-20/18′).
9. Compound **3**: colorless solid; HRFABMS (pos, glycerol–thioglycerol–HCl = 100:100:0.1) m/z 531.4681 $[M-H]^+$ ($\Delta+0.3$ mmu, calcd for $C_{37}H_{59}N_2$); UV (MeOH) λ_{max} 204 (ε 19000), 215 (sh) (9400), 269 (8600), and 274 (sh) (7200) nm; ^{13}C NMR (CD_3OD, 125 MHz, 300 K) δ 145.2 (C-2/2′), 145.6 (C-3/3′), 146.8 (C-4/4′), 129.1 (C-5/5′), 143.3 (C-6/6′), 63.0 (C-7/7′), 32.2 (C-8/8′), 26.8 (C-9/9′), 30.0–30.5 (C-10/17/10′–16′), 30.6 (C-11/16), 28.0 (C-12/15), 130.6 (C-13/14), 29.7 (C-18/17′), 31.2 (C-19/18′), 33.4 (C-20/19′); ^{1}H NMR (CD_3OD, 600 MHz, 300 K) δ 8.92 (2H, br s, H-2/2′), 8.47 (2H, d, $J=7.7$ Hz, H-4/4′), 8.03 (2H, br t, $J=7.3$ Hz, H-5/5′), 8.83 (2H, d, $J=6.2$ Hz, H-6/6′), 4.62 (4H, br t, $J=8.1$ Hz, H-7/7′), 2.02 (4H, m, H-8/8′), 1.27 (H-9/9′), 1.26–1.35 (H-10/17/10′–16′), 1.36 (H-11/16), 2.00 (4H, m, H-12/15), 5.33 (2H, m, H-13/14), 1.36 (H-18/17′), 1.75 (4H, m, H-19/18′), 2.91 (4H, br t, $J=7.5$ Hz, H-20/19′).
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12. The ion at m/z 549 was most likely derived from a diol, because the dial does not give this ion peak after Hoffman-type fragmentation. It is likely that the reaction product contained both the diol and the dial.
13. The FAB-MS/MS spectrum of **3** showed two daughter ions at m/z 272 and 260 and the $OsO_4/NaIO_4$ oxidation product gave prominent ion peaks at m/z 576 (HI adduct of the ion at 451), 563, and 451 in the FABMS. The ion at m/z 451 was a methylene homologue of the ion at 437 in the mass spectrum of the oxidation product of **2**. These data proved that **2** and **3** have an identical alkyl chain with one double bond and that **3** is one methylene carbon longer than **2** in the saturated chain.
14. Inhibitory activity against histone deacetylase was determined as follows. Human HDAC was partially purified from K562 cells (human chronic myeloid leukemia cell line) by a modification of the method of Yoshida et al.[15] In brief, cultured cells were collected and resuspended in HDA buffer (15 mM potassium phosphate pH 7.5, 5% glycerol, 0.2 mM EDTA) and homogenized with a Dounce homogenizer. Nuclei were collected and suspended in HDA buffer containing 1 M ammonium sulfate, and the suspension was sonicated to reduce its viscosity. After collecting the supernatant by centrifugation at 500g for 15 min at 4 °C, ammonium sulfate was added to the supernatant to attain a final concentration of 3.5 M. The precipitated protein was collected, dissolved in HDA buffer, and then dialyzed against the same buffer at 4 °C. The dialyzate was loaded onto a Q-Sepharose FF (Amersham Bioscience) column equilibrated with HDA buffer, and eluted with a linear gradient of 0–1 M NaCl in HDA buffer. HDAC activity was eluted between 0.3 and 0.4 M NaCl. The fraction associated with HDAC activity was collected, dialyzed against HDA buffer, and stored at 4 °C. Biotinylated [^{3}H] acetyl-histone H4 peptide (Biotin-Gly-Ala-[^{3}H-acetyl] Lys-Arg-His-Arg-[^{3}H-acetyl] Lys-Val-amide, 90 Ci/mmol; Amersham Bioscience) was used as a

substrate for HDAC.[15,16] Various concentrations of compounds (2 μL) was incubated with 25 μL of [^{3}H] acetylhistone H4 peptide in HDA buffer at room temperature and then 25 μL of HDAC enzyme solution was added to the substrate solutions and incubated at room temperature for 2 h. Each reaction was stopped with 50 μL of 1 M HCl and the released [^{3}H] acetic acid was extracted with 1 mL of ethyl acetate. After centrifugation at 12,000*g* for 1 min, the radioactivity in the organic layer (0.8 mL) was measured by a liquid-scintillation counter in 5 mL Aquasol-2 (PACKARD).

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