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Cyclostellettamines A-F, Pyridine Alkaloids Which Inhibit Binding of Methyl Quinuclidinyl Benzilate (QNB) to Muscarinic Acetylcholine Receptors, from the Marine Sponge, *Stelletta maxima*[1]

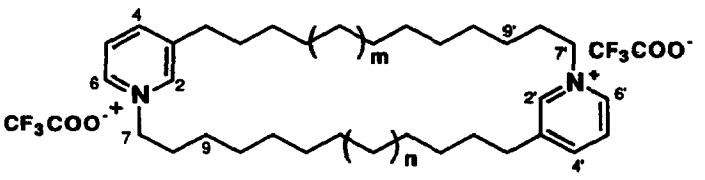
Nobuhiro Fusetani,* Naoki Asai, and Shigeki Matsunaga
Laboratory of Marine Biochemistry, Faculty of Agriculture,
The University of Tokyo, Bunkyo-ku, Tokyo, 113, Japan

Kazuo Honda and Kenichi Yasumuro
Central Laboratories, Yamanouchi Pharmaceutical Co. Ltd.
Itabashi-ku, Tokyo, 175, Japan

Abstract: Six new pyridine alkaloids, cyclostellettamines A-F were isolated as muscarinic receptor binding inhibitors from the marine sponge *Stelletta maxima* , and their structures were elucidated on the basis of spectroscopic data.

Muscarinic acetylcholine receptors play important roles in various physiological functions including memory and learning.[2] Molecular cloning studies have shown the presence of five distinct muscarinic receptor proteins which are related to pharmacological heterogeneity. Muscarinic receptors are now known to be correlated with some disease states; their agonists or antagonists may be potential drugs.[3] In our screening of muscarinic receptor antagonists from Japanese marine invertebrates, the hydrophilic extract of the sponge *Stelletta maxima*[4] collected off the Sata Peninsula, Shikoku, inhibited binding of [^{3}H]-methyl quinuclidinyl benzilate (QNB), a selective antagonist, to muscarinic receptors. Purification guided by the radioligand binding assay[5] afforded six new compounds, cyclostellettamines A-F (**1**-**6**), which are macrocyclic bis-pyridines linked through two C_{12}-C_{14} alkyl chains. We describe the isolation and structure elucidation of these metabolites.

The EtOH extract of the frozen sponge (1.0 kg, wet weight) was partitioned between ether and water; the aqueous phase was extracted with *n*-BuOH. The ether soluble portion was further partitioned between aqueous methanol and the solvent series of *n*-hexane, CCl_4, and CH_2Cl_2. The aqueous methanol and *n*-BuOH layers were combined and gel-filtered on Sephadex LH-20 with MeOH. The active fractions were separated on an Al_2O_3 column ($CHCl_3$/MeOH/H_2O, 7:3:0.5), followed by reversed phase HPLC (47% MeCN, 0.1% TFA) to yield cyclostellettamines A (**1**, 0.22 mg yield), B (**2**, 0.33 mg), C (**3**, 0.34 mg), D (**4**, 0.36 mg), E (**5**, 0.31 mg), and F (**6**, 0.22 mg), presumably as TFA salts.



1	**m=1, n=1**
2	**m=1, n=2**
3	**m=2, n=2**
4	**m=1, n=3**
5	**m=2, n=3**
6	**m=3, n=3**

We tried to elucidate the structure of cyclostellettamine C which gave a simple FABMS pattern. The ^{1}H NMR spectrum of cyclostellettamine C exhibited signals of mutually coupled pyridine protons [δ 8.87 (brs),

8.81 (d, J=6.1 Hz), 8.45 (d, J=7.9 Hz), and 8.02 (dd, J=7.9, 6.1 Hz)] whose chemical shifts and coupling constants were reminiscent of a 1,3-disubstituted pyridinium moiety.[6] Although the presence of three contiguous methylene units at N1 and C3 was established on the basis of COSY and HMBC data, no other signal than a huge methylene envelope at δ 1.20-1.40 could be observed in the ^{1}H NMR spectrum, indicating that **3** was a symmetrical oligomer composed of disubstituted pyridine rings and long aliphatic chains. Signal integration in the ^{1}H NMR spectrum suggested the length of methylene chain to be 13-15.

In the positive FABMS,[7] **3** gave charasteristic ions at m/z 519 (two monomer units-H$^+$), 555 (two monomer units+Cl$^-$) and 633 (two monomer units+CF_3COO^-), which demonstrated that **3** was a cyclic dimer with two positively charged nitrogens. The generation of the [M-H]$^+$ ion can be rationalized as the result of Hoffmann-type elimination during the FAB ionization process.[8] The size of alkyl chains in **3** was unambiguously established by an FAB-MS/MS experiment, in which an intense daughter ion was observed at *m/z* 260 as shown in Fig.1 (a), thereby confirming a symmetrical structure. Thus, the structure of cyclostellettamine C was as shown. It should be noted that synthetic cyclostellettamine C was identical with the natural product in HPLC, FABMS and ^{1}H NMR.[9]

Once the methodology for structure elucidation of this type of compounds was established, the structure elucidation of the remaining compounds was straightforward. They exhibited almost identical ^{1}H NMR spectra, suggesting that the structural difference among **1-6** lay in the size of alkyl chains.

Cyclostellettamine A (**1**),[10] with the shortest retention time in the reversed phase HPLC, had a molecular formula of $C_{34}H_{56}N_2$ which was determined by a HRFAB mass spectrum, thus suggesting that **1** was two methylene units smaller than **3**. The FAB-MS/MS spectrum showed a characteristic fragment peak at *m/z* 246, thereby confirming the structure of **1**.

The molecular formula of cyclostellettamine B (**2**),[11] $C_{35}H_{58}N_2$, indicated that one of alkyl chains had an odd number of methylene units, while the other an even number of methylene units, thus indicating an

Table 1. NMR Data of Cyclostellettamine C (**3**) in CD_3OD

	^{1}H	^{13}C[a]	^{1}H-^{1}H COSY correlations	HMBC correlations
2,2'	8.87 (1H, brs)	144.5 d		C-4,4', C-6,6'
3,3'		146.2 s		
4,4'	8.45 (1H, d, 7.9)	146.2 d	H-5,5'	C-2,2', C-6,6'
5,5'	8.02 (1H, dd, 7.9, 6.1)	128.3 d	H-4,4', H-6,6'	C-3,3', C-6,6'
6,6'	8.81 (1H, d, 6.1)	142.5 d	H-5,5'	C-2,2', C-4,4' C-5,5'
7,7'	4.61 (2H, t, 6.7)	62.7 t	H-8,8'	C-2,2', C6,6'
8,8'	2.00 (2H, quint, 7.0)	31.9 t	H-7,7', H-9,9'	
9,9'	1.23 (m)	26.4 t	H-8,8'	
10-15, 10'-15'	1.20-1.40 (br)	29-31 br		
16,16'	1.26 (m)	29-31 br	H-17,17'	
17,17'	1.73 (2H, quint, 7.2)	30.8 t	H-16,16' H-18,18'	
18,18'	2.89 (2H, t, 7.1)	33.0 t	H-17,17'	C-2,2', C-3,3' C-4,4'

[a] ^{13}C NMR chemical shift values were determined by tracing HMQC or HMBC spectrum.

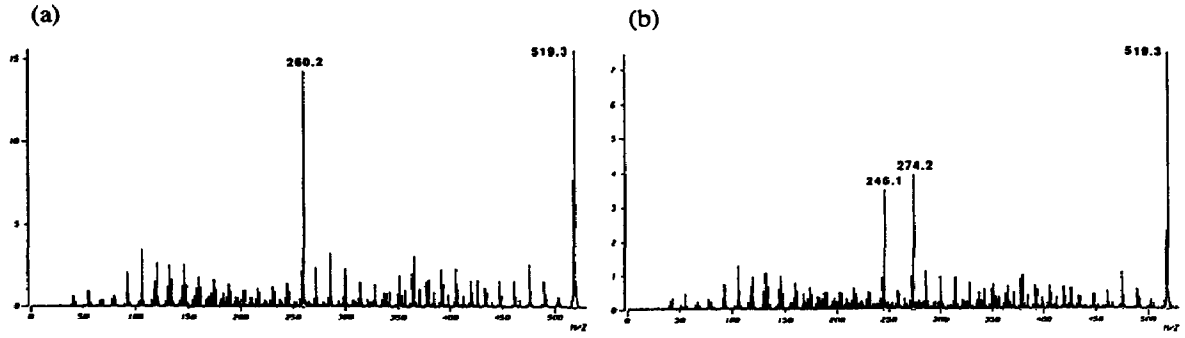


Figure 1. Positive FAB-MS/MS spectra of 3 (a) and 4 (b).

asymmetric structure. In fact, the FAB-MS/MS displayed two intense ions at *m/z* 246 and 260, thereby revealing that **2** had C_{12} and C_{13} alkyl chains.

Cyclostellettamine D (**4**)[12] was eluted immediately after **3** in reversed phase HPLC. It had the same molecular formula as **3**, since the FAB-MS/MS spectrum exhibited two intense daughter ions at *m/z* 246 and 274 as depicted in Fig.1 (b); hence **4** was a hetero-dimer composed of C_{12} and C_{14} alkyl chains.

The HRFAB mass spectrum of cyclostellettamine E (**5**)[13] gave an $[M-H]^+$ ion peak at *m/z* 533.4792 ($C_{37}H_{62}N_2$, $[M-H]^+$, Δ -4.3mmu); one CH_2 unit larger than **3**. Prominent ion peaks at *m/z* 260 and 274 in the FAB-MS/MS revealed that **5** had C_{12} and C_{13} alkyl chains.

Cyclostellettamine F (**6**),[14] having the longest retention time in the reversed phase HPLC, had a molecular formula of $C_{38}H_{64}N_2$ as determined by HRFABMS. A symmetrical structure was assigned for **6** on the basis of an intense ion at *m/z* 274 in the FAB-MS/MS.

Table 2. Activity of Cyclostellettamines A-F (IC_{50}, mg/mL)

	M_1	M_2	M_3
Cyclostelletamine A	0.068	0.026	0.071
B	0.081	0.031	0.109
C	0.121	0.054	0.144
D	0.174	0.059	0.211
E	0.212	0.133	0.257
F	0.364	0.150	0.474

Cyclostelletamines blocked the binding of [^{3}H]-methyl quinuclidinyl benzilate (QNB) to the muscarinic receptor subtypes M_1 (rat brain), M_2 (rat heart), and M_3 (rat salivary gland) as exhibited in Table 2. Obviously, positively charged pyridinium ions of cyclostellettamines participate in binding to TM III Asp residue[15] in the ligand-binding domain of muscarinic receptors. Cyclostellettamines are biogenetically related to the halitoxins,[16] the haliclamines,[17] and other monomeric b-substituted alkyl pyridines.[18] Quite recently polymeric 1, 3-dialkylpyridinium has been reported from the Mid-Pacific marine sponge *Callyspongia fibrosa*[8] as an EGF antagonist.

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7. **3**: HRFABMS (pos) *m/z* 519.4673 $(M-H)^+$, Δ -0.5 mmu; FABMS (pos) *m/z* 633 $(M+CF_3COO^-)^+$, 555 $(M+Cl^-)^+$, 519 $(M-H)^+$, 260 $(M/2)^+$.
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9. Synthesis of cyclostellettamines will be reported elsewhere.
10. **1**: HRFABMS (pos) *m/z* 491.4346 $(M-H)^+$, Δ -1.9 mmu; UV (MeOH) λmax 266nm; ^1H NMR (CD_3OD) δ 8.89 (s, H2/2'), 8.81 (d, 5.8, H6/6'), 8.45 (d, 7.6, H4/4'), 8.02 (t, 6.9, H5/5'), 4.61 (t, 6.8, H7/7'), 2.89 (t, 7.2, H18/18'), 2.00 (brs, H8/8'), 1.73 (quint, 7.4, H17/17'), 1.4-1.2 (br, H9/9'-H16/16').
11. **2**: HRFABMS (pos) *m/z* 505.4500 $(M-H)^+$, Δ +4.5 mmu; UV (MeOH) λmax 268nm; ^1H NMR (CD_3OD) δ 8.89 (s, H2/2'), 81 (d, 5.8, H6/6'), 8.45 (d, 7.2, H4/4'), 8.01 (t, 6.9, H5/5'), 4.61 (t, 6.7, H7/7'), 2.89 (t, 6.2), 2.00 (quint, 7.0, H8/8'), 1.73 (quint, 6.2), 1.4-1.2 (br).
12. **4**: HRFABMS (pos) *m/z* 519.4663 $(M-H)^+$, Δ -1.5 mmu; UV (MeOH) λmax 267nm; ^1H NMR (CD_3OD) δ 8.89 (s, H2/2'), 8.81 (d, 5.7, H6/6'), 8.45 (d, 6.9, H4/4'), 8.01 (t, 6.9, H5/5'), 4.61 (t, 5.4, H7/7'), 2.89 (t, 6.8), 2.00 (brs, H8/8'), 1.73 (brs), 1.4-1.2 (br).
13. **5**: HRFABMS (pos) *m/z* 533.4792 $(M-H)^+$, Δ -4.3 mmu; UV (MeOH) λmax 268nm; ^1H NMR (CD_3OD) δ 8.89 (s, H2/2'), 8.81 (d, 5.6, H6/6'), 8.45 (d, 7.9, H4/4'), 8.01 (t, 7.0, H5/5'), 4.61 (t, 7 .2, H7/7'), 2.89 (t, 6.3), 2.00 (brs, H8/8'), 1.74 (brs), 1.4-1.2 (br).
14. **6**: HRFABMS (pos) *m/z* 547.5012 $(M-H)^+$, Δ +2.0 mmu; UV (MeOH) λmax 267nm; ^1H NMR (CD_3OD) δ 8.89 (s, H2/2'), 8.81 (d, 5.5, H6/6'), 8.45 (d, 6.6, H4/4'), 8.01 (t, 6.4, H5/5'), 4.61 (t, 6.9, H7/7'), 2.89 (t, 7.3), 2.00 (brs, H8/8'), 1.74 (quint, 7.2), 1.4-1.2 (br).
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