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Structure of Lipogrammistin-A, a Lipophilic Ichthyotoxin Secreted by the Soapfish *Diploprion bifasciatum*

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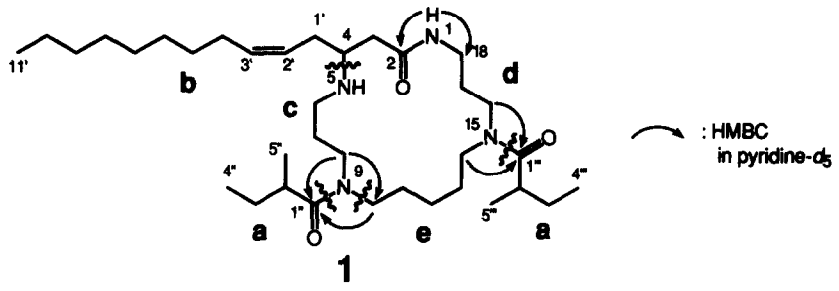
Abstract: The lipophilic ichthyotoxin, lipogrammistin-A (1), has been isolated from the mucus secreted by the soapfish *Diploprion bifasciatum*, and its planar structure was elucidated on the basis of spectroscopic data. 1 exists as a mixture of four conformers arising from the amide bonds in the solution state.

Chemical defense is employed by fishes referred to as ichthyocrinotoxic and belonging to more than ten families.[1] They often lack scales, are largely sessile, and when molested discharge mucous secretions toxic to fish and other marine organisms. Chemical studies of the toxins are limited; pahutoxins[2] have been isolated from boxfishes; pavoninins[3], mosesins[4] and pardaxins[5] from flatfishes of the genus *Pardachirus*. Soapfishes are members of a well-known ichthyocrinotoxic family, which when stimulated secrete copious amounts of mucus that acts like soap in the surrounding water. The mucous secretions contain hydrophilic toxins, grammistins, which are composed of peptidic and unknown lipophilic portions.[1,6] Lipophilic toxins were also detected in the mucus.[1] We now report isolation and structure elucidation of a lipophililc toxin, lipogrammistin-A, from the mucous secretion of the soapfish *Diploprion bifasciatum*.

The acetone extract of the mucus (52.1 g) collected from nine specimens of *D. bifasciatum*, caught by a hand-net off Amami-oshima Island, 1200 km southwest of Tokyo, was partitioned between water and ethyl acetate. The ethyl acetate layer, which was hemolytic against the rabbit erythrocytes, was fractionated on an alumina column ($CHCl_3$/MeOH) followed by repeated reversed-phase HPLC on ODS to yield 277 mg of lipogrammistin-A (yield 0.5% based on wet mucus) as a pale yellow viscous oil.

Lipogrammistin-A (1),[7] $[\alpha]_D$ +18.5° (*c* 0.86, MeOH), had a molecular formula of $C_{35}H_{66}N_4O_3$ which was established by high resolution EI mass spectrum (*m/z* 590.5104, Δ=-3.1 mmu). The presence of three amide groups was inferred from NMR and IR (1624 cm^{-1}) data. The compound reacted positive to Dragendorff's reagent. The ^{13}C NMR spectrum displayed many sets of weak signals. Despite overlapped signals, interpretation of the CH-COSY spectrum revealed that each set of the small signals, corresponding to one carbon, consisted of up to four signals. Allowing for this multiplicity, a DEPT spectrum revealed that the 35 carbons in lipogrammistin-A consisted of five methyls, 22 methylenes, five methines, and three carbonyls. Relative

intensity of these carbon signals within a set was temperature-dependent; broadening of some ^{1}H NMR signals was also observed at higher temperature (333 K in DMSO-d_6 or pyridine-d_5), thereby indicating that lipogrammistin-A was a mixture of conformers.



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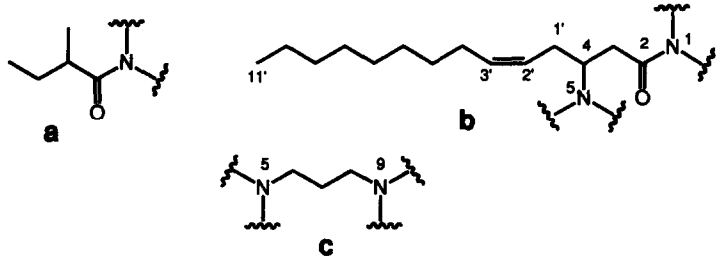
Extensive 2D-NMR analyses (CH-COSY, HH-COSY, HMBC, and HOHAHA) in CD_3OD allowed us to assign partial structures **a**-**c**: two 2-methylbutyramide moieties (**a**), one δ,ε-unsatuated β-amino amide **b**, and one 1,3-propanediamine (**c**). Connectivity of **b** and **c** was established by an HMBC crosspeak between the single methine carbon (δ 56.4) in **b** and the methylene protons (δ 2.7-2.9) in **c**. The size of the aliphatic side-chain was confirmed by ozonolysis, followed by $NaBH_4$ reduction to 1-nonanol, consistent with a prominent fragment ion peak at *m/z* 437 (M^+-$C_{11}H_{21}$) in the EI mass spectrum, which arises by fission of the C_{11} side-chain. *Z*-Geometry of the double bond was secured by the NOESY crosspeaks between olefinic protons (δ 5.36 and 5.58). Though there were no HMBC crosspeaks between the carbonyl carbons in **a** and protons in **b** or **c**, location of two exchangeable NH protons was substantiated by isotope shifts of neighboring carbons when ^{13}C NMR were measured in CD_3OH (Δδ 0.1-0.5).



In order to confirm this result and to establish connectivities of the remaining methylene signals, we measured NMR spectra in pyridine-d_5, which provided well-separated signals due to the anisotropic effect of pyridine, and generated signals for the amide protons. Interpretation of the HMBC spectrum in pyridine-d_5 together with ^{13}C and ^{1}H NMR data (Table 1) led to additional two partial structures **d** and **e**: 1) a three-methylene unit **d** linked to the amide nitrogen in segment **b**, in which the amide proton at N1 (δ 8.5-8.7) correlated with a carbonyl carbon (δ 172) and C-18 methylene (δ_C 37.5, δ_H 3.2-3.6); 2) a five-methylene unit **e** connected to partial structure **c**; this was supported by crosspeaks between C-8 methylene (δ_C 45-46, δ_H 3.3-3.6) and C-10 methylene (δ_C 48-49, δ_H 3.1-3.2). The HMBC spectrum also displayed crosspeaks between two amide carbons (δ 176 x 2) of 2-methylbutyramides and four methylene protons attached to nitrogen atoms (H_2-8, -10,

-14 and -16). Thus, the gross structure of lipogrammistin-A was completed. Stereochemistry of the three chiral centers remains to be elucidated.

Table 1. NMR Data of Lipogrammistin-A (**1**) in Pyridine-d_5[a]

	^{1}H	^{13}C	HMBC correlation[b]
1	8.53, 8.60, 8.65, 8.71 brt		2, 18
2		172.1, 172.2, 172.2 s	
3	2.27, 2.44 m	39.7, 40.0, 40.2, 40.4 t	2, 4, 1'
4	3.08 brm	55.5, 55.6 d	
6	2.56, 2.74 m	44.0, 44.2, 44.2, 44.4 t	4, 7, 8
7	1.65-1.78 m	28.7, 28.9, 30.8, 30.9 t	6, 8
8	3.28-3.63 m	45.5, 45.5, 45.8 t	6, 7, 10, 1"
10	3.10-3.21 m	48.2, 48.4, 48.8 t	8, 12, 1"
11	1.40-1.51 m	29.7-29.9 t	10, 12, 13
12	1.08-1.26 m	23.6, 23.8, 24.1 t	10, 14
13	1.40-1.51 m	27.8-28.1 t	11, 12, 14
14	3.21-3.36 m	46.6-47.3 t	12, 13, 16, 1"'
16	3.21-3.36 m	46.6-47.3 t	14, 17,18, 1"'
17	1.75 m	29.6, 31.3 t	16, 18
18	3.15, 3.40, 3.52 m	37.0, 37.5 t	2, 16, 17
1'	2.12, 2.27 m	31.6, 31.7, 32.0 t	3, 4, 2', 3'
2'	5.26-5.31 m	125.9, 126.1 d	4, 1', 4'
3'	5.32 m	132.7, 132.8, 132.8, 132.9 d	1', 4', 5'
4'	1.84 m	27.6 t	5' or 6'
5'	1.10 m	29.7-29.9 t	3', 4'
6'-8'	0.98-1.02 m	29.5-29.9 t	
9'	0.98-1.02 m	32.0 t	10'
10'	1.04 m	22.8 t	9', 11'
11'	0.64 (t; J=7.0 Hz)	14.2 q	9', 10'
1", 1"'		176.0, 176.0, 176.1, 176.1, 176.3 sx2	
2", 2"'	2.38-2.47 m	37.3-37.5 dx2	1", 3", 4", 5"[c]
3", 3"'	1.21 m, 1.65 m	27.8-28.1 tx2	1", 2", 4", 5"[c]
4", 4"'	0.64-0.74 m	12.2 qx2	2", 3"[c]
5", 5"'	0.91-0.98 m	18.2, 18.3, 18.4 qx2	1", 2", 3"[c]

[a] Measured at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Chemical shifts are referenced to solvent peaks: δ_H 8.50 for Cα-H for residual pyridine-d_4 and δ_C 149.8 for Cα of pyridine-d_5.

[b] Correlated carbon signals are presented for each proton signal.

[c] Corresponding correlations were also observed for H-2"'-H-5"'. They are not shown in this table due to limited space.

To our knowledge, this is the first isolation of a macrocyclic polyamine from an animal; cytotoxic pithecolobine alkaloids are known from terrestrial plants.[8,9] Interestingly, budmunchiamines[8], members of

pithecolobine alkaloides, have strong affinity to DNA, which exerts cytotoxic property. Our compound is also expected to have a similar property. Lipogrammistin-A is lethal to medaka fish (*Oryzias latipes*) at 10 ppm (time-to-death 50 min), and to mice at an *i. p.* dose of 100 mg/kg (14 min). It is also cytolytic against the rabbit erythrocytes (0.70 SU/mg)[10] and fertilized sea urchin eggs (10 μg/mL).

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7. **1**: IR data (film): ν_{max} 3284, 2958, 2925, 2854, 1624, 1554, 1464, 1375, 1244, 1086 cm^{-1}. EIMS data: *m/z* (rel. intensity) 590 (1.4), 533 (0.8), 505 (1.9), 437 (50), 356 (8.3), 353 (8.1), 142 (17), 98 (15), 85 (27), 57 (100). CIMS data (isobutane): *m/z* (rel. intensity) 591 (100), 437 (89).
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10. Hemolytic activity is expressed by SU/mg, where 1 SU/mg is equivalent to the potency of Merck saponin (Monograph No. 8328).

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