Azaspiracid, a New Marine Toxin Having Unique Spiro Ring Assemblies, Isolated from Irish Mussels, *Mytilus edulis*

Masayuki Satake,[†] Katsuya Ofuji,[†] Hideo Naoki,[‡] Kevin J. James,[§] Ambrose Furey,[§] Terry McMahon,[∥] Joe Silke,[∥] and Takeshi Yasumoto*,[†]

Faculty of Agriculture, Tohoku University Tsutsumidori-Amamiya, Aoba-ku Sendai 981-8555, Japan
Suntory Institute for Bioorganic Chemistry Wakayamadai, Shimamoto-cho Mishima-gun, Osaka, Japan
Chemistry Department, Cork Institute of Technology Bishopstown, Cork, Ireland
Marine Institute, Fisheries Research Centre Abbotstown, Dublin 15, Ireland

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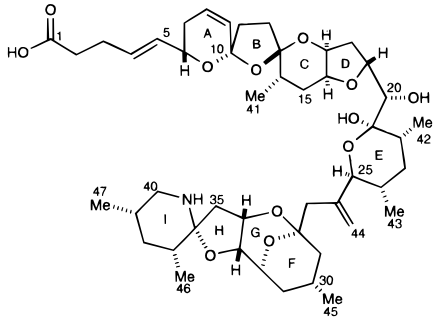
In November, 1995, at least eight people in the Netherlands became ill after eating mussels (*Mytilus edulis*) cultivated at Killary Harbor, Ireland. Although human symptoms such as nausea, vomiting, severe diarrhea, and stomach cramps were reminiscent of diarrhetic shellfish poisoning (DSP), contaminations of the major DSP toxins okadaic acid (OA) and dinophysistoxins (DTXs) were very low.[1] These observations prompted us to explore the causative toxin in the mussels for structural studies. In this paper we report the structure of azaspiracid (**1**),



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a new marine toxin having two spiro ring assemblies, a cyclic amine and a carboxylic acid, as the major causative agent of that incident.

Whole mussel meat (20 kg) collected at Killary Harbor in February 1996 was extracted with acetone three times. After the acetone was evaporated, the residue was partitioned between hexane and 85% aq MeOH. The major toxin obtained in the MeOH phase was chromatographed on silica gel with acetone, acetone/MeOH/AcOH (180:20:1), and MeOH in this order. The toxin eluted in the second fraction was applied to gel permeation chromatography over Toyopearl HW-40 super fine (Tosoh) with 1% AcOH/PrOH (3:7). The toxic residue was dissolved in H_2O/PrOH (1:1) and charged onto a CM-Toyopearl 650M (Tosoh) column. The column was washed with 50% PrOH and then the toxin was eluted with 1% AcOH/PrOH (3:7). The toxin was next adsorbed on DEAE-Toyopearl from 50% PrOH solution and then eluted with 1% AcOH/PrOH (3:7). Finally, azaspiracid was purified on Toyopearl HW-40 with 1% AcOH/PrOH (3:7). Throughout the purification, elution of azaspiracid was monitored with a diode array detector at 210 nm and by mouse toxicity assay.[2]

Azaspiracid (2 mg) was obtained as a colorless amorphous solid: $[\alpha]^{20}_{D}$ −21 (*c* 0.10, MeOH); no UV absorption maxima above 210 nm; mouse lethality (ip) 0.2 mg/kg.[2] The presence of nitrogen was indicated by the positive test to ninhydrin reagent. HR-FAB MS suggested a molecular formula of $C_{47}H_{71}NO_{12}$ ([M + H]$^+$ *m*/*z* 842.5077 +2.2 mmu). Structural elucidation was performed mainly by NMR experiments.[3] ^{1}H NMR, ^{13}C NMR and HSQC spectra showed that azaspiracid contained 6 methyls, 15 methylenes, 19 methines, and 7 quaternary carbons. The carboxyl carbon was not directly observed on ^{13}C NMR but was deduced from HMBC correlation from H2a,b to C1 at 180.3 ppm and an intense IR absorption band observed at 1721 cm^{-1}. Detailed analysis of ^{1}H−^{1}H COSY and TOCSY spectra in CD_3-OD led to elucidation of 7 partial structures (H2a,b-H9, H11a,b-H12a,b, H14-H20, H22-H25, H27a,b, H29a,b-H35a,b, and H37-H40a,b) which were interrupted by quaternary carbons.[4] Assembling the partial structures was mainly accomplished by HMBC experiments. Cross-peaks were observed for H2a,b/C1, H7a/C10, H11a/C10, H12a/C13, Me41/C13, H20/C21, Me42/C21, H25/C26, H44a,b/C26, H27a,b/C26, H27a,b/C28, H29a,b/C28, H35a,b/C36, and Me46/C36, allowing the carbon skeleton to be traced from C1 to C40. The ^{13}C NMR, HSQC, HMBC, and HR-FAB MS suggested that 4 exchangeable protons existed in the molecule of azaspiracid. One belonged to the carboxylic acid moiety. Isotope shifts in ^{13}C NMR signals as observed by the chemical-shift differences (see Table 1) between CD_3OD and CD_3OH solutions led to identification of hydroxy-bearing carbons; significant shifts (approximately 0.1 ppm) were observed for C20 and C21, indicating the presence of two hydroxyl groups. ^{13}C chemical shifts 77.6 (C20) and 101.1 (C21) ppm matched well those of a hydroxy methine and a hemiacetal carbon, respectively. The rest of exchangeable proton was in an amino group. The location of the amino group was deduced from the chemical shifts of H40a,b (δ 2.84, 2.91) and C40 (δ 46.9) which were typical for a methylene adjacent to nitrogen in piperidine. The shift of the C40 signal to over 0.1 ppm in the isotope shift experiment further supported the position of the nitrogen atom and the existence of an amino proton. The degree of unsaturation derived from the molecular formula and the structural features described above suggested the presence of 9 rings. HMBC correlations from H16 to C19, H25 to C21, H32 to C28, H33 to C36, and H40b to C36 led to constructing rings D, E, F, H, and I. The chemical shifts of C10 (107.9 ppm) and C13 (112.1 ppm) and NOE correlations between H6 and Me41 and between H12b and H17 allowed us to construct a 6,5,6-trispiroacetal moiety (rings A, B, and C) similar to those in pinnatoxin[5] and spirolides.[6] The remaining G ring was supported by large NOE correlations from H29b and H30 to H34.

The proposed structure was further supported by both negative and positive ion FAB MS/MS studies.[7] A collision-induced dissociation (CID) MS/MS experiment was carried out on negative

[†] Tohoku University.
[‡] Suntory Institute for Bioorganic Chemistry.
[§] Cork Institute of Technology.
[∥] Fisheries Research Centre.

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(2) The toxicity was assayed by intraperitoneally injecting respective doses into two mice (male, ddY, 14−15 g).

(3) NMR spectra were measured with Varian Unity INOVA 600 and 500 spectrometers at 20 °C in CD_3OD except for isotope shifts experiments.

(4) For the assignment of methylene protons, high field protons were suffixed by a (e.g., Ha), while low field protons were suffixed by b (e.g., Hb).

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(7) Negative ion FAB MS/MS experiments were carried out on a JMS HX-110/HX-110 instrument (JEOL) with use of 2, 2′-dithiodiethanol as a matrix at a resolution of 2000.

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Table 1. NMR Chemical Shifts (δ) of Azaspiracid[a]

pos	^{1}H	^{1}H	^{13}C	pos	^{1}H	^{1}H	^{13}C
1			180.3	25	4.00		80.4
2	2.31	2.31	37.4	26			149.1
3	2.33	2.33	30.3	27	2.26	2.43	50.4
4	5.74		133.8	28			99.5
5	5.46		131.8	29	1.37	2.05	44.9
6	4.81		73.2	30	2.23		27.2
7	2.15	2.49	36.5	31	1.54	1.84	36.1
8	5.76		124.1	32	4.38		73.6
9	5.65		130.1	33	4.08		82.3
10			107.9	34	5.02		75.6
11	1.68	2.33	33.9	35	2.50	2.64	42.5
12	1.97	2.16	38.3	36			97.4
13			112.1	37	1.99		36.4
14	2.02		31.7	38	1.31	1.70	38.4
15	1.77	1.85	33.4	39	1.89		30.2
16	3.89		79.1	40	2.84	2.91	46.9
17	4.25		74.2	41	0.94		17.4
18	2.00	2.01	37.8	42	0.91		17.2
19	4.44		79.9	43	0.84		18.8
20	3.94		77.6	44	5.18	5.36	117.2
21			101.1	45	0.96		24.3
22	2.09		37.6	46	0.98		16.2
23	1.44	1.44	38.9	47	0.95		19.3
24	1.35		43.1				

[a] CHD_2OD taken as 3.31 ppm. $^{13}CD_3OD$ taken as 49.8 ppm.

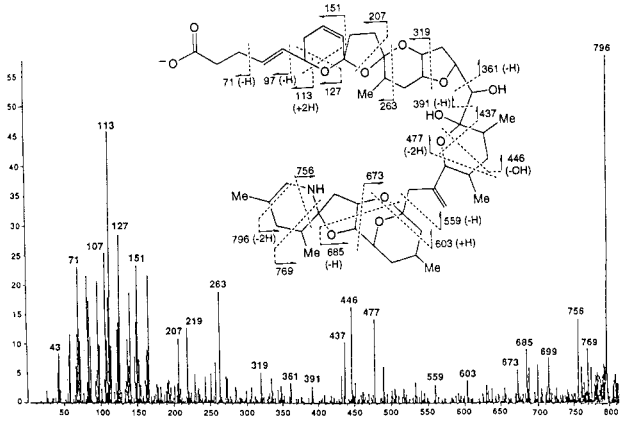

Figure 1. Negative ion FAB CID MS/MS spectrum and fragmentation patterns of azaspiracid (**1**).

ions by choosing the $(M - H)^-$ ion (*m*/*z* 840) as the precursor ion (Figure 1). The position of the amino group was supported by product ions at *m*/*z* 769 and 756. Prominent product ions at *m*/*z* 319, 263, 207, 151, 127, and 113 confirmed the 6,5,6-trispiroacetal structure. Bond cleavages between the hydroxy carbon and α carbons (C21−C20 and C20−C19) were evident by product ions at *m*/*z* 391 and 361. Other prominent product ions were generated by bond cleavage at the sites characteristic of ether rings, as established in previous experiments on amphidinol,[8] yessotoxin,[9] and maitotoxin.[10] In the positive ion CID MS/MS experiment carried out on the $(M + H)^+$ ion (*m*/*z* 842), product ions observed at *m*/*z* 448, 350, 280, 168, and 125 were well-explained by the structure at ether rings from C21 to C40 portion of **1** (see Supporting Information). These MS/MS data thus supported the proposed structure of azaspiracid.

The relative stereochemistry of **1** was deduced from NOE correlations and proton coupling constants (Figure 2). Rings C and D were deduced to be cis-fused from both NOEs and a small coupling constant between H16 and H17. Ring C should be in a chair conformation because coupling constants (13, 13, 3 Hz) of H15a were typical of those for diaxial (H14/H15a), geminal (H15a/H15b), and axial−equatorial (H15a/H16) protons on a

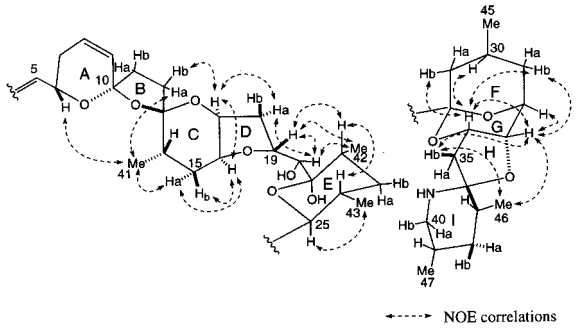

Figure 2. Interpretation of NOE correlations observed in ROESY spectra for azaspiracid (**1**).

chair-form tetrahydropyran ring. Unobserved NOE between Me41/H16 also supported the chair conformation and equatorial disposition of Me41. The anti arrangement for H6 and CH_2-11 was inferred because no NOE was observed between H6 and H11a,b. A significant NOE correlation between H12b and H17 was indicative of an axial orientation of C12. A large coupling (9 Hz) of H24/H25 and an NOE correlation between H22 and H24 indicated a diaxial arrangement for H24 and H25. Hence substitution of both Me42 and Me43 was equatorial. A coupling constant of 5 Hz between H19 and H20 as well as NOE correlations between H19/H20, H19/Me42, H19/H22, and H18a/H20 put rotational constraints along the C19−C20 and C20−C21 bonds, allowing us to interrelate the relative stereochemistries of ring D, C20, and ring E as shown in Figure 2. On the basis of a large coupling constant (12 Hz) between H29a and H30, Me45 was assigned as equatorial. The fusing manner of rings G and H was inferred to be cis from an NOE correlation between H33/H34 and a small coupling constant (4 Hz) of H33/H34. NOE correlations between H30/H34 and H32/H33 established dispositions of H32, H33, and H34 to be equatorial, equatorial, and axial, respectively. Both Me46 and Me47 were assigned as equatorial on the basis of large couplings (13 Hz) between H37 and H38a and between H39 and H40a. Equatorial orientation of the C35−C36 bond was determined by NOE correlations between Me46/H33 and Me46/H35b. All of these data allowed us to assign the relative stereostructure of azaspiracid as **1**.

Azaspiracid is characterized by a trispiro assembly, an unusual azaspiro ring structure fused with a 2,9-dioxabicyclo[3.3.1] nonane ring, and a carboxylic acid. Azaspiracid differs from any of the previously known nitrogen-containing toxins from shellfish or dinoflagellates, e.g., prorocentrolide,[11] pinnatoxin,[4] gymnodimine,[12] and the spirolides.[5] Azaspiracid has a cyclic amine instead of a cyclic imine, and a carbocyclic or lactone ring was absent in the molecule. The ultimate origin of azaspiracid is most probably a dinoflagellate because of the highly oxygenated polyether structure and seasonal occurrence. However, none of the known toxic phytoplanktons was observed in water samples collected. The structural information allows us to develop an LC/MS determination method for azaspiracid in mussels and eventually may lead to identification of the biogenetic origin of this intriguing compound.

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Supporting Information Available: 1D ^{1}H NMR, ^{1}H−^{1}H COSY, TOCSY, ROESY, HSQC, isotope shifts of ^{13}C NMR, HMBC, and positive ion CID MS/MS spectra (12 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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