Spirolides B and D, Two Novel Macrocycles Isolated from the Digestive Glands of Shellfish

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Two polar lipid-soluble macrocycles **1** and **2**, containing a spiro-linked tricyclic ether ring system and an unusual seven-membered spiro-linked cyclic iminium moiety, have been isolated from the digestive glands of mussels (*Mytilus edulis*) and scallops (*Placopecten magellanicus*).

During chemical investigations of polar bioactive molecules from microalgae and shellfish,[1] we isolated from the digestive glands of both mussels (*Mytilus edulis*) and scallops (*Placopecten magellanicus*), a family of novel macrocycles which we have named spirolides A–D. Here we report the structural elucidation of the two major components, spirolides B and D.

Spirolide B **1** and D **2** (Fig. 1) were purified from methanolic extracts of frozen digestive glands of shellfish collected from sites along the eastern shore of Nova Scotia.† High resolution LSIMS determined the molecular formulae for **1** and **2** to be $C_{42}H_{63}NO_7$ (MH$^+$ 694.4651, δ 4.5) and $C_{43}H_{65}NO_7$ (MH$^+$ 708.4831, δ 1.1) respectively, and the latter was confirmed also by high resolution DEI (M$^{+\cdot}$ 707.4975, δ 1.8). These data were supported by the ^{13}C NMR spectra for **2** (Table 1), in which 10 quaternary carbons, 10 CH, 16 CH_2 and 7 Me groups were identified. The strong IR absorption bands at 3460 cm^{-1} in the IR spectra of both compounds suggested the presence of hydroxy groups, and the number of exchangeable protons in **2** was established by briefly warming the compound in D_2O and recording the molecular isotopic cluster using DEI at a low ionizing electron energy. The pattern of the cluster was consistent with the existence of three exchangeable protons.‡ In both compounds IR absorption bands at 1771 and 1676 cm^{-1} indicated the presence of a γ-lactone ring system as well as a double bond between carbon and an oxygen or nitrogen, consistent with ^{13}C resonances at δ 182.3 and 178.9. Signals at δ 111.2 (CH_2) and 147.8 (quaternary C) indicated a vinyl double bond, and four additional resonances in the range δ 124–145 established another two double bonds. Together, these data account for 5 of the 12 degrees of unsaturation in **1** and **2** as required by the molecular formulae, and hence both molecules must contain 7 rings.

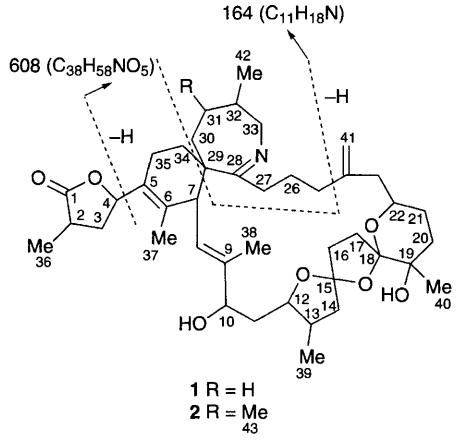

Fig. 1 Structures of spirolides B **1** and D **2**. Two major fragment ions from the LSIMS, MS/MS spectrum of **2** are also shown. The elemental compositions were determined by accurate mass measurements in the DEI spectra, and analogous fragments 14 mass units lower were observed in **1**.

Analysis of the TOCSY and COSY spectra for **2** established the connectivities of six ^1H-^1H spin systems corresponding to the partial structures *a–f* (Fig. 2). The γ-lactone partial structure *a* was identified by the characteristic shifts of C-1, C-4 and H-4 (δ 5.39) and by several key connectivities in the HMBC spectra including C-1 to H-2, H-36, H-3 and H-4, and C-3 to H-2 and H-36. Additional HMBC including C-5, C-6 and C-35 to H-4 and NOE correlations between H-37 and H-3 and H-4, attached this γ-lactone ring to partial structure *b* at position C-5. In addition, the LSIMS MS/MS fragmentation data for **2** showed a fragment ion at *m/z* 608 (DEI: *m/z* 608.4365, $C_{38}H_{58}NO_5$) corresponding to loss of the pendant lactone ring (Fig. 1).

Table 1 NMR Data[a] for spirolide D **2**

C	δ_C	δ_H	C	δ_C	δ_H	C	δ_C	δ_H
1	182.3 (s)		16	36.5 (t)	2.04, 2.23	31	36.7 (d)	1.17
2	36.9 (d)	2.80	17	31.5 (t)	1.75, 2.14	32	41.3 (d)	1.38
3	36.2 (t)	1.66, 2.53	18	112.5 (s)		33	53.3 (t)	3.47, 3.83
4	79.8 (d)	5.39	19	71.0 (s)		34	32.7 (t)	1.59, 1.90
5	129.3 (s)		20	35.8 (t)	1.49, 1.84	35	20.8 (t)	1.97, 2.32
6	132.2 (s)		21	30.2 (t)	1.25, 1.59	36	15.1 (q)	1.23
7	48.7 (d)	3.57	22	69.3 (d)	4.01	37	16.9 (q)	1.63
8	124.4 (d)	5.18	23	47.7 (t)	2.04, 2.38	38	12.3 (q)	1.83
9	144.4 (s)		24	147.8 (s)		39	15.7 (q)	1.20
10	76.8 (d)	4.15	25	35.9 (t)	1.59, 2.12	40	22.4 (q)	1.20
11	39.6 (t)	1.58, 2.17	26	23.3 (t)	1.40, 2.08	41	111.2 (t)	4.78, 4.81
12	81.7 (d)	4.33	27	35.2 (t)	2.34, 2.34	42	19.4 (q)	1.00
13	35.2 (d)	2.42	28	178.9 (s)		43	21.2 (q)	0.97
14	45.9 (t)	2.15, 2.27	29	50.8 (s)				
15	117.4 (s)		30	38.3 (t)	1.58, 1.78			

[a] (s) = C, (d) = CH, (t) = CH_2, (q) = CH_3. Spectra were recorded at 600.13 MHz (^1H) and 125.7 MHz (^{13}C) using CD_3OD as solvent. Chemical shifts δ_C and δ_H (ppm) were referred to CHD_2OD = 3.30 ppm (^1H), CD_3OD = 49.0 ppm (^{13}C). The NMR data for **1** were essentially the same except the resonances for positions 30 (δ_C 28.1, δ_H 1.68, 1.91), 31 (δ_C 32.2, δ_H 1.08, 1.78) and 32 (δ_C 33.7, δ_H 1.88).

In partial structure *b* (Fig. 2) the HMBC of C-5 to H-34 and H-37 and C-6 to H-7 and H-37 as well as homoallylic coupling between H-7 and H-35 confirmed the connectivity around the 5,6 double bond. Additional HMBC from C-8 and C-9 to H-38, and a large $J_{7,8}$ (11.54 Hz), fixed the connectivity around Δ 8,9, while a strong NOE between H-8 and H-10 established the *E*-configuration. The nature of the cyclohexene ring system, which includes the quaternary carbon C-29 (δ 50.8), was revealed by the HMBC data C-7 to H-34 and C-29 to H-7 and H-34.

The three partial structures *c*, *d*, and *e* respectively include H-10 to H-14 (and methyl H-39), H-16 to H-17, and H-20 to H-27 (and exomethylene H-41). The HMBC of C-8 to H-10, and C-10 to H-8 and H-38, linked partial structures *b* and *c* at C-9. Extension of the structure beyond C-14 into *d* through the oxygen-bearing quaternary carbon C-15 (δ 117.4) was established by the HMBC of C-14 to H-16 and C-15 to H-13, and H-17. Similarly, the correlations C-18 to H-16, H-17, and H-40, C-19 to H-17, H-20 and H-40, and C-20 to H-40, positioned the methyl group at the oxygen-bearing carbon C-19 (δ 71.0) and linked partial structures *d* and *e* through the oxygen-bearing quaternary carbon C-18 (δ 112.5).

The nature of the oxygen-bearing carbons in the portion of the molecule that includes the partial structures *c*, *d* and *e* was determined by recording the ^{13}C NMR spectrum of **2** first in CD_3OH and then in CD_3OD.[2] The resonances of the oxygen-bearing carbons at C-10 (methine carbon) and C-19 (quaternary carbon), were shifted upfield by 0.11 and 0.10 ppm in CD_3OD, indicating secondary and tertiary hydroxy groups, respectively. The remaining four oxygen-bearing carbons in *c*, *d* and *e* showed shifts of less than 0.02 ppm, suggesting that they were present as ether linkages. On the basis of the HMBC and ^{13}C chemical shift data for spiroketal carbons,[3] these oxygen-bearing carbons were assigned as C-12 (δ 81.7), C-15 (δ 117.4), C-18 (δ 112.5) and C-22 (δ 69.3).

The position of the single nitrogen remained to be determined though it had to be contained in the remaining partial structure *f*, which includes H-30–33 plus H-42 (in **1** and **2**) and H-43 (in **2**), as well as the quaternary C-28. The nitrogen was located in this moiety by the characteristic chemical shift of the C-33 methylene (δ_C 53.3, δ_H 3.47, 3.83), and the C-28 imine carbon (δ 178.9). From this, a seven-membered nitrogen-containing ring system was established by the HMBC of C-28 to H-33 and H-30, and C-29 to H-30. The fact that the quaternary carbon C-29 (δ 50.8) was incorporated in *both* the six-membered and seven-membered rings, indicated that these two rings are spiro-linked at this position, and this was further supported by the HMBC of C-7 to H-30 and C-30 to H-7. The location of the C-42 and C-43 methyl groups in **2** was established by HMBC of C-31, C-32 and C-33 to H-42 and of C-30, C-31 and C-32 to H-43.

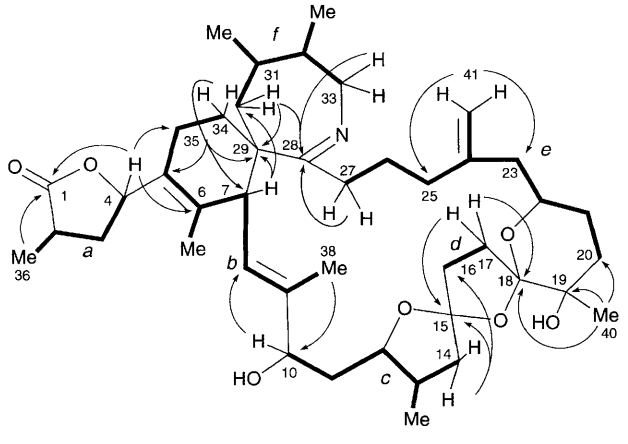

Fig. 2 Partial structures (*a–f*) in spirolide D **2**, showing some key HMBC (⌒) correlations. Bold lines represent ^1H-^1H spin systems identified from TOCSY and COSY spectra.

The molecular formula for spirolide B **1** indicated one less methyl group than in **2**, and the NMR data showed loss of the methyl group C-43 from position 31 and its replacement by a proton (δ_H 1.08, 1.78; δ_C 32.2) in partial structure *f*. Furthermore, the LSIMS MS/MS spectra of the MH^+ ion of **2** contains a series of fragment ions following opening of the macrocycle adjacent to C-29. An analagous series of ions 14 mass units less was observed for **1**, and the base fragment ion at *m/z* 164 (DEI: 164.1449, $C_{11}H_{18}N$) in **2**, (*m/z* 150 in **1**), verifies that the methyl group is located on the fragment containing the iminium ring system (Fig. 1).

The third exchangeable proton was also identified by the isotope-shift ^{13}C NMR experiment when three non-oxygen-bearing carbons C-26, C-27 and C-28 were shifted upfield ($\Delta\delta_C$ 0.12, 0.38 and 0.11 ppm respectively). Integration of the ^1H NMR spectrum of **2** recorded in CD_3OD revealed that only one proton (δ 2.34) was retained at position 27, indicating that iminium tautomerization had resulted in a stereoselective deuterium exchange with the solvent. The $\Delta\delta_C$ for C-27 was consistent with direct substitution of one deuterium at this position, while the upfield shifts of C-28 and C-26 are explained by a β-shift.

The spiro-linked cyclic iminium group is extremely rare, and to our knowledge has only been reported in pinnatoxin A, isolated from the digestive glands of shellfish from sub-tropical waters around Okinawa.[4] A cyclic iminium group is present in prorocentrolide isolated from the marine dinoflagellate *Prorocentrum lima*.[5] The fact that the spirolides have been found in the digestive glands of shellfish, coupled with the observation that they occur on a seasonal basis, usually June–July, indicates a microalgal origin for these compounds and their spiro-linked polyether structure might suggest a dinoflagellate source.§ The spirolides cause potent and characteristic symptoms in the mouse bioassay (LD_{100} 250 μg kg^{-1} i.p.), and their toxicological properties are under investigation.¶

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Footnotes

† Frozen scallop digestive glands (3.9 kg) were extracted with aqueous methanol and the extract successively partitioned against hexane and chloroform. The chloroform-soluble fraction gave a positive response in the mouse bioassay and the oily residue was purified by bioassay-guided fractionation using a combination of normal phase silica gel, reversed phase C_{18} and gel permeation (Sephadex LH 20, MeOH eluent) chromatography. Final clean-up was achieved by reversed phase C_{18} HPLC (30% aq. MeCN with 0.1% TFA as eluent) to yield four compounds (A–D), rrt. 1.00, 1.12, 1.18 and 1.3. The major components were spirolide B **1** (*ca.* 200 μg) and D **2** (450 μg), displaying MH^+ ions at *m/z* 694 and 708 respectively by LC-ionspray MS.

‡ Assuming that the ratio MH^+/M observed in non-deuterated solvent is the same in deuterated solvent, the isotope distribution calculated from the observed pattern was found to be 1 : 4.1 : 5.5 : 2.9 : 0.3 for $D_0 : D_1 : D_2 : D_3 : D_4$. Exchangable protons are C10(OH), C19(OH), and one proton at C27 (see text).

§ Purified fractions obtained from a chloroform-soluble extract of a heterogeneous plankton sample, obtained during a period when spirolides were present in shellfish, were found to contain spirolides by the bioassay data, as well as TLC, HPLC and LC-MS analyses.

¶ *In vitro* assays have shown that spirolides do not affect NMDA, AMPA or kainate receptors, nor do they inhibit PP1 and PP2A phosphatases. They do not activate or block voltage dependant Na channels, although they are weak activators (1.7 μmol dm^{-3}) of type L Ca channels.

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