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Characterization of Biologically Inactive Spirolides E and F: Identification of the Spirolide Pharmacophore

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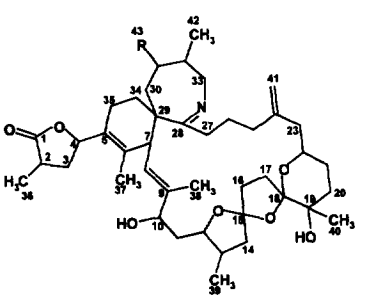
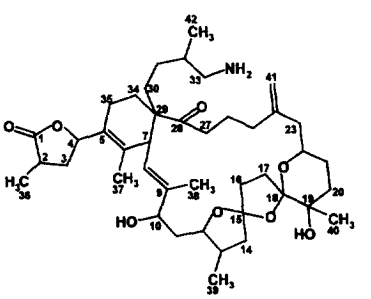
Abstract: Two new spirolide derivatives, E and F, have been isolated in low yield from shellfish extracts. Absence of activity in the mouse bioassay of these derivatives, and of the secondary amine reduction product of spirolide B, identifies the spirolide pharmacophore as the cyclic imine moiety.

Recently we reported the isolation of four unusual compounds, spirolides A-D, from the digestive glands of shellfish and described the structures of two, spirolides B (**1**) and D (**2**).[1] These compounds contain several unusual structural features that are also found in the recently reported pinnatoxins[2] and gymnodimine[3]. Together, these compounds form the basis of a new class of marine toxin, whose biological properties are characterized by the rapid onset of symptoms in the mouse bioassay. At the moment the pharmacological activity of these compounds has not been defined, though there is some evidence to indicate that spirolides may affect Ca channels.[1] In this paper we report the isolation and structural characterization of two keto amine hydrolysis derivatives of the spirolides, named spirolide E (**3**) and spirolide F (**4**). These compounds, which are also found in shellfish extracts, are inactive in the mouse bioassay, suggesting that the common pharmacophore in this class of toxins is the cyclic imine moiety.

1: R = H
2: R = CH_3





3: $\Delta^{2,3}$
4

During isolation of the spirolides from the digestive glands of shellfish, two peaks eluting somewhat later than spirolides A-D were observed in the final HPLC step.[4] Repeated HPLC runs yielded small amounts of two compounds, **3** (< 200 μg) and **4** (< 400 μg). Interestingly, neither compound produced a response in the mouse bioassay, although preliminary examination of their ^{1}H NMR spectra revealed a close resemblance to **1**

(Table 1). The IR data for **4**, unlike those for **1**, showed no imine absorption at 1641 cm^{-1}, but had instead a new peak at 1696 cm^{-1} suggestive of a ketone, supported by the ^{13}C NMR data for **4** which included a new resonance at 218.7 ppm. In addition, **4** gave a pinkish stain upon spraying with ninhydrin, consistent with the presence of a primary amine. The HR electrospray MS data for **4** indicated the molecular formula $C_{42}H_{65}NO_8$ (MH^+ 712.4775, Δ=1.9ppm), corresponding to the addition of water to **1** (MW 693).

Table 1. NMR Data[a]

	Spirolide B (1)		Spirolide F (4)		Dihydrospirolide B (5)[b]	
#C	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR
1	182.3 (s)		182.2 (s)		179.7 (s)	
2	36.9 (d)	2.80	36.9 (d)	2.81	36.3 (d)	2.66
3	36.2 (t)	1.66, 2.52	36.0 (t)	1.69, 2.53	35.5 (t)	1.66, 2.39
4	79.7 (d)	5.38	79.6 (d)	5.41	78.4 (d)	5.26
5	129.1 (s)		129.3 (s)		127.3 (s)	
6	132.3 (s)		132.0 (s)		133.0 (s)	
7	48.6 (d)	3.55	47.9 (d)	3.40	46.2 (d)	2.89
8	124.6 (d)	5.34	123.6 (d)	5.26	126.0 (d)	5.61
9	144.1 (s)		144.2 (s)		140.3 (s)	
10	76.6 (d)	4.16	76.2 (d)	4.05	76.1 (d)	4.26
11	39.7 (t)	1.62, 2.14	38.8 (t)	1.57, 2.05	37.4 (t)	1.67, 2.13
12	81.7 (d)	4.33	81.4 (d)	4.26	80.4 (d)	4.29
13	35.2 (d)	2.41	35.2 (d)	2.40	34.4 (d)	2.42
14	45.2 (t)	2.14, 2.27	44.6 (t)	2.16, 2.31	45.7 (t)	1.93, 2.28
15	117.3 (s)		117.0 (s)		116.4 (s)	
16	36.5 (t)	2.04, 2.20	37.5 (t)	1.95, 2.07	36.5 (t)	1.98, 2.19
17	31.5 (t)	1.76, 2.15	31.9 (t)	1.80, 2.22	31.4 (t)	1.75, 2.19
18	112.5 (s)		112.9 (s)		111.7 (s)	
19	71.1 (s)		71.5 (s)		70.4 (s)	
20	35.7 (t)	1.49, 1.84	35.7 (t)	1.55, 1.95	36.2 (t)	1.58, 1.74
21	30.2 (t)	1.27, 1.60	30.8 (t)	1.30, 1.60	30.2 (t)	1.29, 1.66
22	69.4 (d)	4.02	69.9 (d)	4.10	69.1 (d)	3.97
23	47.5 (t)	2.03, 2.36	45.2 (t)	2.02, 2.33	45.5 (t)	2.05, 2.26
24	147.9 (s)		147.5 (s)		147.3 (s)	
25	35.8 (t)	1.58, 2.16	35.3 (t)	1.83, 2.08	37.9 (t)	2.02, 2.02
26	23.5 (t)	1.38, 2.01	22.9 (t)	1.60, 1.60	21.4 (t)	1.34, 1.74
27	35.2 (t)	2.31, 2.31	38.9 (t)	2.55, 2.63	29.8 (t)	1.01, 1.54
28	178.7 (s)		218.7 (s)		51.3 (d)	b
29	51.3 (s)		49.8 (s)		43.9 (s)	
30	28.1 (t)	1.68, 1.91	25.3 (t)	1.43, 2.02	26.6 (t)	1.37, 1.42
31	32.2 (t)	1.08, 1.78	30.0 (t)	1.01, 1.43	28.1 (t)	1.37, 1.65
32	33.7 (d)	1.88	34.1 (d)	1.72	33.5 (d)	1.79
33	53.0 (t)	3.50, 3.70	46.6 (t)	2.73, 2.86	56.4 (t)	2.52, 2.63
34	32.5 (t)	1.63, 1.90	38.7 (t)	1.85, 1.99	31.2 (t)	1.37, 1.75
35	20.8 (t)	1.95, 2.29	20.8 (t)	2.00, 2.03	21.0 (t)	1.94, 2.13
36	15.1 (q)	1.23	15.1 (q)	1.25	14.9 (q)	1.24
37	16.8 (q)	1.62	16.7 (q)	1.61	16.9 (q)	1.56
38	12.2 (q)	1.85	11.3 (q)	1.65	11.7 (q)	1.68
39	15.8 (q)	1.21	15.1 (q)	1.21	14.8 (q)	1.18
40	22.4 (q)	1.20	22.7 (q)	1.25	21.1 (q)	1.20
41	111.2 (t)	4.77, 4.79	111.9 (t)	4.74, 4.78	111.5 (t)	4.70, 4.81
42	21.0 (q)	0.93	17.1 (q)	1.05	19.3 (q)	0.90

[a] (s) = C, (d) = CH, (t) = CH_2, (q) = CH_3. Samples of **1** and **4** were dissolved in CD_3OD (reference to ^1H 3.30, ^{13}C 49.0). Carbon resonances were correlated with those of directly bonded protons by HMQC spectra. [b] NMR data for **5** were obtained from the borodeuteride reduction product of spirolide B, dissolved in CD_2Cl_2 (reference to ^1H 5.32, ^{13}C 54.0).

From ^1H TOCSY and DQF-COSY NMR data for **4** it was possible to identify 6 partial structures, which were essentially identical with the equivalent partial structures in **1**[1]. The only differences appeared around C28: For example, the ^1H and ^{13}C resonances for position 33 in **1** are shifted upfield in **4** consistent with conversion of the imine group to a primary amine.[5] In addition, the H27 resonance in **1** was shifted downfield in **4** (Table 1), in line with hydrolysis of an imine to a keto group. HMBC's in accord with structure **4** were found from H27 (δ 2.55) to C29, and from H42 to C31, C32 and C33.

The absence of the imine moiety was also reflected by dramatic differences between the electrospray MS/MS spectra of **1** and **4**. The former displays a base fragment ion at m/z 150 as described earlier,[1] while the latter shows extensive fragmentation due to the lack of charge stabilization (Figures 1a and 1b). Together, these NMR, MS and other spectral data suggested that spirolide F (**4**) is the keto-amine precursor of the final Schiff base biosynthetic product, spirolide B (**1**). This was confirmed by the acid catalyzed hydrolysis of **1** to 4,[6] the product being identified as **4** by comparison of HPLC retention times, ^1H NMR data (Table 1), and MS data including identical MS/MS spectra of the MH^+ ion, (Figure 1b).

The HR electrospray MS data for the second inactive product spirolide E (**3**) (MH^+ $C_{42}H_{64}NO_8$, 710.4632, Δ 0.0 ppm), suggested that this compound was the keto-amine derivative of spirolide A, the $\Delta^{2,3}$

derivative of **1**.[6] The location of the extra double bond was confirmed by comparison of the MS/MS spectra of the MH^+ ions of **3** and **4** (Xe CID) which share common fragment ions at m/z 613 (fragmentation between C-4 and C-5) and m/z 642 (fragmentation between C-3 and C-4 and between C-1 and O). The ^1H NMR and IR data of **3** were consistent with this assignment and were virtually identical to the data for **4**.[7] This structure was confirmed by the acid catalyzed hydrolysis of spirolide A to **3**.

The observation that **3** and **4** are inactive in the mouse bioassay is significant, and suggests that the imine group is essential for activity. To substantiate this further, the imine function in **1** was reduced to the secondary amine using borohydride.[8] The reduction product **5**, which eluted marginally faster than **4**, gave a greenish yellow stain upon spraying with ninhydrin, in accord with the presence of a secondary amine. The IR data for **5** (3474, 3393, 2929, 1765, 1453, 1170, 1006 cm^{-1}) indicated that the lactone was intact, but the very low absorption in the region 1600-1700 cm^{-1} compared with that of **1**, confirmed reduction of the imine moiety. Finally, HRMS data provided a molecular formula $C_{42}H_{65}NO_7$ (MH^+ 696.4818, Δ 3.1 ppm), establishing the addition of two hydrogens.[9] NMR data (Table 1) including TOCSY/COSY - defined partial structures **a** - **f** (Fig. 2) were concordant with **5**.

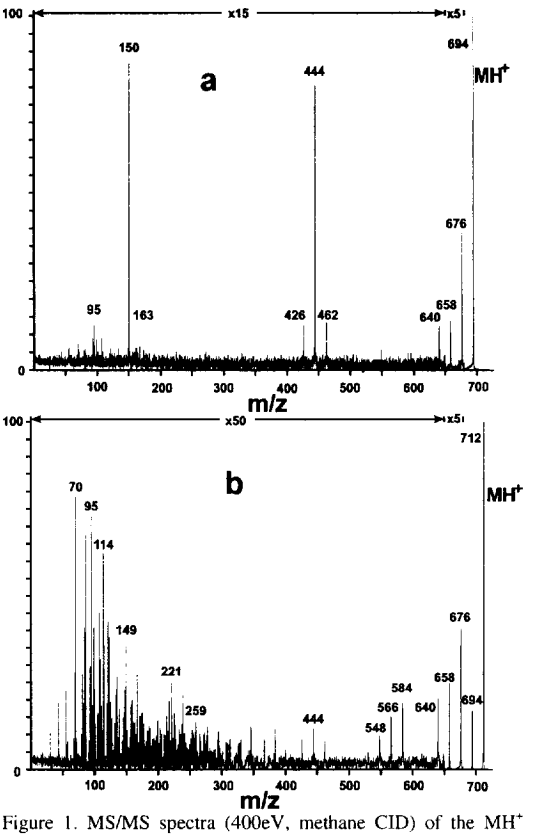


Figure 1. MS/MS spectra (400eV, methane CID) of the MH^+ ions of (a) spirolide B (**1**) and (b) spirolide F (**4**).

When the secondary amine **5** was administered in the bioassay it also failed to elict any toxic effects even at four times the equivalent spirolide B (**1**) dose.[10] Thus the combined bioassay data for **3**, **4** and **5** underscore the importance of the imine group for activity, and pinpoint this moiety as the spirolide pharmacophore. The absence of activity in the keto amines also rules out a ligand-receptor mechanism in which the imine group is first hydrolyzed to the keto-amine which then reacts with the receptor. To ensure that the loss of activity in **4** and **5** is not due to conformational changes elsewhere in the molecule following reduction or opening of the C28-N bond, energetically-minimized molecular models of **1**, **4** and **5** were constructed.[11] All three models were almost superimposable (Figure 3), suggesting that the loss of activity in **4** and **5** compared with **1** was not attributable

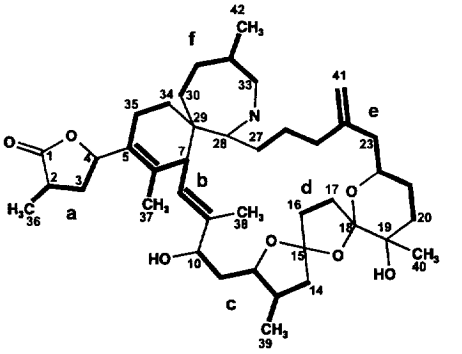


Figure 2. Partial structures (**a** - **f**) in **5**, with bold lines showing individual spin systems.

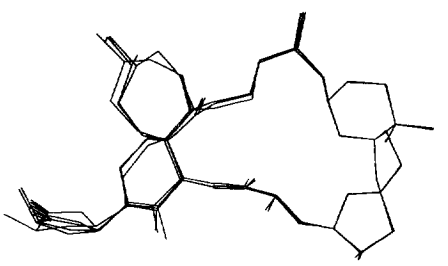


Figure 3. Superimposed structures of spirolide B (**1**), spirolide F (**4**) and dihydrospirolide B (**5**).

to significant conformational differences. The relative stereochemistry used in these models was obtained from ^{1}H NOESY data by application of the ConGen procedure,[12] and will be reported elsewhere.

Several different marine toxins induce identical symptoms in the bioassay: the structurally related spirolides, pinnatoxins and gymnodimine discussed above, as well as the structurally dissimilar macrocycles prorocentrolide,[13] and prorocentrolide B.[14] Despite gross structural differences between these two classes of compounds, all of them contain a cyclic imine group, lending further support to the proposal that this moiety is the pharmacophore.

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References and Notes

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4. The isolation and purification procedures were the same as described in reference 1. Final clean-up was achieved by reversed phase HPLC (Vydac 201TP® C18 column, eluted with CH_3CN/H_2O/TFA 30:70:0.1) to yield six compounds, spirolides A-F. Spirolides A and C have been isolated previously with B and D. The structures of A and C, which will be reported elsewhere, were identified respectively as $\Delta^{2,3}$ derivatives of spirolides B and D. Pure spirolide B, subjected to the same HPLC treatment, showed no trace of conversion to spirolide F, confirming the latter is not an artifact of purification. LCMS showed both cyclic imine and keto-amine forms of spirolide were present in the initial methanol shellfish extract.
5. The ^{1}H and ^{13}C resonances of CH_2 next to the NH_2 in 2-ethylhexylamine (Sadtler NMR Spectra: δH 2.61 and δC 45.0) are consistent with those of H33 (δ 2.73, 2.86) and C33 (δ 46.6) in **4**.
6. Spirolide B (500 μg) was dissolved in a mixture of tetrahydrofuran (0.5 ml) and an aqueous saturated solution of oxalic acid (0.5 ml), and heated (60°C) for 12 hours. The reaction mixture was directly injected into semi-preparative HPLC (Vydac 201TP® C18 column, eluted with CH_3CN/H_2O/TFA 40:60:0.1), and the hydrolyzed product collected (*ca.* 200μg, 40% yield). The latter was identical with **4** by HPLC retention time, ionspray MS data, and extensive 1D and 2D ^{1}H NMR data.
7. Spirolide E (**3**) gave a pink stain with ninhydrin. IR: 3354, 2963, 1758 (γ-lactone C=O), 1700 (C=O), 1585, 1262, 1090 cm^{-1}; δH (ppm) for resonances of **3** in CD_3OH were within 0.02 ppm of those for **4** except for 7.14 (H3), 5.95 (H4), 2.65 & 2.80 (H33), 2.28 (H14), 1.90 (H-36), 1.71 (H37), 0.97 (H31), 1.01 (H42). Insufficient material was available for ^{13}C NMR.
8. Spirolide B (200 μg) was dissolved in tetrahydrofuran (1.5 ml), $NaBH_4$ (10mg) was added and the mixture stirred for 4h at room temperature. The reaction mixture was filtered, neutralized and, after addition of H_2O (5.0 ml), extracted with CH_2Cl_2. The total extract was purified by semi-preparative HPLC (same conditions as in 4 above), yielding ca. 100 μg of pure reduced compound, which was identified as dihydrospirolide B (**5**) by HRMS and 1D and 2D NMR data (Table 1).
9. MS/MS data for the MH$^+$ ion of **5** confirmed the absence of the imine moiety in an analagous way to **4** (Fig. 1). ^{1}H NMR data for **5** were remarkably similar in many respects to those of **1** (Table 1). Chemical shift considerations and comparison of the COSY and TOCSY data established the partial structures **a** - **f** as shown in Fig. 2. Of particular note, the resonance at δ 51.3 was assigned to the reduced methine carbon at C-28. Further proof of the structure was obtained by repeating the reduction reaction using sodium borodeuteride to yield a monodeuterated product (MH$^+$ 697.4887, $C_{42}H_{65}NO_7D$, Δ 2.2 ppm). The ^{1}H resonance for H-28 was not observed in this product. The MS/MS spectra of labeled and unlabeled **5** were identical except that every high mass fragment ion in $^2H_1$-labeled **5** was 1Da higher. This, together with the knowledge that reduction took place solely at the imine group, indicates that the deuterium atom is located at C-28.
10. Each spirolide (5 μg) was dissolved in 1% Tween 80 (1.0 ml) and injected i.p. into mice. Death occurred within seven minutes after injection of spirolides A-D. In the case of **3** and **4**, both 5 μg and 20 μg doses failed to induce any symptoms.
11. Molecular models were constructed using Hyperchem®, (Hypercube Inc., 419 Phillip St., Waterloo, Ontario, Canada, N2L 3X2) minimized using the MM+ force field to a gradient of 0.1 kcal/(A mol).
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