Stolonoxides E and F, Cytotoxic Metabolites from the Marine Ascidian *Stolonica socialis*

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Two new members of the stolonoxide family, stolonoxides E (**1**) and F (**2**), were isolated from samples of the marine ascidian *Stolonica socialis* collected in Cádiz (Spain). Their structures were determined by a combination of techniques, including (+)-HRESIMS, 1D and 2D NMR spectroscopy, and comparison with published data for the structurally related stolonoxides A−D (**3**−**6**). Both compounds displayed cytotoxicity against a panel of three human tumor cell lines.

The stolonoxides and stolonic acids are a family of natural aliphatic endoperoxides obtained from samples of marine acidians belonging to the genus *Stolonica*. Stolonoxide A (**3**), the first member of the series, was isolated as its methyl ester from the Et_2O extract of the tunicate *Stolonica socialis*.[1] A second investigation conducted on the same species yielded stolonoxides B−D (**4**−**6**), with strong cytotoxic activity against a panel of five tumor cell lines.[2] Stolonoxides A and C were also found to be inhibitors of the mitochondrial respiratory chain, affecting specifically the functionality of complex II (succinate:ubiquinone oxidoreductase) and complex III (ubiquinol:cytochrome *c* oxidoreductase) in mammalian cells.[3] In addition, two new members of this structural class possessing a longer aliphatic chain, stolonic acids A and B (**7**, **8**), were isolated from CH_2Cl_2−MeOH (1:1) extracts of an undescribed ascidian species, *Stolonica* sp., collected off the Maldives Islands in the Indian Ocean. Potent antiproliferative activity against selected human melanoma and ovarian tumor cell lines was described for both compounds.[4]

In an attempt to reisolate some members of this family and further assess their potential as antitumor agents, a new collection of *S. socialis* was carried out in Cádiz (Spain) in September 2003. The frozen tunicate was extracted with 2-propanol, and to our surprise, and as observed by NMR, this extract did not contain any of the stolonoxides previously described from this species. In contrast, fractionation of the extract by solvent partition, silica gel chromatography, and reversed-phase HPLC afforded two new compounds of this structural class, the stolonoxides E (**1**) and F (**2**).

A pseudomolecular ion at $[M + H]^+$ 405.2624 in its (+)-HRESIMS and the presence of 24 signals in the ^{13}C NMR spectrum established a molecular formula of $C_{24}H_{36}O_5$ for compound **1**, requiring seven degrees of unsaturation. The "stolonoxide-like" nature of the compound was evident from ^1H and ^{13}C NMR signals (Table 1) corresponding to four oxymethine groups (δ_H 4.55, 4.08, 3.89, and 3.89) placed at C-3, C-6, C-7, and C-10 according to correlations observed in the COSY and HMBC spectra. Examination of the 2D NMR spectra allowed the assignment of the sequence from C-1 to C-12 and confirmed it to be the same as in the structure of stolonoxide A (**3**). Major differences between the compounds were found in the olefinic region of the NMR spectra. Signals for six olefinic methine hydrogens were observed for **1**, excluding therefore the presence of the terminal vinyl group present in stolonoxide A (**3**). Additionally, the presence in stolonoxide E of an sp^3 methine hydrogen, the absence in the spectra of methyl groups, and the requirement for an extra unsaturation with respect to stolonoxide A, as indicated by the molecular formula, clearly pointed to the existence of an extra ring in the structure of **1**. Analysis of the correlations observed in the COSY and HMBC spectra (Figure 1) were indicative of the presence of a substituted 1,3,5-octatriene substructure placed at the end of the aliphatic chain and further supported by 1D TOCSY spectra obtained by irradiating H-17, H-18, and H_2-24 protons. NMR chemical shifts observed for this part of the molecule were in agreement with those described for 7-methyl-1,3,5-octatriene,[5] previously described as a trace component of the volatile compounds produced by the brown alga *Cutleria multifida*.[6] Although the presence of a major product was evident in the NMR spectra of **1**, the existence of minor amounts of the corresponding bicyclo[4.2.0]octa-2,4-diene tautomers in equilibrium with the 1,3,5-octatriene ring has been described and therefore cannot be discarded.[5,6] A plausible origin for this 1,3,5-octatriene ring in the structure of stolonoxide E comes from the cyclization of an unstable tetraene function located at the end of its aliphatic chain (Figure 2). ROESY correlations between H-3 and both H-4eq (δ 1.94) and H-5ax (δ 1.76) established the H-3 axial orientation. In a similar manner, the axial orientation of H-6 was defined by a ROESY cross-peak with H-4ax (δ 1.58). Due to

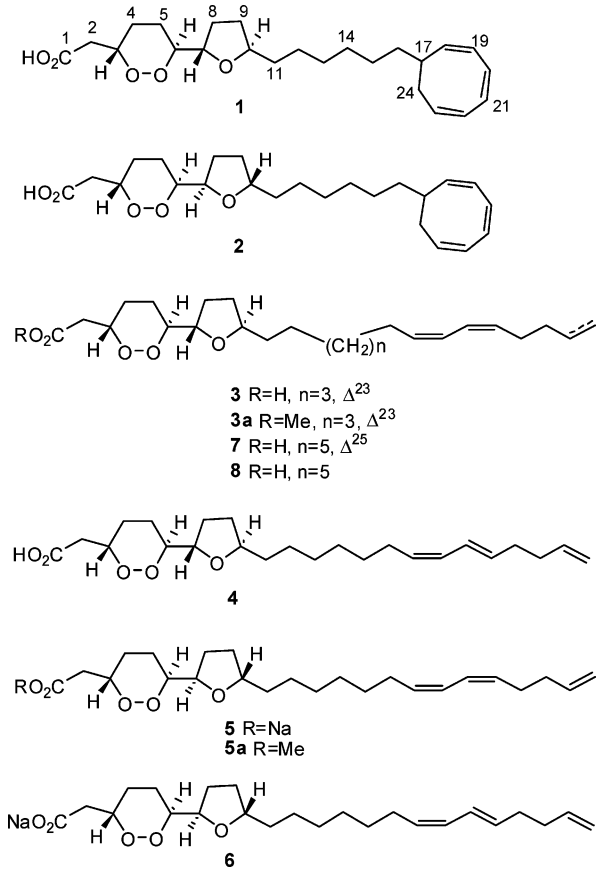

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Table 1. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for Stolonoxides E (**1**) and F (**2**)

	1		**2**	
position	δ_C, mult.	δ_H, mult. (*J* in Hz)	δ_C, mult.	δ_H, mult. (*J* in Hz)
1	173.4, C		174.3, C	
2	38.0, CH_2	2.52, dd (15.9, 7.5) 2.43, dd (15.9, 5.6)	37.9, CH_2	2.53, dd (16.0, 7.5) 2.46, dd (16.0, 5.5)
3	77.4, CH	4.55, m	77.3, CH	4.57, m
4	29.0, CH_2	1.94, m; 1.58, m	28.7, CH_2	1.95, m; 1.58, m
5	25.1, CH_2	1.76, m; 1.72, m	25.6, CH_2	1.95, m; 1.70, m
6	83.9, CH	4.08, ddd (11.1, 5.3, 2.5)	83.7, CH	4.08, m
7	78.5, CH	3.89, m	78.4, CH	3.84, dd (13.5, 7.0)
8	27.7, CH_2	1.95, m; 1.79, m	28.0, CH_2	2.01, m; 1.82, m
9	31.7, CH_2	2.00, m; 1.44, m	31.6, CH_2	2.02, m; 1.46, m
10	80.0, CH	3.89, m	79.9, CH	3.89, m
11	35.6, CH_2	1.58, m; 1.38, m	35.6, CH_2	1.58, m; 1.40, m
12	26.0, CH_2	1.30, m, 2H	26.1, CH_2	1.30, m, 2H
13	29.7, CH_2	1.26, m, 2H	29.7, CH_2	1.28, m, 2H
14	29.7, CH_2	1.26, m, 2H	29.7, CH_2	1.28, m, 2H
15	27.1, CH_2	1.30, m, 2H	27.1, CH_2	1.30, m, 2H
16	37.0, CH_2	1.34, m, 2H	37.0, CH_2	1.34, m, 2H
17	37.9, CH	2.74,m	37.9, CH	2.76, m
18	140.9, CH	5.70, dd (12.0, 6.2)	140.9, CH	5.71, dd (12.0, 6.0)
19	125.4, CH	5.87, m	125.4, CH	5.88, m
20	126.6,[a] CH	5.74, m	126.6,[a] CH	5.75, m
21	126.3,[a] CH	5.77, m	126.3,[a] CH	5.78, m
22	126.4,[a] CH	5.87, m	126.4,[a] CH	5.88, m
23	134.1, CH	5.91, m	134.1, CH	5.92, m
24	34.2, CH_2	2.31, m, 2H	34.2, CH_2	2.32, m, 2H

[a] Assignments interchangeable.

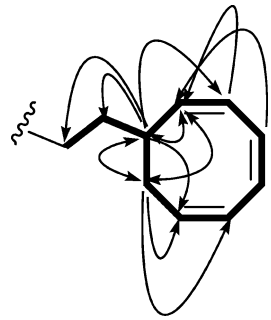

Figure 1. COSY (—) and key HMBC (→) correlations observed in the 1,3,5-octatriene ring of **1**.

the coincidence in chemical shifts between H-7 and H-10, no considerations about their relative orientation could be established on the basis of ROESY experiments. Nonetheless, the relative configuration of the C-3, C-6, C-7, and C-10 stereogenic centers was assumed to be the same as in stolonoxide A due to the nearly identical ^1H and ^{13}C NMR chemical shifts observed for this part of the molecule in both compounds. In addition, the specific rotation of **1** ($[\alpha]^{25}_D$ −33.4, *c* 0.09, $CHCl_3$) was also in close agreement with that reported for stolonoxide A methyl ester (**3a**) ($[\alpha]^{25}_D$ −33.3, *c* 0.1, $CHCl_3$), and therefore the same absolute configuration can be proposed for the four above-mentioned stereogenic centers of stolonoxide E.

Compound **2** had a molecular formula of $C_{24}H_{36}O_5$, according to its (+)-HRESIMS (*m/z* M^+ 405.2622, calcd for $C_{24}H_{37}O_5$, 405.2635) and the presence of 24 signals in its ^{13}C NMR spectrum, being therefore an isomer of **1**. The major differences found in the NMR spectra of **2** with respect to **1** were located in the region around the oxygenated C-3, C-6, C-7, and C-10 centers. H-7 was upfield shifted in **2** as a consequence of the change in the configuration of the stereogenic centers at C-7 and C-10 with respect to C-3 and C-6. In addition, no correlations were observed in the NOESY spectrum between H-7 and H-10, supporting the *trans* orientation of both protons. Similar changes were observed when comparing the NMR spectra of stolonoxide A methyl ester (**3a**)[1] with those of its stereoisomer stolonoxide C methyl ester (**5a**).[3] The positive value of the specific rotation measured for **2** ($[\alpha]^{25}_D$ +18.5, *c* 0.13, $CHCl_3$) is also in favor of this structural change ($[\alpha]^{25}_D$ +28.0, *c* 0.1, $CHCl_3$ for **5a**), and therefore, the same absolute configuration can be assumed for the C-3, C-6, C-7, and C-10 stereogenic centers in stolonoxides F and C.[2]

The cytotoxic activity of compounds **1** and **2** was tested against three human tumor cell lines, including lung (A549), colon (HT29), and breast (MDA-MB-231). Both compounds exhibited moderate activity, with GI_{50} values in the micromolar range, and no selectivity among the lines tested (Table 2). The activity displayed by both compounds is 1−2 orders of magnitude weaker than that measured for their analogues stolonoxides A−D and stolonic acids A and B, indicating perhaps that the presence of the diene and the vinyl terminal group in these latter compounds plays a key role in the cytotoxic activity displayed by members of this family.

In conclusion, two new cyclic peroxides with cytotoxic properties have been isolated from a re-collection of samples of the ascidian *S. socialis*. While being members of the stolonoxide family, with clear precedent in the literature, they incorporate a 1,3,5-octatriene ring in their molecules, an element of structural novelty with only one precedent in marine natural products. This ring seems to originate from an unstable tetraene at the end of the aliphatic chain of a potential precursor. Whether this 1,3,5-octatriene ring is formed inside the animal or during the process of extraction and purification of the compounds needs to be explored.

Experimental Section

General Experimental Procedures. Optical rotations were determined using a Jasco P-1020 polarimeter. UV spectra were obtained with an Agilent 1100 DAD. NMR spectra were recorded on a Varian "Unity 500" spectrometer at 500/125 MHz ($^1H/^{13}C$). Chemical shifts were reported in ppm using residual $CDCl_3$ (δ 7.26 for ^1H and 77.0 for ^{13}C) as internal reference. HMBC experiments were optimized for a $^3J_{CH}$ of 8 Hz. ROESY spectra were measured with a mixing time of 350 ms. (+)-HRESIMS was performed on a Applied Biosystems QSTAR Pulsar i hybrid Q-TOF spectrometer. ESIMS were recorded using an Agilent 1100 Series LC/MSD spectrometer.

Animal Material. *S. socialis* was collected in September 2003 by scuba at a depth between 3 and 15 m in Las Lajas (Cádiz, Spain) (36°06′11″ N, 5°25′47″ W). The animal material was identified by Dr. Santiago Naranjo from PharmaMar. A voucher specimen is deposited at PharmaMar (ORMA028621).

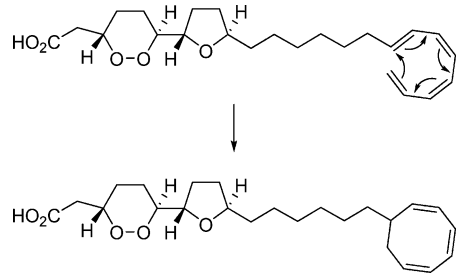

Figure 2. Possible explanation for the formation of the 1,3,5-octatriene ring of **1**.

Table 2. Cytotoxic Activity (μM) of Stolonoxides E (**1**) and F (**2**) and Doxorubicin

cell line		**1**	**2**	doxorubicin
MDA-MB-231	GI_{50}	4.94	4.70	0.038
	TGI	5.44	5.44	0.31
	LC_{50}	6.18	6.18	2.41
HT29	GI_{50}	3.96	2.72	0.066
	TGI	5.19	3.21	0.40
	LC_{50}	6.67	3.71	>17.2
A549	GI_{50}	7.91	5.44	0.062
	TGI	8.16	5.93	0.26
	LC_{50}	8.65	6.67	1.57

Extraction and Isolation. The frozen organism (1.94 kg) was triturated and extracted with 2-propanol (3 × 1 L) at room temperature. The organic extract was evaporated under reduced pressure to yield a crude of 129.92 g. This material was partitioned against hexanes (3 × 300 mL) and EtOAc (3 × 300 mL). The EtOAc fraction (1.0 g) was chromatographed on silica gel (hexanes−EtOAc mixtures). Fractions eluting from this chromatography with 3:2 hexanes−EtOAc were subjected to preparative HPLC (SymmetryPrep C18, 19 × 150 mm, isocratic H_2O + 0.1% TFA:MeOH + 0.1% TFA, 17:83, flow 15 mL/min, UV detection at 254 nm) to yield impure compounds **1** and **2**. Final purification of compound **1** was achieved by semipreparative HPLC (X-Terra RP-18, 10 × 150 mm, gradient H_2O + 0.1% TFA/CH_3CN + 0.1% TFA from 47% to 85% organic in 18 min, flow 3.5 mL/min, UV detection at 254 nm) to yield 18.9 mg of compound. **2** (13.8 mg) was obtained by semipreparative HPLC in a similar manner (X-Terra RP-18, 10 × 150 mm, gradient H_2O + 0.1% TFA/CH_3CN + 0.1% TFA from 47% to 90% organic in 25 min, flow 3.5 mL/min, UV detection at 254 nm).

Stolonoxide E (1): colorless oil; $[\alpha]^{25}_D$ −33.4 (*c* 0.09, $CHCl_3$); ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 1; (+)-APCIMS *m/z* 405 $[M + H]^+$, 387 $[M + H - H_2O]^+$, 343 $[M + H - H_2O - CO_2]^+$; (+)-HRESIMS *m/z* 405.2624 $[M + H]^+$ (calcd for $C_{24}H_{37}O_5$, 405.2635)

Stolonoxide F (2): colorless oil; $[\alpha]^{25}_D$ +18.5 (*c* 0.13, $CHCl_3$); ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 1; (+)-APCIMS *m/z* 405 $[M + H]^+$, 387 $[M + H - H_2O]^+$, 343 $[M + H - H_2O - CO_2]^+$; (+)-HRESIMS *m/z* 405.2622 $[M + H]^+$ (calcd for $C_{24}H_{37}O_5$, 405.2635).

Biological Activity. A549 (ATCC CCL-185, lung carcinoma), HT29 (ATCC HTB-38, colorectal carcinoma), and MDA-MB-231 (ATCC HTB-26, breast adenocarcinoma) cell lines were obtained from the ATCC. Cell lines were maintained in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 U/mL penicillin and streptomycin, at 37 °C and 5% CO_2. Triplicate cultures were incubated for 72 h in the presence or absence of test compounds (at 10 concentrations ranging from 10 to 0.0026 μg/mL). For quantitative estimation of cytotoxicity, the colorimetric sulforhodamine B (SRB) method was used.[7] Briefly, cells were washed twice with PBS, fixed for 15 min in 1% glutaraldehyde solution, rinsed twice in PBS, and stained in 0.4% SRB solution for 30 min at room temperature. Cells were then rinsed several times with 1% acetic acid solution and air-dried. Sulforhodamine B was then extracted in 10 mM trizma base solution and the absorbance measured at 490 nm. Results are expressed as GI_{50}, the concentration that causes 50% inhibition in cell growth after correction for cell count at the start of the experiment (NCI algorithm). Doxorubicin and DMSO (solvent) were used as the positive and negative controls in this assay. Prism 3.03 from GraphPad was used for the statistical analysis of the cell growth inhibition results.

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Supporting Information Available: ^1H and ^{13}C NMR spectra for compounds **1** and **2** and an underwater picture of the organism are available free of charge via the Internet at http://pubs.acs.org.

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