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Minor Metabolites from the Ascidian *Stolonica socialis* and Cytotoxicity of Stolonoxides

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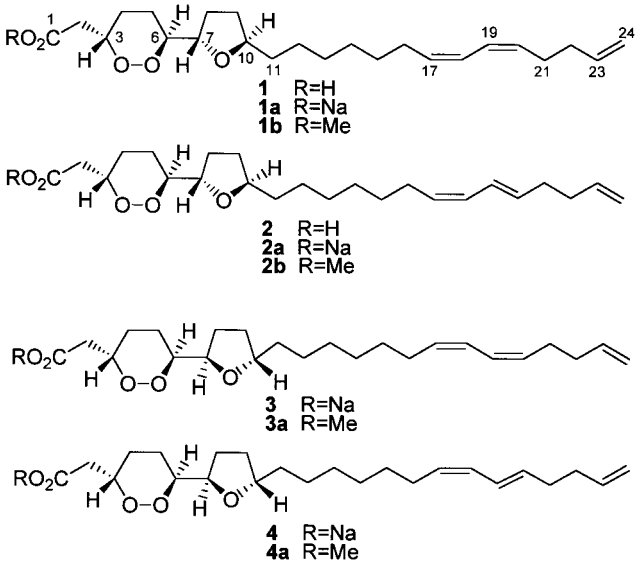
Abstract—Bioassay guided isolation of the metabolites of the ascidian *Stolonica socialis* from Tarifa Island (Cádiz, Spain) led to a series of cyclic peroxides. A 9:1 mixture of stolonoxide A and the new stolonoxide B, a 6:4 mixture of their corresponding sodium salts, and a minor 6:4 mixture of the new stolonoxides C and D obtained as their sodium salts. Their structures were established by spectroscopic study of both the natural mixtures, whose constituents differ in the geometry of a double bond, and the corresponding methyl esters. The strong cytotoxicity exhibited by the stolonoxides against five tumor cell lines is presented and discussed.

Marine ascidians continue to focus interest of both marine chemical and biomedical research. The chemistry of ascidians is dominated by amino acid derived compounds either peptides or alkaloids. However, the non-nitrogenous metabolites from ascidians, although considerably minor in number, are by no means less important.[1]

As a part of the research project carried out in our laboratories aimed to examine the biomedical potential of new metabolites from ascidians of the southern coast of Spain,[2] we collected specimens of the ascidian *Stolonica socialis* (Hartmeyer, 1903) belonging to the Family Styelidae. Specimens of *S. socialis* were collected by hand using SCUBA off Tarifa Island (Cádiz, Spain) and immediately frozen. The frozen material was extracted with an acetone: methanol mixture (1:1). After evaporation of the solvent the aqueous residue was extracted with Et_2O to yield a cytotoxic extract active against the tumor cell lines of mouse lymphoma P-388, human melanoma MEL-28, human prostate carcinoma DU-145, human lung carcinoma A-549, and human colon carcinoma HT-29 (IC_{50}=5 μg/mL). Column chromatography of the Et_2O soluble material yielded six fractions of which fifth fraction had specific activity against the mentioned tumor cell lines (IC_{50}=0.05 μg/mL). Further purification of this fraction using reversed phase HPLC allowed isolation of three pairs of compounds: a 9:1 mixture of stolonoxide A (**1**) and stolonoxide B (**2**), a 6:4 mixture of their corresponding sodium salts (**1a/2a**), and a 6:4 mixture of the sodium salts of stolonoxide C and stolonoxide D (**3/4**). The individual components of each of these three pairs of compounds were unseparable under all the HPLC conditions assayed and therefore it was necessary to derive the natural constituents. Once the individual components of each mixture were identified, attempts at further purification were abandoned in order to preserve material for biological assessment.



The NMR and MS data of the mixture of stolonoxides A (**1**) and B (**2**), together with the IR absorptions at 3460 and 1745 cm^{-1}, suggested the presence of two isomeric carboxylic acids, which differed in their olefinic pattern. Treatment of this mixture with diazomethane afforded the methyl esters **1b** and **2b**, that could be isolated after repeated HPLC.

Keywords: marine metabolites; biologically active compounds; peroxides.
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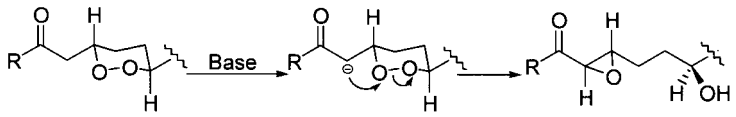
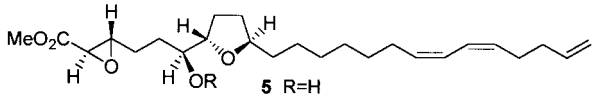


Scheme 1. A proposed mechanism for the oxirane ring formation under basic conditions.

The high resolution mass measurement indicated that the major methyl ester **1b** had the molecular formula $C_{25}H_{40}O_5$ which implies six degrees of unsaturation. The presence of the methyl ester group was confirmed by the ^{1}H NMR singlet at δ 3.69 (s, 3H) and the ^{13}C NMR signals at 170.4 (s) and 51.9 (q). The ^{13}C NMR spectrum contained six olefinic carbon signals at δ 138.2 (d), 132.4 (d), 130.8 (d), 124.0 (d), 123.4 (d), and 114.7 (t) attributable to a monosubstituted and two disubstituted double bonds. Furthermore, four doublets of methine carbons bearing oxygen at δ 83.8 (d), 79.9 (d), 78.5 (d), and 77.6 (d), together with the absence of further carbonyl, methine, or quaternary carbon signals in the ^{13}C NMR spectrum, indicated that **1b** was the methyl ester of a C_{24} acid containing three double bonds and two oxygenated rings, which gave rise to the four methines bearing oxygen, and accounted for the remaining two degrees of unsaturation.

While this research was in progress we were aware of the publication of the structure of stolonoxide A, a novel cyclic peroxide isolated as its methyl ester from *Stolonica socialis* collected at the same location where our specimens had been obtained.[3] Comparison of the spectroscopic data of **1b** with those reported for stolonoxide A methyl ester proved that the compounds were identical.

The absolute stereochemistry of stolonoxide A (**1**) has been suggested as 3*S*, 6*S*, 7*S*, 10*R* by application of Mosher's method to the C-3, C-6 diol obtained through peroxide ring opening by catalytic hydrogenation of the methyl ester **1b**.[3] To avoid the apparently confusing results arising from the double MTPA esters we employed Mosher's chiral reagent on the monohydroxy derivative **5**.



5 R=H
5a R=(*R*)-MTPA
5b R=(*S*)-MTPA

The epoxide **5** was obtained by treatment of the methyl ester **1b** with NaOH followed by re-esterification with diazomethane. A proposed mechanism for the formation of the oxirane ring in **5** is shown in Scheme 1. The molecular formula of **5**, $C_{25}H_{40}O_5$, was obtained from the high resolution mass measurement. The IR absorption at 1755 cm^{-1} together with the NMR signals at δ_H 3.77 (3H, s) and δ_C 169.7 (s) and 52.4 (q) confirmed that **5** was a methyl ester. A comparison between the spectral data of **5** and those of methyl ester **1b** clearly stated that **5** lacked the methylene adjacent to the methoxy carbonyl group and the peroxide ring present in the methyl ester **1b**. The presence of an oxirane ring in **5** appeared evident from the ^{13}C NMR doublets at δ 58.1 and 53.0. Furthermore, these carbon signals were correlated in the HMQC spectrum with the proton signals at δ 3.23 (1H, ddd, J=6.3, 4.9 and 1.9 Hz) and 3.27 (1H, d, J=1.9 Hz), respectively. The coupling constant observed between the oxirane ring protons (J=1.9 Hz) requires a *trans* stereochemistry for the three-membered ring.[4]

The secondary hydroxyl group present in **5** gave rise in the IR spectrum to the absorption at 3465 cm^{-1} and to a ^{13}C NMR doublet at δ 73.0 that was correlated in the HMQC spectrum with the ^{1}H NMR signal at 3.43 (1H, m). COSY, LR COSY and HMBC spectra provided confirmation to the location of both the hydroxyl group at C-6 and the oxirane ring at C-2, C-3. All these spectral features are consistent with the structure **5**. It is worth noting that derivative **5** bears a single hydroxyl group and retains the absolute configuration at all the chiral centers of the natural compound.

The (*R*)- and (*S*)-MTPA esters **5a** and **5b** were obtained by treatment of **5** with (*R*)- and (*S*)-MTPA acids, respectively. Positive $\Delta\delta$ ($\delta_S-\delta_R$) values were found for H-8, H-9 and H-10 while negative $\Delta\delta$ values were found for H-2, H-3, H-4, and H-5 (Fig. 1). Following the MTPA rules these data indicated an *S* configuration for C-6 and therefore an absolute stereochemistry 2*S*, 3*S*, 6*S*, 7*S*, 10*R* for the epoxide derivative **5**. This result unambiguously confirms an absolute stereochemistry 3*S*, 6*S*, 7*S*, 10*R* for stolonoxide A (**1**).

The minor methyl ester **2b**, obtained by methylation of the natural mixture of **1** and **2**, was isolated as a colorless oil of molecular formula $C_{25}H_{40}O_5$ as indicated by the high resolution mass measurement. A general inspection of the ^{1}H and ^{13}C NMR of both isomers **1b** and **2b**, clearly showed that their structures were closely related and that they shared the same carbon skeleton and functionalities. However, slight differences were observed in the signals corresponding to the conjugated disubstituted double bonds. Thus, the ^{13}C NMR spectrum of **2b** exhibited the signals of the olefinic carbons at δ 130.5 (d), 128.4 (d), 126.1 (d), and 133.5 (d) which in the HMQC spectrum were correlated with the proton signals at δ 5.31 (1H, dt, J=11.0 and 7.6 Hz), 5.94

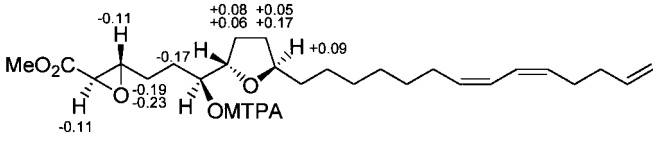


Figure 1. Chemical shifts differences ($\Delta\delta$) between the MTPA derivatives of **5**.

Table 1. ^{1}H NMR data recorded in $CDCl_3$ for the natural metabolites of *Stolonica socialis* (assignments were aided by COSY, LR COSY and HMQC experiments)

	1[a]	2[a]	1a	2a	3	4
2	2.49 (dd, 15.8, 7.2) 2.44 (dd, 15.8, 5.3)		2.26 (m)		2.34 (m)	
3	4.52 (m)		4.45 (m)		4.48 (m)	
4	1.95 (m, Heq) 1.60 (m, Hax)		1.90 (m, Heq) 1.49 (m, Hax)		1.91 (m, Heq) 1.52 (m, Hax)	
5	1.73 (m)		1.60 (m)		1.65 (m)	
6	4.07 (m)		4.00 (m)		4.02 (m)	
7	3.87 (m)		3.79 (m)		3.82 (bd, 6.9)	
8	1.95 (m) 1.77 (m)		1.92 (m) 1.66 (m)		1.95 (m) 1.70 (m)	
9	2.00 (m) 1.44 (m)		1.97 (m) 1.43 (m)		1.97 (m) 1.43 (m)	
10	3.87 (m)		3.88 (m)		3.88 (m)	
11	1.58 (m) 1.33 (m)		1.60 (m) 1.32 (m)		1.60 (m) 1.34 (m)	
12	1.30–1.20 (m)		1.30–1.20 (m)		1.30–1.20 (m)	
13	1.30–1.20 (m)		1.30–1.20 (m)		1.30–1.20 (m)	
14	1.30–1.20 (m)		1.30–1.20 (m)		1.30–1.20 (m)	
15	1.30–1.20 (m)		1.30–1.20 (m)		1.30–1.20 (m)	
16	2.15 (m)		2.14 (m)		2.15 (m)	
17	5.44 (m)	5.30 (m)	5.44 (m)	5.30 (m)	5.44 (m)	5.31 (dt, 10.9, 7.4)
18	6.25 (m)	5.96 (bt, 10.8)	6.24 (m)	5.94 (bt, 10.6)	6.24 (m)	5.93 (bt, 10.9)
19	6.25 (m)	6.30 (m)	6.24 (m)	6.31 (m)	6.24 (m)	6.31 (bdd, 15.2, 10.9)
20	5.44 (m)	5.65 (m)	5.44 (m)	5.65 (m)	5.44 (m)	5.65 (bdt, 15.2, 6.6)
21	2.27 (bdd, 14.7, 7.5)	2.17 (m)	2.26 (m)	2.17 (m)	2.27 (bdd, 14.8,7.5)	2.18 (m)
22	2.13 (m)		2.14 (m)		2.15 (m)	
23	5.82 (ddt, 17.0, 10.2, 6.5)		5.82 (ddt, 16.9, 10.3, 6.5)		5.82 (ddt, 17.0, 10.2, 6.5)	
24	5.03 (ddd, 17.0, 1.7, 1.6) 4.96 (bd, 10.2)		5.03 (bd, 16.9) 4.97 (bd, 10.3)		5.03 (bd, 17.0) 4.96 (bd, 10.2)	

[a] Assignments were aided by an HMBC experiment.

Table 2. ^{13}C NMR data recorded in $CDCl_3$ for the natural metabolites of *Stolonica socialis* (assignments were aided by an HMQC experiment)

	1[a]	2[a]	1a	2a	3	4
1	175.1 (s)		177.1 (s)		176.0 (s)	
2	38.4 (t)		41.3 (t)		40.0 (t)	
3	77.4 (d)		79.2 (d)		78.5 (d)	
4	29.0 (t)		29.0 (t)		29.0 (t)	
5	25.1 (t)		25.1 (t)		25.1 (t)	
6	83.8 (d)		84.0 (d)		84.0 (d)	
7	78.5 (d)		78.7 (d)		78.6 (d)	
8	27.7 (t)		28.1 (t)		28.0 (t)	
9	31.6 (t)		31.6 (t)		31.6 (t)	
10	80.0 (d)		79.9 (d)		79.9 (d)	
11	35.5 (t)		35.6 (t)		35.6 (t)	
12	26.0 (t)		25.9 (t)		26.0 (t)	
13	29.6 (t)[b]		29.7 (t)[b]		29.7 (t)[b]	
14	29.5 (t)[b]		29.6 (t)[b]		29.6 (t)[b]	
15	29.2 (t)[b]		29.3 (t)[b]		29.3 (t)[b]	
16	27.4 (t)	27.7 (t)	27.5 (t)	27.7 (t)	27.5 (t)	27.8 (t)
17	132.4 (d)	130.4 (d)	132.3 (d)	130.4 (d)	132.4 (d)	130.4 (d)
18	123.4 (d)	128.4 (d)	123.5 (d)	128.5 (d)	123.5 (d)	128.5 (d)
19	124.0 (d)	126.0 (d)	124.0 (d)	126.0 (d)	124.0 (d)	126.1 (d)
20	130.8 (d)	133.4 (d)	130.8 (d)	133.5 (d)	130.8 (d)	133.5 (d)
21	26.8 (t)	32.2 (t)	26.9 (t)	32.2 (t)	26.9 (t)	32.2 (t)
22	33.7 (t)		33.7 (t)		33.7 (t)	
23	138.2 (d)		138.2 (d)		138.2 (d)	
24	114.7 (t)		114.8 (t)		114.8 (t)	

[a] Assignments were aided by an HMBC experiment.
[b] Values with the same superscript in the same column may be interchanged.

(1H, t, J=11.0 Hz), 6.32 (1H, dd, J=15.1 and 11.0 Hz), and 5.65 (1H, dt, J=15.1 and 6.7 Hz), respectively. The coupling constants observed are consistent with a different geometry for each of the two conjugated double bonds. Since the *trans* double bond proton signal at δ 5.65 was correlated in the COSY spectrum with an allylic methylene proton signal at δ 2.20 (2H, m) which was in turn coupled with the signal at δ 2.17 (2H, m) due to the methylene allylic to the terminal double bond, the *trans* double bond must be located at C-19, C-20. The ^{13}C NMR chemical shifts of the allylic methylene carbons C-21 at δ 32.2 and C-16 at δ 27.7 confirmed the proposed geometry for the diene.[5] It was characterized the structure of the methyl ester as **2b** and therefore structure **2** was proposed for stolonoxide B. In the absence of an independent determination of the absolute stereochemistry of **2** it was assumed, based on the similar optical rotations, an identical configuration of the stereogenic centers C-3, C-6, C-7, and C-10 as that determined for stolonoxide A (**1**).

It is worth noting that the structure determination of esters **1b** and **2b** aided assignation of the NMR data of the natural mixture of stolonoxides A (**1**) and B (**2**) to each of the individual compounds (Tables 1 and 2).

Careful separation on reversed phase HPLC allowed isolation of a mixture of the sodium carboxylate salts **1a** and **2a**. The inspection of the ^{1}H and ^{13}C NMR spectra of **1a** and **2a**, although clearly indicated that their structures were closely related to those of stolonoxides A (**1**) and B (**2**), showed some diagnostic differences. In the ^{13}C NMR spectrum the resonances attributed to C-1, C-2, and C-3 at δ 177.1 (s),

Table 3. Cytotoxicity assay results against five tumor cell lines for the stolonoxides and derivatives (IC_{50}, μg/mL)

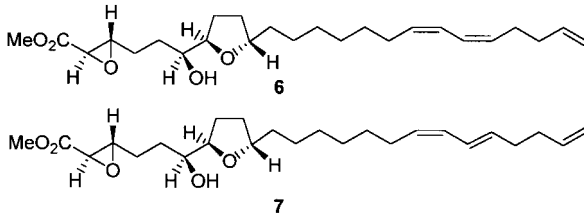
	P-388	A-549	HT-29	MEL-28	DU-145
1/2	0.01	0.10	0.10	0.10	0.10
1a/2a	0.05	0.10	0.10	0.10	0.10
1b	0.10	0.10	0.10	–	–
2b	0.50	0.10	0.10	0.50	0.10
3/4	0.01	0.01	0.05	0.10	0.10
5	>1	>1	>1	>1	>1
Doxorubicin[a]	0.02	0.002	0.05	0.02	–

[a] Standard compound.

41.3 (t) and 79.2 (d), respectively, were downfield shifted with respect to the resonances observed for these carbons in compounds **1** and **2**. On the other hand, in the ^{1}H NMR spectrum the signals of the methylene protons H-2 and of the methine proton H-3 at δ 2.26 (2H, m) and 4.45 (1H, m), respectively, were upfield shifted with respect to those observed for these protons in **1** and **2**. These effects can be explained by the presence in **1a** and **2a** of a carboxylate group at C-1 instead of the corresponding carboxylic group.[5] The IR absorption at 1579 cm^{-1} was in complete agreement with this assignment. Treatment of the mixture with HCl and, subsequently, with diazomethane afforded a mixture of the methyl esters **1b** and **2b** indicating that **1a** and **2a** were carboxylate salts of **1** and **2**. As the ESIMS of the mixture **1a** and **2a** displayed an $[M-Na]^-$ ion at *m/z* 405 in the negative mode and the $[M+H]^+$ and $[M+Na]^+$ ions at *m/z* 429 and 451 in the positive mode, it was concluded that the compounds **1a** and **2a** isolated from *S. socialis* were the corresponding sodium carboxylate salts of the stolonoxide A (**1**) and B (**2**).

The more polar component of the constituents of *S. socialis* was a minor 6:4 mixture of stolonoxides C and D isolated as their corresponding sodium salts (**3/4**). The IR absorption of **3** and **4** at 1584 cm^{-1} together with the ^{1}H and ^{13}C NMR spectra suggested that the constituents had to be quite similar to those present in the mixture of stolonoxides A and B sodium carboxylates (**1a/2a**). The ESIMS ion $[M+H]^+$ in the positive mode at *m/z* 429 were consistent with a molecular formula $C_{24}H_{37}O_5Na$. Treatment of the mixture of **3** and **4** with HCl, followed by diazomethane afforded the corresponding methyl esters **3a** and **4a** that could not be separated by HPLC. However, the structure of the methyl esters could be deduced by the spectroscopic study of the mixture allowing, in addition, identification of the natural components **3/4**. Thus, the molecular formula $C_{25}H_{40}O_5$, obtained from the high resolution mass measurement, indicated that the methyl esters **3a** and **4a** were isomers of the stolonoxides A and B methyl esters (**1b**, **2b**). Furthermore, the comparison of the ^{1}H and ^{13}C NMR spectra indicated that they shared an identical gross structure and that the structural differences were due to minor differences in stereochemistry.

The relative stereochemistry at the stereogenic centers C-3, C-6, C-7, and C-10 was established as follows. The axial orientation of H-3 was deduced upon observation of a coupling constant of 11.0 Hz between H-3 and H-4ax signals and by the correlations exhibited in the ROESY spectrum between the H-3 signal with the H-4eq and H-5ax signals. The axial orientation of H-6 was clear from the observation of a coupling constant of 10.9 Hz between H-6 and H-5ax signals and by the cross peaks observed in the ROESY spectrum between the H-6 signal and the H-5eq and H-4ax signals. Furthermore, the analysis of the correlations observed in the ROESY spectrum and a series of NOE difference spectroscopy experiments required H-7 and H-10 to be oriented towards opposite sides of the tetrahydrofuran ring. However, the stereochemical relationship between both oxygenated rings could not be unequivocally established by NMR study of the methyl esters **3a** and **4a**. This stereochemical assignment was deduced by a careful study of the unseparable mixture of epoxides **6** and **7** arising by treatment of the mixture of the methyl esters **3a** and **4a** with NaOH, followed by diazomethane. Basically the ^{1}H and ^{13}C NMR spectra of **6** and **7** were quite similar to those of the epoxide **5** discussed above excepting for the ^{1}H NMR signal of the H-6 at δ 3.79 (1H, m) and the ^{13}C NMR doublet of C-6 at δ 71.3. These chemical shifts fit better for an *erythro* orientation of the substituents around C-6 and C-7 stereogenic centers rather than the alternative *threo* orientation present in epoxide **5**.[6] Based on this result the relative stereochemistry 3*S**, 6*S**, 7*R**, 10*S** for the stolonoxides C and D sodium salts (**3/4**) was proposed.



Bioassays aimed to identify the compounds responsible for the cytotoxicity of the crude extract and the most active fraction were performed using cultures of mouse lymphoma (P-388), human lung carcinoma (A-549), human colon carcinoma (HT-29), human melanoma (MEL-28), and human prostate carcinoma (DU-145). In general the stolonoxides (**1**–**4**) are strongly cytotoxic compounds as stated by the IC_{50} values (μg/mL), which are displayed in Table 3 along with the values found for the standard doxorubicine. The natural mixture 9:1 of stolonoxides A (**1**) and B (**2**) exhibited significant and selective cytotoxicity against P-388 cell line (IC_{50}=0.01 μg/mL) with their corresponding sodium salts mixture (**1a/2a**) being less cytotoxic than the corresponding free acids. However, the mixture of stolonoxides C and D sodium salts (**3/4**) was also strongly cytotoxic against P-388 and A-549 cell lines (IC_{50}=0.01 μg/mL). Interestingly, the synthetic epoxide derivative **5** was inactive against the five tumor cell lines tested. This result states the importance of the presence of the peroxide ring in relation with the cytotoxic activity.

Experimental

General

Optical rotations were measured on a Perkin–Elmer 241 polarimeter. IR and UV spectra were recorded on a Genesis

Series FT IR Mattson and Philips PU 8710 spectrophotometer, respectively. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Varian Unity 400 spectrometer using $CDCl_3$ as solvent. Proton chemical shifts were referenced to the residual $CHCl_3$ signal at δ 7.26 and ^{13}C NMR spectra were referenced to the central peak of $CDCl_3$ at δ 77.0. ^{1}H–^{1}H-COSY, LR COSY, HMQC and HMBC were performed using standard VARIAN pulse sequences. Assignments marked with an asterisk may be interchanged. Mass spectra were recorded on a VG Autospec spectrometer. Column chromatography was carried out using Merck Silica gel 60 (70–230 mesh). HPLC separations were performed on a LaChrom-Hitachi apparatus equipped with LiChrosorb RP-18 (Merck) and LiChrosorb Si 60 (Merck) columns using a differential refractometer RI-71. All solvents were spectral grade or were distilled from glass prior to use.

Collection, extraction, and isolation procedures

Specimens of *Stolonica socialis* (76 g dry weight) were collected by hand using SCUBA off Tarifa Island in May 1996 and immediately frozen. A voucher specimen is deposited in the Benthic Invertebrate Collection at Laboratorio de Biología Marina, Universidad de Sevilla (#LLMA3496). The frozen samples were extracted with acetone–methanol (1:1) at room temperature. The filtered solution was evaporated under reduced pressure to yield an aqueous residue that was further extracted sequentially with Et_2O (4×450 mL) and *n*-BuOH (3×500 mL). The Et_2O extract was filtered and concentrated to yield 5.7 g of an orange cytotoxic oil (5.7 g) which was chromatographed on a SiO_2 column eluting with mixtures of increasing polarities from hexane to Et_2O and, subsequently, EtOAc, $CHCl_3$/MeOH (1:1) and MeOH. Selected fractions were subjected to repeated reversed phase HPLC separations on a preparative LiChrosorb RP-18 column eluting with MeOH/H_2O (9:1) to afford in order of elution: stolonoxides C and D sodium salts (**3/4**, 17 mg, 0.022% dry wt), stolonoxides A and B sodium salts (**1a/2a**, 58 mg, 0.076% dry wt), and stolonoxides A and B (**1/2**, 80 mg, 0.105% dry wt). Final purification was accomplished by repeated HPLC on reversed phase mode using mixtures of MeOH/H_2O.

Stolonoxides A and B (1/2). Amorphous powder; $[\alpha]_D^{25}=-62.9°$ (*c* 0.49, $CHCl_3$); IR (film) 3460, 1745, 1237, 1064 cm^{-1}; UV (MeOH) λ_{max} 234 (ϵ=20400) nm; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 2; EIMS (70 eV) *m/z* (rel. int.) 406 (18), 388 (7), 344 (5), 261 (10), 219 (5), 135 (15), 121 (23), 95 (50), 67 (100); HREIMS *m/z* 406.2734, $C_{24}H_{38}O_5$ requires *m/z* 406.2719.

Stolonoxides A and B sodium salts (1a/2a). Amorphous powder; $[\alpha]_D^{25}=-76.8°$ (*c* 0.19, $CHCl_3$); IR (film) 1579, 1435, 1070 cm^{-1}; UV (MeOH) λ_{max} 234 (ϵ=19600) nm; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 2; (–)LRESIMS *m/z* (rel. int.) 405 $[M-Na]^-$ (100), (+)LRESIMS *m/z* (rel. int.) 429 [M ($C_{24}H_{37}O_5Na$)+H]$^+$ (100), 451 $[M+Na]^+$ (5); HREIMS *m/z* 406.2766, $C_{24}H_{38}O_5$ requires *m/z* 406.2719.[7]

Stolonoxides C and D sodium salts (3/4). Amorphous powder; $[\alpha]_D^{25}=+38.0°$ (*c* 0.2, $CHCl_3$); IR (film) 1584, 1440, 1057 cm^{-1}; UV (MeOH) λ_{max} 234 (ϵ=22500) nm; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 2; (–)LRESIMS *m/z* (rel. int.) 405 $[M-Na]^-$ (100), (+)LRESIMS *m/z* (rel. int.) 429 [M ($C_{24}H_{37}O_5Na$)+H]$^+$ (100); HREIMS *m/z* 388.2639, $C_{24}H_{36}O_4$ requires *m/z* 388.2614.[7]

Methylation of the mixture of stolonoxides A (1) and B (2). Excess of CH_2N_2 was added to a solution of the mixture of stolonoxide A (**1**) and B (**2**) (22 mg) in Et_2O (2 mL) at room temperature. After 1 h, the solvent was removed under reduced pressure and the crude of reaction subjected to repeated HPLC separations to yield stolonoxide A methyl ester (**1b**, 18 mg) and stolonoxide B methyl ester (**2b**, 2 mg).

Methylation of the mixtures 1a/2a and 3/4. Methylation of these mixtures was performed using the same procedure described above but the mixtures were previously acidified with a solution of 1 N HCl.

Stolonoxide A methyl ester (1b). colorless oil; $[\alpha]_D^{25}=-50.8°$ (*c* 0.39, $CHCl_3$); IR (film) 1745, 1640, 1200, 1060 cm^{-1}; UV (MeOH) λ_{max} 235 (ϵ=22000) nm; HREIMS *m/z* 420.2865, $C_{25}H_{40}O_5$ requires *m/z* 420.2876.

Stolonoxide B methyl ester (2b). colorless oil; $[\alpha]_D^{25}=-37.7°$ (*c* 0.26, $CHCl_3$); IR (film) 1745, 1200, 1090 cm^{-1}; UV (MeOH) λ_{max} 233 (ϵ=19900) nm; ^{1}H NMR ($CDCl_3$) 6.32 (dd, *J*=15.1, 11.0 Hz, 1H, H-19), 5.94 (t, *J*=11.0 Hz, 1H, H-18), 5.82 (ddt, *J*=16.9, 10.3, 6.6 Hz, 1H, H-23), 5.65 (dt, *J*=15.1, 6.7 Hz, 1H, H-20), 5.31 (dt, *J*=11.0, 7.6 Hz, 1H, H-17), 5.03 (ddd, *J*=16.9, 1.6, 1.3 Hz, 1H, H-24), 4.97 (bd, *J*=10.3 Hz, 1H, H-24), 4.54 (dddd, *J*=11.2, 7.7, 6.0, 2.3 Hz, 1H, H-3), 4.06 (ddd, *J*=11.0, 5.6, 2.6 Hz, 1H, H-6), 3.87 (m, 2H, H-7 and H-10), 3.69 (s, 3H, CH_3O–), 2.47 (dd, *J*=15.6, 7.7 Hz, 1H, H-2), 2.36 (dd, *J*=15.6, 6.0 Hz, 1H, H-2), 2.20 (m, 2H, H-21), 2.17 (m, 2H, H-22), 2.14 (m, 2H, H-16), 1.99 (m, 1H, H-9), 1.95 (m, 1H, H-8), 1.92 (m, 1H, H-4eq), 1.77 (m, 2H, H-5ax and H-8), 1.70 (m, 1H, H-5eq), 1.58 (m, 1H, H-11), 1.54 (m, 1H, H-4ax), 1.42 (m, 1H, H-9), 1.34 (m, 1H, H-11), 1.33 (m, 1H, H-12), 1.30 (m, 6H, H-13, H-14 and H-15), 1.28 (m, 1H, H-12); ^{13}C NMR ($CDCl_3$) 170.4 (s, C-1), 138.2 (d, C-23), 133.5 (d, C-20), 130.5 (d, C-17), 128.4 (d, C-18), 126.1 (d, C-19), 114.7 (t, C-24), 83.8 (d, C-6), 79.9 (d, C-10), 78.5 (d, C-7), 77.6 (d, C-3), 51.2 (q, CH_3O–), 38.4 (t, C-2), 35.6 (t, C-11), 33.6 (t, C-22), 31.7 (t, C-9), 32.2 (t, C-21), 29.6 (t, C-13)*, 29.6 (t, C-14)*, 29.2 (t, C-15)*, 29.1 (t, C-4), 27.7 (t, C-8), 27.7 (t, C-16), 26.0 (t, C-12), 25.2 (t, C-5); EIMS (70 eV) *m/z* (rel. int.) 420 (2), 402 (17), 261 (27), 219 (9), 159 (17), 143 (76), 121 (61), 67 (100); HREIMS *m/z* 420.2866, $C_{25}H_{40}O_5$ requires *m/z* 420.2876.

Stolonoxides C and D methyl esters (3a/4a). Colorless oil; $[\alpha]_D^{25}=+41.7°$ (*c* 0.3, $CHCl_3$); IR (film) 1745, 1200, 1060 cm^{-1}; UV (MeOH) λ_{max} 234 (ϵ=19600) nm; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 2; EIMS (70 eV) *m/z* (rel. int.) 420 (2), 402 (23), 261 (23), 219 (6), 159 (10), 143 (27), 121 (35), 67 (100); HREIMS *m/z* 420.2863, $C_{25}H_{40}O_5$ requires *m/z* 420.2876.

Methyl ester 3a. ^{1}H NMR ($CDCl_3$) 6.25 (m, 2H, H-18 and

H-19), 5.82 (ddt, *J*=17.0, 10.2, 6.6 Hz, 1H, H-23), 5.44 (m, 2H, H-17 and H-20), 5.03 (bd, *J*=17.0 Hz, 1H, H-24), 4.97 (bd, *J*=10.2 Hz, 1H, H-24), 4.54 (dddd, *J*=11.0, 7.7, 5.9, 2.2 Hz, 1H, H-3), 4.03 (ddd, *J*=10.9, 6.0, 2.1 Hz, 1H, H-6), 3.86 (m, 1H, H-10), 3.80 (q, *J*=6.0 Hz, 1H, H-7), 3.69 (s, 3H, CH_3O–), 2.47 (dd, *J*=15.6, 7.7 Hz, 1H, H-2), 2.37 (dd, *J*=15.7, 5.9 Hz, 1H, H-2), 2.28 (bdd, *J*=14.7, 7.4 Hz, 2H, H-21), 2.16 (m, 2H, H-16), 2.14 (m, 2H, H-22), 2.00 (m, 1H, H-8), 1.98 (m, 1H, H-9), 1.94 (m, 1H, H-5eq), 1.92 (m, 1H, H-4eq), 1.80 (m, 1H, H-8), 1.65 (m, 1H, H-5ax), 1.56 (m, 1H, H-11), 1.55 (m, 1H, H-4ax), 1.45 (m, 1H, H-9), 1.38 (m, 1H, H-12), 1.37 (m, 1H, H-11), 1.28 (m, 1H, H-12), 1.30-1.20 (m, 6H, H-13, H-14 and H-15); ^{13}C NMR ($CDCl_3$) 170.4 (s, C-1), 138.2 (d, C-23), 132.4 (d, C-17), 130.9 (d, C-20), 124.0 (d, C-19), 123.5 (d, C-18), 114.8 (t, C-24), 83.7 (d, C-6), 79.9 (d, C-10), 78.5 (d, C-7), 77.8 (d, C-3), 51.9 (q, CH_3O–), 38.4 (t, C-2), 35.6 (t, C-11), 33.7 (t, C-22), 31.6 (t, C-9), 29.6 (t, C-13)*, 29.5 (t, C-14)*, 29.2 (t, C-15)*, 28.9 (t, C-4), 28.0 (t, C-8), 27.5 (t, C-16), 26.9 (t, C-21), 26.1 (t, C-12), 25.7 (t, C-5).

Methyl ester 4a. ^{1}H NMR ($CDCl_3$) 6.32 (m, 1H, H-19), 5.94 (bt, *J*=10.9 Hz, 1H, H-18), 5.82 (ddt, *J*=17.0, 10.2, 6.6 Hz, 1H, H-23), 5.66 (dt, *J*=14.4, 6.4 Hz, 1H, H-20), 5.30 (dt, *J*=10.9, 7.3 Hz, 1H, H-17), 5.03 (bd, *J*=17.0 Hz, 1H, H-24), 4.97 (bd, *J*=10.2 Hz, 1H, H-24), 4.54 (dddd, *J*=11.0, 7.7, 5.8, 2.2 Hz, 1H, H-3), 4.03 (ddd, *J*=10.9, 6.0, 2.1 Hz, 1H, H-6), 3.86 (m, 1H, H-10), 3.80 (q, *J*=6.0 Hz, 1H, H-7), 3.69 (s, 3H, CH_3O–), 2.47 (dd, *J*=15.7, 7.7 Hz, 1H, H-2), 2.37 (dd, *J*=15.7, 5.8 Hz, 1H, H-2), 2.19 (m, 2H, H-21), 2.14 (m, 2H, H-22), 2.13 (m, 2H, H-16), 2.00 (m, 1H, H-8), 1.98 (m, 1H, H-9), 1.94 (m, 1H, H-5eq), 1.92 (m, 1H, H-4eq), 1.80 (m, 1H, H-8), 1.65 (m, 1H, H-5ax), 1.56 (m, 1H, H-11), 1.55 (m, 1H, H-4ax), 1.45 (m, 1H, H-9), 1.38 (m, 1H, H-12), 1.37 (m, 1H, H-11), 1.28 (m, 1H, H-12), 1.30–1.20 (m, 6H, H-13, H-14 and H-15); ^{13}C NMR ($CDCl_3$) 170.4 (s, C-1), 138.2 (d, C-23), 133.5 (d, C-20), 130.4 (d, C-17), 128.5 (d, C-18), 126.0 (d, C-19), 114.8 (t, C-24), 83.7 (d, C-6), 79.9 (d, C-10), 78.5 (d, C-7), 77.8 (d, C-3), 51.9 (q, CH_3O–), 38.4 (t, C-2), 35.6 (t, C-11), 33.6 (t, C-22), 32.2 (t, C-21), 31.6 (t, C-9), 29.6 (t, C-13)*, 29.5 (t, C-14)*, 29.2 (t, C-15)*, 28.9 (t, C-4), 28.0 (t, C-8), 27.7 (t, C-16), 26.1 (t, C-12), 25.7 (t, C-5).

Synthesis of the epoxide 5. To a solution of **1b** (20 mg) in MeOH (2 mL) was added a solution of NaOH 1 M (1 mL). The mixture was stirred for 3 h at room temperature and the solvent was evaporated under reduced pressure to yield an aqueous residue that was filtered through a small silica gel column. The reaction crude obtained was treated with CH_2N_2 as described above to give **5** (16 mg).

Epoxide 5. Colorless oil; $[\alpha]_D^{25}=-23.5°$ (*c* 0.46, $CHCl_3$); IR (film) 3465, 1755, 1640, 1200, 1070 cm^{-1}; UV (MeOH) λ_{max} 235 (ϵ=20800) nm; ^{1}H NMR ($CDCl_3$) 6.25 (m, 2H, H-18 and H-19), 5.82 (ddt, *J*=16.9, 10.3, 6.6 Hz, 1H, H-23), 5.44 (m, 2H, H-17 and H-20), 5.03 (bd, *J*=16.9 Hz, 1H, H-24), 4.97 (bd, *J*=10.3 Hz, 1H, H-24), 3.87 (m, 1H, H-10), 3.77 (s, 3H, CH_3O–), 3.77 (m, 1H, H-7), 3.43 (m, 1H, H-6), 3.27 (d, *J*=1.9 Hz, 1H, H-2), 3.23 (ddd, *J*=6.3, 4.9, 1.9 Hz, 1H, H-3), 2.27 (bdd, *J*=14.7, 7.5 Hz, 2H, H-21), 2.16 (m, 2H, H-16), 2.14 (m, 2H, H-22), 2.02 (m, 1H, H-9), 1.96 (m, 1H, H-8), 1.83 (m, 1H, H-4), 1.80 (m, 1H, H-4), 1.62 (m, 1H, H-8), 1.57 (m, 1H, H-11), 1.55 (m, 2H, H-5), 1.51 (m, 1H, H-9), 1.40 (m, 1H, H-11), 1.39 (m, 1H, H-12), 1.30–1.20 (m, 7H, H-12, H-13, H-14 and H-15); ^{13}C NMR ($CDCl_3$) 169.7 (s, C-1), 138.2 (d, C-23), 132.3 (d, C-17), 130.9 (d, C-20), 123.9 (d. C-19), 123.5 (d, C-18), 114.7 (t, C-24), 81.7 (d, C-7), 79.4 (d, C-10), 73.0 (d, C-6), 58.1 (d, C-3), 53.0 (d, C-2), 52.4 (q, CH_3O–), 35.6 (t, C-11), 33.6 (t, C-22), 32.4 (t, C-9), 29.5 (t, C-13)*, 29.5 (t, C-14)*, 29.2 (t, C-15)*, 28.8 (t, C-5), 28.3 (t, C-8), 27.4 (t, C-16), 27.3 (t, C-4), 26.8 (t, C-21), 26.1 (t, C-12); HREIMS *m/z* 421.2953, $C_{25}H_{41}O_5$ requires *m/z* 421.2954.

Synthesis of epoxides 6 and 7. A mixture of **3a/4a** (3 mg) was treated following the same procedure described above for the formation of **5** to give a mixture of epoxides **6** and **7** (1 mg).

Epoxide 6. ^{1}H NMR ($CDCl_3$) 6.25 (m, 2H, H-18 y H-19), 5.82 (ddt, *J*=17.0, 10.3, 6.5 Hz, 1H, H-23), 5.45 (m, 2H, H-17 and H-20), 5.03 (bd, *J*=17.0 Hz, 1H, H-24), 4.97 (bd, *J*=10.3 Hz, 1H, H-24), 3.94 (m, 1H, H-10), 3.87 (m, 1H, H-7), 3.79 (m, 1H, H-6), 3.77 (s, 3H, CH_3O–), 3.28 (d, *J*=1.9 Hz, 1H, H-2), 3.23 (m, 1H, H-3), 2.28 (bdd, *J*=14.7 and 7.5 Hz, 2H, H-21), 2.16 (m, 4H, H-22 and H-16), 2.04 (m, 1H, H-9), 1.85 (m, 1H, H-4), 1.82 (m, 2H, H-8), 1.78 (m, 1H, H-4), 1.58 (m, 1H, H-11), 1.56 (m, 2H, H-5), 1.52 (m, 1H, H-9), 1.40 (m, 1H, H-11), 1.38 (m, 1H, H-12), 1.32 (m, 1H, H-12), 1.30–1.20 (m, 6H, H-13, H-14 and H-15); ^{13}C NMR ($CDCl_3$) 169.7 (s, C-1), 138.2 (d, C-23), 132.4 (d, C-17), 130.9 (d, C-20), 123.9 (d. C-19), 123.5 (d, C-18), 114.8 (t, C-24), 81.3 (d, C-7), 80.3 (d, C-10), 71.3 (d, C-6), 58.1 (d, C-3), 53.0 (d, C-2), 52.5 (q, CH_3O–), 36.0 (t, C-11), 33.6 (t, C-22), 32.2 (t, C-9), 29.5 (t, C-13)*, 29.5 (t, C-14)*, 29.2 (t, C-15)*, 28.0 (t, C-5), 27.9 (t, C-4), 27.4 (t, C-16), 26.8 (t, C-21), 26.1 (t, C-12), 25.3 (t, C-8).

Epoxide 7. ^{1}H NMR ($CDCl_3$) 6.32 (m, 1H, H-19), 5.94 (bt, *J*=10.7 Hz, 1H, H-18), 5.82 (ddt, *J*=17.0, 10.3, 6.5 Hz, 1H, H-23), 5.66 (m, 1H, H-20), 5.31 (m, 1H, H-17), 5.03 (bd, *J*=17.0 Hz, 1H, H-24), 4.97 (bd, *J*=10.3 Hz, 1H, H-24), 3.94 (m, 1H, H-10), 3.87 (m, 1H, H-7), 3.79 (m, 1H, H-6), 3.77 (s, 3H, CH_3O–), 3.28 (d, *J*=1.9 Hz, 1H, H-2), 3.23 (m, 1H, H-3), 2.18 (m, 2H, H-21), 2.16 (m, 2H, H-22), 2.14 (m, 2H, H-16), 2.04 (m, 1H, H-9), 1.85 (m, 1H, H-4), 1.82 (m, 2H, H-8), 1.78 (m, 1H, H-4), 1.58 (m, 1H, H-11), 1.56 (m, 2H, H-5), 1.52 (m, 1H, H-9), 1.40 (m, 1H, H-11), 1.38 (m, 1H, H-12), 1.32 (m, 1H, H-12), 1.30–1.20 (m, 6H, H-13, H-14 and H-15); ^{13}C NMR ($CDCl_3$) 169.7 (s, C-1), 138.2 (d, C-23), 133.5 (d, C-20), 130.4 (d, C-17), 128.4 (d, C-18), 126.0 (d, C-19), 114.8 (t, C-24), 81.3 (d, C-7), 80.3 (d, C-10), 71.3 (d, C-6), 58.1 (d, C-3), 53.0 (d, C-2), 52.5 (q, CH_3O–), 36.0 (t, C-11), 33.6 (t, C-22), 33.5 (t, C-21), 32.2 (t, C-9), 29.5 (t, C-13)*, 29.5 (t, C-14)*, 29.2 (t, C-15)*, 28.0 (t, C-5), 27.9 (t, C-4), 27.7 (t, C-16), 26.1 (t, C-12), 25.3 (t, C-8).

Synthesis of the (*R*)-MTPA ester (5a). A solution of **5** (4 mg) in CH_2Cl_2 was treated with CH_2Cl_2 solutions of dicyclohexylcarbodiimide (16 mg in 0.5 mL), *N*,*N*-dimethylaminopyridine (2.3 mg in 0.5 mL) and (*R*)-α-methoxy-α-trifluoromethylphenylacetic acid (8.5 mg in 0.5 mL) and the mixture was stirred at room temperature for 48 h. After evaporation of the solvent under reduced pressure the residue was purified on HPLC to afford the (*R*)-MTPA ester

5a (4 mg). ^{1}H NMR ($CDCl_3$, 400 MHz) (selected data, assignments aided by a COSY experiment): 5.09 (m, 1H, H-6), 4.03 (bq, J=7.0 Hz, 1H, H-7), 3.78 (m, 1H, H-10), 3.78 (s, 3H, CH_3O-), 3.23 (d, J=1.9 Hz, 1H, H-2), 3.20 (m, 1H, H-3), 1.92 (m, 1H, H-8), 1.81 (m, 4H, H-4, H-5 and H-9), 1.54 (m, 1H, H-4), 1.50 (m, 1H, H-8), 1.40 (m, 1H, H-9).

Synthesis of the (*S*)-MTPA ester (5b). Treatment of **5** (4.4 mg) with CH_2Cl_2 solutions of dicyclohexylcarbodiimide (16.2 mg in 0.5 mL), *N*,*N*-dimethylaminopyridine (2.3 mg in 0.5 mL) and (*S*)-α-methoxy-α-trifluoromethylphenylacetic acid (8.9 mg in 0.5 mL) as described previously yielded the (*S*)-MTPA ester **5b** (5.5 mg). ^{1}H NMR ($CDCl_3$, 400 MHz) (selected data, assignments aided by a COSY experiment): 5.10 (ddd, J=7.5, 7.5, 4.0 Hz, 1H, H-6), 4.01 (bq, J=7.0 Hz, 1H, H-7), 3.87 (m, 1H, H-10), 3.77 (s, 3H, CH_3O–), 3.12 (d, J=1.9 Hz, 1H, H-2), 3.09 (m, 1H, H-3), 2.00 (m, 1H, H-8), 1.98 (m, 1H, H-9), 1.64 (m, 2H, H-5), 1.58 (m, 1H, H-4), 1.56 (m, 1H, H-8), 1.45 (m, 1H, H-9), 1.35 (m, 1H, H-4).

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