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Lissoclinotoxins E and F, novel cytotoxic alkaloids from a Philippine didemnid ascidian

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Abstract—Bioassay-guided fractionation of the MeOH extract from a Philippine didemnid ascidian resulted in the isolation of two new dimeric alkaloids, lissoclinotoxins E (**1**) and F (**2**). The polysulfide structures for compounds **1** and **2** were determined by interpretation of spectroscopic data and chemical degradation. Computational chemistry studies suggested that the *N*-alkyl chains about the tricyclic systems of lissoclinotoxins E and F had *trans* and *cis* orientations, respectively. Alkaloids **1** and **2** displayed IC_{50} values of 2.3 and 1.5 μg/mL, respectively, towards the PTEN-deficient human breast carcinoma cell line, MDA-MB-468.

1. Introduction

PTEN is a recently identified tumor suppressor gene located on the human chromosome 10q23.3.[1–3] Mutations and deletions in PTEN occur frequently in advanced cancers such as malignant melanoma, breast cancer, bladder carcinomas, small cell lung cancer and endometrioid ovarian cancer.[3–9] It is known that PTEN is a negative regulator of the anti-apoptotic PI3-K/AKT/mTOR cellular signaling pathway and that loss of PTEN function can lead to cancerous growth.[10] Identifying compounds that inhibit PTEN-deficient ($PTEN^{-/-}$) cell lines would be advantageous in the development of new anti-tumor agents. As part of our continuing search for new anti-cancer compounds from the marine environment, we tested our extract library in a PTEN-deficient human breast carcinoma cell line (MDA-MB-465) assay. The crude MeOH extract from a Philippine didemnid ascidian demonstrated significant inhibition towards this particular breast carcinoma cell line. Bioassay-guided fractionation of this extract yielded two new dimeric polysulfide alkaloids, that we have named lissoclinotoxins E (**1**) and F (**2**).

2. Results and discussion

The MeOH extract of the didemnid ascidian was evaporated to dryness then subjected to a solvent partitioning scheme resulting in hexanes, $CHCl_3$ and 30% aqueous MeOH-soluble fractions. The bioactive $CHCl_3$-soluble material was initially chromatographed over a column of Sephadex LH-20 (MeOH/$CHCl_3$). Further purification of the active fractions by C_{18} HPLC using 0.1% aqueous TFA and increasing amounts of MeOH afforded the bis-TFA salt of lissoclinotoxin E (**1**, 5.5 mg). An earlier eluting fraction from the C_{18} HPLC work was further fractionated by phenyl-hexyl HPLC using a 0.1% aqueous TFA/MeOH gradient to yield the bis-TFA salt of lissoclinotoxin F (**2**, 3.6 mg).

The major metabolite, lissoclinotoxin E (**1**) was isolated as a light brown film. A pseudomolecular ion in the (+)-HRCIMS at *m/z* 571.17810 allowed a molecular formula of $C_{26}H_{38}N_2O_4S_4$ to be assigned to **1**. The LRCIMS isotopic pattern suggested the presence of four sulfur atoms. The ^{1}H NMR spectrum (Table 1) of **1** contained two methoxyl singlets [δ 3.92 (3H) and 3.94 (3H)], two methylene multiplets [δ 3.22 (2H) and 3.76 (2H)], and an *N*-methyl [δ 3.06 (6H)] and *S*-methyl [δ 2.44 (3H)] singlet. The ^{13}C NMR spectrum displayed only 12 resonances, and DEPT analysis revealed signals for a two-carbon methoxyl at 61.3 ppm, two methylenes at 30.0 and 58.3 ppm, a two-carbon *N*-methyl at 43.7 ppm, and an *S*-methyl at 19.3 ppm. The HMQC spectra enabled all the proton signals to be assigned to their directly attached carbons. Analysis of the gCOSY spectrum allowed the assignment of an ethylene

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Table 1. NMR data for lissoclinotoxin E (**1**)

Position	^{13}C (δ)	^{1}H (δ, mult., J in Hz)	gCOSY	gHMBC	ROESY
1	131.5[a]				
2	133.8				
3	151.9				
4	156.1				
5	133.3				
6	135.1[a]				
7	30.0	3.76 (m)	8	1, 5, 6, 8	8, 12, 11
8	58.3	3.22 (m)	7	7, 12	7, 12
9	61.3	3.92 (s)		3	
10	61.3	3.94 (s)		4	11
11	19.3	2.44 (s)		5	7, 10
12	43.7	3.06 (s)		8, 12	7, 8

Spectra were recorded in CD_3OD at 26°C.
[a] Signals are interchangeable.

moiety that was substituted at one end by an *N*,*N*-dimethylamino group, based on strong HMBC correlations from H-8 (δ 3.22) to the *N*-methyl carbons, C-12 (43.7 ppm). This *N*-alkyl substructure was attached to an aromatic ring due to HMBC correlations from H-7 (δ 3.76) to C-1 (131.5 ppm), C-5 (133.3 ppm) and C-6 (135.1 ppm). The *S*-methyl substituent was attached to C-5 of the benzenoid system based on an HMBC correlation to the carbon resonance at 133.3 ppm and a ROESY correlation (ROE) to H-8. The methoxyl protons at δ 3.92 and 3.94 showed one HMBC correlation each to carbons at 151.9 and 156.1 ppm, respectively. An *ortho* orientation on the benzene ring between the *S*-methyl (δ 2.44) and *O*-methyl (δ 3.94) group was determined based on a strong ROE between these two methyl singlets. The remaining methoxyl group (δ 3.92) was positioned at C-3 due to the obvious structural and biosynthetic relationship of compound **1** to the previously reported metabolites from the *Lissoclinum* genus.[11–14] By default the only remaining carbon signal at 133.8 ppm was assigned to C-2. Hence, two identical DOPA-derived substructures were defined for **1**. Assemblage of these two partial units via two thioether linkages established the gross structure for lissoclinotoxin E (**1**).

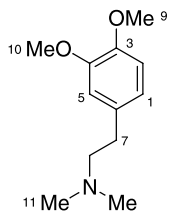
The minor metabolite, lissoclinotoxin F (**2**) was isolated as a light brown film. An $[M+H]^+$ ion in the (+)-HRCIMS at m/z 603.15112 allowed a molecular formula of $C_{26}H_{38}N_2O_4S_5$ to be assigned to **2**. The LRCIMS isotopic pattern suggested the presence of five sulfur atoms. Analysis of the ^{1}H and ^{13}C NMR spectra (Table 2) showed that lissoclinotoxin F (**2**) contained the same number and type of signals compared to **1**, with only minor chemical shift discrepancies identified (^{1}H: $<$0.17 ppm; ^{13}C: $<$5.3 ppm). The extra sulfur atom present in **2** suggested that lissoclinotoxin F contained a disulfide linkage between the aryl moieties. The presence of the disulfide bond was confirmed by MS analysis of a mixture of compound **2** and Cleland's reagent (dithiothreitol). Under these reducing conditions the (+)-LRESMS showed a strong ion at m/z 605 consistent with disulfide bond reduction. Hence, two identical DOPA-derived substructures were defined for **2** and were joined via a thioether and disulfide linkage. This established the gross structure for lissoclinotoxin F (**2**). Although the reduction and methylation[11] of **2** to the tetramethylthio ether may have possibly assisted in the definitive positioning of the disulfide linkage following ROESY analysis, this reaction was not attempted in order to preserve material for biological evaluation.

Table 2. NMR data for lissoclinotoxin F (**2**)

Position	^{13}C (δ)	^{1}H (δ, mult., J in Hz)	gCOSY	gHMBC	ROESY
1	136.6[a]				
2	133.4				
3	157.2[b]				
4	154.2[b]				
5	132.8				
6	136.4[a]				
7	28.7	3.59 (m)	8	1, 5, 6, 8	8, 11, 12
8	57.7	3.17 (m)	7	7, 12	7, 11, 12
9	61.5[c]	3.95[d] (s)		3	
10	61.3[c]	3.94[d] (s)		4	11
11	19.2	2.46 (s)		5	7, 8, 10
12	43.5	3.01 (s)		8, 12	7, 8

Spectra were recorded in CD_3OD at 26°C.
[a] Signals are interchangeable.
[b] Signals are interchangeable.
[c] Signals are interchangeable.
[d] Signals are interchangeable.

The structural relationship between compounds **1** and **2** was confirmed by desulfurization of a mixture of these metabolites using Raney Ni in MeOH at 90°C.[15,16] Purification of the reaction product by C_{18} flash chromatography [MeOH/aqueous TFA (0.1%)] yielded the pure TFA salt of 2-(3,4-dimethoxyphenyl)-*N*,*N*-dimethylethanamine (**3**), which has been previously reported in the literature both as a synthetic compound[17,18] and as a natural product.[19–21]



3

Although the gross structures for compounds **1** and **2** had been conclusively proven by spectroscopic and chemical degradation methods, the determination of stereochemistry about both tricyclic systems had not been established. Two possible geometric isomers existed for lissoclinotoxin E (**1**) with the *N*-alkyl chains orientated either *trans* or *cis* to each other about the tricyclic system. For lissoclinotoxin F (**2**) three possible geometric isomers existed (two *cis* and one *trans*), due to the presence of the disulfide linkage. The previously isolated polysulfide dimers, lissoclinotoxin D (**4**)[14] and lissoclin disulfoxide (**5**)[13] were assigned to the *trans* and *cis* isomer, respectively, although these geometric orientations were not proven by any chemical or spectroscopic techniques. Lissoclinotoxin D (**4**) was assigned *trans* stereochemistry on the basis that this isomer would be thermodynamically more favorable,[14] while the geometric assignment of lissoclin disulfoxide (**5**) was not discussed.[13] In order to determine which geometric isomer of lissoclinotoxins E and F was most likely to exist based on energy considerations, we performed computational chemistry studies using MacroModel® software.[22] Montecarlo conformational searching in vacuo using a MM2 force field was

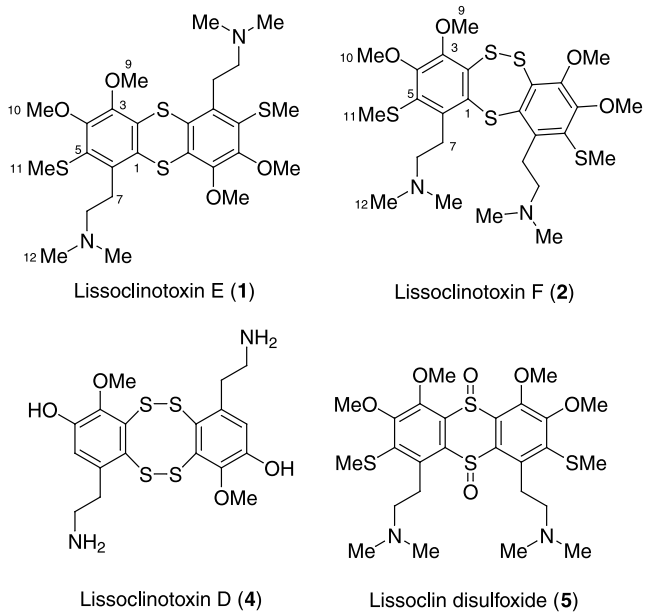


Figure 1. Dimeric polysulfide ascidian metabolites.

employed on all non-charged isomers of **1**, **2**, **4** and **5**. Global energy minima calculations showed that the *trans* isomers were thermodynamically preferred for both **1** and **5**, while the *cis* isomers were more energetically stable for **2** and **4**. Global energy minima values for the *trans* isomers of **1** and **5** were shown to be 0.4 and 1.0 kcal/mol lower than their corresponding *cis* isomer, respectively. The *cis* isomer of **2** (as drawn in Fig. 1) had a global energy minimum value that was 3.0 and 1.5 kcal/mol lower than the other possible *cis* and *trans* isomers, respectively. The difference in *cis* and *trans* energy minima values for **4** was determined to be only 0.2 kcal/mol.

On the basis of these modeling data we assigned lissoclinotoxins E and F to the *trans* and *cis* isomers respectively, however, due to the small discrepancies in the calculated global energy minima values the other isomer(s) cannot be excluded.

It should also be noted that the stereochemical assignments obtained from the modeling studies are tenuous, since biosynthetic enzymes introduce the potential for the production of natural product isomers that would not be expected based on thermodynamic considerations. Unfortunately, attempts to obtain crystalline material suitable for X-ray analysis to resolve this issue have been unsuccessful. In fact there are no reports in the literature of crystalline members from the lissoclinotoxin family of natural products or derivatives.

Compounds **1**–**3** were all tested for cytotoxicity against the MDA-MB-468 human breast carcinoma cell line; **1** and **2** displayed IC_{50} values of 2.3 and 1.5 μg/mL, respectively, while **3** was inactive at 50 μg/mL. Due to the promising results for lissoclinotoxins E and F further biological evaluations were performed. In an attempt to determine whether our compounds selectively inhibited the PI3-K/AKT/mTOR cellular signaling pathway, we tested **1** and **2** in the MDA-MB-435S ($PTEN^{+/+}$) human breast carcinoma cell line. This tumor cell line is known to possess a wild-type PTEN protein, hence if a drug were to affect part of the PI3-K/AKT/mTOR pathway then it would be expected to have a lower IC_{50} value in the MDA-MB-468 ($PTEN^{-/-}$) cell line compared to the MDA-MB-435S ($PTEN^{+/+}$) cell line.[23] Lissoclinotoxins E and F displayed cytotoxicity against the MDA-MB-435S human breast carcinoma cell line with IC_{50} values of 2.1 and 4.2 μg/mL, respectively. These data showed that lissoclinotoxin E (**1**) had no selectivity between the two carcinoma cell lines, while lissoclinotoxin F (**2**) showed approximately threefold greater potency against the PTEN deficient cell line. Further biological evaluations on compound **2** are required in order to determine whether this dimeric alkaloid is a specific inhibitor of the PI3-K/AKT/mTOR cellular signaling pathway.

3. Conclusion

Although more than 10 monomeric cyclic polysulfides have been published to date,[11,12,14,24,25] lissoclinotoxins E and F represent only the third and fourth dimeric polysulfides to be isolated and characterized from a marine source. Montecarlo conformational searching using a MM2 force field suggested that the *trans* and *cis* isomer are energetically preferred for lissoclinotoxins E and F, respectively. Compounds **1** and **2** were shown to display potent cytotoxicity towards the MDA-MB-468 ($PTEN^{-/-}$) and the MDA-MB-435S ($PTEN^{+/+}$) cell lines. Lissoclinotoxin F (**2**) showed threefold greater cytotoxicity towards the PTEN deficient cell line.

4. Experimental

4.1. General procedures

NMR spectra were recorded at 26°C on a Varian Unity 500 MHz spectrometer at 500.620 MHz for ^1H and 125.893 MHz for ^{13}C. The ^1H and ^{13}C chemical shifts are reported in parts per million relative to the reference solvent signals at δ 3.30 and 49.00 ppm for CD_3OD. FTIR and UV spectra were recorded on a Jasco FT/IR-420 spectrophotometer and a Hewlett–Packard 8452A diode array spectrophotometer, respectively. High- and low-resolution chemical ionization mass spectral measurements were made on a Finnegan MAT 95 high-resolution spectrometer. Size-exclusion chromatography was performed using a glass column (25×560 mm^2) packed with Sigma Lipophilic Sephadex LH-20 that was connected to a Spectra/Chrom CF-1 fraction collector. A Beckman Gold solvent module equipped with a 7725i Rheodyne injector and a Beckman 168 PDA detector were used for HPLC separations. A Phenomenex Luna $C_{18}(2)$ 5 μm 100 Å column (10×250 mm^2) and a Phenomenex Luna phenyl-hexyl 5 μm 100 Å column (10×250 mm^2) were used for semi-preparative HPLC. A SPE cartridge (10×30 mm^2) packed with J.T. Baker Bakerbond C_{18} (40 μm, 60 Å) was used for desulfurization reaction purification. All solvents used for HPLC, UV and MS were Fisher HPLC grade, and the H_2O used was Barnstead E-pure 0.2 μm filtered. All synthetic reagents used were purchased from Sigma-Aldrich.

Computational chemistry studies were performed using MacroModel® version 6.0 software[22] on a Silicon Graphics workstation. Montecarlo conformational searching in vacuo with a MM2 force field was employed for all global energy minima calculations.

4.2. Animal material

A specimen of the didemnid ascidian was collected during April of 1999 by SCUBA diving (−10 m) at Sabtang Reef, Batanes Islands, Philippines, and immediately steeped in MeOH. Voucher specimen DZ-UFPR DID 148 has been deposited at the Departamento de Zoologia, Universidade Federal do Paraná, C.P. 19020, 81.531-980, Curitiba, Brazil.

4.3. Extraction and isolation

The MeOH extract from the didemnid ascidian was concentrated under vacuum to yield a dark brown gum (320 mg). This material was dissolved in 90% MeOH/10% H_2O (50 mL) and partitioned with 100% hexanes (3×50 mL). The aqueous phase had H_2O (14.3 mL) added and the resulting 30% aqueous MeOH fraction was partitioned with 100% $CHCl_3$ (3×50 mL). The hexanes, $CHCl_3$ and 30% aqueous MeOH fractions were all evaporated to dryness under reduced pressure and yielded 19, 63, and 238 mg of material, respectively. The $CHCl_3$-soluble material was subjected to gel permeation chromatography using a Sephadex LH-20 column with 1:1 MeOH/$CHCl_3$ as the eluant at a flowrate of 2.0 mL/min. The alkaloidal fraction was further purified by semi-preparative C_{18} HPLC with initial isocratic conditions of 50:50 MeOH/0.1% aqueous TFA for the first 2 min followed by a linear gradient to 80:20 MeOH/0.1% aqueous TFA in 15 min at a flowrate of 4 mL/min. After evaporation of solvents this yielded the pure bis-TFA salt of lissoclinotoxin E (**1**, 5.5 mg). An earlier eluting fraction from the C_{18} HPLC work was further fractionated by semi-preparative phenylhexyl HPLC with initial isocratic conditions of 50:50 MeOH/0.1% aqueous TFA for the first 2 min followed by a linear gradient to 80:20 MeOH/0.1% aqueous TFA in 15 min at a flowrate of 4 mL/min. Removal of the solvents under reduced pressure afforded the bis-TFA salt of lissoclinotoxin F (**2**, 3.6 mg).

4.3.1. Bis-TFA salt of lissoclinotoxin E (1). Stable light brown film; UV (MeOH) λ_{max} 210 (ε 15 000), 232 (sh, ε 9000), 254 (ε 7000), 272 (ε 12 000), 312 nm (ε 3000); IR ν_{max} (NaCl) 1685, 1448, 1395, 1362, 1280, 1206, 1176, 1132, 1063, 1025, 961, 838, 800, 723 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; (+)-LRCIMS *m/z* (rel. int.) 523 (25), 557 (15), 571 (100); (+)-HRCIMS *m/z* 571.17810 ($C_{26}H_{39}N_2O_4S_4$ $[M+H]^+$, requires 571.17926).

4.3.2. Bis-TFA salt of lissoclinotoxin F (2). Stable light brown film; UV (MeOH) λ_{max} 210 (ε 13 000), 238 (sh, ε 11 000), 268 (ε 11 000), 320 nm (ε 3000); IR ν_{max} (NaCl) 1678, 1450, 1386, 1362, 1273, 1200, 1181, 1130, 1062, 1021, 959, 832, 799, 721 cm^{-1}; ^1H and ^{13}C NMR data see Table 2; (+)-LRCIMS *m/z* (rel. int.) 523 (10), 557 (15), 571 (100), 603 (50); (+)-HRCIMS *m/z* 603.15112 ($C_{26}H_{39}N_2O_4S_5$ $[M+H]^+$, requires 603.15133).

4.3.3. Desulfurization of 1 and 2. Raney 2800 nickel (50% slurry in H_2O; 4.5 mg, 75 μmol) was added to ~1:2 mixture of **1** and **2** (0.9 mg, 1.5 μmol) in MeOH (1 mL). The resulting black suspension was heated in a sealed vial at 90°C for 2 h.[15,16] Upon cooling, the solution was purified on a C_{18} SPE cartridge using 90:10 MeOH/0.1% aqueous TFA as the eluant. This yielded the pure TFA salt of 2-(3,4-dimethoxyphenyl)-*N*,*N*-dimethylethanamine (**3**, 0.6 mg, 92% yield).

4.3.4. TFA salt of 2-(3,4-dimethoxyphenyl)-*N*,*N*-dimethylethanamine (3). Stable clear film; UV (MeOH) λ_{max} 206 (ε 1200), 228 (ε 400), 278 nm (ε 200); IR ν_{max} (NaCl) 3600–3100, 1679, 1442, 1206, 1138, 1021, 844, 802, 725 cm^{-1}; ^1H (500 MHz, CD_3OD) δ 2.90 (6H, s, H-11), 2.97 (2H, m, H-7), 3.33 (2H, m, H-8), 3.79 (3H, s, H-10) 3.82 (3H, s, H-9), 6.83 (1H, dd, *J*=8.0, 1.5 Hz, H-1), 6.90 (1H, d, *J*=1.5 Hz, H-5), 6.91 (1H, d, *J*=8.0 Hz, H-2); ^{13}C NMR (125 MHz, CD_3OD) δ 31.4 (C-7), 43.6 (2C, C-11), 56.5 (2C, C-9, C-10), 60.0 (C-8), 113.4 (C-2), 113.7 (C-5), 122.2 (C-1), 130.0 (C-6), 149.8 (C-3), 150.9 (C-4); (+)-LRCIMS *m/z* (rel. int.) 210 (100); (+)-HRCIMS *m/z* 210.14753 ($C_{12}H_{20}NO_2$ $[M+H]^+$, requires 210.14940).

4.3.5. Cells and culture conditions. MDA-MB-468 (ATCC # HTB-132) and MDA-MB-435S (ATCC # HTB-129) human breast tumor cell lines were obtained from the American Type Culture Collection. Cells were maintained in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM MEM sodium pyruvate, 50 units/mL penicillin and 50 μg/mL streptomycin. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere in T-75 cm^2 tissue culture flasks.

4.3.6. Cell proliferation assay. MDA-MB-468 and MDA-MB-435S cells were seeded in 96-well microtiter plates at 5000 cells/well. After 24 h, the compounds were added to the cells. After 48 h of drug treatment, cell viability was determined by measuring the metabolic conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into purple formazan crystals by active cells.[26,27] MTT assay results were read using a Labsystem multiscan plate reader at 570 nm. All compounds were tested in sextuplicate and were solubilized in 100% DMSO with a final DMSO concentration of 1% in each well. LY-294002 was used as the positive control for both cell lines. IC_{50} values were calculated using Prism version 3.02 software.

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