Notes

Cytotoxic Staurosporines from the Marine Ascidian *Cystodytes solitus*

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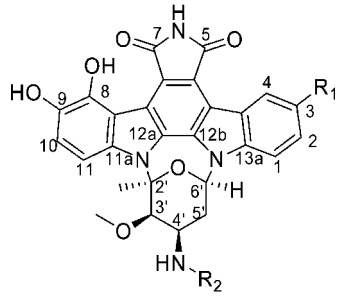
Two new indolocarbazole alkaloids, 7-oxo-3,8,9-trihydroxystaurosporine (**1**) and 7-oxo-8,9-dihydroxy-4′-*N*-demethylstaurosporine (**2**), were isolated from samples of the marine ascidian *Cystodytes solitus*. Their structures were determined by a combination of spectroscopic techniques, including (+)-HRMALDITOFMS and 1D and 2D NMR spectroscopy, and comparison with published data for related structures. Both compounds displayed strong cytotoxicity against three human tumor cell lines.

The staurosporines are a group of complex structurally related indolo[2,3-*a*]carbazole alkaloids obtained from culture broths of actinomycetes[1] and extracts of the marine tunicate *Eudistoma* sp.,[2,3] the prosobranch mollusk *Coriocella nigra*,[4] and the colonial tunicate *Eudistoma toalensis* and its predatory flatworm *Pseudoceros* sp.[5,6] Cytotoxic and antitumor properties, as well as strong inhibition of protein kinases, are among the most relevant biological properties exhibited by compounds of this structural class.[1]

As part of our continuing program in search of new anticancer agents, we have investigated the chemical composition of samples of the ascidian *Cystodytes solitus* Monniot (1988) (phylum Chordata, family Polycitoridae, order Enterogona, class Ascidiacea), collected in Tanzania, due to the cytotoxicity displayed by their organic extracts. Herein we report the isolation, structural characterization, and cytotoxic activity of two new staurosporine derivatives (**1** and **2**), obtained by bioassay-guided fractionation of extracts of this tunicate. Frozen samples of the marine organism were extracted with a 1:1 mixture of $MeOH/CH_2Cl_2$, and the extract was subjected to reversed-phase VLC on Polygoprep C18 silica gel and semipreparative HPLC to yield compounds **1** and **2** as their TFA salts.



1 R_1= OH, R_2= Me
2 R_1= R_2= H

A molecular ion at M^+ 528.1632 in its (+)-HRMALDITOFMS and the presence of 28 signals in the ^{13}C NMR spectrum established a molecular formula of $C_{28}H_{24}N_4O_7$ for compound **1**. The "staurosporine-like" nature of the compound was evident from absorptions in the UV spectrum and ^1H and ^{13}C NMR signals (Table 1) corresponding to the aromatic rings and the aminopyranose moiety present in other compounds belonging to the same structural class. The chemical shifts of signals for the 2′-Me, 3′-OMe, and 4′-NMe (Table 1) were assigned with the help of HSQC and HMBC experiments. The unusual high-field shift for the 3′-OMe (δ 2.30 ppm) can be explained by ring currents and charge effects occurring in the boat conformation adopted by the compound in its protonated form. This effect has been previously described for other staurosporine derivatives isolated from ascidians of the genus *Eudistoma* and flatworms of the genus *Pseudoceros*.[5,6] Signals of the aromatic region of the ^1H NMR spectrum accounted for the presence in the molecule of two different proton-bearing aromatic ring systems. A 1,2,4-trisusbtituted substitution pattern was inferred for the first of these rings from ^1H and COSY spectra. Chemical shifts and coupling constants measured for protons of this aromatic system were in agreement with the placement of a hydroxyl group at C-3. The chemical shift of C-3 (153.5 ppm), HMBC correlations from H-1 to C-3 and C-4a, from H-2 to C-13a, and from H-4 to C-2, C-3, and C-13a, and a ROESY correlation between H-1 and H-6′ corroborated this proposal.

The second proton-bearing aromatic ring was a 1,2,3,4-tetrasubstituted one, as confirmed by the presence in the ^1H NMR spectrum of two doublets with a coupling constant of 8.9 Hz. Both aromatic protons were located at C-10 and C-11 on the basis of the observation of cross-peaks between H-11 and H-3′, 2′-Me, and 3′-OMe in the ROESY spectrum. The chemical shifts of C-8 (141.3 ppm) and C-9 (141.1 ppm) and HMBC correlations between H-10 and C-8 and C-11a and between H-11 and C-9, C-7c, and C-10 confirmed the placement of two hydroxyl groups at the C-8 and C-9 positions and the proposed substructure for this part of the molecule. No signals for the AB system of two methylene protons at C-7 characteristic of the staurosporines were observed in the ^1H NMR of compound **1**. Instead of them, a ^{13}C signal at δ 176.9 ppm accounted for the presence of a carbonyl group at that position. It is worth mentioning that the formation of a hydrogen bond between C-7 and the C-8 hydroxyl group causes a deshielding of C-7 that allows the differentiation between the chemical shifts of this carbon and C-5.[7] As mentioned earlier, the aminopyranose ring of the staurosporines adopts a boat conformation in its protonated form. ROESY correlations observed for this ring (Figure 1) corroborated the relative configuration proposed. Compound **1** was therefore identified as 7-oxo-3,8,9-trihydroxystaurosporine.

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Table 1. NMR Spectroscopic Data (500 MHz, CD_3OD) for Compounds **1** and **2**

	1		**2**	
position	δ_C, mult.	δ_H, mult. (*J* in Hz)	δ_C, mult.	δ_H, mult. (*J* in Hz)
1	110.4, CH	7.18, br d (8.9)	109.8, CH	7.34, br d (8.2)
2	117.8, CH	7.09, br d (8.9)	128.6, CH	7.57, ddd (8.2, 7.3, 1.1)
3	153.5, qC		122.3, CH	7.38, ddd (8.1, 7.3, 0.8)
4	111.9, CH	8.58, br s	127.1, CH	9.09, br d (8.1)
4a	123.9, qC		122.9, qC	
4b	117.2, qC		117.4, qC	
4c	117.2, qC[a]		117.7, qC[a]	
5	170.9, qC		170.7, qC	
7	176.9, qC		176.8, qC	
7a	122.0, qC[a]		121.6, qC[a]	
7b	114.6, qC		114.8, qC	
7c	114.1, qC		114.1, qC	
8	141.3, qC		141.4, qC	
9	141.1, qC		141.1, qC	
10	117.2, CH	7.09, d (8.9)	117.2, CH	7.10, d (8.9)
11	103.0, CH	7.23, d (8.9)	103.0, CH	7.22, d (8.9)
11a	136.3, qC		136.4, qC	
12a	132.8, qC		132.7, qC	
12b	132.4, qC		131.7, qC	
13a	134.0, qC		139.4, qC	
2′	94.1, qC		94.2, qC	
3′	81.0, CH	4.23, br s	82.2, CH	4.07, br d (2.0)
4′	55.7, CH	3.94, m	48.0, CH	4.01, ddd (11.3, 7.1, 2.0)
5′	28.7, CH_2	3.24, m 2.20, br dd (12.2, 12.2)	30.1, CH_2	3.13, ddd (13.1, 9.3, 7.1) 2.27, ddd (13.1, 11.3, 2.7)
6′	82.0, CH	6.60, br d (8.7)	82.0, CH	6.67, dd (9.3, 2.7)
2′-Me	29.2, CH_3	2.39, s	29.4, CH_3	2.36, s
3′-OMe	61.0, CH_3	2.30, s	61.0, CH_3	2.35, s
4′-NMe	31.4, CH_3	2.81, s		

[a] Assignments interchangeable.

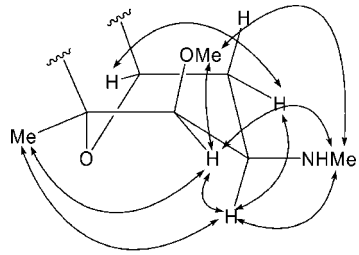

Figure 1. Key ROESY correlations observed in the aminopyranose ring of **1**.

Compound **2** had a molecular formula of $C_{27}H_{22}N_4O_6$, according to its (+)-HRMALDITOFMS (*m/z* M^+ 498.1525, calc for $C_{27}H_{22}N_4O_6$, 498.1534) and the presence of 27 signals in its ^{13}C NMR spectrum. The major differences found in the ^1H NMR spectrum of **2** with respect to **1** were the absence of the 4′-NMe group and changes in the aromatic region. Signals observed in the ^1H NMR (Table 1) and COSY spectra for a 1,2-disubstituted aromatic ring were in agreement with the disappearance of the hydroxyl group at the C-3 position in **1**. The chemical shifts of carbons of the central aromatic ring C-4b, C-12b, and C-7b were determined through correlations observed in the HMBC spectrum to H-4, H-6′, and H-11 (4-bonds distance), respectively. The chemical shift of carbon C-12a was deduced from its nitrogen substitution. However, despite the great difference between the chemical shifts of C-4c and C-7a, no correlations were observed that allowed us to deduce their chemical shifts, and therefore both assignments remained interchangeable. Compound **2** lacked therefore the 4′-NMe and 3-OH groups present in **1** and was confirmed to be 7-oxo-8,9-dihydroxy-4′-*N*-demethylstaurosporine.

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 strong activity with GI_{50} values in the submicromolar range: 26.6 nM (A549), 68.1 nM (HT29), and 28.4 nM (MDA-MB-231) for compound **1** and 17.5 nM (A549), 90.3 nM (HT29), and 32.1 nM (MDA-MB-231) for compound **2**. Staurosporine gave GI_{50} values of 2.4 nM (A549), 10.9 nM (HT29), and 7.1 nM (MDA-MB-231) when tested as a positive control under the same conditions. Currently, studies are under way to assess the potential of these compounds as protein kinase inhibitors.

In conclusion, we have isolated two new staurosporines with strong cytotoxic properties from samples of the hitherto uninvestigated ascidian *C. solitus*. It is important to mention that this note constitutes the first report of members of this structural class isolated from specimens of this genus. The isolation of staurosporine and other derivatives from marine actinomycetes and other tunicates belonging to the genus *Eudistoma* strongly supports the hypothesis that associated microorganisms are the actual producers of compounds **1** and **2** in *C. solitus*. Whether the compounds are produced by the tunicate or by the microbial fauna/flora associated with the organism is something that needs to be verified.

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 CD_3OD (δ 3.31 for ^1H and 49.0 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. (+)-HRMALDITOFMS was performed on a Applied Biosystems 4700 proteomics analyzer spectrometer employing a ditranol matrix. ESIMS were recorded using an Agilent 1100 Series LC/MSD spectrometer.

Animal Material. *Cystodytes solitus* was collected in December 2005 by scuba at a depth between 6 and 32 m at Mafia Island (Tanzania) (7°40′43″ S, 39°54′59″ E). The material was identified by Dr. Xavier Turón from the University of Barcelona (Spain). A voucher specimen is deposited at PharmaMar (ORMA039954).

Extraction and Isolation. The frozen organism (65 g) was triturated and extracted with a 1:1 mixture of CH_2Cl_2/MeOH (3 × 200 mL). The extract was concentrated to yield a red crude of 2.45 g. This material was subjected to VLC on Polygoprep RP-18 with a stepped gradient from H_2O to MeOH. The fraction eluted with MeOH (54.7 mg) was

subjected to semipreparative HPLC (X-Terra RP-18, 10 × 150 mm, gradient H_2O + 0.1% TFA: CH_3CN + 0.1% TFA from 25% to 38% organic in 20 min, UV detection, 3.7 mL/min) to yield compounds **1** (3.3 mg) and **2** (8.2 mg) as their TFA salts.

Compound 1: red, amorphous solid; $[\alpha]^{25}_D$ +37.1 (*c* 0.040, MeOH); UV (HPLC-DAD) ν_{max} 243, 300, 313, 340, 358 nm; ^{1}H (500 MHz) and ^{13}C NMR (125 MHz), see Table 1; ESIMS *m*/*z* 529 $[M + H]^+$, 1057 $[2M + H]^+$; (+)-HRMALDITOFMS *m*/*z* 528.1632 M^+ (calcd for $C_{28}H_{24}N_4O_7$, 528.1640).

Compound 2: orange, amorphous solid; $[\alpha]^{25}_D$ +47.7 (*c* 0.068, MeOH); UV (HPLC-DAD) ν_{max} 246, 300, 313, 323, 353 nm; ^{1}H (500 MHz) and ^{13}C NMR (125 MHz), see Table 1; ESIMS *m*/*z* 499 $[M + H]^+$, 997 $[2M + H]^+$; (+)-HRMALDITOFMS *m*/*z* 498.1525 M^+ (calcd for $C_{27}H_{22}N_4O_6$, 498.1534).

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 (FCS), 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.[8] 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).

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Supporting Information Available: ^{1}H 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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