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Staurosporine aglycone (K252-c) and arcyriaflavin A from the marine ascidian, *Eudistoma* sp.

P. A. Horton, R. E. Longley, O. J. McConnell* and L. M. Ballas[a]

Divison of Biomedical Marine Research, Harbor Branch Oceanographic Institution, Inc., 5600 U.S. Highway 1 North, Ft. Pierce (Florida 34946, USA), and [a]Sphinx Pharmaceuticals Corporation, Two University Place, P.O. Box 52330, Durham (North Carolina 27717, USA)

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Abstract. Staurosporine aglycone (K252-c) (compound **1**) and arcyriaflavin A (**2**) were isolated from a specimen of the marine ascidian, *Eudistoma* sp., collected off the coast of West Africa. In addition to expressing micromolar and submicromolar inhibition of enzyme activity against seven protein kinase C isoenzymes and inhibition of proliferation of the human lung cancer A549 and P388 murine leukemia cell lines, **1** also inhibited cell adhesion of the EL-4.IL-2 cell line and expressed activity in the K562 bleb and neutrophil assays.

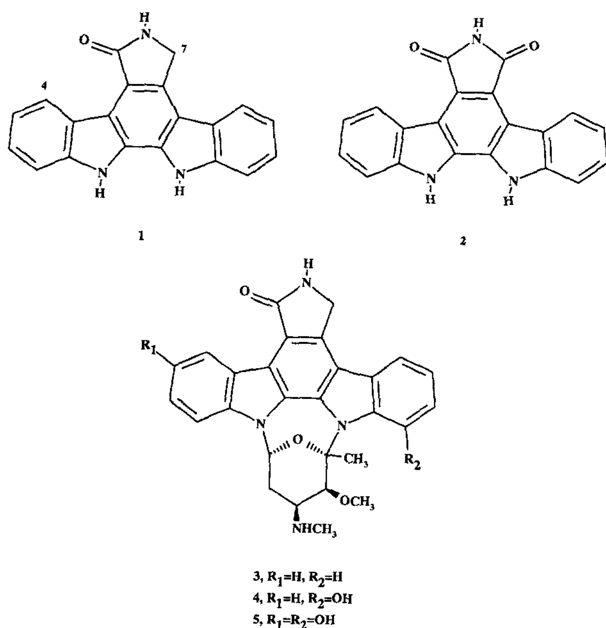
Key words. Staurosporine aglycone; K252-c; arcyriaflavin A; ascidian; *Eudistoma*; protein kinase C; cytotoxicity.

As part of an ongoing search for marine natural products which exhibit cytotoxicity through signal transduction pathways[1,2], we investigated a specimen of the ascidian, *Eudistoma* sp. collection by scuba in 1990 off the coast of West Africa. In this note, we report the bioassay-guided isolation and identification of two indolo[2,3-a]carbazoles, staurosporine aglycone (K252-c) (compound **1**) and arcyriaflavin A (**2**), reported previously as fungal metabolites obtained from the fungi, *Nocardiopsis* sp.[3,4] and *Arcyrai denudata*[5] respectively. Further, we report the combined signal transduction and cytotoxic activities for **1**.

Methods and results

The sample of *Eudistoma* sp. that was used in this study (HBOI no. 6-IX-90-2-1) was collected by scuba at a depth of 75 feet on the east shore of Baia de Santa Clara, which is off the west coast of Africa at Santiago, Cape Verde. Extraction of a thawed portion of the ascidian (112 g) with EtOH yielded a crude extract (4.19 g), a portion of which (4.12 g) was partitioned between EtOAc and H_2O. Most of the EtOAc soluble material (0.33 g out of 0.36 g) was then successively partitioned between equal volumes of aqueous MeOH (% water adjusted to give a biphasic solution) and hexane (vs 10% aq. MeOH) then CH_2Cl_2 (vs 40% aq. MeOH), to generate three fractions (hexane, 0.19 g; CH_2Cl_2, 0.08 g; and 40% aq. MeOH, 0.06 g, respectively). Biological evaluation in cytotoxicity assays using the human lung cancer cell line A-549 and P388 murine leukemia[6], protein kinase C assays using the isoenzymes alpha, betaI, betaII, delta, epsilon, gamma, and eta[7,8], and a cell adhesion assay using the EL-4 cell line[6], which is our functional, whole cell assay for PKC activity, revealed that the biological activity of the crude extract was concentrated in the CH_2Cl_2 solvent partition. Bioassay-guided purification-vacuum liquid chromatography (VLC) with reversed-phase C-18 stationary phase (step gradient of 20–100% MeOH/H_2O) followed by reversed-phase HPLC of the VLC fraction that eluted with 30% MeOH/H_2O (HPLC conditions: semi-prep reversed-phase C-18 column (10 mm × 250 mm); elution solvent – 70% MeOH/H_2O, 1.5 mL/min. flow rate) – afforded **1** as a yellow solid (2 mg or approximately 0.05% of the crude EtOH extract). A separate VLC fraction (20% MeOH/H_2O) yielded, after HPLC (same conditions as for **1**), **2** also as a pale yellow material (0.6 mg or approximately 0.006% of the crude extract).

The molecular formula of **1** was deduced as $C_{20}H_{13}N_3O$ from the high resolution FABMS (thioglycerol, *m/z* 312.107 $(M+H)^+$, Δ −6.4 mmu); it requires sixteen degrees of unsaturation. Analysis of the ^1H NMR and COSY[9] spectra (in d_4-MeOH) of **1** led to the identification of two sets of four contiguous aromatic protons: δ7.61 (d, $J=8.1$), 7.42 (td, $J=8.0$, 1.1), 7.23 (td, $J=7.8$, 1.3), and 9.18 (d, $J=7.8$), and δ8.00 (d, $J=7.9$), 7.31 (t, $J=7.3$), 7.47 (dt, $J=7.3$, 1.2), and 7.67 (d, $J=7.9$). The deshielded H-4 proton observed at δ9.18, which is in proximity to the amide carbonyl, is characteristic of staurosporine (**3**) and related metabolites that lack substituents in the indole groups. Further, there was a proton resonance observed at approximately δ5.00 that was partially obscured by the HDO peak and corresponds to H-7. The presence of an amide functionality in **1** was indicated by the ^{13}C NMR resonance observed at δ175.6 and a carbonyl stretching frequency of 1649 cm^{-1} in the IR spectrum. These data as well as the UV data (λ_{max} (EtOH): 231, 288, 331, 341, and 357 nm) are consistent with data reported for both the natural[3,4] and synthetic staurosporine aglycone[10–13].



The molecular formula of **2** was deduced as $C_{20}H_{11}N_3O_2$ from the high resolution FABMS (thioglycerol, *m/z* 326.0874 $(M+H)^+$, Δ −5.5 mmu); it requires seventeen degrees of unsaturation. The ^1H NMR spectrum of **2** contained only four aromatic protons indicating symmetry within the molecule, and analysis of the ^1H NMR spectrum (in d_6-DMSO) indicated that, similar to **1**, the four aromatic protons in **2** were contiguous: δ8.97 (d, $J=7.9$), 7.75 (d, $J=8.2$), 7.52 (t, $J=7.6$), and 7.32 (d, $J=7.5$). The N*H* protons were observed at δ11.72 and 10.91; **2** lacked the proton resonance observed in **1** at approximately δ5. These spectral data are also consistant with those reported for both the natural[5] and synthetic A[14–15]; upon NMR and thin layer chromatographic comparison, our sample of **2** proved to be identical with a synthetic sample.

Staurosporine aglycone (K252-c) (**1**) expressed potent in vitro cytotoxicity: lung cancer A549 $IC_{50}=2.0$ μM; P388 $IC_{50}=3.2$ μM. As expected[3–4], **1** also inhibited enzyme activity against seven of eight cloned PKC isoenzymes[7,8] – IC_{50} values (μM): alpha, 1.3; beta I, 0.6; beta II, 0.5; delta, 1.2; epsilon, 1.1; eta, 0.8; gamma, 1.5; and zeta, > 6.4. (The reported inhibition of PKC by **1** is $IC_{50}=0.2$ μM[4]). Further, **1** inhibited PKC activity in two whole cell assays by inhibiting cell adhesion in the EL-4 cell line with an $IC_{50}=30$ μM, and by inhibiting 'blebbing' in the K562 bleb assay[16] with an $IC_{50}=0.9$ μM. Finally, **1** inhibited oxygen release (as detected by the presence of lucigenin) in a neutrophil burst assay[17] with an $IC_{50}=0.5$ μM.

Discussion

Protein kinase C, an important growth regulatory enzyme in neoplastic and normal cells, continues to be pursued as a viable target for the discovery and design of novel therapeutics for the treatment of various human cancers. This paradigm includes the notion that perturbations in signal transduction pathways lead to uncontrolled growth of neoplastic cells due to faulty regulatory signals which are under the control of protein kinase C (ref. 18). In some cases, agents which inhibit protein kinase C and tumor cell growth in vitro may be effective in inhibiting tumor cell growth in vivo. While staurosporine itself is capable of controlling tumor cell growth in experimental tumor models, the compound exhibits cumulative toxicity[19–21], however, derivatives of staurosporine have been shown to be particularly efficacious in the treatment of experimental tumors and exhibit considerably less toxicity compared to the parent compound[19–21]. Thus, the rationale to search for inhibitors of protein kinase C which are related to the staurosporine class of compounds continues to be valid.

Ascidians have proven to be a rich source of nitrogen-containing secondary metabolites[22]; in particular, a large array of diverse alkaloids have been reported. However, despite the large number of alkaloids isolated, until recently no examples of indolo[2,3-1]carbazole were reported from ascidians or any other marine source. This is in contrast to the numerous indolocarbazoles that have been reported from terrestrial microorganisms[23], slime molds[24], and from fresh water blue-green algae[25,26]. Recently, 11-hydroxystaurosporine (**4**) and 3,11-hydroxystaurosporine (**5**), which are structurally related to the *Nocardiopsis* metabolite staurosporine (**3**)[3,4], were obtained from a brown ascidian, *Eudistoma* sp., which was collected off Hawaii[27]; thus, **1, 2, 4** and **5** represent the first examples of indolo[2,3-a]carbazoles isolated from a marine organism. Their presence in certain *Eudistoma* spp. provide additional examples of terrestrial microorganism-derived metabolites that are also found in marine macroorganisms. Determining whether parallel biosynthetic pathways exist in ascidians and microorganisms or whether **1** and **2** are actually produced by microorganisms associated with *Eudistoma* spp. should continue to prove to be a fruitful area of research.

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* To whom correspondence should be addressed.

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