# Staurosporine Derivatives from the Ascidian *Eudistoma toealensis* and Its Predatory Flatworm *Pseudoceros* sp.[▽]

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Two new indolocarbazole alkaloids, 3-hydroxy-3′-demethoxy-3′-hydroxystaurosporine (**5**) and 11-hydroxy-4′-*N*-demethylstaurosporine (**6**), were isolated from the marine ascidian *Eudistoma toealensis* and its predator, the marine flatworm *Pseudoceros* sp. In addition, five known derivatives were isolated in their protonated states, which caused the pyran-ring system to adopt a boat conformation. The structures were determined by 1D and 2D homonuclear and $^1$H-detected heteronuclear NMR spectroscopy and from comparisons with published data. The heteronuclear correlations were necessary to establish reliable data for the structure elucidation.

The white colonial ascidian *Eudistoma toealensis* was observed in high densities on mangrove roots in areas of Chuuk, Micronesia. Despite its high abundance and lack of visible morphological defenses, only the flatworms *Pseudoceros* sp. and *Pseudoceros tristriatus* were observed feeding on the ascidians. Sufficient quantities of the ascidian *E. toealensis* and the flatworm *Pseudoceros* sp. were collected via snorkeling for extraction of metabolites. *P. tristriatus* was not collected, as it was only occasionally present on the ascidian, unlike *Pseudoceros* sp., which was found in large numbers.

In this paper we describe the isolation and structure elucidation of two new, as well as five known, staurosporine derivatives. To date there have only been two reports of staurosporine derivatives from marine organisms, both from ascidians of the genus *Eudistoma*.[1,2] One of the *Eudistoma* species was collected in a similar habitat in Pohnpei, Micronesia,[1] while the other was collected in deep water off the west coast of Africa at Santiago, Cape Verde.[2] In both cases only two staurosporine derivatives were isolated. On the other hand, numerous derivatives have been found in terrestrial microorganisms such as actinomycetes of the genus *Saccharothrix*.[3−5] Whether staurosporine and the derivatives are produced by the ascidian *E. toealensis* or associated microorganisms needs to be verified.

Staurosporine and its derivatives have aroused considerable interest as they were shown to possess strong inhibitory activity against protein kinase C.[6] Staurosporine also inhibits several other kinases such as protein kinases A and G, myosin light-chain kinase, and tyrosine kinases. The mode of action for kinase inhibition seems to vary among the different kinases and is not yet fully understood.[5,7] The search for new derivatives was further intensified after the discovery that staurosporine inhibits platelet aggregation and smooth muscle contraction and blocks certain phases of the cell-growth cycle.[5] The most promising activity is the reversal of multidrug resistance by some derivatives.[8,9]

Here we present spectroscopic data that allow structure elucidation of two new and five known staurosporine congeners. The assessment of the biological activities of all isolated compounds is in progress and will be reported in a separate publication.

## Results and Discussion

The marine ascidian *E. toealensis* and the marine flatworm *Pseudoceros* sp. were collected by snorkeling at depths of 1 to 2 m. Samples were frozen immediately and freeze-dried prior to extraction. Compounds **1**−**7** were isolated from the methanol-soluble material using column chromatography and semipreparative HPLC. The structures of **1**−**7** were identified by comparing the respective spectroscopic data with literature data.[10−14] The $^1$H NMR data of **1**−**4** are included in Table 1, as all the isolated compounds existed in the protonated form (in a boat conformation, **B**) and not as the free base (chair conformation, **A**), which has been extensively reported in the literature.[12−15] In **1**−**4**, there was a large $^1$H downfield shift for the NH-methyl group and upfield shift for the *O*-methyl group of the pyran moiety when compared with literature values. An incorrect assignment of these proton signals could be excluded, as one-bond and long-range $^{13}$C−$^1$H correlations were observed for these signals in the HMQC[17] and HMBC[18] spectra, respectively, of **1**−**4**. The $^{13}$C shifts of the methoxyl and *N*-methyl groups were as expected at ca. 61 and 31 ppm, respectively, and these provided the unambiguous identification of these groupings. The corresponding $^1$H shifts were unusual and initially puzzling. However, Davis et al. have explained these changes in chemical shifts with ring current and charge effects occurring in the boat form relative to the chair conformation.[15] Clearly, it is imperative for correct structure elucidation that the $^{13}$C shifts of the relevant methyl groups be identified, as the $^1$H shifts are unreliable in this respect. Consequently, even though only small amounts of material

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**Table 1.** $^1$H NMR Data of Staurosporine (**1**) and Its Derivatives **2**−**7**

| H no. | **1**[a] | **2**[a] | **3**[a] |
|---|---|---|---|
| 1 | 7.23 dd (1.1, 7.2) | 7.24 br d (8.1) | 7.02 d (8.8) |
| 2 | 7.46 ddd (1.3, 7.2, 7.8) | 7.46 br t (7.6) | 6.98 dd (8.7, 2.1) |
| 3 | 7.30 ddd (1.1, 7.2, 7.8) | 7.30 br t (7.5) | |
| 4 | 9.27 dd (1.3, 7.2) | 9.27 br d (7.9) | 8.71 d (1.8) |
| 7 | 7A 4.81 | 7A 4.84 (17.3) | 7A 4.71 (17.8) |
| | 7B 4.54 (17.8) | 7B 4.62 (17.3) | 7B 4.39 (17.8) |
| 8 | 7.88 dd (1.4, 7.8) | 7.90 br d (7.7) | 7.78 br d (7.8) |
| 9 | 7.41 ddd (1.4, 7.8, 8.6) | 7.41 br t (7.4) | 7.37 br t (7.5) |
| 10 | 7.56 ddd (1.4, 7.2, 8.6) | 7.56 br t (7.8) | 7.52 ddd (1.4, 7.3, 8.6) |
| 11 | 8.00 dd (1.4, 7.2) | 8.00 br d (8.5) | 7.95 br d (8.7) |
| 3' | 4.26 d (2.4) | 4.12 d (2.6) | 4.21 d (2.4) |
| 4' | 4.00 ddd (2.3, 6.9, 11.8) | 4.07 ddd (2.4, 6.5, 10.8) | 3.94 ddd (2.3, 7.0, 11.5) |
| 5'a | 3.24 ddd (6.9, 9.3, 12.8) | 3.08 ddd (6.8, 9.1, 13.5) | 3.17 ddd (6.8, 9.8, 13.2) |
| 5'b | 2.17 ddd (2.9, 11.7, 12.8) | 2.21 ddd (3.2, 10.8, 13.4) | 2.08 m |
| 6' | 6.53 dd (2.8, 9.4) | 6.58 dd (3.2, 9.3) | 6.41 dd (3.0, 9.4) |
| 2'-$CH_3$ | 2.57 s | 2.55 s | 2.52 s |
| 3'-$OCH_3$ | 2.21 s | 2.35 s | 2.15 s |
| | | 3.07 s | |
| 4'-$NCH_3$ | 2.83 s | | 2.80 s |

| H no. | **4**[a] | **5**[a] | **6**[a] | **7**[b] |
|---|---|---|---|---|
| 1 | 7.54 m | 7.42 d (8.7) | 7.44 br d (8.0) | 7.63 br d (8.8) |
| 2 | 7.54 m | 7.09 dd (2.4, 8.7) | 7.54 br t (7.8) | 7.40 br d (8.2) |
| 3 | 7.35 ddd (1.5, 6.7, 8.1) | | 7.36 br t (7.5) | 7.22 br d (7.6) |
| 4 | 9.30 dd (1.0, 7.6) | 8.74 d (2.4) | 9.31 br d (8.0) | 9.41 br d (7.9) |
| 7 | 7A 5.11 (17.6) | 7A 5.08 (17.5) | 7A 5.10 (17.9) | 5.06 s |
| | 7B 5.06 (17.6) | 7B 5.04 (17.5) | 7B 5.01 (17.9) | |
| 8 | 8.09 dd (1.3, 8.0) | 8.07 br d (7.7) | 7.61 d (7.5) | 8.05 br d (8.1) |
| 9 | 7.43 br t (7.4) | 7.42 m | 7.29 t (7.7) | 7.30 br t (7.5) |
| 10 | 7.54 m | 7.54 br t (7.6) | 7.03 d (7.9) | 7.45 br t (7.6) |
| 11 | 7.97 br d (8.5) | 7.95 br d (8.1) | | 7.71 br d (7.8) |
| 3′ | 4.82 br s | 4.84 d (4.1) | 4.65 br s | |
| 4′ | 3.94 m | 3.95 ddd (3.9, 3.9, 12.2) | 4.10 m | |
| 5′a | 2.99 m | 2.95 m | 3.17 m | |
| 5′b | 2.36 m | 2.35 ddd (12.2, 12.2, 6.9) | 2.68 ddd (12.5, 12.5, 4.4) | |
| 6′ | 6.95 dd (6.9, 8.1) | 6.86 dd (6.9, 8.3) | 6.73 dd (4.4, 9.5) | |
| 2′-$CH_3$ | 2.42 s | 2.41 s | 2.52 s | |
| 3′-$OCH_3$ | | | 3.07 s | |
| 4′-$NCH_3$ | 2.87 s | 2.85 s | | |

[a] Measurements were performed in $CD_3OD$. [b] Measurements were performed in $Me_2CO$-$d_6$.

**Table 2.** $^{13}$C NMR Data of Staurosporine **1** and Its Derivatives **2**−**6** in $CD_3OD$

| C no. | **1** | **2** | **3** | **4**[a] | **5**[a] | **6**[a] |
|---|---|---|---|---|---|---|
| 1 | 109.4 d | 109.3 d | 109.7 d | 108.9 | 109.3 | 109.0 |
| 2 | 126.5 d | 126.6 d | 116.0 d | 126.6 | 116.0 | 126.6 |
| 3 | 120.8 d | 120.8 d | 152.0 d | 120.7 | 152.1 | 120.9 |
| 4 | 127.2 d | 127.2 d | 111.9 d | 127.0 | 111.4 | 127.1 |
| 4a | 124.5 s | 124.5 s | 125.2 s | 124.3 | 125.0 | 124.6 |
| 4b | 116.9 s | 117.0 s | 116.4 s | 117.3 | | 117.1 |
| 4c | 120.3 s | 120.3 s | 120.2 s | | | |
| 5 | 175.1 s | 175.1 s | 175.2 s | 175.1 | | 175.1 |
| 7 | 46.9 t | 46.9 t | 46.8 t | 46.9 | 46.9 | 47.0 |
| 7a | 133.8 s | 133.9 s | 133.3 s | 134.3 | 133.8 | 134.1 |
| 7b | 115.8 s | 115.9 s | 115.5s | | 115.6 | |
| 7c | 125.9 s | 125.9 s | 125.9 s | 125.9 | 125.9 | 128.2 |
| 8 | 122.8 d | 122.7 d | 122.6 d | 122.1 | 122.1 | 113.6 |
| 9 | 122.0 s | 122.0 s | 121.9 s | 121.4 | 121.5 | 122.9 |
| 10 | 126.5 s | 126.6 s | 126.4 s | 126.0 | 125.7 | 113.0 |
| 11 | 113.4 s | 113.6 s | 113.3 s | 114.7 | 114.6 | 143.6 |
| 11a | 139.5 d | 139.7 d | 139.4 d | 140.5 | 140.4 | 129.1 |
| 12a | 127.7 s | 131.4 s | 131.5 s | | | |
| 12b | 131.5 s | 127.5 s | 128.3 s | 126.7 | 127.2 | |
| 13a | 137.8 s | 137.9 s | 132.6 s | 137.9 | 132.7 | 138.8 |
| 2′ | 94.2 s | 94.2 s | 94.1 s | 95.7 | 95.6 | 95.6 |
| 3′ | 81.4 d | 82.5 d | 81.4 d | 70.2 | 70.1 | 81.5 |
| 4′ | 55.9 d | 47.9 d | 55.9 d | 55.5 | 55.7 | |
| 5′ | 28.7 t | 30.3 t | 28.8 t | 27.4 | 27.0 | |
| 6′ | 82.0 d | 82.0 d | 81.9 d | 82.0 | 82.0 | |
| 2′-$CH_3$ | 28.8 q | 29.0 q | 28.7 q | 29.5 | 29.4 | 29.7 |
| 3′-$OCH_3$ | 62.2 q | 60.8 q | 60.6 q | | | 60.7 |
| 4′-$NCH_3$ | 31.3 q | | 31.3 q | 31.1 | 30.8 | |

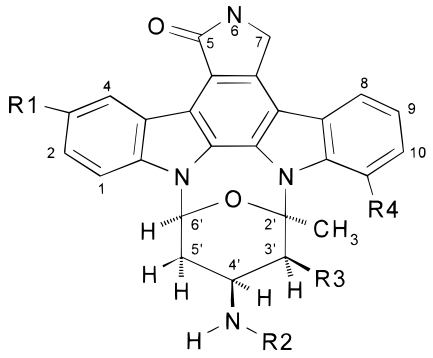
[a] The $^{13}$C shifts were taken from the cross-peaks in the HMBC spectrum, and consequently not all shifts could be accessed.

were available for **5** and **6** $^1$H-detected $^{13}$C−$^1$H correlations provided the necessary information.

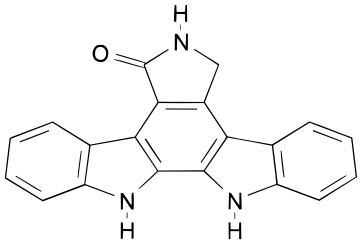
Compounds **5** and **6** exhibited the same UV spectrum as those of **1**−**4**, indicating that **5** and **6** were also indolocarbazole compounds of the staurosporine type. The MS indicated a relative mass of 468 for both compounds. The structures of **5** and **6** followed directly from the shift data (Tables 1 and 2) and long-range correlations in the HMBC spectra. Support for the structure was afforded by comparisons with compounds **1**−**4**, as well as with literature data.[1,10−14,16]

The $^1$H NMR spectrum of **5** showed a characteristic three-proton aromatic coupling pattern (2D COSY) in which there was an upfield shift of H-4 (8.74 ppm) and only one small coupling constant (2.4 Hz) compared to the values in **1**, **2**, **4**, and **7**. The absence of H-3 and the corresponding upfield shifts of H-4 and H-2 were compatible only with an additional hydroxyl group at C-3. In addition, the 3′-$OCH_3$ group of the pyran ring was absent, although the lowfield shift of H-3′ (4.84 ppm) and its direct correlation with a signal at 70.1 ppm clearly indicated that C-3′ was attached to a hydroxyl group. These changes are comparable to those found in **4** and indicate that **5** must be 3-hydroxy-3′-demethoxy-3′-hydroxystaurosporine.

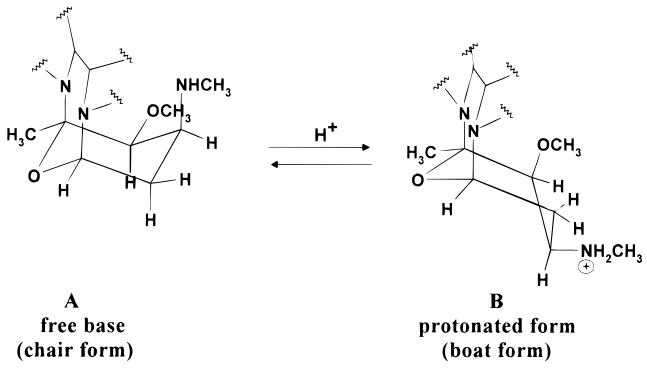
The $^1$H chemical shifts for protons H-1 to H-4 of **6** and their coupling pattern (Table 1) were the same as in staurosporine (**1**), while the coupling pattern (2D COSY) of the three remaining aromatic protons, which must be adjacent to one another, their shifts, and long-range correlations in the HMBC spectrum were only compatible with the introduction of a hydroxyl group into the second proton-bearing aromatic ring system. Comparison of **6** with **5** showed that the shifts of H-7A and H-7B remained unchanged, while those of H-3′ and the quaternary methyl



| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| **1** | H | $CH_3$ | $OCH_3$ | H |
| **2** | H | H | $OCH_3$ | H |
| **3** | OH | $CH_3$ | $OCH_3$ | H |
| **4** | H | $CH_3$ | OH | H |
| **5** | OH | $CH_3$ | OH | H |
| **6** | H | H | $OCH_3$ | OH |



**7**



**A**
**free base**
**(chair form)**

**B**
**protonated form**
**(boat form)**

were considerably different. Consequently, the aromatic hydroxyl function must reside at C-11 as opposed to the alternative at C-8. This was confirmed from the long-range correlations observed in the HMBC spectrum, which showed upfield shifts of C-10 and C-11a and a corresponding downfield shift of C-11 compared to those found for **1**−**4** (Table 2). This same spectrum indicated that there was still a methoxyl group attached to C-3′, although the $^1$H shift was to lower field than those found in **1**−**3**, but still to highfield of those normally associated with aliphatic methoxyl groups. The absence of signals for a *N*-methyl group was compatible with the MS data and indicated that **6** is 11-hydroxy-4′-*N*-demethylstaurosporine.

The concentrations of the staurosporine derivatives were determined by HPLC quantification. For statistical analysis we extracted three samples (0.3 g dry wt each) of the ascidian and the flatworm. The crude extracts from the ascidian and the flatworm were diluted with methanol and injected into an HPLC-system (Gynkotec, Germany). Separation was achieved by applying a linear gradient from 100% water (with 0.1% TFA) to 45% acetonitrile over 55 min. The compounds were detected at their UV maximum (292 nm). We used previously isolated staurosporine as an external standard for quantification. The concentrations of the derivatives were calculated as staurosporine equivalents.

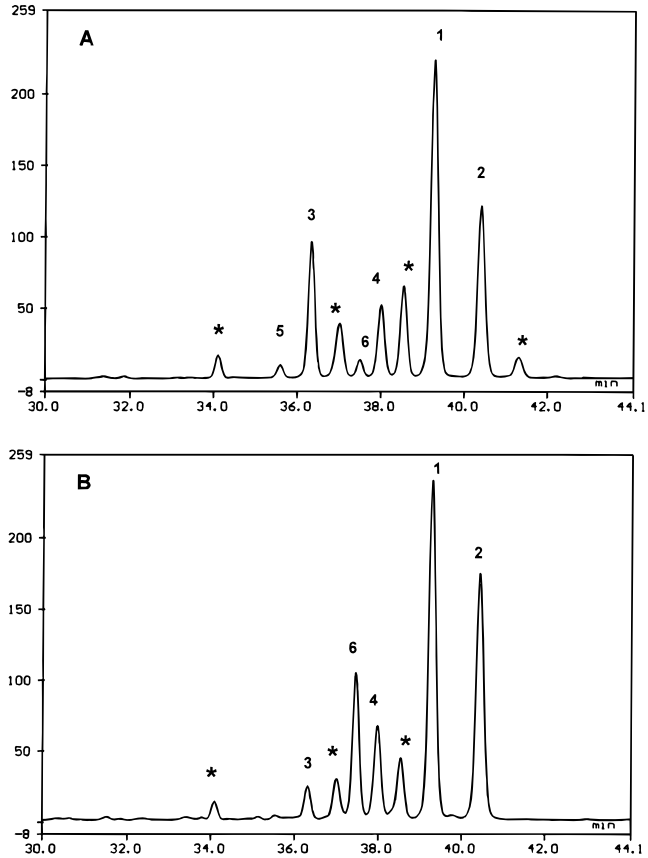



**Figure 1.** HPLC charts of the extracts of the flatworm *Pseudoceros* sp. (**A**) and the ascidian *Eudistoma toealensis* (**B**). The extract of **A** was diluted 6-fold. Peak identification: * = unknown, **1** = staurosporine, **2** = *N*-demethylstaurosporine, **3** = 3-OH-staurosporine, **4** = 3′-OH-3′-demethoxystaurosporine, **5** = 3-OH-3′-demethoxy-3′-OH-staurosporine, **6** = 11-OH-4′-*N*-demethylstaurosporine.

The flatworm *Pseudoceros* sp. accumulated the isolated staurosporine derivatives (Figure 1A). Concentrations of staurosporine and its derivatives in the flatworm showed a 4- to 25-fold increase compared to the ascidian (Figure 1B), with the exception of **6**. 11-Hydroxy-4′-*N*-demethylstaurosporine (**6**) was present in only two-thirds of the concentration found in the ascidian. Compound **5** and another, unidentified derivative were only found in the flatworm. One possible explanation for this divergence is that the concentrations of the latter two compounds in the ascidian were too low for detection. Another possibility is the chemical modification of staurosporine or its derivatives by the flatworm to produce the two compounds. Compound **7** was not detected in the crude extract of either the ascidian or the flatworm. Either the concentration of the compound is too low in the crude extract of both animals or the aglycon is an artifact from the extraction process.

## Experimental Section

**General Experimental Procedures.** $^1$H (1D and 2D COSY) and $^{13}$C (1D and 2D $^1$H-detected heteronuclear one-bond HMQC[17] and multiple-bond HMBC[18] correlations) NMR spectra (chemical shifts in ppm) were recorded on Bruker DPX 300, ARX 400, or DMX 600 NMR spectrometers using standard Bruker software. Mass spectra (ESIMS) were recorded on a Finnigan MAT TSQ-7000 triple-stage quadrupole mass spectrometer. The temperature of the heated capillary (20 V) was 200 °C, and the electrospray capillary voltage was set to 3.5

kV. Nitrogen served both as sheath (70 psi) and auxiliary gas; argon served as collision gas. For HPLC analysis, samples were injected into a HPLC system equipped with a photodiode-array detector (Gynkotek, Germany). Routine detection was at 254 and 292 nm. The separation column (125 × 4 mm, i.d.) was prefilled with Eurospher 100-$C_{18}$, 5 $\mu$m (Knauer GmbH, Germany). Separation was achieved by applying a linear gradient from 100% $H_2O$ (with 0.2% TFA) to 100% MeCN over 40 min. Semipreparative HPLC was conducted on a Merck Hitachi, La-Chrome L-7100 pump and Merck Hitachi, La-Chrome L-7400 UV detector. Chromatograms were recorded on a Merck Hitachi, D-2000 Chromato-Integrator. Separation columns (300 × 8 mm, i.d.) were prefilled with Eurospher 100-$C_{18}$, 7 $\mu$m (Knauer GmbH, Germany). Compounds were purified by isocratic separation with 33% MeCN, 67% $H_2O$ (with 0.2% TFA) for 40 min. TLC was performed on precoated TLC plates with Si gel 60 $F_{254}$ and Si gel RP-18 $F_{254}$ (Merck, Darmstadt, Germany). Compounds were detected from their UV absorbance at 254 and 366 nm. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements.

**Animal Material.** *E. toealensis* is a colonial ascidian first described by Monniot and Monniot in 1996.[19] The zooids are whitish and slightly transparent. Several zooids converge, sitting on small, 2−4 cm short, brownish branches, which are attached to mangrove roots. A voucher specimen is kept under MNHN A3 Eud 134 at the Museum National d′Histoire Naturelle, Paris, France.

*Pseudoceros* sp. is a new species often confused with *Pseudoceros concinnus* (Collingwood 1876) (L. Newman personal communication). However, it lacks the median blue stripe, and the margin is clearly composed of spots and not a uniform stripe. *Pseudoceros* sp. has a cream-colored body with uneven royal blue to dark purple spots along the margin. The spots continue over the pseudotentacles. Sizes ranged from 6 × 3 mm (immature) to 40 × 29 mm (mature), with an average length of 23 × 8 mm ($n$ = 44, STD = 1.4 mm and 0.3 mm, respectively). It was found in large numbers on colonies of *E. toealensis* at depths of 1 to 2 m. A voucher specimen (QM # G211100) is deposited at the Queensland Museum, Brisbane, Australia.

**Extraction and Isolation.** The freeze-dried samples of the ascidian *E. toealensis* (94 g) were extracted exhaustively with MeOH. The extract was evaporated under reduced pressure to give a residue of 19.26 g. The MeOH fraction was then partitioned sequentially between hexane, EtOAc, butanol, and $H_2O$. HPLC chromatograms showed that only the EtOAc fraction contained the compounds with the characteristic UV spectra. This fraction was chromatographed on RP-18 Si gel columns [elution with MeOH−$H_2O$−TFA (80:20:0.2)] yielding eight fractions. Final purification of the major UV-active fractions was achieved with semipreparative HPLC [MeCN−$H_2O$−TFA (33:67:0.2)]. This yielded compounds **1** (3.2 mg, 0.003%), **4** (2.7 mg, 0.003%), **6** (3.4 mg, 0.003%), and **7** (1.5 mg, 0.002%).

*Pseudoceros* sp. was also freeze-dried (60 g), then extracted with MeOH, and the MeOH extract was partitioned with $CH_2Cl_2$. Extracts were evaporated under reduced pressure to yield 6 g of the $CH_2Cl_2$ and 18 g of the MeOH fraction. HPLC chromatograms showed that only the $CH_2Cl_2$ fraction contained the compounds with the characteristic UV spectra. The $CH_2Cl_2$ fraction was chromatographed on RP-18 Si gel columns [elution with MeOH−$H_2O$−TFA (80:20:0.2)] yielding 13 fractions. Again the major UV-active fractions were purified with semipreparative HPLC [MeCN−$H_2O$−TFA (33:67:0.2)]. This yielded compounds **1** (7.1 mg, 0.012%), **2** (5.9 mg, 0.01%), **3** (3.8 mg, 0.006%), and **5** (2.6 mg, 0.004%). Other fractions also contained staurosporine and its derivatives; however, these fractions were not purified further.

**3-Hydroxy-3′-demethoxy-3′-hydroxystaurosporine (5):** yellowish amorphous powder; 100% purity (as estimated by HPLC analysis); UV max (MeOH) 237 ($\epsilon$ 8350), 251 ($\epsilon$ 6600), 270 ($\epsilon$ 7120), 297 ($\epsilon$ 13 380), 342 ($\epsilon$ 3790), 366 ($\epsilon$ 2400), 384 ($\epsilon$ 2420); ESIMS: $m/z$ 469 $[M + H]^+$; ESIMS−MS (collision energy 29 eV, 2 mTorr Argon) $m/z$ 354 (469−115); product ion $m/z$ 354 characteristic for the oxygenated indolocarbazole moiety.

**11-Hydroxy-4′-*N*-demethylstaurosporine (6):** yellowish amorphous powder; 100% percent purity (as estimated by HPLC analysis); UV max (MeOH) 290 ($\epsilon$ 1040), 356 ($\epsilon$ 180), 373 ($\epsilon$ 180); ESIMS: $m/z$ 469 $[M + H]^+$; ESIMS−MS (collision energy 29 eV, 2 mTorr Argon) product ion $m/z$ 354 characteristic for the oxygenated indolocarbazole moiety.

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