New pigments from the terrestrial cyanobacterium Scytonema sp. collected on the Mitaraka inselberg, French Guyana

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New Pigments from the Terrestrial Cyanobacterium *Scytonema* sp. Collected on the Mitaraka Inselberg, French Guyana

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Inselbergs are hills rising abruptly from the surrounding plains where cyanobacteria are the only living organisms under conditions of intense solar radiation. A survival mechanism to prevent UV-damage has been associated with synthesis of the ultraviolet-screening, photostable sheath pigment scytonemin. The organic extract of *Scytonema* sp., collected on the Mitaraka inselberg, French Guyana, yielded three new pigments, tetramethoxyscytonemin (**1**), dimethoxyscytonemin (**2**), and scytonine (**3**), derived from the scytoneman skeleton of scytonemin. These structures were assigned mainly on the basis of 1H and 13C NMR and MS experiments.

Cyanobacteria have drawn attention for their ability to produce an immense number and variety of bioactive secondary metabolites, ranging from notorious toxins to potential therapeutic agents. They are an ancient, diverse group of microorganisms and are able to inhabit and thrive in an incredible variety of environments.

Inselbergs are isolated rocks, mountains, or groups of mountains (the so-called "island mountains") rising abruptly from the surrounding plains in humid (forest) to semiarid (savanna) locations. They are often dome-shaped, consisting of granite and gneiss, partially covered by a thin layer of organic substrates. They possess a unique vegetation that differs in species composition from that of the surroundings.

In an ongoing program devoted to the study of plant succession, we investigated the Mitaraka inselberg in French Guyana, in particular granite-collected samples where cyanobacteria are the only living organisms under conditions of intense solar radiation. The survival ability of cyanobacteria under these specific conditions

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to prevent UV-damage has been associated with synthesis of the ultraviolet screening, photostable sheath pigment scytonemin.[1]

Scytonemin is a yellow-brown dimeric pigment with potent ultraviolet-absorbing properties, located in the extracellular polysaccharide sheath of some cyanobacteria, characterized by Proteau and co-workers in 1993.[2] To date, it is the only sunscreen pigment identified from this series. The occurrence of scytonemin restricted to cyanobacteria is widespread among this diverse group, and more than 300 species with sheaths colored with yellow to brown pigments have been described.[3,4] Instead of scytonemin, some cyanobacteria contain a red to purple pigment, gloeocapsin, whose structure remains unknown. The production of such molecules can be related to those of other known sunscreens, such as mycosporin-like amino acids in phytoplankton and fungi,[5] animal melanins,[6] and plant phenylpropanoids.[7,8]

We report, herein, the structure of three new pigments, **1**-**3**, related to the scytoneman skeleton. These molecules derive from condensation of tryptophanyl- and tyrosylderived subunits with a linkage between these units unique among natural products. Compound **1** has been termed tetramethoxyscytonemin; compound **2**, dimethoxyscytonemin; and compound **3**, scytonin. Their isolation and structure determination is now presented.

The intensly colored compounds exhibited typical spectroscopic properties of scytonemin.[1]

The 1H NMR spectrum (Table 1) of purple compound **1** ($C_{40}H_{34}N_2O_8$ by HRFABMS) indicated two tertiary methyl groups resonating as singlets at δ 2.95 and 3.15, a singlet for a methine at δ 4.65, a typical AB system for a para-substituted phenol with a hydroxyl proton at δ 9.23, and signals for one disubstituted indole ring and an NH protonat δ 12.12.

The ^{13}C NMR spectrum showed a carbonyl signal at δ 203.0, a methine at δ 85.3, a quaternary carbon at δ 84.5, and two methoxy signals at δ 52.4 and 56.6. Among the aromatic carbons was observed an olefinic quaternary carbon signal at δ 157.1, indicating a phenol function. Owing to the molecular formula indicating 40 carbons and the relatively simple aspect of both its ^{1}H and ^{13}C NMR spectra, compound **1** has several elements of symmetry.

2D-NMR analysis led to the structural assignment of the molecule. Starting from the long-range correlations of H-11/H-15, we were able to link the *para*-substituted phenol to the CH-9 bearing a methoxy. A second spin system was built from HMBC correlations of H-9 with C-2, C-3 bearing O-CH_3-17, C-3a, and C-10. H_3-17 showed long-range correlations with C-3 and C-9. The disubstituted indole ring was established by COSY

and HMBC correlations of H-5 to H-8 and the long-range correlations observed for H-4 with C-3a, C-4a, C-8a, and C-8b. The remaining two quaternary carbons, C-1 and C-1', provided the connection between the two dimeric units. The geometry of the tetrasubstituted olefin in **1** is predicted as *E* by inspection of molecular models and was confirmed as the lower isomer by MM2 calculation.

The ^{1}H NMR spectrum (Table 2) of the dark red compound **2** ($C_{38}H_{28}N_2O_6$ by HRFABMS) indicated two tertiary methyl groups resonating as singlets at δ 3.05 and 3.18, a singlet for a methine at δ 4.56, two typical AB systems for *para*-substituted phenols with two hydroxyl protons at δ 9.28 and 10.28, and signals for two disubstituted indole rings and two NH protons at δ 10.60 and 12.18. The ^{13}C NMR spectrum showed two carbonyls at δ 194.5 and 199.7, a methine at δ 84.6, a quaternary carbon at δ 102.2, and two methoxy groups at δ 50.9 and 56.4. Among the aromatic carbons, there were two olefinic quaternary carbons at 157.1 and 160.1 ppm (bearing the phenol functions) and two olefinic carbons at δ 144.2 and 130.6. Detailed 2D-NMR analysis led to the full assignment of two parts of the molecule. The first part of the molecule was assigned as in **1**. The second part was built, starting from the H-11/H-15 signal, observing HMBC correlations with the quaternary olefinic C-3 and with C-10, in addition to those of H-9 with C-11/15, C-3, and the carbonyl C-2. The indole ring was established by the COSY and HMBC correlations of proton H-5 to H-8 and the long-range correlations observed for NH-4 with C-3a, C-4a, C-8a, and C-8b. The *E*-configuration of the 3-9 double bond was deduced by observing NOESY correlation between H-11/H-15 and H-12/H-14 and NH-4.

As in **1**, the remaining two quaternary carbons (C-1 and C-1') provided the connection between the dimeric units. The geometry of the tetrasubstituted olefin in compound **2** was predicted to be *E* by inspection of molecular models and was confirmed as the lower isomer by MM2 calculation.

The 1H NMR spectrum (Table 3) of the brown compound **3** ($C_{31}H_{22}N_2O_6$ by HRFABMS) indicated two tertiary methyl groups resonating as singlets at δ 3.55 and 3.65, a singlet for an olefinic methine at δ 7.67, signals of a typical AB system for a *para*-substituted phenol with a hydroxyl proton at δ 9.35, and signals for disubstituted indole rings and two NH protons at δ 11.24 and 11.62.

The first disubstituted indole ring was established by the COSY and HMBC correlations of protons H-5 to H-8. Using the long-range correlations observed for H-4 with a quaternary carbon at δ 150.7 and a carbonyl at 170.2, we were able to locate C-2 and C-3, respectively.

Starting from the long-range correlations of H-11/H-15, we were able to link the *para*-substituted phenol to the olefinic CH-9. A second spin system was built by the HMBC correlations of H-9 with C-2', C-3'a, C-15, and C-3' bearing the carbonyl C-2', in addition to CH3-17 protons showing long-range correlations with the carbonyl C-2'. The disubstituted indole ring was established by the COSY and HMBC correlations of proton H-5' to H-8' and the long-range correlations observed for H-4' with C-2, C-3'a, C-4'a, C-8'a, and C-8'b. The second part of the molecule was constituted and linked to the first indole ring owing to the HMBC correlation observed for NH-4' with C-2.

The *E*-configuration of the 3'-9 double bond was deduced from NOE data (NOESY experiment) particularly the correlation between the methoxy protons H3-17 and H-9 and the correlation between H-11/H-15 and H-12/H-14 and NH-4'.

A possible route for biosynthesis of compound **3**, starting from reduced scytonemin, is proposed. The first step consists of loss of one *para*-substituted phenol unit. The cyclopentenone rings may then be opened, and successive methoxylation can occur before cyclization.

Scytonemin demonstrated interesting anti-inflammatory activity in a model of PMA-induced mouse ear edema and anti-proliferative activity by the inhibition of rhPKCβ1 activity.[9]

Compounds **1**-**3** were tested for their cytotoxicity (KB cells); they were atoxic even at 10^{-5} M. These compounds did not inhibit the growth of the Gram-positive bacterium *Staphylococcus aureus* (ATCC 6538), the Gram-negative *Escherishia coli* (ATCC 8739), and the fungi *Candida tropicalis* (IP 201.73) even at 1 μM.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet FTIR in MeOH. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance 400 spectrometer with standard pulse sequences operating at 400 and 100 MHz, respectively; the chemical shift values are reported as δ (ppm units) and the coupling constants in Hz. J_{mod}, NOESY, HSQC (optimized for $^{1}J_{CH}$) 140 Hz), and HMBC ($^{n}J_{CH}$ = 7 Hz) experiments were recorded using standard Bruker pulse sequences. ESI-QqTOF spectra were acquired in positive mode on a Q-Star Applied Biosystem. HRMS (positive mode) were measured on a JEOL 700 spectrometer. Si gel CC was carried out using Kieselgel 60 (230-400 mesh, E. Merck), and RP-18 gel CC was carried out using Polygoprep

60-50 (Macherey-Nagel). Fractionations were monitored by TLC using aluminium-backed sheets (Si gel 60 F-254, 0.25 mm thick and RP-18WUV$_{254}$, Macherey-Nagel, 0.25 mm thick) with visualization at 254 and 366 nm and Liebermann, or phosphomolybdic acid, spray reagent. All solvents were distilled. Semipreparative reversed-phase HPLC (Akzo Nobel RP-18 column, 7.5 x 250 mm) was performed with a L-6200A pump (Merck-Hitachi) equipped with an L-4250C UV-vis detector (Merck-Hitachi) and a D-2500 chromato-integrator (Merck-Hitachi).

Biological Material. The cyanobacterium *Scytonema* sp. was collected in the dry state in March 2001, on the Mitaraka inselberg (Tumuc Hamac) in French Guyana, and stored in the dark at room temperature. This cyanobacterium grows on granite as colonies, i.e., as mats with an area of several square centimeters even under full sunlight in semiarid habitats. The material was identified by Prof. Couté, and a specimen is deposited at Museum National d'Histoire Naturelle (Paris, France, no. SC2002). The crust consists of a close association between soil mineral particles and cyanobacteria, living on granite substrate; a minute collection yielded several samples of *Scytonema* sp. The crusts constitute the first step before the development of humus and provides niches for the establishment by seed of several plant species.

Extraction and Isolation. Extraction of SC2002 with CH_2-Cl_2/MeOH (v/v) yielded 2 g of crude material. Purification of this extract by chromatography over a silica gel column (CH_2-Cl_2 to MeOH) led to two fractions containing pigments eluted with 10% MeOH in CH_2Cl_2. Repeated chromatographic separations of the first, over RP18 TLC, afforded **1** and **3.** Compound **1** (6.3 mg) eluted with 40% TFA 0.1% in MeCN, R_f 0.56. Elution of the fraction R_f 0.40 obtained after the first TLC purification with 20% TFA 0.1% in MeOH yielded compound **3**, R_f 0.42 (4.6 mg).

The second pigments-containing fraction was subjected to open column reversed-phase RP-18 and semipreparative reversed-phase HPLC (Akzo Nobel RP-18 column, 7.5 x 250 mm, 2 mL/min 50% TFA 0.1% in MeCN; λ = 386 nm), accomplishing the separation and final purification of **2** (5.6 mg) eluted at t_R 21 min.

Tetramethoxyscytonemin (1): purple amorphous solid; UV (MeOH) λ_{max} nm (ε), 212 (35928), 562 (5944); IR (MeOH) 3652, 3541, 2978, 2831, 1696, 1514, 1448, 1413, 1025; ^{1}H and ^{13}C NMR data, see Table 1; ESI-QqTOF-MS *m*/*z* [M + H]+ 671; *m*/*z* [M + Na]+ 693; *m*/*z* [M + K]+ 709; FABHRMS *m*/*z* [M + H]+ 671.2396 (calcd for $C_{40}H_{35}N_2O_8$, 671.2384).

Dimethoxyscytonemin (2): dark red amorphous solid; UV (MeOH) λ_{max} nm (ε), 215 (60354), 316 (18143), 422 (23015); IR (MeOH) 3662, 3533, 2970, 2900, 2878, 1695, 1602, 1452, 1401, 1025; ^{1}H and ^{13}C

NMR data, see Table 2; ESI-QqTOF-MS *m*/*z* [M + H]+ 609; *m*/*z* [M + Na]+ 631; *m*/*z* [M + K]+ 647; FABHRMS *m*/*z* [M + H]+ 609.2025 (calcd for$C_{38}H_{29}N_2O_6$, 609.2018).

Scytonine (3): brown amorphous solid; UV (MeOH) λ_{max} nm (ε), 207 (38948), 225 (37054), 270 (22484); IR (MeOH) 3661, 3536, 2971, 2878, 1690, 1602, 1510, 1440 cm-1; ^{1}H and ^{13}C NMR data, see Table 3; ESI-QqTOF-MS *m*/*z* [M + H]+ 519; *m*/*z* [M + Na]+ 541; *m*/*z* [M + K]+ 557; FABHRMS *m*/*z* [M + H]+ 519.1564 (calcd for $C_{31}H_{23}N_2O_6$, 519.1550).

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Legends of figures

Figure 1. Proposed biosynthetic pathway for **3**.

Table 1. ^{1}H and ^{13}C NMR Data for **1** in DMSO-d_6[a]

no.	δ ^{1}H (m, *J* Hz)	δ ^{13}C
1		122.0
2		203.0
3		84.5
3a		143.3
4	12.12 (s, 1H)	
4a		140.2
5	7.05 (d, 8.1, 1H)	125.3
6	7.25 (ddd, 7.6, 7.6, 1.1, 1H)	123.4
7	7.15 (ddd, 7.6, 7.6, 0.9, 1H)	119.9
8	7.54 (d, 8.1, 1H)	112.6
8a		122.1
8b		126.9
9	4.65 (s, 1H)	85.3
10		125.7
11	6.67 (m, 8.6, 1H)	129.4
12	6.45 (m, 8.6, 1H)	114.7
13		157.1
14	6.45 (m, 8.6, 1H)	114.7
15	6.67 (m, 8.6, 1H)	129.4
16	9.23 (s, 1H)	
17	2.95 (s, 3H)	52.4
18	3.15 (s, 3H)	56.6

a ^{1}H 400 MHz; ^{13}C 100 MHz; 298 K.

Table 2. ^{1}H and ^{13}C NMR Data of **2** in DMSO-d_6[a]

no.	δ ^{1}H (m, *J* Hz)	δ ^{13}C
1		123.0
2		194.5
3		144.2
3a		132.2
4	12.18 (s, 1H)	
4a		122.7
5	7.38 (d, 7.9, 1H)	127.5
6	7.28 (dd, 7.9, 7.4, 1H)	122.1
7	7.35 (dd, 7.3, 6.3, 1H)	125.5
8	7.58 (d, 7.6, 1H)	123.3
8a		124.3
8b		127.7
9	7.09 (s, 1H)	130.6
10		134.3
11	7.21 (m, 8.4, 1H)	129.6
12	6.92 (m, 8.4, 1H)	116.3
13		160.1
14	6.92 (m, 8.4, 1H)	116.3
15	7.21 (m, 8.4, 1H)	129.6
16	10.28 (s, 1H)	
1		123.2
2		199.7
3		102.2
3'		139.7
4	10.60 (s, 1H)	
4'		130.4
5	7.03 (d, 8.5, 1H)	130.2
6	6.89 (m, 3.7, 1H)	109.6
7	6.82 (dd, 7.4, 7.4, 1H)	121.5
8	6.97 (d, 7.4, 1H)	123.3
8'		129.5
8'		144.1
9	4.56 (s, 1H)	84.6
10		126.1
11	7.22 (m, 8.4, 1H)	130.8
12	6.65 (m, 8.4, 1H)	114.3
13		157.1
14	6.65 (m, 8.4, 1H)	114.3
15	7.22 (m, 8.4, 1H)	130.8
16	9.28 (s, 1H)	
17	3.05 (s, 3H)	56.4
18	3.18 (s, 3H)	50.9

a ^{1}H 400 MHz; ^{13}C 100 MHz; 298 K.

Table 3. ^{1}H and ^{13}C NMR Data for **3** in DMSO-d_6[a]

no.	δ 1H (m, *J* Hz)	δ ^{13}C
1		144.2
2		150.7
3		170.2
3a		137.2
4	11.62 (s, 1H)	
4a		136.1
5	7.42 (d, 7.8, 1H)	113.8
6	7.19 (dd, 7.7, 5.2, 1H)	123.0
7	7.36 (dd, 8.2, 5.2, 1H)	127.5
8	7.90 (d, 8.2, 1H)	123.3
8a		139.1
8b		122.2
9	7.67 (s, 1H)	143.5
10		124.8
11	6.95 (m, 8.7, 1H)	132.7
12	6.50 (m, 8.7, 1H)	115.1
13		159.2
14	6.50 (m, 8.7, 1H)	115.1
15	6.95 (m, 8.7, 1H)	132.7
16	9.35 (s, 1H)	
17	3.55 (s, 3H)	59.5
18	3.65 (s, 3H)	51.8
19		167.3
2		167.1
3		131.8
3'		119.2
4	11.24 (s, 1H)	
4'		135.7
5	7.35 (d, 8.1, 1H)	111.4
6	7.11 (dd, 8.1, 7.0, 1H)	126.1
7	7.02 (d, 7.0, 1H)	119.2
8	7.37 (d, 5.1, 1H)	119.6
8'		129.1
8'		106.3

a ^{1}H 400 MHz; ^{13}C 100 MHz; 298 K.

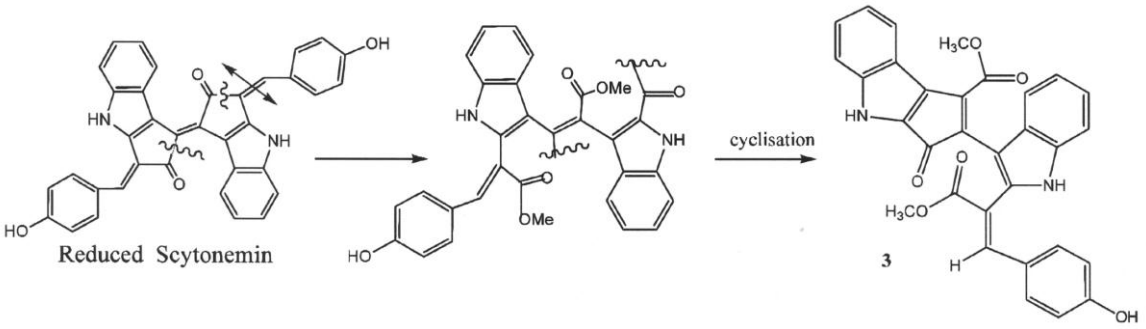
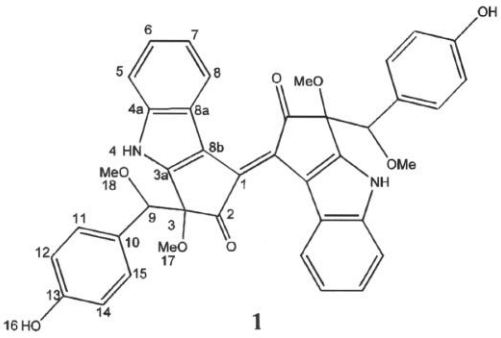


Fig. 1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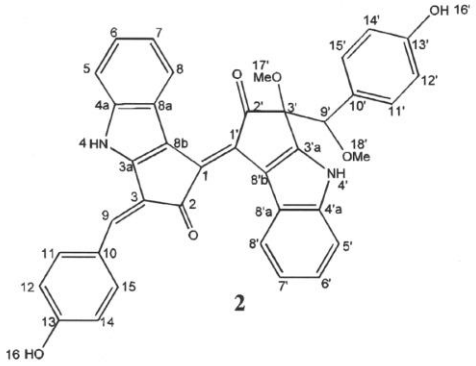
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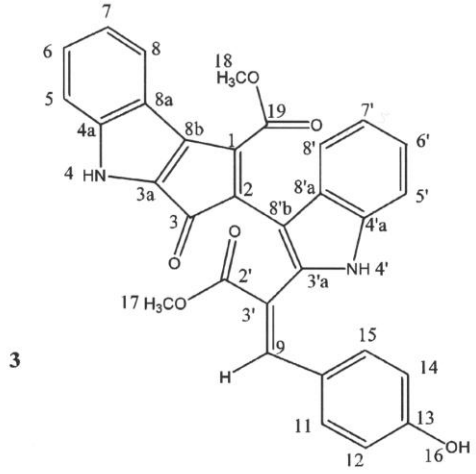
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