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ARTICLE TYPE

On the Photostability of Scytonemin, Analogues Thereof and Their Monomeric Counterparts

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As a part of their sun-protective strategy, cyanobacteria produce the natural UV-screener scytonemin. Its accumulation in the extracellular sheaths allows the bacteria to thrive in inhospitable locations highly exposed to solar radiation. Scytonemin is often referred to as photostable and has been reported to be non-fluorescent. Taken together, these properties indicate inherently fast non-radiative relaxation processes. Despite these interesting traits, the photophysics of scytonemin is as yet almost completely unexplored. In this study, we have compared the steady-state photophysics of scytonemin itself and four derivatives thereof. Furthermore, the *in vitro* photostability of scytonemin was studied in different solvents using a solar simulation system. Scytonemin and the investigated derivatives demonstrated a more rapid photoinduced decay in comparison with two commercial UV-screening agents. The photostability could be modulated by varying the solvent, with the protic solvent ethanol providing the most stabilizing environment.

Introduction

The extracellular cyanobacterial pigment scytonemin is believed to be essential for the notable survival ability of many strains of cyanobacteria. Its vital importance to these organisms is accentuated by the fact that despite the structural complexity of scytonemin, the compound is found in vast amounts in cyanobacteria cultures, accounting for up to 5% of the dry weight,[1, 2] and the pigment has been observed in more than 300 species of cyanobacteria.[3] Additional support for the importance of scytonemin has been provided by genomic comparisons, which reveal that the biosynthetic gene cluster of scytonemin is highly conserved between evolutionary diverse strains of cyanobacteria.[4, 5]

The principal role of scytonemin is believed to be as a passive UV-sunscreen that protects cyanobacteria against harmful UV-radiation. The proposed UV-protective role is corroborated by the demonstration that transcription of the biosynthetic gene cluster and the biosynthesis of scytonemin is induced by UV-irradiation.[2, 4] In addition, scytonemin is often found in the upper layers of microbial mats that thrive in areas exposed to intense solar irradiation. It is a greenish brown pigment and absorbs light only weakly in the visible region but shows strong absorption of UV-light. Thus, scytonemin efficiently shields the cyanobacteria from high-energy radiation, while at the same time allows penetration of light of the correct wavelengths necessary to support the vital photosynthesis. This UV-defence mechanism has enabled cyanobacteria to occupy diverse ecological niches and to adapt to various extreme environments such as soil surfaces, rocks, as well as in intertidal zones where the intensity of solar irradiation is high.[6, 7]

Scytonemin has often been referred to as an exceptionally stable or photostable compound.[3, 8-10] It is reported to remain largely intact in the sheaths of desiccated bacteria even after exposure to UVA for two months.[11] The observed photodegradation of scytonemin was shown to be orders of magnitude slower *in vivo* than that of chlorophyll, another essential biological pigment.

Scytonemin has also been proposed to provide the cyanobacteria with a high tolerance to desiccation. This ability has not only been ascribed to the UV-protection capacity but also to the antioxidative properties of scytonemin.[12, 13] Measurements have shown that scytonemin has a strong but slow radical scavenging activity, and it has been suggested to be the main hydrophobic antioxidant in the extracellular matrix of cyanobacteria.[12] Therefore, scytonemin is proposed to play multiple roles in the defence mechanisms activated in response to environmental stress such as UV radiation and desiccation.[13]

Scytonemin is characterized by a homodimeric core structure that is unique among natural products, and is composed of two cyclopenta[*b*]indol-2(1H)-one moieties linked at the 1 and 1' positions. The core is symmetrically appended by 4-hydroxybenzylidene in the 3-position of the fused tricyclic cyclopenta[*b*]indol-2(1H)-one system. The parent skeleton comprising the eight rings has been given the trivial name scytoneman by Proteau et al. who in 1993 were the first to elucidate the chemical structure of the scytonemin pigment.[14]

The ascertained role as a "natural UV-sunscreen" has sparked the interest of using scytonemin as a UV-absorbing agent (UVA)/sunscreen agent in consumer products or as a starting point for the development of new UVAs.[9, 15] This interest has certainly been nurtured by the reported stability of the pigment.

The focus of this report is on the intrinsic photostability of scytonemin, i.e. the photostability of scytonemin isolated from its natural environment. Thus, in this study, we report on the steady-state electronic spectroscopic properties of scytonemin and its photostability *in vitro*.

Experimental

Chemicals

Reagents and solvents were purchased from commercial vendors and used as received for chemical synthesis, unless otherwise stated. Dry tetrahydrofuran (THF) was obtained via distillation over sodium under a nitrogen atmosphere. 2-Methyl-THF was distilled to remove stabilizer prior to spectroscopic measurements. EtOH (95%) and ethyl acetate were used as received for the spectroscopic measurements.

Instrumentation and modes of analyses

Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm Merck silica gel plates (60–F_{254}) using a mercury vapour UV-lamp to visualize the analytes. Fisher silica gel (particle size 40 – 63 µm) was used for flash column chromatography.

Absorption spectra were recorded on a Cary 5000 or Cary 4000 spectrophotometer, while fluorescence measurementes were performed on a SPEX fluorolog 3 spectrofluorimeter (JY Horiba). Measurements were performed at room temperature using 1x1 cm quartz cuvettes for all experiments except the photolysis/HPLC experiments where 1 mm quartz cuvettes were used. Concentrations were typically set to give an optical density of approximately 0.1 which corresponds to a concentration of approximately 1.5 µM as estimated using the extinction coefficient of 112.6 L g^{-1} cm^{-1} for scytonemin in acetone.[10]

Photolysis experiments were also performed in a Solarlux™ Solar Simulation system from EYE Lighting International / Iwasaki Electric that generates the same spectral distribution as the sun. Samples were kept in quartz cuvettes set up at an angle so that the fronts of the cuvettes were directly exposed to the light. The setup was adjusted to expose samples to an intensity of 1000 W/m^2, which corresponds to 1 sun. The temperature under the lamp reached approximately 50 °C during the experiments. A more detailed description of the solar simulation system is available on the homepage (http://www.eyesolarlux.com/) of the EYE Lighting International / Iwasaki Electric of North America.

HPLC-MS analyses were performed using electrospray ionization on an Agilent 1100 HPLC-MS. The system included a vacuum degasser, a binary pump, an autoinjector, a column thermostat, a diode array detector, and a single quadrupole mass spectrometer. The electrospray interface was used with the following spray chamber settings: nebulizer pressure, 35 psig; capillary voltage, 3000 V; drying gas temperature, 350 °C; and drying gas flow rate, 12 L/min. For mass spectral analysis, the mass spectrometer was used in the scan mode detecting ions with m/z ranging from 100 to 1000. Mass spectral analysis was performed in positive ionization mode with fragmentor voltage of 70 V. A Hypersil-Keystone HyPurity C18 column (150 mm × 3 mm, 3 µm particles, Thermo Scientific) was used, and the column temperature was set to 30 °C. Mobile phase A consisted of 0.1% formic acid in Milli-Q water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. Aliquots of 5 µL were injected onto the column and eluted with a gradient flow of 0.30 mL/min. A linear gradient from 10 to 100% B in 20 min was followed by 10 min of isocratic elution at 100% B. The column was equilibrated with 10% B for 10 min between each run.

NMR spectra were recorded on samples dissolved in deuterated chloroform (Chloroform-d). ^{1}H-NMR spectra of compounds **1b**, **M1b** and **M1c** and ^{13}C-NMR spectra of compounds **M1b** and **M1c** were recorded on an Agilent 400 MHz (101 MHz for ^{13}C) spectrometer. ^{13}C-NMR spectrum for compound **1b** was recorded on an Oxford 800 MHz (201 MHz for ^{13}C) 4.2K magnet with Bruker Avance III HD and 5mm TXO cryoprobe. Residual non-deuterated chloroform was used as internal reference: ^{1}H, δ = 7.26 ppm; ^{13}C, δ = 77.0 ppm. The following abbreviations, or a combination thereof, were used to characterize the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Melting points (mp) were recorded on a Mettler FP 90/82 melting point apparatus and are uncorrected. IR spectra were recorded on a Bruker IFS 66v/S FT-IR spectrometer using KBr pellet sample preparation. HRMS analyses were performed on either a QSTAR XL or a Waters Micromass Q-Tof micro instrument, using positive electrospray ionization and a tandem quadropole time-of-flight mass analyzer (ESI+ qTOF).

Synthesis of di-*O*-methyl scytonemin 1b

To a mixture of diastereoisomers **2** (50 mg, 0.09 mmol) in dry THF (5.5 mL, 0.015 M) under air atmosphere was added neat DDQ (60 mg, 0.26 mmol). The solution was stirred for 1 min, diluted with EtOAc (40 mL) and washed with $NaHCO_3$ (4 × 10 mL) to remove DDQ-adducts. The organic phase was further washed with brine (10 mL) and a dark precipitate was observed. The layers were separated and the remaining precipitate was dissolved in acetone (30 mL) and EtOAc (30 mL). The resulting solution was washed with brine (15 mL) and combined with the previously obtained organic layer. The combined layers were dried over Na_2SO_4, filtered and concentrated onto SiO_2 (1 g). The silica-bound crude product was purified by column chromatography (ø = 4 cm, 30 g SiO_2, DCM/EtOAc 1:0 → 3:1) to furnish a mixture of two similar compounds, as judged by NMR. One compound gradually transformed into the other, indicating an isomerization process. The stable isomer **1b** was obtained as a dark solid (19 mg, 37%). An analytical sample was prepared by the following procedure: $CHCl_3$ (2 mL) was saturated with **1b** and transferred into a vial (ø = 1 cm). A layer of benzene (2 mL) was added on top and the resulting biphasic system was left for seven days. A gel was formed at the boundary layer and after pipetting off the solvent and drying the gel under reduced pressure, pure di-*O*-methyl scytonemin was obtained; R_f = 0.2 (DCM); mp > 300°C ($CHCl_3$/benzene); ν_{max}/cm^{-1} 1711 (CO); δ_H(400 MHz, Chloroform-d) 8.66 (AA' part of a AA'XX' system, 4H), 7.63 (dt, *J* = 7.7, 0.9 Hz, 2H), 7.49 (td, *J* = 7.7, 1.3 Hz, 2H), 7.45 (ddd, *J* = 7.5, 1.2, 0.6 Hz, 2H), 7.16 (td, *J* = 7.5, 1.0 Hz, 2H), 7.08 (XX' part of a AA'XX' system, 4H), 3.93 (s, 6H); δ_C(201 MHz, Chloroform-d) 194.0, 173.4, 163.4, 162.8, 158.5, 138.8, 135.5, 134.8, 129.2, 128.7, 127.2, 126.5, 124.8, 122.0, 119.1, 114.5, 55.5.; HRMS (ESI+ qTOF) calcd for $C_{38}H_{25}N_2O_4$ [M + 1] 573.1814, found. 573.1816.

Synthesis of monomer M1b

To reduced monomer **L1b** (16 mg, 0.05 mmol) in dry THF (1.1 mL, 0.05 M) at 0 °C under air atmosphere was added neat DDQ (13 mg, 0.06 mmol). The solution was stirred for 10 seconds, then diluted with EtOAc (15 mL) and washed with $NaHCO_3$ (5 × 5 mL) to remove DDQ-adducts. The organic layer was further washed with brine (5 mL), dried over Na_2SO_4, filtered and concentrated. The orange residue was purified by column chromatography (ø = 4 cm, 16 g SiO_2, DCM) to yield monomer **M1b** as a red crystalline solid (4 mg, 28%). R_f = 0.20 (hexane/DCM 1:3); mp decomposition ~ 150°C (concentrated from DCM); ν_{max}/cm^{-1} 1709 (CO); δ_H(400 MHz, Chloroform-d) 8.58 (AA' part of AA'XX' system, 2H), 7.71 – 7.68 (m, 1H), 7.64 (dt, *J* = 7.8, 0.8 Hz, 1H), 7.54 (s, 1H), 7.51 (td, *J* = 7.7, 1.3 Hz, 1H), 7.23 (td, *J* = 7.5, 1.0 Hz, 1H), 7.05 (XX' part of AA'XX' system, 2H), 6.87 (d, *J* = 0.6 Hz, 1H), 3.91 (d, *J* = 0.9 Hz, 3H); δ_C(101 MHz, Chloroform-d) 197.2, 174.7, 162.7, 162.5, 160.2, 137.3, 135.1, 134.1, 127.1, 127.1, 126.3, 125.9, 124.2, 121.9, 120.1, 114.3, 55.5; HRMS (ESI+ qTOF) calcd for $C_{19}H_{14}NO_2$ [M + 1] 288.1025, found 288.1014.

Synthesis of monomer M1c

To reduced monomer **L1c** (29 mg, 0.11 mmol) in dry THF (2.3 mL, 0.05 M) at 0 °C under air atmosphere was added neat DDQ (55 mg, 0.24 mmol). The solution was stirred for 40 seconds, then diluted with EtOAc (25 mL). The solution was washed with $NaHCO_3$ (3 × 10 mL) to remove DDQ-adducts. The organic layer was further washed with brine (10 mL), dried over Na_2SO_4, filtered and concentrated onto SiO_2 (1.5 g). The silica-bound crude product was purified by column chromatography (ø = 4 cm, 16 g SiO_2, hexane/DCM 1:1) to yield monomer **M1c** as an orange crystalline solid (19 mg, 62%). An analytical sample was prepared by the following recrystallization procedure: The compound was dissolved in minimal amount of boiling MeCN, cooled to room temperature and then cooled further to -20°C for 22h. The mother liquid was removed by pipette, leaving pure crystals of the monomer; R_f = 0.55 (hexane/DCM 1:1); mp decomposition ~ 170°C (MeCN); ν_{max}/cm^{-1} 2924 (CH), 1706 (CO); δ_H(400 MHz, Chloroform-d) 8.59 – 8.54 (m, 2H), 7.73 – 7.70 (m, 1H), 7.68 (dt, *J* = 7.8, 0.9 Hz, 1H), 7.59 (s, 1H), 7.57 – 7.47 (m, 4H), 7.26 (td, *J* = 7.6, 1.0 Hz, 1H), 6.94 (s, 1H); δ_C(101 MHz, Chloroform-d) 196.8, 174.0, 162.5, 160.6, 137.5, 134.3, 133.9, 132.8, 131.6, 128.8, 127.5, 126.6, 126.0, 124.1, 122.6, 122.2; HRMS (ESI+ qTOF) calcd for $C_{18}H_{12}NO$ [M + 1] 258.0919, found. 258.0905.

Computational details

Molecular geometry optimizations and energy calculations were carried out using the Gaussian03 suite of programs.[16] The ground-state geometries were optimized using density functional theory (DFT) with the B3LYP hybrid functional and the 6-31G(d) basis set. The vertical transition energies and oscillator strengths were calculated from the optimized ground state geometries using time-dependent density functional theory (TD-DFT) with the same hybrid functional and basis set as applied in the geometry optimizations. The calculated data correspond to isolated molecules in gas phase at 0K. Zero-point vibrational energies are not included in the calculations and no corrections are made.

Results

Chemical synthesis

Scytonemin, two analogues thereof and the corresponding monomers have been synthesized. Scytonemin and its unsubstituted analogue scytoneman were prepared according to our previously published routes to these compounds.[17] Di-*O*-methyl scytonemin was prepared from the diastereoisomeric mixture **2** (Scheme 1), synthesized as previously described by us.

The monomeric tricyclic ketones **L1a-c** were all key intermediates in the syntheses of the dimeric structures **1a-c**. Two of the monomeric tricyclic ketones, **L1b-c**, were oxidized by DDQ under anhydrous conditions to the fully conjugated compounds **M1b-c** (Scheme 1). These are the actual monomers of the dimeric pigment scytonemin and its analogues. The monomer of scytonemin itself, i.e. **M1a**, was inaccessible due to the chemical instability caused by the free phenolic group.

Steady-state spectroscopy

Steady-state absorption spectra were recorded for scytonemin (**1a**), di-*O*-methyl scytonemin (**1b**) and scytoneman (**1c**) in several solvents. Figure 1 shows that there is no major difference between the spectra recorded in different types of solvents, e.g. polar aprotic and polar protic solvents. It also shows that the spectra of scytonemin and di-*O*-methyl scytonemin approximately overlap, while the maximum absorption band of scytoneman is blue-shifted roughly 40 nm as compared to the other two compounds. The absorption spectra of all dimeric structures feature a characteristic broad, weak absorption band in the visible region, and a strong absorption band in the blue end of the visible region and the UVA region.

The absorption spectra of the monomers **M1b-c** all have similar characteristic absorption features as the dimeric structures, although the absorption maxima are all blue-shifted, see Figure 1.

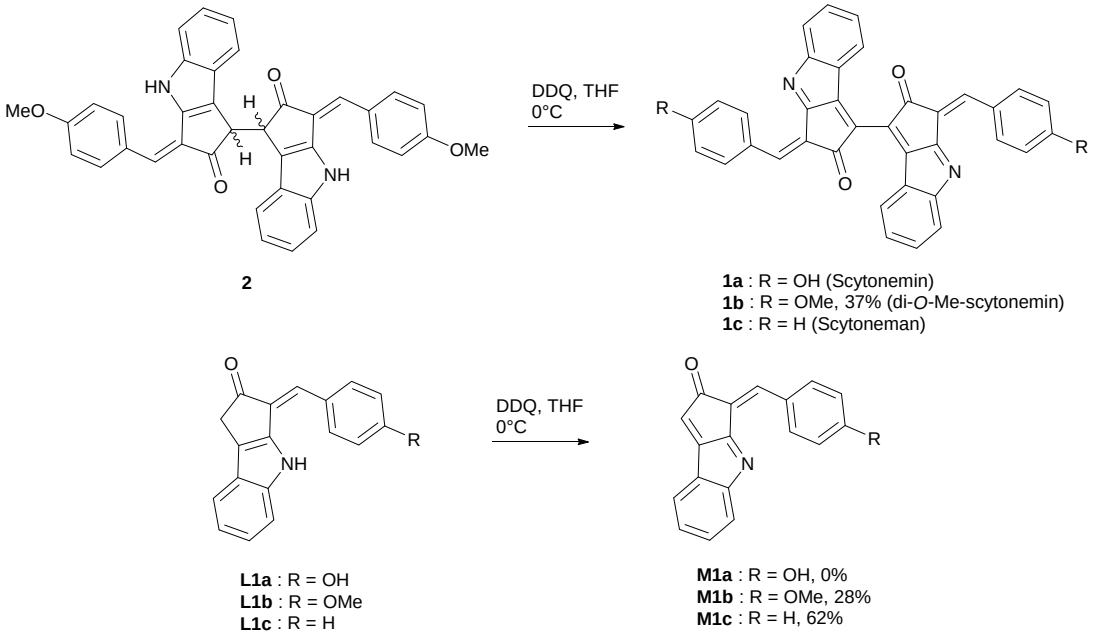
We were unable to detect fluorescence from any of the dimeric structures, and the monomeric structures proved to be too unstable to get any reliable results.

Photostability

Samples of scytonemin, its analogues and two UV-absorbing agents used in sunscreen products were irradiated by a Solar Simulation system, and the time-evolution of their absorption spectra were monitored. The characteristic absorption spectrum of scytonemin showed a dramatic change and decrease over the time-span of irradiation, while the spectra for the two UV absorbing agents, 4-*tert*-butyl-4'-methoxydibenzoylmethane (avobenzone) and bis-ethylhexyloxyphenol methoxyphenyl triazine (2,2′-[6-(4-methoxyphenyl)-1,3,5-triazine-2,4-diyl]bis 5-[(2-ethylhexyl)oxy]phenol, bemotrizinol), were virtually unaffected, see Figure 2. The time evolution of the spectra for scytoneman and di-*O*-methyl-scytonemin parallels that of scytonemin (no data shown).

The concentration of scytonemin as a function of time during irradiation by the Solar Simulation system in EtOAc and ethanol was measured by HPLC-MS, see Figure 2. In EtOAc, the concentration decreases seemingly linearly with exposure time.

The concentration is below the detection limit after 5 hours of irradiation. The decrease in scytonemin concentration is approximately 2.5 times slower in ethanol than in EtOAc.



Scheme 1 Structures of the investigated compounds. Oxidation of the reduced di-*O*-Me-scytonemin compound **2** into di-*O*-Me-scytonemin and oxidation of reduced monomers **L1b-c** into monomers **M1b-c**. Oxidation of **L1a** was unsuccessful.

The oxygen dependence was studied by comparing the time-evolution of the spectra for argon-purged samples as well as air-saturated samples being exposed to the Solar Simulation system, see Figure 3. In both 2-methyl-THF and EtOAc no significant effect of oxygen was observed; the spectral shape and the time evolution were the same in both the absence and the presence of air. Figure 3 also show that the stability of scytonemin in 2-methyl-THF is significantly lower as compared to EtOAc.

Discussion

Chemical synthesis

The syntheses of the scytonemin, scytoneman and di-*O*-methyl-scytonemin have been discussed thoroughly in a previous publication by us.[17] The corresponding monomers were obtained by DDQ-oxidation under anhydrous conditions of the precursors to the corresponding reduced dimeric structures. The starting materials for the monomers were therefore available via the routes developed for the syntheses of the dimeric structures. The oxidation required only a short reaction time for completion. Too long reaction times resulted in over-oxidation and excessive byproduct formation. The obtained fully conjugated structures decompose over the time of weeks/months on storage at ambient temperature. These compounds were for this reason freshly prepared just before spectroscopic measurements.

Steady-state spectroscopy

Our recorded electronic absorption spectra of scytonemin are in accordance with previously reported spectra.[3] The spectrum of the di-*O*-methyl analogue is almost identical to that of scytonemin, whereas that of the parent structure scytoneman is distinctly different. The strong absorption band of scytoneman in the UVA and the blue end of the visible regions has a different shape and shows more fine structure than that of the other two. The effect of the two phenolic oxygens on the electronic absorption spectra of scytonemin and the di-*O*-methyl analogue seems to be two-fold. First, the absorption bands are slightly red-shifted by the oxygen atoms. Second, the shape of the band in the UV-blue region is altered. Scytoneman has a blue-shifted main maximum compared to scytonemin, and a weaker but more pronounced shoulder on the low-energy side of the main peak in the UVA-blue region.

The absorption spectra of the two monomers **M1b** and **M1c** are blue-shifted compared to the spectra for the dimeric structures. Addition of an oxygen atom in the para position of the exocyclic phenyl group red shifts the entire spectrum, analogously to what was observed for the dimeric structures. However, the shape of the intense band in UVA-blue region is unaffected. Although weaker in the monomers, the characteristic broad, low-energy band observed for scytonemin and its analogues is still present and is therefore not a direct consequence of its dimeric structure. The low-energy bands of the monomers can, based on the results of the TD-DFT calculations, be assigned as single weakly allowed electronic HOMO-LUMO transitions. The same calculations also suggest that the low-energy bands for the dimeric structures are composed of two transitions each: a

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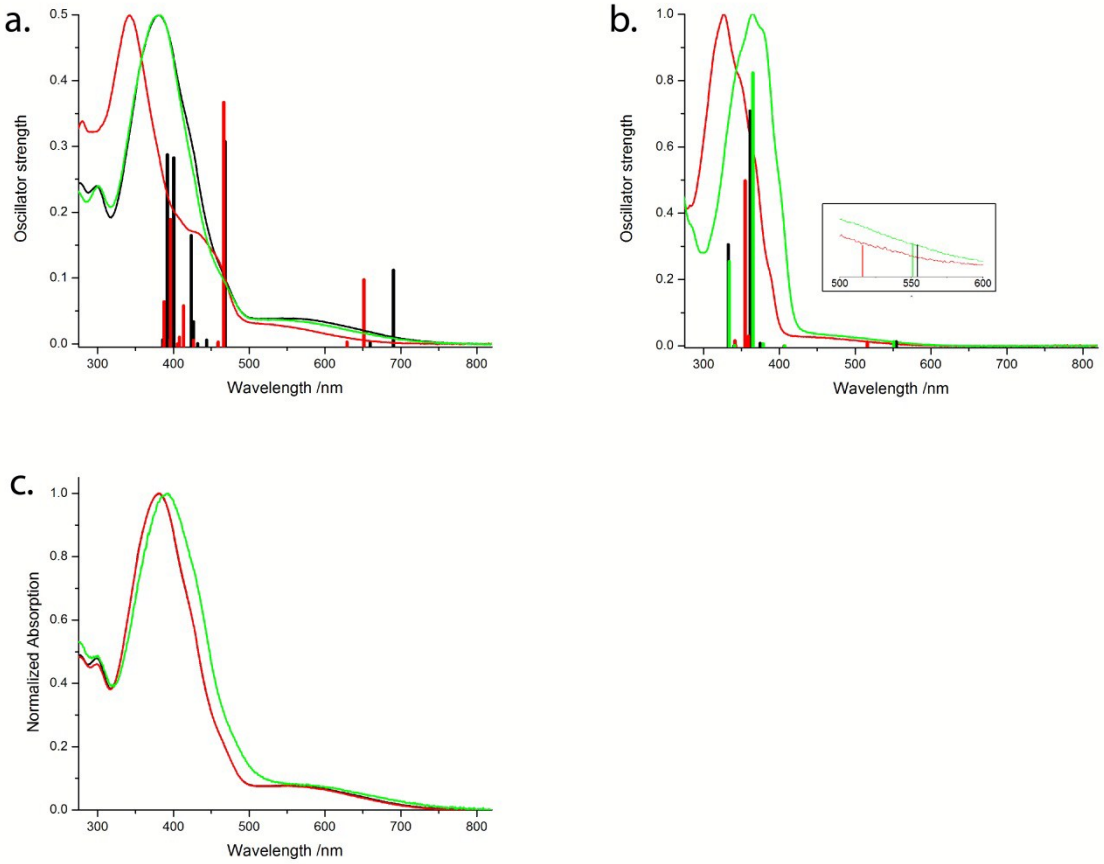

Fig. 1 a) The electronic absorptions spectrum measured in 2-methyl-THF for scytonemin (black, λ_{max} = 380nm), di-*O*-methyl-scytonemin (green, λ_{max} = 380nm) and scytoneman (red, λ_{max} = 342nm) together with calculated oscillator strengths for scytonemin (black) and scytoneman (red). b) Electronic absorptions spectra measured in 2-methyl-THF for the methoxy monomer **M1b** (green, λ_{max} = 365nm) and the scytoneman monomer **M1c** (red, λ_{max} = 326nm) together with calculated oscillator strengths for **M1a** (black), **M1b** (green) and **M1c** (red). c) Electronic absorption spectrum for scytonemin in 2-methyl-THF (black), EtOAc (red, λ_{max} = 380nm) and EtOH (green, λ_{max} = 390nm). All measured spectra are normalized at the respective absorption maximum.

HOMO-LUMO and a (HOMO-1)-LUMO transition. In effect, scytonemin behaves as a weakly coupled system of two identical chromophores, i.e. a composite-molecule.

Photostability

Scytonemin has been hypothesized to be essential for sustaining the observed long-term viability, 100 years or more, of cyanobacteria in the desiccated state.[12] Indeed, very old samples of this pigment have been collected.[18] It has even been proposed as an important biomarker for extinct life, both on Earth and on other planets such as Mars.[19] However, scytonemin is not completely inert. It is easily reduced to a compound with a characteristic bright-red colour, red-scytonemin, which in turn is readily reoxidized back to scytonemin, e. g. in air.[3] Further, although substantially more photostable than other essential biologically molecules such as chlorophyll, scytonemin has been shown to degrade linearly when exposed to light.[11]

The results from our stability tests of scytonemin *in vitro* show that irradiation of scytonemin in solution by a solar simulation system results in degradation over just a few hours. The concentration of scytonemin, as monitored by HPLC-MS, appears to decrease linearly with irradiation time. The time evolution of the absorption spectra recorded during the stability test follows a more complicated dynamics than indicated by the concentration measurements by HPLC-MS. After 5 hours, there is still absorption in the visible region showing that the initially formed degradation products have absorption spectra that overlap with the parent scytonemin. The formed degradation products in turn decompose further into species with absorption at shorter wavelengths. Only minute peaks, most likely corresponding to these transient intermediates, could be discerned in the HPLC chromatograms. However, none of these were accumulated in

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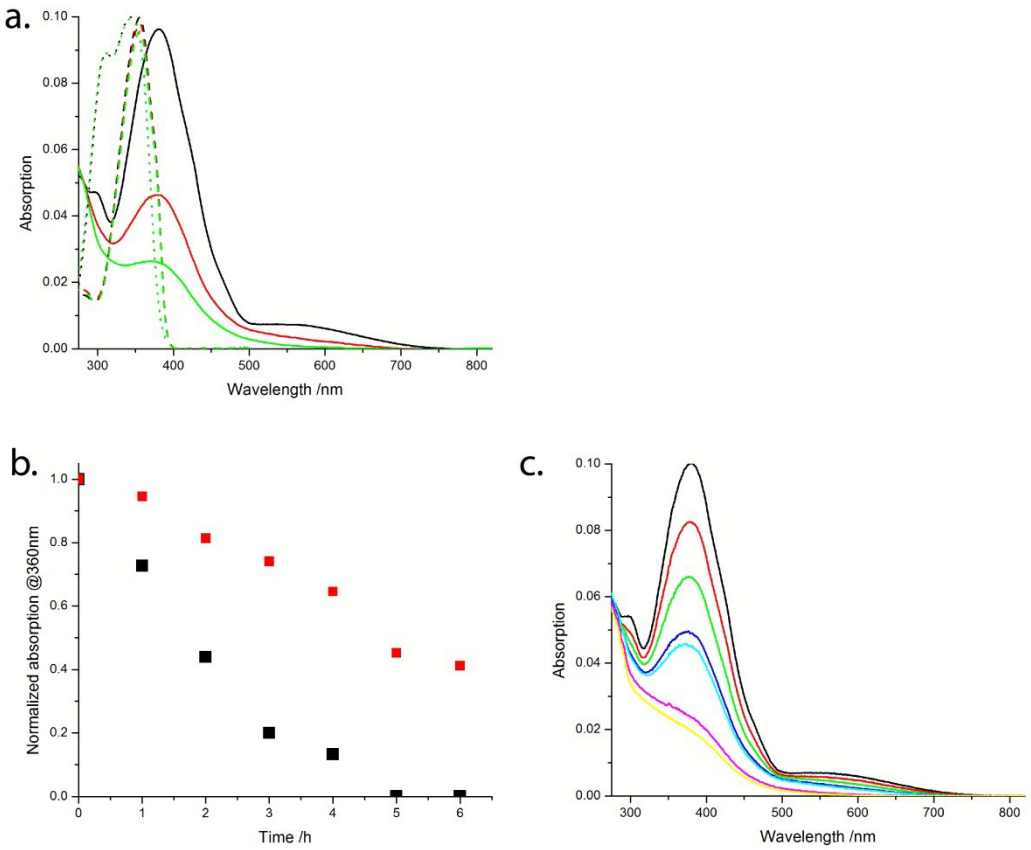

Fig. 2 a) Electronic absorption spectra of scytonemin (—), 4-*tert*-butyl-4'-methoxydibenzoylmethane (- - -) and bis-ethylhexyloxyphenol methoxyphenyl triazine (·····) recorded after irradiation by a solar simulation lamp for 0 h (black), 3 h (red), and 6 h (green). b) The concentration of scytonemin in EtOAc (black) and EtOH (red) as a function of time of irradiation, measured by HPLC-UV. The concentrations are normalized to the initial concentrations. c) Corresponding electronic absorption spectrum to the sample in EtOAc shown in b.

sufficiently high concentrations to be assigned specific mass-to-charge ratios with any certainty. Neither could any end-products be identified. This suggests that the degradation of scytonemin occurs in several steps where also the intermediates are unstable under the present conditions.

A clear solvent dependence was observed for the degradation. Complete degradation of scytonemin was achieved within 5 hours of irradiation in the polar solvent EtOAc. In the protic polar solvent ethanol the degradation was approximately 2.5 times slower. This may suggest that hydrogen bonding increases the photostability of scytonemin. The related natural product nostodione A has been proposed to undergo cis/trans-isomerization via its quinoidal enol tautomer, formed via proton transfer.[20] Proton transfer reactions are important processes in providing efficient excited-state relaxation pathways and to render compounds photostable, e.g. 4-*tert*-butyl-4'-methoxydibenzoylmethane and bis-ethylhexyloxyphenol methoxyphenyl triazine.[21, 22] Formation of the quinoidal enol tautomer of both nostodione A and scytonemin requires that the carbonyl oxygen is protonated and that the phenolic proton is removed. The latter step is not possible in either di-*O*-methyl-scytonemin or in scytoneman. The former cannot form a neutral quinoide structure because a methyl group replaces the phenolic hydrogen. The latter, in turn, cannot form a quinoid structure because of the lack of a para substituent. However, neither the substitution of the phenolic hydrogen for a methyl group, nor the substitution of the phenolic hydroxyl group by hydrogen seems to be inimical to the photostability.

The photostability of scytonemin *in vitro* can be contrasted to that of the two sunscreen agents 4-*tert*-butyl-4'-methoxydibenzoylmethane and bis-ethylhexyloxyphenol methoxyphenyl triazine, see Figure 2. Under identical experimental conditions and on the same time-scale of complete degradation of scytonemin, both dibenzoylmethane and diphenol triazine were both virtually unaffected.

As scytonemin has been shown to act as an antioxidant, it is

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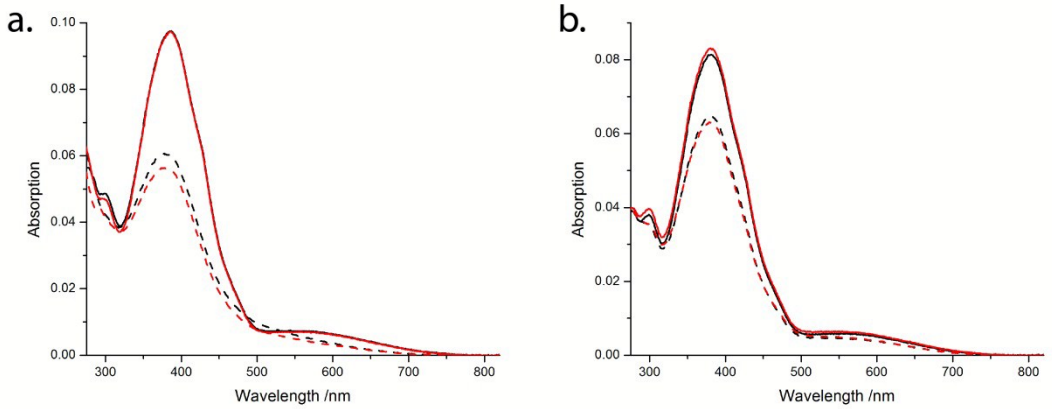

Fig. 3 a) Electronic absorption spectrum of scytonemin in Ar-bubbled 2-methyl-THF (black) and 2-methyl-THF (red) before (—) and after 1h under the solar simulation lamp (- - -). Spectra were corrected for differences in initial concentration due to Ar-bubbling and evaporation of solvent. b) Electronic absorption spectrum of scytonemin in Ar-bubbled EtOAc (black) and EtOAc (red) before (—) and after 3h under the solar simulation lamp (- - -).

expected that the absence or presence of oxygen would affect degradation rates. The difference could be expected to be especially pronounced in ethers such as 2-methyl-THF which are known to rapidly form hydroperoxides in the presence of oxygen and light.[23] However, our results show no significant effect of oxygen on the degradation rate. The lack of effect of oxygen can be a result of the slow radical scavenging activity reported for scytonemin[12] in combination with the fast photodegradation under the experimental conditions used in this study.

To the best of our knowledge there is only one study on the stability of scytonemin *in vivo*.[11] The content of scytonemin in the sheaths of desiccated *Nostoc punctiforme* was demonstrated to decrease slowly when continuously irradiated by light. The content was reduced to 93% of the original value by continuous irradiation by 55 W m^{-2} of visible light, and to 84% by continuous irradiation by 5 W m^{-2} of UVA radiation for 50 days. The energy used was approximately 20 times lower compared to the present study as well as to what is approximately found naturally outside on a clear day. Nevertheless, extrapolation of their results suggests that scytonemin is more stable *in vivo* than in the *in vitro* systems studied here. However, to verify if scytonemin can be considered "stable" under natural conditions, further studies on the biological systems under simulated sunlight are needed.

Scytonemin is located in the extracellular viscous polysaccharide sheaths of cyanobacteria.[3] Apart from the matrix bulk of glycan polysaccharides,[24] there are also proteins and metal ions present.[25] While the hydrophilic glycans offer abundant possibilities for hydrogen bonding, the proteins and metal ions can offer more unique interactions with scytonemin. For instance, the water stress protein WspA, which is expressed at high levels in the extracellular matrix of *Nostoc commune*, has been demonstrated to interact in a non-covalent manner with scytonemin. Furthermore, the synthesis of WspA is promoted by exposure to desiccation and UV light, which is also true for scytonemin.[25] In computational studies, scytonemin has been proposed to chelate with iron, forming a "sandwich" type of complex.[26] Taken together, the extracellular matrix offers a unique environment for scytonemin. Consequently, the *in vivo* photostability of scytonemin may be highly dependent on interactions with components of the extracellular matrix, explicit interactions that are not available upon dissolution in organic solvents.

Conclusions

Surprisingly, despite the general consideration that scytonemin is a stable molecule, we found that it easily degrades under simulated sunlight in a matters of hours. The degradation was not dependent on the presence of air, while highly dependent on the solvent, with ethanol offering the most stabilizing environment. Furthermore, neither removing the phenolic hydroxyl group of scytonemin, nor substituting the hydrogen for a methyl, affected the photostability in organic solvents.

Although this study was performed using organic solvents and thereby creating an artificial environment, the results clearly suggest that the promise of scytonemin as an ideal UV-protection agent can be questioned. However, the environment surrounding scytonemin in its native state may play a key role by significantly altering the photostability of scytonemin. Further studies where scytonemin in its natural environment is exposed to light, reflecting the conditions found in nature, are indeed needed to properly assess the photostability of scytonemin observed in its natural context.

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Notes and references

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† Electronic Supplementary Information (ESI) available: NMR specta for the synthesized compounds. See DOI: 10.1039/b000000x/

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Observation of unexpectedly rapid photodegradation of the cyanobacterial UV-screener scytonemin *in vitro*.