Photochemical & Photobiological Sciences

PAPER

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Cite this: *Photochem. Photobiol. Sci.*, 2014, **13**, 407

Photochemical studies of a fluorescent chlorophyll catabolite – source of bright blue fluorescence in plant tissue and efficient sensitizer of singlet oxygen†

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Received 12th November 2013,
Accepted 19th December 2013

DOI: 10.1039/c3pp50392e

www.rsc.org/pps

Fluorescent chlorophyll catabolites (FCCs) are fleeting intermediates of chlorophyll breakdown, which is seen as an enzyme controlled detoxification process of the chlorophylls in plants. However, some plants accumulate large amounts of persistent FCCs, such as in senescent leaves and in peels of yellow bananas. The photophysical properties of such a persistent FCC (Me-*s*FCC) were investigated in detail. FCCs absorb in the near UV spectral region and show blue fluorescence (max at 437 nm). The Me-*s*FCC fluorescence had a quantum yield of 0.21 (lifetime 1.6 ns). Photoexcited Me-*s*FCC intersystem crosses into the triplet state (quantum yield 0.6) and generates efficiently singlet oxygen (quantum yield 0.59). The efficient generation of singlet oxygen makes fluorescent chlorophyll catabolites phototoxic, but might also be useful as a (stress) signal and for defense of the plant tissue against infection by pathogens.

Introduction

Breakdown of chlorophyll is a colorful process accompanying leaf senescence and fruit ripening.[1–4] Basic features of this metabolically controlled degradation of the green plant pigment appear to be largely established.[4,5] Chlorophylls (*a* and *b*) are broken down in higher plants to colorless linear tetrapyrroles, most notably to the nonfluorescent chlorophyll catabolites (NCCs).[6,7] A key intermediate, still formed in the chloroplast in this process, is the 'primary' fluorescent catabolite (*p*FCC, Scheme 1).[8] FCCs, such as *p*FCC, exhibit a characteristic blue luminescence.[8,9] However, in most plants, FCCs exist only fleetingly, since they are imported into the vacuole,[1,3] where they undergo acid-catalyzed isomerization to NCCs.[10] The rapid degradation of the photoactive, colored chlorophylls to the colorless and basically photo-inactive NCCs shows the molecular features of a metabolic detoxification program.[11] Thus, the typical rapid disappearance of most FCCs[10] has also been suggested to serve the purpose of eliminating such still photoactive chlorophyll-derived pigments.[4]

Strikingly, in yellow banana peels FCCs accumulate, so that such bananas show blue fluorescence under UV illumination, observable by eye.[9,12] A characteristic of the FCCs in banana peels is their propionic acid ester function. Esterification of FCCs inhibits their isomerization to NCCs and makes such 'hypermodified' FCCs (*hm*FCCs) persistent.[9,13] Thus, the intriguing accumulation of *hm*FCCs in banana peels and in a variety of senescent leaves,[14–16] could be explained by their chemical persistence.

On the other hand, NCCs and FCCs are candidates for biological roles,[5,9,16–18] and the physiological impact of the puzzling accumulation of FCCs remains to be investigated.[5,9] Relevant clues in this respect are to be expected from their largely unexplored photochemical properties. Thus, as reported here, we prepared Me-*s*FCC (Scheme 1), the artificial persistent methyl ester analog of natural *hm*FCCs from bananas,[17] and studied its photochemistry.

Results and discussion

Me-*s*FCC, the methyl ester of the 'secondary' FCC (*s*FCC) from banana peels is a persistent *hm*FCC, identified in extracts from banana peels (as *Mc*-FCC-71).[17] In these extracts Me-*s*FCC was formed from spontaneous, slow trans-esterification of natural *hm*FCCs with the solvent methanol.[17] In the present work, Me-*s*FCC was prepared by deliberate methanolysis of two anomeric *hm*FCCs (named *Ma*-FCC-63 and *Ma*-FCC-64), which are abundant in senescent leaves of the banana plant (*Musa acuminata*).[16]

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† Dedicated to the memory of Professor Nicholas J. Turro.

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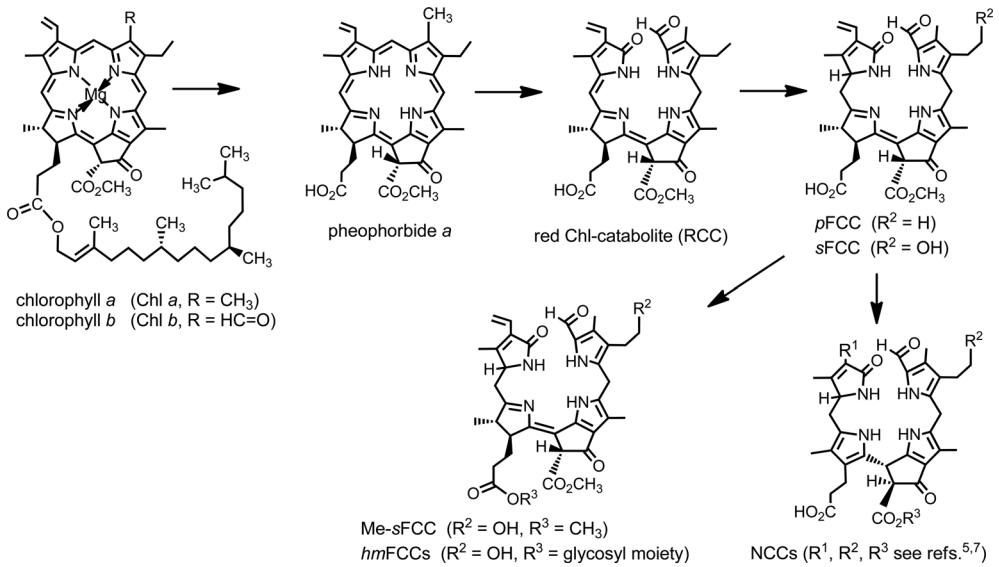

Scheme 1 Outline of chlorophyll breakdown in higher plants with structural formulas and names of key compounds (see ref. 5, 7 and 18 for more details).

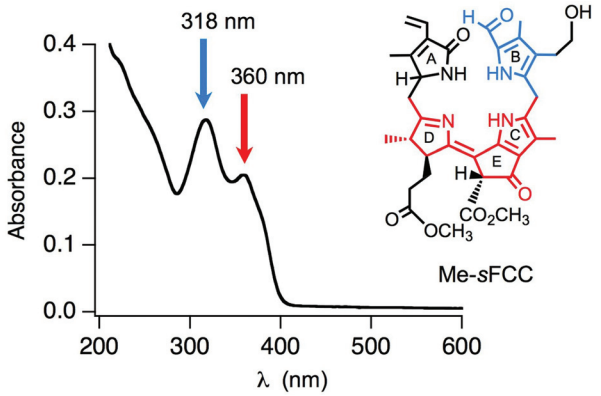

Fig. 1 Absorption spectrum of Me-*s*FCC in EtOH at room temperature. The band at 360 nm was assigned to the chromophore of a conjugated system (marked in red) extending over the two rings (C and D) in the 'southern' moiety.[8,19] The absorption band at 318 nm was assigned to the α-formyl-pyrrole chromophore (ring B, marked in blue).[6,8]

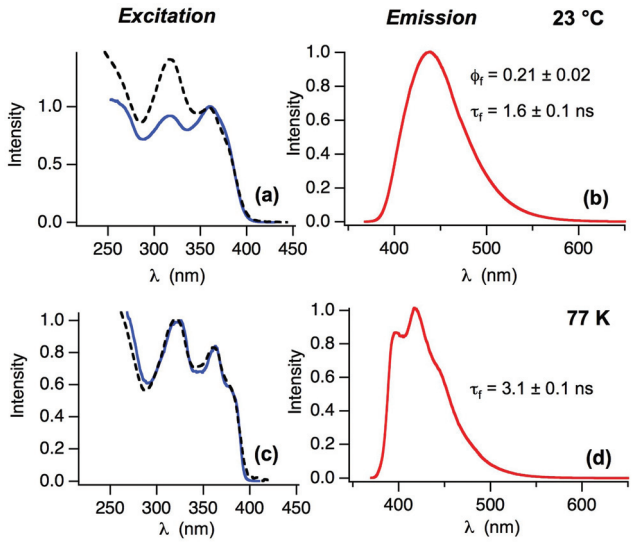

Fig. 2 Fluorescence excitation (blue; a, c) and emission (red; b, d) spectra of Me-*s*FFC in EtOH at room temperature (a, b) and EtOH glass at 77 K (c, d). λ_{ex} = 360 nm. The absorption spectra of **1** at room temperature (a) and at 77 K (c) are shown as black dashed lines.

The absorption spectrum of Me-*s*FCC in ethanol (EtOH) solution is shown in Fig. 1. In contrast to chlorophyll, Me-*s*FCC does not absorb light in the visible spectral region, since the conjugated chromophore of the chlorophylls is disrupted at three key positions. Two main chromophores of Me-*s*FCC are highlighted, which both absorb in the UV spectral region (see Fig. 1).

Photo-excitation of an ethanolic solution of Me-*s*FCC at 360 nm led to blue fluorescence with a maximum at 437 nm (Fig. 2b, solid line). The excitation spectrum for this fluorescence (Fig. 2a) matched largely (but not completely) the absorption spectrum (see Fig. 1 and 2b, dashed line). The absorbance at 318 nm is due to the non-luminescent α-formyl-pyrrole moiety (ring B): photonic excitation of ring B of Me-*s*FCC appears to contribute significantly less at room temperature, than at 77 K, to the observed fluorescence emission of Me-*s*FCC, indicating incomplete transfer of electronic excitation to the lumophore of Me-*s*FCC. The fluorescence excitation and absorption spectra of Me-*s*FCC in frozen matrix match well (Fig. 2c) which suggests efficient energy transfer from the α-formyl-pyrrole ring to the main chromophore at low temperature. In frozen solution, at 77 K, the vibrational bands of the fluorescence of Me-*s*FCC became observable (Fig. 2c and d). The singlet excited state energy of Me-*s*FCC was estimated as amounting to 308 kJ mol^{-1}, from the interception of the low

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temperature fluorescence and excitation spectra. Using 9,10-diphenylanthracene as standard,[20] the quantum yield of room temperature fluorescence was determined (Φ_f = 0.21). The remaining fraction of the singlet excited state (0.79) would undergo radiation-less deactivation to the ground state, photochemical reactions, such as double-bond *cis–trans* isomerization (not documented, so far, for an FCC), or intersystem cross into the triplet state.

When frozen in a solid matrix (77 K) radiation-less deactivation and *cis–trans* isomerization would be inhibited by the rigid matrix and become insignificant. Therefore, deactivation of the excited singlet state of Me-*s*FCC at 77 K would be dominated by fluorescence and intersystem crossing. Accurate fluorescence quantum yields are technically difficult to measure at 77 K. However, we were able to measure changes in fluorescence lifetimes from room temperature to 77 K which could be used to estimate the fractions of decay pathways of the singlet excited state. At room temperature (and in both, EtOH and perdeuterated ethanol) the fluorescence decayed mono-exponentially with a lifetime of τ_f = 1.6 ns whereas at 77 K the lifetime increased to 3.1 ns. From this increase of the lifetime, the fluorescence quantum yield can be estimated to be about 0.4 at 77 K. Thus, the quantum yield of intersystem crossing from the singlet excited state to the triplet state is estimated to be as high as 0.6.

We note that the fluorescence lifetime of 1.6 ns is too short to be efficiently quenched by molecular oxygen in air saturated solutions. However, triplet states are generally long-lived and are efficiently quenched by atmospheric oxygen, providing an effective path for the generation of $^1O_2$.[21]

The persistent Me-*s*FCC turned out to be a very effective $^1O_2$ sensitizer. At room temperature, the characteristic emission of $^1O_2$ was directly observed at 1270 nm (Fig. 3). Excitation of Me-*s*FCC in oxygen saturated perdeuterated ethanol solution produced the emission spectrum shown in Fig. 3 (left). There is only negligible emission when oxygen is removed from the solution by purging with argon. Based on these results we conclude that a long-lived triplet of Me-*s*FCC is produced by intersystem crossing at room temperature, which then sensitizes the formation of a significant amount of $^1O_2$. To quantify the amount of $^1O_2$ generated, the quantum yield was determined. With pulsed laser excitation at 355 nm and using phenalone (Φ_{ref} = 0.98) as standard[22] a $^1O_2$ quantum yield of 0.59 was obtained for Me-*s*FCC from the phosphorescence intensity monitored at 1270 nm at the end of the laser pulse. Thus, the quantum yields of $^1O_2$ formation (Φ_{1O2} = 0.59) and of intersystem crossing from the singlet excited state to the triplet state (Φ_{isc} = 0.6) are estimated to match quantitatively.

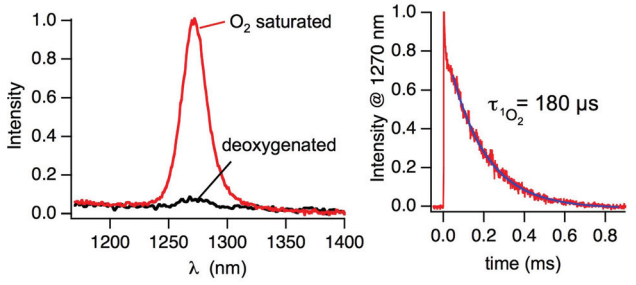

Fig. 3 Left: Phosphorescence of $^1O_2$ generated by photo-excitation of Me-*s*FCC in oxygen saturated (red) and deoxygenated (black) perdeuterated ethanol solution under steady-state excitation at 360 nm. Right: decay trace of $^1O_2$ monitored at 1270 nm after pulsed laser excitation at 355 nm.

As reported here, the blue fluorescent catabolite Me-*s*FCC exhibits a high quantum yield of fluorescence, and is an efficient sensitizer of the formation of $^1O_2$, as well. The first finding justifies, indeed, the qualification of Me-*s*FCC as a 'fluorescent' chlorophyll catabolite (FCC).[8,17] The remarkable bright 'blue glow' of yellow bananas was shown to have a natural endogenous basis, and to be due, largely, to the strongly blue fluorescent *hm*FCCs.[9] This striking visual effect was suggested to be relevant as optical feature of the fruit, visible in the near UV-range, that may help to specifically attract certain frugivors.[9,12]

As also shown here, Me-*s*FCC exhibits a remarkable capacity for photosensitization of the formation of $^1O_2$ in oxygenated solution: $^1O_2$ is estimated to be formed with nearly 100% quantum yield from the triplet excited state of Me-*s*FCC. This chemical feature raises several questions in the context of biological issues of chlorophyll breakdown. The "cell poison" $^1O_2$ leads to degradation of vital constituents in plant cells, and induces stress.[23–25] As photosensitizers of the formation of $^1O_2$, natural FCCs would be phototoxic, and have thus harmful effects for the vitality of a plant cell. FCCs are indeed generally eliminated rapidly in senescent leaves by isomerization to NCCs, which is seen as the 'last' stage of the important 'detoxification' path of chlorophyll breakdown.[1,3] Along these lines, the accumulation in defective mutants of *Arabidopsis thaliana* of red chlorophyll catabolite (RCC),[26] the direct natural precursor of *p*FCC,[5,8] was associated with the presumed related phototoxic properties of RCC.[27] Clearly, in contrast to RCC and the known phototoxic green tetrapyrroles, such as chlorophyll *a* and pheophorbide *a*,[28] which generate singlet oxygen with quantum yields of Φ_{1O2} = 0.24 and Φ_{1O2} = 0.59, respectively,[29,30] the colorless FCCs absorb only a minor fraction of solar radiation.

In striking opposition, apparently, to the postulated basic role of chlorophyll breakdown in the 'detoxification' of the green plant pigments,[1,3,28] large amounts of persistent *hm*FCCs are found *e.g.* in banana fruit[9] and in some senescent leaves (such as of bananas[14,16] and of the Peace Lily, a tropical evergreen[15]). Efficient formation of $^1O_2$ is to be expected with near UV-light as an important consequence of *hm*FCC-accumulation in living plant cells. This aspect raises questions as to the intracellular location of *hm*FCCs and their eventual binding and possible protection by macromolecules. Indeed, while *hm*FCCs are presumably biosynthesized in the cytosol of the aging plant cells, their intracellular transport and further location still are elusive.[5] Interestingly, the aggressive chemical $^1O_2$ is not only a general "cell poison" in the plant cell. $^1O_2$ is also useful for defense against infection by pathogens,[31,32] and

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it is also a noted diffusible, yet only fleetingly existent molecular signal in plants.[23,24,33] Thus, the discovery of the efficient FCC-sensitized generation of $^1O_2$ in oxygenated solution induces basic questions on the fate and biological roles of colorless chlorophyll catabolites, and invites studies of *in vivo* effects of FCCs in plant tissue.

Experimental

Spectroscopy and measurements

UV/vis-spectra: Agilent 8453 or Hitachi U 3000 spectrometers. CD-spectra JASCO J715 spectrometer. Nuclear magnetic resonance (^{1}H-NMR) spectra: Bruker 300 spectrometer at 300 K. For absorbance measurements in frozen matrix at 77 K, a liquid-N_2 cooled Oxford cryostat was used. Steady-state luminescence spectra were recorded on a Fluorolog-3 fluorometer (HORIBA Jobin Yvon). Fluorescence lifetimes were measured by time correlated single photon counting on an OB920 spectrometer (Edinburgh Analytical Instruments). All absorbance and fluorescence measurements were performed in 1 × 1 cm quartz cells. Singlet oxygen phosphorescence measurements were performed on a modified Fluorolog-2 spectrometer (HORIBA Jobin Yvon) in conjunction with a NIR sensitive photomultiplier tube (H9170-45, Hamamatsu).[34] A 450 W Xe was use for steady-state excitation to record singlet oxygen phosphorescence spectra and a Spectra Physics GCR-150-30 Nd:YAG laser (355 nm, *ca.* 5 mJ per pulse, 5 ns) was used for pulsed excitation to collect phosphorescence decay kinetics at 1270 nm.

Materials

Me-*s*FCC was obtained from partial synthesis. All other materials were purchased from commercial sources and used as received.

Preparation of Me-*s*FCC

A lyophilized sample of the epimeric *Ma*-FCC-63 and *Ma*-FCC-64 (obtained in roughly equal amounts by HPLC-purification of a banana leaf extract) was dissolved in 5 mL of MeOH (HPLC grade, stored over 4 Å molecular sieves), then left to stir under protection from light at room temperature. The light yellow solution turned to orange color overnight, and accumulated two non-polar FCCs according to HPLC analysis (using an RP-18 column). After a reaction time of 15.5 h, the mixture was frozen using liquid nitrogen and dried by lyophilization under high vacuum. The raw reaction mixture was purified by HPLC (solvent composition: solvent A = 20 mM $NEt_3{\cdot}H_3PO_4$ (pH 7.0); solvent B = MeOH; A/B = 50/50 from 0 to 15 min; from 15 to 45 min: constant gradient from A/B = 50/50 to 40/60; followed by washing steps). Two major non-polar fractions with retention times (t_R) of 40.1 and 46.4 min were isolated by semi-preparative HPLC. The two FCC fractions were isolated and identified as isomeric FCC methyl esters by mass spectrometry and NMR-spectroscopy. Me-*s*FCC, the less polar fraction (t_R = 46.4 min), was identified as *Mc*-FCC-61 from extract of banana peels (by UV/Vis-, CD-, ^{1}H-NMR- and mass-spectra),[17] and was used for the photochemical study reported here.

Acknowledgements

The authors at Columbia University thank the National Science Foundation (NSF grant: CHE 1025508), those in Innsbruck the Austrian Science Foundation (FWF-projects P-19596 and I-563) for generous support of this research.

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