

Novel Raman signature evidences an unknown cyanobacterial pigment

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ABSTRACT

From the first botanical characterization of cyanobacteria about two centuries ago to contemporary studies, only two categories of extracellular UV-screening pigments have been considered: scytonemin which is very well described and gloeocapsin, which remains enigmatic.

In this study, we use Raman microspectroscopy to compare the extracellular EPS-associated pigment produced by the strain *Chamaesiphon polonicus* SAG 32.87 with gloeocapsins, scytonemins and other natural and synthetic pigments. Chemometrics analyses of Raman data reveal the existence of a third and new category of cyanobacterial extracellular pigment.

Keywords

Microbial pigments, cyanobacteria, exopolymeric substance (EPS), Raman microspectroscopy

1. Introduction

From the emergence of their oxygenic photosynthetic ancestor – probably during the early Precambrian [1] – cyanobacteria colonized a wide range of habitats that include rock surfaces, intertidal zones, open waters, equatorial deserts and polar ecosystems among others [2, 3]. Due to the absence of a protective ozone layer in the early atmosphere, they may have developed different strategies to survive lethal UV radiations [4, 5]. One of these strategies consists in the biosynthesis of molecules that contain UV-absorbing function(s) and that are stored in the cell or exported through the

cell wall and condensed in the extracellular polymeric substance (EPS) envelope.

Scytonemin and gloeocapsin are the only cyanobacterial extracellular (UV-screening) pigments reported so far. Both molecules represent promising biosignature candidates for the search of early traces of cyanobacteria, if they cannot be formed abiotically and are unique to this clade [5, 6]. Indeed, their preservation in fossil cell-like or sheath-like shape or other morphological traces of life in a suitable environmental context would permit to accurately identify microfossils even when they have simple morphologies. Indeed, the evidence of their occurrence in the fossil record when associated with cell-like or sheath-like shape or other morphological traces of life and preserved in a suitable environmental context permit to accurately identify microfossils even when they have simple morphologies [5, 7].

Across the cyanobacterial phylogeny, the biofilm-forming genus *Chamaesiphon* (Gomontiellales) [8] was first described by Alexander Braun in 1864 [9]. It is characterized by a thin sheath (also called pseudovagina) that can be slightly lamellate and colorless or yellowish or brownish, in which a mother cell divides into smaller cells called exocytes that are expelled by the sheath apex [10]. Colonies of the *Chamaesiphon* genus are abundant in mountain rivers where they form conspicuous colorful biofilms on rocky surfaces, but can also be found as epiphytes on algae, and as biofilms on rocky surfaces from rainforest streams [11, 12], from the Antarctic continent [13, 14], to the arctic biosphere [15]. Except for one strain (*C. incrustans* N°1036), 16S rRNA sequences phylogeny analysis suggests that all strains of the genus had a common ancestor [16]. The production of UV-screening pigment by *Chamaesiphon* spp. was investigated by UV-HPLC in three morphospecies sampled in the Alps from two shaded and exposed sites with different geochemistries and altitudes [17]. Absorption spectra analyses suggested the presence of scytonemin in *C. starmachii* and *C. geilteri* whereas the identification of the pigment produced by *C. polonicus* was more enigmatic and somehow seemed more related to a carotenoid [17]. More recently, Velichko and colleagues [18]

characterized a new strain isolated from a dark crimson (brownish-red) forest spring biofilm. While characterizing their new isolates, the authors highlighted a complementary chromatic adaptation under low light leading to the dark crimson phenotype observed in that environment and which is mostly related to the cell phycobilisome composition [18].

Raman spectroscopy is a non-destructive technique that allows the characterization of molecules based on their vibrational behavior under monochromatic excitation [19]. High resolution Raman spectroscopy performed at the micron scale allows to differentiate microbial pigments at a cellular level [5, 20, 21, 22, 23, 24] and to characterize their cellular distribution. Recently, while investigating microbial epilithic and endolithic communities from polish gypsum, Edwards and colleagues [25] were able to isolate the Raman signal of scytonin for the first time, enabling the extension of scytonemin family signatures, showing that Raman spectroscopy is a useful method to study the still poorly known molecular diversity of cyanobacterial UV-screening pigments.

In this study, we used a Raman microspectroscopy approach to investigate the Raman signature of the extracellular pigment produced by *C. polonicus* SAG 32.87. Its comparison with the Raman signature of cyanobacterial, microbial and artificially produced pigments strongly suggests the existence of an unknown family of cyanobacterial extracellular pigments, strengthening the importance for further exploration of the diverse molecular strategies developed by cyanobacteria in photoprotection. In addition to the interest for fundamental and applied microbiology, such pigments might also provide new and potentially unique taxonomic signatures useful in paleobiology and astrobiology.

Results

Morphological characterization of *Chamaesiphon polonicus* SAG 32.87

The cyanobacterial strain SAG 32.87 morphology corresponds to the description of *Chamaesiphon polonicus* [10]. Cells are spherical, oblong and sometimes appear with a pear or skittle-shape (Fig. 1c, f). They are solitary,

pale blue-green and may contain large granules (Fig. 1f). Cells may be surrounded by a mucilaginous concentric, firm, pale orange to brown-colored multilayered envelope called pseudovagina or EPS sheath (Fig. 1). Exocytes released by the mother cell are observed (Fig. 1c). The orange-brownish pigment is never observed inside the cells, but in the EPS sheath.

Characterization of extracellular pigment using both single point and Raman mapping

To characterize the Raman signature of the enigmatic pigment produced by *Chamaesiphon polonicus* SAG 32.87, we first performed single point Raman microspectroscopy on *in vivo* cells with EPS sheath and EDTA treated cell-free EPS sheath using 514.5 nm light laser (Fig. 2, Supplementary Fig. S1). Fifteen Raman bands were observed and assigned based on literature (Table 1). Bands with strongest intensities were observed at 1740 cm^{-1} assigned to C=O stretching vibration that can be found for cholesterol ester or triglycerides, at 1636 cm^{-1} potentially assigned to C-CH, CNH bending or C=C stretching vibration, and at 644-648 cm^{-1} potentially assigned to a ring deformation. Two of the strongest and one medium strong band were specific to the main skeletal features of carotenoids ν_1, ν_2 and ν_3 , respectively observed at 1519 cm^{-1} assigned to C=C stretching, 1155 cm^{-1} C-C stretching and 1004 cm^{-1} C-CH bending. Four bands with medium strong intensities were potentially interpreted as a C-H bending or indole ring vibration at 1490 cm^{-1} , as quadrant C-N-C at 1407 cm^{-1} , C-C ring or indole ring at 1330 cm^{-1} , as C-H bending at 970 cm^{-1} . Additional less intense bands were observed at 1550 cm^{-1} that can be assigned to potential C-CH aromatic ring stretch, and at 1435 cm^{-1} and 1225 cm^{-1} , 1195 cm^{-1} , which vibrations may be related to C-N bonds.

Spatial distribution characterization of the Raman spectral signature on the sample that contained cells with their EPS sheath show its co-localization with the EPS sheath (Fig. 3, Supplementary Fig. S2). Band intensities at 648 cm^{-1} , 970 cm^{-1} , 1330 cm^{-1} , 1407 cm^{-1} , 1636 cm^{-1} , 1740 cm^{-1} related to the pigment coincides with the pigment distribution in the EPS sheath, but also on an exocyte (Fig. 3., a, c, h). In contrast, mapping of the band intensities

related to carotenoids at 1004, 1155, and 1519 cm^{-1} display intracellular content of elongated cells and an exocyte (Fig. 3., e, f, i).

Comparison of the Raman signature of the enigmatic pigment with known natural and synthetic pigments signatures using multivariate statistics

Raman signatures of twelve natural and two synthetic pigments that are or can be produced in the environment by microorganisms were investigated using a green laser (514 nm for coumarin, gloeocapsin, pyocyanin, quercetin, scytonemin and violacein), an orange laser (633 nm for anthraquinone) or a red laser (785 nm for 2-hydromethyl anthraquinone, emodin, emodin-8-glucoside, methyl-amino-anthraquinone, and parietin) when the Raman fluorescence background was too intense to observe actual Raman bands (Fig. 4.). Non-metric multidimensional scaling (NMDS) and hierarchical cluster analyses were performed to classify pigments based on their spectral similarities (Fig. 5., a). The NMDS shows that *C. polonicus* SAG 32.87 pigment signature does not cluster with any other pigments. Generalized additive model fitting generated higher restricted maximum likelihood (REML) scores for the complexity computed index (REML=58.3), the molecular weight (REML=51.1) and the topological polar surface area (REML=46.7) (Supplementary Table S1). In contrast, significant Pearson correlation coefficients were obtained between NMDS axis 1 and topological polar surface area ($R^2=0.83$, p-value=0.002), molecular weight ($R^2=0.80$, p-value= 0.003), heavy atom count ($R^2= 0.79$, p-value=0.003), number of OH function ($R^2= 0.78$, p-value= 0.004), number of C-O bonds ($R^2= 0.75$, p-value= 0.006), and complexity computed index ($R^2=0.71$, p-value = 0.01) (Supplementary Table S2). No significant correlation was found for the NMDS axis 2. Similarities between four pairs of compounds are observed: anthraquinone and 2-hydroxy-methyl-antrhaquinone share nine bands ($\pm 6 \text{ cm}^{-1}$), emodin and methylamino-anthraquinone share eight bands ($\pm 6 \text{ cm}^{-1}$), scytonemin signals from *Nostoc* and *S. informe* share more than nine bands ($\pm 1 \text{ cm}^{-1}$).

¹), and gloeocapsins from *G. alpina* and *P. nigrescens* share seven bands ($\pm 6\text{ cm}^{-1}$) (Table 2 and 3).

Anthraquinone derivatives are organized along the NMDS-1 axis from the simplest form, the anthraquinone, to the most structurally complex form, the emodin-8-glucoside. Gloeocapsins share the same ordinates intervals as the anthraquinone derivatives. Some of the gloeocapsin bands are also common to one of the other analyzed anthraquinone derivatives. All Hierarchical Cluster Analysis (HCA) resulting dendrograms, regardless of the distance index and the clustering methods, grouped the SAG 32.87 Raman signature with the quercetin signature relatively far from the signatures of the other pigments (Fig. 5, b, c; Supplementary Fig. S3). Quercetin and SAG 32.87 pigment share five bands ($640\text{-}648\text{ cm}^{-1}$, $1220\text{-}1225\text{ cm}^{-1}$, 1330 cm^{-1} , $1400\text{-}1407\text{ cm}^{-1}$, 1435 cm^{-1}) which may explain the clustering of the two Raman signatures.

Discussion

To our knowledge, the Raman signatures of *C. polonicus* extracellular pigment were not characterized before this study. The present analysis shows that the pigment is not related to any of the other prokaryotic pigments elucidated so far and suggests the existence of a third class of cyanobacterial extracellular pigment beside scytonemin (including scytonin) and gloeocapsin.

Although the orange color of the targeted pigment (Fig. 1) as well as the published absorption spectrum suggest that it may belong to the carotenoid group [17], the general profile of the Raman spectrum (Fig. 2) obtained during this study seems to exclude this hypothesis.

The overall signature bands suggest some aromaticity of the compound, and several bands seem to be related to the presence of at least one nitrogen atom in the molecular composition of the pigment (Table 1). Besides pigments, EPS envelopes are mainly composed of polysaccharides as well as other compounds such as various proteins, DNA, metals and lipids. This nanoscale molecular heterogeneity may imply strong interactions like hydrogen bonds between the different compounds which

may be related to the broadening of Raman bands (Fig. 2). In this study, the spot size of Raman analyses (700 nm) does not allow to isolate molecular species but represents a large surface which may contain various molecules. In addition, a potential amorphous state of the pigment may also explain broadened Raman bands. Four of the bands were only related to a carbon-nitrogen interaction suggesting the presence of a carbon ring containing a nitrogen atom such as an indole group. Comparison of bands wavenumbers with those of already elucidated microbial pigments shows little resemblance N-containing cyanobacterial compound scytonemin and scytonin, violacein from the Pseudomonadota *Chromobacterium violaceum* or prodigiosin from *Serratia marcescens* (Table 2, Fig. 4). Interestingly, the targeted compound exhibits a strong band at 1740 cm^{-1} that is possibly related to the C=O stretching vibration of ester groups of cholesteryls [26], or of a ketone group, both allowing the association of an aromatic backbone together with a side chain. Reported pigment esters in microbes include carotenoid-retinol derivatives, such as astaxanthins or the flexirubin pigments [27, 28, 29]. Their Raman spectra are recognizable by the dominance of $\nu\text{ C}=\text{C}$ and $\nu\text{ C}-\text{C}$ stretching distinctive of carotenoids [29], which is not the case here.

UV-screening pigments are also synthesized by fungi. Orange rubropunctatin and monascorubin, red and orange red sequoiamonascins fungal pigments are produced by *Aspergillus parasiticus*, *Monascus* spp., *Penicillium* spp. or *Talaromyces purpureogenus* for examples [30, 31]. These pigments are polyketides that share a pyrano-quinone bicyclic core specific to azaphilone pigments [32]. Among these compounds, nitrogenated azaphilones can be generated during cultivation in presence of endogenous ammonia or exogenous amino compounds, and some may harbor a side chain linked by a ketone function such as the rubropunctatin [33]. Cyanobacteria are known to produce secondary metabolites via the polyketide synthase pathway, therefore the azaphilone nature of the target pigment could be speculated. However, to our knowledge, azaphilone biosynthesis has never been reported in cyanobacteria so far or any other

bacteria. Future analyses shall include Raman characterization of this class of molecules.

The NMDS classification analysis (Fig. 5, a) was performed to visualize similarities between the strain SAG 32.87 novel pigment signature, some reference purified and *in vivo* bacterial UV-screening pigments and anthraquinone derivative naturally synthesized by lichens or artificially obtained, as well as the two aromatic compounds coumarin and quercetin synthesized by plants. The novel pigment does not seem to cluster with any of the microbial pigments. Despite the limited number of fully characterized molecules present in our dataset, the first NMDS axis exhibited significant relationships with chemical and physical properties of the purified compounds. The best GAM (REML score = 58.3) fit obtained for complexity computed index can be illustrated by the distribution of anthraquinone derivative horizontally arranged along the NMDS-1 axis from the simple anthraquinone to the most structurally complex form, the emodin-8-glucoside (Fig. 5, a). Scytonemins from EPS envelopes of *Nostoc* sp. and *S. informe* have close location and seem not to agree with GAM fit as shown by a complexity index of purified scytonemin of 1290 (Fig. 5, a).

Gloeocapsins are grouped together and quite distant from the other compounds which may be explained by the large width of the bands between 1000 and 1600 cm^{-1} potentially evidencing the amorphous nature of the extracted gloeocapsins.

Chemometrics, more precisely hierarchical cluster analysis, has been successfully used previously to differentiate anthocyanins, carotenoid, and flavonols [34]. Dendrograms resulting from our hierarchical cluster analyses are separating gloeocapsin from the rest of the compounds (Fig. 5 b, c). All topologies are regrouping the SAG 32.87 Raman signature with the signature of quercetin. Quercetin has been reported once in chloroform extracts of *Anabaena aequalis* [35], and other flavonoids are known to be produced by cyanobacteria [36] but to our knowledge were never described as an extracellular protective pigment.

To date, microbial pigments are known to absorb photonic radiations to perform photosynthesis or other phototrophic processes, to perform

photosensitivity reactions, to protect from lethal radiations, to prevent from virulence or predation, or to prevent from oxidative stress [37]. In the case of *C. polonicus*, the extracellular distribution of the pigment obviously excludes a photosynthetic role. In nature, *Chamaesiphon* spp. are found in both streams and eulittoral zone of lakes forming conspicuous biofilms exposed to desiccation, high light and UV-radiations stresses. Therefore, a likely hypothesis is that the extracellular pigment found in *C. polonicus* SAG 32.87 sheath may play a role in light absorption or UV- screening as in the case of the gloeocapsin and scytonemin pigments [5, 20, 38]. The UV-screening properties of environmental *C. polonicus* extracellular pigment were questioned due to its carotenoid-like absorption spectrum with a maximum at 470 nm and the absence of absorption in the UVA-range [17]. However, the strong absorbance at 322 nm of SAG 32.87 pigmented cell-free sheath (Supplementary Fig. S4) does suggest a UV protective role of at least one molecule contained in the sheath or by the association of polysaccharide matrix and potential lipopolysaccharide, protein or another molecule that may be present in the sheath. Moreover, it was previously shown that *C. polonicus* was more resistant to UV exposition than freshwater planktonic taxa and could survive up to 4h irradiation during laboratory experiments [39]. Therefore, it is possible that the compound present in *C. polonicus* SAG 32.87 extracellular envelope/sheath differs from the one isolated from the Isar Spring specimen [17] and does play a UV-protective role.

Flavonoids are known to have allelopathic, antibiotic and anti-predatory activities.

Further chromatographic, spectroscopic, mass spectrometry, and NMR analysis will allow to better hypothesize on the ecological role of this enigmatic compound.

Pigments have also critical implications in life evolution because of their role in light harvesting for photosynthesis or phototrophy or to prevent from damages provoked by extreme radiations exposure. Their great diversity in different clades of prokaryotes [40] suggests their early emergence and important functions. UV-screening pigments are widespread in the tree of

life, although not yet reported in *archaea* [41] in contrast to fungi or cyanobacteria.

The Proterozoic fossil record of cyanobacteria includes abundant microfossils with an identification ranging from possible, to probable, to unambiguous cyanobacteria [7, 42]. Among the unambiguous taxa, the endolithic *Eoentophysalis belcherensis* shows the accumulation of organic matter in some regions of its fossilized EPS envelopes resembling scytonemin localization in its modern counterparts [43]. Previous work has demonstrated that porphyrins derived from cyanobacterial chlorophylls can be preserved within fossil cells for more than 1 billion years (Ga) [44] or in 1.1 billion year old fossil oil [45], as well as carotenoids derived from anoxygenic photosynthetic bacteria and or cyanobacteria in up to 1.73 Ga bitumen [46]. Therefore, it is possible that molecular remains of UV-screening pigments may be preserved in the rock record but this remains to be evidenced.

The presence or resemblance of microfossils to *Chamaesiphon* genera in the geological record is quite rare and may remain ambiguous. Populations of epibionts on possible cyanobacterial filaments harboring morphological resemblance with *Chamaesiphon* have been described in Mesoproterozoic silicified stromatolites and tentatively identified based on their asymmetric, linearly oriented cell division [47]. Evidencing the molecular signatures of the pigment specific to this taxon in such a microfossil would remove ambiguities towards its biological affinity.

So far, Raman signatures of UV-screening pigments in the fossil record included carotenoid signatures obtained from various ancient samples of different ages, in halite brine inclusions of 9 Ka to 1.44 Ma borehole cores from three sites in California [48], in a Miocene Fe-Rich fossil vents [49], in fossil photosynthetic mats from an East Antarctic lake [50]. Scytonemin signature was recovered from 4530-year-old filaments isolated from an Antarctic lacustrine fossil mat [50].

Other features such as the preservation of thylakoid membranes [51] or pigments within fossil cells and their particular division pattern and morphology [44] are important diagnostic criteria as well but not the topic

of this work focused on extracellular UV-screening pigments. The unambiguous interpretation of microfossils would then allow to compute time calibrated phylogenetic trees with higher resolution, providing new insights on the evolution of cyanobacteria, on the evolution of bacteria and chloroplast, on the evolution of their pigment biosynthetic gene clusters as well as genes from the same family [5].

In contrast to carotenoids, UV-screening pigments are not necessarily widely distributed in the tree of life and may represent a potential diagnostic taxonomic signature for modern and fossil samples, such as scytonemin for cyanobacteria and possibly gloeocapsin.

Conclusion

In this study, we identified the Raman signature of a yet undescribed extracellular pigment produced by the cyanobacterium *Chamaesiphon polonicus* SAG 32.87. Bands related to the pigment differ from gloeocapsin and scytonemin derivatives. The pigment spectrum was differentiated from fourteen other compounds (including scytonemin and gloeocapsin) using chemometrics analyses. These results suggest the occurrence of a third category of extracellular pigment in the cyanobacterial phylum.

The discovery of this potential third class of extracellular cyanobacterial pigment shows the relevance of Raman signatures as diagnostic tools for probing and discovering new microbial pigments for future ecological, evolutionary and paleobiological studies.

Methods

Cell culture and preparation of cyanobacterial sheath and of pigment standards

The strain *Chamaesiphon polonicus* SAG 32.87 was purchased at the University of Göttingen culture collection of algae (SAG). SAG 32.87 was grown in BG11 liquid medium at 20°C under constant illumination for 3 months using a hyperspectral LED bar which covered 400-900 nm from the company EFFILUX (Les Ulis, France).

Emptied sheaths were obtained from grinding dried biomass from old cultures. Sheaths were washed in EDTA to expel potential remaining cells out of the sheath as described by Lepot and colleagues [50]. Cells and purified sheaths were placed onto glass slides used for analyses.

Ten commercial references of [natural](#) and synthetic pigments for which chemical and physical computed properties are available, listed in Supplementary [Table S3](#), were purchased at Merck Life Science BV (Belgium). Two scytonemin spectra were obtained from the analysis of the extracellular sheath associated pigment of *Stigonema informe* filaments from the specimen CBFS-A34 of the CBFS herbarium of the Botany department of the University of South Bohemia, and from extracellular sheath of an environmental colony of *Nostoc commune* collected in La Chartreuse park (N50°37'49.468", E5°35'47.972") Grivegnée, Belgium.

Raman single spectrum acquisitions and High-Resolution Raman spatial analysis

The samples were deposited on ZnSe windows. Raman investigations targeting SAG 32.87 pigments were performed using a Renishaw inVia Raman spectrometer coupled to a Leica DM 2500 confocal microscope equipped with [a 100X](#) objective and connected to a RL514C100 green laser source of 514 nm (maximum power= 45 W), with a 1800 L/mm grating and an air cooled (-70°) 1040x256-pixel CCD array detector at the Early Life Traces & Evolution-Astrobiology Lab at ULiege. Single spectrum acquisitions were performed with 514 nm confocal laser with an exposure time of 1 second, 10 accumulations for the pigment before EDTA treatment at a laser power of 5 mW on the sample, and 20 accumulations after EDTA treatment at a laser power of 0.6 mW on the sample.

Raman maps were acquired using the Edge Stream HR mode, at objective 100X with 10% of the maximum power of the 514 nm laser beam, and an exposure time of 2s, [and a spot size of 700 nm](#). Spectra were acquired using Renishaw software WIRE 5.1. The acquired spectral range was from 100 to 2000 cm^{-1} , with a spectral resolution of 1 cm^{-1} . Spectral band assignments were determined based on reference listed in Table 1.

Classification analyses

Spectra were processed using R package hyperSpec (version 0.100.2). Baselines were subtracted using polynomial function using least square fitting approach. The conserved spectral region was from 350 to 2000 cm^{-1} . After normalization by subtracting the minimum intensity and subsequently dividing by the maximum intensity for each spectrum using function *normalize01* from hyperspec R package, an Euclidean distance matrix was computed between the fifteen spectra to perform a non-metric multidimensional scaling (NMDS) using *metaMDS* function from vegan package (version 2.6-10). Chemical and physical computed properties were fitted on the ordination using generalized additive modelling (GAM) [52] implemented the function *ordisurf* as described in [53]. Best GAM is characterized by higher restricted maximum likelihood (REML) values. The function *cor.test* from R package stats (version 3.6.2) was used to compute Pearson correlation coefficients and their p-value between chemical properties and computed properties retrieved from PubChem database (molecular weight, complexity index computed used as a proxy estimating how complex is the molecular structure using Bertz/Hendrickson/Ihlenfeldt formula [54, 55], heavy atom count, topological polar surface area in square Angström) and numbers of atom-atom chemical bonds with NMDS ordinates for elucidated compounds. Distances matrices for hierarchical clustering analyses was calculated with absolute distances, Euclidean distances, and Minkowski distances. Dendrograms were computed using *dendrogram* function from the R package stats (version 3.6.2) with Ward [56], UPGMA and WPGMA methods. Bootstraps and p-value percentage were computed with 10 000 iterations with the function *pvclust* from pvclust R package.

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Author contributions

YJL designed the project and performed the analyses. YJL and AL cultivated the cyanobacterial strain. AL helped with Raman analyses of standards. YJL wrote the original draft and YJL, AL, EJJ discussed the results and contributed to the final manuscript. EJJ supervised the project.

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Data availability statement

Data supporting this study are available within the article and supplementary information. Raw data are available from corresponding author and last author upon reasonable request.

Additional Information (including a Competing Interests Statement)

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported.

Figure legends (these are limited to 350 words per figure)

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Table 1, Potential assignment of characteristic bands of SAG 32.87 extracellular pigment.

Wavenumber	Potential assignment	References
1740 s	C=O (ester, ketone, or aldehyde group)	[57, 58]
1636 s	CCH, CNH, C=C	[59, 60]

1605 sh	C=CH vinyl stretch, indole ring/ $\nu(\text{C=CH})$ attached to cyclopentene ring	[25, 61]
1550 mw	CCH p-disubstituted aromatic ring stretch, C=C unit in the diketoalkene, OH bend	[57, 61]
1490 ms	C-H bending	[57]
1435 w	quadrant C-N-C, OH- bend	[57]
1407 mw	quadrant C-N-C	[57]
1330 ms	C-C ring or indole ring	[25, 61]
1225 mw	CN stretch, CNH deformation, ring deformation	[57]
1195 mw	C-N stretch	[25]
970 ms	C-H bending	[62]
648 s	ring deformation, aromatic ring C-C and CCC bending	[57]

's' stands for strong, 'sh' for shoulder, 'm' for medium, 'w' for weak, 'mw' for medium weak, and 'ms' for medium strong.

Table 2, Summary table of main bands of SAG 32.87 pigment compared to extracellular non carotenoid non photosynthetic bacterial pigments.

Pigments	unknown compound	Scytonin	Scytonemin	Gloeocapsin	Gloeocapsin	Violacein	Prodigiosin chloride	Approximate assignment of vibrational mode
References	(This study)	[25]	[61]	[5]	[5]	[59]	[60]	
Organisms	<i>Chamaesiphon polonicus</i> SAG 32.87	mixture	<i>Lyngbya cf aestuarii</i>	<i>P. nigrescens</i> ULC007	<i>G. alpina</i>	<i>Chromobacterium violaceum</i>	<i>Serratia marescens</i>	
Lasers	514 nm 1740 s	445 nm	1064 nm	514 nm	514 nm	514 nm	514 nm	
			1710 w			1730 w		
		1676 w, sh 1660 mw, sh 1649 m	1656 mw, sh	1675 m	1677 ms	1678vw		
	1636 s					1636 w 1618 w	1640 s	C=O ester, C=O ketone CC aromatic stretch
	1605 sh	1603 s	1605 mw, sh 1590 s					C=CH vinyl stretch, indole ring CCH aromatic ring quadrant stretch C=CH vinyl stretch, indole ring, CC aromatic stretch indole ring indole ring C=C unit in the diketoalkene, CCH stretch p-disubstituted aromatic ring
		1585 ms 1568 sh 1559 s		1580 ms	1565 ms			
	1550 mw		1549 ms			1552 s		

					1542 m sh		pyrrole ring CC and C-N N=C-C=C ring mode stretch indole ring
		1525 w					
	1518 mw						
1490 ms							
			1479 w		1483 m 1466 m		CH deformation
				1456 w			
1435 w	1435 ms						C=CH bend, ring CH bend
		1444 mw	1441 w,sh				C=CH bend indole ring
	1424 ms				1429 m 1414 m		
1407 ms							
	1399 m						
		1384 w	1381 ms				CCN indole ring stretch CCN indole ring stretch
		1375 vw					
					1369 m		
	1362 mw 1342 mw			1363 ms			indole ring, C-C
1330 ms							
	1320 m	1323 ms	1323 s				CC ring, CCN indole ring stretch CC bridge bending CC aromatic ring stretch CC aromatic ring: p-disubstituted benzenes CCH, CNH bending
				1305 s		1303 w	
	1294 m				1297 m		
		1283 mw					
			1281 s				
	1272 m			1269 s		1263 w	

		1259 m	1245 mw						CC aromatic ring stretch: p-disubstituted benzenes CN stretch, CNH deformation
	1225 mw				1207 mw		1228 m		
	1195 mw	1185 mw	1172 ms		1177 w				C-N stretch C=C—C=C system (trans) stretch CC ring breathing pyrrole stretch
		1157 mw	1163 m, sh				1149 m, sh		C-C stretch
							1140 s	1142 m	C-C stretch, C-O methoxy stretch
							1126 w, sh		C-C stretch
								1123 m	COC ether bending, CCH bending
			1102 w						COH phenolic bending
			1090 w						C-O phenolic stretching
							1082 vw		
								1070 m	CCH bend, twist CH ₂
		1052 mw		1062 w					O-Me, C-C=O bend, indole
								1047 w	
			1024 w	1021 w	1017 w				C-O phenolic stretching
					996 w			991 w	
			984 w						CNC bending
	970 ms								

				957 m	960 vw	aliphatic CH ₂ bending and rocking C=C aromatic
			943w 909 w	947 m		
		889 w			889w	CCC bending C=C aromatic CCH breathing
		835 w		866 w		CCH breathing disubstituted aromatic ring bend CNO deformation
				806 w	843 s	
		754 w				aromatic ring C-C and CCC bending aromatic ring C-C and CCC bending
				727 s		
	648 s	677 w		679 m		
				620 w		aromatic ring C-C and CCC bending
		565 w 539 w 500 w				
			474 mw 462 mw			
				462 ms		
		450 w				

's' stands for strong, 'sh' for shoulder, 'm' for medium, "vw" very weak, 'w' for weak, 'mw' for medium weak, and 'ms' for medium strong.

Table 3, Potential assignment of characteristic bands of natural and synthetic pigments measured during this study for chemometrics analyses.

Category	Cyanobacterial compounds			Other bacterial compounds			
Compound	Scytonemin	Scytonemin	Approximate vibrational assignment	Pyocyanin	Approximate vibrational assignment	Violacein	Approximate vibrational assignment
Organism/ Nature Laser	<i>S. informe</i> A34	<i>Nostoc commune</i>		<i>Pseudomonas aeruginosa</i>		<i>Janthinobacterium lividum</i>	
	514 nm	514 nm		514 nm		514 nm	
	1710 w	1710 w	C=O stretching	1700 w	C=O stretching		
	1630 m	1630 m		1635 mw			
	1595 s	1595 s	CCH aromatic ring quadrant stretch	1610 m	CCH aromatic ring quadrant stretch	1600 mw	CCH aromatic ring quadrant stretch
	1555 m	1555 m	CCH aromatic ring quadrant stretch	1565 ms	CCH aromatic ring quadrant stretch	1545 s	C=C unit in the diketoalkene
	1520 w, sh	1520 w, sh	N=C-C=C ring mode stretching	1520 m	N=C-C=C ring mode stretching	1495 w	
		1465 w		1475 mw		1460 w	Pyrrole ring C=C and C-N
	1435 w	1445 w	C=CH bending	1430 mw			
	1385 w	1385 w	CCN indole ring stretching	1405 s	Quadrant CNC stretching	1405 m	Quadrant CNC stretching

	1355 w	1355 w	CCN indole ring stretching	1360 w		1345 mw	Quadrant CNC stretching
	1325 w	1320 w	CCN indole ring stretching	1315 w		1295 m	
	1235 w		C=C—C=C system (trans) stretch	1280 w		1270 m	
	1170 ms	1170 ms	CC ring breathing	1220 w		1240 w	
	1095 w	1095 w	pyrrole stretch				
			C-O phenolic stretching	1165 mw		1180 w	C-C stretching
	1020 w	1025 w	C-O phenolic stretching	1080 w		1150 m	C-C stretching
	985 w	980 w	Bending CNC	1000 w		1130 m	C-C stretching
	935 w					1010 mw	
	910 w	910 w		950 w		955 mw	
	825 w			880 w		870 m	C-N stretching
		780 w		865 w	C-N stretching	805 mw	
			Bending CCC/CCN indole ring-puckering mode	820 w		730 m	
	675 w	675 w	Bending CCN pyrrole ring-puckering mode	800 w		680 w	
		660 w		720 mw		620 w	

	630 w				650 w				495 w				CCO and CCN rocking and bending modes	
	575 w		575 w		610 mw				450 w				CCO and CCN rocking and bending modes	
			540 w		575 w				425 w				CCO and CCN rocking and bending modes	
			495 w		555 mw 535 mw				385 w					
					490 w				CCO and CCN rocking and bending modes					
					415 mw									
Category	Anthraquinone derivatives							Other Plants coumponds						
Coumpo nd	Anthraquino ne	2- hydroxymeth yl- anthraquino ne	Parieti n	Emodi n	Methyl- amino- anthraquino ne	Emodin -8- glucosi de	Approxima te vibrational assignmen t	Coumari n	Approximate vibrational assignment	Quercet in	Approxima te vibrational assignmen t			
Organis m/ Nature	synthetic	Plants	Liche n	Plants	synthetic	Plants		plants		plants				
Laser	633 nm	785 nm	785 nm	785 nm	785 nm	785 nm		514 nm		514 nm				
								1755 w 1725 mw	C=O stretching					
					1705 w			1710 m	C=O stretching					
	1666 s	1660 s	1670 m	1660 m	1665 mw	1676 m	C=O stretching	1675 w	C=O stretching	1660 mw	C=O stretching			

[illegible]

Wavenumber (cm ⁻¹)	Assignment
1195 mw	C-CH ₃ stretch
1185 mw	Ring stretch, C-C
1180 m	Ring stretch, C-C
1170 mw	C-CH ₃ stretch
1155 mw	C-CH ₃ stretch
1154 mw	C-CH ₃ stretch
1090 w	C-CH ₃ stretch
1132 w	C-CH ₃ stretch
1070 w	C-CH ₃ stretch
1084 m	C-CH ₃ stretch
1024 w	C-CH ₃ stretch

[illegible]

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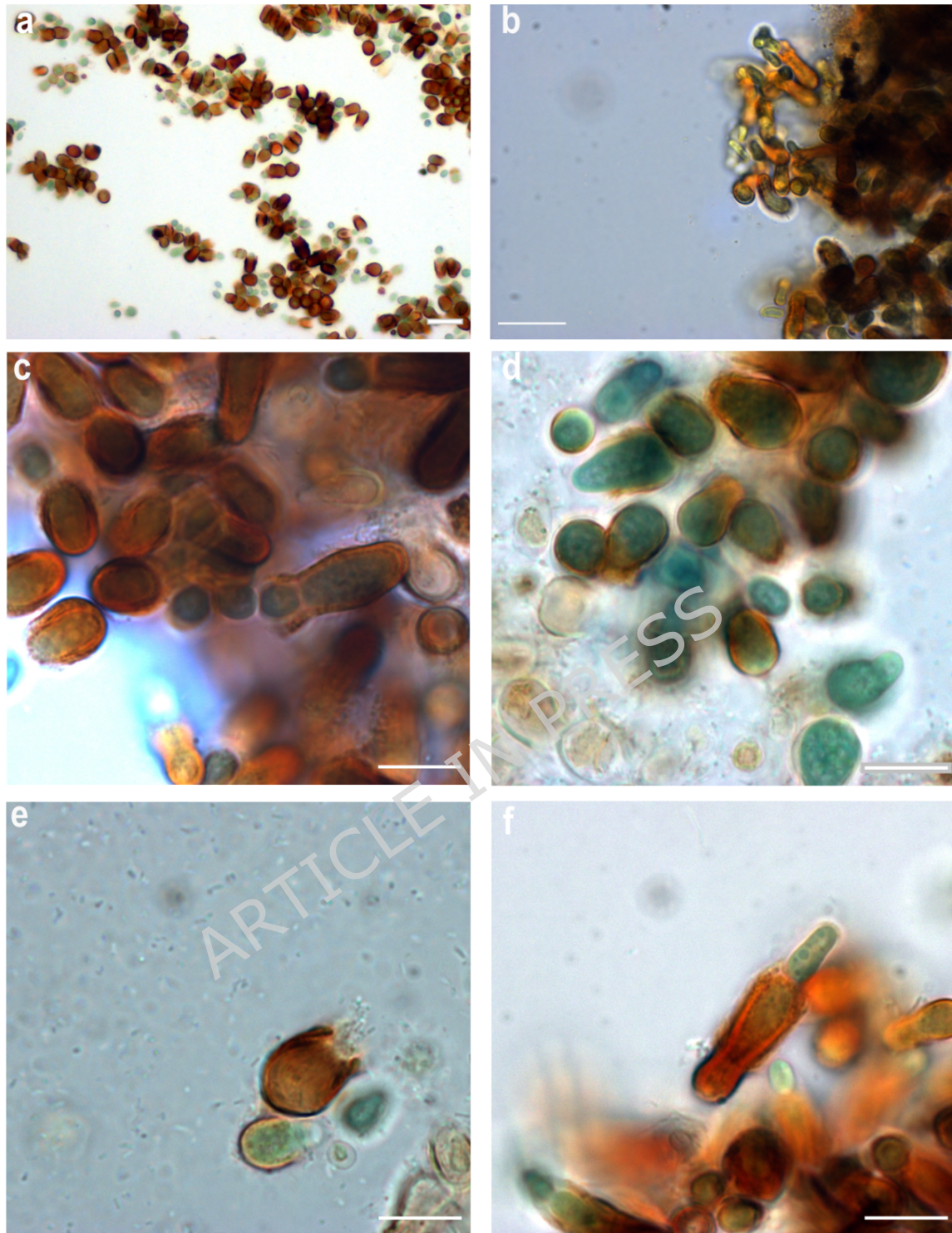


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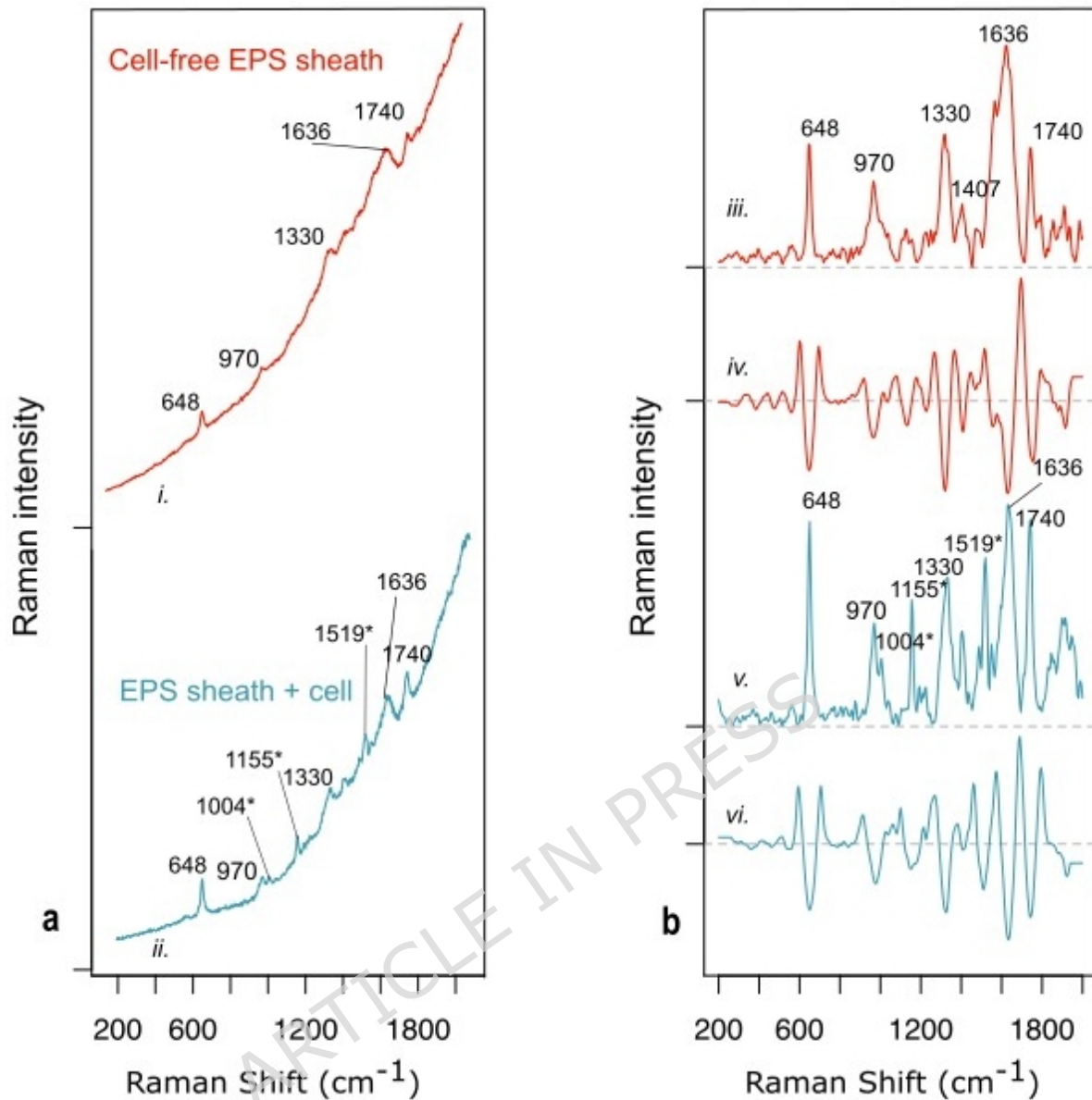


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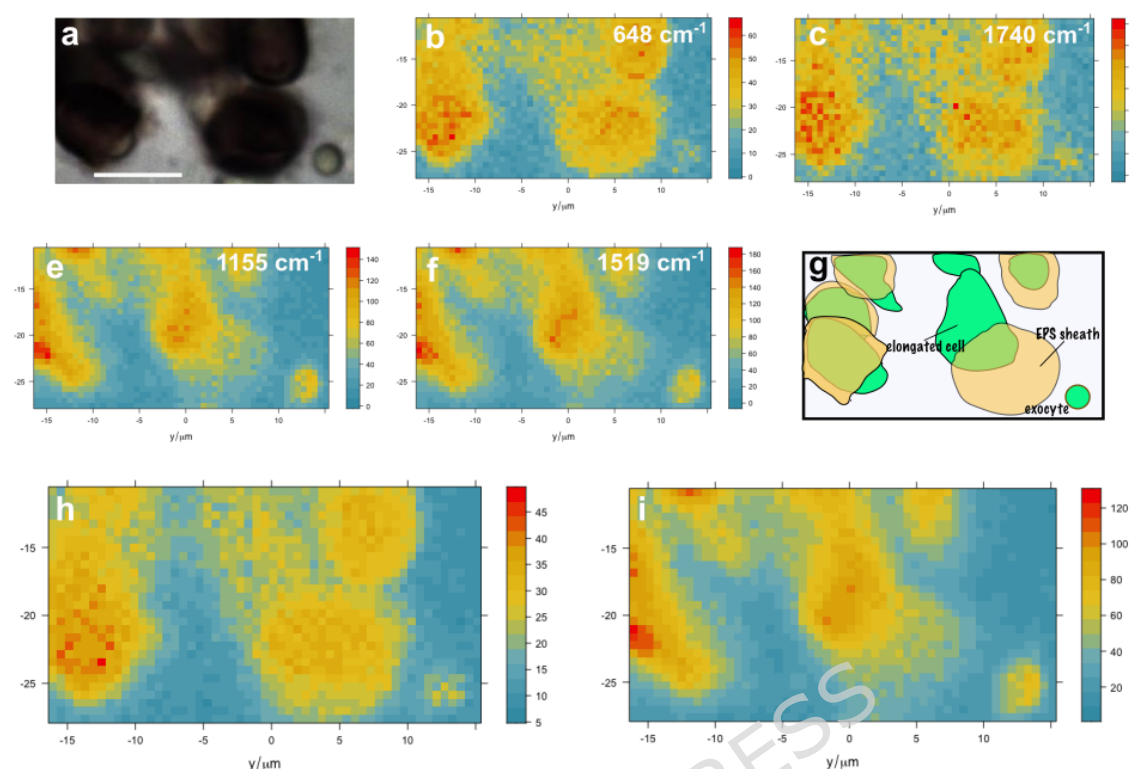


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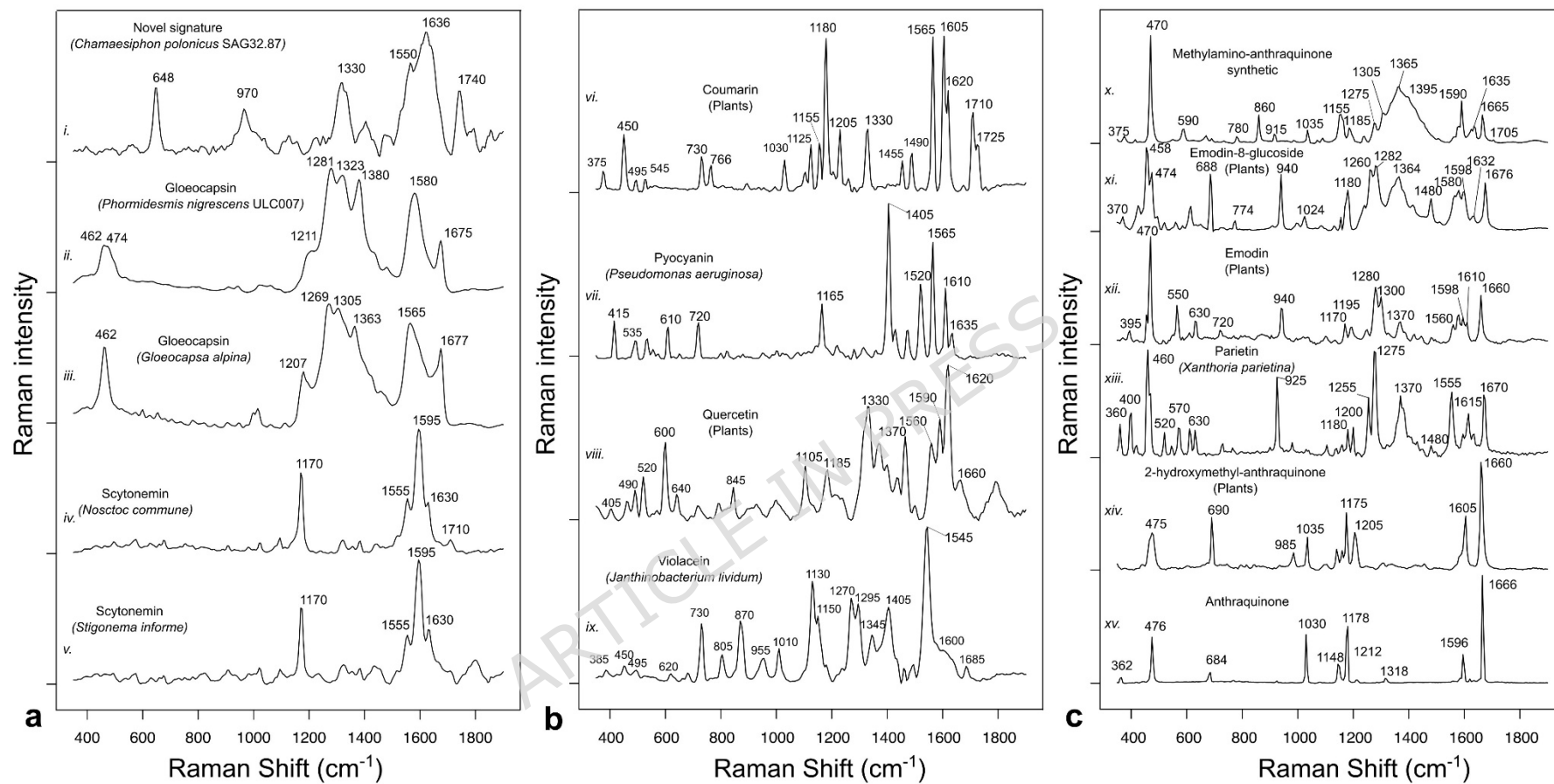


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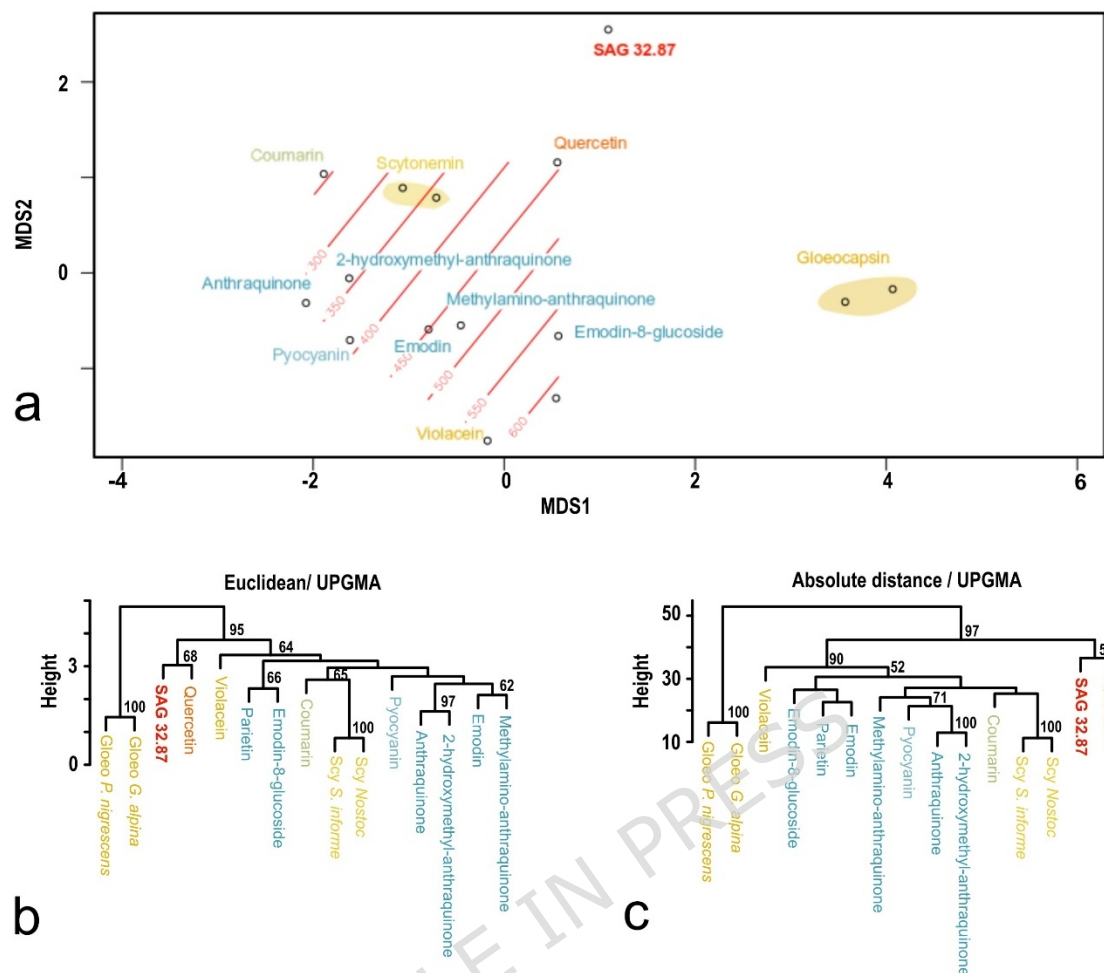


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