Metabolites from the Euryhaline Ciliate *Pseudokeronopsis erythrina*

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Abstract: Three new secondary metabolites (named erythrolactones A2, B2 and C2), that are characterized by a central 4-hydroxy-unsaturated δ-lactone ring bearing an alkyl saturated chain at C(2) and a butyl-benzenoid group at C(5), together with their respective sulfate esters (erythrolactones A1, B1 and C1), have been isolated from cell cultures of *Pseudokeronopsis erythrina*, clone TL-1. The structures are assigned on the basis of extensive spectroscopic measurements (1D and 2D NMR, UV, IR and HR-MALDI-TOF). A plausible biogenetic route for their formation is also suggested. Cold-shock treatment was performed in order to induce the discharge of the metabolites contained in pigment granules lying on the ciliary organelles of this microorganism. HPLC-ESI-MS analysis of this granule discharge reveals that erythrolactones A2-C2 are actually therein contained, strongly suggesting a possible role for these metabolites in the chemical defence strategy of *P. erythrina*.

Introduction

Freshwater and marine protozoa are known for their ability to produce a vast and chemically diverse array of secondary metabolites that are involved in different ecological functions. Among those, low molecular weight bioactive compounds, which are stored in specialized ejectable membrane-bound organelles generally called extrusomes,[1,2] can inhibit cell division, kill a prey or can be used as a chemical defence.[3,4–7] The ciliated protozoan *Blepharisma japonicum* produces blepharismins, a mixture of five similar red compounds that act as UV radiation screens and photosensors,[8,9] and are also involved in defence against predators.[3,4–7] Similarly, *Stentor coeruleus* produces stentorins, blue UV-screen compounds that also exert defensive functions against other ciliates.[10,11] Blepharismins and stentorins are both characterized by a hypercin-like skeleton. Similar compounds have been recently isolated, including maristentorin from the marine ciliate *Maristentor dinaferus*,[15] and amethystin from the freshwater ciliate *Stentor amethystinus*.[16]

Differently from *Blepharisma* and *Stentor*, *Climacostomum viridens* and *Spirostomum teres* produce, respectively, the major represented colourless compounds climacostol and spirostomin, which appear to be exclusively related to predator-prey interactions.[17–19] In addition, *Spirostomum ambiguum* and *Coleps hirsutus*, have been, respectively, demonstrated to produce the defensive molecule mono-prenyl hydroquinone and a cocktail of free fatty acids that assist in carnivorous feeding.[7,20] Two of these compounds, spirostomin and climacostol, have been chemically synthesized[18,19,21,22] and synthetic climacostol has also been studied for its antibiotic, cytotoxic and proapoptotic effects on pathogen prokaryotes, protists and human cancer cell lines. Experiments performed with this compound and plasmid DNA indicate that the mechanism of action of climacostol involves Cu<sup>2+</sup>-mediated oxidative DNA damage.[23–27]

Among marine ciliates, morphospecies belonging to the genus *Euplotes* have been extensively studied for their ability to produce chemically diverse secondary metabolites. Interestingly, it was found that strains belonging to the same genetic clade were characterized by a different profile of bioactive compounds.[6] For example, the morphospecies *E. vannus* is characterized by great biodiversity on genetic scale, which is reflected, from a metabolic point of view, by the production of different secondary metabolites. Tropical strains are known to produce vannusal A and B, whereas other strains produce the sesquiterpenoids prevannusal A and B and hemivannusal. *E. crassus* has been widely investigated for its ability to produce euplotin A, B and C. Euplotin C, in particular, has shown powerful cytotoxic effects against other *Euplotes* morphospecies and tumour cells in addition to antimicrobial activities.[28–31]

Keronopsins are another group of pigments and defensive molecules, characterized by a β-bromide-substituted pyrrole linked to a sulfate pyrrole through a conjugated acyl chain, that were isolated from *Pseudokeronopsis rubra* in 1994.[32] Recently,
a third group of pigments called keronopasamides has been isolated from the marine ciliate Pseudokeronopsis ricci. [3]

Pseudokeronopsis erythrina (Ciliophora, Hypotricha) belongs to the family of Pseudokeronopsidae and was recently described as an estuarine ciliate species. [13] The strain used in this study was collected from a freshwater environment (Lake Trasimeno, Italy) and perfectly adapted to be cultured in the laboratory using the same conditions established for other freshwater species. For this reason, we propose to classify this organism as an euryhaline ciliate. *P. erythrina* displays an elliptical shape and an elongated body (120–200 × 20–50 μm), and is equipped with spherical, dark-reddish, brown or brick red coloured pigment granules (about 1 μm in diameter) that are mainly arranged around ciliary organelles. [23]

In this work we report on the characterization of new pigments produced by cell cultures of *P. erythrina* using different analytical approaches such as Nuclear Magnetic Resonance (NMR), High Resolution/Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (HR-MALDI-TOF-MS) and High Performance Liquid Chromatography-Electrospray Ionization-Ion Trap-Mass Spectrometry coupled to a Diode Array Detector (HPLC-ESI-IT-MS/DAD). Cold-shock treatments of the producing organism induced metabolite discharge from pigment granules and the contents of this discharge were characterized using the same analytical procedures noted above.

## Results and Discussion

To investigate the chemical composition of compounds produced by massive cell cultures of *Pseudokeronopsis erythrina*, we analyzed the first fraction eluted from RP18 cartridges loaded with crude ethanol extracts using methanol/water (9:1). When subjected to HPLC-ESI-IT-MS/DAD analysis, the chromatogram of this fraction showed the presence of six major peaks, all with a strong UV absorption around 305 nm (Figure 1).

![Figure 1. Chromatogram of *P. erythrina* crude extract obtained from cell cultures with UV detection at 305 nm.](image)

The nominal masses of the [M - H] pseudo-molecular ions of metabolites 1-3 differed from each other by 14 Da (m/z 437, 451 and 465, respectively); the same mass difference was also found among metabolites 4-6 (357, 371 and 385, respectively). More strikingly, the molecular weights of the most polar metabolites 1-3 were found to be 80 Da mass-shifted with respect to the less polar metabolites 4-6, thus defining two classes of homologous metabolites with different polarities. We hypothesized that this mass shift could be attributable to a -SO_3 group; 1-3 should simply be the sulfonated analogues of 4-6 metabolites. ESI(−) MS and MS^3 analyses confirmed this hypothesis. In fact, isolation and fragmentation (Figure 2, A) of the parent ion [M - H] for peak 2 (m/z 451.3, eluting at 7.8 min) led to a daughter ion at m/z = 371.3 (through the loss of an 80 Da neutral fragment), the same mass value observed for the parent ion of peak 5, eluting at 16.3 min. Even more convincingly, the further isolation and fragmentation of this ion at m/z = 371.3 (MS^3 spectrum of 2 in Figure 2, B) is almost superimposable with the MS^3 spectrum of the parent ion of peak 5, (MS^2 of 5 in Figure 2, C). Similar MS^n measurements carried out on the parent ions of peaks 1 and 3 confirmed their structural relationship with peaks 4 and 6, respectively. Molecular formulas of the parent ions for peaks 1-6 were established by high resolution MALDI-TOF mass measurements carried out by using the TOF analyser in reflectron mode. Thus, for instance, the parent ion of compound 2 was found to have a molecular formula of C_{32}H_{27}O_5S (accurate mass 451.1450 Da) and that of compound 5 was found to be C_{27}H_{27}O_5S at (371.1838 Da). Hence, HR-MALDI-MS measurements established that this +80 Da mass shift was actually due to the SO_3 group; metabolites 1-3 being the sulfate esters of 4-6, a finding also in agreement with their observed higher polarity (shorter retention time on reversed-phase chromatography).

![Figure 2. A) ESI(−) MS^2 spectrum of m/z 451 (parent ion of peak 2); B) ESI(−) MS^3 spectrum of m/z 451–371 of peak 2; C) ESI(−) MS^3 spectrum of the parent ion of peak 5 (m/z 371).](image)

Another striking difference observed in the spectroscopic properties of the 1-3 series (sulfate esters) with respect to the 4-6 (hydroxyl) series of metabolites was found in their corresponding UV spectra taken on-line during the chromatographic runs. Although all compounds shared the same absorption band at 250 nm, the UV spectra of compound 1-3 showed a bathochromic shift of the absorption maximum around 300 nm (λ = 307±1 nm in 1-3 and λ = 301±1 nm in 4-6). This finding
strongly suggests that the sulfonic group must be somehow conjugated to the main chromophoric system of these compounds.

In positive ion mode, MS spectra of sulfate esters 1-3 showed intense signals mainly attributable to i) mono- and disodiated adducts of the parent ion, and ii) their corresponding desulfonated ion fragments. ES(+) MS spectra of 4-6, instead, showed intense signals only for their protonated and mono-sodiated adducts. Since the sulfonic group exists in aqueous solution as an anionic -SO₃ group, we can consider the molecular formula of neutral 1 (M) to be C₃₂H₃₄NaO₁₆S, corresponding to the mono-sodiated salt. Thus, in positive ion-mode EI measurements of compounds 1-3, only one sodium ion is required to give an overall positive charge to the parent ion adduct M, which, however, already contains a sodium ion. Following this definition, we have to consider [M + Na]⁺ parent ions which eventually lose a neutral SO₃ molecule leading to other abundant fragments ions [i.e. M + Na - SO₃]⁺. Noteworthy is that, as soon as the SO₃ moiety is lost, compounds 1-3 become 4-6 and the same expected ESI (+) adduct ions are observed.

The structural elucidation of these compounds was performed through extensive 1D and 2D NMR analysis. We describe here NMR details only for compound 5 (erythrolactone B2) and its analogue 2 (erythrolactone B1) since they are comprehensive of all the NMR features of these metabolites. As outlined above, the high resolution measurement (371.1838 Da) determined by TOF analyser on the monoisopic peak of the [M - H]⁺ parent ion revealed the formula of 5 to be C₁₉H₂₁NO₆ Sug. implying nine degrees of unsaturation. Analysis of 1D and 2D NMR spectra revealed the presence of i) a substituted 1,4-hydroquinone ring conjugated to a disubstituted carbon-carbon double bond, ii) a trisubstituted pyranone ring and iii) a short linear alkyl chain. The presence of one 1,4-hydroquinone moiety (arbitrary numbering as defined in Scheme 1) was established by the δ values of aromatic protons, their characteristic J coupling pattern and, mainly, by their correlation with the corresponding δ values obtained by HSQC experiments. The HMBC spectrum not only confirmed this partial structure, but also allowed us to ascertain the substituent at position 6 as a disubstituted C(7)=C(8) double bond with E stereochemistry (J₇,₈ = 15.9 Hz). Notably, the C(7) at δC = 127.71 ppm showed ¹J hetero-correlations only with H-C(5) at δH = 6.79 ppm, whereas the C(6) at δC = 126.38 ppm showed ¹J hetero-correlations with H-C(2) at δH = 6.59 ppm.

The analysis of homonuclear correlations (COSY) spectra allowed us to easily extend the atom connectivity of this alkyl chain as far as C(10). The ¹J hetero-correlation of C(10) at δC = 34.42 ppm with the proton singlet at δH = 0.03 ppm and of the latter with the characteristic singlets at δC = 167.70, 164.31 and 104.06 ppm allowed us to assign the remaining 4 points of unsaturation to a 2,6-dialkyl-substituted, 4-hydroxy-2H-pyranone ring. Whereas HSQC and HMBC measurements clearly indicated C(10) to be linked at position 11, the linear heptyl chain must be located at position 14 of the pyranone moiety as noted in Scheme 1. In fact, NMR spectra enabled full assignment of the protons and carbons of this chain indicating that deshielded triplet protons 2H-(C1) at δH = 2.37 ppm (δC = 23.86 ppm) were ¹J (H,C) hetero-correlated with carbon singlets at δC = 168.84 ppm [C(15)] and 167.70 ppm [C(13)] and ²J (H,C) hetero-correlated with the carbon atom singlet at δC = 104.06 ppm [C(14)].

The NMR spectra of compound 4 (erythrolactone A2) and 6 (erythrolactone C2) were almost superimposable with those of 5 (erythrolactone B2); the only difference pertained to the relative integration area of the multiplet at δH = 1.29 ppm. The high resolution mass measurements on these metabolites showed that they possessed one methylene group (CH₂–) greater (4) or less (6) than heptyl-bearing 5 whereas the NMR data indicated that 4 was the n-hexyl analogue of 5 and that 6 was the n-octyl analogue of 5.

On the other hand, a few but characteristic differences were displayed by compounds 1-3 with respect to 4-6. In particular, in compound 2, H-C(12) was found to be more than 0.8 ppm deshielded (δH = 6.87 ppm) relative to the same moiety in 5 and almost all the ¹³C resonances (but only these) of the pyranone ring system were significantly affected. Since the high resolution measurement (451.150 Da) of the monoisopic peak of the [M - Na] parent ion revealed the molecular composition of 2 as C₃₀H₃₂O₇S (i.e. 5 + SO₃), it became evident not only that we were dealing with the sulfate analogue of 5, but also that the sulfonated hydroxyl group is linked to C(13). Following similar lines of reasoning as discussed above, it was straightforward to assign structures 1 (erythrolactone A1) and 3 (erythrolactone C1) as, respectively, the n-hexyl and n-octyl analogues of 2 (Figure 3).

![Figure 3. Structures of erythrolactones 1-6 isolated from massive cell cultures of Pseudokeronopsis erythrina.](image-url)

Chemical Characterization of Erythrolactones from Cold-Shock Treatment

Cold-shock treatment proved to be an efficient method of triggering release of extrusomal contents. We performed a quick extraction from a small portion of the lyophilized fraction by adding a few drops of ethanol and subjecting this micro-extract to prompt HR-MALDI-TOF analysis. The remaining part of the same lyophilized fraction was then subjected to an extended ethanol/acetone extraction followed by HPLC-ESI-IT-MS/DAD and NMR measurements. Comparisons of experimental data obtained by these two different procedures allowed us to clearly establish that no significant differences were apparent in the metabolite distributions of both extracts; we therefore felt confident to that the “native” profile was unaffected by ex-
tractive work-up. As can be appreciated in the chromatogram shown in Figure 4, this extract contains significant amounts of erythrolactones 4–6. Some minor metabolites were also detected by LC-MS, (also present in the crude extract obtained from cell cultures) but the material in our hands was insufficient for chemical characterization of these minor constituents. However, a careful comparison of MS spectra of minor metabolites isolated from cell culture with those present in the cold-shock treatment revealed that the latter contained merely de-sulfonated molecules.

![Figure 4. UV chromatogram of fraction obtained by cold-shock treatment of *P. erythrina* cells (detection at 305 nm).](image)

The genus *Pseudokeronopsis* is known for the production of secondary metabolites that exist both as free or as esterified (sulfonic) forms. For example, keronopsamides B and C isolated from *Pseudokeronopsis ricci* have been found as sulfate esters whereas keronopsamide A is the non-sulfonated analogue of keronopsamide B. Similarly, *P. rubra* is known to produce keronsins A₁ and A₂, which are the sulfate esters of keronsins B₁ and B₂ respectively. The question of why the toxin-enriched fraction (TES) contains only non-sulfonated molecules has not yet been definitively answered. The sulfation of endogenous molecules is important, especially in eukaryotes; the addition of highly charged sulfate groups increases water solubility and can influence conformational changes as well as downstream biotransformations of low molecular weight compounds.

### Biogenetic Considerations

The biosynthesis of erythrolactones may be supposed (Scheme 1) to occur from the Claisen condensation of two intermediates, an extended 2,5-dihydroxycinnamoyl-CoA (A) and nonanoyl-CoA, affording the key intermediate C. 2,5-Dihydroxycinnamoyl-CoA can be elongated via classical fatty acid synthetic machinery (using 2 equiv. acetyl CoA) whereas the nonanoyl-CoA can arise from oxidative scission of the C9=C10 double bond of oleic acid (18:1, ω = 9). That the saturated alkyl chains can derive from this C-C bond breaking easily explains the structural composition of erythrolactones A2–C2 (as well as erythrolactone sulfates A1–C1); the C6, C7 and C8 alkyl chains are derived, respectively, from the oxidative breaking of the very common 16:1, 18:1 and 20:1 acyl chains. Lactonization followed by tautomerization of intermediate C eventually provides the erythrins themselves.

### Conclusions

Three new molecules, erythrolactones A2, B2 and C2, and their respective sulfate esters (A1, B1, C1), were isolated from cell cultures of *Pseudokeronopsis erythrina*. Their structures were determined by NMR spectroscopy and MS data generated using HR-MALDI-TOF and ESI-MS. Very interestingly, cold-shock treatments provided evidence that only non-sulfonated molecules are stored in the pigment granules.

![Scheme 1. Proposed biogenetic path for the biosynthesis of erythrolactone B2 isolated from marine ciliates of the genus *P. erythrina*.](image)
Consistent with our suggestions, these new findings underscore i) the high skeletal diversity found in the secondary metabolites of this phylum, and ii) the high biodiversity and adaptive ability of ciliates. To date, the only investigated species, *P. rubra*, *P. ricciui* and *P. erythrina*, produces significantly different metabolites by exploiting different metabolic pathways. Further studies, currently in progress, will highlight the biological and ecological roles of the erythrolactone.

**Experimental Section**

**General Methods:** HPLC grade acetonitrile, chloroform, ethanol, ethyl acetate, methanol, *n*-hexane and LC-MS grade methanol were purchased from VWR (VWR International PBI, Milan, Italy); deionized water filtered at 0.2 μm was obtained from Elix Water Purification System (Merck Millipore, Billerica, MA, USA). LC-MS grade formic acid was purchased from Fisher Scientific (Fisher Scientific, Illkirch, France). [D₆]methanol for NMR spectroscopy was purchased from Merck and had a degree of deuteration of min 99.8%. 9-Aminoacridine hydrochloride hydrate (9AA) for MALDI was purchased from Alfa Aesar (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany). Kuromin chloride and myrtillin chloride were purchased from Extranaytres (Extranaytres, Lyon, France).

Infrared spectra (IR) were recorded using a FT-IR Equinox 55 Bruker spectrometer (ATR configuration) at 1 cm⁻¹ resolution in the absorption region Δν 4000-1000 cm⁻¹. A thin solid layer is obtained by evaporation of a methanol solution of erythrolactone B1 (2) and erythrolactone B2 (5). The instrument was purged with a constant dry air flow and clean ATR crystal as background was used. Spectral processing was accomplished using Opus software.

¹H NMR (400 MHz) and ¹³C NMR (100 MHz) analyses were conducted with a Bruker-Avance 400 MHz NMR spectrometer by using a 5 mm BBI probe equipped with pulsed-gradient field utility; the system was controlled by TopSpin software 2.1. The ¹H-90° proton pulse length was 9.3 μs with a transmission power of 0 dB. Spectra were acquired at 300 K. The chemical shift (δ ppm) was calibrated: i) for ¹H-spectra on the residual proton signal of methanol at δH = 3.310 ppm, and ii) for ¹³C-spectra on the ¹³C-NMR resonance of [D₂]MeOH at δC = 49.00 ppm. Proton-proton scalar correlation (¹H-¹H DQ COSY), proton-carbon single bond correlation (¹H-¹C HSQC), and proton-carbon multiple bond correlation (¹H-¹C HMBC) were also recorded. NMR spectra were processed also by using MestreNova 9.1 software (MestreLab Research S.L., Escondido, CA).

HR-MALDI-TOF-MS analyses were performed with a Bruker Daltonics Ultraflex II instrument operated by FlexControl 3.0 software (Bruker Daltonik GmbH, Leipzig, Germany). Spectra were acquired in reflectron negative mode at a laser frequency of 20 Hz in the mass range from 0-1000 Da. Ion source 1 (IS1) voltage was set at 20.0 kV, IS2 at 17.5 kV, lens at 7.0 kV, reflectron 1 at 21.0 kV, reflectron 2 at 11.0 kV. Laser power level was adjusted to ensure high signal-to-noise ratios and low fragmentation. Detector gain was 10.2 ×, pulsed ion extraction was 50 ns and electronic gain 100 mV.

For each sample point, one spectrum was recorded after accumulation of 500 measurements on different spot locations. The matrix was 9AA 4 mg/mL in acetonitrile/water (1:1). MALDI was internally calibrated at each measurement on the mono-isotopic peak of [M+H]⁺ signals of 9AA (m/z: 193.0765 Da), kuromin aglycon (m/z: 285.0399 Da), kuromin (m/z: 447.0927 Da), myrtillin aglycon (m/z: 301.0399 Da), myrtillin (m/z: 463.0876 Da) and daphnoretin (m/z: 351.0504 Da). Aliquots (0.5 μL) of isolated metabolites were spotted onto MALDI plates (BrukerDaltonik GmbH, Leipzig, Germany), allowed to air dry, covered with an equal amount of standard solution, dried and then covered with 0.5 μL of 9AA.

HPLC-ESI-MS/DAD analyses were conducted both in positive and negative ion mode using a Hewlett-Packard Model 1100 Series liquid chromatography (Hewlett-Packard Development Company, L.P., Palo Alto CA, USA) coupled both to a Bruker Esquire-LC quadrupole ion-trap mass spectrometer (IT-MS) equipped with an electrosprey source (Bruker Optik GmbH, Ettlingen, Germany) and to a photo diode-array detector (Agilent Technologies, Milan, Italy, Agilent 1100). Chromatographic separation was conducted on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm i.D., particle size: 3.5 μm) purchased by Agilent (Agilent Technologies, Santa Clara, CA); solvent A consisted of 0.1 % formic acid in water and B, 0.1 % formic acid in methanol. Elution program was: 40 %A/60 %B for 3 min, then %B was increased to 75 % in 20 min and then to 100 % B in 5 min; operating flow was 1.0 mL/min. The following parameters were used: scan range: 100-1200 m/z at 13000 m/z s⁻¹; high purity nitrogen was used at a pressure of 35 psi, a temperature of 300 °C and at a flow rate of 7 L min⁻¹; high voltage capillary was set at 4000 V for positive ionization mode and -4000 V for negative mode. Injection volumes were set at 10 μL. The same parameters were also used for MS² and MS³ analyses.

**Cultures and Taxonomic Identification of *P. erythrina*:** *P. erythrina* (clone TL-1) was isolated from Lake Trasimeno (Perugia, Italy). Cells were cultured in a balanced salt solution [SBM: (1.5 m NaCl, 0.05 m KCl, 0.4 m CaCl₂, 0.05 m MgCl₂, 0.05 m MgSO₄, 2 m Na-phosphate buffer pH 6.8, 2 10⁻³ m EDTA)] (Miyake, 1981) and fed with the flagellate *Chlorogonium elongatum*, grown as described in ref.[16]

The taxonomic identification of *P. erythrina* was performed using both molecular and morphological data. DNA was extracted from cells which were re-suspended for at least one week without food in fresh culture medium, and pelletted by centrifugation. The extractions were performed using the QiAamp® DNA Micro Kit (Qiagen, Milan, Italy) in accord with manufacturer instructions, and the DNA concentrations were measured with a DU 640 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The small subunit (SSU) rRNA gene was PCR amplified using the universal eukaryotic forward primer 18S F9 5'-CTGTTGATACCGTCCAG-3' and the 18S R1513 Hypo reverse primer 5'-TACCCCTYGCAGGTTC-3' [18] PCR amplifications were performed by adding DNA aliquots (100 ng) to 50 μL of reaction mixture containing 2 μL MgCl₂, 250 μL of dNTP, one unit of Taq DNA polymerase (Polymed, Florence, Italy) and 0.2 μL of each primer. Amplifications were run in a GenAmp PCR system 2400 (Applied Biosystems, Foster City, CA, USA), following a standard program (30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 120 s at 72 °C), with an initial denaturation step of 5 min at 94 °C and a final extension step of 5 min at 72 °C. Amplified products were purified using Quantum Prep PCR Kleen Spin columns (Bio-Rad, Hercules, CA, USA) and sequenced in both directions with an ABI Prism 310 automated DNA sequencer (Applied Biosystems). To minimize amplification errors, sequences of two different amplicons were compared. The correct assignment of the species was further verified by means of a morphological analysis on in vivo and fixed specimens using previously described methods.[33]

**Extraction, Isolation and Purification of Secondary Metabolites from *P. erythrina* Cell Cultures:** *P. erythrina* lyophilized cell cultures (about 5.5 × 10⁶ cells) were extracted three times with ethanol (50 mL), three times with acetone (50 mL) and three times with methanol/chloroform (50 mL, 1:2, v/v) in glass vials, until the cell
cultures appeared colourless. After solvent addition, cultures were sonicated 15 min in an ultrasonic bath (Sonorex Super, Bandelin electronics, Berlin, Germany) and centrifuged 10 min at 3000 x g at room temperature. Each fraction was concentrated in a rotary evaporator and then small aliquots were loaded onto TLC silica gel 60F254 plates (Merck KgaA, Darmstadt, Germany), developed with n-hexane/ethyl acetate (1:1, v/v), and TLC silica gel 60 RP18 F254 (Merck KgaA, Darmstadt, Germany), which were developed with methanol/water (9:1, v/v). Extracts (15.6 mg) were combined together, dried in an evaporator, dissolved in methanol (200 μL) and loaded to a 2 g RP18 SPE cartridge (Supelco Analytical, Bellefonte, PA, USA) and eluted with mixtures of water and methanol. Fractions (5 mL) were collected and tested on TLC as described above. Metabolites of interest were collected in fractions 1 and 2, eluting before chlorophylls. These fractions were purified by reversed-phase Agilent Zorbax Eclipse XDB-C18 column (150 x 4.6 mm, 3.5 μm) eluting as described above for HPLC-ESI-MS/DAD and following the chromatogram as recorded with detection at 305 nm. Purified compounds were collected, concentrated and analyzed by NMR and HPLC-ESI-MS.

**Erythrolactone A1 (1):** Pale yellow, amorphous solid. UV (methanol): $\lambda_{max} = 250, 306 \text{ nm}$. 1H NMR (400 MHz, [D6]MeOH): $\delta = 6.86$ [s, 1 H, H-C(12)], 6.786 [d, $J = 2.9$ Hz, 1 H, H-C(5)], 6.684 [d, $J = 16.0$ Hz, 1 H, H-C(7)], 6.593 [d, $J = 8.6$ Hz, 1 H, H-C(2)], 6.497 [dd, $J = 8.6, 2.9$ Hz, 1 H, H-C(3)], 6.148 [dt, $J = 15.9, 6.8$ Hz, 1 H, H-C(8)], 2.645 [t, $J = 7.1$ Hz, 2H, H-C(10)], 2.544 [q, $J = 6.7$ Hz, 2H, H-C(9)], 2.375 [m, 2 H, H-C(11)], 1.453 [m, 2 H, H-C(2)], 1.297 [m, 10 H, H-C(3): H-C(7)], 0.893 [t, $J = 6.9$ Hz, 3 H, H3-C(7)] ppm. 13C NMR (100 MHz, [D6]MeOH): $\delta = 164.42$ [s, C(15)], 162.80 [s, C(13)], 164.31 [s, C(1)], 162.80 [s, C(6)], 127.71 [s, C(7)], 126.38 [s, C(6)], 118.73 [d, C(2)], 116.00 [d, C(3)], 113.38 [d, C(5)], 111.11 [s, C(4)], 114.19 [t, C(9)], 30.48 [t, C(8)], 28.97 [t, C(2)], 23.86 [t, C(1)], 23.71 [t, C(6)], 14.42 [q, C(7)] ppm. HR-MArDI-TOF-MS: $m/z$ 451.1450 [M - Na]$^+$ (calcd. for C21H25O2S: 451.1427; M - Na) ppm. ESI-MS (positive ion mode detection) $m/z$: 373.3 $[M + H]^+$, 395.3 $[M + Na]^+$. ESI-MS (negative ion mode detection) $m/z$: 371.0 $[M - H]^-$.

**Erythrolactone A2 (4):** Orange, red-brick amorphous solid. UV (methanol): $\lambda_{max} = 250, 302 \text{ nm}$. 1H NMR (400 MHz, [D6]MeOH): $\delta = 6.86$ [s, 1 H, H-C(12)], 6.786 [d, $J = 2.9$ Hz, 1 H, H-C(5)], 6.684 [d, $J = 16.0$ Hz, 1 H, H-C(7)], 6.593 [d, $J = 8.6$ Hz, 1 H, H-C(2)], 6.497 [dd, $J = 8.6, 2.9$ Hz, 1 H, H-C(3)], 6.148 [dt, $J = 15.9, 6.8$ Hz, 1 H, H-C(8)], 2.645 [t, $J = 7.1$ Hz, 2H, H-C(10)], 2.544 [q, $J = 6.7$ Hz, 2H, H-C(9)], 2.375 [m, 2 H, H-C(11)], 1.453 [m, 2 H, H-C(2)], 1.297 [m, 10 H, H-C(3): H-C(7)], 0.893 [t, $J = 6.9$ Hz, 3 H, H3-C(7)] ppm. 13C NMR (100 MHz, [D6]MeOH): $\delta = 164.42$ [s, C(15)], 162.80 [s, C(13)], 164.31 [s, C(1)], 162.80 [s, C(6)], 127.71 [s, C(7)], 126.38 [s, C(6)], 117.37 [s, C(2)], 116.00 [d, C(3)], 113.38 [d, C(5)], 99.78 [s, C(14)], 100.20 [d, C(12)], 34.22 [t, C(10)], 33.02 [t, C(4)], 31.61 [t, C(9)], 30.48 [t, C(8)], 28.97 [t, C(2)], 23.86 [t, C(1)], 23.71 [t, C(6)], 14.42 [q, C(7)] ppm. HR-MArDI-TOF-MS: $m/z$ 451.1450 [M - Na]$^+$ (calcd. for C21H25O2S: 451.1427; M - Na) ppm. ESI-MS (positive ion mode detection) $m/z$: 373.3 $[M + H]^+$, 395.3 $[M + Na]^+$. ESI-MS (negative ion mode detection) $m/z$: 371.0 $[M - H]^-$.
(100 MHz, D$_2$O methanol);  δ = 164.42 [s, C(15)], 162.80 [s, C(13)], 164.31 [s, C(11)], 151.21 [s, C(4)], 148.67 [s, C(1)], 128.64 [d, C(8)], 127.71 [d, C(7)], 126.38 [s, C(6)], 117.37 [d, C(2)], 116.00 [d, C(3)], 113.38 [d, C(5)], 99.78 [s, C(14)], 100.20 [d, C(12)], 34.42 [t, C(10)], 33.02 [t, C(6)], 31.61 [t, C(9)], 30.48 [t, 3 C. C(3) + C(4) + C(5)], 30.29 [t, C(3) or C(4)], 28.97 [t, C(2)], 23.86 [t, C(1)], 23.71 [t, C(7)], 14.42 [t, C(8)] ppm. HR-MALDI-TOF-MS: m/z 385.2030 [M – H]$^-$ (calcd for C$_{23}$H$_{29}$O$_2^-$: 385.2015 A. 3.89 ppm). ESI-MS (positive ion mode detection) m/z: 387.3 [M + H]$,^+$, 409.3 [M + Na]$^+$, ESI-MS (negative ion mode detection) m/z: 385.0 [M – H]$^-$: t$_{R}$ 20.4 min (2 mg).

Induction of Erythrolactone Discharge: The cold-shock treatment$^{[19]}$ was applied to P. erythrina to obtain the metabolite-containing discharge. Briefly, a dense suspension of ciliates (about 400,000 cells/mL) were quickly mixed in a 1:5 ratio with ice-cooled SMB, at 0 °C for 30 or 5 s, and then centrifuged at about 50 g to separate the cells from the supernatant. Precipitated cells were washed twice, re-suspended in SMB at room temperature for 2 h, and then used in experiments. The pigment granule deficient cells obtained by this procedure (with 5 s of exposition) were as healthy as control cells (data not shown). The supernatant containing the metabolite-discharge was adsorbed onto a Sep-Pak C18 cartridge (Water, Milford, MA), which was then washed with distilled water and eluted with 60% 2-propanol (2 mL) followed by 100% 2-propanol (1 mL). The eluted fraction containing the discharge from the pigment granules was dried by vacuum centrifuge, weighed, and then stored at -20 °C until use.

Supporting Information (see footnote on the first page of this article): 400 MHz NMR (CD$_3$OD) spectra of erythrolactone B2. $^1$H NMR (Figure S1), COSY full spectrum (Figure S2), HSQC, HMBC, $^{13}$C NMR.

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