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# *In vitro* Conversion of Vinyl to Formyl Groups in Naturally Occurring Chlorophylls

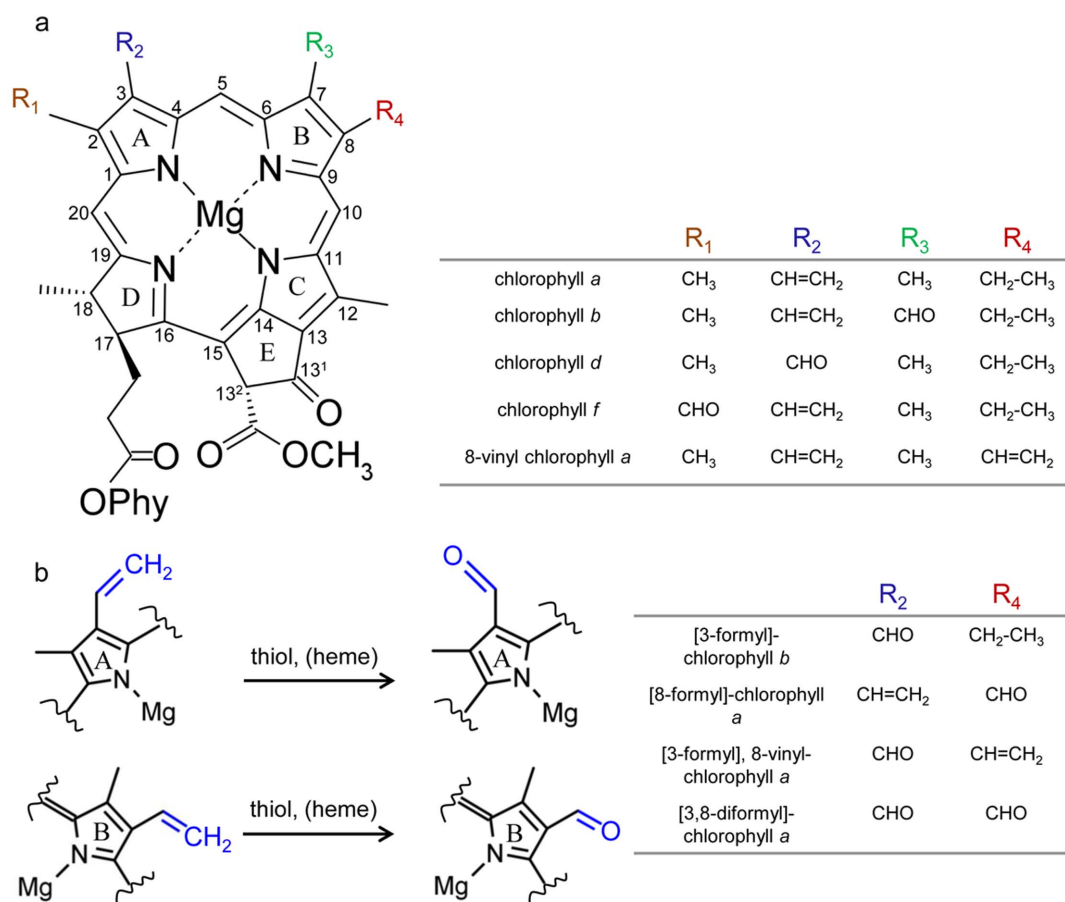
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The chemical structural differences distinguishing chlorophylls in oxygenic photosynthetic organisms are either formyl substitution (chlorophyll *b*, *d*, and *f*) or the degree of unsaturation (8-vinyl chlorophyll *a* and *b*) of a side chain of the macrocycle compared with chlorophyll *a*. We conducted an investigation of the conversion of vinyl to formyl groups among naturally occurring chlorophylls. We demonstrated the *in vitro* oxidative cleavage of vinyl side groups to yield formyl groups through the aid of a thiol-containing compound in aqueous reaction mixture at room temperature. Heme is required as a catalyst in aqueous solution but is not required in methanolic reaction mixture. The conversion of vinyl- to formyl- groups is independent of their position on the macrocycle, as we observed oxidative cleavages of both 3-vinyl and 8-vinyl side chains to yield formyl groups. Three new chlorophyll derivatives were synthesised using 8-vinyl chlorophyll *a* as substrate: 8-vinyl chlorophyll *d*, [8-formyl]-chlorophyll *a*, and [3,8-diformyl]-chlorophyll *a*. The structural and spectral properties will provide a signature that may aid in identification of the novel chlorophyll derivatives in natural systems. The ease of conversion of vinyl- to formyl- in chlorophylls demonstrated here has implications regarding the biosynthetic mechanism of chlorophyll *d* *in vivo*.

Chlorophylls are cyclic tetrapyrroles essential for photosynthesis, participating in both photon capture and conversion into chemical energy. Naturally occurring chlorophylls are distributed across oxygenic photosynthetic organisms, with Chl *a* predominating in most of these organisms. All chlorophylls have similar chemical structure with one or two side group substitutions. Chl *b*, *d* and *f* differ from Chl *a* by a formyl substitution at the C7, C3 and C2 positions, respectively (Figure 1a). Chl *b* is largely found in higher plants as well as prochlorophytes and acts as an accessory pigment. The marine cyanobacterium *Prochlorococcus* uniquely utilises 8-vinyl Chl *a* (Figure 1a) and 8-vinyl Chl *b* for oxygenic photosynthesis<sup>1</sup>. Both 8-vinyl Chl *a* and 8-vinyl Chl *b* have an unreduced vinyl side group at the C8 position in addition to the vinyl group at the C3 position and are also called 3,8 divinyl chlorophylls. The unicellular cyanobacterium *Acaryochloris marina* has replaced Chl *a* with Chl *d* for almost all photosynthetic functions<sup>2,3</sup>. The most recently discovered naturally occurring chlorophyll, Chl *f*, is a minor pigment that has been found in two disparate cyanobacterial lineages<sup>4,5</sup>. Replacement of either methyl or vinyl side chains of Chl *a* for formyl groups manifest themselves in different spectral and chemical manners, depending on the change in the distribution of electrons across the macrocycle<sup>6</sup>.

Chl *b* is synthesised from chlorophyll(ide) *a* via the enzyme CAO (chlorophyll(ide) *a* oxidase)<sup>7-9</sup>. CAO catalyses two hydroxylations at the C7 position of chlorophyll(ide) *a*, yielding an aldehyde hydrate which spontaneously forms a formyl group to yield Chl *b*. Chl *d* is also most likely synthesised from chlorophyll(ide) *a*<sup>10</sup>, but the enzyme(s) responsible are unknown. The biosynthesis of Chl *d* from Chl *a* must involve a different mechanism from the characterised CAO enzyme as it requires the conversion of the 3-vinyl group of Chl *a* to a 3-formyl group in Chl *d* rather than a methyl to formyl conversion as is the case for the synthesis of Chl *b* from Chl *a*. The oxidative cleavage of the 3-vinyl in Chl *a* (or its demetallated/dephytylated derivatives) to yield a formyl group can be achieved chemically in solvents using a strong oxidising agent<sup>11,12</sup> or using thiol-containing compounds under acidic conditions<sup>13-15</sup>. Additionally, Chl *d* has been synthesised non-enzymatically in the presence of the cysteine protease papain<sup>16,17</sup>. Although chemical syntheses of 2-formyl chlorins have recently been described<sup>18,19</sup>, as yet there are no published data regarding the biosynthesis of Chl *f*.

Here we describe the oxidative cleavage of vinyl side chains of naturally occurring chlorophylls to yield formyl groups. Although not enzyme catalyzed, it progresses in a 'biological-like' environment: in an aqueous buffer at a moderate pH and at room temperature in the presence of a thiol containing compound and heme. When



**Figure 1** | Chemical structures of chlorophylls and schematic of the vinyl to formyl conversion described in this study. (a) Structures of naturally occurring chlorophylls. Carbon skeleton atoms are numbered according to the IUPAC system and rings A–E on the macrocycle are indicated. (b) partial structures of chemically synthesised formyl chlorophyll derivatives on ring A (C3) and ring B (C8). Vinyl and formyl groups are highlighted in blue. Phytol (C<sub>20</sub>H<sub>39</sub>).

methanol is used as a solvent, only the thiol-containing compound is vital for this oxidative cleavage reaction. As well as demonstrating the synthesis of Chl *d* from Chl *a*, we show this oxidative cleavage mechanism can be extended to the vinyl side group at other positions of chlorophylls, such as the 3-vinyl group in Chl *b* and 8-vinyl group in 8-vinyl chlorophyll *a*, to give formyl substitution derivatives, including [3-formyl]-Chl *b*, [8-formyl] Chl *a*, [3-formyl], 8-vinyl Chl *a*, and [3,8-diformyl] Chl *a* (or 8-formyl Chl *d*) under these reaction conditions (Figure 1b).

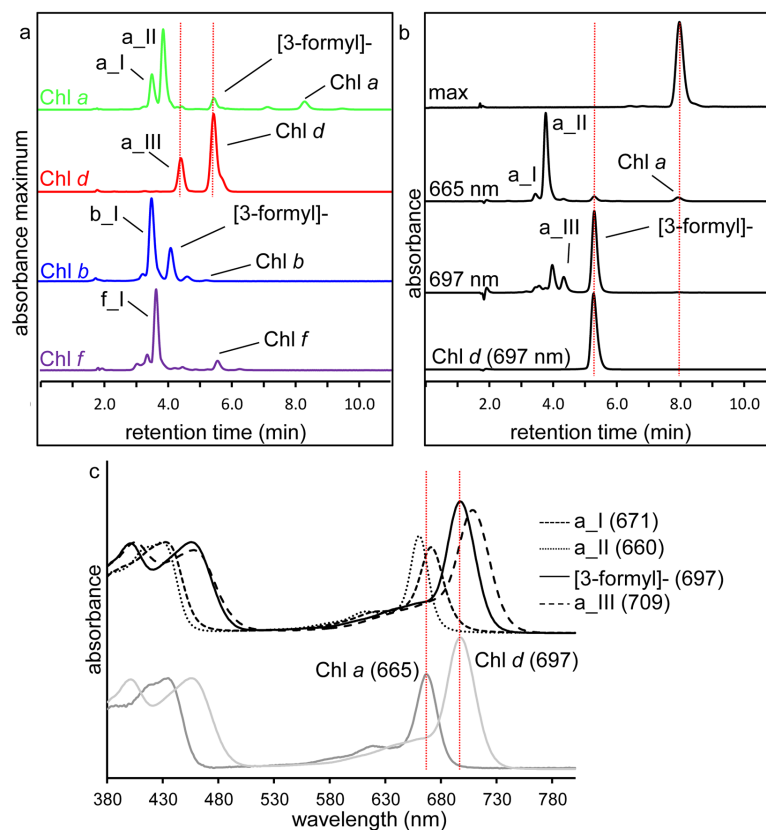
## Results

**Synthesis of [3-formyl]-Chl *a* (also named as Chl *d*) from Chl *a*.** To characterise the reaction mechanism for the biosynthesis of Chl *d* from Chl *a*, we tested a number of *E. coli*-expressed *Acaryochloris* enzymes along with various cofactors, initially basing the reaction conditions on those reported to characterise the CAO enzyme *in vitro*<sup>8</sup>. However, rather than identifying the enzymatic synthesis of Chl *d* from Chl *a*, we determined the requirements for the non-enzymatic synthesis of Chl *d* from Chl *a* in an aqueous buffer. The minimal reaction conditions in 50 mM Tris, pH 8.0 were detergent-solubilised Chl *a*, heme and  $\beta$ -mercaptoethanol which typically yielded approximately 10% Chl *d* after 18 h at room temperature with stirring (Figure 2a, top panel). Replacing the aqueous buffer with methanol accelerated this reaction with almost all of the Chl *a* substrate consumed after 4 h (Figure 2b). Interestingly, under these conditions heme was not required for the reaction to proceed. The accelerated reaction rate observed is likely due to the increased solubility of Chl *a* in methanol and the 10 fold higher solubility of

oxygen in methanol compared to in an aqueous buffer<sup>20</sup>. As conversion of the 3-vinyl group of Chl *a* to the 3-formyl group of Chl *d* was achieved in the presence of  $\beta$ -mercaptoethanol alone in methanol but not in the aqueous buffer we suggest that oxygen-bound heme is the essential reactant in the aqueous reaction mixture. Addition of hydrogen peroxide to the reaction mix accelerated the reaction significantly, most likely due to re-reduction of any oxidised heme, and when heme was removed from the reaction mix or replaced with protoporphyrin IX, almost all of the Chl *a* remained unreacted (data not shown).

In both methanolic or the aqueous buffer the same major products were formed, although the relative amounts of each product formed was somewhat different (Figure 2). Four detectable products were observed under both reaction conditions- products of a\_I, a\_II, a\_III and Chl *d* with a small amount of unreacted Chl *a* remaining (Figure 2a and 2b). Chemically synthesised Chl *d* (or [3-formyl]-Chl *a*) was positively identified based on comparison of its absorption spectrum, retention time and mass with that of *bona fide* Chl *d* from *Acaryochloris* (Figure 2b and 2c, Table 1).

The major byproduct, a\_II, had a slightly blue-shifted Q<sub>y</sub> maximum (*c.f.* Chl *a*) (Figure 2c) and a molecular mass of 986 Da (Table 1). Based on <sup>1</sup>H-<sup>1</sup>H, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) spectroscopy analysis, product a\_II was identified as the sulfoxide derivative of [3<sup>1</sup>- $\beta$ -mercaptoethanol]-Chl *a* (Table S1, Figure S1). The most polar Chl *a* derivative, product a\_I, had a slightly red-shifted Q<sub>y</sub> maximum relative to Chl *a* (Figure 2c) and a mass of 1062 Da (Table 1). This product was not characterised further, how-

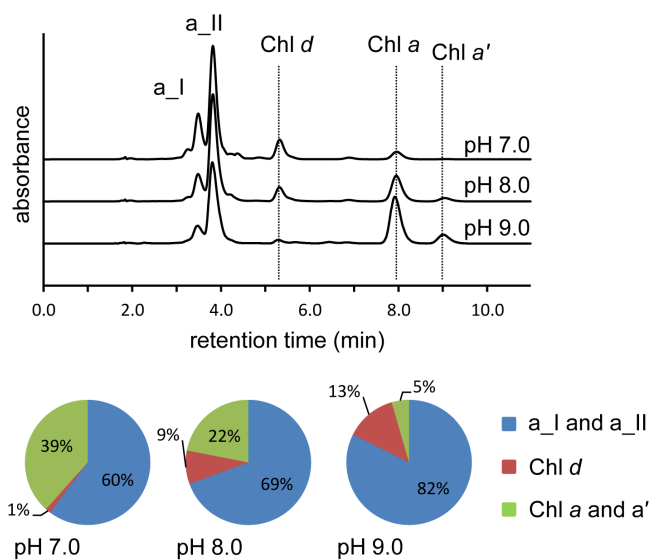


**Figure 2 | Reaction of monovinyl chlorophylls with  $\beta$ -mercaptoethanol in aqueous and methanolic environments.** (a) Spectrum maximum (maximum absorbance reading at the range of 630–710 nm) RP-HPLC chromatograms of the aqueous buffer reaction mixture after reaction with  $\beta$ -mercaptoethanol using purified Chl *a*, Chl *d*, Chl *b* or Chl *f* as reactant. Dotted vertical lines mark the retention time of product a\_III and Chl *d*. For more details regarding products a\_I, a\_II, a\_III, b\_I and f\_I refer to the text and Table 1. (b) RP-HPLC chromatograms of Chl *a* prior to (top panel) and following (two middle panels) reaction with  $\beta$ -mercaptoethanol in methanol, detected at indicated wavelengths. Chromatogram of Chl *d* from *Acaryochloris* (lower chromatogram) is shown for comparison with the chemically synthesised [3-formyl]-Chl *a* (Chl *d*). Dotted vertical line marks the retention time of Chl *d* and Chl *a*. (c) Online spectra of the major derivatives synthesised from Chl *a*. The absorption spectra of Chl *a* and Chl *d* are plotted and their  $Q_y$  maxima are marked with vertical lines for comparison with synthesised products.  $Q_y$  maxima are indicated (in nm).

**Table 1 | Spectral and mass properties for Chl *a*, Chl *b*, Chl *f* and 8-vinyl Chl *a* (in bold) and their major derivatives following reaction with  $\beta$ -mercaptoethanol**

Chlorophyll (Chl) and Derivatives	Soret (nm)	$Q_y$ (nm)	Soret: $Q_y$ ratio (intensity)	Mass (Da)
<b>Chl <i>a</i></b>	433	666	0.97	892
a_I	432	671	1.06	1062
a_II ([3 <sup>1</sup> - $\beta$ ME]-Chl <i>a</i> )	429	660	0.88	986
a_III	406	709	0.74	970
[3-formyl]-Chl <i>a</i> (Chl <i>d</i> )	402, 457	697	0.74	894
<b>Chl <i>b</i></b>	470	653	2.4	906
b_I ([3 <sup>1</sup> - $\beta$ ME]-Chl <i>b</i> )	468	648	2.44	1000
[3-formyl]-Chl <i>b</i> ([3,7-diformyl]-Chl <i>a</i> )	484	674	2.54	908
<b>Chl <i>f</i></b>	407	707	0.90	906
f_I ([3 <sup>1</sup> - $\beta$ ME]-Chl <i>f</i> )	404	704	0.94	1000
<b>8-vinyl Chl <i>a</i></b>	443	667	1.15	890
[3 <sup>1</sup> ,8 <sup>1</sup> -di $\beta$ ME]-Chl <i>a</i>	435	659	1.20	1078
[3 <sup>1</sup> - $\beta$ ME, 8-formyl]-Chl <i>a</i>	462	671	2.65	986
[3-formyl,8 <sup>1</sup> - $\beta$ ME]-Chl <i>a</i>	462	693	1.04	986
[3 <sup>1</sup> - $\beta$ ME,8-vinyl]-Chl <i>a</i>	440	661	1.06	984
[3,8-diformyl]-Chl <i>a</i>	487	687	2.34	894
[8 <sup>1</sup> - $\beta$ ME]-Chl <i>a</i>	438	664	1.01	984
[8-formyl]-Chl <i>a</i>	465	675	2.34	892
[3-formyl, 8-vinyl]-Chl <i>a</i> (8-vinyl Chl <i>d</i> )	467	695	0.99	892

Absorption maxima (in nm) are given according to HPLC online absorption spectra in 100% methanol; Soret: $Q_y$  ratios and mass (in Da) of the formyl and sulfoxide of  $\beta$ -mercaptoethanol ( $\beta$ ME) derivatives are given. The chemical subunits in square parentheses represent the substituted side groups and the chemical names in normal brackets represent the alternative designated name for synthesised chlorophyll derivatives.

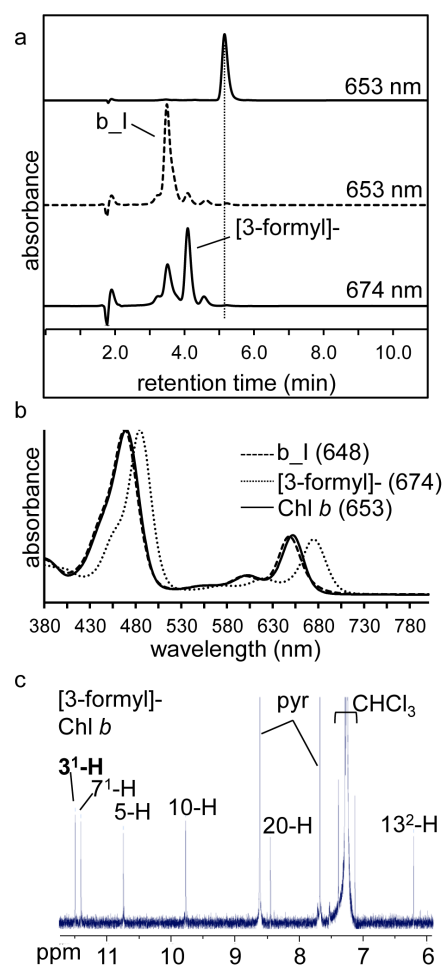


**Figure 3 | pH dependence of Chl *d* chemical synthesis from Chl *a*.** Chromatograms (spectrum maximum plots) of detergent solubilised Chl *a* reacted with  $\beta$ -mercaptoethanol and heme in a 50 mM Tris buffer at the indicated pH. Unreacted Chl *a*, Chl *d* and 3<sup>1</sup>-sulfoxide of  $\beta$ -mercaptoethanol derivatives (a\_I and a\_II) are marked. Proportions of the major products and remaining Chl *a* and Chl *a'* (the C13<sup>2</sup>-Chl *a* epimer) are indicated in the pie charts for each pH tested.

ever under the same reaction conditions as described above, product a\_I was synthesised from HPLC-purified product a\_II (and no Chl *d* was produced). Another product, a\_III, had a mass of 970 Da and a significantly red-shifted absorption spectrum with a  $Q_y$  maximum of 709 nm (Figure 2, Table 1). Again, this product was not examined further, however it was also derived from Chl *d* under these reaction conditions, indicating Chl *d* is an intermediate product in the synthesis of a\_III (Figure 2a). In order to confirm the reactive properties of 3-vinyl group of chlorophylls, both Chl *b* and Chl *f* also reacted with  $\beta$ -mercaptoethanol, with both yielding probable sulfoxide of [3<sup>1</sup>- $\beta$ -mercaptoethanol]- derivatives based on their masses, retention times and spectral properties relative to the substrate (Figure 2a, products b\_I and f\_I, Table 1). Strikingly, although [3-formyl]-Chl *b* was identified when Chl *b* was substrate (see below), no [3-formyl]-Chl *f* was observed.

As pH affects the equilibrium protonation state of thiol reagents and therefore their function<sup>15</sup>, we examined the effect of pH on the conversion of Chl *a* and using  $\beta$ -mercaptoethanol as the thiol reagent. There was an increase in the Chl *d* yield (from 9% up to 13%) when the pH was reduced from 8.0 to 7.0 (Figure 3), however when the pH was dropped further to 5.6, demetallation and dephytylation of Chl *a* were observed (data not shown). Increasing the pH to 9.0 reduced Chl *d* yields significantly with Chl *d* making up only 1% of the chlorophyll derivatives and ~40% of the pigment remaining as Chl *a* or the C13<sup>2</sup>-epimer, Chl *a'* (Figure 3).

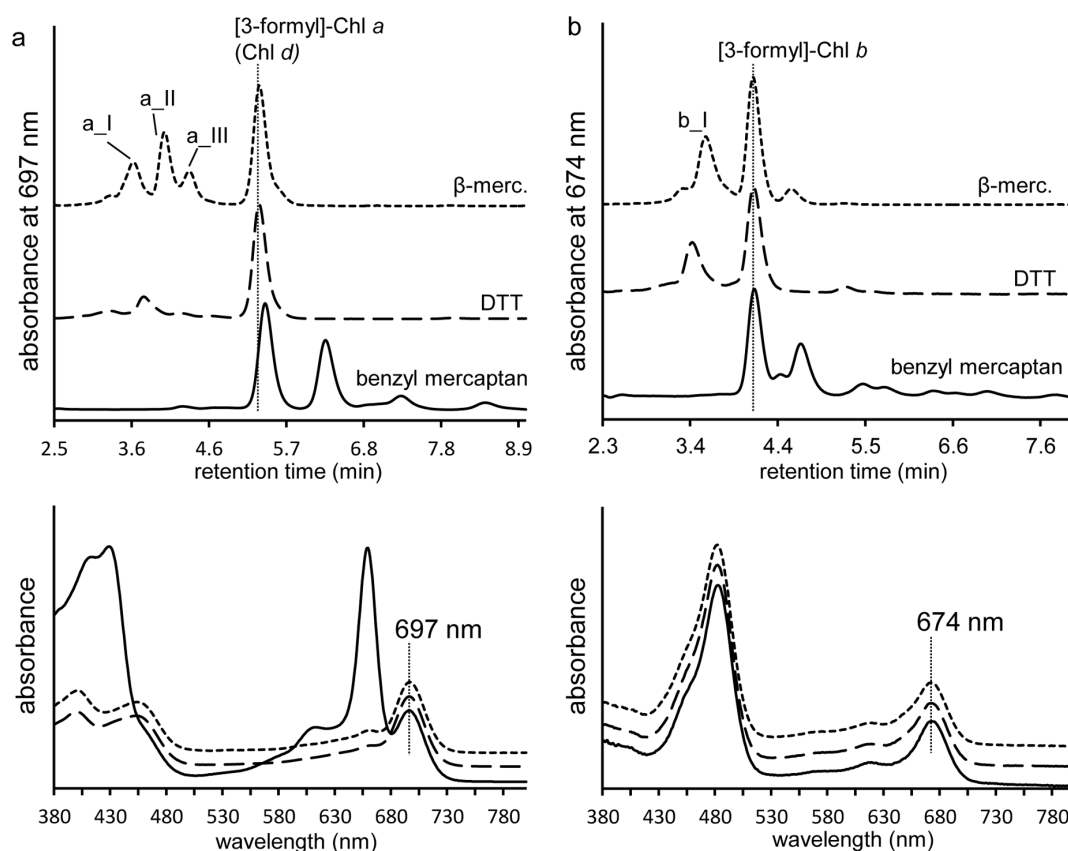
**[3-formyl]-Chl *d* is synthesised from Chl *b*.** Chl *b* differs from Chl *a* by a 7-methyl group of Chl *a* being replaced by a formyl group in Chl *b* (Figure 1a). The presence of the 3-vinyl group is common to both Chl *a* and Chl *b*. When Chl *b* was used as substrate, we also observed the conversion of its 3-vinyl to a 3-formyl group, yielding [3-formyl]-Chl *b* (Figure 4). [3-formyl]-Chl *b* showed a characteristic red shift of both the Soret and  $Q_y$  peaks (*cf.* Chl *b*), with a  $Q_y$  maximum of ~674 nm (Figure 3b), which is the same as that of the previously described [3-formyl]-Chl *b* ([7-formyl]-Chl *d*) synthesised in a *Prochlorothrix hollandica* CAO (*PhCAO*)-expressing *Acaryochloris* mutant<sup>21</sup>. The mass (908 Da, Table 1) and <sup>1</sup>H-NMR spectrum of the [3-formyl]-Chl *b* confirmed the presence of both the 3-formyl and 7-



**Figure 4 | Synthesis of [3-formyl]-Chl *b* from Chl *b*.** (a) RP-HPLC chromatograms of Chl *b* prior to reaction (upper panel) and following reaction (lower panels), detected at indicated wavelength. Dotted vertical line marks the retention time of Chl *b*. (b) Online spectra of Chl *b*, chemically synthesised [3-formyl]-Chl *b* and product b\_I. For more detail regarding product b\_I refer to the text and Table 1.  $Q_y$  maxima are as indicated (in nm). (c) Partial <sup>1</sup>H-NMR spectrum of [3-formyl]-Chl *b* with the 3<sup>1</sup> proton in bold. Peaks corresponding to CHCl<sub>3</sub>, and pyridine (pyr) are indicated.

formyl groups (Figure 4c). Surprisingly, the efficiency of [3-formyl]-Chl *b* synthesis was around 30%, assuming the extinction coefficients of the Chl *b* derivatives are equal, which is significantly more than the 10–12% yield observed for Chl *d* ([3-formyl]-Chl *a*) synthesis using Chl *a* as substrate. Additionally, the synthesis of [3-formyl]-Chl *b* yielded fewer byproducts compared to Chl *d* synthesis, with a single major byproduct, b\_I, formed (Figure 2a and 4a). As was the case with the major byproduct formed with Chl *a* as the substrate (a\_II), this b\_I had the same mass shift of plus 94 Da, reduced retention time and an absorption spectrum relative to the substrate (Chl *b*) and so is likely the sulfoxide derivative of [3<sup>1</sup>- $\beta$ -mercaptoethanol]-Chl *b* (Figure 4b, Table 1).

To examine the role of the thiol compound in the conversion of vinyl to formyl, different thiol compounds were tested under the same reaction conditions using either Chl *a* or Chl *b* as substrate. Replacing  $\beta$ -mercaptoethanol with either dithiothreitol or benzyl mercaptan also yielded the conversion of the 3-vinyl group of Chl *a* and Chl *b* to a 3-formyl group (Figure 5), and in the absence of a thiol compound no formyl group was formed (data not shown), confirming the indispensable role of the thiol group in this co-oxidative reaction.



**Figure 5** | Dithiothreitol and benzyl mercaptan also facilitate the conversion of the C3 vinyl of Chl *a* (a) and Chl *b* (b) to a formyl. RP-HPLC chromatograms (top panels) demonstrate retention times of the 3<sup>1</sup>-formyl derivatives of Chl *a* and Chl *b* were identical and are marked with vertical lines. Spectra (bottom panels) were identical to the 3<sup>1</sup>-formyl derivatives formed in the presence of β-mercaptoethanol (β-merc.). Note, in the case of the reaction of Chl *a* with benzyl mercaptan, another product spectrally similar to Chl *a* has a similar retention time to Chl *d*, causing the mixed online absorption spectrum. Q<sub>y</sub> for [3-formyl]-Chl *a* (Chl *d*) and [3-formyl]-Chl *b* are marked. Dotted line, β-mercaptoethanol; dashed line, dithiothreitol; solid line, benzyl mercaptan.

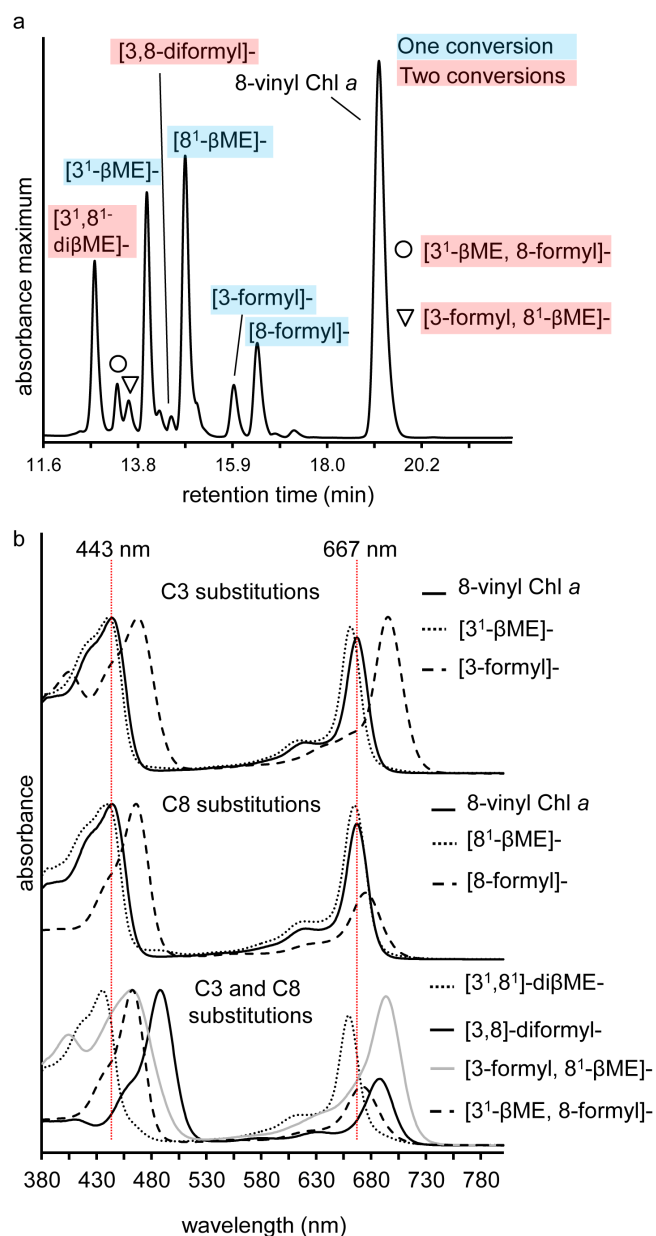
**The thiol-driven reaction targets both the C3 and C8 vinyl of 8-vinyl Chl *a*.** For both Chl *a* and Chl *b*, the initial reaction target is the 3-vinyl under our reaction conditions. To test whether this reaction was structurally specific to the location of vinyl group on the chlorophyll macrocycle, we reacted 8-vinyl Chl *a* (two vinyls, at the C3 and the C8 position, Figure 1a) with β-mercaptoethanol in methanol. A large number of polar products with varying absorption spectra were produced (Figure 6). Mass spectrometry and <sup>1</sup>H-NMR spectroscopy confirmed two of these products corresponded to two novel formyl chlorophylls: [3-formyl], 8-vinyl Chl *a* (or 8-vinyl Chl *d*) and [8-formyl]-Chl *a* (Figure 7a, Table 1, Figure S2). These results demonstrate that the thiol-driven oxidative cleavage of the vinyl is not specific to a particular position on the macrocycle. When HPLC-purified [3-formyl], 8-vinyl Chl *a* or [8-formyl]-Chl *a* was reacted with β-mercaptoethanol again, both yielded the same product with a mass of 894 Da, plus 2 relative to the substrate, which we conclude is the [3,8-diformyl]-Chl *a* (or 8-formyl Chl *d*; Figure 7b, Table 1, Figure S2). This was confirmed by <sup>1</sup>H-NMR spectroscopy, with key proton resonances for the [3,8-diformyl]-Chl *a* being δ<sub>H</sub>(600 MHz; CDCl<sub>3</sub>; Me<sub>4</sub>Si) 6.23 (1 H, s, C<sub>13-2</sub>), 8.52 (1 H, s, C<sub>20</sub>), 10.37 (1 H, s, C<sub>5</sub>), 10.54 (1 H, s, C<sub>10</sub>), 11.31 (1 H, s, C<sub>8-1</sub>), 11.37 (1 H, s, C<sub>3-1</sub>) inter alia (Figure 7a). This [3,8-diformyl]-Chl *a* product was also formed when 8-vinyl Chl *a* was reacted with β-mercaptoethanol (Figure 4) however at the completion of the reaction the yield of [3,8-diformyl]-Chl *a* was only ~2% of the chlorophylls synthesised. Interestingly, this chlorophyll derivative shares spectral characteristics of both the single 3- and 8-formyl chlorophyll derivatives, with a Q<sub>y</sub> maximum

of 687 nm, intermediate between the two, and an increased Soret:Q<sub>y</sub> ratio, similar to the [8-formyl]-Chl *a* (Figure 6b, Table 1).

Having a formyl group at both the C3 and C8 positions also bathochromically shifts the Soret peak by approximately 20 nm relative to either of the single formyl chlorophylls (from 466 nm to 487 nm). As was the case for Chl *a*, Chl *b* and Chl *f*, based on mass, relative retention times and spectral characteristics, we also observed the formation of probable sulfoxide of β-mercaptoethanol (βME) derivatives as well as formyl groups, at the C3 and C8 positions of 8-vinyl Chl *a*. These were as follows: [8<sup>1</sup>-βME]-Chl *a*, [3<sup>1</sup>-βME], 8-vinyl-Chl *a*, [3-formyl, 8<sup>1</sup>-βME]-Chl *a*, [3<sup>1</sup>-βME, 8-formyl]-Chl *a* and [3<sup>1</sup>, 8<sup>1</sup>-βME]-Chl *a* (Figure 6, Table 1), with approximately 60% of the chlorophyll at the end of the reaction corresponding to [3<sup>1</sup>, 8<sup>1</sup>-βME]-Chl *a* (data not shown). The 3<sup>1</sup>-βME derivative and the 8<sup>1</sup>-βME derivatives of Chl *a* were distinguished by reacting these derivatives with β-mercaptoethanol to form the [3<sup>1</sup>-sulfoxide, 8-formyl]- and [3-formyl, 8<sup>1</sup>-sulfoxide]- derivatives respectively and comparing them to products formed from the structurally confirmed [3-formyl], 8-vinyl Chl *a* (8-vinyl Chl *d*) and [8-formyl]-Chl *a* (Figure 7b, Table 1).

## Discussion

Interaction between chlorophylls and either a thiol reagent and heme in an aqueous buffer environment or thiol alone in a methanol environment leads to the synthesis of novel C3 and C8 position chlorophyll derivatives. Both Chl *a* and Chl *b* have a vinyl group at the C3 position and two reaction pathways result in the formation of either



**Figure 6** | RP-HPLC chromatogram and online absorption spectra of 8-vinyl Chl *a* formyl and sulfoxide of  $\beta$ -mercaptoethanol derivatives. (a) RP-HPLC spectrum maximum plot of an incomplete 8-vinyl Chl *a* reaction after 2 h. Products with either a C3 or C8 conversion (one conversion) are marked in blue, and those products where both the C3 and C8 vinyl have reacted (two conversions) are marked with red. (b) Online spectra of 8-vinyl Chl *a* and the major formyl and sulfoxide of  $\beta$ -mercaptoethanol derivatives. The Soret and Q<sub>y</sub> maxima of 8-vinyl Chl *a* are marked with vertical red lines. Spectra are arithmetically shifted for clarity.  $\beta$ ME, sulfoxide of  $\beta$ -mercaptoethanol.

3<sup>1</sup>-sulfoxide  $\beta$ -mercaptoethanol derivatives, or 3-formyl derivatives (Figure 8). In the case of 8-vinyl Chl *a*, these reactions are extended to the C8-vinyl group.

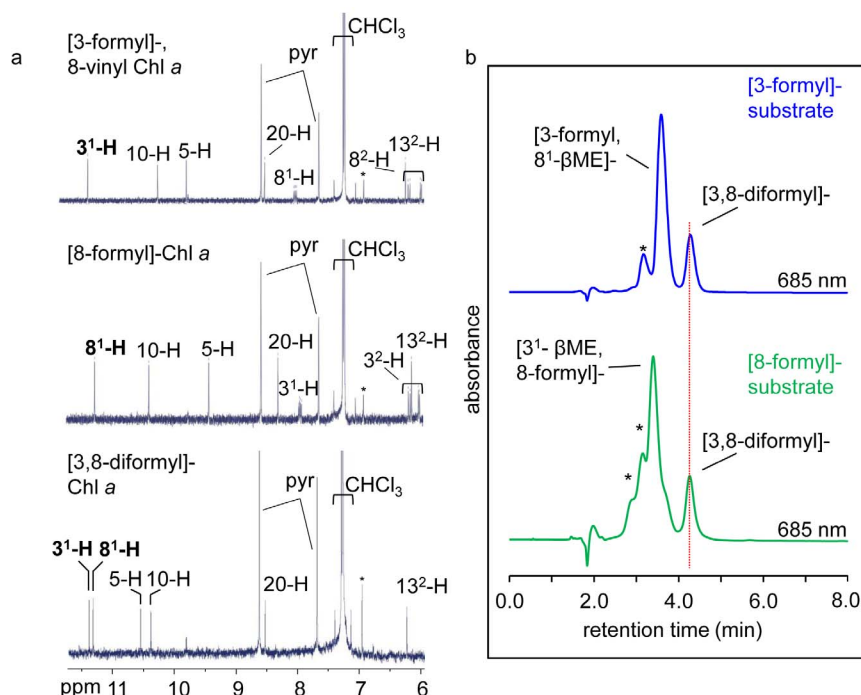
Using the HPLC-purified 3<sup>1</sup>-sulfoxide derivative (a\_II) as a reactant under the same reaction conditions, only product a\_I was produced, so therefore the 3<sup>1</sup>-sulfoxide derivative is not a precursor to the formation of the 3-formyl group, which is consistent with the observation by Oba *et al.*<sup>14</sup>. Incubation of Chl *d* under these reaction conditions also yielded another product, product a\_III (Figure 2a). Relative to their precursors, both a\_I and a\_III have a red-shifted Q<sub>y</sub> maximum and an increased mass of 76 Da. Unfortunately insuf-

ficient product was yielded for structural analysis of these derivatives. Co-oxidation of thiols and olefins, particularly conjugated olefins, has previously been observed, and an anti-Markovnikov reaction mechanism has been suggested<sup>22</sup>. This reaction scheme has been used to explain the thiol attack on the 3-vinyl group of Chl (or pheophorbide) *a* and formation of the 3<sup>1</sup>-formyl<sup>13,15</sup>. We have shown here that these two reaction pathways are independent of the position of vinyl group at the macrocycle, *i.e.* both the C3 and the C8 vinyl group in 8-vinyl Chl *a* may be oxidatively cleaved to give the corresponding formyl derivative as well as the sulfoxide products (Figure 6). These data support the fact that there are different reaction mechanisms for the formation of the formyl groups converted from vinyl group at the C3 (or C8 *in vitro*) position compared to the methyl group at the C7 or C2 positions, corresponding to the naturally occurring Chl *d*, Chl *b* and Chl *f* respectively.

The reactivity of the vinyl group of chlorophylls with  $\beta$ -mercaptoethanol was strongly dependent on the presence of heme in an aqueous buffer whereas in methanol heme was not required for the formation of the formyl group and other  $\beta$ -mercaptoethanol derivatives. Oxygen is approximately 10 times less soluble in water than methanol<sup>20</sup>. This suggests that it is the ability of heme to promote oxygen radical formation in water as well as its ability to deliver the oxygen to the chlorophyll molecule, that allows these thiol driven reactions to take place in an aqueous, relatively low oxygen environment. A number of lines of evidence support this hypothesis: No Chl *d* was synthesised from Chl *a* when the heme was either removed or replaced with protoporphyrin IX in the aqueous reaction mixture. Reaction kinetics were accelerated when hydrogen peroxide was added to the reaction mix. Furthermore, it has been shown previously that the presence of the radical scavenger ascorbic acid significantly reduces the synthesis of pheophorbide *d* from pheophorbide *a* under similar reaction conditions<sup>15</sup>.

Previously, it has been shown that under acidic conditions, pheophorbide *d* can be produced from pheophorbide *a* in either a micellar<sup>15</sup> or organic solvent<sup>14</sup> environment in the presence of hydrogen sulfide or thiophenol respectively. More recently, it has been shown that under less acidic conditions, demetallation and dephytylation of the chlorophyll can be avoided, allowing the synthesis of Chl *d* from Chl *a* in organic solvents<sup>13</sup>, which agrees well with the optimal pH range of 7–8 observed in our experiments. Our results expand on these previous reports with the observation that other thiol-containing reagents such as  $\beta$ -mercaptoethanol, benzyl mercaptan and dithiothreitol can drive this reaction under mild conditions, either for detergent-solubilised chlorophyll in aqueous reaction buffer or chlorophyll in a methanolic reaction mixture.

Three new [formyl-] chlorophyll derivatives are synthesised using 8-vinyl Chl *a* as the reactant, 8-vinyl Chl *d* (or [3-formyl]-8-vinyl-Chl *a*), [8-formyl]-Chl *a* and [8-formyl]-Chl *d* (or [3,8-diformyl]-Chl *a*) (Figure 6). The different spectral profiles of the formyl derivatives of chlorophylls synthesised in this study can be explained by shifts in the electronic configuration caused by the electronegative oxygen atom<sup>23</sup>. The 8-vinyl Chl *d* has a similar Q<sub>y</sub> absorption peak to (8-ethyl) Chl *d*, due to the electron withdrawing effect of the oxygen atom, along the Y axis of the chlorophyll. Distinctly though, the Soret peak is bathochromically shifted ~10 nm toward the green window region, which is analogous to the spectral changes from Chl *a* to 8-vinyl Chl *a*<sup>1</sup>. It is believed that 8-vinyl Chl *a* and 8-vinyl Chl *b* in *Prochlorococcus* confer an advantage, enabling the organism to thrive in the open ocean at depths down to 200 m due to the approximately 10 nm bathochromic shift in their Soret-bands matching the available blue light there<sup>24</sup>. This bathochromic shift in the Soret peak is even more pronounced in both the [3,8-diformyl]-Chl *a* and [3,7-diformyl]-Chl *a* with Soret maxima of 487 and 484 nm respectively (Table 1). At least in the case of the [3,7-diformyl]-Chl *a* ([3-formyl]-Chl *b* or [7-formyl]-Chl *d*), synthesis of this chlorophyll in *Acaryochloris* leads to a modest increase in the *in vivo* absorption

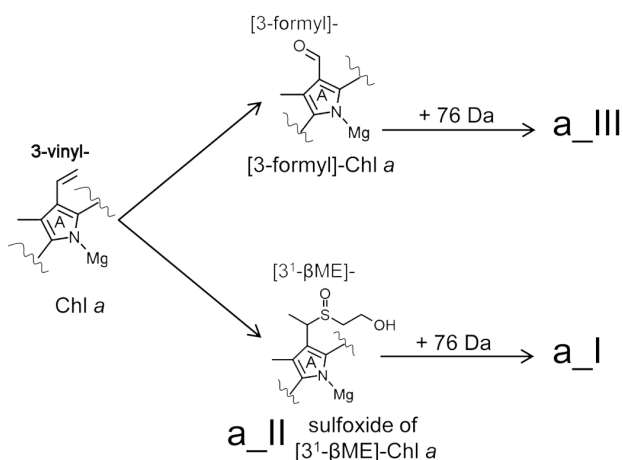


**Figure 7 | Confirmation that both the C3 and C8 vinyl groups of 8-vinyl Chl *a* are converted to formyl groups.** (a) Partial <sup>1</sup>H-NMR spectra of [3-formyl, 8-vinyl]-Chl *a* (8-vinyl Chl *d*), [8-formyl]-Chl *a* and [3, 8-diformyl]-Chl *a*. Formyl associated protons are in bold, CHCl<sub>3</sub>, pyridine (pyr) and solvent contaminant (\*) peaks are indicated. (b) RP-HPLC chromatogram (detected at 685 nm) of completed reaction mixtures using either [3-formyl], 8-vinyl-Chl *a* (8-vinyl Chl *d*, upper panel) or [8-formyl]-Chl *a* (lower panel) as substrate. The peak corresponding to the [3,8-diformyl]-Chl *a* product is marked with a vertical dotted line. \*, unknown products.

at around 480 nm<sup>21</sup>, and the chlorophyll is incorporated into the antenna of photosystem II<sup>25</sup>. So although as yet these formyl chlorophylls have not yet been identified in nature, it would appear that at least spectrally, they may provide a competitive advantage in some instances, absorbing wavelengths of light not absorbed by other

organisms. Our data provide a spectral signature for these novel formyl chlorophylls, which should aid in their identification in nature, if they are present. We observed that any formyl group substitution on ring B (either at position C7 or C8) caused a dramatic decrease in the intensity of the Q<sub>y</sub> peak, which is also the case with the C7 formyl substitution in Bchl *e* and *f* from Bchl *c* or *d* respectively<sup>26</sup>. The red shift in the Q<sub>y</sub> peak of [8-formyl]-Chl *a* relative to the 8-vinyl Chl *a* was initially surprising as it is the opposite of the blue shifted Q<sub>y</sub> peak of the structurally similar Chl *b* (Table 1). The transition dipole moment of the Q<sub>y</sub> axis in Chl *a* lies almost along the line bisected by C2 and C11 axis<sup>27</sup>. Thus adding an electron withdrawing formyl group along the axis, such as in Chl *d* and Chl *f*, would be expected to red shift the Q<sub>y</sub> absorbance maxima while adding an electron withdrawing substituent at right angles to this axis such as in Chl *b* would blue shift the Q<sub>y</sub> absorbance maxima. As an 8-formyl group is close to being along this Q<sub>y</sub> axis this would explain the observed red shift in of [8-formyl]-Chl *a*.

Here for the first time we show that [3-formyl]-Chl *b* can also be synthesised from Chl *b* in the presence of a thiol (Figure 4). Although the 10% yield of Chl *d* synthesised from Chl *a* under our reaction conditions was less than the 31% reported by Fukusumi et al.<sup>13</sup>, our results for [3-formyl]-Chl *b* synthesis (30% yield) were comparable, and this is achieved for detergent-solubilised Chl *b* rather than in an organic solvent such as chloroform or the alcohol methanol. Reaction kinetics can be increased around 3-fold in the methanolic reaction mixtures instead of the aqueous reaction mixture, however the relative yields of each product remain largely unaffected. A series of novel chlorophyll derivatives are formed by using 8-vinyl Chl *a* as substrate due to the oxidative cleavage independently at either the 3-vinyl or 8-vinyl position of 8-vinyl Chl *a* (Figure 6). Yield of the 3- and 8-formyl derivatives of 8-vinyl Chl *a* was difficult to measure as they were both intermediates, reacting further with the β-mercaptoethanol. Assuming the extinction coefficient of Chl *d* for the 3-formyl derivative and that of Chl *b* for the 8-formyl derivative



**Figure 8 | Substitutions at the C3 position of ring A of Chl *a* mediated by β-mercaptoethanol.** The C3 vinyl position of Chl *a* is targeted by the thiol group of β-mercaptoethanol yielding a sulfoxide derivative of [3<sup>1</sup>-β-mercaptoethanol] Chl *a* (product a<sub>II</sub>), which can react further with β-mercaptoethanol to yield an unknown product (a<sub>I</sub>) with a mass of a<sub>II</sub> plus 76 Da. An alternate, competing reaction also targets the C3 vinyl group of Chl *a*, oxidatively cleaving it to yield [3-formyl]-Chl *a* (Chl *d*). As is the case for product a<sub>II</sub>, [3-formyl]-Chl *a* also reacts further with β-mercaptoethanol to yield an unknown product (a<sub>III</sub>) with a mass of [3-formyl]-Chl *a* plus 76 Da. Structure of product a<sub>II</sub> and synthesized Chl *d* is confirmed by NMR analysis (details see supplementary information).



(which it resembles spectrally and likely has a similar molecular extinction coefficient to) the average ratio of formation of formyl group at C3 and C8 positions is 0.6:1. It may be that the C8 vinyl is more reactive than the C3 vinyl, as a similar trend is observed when comparing the relative synthesis of 3' and 8' sulfoxide of  $\beta$ -mercaptoethanol derivatives.

Despite the obvious advantage of being able to exploit far red light for oxygenic photosynthesis, increasing available photons by approximately 14%<sup>28</sup>, as yet only the monophyletic *Acaryochloris* has been shown to utilise Chl *d*. We have demonstrated, at least *in vitro*, the simplicity with which Chl *d* can be synthesised from Chl *a* in an aqueous buffer. Whether these thiol-driven reactions shed light on the *in vivo* synthesis of formyl groups from vinyl on the chlorophyll macrocycle remains uncertain. Clearly, in addition to the synthesis of Chl *d*, *Acaryochloris* must have had to acquire the ability to efficiently utilise this chlorophyll in its photosystems. For example the *Acaryochloris* D1 protein, located in the reaction center of photosystem II, has a cysteine residue in place of a conserved glycine in Chl *a*-utilising photosynthetic organisms<sup>29</sup>. Interestingly, 8-vinyl Chl *a*-utilising *Prochlorococcus* also have a cysteine residue at this position. *Acaryochloris* has an unusually large genome for a unicellular cyanobacterium<sup>30</sup> so perhaps this has allowed a certain level of gene plasticity, leading to neofunctionalisation of the 'Chl *d* synthase' and other associated changes to its photosynthetic apparatus which would be required for efficient utilisation of Chl *d*.

## Methods

**Chlorophyll isolation and quantification.** All pigment extraction and purification steps were performed in a darkened room or under weak green light to avoid photodamage. All solvents used were HPLC grade. Chl *a* and *b* were isolated from *Arabidopsis* seedlings by grinding leaf tissue in 100% acetone. Chl *d* and Chl *f* were extracted from *Acaryochloris marina* MBIC 11017 and *Halomicronema hongdechloris* cells respectively using 100% methanol. 8-vinyl Chl *a* was isolated from a  $\Delta$ slr1923 *Synechocystis* PCC 6803 mutant<sup>31</sup> using 100% methanol. All chlorophylls were purified from total pigment using a Strata C18-T reverse phase column (200 mg/3 ml, Phenomenex, USA) and isolated from one another using a 4.6  $\times$  150 mm Synergi 4  $\mu$ m Max-RP column (Phenomenex, Australia) on a Shimadzu HPLC (model 10A series) using 100% methanol (Fisher Scientific, USA) at a flow rate of 1 ml/min. Eluted pigments were detected with a diode array absorption detector (SPD-M10Avp; Shimadzu, Japan). Purified chlorophylls were vacuum dried and stored at  $-80^\circ\text{C}$ . Chl *a* and *b* products, following their reaction with the thiol compound, were also separated using this HPLC program. Separation of 8-vinyl Chl *a* reaction products was performed as described above except a 15 min linear gradient (0–100%) of solvent A (85% (v/v) methanol in 150 mM ammonium acetate) and solvent B (methanol) followed by 100% solvent B for 20 min at a flow rate of 0.9 ml/min was used. Chlorophyll concentrations were quantified by spectroscopy in 100% methanol using the following molar extinction coefficients: Chl *a*,  $\epsilon_{665\text{ nm}} = 70.02 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ ; Chl *d*,  $\epsilon_{697\text{ nm}} = 63.68 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ ; Chl *f*,  $\epsilon_{707\text{ nm}} = 71.11 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$  and Chl *b*,  $\epsilon_{652\text{ nm}} = 38.55 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ <sup>32,33</sup>. The dried chlorophylls were resuspended in a small volume of 100% acetone immediately prior to setting up reactions. Yields of chlorophyll derivatives were determined based on peak area from the HPLC and either the published molar extinction coefficients described above or estimated based on the extinction coefficient of either the parent chlorophyll or spectrally similar chlorophyll as stated in the text.

**Reaction conditions.** Under standard reaction conditions, 10  $\mu$ l of acetone dissolved chlorophyll was added to 3 ml of 0.02% (w/v) n-dodecyl- $\beta$ -D-maltoside (Sigma) or 0.02% (v/v) Triton X-100 in 50 mM Tris buffer, pH 8.0 plus 10  $\mu$ M hemin chloride (MP Biochemicals) and 50 mM  $\beta$ -mercaptoethanol (Sigma). Where stated, hemin chloride was omitted or replaced with 10  $\mu$ M protoporphyrin IX (Sigma), and  $\beta$ -mercaptoethanol was replaced with 50 mM benzyl mercaptan (Sigma) or 50 mM dithiothreitol (Sigma). For the pH experiments, pH was maintained using a 50 mM Tris buffer for pH 7–9 or 50 mM MES buffer for pH 5.6. After 18 h shaking at room temperature, unless stated otherwise, the chlorophylls and their derivatives were extracted from the reaction mix with saturated NaCl solution and 100% butanol, which was transferred to a new tube and evaporated in a vacuum concentrator. Pigments were resuspended in 100% methanol and immediately subjected to HPLC. The separated pigments were collected from the HPLC, dried using a vacuum concentrator or nitrogen gas stream, and stored at  $-80^\circ\text{C}$  prior to mass spectrometry or NMR. For  $\beta$ -mercaptoethanol reactions in methanol, a small volume of acetone-solubilised chlorophyll (20  $\mu$ M) was added to 50 mM  $\beta$ -mercaptoethanol dissolved in 100% methanol and incubated with shaking for 2–4 h. Reactions were concentrated using a nitrogen gas stream before performing HPLC.

**NMR spectroscopy.** All NMR experiments were performed using a Bruker Avance III-800 or Bruker Avance III-600 spectrometer at the School of Molecular Biology, University of Sydney. Dried HPLC purified samples were resuspended in chloroform-*d* (Sigma) with 0.5% (v/v) pyridine-*d*<sub>5</sub> (Cambridge Isotope Laboratories, Inc).

**Mass spectrometry.** Mass spectrometry was performed as described previously<sup>10</sup>.

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## Author contributions

P.C.L. performed the experiments and analysed the data. R.D.W. performed the NMR experiments and data processing. P.C.L., R.D.W. and M.C. conceived of and designed experiments and wrote the manuscript.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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