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The Addition of Methyl-2-mercaptoacetate to Phycocyanobilin Dimethyl Ester: A Model Reaction for Biliprotein Biosynthesis?

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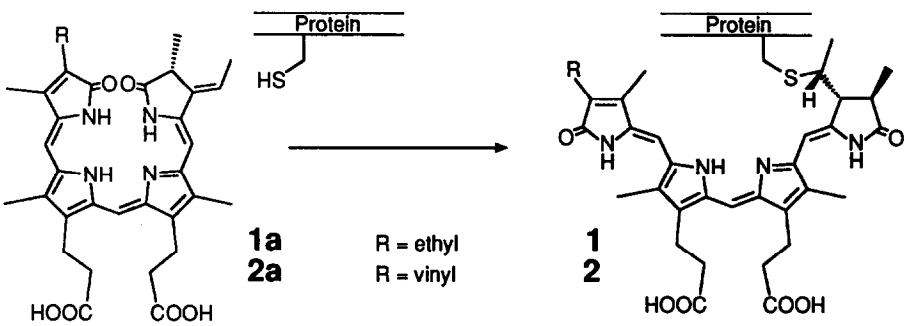
Abstract: Addition of methyl-2-mercaptoacetate to the ethylidene double bond of phycocyanobilin dimethyl ester **3** results in quantitative formation of the two diastereomeric adducts **4** and **5**. Their structural and chemical properties correspond to those found for the protein bound chromophores of biliproteins.

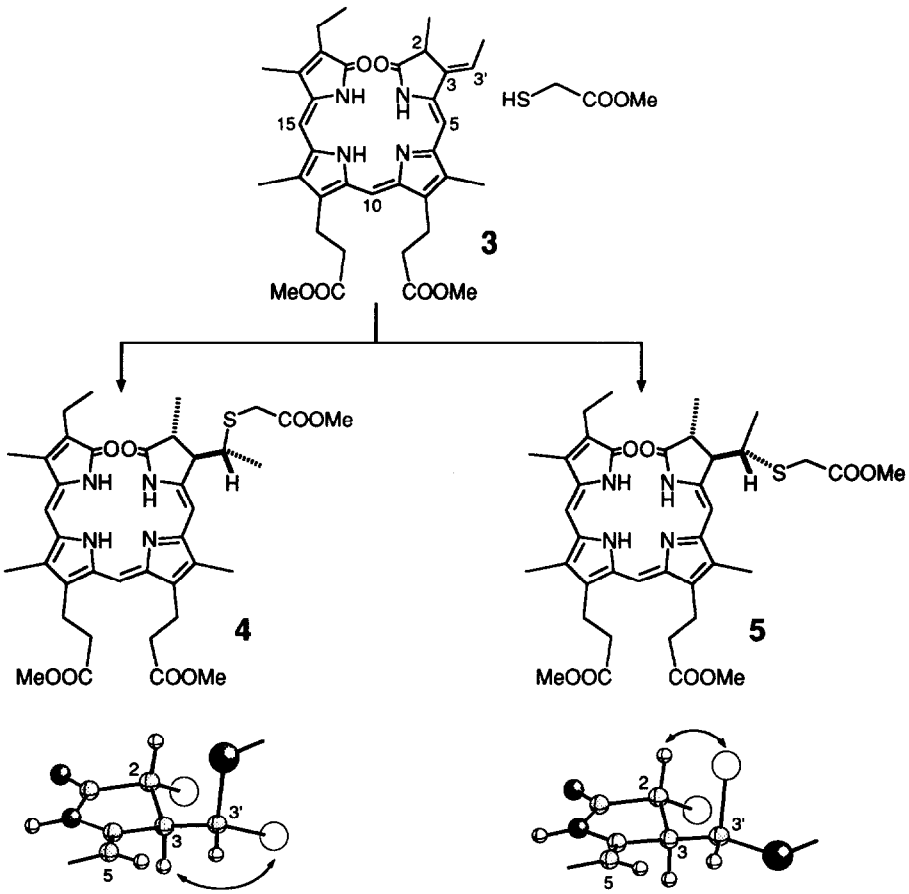
Phycocyanin **1** and phythochrome **2** are the most intensively studied biliproteins[1]. Phycocyanin acts as a photosynthetic antenna pigment in various types of algae, whereas phytochrome initiates morphogenic processes in higher plants. Their biological activities are based on photophysical and photochemical properties of their bilindione chromophores[2], which are bound covalently to the protein moieties by thioether linkages. Chromophore attachment to the apoprotein results from addition of cysteinyl thiols to the ethylidene double bond of the *in vivo* precursors phycocyanobilin **1a** or phytochromobilin **2a** during the last steps of biliprotein biosynthesis[3].



In vitro attachments of bilins to apoproteins have been accomplished in principle[4,5]. However, detailed structure determinations of phycocyanobilin adducts showed the addition to be incomplete and the oxidation level of the chromophores to be increased[4]. Simple thiols also could be attached. Under basic conditions cysteine and glutathione have been added to **1a**[6], whereas ethanethiol has been added to the dimethyl ester **3** under acidic conditions[7]. In both cases large excesses of thiol were necessary to form the adducts. In addition, preparation of phycocyanobilin-cysteine adducts also succeeded along the lines of organic synthesis[8].

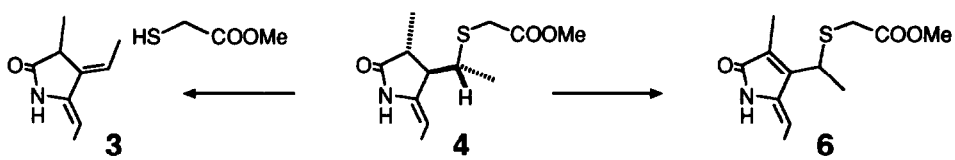
In this letter we report a biomimetic attachment model: the addition of methyl-2-mercaptoacetate (MMA) to racemic phycocyanobilin dimethyl ester **3** at room temperature without additional catalysts in quantitative yield[9]. Furthermore, reaction mechanistic features of the addition reaction are presented.

MMA (1.5 µl; 16.8 µmol) adds to **3** (5.0 mg; 8.2 µmol) at 25 °C in chloroform (380 µl) within 24 hours yielding a mixture of the two racemic diastereomeric adducts **4** and **5** (5.6 mg; 96%; **4** : **5** = 1 : 1.4) after column chromatography on silica (dichloromethane/acetone = 100/6). **4** and **5** can be separated by repeated



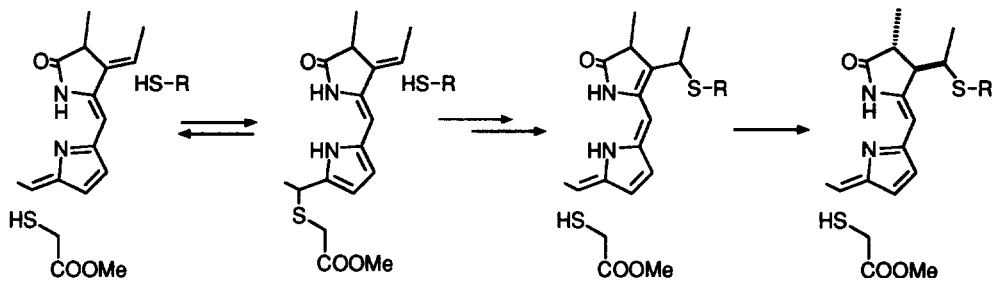
thin layer chromatography (silica; toluene/methanol/THF = 50 / 2 / 1; R_f: **4** < **5**). Diastereomer **5**[11] is a rather stable compound, whereas **4**[10] is not. It tends to be oxidized or to eliminate MMA, yielding **6**[12] or **3**, thus preventing successful purification. Therefore, all spectral data of **4** have been recorded on mixtures of 90 % maximum purity.

Stereochemical assignments of **4** and **5** succeeded in finding the *trans* configuration of H-2 and H-3 together with the close neighborhood of H-3, H-3' and H-5 in both diastereomers from NOESY-spectra. Significant geometrical differences could be derived from the occurrence (curved arrows) or absence of the corresponding cross peaks. Accordingly, stereochemical differences arise from the opposite configuration of carbon 3'. Monitoring the reaction by means of ^{1}H-NMR spectroscopy revealed **4** as the kinetically and **5** as the thermodynamically controlled adduct. Furthermore, purified **4** can be epimerized to equilibrium with **5** in



chloroform after adding catalytic quantities of MMA. These findings are in good accordance with steric approach models and stereoelectronic concepts. Attack of thiol is preferred opposite to the methyl group at carbon atom 2. Ease of thiol elimination of **4** is due to the *antiperiplanar* orientation of the S-C(3') and H-C(3) bonds.

In principle, addition works with stoichiometric quantities of MMA, but fails using equivalent amounts of ethanethiol. However, addition of ethanethiol at stoichiometric level can be achieved by use of catalytic quantities of MMA. These results indicate, that successful addition to the ethylidene double bond needs a preceding addition to the azafulvenyl moiety. Additions of this kind are known to be reversible[13]. They depend on temperature and solvent. Thiols of low pK_a like MMA (pK_a = 7.9) are well suited for this kind of addition, whereas ethanethiol (pK_a = 10.5) is not. In comparison with MMA ethanethiol has to be used in large excess to provide sufficient amounts of azafulvene adducts, which are the key intermediates of the reaction sequence. They enable covalent binding of thiol at carbon atom 3'. Thus thiol at carbon atom 10 is substituted vinylogously. Again MMA is advantageous due to its good leaving group activity. Final tautomerization transfers a proton to carbon atom 3 favoring 2,3-*trans* geometry.



In conclusion, an addition-substitution sequence is characterizing these model reactions of thiol addition to 3-ethylidenebilindiones. A mechanism like this is able to explain the site unspecific chromophore binding to apoproteins using azide[14], and parallels the mechanism discussed for biliverdinreductase[15]. It gives rise to the assumption of covalently bound intermediates during the course of bilindione attachment *in vivo*. Furthermore, stereochemical results fit well to latest crystal structure results of phycocyanins[16]: relative configurations (*l,l*) and (*l,u*) of **4** and **5** correspond to the (2*R*,3*R*,3'*R*) and (2*R*,3*R*,3'*S*) absolute configurations of the α-82, β-84 and β-155 chromophores in phycocyanin of cyanobacterium *Fremyella diplosiphon*. Comparing these findings with the incomplete *in vitro* attachment of **1a**[4] it becomes apparent that exclusive attachment to α-82 and β-84 cysteinyl thiols is governed by kinetic control. Only chromophores corresponding to **4** could be formed rapidly and oxidized easily yielding chromophores like **6** after attachment. Finally, oxidation of **4** to **6** may be a key step in the biosynthesis of pigments recently found in cryptophycean algae[17].

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10. **4**: ^{1}H-NMR (360 MHz; $CDCl_3$): δ = 1.09 (t, 3H, J = 7.6 Hz, CH_3-(C18')); 1.28 (d, 3H, J = 7.5 Hz, CH_3-(C2)); 1.43 (d, 3H, J = 6.9 Hz, CH_3-(C3'); 2.03 (s, 3H, CH_3-(C7)); 2.10 (s, 3H, CH_3-(C13)); 2.12 (s, 3H, CH_3-(C17)); 2.32 (q, 2H, J = 7.6 Hz, CH_2-(C18)); 2.52 (covered dq, 1H, J = 5.3 Hz, J = 7.5 Hz, H-(C2)); 2.55 (triplet like, 4H, CH_2-(C8') + CH_2-(C12')); 2.89, 2.93 (2 x triplet like, 4H, CH_2-(C8) + CH_2-(C12)); 3.11 (br t, J = 5.3 Hz, J = 3.5 Hz, J = 0.9 Hz, 1H, H-(C3)); 3.26, 3.36 (AB-system, 2H, J = 14.9 Hz, CH_2-S); 3.45 (dq, 1H, J = 3.5 Hz, J = 6.9 Hz, H-(C3')); 3.66, 3.68 (2s, 6H, CH_3O-(C8''') + CH_3O-(C12''')); 3.71 (s, 3H, CH_3O-(C-C-S)); 5.77 (d, 1H, J = 0.9 Hz, H-(C5)); 5.97 (s, 1H, H-(C15); 6.65 (s, 1H, H-(C10)). ^{13}H-NMR (90 MHz; $CDCl_3$): δ = 111.97 (C10); 96.04 (C15); 93.09 (C5); 51.82 (C3); 42.97 (C3'); 38.67 (C2); 32.92 (H_2C-S); 18.64 (C3''); 16.41 (C2').
11. **5**: ^{1}H-NMR (360 MHz; $CDCl_3$): δ = 1.09 (t, 3H, J = 7.4 Hz, CH_3-(C18')); 1.25 (d, 3H, J = 6.7 Hz, CH_3-(C3')); 1.30 (d, 3H, J = 7.3 Hz, CH_3-(C2); 2.02 (s, 3H, CH_3-(C7)); 2.10 (s, 3H, CH_3-(C13)); 2.12 (s, 3H, CH_3-(C17)); 2.31 (q, 2H, J = 7.4 Hz, CH_2-(C18)); 2.52 (covered dq, 1H, J = 4.5 Hz, J = 7.3 Hz, H-(C2)); 2.54 (triplet like, 4H, CH_2-(C8') + CH_2-(C12')); 2.90, 2.93 (2 x triplet like, 4H, CH_2-(C8) + CH_2-(C12)); 3.17 (br t, J = 4.5 Hz, J = 3.8 Hz, J = 1.3 Hz, 1H, H-(C3)); 3.31, 3.36 (AB-system, 2H, J = 14.5 Hz, CH_2-S); 3.45 (dq, 1H, J = 3.8 Hz, J = 6.7 Hz, H-(C3')); 3.66, 3.68 (2s, 6H, CH_3O-(C8''') + CH_3O-(C12''')); 3.78 (s, 3H, CH_3O-(C-C-S)); 5.46 (d, 1H, J = 1.3 Hz, H-(C5)); 5.97 (s, 1H, H-(C15); 6.66 (s, 1H, H-(C10)). ^{13}H-NMR (90 MHz; $CDCl_3$): δ = 112.15 (C10); 96.11 (C15); 92.34 (C5); 50.31 (C3); 42.71 (C3'); 38.49 (C2); 32.65 (H_2C-S); 17.55 (C2'); 15.51 (C3''). UV-Vis ($CHCl_3$): nm (ε) = 276 (14600); 349 (28800); 587 (12800).
12. **6**: ^{1}H-NMR (360 MHz; $CDCl_3$): δ = 1.06 (t, 3H, J = 7.6 Hz, CH_3-(C18')); 1.67 (d, 3H, J = 7.4 Hz, CH_3-(C3')); 1.90 (s, 3H, CH_3-(C2); 2.08 (s, 3H, CH_3-(C7)); 2.09 (s, 6H, CH_3-(C13) + CH_3-(C17)); 2.25 (q, 2H, J = 7.6 Hz, CH_2-(C18)); 2.55 (triplet like, 4H, CH_2-(C8') + CH_2-(C12')); 2.92 (triplet like, 4H, CH_2-(C8) + CH_2-(C12)); 3.23, 3.36 (AB-system, 2H, J = 15.5 Hz, CH_2-S); 3.67 (s, 6H, CH_3O-(C8''') + CH_3O-(C12''')); 3.71 (s, 3H, CH_3O-(C-C-S)); 4.43 (q, 1H, J = 7.4 Hz, H-(C3')); 5.88 (s, 1H, H-(C5)); 6.42 (s, 1H, H-(C15); 6.75 (s, 1H, H-(C10)). UV-Vis ($CHCl_3$): nm (ε) = 310 (15800); 374 (42300); 649 (13500).
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