

Isolation and Synthesis of New Bile Pigments Possibly Related to the Biosynthesis of the Phycocyanin Chromophore

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After incubation with 5-aminolevulinic acid and cysteine or 5-amino-levulinic acid and glutathione, *Cyanidium caldarium* excreted two new bile pigments **1** and **2**. Their structures are identified by spectroscopical methods. By chemical addition of cysteine, glutathione and hydrogen sulfide to the ethylen double bond of *E*-phycocyanobilin, the bile pigments **3–6** were obtained.

Introduction

Phycocyanins and phycoerythrins are light-harvesting components of the photosynthetic apparatus of red algae, cryptomonad algae and cyanobacteria [1]. Phytochrome is a photoreceptor of photomorphogenesis in higher plants [2, 3]. Phycocyanins, phycoerythrins and phytochrome are proteins containing one or several bile pigments as prosthetic groups. So far, the chromophore biosynthesis of these important chromoproteins is only partly known. Most of the investigations have been carried out with the unicellular acidophilic and thermophilic organism *Cyanidium caldarium*, which can be grown heterotrophically in the dark when glucose is added to the culture medium. Dark grown cultures of *Cyanidium caldarium* lack chlorophyll *a* and phycocyanin. By incubation with 5-aminolevulinic acid, however, the cells produce precursors of phycocyanobilin and excrete these compounds into the medium [4, 5]. Besides porphobilinogen and porphyrins the bile

pigments biliverdin IX α [6], *E*-phycocyanobilin [5, 7] and 3¹-OH-mesobiliverdin IX α [8] could be isolated. By radioactive incubation experiments, haem [9] and biliverdin IX α [10, 11] were established to be precursors of the phycocyanin chromophore. *In vitro* studies with a crude enzyme extract gave hints that *Z*-phycocyanobilin is the precursor of *E*-phycocyanobilin [12]. Interestingly, a possible intermediate with a hydrogenated ring A and an vinyl-group at C(3) which was synthesized chemically was shown to isomerize rapidly to yield *Z*-phycocyanobilin, which itself isomerizes slowly to *E*-phycocyanobilin [13].

There is still a question which chromophore is added to the binding cystein residue and furthermore, whether the thioether linkage is formed before translation, co-translational, or after formation of the complete apoprotein. Investigations with apophycocyanin and phycocyanobilin or phycoerythrobilin showed that addition to the apoprotein occurs *in vitro* without the enzyme. Under these conditions, however, the chromophores undergo oxidation at ring A to yield a mesobiliverdin bonded to the apoprotein [14–16]. Phycocyanobilin also adds to apophytochrome, in this case, however, ring A remains hydrogenated [17].

To get more information about the last steps of phycocyanin biosynthesis, we incubated dark-grown cells of *Cyanidium caldarium* with 5-amino-levulinic acid in the presence of cysteine or glutathione. In both cases we were able to isolate two new bile pigments. We also added cysteine and glutathione chemically to *E*-phycocyanobilin as well as hydrogen sulfide. The results are described in this report.

Abbreviations: COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; E^{UV}, extinction, ultraviolet; E^{VIS}, extinction, visible; FAB-MS, fast atom bombardment – mass spectrum; HETCOR, heteronuclear shift correlation; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; UV/VIS, ultraviolet/visible.

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Experimental Procedures

Materials

Chemicals were obtained from E. Merck (Darmstadt, F.R.G.). 5-Aminolevulinic acid was synthesized as described in [18]. Preparative thin-layer chromatography was carried out on plates (20 × 20 cm) precoated with silica gel 60 PF₂₅₄₊₃₆₆; the products were eluted with acetone. UV/VIS: Perkin Elmer 320 spectrophotometer; NMR: ¹H NMR spectroscopy: 400 MHz, Varian XL-400, ¹³C NMR: 100 MHz, Varian XL-400.

Isolation of pigments 1 and 2

Cultures of *Cyanidium caldarium*, mutant III-D-2 were grown in Fernbach flasks with 1 l of medium each [19], containing 1% glucose at 28 °C in the dark under shaking. After 8–10 days the cells were collected by centrifugation under sterile conditions. The cells of 4–5 l were resuspended in 1 l medium containing 2.0 g 5-aminolevulinic acid and 2.1 g glutathione or 2.0 g 5-aminolevulinic acid and 0.45 g L-cysteine hydrochloride. After 5 to 7 days of incubation in the dark at 28 °C on a shaker the cells were harvested by centrifugation and the blue supernatant extracted with butanol-1, the organic phase washed with water and evaporated with a rotary evaporator (bath temperature: 40 °C). When methylene chloride was used instead of butanol-1 and evaporated at about 20 °C, the same pigments were obtained, however, the pigments are better soluble in butanol-1 than in methylene chloride. The pigments were esterified with 4% (v/v) sulfuric acid/methanol for 1 h at room temperature or with N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimid-hydrochloride in methanol, which yielded the same pigments. Purification was carried out by thin layer chromatography with the solvent system ethyl acetate/methylene chloride/methanol = 2:2:1. Pigment 1 was purified again with the same solvent system, pigment 2 was further purified by using chloroform/acetone/pyridine = 50:50:1 as solvent system.

Pigment 1

Ultraviolet/visible spectrum. λ_{\max} /nm (relative intensity) in CH₃OH 583 (1), 360 (0.77), 310 (1.31); in CH₃OH + H₂SO₄ 625 (1), 328 (0.72); CH₃OH + (CH₃CO₂)₂Zn 662 (1); 370 (0.55), 335 (0.72).

¹H NMR spectrum. δ /ppm in d₅-pyridine: 7.37 [s, 1 H, HC(10)]; 6.12 [s, 1 H, HC(15)]; 5.75 [qxd, 1 H, $J(3^1, 3^2) = 7.62$ Hz, $J/2, 3^1 = 1$, HC(3¹)]; 3.59, 3.55 [s, each 3 H, H₃C(8⁴) + H₃C(12⁴)]; 3.20–2.98 [m, 5 H, H₂C(8¹) + H₂C(12¹) + HC(2)]; 2.78–2.75 [m, 2 H, H₂C(8²) or H₂C(12²)]; 2.65–2.62 [m, 2 H, H₂C(8²) or H₂C(12²)]; 2.10, 1.84 [s, each 3 H, H₃C(13¹) + H₃C(17¹)]; 2.03–1.98 [m, 2 H, H₂C(18¹)]; 1.35 [d, $J(2, 2^1) = 5.10$, 3 H, H₃C(2¹)]; 0.74 [t, $J(18^1, 18^2) = 7.59$, 3 H, H₃C(18²)]; 1.90 [s, 3 H, H₃C(7¹)]; 1.36 [d, $J(3^1, 3^2) = 7.62$ Hz, 3 H, H₃C(3²)].

FAB mass spectrum. m/z in glycerol: 646 (48%), M⁺.

An additional peak at 727/728 cannot be explained.

Pigment 2

Ultraviolet/visible spectrum. λ_{\max} /nm (relative intensity) in CH₃OH 597–590 (1), 342 (2.00); in CH₃OH + H₂SO₄ 660–652 (1), 348 (0.93); in CH₃OH + (CH₃CO₂)₂Zn 642 (1), 378 (0.79), 346 (0.88).

¹H NMR spectrum. δ /ppm in d₅-pyridine: 7.08 [s, 1 H, HC(10)]; 6.43 [s, 1 H, HC(5)]; 6.05 [s, 1 H, HC(15)]; 4.68 [q, $J(3^1, 3^2) = 7.20$, 1 H, HC(3¹)]; 3.92 [q, $J(2, 2^1) = 7.45$, 1 H HC(2)]*; 3.60 [s, 6 H, H₃C(8⁴) + H₃C(12⁴)]; 3.06, 2.93 [t, $J = 7.34$ and 7.61 Hz, each 2 H, H₂C(8¹) + H₂C(12¹)]; 2.68, 2.61 [t, $J = 7.34$ and 7.61 Hz, each 2 H, H₂C(8²) + H₂C(12²)]; 2.50–2.40 [m, 2 H, H₂C(18¹)]; 2.07, 2.05 [s, each 3 H, H₃C(13¹) + H₃C(17¹)]; 1.84 [s, 3 H, H₃C(7¹)]; 1.52 [d, $J(3^1, 3^2) = 7.20$, 3 H, H₃C(3²)]; 1.42 [d, $J(2, 2^1) = 7.45$, 3 H, H₃C(2¹)]; 1.24 [t, $J(18^1, 18^2) = 7.53$, 3 H, H₃C(18²)].

FAB mass spectrum. m/z in glycerol: 665 (30%), M⁺ + 1, 645/647 (each 14%), M⁺ + 1 – H₂O – H₂, M⁺ + 1 – H₂O, 615 (10%), M⁺ + 1 – SH – OH.

Preparation of pigments 3 and 4

A spray-dried powder of *Spirulina geitleri* was extracted with methanol to remove chlorophyll a and carotenoids. Then the powder was boiled under reflux several times for about 6 h to split off phycocyanobilin from the protein. The crude extract was evaporated with a rotatory evaporator and brought on a silica gel column. To remove

* The assignments may be vice versa.

parts of chlorophyll *a* and carotenoids, the column was first eluted with ethyl acetate, then with methanol to elute *E*-phycocyanoblin.

For the addition of L-cysteine to *E*-phycocyanoblin, the chromophore was dissolved in methanol + 10% triethyl amine. Under stirring at room temperature solid-L-cysteine methyl ester was added until the blue colour of the solution changed to yellow. After 1–2 h sulfuric acid in methanol was added to the solution to a final concentration of 4% (v/v) of sulfuric acid methanol. After about 1 h at room temperature the esterified pigments were extracted with methylene chloride/water and purified by thin layer chromatography with the solvent system methylene chloride/ethyl acetate/acetone = 20/20/7. The R_f was about 0.29.

To add glutathione a solution of *E*-phycocyanobilin in methanol + 10% triethyl amine was stirred at room temperature and solid glutathione was added until the colour was yellow. After 2 h stirring, sulfuric acid was added to a final concentration of 20% sulfuric acid in methanol. Esterification was finished after about 6 h in the dark at room temperature. Instead of 20% sulfuric acid N-(3-dimethylaminopropyl)-M'-ethyl-carbodiimid-hydrochlorid in methanol can be used. With only 4% sulfuric acid as in the case of cysteine, one carboxylic acid of glutathione was not esterified as shown by FAB-MS. Purification was carried out by thin layer chromatography with the solvent system ethyl acetate/methylene chloride/methanol = 2/2/1, the R_f was about 0.5.

Pigment 3

Ultraviolet/visible spectrum. λ_{\max}/nm (relative intensity) in CHCl_3 580 (**1**), 358 (2.33); in CH_3OH 582 (**1**), 350 (2.38); in $\text{CH}_3\text{OH} + \text{H}_2\text{SO}_4$ 655 (**1**), 357 (1.18); in $\text{CH}_3\text{OH} + (\text{CH}_3\text{CO}_2)_2\text{Zn}$ 635 (**1**), 372 (1.08), 340 (1.05).

^1H NMR spectrum. δ/ppm in CDCl_3 : 6.66 [s, 1H, HC(10)]; 5.97 [s, 1H, HC(15)]; 5.68 + 5.46 + 5.44 [each s, together 1H, HC(5)]; 3.80–3.67 [m, 10H, HC(3') + $\text{H}_3\text{C}(3'')$ + $\text{H}_3\text{C}(8'')$ + $\text{H}_3\text{C}(12'')$]; 3.33–2.85 [m, 8H, HC(3) + HC(3') + $\text{H}_2\text{C}(3'')$ + $\text{H}_2\text{C}(8'')$ + $\text{H}_2\text{C}(12'')$]; 2.57–2.51 [m, 5H, HC(2) + $\text{H}_2\text{C}(8'')$ + $\text{H}_2\text{C}(12'')$]; 2.32–2.25 [m, 2H, $\text{H}_2\text{C}(18'')$]; 2.12–2.02 [m, 9H, $\text{H}_3\text{C}(7'')$ + $\text{H}_3\text{C}(13'')$ + $\text{H}_3\text{C}(17'')$]; 1.43–1.19 [m, 6H, $\text{H}_3\text{C}(2'')$ + $\text{H}_3\text{C}(3'')$]; 1.10–1.05 [m, 3H, $\text{H}_3\text{C}(18'')$].

FAB mass spectrum. m/z (intensity) in glycerol: 750 (95%), $\text{M}^+ + 1$; 615 (100%), $\text{M}^+ + 1$ -cysteine methyl ester.

Pigment 4

Ultraviolet/visible spectrum. λ_{\max}/nm (relative intensity) in CHCl_3 588 (**1**), 350 (2.32); in CH_3OH 584 (**1**); 347 (2.34); in $\text{CH}_3\text{OH} + \text{H}_2\text{SO}_4$ 647 (**1**), 350 (1.06), 335 (1.05); in $\text{CH}_3\text{OH} + (\text{CH}_3\text{CO}_2)_2\text{Zn}$ 634 (**1**), 378 (1.09), 342 (1.19).

FAB mass spectrum. m/z (intensity) in 2-nitrophenyl-octylether: 951 (100%), $\text{M}^+ + 2$, 615 (68%), $\text{M}^+ + 1$ -glutathione dimethyl ester.

Preparation of pigments *rac. 5* and *rac. 6*

rac. E-Phycocyanobilin dimethyl ester was dissolved in methanol + 10% ammonia. The reaction took also place in methanol, ethanol and methylene chloride, however, the reaction time was longer. To this solution hydrogen sulfide was passed through until the colour changed to yellow. After 1–2 h in the dark at room temperature the solution was poured into methanol/10% sulfuric acid so that the colour changed again to blue. The pigments were extracted with methylene chloride/water. To remove all traces of hydrogen sulfide, the methylene chloride solution was acidified several times and washed neutral again. Purification by thin layer chromatography with the solvent system ethyl acetate/methylene chloride = 1:1 yielded two new pink products with R_f about 0.67 (**5**) and 0.60 (**6**) and a ratio of about 2:3.

Pigment *rac. 5*

Ultraviolet/visible spectrum. λ_{\max}/nm (relative intensity) in CHCl_3 564 (**1**), 328 (1.46); in CH_3OH 554 (**1**), 324 (1.54); in $\text{CH}_3\text{OH} + \text{H}_2\text{SO}_4$ 572 (**1**); 328 (1.05); in $\text{CH}_3\text{OH} + (\text{CH}_3\text{CO}_2)_2\text{Zn}$ 626 (**1**), 578 (sh), 338 (0.98).

^1H NMR spectrum. δ/ppm in CDCl_3 : 8.62 [br s, 1H, NH]; 6.76 [s, 1H, HC(10)]; 5.90 [s, 1H, HC(15)]; 3.64, 3.61 [s, each 3H, $\text{H}_3\text{C}(8'')$ + $\text{H}_3\text{C}(12'')$]; 3.45–3.30 [m, 1H, HC(3')]; 3.41 [s, 2H, $\text{H}_2\text{C}(5'')$]; 2.95–2.85 [m, 4H, $\text{H}_2\text{C}(8'')$ + $\text{H}_2\text{C}(12'')$]; 2.53–2.48 [m, 4H, $\text{H}_2\text{C}(8'')$ + $\text{H}_2\text{C}(12'')$]; 2.41–2.31 [m, 2H, $\text{H}_2\text{C}(18'')$]; 2.211–2.14 [m, 1H, HC(2)]; 2.08 [s, 3H, $\text{H}_3\text{C}(17'')$]; 2.08–2.02 [m, 1H, HC(3)]; 2.03 [s, 3H, $\text{H}_3\text{C}(13'')$]; 2.02 [s, 3H, $\text{H}_3\text{C}(7'')$]; 1.51 [d, $J(3', 3'') = 6.84 \text{ Hz}$, 3H, $\text{H}_3\text{C}(3'')$];

1.11 [t, $J(18^1, 18^2) = 7.57$ Hz, 3 H, $H_3C(18^2)$]; 0.64 [d, $J(2, 2^1) = 7.57$ Hz, 3 H, $H_3C(2^1)$].

^{13}C NMR spectrum. δ /ppm in $CDCl_3$: 178.29 [C(1) or C(19)]; 173.44, 173.40, 173.18 [$C(8^3) + C(12^3) + C(1)$ or C(19)]; 162.75, 147.27, 143.34, 142.25, 140.93, 137.62, 135.63, 133.47, 131.77, 130.34, 120.86 [C(6), C(7), C(8), C(9), C(11), C(12), C(13), C(14), C(16), C(17), C(18)]; 116.30 [C(10)]; 98.17 [C(15)]; 87.04 [C(4)]; 64.03, 60.94 [C(3) + C(3¹)]; 51.93 [$C(8^4) + C(12^4)$]; 44.36 [C(2)]; 39.32 [C(5)]; 35.49 [C(8²) + C(12²)]; 20.17, 20.13 [C(8¹) + C(12¹)]; 19.56, 16.69, 13.62 [C(2¹) + C(3²) + C(18²)]; 17.13 [C(18¹)]; 9.88, 9.70, 9.64 [C(7¹) + C(13¹) + C(17¹)].

FAB mass spectrum. m/z in glycerol (intensity): 681 (11.8%), $M^+ + 1$; 649 (1.5%), $M^+ + 1 - S$; 647 (1.7%), $M^+ + 1 - H_2S$; 615 (3.5%), $M^+ + 1 - H_2S_2$.

Pigment rac. 6

Ultraviolet/visible spectrum. λ_{max} /nm (relative intensity) in $CHCl_3$ 562 (1), 328 (1.42); in CH_3OH 552 (1), 326 (1.46); in $CH_3OH + H_2SO_4$ 572 (1), 328 (0.92); in $CH_3OH + (CH_3CO_2)_2Zn$ 626 (1), 578 (sh), 338 (0.84).

1H NMR spectrum. δ /ppm in $CDCl_3$: 7.98 [br s, 1 H, NH]; 7.23 [s, 1 H, HC(10)]; 6.75 [s, 1 H, HC(15)]; 3.87 [dxq, $J(3, 3^1) = 5.24$ Hz, $J(3^1, 3^2) = 7.15$ Hz, 1 H, HC(3¹)]; 3.63, 3.60 [s, each 3 H,

$H_3C(8^4) + H_3C(12^4)$]; 3.34 [s, 2 H, $H_2C(5)$]; 2.94–2.60 [m, 4 H, $H_2C(8^1) + H_2C(12^1)$]; 2.56–2.49 [m, 6 H, HC(2) + HC(3) + $H_2C(8^2) + H_2C(12^2)$]; 2.41–2.36 [m, 2 H, $H_2C(18^1)$]; 2.08 [s, 3 H, $H_3C(17^1)$]; 2.03 [s, 3 H, $H_3C(13^1)$]; 2.00 [s, 3 H, $H_3C(7^1)$]; 1.33 [d, $J(3^1, 3^2) = 7.15$ Hz, 3 H, $H_3C(3^2)$]; 1.11 [t, $J(18^1, 18^2) = 7.43$, 3 H, $H_3C(18^2)$]; 0.78 [d, $J(2, 2^1) = 7.16$ Hz, 3 H, $H_3C(2^1)$].

^{13}C NMR spectrum. δ /ppm in $CDCl_3$: 178.20 [C(1) or C(19)]; 173.15, 172.97, 172.81 [$C(8^3) + C(12^3) + C(1)$ or C(19)]; 161.87, 146.69, 143.08, 141.76, 140.60, 138.22, 135.34, 133.38, 131.31, 130.54, 120.55 [C(6) + C(7) + C(8) + C(9) + C(11) + C(12) + C(13) + C(14) + C(16) + C(17) + C(18)]; 116.04 [C(10)]; 97.58 [C(15)]; 87.56 [C(4)]; 61.15 [C(3)]; 58.38 [C(3¹)]; 51.69 [$C(8^4) + C(12^4)$]; 38.29 [C(5)]; 37.21 [C(2)]; 35.27 [C(8²) + C(12²)]; 19.98, 19.88 [C(8¹) + C(12¹)]; 17.29 [C(2¹)]; 16.91 [C(18¹)]; 14.00 [C(3²)]; 13.37 [C(18²)]; 9.64, 9.52, 9.46 [C(7¹) + C(13¹) + C(17¹)].

FAB mass spectrum. m/z in glycerol (intensity): 681 (19.2%), $M^+ + 1$; 649 (1.7%), $M^+ + 1 - S$; 648 (2.2%), $M^+ + 1 - HS$; 647 (2.5%), $M^+ + 1 - H_2S$; 615 (7.0%), $M^+ + 1 - H_2S_2$.

Results

By incubation of a culture of *Cyanidium caldarium* III-D-2 with 5-aminolevulinic acid (2 g/l) and

Table I. Nuclear-Overhauser effects with pigments rac. 5 and 6.

Compound	Irradiated at	NOE observed
5	HC(3 ¹) + $H_2C(5)$	HC(2), HC(3), $H_3C(7^1)$, $H_3C(3^2)$
	$H_2C(8^1) + H_2C(12^1)$	HC(10), $H_3C(7^1)$, $H_3C(13^1)$, $H_2C(8^2)$, $H_2C(12^2)$
	$H_2C(8^2) + H_2C(12^2)$	HC(10), $H_2C(8^1)$, $H_2C(12^1)$
	HC(2)	HC(3 ¹) [strong], $H_3C(2^1)$, $H_3C(3^2)$ [weak]
	$H_3C(3^2)$	HC(3 ¹), HC(3)
	$H_3C(2^1)$	HC(2), HC(3)
6	HC(10)	$H_2C(8^1)$, $H_2C(12^1)$, $H_2C(8^2)$, $H_2C(12^2)$
	HC(15)	$H_3C(13^1)$, $H_3C(17^1)$
	HC(3 ¹)	HC(3), $H_3C(3^2)$
	$H_2C(5)$	HC(3), $H_3C(7^1)$
	$H_2C(8^1) + H_2C(12^1)$	$H_2C(8^2)$, $H_2C(12^2)$, HC(15)
	$H_2C(8^2) + H_2C(12^2) + HC(2) + HC(3)$	$H_2C(8^1)$, $H_2C(12^1)$, HC(3 ¹), $H_3C(7^1)$, $H_3C(2^1)$, $H_3C(3^2)$ [very weak], $H_2C(5)$ [very weak]
	$H_2C(18^1)$	$H_3C(18^2)$, $H_3C(17^1)$
	$H_3C(17^1)$	HC(15)
	$H_3C(13^1)$	HC(15)
	$H_3C(7^1)$	$H_2C(5)$, HC(3)
	$H_3C(3^2)$	HC(3 ¹), HC(2) [very strong], HC(3) [weak], $H_2C(18^1)$
	$H_3C(2^1)$	HC(2) [very strong], HC(3 ¹) [weak]

glutathione (2.1 g/l) or L-cysteine (0.45 g/l), two new polar bile pigments **1** and **2** could be isolated. Interestingly under these conditions no 3¹-hydroxy-mesobiliverdin IXa [7] could be observed although all the other pigments described in [7] (porphyrins, *E*-phycocyanobilin, biliverdin IXa) were detectable. By adding smaller amounts of glutathione or cysteine, the hydroxy-bile pigment could be isolated besides small amounts of the new pigments or no new pigments at all. Higher amounts of glutathione or cysteine yielded the same pigment composition as with the conditions used, however, pigment excretion started later. Our first assumption was, that the new chromophores were the products of addition of cysteine respectively glutathione at the ethylidene double bond of phycocyanobilin to give the compounds **3** and **4** (Fig. 1). However, chemical addition of cysteine

and glutathione to *E*-phycocyanobilin clearly showed, that this was not the case (see last part of this paper). By spectroscopical methods we identified the pigments **1** and **2** as *E*-phycocyanobilin derivatives, containing one SH-group at C5 (compound **1**); and one SH and one OH-group at C3 and C3¹ (compound **2**), respectively (Fig. 1).

Structure elucidation of pigment **1**

Chromophore **1** is rather easily protonated. Therefore, we were not able to run a UV/VIS spectrum of the free base in chloroform, even when the solvent was filtered over basic alumina. Absorption maxima at 310, 360 and 583 nm could be observed in methanol. By adding a drop of sulfuric acid only two maxima were obtained, at 328 and 625 nm. The zinc complex yielded absorption

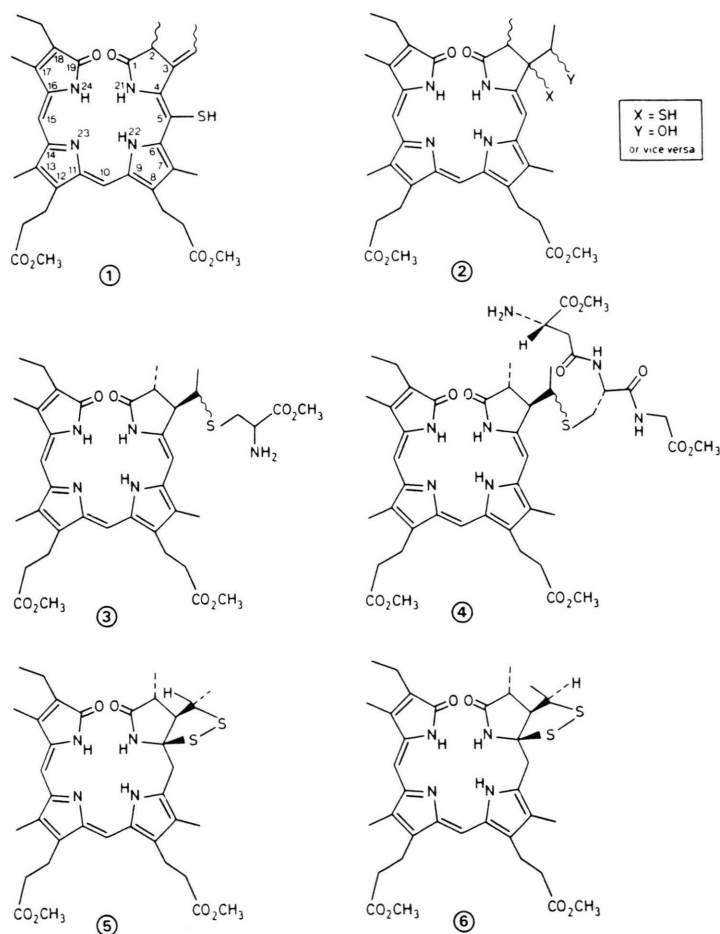


Fig. 1. Structures of bile pigments possibly related to phycocyanin biosynthesis. Bile pigment **1** and **2** were isolated from the culture medium of *Cyanidium caldarium* III-D-2 after incubation with 5-aminolevulinic acid and cysteine or glutathione. Bile pigments **3**–**6** were obtained chemically from *E*-phycocyanobilin.

maxima at 335, 370 and 662 nm. In contrast to other bile pigments like verdins and violins, the bathochromic shift of the protonated form is smaller than that of the zinc complex. The ratio E^{UV}/E^{VIS} in methanol is smaller compared with *E*-phycocyanobilin or biliverdins, which show a helical conformation. This might be a hint that chromophore *1* is not completely helical but somewhat extended. A FAB-MS in glycerol yielded a M^+ at 646, which fits to a phycocyanobilin-chromophore containing one sulfur atom.

As we could not run NMR-spectra in chloroform, we used d_5 -pyridine as solvent. Unfortunately, we were not able to purify the chromophore further because of its instability. The 1H NMR (Fig. 2) shows two singlets at 7.37 and 6.11 ppm which were assigned to HC(10) and HC(15) by NOE-experiments (Fig. 2). The signals for the propionic acid side chains at C(8) and C(12) appear at 3.59 and 3.55 ppm ($H_3C(8^4) + H_3C(12^4)$), 3.20–2.98 ($H_2C(8^1) + H_2C(12^1)$), 2.78–2.75 and 2.65–2.62 ppm ($H_2C(8^2) + H_2C(12^2)$). The ethyl side chain shows signals at 2.03–1.98 ppm ($H_2C(18^1)$) and 0.76–0.72 ppm ($H_3C(18^2)$), which are high

field shifted compared to other bile pigments like phycocyanobilin and biliverdins. Three singlets at 2.10, 1.90 and 1.84 ppm were assigned to the three methyl groups $H_3C(13^1)$, $H_3C(17^1)$ and $H_3C(7^1)$; by NOE-experiments, only the signal at 1.90 ppm could be assigned to $H_3C(7^1)$. Furthermore, there are two methyl groups at 1.34 and 1.35 ppm, each being a doublet. The first one shows a coupling with a signal at 5.74 ppm, the latter one with a peak at about 3.00 ppm. Due to the low field shift to 5.74 ppm we assigned this signal belonging to $HC(3^1)$ which could therefore be at a double bond. The other signals at about 3.00 ppm and at 1.35 ppm could therefore be assigned to $HC(2)$ and $H_3C(2^1)$, respectively. As a signal for the methine bridge $HC(5)$ could not be detected – the NMR spectrum in d_6 -acetone clearly showed that there is no other signal under the water peak – the sulfur-substituent was placed at C(5). By NOE-experiments it was not possible to decide whether the ethylenidene-group at C(3) has *Z* or *E*-configuration. Also the stereochemistry at C(2) is unclear, however, as phycocyanobilin shows 2*R* configuration it might be also 2*R*.

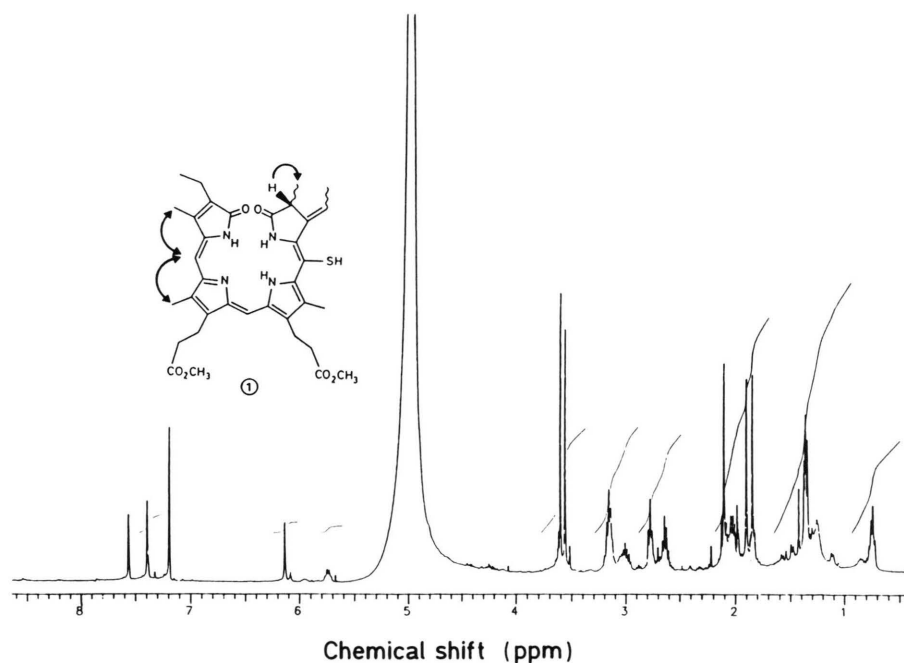


Fig. 2. 1H NMR spectrum (400 MHz) for **1** in d_5 -pyridine. NOE are indicated by arrows (irradiation = end of arrow; enhancement obtained = top of arrow). By irradiation at all the other signals no NOE was obtained.

Structure elucidation of pigment 2

Like pigment **1** the other new chromophore is also sensitive to small amounts of acid. The UV/VIS spectrum in methanol shows two maxima at 342 and 590–597 nm. By addition of a drop of sulfuric acid, a shift to 348 respectively 652–660 nm and an enhancement of the long-wavelength absorption were observed. The zinc complex yielded shifts to 348, 378 and 642 nm.

FAB-MS in glycerol yielded a $M^+ + 1$ -peak at 665, which is in agreement with structure **2**. A small peak at 647 is due to $M^+ + 1 - H_2O$.

As in the case of compound **1** we chose d_5 -pyridine as solvent for NMR-investigations (Fig. 3). Three singlets at 7.08, 6.43 and 6.05 ppm were assigned to HC(10), HC(5) and HC(15) by NOE-experiments. The signals of the two propionic acid side chains appear at 4.60 ppm ($H_3C(8^4) + H_3C(12^4)$), 3.06 and 2.93 ($H_2C(8^1) + H_2C(12^1)$) and 2.68 and 2.61 ($H_2C(8^2) + H_2C(12^2)$). The signals at 3.06 and 2.68 ppm couple with each other as well as the signals at 2.93 and 2.61 ppm. The ethyl group at ring D was assigned to the signals at 2.50–2.40 ppm ($H_2C(18^1)$) and 1.24 ppm

($H_3C(18^2)$). Three methyl groups appear as singlets at 2.07, 2.05 and 1.84 ppm. Due to NOE-experiments (Fig. 3) we could identify the signal at 1.84 ppm belonging to $H_3C(1^1)$, the other two methyl groups are therefore $H_3C(13^1)$ and $H_3C(17^1)$. The two methyl groups $H_3C(2^1)$ and $H_3C(3^2)$ appear as doublets at 1.52 and 1.42 ppm. The doublet at 1.52 ppm shows coupling with the signal at 4.68 ppm, the one at 1.42 ppm with the signal at 3.92 ppm. A definite assignment for the side chains of C2 and C3 was not possible, but by comparison with similar compounds it is rather certain that the signals at lower field belong to C3. A NMR spectrum in d_6 -acetone clearly showed that there is no further signal under the huge water peak.

There is still the question at which position the OH- respectively the SH-group has to be placed. We were not able to elucidate this by spectroscopy. Due to the known 3^1 -OH-mesobiliverdin IX α we speculate that the hydroxy-group might be at position 3^1 and the thiol-group at C3.

Chromophore **2** has 3 asymmetric carbons, therefore 3 diastereomers could be expected: 2 *RS*, 3 *RS*, 3^1 *RS*, 2 *RS*, 3 *SR*, 3^1 *RS* and 2 *RS*, 3 *SR*, 3^1 *SR*. When compounds like methanol [20], cysteine or

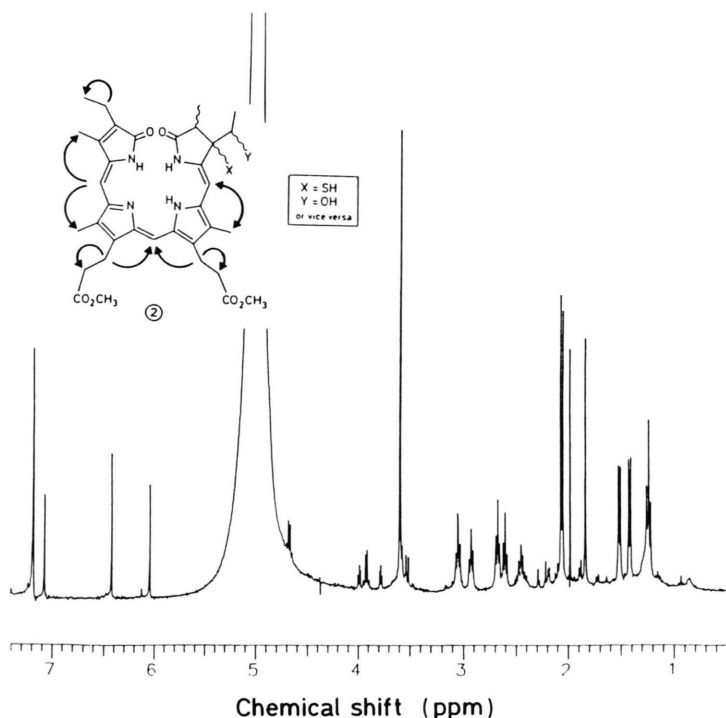


Fig. 3. 1H NMR spectrum (400 MHz) of **2** in d_5 -pyridine. NOE are indicated by arrows (irradiation = end of arrow; enhancement obtained = top of arrow). By irradiation at all the other signals no NOE was obtained.

glutathione (see above) are added chemically at the ethylenedouble bond of phycocyanobilin, one always obtains trans-configuration in ring A and both diastereomers of C(3') which show different NMR spectra. In the case of chromophore **2**, however, the ^1H NMR spectrum looks like that of a single compound. This might be a hint that compound **2** was synthesized enzymatically and not by chemical addition of water and hydrogen sulfide to excreted phycocyanobilin or another chromophore. This possibility had to be considered because a strong odour of hydrogen sulfide always evolved when algae were incubated with cysteine or glutathione.

To get further confirmation of the enzymatical formation of **1** and **2** we did the following experiments. A culture of *Cyanidium caldarium* was incubated only with 5-aminolevulinic acid to obtain the "normal" pigments [6, 8]. Other cultures were incubated only with cysteine for 1, 2 or 3 days. At the end of the incubation with cysteine, we split each culture into 2 equal parts and added either the crude pigment-extract of the first incubation or *E*-phycocyanobilin isolated from *Spirulina geitleri*. Immediately after addition of the pigments, the algal cells were removed from aliquots of each sample by centrifugation. The supernatants and the remaining cultures were further incubated under identical conditions for 5 days. In no one of the cell-free supernatants we could detect pigment **1** or

2 whereas the control samples with the algal cells formed **1** and **2** under the same conditions. Therefore we believe that the algae biosynthesized the two new compounds.

When we knew the structure of pigments **1** and **2** we tried to synthesize them chemically by addition of hydrogen sulfide to rac. *E*-phycocyanobilin dimethyl ester. However, by passing a stream of hydrogen sulfide through a solution of rac. *E*-phycocyanobilin dimethyl ester in methanol + 10% ammonia we obtained two new violet chromophores which turned out to be the diastereomers rac. **5** and rac. **6** (Fig. 1).

Structure elucidation of pigments rac. **5** + rac. **6**

The UV/VIS spectra of the chromophores rac. **5** + rac. **6** in methanol, acidic methanol and methanol containing Zn-acetat are very similar and typically for violins, *e.g.* chromophores with 3 fully conjugated rings (for data see experimental part).

A FAB-mass spectrum of rac. **5** and rac. **6** showed the same $M^+ + 1$ peak at 681, which corresponds to a phycocyanobilin dimethyl ester chromophore containing 2 sulfur atoms. Moreover, there were signals at $m/z = 648$ and 615 which belongs to $M^+ + 1 - \text{HS}$ respectively $M^+ + 1 - \text{H}_2\text{S}_2$. The ^1H NMR spectra of rac. **5** (Fig. 4) and rac. **6** (Fig. 5) are also very similar. By decoupling and NOE-experiments (Table I) we

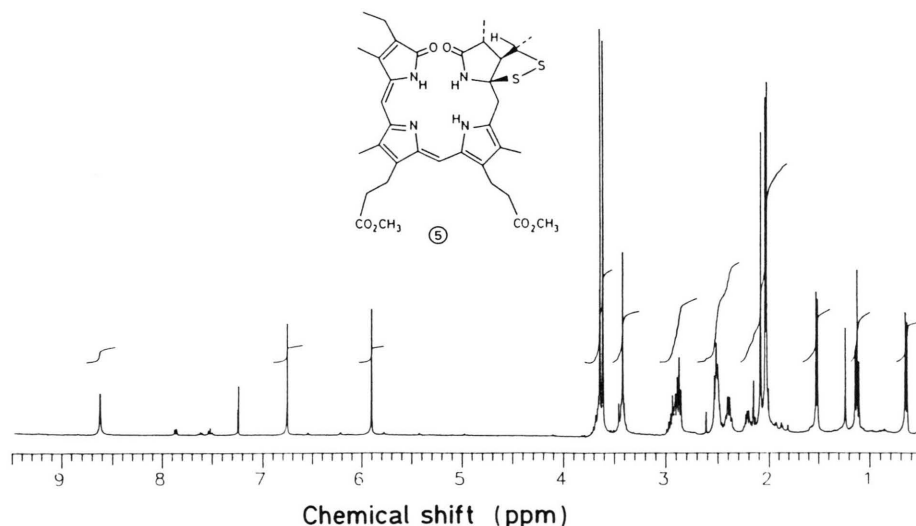


Fig. 4. ^1H NMR spectrum (400 MHz) of rac. **5** in CDCl_3 .

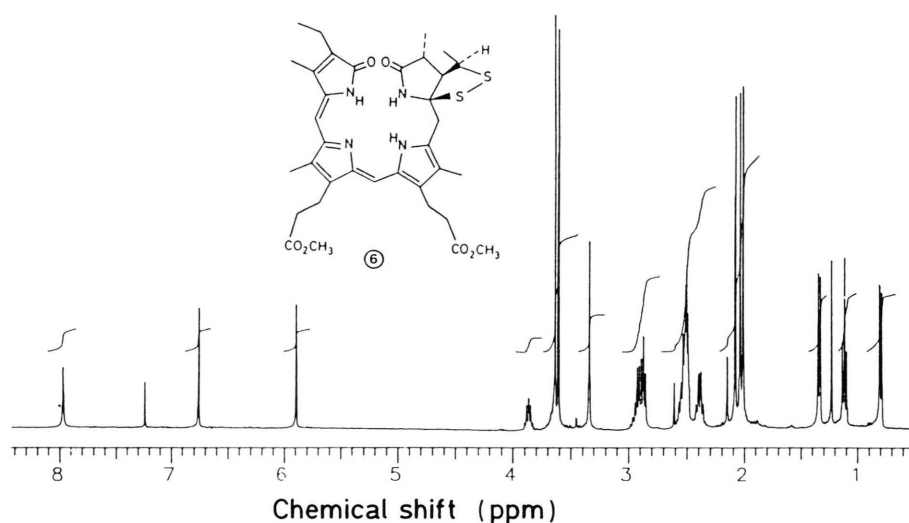


Fig. 5. ¹H NMR spectrum (400 MHz) of rac. 6 in CDCl₃.

could assign almost all signals. The only question was whether the signal at 3.34 ppm was due to a CH₂-bridge between ring A and B or due to two CH-protons at C(4) and C(5). However, in the latter case we would have expected a coupling of HC(4) with HC(3). To decide between the two

possibilities we applied ¹³C NMR spectroscopy. A DEPT-¹³C NMR spectrum of pigment rac. 6 and a HECTOR of pigment rac. 5 clearly showed that there was a CH₂-group. From the ¹H NMR spectrum we already knew that one sulfur had to be placed at position 3¹ because of the chemical shift

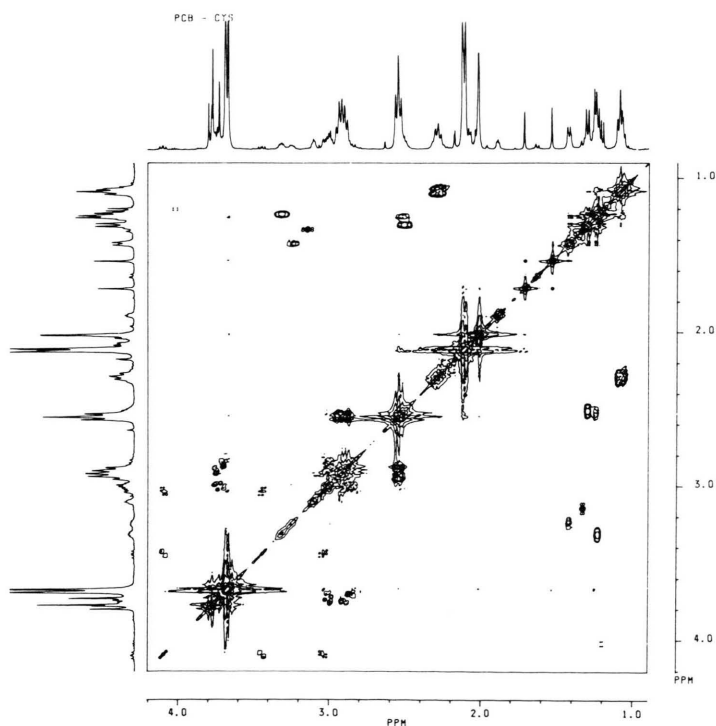


Fig. 6. ¹H NMR spectrum and COSY (400 MHz) of rac. 3 in CDCl₃. The cross-peaks of HC(2) with HC(3) and HC(3) with HC(3¹) cannot be seen in the chosen plot, however, they were visible by choosing a lower contour level and moreover their assignment was done by decoupling experiments.

of HC(3¹) and the typical coupling. When we learned that there was a methylene bridge between ring A and B the only possibility to place the second sulfur atom was to form a disulfide between C(3¹) and C(4), which yielded a 5-membered ring.

By NOE-experiments (Table I) we were able to figure out the stereochemistry of rac. **5** and rac. **6**: both compounds have the *trans*-configuration at ring A. It is known that adding methanol to the ethylenic-double bond of *E*-phycocyanobilin yields the *trans*-configuration of ring A [20]. Therefore, only two diastereomers are possible: 2*RS*, 3*SR*, 3¹*SR*, 4*RS* and 2*RS*, 3*SR*, 3¹*RS*, 4*RS*. In case of the first diastereomer we could expect a NOE between CH(2) and HC(3¹), in case of the other diastereomer NOE between HC(2) and H₃C(3²) should be obtained besides other NOEs which are not interesting for the determination of the two diastereomers. When irradiated in HC(2) of compound rac. **5** we observed a strong NOE at HC(3¹) and vice versa. Compound rac. **6** yielded strong NOE between HC(2) and H₃C(3²). Compound rac. **5** is, therefore, the diastereomer with 2*RS*, 3*SR*, 3¹*SR*, 4*RS* and compound rac. **6** the one with 2*RS*, 3*SR*, 3¹*RS*, 4*RS*-configuration.

Normally, 5-membered ring systems with two adjacent sulfur atoms are unstable. In our case, however, pigments rac. **5** and rac. **6** are as stable as other bile pigments like *E*-phycocyanobilin.

Synthesis of phycocyanobilin-cysteine-adduct (3) and phycocyanobilin-glutathione-adduct (4)

By adding cysteine or glutathione to the ethylenic double bond of phycocyanobilin, several diastereomers are possible. In the case of cysteine, we first used 2*R-E*-phycocyanobilin and S-cysteine methyl ester. We expected only two diastereomers, 2*R*, 3*R*, 3¹*R*, 3²*S* and 2*R*, 3*R*, 3¹*S*, 3²*S*. A ¹H NMR spectrum, however, showed that we got at least 3 diastereomers. When racemization of either phycocyanobilin or cysteine took place during the reaction, one can theoretically expect 4 diastereomers in each case, when both educts isomerize also 4 diastereomers are possible, however, each is racemic. By using rac. *E*-phycocyanobilin dimethyl ester as starting material and S-cysteine, we obtained the same NMR spectrum, so that we cannot decide whether only phycocyanobilin racemized in our first experiment or both compounds. A separation of the diastereomers was not possible due to

the instability of the phycocyanobilin-cysteine-adduct. Cysteine adds very easily at the double bond but also eliminates quite easily to yield phycocyanobilin back. When the product was eluted from the silica gel, elimination was always observed. Two-dimensional chromatography, however, showed that the adduct is stable on wet silica gel.

In the case of glutathione addition the problem is even more difficult. The yield of the addition is very poor, moreover purification is difficult because of the polarity of the adduct. Also the compound is not very stable, different unidentified degradation products were obtained, but no elimination of glutathione to yield phycocyanobilin was observed. Unfortunately we were not able to obtain a reasonable ¹H NMR spectrum. By FAB mass spectroscopy and comparison the UV/VIS data of the cysteine-adduct **3** and the glutathione-adduct **4** we propose the analogous structure **4**.

The UV/VIS spectra of pigment **3** and **4** in chloroform, methanol, methanol + sulfuric acid and methanol + zinc acetate are almost identical and typical for bile pigments with 3 conjugated rings and an additional conjugated double bond. *E.g.* the UV/VIS spectra of the well-known phycocyanobilin-methanol-adducts [20] are very similar with those of pigments **3** and **4**.

A FAB mass spectrum of **3** and **4** exhibits the M⁺ + 1 at 750 (pigment **3**) and a M⁺ + 2-peak at 951 (pigment **4**). In both cases also a peak at 615 was present, which is due to phycocyanobilin dimethyl ester.

The ¹H NMR spectrum and a COSY-NMR spectrum of compound **3** is shown in Fig. 6. Evidence for the presence of at least 3 diastereomers can be seen at the signals of ring A (*e.g.* at least 6 doublets for H₃C(2¹) and H₃C(3²)) and also the signal of HC(5), which appears at 3 different positions. The assignment of the bile pigment part is very clear and shown in Fig. 6. The resonances for the cysteine are not well separated but fall together with other signals. With the help of a COSY-NMR-experiment, however, the methylene-group H₂C(3¹) and the α-proton HC(3²) were assigned to 3.33–2.85 ppm and 3.80–3.67 ppm, respectively.

Discussion

The structures of compounds **1** and **2** which are produced by incubation of *Cyanidium caldarium*

cells with 5-aminolevulinate and cysteine or glutathione are unexpected and may lead to various speculations. The expected addition products of phycocyanobilin with cysteine (**3**) or glutathione (**4**) can be prepared *via* chemical reaction as described in this paper. These products are not detectable in the mixture of pigments excreted by the algal cells even after varying the conditions of incubation. If cysteine or glutathione would serve as obligatory “carriers” for chromophore insertions into the apoprotein, one would expect enzymatic formation of these adducts. Our results do not support such a possibility. It is, however, questionable whether cysteine or glutathione can penetrate the membranes of algal cells without decomposition. It has been reported that cysteine is metabolized by algae and can serve as the only source for sulfur [21]. Our observation that hydrogen sulfide is liberated from cysteine and glutathione also points to metabolization of these compounds by *Cyanidium caldarium* although we did not investigate whether this occurs inside the cell or by exoenzymes.

The production of hydrogen sulfide by the algae prompted us to investigate chemical addition of this compound to phycocyanobilin. The bile pigments which were produced by this means are different from the compounds produced by the algae. Surprisingly, the chemical reaction apparently involves addition of 2 moles hydrogen sulfide per chromophore and subsequent dehydrogenation.

There still remains the questions of the mechanism of disulfide formation. Did one sulfur atom add first at C(3¹) and another one at C(4) followed by oxidative ring closure or did the second sulfur atom add to the first sulfur atom and close the ring later? Did this occur in the algal cell or only during workup?

Bile pigments with an additional 5-membered heterocycle between C(3¹) and C(4) were still unknown. However, in the case of dipyrinones there are some derivatives described with a condensed tetrahydrofuran between C(3¹) and C(4) [13, 22–24].

Grubmayr and Wagner [24] assume at first the nucleophilic addition (of benzylmercaptan) at C-3¹

and in a second step (of either a second benzylmercaptane or a hydroxy group) at C-4 with an ethylidene-substituted dihydrodipyrinone. This order of reactions might also occur with hydrogen sulfide. It should be mentioned here that we did not find dehydrogenation of ring A in any of our addition products, contrary to the findings with addition of the apoprotein [14–16]. We find dehydrogenation instead of the thiol functions under formation of the disulfide ring structure.

Although the cells produce hydrogen sulfide from added cysteine or glutathione, the products **1** and **2** obtained from phycocyanobilin (produced from ALA by the algae) and the same sulfur compounds do not resemble any of the products of the chemical addition reactions **3**–**6**. We take this fact as one argument for the formation of **1** and **2** by an enzymatic reaction or by chemical reaction with an intermediate other than phycocyanobilin. Further arguments are (1) the lack of diastereomers for **2** which we find in all our chemical addition reactions and (2) the failure of formation of **1** and **2** with cell-free supernatants, even after (separate) incubation of algae with cysteine (or glutathione) and ALA. Whereas the structure of product **2** is in agreement with a nucleophilic addition, compound **1** looks rather like a product of an electrophilic or radicalic addition. Examples of such reactions have been described [1, 25]. However, we cannot yet deduce any enzymatic mechanism from our results nor solve the question whether products **1** and **2** are members of the normal biosynthetic chain of biliproteins or rather of a metabolic side way.

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