(2) (a) Enzymes that catalyze addition at C-3 of PEP are pyruvate kinase (EC 2.7.1.40), pyruvate, orthophosphate dikinase (EC 2.7.9.1), PEP synthase (EC 2.7.1.dd), phosphoenolpyruvate carboxylase (EC 4.1.1.3.1), phosphoenolpyruvate carboxykinases (EC 4.1.1.32, 4.1.1.a), phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38), and phospho-2-keto-3-deoxy-heptonate aldolase (EC 4.1.2.15). (b) Enolpyruvate transferase (EC 2.5.1.7) catalyzes substitution for the $PO_3^{2-}$ group of PEP. (c) Substitution at C-3 H of pyruvate occurs in pyruvate carboxylase (EC 6.4.1.1), methylmalonyl-CoA:pyruvate carboxyltransferase (EC 2.1.3.1), malate dehydrogenases (decarboxylating) (EC 1.1.1.38 and .40), (4*S*)-4-hydroxy-2-ketoglutarate aldolase (4.1.2.e), and oxaloacetate decarboxylases (4.1.1.3, biotin-dependent or non-biotin-dependent).
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# Chromopeptides from *C*-Phycocyanin. Structure and Linkage of a Phycocyanobilin Bound to the $\beta$ Subunit

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**Abstract:** The smallest cyanogen bromide fragment derived from the $\beta$ subunit of *Synechococcus* sp. 6301 *C*-phycocyanin, the blue heptapeptide **2**, has been investigated by 360-MHz $^1$H NMR spectroscopy. The peptide portion, heptapeptide **3**, was synthesized independently and used in comparative spectroscopic analysis. These studies have led to complete assignment of the structure of the peptide-linked phycocyanobilin and elucidation of the nature of the thioether chromophore–peptide linkage.

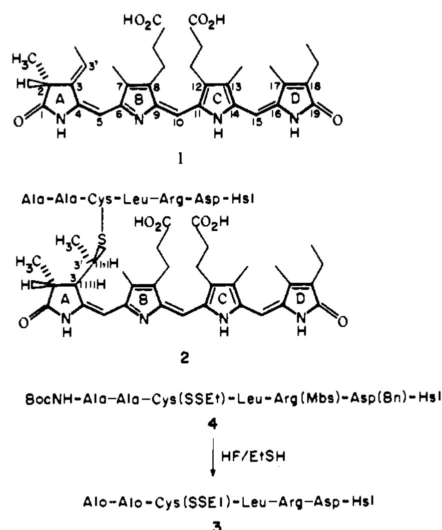
The intensely blue protein, *C*-phycocyanin, is a major light harvesting component of the photosynthetic apparatus of cyanobacteria (blue-green algae) and of certain red algae.[1] Composed of two distinct polypeptide chains, $\alpha$ and $\beta$ subunits,[2] the monomer of *C*-phycocyanin contains three distinct covalently bound prosthetic groups known as phycocyanobilins, one on the $\alpha$ chain and two on the $\beta$ chain.[3] Since the initial work in the 1930's,[4] intensive study of the phycocyanobilins has not yet provided the unambiguous assignment of the structure of the natural prosthetic groups or the precise nature of the covalent linkage to the apoprotein.[5,6]

The methodology most widely used to examine the structure of these pigments has entailed the cleavage of the chromophore–protein linkages of *C*-phycocyanin through treatment under various conditions.[7-14] From these studies a number of degradation products and phycobilins released from the protein have been characterized spectroscopically, including the "blue pigment" **1**, whose dimethyl ester has been synthesized recently.[15] It is clear, however, that all of these pigments, while derived from the various native prosthetic groups, are products whose nature is dependent on the cleavage conditions[5] and the potential of the latter for introducing artifacts.

The second experimental approach, which requires the chromophore–protein linkage to be maintained, has been applied to the determination of the amino acid sequences about the sites of attachment of the three phycocyanobilins.[16-19] From such analyses of proteolytically prepared oligopeptides from *C*-phycocyanin, chromophore–protein linkages involving the side chains of serine,[16] aspartic acid,[17] cysteine,[17-19] and tyrosine[19] have been proposed. Two recent studies of highly purified chromopeptides from the *C*-phycocyanins of *Mastigocladus laminosus*[18] and *Synechococcus* sp. 6301,[20] however, have unambiguously established the linkage of a cysteine residue to each of the three phycocyanobilins. Although substantial evidence implicates a thioether linkage,[17,18,20-22] direct proof of the structure of any proposed chromoprotein linkage in *C*-phycocyanin is lacking.

In the present study, we report the structure determination of one of the three peptide-bound phycocyanobilins, namely $\beta_1$-phycocyanobiliheptapeptide **2** obtained from the cyanogen bromide cleavage of *Synechococcus* sp. 6301 *C*-phycocyanin.[20,23] A comparison of the $^1$H NMR spectra of this chromopeptide **2** with those of synthetic peptide **3** has permitted the direct assignment of the structure of the $\beta_1$-phycocyanobilin as well as that of the chromoprotein linkage.

Chromopeptide **2**, derived from residues 79–85 of the $\beta$ chain,[20] was chosen as the substrate to demonstrate a new methodology for the analysis of the prosthetic groups of bil-

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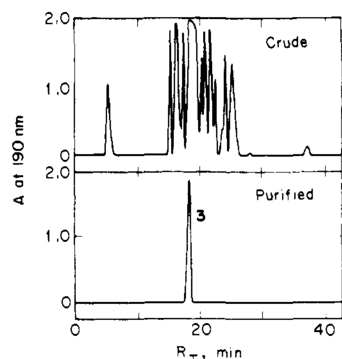
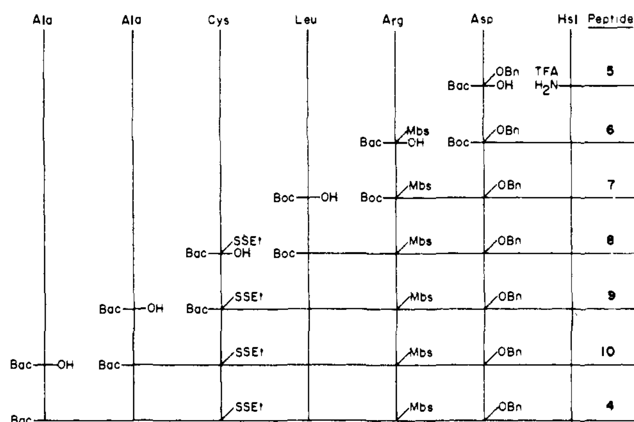



**Figure 1.** Reversed phase LC (system C) of heptapeptide **3** as the crude reaction product and after purification.

**Chart I.** Repetitive Excess Mixed Anhydride Synthesis of the Protected Heptapeptide **4**



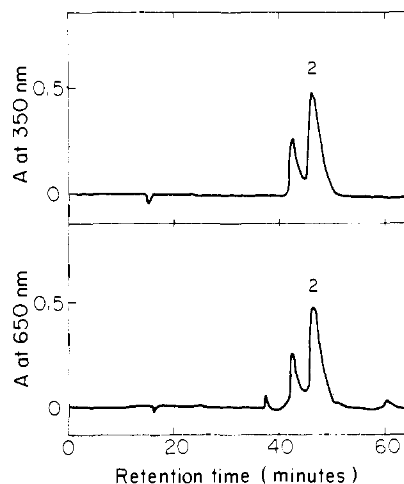




**Figure 2.** Reversed phase LC (system D) of crude chromopeptide **2**.

iproteins. Our approach required that the conditions used in obtaining the substrate for structural investigation should not alter the native substrate of the prosthetic group or its linkage to the protein. The cyanogen bromide cleavage procedure satisfied these requirements and in addition provided a good yield of the chromopeptide **2**. Furthermore, the development of an effective high performance liquid chromatographic (LC) system for the separation of this type of chromophore-linked peptide assured the homogeneity of the cleavage product. Using this methodology and the comparative $^1H$ NMR spectroscopic analysis described in this report, the direct proof of the structure of an intact phycocyanobilin and its thioether linkage has now been realized.

## Results and Discussion

**Synthesis of the Heptapeptide 3.** To facilitate the interpretation of the $^1H$ NMR spectrum of the chromopeptide **2**, authentic heptapeptide **3** was necessary and it was obtained in quantity by synthesis. We chose first to prepare the protected heptapeptide **4**,[24] which subsequently could be deblocked with acid to afford **3**.

The synthesis of the protected heptapeptide **4** was accomplished with the repetitive mixed anhydride method (Chart I).[25] This process was chosen in preference to the solid-phase method[26] as the intermediate peptides (**6–10**) could be highly purified after each condensation step. Furthermore, the deblocked peptides Leu-Arg-Asp-Hsl (**11**) and Cys(SSEt)-Leu-Arg-Asp-Hsl (**12**), which were obtained from intermediates **8** and **9**, respectively, were necessary for the interpretation of the NMR spectra of heptapeptides **2** and **3**.

The lactone of homoserine (Hsl) proved to be an effective protecting group for the C-terminal residue. It was rapidly formed as the crystalline trifluoroacetic acid salt upon dissolution of L-homoserine in anhydrous trifluoroacetic acid, and throughout the synthesis of the heptapeptide **4** the homoserine lactone moiety remained quite stable.[27] With one exception the yields of the purified peptide after each condensation were >90%. This exception was the low yield of tripeptide **7**, for which the cyclization of the mixed anhydride intermediate of the arginine derivative was responsible. Since this side product can be easily separated from the desired tripeptide, the use of excess arginine derivative should improve this conversion.

For the synthesis of the hexa- and heptapeptides **10** and **4**, the *t*-Boc group was removed with 4 N HCl in anhydrous dioxane. This method was preferred because of the reported instability of the ethyl disulfide in anhydrous trifluoroacetic acid.[28] Final removal of the protecting groups was accomplished with anhydrous hydrogen fluoride for 0.5 h at 0 °C.[29] Ethyl mercaptan, instead of anisole, served to scavenge the carbonium ion and to prevent acid-catalyzed disulfide interchange. Despite this precaution, a complex mixture of side products accompanied the heptapeptide **3** which, after LC purification (Figure 1), was isolated in 30% yield. The side reactions encountered during the HF cleavage probably involve the disulfide moiety. Similar reaction of the protected tetrapeptide **8** with HF/EtSH provided pure material without need for further purification; however, the pentapeptide **9** also gave a complex mixture of products which required purification by LC.

LC proved to be an effective tool for the analysis and purification of the peptide mixtures. Using reversed-phase C18 chromatography, all of the synthetic oligopeptides could be separated. The protected peptides **4–10** were eluted with 60:40 $CH_3CN/H_2O$ and detected at 225 nm; they showed increased retention times with increased size. The deblocked peptides were detected at 190 nm using a gradient eluent containing a mixture of acidic phosphate buffer and acetonitrile (see Experimental Section). With the latter system both the chromopeptide **2** (Figure 2) and the synthetic heptapeptide **3** (Figure 1) were purified on a preparative scale for $^1H$ NMR spectroscopy.

**Comparative $^1H$ NMR Spectral Analysis.** In order to determine the chromophore structure and mode of linkage to the peptide, the 360-MHz $^1H$ NMR spectra of the chromopeptide **2** and the synthetic peptide **3** (Figures 3 and 4), in $D_2O$ and in pyridine-$d_5$, were compared.

Complete assignment of the $^1H$ NMR spectra of the synthetic heptapeptide **3** has involved extensive spin decoupling experiments and comparative NMR studies with synthetic tetra- and pentapeptide, **11** and **12**. Parallel analysis of the NMR spectra of chromopeptide **2** reveals the expected similarity of the resonant frequencies of the corresponding amino acid residues of **3** (Tables I and II).

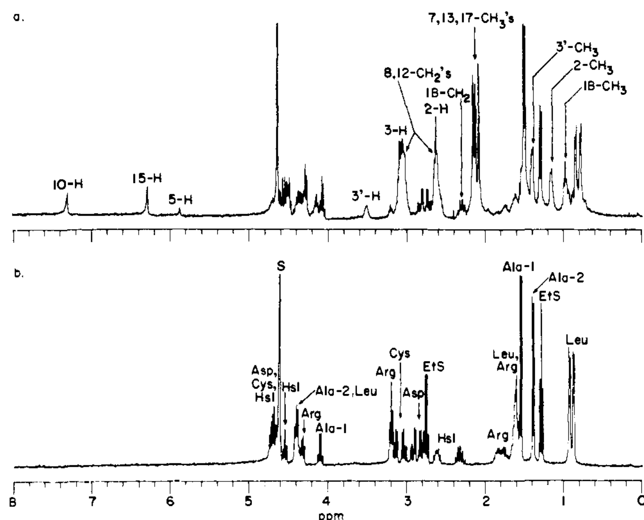



Figure 3. (a) The 360-MHz $^1$H NMR spectrum of chromopeptide **2** at 40 °C (2.4 mM, $D_2O$). (b) The 360-MHz $^1$H NMR spectrum of synthetic heptapeptide **3** at 40 °C (2.55 mM, $D_2O$).

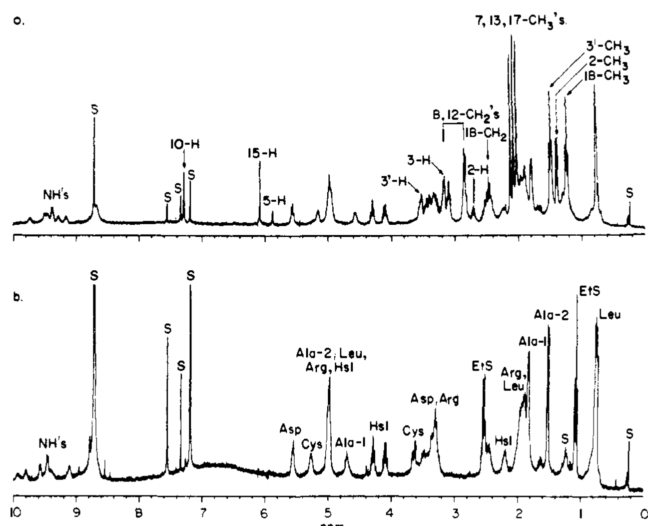



Figure 4. (a) The 360-MHz $^1$H NMR spectrum of chromopeptide **2** at 25 °C (2 mM, pyridine-$d_5$). (b) The 360-MHz $^1$H NMR spectrum of synthetic heptapeptide **3** at 25 °C (1 mM, pyridine-$d_5$).

Table I. 360-MHz $^1$H NMR Assignments for the Peptide Moieties of Chromopeptide **2** and Synthetic Peptide **3** in $D_2O$ at 40 °C

| chem shift[a] | | no. of | multiplicity, | |
|---|---|---|---|---|
| 2 | 3 | H's | J, Hz | assignment |
| 4.07 | 4.07 | 1 | q, 7.2 (6.8)[c] | Ala(1) α-CH[d] |
| 1.51 | 1.53 | 3[b] | d, 7.2 (6.8) | Ala(1) β-$CH_3$ |
| 4.27 | 4.37 | 1 | q, 7.2 (6.9) | Ala(2) α-CH |
| 1.29 | 1.37 | 3 | d, 7.2 (6.9) | Ala(2) β-$CH_3$ |
| 4.50 | 4.67 | 1 | dd, 6.8, 7.6 | Cys α-CH |
| 3.02 | 3.00 | 1[b] | dd, 7.6, −13.9 | Cys $β_1$-CH |
| 3.11 | 3.11 | 1 | dd, 6.8, −13.9 | Cys $β_2$-CH |
| 4.26 | 4.41 | 1 | dd | Leu α-CH |
| 1.51 | 1.61 | 3[b] | m | Leu β-$CH_2$, γ-CH |
| 0.77 | 0.87 | 3 | d, 5.3 (4.1) | Leu $δ_1$-$CH_2$ |
| 0.83 | 0.92 | 3 | d, 5.3 (4.1) | Leu $δ_2$-$CH_2$ |
| 4.15 | 4.30 | 1 | dd, 6.0, 7.8 | Arg α-CH |
| 1.64 | 1.81 | 1[b] | m | Arg $β_1$-$CH_2$ |
| 1.74 | 1.81 | 1[b] | m | Arg $β_2$-$CH_2$ |
| 1.54 | 1.61 | 2[b] | m | Arg $γ_2$-$CH_2$ |
| 3.11 | 3.19 | 2 | t, 6.8 | Arg δ-$CH_2$ |
| 4.63 | 4.70 | 1 | dd, 5.9, 7.8 | Asp α-CH |
| 2.77 | 2.80 | 1 | dd, 7.8, −16.8 | Asp $β_1$-$CH_2$ |
| 2.87 | 2.92 | 1 | dd, 5.9, −16.8 | Asp $β_2$-$CH_2$ |
| 4.58 | 4.63 | 1 | dd, 9.4, 10.4 | Hsl α-CH |
| 2.30 | 2.32 | 1 | m | Hsl $β_1$-$CH_2$ |
| 2.59 | 2.60 | 1 | m | Hsl $β_2$-$CH_2$ |
| 4.37 | 4.38 | 1 | m | Hsl $γ_1$-$CH_2$ |
| 4.53 | 4.54 | 1 | m | Hsl $γ_2$-$CH_2$ |

[a] The chemical-shift values are in parts per million from sodium 4,4-dimethyl-4-silapentane-5-sulfonate (DSS), and $CHCl_3$ was used as the internal chemical-shift marker. The chemical shift of 10 mM $CHCl_3$ in $D_2O$ is 7.66 ppm relative to DSS. [b] Overlapping resonances were observed. [c] The coupling constants in parentheses represent values obtained for the chromopeptide **2** that differed from those of heptapeptide **3**. [d] Ala(1) designates the N-terminal alanine.

In $D_2O$, minor differences in the spectra of the two heptapeptides are evident in the resonances attributed to the cysteine, leucine, and arginine residues (Figure 3, Table I). The positions of the α hydrogens of these three amino acids in **2** are displaced 0.10–0.20 ppm upfield from those in synthetic peptide **3**. Smaller (0.1 ppm) upfield shifts are also observed in some of the resonances of the leucine and arginine side chains of chromopeptide **2**. Little difference in the chemical shifts of the N-terminal alanyl, C-terminal homoserine lactone, and aspartic acid side chains of the two peptides in $D_2O$ is evident.

Table II. 360-MHz $^1$H NMR Assignments for the Peptide Moieties of Chromopeptide **2** and Synthetic Peptide **3** in Pyridine-$d_5$ at 25 °C

| chem shift, ppm[a] | | no. of | multiplicity, | |
|---|---|---|---|---|
| 2 | 3 | H's | J, Hz | assignment[b] |
| 4.57 | 4.70 | 1 | m | Ala(1) α-CH |
| 1.80 | 1.84 | 3 | d, 6.3 (5.5) | Ala(1) β-$CH_3$ |
| 4.92-5.00 | 4.94-5.04 | 4[c] | m | Ala(2) α-CH |
| 1.50 | 1.53 | 3 | d, 7.0 (7.4) | Ala(2) β-$CH_3$ |
| 5.14 | 5.26 | 1 | m | Cys α-CH |
| 3.42 | 3.49 | 1 | dd, 7.8, −13.6 | Cys $β_1$-$CH_2$ |
| 3.54 | 3.64 | 1 | dd, 4.9, −13.6 | Cys $β_2$-$CH_2$ |
| 4.92-5.00 | 4.94-5.04 | 4[c] | m | Leu α-CH |
| 1.89-2.00 | 1.89-2.00 | 7[c] | m | Leu β-$CH_2$ |
| 1.90 | 1.90 | 7[c] | m | Leu γ-CH |
| 0.77 | 0.75 | 3 | d, 5.7 | Leu $δ_1$-$CH_3$ |
| 0.77 | 0.78 | 3 | d, 4.3 | Leu $δ_2$-$CH_3$ |
| 4.92-5.00 | 4.94-5.04 | 4[c] | m | Arg α-CH |
| 1.89-2.00 | 1.89-2.00 | 7[c] | m | Arg β-$CH_2$ |
| 1.89-2.00 | 1.89-2.00 | 7[c] | m | Arg γ-$CH_2$ |
| 3.33 | 3.30 | 2 | m | Arg δ-$CH_2$ |
| 5.56 | 5.56 | 1 | m | Asp α-CH |
| 3.25 | 3.27 | 1 | dd, 7.3, −17.0 | Asp $β_1$-$CH_2$ |
| 3.41 | 3.39 | 1 | dd, 5.9, −17.0 | Asp $β_2$-$CH_2$ |
| 4.92-5.00 | 4.94-5.04 | 4[c] | m | Hsl α-CH |
| 2.23 | 2.20 | 1 | m | Hsl $β_1$-$CH_2$ |
| 2.43 | 2.46 | 1 | m | Hsl $β_2$-$CH_2$ |
| 4.09 | 4.09 | 1 | m | Hsl $γ_1$-$CH_2$ |
| 4.29 | 4.29 | 1 | m | Hsl $γ_2$-$CH_2$ |

[a] The chemical-shift values are in parts per million from $Me_4Si$ and were determined from a residual proton of pyridine (7.81 ppm from $Me_4Si$ at 25 °C). [b] The NH's were not assigned. [c] Multiple resonances occurred at these frequencies.

These upfield shifts in the spectrum of chromopeptide **2** can arise from the local magnetic ring current field of the bound phycocyanobilin and from phycocyanobilin-induced conformational change in the peptide backbone. In pyridine-$d_5$, the similarity of the spectra of the two heptapeptides **2** and **3** is even more striking with respect to the peptide moieties (Figure 4, Table II). Only the resonances attributed to the α and β hydrogens of cysteine and the α hydrogen of the N-terminal alanine are shifted significantly (0.10 ppm upfield) from their assigned frequencies in the spectrum of **3**. The chemical shifts of the other amino acid residues of **2** and **3** fall within 0.03 ppm of each other in pyridine-$d_5$.

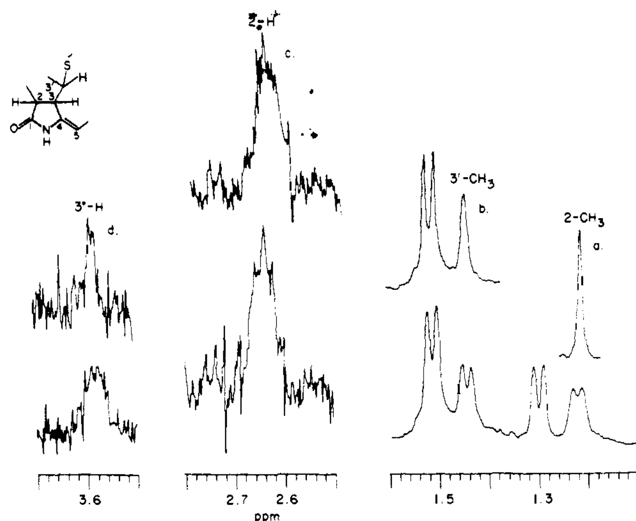



**Figure 5.** The partial 360-MHz $^1$H NMR spectrum of the chromopeptide **2** taken at 40 °C in $D_2O$: (a) after irradiation of the multiplet at 2.69 ppm; (b) after irradiation of the multiplet at 3.58 ppm; (c) after irradiation of the doublet at 1.21 ppm; (d) after irradiation of the multiplet at 1.44 ppm.

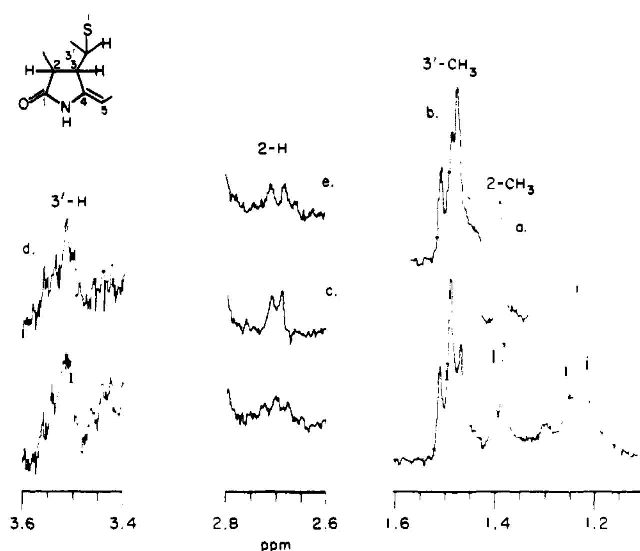



**Figure 6.** The partial 360-MHz $^1$H NMR spectrum of the chromopeptide **2** taken at 25 °C in pyridine-$d_5$. Lower spectra, coupled. Upper spectra, (a) after decoupling the multiplet at 2.70 ppm; (b) after irradiation of multiplet at 3.52 ppm; (c) after irradiation of doublet at 1.39 ppm; (d) after decoupling the doublet at 1.48 ppm; (e) after irradiation of the multiplet at 3.15 ppm.

**Table III.** 360-MHz $^1$H NMR Assignments of the Bilin Moiety of Chromopeptide **2** in $D_2O$ and Pyridine-$d_5$ and the Blue Pigment **1** (BP) in Pyridine-$d_5$

| chem shift[a] | | | no. of H's | multiplicity, J, Hz | assignment |
|---|---|---|---|---|---|
| **2** ($D_2O$) | **2** (pyr-$d_5$) | **1** (pyr-$d_5$)[b] | | | |
| 1.02 | 1.23 | 1.11 | 3 | t, 7.4 | 18-$CH_2CH_3$ |
| 1.21 | 1.39 | 1.34 | 3 | d, 7.3 | 2-$CH_3$ |
| 1.44 | 1.48 | 1.58 | 3 | d, 7.1 | 3′-$CH_3$ |
| 2.11 | 2.02 | 1.89 | 3 | s | 7,13,17-$CH_3$'s |
| 2.15 | 2.07 | 1.95 | 3 | s | |
| 2.17 | 2.12 | 2.01 | 3 | s | |
| 2.23 | 2.48 | 2.34 | 2 | q | 18-$CH_2CH_3$ |
| 2.69 | 2.83, 2.85 | 2.70 | 4 | m | 8,12-$CH_2CH_2$-$CO_2H$ |
| 2.69 | 2.70 | 2.70 | 1 | dq | 2-H |
| 3.11 | 3.09, 3.17 | 2.97 | 4 | m | 8,12-$CH_2CH_2$-$CO_2H$ |
| *c* | 3.15 | | 1 | m | 3-H |
| 3.58 | 3.52 | 6.17 | 1 | m | 3′-H |
| 5.94 | 5.87 | 5.71 | 1[d] | s | 5-H |
| 6.32 | 6.08 | 5.92 | 1 | s | 15-H |
| 7.41 | 7.29 | 7.09 | 1 | s | 10-H |

[a] The chemical shifts in $D_2O$ and pyridine-$d_5$ are reported in parts per million from DSS and $Me_4Si$, respectively, as described in Tables I and II. [b] From ref 10. [c] The chemical shift attributed to the $C_3$ methine was not determined in $D_2O$. [d] Slow exchange of the $C_5$ methine with $D_2O$ was responsible for the reduced size of the signal.

The observation that the differences in the spectra of the two heptapeptides in both solvents are the largest for amino acid residues nearest the cysteine is consistent with the proposed thioether linkage of phycocyanobilin to the peptide chain.[17,18,20-22] Moreover, the essential identity in chemical shifts of the arginine δ-methylenes and aspartic acid β-methylenes in the two peptides in both $D_2O$ and pyridine-$d_5$ discounts the possibility of a second linkage involving the side chain of these amino acids.

From the previous spectral analysis of the peptide moiety of **2**, the assignment of the remaining resonances to the phycocyanobilin prosthetic group can be made. Shown in Table III, the $^1$H NMR spectral assignments of the bilin moiety of **2** show great similarity with those reported for the blue pigment **1**.[10] In particular, the resonances attributed to the aromatic methyls of C-7, -13, and -17, olefinic methines of C-5, -10, and -15, the ethyl group of C-18, and the propionic acid methylenes at C-8 and -12 are salient features of the spectra of both pigments (**1** and **2**).

The complex spin system of ring A of **2** was deciphered with a number of double resonance experiments (Figures 5 and 6). Two broad doublets at 1.21 and 1.44 ppm in the spectrum of chromopeptide **2** in $D_2O$ (Figure 5) have been assigned to the C-2 and C-3′ methyl groups, respectively. The doublet at 1.21 ppm is coupled with a multiplet at 2.69 ppm (Figure 5a), while irradiation of a multiplet at 3.58 ppm collapses the doublet at 1.44 ppm to a singlet (Figure 5b). In pyridine-$d_5$, the two doublets at 1.39 and 1.48 ppm are coupled with multiplets at 2.70 and 3.52, respectively (Figure 6a,b).

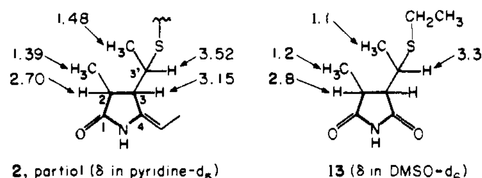
Converse double resonance experiments have provided conclusive evidence for the proposed thioether linkage to ring A of chromopeptide **2**. Irradiation of the higher field methyl doublets, 1.21 ppm in $D_2O$ and 1.39 ppm in pyridine-$d_5$, effects a change in the corresponding multiplets at 2.69 (Figure 5c) and 2.70 ppm (Figure 6c). Owing to overlapping resonances at 2.69 ppm in the $D_2O$ spectrum of **2**, the precise nature of this change cannot be ascertained. However, the analogous irradiation of the doublet at 1.39 ppm in the pyridine-$d_5$ spectrum of **2** clearly brings about the collapse of the multiplet at 2.70 ppm to a doublet with $J = 5.5$ Hz (Figure 6c).

When the lower field methyl doublets at 1.44 ppm in $D_2O$ and 1.48 ppm in pyridine-$d_5$ are irradiated, the collapse of resonances at 3.58 (Figure 5d) and 3.52 ppm (Figure 6d) is observed. In this case, the effect of these decoupling irradiations on the multiplet at 3.52 ppm in pyridine-$d_5$ (Figure 6d) is obscured by overlapping resonances, whereas the multiplet at 3.58 ppm in $D_2O$ becomes a doublet (Figure 5d).

The results of these decoupling experiments confirmed the presence of two α-substituted ethyl groups, $CH_3CH(X)$–, each adjacent to a methine. Upon irradiation of a multiplet at 3.15 ppm in the pyridine-$d_5$ spectrum of **2**, a change in the multiplicity of the resonances at 2.70 (C-2–H) and 3.52 ppm (C-3′–H) was observed. Owing to the presence of overlapping signals, the precise effect of this irradiation on the multiplet at 3.52 ppm could not be determined; however, the collapse of the multiplet at 2.70 ppm to a quartet ($J = 7.2$ Hz) was clearly

discernible (Figure 6e). These data show that the two ethyl moieties which include C-2 and C-3′ are adjacent to the same methine hydrogen, C-3-H, $\delta$ 3.15 in pyridine-$d_5$.

The assignment of the higher field methyl doublet (1.21 ppm in $D_2O$ and 1.39 ppm in pyridine-$d_5$) to the C-2 methyl group is based on comparison with model compounds. In a recent study on bilin-protein linkages, the synthesis and NMR spectrum (in $Me_2SO$-$d_6$) of the thioether-containing succinimide **13** were reported,[30] and the chemical shifts of methines C-2 and C-3′ were 2.8 and 3.3 ppm, respectively. The analogous resonances of the C-2 and C-3′ methines of the $\beta_1$-phycocyanobilin in **2**, 2.69 and 3.58 ppm in $D_2O$ (2.70 and 3.52 ppm in pyridine-$d_5$), respectively, similarly reflect the assignment of the thioether methine resonance to the lower field value. Since the lower field resonance is coupled with the doublet at 1.44 ppm in $D_2O$ (1.48 ppm in pyridine-$d_5$), this doublet has been assigned to the C-3′-methyl, $\alpha$ to the sulfur (Table III).



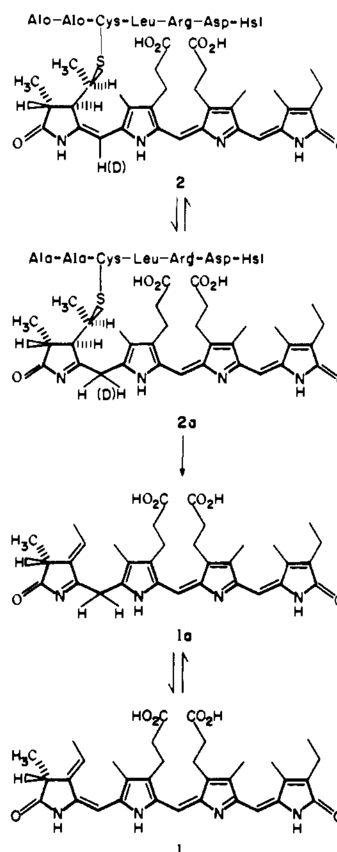

**Stereochemistry of the Chromophore–Protein Linkage.** The assignment of the stereochemistry of the thioether linkage shown in **2** requires three assumptions. If one assumes that (a) the absolute stereochemistry of C-2 is *R*,[31] (b) the geometry of ring A is trans,[32] and (c) the cleavage of **1** from *C*-phycocyanin involves a concerted trans-periplanar elimination requiring the relative stereochemistry at C-3 and C-3′ to be *RR* or *SS*,[33] then the assignment of the *RRR* stereochemistry at C-2, -3, and -3′ can be made. Although this stereochemical assignment for the $\beta_1$-phycocyanobilin-peptide linkage is probable, the stereochemical questions have not been unambiguously answered.

Our proposed structure **2** is consistent with the observation that the blue pigment **1** is the major pigment obtained from *C*-phycocyanin under a variety of conditions.[12] As shown in Scheme I, a tautomerization-$\beta$-elimination mechanism could explain these experimental results. The deuterium exchange of the C-5 methine observed in the NMR spectrum of **2** provides clear evidence for the equilibrium between tautomers **2** and **2a**. This tautomerization would increase the acidity of the C-3 hydrogen of **2a** (pyrrolinone form) through conjugation with the C-1 carbonyl, and thus promote the $\beta$ elimination of the peptide. Presumably a rapid equilibrium between the resulting pigment **1a** and the blue pigment **1** would then be established.[34] Owing to the additional double bond (C-3–C-3′), it is plausible to assume the position of this equilibrium favors the blue pigment **1**. Whether the pigment can indeed be released from the chromopeptide **2** under the same conditions used to liberate it from the intact protein[13] remains to be determined.

**Conformation and Complex Formation of 2.** A large difference in the temperature dependence of the $^1H$ NMR spectra of the synthetic peptide **3** and the chromopeptide **2** in $D_2O$ was observed. While the spectrum of **3** was relatively unaffected by changes in temperature between 15 and 40 °C, the effect on the spectrum of the chromopeptide **2** was significant. In contrast to **3**, at 15 °C many of the resonances of the chromopeptide **2** were so broad that much of the fine structure was not discernible. This was especially the case for proton resonances of the $\beta_1$-phycocyanobilin group of **2**. Although the line width and resolution greatly improved at 40 °C, many of the resonances due to the prosthetic group of **2** remained broad.

These temperature effects on the NMR spectrum of **2** in $D_2O$ can be interpreted to represent interconversions between different molecular conformations or aggregations of the chromopeptide. The observed line broadening in $D_2O$ may be attributed to a large number of interconverting prototropic forms and geometric isomers of the bilin chromophore. These interconversions would result in a concomitantly large number of magnetically nonequivalent environments for individual protons of the phycocyanobilin. Since the line width depends on the rate of interconversion between such isomers, the temperature dependence of the NMR spectrum can thus be explained.

**Scheme I.** Proposed Tautomerization and Trans-Periplanar $\beta$ Elimination of $\beta$-Phycocyanobiliheptapeptide **2**




Concentration dependence in the circular dichroism (CD) spectrum of **2** in $D_2O$ (Figure 7) suggests that dimerization of the phycocyanobilin moiety (perhaps through $\pi$–$\pi$ complex formation) is occurring at the concentration at which the NMR spectrum was obtained (2.4 mM in $D_2O$). Comparative analysis of the NMR spectra of **2** and **3** in $D_2O$ also shows that the phycocyanobilin prosthetic group effects a conformational change in the peptide backbone of **2**. The influence of the bilin moiety is particularly evident in amino acid residues closest to the thioether linkage as discussed earlier. On the other hand, the great similarity in the NMR spectra of **2** and **3** in the aromatic solvent pyridine-$d_5$[35] shows that the chromopeptide **2** exists as a monomeric species in this solvent. This observation is consistent with a $\pi$–$\pi$ interaction in $D_2O$ suggested by CD data. The exact nature of the complex formation in $D_2O$ warrants further investigation that may shed light on the orientation and interaction of the three bilins within native *C*-phycocyanin.

**Chromopeptide Stability.** The NMR and electronic absorption spectral data indicate that chemical modification of the chromopeptide has not occurred during the cyanogen bromide cleavage. A comparison of the absorption spectra of *C*-phycocyanin and chromopeptide **2** in 30% acetic acid (Figure 8) shows an insignificant difference in the spectrum of the phycocyanobilin prosthetic group after cleavage. The

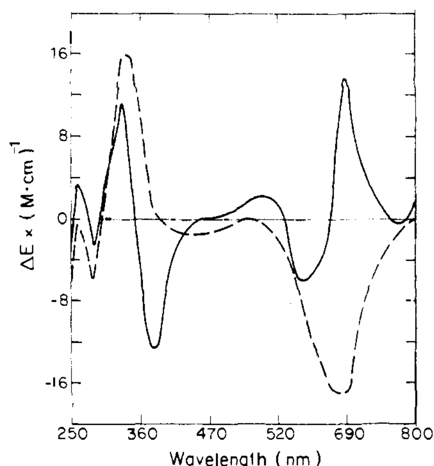



**Figure 7.** The circular dichroism spectrum of the chromopeptide **2** in $D_2O$: (- - -) $5.7 \times 10^{-5}$ M; (—) $2.21 \times 10^{-3}$ M.

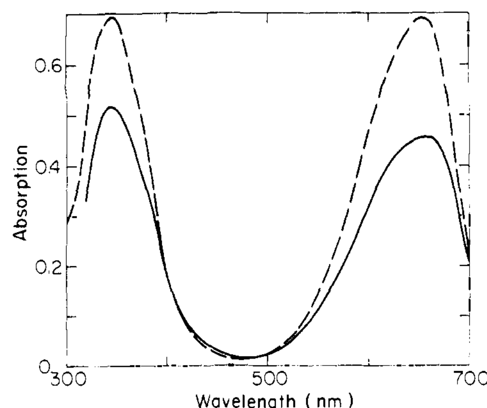



**Figure 8.** Absorption spectra in 25% HOAc of LC purified chromopeptide **2** [(- - -) $2.2 \times 10^{-5}$ M] and *C*-phycocyanin from *Synechococcus* sp. 6301 [(—) $5.7 \times 10^{-6}$ M].

difference in absorption maxima, $\lambda_{max}$ 655 nm for **2** and 660 nm for denatured *C*-phycocyanin, is small considering the presence of three phycocyanobilins attached to different peptide sequences in *C*-phycocyanin.[3,20] Thus, we conclude that the conditions of the cyanogen bromide cleavage leave the phycocyanobilin group unmodified.

Comparative NMR analysis of the two heptapeptides **2** and **3** shows no evidence for a second chromopeptide linkage (see earlier discussion). The conditions used for the isolation of chromopeptide **2** limit any second linkage to one that would be cleaved by cyanogen bromide in 70% formic acid at 0 °C for 18 h and by subsequent chromatographic procedures. These limitations rule out both an ester linkage involving seryl[16] or tyrosyl[19] hydroxyl groups, and an amide linkage to the propionic acid side chains of the $\beta_1$-phycocyanobilin moiety. The proposed[17] aspartic acid–enol ester linkage is of doubtful stability even in the native protein. It also requires ring A to exist as a less stable (relative to pyrrole) tautomeric pyrrolenine. For the $\beta_1$-phycocyanobilin prosthetic group, the thioether linkage appears to be the only linkage to the protein.

## Summary


In this report we present a methodology for the analysis of one of three phycocyanobilins of *C*-phycocyanin. This approach, which involves the isolation, LC purification, and spectroscopic analysis of small chromopeptides, will be applied to the study of the other two peptide-linked phycocyanobilins of *C*-phycocyanin. In this way, the structures and linkages of all the pigment prosthetic groups associated with an individual biliprotein should be established. Such data hopefully will provide the fundamental structural information for further studies of the arrangement of the bilin prosthetic groups within the protein that are responsible for the light-harvesting effectiveness of algal biliproteins


## Experimental Section

**Methods.** All reactions were performed under $N_2$. Solutions were dried over $Na_2SO_4$ and evaporations were done in vacuo with a Buchi rotary evaporator. Uncorrected melting points were determined on a Thomas-Hoover capillary MP apparatus and a Kofler micro hot stage ($\mu$mp). $^1$H NMR spectra of the protected peptides **5–9** were taken in $CDCl_3$ solution using internal $Me_4Si$ ($\delta$ 0.0) on a homemade spectrometer[36] based on a Bruker 63 KG magnet operating at 270 MHz with a Nicolet 1180 data system. The 360-MHz $^1$H NMR spectra of the deblocked peptides **3**, **11**, and **12** and chromopeptide **2** in $D_2O$ and in pyridine-$d_5$ were taken at the Stanford Magnetic Resonance Laboratory. TLC was done on silica gel 60 (EM reagents) with the following solvent systems: A (BuOH/HOAc/$H_2O$, 4:1:5, upper phase); B (benzene/acetone, 2:1); C (benzene/$Et_2O$, 1:1); D (benzene/acetone, 1:1). LC was done using a Spectra Physics 8000 instrument equipped with a Schoeffel 770 variable wavelength detector. The following column and condition systems were used: A [10 $\mu$m Spherisorb ODS (3 × 250 mm), isocratic (60:40, $CH_3CN/H_2O$), flow rate (*F*) 0.5 mL/min]; B [10 $\mu$m Spherisorb ODS (3 × 250 mm), isocratic (25:75, $CH_3CN$/0.01 M $KH_2PO_4$, pH 2.1), *F* 0.5 mL/min]; C [10 $\mu$m LiChrosorb RP 18 (10 × 250 nm), gradient ($t$ = 0: 0.01 M $KH_2PO_4$, pH 2.1, to 15 min; 25:75, $CH_3CN$/0.01 M $KH_2PO_4$, pH 2.1, remaining isocratic to 30 min), *F* 2.0 mL/min]; D [10 $\mu$m LiChrosorb RP 18 (9 × 500 nm, all glass system),[37] same eluent as C, *F* 0.8 mL/min]. Absorption spectra were taken on a Cary 118 spectrometer and are base line corrected. CD spectra were taken on a homemade spectrometer.[38] Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley (see Table IV). Amino acid analyses were performed on a Beckman 120C analyzer.

**Materials.** The following solvents were routinely distilled immediately prior to use: THF and dioxane from sodium benzophenone ketyl and dimethylacetamide from Linde 4A molecular sieves at reduced pressure. LC grade solvents from Burdick and Jackson Laboratories and water purified with a Milli-Q System (Millipore Corp.) were used for LC. Amino acid derivatives of L-alanine and L-aspartic acid were purchased from Sigma Chemical Co.

**360-MHz $^1$H NMR Sample Preparation.** After LC purification (systems C and D), the peptide solutions were concentrated to ~3 mL and desalted with Sephadex G10 (15 × 500 mm), eluting with 25% HOAc. Following lyophilization, the peptide residues were dissolved in 0.01 M TFA-*d* in 99.8% $D_2O$ and lyophilized again. The solutions for NMR analysis were prepared in 99.996% $D_2O$ or 99.96% pyridine-$d_5$ (Aldrich Chemical Co.) in a dry box (Vacuum Atmosphere Corp.) under argon.

**$\beta_1$-Phycocyanobiliheptapeptide (2).** Pure *Synechococcus* sp. 6301 *C*-phycocyanin was prepared as previously described.[20] The protein preparation was dialyzed exhaustively against deionized, glass-distilled water and lyophilized. Lyophilized phycocyanin (450 mg, ~12 $\mu$mol) was dissolved in 14.5 mL of water, formic acid (98–100%, 34.5 mL) was slowly added to this solution with constant swirling, and then cyanogen bromide (600 mg, 5.7 mmol) was added and the mixture swirled for several minutes to dissolve it. Reaction was allowed to proceed in the dark, under nitrogen, for 18 h at room temperature. The reaction mixture was then applied directly to a column of Sephadex G50 (Fine, 5 × 53 cm), equilibrated, and developed with a 30% v/v aqueous acetic acid. The appropriate blue fractions were pooled, concentrated, and purified by LC (system D, Figure 2). The bilin-carrying heptapeptide **2** was obtained in a yield of 60%, as determined from the absorbance at 650 nm ($\epsilon_M$ 32 000 $M^{-1}$ $cm^{-1}$, Figure 8); $^1$H NMR ($D_2O$ and pyridine-$d_5$, Figures 3 and 4); amino acid analysis and sequence analysis as in ref 20.

**$\alpha$-L-Amino-$\gamma$-butyrolactone (L-Homoserine Lactone, 5).** L-Homoserine was quantitatively converted to the lactone salt within 24 h at room temperature in anhydrous TFA. Evaporation of the solvent afforded a white solid: mp 122–125 °C; $[\alpha]_D^{25}$ −33.9° (*c* 1.31, TFA).

**Peptide Synthesis. General Procedure. *N-tert*-Butoxycarbonyl-$O^\beta$-benzyl-L-aspartyl-L-homoserine Lactone (6).** *N-tert*-Butoxy-

Table IV. Elemental Analyses

| compd | mol formula | calcd C | calcd H | calcd N | calcd S | found C | found H | found N | found S |
|---|---|---|---|---|---|---|---|---|---|
| **5** | $C_6H_8NO_4F_3$ | 33.50 | 3.75 | 6.51 | | 33.22 | 3.95 | 6.46 | |
| **6** | $C_{20}H_{26}N_2O_7$ | 59.10 | 6.45 | 6.89 | | 58.84 | 6.42 | 6.73 | |
| **7** | $C_{33}H_{44}N_6O_7S$ | 54.08 | 6.05 | 11.47 | 4.38 | 53.79 | 6.01 | 11.20 | 3.99 |
| **8** | $C_{39}H_{55}N_7O_{12}S$ | 55.37 | 6.55 | 11.59 | | 54.97 | 6.49 | 11.49 | |
| **9** | $C_{44}H_{64}N_8O_{13}S_3$ | 52.36 | 6.39 | 11.10 | 9.53 | 52.54 | 6.27 | 11.07 | 9.96 |
| **10** | $C_{47}H_{69}N_9O_{14}S_3$ | 52.25 | 6.44 | 11.67 | | 52.19 | 6.29 | 11.60 | |
| **4** | $C_{50}H_{74}N_{10}O_{15}S_3$ | 52.16 | 6.48 | 12.17 | 8.36 | 51.86 | 6.52 | 12.04 | 8.09 |

carbonyl-$O^{\beta}$-benzyl-L-aspartic acid (3.23 g, 10 mmol) and *N*-methylmorpholine (1.01 g, 10 mmol) were dissolved in 50 mL of THF.[39] After cooling to −15 °C, isobutyl chloroformate (1.37 g, 10 mmol) was rapidly introduced, and 1 min later a solution of L-homoserine lactone trifluoroacetic acid salt (2.15 g, 10 mmol) and triethylamine (1.01 g, 10 mol) in 25 mL of THF was added. The cooling bath was then removed and the mixture was stirred for an additional 1.5 h, filtered, and evaporated. The residue was dissolved in 100 mL of ethyl acetate, washed with $H_2O$ (3 × 50 mL), saturated $NaHCO_3$ (3 × 50 mL), 0.3 N HCl (3 × 50 mL), and saturated NaCl (1 × 50 mL), dried, and evaporated to give the dipeptide **6** as an oil weighing 3.87 g (95%): TLC (B) $R_f$ 0.55; LC (A), $t_R$ 4.1 min; $^1$H NMR (see supplementary material paragraph at end of paper).

**$N^{\alpha}$-*tert*-Butoxycarbonyl-$N^G$-*p*-methoxybenzenesulfonyl-L-arginyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine Lactone (7).** Dipeptide **6** (3.63 g, 9.0 mmol) was dissolved in 40 mL of anhydrous TFA containing 0.5 mL of anisole at 0 °C. After 0.5 h, the solvent was evaporated and the oily residue was crystallized by triturating with $Et_2O$, affording 3.62 g, 96% yield of the TFA salt, mp 138–140 °C. Following the synthetic procedure outlined above, the TFA salt (2.88 g, 6.9 mmol) and $N^{\alpha}$-*tert*-butoxycarbonyl-$N^G$-*p*-methoxybenzenesulfonyl-L-arginine (3.05 g, 6.9 mmol)[40] were condensed. After chromatography (B, 450 g of $SiO_2$, 4 × 74 cm), pure tripeptide **7** (2.79 g, 55%) was obtained: mp 78–81 °C; LC (A), $t_R$ 4.0 min; $^1$H NMR (see supplementary material paragraph at end of paper).

***N*-*tert*-Butoxycarbonyl-L-leucyl-$N^G$-*p*-methoxybenzenesulfonyl-L-arginyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine Lactone (8).** Removal of the *t*-Boc protecting group from tripeptide **7** was accomplished with TFA/anisole at 0 °C. Condensation of the tripeptide salt (2.42 g, 2.8 mmol) with *N*-*tert*-butoxycarbonyl-L-leucine (0.684 g, 2.96 mmol),[41] as described above, afforded tetrapeptide **8** as a glass weighing 2.35 g (99%): μmp 98 °C; LC (A), $t_R$ 4.7 min; TLC (D), $R_f$ 0.4; $^1$H NMR (see supplementary material paragraph at end of paper).

***N*-*tert*-Butoxycarbonyl-*S*-ethylmercapto-L-cysteinyl-L-leucyl-$N^G$-*p*-methoxybenzensulfonyl-L-arginyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine Lactone (9).** Deblocking of the tetrapeptide **8** with TFA/anisole afforded the crystalline TFA salt after trituration with $Et_2O$. This salt (2.2 g, 1.9 mmol) was condensed with *N*-*tert*-butoxycarbonyl-*S*-ethylmercapto-L-cysteine (0.57 g, 2.0 mmol)[41,42] to give **9** as a glass weighing 1.89 g (97%): μmp 108 °C; LC (A), $t_R$ 6.3 min; TLC (D), $R_f$ 0.43; $^1$H NMR (see supplementary material paragraph at end of paper).

***N*-*tert*-Butoxycarbonyl-L-alanyl-*S*-ethylmercapto-L-cysteinyl-L-leucyl-$N^G$-*p*-methoxybenzenesulfonyl-L-arginyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine Lactone (10).** The pentapeptide **9** (1.78 g, 1.8 mmol) was dissolved in a premixed solution of 7 mL of 4 N HCl in dioxane and 2.5 mL of ethyl mercaptan. After 30 min, 40 mL of $Et_2O$ was added, and the resulting solid was filtered and triturated with ethyl acetate. A solution of the dihydrochloride salt (1.45 g, 1.5 mmol) thus obtained and triethylamine (0.30 g, 3.0 mmol) in 7.5 mL of dimethylacetamide was added to a mixture of the preformed mixed anhydride of *N*-*tert*-butoxycarbonyl-L-alanine (0.29 g, 1.6 mmol) at −15 °C as before. After purification, the hexapeptide **10** (1.5 g, 94%) was obtained: mp 131–145 °C; LC (A), $t_R$ 7.2 min; TLC (D), $R_f$ 0.36; $^1$H NMR (in acetone-$d_6$; see supplementary material paragraph at end of paper).

***N*-*tert*-Butoxycarbonyl-L-alanyl-L-alanyl-*S*-ethylmercapto-L-cysteinyl-L-leucyl-$N^G$-*p*-methoxybenzenesulfonyl-L-arginyl-$O^{\beta}$-benzyl-L-aspartyl-L-homoserine Lactone (4).** Removal of the *t*-Boc group of **10** with HCl/dioxane/EtSH was accomplished as above. The heptapeptide **4** was prepared in 98% yield (1.26 g) from the hexapeptide dihydrochloride salt (1.31 g, 1.2 mmol). Recrystallization of the crude product from acetone gave analytically pure material: μmp 167 °C; LC (A), $t_R$ 7.8 min. Amino acid analysis—(a) 6 N HCl, 110 °C, 16 h (amino acid, experimental value (calculated number of residues)): Ala, 2.00 (2); ½-Cys, 0.57 (0.50); Leu, 0.98 (1); Arg, 1.01 (1); Asp, 1.01 (1); Hse/Hsl, 0.95 (1); (b) $HCO_3H$, 0 °C, 4 h, followed by 6 N HCl, 110 °C, 16 h: Ala, 2.00 (2); Cys acid, 1.00 (1); Leu, 0.90 (1); Arg, 0.92 (1); Asp, 0.93 (1); Hse/Hsl, 0.88 (1).

**HF Deblocking. General Procedure. L-Alanyl-L-alanyl-*S*-ethylmercapto-L-cysteinyl-L-leucyl-L-arginyl-L-aspartyl-L-homoserine Lactone (3).** The blocked heptapeptide **4** (27.0 mg, 23 mmol) and ethyl mercaptan (300 μL, 4.0 mmol) were dissolved in 1.5 mL of anhydrous hydrogen fluoride freshly distilled from $CoF_3$. After 0.5 h stirring at 0–5 °C, the HF was removed in vacuo. The residue was dissolved in 2 mL of $H_2O$, washed with EtOAc (3 × 2 mL), and lyophilized. Following LC purification (system B, Figure 1) and desalting with Sephadex G10, the heptapeptide **3** was obtained as the di-TFA salt in 30% yield: μmp 173 °C; LC (B), $t_R$ 20.9 min; $^1$H NMR ($D_2O$; see Figure 3).

**L-Leucyl-L-arginyl-L-aspartyl-L-homoserine Lactone (11).** The tetrapeptide **8** (11.3 mg, 13.3 μmol) was deblocked in an analogous manner. The crystalline tetrapeptide salt (10 mg, 100%) of **11** thus obtained was chromatographically and spectroscopically pure: μmp 114 °C; LC (B), $t_R$ 11.2 min; TLC (A), $R_f$ 0.17; $^1$H NMR ($D_2O$ see supplementary material paragraph).

***S*-Ethylmercapto-L-cysteinyl-L-leucyl-L-arginyl-L-aspartyl-L-homoserine Lactone (12).** The deblocking of the pentapeptide **9** (33 mg, 34 mol) was accomplished as described previously and the crude product (15.6 mg, 53%) was purified by LC: LC (B), $t_R$ 18.5 min; mp 123 °C; $^1$H NMR ($D_2O$, see supplementary material paragraph).

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**Supplementary Material Available:** Full details of the $^1$H NMR spectra of dipeptide **6**, tripeptide **7**, tetrapeptide **8**, pentapeptide **9**, hexapeptide **10**, tetrapeptide salt **11**, and pentapeptide salt **12** (3 pages). Ordering information is given on any current masthead page.

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(23) The chromopeptide **2** obtained by CNBr cleavage of *C*-phycocyanin and the subject of this report corresponds to the peptide CNBr-2 of ref 20.
(24) We have used the following abbreviations for structural simplicity: SSEt to represent the *S*-ethylmercapto protecting group for the cysteine SH; Mbs to represent the *p*-methoxybenzenesulfonyl protecting group for the arginylguanidine moiety; Bn for benzyl; Hse and Hsl to represent L-homoserine and its lactone; TFA for trifluoroacetic acid.
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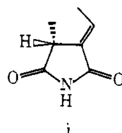
(31) The assignment of *R* stereochemistry for C-2 made in ref 22 has been based on the isolation of optically active (*E*)-2-ethylidene-3-methylsuccinimide (i) from the chromic acid degradation of *C*-phycocyanin. However, the actual optical activity data for the succinimide cleavage product obtained in this way are unavailable in the literature. In another study, a levorotatory enantiomer of succinimide i has been reported as resulting from the chromate degradation of the blue pigment **1**: Knobloch, G. Doctoral Thesis, Technical University, Braunschweig, 1972. By comparison with the authentic + and − isomers, the assignment of absolute stereochemistry at C-2 has been made: Brockman, H., Jr.; Knobloch, G. *Chem. Ber.* **1973**, *106*, 803. Since the blue pigment **1** may not be representative of all three phycocyanobilin moieties of *C*-phycocyanin, the assignment of absolute stereochemistry at C-2 based on the optical activity of succinimide cleavage products obtained from **1** or from the native biliprotein requires the demonstration of the stereochemical identity of all three phycocyanobilins.

i


(32) Before the present study, no direct experimental evidence had appeared regarding the geometry of ring A of phycocyanobilin. Based on biosynthetic analogy with other tetrapyrrolic pigments with trans dihydro rings, trans stereochemistry for ring A of phycocyanobilin has been proposed (ref 22). The coupling constant of 5.0 Hz, which we observe for $^3J_{2H-3H}$ of **2**, closely agrees with the value of this coupling constant in *trans*-succinimide models, the cis coupling constant being somewhat larger (ref 22 and 30). However, only when the appropriate bilitriene models with cis and trans dihydro ring A are compared, can the validity of the stereochemical assignment be ascertained.
(33) In ref 22 and 30 a concerted trans-periplanar elimination mechanism is demonstrated for model succinimides. The authors argue that the obtention of only (*E*)-2-ethylidene-3-methylsuccinimide from chromic acid treated *C*-phycocyanin, contaminated with none of the *Z* isomer, suggests that the same mechanism must be occurring with the biliprotein. This requires the relative stereochemistry of C-3 and C-3′ be *RR* or *SS*.
(34) In a preliminary study of *C*-phycocyanin, we have observed that in addition to the blue pigment **1**, other pigments are obtained from the methanol-treated biliprotein. The nature of these pigments is under further investigation.
(35) The slight upfield shifts of the N-terminal alanine residue of **2** have been interpreted to represent internal salt formation with one of the propionic acid side chains of the $\beta_1$-phycocyanobilin chromophore in pyridine-$d_5$. The differences in the chemical shifts of the homologous C-8 and C-12 methylenes of **2** (Table III) support this hypothesis.
(36) The 270-MHz spectrometer was designed and constructed by Willy C. Smith of the Laboratory of Chemical Biodynamics, University of California—Berkeley.
(37) When a brilliantly blue solution of chromopeptide **2** was passed through a stainless steel Millipore filter, a multi-hued solution emerged. Thus, an all-glass and Teflon system was devised to avoid the presumed, metal-catalyzed decomposition of the chromopeptide **2**.
(38) Sutherland, J. C.; Vickery, L. E.; Klein, M. P. *Rev. Sci. Instrum.* **1974**, *45*, 1089.
(39) For the syntheses of the penta-, hexa-, and heptapeptides (**9**, **10**, and **4**, respectively), *N*,*N*-dimethylacetamide was used instead of THF because of its better solvent properties.
(40) Nishimura, O.; Fujino, M. *Chem. Pharm. Bull.* **1976**, *24*, 1568.
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(42) Weber, U. *Hoppe-Seyler's Z. Physiol. Chem.* **1969**, *350*, 1421.