Chromopeptides from Phycoerythrins. Structure and Linkage of a Phycoerythrobilin Tryptic Tripeptide Derived from a B-Phycoerythrin

Robert W. Schoenleber, See-Lap Leung, Daniel J. Lundell, Alexander N. Glazer, and Henry Rapoport*

Contribution from the Department of Chemistry, Department of Microbiology and Immunology, and Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720.
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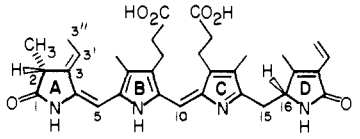
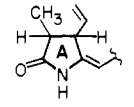
Abstract: Phycoerythrobilin peptides were prepared from *Porphyridium cruentum* B-phycoerythrin by tryptic digestion and isolated under mild conditions without alteration in the spectroscopic properties of the bilin. High-resolution ^{1}H NMR spectral analysis of the smallest trypsin fragment derived from the α subunit of B-phycoerythrin, the α_1-B-phycoerythrobilitripeptide (**3**), has provided direct proof that the bilin is linked to the peptide through a single bond, a thioether linkage at the 3′-position of ring A. Two linkages between the bilin and peptide have been proposed by others. The present study confirms previous reports on the major structural features of the free phycoerythrobilin chromophore and indicates probable stereochemical assignments for all of the chiral centers.

The intensely red phycoerythrins serve as major light harvesting proteins in numerous cyanobacteria as well as in two algal groups, the Rhodophyta (red algae) and the cryptomonads.[1] Rhodophytan phycoerythrins carry two types of covalently bound prosthetic groups, phycoerythrobilins and phycourobilins.[2] Substantial information is available on the structure of phycoerythrobilin, while much less is known about phycourobilin. Treatment of phycoerythrins with boiling methanol results in the release of a purple pigment, phycoerythrobilin (**1**).[3] The structure





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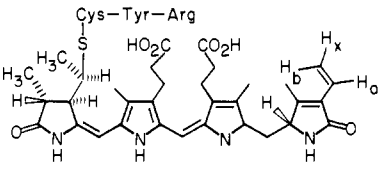

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of this pigment has been established by nuclear magnetic resonance and mass spectrometry[4,5] as well as by chemical synthesis.[6,7]

The presence of a thioether linkage between a cysteinyl residue and the bilin has been demonstrated in all chromopeptides thus far studied from both cyanobacterial[8] and red algal phycoerythrins.[9,10] There is suggestive evidence, based on degradation studies, that the thioether bond is to the ethyl group on ring A of the bilin.[3] Upon chromic acid oxidation, the free bilin yields only 2-(*E*)-ethylidene-3-methylsuccinimide among the degradation products, whereas a mixture of the *Z* and *E* isomers of this imide was isolated on degradation of B-phycoerythrin under the same conditions.[11] The mechanism of the methanol cleavage and generation of the purple pigment is believed to involve elimination of the thiol group, generating the ethylidene of the released pigment.[3] Release of phycoerythrobilin (**1**) can also be achieved with concentrated HCl at room temperature.[12] The free bilin obtained under these conditions has spectroscopic properties similar to those of denatured phycoerythrin.[12] On the basis of indirect evidence, the structure proposed for the pigment released by acid is **2**.[13]

The presence of a second linkage between the phycoerythrobilin and the polypeptide has been proposed by several authors on the basis of degradation studies.[14] This second bond has been suggested to be an ester linkage from a seryl or threonyl residue to a propionic acid group.[14] The evidence in support of such a second linkage is not convincing, and available amino acid sequence data on phycoerythrobilin chromopeptides argue against its presence.[8,9]

Phycoerythrins are complex macromolecules that carry numerous prosthetic groups. For example, B-phycoerythrin of the unicellular red alga, *Porphyridium cruentum*, the source of the bilin peptide we have investigated, has the polypeptide composition $(\alpha\beta)_6\gamma$, with α and β subunits of 19000 daltons and the γ subunit of 30000 daltons.[15] Spectroscopic studies indicate that the α subunit carries two phycoerythrobilins, the β subunit carries four phycoerythrobilins, and the γ subunit carries two phycoerythrobilin and two phycourobilin groups.[15] It is important to appreciate, therefore, that isolation and characterization of 10 distinct chromopeptides is required to fully establish the structure of all of the polypeptide-linked bilins.

In an earlier study, we demonstrated that the structure of a phycocyanobilipeptide could be unambiguously determined by high-resolution NMR.[16] In the present study, we have extended this methodology to the structure determination of a phycoerythrobilin chromopeptide **3**.

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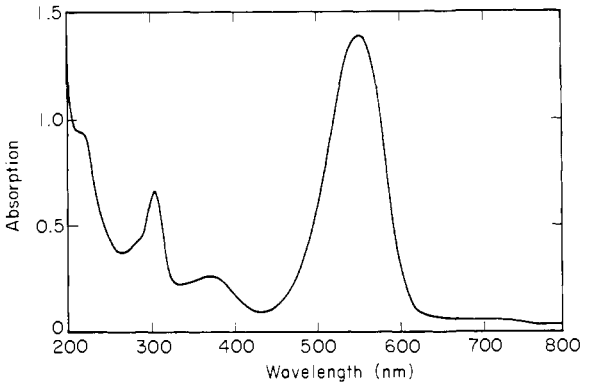
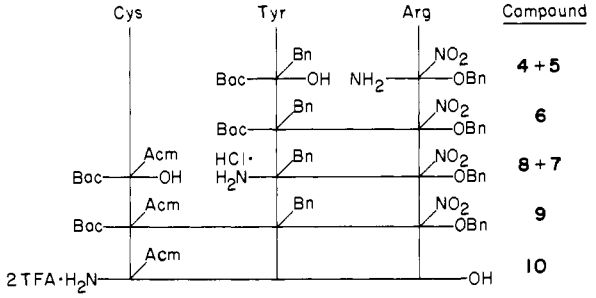


Figure 1. Absorption spectrum in 10 mM aqueous TFA of LC purified chromopeptide **3** (3.0×10^{-5} M).

Chart I. Repetitive Mixed Anhydride Synthesis of Tripeptide 10



Results and Discussion

Isolation and Purification of α_1-B-Phycoerythrobilitripeptide 3. B-Phycoerythrin was isolated and purified from *P. cruentum* by a combination of ammonium sulfate precipitation, DEAE-cellulose chromatography, and gel filtration.[15] The B-phycoerythrin was free from other biliproteins at this point. To obtain enough material for analysis, the entire protein was subjected to trypsin cleavage. Gel filtration followed by ion-exchange chromatography of the resultant peptide fragments allowed the isolation of the desired α_1-B-phycoerythrobilitripeptide in 90% purity.

In order to obtain a pure sample of the chromotripeptide for NMR analysis, preparative HPLC of the Sephadex fraction was necessary. Reverse-phase chromatography was done by using an isocratic sodium phosphate buffer–acetonitrile mixture on an Ultrasphere ODS column. Our studies indicated that the high buffer salt concentration was essential for the requisite resolution. The conditions, however, necessitated a desalting step before concentration. After removal of the acetonitrile, the chromopeptide solution was applied to a LiChrosorb C-18 column, washed with a 10 mM aqueous trifluoroacetic acid solution, and eluted by stepping to a 50% acetonitrile mixture with this same aqueous acid. The use of this volatile acid allowed us to control the acid concentration of the final NMR sample without the introduction of interfering proton resonances. During the desalting procedure, some chromophore cleavage (~0.5%) was seen, although both free chromophore and free peptide were well separated from the chromopeptide fraction. The UV/vis spectrum (Figure 1) shows that no alteration in the chromophore structure has occurred during the isolation and purification steps.[15]

The NMR spectra were taken in 10 mM TFA in D_2O. Although we considered that aggregation might be a problem, the CD spectra showed no concentration effects in aqueous buffer solutions up to the concentration at which the NMR spectra were recorded.[16] Previous results with phycocyanobilin-bearing peptides suggested that pyridine might be a better NMR solvent,[16,17] but isomerization of the phycoerythrin to a urobilin-type structure prevented meaningful NMR analysis in pyridine.

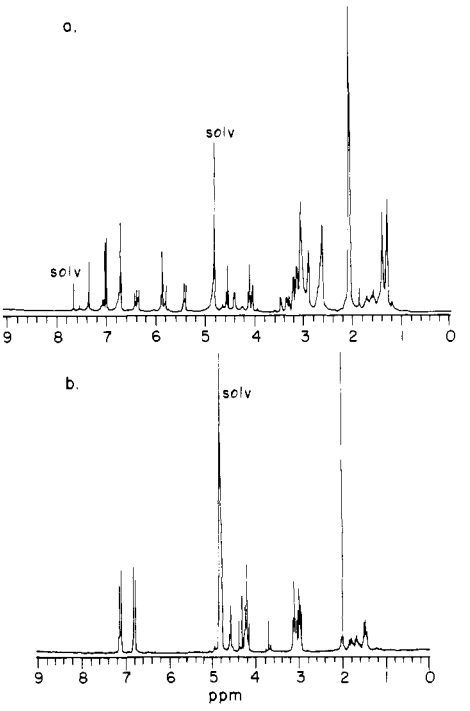


Figure 2. (a) The 360-MHz ^1H NMR spectrum of chromopeptide **3** at 25 °C (4.4 mM, 10 mM TFA in D_2O). (b) The 250-MHz ^1H NMR spectrum of synthetic tripeptide **10** at 25 °C (5.0 mM, 10 mM TFA in D_2O).

Table I. ^1H NMR Assignments for the Peptide Moiety of α_1-B-Phycoerythrobilitripeptide (3) and Synthetic Peptide 10 in 10 mM TFA in D_2O at 25 °C

chemical shift, ppm			
3	10	multiplicity, J,[a] Hz	assignment
6.98	7.11	d, 8.4 (8.5)	Tyr 2,6-H
6.71	6.78	d, 8.4 (8.5)	Tyr 3,5-H
4.55	4.54	t, 7.2 (7.7)	Cys α-CH
4.10	4.20	t, 6.3 (4.5)	Tyr α-CH
4.03	4.23	dd, 5.1, 8.5 (t, 5.0)	Arg α-CH
3.2	3.1	m (t, 6.9)	Arg δ-CH_2
3.14	3.04, 2.99	m (d, 5.6; d, 7.2)	Tyr β-CH_2
2.90	2.98	d, 7.2 (7.7)	Cys β-CH_2
1.55–1.75	1.47–1.77	m	Arg β,γ-CH_2

[a] Values in parentheses represent multiplicity or coupling constant differences seen in the synthetic tripeptide.

Synthesis of Tripeptide 10. Amino acid sequencing data on the purified chromopeptide established that the sequence was Cys-Tyr-Arg. In order to facilitate NMR analysis and to see what effects might be caused by chromophore–peptide interactions, the tripeptide was synthesized (Chart I). The synthesis was achieved by using a mixed anhydride method on fully protected amino acid residues to satisfy the purity requirements of the high-resolution NMR study.[18]

The amino acid derivatives were synthesized according to known methods.[19-23] The couplings were done in such a manner as to avoid racemization which would result in diastereomeric mixtures complicating the NMR analysis, and the protected di- and tripeptides were purified by HPLC. Final deprotection with an-

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Table II. ^{1}H NMR Assignments of the Bilin Moiety of Chromopeptide 3 and the Purple Pigment 1 in 10 mM TFA in D_2O at 25 °C

chemical shift, ppm			
1[a]	3	multiplicity, J,[b] Hz	assignment
6.68	7.34	s	10-H
6.40	6.37	dd, 11.7, 17.9 (12, 18)	18-H_a
6.17	5.82	dd, 1.4, 17.9 (2, 18)	18-H_b
5.84	5.86	s	5-H
5.36	5.40	dd, 1.4, 11.7 (2, 12)	18-H_x
4.28	4.40	dd, 4.2, 7.9 (m)	16-H
	3.44	m, 3.0, 7.0	3'-H
2.8–3.0	3.30	dd, 4.2, 14.1 (m)	15-H
	3.10	3.0, 3.5[c]	3-H
2.8–3.0	3.07	m	8,12-$CH_2CH_2CO_2H$
2.8–3.0	2.93	7.9, 14.1[c]	15-H
3.22	2.69	m, 3.5, 7.4 (q, 7)	2-H
2.4–2.7	2.64	m	8,12-$CH_2CH_2CO_2H$
1.98	2.055	s	
2.03	2.06	s	7,13,17-CH_3
2.03	2.08	s	
	1.39	d, 7.0	3'-CH_3
1.42	1.28	d, 7.4 (7)	2-CH_3

[a] From ref 7. [b] Values in parentheses represent multiplicity or coupling constant differences seen in the free pigment 1. [c] Overlapping resonances are seen, but coupling constants are abstracted from the coupled protons.

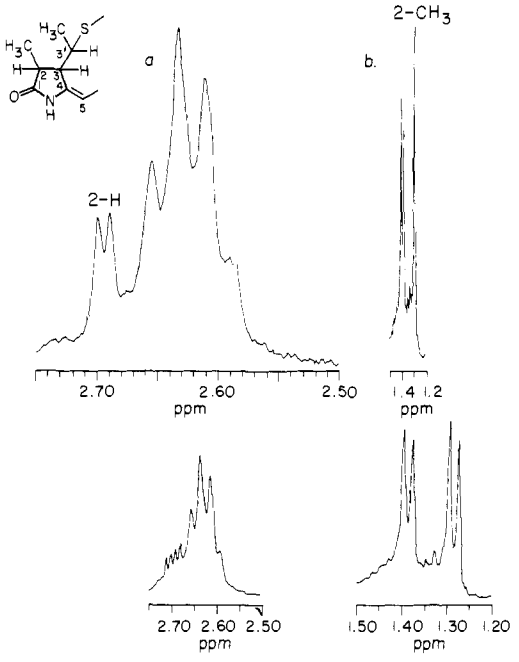

Figure 3. Partial 360-MHz ^{1}H NMR spectrum of chromopeptide 3 taken at 25 °C in 10 mM TFA in D_2O: (a) after irradiation of the doublet at 1.28 ppm; (b) after irradiation of the multiplet at 2.69 ppm.

hydrous hydrogen fluoride removed all blocking groups except the acetamidomethyl, which was chosen to avoid any complicating thiol oxidation.[19,24] Although the yields were generally low, the HPLC analyses showed high purity, indicating that the losses were probably due to the extensive handling necessary in the rigorous purification procedures.

^{1}H NMR Analysis. The NMR spectra of the chromopeptide and the synthetic peptide taken in the same solvent are shown in Figure 2. In contrast to what occurs with phycocyanobilipeptides in D_2O, very little broadening of the resonances due to the chromophore is seen.[16] The corresponding chemical shifts of the amino acid residues all fall within 0.2 ppm (Table I). Considering the possible effects of such a highly conjugated system in close proximity to the peptide, the chemical shift agreement is surprisingly close. However, because of this agreement, little speculation on conformational features can be made. There is a general pattern of shielding of the peptide resonances, indicating that some internal hydrogen bonding may hold the peptide chain over the tetrapyrrole in the shielding region of the ring current effects. However, the pattern is not absolute, and no data on the conformation of the bilin in such a complex or in such a solvent system are available.[25]

The close agreement of the chemical shifts of the tyrosine aromatic resonances precludes the possibility of an ester linkage between the phenolic oxygen and a propionic acid side chain. Therefore, since a free arginine guanidine group is essential for trypsin cleavage, the thioether linkage is shown to be the only covalent bond to the chromophore.

Detailed NMR spectral analysis of the chromophore resonances provides information not previously available (Table II). The singlets at 7.34 and 5.86 ppm represent the methine protons at C-10 and C-5, respectively. No deuterium exchange is seen at these or any other possibly enolizable protons under the aqueous acid conditions employed. This is in marked contrast to the exchange seen in the phycocyanobilin chromopeptide[16] and a previous report on the phycoerythrobilin chromophore.[26] The lability of certain protons having been documented, we interpret our results as an indication that no isomerization has occurred during the mild conditions used in this study.

The ABX pattern at 6.37, 5.82, and 5.40 ppm with coupling constants of 1.4, 11.7, and 17.9 Hz, respectively, is direct proof of the presence of a side chain vinyl group in the native chromophore. The mild procedure used for isolation of the chromopeptide precludes the possible generation of this vinyl group, as evidenced by the intact nature of the thioether linkage. The chemical shifts and coupling constants correspond well with those found for the free chromophore despite the solvent differences.[4,6,7]

From a comparison with the synthetic peptide, the α-protons of the amino acid residues can be assigned. Further comparisons and decoupling experiments then allow the complete assignment of the amino acid resonances (Table I). By difference, we can assign the doublet of doublets at 4.40 ppm (J = 4.2, 7.9 Hz) to the proton at C-16, indicating the saturated nature of the adjacent methylene position. Double irradiation experiments show that the protons at C-15 lie at 3.30 and approximately 2.9 ppm. The higher field C-15 proton is a doublet of doublets with coupling constants of 4.2 Hz ($J_{vicinal}$) and 14.1 Hz ($J_{geminal}$). The other C-15 proton is obscurred by overlapping resonances, although the decoupling data allow the approximate chemical shift assignment, as irradiation at 2.9 ppm collapses both the C-16 proton and the other C-15 proton. Its coupling constants must be 7.9 Hz ($J_{vicinal}$) and 14.1 Hz ($J_{geminal}$). The line widths of the C-15 and C-16 protons are considerably larger than those seen for the other protons, which indicates restricted rotation about this bond, consistent with a rigid preferred conformation due to internal hydrogen bonding despite the aqueous acid solvent.

With the exception of those of ring A, the remaining tetrapyrrole substituents are assigned by comparison with phycoerythrobilin NMR data previously published.[4,6,7] The propionic acid methylenes appear as unresolved multiplets centered at 3.07 ppm for the α- and 2.64 ppm for the β-methylene groups. The three methyl groups at C-7, C-13, and C-17 are not completely resolved in our spectra. A resolution enhancement technique (sinusoidal multiplication of the free induction decay curve) shows the presence of the three at 2.055, 2.06, and 2.08 ppm.

The substitution pattern about ring A was ascertained by decoupling experiments. Previous results[2] suggest that a methyl group is at C-2 and an ethyl group is at C-3, with a thioether linkage at C-3'. We provide the first direct proof of this assignment. The C-2 methyl group appears as a doublet at 1.28 ppm as determined by correlation with the phycocyanin work.[16] Irradiation at this position causes the C-2 proton at 2.69 ppm

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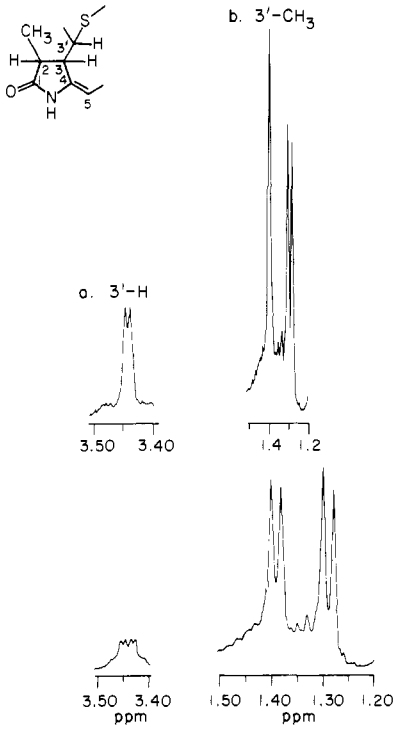

Figure 4. Partial 360-MHz ^1H NMR spectrum of chromopeptide **3** taken at 25 °C in 10 mM TFA in D_2O: (a) after irradiation of the doublet at 1.39 ppm; (b) after irradiation of the multiplet at 3.44 ppm.

(Figure 3) to collapse to a doublet. The observed coupling constant between the C-2 and C-3 protons is 3.5 Hz. The C-3 proton is obscurred by the propionic acid α-methylene protons. However, irradiation at 3.1 ppm causes a small change in the appearance of the C-2 proton, indicating the approximate chemical shift of the C-3 proton. The overlapping resonances do not allow us to see a change in the C-3 proton when the C-2 proton is irradiated; however, the C-2 methyl group does collapse to a singlet when this irradiation is done (Figure 3). The C-2 methyl doublet allows the direct reading of the coupling constant between the C-2 methyl and proton as 7.4 Hz.

A similar pattern is seen for the C-3′ proton at 3.44 ppm. This proton initially appears as a multiplet with an apparent four-line pattern (Figure 4). Irradiation at the C-3 proton only causes slight changes in the appearance of the C-3′ proton. The collapse should be to a quartet, but the imprecise nature of the C-3 proton irradiation, because of overlapping resonances, did not allow us to make this direct correlation. Irradiation of the C-3′ methyl group at 1.39 ppm collapses the C-3′ proton to a doublet with $J_{3,3'}$ = 3.0 Hz. Irradiation of the C-3′ proton shows no change in the C-3 proton region but does collapse the C-3′ methyl group signal to a singlet. Direct reading of the coupling constant of the initial doublet gives a value of 7.0 Hz. Therefore, we have the first direct proof of the presence and position of the thioether linkage.

Stereochemistry. The absolute stereochemistry at the C-2 position in the cleaved chromophore has been determined by other degradation and CD studies to be the R configuration.[7] The CD study of the two epimers at this position shows only slight shifts in the short-wave region of the spectra.[7] Degradation studies allow the isolation of (*R*)-2-(*E*)-ethylidene-3-methylsuccinimide, although some epimerization at C-2 may have occurred during isolation.[7] This proof is not as rigorous as one would like, although the isolation of the optically active succinimide is reasonably conclusive.

Assignment of the C-3 to C-3′ relative stereochemistry is based on the proposed antiperiplanar elimination of the sulfone group under oxidative degradation conditions to give the (*E*)-ethylenesuccinimide.[27,28] Although the reaction conditions are harsh and the yields are generally low, the model studies and the preponderance of evidence suggest the acceptability of this assignment.[2,27,28]

Our data supply the final piece of evidence for the complete absolute stereochemical assignment of the chromophore–protein complex, with the above caveats. Until now, no data on the relative stereochemistry between the C-2 and C-3 protons were available. The low value of the coupling constant for these protons (3.5 Hz) combined with values obtained from model work is an indication that the relative stereochemistry is probably trans.[29] Accepting this final conclusion allows the complete stereochemical assignment for the C-2, C-3, and C-3′ positions to be made as *R*, *R*, and *R*. The stereochemistry at the C-16 position is clearly *R* from the CD studies.[7] Thus, all four stereo centers have been assigned.

Experimental Section

Methods. All reactions were performed under N_2. Evaporations were done in vacuo with a Berkeley rotary evaporator. Uncorrected melting points were determined on a Buchi capillary melting point apparatus. ^1H NMR spectra of the synthetic peptides **6–10** were taken at 250 MHz and chemical shifts are expressed in ppm relative to an internal Me_4Si standard for $CDCl_3$ and relative to an external TSP standard for D_2O. The NMR spectra of chromopeptide **3** were taken on the University of California, Davis, 360-MHz NMR spectrometer. High-pressure liquid chromatography (HPLC) was done with an Altex system consisting of two Model 110A pumps, a Model 115-10 UV/vis detector, and a Model 420 microprocessor. Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification after verification. Dimethylformamide was distilled from P_2O_5 under reduced pressure and stored over 3-Å sieves. Hydrogen fluoride was distilled from CoF_3 immediately before use.

Preparation of B-Phycoerythrin. The unicellular red alga *P. cruentum* strain B was obtained from the Marine Biology Research Division, Scripps Institution of Oceanography, La Jolla, CA, and the cells were cultured as described.[15] The previously described procedure for B-phycoerythrin purification was followed through the second $(NH_4)_2SO_4$ precipitation step. The protein precipitate from 100 g (wet weight) of cells in 65% saturated $(NH_4)_2SO_4$ at 25 °C was slurried with 800 mL of DEAE-cellulose (Whatman DE-52, microgranular) in the same solution, and the mixture was layered onto a 200-mL bed of DEAE-cellulose in a 5 cm diameter column. The column was washed with 65% saturated $(NH_4)_2SO_4$–50 mM sodium phosphate, pH 7.0, until the resin had settled to a stable bed volume. The column was then developed with decreasing concentrations of ammonium sulfate in 50 mM sodium phosphate, pH 7.0. R-Phycocyanin, allophycocyanin, and b-phycoerythrin were eluted between 20% and 15% saturated $(NH_4)_2SO_4$; elution of B-phycoerythrin was initiated with 10% saturated $(NH_4)_2SO_4$ and was completed with 0.2 M sodium phosphate, pH 7. B-Phycoerythrin was precipitated with 65% saturated $(NH_4)_2SO_4$, centrifuged after standing overnight at 4 °C, dissolved in the minimum volume of 20 mM sodium phosphate, pH 7, and dialyzed overnight at 4 °C to equilibrium. The protein was further purified by gel filtration in two batches on a Sephadex G-200 column (5 × 50 cm) equilibrated with 20 mM sodium phosphate, pH 7. The B-phycoerythrin was >95% pure after gel filtration and was free of other biliproteins.

Preparation and Purification of B-Phycoerythrobilipeptides. B-Phycoerythrin (~300 mg; representing 7.5 μmol each of α and β subunits) was dissolved at 10 mg/1 mL in 1 mM sodium phosphate, pH 7, and dialyzed overnight against the same buffer at 4 °C. All subsequent steps were performed at room temperature. The protein solution was adjusted to 20 mM HCl by addition of 1 M HCl to denature the protein. Acidification caused an immediate change in the color of the protein solution from bright red to red-violet. The pH was quickly raised to 8 by the addition of NH_4HCO_3 to 0.1 M, with final pH adjustment with 2 M NaOH. Trypsin was added to give an enzyme/substrate ratio of 1%, by weight; the sample was sealed under N_2 and incubated in the dark at 30 °C. Additional trypsin, in 0.5% (w/w) increments, was added at 2-h intervals to a final trypsin concentration of 2.5% (w/w). After 8 h of digestion the sample was acidified with acetic acid to ~pH 4 and lyophilized. The lyophilized digest was dissolved in 15 mL of 80% (v/v) formic acid and was fractionated in two batches on a column of Sephadex G-50 (2.5 × 110 cm) equilibrated in 30% (v/v) acetic acid. Three major phycoerythrobilin-containing peaks were eluted. The last peak eluted

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(29) Reference 16, footnote 32.

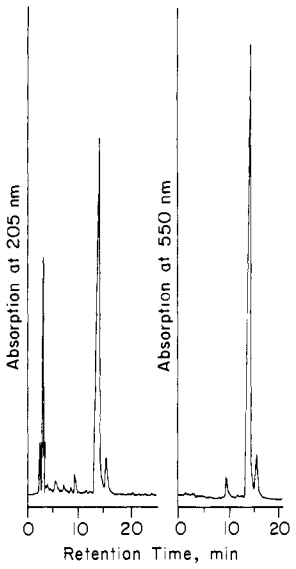

Figure 5. High-performance liquid chromatography of α_1-B-phycoerythrobilitripeptide (**3**) on an Ultrasphere ODS column.

contained the small bilipeptides. This material was concentrated to near dryness by rotary evaporation, diluted with 20 mL of 50 mM sodium phosphate, pH 2.5, and applied to a column of SP-Sephadex C-25 (1.7 × 22 cm) equilibrated in the same buffer. The column was immediately developed with a 1-L linear gradient from 0 to 0.55 M NaCl in 50 mM sodium phosphate, pH 2.5, at a flow rate of 30 mL/h. Fractions of 6 mL were collected. Three major bilin-containing peaks were eluted. The last eluted peak from the SP-Sephadex column was adsorbed onto a column of LiChrosorb C-18, washed extensively with 10 mM formic acid, and then eluted with 60% (v/v) acetonitrile–10 mM formic acid. The acetonitrile was removed under a stream of N_2, and the residue was further purified by HPLC to give chromopeptide at this point in 75% yield.

The HPLC purification was done on an Ultrasphere ODS column (5 μm, 10 × 250 mm), using 22% CH_3CN and 78% of a 0.10 M NaH_2PO_4 buffer adjusted to pH 2.1 with concentrated H_3PO_4 (Figure 5) at a flow rate of 2.0 mL/min and with detection at 550 nm. The purification was done by multiple injections of 0.5 μmol/injection, collecting the major peak as the desired product. Of the applied chromophoric material, 60% was recovered.

After the acetonitrile was removed by rotary evaporation at 5 torr and room temperature, the remaining aqueous solution was applied to a LiChrosorb C-18 column (10 μm, 3.2 × 250 mm) and washed with 150 mL of 10 mM aqueous TFA to desalt. Elution using 50% acetonitrile and 50% 10 mM aqueous TFA then gave a 98% recovery of chromophoric material after desalting, along with 0.5% of free chromophore in a later fraction. The chromopeptide solution was again evaporated as above to remove the acetonitrile and then lyophilized.

Sequence of the α_1-B-Phycoerythrobilitripeptide (3). Amino acid analysis of the chromopeptide after acid hydrolysis (6 N HCl, 110 °C, 20 h, under vacuum) yielded tyrosine and arginine in equimolar amounts. Hydrolysis under the same conditions, but with 6 N HCl containing 1.5% (v/v) dimethyl sulfoxide, yielded cysteic acid and arginine in equimolar amounts. These conditions of hydrolysis convert bilin-linked cysteinyl residues quantitatively to cysteic acid;[10] tyrosine is destroyed. The sequence of the peptide was determined by subtractive Edman degradation.[30] After one cycle of the Edman degradation, the red color was extracted into the organic phase. Acid hydrolysis of the residual peptide yielded tyrosine and arginine in unaltered amounts. A second cycle of the Edman degradation, followed by acid hydrolysis of the residual peptide and amino acid analysis, showed only arginine remaining. The sequence of this peptide is therefore H_2N-Cys(phycoerythrobilin)-Tyr-Arg-COOH. Tryptic digests of separated α and β subunits of B-phycoerythrin[15] showed that this tripeptide was derived from the α subunit.

Sample Preparation for NMR. The lyophilized chromopeptide was dissolved in 1.0 mL of 10 mM TFA in 99.8% D_2O (made by adding 10 μmol of trifluoroacetic anhydride to 1.0 mL of D_2O). The solution was stirred at room temperature for 1 h and lyophilized, and this process was repeated 2 more times. The same procedure was repeated twice with 99.96% D_2O. Finally, the NMR sample was prepared in 10 mM TFA in 99.996% D_2O at a concentration of 4.4 mM.

(*N*-*tert*-Butyloxycarbonyl-*O*-benzyl-L-tyrosyl)-N^{ϵ}-nitro-L-arginine Benzyl Ester (6). *N*-*tert*-Butyloxycarbonyl-*O*-benzyl-L-tyrosine (**4**)[20,21] (0.26 g, 0.70 mmol) was dissolved in 2 mL of DMF and cooled to −20 °C before 80 μL (0.73 mmol) of *N*-methylmorpholine and 95 μL (0.73 mmol of isobutyl chloroformate were added. After a 20-min activation, N^{ϵ}-nitro-L-arginine benzyl ester (**5**)[23] (0.27 g, 0.87 mmol) in 2 mL of DMF was added and the mixture stirred for 3 h at −20 °C. The solvent was removed by rotary evaporation at 0.1 torr and room temperature, and the residue was taken up in 25 mL of ethyl acetate and 10 mL of H_2O. The organic layer was washed successively with 10 mL of 1 M citric acid, 10 mL of water, 10 mL of aqueous saturated sodium bicarbonate, 10 mL of water, and 10 mL of aqueous saturated sodium chloride, then dried over Na_2SO_4, filtered, and evaporated to give an oily product which was purified by HPLC. A LiChrosorb Si-60 column (10 μm, 10 × 250 mm) was used with elution with 2% methanol in chloroform at 2.8 mL/min, detection at 280 nm, and multiple injections of 10 mg each. A single product with a retention time of 5.1 min was present and was collected. Solvent was evaporated to give a clear oil which was crystallized from ethyl acetate/ether: 0.17 g (37%) of **6** as an amorphous white powder; mp 156–157 °C; NMR ($CDCl_3$) δ 1.37 [s, 9 H, $(CH_3)_3C$)], 1.5–1.9 (m, 4 H, Arg β,γ-CH_2), 2.95 (m, 1 H, Tyr β-CH_2), 3.20 (m, 2 H, Arg δ-CH_2), 3.50 (m, 1 H, Tyr β-CH_2), 4.30 (m, 1 H, Arg α-CH), 4.60 (m, 1 H, Tyr α-CH), 5.00 (s, 2 H, $PhOCH_2Ph$), 5.15 (s, 2 H, CO_2CH_2Ph), 6.90 (d, 2 H, Tyr Ar-3,5H), 7.10 (d, 2 H, Tyr Ar-2,6H), 7.35 (m, 10 H, Ar). Anal. Calcd for $C_{34}H_{42}N_6O_8 \cdot H_2O$: C, 60.0; H, 6.5; N, 12.4. Found: C, 60.4; H, 6.2; N, 12.4.

[*N*-*tert*-Butyloxycarbonyl-*S*-(acetamidomethyl)-L-cysteinyl](*O*-benzyl-L-tyrosyl)-N^{ϵ}-nitro-L-arginine Benzyl Ester (9). The BOC group was removed from dipeptide **6** (96 mg, 0.14 mmol) by dissolution in 25 mL of dioxane previously saturated with HCl. After the mixture was stirred for 30 min at room temperature, the volatile components were evaporated and the residue was triturated with anhydrous ether to give 79 mg (94%) of the HCl salt **7** as a white powder. *N*-*tert*-Butoxycarbonyl-*S*-(acetamidomethyl)-L-cysteine (**8**)[19,20] (46.2 mg, 0.16 mmol) was dissolved in 1.5 mL of DMF and cooled to −25 °C, and 21 μL (0.16 mmol) of isobutyl chloroformate and 17.4 μL (0.16 mmol) of *N*-methylmorpholine were added. After a 25-min activation time, a solution of the dipeptide salt (**7**, 79 mg, 0.13 mmol) and 14.5 μL (0.13 mmol) of *N*-methylmorpholine in 1.5 mL of DMF was added. After 3 h at −25 °C, the solvent was evaporated and isolation proceeded as described for **6** above. HPLC purification was done under the same conditions giving one peak: R_t 4.2 min; yield, 76 mg, 70%, of **9** as a clear foam; NMR ($CDCl_3$) δ 1.40 [s, 9 H, $(CH_3)_3C$], 1.55–1.85 (m, 4 H, Arg β,γ-CH_2), 2.00 (s, 3 H, $COCH_3$), 2.75 (m, 2 H, Cys β-CH_2), 2.90 (m, 1 H, Tyr β-CH_2), 3.04 (m, 2 H, Arg δ-CH_2), 3.20 (m, 1 H, Tyr β-CH_2), 4.20 (m, 1 H, Arg α-CH), 4.35 (m, 1 H, Tyr α-CH), 4.55 (m, 3 H, Cys α-CH, SCH_2NH), 5.00 (s, 2 H, $PHOCH_2Ph$), 5.13 (s, 2 H, CO_2CH_2Ph), 6.87 (d, 2 H, Tyr Ar-3,5H), 7.10 (d, 2 H, Tyr Ar-2,6H), 7.35 (m, 10 H, Ar). Anal. Calcd for $C_{40}H_{52}N_8O_{10}S \cdot H_2O$: C, 56.2; H, 6.4; N, 13.1. Found: C, 56.5; H, 6.2; N, 13.2.

[*S*-(Acetamidomethyl)-L-cysteinyl]-L-tyrosyl-L-arginine Bis(trifluoroacetate) (10). The protected tripeptide **9** (54 mg, 64 μmol) along with 70 μL of anisole was treated with anhydrous hydrogen fluoride at 0 °C for 30 min after which the HF was evaporated. The residue was taken up in water (7 mL), washed with ethyl acetate (2 × 3 mL), and lyophilized. The residue was purified by HPLC on a Spherisorb ODS column (10 μm, 10 × 250 mm) by using 25% CH_3CN and 75% 10 mM aqueous TFA as the eluent at a flow rate of 4 mL/min with detection at 270 nm and multiple injections of 5 mg each. One minor and one major (R_t = 10 min) product were seen. The major peak was collected and lyophilized to give 22 mg (46%) of the tripeptide bis(trifluoroacetate) **10**. The NMR sample was prepared as described for chromopeptide **3** and was determined at 5.0 mM in 10 mM TFA in D_2O (Figure 2).

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(30) Konigsberg, W. *Methods Enzymol.* **1972**, *25B*, 326.