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STRUCTURE OF PLANT BILE PIGMENTS

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"Structure of Plant Bile Pigments"

by

Robert William Schoenleber

Abstract

Selective peptide cleavage has provided a general procedure for the study of the structure, including stereochemistry, of plant bile pigments. The information derived from the synthesis and spectral analysis of a series of 2,3-dihydrodioxobilins allows the determination of the trans relative stereochemistry for ring A of the β_1-phycocyanobilin from C-phycocyanin as well as for ring A of phytochrome. A complete structure proof of the five phycoerythrobilins attached to the α and β subunits of B-phycoerythrin is described. One of these tetrapyrroles is doubly-peptide linked to a single peptide chain through two thioethers at the C-3' and C-18' positions. The four remaining phycoerythrobilins are singly-linked to the protein through thioethers at the C-3' position and all possess the probable stereochemistry C-2(R), C-3(R), C-3'(R), and C-16(R).

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Dedication

To my parents....

Part One

The Relative Stereochemistry of the A Ring of Plant Bile Pigments

Abstract

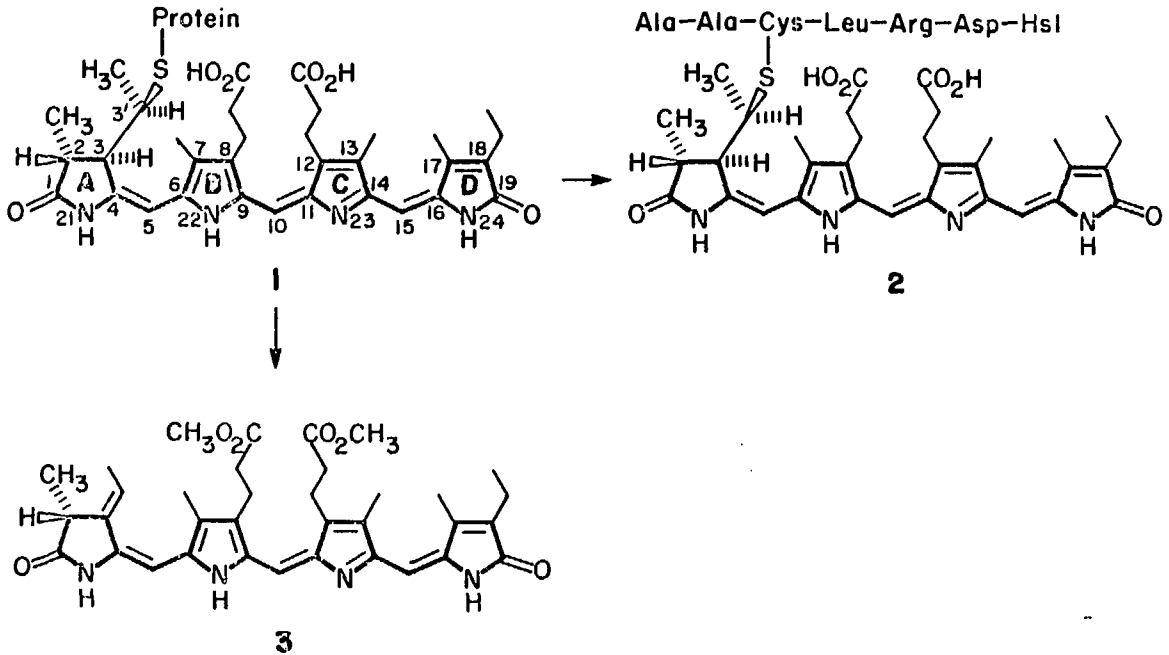
The synthesis and characterization, including the stereochemistry, of a series of 3,4-dihydropyrromethenones and 2,3-dihydrodioxobilins are described. High resolution ^{1}H NMR spectral analysis allows the determination of the A ring coupling constants for a series of cis and trans model compounds. From these data and correlations, the relative stereochemistry in the A ring of phycocyanin and similar bile pigment structures can be concluded.

Complete structure elucidation of plant bile pigments (1) has been limited by the common practice of cleaving the linear tetrapyrrole (3) from its covalently bound protein (Scheme I).[1-6] This process has been a valuable tool for gross structural determination, as the resulting product can be easily studied by spectroscopy,[7] chemical degradation,[8] and total synthesis.[9,10] The approach, however, suffers from several disadvantages. Firstly, all direct information on the chromophore-protein linkage, including two stereo centers, is lost.[1] Secondly, side reactions leading to complex mixtures of unnatural chromophores have been observed.[11] Finally, the yield in the cleavage step is frequently low.[4,5] These limitations, when combined with the fact that complex proteins are involved containing multiple chromophores with possible structural variations, indicate a more controlled method for structural study is necessary.

In previous studies, we have developed methodology to conclusively ascertain the nature of the protein-tetrapyrrole covalent bond.[12-14] By mild, selective degradation of the protein moiety without alteration of the chromophore or its attachment site, the three chiral centers about the A ring of the bile pigment are retained (Scheme I). Therefore, we are in a unique position to study the stereochemistry at these centers. For simplicity, we have chosen to concentrate on the phycocyanin series of bile pigments, as it contains only three chiral centers, all contiguous about the A ring.

The proposed structure for the peptide-pigment from the N-terminal portion of the β-subunit of C-phycocyanin from Synechococcus sp. 6301 is shown in structure 2.[12] Assignment of stereochemistry at C-2 is based on the report that chromic acid oxidation of C-phycocyanin results in the

Scheme I. Bile Pigment Degradation Approaches.



formation of (E)-2-ethylidene-3(R)-methylsuccinimide.[15] Unfortunately, the actual analytical and spectroscopic data for the compound obtained from the bile pigment degradation are unavailable in the literature. Also, C-phycocyanin contains three phycocyanobilins, each with possible structural differences.[16] Although it seems reasonable that the three chromophores have the same stereochemistry, until this determination is made for each chromophoric unit, the question remains unanswered.

Assignment of the relative stereochemistry between C-3 and C-3' also is based on degradative evidence. Mechanistic interpretations indicate that an antiperiplanar elimination of the sulfone resulting from thioether oxidation gives the observed (E)-ethylidene succinimide.[17] Although the findings have been corraborated by thiol elimination to the same product on numerous occasions, no detailed mechanistic studies on this reaction have been done.[15] Despite the evidence presented, the multiple chromophore problem casts doubts on the utility of these data.

The remaining stereochemical relationship to be addressed, then, is the relative stereochemistry between the C-2 and C-3 centers. Having two adjacent protons in a rigid five membered ring suggests as a solution the homonuclear coupling constant determination of the dihedral angle relationship. Conclusions would require the proper model compounds, as the system is too complex to approach theoretically.[18] Although some model systems are available, the information cannot be directly related to the natural product.[17,19] Possible solvent effects and complicating side chain functionality have prevented a definitive interpretation.

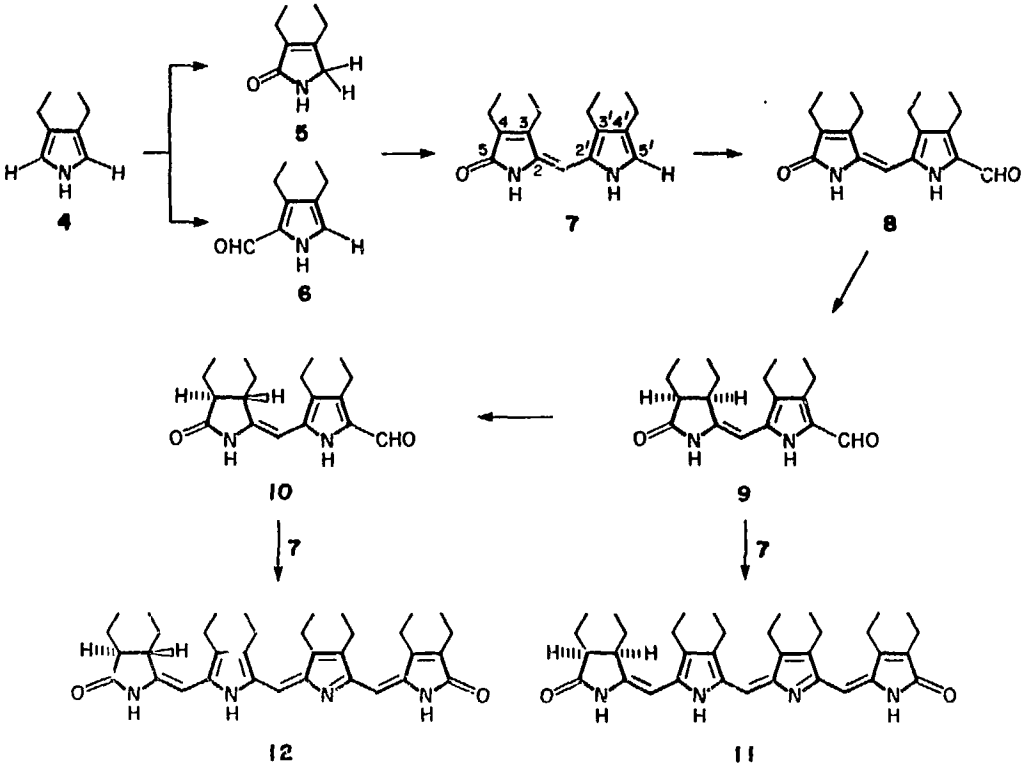
We present a systematic study of model 2,3-dihydrodioxobilins and correlate their NMR properties to those of natural bile pigments. The information gained, if used with properly collected degradative data,

should allow the complete absolute stereochemical assignment of any phycocyanobilipeptide.

Our initial synthetic goal then, was to gain entry into symmetrically substituted octaalkyl dihydrobiliverdins (Scheme II). The octaethyl series was chosen to avoid any solubility problems which might restrict the analysis. We began with the 1,3-dipolar addition of the tosylmethyl-isocyanide anion to an α,β-unsaturated ketone that allows for rapid and facile entry into 3,4-dialkylpyrroles, after reduction of the carbonyl group.[20,21] The 3,4-diethylpyrrole (4) thus obtained served as the starting material for all four pyrrolic rings. This pyrrole could be formylated under Vilsmeier conditions to give an appropriately functionalized unit 6 for the B and C rings.[22] The diethylpyrrole could also be oxidized in a stepwise manner, first to give 3,4-diethyl-3-pyrrolin-2-one (5) and then further to give 3,4-dimethylmaleimide.[22,23] The pyrrolinone was the A and D ring precursor, while the maleimide was a necessary compound for the stucture proof.

Base catalyzed condensation of pyrrolinone 5 and formylpyrrole 6 gave 3,3',4,4'-tetraethyl-5(1H)-pyrromethenone (7), again a common intermediate.[22] Formylation, this time with trimethyl orthoformate gave the key pyrromethenone 8 into which the 3,4-dihydro feature could be introduced.[10] This catalytic reduction of the 5'-formylpyrromethenone 8 gave a mixture of products reduced at the 3,4- and the methine double bonds, as well as the tetrahydro material.[24,25] The desired 3,4-dihydro compound was the major product and could be easily separated by chromatography. Cis-3,4-dihydro-5'-formylpyrromethenone (9) obtained from the catalytic reduction was epimerized completely to the trans isomer by refluxing in methanolic sodium hydroxide.[24] Operationally, it is easier

Scheme II. Synthesis of cis- and trans-2,3-Dihydrooctaethyl-biliverdins 11 and 12.



to carry out the epimerization on the crude reduction mixture and then do a single purification to obtain the trans compound.

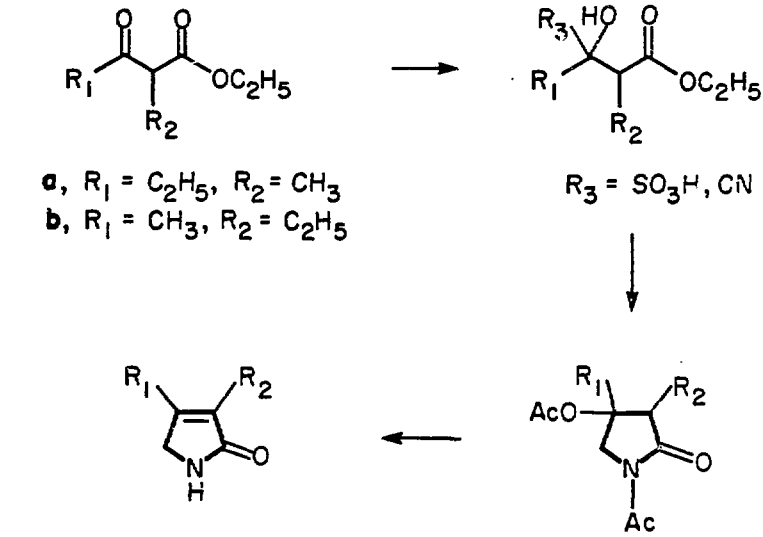
The final synthetic transformation in this series was to couple the aldehydic pyrromethenone 9 and 10 with the 5'-H pyrromethenone 7 to give the dioxotetrapyrroles 11 and 12 without epimerization at either C-2 or C-3.[26] A final HPLC of purification of the resulting product showed that no epimerization had occurred, especially if one uses only 33 mol% of $POBr_3$ and 100 mol% of 2,5-di-t-butyl-4-methylpyridine for the condensation reaction.

Although the octaalkyl series gave us a valid 2,3-dihydro model system, a more appropriately functionalized linear tetrapyrrole was necessary for direct correlation with the natural pigments. Due to the inherent dissymmetry of the bile pigments, a more elaborate synthetic scheme to the ring precursors was required. However, the synthesis is still quite convergent, and the key synthetic transformations to obtain the 2,3-dihydro feature and to couple to the ultimate dioxotetrapyrroles were retained.

The necessary pyrrolinones 13a and 13b were synthesized through their corresponding cyanohydrins (Scheme III).[27] We found that the intermediacy of the bisulfite adduct avoided the need for large amounts of anhydrous HCN.[28] Attempts at reducing the cyanohydrin with platinum failed, however, the now standard Raney nickel reduction led smoothly to the desired compounds.[27]

The formyl pyrrole 14 came from an initial Knorr condensation followed by extensive functional group transformations to obtain the necessary substituent pattern.[29,30] Base catalyzed condensation to pyrromethenones 15 was done in methanolic KOH as in the ethyl series

Scheme III. Synthesis of Ethylmethylpyrrolinones 13a and 13b.



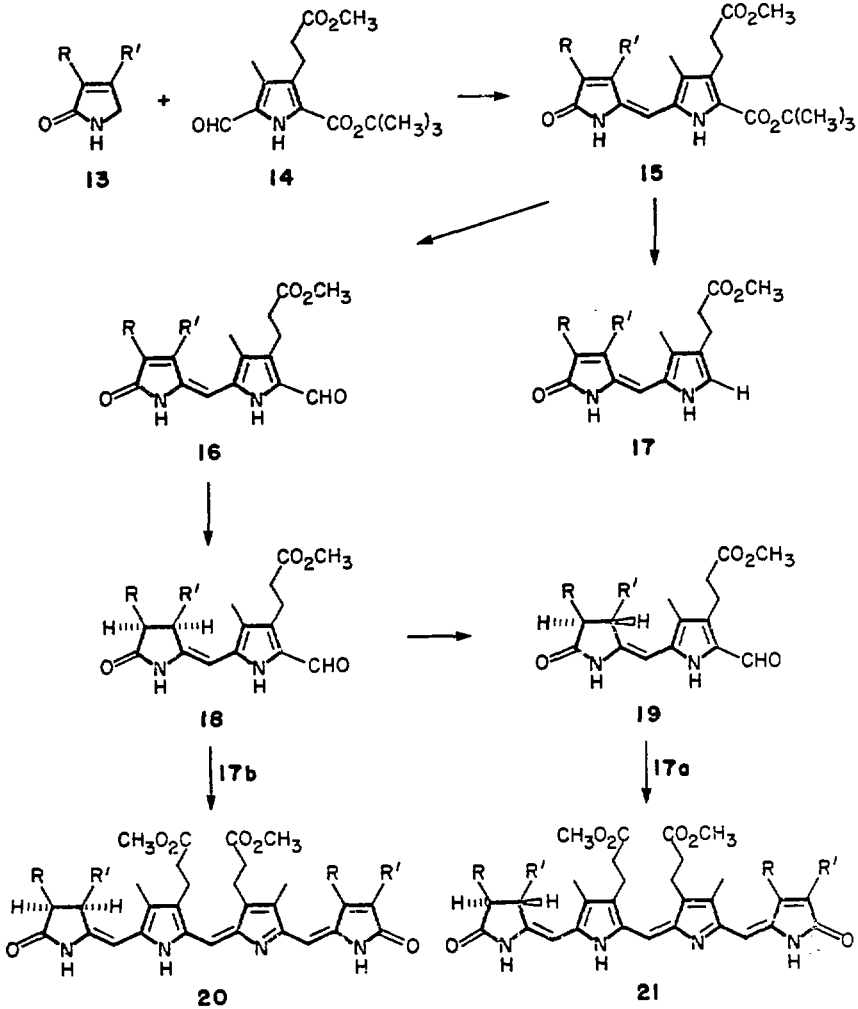
(Scheme IV).[30] This required a subsequent re-esterification with diazomethane. Attempts to carry out the condensation with sodium methoxide resulted in significantly lower yields.

The 5'-t-butoxycarbonyl group served as a stabilizing group during the pyrrole synthesis. Although catalytic reduction to the dihydro series can be done on the ester,[24] we wanted to minimize the number of transformations after the reduction in order to avoid exposure to epimerization. Therefore, an acid catalyzed decarboxylative-formylation was used and gave the 5'-formylpyrromethenone 16 directly. On the other hand, treatment of the t-butyl ester with neat trifluorooacetic acid for five minutes allows one to isolate the 5'-acid in good yield, while a longer reaction time (two hours) effects decarboxylation to give the 5'-H-pyrromethenone 17. This latter compound is the precursor of the C,D-ring moiety.

The remainder of the synthesis was directly analogous to the ethyl series, and led to the synthesis of the four 2,3-dihydrodioxobilins 20a, 20b, 21a, and 21b as shown in Scheme IV. The side chain methyl ester introduced some problems, as some hydrolysis was seen in both the epimerization and coupling steps. However, in both cases the acid could easily be separated from the ester and re-esterified with stoichiometric diazomethane. The final products were purified by HPLC, and again the coupling was effected without epimerization.

It has always been assumed that catalytic hydrogenation gives cis hydrogen addition to the pyrromethenones. However, the empirical nature of the experimental results makes such a simplistic assumption tenuous. Since the methine double bond is trisubstituted while the 3,4-double bond is tetrasubstituted, it is surprising that the 3,4-dihydro compound

Scheme IV. Synthesis of 2,3-Dihydrodioxobilins 20a, 20b, 21a, and 21b.



is the major product. Furthermore, if the reduction is done under alkaline conditions or with other catalysts, only the pyrromethane is seen.[24] Due to uncertain mechanistic paths in such a complex system, we concluded that a more rigorous stereochemical proof was required in our dioxobilin series.

Diethylmaleimide and ethylmethylmaleimide were independently synthesized by oxidation of the corresponding pyrroles.[22,23] Catalytic hydrogenation of these compounds over PtO_2 gives the cis-succinimide in nearly quantitative yield.[31] The trans-succinimides were then obtainable from a strong base-catalyzed epimerization.

Each of the dioxobilins was then subjected to the now standard chromic acid oxidation.[32] In the octaethyl series, diethylmaleimide was generated from the B, C, and D rings. From the trans-tetrapyrrole, only the trans-succinimide resulting from A ring oxidation was seen by GC analysis; no cis-succinimide was present. The cis-tetrapyrrole likewise gave diethylmaleimide and only cis-diethylsuccinimide. Although no rigorous detection limits were established, less than ten percent of the isomeric succinimide would easily have been seen in each case.

The bile pigment degradation products are more easily observed by TLC analysis, using the sensitive chlorine/o-anisidine reagent for visualization.[33,34] Chromic acid oxidation of each of the four compounds 20a, 20b, 21a and 21b gave poor yields, as usual, but no interfering side products. Each dioxotetrapyrrole gave positive tests for ethylmethylmaleimide and hematinic acid methyl ester, resulting from the B, C, and D rings. From both trans-tetrapyrroles 21a and 21b only trans-ethylmethylsuccinimide was obtained. The cis-tetrapyrroles 20a and 20b gave cis-ethylmethylsuccinimide with a barely detectable trace of the

trans succinimide. Considering the sensitivity of the visualization process, the trace amounts are extremely small. Since the dioxotetrapyrroles 20a and 20b, having been purified by preparative HPLC prior to degradation, were free from contaminating trans-isomer, this indicates that a small amount of epimerization did occur in the oxidation step.[35] The results, however, leave no doubt that the correct cis and trans assignments have been made.

The ^{1}H NMR assignments for the cis and trans-3,4-dihydro-5'-formylpyrromethenones 18a, 18b, 19a, and 19b in $CDCl_3$ are shown in Table I. Decoupling experiments allowed us to assign all proton resonances and to determine the 3-H/4-H coupling constants (Table IV) despite a few overlapping resonances. This analysis greatly facilitated the interpretation of the more complicated tetrapyrrole spectra.

The ^{1}H NMR assignments for the dioxotetrapyrroles 20 and 21 in $CDCl_3$, along with shifts reported in a previous study,[25] are found in Table II. Compound 21b is included in this study for completeness, as we believe that conclusive evidence for all bile pigment attachments through the A ring has yet to be presented. Our values agree well (0.05 ppm) with those reported[25] with the exception of the C-2-H, C-2-CH_3, and C-3-H. These resonances show the most variation when comparing the pyrromethenones and the dioxotetrapyrroles as well. The decoupling experiments, though, leave no doubt about the assignments made. The chemical shift variation may reflect differing protonation states due to trace amounts of acid in the solvent or concentration effects, as these molecules are known to exhibit complex solution conformational behavior.

In our study of the β_1-phycocyanobiliheptapeptide,[12] we found

Table I. ^1H NMR Assignments for the 3,4-Dihydro-5'-formyl cis (18) and trans (19) Pyrromethenones in $CDCl_3$.

assignment	18a (cis)	18b (cis)	19a (trans)	19b (trans)
5'-CHO	9.54	9.54	9.54	9.53
meso-H	5.37	5.35	5.31	5.31
4'-$CH_2CH_2CO_2CH_3$	3.68	3.68	3.68	3.67
4'-$CH_2CH_2CO_2CH_3$	3.06	3.10	3.06	3.06
3-H	2.95	3.26	2.65	2.85
4'-$CH_2CH_2CO_2CH_3$	2.58	2.57	2.58	2.58
4-H	2.79	3.05	2.42	2.17
3'-CH_3	2.03	2.02	2.02	2.03
3 or 4-CH_2CH_3	1.6-1.8	1.5-2.0	1.5-2.0	1.5-2.0
3 or 4-CH_3	1.21	1.23	1.32	1.36
3 or 4-CH_2CH_3	1.01	1.05	1.02	1.03

Table II. ^{1}H NMR Assignments for the 2,3-Dihydrodioxobilins 20 (cis) and 21 (trans) in $CDCl_3$.

assignment	20a (cis)	20b (cis)	21a (trans)	21b (trans)
10-H	6.65 (6.64)[a]	6.64 (6.65)	6.64	6.65 (6.66)
15-H	5.98 (5.98)	6.00 (6.01)	5.98	6.01 (6.01)
5-H	5.49 (5.47)	5.47 (5.47)	5.46	5.47 (5.48)
8,12-$CH_2CH_2CO_2CH_3$	3.68 (3.68)	3.68 (3.68)	3.68	3.68 (3.68)
	3.66 (3.67)	3.67 (3.67)	3.67	3.67 (3.67)
8,12-$CH_2CH_2CO_2CH_3$	2.94 (2.94)	2.94 (2.93)	2.94	2.95 (2.92)
	2.90 (2.90)	2.90 (2.93)	2.90	2.90 (2.92)
3-H	3.14 (2.75)	3.38 (2.93)	2.79	2.94 (2.14)
8,12-$CH_2CH_2CO_2CH_3$	2.55 (2.55)	2.55 (2.54)	2.55	2.56 (2.54)
	2.54 (2.54)	2.54 (2.54)	2.54	2.54 (2.54)
2-H	2.85 (2.32)	2.6[b] (2.1)	2.35[b]	2.5[b] (2.92)
17 or 18-CH_2CH_3	2.32 (2.32)	2.55[b] (2.54)	2.32	2.55[b] (2.54)
7,13,17 or 18-CH_3	2.12 (2.12)	2.12 (2.12)	2.12	2.12 (2.12)
	2.11 (2.12)	2.02 (2.02)	2.11	2.02 (2.02)
	2.02 (2.02)	1.85 (1.84)	2.01	1.85 (1.85)
2 or 3-CH_2CH_3	1.5-2.0 (1.7-2.0)	1.4-2.0 (1.6-1.8)	1.6-2.0	1.5-1.9 (1.6-1.8)
2 or 3-CH_3	1.12 (1.24)	1.27 (1.42)	1.24	1.43 (1.43)
17 or 18-CH_2CH_3	1.10 (1.10)	1.22 (1.22)	1.10	1.22 (1.22)
2 or 3-CH_2CH_3	1.01 (1.06)	1.02 (0.99)	1.06	0.99 (0.99)

[a]Chemical shifts in parentheses are from reference 25.

[b]Multiple resonances occur at these frequencies, but assignments can be made from decoupling and integration studies.

pyridine to be an excellent solvent for avoiding aggregation and conformational effects. The basicity of the pyridine, present in large excess, precludes differential protonation of the substrate, and the aromatic nature of this solvent encourages intercalation between substrate molecules, preventing aggregation. Thus, the conformations adopted by the dioxotetrapyrroles appear to be uniform.

To complete our present study, therefore, the 360 MHz ^{1}H NMR spectra of the dioxobilins were recorded in pyridine-d_5 (Table III). Not only does this serve to avoid the problems mentioned above, but it also allows us to make a direct comparison with previous natural products studied in pyridine. There is much less variation in resonances for the model compounds and several patterns appear. The C-15-H in the 2-ethyl-3-methyl series is shifted downfield approximately 0.1 ppm from that in the 3-ethyl-2-methyl series. The A ring methyl group for the former series is also shifted downfield 0.1 ppm. Finally, although the C-2-H and C-3-H still show the most variation in chemical shift, the 2-ethyl-3-methyl cis and trans compounds show a significant (0.2-0.3 ppm) upfield shift of the C-2-H as compared to the corresponding 3-ethyl-2-methyl compounds.

That our models truly reflect the actual case is shown by the excellent agreement between the shifts for 21a, the most likely model, and the β_1-phycocyanobiliheptapeptide 2. Where minimal perturbation of structure is seen, the agreement is within 0.05 ppm. Of particular importance is the correlation of the C-2-CH_3 and even the C-2-H. The substituents on the B, C, and D rings likewise agree well, although the presence of the acid rather than the ester causes slightly larger shift differences in the propionic acid resonances. The methine protons also show a substantial difference in shift as has been seen before when

Table III. ^{1}H NMR Assignments for the 2,3-Dihydrodioxobilins 20 (cis) and 21 (trans) in Pyridine-d_5.

assignment	20a (cis)	20b (cis)	21a (trans)	21b (trans)
10-H	7.09	7.07	7.08 (7.29)[a]	7.09
15-H	6.07	6.15	6.07 (6.08)	6.17
5-H	5.47	5.47	5.44 (5.87)	5.47
8,12-$CH_2CH_2CO_2CH_3$	3.64	3.64	3.64 (b)	3.64
	3.62	3.62	3.62 (b)	3.62
8,12-$CH_2CH_2CO_2CH_3$	3.09	3.08	3.09 (3.17)[b]	3.09
	3.00	3.00	3.00 (3.00)[b]	3.00
3-H	c	3.28	2.7[d] (3.15)[b]	2.93
8,12-$CH_2CH_2CO_2CH_3$	2.72	2.72	2.71 (2.85)[b]	2.72
	2.69	2.68	2.69 (2.83)[b]	2.69
2-H	3.03[d]	2.71	2.62 (2.70)	2.40
17 or 18-CH_2CH_3	2.52	2.55	2.51 (2.48)	2.55
7,13,17 or 18-CH_3	2.11	2.10	2.11 (2.12)	2.11
	2.09	2.05	2.09 (2.07)	2.05
	1.95	1.94	1.96 (2.02)	1.96
2 or 3-CH_2CH_3	1.6-1.7	1.4-1.7	1.6-2.0 (e)	1.6-2.0
2 or 3-CH_3	1.24	1.33	1.36 (1.39)	1.47
17 or 18-CH_2CH_3	1.30	1.24	1.28 (1.23)	1.24
2 or 3-CH_2CH_3	0.97	1.08	1.03 (1.48)[e]	1.03

[a] Chemical shifts in parentheses are for the β_1-phycocyanobiliheptapeptide 2 from reference 12.

[b] This chemical shift is not strictly analogous as compound 2 is present as the acid.

[c] Assignment could not be made due to obscurring resonances.

[d] Multiple resonances occur at these frequencies, but assignments could be made from decoupling and integration studies.

[e] This chemical shift is not strictly analogous due to the presence of the thioether.

comparing a free dioxotetrapyrrole to one which is peptide bound.[12] The nature of this difference is still unknown and, to be deciphered, will require synthesis of a more appropriate model incorporating the amino acid thioether moiety. The presence of the thioether likewise destroys any possible comparison at the C-3-H or any of the C-3-ethyl protons.

The most important stereochemical information comes from the pattern of A ring coupling constants seen in Table IV. The $J_{3,4}$ for the cis-dihydropyrromethenones 18 centers around 8.1 Hz in $CDCl_3$ while the trans compounds 19 show $J_{3,4} \simeq 5.0$ Hz. The ethyl compounds show coupling constants quite a bit lower (6.8 and 2.9 Hz, respectively) due to the steric interaction between the C-3 and C-3' ethyl groups which must distort the conformation. The dioxotetrapyrroles show slightly higher J values, as now the tendency to form helical conformations removes some of the C-3/C-7 steric interaction. In pyridine, the coupling constants show the same behavior. For the cis-dioxotetrapyrroles 20 one sees $J_{2,3} \simeq$ 8.3 Hz while the trans compounds 21 exhibit $J_{2,3} \simeq 5.8$ Hz. The coupling constants are not nearly as sensitive to solvent effects as are the chemical shifts. Again, as witnessed by the ethyl series, steric bulk about the C-3 and C-7 centers causes a pronounced (1.0-1.5 Hz) lowering of the coupling constant.

The pattern is clear. Based on the models with the appropriate substitution, 20a and 21a, one expects the 2-H/3-H coupling constant in pyridine to be 8.2 Hz in the cis configuration and 5.7 Hz in the trans configuration. Returning to the β_1-phycocyanobiliheptapeptide, we observe a coupling constant of 5.0 Hz for these protons.[12] As the steric effect of the thioether undoubtedly causes a slight lowering of the coupling constant, we can now conclusively state that this bile

Table IV. ^{1}H Coupling Constants[a] Observed for Ring A.

compound	stereochemistry	J (Hz, $CDCl_3$)	J (Hz, pyridine-d_5)
18a	cis	8.2	—
18b	cis	8.0	—
9	cis	6.8	—
20a	cis	8.5	8.2
20b	cis	8.2	8.5
11	cis	6.5	7.6
19a	trans	4.9	—
19b	trans	5.1	—
10	trans	2.9	—
21a	trans	5.4	5.7
21b	trans	6.0	5.8
12	trans	3.7	4.1

[a]These values represent the 3-H/4-H coupling constants for the 3,4-dihydropyrromethenones and the 2-H/3-H coupling constants for the 2,3-dihydrodioxotetrapyrroles.

pigment has a trans relationship between the C-2-H and the C-3-H. Given the previously mentioned caveats in regard to the assignment of the stereochemistry at C-2 and C-3', our results also can serve to assign the absolute stereochemistry at the C-2, C-3, and C-3' centers as R,R,R.

These data are applicable to the phytochrome structure as well, as the pigment structure differs only in the substitution of a vinyl for an ethyl group at C-18. The chemical shift correspondence substantiates this claim.[13] Again, a coupling constant of 5.0 Hz was seen for the 2-H/3-H interaction. By direct analogy, a trans-dihydro A ring must be present. This completes the relative stereochemical assignment of the phytochrome tetrapyrrole. The absolute stereochemistry has yet to be determined; therefore, the possibilities for the C-2, C-3, and C-3' centers remain R,R,R or S,S,S.

Although drawing the analogy to the phycoerythrin as well as other bile pigment series would seem straightforward, it is clear that the stereochemistry at C-16 has a profound effect on the spectroscopic properties of this chromophore.[36] In our studies in this series, we found a 2-H/3-H coupling constant of 3.5 Hz in D_2O.[14] This low value is the first good evidence for the trans configuration in that series. However, because of possible solvent differences and until the phycoerythrin model series is synthesized, no stronger argument can be made.

Experimental Section

General Methods. Tetrahydrofuran (THF) was distilled from sodium/benzophenone, methanol was distilled from magnesium, methylene chloride was distilled from phosphorus pentoxide, t-butanol and ethyl acetate were distilled from calcium hydride, pyridine was distilled from p-toluenesulfonyl chloride then from calcium hydride, and trimethyl orthoformate was freshly distilled before use. Potassium t-butoxide was freshly sublimed before use. Sodium methoxide was prepared immediately before use, and all other reagents were made as referenced or used directly from commercial suppliers after verification.

Melting points were measured on a Buchi capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 137 spectrophotometer with polystyrene film for calibration (1601.4 cm^{-1} absorption). UV/vis spectra were recorded in methanol with a Perkin-Elmer 522A spectrophotometer. Unless otherwise noted, the ^1H NMR spectra were determined on the UCB-200 spectrometer (a home-made FT instrument operating at 201.95 MHz). The ^1H NMR spectra in pyridine-d_5 were recorded on the University of California, Davis, 360 MHz NMR spectrometer. All chemical shifts are expressed in parts per million (δ) downfield from an internal Me_4Si standard. The tabulated chemical shifts were recorded on approximately 15 mM samples. Multiplicities are not included for the sake of clarity, but are easily inferred from the data given. High-resolution (exact mass) mass spectra were obtained on a Kratos MS-50. Elemental analyses were performed by the Analytical Laboratory, College of Chemistry, University of California, Berkeley.

Gas chromatography (GC) was done with a Hewlett-Packard 402 gas

chromatograph with a He flow rate of 80-100 mL/min on a 1.8m 5% Dexsil 300 on 90/100 Anakrom Q column (6 mm i.d.) operating at 160°C. High pressure liquid chromatography (HPLC) was done on an Altex analytical system consisting of two Model 110A pumps, a 155-10 UV/vis detector, and a Model 420 microprocessor controller/programmer. An Altex 10x250 mm, 10 μm LiChrosorb Si60 silica gel column was used with detection at 340 nm with the following solvent compositions (v/v): (A) 60% isooctane/40% ether; (B) 0.75% methanol/chloroform; (C) 1.25% methanol/chloroform; (D) 55% ether/45% hexane. Analytical thin layer chromatography (TLC) was done on aluminum-backed silica plates activated at 100°C for 2 hours. A three-fold multiple development was done using the solvent system chloroform/ethyl acetate/ cyclohexane (32:9:1)[37] and the R_F values recorded are after three developments. Visualization was by a modification of the chlorine/benzidine procedure,[33,34] substituting o-anisidine for benzidine.

Unless otherwise noted, all reaction were conducted under a nitrogen atmosphere. Evaporations were done on a Berkeley rotary evaporator after drying over Na_2SO_4, using a water aspirator followed by static evaporation with an oil pump.

3,3',4,4'-Tetraethyl-5'-formyl-5(1H)-pyrromethenone (8). 3,3',4,4'-Tetraethyl-5(1H)-pyrromethenone (7, 300 mg, 1.1 mmol)[22] was dissolved in 6 mL of trifluoroacetic acid at room temperature, trimethyl orthoformate (3 mL, 27 mmol) was added at once, and the solution was stirred for five minutes at room temperature. Rapid addition of 40 mL of ice water was followed by extraction with CH_2Cl_2 (4x10 mL), and the combined organic layer was dried and evaporated. The residue was recrystallized from 50 mL of methanol to give 302 mg (91%) of 8 as yellow needles: mp 194-195°C;

UV/vis λ_{max} (log ε) 415 (sh), 394 (3.94), 268 (3.94), 261 (sh); NMR ($CDCl_3$) δ 1.1-1.3 (4xt, 12H, 4xCH_3), 2.43 (q, 2H, CH_2CH_3, J=7.5Hz), 2.57 (q, 4H, 2xCH_2CH_3, J=7.5Hz), 2.78 (q, 2H, CH_2CH_3, J=7.6Hz), 6.10 (s, 1H, meso-H), 9.60 (s, H, CHO), 10.77 (s, 1H, NH), 10.85 (s, 1H, NH); Anal. Calcd for $C_{18}H_{24}N_2O_2$: C, 72.0; H, 8.0; N, 9.3. Found: C, 72.3; H, 8.0; N, 9.4.

cis-3,4-Dihydro-5'-formyl-3,3',4,4'-tetraethyl-5(1H)-pyrromethenone (9). Pyrromethenone 8 (200 mg, 0.67 mmol) was suspended in 50 mL of methanol, 200 mg of 3% $PdCl_2/SrCO_3$[38] was added, and one atmosphere of hydrogen was applied for four hours, or until the solution turned from yellow to colorless. Filtration and evaporation left a residue which was purified by preparative HPLC: R_t (solvent A, 4.2 mL/min) 8.4 min, dihydropyrromethanone 28.8 min, pyrromethanone 42.0 min. The product 9 (104 mg, 52%) was a slightly yellow solid and was recrystallized from methanol: mp 166-170°C; UV/vis λ_{max} (log ε) 360 (4.10), 242 (4.16); NMR ($CDCl_3$) δ 1.00, 1.04, 1.11, 1.23 (4xt, 12H, 4xCH_3, 4xJ=7.6Hz), 1.4-1.7 (m, 2H, $CHCH_2CH_3$), 1.8-1.95 (m, 2H, $CHCH_2CH_3$), 2.47 (q, 2H, CH_2CH_3, J=7.6Hz), 2.5-2.65 (m, 1H, 4-H), 2.73 (q, 2H, CH_2CH_3, J=7.6Hz), 2.9-3.0 (m, 1H, 3-H), 5.38 (s, 1H, meso-H), 9.51 (s, 1H, CHO). Anal. Calcd for $C_{18}H_{26}N_2O_2$: C, 71.5; H, 8.7; N, 9.3. Found: C, 71.6; H, 8.7; N, 9.2.

trans-3,4-Dihydro-5'-formyl-3,3',4,4'-tetraethyl-5(1H)-pyrromethenone (10). The cis compound 9 (100 mg, 0.33 mmol) was dissolved in 40 mL of 1N NaOH in MeOH and refluxed for 2.5 h. The methanol was evaporated, 20 mL of 1M aqueous H_3PO_4 was added, the aqueous solution was extracted with CH_2Cl_2 (4x10 mL), and the combined CH_2Cl_2 solution was washed with 10 mL each of H_2O and saturated aqueous NaCl and dried. After filtration and solvent evaporation, the residue was purified by HPLC as described

for cis compound 9 to give 10 as a slightly yellow solid (76 mg, 76%): mp 181-185°C; UV/VIS λ_{max} (log ε) 366 (4.09), 239 (4.14); NMR ($CDCl_3$) δ 0.99, 1.01, 1.11, 1.23 (4xt, 12H, 4xCH_3, 4xJ=7.5Hz), 1.6-1.9 (m, 4H, 2x $CHCH_2CH_3$), 2.25-2.35 (m, 1H, 4-H), 2.46 (q, 2H, CH_2CH_3, J=7.6Hz), 2.73 (q over m, 3H, CH_2CH_3 and 3-H, J_q=7.6Hz), 5.32 (s, 1H, meso-H), 9.48 (s, 1H, CHO), 10.92 (s, 1H, NH), 10.96 (s, 1H, NH); exact mass calcd for $C_{18}H_{26}N_2O_2$ 302.1995, found m/e 302.1991 (M^+).

cis-1,2,3,19,21,24-Hexahydro-2,3,7,8,12,13,17,18-octaethyl-1,19-dioxobilin (11). The cis-3,4-dihydro-5'-formyl-pyrromethenone 9 (6.8 mg, 22 μmol) and the 5'-H-pyrromethenone 7 (6.1 mg, 22 μmol) were dissolved in 1.0 mL of CH_2Cl_2, 2,6-di-t-butyl-4-methylpyridine[39] (4.5 mg, 22 μmol) and $POBr_3$[40] (2.0 mg, 7.3 μmol) were added, and the reaction was left for one hour at room temperature. The reaction then was diluted with 5 mL of CH_2Cl_2, washed successively with 2.5 mL each of saturated aqueous $NaHCO_3$, 1M aqueous H_3PO_4, H_2O, and saturated aqueous NaCl, dried and evaporated. The residue was purified by HPLC to give 5.7 mg (46%) of a blue solid: R_t (solvent B, 2.6 mL/min) 18 min; UV/vis λ_{max} (log ε) 580 (4.01), 358 (4.50), 270 (4.26); NMR ($CDCl_3$) δ 1.00 (t, 3H, CH_2CH_3, J=7.5Hz), 1.05-1.3 (m, 21H, 7xCH_2CH_3), 1.41.8 (m, 4H, 2x$CHCH_2CH_3$), 2.3-2.7 (m, 13H, 6xCH_2CH_3 and 4-H), 3.05-3.15 (m, 1H, 3-H), 5.50, 6.01, 6.60 (3xS, 3H, 3x meso-H); NMR (pyridine-d_5) 1.0-1.4 (m, 24H, 8xCH_2CH_3), 1.6-2.0 (m, 4H, 2x$CHCH_2CH_3$), 2.3-2.8 (m, 13H, 6xCH_2CH_3 and 4-H), 3.05-3.10 (m, 1H, 3-H), 5.50, 6.23, 6.83 (3xS, 3H, 3x meso-H); exact mass calcd for $C_{35}H_{48}N_4O_2$ 556.3777, found m/e 556.3769 (M^+).

trans-1,2,3,19,21,24-Hexahydro-2,3,7,8,12,13,17,18-octaethyl-1,19-dioxobilin (12). The trans-3,4-dihydro-5'-formylpyrromethenone 10 was coupled and purified as described for 11. The product was a blue solid

(37%): R_t (solvent B, 2.6 mL/min) 18 min; UV/vis λ_{max} (log ϵ) 584 (4.00), 354 (4.54), 274 (4.30); NMR ($CDCl_3$) δ 0.95, 1.04 (2xt, 6H, $2xCH_2\underline{CH_3}$, 2xJ=7.5 Hz), 1.1-1.3 (m, 18H, $6xCH_2\underline{CH_3}$), 1.5-1.9 (m, 4H, 2x $CH\underline{CH_2}CH_3$), 2.2-2.7 (m, 13H, $6x\underline{CH_2}CH_3$ and 4-H), 2.80 (m, 1H, 3-H), 5.47 (d, 1H, meso-H, J=1.2 Hz), 6.00, 6.58 (2xS, 2H, meso-H); NMR (pyridine-d_5) 0.8-1.5 (m, 24H, $8xCH_2\underline{CH_3}$), 1.6-2.0 (m, 4H, $2xCH\underline{CH_2}CH_3$), 2.3-2.7 (m, 13H, $6x\underline{CH_2}CH_3$ and 4-H), 2.75-2.83 (m, 1H, 3-H), 5.50, 6.23, 6.83 (3xS, 3H, meso-H); exact mass calcd for $C_{35}H_{48}N_4O_2$ 556.3777, found m/e 556.3795 (M^+).

4-Ethyl-3-methyl-3-pyrrolin-2-one (13a).[27] The cyanohydrin of ethyl 2-methyl-3-oxopentanoate[41] was prepared through the bisulfite adduct[28] without purification and carried on directly to the pyrrolinone 13a: mp 79-81°C (lit.[27] mp 81-83°C); NMR ($CDCl_3$) δ 1.10 (t, 3H, 4-$CH_2\underline{CH_3}$, J=7.5Hz), 1.77 (s, 3H, 3-CH_3), 2.38 (q, 2H, 4-$\underline{CH_2}CH_3$, J=7.5Hz), 3.82 (s, 2H, 5-CH_2), 7.68 (br s, 1H, NH).

3-Ethyl-4-methyl-3-pyrrolin-2-one (13b).[23] Ethyl 2-ethyl-3-oxobutanoate[42] was converted as above to pyrrolinone 13b:[26] mp 101-102°C (lit.[23] mp 101°C); NMR ($CDCl_3$) δ 1.06 (t, 3H, 3-$CH_2\underline{CH_3}$, J=7.5Hz), 1.97 (s, 3H, 4-CH_3), 2.25 (q, 2H, 3-$\underline{CH_2}CH_3$, J=7.5Hz), 3.80 (s, 2H, 5-CH_2), 8.14 (br s, 1H, NH).

t-Butyl-3',4-Dimethyl-3-ethyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone-5'-carboxylate (15a). To a solution of 0.692 g (2.36 mmol) of t-butyl 5-formyl-3-(2-methoxycarbonylethyl)-4-methylpyrrole-2-carboxylate (14)[30] and 0.433 g (3.47 mmol) of 4-ethyl-3-methylpyrrolin-2-one (13a) in 1.3 mL of MeOH was added 1.71 g (30.5 mmol) KOH in 6.5 mL of H_2O at room temperature. The reaction was stirred for 18 h, then diluted with 26 mL of H_2O and SO_2 (g) was passed through until the pH registered 3.

The precipitate formed during the acidification was filtered, washed with 10 mM aqueous TFA, and dried to give 0.870 g (95%) of the 4'-propionic acid-pyrromethenone derivative. This acid was directly esterified by adding 1.2 mL of MeOH and then 23.6 mL of 0.1M CH_2N_2 in ether[43] and stirring for one hour at room temperature. The heterogeneous reaction was filtered and the solid recrystallized from MeOH/ether/hexane to give 3.10 g (86%) of a yellow solid. The filtrate was evaporated and the residue was recrystallized from the same solvent to give an additional 0.26 g of product for a total yield of 15a of 93%: mp 188-190°C (dec); UV/VIS λ_{max} (log ε) 400 (sh), 380 (4.38), 257 (4.32), 250 (sh); NMR ($CDCl_3$) δ 1.18 (t, 3H, 3-$CH_2\underline{CH}_3$, J=7.5Hz), 1.56 (s, 9H, $C(CH_3)_3$), 1.97 (s, 3H, 4-CH_3), 2.10 (s, 3H, 3'-CH_3), 2.45-2.65 (m, 4H, 3-$\underline{CH}_2CH_3$ overlapping 4'-$CH_2\underline{CH}_2CO_2CH_3$), 3.01 (t, 2H, 4'-$\underline{CH}_2CH_2CO_2CH_3$, J=7.5Hz), 3.68 (s, 3H, OCH_3), 5.98 (s, 1H, meso-H); exact mass calcd for $C_{22}H_{30}N_2O_5$ 402.2155, found m/e 402.2158 (M^+).

t-Butyl 3,3'-Dimethyl-4-ethyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone-5'-carboxylate (15b).[30] This compound was prepared as described.[30] The filtrate was evaporated and the residue was recrystallized from MeOH/ether/hexane to give 15b in a total yield of 92%: mp 205-207°C (lit.[30] mp 206-208°C); exact mass calcd for $C_{22}H_{30}N_2O_5$ 402.2155, found m/e 402.2141 (M^+).

3',4-Dimethyl-3-ethyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone (17a).[44] The 5'-t-butyloxycarbonylpyrromethenone 15a (0.50 g, 1.2 mmol) was added to 20 mL of anhydrous trifluoroacetic acid and stirred for two hours at room temperature. The solution was poured into 200 mL of water and filtered, and the precipitate was washed with water and dried to give 0.37 g (98%) of 17a as a greenish solid: mp 172-174°C (lit.[44] mp 175-

176°C); UV/vis λ_{max} (log ε) 395 (5.32), 261 (4.83), 231 (4.77); NMR ($CDCl_3$) δ 1.18 9t, 3H, 3-$CH_2\underline{CH_3}$, J=7.6 Hz), 1.94 (s, 3H, 4-CH_3), 2.16 (s, 3H, 3'-CH_3), 2.5-2.65 (m, 4H, 3-$\underline{CH_2}CH_3$ overlapping 4'-$CH_2\underline{CH_2}CO_2CH_3$), 2.77 (t, 2H, 4'-$\underline{CH_2}CH_2CO_2CH_3$, J=7.5Hz), 3.69 9s, 3H, OCH_3), 6.33 (s, 1H, meso-H), 6.89 (d, 1H, 5'-H, J=2.7Hz), 10.05 (br s, 1H, NH), 11.63 (br s, 1H, NH).

3,3'-Dimethyl-4-ethyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone (17b).[44] The 5'-t-butyloxycarbonylpyrromethenone 15b was converted to the 5'-H compound as described above in 99% yield: mp 200-202°C (lit.[44] mp 203.5-205°C); UV/vis λ_{max} (log ε) 394 (5.30), 259 (4.80), 230 (4.74); NMR ($CDCl_3$) 1.15 (t, 3H, 4-$CH_2\underline{CH_3}$, J=7.5Hz), 2.13 (s, 6H, 3-CH_3 and 3'-CH_3), 2.41 (q, 2H, 4-$\underline{CH_2}CH_3$, J=7.5Hz), 2.60 (t, 2H, 4'-$CH_2\underline{CH_2}CO_2CH_3$, J=7.5Hz), 2.79 (t, 2H, 4'-$\underline{CH_2}CH_2CO_2CH_3$, J=7.5Hz), 3.70 (s, 3H, OCH_3), 6.13 (s, 1H, meso-H), 6.78 (d, 1H, 5'-H, J=2.8Hz).

3',4-Dimethyl-3-ethyl-5'-formyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone (16a).[45,46] The 5'-t-butyloxycarbonylpyrromethenone 15a (0.465 g, 1.16 mmol) was added to 2.3 mL of anhydrous trifluoroacetic acid and stirred for 2.5 h at room temperature. Trimethyl orthoformate (0.70 mL, 6.4 mmol) was added all at once and, after five minutes of stirring, the reaction was quenched by pouring it into 23 mL of water. The aqueous phase was extracted with 25% (v/v) isopropyl alcohol in chloroform (6x10 mL) and the combined organic phases were washed with 20 mL of water and 20 mL of saturated aqueous sodium chloride and dried. Evaporation and recrystallization of the residue from methanol/hexane gave 0.310 g (81%) of 16a as a yellow solid: mp 207-209°C (lit.[45] mp 205.5°C, lit.[46] mp 211°C); NMR ($CDCl_3$) δ 1.20 (t, 3H, 3-$CH_2\underline{CH_3}$, J=7.6Hz), 2.00 (s, 3H, 4-CH_3), 2.14 (s, 3H, 3'-CH_3), 2.55 (q, 2H, 3-$\underline{CH_2}CH_3$, J=7.6Hz), 2.61 (t, 2H, 4'-$CH_2\underline{CH_2}CO_2CH_3$, J=7.7Hz), 3.09 (t, 2H, 4'-$\underline{CH_2}CH_2CO_2CH_3$,

J=7.7Hz), 3.68 (s, 3H, OCH_3), 5.99 (s, 1H, meso-H), 9.77 (s, 1H, CHO), 10.69 (br s, 1H, NH), 10.90 (br s, 1H, NH); exact mass calcd for $C_{18}H_{22}N_2O_4$ 330.1580, found m/e 330.1583 (M^+).

3,3'-Dimethyl-4-ethyl-5'-formyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone (16b).[9,45,46] The above procedure was used on the 5'-t-butyloxycarbonylpyrromethenone 15b to give a 76% yield of 16b as a yellow solid: mp 201-203°C (lit.[9] mp 202°C, lit.[45] mp 218-220°C, lit.[46] mp 212°C); NMR ($CDCl_3$) δ1.12 (t, 3H, 4-CH_2CH_3, J=7Hz), 2.13 (s, 6H, 3-CH_3 and 3'-CH_3), 2.45 (q, 2H, 4-CH_2CH_3, J=7.5Hz), 2.60 (t, 2H, 4'-$CH_2CH_2CO_2CH_3$, J=7.6Hz), 3.08 (t, 2H, 4'-$CH_2CH_2CO_2CH_3$, J=7.6Hz), 3.67 (s, 3H, OCH_3), 5.96 (s, 1H, meso-H), 9.74 (s, 1H, CHO), 10.58 (br s, 1H, NH), 10.78 (br s, 1H, NH); exact mass calcd for $C_{18}H_{22}N_2O_4$ 330.1580, found m/e 330.1576 (M^+).

cis-3,4-Dihydro-3',4-dimethyl-3-ethyl-5'-formyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone (18a).[25,26] The 5'-formylpyrromethenone 16a was reduced as described for the tetraethylpyrromethenone 8 above. The product was purified by HPLC to give 18a as a slightly yellow solid in 37% yield: R_t (solvent C, 2.0 mL/min) 34.8 min (the corresponding pyrromethanone and dihydropyrromethanone were not eluted under these conditions); UV/vis λ_{max} (log ε) 350 (4.11), 240 (4.10). The NMR spectral assignments in $CDCl_3$ are found in Table I and the 3-H/4-H coupling constant is found in Table IV. Exact mass calcd for $C_{18}H_{24}N_2O_4$ 332.1736, found m/e 332.1731 (M^+).

cis-3,4-Dihydro-3,3'-dimethyl-4-ethyl-5'-formyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone (18b).[25,26] The above procedure was carried out on pyrromethenone 16b to give a 44% yield of 18b after preparative HPLC purification: R_t (solvent C, 2.0 mL/min) 34.8 min (the corresponding pyrromethanone and dihydropyrromethanone were not eluted under these

conditions); UV/vis λ_{max} (log ε) 354 (4.12), 241 (4.11). The NMR spectral assignments are found in Table I and the 3-H/4-H coupling constant is found in Table IV. Exact mass calcd for $C_{18}H_{24}N_2O_4$ 332.1736, found m/e 332.1738 (M^+).

trans-3,4-Dihydro-3',4-dimethyl-3-ethyl-5'-formyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone (19a).[25,26] The corresponding cis-dihydropyrromethenone 18a (0.100 g, 0.30 mmol) was heated under reflux in 30 mL of 0.5M sodium methoxide in methanol for 2.5 h then cooled, and 15 mL of 1.0M aqueous KH_2PO_4 was added and the methanol was evaporated. The residue was extracted with 25% (v/v) isopropyl alcohol in methanol (6x15mL) and the combined organic phase was washed with 15 mL of water and 15 mL of saturated aqueous NaCl and dried. Evaporation left a residue which was purified by preparative HPLC to give 19a as a slightly yellow solid in 48% yield (48 mg): R_t (solvent C, 2.0 mL/min) 31.3 min (the corresponding pyrromethenone was not eluted under these conditions); UV/vis λ_{max} (log ε) 351 (4.11), 242 (4.09). The NMR spectral assignments in $CDCl_3$ are found in Table I and the 3-H/4-H coupling constant is found in Table IV. Exact mass calcd for $C_{18}H_{24}N_2O_4$ 332.1736, found m/e 332.1727 (M^+).

trans-3,4-Dihydro-3,3'-dimethyl-4-ethyl-5'-formyl-4'-(2-methoxycarbonylethyl)-5(1H)-pyrromethenone (19b).[25,26] The procedure as described for 19a above was used to give a 45% yield of 19b: R_t (solvent C, 2.0 mL/min), 31.3 min (the corresponding pyrromethanone was not eluted under these conditions); UV/vis λ_{max} (log ε) 353 (4.13), 241 (4.10). The NMR spectral assignments in $CDCl_3$ are found in Table I and the 3-H/4-H coupling constant is found in Table IV. Exact mass calcd for $C_{18}H_{24}N_2O_4$ 332.1736, found m/e 332.1726 (M^+).

cis-3,18-Diethyl-1,2,3,19,21,24-hexahydro-2,7,13,17-tetramethyl-1,19-dioxobilin-8,12-dipropionic acid Methyl Ester (20a).[25] This compound was prepared by coupling 18a and 17b as described for dioxobilin 11. The product was purified by preparative HPLC to give 41% of 20a as a blue solid: R_t (solvent D, 2.0 mL/min) 30 min. NMR spectral properties are found in Tables II, III, and IV. Exact mass calcd for $C_{35}H_{44}N_4O_6$ 616.3261, found m/e 616.3270 (M^+).

cis-2,17-Diethyl-1,2,3,19,21,24-hexahydro-3,7,13,18-tetramethyl-1,19-dioxobilin-8,12-dipropionic Acid Methyl Ester (20b)[25] This compound was prepared by coupling 18b and 17a as described for dioxobilin 11. The product was purified by preparative HPLC to give 45% of 20b as a blue solid: R_t (solvent D, 2.0 mL/min) 32 min. NMR spectral properties are found in Tables II, III, and IV. Exact mass calcd for $C_{35}H_{44}N_4O_6$ 616.3261, found m/e 616.3236 (M^+).

trans-3,18-Diethyl-1,2,3,19,21,24-hexahydro-2,7,13,17-tetramethyl-1,19-dioxobilin-8,12-dipropionic Acid Methyl Ester (21a)[26] This compound was prepared by coupling 19a with 17b as described for dioxobilin 11. The product was purified by preparative HPLC to give 58% of 21a as a blue solid: R_t (solvent D, 2.0 mL/min) 25 min; UV/vis λ_{max} (log ε) 583(4.06), 343 (4.45), 272 (4.25). NMR spectral properties are found in Tables II, III, and IV. Exact mass calcd for $C_{35}H_{44}N_4O_6$ 616.3261, found m/e 616.3233 (M^+).

trans-2,17-Diethyl-1,2,3,19,21,24-hexahydro-3,7,13,18-tetramethyl-1,19-dioxobilin-8,12-dipropionic Acid Methyl Ester (21b)[25,26] This compound was prepared by coupling 19b with 17a as described for dioxobilin 11. The product was purified by preparative HPLC to give 36% of 21b as a blue solid: R_t (solvent D, 2.0 mL/min) 25 min. NMR spectral properties are

found in Tables II, III, and IV. Exact mass calcd for $C_{35}H_{44}N_4O_6$ 616.3261, found m/e 616.3247 (M^+).

3,4-Diethyl-1H-pyrrole-2,5-dione(3,4-Diethylmaleimide).[47,48] 3,4-Diethylpyrrole[20,21,49] (10 g, 81 mmol) in 15 mL of pyridine containing 12 mL of 30% aqueous H_2O_2 was heated at reflux for five minutes. Another 3 mL of H_2O_2 was added and heating continued for ten more minutes.[22] The solvent was evaporated and the residue was taken up in 100 mL of $CHCl_3$. The organic phase was washed with 30 mL of 1M NaOH, dried, and cautiously evaporated, leaving the crude pyrrolinone (10.0 g, 97%). This crude product was dissolved in 175 mL of acetone, a solution of 17.1 g (171 mmol) of CrO_3 in 170 mL of 2M H_2SO_4 was added, and the reaction was heated at 50°C for one hour. After cooling the solution to room temperature, the acetone was evaporated and the aqueous residue was extracted with EtOAc (7x100 mL). The combined organic phase was washed with 100 mL of saturated aqueous NaCl, dried, and evaporated, and the residue was purified by silica gel chromatography using isooctane/ether, 1/1. to give 5.8 g (47%) of 3,4-diethylmaleimide: mp 69-70°C (lit.[47] mp 68°C, lit.[48] mp 68-70°C); R_t (GC) 3.30 min.

cis-3,4-Diethylpyrrolidine-2,5-dione(cis-3,4-Diethylsuccinimide). To 180 mg (0.85 mmol) of the above 3,4-diethyl-1H-pyrrole-2,5-dione in 10 mL of ethyl acetate was added 18 mg PtO_2. Hydrogen at 50 psi was applied for 6 hours,[31] the solution was filtered through celite, and the filtrate was evaporated to give 132 mg (100%) of residue. GC analysis showed a 95/5 mixture of cis/trans isomers which may be due to epimerization during analysis, as the cis compound was never found completely free of the trans by GC analysis.[50] The product was recrystallized from MeOH/H_2O and sublimed at 70°C/1 torr to give a 98/2 ratio of cis/trans 3,4-diethyl-

succinimide as white crystals: mp 87-89°C; R_t (GC) cis 5.16 min, trans 4.17 min; UV λ_{max} (log ε) 246 (1.93), 221 (2.28); IR (KBr) 3130, 1670, 1340 cm^{-1}; NMR ($CDCl_3$, 90 MHz) δ 1.02 (t, 6H, $2xCH_2\underline{CH_3}$, J=7.5 Hz), 1.54-1.77 (m, 4H, $2xC\underline{H_2}CH_3$), 2.70-2.83 (m, 2H, 2xCH), 8.32 (br s, 1H, NH). Anal. Calcd for $C_8H_{13}NO_2$: C, 61.9; H, 8.4; N, 9.0. Found: C, 61.9; H, 8.4; N, 8.8.

trans-3,4-Diethylpyrrolidine-2,5-dione(trans-3,4-Diethylsuccinimide). To the above cis-3,4-diethylpyrrolidine-2,5-dione (153 mg, 1.00 mmol) was added a solution of 2.24 g (2.00 mmol) of potassium t-butoxide in 60 mL of t-butanol. After refluxing for two hours, the solution was cooled and the t-butyl alcohol was evaporated. Aqueous 1M H_3PO_4 (20 mL) was added and the aqueous phase was extracted with CH_2Cl_2 (5x10 mL) which was dried and evaporated to give 143 mg (93%) of trans-3,4-diethylsuccinimide, mp 59-60°C R_t (GC) 4.17 min; UV λ_{max} (log ε) 244 (1.64), 220 (2.22); IR (KBr) 3140, 1670, 1175 cm^{-1}; NMR ($CDCl_3$, 90 MHz) δ 1.00 (t, 6H, $2xCH_2\underline{CH_3}$, J=7.5 Hz), 1.60-1.90 (m, 4H, $2xC\underline{H_2}CH_3$), 2.42-2.53 (m, 2H, 2xCH), 8.80 (br s, 1H, NH). Anal. Calcd for $C_8H_{13}NO_2$: C, 61.9; H, 8.4; N, 9.0. Found: C, 62.2; H, 8.4; N, 8.9.

3-Ethyl-4-methyl-1H-pyrrole-2,5-dione(3-Ethyl-4-methylmaleimide).[15,47,51] This compound was prepared from 3-ethyl-4-methylpyrrole[20,21,52] as described for the 3,4-diethyl-1H-pyrrole-2,5-dione above in 45% yield: mp 66-68°C (lit.[15] mp 66-67°C, lit.[47] mp 67-68°C, lit.[51] mp 68°C); R_f (TLC) 0.89.

cis-3-Ethyl-4-methylpyrrolidine-2,5-dione(cis-3-Ethyl-4-methylsuccinimide).[15,31] This compound was prepared as described[15] in quantitative yield: mp 48-50°C (lit.[31] mp 50°C); R_f (TLC) cis 0.20, trans 0.30; R_t (GC) cis 1.9 min, trans 1.3 min.[50]

trans-3-Ethyl-4-methylpyrrolidine-2,5-dione(trans-3-Ethyl-4-methyl-

succinimide).[31] This compound was prepared in an analogous manner to the trans-3,4-diethyl-pyrrolidine-2,5-dione above in 64% yield. mp 57-59°C (lit.[31] mp 60-61°C); R_f (TLC) 0.30; R_t (GC) 1.3 min.

Bile Pigment Oxidative Degradations.[32] To a solution of 250 mg of $Na_2Cr_2O_7$ in 6 mL of H_2O was added 10 mg of the bile pigment in 2 mL of THF. The oxidation was allowed to proceed at room temperature for 2 h, then 10 mL of H_2O was added and the mixture was extracted with CH_2Cl_2 (5x10 mL). The combined organic phase was dried and evaporated. The residue of oxidation products from the octaethyldioxotetrapyrrole was analyzed by GC. In addition to the succinimide and maleimide products, an incomplete degradation product appears with a R_t 8.30 min. The oxidation products from the bile pigments 20a, 20b, 21a, and 21b were analyzed by TLC.[33,34,37] In addition to the succinimide and 3-ethyl-4-methylmaleimide, 3-(2-methoxycarbonylethyl)-4-methyl-1H-pyrrole-2,5-dione (hematinic acid methyl ester) was seen at R_f 0.50.

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Part Two

Bilin Attachment Sites in the α and β Subunits of B-Phycoerythrin: Structural Studies on the Singly-Linked Phycoerythrobilins

Abstract

Five phycoerythrobilin peptides were prepared from Porphyridium cruentum B-phycoerythrin by a combination of tryptic and thermolysin digestion and isolated under mild conditions without alteration in the spectroscopic properties of the bilin. High resolution ^1H NMR spectral analysis of four of the chromopeptides has provided direct proof that all of the singly-linked phycoerythrobilins contain a thioether bond to the 3'-position of ring A. The present study confirms the major structural features of the phycoerythrobilin chromophore and indicates the probable stereochemical assignments for all of the chiral centers for every singly-linked phycoerythrobilin on the α and β subunits of B-phycoerythrin.

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] B-Phycoerythrin from the unicellular red alga Porphyridium cruentum, the source of the bilin peptides we have investigated, has the polypeptide composition $(\alpha\beta)_6\gamma$, with α and β subunits of 17,000 daltons and γ subunit of 30,000 daltons.[3-5] The α subunit contains two phycoerythrobilins, the β subunit contains three phycoerythrobilins, and the γ subunit contains two phycoerythrobilins and two phycourobilins as determined by spectroscopic studies of the isolated subunits.[4] It is important to appreciate, then, that isolation and characterization of 9 distinct peptides is required to fully establish the structure of all of the polypeptide linked bilins.

All of the phycoerythrobilins on the α and β subunits are accounted for in five chromopeptides derived from trypsin digestion of B-phycoerythrin.[6] One of these chromopeptides, β-3T is the subject of part three of this thesis. Herein, we report a detailed structural study of the four remaining phycoerythropeptides obtained upon trypsin cleavage of the α and β subunits of B-phycoerythrin.

B-Phycoerythrin was isolated and purified from Porphyridium cruentum by a combination of ammonium sulfate precipitation, DEAE-cellulose chromatography, and gel filtration.[4] Trypsin digestion was carried out on the intact $(\alpha\beta)_6\gamma$ complex of purified B-phycoerythrin.[6] From work on the isolated subunits, the origin of the chromopeptides could be ascertained from the Sephadex elution profiles. Thus, the α-1, α-2, and β-1 chromopeptides were isolated. The β-2 chromopeptide

from trypsin cleavage was further digested with thermolysin and fractionated on Sephadex to give the β-2T chromopeptide investigated here.

Amino acid analyses and sequential Edman degradations served to establish the amino acid sequences of the chromopeptides as follows:

α-1: Cys-Tyr-Arg
PEB

α-2: Leu-Cys-Val-Pro-Arg
PEB

β-1; Met-Ala-Ala-Cys-Leu-Arg
PEB

β-2T: Phe-Ala-Ala-Gly-Asp-Cys-Thr-Ser
PEB

β-3T: Ala-Ser-Cys—PEB—Met-Ile-Cys-Glu-Asn-Pro-Gly

where the designations α and β refer to the subunit from which the peptide was derived, the number refers to the relative N-terminal position of the phycoerythrobilin (PEB) peptide fragment found, and the T indicates a thermolysin cleavage in addition to trypsin cleavage. In addition to the sequence data, the Edman degradations also indicated the attachment site on the peptide of the phycoerythrobilin. The loss of the red color due to the tetrapyrrole was always concomitant with the loss of the indicated cysteine residues suggesting a thioether linkage between the

peptide and the chromophore. The absence of any change in the UV/vis spectra shows that no alteration in the chromophore structure has occurred during the enzymatic cleavages.

The chromophore structure and peptide linkage of the β-3T chromopeptide is discussed in part three of this thesis. The remaining four peptides were further purified by HPLC (Figures 1 and 2). Reverse-phase chromatography was done using an isocratic sodium phosphate buffer-acetonitrile mixture on an Ultrasphere ODS column. Our studies indicated that a high buffer salt concentration and isocratic conditions were essential for the requisite resolution. The α-2 and the β-1 chromopeptides were incompletely separated by gel filtration, but reverse phase chromatography yielded very pure fractions for NMR analysis. The β-2T chromopeptide contained a major impurity (20%) of unknown origin which was removed upon HPLC purification. The chromatography elution profiles at 200 nm showed that the isolated chromopeptides were the major peptide containing fractions. Each HPLC fraction was desalted by adsorption onto a LiChrosorb C-18 column equilibrated with 10 mM aqueous TFA and eluted with a mobile phase of 50% acetonitrile and 50% aqueous 10 mM TFA. The use of this volatile acid allowed us 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 fractions. The UV/vis spectra conclude the integrity of the chromophore at this final purification stage as well as provide evidence for the identity of the tetrapyrrole in all four chromopeptides (Figures 3 and 4).

Amino acid sequencing data on the purified α-1 chromopeptide

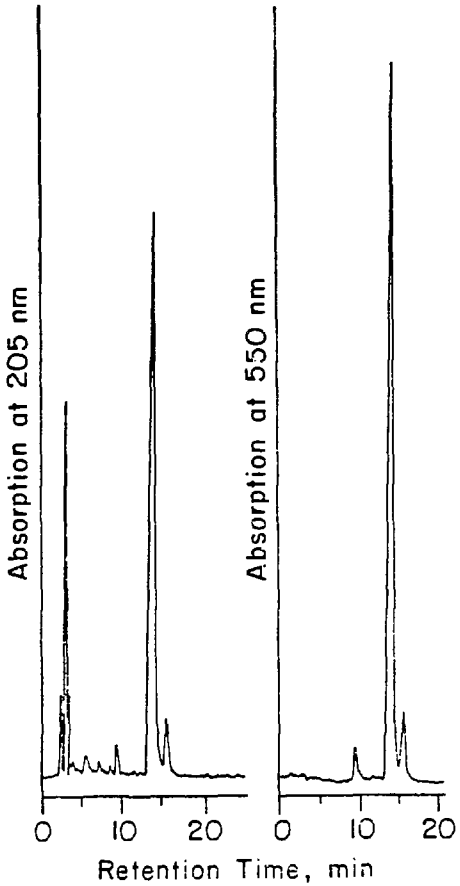


Figure 1. High pressure liquid chromatography of the α-1 phycoerythrobilitripeptide 8 on an Ultrasphere ODS column (4.6x250 mm, 5 μm) using 22.5% CH_3CN and 77.5% 0.10 M NaH_2PO_4 buffer, pH 2.1 with a 1.0 mL/min flow rate.

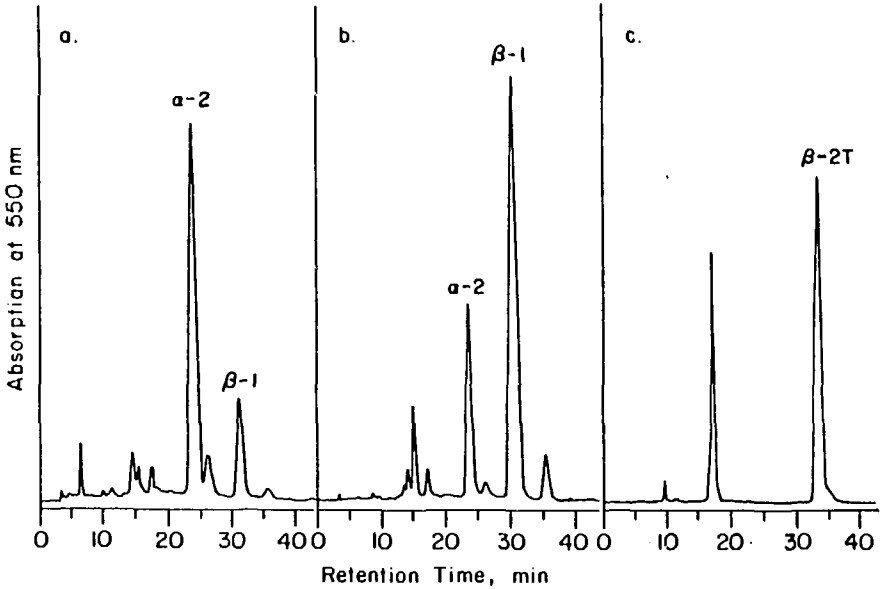


Figure 2. High pressure liquid chromatography of phycoerythrobilipeptides (a) α-2, (b) β-1, (c) β-2T on an Ultrasphere ODS column (4.6x250 mm, 5 μm) using 22.5% CH_3CN and 77.5% 0.10 M NaH_2PO_4 buffer, pH 2.1 with a 1.0 mL/min flow rate and detection at 550 nm.

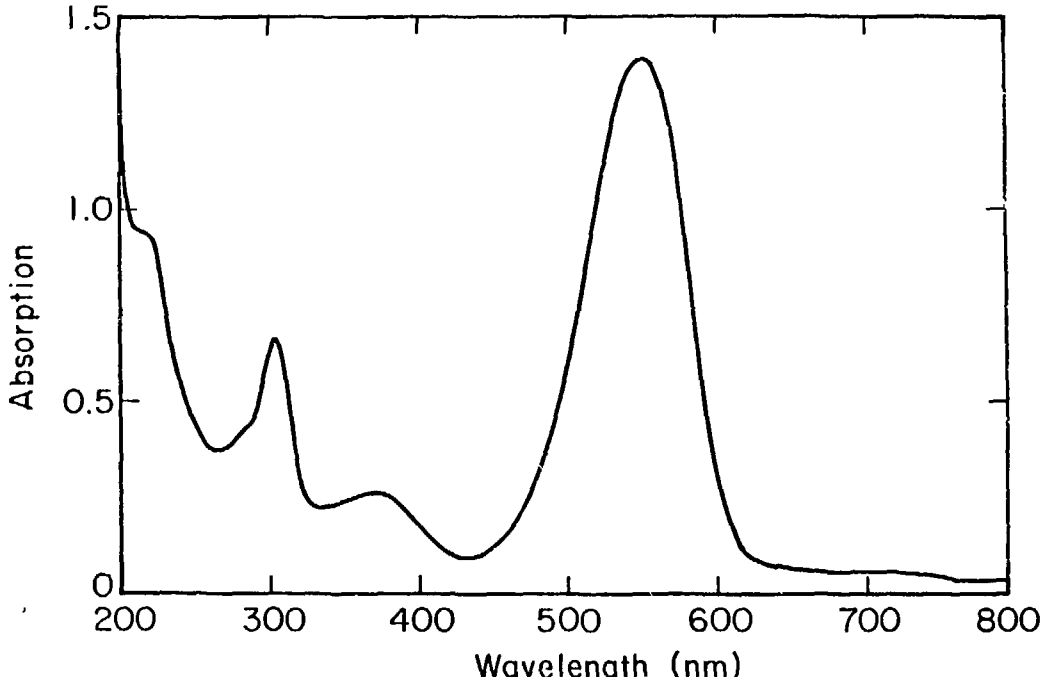


Figure 3. The absorption spectrum in 10 mM aqueous TFA of the HPLC purified α-1 phycoerythrobilitripeptide 8 (c=30.0 μM).

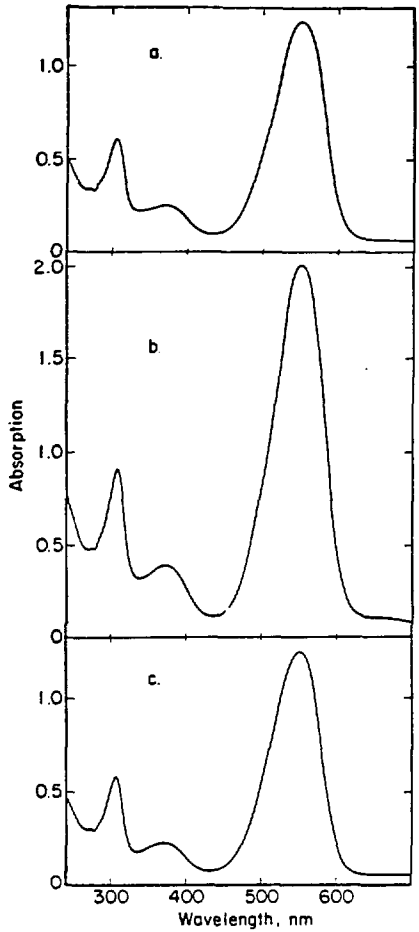


Figure 4. The absorption spectra in 10 mM aqueous TFA of the HPLC purified phycoerythrobilipeptides (a) α-2, c=26.2 μM; (b) β-1, c=42.2 μM; (c) β-2T, c=26.4 μM.

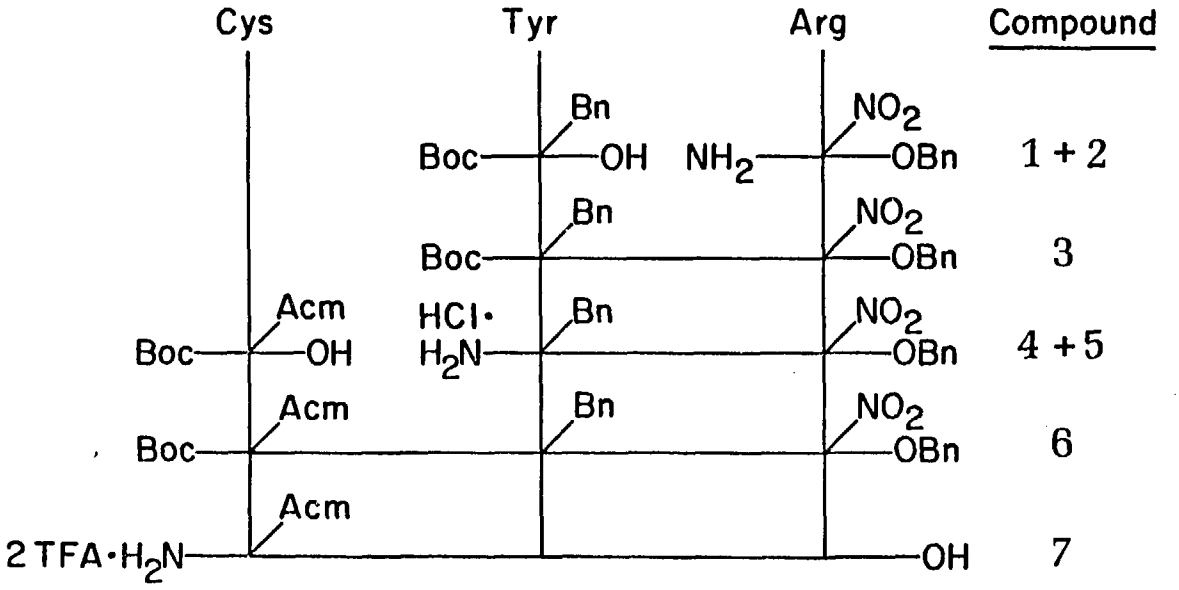
established that the sequence was Cys-Tyr-Arg. In order to facilitate NMR analysis and to see what effects might be caused by chromophore-peptide interactions, the tripeptide was synthesized (Chart 1). The synthesis was achieved using a mixed anhydride method on fully protected amino acid residues to satisfy the purity requirements of the high resolution NMR study.[7]

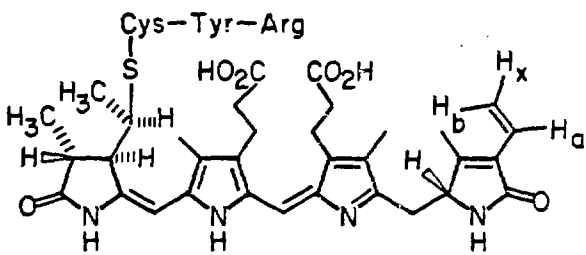
The amino acid derivatives were synthesized according to known methods.[8-12] The couplings were done in such a manner as to avoid racemization which would result in diastereomeric mixtures complicating the NMR analysis, and the protected di- and tri-peptides were purified by HPLC. Final deprotection with anhydrous hydrogen fluoride removed all blocking groups except the acetamidomethyl, which was chosen to avoid any complicating thiol oxidation.[8,13] 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.

The NMR spectra were taken in 10 mM TFA in D_2O. Although we considered that aggregation might be a problem, the circular dichroism spectra showed no concentration effects in aqueous buffer up to the concentration at which the NMR spectra were recorded.[14] Previous results with phycocyanobilin-bearing peptides suggested that pyridine might be a better NMR solvent,[14,15] but isomerization of the phycoerythrin to a urobilin-type structure prevented meaningful NMR analysis in pyridine.

The 360 MHz NMR spectra of the chromopeptide α-1 (8) and the corresponding synthetic tripeptide 7 are shown in Figure 5. In contrast to what occurs with phycocyanobilipeptides in D_2O, very little broadening

Chart I. Repetitive Mixed Anhydride Synthesis of Tripeptide 7.





8

α-1 Phycoerythrobilitripeptide

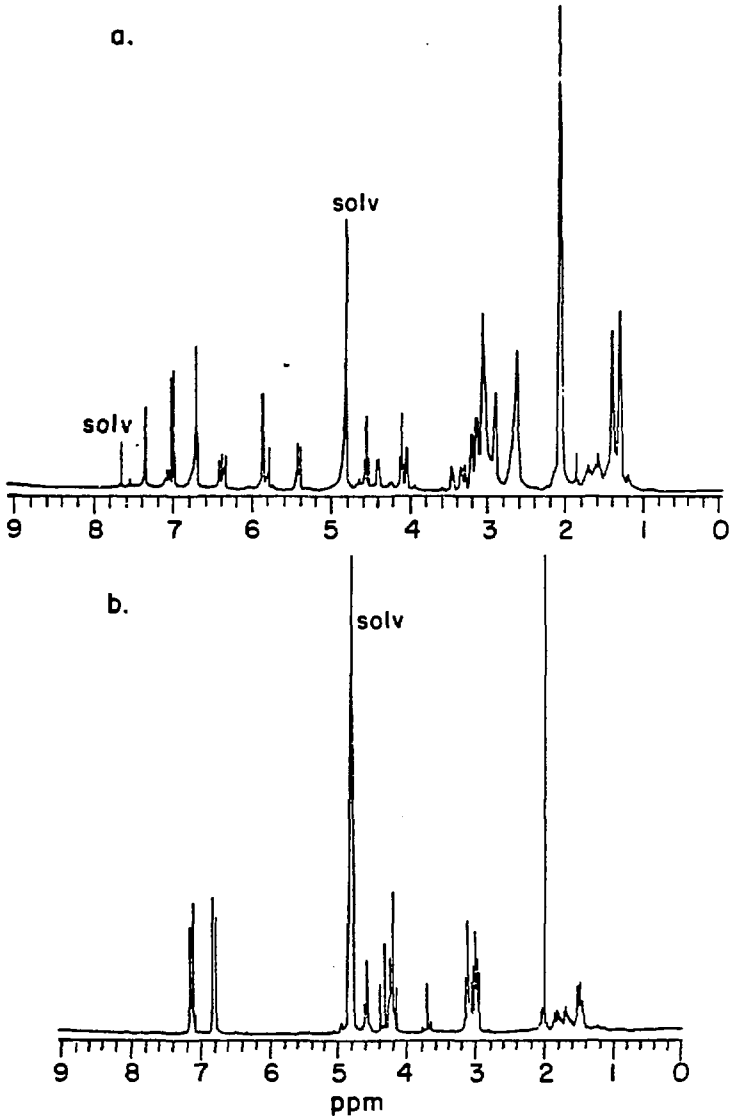


Figure 5. (a) The 360 MHz ^{1}H NMR spectrum of the α-l phycoerythrobilitripeptide 8 (c=4.4 mM) in 10 mM TFA in D_2O at 25°C; (b) The 250 MHz ^{1}H NMR spectrum of synthetic tripeptide 7 (c=5.0 mM) in 10 mM TFA in D_2O at 25°C.

of the resonances due to the chromophore is seen.[14] The corresponding chemical shifts of the amino acid residues for chromopeptide α-1 and the synthetic tripeptide 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 or hydrophobic interactions 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 are available.[16]

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 in the α-1 chromopeptide.

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[14] and a previous report on the phycoerythrobilin chromophore.[17] The lability of certain protons having been documented, we interpret our results as an indication that no isomerization has occurred during the mild conditions

Table I. ^1H NMR Assignments for the Peptide Moiety of the α-1 Phycoerythrobilipeptide 8 and the Synthetic Peptide 7 in 10 mM TFA in D_2O at 25°C.

8	7	assignment
6.98 (d, 8.4)	7.11 (d, 8.5)	Tyr 2,6 Ar-H
6.71 (d, 8.4)	6.78 (d, 8.5)	Tyr 3,5 Ar-H
4.55 (t, 7.2)	4.54 (t, 7.7)	Cys α-CH
4.10 (t, 6.3)	4.20 (t, 4.5)	Tyr α-CH
4.03 (dd, 5.1, 8.5)	4.23 (t, 5.0)	Arg α-CH
3.2 (m)	3.1 (t, 6.9)	Arg δ-CH_2
3.14 (m)	3.04 (d,5.6); 2.99 (d, 7.2)	Tyr β-CH_2
2.90 (d, 7.2)	2.98 (d, 7.7)	Cys β-CH_2
1.55-1.75 (m)	1.47-1.77 (m)	Arg β,γ-CH_2

Table II. ^{1}H NMR Assignments of the Bilin Moiety of the α-1 Phycoerythrobilitripeptide 8 in 10 mM TFA in D_2O and the Free Pigment 9 in $CDCl_3$ at 25°C.

8	9[a]	assignment
7.34 (s)	6.68 (s)	10-H
6.37 (dd, 11.7, 17.9)	6.40 (dd, 12,18)	18-H_a
5.86 (s)	5.84 (s)	5-H
5.82 (dd, 1.4, 17.9)	6.17 (dd, 2, 18)	18-H_b
5.40 (dd, 1.4, 11.7)	5.36 (dd, 2, 12)	18-H_x
4.40 (dd, 4.2, 7.9)	4.28 (m)	16-H
3.44 (m, 3.0, 7.0)	b	3'-H
3.30 (dd, 4.2, 14.1)	2.8-3.0 (m)	15-H
3.10 (m, 3.0, 3.5)[c]	b	3-H
3.07 (m)	2.8-3.0 (m)	8,12-$CH_2CH_2CO_2H$
2.93 (m, 7.9, 14.1)[c]	2.8-3.0 (m)	15-H
2.69 (m, 3.5, 7.4)	b	2-H
2.64 (m)	2.4-2.7 (m)	8.12-$CH_2CH_2CO_2H$
2.08 (s)	2.03 (s)	
2.06 (s)	2.03 (s)	7, 13, 17-CH_3
2.055 (s)	1.98 (s)	
1.39 (d, 7.0)	b	3'-CH_3
1.28 (d, 7.4)	1.42 (d, 7.0)[b]	2-CH_3

[a] From reference 20.

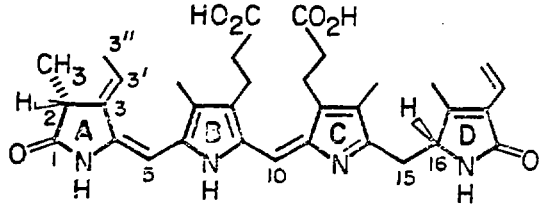
[b] This chemical shift is not strictly analogous or no longer exists due to the presence of the ethylidene.

[c] Overlapping resonances are seen, but coupling constants are abstracted from the coupled protons.

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 9 despite the solvent differences.[18,19,20]

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 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. Perhaps a more plausible explanation, however, would be that lypophilic interactions



9

Phycoerythrobilin

between the two end rings of the tetrapyrrole itself are the cause of the rotational constraints.

With the exception of those of ring A, the remaining tetrapyrrole substituents are assigned by comparison with phycoerythrobilin NMR data previously published.[18,19,20] The propionic acid methylenes appear as unresolved multiplets centered at 3.07 ppm for the β- and 2.64 ppm for the α-methylene groups. The three methyl groups at C-7, C-13, and C-17 are not completely resolved in our spectra. A resolution enchancement 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.[14] Irradiation at this position causes the C-2 proton at 2.69 ppm (Figure 6) 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 β-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 6). 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

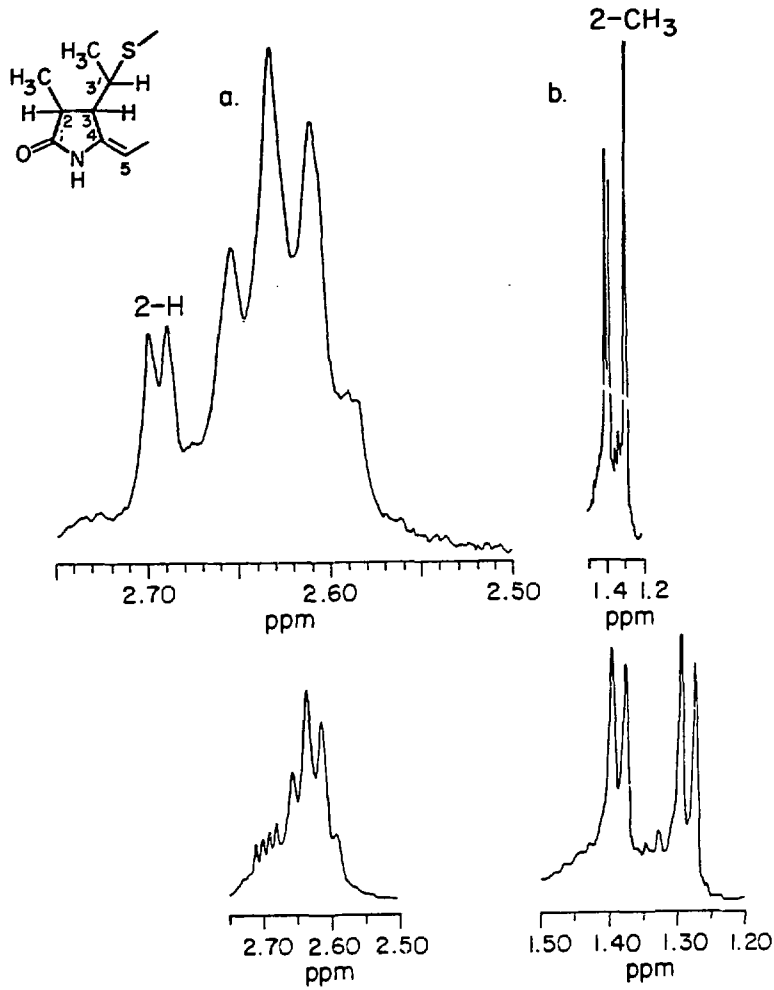


Figure 6. The partial 360 MHz ^1H NMR spectrum of the α-1 phyco erythrobilitripeptide 8 in 10 mM TFA in D_2O at 25°C (a) after irradiation of the doublet at 1.28 ppm; (b) after irradiation of the multiplet at 2.69 ppm.

proton initially appears as a multiplet with an apparent four line pattern (Figure 7). 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 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 provided the first direct proof of the presence and position of the thioether linkage in a phycoerythrobilipeptide.

The 500 MHz ^{1}H NMR spectra of chromopeptides α-2, β-1, and β-2T are shown in Figure 8. Again, the aqueous acid solvent provides excellent stability for the chromophore and avoids any problems associated with aggregation or with interconverting prototropic isomers of the tetrapyrrole. The chemical shift and coupling constant assignments for the bilin portion of all four chromopeptides are shown in Table III. The correspondence between the α-1, α-2, and β-1 chromopeptides is excellent. The largest chemical shift discrepancy is a 0.12 ppm difference in the 3-H, while all other chemical shifts agree within 0.04 ppm. Likewise, all coupling constants agree well, with the largest difference (1.1 Hz) seen in the $J_{15,16}$. Clearly, no gross structural differences are seen. The presence of the C-18 vinyl group and the saturation at the C-15/C-16 position of the α-2 and β-1 chromopeptides can be inferred by analogy to the detailed α-1 study. Decoupling studies confirm these assignments as well as allow assignments for the 2-CH_3, 3'-CH_3, 2-H, 3-H, and 3'-H. The exact

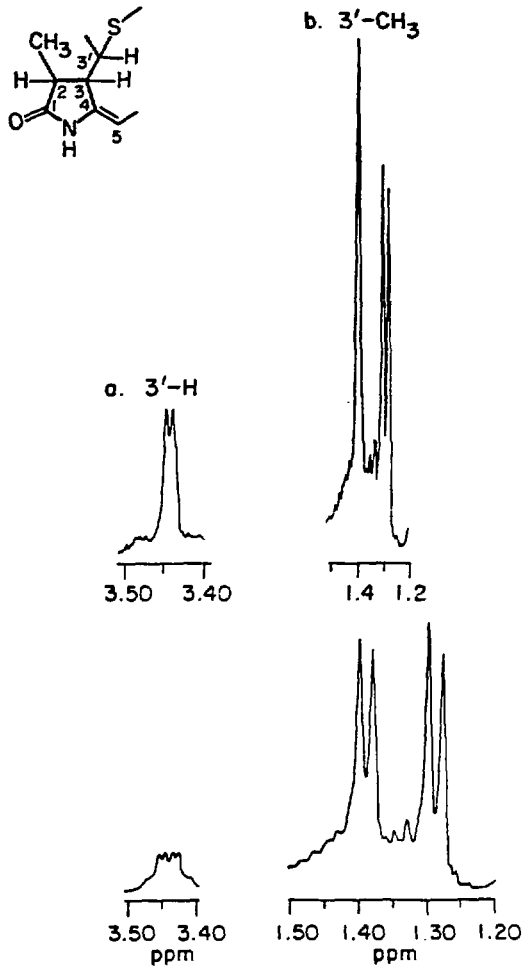


Figure 7. The partial 360 MHz ^1H NMR spectrum of the α-1 phycoerythrobilitripeptide 8 in 10 mM TFA in D_2O at 25°C (a) after irradiation of the doublet at 1.39 ppm; (b) after irradiation of the multiplet at 3.44 ppm.

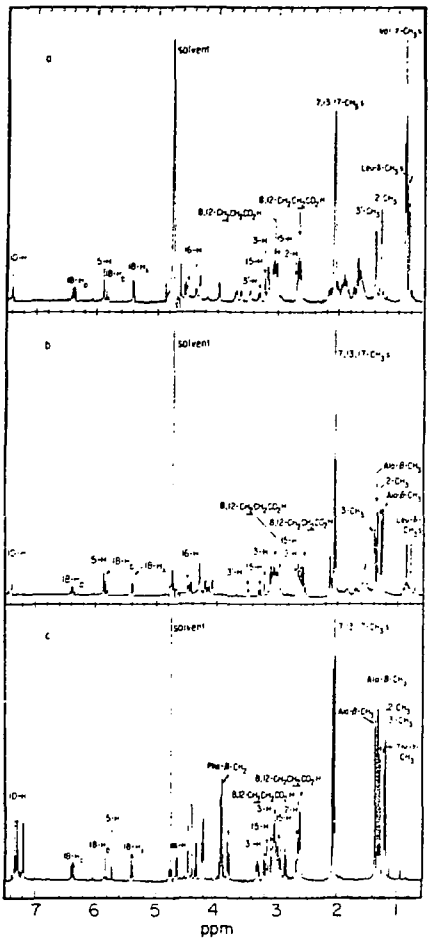


Figure 8. The 500 MHz ^{1}H NMR spectra of the phycoerythrobilipeptides (a) α-2, c=5.2 mM; (b) β-1, c=4.2 mM; (c) β-2T, c=2.6 mM in 10 mM TFA in D_2O at 25°C.

Table III. 500 MHz ^{1}H NMR Assignments for the Bilin Portion of the Phycoerythrobilipeptides α-1 (4.4 mM), α-2 (5.2 mM), β-1 (4.2 mM), and β-2T (2.6 mM) in 10 mM TFA in D_2O at 25°C.

α-1[a]	α-2	β-1	β-2T	Assignment
7.34 (s)	7.39 (s)	7.39 (s)	7.34 (s)	10-H
6.37 (dd, 11.7, 17.9)	6.38 (dd, 11.6, 17.8)	6.39 (dd, 11.6, 18.0)	6.37 (dd, 11.7, 18.0)	18-H_a
5.86 (s)	5.89 (s)	5.89 (s)	5.73 (s)	5-H
5.82 (dd, 1.4, 17.9)	5.83 (dd, 1.5, 17.8)	5.83 (dd, 1.5, 18.0)	5.82 (dd, 1.7, 18.0)	18-H_b
5.40 (dd, 1.4, 11.7)	5.39 (dd, 1.5, 11.6)	5.40 (dd,1.5, 11.6)	5.39 (dd, 1.7, 11.7)	18-H_x
4.40 (dd, 4.2, 7.9)	4.50 (dd, 3.6, 8.5)	4.49 (dd, 4.7, 8.0)	4.65 (dd, 6.6, 7.4)	16-H
3.44 (m, 3.0, 7.0)	3.45 (m, 3.2, 7.0)	3.48 (m, 3.0, 6.9)	3.32 (m, 4.0, 7.0)	3'-H
3.30 (dd, 4.2, 14.1)	3.30 (dd, 3.6, 13.6)	3.30 (dd, 4.7, 14.5)	3.13 (dd, 6.6, 13.9)	15-H
3.10 (m, 3.0, 3.5)	3.22 (m, 3.2, 3.8)	3.22 (m, 3.0, 3.6)	3.04 (m, 3.4, 4.0)	3-H
3.07 (m)	3.05 (m)	3.04 (m)	3.03 (m)	8,12-$CH_2CH_2CO_2H$
2.93 (m, 7.9, 14.1)	3.00 (m, 8.5, 13.6)	3.02 (m, 8.0, 14.5)	2.97 (m, 7.4, 13.9)	15-H
2.69 (m, 3.5, 7.4)	2.68 (m, 3.8, 7.4)	2.67 (m, 3.6, 7.4)	2.67 (m, 3.4, 7.4)	2-H
2.64 (m)	2.63 (2xt, 7.2, 7.6)	2.63 (2xt, 7.3, 7.6)	2.62 (2xt, 7.2, 7.4)	8,12-$CH_2CH_2CO_2H$
2.08 (s)	2.08 (s)	2.06 (s)	2.07 (s)	7,13,17-CH_3
2.06 (s)	2.07 (s)	2.055 (s)	2.05 (s)	
2.055 (s)	2.05 (s)	2.04 (s)	2.04 (s)	
1.39 (d, 7.0)	1.37 (d, 7.0)	1.39 (d, 6.9)	1.21 (d, 7.0)	3'-CH_3
1.28 (d, 7.4)	1.28 (d, 7.4)	1.28 (d, 7.4)	1.28 (d, 7.4)	2-CH_3

[a] These values were recorded at 360 MHz.

correspondence of the chemical shifts of the 2-CH_3, the 3'-CH_3, and the C-18 vinyl protons, in conjunction with the α-1 and synthetic studies, conclusively proves the thioether linkage and strongly suggests its presence at the A ring 3'-position.[20] The absence of any anomolous chemical shift differences in either the bilin or the proton resonances, in conjunction with the Edman degradation results, precludes any possible second covalent linkage between the peptides and the tetrapyrrole.

The same gross structural details are seen in the β-2T chromopeptide. The UV/vis spectrum and the ^1H NMR chemical shifts of the C-18 vinyl group and the 2-CH_3 evidence the A ring peptide linkage. The 3'-CH_3 doublet proves the thioether attachment site at the C-3' position. However, we see chemical shift differences in the 3'-CH_3, 3-H, 3'-H, 5-H, 15-H, and 16-H resonances (Table III). In addition, the β-CH_2 resonances at 3.75-4.0 ppm are downfield from those expected for phenylalanine.[21] The chemical shift differences are in the range of 0.15 ppm and are accompanied by some coupling constant differences in the 1-2 Hz range. The most plausible explanation is that the phenylalanine, being four amino acid residues removed from the cysteine attachment site can coil to interact with the tetrapyrrole. As a result, the 3-H, 3'-H, and 3'-CH_3 sit in the shielding region of the phenylalanine aromatic ring and are shifted upfield of their normal positions. The β-CH_2 position now lies in the deshielding region of the tetrapyrrole causing the observed shifts. This interaction must also slightly distort the tetrapyrrole conformation. The 5-H shows an upfield shift and now shows a slight (0.8 Hz) allylic coupling to the 3-H. In addition, the conformationally sensitive 15-H and 16-H show chemical shift and coupling constant distortions.

In his synthetic studies, Gossauer has shown that the absolute

stereochemistry at C-16 has a dominant affect on the chiroptic properties of the phycoerythrobilin chromophore.[20] That is, the R configuration at C-16 causes a large positive Cotton effect in the long wavelength region of the circular dichroism spectrum. The CD spectra of the four singly-linked phycoerythropeptides are shown in Figure 9. All of the chromopeptides show the large positive Cotton effect in the 585 nm region concluding, for the first time, the R configuration at C-16 for each of the singly-linked phycoerythrobilins in B-phycoerythrin's α- and β-subunits.

The absolute stereochemistry at C-2 has been determined for isolated phycoerythrobilin.[20] Synthetic C-2(R) and C-2(S) phycoerythrobilin dimethyl esters show very slight differences in the short wavelength region of their CD spectra with the C-2(R) spectrum corresponding to that found for the tetrapyrrole isolated from C-phycoerythrin.[20] Chromic acid oxidation of these materials allows the isolation of 2-(E)-ethylidene-3(R)-methylsuccinimide and 2-(E)-ethylidene-3(S)-methylsuccinimide from the corresponding tetrapyrroles. The oxidation of phycoerythrobilin dimethyl ester isolated from C-phycoerythrin yields 2-(E)-ethylidene-3(R)-methylsuccinimide, suggesting the R configuration at C-2.[20] The presence of more than one phycoerythrobilin in these large protein complexes as well as the finding of some C-2 epimerization during the isolation of phycoerythrobilin dimethyl ester from natural sources casts some doubt on the utility of these data.[20]

The CD spectra of chromopeptides α-1 and β-2T show a decidedly different pattern in the 300 nm region than that seen for α-2 and β-1. However, the α-1 and β-2T chromopeptides contain aromatic amino acid residues whose absorption properties may influence the inter-

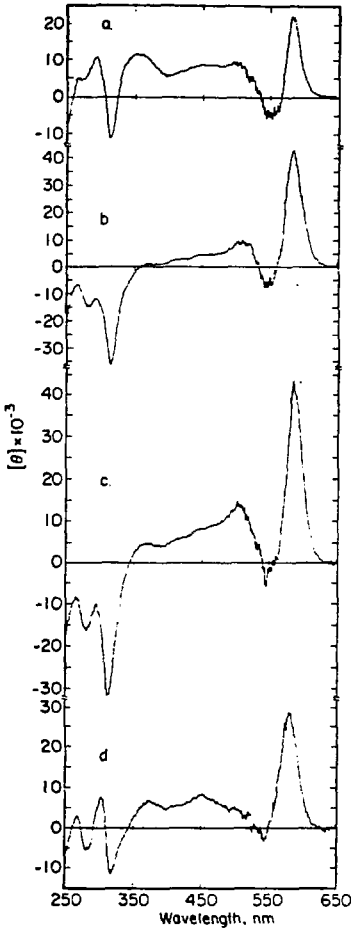


Figure 9. The circular dichroism spectra of the phycoerythrobilipeptides (a) α-1, c=54.2 μM; (b) α-2, c=39.5 μM; (c) β-1, c=43.6 μM; (d) β-2T, c=29.5 μM in 10 mM aqueous TFA at 25°C.

pretation of these differences. Therefore, our data neither supports nor refutes any C-2 absolute stereochemical assignments. By convention we have chosen to represent our structures as having the C-2(R) configuration, with the reservation that any stereochemical proof originate from a single chromophore molecule such as those studied here.[22]

The assignment of the C-3 to C-3' relative stereochemistry is based on the antiperiplanar elimination of the proposed sulfone group under oxidative degradation conditions of C-phycoerythrin to give the (E)-ethylidene succinimide.[23,24] A detailed analysis of further evidence on this question casts doubts on the validity of any conclusions based on other data.[22] Unfortunately, the CD and NMR results presented do not bear on this point and the C-3(R)/C-3'(R) stereochemical relationship must be adopted until further evidence is presented in proof.

Our study on the C-2/C-3 relative stereochemistry demonstrates that the C-2/C-3 coupling constant for cis-3,18-diethyl-1,2,3,19,21,24-hexahydro-2,7,13,17-tetramethyl-1,19-dioxobilin-dipropionic acid methyl ester is 8.5 Hz while the trans compound exhibits a 5.4 Hz coupling constant (see part one of this thesis). These compounds represent the first evidence for a trans dihydro A ring in the phycocyanin series of bile pigments.[23] The corresponding A ring coupling constants in the phycoerythrochromopeptides (α-1: 3.5 Hz, α-2: 3.8 Hz, β-1: 3.6 Hz, β-2T: 3.4 Hz) are all found in the low, trans region. As the stereochemistry at C-16 has a profound effect on the spectroscopic properties of the phycoerythrobilin chromophore not found in that of phycocyanobilin, these data are suggestive, not conclusive, of the A ring relative stereochemistry. For the first time, then, we are able to assign the probable stereochemistry of C-2(R), C-3(R), C-3'(R), and C-16(R) for each of the

individual, singly-linked tetrapyrroles on the α- and β-subunits of B-phycoerythrin.

Experimental Section

General Methods. 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 1-7 were taken at 250 MHz and chemical shifts are expressed in ppm relative to an internal TMS standard for $CDCl_3$ and relative to an external TSP standard for D_2O. The NMR spectra of the chromopeptides were taken on the University of California, Davis, 360 MHz or 500 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. UV/vis spectra were recorded on a Cary Model 219 spectrophotometer. Circular dichroism spectra were recorded on a Jasco J-500-C spectropolarimeter equipped with a Jasco DP-500N data processor. 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 3Å sieves. Hydrogen fluoride was distilled from CoF_3 immediately before use.

Preparation of B-Phycoerythrin. The unicellular red alga Porphyridium cruentum strain B was obtained from the Marine Biology Research Division, Scripps Institution of Oceanography, La Jolla, California, and the cells were cultured as described.[4] 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° 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 phoshate, 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 x 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 of Phycoerythrin Peptides α-1, α-2, β-1, and β-2T.

The conditions employed for isolation and amino acid sequence studies of chromopeptides α-1, α-2, β-1, and β-2T were those described by Lundell et al.[6] For each chromopeptide, the eluate from the SP-Sephedex column was adsorbed onto a column of LiChrosorb C-18, washed extensively with 10 mM formic acid, and then eluted with 60% acetonitrile/40% 10 mM formic acid. The acetonitrile was removed under a stream of N_2 and HPLC analysis was done using a 4.6x250 mm Ultrasphere ODS (5 μm) column eluting with 22.5% CH_3CN and 77.5% of a 0.10M NaH_2PO_4 buffer adjusted to pH 2.1 with concentrated H_3PO_4 (Figures 1 and 2). A flow rate of 1.0

mL/min was used with detection at both 200 nm and 550 nm. Preparative HPLC was carried out on each peptide fraction using a 10x250 mm Ultrasphere ODS (5 μm) column at a flow rate of 2.0 mL/min and a solvent composition of 22% CH_3CN and 78% of 0.10 M NaH_2PO_4, pH 2.1 aqueous buffer. The purification was done by multiple injections of 0.5 μmol/ injection with detection at 550 nm.

After the acetonitrile was removed by rotary evaporation at 5 torr and room temperature, the remaining aqueous solution for each chromopeptide was applied to a LiChrosorb C-18 column (10 μm, 3.2 x 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.

Sample Preparation for UV/vis and CD Spectral Analysis. Each chromopeptide fraction was concentrated to 0.5 mL by rotary evaporation and diluted with 10 mM aqueous TFA to appropriate concentrations for UV/vis and CD spectral analyses (Figures 3,4, and 9).

Sample Preparation for NMR Spectral Analysis. Each chromopeptide fraction in 10 mM aqueous TFA was concentrated to 0.5 mL by rotary evaporation and diluted with 10 mL of 10 mM trifluoroacetic acid in 99.8% D_2O (made by adding 50 μmol of trifluoroacetic anhydride to 10 mL of D_2O). The solution was concentrated to 0.5 mL as before and this same procedure was repeated three times, twice utilizing 1.0 mL of 10 mM TFA in 99.96% D_2O, and once using 1.0 mL of 10 mM TFA in 99.996% D_2O. Finally, the NMR samples were prepared in 1.0 mL of 10 mM TFA in 99.996% D_2O at the following concentrations: α-1 4.4 mM, α-2 5.2 mM, β-1 4.2 mM, and β-2T 2.6 mM.

N-tert-Butyloxycarbonyl-O-benzyl-L-tyrosyl-N^{ϵ}-nitro-L-arginine Benzyl Ester (3). N-tert-Butyloxycarbonyl-O-benzyl-L-tyrosine (1)[9,10] (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 twenty minute activation, N^{ϵ}-nitro-L-arginine benzyl ester (2)[12] (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 1M 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 x 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 minutes 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 3 as an amorphous white powder, mp 156-157°C; NMR ($CDCl_3$) 1.37 (s, 9H, $(CH_3)_3C$)), 1.5-1.9 (m, 4H, Arg β,γ-CH_2), 2.95 (m, 1H, Tyr β- CH_2), 3.20 (m, 2H, Arg δ-CH_2), 3.50 (m, 1H, Tyr β-CH_2), 4.30 (m, 1H, Arg α-CH), 4.60 (m, 1H, Tyr α-CH), 5.00 (s, 2H, $PhOCH_2Ph$), 5.15 (s, 2H, CO_2CH_2Ph), 6.90 (d, 2H, Tyr Ar-3,5H), 7.10 (d, 2H, Tyr Ar-2,6H), 7.35 (m, 10H, 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 (6). The Boc group was

removed from dipeptide 3 (96 mg, 0.14 mmol) by dissolution in 25 mL of dioxane previously saturated with HCl. After 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 5 as a white powder. N-tert-Butyloxycarbonyl-S-acetamidomethyl-L-cysteine (4)[8,9] (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 (5, 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 3 above. HPLC purification was done under the same conditions giving one peak, R_t 4.2 min: yield, 76 mg, 70%, of 6 as a clear foam; NMR($CDCl_3$) 1.40 (s, 9H, $(CH_3)_3C$), 1.55-1.85 (m, 4H, Arg β,γ-CH_2), 2.00 (s, 3H, $COCH_3$), 2.75 (m, 2H, Cys β-CH_2), 2.90 (m, 1H, Tyr β-CH_2), 3.04 (m, 2H, Arg δ-CH_2), 3.20 (m, 1H, Tyr β-CH_2), 4.20 (m, 1H, Arg α-CH), 4.35 (m, 1H, Tyr α-CH), 4.55 (m, 3H, Cys α-CH, SCH_2NH), 5.00 (s, 2H, $PhOCH_2Ph$), 5.13 (s, 2H, CO_2CH_2Ph), 6.87 (d, 2H, Tyr Ar-3,5H), 7.10 (d, 2H, Tyr Ar-2,6H), 7.35 (m, 10H, Ar). Anal. Calcd for $C_{40}H_{52}N_8O_{10}S\ H_2O$: 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 Ditrifluoroacetate (7). The protected tripeptide 6 (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 x 3 mL), and lyophilized. The residue was purified by HPLC on a Spherisorb ODS column (10 μm, 10 x

250 mm) using 25% CH_3CN and 75% 10 mM aqueous TFA as 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) 7. The NMR sample was prepared as described for chromopeptides and was determined at 5.0 mM in 10 mM TFA in D_2O (Figure 4).

Acknowledgement

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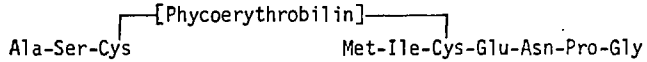
Part Three

Bilin Attachment Sites in the α and β Subunits of B-Phycoerythrin: Structural Studies on a Doubly Peptide-Linked Phycoerythrobilin

Abstract

A bilin-containing fragment, β-3T, from Porphyridium cruentum B-phycoerythrin produced by cleavage with trypsin and thermolysin was shown to have the structure:



Secondary ion mass spectrometry of this bilin-peptide yielded a protonated molecular ion of 1629 mass units corresponding to that predicted from the composition of the fragment, and indicated that the heptapeptide is linked to ring A and the tripeptide to ring D. NMR spectra provided definitive evidence for a thioether linkage at the C-3' of ring A and a second thioether linkage at C-18' of ring D of the bilin. This is the first documented report of a bilin linked through two thioether covalent bonds to a polypeptide.

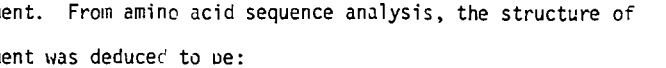
The phycobiliprotein B-phycoerythrin from the unicellular red alga Porphyridium cruentum has the subunit composition $(\alpha\beta)_6\gamma$.[1-3] The α and β subunits carry only phycoerythrobilin chromophores whereas the γ subunit carries both phycoerythrobilins and phycourobilins.[2] All of the phycoerythrobilin groups on the α and β subunits can be accounted for in five tryptic peptides each carrying one phycoerythrobilin, two derived from the α subunit and three from the β subunit.[4] In four of these peptides, the bilin is attached by a single thioether linkage to a cysteinyl residue, however, in the fifth peptide, β-3, sequence analysis showed the presence of two thioether linkages to the bilin from cysteinyl residues ten residues apart in the linear sequence. The sequence of β-3 was determined to be

Leu-Asp-Ala-Val-Asn-Ser-Ile-Val-Ser-Asn-Ala-Ser-Cys*-Met-
Val-Ser-Asp-Ala-Val-Ser-Gly-Met-Ile-Cys*-Glu-Asn-Pro-Gly-
Leu-Ile-Ser-Pro-Gly-Gly-Asn-Cys-Tyr-Thr-Asn-Arg

where the asterisks mark the sites of bilin attachment.[4]

The finding of this doubly-linked phycoerythrobilin was unexpected. Previous studies of phycoerythrin-peptides from cyanobacterial C-phycoerythrins had shown a single thioether linkage to the bilin in each peptide.[5,6]

Thermolysin digestion of the β-3 chromopeptide gives a smaller β-3T fragment. From amino acid sequence analysis, the structure of this fragment was deduced to be:



The UV/vis spectra showed that no alteration of the chromophore took place during the thermolysin cleavage.[4] The loss of the characteristic

red color of the tetrapyrrole during Edman degradation was seen only as both indicated cysteine residues were removed, suggesting the doubly-linked nature of this chromophore.

The secondary ion mass spectrum (SIMS) of the bilin-peptide β-3T was recorded using a cesium ion primary beam.[7] The high mass region (Table I) shows a strong molecular ion peak along with a number of confirming salt adducts. As shown in Tables I and II, each mass is actually represented by a cluster of peaks. Generally, the primary mass is the unprotonated species. An exception to this is seen in Table I where the base peak represents $(M+H^+)^+$. The protonated molecular ion at 1629 m.u. is the result of rounding up (above 0.59) of the exact mass measured at 1628.94 m.u. From compositional data, the calculated exact mass of $(M+H^+)^+$ is 1628.68 m.u., and this agreement is within the accuracy of the instrumentation ($\pm$ 0.3 m.u.).

In addition to the molecular ion information, the positive SIMS showed a number of useful fragments. Small peaks, at 1497 m.u. and 1519 m.u., represent $(M-Met)^+$ and $(M-Met-H^++Na^+)^+$, respectively, resulting from the loss of the amino-terminal methionyl residue from the heptapeptide portion of β-3T.[8,9] A small peak at 1349 m.u. attests to the presence of the $(M-tripeptide)^+$ fragment resulting from the loss of Ala-Ser-Cys. However, all of the major fragments result from the loss of the heptapeptide moiety from β-3T (Table II). The heptapeptide itself is seen in the 763, 785, and 801 m.u. peak series. The most abundant fragment, 743 m.u., is derived from the heptapeptide by the loss of $CH_2CO_2^-$ from the C-terminal glycine leaving a terminal amide-containing fragment.[8] The other two major fragments in the low mass fragmentation region contain the bilin as $(tetrapyrrole + H^+)^+$ at 587 m.u. and as

Table I. High Mass Region of the Positive Secondary Ion Mass Spectrum of the β-[illegible]coerythrobilipeptide.

Mass	Relative Intensity (%)	Assignment
1628	79	M^+
1629	100	$(M + H^+)^+$
1650	60	$(M - H^+ + Na^+)^+$
1666	36	$(M - H^+ + K^+)^+$
1672	23	$(M - 2H^+ + 2Na^+)^+$
1688	15	$(M - 2H^+ + Na^+ + K^+)^+$
1742	14	$(M + TFA)^+$
1764	14	$(M - H^+ + Na^+ + TFA)^+$

Table II. Low Mass Fragmentation Region of the Positive Secondary Ion Mass Spectrum of the β-3T Phycoerythrobilipeptide.

Mass	Relative Intensity (%)[a]	Assignment
866	20	$(M - \text{heptapeptide} + H^+)^+$
801	14	$(\text{Heptapeptide} - H^+ + K^+)^+$
785	25	$(\text{Heptapeptide} - H^+ + Na^+)^+$
763	12	$(\text{Heptapeptide} + H^+)^+$
743	94	$(\text{Heptapeptide} - CH_2CO_2^- + K^+)^+$
727	12	$(\text{Heptapeptide} - CH_2CO_2^- + Na^+)^+$
587	22	$(\text{Tetrapyrrole} + H^+)^+$

[a]The base peak was 115 m.u. $(\text{glycerol} - H^+ + Na^+)^+$ derived from the glycerol matrix.

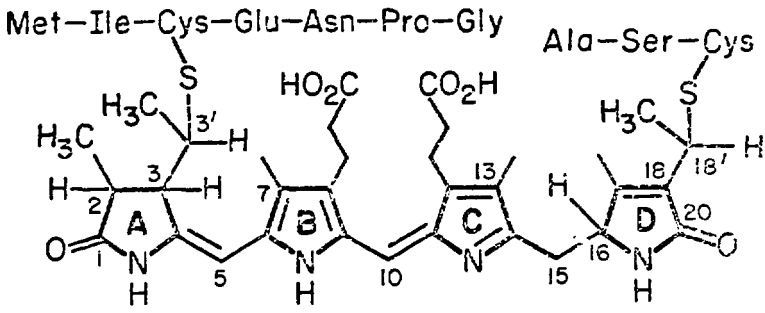
$(M\text{-heptapeptide} + H^+)^+$ at 866 m.u.

Calibration problems prevented full interpretation of the negative SIMS. However, the only major peaks seen corresponded to the molecular ion and the free heptapeptide.

The molecular ion information presented above give excellent confirmation of structure 1. In conjunction with the NMR data (see below), the doubly-linked nature of this bilin is conclusively demonstrated. The saturation at C-15,16 and the unsaturation at C-17,18 are further documented. Also, the mass spectrum precludes any possible ester linkage as is often postulated for bilin-peptides.[10]

The fragmentation gives additional information about the peptide moieties. Losses of methionine and glycine establish their positions as N- and C-terminal, respectively, in the peptide moieties. Although computer analysis of some of the minor peaks might give further sequence data, this information was more readily available by other methods.[4] The fragmentation appears to be indicative of the relative lability of the two peptide-tetrapyrrole linkages. The major 866 m.u. fragment indicates the loss of the heptapeptide through a two electron process with additional loss of hydrogen generating a double bond in the tetrapyrrole. The 587 m.u. peak for the protonated tetrapyrrole and the minor 1349 m.u. peak for the loss of the tripeptide imply that the tripeptide is cleaved through this same type of mechanism.

This is the first report of a D ring thioether attachment. Mechanistic studies of tetrapyrrole-peptide cleavages in solution show that the rather facile thioether elimination at ring A involves the loss of the tertiary allylic hydrogen at C-3, perhaps assisted by favorable stereochemistry.[11] Such data, of course, do not exist for elimination



1

β-3T Phycoerythrobilipeptide

of the thioether at ring D. However, the structure as assigned in 1 indicates that such an elimination should be more difficult at ring D than at ring A. Thus elimination of the D ring thioether group could involve any of three hydrogens: (a) a primary homoallylic hydrogen from the C-18'-CH_3, (b) a primary allylic hydrogen from the C-17-CH_3, or (c) the tertiary allylic hydrogen at C-16. Processes involving (a) or (b) would be less favorable than one involving (c). Even the latter case, however, requiring a 1,4-elimination and resulting in a less conjugated system, appears less facile than elimination from ring A.

At this point, our evaluation of the relative ease of elimination at the two sites is conjectural and will be resolved only by direct comparison of reactivity with specific, authentic structures. However, the striking dominance of all major fragments originating from the loss of the heptapeptide clearly demonstrates that there is a significant difference between the two modes of elimination. We find this difference is best accommodated by the assignment of structure 1.

The NMR spectrum of the bilin-peptide B-3T in 10 mM TFA in D_2O is shown in Figure 1. This solvent was chosen because the chromophore has good stability in it and because its acidic nature precludes the existence of interconverting prototropic isomers. The peptide resonances show very good resolution while the tetrapyrrolic signals show severe line broadening, especially the 5-H and 10-H resonances. The same phenomenon has been seen to a lesser degree in previous NMR studies of bilin-peptides.[12] Evidently the peptide portions, while themselves free to extend into solution, provide steric constraints on the conformational flexibility of the bilin.

The line broadening in conjunction with overlapping signals pre-

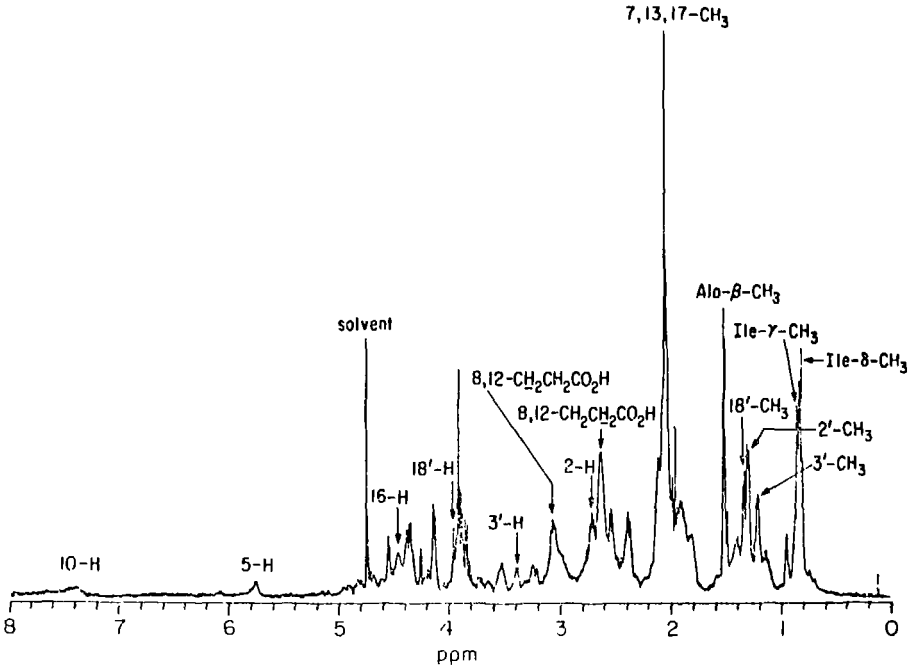
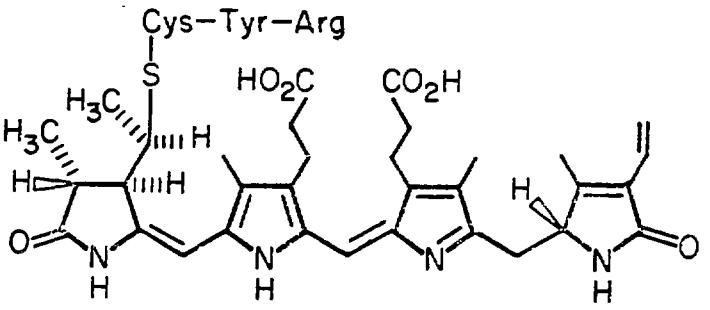


Figure 1. The 500 MHz ^1H NMR spectrum of the phycoerythrobilipeptide β-3T (c=2.2 mM) in 10 mM TFA in D_2O at 25°C.

cludes complete assignment of all of the tetrapyrrolic-peptide resonances. In particular, the 3-H and 15-H protons could not be assigned even by difference decoupling. The line broadening also prevented the assessment of the 2-H/3-H coupling constant and no relative stereochemical information could be extracted.

The ^{1}H NMR assignments for the bilin moiety, together with those for the phycoerythrobilin bearing α-1 tripeptide 2, Cys(PEB)-Tyr-Arg (see part two of this thesis), are shown in Table III. The general substitution pattern of the tetrapyrrole is assumed by analogy to other studies on phycoerythrobilins.[13] Comparison with the data on the tripeptide permits straightforward assignment of most of the bilin resonances. The key to the important assignments in the A and D rings lies in the upfield (0.7-1.6 ppm) region. Decoupling experiments allow us to assign the isoleucine δ-methyl triplet at 0.81 ppm and the isoleucine γ-methyl doublet at 0.84 ppm. The alanine methyl doublet appears at 1.51 ppm. These assignments leave three broadened (relative to the peptide resonances) doublets attributable to the bilin. The doublet at 1.29 ppm is coupled to the proton at 2.7 ppm. These values correspond to resonances assigned to 2-CH_3 and 2-H in the tripeptide. Such agreement would be expected because the 2-position is least influenced by conformational effects.

The two remaining upfield doublets provide direct evidence for the two thioether linkages at the 3'- and 18'-positions. The methyl doublet lying further downfield is coupled to the proton at 3.9 ppm. The presence of three aromatic methyl groups in the 2.04 ppm region and the absence of another methyl doublet shows that the C-17/C-18 double bond is still intact. As the 3.9 ppm proton is allylic and



2

α-l Phycoerythrobilitripeptide

Table III. ^{1}H NMR Assignments for the Bilin Moiety of Phycoerythrobilipeptide β-3T 1 and the α-1 Phycoerythrobilitripeptide 2 in 10 mM TFA in D_2O at 25°C.

1	2[a]	assignment
7.4 (s)	7.34 (s)	10-H
b	6.37 (dd, 11.7, 17.9)	18-H_a
5.75 (s)	5.86 (s)	5-H
b	5.82 (dd, 1.4, 17.9)	18-H_b
b	5.40 (dd, 1.4, 11.7)	18-H_x
4.46 (m)	4.40 (dd, 4.2, 7.9)	16-H
3.9 (m)	b	18'-H
3.40 (m)	3.44 (m, 3.0, 7.0)	3'-H
c	3.30 (dd, 4.2, 14.1)	15-H
c	3.10 (m,3.0, 3.5)	3-H
3.05 (m)	3.07 (m)	8,12-$CH_2CH_2CO_2H$
c	2.93 (m, 7.9, 14.1)	15-H
2.7 (m)	2.69 (m, 3.5, 7.4)	2-H
2.6 (m)	2.64 (m)	8,12-$CH_2CH_2CO_2H$
2.05 (s)	2.08 (s)	7,13,17-CH_3
2.04 (s)	2.06 (s)	
2.03 (s)	2.055 (s)	
1.33 (d, 7.2)	b	18'-CH_3
1.29 (d, 6.6)	1.28 (d, 7.4)	2-CH_3
1.21 (d, 5.5)	1.39 (d, 7.0)	3'-CH_3

[a]Data from part two of this thesis.

[b]Assignment is not applicable as th s functionality differs in this compound.

[c]Overlapping resonances and line broadening prevent assignment.

therefore expected to be shifted further downfield, we assign this resonance to the C-18' proton. The C-3' methyl, then, appears at 1.21 ppm and is coupled to the C-3'-H at 3.4 ppm. The difference in the chemical shift of the C-3' methyl (0.18 ppm upfield from that in the tripeptide) along with the severely broadened nature of the signal indicate that it is sterically constricted in the shielding region of the chromophore.

The absence of attributable vinyl resonances provides confirmatory evidence of the second thioether linkage at C-18'. Structure 1 is drawn with the peptide unit originally closer to the amino-terminus of the protein as bound to ring D since the mass spectral data supports this assignment. The finding of a doubly-linked phycoerythrobilin mandates the re-evaluation and indicates the limitations of other studies of linkages to bilins.[14] Our study of the phycoerythrobilin-peptides from the α and β subunits of P. cruentum B-phycoerythrin definitively shows that four of the bilins are singly-linked through the A ring and the remaining bilin is doubly-linked.[13] The existence of a bilin singly-linked through the D ring has never been proven but seems likely in light of the present results. The biological significance of these different linkages must now be explored.

Experimental Section

General Methods. Evaporations were done on a Berkeley rotary evaporator using an oil pump for reduced pressure. The NMR spectra were obtained on the University of California, Davis, 500 MHz spectrometer and the chemical shifts are expressed in ppm relative to an external TSP standard. High pressure liquid chromatography (HPLC) was done on an Altex system consisting of two Model 110A pumps, a Model 115-10 UV/vis detector, and a Model 420 microprocessor.

Preparation of the Phycoerythrobilin Peptide β-3T. The conditions employed for the tryptic digestion of Porphyridium cruentum B-phycoerythrin and for the isolation and carboxymethylation of peptide β-3 were those described by Lundell et al.[4] Carboxymethylated peptide β-3 (4.9 μmol) in 1 M acetic acid was dialyzed overnight at 4°C against 10 mM HCl (Spectra/Por dialysis tubing, MW cut-off 3,500). After dialysis. the peptide solution (12 mL) was adjusted to pH 7.6 by the addition of 1/10 volume of 1 M Tris-Cl, pH 8, and calcium chloride was added to a final concentration of 5 mM. Thermolysin (375 μg) was added and the mixture was incubated under N_2 in the dark at 37°C for 190 min. A second aliquot of thermolysin (375 μg) was then added and the digestion was allowed to proceed for a further 180 min under the same conditions. The reaction was terminated by acidification to pH 2 with 1 M HCl.

The digest was applied to a column (1.5 x 20 cm) of SP-Sephadex C-25 in 0.05 M sodium phosphate, pH 2.5, and the column was developed with a linear gradient (600 mL) of 0.0 to 0.4 M NaCl in 0.05 M sodium phosphate, pH 2.5 at a flow rate of 30 mL/hr. A single peak of bilin-containing material was eluted at approximately 0.21 M NaCl. Amino acid

analysis data and UV/vis spectral analysis are available.[4]

For further studies, the eluate from the SP-Sephadex column was adsorbed onto a column of LiChrosorb C-18, washed extensively with 10 mM aqueous formic acid, and then eluted with 60% acetonitrile/40% 10 mM aqueous formic acid. The acetonitrile was removed under a stream of nitrogen. HPLC analysis of the desalted peptide ß-3T is shown in Figure 2.

Sample Preparation for NMR. The chromopeptide in 10 mM aqueous formic acid was concentrated under reduced pressure by rotary evaporation to 0.5 mL and diluted with 10 mL of 10 mM trifluoroacetic acid (TFA) in 99.8% D_2O (made by adding 50 µmol of trifluoroacetic anhydride to 10 mL of 99.8% D_2O). The solution was stirred for 1 hr at room temperature and concentrated as before to 0.5 mL. This same procedure was repeated twice and then twice using 1.0 mL of 99.96% D_2O. Finally, the NMR sample was prepared in 1.0 mL of 10 mM TFA in 99.996% D_2O at a concentration of 2.2 mM.

Secondary Ion Mass Spectrometry. An aliquot of the chromopeptide in 10 mM aqueous TFA representing approximately 3 µg was applied directly to the glycerol matrix. Scans were done at 30 seconds/decade.[7]

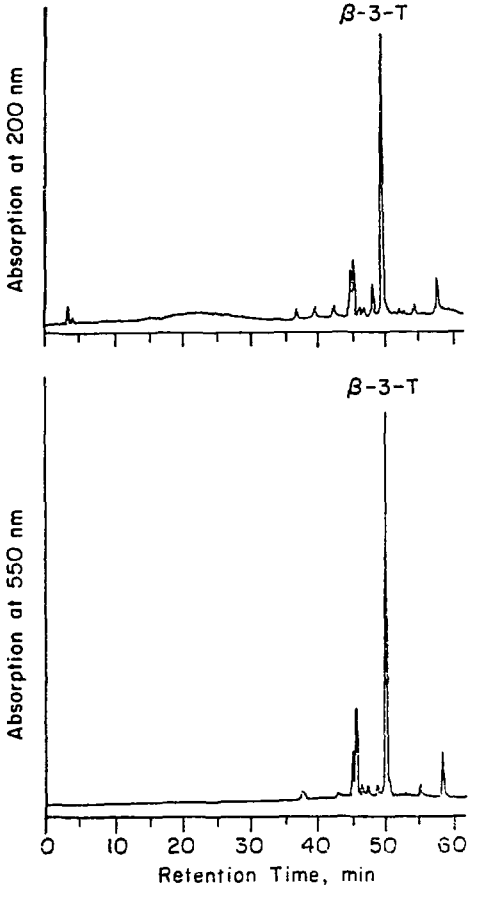


Figure 2. High pressure liquid chromatography of the phycoerythrobilipeptide β-3T on an Ultrashere ODS column (4.6x250 mm, 5 μm) using a 60 minute gradient (10%-30%) of CH_3CN in 0.10 M NaH_2PO_4 buffer, pH 2.1 with a 1.0 ml/min flow rate.

Acknowledgement

This research was supported in part by the Division of Biological Energy Conversion and Conservation, U.S. Department of Energy under Contract No. DE-AC03-76SF00098, the National Institute of General Medical Sciences, DHHW, grant GM 28994, and National Science Foundation grant NSF PCM 82-08158. The 500 MHz NMR studies were carried out at the University of California, Davis, NMR Facility, under the auspices of National Science Foundation grant CHE 79-04932. The secondary ion mass spectral data were obtained from the Bioorganic Biomedical Mass Spectrometry Resource (A.L. Burlingame, Director), University of California, supported by NIH Division of Research Resources grant RR01614.

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