Chromopeptides from Phycoerythrocyanin. Structure and Linkage of the Three Bilin Groups

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Abstract: Phycoerythrocyanin carries two covalently attached phycocyanobilin (PCB) groups on the β subunit and a phycobiliviolinoid (PXB) group on the α subunit. Three distinct bilipeptides were obtained by proteolytic digestion of this protein: Asn-Gln-Ala-Ala-Cys(PCB)-Ile-Arg, Gly-Asp-Cys(PCB)-Ser-Gln, and Cys(PXB)-Val-Arg. Correlative 500-MHz ¹H NMR analyses showed that the heptapeptide and pentapeptide were attached by cysteinyl thioether linkage to the A ring of the PCB moiety. ¹H NMR and mass spectrometry determinations led to structural assignment for the hitherto uncharacterized PXB moiety, with peptide–thioether bonding possible to either ring A or D. Amino acid sequence homologies strongly favor A-ring linkage.

Phycobiliproteins are a class of brilliantly colored, intensely fluorescent photosynthetic proteins found in numerous cyanobacteria and red algae.[1] Arranged into units called phycobilisomes, these antenna pigments are located on the thylakoid membrane and serve the function of trapping and transferring light energy to chlorophyll *a* with an efficiency approaching 100%.[2] While phycobiliproteins have been of interest to chemists and biologists for over 150 years, it was not until 50 years ago that the presence of covalently attached linear tetrapyrrole or bilin groups was discovered, accounting for the various colors of the proteins.[3-5] Since then, many structural studies have been reported that attempted to delineate the double-bond network of the bilin moiety, the absolute stereochemistry of groups on the tetrapyrrole skeleton, and the nature of the protein–chromophore linkage. Such studies have typically relied on the chemical degradation of the biliprotein and identification of the various degradation products. Unfortunately, this methodology is inherently destructive, and the many resulting erroneous structure assignments reflect its imprecise nature.

The experimental approach we have employed involves proteolytic digestion of the biliproteins, resulting in bilipeptides of sufficient simplicity to be analyzed by ¹H NMR spectroscopy. Such methodology has allowed direct observation of the intact peptide-bound tetrapyrroles. In this manner, we have determined the structures of the three phycocyanobilin (PCB)[6] groups from *Synechococcus* sp. 6301, C-phycocyanin,[7,8] the phycoerythrobilin (PEB) groups from three algal sources, *Porphyridium cruentum* B-phycoerythrin,[9-12] *Gastroclonium coulteri* R-phycoerythrin,[12,13] and *P. cruentum* R-phycocyanin,[12] and three phycourobilin (PUB) groups from *G. coulteri* R-phycoerythrin.[13] Excluding the absolute stereochemical assignments to be discussed below, these studies not only identified the structures of the chromophores, but they also proved the existence of thioether linkages between cysteine and the bilin moieties in all instances and conclusively ruled out additional linkages involving threonyl, aspartyl, or tyrosyl residues. Two bilipeptides, β-3T PEB and β-3T PUB, were found to be doubly linked, with cysteinyl thioether linkages at C-3′ and C-18′. The structures of the isomeric A-ring-linked PCB, PEB, and PUB groups are shown with their corresponding visible absorbance maxima in Figure 1.

Phycoerythrocyanin is a phycobiliprotein whose occurrence is limited to certain genera of filamentous cyanobacteria.[14] The α subunit of this protein carries a biliviolinoid chromophore (PXB) of unknown structure, which exhibits a distinctive visible absorption

Table I. Amino Acid Composition of Bilin-Containing Peptides Isolated from *A. variabilis* PEC

amino acid	α-1 PXB	β-1 PCB	β-2T PCB
Cys-X[a]	0.9 (1)[b]	0.8 (1)	0.8 (1)
Asp		1.0 (1)	1.0 (1)
Ser[c]			0.8 (1)
Glu		1.0 (1)	1.2 (1)
Gly			1.4 (1)
Ala		1.8 (2)	
Val	1.0 (1)		
Ile		1.1 (1)	
Arg	1.0 (1)	0.9 (1)	

[a] Cys-X represents bilin-linked cysteine. The value given is that for cysteic acid recovered after hydrolysis in 6 N HCl containing 0.21 M Me₂SO. A 10% correction for destruction during acid hydrolysis is included. [b] Values in parentheses are the number of residues obtained in the sequence determination. [c] A 10% correction for destruction during acid hydrolysis is included.

maximum at 590 nm.[15] The β subunit of phycoerythrocyanin carries two PCB chromophores.[15-17] The amino acid sequence

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(6) The abbreviations used are PCB, phycocyanobilin; PEB, phycoerythrobilin; PUB, phycourobilin; Pᵢ, phosphate; SP-Sephadex, sulfopropyl-Sephadex C 25. PXB refers to the pigment showing a visible absorbance maximum at 590 nm. This chromophore has previously been referred to as a phycobiliviolinoid pigment. (See ref 14 and 15). PEC refers to the native protein, phycoerythrocyanin.

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(16) Füglistaller, P.; Suter, F.; Zuber, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1983**, *364*, 691.

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Table II. Fast Atom Bombardment Mass Spectrum of α-1 PXB

mass	% rel intensity	elemental composition		measured mass[a]	assignment
		theoretical	found		
985	11	$C_{47}H_{66}N_{10}O_{10}SNa$	b	b	$(M + Na)^+$
963	52	$C_{47}H_{67}N_{10}O_{10}S$	$C_{47}H_{67}N_{10}O_{10}S$	963.4755	$(M + H)^+$
587	13	$C_{33}H_{39}N_4O_6$	b	b	(tetrapyrrole) cation
500	20	$C_{21}H_{38}N_7O_5S$	b	b	(monopyrrole + tripeptide + 2H) cation
498	14	$C_{21}H_{36}N_7O_5S$	b	b	(monopyrrole + tripeptide) cation
464	34	$C_{26}H_{30}N_3O_5$	$C_{26}H_{30}N_3O_5$	464.2193	(tripyrrole) cation
377	100	$C_{14}H_{29}N_6O_4S$	$C_{14}H_{29}N_6O_4S$	377.1979	(tripeptide + 2H) cation
375	37	$C_{14}H_{27}N_6O_4S$	b	b	(tripeptide) cation

[a] Determined by peak matching against glycerol. [b] Not determined.
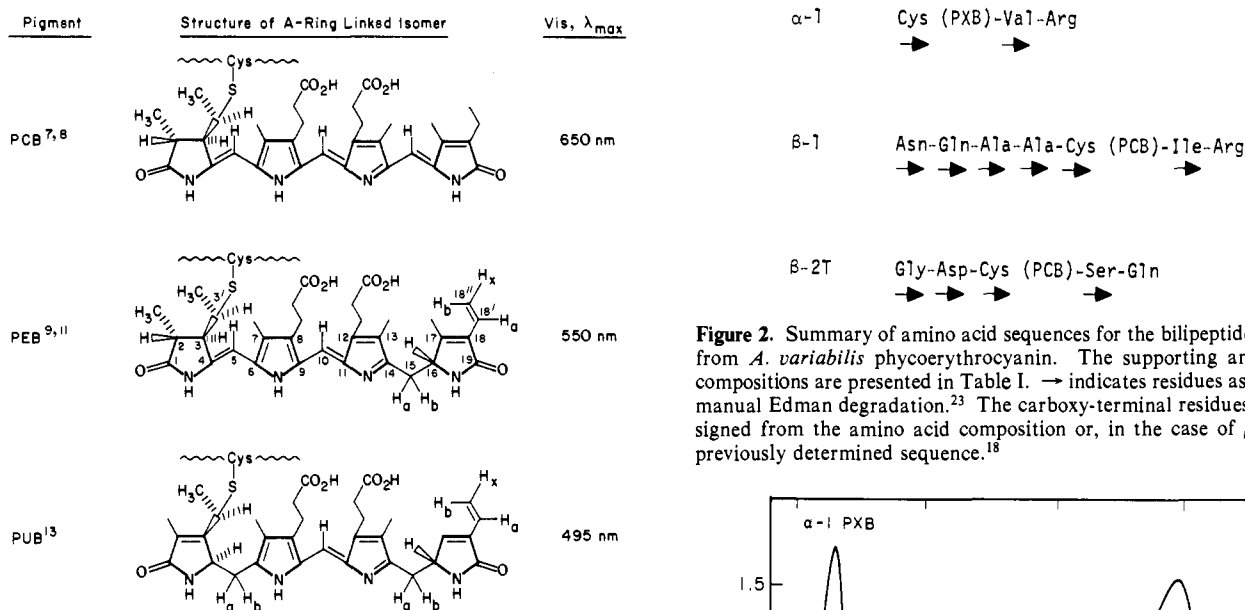

Figure 1. Structures of A-ring-linked phycobilipeptides and their visible absorbance maxima in acid solution.

of *Mastigocladus laminosus* PEC has been fully determined and that of *Anabaena variabilis* PEC partially established.[16-18] In this study, we present the structure assignment for the two peptide-linked PCB groups and provide two possible structures for the previously uncharacterized PXB group in peptides isolated from both *A. variabilis* and *M. laminosus* PEC.

Results and Discussion

Isolation and Purification. *A. variabilis* phycoerythrocyanin was thoroughly digested with trypsin and then subjected to gel filtration, and the bilin-containing peptides were eluted in 50–70% yield. The PXB tripeptide, known to be derived from the α subunit,[15] was labeled α-1 PXB, the smaller of the two PCB-containing peptides was found to contain seven amino acid residues and was labeled β-1 PCB based on sequence homologies, and the remaining PCB-containing peptide with an amino acid composition that matched the β-2 fragment from PEC was accordingly labeled β-2 PCB.[16,18] This latter bilipeptide was digested with thermolysin, providing a bilipentapeptide labeled β-2T PCB. The amino acid composition (Table I) and sequence (Figure 2) of the α-1, β-1, and β-2T peptides agree with previous work on *A. variabilis* PEC and exhibit perfect homology to those reported for *M. laminosus* PEC.[16,18]

M. laminosus PEC was similarly subjected to trypsin digestion and gel filtration. The amino acid analysis and sequence of the PXB-containing tripeptide were identical with the data obtained from *A. variabilis* α-1 PXB.

UV–Vis Spectroscopy. All bilipeptides were rigorously purified by reverse-phase HPLC before spectroscopic analysis. The UV–vis

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Figure 2. Summary of amino acid sequences for the bilipeptides derived from *A. variabilis* phycoerythrocyanin. The supporting amino acid compositions are presented in Table I. → indicates residues assigned by manual Edman degradation.[23] The carboxy-terminal residues were assigned from the amino acid composition or, in the case of β-2T, the previously determined sequence.[18]
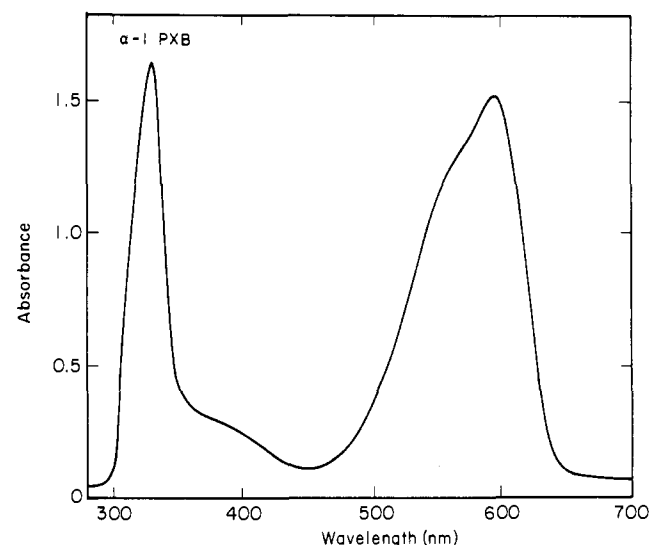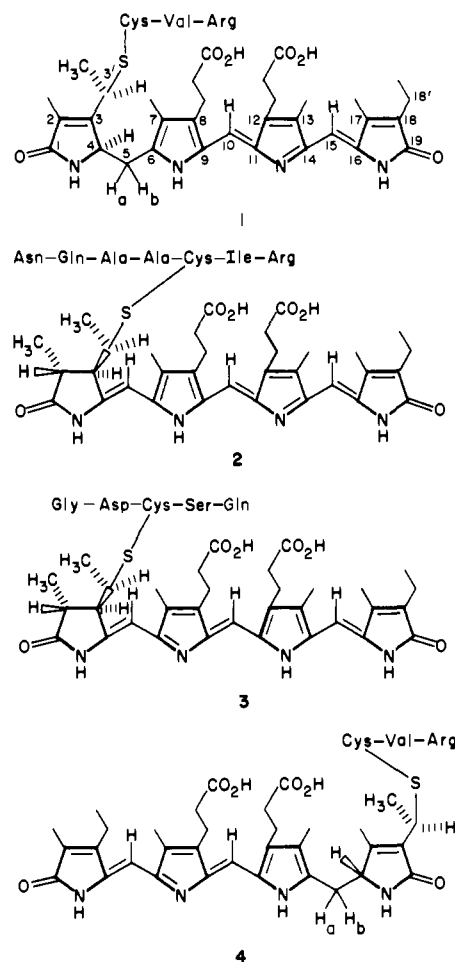

Figure 3. UV–vis spectrum of α-1 PXB in 10 mM aqueous TFA.

spectra of β-1 and β-2T PCB were identical with those obtained from C-phycocyanin,[8] while that of α-1 PXB in 10 mM aqueous TFA (Figure 3) showed absorbance maxima at 328 and 590 nm. The shoulder at 570 nm grew more pronounced after storage and may result from a further transformation product.

Mass Spectroscopy. The fast atom bombardment (FAB) mass spectral data obtained from α-1 PXB appear in Table II. The elemental composition of several critical peaks was determined by peak matching against glycerol adducts. The peak at m/z 963, for which the elemental composition $C_{47}H_{67}N_{10}O_{10}S$ was calculated, corresponds to the monoprotonated tripeptide attached to a tetrapyrrolic moiety with a mass identical with that of the previously studied PCB,[8] PEB,[12] and PUB[13] groups (Figure 1). A sodium salt adduct is observed at m/z 985. Because the PXB chromophore has an absorbance maximum between those of PCB and PEB with eight and six conjugated double bonds, respectively, these data led us to consider isomeric structures containing seven conjugated double bonds. On the basis of further mass and ^{1}H

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Figure 4. Structures of α-1 PXB (**1**), β-1 PCB (**2**), and β-2T PCB (**3**) from *A. variabilis* phycoerythrocyanin, and a possible alternate structure for α-1 PXB with D-ring linkage (**4**).

NMR spectroscopic data, the possible structures for the α-1 PXB tripeptide are shown in Figure 4 (**1** and **4**).

Analyzing the fragmentation patterns, one first notices the facile release of pigment from the tripeptide. The peak at m/z 375 corresponds to the unprotonated tripeptide, while m/z 377, for which the elemental composition $C_{14}H_{29}N_6O_4S$ was determined, corresponds to the diprotonated form. The liberated tetrapyrrole is seen at m/z 587. However, the most interesting fragments result from the benzylic-type cleavage of the C-4–C-5 (or C-14–C-15) bond, leaving a free tripyrrole and a tripeptide attached to the remaining pyrrole unit. The peak at m/z 464 is assigned to the tripyrrole and the elemental composition, $C_{26}H_{30}N_3O_5$, was confirmed by peak matching. The unprotonated (monopyrrole-tripeptide) cation peak is observed at m/z 498 followed by the diprotonated species at m/z 500. These data support the conclusion that the thioether linkage is positioned on a ring which is attached to its neighboring ring by a saturated bridge. The only structures for α-1 PXB consistent with these mass spectral data are shown in Figure 4 (**1** and **4**). The problem of differentiating between these two possible structures is addressed below.

In summary, the mass spectral data prove that PXB is an isomer of PCB, PEB, and PUB and show that the thioether linkage is on the pyrrole ring isolated from the seven conjugated double bond system by a saturated methylene bridge.

¹H NMR Spectrum of α-1 PXB. The ¹H NMR spectra of α-1 PXB derived from *M. laminosus* PEC and that from *A. variabilis* PEC were identical. The ¹H NMR spectral analysis of α-1 PXB from either phycoerythrocyanin was complicated by line broadening in 10 mM TFA/D₂O and instability of the compound in pyridine-d_5. Incrementally increasing the concentration of the TFA/D₂O solution to 1 M did little to the resolution but shift the residual HOD peak downfield. Although far from perfect,

Table III. ¹H NMR (500-MHz) Assignments for α-1 PXB in 10 mM TFA/D₂O at 25 °C

chem shift	multiplicity, J_{H-H}, Hz	no. of Hs	assignment
7.69	s	1	10-H
6.39	s	1	15-H
4.63	br dd	1	4-H
4.27	t, 7.1	1	Cys α-CH
4.23	d, 7.4	1	Val α-CH
4.20	br d, 8.4	1	Arg α-CH
4.06	br m	1	3'-H
3.69	br dd	1	5-H
3.11[a]	m	2	Cys β-CH₂
3.09[a]	m	4	8,12-CH_2CH₂CO₂H
3.08[a]	m	2	Arg δ-CH₂
2.69[a]	m	4	8,12-CH₂CH_2CO₂H
2.67[a]	m	1	5-H
2.35	br m	2	18-CH_2CH₃
2.19	s ⎫		
2.17	s ⎬	12	2,7,13,17-CH₃[b]
2.14	s ⎭		
1.93	m	1	Val β-CH
1.4–1.7	m	4	Arg β,γ-CH₂
1.45	d, 7.1	3	3'-CH₃
1.01	br t, 7.1	3	18-CH₂CH_3
0.86	dt, 7.4, 1.2	6	Val γ-CH₃

[a] Overlapping resonances prohibit precise chemical shift values. [b] Four methyl groups are seen as three overlapping singlets.

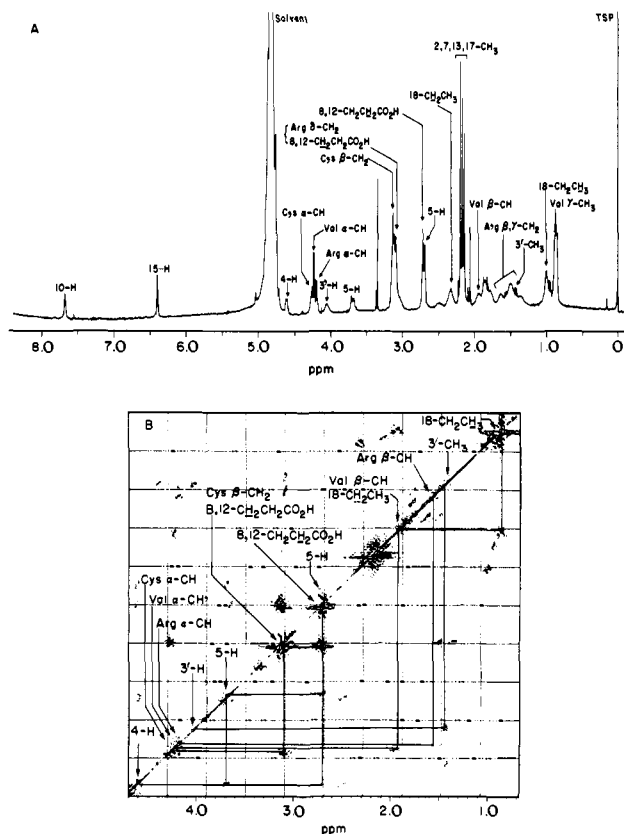**Figure 5.** (A) 1-D and (B) 2-D COSY ¹H NMR spectra of α-1 PXB in 10 mM TFA/D₂O at 25 °C.

the NMR solvent of choice for α-1 PXB was 10 mM TFA/D₂O.

The ¹H NMR data support structure **1** shown in Figure 4 and are discussed in relation to this structure, although the corresponding D-ring-linked structure **4** is not excluded from consideration. All assignments are made by using the A-ring linkage numbering scheme and are given in Table III. The full 1-D and 2-D phase sensitive homonuclear correlated (COSY) ¹H NMR spectra are shown in Figure 5.

Analyzing the downfield region of the spectrum, one observes the two vinylic 10-H and 15-H resonances at 7.69 and 6.39 ppm,
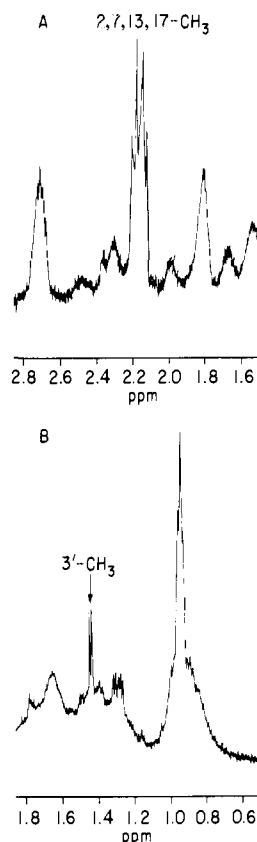
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A   ?,?,13,17-CH₃

B

3'-CH₃

2.8 2.6 2.4 2.2 2.0 1.8 1.6
ppm

1.8 1.6 1.4 1.2 1.0 0.8 0.6
ppm

Figure 6. (A) Upfield region from the Gaussian–Lorenzian apodization spectrum of α-1 PXB in 10 mM TFA/D₂O. (b) Upfield region of α-1 PXB in 1.0 M TFA/D₂O at 25 °C.

Table IV. ^{1}H NMR (500-MHz) Assignments for β-1 PCB in Pyridine-d_5 at 25 °C

chem shift	multiplicity, J_{H-H}, Hz	no. of Hs	assignment
7.29	s	1	10-H
6.07	s	1	15-H
5.94	s	1[a]	5-H
5.17	br m, 5.8	1	Cys α-H
5.11	t, 5.9	1	Asn α-H
5.06	br m	1	Arg α-H
4.88	br m	1	Gln α-H
4.83	t, 7.5	1	Ile α-H
4.76	t, 6.8	1	Ala(1) α-H
4.70	t, 6.7	1	Ala(2) α-H
3.69	m, 10.3, 6.3	2	Asn β-CH₂
3.59	m, 7.3	1	3'-H
3.51	m	1	Arg δ-CH₂
3.45	dd, 9.5, 3.8	2	Cys β-CH₂
3.38	m	1	Arg δ-CH₂
3.19[b]	m	1	3-H
3.16[b]	m	2	8,12-C*H₂*CH₂CO₂H
3.09	m, 6.8, 6.2	2	8,12-C*H₂*CH₂CO₂H
2.83	m, 9.2, 7.6	4	8,12-CH₂C*H₂*CO₂H
2.76[b]	m	2	Gln γ-CH₂
2.74[b]	m	1	2-H
2.56	m, 6.2, 7.5	2	Gln β-CH₂
2.46	q, 7.2	2	18-C*H₂*CH₃
2.23	m	1	Ile β-CH
2.09	s ⎫		
2.06	s ⎬	9	7,13,17-CH₃
2.04	s ⎭		
2.01	m	2	Arg β-CH₂
2.00	m	2	Arg γ-CH₂
1.80	m	1	Ile γ-CH₂
1.60	d, 7.3	3	Ala(1)-CH₃
1.52	d, 6.8	3	3'-CH₃
1.48	d, 7.2	3	Ala(2)-CH₃
1.38	d, 7.3	3	2-CH₃
1.31	m, 8.1, 6.3	1	Ile γ-CH₂
1.23	t, 7.6	3	18-CH₂C*H₃*
1.10	d, 6.7	3	Ile β-CH₃
0.75	t, 7.4	3	Ile γ-CH₃

[a] Deuterium exchange is observed at this position. [b] Overlapping resonances prohibit precise assignment of chemical shift values.

respectively. These peaks correspond well with the 10-H and 15-H singlets previously observed in A-ring-linked β-1 PCB from C-phycocyanin.[7] Noticeably absent, however, is a downfield vinylic resonance corresponding to 5-H. The β-1 PCB group from C-phycocyanin exhibits this proton as a singlet at 5.94 ppm.[7] α-1 PXB shows no such resonance, and this is taken as proof of saturation between the A and B rings. Because the mass spectral data prove the isomeric relationships of PXB and PCB, this double bond must exist elsewhere on the PXB–tetrapyrrole moiety. Previous studies showed that the PEB and PUB chromophores have an exocyclic vinyl group attached at C-18,[12,13] typically manifested as a downfield ABX pattern in the ^{1}H NMR spectrum. No such group is observed in the spectrum of PXB. The only alternate position for this unsaturation is between C-2 and C-3, which is corroborated by the ^{1}H NMR spectrum and the *m/z* 498 peak for the (monopyrrole–tripeptide) cation fragment. Although only 3 vinylic methyl singlets are seen between 2.1 and 2.2 ppm, integration establishes a total of 12 protons; therefore, 4 methyl groups are actually present. A Gaussian–Lorenzian apodization of the FID produced the spectrum shown in Figure 6A. The enhanced resolution, at the sacrifice of the signal-to-noise ratio, clearly shows four singlets, confirming the sp² hybridization state of C-2, C-7, C-13, and C-17 and proving the presence of a double bond between C-2 and C-3.

Although the C-4/C-5 ABM pattern was not well resolved, assignments can be made chiefly from the 2-D COSY spectrum. The broad doublet of doublets at 4.63 ppm is assigned to 4-H, and coupling with the two nonequivalent 5-H protons at 3.69 and 2.67 ppm can clearly be seen. Line broadening also made it difficult to observe the 3'-H and 3'-CH₃ resonances. Normally, the 3'-CH₃ group is observed between 1.0 and 2.0 ppm as a strong doublet.[13] The arginine methylene resonances unfortunately were very broad and severely obscured this region of the spectrum. Increasing the TFA concentration to 1.0 M sharpened this area and allowed assignment of the 3'-CH₃ doublet (Figure 6B). Coupling with the 3'-H resonance at 4.06 ppm can be seen in the

COSY spectrum. The resonances from the propionyl side chains are found at 3.09 and 2.69 ppm. The ethyl group, although quite broadened, is seen as a multiplet at 2.35 ppm (18-C*H₂*CH₃) coupled to a triplet at 1.01 ppm (18-CH₂C*H₃*). The peptide protons of the molecule, with the exception of the broad arginine resonances, are quite well resolved, and all assignments were verified by the COSY spectrum.

^{1}H NMR Spectrum of β-1 PCB. The ^{1}H NMR spectrum of the β-1 PCB heptapeptide (Figure 4, **2**) was recorded in 10 mM TFA/D₂O; however, line broadening made assignments difficult. The 1-D and 2-D COSY NMR spectra in pyridine-d_5 are shown in Figure 7, and the assignments are found in Table IV. This bilipeptide shows the best spectral resolution observed to date. All seven amino acid α protons appear between 4.6 and 5.2 ppm. Couplings between these resonances and the β protons are seen in the 2-D COSY spectrum allowing the correct assignment of the alanine methyl doublets in the upfield region, such that confusion with the critical bilin methyl doublets is avoided.

With the exception of the vinylic 5-H, 10-H, and 15-H resonances, the 3'-H peak at 3.59 ppm is the most downfield bilin resonance due to its proximity to the sulfur atom. The COSY spectrum verifies coupling between this proton and the 3'-CH₃ doublet at 1.52 ppm. The 2-CH₃ doublet at 1.38 ppm is similarly seen coupled to the 2-H resonance at 2.74 ppm. The remaining upfield doublet, Ile β-CH₃, is identified by the coupling of the β proton at 2.23 ppm to the resonance at 1.10 ppm.

The correct assignment of the 2-CH₃ and 3'-CH₃ groups is particularly important. Correlation with the previously reported NMR data made assignment of the linkage ring possible after all the upfield doublets were correctly assigned. The 2-CH₃ resonance at 1.38 ppm is in close agreement with the chemical
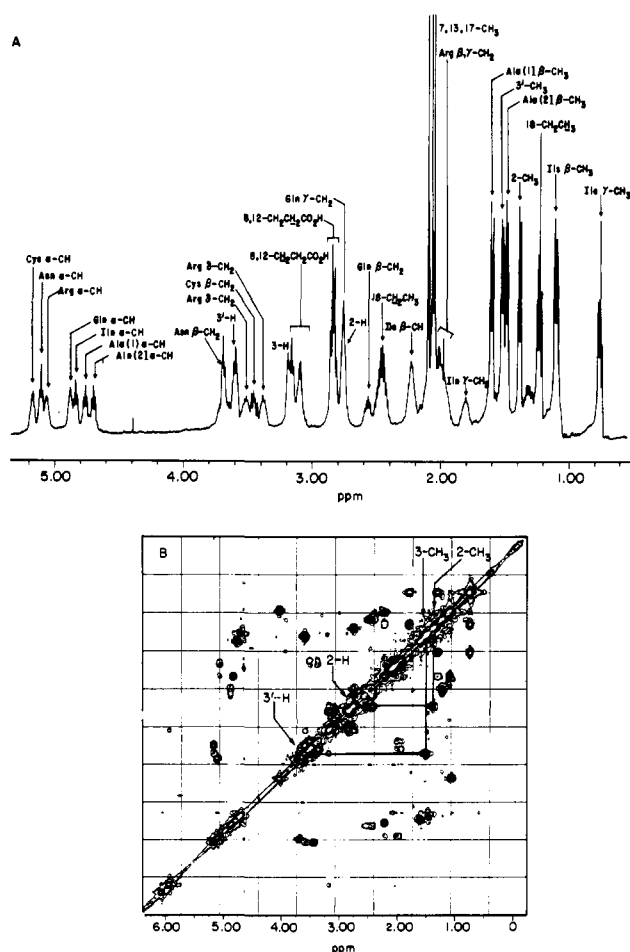
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Figure 7. (A) 1-D and (B) 2-D COSY ^{1}H NMR spectra of β-1 PCB in pyridine-d_5 at 25 °C (upfield region).

shifts found for this group in the analogous A-ring-linked peptides α-1 PCB (1.34 ppm) and β-1 PCB (1.39 ppm) derived from C-phycocyanin.[8] These data provide the basis for the assignment of A-ring linkage for the β-1 PCB heptapeptide from phyco-erythrocyanin.

^{1}H NMR Spectrum of β-2T PCB. The 1-D and 2-D COSY ^{1}H NMR spectra of the β-2T PCB pentapeptide **3** in pyridine-d_5 are shown in Figure 8, and the assignments are given in Table V. Although the relatively low sample concentration made the 1-D spectrum difficult to analyze, the 2-D COSY spectrum confirmed all assignments. This peptide is closely related in amino acid sequence to the previously described C-phycocyanin peptide β-2T PCB.[8] Our previous work on the latter peptide had led us to the conclusion that the bilin was D-ring linked, based on dif-ferences between peptide β-2T and the A-ring-linked β-1 PCB peptide[8] in the ^{1}H NMR spectra, the CD spectra, and the chemical release behavior of bilin. However, more recent experiments, including NOESY data, have shown that the PCB in the C-phycocyanin peptide β-2T is also A-ring linked and that the ^{1}H NMR and CD spectra are influenced by conformational and/or configurational differences.[19]

The chemical shifts of the bilin resonances of the phyco-erythrocyanin pentapeptide β-2T PCB agree to within 0.03 ppm with the values found for the corresponding C-phycocyanin peptide. The obscured 2-H resonance, which deviates by 0.05 ppm, is the only exception. The correct assignment of the 2-CH$_3$ and 3'-CH$_3$ groups again was critical. The downfield 3'-H resonance is observed at 3.71 ppm and the 2-D COSY spectrum clearly verifies its connectivity to the upfield 3'-CH$_3$ doublet at 1.36 ppm. The other methyl doublet, 2-CH$_3$, is similarly connected to the
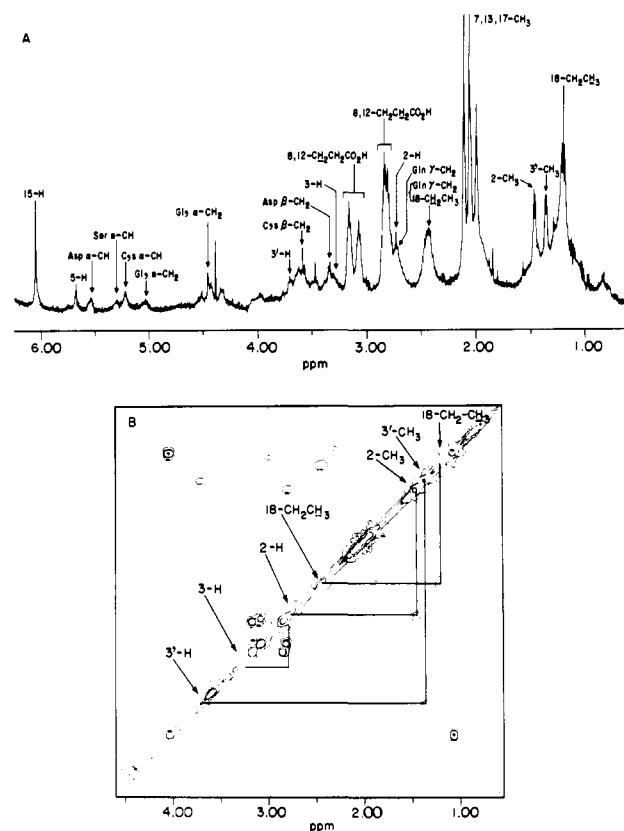
(19) Detailed configurational and conformational studies will be presented in a forthcoming publication.

Figure 8. (A) 1-D and (B) 2-D COSY ^{1}H NMR spectra of β-2T PCB in pyridine-d_5 at 25 °C (upfield region).

Table V. ^{1}H NMR (500-MHz) Assignments for β-2T PCB in Pyridine-d_5 at 25 °C

chem shift	multiplicity, J_{H-H}, Hz	no. of Hs	assignment
7.27	s	1	10-H
6.06	s	1	15-H
5.68	s	1[a]	5-H
5.54	m	1	Asp α-H
5.30	m	1	Ser α-H
5.22	m	1	Cys α-H
2.04	m	1	Gln α-H
4.47	br s	2	Gly α-CH$_2$
4.33–4.42	m	2	Cys β-CH$_2$
3.71	m	1	3'-H
3.32	m	1	3-H
3.08–3.17	dt, 6.2, 7.2	4	8,12-CH_2CH$_2$CO$_2$H
2.83	m	4	8,12-CH$_2$CH_2CO$_2$H
2.78[b]	m	1	2-H
2.75[b]	m	2	Gln γ-CH$_2$
2.46[b]	m	2	18-CH_2CH$_3$
2.46[b]	m	2	Gln β-CH$_2$
2.11	s		
2.06	s	9	7,13,17-CH$_3$
2.00	s		
1.46	d, 6.7	3	2-CH$_3$
1.36	d, 6.3	3	3'-CH$_3$
1.20	t, 7.5	3	18-CH$_2$CH_3

[a] Deuterium exchange is observed at this position. [b] Overlapping resonances prohibit precise assignment of the chemical shift.

2-H resonance at 2.78 ppm and is observed at 1.46 ppm. This 2-CH$_3$ resonance agrees very well with that seen in the spectrum of C-phycocyanin peptide β-2T PCB (1.48 ppm).[8]

Circular Dichroism Spectroscopy. The origins of the circular dichroism features seen in the spectra of different bilipeptides are not fully understood. However, it is likely that the factors that contribute to the variation in the NMR spectra also strongly influence the circular dichroism spectra of A-ring-linked bilins attached to different peptides. The CD spectra of the three *A. variabilis* PEC bilipeptides, α-1 PXB, β-1 PCB, and β-2T PCB,
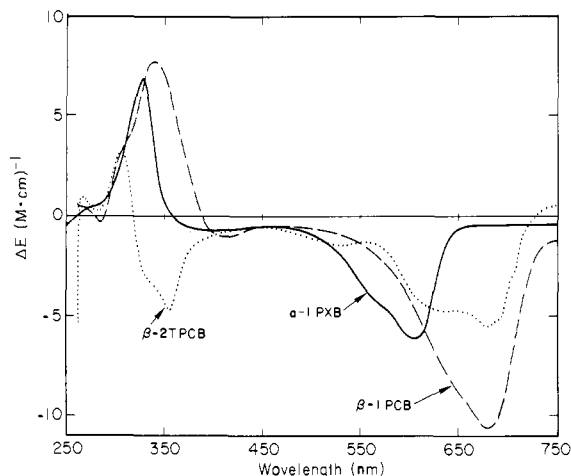
Figure 9. CD spectra of 35.4 μM α-1 PXB (—), 55.3 μM β-1 PCB (- - -), and 56.3 μM β-2T PCB (···) from *A. variabilis* PEC. All spectra were obtained in 10 mM aqueous TFA at 22 °C.

in 10 mM aqueous TFA are shown in Figure 9. The α-1 PXB group from *M. laminosus* PEC exhibited a CD spectrum identical with that from *A. variabilis*.

The β-1 PCB group shows an intense negative Cotton effect at 680 nm followed by a strong positive Cotton effect at 340 nm. This behavior is strongly reminiscent of the spectrum obtained from the A-ring-linked β-1 PCB group of C-phycocyanin[7,8] and is compatible with A-ring linkage for the PEC-derived pigment.

The PEC-derived β-2T PCB group does not share similarity with the corresponding C-phycocyanin pigment in the high-wavelength region of the CD spectrum: a negative Cotton effect is observed between 600 and 700 nm, whereas the latter pigment showed a negative Cotton effect at 680 nm and a relatively strong positive Cotton effect at approximately 620 nm.[8] It has been reported that the CD behavior of bilipeptides is extremely concentration dependent, and the formation of different aggregation states appears to have a pronounced effect on the sign of the visible CD peak. The peaks in the near-UV region are, however, less concentration dependent. Here, the PEC-derived β-2T PCB group shows a strong negative Cotton effect at 364 nm like that seen with the β-2T of C-phycocyanin.[8] Two well-defined peaks are observed in the CD spectrum of α-1 PXB: an intense positive Cotton effect at 328 nm and an almost equally intense negative Cotton effect in the visible region (604 nm).

Peptide Homology. As noted before, the spectroscopic data on α-1 PXB do not lead to a differentiation between the A-ring (Figure 4, **1**) and D-ring (Figure 4, **4**) structures. The peptides sequence of α-1 PXB bears strong homology to several bilin peptides containing A-ring-linked phycocyanobilin or phycoerythrobilin.[9,11,12,13] This comparison suggests that the PXB moiety is also linked through ring A (Figure 4, **1**).

Stereochemistry. The structures of the bilin groups presented in this study are shown with a linear, all-Z arrangement of pyrrole groups, although it has been theorized that the bilin assumes a helical conformation in solution.[2] The structures also show assignments of absolute stereochemistry. These assignments are in accord with the existing data;[20] however, they are nonetheless provisional.

The present data suggest that the absolute stereochemistry of the linkage ring of β-1 PCB is 2R,3R,3'R.[21] This assignment carries the reservations discussed previously[8] and is further complicated by the existence of the two spectroscopically and chemically distinct types of pigment found in the native protein.

The stereochemistries of C-3' and C-4 on the PXB moiety have not been determined. The R assignment at C-3' is based solely

on the studies conducted on phycocyanobilin cited above, while the assignment of R stereochemistry at C-4 is made based on the synthetic studies of optically active phycoerythrobilins, which have been shown to have an R absolute stereochemistry at the analogous C-16 position.[22] Clearly, the determination of absolute stereochemistry on all of the bilin groups must be established by synthesis of the various diastereomeric peptide-bound chromophores.

Summary

The isolation and structure of the PXB chromophore from the α subunit of *A. variabilis* phycoerythrocyanin has been described for the first time. The corresponding PXB group from *M. laminosus* phycoerythrocyanin has been found to be spectroscopically and, therefore, structurally identical.

The two PCB groups from the β subunit of *A. variabilis* PEC, β-1 and β-2T, were also studied and the chromophore structures were found to be identical with the bile pigments derived from the β subunit of C-phycocyanin.

Experimental Procedures

General Methods. Amino acid compositional analysis, sequence analysis, peptide desalting, and HPLC were performed as previously described.[23] Phycocyanobilin-containing peptide concentrations were determined by using the extinction coefficient of 33.8 mM^{-1} cm^{-1} at 650 nm.[24] PXB-containing peptide concentrations were determined by using an extinction coefficient of 38.6 mM^{-1} cm^{-1} at 590 nm, determined in 10 mM aqueous TFA by quantitative amino acid analysis of the α-1 peptide utilizing a norleucine internal standard. PXB chromophore determinations in crude mixtures were corrected for the PCB contribution at 590 nm by subtracting 48% of the absorbance at 650 nm.[24]

Isolation of Bilipeptides α-1 PXB, β-1 PCB, and β-2T PCB from *A. variabilis* Phycoerythrocyanin. Phycoerythrocyanin Tryptic Digestion and Initial Fractionation. Phycoerythrocyanin (A_{568}/A_{615} = 1.51) was purified from *A. variabilis* as previously described.[15] The purified phycoerythrocyanin (2.7 μmol [αβ], 97 mg) was exhaustively dialyzed against 1 mM NaPi, pH 6.8. For trypsin digestion, the protein solution 7.8 mg/mL) was adjusted to pH 2 by the addition of 1 M HCl to 20 mM, TPCK–trypsin (Worthington) was added to 2% (w/w), solid NH$_4$HCO$_3$ was added to 100 mM, and the pH was adjusted to 8 with 2 M NaOH. The solution was sealed under N$_2$, incubated in the dark at 34 °C for 2 h, and twice spiked with additional TPCK–trypsin (2 w/w) at 2-h intervals. After 6 h of digestion, the incubation was acidified by adding glacial acetic acid to 30% (v/v), the digest was clarified by centrifugation, and the soluble portion was subjected to gel filtration (2.5- × 110-cm Sephadex G-50 column, 30% (v/v) aqueous acetic acid). Two colored peaks were eluted: a medium-sized phycocyanobilin-containing peptide fraction and a fraction of small peptides containing both PEB and PXB chromophores. The first and second colored peptide peaks were labeled fraction II and fraction III, respectively, by analogy of their elution position relative to our previous purification of bilipeptides.[12,23]

Purification of Fraction III Bilin-Containing Peptides. The fraction III peptide solution (approximately 3 μmol PCB and 2 μmol PXB) was concentrated to a small volume by rotary evaporation, diluted to the original volume with 10 mM acetic acid, and reconcentrated to approximately 12 mL. The sample was clarified by centrifugation and diluted with an equal volume of 50 mM NaP$_i$,[6] pH 2.5 and then chromatographed on a 18 mL SP-Sephadex column which had been equilibrated in 50 mM NaP$_i$, pH 2.5, and immediately developed with a 450-mL linear gradient of 0–0.55 M NaCl in the same buffer. Two peaks were eluted after the middle of the gradient: (1) a PCB-containing fraction (labeled β-1) and (2) a PXB-containing fraction (labeled α-1). These components were recovered in equimolar amounts. The α-1 and β-1 bilipeptides were purified each to a single component by high-pressure liquid chromatography under isocratic conditions in 26% and 27% acetonitrile, respectively, and then desalted by chromatography on C-18 columns and stored at 4 °C.

Thermolysin Subdigestion of Fraction II. Fraction II (2 μmol PCB), labeled β-2, was rotary evaporated to complete dryness and dissolved in 5 mL of 10 mM HCl. The peptide solution was adjusted to 5 mM CaCl$_2$, thermolysin was added to 3% (w/w), solid NH$_4$HCO$_3$ was added to 100 mM, and the pH was adjusted to 8 with 2 M NaOH. The reaction mixture was sealed under N$_2$ and incubated in the dark at 30 °C for 3

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Chromopeptides from Phycoerythrocyanin

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h and then spiked with fresh thermolysin (3% w/w) and incubated for a second 3-h period. The reaction was stopped by adjusting the pH to 2 with 1 M HCl, and the mixture was clarified by centrifugation, diluted with one volume of 10 mM HCl, and chromatographed on an 18-mL SP-Sephadex column which had been equilibrated in 50 mM NaP_i, pH 2.5. The column was immediately developed with a 400-mL linear gradient of 0–0.3 M NaCl in the same buffer. The phycocyanobilin-containing peptide peak which eluted near 0.15 M NaCl was collected, labeled β-2T, and purified to a single component by high-pressure liquid chromatography under isocratic conditions in 26% acetonitrile. The purified chromopeptide was desalted by chromatography on a C-18 column and stored at 4 °C.

Isolation of α-1 PXB from *M, laminosus* Phycoerythrocyanin. *M. laminosus* phycobilisomes were obtained as previously described[25] and stored at −25 °C as a 50% saturation ammonium sulfate precipitate. Precipitated phycobilisomes (A_{625} = 80 cm⁻¹, 50 mL) were diluted 10-fold with 5 mM potassium phosphate, 1 mM sodium azide, and 1 mM phenylmethanesulfonyl fluoride, pH 7.05, and concentrated to 40 mL by ultrafiltration (Diaflo, PM-10). The protein solution was brought to 5 mM potassium phosphate and 1 mM sodium azide, pH 7.05, by gel filtration on a column (2.8 × 51 cm) of Sephadex G-25 (superfine). The phycobiliprotein aggregates were separated on a Cellex-D anion-exchange column (2.7 × 30 cm), which had previously been equilibrated in 5 mM potassium phosphate and 1 mM sodium azide, pH 7.05. Phycoerythrocyanin eluted with the application buffer. Formic acid was added to the phycoerythrocyanin solution to 50% by volume. The solution was concentrated to 10 mL on a rotary evaporator and then chromatographed on two Bio-Gel P-100 (100–200-mesh) columns in 50% formic acid. The intensely colored α and β subunits eluted in one peak. After lyophilization, approximately 45 mg of pure phycoerythrocyanin was obtained.

Lyophilized phycoerythrocyanin (360 mg) was suspended in 20 mL of 100 mM ammonium bicarbonate, pH 8.6. L-(1-(Tosylamido)-2-phenyl)ethyl chloromethyl ketone treated trypsin (2380 u/mg) was dissolved in 1 mM HCl to give a stock solution of 20 mg/mL. After 0, 1, and 2 h, 3% (w/w) of trypsin from the stock solution was added to the phycoerythrocyanin suspension. The digestion was carried out at 37 °C in the dark under Freon and was stopped after 4 h by adjusting the pH to 2.5 with concentrated formic acid.

The peptides were applied to four Bio-Gel P-10 (<400-mesh) columns, equilibrated with 63 mM formic acid. The chromopeptide from the α subunit was diluted with formic acid (1:1), concentrated to 2 mL, and rechromatographed using 50% formic acid.

Final purification of the α-1 PXB tripeptide was done by the HPLC and desalting procedures previously described.[23]

Mass Spectra. The fast atom bombardment (FAB) mass spectrum of the α-1 PXB tripeptide (200 μg/mL in 10 mM aqueous TFA solution) was obtained by dissolving a few microliters of solution in a matrix of glycerol and then scanning at 8 kV by using xenon gas on a Kratos MS-50 instrument.[26] The elemental composition of certain peaks was determined by peak matching against glycerol adducts.

¹H NMR Spectra. Each chromopeptide in approximately 60% acetonitrile/40% 10 mM aqueous TFA was concentrated to near dryness in the dark. TFA (10 mM) in 99.8% D_2O (made by adding 50 μmol of trifluoroacetic anhydride to 10 mL of D_2O) was then added to a final volume of 10 mL. The solution was concentrated to near dryness as before, and this procedure was repeated 3 times, twice by using 1.0 mL of 10 mM TFA in 99.96% D_2O and once by using 1.0 mL of 10 mM TFA in 99.996% D_2O.

The final samples were prepared in a glove bag under an atmosphere of dry nitrogen. The NMR samples in 10 mM TFA/D_2O were made by adding 0.5 mL of 10 mM TFA in 99.996% D_2O, while those in pyridine-d_5 were prepared by adding 0.5 mL of 100.0% pyridine-d_5. NMR samples were at the following concentrations: α-1 PXB, 1.6 mM; β-1 PCB, 2.2 mM; β-2T PCB, 1.2 mM.

The NMR spectra were obtained on a Bruker 500-MHz spectrometer at the University of California, Berkeley. Chemical shifts are expressed in parts per million (ppm) relative to an internal sodium 3-(trimethylsilyl)propionate-d_4 (TSP) standard for spectra taken in D_2O and relative to a residual pyridine proton resonance calibrated at 7.18 ppm (relative to Me_4Si) for spectra recorded in pyridine-d_5.

Circular Dichroism Spectra. After determining concentrations on a Perkin-Elmer 552A UV–vis spectrophotometer, CD spectra were taken in 10 mM aqueous TFA on a Jasco J-500C spectropolarimeter, at 22 °C.

Registry No. PXB, 10897-67-1; PCB, 20298-86-6.

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