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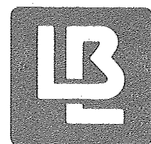

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CHROMOPEPTIDES FROM PHYTOCHROME. THE STRUCTURE AND LINKAGE OF THE P_R FORM OF THE PHYTOCHROME CHROMOPHORE

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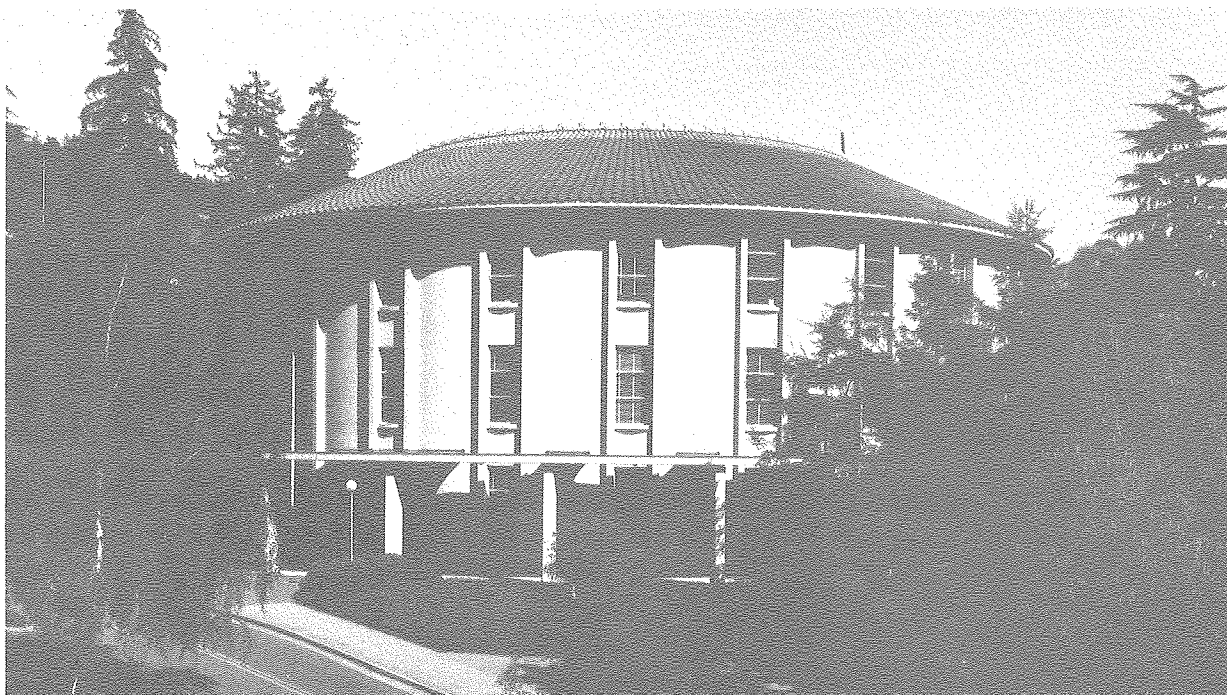

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Chromopeptides from Phytochrome. The Structure and Linkage of the P_R Form of the Phytochrome Chromophore.

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Abstract. The isolation and chromatographic purification of chromophore-containing peptides from the P_R form of phytochrome treated with pepsin and thermolysin are described. From the amino acid sequence and ^{1}H NMR spectral analysis of phytochromobiliundecapeptide (2), the structure of the P_R phytochrome chromophore and the nature of the thioether linkage joining pigment to peptide have been established. Confirmatory evidence was obtained from similar analysis of phytochromobilioctapeptide (3). The implications of this structural assignment with respect to the mechanism of the P_R to P_{FR} phototransformation is considered.

1 Owing to the wide range of light-controlled developmental and
2 metabolic processes in green plants believed to be mediated by
3 phytochrome, this biliprotein has been exhaustively studied by plant
fn 1 4 physiologists for many years.[1] Phytochrome has also received extensive
5 study by physical and biological chemists because it exists in two
6 spectrally distinct forms P_R (λ_{max} 665 nm) and P_{FR} (λ_{max} 720 nm)
fn 2 7 which are interconvertible upon absorption of light.[2] Despite the
8 tremendous interest in this unusual photoreceptor, neither the
9 chemical structure nor the precise nature of the chromophore-protein
10 linkage of the P_R or P_{FR} chromophore has been definitively
11 established.[2]

12 The numerous structures proposed for the phytochrome chromo-
13 phore have been based primarily on degradative approaches which have
14 involved spectroscopic analyses of altered forms of the chromophore,
fn 3 15 released from phytochrome after treatment with refluxing methanol[3]
fn 4 16 or with chromic acid.[4] In contrast to these previous studies of the
17 phytochrome chromophore, our approach is based on the chromophore
18 as well as the chromophore-protein linkage remaining unchanged
19 throughout the analysis. Previously we have successfully applied
20 this methodology to the structure elucidation of the β_1-phycocyano-
fn 5 21 biliheptapeptide (1) isolated from C-phycocyanin.[5] Now we provide
22 ^{1}H NMR spectroscopic evidence for the structure and linkage of the
23 P_R form of the phytochrome chromophore.

24 Fry and Mumford (1971) partially determined the amino acid sequence
25 of a phytochromobiliundecapeptide isolated from "small" oat phytochrome
fn 6 26 treated with pepsin.[6] In the present investigation, we describe the
27 isolation of phytochromobiliundecapeptide (2) and phytochromobiliocta-

1 peptide (3) following the sequential pepsin-thermolysin digestion of
2 oat phytochrome in the P_R form. ^1H NMR spectra were obtained, and
3 their analyses provided proof of the structure and thioether linkage
4 of the P_R form of the phytochrome chromophore.
5
6 Results and Discussion

7 Phytochrome Purification. The routine isolation of 50-60 mg
8 of brushite-purified oat phytochrome with a specific absorption ratio
9 (SAR=A667nm/A280nm) of 0.07[2a] from 4 kg batches of etiolated oat
fn 7 10 seedlings was accomplished as described.[7] Crude phytochrome fractions
11 eluted from brushite chromatography were assayed by measuring the
fn 8 12 double difference spectra with a modified Cary 118 spectrometer.[8]
Fig. 1 13 As shown in Figure 1, this low purity phytochrome was phototransform-
14 able, although a dramatic increase in turbidity accompanied the P_R
15 to P_{FR} conversion.

16 Pepsin-Thermolysin Digestion of Phytochrome. The isolation of
17 a chromopeptide fragment from the pepsin digest of brushite-purified
18 phytochrome in the P_R form was accomplished according to the reported
19 procedure[6] with several modifications. First, the phytochrome
20 used for this study was large, crude phytochrome (SAR=0.07) instead
21 of small purified phytochrome (SAR=0.2-0.9) employed in the previous
fn 9 22 study.[6,9] A second modification was the pretreatment of the BioGel P4
23 column with a mixture of 0.1N ascorbic acid and 0.01N EDTA. Unless
24 this precaution was taken before application of the digest mixture,
25 a decomposition product (λ_{max} 415 nm) appeared on the column at the
26 expense of compounds with longer wavelength absorptions. During
27 chromatography the majority of the blue color remained attached to

1 the BioGel P4. Even after three column volumes of 1.3M formic acid,
2 these chromopeptides were not removed from the column. Step elution
3 with 25% aq. acetic acid proved necessary to desorb the blue peptides
4 from the column. With these modifications, a chromopeptide with
Fig. 2 5 spectral characteristics similar to that reported[6] (Figure 2) was
6 obtained in 58% yield. This yield was based on absorbance, where the
7 extinction coefficient for P_R at 665 nm was $7.0x10^4$ L $mol^{-1}cm^{-1}$ at
fn 10 8 pH 7.8[10] and for phytochromobilipeptides at 665 nm was $3.2x10^4$ L mol^{-1}
9 cm^{-1} in 25% aq. acetic acid.[6]

Table I 10 As shown in Table I, the amino acid composition of our chromo-
11 peptide fraction is similar to that reported.[6] The presence of
fn 11 12 cysteine in this chromopeptide fraction was clearly established.[11]
13 However, the presence of small amounts of threonine, glycine, iso-
14 leucine, and phenylalanine, in addition to the low value of arginine,
15 indicated the inhomogeneity of this chromopeptide fraction. The
16 ^{1}H NMR spectrum of this fraction also showed the presence of a con-
17 taminant which appeared to be derived from the polyacrylamide matrix
18 of the BioGel P4 column (see supplementary materials, Figure 1).

19 The similarity of the amino acid composition of this pepsin
20 chromopeptide fraction with that of the undecapeptide reported
21 previously[6] suggested that a thermolysin cleavage would result in
22 shortening of the peptide chain. Thermolysin has been an effective
23 tool for sequence analysis of chromopeptides from C-phycocyanin,
24 through selective cleavage at the amino termini of leucine or iso-
fn 12 25 leucine residues.[12] Thus we subjected our pepsin chromopeptide
26 fraction to thermolysin digestion with the following modification of
27 the reported procedure. Before incubation at 37°C for 4 h, the

1 initial chromopeptide mixture was dissolved in 0.1N ammonium bicar-
2 bonate with the addition of 3% thermolysin (w/w), degassed by freeze-
3 thawing, and sealed under vacuum. Taking these precautions and
4 avoiding exposure of the sample to light virtually eliminated the
5 color changes which accompany chromophore decomposition. After
6 digestion, the mixture was applied to a Sephadex G50 column equili-
7 brated with 25% acetic acid. Elution with 25% acetic acid afforded
8 a 47% overall recovery of a chromopeptide fraction with unchanged
9 absorption properties (see supplementary materials, Figure 2).

fn 13 10 Subsequent high performance liquid chromatography (HPLC)[13] of
Fig. 3 11 this fraction resolved five major chromopeptide components (Figure 3)
12 with indistinguishable absorption spectra in 30% overall yield. The
13 amino acid composition of fractions 2 through 5 were next determined
14 (Table I). These results showed that the four fractions were different
15 sized peptides derived from the same polypeptide chain. The peptide
16 obtained in the largest quantity and the purest, fraction 5, was an
17 undecapeptide **2** with the same amino acid composition as the reported
18 phytochromopeptide.[6] The absorption spectrum of the undecapeptide **2**
Fig. 4 19 is shown in Figure 4.

20 Fraction 3 was an octapeptide **3** with an amino acid composition
21 identical to that of the undecapeptide less the three residues leucine,
22 glutamine, and tyrosine. Although fractions 2 and 4 were not as
23 pure as the octa- and undecapeptides, these fractions appear to be
24 a heptapeptide and a decapeptide, respectively. The similarity in
25 the core composition of all four chromopeptides showed that these
26 peptides are all derived from the same region of the phytochrome
fn 14 27 polypeptide chain.[14] Although conventional hydrolysis of fractions

1 3 and 5 gave low yields of half cystine, oxidation for 20 h with 0.2M
2 DMSO/6N HCl released 0.8 residue of cysteic acid.[11] This result
3 confirmed the presence of one residue of cysteine in these chromo-
4 peptides.

5 Sequence analysis of the phytochromobiliundecapeptide (2)
6 established the amino acid sequence to be Leu-Arg-Ala-Pro-His-Ser-
7 Cys-His-Leu-Gln-Tyr. By analogy, the sequence of the octapeptide 3
8 was ascertained. The results of the Edman degradation of 2 are
Table II 9 summarized in Table II. With the exception of the cysteine derivative,
10 the PTH derivatives of the cleaved amino acids were determined by TLC,
11 HPLC, and mass spectrometry. Back hydrolysis of the sixth step
12 yielded alanine as a confirmation for the initial presence of serine
13 at this step. After the seventh step of the Edman degradation the
14 blue color, which mostly remained in the sequenator cup throughout the
15 analysis, was extracted into the butyl chloride washes. That cysteine
16 was removed during this step was confirmed by hydrolysis of the
17 evaporated butyl chloride extracts with 0.2M DMSO in 6N HCl for 20 h.
18 Amino acid analysis of this hydrolysate showed a good recovery of
19 cysteic acid.

20 <u>^{1}H NMR Spectral Analysis</u>. The 270 MHz ^{1}H NMR spectrum of phyto-
fn 15 Fig. 5 21 chromobiliundecapeptide (2) in [$^2H_5$]-pyridine[15] is shown in Figure 5.
22 Complete analysis of this spectrum shows that with respect to the
23 chromophore moiety this chromopeptide is quite similar to β_1-phyco-
fn 16 24 cyanobiliheptapeptide (1).[5,16]

25 The major difference between the two spectra can be explained
26 by the replacement of the ethyl group of 1 with a vinyl group as
27 in structure 2. A new ABX pattern in the spectrum of undecapeptide

2 has replaced the high field A_3X_2 pattern of the C-18 ethyl group of 1. The assignments of these terminal olefin resonances of 2 shown in Table III were confirmed by computer simulation. As illustrated in Figure 6, excellent agreement between the experimental spectrum of the vinylic ABX pattern and the computer-simulated spectrum was observed. Furthermore, the small differences (<0.1 ppm) between the spectra of the bilin moieties of 1 and 2 (i.e., the lower field values of the C-15 methine bridge hydrogen and the C-17 methyl group) can be attributed to the effect of the vinyl group in 2 on the electron density, especially in ring D.

The assignment of the structure of the dihydro A-ring and the 3'-thioether linkage of 2 was based on double irradiation experiments illustrated in Figure 7. By analogy to the assignments for 1,[5] the two doublets at 1.38 and 1.43 ppm in the spectrum of chromopeptide 2 have been assigned to the C-2 and C-3' methyl groups. Collapse of the multiplet at 2.64 ppm to a doublet with 5.0 Hz spacing, after irradiation of the C-2 methyl doublet at 1.38 ppm (insert c, Figure 7) supported the dihydro A-ring structure for 2. Irradiation of the 2.64 ppm multiplet lead to changes in the multiplicity of the signal at 3.18 ppm (insert b, figure 7) while also collapsing the 1.38 ppm doublet to a singlet (insert f, Figure 7). The assignment of the C-3-H resonance to 3.18 ppm was confirmed when the C-2 proton multiplet at 2.64 ppm became a quartet with J=7.3 Hz during double irradiation at 3.18 ppm (insert d, Figure 7). The vicinal relationship of the C-3' proton at 3.50 ppm and the C-3' methyl at 1.44 ppm was similarly established by spin decoupling experiments (inserts a,e, Figure 7).

A second linkage involving the propionic acid side chains of 2

1 is highly unlikely due to the near identity of the chemical shifts
2 of the propionic acid methylenes (C-8 and C-12) of the two chromo-
3 peptides **1** and **2**. An ester linkage through the tyrosine phenolic
4 group can be clearly ruled out because of the isolation of chromo-
5 peptides lacking tyrosine. The ^{1}H NMR spectrum of one of these
6 peptides, phytochromobilioctapeptide (**3**), was obtained and showed no
7 difference in the chemical shifts of the C-8 and C-12 methylenes (see
8 supplementary materials, Figure 3). With respect to the thioether
9 linkage in ring A, the spectra of the two phytochromobilipeptides **2**
10 and **3** were the same as well. Furthermore, by comparison of the ^{1}H
11 NMR spectrum of **2** with **1** and other chromopeptides from C-phycocyanin
12 and R-phycoerythrin, bilin-peptide linkages through the side chains
fn 17 13 of arginine, serine and glutamine can be ruled out.[17]

14 Since it is improbable that an ester or amide linkage would be
15 cleaved during the proteolytic digests and subsequent purification
16 procedures used in this study, we conclude that P_R phytochrome is
17 singly bound to the apoprotein through a thioether linkage. Owing
18 to the near identity of the ^{1}H NMR spectra of the two chromopeptides
19 **1** and **2** with respect to the bilin moieties, we have assigned this
20 linkage through ring A of the phytochromobilin in **2** in preference
21 to a ring D linkage. Experiments to provide direct proof for this
22 assignment are in progress.

23 Stereochemistry. The similarity of the ^{1}H NMR spectra of the
24 bilin moieties of phytochromobiliundecapeptide (**2**) and β_1-phycocyano-
25 biliheptapeptide (**1**)[5] suggests that the dihydro A-ring of both bilins
fn 18 26 has the trans stereochemistry.[18] The relative stereochemistry at
27 C-3, C-3' was proposed to be R,R (or S,S) based on the isolation of

(E)-2-ethylidene-3-methylsuccinimide (4) from chromic acid treated phytochrome.[4e] Since none of the Z isomer was detected after this treatment, a concerted trans-periplanar elimination of the cysteine thioether linkage was proposed.[4e] Assuming this mechanism, the isolation of the E isomer 4 therefore required the stereochemistry at C-3,C-3' to be R,R (or S,S).[4e] Based on these assumptions, the stereochemistry of the dihydro A-ring of phytochromobilin (C-2, C-3, C-3') is R,R,R (or S,S,S). The R,R,R representation has been incorporated into structures 1, 2, and 3. Since the optical activity of E-succinimide 4 has not yet been reported, the absolute stereochemistry of the phytochrome chromophore remains in doubt. These questions will be addressed in a future report on the stereochemistry of biliprotein chromophores.

Phototransformation mechanism $P_R \rightleftarrows P_{FR}$. Many different structural possibilities have been proposed to account for the P_R to P_{FR} phototransformation. Most of these hypotheses are incompatible with the experimental evidence from the present study. Nonetheless, the more popular proposals illustrated in Scheme I[4a,b,e,19,20,21] have all received support from quantum mechanical calculations to explain the spectral differences of P_R to P_{FR}.[22,23,24] A number of postulates[19,20,4a,4b], Scheme Ia-d respectively, are irreconciliable with the ^{1}H NMR spectral data of the phytochromobilipeptides 2 and 3. On the other hand, P_R chromophore structures which are consistent with our experimental results have been proposed as models for phytochrome phototransformation,[4e,21] Scheme I e and f. These proposals differ with respect to the structure of P_{FR} where Klein, et al[4e] propose an anionic biliveridin chromophore and Song, et al[21] suggest a bili-

1 verdin type prosthetic group for P_{FR}. Both of these models for P_{FR}
2 are based on spectral analysis of native and denatured P_{FR} and have
3 stimulated theoretical and spectroscopic analysis of model compounds
4 including various tripyrrinones such as mesobiliviolin 5,[4c,23] and
fn 25 5 biliverdin 6[21,22,25] in support of the former[4e] and latter hypotheses,[20]
6 respectively. Unfortunately both proposals fail to adequately explain
7 the results of Fry and Mumford,[6] who isolated the spectrally identical
8 peptide from both P_R and P_{FR}. On the basis of pKa considerations it
9 is difficult to conceive of a chromophore-protein interaction which
10 would stabilize the anionic biliviolin structure for P_{FR} also pro-
11 posed,[4c] Scheme Ie. Theoretical calculations[21] as well have cast
Scheme I here 12 considerable doubt on the validity of this proposal.

13 Perhaps the experimental results[4c] which led to the biliviolin
14 P_{FR} structural hypothesis could be reinterpreted. In an earlier
15 study[5] we observed the incorporation of deuterium at the C-5 methine
16 bridge of β_1-phycocyanobiliheptapeptide (1). This observation led to
17 a mechanistic proposal for the elimination of the blue pigment 7 from
18 C-phycocyanin during methanolysis.[5] The proposed intermediate 8 in
19 this process, an isomer of 1, has a biliviolin type chromophoric
20 system, a fact we used to explain the occurrence of purple pigments
21 (λ_{max} ~590 nm at acidic pH's) which often accompany the purification
22 procedure. The biliviolin type spectrum obtained after denaturing
23 P_{FR} phytochrome in acidic 8M guanidinium chloride (λ_{max} ~610 nm, pH
24 1.5) could be due to such an artifactual isomerization product derived
25 from the native P_{FR} chromophore. In our hands these types of bili-
fn 26 26 triene peptides show a pronounced tendency to isomerize.[26] The
27 previous results[4c] therefore could indicate that P_{FR} is more labile

with respect to double bond isomerization than P_R.

While transformation from a dihydrobiliverdin to a biliverdin-type chromophore as proposed in Scheme If[21] seems reasonable, the reverse conversions appears to be energetically unfavorable. The reported instability of the P_{FR} form of phytochrome and its reversion to P_R is not consistent with this proposal, neglecting any protein stabilization. Furthermore, this proposal of Scheme If requires cleavage of the thioether linkage during phototransformation. Based on our experimental evidence for a single linkage, that of the thioether, this mechanism would lead to the complete covalent release of the phytochrome chromophore from the apoprotein during phototransformation. Therefore in principle, this P_{FR} chromophore should be easily extractable with organic solvents. There is no indication from other studies on phytochrome that this is the case.[2]

Because of the instability of the P_{FR} form of phytochrome, indicated by its reversion to P_R under a variety of conditions, and the fact that small chromopeptides derived from phytochrome are not photoreversible,[2] it is generally accepted that the role of the apoprotein in stabilizing the P_{FR} form is an important one. What effect phototransformation has on the apoprotein structure and how this relates to the physiological responses elicited by phytochrome in plants is an area of much speculation.

We would like to suggest three hypotheses (Scheme IIa,b,c)[27] for the phototransformation of phytochrome based on the above considerations and the evidence presented in this report. These mechanisms show the chromophoric system in linear conformations for simplicity. We do not intend to imply the absence of conformational changes of

1 the chromophore accompanying phototransformation, which is an area of
2 controversy among theoreticians.[21-24] The bond making/bond breaking
3 hypothesis of Scheme IIa has support from studies which show that 1.7
4 additional cysteine-SH groups become surface labelable after P_R to
fn 28
5 P_{FR} phototransformation.[28] Whether this indicates the cleavage of
6 the cysteine thioether linkage remains to be determined. To give the
7 spectral shift for P_{FR} and to insure that P_{FR} is covalently linked,
8 the Schiff's base type linkage, perhaps via lysine or histidine
9 residues, is proposed in Scheme IIa.

10 In Scheme IIb the spectral red shift for P_{FR} is rationalized by
11 means of an acyl enol linkage (perhaps via aspartic acid or glutamic
12 acid) giving rise to an additional double bond in the chromophoric
13 system, as well as a positive charge migration from ring C to ring A.
14 Both of these hypotheses suggest experiments which can establish
15 the nature of any new chromophore-protein linkages, and such experi-
16 ments are actively being considered.

17 A third postulate for phytochrome phototransformation is shown in
18 Scheme IIc. This mechanism results in the movement of positive charge
19 from the center of the chromophoric system to the terminal ring A as
20 well as an increase in conjugation by lactam-lactim interconversion.
21 Accompanying this reorientation of charge is the change in association
22 of the chromophore with the apoprotein. One possibility for this assoc-
23 iation could involve the imidazole side chains of histidine, which
24 can act as both a nucleophile (Nu) and an electrophile (E). This could
25 explain the unusual occurrence of two histidines in the peptide back-
26 bone immediately adjacent to the chromophore. The loss of a proton
27 from ring C during phototransformation to P_{FR} would remove the steric

fn 29 effect restricting partial cyclization of the bilitriene.[29] The smaller extinction coefficient of P_{FR} versus that of P_R has been interpreted as supporting that P_{FR} assumes a more cyclic conformation from the extended, linear form of P_R during phototransformation.

Recent evidence from protein surface labeling experiments of highly purified phytochrome has shown differences in the surface properties of P_R and P_{FR}.[28] Selective chemical modification of the P_R and P_{FR} chromophores in a similar manner might provide some understanding of the differences in the chemical and physical association of the phytochrome chromophore with the apoprotein. Such experiments also are being considered.

1 Experimental Section

2 Materials. Oats (Avena sativa L., cv. Garry), obtained
3 from Whitney, Dickinson Seeds, Inc. Buffalo, N.Y., were grown and
4 harvested as previously described,[7] and stored at -20°C until extracted.
5 Pepsin (Worthington Biochemicals, Anson activity grade 2500-3000 units)
6 and thermolysin (Sigma Chemical Co., activity 63 units/mg solid) were
7 used for proteolysis experiments. HPLC grade CH_3CN from Burdick and
8 Jackson and water purified with a Milli-Q system (Millipore Corp.)
9 were used for HPLC.

10 Instrumentation. ^1H NMR spectra of the chromopeptides were
fn 30 11 taken in $[^2H_5]$-pyridine solution at 23-25°C on a homemade spectrometer.[30]
12 HPLC was done using a Spectra Physics 3500 B instrument equipped with
13 a Schoeffel 770 variable wavelength detector. Absorption spectra and
14 phytochrome spectral assays were taken on a modified Cary 118 spectro-
15 meter.[8] Amino acid analysis were performed on a Beckman 120C Analyser
16 by the Analytical Laboratory, Department of Chemistry, University of
17 California, Berkeley. Amino acid sequence analysis were obtained on
18 a Beckman 890C Sequencer by Dr. Al Smith at the Department of Bio-
19 chemistry and Biophysics, University of California, Davis.

20 Phytochrome preparation. The handling of plants and the purifi-
fn 31 21 cation procedure were performed under green safelight.[31] Undegraded
22 phytochrome (MW 120,000) was repetitively isolated from 4 kg etiolated
23 oat seedlings according to the method of Hunt and Pratt.[7] Crude
24 extracts were partially purified by brushite chromatography (bed
25 volume ~1.5 ℓ, 13 cm diameter).[7] After spectral assay,[8] the phytochrome
26 containing fractions (>5 μg/ml) were combined and precipitated with
27 200 g/ℓ solid $(NH_4)_2SO_4$. The precipitate was then resuspended in

1 ~50 ml 0.1M K_2HPO_4/KH_2PO_4 buffer, pH 7.8, and measured by spectral
2 assay.[8] The typical yield of brushite phytochrome from 4 kg etiolated
3 oats was 50-60 mg (SAR=0.07, Figure 1). After a saturating far red
4 irradiation (using a Sylvania 150W flood lamp impinging on an Optical
5 Industries 720 nm interference filter, 10 nm bandwidth), the brushite
6 phytochrome solution was stored frozen at -20°C in the dark.

7 Phytochromobilipeptides. Pepsin Digestion. This procedure was
8 performed under green safelight[31] at 4°C using a modification of the
9 published procedure.[6] Brushite P_R phytochrome (614 mg, 5.1 μmoles,
10 SAR=0.06) in 1ℓ 0.1M K_2HPO_4/KH_2PO_4 buffer, pH 7.8, was precipitated
11 with 300 g solid $(NH_4)_2SO_4$. After centrifugation (15 min, 20,000X g)
12 the pellet was suspended in 1.3M HCOOH (200 ml) and stirred overnight.
13 Nine hours later this suspension was centrifuged (15 min, 20,000X g).
14 The precipitate was resuspended in 1.3M HCOOH (131 ml) to which a
15 solution of pepsin (32 ml, 8.9 mg/ml in 1.3M HCOOH) was added. The
16 mixture was then incubated with stirring for 4.5 hr at 37°C under
17 Ar. After digestion, the mixture was centrifuged (15 min, 20,000X g),
18 rotary evaporated to 25 ml, and applied to a Bio Gel P4 column (2.5x
19 33.5 cm, flow rate 45 ml/h; preequilibrated with 1.3M HCOOH).[32]
20 The column was then washed with 550 ml 1.3M HCOOH while most of the
21 blue material remained adsorbed to the first 2/3 of the column. This
22 blue fraction was eluted from the column with 25% aq. HOAc and col-
23 lected in a 168 ml volume. Figure 2 shows the absorption spectrum
24 of this fraction, which represents 3.1 μmol (58% yield) of phytochromo-
25 bilin (with $\varepsilon_{665\ nm} = 3.2 \times 10^4\ L mol^{-1} cm^{-1}$).[6] The amino acid composition
26 of this fraction is compiled in Table 1. The ^1H NMR spectrum is illus-
27 trated in Figure 1 of supplementary materials.

in 32

Thermolysin Digestion. To minimize photochemical side reactions, this procedure was performed under green safelight[31] or in the dark whenever possible. The pepsin-cleaved phytochromobilin peptide fraction was lyophilized in two equal portions in 5 ml ampules. The dry, blue residues were then dissolved in 1.0 ml 0.1N NH_4HCO_3 to which 200 µl thermolysin solution (0.51 mg/ml in 0.1N NH_4HCO_3) was added. After freeze-thaw degassing twice, the ampules were sealed under vacuum. The mixtures were incubated at 37°C for 4 h, cooled in ice, and 300 µl glacial acetic acid was introduced into each ampule.

The resulting dark blue solution was applied to a Sephadex G50 column (medium, 2.5x50 cm, flow rate 49 ml/h, pre-equilibrated with 25% aq. EOAc) and eluted with 25% aq. HOAc. A colorless 134 ml fraction was collected before the phytochromobilipeptide fraction eluted within 64 ml. Based on the absorption spectrum of this fraction, the recovery was determined as 2.4 µmol, 47% overall yield, of phytochromobilipeptides (see supplementary materials, Figure 2).

HPLC of this thermolysin chromopeptide mixture was accomplished on a C_{18} reversed phase column. As shown in Figure 3, five major phytochromobilipeptides, fractions 1-5, and three cleaved pigments, fractions 6-8, were obtained after HPLC. In Tables I and II, the amino acid composition of fractions 2-5 and the sequence data for fraction 5 are tabulated. The absorption and ^1H NMR spectra of fraction 5, phytochromobiliundecapeptide (2), are illustrated in Figures 4-7 and Table III.

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Supplementary Material Available: Full details of the ^{1}H NMR spectra of the peptide moiety of phytochromobiliundecapeptide (2), the pepsin peptide from phytochrome, and the phytochromobiliocta-peptide (3), and the adsorption spectrum of the chromopeptide fraction after thermolysin digestion (5 pages). Ordering information is given on any current masthead page.

References and Notes

(1) Phytochrome mediated physiological responses in plants have been reviewed by Satter, R. L.; Galston, A. W. in "Chemistry and Bio-Chemistry of Plant Pigments", Goodwin, T. W. Ed.; Academic Press: New York, 1976; Vol. 1, pp 680-735.

(2) For recent reviews of phytochrome biochemistry and chromophore chemistry see: (a) Pratt, L. H. Photochem. Photobiol. 1978, 27, 81-105; (b) Kendrick, R. E.; Spruit, C. J. P. Photochem. Photobiol. 1977, 26, 201-14.

(3) Siegelman, H. W.; Turner, B. C.; Hendricks, S. B. Plant Physiol. 1966, 41, 1289.

(4) (a) Rudiger, W.; Correll, D. L. Liebigs Ann. Chem. 1969, 723, 208; (b) Rudiger, W. in "Phytochrome", Mitrakos, K.; Shropshire, W. Eds.; Academic Press: New York, 1972; pp 129-141; (c) Grombein, S.; Rudiger, W.; Zimmermann, H. Z. Physiol. Chem. 1975, 356, 1709; (d) Schoch, S.; Rudiger, W. Liebigs. Ann. Chem. 1976, 559; (e) Klein, G.; Grombein, S.; Rudiger, W. Z. Physiol. Chem. 1977, 358, 1077.

(5) Lagarias, J. C.; Glazer, A. N.; Rapoport, H. J. Am. Chem. Soc. 1979, 101, 5030.

(6) Fry, K. T.; Mumford, F. E. Biochem. Biophys. Res. Comm. 1971 45, 1466.

(7) Hunt, R. E.; Pratt, L. H. Plant Physiol. 1979, 64, 327, 332.

(8) Jung, J.; Song, P.-S. Photochem. Photobiol. 1979, 29, 419.

(9) By using brushite purified photochrome, the large losses of phytochrome, which occur at each step of the various published purification procedures (ref. 2), could be avoided. Previously

the lack of sufficient material has prevented this type of spectroscopic analysis of chromopeptides derived from photochrome. The use of brushite-phytochrome, which can be obtained rapidly in quantity, has greatly reduced the amount of material and labor necessary for this undertaking.

(10) Tobin, E. M.; Briggs, W. R. Photochem. Photobiol. 1973, 18, 487.

(11) Glazer, A. N.; Hixson, C. S.; DeLange, R. J. Anal. Biochem. 1979, 92, 489.

(12) Williams, V. P.; Glazer, A. N. J. Biol. Chem. 1978, 253, 202.

(13) As described in reference 5, an all glass and teflon HPLC system was used due to the instability of these chromopeptide when exposed to metals.

(14) This result supports the prediction that there is a single bilin chromophore per monomer chain of phytochrome (MW 120,000). The proof of this hypothesis requires a complete structural analysis of immunoaffinity-purified phytochrome (ref. 7) like that described for the analyses of algal biliproteins (ref. 12).

(15) ^{1}H NMR spectra of the phytochromobilipeptides were also recorded in D_2O solutions. Aggregation of these peptide, as observed for **1** (ref 5), made these spectra difficult to interpret. For this reason, the D_2O spectral data will not be dealt with in this report.

(16) The assignment of the ^{1}H NMR spectrum of the peptide moiety is included in Table 1 of the supplementary materials.

(17) Data obtained from phycobilipeptides in addition to β_1-phycocyanobiliheptapeptide **1** (ref. 5); in preparation.

(18) The coupling constant of 5.0 Hz which we observe for $^{3}J_{2H-3H}$ in both **1** and **2** agrees well with the value of this coupling constant in trans succinimide models, the cis coupling constant being somewhat larger (unpublished work, This Laboratory); see (a) Klein, G.; Rudiger, W. *Justus Liebigs. Ann. Chem.* **1978**, 267; (b) Schoch, S.; Klein, G.; Linsenmeier, U.; Rudiger, W. *Justus Liebigs Ann. Chem.* **1976**, 549; (c) reference 5 for discussion of the stereochemistry of **1**.

(19) Siegelman, H. W.; Chapman, D. J.; Cole, W. J. in "Porphyrins and Related Compounds", Goodwin, T. W. Ed.; Academic Press: New York, 1968, 107-120.

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(21) (a) Song, P.-S; Chae, Q.; Gardner, J. D. *Biochim. Biophys. Acta* **1979**, *576*, 479; (b) Song, P-S.; Chae, Q. *Photochem. Photobiol.* **1979**, *30*, 117.

(22) Burke, M. J.; Pratt, D. C.; Moscowitz, A. *Biochem.* **1972**, *11*, 4025.

(23) Pasternak, R.; Wagniere, G. *J. Am. Chem. Soc.* **1979**, *101*, 1662.

(24) Sugimoto, T.; Oishi, M.; Suzuki, H. *J. Phys. Soc. Japan*, **1977**, *43*, 619, and previous papers in the series.

(25) (a) Lehner, H.; Braslavsky, S. E.; Schaffner, K. *Justus Liebigs Ann. Chem.* **1978**, 1990; (b) Holzwarth, A. R.; Lehner, H.; Braslavsky, S. E.; Schaffner, K. *Justus Liebigs Ann. Chem.* **1978**, 2002; (c) Margulies, L.; Stockburger, M. *J. Am. Chem. Soc.* **1979**, *101*, 743; (d) Sheldrick, W. S. *J. Chem. Soc.* **1976**, 1457.

(26) We also observed a nearly complete phototransformation of the β_1-phycocyanobiliheptapeptide (**1**) in the CD spectrometer from a blue form (λ_{max} 650 nm in anhydrous trifluroacetic acid) to a

purple form (λ_{max} 590 nm). The characterization of this purple pigment and its relevance to the proposed C-5 isomerization hypothesis is currently being studied.

(27) In Scheme II we have chosen to represent the P_R form of phytochrome as the protonated structure. Spectrophotometric titration has shown the pKa of denatured P_R phytochrome to be 5.4 (reference 4c). Furthermore the similarity of the absorption spectral properties of phytochromobiliundecapeptide (2) and denatured P_R (reference 4c) in acidic solvent (λ_{max} 665 nm in 0.01N trifluoroacetic acid, Figure 4) with those of native P_R phytochrome (λ_{max} 662 nm, Figure 1) suggests that the phytochrome chromophore is protonated. The difference in the absorption spectrum of P_R with that of the free base of 2 (λ_{max} 600-610 nm) is dramatic.

(28) Hunt, R. E.; Pratt, L. H. Abstracts of the 7th Annual American Society for Photobiology, 1979, June 24-28, Asilomar Conference Grounds, Pacific Grove, CA.

(29) In general the free bases of bilitrienes assume more cyclic conformations than those of the protonated forms as shown by the decrease in the ratio of the red absorption to the blue absorption bands of bilitrienes upon deprotonation. This is shown more elegantly by MO calculations in references 22-25.

(30) The NMR spectrometer was designed and constructed by Dr. Willy C. Shih, Laboratory of Chemical Biodynamics, University of California, Berkeley. Instrumentation documentation is provided in Shih, W. C. Ph.D. Thesis, 1979, Univ. of California, Berkeley.

(31) The green safelight used for harvesting oats and for phytochrome isolation was obtained by wrapping green fluorescent

tubes (Sylvania No. F40 Green) with one sheet each of a medium blue green plastic (Roscolene No. 877) and medium green plastic (No. 874) available from Rosco Laboratories, Hollywood, CA.

(32) The Bio Gel P4 column was prewashed with a mixture of 0.01N EDTA and 0.1N L-ascorbic acid to remove any trace metal or other oxidizing contaminants in the gel.

Table I. Amino Acid Analyses.

amino acid	pepsin digest, Biogel P4 chromopeptide fraction,[a] nmols	thermolysin digest, HPLC fraction,[b] nmols			
		2	3	4	5
His	21	54	121	79	102(4)
Arg	5(8)	19	62(55)	36	46(43)
Cya[c]	(13)		(57)		(42)
Asp	4(7)	4	<1(<2)	5	
Thr	1(2)	tr		tr	
Ser	14(20)	29	47(54)	44	36(47)
Glu	14(17)	23	18(21)	34	46(47)
Pro	tr[c]	31	63(62)	39	52(48)
Gly	3(7)	7	14(16)	9	(2)
Ala	14(16)	40	67(74)	46	49(56)
1/2 Cys		7		11	6
Val	1(4)	tr	(<1)		
Met		2		tr	
Ile	4(<2)	5	(3)	<2	
Leu	26(35)	53	66(71)	60	109(100)
Tyr	16	17	14	26	41
Phe	4	6		tr	

[a] Hydrolyzed 27 nmoles (based on $\varepsilon_{665}=3.2\times10^{4}$ L mol^{-1}cm^{-1}) in 6N HCl plus 50 μL 5% phenol at 110°C for 20 h. Values in parentheses are amino acid yields after 20 h, 110°C hydrolysis in 6N HCl plus 30 μL DMSO (ref. 11). [b] Hydrolysis as in a: Fraction 2 (41 nmol), 3 (68 nmol), 4 (48 nmol), and 5 (55 nmol). [c] Abbreviations: tr=trace, cya=cysteic acid.

Table II. Amino Acid Analysis of Phytochromobiliundecapeptide (2, HPLC Fraction 5, Figure 3) and Recovery of PTH Amino Acid Derivatives at Each Step of the Edman Degradation.

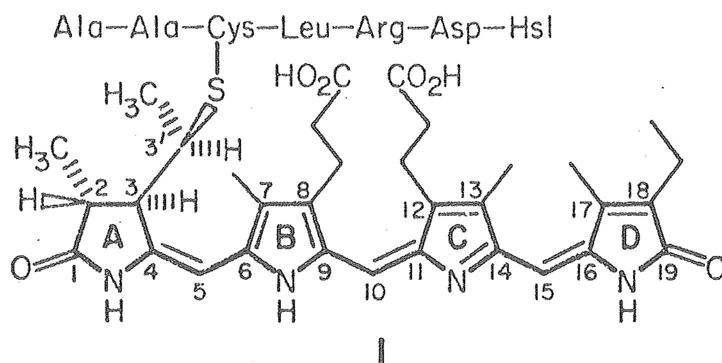
amino acid	original analysis[a]	PTH derivative recovered after each step of the Edman degradation										
		1	2	3	4	5	6	7	8	9	10	11
His	2.0					+			+			
Arg	0.9		+									
Cya[c]	0.9						+					
Ser	0.7							+				
Gln	0.9										+	
Pro	1.0				+							
Ala	1.0			+								
Leu	2.1	+								+		
Tyr	0.8											+

[a] Results from Table I. Relative residue amounts of each amino acid based on Ala=1.0 residue. [b] PTH derivatives identified by TLC, HPLC and MS. [c] Cysteic acid determination using the method of reference 11.

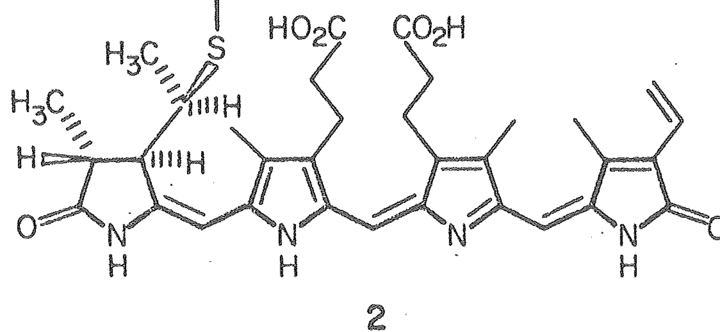
Table III. 270 MHz ^{1}H NMR Assignments for the Bilin Moieties of Phytochromobiliundecapeptide (2) and β_1-Phycocyanobiliphepta-peptide (1)[a] in [$^{2}H_5$]-Pyridine at 23°C.

chem. shift[b] 2	chem. shift[b] 1	no. of H's	multiplicity J, Hz for 2	assignment
–	1.23	3	t, 7.3	18-CH_2CH_3
1.38	1.39	3	d, 7.3	2-CH_3
1.43	1.48	3	d, 7.3	3'-CH_3
2.03	2.02	3	s	7-CH_3
2.11	2.07	3	s	17-CH_3
2.13	2.12	3	s	13-CH_3
–	2.48	2	q, 7.3	18-CH_2CH_3
2.64	2.70	1	dd, 5.0, 7.3	2-H
2.81	2.83	2	t, 7.2	8,12-$C\underline{H}_2CH_2COOH$
2.84	2.85	2	t, 7.2	
3.09	3.09	2	t, 7.2	8,12-$CH_2C\underline{H}_2COOH$
3.17	3.17	2	t, 7.2	
3.18	3.15	–	c	3-H
3.50	3.52	–	c	3'-H
5.51	–	1	dd, 2, 12 (ABX)	18-H_X (vinyl)
5.90	5.87	<1[d]	s	5-H
6.16	6.08	1	s	15-H
6.71	–	1	m, 2, 18 (ABX)	18-H_B (vinyl)
6.73	–	1	m, 12, 18 (ABX)	18-H_A (vinyl)
7.26	7.29	1	s	10-H

[a] Assignments from ref. 5. [b] The chemical shift values are in parts per million from Me_4Si and were determined from a residual proton of pyridine (7.81 ppm from Me_4Si at 23°C). [c] Overlapping resonances attributed to the peptide obscured these signals. [d] The low integral for the C-5 proton appears to be due to deuterium exchange as was observed for 1 (reference 5); before ^{1}H NMR spectroscopy undecapeptide 2 was exchanged in D_2O.



1



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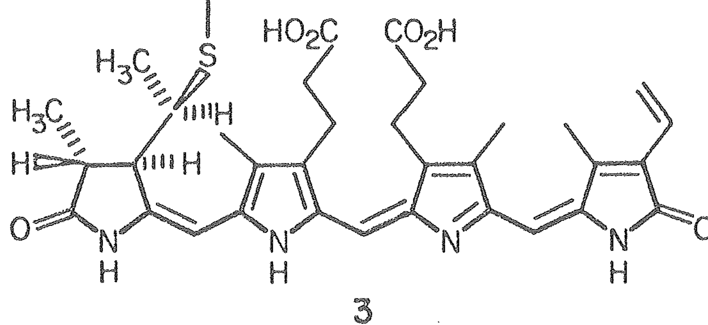

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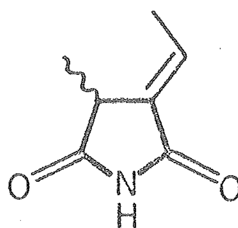

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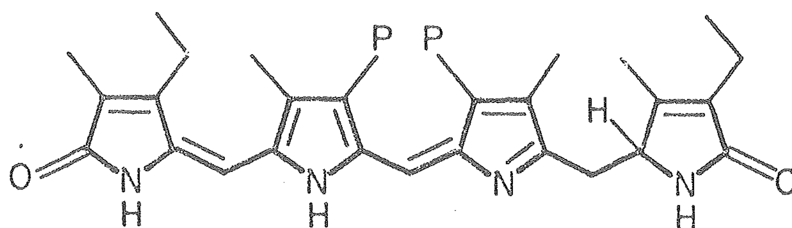

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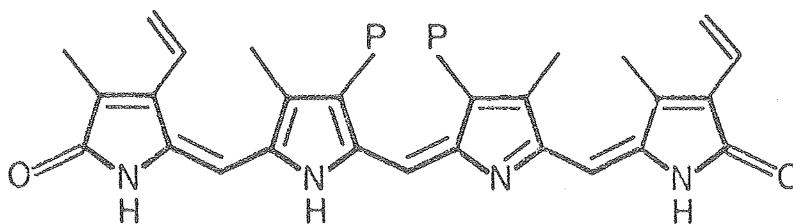

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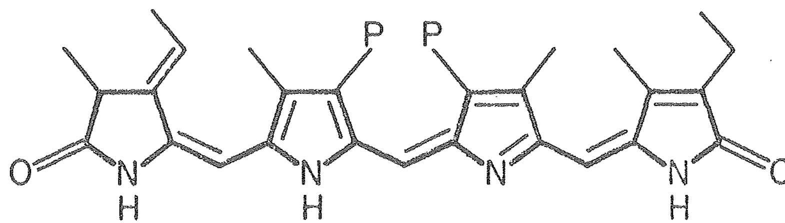

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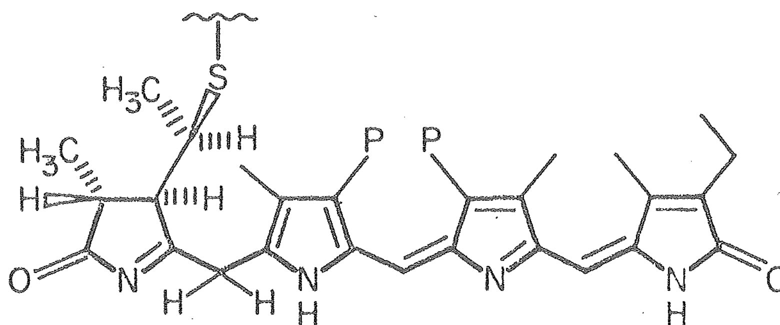
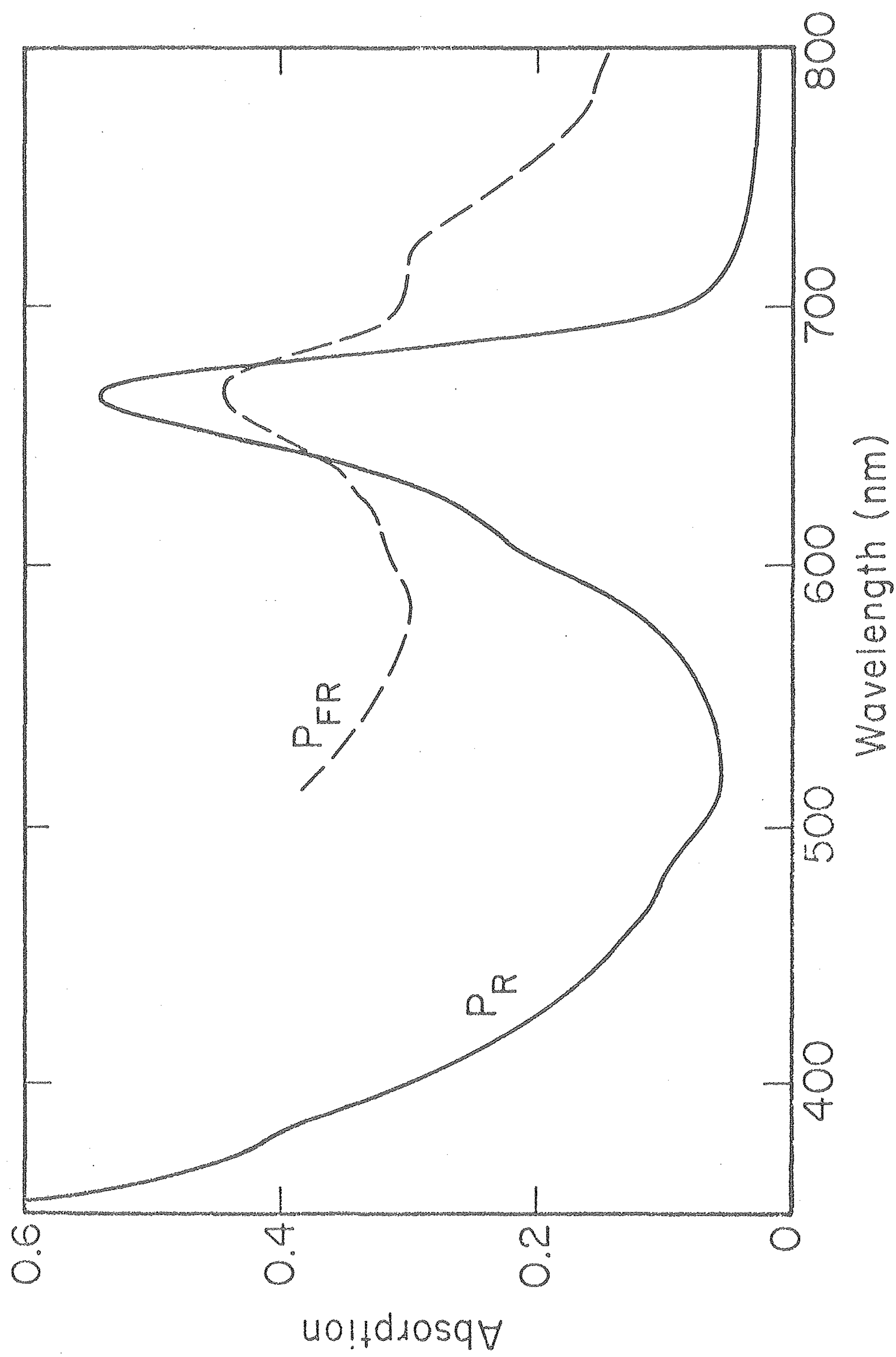


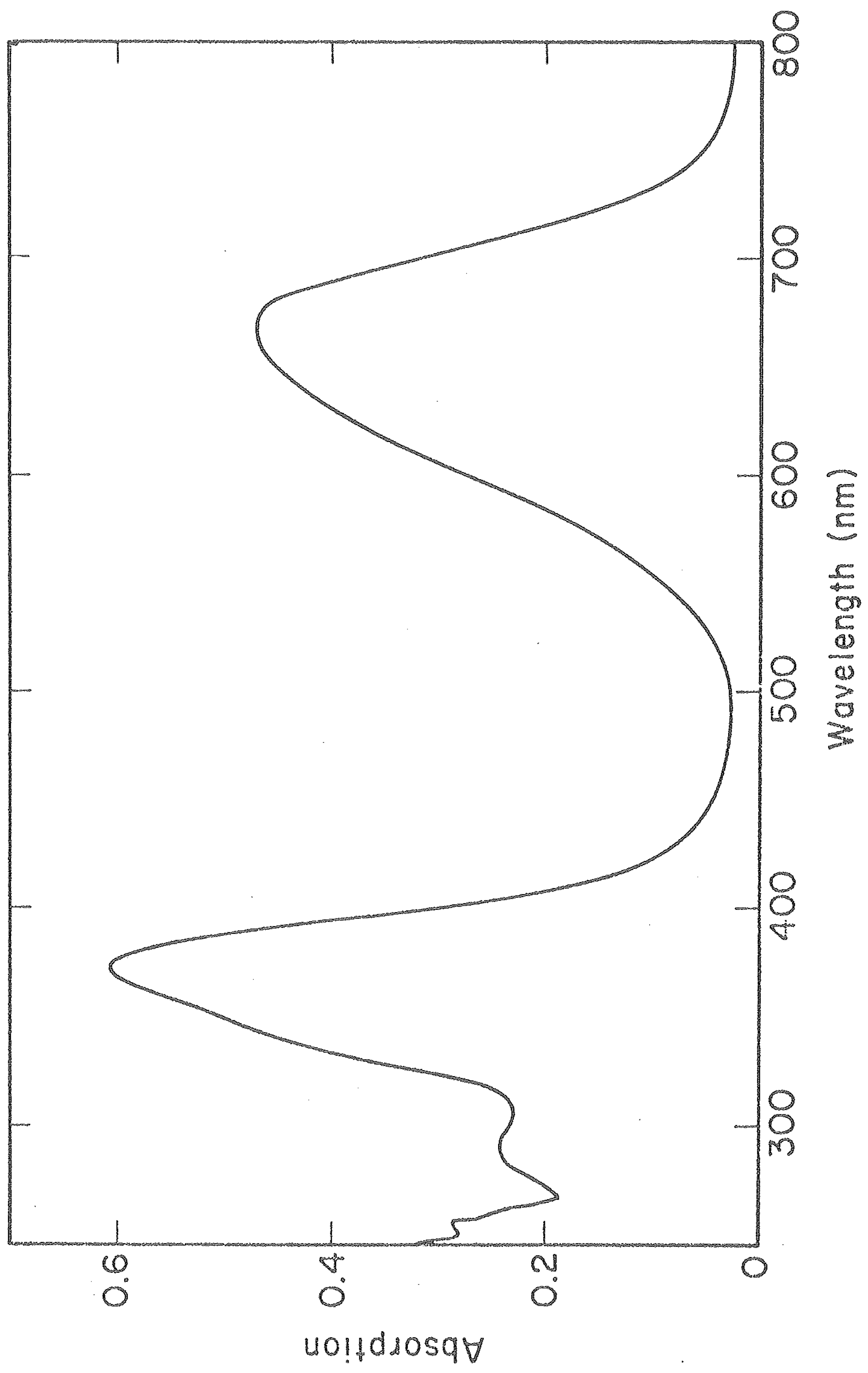
8

Figure 1. Absorption spectrum of brushite-prepared phytochrome used in this study (c 7.33×10^{-6}M in 0.1M K_2HPO_4/KH_2PO_4, pH 7.8, SAR=0.07); —— P_R after irradiation at 720 nm; ---- P_{FR} after irradiation at 660 nm.



0.6
0.4
0.2
0
Absorption
400
500
600
700
800
Wavelength (nm)
P_FR
P_R

Figure 2. Absorption spectrum of the pepsin-cleaved chromopeptide, fraction 4 from Bio Gel P4 chromatography (c 1.80×10^{-5}M, 25% HOAc).



Absorption
0.6
0.4
0.2
0
300
400
500
600
700
800
Wavelength (nm)

Figure 3. HPLC of the pepsin chromopeptide fraction after thermolysin digestion. Column: LiChroprep RP18, 25-40μ, 15x500 mm; mobile phase: 30% CH_3CN/70% 0.01N aq. trifluoroacetic acid, changed to 44% CH_3CN/56% 0.01N TFA at the arrow; flow rate 3 ml/min; injection volume 1.5 ml.

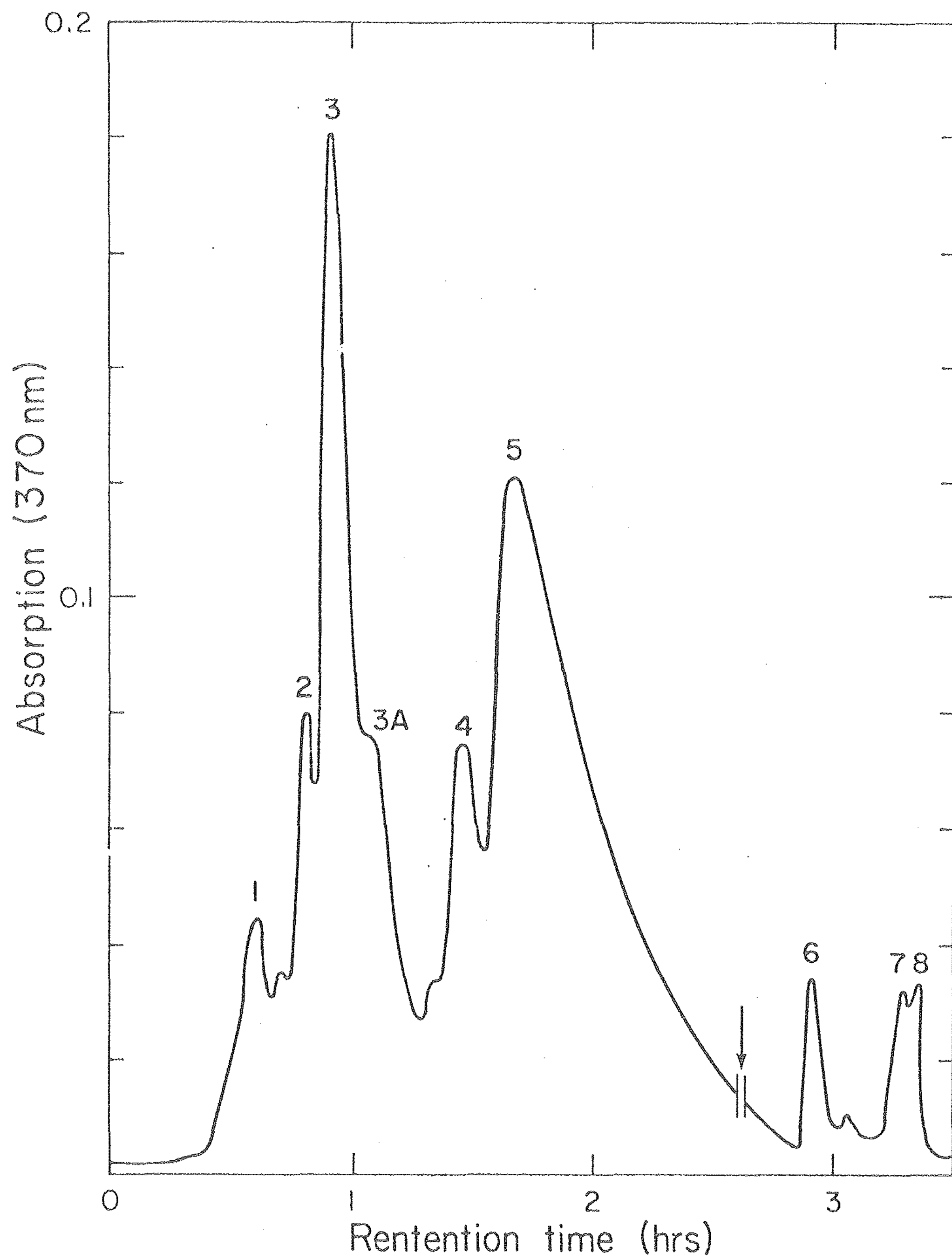

0.2
0.1
Absorption (370nm)
1
2
3
3A
4
5
6
7 8
0
1
2
3
Rentention time (hrs)

Figure 4. Absorption spectrum of phytochromobiliundecapeptide (2, HPLC fraction 5); —— 0.01N trifluoroacetic acid, c 1.72×10^{-5}M; ---- pyridine, c 2.15×10^{-5}M.

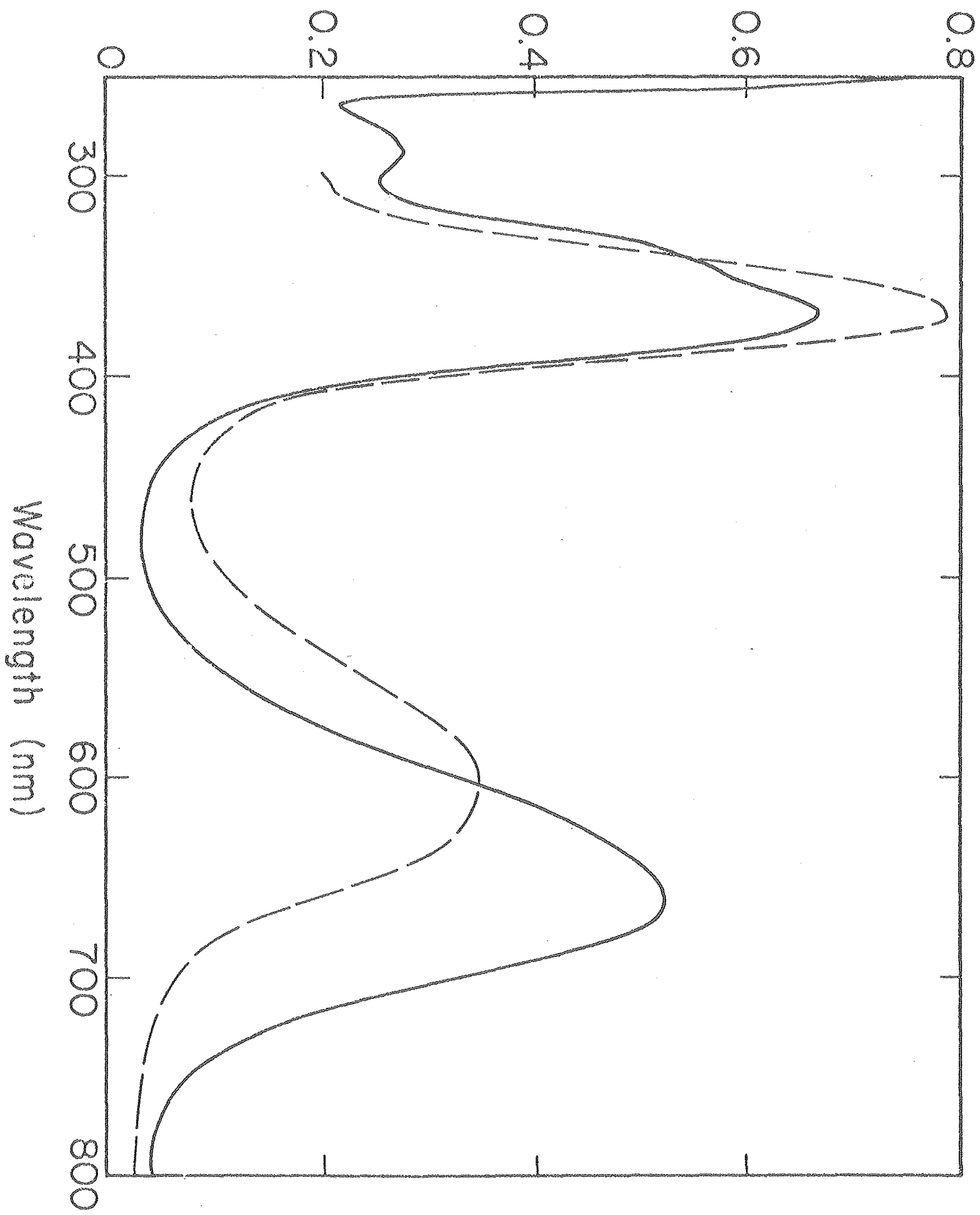
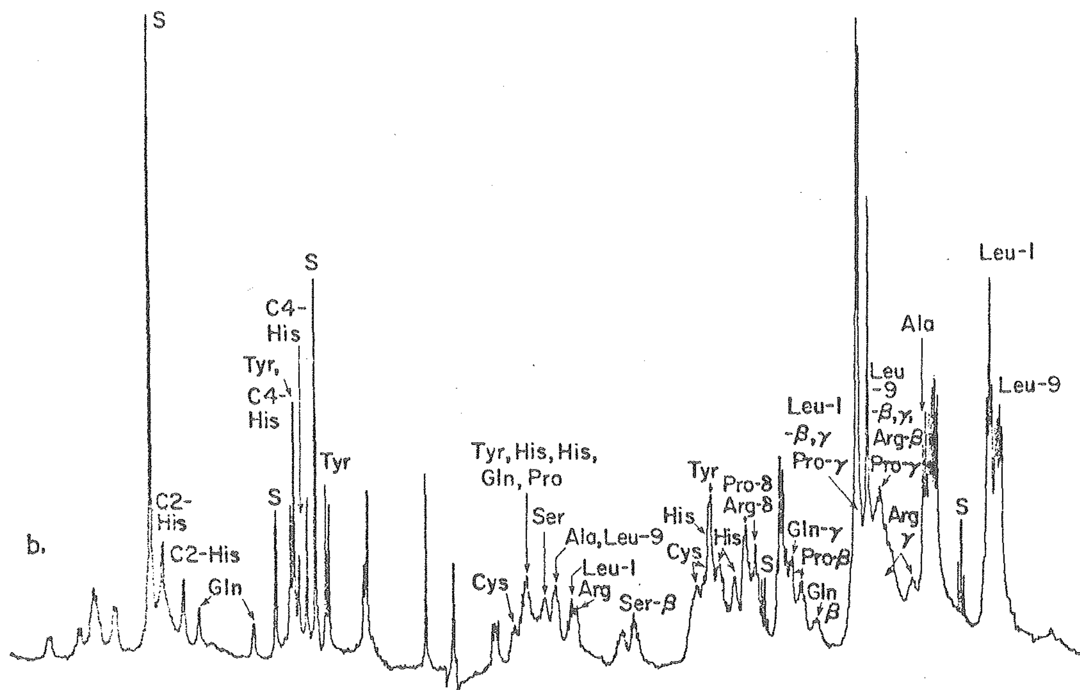

Absorption
0
0.2
0.4
0.6
0.8
300
400
500
600
700
800
Wavelength (nm)

Figure 5. The 270 MHz ^{1}H NMR spectrum of phytochromobiliundecapeptide (2) in $[^{2}H_5]$-pyridine at 25°C; (a) chromophore assignments; (b) peptide assignments.


S
S
C4-
His
Tyr,
C4-
His
Tyr
S
C2-
His
C2-His
Gln
b.
Tyr, His, His,
Gln, Pro
Ser
Ala, Leu-9
Cys
Leu-I
Arg
Ser-β
Tyr
His
Cys
Pro-δ
Arg-δ
His
S
Leu-I
-β,γ
Pro-γ
Gln-γ
Pro-β
Gln
β
Leu
-9
-β,γ,
Arg-β
Pro-γ
Arg
γ
Ala
S
Leu-I
Leu-9

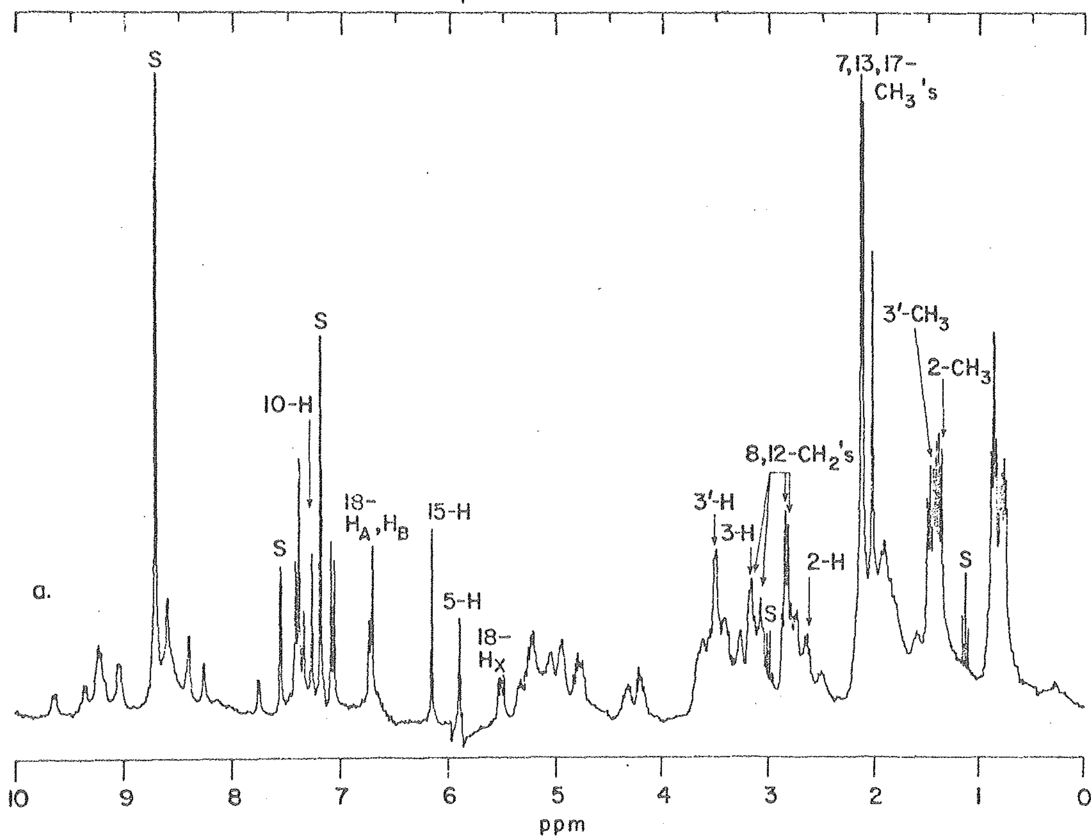
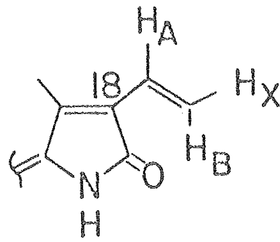
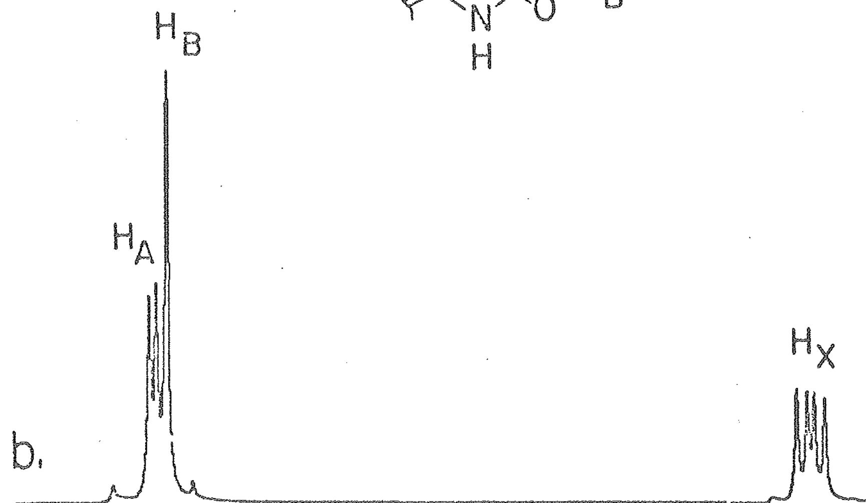

S
7,13,17-
CH3's
S
10-H
S
18-
HA, HB
15-H
5-H
18-
HX
a.
3'-H
3-H
8,12-CH2's
S
2-H
3'-CH3
2-CH3
S
10
9
8
7
6
5
4
3
2
1
0
ppm

Figure 6. Low field region of the 270 MHz ^{1}H NMR spectrum of 2. (a) Experimentally observed spectrum; (b) computer-simulation of the 18-vinyl ABX pattern with J_{AB} = 18 Hz, J_{AX}=12 Hz, and J_{BX} = 2Hz; line width assumed in spectrum simulation, 2.0 Hz.


H_A
18
H_X
H_B
N
O
H


H_B
H_A
H_X
b.

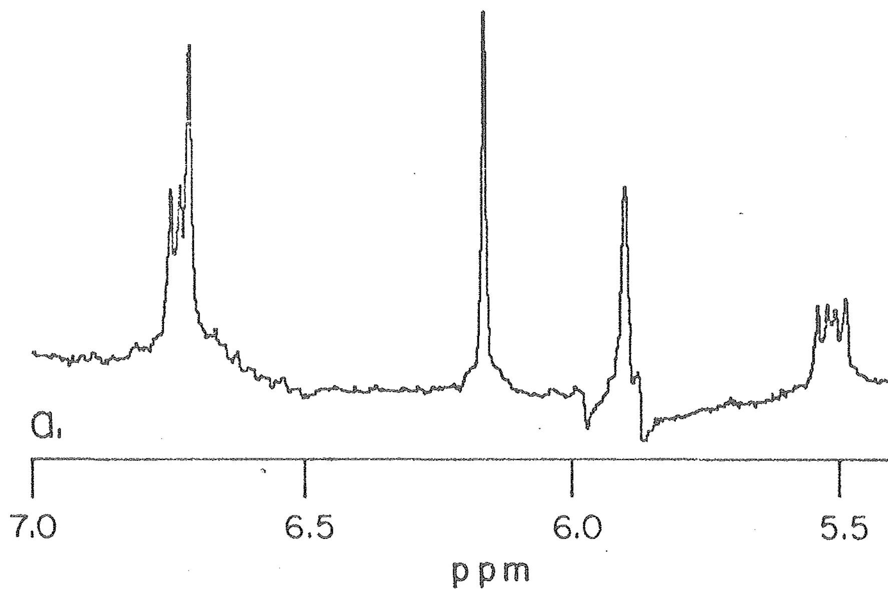
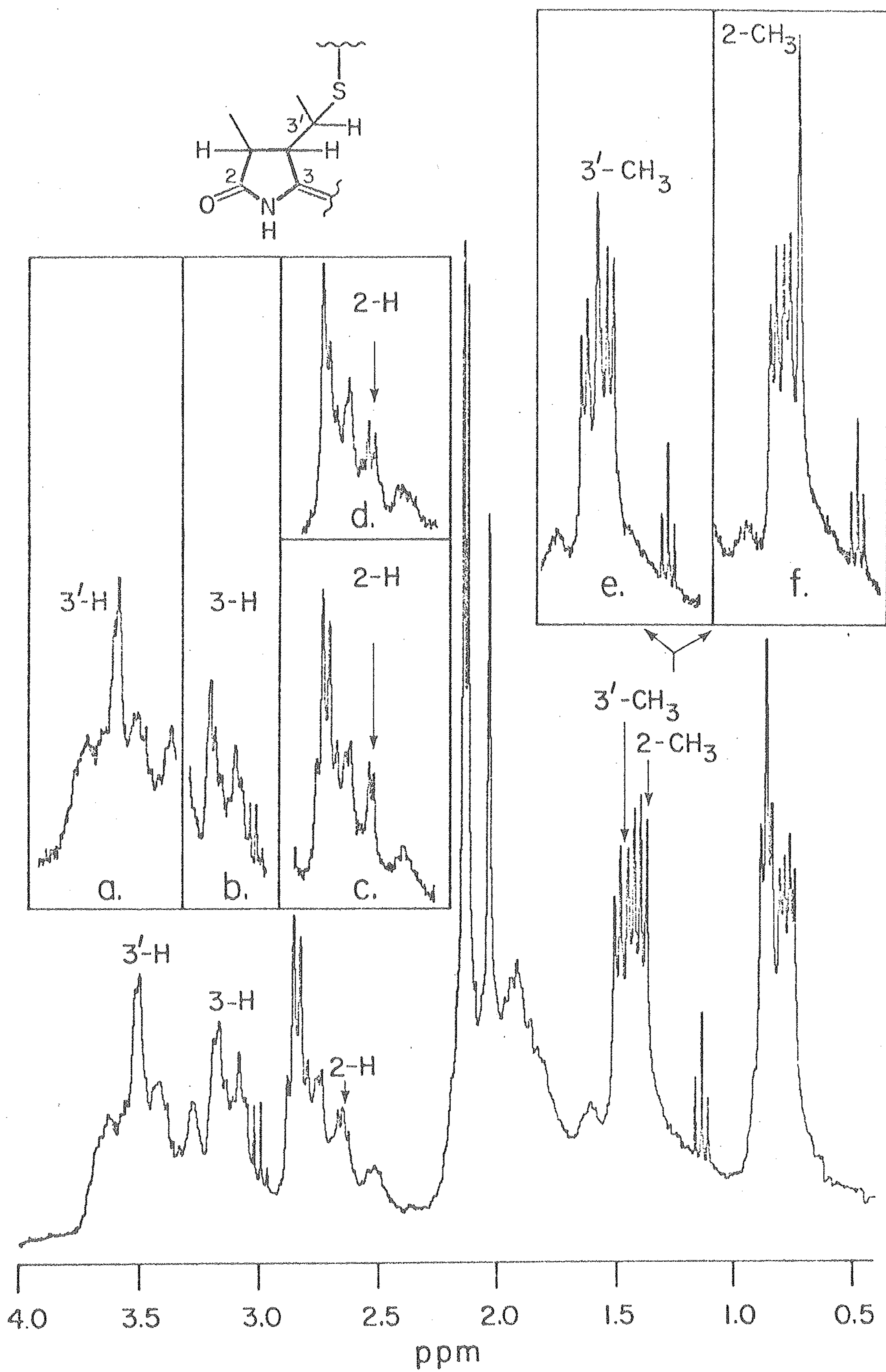

a.
7.0
6.5
6.0
5.5
ppm

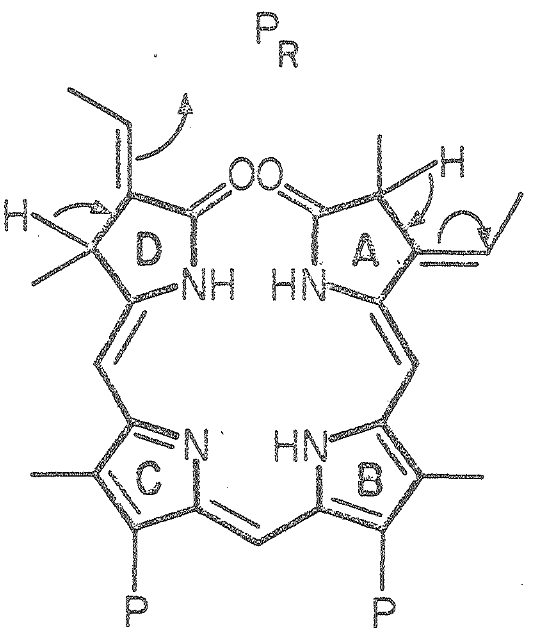
Figure 7. High field region of the 270 MHz ^{1}H NMR spectrum of 2. Inserts show spectra after double irradiation experiments: irradiation of (a) doublet at 1.43 ppm; (b) and (f) multiplet at 2.64 ppm; (c) doublet at 1.38 ppm; (d) multiplet at 3.18 ppm; (e) multiplet at 3.50 ppm.

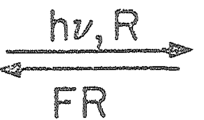


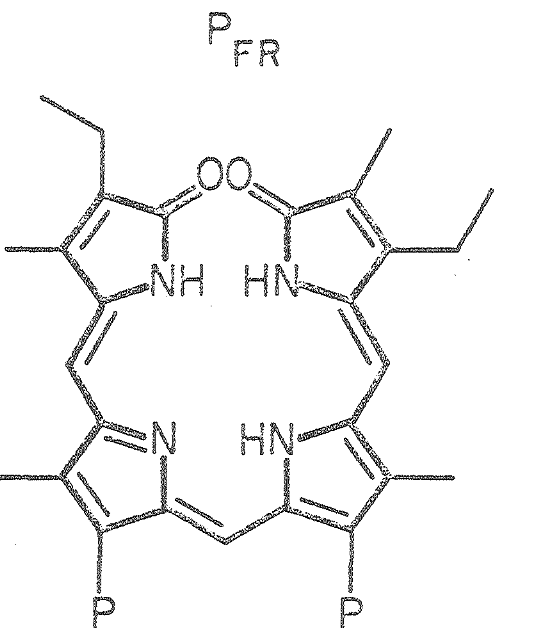
S
3'
H
H
H
2
3
O
N
H
2-CH3
3'- CH3
e.
f.
2-H
d.
2-H
c.
3'-H
a.
3-H
b.
3'-CH3
2-CH3
3'-H
3-H
2-H
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm

Scheme I. Previous Hypotheses for P_R to P_{RF} Phototransformation: From (a) reference 18; (b) reference 19; (c) reference 4a; (d) reference 4b; (e) reference 4e); (f) reference 20. (Asterisks represent covalent linkages to the apoprotein. These structures are written in the cyclic form and are not meant to suggest proposed conformations except in (f). P = propionic acid side chain).

a.







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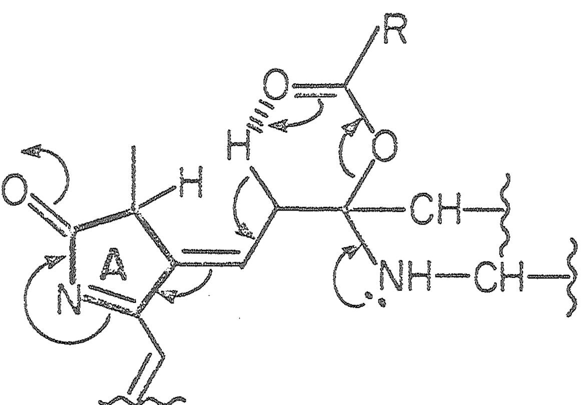
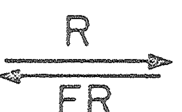
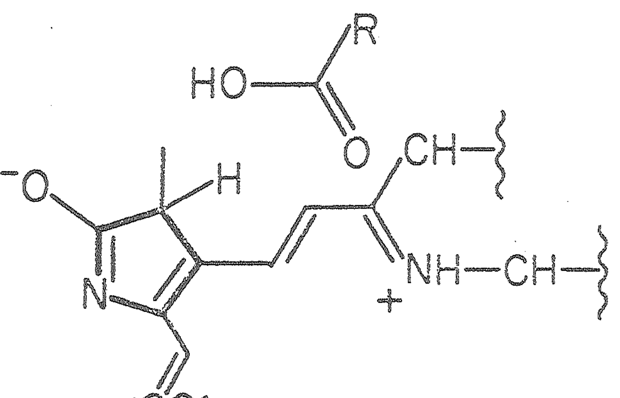
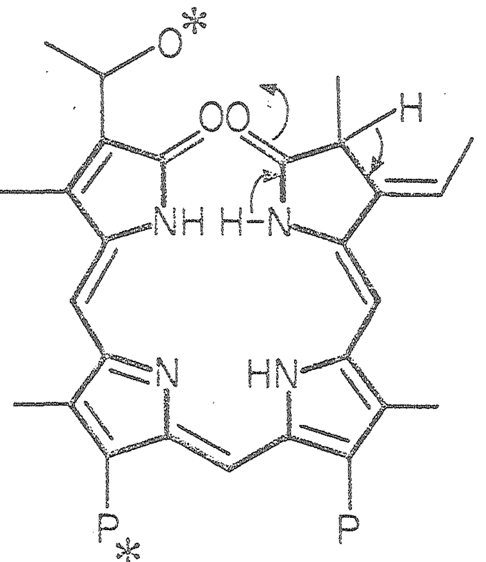
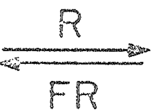


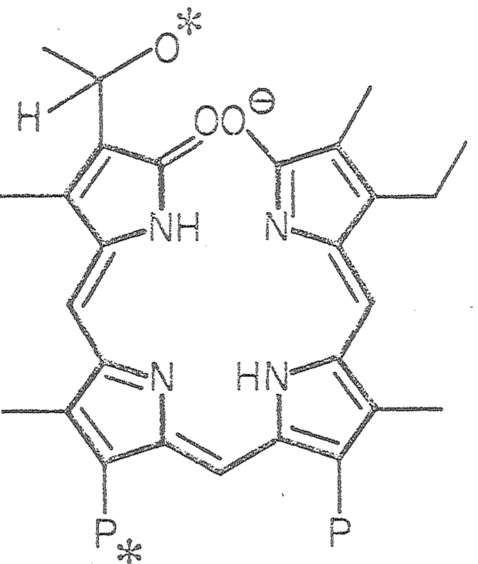




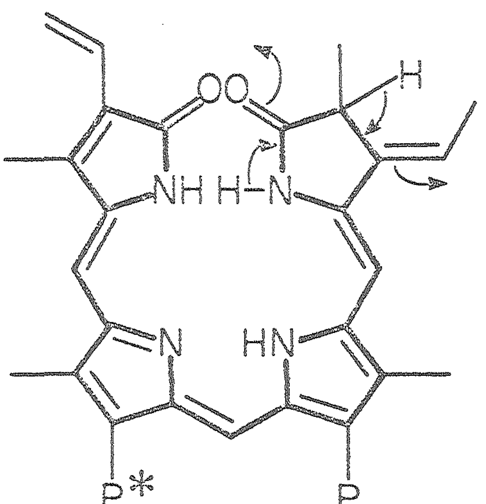
c.

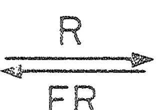


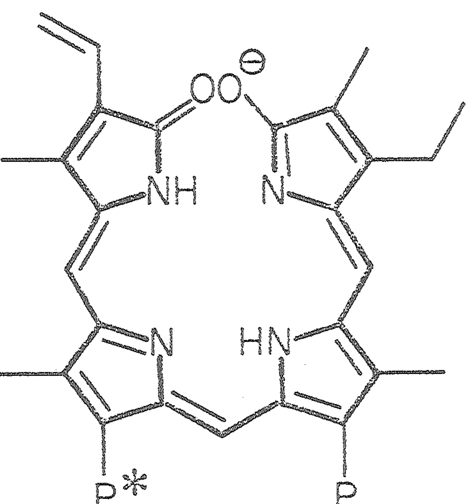




d.







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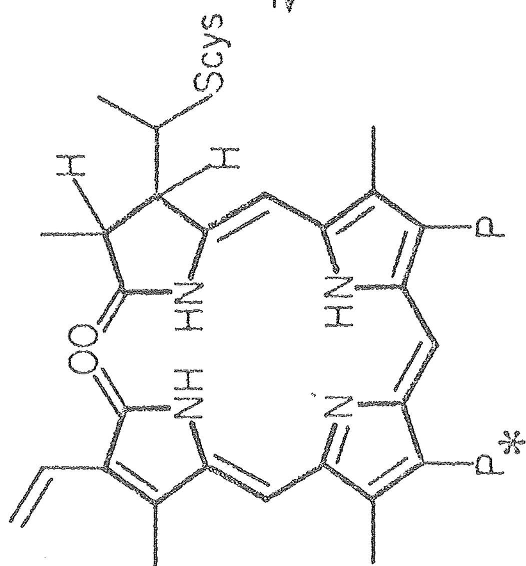

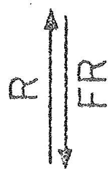

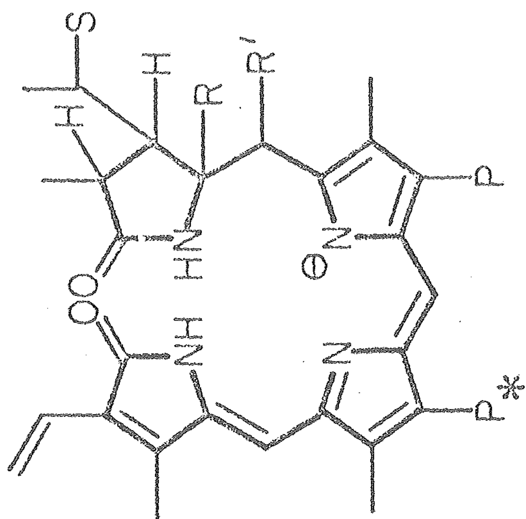

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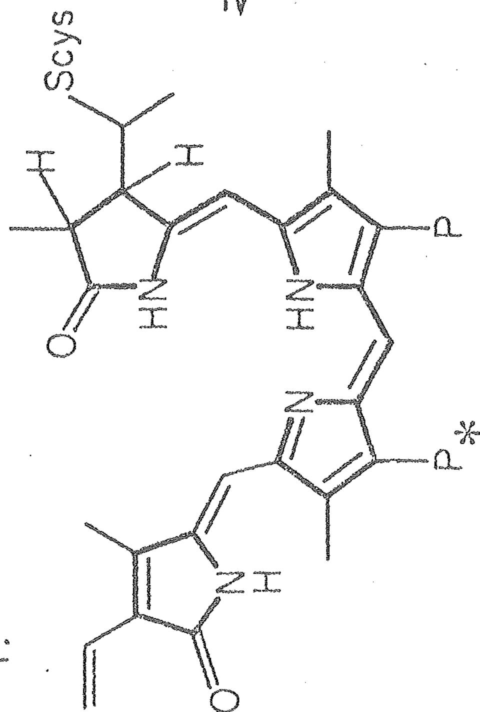
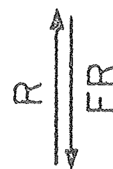
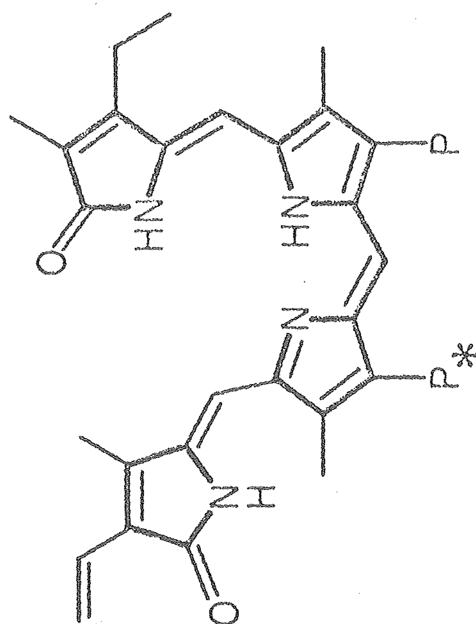




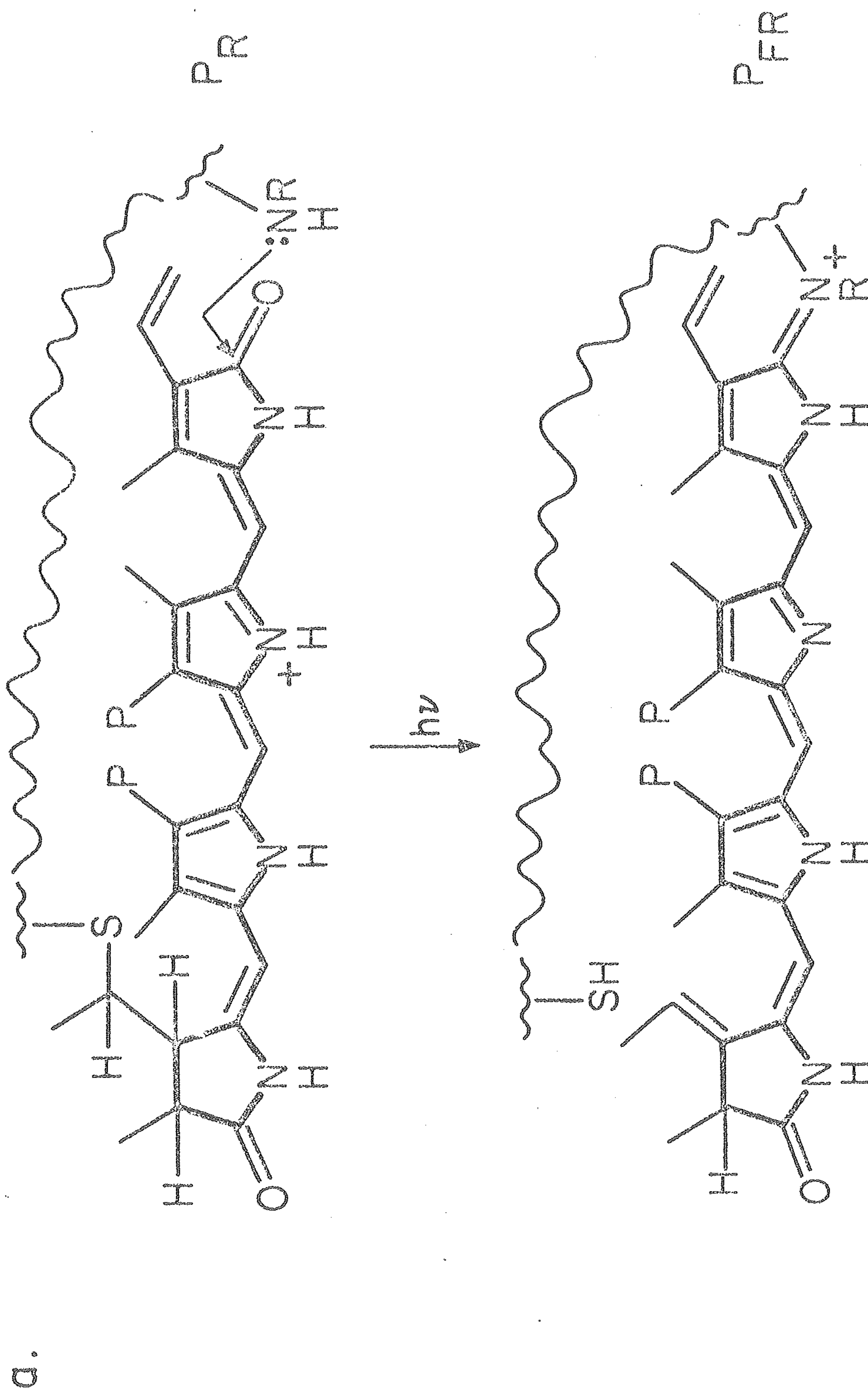


Scheme II. New Proposals for P_R to P_{FR} Phototransformation:

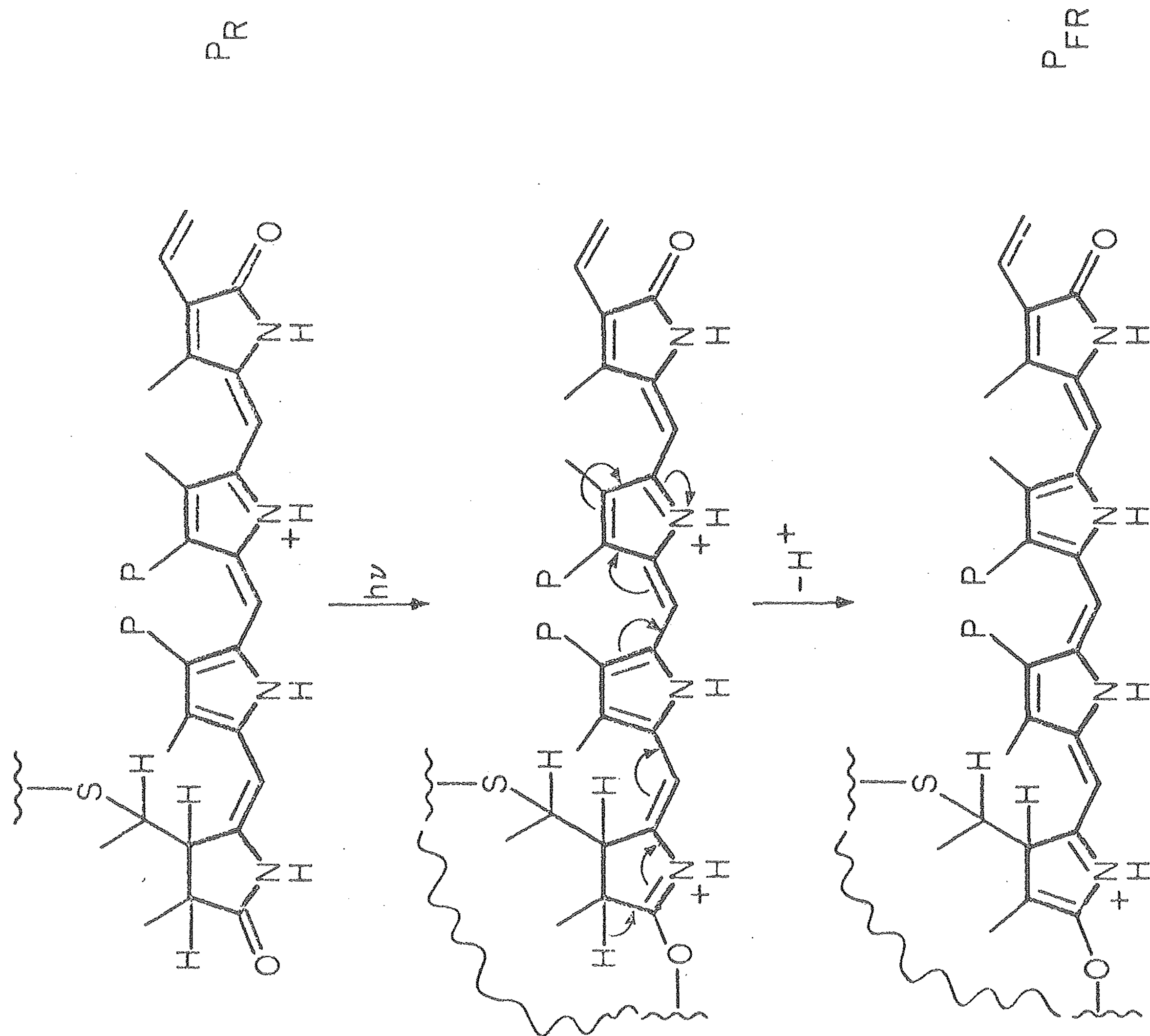
(a) with Thioether cleavage and Schiff's Base Formation;

(b) with Enol Acylation and Migrating Positive Charge; and

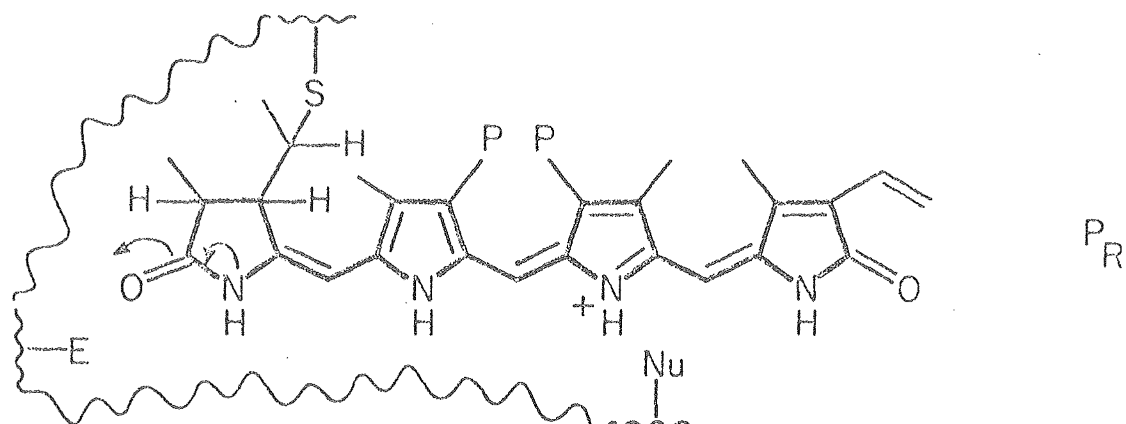
(c) with Lactam-Lactim Interconversion Accompanied by Deprotonation.



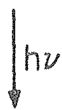
a.

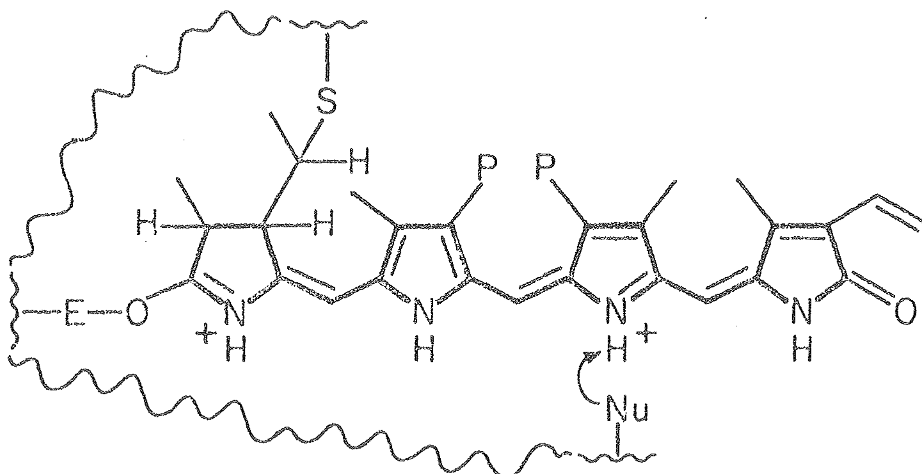

b.
P_R
hν
−H+
P_FR

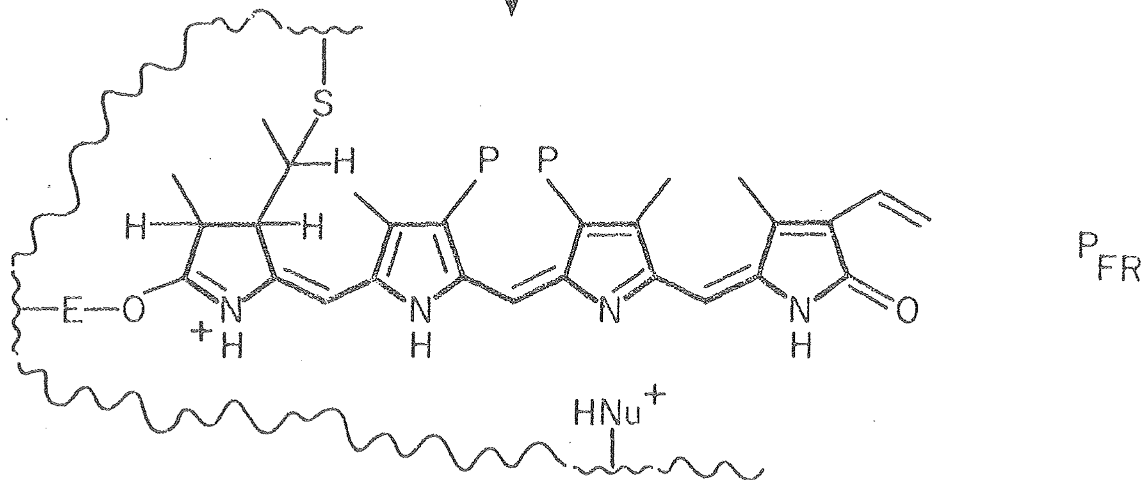
c.



P_R







P_{FR}

Supplementary Material.

Table I. 270 MHz ^{1}H NMR Assignment for the Peptide Moiety of Phytochromobiliundecapeptide (2) in [$^{2}H_5$]-Pyridine at 23°C.

Chemical Shift	No. of H's	Multiplicity J, Hz	Assignment[a]
4.80	1	m	Leu(1) α-CH
2.11-2.13	b	m	Leu(1) β-CH_2, γ-CH
0.84	3	d, 6.1	Leu(1) δ_1-CH_3
0.87	3	d, 6.1	Leu(1) δ_2-CH_3
4.75	1	m	Arg α-CH
1.79-1.98	b	m	Arg β-CH_2
1.57-1.82	b	m	Arg γ-CH_2
3.14-3.18	b	m	Arg δ-CH_2
4.85-5.00	b	m	Ala α-CH
1.49	3	d, 7.0	Ala β-CH_3
5.1-5.3	b	m	Pro α-CH
2.61-2.75	b	m	Pro β-CH_2
1.79-1.98, 2.11-2.13	b	m	Pro γ-CH_2
3.14-3.18	b	m	Pro δ-CH_2
5.1-5.3	b	m	His (5,8) α-CH's
3.27-3.58	b	m	His (5,8) β-CH_2's
8.40, 8.60	1,1	s	His (5,8) 2H
7.34, 7.39	1,1	s	His (5,8) 4H
5.02-5.08	b	m	Ser α-CH
4.21, 4.33	1,1	m	Ser β-CH_2
5.33	1	m	Cys α-CH
3.59-3.70	b	m	Cys β-CH_2
4.85-5.00	b	m	Leu (9) α-CH

Table I. (continued)

Chemical Shift	No. of H's	Multiplicity J, Hz	Assignment[a]
1.79-1.98	b	m	Leu(9) β-CH_2, α-CH
0.75	3	d, 5.9	Leu(9) δ_1-CH_3
0.79	3	d, 5.9	Leu(9) δ_2-CH_3
5.1-5.3	b	m	Gln α-CH
2.50	2	m	Gln β-CH_2
2.75	2	m	Gln γ-CH_2
7.76, 8.26	1,1	s	Gln ε-NH_2
5.1-5.3	b	m	Tyr α-CH_2
3.50	b	m	Try β-CH_2
7.08	2	d, 8.3	Try 3,5H
7.40	2	d, 8.3	Try 2,6H

(a) Assignments have been based on chemical shifts, results of decoupling experiments and by comparison with other peptides in [$^2H_5$]-pyridine. (b) These resonances are obscured by others in the peptide.

Figure 1. 270 MHz ^{1}H NMR spectrum of the BioGel P4 purified pepsin peptide from phytochrome in [$^2H_5$]-pyridine at 23°C.

S4a

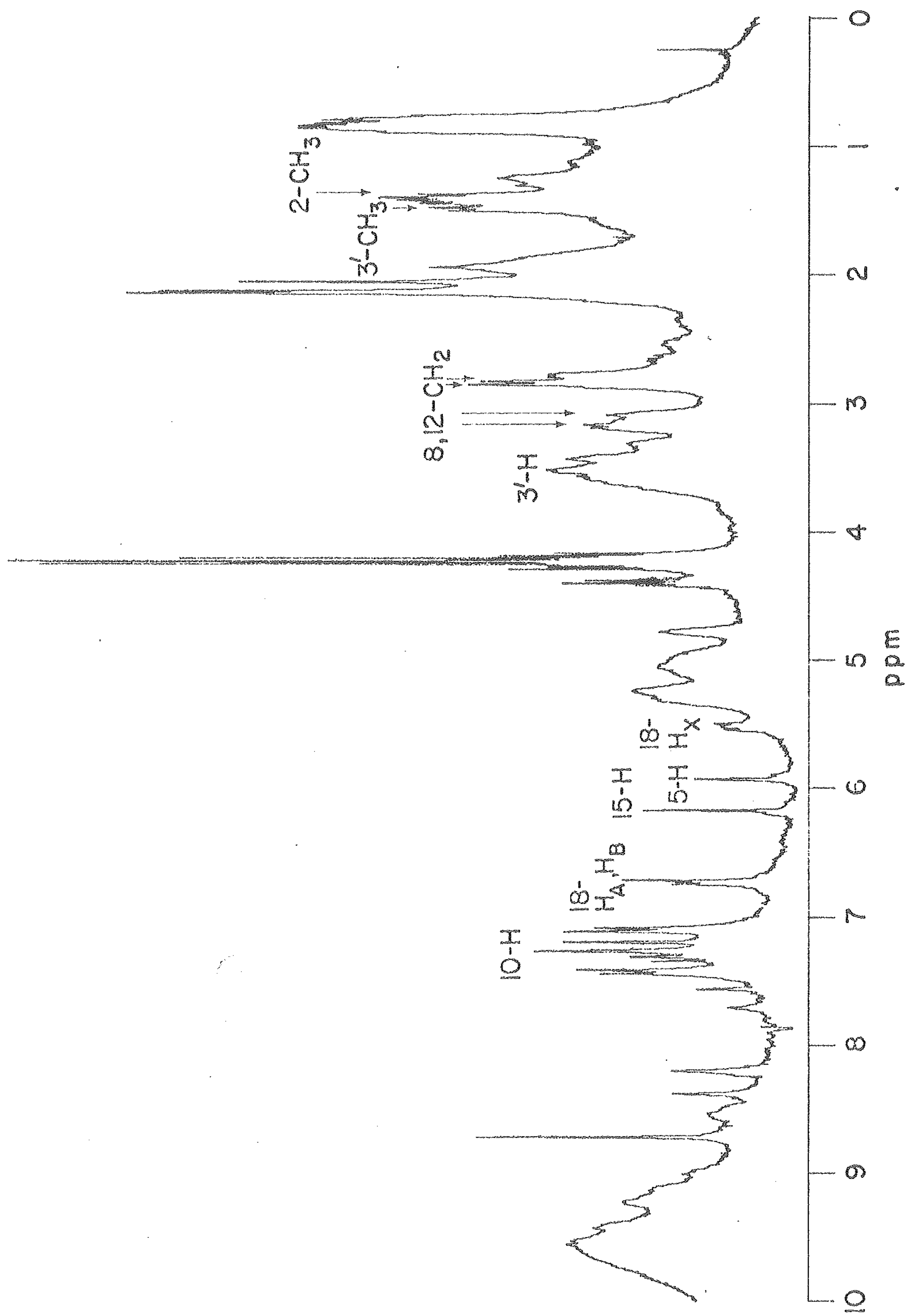
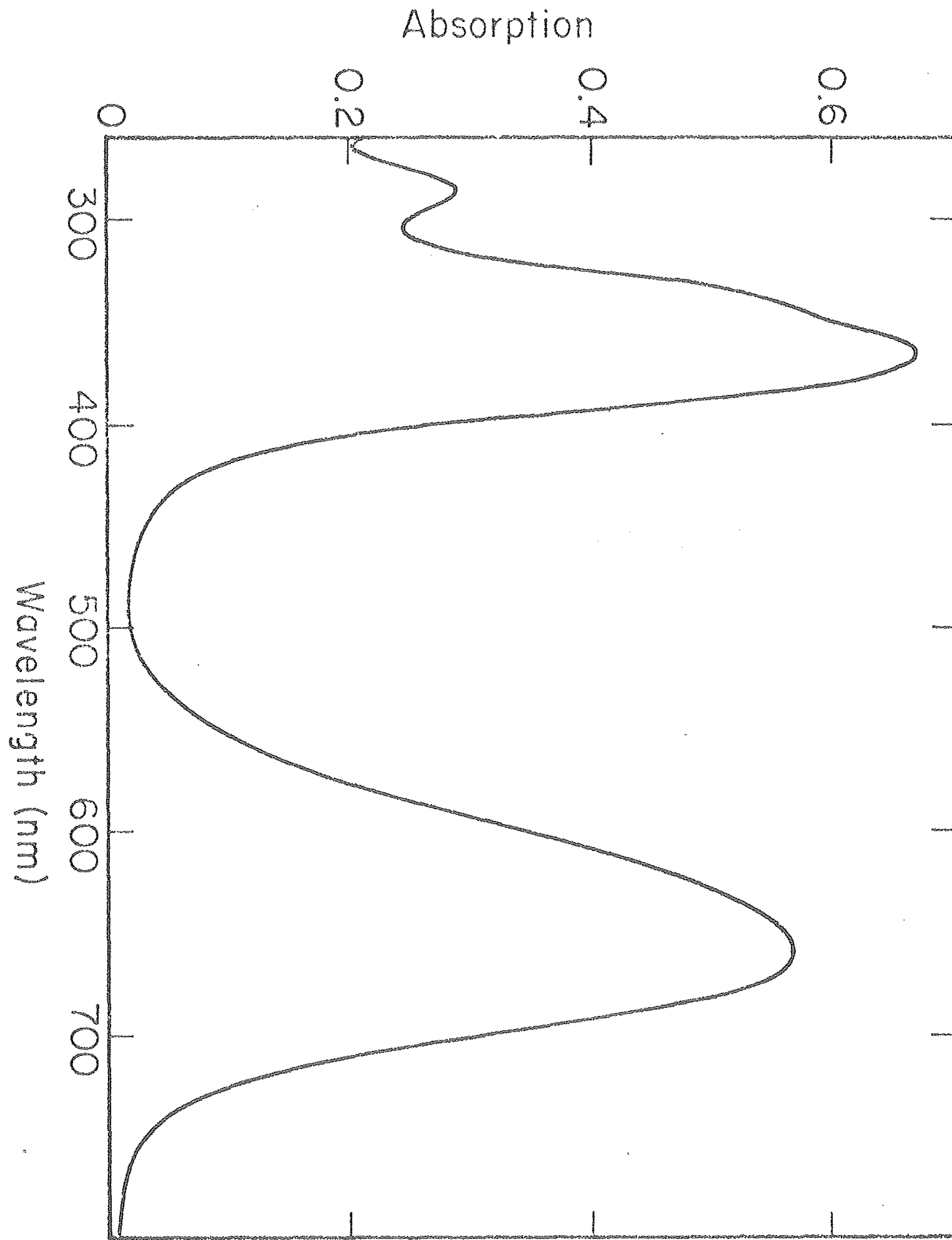


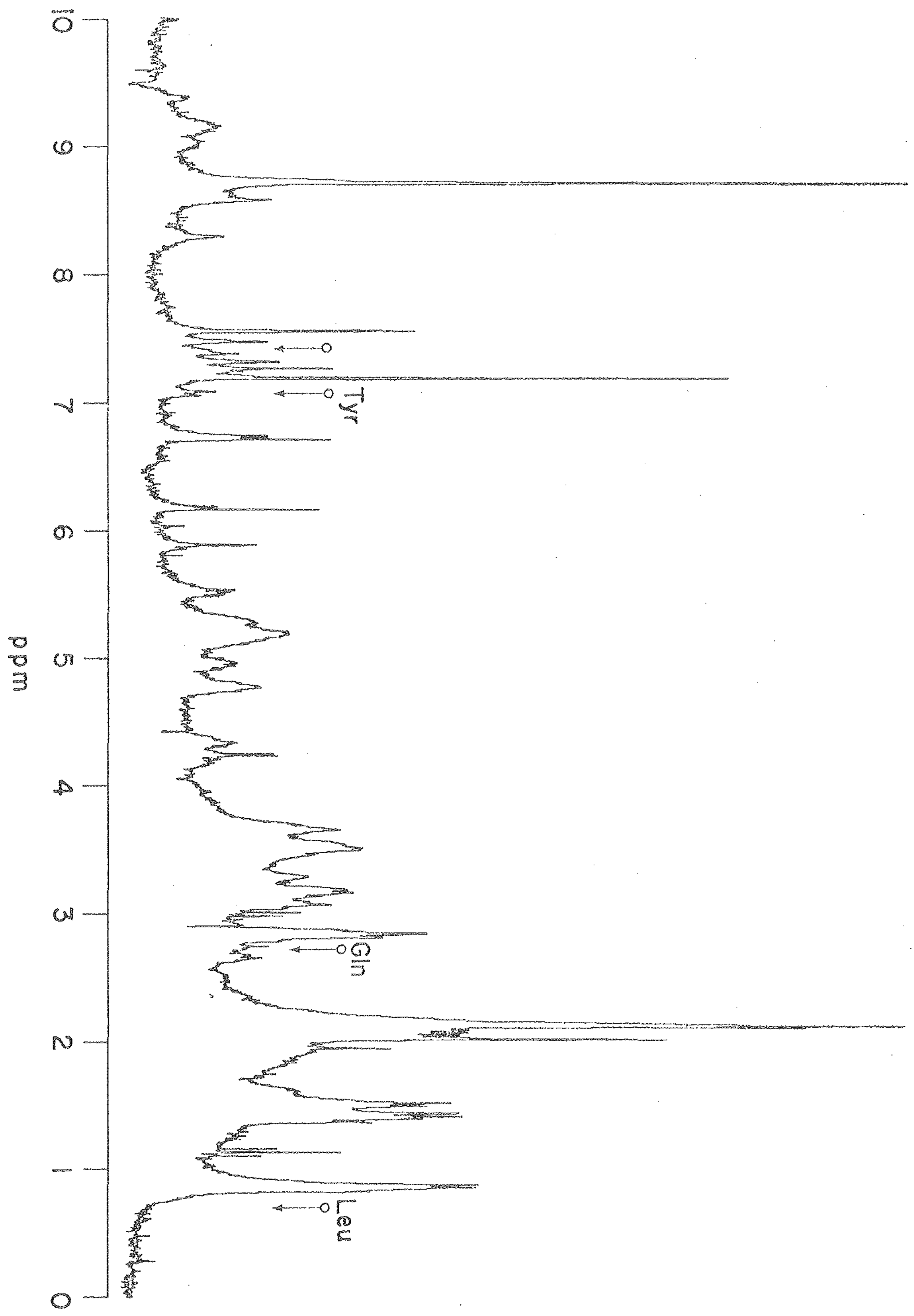
Figure 2. Absorption spectrum of the chromopeptide fraction obtained after Sephadex G50 purification of the thermolysin digestion of the pepsin peptides, c 2.44×10^{-5}M in 25% HOAc.



XBL 7910-5037

Figure 3. The 270 MHz ^{1}H NMR spectrum of phytochromobiliocta-peptide (3) in [$^{2}H_{5}$]-pyridine at 23°C. Arrows indicate the absence of resonances from the three cleaved residues -Leu-Gln-Tyr.

S6a



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