Isolation of the Phytochrome Chromophore. The Cleavage Reaction with Hydrogen Bromide

Wolfhart Rüdiger, Thomas Brandlmeier, Inge Blos

Botanisches Institut der Universität München, Menzinger Str. 67, D-8000 München 19

Albert Gossauer, and Jens-Peter Weller

Institut für Organische Chemie der Technischen Universität Berlin, Straße des 17. Juni 135, D-1000 Berlin 12

Z. Naturforsch. **35 c,** 763–769 (1980); received July 11, 1980

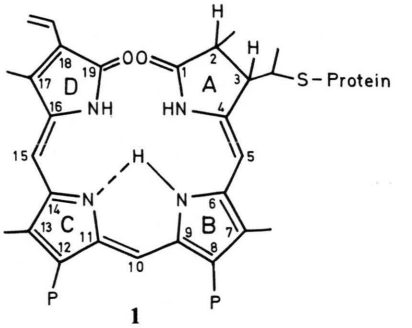
Dedicated to Professor Dr. R. Huisgen *on the Occasion of His 60th Birthday*

Phytochrome, Bile Pigments, Phytochromobilin, Thioether Cleavage, C-Phycocyanin

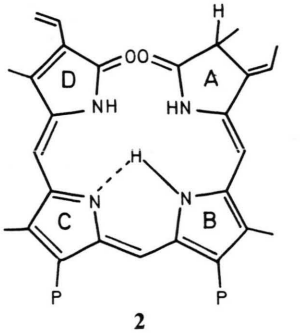
The cleavage of the bilin chromophore from C-phycocyanin with hydrogen bromide yields 3E-configurated phycocyanobilin (**4**) as the major and 3Z-configurated phycocyanobilin (**5**) as the minor reaction product. The reaction of synthetic 3E-configurated phytochromobilin (**2**) with hydrogen bromide and methanol leads only to a methanol adduct at the C-18 side chain (**7**) whereas the same reaction with the 3Z-configurated phytochromobilin (**3**) leads to **7** and **2**. The bilin chromophore was cleaved also from phytochrome after preparation of phytochromobilin peptides. The detection of **2** and **7** suggested that 3Z- and 3E-configurated phytochromobilin were the primary products of cleavage from phytochrome. A reaction scheme is given which can explain the results of the reaction with hydrogen bromide and methanol.

Phytochrome, the most important photoreceptor for photomorphogenesis in higher plants (reviews: [1–3]), is a biliprotein. Proposals for the chemical structure (**1**)* of the bile pigment chromophore, phytochromobilin and its covalent protein linkage were based only on indirect evidence. Spectrophotometric comparison of phytochrome (after unfolding of the peptide chain) and model chromophores suggested an A-dihydrobiliverdin structure [4, 5]. Oxidative degradation of phytochrome allowed the extraction and identification of defined breakdown products of the chromophore [6–8]. However, isolation and characterization of free phytochromobilin were hampered by the poor yield of the cleavage reaction either with boiling methanol [9] or with cold hydrogen bromide [10]. This sharply contrasted the situation with other biliproteins *e.g.* phycocyanin where the HBr method cleaved the bilin chromophore in almost quantitative yield [11].

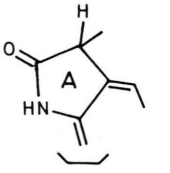
The total synthesis of E-phytochromobilin (**2**) and Z-phytochromobilin (**3**) [12] enabled us to study the reactivity of these pigments towards HBr. We report here on this reactivity and on an improved cleavage of the phytochrome chromophore derived from this study. This enabled us to identify bile pigments derived from natural phytochrome with pigments obtained by total synthesis.



1



2



3

rings B,C,D as in 2

* P in this and all subsequent formulas means $CH_2CH_2CO_2H$ for natural free carboxylic acids and $CH_2CH_2CO_2CH_3$ for dimethyl esters investigated in this paper.

Abbreviations: TLC, thin layer chromatography.

Reprint requests to Professor Dr. W. Rüdiger.

0341-0382/80/0900-0763 $ 01.00/0

Materials and Methods

Small phytochrome (60000 daltons) was isolated from 3.5 days old etiolated oat seedlings [13] according to Rice *et al.* [14] and Pratt [2]. After chromatography on brushite, DEAE-cellulose and Sephadex G-200 and dialysis against 10 mM tris-HCl, pH 7.4, containing 10 mM KCl, 1 mM NaN_3, and 1 mM EDTA, the product had a purity index $A_{665}:A_{280} = 1:9$. *Chromopeptides* were prepared by pepsin digestion of this phytochrome according to Fry and Mumford [15] but omitting the ammonium sulfate precipitation step. Purification was performed on a column (1.6 × 30 cm) filled with Bio-Gel P-2 with 5% formic acid (Fig. 2). The blue fractions (20 to 100) were collected, recovery of chromopeptides was 76%. The acidic solution was then lyophilized, recovery of this step was 71% as determined by spectrophotometry after dissolving an aliquot of the blue powder in 5% formic acid.

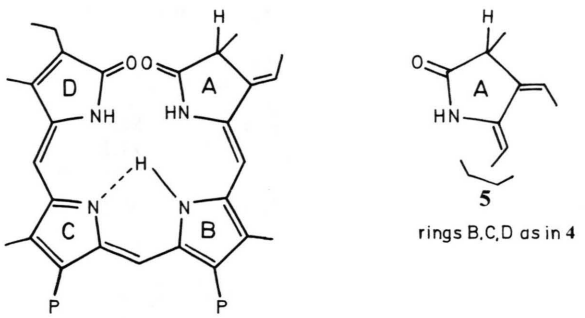
For the cleavage of *phytochromobilin,* 60 nmol of the blue chromopeptide lyophilisate were dissolved in 3 ml trifluoroacetic acid. Air was removed from the flask by bubbling the solution with nitrogen gas for 10 min. Dry hydrogen bromide gas was then passed into the solution for 10 min. The solution turned yellow during this time. Hydrogen bromide and trifluoroacetic acid were then removed by a stream of nitrogen (whereby the flask was kept at 30 °C in a water bath). The dry greenish residue was dissolved in 2 ml precooled methanol containing 7.5% sulfuric acid. The blue solution was kept at −20 °C for 15 h to complete esterification. Dimethylesters of the bile pigments were extracted into dichloromethane (3 × 1 ml) from which acid and methanol were removed with water (4 × 5 ml). The solution was dried with sodium sulfate. Yield: 15 nmol (25%) esterified product, which consists according to TLC analysis of phytochromobilin (**2**) and the methanol adduct **7** (R_F values in Table II). Yield was determined with absorption coefficients of chromopeptide [15] and phytochromobilin [12].

Methanol adduct **7**: 46.6 mg (76 µmol) phytochromobilin dimethylester (**2**) were dissolved in 3 ml anhydrous trifluoroacetic acid and treated with hydrogen bromide (for 20–25 min) after removal of air with nitrogen gas as above. The dry residue was dissolved in 2 ml methanol and mixed with 20 ml 5% precooled methanolic sulfuric acid. This treatment served to reesterify some eventually saponified pigment. After extraction with dichloromethane and water as above, the concentrated pigment solution (~ 1 ml) was applied to 5 TLC plates (20 × 20 cm) coated with silica gel G (HPTLC, Merck, Darmstadt). Chromatograms were developed with carbon tetrachloride/ethyl acetate (1:1, v/v). A minor blue band ($R_F = 0.36$) was not further investigated. The main blue band ($R_F = 0.31$) was eluted with acetone. The residue of this eluate (15.7 mg = 24.5 µmol) consisted of the methanol adduct **7** ($\lambda_{max}^{methanol}$ = 600 nm, $\varepsilon_{max} = 16700$; $\lambda_{max}^{methanol/H^+}$ = 660 nm, $\lambda_{max}^{methanol/Zn^{2+}}$ = 635 nm). It was used for mass spectroscopy (Fig. 1) and ^{1}H-NMR spectroscopy (Table I) without further purification.

Cleavage of phycocyanobilin from C-phycocyanin: 1 mg C-phycocyanin (isolated from *Spirulina platensis* [16]) were dissolved in 3 ml trifluoroacetic acid and treated with hydrogen bromide as above. After workup with methanol, methanolic sulfuric acid, dichloromethane and water as usual, the product was applied to TLC (HPTLC plates coated with silica gel G, Merck, Darmstadt) with carbon tetrachloride/ethyl acetate as solvent. Besides the major zone of E-phycocyanobilin dimethylester (**4**) a minor zone of Z-phycocyanobilin dimethylester (**5**) was detected (R_F values in Table II).

Results and Discussion

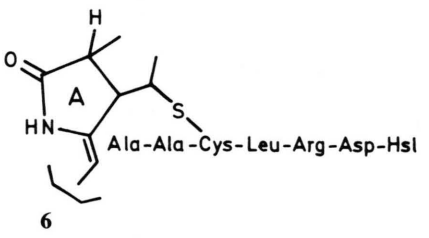
The key for the isolation of free phytochromobilin (**2**) from phytochrome should be a good cleavage reaction. Because free phycocyanobilin (**4**) had been obtained from phycocyanin in almost quantitative yield with hydrogen bromide in trifluoroacetic acid [11] we checked this reaction first. The chromophore cleavage was quantitative also in our hands with phycocyanin: after reaction with HBr for only 10 min all blue colour was extracted into the organic layer whereas the aqueous phase which contained the protein was colourless. After esterification with methanolic sulfuric acid (see Materials and Methods) the esterified blue pigments were investigated by TLC. The pigment fraction contained besides the well-known dimethylester of E-phycocyanobilin (**4**) a faster moving pigment in small amounts which was identified as Z-phycocyanobilin dimethylester (**5**) by the following experiments: 1. **5** prepared by photoisomerization from **4** as described earlier [12] was identical in cochromatography. 2. **5** isolated by TLC was converted



4

5

rings B,C,D as in 4



6

rings B,C,D as in 4

into **4** except for some unreacted **5** by acid treatment (*e.g.* with sulfuric acid or hydrogen bromide). 3. This conversion leads to an equilibrium because some **5** was also obtained by treatment of crystalline, synthetic **4** with hydrogen bromide. Thus the reaction with HBr is possibly not stereospecific with regard to the configuration of the ethylidene group.

The next question was to check the reaction of HBr with E-phytochromobilin (**2**). Synthetic **2** reacted with HBr to yield – after workup with methanol, see Materials and Methods – a new product in quantitative yield which was identified as the methanol adduct (**7**) as follows. The mass spectrum (Fig. 1) shows a parent peak at *m/e* 644 which corresponds to addition of one molecule of methanol to phytochromobilin dimethylester ($M = 612$). The fragmentation pattern is characterized by loss of methanol and subsequent fragmentation of the propionic acid side chains. The position of the new methoxy substituent follows from the ^{1}H-NMR-spectrum (Table I). Nearly all signals of the methanol adduct (**7**) can also be found in a recently investigated phycocyanobilin peptide (**6**) [17] and in phytochromobilin (**2**) except those at 1.427/1.444, 3.302/3.315 and 4.352/4.359 ppm. These signals correspond to a $CH(OCH_3)CH_3$ group [18] whose chemical shifts, however, do not agree with the values found for the $CH(OCH_3)CH_3$ group of the trans-configurated methanol adducts of phycocyanobilin dimethylester which have been synthesized and characterized unequivocally [19]. With HBr in methanol as described here, no methanol adduct was obtained from phycocyanin or phycocyanobilin. Furthermore, the methanol adduct obtained from phytochromobilin still contains the ethylidene group as shown by the corresponding ^{1}H-NMR signals (Table I). The signals of the 3-ethylidene group at 1.905 and 6.407 ppm are typical for the E-configuration and clearly different from signals at 2.12 and 5.86 ppm determined for 3(Z)-phytochromobilin (**3**) or at 2.08 and 5.84 ppm determined for 3(Z)-phycocyanobilin (**5**) [12]. The only signals of phytochromobilin (**2**) which are not found in the methanol adduct (**7**) are those of the vinyl group at 5.41, 6.22 and 6.52 ppm [12], not given in Table I. It is obvious therefore that the vinyl group at C-18 has added methanol.

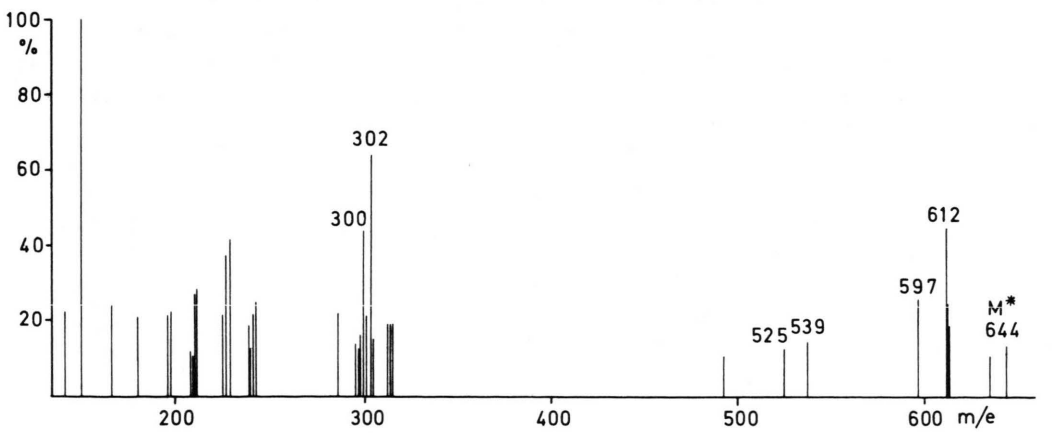


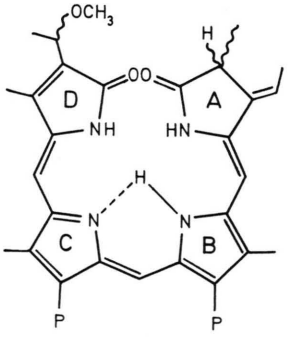
Fig. 1. EI Mass spectrum of the methanol adduct **7**. Direct inlet measurement at 70 eV in a mass spectrometer IMS-D 100 (JEOL), source temperature 250 °C, sample temperature 110 °C.

Table I. 270-MHz ^{1}H-NMR assignments for the methanol adduct **7** in $CDCl_3$ compared with the corresponding signals of phytochromobilin dimethylester (**2**) and a phycocyanobilinpeptide (**6**).

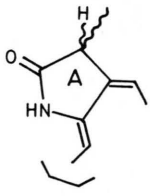
7 ($CDCl_3$)		Assignment	**2** ($CDCl_3$) [a]		**6** (pyridin-d_5) [b]	
ppm	multiplicity *J* [Hz]		ppm	Multiplicity *J* [Hz]	ppm	Multiplicity *J* [Hz]
1.339 [c] / 1.359	d; 8	2-CH_3	1.30	d; 8	1.39	d; 7.3
1.427 [c] / 1.444	d; 7	18-CH(OCH_3)CH_3	–	–	–	–
1.905	d; 7	3-CH–CH_3	1.81	dd; 8 and 1	–	–
2.046	s	7.13-CH_3	2.03	s	2.02	s
2.126	s		2.12	s	2.07	s
2.270 [c] / 2.274	s	17-CH_3 [e]	2.20	s	2.12	s
2.553 [d]	m	8.12-$CH_2CH_2CO_2R$	2.4–2.7	m	2.83; 2.85	m
2.86–2.98	m	8.12-$CH_2CH_2CO_2R$	2.8–3.2	m	3.09; 3.17	m
3.302 [c] / 3.315	s	18-CH(OCH_3)CH_3	–	–	–	–
3.674 / 3.685	s / s	8.12 propionic ester OCH_3	3.68 / 3.70	s / s	– / –	– / –
4.352 [c] / 4.359	m	18-CH(OCH_3)CH_3	–	–	–	–
5.830	s	5-H	5.82	s	5.87	s
6.065	s	15-H	6.08	s	6.08	s
6.407	dq; 7 and 2	3- CH–CH_3	6.38	dq 7 and 2.2	–	–
6.627	s	10-H	6.63	s	7.29	s

[a] 100 MHz ^{1}H-NMR; data from Weller and Gossauer [12].
[b] 360 MHz ^{1}H-NMR; data from Langarias *et al.* [17].
[c] Two sets of signals because of mixture of stereoisomers.
[d] Presumably contains also multiplet of 2-H.
[e] Assignment at 17-CH_3 because stereoisomers at C-18 side chain should preferably cause 2 signals at this position but not at 7-CH_3 and 13-CH_3.

The reaction with HBr and methanol was also applied to 3(Z)-phytochromobilin (**3**) which had been obtained from **2** by photoisomerization [12] and isolated by TLC (R_F values see Table II). Surprisingly, two products were obtained in a ratio of about 2:1 which were identified by TLC with **7** and **2**, respectively. No unreacted Z-phytochromobilin (**3**) was left. The minor product was further characterized by a subsequent reaction with HBr and methanol which quantitatively yielded (**7**) as expected. This means that E-phytochromobilin (**2**) has not been present when HBr was introduced but was formed later, presumably when methanol reacted with the mixture. Because the identity of (**7**) was checked only by TLC in these experiments the question arose whether the Z-configurated methanol adduct (**8**) would indeed be separated from the E-configurated isomer **7**. We therefore applied conditions for photoisomerization [12] also to **7**. The resulting mixture contained – besides traces of **2**



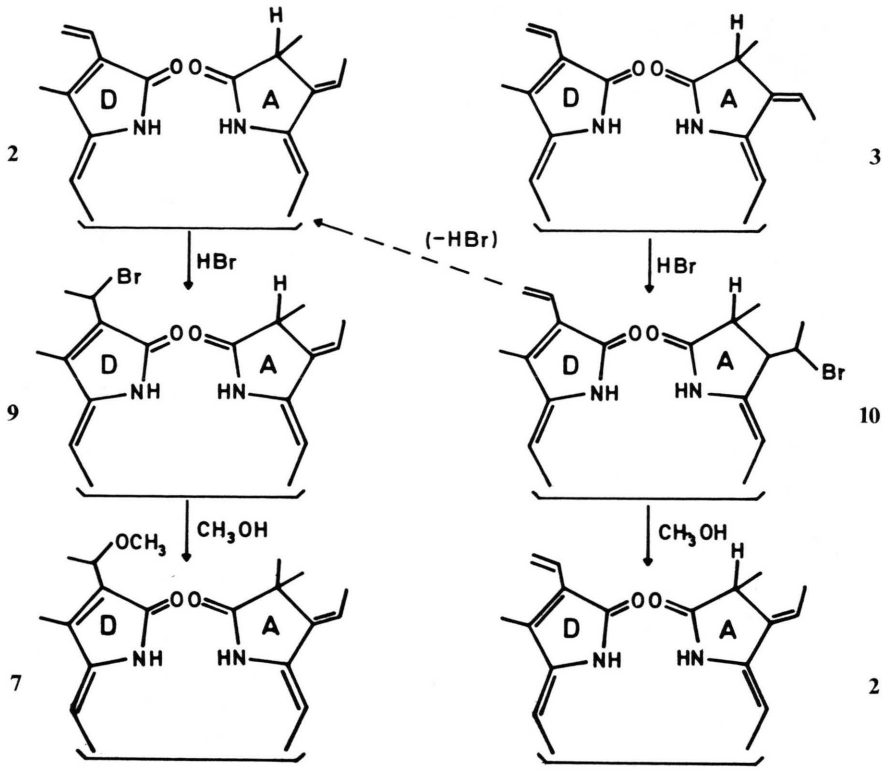
7



8

rings B,C,D as in **7**

Scheme 1. Proposed sequence of the reactions of E- and Z-phytochromobilin (**2** and **3**) with hydrogen bromide and methanol.



and **3** – a 1:1 mixture of **7** and a new pigment which was presumably **8**. It was isolated by TLC (R_F values see Table II). The new pigment was easily reconverted to **7** either photochemically or thermally.

The most probable explanation is given in Scheme 1: HBr adds according to this assumption not only at the vinyl group to product **9** but also at the ethylidene group to product **10**. The subsequent reaction with methanol would be either elimination of HBr at the side chain of ring A to yield the E-ethylidene group (product **2**) or nucleophilic substitution to yield the methoxy substituent at ring D (product **7**). The experimental results can be explained 1. if reactivity towards HBr decreases in the order Z-ethylidene > vinyl > E-ethylidene and 2. if addition of one HBr excludes the addition of a second HBr. These predictions are experimentally tested at the moment.

The concept of highly reactive, brominated intermediates (structure **9**) led to the assumption that the small yield of free phytochromobilin from phytochrome could be due to a secondary reaction with nucleophilic functional groups of the protein. This would lead to irreversible binding of the chromophore with the protein. Hence the problem was to remove most of the reactive functional groups of the protein before the cleavage reaction. We therefore prepared chromopeptides from phytochrome according to a described method [15]. The yield of chromopeptides after pepsin digestion was about 90%. Separation of the chromopeptides from most of the colourless peptides was achieved by a chromatography step (Fig. 2). The chromopeptide fraction (chromophore yield 76%) was then lyophilized, dissolved in anhydrous trifluoroacetic acid and

Table II. R_F values of bile pigments related to phytochromobilin HPLC-plates (Merck Darmstadt) coated with silica gel G, solvent A: carbon tetrachloride/ethyl acetate 1:1 (v:v), solvent B: carbon tetrachloride/acetic acid 1:1 (v:v).

	A	B
E-phytochromobilin (**2**)	0.40	0.41
Z-phytochromobilin (**3**)	0.45	0.48
E-phycocyanobilin (**4**)	0.35	0.37
Z-phycocyanobilin (**5**)	0.41	0.43
E-methanol adduct (**7**)	0.27	0.35
Z-methanol adduct (**8**)	0.33	0.43

Table III. Spectral data of phytochromobilin and related bile pigments. The pigments were dissolved in ethyl acetate. The maxima of the broad long-wavelength absorption band were determined from the 2. derivative of the absorption curve.

Pigment	Absorption maxima [nm]	
A-dihydrooctaethyl biliverdin [5]	617	566
E-phycocyanobilin (**4**)	641	587
E-methanol adduct (**7**)	636	582
E-phytochromobilin (**2**)	653	600
pigment mixture obtained from phytochrome	639–649	603, 582
difference spectrum [a]	651	603

[a] Difference spectrum = spectrum of pigment mixture obtained from phytochrome minus spectrum of **7**. Calculation based on 1:1 mixture of two compounds as revealed by TLC.

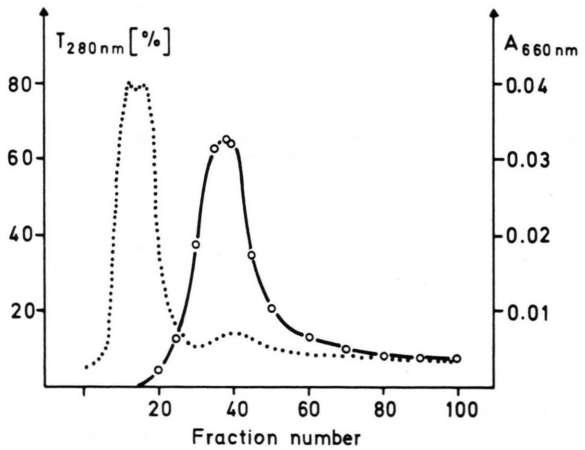


Fig. 2. Chromatography of peptide mixture obtained by peptic digestion of phytochrome on Biogel P-2 with 5% formic acid. (· · · ·) $T_{280\,nm}$, indicating peptides; (O–O–O) $A_{660\,nm}$, indicating phytochromobilin peptides.

treated with hydrogen bromide. The solution turned greenish-yellow first and then blue again. Analysis of the chromophore fraction – after esterification – by TLC revealed the presence of E-phytochromobilin (**2**) and E-configurated methanol adduct (**7**) in the ratio of about 1:1. *This is the first preparation of defined bile pigments from phytochrome.* Siegelman *et al.* [9] were able to cleavage a small amount of bile pigment from phytochrome by boiling methanol. But the yield was much smaller than that of the present preparation. Furthermore, no characterization of the bile pigment was achieved at that time because of lack of authentic phytochromobilin.

Bile pigments can be characterized by the position of the long-wavelength absorption-band [20]. In the case of neutral bilatrienes, broad bands with shoulders or two peaks of varying intensity are observed [12, 20, 21]. This is possibly due to the presence of various conformers with different spectral properties [22]. For the characterization of the bile pigments delt with in this paper, we used the second derivative of the absorption spectrum to exactly localize the absorption peaks (Table II). The chromophore system of the methanol adduct (**7**) is the same as that of phycocyanobilin (**4**). Therefore the position of the maxima is nearly the same in **4** and **7**. Loss of the ethylidene group leads to a hypsochromic shift of about 20 nm (comparison with A-dihydrobiliverdin) whereas introduction of a vinyl group leads to a bathochromic shift of 15 to 20 nm (comparison with phytochromobilin **2**). The pigment mixture obtained from phytochrome has 2 peaks at 582 and 603 nm and a broad absorption band at 639–649 nm which certainly contains 2 more peaks. Because TLC analysis had revealed a 1:1 mixture of **2** and **7** we calculated the spectrum of one component after subtraction of the spectrum of **7**. The difference spectrum so obtained agrees very well with the spectrum of phytochromobilin (**2**) (see Table III). This can be taken as further evidence for the presence of **2** and **7** in the pigment mixture obtained from phytochrome.

The result of the present investigation can best be explained by a cleavage of the thioether linkage [8] in such a way that a mixture of Z- and E-configurated phytochromobilin (**2** and **3**) are formed first. This would correspond to the result with phycocyanin. **2** would then further react with HBr and methanol to yield E-configurated methanol adduct (**7**) whereas **3** would react to **2** and **7** (scheme 1). Therefore **2** and **7** are the only products observed after completion of the reaction sequence.

Acknowledgements

We thank Dr. R. Zeisberg (TU Berlin) for the measurement of the 270 MHz ^{1}H-NMR spectrum and Dr. H. Scheer (Univ. München) for a gift of pure C-phycocyanin. We thank the DFG, Bonn-Bad Godesberg, for financial support of this research.

[1] H. Smith, Phytochrome and Photomorphogenesis, McGraw-Hill, London 1975.
[2] L. H. Pratt, Photochem. Photobiol. **27,** 81–105 (1978).
[3] W. Rüdiger, Structure and Bonding **40,** 101–140 (1980).
[4] S. Grombein, W. Rüdiger, and H. Zimmermann, Hoppe-Seyler's Z. Physiol. Chem. **356,** 1709–1714 (1975).
[5] H. Scheer, Z. Naturforsch. **31 c,** 413–417 (1976).
[6] W. Rüdiger and D. L. Correll, Liebigs Ann. Chem. **723,** 208–212 (1969).
[7] W. Rüdiger, Phytochrome (K. Mitrakos and W. Shropshire, Jr., eds.), pp. 129–141, Academic Press, New York 1972.
[8] G. Klein, S. Grombein, and W. Rüdiger, Hoppe-Seyler's Z. Physiol. Chem. **358,** 1077–1079 (1977).
[9] H. W. Siegelman, B. C. Turner, and S. B. Hendricks, Plant Physiol. **41,** 1289–1292 (1966).
[10] H. H. Kroes, Meded. Landbouwhogeschool Wageningen **70-18,** 1–112 (1970).
[11] B. L. Schram and H. H. Kroes, Eur. J. Biochem. **19,** 581–594 (1971).
[12] J.-P. Weller and A. Gossauer, Chem. Ber. **113,** 1603–1611 (1980).
[13] S. Grombein and W. Rüdiger, Hoppe-Seyler's Z. Physiol. Chem. **357,** 1015–1018 (1976).
[14] H. V. Rice, W. R. Briggs, and C. J. Jackson-White, Plant Physiol. **51,** 917–926 (1973).
[15] K. T. Fry and F. E. Mumford, Biochem. Biophys. Res. Commun. **45,** 1466–1473 (1971).
[16] H. Scheer and W. Kufer, Z. Naturforsch. **32 c,** 513–519 (1977).
[17] J. C. Lagarias, A. N. Glazer, and H. Rapoport, J. Amer. Chem. Soc. **101,** 5030–5037 (1979).
[18] As two sets of signals are obtained for each type of proton, both possible stereoisomeres must be present in the obtained product as indicated in formula **7**.
[19] A. Gossauer, R.-P. Hinze, and R. Kutschan, Chem. Ber., in press (1980).
[20] W. Rüdiger, Fortschr. Chem. Organ. Naturst. **29,** 59–139 (1971).
[21] G. Klein and W. Rüdiger, Z. Naturforsch. **34 c,** 192–195 (1979).
[22] S. E. Braslavsky, A. R. Holzwarth, E. Langer, H. Lehner, J. J. Matthews, and K. Schaffner, Liebigs Ann. Chem., in press (1980).