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STUDIES ON THE STEREOCHEMISTRY OF BILIPROTEIN CHROMOPHORES AND RELATED MODEL COMPOUNDS

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Abstract—The problems related to the assignment of the absolute configuration of chiral biliprotein chromophores (e.g. phycocyanins, phycoerythrins and phytochrome) are summarized and considered critically in the light of the experimental data currently available.

THE origin of phototrophic prokaryotes capable of producing molecular oxygen from water, to which blue-green algae belong, is estimated at about 3.5×10^9 years ago.[1] Blue-green algae (Cyanobacteria) as well as the eucaryotic red algae (Rhodophyta) and some Cryptophyta (cryptomonad algae) contain besides chlorophyll, as the essential photosynthetic pigment, several chromoproteins (termed phycobiliproteins) which perform important functional roles as light harvesters and light sensors.[2] In the living algae, these accessory pigments are organized into macromolecular assemblies—so called phycobilisomes—regularly arranged on the thylakoid membrane, thus enabling the transfer of light energy to chlorophyll with high efficiency.[3] The corresponding monomers, in turn, contain in most cases two physically separable polypeptide subunits—defined as α and β—which differ both in their molecular weight and in their content of chromophore molecules.

On the basis of their coloration algal biliproteins are differentiated into two main groups: phycoerythrins are a clear red by transmitted light and emit a brilliant orange-yellow fluorescence; phycocyanins are blue with a strong red fluorescence. In a few individual cases, the structures of the corresponding chromophores have been unequivocally assigned as shown in Fig. 1. Thus, regardless of their stereochemistry, the different types of biliprotein chromophores are characterized by the structure of ring IV of the bile pigment molecule (*vide infra*). Most likely, the chromophores represented in Fig. 1 occur in most phycobiliproteins although other less frequent chromophores have also been found, whose structures, however, have not yet been elucidated.

Most species of red, blue-green, and cryptomonad algae contain both a phycoerythrin and one or more phycocyanins, although a single biliprotein usually predominates thus giving rise to the colour of the particular species. Depending on their origin, both phycoerythrins and phycocyanins are differentiated by capital letters set as prefixes, thus C-, R- and B-stand for Cyanobacteria, Rhodophyta and Bangiophyceae (a subclass of the lower red algae) respectively. However, such a correspondence with the taxonomic pattern is now known to be only partly fulfilled.

A peculiar feature of phycobiliproteins is the covalent linkage between the chromophore and the apoprotein (cf. Fig. 1). In this respect they differ from other photosynthetic pigments, e.g. chlorophylls and carotenoids, which are readily released from algal cells by extraction into organic solvents. The cleavage of the phycobilin-apoprotein linkages requires relatively drastic hydrolytic conditions, and the lability of the bilins during such treatment has severely hampered

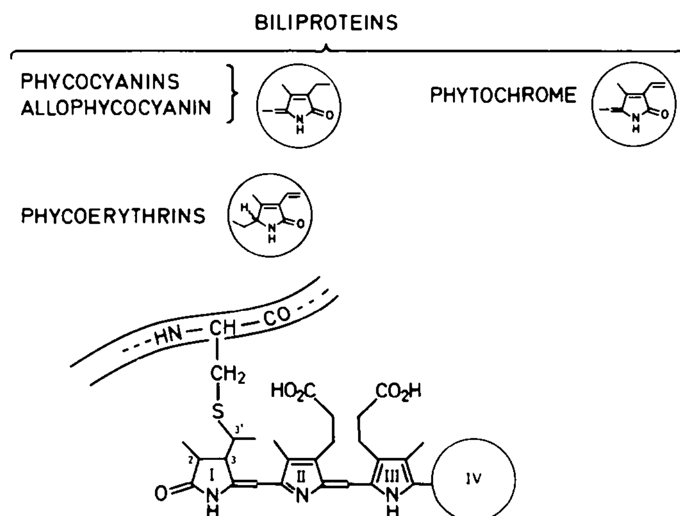


Fig. 1. Structural relationship between the different types of biliprotein chromophores.

and confused structural studies in this area. On the basis of the visible spectral data of the phycobiliproteins themselves and of the pigments obtained therefrom by acid hydrolysis, Lemberg[4] deduced that both phycoerythrins and phycocyanins contain bile pigment-like chromophores (i.e. phycobilins), for which he coined the names phycoerythrobilin and phycocyanobilin respectively. This correspondence is, however, not always fulfilled. Lemberg himself realized later[5] that some algal biliproteins (e.g. R-phycocyanin) contain both phycoerythrobilin and phycocyanobilin, and this has been confirmed by other workers.[6,7] Moreover, as both phycoerythrobilin and phycocyanobilin are covalently attached to the apoprotein, the isolated prosthetic groups have necessarily a structure different from that of the corresponding "native" chromophores, so that for a long time some disagreement prevailed in the literature concerning the nomenclature of phycobilins.[8]

A further source of confusion arose in the pioneering days of phycobilin chemistry from the fact that several pigments (e.g. phycobilins 608, 630 and 655) were obtained from the biliproteins depending on the conditions of isolation and purification of the prosthetic groups. Some of them have been recognized later to be artefacts[9] generated by formal substitution of the peptide chain by nucleophiles.[10] At this stage, and by way of definition, it seems appropriate to assign arbitrarily the names phycocyanobilin and phycoerythrobilin to compounds **1a** and **2a**, respectively.[11]

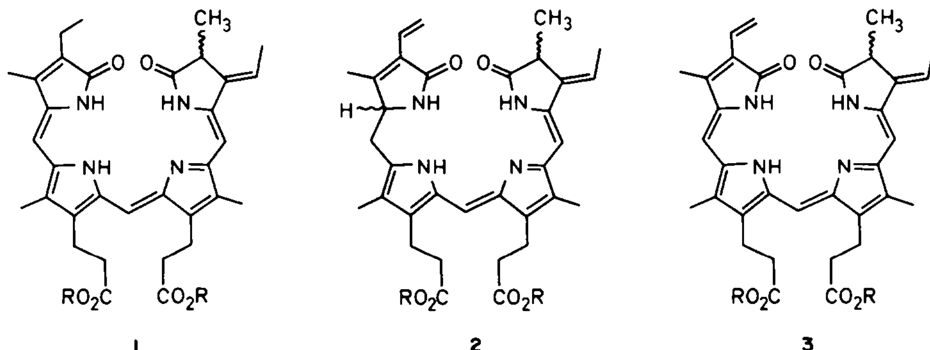
Until now, phycocyanobilin dimethyl ester (**1b**) has been isolated from C- and R-phycocyanin as well as from allophycocyanin (a quantitatively minor component of most—possibly all—red algae and blue-green algae).[12,13] Phycoerythrobilin dimethyl ester **2b**) has been isolated from B-, C- and R-phycoerythrin and – as mentioned before—from R-phycocyanin.[14,15] Their structures have been elucidated by means of spectroscopic[16-19] and degradation studies[20,21] and confirmed by total syntheses.[22-25] Moreover, the complete amino acid sequences of the β subunit of C-phycocyanin from the cyanobacterium *Synechococcus* sp. 6301[26,27] as well as of both α- and β-subunits of C-phycocyanin from *Mastigocladus laminosus*[28] have been determined. These fundamental studies demonstrated conclusively that each of the three bilin moieties of C-phycocyanin (one located in the α subunit and two in the β subunit) is linked to the polypeptide chain through a cysteinyl thioether linkage. No evidence was found, however, for a second linkage between the apoprotein and the chromophores, as postulated earlier.[29] A thioether bond between the thiol group of cysteine and ring I of the phycobilin chromophore has also been demonstrated at least for one, but certainly not for all, of the prosthetic groups[30] of B-phycoerythrin (from the red alga *Porphyridium cruentum*) by Edman degradation of a tripeptide isolated by peptic digestion of the algal protein.[31]

On the other hand, a heptapeptide derived by cleavage with cyanogen bromide from the β-subunit of C-phycocyanin (from *Synechococcus* sp. 6301) has been investigated by 360 MHz ^{1}H-NMR spectroscopy.[32] On the basis of these studies both the structure of the peptide-linked phycobilin and the nature of the thioether chromophore-peptide linkage could be elucidated unequivocally for the first time. However, in order to assign the absolute configuration at the three asymmetric carbon atoms of the chromophore some assumptions had to be made which cannot be considered conclusive at present.

Irrespective of the inherent significance which must be attributed to a detailed knowledge of the stereochemistry of the phycobiliprotein chromophores, the assignment of the absolute configuration of phycobilins may contribute to a better understanding of the dependence of the spectroscopic properties of the chromophores on the tertiary structure of the apoprotein in biliproteins. Such a dependence deserves particular interest in the case of phytochrome, a light receptor which governs plant morphogenesis.[33]

In the living plant, phytochrome occurs in two modifications, a red absorbing form (P_r) and a far red absorbing form (P_{fr}), which are interconverted by irradiation with light of λ_{max} 665 and 725 nm respectively. So far, only the structure of the thermodynamically more stable P_r chromophore is known (*vide infra*), whereas the structure of P_{fr} remains still a matter of speculation. However, most of the mechanisms suggested for the phototransformation $P_r \rightleftharpoons P_{fr}$ involve changes in the stereochemistry of the chromophore, so that the knowledge of the configurations actually present at both the chirality centres and the exocyclic double bonds of the chromophores has to be considered as a prerequisite for a better understanding of its photochemical properties.

The relationship of the phytochrome chromophore with the phycobilins was recognized as early as 1950



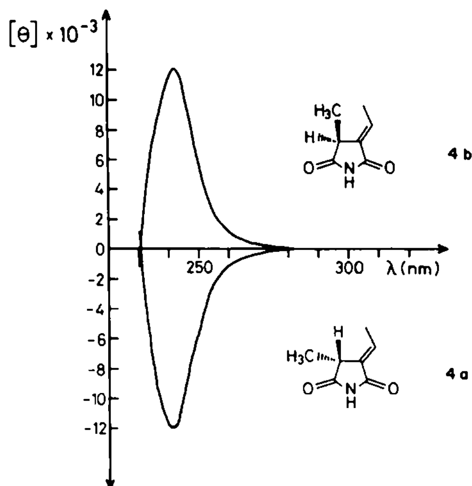


Fig. 2. CD-spectra of (+) and (−)- 2-methyl-3-(*E*)-ethylidene-succinimide (10^{-3} M in methanol).

by Borthwick *et al.*[34,35] On the basis of oxidative degradation studies, Rüdiger[36,37] assigned to the chromophore of P_r the structure depicted in Fig. 1. This structure has been recently confirmed by the total synthesis of phytochromobilin dimethyl ester (**3b**)[38] which was identified with the blue pigment isolated from P_r by pepsin digestion and subsequent acidic hydrolysis,[39] as well as by the 270 MHz ^{1}H-NMR spectrum of a chromophore-containing undecapeptide isolated from the red form of phytochrome by enzymatic degradation.[40] In the latter work the relative configuration of the P_r chromophore was assumed to be the same as in C-phycocyanin owing to the similarity of the high resolution ^{1}H-NMR spectra of both prosthetic groups.

In the following, the evidence for the assignments of the configurations at the different asymmetric carbon atoms present in phycobilin molecules will be discussed separately for the three known biliprotein chromophores in the light of the experimental data now available. For the sake of objectivity, no generalizations will be made, even though some of them might seem to be evident to the reader owing to the high degree of homology present within phycobiliproteins.[41]

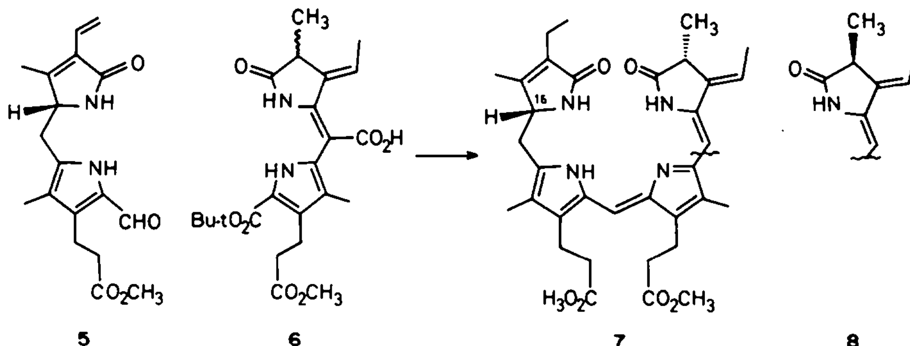
Configuration at C-2 (cf. Fig. 1)

The absolute configuration of the C-2 atom in C-phycocyanin (from *Phormidium luridum*) has been found to be *R* by Brockmann and Knobloch.[42] This assignment is based on the chiroptical data of the optically active (−)-2-methyl-3-(*E*)-ethylidenesuccinimide (**4a**) isolated by chromic acid oxidation of phycocyanobilin dimethyl ester which was obtained by cleavage of the chromophore from the algal biliprotein with boiling methanol. The absolute configuration of dextrorotatory 2-methyl-3-(*E*)-ethylidenesuccinimide (**4b**) which had been partially separated from the racemate by chromatography on cellulose acetate, was assigned by transformation into optically active 2-methyl-3-ethylsuccinimide of known absolute configuration.[43] Considerably purer samples of both (+) and (−)-2-methyl-3-(*E*)-ethylidenesuccinimides were obtained later by oxidative degradation of synthetic phycoerythrobilin dimethyl ester[25] (cf. Fig. 2).

Laevorotatory 2-methyl-3-(*E*)-ethylidenesuccinimide was also isolated by Rüdiger[44] on oxidative degradation of aplysioviolin, the main violet pigment from the defensive excretion of the sea hare *Aplysia limacina*. As aplysioviolin arises probably from the phycoerythrins contained in the food (red algae) of these Mediterranean snails,[45] the *R*-configuration was inferred for the phycoerythrins.[46] A more straightforward proof was obtained in the course of the total synthesis of optically active phycoerythrobilin dimethyl ester (**2b**).[25] Only one of the two diastereomeric synthetic phycobilins obtained by condensation of the enantiomerically pure aldehyde **5** with racemic **6** proved to be identical (by CD- and ORD-spectroscopy) with phycoerythrobilin dimethyl ester isolated by treatment of C-phycoerythrin (from *Phormidium persicinum*) with boiling methanol. Oxidative degradation of 7 afforded laevorotatory (i.e. *R*) 2-methyl-3-(*E*)-ethylidenesuccinimide, in 56% yield, whereas the corresponding antipode was obtained from diastereomer **8** (cf. Fig. 2). It must be inferred, therefore, that C-phycoerythrin, as C-phycocyanin, has the *R* configuration at C-2. In the course of the same work, the absolute configuration of C-phycoerythrin at C-16 was deduced to be *R*. The latter assignment implies the validity of Moscowitz's rule for optically active urobilinoids[47,48] which has not yet been proved for any compound whose absolute configuration is firmly established (cf. ref. 25).

Since the optical activity of the 2-methyl-3-(*E*)-ethylidenesuccinimide isolated by chromic acid oxidation of both phytochrome forms[49] has not been reported, the absolute configuration of the C-2 atom of the phytochrome chromophore remains undetermined.

The above results undoubtedly show a uniformity



in the stereochemistry of biliproteins from different origins. However, as has been pointed out by Rapoport *et al.*,[32] the assignment of the absolute configuration at C-2 based on the optical activity of succinimide cleavage products obtained directly or indirectly from native biliproteins requires the demonstration of the stereochemical identity of all chromophore molecules present in the biliprotein. Of course, the same limitation holds true for any of the other chirality centres within this class of compounds.

Relative configuration at C-2 *and* C-3

Since reliable ^{1}H-NMR data are available for the chromophores of C-phycocyanin[32] and phytochrome P_r,[40] the assignment of the relative configuration at the C-atoms of the protein-bonded ring of the bilin moiety in the biliproteins should be possible, in principle, by inspection of the signals attributed to the protons bonded to the corresponding chirality centres of the chromophores. Thus, for both C-phycocyanin[32] and phytochrome P_r,[40] a coupling constant of 5.0 Hz has been reported; this does not disagree with the *trans* arrangement of the substituents which has been assumed in the literature by analogy with other tetrapyrrolic pigments bearing dihydro rings (e.g. stercobilin, chlorophylls and bacteriochlorophylls) which occur in nature.[46] However, owing to the very different origins of these pigments and hence the different enzymes which presumably participate in their formation, the biogenetic argument cannot be considered seriously in connection with the problem of phycobilin stereochemistry. On the other hand, the practicability of NMR studies has been thwarted until now by the lack of appropriate reference compounds with known stereochemistry.

Only a few bile pigments related to chromophores of biliproteins are known whose relative configurations at the C-2 and C-3 atoms have been proved convincingly. These are: *cis*- and *trans*-mesobilirhodin dimethyl ester (*rac.* **9a** and *rac.* **9b**, respectively),[50,51] *cis*- and *trans*-isomesobilirhodin dimethyl ester[51] and the *trans* methanol adducts of phycocyanobilin dimethyl ester, *rac.* **10a** and *rac.* **10b**.[10] Unfortunately, however, the complexity of the multiplets observed for the ring protons at C-2 and C-3, as well as their overlapping with other signals (chiefly those of the propionic acid chains), complicates the determination of the coupling constants between them. Moreover it

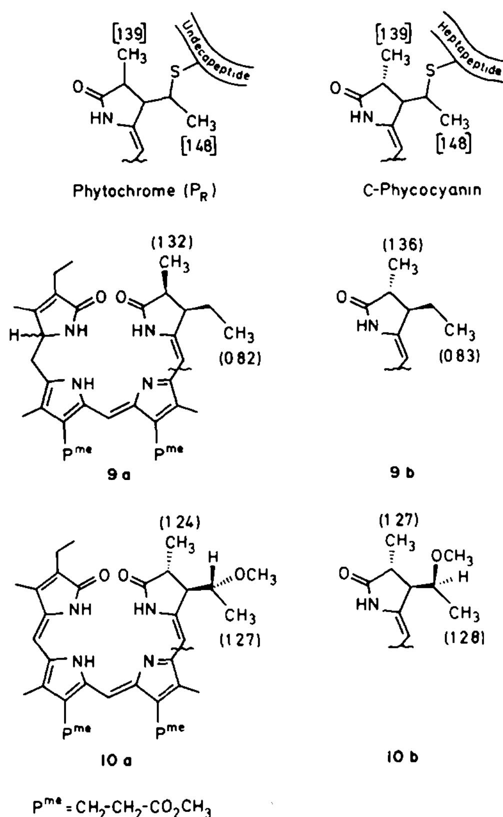
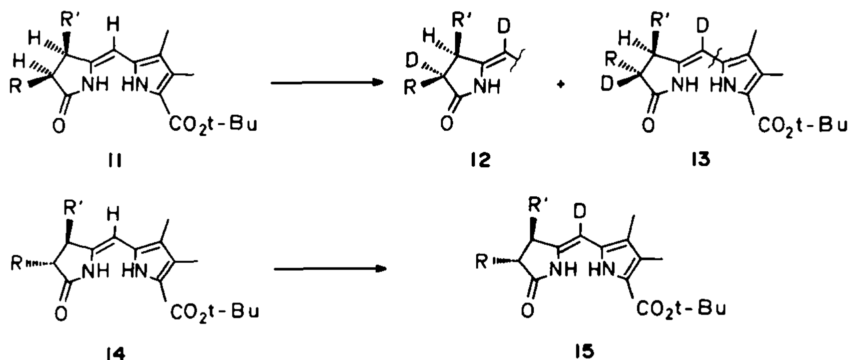


Fig. 3. ^{1}H-NMR chemical shifts of methyl protons in structurally related bile pigment chromophores. In brackets: δ-values from tetramethylsilane, [ ] in pyridine-d_5, ( ) in $CDCl_3$.

is known that the Karplus relationship turns out to be insufficiently discriminating when applied to elucidating the stereochemistry of five-membered heterocycles.[52]

Within a series of structurally related succinimides, Bode and Brockmann[53] observed that non-bonding interactions may be relevant for the *cis*/*trans* relationship of alkyl groups attached to the β-position of the ring. Thus for instance, the chemical shift of the methyl group protons in ethylmethylsuccinimide moves from δ 1.22 to δ 1.35 on changing the relative configuration from *cis* to *trans*. This regularity has



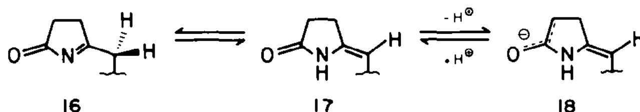


Fig. 4. Proposed tautomerization of 2,3-dihydro-1(10*H*)-dipyrrinones in basic medium (for the sake of clarity, substituents are omitted).

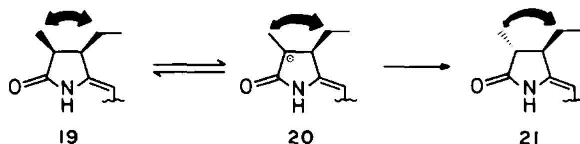


Fig. 5. Steric interactions in *cis*- and *trans*-2,3-dihydro-1(10*H*)-dipyrrinones and their enolates (the size of the arrow head symbolizes the magnitude of steric repulsion).

been confirmed in the case of the *erythro*- and *threo*-isomers of both meso- and isomesobilirhodin.[50,51] When compared with the chemical shifts of the corresponding Me group protons in both (*trans*) methanol adducts of phycocyanobilin dimethyl ester *rac*. **10a** and *rac*. **10b**,[10] however, the differences turn out to be too small to allow an assignment of the relative configurations of such molecules to be made (see Fig. 3). For this reason, the ^{1}H-NMR spectra of two epimeric *trans*-1-(ethylsulfonyl)ethyl-methylsuccinimides were originally misinterpreted[54] as though they were *cis-trans* isomers. The correct structures were demonstrated later by X-ray analysis[55] (cf. ref. 46).

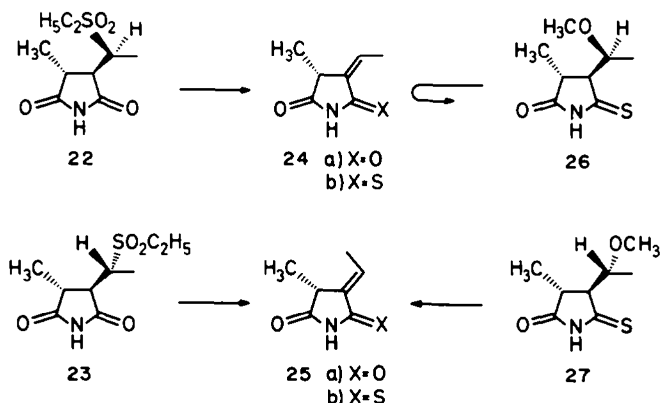
From the energetic point of view, it seems evident that a *trans* arrangement of the substituents at the dihydro ring of the bilin molecule should be thermodynamically favoured. An interesting observation in this connection was made during a study of the properties of reduced dipyrrinone derivatives. Thus, when refluxed in NaOD-CH_3OD, the *cis* compounds *rac*. **11a** c exchange *both* the proton at the methine bridge and that at the C-2 position for deuterium. Therefore, the reaction product consists of a mixture of the *cis*- and *trans*-deuterated compounds *rac*. **12a** c and *rac*. **13a**–c. On the other hand, the corresponding *trans* isomers *rac*. **14a** c exchange, under the same conditions, *only* the proton at the methine bridge, yielding compounds *rac*. **15a** c.

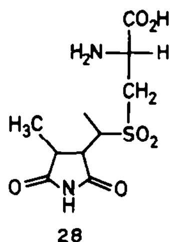
The behaviour in the *cis* series can be straightforwardly explained by the competition between both equilibria represented in Fig. 4. In the *trans* series, however, only the equilibrium **16**$\rightleftharpoons$**17** seems to operate. A possible rationale for this observation is depicted in Fig. 5. As the dihedral angle defined by the planes containing the β-substituents at the lactam ring *increases* when the *cis* isomers (*rac*. **19**) are transformed into the corresponding enolates (*rac*. **20**), steric repulsion should be less in the latter than in the precursors, thus favouring epimerization (and exchange for deuterium) at the C-2 position. On the contrary, enolate formation from the *trans* isomers (*rac*. **21**) would bring about an *increase* of steric repulsion which is apparently severe enough to prevent the abstraction of the proton at C-2. The above results indicate that the *trans* isomers are not only thermodynamically but also *kinetically* stable.

Presumably according to equilibrium **16**$\rightleftharpoons$**17**, the chromophore of C-phycocyanin exchanges the proton at C-5 for deuterium when its ^{1}H-NMR spectrum is recorded in D_2O.[32] However, the signal of the proton at C-2 remains unchanged under these conditions. As the conditions used for H-D exchange experiments in the dipyrrinone series (Experimental) are substantially more drastic than those used for the peptide-linked phycobilin, no conclusion may be drawn, at present, from the above observation. Nevertheless, in the light of the above results, a comparative study with both series of compounds might be helpful in elucidating the stereochemistry of such molecules.

Relative configuration at C-3 *and* C-3^1

During a study of substituted succinimides as models for the chromophore-protein linkage in biliproteins, Rüdiger *et al.*[46,54] made the remarkable observation that on treatment of both C-3^1 epimers of the *trans* isomers (e.g. *rac*. **22** and *rac*. **23**) with ammonia at room temperature, an elimination reaction occurs which leads stereospecifically to *rac*. **24a**





and *rac*. **25a** respectively. These results were confirmed in the course of a stereospecific total synthesis of the methanol adducts of phycocyanobilin dimethyl ester[10] for two epimeric monothiosuccinimides *rac*. **26** and *rac*. **27**. Thus, on heating the latter for 2 min at 90° in dry pyridine, (*E*)-ethylidene-methyl-monothiosuccinimide (*rac*. **25b**) is obtained in 40% yield, and 60% of the starting material is recovered. In contrast, *rac*. **26**, which would lose methanol to give the thermodynamically less stable (*Z*) isomer, *rac*. **24b**, is recovered quantitatively under the same conditions.

Since ethylidene-methylsuccinimide is not released from phycobiliproteins after chromic acid oxidation at room temperature but only after heating,[29] or treatment with ammonia,[46,54] it seems reasonable to deduce that the chromophore-protein bond survives the oxidation conditions (probably as a $C–SO_2$ bond). However, the sulfone of an adduct of cysteine to ethylidene-methylsuccinimide (**28**), which ought to represent a good model for the oxidized biliproteins, was found to be too unstable to be purified.[46] Nevertheless, on treatment of the protein precipitate obtained after chromic acid oxidation of C-phycocyanin,[46] C-phycoerythrin,[46] and phytochrome P_r[49] with ammonia, only (*E*)-ethylidene-methylsuccinimide was obtained, thus suggesting that the relative configurations at C-3 and C-3^1 in the biliproteins should be the same as in the case of the model compound (**22**).

Provided that the same mechanism of elimination assumed for the above succinimides operates in the case of the denatured biliproteins, the configuration at the ethylidene group of the released phycobilins should also reveal the relative configuration at the protein-bonded C-atom. This assumption, however, has not been confirmed experimentally.

In fact, when boiling methanol is used to cleave the chromophores of C-phycocyanin[43] and R-phycoerythrin[24] the phycobilins obtained (i.e. phycocyanobilin and phycoerythrobilin, respectively) have the *E* configuration at the ethylidene double bond. Using hydrogen bromide in trifluoroacetic acid, however, both C-phycocyanin and phytochrome P_r yield phycocyanobilin and phytochromobilin, respectively, as a mixture of *Z* and *E* stereoisomers.[39] Since (*Z*)-phycocyanobilin dimethyl ester is readily available by photoisomerization of the corresponding *E* isomer[38] it

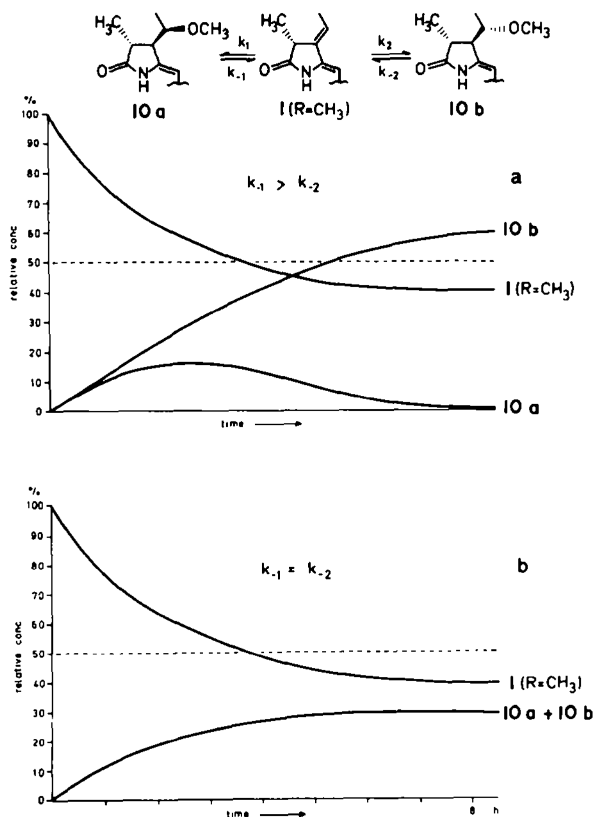
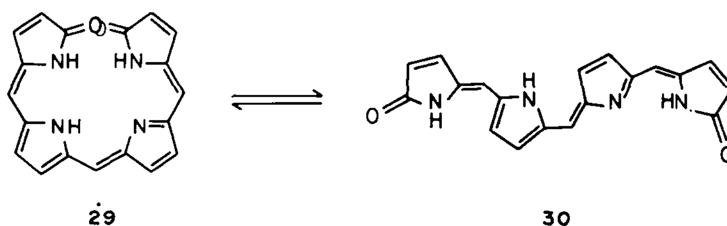


Fig. 6. (a) Hypothetical curves calculated assuming a preferential cleavage of methanol from the phycocyanobilin dimethyl ester adduct (*rac*. **10a**). (b) Experimental curves obtained by monitoring the reaction of phycocyanobilin dimethylester by high-resolution ^{1}H-NMR spectroscopy (see experimental part).



could be demonstrated that the Z isomers are converted quantitatively into the E isomers by boiling methanol and that both Z and E isomers are present in equilibrium when the pure compounds are treated with acid. Therefore, even if (Z)-phycocyanobilin were formed stereospecifically from the biliprotein, it could not be detected as the sole isomer in the reaction mixture. On the other hand, a stereospecific elimination of the protein moiety which would yield exclusively (E)-phycocyanobilin has to be regarded as very improbable on the basis of following experiment.

When heated in methanol (E)-phycocyanobilin dimethyl ester adds a molecule of solvent at the ethylidene double bond[9,42] yielding a nearly equimolecular mixture of only *two* components whose structures have been elucidated unequivocally.[10] Both adducts (*rac.* **10a** and *rac.* **10b**) which are epimers at C-3^1, with the *trans* configuration at the reduced ring, liberate a molecule of methanol when treated with boiling methanol, yielding back (E)-phycocyanobilin dimethyl ester. Bearing in mind that after rather long reaction periods, equimolecular amounts of both adducts are present in the reaction mixture, the possibility of a stereospecific cleavage of methanol—as observed in the case of the monothiosuccinimide (*rac.* **27**) can be ruled out. If, after the formation of the phycocyanobilin adducts, the cleavage of methanol from the epimer whose relative configuration at C-3 and C-3^1 is unfavourable for an E2 reaction (e.g. *RS* or *SR*) became rate-determining, the concentration of the latter (*rac.* **10b**) in the reaction mixture should increase with the passage of time. As both epimers (*rac.* **10a** and *rac.* **10b**), however, are present in nearly equimolecular amounts, the cleavage of methanol from both adducts must take place, irrespective of their relative configurations at C-3 and C-3^1, at approximately the same rate. The above considerations are represented schematically in Fig. 6.

Configuration at the exocyclic double bonds

In the solid state, the X-ray analysis of biliverdin IXα dimethyl ester shows that the bilin chromophore, whose exocyclic double bonds have the all-Z configuration, takes up a nonplanar helical-shaped all-*syn* conformation (**29**) involving a considerable degree of bond fixation within the molecule skeleton.[56,57] In solution, an equilibrium between the energetically most favourable cyclic form and a population of more or less "stretched" conformers (e.g. **30**) is present.[58]

As the electronic absorption spectra of denatured biliproteins and those of the corresponding chromophores are quite similar (particularly the UV band is more intense than the visible band), Scheer and Kufer[59] deduce that the bilin molecules are helix shaped in both. In contrast, native biliproteins show not only a significantly enhanced absorption in the visible range of the spectrum but also a very intense fluorescence which is almost absent in the free chromophores. On the basis of these observations, the interesting suggestion has been made that in the protein template the bilin molecules occur as some kind of non-cyclic chromophores whose conformations (and possibly also configurations at the exocyclic double bonds?) differ from those of the denatured biliprotein.[59] In this connection, the recent syntheses of bilins possessing E exocyclic double bonds either by photoisomerization of the all-Z isomers[60,61] or by condensation of suitable precursors[62] open up the possibility of more detailed studies on the relationship between geometry and photochemistry in the bile pigment field.

CONCLUSIONS

On the basis of the foregoing considerations, taken as a whole, Rüdiger's assignment of the absolute configuration of biliprotein chromophores (i.e. 2R, 3R, 3^1R)[46,49] has to be regarded intuitively as the most plausible one. On the other hand, the remarkable photochemical properties of native biliproteins emphasize the need for more detailed studies on the stereochemistry of bilins at the exocyclic double bonds.

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage m.p. apparatus (Reichert) and are uncorrected. ^{1}H-NMR spectra were recorded on Bruker instruments Models HFX-90 and WM 400 using $CDCl_3$ solns. Chemical shifts (δ) are expressed in ppm downfield from internal TMS and coupling constants (J values) in Hertz. Spin multiplicities are indicated by symbols s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Mass spectra (MS) data were obtained at an ionizing voltage of 70 eV on AEI MS 9 and MS 30 instruments. The assignments of particular peaks are made on the basis of plausible fragmentation mechanisms which are compatible with the structure of the molecules taken into consideration. They have not been verified, however, either by high resolution mass spectrometry or by means of isotope-labelled derivatives.

Base catalyzed H/D exchange experiments

General procedure. A soln of the dipyrrinone compound (*rac.* **11a**–**c** or *rac.* **14a**–**c**[63]) (100 mg) in MeOD (9 ml) containing NaOD (370 mg) was refluxed for 150 min under N_2. After cooling to room temp, the mixture was neutralized with SO_2, and the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2, the soln shaken with water, and the solvent evaporated *in vacuo*. In the *trans* series (*rac.* **14a**–**c**) the product obtained (**15**) was recrystallized from CH_2Cl_2–n-hexane. In the *cis* series (**11**), the two components of the mixture (*rac.* **12a**–**c** and *rac.* **13a**–**c**) were previously separated by preparative TLC on silica gel 60 $PF_{254+366}$ (E. Merck, Darmstadt) eluting with a mixture of n-hexane, EtOAc, and i-PrOH (44 : 5 : 1). After 5 fold development of the chromatographic plates, the *trans* isomers (*rac.* **13a**–**c**) could be separated from the slightly slower moving *cis* isomers (*rac.* **12a**–**c**).

t-Butyl (Z)-cis-1,2,3,10-*tetrahydro*-2,3,7,8-*tetramethyl*-1-*oxo*-11H-*dipyrrin*-2,5-d_2-9-*carboxylate* (*rac.* **12a**),[64] yield 37 mg (37%). M.p. 172–173°. ^{1}H-NMR (90 MHz): δ = 1.14 (s, 2-CH_3), 1.18 (d, J = 7; 3-CH_3), 1.51 (s, *t*-butyl), 1.89 (s, 7-CH_3), 2.19 (s, 8-CH_3), 3.15 (q, J = 7; 3-H), 8.11 and 8.72 ppm (each br. s, NH). MS *m/e* (rel. intensity): 320 (33) M^+, 264 (100) M^+-isobutylene, 246 (26) M^+ − *t*-BuOH, 231 (9), 218 (8) M^+ − (CO + *t*-BuOH), 203 (8).

t-Butyl (Z)-cis-2-*ethyl*-1,2,3,10-*tetrahydro*-3,7,8-*trimethyl*-1-*oxo*-11H-*dipyrrin*-2,5-d_2-9-*carboxylate* (*rac.* **12b**),[64] yield 26 mg (26%). M.p. 166–167°. ^{1}H-NMR (90 MHz): δ = 1.04 (t, J = 7; ethyl-CH_3), 1.22 (d, J = 7; 3-CH_3), 1.55 (s, *t*-butyl), 1.66 (q, J = 7; ethyl-CH_2), 1.91 (s, 7-CH_3), 2.22 (s, 8-CH_3), 3.17 (q, J = 7; 3-H), 7.65 and 8.48 (each br. s, NH). MS *m/e* (rel. intensity): 334 (31) M^+, 278 (100) M^+-isobutylene, 261 (14) M^+ − *t*-BuO, 260 (19) M^+ − *t*-BuOH, 232 (14) M^+ − (CO + *t*-BuOH), 231 (35).

t-Butyl (Z)-cis-3-*ethyl*-1,2,3,10-*tetrahydro*-2,7,8-*trimethyl*-1-*oxo*-11H-*dipyrrin*-2,5-d_2-9-*carboxylate* (*rac.* **12c**),[64] yield 54 mg (54%). M.p. 165–167°. ^{1}H-NMR (90 MHz): δ = 0.97 (t, J = 7; ethyl-CH_3), 1.14 (s, 2-CH_3), 1.52 (s, *t*-butyl), 1.4–1.7 (m, ethyl-CH_2), 1.89 (s, 7-CH_3), 2.19 (s, 8-CH_3), 2.89 (t, J = 7; 3-H), 7.95 and 8.68 (each br. s, NH). MS *m/e* (rel. intensity): 334 (22) M^+, 278 (100) M^+-isobutylene, 261 (22) M^+ − *t*-BuO, 260 (41) M^+ − *t*-BuOH, 232 (29) M^+ − (CO + *t*-BuOH), 231 (52).

t-Butyl (Z)-trans-1,2,3,10-*tetrahydro*-2,3,7,8-*tetramethyl*-1-*oxo*-11H-*dipyrrin*-2,5-d_2-9-*carboxylate* (*rac.* **13a**),[64] yield 50 mg (50%). M.p. 179–181°. ^{1}H-NMR (90 MHz): δ = 1.25 (s, 2-CH_3), 1.32 (d, J = 7; 3-CH_3), 1.52 (s, *t*-butyl), 1.91 (s, 7-CH_3), 2.20 (s, 8-CH_3), 2.67 (q, J = 7; 3-H), 8.14 and 8.74 (each br. s, NH). MS *m/e* (rel. intensity): 320 (36) M^+, 264 (100), M^+-isobutylene, 246 (32) M^+-*t*-BuOH, 231 (12), 218 (11), M^+ − (CO + *t*-BuOH), 203 (10).

t-Butyl (Z)-trans-2-*ethyl*-1,2,3,10-*tetrahydro*-3,7,8-*trimethyl*-1-*oxo*-11H-*dipyrrin*-2,5-d_2-9-*carboxylate* (*rac.* **13b**),[64] yield 26 mg (26%). M.p. 166–167°. ^{1}H-NMR (90 MHz): δ = 1.04 (t, J = 7; ethyl-CH_3), 1.22 (d, J = 7; 3-CH_3), 1.55 (s, *t*-butyl), 1.66 (q, J = 7; ethyl-CH_2), 1.91 (s, 7-CH_3), 2.22 (s, 8-CH_3), 3.17 (q, J = 7; 3-H), 7.65 and 8.48 (each br. s, NH). MS *m/e* (rel. intensity): 334 (31) M^+, 278 (100) M^+-isobutylene, 261 (14) M^+ − *t*-BuO, 260 (19) M^+ − *t*-BuOH, 323 (14) M^+ − (CO + *t*-BuOH), 231 (35).

t-Butyl (Z)-trans-3-*ethyl*-1,2,3,10-*tetrahydro*-2,7,8-*trimethyl*-1-*oxo*-11H-*dipyrrin*-2,5-d_2-9-*carboxylate* (*rac.* **13c**),[64] yield 41 mg (41%). M.p. 158–159°. ^{1}H-NMR (90 MHz): δ = 0.99 (t, J = 7; ethyl-CH_3), 1.27 (s, 2-CH_3), 1.52 (s, *t*-butyl), 1.4–1.9 (m, ethyl-CH_2), 1.90 (s, 7-CH_3), 2.21 (s, 8-CH_3), 2.5–2.7 (m, 3-H), 8.17 and 8.77 (each br. s, NH). MS *m/e* (rel. intensity): 334 (22) M^+, 278 (100) M^+-isobutylene, 261 (21) M^+ − *t*-BuO, 260 (36) M^+ − *t*-BuOH, 232 (26) M^+ − (CO + *t*-BuOH), 231 (47).

t-Butyl (Z)-trans-1,2,3,10-*tetrahydro*-2,3,7,8-*tetramethyl*-1-*oxo*-11H-*dipyrrin*-5-d-9-*carboxylate* (*rac.* **15a**),[64] yield 93 mg (93%). M.p. 167–168°. ^{1}H-NMR (90 MHz): δ = 1.27 (d, J = 7; 2-CH_3), 1.34 (d, J = 7; 3-CH_3), 1.55 (s, *t*-butyl), 1.92 (s, 7-CH_3), 2.22 (s, 8-CH_3), 2.1–2.5 (m, 2-H), 2.5–2.9 (m, 3-H), 8.16 and 8.75 (each br. s, NH). MS *m/e* (rel. intensity): 319 (29) M^+, 263 (100) M^+-isobutylene, 246 (27) M^+ − *t*-BuO, 245 (30) M^+ − *t*-BuOH, 230 (21), 217 (15) M^+ − (CO + *t*-BuOH), 202 (20).

t-Butyl (Z)-trans-2-*ethyl*-1,2,3,10-*tetrahydro*-3,7,8-*trimethyl*-1-*oxo*-11H-*dipyrrin*-5-d-9-*carboxylate* (*rac.* **15b**),[64] yield 91 mg (91%). M.p. 159–161°. ^{1}H-NMR (90 MHz): δ = 1.01 (t, J = 7; ethyl-CH_3), 1.33 (d, J = 7; 3-CH_3), 1.54 (s, *t*-butyl), 1.5–1.9 (m, ethyl-CH_2), 1.91 (s, 7-CH_3), 2.22 (s, 8-CH_3), 2.0–2.3 (m, 2-H), 2.6–2.9 (m, 3-H), 8.10 and 8.70 (each br. s, NH). MS *m/e* (rel. intensity): 333 (18) M^+, 277 (100) M^+-isobutylene, 260 (36) M^+-*t*-BuO, 259 (42) M^+ − *t*-BuOH, 231 (65) M^+ − (CO + *t*-BuOH), 230 (79).

t-Butyl (Z)-trans-3-*ethyl*-1,2,3,10-*tetrahydro*-2,7,8-*trimethyl*-1-*oxo*-11H-*dipyrrin*-5-d-9-*carboxylate* (*rac.* **15c**),[64] yield 88 mg (88%). M.p. 153–156°. ^{1}H-NMR (90 MHz): δ = 100 (t, J = 7; ethyl-CH_3), 1.29 (d, J = 7; 2-CH_3), 1.53 (s, *t*-butyl), 1.5–2.0 (m, ethyl-CH_2), 1.91 (s, 7-CH_3), 2.22 (s, 8-CH_3), 2.2–2.5 (m, 4-H), 2.5–2.7 (m, 3-H), 8.14 and 8.77 (each br. s, NH). MS *m/e* (rel. intensity); 333 (18) M^+, 277 (100) M^+-isobutylene, 260 (36) M^+ − *t*-BuO, 259 (42) M^+ − *t*-BuOH, 231 (65) M^+ − (CO + *t*-BuOH), 230 (79).

Addition of methanol to phycocyanobilin dimethyl ester

Synthetic **1b**[23] (200 mg) was dissolved in MeOH (100 ml) and the soln was refluxed under argon for 8 hr. From time to time a sample was withdrawn, evaporated to dryness, and the residue was dissolved in pyridine-d_5. The course of the reaction was monitored by 400 MHz ^{1}H-NMR-spectroscopy using the absorption signals assigned to the OMe groups of the MeOH adducts of phycocyanobilin dimethyl ester (*rac.* **10a** and *rac.* **10b**) at δ 3.32 and 3.35 respectively, as well as the characteristic doublet arising from the ethylidene Me group of the starting material at δ 1.72. After a steady state had been attained, the solvent was removed under reduced pressure and the components of the mixture were separated by preparative TLC on silica gel with CCl_4 : EtOAc (9 : 1). The mixture of epimeric MeOH adducts (*rac.* **10a** and *rac.* **10b**) (60 mg) thus obtained was dissolved in MeOH, and the soln was refluxed under argon. In the course of time, an increasing concentration of **1b** was detected by TLC under the conditions mentioned above.

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[64]For the sake of consistency with the nomenclature and numbering of other linear polypyrrolic compounds (e.g. tripyrrins and bilins)[11] the name 11*H*-dipyrrin is used throughout this work for the following parent compound: