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Circular Dichroism of Chromopeptides from Phycocyanin

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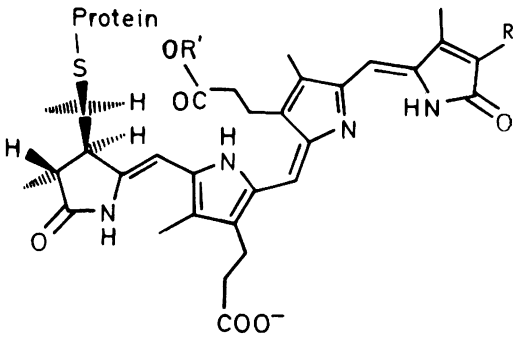
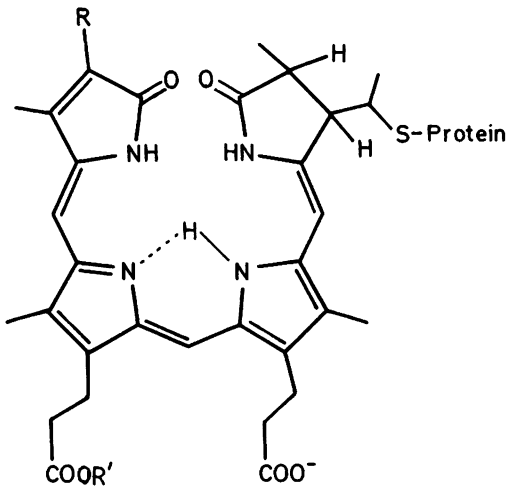
The circular dichroism of bilipeptides from *Spirulina geitleri* phycocyanin is strongly solvent and pH dependent. Maximum optical activity has been observed in aqueous solutions containing urea (8 M). In aqueous buffer, a sign reversal occurred upon the change from neutral to acidic pH; in methanolic solutions shows the optical activity a strong pH dependence both with respect to sign and magnitude. These findings have been rationalized by the presence of chromophore-peptide interactions, which are minimized in the presence of urea. Molecular orbital calculations indicate that the observed sign reversal is not necessarily due to a reversal of the chirality of the entire chromophore, but may also result from more localized conformational changes.

Introduction

Plant "bile" pigments containing linear tetrapyrrole chromophores serve important functions in the light reception [1–4] and perception [5, 6] of plants and microorganisms. The chromophores of these pigments are covalently bound to proteins. Additional non-covalent protein chromophore interactions are critical to the function of these pigments, but hitherto only partly understood (see Ref. 2 for leading references). In phycobiliproteins, like phycocyanin, (PC)*, and in phytochrome conformational changes (*e.g.* **1a** and **1b**) have been proposed for native and denatured PC, respectively, as one major source for different interactions. Chiroptical studies have been substantial in their analysis. The chomophores have been used as built-in monitors for the state of the protein in controlled denaturation studies [7–9]. Based on the pioneering work of Moscowitz *et al.* [10, 11], circular dichroism (cd) has also been used to extract information on the chromophore conformation [12–15], and there are finally several yet inconclusive studies on protein-induced chromophore-chromophore interactions in biliproteins [9, 16, 17].



Formula **1a** (PC_{native}): R = ethyl, R′ = H
2a ($P_{r,native}$): R = vinyl, R′ = H or protein



Formula **1b** ($PC_{denatured}$): R = ethyl, R′ = H
2b ($P_{r,denatured}$): R = vinyl, R′ = H or protein

* *Abbreviations:* PC, C-phycocyanin; Pr, phytochrome in the red form; cd, circular dichroism; uv-vis, absorption in the ultraviolet and visible spectral range; MO, molecular orbit; SCF, self consistent field; ci, configuration interaction; tris, tris-hydroxymethylaminomethane.

Reprint requests to Prof. Dr. H. Scheer.

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There remains still a considerable lack of knowledge on the inherent chiroptical properties of the chromophores and on the details of their interactions with the protein. It has been concluded that free bile pigments of the biliverdin type are conformationally heterogeneous, and that they predominantly assume an inherently dissymmetric, cyclic-helical conformation, (*e.g.* **1b**). Furthermore, this equilibrium between the two forms of opposite helicity is dependent on the presence of asymmetric centers within the molecule (inherent cd) or in its neighborhood (induced cd) [see 2, 12, 13, 15, 18, 19 for leading references].

Induction of optical activity in achiral bile pigments is possible by chiral solvents, but also by proteins. We are currently investigating, whether such induction can already be observed in peptides derived from biliproteins, which still carry the covalently bound chromophore. Here we wish to report the cd spectra of phycocyanin-peptides from the cyanobacterium *Spirulina geitleri*, which exhibit a strong solvent and pH dependence.

Materials and Methods

Chromopeptides from Spirulina geitleri

C-phycocyanin was extracted from spray-dried *Spirulina geitleri* (SOSA Texcoco, Mexico City, Mexico), freed from chlorophyll and other particulate material by centrifugation at $60\,000 \times g$ [20] and precipitated with 60% ammonium sulfate. The dialyzed and lyophilized PC (100 mg) was digested for 3 h at 37° with pepsin (10 mg) in dilute formic acid (5%, 25 ml) under aspirator vacuum (≈ 12 Torr). The cooled mixture was filtered, and the filtrate divided into 12 portions each sufficient for one experiment. The portions were freeze dried, stored in the dark in the freezer and used within less than one week.

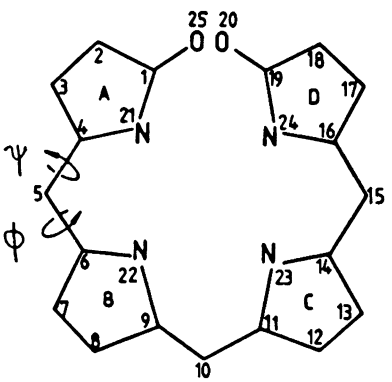
Cd-spectra were recorded on a dichrograph model V (Instruments SA, München, FRG) equipped with a data handling system (Leanord, Lille, France). The optical pathlength was 1 cm throughout, and the optical density at the maxima of absorption was kept below 0.5. All spectra are baseline corrected. Ellipticities (θ) are given in molar units ($° \cdot M^{-1} \cdot cm^{-1}$).

The time to record a spectrum was generally less than 0.5 h, and the integrity of the readily autoxidized bilipeptides was checked before and after each cd measurement by uv-vis spectroscopy on a model 320 photometer (Perkin Elmer, Überlingen, Germany).

Solutions of the chromopeptides were prepared immediately before each measurement, and two parallel experiments were carried out in each of the solvent systems given in Table I. Since the pH of neutral and alkaline solutions was lowered by up to three units upon addition of the chromopeptides, it was readjusted with solid tris and checked prior to each measurement.

Calculations of circular dichroism were done with a FORTRAN program developed and applied previously to conformational studies of bile pigments by Wagnière and coworkers [19, 21, 22]. The computation of ground and excited states follows the usual CI scheme (singly excited configurations) in the frame of an adapted Pariser-Parr-Pople approximation, assuming a local $\sigma - \pi$ separation. The crude Born-Oppenheimer wavefunctions thus obtained were used to calculate the oscillator and rotatory strengths by published procedures [23–25].

Structure **3** lacking all side chains has been chosen to represent the chromophore of the PC peptides. The angles φ and ψ correspond to rotations around the C5–C6 and C4–C5 bonds, respectively (see formula **3** for the numbering scheme). Positive signs are defined for counterclockwise rotations viewing from C5. The set of parameters developed for biliverdin by Wagnière *et al.* was used with minor modifications chosen in analogy with our earlier MO calculations [26, 27]. N22 is considered to be of the "pyridine", the other three nitrogen atoms (N21, N23 and N24) to be of the "pyrrole" type. The former contributes only one electron to the π system; for this atom (N22) we took $\gamma(NC) = 7.35$ eV (instead of 7.65 eV), $\gamma(NN) = 11.30$ eV (12.27 eV),



Formel 3

β(NC) = − 2.63 eV (− 2.50 eV). In order to take into account the effect of hyperconjugation, C2 and C3 in the hydrogenated ring A were represented by doubly occupied atomic orbitals with the following parameters: U(CC) = − 24 eV, γ(CC′) = 7.17 eV, γ(CC) = 12.76 eV, β(CC′) = − 0.49 eV. The resonance integrals of the twisted bonds were multiplied by abs(cos φ) or abs(cos ψ). The configuration interaction was limited to the lowest 36 singly excited configurations, which are derived from the highest six occupied and lowest six unoccupied MO's. Test runs with only 16 interacting configurations generally yielded roughly similar results. An exception are very weak cd-bands which may change sign due to the mixing-in of higher configurations.

Results and Discussion

Absorption spectra

Depending on the protonation state of the nitrogen atoms, bile pigments of the dihydro-bilindion conjugation type like **3** can be present in the physiological pH range as free bases, cations and anions [28]. The status can be assessed by uv-vis spectroscopy, which has been used here to ascertain the full conversion of the chromopeptide chromophores to the free base or cationic form after titration with base or acid, respectively. It should be noted that a higher (apparent) pH is required to fully convert the chromophores to their free base form in urea solution.

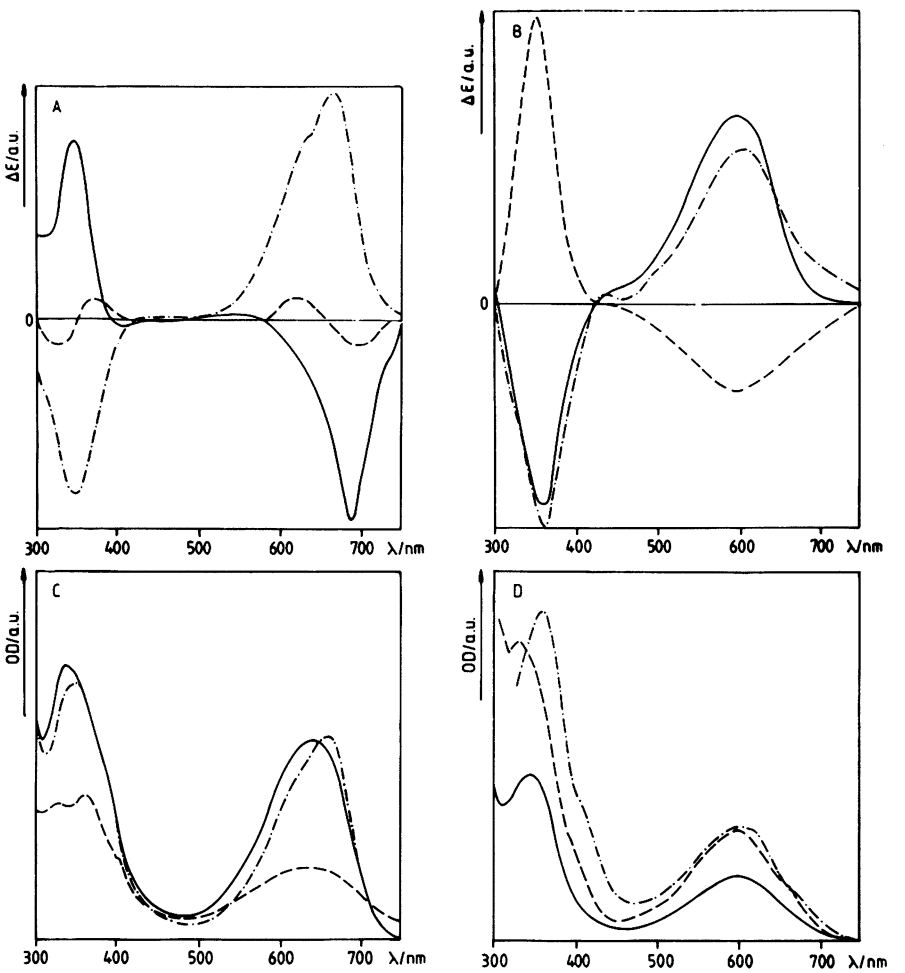
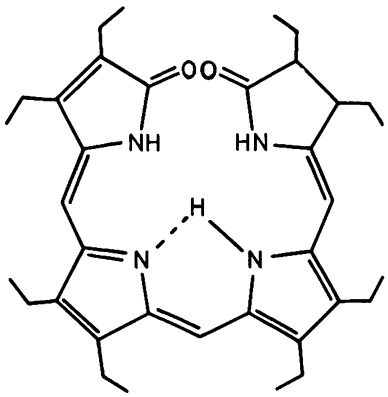


Fig. 1. Circular dichroism (A, B) and uv-vis absorption spectra (C, D) of C-phycocyanin chromopeptides from *Spirulina geitleri*. Solvent dependence: --- methanol, —— tris buffer, —·—·— tris buffer with urea (8 M). PH dependence: Spectra B and D show the free base chromophores, spectra A and C the protonated chromophores. See Table I for details.



Formel **4**

The absorption spectra of the free base chromophores (pH ≈ 7) are rather similar in the three solvents (Table I, Fig. 1). The absorption maxima and intensities are likewise similar to those of the urea deatured integral chromoprotein, PC, and to those of the free bile pigment **4** bearing the identical conjugation system. In acidic solution, the typical changes [28, 29] for protonation are observed (long wavelength shift and increase in intensity of the "red" maximum), and the differences between the three solvents are somewhat more pronounced (Table I, Fig. 1). None of the spectra indicates significant amounts of oxidation products, which give rise to more or less developed shoulders at the short wavelength side of the red absorption band (see ref. 2).

Solvent dependent spectral changes are well known in bile pigments. They have been assigned by Holzwarth *et al.* [18] to changed proportions of different chromophore states, *e.g.* conformers, tautomers or the like. The differences observed here remain well within this range. According to band position and in particular the intensity ratio of the near uv and the red bands [21, 22, 27], the chromophores are predominantly present in cyclic-helical conformations. It should be noted, however, that uv-vis spectroscopy is insufficient to analyze conformer mixtures in bile pigments.

Circular dichroism of the free base chromopeptides

All cd bands above 320 nm are in biliproteins generally asigned to $\pi \rightarrow \pi^*$ transitions of the chromophores. In contrast to what was said about the similar absorption spectra of the bilipeptides in various solvents, are the cd-spectra considerably different. Only the spectrum in tris buffer containing 8 M urea is comparable to that of urea denatured PC. The band positions, relative signs and intensity ratios of the chromopeptides in this solvent are also similar to the induced circular dichroism of the achiral free bilindion **4** in the optically active solvent, ethyl lactate [13]. The cd signals of the chromopeptides decrease in tris buffer, in particular that of the near uv band, and they decrease further in magnitude and change even their sign in methanol (Table I, Fig. 1). The cd extrema coincide in aqueous solutions with the absorption maxima, but deviate considerably in methanolic solutions. Lehner *et al.* [12] have observed similar deviations in solutions of biliverdin dimethylesters in different solvents and related them to the presence of more than one species, of which only one (with probably cyclic-helical conformation) is strongly cd active. Irrespective of the assignment to any particular conformation indicate the different cd-spectra a rather pronounced solvent dependence of the chromophore conformations of bilipeptides.

A tentative explanation for the aqueous system relates to the well known properties of urea as a denaturing agent by breaking hydrogen and electrostatic bonds. Moscowitz *et al.* [10, 11] have emphasized the importance of hydrogen bonding on the conformation of a different class of bile pigments, the urobilins.

Some of the isomers show a very high optical activity related to inherently dissymetric chromophores. Their existence can be rationalized by the cooperation of two factors, a) the formation of cyclic-helical chromophores by hydrogen bonding and b) the sterically induced preference of only one of the wo possible helices, depending on the relative and absolute configuration(s) of asymmetric centers within the molecule. In hydrogen bonding solvents, this situation is complicated by an interplay between *intra-* and *inter*molecular hydrogen bonding which may even give rise to a solvent and temperature dependent cd sign reversal [11]. Moreover has the influence of asymmetric centers within the chromophore on the preferential formation of one type of helix been demonstrated for the chromophores of biliproteins [9, 13, 27].

In bilipeptides the interactions with the asymmetric peptide residue provide an additional constraint on the chromophore conformation. Of the three solvents used in this study, 8 M urea in tris

Table I. Circular dichroism (ellipticities θ in molar units) and absorption maxima of C-phycocyanin chromopeptides from *Spirulina geitleri*. Solvent and pH dependence. The actual spectra are shown in Fig. 1.

Solvent	pH	Concentration	Red band				Near-uv band			
		[μM][a]	λ_{max} (abs) [nm]	λ'_{max} (cd) [nm]	$[\theta] \cdot 10^{-3}$ [° · M^{-1} · cm^{-1}]	g[b] · 10^3	λ_{max} (abs) [nm]	λ'_{max} (cd) [nm]	$[\theta] \cdot 10^{-3}$ [° · M^{-1} · cm^{-1}]	g[b] · 10^3
Tris-buffer (20 mM)	6.8	4.7	600	600	+42.8	0.76	355	355	−43.5	−0.24
Methanol (+ ampholin)	7.0	7.0	600	585	−12.7	−0.23	328	355	+42.0	+0.33
Aqueous urea (8 M)	8.8	24	600	600	+57.8	1.02	355	355	−80.2	−0.60
Formic acid (5%)	1.9	15.9	640	685	−21.4	−0.19	333	350	+18.5	+0.12
Methanolic HCl (6.5%)[d]	n.d.[c]	5.6	640	625 705	+ 4.7 − 4.7	+0.04 −0.04	360	325 370	− 4.7 + 3.5	−0.021 +0.016
Formic acid (5%) + urea (8 M)	2.2	17.3	660	670	+50.2	0.45	350	350	−38.2	−0.27

[a] Determined spectroscopically with $\varepsilon = 17000$ amd 34000 M^{-1} cm^{-1} for the red absorption band of the free base and protonated chromophores, respectively.
[b] Anisotropy factor g. Irrespective of deviations between λ(max) of the absorption and λ'(max) of the circular dichroism spectra is g given as the ratio of $\Delta E(\lambda'(\max))/E(\lambda(\max))$.
[c] PH not determined, titrated with methanolic HCl to complete conversion to the protonated chromophore.
[d] The optical activity is very weak and seems to result from the presence of two species with overlapping cd spectra of opposite sign.

buffer is expected to minimize these interactions. Under such conditions are the intra-chromophore interactions expected to be maximum. It is noteworthy that the cd spectrum of the peptide in this solvent is very similar to that of urea denatured PC [9], although the absolute ellipticities of the chromopeptide are smaller than in the denatured chromoprotein. The band positions and the intensity ratios are identical for both the free base and the protonated chromophores bound to the peptide. The different optical activity in tris buffer would then indicate a partial restoration of the peptide-chromophore interactions in water. Brandlmeier *et al.* [8] have likewise observed a strong decrease in optical activity of phytochrome Pr when comparing aqueous solutions with and without urea. The chromophore-peptide interactions are even more pronounced in methanol, where they give rise to a sign reversal, which *may* correspond to a reversal of the helicity of the tetrapyrrole (see below). If these arguments were correct, different cd spectra are expected for peptides with a different amino acid sequence. C-phycocyanins contain three different chromophore binding sites [30–32], and the chromopeptides used in this study are a mixture of at least five different species. This mixture is currently separated to test the above hypothesis.

The circular dichroism of the protonated chromopeptides follows a similar pattern, but the solvent induced differences are even more pronounced. There is again only a good correlation between the spectrum of urea denatured PC at pH 1.5 [9] and that of the chromopeptide in acidic aqueous urea. Drastic differences occur in the absence of urea and in acidic methanol, and the sign inversion of both cd bands occurs already after removal of urea. This enhanced differences between solutions with and without urea, respectively, can be rationalized by the presence of positive charges on both the chromophore and the peptide moiety. They give rise to electrostatic interactions between the two, which are only masked in the solution containing urea. The rather large differences between the positions of the cd extrema and the absorption maxima indicate that (except for the 8 M urea solution) the cd active chromophores are probably not the predominant species. As compared to the free base, relatively little is known about the conformation of protonated bile pigments in solution, and about the relation between uv-vis absorption spectra and conformation. Falk and Thirring [15] have observed the "normal" spectral changes (red shift and increased intensity of the visible band) in a N21–N24-methanobilindion which is restricted to cyclic conformations. MO calculations predict as well a red shift, but by contrast a decreased intensity for a cyclic-helical protonated bilindione [27]. If one assumes that only helical conformations have large cd signals, such a conformation would be predominant in the 8 M urea solution, but not in other solvents.

Molecular orbital calculations of optical activity

From the similarity of the absorption spectra at neutral pH we conclude that the chromophore conformation of the bilipeptides remains of the same type in the three solvent systems. The extinction ratios of the visible to near uv band (Table I) are characteristic for a cyclic or at most semi extended conformation of the chromophore (see *e.g.* ref. [22, 27]). On the other hand is the circular dichroism rather different in both sign and magnitude (Fig. 1). One possibility to induce such changes, and in particular a sign reversal of the cd-bands, is an inversion of the chirality of the entire chromophore, *e.g.* from a P to M helix [10, 12, 21, 27]. There is, however, in principle another possibility, *viz.* more localized changes in only one part of the tetrapyrrole skeleton without concomitant changes in the remainder. Conformational changes of this type are *ad hoc* expected to affect the magnitude of the cd signals. It was not clear, however, from the literature if they could also result in sign inversions. To test this possibility, we have theoretically investigated the influence of such variations on the chiroptic properties of the 2,3-dihydrobilindione system **3** contained in the chromophore system of PC. This particular conjugation system has already been studied theoretically to elucidate the influence of conformational changes on its absorption [22, 27] and circular dichroism [22]. In the past we have studied in particular the effect of the rotation of only one ring (A) on the absorption spectra [27], and the identical geometries were now taken to calculate the corresponding cd spectra. These studies are as yet incomplete and shall be published later in detail, but one interesting result with regard to the above question is already apparent.

To this end, only two special cases are presented here, *e.g.* the nearly cyclic structures ($\varphi = 40°$, Fig. 2) and the semi-open geometries ($\varphi = 180$ and 220°, Fig. 3). For each of the basic geometries, the second torsional angle ψ has been varied between 40 and 320 ($= -40$)°. The ring B,C,D-fragment has been fixed in a slightly left-handed chirality, with all individual rings being planar, and the twist distributed over the three methine bridges. In first approximation, one would therefore expect a negative long-wavelength cd band for all geometries investigated [12, 22] although the concept of an inherently dissymmetric chromophore is difficult to apply with a non-uniformly twisted chromophore.

The absorption spectra calculated with the set of parameters developed by Wagnière *et al.* [22] agree satisfactorily with our earlier published data [27]. There are two main absorption bands for both kinds of geometries (Figs. 2, 3), one in the red spectral range due to the first and one in the near uv due to the fifth transition. Additional transitions in the blue region around 400 nm are only prominent in the semi-open forms. The ratios of the oscillator strengths $f(\text{uv})/f(\text{vis})$ are about 9 for the cyclic-helical geometry. They decreases to 4–5 for the semi-open geometries, due to an increase of the visible band.

The most characteristic feature of the calculated cd spectrum for the cyclic-helical geometries are remarkably large anisotropy factors for the first transition. They are caused by only a small electric, but a large magnetic dipole transition moment parallel to the axis of the helix. The near uv spectral range is dominated by one strong positive cd band, which is accompanied by smaller bands of either sign. The spectra show, as expected, only minor variations with any rotation around the C4–C5 bond (Fig. 2).

The optical activity of the two main bands is generally decreased in the semi-open geometries (Fig. 3), despite of an increase of the electric transition dipole moment. This is less important for the long wavelength band corresponding to one well separated transition. It leads, however, to problems in the sign determination of the near uv band, because this is no longer dominated by a single one of the several transitions in this range, and the sign of the small bands may even change with the

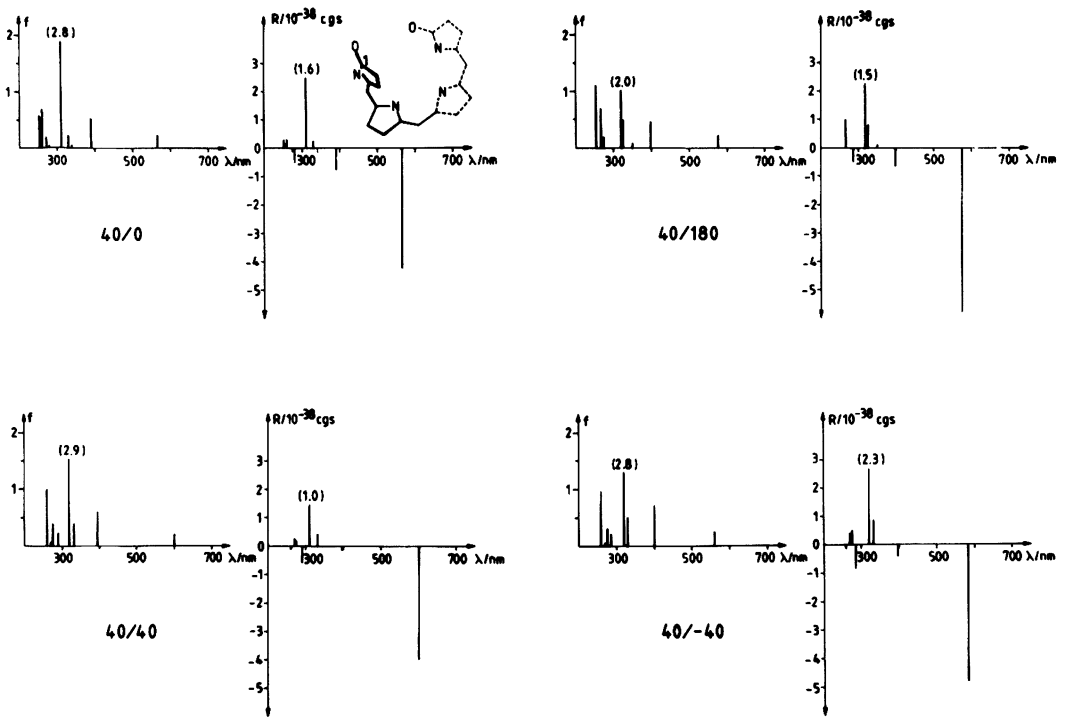


Fig. 2. Calculated oscillator strengths (f) and rotary strengths (R) for cyclic-helical geometries of the dihydrobilindion chromophore (**3**). Schematic representation of the effect of a varying angle ψ, with φ fixed at 40°.

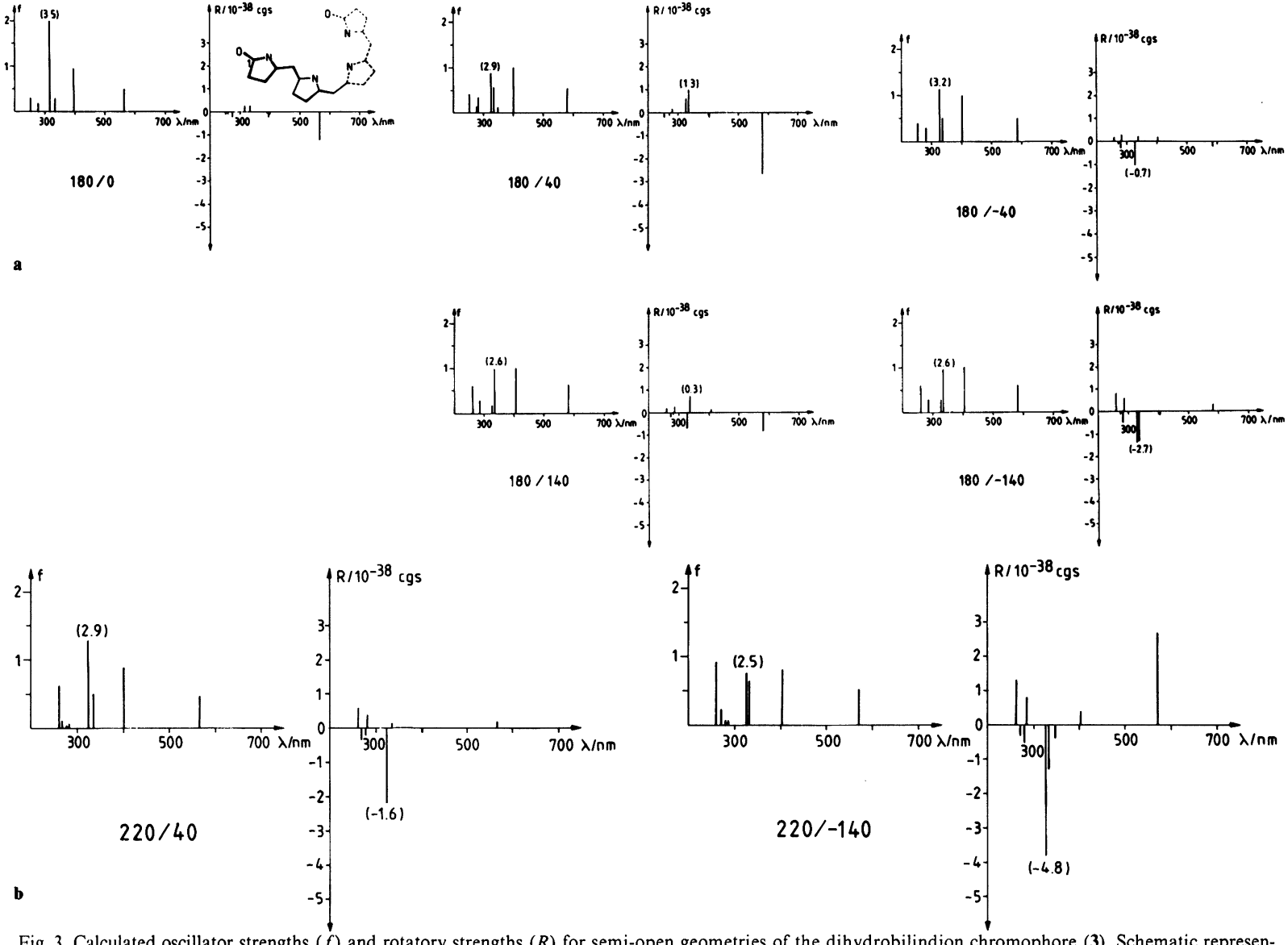


Fig. 3. Calculated oscillator strengths (f) and rotatory strengths (R) for semi-open geometries of the dihydrobilindion chromophore (**3**). Schematic representation of the effect of a varying angle ψ, with φ fixed at 180° (3a) and 220° (3b), respectively.

number of interacting configurations used in the calculation (see also Ref. [25] for the latter problem). Irrespective of these difficulties, it is apparent that the signs of both the isolated long-wavelength and that of the more complex near-uv band are no longer dictated by the fixed twist of the rings B,C,D fragment. They may change in response to the rotation around C4–C5, as seen *e.g.* in the pair $\varphi/\psi = 220/-140°$ and 180/40°. For 180/−40° the sign is the same for both bands.

Inspection of molecular models shows, that the semi-open structures contain helical fragments of opposite chirality. In such structures, one may therefore expect that the calculated sign reversal of the cd bands can originate from rather small and localized conformational changes. Thümmler *et al.* [33] have recently observed a sign reversal for *Z,Z,Z*-configurated PC peptides depending on their history. One possibility is the formation of peptide-stabilized atropisomers during the $Z \rightarrow E \rightarrow Z$ isomerization cycle to which one of the peptides had been subjected. Since this study deals as ours with chromopeptide mixtures, a more detailed investigation of the pure chromopeptides with known sequence is necessary to obtain further insight into the chromophore-peptide interactions.

Acknowledgements

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