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Absolute Sense of Twist of the C12C13 Bond of the Retinal Chromophore in Bovine Rhodopsin Based on Exciton-Coupled CD Spectra of 11,12-Dihydroretinal Analogues

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ever, they are built up by *inverted micelles* and appear in the *absence of any solvent*. Thus, the cubic lattice of the compound **3**, $n = 8$, should be built up by short rodlike micelles, each consisting of approximately 44 individual molecules.^[11] The proposed model (Figure 3) requires that two micelles are arranged side by side at each cell face of the cubic lattice (edge length 6.9 nm). Therefore the short diameter d_1 of these rods ($d_1 = a_{\text{cub}}/2$) should not exceed 3.45 nm. In order to organize 44 molecules in each of these closed cylindrical micelles (see Figure 3) with a diameter of about 3.4 nm, these micelles should be about 5 nm long (d_2). Thus, an axial ratio $d_2:d_1$ of the order of about 1.5:1 results.^[12]

Remarkably the short diameter (d_1) of the rodlike micelles in the cubic mesophase is nearly identical with the diameter of the columns in the hexagonal columnar mesophase of **3**, $n = 8$, formed at lower temperature ($a_{\text{hex}} = 3.3$ nm at 60 °C). Thus, we conclude that the short rodlike micelles of the cubic mesophase result from the collapse of the extended columns of the hexagonal columnar phase into small segments. Probably the arrangement of eight rodlike micelles in such a cubic lattice represents an energetic minimum that could be stabilized by quadrupole interactions^[9] and allows an efficient packing of the molecules. If spherical micelles are formed, their packing coefficient in this arrangement would amount to only 0.52. Therefore, it is expected that spherical micelles preferably arrange in a body-centered (0.68) or in a face-centered lattice (0.74), which allow more efficient packing.

A related model was recently proposed for thermotropic cubic phases of some dendrimers.^[7] A nearly spherical shape was assumed for the micelles of these cubic phases.^[13] However, since both compounds differ in their chemical structure, they could not be compared directly. Further detailed investigations are necessary to elucidate the general principles for the directed design of thermotropic cubic mesophases.

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Absolute Sense of Twist of the C12–C13 Bond of the Retinal Chromophore in Bovine Rhodopsin Based on Exciton-Coupled CD Spectra of 11,12-Dihydroretinal Analogues**

Qiang Tan, Jihong Lou, Babak Borhan, Elena Karnaukhova, Nina Berova, and Koji Nakanishi*

Rhodopsin, the photoreceptor responsible for dim-light vision in vertebrate species, is located in the outer segment of rod cells. This membrane protein is composed of seven transmembrane α -helices and contains the 11-*cis*-retinal chromophore **1** (Figure 1), which is attached to the ϵ -amino group of Lys(K) 296 on helix G through a protonated Schiff base (PSB).^[1, 2] Glu 113 serves as a counterion to stabilize the positive charge of the PSB.^[3] The 11-*cis* \rightarrow *trans* isomerization triggered by light initiates changes in the protein conformation leading to a cascade of events referred to as the visual transduction process.^[4] The structure of the protonated Schiff base (PSB) of 11-*cis*-retinal, with the long conjugated polyene side chain, five methyl groups, the cyclohexene ring, and the C11–C12 double bond, together with the nonplanar 6-*s-cis* and 12-*s-trans* arrangements, is ideally suited for the very subtle regulation of the pigment absorption maximum.^[5] Depending on the fit of the pigment within the rhodopsin binding site, this single chromophore can cover the entire range from the UV region up to wavelengths of around 640 nm, a range most suited for the environment surrounding the organism.^[5]

The UV/Vis and circular dichroism (CD) spectra of bovine rhodopsin in octyl glucoside are shown in Figure 1. Native rhodopsin ($\lambda_{\text{max}} = 500$ nm) exhibits two positive Cotton effects in its CD spectrum at 480 nm ($\Delta\epsilon = +2.8$, α -band) and 337 nm ($\Delta\epsilon = +9.8$, β -band). A rhodopsin incorporating a retinal analogue in which planes B and C are coplanar due to a five-membered ring (linkage of C10 and 13-methyl group) exhibit CD spectra with a negligible α -band. Similarly, the pigment formed from a retinal with coplanar A and B planes as a result of a bridged five-membered ring between the 5-methyl group and C8 showed weak β -band. Based on these findings, Ito and co-workers^[6] have assigned the origin of the α - and β -bands to distortions around 12-*s-trans* and 6-*s-cis* bonds, respectively. Although the two positive Cotton effects reflect the interaction between the twisted chromophore and its environment, at this stage it is not possible to gain further information regarding the sense of twist around the 6-*s-cis* and 12-*s-trans* bonds, that is, the arrangement of planes A, B, and C in Figure 1. We have

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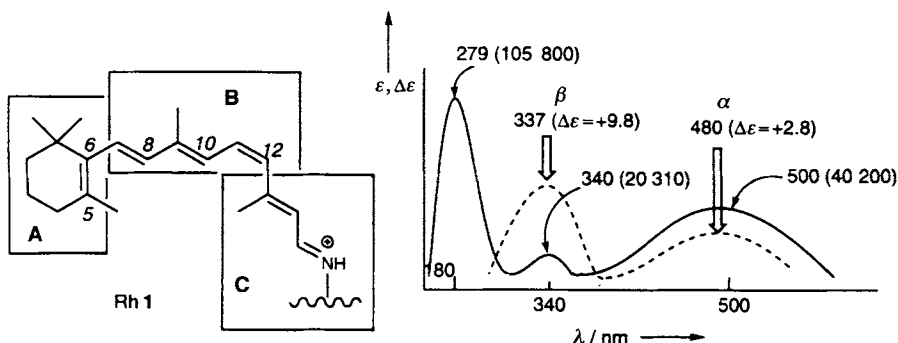


Figure 1. The 11-*cis*-retinal chromophore **1** in native rhodopsin attached in PSB form, and the UV/Vis (—, ϵ) and CD spectrum (---, $\Delta\epsilon$) of bovine rhodopsin in octyl glucoside.

therefore disrupted the conjugated polyene system by a single bond so that the two polyene moieties flanking the single bond would couple. The sign of the resulting bisignate couplet would reflect the twist in a nonempirical manner. Here we present data that determine the absolute sense of twist of planes B and C planes.

Previous photoaffinity labeling experiments have shown that the C3 and C4 atoms of the ionone ring lie very close to the transmembrane helix F of rhodopsin (Figure 2).^[7,8] The next essential structural feature to be clarified is the absolute sense of twist of the retinal chromophore, that is, the twists between planes A and B and planes B and C. The polyene side chain comprising C7 to C12 (plane B) is perpendicular to the axis of the transmembrane helices of rhodopsin (Figure 2). By establishing the absolute twist around the C12–C13 bond one can determine whether plane C is tilted up or down with respect to plane B. We have now established the helicity between planes B and C by incorporating 11,12-dihydroretinals into opsin and measuring their CD spectra. To our knowledge this is the first time that exciton-coupled CD spectroscopy has been applied to elucidate subtle conformational details of protein-bound substrates.

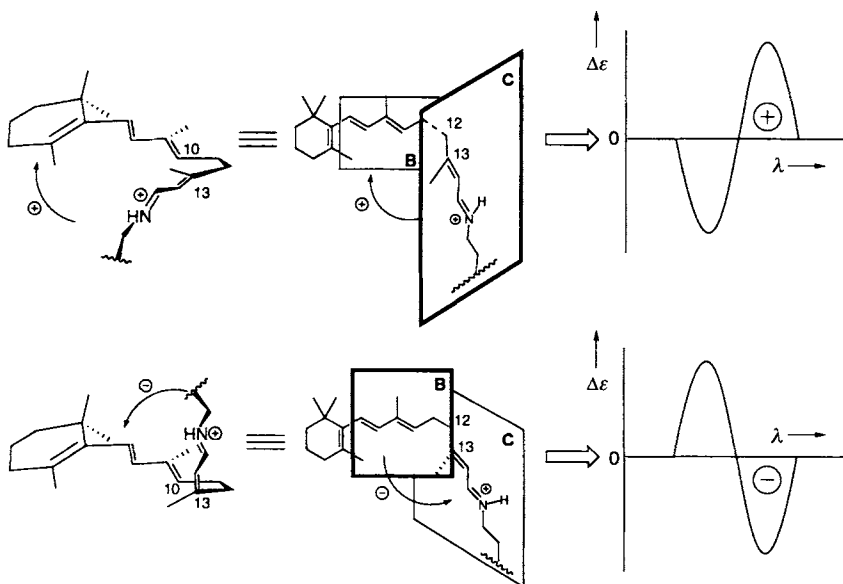


Figure 3. Absolute sense of twist between planes B and C and the predicted CD exciton couplets. The exciton-coupled CD curve is defined as positive if the sign of the first Cotton effect of the couplet at longer wavelength is positive, and vice versa.

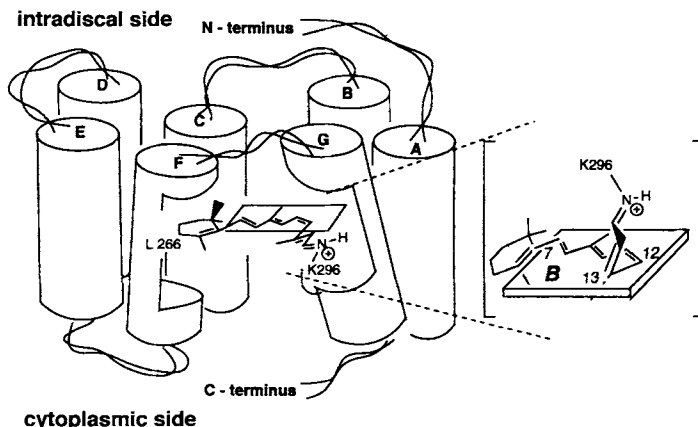


Figure 2. Schematic drawing of rhodopsin showing the location and twist of the chromophore. The residue cross-linked in the photoaffinity labeling study^[7], L266 on helix F, is indicated. The triene moiety C7 to C12 is in plane B, which is parallel to the lipid bilayer membrane. The PSB moiety is twisted around the C12–C13 bond so that it is directed toward the intradiscal side of the membrane.

adopts a conformation similar to that of 11-*cis*-retinal in rhodopsin, the exciton coupling between the triene and PSB should appear as either a “positive” or “negative” CD couplet depending on whether planes B and C assume a positive or negative twist (Figure 3).^[9,10]

The triene and enal moieties of 11,12-dihydroretinal (2H-ret, **2**)^[11] absorb at 255 nm (shoulder) and 236 nm, respectively. The triene absorption of the corresponding Schiff base (2H-PSB, **3**) at 255 nm overlaps with the red-shifted PSB absorption at 270 nm (Figure 4). The UV spectrum of 11,12-dihydroretinal (2H-Rh, **4**) in octyl glucoside has one broad maximum centered around 279 nm;^[12] the accompanying CD spectrum shows negative and positive Cotton effects at 295 nm ($\Delta\epsilon = -1.2$) and 275 nm ($\Delta\epsilon = +2.5$), respectively; the difference between the extrema or A value is -3.7 . In contrast the CD spectrum of native rhodopsin shows two positive Cotton effects (Figure 1). Thus the CD spectrum of **4** with negative and positive Cotton effects can be ascribed to the expected exciton coupling between the triene and PSB moieties, which absorb around

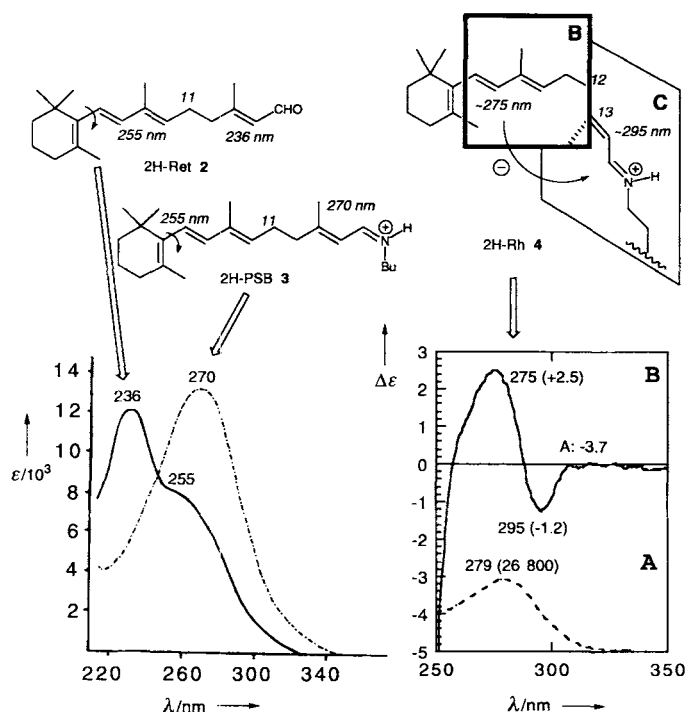
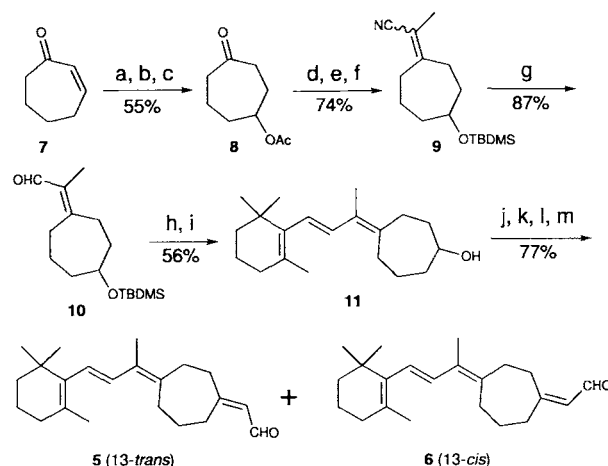


Figure 4. Left: 11,12-Dihydroretinal (**2**), its protonated Schiff base **3** obtained with *n*-butylamine, and their UV spectra in methanol. Right: Dihydrorhodopsin **4** and its UV (A) and CD spectra (B) in octyl glucoside. The chromophore analogue was bound by incubating 3.5 molequiv of analogue with a suspension of bovine opsin in 67 mM phosphate buffer (pH 7.0) at 25°C for 5 h. All spectra were recorded in 23 mM octyl glucoside solution at pH 7.0 with opsin as a control.

275 and 295 nm, respectively. The red shift of the PSB band from 270 nm in **3** to roughly 295 nm in **4** is due to the effect of the electrostatic charges operating within the binding site^[13] and is not unexpected. The extremum at 275 nm represents the positive Cotton effect of the negative couplet; the contribution from the opsin should be negligible since the CD spectrum depicted in Figure 4 is a difference spectrum between that of the pigment and that of the opsin. Thus it appears that the triene band in pigment **4** is red-shifted by 20 nm relative to the band in **3** (255 nm in methanol). This implies that the twist around the 6-*s-cis* bond in pigment **4** is smaller than in **3** in solution; in other words, planes A and B (Figure 1) adopt a more planar arrangement in the protein due to constraints within the binding site. The conclusion arising from the negative couplet of **4** is that planes B and C are twisted as depicted in Figure 4.

It is conceivable that the 11,12-dihydrochromophore is flexible and subject to many degrees of freedom, which ultimately lead to the weak amplitudes observed in the exciton-coupled CD spectra. To check the validity of the conclusion reached from studies with the dihydropigment **4**, the same set of experiments were performed with the 13-*trans*- and 13-*cis*-11,12-dihydrocycloheptatrienylidene retinal (13-*trans*-2H-ret7 **5** and 13-*cis*-2H-ret7 **6**). The synthesis of the retinal analogues is outlined in Scheme 1. After HPLC separation, **5** and **6** were characterized by their NMR and mass spectra.^[14] The rationale to pursue a dihydro analogue of pigment **4** in which the conformation is locked with a seven-membered ring lies



Scheme 1. Synthesis of retinal analogues **5** and **6**. a) *N*-bromosuccinimide (NBS), benzoyl peroxide, CCl_4 ; b) potassium acetate, 3-methyltriocetylammmonium chloride (Aliquat 336), H_2O ; c) H_2 , Pd/C, acetic acid, CH_2Cl_2 ; d) diethyl 1-cyanoethyl phosphonate, NaH, THF; e) potassium carbonate, methanol, H_2O ; f) *tert*-butyldimethylsilyl chloride (TBDMS-Cl), imidazole, DMF; g) diisobutylaluminum hydride (DIBAL-H), CH_2Cl_2 , 1:1 mixture of isomers separated by column chromatography; h) β -cyclocitryl triphenylphosphonium bromide, potassium *tert*-butoxide, THF; i) $\text{Bu}_4\text{N}^+\text{F}^-$, THF; j) tetra-*N*-propylammmonium perruthenate (TPAP), 4-methylmorpholine *N*-oxide (NMO), finely powered 4 Å molecular sieves, CH_2Cl_2 ; k) diethyl cyanomethyl phosphonate, NaH, THF; l) DIBAL-H, CH_2Cl_2 ; m) HPLC separation of 1:1 mixture of isomers^[15].

in earlier data obtained with cycloheptatrienylidene retinal (ret7 **12**). The UV and CD spectra of the pigment obtained from **12** and opsin closely resemble the UV and CD spectra of native rhodopsin.^[15] It was therefore concluded that the geometry of **12** within the binding site and the corresponding protein–substrate interactions are very similar to those of 11-*cis*-retinal in the native rhodopsin. Based on these observations we felt that 13-*trans*-2H-ret7 **5** would be a more appropriate choice for a dihydro-11-*cis* locked retinal analogue of 11,12-dihydroretinal (Figure 5).

The enal moiety in **5** absorbs at 244 nm in methanol. The absorption of the nonplanar triene moiety was assigned to be around 266 nm (the synthetic intermediate **11** with the same triene system exhibits an absorption maximum at 266 nm) and appears as a shoulder around 270 nm (Figure 5; the UV and CD spectra of **6** are very similar). In the protonated Schiff base **13**

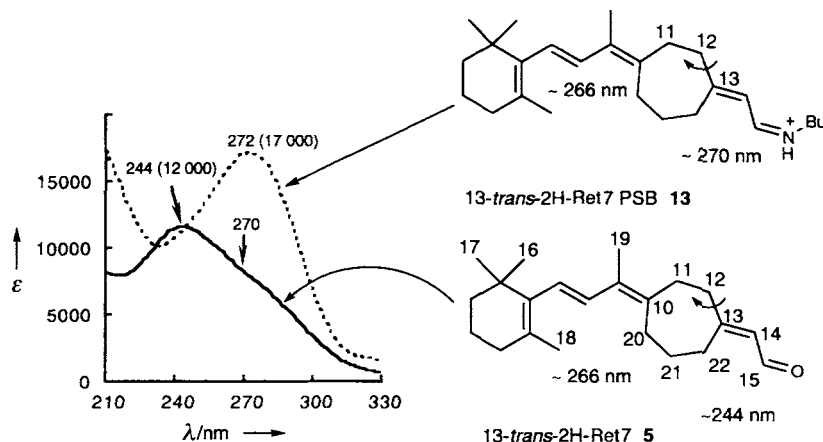


Figure 5. 13-*trans*-2H-ret7 **5**, its PSB derivative **13**, and their UV spectra in methanol. The UV spectra of 13-*cis*-2H-ret7 **6** and its PSB derivative are very similar to those of the *trans* isomer.

obtained with *n*-butylamine, the absorption maximum of the enal unit is shifted to around 270 nm (Figure 5) and overlaps with the triene absorption.

Rhodopsin analogues **14** and **15**, which incorporate **5** and **6**, respectively, have absorption maxima at 283 nm ($\epsilon = 28000$) and 284 nm ($\epsilon = 29000$), respectively (Figure 6).^[16] Repeated addition of 11-*cis*-retinal to incubation mixtures of each failed to generate the native rhodopsin as determined by the absorption at 500 nm. Therefore, it was concluded that no opsin was left and the analogues bound to opsin could not be replaced by 11-*cis*-retinal.

13-*trans*-2H-Rh7 **14** exhibits a negative split CD band at 297 nm ($\Delta\epsilon = -6.5$) and 273 nm ($\Delta\epsilon = +5.2$; $A = -11.7$). Rhodopsin analogue **15** also shows a negative couplet with a slightly larger A value of -14.1 at similar wavelengths, 298 nm ($\Delta\epsilon = -7.5$) and 272 nm ($\Delta\epsilon = +6.6$; Figure 6). The

most probably due to the flexibility of the side chain leading to conformations in which the angles between the two interacting chromophores are smaller and/or the chromophores are more distal. Nevertheless, the fact that the CD spectrum of 11,12-dihydrorhodopsin (**4**) displays a negative bisignate curve is in agreement with the results obtained with 13-*trans*-2H-Rh7 **14** and 13-*cis*-2H-Rh7 **15**.

The experimental results indicate that the negative helicity between planes B and C is as depicted in Figure 3; they also suggest that the twist between planes A and B of chromophores bound to opsin is smaller than in the same chromophore in solution. The handedness of the arrangement of the planes B and C agrees with the theoretical prediction of Kakitani and co-workers,^[18] according to which the C12–C13 bond in rhodopsin is twisted by -130 to -150° when the torsion angle is measured clockwise from the planar *cis* conformation. (Han

and Smith have also arrived at the same conclusion by theoretical considerations.^[19]) It is remarkable that the conformation of the chromophore in the binding pocket of a large protein can be determined from the sign of the bisignate exciton-coupled split CD spectra. As mentioned above, the twist of the chromophore is reflected in the CD spectrum of the original rhodopsin by the presence of the α - and β -bands. The characteristics shape and sign of these bands are dictated by the interaction between the chromophore and the opsin binding site. Therefore, in other visual pigments that exhibit similar positive α - and β -bands, it can be postulated that the chromophoric twist along their polyene chain will be similar to that in bovine rhodopsin.

These results further demonstrate the versatility of the exciton-coupled split CD method in probing subtle conformational aspects of ligand–receptor interactions. Further studies directed toward determining the absolute sense of twist between planes A and B and clarifying the mechanism of the visual transduction process on a molecular structural basis are in progress.

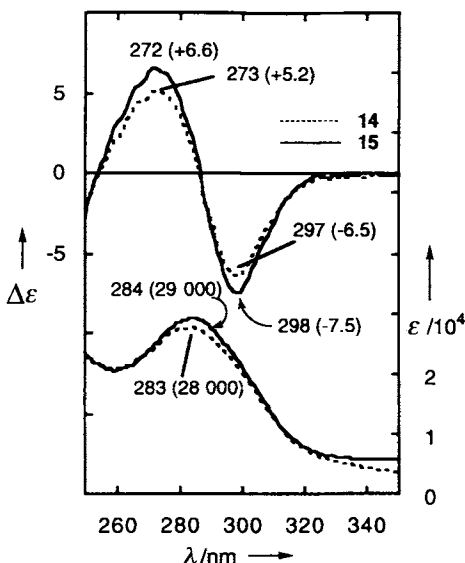
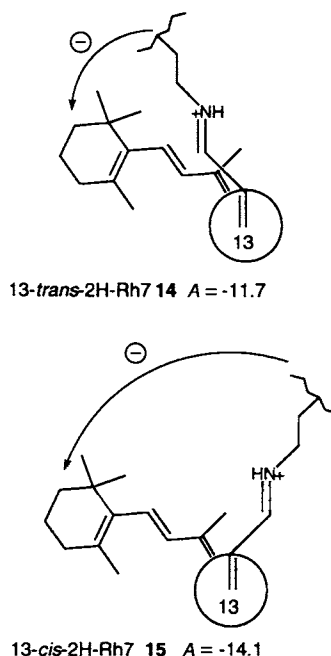


Figure 6. UV and CD spectra of 13-*trans*-2H-Rh7 **14** and 13-*cis*-2H-Rh7 **15**. The projected structures of the chromophores show that the angle between the coupled chromophores is larger for the 13-*cis* pigment, and this is reflected in the slightly larger amplitude in its CD spectrum. The chromophore analogues were bound by incubating 1.5 molequiv of analogue with a suspension of bovine opsin in 67 mM phosphate buffer (pH 7.0) at 25 °C for 5 h. All spectra were recorded in 23 mM octyl glucoside solution at pH 7.0 with opsin as a control.



bisignate CD spectra can again be ascribed to the coupling between the triene and protonated Schiff base (PSB) moieties, thus confirming the conclusion resulting from experiments with analogue **4**. To secure further support for the origin of the couplet, the amplitudes of the split CD bands of 13-*trans* and 13-*cis* isomers **14** and **15** were compared. It is known that the A value of bisignate exciton-coupled split CD curves increases as the projection angle of the two interacting chromophores approaches 70° .^[9, 10] The estimated angle between the triene and protonated Schiff base moieties is roughly 60° for the 13-*cis* isomer and 20° for the 13-*trans* isomer based on molecular modeling calculations (MM2 force field).^[17] This is in agreement with the observed larger A value for the 13-*cis* isomer (-14.1) relative to that for the 13-*trans* isomer (-11.7), and supports the conclusion that the observed split CD band is due to exciton coupling between the two chromophores within the binding site.

The smaller A value of the pigment incorporating 11,12-dihydrorretinal (**2**), in which the 11-*s-cis* conformation is not fixed, is

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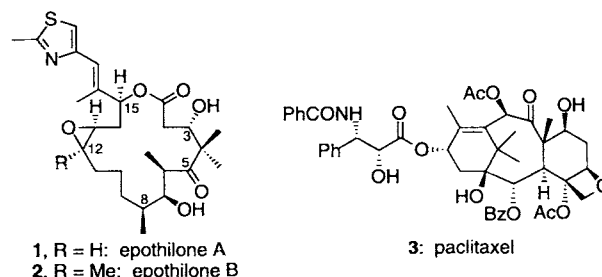
Keywords: chirality • circular dichroism • exciton coupling • retinal analogues • rhodopsin

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Structure–Activity Relationships of the Epothilones and the First In Vivo Comparison with Paclitaxel**

Dai-Shi Su, Aaron Balog, Dongfang Meng, Peter Bertinato, Samuel J. Danishefsky,* Yu-Huang Zheng, Ting-Chao Chou, Lifeng He, and Susan B. Horwitz

The discovery and characterization of the bacterial natural products epothilones A (**1**) and B (**2**) have evoked a great deal of interest.^[1] Their high levels of cytotoxicity and their potent stabilization of microtubules are reminiscent of the biological activity of paclitaxel (**3**), a clinically valuable resource in cancer



chemotherapy.^[2] Paclitaxel (**3**), which has been in use for the treatment of ovarian and breast carcinomas, is also being evaluated against a variety of other tumors. Nonetheless, its application is hampered by difficulties in formulation and susceptibility to multiple drug resistance (MDR). Though a massive amount of analog synthesis based on the paclitaxel structure has been accomplished, the framework appears to be intolerant to major simplification with maintenance of biological activity. To the best of our knowledge, no compound which is of a significantly lower level of structural complexity than paclitaxel has been of clinical interest as a replacement for the parent drug.

Since the epothilones are more water-soluble than paclitaxel and, in preliminary in vitro studies, seemed to perform better against several MDR cell lines, this series warranted evaluation. Our laboratory and several others have attacked the problem of the total synthesis of **1** and **2**.^[3–9] This goal, in the case of **1**, was first accomplished in a stereocontrolled fashion by using a boron-alkyl Suzuki coupling strategy to establish the C11–C12

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- [14] Compounds **5** and **6** were separated by HPLC on a YMC-Pack SIL column (250 × 10 mm i.d.). The chromophores were eluted with ethyl acetate/hexane (5/95, v/v) at a flow rate of 2.0 mL min⁻¹. The retention times for **5** and **6** were 22.6 and 21.0 min, respectively. ¹H NMR (CDCl₃, TMS): **5**: δ = 1.00 (s, 6H, 1-CH₃), 1.46 (m, 2H, 2-H), 1.61 (m, 2H, 3-H), 2.00 (t, J = 6.4 Hz, 2H, 4-H), 1.68 (s, 3H, 5-CH₃), 1.83 (s, 3H, 9-CH₃), 6.06 (d, J = 15.8 Hz, 1H, 7-H), 6.38 (d, J = 15.8 Hz, 1H, 8-H), 5.85 (d, J = 8.2 Hz, 1H, 14-H), 9.95 (d, J = 8.2 Hz, 1H, 15-H), 2.52 (t, J = 5.8 Hz, 2H, 11-H), 2.48 (t, J = 5.8 Hz, 2H, 12-H), 2.45 (t, J = 6.5 Hz, 2H, 20-H), 1.75 (m, 2H, 21-H), 2.72 (t, J = 6.4 Hz, 2H, 22-H); **6**: δ = 1.00 (s, 6H, 1-CH₃), 1.46 (m, 2H, 2-H), 1.60 (m, 2H, 3-H), 2.00 (t, J = 6.4 Hz, 2H, 4-H), 1.68 (s, 3H, 5-CH₃), 1.82 (s, 3H, 9-CH₃), 6.06 (d, J = 16.3 Hz, 1H, 7-H), 6.39 (d, J = 16.3 Hz, 1H, 8-H), 5.85 (d, J = 8.1 Hz, 1H, 14-H), 9.96 (d, J = 8.1 Hz, 1H, 15-H), 2.57 (t, J = 6.6 Hz, 2H, 11-H), 2.91 (t, J = 6.6 Hz, 2H, 12-H), 2.45 (t, J = 6.3 Hz, 2H, 20-H), 1.71 (m, 2H, 21-H), 2.37 (t, J = 6.3 Hz, 2H, 22-H); positive-ion CI-MS (NH₃): m/z: 313 [M + H]⁺, 331 [M + NH₄]⁺.
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- [16] Bovine rod outer segments (ROS) were isolated and bleached according to published procedures^[20]. The artificial pigments **4**, **14**, and **15** were prepared at 25 °C in the dark. Screw-capped vials equipped with magnetic stirrer bars were charged with 1 OD of opsin suspended in 67 mM sodium phosphate buffer (pH 7.0, 500 μL) per vial. Retinal analogue **2** (3.5 molequiv for making pigment **4**), or retinal analogue **5** or **6** (1.5 molequiv for making pigment **14** or **15**) in EtOH (5 μL) was added to each vial containing opsin. The control sample was prepared by adding EtOH (5 μL) to 1 OD of opsin. All samples were stirred for 5 h then centrifuged at 50 000 g for 15 min. The pellets were dissolved in 23 mM octyl glucoside in phosphate buffer (67 mM, pH 7.0, 1 mL) and centrifuged at 50 000 g for 10 min. The supernatants were used to obtain UV and CD spectra. All UV and CD spectra of the rhodopsin pigments were corrected by subtraction of the UV and CD spectra of the control sample.
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