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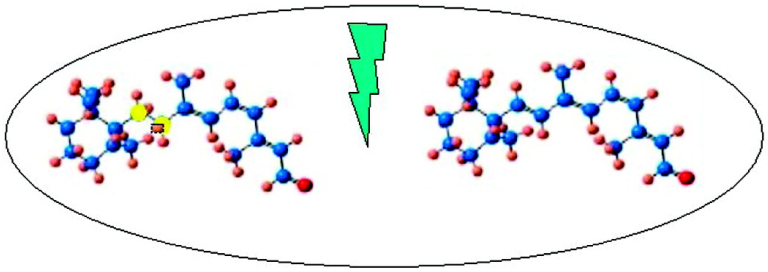

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# 7,8-Dihydro Retinals Outperform the Native Retinals in Conferring Photosensitivity to Visual Opsin

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***Abstract:*** The visual pigment rhodopsin presents an astonishing photochemical performance. It exhibits an unprecedented quantum yield (0.67) in a highly defined and ultrafast photoisomerization process. This triggers the conformational changes leading to the active state Meta II of this G protein-coupled receptor. The responsible ligand, retinal, is covalently bound to Lys-296 of the protein in a protonated Schiff base. The resulting positive charge delocalization over the terminal part of the polyene chain of retinal creates a conjugation defect that upon photoexcitation moves to the opposite end of the polyene. Shortening the polyene as in 5,6-dihydro- or 7,8-dihydro analogues might facilitate photoisomerization of a 9-*Z* and an 11-*Z* bond. Here we describe pigment analogues generated with bovine opsin and 11-*Z* 7,8-dihydro retinal or 9-*Z* 7,8-dihydro retinal. Both isomers readily generate photosensitive pigments that differ remarkably in spectral properties from the native pigments. In addition, in spite of the more flexible 7,8 single bond, both analogue pigments exhibit strikingly efficient photoisomerization while largely maintaining the activity toward the G-protein. These results bear upon the activation of ligand-gated signal transducers such as G protein-coupled receptors.

## Introduction

The visual pigment rhodopsin, responsible for dim-light vision, exhibits an astonishing photochemical performance.[1,2] It couples an unprecedented quantum yield (0.67) to a highly defined reaction pathway (11-*Z* → *all-E*) in an ultrafast (200 fs) photoisomerization process that triggers the conformational changes leading within milliseconds to the active-state meta-(rhodopsin) II. This proceeds through a series of intermediates (Batho ↔ BSI → Lumi → Meta I ↔ Meta II) that differ in lifetime and in spectral band position.[1−3] Because of its relatively well-defined activation mechanism and the availability of crystal structures of the inactive state[4,5] and several photointermediate states,[6−8] rhodopsin has become a paradigm for the ubiquitous family of signal-transducing G protein-coupled receptors.

The ligand of vertebrate visual pigments is the isoprenoid compound retinal, the aldehyde derivative of vitamin A (Chart 1; carbon numbering in Chart S1). It is covalently bound to Lys-296 of the protein (opsin) in a protonated Schiff base and is also responsible for the absorbance band of the pigment in the visible region of the electromagnetic spectrum.[1] It is mainly found as the 11-*Z* isomer (rhodopsin, $\lambda_{max} = 498$ nm), but occasionally as the 9-*Z* isomer (isorhodopsin, $\lambda_{max} = 485$ nm). In order to investigate ligand−receptor interactions responsible for spectral tuning, receptor activation, or the exquisite photochemical properties, many studies have analyzed the properties of photoreceptor pigments generated with a large variety of retinal analogues.[2,9−21] Although a wealth of information has

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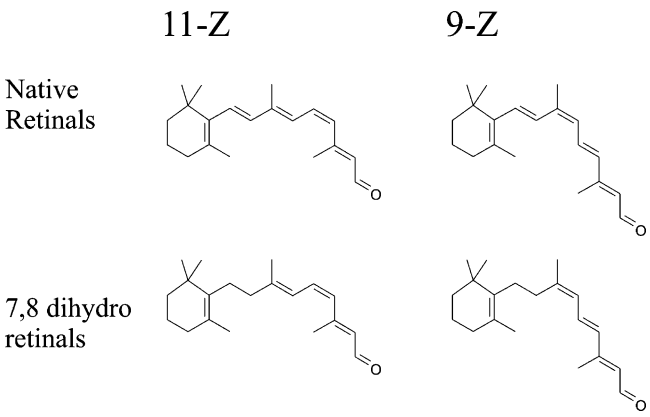
***Chart 1.*** Chemical Structure of the Retinal Derivatives Used in This Study




been excavated in this way on binding pocket constraints and agonistic properties of ligand derivatives, so far no combination has been reported that equaled, let alone surpassed, the photochemical properties of the native pigment. This also is a severe limitation in therapeutic supplementation with retinal derivatives in the case of genetic defects in retinoid metabolism, where good binding properties, low metabolic stress, and high photosensitivity are important factors.[22]

The positive charge delocalization over the terminal part of the polyene chain creates a conjugation defect, a polaron that upon photoexcitation moves to the opposite end of the polyene.[2,23,24] We reasoned that shortening the polyene as in 5,6-dihydro- or 7,8-dihydro analogues would stabilize the polaron in the isomerization region, enhance the inversion of the $\pi$-distribution, and thereby might facilitate photoisomerization of a 9-*Z* and an 11-*Z* bond. Since ring modifications as in 5,6-dihydro may affect ligand−receptor interaction[25] and scattered reports indicated that 7,8-dihydro retinals (Chart 1) can generate rhodopsin analogues that appear to be functional,[12,26,27] we opted for the 7,8-dihydro modification.

Here we describe pigment analogues generated with bovine opsin and 11-*Z* 7,8-dihydro retinal or 9-*Z* 7,8-dihydro retinal. In spite of the more flexible 7,8 single bond, both analogue pigments exhibit strikingly efficient photoisomerization while largely maintaining the activity toward the G-protein.

## Experimental Section

Since 11-*Z* 7,8-dihydro retinal could not be prepared photochemically from the *all-E* isomer, a*ll-E*, 9-*Z*, and 11-*Z* 7,8-dihydro retinals were synthesized by a stereoselective procedure as described for 4,5-dehydro-5,6-dihydro retinal.[28] The 9-*E* and 9-*Z* 7,8-dihydro-C11 aldehyde intermediates had to be purified by HPLC, but the final all-*E* and 11-*Z* or 9-*Z* 7,8-dihydro retinals could equally well be purified by conventional column chromatography, as described.[28] The same procedure was used for native 9-*Z* retinal. Native 11-*Z* retinal was a gift from Dr. R. K. Crouch (Medical University of South Carolina). The analytical data of the isomers fully comply with their proposed structure. The electronic properties of the *all-E* and 9-*Z* isomer agree with those reported in the literature.[26] UV−vis spectra of the 9-*Z* and 11-*Z* isomers of native and 7,8-dihydro retinal are presented in Figure 1. $^1$H NMR data for the 11-*Z* 7,8-dihydro isomer are given in Table 1. The chemical shift assignments were supported by COSY and NOESY analyses.

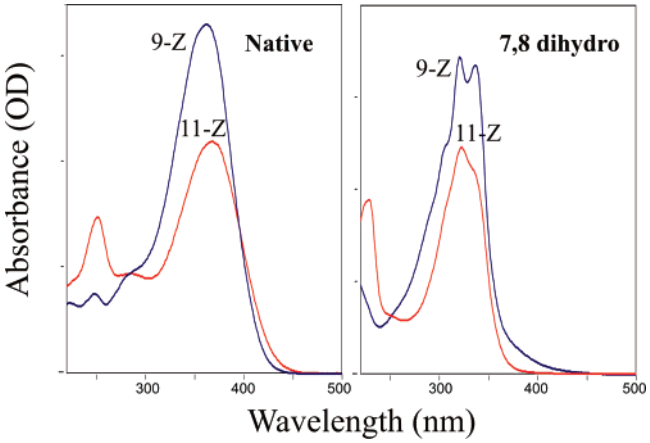



***Figure 1.*** Spectral properties of the retinals used in this study. UV−visible spectra were taken in hexane solution at room temperature and show native (left panel) and 7,8-dihydro derivatives (right panel) of 11-*Z* (red curves) and 9-*Z* (blue curves) retinals. $\lambda_{max}$: in nm: 365 (11-Z), 362 (9-Z), 323 (11-*Z* 7,8-dihydro), and 321 (9-*Z* 7,8-dihydro).

***Table 1.*** $^1$H Chemical Shifts and *J*-Couplings of 11-*Z* 7,8-Dihydro Retinal

| H-atom | chemical shift (ppm) | *J*-coupling (Hz) |
|---|---|---|
| 15-H | 10.07 | 7.04 |
| 14-H | 6.07 | 7.04 |
| 12-H | 5.83 | 11.7 |
| 11-H | 6.53 | 13.0 + 11.7 |
| 10-H | 6.37 | 13.0 |
| 7,8-2 × $H_2$ | 2.1−2.2 | 4 × H AA′BB′ spectrum |
| 4-$H_2$ | 1.92 | 2 × H triplet |
| 16,17-$CH_3$ | 1.01 | |
| 18-$CH_3$ | 1.61 | |
| 19-$CH_3$ | 1.87 | |
| 20-$CH_3$ | 2.35 | |

Isolation of opsin membranes, incubation with a 5-fold excess of retinal, removal of excess retinal, spectral analysis by UV/vis and FTIR spectroscopy, and determination of the photoisomerization quantum yield were performed as described.[13,14] The G protein transducin activation was assayed using fluorescence emission enhancement of an endogenous tryptophan residue in the α-subunit upon binding of GTP$\gamma$S, as described.[13] For calculation of the quantum yield, $\Phi$, rhodopsin was taken as a reference compound ($\Phi = 0.67 \pm 0.02$[29,30]). Illumination of the pigments was performed under identical conditions through a Schott band filter (452 nm, bandwidth 5 nm), and the rate of pigment decay was related to that of rhodopsin.[13,14] The molar absorbance of the pigments at 452 nm was calculated from the absorbance curves and the molar absorbance at the $\lambda_{max}$ ($\epsilon_{max}$).

The $\epsilon_{max}$ of the 7,8-dihydro pigments was estimated from the optical density, obtained for the same amount of opsin relative to that with native 11-*Z* retinal (90 ± 5% for 11-*Z* 7,8-dihydro retinal, 125 ± 6%

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***Table 2.*** Spectral and Photochemical Properties of Native and 7,8-Dihydro Analogues of Rhodopsin and Isorhodopsin[a]

| | rhodopsin (11-*Z*) | | isorhodopsin (9-*Z*) | |
|---|---|---|---|---|
| | native | 7,8-dihydro | native | 7,8-dihydro |
| $\lambda_{max}$ (nm) | 498 ± 1 | 426 ± 2 | 485 ± 2 | 428 ± 2 |
| $\epsilon_{max}$ (L/M·cm) | 40,500 ± 800 | 37,000 ± 4,000 | 43,000 ± 1,000 | 51,000 ± 4,000 |
| quantum yield | 0.67 ± 0.02 | 0.68 ± 0.06 | 0.27 ± 0.03 | 0.39 ± 0.04 |
| p$K_a$ Meta I/Meta II | 7.4 ± 0.2 | ≤6.5 | 7.4 ± 0.2 | ≤6.5 |
| G-protein activation | ≡100 | 75 ± 15 | 98 ± 10 | 68 ± 6 |

[a] Native quantum yields have been reported.[13,29,30] G protein activation rate of rhodopsin was set at 100. Data pertain to 293 K and are given as average ± SD with number of independent experiments varying from 2 to 5.

for 9-*Z* 7,8-dihydro retinal) in combination with the ratio of the relative amount of oxime generated upon illumination of the 7,8-dihydro pigments (11-*Z*/9-*Z* = 1.35 ± 0.05).

## Results and Discussion

Since 11-*Z* 7,8-dihydro retinal could not be photochemically prepared from the *all-E* isomer, we applied a stereoselective procedure to synthesize the 11-*Z*, *all-E*, and 9-*Z* isomers with a final purification by means of liquid chromatography.[28] This afforded the 11-*Z* and 9-*Z* 7,8-dihydro retinals in high purity. Novel spectral data are presented in Figure 1 and Table 1. Due to the absence of the $C_7$═$C_8$ double bond the 9-*Z* 7,8-dihydro retinal isomer now carries a straight polyene chain (Chart 1). Such compounds usually show fine structure in their electronic spectra,[31] and this also is evident in the spectrum of 9-*Z* 7,8-dihydro retinal (Figure 1, right panel). In fact, also the 11-*Z* isomer exhibits subtle signs of fine structure.

Upon incubation with bovine opsin both 9-*Z* and 11-*Z* 7,8-dihydro retinals generated the full equivalent of analogue pigments (7,8-dihydro isorhodopsin and 7,8-dihydro rhodopsin, respectively) since no additional rhodopsin was formed upon subsequent incubation with 11-*Z* retinal. These analogue pigments display some remarkable spectral features (Table 2). Both show a large blue-shift relative to the native pigments, which is expected because of the shortening of the polyene and agrees with earlier reports.[12,26,27] However, the analogue pigments have about the same absorbance maxima (426 ± 2 nm for the 11-*Z* pigment and 428 ± 2 nm for the 9-*Z* pigment), in strong contrast to rhodopsin (498 nm) and isorhodopsin (485 nm) (Figure 2). This suggests that the two isomers now sense a similar protein environment and that the structural distortion of 9-*Z* retinal, because of its "misfit" in the opsin binding site,[32−34] can be relieved by allowing structural flexibility in the 7−9 segment of the retinal polyene chain. Another feature is the molar absorbance coefficient (Table 2). The $\epsilon_{max}$ of the 11-*Z* analogue pigment (37,000 L/mol·cm) is close to that of native rhodopsin (40,500), but the $\epsilon_{max}$ of the 9-*Z* pigment (51,000) is much higher than the native one (43,000). This also can be explained by the absence of the 7,8 double bond, since a straightly extending polyene configuration usually exhibits a higher oscillator strength in the lowest electronic transition.[31] The tendency of a straight polyene to display spectral fine structure is not only observed in the spectrum of 9-*Z* 7,8-dihydro retinal at room temperature (Figure 1) but also in that of 7,8-dihydro isorhodopsin at 80 K.[27]

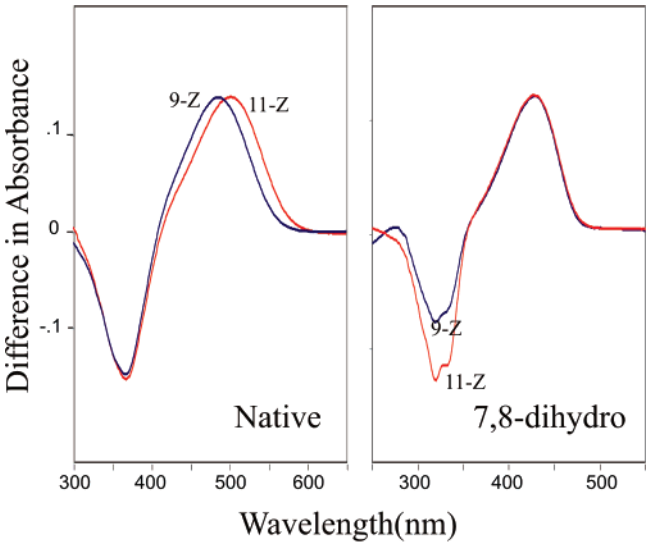


***Figure 2.*** Spectral properties of native and 7,8-dihydro pigments compared in this study. (A) UV−visible difference spectra of native rhodopsin (11-*Z*) and isorhodopsin (9-*Z*), (B) UV−visible difference spectra of 7,8-dihydro rhodopsin (11-*Z*) and 7,8-dihydro isorhodopsin (9-*Z*). Difference spectra were obtained by subtracting the spectrum after illumination from the dark-state spectrum. Illumination was done in the presence of 50 mM hydroxylamine to accomplish full conversion of pigment, with production of the *all-E* retinal oxime derivative.

The most striking observation is the highly efficient photoisomerization of both analogue pigments (Table 2). The 11-*Z* 7,8-dihydro pigment is the first to equal native rhodopsin with respect to quantum yield Φ (0.68 versus 0.67), while the 9-*Z* 7,8-dihydro pigment is significantly more efficient (Φ = 0.39) than native isorhodopsin (0.27). As a result, the photosensitivities (Φ × $\epsilon_{max}$) of both analogue pigments approach that of native rhodopsin (92 ± 10% for 7,8-dihydro rhodopsin and 73 ± 8% for 7,8-dihydro isorhodopsin). The quantum yield of rhodopsins is related to the rate of curve crossing from the excited-state to the distorted Batho ground state via the highly distorted *all-E* photoproduct photorhodopsin.[2,35] In the case of the 11-*Z* pigment this process has been superbly optimized by natural selection, and shortening the polyene segment to allow rapid transfer of the polaron into the isomerization region upon photoexcitation apparently cannot give a further boost. This lends support to the concept that the torsion in the $C_9$−$C_{13}$ segment of the polyene, which is fixed by the anchoring 9- and 13-methyl groups and the Schiff base, coupled to high-frequency vibrational coordinates in this region, are the prime determinants of the high Φ in rhodopsin.[35,36] This "primes" rotational motion of the $C_{12}$−H unit toward $C_{11}$═$C_{12}$ bond isomerization.[36] On

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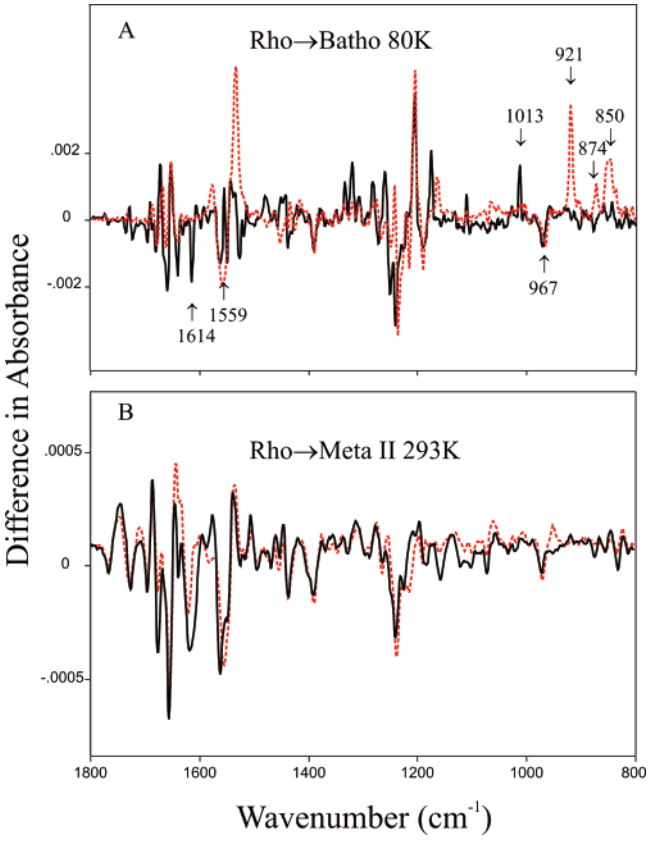


***Figure 3.*** FTIR difference spectra of native and 7,8-dihydrorhodopsin and photointermediates. Difference spectra were constructed by subtracting the dark-state spectrum from the spectrum after illumination. Dotted curves represent the native and solid curves, the 7,8-dihydro pigments. Negative peaks represent vibrational bands characteristic for the dark state; positive peaks represent vibrational bands characteristic for the photoproduct. (A) Rho → Batho transition obtained by illumination at 80 K. The vibration at 967 $cm^{-1}$ represents the major coupled HOOP vibration of the 11-*Z* configuration. The typical 7,8-dihydro vibrations at 1013 and 1614 $cm^{-1}$ have not yet been assigned. (B) Rho → Meta II transition obtained by illumination at 293 K and an approximate pH of 5.5.

the other hand, the lower efficiency in isorhodopsin is probably related to the suboptimal positioning in the binding site and the reduced torsion around the 9-*Z* bond. In the 7,8-dihydro case, the better fit with the protein that should increase the torsion and the vibrational coordinates in the $C_9-C_{13}$ segment in combination with the polaron switch to the isomerization site apparently can enhance the rotational motion of the $C_{10}-H$ unit and thereby increase the isomerization rate in 7,8-dihydro isorhodopsin relative to that of native isorhodopsin.

Important next questions are whether the increased flexibility of the $C_7-C_9$ segment in the 7,8-dihydro pigments will reduce the double-bond distortion of the polyene segment in the first stable photoproduct bathorhodopsin and subsequently will affect the thermal transitions leading to the active-state metarhodopsin II. Typical markers of this distorted *all-E* configuration in Batho are the strong and partially uncoupled hydrogen-out-of-plane (HOOP) vibrations of the polyene hydrogens in the 800−1000 $cm^{-1}$ region of the infrared spectrum[37,38] (Figure 3A, dotted curve). We find that these amplified HOOPs are largely absent in the 7,8-dihydro Batho spectrum (Figure 3A, solid curve), indeed indicating that the conformational strain is at least partially absorbed by the $C_7-C_9$ segment. In that case we would not expect the same large red-shift of the 7,8-dihydro Batho spectrum as displayed by native Batho. The rhodopsin to Batho red-shift (508 → 543 nm at 80 K) is reflected in the major C═C stretch vibration that shifts from 1559 to 1535 $cm^{-1}$ (Figure 3A). In contrast, the 7,8-dihydro spectrum displays hardly any shift of this peak, suggestive of at most only a small red-shift in the transition to 7,8-dihydro Batho. This agrees with cryospectral[27] as well as with time-resolved studies at room temperature[39] of 7,8-dihydro isorhodopsin that indicate strong spectral overlap between the ground state and the first photoproduct. It could be argued that the differences in Figure 3A between the native and the 7,8-dihydro pigments are due to lower stability of the Batho and BSI-like intermediates of the 7,8-dihydro pigment, because of the flexibility in their $C_7-C_9$ segment, resulting in generation of a Lumi-like intermediate already at 80 K, instead of at about 130 K as in the native state. However, this can be excluded on several grounds. First, the time-resolved studies mentioned above could show that a blue-shifted 7,8-dihydro Lumi-like photointermediate ($\lambda_{max} \approx 415$ nm) is generated in the same time frame as native Lumi, subsequent to a first photoproduct with spectral properties very similar to those of the ground state.[39] Second, the FTIR difference spectra in Figure 3A exhibit a very similar peak distribution in the chromophore fingerprint region (1150−1250 $cm^{-1}$) and in the protein amide I region (1600−1700 $cm^{-1}$), whereas these patterns differ markedly from those in the Rho to Lumi transition (Figure S1). These patterns are quite different as well from that of the transition to BSI.[25] Hence, we feel it is safe to conclude that the first stable 7,8-dihydro photoproduct is a Batho analogue.

The subtle changes in protein structure in the Rho → Batho transition, as reflected in the small shifts in the protein backbone region (1600−1700 $cm^{-1}$) and some carboxyl side chains (1700−1800 $cm^{-1}$), actually appear somewhat amplified in the 7,8-dihydro pigment. This suggests that in 7,8-dihydro Batho the strain in the isomerized chromophore is distributed differently but also triggers conformational changes in the protein.

According to our FTIR data the 7,8-dihydro Batho in fact proceeds through similar structural transitions via Meta I (not shown) to Meta II (Figure 3B) as the native pigments. The corresponding spectra of the Rho → Meta II transition are virtually identical (Figure 3B). This is a strong indication that the activated 7,8-dihydro pigments would be able to activate the cognate G protein transducin. This was indeed confirmed in a transducin activation assay, albeit the rate was about 70% of that of the native pigments (Table 1). This modest reduction in rate is probably explained by our preliminary observations that the Meta I to Meta II transition in the 7,8-dihydro pigments has a different thermodynamic profile, resulting in a lower $pK_a$ (Table 1) and a different temperature dependence. For instance, in the native system at 283 K and pH 5.0 exclusively Meta II is formed, whereas under these conditions 40−50% 7,8-dihydro Meta I persists (not shown).

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While the 11-*Z* and 9-*Z* 7,8-dihydro retinals confer excellent photoisomerisation activity upon the corresponding analogue pigments, the resulting *all-E* isomer cannot activate the opsin to the same extent as its native counterpart. Hence, 7,8-dihydro retinal should be classified as a partial agonist. It has been shown before that partial agonism of retinal analogues can be the result of a shift in the protonation switch equilibrium Meta I ↔ Meta II toward the inactive Meta I state,[14−19] and our preliminary data point in the same direction. There is increasing evidence that the dependence of receptor activity on a critical equilibrium node between active and inactive state(s) may be a more general phenomenon in G protein-coupled receptors.[40−42] Our results support the concept that modulation of this equilibrium is one of the modes a ligand can exploit to achieve a certain agonistic potential. Our data further exemplify that very subtle modifications in ligand structure, sufficient to evoke a redistribution of charge and/or conformational strain, can be communicated to modulate receptor dynamics.

## Conclusions and Prospects

We here show that the visual pigment analogues 7,8-dihydro rhodopsin and 7,8-dihydro isorhodopsin perform equally as well as or superior to their native counterparts. They exhibit strikingly efficient photoisomerization, and their photosensitivities approach that of rhodopsin. In addition, they activate the cognate G protein transducin almost as efficiently as the native pigments. It appears that a 7,8 single bond can absorb structural distortion in the chromophore without major implications for receptor function. We anticipate that 7,8-dihydro retinals will help to further unravel the remarkable photochemical properties and conformational dynamics of natural visual pigments. In addition, they may offer interesting prospects as therapeutic supplements in the treatment of retinal pathologies.[22,43,44] First of all, their effectiveness can be easily assessed thanks to the blue-shift of the corresponding pigments. In fact, this blue-shift can probably be modulated by additional modifications such as introduction of a 14-F substituent that will partially reverse the blue-shift.[20] Second, the 7,8 single bond will allow alternative catabolic pathways, thus avoiding overcrowding of normal retinoid metabolic routes and storage pathology.[21,45] Such oral therapies still need careful pharmacometabolic studies,[22,45] but for dietary supplementation the 9-*Z* 7,8-dihydro isomer may be very suitable, since it combines good chromophore properties with low sensitivity to thermal isomerization.

The partial agonism of 7,8-dihydro retinal probably relates to its ability to shift the pH and/or temperature dependence of the Meta I ↔ Meta II equilibrium as observed in the native pigment, in favor of the inactive Meta I state. Modulation of such a critical equilibrium node may represent a more general mechanism in G protein-coupled receptors to regulate ligand activity.

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**Supporting Information Available:** Figure S1, comparing the Batho and Lumi FTIR difference spectra, and Chart S1, showing the carbon numbering scheme in retinal. This material is available free of charge via the Internet at http://pubs.acs.org.

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