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Cyclopropyl and Isopropyl Derivatives of 11-*cis* and 9-*cis* Retinals at C-9 and C-13: Subtle Steric Differences with Major Effects on Ligand Efficacy in Rhodopsin

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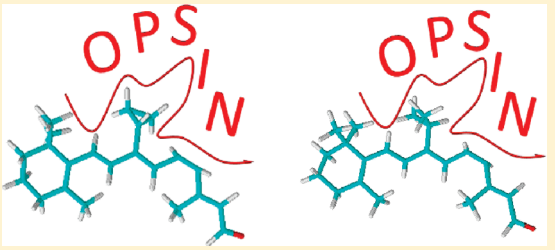
ABSTRACT: Retinal is the natural ligand (chromophore) of the vertebrate rod visual pigment. It occurs in either the 11-*cis* (rhodopsin) or the 9-*cis* (isorhodopsin) configuration. In its evolution to a G protein coupled photoreceptor, rhodopsin has acquired exceptional photochemical properties. Illumination isomerizes the chromophore to the all-*trans* isomer, which acts as a full agonist. This process is extremely efficient, and there is abundant evidence that the C-9 and C-13 methyl groups of retinal play a pivotal role in this process. To examine the steric limits of the C-9 and C-13 methyl binding pocket of the binding site, we have prepared C-9 and C-13 cyclopropyl and isopropyl derivatives of its native ligands and of α-retinal at C-9. Most isopropyl analogues show very poor binding, except for 9-*cis*-13-isopropylretinal. Most cyclopropyl derivatives exhibit intermediate binding activity, except for 9-*cis*-13-cyclopropylretinal, which presents good binding activity. In general, the binding site shows preference for the 9-*cis* analogues over the 11-*cis* analogues. In fact, 13-isopropyl-9-*cis*-retinal acts as a superagonist after illumination. Another surprising finding was that 9-cyclopropylisorhodopsin is more like native rhodopsin with respect to spectral and photochemical properties, whereas 9-cyclopropylrhodopsin behaves more like native isorhodopsin in these aspects.



The visual pigment rhodopsin is the sensory element of the rod photoreceptor cell, which mediates dim-light vision in the vertebrate retina.[1−3] Rhodopsin is a member of the superfamily of G protein-coupled receptors. It evolved for the reception of photons by adopting a photosensitive ligand (chromophore) that has become covalently bound to the apoprotein, opsin, and provides the visual photosensitivity of the holoprotein (λ_{max} = 498 nm). This physiological ligand is 11-*cis*-retinal (Figure 1), a derivative of vitamin A, and it is attached in the opsin binding site to Lys-296 through formation of a protonated Schiff base.[1,4−6] The latter is stabilized by a complex counterion positioned around a glutamate residue of opsin (Glu-113).[7−9] Under certain pathological conditions, however, also the 9-*cis* configuration of retinal is observed, which generates isorhodopsin (λ_{max} = 485 nm).[10] Both the 9-*cis* and the 11-*cis* isomer of retinal act as potent inverse agonists of opsin, practically eliminating its basal activity, and are converted by light into the all-*trans* configuration that acts as a full agonist.[2,11] The photoisomerization of the ligand induces conformational changes in the protein, which are driven by about 35 kcal of photon energy stored in the first photoproduct, Batho.[12−14] These conformational steps in the protein culminate within several milliseconds in the formation of the active state, Meta II (λ_{max} = 380 nm), which binds and activates its cognate G protein transducin (G_t).[11,15]

The photochemical performance of the 11-*cis*-retinal−opsin couple is exceptional, showing a photoisomerization quantum yield of 0.65 ± 0.02 and a fully selective reaction pathway (11-*cis* → all-*trans*) and generating within 200 fs a vibrationally hot intermediate (photorhodopsin) with a highly distorted, but already all-*transoid* chromophore. Within 1 ps photorhodopsin relaxes to Batho, which still contains a highly strained all-*trans* chromophore.[16−22] Isorhodopsin exhibits similar features, except that its photochemistry is less efficient (slower kinetics and lower quantum yield of 0.26 ± 0.03).[23−25]

The crux of this top performance lies in an optimal nonbonding communication between ligand and protein. This aspect has been investigated in a large number of experimental studies, employing modified retinals that generate (iso)rhodopsin analogue pigments.[26−44] These studies have vastly expanded our insight in the binding site requirements. Relevant in the context

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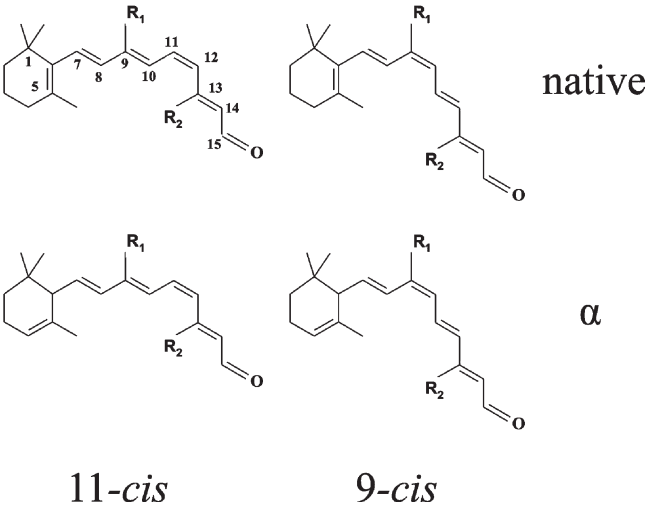

Figure 1. Chemical structure of 11-*cis*- and 9-*cis*-retinals and α-retinals. The structures are shown in the 12-*s trans* conformation, which is dictated by the opsin binding site. The following derivatives are used in this study: natural: R_1 = R_2 = methyl; 9-cyclopropyl: R_1 = cyclopropyl, R_2 = methyl; 9-isopropyl: R_1 = isopropyl, R_2 = methyl; 13-cyclopropyl: R_1 = methyl, R_2 = cyclopropyl; 13-isopropyl: R_1 = methyl, R_2 = isopropyl.

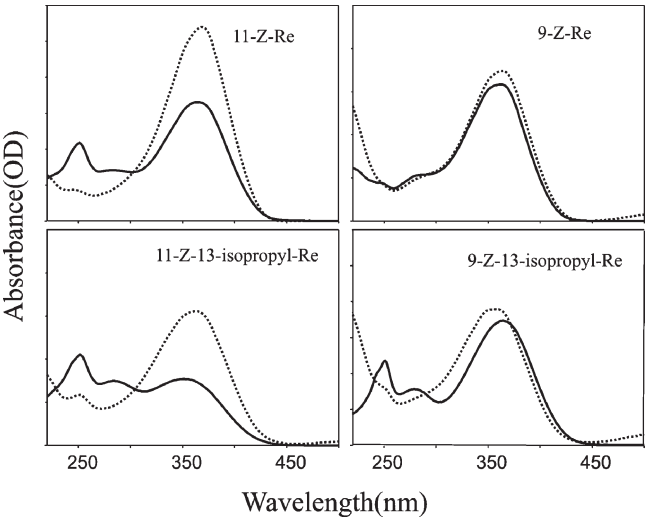

Figure 2. Spectral properties of native and selected 11-*cis*- and 9-*cis*-retinals in *n*-hexane solution (continuous curves). After illumination in the presence of a trace of iodine the dotted curves are obtained, which mainly represent the all-*trans* and 13-*cis* isomers.

of this report is the outcome that the C-9 and C-13 methyl groups are pivotal elements in this ligand−receptor communication. For instance, 9- and 13-demethylretinals still yield analogue pigments, but with an increase in constitutive activity and/or much reduced physiological activity.[33,34,41,45−53]

Indeed, the crystal structure of rhodopsin as well as various biochemical and biophysical evidence agree on quite a defined pocket for the 9-methyl substituent.[54−60] In a recent study we probed the spherosymmetrical boundaries of this pocket using 9-halogen substituents[60] and observed that all bind readily except for a sluggish reaction with the 9-iodo substituent, which, in fact, is somewhat bulkier than the methyl group (van der Waals radii of about 0.215 and 0.20 nm, respectively). This would agree with the absence of any binding data for the 9-*tert*-butyl derivatives (van der Waals radius of about 0.24 nm). The latter derivatives have been prepared synthetically, and in solution they already have a distorted conformation due to severe intramolecular steric hindrance.[61−63] On the other hand, preliminary binding data have been reported for ethyl, *n*-propyl, isopropyl, and even *n*-pentyl substituents at C-9.[28,64] This suggests that the 9-methyl binding pocket can accommodate larger substituents, if they are not spherosymmetrical. Another interesting aspect is that in the 9-*cis*-retinal configuration the 9-methyl group does not comfortably fit into its pocket,[59,65,66] which also results in poorer binding of the 9-iodo derivative.[60] Remarkably, this situation is reversed in the α-retinals, where apparently the poorer fit in the β-ionone binding pocket is partially compensated for by the 9-*cis* geometry.[43,60]

The 13 position has been less well investigated in this respect. Binding data have been reported for the 13-ethyl and *n*-propyl derivatives, showing decreasing affinity, respectively,[67,68] but 13-ethylrhodopsin still having full receptor activity. The 13-*n*-butyl derivative apparently is not able to generate a pigment analogue, however.[28]

In order to further clarify these issues, we have investigated the ligation capability of C-9 and C-13 cyclopropyl and isopropyl substituents, for both the natural retinals and the α-retinals, in the 11-*cis* as well as the 9-*cis* configuration. The cyclopropyl and isopropyl groups are not spherosymmetical, but the cyclopropyl group is somewhat less bulky (ca. 56 versus ca. 63 Å^3). We report here some spectral data, the pigment formation capacity of the various retinal derivatives, and the resulting maximal absorbance, and where relevant some photochemical properties of the analogue pigments and their signaling activity, measured as capability to activate the G protein transducin.

RESULTS AND DISCUSSION

Retinal Derivatives. The spectral properties of most retinal derivatives used in this study were quite similar to the parent 11-*cis* and 9-*cis* compounds, shown in the top panels of Figure 2. The native 11-*cis* isomer (continuous curve, top left panel) shows a main band at 365 nm with a pronounced β-band at 250 nm, indicative of the presence of a twisted 12-*s*-*cis* conformation because of steric hindrance between the C-10 hydrogen and the 13-methyl group. Illumination in the presence of a trace of iodine generates a mixture of all-*trans* and 13-*cis* isomers, which adopt the 12-*s*-*trans* conformation, with concomitant reduction of the β-band intensity and increase in main band intensity. The same behavior is observed for the 11-*cis* derivatives, except that the main band intensity slowly decreases and the β-band intensity gradually increases in the order 9-cyclopropyl < 9-isopropyl < 13-cyclopropyl < 13-isopropyl. This reflects the higher abundance of twisted conformations and at the same time accentuates the lower steric perturbation inflicted by the cyclopropyl substituent. As an example, the spectrum of 11-*cis*-13-isopropylretinal is presented in the lower left panel of Figure2 (continuous curve). The 9-*cis*-retinals are relatively unperturbed, as demonstrated by the absence of a strong β-band and little change in intensity of the main band upon illumination (Figure2, top right panel), except for the 13-isopropyl derivative, which shows a pronounced β-band (Figure2, lower right panel). The latter probably reflects the presence of a twisted 12-*s*-*cis* conformation due to steric hindrance between the C-13 isopropyl group, the C-5 methyl group, and the C-8 hydrogen.

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Table 1. Spectral and Photochemical Properties and Relative G Protein Activation Rates of Generated Pigments

	retinal derivative	regeneration (±10%)	λ_{max} (±2 nm)	quantum yield Φ (±0.04)[a,b,c]	transducin activation[a,b]
11-*cis*	unmodified	≡100	498	0.65	≡100
	9-cyclopropyl	30	492	0.08	83 ± 7
	9-isopropyl	2	504	–	–
	13-cyclopropyl	28	506	0.18	–
	13-isopropyl	6	500	–	–
9-*cis*	unmodified	100	486	0.26	104 ± 8
	9-cyclopropyl	36	504	0.39	60 ± 18
	9-isopropyl	5	485	–	–
	13-cyclopropyl	81	488	0.10	53 ± 7
	13-isopropyl	31	490	0.23	139 ± 6
11-*cis*	α-	50	469	ND	ND
	α-9-cyclopropyl	5	476	–	–
9-*cis*	α-	90	461	0.28	ND
	α-9-cyclopropyl	10	485	–	–

[a] –not determined because of too low production of pigment or lack of retinal derivative. [b] ND, not determined. [c] The Φ value of the unmodified pigments has been determined before.[23,24,69,70]

Similar behavior is observed for the α-retinals (not shown). For spectra of α-retinals we refer to Wang et al.[43]

Pigment Formation and Spectral Properties. The data obtained for the pigments discussed in this study are collected in Table 1. We include relevant data on the α-retinals, as in that case the opsin binding site shows clear preference for the 9-*cis* geometry, as well.[43] However, we observed that the 9-cyclopropyl and 9-isopropyl derivatives of α-retinal are very poor ligands. In fact, the 9-isopropyl-α-retinals did not generate detectable quantities of analogue pigment. We have therefore restricted the analysis of α-pigments and did not test α-retinal modified at C-13.

The general finding is that, as expected, the less bulky cyclopropyl derivatives present a larger rate of pigment formation and a higher incorporation level. It should be noted that in the case of 11-*cis*-retinal the 9- and 13-cyclopropyl derivatives are almost equally effective, and the 9- and 13-isopropyl derivatives almost equally ineffective. In the case of 9-*cis*-retinal, the C-13 derivatives are much more effective than the C-9 derivatives and also much more effective than the C-13 derivatives of 11-*cis*-retinal. The 9-*cis*-13-cyclopropylretinal derivative stands out in this respect, being capable of regenerating about 80% of the available aporeceptor into photopigment, while the other cyclopropylretinal derivatives do not reach further than about 30%.

In addition, we observed that most derivatives effectuate no or a small red-shift in the main absorbance band of the analogue pigments relative to that of the parent pigment, as is evident from their λ_{max}. Exceptions are the 9-*cis*-9-cyclopropyl derivatives of retinal and α-retinal, which induce red-shifts of about 20 nm, and the 11-*cis*-9-cyclopropylretinal, which induces a small blue-shift (Table 1).

Primary Photochemistry. The primary photoreaction of selected analogue pigments was documented by their photoisomerization quantum yield (Table 1) and the photoproduct generated at 80 K, where native pigments produce a stable Batho intermediate (Figure 3).[1]

Rhodopsin is distinguished by an unusually high photoisomerization quantum yield (Φ),[23,24,69,70] while isorhodopsin is less exceptional in this respect, but still significantly enhanced over the free retinals (0.15−0.20).[71] The Φ value of analogue pigments was only determined if sufficient material was available. Conspicuous is the marked reduction in the Φ value of the 13-cyclopropyl derivatives and, again, the large and opposite effects of the 9-cyclopropyl substituent. A strong increase in Φ of isorhodopsin analogue pigments has so far only been reported for the 7,8-dihydro analogue,[42] and that value was similar to the one now observed for 9-cyclopropylisorhodopsin (0.39). The Φ value measured for 9-cyclopropylrhodopsin (0.08) presents a spectacular decrease relative to rhodopsin itself (0.65), which is in glaring contrast to the exceptional increase observed for its 9-*cis* counterpart.

The photoreaction of the parent and analogue pigments was analyzed at 80 K by Fourier transform infrared (FTIR) difference spectroscopy (Figure 3). Negative signals in these spectra correspond to vibrational features in the pigment that change in frequency or intensity or disappear upon formation of the all-*trans* photoproduct, while the positive signals are characteristic for the photoproduct and can be used for identification.[72−75] In the native pigments illumination at this temperature generates a stable Batho intermediate.[1,72] At this stage most of the changes in vibrational pattern reflect the isomerization of the chromophore and associated aborbance band shifts.[76,77] Most prominent are those of C=C and C=N stretches (1500−1600 cm^{-1}), C−C stretches (1100−1360 cm^{-1}, fingerprint region), and wag vibrations (800−1050 cm^{-1}).[78,79]

Very distinct for Batho is the isolated hydrogen-out-of-plane (HOOP) vibrational pattern between 800 and 940 cm^{-1}, which reflects the highly strained structure of the all-*trans* chromophore in Batho.[18] The small tridentate feature around 1650 cm^{-1} represents small changes in protein secondary structure,[80] indicative of the very minor rearrangement of the opsin structure occurring at this first stage of rhodopsin photoactivation.

The isorhodopsin to Batho transition (labeled 9-Z-Rho) presents the same distinct HOOP pattern for Batho, but the other changes show a different pattern, reflecting the fact that here it concerns a 9-*cis* to all-*trans* isomerization. For instance, the combination C−H wag of the polyene chain absorbs at 959 cm^{-1} in isorhodopsin and at 967 cm^{-1} in rhodopsin. In fact, the protein pattern around 650 cm^{-1} is still small but also clearly different from the rhodopsin to Batho transition.

The difference spectra of the analogue pigments globally show the same pattern. The 9-cyclopropylrhodopsin analogue produces a very typical Batho spectrum. Small changes like the upshift and split of the polyene C−H wag to 977 and 988 cm^{-1} reflect an effect of the cyclopropyl group. Interestingly, the structural changes in the protein seem to be somewhat enlarged. The isorhodopsin analogues show a typical 9-*cis* to all-*trans* difference pattern in the fingerprint region and protein changes quite similar to the parent pigment. The polyene C−H wag is again shifted, from 959 to 974 cm^{-1}, and the C=C stretch is split. In the isorhodopsin analogues modified at C-13 the HOOP pattern of the photoproduct is less intense and shifted, probably due to the large C-13 substituent, but we are confident to identify it as the corresponding Batho intermediate. The 9-cyclopropyl analogue of isorhodopsin produced very noisy spectra, which may be due to large spectral overlap between the pigment and the photoproduct. Most features look similar to the C-13 analogues, except for a seemingly different pattern in the HOOP region, which however suffers from a very poor signal-to-noise ratio.

Signaling Activity. In the majority of analogue pigments the transducin activation rate (the signaling activity toward the

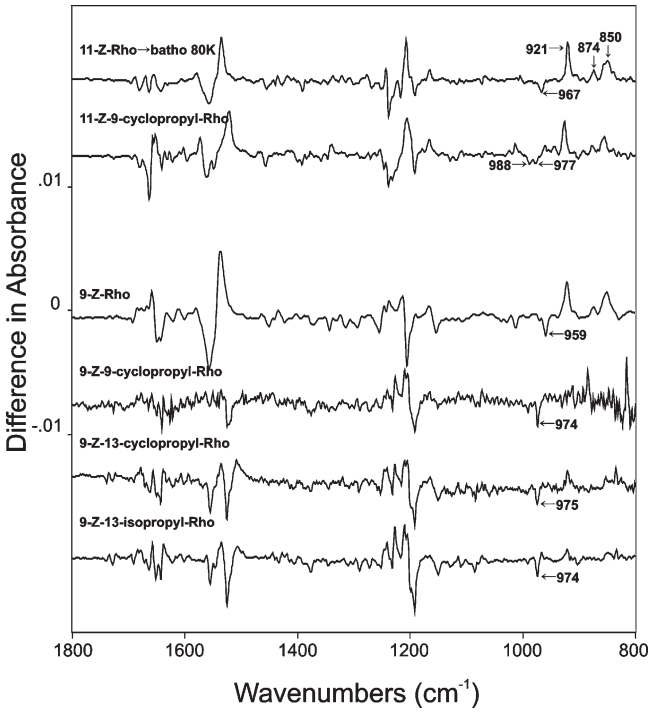

Figure 3. FT-IR difference spectra of selected 11-*cis* and 9-*cis* pigments and photoproducts generated by illumination at 80 K. Difference spectra were constructed by subtracting the dark-state spectrum from the spectrum obtained during two minutes after illumination. Negative bands represent vibrational bands characteristic for the pigment; positive bands represent characteristic vibrations of the photoproduct. The native rhodopsin → Batho and isorhodopsin → Batho difference spectra present the typical, isolated HOOP pattern of Batho between 800 and 930 cm^{-1}, reflecting the highly strained structure of the all-*trans* chromophore in Batho.

cognate G protein transducin) is reduced; that is, the ligand behaves as a partial agonist at most. Usually this at least partially entails a marked downshift of the pH dependence of the Meta I ↔ Meta II equilibrium, which leads to a much lower production of the active state (Meta II) at physiological pH and hence a lower signaling activity.[11,75] We determined the pH dependence of this transition for some analogues (Figure 4). Both the 11-*cis* and 9-*cis* α-pigments exhibit a significant downshift, with quite a steep pH dependence, reminiscent of the profile reported for the 10-F and 12-F analogues.[44] The 9- and 13-cyclopropylrhodopsins behave quite differently. The profile of the 9-cyclopropyl analogue is very similar to the parent compound with pK_a's of 7.4 and 7.3, respectively. The 13-cyclopropyl analogue actually produces very little Meta I even at higher pH, and because of the low stability of Meta I at higher pH, a pK_a could not be determined. This is reminiscent of the behavior of 14-F pigments.[40,44]

Considering that the pK_a of the Meta I ↔ Meta II equilibrium is independent of whether originating in rhodopsin or in isorhodopsin, we would expect that, except for the α-analogues, the signaling activity of the (iso)rhodopsin analogues would approach that of the parent pigment. The transducin activation rate of the cyclopropyl analogues indeed is quite high for an analogue pigment, ranging from 50% to 80% of that of the native pigments (Table 1). Surprisingly, the activity of the 13-isopropyl-isorhodopsin analogue pigment is significantly higher than the parent compound (ca. 140%), classifying its all-*trans* chromophore

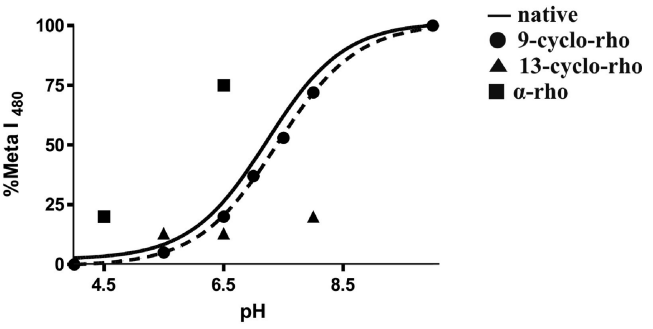

Figure 4. pH dependence of the Meta I ↔ Meta II equilibrium in native rhodopsin (solid line without data points) and selected derivatives (9-cyclopropylrhodopsin, 13-cyclopropylrhodopsin, and α-rhodopsin) measured at 10 °C (283 K). The rhodopsin and 9-cyclopropylrhodopsin data could be fitted with a regular Henderson−Hasselbalch function, yielding a pK_a of 7.3 and 7.4, respectively. α-Rhodopsin behaves irregularly, while the 13-cyclopropylrhodopsin data suggest a significant upshift of the pK_a, but this could not be experimentally verified because of the low stability of Meta I at higher pH.

as a "superagonist". Owing to limited quantities we have not been able to determine the pK_a of the Meta I ↔ Meta II equilibrium, but this analogue pigment certainly deserves further investigation.

While the signaling activity of the active analogue pigments is not much reduced relative to rhodopsin, Φ is markedly lower, varying from 0.08 to 0.39, i.e., 13% to 60% of that of rhodopsin. Thus, when these analogues would need to function in a native setting, i.e., the rod photoreceptor cell, their physiological activity would exhibit a shift in their stimulus/response curve relative to rhodopsin. Their maximum response would probably not be much lower than in the case of native rhodopsin, but they would show a lower photosensitivity; that is, they would need brighter light to elicit the same response.

Interpretation. The C-9 and C-13 methyl groups of 11-*cis*-retinal play a pivotal role in the exquisite interaction between ligand and receptor responsible for the exceptional photochemical performance of this photoreceptor couple.[33,34,41,45−53] This suggests the presence of well-defined binding pockets for these structural elements as part of the global ligand binding site. There are several lines of evidence supporting such a pocket for the 9-methyl group,[54−57,59,60] and a first delineation of its steric requirements has been described using 9-halogen substituents.[60] From the latter study one can estimate an upper limit of ca. 2.2 nm for the radius of a spherical ligand. Nevertheless, preliminary data on pigment formation indicate that this pocket also accommodates isopropyl and *n*-pentyl substituents at C-9.[28] Hence, this pocket may not be spherosymmetrical but more elongated in shape. Evidence for a defined pocket for the 13-methyl group is less substantial. The 13-ethyl and 13-*n*-propyl groups can be accommodated, albeit with much lower binding kinetics,[67,68] but the 13-*n*-butyl group does not seem to give a stable pigment.[28]

To further clarify these issues, we have prepared the potential ligands with cyclopropyl and isopropyl substituents at C-9 or C-13, presented in Figure 1. The cyclopropyl and isopropyl groups both are not spherosymmetical, but the cyclopropyl group is somewhat less bulky (ca. 56 versus ca. 63 $Å^3$) and hence slightly more asymmetric.

The spectral properties of the prepared retinals are outlined in Figure 2 and are in line with the properties of the substituent. A *tert*-butyl substituent at C-9 or C-13 generates substantial

intramolecular steric hindrance, thereby stabilizing a highly twisted conformation.[61,63] This effect will increase in the succession cyclopropyl < isopropyl < *tert*-butyl and explains the gradual intensity changes in the β-band and main band of the 11-*cis*-retinal analogues. The 9-*cis* configuration is less perturbed by these modifications, but does show a pronounced β-band in the case of the 13-isopropyl substituent, probably reflecting torsion around the 12-*s* bond to minimize steric hindrance between the C-13 isopropyl, the C-5 methyl, and the C-8 hydrogen.

Pigment formation with this panel of retinal analogues yielded several unexpected results. None of the analogues were able to bind the full complement of aporeceptor present, even when added in 10−20-fold molar excess (Table 1). The data in this table are based on the assumption that the molar absorbance of the analogue pigments is similar to that of rhodopsin (40 600 M^{-1} cm^{-1}). However, even if this value would be 40% lower (24 000 M^{-1} cm^{-1} is the lowest value reported for an analogue pigment[32]), all retinal analogues in Table 1 would still yield less than 50% regeneration, except for the 9-*cis*-9-cyclopropyl derivative. The phenomenon of only partial pigment formation is quite common in the retinal analogue field.[28,46,81] This is not just a matter of affinity or equilibrium distribution, since it does not depend on the molar excess of analogue, and sometimes the remaining opsin can still bind 11-*cis*-retinal to generate native rhodopsin. Lower pigment formation usually is accompanied by a substantially lower binding rate, and that is also what we observe for these retinal analogues. We have extensively discussed this aspect before[60] and have suggested that the binding process is more complicated and may involve at least a two-step process involving secondary binding sites, for which there is some evidence.[82−84] Nevertheless, a lesser fit of the ligand in the binding site will manifest itself as a lower rate of pigment formation and, hence, a lower extent of pigment formation. Since we observe strongly reduced rates of pigment formation for all analogues, we assume that the data given for the percentage of regeneration are a good approximation.

As such, it can be concluded that the combination of an α-ionone ring with a cyclopropyl or isopropyl moiety at C-9 is poorly accommodated by the binding site. In the case of the normal retinals the cyclopropyl group is much better accepted for binding than the isopropyl group. This again demonstrates that a small difference in steric properties can make a large difference in ligand effectivity and further supports the concept that the 9-methyl binding pocket is not spherosymmetrical. It is therefore not surprising that the 9-*n*-propyl derivative can generate a pigment analogue.[64] Binding of the 9-*n*-pentyl analogue[28] remains surprising, however, and deserves a more detailed study. With respect to the 13-substituent it is obvious that the binding site can only with some difficulty accommodate an isopropyl group, and it is not surprising that an *n*-butyl group is not accepted.[28] The most salient is our observation that the 13-cyclopropyl group is much better accommodated in the opsin binding site in combination with the 9-*cis* configuration than in combination with the 11-*cis* configuration. Possibly, the slight structural adaptation the binding site has to undergo to accommodate 9-*cis*-retinal[54,59] may lead to small rearrangements in the protein surface near C-13. Hence, this would suggest a defined binding site for the 13-methyl group, as well.

Most analogues have no effect on the main absorbance band of the resulting photopigment or generate a small red-shift (Table 1). In this context, a cyclopropyl group at C-9 presents a striking exception. In combination with the 11-*cis* geometry it induces a small blue-shift, but with the 9-*cis* geometry it gives rise to large red-shifts of 18 and 24 nm. We offer the following explanation: In the 9-*cis* geometry the polyene chain is straight from C-9 on. An electron-dense substituent like the cyclopropyl group then is in an optimal position to conjugate with the polyene system, which has positron properties,[85] and to induce a significant bathochromic shift. Such a strong effect cannot occur with the 11-*cis* geometry. The small blue-shift, observed instead for the 11-*cis*-retinal derivative, must arise in a specific interaction with the protein, since the same derivative in 11-*cis*-α-retinal again shows a small red-shift like most other analogue pigments in Table 1.

Analogue pigments usually show a decrease in Φ,[32,39,42,44,68,86] probably reflecting suboptimal cooperation with the protein in the photochemical isomerization process. Hence, it is not surprising that most analogue pigments we report here comply with this general behavior. In calculating the Φ value we again assume the same molar absorbance for all pigments in Table 1, but also in this case an increase of 40% for the analogue data would not change the general trend. The most striking observation is again the quite opposite effect of the 9-cyclopropyl group in the 9-*cis* configuration (large increase relative to the native pigment) versus the 11-*cis* configuration (dramatic decrease). The large decrease for the 11-*cis* derivative is difficult to explain. Since the FTIR analysis indicates that upon Batho formation in this analogue pigment, somewhat larger conformational changes occur in the protein elements lining the binding site (Figure 3, second spectrum from the top), an additional energy barrier might kick in and lead to the observed effect. The large increase in the case of the 9-*cis* derivative is even more difficult to explain. The high Φ value in native rhodopsin is facilitated by the fixed torsion in the C-10−C-13 segment of the polyene chain of its chromophore.[22,54,85,87] This torsion is largely absent in the C-8−C-11 segment of the chromophore of native isorhodopsin. Possibly, the bulky 9-cyclopropyl substituent is tightly fixed in its binding pocket and can induce a twist in the Δ^9 double bond and/or facilitate isomerization electronically. Femtosecond vibrational and electronic spectroscopy and molecular dynamics will hopefully shed more light on these fascinating effects.

The present evidence indicates that the retinal analogues in this study that generate fair levels of analogue pigment do not downshift the pK_a of the Meta I ↔ Meta II equilibrium (Figure 4) and have relatively high partial agonist activity. A remarkable exception is the 13-isopropylisorhodopsin analogue, the only isopropyl derivative that can be reasonably well accommodated in the binding site. This analogue induces a transducin activation rate that is about 140% of that of the parent compound, and hence its all-*trans* photoproduct can be classified as a superagonist. This analogue pigment certainly deserves more detailed investigation, as well.

CONCLUSION

To examine the steric limits of the 9-methyl and 13-methyl binding pocket of opsin, we have prepared cyclopropyl and isopropyl derivatives of its native ligands, 11-*cis*- and 9-*cis*-retinal, at C-9 and C-13, and of α-retinal at C-9. Most isopropyl analogues show very poor binding, except for 9-*cis*-13-isopropylretinal. Most cyclopropyl derivatives exhibit intermediate binding activity, except for 9-*cis*-13-cyclopropylretinal, which presents good binding activity. In general, the opsin binding site has preference for the 9-*cis* over the 11-*cis* analogues. One of

several surprising findings was that 9-cyclopropylisorhodopsin is more like native rhodopsin with respect to spectral and photochemical properties, whereas 9-cyclopropylrhodopsin behaves more like native isorhodopsin in these aspects.

Our data are in line with the growing body of evidence that the interplay between a receptor and its ligand is very finely tuned. Small modification of a ligand can already alter this interplay and thereby redirect the conformational space of a receptor, leading to a different activity profile. Thus, full agonist activity can readily be modified into partial agonist or even neutral antagonist activity.[39,88,89] This emphasizes the need of very accurate mapping of the *in situ* ligand structure and nonbonding ligand−protein communication to allow proper structure-based drug design.

EXPERIMENTAL SECTION

Materials. All chemicals were of analytical grade. Detergents were obtained from Anatrace (Maumee, OH, USA). Native 11-*cis*-retinal was provided by Dr. Rosalie Crouch (Medical University of South Carolina, Charleston, USA) through financial support from the National Eye Institute (NEI).

Synthesis of Retinals. The 9-cyclopropyl and 9-isopropyl derivatives of retinal and α-retinal were prepared in their isomerically pure 9-*cis* or 11-*cis* configuration as described before.[90] The 13-cyclopropyl and 13-isopropyl derivatives were obtained as a mixture of isomers, employing a C_{15} + "C_5" scheme with isopropyl- or cyclopropyl-functionalized trifluoroethyl C_5-phosphonates to increase the yield of the 11-*cis* isomers.[91] The corresponding 11-*cis* and 9-*cis* isomers were purified by preparative HPLC.[92] The purity of the compounds was verified using ^{1}H and ^{13}C NMR spectroscopy and was always better than 98%. UV−vis absorbance spectra were recorded in *n*-hexane solution on a Perkin-Elmer lambda 18 double-beam spectrophotometer at a concentration between 2 and 4 μM.

Isolation of Bovine Opsin and Generation of Analogue Pigments. Bovine rod outer segment membranes in the opsin form (opsin membranes) were prepared from fresh, light-adapted cattle eyes as described.[32,93] The regeneration capacity of these preparations was estimated from the A_{280}/A_{500} ratio measured after subsequent incubation with a 2−3-fold excess of 11-*cis*-retinal, whereby a ratio of 2.1 ± 0.1 was taken to represent membranes with maximal rhodopsin content. Rhodopsin, isorhodopsin, and the analogue pigments were generated with opsin membranes, showing a regeneration capacity in the range 90−100%. Regeneration and further manipulations with the pigments were done under dim red light (>620 nm, Schott RG620 cutoff filter).

Analogue pigments were generated by incubating a suspension of opsin membranes (50−100 μM opsin in buffer A: 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.1 mM EDTA, 1 mM dithioerythritol, pH 6.5) with a 5−10-fold molar excess of the retinal derivative at room temperature. After two hours a small aliquot was assayed for the extent of regeneration by addition of 11-*cis*-retinal in a 2:1 molar ratio to the original opsin. With most retinal analogues regeneration had not yet reached its maximal level. Thus, an additional aliquot of the retinal derivative was added, and the incubation continued for at least overnight at 4 °C until pigment formation had leveled. Excess retinal was then converted into the corresponding oxime by addition of a 1 M hydroxylamine solution (pH 6.5) to a final concentration of 10 mM. After cooling on ice and 30 min incubation, the oxime was largely removed by two extractions with 50 mM heptakis(2,6-di-*O*-methyl)-β-cyclodextrin,[32] which, however, also removes some lipids, thereby perturbing the Meta I to Meta II transition. To restore a native lipid/protein ratio, the membrane pellet was dissolved in 20 mM nonylglucoside in buffer A (to ca. 50 μM of pigment) by incubation for 1 h on ice. Undissolved material was removed by centrifugation (30 min, 80000*g*, 4 °C), and the supernatant was mixed with a solution of asolectin (100 mg/mL in 50 mM nonylglucoside in buffer A) to achieve a 50-fold molar excess of asolectin with respect to pigment. After 15 min incubation on ice detergent was extracted by addition of solid β-cyclodextrin to a slight excess over nonylglucose, and the resulting proteoliposomes were isolated by overnight sucrose step-gradient centrifugation at 200000*g* and 4 °C as described before.[94] The proteoliposome band was recovered from the 20%/45% sucrose interface, diluted with 2 volumes of doubly distilled H_2O, pelleted by centrifugation (60 min, 200000*g*, 4 °C), and stored in aliquots under Ar in a light-tight container at −80 °C.

UV/Vis Spectroscopy. The spectral properties of the pigments were determined in micellar solution, by solubilization to about 2.5 μM in 20 mM dodecylmaltoside (DDM) in buffer A containing 10 mM hydroxylamine. The wavelength of maximal absorbance in the visible region (λ_{max}) was determined as the peak position in the difference spectrum obtained after subtraction of the spectrum after illumination (300 s; 150 W halogen light through Schott OG530 cutoff filter and fiber optics) from the dark-state spectrum.

The Meta I ↔ Meta II equilibrium was analyzed in proteoliposomes using the end-on photomultiplier setup of a Perkin-Elmer Lambda 18 spectrophotometer. Pigments were suspended to about 1 μM in buffer of various pH as indicated in the text using MES, MOPS, or bis-tris propane as buffering compound. Samples were maintained at 10 °C by means of a circulating water bath. Spectra were recorded before and after illumination (10 s; conditions as above) and then every 5 min up to 30 min after illumination to check for the stability of the Meta photoproducts. Finally, 1 M hydroxylamine was added to a final concentration of 50 mM to convert all photointermediates into the all-*trans*-retinaloxime derivative. The relative amount of Meta I at 480 nm remaining immediately after illumination was calculated as described before.[95]

FT-IR Spectroscopy. FT-IR analyses were performed on a Bruker IFS 66/S spectrometer, equipped with a liquid-nitrogen-cooled, narrow-band HgCdTe (MCT) detector as described.[32,39] In brief, sample temperature was under computer control using a variable-temperature helium-cooled cryostat (Heliostat, APD Cryogenics Inc.), covered with a set of NaCl windows in the IR light path. Membrane films were prepared by isopotential spin drying[96] 2−3 nmol of pigment in proteoliposomes on AgCl windows (Crystran Limited, UK). The membrane film was rehydrated, sealed using a rubber O-ring spacer and a second AgCl window, and screwed tight in the sample holder of the cryostat. Samples were illuminated in the spectrometer using a modified fiberoptics ring illuminator (Schott) fed by a 150 W halogen light filtered through a 488 ± 10 nm interference filter and a long-pass filter (Schott). Routinely, six consecutive blocks of 1280 scans each were recorded at 4 cm^{-1} resolution, taking about 120 s acquisition time per block to generate a spectrum, both before and after 2 min of illumination. No differences in pattern for the subsequent spectra were detected, and routinely the last dark-state spectrum was subtracted from the first spectrum of the photoproduct to generate a difference spectrum. Difference spectra were measured at 80 K, where Batho is sufficiently stable to allow analysis. Temperature stability was ±0.2 K.

Determination of Quantum Yield. The photoisomerization quantum yield (Φ) of selected analogue pigments was determined relative to rhodopsin, of which the Φ (0.65 ± 0.02) and molar absorbance (ε_{498} = 40 600 ± 500 M^{-1} cm^{-1}) are well established.[23,24,69,70] Rhodopsin, isorhodopsin, and analogue pigments were solubilized in buffer A containing 10 mM DDM and 10 mM hydroxylamine to give an OD/cm at 500 nm of 0.100 ± 0.07. Samples were kept at 10 °C and showed no significant decrease of A_{500} after 2 h in the dark. Samples were illuminated through a 497 ± 5 nm interference filter (Schott) such that the half-time of bleaching was ≥30 min. Spectra were recorded at intervals of 2−10 min. The data were converted to a straight

line, the slope of which (S) is a measure of the photosensitivity $\varepsilon_{497}\Phi$, as described before.[32,69] Using the S of rhodopsin measured under identical conditions allows the calculation of the Φ value of the analogue pigment.[32,69]

Transducin Activation. Activation of the rhodopsin-associated G protein transducin was determined at 20 °C using a fluorescence assay as described.[97,98] A hypotonic extract of isotonically washed bovine rod outer segments served as the source of transducin.[15] Samples contained 2, 5, or 10 nM of pigment and about 100 nM of transducin in 20 mM HEPPS, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTE, and 0.01% (w/v) DDM, pH 7.4. Immediately before data acquisition a sample was illuminated for 5 min in bright white light, and when a steady fluorescence level was reached, GTPγS was added to a final concentration of 2.5 μM. The initial rate in the increase in tryptophan fluorescence of the α-subunit of transducin, induced by binding of GTPγS, was plotted against the pigment concentration, and the slope of the resulting straight line was rated against the slope obtained for rhodopsin in the same set of experiments.

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DEDICATION

Dedicated to Dr. Koji Nakanishi of Columbia University for his pioneering work on bioactive natural products.

REFERENCES

(1) Wald, G. *Nature* **1968**, *219*, 800–807.
(2) Ernst, O. P.; Hofmann, K. P.; Palczewski, K. In *Photoreceptors and Light Signalling*; Batschauert, A., Ed.; Royal Society of Chemistry: Cambridge, U.K., 2003; Vol. 3, pp 77−123.
(3) Palczewski, K. *Annu. Rev. Biochem.* **2006**, *75*, 743–767.
(4) Bownds, M. D. *Nature* **1967**, *216*, 1178–1181.
(5) Eyring, G.; Mathies, R. A. *Proc. Natl. Acad. Sci. U. S. A.* **1979**, *76*, 33–37.
(6) Wang, K.; McDowell, J. H.; Hargrave, P. A. *Biochemistry* **1980**, *19*, 5111–5117.
(7) Zhukovsky, E. A.; Oprian, D. D. *Science* **1989**, *246*, 928–930.
(8) Sakmar, T. P.; Franke, R. R.; Khorana, H. G. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86*, 8309–8313.
(9) Nathans, J. *Biochemistry* **1990**, *29*, 9746–9752.
(10) Fan, J.; Rohrer, B.; Moiseyev, G.; Ma, J.-X.; Crouch, R. K. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 13662–13667.
(11) Hofmann, K. P. In *Molecular Mechanisms in Visual Transduction*; Stavenga, D. G.; DeGrip, W. J.; Pugh, E. N., Jr., Eds.; Elsevier Science Pub.: Amsterdam, The Netherlands, 2000; Vol. 3, Chapter 3, pp 91−142.
(12) Cooper, A. *Nature* **1979**, *282*, 531–533.
(13) Schick, G. A.; Cooper, T. M.; Holloway, R. A.; Murray, L. P.; Birge, R. R. *Biochemistry* **1987**, *26*, 2556–2562.
(14) Birge, R. R.; Vought, B. W. *Methods Enzymol.* **2000**, *315*, 143–163.
(15) Kühn, H. *Prog. Retinal Res.* **1984**, *3*, 123–156.
(16) Schoenlein, R. W.; Peteanu, L. A.; Mathies, R. A.; Shank, C. V. *Science* **1991**, *254*, 412–415.
(17) Haran, G.; Morlino, E. A.; Matthes, J.; Callender, R. H.; Hochstrasser, R. M. *J. Phys. Chem. A* **1999**, *103*, 2202–2207.
(18) Mathies, R. A.; Lugtenburg, J. In *Molecular Mechanisms in Visual Transduction*; Stavenga, D. G.; DeGrip, W. J.; Pugh, E. N., Jr., Eds.; Elsevier Science Pub.: Amsterdam, The Netherlands, 2000; Vol. 3, Chapter 2, pp 55−90.
(19) Kandori, H.; Furutani, Y.; Nishimura, S.; Shichida, Y.; Chosrowjan, H.; Shibata, Y.; Mataga, N. *Chem. Phys. Lett.* **2001**, *334*, 271–276.
(20) Kukura, P.; McCamant, D. W.; Yoon, S.; Wandschneider, D. B.; Mathies, R. A. *Science* **2005**, *310*, 1006–1009.
(21) Nakamichi, H.; Okada, T. *Angew. Chem., Int. Ed.* **2006**, *45*, 4270–4273.
(22) Polli, D.; Altoè, P.; Weingart, O.; Spillane, K. M.; Manzoni, C.; Brida, D.; Tomasello, G.; Orlandi, G.; Kukura, P.; Mathies, R. A.; Garavelli, M.; Cerullo, G. *Nature* **2010**, *467*, 440–443.
(23) Hurley, J. B.; Ebrey, T. G.; Honig, B.; Ottolenghi, M. *Nature* **1977**, *270*, 540–542.
(24) Birge, R. R.; Einterz, C. M.; Knapp, H. M.; Murray, L. P. *Biophys. J.* **1988**, *53*, 367–385.
(25) Kochendoerfer, G. G.; Verdegem, P. J. E.; Van der Hoef, I.; Lugtenburg, J.; Mathies, R. A. *Biochemistry* **1996**, *35*, 16230–16240.
(26) Becker, R. S. *Photochem. Photobiol.* **1988**, *48*, 369–399.
(27) Balogh-Nair, V.; Nakanishi, K. *Methods Enzymol.* **1982**, *88*, 496–506.
(28) Liu, R. S. H.; Asato, A. E. In *Chemistry and Biology of Synthetic Retinoids*; Dawson, M. I.; Okamura, W. H., Eds.; CRC Press, Inc.: Boca Raton, FL, USA, 1990; Chapter 3, pp 52−75.
(29) Calhoon, R. D.; Rando, R. R. *Biochemistry* **1985**, *24*, 6446–6452.
(30) Asato, A. E.; Zhang, B.-W.; Denny, M.; Mirzadegan, T.; Seff, K.; Liu, R. S. H. *Bioorg. Chem.* **1989**, *17*, 410–421.
(31) Okada, T.; Kandori, H.; Shichida, Y.; Yoshizawa, T.; Denny, M.; Zhang, B.-W.; Asato, A. E.; Liu, R. S. H. *Biochemistry* **1991**, *30*, 4796–4802.
(32) DeLange, F.; Bovee-Geurts, P. H. M.; VanOostrum, J.; Portier, M. D.; Verdegem, P. J. E.; Lugtenburg, J.; DeGrip, W. J. *Biochemistry* **1998**, *37*, 1411–1420.
(33) Lou, J. H.; Tan, Q.; Karnaukhova, E. N.; Berova, N.; Nakanishi, K.; Crouch, R. K. *Methods Enzymol.* **2000**, *315*, 219–237.
(34) Meyer, C. K.; Böhme, M.; Ockenfels, A.; Gärtner, W.; Hofmann, K. P.; Ernst, O. P. *J. Biol. Chem.* **2000**, *275*, 19713–19718.
(35) Crouch, R. K.; Kefalov, V. J.; Gärtner, W.; Cornwall, M. C. *Methods Enzymol.* **2002**, *343*, 29–48.
(36) Kuksa, V.; Franz, B.; Maeda, T.; Jang, G.-F.; Ritter, E.; Heck, M.; VanHooser, J. P.; Liang, Y.; Filipek, S.; Gelb, M. H.; Hofmann, K. P.; Palczewski, K. *J. Biol. Chem.* **2002**, *277*, 42315–42324.
(37) Vogel, R.; Siebert, F.; Lüdeke, S.; Hirshfeld, A.; Sheves, M. *Biochemistry* **2005**, *44*, 11684–11699.
(38) Bartl, F. J.; Fritze, O.; Ritter, E.; Herrmann, R.; Kuksa, V.; Palczewski, K.; Hofmann, K. P.; Ernst, O. P. *J. Biol. Chem.* **2005**, *280*, 34259–34267.
(39) Verhoeven, M. A.; Bovee-Geurts, P. H. M.; de Groot, H. J. M.; Lugtenburg, J.; DeGrip, W. J. *J. Mol. Biol.* **2006**, *363*, 98–113.
(40) Vogel, R.; Siebert, F.; Yan, E. C. Y.; Sakmar, T. P.; Hirshfeld, A.; Sheves, M. *J. Am. Chem. Soc.* **2006**, *128*, 10503–10512.
(41) Vogel, R.; Lüdeke, S.; Siebert, F.; Sakmar, T. P.; Hirshfeld, A.; Sheves, M. *Biochemistry* **2006**, *45*, 1640–1652.
(42) DeGrip, W. J.; Bovee-Geurts, P. H. M.; Van der Hoef, I.; Lugtenburg, J. *J. Am. Chem. Soc.* **2007**, *129*, 13265–13269.
(43) Wang, Y.-J.; Bovee-Geurts, P. H. M.; Lugtenburg, J.; DeGrip, W. J. *Photochem. Photobiol.* **2008**, *84*, 889–894.

(44) Bovee-Geurts, P. H. M.; Fernández Fernández, I.; Liu, R. S. H.; Mathies, R. A.; Lugtenburg, J.; DeGrip, W. J. *J. Am. Chem. Soc.* **2009**, *131*, 17933–17942.
(45) Blatz, P. E.; Lin, M.; Balasubramaniyan, P.; Balasubramaniyan, V.; Dewhurst, P. B. *J. Am. Chem. Soc.* **1969**, *91*, 5930–5931.
(46) Kropf, A.; Whittenberger, B. P.; Goff, S. P.; Waggoner, A. S. *Exp. Eye Res.* **1973**, *17*, 591–606.
(47) Ebrey, T. G.; Tsuda, M.; Sassenrath, G.; West, J. L.; Waddell, W. H. *FEBS Lett.* **1980**, *116*, 217–219.
(48) Ganter, U. M.; Schmid, E. D.; Perez-Sala, D.; Rando, R. R.; Siebert, F. *Biochemistry* **1989**, *28*, 5954–5962.
(49) Ganter, U. M.; Gärtner, W.; Siebert, F. *Eur. Biophys. J.* **1990**, *18*, 295–299.
(50) Corson, D. W.; Crouch, R. K. *Photochem. Photobiol.* **1996**, *63*, 595–600.
(51) Wang, Q.; Kochendoerfer, G. G.; Schoenlein, R. W.; Verdegem, P. J. E.; Lugtenburg, J.; Mathies, R. A.; Shank, C. V. *J. Phys. Chem. B* **1996**, *100*, 17388–17394.
(52) Vogel, R.; Fan, G.-B.; Sheves, M.; Siebert, F. *Biochemistry* **2000**, *39*, 8895–8908.
(53) Corson, D. W.; Kefalov, V. J.; Cornwall, M. C.; Crouch, R. K. *J. Gen. Physiol.* **2000**, *116*, 283–297.
(54) Okada, T.; Sugihara, M.; Bondar, A.-N.; Elstner, M.; Entel, P.; Buss, V. *J. Mol. Biol.* **2004**, *342*, 571–583.
(55) Li, J.; Edwards, P. C.; Burghammer, M.; Villa, C.; Schertler, G. F. X. *J. Mol. Biol.* **2004**, *343*, 1409–1438.
(56) Han, M.; Groesbeek, M.; Smith, S. O.; Sakmar, T. P. *Biochemistry* **1998**, *37*, 538–545.
(57) Creemers, A. F. L.; Kiihne, S. R.; Bovee-Geurts, P. H. M.; DeGrip, W. J.; Lugtenburg, J.; de Groot, H. J. M. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 9101–9106.
(58) Stenkamp, R. E.; Teller, D. C.; Palczewski, K. *ChemBioChem* **2002**, *3*, 963–967.
(59) Creemers, A. F. L.; Bovee-Geurts, P. H. M.; DeGrip, W. J.; Lugtenburg, J.; de Groot, H. J. M. *Biochemistry* **2004**, *43*, 16011–16018.
(60) Wang, Y.-J.; Bovee-Geurts, P. H. M.; Lugtenburg, J.; DeGrip, W. J. *Biochemistry* **2004**, *43*, 14802–14810.
(61) Ernst, L.; Hopf, H.; Krause, N. *J. Org. Chem.* **1987**, *52*, 398–405.
(62) De Lera, A. R.; Okamura, W. H. *Tetrahedron Lett.* **1987**, *28*, 2921–2924.
(63) Norman, T. C.; De Lera, A. R.; Okamura, W. H. *Tetrahedron Lett.* **1988**, *29*, 1251–1254.
(64) Han, M.; Groesbeek, M.; Sakmar, T. P.; Smith, S. O. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 13442–13447.
(65) Nakamichi, H.; Okada, T. *Photochem. Photobiol.* **2007**, *83*, 232–235.
(66) Sekharan, S.; Sugihara, M.; Weingart, O.; Okada, T.; Buss, V. *J. Am. Chem. Soc.* **2007**, *129*, 1052–1054.
(67) Nakanishi, K. *Pure Appl. Chem.* **1985**, *57*, 769–776.
(68) Karnaukhova, E. N.; Hu, S. H.; Boonyasai, R.; Tan, Q.; Nakanishi, K. *Bioorg. Chem.* **1999**, *27*, 372–382.
(69) Dartnall, H. J. A. In *Photochemistry of Vision*; Dartnall, H. J. A., Ed.; Springer-Verlag: Berlin, GFR, 1972; Vol. VII/I, Chapter 4, pp 122−145.
(70) Kim, J. E.; Tauber, M. J.; Mathies, R. A. *Biochemistry* **2001**, *40*, 13774–13778.
(71) Nakanishi, K. *Pure Appl. Chem.* **1977**, *49*, 333–339.
(72) Rothschild, K. J.; Cantore, W. A.; Marrero, H. *Science* **1983**, *219*, 1333–1335.
(73) DeGrip, W. J.; Gray, D.; Gillespie, J.; Bovee-Geurts, P. H. M.; VanDenBerg, E. M. M.; Lugtenburg, J.; Rothschild, K. J. *Photochem. Photobiol.* **1988**, *48*, 497–504.
(74) DeGrip, W. J.; Rothschild, K. J. In *Molecular Mechanisms in Visual Transduction*; Stavenga, D. G.; DeGrip, W. J.; Pugh, E. N., Jr., Eds.; Elsevier Science Pub.: Amsterdam, The Netherlands, 2000; Vol. 3, Chapter 1, pp 1−54.
(75) Mahalingam, M.; Martínez-Mayorga, K.; Brown, M. F.; Vogel, R. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 17795–17800.
(76) Mathies, R. A.; Smith, S. O.; Palings, I. In *Resonance Raman Spectra of Polyenes and Aromatics*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1987; Vol. 2, pp 59−108.
(77) Touw, S. I. E.; de Groot, H. J. M.; Buda, F. *J. Phys. Chem. B* **2004**, *108*, 13560–13572.
(78) Palings, I.; Pardoen, J. A.; VanDenBerg, E. M. M.; Winkel, C.; Lugtenburg, J.; Mathies, R. A. *Biochemistry* **1987**, *26*, 2544–2556.
(79) Palings, I.; VanDenBerg, E. M. M.; Lugtenburg, J.; Mathies, R. A. *Biochemistry* **1989**, *28*, 1498–1507.
(80) Pistorius, A. M. A.; DeGrip, W. J. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 1040–1045.
(81) Balogh-Nair, V.; Nakanishi, K. In *Stereochemistry*; Tamm, T., Ed.; Elsevier Biomedical Press: Amsterdam, 1982; Chapter 7, pp 283−334.
(82) Heck, M.; Schädel, S. A.; Maretzki, D.; Hofmann, K. P. *Vision Res.* **2003**, *43*, 3003–3010.
(83) Schädel, S. A.; Heck, M.; Maretzki, D.; Filipek, S.; Teller, D. C.; Palczewski, K.; Hofmann, K. P. *J. Biol. Chem.* **2003**, *278*, 24896–24903.
(84) Hildebrand, P. W.; Scheerer, P.; Park, J. H.; Choe, H. W.; Piechnick, R.; Ernst, O. P.; Hofmann, K. P.; Heck, M. *PLoS ONE* **2009**, *4*, e4382-1–9.
(85) Buda, F.; de Groot, H. J. M.; Bifone, A. *Phys. Rev. Lett.* **1996**, *77*, 4474–4477.
(86) Liu, R. S. H.; Denny, M.; Zingoni, J. P.; Mead, D.; Matsumoto, H.; Asato, A. E. In *Biophysical Studies of Retinal Proteins*; Ebrey, T. G.; Frauenfelder, H.; Honig, B.; Nakanishi, K., Eds.; University of Illinois Press: Chicago, IL, USA, 1987; pp 59−63.
(87) Weingart, O. *J. Am. Chem. Soc.* **2007**, *129*, 10618–10619.
(88) Kristiansen, K. *Pharmacol. Ther.* **2004**, *103*, 21–80.
(89) Swaminath, G.; Deupí, X.; Lee, T. W.; Zhu, W.; Thian, F. S.; Kobilka, T. S.; Kobilka, B. K. *J. Biol. Chem.* **2005**, *280*, 22165–22171.
(90) Wang, Y.-J.; Lugtenburg, J. *Eur. J. Org. Chem.* **2004**, 3497–3510.
(91) Creemers, A. F. L.; Lugtenburg, J. *J. Am. Chem. Soc.* **2002**, *124*, 6324–6334.
(92) Lugtenburg, J. *Pure Appl. Chem.* **1985**, *57*, 753–762.
(93) DeGrip, W. J.; VanOostrum, J.; Bovee-Geurts, P. H. M.; VanDerSteen, R.; VanAmsterdam, L. J. P.; Groesbeek, M.; Lugtenburg, J. *Eur. J. Biochem.* **1990**, *191*, 211–220.
(94) DeGrip, W. J.; VanOostrum, J.; Bovee-Geurts, P. H. M. *Biochem. J.* **1998**, *330*, 667–674.
(95) DeLange, F.; Merkx, M.; Bovee-Geurts, P. H. M.; Pistorius, A. M. A.; DeGrip, W. J. *Eur. J. Biochem.* **1997**, *243*, 174–180.
(96) Clark, N. A.; Rothschild, K. J.; Simon, B. A.; Luippold, D. A. *Biophys. J.* **1980**, *31*, 65–96.
(97) Phillips, W. J.; Cerione, R. A. *J. Biol. Chem.* **1988**, *263*, 15498–15505.
(98) Fahmy, K.; Sakmar, T. P. *Biochemistry* **1993**, *32*, 7229–7236.