similarities, metabolism of Asp and Glu could be independent from CSA metabolism. This is of interest because these amino acids could play an important neuromodulator and/or neurotransmitter role in the central nervous system.

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Aromatic Retinal Analogues and Their Interaction with Cattle Opsin[†]

Hiroyuki Matsumoto, Alfred E. Asato, Marlene Denny, Bruce Baretz, Yao-Pin Yen, Dominic Tong, and Robert S. H. Liu*

ABSTRACT: The preparation of seven aromatic analogues of retinal, the isolation and characterization of their geometric isomers, and the interaction of these isomers with cattle opsin are reported. Within certain limitations, it has been demonstrated that stable aromatic rhodopsin analogues can be prepared. In general, the stereoselectivity of isomers of these compounds in their interaction with opsin and the absorption properties of the resultant pigments are similar to those of the parent retinal.

The approach of using structurally modified retinal, whether in the form of geometrical isomerism or chain or ring modifications, for probing for information on structure and binding specificity of opsin has been described in many reports in the literature. The classical work of Wald (Hubbard & Wald, 1952) was followed by more recent work from several different laboratories (Blatz et al., 1969; Kropf et al., 1973; Ebrey et al., 1975; Nakanishi et al., 1976; Arnaboldi et al., 1979; DeGrip et al., 1976; Asato et al., 1978; Kini et al., 1979). It appears that there is a general agreement that within certain limitations, e.g., the longitudinal restriction (Matsumoto & Yoshizawa, 1978), the binding site of opsin is fairly flexible, being able to accommodate retinal isomers of varied geometry and analogues which have undergone much chain and ring modifications. An exception is retinal analogues containing an aromatic end group. As far as we are aware only one report describing results on one system has appeared in the literature (Kropf et al., 1973). A photoisomerized mixture of the simple aromatic aldehyde 9-phenyl-2,4,6,8-nonatetraenal (I)[1] was

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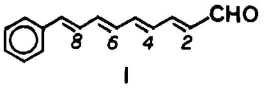

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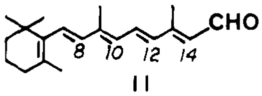

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found not to interact, perhaps somewhat unexpectedly, with cattle opsin to give an identifiable stable pigment. Because of the possibility of preparing in aromatic systems pigment analogues with considerably modified absorption properties, we have carried out a more detailed study first to establish the possible existence of aromatic rhodopsin analogues and then with the hope to change absorption maxima by further calculated structural modifications. In this paper we describe preliminary results with several aromatic systems.

Materials and Methods

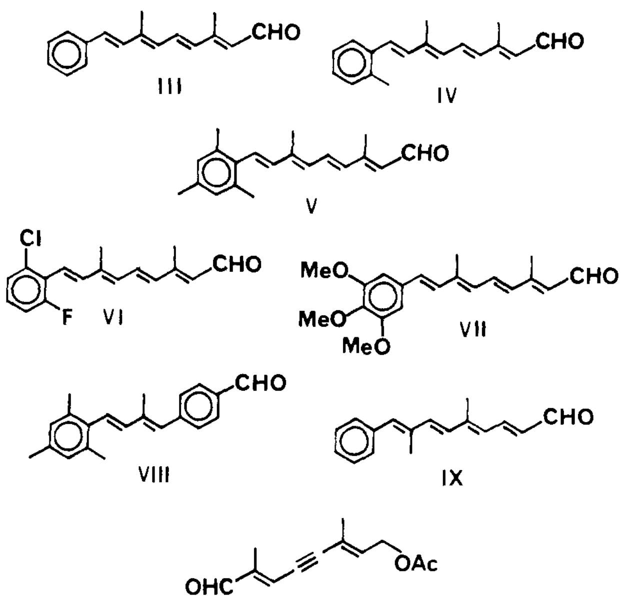
All aromatic retinals were synthesized according to the $C_{15} + C_5$ route (Bharucha & Weedon, 1953). The following is a brief description of the general procedure. The preparation of 5-aryl-3-methyl-2,4-pentadienonitriles was effected in good yields (60–85%) by the reaction of appropriately substituted benzaldehydes with the lithio derivative of 4-(diethylphosphono)-3-methylcrotononitrile ($E/Z = 1$) in H_4furan[2]–HMPA–hexane. Reduction of the 5-aryldienonitriles with diisobutylaluminum hydride (DIBAH) in benzene–hexane (1.5 h, 0 °C to room temperature), followed by a wet silica gel workup (Kini et al., 1979), afforded the corresponding 5-aryl-3-methyl-2,4-pentadienals in moderate yields (50–65%). Subsequent repetition of the chain extension reaction of the above dienals with the phosphonate $(EtO)_2POCHC(CH_3)=CHCN$, followed by DIBAH reduction to the desired aromatic retinal analogues VI and VII, was effected in fair overall yields (after recrystallization from benzene–hexane or ethyl acetate–hexane).

Aldehyde V was prepapred by the condensation of (2*E*,4*E*)-3-methyl-5-(2,4,6-trimethylphenyl)-2,4-pentadienal with the lithium derivative of methyl (*E*)-3-methyl-4-(diethylphosphono)crotonate, followed by (a) lithium aluminum hydride reduction and (b) manganese dioxide oxidation and purification by recrystallization from acetonitrile.

Aldehyde VIII was prepared by condensation of *p*-cyanotolylphosphonate with the mesityl analogue of β-ionone, followed by partial reduction with DIBAH. The starting material for the synthesis of IX was α-methylcinnamaldehyde.

Cattle Opsin and Its Interaction with Aromatic Retinals. Rod outer segments (ROS) were purified by a conventional method (Hubbard et al., 1971; Matsumoto et al., 1978) from frozen cattle retinas purchased from Hormel. The purified ROS were washed twice with distilled water and lyophilized. Then they were washed with chilled petroleum ether several times. Opsin was extracted from the resultant ROS with 1% digitonin dissolved in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.0.

Chart I



Opsin was assayed by incubating with it an excess amount of 11-*cis*-retinal (11*Z*) for 2 h at room temperature, followed by the addition of NH_2OH to a final concentration of 50 mM and the measurement of the difference absorption spectrum before and after irradiation with yellow light (>500 nm). All spectra were measured with a Hitachi Perkin-Elmer 124 recording spectrophotometer. The regenerated amount of rhodopsin was calculated by using 42 000 cm^{-1} mol^{-1} as ϵ (Shichi et al., 1969).

Reaction mixtures contained ca. 5–20 μM opsin (i.e., about 0.2–0.8 OD unit of rhodopsin at 500 nm) and a 2× excess OD unit of each isomer of aromatic retinals in 2 mL of 0.5% digitonin (10 mM Hepes buffer, pH 7.0). The isomers were added to the reaction mixtures in ethanol, the latter concentration not exceeding 3%. The reaction mixtures were incubated at room temperature for 10–20 h to complete pigment formation. All the aromatic retinal pigments tested in this work seem to have much slower formation rates than the parent pigment, rhodopsin (Matsumoto et al., 1978). Hydroxylamine was then added to a final concentration of 50 mM. Immediately after the addition of NH_2OH, the absorbance decrease at 440 nm (A_{440}) was measured to monitor the quenching process of random Schiff bases by NH_2OH. In a control experiment with 11-*cis*-retinal, A_{440} ceased to decrease within 30 min under similar conditions. In the case of the aromatic retinal pigments, A_{440} continued to decrease after 30 min from the addition of NH_2OH, suggesting instability of pigments in NH_2OH. This was confirmed by measuring the absorption spectral change from 400 to 600 nm during the quenching process. To take the difference absorption spectrum of such an unstable pigment, we measured a spectrum at 30–45 min after the addition of NH_2OH. Then the sample was irradiated by yellow light (>500 nm) for 5 min with a 50-W tungsten lamp and a glass cutoff filter. After the irradiation the spectrum was measured again. The difference between the two spectra measured before and after the irradiation was calculated. The approximate yield of an aromatic retinal pigment was based on a spectrum obtained ~15 min after the addition of NH_2OH.

Results

The aromatic retinal analogues III–IX, shown in Chart I, were synthesized. In general, the initial synthetic mixture contained primarily the all-*E* isomer. The mono-*Z* isomers were subsequently produced by photoisomerization followed by separation by preparative high-pressure liquid chromatography.

Synthesis. An early attempt to prepare the aromatic retinals by coupling of the C_{10} fragment X with the Wittig salt of

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[1] For compound I and all other aromatic retinals described in this paper, we use the IUPAC numbering system for polyenes instead of those commonly used for vitamin A and its derivatives II, including the *E*,*Z* designation for polyene geometry.

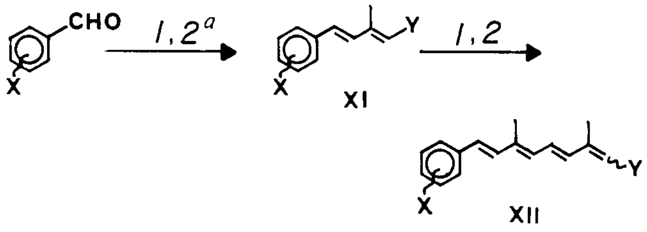
[2] Abbreviations used: H_4furan, tetrahydrofuran; HMPA, hexamethylphosphoramide.

Table I: ^{1}H NMR Data of Aromatic Aldehydes[a]

		chemical shift, δ (ppm)									coupling constant (Hz)		
compd	isomer	CH_3-3	CH_3-7	H-1	H-2	H-4	H-5	H-6	H-8	H-9	$J_{4,5}$	$J_{5,6}$	$J_{8,9}$
III	all-*E*	2.32	2.09	10.13	5.96	6.40	7.32?	6.33	6.85	6.70	15	12	16
	2*Z*,4*E*,6*E*,8*E*	2.16	2.10	10.23	5.87	7.4	7.05	6.39	6.90	6.75	15	11	16
	2*E*,4*Z*,6*E*,8*E*[b]	2.37	2.05	10.12	5.95				6.90	6.74			
	2*E*,4*E*,6*Z*,8*E*	2.35	2.09	10.15	5.98	6.36	7.4	6.2	7.3	6.75			16
IV	all-*E*	2.37	2.14	10.09	5.95	6.8-6.9		6.33	6.86	6.45			
	2*Z*,4*E*,6*E*,8*E*	2.18	2.14	10.17	5.82	7.1	7.38	6.38	6.78	6.9		11	
	2*E*,4*Z*,6*E*,8*E*[b]	2.43	2.12	10.10	6.03								
	2*E*,4*E*,6*Z*,8*E*	2.38	2.14	10.09	5.92			6.16		6.36			16
V	all-*E*	2.35	2.16	10.14	5.99	6.44	7.18	6.25	6.42	6.62	14.5	11.5	16.3
	2*Z*,4*E*,6*E*,8*E*	2.18	2.14	10.21	5.88	7.32	7.1	6.32	6.44	6.74	15	10	16.4
	2*E*,4*Z*,6*E*,8*E*[b]	2.41	2.17						6.38				
	2*E*,4*Z*,6*E*,8*E*[c]			9.99	6.13	5.64	6.40	6.58	6.31	6.65	11.5	11.8	16.5
	2*E*,4*E*,6*Z*,8*E*	2.30	2.15	10.10	5.97	6.37	7.23	6.19	6.90	6.77	15.2	11.5	16.5
VI	all-*E*	2.26	2.13	10.16	6.01	6.45	7.19	6.40		6.87	15.1	11.5	16.7
	2*Z*,4*E*,6*E*,8*E*	2.16	2.12	10.24	5.90	7.38	7.1	6.45		6.84	15.5	11.4	16.2
	2*E*,4*Z*,6*E*,8*E*[b]	2.37	2.07	10.09	5.99					6.8			
	2*E*,4*E*,6*Z*,8*E*[b]	2.26	2.14	10.10	5.93	6.38	7.3	6.25	7.66	6.83	16	11	16.2
VII	all-*E*	2.34	2.09	10.13	5.99	6.44	7.16	6.37	6.65	6.79	14.5	11.6	16.2
	2*Z*,4*E*,6*E*,8*E*	2.09	2.05	10.16				6.20	7.25	6.7			
	2*E*,4*Z*,6*E*,8*E*[b]	2.36	2.16	10.24									
	2*E*,4*E*,6*Z*,8*E*	2.36	2.10	10.12	6.01	6.38	7.35						16
			CH_3-2	CHO				H-1	H-3	H-4			
VIII	1*E*,3*E*[c,d]		1.96	9.64				6.28	6.76	6.74			16.1
	1*Z*,3*E*[c,d]		1.90	9.69				6.32	6.37	6.64			17
		CH_3-5	CH_3-8	H-1	H-2	H-3	H-4	H-6	H-7	H-9	$J_{2,3}$	$J_{3,4}$	$J_{6,7}$
IX[e]	all-*E*	2.14	2.09	9.63	6.15	7.72	6.49	6.56	6.86	6.78	15	12	16
	2*Z*,4*E*,6*E*,8*E*	2.18	2.18	10.24	5.79	7.65	6.78	6.60	6.83	6.79	12	10	16
	2*E*,4*Z*,6*E*,8*E*	2.12	2.07	9.63	6.05	7.87	6.25	6.74	7.10	6.72	15	12	16
	2*Z*,4*Z*,6*E*,8*E*	2.17	2.17	10.26	5.77	7.70	6.73	6.80	7.11	6.78	12	11	16

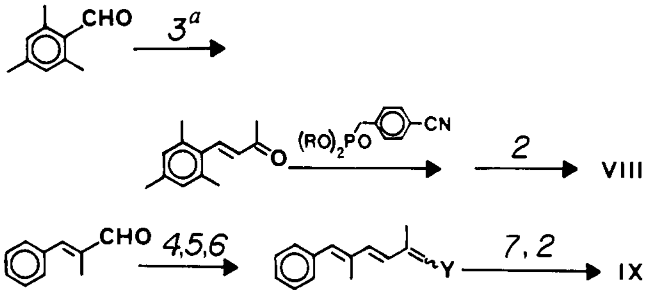
[a] All spectra recorded on a Varian XL-100 spectrometer with $CDCl_3$ as solvent unless specified otherwise. [b] With CCl_4 and 5% dioxane-d_8. [c] With C_6D_6 as solvent. [d] Recorded on a Bruker 270-MHz spectrometer. [e] With CD_3CN as solvent.

appropriately substituted benzylphosphoranes had to be abandoned because selective hydrogenation over Lindlar catalyst of the coupled product failed to give cleanly the desired 4*Z* isomer. The reaction was generally irreproducible, ranging from no reaction to complex mixtures. The route we subsequently followed was essentially that used for the synthesis of *o*-tolyl and mesityl analogues of vitamin A acid (Bharucha & Weedon, 1953). The synthetic mixture usually contained the all-trans and the 13-cis isomers. For compounds VIII and IX, the synthetic sequences shown in Scheme I were followed.



[a] Step 1, $(EtO)_2POCHC(CH_3)=CHCN$ (C_5 phosphonates); step 2, diisobutylaluminum hydride (DIBAH). For XI, Y = CN; for XII, Y = CHO.

Scheme I



[a] Step 2, DIBAH; step 3, acetone, base; step 4, $(EtO)_2POCHC(CH_3)=CHCO_2Et$ (C_5 phosphonate); step 5, $LiAlH_4$; step 6, MnO_2; step 7, $(EtO)_2POCHCN$ (C_2 phosphonate).

Photoisomerization and Structural Identification. The all-*E* isomer or, in some cases, a mixture of the all-*E* and 2*Z* isomers obtained from the synthetic reactions was dissolved in acetonitrile and irradiated with light ≥390 nm (Corning 0-51 filter) (Denny & Liu, 1977). In most cases the initial product mixture as indicated by high-pressure LC chromatographic analyses contained only three major new isomers in addition to the all-*E* isomer. The relative ratio of the three isomers changed significantly depending on the nature of the substituents on the phenyl ring. In one case the presence of a *Z*,*Z* isomer (see Materials and Methods) was also detected even during early stages of irradiation. All new isomers were isolated by preparative high-pressure LC. The structural assignments were made on the basis of their proton magnetic resonance spectra augmented by their UV-vis absorption spectra. The high-pressure LC retention time was also found as reliable supporting evidence for the stereochemical assignments: isomers of all compounds elute in the order of (similar to the parent retinal) 2*Z*, 4*Z*, 6*Z*, and all-*E* (Lichrosorb columns, ether-hexane solvent mixtures).

The key NMR data are listed in Table I. Discussions on the use of such data for stereochemical assignments were presented in some detail in several recent papers (Patel, 1969; Rowan et al., 1974; Asato et al., 1978). They will not be repeated here. Part of the UV data is listed in Table II. The absorption characteristics follow those of retinal isomers; e.g., the presence of a cis band in the 4*Z* isomer and the hypsochromic shift of the main band of the hindered isomer vs. other nonhindered isomers are similar to those of 11-*cis*-retinal (Sperling, 1973). We also noticed that the main band of the

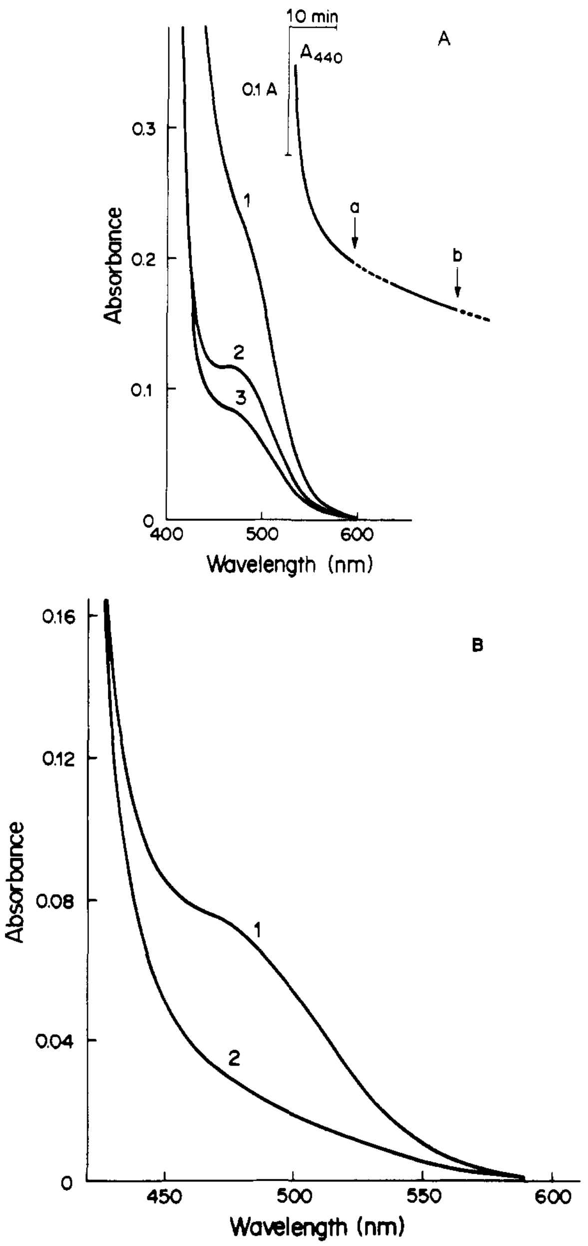


FIGURE 1: Determination of absorption spectrum of the mesityl analogue of rhodopsin, an unstable pigment analogue. (A) Curve 1 was recorded after incubating (2*E*,4*Z*,6*E*,8*E*)-V with opsin for 10 h. Curve 2 was recorded 12 min after addition of NH_2OH (final concentration of 50 mM). Curve 3 was recorded 40 min after addition of NH_2OH. The insert shows the decrease of absorption at 440 nm of the pigment mixture after addition of NH_2OH; curves 2 and 3 were recorded at points a and b (see text for details). (B) Curve 1 shows the absorption of the mixture recorded 45 min after addition of NH_2OH. Curve 2 shows the absorption of the same mixture after irradiation with yellow light.

nonhindered isomers of those compounds without the ortho substituents exhibits fine structure presumably associated with the planarity of the chromophore.

Interaction with Cattle Opsin. The procedure for opsin isolation from cattle retinas and subsequent interaction with retinal analogues was identical with those already described in the literautre (Matsumoto et al., 1978, 1979). Briefly, the formation of a visual pigment analogue was detected by the appearance of a long-wavelength absorption band upon mixing of the retinal analogue with an equivalent amount of opsin solubilized in a detergent such as digitonin. Since most of the pigments were only moderately stable in an excess of hydroxylamine, additional effort was necessary to distinguish between absorption of the pigment analogues and any random Schiff bases. Below we discuss the case of (4*Z*)-V in detail, a typical case of an aromatic retinal.

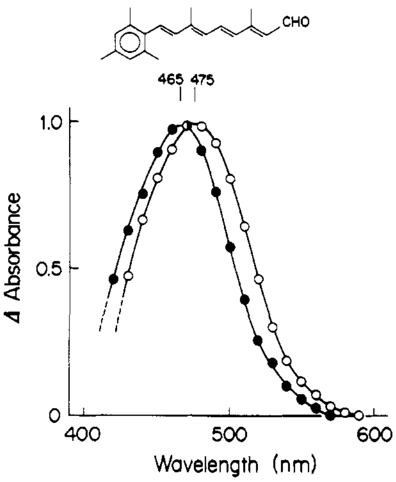


FIGURE 2: Difference absorption spectrum of the mesityl analogue of rhodopsin obtained from plotting the difference of spectra in Figure 1A or other equivalent spectra: (○) 4*Z* (11-cis); (●) 6*Z* (9-cis).

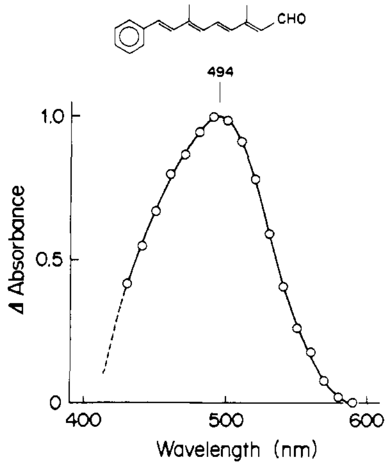


FIGURE 3: Difference absorption spectrum of the phenyl analogue of rhodopsin (from the 4*Z* isomer only).

Curve 1 in Figure 1 was obtained after incubation of (4Z)-V with opsin for 10 h at room temperature. The addition of 50 mM NH_2OH to the mixture caused a rapid decrease of pigment absorption. This process was monitored at 440 nm, as shown in thee insert in Figure 1A. Clearly, the rapid absorbance decrease within the first 5 min was accompanied by a slower rate of decrease. We interpreted the result to mean that the first part was due to removal of random Schiff bases and the latter to reaction of the unstable pigment analogue. Consequently, absorption curves 2 and 3, measured at arrows a and b, respectively, must not have much of the random Schiff bases left. (In a control experiment with the parent 11-*cis*-retinal it was shown that 10 min after the addition of NH_2OH, <5% of random Schiff bases still remained.) They clearly show that the pigment is unstable in excess NH_2OH. To be sure of complete removal of the random Schiff bases, we recorded the final spectrum 45 min after addition of NH_2OH on an expanded scale (curve 1 in Figure 1B). After irradiation with yellow light, absorption curve 2 was obtained. The absorption spectrum of the aromatic pigment analogue is therefore the difference between these two curves.

In this way, the difference spectra of all the aromatic pigment analogues were obtained. They are shown in Figures 2–4. In Table II are summarized some of the properties of

Table II: Absorption Properties of Isomers of Aromatic Retinal and Corresponding Rhodopsin Analogues

		retinal absorption (nm)		aromatic pigment analogues		
compd	isomer	hexane	EtOH	yes/no	yield (%)	λ_{max} (nm)
III	2*E*,4*E*,6*E*,8*E* (trans)	375	390	no		
	2*E*,4*E*,6*Z*,8*E* (9-cis)	370	383	no	*b*	
	2*E*,4*Z*,6*E*,8*E* (11-cis)	374	384	yes	~7	494
	2*Z*,4*E*,6*E*,8*E* (13-cis)	370	388	no		
IV	2*E*,4*E*,6*E*,8*E* (trans)	375	387	no		
	2*E*,4*E*,6*Z*,8*E* (9-cis)	372	380	yes	~10	482
	2*E*,4*Z*,6*E*,8*E* (11-cis)	368	379	yes	~10	496
	2*Z*,4*E*,6*E*,8*E* (13-cis)	373	381	no		
	2*Z*,4*E*,6*Z*,8*E* (9-cis,13-cis)	372	374	no		
V	2*E*,4*E*,6*E*,8*E* (trans)	368	380	no		
	2*E*,4*E*,6*Z*,8*E* (9-cis)	355	368	yes	15	470
	2*E*,4*Z*,6*E*,8*E* (11-cis)	364	375	yes	40	480
	2*Z*,4*E*,6*E*,8*E* (13-cis)	363	374	no		
VI	2*E*,4*E*,6*E*,8*E* (trans)	371	378	no		
	2*E*,4*E*,6*Z*,8*E* (9-cis)	366	370	yes	~6	470
	2*E*,4*Z*,6*E*,8*E* (11-cis)	368	373	yes	~8	460
	2*Z*,4*E*,6*E*,8*E* (13-cis)	365	371	no		
VII	2*E*,4*E*,6*E*,8*E* (trans)	393		*a*		
	2*E*,4*E*,6*Z*,8*E* (9-cis)	383		no		
VIII	1*E*,3*E* (trans)	350		no		
	1*Z*,3*E* (9-cis)	340		no		
IX	2*E*,4*E*,6*E*,8*E* (trans)	365		no		
	2*E*,4*Z*,6*E*,8*E* (11-cis)	357	368	no		
	2*Z*,4*Z*,6*E*,8*E* (11-cis,13-cis)	357		*a*		

[a] Not tested. [b] Possibly a pigment formed in <2% yield.

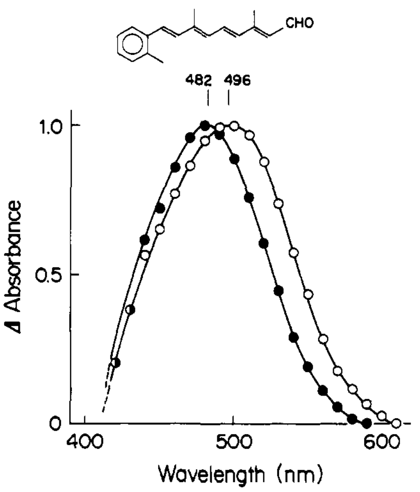


FIGURE 4: The difference absorption spectrum of the tolyl analogue of rhodopsin: (○) 4*Z* (11-cis); (●) 6*Z* (9-cis).

the retinals and the associated pigments.

In all cases the yields of the pigment analogues were far from quantitative; in fact, in several cases they were below 10%. We suspect the main contributing factor to the low yield is the slow rate of pigment regeneration which in some cases must be unfavorable in competition with opsin degradation under the conditions of incubation.[3] Nevertheless, it should be noted that our analytical method limits detection of pigment yields >2%. The negative results listed in Table II should therefore be read accordingly.

While we have not rigorously demonstrated the retention of the polyene geometry in the pigments, the different absorption properties of the isomeric pigments make the possiblity of prior isomerization of the retinals before pigment formation highly unlikely.

Discussion

Results in Table II clearly show that stable aromatic analogues of rhodopsin, within certain limitations, can be prepared. Compounds III–VI, which all contain relatively small substituents on the phenyl ring and unaltered side chain, show stereoselectivity similar to the parent retinal in their interaction with cattle opsin; namely, the all-*E* (all-trans) and the 2*Z* (13-cis) isomers fail to form stable pigments while the 4*Z* (11-cis) and the 6*Z* (9-cis) isomers do. The only major difference appears to be the relatively low yield of these aromatic pigment analogues, which is believed to be due to the slow rate of pigment formation with these modified retinals. Since the ring–CHO distances (Matsumoto & Yoshizawa, 1978) in these aromatic aldehydes are not significantly different from those in parent retinals, the slow rate is most likely due to unfavorable hydrophobic interaction. Another difference is the failure of (2*Z*,6*Z*)-IV to give a pigment analogue. Considering that 9-*cis*,11-*cis*-retinal reacts at a slower rate than 11-*cis*- and 9-*cis*-retinal with opsin (Crouch et al., 1975), it is perhaps not surprising to find that an aromatic analogue with the same geometry failed to give a detectable pigment with opsin.

Any further structural modifications other than the replacement of the trimethylcyclohexenyl ring by a phenyl group apparently further reduce the rate of pigment formation to an extent no longer competitive with protein degradation.[3] The negative results of compounds VII and VIII must be due to the steric bulk at the ring or at the side chain, making the compounds less compatible with the binding site of opsin. Alternatively, it is possible that unfavorable hydrophobic interaction in VII and incorrect side-chain "conformation" in VIII are further detrimental factors for pigment formation. The negative result of IX is somewhat unexpected. The introduction of the 8-methyl is intended as a strategic replacement of the ring methyls because the steric interaction of the added methyl with ortho hydrogens should lead to a similar amount of ring–chain distortion as in 11-*cis*-retinal or (4*Z*)-V, an apparently favorable conformation for interaction with opsin as suggested by the result of 5-demethylretinal (Kropf, 1976).

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[3] Added in Proof. We have since observed that some of the retinal analogues exhibit on inhibitory effect toward opsin in its interaction with 11-*cis*-retinal. The low yield could be due to effects other than protein degradation.

We suspect that the negative result is partially due to insufficient steric interaction near the end of the side chain, resulting in a planar conformation near the C-2,3,4,5 portion of the molecule. It is now well-known that the corresponding portion of the molecule in 11-*cis*-retinal is twisted (Honig & Karplus, 1971). Furthermore, the absence of 3-methyl could have further reduced the rate of pigment formation as suggested by the results of 13-demethylretinal (Nelson et al., 1970), making this aromatic retinal totally unreactive.

On the basis of the above results, one can rationalize the negative results of the bare-bone aromatic retinal analogue I: the ring modification, the unfavorable ring–chain and side-chain conformations, and the absence of 13-methyl are all detrimental factors for favorable interaction with opsin. Consequently the rate of interaction is probably too miniscule for it to compete with protein degradation.

At this stage only a tentative statement can be made as to the feasibility of using an appropriately modified aromatic retinal analogue for attenuation of the absorption properties of visual pigments, particularly into the longer wavelengths. The change to a phenyl end group which resulted in a lower pigment yield did not seem to have caused a significant change of the λ_{max}. On the other hand, steric restriction appears to rule out the addition of other substituents that are known to cause bathochromic shift in aromatic compounds. A possible alternative will be the use of other smaller aromatic systems.

Lastly, we note that all aromatic pigment analogues reported in this paper are photobleachable, including those without any benzylic hydrogens on the ring (III and VI). Hence any mechanism involving hydrogen abstraction as the primary photochemical reaction in the photobleaching process (van der Meer et al., 1976; Peters et al., 1977) of these systems can be safely ruled out.

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