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Fluorinated Phenylrhodopsin Analogs. Binding Selectivity, Restricted Rotation and ^{19}F-NMR Studies.

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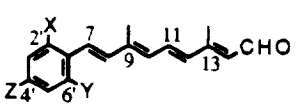
Abstract: Results from interactions of the 11-*cis* and 9-*cis* isomers of eleven fluorinated phenylretinal analogs, prepared from fluorinated benzaldehydes, with bovine opsin have been examined. Five of these (2',6'-bis-CF_3, 2',4',6'-tris-CF_3, 2'-CF_3-6'-F, 2'-CF_3-7-methyl and 2'-CF_3,6'-F,8-F) formed pigments in moderate to high yields, thus allowing recording of their ^{19}F-NMR spectra which revealed inhibited conformational equilibration when protein bound. Possible causes for binding selectivity and fluorine chemical shifts are discussed.

INTRODUCTION

Recently we reported the ^{19}F-NMR spectra of a series of rhodopsin analogs with the label located at the polyene side-chain of the retinyl chromophore.[1] Several cases of unusual fluorine opsin shift (FOS), defined as the difference between the chemical shifts of the pigment analog and the corresponding protonated Schiff base (PSB) were attributable to local protein perturbation.[2] Since such a reporting group is located one bond away from the carbon framework of the retinyl chromophore, structural information so derived should not duplicate those from the better established NMR methods employing isotopically labeled (^{13}C and ^{15}N) retinal analogs.[3]

We have now carried out a parallel study using ring fluorinated rhodopsin analogs. Possible preparation of substituted phenylretinal rhodopsin analogs was demonstrated earlier with several alkylated phenylretinals. Eleven fluorine-labeled phenylretinals (compounds **1**-**11**) have been prepared and used for examination of



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3: X = Y = F, Z = H
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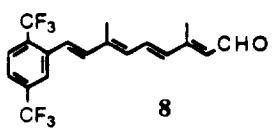

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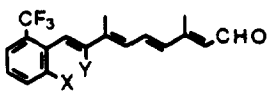
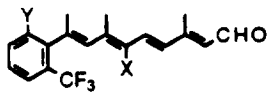


5: X = CF_3, Y = F
6: X = F, Y = H
7: X = F, Y = F



9: X = H, Y = H
10: X = F, Y = H
11: X = H, Y = F

binding selectivity of the hydrophobic pocket of bovine opsin and for studies of ^{19}F-NMR characteristics of the corresponding pigment analogs.

RESULTS

Pigment formation. Varying amounts of pigment analogs were obtained from mixing the synthetic fluorophenylretinal isomers with bovine opsin in 2% CHAPS solution (Table 1). In general only analogs with two ortho substituents (analogs **1**, **2**, **6** and **7**) gave pigments in moderate to high yields. Interestingly, the low yields of the singly *o*-substituted analogs (**4** and **8**) could be reversed by adding the 7-methyl substituent (**9**-**11**). UV-Vis absorption maxima of all the pigment analogs were compared with the corresponding values for the protonated Schiff bases (PSB), the differences are the (UV) opsin shifts[4] (Table 1).

Room temperature photobleaching characteristics of the pigments from analogs **1**-**7** were similar to those of rhodopsin. The more extensively modified analog **9** exhibited a different trend: upon irradiation with >460 nm light, the 461 pigment peak shifted initially to 446 nm with an increase in intensity, followed by a slow thermal degradation to opsin and the free retinal.

F-NMR studies. The observed linewidths for these detergent solubilized pigment analogs (membrane proteins) are of the order of 0.3 - 1.0 ppm, causing a low signal to noise ratio, making the method somewhat insensitive. Also, the necessary longer total data acquisition time limits the current NMR study to those pigment analogs obtained in moderate to high yields. The F-NMR spectra of the pigment analogs from **1**, **2**, **6** and **9**, before and after photobleaching, are shown in Figures 1-4. The chemical shifts of the F-signals in each spectrum are tabulated in Table 2 (see below) along with those of the corresponding PSB's for comparison. Also listed are the fluorine opsin shift (FOS) data. It should be noted that the organic solvents used for the model PSB are more appropriate mimic than water for the hydrophobic nature of the binding cavity.

DISCUSSION

Selectivity of the binding site of opsin for phenylretinals. High pigment yields were obtained from analogs substituted at both ortho positions. This is consistent with the notion that the opsin binding site favors a twisted ring-chain conformation of the retinyl chromophore, as reflected in earlier studies with demethylated retinal chromophores[5] and simple phenyl or *o*-tolyl retinals.[3] *o*-Fluorine substituents alone (**3**), apparently are not

Table 1. ^{1}H-NMR[a] and UV-Vis[b] data of fluorinated phenylretinals, pigment analogs (yield), opsin shifts and calculated ring-chain $C_{5,6}$-$C_{7,8}$ dihedral angle of the energy minimized structure of PSB's.

Retinal	H-NMR								Absorption max, nm[b]				OS	Dihed.
	H-7	H-8	H-10	H-11	H12	H14	H-15	$J_{11,12}$	Ald	PSB	Rhod.(yd)[f]		cm^{-1}	angle
1, 11-*cis*	6.84	6.38	6.62	6.70	6.04	6.05	10.07	12.5	350	403	456	(58)	2,880	55.2
9-*cis*	6.91	6.91	6.26	7.14	6.36	5.98	10.10	15.2	352	404	442	(53)	2,130	53.5
9,11-*cis*	6.89	6.89	6.57	6.69	5.92	6.07	10.10	11.4	345	401	442	(9)	2,310	
2, 11-*cis*[c]	6.57	6.13	6.56	6.21	5.56	6.00	9.84	11.9	351	398	443	(63)	2,550	59.6
3, 11-*cis*[d]	6.74	7.25	6.88	6.81	6.18	5.96	10.12	11.4	368	430	454	(3)	1,230	32.3
9-*cis*[d]	6.74	7.75	6.27	7.30	6.38	6.00	10.13	15.1	369	425	435	(6)	540	33.0
4, 11-*cis*[g]	7.03	7.36	7.06	6.82	6.24	5.97	10.12	11.5	376	420	--	(0)	--	31.5
9-*cis*	7.08	7.41	6.33	7.27	6.42	6.03	10.14	15.5	376	421	--	(0)	--	30.6
5, 11-*cis*	6.15	-111.6[e]	7.08	6.63	6.16	6.03	10.08	12.0	344		--	(0)	--	61.6
6, 11-*cis*[g]	6.77	7.10	6.90	6.81	6.20	5.96	10.11	11.1	367	421	454	(51)	2,060	40.8
9-*cis*	6.77	7.57	6.29	7.27	6.39	6.00	10.12	15.1	369	416	451	(32)	1,870	39.1
7, 11-*cis*	6.05	-109.9[e]	7.15	6.64	6.16	6.05	10.09	12.3	346	394	437	(44)	2,650	53.0
8, 9-*cis*	7.07	7.36	6.33	7.28	6.43	6.01	10.14	15.3	370	419	450	(3)	1,640	30.1
9, 11-*cis*	--	6.17	6.36	6.45	5.84	5.94	10.05	12.1	362	426	454	(45)	1,450	58.2
9-*cis*	--	6.72	5.94	7.24	6.28	5.91	10.10	15.0	362	414	452	(60)	2,030	57.6
10, 9-*cis*	--	6.39	-120.2[e]	6.93	6.55	6.05	10.15	15.5	362	406	458	(63)	2,800	57.1
11, 9-*cis*[h]	--	6.78	5.93	7.17	6.25	5.92	10.12	15.1	343[i]		445	(21)		61.3

a. Chemical shift in δ, ppm, coupling constant in Hz; in $CDCl_3$. b. In ethanol (aldehyde & PSB).
c. C_6D_6. d. Provided by R.-L. Chen. e. F-shift. f. Pigment yield in CHAPS. g. In acetone-d_6.
h. From T. Hong. i. In 12 % ether-hexane, with ~50% of the 7-*cis* isomer.

sufficiently bulky to cause a substantial ring-chain twist necessary for pigment formation. On the other hand, two close lying fluorine atoms (such as *o*-CF_3 and 8F) as in **5** may introduce sufficient repulsive energy to give a dihedral angle too large for ready pigment formation.

For analogs containing only one *o*-CF_3 substituent, the introduction of a methyl group at the 7-position on the side chain (**9-11**) apparently successfully restores the preferred twisted ring-chain conformation, again giving pigment analogs in moderate to high yields. However, some differences still remain because for these analogs, the 9-*cis* isomer gives better pigment yields than the 11-*cis*. There is also a change in the photobleaching characteristics of such a modified pigment as noted above.

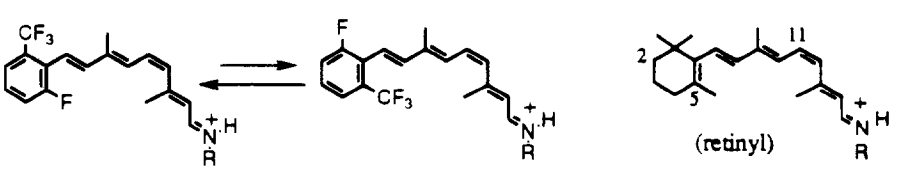
Results from molecular mechanics calculations (MMP2) of the energy minimized structure of these phenylretinal analogs are in agreement with the above analysis. The combined data of ring chain dihedral angles and pigment yields (Table 1) suggest that the opsin binding site prefers chromophores twisted -40 to -60° (bracketing the calculated -52.0 and -51.0° for the parent 11-*cis*-and 9-*cis*-retinyl chromophore).[6]

The absorption maxima of the fluorinated phenylrhodopsin analogs fall within the narrow range of 440-460 nm, considerably blue shifted from those of the parent rhodopsin (498 nm) and 9-*cis*-rhodopsin (483 nm), but not different from other phenylrhodop-sin analogs reported earlier.[7] The UV opsin shift values (1,900-2,900 cm^{-1} for the high yield pigments), remain similar to those of the natural occurring pigments (2,600 for rhodopsin and 2,400 for 9-*cis*-rhodopsin).[4]

Hindered rotation in the binding site of opsin. The most obvious feature in the ^{19}F-NMR spectra of the fluorinated pigments from analogs **1** & **2** (Figures 1 & 2) is the extra peak for the pigment relative to the respective PSB chromophore in solution. Thus, the equivalent 2',6'-bis-CF_3 signals of **1** in solution split into two singlets (one up field and one down field) for its pigment analog. And, the 2 : 1 ortho to para signals in **2** in solution change to three broad singlets of equal intensity when protein bound. These observations clearly demonstrate an increase for barrier of rotation of the phenyl ring due to interactions with nearby protein residues. It is consistent with the postulated twisted 6-S-*cis* conformation in rhodopsin, recently conclusively demonstrated through a photoaffinity labelling and molecular modeling study although the exact amount of twist is yet to be defined.[8]

The narrow temperature range (0 - 25°C) available to us (limited by solvent viscosity and protein stability) unfortunately precluded any dynamic NMR experiments for determination of the barrier for the restricted rotation process. But the data allow us to assign a lower limit of 15 kcal/mole for free energy of activation for ring-chain conformational equilibration within the protein cavity.

That only one set of F-signals was detected for both pigments from analogs **6** and **9** (Figures 3 & 4) is interesting. Preferential formation of one conformer in pigment **9** must be due to the directing property of the 7-methyl group to orient the CF_3 group to the 5-methyl position in the 6-S-cis retinyl chromophore.[3c] For the pigment from 11-*cis* **6**, two possible twisted chromophore exist (Table 1). But the one with the CF_3 group



occupying the more open 1,1-dimethyl position should be the dominant conformer in solution and in the protein pocket (see above).

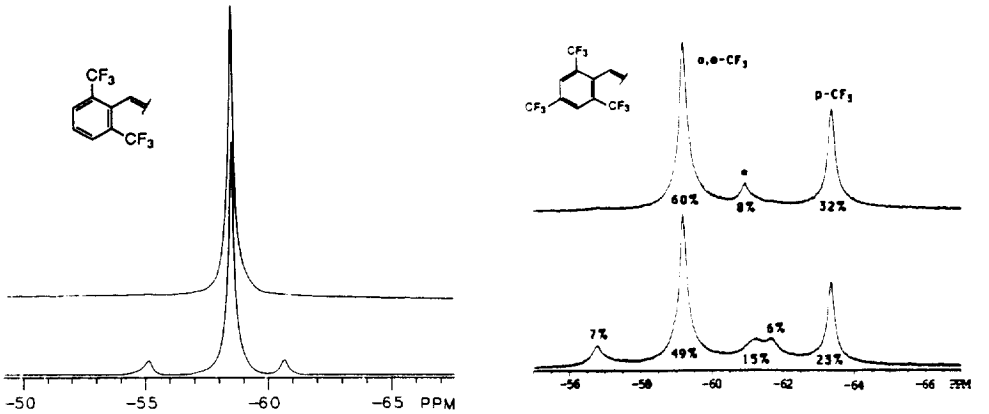


Figure 1. (left) ^{19}F-NMR spectra (F-chemical shift in δ, -49 to -67 ppm) of the pigment analog derived from 11-*cis*-**1**. Lower: the pigment (-55.2 and -60.7 ppm) in the presence of excess retinal (-58.7 ppm) before photobleaching; upper: after photobleaching, 2 h of irradiation with >460 nm light.

Figure 2. (right) ^{19}F-NMR spectra (-57 to -67 ppm) of the pigment analog derived from 11-*cis*-**2**. Lower: the pigment (-56.8, -61.2. & -61.7 ppm) in the presence of excess retinal (-59.2 & -63.4 ppm) before photobleaching; upper: after photobleaching with >460 nm light. The peak marked with * (upper) is due to an unidentified impurity that was apparently not photo-sensitive, present originally (lower) as part of the broad peak (15%) along with one of the CF_3 signal of the pigment.

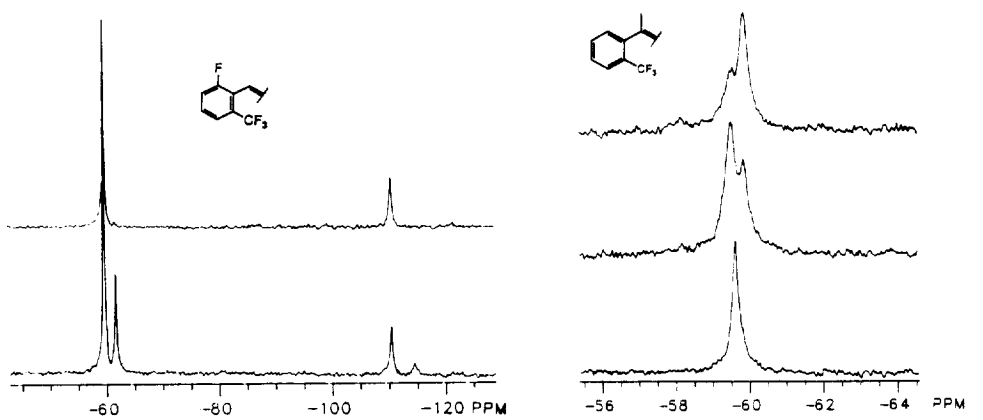


Figure 3. (left) ^{19}F-NMR spectra (-45 to -130 ppm) of the pigment analog derived from 11-*cis*-**6**. Lower: the pigment (-61.6 & -114.5 ppm) in the presence of excess retinal (-59.6 & -110.5 ppm) before photobleaching; upper: after photobleaching with >460 nm light.

Figure 4. (right) ^{19}F-NMR spectra (-55 to -64.5 ppm) of the pigment analog derived from 9-*cis*-**9**. The pigment (-59.6 ppm, lower), and after 2 h (middle) and 8 h (upper) photobleaching with >460 nm light.

Assignments of F-signals in pigment analogs. Fluorine labels on substrates imbedded in a protein cavity,[9] including most of the chain-labelled visual pigment analogs,[1] exhibit a substantial down field shift compared to the same substrate in solution. For some time, such deshielding was believed accountable only by a combination of local electronic perturbation effects and second order effects such as van der Waals (vdW) dispersion interactions.[9,10] But recent calculated results[11] on several systems using refined structural parameters suggest the contrary in that first order electronic perturbations are sufficient to account for the observed perturbed shifts (including the successful duplication[12] of the unusually large down field F-shift of 1-fluoro-8-t-butyl naphthalene, considered a strong case for vdW dispersion interactions)[13] obviating the need to postulate second order perturbations. Experimentally, it is clear that any unusual down field shift for the F-label of a protein bound substrate is a useful handle indicating the presence of nearby perturbing group(s).

The FOS values for the CF_3 ring-labeled rhodopsin analogs are small (3.6 to -1.9 ppm). And, their calculations are complicated by the fact that there is not a conformationally rigid PSB molecule suitable as model for the two magnetically non-equivalent *o*-signals of the protein sample (see above). The FOS values listed in Table 2 were obtained after comparing with the averaged (observed) solution value for the two *o*-substituents. One might logically ask whether the $\delta\Delta$ for the two *o*-signals in a protein sample (**1** & **2**) is due to their different protein environment or simply a reflection of the different internal environment for the two substituents in a frozen chromophore.

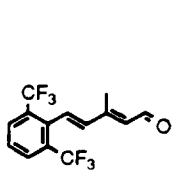
Table 2. F-chemical shift[a] data of retinals, PSB, pigments, opsin shift values and assignments.

Analog	-CHO	PSB	Rhod	FOS[b]	Assm[e]
1, 11-*cis*	-58.7	-58.8	-55.2	3.6	5-Me
			- 60.7	-1.9	1-Me
2, 11-*cis*	-59.0	-59.6	-56.8	2.8	5-Me
	-63.3		-61.2[c]	-1.6	1-Me
		-64.0	-61.7[c]	2.3	
6, 11-*cis*	-60.0	-60.2	-61.6	-1.4	1-Me
	-111.6	-112.4	-114.5	-2.1	5-Me
9, 9-*cis*	-59.9	(-59.9)[d]	-59.6	0.3	5-Me
10, 9-*cis*	-60.0	-59.2	-59.6	0.4	5-Me
	-120.2	-119.0	-116.2	2.8	

a. In δ, ppm, CHAPS. b. δ-Rhod - δ-PSB. c. May be reversed. d. That of retinal. e. See text.

To distinguish between these possibilities we have estimated by calculation the $\delta\Delta$ of the two *o*-CF_3 substituents of the energy minimized structure of the 2,6-bis-CF_3-phenyldienal analog **12**. Its geometry was first optimized; then, energy and chemical shift calculations (full *ab initio* using the DZ + P basis set) were carried out with Turbomole and TurboNMR programs from Biosym Technologies. The calculated shifts for the three F-atoms of each CF_3 group were averaged to give its value. The difference of the shifts ($\delta\Delta$) was found to be 4.99 ppm with the "top" CF_3 group, i.e. the one facing H-7, being more shielded. To test sensitivity of

δΔ on molecular geometry, we repeated the same calculation for a conformer of a smaller dihedral angle (57°) which is 0.13 kcal/mole higher in energy than that of the optimized (66°), giving a δΔ value of 6.31 ppm.



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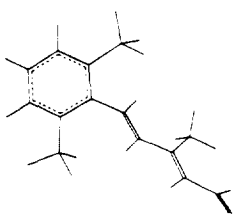

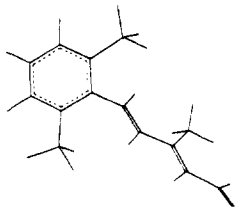

Figure 5. Model substituted phenyldienal **12** and its optimized structure in stereo-view (right).

These values suggest that the observed δΔ values in protein samples of **1** & **2** are largely accountable by local chromophore effects although protein effects near the two *o*-substituents are clearly not identical as suggested by the fact that the averaged FOS values for the *o*-substituents are not zero. For the unsymmetrical analog **6**, the direction of the FOS value and the magnitude of the F-shift suggest that the CF_3 group occupies the Me-1 position. Furthermore, the location of the *p*-CF_3 group (**2**) is such that it should not be sensitive to any chromophore conformational changes, yet it exhibits an FOS of 2.3 ppm. We suggest that this is due to its close contact with a protein residue(s). A recent photoaffinity labelling study with the reactive label located at the C-3 position, suggesting that the likely nearby amino acid unit is either Trp-265 or Leu-266.[8]

The conclusion that internal conformational effects, rather than the protein pocket exerting a dominant influence on F-shift, is somewhat surprising especially in view of the fact that the two *o*-substituents are expected to face different protein environments (corresponding to the 1,1-dimethyl groups and the 5-methyl group of the native system).[6] We have therefore prepared the two conformationally less flexible analogs, **9** & **10**, in which the 7-methyl group forces the CF_3 group into the 5-methyl position in solution as well as in the protein. The small FOS values (~.4 ppm) agree that indeed the protein effect is minor. But, one should also be aware of the seemingly contradictory result that the non-equilibrating *o*-CF_3 groups of the hindered 2,4,6,2',4',6'-hexatrifluoromethyl-*cis*-stilbene differ only by ~.5 ppm.[14]

CONCLUSION

The series of ring-fluorinated rhodopsin analogs have yielded new information related to the binding pocket of the visual protein. A twisted (40-60°) chromophore is a necessary feature for pigment formation. The non-equivalent ortho substituents in the analogs clearly demonstrate inhibited conformational equilibration of the

imbedded chromophore of a substrate. The observed chemical shifts were found to be consistent with the postulated structural features of the binding cavity of opsin. We are hopeful that the method will become equally useful for obtaining similar information of other labeled proteins.

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EXPERIMENTAL

Materials. The fluorinated benzaldehydes or their precursors were obtained from Lancaster or Aldrich chemical companies. 2',6'-Bis-CF_3-benzaldehyde was prepared by esterification of the corresponding benzoic acid with diazomethane followed by DIBAH reduction and Swern oxidation. 2',4',6'-Tris-CF_3-benzaldehyde was prepared by lithiation and carbonylation of nonafluoromesitylene,[15] followed by the same sequence of esterification, reduction and oxidation. Procedures for chain extension to the retinal analogs were those reported.[16] Preparation of the 8-F analog of the 2'-F,6'-CF_3-derivative was modified by first reacting the benzaldehyde with the fluoro-C_2-phosphonate. Conversion to the corresponding ionone analog was achieved through methylenation of the ester with Cp_2TiMe_2 followed by acid hydrolysis.[17] For 2,4- and 2,5-bis-CF_3-benzoic acids, conversion to the corresponding benzaldehydes was achieved by the standard sequence of reduction[18] and partial oxidation with MnO_2.

The 9-*cis* and 11-*cis* isomers of **3**, **4** & **8** were isolated by preparative HPLC from irradiated mixtures of the synthetic products.[16] For others, the 11-cis isomer was prepared by way of the Still's procedure for *cis*-selective reaction of a modified C_5-phosphonate.[19] The vinyl signals (Table 1) in the ^{1}H-NMR spectra provided the necessary information for configurational assignments.

Methods. *PSB data.* Butyl Schiff bases (SB) of purified retinal isomers were prepared in the same manner as described before.[1] Protonation of the SB was carried out in ethanol with camphor sulfonic acid.

Pigment analogs. Procedures for extraction of bovine opsin and formation of the pigment analogs were those reported.[20] Absorption maxima of the pigments listed in Table 1 were obtained from the difference spectra between those before and after photobleaching (>460 nm, Corning 3-71-filter). Hydroxyl amine was not added because all the phenyl rhodopsin analogs were found to react in varying degrees with this reagent.

F-NMR spectra. Samples for F-NMR studies were prepared and concentrated in the following manner. A solution of the reconstituted pigment analog of ~0.1 mM, prepared from an excess of the fluorinated retinal in

ethanol (0-2 absorbance unit) and 6-8 ml of an aqueous (20% D_2O) opsin solution with 2% CHAPS, was transferred to an Omega cell ultrafiltration assembly, filtered under 40-45 psi of nitrogen to a final volume of ~2.5 ml, then transferred to a 10 mm NMR sample tube. Pigment concentrations ranged from 1-5 x 10^{-4} M.

F-NMR spectra were recorded on a NT-300 spectrometer at ambient temperature. Typical parameters for data acquisition were: pulse width, 30-40 μs; delay time 1-3 s; 16k data sets at spectrometer frequency of 283.063-283.070 MHz; total number of pulses, 400 to 10,000. In the case of analog **2**, parameters used were: 10 μs, 4 s and 470.548 MHz respectively. The sample was then irradiated outside the probe with >460 nm light (Corning 3-71 filter). Progress of the photobleaching process was monitored by UV-Vis absorption spectra. Upon completion, the F-NMR spectrum was again recorded.

Chromophore extraction experiments (modified from that of Pilkiewicz et. al.)[21] were carried out for confirmation of assignment of the F-resonance signals. An NMR sample was lyophilized for >12 h and extracted with dichloromethane with the aid of a homogenizer. The extract, dried with anhydrous sodium sulfate, was subjected to preparative hplc separation. The isomers were characterized by comparison with UV-Vis absorption spectra and hplc retention time of authentic compounds.

Calculation of energy minimized structures of phenylretinals and fluorine shifts. Molecular mechanics calculations (MMP2) were performed to obtain the minimized structures of isomers of the ethyl PSB of the fluorinated phenylretinals, **1-11**. The procedure was essentially the same as that described for retinal isomers.[6] In the present case the PCModel Version 4 program installed on a Sun Sparcstation computer was used. The $C_{11,12}$-$C_{13,14}$ dihedral angle is positive while the ring-chain angles ($C_{5,6}$-$C_{7,8}$) are all negative.

Energy minimization of the model compound was done using MOPAC on an IBM 6000-520 computer. Chemical shielding calculations were conducted with Turbomol and TurboNMR programs from Biosym Technologies of San Diego. For the optimized structure where ring chain dihedral angle is 66°, the averaged isotropic shieldings of 286.36 (279.30, 296.38 and 283.39) and 281.37 ppm (284.99, 294.75 and 264.38) for the "top" and "below" CF_3's gave a $\delta\Delta$ of 4.99 ppm. The corresponding values for a second conformer of a dihedral angle of 57° are 286.55 (280.81, 296.64, 282.21), 280.24 (286.76, 292.27, 261.70) and 6.13 ppm.

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