11,12-Difluororhodopsin and Related Odd-Numbered Fluororhodopsins. The Use of $J_{F,F}$ for Following a Cis−trans Isomerization Process

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Fluorine-19 NMR spectroscopy has become popular in studies of complex molecules mainly because of the natural abundance and the high sensitivity of the ^{19}F nucleus and its wide range of chemical shifts, extending beyond 300 ppm. The sensitivity of the ^{19}F nucleus to changes in the environment further enhanced its appeal as a label in complex biochemical systems.[1] However, possible use of the stereochemically sensitive vicinal F,F couplings[2] in structural studies have not been fully explored.

As part of an extended study of F-labeled retinals and retinal binding proteins,[3] the possible incorporation of vicinal fluorine probes into a retinal analogue (13,14-difluoro-13-demethylretinal)[4] was demonstrated earlier by following the Middleton procedure for vicinal difluorination:[5] reaction of ethyl acetoacetate (or a β-diketone) with diethylaminosulfur trifluoride (DAST) in an aprotic dipolar solvent.[4] In the process, we took advantage of the unusual but well-known stability of the cis isomers of vicinal difluoroalkenes,[6] as revealed in the allylic bromination in the scheme below, that allowed introduction of the cis-difluoro linkage into the polyene chain. Now, after employing a modified sequence of reactions (Scheme 1), we have completed a stereoselective synthesis of the important 11-*cis*-11,12-difluororetinal (**1**) and a related analogue 11-*cis*-11,12-F$_2$-13-demethylretinal (**2**). The synthesis of odd-numbered F retinals was reported recently.[7]

The cis isomer of 11,12-F$_2$-retinal is characterized by two sets of signals in its F NMR spectrum (CD$_2$Cl$_2$): d × d at −118.5 (F-11, with a small $J_{F,F}$ = 7.5 and $J_{H,F}$ = 25.6) and d at −138.3 ppm (F-12) while the trans isomer is characterized by d × d at −135.5 (F-11 with a large $J_{F,F}$ = 107.5 and $J_{H,F}$ = 31.9 Hz) and d at −149.0 ppm (F-12). Detailed F NMR data including that for other F retinals, their Schiff bases (SB), and protonated Schiff bases (PSB) are listed in Table 1.

To demonstrate the possible use of three-bond F,F coupling constants in following a cis to trans isomerization reaction, Figure 1 shows the F NMR spectrum of the configurationally unstable hydrobromide of the *n*-butyl Schiff base (PSB) of 11-*cis*-11,12-F$_2$-retinal. Immediately after addition of HBr to the Schiff base, the room-temperature spectrum shows a mixture of the anti (major) and the syn (minor) isomers (assignments based on $J_{15,16}$

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Scheme 1. (a) N-Bromosuccinimide. (b) C$_{15}$-phenyl sulfone + BuLi.[8] (c) MeN−OMe Li$^+$. (d) MeLi.[9] (e) Cyanophosphono-acetate, base. (f) Dibal-H.
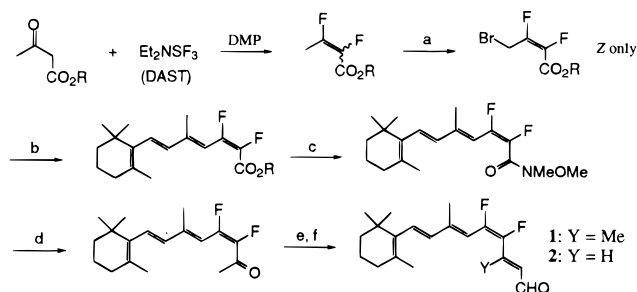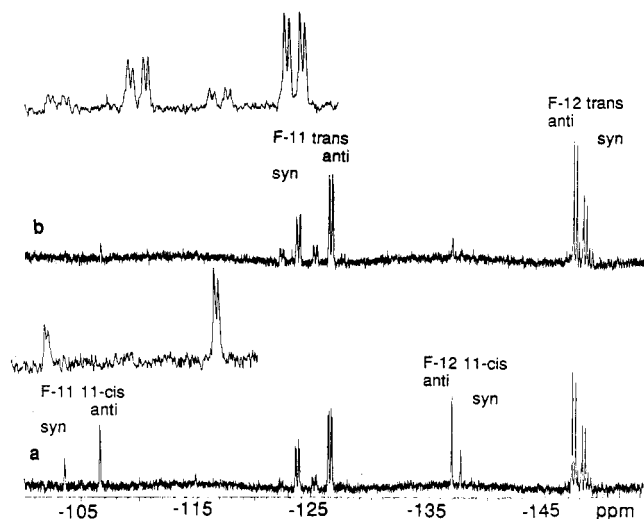

Table 1. F NMR^a and UV−vis Data for the 11,12-F$_2$-, 9-F-, 11-F-, and 13-F-retinal and their SB, PSB, and Bovine Rhodopsin Analogues

retinal		F shift, ppm					Rh, UV-vis λ_{max}^c
		CHO	SB	PSB	Rh	FOS^b	
11,12-F$_2$							
11-*cis*	11-F	−118.5	−129.0	−103.4	−101.6	1.8	503.5
		$J_{10,11}$ = 25.6	$J_{10,11}$ = 25.6	$J_{10,11}$ = 25.6			
	12-F	−138.3	−136.9	−136.1	−117.1	19	
		$J_{11,12}$ = 7.5	$J_{11,12}$ = 9.1	$J_{11,12}$ = 0			
all-*t*	11-F	−135.5	−143.8	−123.7	NA	NA	NA
		$J_{10,11}$ = 32.1	$J_{10,11}$ = 29.5	$J_{10,11}$ = 29.5			
	12-F	−149.0	−148.7	−147.5			
		$J_{11,12}$ = 107.5	$J_{11,12}$ = 108.8	$J_{11,12}$ = 103.9			
11,12F$_2$−13-deMe							
11-*cis*	11-F	−122.1	−130.2		no	NA	NA
		$J_{10,11}$ = 28.1	$J_{10,11}$ = 28.4		pigm.		
	12-F	−147.2	−146.0				
		$J_{11,12}$ = 8.1	$J_{11,12}$ = 7.7				
		$J_{12,13}$ = 27.4	$J_{12,13}$ = 26.4				
9-F							
9-*cis*	9-F	−110.8	−115.0	−103.3	−96.4	6.9	463
		$J_{8,9}$ = 27.6	$J_{8,9}$ = 28.6	$J_{8,9}$ = 27.1			
		$J_{9,10}$ = 18.7	$J_{9,10}$ = 19.3	$J_{9,10}$ = 18.1			
11-F							
9-*cis*	11-F	−95.9	−104.1	−87.1	−81.0	6.1	474
		$J_{10,11}$ = 31.0	$J_{10,11}$ = 29.6	$J_{10,11}$ = 32.2			
		$J_{11,12}$ = 36.9	$J_{11,12}$ = 37.6	$J_{11,12}$ = 35.7			
11-*cis*	11-F	−90.0	−97.6	−78.0	−75.9	2.1	488.5
		$J_{10,11}$ = 28.6	$J_{10,11}$ = 28.6	$J_{10,11}$ = 29.7			
		$J_{11,12}$ = 22.4	$J_{11,12}$ = 23.2	$J_{11,12}$ = 23.0			
all-*t*	11-F	−97.7	−106.0	−88.0	NA	NA	NA
		$J_{10,11}$ = 29.9	$J_{10,11}$ = 29.6	$J_{10,11}$ = 32.8			
		$J_{11,12}$ = 36.8	$J_{11,12}$ = 37.6	$J_{11,12}$ = 36.0			
13-F							
11-*cis*	13-F	−100.8	ND^d	NA			501.5
		$J_{12,13}$ = 33.9					
		$J_{13,14}$ = 32.4					

^{a} For CHO, SB (*n*-butyl), and PSB (trichloroacetic acid), in CD$_2$Cl$_2$, while for Rh, in 2% CHAPS. Coupling constants in Hz. ^{b} F shift (Rh) − F shift (PSB) in ppm. ^{c} In nm. ^{d} ND, not determined due to overwhelming intensity of −121 ppm peak.

values: 15.7 Hz for anti and 10.6 Hz for syn).[10] Upon standing, this was displaced by a new set of signals of all-*trans*-PSB of the same anti/syn ratio. The changes in peak structure reflect the increase of the F,F coupling constants from ∼0 Hz for the 11-cis isomers to 103.6 and 104.6 Hz (anti and syn) for the trans. The large change in coupling constant has been put to use in following the cis-to-trans photoisomerization of a visual pigment analogue where excessive line width is commonly encountered in NMR studies of detergent-solubilized solutions of proteins of this size.

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Figure 1. F NMR spectra (376.4 MHz) showing isomerization of 11-*cis*-*n*-butyl-11,12-F$_2$-retinal-PSB to the trans isomer (a) 8 min after addition of HBr to the Schiff base and (b) 20 min after addition. The minor peaks in the upper spectrum have not been assigned; however, we suspect they are due to the 9-cis isomers. The inserts show expansions of the F-11 signals for the 11-cis isomer (lower) and the trans isomer (upper), clearly revealing the large change in $J_{F,F}$.
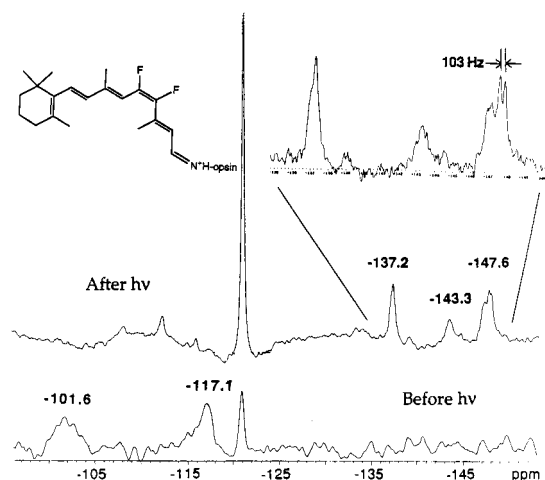

Figure 2. F NMR spectra (376.4 MHz) of 11,12-F$_2$-rhodopsin and photobleaching products. Lower: spectrum of a concentrated sample of pigment in a @Shigemi sample tube (0.5 mL, ~10^{-4} M) before photoirradiation (spectrometer parameters: 23,216 scans; LB, 200 Hz; pulse delay, 2 s). Upper: after photobleaching. Upfield signals are expanded (insert).

11,12-Difluoro bovine rhodopsin analogues, solubilized in 2% CHAPS, were prepared[11] in good yield (80%).[12] For comparison, we have also prepared rhodopsin analogues from three other odd-numbered F retinal analogues (9F, 11F, and 13F). The absorption maxima are listed in Table 1. Concentrated NMR samples were also prepared in the same manner as described before.

The F NMR spectrum for 11,12-F$_2$-rhodopsin, shown in Figure 2a, consists of two broad peaks at −101.6 and −117.1 ppm and a sharp peak at −120.9 ppm. Assignment of these signals was made by comparison with related F rhodopsins. The F shifts for 12-F-rhodopsin and the corresponding PSB were reported earlier,[3b] giving a large FOS value[13] (F-pigment shift minus F-PSB shift,

thus a measure of the effect of the protein host on the F label of the substrate) of 13.2 ppm. We have now obtained the F-shift data for 11-F-rhodopsin and the PSB giving a small[13] FOS of 2.1 ppm (Table 1). FOS values for the −117.1 and −101.6 ppm peaks (19 and 1.8, ppm, respectively) clearly suggest that the two peaks are, respectively, due to F-12 and F-11 of the pigment.

Photoirradiation of this pigment (>520 nm) resulted in the disappearance of the two broad peaks, replaced by two broad singlets at −143.3 and −137.2 ppm and an unsymmetrical doublet ($J = 103$ Hz) near −147 ppm, and intensification of the −120.9 ppm peak. Recalling the long-accepted notion that the chromophore freed from the binding site exists as a mixture of all-*trans*-retinal and the corresponding random Schiff bases (from random linkage between the extruded retinal and amino units outside the binding pocket)[14] and by comparison of chemical shifts of authentic samples of all-*trans*-11,12-F$_2$-retinal and its butyl Schiff base (Table 1), we conclude that the three upfield peaks can be safely assigned to those of the retinal (F-11 at −137.2 and F-12 at −147.6 ppm) and the random Schiff base (F-11 at −143.3 and F-12 at −147 ppm). It is interesting to note that only the shifts of F-11 (and not F-12) are sufficiently different to allow measurements of aldehydes and random Schiff bases in the product mixture. The observation of a distinct doublet for F-12 in the photoproduct retinal[15] (despite the broad line width, $W_{1/2}$ = ~300 Hz, for the protein-containing sample) from, initially, a broad singlet has successfully demonstrated the use of three-bond F,F coupling constants for following the cis-to-trans photoisomerization process of the visual pigment analogue. The large three-bond $J_{F,F}$, in contrast to the smaller $J_{H,H}$ (<18 Hz) or $J_{H,F}$ (<40 Hz), is clearly needed to study of proteins of broad line width.

An unusually narrow signal near −121 ppm was observed in all samples in aqueous solution. The peak was apparently associated with the free chromophore because, in the initial rhodopsin sample, the peak was weak and intensified dramatically after photobleaching. In addition, a similar peak was present in the spectrum of an aqueous solution of all-*trans*-11,12-F$_2$-retinal solubilized with CHAPS. The sharpness of the line suggests that it is a free small species. Subsequently we demonstrated that the signal is due to fluoride ion because addition of NaF to these solutions increased the intensity of the −121 ppm peak.

That the chemical processes led to the formation of the fluoride ion is suggested by the observation that in previous studies of even-number-substituted F rhodopsins,[3b] the −121 ppm peak was not detected, whereas in the current study of the odd-numbered F rhodopsins, the ease of formation of the fluoride ion parallels proximity of the substituent to the electron-withdrawing end group (i.e., extensive loss of 13-F-retinal and minimal loss of 9-F-retinal). Hence, we suspect a process involving nucleophilic addition and fluoride elimination must have taken place, in competition with pigment formation. The generality of this reaction in studies with F-labeled retinoid binding proteins will be examined in a separate study. It will also be of interest to devise conditions for low-temperature F NMR studies so that the coupling constant values can be applied to examine structural details of the photobleaching intermediates.[16]

(11) Following published procedures for pigment formation and sample concentration.[3]

(12) It is interesting to note that while the extra 11-F substituent does not affect formation of the 11,12-F$_2$-pigment, the slight increase in steric interaction between 9-Me and F-11 is reflected in the shifts of λ_{max} of 11-*cis*-11-F-retinal (364.5 nm) and 11-*cis*-11,12-F$_2$-retinal (374.5 nm) from those of 11-*cis*-retinal (380) and 11-*cis*-12-F-retinal (382 nm, all in ethanol) and the failure of pigment formation for 11-*cis*-11,12-F$_2$-13-demethylretinal (relative to the positive binding result of 13-demethylretinal).

(13) The large FOS of F-12 was attributed to its proximity to the counterion.[3b] The small FOS for F-11 must, therefore, be due to the orientation of the 11-F substituent, directed away from the counterion toward a more open space of the binding pocket. This interpretation is consistent with the currently accepted model for rhodopsin, see, e.g., Shieh, T.; Han, M.; Sakmar, T. P.; Smith, S. O. *J. Mol. Biol.* **1997**, *269*, 373−384.

(14) See, e.g., (a) Wald, G.; Brown, P. K. *J. Gen. Physiol.* **1953**, *37*, 189−200. (b) Rao, V. R.; Cohen, G. B.; Oprian, D. D. *Nature* **1994**, *367*, 639−642.

(15) F-12 signals for the random Schiff bases appear as an unresolved broad band slightly downfield from the retinal doublet.

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