# $^{19}$F NMR Evidence for Restricted Rotation of the Retinyl Chromophore in Doubly Labeled Visual Pigment Analogs

Leticia U. Colmenares and Robert S. H. Liu*

*Department of Chemistry, University of Hawaii*
*2545 The Mall, Honolulu, Hawaii 96822*
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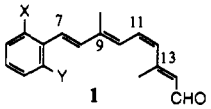
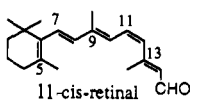
Ring/chain rotational equilibration of the retinyl chromophore in solution is known to be a rapid process.[1] However, a similar process in a protein-bound chromophore is suspected to be impeded, as evidenced from indirect observations such as CD activities of the protein-bound chromophore[2] and s-cis or s-trans preference of the chromophore for visual and bacteriorhodopsin (BR) pigments, respectively.[3] We now wish to report direct NMR evidence for the hindered rotation.

We have prepared the 11-cis isomer of three phenylretinal analogs with fluorine labels located at both ortho positions (**1a–c**).[4]




**1**

a : X = Y = $CF_3$
b : X = $CF_3$; Y = F
c : X = Y = F




11-cis-retinal

All three form stable visual pigment analogs upon incubation with bovine opsin. Pigment yields (based on rhodopsin formation with a limiting amount of opsin), however, varied considerably: moderately high for $CF_3$,$CF_3$ (**1a**, 59%, $\lambda_{max}$ = 456 nm in CHAPS) and for F,$CF_3$ (**1b**, 51%, 461 nm) and low for F,F (**1c**, 3%, 454 nm), but the trend for the two analogs with symmetrically substituted phenyl rings is consistent with the notion that opsin prefers to interact with a twisted chromophore, as established in earlier studies with phenyl- and mesitylretinal analogs.[6] Furthermore, one may surmise that the substantial amount of pigment from the unsymmetrically substituted phenylretinal (**1b**) probably results from selective interaction of opsin with the less stable (twisted) conformer (left structure in the equilibrium shown) where the $CF_3$ group occupies the 5-methyl position of the parent retinal.


We have recorded $^{19}$F NMR spectra of pigments derived from **1a,b** (the yield of pigment **1c** was too low for NMR studies). The spectrum of the pigment with two $CF_3$ groups, formed in the presence of a large excess of the retinal, along with that recorded after photobleaching are shown in Figure 1. Pigment formation was revealed by the appearance of two new peaks of equal intensity on either side of the main band. That these peaks are associated with the pigment is reflected by their disappearance upon irradiation with orange light (>460 nm).

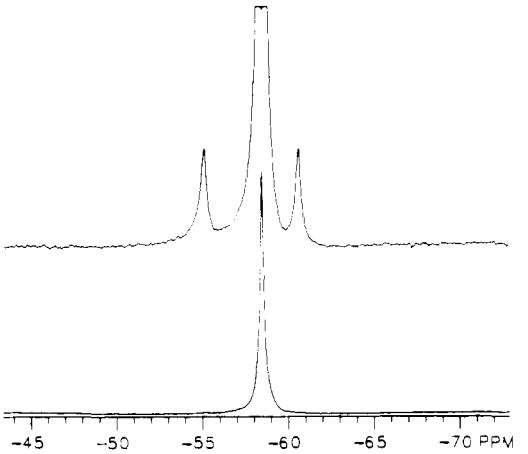



**Figure 1.** (upper) $^{19}$F NMR spectrum of the visual pigment (approximately $10^{-4}$ M in CHAPS) from the *o,o*-bis(trifluoromethyl)phenyl retinal analog **1a** in the presence of an excess of the fluororetinal (sample prepared according to the procedure outlined in ref 8) (upper spectrum). The chemical shifts for the three peaks are −55.2 (LW = 88 Hz), −58.5 (50 Hz, free excess fluororetinal), and −60.7 (72 Hz) ppm with $CF_3CCl_3$ as an external standard. Spectrometer (NT-300) parameters are NA = 400, D5 = 3.00 s, P2 = 28 μs, LB = 20 Hz, probe temp = 20 °C. Line width was determined by Lorentzian curve fitting. Spectrum of the same sample after photobleaching with orange light (>460 nm) (lower spectrum); NA = 800.

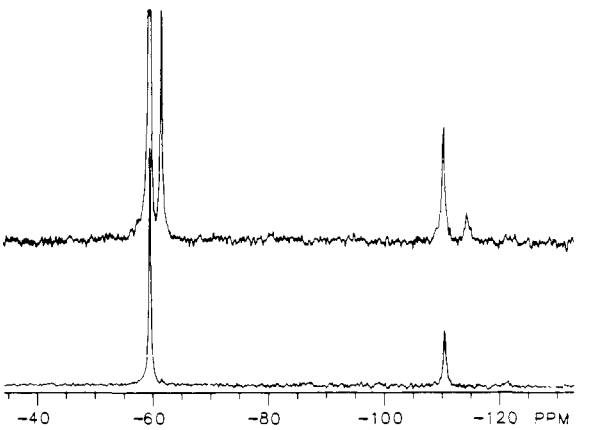



**Figure 2.** $^{19}$F NMR spectrum of the rhodopsin analog reconstituted from the 11-cis *o*-fluoro-*o*-(trifluoromethyl)phenyl retinal (**1b**) (approximately $10^{-4}$ M in CHAPS) in the presence of an excess of fluororetinal (upper spectrum) (the two pigment signals are centered at −61.6 and −114.5 ppm and the two free fluororetinal signals at −59.6 and −110.5 ppm). Spectrum of the same sample after photobleaching with orange light (>460 nm) (lower spectrum).

It is clear that only a single peak is present for a retinyl chromophore in solution.[7] When protein bound, two peaks appeared. Clearly, the ring/chain rotational equilibration is stalled, with the two $CF_3$ groups now facing different magnetic environments. In fact, one may further deduce that the upfield signal (negative fluorine opsin shift, FOS)[8] corresponds to the $CF_3$ occupying the same position as the $CH_3$-5 in the parent retinal, which from available evidence is known to occupy an open region of the binding site. For example, a series of pigment analogs with bulkier substituents at this site (e.g., 5-isopropyl and 5-butyl) has been prepared.[5] The signal of 8-fluororhodopsin, with the sub-

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(2) See, for example: Yoshizawa, T.; Shichida, Y. *Methods Enzymol.* **1982**, *81*, 634–641.

(3) (a) For visual pigment, see: Mollevanger, L. C. P. J.; Kentgens, A. P. M.; Pardoen, J. A.; Veeman, W. S.; Lugtenburg, J.; deGrip, W. J. *FEBS Lett.* **1987**, *163*, 9–14. (b) For BR, see: van der Steen, R.; Biesheuvel, P. L.; Mathies, R. A.; Lugtenburg, J. *J. Am. Chem. Soc.* **1986**, *108*, 6410–6411. (c) Nakanishi, K. *Pure Appl. Chem.* **1991**, *63*, 161–170.

(4) Prepared with either the corresponding benzoic acid or benzaldehyde as the starting material by following established chain extension reactions for retinoids, including stereoselective construction of the 11-cis double bond (see, for example, ref 5). Key $^1$H NMR data (δ for 10, 11, 12 and $J_{10,11}$ and $J_{11,12}$, in $CDCl_3$) of the 11-cis isomers: **1a**, 6.62, 6.70, 6.04 ppm, 12.2 and 12.5 Hz; **1b**, 6.90, 6.81, 6.20 ppm, 12.4 and 11.1 Hz; **1c**, 6.88, 6.81, 6.18 ppm, 12.4 and 11.4 Hz; in $CDCl_3$ for **1a** and acetone-$d_6$ for **1b,c**.

(5) Liu, R. S. H.; Asato, A. E. In *Chemistry and Biology of Synthetic Retinoids*; Dawson, M. I., Okamura, W. H., Eds.; CRC Press: Boca Raton, FL, 1990; p 51–75.

(6) (a) Kropf, A. *Nature (London)* **1976**, *264*, 92–94. (b) Matsumoto, H.; Asato, A. E.; Denny, M.; Baretz, B.; Yen, Y.-P.; Tong, D.; Liu, R. S. H. *Biochemistry* **1980**, *19*, 4589–4594. (c) Balogh-Nair, V.; Nakanishi, K. In *Chemistry and Biology of Synthetic Retinoids*; Dawson, M. I., Okamura, W. H., Eds.; CRC Press: Boca Raton, FL, 1990; p 147–176.

(7) The chemical shift of the fluorine labels on the aromatic ring is insensitive to the nature of the end group on the chain. Thus, the aldehyde, the Schiff base, and the protonated Schiff base of a given retinal analog have identical F shift values. In a separate experiment, we showed that pigment photobleaching was followed by partial regeneration, presumably due to reaction of opsin with the remaining 11-*cis*-fluororetinal.

(8) Colmenares, L. U.; Asato, A. E.; Denny, M.; Mead, D.; Zingoni, J. P.; Liu, R. S. H. *Biochem. Biophys. Res. Commun.* **1991**, *79*, 1337–1343.

0002-7863/92/1514-6933$03.00/0 

stituent facing the same open space as 5-methyl, also exhibited a high-field shift (FOS = 1.8 ppm rather than 4–8 ppm for pigments of normal hydrophobic shifts).[8,9] The low-field signal, therefore, corresponds to the $CF_3$ occupying the 1,1-dimethyl position of the parent retinal. The presence of a recognition site for the latter moiety in the opsin binding site is well known.[5] Close nonbonding interaction of the $CF_3$ group with those amino acid residues constituting the recognition site probably caused this increased protein (down field) shift.

It is interesting to note that the low-field signal exhibited a broader line width (88 vs 72 Hz) consistent with a more crowded environment. From 0 to 25 °C (limited by thermal stability of the sample and freezing point of the medium), the relative line width of the two peaks remained the same.

For the fluoro(trifluoromethyl)phenyl sample (**1b**), the pigment was characterized by the appearance of two peaks of a 3:1 ratio in intensity, both at a higher field than the corresponding signals in the free chromophore (Figure 2). The appearance of only one set of such signals indicates that the pigment is conformationally homogeneous. The high-field shift of the $CF_3$ group is consistent with the above interpretation that the $CF_3$ group occupies the more open 5-methyl position, and the similar shift of the F signal (rather than the downfield shift for the bis-$CF_3$) is consistent with its smaller size, no longer in close contact with the recognition site for the 1,1-dimethyl groups, and consequently reduced van der Waals interactions.

In summary, $^{19}F$ NMR spectroscopy has provided direct evidence for the suspected conformational restriction of the cyclohexenyl ring and the polyene chain of the retinyl chromophore in visual pigment analogs.[10] Studies of analogs with fluorine labels directed toward the remaining regions of the hydrophobic pocket are in progress.

**Acknowledgment.** The work was supported by a grant from the U. S. Department of Health and Human Services (DK-17806). (*o,o*-Difluorophenyl)retinonitrile was provided by R.-L. Chen.

(9) See, for example: Gerig, J. *Methods Enzymol.* **1989**, *77*, 3–22.
(10) A similar observation was made in a preliminary experiment with the *m,m*-bis(trifluoromethyl)phenyl retinal analog of BR: Mead, D. Ph.D. Thesis, University of Hawaii, Honolulu, HI, 1985.

## A Broad-Substrate Analogue Reaction System of the Molybdenum Oxotransferases

Stephen F. Gheller,[1a] Brian E. Schultz,[1b] Michael J. Scott, and R. H. Holm*

*Department of Chemistry, Harvard University*
*Cambridge, Massachusetts 02138*

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Previously, we have developed reaction system I, $MoO_2(L\text{-}NS_2) + X \rightleftarrows MoO(L\text{-}NS_2)(DMF) + XO$,[2-5] as a functional model for the molybdenum oxotransferases,[6] which catalyze the overall transformation $X + H_2O \rightleftarrows XO + 2H^+ + 2e^-$. Others have also

**ANALOGUE REACTION SYSTEM**

$Mo^{VI}O_2(t\text{-}BuL\text{-}NS)_2 + X \rightleftharpoons Mo^{IV}O(t\text{-}BuL\text{-}NS)_2 + XO$ (II)

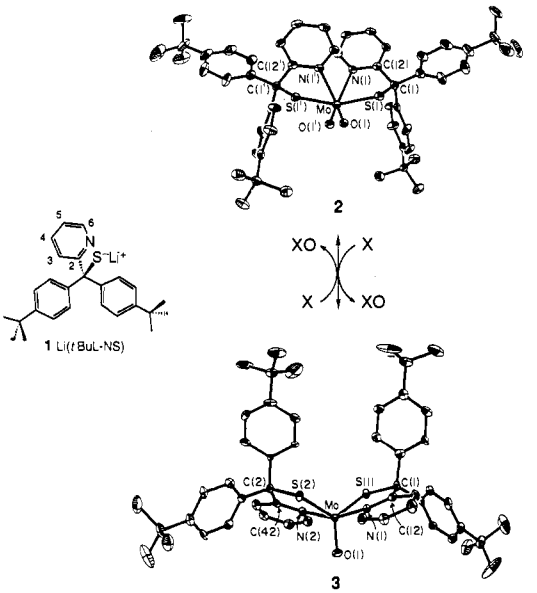


**Figure 1.** Schematic representation of analogue reaction system II of molybdenum oxotransferases, including the formula of Li(*t*-BuL-NS) (**1**) and the structures of $Mo^{VI}O_2(t\text{-}BuL\text{-}NS)_2$ (**2**) and $Mo^{IV}O(t\text{-}BuL\text{-}NS)_2$ (**3**). Substrates X/XO are given in the text. For **2**, primed and unprimed atoms are related by a 2-fold axis. Selected bond distances (Å) and angles (deg) are noted. Compound **2**: Mo–O(1), 1.696 (4); Mo–S(1), 2.418 (2); Mo–N(1), 2.411 (5); O(1)–Mo–O(1′), 107.7 (3); S(1)–Mo–S(1′), 159.8 (1); N(1)–Mo–N(1′), 76.2 (2); S(1)–Mo–N(1), 73.6 (2); O(1)–Mo–N(1), 89.8 (2). Compound **3**: Mo–O(1), 1.681 (5); Mo–S, 2.313 (3), 2.330 (3); Mo–N, 2.175 (7), 2.173 (7); S(1)–Mo–S(2), 124.3 (1); S(1)–Mo–O(1), 116.2 (3); S(2)–Mo–O(1), 119.5 (3); N–Mo–S, 80.3 (2)–92.1 (2); N(1)–Mo–N(2), 160.5 (3).

advanced pertinent model systems.[7,8] Under the oxo transfer hypothesis,[5] the substrate oxidation/reduction step involves atom transfer from/to the molybdenum center, as in $Mo^{VI}O_2 + X \rightleftarrows Mo^{IV}O + XO$. Other redox steps in the cycle restore the enzyme to its catalytic state.[5,6,9] While system I has most effectively demonstrated oxo transfer as a possible enzymatic pathway,[5] it does not fulfill several important criteria for optimal utility as an analogue reaction system. Its complexes are not stable in the presence of certain substrates, the structure of the reduced complex has not been established by X-ray diffraction, and rate constants and activation enthalpies for substrate reduction (nitrate, *N*-oxide, *S*-oxide) are sufficiently similar (ca. 1.5 (1) × $10^{-3}$ $s^{-1}$ (298 K), 23 (1) kcal/mol)[3,5] as to indicate a common early transition state without appreciable X–O bond weakening. Such a situation is uninformative with respect to the atom transfer event itself. Given the demonstration of the oxo transfer pathway for one enzyme (xanthine oxidase[10]) and its likelihood for others, we have sought an improved reaction system. As before, we utilize an anionic nitrogen–thiolate ligand to maintain an extent of consistency with Mo EXAFS results.[11]

(1) (a) Present address: Research School of Chemistry, Australian National University, Canberra, Australia. (b) National Science Foundation Predoctoral Fellow, 1989–1992.
(2) Abbreviations: acac, acetylacetonate(1−); *t*-Bu-LNS, bis(*p-tert*-butylphenyl)-2-pyridylmethanethiolate(1−); $Et_2$dtc, *N,N*-diethyldithiocarbamate(1−); L-$NS_2$, 2,6-bis(2,2-diphenyl-2-thioethyl)pyridinate(2−); X/XO, oxygen atom (oxo) acceptor/donor.
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