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On the mechanism of isomerization of all-*trans*-retinol esters to 11-*cis*-retinol in retinal pigment epithelial cells: 11-Fluoro-all-*trans*-retinol as substrate/inhibitor in the visual cycle

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Submitted in honor of Professor Koji Nakanishi on the occasion of his 80th birthday and his being awarded the Tetrahedron Prize.

Abstract—The synthesis of 11-fluoro-all-*trans*-retinol (11-F-tROL), which is shown to be an excellent substrate for processing by visual cycle enzymes, is described. It is isomerized to its 11-*cis* congener subsequent to its esterification by lecithin retinol acyl transferase (LRAT) approximately as well as is vitamin A itself. The enzymatic turnover of 11-F-tROL is unaccompanied by enzyme inhibition. The previously reported lack of isomerization of this substrate had been suggested as evidence for a carbonium mechanism in the critical enzymatic isomerization pathway in vision. The mechanism of this process remains unknown.

1. Introduction

The primary event in vision is the photochemical *cis* → *trans* isomerization of the 11-*cis*-retinal Schiff base chromophore of rhodopsin. This isomerization reaction initiates the phototransduction cascade, which results in the visual response.[1] Continued vision in vertebrates depends, of course, on the enzymatic reverse *trans* → *cis* isomerization to regenerate the 11-*cis*-retinal and thereby complete the visual cycle. A diverse group of retinoid binding proteins and enzymes, largely confined to the retinal pigment epithelium (RPE), are required for the operation of the overall visual cycle.[2,3]

The endergonic *trans*-to-*cis* retinoid isomerization reaction is of special interest. Despite the fact that the overall retinoid isomerization pathway is known and conditions for an in vitro system capable of carrying out 11-*cis*-retinoid biosynthesis were developed almost 20 years ago,[4] the enzyme component(s) responsible for the key 11-*trans* to *cis* conversion have not been identified nor has a complete molecular mechanism been established.

It is known, however, that this process requires a minimal three-component system in the RPE (Scheme 1) comprised of lecithin retinol acyl transferase (LRAT), mRPE65, a chaperone for all-*trans*-retinyl esters (tREs),[5,6] and the isomerohydrolase (IMH), which processes tREs into 11-*cis*-retinol.[7] The latter reaction can occur via a variety of mechanisms, which include S_N2' and carbonium ion mechanisms.[8] In either mechanism, the driving force for reducing the bond order at C11–C12 involves C–O bond cleavage, in the process of which it expels the palmitate moiety (see Scheme 2). The resulting formal positive charge could either be spread out over the odd numbered carbons along the retinoid (carbonium ion) or be neutralized by specific enzymatic nucleophilic addition.

It was postulated that if isomerization is active through an S_N2' mechanism then an isostere of retinol containing a fluorine atom at C11 instead of a hydrogen could allow for irreversible covalent attachment of the retinoid to the IMH (see Scheme 3), making it a potential suicide inhibitor of isomerization. This is an appealing prospect,

Abbreviations: tROL, all-*trans*-retinol; 11-F-tROL, 11-fluoro-all-*trans*-retinol; tREs, all-*trans*-retinyl esters; LRAT, lecithin retinol acyl transferase; IMH, isomerohydrolase; RPE, retinal pigment epithelium; CRALBP, cellular retinaldehyde binding protein; BSA, bovine serum albumin.

Keywords: Visual cycle; 11-Fluoro-retinol; Isomerohydrolase mechanism.

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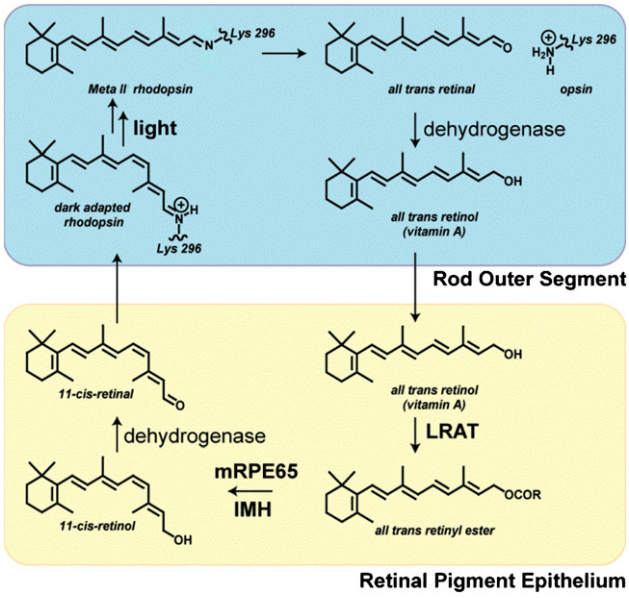


Scheme 1. Mammalian visual cycle.

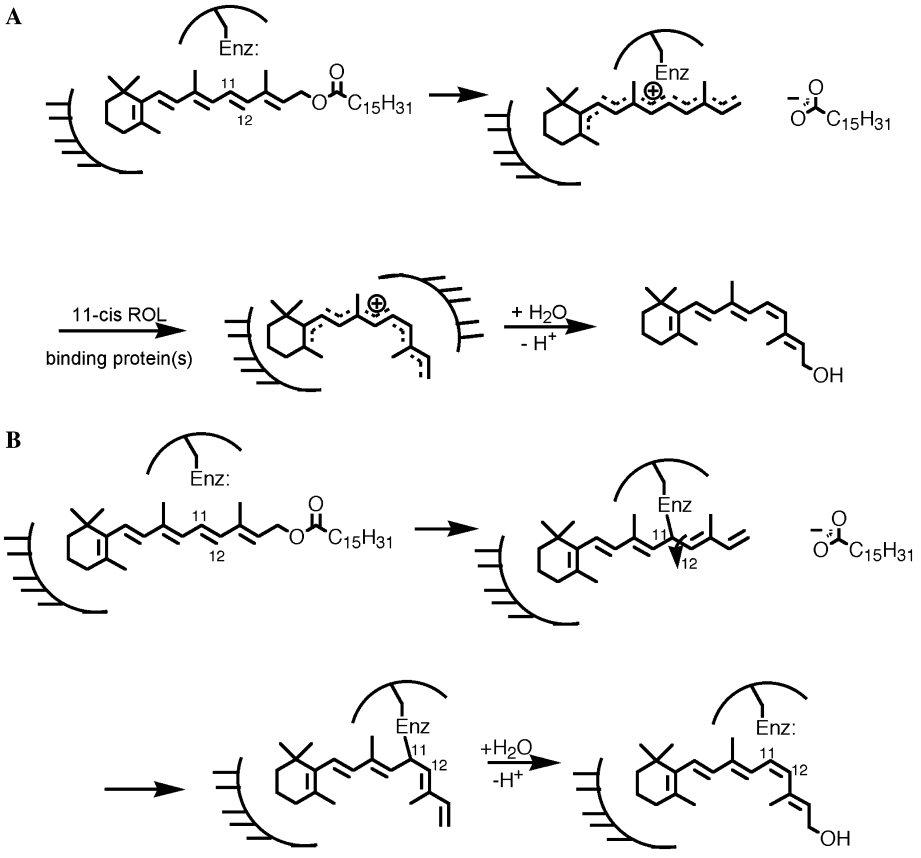
in that one retinol analogue might provide both mechanistic information on the IMH mode of action and also serve as the basis for a chemical affinity labeling agent for isolation of the IMH. In possible support of this notion is the reported observation that 11-F-all-*trans*-retinol (11-F-tROL), after conversion to 11-F-tRE, is *not* isomerized by RPE membranes.[9] The electronegativity of the 11-F was suggested to suppress carbonium ion formation, thus implicating it as a mechanism in the isomerization reaction. However, the more interesting possibility that 11-F-tRE might inactivate IMH was not suggested or experimentally tested. For this reason, we reinvestigated this problem.

11-F-tROL **1** (Scheme 4) was synthesized using a new and unambiguous route, and was tested for its ability to irreversibly inhibit IMH activity. Compound **1** was tested in vitro and was found to be a competitive inhibitor of 11-*cis*-retinol formation with vitamin A as the substrate, but, within experimental limits, did not irreversibly inactivate IMH. But contrary to a previous report,[9] we found that 11-F-tROL *is* an excellent substrate for the IMH, and conversion to 11-F-11-*cis*-ROL takes place with comparable kinetics to vitamin A (tROL).

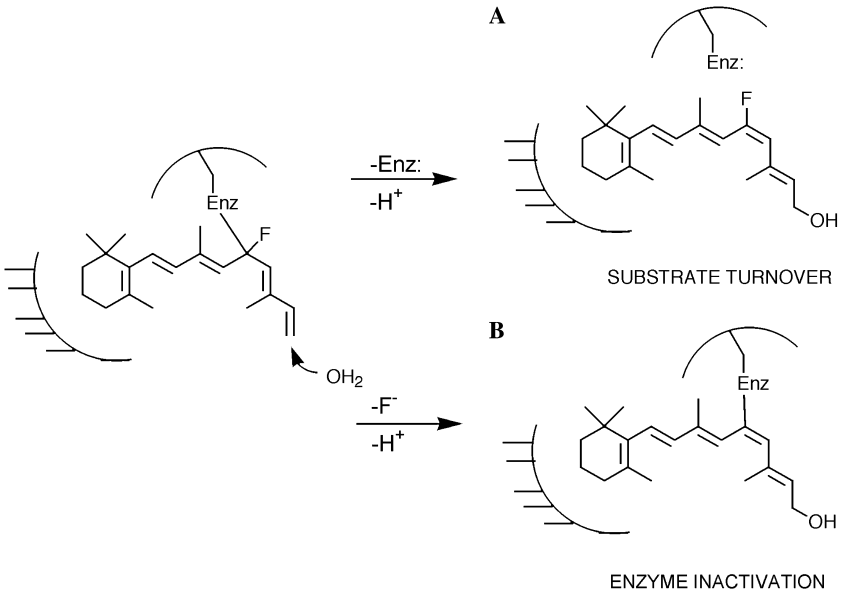
2. Results

2.1. Synthesis of (7*E*,9*E*,11*Z*,13*E*)-11-fluoro-retinol (all-*trans*)

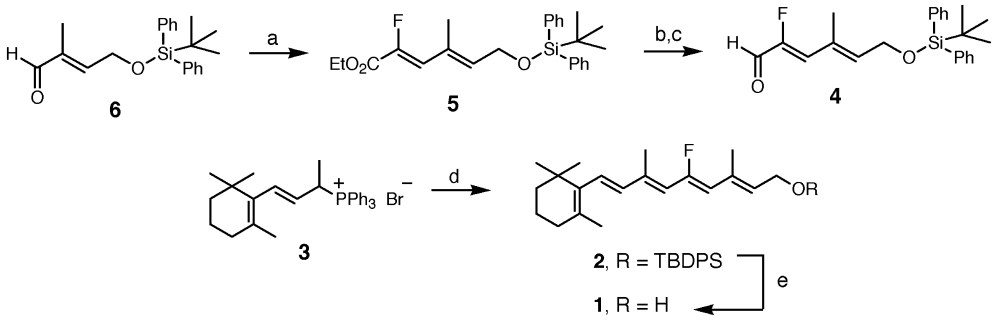
A synthesis of 11-F-11-*cis*-ROL was reported sometime ago in the literature;[10] however, a direct route to the 11*Z*



Scheme 2. Carbonium ion mechanism (A) as well as an S_N2' mechanism (B) are consistent with retinoid labeling experiments done on the isomerohydrolase.



Scheme 3. Potential S_N2' mechanism by which 11-F-ROL could either act as a substrate (A) or irreversibly inactivate the isomerohydrolase (B).



Scheme 4. Reagents and conditions: (a) (fluorocarbethyoxymethyl)triphenylphosphonium bromide, *n*BuLi, CH_2Cl_2, −78 °C, then 25 °C, 2 h, 72%. (b) DIBAL-H, THF, −78 °C, then 0 °C, 1 h. (c) $BaMnO_4$, CH_2Cl_2, 25 °C, 12 h, 80% two steps. (d) *n*BuLi, THF, −78 °C, then aldehyde **4**, then 25 °C, 3 h, 62%. (e) nBu_4NF, Et_2O, 25 °C, 30 min, 70%.

congener was never established. De Lera et al. report the conversion of 11-F-11-*cis*-ROL to the corresponding all-*trans* aldehyde using $BaMnO_4$; however, the oxidation/reduction procedure did not work reliably in our hands. A route was therefore employed that sets the 11*Z* stereochemistry much earlier in the synthesis. This route to compound **1** is outlined in Scheme 4. Known protected aldehyde **6**[11] was reacted with (fluorocarbethoxymethyl)triphenylphosphonium bromide[12] in a Wittig coupling. Diene **5** was carried through to 11-F-tROL **1** using essentially the same procedure reported for the 11*E* isomer.[10] The final product was obtained as a mixture of 9*E*/*Z* isomers and was purified by semi-preparative HPLC (YMC-PACK, PVA-SIL, 250 × 10 mm, flow 4 ml/min, hexanes/dioxane 90:10) with the desired 9*E*,11*Z* isomer (all-*trans*) eluting first. Due to its lability to oxygen, compound **1** was stored in aliquots at −80 °C in the presence of 10 mol% BHT. The UV spectra of compound **1**, as well as its 11*E* isomer, were measured in hexane and are shown normalized in Figure 1.

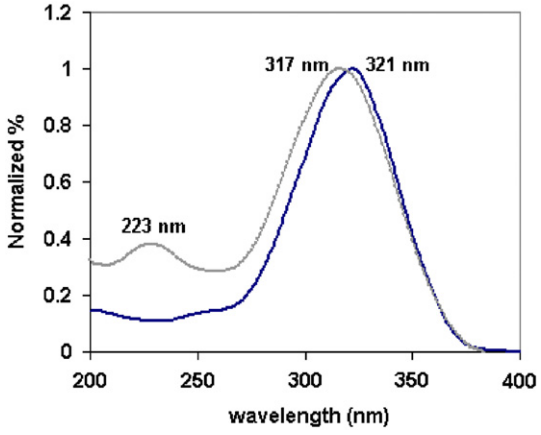


Figure 1. UV spectra of 11-F-tROL (black line) and 11-*cis*-11-F-ROL (gray line) measured in hexane. The shoulder at 223 nm is diagnostic of 11-*cis*-11-F-ROL as the corresponding shoulder peak of native 11-*cis*-ROL is red shifted to 233 nm.

2.2. 11-F-tROL does not inhibit IMH activity irreversibly

A 3 μM solution of either tROL or **1** was incubated with RPE membranes at time points between 0 and 90 min at room temperature. IMH activity was then measured at each time point with all-*trans*-[^{3}H]retinol (0.067 μM).

Membranes treated with 11-F-tROL were just as active as those incubated with tROL at each time point tested (data not shown). Had compound **1** been a potent irreversible inhibitor (through covalent attachment to IMH), it would be expected that tracer conversion to 11-*cis*-retinol would drop off significantly with time compared to the membranes incubated with native tROL. This was the case with 11-*cis*-retinyl bromoacetate (cRBA), a specific and potent irreversible inhibitor of isomerohydrolase activity.[13] Thus, 11-F-tROL is not an irreversible inactivator of IMH. We then sought to determine whether 11-F-tROL is a substrate for IMH, after conversion into the corresponding tRE.

2.3. 11-F-tROL is a substrate for IMH

When RPE membranes are incubated with **1**, several new enzymatic products are generated. One of the major compounds produced in this reaction is identified as 11-F-11-*cis*-ROL based on an identical HPLC retention time and UV spectrum to an authentic standard.[10] Figure 2 shows a control experiment in which compound **1** is incubated without membranes in pH 8.0 buffer for 45 min at 37 °C. As can be seen in panel A, no other 11-F retinol isomers are produced under these conditions. However, when RPE membranes are added under otherwise identical conditions, about 20% of compound **1** is converted to 11-F-11-*cis*-ROL (Fig. 2, panel B). Relatively small amounts of 11-F-13-*cis*-ROL as well as 11-F-11,13-*dicis*-ROL are also formed in the reaction. A control experiment in which the RPE membrane proteins were denatured (100 °C, 5 min) prior to carrying out the reaction showed no conversion to the 11-*cis* isomer. This demonstrates that isomerization of compound **1** is enzymatic and not simply a background thermal reaction. 11-F-retinyl esters (labeled in Fig. 2, panel B) are also produced from compound **1** at both 37 °C and room temperature in the presence of membranes, showing that **1** is a substrate for LRAT and, as expected, the isomerization also proceeds through a retinyl ester intermediate.

Figure 3 shows the percentage of retinyl esters 11-*cis* and 13-*cis*, and all-*trans* isomers produced after incubation with RPE membranes for 45 min IMH reaction using either compound **1** or tROL as the substrate. LRAT does not seem to distinguish between tROL and 11-F-tROL, and it appears that the 11-F isostere is as effective an overall substrate for LRAT/IMH as native tROL. This was further investigated by measuring compound **1** conversion to the 11-*cis* isomer as a function of time. The time course of the IMH activity is shown in Figure 4. The initial rate of conversion of compound **1** to 11-F-11-*cis*-ROL was about 80% of that for the IMH reaction with tROL. It also appears that saturation occurs at only about 75% the activity of tROL. This may be partly due to the fact that at longer reaction times beyond the linear part of the curve, a significant amount of 13-*cis* isomer is produced as a byproduct.

Along these lines, we tested the recombinant cellular retinaldehyde binding protein (CRALBP)[14] for its ability to stabilize and pull out of equilibrium the 11-F-11-*cis*-ROL formed in the isomerohydrolase reaction. Carrying

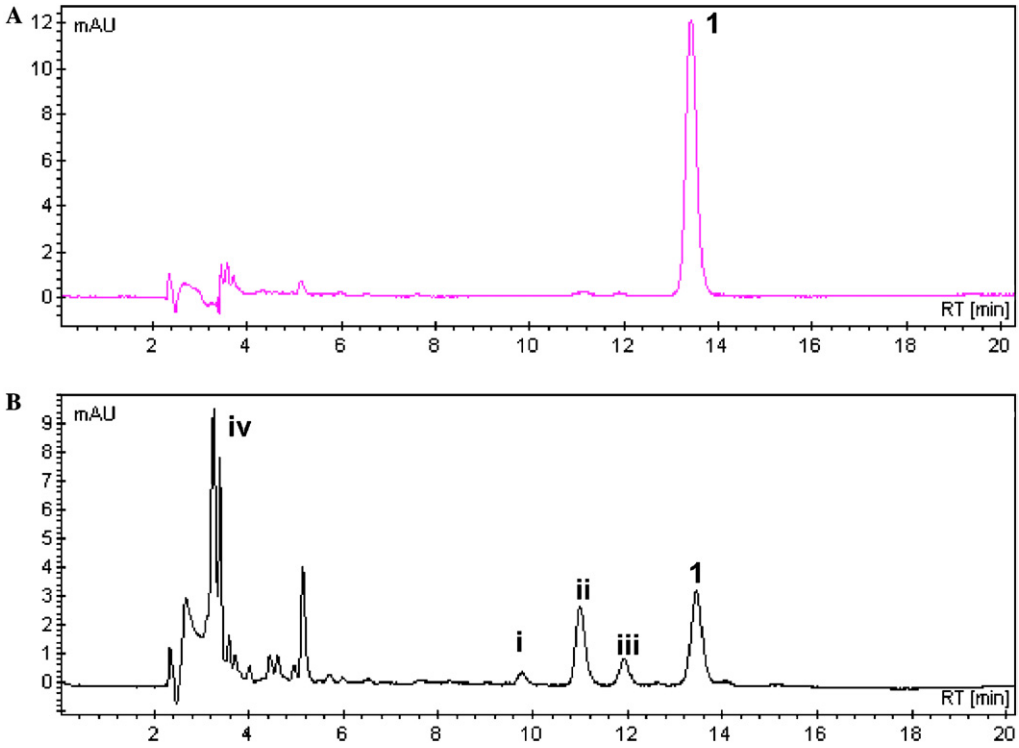


Figure 2. Normal phase HPLC profile of (A) heat control with 11-F-tROL **1** (pH 8.0 buffer, 37 °C, 45 min) without RPE membranes and (B) reaction of **1** with buffered membranes under the same conditions. (i) 11,13-*dicis*-11-F-ROL, (ii) 11-*cis*-11-F-ROL, (iii) 13-*cis*-11-F-ROL, and (iv) 11-F REs.

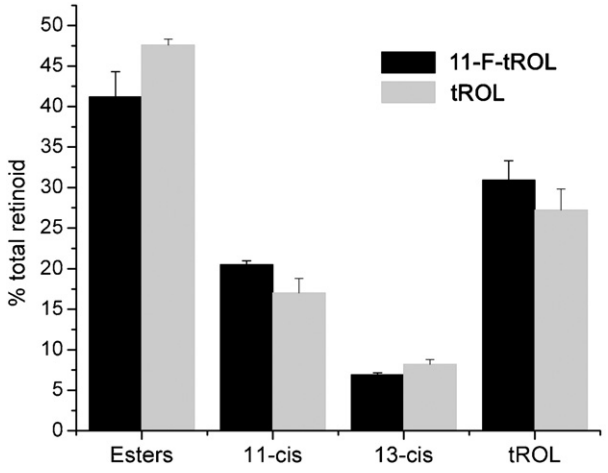


Figure 3. Retinoid product distribution for 11-F-tROL and tROL after a 45 min reaction with buffered membranes at 37 °C. Substrate concentrations in the reactions were 3 μM.

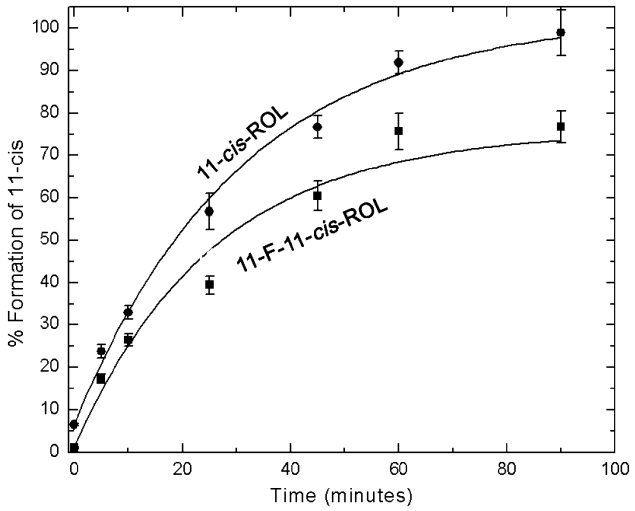


Figure 4. Time course for the isomerization of 11-F-tROL and native tROL to their corresponding 11-*cis* congeners. Activity is reported as percent of maximal 11-*cis* retinoid formed.

out the reaction of compound **1** with membranes in the presence of 60 μM CRALBP did have a significant effect on the ratio of 11-*cis*:13-*cis* isomer produced; ~3:1 in the case of bovine serum albumin (BSA) versus 8:1 using CRALBP. This result is consistent with the reported ability of 11-F-11-*cis*-ROL to bind to CRALBP with 80% the affinity of native 11-*cis*-ROL.[9]

3. Discussion

The previously reported negative result of compound **1** not being an IMH substrate was used to support the notion that isomerization takes place through a carbonium ion mechanism, as shown in Scheme 2. It was argued that an electron-withdrawing fluorine atom would destabilize a putative carbocation intermediate and therefore make the isomerization improbable. However, the present findings show quite clearly that 11-F-tROL is a substrate for the reaction. It should be noted that there was no characterization offered of the highly unstable 11-F-tROL in the previous study, so a direct comparison of the two studies cannot be made.[9]

The fact that 11-F-tROL is an excellent substrate for IMH after conversion to ester does not necessarily disprove the carbonium ion mechanism. For example, fluorine may compensate for its destabilization of a neighboring positive charge through its inductive effect with its ability to stabilize the charge through n–π back donation.[15] In addition, through resonance, any resulting positive charge on the retinoid polyene would likely be spread over all of the odd numbered carbons and not localized at C11. Indeed, some experimental justification for this comes from the UV spectra of 11-fluoro-retinal and its corresponding protonated Schiff base whose λ_{max} values are only slightly blueshifted (~10 nm) compared to the non-fluorinated compounds.[10] Thus, the question of the mechanism of IMH action remains undecided.

Under the conditions tested, **1** did not act as an irreversible inhibitor of IMH activity. This result does not, though, rule out the possibility of an S_N2' mechanism for isomerization. The substrate could simply be turning over many times before a fluoride ion gets released preferentially to the enzymatic nucleophile, making covalent attachment improbable.

In any event, the fact that compound **1** is shown here to be a tROL surrogate suggests that **1** could be a useful compound to further probe interactions between retinol and such RPE proteins as LRAT and RPE65 using ^{19}F NMR perturbation experiments.[16]

3.1. Experimental

3.1.1. Ethyl (2*Z*,4*E*)-6[(*tert*-Butyldiphenylsilyl)oxy]-2-fluoro-4-methylhexa-2,4-dienoate (5). (Fluorocarbethoxymethyl)triphenylphosphonium bromide (4.56 g, 0.01 mol) was dissolved in 100 ml dry CH_2Cl_2 and cooled to −78 °C. *n*BuLi (11.3 ml, 1.6 M in hexanes) was added dropwise over 20 min. After 30 min, aldehyde **6** (4.0 g, 0.0118 mol) dissolved in dry CH_2Cl_2 was added dropwise over 45 min, and the reaction mixture was allowed to warm to room temperature. After 2 h, water was added to the orange slurry, followed by 200 ml hexanes. The organic phase was washed with satd NaCl and dried with $MgSO_4$. The solvent was removed in vacuo and the resulting orange oil was purified by flash column chromatography (SiO_2, hexanes/EtOAc 10:1) to give 3.63 g of **5** (72% yield) as a colorless oil.

^1H NMR (200 MHz, $CDCl_3$): δ 1.08 (9H, s), 1.35 (3H, t, J = 7 Hz), 1.79 (3H, s), 4.34 (2H, q, J = 7 Hz), 4.41 (2H, q, J = 6 Hz), 6.02 (1H, dt, $^3J_{H-H}$ = 6 Hz, $^4J_{H-H}$ = 1.2 Hz), 6.52 (1H, d, $^3J_{H-F}$ = 39 Hz), 7.4–7.5 (6H, m), 7.7–7.8 (4H, m).

Compound **5** was carried through to the desired 11-F-retinol **1** using the same literature procedure[10] used for the corresponding *E* isomers. The ^1H NMR spectra are reported below for the new compounds.

3.1.2. (2*Z*,4*E*)-6[(*tert*-Butyldiphenylsilyl)oxy]-2-fluoro-4-methylhexa-2,4-dien-1-ol (4). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.06 (9H, s), 1.76 (3H, s), 4.09 (2H, d, $^3J_{H-F}$ = 13.2 Hz), 4.36 (2H, d, J = 6.3 Hz), 5.44 (1H, d, $^3J_{H-F}$ = 40.2 Hz, 5.73 (1H, t, J = 6.3 Hz), 7.4–7.5 (6H, m), 7.7–7.8 (4H, m).

3.1.3. (2*Z*,4*E*)-6[(*tert*-Butyldiphenylsilyl)oxy]-2-fluoro-4-methylhexa-2,4-dienal. ^{1}H NMR (200 MHz, CD_3COCD_3): δ 1.05 (9H, s), 1.78 (3H, s), 4.59 (2H, d, J = Hz), 6.11 (1H, dt, $^3J_{H-H}$ = 6 Hz, $^4J_{H-H}$ = 1.2 Hz), 6.98 (1H, d, $^3J_{H-F}$ = 25 Hz), 7.4–7.5 (6H, m), 7.6–7.8 (4H, m), 9.86 (1H, d, $^3J_{H-F}$ = 21 Hz).

3.1.4. (7*E*,9*E*,11*Z*,13*E*)-*tert*-Butyldiphenylsilyl 11-fluoro retinyl ether (2). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.02 (6H, s), 1.05 (9H, s), 1.4–1.68 (4H, m), 1.70 (3H, s) 1.78 (3H, s), 1.94–2.05 (2H, m), 2.1 (3H, s), 4.41 (2H, d, J = 6.4 Hz), 5.49 (1H, d, $^3J_{H-F}$ = 38.6 Hz), 5.79 (1H, t, J = 6.4 Hz), 5.87 (1H, d, $^3J_{H-F}$ = 30 Hz), 6.12 (1H, d, J = 16 Hz), 6.32 (1H, d, J = 16 Hz), 7.4–7.5 (6H, m), 7.7–7.8 (4H, m). HRMS calculated 542.3380, found 543.3382 EI-MS.

3.1.5. (7*E*,9*E*,11*Z*,13*E*)-11-Fluoro-all-*trans*-retinol (1). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.07 (6H, s, H16 and H17), 1.4–1.65 (4H, m, H2 and H3), 1.68 (3H, s, H18) 1.90 (3H, s, H20), 1.94–2.05 (2H, m, H4), 2.08 (3H, s, H19), 4.18 (2H, d, J = 6.2 Hz, H15), 5.46 (1H, d, $^3J_{H-F}$ = 39 Hz, H12), 5.70 (1H, t, J = 6.2 Hz, H14), 5.87 (1H, d, $^3J_{H-F}$ = 30 Hz, H10), 6.10 (1H, d, J = 16 Hz, H8), 6.28 (1H, d, J = 16 Hz, H7). Calculated for $C_{20}H_{29}FONa$ = 327.2095, ESI $[M + Na]^+$ = 327.2107. UV λ_{max} = 321 nm (in hexane), ε = 39,650 cm^{-1} M^{-1}.

3.1.6. IMH inhibition assay. Bovine RPE membranes were prepared from whole eye cups as described.[17] A buffered membrane suspension (100 mM Tris/HCl, pH 8.0 and 200 μg protein) was incubated with either **1** or native all-*trans*-retinol (3 μM) (DMSO vehicle) at room temperature. Aliquots were removed at 0, 5, 15, 25, 45, 60, and 90 min, and all-*trans*-retinol [11,12-$^3H_2$], (0.067 μM), DPPC (100 μM), and BSA (2% by wt) were added. Each reaction was allowed to proceed at 37 °C for 45 min and then quenched with MeOH. The retinoids were extracted in hexanes and were analyzed as previously reported.[18] The amount of radioactive 11-*cis*-retinol formed was used as a measure of isomerohydrolase activity.

3.1.7. Substrate conversion assay. IMH reactions were carried out on a 250 μl scale with 20 μg buffered RPE membranes (pH 8.0), 100 μM DPPC, 2% BSA or 60 μM CRALBP, and 3 μM **1** or native all-*trans*-retinol as the substrate. Reactions were quenched with MeOH at the indicated time points in Figure 4, extracted with hexanes, concentrated under a stream of argon, and then analyzed by HPLC. A UV profile of each eluted peak was collected by an online PDA detector (Varian Pro Star, Wakefield, RI). Isomers of native retinol were quantified using known molar absorptivity coefficients.[19] Compound **1** was quantified using λ_{max} = 321 nm (in hexane), ε = 39,650 cm^{-1} M^{-1} and the 11-*cis*-11-fluoro-retinol using λ_{max} = 317 nm (in hexane), ε = 28,900 cm^{-1} M^{-1}. All measurements were repeated in triplicate and the average values were used for analysis. Standard deviations are reflected in the error bars in Figures 3 and 4. All manipulations were carried out under dim red light.

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