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Stereospecificity of Retinol Saturase: Absolute Configuration, Synthesis, and Biological Evaluation of Dihydroretinoids

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Mouse RetSat catalyzes the saturation of the C13−C14 double bond of all-*trans*-retinol to produce all-*trans*-13,14-dihydroretinol **8**[1] (Figure 1). A related enzyme in zebrafish catalyzes the saturation of the C7−C8 double bond in addition to the C13−C14 double bond of all-*trans*-retinol to produce both **8** and all-*trans*-7,8-dihydroretinol.[2] Further oxidation of **8** and of all-*trans*-7,8-dihydroretinol by retinol dehydrogenases and then by retinaldehyde dehydrogenase enzymes leads to formation of all-*trans*-13,14-dihydroretinoic acid **9** and all-*trans*-7,8-dihydroretinoic acid, compounds whose levels are exquisitely controlled in vivo by the enzymes that catalyze their synthesis and breakdown.[2,3] Both **9** and all-*trans*-7,8-dihydroretinoic acid are highly selective agonists in activating the retinoic acid receptor (RAR) but not the retinoid X receptor (RXR).[3,4] Because 13,14-dihydroretinoids are chiral compounds it is important to determine their absolute configuration and evaluate how the different enantiomers interact with binding proteins, receptors, and enzymes. In this study we investigated the absolute configuration of biologically derived **8** and consequently of **9** and evaluated the activation of RAR by the enantiomers of **9**.

To establish the absolute configuration of **8** we examined the products of mouse and zebrafish RetSat by chiral HPLC. We also examined the endogenous form of compound **8** purified from livers of mice gavaged with all-*trans*-retinyl palmitate. For our analyses by chiral HPLC we established authentic standards by stereospecific syntheses of the two enantiomers of **8** (Scheme 1 and Supporting Information Scheme S-2). The synthetic scheme leading to the biological material (*R*)-all-*trans*-13,14-dihydroretinol (*R*)-**8** is depicted in Scheme 1. The chirality was transferred from that of the γ-alkoxy group in (4*S*)-4,5-(*O*-isopropylidene)pent-2-enoate enantiomer (4*S*)-**1**,[5] and Suzuki coupling was selected as the connective method to construct the polyene skeleton.[6] An analogous sequence produced (*S*)-all-trans-13,14-dihydroretinol (*S*)-**8** from (4*R*)-4,5-(*O*-isopropylidene)pent-2-enoate (4*R*)-**1**. Determination of the enantiomeric excess of the target compounds was based on HPLC separation by a Chiracel OD-H 0.46 cm × 15 cm column that afforded an enantiomeric excess (ee) value of >96% for each enantiomer of **8**.

We analyzed compound **8** purified from previously described human embryonic kidney cells (HEK) 293 cells that express mouse or zebrafish RetSat (HEK-mRetSat and HEK-zRetSat, respectively).[1,2] This analysis showed that both mouse and zebrafish RetSat produce the (*R*)-**8** enantiomer as it had chromatographic properties on chiral HPLC identical to synthetic (*R*)-**8** but distinct from (*S*)-**8** (Figure 1 and Figure S-1). Therefore, both mouse and zebrafish RetSat have the same stereospecificity at the C13−C14 double bond. We also examined the endogenous form of compound **8** purified from the livers of mice gavaged with all-*trans*-retinyl palmitate. Chiral HPLC analysis of compound **8** found in vivo indicates it is predominantly (*R*)-**8** (Figure 1). A smaller peak that co-migrates with (*S*)-**8** may represent a racemization product of (*R*)-**8** or a saturation product of all-*trans*-retinol that occurs by a pathway independent of RetSat (Figure 1, marked by asterisk). We employed a second chromatographic chiral separation method by

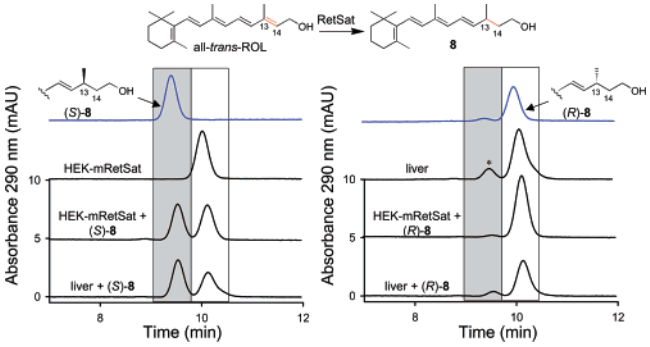
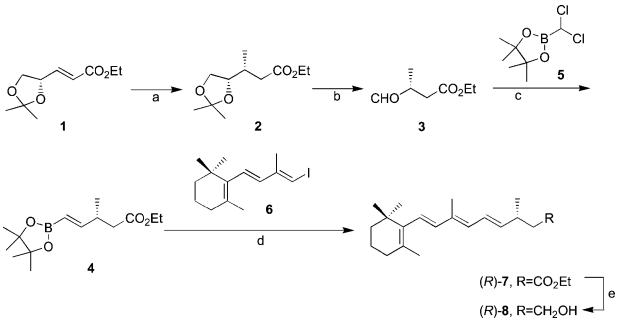


Figure 1. Chiral HPLC analysis of compound **8** product of mouse RetSat and of compound **8** purified from mouse liver in comparison with synthetic (*R*) and (*S*)-**8** standards. Compounds were analyzed separately or in combination as indicated. Blue line chromatograms depict the chiral HPLC analysis of the synthetic standards.

Scheme 1. Preparation of (*R*)-All-*trans*-13,14-dihydroretinol, (*R*)-**8**[a]



[a] Reagents and reaction conditions: (a) MeLi, THF, −78 °C. (b) H_5IO_6, 1:1 THF/Et_2O, 25 °C, 14 h, 68%. (c) $CrCl_2$, THF, 2-(dichloromethyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane **5**, LiI, 25 °C, 14 h, 68%. (d) Iodide **6**, $Pd(PPh_3)_4$, THF, 10% aq TlOH, 25 °C, 2 h, 55%. (e) DIBAL-H, THF, −78 °C, 2 h, 73%.

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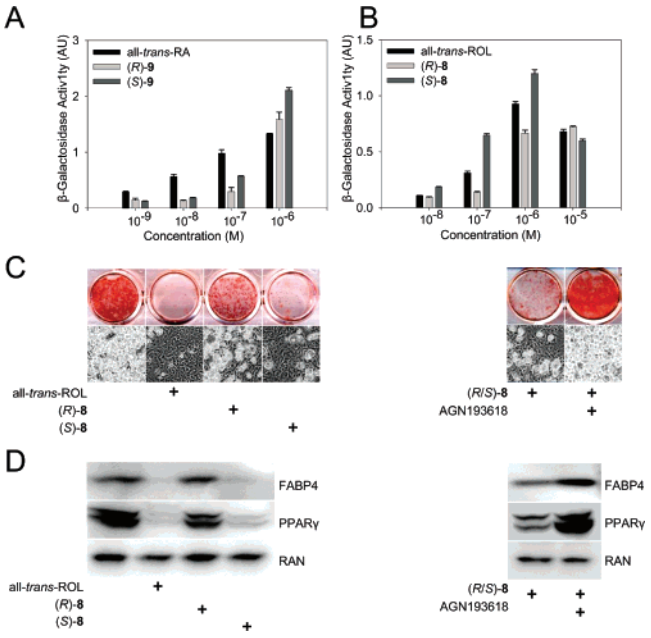

Figure 2. (A,B) Stereospecificity of RAR activation by (*R*)- and (*S*)-13,-14-dihydroretinoids. Activation of RAR by enantiomers of **8** or **9** was evaluated by assaying the level of β-galactosidase whose expression is controlled by a retinoic acid response element. (C,D) Effect of enantiomers of **8** (1 μM final concentration) on adipocyte differentiation (left panels). Inhibition of adipocyte differentiation by a racemic mixture of **8** (500 nM) was relieved by the RARα antagonist AGN193618 (2 μM) (right panels). Accumulation of neutral lipids was evaluated by Oil Red-O staining and differentiated cells were observed by phase contrast (C, bottom panels). Expression of adipocyte specific markers (FABP4 PPARγ) was evaluated by immunoblotting and compared to the loading control protein RAN (D).

derivatizing the synthetic compounds (*R*)-**8** and (*S*)-**8** and the compound **8** purified from HEK-mRetSat with (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) chloride to generate the MTPA esters. Following separation of the MTPA esters of **8** by normal phase HPLC we found that the MTPA ester of purified endogenous **8** co-migrates with the MTPA ester of (*R*)-**8** but not with the MTPA ester of (*S*)-**8** (Figure S-2).

Previous studies have shown that a racemic mixture of compound **9** can activate RAR but not RXR.[3] In this study we investigated the activation of RAR by the enantiomers of **9** with a cell-based RAR transactivation assay.[7] We found that (*S*)-**9** is more potent in activating RAR than (*R*)-**9**, yet both activate RAR less than all-*trans*-retinoic acid at physiological (nanomolar) concentrations (Figure 2A). Surprisingly, at supraphysiological concentrations (*S*)-**9** is a more potent activator of RAR than all-*trans*-retinoic acid. Similar results were obtained with enantiomers of **8** which are converted by cells to the corresponding acids. In these experiments, the RAR activation potency of compounds is (*S*)-**8** > all-*trans*-retinol > (*R*)-**8** (Figure 2B). Therefore, endogenous dihydroretinoids in the (*R*) configuration are the least active RAR agonists compared to the (*S*) enantiomers or all-*trans*-retinoic acid. All-*trans*-retinoic acid plays an important role in adipose differentiation acting through the RAR to prevent differentiation.[8] RetSat is an enzyme important in adipose differentiation (Schupp et al., unpublished) so we investigated the role of dihydroretinoids in adipose differentiation. We found that while all retinoids inhibit differentiation, (*R*)-**8** inhibits adipose differentiation much less than (*S*)-**8** or all-*trans*-retinol. This was evidenced by the accumulation of neutral lipids stained with Oil Red-O as well as by the expression of adipocytic markers FABP4 and PPARγ (Figure 2, parts C and D). The inhibition of adipogenesis by a racemic mixture of **8** can be relieved by the addition of AGN193618, a specific antagonist of RAR.[9] Therefore, **8** inhibits adipogenesis through activation of RAR. The human RARγ ligand binding pocket can discriminate between the active agonist BMS270394 and its inactive enantiomer BMS270395.[10] On the basis of the crystal structure of RARγ bound to each enantiomer, this selectivity was proposed to result from the unfavorable contacts between the ligand and the ligand binding pocket. In the case of the (*R*) and (*S*) enantiomers of **9** we believe a similar mechanism may occur where (*R*)-**9** interacts unfavorably with several residues, that may include those that interact with carboxylate (Figure S-5). Further crystallographic studies could shed light on the interactions of the (*R*) and (*S*) enantiomers of **9** with RAR.

We present data that establishes the stereospecificity of RetSat and the absolute configuration of its product as (*R*)-**8**. The (*R*) configuration renders dihydroretinoids weaker agonists of RAR than the (*S*) enantiomers. These studies should help clarify the physiological function of RetSat as its expression in adipose tissue would result in conversion of an inhibitor of adipose differentiation, all-*trans*-retinol, into a much weaker inhibitor of differentiation, that is, (*R*)-**8**. Our findings are also relevant to understanding the interaction of RAR with chiral agonists.

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Supporting Information Available: Experimental procedures, analysis of the stereospecificity of zebrafish RetSat, chiral separation of (*R*)- and (*S*)-**8** after derivatization with MTPA, preparation of (*R*)- and (*S*)-**8**, chromatographic separation and CD analysis of (*R*)- and (*S*)-**7**, CD analysis of (*R*)- and (*S*)-**8**, structural modeling of (*R*)- and (*S*)-**9** inside RARγ. This material is available free of charge via the Internet at http://pubs.acs.org.

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