CHAPTER NINE

Natural ligands of RXR receptors

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Abstract

Given the role of retinoid X receptors (RXRs) as promiscuous partners of heterodimeric complexes with other members of the Nuclear Receptor (NR) superfamily, RXR ligands (rexinoids) play fundamental roles in gene transcription, since upon ligand binding either transcriptionally activate the "permissive" subclass of heterodimers or synergize with partner ligands in the "non-permissive" subclass of heterodimers. The collection of natural products thus far reported to bind RXR are described, including those discovered by high-throughput screening (HTS), mere serendipity, and a combination of those. Detailed protocols for the diastereo- and enantioselective synthesis of (*R*)-9-*cis*-13,14-dihydroretinoic acid, a putative natural RXR ligand, are provided.

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Abbreviations

AF	activation function
ATRA	all-*trans*-retinoic acid
CADD	computer-aided drug design
CAR	constitutive androstane receptor
ChEMBL	European Molecular Biology Laboratory Chemical Database
CoA	co-activator
Co-R	co-repressor
DHA	docosahexaenoic acid
EBI	European Bioinformatics Institute
FXR	farnesoid X receptor
GPCR	G-protein coupled receptor
GGA	geranyl geranoic acid
HETE	hydroxyeicosatetraenoic acid
LBD	ligand binding domain
LBP	ligand binding pocket
LXR	liver X receptors
NOE	nuclear Overhauser effect
NR	nuclear receptor
PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
RAR	retinoic acid receptor
RXR	retinoid X receptor
RetSat	retinol saturase
SAR	structure–activity relationships
SMRT	silencing mediator for retinoid and thyroid hormone receptors
TR	thyroid hormone receptor
VDR	vitamin D receptor

1. Introduction. Structure of RXR receptors

Retinoid X receptors RXRα, β, and γ (NR2B1, NR2B2 and NR2B3) are members of the nuclear receptor (NR) superfamily of ligand-dependent transcriptional regulators that bind DNA response elements associated with target genes (Germain et al., 2006; Laudet & Gronemeyer, 2002). Similar to other NRs, RXRs feature a modular structure, which can be dissected in several domains with associated functions, most notably the N-terminal activation function (AF-1), the central DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD).

The LBD is a single protein domain of 11–13 helices and a short β-turn arranged in three layers to form the so-called anti-parallel alpha-helical

sandwich (Huang, Chandra, & Rastinejad, 2014). The multifunctional nature of this NR domain is shown not only by the control of ligand binding and receptor dimerization, but also by the interaction with the so-called co-regulators (co-repressors, CoRs, or co-activators, CoAs) through a ligand-dependent activation function (AF-2). Mechanistically, ligand binding induces the repositioning of the C-terminal helix H12 of the LBD due to alterations on the interaction surfaces with co-regulators (Huang et al., 2014; le Maire et al., 2012).

In addition, agonist ligand binding induces an exchange of CoR by CoA protein complexes and also of chromatin-remodeling enzymes. As a consequence, the condensed transcriptionally inactive state is transformed into the activated accessible state, leading ultimately to gene transcription. Other structural alterations on these complexes due to the action of ligands that act as antagonists, partial agonists and inverse agonists have also been revealed in the last few years (le Maire et al., 2012; Xu et al., 2001).

Being the common heterodimerization partner of multiple NRs, RXR occupies a central position in NR signaling (Mangelsdorf & Evans, 1995), since it can act as an active or as a silent transcription partner, and therefore the corresponding RXR-NR heterodimers are called permissive or non-permissive, respectively, if they are activated by the RXR ligand or by the partner's ligand (although synergistic effects are usually observed if both partners of the heterodimer are bound to their ligands), or only by the partner's ligand whereas ligand-bound RXR is silent or subordinated (Germain, Iyer, Zechel, & Gronemeyer, 2002).

2. Structural determinants for ligand binding to RXR receptors

As in other NRs, the CoR- or the CoA-bound states of RXR are exquisitively sensitive to ligand action (as agonists, antagonists or inverse agonists) and therefore, to their structure (Bourguet & Moras, 2015; Dominguez, Alvarez, & de Lera, 2017; Huang et al., 2014; Mendoza-Parra, Bourguet, de Lera, & Gronemeyer, 2015).

The crystal structures of RXR-ligand complexes (Bourguet & Moras, 2015; Huang et al., 2014) clearly show that the most selective RXR ligands feature a bent structure (see Fig. 1), which is enforced by a tetrahedral carbon, a 1,1-disubstituted olefin, or a twisted polyene side-chain for optimizing binding to the L-shaped RXR LBP. This sharp turn on the LBP volume is induced by the (highly conserved) conformation of a lysine residue in H11

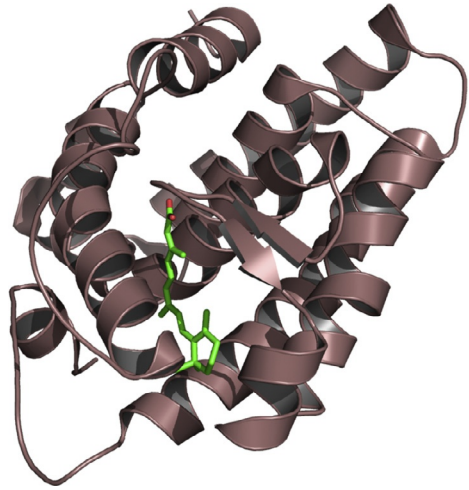


Fig. 1 RXRα–9-*cis*-retinoic acid (**31**, see Fig. 4) crystal structure (PDB code: 1k74) (Germain et al., 2009).

(residue L436 in human RXRα) (Huang et al., 2014). As a consequence, the shape of the RXR LBP is very restrictive to ligand binding, and selects those ligands that are either conformationally or configurationally twisted or sufficiently flexible to twist around single bonds. Such bending is not structurally attained by the elongated fully conjugated polyenes (although not for partially unsaturated analogs), such as all-*trans*-retinoic acid.

More than 500 RXR agonists and antagonists (with IC/IC_{50} lower than 50 μM) have been annotated in ChEMBL, the database dependent on the European Bioinformatics Institute (EBI). From the collection of RXR modulators, going from naturally occurring compounds, those discovered by high throughput screening or mere serendipity, only a few belong to the first group. The rationale for the structure-based design of the most important classes of RXR modulators has been described elsewhere (Bourguet & Moras, 2015; Dominguez et al., 2017; Hiebl, Ladurner, Latkolik, & Dirsch, 2018; Mendoza-Parra et al., 2015).

Natural ligands are compounds present in Nature, which bind their receptors with high potency (ideally, in the nanomolar range) usually leading to conformational changes in the complex structure, inducing thereby relevant physiological activities. We will discuss the biological relevance of the natural ligands reported for RXR. Needless to say, the relevant question of their presence at the right concentration on cells and tissues remains in most cases uncertain. Some of these compounds have been detected under

supraphysiological conditions or nutritional intervention and their relevance for the control of biological processes dependent on RXR remains uncertain.

3. Natural RXR ligands

3.1 Dietary compounds. Natural rexinoids (Fig. 2)

A collection of flexible unsaturated natural fatty acids acquired in the diet have been reported to bind to and activate RXR (Dominguez et al., 2017; Hiebl et al., 2018). The promiscuity of RXR on binding several metabolites indicates a putative role for this receptor as an intracellular sensor of the cell metabolic status, binding either the natural ligand or fatty acids, depending upon their supply and local concentration.

Within the series of flexible unsaturated fatty acids, both the endogenous and those acquired from the diet, that bind to and activate RXR, docosahexaenoic acid (DHA) **1** (Fig. 2) isolated from mouse brain, was the first reported activator of RXR in cell-based assays (de Urquiza et al., 2000). DHA **1** is a major constituent of nutrients and is considered as a conditionally essential fatty acid. DHA **1** is highly abundant (up to 30–50% of the total fatty acid content) in mammalian brain cells, where it is found associated to membrane phospholipids, and accumulates in the retina. It is required for brain maturation in rodents and humans, and its deficiency results in abnormalities similar to those in RXRα-deficient mice. Despite these findings, it has not been demonstrated that DHA **1** is required for RXR-RAR retinoid signaling or any other RXR-NR nonretinoid signaling pathway in which RXR may be involved (Mic, Molotkov, Benbrook, & Duester, 2003; Wietrzych-Schindler et al., 2011).

DHA **1** easily adapts to the LBP of RXR, and the crystal structure of RXRα bound to DHA **1** and a co-activator peptide in a homodimeric association (Egea, Mitschler, & Moras, 2002), indicates an occupancy of about 80% of the LBP volume (compared to 72% for 9-*cis*-retinoic acid **31**, see Fig. 1). All atoms of the ligand are in direct van der Waals contact with the protein and in particular those filling the hydrophobic cavity occupied by the β-ionone ring-binding subpocket of 9-*cis*-retinoic acid (**31**, see Fig. 4) bound to RXRα. Since the endogenous levels of DHA (which range from 0.1 to 0.01 μM in animal and human serum or tissue samples) are not sufficient for transcriptional activation, which requires at best a concentration range of 5–10 μM (Lengqvist et al., 2004), its role as physiological ligand

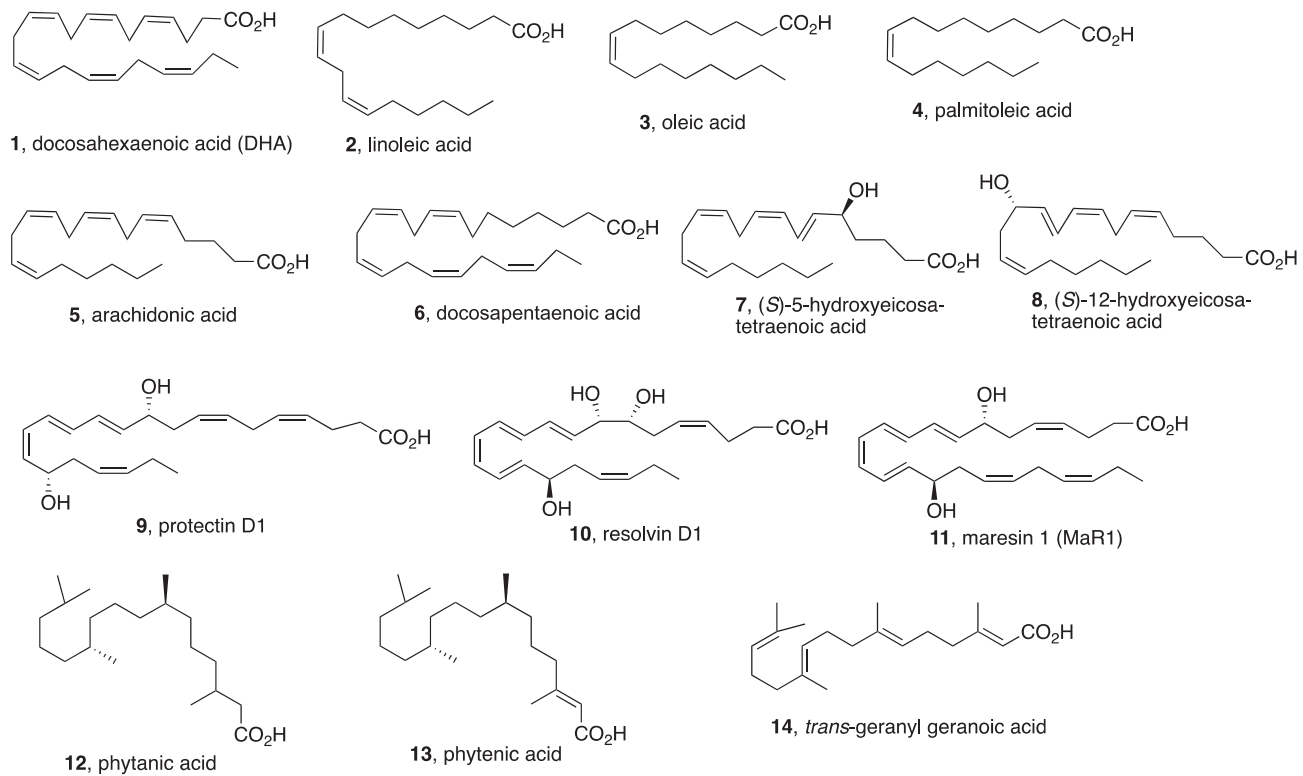


Fig. 2 Natural and dietary (unsaturated) fatty acids. Arbitrary conformations have been selected.

is questionable, despite the reported ability of vertebrates to synthesize DHA from α-linolenic acid as precursor of plant origin.

Additional fatty acids isolated from rat tissue total lipid extracts were also shown to activate RXR (Goldstein, Dobrzyn, Clagett-Dame, Pike, & DeLuca, 2003). Out of the large number of family members only the highly unsaturated fatty acids were able to bind the receptor with high affinity as shown by competition experiments with tritiated 9-*cis*-retinoic acid for binding to mRXRγ. Notably, linoleic acid **2**, which is one of the essential fatty acids, and also oleic acid **3**, palmitoleic acid **4**, arachidonic acid **5** and docosapentaenoic acid **6**, which have been isolated mainly from testis and heart, were found to activate mRXRγ at high ($EC_{50} > 200\,\mu M$) concentrations.(Lengqvist et al., 2004) Other eicosanoids, most notably hydroxyeicosatetraenoic acids such as (*S*)-5-HETE **7** and (*S*)-12-HETE **8** were reported to mimic some actions of the retinoids in cell-based assays (Eager, Brickell, Snell, & Wood, 1992).

It is not unusual to find unsaturated fatty acids bound to the LBP in the corresponding crystal structures of holo NRs (Huang et al., 2014), which are present due to the competing biosynthetic pathways of the bacterial expression host. This is the case of compound **3**, which was present in the crystal structure of the LBP of the ligand-bound RARα/F318A RXRα heterodimer complex. Oleic acid **3** conformationally adapts to the U-shape and resembles 9-*cis*-retinoic acid in the extended volume of the RXRα binding pocket due to the F318A mutation (Bourguet et al., 2000).

Several other natural products termed protectins, resolvins and maresins (**9**–**11**, Fig. 2), which are bis-, and tris-hydroxylated DHA metabolites (Serhan & Petasis, 2011), have also been found endogenously in vertebrates including humans (Mas, Croft, Zahra, Barden, & Mori, 2012), some of them even at higher levels than the parent DHA. They have been reported to display a large variety of biological activities through blocking or activation of various GPCRs, as well as anti-inflammatory and neuro-protective effects, similar to those regulated by RXRs (Bazan, 2018; Krishnamoorthy et al., 2010). Different mechanisms have been proposed to explain the numerous biological activities of these compounds, termed collectively Specialized Proresolving Mediators (SPMs) although binding and transactivation of RXRs has been rarely investigated (Krishnamoorthy et al., 2010; Serhan, 2014).

Phytanic acid **12** (Fig. 2), a metabolite of the phytol fragment of chlorophyll that can be obtained from dietary sources, was reported to bind RXR, but not RAR, and elicit transactivation, most likely through

RXR homodimers (Kitareewan et al., 1996; LeMotte, Keidek, & Apfel, 1996). It promoted transactivation of RXRα activities at 10 μM, and entered into competition with [^{3}H]-9-*cis*-retinoic acid for RXRα binding with a Ki of 4 μM, ca. 200-fold less efficient than 9-*cis*-retinoic acid (Zomer et al., 2000). RXR transactivation by phytanic acid **12** (at 4–10 μM) could take place under physiological levels in plasma (1–6 μM) but in mouse tissue 10–100 lower levels were measured. Therefore, physiological RXR-activation could be cell-type specific and depend on subcellular availability (including nucleus) of both this natural product and its putative metabolites. Since humans do not efficiently absorb chlorophyll, the metabolic precursor of phytanic acid, its presence in the human body is due to the digestion of meat and milk products on the diet, and therefore it cannot be considered as an endogenous ligand in humans. Thus, phytanic acid **12** should be considered as a RXR ligand candidate of nutritional origin, but its physiological role in RXR activation remains uncertain.

Its unsaturated precursor phytenic acid **13** (Fig. 2), which has been detected in bovine serum extracts, is likewise considered as RXR ligand since it was found to also displace tritiated 9-*cis*-retinoic acid **x** from the receptor in competition assays, whereas its *Z* isomer was inactive (Kitareewan et al., 1996; LeMotte et al., 1996).

Additional C20-fatty acids, like geranyl geranoic acid derivatives (GGA, **14**), which are present in diet relevant herbs have been reported as potent RXR activators (Araki, Shidoji, Yamada, Moriwaki, & Muto, 1995), although they could not be identified endogenously.

In summary, there is a strong link between DHA **1** and RXR-mediated signaling, although a clear picture of how it operates in vivo is still missing. Since the potency of these unsaturated compounds is not sufficient to make them qualify as true endogenous RXR ligands, they should instead be considered as partial RXR agonists (Bourguet et al., 2000). It appears that the promiscuity of RXR with several unsaturated metabolites reflects its fundamental role as intracellular sensor of the cell metabolic status, binding fatty acids and analogous compounds depending on their supply and their local concentrations. All together, these results underline the potential involvement of RXR in lipid homeostasis through complex feedback mechanisms in potent association with other NRs (such as PPAR or FXRs).

3.2 Rexinoids from plants (Fig. 3)

Natural product honokiol **15** (Fig. 3), isolated from *Magnolia obovata*, and positional isomer magnolol **16**, from *Magnolia officinalis*, used in traditional

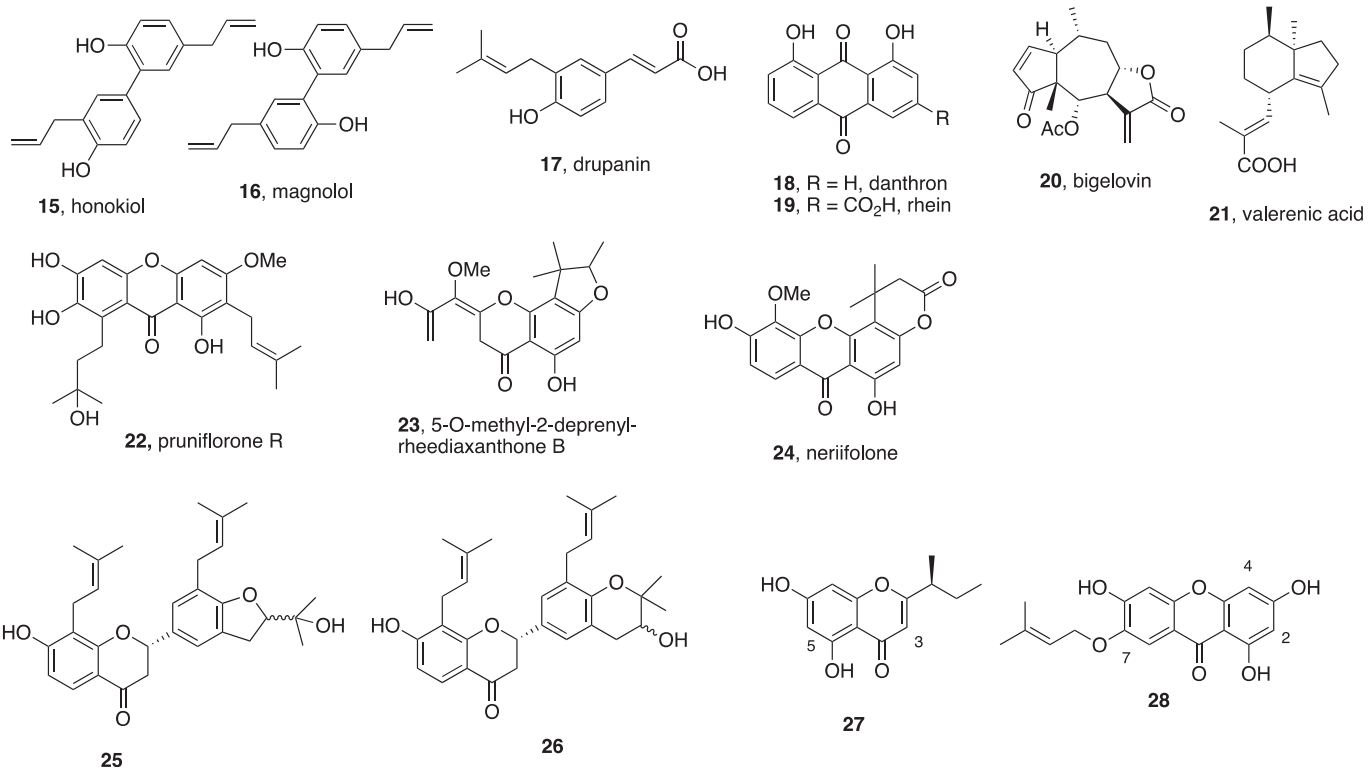


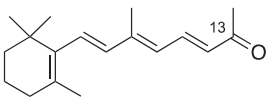
Fig. 3 Naturally occurring RXR ligands from plants with phyto-pharmacological relevance.

Chinese medicine to cure cough, diarrhea and allergic rhinitis (also metabolic syndrome) have been reported as RXR modulators (Kotani, Tanabe, Mizukami, Makishima, & Inoue, 2010; Zhang et al., 2011). Honokiol **15** was found to induce the transactivation of the PPARγ/RXRα heterodimer, but not that of the RXRα/RXRα homodimer (Kotani et al., 2010). The crystal structure of the ligand-bound RXRα revealed that the allyl phenol moieties of magnolol **16** occupy the L-shaped arms of the LBD and, since it does not contain a carboxylic acid, stabilization involves formation of a hydrogen bond with Asn306 on N-terminal helix H5 by one of the phenol groups. Ligand binding to RXRα and PPARγ was estimated as EC_{50} ~40 μM and EC_{50} ~2 μM, respectively, for **16**. The data might explain its preference for activation of RXR/PPARγ transcription at EC_{50} ~10 μM (Zhang, Xu, et al., 2011). Likewise, the reported anti-inflammatory, neuroprotective or antitumor activities of honokiol **15** in animal models of Alzheimer's or Parkinson's disease (Kotani et al., 2010) may be related to the proposed stimulation of RXRα (Wang, Dong, & Wang, 2018).

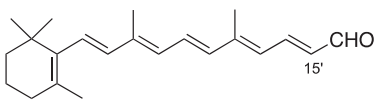
More recent Molecular Modeling and NMR spectroscopy concluded that honokiol **15** targets RXR at both sides of the interface, acting on the coactivator side of the dynamic activation function (AF2) or alternatively it switches from one to the other side of the interface (Scheepstra et al., 2014). Rational design allowed to split the dual-binding properties of the natural product honokiol **15** at the dynamic nuclear receptor interface AF2, and led to derivatives that either stabilize AF2 through binding at the LBP or destabilize AF2 through binding at the coactivator solvent-exposed side of the interface (Scheepstra et al., 2014).

Drupanin **17** (Fig. 3) isolated from green propolis (bee glue), a resinous mixture produced by honey bees, has shown selective, dual RXR/PPARγ agonistic profile in transactivation and binding assays (no binding to RARs, LXRs, VDR was observed), and also increased Ap2/FABP4 expression and accelerated lipid accumulation in differentiated 3T3-L1 cells. The dual RXR/PPARγ agonistic profile was similar to that described for magnolol **16**, although drupanin **17** can bind with higher efficacy to all RXR isotypes (EC_{50} = 2–7 μM) than to PPARγ (EC_{50} ~15 μM) (Nakashima, Murakami, Tanabe, & Inoue, 2014).

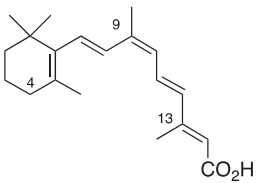
Naturally occurring anthraquinones danthron and rhein (**18**, **19**), isolated from Chinese rhubarb *Rheum palmatum* have been reported to function as antagonists of 9-*cis*-retinoic acid **31** (Fig. 4) in RXRα-LBD, Gal4-luciferase transactivation assays with IC_{50} values of 10 and 70 μM,



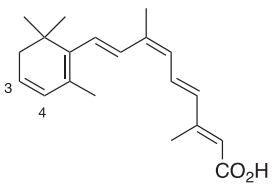
29, β-apo-13-carotenone



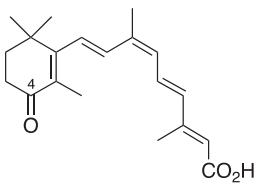
30, β-apo-14'-carotenal



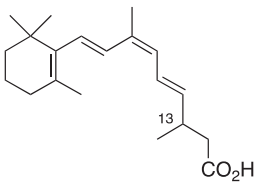
31, 9-*cis*-retinoic acid



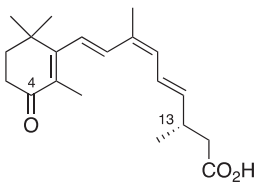
32, 9-*cis*-3,4-dihydroretinoic acid



33, 9-*cis*-4-oxoretinoic acid



34, 9-*cis*-13,14-dihydroretinoic acid



35, (*S*)-9-*cis*-4-oxo-13,14-dihydroretinoic acid

Fig. 4 Structures of apocarotenoids (including retinoids).

respectively (Zhang et al., 2011; Zhang, Chen, Chen, Jiang, & Shen, 2011). A crystal structure of tetrameric RXR was found to be stabilized by the anthraquinones as an inactive form with the recruitment of SMRT (silencing mediator for retinoid and thyroid hormone receptors) corepressor, and showed a strong displacement of H12 helix, making it inaccessible for formation of transcriptionally functional homo- or heterodimers. Danthron **18** and rhein **19** inhibited RXR-mediated transactivation through not only RXR-homodimers, but also through heterodimers of RXR with FXR, LXR or PPARγ.

Sesquiterpene lactone bigelovin **20** (Fig. 3), isolated from the flowers of *Inula helianthus-aquatica* or *Inula hupehensis*, which are used in folk medicine for their anti-cancer potential, binds to RXRα-LBD (no binding to LXRα, FXR or PPARγ was observed), but transactivates through the RXRα-PPARγ heterodimers (not through RXRα-FXR heterodimers) and suppresses RXR-homodimers or RXRα-LXR heterodimers on their respective response elements in the 1–10 μM concentration range (Zhang et al., 2011). The ligand-mediated remodeling of LBD observed in the crystal structure of RXR-LBD was proposed to rationalize the discriminatory activities between RXR-homodimer and heterodimer interactions, and the opposing activities of specific RXR heterodimers.

Computer-aided drug design (CADD) has also been applied to RXR receptors, leading to the discovery of rexinoids that modulate the activities of RXR in a canonical and in a non-canonical way, through binding to the receptor surface or attachment to the co-regulatory binding surface (co-activator binding site and dynamic protein interfaces).

Screening the Dictionary of Natural Products based on isomimetics of natural RXR ligands led to identification of valerenic acid **21** as new natural RXR ligand (Merk, Grisoni, Friedrich, Gelzinyte, & Schneider, 2018). This sesquiterpenoid, isolated from *Valeriana officinalis* (a flowering plant mainly present in Europe and Asia), showed agonistic activity in the micromolar range, which was reflected in the efficient induction of the RXR transcription target genes ABCA1 and ApoE. The Gal4-based reporter assays performed in HEK293T cells revealed intriguing partial selectivity for RXRβ, since valerenic acid showed better EC_{50} (5 μM) and about 10-times higher transactivation of RXRβ as compared to RXRα ($EC_{50} = 27$ μM), and RXRγ ($EC_{50} = 43$ μM). In addition, no activation of RARs, PPARs, LXRs, FXR, CAR, VDR and PXR was observed. Molecular modeling studies suggested similar binding modes of **21** to RXRα and RXRβ as classical rexinoids (Merk et al., 2018).

Among other natural products analogs isolated from *Cratoxylum formosum* ssp. pruniflorone R **22** (Fig. 3) was found to be active on RXRα, when CV-1 cells were transiently transfected with the TREpal-tk-CAT reporter (Duan et al., 2010). Xanthone **23** was the most potent of the family of analogues isolated from *Cratoxylum formosum* ssp. *pruniflorum* and assayed using the transcriptional activities of RXRα in CV-1 cells co-transfected with TREpal-tk-CAT (Duan et al., 2011). Neriifolone B **24**, isolated from the stems of *Cratoxylum cochinchinense*, was also reported as an inhibitor of the RXRα transcriptional activity (Shen et al., 2014). Similar to other derivatives and analogues, it showed concentration-dependent RXRα inhibitory activities.

RXR agonistic activity has been reported for two prenylated flavonones isolated from *Sophora tonkinensis*, which is used in traditional Chinese medicine (Inoue, Tanabe, Nakashima, Ishida, & Kotani, 2014). Both natural products (**25**, **26**) were found to bind at the nanomolar range (EC_{50} 0.77 and 0.78 μM, respectively) to the three RXR isotypes (no binding to RARα, LXRα or PPARα, β/δ or γ), and to transactivate through PPARγ/RXR, PPARδ/RXR, RXR/LXR, and RXR/RAR heterodimers, with the ability to potentiate the activity of agonists for the partner NRs. However, the concentration required for inducing a similar transcriptional activity than bexarotene (LDG1069) was 10 times higher. In addition, the prenylated flavonones were shown to induce the preferential expression of lipoprotein lipase, angiopoietin-like protein 4, and heme oxygenase-1, which are involved in prevention of heart diseases, diabetes, insulin resistance, and atherosclerosis. Therefore, the transcriptional activity of these ligands on RXR heterodimers with LXR, PPARβ/δ, PPARγ are different to those induced by bexarotene, and further studies on these prenylated flavonones are required in order to clarify their potential utility.

Xanthones **27** and **28** were reported as RXRα agonists, with K_D to RXRα-LBD of 68.3 and 14.0 μM, respectively, and showed selective cytotoxicity against HeLa cells, most likely by extrinsic pathways. Apoptosis was shown to be promoted through PARP cleavage and caspase-8 activation. A fluorescence quenching assay suggested that these two xanthones can interact with the Arg316 of the RXRα-LBD and directly bind to RXRα. Molecular docking studies taking into account the topology of these two compounds indicated their structural adaptation to occupy the L-shaped binding pocket of RXRα (Qiu et al., 2019). The proposal is in agreement with SAR studies showing that the hydroxyl group at C5 and an aliphatic chain at C2 in chromone-type compounds as well as a free

3-OH and an aliphatic chain substituted at the 7-OH in these xanthones are essential for their potent RXRα transcriptional inhibitory activities. Likewise, it is consistent with the finding that prenylated xanthones exhibited more potent effects on the RXRα transcriptional inhibitory activities than simple xanthones (Duan et al., 2010, 2011; Shen et al., 2014).

3.3 Endogenous RXR ligands (Fig. 4)

β-Apo-13-carotenone **29** (Fig. 4), has been recently demonstrated to stabilize inactive tetrameric RXR, instead of the transcriptionally active RXRα dimers (Eroglu, Hruszkewycz, Curley, & Harrison, 2010; Sun, Narayanasamy, Curley, & Harrison, 2014). Ki values for RXRα of 8 nM in a 9-*cis*-retinoic acid **31** competitive displacement assay were measured, although different modes of binding are suggested by in silico ligand docking studies with RXRα-LBD (Sun et al., 2014). In addition, this apocarotenoid appears to also act as a competitive inhibitor of RARs with a Ki of 4 nM (Eroglu et al., 2012), and to reduce ATRA-RAR-mediated activity by about 70%. Lower levels of β-apo-13-carotenone **29** (1 nM) following chronic consumption of β-carotene-enriched diets has been reported (Cooperstone et al., 2017). Since β-apo-13-carotenone **29** is generated by thermal degradation and oxidation of β-carotene during food processing (it is also found only in some fresh fruits), it cannot be produced in mammals (Harrison & Quadro, 2018). Similarly, β-apo-14′-carotenal **30** (Fig. 3) has been reported to act as a potential inhibitor of RXRα as well as of selected heterodimeric partners (PPARα, PPARβ/δ, LXRα and LXRβ, but not RARs). This apocarotenoid can be found in several melons, but its detection in human plasma was not consistent (Harrison & Quadro, 2018). It is therefore unlikely that those apocarotenoids are physiologically meaningful, despite their in vitro inhibitory activities.

Although it is traditionally considered as a natural RXR ligand, it is important to indicate that 9-*cis*-retinoic acid **31** (Fig. 4) has not been conclusively shown to be endogenously present, except in pancreatic β-cells, and recently in human serum in very low concentrations (Arnold, Amory, Walsh, & Isoherranen, 2012). Either its concentration is under the detection limit, it is present transiently or it accumulates in local cell populations (Kane, 2012).

Additional biologically potent endogenous vitamin A derivatives, among them 9-*cis*-3,4-didehydroretinoic acid **32** and 9-*cis*-4-oxo-retinoic acid **33**, have been demonstrated to bind to and activate RXR in vitro

(Mangelsdorf & Evans, 1995; Mangelsdorf et al., 1995). 9-*cis*-3,4-didehydroretinoic acid **32** could be biosynthetically derived from the precursor vitamin A_2 (all-*trans*-3,4-didehydroretinol) following similar routes to those of **31** from vitamin A (all-*trans*-retinol). 9-*cis*-4-oxo-retinoic acid **33** has been isolated from *Xenopus* embryos, where it was found to modulate axial pattern formation (Pijnappel et al., 1998), after its likely formation by isomerization of the all-*trans* isomer, which is endogenously present in the same organism (Pijnappel et al., 1993). Metabolite **33** was shown to bind RARα,β,γ and RXRα,β,γ, with IC_{50} values about 5–10 times higher than those of 9-*cis*-retinoic acid **31**, and to induce a synergistic activation of the RAR/RXR heterodimers, but not the RXR homodimers (in contrast to 9-*cis*-retinoic acid **31**) (Pijnappel et al., 1998).

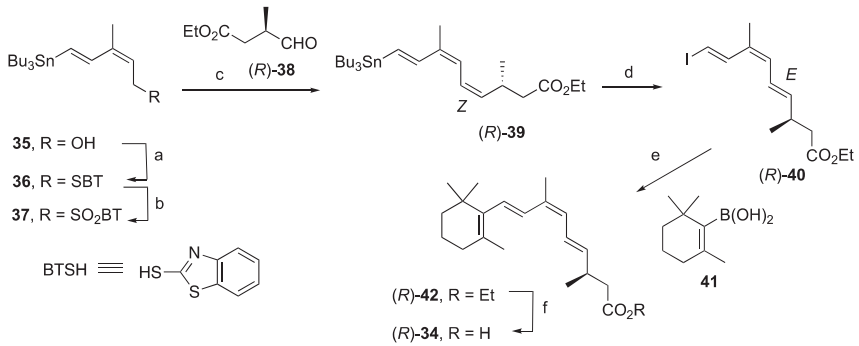
The endogenous presence of 9-*cis*-13,14-dihydroretinoic acid **34** and its all-*trans* isomer, has recently been proven in several mice organs (liver, serum, brain) by HPLC-MS-MS and UV and quantified (quantitative binding affinity of K_d 90 ± 20 nM; cf. 20 ± 10 nM for 9-*cis*-retinoic acid **31** by fluorescence quenching assays) to be sufficient to maintain RXR activities (Rühl et al., 2015). Titration experiments monitored by ESI-MS showed that the affinity of RXR for the *S* enantiomer was about 65% lower than that for the *R* enantiomer. Crystal structure studies on the *R* enantiomer bound to hRXRα LBD and a 13-residue peptide comprising the nuclear receptor binding surface NR2 of NCoA2 confirmed binding to the canonical binding pocket, adopting a similar orientation than 9-*cis*-retinoic acid **31**, which is consistent with their similar transcriptional activities on several nuclear receptor-signaling pathways, possibly through the corresponding permissive heterodimers. Therefore, 9-*cis*-13,14-dihydroretinoic acid **34** was considered as the first endogenous and physiologically relevant retinoid RXR ligand in mammals (Rühl et al., 2015). This ligand and other dihydroretinoids are presumably produced by retinol saturase (RetSat) (Moise et al., 2009). The structure of 9-*cis*-13,14-dihydroretinoic acid **34** had previously been assigned to a metabolite detected in rats, together with its taurine derivative, after being fed with high doses of 9-*cis*-retinoic acid **31** (Shirley et al., 1996), but in-depth characterization of its structure and function was not pursued. 9-*cis*-retinol might be the metabolic precursor of RetSat action to generate **34** following similar biosynthetic pathways as (*R*)-all-*trans*-13,14-dihydroretinol to all-*trans*-13,14-dihydroretinal (Moise et al., 2008; Moise, Kuksa, Blaner, Baehr, & Palczewski, 2005; Moise, Kuksa, Imanishi, & Palczewski, 2004), and further metabolism to the carboxylic acid. The specific all-*trans* to 9-*cis* isomerization process is likely

to occur in a fully conjugated polyene precursor, namely a carotenoid or a C9-C10-dihydrocarotenoid. Nevertheless, the precise mechanistic details and the possibility that the isomerization step could be enzymatically driven are yet unknown.

A putative metabolite of **34**, namely (*S*)-9-*cis*-4-oxo-13,14-dihydroretinoic acid **35** has been shown to be present in the liver and other tissues of both wild-type mice and rats fed a standard laboratory diet, as well as in humans (Schmidt, Volland, Hamscher, & Nau, 2002). This compound activated RAR-dependent gene transcription via RAR–RXR heterodimers, although it was less potent than all-*trans*-retinoic acid, despite its higher concentration in mice liver, but showed no transcriptional activity with either RXRα or RXRβ (Schuchardt et al., 2009).

4. Diastereo- and enantioselective synthesis of (*R*)-9-*cis*-13,14-dihydroretinoic acid 34

The preparation of (*R*)-9-*cis*-13,14-dihydroretinoic acid (*R*)-**34** was based on the Suzuki cross-coupling of enantiopure trienyliodide **40** and boronic acid **41** (Scheme 1). The synthesis of (*R*)-**3** started with (2*Z*,4*E*)-stannyldienol **35** (Domínguez, Pazos, & de Lera, 2000; Pazos & de Lera, 1999) which was transformed into the benzothiazolyl allyl sulfide **36** by Mitsunobu reaction with the corresponding thiol and subsequently oxidized to sulfone **37** with H_2O_2 and a peroxymolybdate (VI) reagent (Schultz, Freyermuth, & Buc, 1963) at −10 °C. The Julia–Kocienski olefination (Aïssa, 2009; Blakemore, 2002) was performed using a slight excess of base



Scheme 1 Reagents and conditions: (a) PPh_3, BTSH, DIAD, CH_2Cl_2, 2 h (98%). (b) $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 30% H_2O_2, EtOH, −10 °C, 17 h (66%). (c) NaHMDS, THF, −78 °C, 30 min (93%). (d) I_2, CH_2Cl_2, 25 °C, 30 min. (e) $Pd(PPh_3)_4$, 10% aq. TlOH, THF, 25 °C, 3 h (42%). (f) 2M KOH, MeOH, 80 °C, 45 min (84%).

(NaHMDS, 1.15 equivalents) and 1.7 equivalents of enantiopure aldehyde (*R*)-**38** (Leonard, Mohialdin, Reed, Ryan, & Swain, 1995; Moise et al., 2008). As anticipated from previous findings on the stereoselectivity of the Julia–Kocienski reactions of allylsulfones and aldehydes (Sorg & Brückner, 2005; Vaz, Alvarez, Souto, & de Lera, 2005), the geometry of the newly formed olefin of trienyl ester (*R*)-**39** is *Z* (which was confirmed by NOE experiments). Treatment of the precursor stannane with a solution of iodine in CH_2Cl_2 produced the iodide (*R*)-**40** via Sn-I exchange and iodine-promoted isomerization of the *Z,Z,E* triene to the desired *E,Z,E* geometric isomer (as confirmed by NOE experiments). The Suzuki reaction (Miyaura & Suzuki, 1995; Suzuki, 2011) of freshly prepared boronic acid **41** and trienyl iodide (*R*)-**40** using $Pd(PPh_3)_4$ as catalyst and 10% aqueous TlOH as base in THF at ambient temperature, followed by immediate work-up afforded ethyl (*R*)-9-*cis*-13,14-dihydroretinoate (*R*)-**42** in 78% yield. Saponification of (*R*)-**42** provided in 84% yield the desired carboxylic acid (*R*)-**34** (Rühl et al., 2015).

5. Materials

Silica gel column chromatography: Merck Kieselgel 60 (230–400 mesh particle size and C18-silicagel.

Aluminum plates for tlc: Merck Kieselgel 60F254.

Visualization for analytical tlc: UV irradiation (254 nm) or staining with an ethanolic solution of phosphomolybdic acid.

5.1 Reagents and solvents

2-Mercaptobenzothiazole, 97%.

Triphenylphosphine (PPh_3), ≥95%.

Tetrahydrofuran (THF), dried and distilled before use.

Acetonitrile, ACS reagent, max 0.0075% H_2O.

Diisopropyl azodicarboxylate (DIAD), 98%.

Ethanol (EtOH), reagent grade.

Ammonium heptamolybdate tetrahydrate $[(NH_4)_6Mo_7O_{24}{\cdot}4H_2O]$, >99%.

Hydrogen peroxide (35% in H_2O).

Diethyl ether (Et_2O), ACS reagent.

Diethyl ether (Et_2O), for synthesis, stabilized with 7 ppm BHT.

Methanol (MeOH), for HPLC ≥99.9%.

Sodium bis(trimethylsilyl)amide solution (NaHMDS, 1 M in THF).

Iodine (I_2), ≥99.8%.

Dichloromethane (CH_2Cl_2) for synthesis, stabilized with amylene.

Sodium dithionite ($Na_2S_2O_3$), ≥82%.

Sodium sulfate (Na_2SO_4), ≥99%, anhydrous powder.

Hexane, synthesis grade.

Triethylamine (Et_3N), ≥99%.

Tetrakis(triphenylphosphine)palladium [$Pd(PPh_3)_4$], 99%.

Thallium hydroxide (TlOH); a 10% aqueous solution, prepared from barium hydroxide, $Ba(OH)_2$ (≥98%) and thallium sulfate, Tl_2SO_4 (≥99.5%).

Celite®, ≥95%.

Potassium hydroxide (KOH), a 2 M aqueous solution.

Hydrochloric acid (HCl), a 10% aqueous solution.

tert-Butyl methyl ether (*t*-BuOMe), ≥99.8%.

Trifluoroacetic acid (TFA), 99%.

Phosphomolybdic acid hydrate, $Na_3[P(Mo_3O_{10})_4]\cdot xH_2O$, technical grade.

6. Methods

6.1 Method for formation of BT-sulfide and BT-sulfone

6.1.1 (2Z,4E)-1-(Benzothiazol-2-yl)sulfonyl-5-(tri-n-butylstannyl)-3-methylpenta-2,4-diene (37)

1. A solution of (2*Z*,4*E*)-3-methyl-5-(tributylstannyl)penta-2,4-dien-1-ol **35** (1.0 g, 2.58 mmol), 2-mercaptobenzothiazole (0.65 g, 3.87 mmol) and PPh_3 (1.10 g, 4.21 mmol) in THF (14 mL) in a 50 mL round bottom flask equipped with a magnetic stirring bar and stirred for 5 min at 0 °C.
2. A solution of DIAD (0.77 mL, 3.87 mmol) in THF (5 mL) was added dropwise to the above flask via syringe and the mixture was stirred for 30 min at 25 °C.
3. The solvent was removed and the residue was purified by column chromatography (C18-silica gel, CH_3CN) to afford 1.11 g (78%) of (2*Z*,4*E*)-1-(benzothiazol-2-yl)sulfanyl-5-(tri-*n*-butylstannyl)-3-methylpenta-2,4-diene **36** as a colorless oil.
4. To a solution of **36** (0.48 g, 0.89 mmol) in EtOH (9 mL), at −10 °C, was added a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (0.44 g, 0.36 mmol) in aqueous hydrogen peroxide (35%, 7.7 mL, 89.1 mmol).
5. Stirring was maintained for 17 h at −10 °C.
6. Work-up: the mixture was quenched with H_2O and extracted with Et_2O (3×). The combined organic layers were washed with brine (3×) and dried (Na_2SO_4), and the solvent was removed.

7. Purification: the residue was purified by chromatography (C18-silica gel, MeOH) to afford 0.33 g (66%) of a colorless oil, which was identified as 37.

6.2 Method for formation of dienylstannane

6.2.1 *Ethyl (3S,4Z,6Z,8E)-3,7-dimethyl-9-(tri-n-butylstannyl)nona-4,6,8-trienoate ((S)-39)*

1. A solution of (2*Z*,4*E*)-1-(benzothiazol-2-yl)sulfonyl-5-(tri-*n*-butylstannyl) 3-methylpenta-2,4-diene **37** (0.115 g, 0.20 mmol) in THF (9 mL) in a 50 mL round bottom flask equipped with a magnetic stirring bar was cooled down to −78 °C and then was treated with NaHMDS (0.23 mL, 1 M in THF, 0.23 mmol).
2. After stirring for 30 min at this temperature, a solution of ethyl (*S*)-3-methyl-4-oxobutanoate **38** (0.044 g, 0.30 mmol) in THF (4.5 mL) was added and the resulting mixture was stirred for 1 h at −78 °C.
3. It was then carefully allowed to reach room temperature for 3 h.
4. After lowering the temperature to 0 °C, Et_2O and water were added and the mixture was allowed to warm up to room temperature.
5. Work up: the reaction mixture was diluted with Et_2O and the layers were separated. The aqueous layer was extracted with Et_2O (3 ×), the combined organic layers were dried (Na_2SO_4) and the solvent was removed.
6. Purification: the residue was purified by column chromatography (C-18 silica gel, MeOH) to afford 0.94 g (93%) of (*S*)-**39** as pale yellow oil.

6.3 Method for stannane-iodine exchange

6.3.1 *Ethyl (3R,4E,6Z,8E)-9-iodo-3,7-dimethylnona-4,6,8-trienoate ((R)-40)*

1. To a solution of ethyl (3*R*,4*Z*,6*Z*,8*E*)-3,7-dimethyl-9-(tri-*n*-butylstannyl) nona-4,6,8-trienoate (*R*)-**39** (0.060 g, 0.121 mmol) in CH_2Cl_2 (5.3 mL) in a 25 mL round-bottomed flask equipped with a magnetic stirring bar was added dropwise a solution of iodine (0.046 g, 0.182 mmol) in CH_2Cl_2 (2.8 mL).
2. The resulting mixture was stirred for 30 min at 25 °C.
3. Work-up: a saturated $Na_2S_2O_4$ solution was then added and the reaction mixture was extracted with Et_2O (3 ×), the combined organic layers were dried (Na_2SO_4) and the solvent was removed.

4. Purification: the residue was purified by column chromatography (silica gel, 97:3 hexane/Et_3N) to afford 0.037 g (92%) of (*R*)-**40** as pale yellow oil.

6.4 Method for Suzuki cross-coupling

6.4.1 Ethyl (9Z,13R)-13,14-dihydroretinoate ((R)-42)

1. To a solution of ethyl (3*R*,4*E*,6*Z*,8*E*)-9-iodo-3,7-dimethylnona-4,6,8-trienoate (*R*)-**40** (0.036 g, 0.107 mmol) in THF (2.3 mL) in a 5 mL round-bottomed flask equipped with a magnetic stirring bar was added $Pd(PPh_3)_4$ (0.013 g, 0.011 mmol).
2. After 5 min at room temperature, 2,6,6-trimethylcyclohex-1-enylboronic acid **41** (0.027 g, 0.161 mmol) was added in one portion followed by a 10% aqueous solution of TlOH (0.75 mL, 0.407 mmol).
3. Stirring was maintained for 3 h at 25 °C.
4. Work-up: Et_2O was added and the reaction mixture was filtered through a short pad of Celite®. The filtrate was washed with $NaHCO_3$ (aqueous saturated solution), the organic layer was dried (Na_2SO_4) and the solvent was removed.
5. Purification: the residue was purified by column chromatography (silica gel, 97:3 hexane/Et_3N) to afford 0.028 g (78%) of a pale yellow oil, which was identified as ethyl (9*Z*,13*R*)-13,14-dihydroretinoate (*R*)-**42**.

6.5 Method for saponification

6.5.1 (9Z,13R)-13,14-dihydroretinoic Acid ((R)-34)

1. To a solution of ethyl (9*Z*,13*R*)-13,14-dihydroretinoate (*R*)-**42** (0.023 g, 0.069 mmol) in MeOH (4.7 mL) in a 5 mL round-bottomed flask equipped with a magnetic stirring bar was added KOH (2 M aqueous solution, 1.1 mL, 2.27 mmol).
2. The reaction mixture was allowed to stir at 80 °C for 45 min.
3. The reaction was allowed to cool down to room temperature.
4. Work-up: CH_2Cl_2 and brine were added and the layers were separated. The aqueous layer was washed with H_2O (3 ×). The combined aqueous layers were acidified with 10% HCl and extracted with CH_2Cl_2 (3 ×). The combined organic layers were dried (Na_2SO_4) and the solvent was removed.
5. Purification: the residue was purified by column chromatography (silica gel, gradient from 95:5 to 90:10 CH_2Cl_2/MeOH) to afford 0.017 g (84%) of (*R*)-**34** as pale yellow oil (Fig. 5).

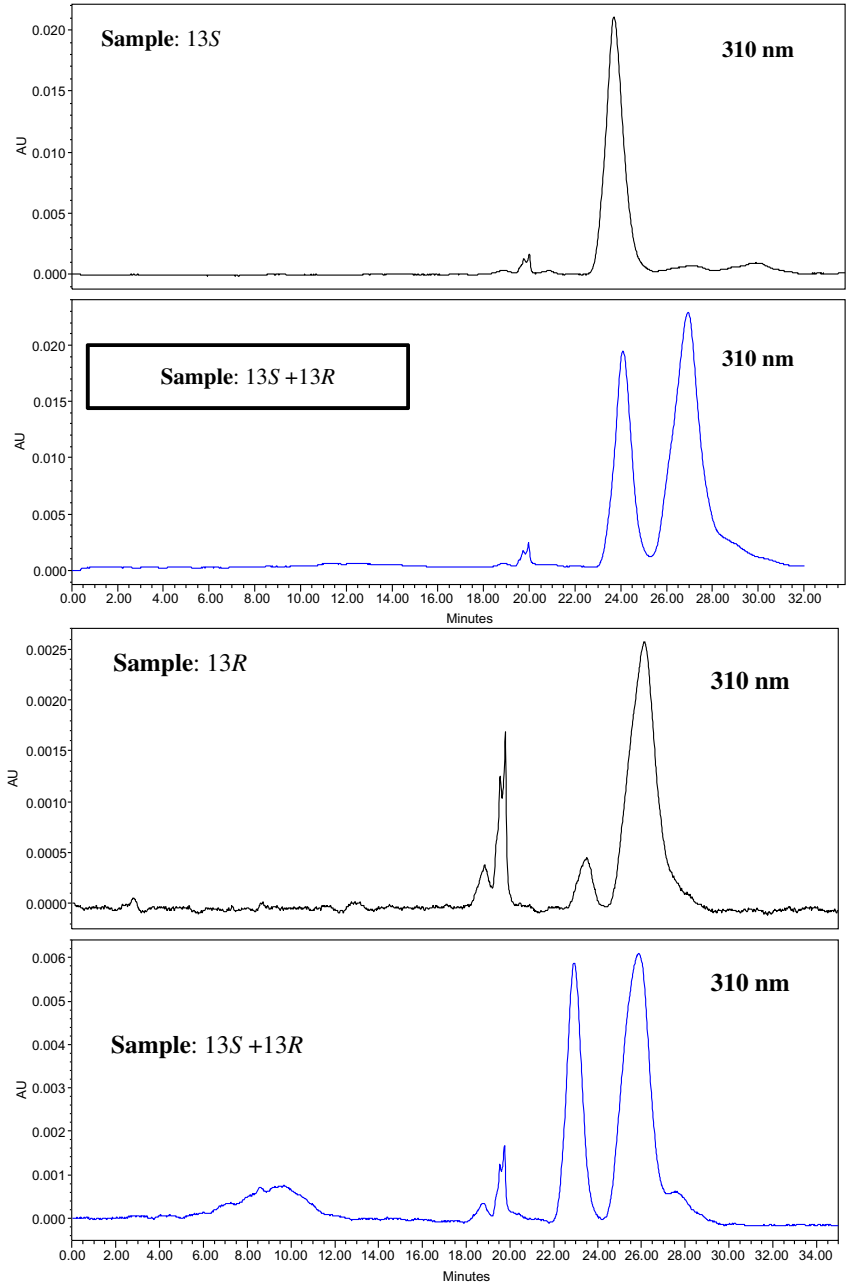


Fig. 5 Separation of 9-*cis*-13,14-dihydroretinoic acid enantiomers.

6.6 Method for enantiomeric excess determination of (9*Z*,13*R*)-13,14-dihydroretinoic acid (9*Z*,13*R*)-34

1. Stationary phase: Chiralpak IA column.
2. Eluent: 92.5:7.5 v/v solvent mixture of hexane (+0.1% TFA)/ *t*-BuOMe.
3. Isocratic mode at a flow of 2 mL/min.
4. Detection by absorption at 310 nm and 254 nm.

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