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Intracellular Signaling Activity of Synthetic (14*R*)-, (14*S*)-, and (14*RS*)-14-Hydroxy-4,14-*retro*-retinol†

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ABSTRACT: 14-Hydroxy-4,14-*retro*-retinol (14-HRR), first isolated from cultures of lymphoblastoid 5/2 and HeLa cells and characterized by NMR, UV, and CD, is a metabolite of retinol which promotes growth of B lymphocytes in culture and activation of T lymphocytes by antigen receptor-mediated signals. It is also produced by various tested cell lines: fibroblasts, leukemia, and *Drosopohila* cells. 14-HRR is the first bioactive *retro*-retinoid to be discovered and, after retinal and retinoic acid, is the third intracellular messenger molecule derived from retinol. Physical properties and intracellular signaling activities of synthetic (14*R*)-HRR, (14*S*)-HRR, and racemic 14-HRR are described. CD spectra indicate that natural 14-HRR isolated previously was a mixture of enantiomers. B-cell survival and T-cell activation assays performed in the optimal range of (7–1.6) × 10^{-7} M surprisingly showed that all 14-HRR compounds exhibit similar activity, with the 14*R* enantiomer exhibiting slightly higher activity in comparison to the 14*S* enantiomer. However, because of the semiquantitative nature of the assays, the conclusion as to which enantiomer is more active and which is the true ligand for the target receptor must await characterization of this protein.

The discovery of a lipophilic extract from egg yolk, which was essential for life (Stepp, 1909), opened an exciting era for chemical and biological research on vitamin A (retinol, **1**) and its derivatives, the retinoids. Since then vitamin A has been found to promote general health and resistance to infections (Sporn et al., 1984). Nearly all mammalian tissues bathe in a constant supply of retinol, and the wide distribution of high-affinity intracellular retinoid-binding proteins suggests that vitamin A exists inside most cells as well (Chytil & Ong, 1984; Noy & Blaner, 1991). However, retinol is not known to be incorporated into structural components of cells and does not bind to any of the nuclear receptors tested so far; thus it seems very likely that vitamin A functions as a precursor of active metabolites.

The best understood metabolic function of vitamin A is its role in vision; pioneered by the studies of Wald on the visual process (Wald, 1935, 1968), research on the biosynthesis of 11-*cis*-retinal (**2**) in the eye (Rando, 1990) and its role in visual transduction (Derguini & Nakanishi, 1986; Nathans, 1992) have led to exciting new pathways owing to recent advances in biochemistry (Khorana, 1992) and spectroscopy (Schoenlein et al., 1991; Mizukami et al., 1993). Another example is retinoic acid (**3**), long known to possess potent teratogenic properties and recently shown to be an endogenous morphogen (Thaller & Eichele, 1987), important for the development of the limbs and the brain (Wagner et al., 1990). Parallel discovery of retinoic acid nuclear receptors as transcriptional regulators helped unravel its mode of action (Giguere et al., 1987; Petkovich et al., 1987; Mangelsdorf et al., 1990).

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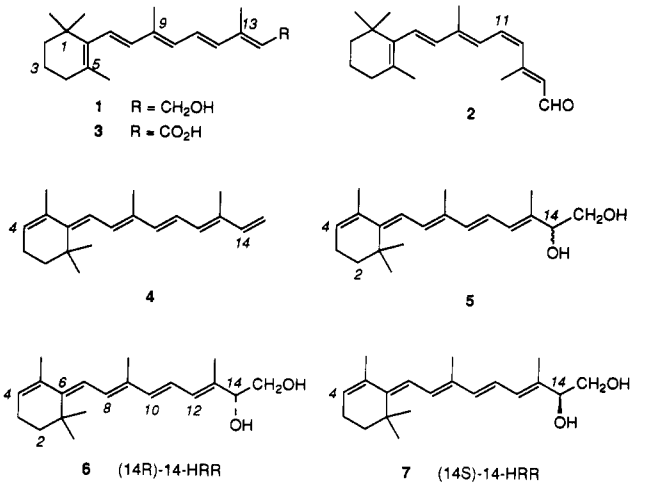


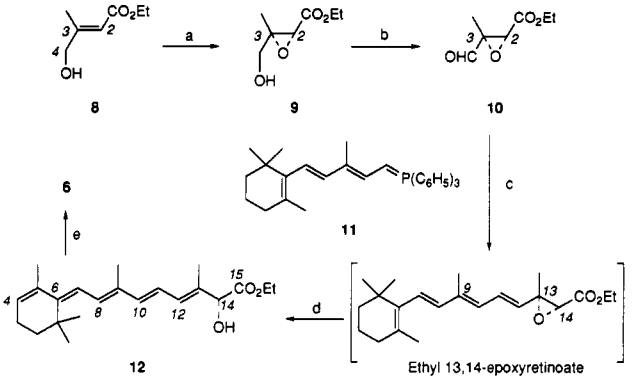
However, it is clear that the role of vitamin A in vision is different from its general function in the body and that although retinoic acid alone may perform some functions of vitamin A, it cannot replace it in vision, in reproduction (Thompson, 1964), or in preventing necrotic cell death of activated immune cells in culture (Buck et al., 1990, 1991a; Garbe et al., 1992). Indeed, biochemical analysis of the intracellular retinoids of B cells led to the identification of known retinoids, i.e., retinol, retinyl esters, and anhydroretinol (AR, **4**) (the latter isolated for the first time from fish liver oils) (Embree, 1939), and to the isolation of two new retinoids. One of them has been characterized as 14-hydroxy-4,14-*retro*-retinol (14-HRR, **5**) (Buck et al., 1991b). 14-HRR, the first described bioactive *retro*-retinoid, was found to be the intracellular mediator of cell proliferation; it sustains the growth of B cells and activation of T cells at 10–30-fold lower concentration than retinol. Although the mechanism of action of 14-HRR is still unknown, it may function, as retinoic acid, through ligand-assisted transcriptional control. Indeed, the hypothesis that 14-HRR might be a ligand for a cytoplasmic or a nuclear receptor was corroborated by the isolation of anhydroretinol (**4**), the second *retro*-retinoid identified in human B cells and also extracted from *Drosophila* and other insect cells; AR functions as a reversible inhibitor of retinol and 14-HRR and thus might bind to the same receptor protein (Buck et al., 1993). 14-HRR and AR constitute the first naturally occurring agonist/antagonist pair of retinoids (or other small lipophilic messenger molecules) to be described.

14-HRR was first isolated from the lymphoblastoid 5/2 cells and then from large-scale cell cultures of HeLa cells (168 μg from 80 L) (Buck et al., 1991b). In this paper we wish to report the physical and biochemical properties of synthetic (14*R*)-**6**, (14*S*)-**7**, and (14*RS*)-14-HRR and a comparison between synthetic and native specimens. Because of the biological importance of 14-HRR as a new second messenger molecule, and the difficulty in its handling due to its lability, the synthesis is reported in some detail. The studies have shown that, unexpectedly, the native compound reported earlier (Buck et al., 1991b) was a mixture of both enantiomers.

MATERIALS AND METHODS

All reactions were conducted under dried argon atmosphere. Unless otherwise stated, reagents were obtained from commercial suppliers and used without further purification. Ethyl *trans*-3-methyl-4-oxocrotonate was purchased from Fluka. Powdered commercially activated 4A molecular sieves and anhydrous *t*ert-butyl hydroperoxide were obtained from Aldrich. CH_2Cl_2 and pyridine were distilled from CaH_2, and

Scheme 1: Synthetic Steps for (14*R*)-14-HRR (**6**)[a]



[a] Steps: (a) $Ti(OiPr)_4$, (+)-DET, tBuOOH, CH_2Cl_2, −30 to −15 °C; (b) CrO_3·2Pyr, CH_2Cl_2; (c) THF; (d) pTsOH, ether; (e) LAH, ether. A similar route was followed for the preparation of (14*S*)-14-HRR.

THF and diethyl ether were distilled from sodium/benzophenone prior to use. Flash chromatography was carried out with E. Merck silica gel 60; alternatively, neutral alumina Brockman activity I from Fischer Scientific was also used, after proper deactivation. Samples for spectroscopic analysis and bioassays were further purified by high-pressure liquid chromatography (HPLC). ^1H NMR spectra were recorded on a Varian VXR-400 spectrometer in $CDCl_3$ or CD_3OD; the chemical shifts are given in parts per million (δ reference peak $CHCl_3$ 7.24 ppm or CH_3OH 3.30 ppm) and coupling constants (J) in hertz. High-resolution electron ionization mass spectra (HRMS) were measured on a JEOL DX-303 HF spectrometer [matrix perfluorokerosine (PFK)]. A Supelco Inc. fused silica capillary column (15 m × 0.25 mm i.d., 0.25-μm film thickness) attached to a Hewlett-Packard gas chromatograph was used for HR-GCMS measurements. Absorption spectra were recorded on a Perkin-Elmer Lambda 4B UV/vis spectrophotometer. Circular dichroism (CD) spectra were measured on a Jasco J-720 spectropolarimeter.

Synthesis. As shown in Scheme 1 for the synthesis of (14*R*)-14-HRR (**6**), the key intermediate is ethyl (2*R*,3*S*)-3-methyl-4-hydroxyepoxycrotonate (α-epoxide, **9**), obtained by Sharpless epoxidation (Gao et al.,1987) of ethyl *trans*-3-methyl-4-hydroxycrotonate (**8**) (prepared by $NaBH_4$ reduction of commercial ethyl *trans*-3-methyl-4-oxocrotonate) in the presence of diethyl L-(+)-tartrate. Oxidation of **9** with dipyridine–chromium(VI) oxide (Collins et al., 1968) led to ethyl (2*R*,3*S*)-3-methyl-4-oxoepoxycrotonate (**10**), which upon condensation with phosphorane **11** (Sarnecki & Pommer, 1960) gave the corresponding ethyl 13,14-α-epoxyretinoate.[1] However, because of the instability of ethyl 13,14-epoxyretinoate (Davalian & Heathcock, 1979), this intermediate was not isolated but converted into ethyl (14*R*)-14-hydroxy-*retro*-retinoate (**12**) by *in situ* treatment with acid; LAH reduction of **12** afforded (14*R*)-14-HRR (**6**).

Ethyl (2R,3S)-3-Methyl-4-hydroxyepoxycrotonate (α-Epoxide) (**9**). Powdered commercially activated 4A molecular sieves (420 mg, 0.2 wt equiv) and 50 mL of CH_2Cl_2 were introduced into a flame-dried three-necked round-bottom flask equipped with a magnetic stirbar, addition funnel, thermometer, and argon inlet. The flask was cooled to −20 °C. A

[1] A similar Witting reaction was reported for the synthesis of racemic ethyl 13,14-epoxyretinoate, from racemic aldehyde **10**, which, in this case, was prepared by ozonization of methyl 3,5-dimethyl-2,3-epoxyhex-4-enoate (Davalian & Heathcock, 1979). The 11,12-ene configuration was depicted as trans; because ethyl 13,14-epoxyretinoate is very unstable, we have not isolated and characterized this intermediate.

solution of freshly distilled diethyl L-(+)-tartrate (358 mg, 1.74 mmol) in 20 mL of CH_2Cl_2 was then added, followed by freshly distilled titanium tetraisopropoxide (0.43 mL, 412 mg, 1.45 mmol, via syringe). The reaction mixture was stirred at −20 °C, while *tert*-butyl hydroperoxide (10 mL, 30 mmol, 3 M in isooctane dried over molecular sieves prior to use) was added dropwise. After 30 min of stirring at −20 °C the reaction mixture was cooled to −30 °C, and a solution of allylic alcohol **8** (2.1 g, 14.5 mmol) in 30 mL of CH_2Cl_2 (dried over molecular sieves prior to addition) was added dropwise while the temperature was kept between −30 and −25 °C. The reaction mixture was stirred for 2 h at −30 °C and then stored without stirring at −15 °C for 20 h. Anhydrous citric acid (278 mg, 1.45 mmol) in acetone/ether (1/9 v/v; 50 mL) was added slowly at −15 °C; the cooling bath was removed and the reaction mixture was stirred for 30 min. After filtration through a Celite pad and evaporation of the solvents, flash chromatography on silica gel (gradient 7/3 to 1/1 hexane/ether) provided a colorless oil [2.4 g, 103%, chemical purity ca. 95% by ^{1}H NMR,[2] 92% ee by ^{1}H NMR shift analysis of the oil or the derived acetate with Eu(hfc)$_3$ (Gao et al., 1987)]: ^{1}H NMR ($CDCl_3$) δ 1.30 (t, J 10, 3H, OCH_2CH_3), 1.36 (s, 3H, 3-Me), 3.70 (s, 1H, 2-H), 3.68/3.78 (d, J 16, each 1H, CH_2OH), 4.26 (m, 2H, OCH_2CH_3); HR-GCMS 160.0745 (M^+), calcd 160.0736.

Ethyl (2R,3S)-3-Methyl-4-oxoepoxycrotonate (**10**). Freshly prepared dipyridine–chromium(VI) oxide (2.4 g, 8.9 mmol) was added to a solution of α-epoxycrotonate **9** (216 mg, 1.35 mmol) in 48 mL of CH_2Cl_2. The suspension was stirred for 1 h at room temperature and then filtered through a Celite pad. Evaporation of the solvent followed by flash chromatography on silica gel (1/1 hexane/ether) gave a colorless oil (125 mg, 60%): ^{1}H NMR ($CDCl_3$) δ 1.31 (t, J 7, 3H, OCH_2CH_3), 1.50 (s, 3H, 3-Me), 3.75 (s, 1H, 2-H), 4.29 (m, 2H, OCH_2CH_3), 8.86 (s, 1H, CHO); HR-GCMS 159.0667 (M + 1), calcd 159.0657.

Ethyl (14R)-14-Hydroxy-4,14-retro-retinoate (14α-OH) (**12**). To a stirred suspension of β-ionylideneethyltriphenylphosphonium bromide (Sarnecki & Pommer, 1960) (490 mg, 0.9 mmol) in 2.5 mL of freshly distilled THF was added at −30 °C *n*-butyllithium (0.56 mL, 0.9 mmol, 1.6 M in hexane). The reaction mixture was stirred for 20 min at −30 °C, and a solution of α-epoxy aldehyde **10** (125 mg, 0.8 mmol) in 2 mL of freshly distilled THF was added. After 1 h at −30 °C, the cooling bath was removed, and the reaction mixture was allowed to warm up to 0 °C. The reaction mixture was diluted with 10 mL of diethyl ether, and *p*-toluenesulfonic acid monohydrate (152 mg, 0.8 mmol) in 40 mL of diethyl ether was added. The reaction mixture was stirred for 5 min at 0 °C and then washed with 5% aqueous $NaHCO_3$ and brine. After the reaction mixture was dried over anhydrous Na_2SO_4 and the solvents were evaporated, chromatography on neutral alumina (activity V; gradient 9/1 to 7/3 hexane/ether) gave (14*R*)-14-hydroxy ester **12** as a yellow oil (226 mg, 82%, ca. 75% all-trans). Further HPLC purification (YMC 5-μm SiO_2 column, 10 × 250 mm; 92/8 hexane/ethyl acetate) gave pure *all-trans*-**12**: UV (MeOH) 366, 348, 332, 316, 300, 252 nm; ^{1}H NMR (CD_3OD) δ 1.26 (t, J 7, 3H, OCH_2CH_3), 1.30 (s, 6H, 1-Me$_2$), 1.52 (t, J 6, 2H, 2-H$_2$), 1.81/1.90/1.93 (s, each 3H, 5/9/13-Me), 2.13 (m, 2H, 3-H$_2$), 4.20 (q, J 7, 2H, OCH_2CH3), 4.20 (1H, 14-H), 5.80 (t, J 4, 1H, 4-H), 6.28 (d, J 10, 1H, 12-H), 6.40 (d, J 12, 1H, 7-H), 6.44 (d, J 15, 1H, 10-H), 6.53 (dd, J 15, 10, 1H, 11-H), 6.80 (d, J 12, 1H, 8-H); HRMS 344.2346 (M^+), calcd 344.2351.

(14R)-14-Hydroxy-4,14-retro-retinol (14α-OH) (**6**). To a solution of hydroxy ester **12** (24 mg, 0.07 mmol) in 0.5 mL of anhydrous diethyl ether was added at 0 °C lithium aluminum hydride (0.25 mL, 0.25 mmol, 1 M solution in THF). After the mixture was stirred for 1 h at 0 °C, reversed-phase TLC analysis (C_{18}, 200 μm; 95/5 CH_3OH/H_2O) showed completion of the reaction. Hydrolysis with 0.1 mL of water was followed by filtration through a pad of wet Celite and washing of the Celite pad with ether. The filtrate was washed with water, dried over Na_2SO_4, and evaporated. Purification of the residue by chromatography on neutral alumina (activity V; gradient 9/1 to 1/1 hexane/ether) gave (14*R*)-14-HRR (**6**) (2340 ODs at 348 nm, 13 mg, 62%, ca. 75% all-trans), while a bright yellow band remained on the top of the alumina column.[3] Further purification by reversed-phase HPLC [Vydac C_{18} reversed-phase column, 10 × 250 mm; 25/75 water/acetonitrile (or methanol)] led to pure *all-trans*-**6**: ^{1}H NMR (CD_3OD) δ 1.30 (s, 6H, 1-Me$_2$), 1.52 (t, J 6, 2H, 2-H$_2$), 1.81/1.90/1.93 (s, each 3H, 5/9/13-Me), 2.13 (m, 2H, 3-H$_2$), 3.5 (m, 1H, 15-H), 3.57 (m, 1H, 15-H), 4.07 (m, 1H, 14-H), 5.77 (t, J 4, 1H, 4-H), 6.21 (d, J 12, 1H, 12-H), 6.37 (d, J 15, 1H, 10-H), 6.38 (d, J 12, 1H, 7-H), 6.55 (dd, J 15, 12, 1H, 11-H), 6.74 (d, J 12, 1H, 8-H); olefinic protons in CD_3CN δ 5.79 (t, J 4, 1H, 4-H), 6.17 (d, J 12, 1H, 12-H), 6.38 (d, J 12, 1H, 7-H), 6.42 (d, J 17, 1H, 10-H), 6.56[4] (dd, J 17, 12, 1H, 11-H), 6.76 (d, J 12, 1H, 8-H); HRMS 302.2256 (M^+), calcd 302.2246.

The synthesis of enantiomeric (14*S*)-14-hydroxy-4,14-*retro*-retinol (**7**) was performed similarly using as key intermediate ethyl (2*S*,3*R*)-3-methyl-4-hydroxyepoxycrotonate (β-epoxide) obtained by using diethyl D-(−)-tartrate.

The synthesis of (14*RS*)-14-hydroxy-*retro*-retinol was carried out by LAH reduction of racemic ethyl 14-hydroxy-4,14-*retro*-retinoate obtained by Darzens reaction (Oediger & Eiter, 1964) between β-C_{18}-ketone (Meyer & Isler, 1971) and ethyl chloroacetate.

Bioassay. Synthetic 14-HRR stereoisomers and natural 14-HRR were tested for biological activation on the lymphoblastoid 5/2 cell line and on murine thymocytes as described (Garbe et al., 1992).

Natural 14-HRR was isolated by reversed-phase HPLC from the retinoid fraction extracted from HeLa cells (Buck et al., 1991b). The 5/2 lymphoblastoid cell line was derived by transformation of B lymphocytes of a normal donor by Epstein–Barr virus (Buck et al., 1990); T cells were obtained from 4–6-week-old male BALB/c mice, from the Sloan-Kettering mouse breeding colony.

RESULTS AND DISCUSSION

Synthesis of (14R)-14-HRR (**6**). Ethyl (14*R*)-14-hydroxy-4,14-*retro*-retinoate (14α-OH, **12**) was obtained in 82% yield as a mixture of isomers where the all-trans analog was predominant (ca. 75%). The 6*E* configuration of the major isomer **12** was established on the basis of an approximately 15% nuclear Overhauser effect (NOE) between 1-Me$_2$ and 8-H; it was also corroborated by the chemical shift of 4-H (5.80 ppm) and 1-Me$_2$ (1.3 ppm), both downfield compared to the reported values of 5.65 and 1.11 ppm, respectively, for

[2] The ca. 5% impurity seen in the NMR spectrum is diethyl tartrate which elutes after, but very closely to, the hydroxycrotonate **9**.

[3] The yield of the reaction varied from 55% to 70% due to rapid decomposition of 14-HRR after concentration of the ether extract and very likely during column chromatography.

[4] Erratum: in Figure 3 of Buck et al. (1991b) the chemical shift of 11-H should read 6.56 ppm and not 5.56 ppm.

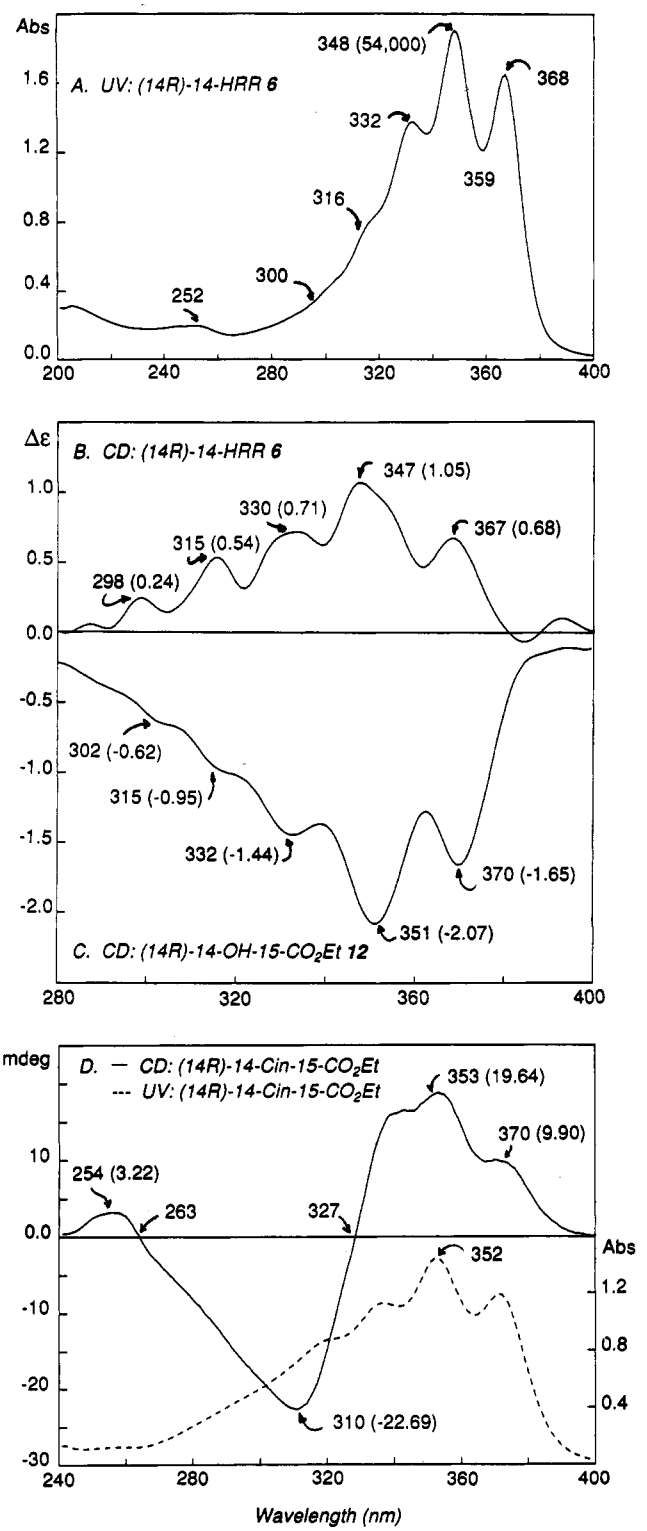


FIGURE 1: (A) Absorption spectrum of all-trans (14*R*)-14-HRR (**6**) in methanol. Circular dichroism spectra of (B) all-trans (14*R*)-14-HRR (**6**), and (C) the corresponding ethyl (14*R*)-14-hydroxy-*retro*-retinoate (**12**) in methanol. (D) CD spectrum of all-trans ethyl (14*R*)-14-(*p*-methoxycinnamoyl)-*retro*-retinoate showing a first positive Cotton effect characteristic of a 14*R* configuration. The Δε values of the CD curves in (B) and (C) were both estimated using a concentration derived from 54 000, the ε value of all-trans 14-HRR.

the 6*Z*-*retro*-retinoids (Vetter et al., 1971; Englert & Vecchi, 1982). The 8*E* configuration was confirmed by a 14% NOE between 8-H and 10-H; the *E* configuration of the 10-ene follows from the observed coupling constants $J_{10,11}$ = 15 Hz; the configuration of the12-ene is deduced from the 6.28 ppm chemical shift of 12-H, downfield compared to 6.16 ppm for the 12*Z* isomer. From ^{1}H NMR analysis of the mixture, the other isomers are tentatively assigned as 6*Z* (< 5%; 4-H moved upfield to 5.65 ppm), 8*Z* (10-H moved downfield to 6.91 ppm, J = 15 Hz; ca. 8%), and 12*Z* (12-H moved upfield to 6.16 ppm, d, J = 11 Hz; 11-H moved downfield to 6.7 ppm, dd, J = 15, 11 Hz; ca. 15%).

LAH reduction of the ethyl *retro*-retinoate mixture led to (14*R*)-14-HRR (**6**), with similar isomeric composition. The all-trans major isomer, isolated by HPLC, exhibited the same ^{1}H NMR spectrum as the natural product (Buck et al., 1991b).[4] 14-HRR is very labile; it decomposes readily when kept neat; however, it can be stored for months in solution in methanol or ether at −78 °C and under argon. Exposure to light isaccompanied by slow double bond isomerization and increase in the 6*Z* isomer ratio.

The preparation of (14*S*)-14-HRR (**7**) proceeded similarly. However, (14*S*)-14-HRR was obtained with a higher optical purity since the key β-epoxide was >98% ee. The synthesis of racemic 14-HRR using the Darzens reaction led to a similar isomeric mixture.

UV and CD Spectra. Figure 1A shows the absorption spectrum of all-trans (14*R*)-14-HRR (**6**) in methanol; it exhibits a small cis peak at 252 nm and a strong peak at 348 nm with a well-resolved vibronic structure, characteristic of the retro structure. Since HPLC-purified 14-HRR partly decomposes upon drying, its extinction coefficient of 54 000 at 348 nm has been estimated by directly computing the ratios of the integrated HPLC peaks of [^{3}H]retinol and [^{3}H]-14-HRR from UV absorption and specific radioactivity. Its isomeric purity can be quickly assessed by the ratio value of 1.40 of the first peak (368 nm) intensity to that of the first valley (359 nm); contamination by cis isomers is accompanied by a significant decrease in this ratio, a more diffuse vibronic structure, a slight hypsochromic shift (348 to 346 nm), and an increase in the 252-nm cis-band intensity.

The UV spectrum of the corresponding all-trans ethyl (14*R*)-14-hydroxy-*retro*-retinoate (**12**) (data not shown) presents similar characteristics. The same observations also apply to (14*S*)- and (14*RS*)-14-hydroxy-*retro*-retinoates and 14-HRR.

The circular dichroism (CD) spectrum of all-trans (14*R*)-14-HRR (**6**) (Figure 1B) exhibits a positive Cotton effect similar to the CD spectrum of natural 14-HRR (Buck et al., 1991b). However, the estimated Δε of the 347-nm peak (1.05) is ca. 10-fold stronger than that of the natural product (0.10); from this we may conclude that the 14-HRR isolated from lymphoblastoid cells (Buck et al., 1991b) was a mixture of enantiomers where the 14*R* isomer predominated only slightly.

The CD spectrum of the corresponding all-trans ethyl (14*R*)-14-hydroxy-*retro*-retinoate (**12**) is shown in Figure 1C. Surprisingly, the sign of the Cotton effect is now negative and opposite to that of (14*R*)-14-HRR (**6**). The reversal in sign upon conversion of the 15-ester to its 14,15-diol clearly shows that hydrogen bonding and other factors are involved and that the 14*R* configuration tentatively assigned to natural 14-HRR (Buck et al., 1991b) on the basis of the "allylic hydroxyl" Cotton effect (Beecham, 1971) was fortuitous. In order to confirm the *R* configuration at this center, we prepared the 14-*p*-methoxycinnamoyl ester (Wiesler et al., 1986) of **12**. The CD spectrum of ethyl 14-(*p*-methoxycinnamoyl)-*retro*-retinoate exhibited a prominent split CD centered at 327 nm due to the exciton coupling (Harada & Nakanishi, 1983) between the polyene and the cinnamate chromophores,

[4] The involvement of CRBP in biochemical reactions is exemplified by the esterification of the retinol–CRBP complex by the retinol acyltransferase LRAT (MacDonald & Ong, 1990).

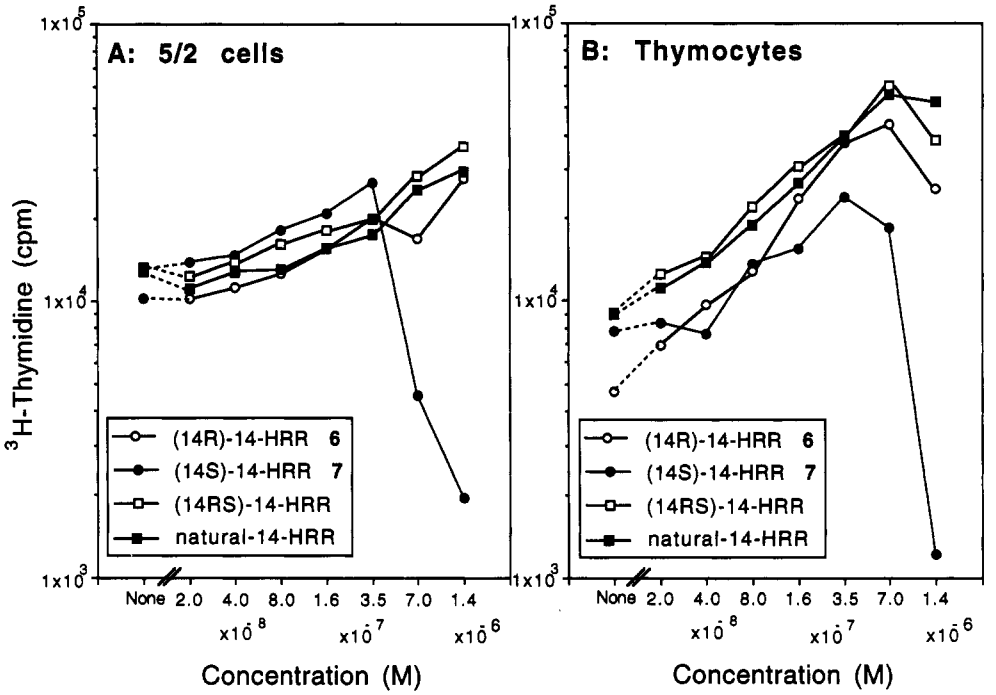


FIGURE 2: The all-trans (14*R*)-, (14*S*)-, and (14*RS*)-HRR and natural all-trans 14-HRR were serially diluted and added to cultures of cells in serum-free ITLB medium at the final concentrations indicated. Target cells were (A) lymphoblastoid 5/2 cells at 25 000 cells per microculture or (B) BALB/c thymocytes at 300 000 cells per microculture, stimulated by plastic-adhered anti-CD3 antibody (clone 2C11) as described (Garbe et al., 1992). B-cell cultures were pulsed with 0.5 μCi of [^{3}H]thymidine after 24 h of culture and harvested for determination of incorporation of thymidine into DNA 24 h later. T-cell cultures were treated likewise, except that the [^{3}H]thymidine pulse was initiated 72 h after onset of culture and terminated 4 h later. All cultures were performed in triplicate, and standard derivations did not exceed 20%.

λ_{max} 348 and 311 nm, respectively (Figure 1D). The positive sign of the first couplet (Gonella et al., 1982) is in full agreement with the 14*R* absolute configuration in **12**.

The CD spectrum of (14*S*)-14-HRR (**7**) showed a negative Cotton effect, while the corresponding ethyl (14*S*)-14-hydroxy-*retro*-retinoate exhibited a positive Cotton effect.

Biological Activity. B lymphoblastoid cells were shown previously to require retinol (10^{-6} M optimum concentration) for sustained growth (Buck et al., 1990, 1991a), and the retinol derivative 14-HRR was found to substitute effectively for retinol at 10–30-fold lower concentration. These findings supported the argument that 14-HRR served as the internal mediator of the retinol effect. We tested natural and synthetic all-trans (14*R*)-, (14*S*)-, and (14*RS*)-14-HRR in two bioassays, the first predicated on vitamin A-starved B lymphocytes and the second on normal murine thymocytes stimulated by a cross-linking monoclonal antibody to the CD3ϵ peptide of the T-cell receptor complex. Surprisingly, all 14-HRR compounds, regardless of their absolute configuration at C-14 or source as natural or synthetic compounds, showed activity and an optimal range from 7×10^{-7} to 1.6×10^{-7} M, in a B-cell survival (Figure 2A), or a T-cell activation assay (Figure 2B). Although the *R* form appears to generate a higher amplitude of stimulation, these types of bioassays are basically semiquantitative in nature, and therefore the conclusion that the *R* form is superior to the *S* form is only tentative.

Moreover, 14-HRR extracted for structural analyses from cultured B cells or HeLa cells was formed under nonphysiological conditions; namely, the concentration of retinol added to the cell culture was ca. 10^{-5} M. Due to the limited buffering capacity of the cell culture medium, the concentration of retinol in the cell pellet of B cells or HeLa cells increases from (1–2) $\times 10^{-7}$ M to about 10^{-3} M, and the concentration of 14-HRR increases from (1.2–2.8) $\times 10^{-8}$ M to (5–10) $\times 10^{-6}$ M. Therefore, under nonphysiological conditions, the cellular retinol concentration overwhelms that of its cellular binding protein, CRBP [(1–3) $\times 10^{-7}$ M], and thus most retinol is free or nonspecifically bound to membranes and nonspecific cellular proteins. If we speculate that the biosynthesis of 14-HRR occurs via epoxidation of the 13,14-ene, epoxidation of CRBP-bound retinol should be stereospecific[5] and should lead to optically pure 14-HRR, whereas free retinol would lead to racemic epoxides and racemic 14-HRR. The CD spectrum reported earlier (Buck et al., 1991b) originates from 9 μg of 14-HRR obtained from lymphoblastoid 5/2 cells grown in medium containing ca. 10^{-6} M retinol; current CD studies of pure (14*R*)- and (14*S*)-14-HRR have shown that this natural specimen was a mixture of enantiomers with a slight excess of the 14*R* species. In order to increase the yield of 14-HRR, all subsequent cultures were carried out with a 10^{-5} M retinol concentration; this led to racemic 14-HRR. Physiologically produced 14-HRR could well be a pure enantiomer; however, the amount extracted under physiological conditions (i.e., cells grown in medium containing 10% serum) is minute (37 ng from a 4-mL cell pellet containing ca. 3×10^9 cells) and does not allow spectroscopic measurements. The decision as to which enantiomer is physiologically produced must await the isolation and characterization of the enzyme system producing 14-HRR and of the 14-HRR receptor. These aspects together with studies on the biosynthetic pathway are currently under investigation.

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