Chemical and biological studies on 5,6-epoxyretinol, retinol, and their phosphoryl esters

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Abstract Studies are reported on chemical synthesis, ultraviolet absorption spectral characteristics, and mass spectral fragmentation of 5,6-epoxyretinol and 5,6-epoxyretinylphosphate. These compounds were separated from each other and from other retinoids by a reverse phase high pressure liquid chromatographic system. A comparative study on the lability to acid of 5,6-epoxyretinylphosphate and retinylphosphate was conducted. The retroretinoid anhydroretinol is formed chemically from retinylphosphate by acid hydrolysis and biologically from retinol in cultured, spontaneously-transformed mouse fibroblasts, 3T12 cells. Similarly, acid hydrolysis of 5,6-epoxyretinylphosphate (absorption maxima 324, 310, 296 nm) in methanol yielded a low polarity retinoid with absorption maxima at 364, 346, and 330 nm, similar to the absorption spectra of retrovitamin A_1 and retrovitamin A_2. Mass spectral analysis was found to be in agreement with a retrostructure and permitted identification of the compound as a methoxyretrovitamin A_1 methyl ether. A similar retroretinoid was formed biologically from 5,6-epoxyretinol in spontaneously-transformed mouse 3T12 cells. Thus, it appeared that these cells have the ability to convert the primary alcohols into retroretinoids, which are also formed by acid treatment of the phosphate esters. The adhesive properties of 3T12 cells were highly enhanced by culturing in the presence of 10^{-6} to 10^{-5} M 5,6-epoxyretinol or -retinoic acid, in analogy with the response of these cells to the parent retinoids. Moreover, in another test of biological activity, 5,6-epoxyretinylphosphate functioned as a highly active acceptor of [^{14}C]D-mannose from GDP-[^{14}C]mannose in a reaction catalyzed by rat liver membranes. Thus, 5,6-epoxyretinoids appear to be as active as the parent retinoids in these in vitro tests of biological activity, even though they do not replace vitamin A in its growth function in vivo.—**Bhat, P. V., P. P. Roller, and L. M. De Luca.** Chemical and biological studies on 5,6-epoxy-retinol, and their phosphoryl esters. *J. Lipid Res.* 1981. **22:** 1069–1078.

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5,6-Epoxy-derivatives of retinol, retinal, and retinoic acid were first synthesized chemically by Jungalwala and Cama (1), Morgan and Thompson (2), and John, Lakshmanan, and Cama (3), but reports of their biological activity were conflicting and they were eventually found to be less active than the intact vitamin in vision and in growth (2–4). More than a decade later, H. F. De Luca and collaborators (5) found 5,6-epoxyretinoic acid to be a normal metabolite of retinoic acid in rat intestine. However, these authors also reported that the biological activity of this compound in vivo was only 0.5% of the activity of vitamin A (6) in maintaining growth.

Here we report that 5,6-epoxy-derivatives of vitamin A are biologically active in enhancing adhesive properties of cultured, spontaneously-transformed mouse fibroblasts. We also report on the metabolism of 5,6-epoxyretinol by these cells, the chemical synthesis of 5,6-epoxyretinylphosphate and its mannosyl acceptor activity in rat liver microsomal membranes. A comparative study of retinylphosphate (RP) and 5,6-epoxyretinylphosphate (eRP) for their lability to acid is also reported.

EXPERIMENTAL PROCEDURES

Preparation of 5,6-epoxymethylretinoate, 5,6-epoxyretinol, and its phosphate

Previously published methods (1–3) were used for the synthetic procedures with minor modifications. The yield in the chemical synthesis of monoperphthalic acid from phthalic anhydride was 65%. Preparation of methyl 5,6-epoxy-methyl retinoate

Abbreviations: RP, retinylphosphate; MRP, mannosylretinylphosphate; AR, anhydroretinol; eRP, 5,6-epoxyretinylphosphate; MeRP, mannosyl 5,6-epoxyretinylphosphate; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.

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from methyl retinoate (from Hoffman-La Roche, Inc., Nutley, NJ) (700 mg) yielded 120 mg of the product.

Reduction of methyl 5,6-epoxyretinoate to 5,6-epoxyretinol

Methyl 5,6-monoepoxyretinoate (20 mg) was treated with $LiAlH_4$ (60 mg) in 40 ml of dry, peroxide-free diethylether at 4°C. The reaction was run for 35 min. Excess $LiAlH_4$ was degraded by cold water at 4°C. The mixture was extracted with diethylether and the ethereal layer was washed and then dried over anhydrous sodium sulfate. The 5,6-epoxyretinol was purified further by column chromatography on 5% (v/w) water-deactivated alumina, with maximum absorbance at 310 and 325 nm in light petroleum as reported previously (1, 2).

Preparation of 5,6-epoxyretinoic acid

Methyl 5,6-epoxyretinoate (30 mg) was saponified with 1.0 ml of ethanolic potassium hydroxide (10% (w/v) for 1 hr under nitrogen. The unsaponified ester was extracted with light petroleum. Acetic acid (1 N) was added dropwise to the aqueous layer with stirring until the solution became acidic. The liberated 5,6-epoxyretinoic acid was extracted with diethylether. The ethereal layer was washed free of acid, dried over sodium sulfate, reduced in volume, and chromatographed on a column (2 × 25 cm) of 10 g of silicic acid. The compound 5,6-epoxyretinoic acid was eluted with 3% diethylether in light petroleum. The yellow crystalline compound was obtained from light petroleum at −30°C. Yield was about 20 mg.

Preparation of 5,6-epoxyretinylphosphate

The equivalent of 40.0 mg of 5,6-epoxyretinol was used for the preparation of 5,6-epoxyretinylphosphate (eRP) following published procedures (7). The yield in eRP expressed in 5,6-epoxyretinol equivalents was about 9% based on UV absorption at 310 nm.

Acid hydrolysis of 5,6-epoxyretinylphosphate and retinylphosphate

5-6-Epoxyretinylphosphate or retinylphosphate (25 μg) was incubated with 0.01 M, 0.1 M, and 0.5 M HCl for 20 min at 37°C in methanol. The reaction mixture was neutralized with sodium hydroxide and made 2:1 (v/v) in chloroform–methanol. The products were washed with water to remove salt, dried, and dissolved in 1.0 ml of absolute methanol; 100 μl was used for spectral studies. Hydrolysis of eRP was measured by the disappearance of absorption at 310 nm and the appearance at 364 nm. RP hydrolysis was followed by disappearance of UV absorption at 325 nm and appearance at 368 nm, or by monitoring the loss of absorption at 325 nm off the ODS column by HPLC. In a separate experiment, 200 μg of eRP was dissolved in 2.0 ml of chloroform–methanol 1:4 at 37°C, rendered 0.1 M in HCl, and incubated at 37°C for 25 min. The sample was brought to 4°C, neutralized with NaOH, and adjusted to contain chloroform–methanol 2:1 by adding 2.45 ml of chloroform. Two phases were obtained by adding 0.8 ml of water after mixing. The lower phase containing the hydrolysis product was dried, and the residue was dissolved in petroleum ether and applied to a column of water-deactivated alumina (5% water, v/w). In this procedure anhydroretinol obtained from acid hydrolysis of RP is eluted in 250 ml of petroleum ether. Free alcohols, such as 5,6-epoxyretinol, are eluted in 25% diethylether in petroleum ether (150 ml) and 5,8-oxyretinol is eluted in diethylether (150 ml).

Alkaline treatment of 5,6-epoxyretinylphosphate and retinylphosphate

To 25 μg of eRP or RP dried under nitrogen from a methanolic solution, 250 μl of chloroform–methanol 1:4 and 25 μl of 1 N NaOH were added as described previously (8) for the hydrolysis of retinylphosphate. The tubes were incubated at 37°C for 20 min. Chloroform (350 μl) was added to bring the ratio of chloroform–methanol to 2:1 and a two-phase system was obtained after addition of 0.1 ml of water. The upper phase was again extracted with chloroform–methanol 2:1 and the lower phase and the wash from the upper phase were combined. A portion was taken for spectrophotometric studies. Another portion was chromatographed on a thin layer of silica gel along with standard RP, eRP, and AR in the solvent system toluene–chloroform– methanol 4:1:1.

Activity of 5,6-epoxyretinol and 5,6-epoxyretinoic acid in promoting the adhesion of spontaneously-transformed mouse fibroblasts

Balb/c 3T12 mouse fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and cultured in T25 flasks (Falcon Plastics, Oxnard, CA) in 3 ml of medium, and the adhesion assay was conducted as described previously (9). All-*trans*-retinoic acid (Eastman Kodak Co., Rochester, NY) was dissolved in DMSO (Pierce Chemical Co., Rockford, IL) at a concentration of 2 mg/ml or less, and the solution (freshly made every week) was stored in the dark at room temperature. Retinoic acid in DMSO, DMSO alone, or nothing was added to the culture medium 24 hr after cells were plated, unless stated otherwise. DMSO concentration in the culture medium was 0.5%. 5,6-Epoxyretinol in DMSO was given to cells at 0.1 μg/ml and 10 μg/ml. A similar

study was conducted using 5,6-epoxyretinoic acid at the same concentrations.

Metabolism of 5,6-epoxyretinol by 3T12-3 fibroblasts

Mouse fibroblasts (3T12 cells) were plated at 10,000 cells/cm² in 12 plastic dishes (15 cm diameter). 5,6-Epoxyretinol (10 μg/ml) (20 ml per dish) was added to the cultures 3 days after plating and the cells were cultured in the presence of the retinoid for 34 hr. At this time, cells were at a density of about 30,000 cells/cm² and were scraped from the plate, pelleted, and rinsed two times with PBS and collected by centrifugation. Cells were resuspended in PBS, homogenized, and lyophilized. The dry lyophilized powder was extracted with 150 ml of 99% methanol. This extract was dried and the residue was dissolved in a small volume of methanol and diluted 100-fold with hexane before application to a column of alumina (5% (v/w) water-deactivated). The column was first eluted with 150 ml of hexane, then with 2% acetone in hexane. The 2% acetone in hexane eluate was evaporated to dryness, redissolved in 2:1 acetone–hexane, and rechromatographed on a short column of alumina. Five fractions were collected: *1*), hexane (50 ml); *2*), 1% diethylether in hexane (50 ml); *3*), 3% ether in hexane eluted a compound with absorption maxima at 364, 346, and 330 nm; *4*), 5% in hexane eluted a small amount of the same compound as in fraction 3 and a major compound having an absorption maxima at 278 nm; *5*), 100% diethylether. HPLC analysis of these metabolites is described later.

Preparation of [^{14}C]mannosylretinylphosphate and [^{14}C]mannosyl-5,6-epoxyretinylphosphate

Retinylphosphate or 5,6-eRP (20 μg) was dried under nitrogen in a test tube to which was added 20 μl each of the following solutions: 5% Triton X-100; 0.025 M EDTA; 0.3 M Tris-HCl buffer, pH 8.5; 0.1 M $MnCl_2$; a solution of 22 mg of ATP/ml H_2O; 1 μCi of guanosine diphospho-[^{14}C]mannose (final conc. 26 μM); and 100 μl of a suspension of 2 mg of microsomal rat liver membrane proteins. The final volume was 200 μl at pH 7.5. Incubation proceeded at 37°C for 30 min. The reaction was stopped by the addition of 19.8 ml of methanol. The volume was brought to 50 ml by 99% methanol and placed on a column (0.5 × 5 cm) of DEAE-cellulose acetate. The column was then eluted with about 150 ml of 99% methanol. [^{14}C]Mannosylretinylphosphate was eluted in 50 ml of 10 mM ammonium acetate in 99% methanol. The 10 mM ammonium acetate eluates were then analyzed by chromatography on thin layers of silica gel in chloroform–methanol–water 60:35:6. The same procedure was used for incubations containing 5 μM UDP-[^{3}H]galactose in which 10 μCi of radioactivity was used. Six incubations were prepared as described, with the following changes: *1*), GDP-[^{14}C]mannose (26 μM), control; *2*), same as *1*, plus (150 μM) RP; *3*), same as *1*, plus 5,6-eRP (150 μM); *4*), UDP-[^{3}H]galactose (5 μM), control; *5*), same as *4*, plus RP (150 μM); *6*), same as *4*, plus 5,6-eRP (150 μM).

Mass spectrometry studies

These were conducted on a JEOL-made JMS-01SG-2 mass spectrometer in the solid probe at the indicated temperature and ionizing voltage.

Separation of 5,6-epoxymethylretinoate; 5,6-epoxyretinol, and 5,6-epoxyretinylphosphate from each other and from other retinoids by high pressure liquid chromatography

The HPLC system used was similar to that utilized to separate various retinoids and described previously (7). High pressure liquid chromatography was performed on an Altex Model 332 MP programmable liquid chromatography system. The sensitivity of this system is dependent on the recorder, a linear Model 300 with a maximum AUFS of 0.01. The UV spectrophotometer was a Hitachi Model 100-30 equipped with a variable wavelength between 195 and 850 nm. Fluorometer Model FS 970 from the Schoeffel Instrument Corporation (Westwood, NJ) was also connected to the HPLC system, in series and before the spectrophotometer. The high pressure liquid chromatography columns were obtained from Whatman Inc., Clifton, NJ. A Partisil-10-ODS column (4.6 mm internal diameter × 25 cm) was used. The column was eluted with acetonitrile–water 55:45 at an initial flow rate of 1.0 ml/min for the first 10 min to elute eRP and retinoic acid; 3.5 ml/minute for the following 20 min eluted 5,6-epoxyretinol, 5,6-epoxymethylretinoate, retinol, retinal, retinylacetate and anhydroretinol. The solvent ratio was then changed to acetonitrile–water 98:2 and the flow rate was adjusted to 1.2 ml/min for an additional 15 min to elute retinylpalmitate.

RESULTS

Preparation of 5,6-epoxymethylretinoate, 5,6-epoxyretinol and its phosphate

Oxidation of methylretinoate to 5,6-epoxymethylretinoate by monoperphthalic acid was monitored by the shift in UV absorption spectrum from an absorption maximum of about 350 nm in petroleum ether to a compound with absorption maximum at 352, 338,

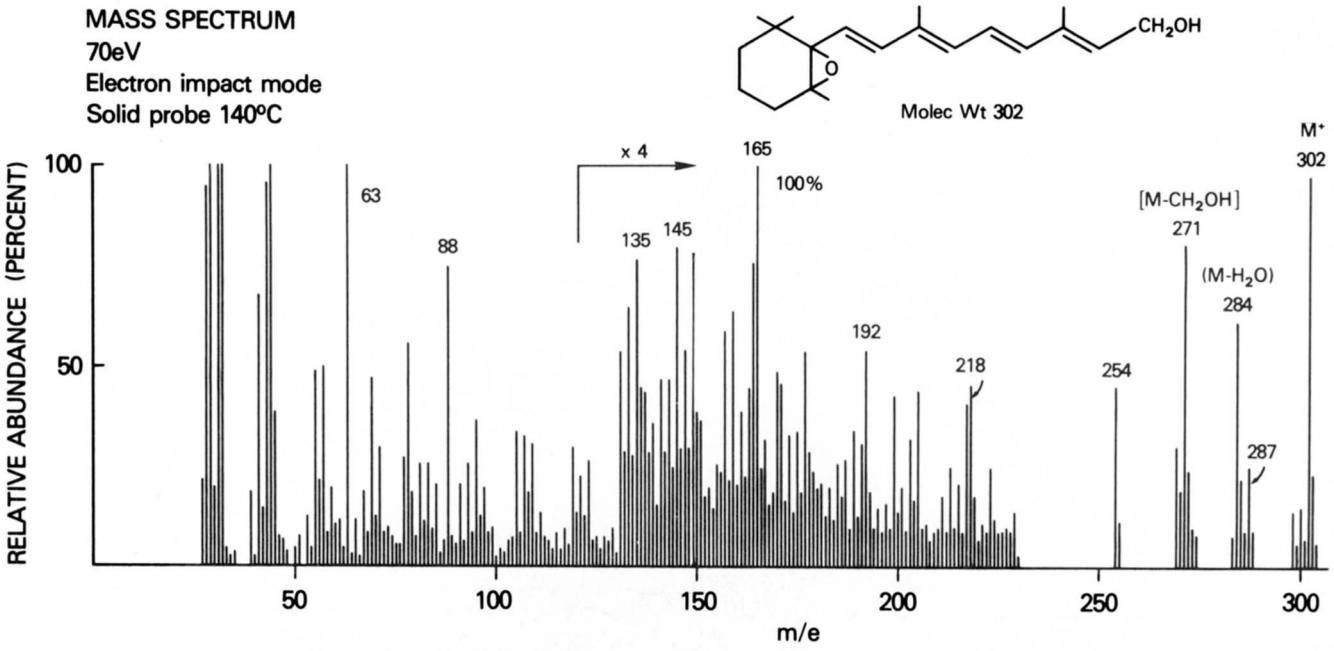


Fig. 1. Mass spectrum of 5,6-epoxyretinol.

and a shoulder of about 324 nm. After purification and crystallization from methanol, the spectrum of the product in cyclohexane showed absorption peaks at 356, 342, and a shoulder at 325–330 nm in agreement with previous reports for 5,6-epoxymethylretinoate (2, 3). The yield was about 20% of the starting material.

Reduction of 5,6-epoxymethylretinoate by $LiAlH_4$ yielded 5,6-epoxyretinol in yields of 75%. Mass spectrometry studies confirmed the structure (**Fig. 1**). The molecular ion was found at m/e 302, and the significant ion at m/e 284 corresponded to M-H_2O. Other ions, known to be characteristic of 5,6-epoxyretinoids (10) were found at m/e 271, 217, 177, 165, 164, 149.

The UV absorption spectrum of 5,6-epoxyretinol in methanol did not change from day 1 to day 4 of storage at 4°C in the dark. The UV absorption spectra showed the typical triplet at 324, 310, and 296 nm (1).

When kept in DMSO (the solvent used for the biological studies), 5,6-epoxyretinol was stable for up to 3 days at room temperature as indicated by its identical UV absorption spectrum read in methanol at day 1 and 3 of storage (results not shown). In light petroleum 5,6-epoxyretinol gives absorption maxima at 310 and 325 mm (1).

Hydrolysis of 5,6-epoxyretinylphosphate and retinylphosphate

Phosphorylation of 5,6-epoxyretinol yielded (9%) 5,6-epoxyretinylphosphate. The UV absorption spectrum of this compound in methanol is shown in **Fig. 2 A** and is identical with the spectrum of 5,6-epoxyretinol. eRP is stable to 0.1 N NaOH (37°C, 20 minutes) as shown in Fig. 2 B and **Table 1.** It is also resistant

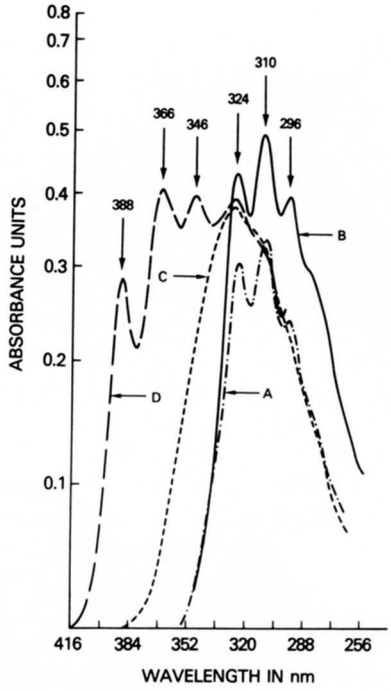


Fig. 2. A) UV absorption spectrum of intact 5,6-epoxyretinylphosphate (2 μg/ml) in methanol before and B) after 0.1 N NaOH treatment. C) UV absorption spectrum of intact retinylphosphate (2 μg/ml) before and D) after 0.1 N NaOH treatment and "neutralization" by acetic acid.

TABLE 1. Studies on base and acid treatment of RP and eRP

		Conditions	% Cleavage RP	% Cleavage eRP
	M			
NaOH	0.1[a]	37°C, 20′	0	0
HCl	0.01[b]	37°C, 20′	100	0–5
	0.1	37°C, 20′	100	90
	0.5	37°C, 20′	100	100
	0.01[c]	37°C, 10′	100	
	0.01	37°C, 5′	100	
	0.01	37°C, 3′	100	
	0.01	37°C, 2′	95	
	0.01	37°C, 1′	90	
CH_3COOH	0.01[d]	37°C, 10′	100	
	0.01	37°C, 5′	100	
	0.01	37°C, 1′	35	
	0.01[e]	20°C, 14′	0	
	0.01	20°C, 5′	0	
	0.005	20°C, 10′	0	

[a,b] Conditions are described under Experimental Procedures.

[c] Ten μg RP was dissolved in 225 μl of chloroform–methanol 1:4. This solution was equilibrated at 37°C before adding 25 μl of an aqueous solution of 0.1 N HCl. The reaction was stopped in dry ice. The extent of cleavage was measured by the loss of absorbance at 325 nm in the area of elution of RP off ODS column by reverse phase HPLC (7).

[d,e] Methods were essentially the same as for *c*.

to 0.01 M HCl (37°C, 20 min) under conditions that caused complete cleavage of RP (UV max 325 nm, Fig. 2 C and Table 1) to anhydroretinol (UV maxima 346, 366, 388 nm, figure 2 D). However, eRP was labile to 0.1 N HCl (37°C, 20 min) (Table 1), yielding products which absorbed maximally at 364, 346, and 278 nm (**Fig. 3 A**). These products could be fractionated by chromatography on a column of water-inactivated alumina (5%) in two peaks. The less polar peak was eluted in 5% ether in hexane and displayed the triplet at 364, 346, and 330 (Fig. 3 B). Mass spectral analysis of this product was performed on a solid probe at 70 eV electron impact and a sample temperature of 145°C. The significant data can be summarized as follows: m/e 330 (57%, M+), 315 (3% M − CH_3), 298 (5%, M − CH_3OH), 285 (100%, M − CH_2OCH_3), 254 [29%, M − (CH_2OCH_3 + OCH_3], 253 [30%, M − (CH_2OCH_3 + CH_3OH], 239 (15%), and 197 (44%) (**Fig. 4**).

Biological activity of 5,6-epoxyretinol

Fig. 5 shows that 5,6-epoxyretinol (A) and 5,6-epoxyretinoic acid (B) are active in inducing adhesive properties of spontaneously-transformed mouse fibroblasts (Balb/c 3T12-3 cells) at concentrations above 10^{-6} M in this assay system. Cells displayed a flatter morphology than DMSO-treated controls. A preliminary report of these data has been published (9).

Metabolism of 5,6-epoxyretinol by 3T12 cells

It was of interest to study the metabolism of 5,6-epoxyretinol by 3T12 cells, inasmuch as these cells can convert retinol into its dehydrated product anhydroretinol (11).

Fig. 6 A shows the UV absorption spectrum of 5,6-epoxyretinol used for the metabolic studies, with the typical maxima at about 324, 310, and 296 nm. The total cellular lipid extract, 34 hr after addition of the retinoid to the cells, gave the spectrum shown in Fig. 6B, with maxima at 366, 324, 310, 290, and 278 nm. Fractionation of this extract on columns of alumina was achieved as described under Experimental Procedures. The least polar fraction eluted with 3% diethylether in hexane gave the spectrum shown in Fig. 6C with maxima at 364, 346, and 330 nm. This spectrum is identical to that obtained for the product of acid hydrolysis of eRP (Fig. 3B). The more polar fraction eluted with 5% ether in hexane displayed an absorption maximum of 278 nm (Fig. 6 D), identical to that of 5,8-oxyretinol, usually formed by acid treatment of 5,6-epoxyretinol (1,2) and also observed as

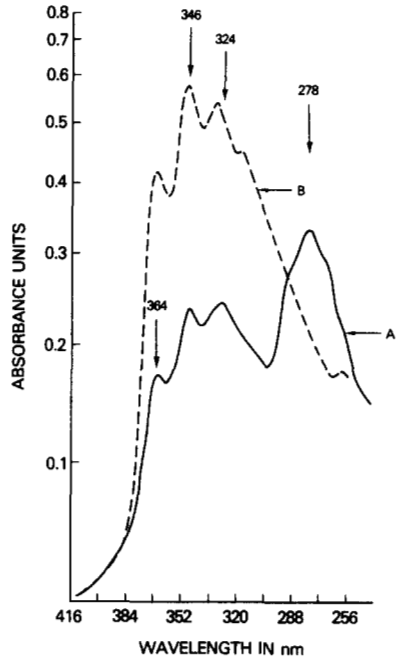


Fig. 3. A, Ultraviolet-absorption spectra of product of acid hydrolysis (0.1 M HCl at 37°C for 20 min) of 5,6-epoxyretinylphosphate. The conditions of hydrolysis are described in Experimental Procedures. B, Absorption spectrum of the fraction obtained from alumina column chromatography by elution with 5% ether in hexane. The mixture of compounds obtained after acid hydrolysis of 5,6-epoxyretinylphosphate was subjected to alumina column chromatography as described in Experimental Procedures and the column was first eluted with hexane and then with 5% ether in hexane. An aliquot of 5% ether in hexane fraction was taken for UV absorption spectroscopy.

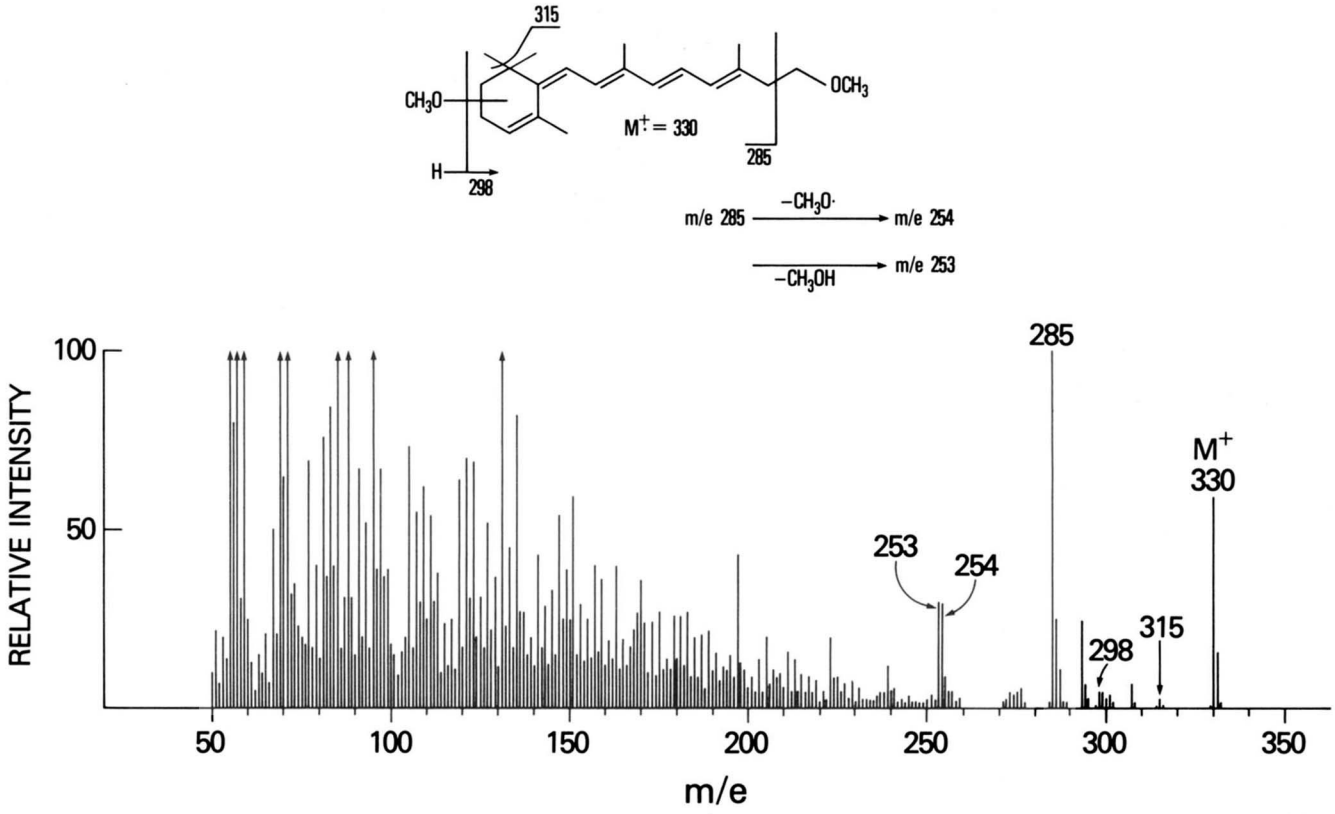


Fig. 4. Mass spectral fragmentation pattern of the product of mild acid hydrolysis of 5,6-epoxyretinylphosphate.

one of the products formed in the acid hydrolysis of eRP (Fig. 3 A).

HPLC analysis studies

Fig. 7 shows the separation of the various retinoids obtained by reverse phase HPLC on a partisil column (ODS (C-18 bonded phase)). eRP is eluted between 2 and 3 min, before retinoic acid at the same position as RP. Its elution was monitored by UV absorption at 350 nm and fluorescence emission at 470 nm. 5,6-Epoxyretinol was eluted at about 11 min and 5,6-epoxymethylretinoate at about 14 min; this latter compound does not fluoresce. The product of mild acid hydrolysis of eRP is eluted at 19 min, as expected for a compound with intermediate polarity between retinol and retinyl acetate. This compound absorbs at 350 nm, but does not fluoresce at 470 nm. The least polar metabolite of 5,6-epoxyretinol obtained from 3T12 cells (absorption maxima at 364, 346, and 330 nm) was eluted with acetonitrile–water 98:2 just before retinylpalmitate, suggesting a fatty acid ester. This compound had the same spectral characteristics as the product of mild acid hydrolysis of eRP.

Mannosyl acceptor activity of eRP and RP

As for RP, eRP is also glycosylated to mannosyl eRP (**Table 2**). Stimulation over endogenous levels was 14-fold for RP and 36-fold for eRP (Table 2). This compound has the same chromatographic behavior as authentic MRP by DEAE-cellulose acetate, from which it is eluted with 10 mM ammonium acetate, and by chromatography on thin layers of silica gel in chloroform–methanol–water 60:35:6 at R_f 0.28. As for RP (12), eRP does not function as an acceptor of galactose from UDP-[^{3}H]galactose, at least in the liver microsomal system. Several [^{3}H]galactolipids were formed and eluted by 10 mM ammonium acetate; these products behaved as gangliosides upon chromatography on thin-layer plates of silica gel. No stimulation by either RP or eRP was seen in any of the products (results not shown).

DISCUSSION

Interest in the 5,6-epoxy derivatives of vitamin A has arisen since McCormick et al. (5) identified 5,6-epoxyretinoic acid as a physiological metabolite of retinoic acid, in the intestinal mucosa of rats. 5,6-Epoxyretinol, -retinal, and -retinoic acid were initially synthesized and characterized by Cama and collaborators (1, 3) and Morgan and Thompson (2). Methyl-5,6-epoxyretinoate given orally was found to be 35%

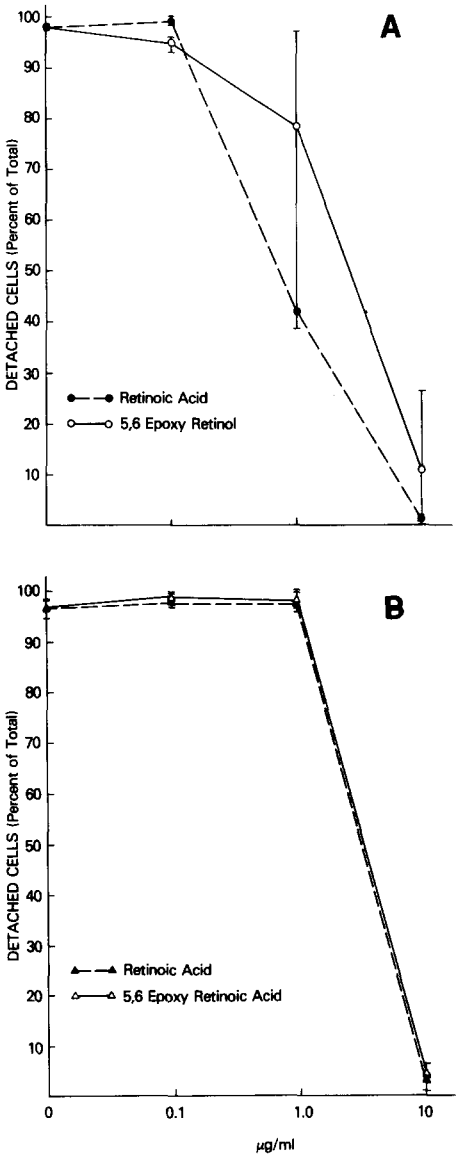


Fig. 5. Study of the effect of 5,6 epoxyretinol (A) and 5,6-epoxyretinoic acid (B) on the adhesive properties of spontaneously-transformed mouse fibroblasts (Balb/c 3T12-3 cells). The assay is described under Experimental Procedures.

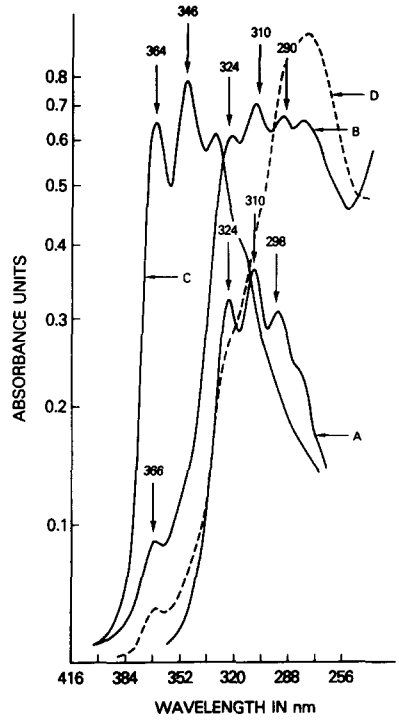


Fig. 6. A, UV absorption spectrum of 5,6-epoxyretinol (1.52 μg/ml) before incubation with 3T12 cells. B, UV absorption spectra of total lipid extract obtained from 3T12 cells after incubation with 5,6-epoxyretinol for 34 hr. Cells were incubated with 5,6-epoxyretinol, as described in Experimental Procedures. C, UV absorption spectrum of the fraction obtained by alumina column chromatography. The mixture of compounds obtained from 3T12 cells incubated with 5,6-epoxyretinol was subjected to alumina column chromatography and the 3% ether in hexane fraction was collected. An aliquot was taken for UV absorption spectroscopy. D, UV absorption spectrum of the 5% ether in hexane fraction obtained by alumina column chromatography.

as active in restoring growth of vitamin A-depleted rats as retinoic acid (2). In a similar experiment, oral 5,6-epoxyretinoic acid was shown to be less active than all-*trans*-retinylacetate (2). However, when the 5,6-epoxyretinoic acid was given intraperitoneally at doses of 0.5, 1.0, and 2.0 μg to vitamin A-deficient rats, it displayed a biopotency of 157% that of retinyl acetate (3). In contrast to these data, Pitt (13) observed that 5,6-epoxyretinal had 4.1% the biological activity of retinol and, upon reinvestigation (4), Krishna Mallia et al. attributed the high biological activity reported previously (3) to contaminating retinol. In concert with this view, a recent publication by Zile

TABLE 2. Synthesis of [^{14}C]mannosylretinylphosphate (MRP) and [^{14}C]mannosyl-5,6-epoxyretinylphosphate (MeRP) from GDP-[^{14}C]mannose by rat liver microsomal membranes

Incubation	Donor	Total [^{14}C]- or [^{3}H]CPM in 10 mM Fraction from DEAE-Sephacel	[^{14}C]MRP/30 min/mg Protein
1. −RP	GDP-[^{14}C]mannose	20,950	700
2. +RP	GDP-[^{14}C]mannose	75,150	10,000
3. +eRP	GDP-[^{14}C]mannose	115,250	25,400
4. −RP	UPD-[^{3}H]galactose	1,222,150	
5. +RP	UPD-[^{3}H]galactose	1,053,650	
6. +eRP	UPD-[^{3}H]galactose	1,074,050	

Conditions of incubation and isolation of products are described under Experimental Procedures. Thin-layer chromatography of the eluate obtained from DEAE-cellulose acetate at 10 mM ammonium acetate was performed in chloroform–methanol–water 60:35:6. The R_f for MRP and MeRP in this system is 0.28 and these compounds represented about 80% of the lipids in incubations 2 and 3. TLC of ^{3}H-labeled galactolipids in the same solvent system resolved several compounds with R_f typical of known gangliosides. None of these compounds was stimulated either by RP or by eRP.

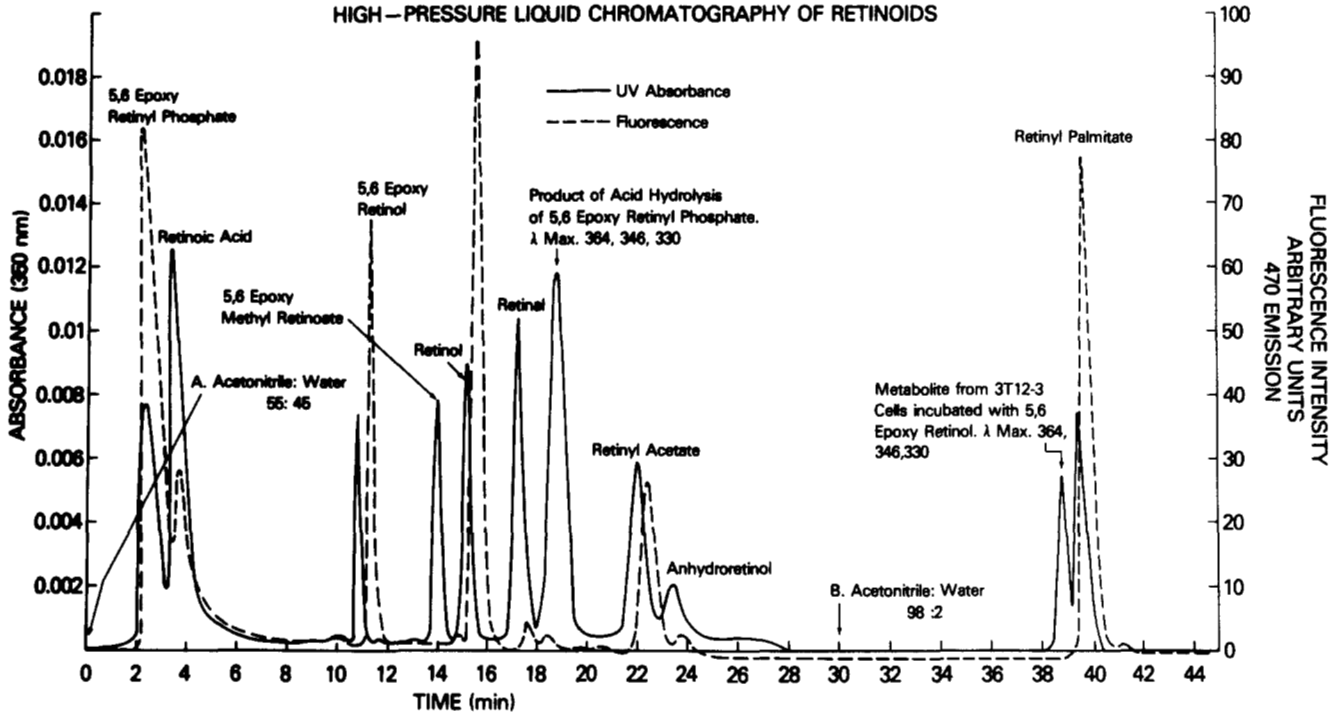


Fig. 7. Reverse phase HPLC separation of retinoids. Conditions are given under Experimental Procedures. Ultraviolet absorption was monitored at 350 nm and fluorescence emission at 470 nm. The amount of each retinoid was 200 to 250 ng.

and DeLuca (6) demonstrates that 5,6-epoxyretinoic acid is inactive in promoting growth of vitamin A-depleted rats.

Recently, we developed an adhesion assay in spontaneously-transformed mouse fibroblasts, which appeared predictive of biological activity of retinoids in other biological systems. In this EDTA-mediated detachment assay, we have found a very high correlation with the biological activity of retinoids in other in vitro systems (14). 5,6-Epoxyretinol and 5,6-epoxyretinoic acid were as active as retinol and retinoic acid in this system of 3T12 cells. Activity was evident at 3.3×10^{-6}M–10^{-5}M, in the absence of any toxic reaction on the cells, insofar as their plating efficiency remained the same; they reverted to their less adhesive state within hours of removal of the retinoids and less than 5% took up vital dyes. These results show that in the case of 5,6-epoxyretinoids, their activity in increasing the adhesive properties of 3T12 cells does not correlate with an in vivo growth promoting activity (6). The UV absorption spectral characteristics of 5,6-epoxymethylretinoate, 5,6-epoxyretinol and 5,6-epoxyretinoic acid were similar to those reported previously (1–3). Thus the λ_{max} in methanol for 5,6-epoxyretinol were found at 324 and 310 nm with a shoulder at 296 nm. Jangalwala and Cama (1) reported λ_{max} at 324 and 310 nm in ethanol. Phosphorylation of the 5,6-epoxyretinol leaves these maxima in methanol unchanged. The reported λ_{max} for 5,6-epoxyretinoic acid in ethanol is at 336 nm (2); we found 332 nm in methanol.

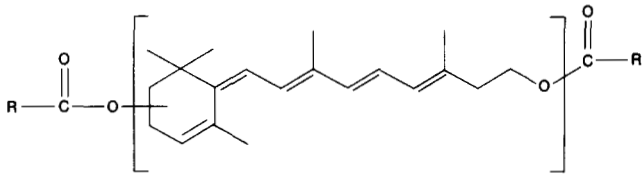
The synthesis, spectral and chromatographic characteristics of 5,6-epoxyretinylphosphate are reported. This compound was very active as an acceptor of mannose from GDP-mannose, and, as for RP (12), it did not accept galactose from uridine-diphosphate [^{3}H]galactose (Table 2).

Retinylphosphate was previously reported to be extremely labile, even to mild alkali, generating anhydroretinol (9, 15). However, in those studies, we routinely treated the alkaline solution with acetic acid, before analysis by chromatography on thin layers of silica gel. Utilizing a recently published HPLC procedure (7), we determined that RP is resistant to the mild alkali (Table 1) and cleaved by acid, and that the acetic acid used in our previous studies to neutralize NaOH in fact caused the cleavage of RP to AR. Table 1 shows the extreme lability of RP to acid. These data are in agreement with those of Dimitrovsky and Poznyakov (16). It is possible that the conversion of RP to AR proceeds through an A-1 reaction similar to the formation of anhydroretinol from retinylacetate (17). The results shown in Table 1 indicate that readiness to acquire the retro structure favors cleavage of

RP to AR, inasmuch as stronger conditions are required when an impediment to the acquisition of this retro structure is introduced in the form of the 5,6-epoxy group. The reverse phase HPLC system achieved a good separation of eRP, retinoic acid, 5,6-epoxyretinol, 5,6-epoxymethylretinoate, retinol, retinal, retinylacetate, anhydroretinol, and retinylpalmitate. Utilizing this system, it was also possible to elute the less polar product of acid hydrolysis of eRP between retinal and retinylacetate.

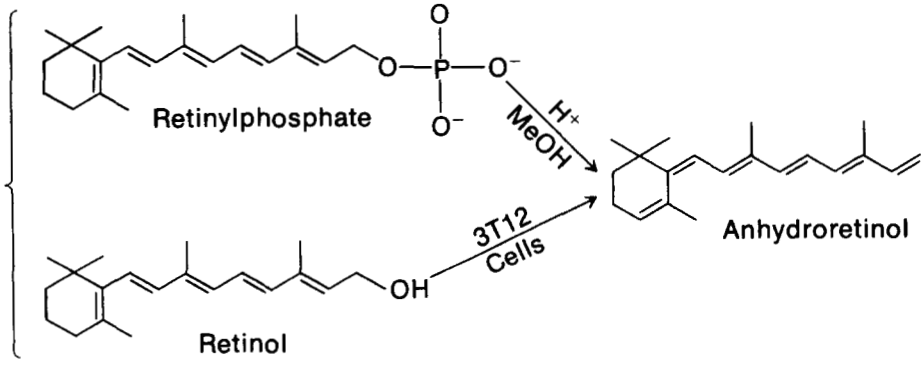
Mass spectrometry analysis of this major product of mild acid hydrolysis of eRP in methanol shows that it has a molecular weight of 330, consistent with a retinoid structure containing two methoxy groups and five double bonds. The significant loss of 45 (CH_2OCH_3) mass units from the molecular ion indicates that one of the methoxy groups is on the terminal methylene carbon (Fig. 4). Ions involving loss of the second methoxy substituent from ion m/e 285 were found at m/e 254 (285 – OCH_3) and at m/e 253 (285 – $HOCH_3$).

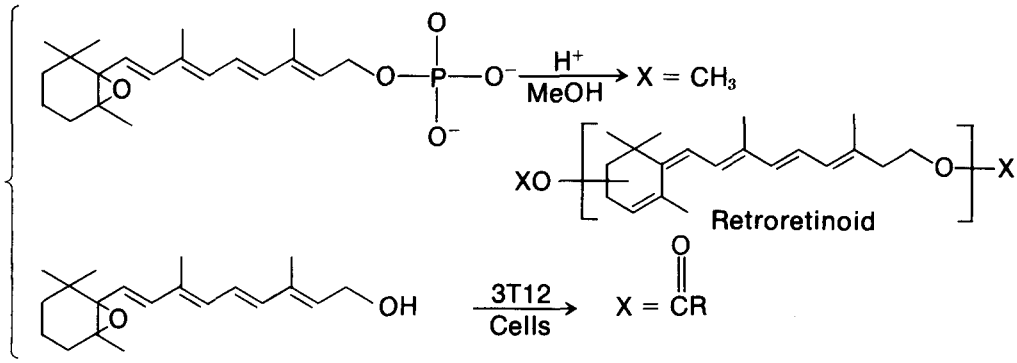
The maxima at 364, 346, and 330 nm of this hydrolysis product are consistent with a retro system of five conjugated double bonds (out of conjugation with the terminal -CH_2-O-CH_3). On the basis of these data and the fragmentation pattern, the retro-structure shown in Fig. 4 is assigned to this compound with elemental composition $C_{22}H_{34}O_2$ (mol wt 330). This compound has UV absorption maxima very close if not identical with those of retro vitamin A_1, (18) and retrovitamin A_2 (19). These retro compounds are formed in vivo, upon administration of the anhydrovitamins to rats (18–20). The metabolite obtained from 3T12-3 cells incubated with 5,6 epoxyretinol has similar λ_{max} at 364, 346, and 330 nm as the product of mild acid hydrolysis of eRP. However, the elution position of the metabolite close to retinylpalmitate (Fig. 6) and elution off alumina at 3% instead of 5% ether in hexane indicates a fatty acid ester instead of the bismethylether. On the basis of the UV adsorption characteristics and polarity, it is possible to suggest a retro structure for this metabolite such as shown below:



Assuming an E 1% cm at 366 nm of 1,107 or equal to that of retrovitamin A_1 (18), the total amount in retroretinoid equivalents (in parenthesis) for this metabolite can be roughly estimated from the absorbance at 364 nm (0.08) compared to the absorbance at 310 nm (0.73) of 5,6-epoxyretinol for which E 1% cm in ethanol is 2,422 (12). The metabolite represents about 13% of the cell associated 5,6-epoxyretinol at 34 hr of incubation. The total amount of 5,6-epoxyretinol associated with 62 × 10^6 cells is about 19.7 μg, the total amount of the retroretinoid shown in parenthesis is about 2.56 μg and the total amount of 5,8-oxyretinol (based on E 1% cm of 1768 at 278 nm) about 28.67 μg. Thus, of the total 1.8 mg of 5,6-epoxyretinol used in twelve 15-cm dishes, only 2.8% was in the cell associated-lipids identified at 34 hr.

In conclusion, these studies demonstrate that 5,6-epoxy retinoids possess biological activity in the adhesion stimulation assay of 3T12 cells and, at least for eRP, as acceptor of mannose from GDP-mannose. Moreover, 5,6-epoxyretinol appears to undergo metabolism to a product similar in chromophoric structure to the retro vitamin A compounds. A similar product is also formed as a result of mild acid treatment of eRP as summarized below.





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