Oxidation and Isomerization of Retinoic Acid by Iodine and Light: A Novel Preparation of All-*trans*- and 13-*cis*-4-Oxoretinoic Acid

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ABSTRACT

A mixture of all-*trans*-retinoic acid and iodine in heptane was irradiated. Two oxidation products were isolated by high performance liquid chromatography and identified as all-*trans*- and 13-*cis*-4-oxoretinoic acid by nuclear magnetic reasonance, ultra violet, Infrared spectroscopy, and mass spectral analysis. Under the same conditions, but without light, a mixture of all-*trans*- and 13-*cis*-retinoic acid resulted. The corresponding methyl esters were obtained when methyl all-*trans*-retinoate was used in place of all-*trans*-retinoic acid.

INTRODUCTION

The metabolism of vitamin A, in general, and retinoic acid in particular has been of interest in our laboratory for several years (1-3). Recent advances in high performance liquid chromatography (HPLC) and the development of high efficiency columns have provided the means for the rapid separation and purification of these labile compounds in the dark, at room temperature and without exposure to air. Procedures for the preparation of geometric isomers and oxidation products, the most commonly encountered derivatives of retinoic acid and related substances, were of particular interest to us. In this regard, Hubbard (4) has reported the isomerization of retinol, retinaldehyde, and retinaldehyde oximes under the influence of light and/or iodine. The major products resulting from the irradiation of all-*trans*-retinoic acid in the absence of iodine have been purified by HPLC and identified as geometric isomers of retinoic acid (5). These products account for at least seven of the eight possible *cis-trans* isomers that involve the 9, 11, and 13 double bond positions of retinoic acid. The present communication is concerned with the isolation and identification of the products generated by the treatment of all-*trans*-retinoic acid with iodine in the presence or absence of light.

EXPERIMENTAL

Distilled in glass solvents were obtained from Burdick and Jackson Laboratories Inc. (Muskegon, MI). Water was deionized, charcoal-filtered, and then glass-distilled. All-*trans*-retinoic acid and all-*trans*-4-oxoretinoic acid were obtained from Dr. W.E. Scott and Dr. B.A. Pawson, Hoffmann-La Roche, Inc. (Nutley, NJ). Methyl esters were prepared from retinoic acid and related products using diazomethane in methanol/diethyl ether solutions, essentially as described by Schlenk and Gellerman (6). Isomerates were prepared by irradiation with a fluorescent lamp (GE F-15-T8-CW). The irradiations were carried out in 22 ml borosilicate glass vials held at a distance of ca. 5 cm from the lamp. Samples were dissolved in heptane, and iodine, dissolved in heptane, was added as indicated. To terminate the iodine reactions, the samples were dried in the dark, with a gentle stream of nitrogen. The vials were partially submerged in a warm water bath to aid in rapid evaporation of the solvent and sublimation of the iodine. Such samples, once dry, were routinely redissolved in diethyl ether and redried to assure removal of iodine. Separations were performed by HPLC on a 0.46 x 25 cm bonded, reverse phase, octadecylsilane (ODS) column (Partisil 10-ODS, Whatman Laboratory Products, Clifton, NJ) with methanol/water, 70:30 (v/v) as solvent as previously described (5). Cochromatography with all-*trans*-4-oxoretinoic acid was on a Partisil PXS ODS-2 column with methanol/0.01 M acetic acid (75:25). Purified samples were subjected to mass spectral analyses by way of the direct probe inlet on a low resolution mass spectrometer (LKB-9000, LKB Instruments, Inc., Rockville, MD) as previously described by Waller (7), Lin et al. (8) and Reid et al. (9). Nuclear magnetic resonance (NMR) spectra were obtained using a Varian XL-100 (15) spectrometer (Varian, Palo Alto, CA). Samples were dissolved in carbon tetrachloride. Infrared spectroscopy (IR) was performed with a Perkin-Elmer (Norwalk, CT) Model 457 grating

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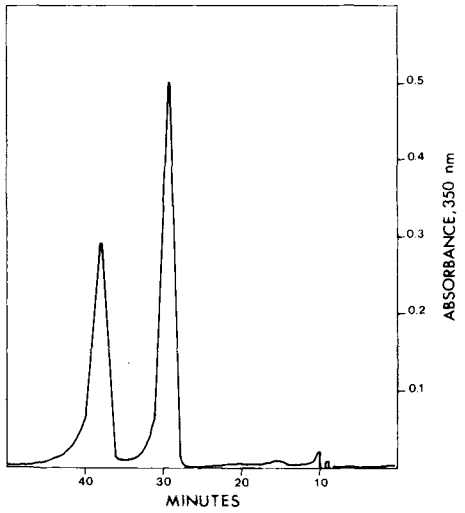

FIG. 1. HPLC of the products resulting from a mixture of all-*trans*-retinoic acid (130 μg/ml) and iodine (25 μg/ml) in heptane incubated for 3 hr in the dark. Compounds were separated on a reverse phase column with methanol/water (70:30) at a flow rate of 0.4 ml/min and 24 C. Compounds eluting at 29 and 38 min were identified as 13-*cis*- and all-*trans*-retinoic acid, respectively.

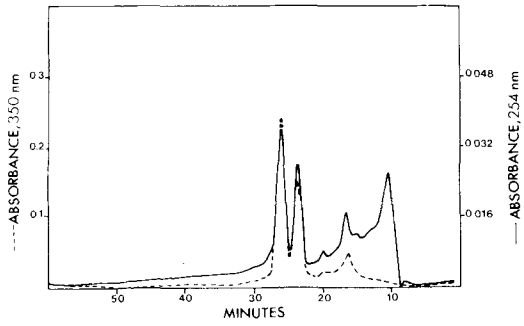

FIG. 2. HPLC of the methylated products resulting from a 2½ hr. irradiation of a mixture of all-*trans*-retinoic acid (130 μg/ml) and iodine (25 μg/ml) in heptane. Chromatography conditions were as described in Figure 1. Compounds eluting at 24 and 27 min were identified as the methyl esters of 13-*cis*- and all-*trans*-4-oxoretinoic acid, respectively.

infrared spectrophotometer using micro KBr pellets.

RESULTS AND DISCUSSION

Iodine-Dark Reaction

In the absence of light and iodine, solutions of either all-*trans*-retinoic acid or methyl all-*trans*-retinoate in methanol, diethyl ether or heptane can be stored at room temperature for extended periods of time without significant change. The addition of iodine to all-*trans*-retinoic acid in heptane in the dark resulted in a rapid change leading to a mixture of two compounds. The two compounds were separated by HPLC (Fig. 1) and identified as the starting material, all-*trans*-retinoic acid, which eluted at 38 min, and 13-*cis*-retinoic acid, which eluted at 29 min. The 13-*cis*-retinoic acid was identified by its UV (λmax 354 nm) and NMR spectra (10,11). The mass spectra of the two compounds (M^+ = *m/e* 300) were indistinguishable and similar to that which has been previously reported (8). Correspondingly, methyl all-*trans*-retinoate and methyl 13-*cis*-retinoate were obtained when methyl all-*trans*-retinoate was the starting material.

The UV spectra of the two acids were of interest. Robeson et al. (12) reported a marked blue shift in the absorbance maximum of retinoic acid in ethanol if great care was not exercised in the preparation of the solvent. This shift was observed in the present study using glass-distilled methanol in which all-*trans*-retinoic acid had an absorbance maximum at 337-338 nm. However, when scanned on-line in aqueous methanol as they passed through the detector, these same samples showed no such shift. On-line UV scans indicated absorbance maxima of 350-351 nm for the all-*trans*-isomer and 353-354 nm for the 13-*cis*-isomer.

Iodine-Light Reaction

When a mixture of retinoic acid and iodine in heptane was irradiated, several products more polar than retinoic acid were separated by HPLC. The same qualitative results were obtained for the iodine-light reaction with either methyl all-*trans*-retinoate or all-*trans*-retinoic acid, followed by subsequent methylation of the reaction products. Since these compounds were more easily handled as methyl esters, the major products were isolated and characterized as methyl esters. A profile obtained with the methylated derivatives of the iodine-light reaction is shown in Figure 2. The compounds eluting at 24 and 27 min were collected and purified by HPLC using methanol/water (60:40) which resulted in retention times of 34 and 40 min, respectively.

The UV spectra of these two compounds were very similar. The on-line scans indicated absorption maxima in the vicinity of 353-355 nm and 278-280 nm for both compounds. The ratio of the peak absorbances ($A_{350}/_{280}$) was about 2.2-2.5. The mass spectral fragmentation patterns of these two compounds were also qualitatively indistinguishable, indicating that they were, in all probability, geometric isomers

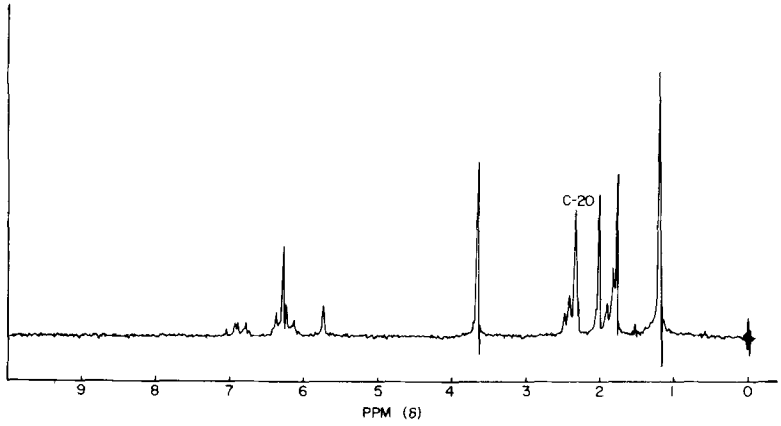

FIG. 3. Nuclear magnetic resonance spectrum of the compound eluting at 27 min in Figure 2 and identified as methyl all-*trans*-4-oxoretinoate.

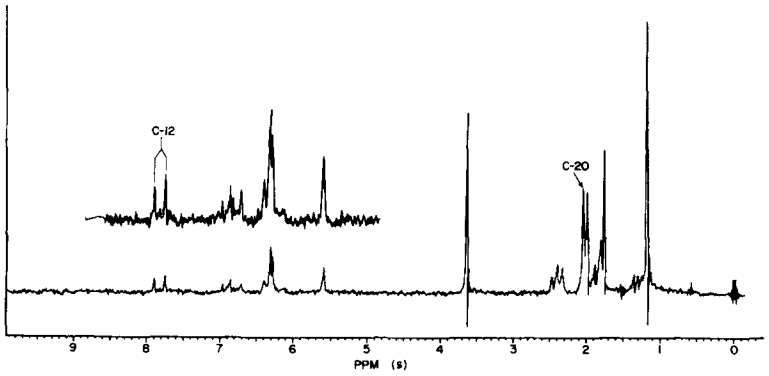

FIG. 4. Nuclear magnetic resonance spectrum of the compound eluting at 24 min in Figure 2 and identified as methyl 13-*cis*-4-oxoretinoate.

of the same compound (8). The molecular ion (*m/e* 328) was consistent with these compounds being oxidation products of methyl retinoate (M^+ = *m/e* 314), which had added a single oxygen atom and lost two protons. The molecular weight and the absorbance at 280 nm are consistent with a ketone structure. The presence of a ketonic function was further supported by the presence of two distinct carbonyl absorbances at 1650 and 1700 cm^{-1} in the infrared spectra. Again, the spectra of the two compounds were essentially identical.

The NMR spectra of the peaks are presented in Figures 3 and 4. Based on these spectra, the site of oxidation is restricted to either carbon 2 or 4 in the cyclohexenyl ring. The location of the oxygen on either carbon 2 or 4 was supported by the conversion of the methylene multiplet of methyl retinoate to a pair of triplets rather than a pair of singlets as would be required by a 3-oxo structure. This triplet character is readily seen in Figure 4, where the upfield shift of the C-20 signal has left a triplet centered at ca. δ 2.4 clearly visible. The second triplet is partially hidden by the C-18 signal and centered at ca. δ 1.8. The assignment of the oxygen to C-4 was based on the fact that the downfield shift (18 Hz) of the *gem*-dimethyl signal (C-16 and C-17) was comparable in extent to that observed in a model 4-oxo compound (13).

Additional support for the 4-oxo structure is provided by Rao et al. (14) who prepared methyl 4-oxoretinoate by exposing methyl retinoate to active manganese dioxide in light petrol. THe NMR spectra shown in Figure 3 is essentially identical to that reported by them for methyl 4-oxoretinoate (14). In addition to the UV, NMR, IR and mass spectral analysis, when all-*trans*-retinoic acid was irradiated in heptane in the presence of iodine as described in Figure 2, the compound identified as all-

trans-4-oxoretinoic acid cochromatographed with the sample obtained from Hoffmann-La Roche, Inc. This compound was one of several 4-oxo derivatives or retinoic acid that were reported to have been isolated from rat urine (15).

In the present study, a comparison of the spectra in Figures 3 and 4 reveals an upfield shift of the C-20 methyl signal from δ 2.33 (Fig. 3) to δ 2.06 (Fig. 4) and a downfield shift of the C-12 doublet from δ 6.4 (Fig. 3) to δ 7.82 (Fig. 4). These changes are characteristic of the 13-*cis*-configuration (10,11). Thus, based on the data presented here and by previous workers, the two compounds eluting at 24 and 27 min (Fig. 2) were assigned the structures of the methyl esters of 13-*cis*- and all-*trans*-4-oxoretinoic acid, respectively.

We have not isolated products resulting from the irradiation of retinol or retinaldehyde in the presence of iodine. These and similar studies can now be simply and fruitfully pursued along many different avenues of interest using HPLC analysis. This potent analytical tool has opened the way to an effective study of this biologically significant family of compounds and of polyene compounds in general.

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